Annual Report
2014

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FROM THE DIRECTOR'S DESK

2014-15 had been a very productive and fruitful year for Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum. With innovative discoveries, high impact publications, successful graduate cadre and efficient collaborations in our credentials, RGCB is turning over a new leaf in our onward scientific expedition.

"Excellence is never an accident. It is always the result of high intention, sincere effort, and intelligent execution; it represents the wise choice of many alternatives - choice, not chance, determines your destiny"

- Aristotle

In an era of cut-throat competition, nothing but our own merits can help us win the struggle. Gone are the days when victory was a relative entity. Today, it is absolute. Our well-deserved victory makes us successful, not the failures of others.

RGCB has built its foundations on the solid ground of commitment and diligence towards victory that is absolute and never relative. Setting our own bar high, our team is constantly and consistently striving to improve our standards in research, academia and community service. Our team can honestly vouch that RGCB has a working environment satiating all the scientific and professional needs of each of us. When perseverance and determination are the means, victory becomes a right than a responsibility. We believe in the collective spirit of our team and keep marching to absolute victory that will be our right on a day not so faraway.

RGCB has been in the news headlines many times this year for scientific and corporate milestones in the timeline of the institution's achievements.

ACHIEVEMENTS TIMELINE

BIO INNOVATION CENTER

The Honorable Union Minister of Science, Technology and Earth Sciences, Dr. Harsh Vardhan dedicated the facilities in over 50,000 Sq. feet of Bio Innovation Center (BIC) to the nation on 17th of May 2015. The center focuses on augmenting fundamental research in disease biology and molecular medicine through translational innovations into tools and products of social significance. Immunotherapeutics and vaccines are our top priorities. Being in a tropical state with high density of population, we have the upper hand in understanding the health needs of a significant cross section of society. BIC campus, situated at KINFRA Park, Kazhakkuttam hosts fully equipped state of the art laboratories of Microbiome and Virology groups as well as Chemical Biology and Bioinformatics. It also caters to the needs of the entire RGCB with Next Generation Sequencing and advanced High Definition Optics.

BIOTECHNOLOGY SKILLS DEVELOPMENT PROGRAM

As pioneers in the state, it is of our prime concern to bridge gaps between academia and industry in biotechnology and pharmaceutical disciplines. As part of a call from the Honorable Prime Minister of India on need for skills development at grass root level as a national priority, a six-month comprehensive training program has been devised to equip graduates and postgraduates in life sciences with career-focused skills. Experts in Molecular Diagnosis, Bio-Imaging, Biotech Instrumentation, Bioinformatics and Analytical Sciences provide hands-on training to familiarize students to needs of the industry and thus bring out their best later in the careers.

FUNDAMENTAL TO TRANSLATIONAL: SCIENTIFIC RESEARCH IS GEARING UP IN RGCB

Though publications are not the sole aim of research, acceptances in internationally acclaimed journals give us immense confidence that our research is definitely heading in the right direction. A quick peek into some of our efforts will undoubtedly ascertain the same.

Laishram RS *et al* identified a phosphorylated residue, Serine 6 in the zinc finger domain of Star- PAP, a nuclear non-canonical poly(A) polymerase with specific mRNA targets. The intranuclear phosphorylation of S6 by CKIα is necessary for the nuclear retention of Star- PAP and its eventual interaction with PIPKIα. Unlike at the catalytic domain, the phosphorylation at ZF domain is independent of oxidative stress. This is suggestive of a signal-mediated regulation of CKIα activity. This conceptually paves way to novel roles for phosphorylation in determining Star-PAP target mRNA specificity. The Department of Biotechnology funded study, which extensively analyzed the Star-PAP phosphorylation in the ZF region and subsequent impact in mRNA 3' end processing and its complex regulation of select set of Star-PAP target messages, was well received by the scientific fraternity worldwide.

We are quite blessed to get the first hand information on the uses of various herbs and plants used in traditional medicines in our region of the planet. Wrightia tinctoria popularly known as *Dandha Pala / Swetha Kudaja* is widely used in our traditional medicine for the effective treatment of dermatological conditions such as psoriasis and herpes. Extrapolating the thought that it's preferential action in skin cells, if proven to have cytotoxic effects, could be used to develop a potential anti-cancer agent for malignant melanoma. This deadly cancer of melanocytes in skin is increasing in incidence across the globe. Anto RJ *et al* was quite successful in extracting a semi-purified fraction from the dichloromethane extract of *W.tinctoria* leaves named DW-F5. This chemical was proven to completely abolish MITF-M through BRAF, Wnt/β-catenin and Akt-NFκB signaling pathways in in vitro and in vivo studies. Elucidating the anticancer properties of the active principle of *W. tinctoria*, this study enriches the scope of developing potential drug formulations from our natural resources.

Despite rigorous efforts to contain the deadly infection of Tuberculosis in India and worldwide, the morbidity and mortality burden of this disease is huge. One of the reasons curbing the efficacy of anti tubercular drugs is the ability of Mycobacterium to stay quiescent in macrophages, insensitive to drugs and later activate during states of compromised immunity of the host. It was by the commendable efforts from

Kumar RA *et al* that deciphered the entire proteomic profile of the pathogen during dormancy and reactivation. Functional correlation between energy metabolism and the relative levels of different proteins during different stages of infectivity is giving us a whole new target for therapeutic intervention to prevent reactivation of latent infections.

In this era of personalized medicine, stem cell therapeutics is gaining a strong foothold. Understanding the molecular mechanisms of cellular differentiation cannot be compromised at any level. Dutta D *et al* delved into the less explored area of hematopoietic differentiation from hemogenic embryonic stem cells in depths. Identifying and analyzing the complex mechanisms of regulation of transcription factor RUNX1 by histone chaperone HIRA newly defined the RUNX1-HIRA axis during the developmental process of blood cells. Leukemogenesis can now be approached in a whole different perspective with the added knowledge.

Analyzing the pathogenesis of oral squamous carcinoma in South Indian population, Banerjee *et al* had positive evidence to support microsatellite instability in D2S123 flanking the hMSH2 gene and polymorphisms in mismatch repair gene human MutS homologue-2. An integrated analytical database on triple negative breast cancer developed and hosted by Pillai MR *et al* is highly referred by breast cancer biologists worldwide. We are very optimistic about our journey forward, thanks to the untiring efforts of our team of researchers.

ESTEEMED GRANTS, NEW FACULTY, WELL-PLACED GRADUATES, STATE OF THE ART INFRASTRUCTURE

RGCB is evolving into one of the best research and academic institutions in the country not by luck, but by the relentless perseverance of our team.

Efficiently progressing studies in cancer biology, tropical infections and metabolic disorders funded by ICMR, DBT, DST, KSCSTE, CSIR and UGC are keeping our team on their tiptoes for the coming years. Twenty-one students graduated from the RGCB Graduate School during 2014-15 with Student Merit Awards and MR Das Career Awards. All of them have been securely placed either in the academia or industry with ample opportunities to make their own mark. Five scientists with research experiences of global standards have joined our team this year. The newly established laboratories focus on cancer metastasis, fat metabolism and microbiome. The centralized instrumentation team provides superior infrastructure comprising Mass Spectrometry, Confocal and Electron Microscopy, Flow Cytometry and Fluorescent Assisted Cell Sorting, Next Generation Sequencing to name a few.

ACHIEVEMENTS TIMELINE

AYURVEDA INSPIRED DISCOVERY

India has one of the richest treasures of traditional medicine through our invaluable knowledge in Ayurveda. It shouldn't be of surprise that the herbs and medicines used in Ayurveda could help us develop pharmaceutical chemicals with unparalleled efficacy and least side effects as this is a system of medicine that stood the trials of time over centuries. None but us can effectively translate this knowledge into marketable products. RGCB proudly entertains the host platform for Ayurveda inspired discovery as evident from the research profile of the institution. We were committed co-organizers of World Ayurveda Congress 2014 and National Arogya Expo 2015 under Department of AYUSH, Government of India.

COLLABORATIONS

Scientific and corporate collaborations are quintessential in the progressive expedition of any system. Understanding our potentials, RGCB has dedicated collaborations with world-class universities like Emory and Mayo Clinic for studies in tropical diseases. We also work hand-in-hand with Kerala State Council for Science, Technology and Environment (KSCSTE), Government of Kerala providing an incubator for the Srinivasa Ramanujam Institute for Basic Sciences.

OF THE PEOPLE AND BY THE PEOPLE; HENCE DEFINITELY FOR THE PEOPLE

Though a high profile institution with top-notch amenities, RGCB doesn't just stand aloof from society. We believe in serving the humanity at community level. In fact, every system ultimately aims for the noble cause of betterment of mankind.

Our Laboratory Medicine and Molecular Diagnostics (LMMD) group dedicatedly works 24x7 to cater to the diagnostic needs of the people of southern Kerala. With high incidence of epidemics in Kerala, LMMD has significantly contributed in accurate outbreak investigations through 42 viral and 4 bacterial tests. This assists patients from various public and private hospitals in the region. Affordability, accuracy, credibility and punctuality have helped us in setting an exemplary standard in faster diagnostics. Additionally, cancer and cardiovascular biomarker panels are being extensively used at the community level at highly subsidized rates for all and even free of cost for the under privileged. The panels of tumor recurrence prediction, anti-tumor drug response as well as pharmacogenomics assay of Tacrolimus® post renal transplantation have been widely accepted by the medical community. With the help of a team of well- chartered bioinformatics professionals, we have the liberty to explore the demography of Kerala to predict outbreaks well in advance and hence move shoulder to shoulder with the government in planning prevention strategies. We hope to continue serving the society by meeting its diagnostic needs at international standards cost effectively.

ACHIEVEMENTS TIMELINE

MICROBIOME RESEARCH GROUP

Celebrated microbiologist and pioneer researcher in Vibrio cholera and its toxins, Dr. G Balkrish Nair joined our team as the Honorary National Chair in Disease Biology to lead the Microbiome Research Group in March 2015. It is with immense pride and joy that we welcome him to our scientific fraternity. A Bhatnagar Awardee, Fellow of all three Science Academies, Foreign Associate of the US National Academy of Sciences, Fellow of the World Academy of Sciences, Fellow of the American Academy of Microbiology and Member of the German National Academy of Sciences, Dr. Nair is no stranger to microbiologists across the globe. He has authored over 500 research papers and several book chapters.

ON THE SOCIAL PLATFORM

RGCB launched à la mode website which gives comprehensive information about the research, academic and diagnostic profile of the institution. We are also active in various social media opening up a wide arena for scientific information exchange and a platform for constructive discussions.

Spanning across three elaborate campuses, RGCB is extending its wings to fly up towards the scientific expansiveness where only sky is our limit.

It is more than just words. None can lead a team without the integrated efforts of each of the contributing elements of the team. I take this opportunity to recognize, appreciate and acknowledge every single person who stood for us.

If asked about the vital factor making RGCB team run like a well-oiled machine, I don't have to think twice to say it is our Governing Council and Scientific Advisory Council. Supporting us for our greater good, being a strong prop when we stumble, guiding us through the right path, the members of these two councils have been indispensible in the day-to-day functioning of RGCB as well as key players in our growth and development. Located towards the southern tip of India, RGCB may pose the unfortunate risk to go unnoticed by the diplomats and administrators of the country in the capital. I cannot express my gratitude in simple words to Professor K VijayRaghavan and his team of dedicated officers for helping us to be seen and heard in the Department of Biotechnology, Government of India for the consistent smooth functioning of RGCB with commendable credentials.

Above all, I have to thank each of my team members for their untiring efforts and dedication to take RGCB to greater heights in research and academia. Our Engineering group has put in an outstanding performance especially in the launch of the new Bio Innovation Center. The RGCB Administration and Finance often have to bear the impacts of many tough decisions taken in interests of the whole institution. But there is no denying that these dedicated officers have ensured that the institution keeps growing ensuring all needed checks and balances while achieving our objectives. Again thanks to them, the staff at RGCB enjoys social security privileges that are among the best in the country.We are thus quite optimistic to make our own mark in the scientific map of the world in the years to come.

Jai Hind

Professor M Radhakrishna Pillai
FRCPath, PhD, FAMS, FASc, FNASc
Director
Rajiv Gandhi Centre for Biotechnology

CANCER RESEARCH PROGRAM
LABORATORY - 1

T.R. Santhosh Kumar

Scientist E II

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Santhosh Kumar took his PhD in Tissue Engineering from Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram and joined RGCB in 2000. His current research interests include understanding molecular signaling involved in cancer drug resistance and cell based assay development for anti cancer drug screening.

Post-Doctoral Fellow

Abitha Murali, PhD

PhD Students

Deepa I.
Krupa Ann Mathew
Asha Lekshmi
Shankara Narayanan V.
Santhik S.L.
.

Project Personnel

Vijith V.
Gin Alexander
Heera Pillai

Manager Technical Services

Indu Ramachandran

Laboratory Technician

Prakash R.

Real-Time Spatio-Temporal Visualisation and Quantitation of Cell Death and Cell Cycle Progression in 3D Culture Models Utilising Genetically Encoded Cell Death and Cell Cycle Probes

Krupa Ann Mathew, Shankara Narayanan V., Ishaque P.K., T. R. Santhosh Kumar

Innovative approaches to study the dynamics of cell growth and cell death in three dimensional models are very important for understanding and modeling tumor progression in culture dishes as well as for identifying tumor drugs with possible in vivo activity. Recent developments in 3D polymeric scaffolds and biological matrices offer a great potential to generate 3D models in dishes utilizing diploid cells as well as cancer cells. However, visualization of spatio-temporal biologically relevant endpoints such as cell cycle and cell death in 3D models require fixation and staining with diverse markers and not suitable for real-time detection or quantification of cell death and cell cycle. Diverse live cell staining methods available for cell death and cell cycle progression are good for 2D models; however, their application in 3D models are not promising because of staining artifacts and toxicity; thereby it is very difficult to translate such methods for real-time quantitative applications. We have recently described a live cell imaging model for real-time monitoring of caspase activation utilizing stable cells expressing FRET probe consisting of donor and acceptor fluorophores linked by a caspase specific amino acid, DEVD. The excellent spatio-temporal resolution for caspase detection in monolayer culture has been demonstrated utilizing several cancer cell line panels from NCI, with adaptation to HTS drug screening. An excellent genetically encoded probe to visualize cell cycle progression utilizing two oscillating nuclear probes, cdt1 and geminin linked respectively to red and green emitting fluorescent protein, was recently described by Atsushi Miyawaki. The red nuclear probe cdt1 is expressed in G1/S phase cells with its complete loss upon entry in to G2 phase. We coupled the ECFP-EYFP FRET probe and cdt1 red probe and engineered cells to express both probes to generate a sensor cell line for both cell death and cell cycle. Several cancer cell lines stably expressing both the probes were utilized to generate 3D culture in dish models. Matrigel and low melting agarose matrices were compatible to generate 3D cultures to visualize both cell death and cell cycle in 3D models. Even though the model showed excellent 3D resolution in confocal imaging, real-time automated quantitation of cells at differing cell death and cell cycle phases were difficult in 3D models because of insufficient spatial separation of cells within the 3D models. To address this challenge, we expressed the FRET probe at the nucleus with nuclear targeting signal (NLS-SCAT3). With this approach we developed several cancer cell lines expressing both the probe at nucleus so that real-time imaging and quatitation becomes easier (Fig. 1A). We have developed a robust and easy method to generate tumor spheres of defined dimension by growing the probe-expressing cells in polyHEMA coated low attachment plate (Fig. 1B). This method can be adapted for drug screening and tumor progression studies in HTS mode in multi well low attachment optical bottom plates. Utilizing laser confocal and white light spinning disc confocal, we could image 3D tumors with a size of 50-70um thickness. Even though 3D culture models represent the in vivo physiology better than conventional two dimensional culture models, accurate monitoring of cell death and cell cycle progression in such models has remained a challenge. With our novel approach of using nuclear targeted fluorescent sensor probes, simultaneous monitoring of cell death and cell cycle progression in a 3D culture format becomes possible. This gives a more physiologically relevant insight into the true cytotoxic activity of potential drug candidates.

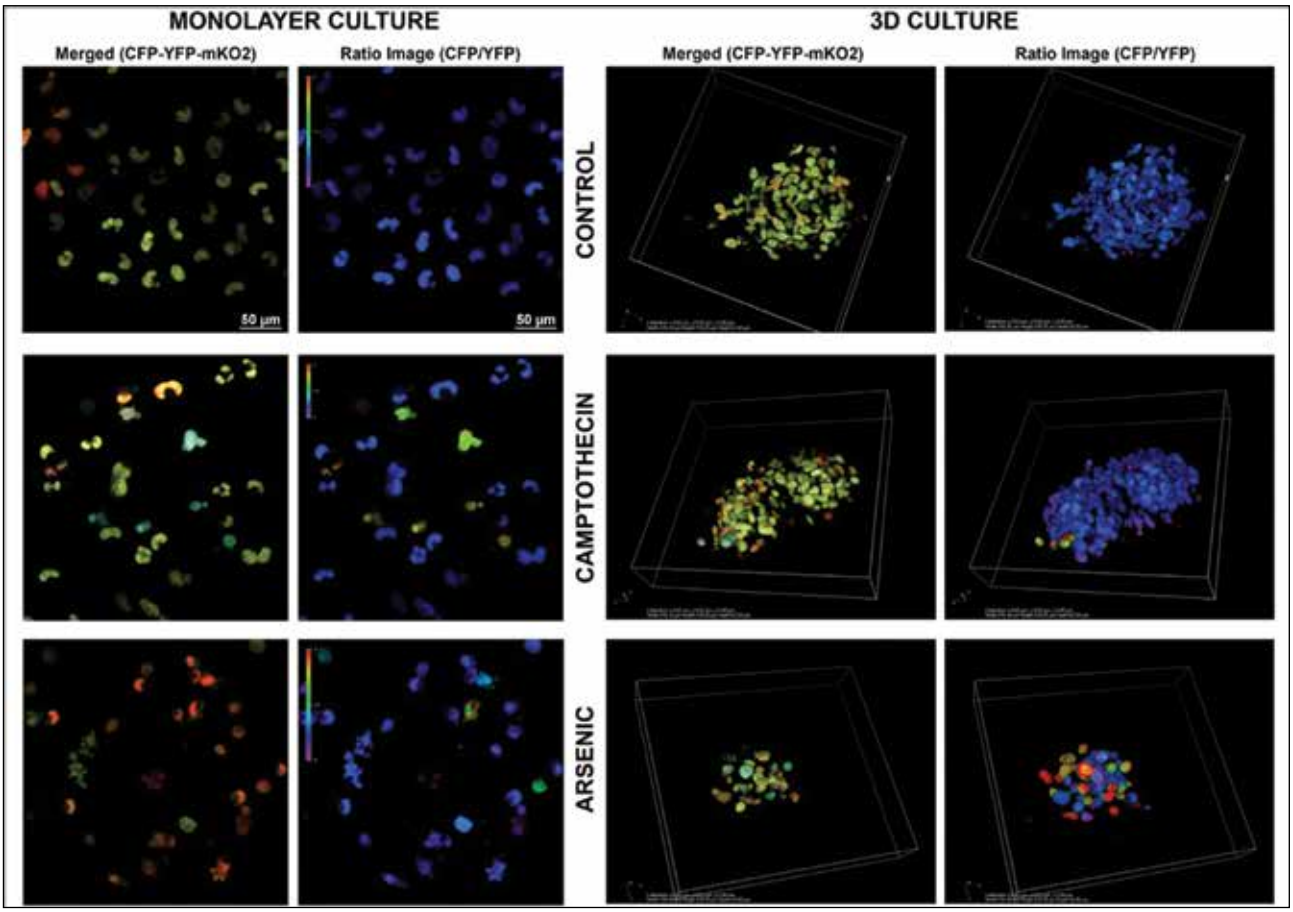


Fig. 1: (A) Representative fluorescence, merged and bright field images of monolayer culture of cells expressing NLS-SCAT3 and Cdt1-mKO2 probes in the nucleus (B) Representative fluorescence, merged and bright field images of spheres of cells expressing NLS-SCAT3 and Cdt1-mKO2.

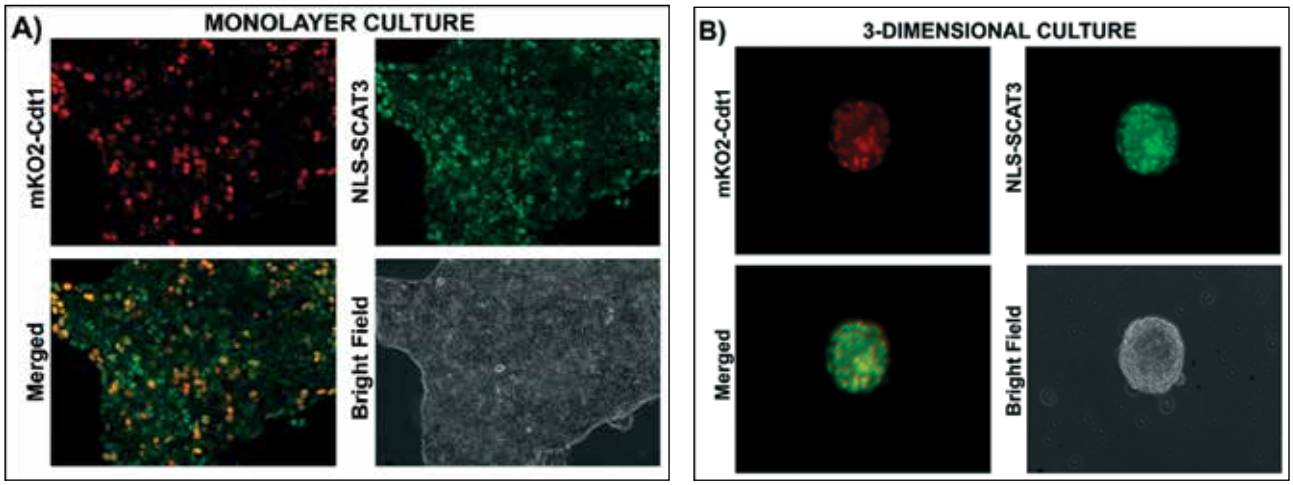


Fig. 2: (A) Real-time imaging of 3D culture of cells expressing a FRET-based caspase sensor probe, NLS-SCAT3 and a G1 phase sensor, Cdt1-mKO2 over a period of 36 hours. (B) Representative merged and ratiomatic images of NLS-SCAT3 and Cdt1-mKO2 expressing cells in 3D format. Merged image was generated using signal from CFP, YFP and red channels. Ratio image was generated using the intensity information from CFP and YFP channels (CFP/YFP ratio) followed by representation in a colour scale ranging from purple (no FRET loss or caspase activation, hence low ratio) to red (FRET loss and caspase activation, hence high ratio).

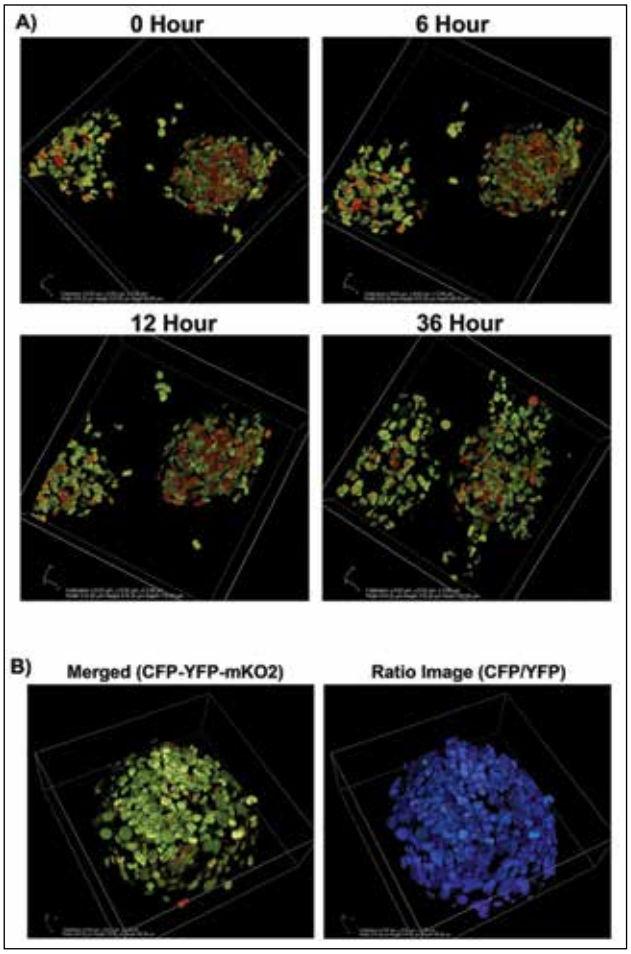


Fig. 3: Comparative analysis of the cell cycle status and apoptosis induction of cells expressing both NLS-SCAT3 and Cdt1-mKO2 as a result of treatment with indicated drugs in monolayer as well as 3D culture.

Persisting Cancer Cell Populations after Drug Treatment: Visualising its Fate and Re - Emergence

Santhik S.L, Shankara Narayanan V, Abdul Jaleel*, Rajesh Raju#, Reshmi G#,
M. Radhakrishna Pillai and T.R. Santhosh Kumar

*Collaborators: *Diabetes Disease Biology Laboratory, Rajiv Gandhi Centre for Biotechnology and #Cancer Research Program-9, Rajiv Gandhi Centre for Biotechnology*

Recurrence of tumor after chemotherapy or radiotherapy impedes successful cancer treatment. Experimental studies using cell lines and breast cancer samples suggest that chemotherapy also allows expansion of drug resistant cells with tumor stem cell like properties in a delayed manner. Molecular events governing the emergence of aggressive chemotherapy resistant cells with stem cell like properties are still poorly defined. Recent reports suggest that molecular events related to the emergence of drug resistant cells after chemotherapy is associated with reactivation of antioxidant defense signaling which help in the transition of these cells from high ROS to low ROS state. But key molecular signature events that contribute to immediate cell survival signaling after a lethal dose of drug or high stress are still poorly understood. Since immediate surviving cells contribute to the

later emergence of drug tolerant cells, we focused our studies to understand the signaling involved in immediate survival of cells. Detailed analysis of cells surviving immediately after the higher dose of anticancer drugs, reveals an unusual secondary acquisition of increased autophagy and mitophagy, coupled with constitutive activation of redox masters. The study suggests that a quick shift to low proteasome activity associated with the induction of autophagy regulators such as LC-3 and adaptor protein p62 prepares a fraction of cells to enter into chronic autophagy followed by parkin dependent mitophagy. Chronic mitophagy appears to promote long-term survival of drug escaped cells in low nutrient condition and intracellular protein stress. This chronic mitophagy-mediated mitochondrial quality control measures eventually lead to the generation of cell fraction enriched in low ROS-stem cells. Using cancer cells stably expressing a sensitive redox sensor allowed us to detect and quantify spontaneous emergence of cells with low intracellular ROS cells in drug exposed cells (Fig. 1). The study reveals unique cascades of signaling and key cell state transitions involved in the evolution of drug resistance. In order to identify key targets in each phase and its dynamics, "immediate drug escaped cells", "emerging drug tolerant colonies" and "drug tolerant expanded colonies" were utilized for transcriptomic analysis. Our study found an oscillation of many proteins, noticeably stem cell markers, during the acquisition of drug resistance by cancer cells (Fig. 2, Fig. 3). An abrupt difference in gene expression pattern was observed in the "immediate drug tolerant cells" with significant up-regulation seen in genes involved in cell cycle regulation and stem cell markers. Interestingly, genes belonging to the forkhead family of transcription factors (shown to play important roles in the expression of genes involved in cell growth, proliferation, differentiation) displayed a considerable down regulation in immediate drug tolerant cells. Proteomics analysis was employed to study the dynamics of "persisters" cells and key cell transitions. Pathways including protein folding, proteasome activity, oxidation-reduction and negative regulation of apoptosis were significantly altered during the process of drug-induced chemoresistance (Fig. 4, Fig. 5).

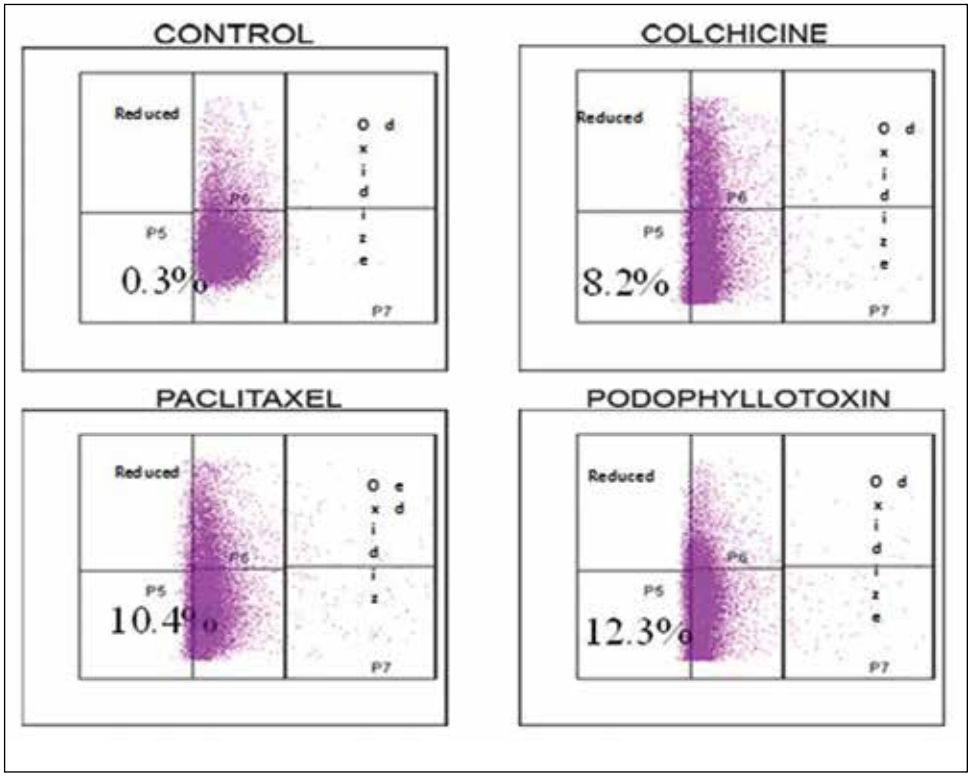


Fig. 1: FACS based ROS analysis using roGFP, a redox sensitive biosensor for iindicating the antioxidant status, in cancer cells. Drug tolerant expanded population shows increased percentage of low ROS cells compared to the control.

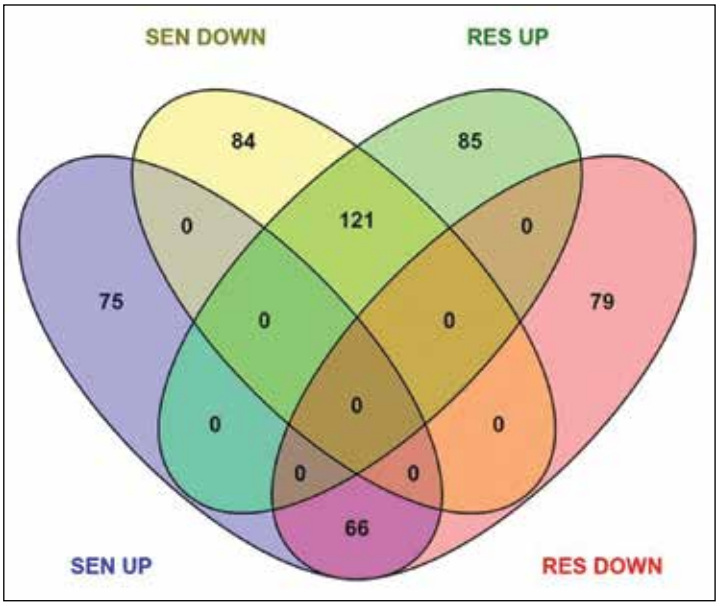


Fig. 2: Genes expressed differentially among drug tolerant senescent cells (SEN) and drug tolerant expanded cells (RES) normalized to control. (SEN UP-Senescent upregulated genes, SEN DOWN-Senescent down regulated genes, RES UP-Resistant upregulated genes, RES DOWN-Resistant downregulated genes)

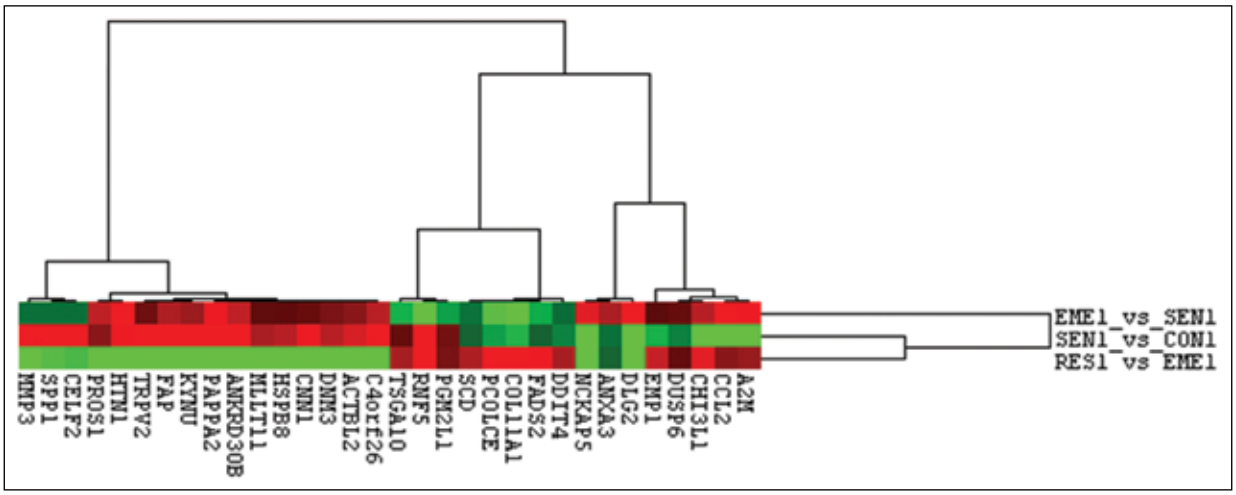
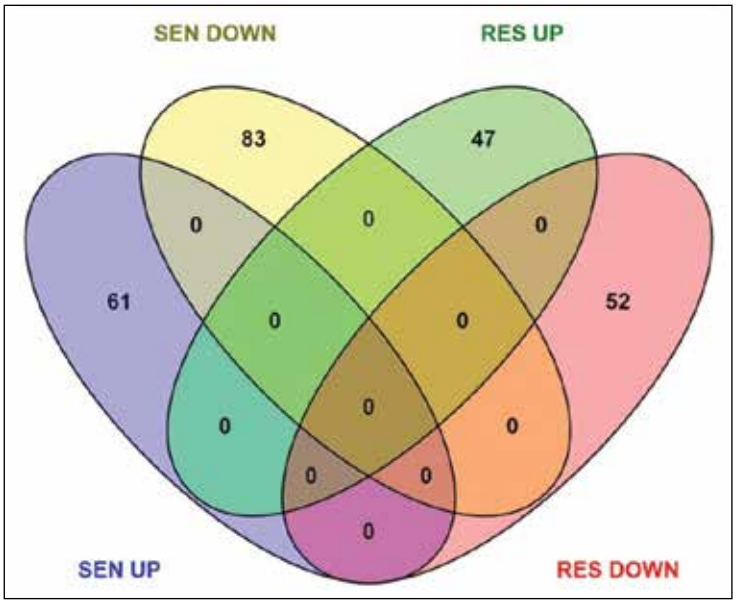


Fig. 3: Transcriptomic profiling - Heat map of genes differentially expressed at various stages of 'persister' cell development.

Fig. 4: Proteins expressed differentially among drug tolerant senescent cells (SEN) and drug tolerant expanded cells (RES) normalized to control.



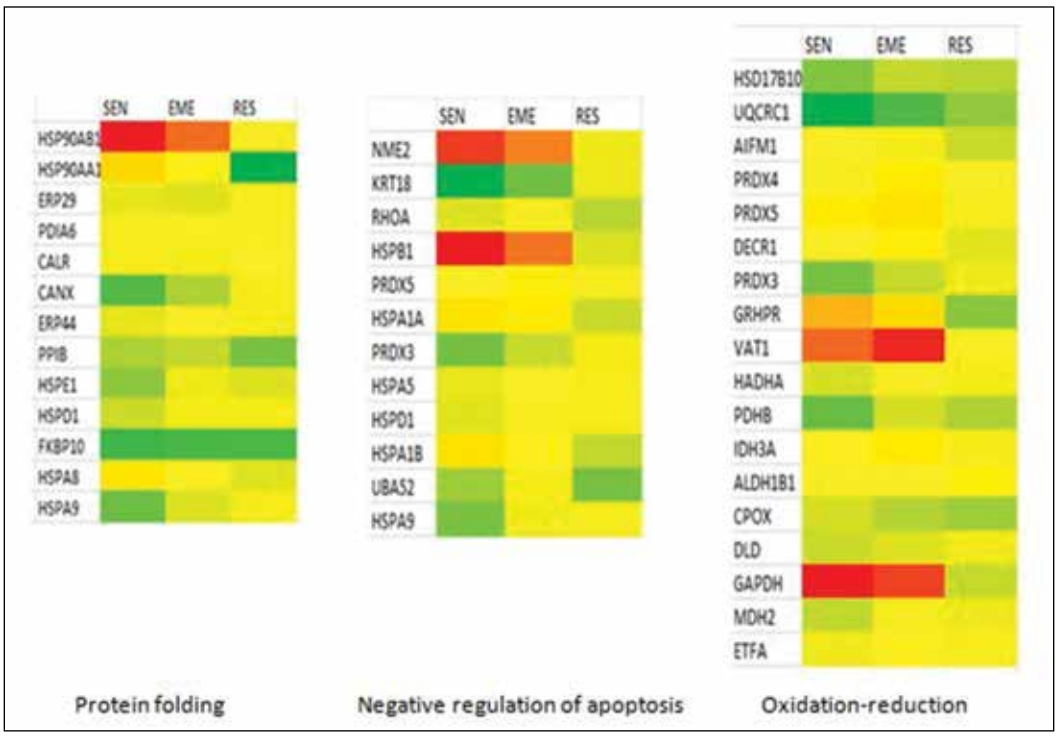


Fig. 5: Proteomic profiling-Heat map of proteins differentially expressed at various stages of 'persistent' cell development.

NRF2, a Key Regulator of Cell Death and Cell Cycle Progression

Asha Lekshmi, Shankara Narayanan V., Vijith V., Prakash R., Indu Ramachandran and T. R. Santhosh Kumar

Previous studies have shown that cancer cells show heterogeneity in their response to various signals. The molecular mechanisms underlying these cell-type-specific differences are not yet clear. At physiological levels, ROS act as an important intracellular messenger in different signalling pathways directing cells towards life or death. Even though ROS was commonly considered to be toxic, during the last decade, evidence suggests that ROS may also play an important role in cell cycle progression. Our earlier studies concluded that the cellular ROS (redox) level oscillates in a cell cycle dependent manner in cancer cells and this oscillation depends on the temporal change in the level of many molecular components involved in maintaining physiological ROS level in the cell. Currently we aim at identifying the key regulators that play a role in cell cycle dependent redox modulation. The transcription factor NF-E2-related factor 2 (Nrf2) is a master regulator of antioxidant defenses that controls a battery of antioxidant and detoxification genes containing cognate-binding sites within their promoters (referred to as antioxidant response elements, AREs). Nrf2 is widely thought to be ubiquitously expressed and has been shown to protect cells against a variety of stress. To address the role of Nrf2 in cell cycle and cell death, we developed a stable cell line expressing Nrf2 tagged with GFP (fig. 1), which will aid in visualization of the dynamics of the redox regulator. The cell line developed was validated both by fluorescence imaging and by immunoblotting of Nrf2 and its downstream effector protein NQO1 (fig. 2).

Functional validation of the cell line was done by analyzing the redox status of the cell using flow cytometry (FACS) (fig. 3). Further studies revealed that the cells over-expressing Nrf2 was found to have an impaired cell cycle progression. BrDU assay and cell cycle analysis by FACS clearly demonstrate that the cells over-expressing Nrf2 experience a prolonged G1 phase when compared to un-transfected cells. In order to confirm the role of Nrf2 in imparting chemoresistance, as reported elsewhere, Nrf2 over-expressing cells were subjected to different chemotherapeutic agents and studied using annexin V staining. The results show that the cells expressing Nrf2-GFP were resistant to most of the chemotherapeutics used. This study gives evidence to the fact that Nrf2 is a key regulator not only for maintaining the intra-cellular redox status, but also for other cellular mechanisms such as proper cell cycle progression and cell death.

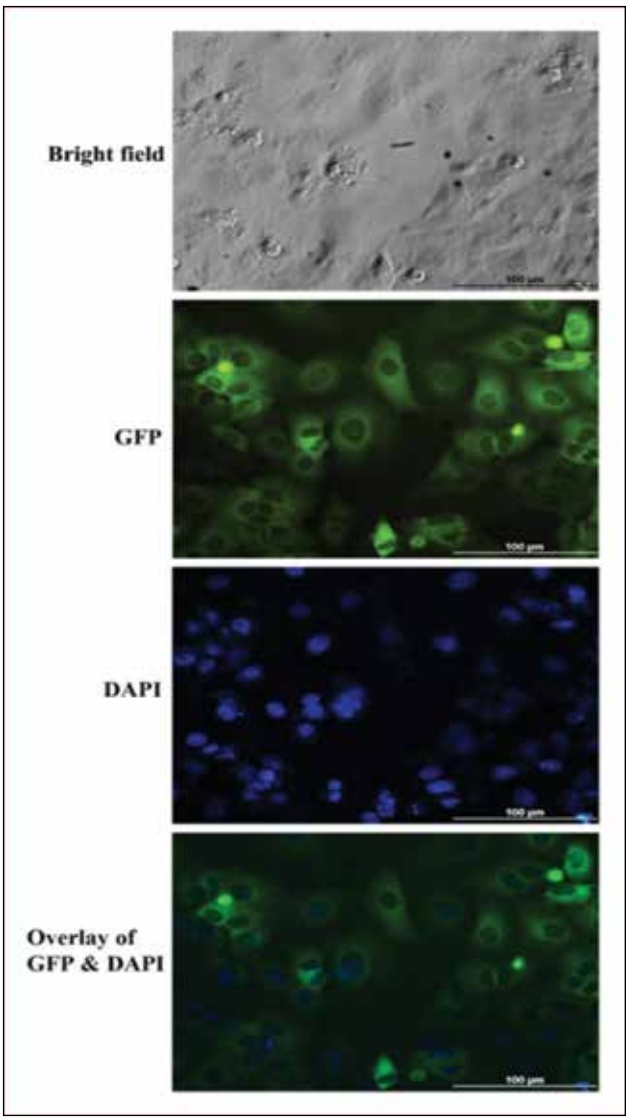


Fig. 1: Fluorescent microscopic image of cell line expressing Nrf2-GFP. Nrf2 is mainly located in the cytosol, upon activation it is translocated to the nucleus.

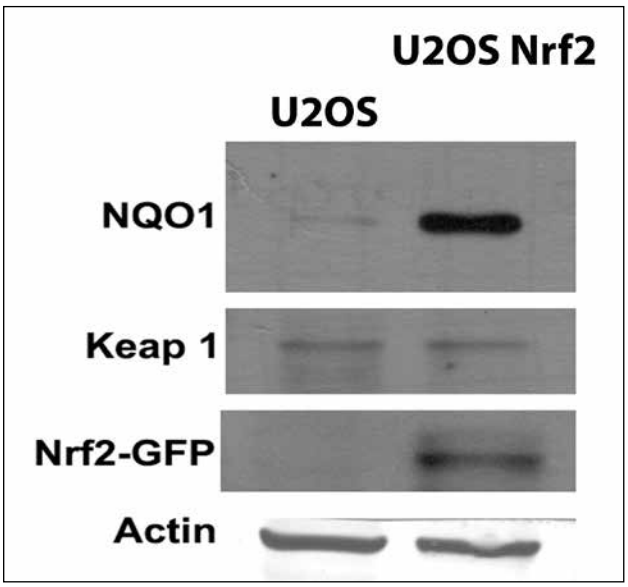


Fig. 2: Immunoblotting of Nrf2 and its downstream effector molecule NQO1 to confirm the over-expression of Nrf2 in cells.

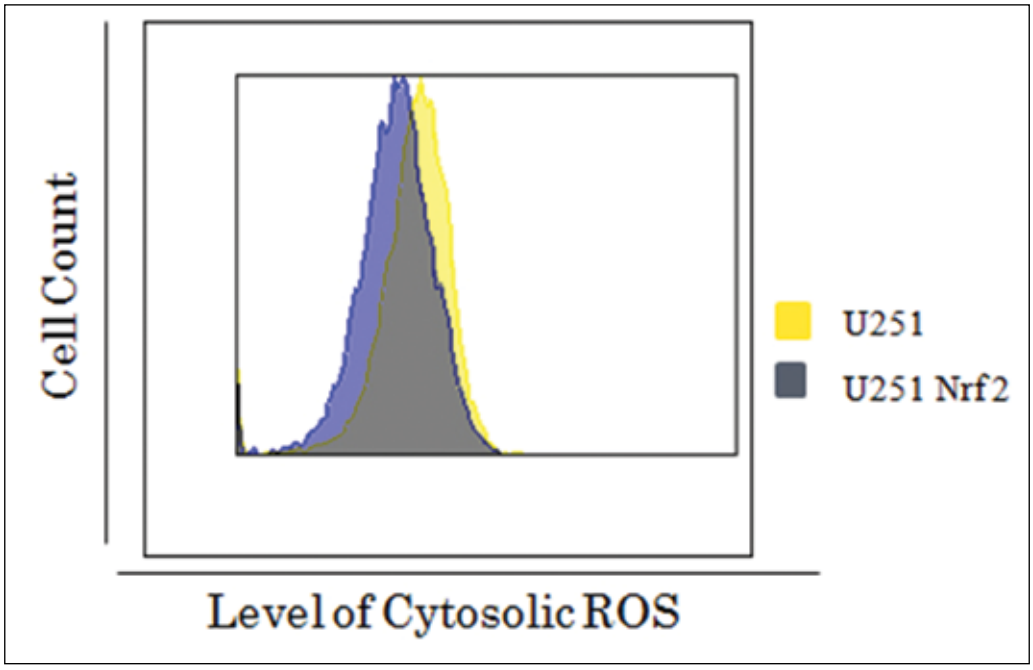


Fig. 3: Analysis of redox status of cells expressing Nrf2 – GFP using FACS

Molecular Targets of Endosulfan in Human Cells

Deepa I., Krupa Ann Mathew, Santhik S.L., Ishaque P.K., Prakash R., Vijith V., Gin Alexander, Abitha Murali, Heera Pillai, George Thomas* and T.R. Santhosh Kumar.

**Collaborator: Plant Biotechnology, RGCB*

The broad-spectrum contact insecticide, Endosulfan, is used as a pesticide in a wide range of crops including cereals, coffee, cotton, fruit, oil seeds, potato, tea and vegetables. Despite previous *in vitro*, animal model and epidemiological studies, the biological effect of endosulfan for the alleged health anomalities noticed among affected population is not known. In the current study, emphasis was given to understand the biological effect of endosulfan and its derivatives on human cells using cellular and molecular approaches. The study, for the first time reports the cytotoxic profile of endosulfan among differentiated human diploid cells and identified several key targets and signaling that could explain few clinical abnormalities observed among affected population including neurotoxic and cardiotoxic effects and developmental abnormalities. Endosulfan induced significant cytotoxity among diverse human cell types isolated from different tissues such as blood vessels, lungs, umbilical cord blood and endothelial cells. This cytotoxicity is associated with loss of mitochondrial transmembrane potential and condensation of chromatin. The study revealed few key cellular targets of endosulfan as the protein stress that culminates in unfolded proteins response (UPR) and the heat shock response. The UPR culminates in ER stress signaling leading to ER calcium release and ER structural disorganization and cell death. Endosulfan increased significant mitochondrial ROS buildup with cytosolic ROS generation. Activation of key antioxidant and defense transcription factor Nrf2 is very much prominent, that also leads to the transcriptional

activation of Nrf2 target genes. The study reveals that endosulfan exerts strong proteotoxic stress subsequent to its direct ability to inhibit proteasome and ubiquitin pathway rather than DNA damaging stress. This non-classical signaling culminates in keap1 independent chronic Nrf2 activation, which allows persistence of cells with severe DNA and mitochondrial damage, a critical process involved in the early stages of carcinogenesis. Fluorescence recovery after photobleaching (FRAP) experiments using Nrf2-stable cells reveals an unusual suppression of the negative regulator of Nrf2 in the cytoplasm compared to control, which augments nuclear recovery of the protein (Fig.1). However, a subsequent experiment reveals this as Keap1 independent. These initial studies also indicate for possible general proteasome inhibition (Fig. 2) and massive up-regulation of PDI, CHOP and GRPs (Fig. 3) that could explain the neurotoxic activity of endosulfan. This study also provides new insights into key molecular signatures that can form markers to test probable toxicological activities of chemicals or derivatives.

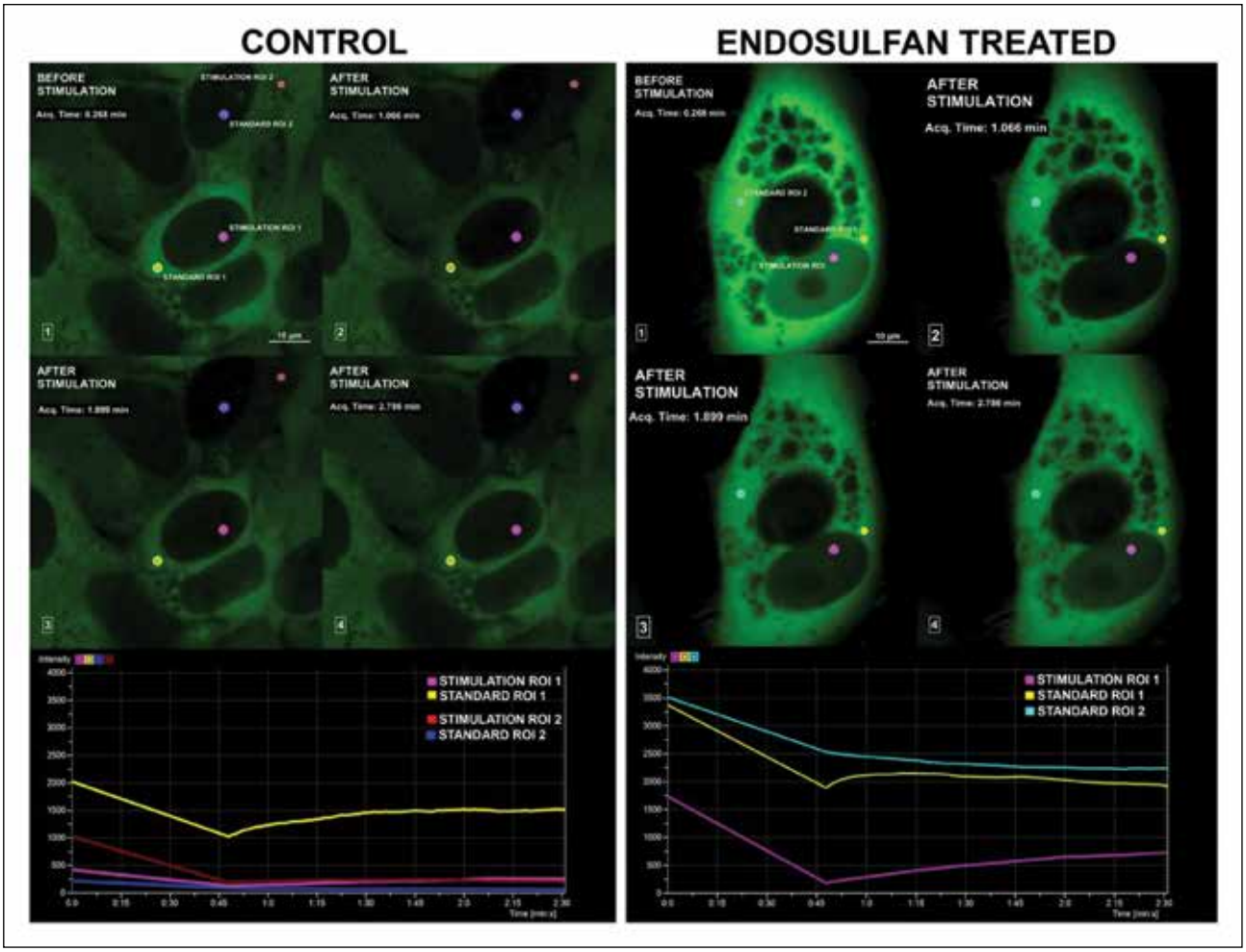


Fig. 1: Fluorescence recovery after photobleaching (FRAP) experiment using Nrf2-GFP expressing cells before and after endosulfan treatment.

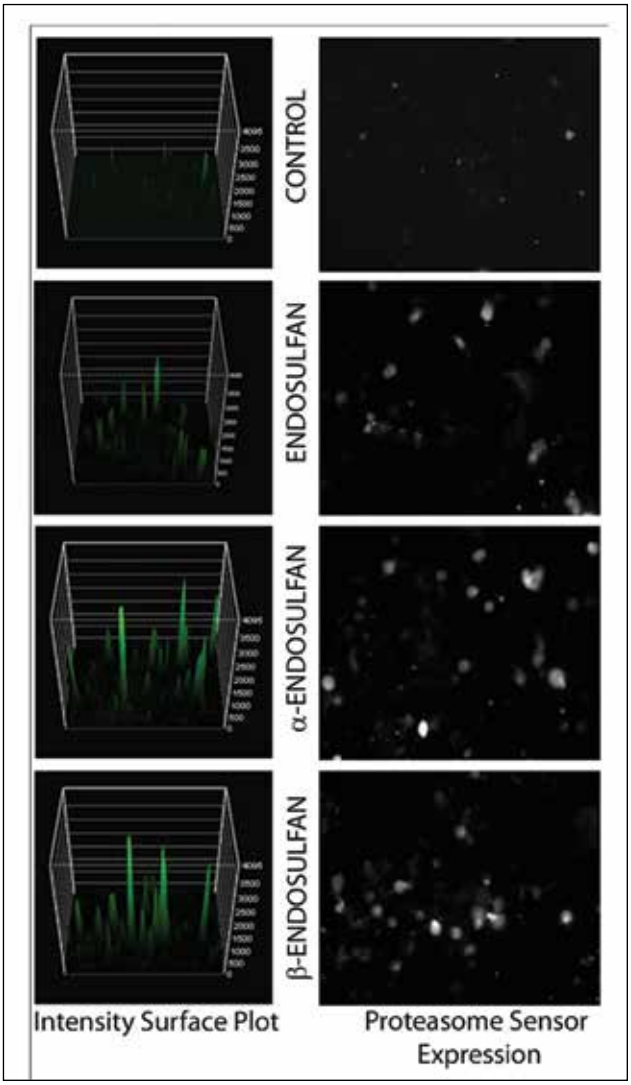


Fig. 2: Intensity surface plots as well as fluorescent images of cells expressing a proteasome sensor probe before and after treatment of endosulfan and its derivatives endosulfan alpha and endosulfan beta

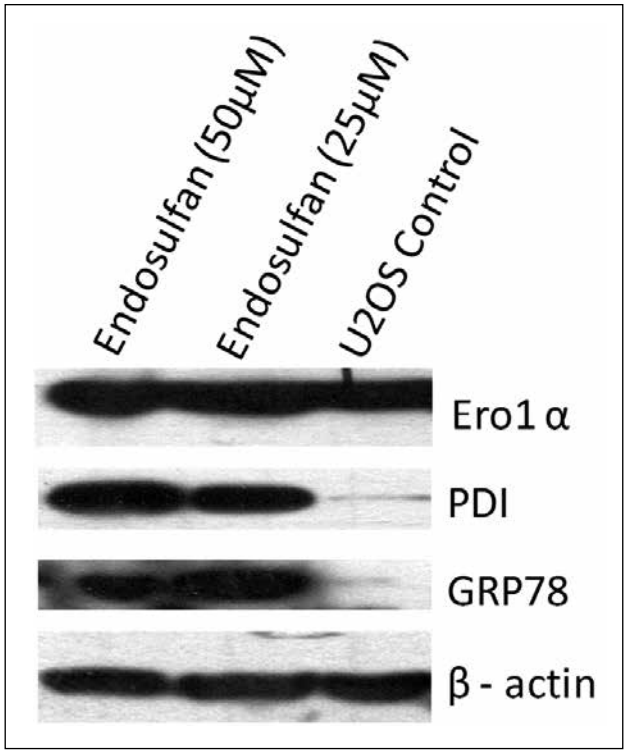


Fig. 3: Western blot of proteins involved in ER stress response after endosulfan treatment.

PUBLICATIONS

- *Mahendra Seervi, Praveen K. Sobhan, Krupa Ann Mathew, Jeena Joseph, Prakash Rajappan Pillai, and T.R. Santhoshkumar.* A high-throughput image based screen for the identification of bax/bak independent caspase activators against drug-resistant cancer cells. *Apoptosis 2014 Jan; 19(1):269-84.*
- *T. R. Santhoshkumar, M. Radhakrishna Pillai.* Tumor stem cell enrichment by anticancer drugs: A potential mechanism of tumor recurrence. In: Perumana S, Oommen V and Pillai MR (eds.), *Perspective in Cancer Prevention – Translational Cancer Research, 9-12, Springer, India, 2014.* doi:10.1007/978-81-322-1533.

PUBLICATIONS WITH COLLABORATORS

- *Suma HK, Kumar V, Senthilkumar U, Kumara PM, Ravikanth G, Santhoshkumar TR, Shanker RU.* Pyrenacantha volubilis Wight, (Icacinaceae) a rich source of camptothecine and its derivatives, from the Coromandel Coast forests of India. *Fitoterapia. 2014 Sep; 97:105-10.*
- *Dhanya R, Arun KB, Syama HP, Nisha P, Sundaresan A, Santhosh Kumar TR, Jayamurthy P.* Rutin and quercetin enhance glucose uptake in L6 myotubes under oxidative stress induced by tertiary butyl hydrogen peroxide. *Food Chem. 2014 Sep 1; 158:546-54.*

EDITORIAL:

- *T.R.Santhosh kumar* (2013) Cancer Research in India : Miles to go: *Health Sciences Vol .2 No.4.*

CANCER RESEARCH PROGRAM

LABORATORY - 2

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HER2- Akt signaling axis regulates the synergistic effect of resveratrol and docetaxel in breast cancer cells.

Haritha H Nair, BS Vinod, Vinod V, Shabna.A and Ruby John Anto

Docetaxel is the most commonly used anti-mitotic drug for breast cancer treatment worldwide, among the taxanes. However, intrinsic or acquired resistance together with adverse side effects are major impediments in the clinical usage of this drug. Previous *in vitro* studies in our laboratory have shown that resveratrol, a plant derived polyphenolic compound enhances docetaxel-induced cytotoxicity in a panel of breast cancer cell lines with maximum effect in SKBR3 and minimum in MDA-MB-231 cells. The divergence in the cytotoxic effect induced by the combination motivated us to do an in depth evaluation of the major characteristics of these cell lines, where we noticed a crucial difference in the expression status of HER2 receptor among these cells. As there were several reports stating the involvement of HER2 in offering resistance against taxane treatment, we investigated the role of HER2 in regulating the synergistic effect of resveratrol and docetaxel together with its connection to the well known Akt and MAPKs pathways which are reported to play major role in docetaxel-induced chemoresistance. Our results indicate that resveratrol sensitizes breast cancer cell lines to docetaxel through HER2 dependent down-regulation of Akt. The results obtained have shown that HER2 is upstream to Akt and regulates the activation of Akt in docetaxel induced signaling pathway.

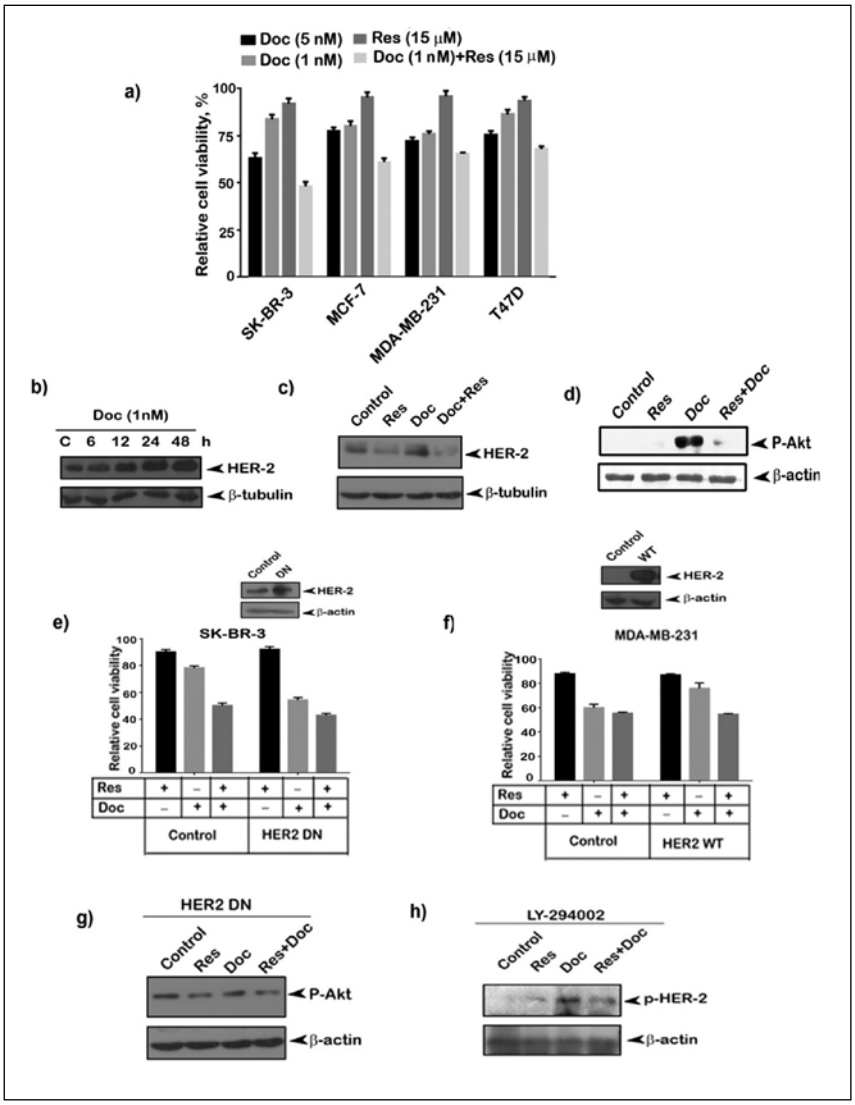

Figure 1: The synergism of docetaxel and resveratrol is regulated by HER2-Akt signaling axis. a) Synergistic cytotoxic effect of combination of docetaxel and resveratrol in panel of breast cancer cells with maximum effect in SKBR3 and minimum in MDA-MB-231. Fig.b & c) Docetaxel induced time dependent activation of HER-2 and the down-regulation of same by resveratrol in SKBR3. Fig. d) Resveratrol down-regulates docetaxel induced up-regulation of Akt. Fig.e & f) HER2 knockdown in SKBR3 abrogates synergism while the ectopic expression of HER2 in MDA-MB-231 restores the same. Fig. g & h) HER2 knock down inhibits the docetaxel induced up-regulation of Akt, while Akt inhibition has no effect on docetaxel induced activation of HER2 in SKBR3.

Chitosan encapsulation enhances the chemopreventive efficacy of curcumin against lung carcinogenesis

Vinod V., Arun Kumar T.T., Vinod Kumar G.S.*, Mohan Shankar G., Shabna A. and Ruby John Anto

**Chemical Biology Group, Rajiv Gandhi Centre for Biotechnology*

Curcumin is considered as an attractive chemopreventive agent. However, the efficacy of curcumin is limited in biological contexts due to its reduced bioavailability. This study demonstrates that the chemopreventive efficacy of curcumin can be enhanced by encapsulating it with chitosan nanoparticles. Chitosan-encapsulated curcumin prevent Benzo[a]pyrene-induced lung carcinogenesis more effectively than normal curcumin in Swiss albino mice. Chitosan-encapsulated curcumin curbs the development of tumor nodules induced by B[a]P in the lungs of the mice, more effectively than free curcumin, which was verified through H & E staining. The molecular mechanisms involved in the Benzo[a] pyrene-induced tumorogenisis, such as MAPK activation and nuclear translocation of NF-κB was blocked more efficiently by chitosan-encapsulated nanocurcumin. Similarly, expression of PCNA, one of the hall marks of a fast dividing tumor cells, in the lungs tissues of Benzo[a] pyrene-treated mice is reduced more effectively by chitosan-encapsulated curcumin compared to that of free curcumin.

Immunohistochemical staining of PCNA, p65 and phospho-Erk was performed in the lung tissues of different treatment groups. A strong positivity of all the survival signals were observed in B[a]P-treated mice while a significant inhibition of all these molecules was observed on curcumin treatment, with a maximum efficacy in chitosan nanocurcumin treated group.

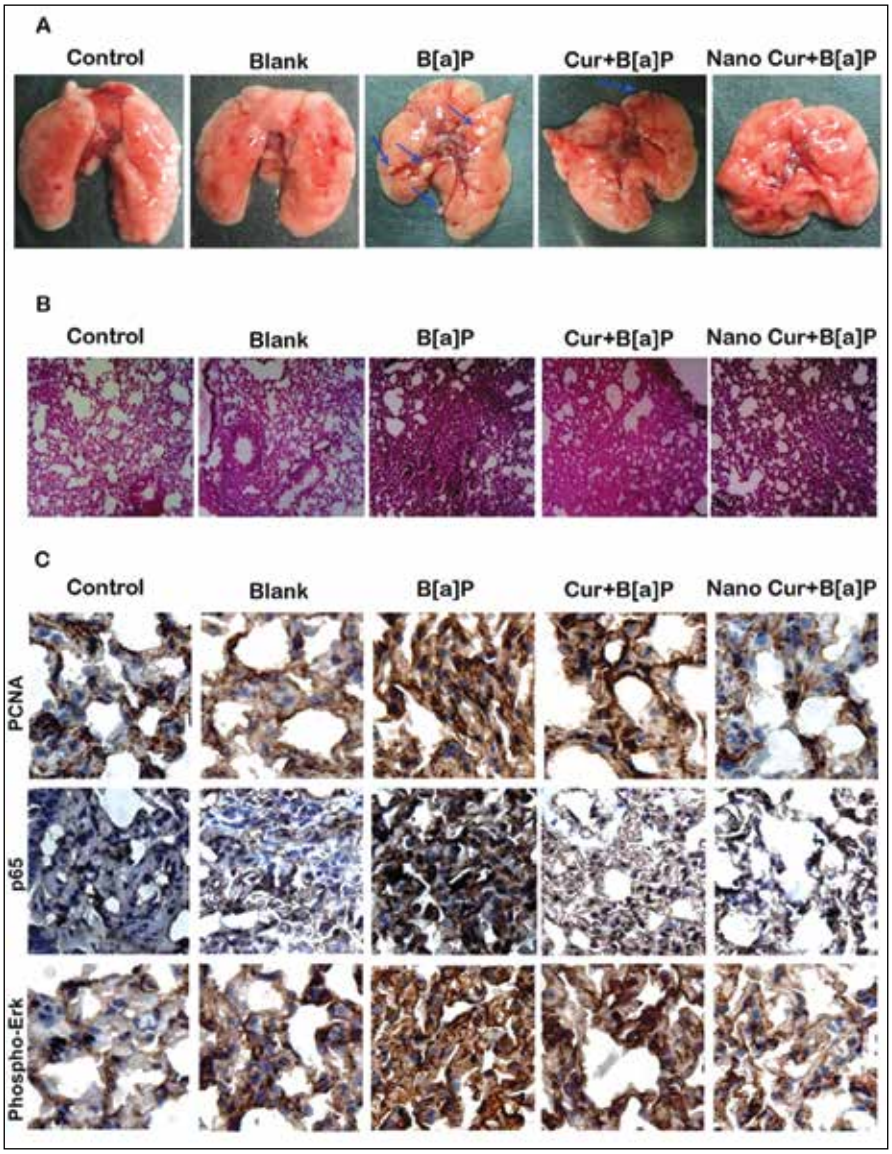

Figure2: Chitosan curcumin nanoparticles are more potent in inhibiting B[a]P-induced lung carcinogenesis in Swiss albino mice, compared to free curcumin

DW-F5: A novel formulation against malignant melanoma from Wrightia tinctoria inhibits development of melanoma tumour in NOD-SCID mice model

Jayesh Antony, Minakshi Saikia, Vinod.V, Lekshmi R Nath, Shabna A, Mohana Rao Katiki*, M.S.R. Murty*, Sophia Margaret Joseph and Ruby John Anto

**Medicinal Chemistry and Pharmacology Division, Indian Institute of Chemical Technology, Hyderabad-500007, India.*

Wrightia tinctoria is a constituent of several ayurvedic preparations against skin disorders, though not yet has been explored for anticancer potential. We found, for the first time, that a semi-purified fraction, DW-F5, from the dichloromethane extract of W. tinctoria leaves against malignant melanoma using an orthotopic xenograft model of melanoma to validate the anticancer efficacy of DW-F5 in vivo (Fig. 3a, b). Two drug treatment strategies were adopted viz. day 15 experiment, where DW-F5 administration was started after 15 days of tumour cell implantation and day 1 experiment, where DW-F5 was administered starting from the next day of tumour cell implantation. Surprisingly, compared to untreated (Group 4A) animals, no measurable tumours were developed till the end of the experiment in group 5 and 6 animals [Group 5 & 6A], which started receiving DW-F5, the very next day after tumor implantation. Hence a set of representative animals from each group were kept [Group 5B and 6B] for two more weeks, without further DW-F5 treatment, along with one set of untreated animals [Group 4B]. Interestingly, tumours developed in these animals, though very small compared to that of untreated controls (Fig. 3a). Group 2 and 3 animals that started receiving DW-F5, 15 days after tumour implantation, developed measurable tumors, the growth of which were also significantly inhibited by DW-F5 (Fig. 3b). Purification of DW-F5 led to the isolation of two cytotoxic components, one being tryptanthrin and the other being an unidentified aliphatic fraction. The overall study predicts Wrightia tinctoria as a candidate plant to be further explored for anticancer properties and DW-F5 as a potential anti cancer compound against malignant melanoma.

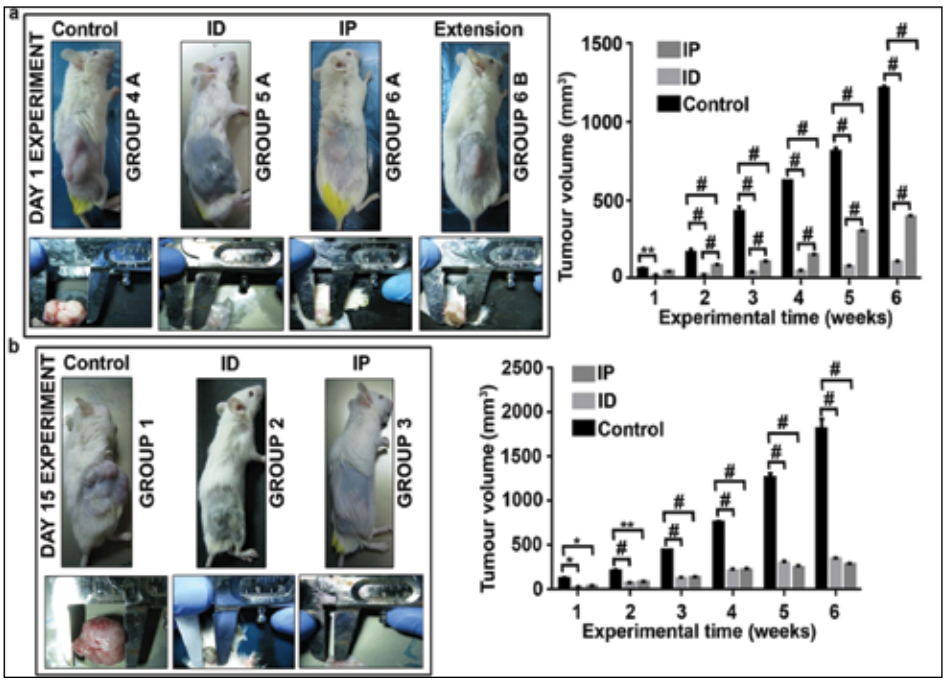

Figure 3. *DW-F5 inhibits melanoma tumourigenesis in NOD-SCID mice.*

*(a) Representative images of tumour bearing mice which received DW-F5 treatment next day after tumour implantation and the graphical representation of the tumour volume. (Day 1 group) (b) Representative images of mice bearing tumour which received DW-F5 treatment 15 days after tumour implantation (Day-15 group) and the graphical representation of the tumour volume. Data represent three independent sets of experiments. The error bars represent ± S.D. Statistical significance was analyzed using ANOVA. * P≤ 0.05; ** P≤ 0.01; # P≤ 0.001.*

Kaempferide isolated fromChromolaena odorata exhibits anticancer properties tocervical cancer cells

Lekshmi.R.Nath, Jaggaiah N. Gorantla*, Ravi S. Lankalapalli*, Arunkumar TT, Jayesh Antony, Vinod V and Ruby John Anto

**National Institute for Interdisciplinary Science & Technology (NIIST), Council of Scientific and Industrial Research (CSIR), Thiruvananthapuram-695019, Kerala, India*

Chromolaena odorata, formerly known as Eupatorium odoratum, is a species of flowering plant belongs to the family Asteraceae. Traditionally the decoction of leaf is used to treat wounds, uterus- related problems and early stages of cancer. The present study was intended to evaluate the anticancer activity of its active principle isolated from the DCM extract of leaves. The organic extracts were screened in five cancer cell lines of various origins for their cytotoxic effect, among which the DCM extract was found to be the most cytotoxic

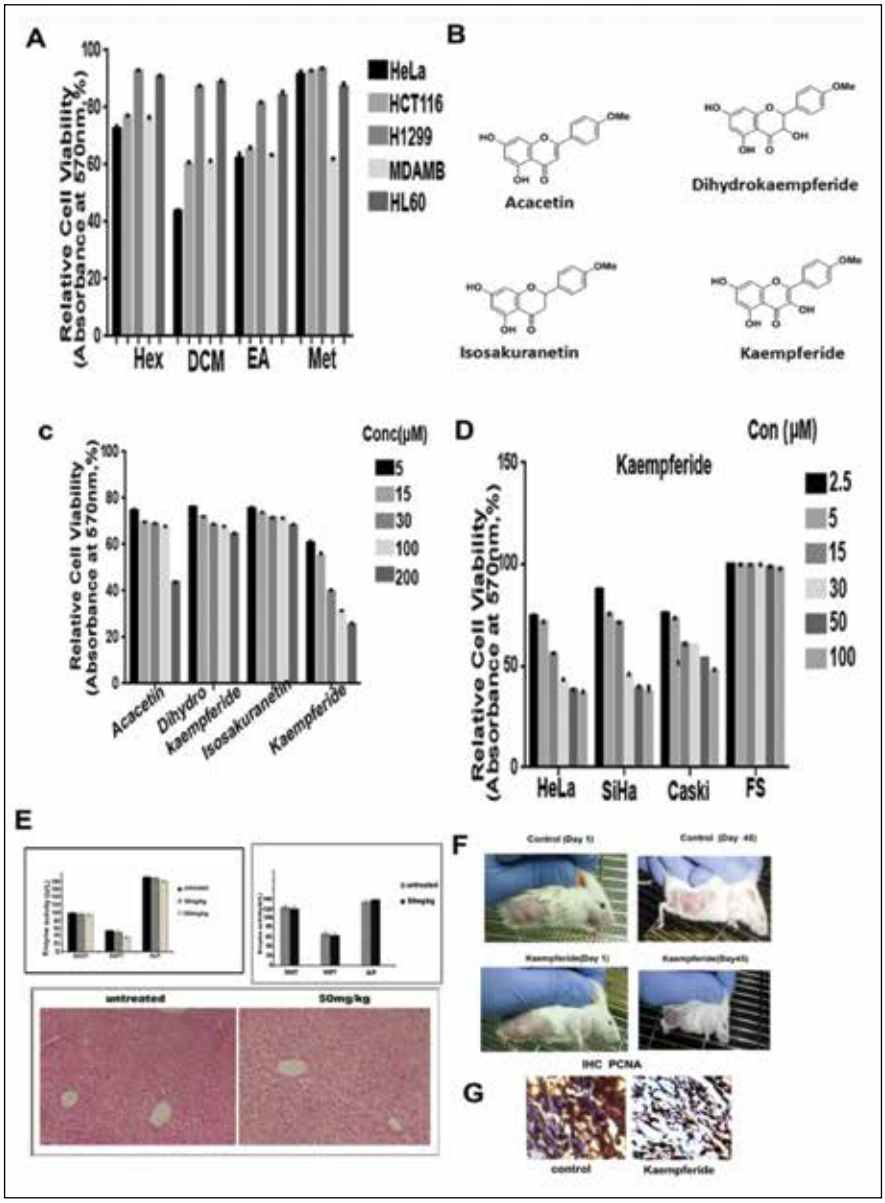

***Figure** 4: Kaempferide isolated from Chromolaena odorata inhibits tumour growth in cervical cancer cells, while being pharmacologically safe as assessed by acute and chronic toxicity studies The most active DCM extract (Fig4A) was purified by silica gel column chromatography to obtain four major compounds, whose chemical structures were characterized by 1H-NMR, 13C-NMR, UV and HR-MS spectroscopic methods as Acacetin, Dihydrokaempferide, Isosakuranetin and Kaempferide (Fig4B). Among the four compounds, kaempferide was found to be most sensitive to cervical cancer cells (IC50:16μM) followed by acacetin, dihydrokaempferide and Isosakuranetin and was selected for further studies(Fig4C). The cytotoxicity of Kaempferide was checked in different cervical cancer cell lines and normal fibroblasts (Fig4D). Kaempferidewas found to be pharmacologically safe as assessed by chronic and acute toxicity models (Fig4E). In vivo validation in HeLa Xenograft model proved the antitumor efficacy of kaempferide (Fig4F&G).*

PUBLICATIONS

Peer Reviewed Journals

- *Haritha H Nair, *Ruby John Anto.* Triple negative breast cancer: The therapeutic windows yet to be opened? *Science Jet, 4:175, 2015*
- *Jayesh Antony, Minakshi Saikia, Vinod.V, Lekshmi. R. Nath, Mohana Rao Katiki, M.S.R. Murty, Anju Paul, Shabna A, Harsha Chandran, Sophia Margaret Joseph, Nishanth Kumar. S, Elizabeth Jayex Panakkal, Sriramya I.V, Sridivya I.V, Sophia Ran,Sankar S, Easwary Rajanand *Ruby John Anto.* DW-F5: A novel formulation against malignant melanoma from Wrightia tinctoria,Scientific Reports.*Published online5:11107|DOI:10.1038/srep11107, 2015.*
- *Jisha J. Pillai, Arun Kumar T. Thulasidasan, Ruby John Anto, Nandan C. Devika, N. Ashwanikumar and G. S. Vinod Kumar.* Curcumin entrapped folic acid conjugated PLGA–PEG nanoparticles exhibit enhanced anticancer activity by site specific delivery, *RSC Adv.,5, 25518-25524. DOI: 10.1039/C5RA00018A. 2015.*
- *Radhakrishnan E K, Smitha V Bava, Sai Shyam Narayanan, Lekshmi R Nath, Arun Kumar Thulasidasan, Soniya E V and *Ruby John Anto.* [6]-Gingerol induces caspase-dependent apoptosis and prevents PMA-induced proliferation in colon cancer cells by inhibiting MAPK/AP-1 signaling, *PLOS ONE, 9(8): e104401. doi:10.1371, 2014.*
- *Jisha Jayadevan Pillai, Arun Kumar Theralikattu Thulasidasan, Ruby John Anto, Devika Nandan Chithralekha, Ashwanikumar Narayanan, and Gopalakrishnapillai Sankaramangalam Vinod Kumar.* Folic acid conjugated cross-linked acrylic polymer (FA-CLAP) hydrogel for site specific delivery of hydrophobic drugs to cancer cells,*J Nanobiotech..12: 25; 2014.*
- *Sasidharan Nishanth Kumar, Sreerag Ravikumar Sreekala, Dileep Chandrasekaran, Bala Nambisan and Ruby John Anto.* Biocontrol of Aspergillus Species on peanut kernels by antifungal diketopiperazine producing Bacillus cereusassociated with entomopathogenic nematode. *PLOS ONE, 9(8): e106041. doi:10.1371, 2014.*
- *M. Sreelekha, NP Anto, Ruby John Anto and PM Shafi.* Cytotoxicity of acetonyl dihydro chelerythrin,arnottianamide and 6-(2.hydroxy propyl)-dihydro chelerythrine towards human cancer cell lines, *Ind. J. Chem. Sec.B, 647-651, 2014.*
- *Jaggaiah N. Gorantla, Jamsheena Vellekkatt, Lekshmi R.Nath, Ruby John Anto and Ravi S. Lankalapalli.* Cytotoxicity studies of semi-synthetic derivatives of theveside derived from the aqueous extract of leaves of'suicide tree' Cerbera odollam, *Natural Product Res, DOI: 10.1080/14786419.2014.913242, 2014.*
- *Kumar SN, Nambisan Bala, Sundaresan A, Mohandas C and Ruby John Anto,* Isolation and identification of antimicrobial secondary metabolites from Bacillus cereus associated with a rhabditid entomopathogenic nematode. *Ann Microbiol.,64, 209–218, 2014.*
- *M. S. R. Murty, Raju Penthala, Lekshmi R. Nath and Ruby John Anto,* Synthesis of Salicylic Acid-Based 1, 3, 4-Oxadiazole Derivatives Coupled with Chiral Oxazolidinones: Novel Hybrid Heterocycles as Antitumor Agents *Letters in Drug Design & Discovery, 11, 1-10, 10.2174 /1570180811666140627004607, 2014.*

Chapters in Text Books

- *# Jayesh Antony, # Minakshi Saikia and *Ruby John Anto.* Phytochemicals from Fruits and Vegetables as potential anti-canceragents: special reference to skin cancer. In: Anticancer properties of fruits and vegetables: A scientific review. Ajay Kunnummakkara (Ed). Pubd: *World ScientificPublishing Co.; 2014 edition, pp 277-307 (# equal authorship)*
- *CN Sreekanth, Smitha VB, Arun Kumar TT, N P Anto, VT Cheriyan, Vineshkumar TP, SG Menon, SD Ravichandran and *Ruby John Anto* - Curcumin: A Potent Candidate to be Evaluated as a Chemosensitizer in Paclitaxel Chemotherapy Against Cervical Cancer. In: *Perspectives in Cancer Prevention-Translational Cancer Research. P Sudhakaran P, Oommen V, Pillai, MR (Eds.). Pubd: Springer; 2014 edition.pp 21-43.*

Non-Peer Reviewed Journals

- *Mohan Shankar G and *Ruby John Anto.* Role of Hedgehog signaling in basal cell carcinoma. *Amala Research Bulletin (2014) 34, 115-121*

CONFERENCE PRESENTATIONS

- *Arun Kumar T Thulasidasan, GS Vinod Kumar, K Lekha Nair, G Deepa andRuby John Anto.* In vitro and in vivo validation of nanoparticle-based drug delivery systems to improve the chemosensitizing efficacy of curcumin in paclitaxel chemotherapy. *6th International Conference on Drug Discovery & Therapy, February 10-12, 2014, Dubai (Sessions Talk).*
- Identification andcharacterization of Tryptanthrin, the active principle from Wrightia tinctoriaandthe validationofitsanti cancer efficacy invitroandinvivo. *Jayesh Antony, Minakshi Saikia and Ruby John Anto, Oral presentation, 26th Kerala Science Congress, 28-31 January 2014, Wayanad, Kerala*
- *Jayesh Antony, MinakshiSaiki, Sophia Margeret Joseph, Vinod.V, Lekshmi.R. Nath, Mohana Rao Katiki, M.S.R. Murty and Ruby John Anto.* In vitro and in vivo validation of anticancer efficacy of tryptanthrin, isolated from

Wrightia tinctoria [Roxb.] R.Br. Poster, *33rd Annual convention of Indian Association for Cancer Research, February 13-15, 2014, Kollam, Kerala.*

- *Arun Kumar T Thulasidasan, GS Vinod Kumar, K Lekha Nair, G Deepa and Ruby John Anto.* Identification of better modes nanoparticle-based drug releasing systems for improving the efficacy of cervical cancer chemotherapy. Poster, *33rd Annual convention of Indian Association for Cancer Research, February 13-15, 2014 Kollam, Kerala.*
- *Lekshmi.R.Nath, Vinod V, Arun Kumar T Thulasidasan, Jaggaiah N. Gorantla, Ravi S. Lankalapalli and Ruby John Anto* Mechanistic evaluation of the anticancer effect of a spirostan-3-ol derivative isolated from Solanum nigrum Linn in liver cancer, Poster, *33rd Annual convention of Indian Association for Cancer Research, February 13-15, 2014 Kollam, Kerala.*
- *Haritha H Nair, Balachandran S Vinod, Jayesh Antony, Minakshi Saikia and Ruby John Anto.* Thymidylate synthase-dependent NF-κB down-regulation plays pivotal role in the efficacy of Curcumin in chemosensitizing breast cancer cells to 5-FU, Poster, *33rd Annual convention of Indian Association of Cancer Research, 13th -15th February 2014, Kollam, Kerala*
- *Arun Kumar T Thulasidasan, Devika Nandan.C, Lekha Nair K, Jisha J Pillai, GS Vinod Kumar, and Ruby John Anto.* Nano Encapsulation of curcumin using PLGA improves its therapeutic potential, *Oral presentation National confrenence organized by dept. of Biochemistry, Bharathidasan University, 6th february 2015 Thiruchirapalli, Tamil Nadu.*
- *Minakshi Saikia, Jayesh Antony, Vinod V, Sophia Margaret Joseph, Lekshmi R Nath and Ruby John Anto.* Evaluation of the anticancer property of Wrightia tinctoria (Roxb.) R.Br. leaves against malignant melanoma. Poster, *7th HOPE Meeting with Nobel laureates conducted by Japan Society for Promotion of Science (JSPS), 1-5 March, 2015, Tokyo, Japan*.

PHD AWARD

- PhD awarded: 2014: Vinod BS; Identification of effective and non-toxic chemosensitizers, which can be used in combination with the conventional chemotherapeutic drugs used for breast cancer treatment

AWARDS & HONORS

- Ruby John Anto. Promotion with Excellence Award for outstanding performance, RGCB Promotion Assessment Committee, 2014
- Haritha H Nair, Balachandran S Vinod, Jayesh Antony, Minakshi Saikia and Ruby John Anto. Thymidylate synthase-dependent NF-κB down-regulation plays pivotal role in the efficacy of Curcumin in chemosensitizing breast cancer cells to 5-FU Best poster award at 33rd Annual convention of Indian Association of Cancer Research, 13th -15th February 2014. Kollam, Kerala.
- Arun Kumar T Thulasidasan, Devika Nandan C, Lekha Nair K, J Jisha Pillai, G.S.Vinod Kumar and Ruby John Anto. Best oral presentation award at National Conference on Challenges and Future Prospects of Applied Research in Life Sciences-2015, Bharathidasan University, Tiruchirappalli.
- Minakshi Saikiaawarded JSPS HOPE fellowship to participate and present a poster on "Evaluation of the anticancer property of Wrightia tinctoria (Roxb.) leaves against malignant melanoma" in the 7th HOPE meeting with Nobel laureates, conducted by the Japan Society for Promotion of Science (JSPS) held at Tokyo (March 1-5, 2015).

LIST OF ONGOING EXTRA MURAL GRANTS

Sl. No	Title of Project	Funding Agency	Duration
1	Isolation and identification of anticancer principle from the mistletoe growing on Chrysophyllum spp	Council for Scientific & Industrial Research, Government of India	2012-2015
2	Comparison of the chemopreventive efficacy of free curcumin and biodegradable polymer based nano curcumin in Benzo[a] pyrene–induced lung carcinogenesis	Department of Science & Technology, Government of India	2013-2016
3	In vivo evaluation of the anticarcinogenic effect and toxicity of the active principle of Wrightia tinctoria	Indian Council of Medical Research, Government of India	2015-2018

CANCER RESEARCH PROGRAM

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Reshma Thamkachy
Rohith Kumar N
J.S.Sreeja.

Mechanism of Resistance of Cancer Cells Against Antimitotic Agents

Smreti Vasudevan and Suparna Sengupta

Acquired drug resistance poses a challenge in cancer therapy. Drug efflux is the most common mechanism of resistance displayed by hydrophobic drugs beyond a certain size, that often gives rise to multidrug resistance. However, target specific changes and imbalance between the pro and anti-apoptotic proteins are also found quite often in many tumors. A number of small antimitotic agents show high potential for multidrug resistant tumors, mainly because they are able to evade the efflux pumps. However, just like any other anticancer agent, these compounds also suffer from resistance upon prolonged treatment. Thus it is important to find out agents that are sensitive to resistant tumors and to know the resistance mechanisms evoked in cancer cells against small molecules so that proper combinations can be planned. We have been trying to understand the basis of the differing anticancer drug resistance mechanisms against large and small tubulin binding agents using taxol, which is a well-known clinically used antimitotic agent of large size and a novel class of antimitotic, potential anticancer compounds of small size – diaminothiazoles.

Model cell lines were generated in the colon carcinoma cell line HCT116 and breast adenocarcinoma cell line MCF7, against the gauge compounds - taxol and a lead diaminothiazole DAT1 [4-amino-5-benzoyl-2-(4-methoxy phenyl amino) thiazole]. Xenograft tumours were derived from them in SCID/NOD mice. Cytotoxicity studies with a panel of anticancer agents showed that the cells resistant to taxol displayed a multidrug resistance profile. In contrast, the cells resistant to DAT1 showed cross-resistance to its own analogues and to some extent to various colchicine site binders, but in general lacked multidrug resistance profile (Table 1). DAT1 showed high efficacy for tumour growth regression in xenografts of multidrug resistant cancers. Mean tumor growth inhibition after DAT1 treatment was 64% and 52% for the treatment groups of 20 and 10 mg/kg body weight respectively (Figure 1).

The resistance against DAT1 was found to be both transient as well as specific, which was further confirmed in the primary cells derived from xenograft tumour models (Table 1).

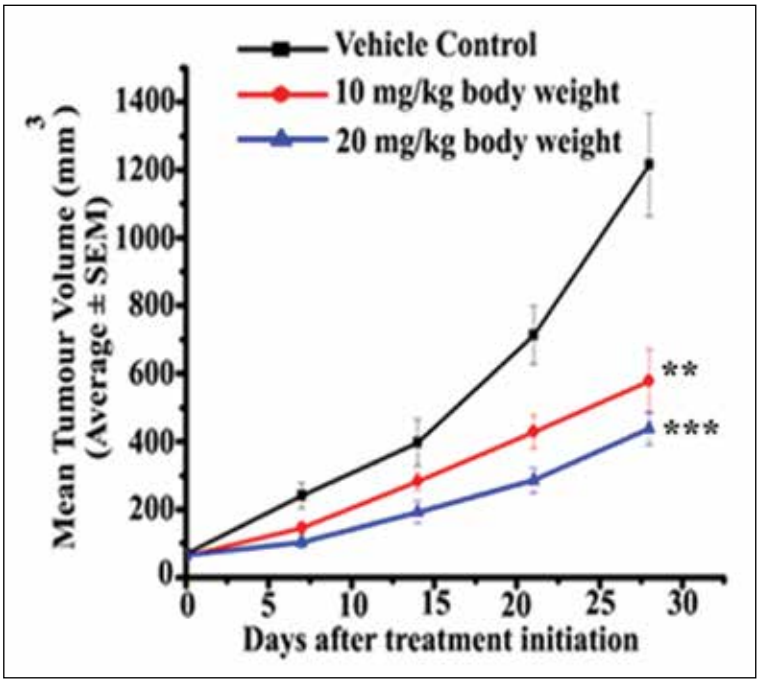
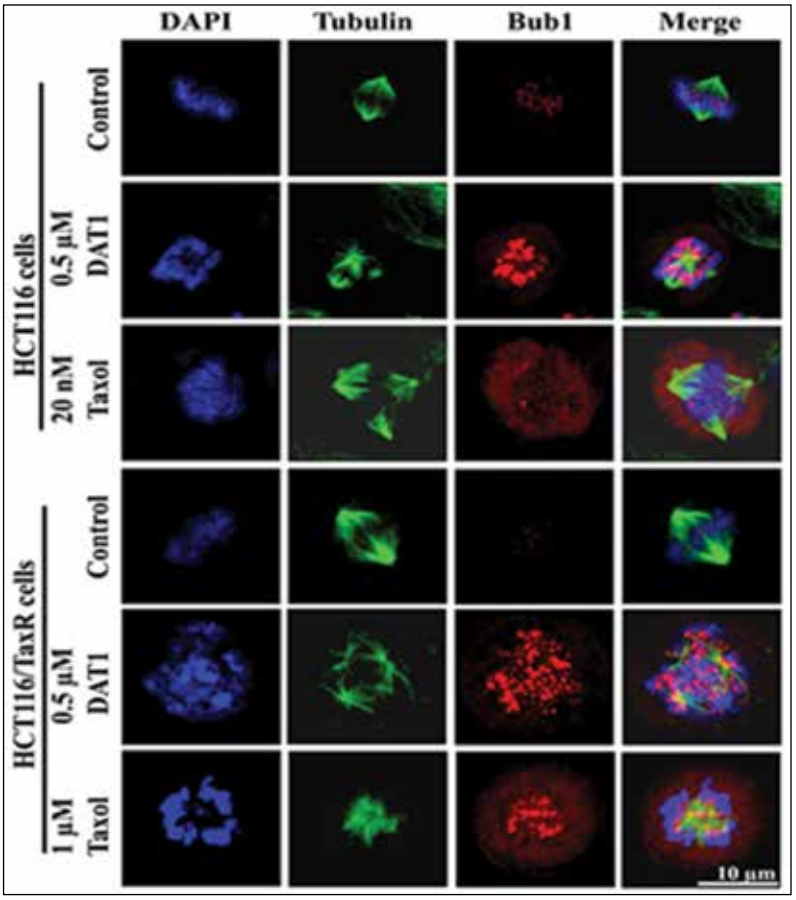


Figure 1. Antitumour activity of DAT1 in multidrug resistant xenograft tumour model

Compound (Molecular weight)	HCT116	HCT116/ TaxR	HCT116/ DAT1R	HCT116/ TaxR cells from tumor	HCT116/ DAT1R cells from tumour
	IC_{50} *(µM)*	IC_{50} *(µM)* (R.I.)	IC_{50} *(µM)* (R.I.)	IC_{50} *(µM) (R.I.)*	IC_{50} *(µM) (R.I.)*
DAT1 (325.42)	0.3±0.09	0 . 3 4 ± 0 . 1 2 (1.13)	12.12±1.41 (40.4)	0.37±0.02 (1.23)	1.69 (5.63)
DAT7 (355)	0.32±0.11	-	6.42±0.15 (20.06)		
DAT22 (385.48)	0.059±0.03	0.051 ± 0.002 (0.86)	0.128 ±0.004 (2.17)		
DAT34 (415.51)	0.013±0.004	0.013 ± 0.002 (1)	0.055 ± 0.013 (4.23)		
Taxol (853.91)	0.012±0.005	1.12 ± 0.23 (93.33)	0.014 ±0.004 (1.16)	1.05±0.27 (87.5)	0.008 (0.66)
Colchicine (399.4)	0.03±0.01	0.101 ± 0.01 (3.36)	0.07 ± 0.01 (2.34)	0.27±0.08 (9)	0.033 (1.1)
Vinblastine (909)	0.005±0.003	0.16 ± 0.05 (32)	0.007 ± 0.0004 (1.4)	0.098 ±0.04 (19.6)	0.003 (0.6)
5-Fluorouracil (130)	8.24±1.73	8.19 ± 0.27 (0.99)	6.64 ± 1.43 (0.81)		
Doxorubicin (579.98)	0.42±0.04	3.16 ± 0.05 (7.52)	0.094 ±0.004 (0.21)		

Table 1. IC50 values of diaminothiazole analogues and some anticancer agents in wild type, resistant sublines and in primary cultures of resistant tumours

Further studies in these model cell lines and the xenograft tumours revealed that P-glycoprotein overexpression was the chief resistance mechanism evoked in cancer cells against large antimitotic agents including taxol, with contributions from κtubulin isotypes, spindle checkpoint and apoptotic proteins in the variant lines. In contrast, the resistance mechanism aroused in cancer cells against diaminothiazoles was irrespective of P-glycoprotein activity and was target specific. Moreover, in the taxol resistant cells, diaminothiazoles could effectively induce mitotic arrest at similar concentrations as in sensitive cells, leading to perturbation in checkpoint proteins like MAD2 and Bub1 eventually triggering resistant cells to undergo apoptosis (Figures 2). Taxol, under similar concentrations, needed 100 times more concentration to induce the checkpoint proteins. DAT1 resistant cells did not have considerable effect on checkpoint proteins.



These studies implicate that the resistance against microtubule binding compounds with higher size like taxol, is broad-spectrum and multifactorial. On the contrary, resistance against small molecules like diaminothiazoles is transient and target specific. Diaminothiazoles show promising antitumor activity in multidrug resistant cancers.

Figure 2. Effect of DAT1/Taxol on Bub1 in HCT116 and HCT116/TaxR cells

Role of p53 on Diaminothiazole induced cell death in colon cancer

Reshma Thamkachy and Suparna Sengupta

Collaborator: K.N.Rajasekharan, University of Kerala

p53 is a tumor suppressor protein found mutated in more than 50% of human cancers. It plays an important role in many steps of apoptosis and cell cycle arrest during DNA damage which explains the reason for failure of many chemotherapeutic drugs in p53 mutated cancers. Antitubulin agents form a major class of drugs used for cancer chemotherapy. Diaminothiazoles are a class of antitubulin agents which are under study in our laboratory due to their potent antimitotic and antiangiogenic properties. They bind to the colchicine binding site of tubulin reversibly. Previous studies from our laboratory show that the lead diaminothiazole DAT1 activates an independent extrinsic pathway and death receptor 5 plays a significant role in this process. The prominent role of p53 in the intrinsic and extrinsic pathway of apoptosis encouraged us to study the role of p53 in DAT1 induced apoptosis. We found that DAT1 causes cell death in colon cancer cell lines irrespective of their p53 status by activating extrinsic pathway of apoptosis. Further, toxicity studies of the compound were performed in Swiss Albino mice. Acute toxicity studies revealed that the compound was tolerated by mice at high concentrations. In sub acute toxicity studies, DAT1 was administered intraperitoneally in mice and serum was collected after 3 months. The serum ALP, ALT, AST, Creatinine and BUN levels did not differ much from the control values which indicated that the drug was non-toxic to liver and kidney tissues (Table 2).

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)	BUN (mg/dL)	Creatinine (mg/dL)
Control	79.92	152.98	457.42	51.36	0.78
vehicle control	185.09	512.12	433.04	48.34	0.64
Group I (DAT1 4 mg/kg)	48.47	114.13	361.11	49.425	0.83
Group II (DAT1 8 mg/kg)	69.45	129.57	422.47	45.54	0.79
Group III (DAT1 16 mg/kg)	111.98	209.89	502.73	54.68	0.58

Table 2: Serum parameters showing minimal toxicity in mice after 3 months of DAT1 administration

In vivo studies in SCID/NOD mice indicated that DAT1 caused tumor regression efficiently in both HCT116 and HCT116 p53 -/- xenografts in mice (Figure 3). Apoptosis induction was more in the tumor tissue derived from DAT1 administered mice as compared to the control tumor tissue (Figure 4). Thus p53 independent activity and minimal toxicity place DAT1 in a favorable position for cancer treatment and should be carried forward for clinical studies.

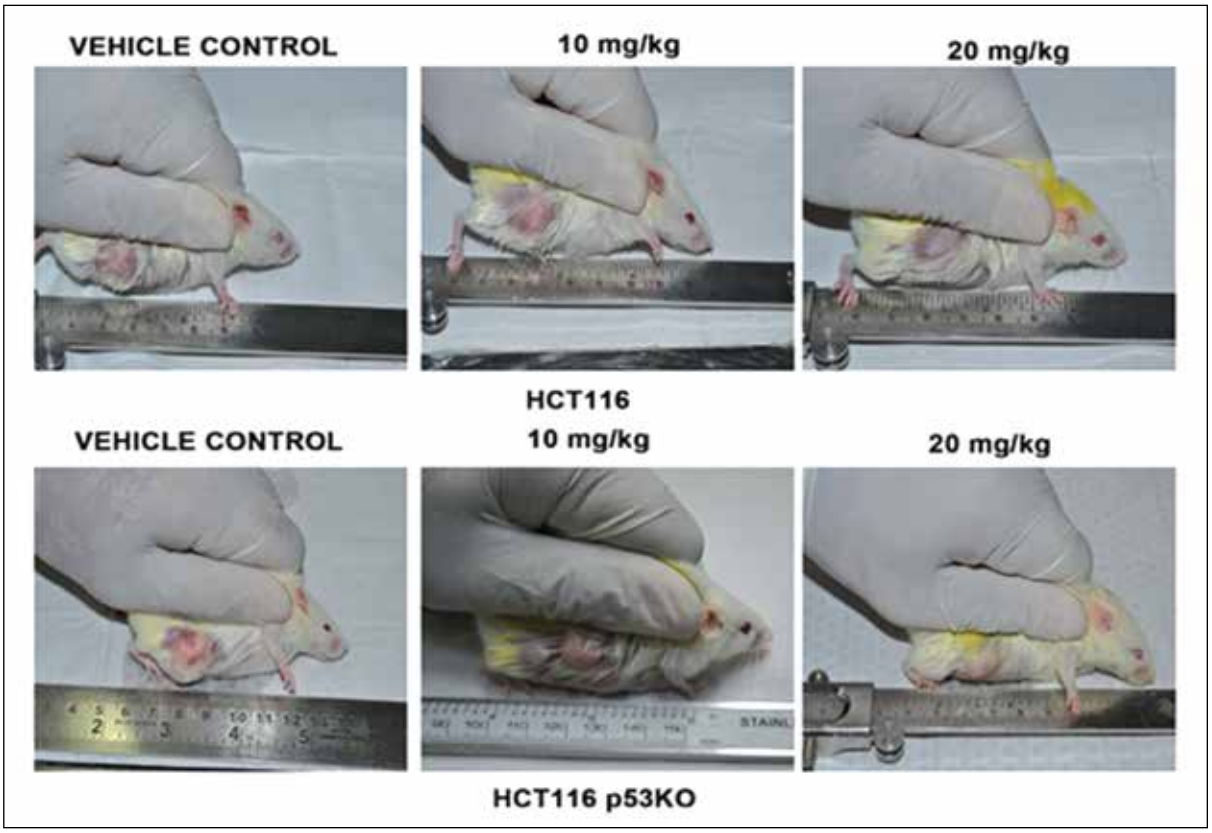


Figure 3: Tumor regression in SCID/NOD mice after 12 doses of DAT1 administration

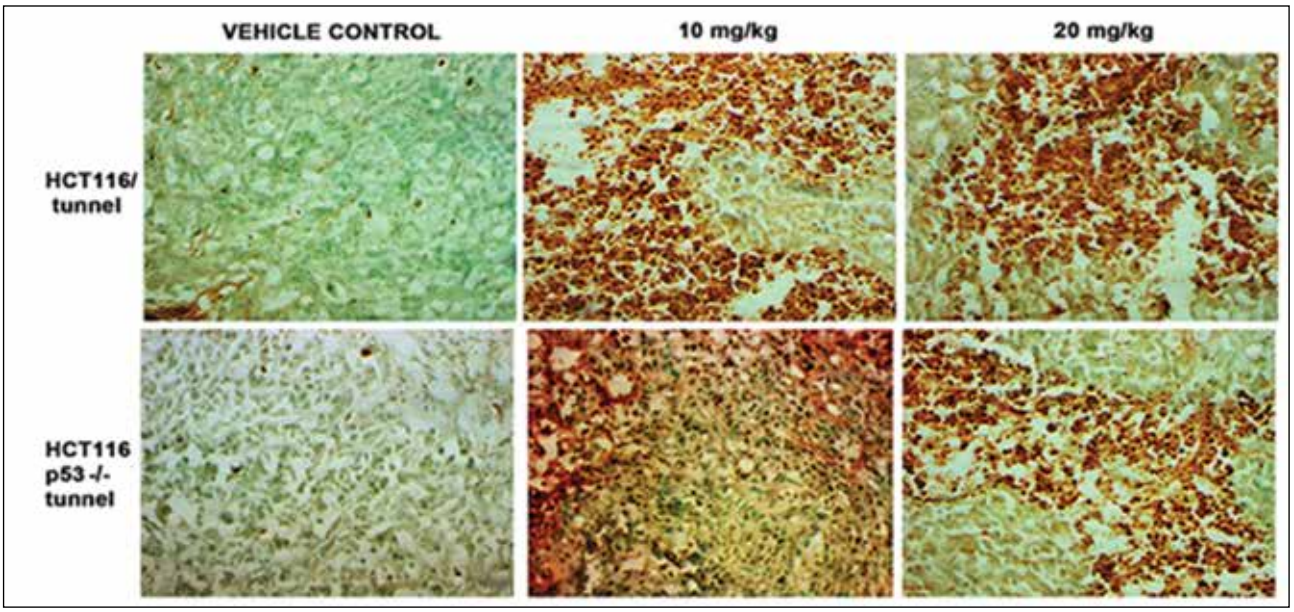


Figure 4: Apoptosis induction by DAT1 in the tumor tissue

Analysis of Fodrin Association with Gamma Tubulin Complex in Mammalian Brain

Microtubules are hollow, tubular cytoskeletal structures that are involved in cell division, cellular transport, maintenance of cell shape and motility. Microtubule nucleation is the process in which several tubulin molecules interact to form a microtubule seed. In eukaryotic organisms, γ tubulin is active as a nucleator of microtubules in association with some other proteins in the form of γ TuRCs (gamma tubulin ring complex). These ring complexes are present throughout the cell but are functionally active only when localized to the centrosome. It is believed that cytoplasmic γ TuRCs are translocated to the centrosome at the onset of mitosis as the amount of gamma tubulin increases several folds in the centrosome during mitosis. During mitosis, the centrosome nucleates microtubules to form the spindle fibres and helps in cell cycle progression. But the mechanism by which the γ- TuRCs are transported remains elusive. Our laboratory has identified fodrin as a part of the κ TuRCs isolated from goat brain. Further fodrin was identified as an associate of gamma tubulin in the centrosomes of IMR32, U373 cell lines which are brain derived cell lines. Colocalization of fodrin with γ tubulin has been observed in primary undifferentiated neuronal cells. We have now found out that fodrin interacts directly with gamma tubulin by far western analysis. I*n silico* analysis suggested a possible domain within alpha fodrin's carboxy terminal region that might be responsible for interaction with gamma tubulin. Since γ TuRCs are active at the centrosome, the amount of γ tubulin localized at the centrosome was determined from immunoflurosence assay after κ tubulin staining. Interestingly, IMR 32 cells which were subjected to alpha fodrin downregulation using shRNA, showed significant decrease in κ tubulin staining at the centrosome when compared to the control shRNA treated cells but the total cellular γ tubulin levels remained same as observed by western blotting. Fodrin downregulated cells also showed considerable disruption of microtubule organization in interphase and gave rise to abnormal mitotic spindle structures. Thus alpha fodrin may play a role in the localization of gamma tubulin at the centrosome and affect its functions.

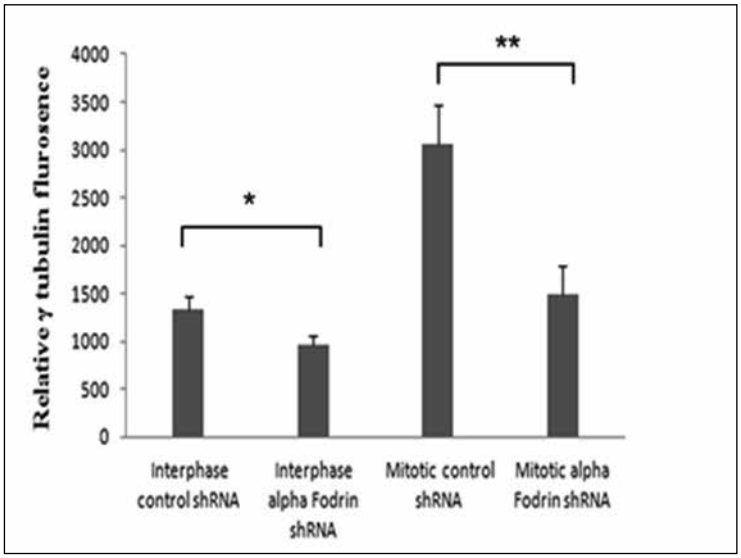


Figure 5: Gamma tubulin levels at centrosome after fodrin downregulation

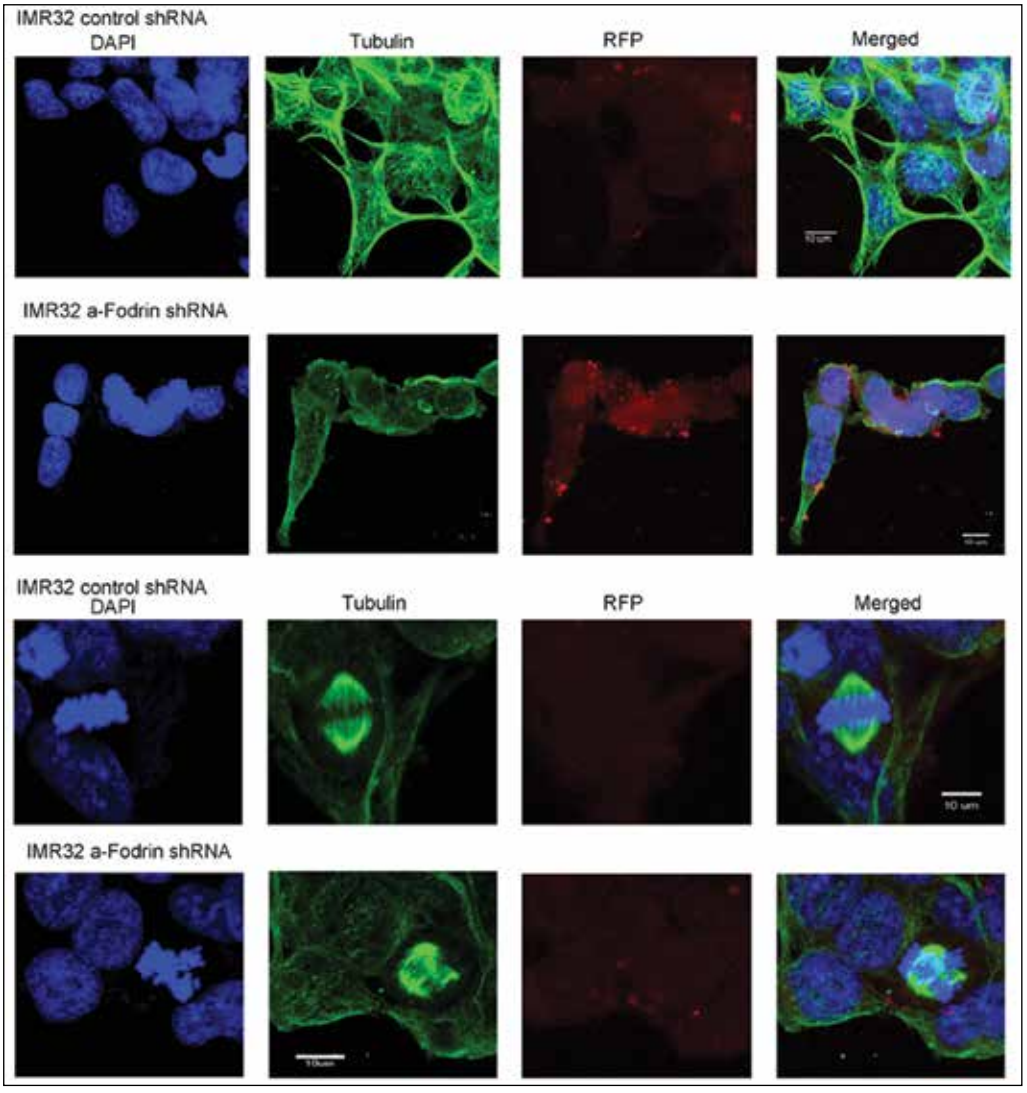


Figure 6: Effect of fodrin downregulation on microtubule organization. Top two panels show interphase cells, bottom two panels show mitotic cells.

Establishing the role of fodrin in gamma tubulin mediated functions

Microtubules are formed in two steps in vitro; the initial slow phase or nucleation phase where the tubulin heterodimers interact to form large oligomers and then the faster phase where the oligomers act as seeds on which further growth of microtubules happens. In cells, for faster nucleation of microtubules, a template is necessary. γ-tubulin performs a critical function of nucleating microtubules from the centrosomes of mammalian cells in the form of a complex in association with some other proteins. This complex localizes in both cell cytoplasm and centrosome, but nucleation generally does not happen from the cytoplasm, which is symptomatic of the presence of a regulatory molecule or mechanism to control the cytoplasmic nucleation. Another interesting aspect is the composition of this complex which varies under the influence of specific spatial and temporal signalling events. It has been previously reported by our lab that the γ-TuRC purified from brain tissue and cell lines of brain origin like IMR32 and U373, contains fodrin as a component. Fodrin is a non-erythroid homologue of spectrin, a cortical cytoskeleton component. Neuronal tissue is peculiar as it contains more number of differentiated or non-proliferating cells as compared to other tissues. These cells have limited or restrained microtubule nucleation and polymerization. Hence specific occurrence of fodrin in brain gamma-tubulin complex indicates that fodrin might have a regulatory role on the nucleation activity of γ-TuRC. The primary objective of this work is to explore the role of fodrin as a controller of the nucleation potential of γ-TuRC. The methodology of the work involves the assessment of nucleation in vitro and in vivo in the presence and absence of fodrin. Towards this end, we have purified tubulin and fodrin from goat brain. γ-TuRC was purified by affinity chromatography conventionally from goat brain. As control, γ-TuRC purified from Hek293T cell line was used as the complex did not contain fodrin as a component. In vitro assessment of microtubule nucleation was done by monitoring the process of polymerization spectrophotometrically by measuring turbidity at 350 nm. Preliminary results indicated that brain γ-TuRC inhibited nucleation whereas Hek293T derived γ-TuRC promoted nucleation. Further, we observed that nucleation was inhibited by the prior incubation of nucleation competent Hek293T derived γ-TuRC with purified fodrin. This is indicative of the fact that fodrin might have an inhibitory role over the nucleation potential of γ-TuRC. To study the role of fodrin in vivo, experiments are underway to establish stable cell lines expressing GFP tagged tubulin and EB1 (microtubule end binding protein). Experiments will be designed to perform live cell imaging in the background of downregulation or over expression of fodrin to draw a comparative analysis of the nucleation potential of γ-TuRC in both cases.

6-shogaol inhibits breast cancer spheroid formation

Anasuya Ray, Smreti Vasudevan and Suparna Sengupta

Shogaols are found in dried ginger and they are primarily the dehydrated product of gingerols. Among the shogaols, 6-shogaol exhibits potent cytotoxic activity against a wide range of cancer cells such as ovarian carcinoma, breast carcinoma, hepatocarcinoma, colorectal carcinoma etc. In this study, the inhibitory effect of 6-shogaol against breast cancer stem cell like spheroids was investigated. The spheroids were formed from breast cancer cells under specific growth conditions and the stem cell properties of the spheroids were verified by checking the CD44/CD24 marker expressions and secondary sphere formation capacity. 6-shogaol showed efficient cytotoxic activity in MCF-7 and MDA-MB-231 spheroids in a condition under which taxol did not show any noticeable cytotoxicity. Earlier studies showed that 6-shogaol inhibited the number and size of primary and secondary spheroids. Besides, cell cycle analysis of breast cancer monolayer and spheroids showed that 6-shogaol induced considerable cell cycle arrest at G2/M phase. We further checked if the cell death observed under 6-shogaol treatment was resulted by apoptosis. A low percentage of apoptototic cells were observed in monolayer MCF-7 cells by chromosome condensation, annexin binding and PARP cleavage. However, we did not find apoptosis in spheroid cells even after prolonged exposure. Thus we have checked if autophagy is induced by 6-shogaol as an alternate cell death mechanism. Our studies showed that 48 hour treatment of 15 µM of 6-shogaol induced a large number of cytoplasmic vacuoles (Acidic Vesicular Organelles (AVO)) in MCF-7 cells as shown in Figure 7. Immunofluorescent staining against microtubule associated protein Light Chain3 (LC3) also showed formation of localized LC3 punctae indicating recruitment of the LC3 to autophagosomes and subsequent proteolytic cleavage and lipidation (Figure 7). The cleavage of LC3 in MCF-7 cells and spheroids was also confirmed by western blotting. These observations proved that autophagy was induced by 6-shogaol in breast cancer cells.

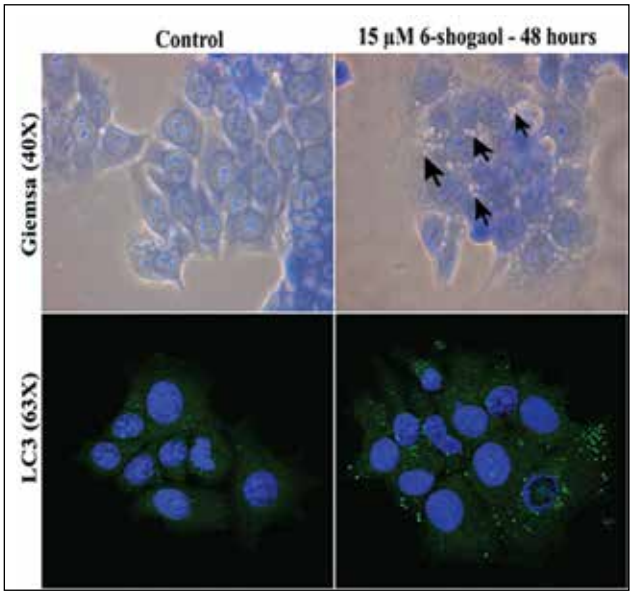


Figure 7: Autophagy in MCF-7 cells upon 6-shogaol treatment. Upper panel: Autophagic vacuole formation; Lower panel: LC3 punctae formation

Earlier we showed that Notch signalling is involved in 6-shogaol mediated inhibition of MCF-7 spheroids. Notch signalling activation usually initiates the γ-secretase mediated proteolytic cleavage of the Notch intracellular domain (NICD) which then translocates into the nucleus and activates variety of Notch target genes such as Cyclin D1. We further investigated the mechanism of inhibition of spheroids by 6-shogaol treatment using DAPT, a γ-secretase inhibitor. After 16 hrs of 6-shogaol treatment no change of Cleaved Notch1 was observed while after 24 hrs of treatment both DAPT and 6-shogaol reduced the cleavage of Notch1 and downregulated Notch target protein Cyclin D1. A combination of DAPT and 6-shogaol had an additive effect and exhibited further reduction in the expression of Cleaved Notch1 and its target protein as shown in figure 8. This observation indicates that 6-shogaol inhibits the breast cancer spheroid formation by altering Notch signalling pathway through γ-secretase inhibition.

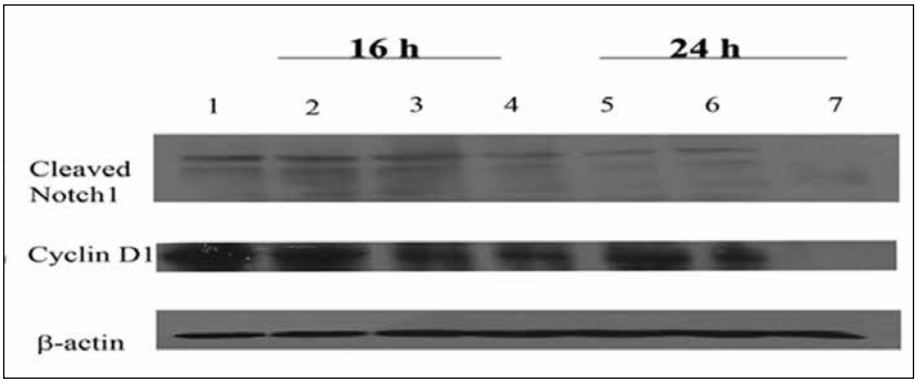


Figure 8: Down regulation of Cleaved Notch1 and Cyclin D1 by 6-shogaol in presence of a γ-secretase inhibitor. 1: no treatment; 2, 5: DAPT; 3, 6: 6-shogaol; 4, 7: DAPT + 6-shogaol; 40 μM 6-shogaol and 50 μM DAPT were used for all the treatments.

CONFERENCE PRESENTATIONS

- *Smreti Vasudevan, Sannu A. Thomas, Kallikat N. Rajasekharan and Suparna Sengupta:* "Promising activity of diaminothiazoles in overcoming multidrug resistance in cancer cells and xenograft tumour models" in the *Proceedings of the 2014 American Society for Cell Biology/ International Federation for Cell Biology Meeting, Philadelphia, Pensylvania, USA, 6-11 December 2014.*
- *Reshma Thamkachy, Rohith Kumar N, K. N. Rajashekharan and Suparna Sengupta:* In vitro and In vivo efficacy of diaminothiazoles is p53 independent: in the *Proceedings of the 2014 American Society for Cell Biology/ International Federation for Cell Biology Meeting, Philadelphia, Pensylvania, USA, 6-11 December 2014.*

CANCER RESEARCH PROGRAM

LABORATORY - 4

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SKP2 Overexpression Leads to an Increase in Phosphorylation of Forkheadbox Transcription Factor, FOXM1

Dhanya K, C.Maharrish and S. Asha Nair

SKP2 is a member of the F box protein family characterized by a 40 amino acid motiff termed as Fbox. This protein regulates cell cycle, immune response, signaling cascade and developmental program by targeting proteins, such as cyclins, cyclin-dependent kinase inhibitors, IkBa and b-catenin. SKP1 recruits the F-box protein SKP2 through a bipartite interface, involving both the F-box and the substrate recognition domain. (Brenda A et al,). The F-box proteins are divided into 3 classes: Fbws containing WD-40 domains, Fbls containing leucine-rich repeats, and Fbxs containing either different protein-protein interaction modules or no recognizable motifs. The protein encoded by this gene belongs to the Fbls class; in addition to an F-box, this protein contains 10 tandem leucine-rich repeats. TIS21 is member of early growth response genes and anti-proliferative gene family. TIS21, an inhibitor of cyclin CDKs is found to be very essential for cell cycle regulation and it is known to regulate the FOXM1 phosphorylation via cyclin CDK complex. SKP2 is a known E3 ubiquitin ligase for TIS21. Expression of SKP2 is regulated via FOXM1. It has been reported that SKP2 over expression could be seen in multitude of cancers (Gstaiger et al). Forkhead box (Fox) proteins are family of evolutionarily conserved transcriptional regulators, which control a wide spectrum of biological processes. A loss or gain of Fox function can alter cell fate and promote tumorigenesis as well as cancer progression. FoxM1 is a proliferation-associated forkhead transcription factor which regulates genes important for G1/S-transition, S-phase progression, G2/M transition and progression through M-phase. Abnormal up regulation of FoxM1 has been reported in majority of solid human cancers including liver, breast, lung, prostate, cervix, colon, pancreas and brain. Cell cycle dependent regulation of FoxM1 is very essential for the controlled expression of cell cycle regulators. Previous studies from our lab have reported that, increased expression of CDK1 occurred when SKP2 was over expressed. This may be because skp2 targets TIS21, an inhibitor of cyclin CDK complex. Based on the previous observation, we went on to elucidate the role of SKP2 and FoxM1 on Cdk1 gene transcription. In order to study the effect of SKP2 on CDK1 expression, HEK 293 cells were transfected with SKP2 as well as control pCDNA3.1. Whole protein as well as nuclear protein isolation followed by co-immunoprecipitation with FOXM1 antibody, led to the precipitation of FOXM1 interactome. Western blot analysis of the immunoprecipitated protein complex showed abundance of phosphorylated FOXM1 compared to the control. This indicates that SKP2 over expression leads to the increased phosphorylation of FoxM1. Besides the increase in phosphorylation of FOXM1, FOXM1 protein as well as mRNA expression also increased upon Skp2 overexpression. Phosphorylation of FOXM1 is very crucial for its function as a transcriptional regulator. So it is obvious that over expression of SKP2 protein leads to the increased phosphorylation of foxm1 thereby inducing the expression of CDK1. SKP2 and FOXM1 over expression have been known to associate with multitude of cancers. In our current study we addressed the cause of increased expression of CDK1 when SKP2 was overexpressed. FoxM1 has been found to be hyper phosphorylated when SKP2 was over expressed which might be the reason for up regulation of CDK1. Previous reports and our data indicate CDK1 to be a downstream target of FoxM1. Phosphorylation in respective amino acid residue of FoxM1 is indispensable for its transcriptional activity. Further studies are being pursued in our lab to understand the regulation cdk1 gene expression by FoxM1. Since the overexpression of these molecules play a pivotal role in cell proliferation and poor prognosis of the disease, targeting them would be a useful therapeutic approach in the future.

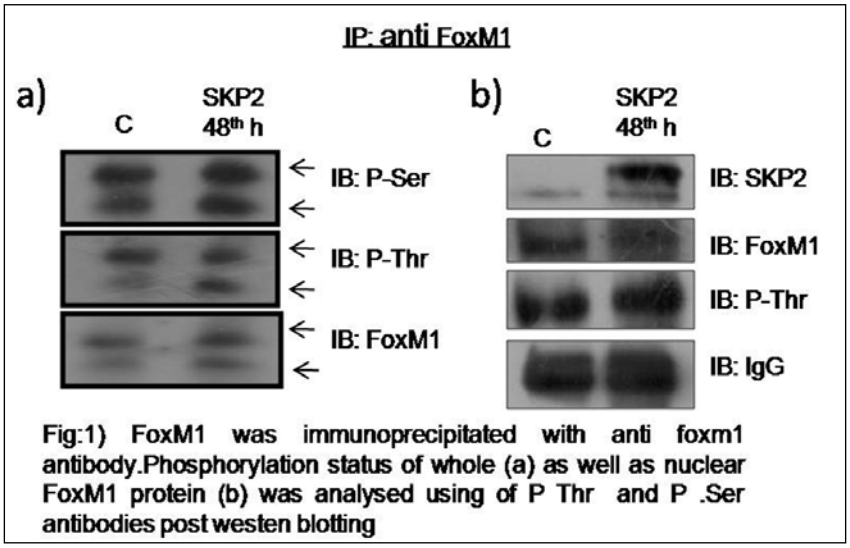


Fig:1) FoxM1 was immunoprecipitated with anti foxm1 antibody.Phosphorylation status of whole (a) as well as nuclear FoxM1 protein (b) was analysed using of P Thr and P .Ser antibodies post westen blotting

STAT3 Reciprocating the FOXM1 Signalling Pathways – An Insight on the Invasiveness of Colorectal Cancer

Chandraprabha M.G. and S. Asha Nair

FoxM1 belongs to a large family of forkhead box (Fox) transcription factors. Unlike the other Fox-transcription factors, FoxM1 is associated with cell proliferation and is expressed only in proliferating cells. The understanding of FoxM1 transcriptional activation and the role of FoxM1 as an oncogene is limited. To date, some studies have revealed that FoxM1 expression can be driven primarily by the Hedgehog signaling pathway in gastric cancer, colorectal cancer, meningoma and breast cancer. Also, significant level of Stat3 expression is predominantly found in colorectal cancer cases. Though, the STAT3 protein was first described as a member of the Jak/Stat signaling pathway, in some cancer cells STAT3 is also activated by non-Jak/Stat proteins, such as BCRABL, c-Abl, MEK1, Src and Smoothened. FoxM1 and STAT3 are often related to cancer and present similar consequences when overexpressed or inhibited. The results of our project so far pertain to the relation between STAT3 /SKP2 expression in correlation with the activation of FOXM1 in colon cells. The preliminary data depicting the STAT3/ SKP2 correlation was carried out using the STAT3 inhibitor, *Stattic*. Further, real time PCR was done to confirm the same at the transcriptional level. FoxM1 has been identified to promote transcription of skp2. Over expression of STAT3 using plasmid construct, pEF-BOSwtSTAT3 significantly increased FOXM1 followed by increase in SKP2 levels in SW620, SW480 and HCT116 cells. Also, suppression of Stat3 activation using siRNA, decreased the expression of FOXM1 target molecules including SKP2, CyclinB1, MMP2, CDC25B. Together, these data might suggest a possible correlation between Stat3 in regulating FOXM1/Skp2 expression in colon cancer cells. Our study may provide the correlation between FoxM1 and STAT3, leading to a conclusion of FOXM1 being the new target of STAT3 transcription factor. Further clarifying its role in proliferation, survival, drug resistance and DNA repair in colon cancer. This elucidation of the signaling pathways involved in FoxM1 expression in colon cancer might be useful to provide new strategies for treatment, drug resistance, and prognosis and disease progression. Stat3 is linked to inflammation-associated tumorigenesis that is initiated by genetic alterations in malignant cells, as well as by many environmental factors, including

chemical carcinogens, infection, cigarette smoking and stress. The mechanism of how Stat3 is initially activated and remains persistently activated in cancer is still vague. Our study will help in addressing that question with the help of the FOXM1 hypothesis. Ultimately, this may provide the rationale for the development of therapeutics inhibiting Stat3/FOXM1 interactions.

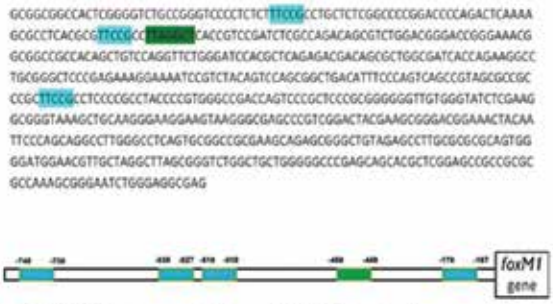


Fig.1.1000 bp sequence from FOXM1 promoter gene from start of transcription (+1), indicating STAT binding sites (blue boxes), STAT3 binding sequence (green box).

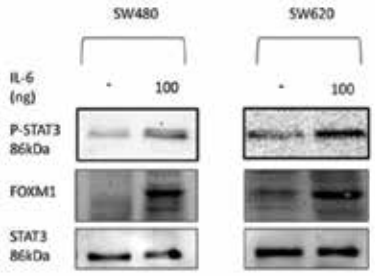


Fig.2. IL-6 activates stat3 and increase the level of p-STAT3, along with the activation of FOXM1

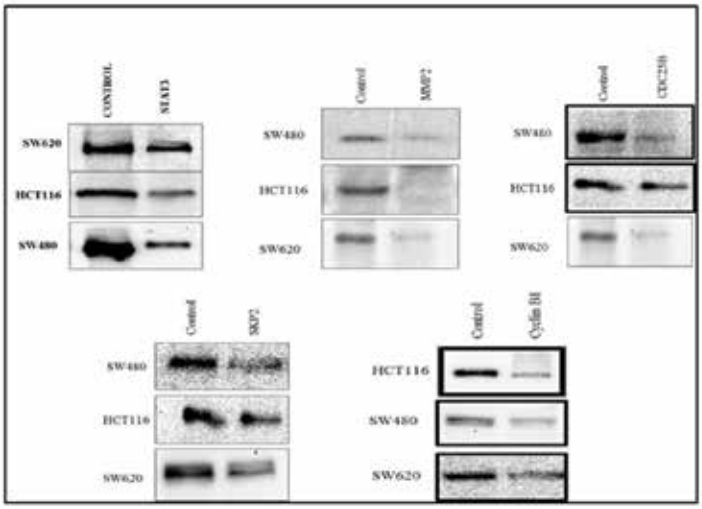


Fig.3. STAT3 knockdown using siRNA led to the down regulation FOXM1 target proteins

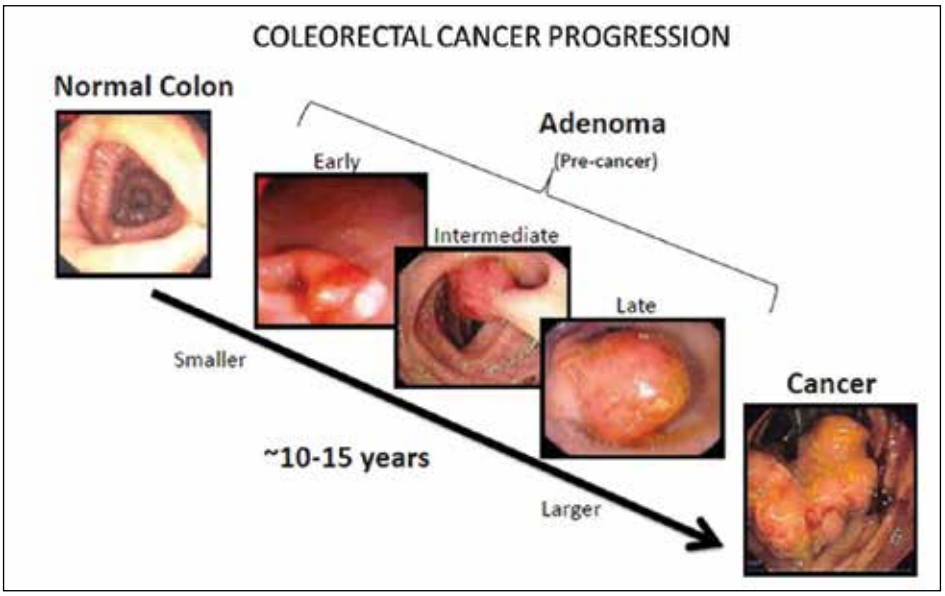
Molecular Mechanisms of Drug Resistance in Colorectal Cancer

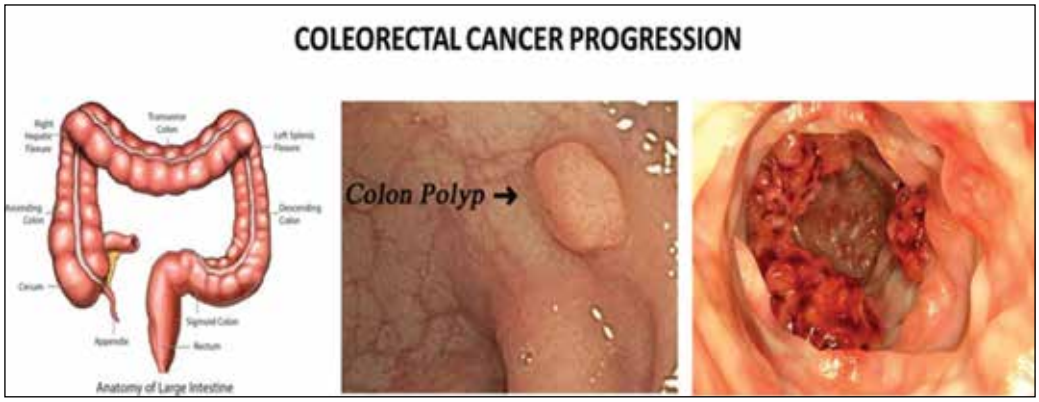
Manu Prasad, Tapas Pradhan, Saneesh Babu. P.S, K.Chandramohan*, S.Asha Nair and M. Radhakrishna Pillai

Collaborator: Regional Cancer Centre, Trivandrum

Colorectal Cancer (CRC) is the third most common cancer in the world and the second leading cause of cancer-related death in the western world. Around a quarter of CRC patients are incurable at diagnosis and half of the patients who undergo potentially curative surgery will ultimately develop metastatic disease? The standard treatment for advanced CRC is based on the administration of 5-fluorouracil (5-FU) combined with oxaliplatin, and the topoisomerase I (TOP1) inhibitor CPT-11 (Irinotecan). Although most patients with advanced CRC are initially responsive to the combined chemotherapy treatment, they later experience disease relapse due to eventual tumor recurrence and emergence of drug-resistant tumor cells. Novel insights in cancer research suggests that the capacity to initiate and sustain tumor growth, is a unique characteristic of a small subset of cancer cells with

stemness properties within the tumor mass, called cancer 'stem cells'(CSCs). CSCs are characterized by high resistance to drugs. Gaining insight of the mechanisms underlying drug resistance and stemness is important to develop more effective therapeutic approaches. Drug resistance is mainly due to the up regulation of several ATP-binding cassette proteins notably MRP1 and ABCG2. We wish to analyze possible pathways associated with drug resistance in tumor samples (Post Chemo-RT as well as primary samples after surgery). We are also developing 5FU resistant cell lines (HCT 116 and SW 620) in our laboratory. Once these 5FU drug resisistant cell line are established, the possible drug resistance mechanisms and genes associated with drug resistance could be identified by transcriptomic and proteomic approach. Several other factors like redox homeostasis, also contribute to drug resistance. Interestingly, we observed upregulation of FOXM1 in tumor samples, which plays a key role in redox homeostasis. Moreover, oxidative stress can affect the efficacy of cancer treatments by multiple mechanisms, which includes chemosensitivity, apoptosis, angiogenesis, metastasis and inflammatory reactions. Further, we aim to focus on finding out a biomarker for early detection and prognosis of CRC. This will be achieved by proteomic approach combined with MALDI imaging of tissue sections. As histological features remain intact throughout the analysis of a section, distribution maps of multiple analytes can be correlated with histological and clinical features. Spatial molecular arrangements can be assessed without the need for target-specific reagents, allowing the discovery of diagnostic and prognostic markers and enabling the determination of effective therapies.





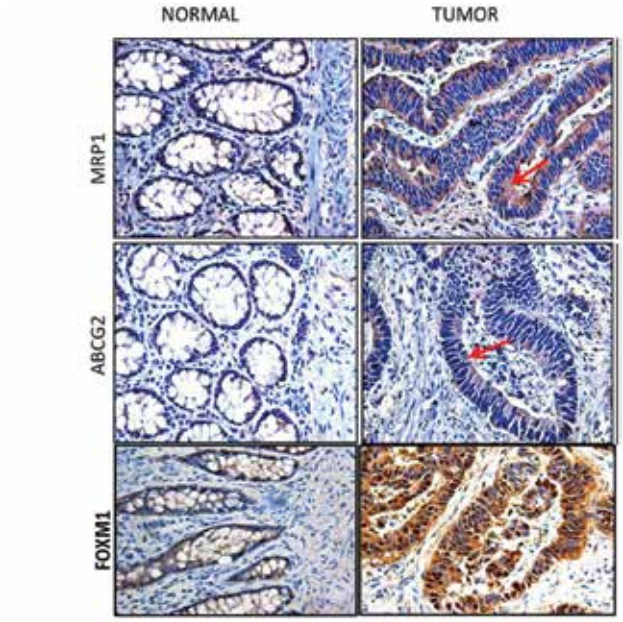


Figure 1:Immunohistochemical analysis of MRP1,ABCG2 and FOXM1 in normal and tumor colorectal tissue sample.

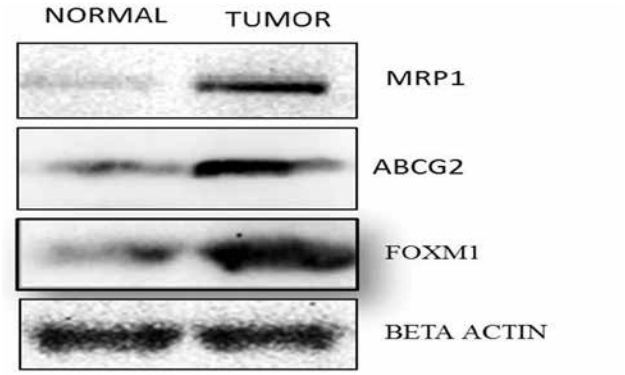


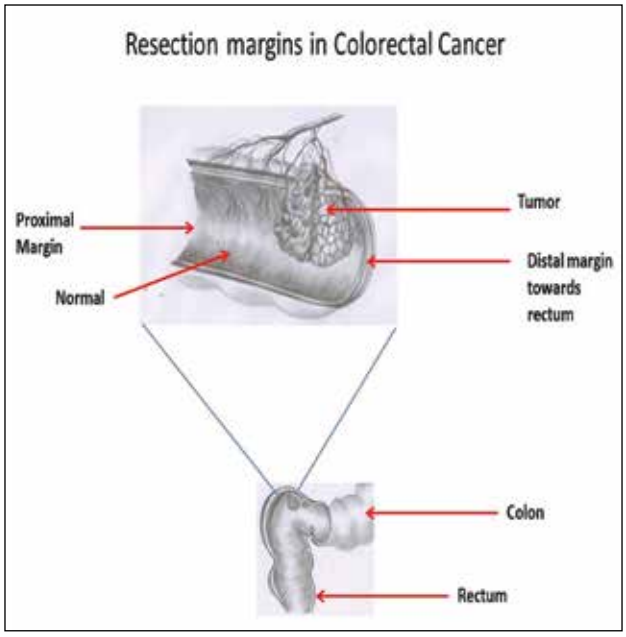
Figure 2: Expression of MRP1, ABCG2 and FOXM1 in normal and tumor colorectal tissue samples.

Surgical margins act as a potential niche for heterogeneous Cancer Stem Cells and drug resistant cells in Colorectal Cancer

Tapas Pradhan, Manu Prasad, K.Chandramohan*, S.Asha Nair and M. Radhakrishna Pillai

**Collaborator: Surgical Oncology, Regional Cancer Centre, Trivandrum*

Advances in the diagnosis and treatment of colorectal cancer (CRC) have had a major impact on management of this malignancy. Developments in screening, prevention, biomarker and genomic analysis, stem-cell research, personalized therapies and chemotherapy have improved detection and mortality statistics. However, despite these advances, many patients with advanced and metastatic tumors will still succumb to the disease. Further diagnosis and treatment advances are therefore needed. Removal of surgical margins in CRC surgery is a usual practice in clinics. Clinical studies have shown that surgical margins do have role in poor prognosis and disease recurrence in CRC. (Nash et al.2010). Preliminary results from our lab show that distal surgical margin from CRC patients harbor cancer stem cells populations. A proper resection length of distal margin is very crucial for removal of residual tumor cells in CRC. So exploring role of surgical margins in disease recurrence and drug resistance could have answer for the present challenges. So we put forth our working hypothesis that these surgical margins could be a potential niche for drug resistant cells (including cancer stem cells) and have very crucial role in tumor phenotypes. In the present study we enrich cancer stem cell population from human tissue, which includes normal, tumor and distal margins of CRC. Specific surface markers (CD133, CD44, EpCAM) were used to identify and analyze



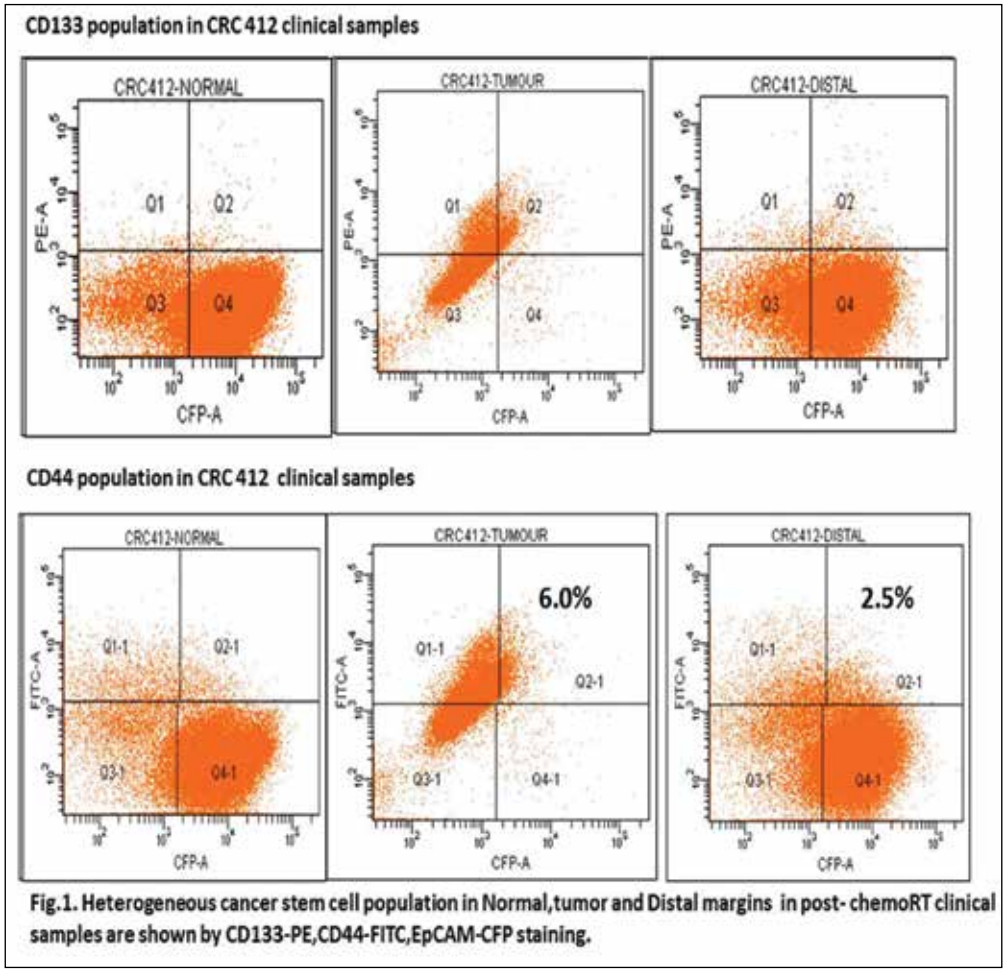


Fig.1. Heterogeneous cancer stem cell population in Normal,tumor and Distal margins in post- chemoRT clinical samples are shown by CD133-PE,CD44-FITC,EpCAM-CFP staining.

cancer stem cells population in CRC samples through FACS analysis. Immuno-histochemical analysis of tissue sections were also done to analyze the expression pattern of key proteins associated with cancer stemness and drug resistance. Taqman Real-time assay was performed in order to find out the important drug resistance and stemness marker expressed in normal, tumor and distal margins in post chemo RT CRC samples. Consistent with our previous preliminary data, we observed that tumor and distal surgical margin are enriched with EpCAM/CD133 positive cells as compared to normal counterpart tissue. From real-time assay, we found that distal surgical margin also expressed high levels of SOX2, OCT4, SNAIL, ABCC1 stemness and drug resistance proteins upon chemo-radiotherapy. These findings were again confirmed by immuno-histo analysis of drug resistance proteins in distal surgical margins. However, interestingly in the case of primary CRC (no chemo-radiotherapy) samples these proteins were not found to be expressed, which indicate that stress produced due to therapy could be the sole reason behind enrichment of cancer stem cells in distal margin. Again from the above results we could speculate that some drug resistance cells could be migrating from tumor towards distal margin upon therapy. Therefore distal margin could be a safe niche for cancer stem cells and drug resistance cells, which could play a potent role in disease recurrence. Further, we are aiming to characterize the heterogeneous cancer stem cells found in tumor and distal margins and aim to further validate their tumorigenic properties both in-vitro and in- vivo.

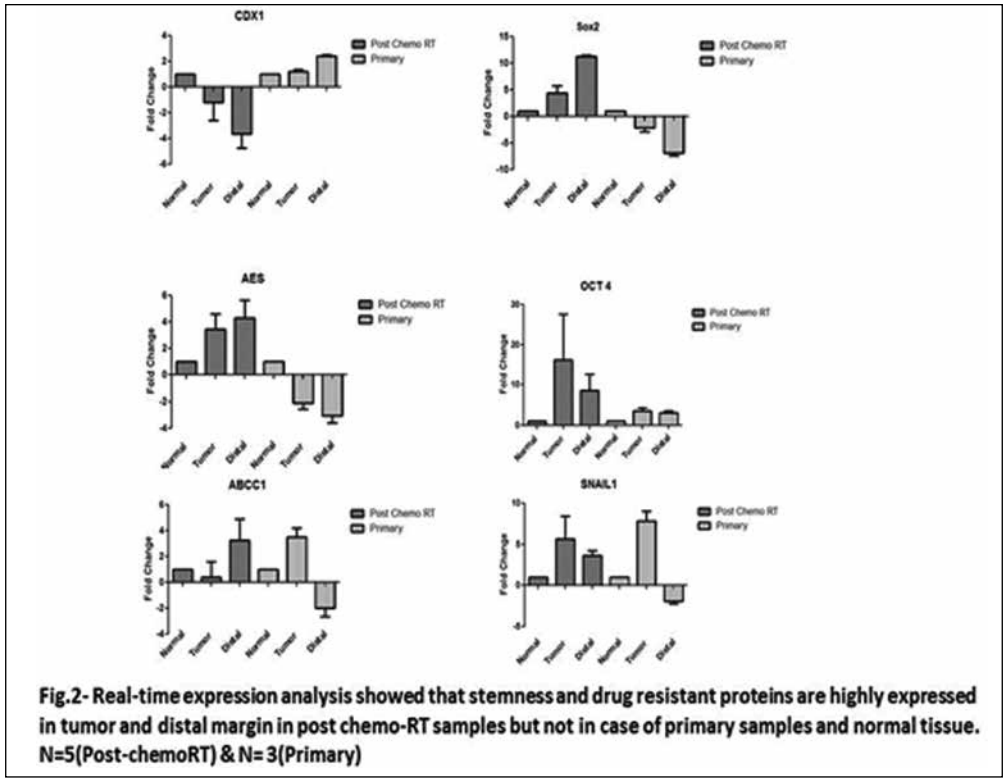


Fig.2- Real-time expression analysis showed that stemness and drug resistant proteins are highly expressed in tumor and distal margin in post chemo-RT samples but not in case of primary samples and normal tissue. N=5(Post-chemoRT) & N= 3(Primary)

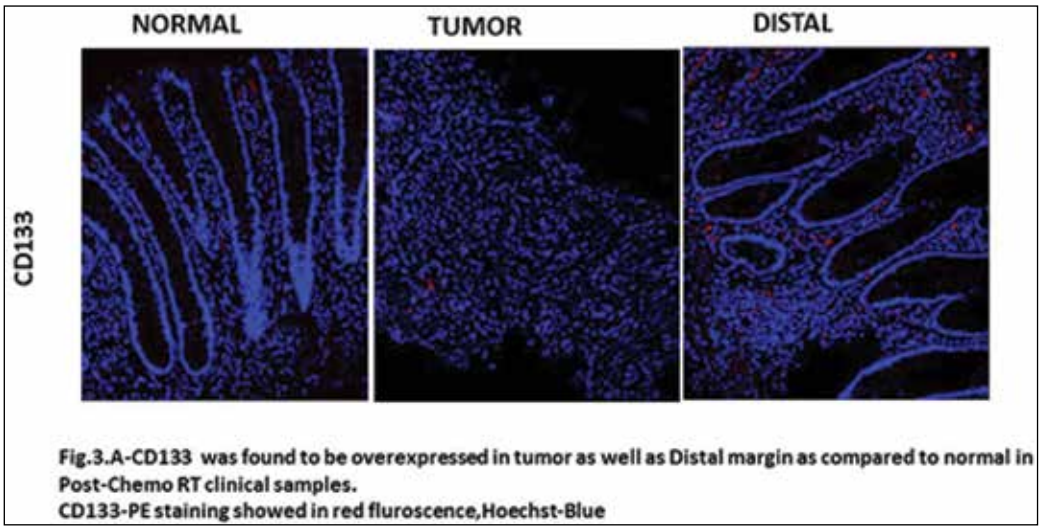


Fig.3.A-CD133 was found to be overexpressed in tumor as well as Distal margin as compared to normal in Post-Chemo RT clinical samples.
CD133-PE staining showed in red fluroscence,Hoechst-Blue

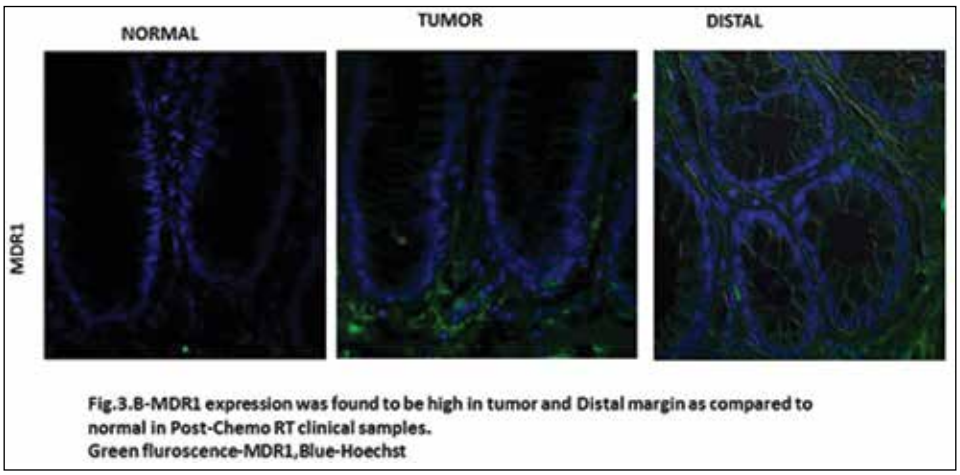


Fig.3.B-MDR1 expression was found to be high in tumor and Distal margin as compared to normal in Post-Chemo RT clinical samples.
Green fluroscence-MDR1,Blue-Hoechst

In vivo validation of synergetic effect of Thisotreptone, a FOXM1 inhibitor in Squaraine based photodynamic therapy (PDT)

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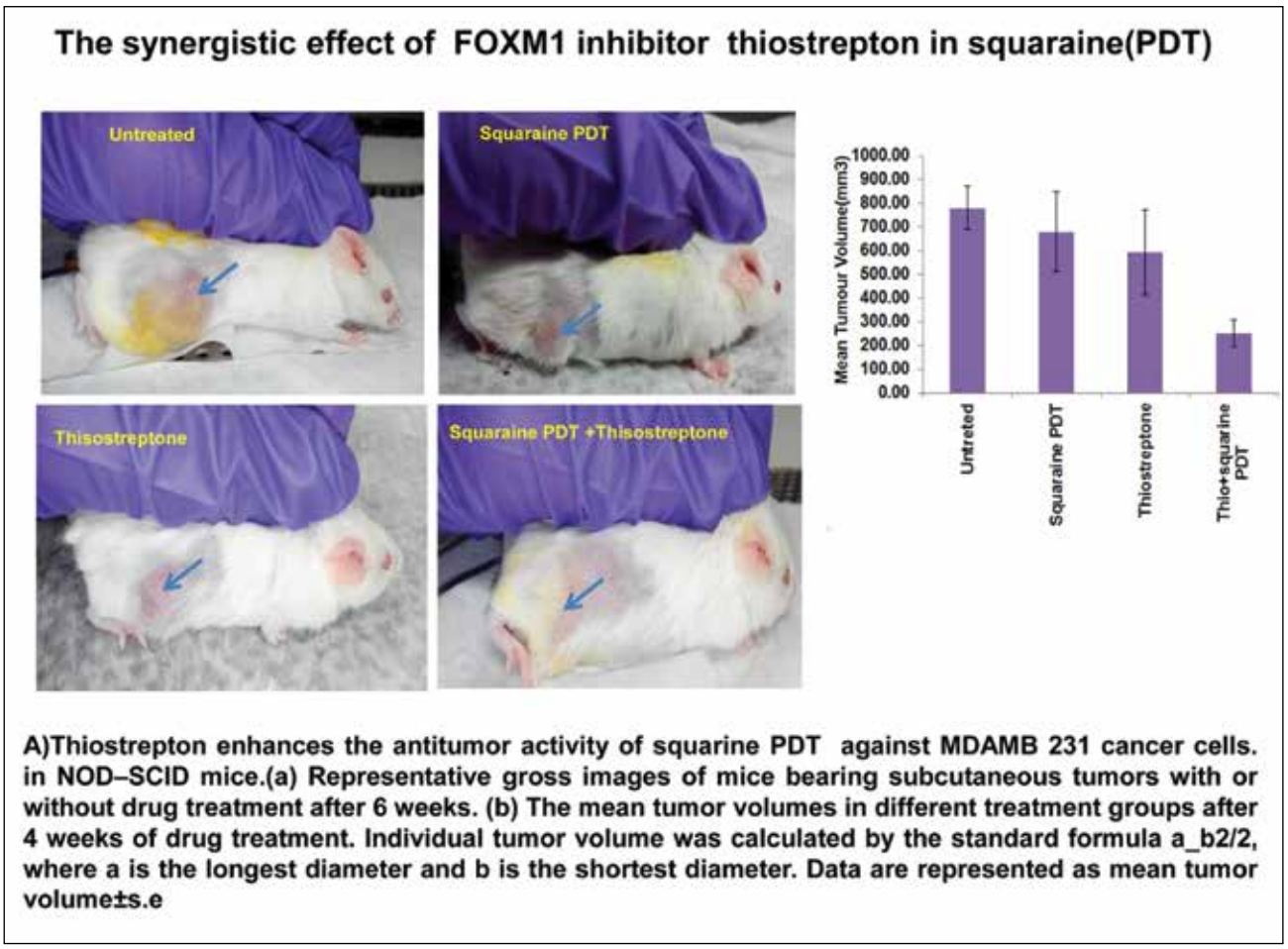
Photodynamic therapy (PDT) is a novel treatment for cancer and certain benign conditions that are generally characterized by overgrowth of unwanted or abnormal cells. The procedure requires exposure of cells or tissues to a photosensitizing drug followed by irradiation with visible light of the appropriate wavelength, usually in the red or near infrared region and readily compatible with the absorption spectrum of the drug. PDT primarily results in a sequence of photochemical events that generate reactive oxygen species (ROS), which induce oxidative damage ultimately causing the killing of cancerous cells or other targets of therapeutic interest. Because of limited light penetration through tissues (resulting from its absorption, scattering and reflection), the antitumor effects of PDT are limited to just a few centimetres. Deeper layers of the tumor receive



A)Thiostrepton enhances the antitumor activity of squarine PDT against MDAMB 231 cancer cells. in NOD–SCID mice.(a) Representative gross images of mice bearing subcutaneous tumors with or without drug treatment after 6 weeks. (b) The mean tumor volumes in different treatment groups after 4 weeks of drug treatment. Individual tumor volume was calculated by the standard formula a_b2/2, where a is the longest diameter and b is the shortest diameter. Data are represented as mean tumor volume±s.e

light doses that are ineffective to fully excite the photosensitizers. Sublethal damage to tumor cells and the vasculature is therefore readily managed by constitutive or induced protective mechanisms. Elucidation of these mechanisms might result in the design of more effective combination strategies to improve the antitumor efficacy of PDT. For investigating this we did a real-time PCR for ROS resistant genes affected by PDT with a sub lethal dose of squarine PDT in MDA MB 231 cells. According to our results, we observed a fold induction of ROS scavenger enzymes (Catalase, MnSOD and PRDX3) and DNA repair genes (BRCA 2 and XRCC1) which are potent candidate genes for PDT resistance. FOXM1 was identified as the transcription factor for these genes in ROS responses. This ROS-regulatory function of FoxM1 protects proliferating tumor cells from oxidative stress and promotes survival. Thiostreptone is a known chemical inhibitor for FOXM1 hence we used this compound for assessing FOXM1 knock down as therapeutic strategy to enhance the effect of PDT. The potential anticancer activity of FOXM1 inhibitors in combination with squaraine PDT was evaluated in NOD/SCID bearing breast tumor xenografts. Female athymic NOD/SCID mice were injected with MDA-MB-231human breast cancer cells in the flank region bilaterally to establish xenograft tumors. After tumors became palpable, their size was measured by caliper and the animals were randomized into the following treatment groups: animals were separated into treatment groups of (1) non-treated control, (2) thiostrepton micelle only 20 mg/kg, IV (3) Squaraine PDT only and (4) combination of thiostrepton micelle 30 mg/kg and Squaraine 10 mg/kg. Animals were injected i.p. with the thiostrepton micelle and photosensitizer (squaraine) 3 times per week followed by laser irradiation (once in a week). Tumor size was recorded weekly. Upon completion of the study, mice were sacrificed by using CO2, and their tumors were excised. The mean of tumor volume was plotted against time in weeks to monitor growth of the breast tumor xenografts. Our result showed enhanced tumour reduction in mice when given a combination of PDT with Squaraine along with Thiostrepton.

EXTRAMURAL GRANTS

Sl No	Project Title	Funding agency	Period
1	Functional significance of fork head box protein, foxm1b in cdk1 turnover-A molecular analysis.	Department of Science & Technology, Government of India	2013-2016
2.	Molecular mechanism of drug resistance in colorectal cancer: tumor stem like cells as unique targets in residual disease	Department of Biotechnology, Government of India	2013-2016
3.	Transcriptional and translational profiling of drug resistance genes following therapeutic intervention in colorectal cancer	Council for Scientific & Industrial Research	2013-2016

PUBLICATIONS

- *Jagadeeshan S, Krishnamoorthy YR, Singhal M, Subramanian A, Mavuluri J, Lakshmi A, Roshini A, Baskar G, Ravi M, Joseph LD, Sadasivan K,Krishnan A, Nair AS, Venkatraman G, Rayala SK* (2014) Transcriptional regulation of fibronectin by p21-activated kinase-1 modulates pancreatic tumorigenesis *Oncogene. 2015 Jan 22;34(4):455-64*
- *Diana David, Sankar Jagadeeshan, Ramkumar Hariharan3, Asha Sivakumari Nair* and Radhakrishna Madhavan Pillai* (2014). Smurf2 E3 ubiquitin ligase modulates proliferation and invasiveness of breast cancer cells in a CNKSR2 dependent manner *Cell Division 9:2*
- *Bugide S, David D, Nair A, Kannan N, Samanthapudi VS, Prabhakar J, Manavathi B* Hematopoietic PBX interacting protein (HPIP) is over expressed in breast infiltrative ductal carcinoma and regulates cell adhesion and migration through modulation of focal adhesion dynamics *Oncogene 2014 doi: 10.1038/onc.2014.389*

CONFERENCE PRESENTATIONS

- *S.Asha Nair, Chithra JS, Rema P, M.Radhakrishna Pillai* "EMT of stem cells in endometrial cancer: role of metastasis tumor antigens" *International Conference On Disease Biology And Therapeutics, 3-5 December 2014*
- *S.Asha Nair, Saneesh Babu.P.S, D. Ramiah, M.Radhakrishna Pillai* "Development of Novel Sensitizers Based on Near Infrared Dyes" *National Conference on Recent Advances Cancer Biology And Therapeutics (ICDBT)-2014, 5th Dec 2014*
- *S.Asha Nair, Saneesh Babu.P.S, D. Ramiah, M.Radhakrishna Pillai* "Squaraine based Photodynamic Therapy induces cancer cell apoptosis by the unfolded protein response" *International Conference On Pharmaceutical Sciences, Jan21-22 DUBAI, UAE*

POSTER PRESENTATIONS

- *Tapas Pradhan, Krishnanand P, K. Chandramohan, S.Asha Nair, M. Radhakrishna Pillai* "Surgical margins: A niche for Heterogeneous Cancer stem cells" at *EMBO, EMBL Symposium On Frontiers In Stem Cells & Cancer, Heidelberg, Germany. EMBL Advanced Training Centre 29th to 31st march 2015.*
- *Dhanya K., Manu Prasad. M, S.Asha Nair,* "Thiostrepton, a FoxM1 and proteasome inhibitor degrades mutant P53 via chaperone mediated autophagy" in the *XXXVIII All India Cell Biology Conference On Cellular Response To Drugs CSIR-CDRI Lucknow during December 10-12, 2014*

CANCER RESEARCH PROGRAM

LABORATORY - 5

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Isolation, Characterization and Therapeutic Interventions of Breast Cancer Stem Cells from BRCA1-defective Breast Cancer

Veena Somasundaram and Priya Srinivas

(In collaboration with Debabrata Mukhopadhyay and, Krishnendu Pal Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN, USA, and S Asha Nair, Cancer Research Program-4, RGCB)

BRCA1 gene defects have profound importance in predisposing to breast as well as ovarian cancer. However this does not cover for the 30-40% patients who possess a functional BRCA1 defect without harboring a mutation due to hypermethylation of the BRCA1 promoter. Majority of the BRCA1-related breast cancers are TNBCs that are high-grade tumors with a high mortality-to-incidence ratio. Early age of onset and dearth of targeted therapy are two other major problems associated with BRCA1-defective cancers. Currently, the most effective method for the management of familial breast cancer is prophylactic mastectomy and oophorectomy, which in many cases can affect the emotional well-being of the patients. The chemotherapeutic regimens that were initially found to be effective in BRCA1-defective breast cancers, including cisplatin and the PARP inhibitors have succumbed to the problem of insensitivity and relapse due to upregulation of efflux pumps in case of cisplatin and secondary mutations that reverse the sensitivity to these drugs. The resistance in such cases as well as tumor relapse could be due to the presence of cancer stem cells (CSCs). There have a few studies on identification and characterization of CSCs from BRCA1-defective cancers. However, these studies have not helped in reaching a consensus about the features of CSCs from BRCA1-defective cancers possibly because the different putative CSC markers have not been compared and contrasted from the perspective of BRCA1 status. The future of treatment of familial breast cancers as well as sporadic breast cancers that exhibit a functional defect in BRCA1 will be to target and abrogate the CSC population responsible for tumor relapse. Prior to targeting, the CSCs have to be identified, and characterized. Earlier work based on in vitro mammosphere cultures has found that ALDH1 positivity could be the best marker for the identification and isolation of BCSCs from BRCA1-defective breast cancer cell lines and also that the CD44+/24-/low profile does not faithfully represent BCSCs. BRCA1 status has also been found to be a determinant of the percentage occurrence of the epithelial-like (ALDH1+) or mesenchymal-like (CD44+/24-/low) BCSCs within breast cancer cell lines with the reconstitution of a full length, wild type BRCA1 in HCC1937 breast cancer cells (5382insC mutation in BRCA1) making them more mesenchymal-like. The robust membrane localization of ABCG2 in HCC1937 as compared to HCC1937/ wt BRCA1 could be a possible reason for the susceptibility of HCC1937 to plumbagin when compared to HCC1937/ wt BRCA1 (Figure 1).

This could however not explain the increase in ALDH1+ BCSCs post-Plumbagin treatment in HCC1937/ wt BRCA1 (Figure 2A). This could possibly be explained based on the generation of ROS (Figure 2B) as treatment with Plumbagin induced pronounced ROS generation in HCC1937 spheres when compared to HCC1937/ wt BRCA1 spheres as observed by DCF-DA staining experiments. Hence, the increase in stem cells could be a hypoxia induced enrichment of BCSCs.

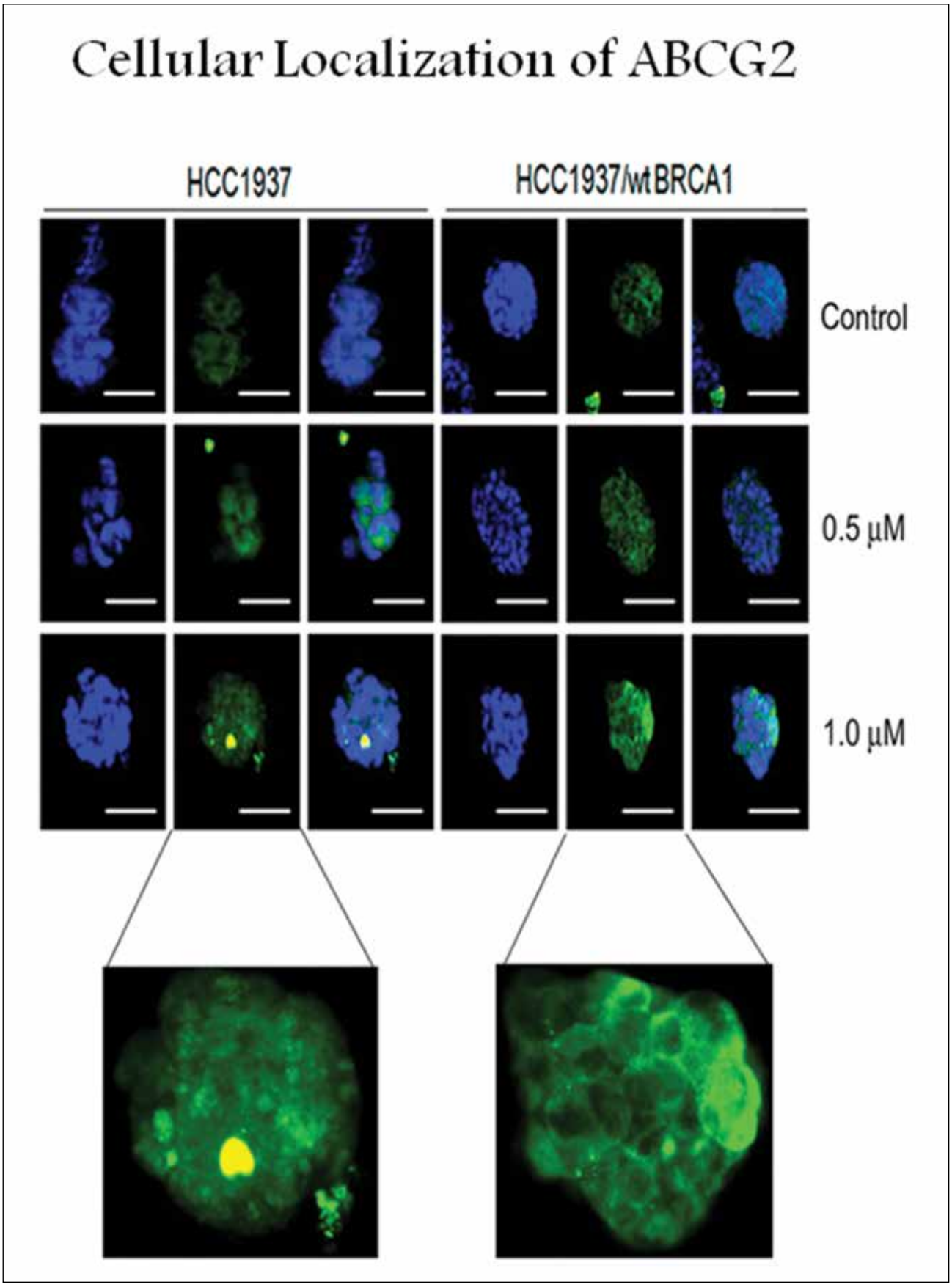
Cellular Localization of ABCG2
HCC1937
HCC1937/wtBRCA1
Control
0.5 µM
1.0 µM

Figure 1

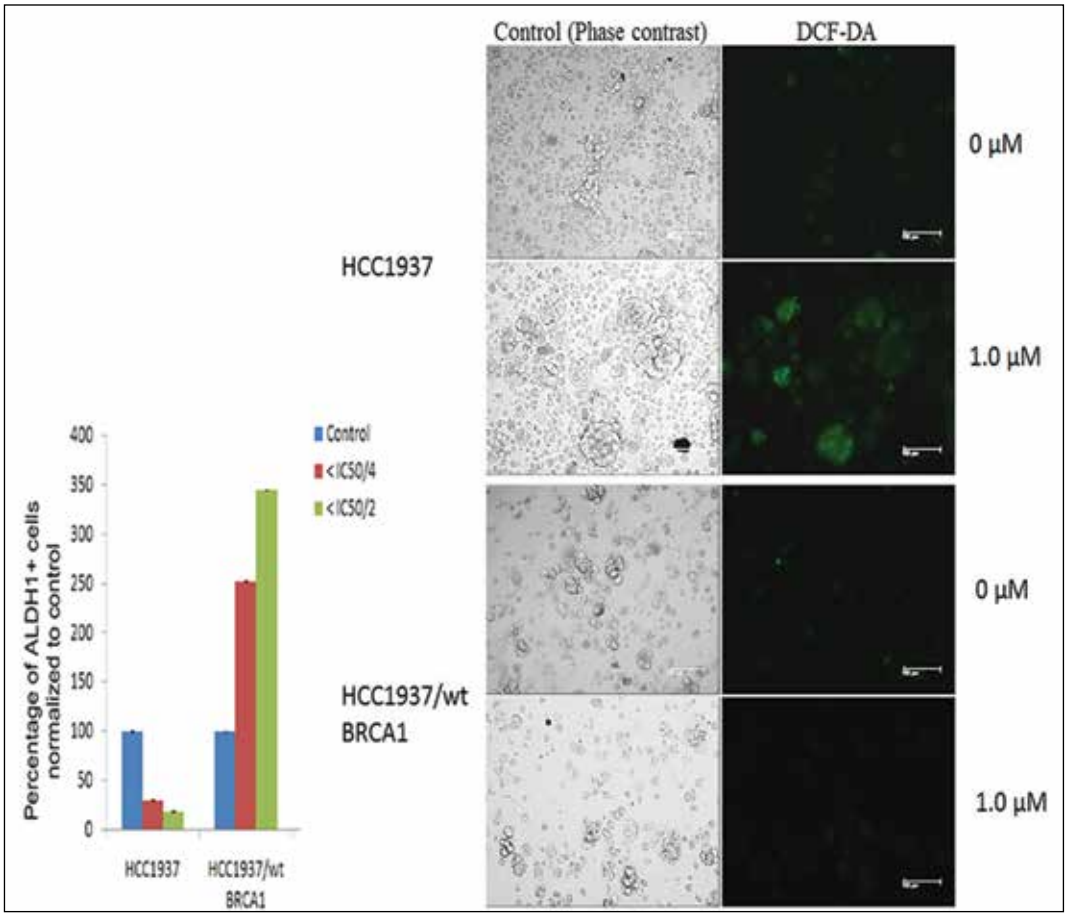

Figure 2

Therapeutic potential of Plumbagin in Castration Resistant Prostate Cancer Stem Cells

Reshma R S, Priya Srinivas

Prostate cancer ranks fourth among the most common cancers worldwide and is the second most frequently diagnosed cancer among men. Resistance to chemotherapy in metastatic CRPC is a result of cellular mechanisms of drug resistance intrinsic to prostate cancer and general mechanisms common to different tumor types. General mechanisms of drug resistance include the existence of subpopulations of cancer cells with cellular mechanisms of resistance, resistance related to interactions between prostate cancer cells and their surrounding microenvironment and impaired drug delivery to the cancer cells. Tumor initiating cells are thought to be responsible for cancer chemo-resistance and relapse, and thus they represent a significant concern for cancer prognosis and therapy. Characterization of cancer stem cells has led to the identification of key cellular activities that may make cancer stem cells vulnerable to therapeutic interventions that target drug-effluxing capabilities, stem cell pathways, anti-apoptotic mechanisms, and induction of differentiation. A subpopulation of both PC 3 cells possess prostatosphere forming ability. Sphere formation in vitro in serum free suspension cultures is increasingly used as a method for enriching putative CSCs as it relies on their characteristic anchorage independent growth. In

contrast to non-tumorigenic cells CSCs are able to form colonies from a single cell and have the ability to grow as spheres in serum free media. To confirm that the prostatospheres were the progeny of individual cells, rather than the aggregation of quiescent cells, characterization using specific stem cell markers was carried out. A common strategy for CSC identification is flow-cytometry using assumed specific CSC surface markers. A subpopulation enriched with CD 44+ / CD 24- cells and CD133+ cells were detected in the in vitro propogation of prostate cancer stem cells of PC 3 cells (Figure 3).

We studied the effect of standard drug finasteride on prostatospheres. We have observed a small percentage of increase in sphere forming ability by prostate cancer stem cells in response to finasteride at an IC50/4 concentration. The comparison of the number of prostatospheres upon Plumbagin and finasteride is shown in Figure 4. Plumbagin, a napthaquinone, reduces the sphere forming efficiency of PC3 cells in specific stem culture media. Plumbagin has the ability to reduce the numbers and sizes of spheres formed. Plumbagin exhibits a progressive dose dependent reduction on prostate cancer stem cell properties.

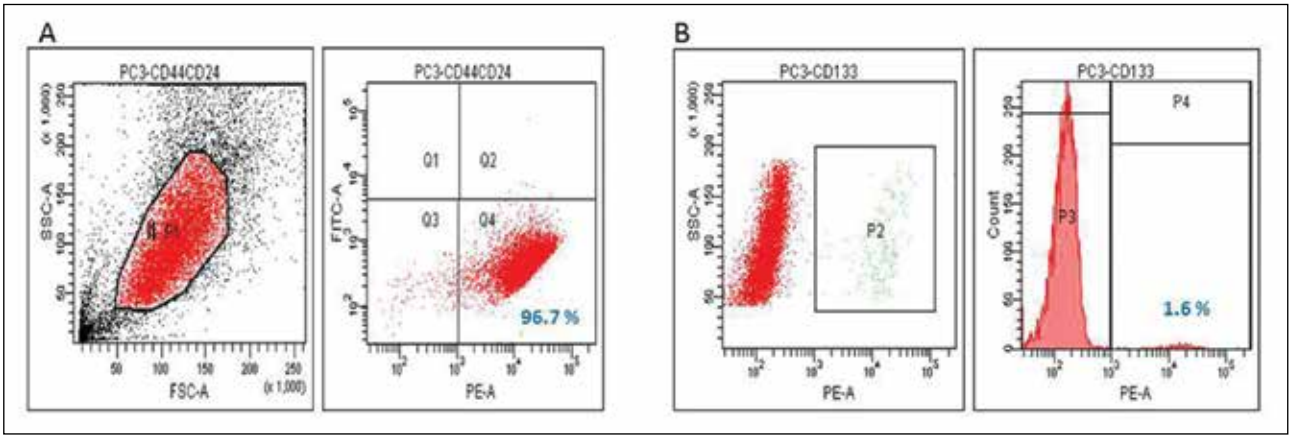

Figure 3

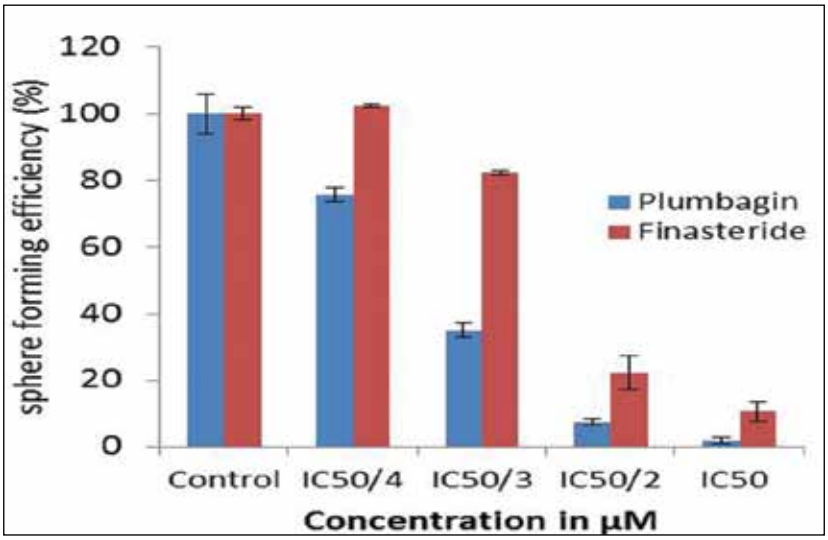

Figure 4

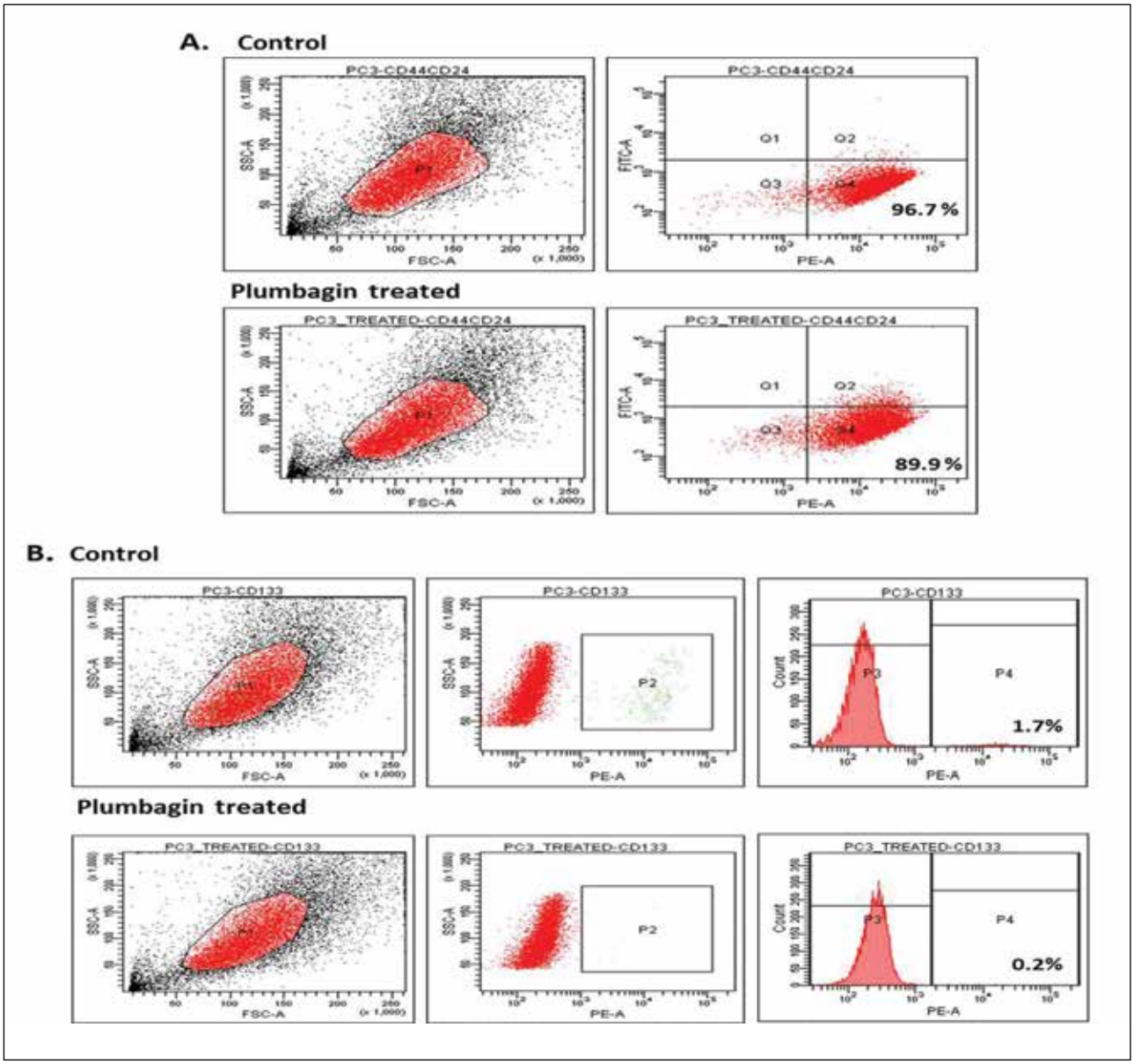

Figure 5

The subpopulation of prostatospheres expressing the stem cell surface marker CD44+/CD24- and stem cell surface marker CD133+ were found to be reduced on treatment with Plumbagin (Figure 5). A decrease in expression of CD44+24- cells as well as CD133+ cells shows the ability of Plumbagin to directly target cells having stemness properties.

In the present work, we demonstrate that treatment with Plumbagin results in a progressive dose dependent cytotoxicity affecting the sphere forming efficiency in prostate cancer cells. A subcytotoxic concentration of Plumbagin causes reduction in the expression of CD 44+/24- and CD133+ cells, the putative prostate cancer stem cells.

Reciprocal interface between BRCA1 defective breast cancers and Cancer Associated Fibroblasts in breast cancer aggression

(In collaboration with Arun Peter Mathew, Department of Surgical Oncology and Dr Thara Somanathan, Department of Pathology, Regional Cancer Centre, Thiruvananthapuram, Kerala).

Parallel to all other cancers the key challenge faced by the breast cancer (the deadliest female cancer) therapies, is the drug resistance acquired by the tumors. One of the decisive roles to this resistance is supposed to be played by the tumor stroma. Among the various stromal components, the altered fibroblast cells take up the major role of assisting the tumors for their progression and are known as Cancer Associated Fibroblast (CAF). Comparative gene expression profiling of tissue-derived normal fibroblasts (NFs) and CAFs and other approaches revealed that CAFs produce a variety of factors, which are lower or not expressed by the normal counterparts. Furthermore, CAFs play an important role in remodeling of the extracellular matrix by expressing a wide variety of matrix-components and matrix-remodeling enzymes such as neuron glial antigen (NG2), tenascin C, type I collagen, fibronectin, or MMP-1/stromelysin-1. We hypothesize that the mutations in the tumor suppressor gene, BRCA1 in the breast cancer cells may activate the CAFs to become more aggressive phenotype called MAF which reciprocates its action in breast cancer aggression. For the study we isolated CAFs and NFs by enzyme digestion method from breast cancer patients who have undergone primary surgery. These stromal fibroblast cells were analyzed for their reciprocal interaction with the breast cancer cells by in vitro co-culture method. Indirect co-culture followed by various assays for analyzing the proliferative, migrating and invasive potential revealed that the breast cancer cells with accumulated mutations particularly BRCA1 mutations are potent in activating CAFs and thereby aggravating the breast cancers leading to metastasis. Further we did a quantitative real time PCR analysis of the co-cultured samples for proliferative and metastatic markers, where we found an upregulation of different relevant genes including metastatic proteins CCL5, angiogenic marker SDF1 and the myoepithelial marker ĸSMA (Figure 6 and 7). Further we will be confirming all our observations by expression studies using western blotting. From the results what we have obtained we may conclude that activated stromal fibroblasts are highly influenced by the breast cancers with the accumulated mutations.

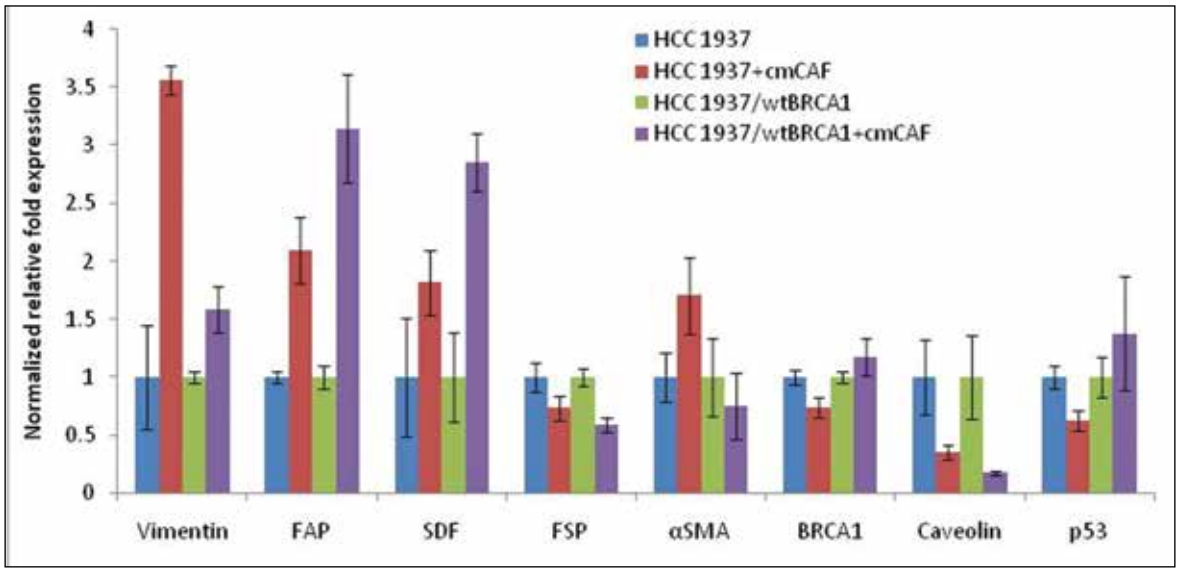

Figure 6

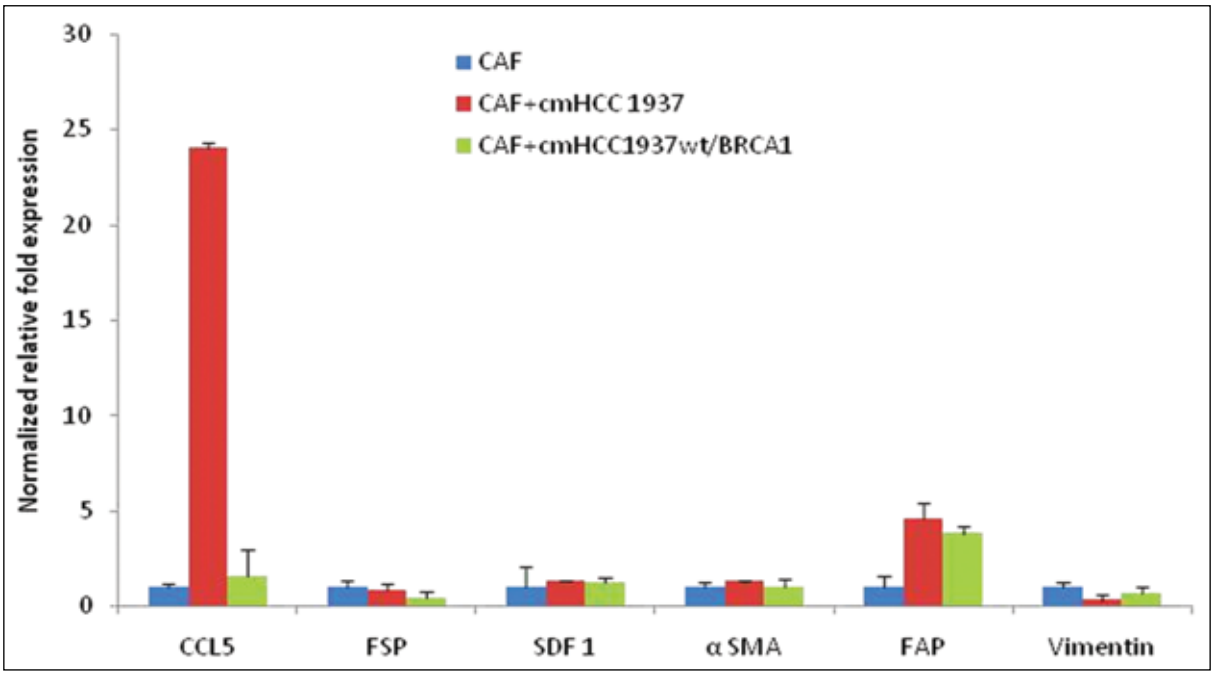

Figure 7

Role of β hCG in triple negative breast cancer: An in vitro study

Satheesh Kumar S and Priya Srinivas

The role of estrogen and progesterone in breast cancer has been studied in detail. Despite the absence of hormonal receptors, triple negative cancers are highly aggressive. It is possible that instead of estrogen and progesterone there could be other factors which could promote the aggressiveness. We analyzed the possible involvement of β-hCG in inducing EMT in triple negative breast cancer. Since epithelial to mesenchymal transition is the prime event for metastasis of any cancer cells, we checked whether EMT is induced in the presence of β-hCG in both HCC1937 and HCC1937/wt BRCA1. The mesenchymal marker, vimentin and the epithelial marker, E-cadherin was assessed to check EMT. In HCC1937, the expression of vimentin is significantly increased in the presence of β-hCG as evidenced by the levels of mRNA and protein (Figure 8). Also the expression of E-cadherin is decreased at mRNA and protein level in the presence of β-hCG. In addition, exogenous supplementation of β-hCG increased the expression of vimentin and decreased the expression of E-cadherin. Also upon silencing endogenous β-hCG, the expression of vimentin is decreased, though significant difference in the expression of E.cadherin was not observed. Similarly in HCC1937/wt BRCA1, though the expression of vimentin is decreased and the expression of E-cadherin is increased at mRNA level, the expression of vimentin is increased and the expression of E-cadherin is decreased at protein level in the presence of β-hCG. In addition, exogenous supplementation of β-hCG increased the expression of vimentin and decreased the expression of E-cadherin. Also upon silencing endogenous β-hCG, the expression of vimentin is decreased, though significant difference in the expression of E cadherin was not observed. All these observations clearly indicate that β-hCG induces the migratory and invasive potential by promoting the process of EMT in HCC1937.

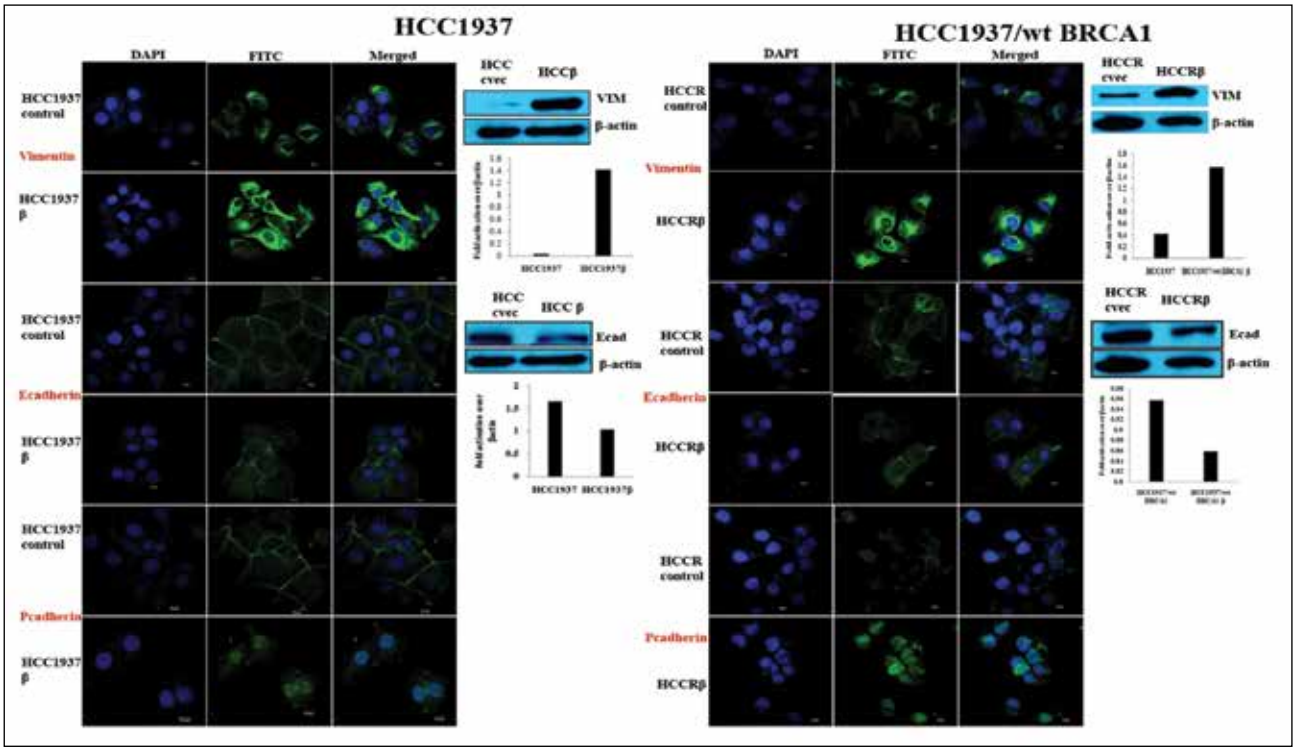

Figure 8

β-hCG and BRCA1 in Gestational Trophoblastic Diseases

Revathy, Krishnapriya RS, Sreevidya P S and Priya Srinivas

(In collaboration with Nirmala C, Department, Department of Obstetrics & Gynecology, SAT Hospital, Medical College, Thiruvananthapuram, Jayshree V Vaman, Department of Obstetrics & Gynecology, T D Medical College, Alappuzha, Santha Sadasivan, Department of Pathology, Medical College, Thiruvananthapuram, Aysha P V, PRS Hospital, Thiruvananthapuram, Balaraman Nair, DDRC SRL Diagnostics, Thiruvananthapuram and Anil Kumar T V, Sree Chitra Tirunal Institute for Medical Sciences & Technology)

Gestational Trophoblastic Diseases (GTD), encompasses an intriguing group of inter-related diseases derived from placental trophoblasts and is characterized by the abnormally elevated levels of β-hCG. The disease is of high incidence in South-East Asia esp India, with a high incidence rate of 5.1/1000 deliveries in Kerala (Sekharanet. al, 2006 &Lybolet.al, 2011). Two of the cohort studies show antagonistic reports about the role of β-hCG exposure to the breast cancer incidence, where in hCG exposure during hydatidiform moles were shown to increase the breast cancer risk (Erlandssonet. al, 2000) and the second study shows that it decreases the breast cancer risk (Gudipudiet. al, 2008). But till date, no studies have been done to analyze this correlation at the molecular level. Also, Though BRCA1 plays a significant role in DNA repair pathways, its particular role in cancer development especially in breast/ovarian cancers is still not known completely. Various positive and negative modifiers of BRCA1 have been identified, the best characterized stimulant being estrogen, thus controlling proliferation and malignancies of hormone regulated tissues like breast, ovary and prostate. In this study, we aim to analyze a possible link for the β-hCG expression to the breast cancer incidence at the molecular level by trying to look into the expression levels of BRCA1, which is a

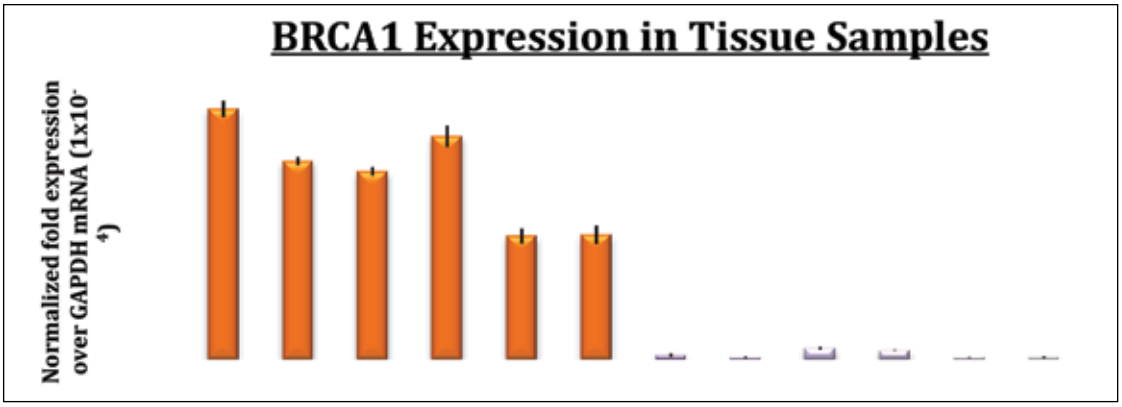

Figure 9

prime tumor suppressor gene associated with the breast cancers. Also, β-hCG being an important player in the scene of very many cancers makes it relevant to study its interactome with the major tumor suppressors/ oncogenes/cell cycle regulators. With all the above factors into consideration, GTD was selected the ideal system for our study. Unraveling the etiopathology of GTD, would help in the prediction of malignant potential and recurrence of the disease and also to improve its treatment modalities. Our studies show the down-regulation of BRCA1 in GTD as compared to the first trimester normal placental controls which are consistent with the cell line studies as well. BRCA1, being a protein with prominent nuclear function, shows a cytoplasmic and cytoplasmic membrane localization in GTD samples as seen in the immunohistochemical and immunocytochemical analysis. The levels of BRCA1 in GTD and normal placental controls were correlated with the serum b-hCG levels. Correlation of b-hCG levels with the BRCA1 level was done in cell lines too by analysing the secretory b-hCG levels by ELISA and BRCA1 by RT PCR (Figure 9, 10, 11). The pathways leading to the altered localization of BRCA1 are currently analyzed in the samples and are in progress. The cytoplasmic localization of the BRCA1 protein in placenta and GTD could also be contributing to the increased proliferation rate of the trophoblastic cells so present in both the tissue samples. This study aiming to correlate BRCA1 and b-hCG in cancers would improvise the treatment modalities not only for GTD but also for BRCA1 related cancers. Also new strategies can be developed to identify the high risk group or recurrent GTD cases/BRCA1 related cancers which would be helpful to the society.

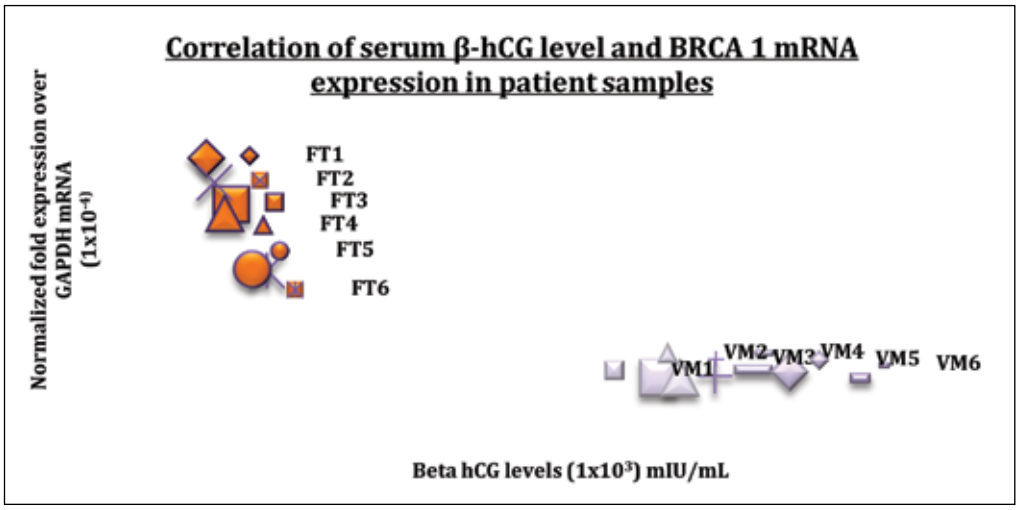

Figure 10

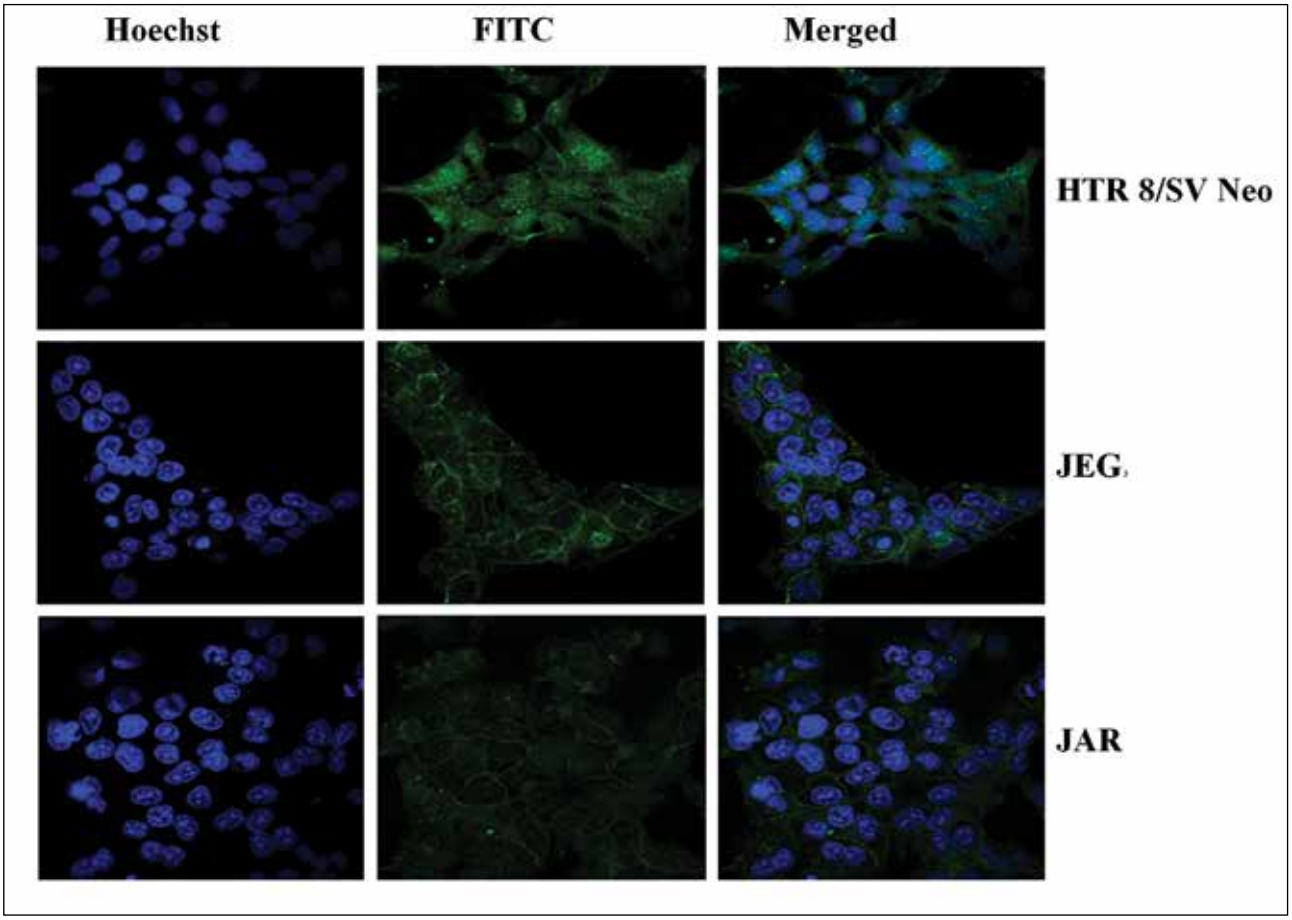

Figure 11

CANCER RESEARCH PROGRAM

LABORATORY - 6

S Sreeja

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Sreeja received PhD in Biotechnology from University of Kerala for work on molecular mechanisms of estrogen action and joined RGCB in 2001

Ph.D Students:

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Lakshmi . M. L

Research Fellows

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Juberiya. M. Azeez
Vini Ravindran
Viji Remadevi

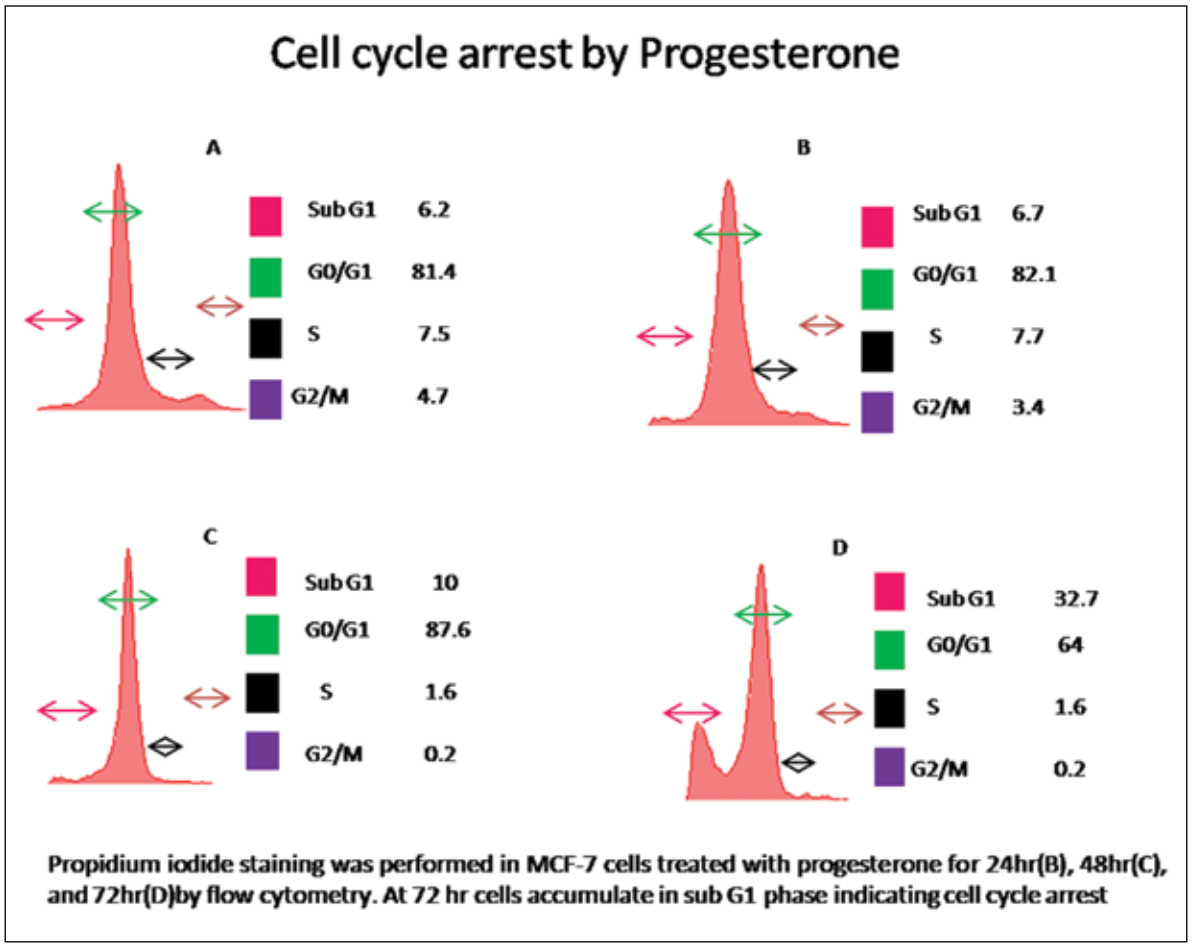
Laboratory Assistant

Savitha R.K

Progesterone regulates the proliferation of breast cancer cells

Juberiya . M. Azeez, Hima Sithul, Viji Remadevi, S Sreeja and M Radhakrishna Pillai

Different studies demonstrated a biphasic progesterone response in breast cancer cells, consisting of an initial proliferative burst, followed by sustained growth arrest. The present study identified Tob-1 as a target for progesterone-mediated signaling through the progesterone receptor, significantly influencing p53, PTEN and p27 expression and down-regulating cyclin D1 and anti-oxidant enzymes. The unique cascade identified from this study appears to result in breast cancer growth inhibition. From our earlier studies we identified differentially expressed genes in each menstrual cycle phase by microarray then were subjected to functional in vitro analyses. Microarray studies disclosed genes that are up-regulated in the luteal phase and follicular phase. TOB1 is a tumor suppressor gene and was expressed exclusively in the luteal phase in our microarray study. Therefore, we further functionally characterized the protein product of TOB1 in vitro. Our studies demonstrate that progesterone can produce reactive oxygen species in MCF-7 cells and the protein product of TOB1 exerts a series of non-genomic interactions, which regulate anti-proliferative activity through modulating the anti-oxidant enzyme MnSOD. Furthermore, this study implicates PTEN as an interacting partner for Tob1, which may regulate the downstream expression of cell cycle control protein p27 by multiple downstream signaling pathways of progesterone through progesterone receptor.



Propidium iodide staining was performed in MCF-7 cells treated with progesterone for 24hr(B), 48hr(C), and 72hr(D)by flow cytometry. At 72 hr cells accumulate in sub G1 phase indicating cell cycle arrest

Estrogen mediated regulatory effect of UCP 2 in Papillary Thyroid Cancer cells

Hima.S, Lakshmi. M. L, Vini Ravindran and Sreeja. S.

Thyroid diseases are more prevalent in women, particularly between puberty and menopause. Carcinomas of the thyroid are three-times more common in women than in men, and the peak rates occur earlier in women. These epidemiological data suggest a role of estrogen in the pathogenesis of thyroid diseases. Oxidative stress is postulated as one of the mechanisms underlying the estrogen carcinogenic effect in Thyroid cancer and estrogens are known to augment mitochondrial-derived reactive oxygen species (ROS) by an unidentified mechanism. Recent studies suggest that UCP2, is up regulated in a number of aggressive human cancers. One mechanism of UCP2 up regulation in these cancers is due to oxidative stress, and elevated UCP2 in turn reduces oxidative stress, which provides a growth advantage for these cancers. In this study we investigate the regulatory effect of estrogen on UCP 2 which may provide a new insight for the reason why thyroid cancer occurs three times more often in females than in males, and the occurrence decreases after menopause. On silencing UCP 2, we found out that Mito ROS level was increased considerably in PTC cells. It seems UCP2 moderates ROS level via Estradiol in Mitochondria. Western Blot Analysis UCP2, NCoR1 and SRC 1 on Nthy and PTC cells after 10nM E2 treatment for 48 hrs along with Control (Fig No 1 A & B)

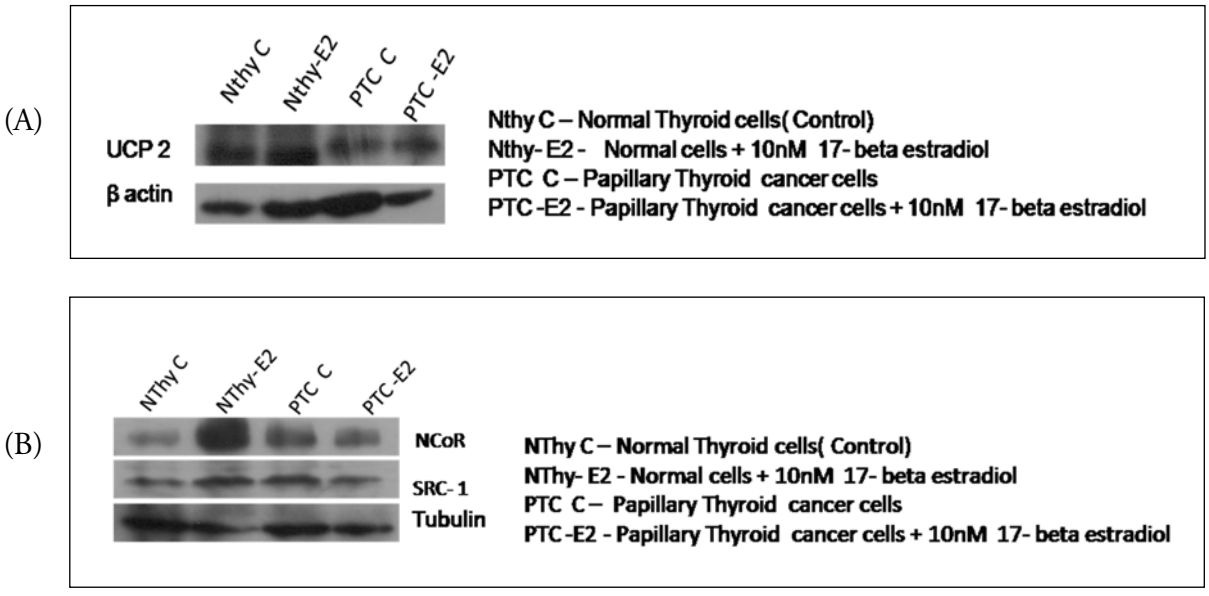


Fig .No: 1

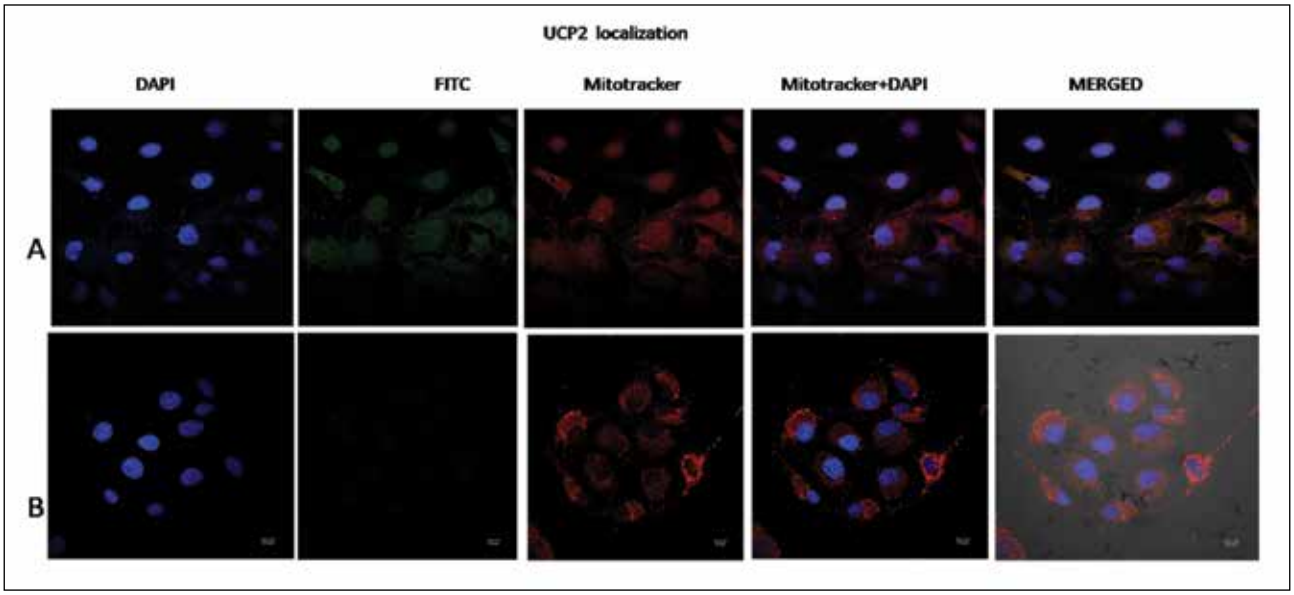


Fig: 2 The cellular localization of UCP2 in Nthy and PTC cells by immuno fluorescent staining with ± 10 nM E2 for 48 hr treatment.

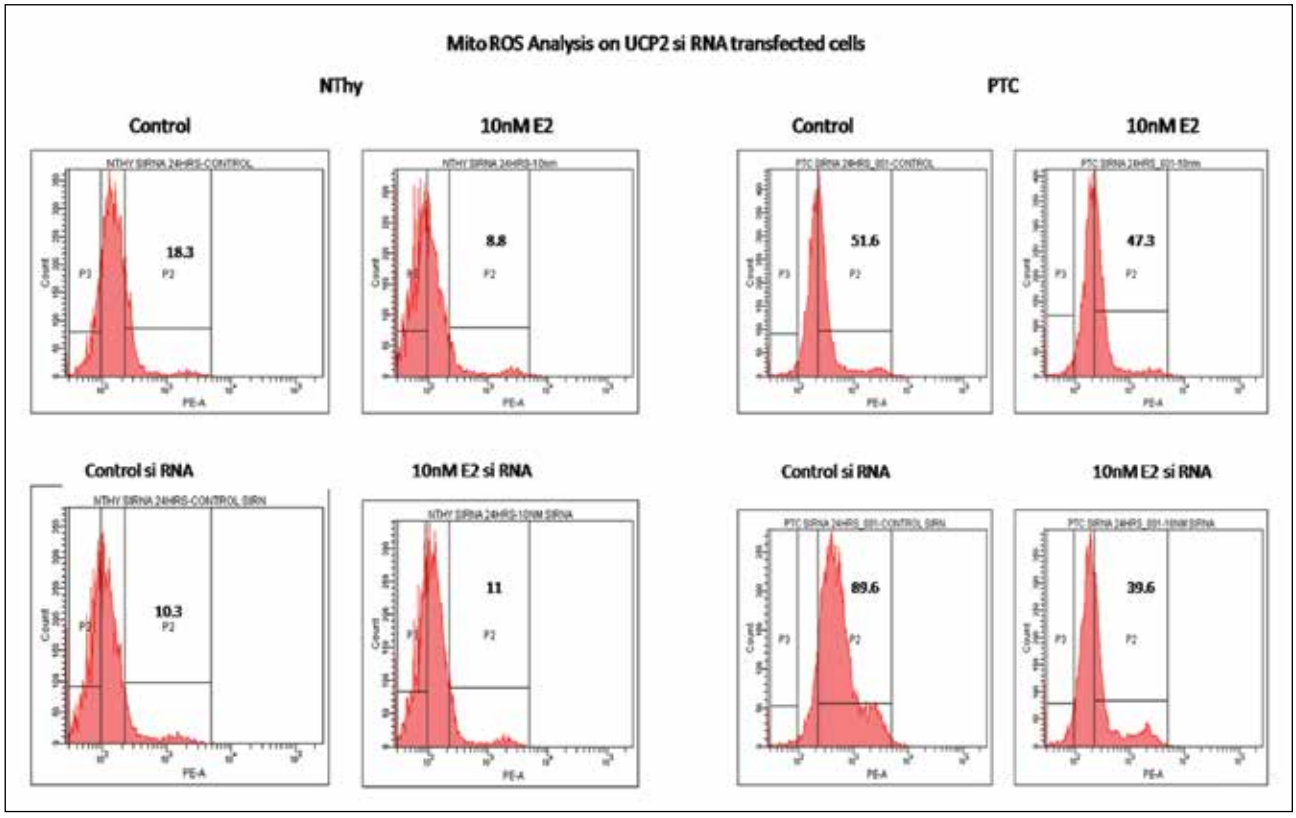


Fig No: 3 - Mito ROS Analysis in UCP2 si RNA transfected cells

Modulatory effect of Pomegranate extract in Endothelial cells

Vini Ravindran, Juberiya M. Azeez and S. Sreeja

Pomegranate an ancient and unique fruit is known to have wide therapeutic applications. Earlier studies from our laboratory proved that methanolic extract of pomegranate could act as a SERM by competing with estradiol in breast cancer cells and reducing the proliferative effect of estrogen ,at the same time have its beneficial effects in other hormone dependent tissue.Further on we also found that administration of PME could bring down the LDL cholesterol and triglycerides in ovaractemized mice .High serum levels of LDL has a major role in development and progression of atherosclerosis, disease of arterial wall. Hence studies were done in vitro in Ea.hy 293 cell line to further understand the possible molecular mechanism of methanolic extract of pomegranate in the critical events like apoptosis, oxidative stress which are associated with atherosclerosis. Since the vascular endothelium, the inner lining of the blood vessel, is involved in various physiological processes, endothelial cell apoptosis (and dysfunction) may constitute an initial step in the pathology of atherosclerosis. Other risk factors of the disease include lipid and non-lipid variables, thrombogenic/hemostatic factors (eg, fibrinogen), and inflammatory markers small, dense low-density lipoprotein particles, oxidized low-density lipoprotein, and apolipoprotein B, oxidative stress etc vascular wall inflammation. Experiments performed to understand the role of PME in TNF α induced apoptosis of endothelial cells clearly indicated that PME could rescue a population of cells from TNF-α induced apoptosis (Fig 1). Experiments with cell ELISA indicated that PME could also down regulate the TNF-α induced expression of adhesion molecules such as VCAM-1, ICAM-1 which plays a critical role in the atherosclerosis. Endothelial dysfunction might also be caused by acute and oxidative stress and leads several cardiovascular disorders. PME could reduce the level of oxo-20-deoxyguanosine levels, which is an important marker of oxidative tissue injury. This clearly indicated absence of DNA adduct formation in endothelial cells. DCFH-DA staining of PME pretreated cells also indicated a down regulation in ROS levels, which is one of the important determinant of oxidative damage to cells.

PUBLICATIONS

- *Ravindran Vini and S.Sreeja,* Punica granatum and its therapeutic implications on breast carcinogenesis : A review. *BioFactors 04/2015; 41(2).DOI;10.1002/biof.1206*

CONFERENCE PRESENTATIONS

- *Vini Ravindran, Juberiya M Azeez and S.Sreeja*, Poster on " Modulatory Effect of Pomegranate extract on Endothelial cells" in *Indo-Cannadian Symposium on Heart Failure Progress and Prospects at Rajiv Gandhi Centre for Biotechnology, Trivandrum(12 th – 14 th March 2015)*
- *Juberiya M Azeez and S.Sreeja,* Oral presentation on "Pomegranate fruit, a natural estrogen selective modulator" at the *UGC sponsored National seminar on cancer-A multi Disciplinary Approach at Kannur University, Dr. P.K.Rajan Memorialcampus, Kasaragod 19th to 21st sept 2014*
- *Lakshmi ML,* Oral presentation on " Mechanistic study of estrogen signaling and reactive oxygen species i papillary thyroid cancer cells" at the *UGC sponsored National seminar on cancer-A multi Disciplinary Approach at Kannur University,Dr.P.K.Rajan Memorial campus,Kasaragod 19thto21st sept 2014*
- *Parvathy Muraleedharan, Sreeja S, MR Pillai and Rakesh Kumar.* P21 Activated Kinase 1 (PAK1) and its substrates are potential therapeutic targets of Oral Cancer , *Indian Genetics Congress (IGC 2015) , Chennai , March 2015.*

AWARD

- *Parvathy Muraleedharan, Sreeja S, MR Pillai and Rakesh Kumar.* P21 Activated Kinase 1 (PAK1) and its substrates are potential therapeutic targets of oral cancer,Indian Genetics Congress (IGC 2015), Chennai, March 2015,(Young scientist Award)

EXTRA MURAL FUNDING

Sl. No.	Investigators	Title	Funding Agency	Duration
1	S. Sreeja	An in-vitro investigation on the role of Estrogen and Reactive Oxygen Species in the invasion of Thyroid Cancer cells with emphasis on TGF – beta signaling	Kerala State Council for Science, Technology and Environment	2013-2016

CANCER RESEARCH PROGRAM

LABORATORY - 7

K B Harikumar

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Harikumar obtained his PhD from the Mahatma Gandhi University while working at Amala Cancer Research Centre. He trained as a Post Doctoral Fellow at MD Anderson Cancer Centre, Houston, USA and then at Virginia Commonwealth University, Rchmond, USA. Harikumar is a recipient of the Department of Biotechnology's Ramalingawamy Re-entry Fellowship.

PhD Students

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Manedra Babu L

Research Fellows

Shirly James
Aparna JS

Spice derived nutraceuticals for colorectal cancer chemoprevention

Shirly James, Aparna JS, Sabira Mohammed and K.B.Harikumar

Colorectal cancer (CRC) is the third most common cancer in men and second in women worldwide. There is a considerable interest in the pharmacological effects of nutraceuticals for cancer treatment and prevention. In this project we mainly focus on nutraceuticals derived from spices. One of the compound is Cardamonin (2E)-1-(2,4-Dihydroxy-6-methoxyphenyl)-3-phenyl-2-propen-1-one, (8CI); Alpinetin chalcone, (E)-2κ 4κDihydroxy-6κmethoxy-chalcone). The compound belongs to the class of chalcones. Azoxymethane induced colorectal cancer in mouse was used as a model system to study the anticancer potential of cardamonin. Oral administration of cardamonin significantly decreased the tumor formation as seen from reduced tumor load and tumor multiplicity and fewer numbers of tumors. Most tumors were small in size in cardamonin treated groups. We are are analysing the molecular mechanisms behind the anti-carcinogenic action of cardamonin.

Nitroxoline: A novel agent for colon cancer chemoprevention:

Manendra Babu L, K.B.Harikumar

We are also interested in identifying new candidates for cancer therapy using drug repurposing approach. Nitroxoline is an FDA approved antibiotic against gram-positive and gram-negative organisms mostly responsible for urinary tract infections. Our initial studies revealed that this nitroquinoline class of compound has anticancer potential. We tested the efficacy in array of colon cancer cell lines and found that this compound inhibited the growth. The IC50 is in the range of 0.73-2.5µM/ml. Moreover Nitroxoline found to potentiate the effect of capecitabine (a standard of care for colon cancer). Further studies are ongoing to understand the mechanism of action.

PUBLICATIONS

- *Oskeritzian CA, Hait NC, Wedman P, Chumanevich A, Kolawole EM, Price MM, Falanga YT, Harikumar K B, Ryan J J, Milstien S, Sabbadini R, Spiegel S.* The sphingosine-1-phosphate/sphingosine-1-phosphate receptor 2 axis regulates early airway T-cell infiltration in murine mast cell-dependent acute allergic responses. *J Allergy Clin Immunol. 2015 Apr; 135(4):1008-18*

CONFERENCE PRESENTATION

- *Shirly James, Manendra Babu L, Aparna JS and Harikumar KB.* Sesamin: a novel agent for colorectal cancer chemoprevention. *International conference on molecular pathways to therapeutics: Paradigms and challenges in Oncology. February 11-13th, 2015. ACTREC, Navi Mumbai.*

TRAINING

- Harikumar K.B: Zebrafish Development and Genetics Course at Marine Biological Laboratory, Woods Hole MA, USA. August 2014.

RESEARCH GRANTS EXTRA-MURAL FUNDING

K.B.Harikumar- Principal Investigator

No	Title	Funding Agency	Duration
1	Spice derived phytochemicals Sesamin and Cardamonin- for colorectal cancer chemoprevention	Department of Biotechnology -Ramalingaswami Fellowship	2012-2017
2	Novel aspects of sphingosine 1-phosphate (S1P) in innate immune responses and host defense mechanism	Department of Science and Technology, Government of India	2013-2016
3	Sphingosine 1-phosphate signaling in pancreatic cancer	Department of Biotechnology, Government of India	2015-2017

CANCER RESEARCH PROGRAM:

LABORATORY - 8

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Debasree has an MTech and PhD in Biotechnology from Jadavpur University. She trained as a Post Doctoral fellow from 2007 to 2011 at the University of Kansas Medical Center in USA and joined RGCB in 2011.

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Sunu Joseph

Ph.D students

Syed Khaja Mohieddin

Role of Histone chaperones in inducing pluripotency

Syed Khaja Mohieddin, Sunu Joseph and Debasree Dutta

The generation of induced pluripotent stem cells (iPSCs) from somatic cells demonstrated that adult mammalian cells can be reprogrammed to pluripotent state with the enforced expression of different transcription factors. In continuation from the last year work, we generated iPSCs from histone chaperone APLF knocked-down MEFs and control ones. The time and efficiency of generation of iPSCs was enhanced in downregulation of APLF (Fig. 1).

We passaged the iPSCs for around 30 times and checked whether they retained the in vitro and in vivo potential of differentiation into different lineages. The iPSCs can differentiate into endoderm, mesoderm and ectoderm and generated teratoma as well (Fig. 2).

The histone chaperone APLF is associated with DNA damage and repair mechanism. So we determined the status of the iPS cells when exposed

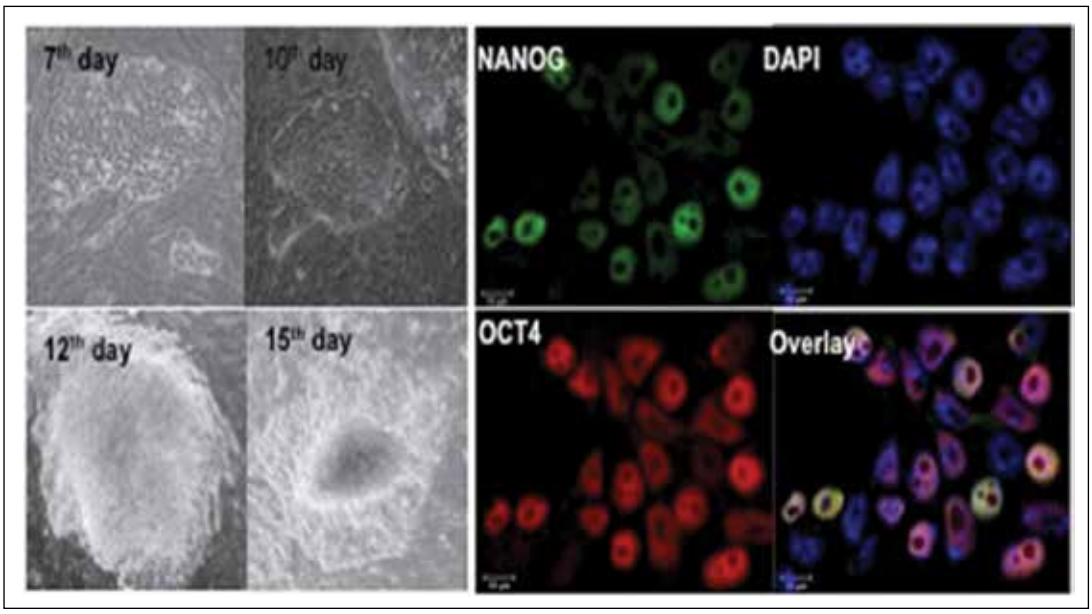


Figure 1. Generation of iPSC colony from MEFs (left panel). IF study for the expression of pluripotent marker in iPSC colony (right panel).

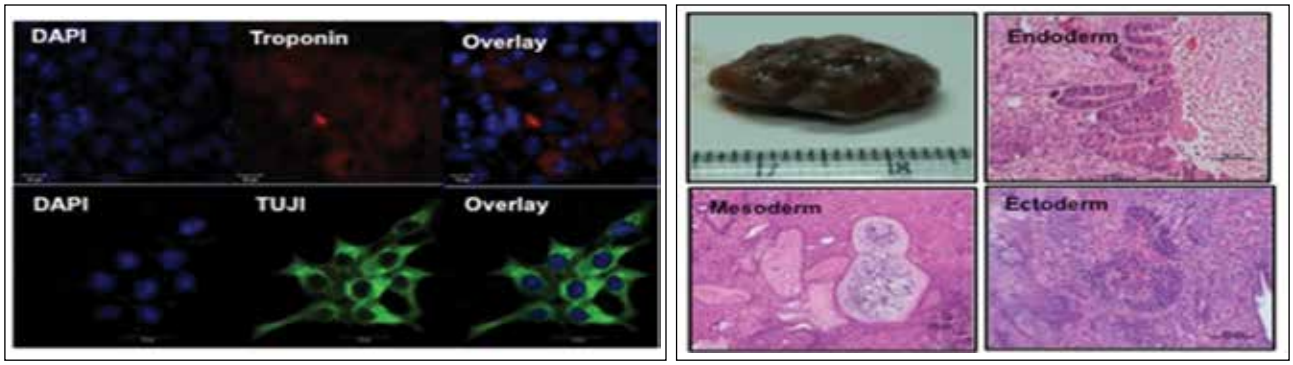
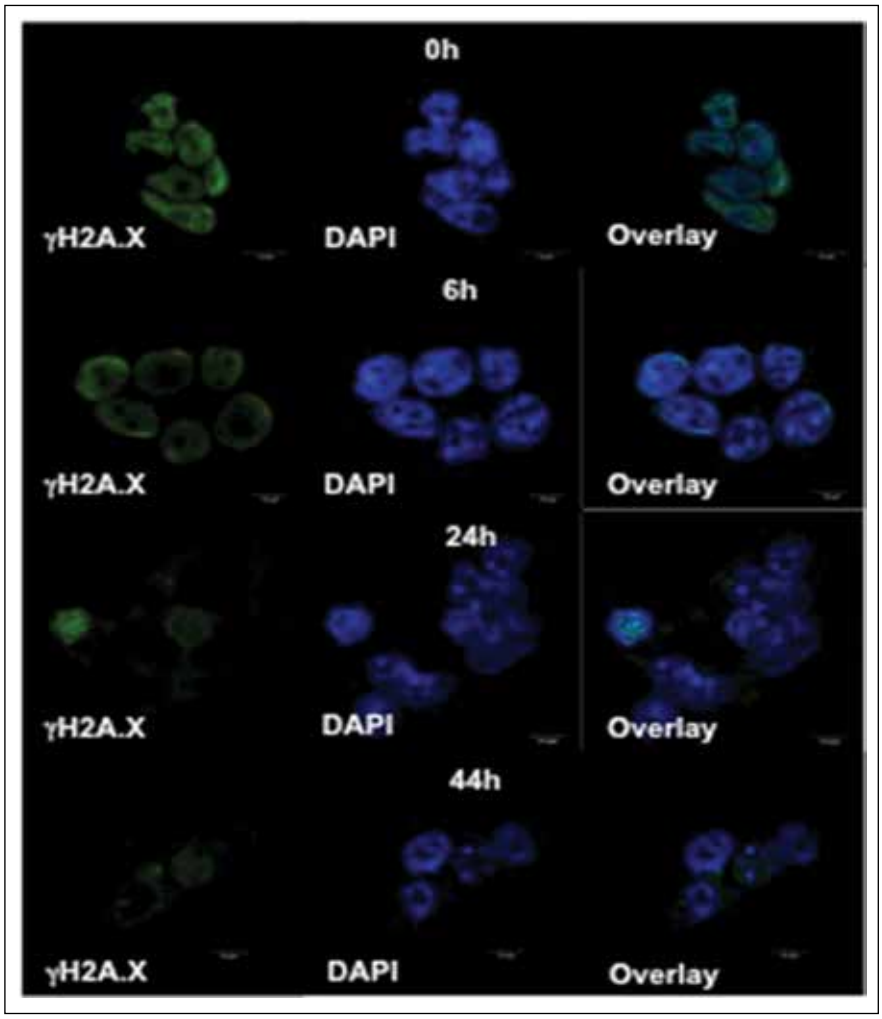


Figure 2. Differentiation potential of iPSCs. IF study for the expression of mesoderm (troponin) and ectoderm (TUJ1) marker (upper panel). Teratoma formation from iPSCs and HE staining for different germ layers in the teratoma (lower panel).



to DNA damaging agent like etoposide. We observed that there is no significant difference in knocked-down iPS cells in comparison to control ES cells. Hence, this extent of knockdown did not compromise the genomic stability or the repairing mechanisms of the cells (Fig. 3).

Figure 3. DNA repair mechanism retained in the APLF-knocked-down iPSCs.

Hemogenic endothelium- regulation and reprograming

Aditi Majumder, Syed Khaja Moheiddin & Debasree Dutta

Hematopoietic stem cells originate from Hemogenic endothelium, composed of endothelial cells. At the molecular level, transcription factor RUNX1 is indispensable for the transition towards HSCs generation. It is very important to study the regulation of RUNX1 to understand the regulation of hemogenic endothelium and the induction of genes implicated in hematopoiesis. So, we investigated the role of Histone chaperones, one of the constituent of the nucleoprotein structure, on they can modulate the regulation of RUNX1. Till last report, we observed that in absence of HIRA, Runx1 is significantly downregulated both in the context of development of embryoid bodies (Fig. 4, upper panel) or in the generation of hematopoietic precursors (Fig. 4, lower panel).

To understand whether absence of HIRA is responsible for the downregulation of Runx1 expression, we ectopically expressed HIRA in null

ES cells and differentiated them towards Hemogenic endothelium or HE. We observed the expression of HIRA in those cells along with the expression of RUNX1 (Fig. 5, upper panel).

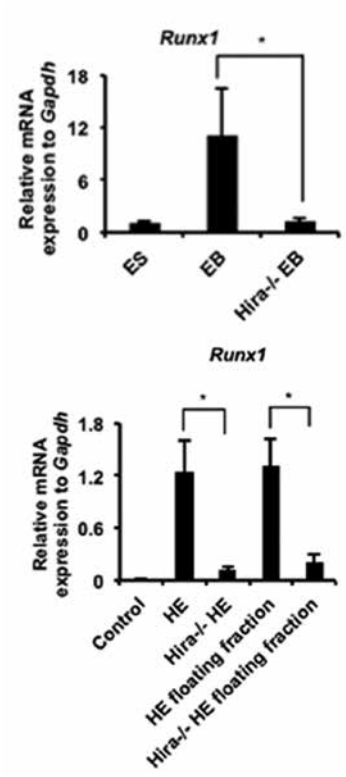


Figure 4. HIRA dependent Runx1 expression

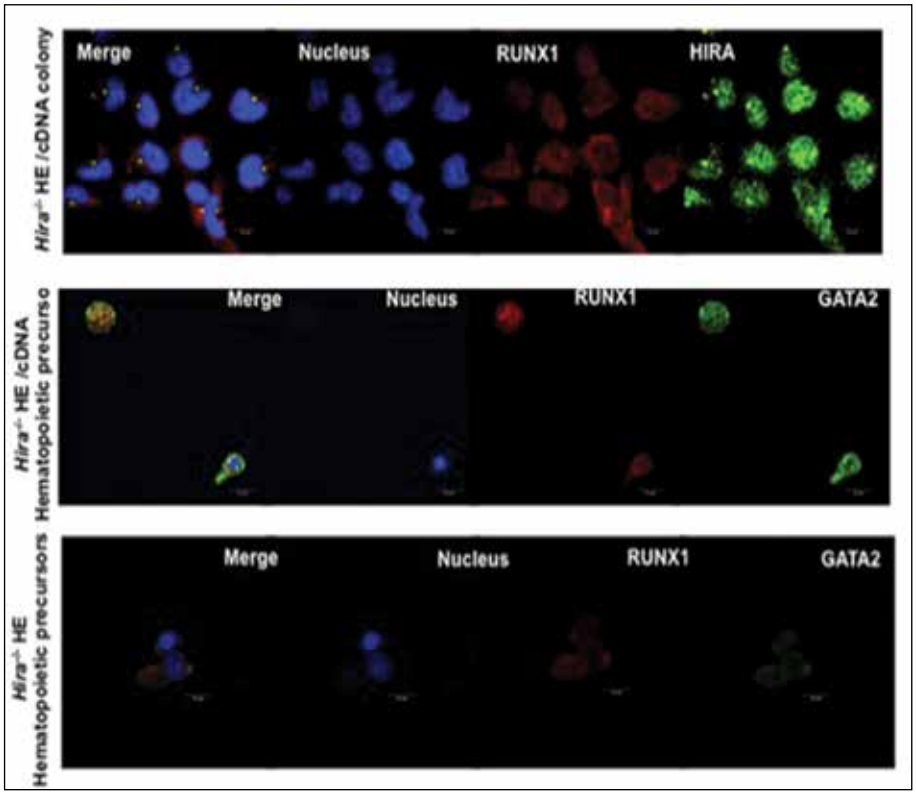


Figure 5.HIRA dependent expression of hematopoietic marker in hematopoietic precurssors

We observed that the hematopoietic precursors devoid of HIRA did not expressed GATA2, a hematopoietic marker, whereas in ectopically expressed HIRA cells, GATA2 was expressed (Fig. 5 lower panels).

We also observed that in absence of HIRA, RUNX1 failed to get recruit within its downstream targets as well regulatory regions of targets responsible for the regulation of definitive hematopoiesis (Figure 6). Physically, HIRA interact with RUNX1 and HIRA along with BRG1 interacts with RUNX1 further to be recruited to its targets

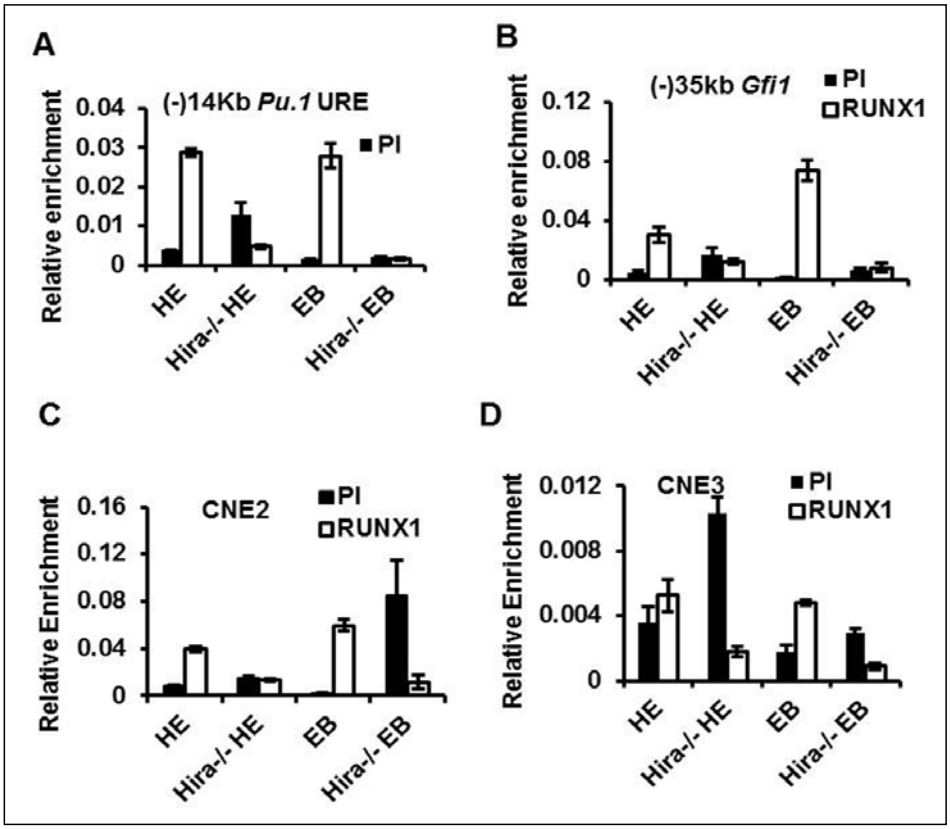


Figure 6. Recruitment of RUNX1 within targets implicated in definitive hematopoiesis

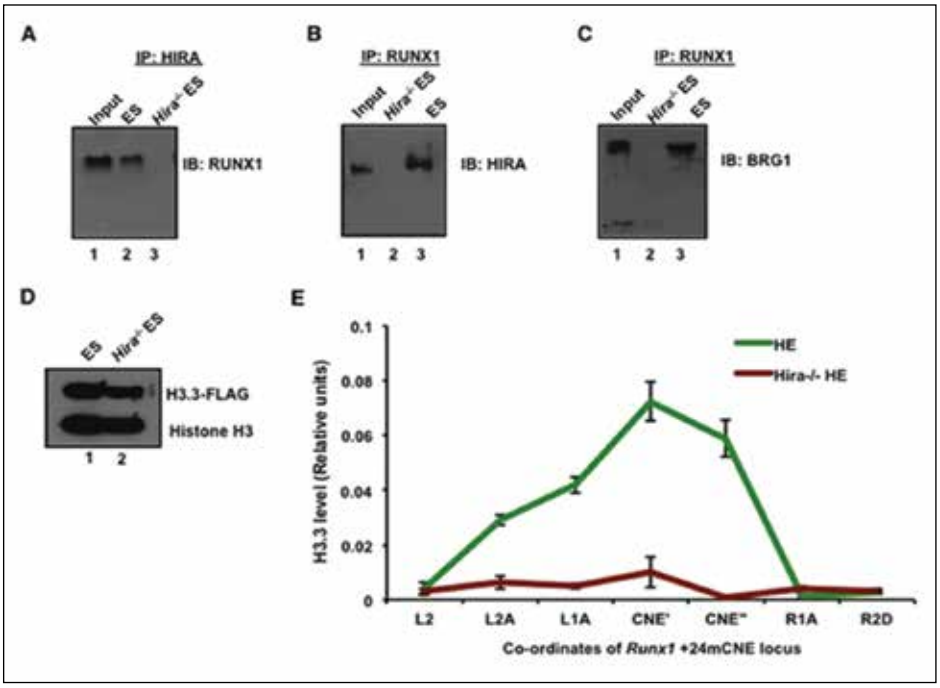


Figure 7. HIRA interacts with RUNX1 and incorporates H3.3 for the activation Runx1 during

(Fig. 7a-7c). During hemogenic to hematopoietic transition, the +24 mouse CNE region of Runx1 locus is activated and we observed that a HIRA dependent incorporation of Histone H3.3 variant (Flag-tagged H3.3 have been used for the study) occurs (Fig. 7c, 7d) within the locus. But, in absence of HIRA, the incorporation is significantly abrogated (Fig. 7c, 7d).

Next, we analysed the HIRA-RUNX1 axis in the context of leukemia. RUNX1 is also termed as AML1 or acute myeloid leukemia 1. We used the AML1/ETO fusion Kasumi-1 cells, harbouring the t(8:21) translocation, for the role of HIRA. We observed that on downregulation of Hira, Runx1 was downregulated whereas the erythroid marker Gata1 was upregulated (Fig. 8B, 8C). This data can be further exploited to use HIRA as a novel target for therapeutic approach.

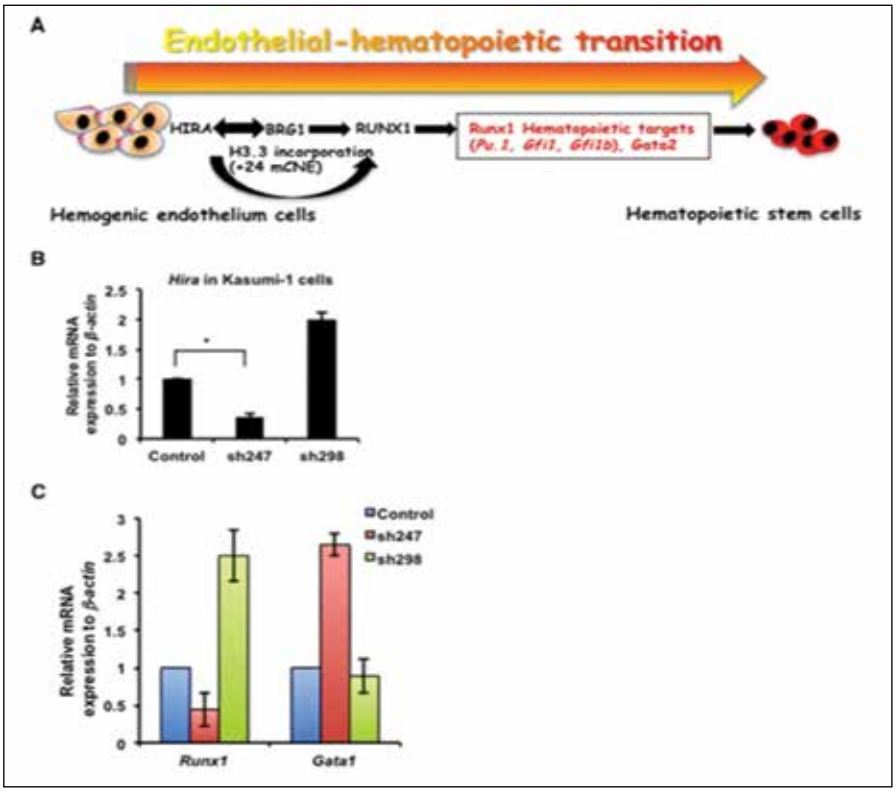


Figure 8. HIRA regulate RUNX1. HIRA-RUNX1 axis in leukemia

PUBLICATION

- *Majumder A, Syed KM, Joseph S, Scambler PJ, Dutta D* (2015). Histone chaperone HIRA in Regulation of Transcription Factor RUNX1. *J Biol Chem. 290:13053-63*

CONFERENCE PRESENTATION

- Invited talk at Central University of Tamil Nadu, Thiruvarur on Feb. 26th 2015.

EXTRA-MURAL FUNDING

DEBASREE DUTTA- PRINCIPAL INVESTIGATOR

No	Title	Funding Agency	Duration
1	Role of Histone chaperones in inducing pluripotency	Department of Biotechnology, Government of India.	2012-2015
2	Transcriptional regulation of VEGFR 3	Council for Scientific & Industrial Research, India.	2013-2016
3	Hemogenic endothelium-regulation and reprogramming	Department of Science & Technology, Government of India.	2013-2016

CANCER RESEARCH PROGRAM
LABORATORY– 9

Professor M Radhakrishna Pillai

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Radhakrishna Pillai joined RGCB in 2005 moving from the Regional Cancer Centre at Trivandrum where he was Professor of Molecular Medicine. Dr. Pillai is a Fellow of the Royal College of Pathologists, London, the National Academy of Medical Sciences, India, the National Academy of Sciences, India and the Indian Academy of Sciences.

Program Scientists

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Sajitha I S
Deepthi Prasad
Parvathy Balachandran
Rahul Sanawar

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Vivekanand A
Bijesh George

Randomized trial of 2 Vs. 3 doses of Human Papillomavirus vaccine – Early results from India

Priya R Prabhu, Janki Mohan Babu, Subha Sankaran, Kannan T R, Anurup K G, Rintu T Varghese, Edwin S, Jinu Austin, Ayswarya R S, Viji S, * R. Sankaranarayanan, *Masimmo Tomassino, *Tarik Gheit **Michael Pawlita, and M. Radhakrishna Pillai

Collaborators: **International Agency for Research on Cancer (IARC), Lyon, France and **German Cancer Research Centre (DKFZ), Heidelberg, Germany*

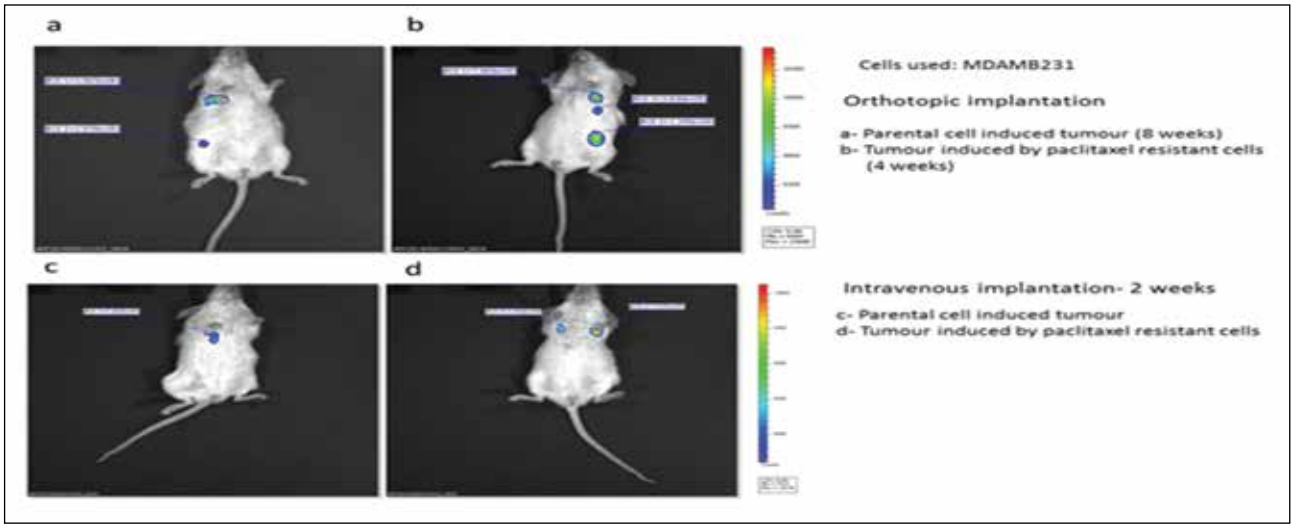
RGCB has been the bio-repository and analysis hub for the HPV vaccination trial initiated by International Agency for Research on Cancer (IARC) and funded by the Bill and Melinda Gates Foundation. The study begain in 2009, recruiting 20,000 unmarried girls aged 10-18 years from different geographical regions of India. The quadrivalent vaccine (Gardasil) containing HPV 16, 18, 6 and 11virus like particles (VLPs) provided free of cost by MERCK™ was administered to the participant girls of the study. After vaccination, blood samples were collected for evaluation of immune response at different time points and a speculum examination of the cervix and collection of cervical cells from these volunteers was done 18 months after their marriage or 6 months after delivery and one year intervals thereafter. The participating clinical centers of this study are Tata Memorial Hospital (Mumbai), Nargis Dutt Memorial Cancer Hospital (Barshi), Jehangir Clinical Development Centre (Pune), Christian Fellowship Community Health Centre (Ambilikai), Gujarat Cancer Research Institute (Ahmedabad), All India Institute of Medical Sciences (New Delhi), MNJ Institute of Oncology and Regional Cancer Centre- (Hyderabad) and Cancer Foundation of India (Kolkata). Volunteers in HPV vaccine trial were followed up for their prolonged antibody response at different time points starting from one month after they receive their last dose till 36 months after vaccination. They are now being followed up for further confirmation of the vaccine efficacy by ruling out infection due to HPV subtypes included in the vaccine by testing their cervical specimen. Our earlier studies proved beyond doubt that 2 dose was non-inferior to the 3 dose regimen in terms of the anti-L1 binding antibodies elicited upon vaccination. For further proof on the efficacy of 2 dose, we continued the study by measuring antigen-antibody binding avidities, which reflects the degree of affinity maturation in the B cells, assesses the quality of the antibody responses following the different dose regimes. Multiplex serology based on a glutathione-S-transferase (GST) capture immunosorbent assay combined with fluorescent bead technology, was modified to analyze antibody avidities using an additional washing step with chaotropic agent- urea, after incubation of sera with antigen-loaded beads. Antibody avidity was calculated as a percentage of ratio of MFI of urea-treated to untreated samples. The geometric mean avidity index, following the 2-dose schedule at 7 months and those for 2-dose, 2 dose/D and 1-dose/D schedules at 18 months, was non-inferior to that of the 3-dose regime. Besides, HPV neutralizing antibodies specific for neutralizing-epitopes in HPV-L1 protein were measured in collaboration with German Cancer Research Centre, DKFZ, Heidelberg and European Molecular Biology Laboratory, Heidelberg, using a highly sensitive, automated, purely add-on, high-throughput pseudovirion-based neutralization assay (PBNA) with excellent repeatability and run-to-run reproducibility. The purpose of the PBNA is to detect the presence of antibodies capable of inhibiting infection of HPV pseudovirions. Bovinepapillomavirus (BPV) pseudovirion assays were run as control to verify that the test serum is not toxic to the cells, which can mimic neutralization. The Lower Limit of Quantitation (LLOQ) for the HPV-PBNA is a

reciprocal dilution of 40. A sample was classified as sero-negative if the PBNA titre was <50; seropositive if the PBNA titre was ≥50 and ≥2 times the BPV titre; or sero-status indeterminate if the PBNA titre was ≥50 and <2 times the BPV titre. The GMT in the 2-dose group for HPV 16/6 was non-inferior to that of the 3-dose group, but was inferior for HPV 18. The GMT following 2-dose/D and 1-dose/D schedules for HPV 16/18/6 was inferior to that of the 3-dose group. Furthermore, collection of cervical cells and HPV testing and genotyping further confirmation of the vaccine efficacy by ruling out infection due to HPV subtypes included in the vaccine by genotyping their cervical cells. The HPV genotyping method involved HPV type-specific E7 PCR bead-based multiplex genotyping (TS-MPG). The multiplex HPV type-specific E7 PCR utilizes HPV type-specific primers targeting the E7 region for the detection of 19 high–risk (HR) / probable HR-HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68a,b, 70, 73, 82), and two low-risk (LR)-HPV types (HPV 6 and11), with detection limits ranging from 10 to 1000 copies of the viral genome. The frequencies and their 95% CI of incident and persistent infections with vaccine targeted HPV and vaccine non-targeted high-risk HPV types were based on the testing of samples from 2649 women and 2 or more samples from 838 women. The frequency of women with HPV 16/18 incident infection was similar across all the dose groups: 0.4% (2/543) in the 3-dose group; 0.8% (4/519) in the 2-dose group; 1.3% (9/717) in the 2-dose/D group and 1.1% (10/870) in the 1-dose/D group during a median follow-up of 4.6 years. There were no persistent HPV16/18 infections in the 4 study groups. The study is still ongoing with the participants being followed up for any further incident /persistent HPV infections by testing their cervical cells at one year intervals.

Elucidation of drug resistance mechanisms in cancer stem cells

Sajitha I.S., T.R. Santhosh Kumar and M. Radhakrishna Pillai

Chemotherapy is a main treatment mode for cancer. However its is usually limited by drug resistance. Multiple mechanisms are involved in acquired drug resistance, which include overexpression of membrane transporters, altered drug metabolism and drug targets. The persistence of cancer stem cells after chemotherapy, which may lead to repopulation of the original tumor is also thought to be associated with the relapse of the tumour. These cells have activated cell survival signaling pathways and inactivation of cell death pathways, which will ultimately lead to drug resistance. The present study aims to elucidate the signaling mechanism involved in drug resistance using both in vitro and in vivo models. Drug resistant cells were developed and both parental and drug resistant cells used for our in vivo study. Tumorigenicity study was done in SCID mice. The difference in the kinetics of tumour growth was analysed by in vivo bioluminescence/ fluorescence imaging, confirmed later by gross pathology and histopathogy. The drug resistant tumours were found to be more invasive and metastatic. We have also used proteomic analysis to identify signaling networks that determine the response of a drug and also to identify possible therapeutic targets that may help to overcome or bypass drug resistance. Preliminary evaluation has identified differential expression of metabolic and ER stress related proteins, conferring a survival advantage for the drug resistant cells. Results are being analysed by bioinformatics and confirmed by immunoblotting and immunohistochemistry.



Regulation of mTOR-DEPTOR axis in cervical cancer

Srinivas K P, Viji Remadevi, Vipin Mohan Dan, I S Sajitha, R Prakash, S Lakshmi*, TR Santhoshkumar and M Radhakrishna Pillai

**Collaborator: Regional Cancer Centre, Thiruvananthapuram*

Cervical cancer is the most common cancer among women in India, of which more than 90% are squamous cell carcinoma (SCC). Of these, almost all are a consequence of persistent high-risk human papillomavirus infection. DEPTOR (DEP domain-containing mTOR-interacting protein) is an endogenous *in vivo* inhibitor of mTOR complexes and is in general thought to be a tumor suppressor. Its overexpression was known to induce apoptosis in pancreatic cancer cells and its loss of function/expression has been detected in pancreatic tissues. However it is also known to be overexpressed and essential for the survival of multiple myeloma, thyroid and hepatocellular carcinoma cells, suggesting a dual nature of DEPTOR. The present study reveals a differential role of DEPTOR in cervical SCC and adenocarcinoma (AC). DEPTOR was found to be overexpressed in both cervical SCC cells and tissues and it's silencing in cervical SCC-derived SiHa and ME-180 cells induced caspase-dependent apoptosis, by inhibiting PI3K/AKT pathway via a feedback inhibition from mTORC1 and S6K. This inhibition resulted in the up-regulation of p53 and PUMA pro-apoptotic proteins, leading to apoptosis. DEPTOR silencing resulted in reduced expression of the nitric oxide synthases iNOS and eNOS, as well as increased activation of ERK1/2 and p38 MAP kinases. The reduction of NOS under DEPTOR silencing conditions clearly indicates and supports a role for NO in cervical carcinogenesis. The results also indicate that DEPTOR is known to regulate ERK in an AKT dependent manner. Reduction in DEPTOR levels in cervical SCC cells induced apoptosis via differential effects on PI3K/AKT, ERK and p38 MAP kinases. DEPTOR regulation was also observed to be independent of HPV E6 and E7 oncoproteins, but it might be a molecular co-factor contributing to cervical carcinogenesis. All these data indicate the complex role of DEPTOR in cell signaling machinery.

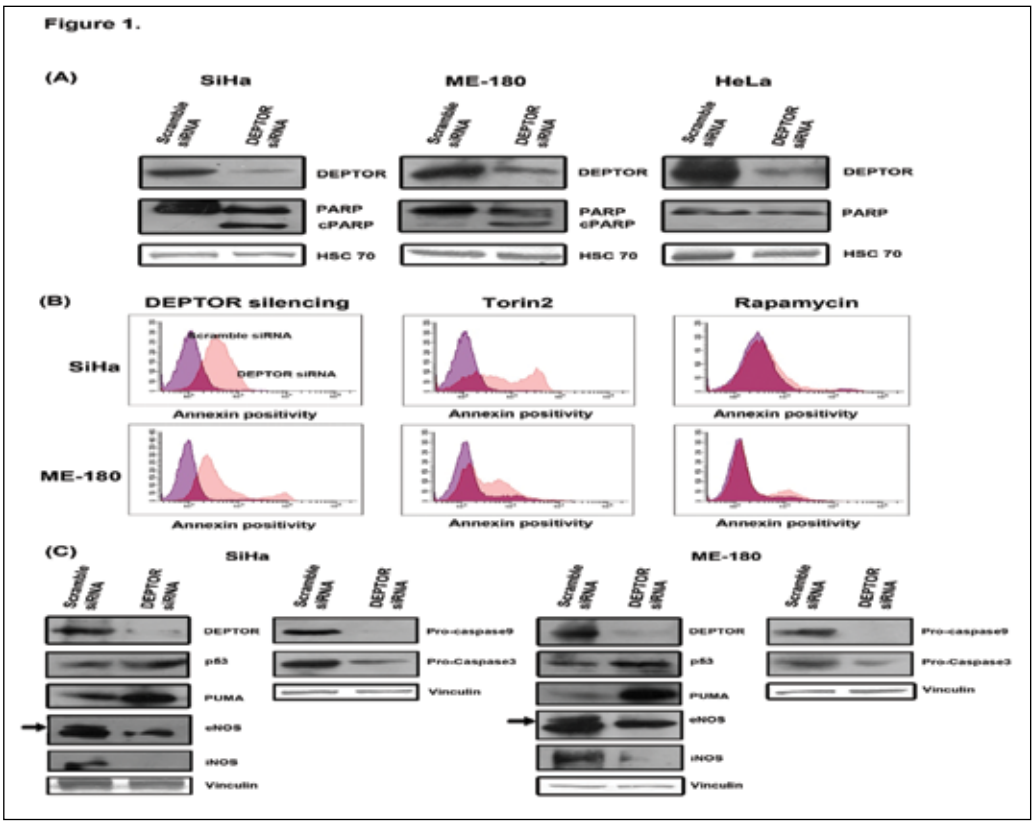


Fig.1: DEPTOR silencing induces apoptosis in cervical SCC cells by up-regulation of p53 and PUMA, and by down-regulation of nitric oxide synthases iNOS and eNOS.

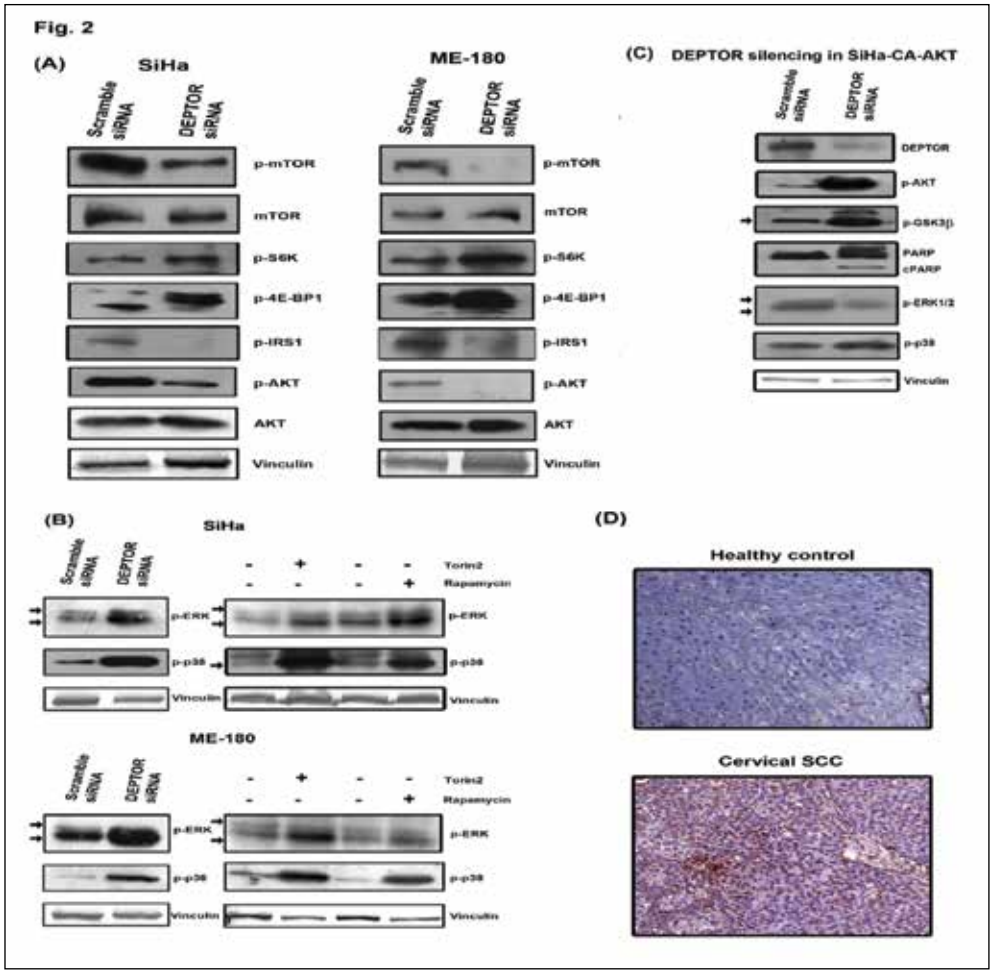


Fig.2: DEPTOR knockdown induced apoptosis is due to inhibition of PI3K/AKT and by up-regulation of ERK and p38MAP kinases. DEPTOR is overexpressed in human primary cervical SCC tissues.

Application of synthetic nucleases for HPV gene editing

Sumitra Shankar, Deepti Prasad and M. Radhakrishna Pillai

In our earlier work on CompoZr Zinc Finger Nucleases, targeting of E6 gene of HPV 16 viral genome showed approximately 4% gene editing activity. Although yeast Mel I assay showed 50% activity (fig 1), its activity was low in both SiHa and Caski cell lines. Upon closer analysis of nucleic acid composition, it was found that HPV 16 E6 gene had about 37% GC content, which led us to conclude that limited GNN rich regions in E6 gene sequence restricted the design options for ZFN. Therefore, we screened other targets for E6 and E7 using TALENs.

SAPTA (scoring algorithm for predicting *TALEN* activity) software was used to screen for TALENs targeting E6 as well as E7. Four pairs of TALENS were designed and synthesized to target the respective positions in E6 and E7 as shown in fig 2.

Next, we performed mismatch endonuclease assay using T7E1 to check for editing activity of each of these pairs. We observed that TALEN targeting E7 (position 524) showed good editing activity in both the cell lines (shown in figure 3). Caski additionally showed some editing activity at position 284 (E6) Further characterizations was done in SiHa and Caski cell lines.

Successively, Immunofluorescence staining for detecting double stand breaks was done using 53bp1 antibody. In SiHa, a single green spot was observed whereas in Caski multiple green spots were seen indicating that SiHa had a single copy of viral genome and caski had multiple copies of HPV 16 genome (shown in figure 4). These results agreed with the literature details of SiHa and Caski cell lines.

Finally gene knockout of E7 was confirmed at RNA and protein level by RT-PCR and western blot respectively (as shown in figure 5). In conclusion, TALEN targeting E7 showed gene-editing activity, which was validated at RNA as well as protein level. Currently we are characterizing the pathway of cell death that is activated after targeting these cell lines by TALEN.

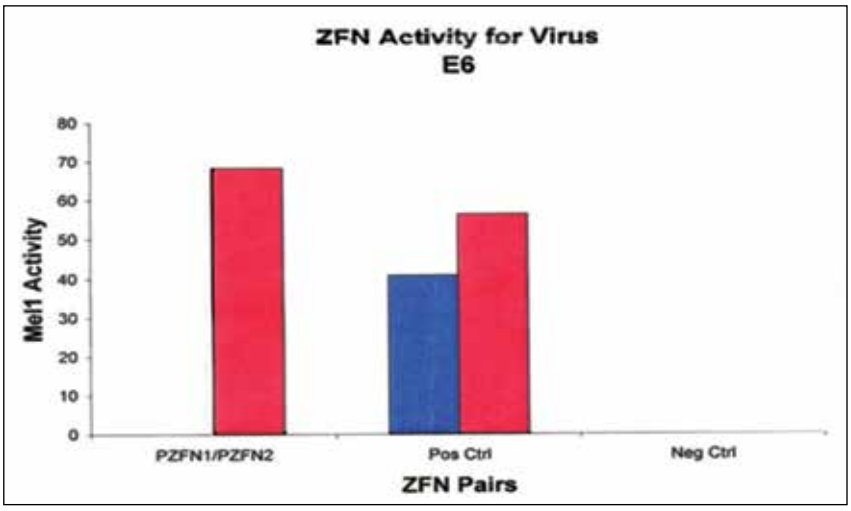


Figure1: Yeast Mel-I assay

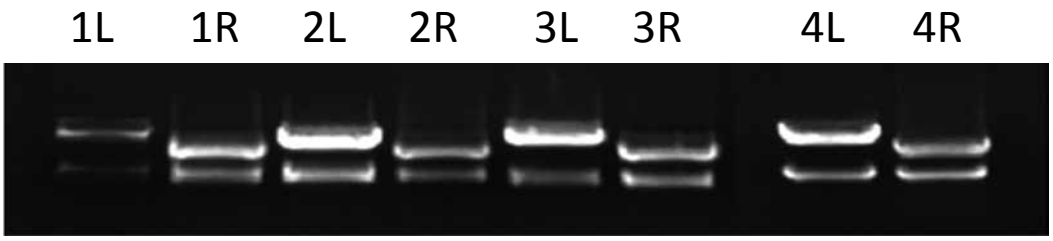


Figure 2: Talen construction, Talen works in pairs: L refers to left and R refers to right

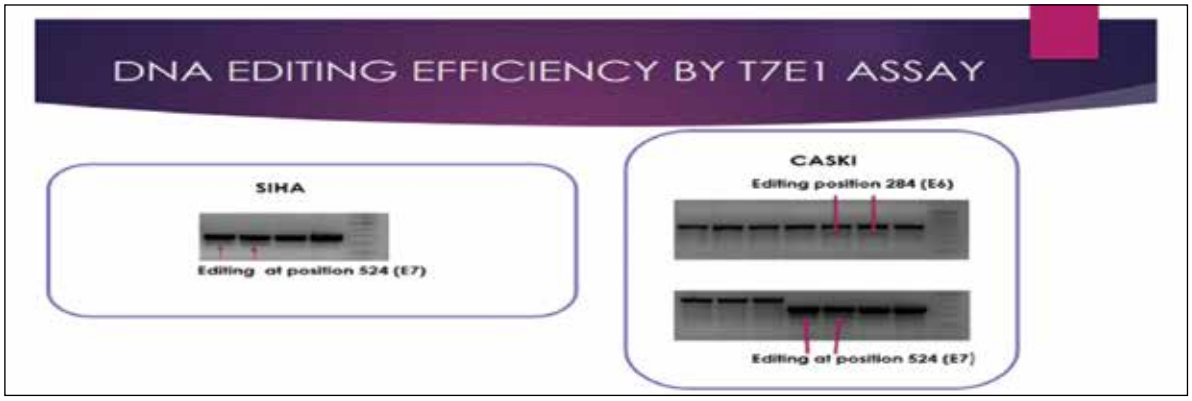


Figure 3: Mismatch Endonuclease assay for screening of editing activity of TALENs in SiHa and Caski cell line

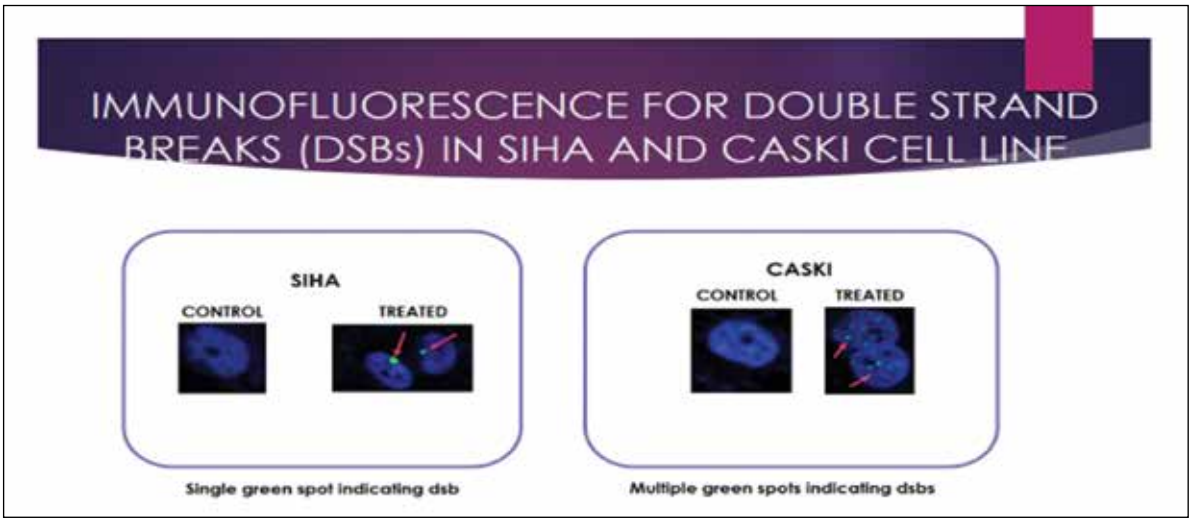


Figure 4: Immunostaining with 53bp1 antibody to show double strand breaks in treated cell lines.

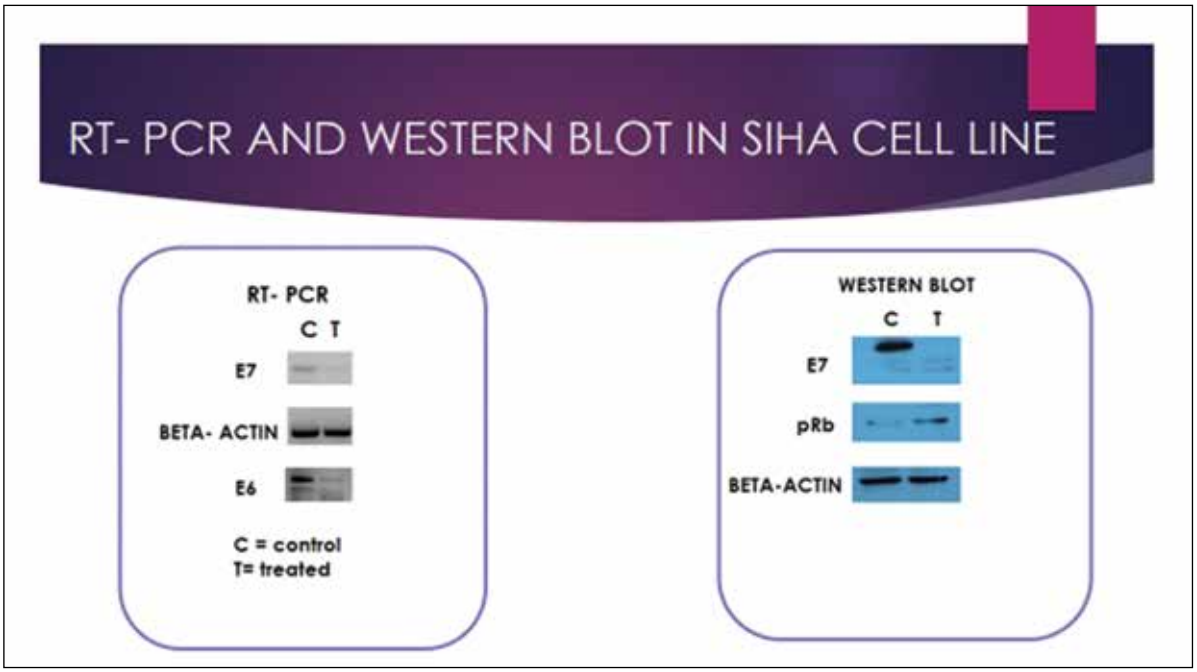


Figure 5: E7 and E6 were downregulated at RNA level and E7 was knocked out at protein level. pRB was upregulated.

Molecular Manifestations of Micronutrient Deficiency in Human Papillomavirus Associated Cervical Cancer

Deepti Prasad and M.Radhakrishna Pillai

Human Papilloma Virus (HPV) is a known etiological agent for development of cervical cancer. HPV integration into the host genome is a necessary, but not a sufficient event for the development of cervical neoplasia. Several co-factors have been suggested, one of which is nutrient deficiency. We have focused on the micronutrient folate because of its role in DNA stability and immune function. Folate deficiency usually leads to the accumulation of homocysteine. Homocysteine has been shown to have complex effects on disease pathogenesis. We have looked at the effects of elevated levels of homocysteine on SiHa cells. Cell cycle analysis of homocysteine-treated cells using Propidium Iodide staining showed no cell cycle arrest or cell death.

In primary rat cardiomyocytes and colon cancer cell lines, folate deficiency and the subsequent hyperhomocysteinemia has been shown to induce cell proliferation and migration. Wound Healing Assay was performed to study the effect of homocysteine on invasive behaviour of SiHa cells. It was seen that the cells became more invasive with increasing concentration of homocysteine.

Further studies are needed to validate the above results and study the expression of the oncogeneic viral proteins E6 and E7 during homocysteine treatment. Also, the effects of homocysteine on HPV-negative cervical cancer cell lines need to be examined.

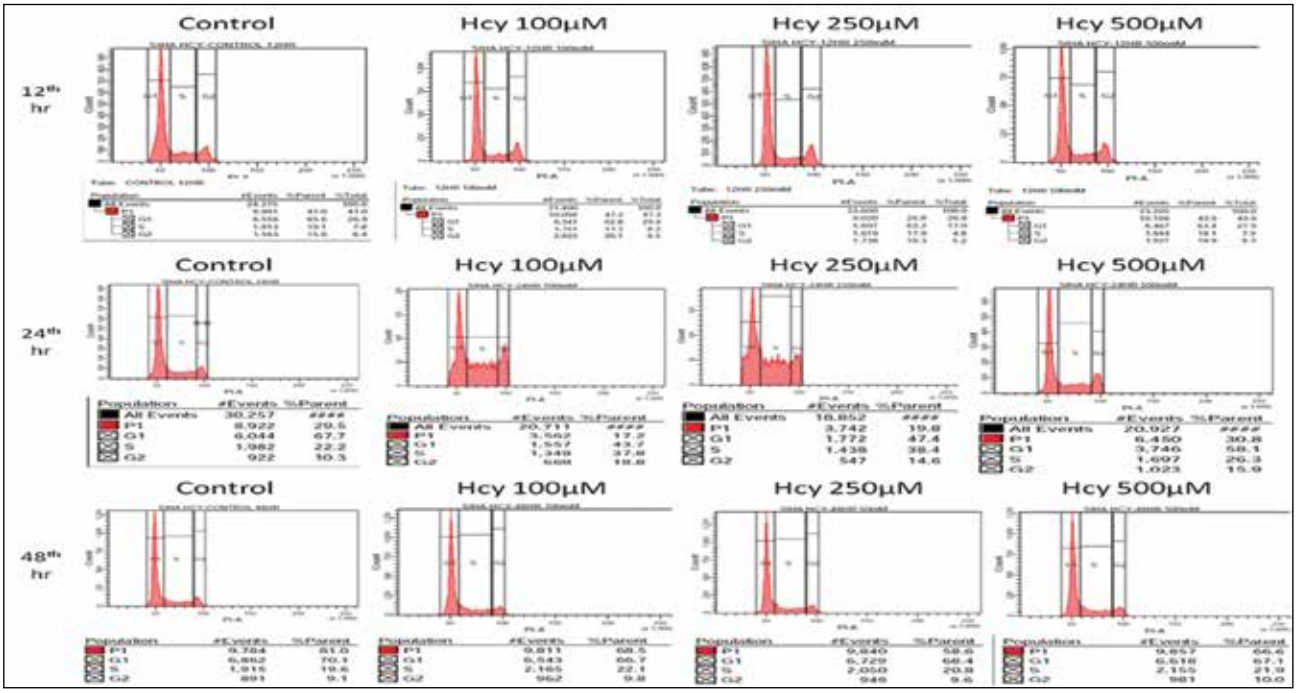


Figure 1: Cell cycle analysis (using Propidium iodide) of SiHa cells treated with L-homocysteine (Hcy) at various time points – P1 represents the cells gated for analysis using PI staining. Row 1: Cell cycle analysis at 12th hour after Hcy treatment: Left-Right: Control cells, Cells treated with 100µM Hcy, 250µM Hcy and 500µM Hcy. Row 2: Cell cycle analysis at 24th hour after Hcy treatment: Left-Right: Control cells, Cells treated with 100µM Hcy, 250µM Hcy and 500µM Hcy. Row3:Cell cycle analysis at 48th hour after Hcytreatment:Left-Right: Control cells, Cells treated with 100µM Hcy, 250µM Hcy and 500µM Hcy.

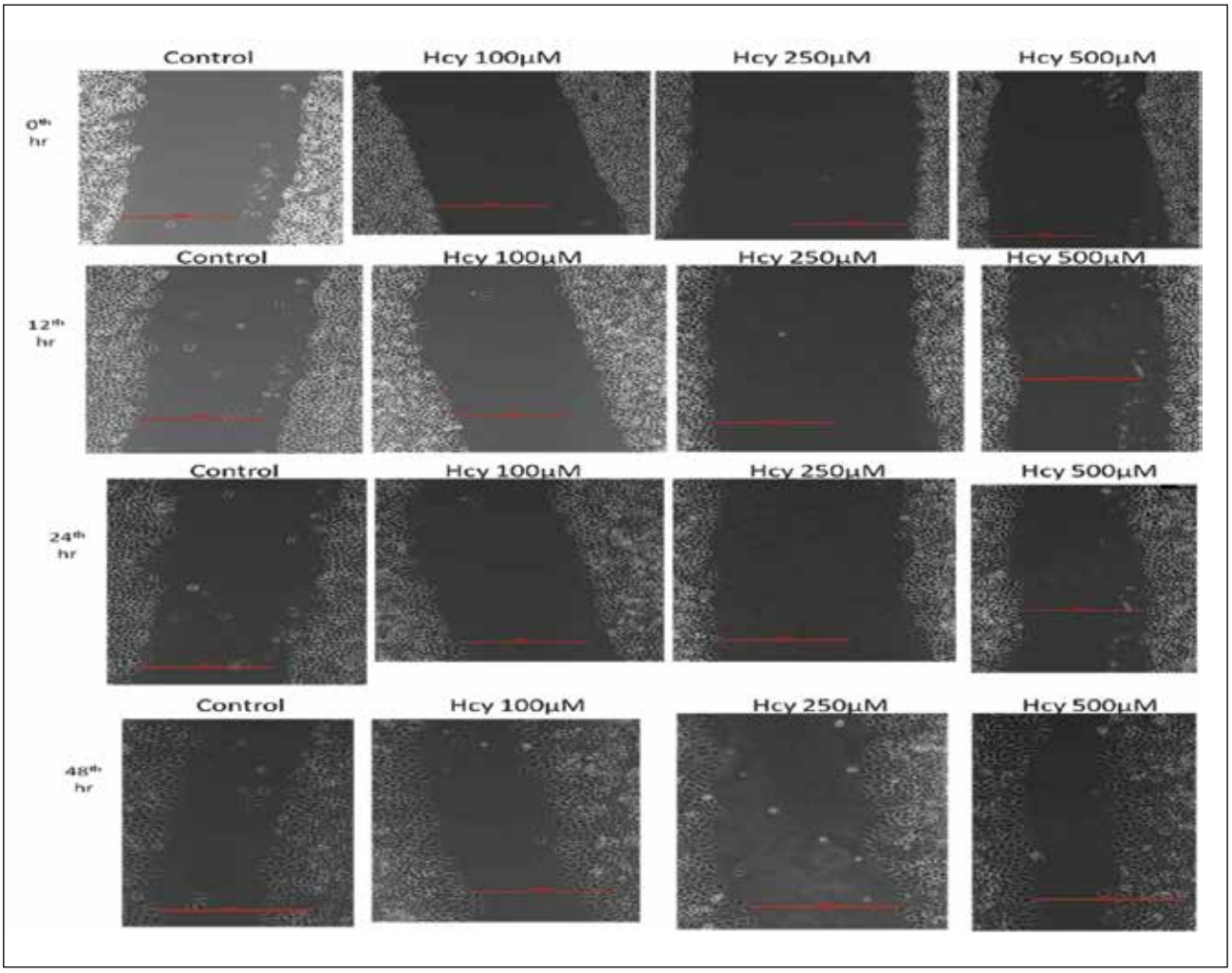


Figure 2: Wound healing assay for SiHa cells treated with different concentrations of Hcy at various time points.Row 1: Cells viewed at 0th hour after Hcy treatment: Left-Right: Control cells, Cells treated with 100µM Hcy, 250µM Hcy and 500µM Hcy. Row 2: Cells viewed at 12th hour after Hcy treatment: Left-Right: Control cells, Cells treated with 100µM Hcy, 250µM Hcy and 500µM Hcy. Row3: Cells viewed at 24th hour after Hcy treatment: Left-Right: Control cells, Cells treated with 100µM Hcy, 250µM Hcy and 500µM Hcy. Row4: Cells viewed at 48th hour after Hcy treatment: Left-Right: Control cells, Cells treated with 100µM Hcy, 250µM Hcy and 500µM Hcy.

Bio prospecting of rare actinomycetes diversity of Kerala Western Ghats forest soils for novel anti-cancer compounds- a high throughput analysis

Vipin Mohan Dan, Balaji M, Srinivas KP, Rahul Sanawar, Ajay Kumar R. and M. Radhakrishna Pillai

Soil microbial flora has been the source of many pharmaceutical drugs in the past. This study focuses on identifying soil microbes and their metabolites with potential anticancer activity. Soil samples were collected from forest regions of Wayanad, Kallar and Ponmudi in the Western Ghats. The bacterial isolates were selected based on their morphological characteristics. Initial screening of 200 isolates was done by cytotoxocity assay. The isolates positive in MTT assay were analyzed for nuclear condensation by Hoechst assay. One isolate designated MP10 with potential activity was selected for further studies. Identification of the potential isolate by FAME analysis revealed that it belonged to Streptomyces sp. In FAME, the isolate gave a similarity index of 0.274 with known microbes thus suggesting that it could be a new species. Based on the FAME library database the most closely related species for MP10 is Streptomyces-violaceusniger-violaceusniger. To study whether apoptosis is triggered by the presence of extracts of MP10, Annexin V-FITC (sigma) apoptotic detection kit was used. FACS analysis via Annexin V-FITC/PI staining was used to observe the induction of apoptosis. In Fig. 1, cells in the lower right quadrant (Q4) indicate Annexin-positive/PI negative, early apoptotic cells. The cells in the upper right (Q2) quadrant indicate Annexin-positive/PI positive, late apoptotic or necrotic cells. The apoptotic cells were significant compared to control in treatment with Streptomycessp MP10. Significant PARP cleavage was observed in Streptomycessp MP10 treated cells after 24hrs. Streptomycessp MP10 ethyl acetate extract inhibited mTOR activity thus suggesting the presence of compound(s) possibly acting on mTOR pathway. Literature survey reveals that Rapamycin is the only known natural compound, obtained from bacterial origin (Streptomyces hygroscopicus), that inhibits the mTOR pathway by complexing with mTORC1. Currently rapamycin derivatives/analogs are in

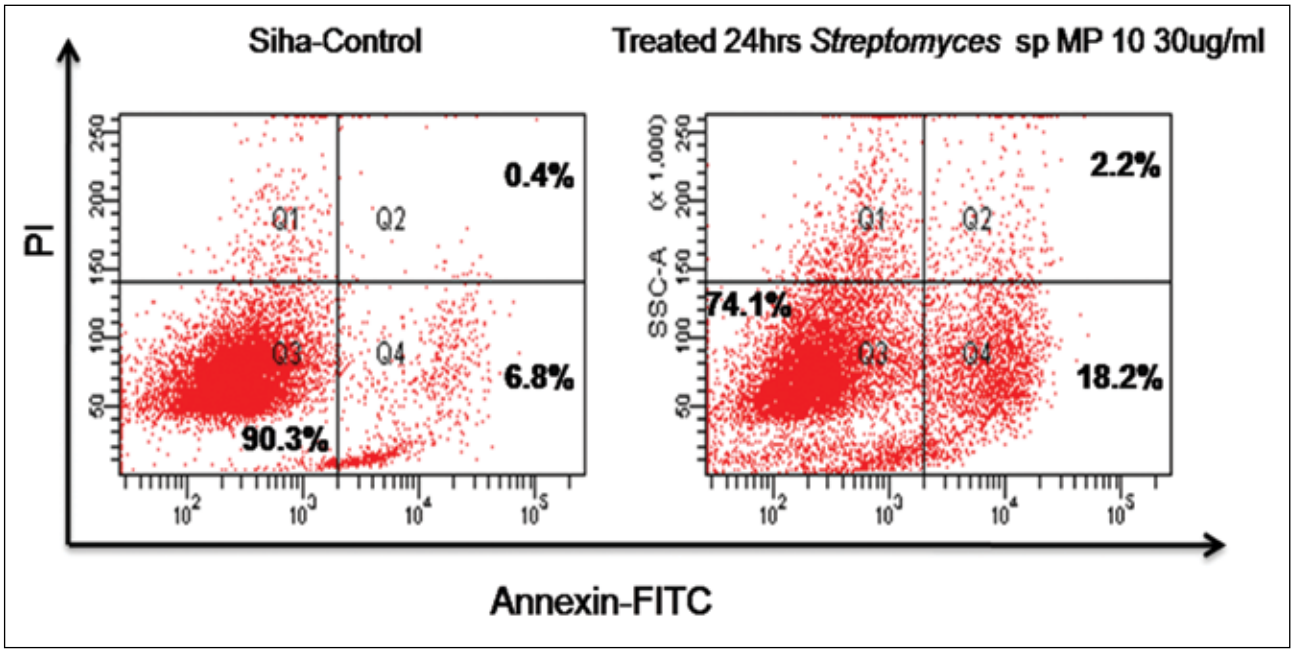


Fig1: FACS analysis via Annexin V-FITC/PI staining

clinical trials for treatment of various cancers. As identification results suggest that Streptomyces sp MP10 has less possibility to be bracketed with already known species, the chances of hitting on a novel mTOR inhibitor from this bacteria is possible.

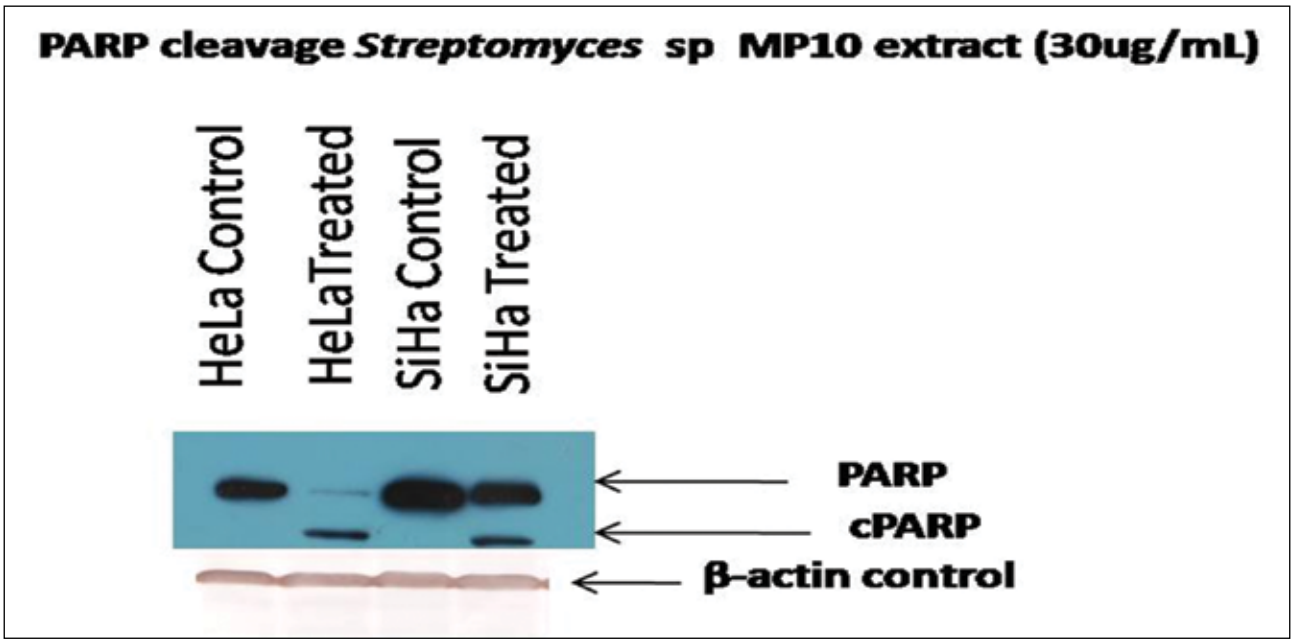


Fig2: PARP cleavage

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A computational approach to analyze microRNA mediated Regulatory Networks in human papillomavirus (HPV) induced Cervical Cancer

Aswathy Mary Paul, Meena Vinay Kumar, Reshmi G and M. Radhakrishna Pillai

Cervical cancer is the second most cancer-related mortality in women worldwide. The human papillomavirus is the principal etiological agent and is associated with deregulation of oncogenic and tumor suppressive miRNAs. We mainly focus on Transcription Factor (TF) and MicroRNA (miRNA) as gene regulators in HPV induced cervical cancer. We planned to construct the regulatory network from the data collected from various sources such as gene expression databases (GEO), SRA data resources, literature surveys and other publically available cervical cancer databases. Since microarray dataset can increase the heterogeneity, the curation was done such that the data can be classified either into cervical cancer cell line, normal cervix and cervical cancer. Level three data has been also curated from TCGA and includes mainly miRNA and mRNA. Data retrieval was followed by normalization to increase integrity of the data and thereby increase the enrichment. Corresponding miRNA and mRNA targets, which are predicted using the algorithms that involved miRNA and mRNA that are not involved in HPV induced cervical cancer, are excluded from our dataset. TF to each target is identified using the sequence overlap in USCS genome browser. The work is progressing in clustering of data to develop a network motif finding algorithm to infer the network and then apply different algorithms to construct the regulatory network and identify the best among the proposed algorithms.

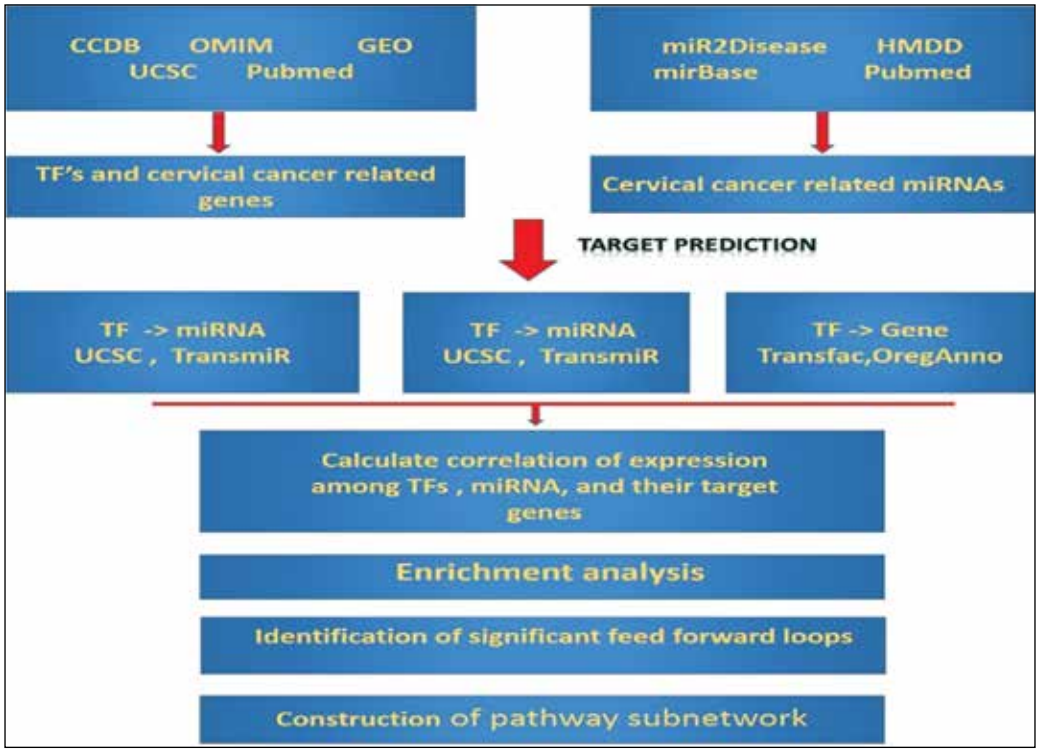
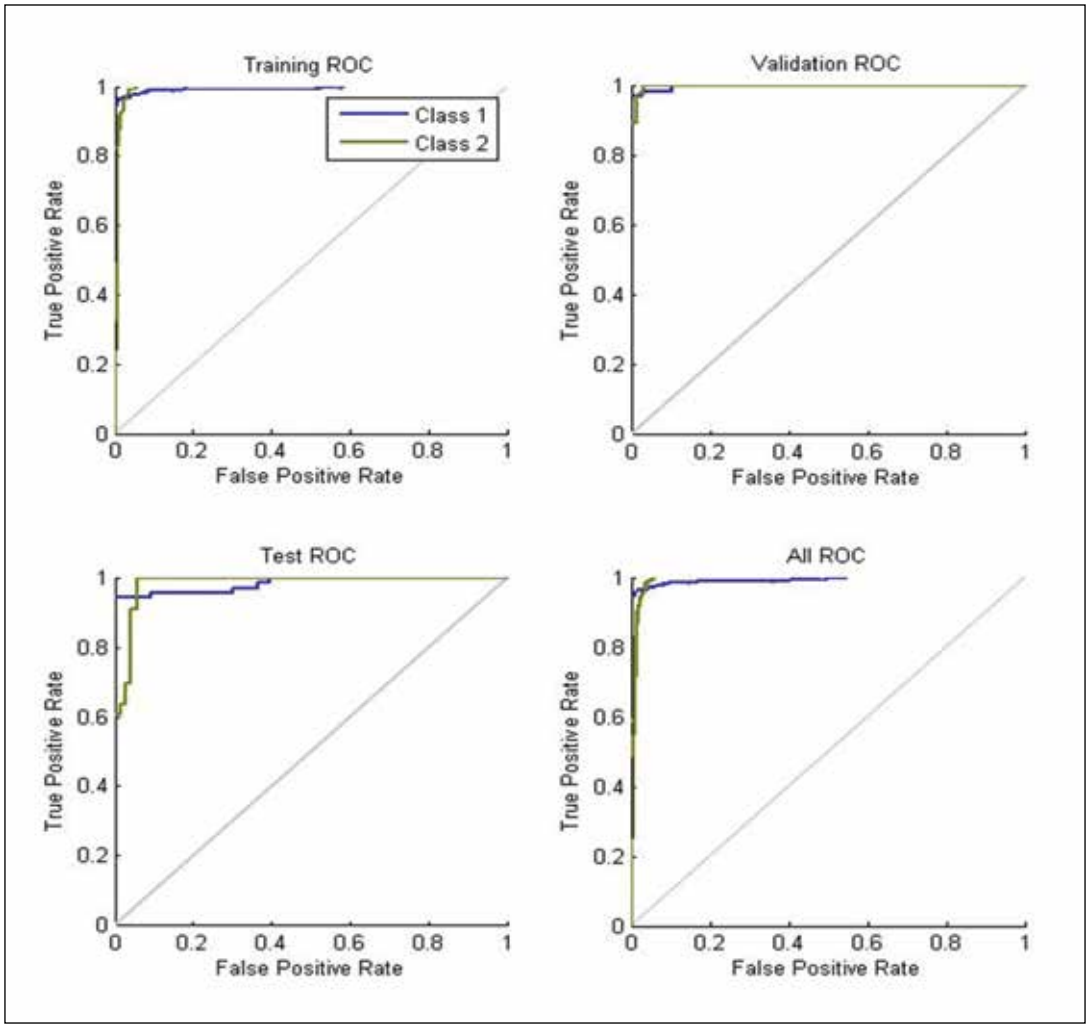


Figure 1: Proposed workflow

Whole genome survey of microRNA target site accessibility based on conserved local RNA secondary structure and protein binding site overlaps: Creating a freely accessible web resource

Vivekanand A, Reshmi G and M Radhakrishna Pillai

Genetic regulation by miRNAs and RNA binding proteins has been studied for over a decade now. However, miRNA-mRNA interaction and target identification in a broad spectrum on basis of experimentally validated results is most challenging. On the other hand, RNA binding proteins have diverse and are crucial in biogenesis, stability, function, transport and cellular localization. We proposed an efficient machine learning model to untangle the relationship between miRNAs and RNA binding proteins.The prediction of RNA binding proteins whole genome needs considerable effort unless a stochastic methods coupled machine learning approach is used. We prefer to use a multilevel Artificial Neural Network (ANN) based architecture for identifying conserved binding regions. The tool was trained by Meta data (RNA binding protein target interaction) as well as CLASH data (miRNA target interactions) from public databases. This model incorporates structural, thermodynamic and positional features of residues in miRNA: mRNA pairs from wet lab proven data sets. The pre-configured score form the training data is used for predicting accurate results. The algorithm matches with the scores generated by the training dataset with the predicted RNA binding protein's parameters and if it fits in the range passed to further processing or else discarded. The predicted RNA binding protein will then pass through the targets identified using CLASH dataset that results in the RNA binding protein and miRNAs have affinity towards a same target. Hence our novel machine learning architecture is found to be more comprehensive than the existing methods in predicting RNA binding proteins, especially in human transcriptome.



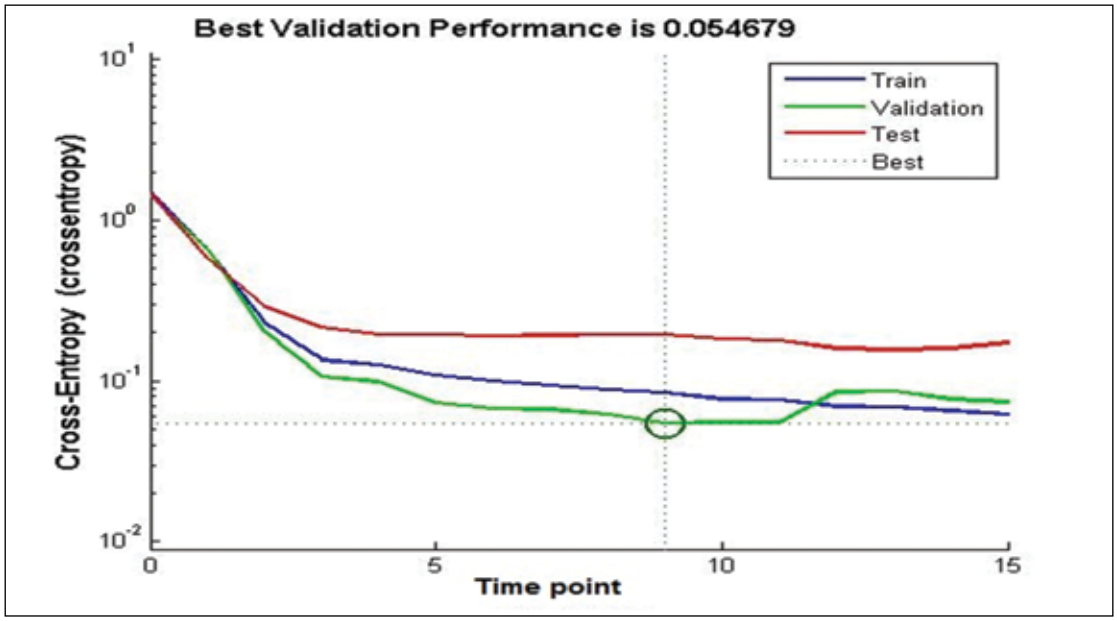
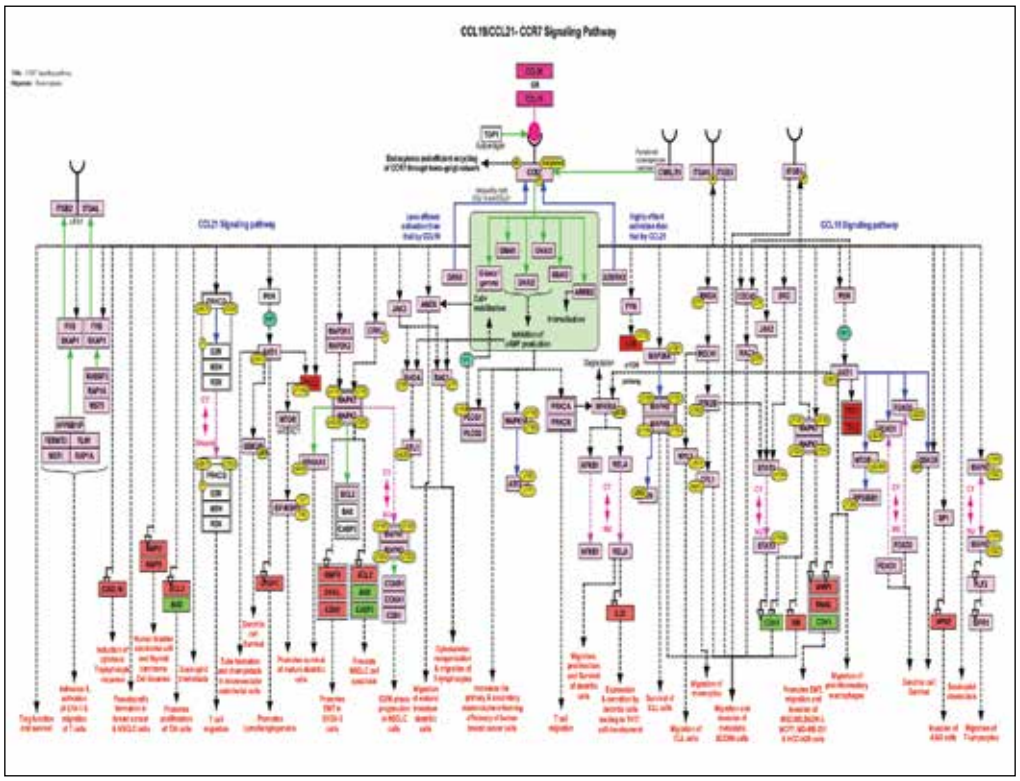


Figure 2: ROC curve developed based on d performance ratio generated with sample data from ANN

Analysis of the differential-ligand signaling network of CCL19/CCL21-CCR7 system

Rajesh Raju and Reshmi G

Chemokine (C-C motif) receptor 7 (CCR7), a class A subtype GPCR, is involved in the migration, activation and survival of multiple cell types including dendritic cells, T cells, eosinophils, B cells, endothelial cells and different cancer cells. Together, CCR7 signaling system has been implicated in diverse biological processes such as lymph node homeostasis, T cell activation, immune tolerance, inflammatory response and cancer metastasis. Elevated expression of the CCL19 and CCL21, the two well-characterized CCR7 ligands, have been reported in diverse disease conditions such as cancers, atherosclerosis, bone disorders and other inflammatory conditions such as asthma, HIV infection and pneumonia. Zidar et al, 2009, have reported the differential and selective activation of the members of the GRK/beta-arrestin system of CCR7 by CCL19 and CCL21, with no substantial bias in the inhibition of adenylatecyclase by the CCR7-Gκ/o system in HEK293 cells. Recently, Corbisier et al, 2015, have reported based on BRET assays that there is a ligand bias in the activation of multiple Gκ/o isoforms of CCR7 in HEK293 cells. Subsequently, a large number of molecules and their involvement in mediating diverse biological processes of CCR7 have been analyzed and reported under stimulation with either or both of these ligands in multiple cell types. In order to analyze the differential signaling of CCR7 ligands further, the experimentally proven molecular reactions induced by CCR7 ligands should be made available in computationally analyzable formats such as BioPAX, SBML or GPML formats in a cell type specific manner. Although the differential ligand signaling through single receptor has been suggested for many receptors including GPCRs, there exists no resource or platform to analyze them globally. Here, first of its kind in the world, we present the cell-type-specific differential signaling network of CCL19/CCL21-CCR7 system for effective visualization and analysis of chemokine/GPCR signaling.



PROGRAM SCIENTIST

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Role of Piwi proteins in the maintenance of cancer stem cells in Human Papillomavirus (HPV) induced epithelial cancers

Epigenetic regulation at the chromatin level plays an important role in tumorigenesis. Mutations or alterations of epigenetic regulators and chromatin modifications cause cancers. Most epigenetic factors such as DNA methyltransferases/ demethylases and histone modification enzymes do not recognize specific DNA sequences and require mediator molecules to guide them to the site of action. The question therefore is how these epigenetic factors are guided to their target site in a genome? Piwi proteins and interacting piRNAs can act as guiding signals for many such epigenetic factors and hence thought to have significant role in tumorigenesis. Moreover, being stem cell factors it is anticipated that they may have a regulatory role in the maintenance of cancer stem cells. Piwi proteins, one of the two sub clades of Argonaute proteins mediate their functions by interacting with special type of non-coding small RNAs called piRNAs which are slightly bigger than miRNAs and siRNAs. Though Piwi is expressed predominantly in the germline cells, recent

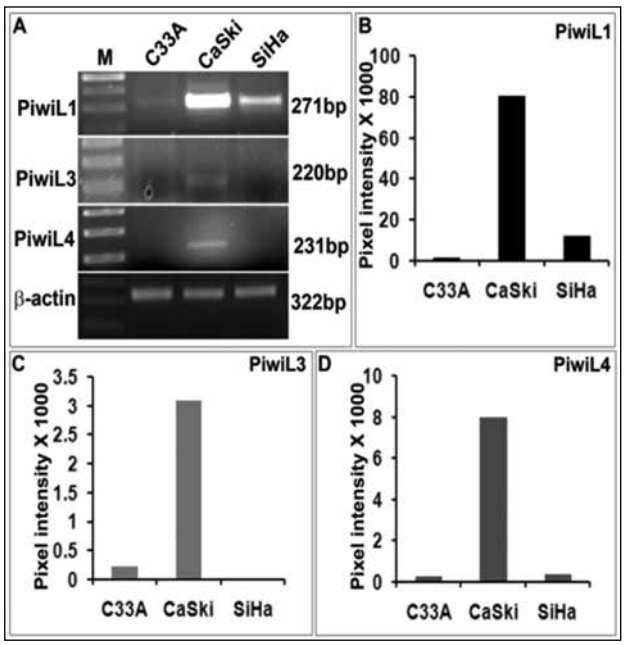


Figure 1. Piwi isoforms are expressed in cervical cancer cells. A) RT-PCR analysis revealed the expression of various Piwi isoforms in cervical cancer cell lines. B) Graph represent the pixel intensity of the amplicons obtained from RT-PCR analysis

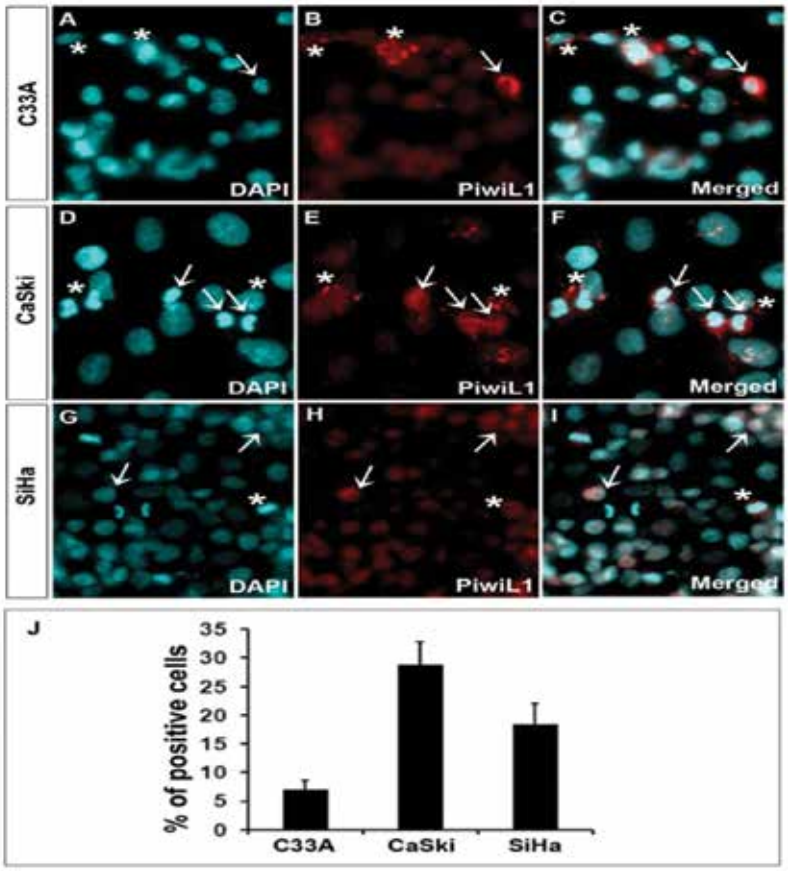


Figure 2. PiwiL1 expression in cervical cancer cells. Immunocytochemical analysis of PiwiL1 in cervical cancer cell lines such as C33A (A-C); CaSki (D-F) and SiHa (G-I). J) Graph depicts the percentage of cells expressing PiwiL1 in cervical cancer cell lines. Arrows indicate the positive cells and stars indicate cells with punctate expression

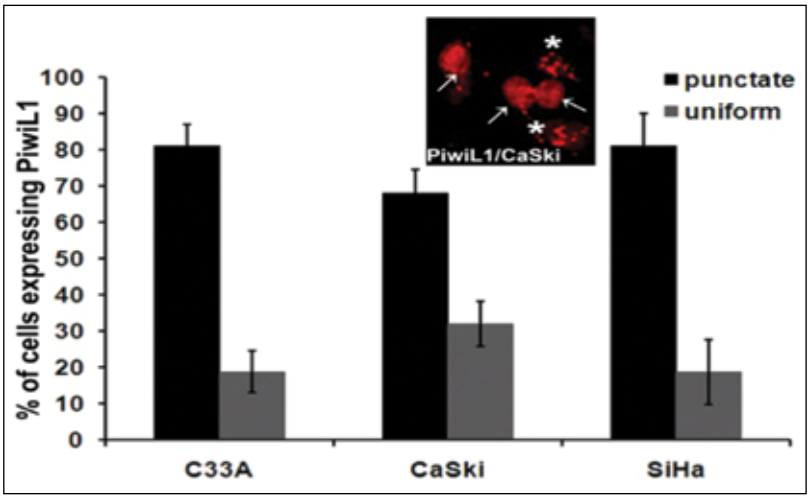


Figure 3. PiwiL1 showed two distinct types of localization in cervical cancer cells. Immunocytochemical analysis of PiwiL1 in cervical cancer cell lines showed two distinct types of cellular distribution. Graph represents the percentage of cells showing punctate and uniform expression. A representation of immunoanalysis of Piwil1 in CaSki shown as inset.

evidence indicates their significant involvement in tumorigenesis since they are aberrantly expressed in various cancers. Since PIWI proteins and associated piRNAs have been shown in HPV-associated expression in cervical cancers and their expression precedes the expression of one of the existing marker, p16, they could be used in combination with the existing biomarkers for early detection of cervical neoplasia. In the present study, we elucidate the role of Piwi proteins in epithelial cancers and also their correlation with HPV infection. Their involvement in the maintenance of cancer stem cells will also be investigated in detail.

We chose cervical and oral cancer as our models since these cancers have shown to be associated with HPV. We evaluated the expression of Piwi proteins in the cervical cancer cell lines selected based on the HPV integration: C33A (HPV-ve), CaSki (HPV+ve High Copy) and SiHa (HPV+ve low copy). There were four homologs of Piwi proteins identified in human (Hiwi/PiwiL1, Hili/PiwiL2, PiwiL3 and Hiwi2/PiwiL4). RT-PCR analysis suggested the isoforms were present in all the cell lines we screened, PiwiL1 found to be highly expressed among all the Piwi variants (Figure1). Further, immune-analysis also revealed the presence of Piwi proteins in all the cell lines we tested with highest expression in CaSki cells (Figure 2). Interestingly, the expression of all the Piwi proteins, especially PiwiL1, showed correlation with HPV as observed that the expression of Piwi proteins was more in CaSki which is known to have high copies of HPV integrated (Figure 1 and Figure 2).

Another important finding is that there were differences in the expression pattern of PiwiL1 proteins in the cervical cancer cell lines. Majority of cells showed punctate pattern of expression and a small population showed uniform intense cytoplasmic expression. There was a slight shift in the percentage of these populations in response to HPV integration (Figure 3). In summary our preliminary observations suggest that Piwi proteins may have important role in HPV-associated tumorigenesis.

PUBLICATIONS

- *Paul Sebastian, Janki Mohan Babu, R Prathibha, Ramkumar Hariharan, and Pillai MR.* Anterior tongue cancer with no history of tobacco and alcohol use may be a distinct molecular and clinical entity. *Journal of Oral Pathology & Medicine 2014, 43 (8): 593-599*
- *Joshi, J.M. Babu, D. Jayalakshmi, V. Kulkarni, U. Divate, R. Muwonge, T.Gheit, M. Tommasino, R. Sankaranarayanan, Pillai MR.* Human papillomavirus infection among human immunodeficiency virus-infected women in Maharashtra, India. *Vaccine 32 (2014) 1079– 1085.*
- *Hezlin Marzook, Deivendran S, Rakesh Kumar, Pillai MR.* Role of MTA1 in Head and Neck cancer. *Cancer and Metastasis Reviews 2014*, 33(4): 953-64,
- *S Deivendran, K Hezlin Marzook, Pillai MR.* The role of inflammation in cervical cancer. *Advances in Experimental Medicine and Biology;* 816:377-99, (2014)
- *Rajesh Raju, Aswathy Paul, Vivekanand Asokachandran, Bijesh George, Lekshmi Radhamony, Meena Vinaykumar, Reshmi Girijadevi and Pillai MR.* The Triple-Negative Breast Cancer Database: an omics platform for reference, integration and analysis of triple-negative breast cancer data. *Breast Cancer Research 2014*, 16:490.

HONORS & AWARDS

- **Best Oral Presentation Award**: *Priya R Prabhu* was awarded the for the presentation titled "*Human Papillomavirus Antibody Patterns in the Indian 2- versus 3-Dose quadrivalent HPV vaccination trial",* at *Carcinogenesis 2015-* International Conference organized by Carcinogenesis Foundation, USA and ACTREC, Tata Memorial Centre, Mumbai, at Navi Mumbai, on 11th to 13thFebruary, 2015.

CONFERENCES PRESENTATIONS

- *T.R. Santoshkumar and Pillai MR* * Persister" cells following Chemotherapy generates tumor stem cells and cell heterogeneity to drive tumor recurrence. *Global Consortium on Cancer Genomics*, University of Kyoto, Japan November 14-15, 2014.
- *Aswathy Mary Paul, Reshmi G and Pillai MR.* A computational approach to analyse microRNA and Transcription Factor Mediated Regulatory Networks in HPV induced Cervical Cancer" at *Asia Pacific Bioinformatics Conference* held at Taiwan, Jan 21st to Jan 23rd 2015.
- *Sajitha I.S., Srinivas K.P., Santhik S.L., T.R.Santhosh Kumar and Pillai MR.*_Elucidation of drug resistance mechanism in a lung cancer model *Carcinogenesis 2015*, 11-13 February 2015, ACTREC, Mumbai.
- *Aswathy Mary Paul, Reshmi G and Pillai MR.* "Intergrated Platform for Triple-Negative Breast Cancer Data Analysis" at *IACR (Indian Association for Cancer Research) conference* held at Jaipur, Feb 19th to Feb 21st 2015
- *Bijesh George, Rajesh Raju, Reshmi G and Pillai MR. "Comprehensive Analysis of HPV-Host Interaction"* at IACR (Indian Association of Cancer Research), held at Jaipur, Rajasthan, February 19- 21, 2015.
- *Vivekanand A, Rajesh Raju, Sreenivas KP, Reshmi G, Pillai MR. "Regulatory Map of Deptor"* at IACR (Indian Association of Cancer Research), held at Jaipur, Rajasthan, February 19- 21, 2015.

INTERNATIONAL TRAVEL GRANT AWARD

- *Aswathy Mary Paul* awarded DBT international travel grant to attend Asia Pacific Bioinformatics Conference held at Taiwan, Jan 21st to Jan 23rd 2015.

RESEARCH GRANTS

Sl No	Title of the project	Investigators	Funding Agency	Duration
1	Whole genome survey of microRNA target site accessibility based on conserved local RNA secondary structure and protein binding site overlaps: Creating a freely accessible web resource.	Reshmi G and M.R Pillai	Deptartment of Biotechnology, Government of India.	2012-2015
2	Integrative Computational Analysis to Drive Discovery of MicroRNA-mediated Regulatory Networks in HPV Induced Cervical Cancer	Reshmi G and M.R Pillai	Department of Biotechnology, Government of India.	2013 -2016
3	Experimental studies on therapy of cancers expressing hCG/ hCCB with a recombinant highly immunogenic vaccine against hCG	M.R. Pillai	Department of Biotechnology, Government of India.	2012 -2014
4	Accurate and satisfactory analysis of all high risks type and some low risks including HPV 6 and 11 antibody titers for the 2-verses 3 dose HPV Vaccination Clinical Trial in India	M.R. Pillai	International Agency for Research on Cancer	2009 - 2014
5	National Virology Network Laboratory (Grade I)	M.R. Pillai	Indian Council of Medical Research	2009 - 2016
6	Role of Human Papiloma Virus infection and other co factors in the aetiology of head and neck cancer in India and Europe	M.R. Pillai	European Commission (ECAS)	2011-2015

VISITING DISTINGUISHED PROFESSOR

Rakesh Kumar, Ph.D
rakeshkumar@rgcb.res.in

Upon recommendation of the RGCB Governing Council, Professor Rakesh Kumar of the Department of Biochemistry, George Washington University, USA was invited to RGCB as Visiting Distinguished Professor of Biotechnology and co-direct a joint research program with Professor M. Radhakrishna Pillai.

Co-mentored PhD students

Hezlin Marzook
Deivendran S
Parvathy Muralidharan
Rahul Sanawar

Upstream Activators and Downstream Effectors of MTA1 Activity during Tumor Progression

Hezlin Marzook, S. Deivendran, T.R. SanthoshKumar, Rakesh Kumar and M. Radhakrishna Pillai

MTA1, the founding member of the MTA family of proteins acts as a dual coregulator and finds widespread importance in invasion and metastasis as well as physiological cellular processes. The level of MTA1 is widely up-regulated in a variety of human cancers, including head and neck, lung, breast, liver, gastrointestinal, pancreatic, ovarian, prostate cancers, melanoma, and lymphomas; and the expression status correlates well with the tumor grade and invasiv5eness as well as a poor prognosis of cancer patients. Recent studies suggest that stress causing factors such as ionizing radiation, ultra-violet radiation, hypoxia and inflammation up-regulate expression of MTA1 in cancer cells, and thus, confer an adaptive mechanism for the cancer cells against stress. Further, MTA1 physically interacts with hypoxic factor HIF1α and stimulates the transcriptional activity of HIF1α, leading to up-regulation of target genes such as VEGF-A. Despite of the paramount importance of MTA1 in stress response, it remains unknown if MTA1 is a direct target of stress signals like hypoxia and whether there is a bi-directional regulatory loop between the MTA1 and stress responses. Because the nature of signaling pathways influencing the status and localization of MTA1 under stressed condition remains poorly understood, the current study is designed to explore these outstanding questions. In our study, we noticed that a substantial amount of MTA1 accumulates in the cytoplasm in breast cancer cell lines namely MCF7 and SKBR3, upon serum starvation and this process is further

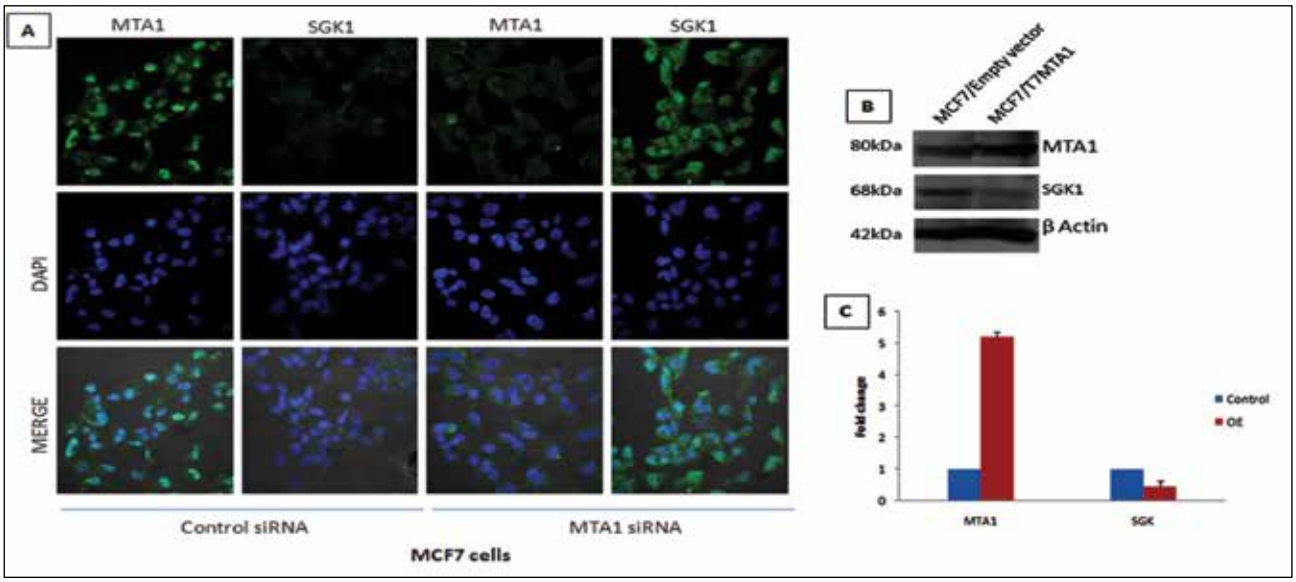


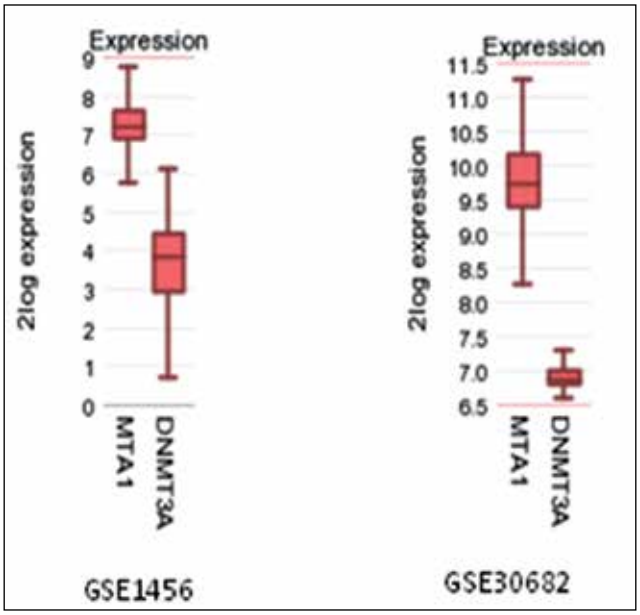
Figure: Regulation of SGK1 by MTA1
A: Immunofluorescence for MTA1 and SGK1 staining in MCF7 cells under knockdown of MTA1
B: Immunoblot to show the expression of SGK1 under the over expression of MTA1
C: qPCR showing fold change decrease in the SGK1 level when MTA1 is over expressed (OE)

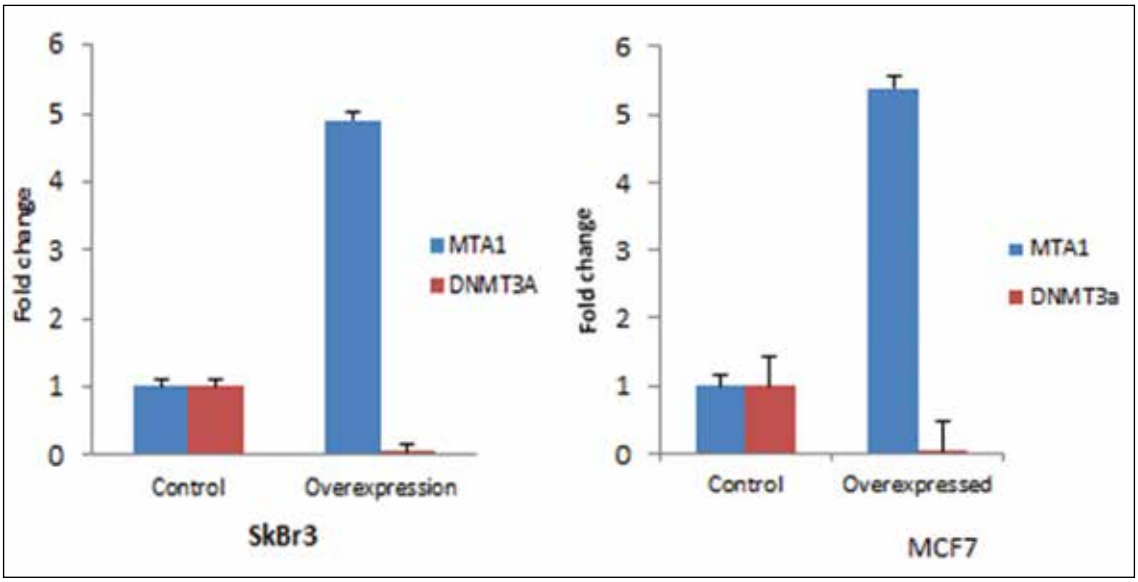
potentiated by hypoxia. MTA1 redistributes from the nucleus to cytoplasm under stress-induced signaling highlights the significance of functional cross talk between distinct pathways. Hypoxia and serum deprivation have an effect on MTA1 status in defining its sub- cellular localization under these conditions. On analyzing the cause of this cytoplasmic accumulation of MTA1, we noticed that MTA1 status undergoes nuclear deprivation via degradation and its translocation to the cytoplasm may not be the primary mechanism to account the noticed cytoplasmic accumulation of MTA1. Experiments conducted with cycloheximide treatments to examine the half-life revealed that MTA1 undergoes degradation in the nuclear compartment within 12hours of hypoxia. We assume that nutrient deprivation or hypoxia could re-set the nuclear functions of MTA1, largely due to its reduced levels in the nucleus. MTA1 tends to act as a stress responsive protein by regulating genes important for survival under stress conditions namely Serum Glucocorticoid regulated Kinase 1 (SGK1) which, in-turn, modulate its target genes which might have some degree of functional overlap with hypoxic responses. Our results also pinpoint a potential negative regulation of SGK1 by MTA1 at the level of mRNA under normoxia. We also found that induction of hypoxia increases the levels of N-Myc Down Regulated Gene 1 (NDRG1), a stress responsive protein under the forced expression of MTA1 when compared to normoxia. Current studies are focused in searching relevant targets of SGK1, including NDRG1, in the action and biology of MTA1. In brief, this study is delineating the role of master coregulator protein under stress conditions in cancer biology.

Regulation of DNMT3a Expression and Functions by MTA1 in cancer cells

Deivendran S, T. R. Santhoshkumar, Rakesh Kumar and M. Radhakrishna Pillai

Metastasis-associated tumor antigen 1 (MTA1), a chromatin modifier overexpressed in human cancers, including, breast, prostate, colon and ovarian. MTA1 is an integral part of the chromatin remodeling NuRD and NURF complexes and function as a dual co-regulator. MTA1 protein contributes to the process of cancer progression and metastasis by modifying the expression of its target genes, presumably due to its ability to interact with co-regulatory proteins and histones. MTA1 contains a BAH, a SANT, and an ELM domains. We postulate a role of MTA1 or MTA1-containing complexes in the expression of target genes by modifying the status or activity of DNA-methylation machinery. While mining the available breast cancer databases, we noticed a negative correlation between the mRNA levels of MTA1 and DNA methyl transferase 3a (DNMT3a) (Fig. 1). The noted relationship was also true for colon cancer, and thus, these observations may have a broader impact in human cancer. Next we experimentally validated these observations and demonstrated that MTA1 overexpression in SKBR3 and MCF-7cells leads to a reduction in the level of DNMT3a mRNA as well as DNMT3a protein while MTA1 knockdown results to an increased expression of DNMT3a in SkBr3breast cancer cells. Consistent with these findings, we found an increase in the levels of DNMT3a in MTA-knockout murine embryonic fibroblasts when compared to wild-type murine embryonic fibroblasts. Using chromatin immunoprecipitation assays, we demonstrated the recruitment of MTA1 (Fig.3). Because MTA1 is a part of co-repressive complex, we explored the possibility of MTA1 interaction with DNMT3a, and preliminary results suggest the presence of DNMT3a in the MTA1 pulled complex. However, studies involving protein-protein interaction between the MTA1 and catalytic domain of DNMT3a revealed no evidence of a direct physical interaction between MTA1 and DNMT3a (data not shown).





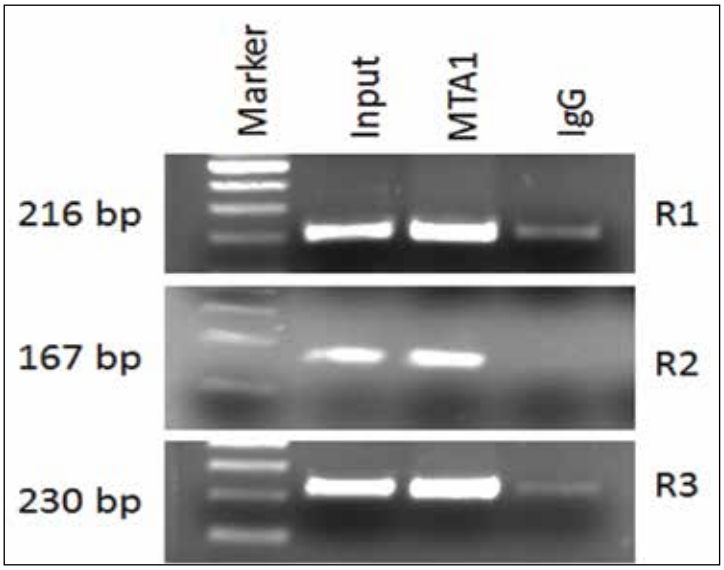


Fig. 1: The analysis of mRNA expression levels of MTA1 and DNMT3A was performed using a public dataset of microarrays GSE1456 and GSE30682 and analysis was performed using R2: Genomics Analysis and Visualization Platform

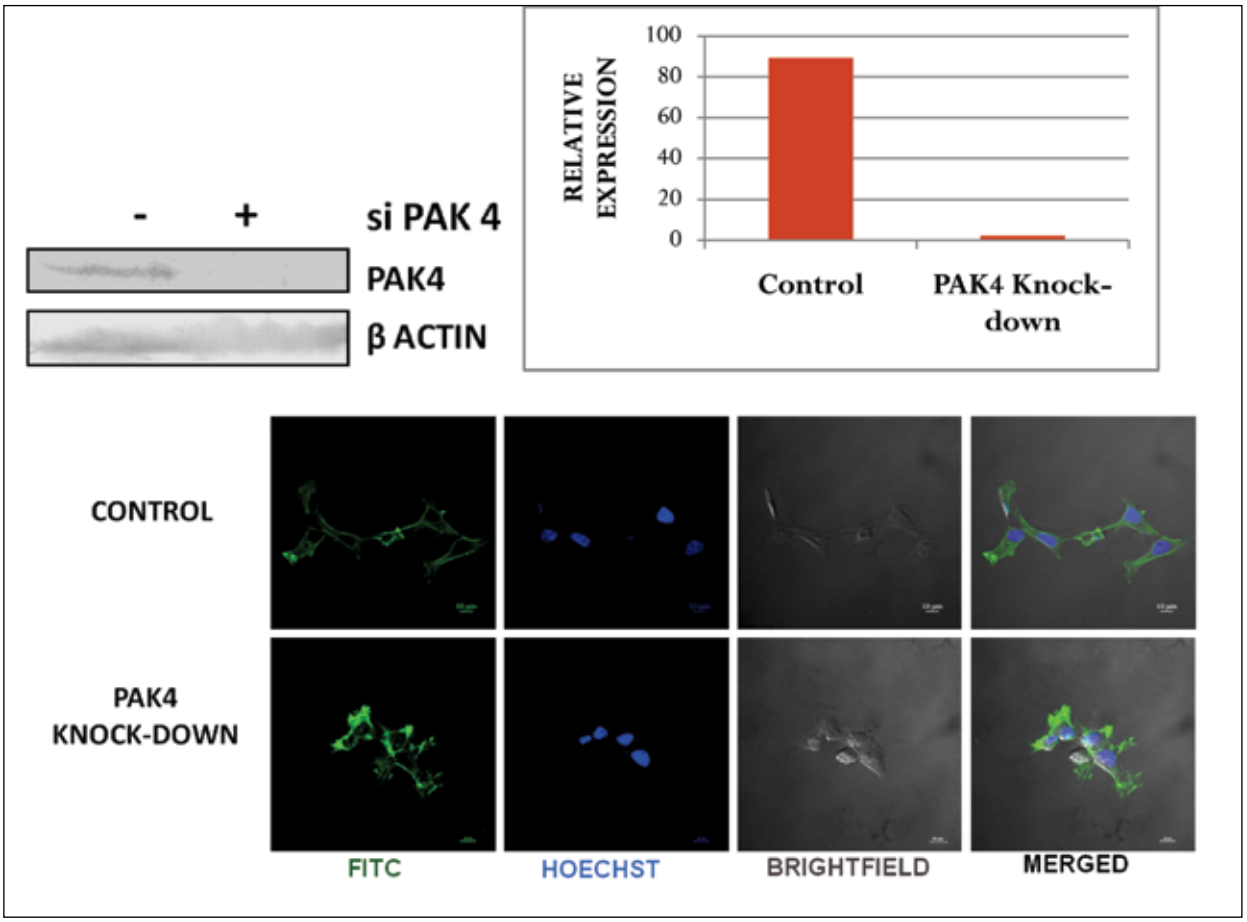
Fig. 2: Quantitative PCR analysis of mRNA expression levels of MTA1 and DNMT3a in MTA1 overexpressed MCF7 and SkBr3 cells.

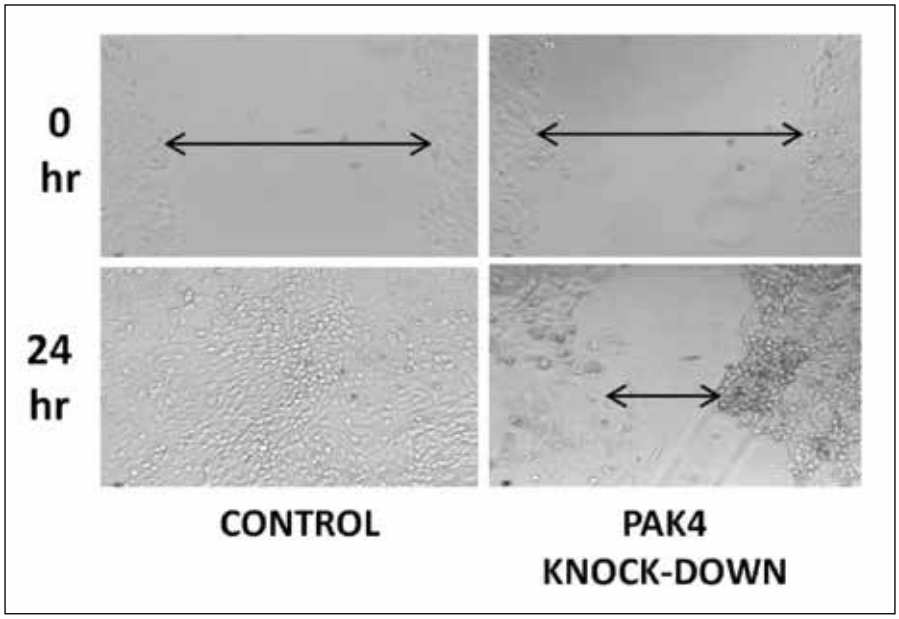
Fig.3: Recruitment of MTA1 onto the Dnmt3a promoter as assayed by chromatin immunoprecipitation.

Functional role of p21-Activated Kinases in Oral Carcinogenesis

Parvathy Muraleedharan, S. Sreeja, Rakesh Kumar and M. Radhakrishna Pillai

Oral Cancer is the second most common cancer among males and fourth-most common cancer among females in India (GLOBOCAN 2012). Even with advent of modern technology and advancements in cancer research, the survival rate of oral cancer patients still remains very low. Hence, search for new and effective therapeutic targets are a priority for oral cancer. A potential set of target molecules is p21-activated kinases or PAKs – family of serine-threonine kinases involved in multiple cellular functions such as cytoskeletal remodelling, motility, angiogenesis, invasion and regulation of cell cycle and mitosis. Because these cellular activities are important for cancer progression, PAKs have potential as possible therapeutic targets. The main objective of this part of the work was to further delineate the functional significance of PAK4 in oral cancer progression. Dynamic changes in the actin structure are important for cancer cells to attain morphology, which will help them to move and invade into neighbouring tissue. To study the role of PAK4 in actin remodelling, motility and invasiveness of OSCC cells, we depleted PAK4 in SAS cells using PAK4 or control siRNA (Fig 1A) and did phalloidin staining, wound-healing and Matrigel invasion assays respectively. It was observed that PAK4 knock-down in SAS cells led to an altered actin structure as compared to the control siRNA transfected cells (Fig 1B). Even after 24 hours of inflicting the wound, PAK4 knock known cells exhibited a compromised wound healing (Fig





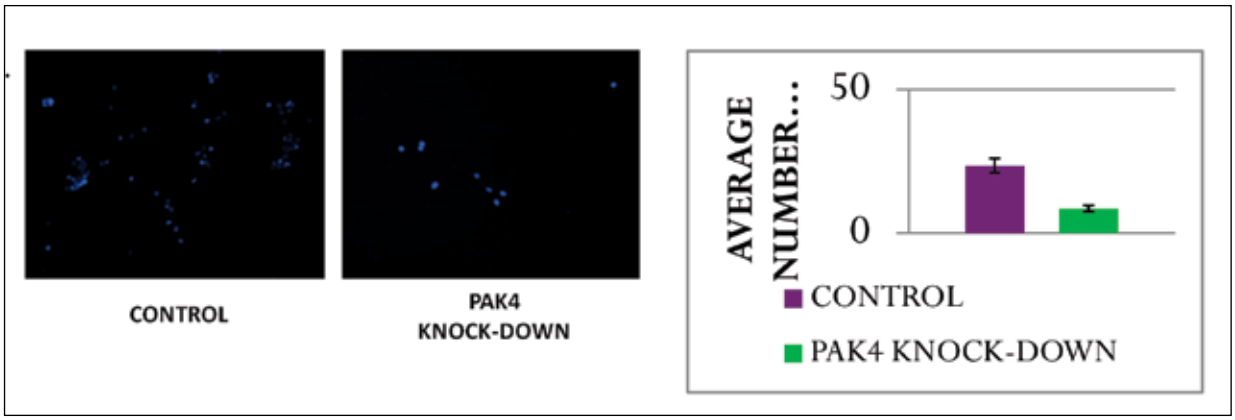


Figure 1: Biological Role of PAK4 in OSCC cells:

Immunoblot analysis of PAK4 expression in control and PAK4 knock-down SAS cells and its densitometric quantitation.

Phalloidin staining showing actin structures in the SAS cells treated with the control or PAK4 siRNA.

Wound healing assay in the SAS cells treated with the control or PAK4 siRNAs.

Fluorescent images obtained after matrigel invasion assay, showing the cells (stained with Hoechst) that have migrated through the matrigel. A quantitative representation of the image data is also illustrated.

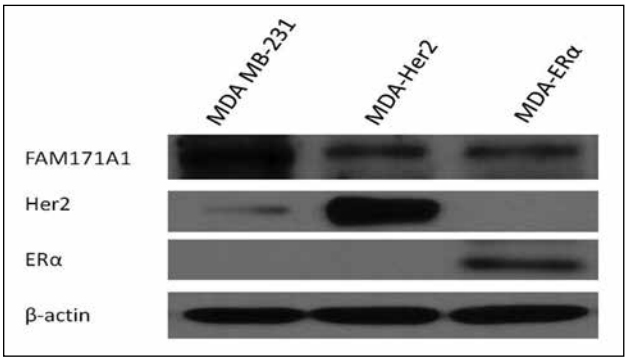
1C), suggesting that the involvement of PAK4 in the directional motility of Oral Squamous Cell Carcinoma (OSCC) cells. Also, in comparison to control siRNA transfected cells, PAK4 knock down cells showed reduced invasiveness (Fig 1D), signifying that PAK4 is involved in the invasiveness of OSCC cells. In brief, these findings reveal the significance of PAK4 in the crucial steps of oral cancer progression, i.e., cytoskeletal remodelling, motility and invasiveness of OSCC cells, and thus, PAK4 represents a potential therapeutic target for Oral Cancer.

The role of FAM171A1 in breast cancer

Rahul Sanawar, TR Santhosh Kumar, Rakesh Kumar and M. Radhakrishna Pillai

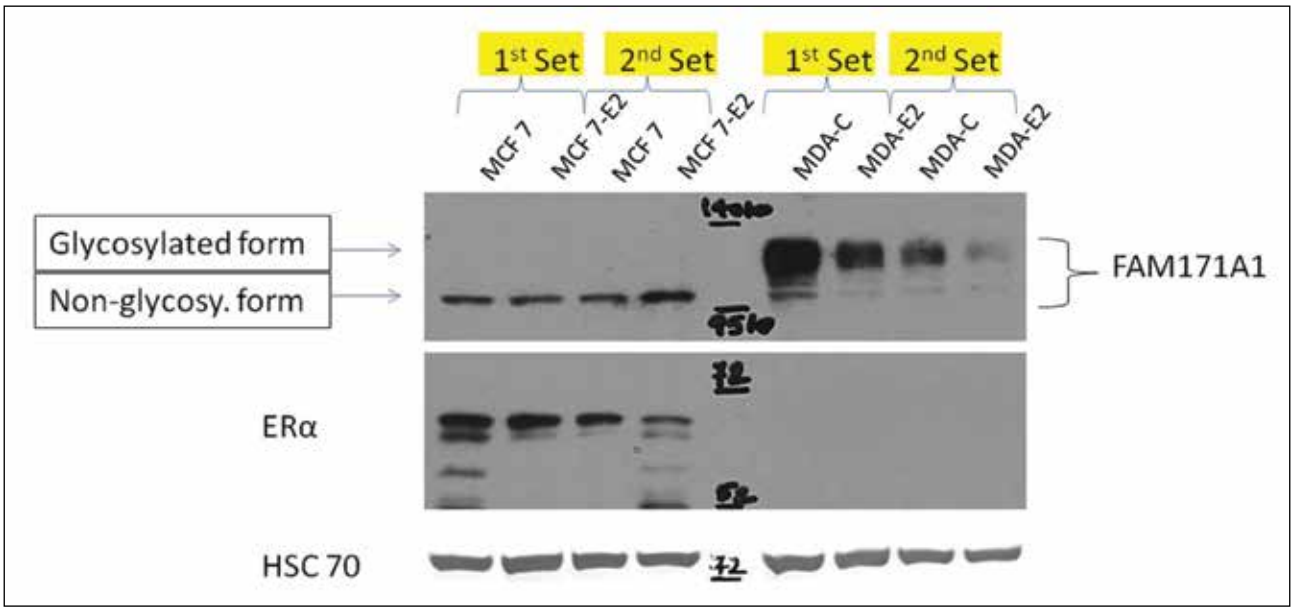
Despite advances in our ability to detect and treat breast cancer, it remains a leading cause of cancer related death in women. Moreover this incidence is rising particularly in case of those tumors that do not express estrogen, progesterone and HER2 receptors. These tumors called triple negative breast cancers (TNBC) are more aggressive owing to non-availability of targeted therapy and its inherent increased invasion and metastasis properties coupled with non-responsiveness to current therapy. It accounts for 15-25% of all breast cancers and characterized by absence of expression of ER, PR and HER2 genes. Microarray expression profiles of breast cancer cell lines such as luminal, basal, TNBC and tumor tissues have been published. Utilizing these database, a bioinformatics based approach was employed to identify unique genes associated with TNBC that show similar expression profiles in both breast cancer cell lines and primary tumor tissues. Adopting this strategy, we shortlisted a gene called "FAM171A1" as a key candidate gene expressed in basal like breast cancer cell lines as compared to luminal type. Extensive data mining analysis suggests that FAM171A1 that was presumed to play a role in cancer invasion, metastasis and proliferation and may also be involved in chemo resistance. Furthermore, in-silico analysis of microarray data for FAM171A1 binding proteins predicts that FAM171A1 may potentially interacts with ETS1 transcription factor and MDM2 (which inhibits the activity of the p53 tumor suppressor). However, the exact function of FAM171A1 in cancer remains unknown at this time. We performed Western blot analysis for FAM171A1 in a panel of cell lines with known receptor status. High expression of FAM171A1 was observed in basal type breast cancer cell lines i.e. MDAMB 231, SUM149, SUM159 (Triple negative cell lines) as well as SKBR3 (here mimicking TNBC status) and OVICAR8 (ovarian cancer cell line) whereas it was found absent in luminal type cell lines(MCF 7 and T47D). From the above experiment, we suspected ERα could negative regulate FAM171A1. To validate this regulatory effect of ERα on FAM171A1, we performed another set of experiment in which we used MDA MB 231 cell line (TNBC cell line showing high FAM171A1 expression) and transfected it with ERα plasmid and studied the effect on FAM171A1 in overexpressing cells. Over expression of Her2 or ER-alpha lead to the decreased expression of FAM171A1, implying a negative regulation of FAM171A1 by ERα and/or HER2.

Based on these results, we focused further on ERα. To examine whether ERα could bind to FAM171A1 promoter, we analyzed 2-kb promoter sequence upstream of transcription start site (TSS) for the predicted transcription factor binding sites using the Transcription factor binding tool -"PROMO" and noticed a perfect ERE consensus sequence (GGTCAnnnTGACC) in the promoter of FAM171A1 (within 2 KB region within the promoter). This raises the possibility that ERα could bind to the FAM171A1 promoter either directly or through a co regulatory complex. To experimentally examine the negative regulation of ERα on FAM171A1, MCF-7(ERα positive) and MDAMB 231(ERα negative as a control) cell lines were treated with 17β-estradiol(E2) treatment. Estradiol did not induce high expression of ER



in MCF-7. In addition ER ligand treatment did not lead to the expression of ERα in MDA MB 231. Moreover in these experiments, we noticed that the FAM171A1 band was seen come above the marker band in MDAMB 231 but not in MCF-7. Hence we suspected that FAM171A1 may undergo post translation modification in TNBC cell lines. It seems to undergo glycosylation (http://www.uniprot.org/uniprot/Q5VUB5) and that this glycosylation is inhibited by 17β-estradiol but independent of ER.

To further confirm whether FAM171A1 undergoes glycosylation, we performed an assay in which we used Tunicamycin(N-glycosylation inhibitor) with and without 17β-estradiol in MDA MB 231 cell line and noticed that glycosylation is inhibited upon tunicamycin treatment.



PUBLICATIONS

- *S Deivendran*, K Hezlin Marzook*, M Radhakrishna Pillai.* The role of inflammation in cervical cancer. *Adv Exp Med Biol 2014 ;816:377-99* (*Equal contribution)
- *Hezlin Marzook*, Deivendran S*, Rakesh Kumar, M Radhakrishna Pillai.* Role of MTA1 in Head and Neck cancer. *Cancer and Metastasis Reviews 2014 33:953–964* (*Equal contribution)

CONFERENCE PRESENTATIONS

- *Hezlin Marzook, Deivendran S, T.R. Santhoshkumar, Rakesh Kumar and M. Radhakrishna Pillai* Decoding the regulatory functions of MTA1, a stress responsive protein under hypoxic conditions*; Carcinogenesis 2015,* 11-13 February 2015, ACTREC, Mumbai
- *Deivendran S, T.R. Santhoshkumar, Rakesh Kumar and M. RadhakrishnaPillai.* Metastatic Tumor Antigen 1, coregulator transcriptionally represses DNA methyl transferase 3a in cancer cells; *Carcinogenesis 2015,* 11-13 February 2015, ACTREC, Navi Mumbai.

AWARDS

Best Poster Award by Biochemical Journal

- *Hezlin Marzook, T. R. Santhoshkumar, Rakesh Kumar and M. Radhakrishna Pillai;* Revelation of physiological stress response of Metastasis Tumor Antigen 1(MTA1) in breast cancer cells; *33rd Annual Convention of Indian Association for Cancer Research IACR 2014,* 13-15 February 2014, Trivandrum

Young Scientist Award

- *Parvathy Muraleedharan, Sreeja S, MR Pillai and Rakesh Kumar.* P21 Activated Kinase 1 PAK1) and its substrates are potential therapeutic targets of oral cancer. Indian Genetics Congress (IGC 2015), Chennai, Tamil Nadu, India. March 2015." at the *Indian Genetics Congress, 2015,* held at SRM University, Chennai.

PROGRAM SCIENTIST

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Annie Agnes Suganya
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Detection of Oral Carcinoma Stem Cells Using Specific Markers

Annie Agnes Suganya, Kochurani, K.J., Madhumathy .G. Nair, Jiss Maria Louis, Balagopal P.G*, Paul Sebastian*, Santhosh Sankaran, Rajagopal, R***., Santhosh Kumar K** and Tessy Thomas Maliekal

** Collaborator: Surgical Oncology, Regional Cancer Centre, Thiruvananthapuram*
*** Collaborator: Chemical Biology, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram.*

Cancer stem cells (CSCs) are a sub-population of heterogeneous tumor, possessing self-renewing ability, tumor initiation capacity, metastatic ability, chemoresistance and radioresistance, regulating the prognosis. We have used sphere culture to ensure the enrichment of self-renewing cancer stem cells in comparison to monolayer culture, which has relatively less number of cancer stem cells. The membrane fractions of sphere cells and monolayer culture were prepared and the proteins were analyzed using Synapt G2 HDMS ESI QTOF. Biological replicates were analyszed and 93 molecules were consistently up-regulated in sphere culture as shown in the Fig. 1a.

The membrane prep was also done using SILAC labeled cells and the membrane fractions were analyzed on a hybrid LTQ–Orbitrap Velos ETD. The up-regulated molecules in all the three experiments were selected for further analysis. The over-expressions of the selected molecules in spheres were confirmed by western blot using antibodies for the specific molecules (Fig. 1b). For further confirmation of membrane localization immunofluorescence was done with or without permeabilization. When the molecules were probed after permeabilization, the expressions of the molecules were limited to the cytoplasm of monolayer cells, while in spheres majority of the molecules were localized to the plasma membrane (Fig. 2). Our observations suggest that the molecules we identified are specifically exported to the plasma membrane in sphere condition, where there is enrichment of self-renewing property. These molecules are being tested as a marker for oral cancer stem cells.

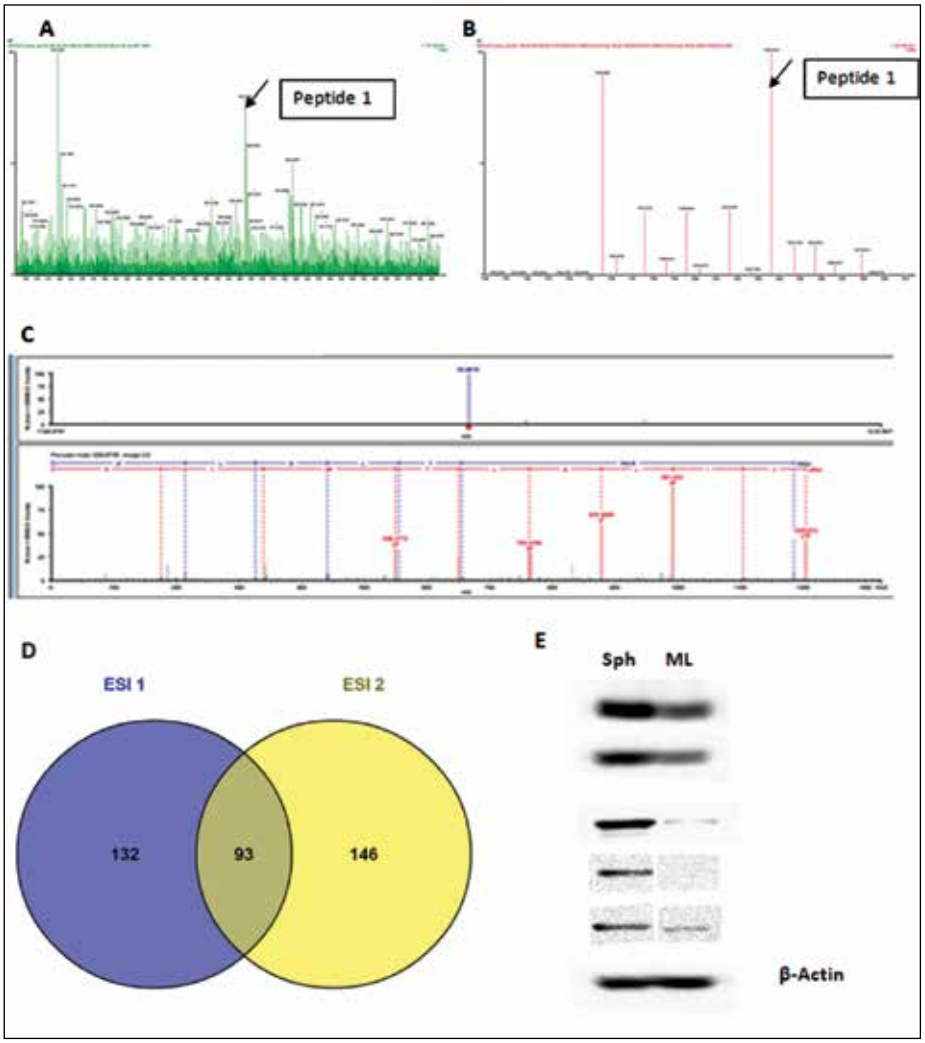

Fig 1. Proteomic analysis for the identification of surface molecules over-expressed in sphere cells.

A. Membrane fractions were made from sphere cells and monolayer cells and they were analysed by Synapt G2 HDMS ESI QTOF to get a chromatogram. Each peptide was deconvoluted as shown in B and identified as shown in C. D. shows the venn diagram of the up-regulated molecules in sphere in biological replicates identified by ESI QTOF.

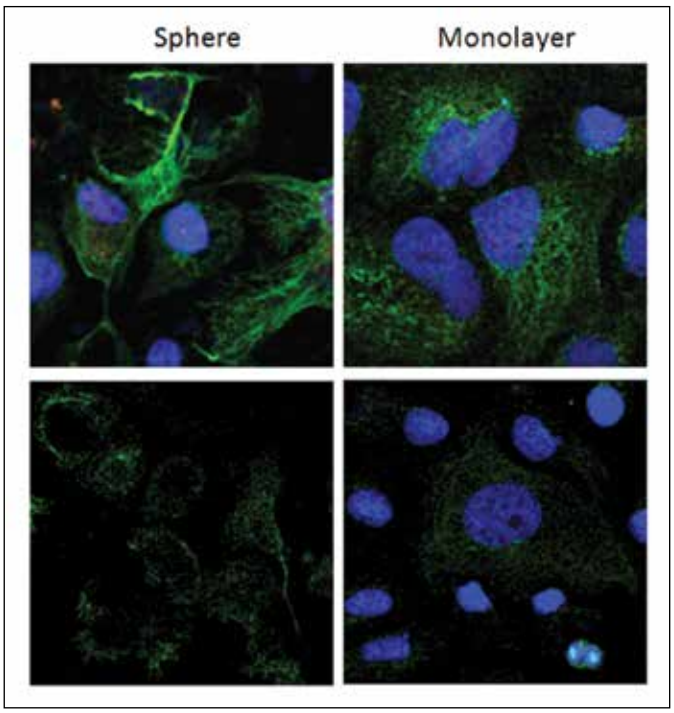

Fig 2 Localization of the identified molecules in oral cancer cells.

Cells were grown either in 10% DMEM or sphere medium for 4 days on a confocal dish and fixed with 4% PFA and permeabilized with 0.3% Triton x100. The different molecules were probed and visualized using confocal microscope.

Characterization and Evaluation of Signaling Pathways Regulating Oral Cancer Stem Cell Properties

Jiss Maria Louis, Annie Agnes Suganya, Madhumathy .G. Nair, Kochurani, K.J., Balagopal P.G*, Paul Sebastian*, Keshava K Datta**, Harsha Gowda**, Ani Das§, Abdul Balagopal§§, Santhosh Sankaran***, Rajagopal, R***., Akhilesh Pandey**and Tessy Thomas Maliekal

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Characterizing the signaling network that sustains oral cancer CSCs

Failure of cancer chemotherapy and radiotherapy largely depends on the survival and expansion of cancer cells that possess self-renewal ability, which are called cancer stem cells (CSCs). Understanding the signaling networks of CSCs that sustain the "CSC-state" will provide insights to the potential targetsthat could be used to develop chemotherapeutic drugs. Since majority of the signaling events are mediated through phosphorylation, a SILAC-based phosphoproteome analysis of sphere cultured cells (with enriched CSCs) in comparison to monolayer cells were performed to understand the signaling events that sustain the CSC characteristics. For SILAC labeling, monolayer cells of oral cancer cell line

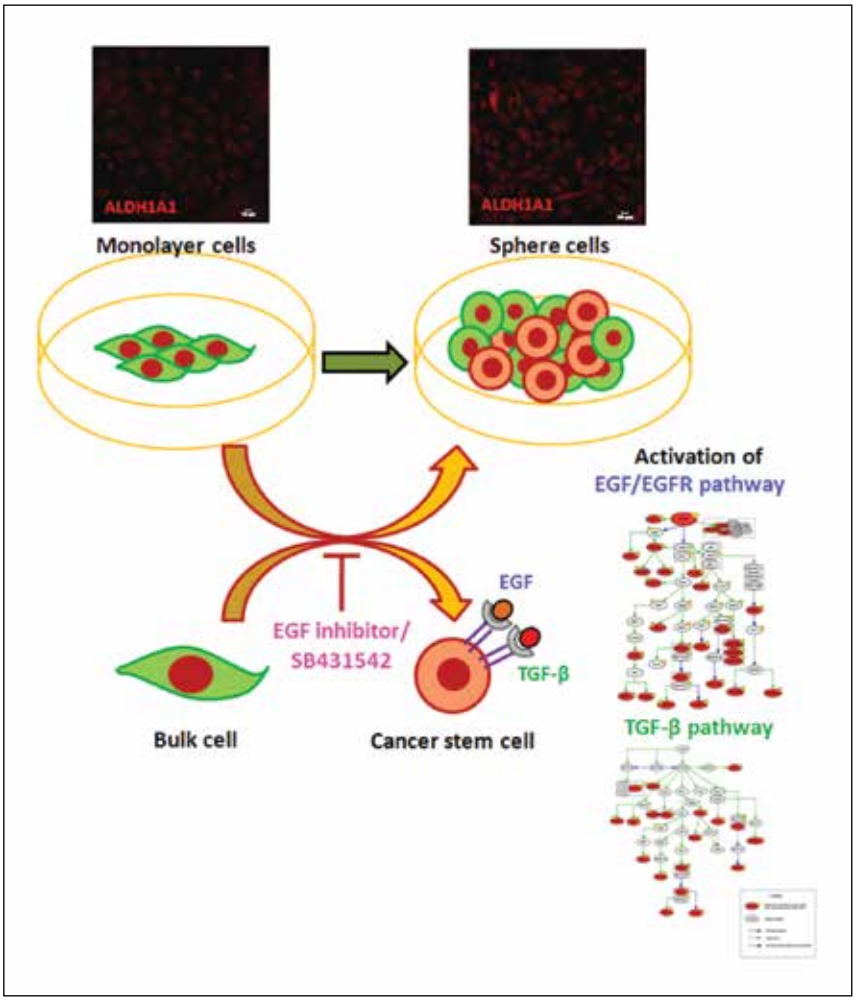

Fig 3 Graphical representation of the result of phosphoproteomic analysis

Cells were grown as monolayer cells or sphere cells show difference in the expression of CSC marker, ALDH1A1 confirming that sphere condition enrich CSCs. The non-self-renewing bulk cell can be converted to self-renewing CSCs by the activation of different pathways that we identified. Two of the pathways are shown in the figure. The red circle in the pathway flow chart indicates the molecules we identified in our analysis

origin were in vivo labeled with 13C labeled lysine and Arginine. Sphere cells of the same origin were grown in unlabeled medium. Samples were analyzed for phosphoproteome in IOB, Bangalore. The phosphoproteome analysis of the sphere culture in comparison to the monolayer culture identified 1210 differentially regulated phosphorylated peptides out of 2464 unique peptides identified. We identified different signaling pathways that are activated in the spheres, which could sustain the CSC-state. We identified several pathways, which included some already known pathways and a few that were not implicated in cancer stem cell regulation. To validate the results we inhibited two of these pathways, TGF-β and EGFR pathways, using chemical inhibitors, which reduced the CSC population, suggesting the vital role of these pathways in the maintenance of oral CSCs (Fig 3).

In vivo evaluation of the role of signaling pathways in the regulation of oral cancer stem cells

TGF-β is reported to be secreted by tumor cells as well as neighboring stromal cells in oral carcinoma It is postulated that the effects of TGF-β signalling are context dependent and influenced by stroma. We hypothesised that tumor niche might play a major role in the regulation of self-renewal. To mark CSC population, an ALDH1A1 reporter vector was constructed by subcloning the promoter region of ALDH1A1 into pCDNA3-mRFP vector (Fig 4A). Oral cancer cells that stably express the construct were selected using G418. To understand the spatio-temporal relation of the activation of the selected pathways and the CSC population, an orthotopic oral cancer model was developed using the ALDH1A1 reporter cells. Immunofluorescence analysis of cryosections of the orthotopic model

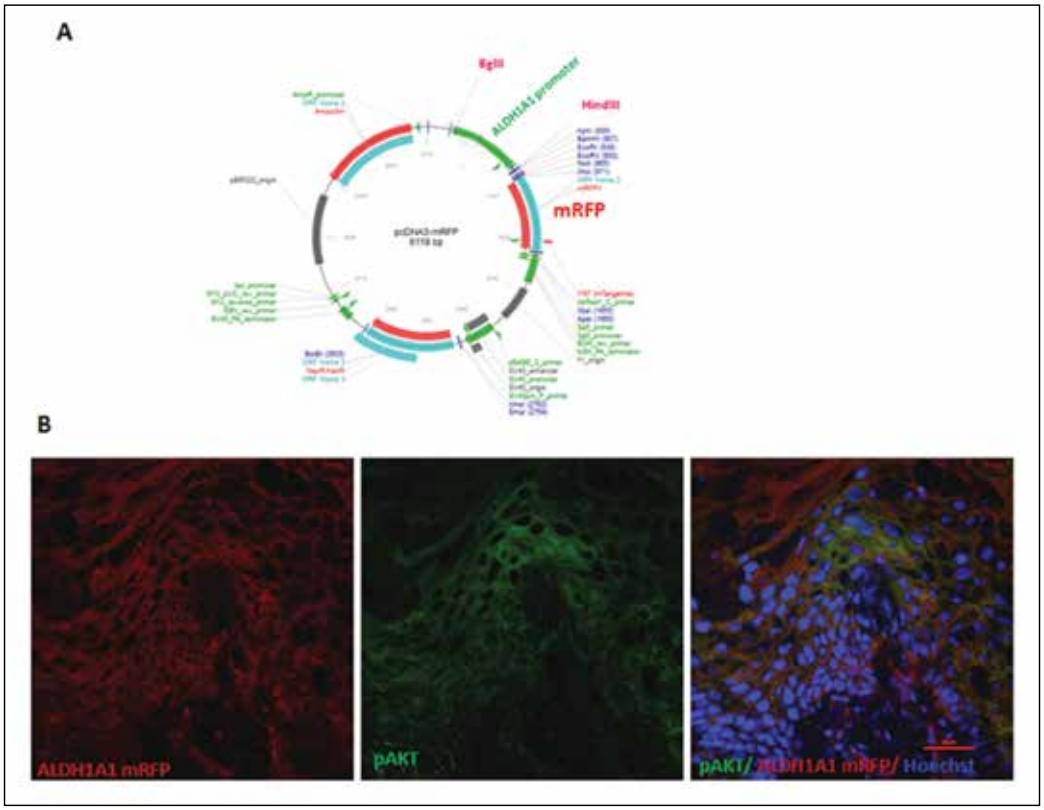

Fig 4 ALDH1A1 reporter cells for in vivo confirmation of the role of signaling pathways

A. The vector map of ALDH1A1 reporter construct B. Oral cancer cells were transfected with the reporter construct and the stable cells were used for the generation of orthotopic model. Cryosections were probed for TGF-β pathway component molecules.

shows that the activation of the TGF-β pathway components is limited to ALDH1A1 expressing cells (Fig 4B). Further analysis confirmed that CSCs have a niche around it supported by different ligand molecules and their activation in the cancer cells converts them to CSCs.

CONFERENCE PRESENTATION: 2014-2015

- GRPR-detecting peptides: A tool for oral carcinoma detection by optical imaging *Annie Agnes Suganya, Kochurani, K.J., Madhumathy .G. Nair, Jiss Maria Louis, Balagopal P.G, Paul Sebastian, Santhosh Sankaran, Rajagopal, R., Santhosh Kumar and Tessy Thomas Maliekal IACR 2014*
- TIF1γ as a master regulator of self-renewal ability of oral cancer stem cells *Jiss Maria Louis, Madhumathy G. Nair, Kochurani, K J., Annie Agnes Suganya, Balagopal P.G, Paul Sebastian, Harsha, Gowda, Datta, K.K., Pandey, A. and Tessy Thomas Maliekal IACR, 2014*
- TGF-β signaling plays a critical role in recurrence after chemotherapy in oral carcinoma *Maria Louis, Madhumathy G. Nair, Kochurani, K J., Annie Agnes Suganya, Balagopal P.G, Paul Sebastian, Harsha Gowda, Datta, K.K., Pandey, A. and Tessy Thomas Maliekal IACR, 2014*

LIST OF ONGOING EXTRA MURAL GRANTS

No	Title of the Project	Funding Agency	Duration
1	Identifying surface marker signature of oral cancer stem cells to develop a prognosis marker for oral squamous cell carcinoma using peptide-based CSC detection	Department of Biotechnology, Government of India	2011- 2014
2	Characterizing the signaling network that sustains oral cancer stem cells for developing a targeted therapy	Department of Science and Technology, Government of India	2011- 2014

CANCER RESEARCH PROGRAM
LABORATORY– 10

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Rakesh Laishram received his PhD working at the Centre for DNA Fingerprinting and Diagnostics, Hyderabad and then trained as a post doctoral fellow at the Department of Phramacology, University of Wisconsin-Madison, USA. Rakesh is a DBT-Welcome Trust Fellow and also has been awarded the Innovative Young Biotechnology Award from the Department of Biotechnology.

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Divya Kandala

Role nuclear non-canonical poly(A) polymerase, Star-PAP in alternative polyadenylation and pre mRNA 3′-end processing

Star-PAP is a newly identified poly(A) polymerase involved in the polyadenylation of select mRNAs involved in oxidative stress response. Genome wide analysis indicated a large numbers of Star-PAP target genes that harbour multiple poly (A) sites suggesting role of Star-PAP in alternative polyadenylation (APA). In APA mediated gene regulation, a single gene encodes multiple mRNA transcripts with different UTR length (encoding the same protein), and the difference in the length of the RNA regulates the expression. APA changes are considered hallmarks of cancer progression and implicated in stem cell development and tissue differentiation, yet the mechanism is obscure. To investigate the role of Star-PAP in genome wide APA, we have analysed the UTR usage pattern from the microarray of all genes containing alternative poly(A) sites using specific probes at

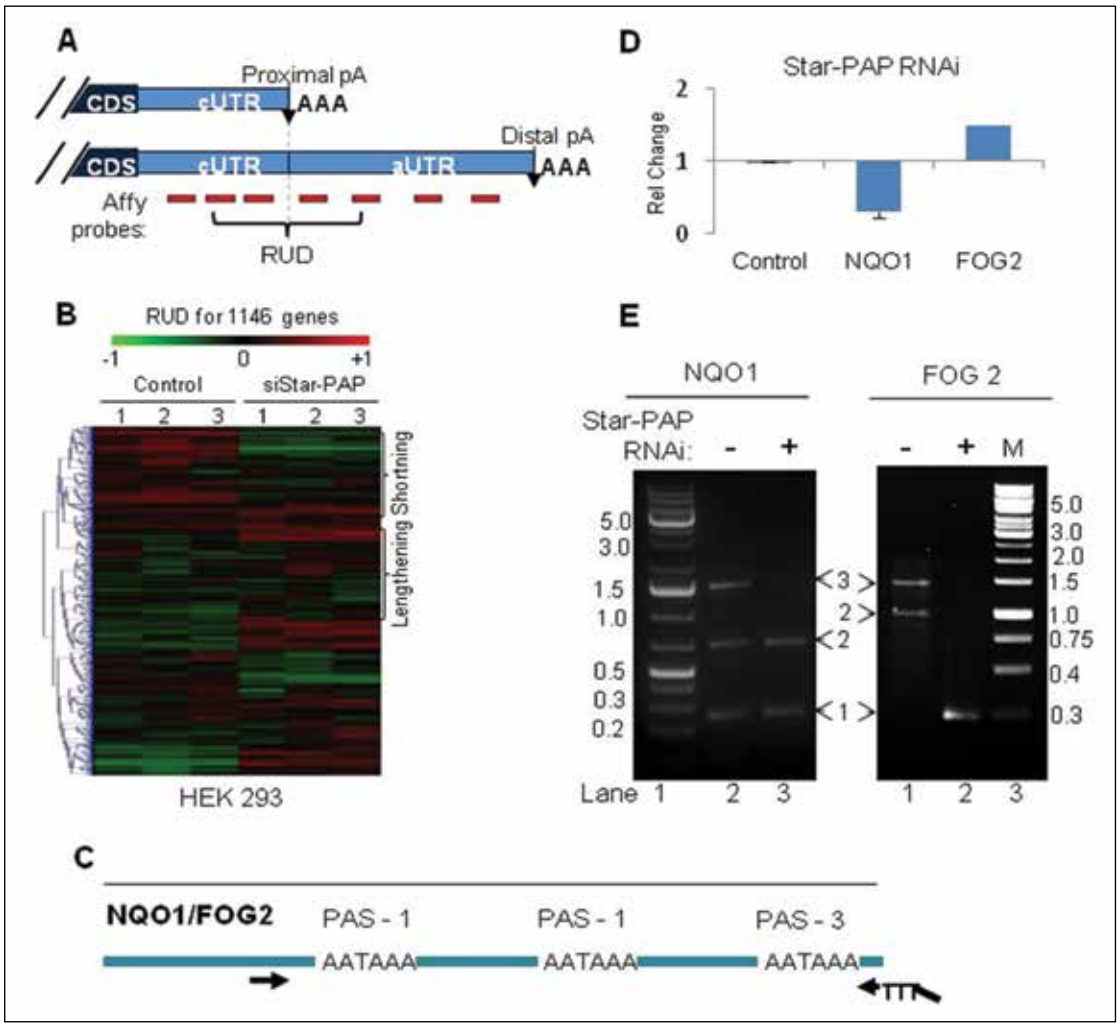

Fig. 1: Role of Star-PAP in alternative polyadenylation. (A-B) Analysis of UTR usage pattern genome wide after Star-PAP knockdown. Star-PAP can use both the proximal and distal poly(A) sites. The schematics are shown in A. (C-D) Confirmation of APA for NQO1 and FOG2 genes. Schematic is shown in C. Both NQO-1 and FOG2 have three poly(A) sites of which Star-PAP regulates distal site in NQO1. In FOG2, two distal sites are functional when Star-PAP is present, and upon knockdown the proximal site becomes functional and exclusively regulates total mRNA expression. (B) Expression profile of a set of NQO1 and FOG2. Star-PAP knockdown resulted in loss of NQO1 and increase expression of FOG2.

the 3'-UTR. We show that knockdown of Star-PAP results in both shortening and lengthening of UTRs of distinct target genes suggesting that Star-PAP can regulate either proximal and distal sites in any of the target genes. The usage of distal site was more compared to the usage of proximal sites. This confirms the regulation of poly(A) site selection by Star-PAP. We further studied the Star-PAP mediated specific poly(A) site selection using genes such as NQO-1 and FOG2, both of which have implication in cardiovascular disease and function. NQO1 has three poly(A) sites but Star-PAP regulates only the distal site specific isoform while the other two proximal sites are likely targets for canonical PAPα. However, quantitative real time PCR demonstrated that Star-PAP controls majority of its mRNA expression suggesting that the distal site specific isoform is the functionally most significant isoform of NQO1. In addition, FOG2 has three poly(A) sites; interestingly, in presence of Star-PAP the most proximal site is repressed and we could detect only the two distal site specific isoforms. Knockdown of Star-PAP resulted in the loss of the two distal site specific isoforms and the proximal site specific appeared suggesting that in absence of Star-PAP the proximal site specific isoform controls majority of FOG2 gene expression. It is shown in Fig. 1. We are in the process of defining the mechanism of APA site selection on these genes using reporter constructs.

Specificity and mechanism of poly(A) site selection by nuclear poly(A) polymerases - Star-PAP vs canonical PAPα:

There are two major nuclear poly(A) polymerases in the nucleus involved in 3'-end processing of mRNAs - canonical PAPα and Star-PAP. We have shown that the two PAPs assemble distinct processing complexes and controls different target genes. However, target mRNA specificities of the two PAPs and how it is achieved is still not understood. Star-PAP directly binds target RNA and recruits cleavage factors CPSF 160 and 73 while PAPα has no such specific RNA recognition, and is recruited on the mRNA by CPSF complex. Therefore, we investigated the following possible mechanism of poly(A) site selection: 1) Distinct cleavage factors associated with Star-PAP and PAPα, 2) Star-PAP specific nucleotide elements on the target RNA, 3) CstF and the suboptimal DSE, while CstF is critical for PAPα, it is dispensable for Star-PAP mediated RNA processing, and 4) Competition between Star-PAP and PAPα for cleavage factor (CPSF) interaction. We have shown that Star-PAP and PAPα has different interacting partners and that both compete for binding to CPSF 160. However, Star-PAP is preferred over PAPα for CPSF interaction. We further confirmed a Star-PAP recognition motif with core -AUA- element that is present in all Star-PAP target mRNAs. We demonstrate the significance of AUA motif in Star-PAP dependent target mRNA 3'-processing and expression using reporter construct, and in vitro by EMSA using mutagenesis approach. We have also demonstrated that CstF is dispensable for Star-PAP target mRNA expression. The suboptimal DSE on the Star-PAP target mRNAs prevent CstF binding onto Star-PAP target genes and as a result it excludes PAPα from accessing Star-PAP target mRNAs. Thus a Star-PAP dependent sequence and a suboptimal downstream sequence endows specificity to Star-PAP for its poly(A) site selection.

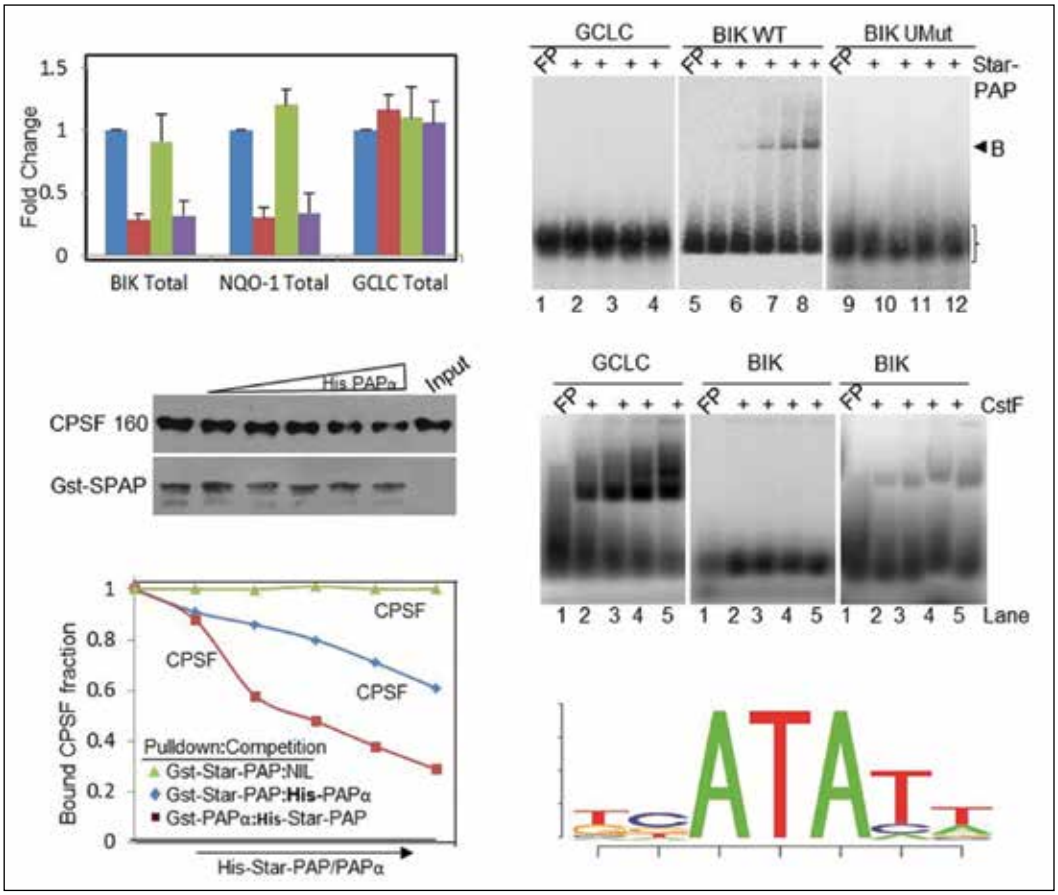

Fig. 2: Star-PAP specificity mechanisms: (A) Star-PAP targets are exclusive to Star-PAP and independent of PAPα. qRT-PCR of BIK, NQO-1 or GCLC from HEK 293 cell total RNA from 1- control, 2-siStar-PAP, 3-siStar-PAP with rescue Star-PAP, 4-siStar-PAP with rescue PAPα. (B) EMSA experiment of GCLC, BIK UTR or with mutation of AUA to GGG on BIK UTR with His-Star-PAP. (C) Star-PAP and PAPα compete with each other for CPSF binding. Gst-pulldown of CPSF 160 with Gst-PAPα in presence of increasing PAPα concentration (D) EMSA of GCLC and BIK with CstF 64. BIK UTR does not bind CstF, however, introduction of U-rich DSE (UUUUUU) at the DSE resulted in strong interaction of CstF with BIK UTR. (E) Preferential binding of Star-PAP to CPSF over PAPα. Loss of CPSF bound to PAPα was more when competed with Star-PAP than those bound to Star-PAP on PAPα competition. (F) Putative Star-PAP binding motif on its target mRNAs.

Signalling regulations of cleavage and polyadenylation: phosphoinositides and phosphorylation:

3'-end processing is highly regulated like at the 5'-end. Different signalling pathways regulate 3'-end processing that includes but not limited to oxidative stress, DNA damage, phosphoinositide and in addition phosphorylation. Star-PAP associates with and regulated by the co-regulator PIPKIα that synthesizes lipid messenger PI4,5P2. Star-PAP activity is also highly induced by addition of nuclear PI4,5P2. Conversely, PIPKIα and PI4,5P2 also regulate Star-PAP target gene expression. Among the two steps of cleavage and polyadenylation, PI4,5P2 does not affect the cleavage reaction but it stimulates polyadenylation. Moreover, both Star-PAP and canonical PAP regulated by phosphorylation. Phosphorylation of PAPα n at the C-terminal end inhibits its activity. PAPα is also phosphorylated by ERK kinase that is required for its activity. Similarly, Star-PAP is phosphorylated by CKI and PKCδ that regulates its activity and target gene expression. CKI mediated

phosphorylation is stimulated by oxidative stress while PKCδ mediated phosphorylation controls DNA damage induced gene expression. The hypothesis is that the stimulations on its target pre-mRNA expression are through phosphorylation by distinct kinases. To identify the phosphorylation sites, we undertook mass spectrometry analysis and identified a phosphorylation site at the serine 6 (S6) under normal and stress induced conditions. Mutation of S6 to alanine (S6A) resulted in the loss of Star-PAP target HO-1 expression suggesting that it is required for the expression of Star-PAP target mRNAs. By RNA immunoprecipitation (RIP) and IP analysis, we have shown that this phosphorylation regulates both the aspects of ZF function i.e. target mRNA binding and PIPKIα interaction. Then we raised an antibody against specific S6-phopsho N-terminal peptide and shown that CKIα as the kinase that phosphorylates S6 on Star-PAP. We show that CKIα phosphorylates Star-PAP within the nucleus and it is required for the retention of Star-PAP in the nucleus. Further using the phospho-deficient S6A and phospho mimetic S6G mutations, we have shown that S6 phosphorylation on Star-PAP regulates select target genes. Among genes studied, S6A mutation affected HO-1, BIK, NQO-1, but not PTEN or CHAC1 which were independent of PIPKIα regulation as well. This demonstrates an example a polymerase where phosphorylation determines its target gene specificity.

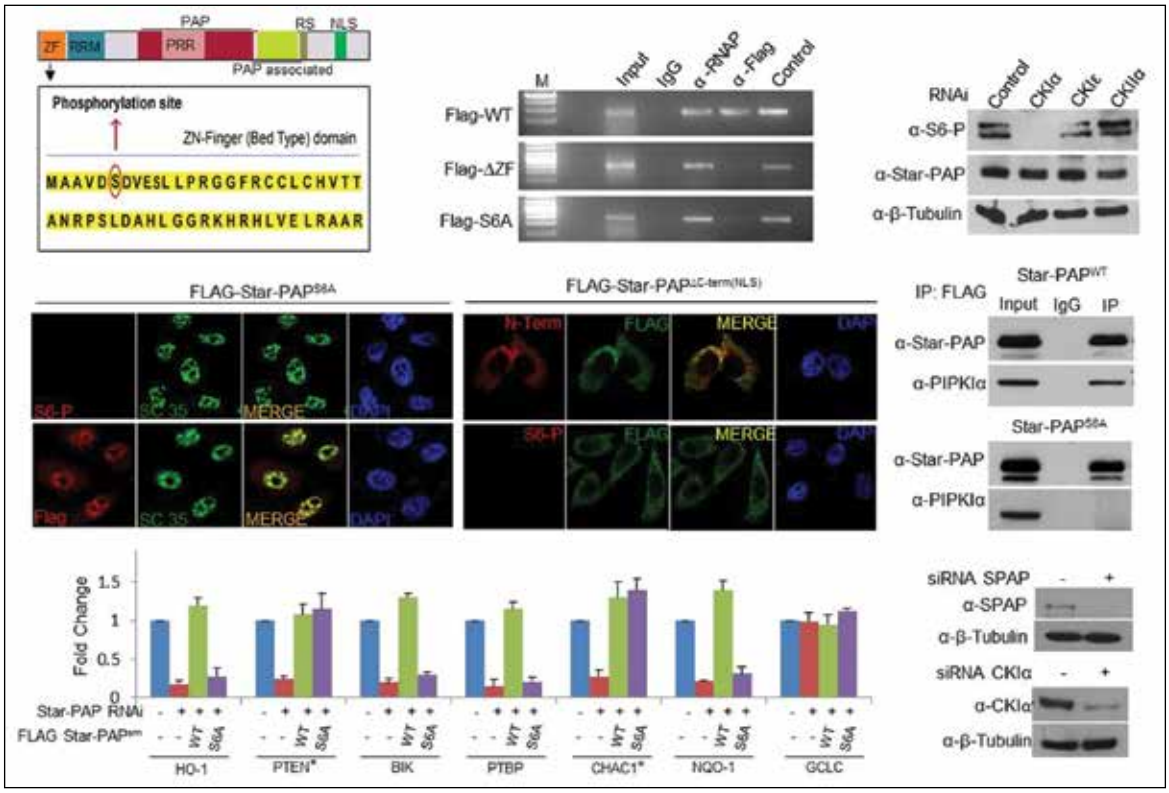

Fig. 3: Star-PAP S6 phosphorylation and its role in Star-PAP regulation: (A) Identification of phosphorylation site by mass spectrometry analysis, (B) RNA immunoprecipitation showing that mutation of S6A resulted in loss of association of Star-PAP with target HO-1 pre-mRNA. (C) Antibody was raised against the N-terminal S6-phopsho peptide and knockdown of CKIα resulted in the loss of S6-phopshor Star-AP with no effect on the non-phospho Star-PAP indicating that CKIα phosphorylates S6 on Star-PAP, (D) Transfection of S6A mutant Star-PAP and detection by IF using FLSG antibody shows no role of S6 phosphorylation on Star-PAP nuclear localization but helps in nuclear retention of Star-PAP, (E) Star-PAP is phosphorylated within the nucleus usng Deletion of NLS on Star-PAP, (F) IP experiment showing that S6 phosphorylation is required for Star-PAP-PIPKIα interaction, (G) qRT-PCR analysis showing that S6-phopsho Star-PAP regulates specific set of mRNA targets. (H) Knockdown Westerns of Star-PAP and CKIα.

Oxidative stress response in 3′-end processing in Cardiovascular Disease and Cancer:

The disruptions of the cellular redox state cause toxic effects through the generation of peroxides and free radicals that damage cell components including proteins, lipids and nucleic acids. Several antioxidant proteins are expressed in the cell that protect against the oxidative stress. In humans, oxidative stress is involved in many diseases including cardiovascular diseases (CVDs) such as myocardial infarction, ischemia, heart failure and stroke. Several genes involved in oxidative stress response such as heme oxygenase-1 (HO-1), NAD(P)H quinine oxidoreductase (NQO-1), aldehyde dehydrogenase (ALDH) etc. are regulated at the 3'-UTR. Star-PAP is one of the polymerase that regulates such genes. We have confirmed the expression profile of several genes critical for cardiovascular system (such as ANAX7, VEGF, CAST, NOS2, FOG2, PAK1, AGTRII, TGFβ, GATA4, HAND2, HO1, NQO1) and cancer (such as ANA7, PTEN, BIK) in presence and absence of Star-PAP knockdown by quantitative real-time PCR analysis. ANXA7 is a tumer suppressor in prostrate cancer and involved in calcium signalling. We showed that ANAX7 expression is controlled by Star-PAP. ANXA7 has two splice isoforms and two poly(A) sites at the 3'-UTR. We observed that both the ANAX7 isoforms are regulated by Star-PAP. Knockdown of Star-PAP resulted in the loss of expression of both the isoforms indicating that the two splice isoforms are not specific to a particular poly(A) site, and both are controlled by Star-PAP. There was 60-80% reduction in the mRNA levels of all the genes studied (such as ANAX7, VEGF, CAST, NOS2, FOG2, PAK1, AGTRII, TGFβ, GATA4, HAND2, HO1, NQO1) critical for cardiovascular function indicating that Star-PAP could act as an important regulator protein in Heart. It is shown in Fig. 4. Further studies are currently under progress as how Star-PAP regulates these genes and also how Star-PAP is involved in human diseases.

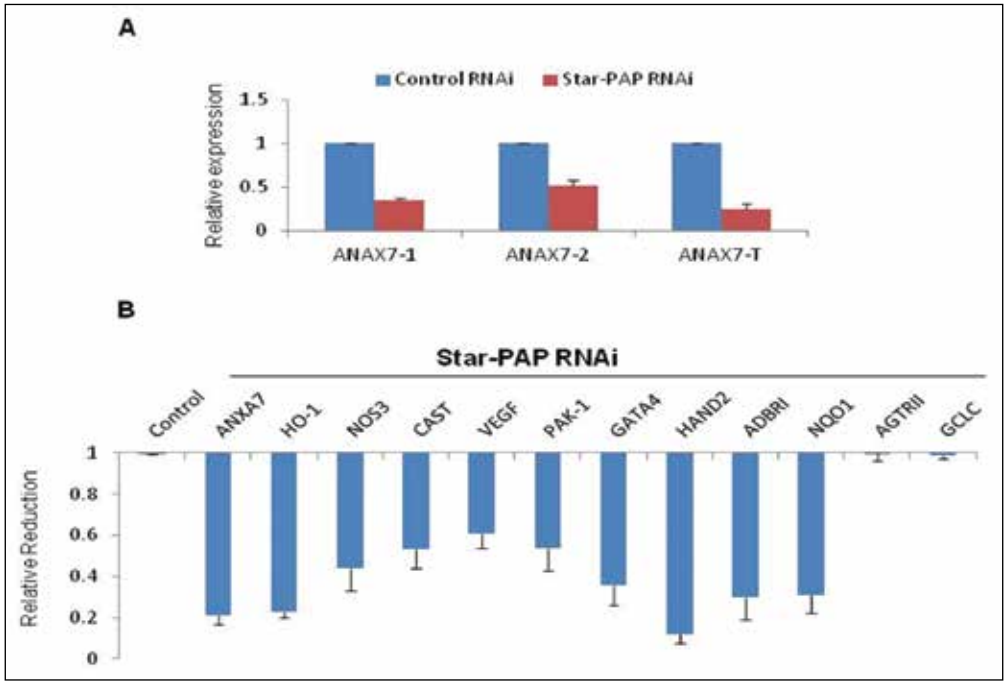

Fig. 4: Expression profile of Star-PAP target genes (A) Real time analysis of ANXA7, a tumer suppressor in prostrate cancer after knockdown of Star-PAP in HEK 293 cells. Both the isoforms of ANXA7 are controlled by Star-PAP (B) Expression profile of a set of Star-PAP target genes critical in oxidative stress response and cardiovascular function.

Molecular function of RNA binding protein, RBM10 in gene expression in the cell:

RBM10 (RNA binding motif 10) is a nuclear protein that belongs to a family proteins having an RNA-binding motif that binds poly(G) or poly(U) stretch and functions in alternate splicing. However, its role in splicing has not been established yet. Defects in this gene have been implicated in human X-linked recessive disorder, TARP syndrome. Recently, role of RBM10 in apoptosis has been proposed. We have identified RBM10 as partner of Star-PAP 3'-end processing complex.

Mass spectrometry analysis of Star-PAP complex purified from the cell has identified RBM10 as a protein associated with Star-PAP. To further investigate the role of RBM10, we undertook co-immunoprecipitation experiment to confirm the association of RBM10 with PAPs. We observed association with both Star-PAP and PAPα suggesting that it could be a common 3'-end processing factor required for cleavage and polyadenylation reactions. Knockdown of RBM10 resulted in a loss of some of the genes but not all studied indicating that RBM10 regulates select set of mRNAs in the cell. Of the tested genes, Star-PAP non target GCLC was also not regulated by RBM10. It is shown in Fig. 5. It is therefore likely that RBM10 plays an important role in the 3'-end processing of select genes from both Star-PAP and PAPα target genes. We are currently undergoing microarray analysis to define the genome wide target genes and its functional significance.

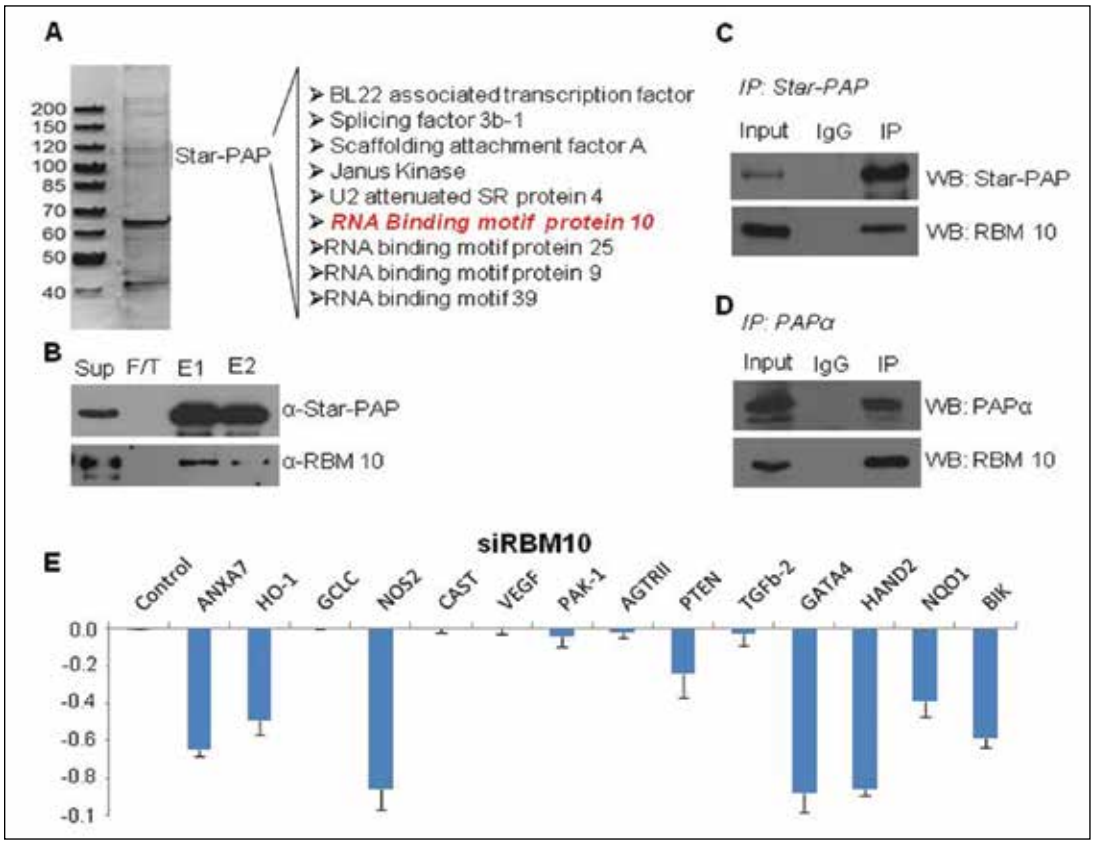

Fig. 5: RNA binding motiff 10, RBM10 and its role in gene expression. (A) Mass spectrometry analysis of a Star-PAP complex. (B) Western blots confirm the association of RBM10 with Star-PAP complex. (C/D) Co-immunoprecipitaion of either Star-PAP or PAPα purifies RBM10 indicating that RBM10 is present in both Star-PAP and PAPα mediated 3'-end processing complex. (E) Expression profile of Star-PAP targets after knockdown of RBM10 suggesting regulation of specific genes.

PUBLICATIONS

- *Rakesh S. Laishram.* 2014. Poly(A) Polymerase (PAP) diversity in the cell: Star-PAP vs canonical PAP. *FEBS Letters.* 588: 2185–2197.

CONFERENCE PRESENTATIONS

- *RNA Meeting,* Kolkata, March 6 - March 8, 2014 (Invited Lecture)
- *Young Investigators Meet,* Hyderabad, January - 2014 (Invited Lecture)
- *CSH meeting on RNA Biology – 2015* Suzhou China, (Speaker)
- *Modern Trends in cardiovascular diseases, 2015* Trivandrum (participant)

RESEARCH GRANTS EXTRA-MURAL FUNDING

Rakesh Laishram - Principal Investigator

No	Title	Funding Agency	Duration
1	Specificity and mechanism of Star-PAP mediated 3'-end processing in gene regulation	Welcome Trust-DBT India Alliance	2012-2017
2	Regulation of 3'-end processing in oxidative stress response and cardiovascular disease	Department of Biotechnology	2013-2015

CARDIOVASCULAR AND DIABETES DISEASE BIOLOGY PROGRAM

CARDIOVASCULAR DISEASE BIOLOGY LABORATORY

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PPIA rs6850:A>G SNP is associated with vascular disease but does not affect plasma cyclophilin A levels

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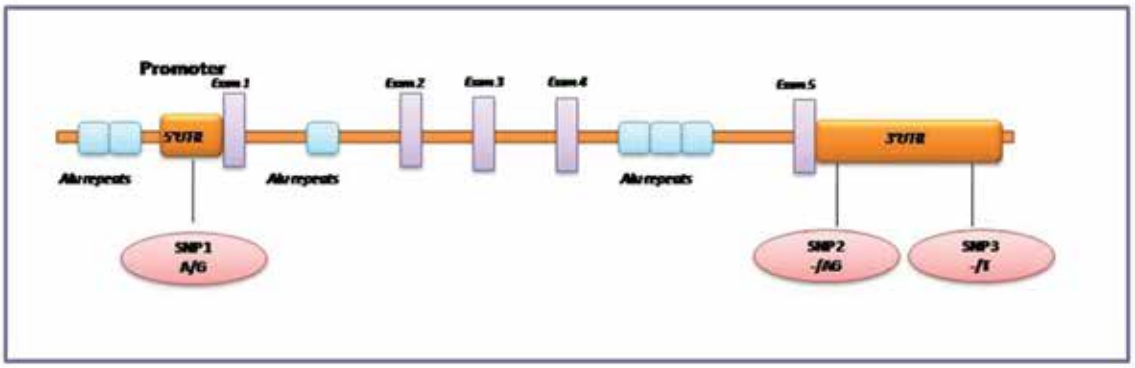
Cyclophilin A, an immunophilin is secreted from human monocytes activated by high glucose. It is part of various intracellular functions, such as intracellular signaling, protein trafficking, and regulating activity of other proteins. Cyclophilin A is also well recognized as a secreted growth factor that is induced by oxidative stress functioning as a mediator of tissue damage associated with inflammation and oxidative stress. We earlier reported proteomic changes occurring in circulating monocytes on activation with high glucose. We also demonstrated secretion of cyclophilin A from monocytes under hyperglycemic conditions. The secretory nature of this protein and its presence in plasma of patients with Diabetes mellitus (DM) and coronary artery disease (CAD) underlines its potential as a marker of disease. The importance of cyclophilin A as a secretory protein is thus clearly evident. There is however a few gap areas in our understanding of the nature of this protein. In order to use this secreted protein as a clinically viable and specific marker of early vascular disease in type 2 diabetes, we need to understand factors affecting its secretion levels. Genetic variations at the gene level may play an important role in its physiological secretion. Cyclophilin A has long been known for its incorporation into HIV-1 virions and its important role in facilitating HIV-1 replication in host cells. Disruption of Cyclophilin incorporation, either by HIV-1 Gag mutations or by cyclosporine A, an immunosuppressive drug that prevents HIV-1Gag binding to Cyclophilin, leads to an attenuation of HIV-1infectivity. Genetic variations, mainly promoter variants in the PPIA gene have been associated with susceptibility to HIV-1 infection and rapid disease progression in HIV-1. Besides HIV, the PPIA variation has also been studied as a risk factor for myocardial infarction. However, the promoter polymorphisms of PPIA gene did not significantly contribute to the risk of suffering from myocardial infarction among patients with atherosclerotic diseased vessels. Even though Cyclophilin A gene variants may not be a causal risk factor for vascular disease, it is possible that promoter based variations may affect the level of secretion of this protein in plasma. In order to exclude this probability, we screened for polymorphisms in the cyclophilin gene and correlated it with levels of the protein in plasma.We examined the effect of single nucleotide polymorphisms (SNPs) within the PPIA gene on status of vascular disease in diabetes. We coupled this with the plasma levels of cyclophilin in the same cohort of patients. The study was carried out in a South Indian cohort of 212 patients constituting healthy controls, patients with diabetes mellitus (DM), coronary artery disease (CAD) and coronary artery disease associated with diabetes (DM+CAD). Using PPIA specific primers the entire PPIA gene was sequenced and analyzed for single nucleotide polymorphisms (SNPs) (Fig 1). We identified three SNPs (SNP1: rs6850:A>G ; SNP 2 : (AG/-) c.*227_*228delAG and SNP 3: (-/T) c.*318_*319insT) (Fig 2). The SNPs were correlated with known blood markers of type 2 diabetes and CAD such as HbA1c, LDL, HDL,

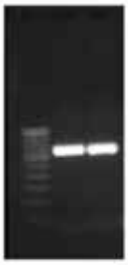
TG, FBS, PPBS as well as Cyclophilin. Welchs two sample t test indicated an association of SNP rs6850:A>G, an SNP located at the putative promoter site of the 5'UTR region with increased plasma levels of cyclophilin. On logistic regression analysis using a multivariate model this association was not confirmed. Presence of rs6850:A>G variant was associated with vascular disease in patients with diabetes mellitus but not with their plasma cyclophilin levels. Furthermore, insilico analysis of the sequence using TRANSFAC software did not predict any significant differential binding sites for SNP1. Our results indicate that cyclophilin A levels in plasma is independent of genetic variations and possibly because of inflammatory vascular disease state.

Fig 1: Gene map and SNPs in the human PPIA gene (cyclophilin A) on Chromosome 7p13

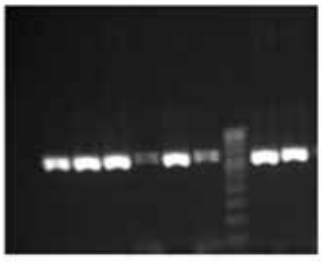


SNP1 is rs6850:A>G
SNP 2 (AG/-) is c.*227_*228delAG
SNP 3 (-/T) is c.*318_*319insT

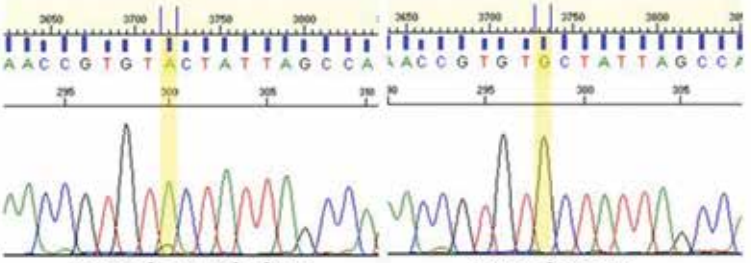
Fig 2: Genotyping of SNPs of PPIA gene using PCR sequencing



SNP-1 PCR product(674bp)

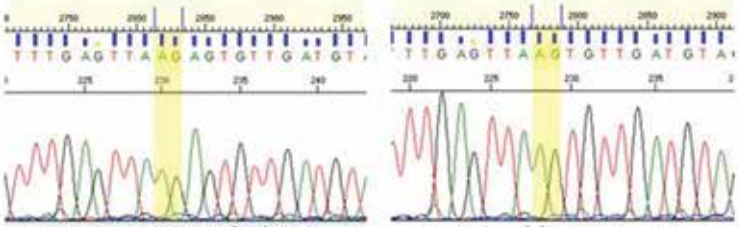


SNP2/SNP3 PCR product (686bp)



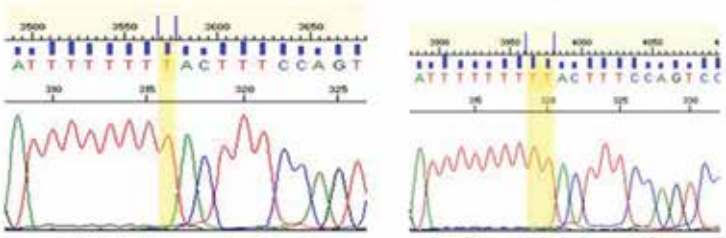
SNP-1 A/G Normal subject

SNP-1 A/G Patient



SNP-2 AG in normal subject

SNP-2 AG deletion in patient



SNP-3 T in normal subject

SNP-2 TT addition in patient

Role of FoxC2 in the pathogenesis of varicose veins

S. Sumi, B.L. RaviKumar*, S.R. Kalpana**, G Kamalapur** and C.C. Kartha

*Collaborators: *Kempegowda Institute of Medical Sciences, Bangalore; **Sri Jayadeva Institute for Cardiovascular Sciences & Research, Bangalore*

The fundamental cause and pathogenesis of primary varicose veins remains vague. Vein wall dilatation and valvular incompetence causing venous reflux has long been postulated as the primary cause of varicose veins. Our earlier studies indicate that transcriptional modulation of FoxC2 expression by regulatory polymorphisms has an impact on the pathogenesis of varicose veins.FoxC2is a regulatory transcription factor and is involved in both lymphatic and vascular development. We have previously reported that c.-512C>T polymorphism of FoxC2 gene is strongly associated with susceptibility to develop varicose veins. It was also observed that this polymorphism increased the expression of FoxC2 at transcriptional and translational level. FoxC2 up regulation in venous endothelial cells resulted in elevated mRNA expression of Notch pathway proteins, specifically arterial endothelial cell markers Dll4 and Hey2 and down regulation of venous marker COUP-TFII. We hypothesize that an altered FoxC2-Notch signaling results in saphenous vein wall remodeling in patients with varicose veins. We performed differential expression analysis for FoxC2 and its downstream targets Dll4 and Hey2 genes in tissue specimens obtained from 22 patients with varicose veins and 25 normal healthy vein specimens obtained during coronary artery bypass graft surgery. Expression levels of FoxC2, Dll4 and Hey2 mRNA were determined in varicose vein relative to control vein samples. Observed values were normalized with GAPDH expression values. There was an elevated expression of FoxC2, Dll4 and Hey2 in vein specimens of patients compared to control vein specimens. Up regulation of FoxC2, Dll4 and Hey2 was confirmed at protein level by western blot analysis (Figure 1).

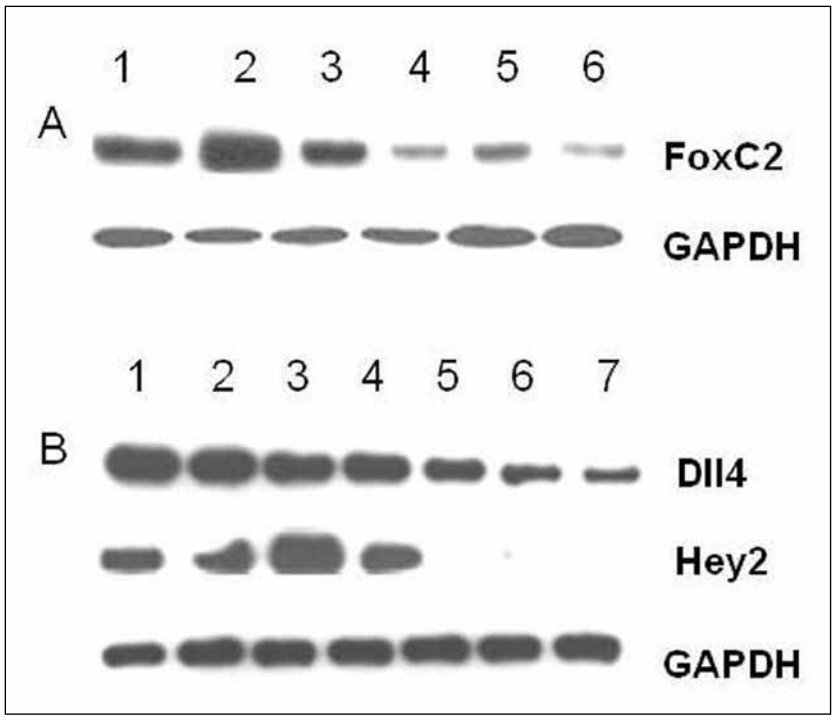


Figure 1: (A) Western blot analysis of FoxC2 and loading control GAPDH in tissue specimens from patients and controls: lanes 1-3 protein homogenates from varicose vein tissues; lanes 4-6 normal saphenous vein protein homogenates. (B) Western blot analysis of Dll4, Hey2 and GAPDH proteins in patients and controls: lanes 1-4 varicose vein protein homogenates; lanes 5-7 protein homogenates from healthy saphenous vein.

We performed immunohisto-chemical staining of sections with specific antibodies against FoxC2, Dll4 and Hey2 proteins. FoxC2, as we reported earlier, was found to be highly expressed in neointima and tunica media of varicose vein tissues compared to its mild expression in tunica media of healthy vein sections. Dll4 was found overexpressed in neointima of varicose vein tissues. In healthy vein sections, we could observe a very weak staining for Dll4 in the

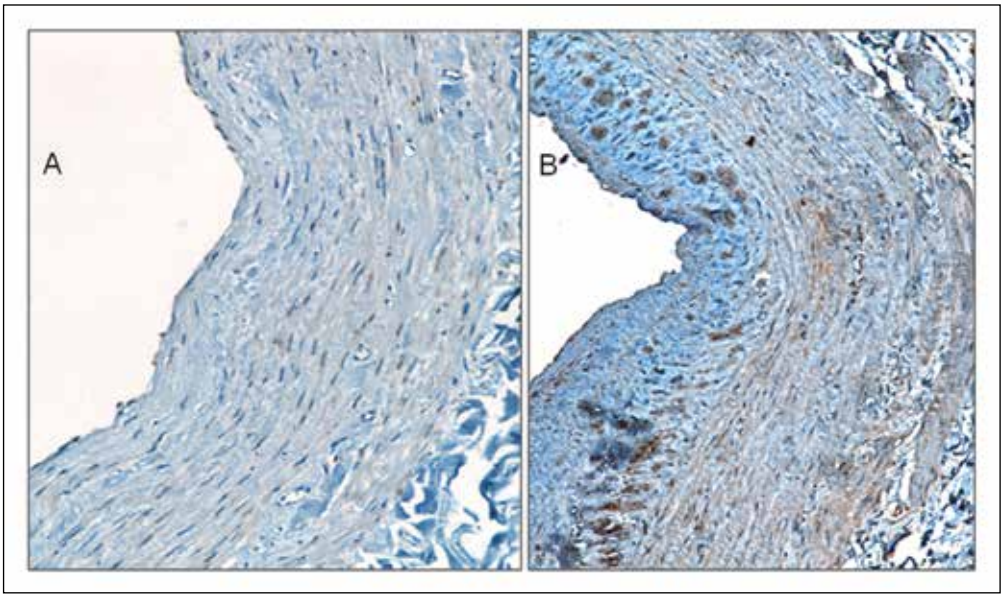


Figure 2: Photomicrograph demonstrating immunohistochemical staining with FoxC2 antibody in venous tissue sections from patients with varicose veins and controls. (A) FoxC2 expression is localized to tunica media in normal venous tissue section. (B) FoxC2 is expressed all over neointima and media of varicose vein tissues (x 10X)

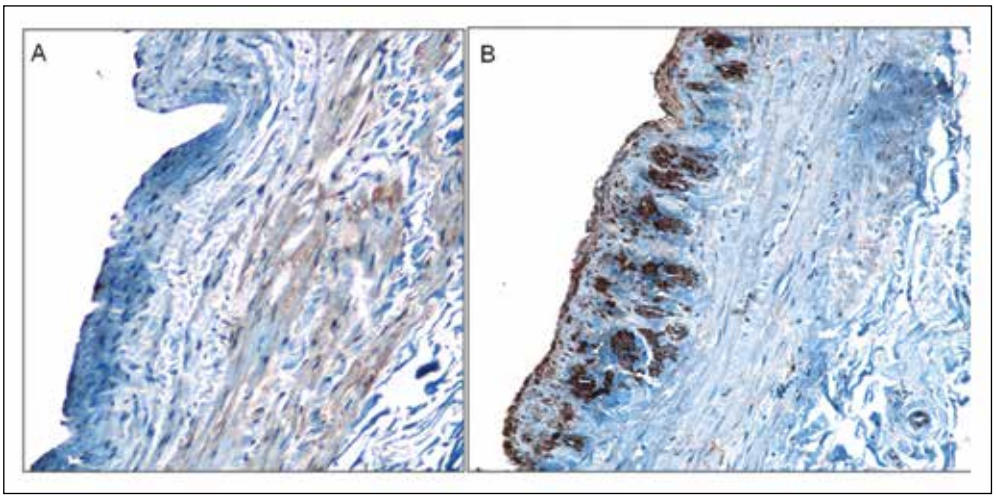


Figure 3: Photomicrograph demonstrating immunohistochemical staining with Dll4 antibody in venous tissue sections from patients with varicose veins and controls. (A) Dll4 expression is localized to tunica media in normal venous tissue section. (B) Dll4 is expressed all over neointima of varicose vein tissues with high staining intensity (x 10X)

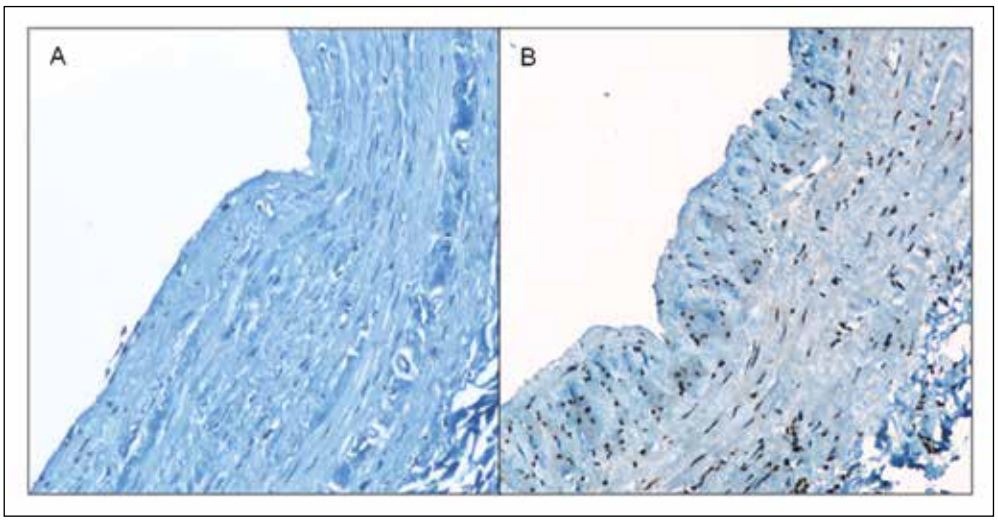


Figure 4: Photomicrograph demonstrating immunohistochemical staining with Hey2 antibody in venous tissue sections from patients with varicose veins and controls. (A) Hey2 was not expressed in normal venous tissue section. (B) Hey2 is over expressed all over intima and media of varicose vein tissues with high staining intensity (x 10X)

medial SMC layer. Hey2 was found expressed both in neointima and media of varicose vein while it was not expressed in healthy vein tissues (Figures 2-4).

This study confirms an altered FoxC2- Notch signalling in the pathogenesis of varicose veins. An altered FoxC2- Notch signaling cascade via upregulation of Dll4 and Hey2 may result in abnormal vein wall architecture and in the remodeling of saphenous vein in patients with varicose veins.

Endocardial endothelial cells resist oxidative stress because of higher endogenous ABCG2

G. S. Ajithkumar, A. Vinitha, S. S. Binil Raj and C. C. Kartha

ATP binding cassette transporters play a pivotal role in the protective mechanism of cells by preventing the accumulation of both extra cellular and intra cellular toxic substances. We have earlier reported that endocardial endothelial cells (EECs), compared to endothelial cells of arteries and veins are relatively resistant to apoptosis induced by anti-cancer agents. But there is no information available regarding the existence of ABC transporter molecules in EECs and their significance in the endocardial endothelial cell survival and maintenance. Here we present evidences that, the relative resistance of EECs against oxidative stress is related to higher endogenous levels of ABCG2, an ATP binding cassette transporter in EECs.Endocardial endothelial cells (PEEC), aortic endothelial cells (PAEC) and left atrioventricular valve endothelial cells (PVEC) of porcine origin at first passage were used for the experiments. Activity of P-glycoprotein, MRP1 and ABCG2 in cultured endothelial cells were assessed by FACS using fluorescent substrates and inhibitors specific to these transporters and also by western blotting analysis. Doxorubicin was used as reactive oxygen species inducer and ROS level in endothelial cells was determined using cellROX reagent. FACS analysis revealed a high efflux of pheophorbide-A in PEEC only (86%), which was inhibited in presence of FTC. No such efflux activity was observed in case of rhodamine 123. This indicated a higher activity of only ABCG2 in primary cultures of EECs. No significant efflux of Calcein-AM, a specific fluorescent substrate of MRP-1, was noticed in all types of endothelial cells. To substantiate this observation, we performed immunoblot analysis in endothelial cells and results emphasized an inherently high level of expression of ABCG2 only in EECs (fig.1&2). FACS analysis of the ROS using cellROX reagent revealed that EECs were relatively resistant to oxidative stress induced by doxorubicin. Decreased levels of ROS were observed in EECs compared to other endothelial cells.But when the ABCG2 inhibitor, FTC is treated along with doxorubicin a significantly elevated quantity of ROS accumulation was noticed in the EECs (fig.3).Our studies revealed an inherently high level of ABCG2 in endocardial endothelial cells compared to aortic and valvular endothelial cells. This could be the basis for increased survival rate of EECs compared to PAEC and PVEC under oxidative stress by preventing the accumulation of ROS.

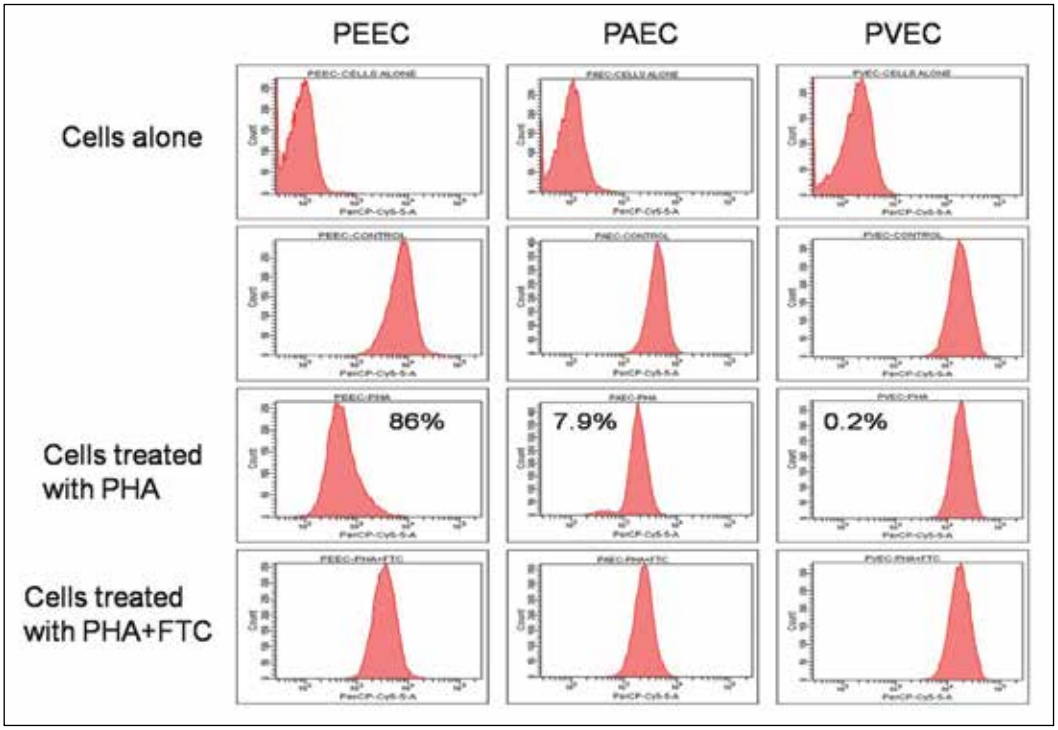


Figure.1: FACS analysis of primary isolates of PEECs, PAECs and PVECs for detecting the expression levels of P-glycoprotein, MRP-1 and ABCG2. A significantly higher efflux activity of ABCG2 was observed in h PEECs (86%) than that in PAECs and PVECs.

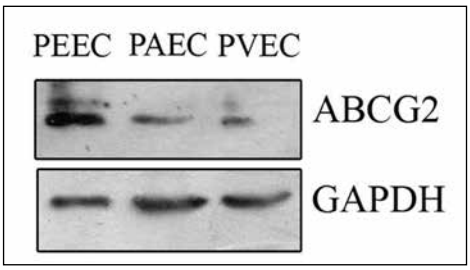


Figure.2: Western blot analysis for detecting the expression levels of ABCG2 in primary cultures of PEEC, PAEC and PVEC. An inherently higher level of expression of ABCG2 was observed in PEEC than that in PAEC and PVEC.

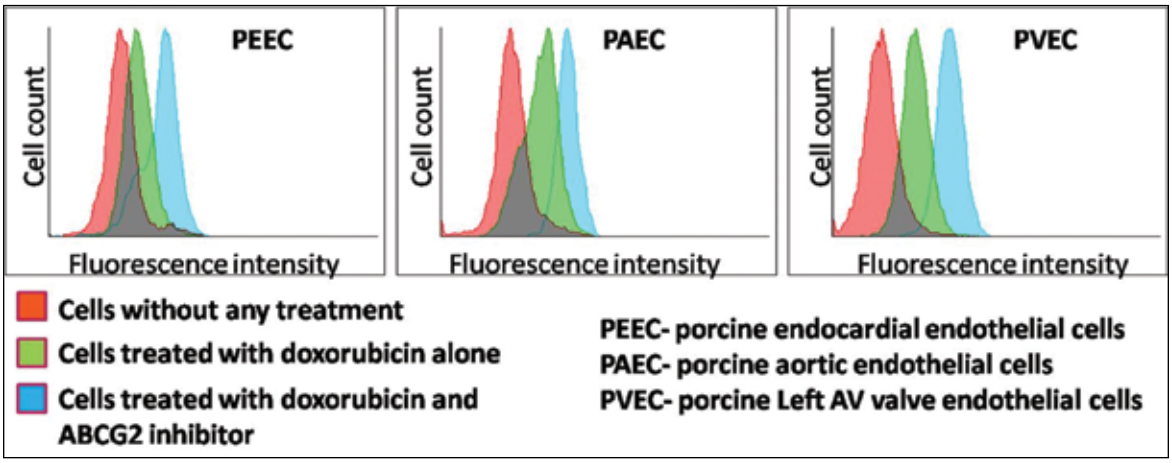


Figure.3: FACS analysis of for detection of ROS level in PEEC, PAEC and PVEC using cellROX reagent. Treatment with doxorubicin revealed a shift of only 20% in PEEC compared to 50% and 80% in PAEC and PVEC respectively. Thus ROS induction by doxorubicin was resisted significantly by PEEC. ROS level in cells after the treatment with ABCG2 inhibitor (FTC) revealed a shift of 80% from the basal level of fluorescence in case PEEC. Thus PEEC are more vulnerable to oxidative stress when ABCG2 activity is inhibited.

IGF-1 down-regulates Kip family proteins through FoxO3a/ Akt pathway and promotes proliferation in murine c-kit^{pos} cardiac stem cells

Ann Mary Johnson and C C Kartha

Cardiomyocyte regeneration following myocardial infarction is a challenging area in the field of translational cardiovascular research. To date, stem cell types such as bone marrow stem cells, endothelial progenitor stem cells, adipose stem cells, epicardial stem cells and cardiac progenitor cells are being studied and evaluated for their efficacy in stem cell therapy. Among them, endogenous cardiac stem cells (CSCs) expressing c-kit receptors are ideal candidates due to their lineage-restricted differentiation into cardiomyocytes, smooth muscle cells and endothelial cells. Growth factors such as insulin-like growth factor (IGF-1), epidermal growth factor, hepatocyte growth factor and basic fibroblast growth factor promote growth of CSCs in in-vitro culture. IGF-1 is a potent growth stimulant and pro- migratory molecule for c-kitpos CSCs. However, the mechanism through which IGF-1 promotes proliferation of CSCs is elusive. A few studies indicate that IGF-1 signaling through Akt is important in cardiac progenitor cell proliferation. One of the downstream targets of IGF-1/Akt-1 signaling is Forkhead (FoxO) family proteins, characterized by a DNA-binding domain called Forkhead box. These factors bind to their cognate FHRE domains and regulate expression of target genes involved in cellular processes such as cell cycle, apoptosis, autophagy, metabolism, differentiation, and response to oxidative stress. Our studies report that IGF-1 mediated cell proliferation is associated with FoxO3a phosphorylation and inactivation of its transcriptional activity. PI3 inhibitors LY294002 and Wortmannin abolished the effects of IGF-1 on FoxO3a phosphorylation. Phosphorylation by IGF-1 signaling also reduced expression of target genes such as p27kip1 and p57kip2. IGF-1 stimulation also increased CyclinD1 expression and cell growth. These results indicate that IGF-1 down- regulates expression of Kip family proteins through FoxO3a pathway and promotes cell proliferation. Also, we investigate the role of FoxO3a in maintaining quiescence of murine c-kitpos CSCs. Understanding the biology of ckitpos cardiac stem cell self-renewal provides an insight into the molecular mechanisms of cardiomyocyte regeneration.

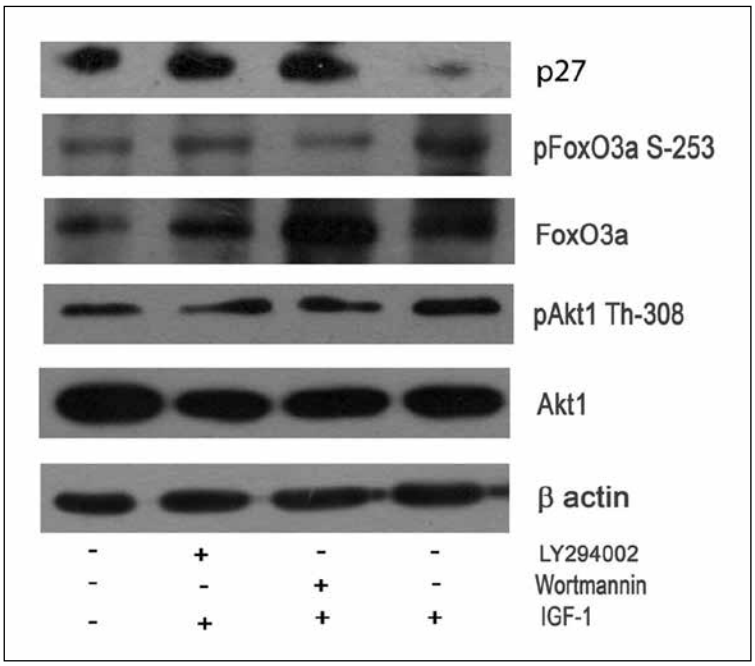


Figure.1: Western blot analyses represent increased phosphorylation of Akt-1 and FoxO3a upon IGF-1 stimulation for 3 h. For inhibiting Akt pathway c-kitpos CSCs were treated with LY294002 (30 μM) and Wortmannin (1 μM) for 30 min prior stimulation with IGF-1.

Molecular basis of remodeling of pulmonary vascular endothelium in left heart failure

SS Binil Raj, GS Ajithkumar, S Santhoshkumar, G Sanjay* and C C Kartha

*Collaborators:*Department of Cardiology, Sree Chitra Tirunal Institute of Medical Sciences and Technology, Thiruvananthapuram*

Pulmonary hypertension associated with left heart disease (PH-LHD) represents the most common form of PH and is characterized by lung endothelial dysfunction and vascular remodeling. Left ventricular or valvular disease results in passive backward transmission of elevated left atrial pressure and partial obstruction to pulmonary venous drainage. This hemodynamic disturbance in circulation causes increased shear stress and turbulent flow in pulmonary circulation. We hypothesize that in LHD induced pulmonary vascular remodeling and PH there appears to be hemodynamic stress induced endothelial dysfunction leading to dysregulation of vasoactive mediators and growth factors resulting in remodeling of pulmonary micro vascular endothelial cells (PMVECs). Proteomic analysis of the PMVECs exposed to disturbed flow and parallel flow identified several proteins, which are involved in the cytoskeletal rearrangement, vascular remodeling and endothelial-to-mesenchyme transition (En-MT). So we studied the role of low shear and disturbed flow induced En-MT in the PMVECs and in lung tissues of rat with left heart disease.The PMVECs seeded in the µ I0.4 leur slides (IBIDI, Germany) was subjected to parallel flow and PMVECs seeded in µ-Y shaped slide was subjected to disturbed flow respectively. The shear stress levels were gradually increased to 10 dyn/cm2 using an IBIDI pump system. for 72 hours. After 72 hrs, the cells were isolated from the slides and the total RNA was isolated using RNeasy Mico Kit (Qiagen).

Our study revealed that in PMVECs exposed to disturbed flow there is a decrease in expression of the CD31 and expression of vimentin and α-SMA (Fig.1). This indicates that PMVECs on chronic exposure to distubed flow results in loss of endothelial characters and acquisition of characters more of a smooth muscle phenotype.

On analyzing the lung tissues of rat post 1st, 2nd and 3rd month of aortic constriction, it was found that the downstream regulator of En-MT, Snail 1 was up regulated (Fig. 2). The markers of En-Mt such as vimentin and α- smooth muscle

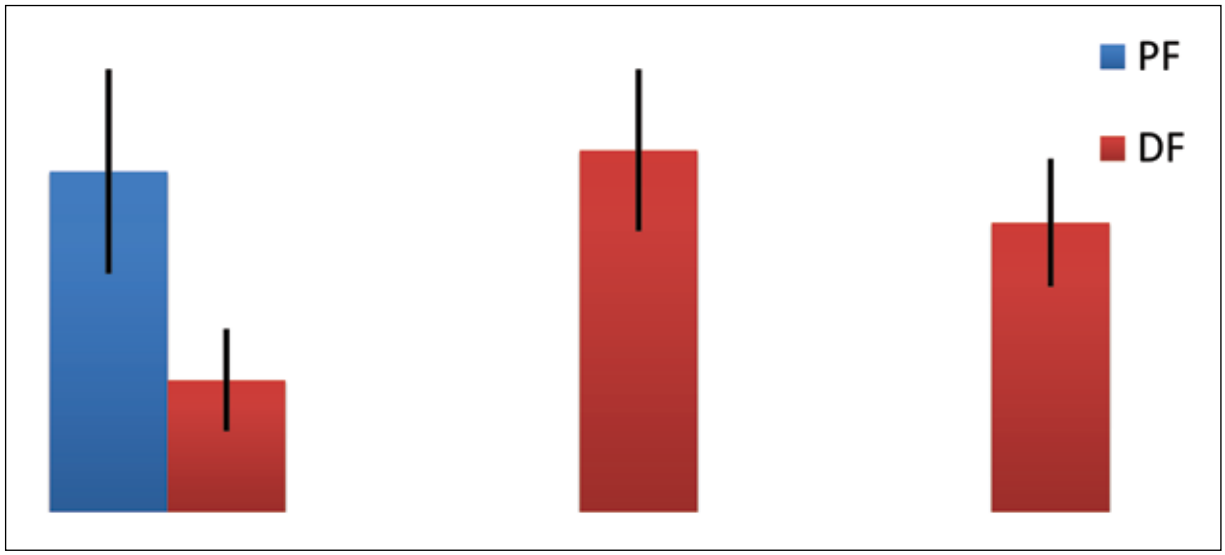


Fig. 1: Relative expression levels of CD31, vimentin and αsmooth muscle actin (αSMA) analyzed (normalised to GAPDH) in PMVECs exposed to parallel flow (PF) and disturbed flow (DF) in comparison to static control.

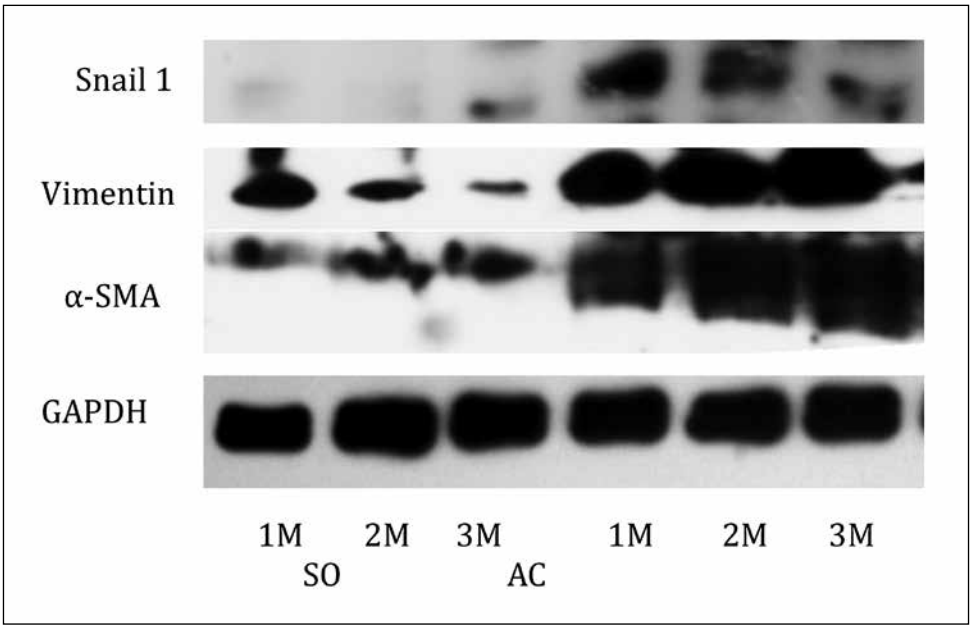


Fig. 2. Expression levels of Snail 1 and vimentin, α- smooth muscle actin in lung tissues of rats that underwent aortic constriction (AC) and sham operation (SO) post 1st, 2nd and 3rd month of surgery.

actin are up regulated. The results from the in vitro studies suggest that exposure of PMVECs to low shear disturbed flow may cause loss of endothelial characters and changing to more or less a smooth muscle like phenotype. Analysis of the lung tissues of rat which were undergone aortic constriction also supports our in vitro data. Our studies suggest that LHD induced disturbed flow in the pulmonary circulation may cause En-MT in the PMVECs leading to vascular remodeling and PH associated LHD. Further studies are required to further characterize the En-MT in vitro and in vivo and also to find out the hemodynamic stress induced initiators of En-MT.

Molecular characteristics of right ventricular outflow tract myocardium in patients with Tetralogy of Fallot

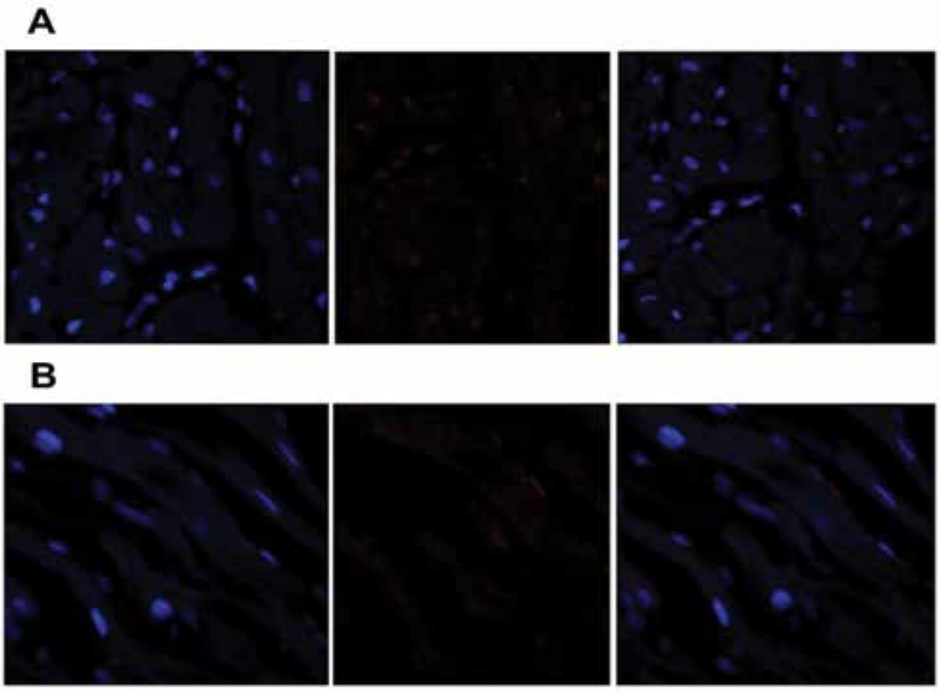
S Shammy, R Suresh Kumar* and C C Kartha

**Collaborators: Frontier Lifeline Hospital, Chennai, Royal Hospital, Oman*

Tetralogy of Fallot (TOF) is a conotruncal heart defect, which accounts for 10% of all cardiac malformations. The basic pathology of TOF is characterized by right ventricular outflow tract obstruction (RVOTO) and a ventricular septal defect (VSD) causing a right-to-left-shunt with low pulmonary blood flow and cyanosis. An understanding of the individual modular steps involved in the development of the conotruncal region is obviously relevant to have a better insight into the origin of the developmental abnormalities that contribute to the morphologic components of TOF. As Tetralogy of Fallot is pertained more to the defects in outflow tract, right ventricle and its septation, secondary heart field's cells and its regulation are most likely to be considered to

play a vital role in the development of the disease. The objective of our study was to analyze the expression pattern of selected transcription factors involved in the regulatory network that operates for the normal development of second heart field cells. This was achieved by semi quantitative RT-PCR and immunoblotting experiments on RVOT myocardium obtained from patients with ToF who underwent corrective surgery (age: 1 to 19 years) and donor healthy human hearts harvested for transplantation (age: 11 to 40 years). A proteomic protein expression analysis was also performed by 2D nano- LC MS /MS to identify the upstream activators and downstream effectors of altered transcription factors. Immunoflurescent staining of tissue sections were also performed for a better understanding of cyto-architectureof infundibular myocardium in ToF.In our earlier studies we have demonstrated an increased gene expression pattern of ISL1. ISL1 is a member of LIM-homeodomain transcription factor family and a marker of resident cardiac progenitor cells that are derived from second heart field (SHF) region. With immunofluorescent staining of tissue sections, we could confirm our findings and was also able to demonstrate that more number of ISL1 positive cells are present in the RVOT myocardium of patients with TOF.From the seminal studies in cardiac development related to Isl1 it is concluded that this marker is predominantly associated with a subset of pacemaker cells, endothelium, myocytes and vascular smooth muscle cells.

On analyzing the proteomic protein expression profile we were able to identify a decreased expression of Retinaldehyde dehydrogenase 2 (ALDH1A2), a key enzyme in retinoic acid biosynthetic pathway.In animal models it has been demonstrated that lack of RA signaling increase cardiac side population numbers and simultaneous reduction in FGF signaling affects the normal development of RVOT. Our study also suggests an involvement of aberrant retinoic acid signaling in ToF. Double immunostaining experiments is being done to analyze whether the increased population of ISL1 positive cells are existing intact or differentiating to any of the cardiovascular lineages in RVOT myocardium and thereby contributing for infundibular muscle stenosis.



Original histological specimen of TOF RVOT myocardium: A. Representative specimen from patient with TOF immuno-stained for Islet-1 (ISL1) (red fluorescence), nuclei are counter-stained in blue. The specific ISL1 staining is indicated by white arrows. **B.** Representative specimen from healthy heart immuno-stained for Islet-1 (ISL1) (red fluo-rescence)

Can Amalaki Rasayana reverse the progressive cardiac remodeling changes with age?

Vikas Kumar, AjithKumar GS, Sanjay G*, Santhosh Kumar TR, Kartha CC

Collaborator: Department of Cardiology, Sree Chithra Thirunal Institute of Medical Science and Technology (SCTIMST), Trivandrum, India

Aging is fundamental biological process of progressive decline in physiological function. Aging causes functional limitations and increased risk of chronic conditions such as inflammation, oxidative stress, mitochondrial dysfunction and impaired proteostasis which contribute to cardiac dysfunction.Therapeutic interventions such as ACEI's (angiotensin converting enzyme inhibitors), aldosterone antagonist, beta-blockers and cardiac resynchronization therapy, presently in practice can reduce death in patients but allow them to survive with a damaged heart which ultimately fails.Developing new therapies with improved efficacy and minimum side effects for prevention and management of heart failure remains a challenge in cardiology. India's traditionally used rejuvenating drugs especially 'Rasayana' groups of drugs belonging to the age old ayurvedic system of medicine may useful in this regard. Amalaki Rasayana; the Indian Gooseberry extract (fruits of Phyllanthus emblica) is considered best among Rasayana group of drugs. The major objective of our studies is to understand the effect of Amalaki Rasayana in reversal of remodeling changes in cardiac failure in a pressure overload model of left ventricular cardiac hypertrophy and age related cardiac dysfunction in rats. We had two objectives, (A) Effect of Amalaki rasayana in physiologically aged rats and (B) Effect of amalaki rasayana in ascending aorta constriction (AAC) induced cardiac hypertrophy in rats. Experiment 1 included4 treatment groups (amalaki rasayana 250, 500, and 750 mg/kg, orally); 3 placebo groups (250, 500, and 750 mg/kg of placebo orally) and a control (untreated) group of animals. Experiment 2 was started with induction of cardiac hypertrophy by constriction of ascending aorta using titanium clip. In order to analyze the effect of amalaki rasayana on cardiac dysfunction associated with cardiac hypertrophy and failure, experimental rats were divided into 3 groups:Amalaki Rasayana (750 mg/kg, orally), placebo (750 mg/kg,

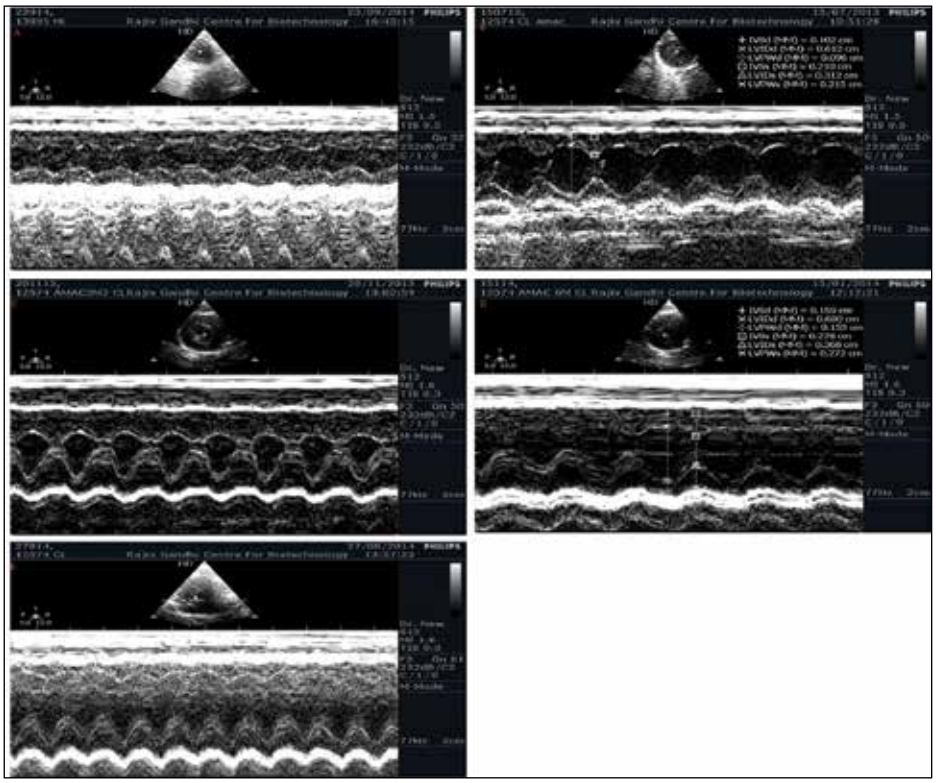


Figure 1: Representative images of M-mode echocardiography analysis of Aortic constricted cardiac hypertrophy a rats treated with amalaki rasayana. A, B, C, D, E represents the Control, 3 months post-surgery, 6 months post-surgery, 750 mg/kg Amalaki treated groups respectively.

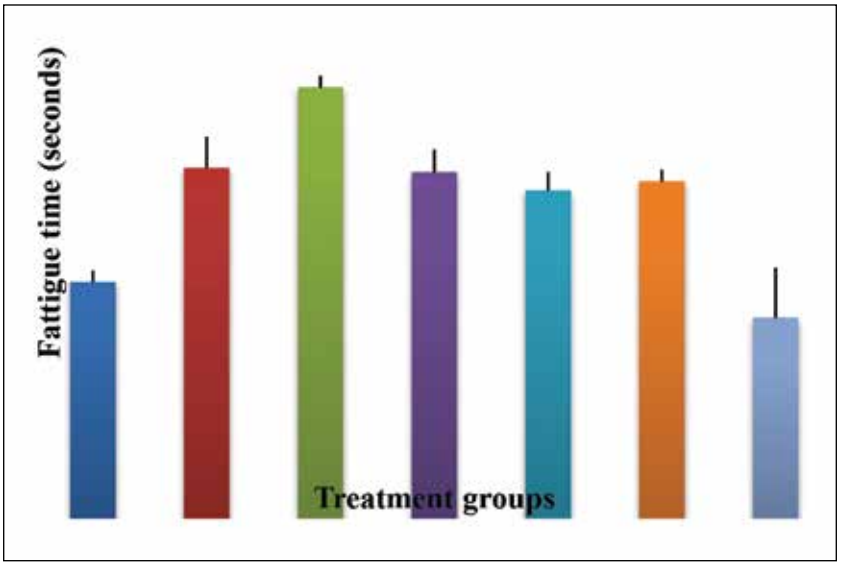


Figure 2. Treadmill exercise tolerance analysis of Aged (2 yrs. of Age) rats treated with Amalaki Rasayana.

orally) treated and untreated AAC rats as control. After 9 months of treatment, M-mode echo was recorded to analyze the effect of amalaki rasayana on cardiac function as shown in figure 1. In addition, to check the cardiac status at functional level, treadmill exercise tolerance analysis was done. Interestingly, fatigue time was found to be increased in Amalaki Rasayana treated rats, when compared to control rats as shown in figure 2. After assessing the heart function with M-mode echocardiography, treadmill test, ECG and blood pressure measurements, animals were sacrificed and samples were collected for molecular biology studies. In treadmill experiments, % efficiency (exercise tolerance/fatigue time) was found to be increased in Amalaki Rasayana treated rats, when compared with untreated groups as shown in table 2. We checked cell survival and ageing related protein expression in cardiac tissues and found upregulation in autophagy regulatory protein beclin-1 expression in aged rats treated with Amalaki Rasayana, when compared with untreated rats. In addition, p53 (a tumor suppressor protein) was found to be up-regulated in Amalaki Rasayana treated rats unlike the control group as shown in figure 3.

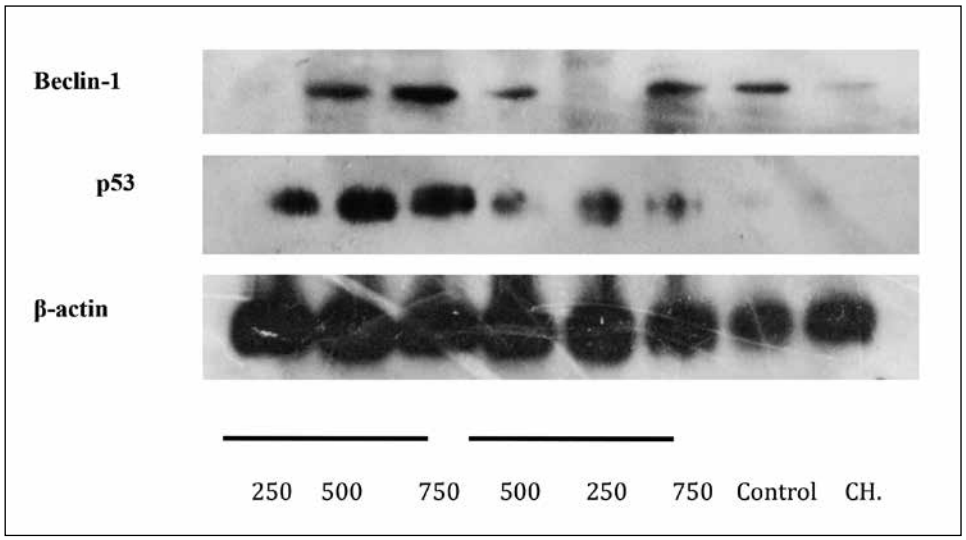


Figure 3, Western blot of heart tissue of Amalaki treated rats indicating the possible molecular changes due to Amalaki Rasayana.

Molecular Basis in the Pathogenesis of Cerebral Arteriovenous Malformation (AVM)

Jaya Mary Thomas, Mathew Abraham *, Sumi S, Arumugam Rajavelu, C C Kartha

*Collaborator:*Sree Chithra Thirunal Institute of Medical Science and Technology, Thiruvananthapuram*

Arteriovenous malformations (AVM) are tangles of dysplastic blood vessels (nidus),which shunt blood from arteries to veins with no intervening capillary bed. AVM can occur in different organs such as liver, lungs, brain etc. Brain or cerebral AVM, are important causal factors of intracranial hemorrhage which may result in permanent disability or death, if not corrected surgically. Sporadic and inherited forms of AVM have been reported suggesting a strong genetic basis and hemodynamic modulation in the disease pathogenesis. The molecular milieu of this disease is complicated and pathogenesis of these malformations remains an enigma. AVM, especially those in brain and spinal cord are the most dangerous and difficult to treat vascular anomalies. Bleeding from an AVM can be devastating and can cause severe and often fatal strokes. The genesis of AVM is not clearly understood. There are no known environmental risk factors for AVMs. Inherited forms of brain AVMs are often reported as secondary features in hereditary hemorrhagic telangiectasia (HHT). Earlier studies report that mutations in genes coding for TGFκ co-receptors, ACVRL1 and Endoglin are strongly associated with HHT. In this context, we explored the association of genetic variants of TGFκ ACVRL1 and Endoglin in 30 sporadic cases of AVM and 26 control samples.Study subjects were recruited from Sree Chithra Thirunal Institute of Medical Science and Technology (SCTIMST), Thiruvananthapuram.DNA was isolated from whole blood samples collected. PCR-Sequencing and data analysis is being performed in the above mentioned genes with specific focus on already reported polymorphisms.

Capillary system in brain have special features when compared to capillaries in other part of body. Brain

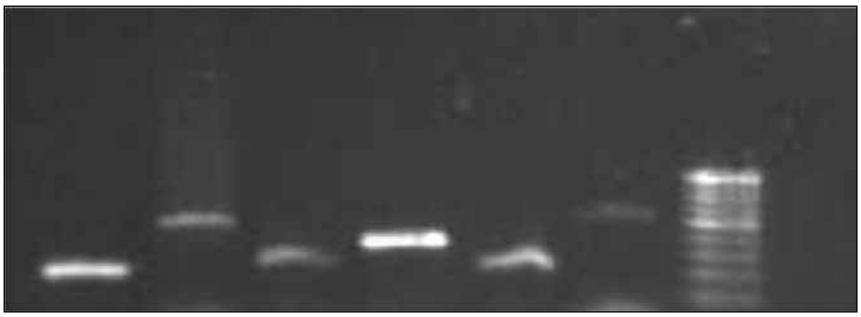


Figure:1. Representative image for amplified product from clinical samples Lane1: ACV1, Lane2:ACV2, Lane3:ENG1, Lane4:ENG2, Lane5:TGF1, Lane6:TGF2, Lane 7:100bpDNA ladder

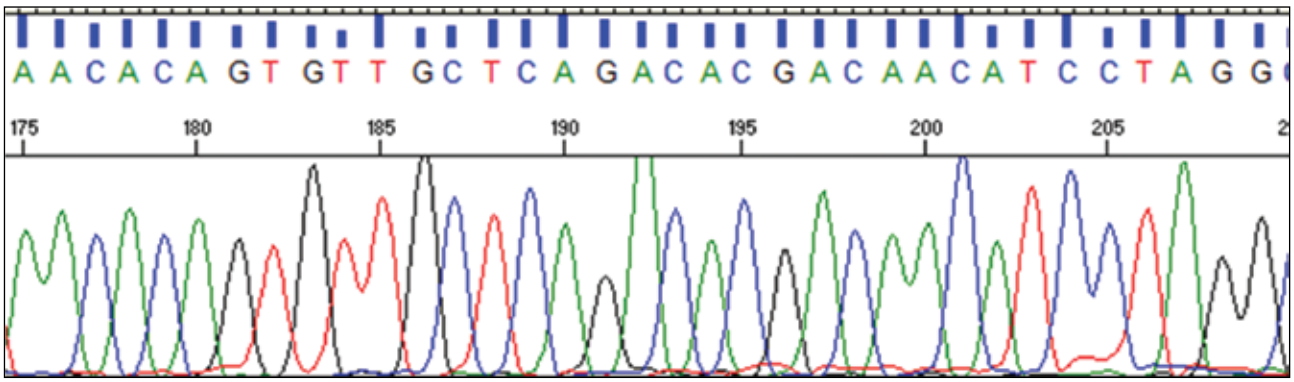


Figure 2: Sequence analysis of ACVRL1

capillaries are featured by particular phenotype which includes tight junction (characterized by proteins like occluidns, claudins, JAM-A, JAM-B and JAM-C), presence of transporters like GGTP, GLUT1, P-glycoprotein etc. AVM is characterized by collection of dysplastic blood vessels instead of normal capillary system.In this context, we have performed immunohistochenical analysis of GLUT1 and GGTP in 6 AVM post surgical tissue samples.AVM tissue samples were collected from patients who have undergone corrective surgery for AVM at Sree Chithra Thirunal Institute of Medical Science and Technology, Thiruvananthapuram. Preliminary results show an increased expression of GLUT1 in endothelial cells of AVM tissue when compared to GGTP. The expression pattern will be further studied in brain capillary endothelial cells of tissues collected from epileptic patients.

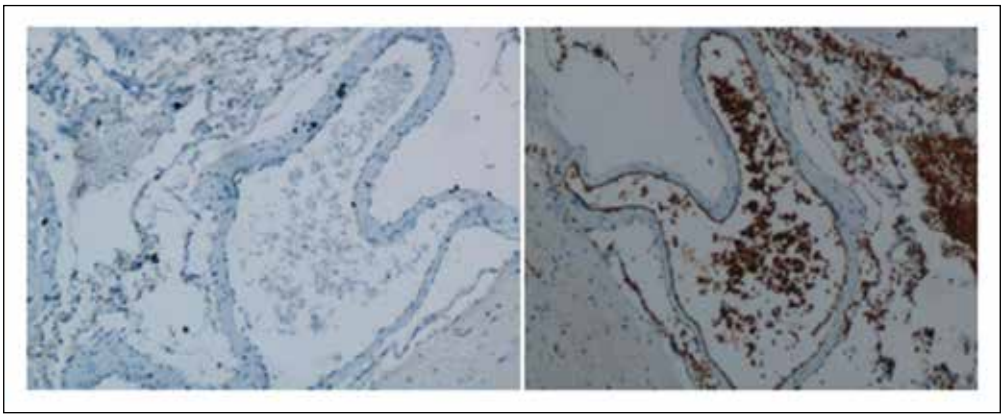


Figure 3: Photomicrograph demonstrating immunohistochemical staining with GGTP and GLUT1 antibody. (A) No GGTP expression was found in AVM tissue sample (x 10X). (B) GLUT1 expression localized to endothelial cell lining and RBC in AVM tissue sample(x 10X)

PUBLICATIONS

Original Publications in Journals

- *GS, Ajithkumar., B, Raj., S, Santhoshkumar., G, Sanjay., C.C, Kartha.* Ascending Aortic Constriction in Rats for Creation of Pressure Overload Cardiac Hypertrophy Model. *J. Vis. Exp (2014), doi:10.3791/50983*
- *Ann Mary Johnson and CC Kartha,* Proliferation of Murine c-kitpos Cardiac Stem Cells Stimulated with IGF-1 is associated with Akt-1 Mediated Phosphorylation and Nuclear Export of FoxO3a and Its Effect on Downstream Cell Cycle Regulators. *Growth Factors. (2014); 32(2):53-62.*
- *Kshemada K, Kartha CC, Mehta JL.* Forensic Sciences and Growth of Cardiology (invited editorial). *J Forensic Res (2014) 5:e115*
- *Shammy S and Kartha CC.* Congenital Heart Defects –Progress and Prospects (invited editorial). *Kerala Heart Journal (2014), 2*

CONFERENCE ABSTRACTS

- *Shammy S, Gayathri AK, Kartha CC, Suresh Kumar R,* Decreased expression of Gata4 and eNOS in the RV outflow tract myocardium of patients with tetralogy of Fallot, *Annals of Pediatric Cardiology (2014) Vol 7;1*
- *Shammy S., Arun S., Gayathri A.K., Jaleel A., Sureshkumar R., Kartha C.C,* Molecular lesions in right ventricular infundibulum in tetralogy of Fallot, *Cardiology in the YoungSupplement (2014)1 volume 24 ppS1-S176.*

CONFERENCE PRESENTATIONS

- *Shammy S, Arun S, Jaleel A, Suresh Kumar R, Kartha CC,* " Molecular characteristics of right ventricular outflow tract myocardium in patients with Tetralogy of Fallot", *International Conference on Current Trends and Genetics in Heart Failure and Cardiac Arrhythmia, at PGIMER on January 31-February1 2015* (oral presentation)
- *SS Binil Raj, GS Ajithkumar, S Santhoshkumar, G Sanjay and CC Kartha.* Role of shear stress sensitive genes in remodeling of pulmonary vascular endothelial cells in rats with left ventricular hypertrophy and associated pulmonary hypertension. *Indo-Canadian Symposium on Heart Failure: Progress & Prospects at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram during March 13-14, 2015.*

- *Shammy S, Arun S, Jaleel A, Suresh Kumar R, Kartha CC,* " Aberrant retinoic acid signaling in right ventricular outflow tract myocardium of patients with Tetralogy of Fallot", *Indo-Canadian symposium on Heart Failure : Progress and Prospects, at Rajiv Gandhi Centre for Biotechnology on March 12-14,2015*
- *Shammy S., Arun S., Gayathri A.K., Jaleel A., Sureshkumar R., Kartha C.C* Molecular lesions in right ventricular infundibulum in Tetralogy of Fallot, at the *48th Annual Meeting of The Association for European Paediatric and Congenital Cardiology (AEPC), May 21 - 24, 2014 in Helsinki.* (Poster presentation)

CONFERENCES ORGANIZED

- First Indo Canadian Symposium on Heart Failure: Progress and Prospects, under the aegis of the International Academy of Cardiovascular Sciences, Winnipeg, Canada was held fromMarch 11-14, 2015. The symposium was sponsored by Kerala State Council for Science Technology and Environment, Indian Council for Medical Research, Department of Biotechnology, Department of Science and Technology and Council for Scientific and Industrial Research.

RESEARCH GRANTS EXTRA –MURAL FUNDING

Sl. No.	Name of Grant	Funding Agency	Duration
1	Can Amalaki rasayana attenuate cardiac dysfunction associated with cardiac failure and aging	Department of Science and Technology	2012-2016
2	How does Cyclophilin A, an oxidative stress induced secretory protein modulate vascular disease progression in type 2 diabetes.	Indian Council of Medical Research	2015-2018
3	Pathogenesis of varicose veins	Dr N Radhakrishnan Foundation Trust for Venous Diseases	2014-2017

CARDIOVASCULAR AND DIABETES DISEASE BIOLOGY PROGRAM

DIABETES DISEASE BIOLOGY LABORATORY

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Abdul Jaleel obtained his PhD from Jamia Millia Islamia, New Delhi while doing his research fellowship at All India Institute of Medical Sciences, New Delhi and then worked as a post-doctoral fellow and faculty at Mayo Clinic, Rochester, MN, USA. He joined RGCB in December 2011.

PhD Students

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Post Doctoral Fellow

Mahesh Krishna, PhD

Metabolic Profiling of Normal Healthy people in Kerala

Aneesh Kumar A, G. Vijayakumar[1], V Raman Kutty[2], and Abdul Jaleel

Collaborators:
1 Medical Trust Hospital & Diabetes Centre, Kulanada, Pathanamthitta
2 Achutha Menon Centre for Health Science Studies, SCTIMST, Trivandrum

The objective of this study is to understand the metabolic transition process associated with the onset of type 2 diabetes (T2D). The metabolic alterations, which may be the foundation for metabolic diseases such as diabetes, could be identified by performing mass spectrometry based metabolomics analysis in the blood of normal healthy study participants who are at the risk of developing T2D (e.g., people having family history of diabetes, Obesity, etc). Such studies are likely to offer substantial data and rationale for developing hypothesis based mechanistic studies. Around 130 normal healthy people were recruited and human studies were performed on them. The normal healthy study participants includes those who have family history diabetes (FHxD) as well as people who have their body mass index (BMI) above than normal. We have also recruited 20 people who are pre-diabetic, for being as positive control in this study. The study participants recruited were matched for their age, sex and BMI. We have previously demonstarted that impact of genetic and environmental factors trigger different routes of mechanism towards the pathogenesis of insulin resistance and type 2 diabetes from the values of inflammatory and diabetes markers in our study groups. Plasma levels of inflammatory markers such as IL-6 and TNF-α are significantly higher in higher BMI group, whereas adiponectin level is significantly lower. However no differences were observed for the FHxD when compared to healthy controls. The levels of diabetes markers such as C-peptide, glucagon and visfatin are significantly high in the higher BMI group. However, basal levels of hormones such as insulin, glucagon, C-peptide, Ghrelin, GLP-1, and visfatin were higher in those who have positive FHxD when compared to people who have no FHxD. These measurements observed with respect to FHxD are in contrast to what is observed for higher BMI. Plasma insulin curve (2 hours postprandial) was not only higher in obese group, but also similar to that of pre-diabetes, implicating insulin resistance and altered metabolism. However the Insulin curve of FHxD group was lower than the normal controls. It is possible to deliniate the altered biochemical pathways and various mechanisms by performing mass spectrometry based metabolomics analsysis. Plasma proteins were removed by precipitation using acetonitrile and methanol mixture and the resultant supernatant were used for the metabolomics analysis. An ultra-performance liquid chromatography, ACQUITY UPLC® System (Waters, Manchester, UK) coupled to a Quadrupole-Time of Flight

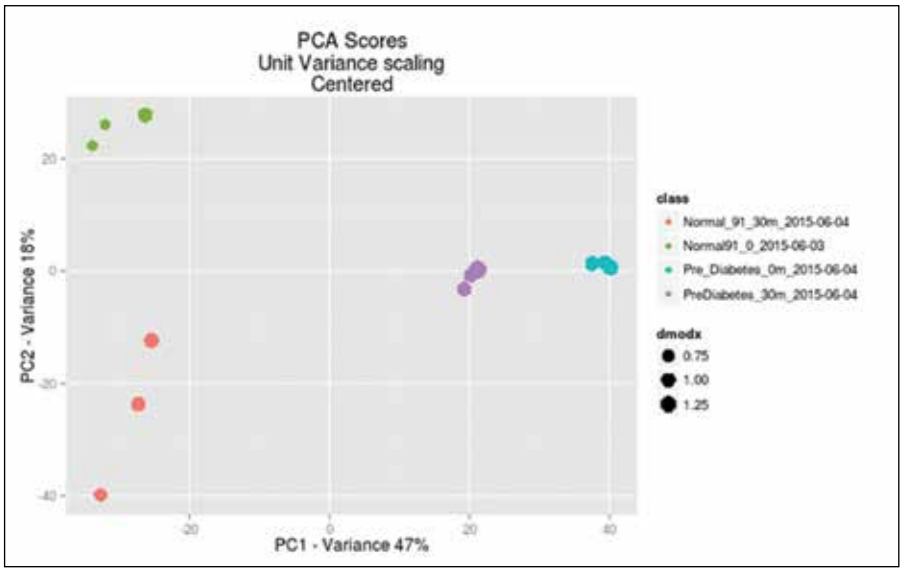

Figure -1. XCMS Principal component analysis scores plot showing the correlation between the samples of normal healthy control and pre-diabetic subjects.

(Q- TOF) mass spectrometer (SYNAPT-G2, Waters) was used for this purpose. The technique was validated using a standard mixture of drugs and amino acids. MassLynx4.1 SCN781 (Waters) was used for data acquisition and collection.The raw data were uploaded to XCMS online and further analyses were done.XCMS integrates areas of each detected peaks in individual samples and performs Welch's t test between two sample groups. It also performs non-parametric tests such as Wilcoxon Rank Sum (paired) and Mann-Whitney (unpaired). The results include, an interactive cloud plot of deregulated features[2] and a feature list table containing observed fold changes between groups, extracted ion chromatographic peak areas (integrated intensities), and p-values. XCMS also integrates to METLIN database and gives the putative identification of metabolites. Preliminary data on the principal component analyses (PCA) results and the cloud plots of samples from pre-diabetic in comparison with healthy controls are shown in figure-1 and figure-2 respectively demonstrating the differences.

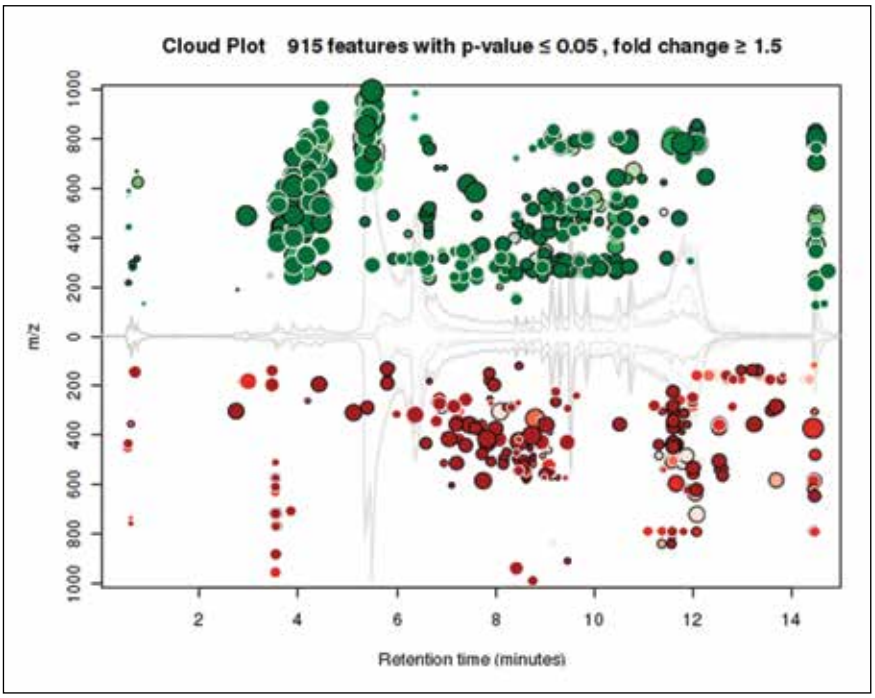

Figure-2. XCMS cloud plot representation of the altered metabolite features in pre-diabetes subjects in comparison with normal controls. Baseline plasma samples between the groups were analysed here. The green bubbles represent up-regulated features and red bubbles represent down-regulated features.

CHEMICAL BIOLOGY PROGRAM

CHEMICAL BIOLOGY LABORATORY - 1

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Smitha Devi

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Aswani Kumar

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Asha. R
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Structure- function studies of peptides identified from the skin secretion of H. temporalis.

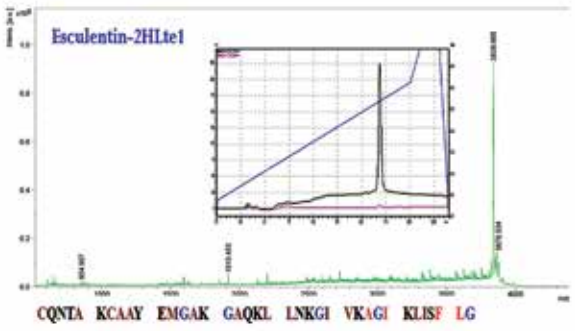
Reshmy V and Santhosh Kumar K

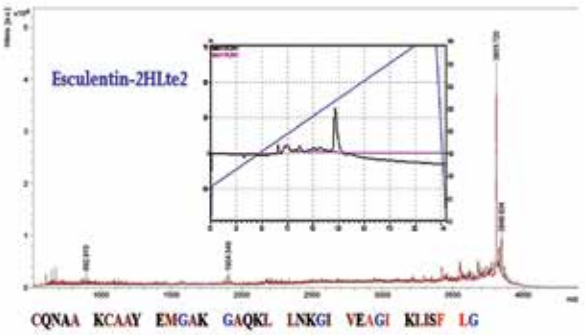
Host defense peptides (HDPs) are endogenous antibiotics secreted by the endocrine glands of living organisms. Antimicrobial peptides (AMPs) can destroy the cell membrane and thus provide the first-line of defence to the animal against the invading pathogens. They are considered as a promising candidate to develop novel anti-infectives. Frog skin is a rich source of AMPs and the variations in their expression in different frogs may be resulted from their co-evolution with pathogenic microorganisms. Twenty-three AMPs were identified from the skin secretion Hylarana temporalis, a frog endemic to Western Ghats by transcriptomic approaches. These peptide amides classified into Brevinin-1 & 2, Esculentin-2, Ranacyclins, Odorranains, Temporalins, Nigroains, Hylaranakinins were synthesized by SPPS and characterized by RP-HPLC and MALDI TOF MS.

Table 1. Peptides sequences encoded by cDNAs cloned from skin cDNA library of H.temporalis

Mature Peptide	Name	N	C	M	G
FFGPLIKIATGVLPNLI CKALGKC	Brevinin-1HLte1	24	+3	2517.1	1.117
FFGPLIKIATGVLPSLICKALGKC	Brevinin-1HLte2	24	+3	2478.1	1.229
FFGPLIKIATGVLPSLICRALGKC	Brevinin-1HLte3	24	+3	2506.1	1.204
GIGSMLLGLAKNVGMSLLNKAQCKISGKC	Brevinin-2HLte1	29	+4	2935.6	0.486
GFMGDTLKGIARNAALALMNAA QCKLSG KC	Brevinin-2HLte2	30	+3	3054.6	0.293
GLGSMFLGLAKNLGMTLLNKAQCKLSGKC	Brevinin-2HLte3	29	+4	2997.6	0.393
GFMGDTLKGIAKNAALALMNAAQCKLSGKC	Brevinin-2HLte4	30	+3	3026.6	0.313
GLFSILKIGAKVIGKNLLKQAGKAGMEYAACK ATNQC	Esculentin-2HLte1	37	+5	3839.6	0.208
GLFSILKIGAEVIGKNLLKQAGKAGMEYAACKAANQC	Esculentin-2HLte2	37	+3	3810.5	0.286
GLFSILKIGAKVIGKSLLKQAGKAGMEYAACKATNQC	Esculentin-2HLte3	37	+5	3812.6	0.281
GLFSILKIGAKVIGKNLLKQAGKAGMEYAACKAANQC	Esculentin-2HLte4	37	+5	3809.6	0.276
AGNSGKVILKRPPGFSPFRFVPASSL AGYSRMIRFPRPPGFTPFRFAPEII*	Hylaranakinin-HLte1 Hylaranakinin-HLte2				
NALVGCWTKSYPPKPCLG	Ranacyclin-HLte1	18	+2	1916.3	-0.116
NALVRCWTKSYPPKPCLG	Ranacyclin-HLte2	18	+3	2015.4	-.0339
TVLRGCWTFTFPPKPCVGKR	Ranacyclin-HLte3	20	+4	2275.7	-0.130
ATAWRRPSTGLLPIKPTYRKPLCGD	Odorranain-HLte1	26	+4	2888.4	-0.175
QNKFYPGAYTPLKQK	Temporalin-HLte1	15	+3	1771.1	-1.380
FTNIWTYYYKITHPEDKKEN	Temporalin- HLte2	20	+2	2584.8	-1.455
SLWETIKNSAKNLFVNILDKVRCK VAGGCKT	Nigroain-HLte1	31	+4	3437.0	-0.058
SPWETIKNSAKNLFVNILDKVRCKVAGGCK	Nigroain-HLte2	31	+4	3415.0	-0.232
FPELSEDALASLLGK	Pleurain-HLte1	15	-2	1583.8	0.240

N- No: of residues; C-Charge; M- Mass; G-GRAVY.


Esculentin-2HLte1
CQNTA KCAAY EMGAK GAQKL LNKGI VKAGI KLISF LG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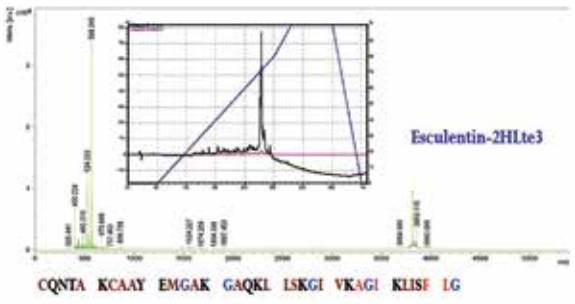
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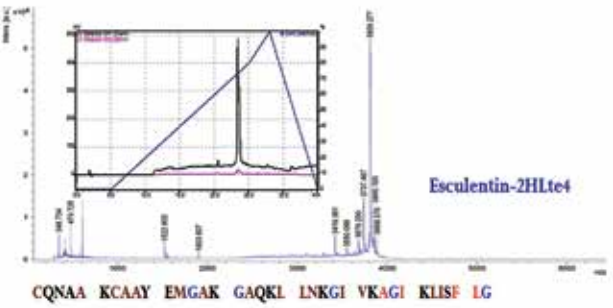
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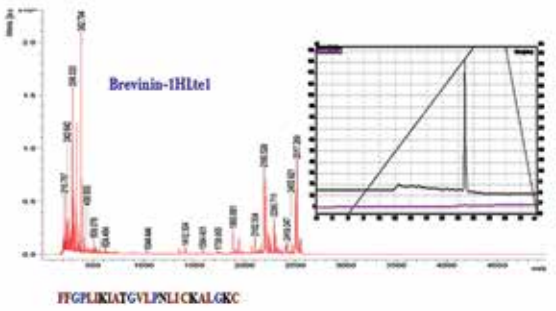
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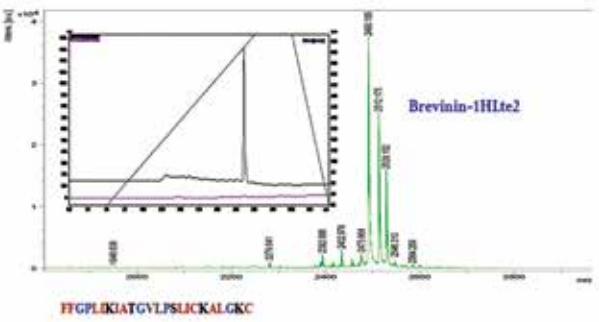
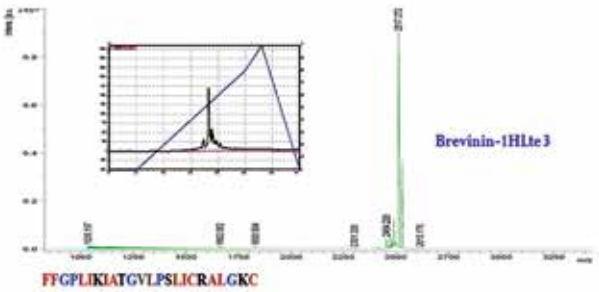

Brevinin-1HLte2
FFGPLIKIATGVLPSLICKALGKC


Brevinin-1HLte3
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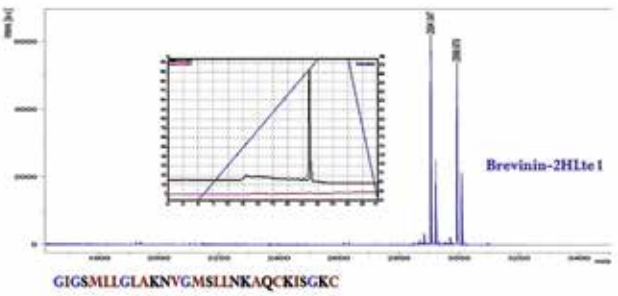
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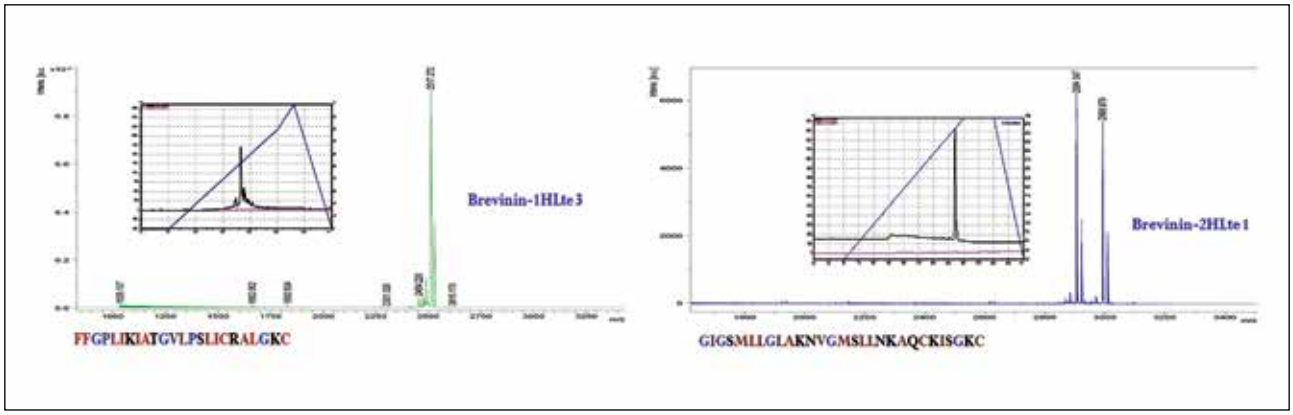


Fig 2. RPHPLC and MALDI TOF MS of the isolated peptides

The esculentins showed higher activity against Gram-positive bacteria *Staphylococcus aureus* and *Bacillus cereus* and Gram-negative bacteria *Pseudomonas Aeruginosa*, *ETEC, JM109, Vibrio cholera* (Clinical strain) compared to their N-terminal truncated sequences showing that this

Table 2. Antimicrobial assay of esculentin and brevinin peptide amides

Peptide	Micro organism (MIC in µM)						
	McVo9	ETEC	P aeurogenosa MTCC 8076	Ecoli 25922	Ecoli JM109	S aureus 9542	B cereus
Esculentin-2HLte1	3	3	25	25	50	1	50
Esculentin-2HLte2	50	100	>100	100	100	25	>100
Esculentin-2HLte3	1	1	25	>100	100	3	50
Esculentin-2HLte4	6.2	12.5	25	12.5	50	1	>100
Kanamycin	6.2	3	50	12.5	50	-	-
Ampicillin	-	-	-	-	-	0.7	>100
Esculentin-2HLte (1-37)	3	3	Esculentin-2HLte (2-37)		McV09 50	ETEC 100	
Esculentin-2HLte (1-32)	25	25	Esculentin-2Hlte (2-32)		100	>100	
Esculentin-2HLte (1-22)	50	>50	Esculentin-2Hlte (2-22)		>100	>100	
Peptide	ETEC	P aeurogenosa	Ecoli 25922	McVO9	Ecoli JM109	S aureus	B cereus
Brevinin-1Hlte1	12	100	25	3			
Brevinin-1HLte2	12	100	6	3	25	3	12
Brevinin-1HLte3	50	>100	50	50	100	25	100
Brevinin-2HLte1	100	>100	>100	50	50	>100	>100
Brevinin-2HLte2	25	>100	50	25	100	50	100
Brevinin-2HLte3	25	100	50	12	25	50	100
Brevinin-2Hlte5	50	100	100	12	25	3	100
Kanamycin	3	50	3	6.2	25	-	-
Ampicillin	-	-	-	-	-	6	>100

region plays an important role in membrane permeation activity. Ampicillin and kanamycin are used as controls. Esculentin-2 peptides were more potent compared to Brevinins and Ranacyclins.

The killing kinetics analysis of the peptide using E. coli and Staphylococcus aureus at their MICs and sub MICs showed these peptides are bactericidal in nature and kills the bacteria within 15 minutes of their addition. 1-N-phenylnaphthylamine (NPN) assay at excitation wavelengths of 350 and emission wavelengths at 420 nm showed that these peptides can permeates the outer membrane of E. coli cells. They are non-toxic to red blood cells and normal eukaryotic cells. NPN (10 µM) was added to the cells, and basal fluorescence level was recorded after 5 seconds. 5µM polymyxin B is used as the positive control. AMPs1 µM, 3 µM, 6 µM, 12 µM,

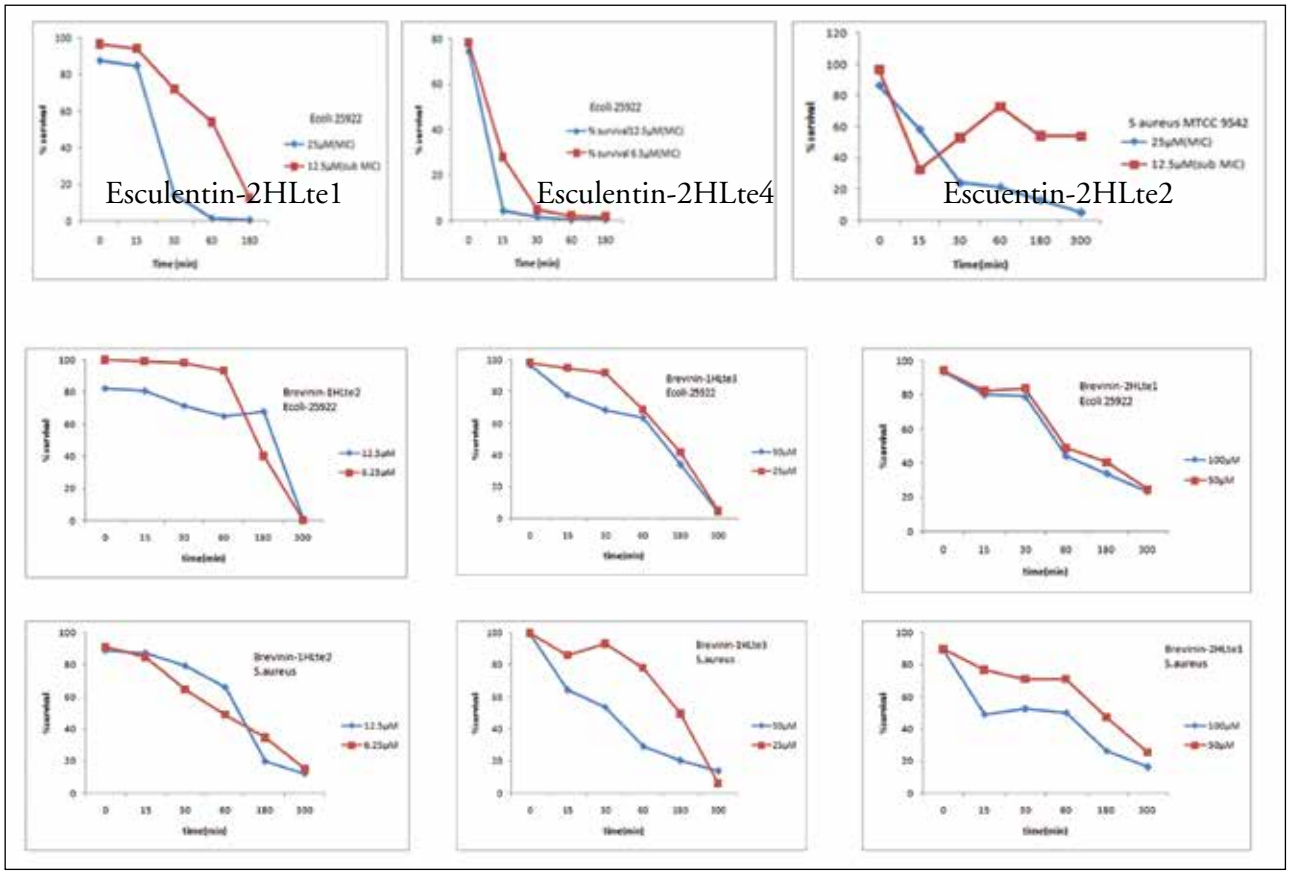


Fig 2 Killing kinetics of Esculentin and brevinin peptides

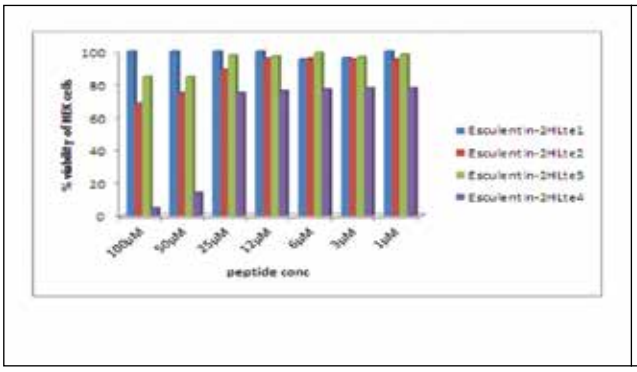


Fig 3. Effect of peptides on viability of HEKcells

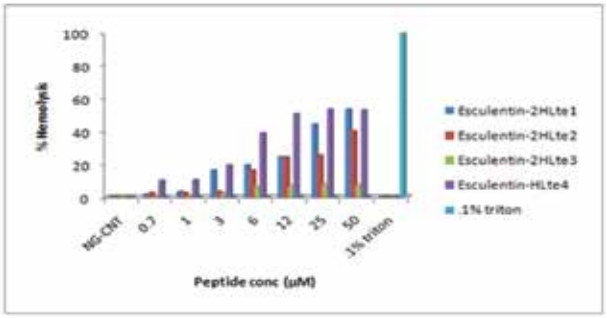
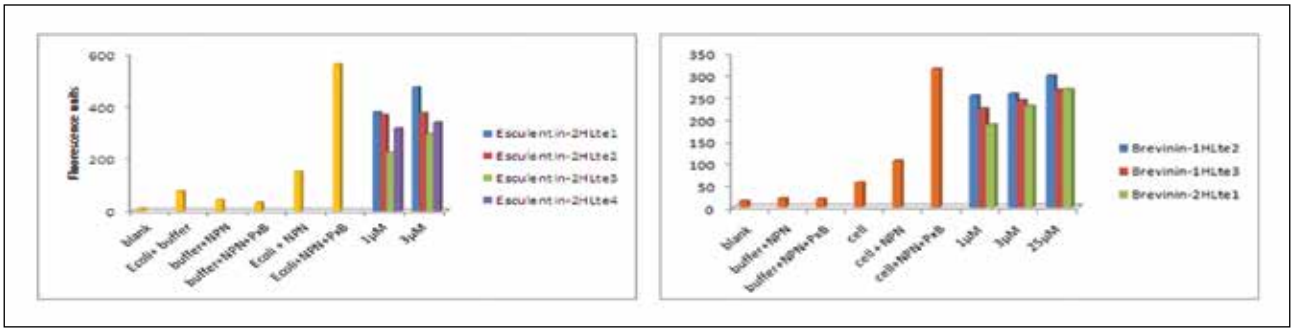


Fig. 4 Effect of peptides on red blood cells



Effect of peptide on outer bacterial membrane

25μM1, 50μM and 100μM concentrations were added and the NPN fluorescence spectra were obtained in the presence of cells. These peptides adopt alpha helical structure in membrane mimetic environment.

Arginine rich cell penetrating peptides and its structure function studies

Asha R and K. Santhosh Kumar

Another class of membrane active molecules are Cell-Penetrating Peptides (CPPs) that have the ability to translocate across biological membranes without causing any damage. These Arg rich molecules can help to introduce molecules into cells to interact with intracellular targets without causing toxicity to it. These peptides can therefore be used to carry site-specific drug, genes and specific molecules for controlling and regulating various biochemical processes within the cell. A thorough understanding of the primary and secondary structural requirements and mechanism of action can help to design short peptides with specific biological action.

Several peptides were synthesised SPPS technique using the templates R_5X_5, R_6X_6, R_7X_7 and R_8X_8 ("X" amino acid other than Arg) and characterized by RPHPLC and MALDI-TOF-MS techniques. When Arg is placed in the alternate positions in R_5V_5, R_5I_5, R_5L_5 and R_5M_5 are very active against both Gram-positive and Gram-negative bacteria. The Arg-Met combinations R_3M_3, R_4M_4, R_5M_5, R_6M_6, R_7M_7 and R_8M_8 also showed excellent antimicrobial property and their MICs are found to increase as the number of Met residues increases. The microscopic visualisation of the effect of R_7M_7 and R_8M_8 on living bacteria showed that though it causes appreciable membrane permeabilisation total cell disruption is not taking place. Sytox green uptake showed a peptide concentration dependant uptake of the dye, indicating that number of cells showing fluorescence increases as the peptide concentration increases.

These peptides induce the membrane depolarisation and that allow the voltage sensitive dye DiBac to penetrate into the cell. SEM images of the untreated bacterial cells showed a bright and smooth surface. But when the peptide was added its showed morphological modifications like surface blebs on the surface which further confirm the membrane damages which leads to the killing of the microbes within a short period of time. Confocal microscopic photographs of rhodamine B conjugated R_8A_8 showed no antimicrobial activity but this peptide can penetrate into the cell and even to the nucleus in a concentration dependent manner.

Peptide	E.coli	Staph aureus	Vibrio cholerae
R5V5	6.25	12.5	100
R5Y5	25	6.25	50
R5I5	50	100	ND
R5M4	>100	>100	ND
R5M5	25	>100	>100
R6M6,	25	50	>100
R7M7	12.5	6	50
R8M8	3	6	12
R8A8	100	>100	>100
Kanamycin	3		50
Ampicillin		0.7	

Fig.1.Antimicrobial activity of the peptides.

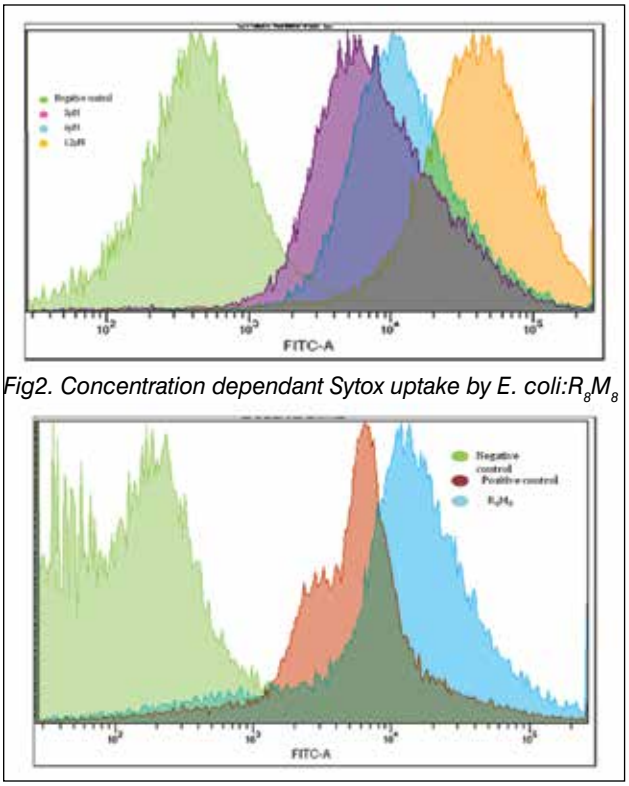


Fig2. Concentration dependant Sytox uptake by E. coli:R_8M_8

Fig.3. Membrane depolarization induced by R_8M_8 on E. coli cells

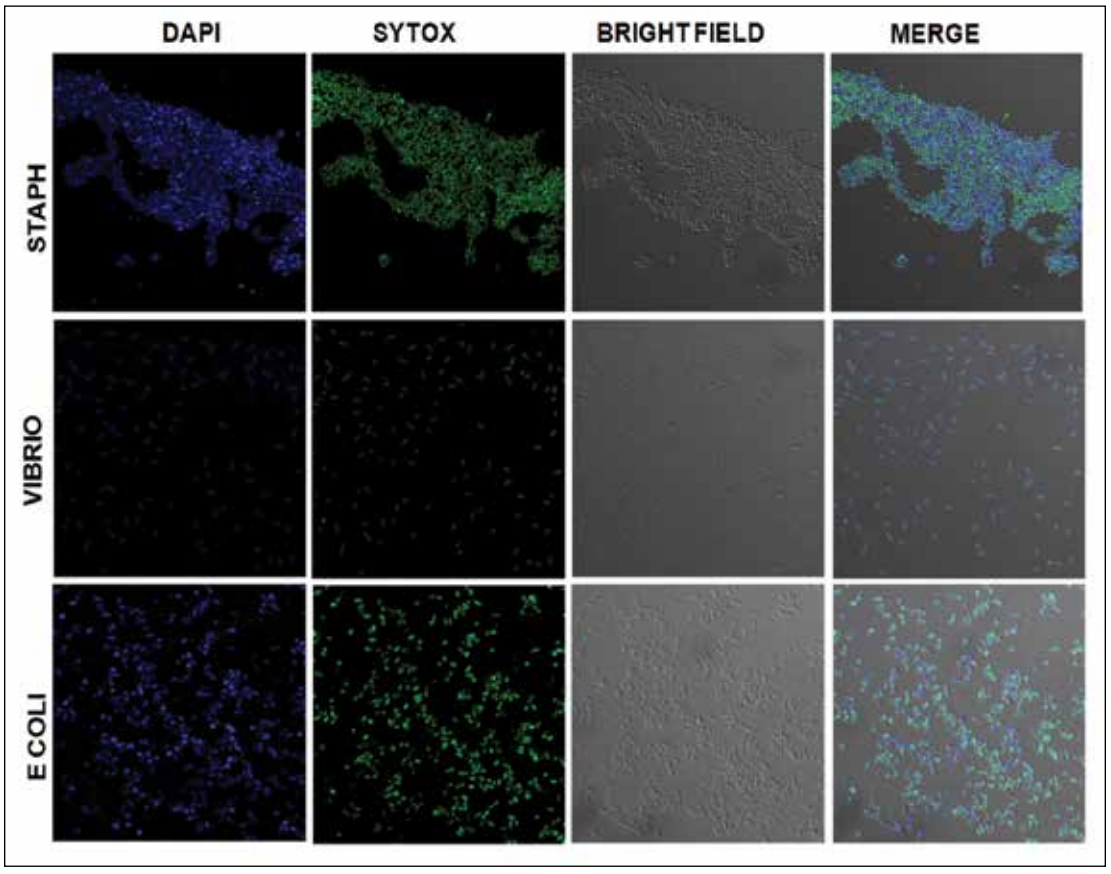


Fig.2. Bacterial membrane permeation induced by R_8M_8, visualized by DAPI and Sytox green

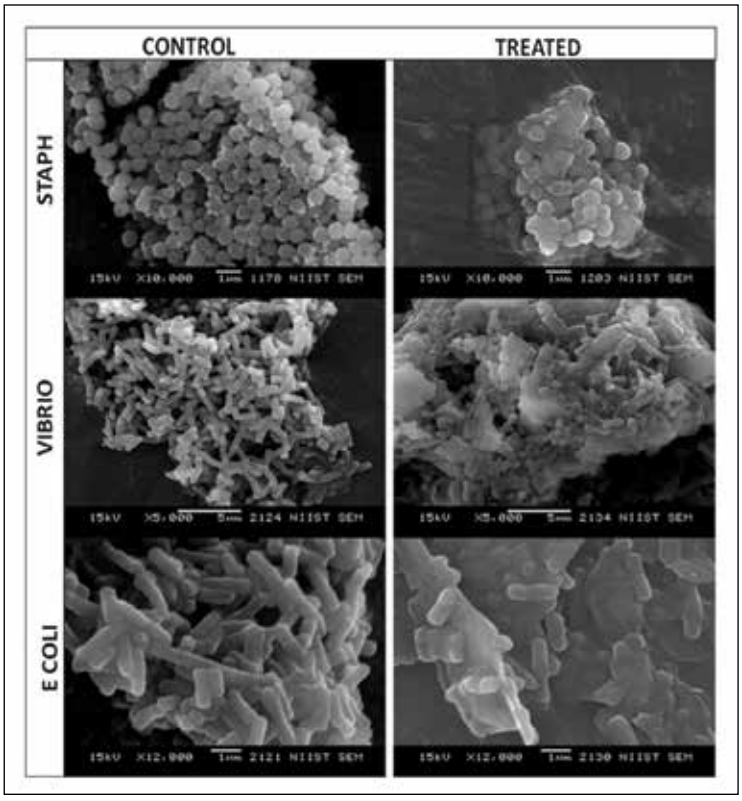


Fig.5. SEM images showing the disruption of cells by the action of R8M8

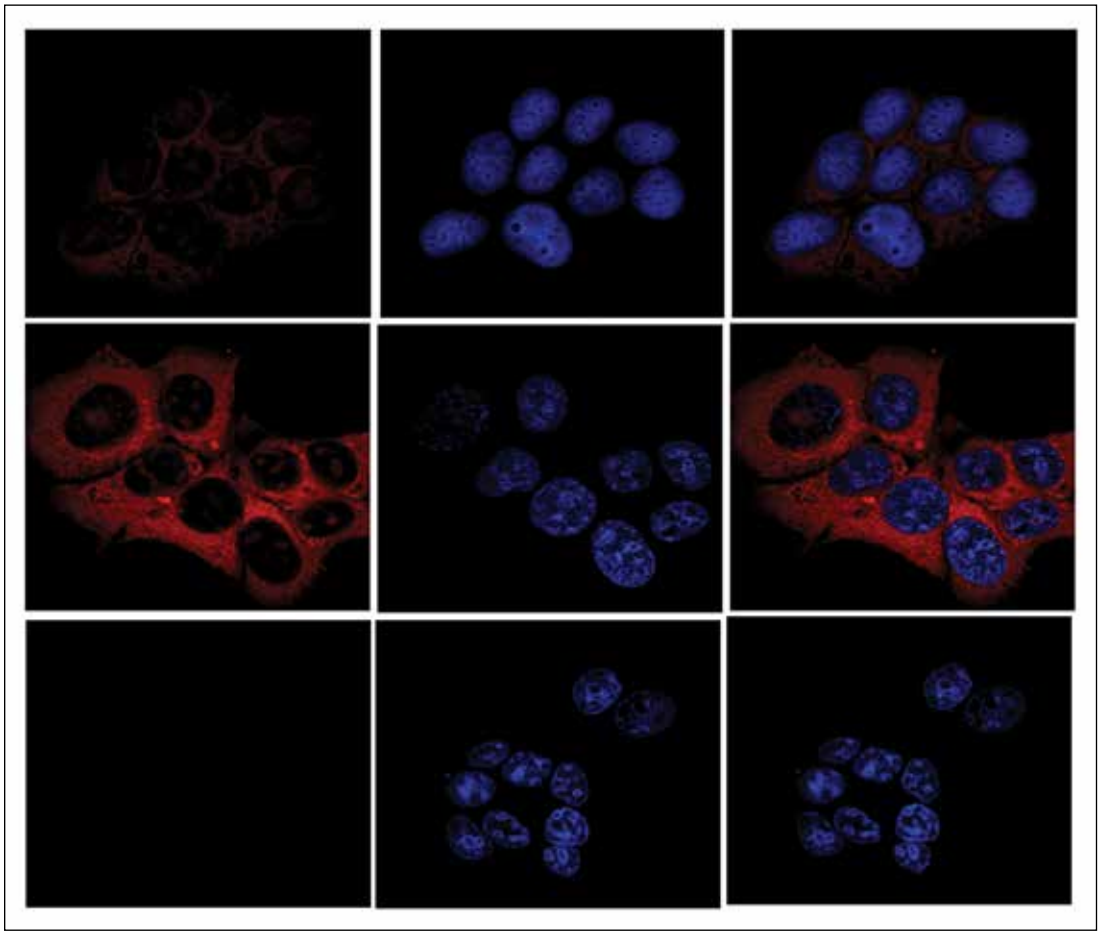


Fig.6.Uptake of R8A8 by MCF7 cells, at 4µm the peptide penetrates well into the nucleus

Dendrimeric Polymer Nanocarriers for Target specific Drug Delivery

Aravind and K. Santhosh Kumar

Tumor targeting and anticancer drug delivery is an interesting approach for the treatment of cancer since the traditional chemotherapy associated with several adverse effects. Many efforts have been made but the poor ligand uptake capacity of target receptors limited its success. A novel multifunctional, globular structured polyalkylether based dendritic nanocarrier was developed for the controlled delivery of the drug by the solid phase synthetic techniques using Michael addition reactions. Dendrimer was synthesised using ethyl-2-(hydroxymethyl)acrylate as the monomer using PEG-200/DABCO medium and then exposing the medium to microwave radiation (Fig.1).

After each generation, cathepsin sensitive tetrapeptide spacer Gly-Phe-Leu-Gly was attached to a fraction of the available functional sites. The amino terminal of the peptide linker was converted to carboxylic group using *cis*-aconitic anhydride and doxorubicin was incorporated to it. The dendrimer synthesis was continued upto the G4 generation. After each generation the drug molecule was incorporated. Short peptide ligands ER5 and ER6 were designed to target overexpressed or abnormally activated tyrosine kinase receptors, EGFR and VEGFR in cancer cells. These peptides were incorporated onto the surface carboxylic group of the G4 dendrimer by the general protocol for SPPS. Free hydroxyl functional sites in the dendrimer were treated with Cs salt of succinic acid to from free carboxylic group to which the drug is attached. Finally the nano carrier was cleaved

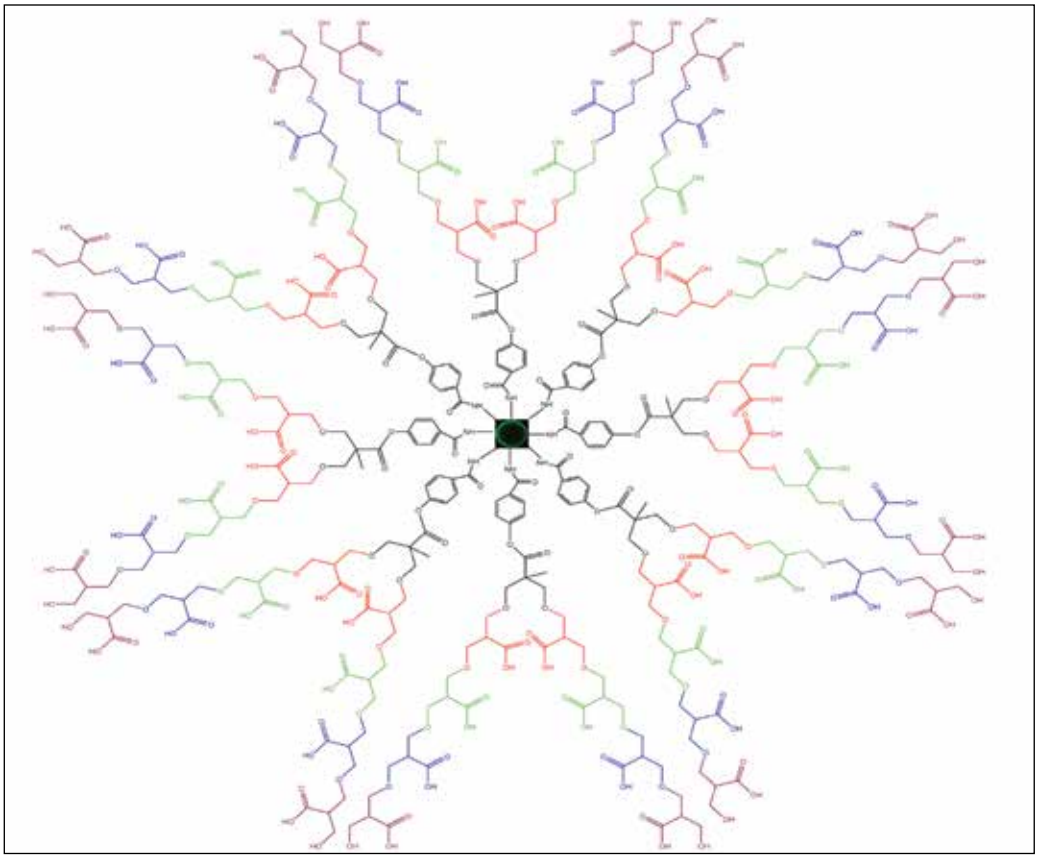


Fig. 1 Dendrimer Synthesis

form the support and analysed by RP HPLC and mass spectrometry. *In-vitro* toxicity analysis showed that the low cytotoxic nature of the new dendrimer is due to the biocompatible nature of the polyethylene oxide skeleton and the minimum interaction between the cell and the peripheral anionic carboxyl and hydroxyl groups (Fig 2). Dendrimer showed dose dependent hemolysis after 1h incubation but it is significantly less compared to commercially available dendrimers suggesting that it can effectively interact with negatively charged erythrocyte membrane. Electrostatic repulsion among red blood cells prevents their self-aggregation and adhesion to the walls of RBC. The hemolytic activity of PAMAM dendrimer is reported due to interaction of positively charged NH_2 group of dendrimer with negatively charged surface of red blood cells.

Acute and 28 days sub acute toxicity analysis of the dendrimer treated Swiss albino mice showed no significant changes in the general behaviour or other physiological activities nano carrier treated mice. Upto 100 mg/Kg, no mortality was observed up to 21st day. Nanocarrier concentration selected for blood analysis was based on results of acute toxicity study, in which no mortality was observed up to 75 mg/Kg. No significant ($p<0.05$) changes observed in the hematological parameters compared to the value of control group and value at 0th day of injection. As the concentration increases the heamoglobin content was found to be significant difference. Similarly RBC, Platelet count and TLC value were also found to have significant differences. These results could be very well correlated with the % survival/ %changes in body weight results. Changes were observed only when higher dose (100 mg/Kg) of dendritic nanocarrier was injected.

The analysis of biochemical parameters of various concentrations of nanocarrier treated animals showed no significant differences ($p<5$) compared with control group. At higher concentration of nanocarrier (75 mg/Kg) treated groups were found to have some difference with control group. Histopathology of the dendrimer treated (25, 50 and 75 mg/Kg) animals after 28 days and compared with that of the control group. It showed no vital changes in normal histopathology of the organs observed during dosing period with poly alkyl ether based dendrimer. Normal tabular

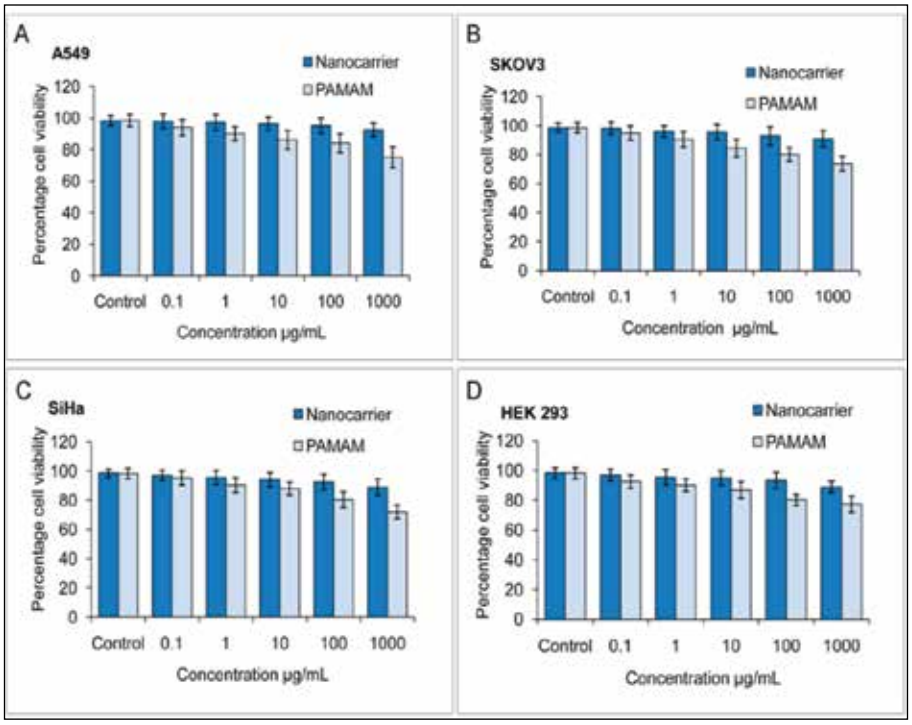


Fig.2 MTT assay with (A) A549 (B) SKOV3 (C) SiHa and (D) HEK-293 after 48h of incubation.

Table.1 Acute and sub-acute toxicity study of PETIM dendrimer

Group	Treatment	Days after administration									
		Percentage survival (%S) / Percentage change in body weight (% BW)									
		1st day		7th day		14th day		21st day		28th day	
		% S	% BW	% S	% BW	% S	% BW	% S	% BW	% S	% BW
1	Control	100	0	100	0	100	1.5	100	1.5	100	2.5
2	G4 (25 mg/Kg)	100	0	100	0	100	1.1	100	1.8	100	2.9
3	G4 (50 mg/Kg)	100	0	100	0	100	1.2	100	1.6	100	2.5
4	G4 (75 mg/Kg)	100	0	100	0	100	1.5	100	2.1	100	2.4
5	G4 (100 mg/Kg)	100	0	100	0	100	1.2	88.3[a]	1.5	88.3[a]	3.7[a]
6	G4 (125 mg/Kg)	100	0	100	0	88.3	1.1	66.3[a]	1.3	66.3[a]	4.4[a]

The data obtained from various groups were statistically analyzed using one-way ANOVA followed by Tukey's Multiple Range Test. P < 0.05, is considered statistically significant. Data represent mean ± SD (n=6) vs control

architecture of hepatocytes was observed in both control and nanocarrier treated groups. But those animals treated with 75 mg/Kg of the nanocarrier showed slight increase in congested blood vessels after 28 days.

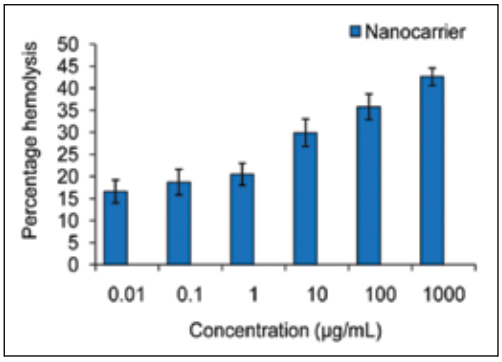


Fig.2 Hemolysis assay at concentrations (0.01-100 µg/mL) incubate at 37± 1.00C for 1h. Mean ± SD (n=3)

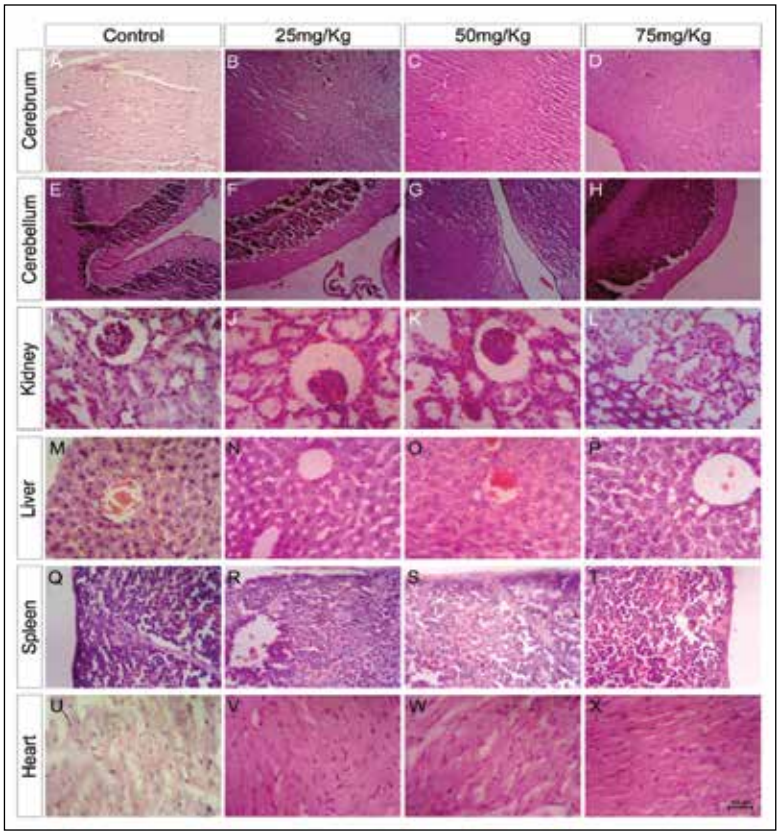


Fig.3 Histopathology of nanocarrier treated mice. Brain (cerebrum- A-D, cerebellum- E-H), Kidney (I-L), Liver (M-P), Spleen (Q-T) and Heart (U-X) at 25, 50, 75 mg/Kg. Control (PBS, pH 7.4), Scale: (40×10X= 400 X)

PUBLICATIONS

- *Abraham P, Sundaram A, R A, V R, George S, Kumar KS* "Structure-Activity relationship and mode of action of a frog secreted antibacterial peptide B1CTcu5 using Synthetically and Modularly Modified or Deleted (SMMD) peptides". *PLoS ONE 2015 10(5)*
- *Parvin Abraham 1, Sanil George 1, K. Santhosh Kumar* "Novel antibacterial peptides from the skin secretion of the Indian bicoloured frog Clinotarsus curtipes" *Biochimie 97 2014 144e151*
- *A Aravind, A Deepu K S Kumar* "Self-assembled cationic cell penetrating peptide nanoparticles as efficient cargoes for antimicrobial agents *BMC Infectious Diseases" 14 (Suppl 3) 2014,19*

CHEMICAL BIOLOGY PROGRAM
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Cationic, amphiphilic dextran nanomicellar clusters as an excipient for dry powder inhaler formulation

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***Collaborator**: * Cardiovascular Disease Biology, Rajiv Gandhi Centre for Biotechnology*

The potential of lung as a port for drug delivery was recognized years ago. The large surface area along with extremely thin and delicate physiological barrier between air and systemic circulation (in alveoli) increases the bioavailability of the administered drugs. Inhalational drug delivery can be a method of choice for ailments affecting lung due to its increased drug availability with minimal systemic side effects. Recently, dry powder inhalation (DPI) formulation gained greater attention over other inhalational formulations as it has increased patient compliance, formulation stability and environment friendliness. The biggest challenge in targeting alveoli is to overcome the effective filtering mechanism of the respiratory system. It is reported that particles of size range 1-5 microns succeed in reaching alveoli, whereas particles of ultrafine size (less than 1 micron) fail to settle in the alveoli and get exhaled out. The most widely accepted strategy in DPI technology is admixing the micronized active pharmaceutical ingredient (API) with lactose. Emitted dose variability and low efficiency in dose delivery (approx. 20% of label claim) are major limitations of this strategy. Dose variability is not a serious problem for the symptomatic treatment of asthma as most of these currently available formulations are meant to target upper respiratory tract. In clinical practice, formulations intended for systemic use can be administered to lung (off-label use) through nebulization. In accordance with this, after preclinical studies Bend research Inc. proposed dextran as a suitable DPI excipient. Hydrophilic polymers are however recognized as poor drug carriers due to its burst release property. Microparticles made of chemically modified, hydrophobic acetylated dextran are observed to have sustained release of entrapped drug on in-vitro release kinetics. We report here synthesis

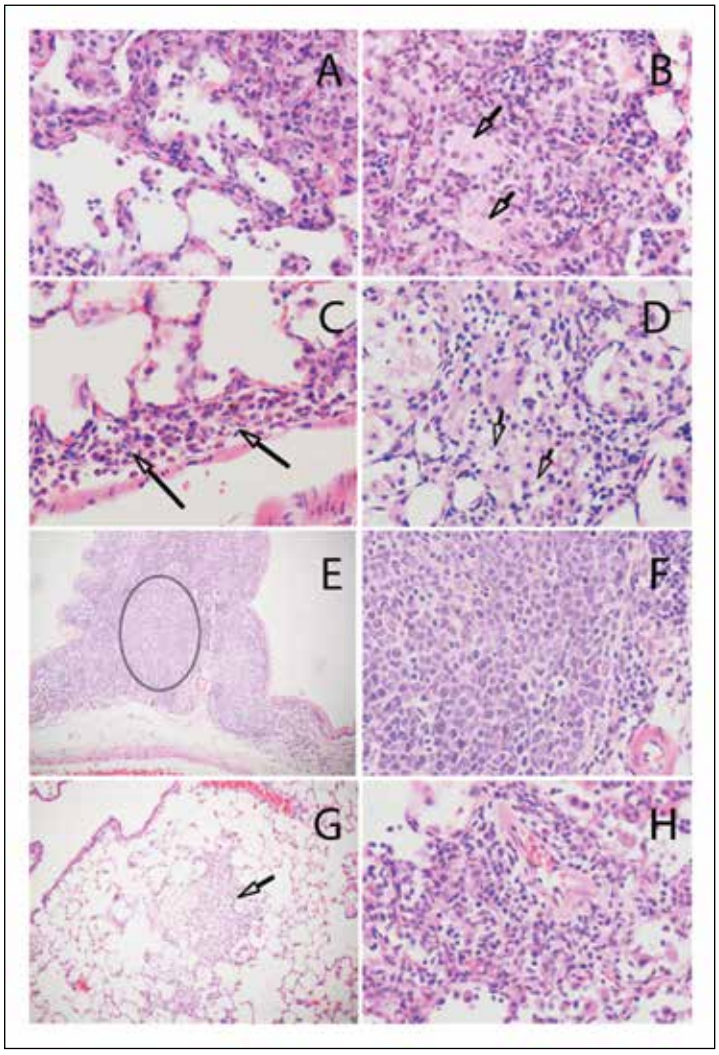

Fig 1. Hematoxylin and Eosin stained microscopic images of rat lung representing different pathological changes observed. (A) Septal hypertrophy with hyperplasia of alveolar epithelium, (B) granulomatous inflammation with multinucleated giant cells (arrows), (C) perivascular inflammation (arrow heads), (D) foamy macrophages inside the alveoli, (E) circled area represents lymphoid hyperplasia and (F) is at higher magnification (40X). (G) neutrophil infiltration into alveolar spaces (alveolitis). (H) perivascular inflammation with alveolitis.

of a cationic amphiphilically modified dextran through grafting hydrophobic stearyl amine in a two step reaction. To study the effect of grafting on physico-chemical as well as safety aspects, two different combinations were synthesized by changing the mass ratio of stearyl amine to dextran. This lipopolymer was further formulated to drug containing inhalable microparticles through spray drying. In the present work, we developed a system having the advantages of both micro and nanoparticulate inhalational systems. The optimum aerodynamic size of spray dried, drug loaded particles will help in the effective alveolar deposition. Further, the presence of lung fluid will steer the deposited microparticles to individual stable nanomicelles due to amphiphilic and cationic property of stearyl amine grafted dextran. This acts like micron sized cluster bomb which releases nano drug carrier after reaching the target site. There are many reports emphasizing the need for inhalation formulation for treating tuberculosis (TB). The widely accepted mode of TB treatment is prolonged intake of fixed dose combination (FDC) tablet containing rifampicin, isoniazid, ethambutol and pyrazinamide. Among the first line of these anti-TB drugs, rifampicin is the only hydrophobic one. This causes dissolution rate limited bioavailability of rifampicin (BCS class II drug). Apart from this, rifampicin often shows chemical and physical incompatibility with the ingredients of FDC tablet. Moreover, avascularity and remodeling of lung in TB patients aggravates the situation through defective drug delivery to the target site. All this made us choose rifampicin as a model drug for the present newly developed DPI formulation. Effective delivery of drugs to alveoli in a controlled manner using hydrophobic polymers as carriers has already been reported. Preclinical studies revealed that toxicity and hydrophobicity are related to each other in pulmonary delivery. Two proportions of lipopolymers were synthesized and physico-chemical characterization was carried out. In-vivo evaluation of sub-acute toxicity of the synthesized lipopolymer in Sparague Dawley rat was carried out for three months. This was followed by a histological evaluation of the sacrificed animal's lung (Fig 1). Further, the synthesized lipopolymer was formulated with drug (Rifampicin) loaded inhalable microparticles through spray drying (Fig 2). The final drug formulation was tested for toxicity and proinflammatory responses in human cell lines. Dose deposition efficiency of the formulation was determined using Anderson Cascade Impactor. The synthesized lipopolymer combinations were found to be biocompatibile in animal model as well as in human in-vitro cell line studies. Self de-aggregation property would aid in wide distribution of administered dose and thus eliminate the risk of dose dumping. Above all, the amphiphilic self assembling system can be used for delivering both hydrophobic and hydrophilic drugs.

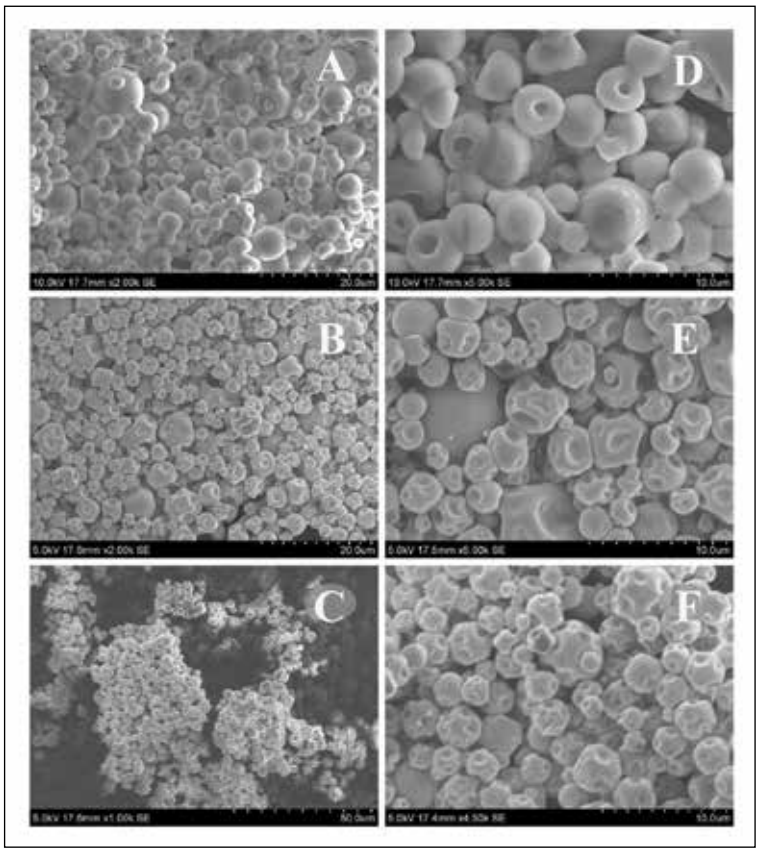

Fig 2. SEM images of spray dried microparticles. A, B and C corresponds to microparticles of Dex, Dex-LS and Dex-HS respectively. D, E and F are images of Dex, Dex-LS and Dex-HS batches at higher magnification.

Self assembling peptide nanotherapeutic cargo for the sustained delivery of 5-fluorouracil

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*Collaborators: * Cancer Research Program, Rajiv Gandhi Centre for Biotechnology and*

*** Distributed Information Sub-Centre, Rajiv Gandhi Centre for Biotechnology*

Self-assembling peptide based biomaterials are important in various fields such as tissue engineering, drug delivery etc. in recent times. They put forward an attractive platform for designing and synthesising biomaterials consisting of hierarchical nanoarchitectures. The molecular structure, available charged groups, and concentration of peptide lead to the effective formation of self-assembling peptide nanofibrous scaffold (SAPNS). Tailoring the amino acid sequence, concentration of the peptide nanofiber; the effective loading and controlled release of drugs from the SAPNS can be achieved successfully. Increasing therapeutic efficacy of such controlled drug delivery system (CDDS) can be achieved by incorporating suitable peptide architecture along with proper drug molecule. Colon cancer continues to be a malignancy with high mortality rate globally. Treatment of colon cancer with F-flurouracil (5-FU) shows several shortcomings including short biological half life, poor absorption due to dihydropyrimidine dehydrogenase enzyme and non-selective action against healthy cells of gastrointestinal tract and bone marrow. To overcome these limitations drug needs to be administered in nano formulations through a biodegradable, biocompatible CDDS. In the present study, we have designed a sequence RADA-F6 [CH_3-CO-RADARFDARADARADA-CO-NH_2]; which exhibited the property of forming SAPNS. The introduction of phenylalanine leads to effective ϖ-ϖ stacking. Self-assembling behaviour, pH stability and 5-FU release from RADA-F6 were analysed by molecular dynamics studies taking RADA-16 as control. As 5-FU contains the aromatic pyrimidine ring, RADA-F6 system is suitable for entrapping an aromatic drug and shows better controlled release. The hydrogel was prepared by dissolving synthesized peptide in Tris HCl buffer at pH =7.4 (Fig 3). Mechanical stability and physical characterisation of the peptide nanofibers was done using Transmission Electron Microscopy (TEM), Circular Dichroism (CD) spectra, Fluorescence spectra etc. Then 5-Fluorouracil (5-FU) was loaded in peptide nano fibre and analysed for *in vitro* drug release studies. Cytotoxicity of drug loaded RADA-F6 was measured by MTT assay and compared to RADA-16. Induction of apoptosis in HCT-116 (Colon adeno carcinoma cell line) was analysed by Western blotting analysis to confirm Poly (ADP-ribose) polymerase (PARP) cleavage. Further flow cytometric analysis imparted the action of drug at different stages of cell cycle. This is the first report explaining physicochemical characterization and biological evaluation studies of a self assembling peptide for the drug release of 5-FU as an anticancer therapeutic. The drug entrapped hydrogel can be given in oral route through a pH sensitive polymer coated capsules. Our biological evaluation vividly depicts the efficacy of drug entrapped peptide for anticancer activity. Apoptosis is a process of an essential tissue homeostasis, and regarded as the ideal way to inhibit cancer cell growth. The induction of apoptosis by RADA-F6+5-FU is significant compared with free 5-FU and RADA-16+5-FU. Western blotting analysis can be viewed as a promising approach for the cancer treatment through peptide based drug delivery systems. The flow cytometric analysis confirms the mechanism of 5-FU at specific phases of cell cycle (Fig 4). The above observations strongly substantiate our hypothesis of RADA-F6 as an ideal DDS to 5-FU.

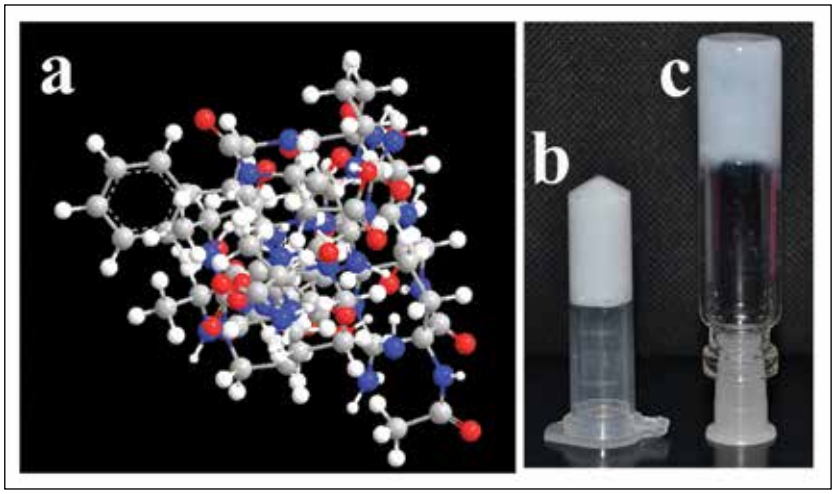

Fig 3. (a) 3D Structure of RADA-F6 (b) 5-FU entrapped RADA-F6 hydrogel (c) Blank RADA-F6 hydrogel.

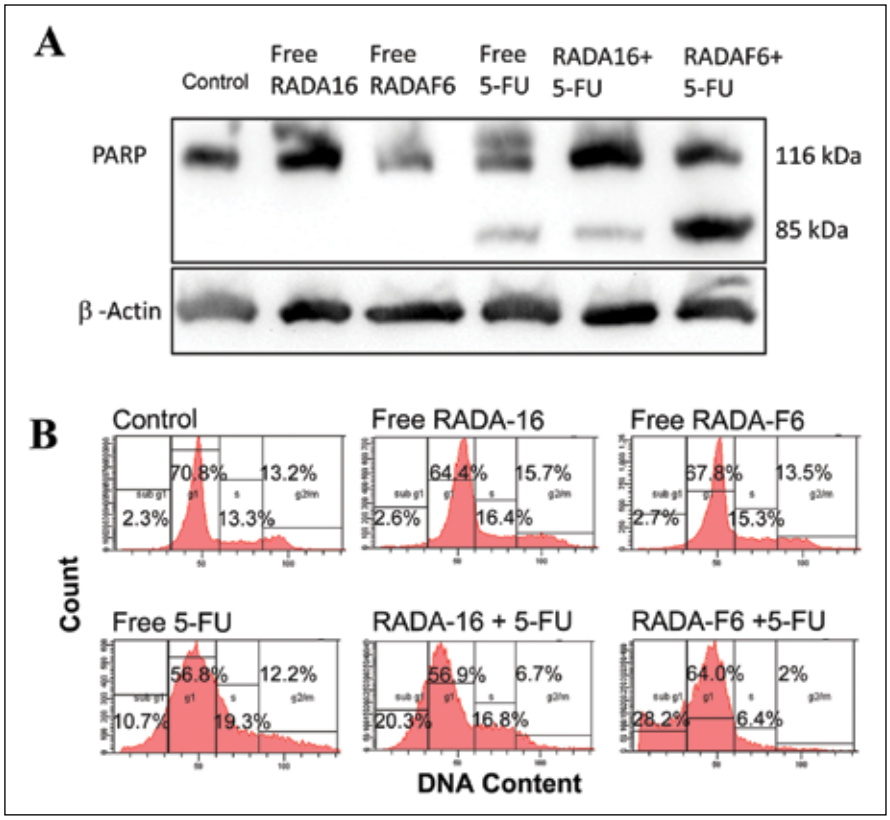

Fig 4. (A) Western blotting analysis of RADA-F6+5-FU to analyse the cleavage of PARP. (B) FACS analysis of RADA-F6 nanofibers containing 5-FU.

PUBLICATIONS

- *Ashwani Kumar N, Nisha Asok Kumar, Asha Nair.S and Kumar GSV.* Dual drug delivery of 5-fluorouracil (5-FU) and methotrexate (MTX) through random copolymeric nanomicelles of PLGA and polyethylenimine demonstrating enhanced cell uptake and cytotoxicity. *Colloids and Surfaces B: Biointerfaces, 2014; 122:520–528.*
- *Ashwani Kumar N, Nisha Asok Kumar, Asha Nair.S and Kumar GSV.* 5-Fluorouracil–lipid conjugate: Potential candidate for drug delivery through encapsulation in hydrophobic polyester-based nanoparticles. *Acta Biomaterialia, 2014; 10:4685–4694.*
- *Ashwanikumar N, Nisha Asok Kumar, Asha Nair.S and Kumar GSV.* Phenylalanine-containing self-assembling peptide nanofibrous hydrogel for the controlled release of 5-fluorouracil and leucovorin. *RSC Advances, 2014; 4:29157–29164.*
- *Jisha Jayadevan Pillai, Arun Kumar Theralikattu Thulasidasan, Ruby John Anto, Devika Nandan Chithralekha, Ashwanikumar Narayanan and Kumar GSV.* Folic acid conjugated cross-linked acrylic polymer (FA-CLAP) hydrogel for site specific delivery of hydrophobic drugs to cancer cells. *Journal of Nanobiotechnology, 2014; 12:25.*
- *Siyad M.A and Kumar GSV.* A class of linker free

amphiphilic PEG grafted polymer support for linear and cyclic peptides. *RSC Advances, 2014; 4:60404–60408.*

- *Lekha Nair K, Sankar Jagadeeshan, Asha Nair.S and Kumar GSV.* Folic acid conjugated d-valerolactone-poly (ethyleneglycol) based triblock copolymer as a promising carrier for targeted doxorubicin delivery. *PLOS ONE, 2013; 8 (8) e70697.*
- *Siyad MA and Kumar GSV.* Synthetic evaluation of disulphide-bonded sarafotoxin on a poly (oxy ether) grafted dendrimeric poly(alkylamine) support for polymer assisted organic synthesis. *Organic & Biomolecular Chemistry, 2013; 11:4860-4870.*
- *Mithun Varghese Vadakkan, Annapoorna K, Sivakumar K.C, Sathish Mundayoor and Kumar GSV.* Dry powder cationic lipopolymeric nanomicelle inhalation for targeted delivery of antitubercular drug to alveolar macrophage. *International Journal of Nanomedicine, 2013; 8:2871-2885.*
- *Siyad MA and Kumar GSV.* PEGylated dendrimer polystyrene support: synthesis, characterisation and evaluation of biologically active peptides. *Amino Acids, 2013;44 (3) :947-959.*
- *Siyad MA and Kumar GSV.* Synthesis, Characterization, and Application of Bisphenol A Glycerolate Dimethacrylate Cross-Linked Polystyrene (PS-BGD): A Novel Support for Gel Phase Peptide Synthesis. *Current Organic Synthesis, 2013; 10 (2): 318-327.*

CONFERENCE PRESENTATIONS

- *Ashwani Kumar N, Nisha Asok Kumar, Asha.S.Nair and G.S.Vinod Kumar.* "Controlled, pH responsive delivery of 5- Fluorouracil by a Polymeric Nanomaterial." *ICAFM-2014 International Conference on Advanced Functional Materials, Trivandrum, 2014.*

RESEARCH GRANTS

Title	Investigators	Funding Agency	Duration
A novel site specifically targeting nanoparticle based oral - drug and siRNA releasing polymer systems for colon cancer	Prof. M.Radhakrishna Pillai (Project Coordinator) G.S. Vinod Kumar (PI) Hari Krishnan (Co-PI) Asha Nair.S (Co-PI)	Department of Biotechnology, Govt. of India	2010-14

CHEMICAL BIOLOGY PROGRAM
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Dna Barcoding of Amphibians of Kerala

Sujith V. Gopalan and Sanil George

Generated DNA barcodes of 50 species of frogs from Kerala part of Western Ghats based on the guidelines provided by International Barcode of Life (iBOL) and (Fig.1) submitted in the Public database BOLD under the project 'GHATS" (http://www.boldsystems.org/index.php/MAS_Management_OpenProject?code=GHATS). This includes 4 critically endangered and 9 endangered frog species as declared by International Union of Conservation of Nature (IUCN). Photographic documentation of these species with GPS coordinates was also performed. This forms the first initiative of barcoding frog species of India.

Fig.1: DNA barcoding of Critically endangered frog species of Western Ghats-BOLD submission

Extension of Distribution Range of Species as Inferred from Dna Barcoding of Tadpoles

Sureshkumar U and Sanil George

Identification of tadpoles collected from the wild is always a challenging task to assign them into their corresponding species. In the present study we analyzed CO1 barcodes of 48 tadpole specimens from Western Ghats, India and identified those into 15 different species by comparing the CO1 barcodes with the corresponding barcodes present in the GenBank. Genetic distance based on p-distance was calculated between barcodes. The maximum likelihood method was used to construct a phylogenetic tree, which grouped all the species into distinct clades (Fig.2). The study also noted that the phylogenetic position of some of the frog species should be revised. DNA barcoding is an effective and rapid tool to identify the tadpoles of a region to provide firsthand information on the regional amphibian diversity.

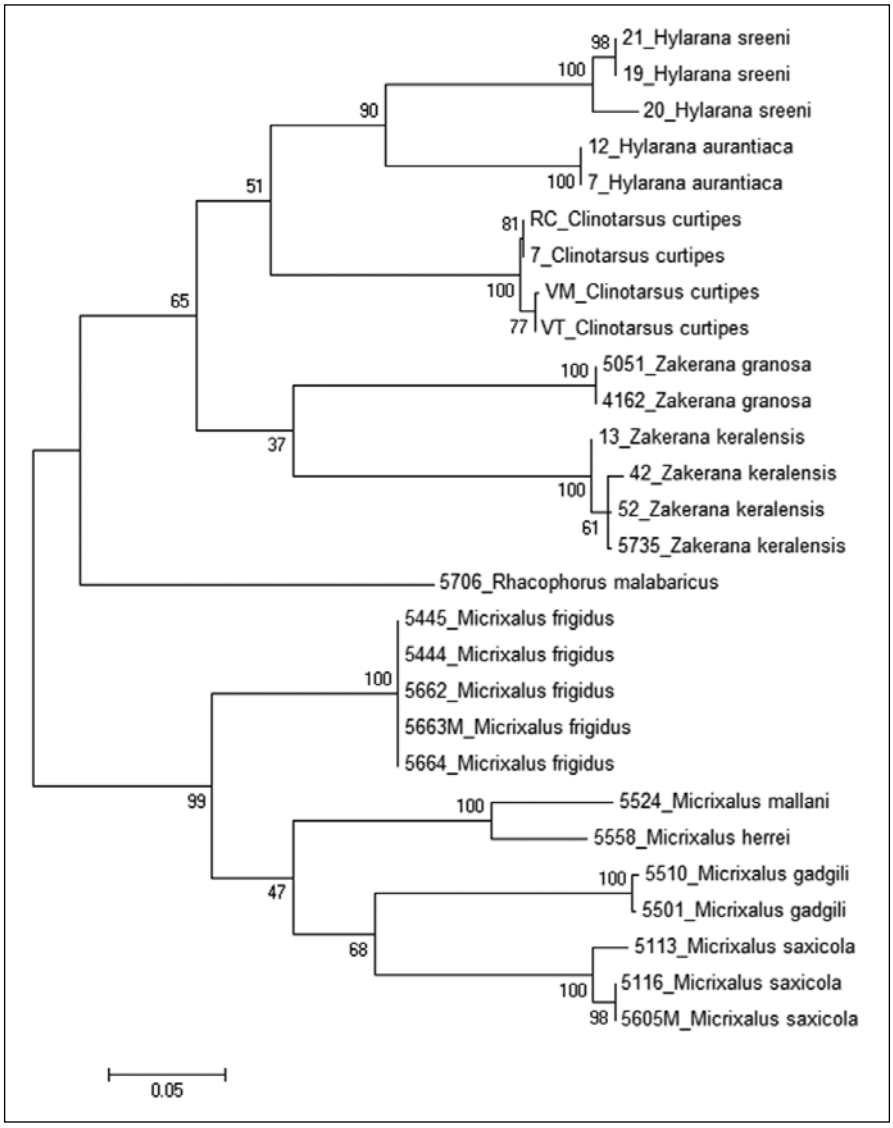

Fig. 2: ML tree based on the CO1 sequences of tadpoles

Multiple Genetic Lineages Challenge: The Monospecific Status of the Ornate Narrow -Mouthed Frog Microhyla Ornata of India.

Kiran S Kumar, Jesmina A.S and George S

Ornate narrow mouthed frog Microhyla ornata is considered as one of the most common Microhyla species in India. Morphology-based species descriptions are known to be problematic in the genus Microhyla because of the high likelihood of sharing common characters and the minute body size. Microhyla species is known to have poses challenges in diagnosing them from their known congeners based on morphology. However, species in this genus are often strongly differentiated in genetic comparisons, facilitating identification of new candidate species. In our study, we have identified a new lineage that (Fig3) can be clearly differentiated from all known species in the genus Microhyla, both by detailed morphological comparisons and by genetic methods.

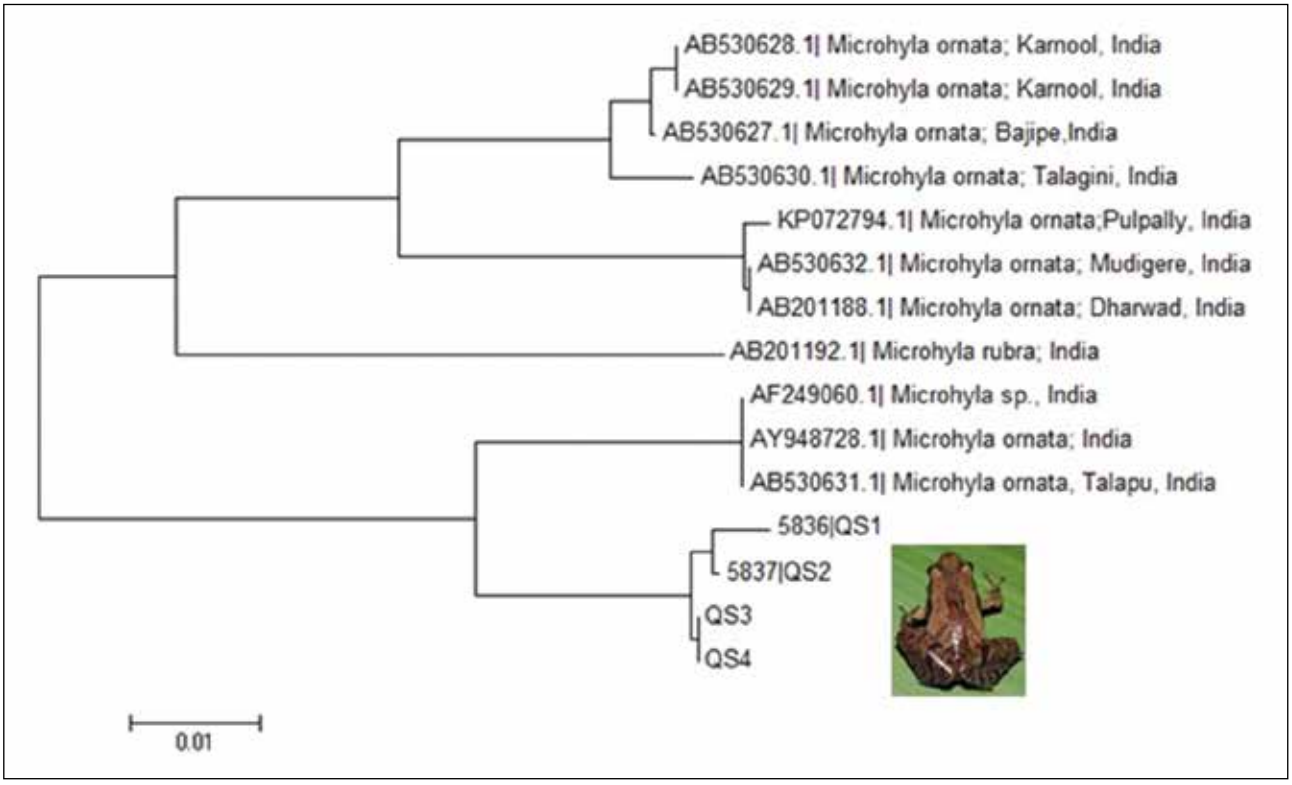

Fig. 3: ML tree based on the Microhylid frogs of India showing unidentified lineage

Genetic Diversity of Endemic Bush Frogs of Western Ghats

Sujith V. Gopalan, Suresh U. Kumar, Kiran S. Kumar, and Sanil George

Samples of endemic endangered frog species, Pseudophilautus wynaadensis, were assessed with the aid of mitochondrial DNA markers in order to study the pattern of intra specific genetic variation of samples lying south of Palghat gap of the Western Ghats, India. Phylogenetic analysis revealed that samples from either side of Palghat gap belonged to a single species (Fig.4). The population of frogs south of Palghat gap showed high haplotype diversity and low nucleotide diversity. The relative gene heterogeneity statistics were low and the gene flow estimates were very high. The AMOVA results showed that 96.05% of the total variations were within the populations. It was concluded that high genetic diversity with relatively little geographic differentiation was found in the populations of P. wynaadensis lying south of Palghat gap.

The major finding with *Pseudophilautus kani* was the lack of genetic structure of populations inferred from the data on haplotype diversity, nucleotide diversity, pairwise genetic distance, phylogenetic tree, neutrality tests and analysis of molecular variance. High levels of gene flow between populations may be correlated with the wide distribution of this species in the Kerala part of Western Ghats, India.

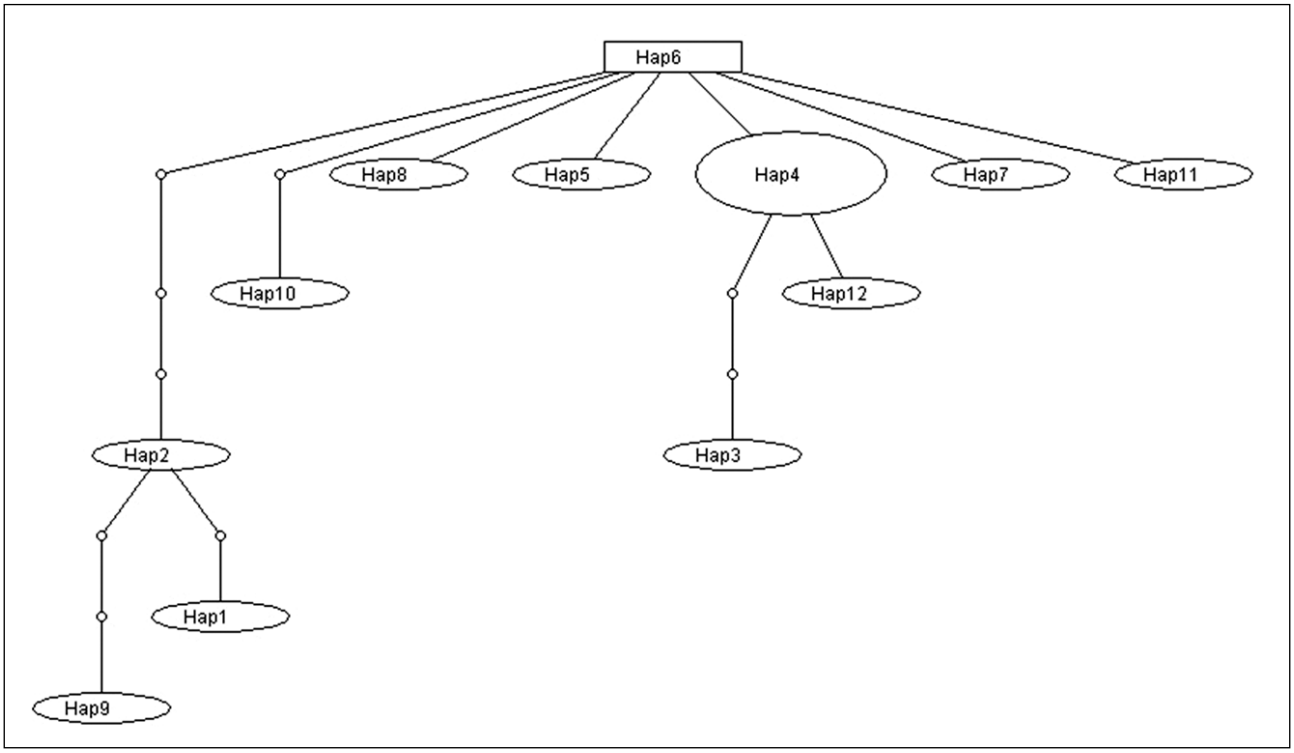

Fig.4. Haplotype network of P. wynaadensis populations south of Palghat gap

Studies on the Host Defence Peptides Isolated from Frog Skin Secretions

Vineethkumar T.V, Shyla Gopal and Sanil George

Amphibian HDPs are gene derived, mostly cationic (facilitates selective interaction with anionic bacterial membranes) and ĸhelical, with 40-50% hydrophobic residues (facilitates Van der Walls interaction with the lipid tails of the membrane) that cluster on one face when they attain helical structure. Apart from the general antimicrobial effect, HDPS have diverse functions, which include anticancer effect, immune system activation, antiviral, antifungal etc. Thirty one novel host defence peptides were isolated Hylarana malabarica, an endemic frog of Western Ghats, Kerala, India, by transcriptomic approaches. Of this 22 peptides belong to 10 known families and 9 belong to new families. Brevinin 1 family (2 paralogs: Brevinin 1 HLmb 1& Brevinin 1 HLmb 2) and Esculentin 2 family (2 paralogs: Esculentin 2 HLmb 1& Esculentin 2 HLmb 2) of peptides were selected for further characterization. The peptides were chemically synthesized in three forms, containing C-terminal carboxyl group (non amidated), C terminal amidated and amidation with disulphide bond at the C terminal, to evaluate the effect of amidation and dilsuphide bond on the biological activity of the peptides. Antimicrobial activity was confirmed by broth dilution method and analysis of killing kinetics. The peptides were tested against a broad range of gram positive and gram negative microbes. Non-amidated Brevinin 1 peptides show high antibacterial potency against gram positive bacteria with MICs ranging from 6.25 to 100 µM and they show negligible activity against gram negative bacteria at a concentration >200µM. Amidation had a great impact on the antimicrobial activity of the Brevinin 1 peptides. The MICs of non amidated peptides against gram positive bacteria was reduced to 2 – 4 times upon amidation. Apart from reducing the MICs against gram positive bugs, the amidated peptides gained activity against the tested gram negative bacteria with MICs ranging from 10 - 40 µM Incorporation of disulphide bond did not increase the activity of the peptides, they exhibited MIC values almost similar to that of amidated peptides. MIC was also determined for two fish pathogens, disulphide containing amidated pepides exhibited strong inhibitory effect on one of the pathogen. In the case of Esculentin 2 peptides, all the three forms show MIC against both gram positive and gram negative bacteria at almost equal range which showed that C terminal modifications did not affect antimicrobial activity of the peptides. The effect of amidation was reflected in the killing kinetics of Brevinin 1, both forms of esculentin 2 exhibited same kinetics (Fig.5). Further elucidation of mechanism of antimicrobial activity was carried

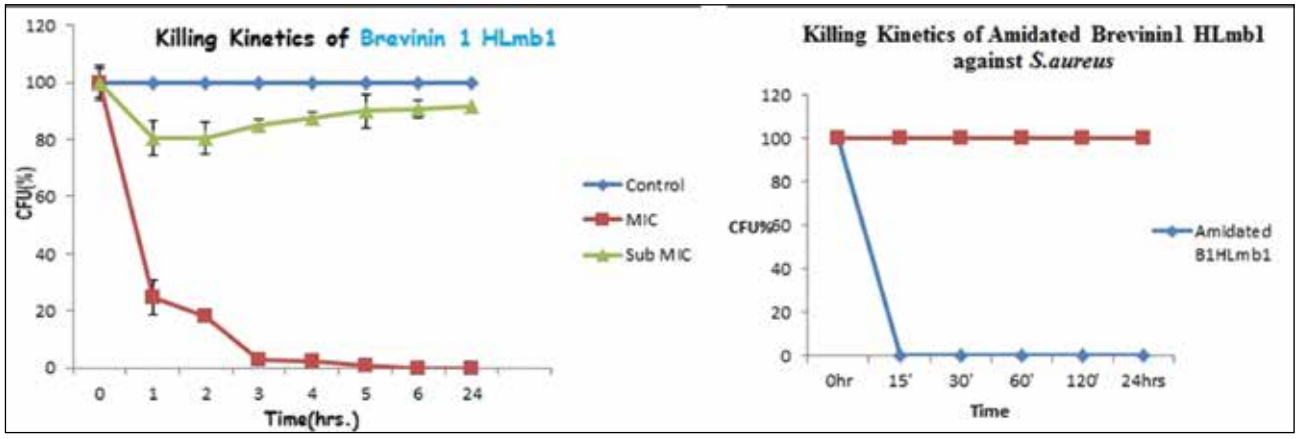

Fig.5. Bacterial killing kinetics under peptide challenge

The effect of divalent (Ca^{2+}, Mg^{3+}) and monovalent (K+, Na^{+}) on the activity of the peptides were evaluated for both negative and gram positive bacteria (Fig. 6). The decrease in the activity of the peptides in the presence of divalent metal ions indicates that they act on the membrane by displacing them. The same was observed for monovalent ions against gram negative bacteria but for gram positive there was no correlation with their activity and the presence of metal ions.

Anticancer activities of amidated and non amidated peptides were evaluated using MTT assay (Fig.7) against two cancer cell lines (Hep 3 B and HEK). It was found that all the peptides show more than 75 % killing at a concentration of 100µM

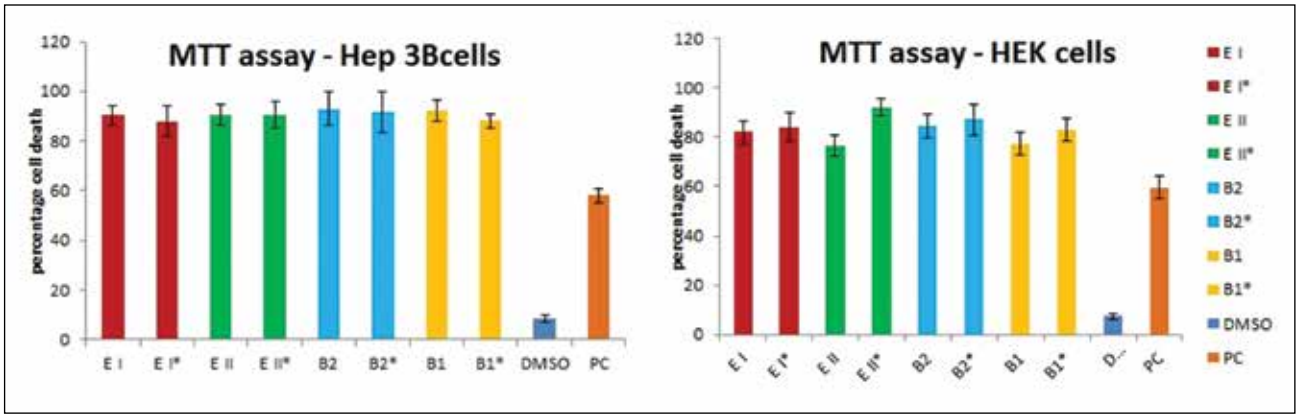

Fig. 6: Anticancer activities of frog skin peptides

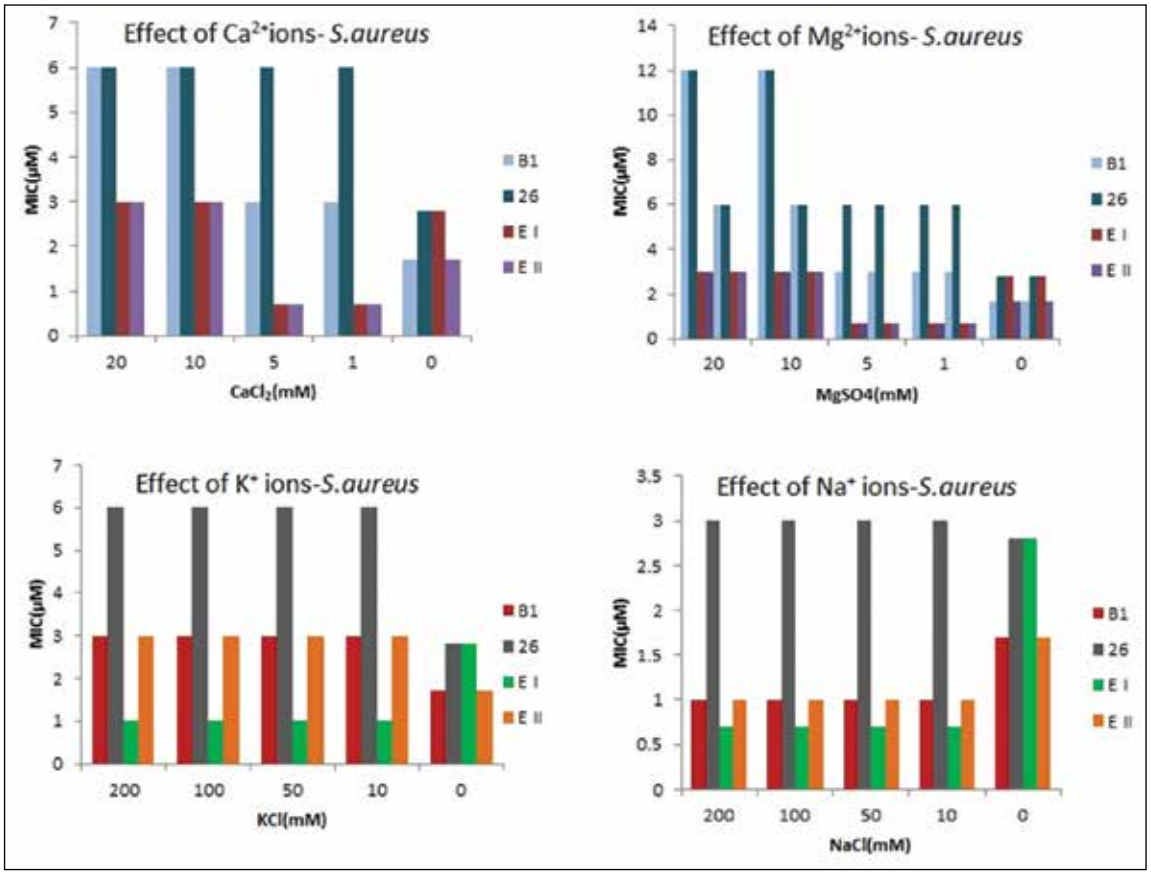

Fig. 7: Effect of Metal ions on the activity of frog skin peptides against microbes

Pore formation on gram negative and gram positive bacterial membranes were confirmed by confocal imaging (CLSM) using SYTOX uptake assay (Fig.8) and was confirmed by SEM analysis (Fig.9). It was observed that SYTOX could penetrate only the cells that were treated with peptides and pores formed and bacterial destruction was visualized by SEM. The concentration dependent pore formation by the peptides was evaluated by FACS analysis.

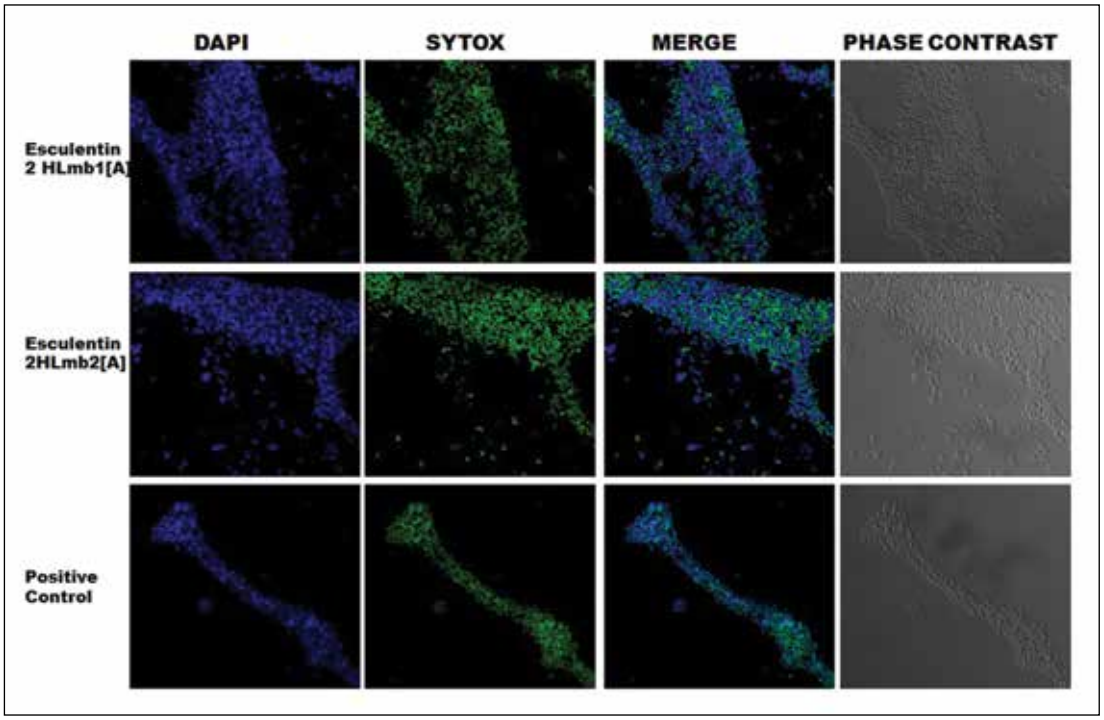

Fig.8: Sytox uptake assay

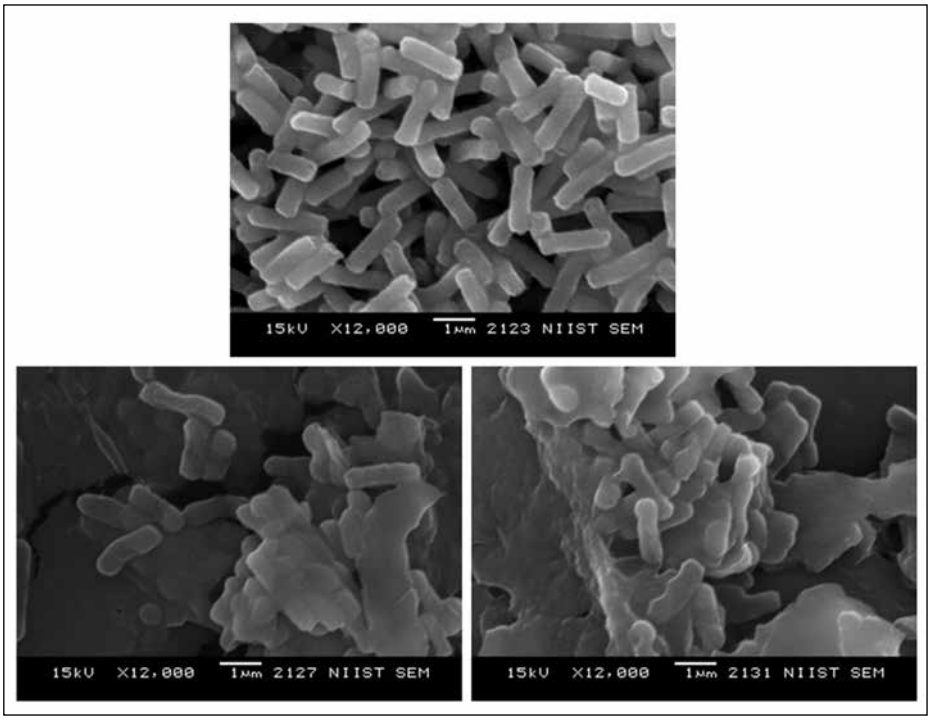

Fig.9: SEM image of V.cholerae under frog skin peptide challenge

GENBANK SUBMISSIONS

KP939044-KP939070 (26 sequences)

KP981222-KP981249 (27 sequences)

KR995130-KR995135 (5 sequences)

KR995268-KR995269 (2 sequences)

BOLD (Barcoding of Life Database) submissions

DNA barcoding of amphibians of Kerala, India: 50 sequences

DNA Barcoding of West African Birds: 19 sequences

PUBLICATIONS

- *Preetha R. Ravinesh R, Bijukumar A, Dhaneesh K.V and George S.* 2014. First record of *Granulifusus poppei* (Mollusca: Fasciolariidae) from Indian coast. *Marine Biodiversity Records, page 1 of 3. # Marine Biological Association of the United Kingdom, 2014 doi: 10.1017/S1755267214000992; Vol. 7; e95; 2014 Published online*
- *Parvin Abraham, Sanil George, K. Santhosh Kumar* 2014. Novel antibacterial peptides from the skin secretion of the Indian bicoloured frog Clinotarsus curtipes. *Biochimie 97 (2014) 144-151.*
- *Sujith V. Gopalan, Sureshkumar U, kiran S. Kumar and Sanil George* 2014. Genetic diversity of an endangered bush frog Pseudophilautus wynaadensis (Jerdon, 1854 "1853') from the south of Palghat gap, Western Ghats, India. *Miotochondrial DNA (In Press)*
- *Vineeth TV Kumar, David Holthausen, Joshy Jacob and Sanil George* 2015. Host Defense Peptides in Asian Frogs as Potential Clinical Therapies. *Antibiotics 2015, 4, 136-159; doi: 10.3390/antibiotics4020136*
- *Bijukumar A, R. Smrithy 2, U. Sureshkumar 3 & S. George* 2015. Invasion of South American suckermouth armoured catfishes Pterygoplichthys spp., (Loricariidae) in Kerala, India- a case study. *Journal of Threatened Taxa 7(3): 6987–6995. (DOI: http://dx.doi.org/10.11609/JoTT.o4133.6987-95)*
- *Parvin Abraham, Anand Sundaram, Asha R, Reshmy V, Sanil George, K.Santhosh Kumar* 2015. Structure-Activity Relationship and Mode of Action of a Frog Secreted Antibacterial Peptide B1CTcu5 Using Synthetically and Modularly Modified or Deleted (SMMD) Peptides. *PLoS ONE 10(5): e0124210. doi: 10.1371/journal.pone.0124210. IF: 3.534*

CHEMICAL BIOLOGY PROGRAM
ENVIRONMENTAL BIOLOGY LABORATORY

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Geetha. S. L (Project Assistant)

In-vitro characterization of purified recombinant L-Asparaginase II from soil metagenomic BAC library

Arjun. J.K and Hari Krishnan. K

Asparaginase producing clones were screened out form the metagenomic library generated from forest soil. Among them, the clone ASP-1 showing the highest enzymatic activity was further characterised. L-Asparaginase II gene present in the BAC clone ASP1 was sub cloned in pET 22b(+) expression vector and the recombinant protein was purified. The specific activity of expressed L-Asparaginase increased from 149 IU/mg to 696.03 IU/mg after Ni-NTA column purification, showing approximately five-fold increase in activity. The activity was found to be higher when compared with the commercially available *E. coli* L-Asparaginase which is 200 IU/mg, proving that the purified recombinant enzyme is of good quality. Preliminary studies on the antitumor activity of the recombinant L-Asparaginase was carried out by MTT assay with SKBr-3 human breast cancer cell lines. Monolayer culture of SKBR-3 cell lines were grown in RPMI-1640 media supplemented with 10% heat inactivated FBS (Gibco) 2% antimycotic antibacterial solution (Gibco) and recombinant L-Asparaginase at concentrations 6.25 µg/ml to 200 µg/ml. The control cultures were supplemented with regular media only. Cells were maintained at fully humid condition at 37°C and at 5% CO_2. After the assay the absorbance at 595nm was measured and the cell viability at different asparaginase concentration was plotted and the IC50 value of the drug was calculated. The IC50 value of the over expressed and partially purified L-Asparaginase was found to be 12.5 µg/ml (Fig: 1) which is comparable with the IC 50 values of *E. coli* and *Erwinia* L-Asparaginase commercially available in the market.

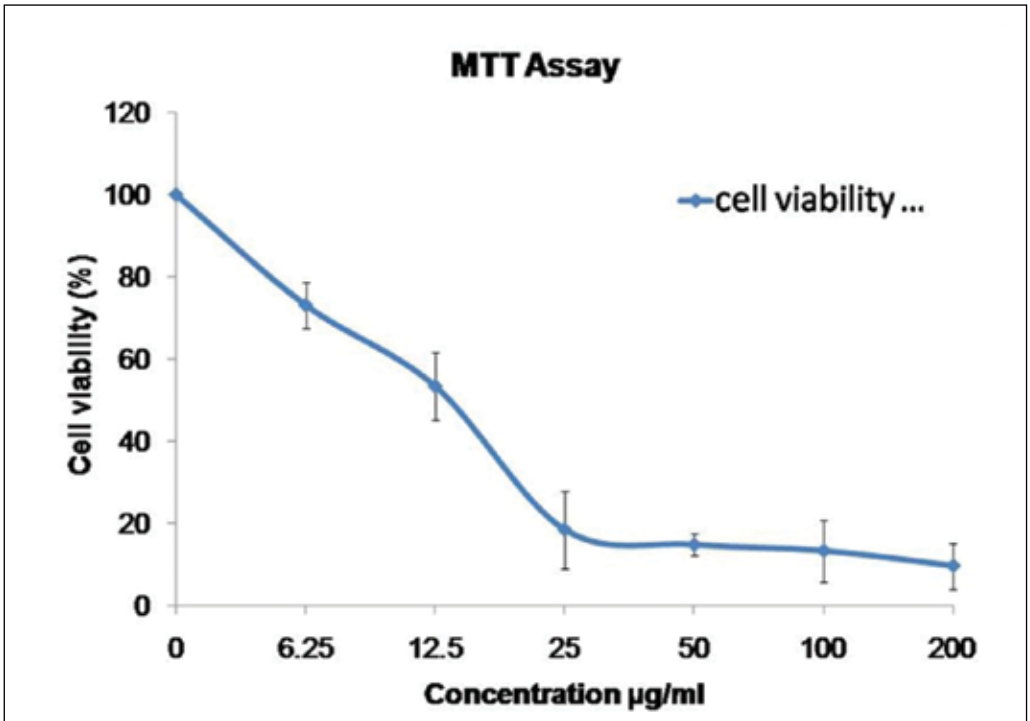

Fig: 1 Cell viability assay of recombinant L-Asparaginase on SKBR-3 cell line

The effect of the enzyme on tumor cell lines (SKBr-3) were checked after treating them with different concentrations of recombinant L-Asparaginase and the commercially available *E. coli* L-Asparaginase as positive control by confocal microscopy using DAPI filter after staining with Hoechst 33242 and photographed (Fig 2). The cells showed typical apoptotic properties, including cell shrinkage, chromatin condensation, and loss of normal nuclear architecture.

The characteristics of the recombinant protein were analyzed using various bioinformatics tools. The secondary structure of the protein was predicted using SOPMA program and found that the amino acids are distributed in alpha helix (35.06 %), beta turn (5.46 %), in random coil (39.37%) and extended strands (20.11 %). The homology model of recombinant protein was analyzed using the Modeller software (Fig: 3). The predicted structure of recombinant L-Asparaginase was superimposed with the L-Asparaginase structure of *Serratia* and found that the recombinant showed 84% homology with L-Asparaginase of *Serratia*.

The ligand protein binding efficiency was predicted by AutoDock software and found that the recombinant protein has better binding efficiency with L-Asparagine and can be inferred that the L-Asparaginase obtained from metagenomic library might be a novel, reliable and efficient candidate for anticancer treatment. The potential antigenic regions in the recombinant protein was predicted following the semi-empirical method

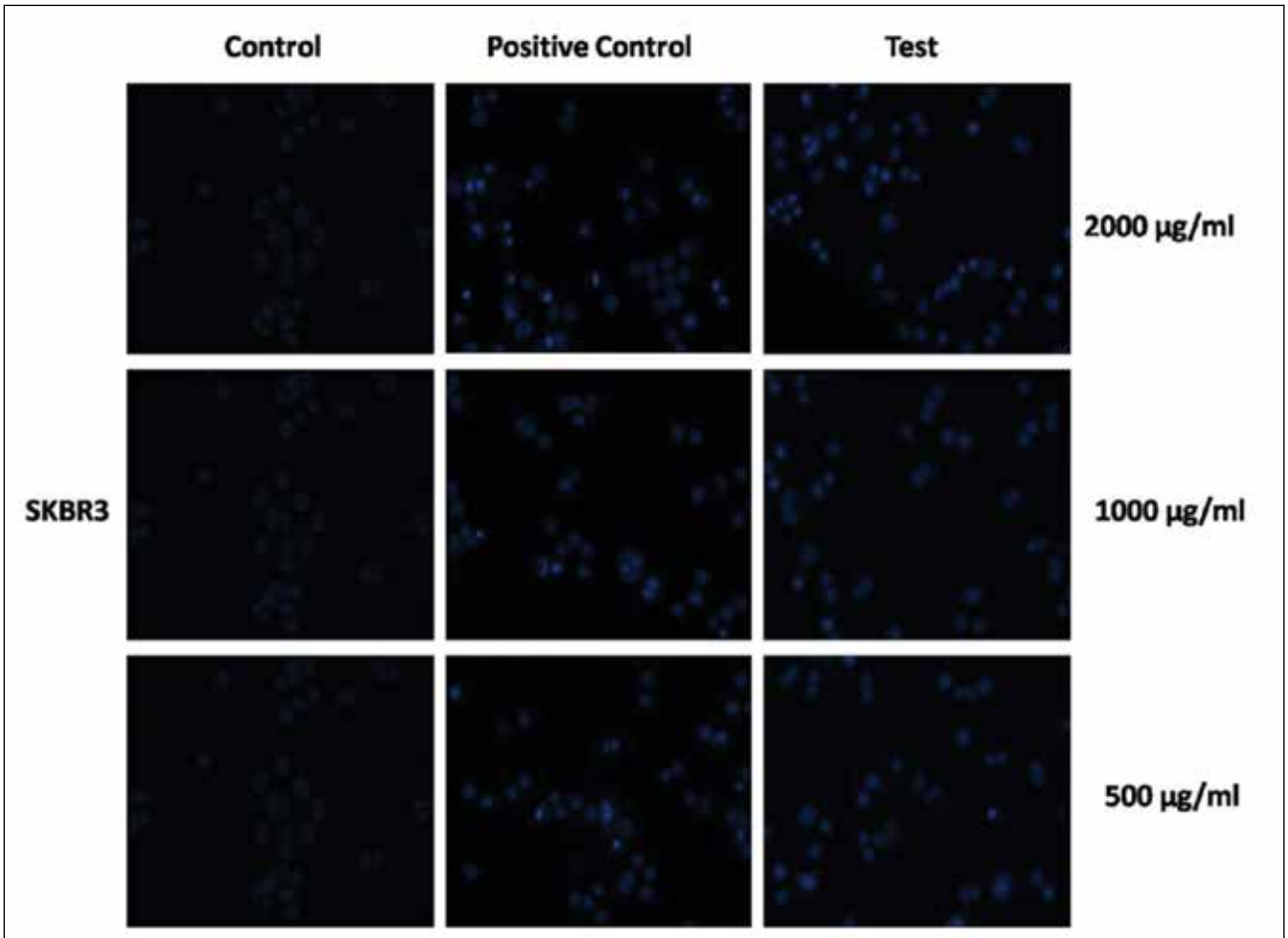

Fig: 2 Confocal micrograph - SKBR-3 cells treated with recombinant L- Asparaginase; Positive control- Native *E. coli* L- Asparaginase; Test- recombinant L-Asparaginase

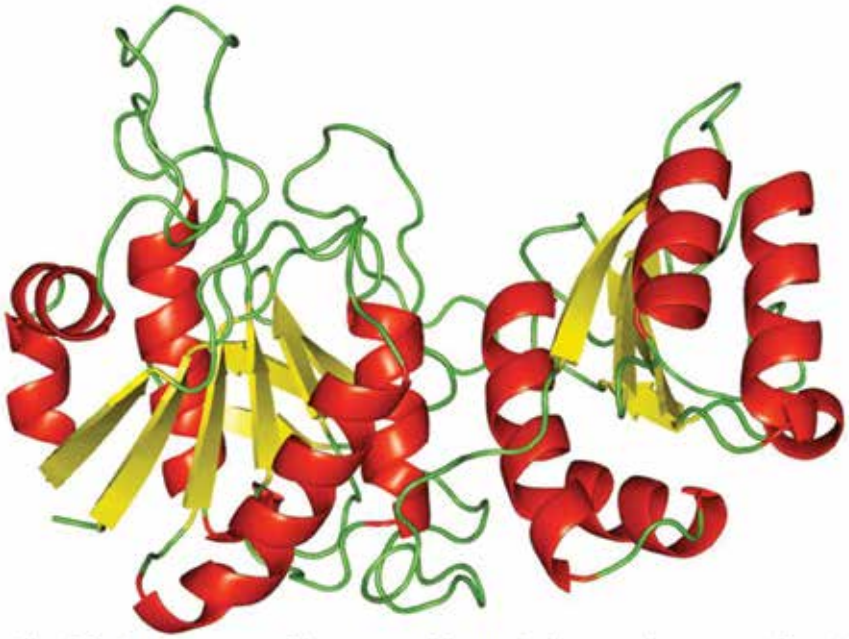

Fig: 3 3-D structure of the recombinant L-Asparaginase predicted using Modeller program

(IEDB analysis tools) and compared with other commercially available drugs in the market (Fig: 4). The average antigenicity of the recombinant L-Asparaginase (score 1.026) was found to be more or less similar to that of commercially available L-Asparaginases. The theoretical isoelectric point of the recombinant protein and the molecular weight of the protein monomer was predicted using ExPasy tool and were found to be pI 8.18 and 36kDa respectively. The protein parameters were analyzed using ProtParam software and found that the protein instability index was computed as 32.98, which classifies the protein as stable.

The findings demonstrate that uncultivable bacterial resources are a powerful source of novel therapeutic enzymes and to explore them through metagenomic tools will provide means to develop improved drug preparations.

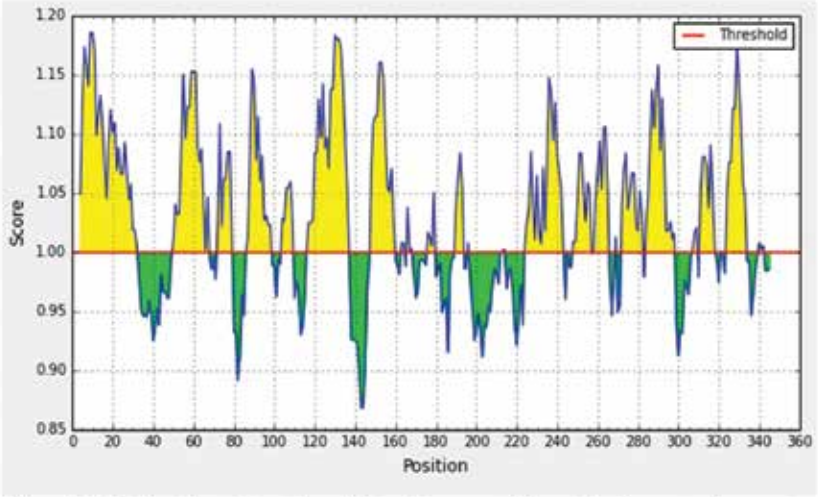

Fig: 4 Distribution of antigenicity of recombinant L-Asparaginase at different amino acid positions predicted by semi-empirical method

Validation of soil metagenomic DNA libraries from forest top soil of Western Ghats, Kerala

Arjun. J.K and Hari Krishnan. K

Culturable bacteria isolated from various environments have traditionally been one of the most productive sources of structurally unique and biologically interesting natural products. Culture-dependent approach for the discovery of natural products approach has likely missed the vast majority of naturally occurring bacterial metabolites since many of them are reluctant to grow in laboratory conditions. To access the unculturable fraction of microbes, the culture independent metagenomic method is appropriate, which solely relies on the genetic information stored in the collective DNA of all microorganisms. During the study, metagenomic libraries were generated from the DNA isolated from the forest top soil. The clones in the libraries were subjected to functional screening to elucidate the biochemical diversity of the libraries (Table: 1). The libraries contained approximately 720Mbp of metagenomic DNA equivalent to 360 bacterial genomes, assuming 2Mbp per bacterial genome. The functional screening of the libraries revealed that the metagenomic DNA in the libraries harbours the genes of various industrially and therapeutically important enzymes.

In order to evaluate the genetic diversity in the BAC metagenomic library, 16S rRNA clone libraries were generated from the pooled DNA of randomly selected clones in the BAC library (n=75). The clones were subjected to colony PCR and RFLP analysis. Based on the RFLP banding pattern the clones were grouped into 14 bacterial groups, subjected to 16S rRNA sequencing and the sequences were compared with the public database (NCBI) for homology analysis. The bacteria belonged to the eight taxonomical groups and the majority (45%) was represented by *Proteobacteria,* which is the dominant bacterial group present in the soil (Fig: 5). The members of *Bacteriodetes* were also well represented in the library which is generally considered as a culture reluctant group in the soil microbial community. The validation of the library clearly shows that the constructed soil metagenomic library possesses a rich bacterial diversity having rich metabolic potential and demonstrates the feasibility of the metagenomic approach to unveil the uncultivable bacterial diversity in the environment.

Table: 1 Functional screening of soil metagenomic libraries

Library Source	Vector	Host	Number of clones	Average insert size (kb)	Library size (kb)	Functional screening	
						Biological activity (Positive clones)	Detection method
Forest soil	pUC 18	Mach 1	3680	5	1.8×10^{4}	Amylase (1) Cellulase (2)	Plate diffusion
Forest soil	BAC	EPI300	1368	35	4.8×10^{4}	Asparaginase (5) DNase (10) Lipase (8)	Plate diffusion
Forest soil	pGEM-3Z	JM 109	2250	2.5	5.6×10^{3}	Nil	Plate diffusion

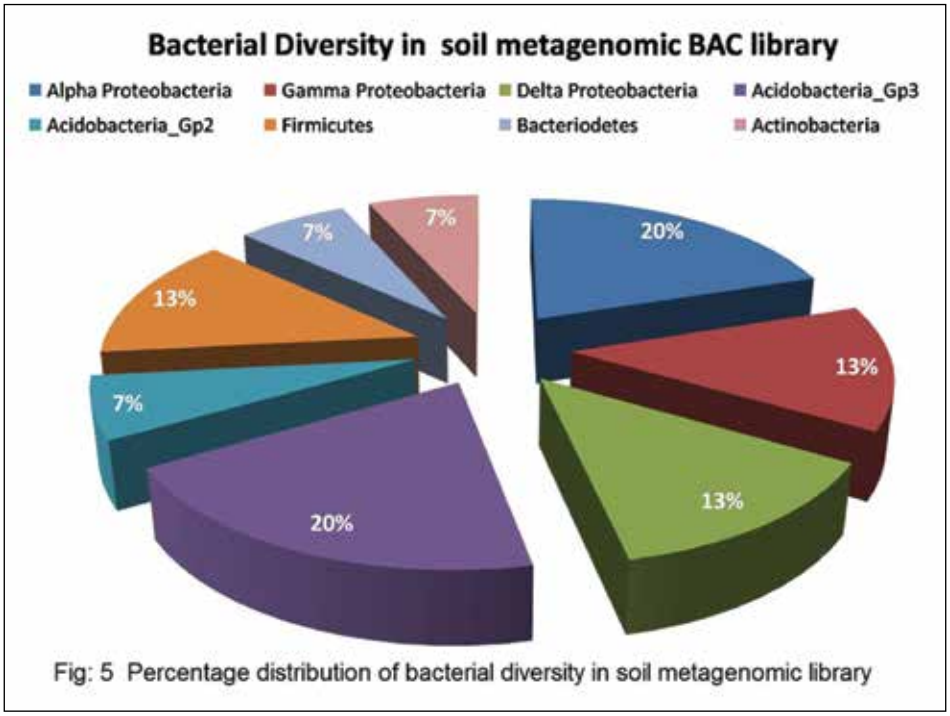

Fig: 5 Percentage distribution of bacterial diversity in soil metagenomic library

Heterologous expression of polyhydroxybutyrate biosynthesis genes and the biopolymer production in Escherichia coli

Aneesh, B and Hari Krishnan. K

Polyhydroxyalkanoates (PHAs) are bacterial storage compounds for carbon and energy. These polymers are accumulated with in cytoplasm as inclusions when bacterial cells are grown in an unbalanced nutrient supply. PHAs are thermoplastics which are biocompatible, biodegradable, hydrophobic materials with high molecular weight ranging from 20,000 - 30 million Daltons. Polyhydroxybutyrate (PHB) is a short-chain-length PHA with wide range of applications such as manufacturing of packaging materials, biomedical implant materials, drug delivery carriers, etc. Even though PHBs have properties comparable to petroleum derived plastics, commercial exploitation of these bacterial polymers is limited due to the high cost of production. Cost of raw materials, maintenance of fermentation conditions, self-utilization of accumulated polymer by the bacterial cells and complications in polymer recovery are the main factors that make biopolymer production non-economical. Utilisation of recombinant *E. coli* for PHB production is a reliable, alternative approach to minimize the polymer production cost, as these bacterial cells can be cultured at high cell density utilizing a wide range of cheap carbon sources, high temperature optimum for growth, minimise the degradation of accumulated polymer due to the absence of PHB catabolic genes and have comparatively fragile cell wall for easier release of the polymer. Our study focussed on the development of a recombinant *E. coli* system for the economical production of PHB. PHB accumulating bacteria were screened out from various environmental conditions and a high yielding *Bacillus* sp. was selected for cloning. PHB biosynthetic gene cluster was PCR amplified from the genomic DNA of this

bacterium and the individual genes were identified and characterised. These gene fragments were ligated with plasmid vector and transformed into *E. coli*. A recombinant bacterium harbouring the genes of interest was screened out from the clone library and was used for heterologous expression of the genes and polymer production. Recombinant *E. coli* cells were stained with Nile Red and the

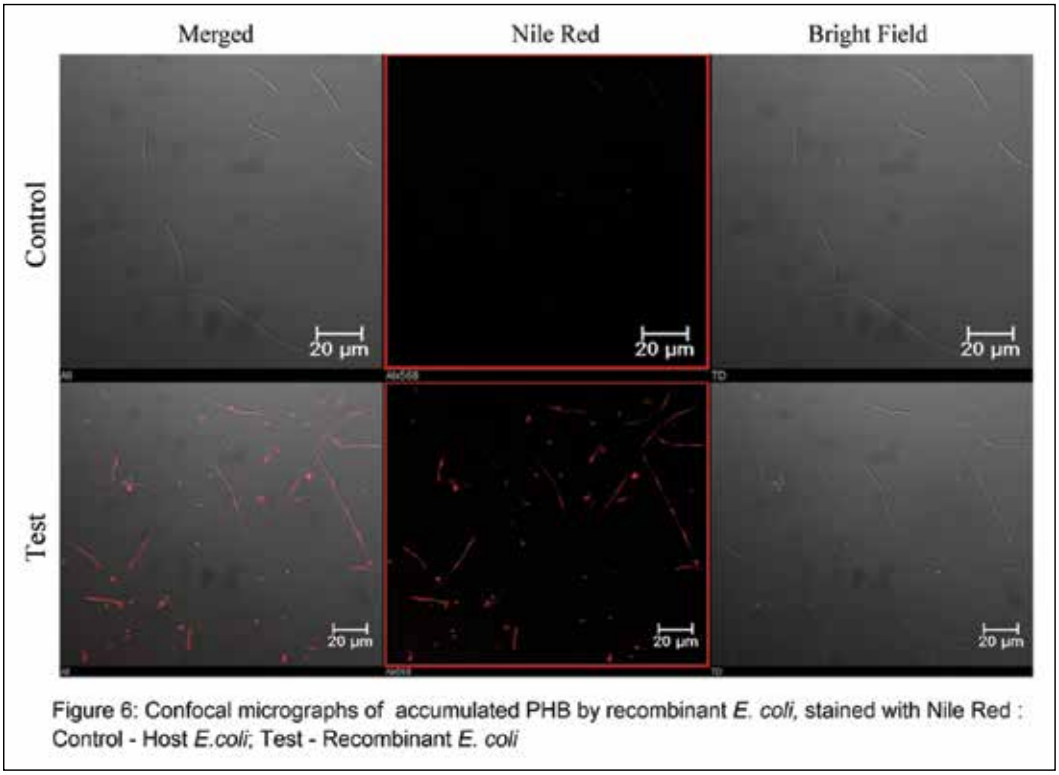

Figure 6: Confocal micrographs of accumulated PHB by recombinant *E. coli*, stained with Nile Red : Control - Host *E.coli*; Test - Recombinant *E. coli*

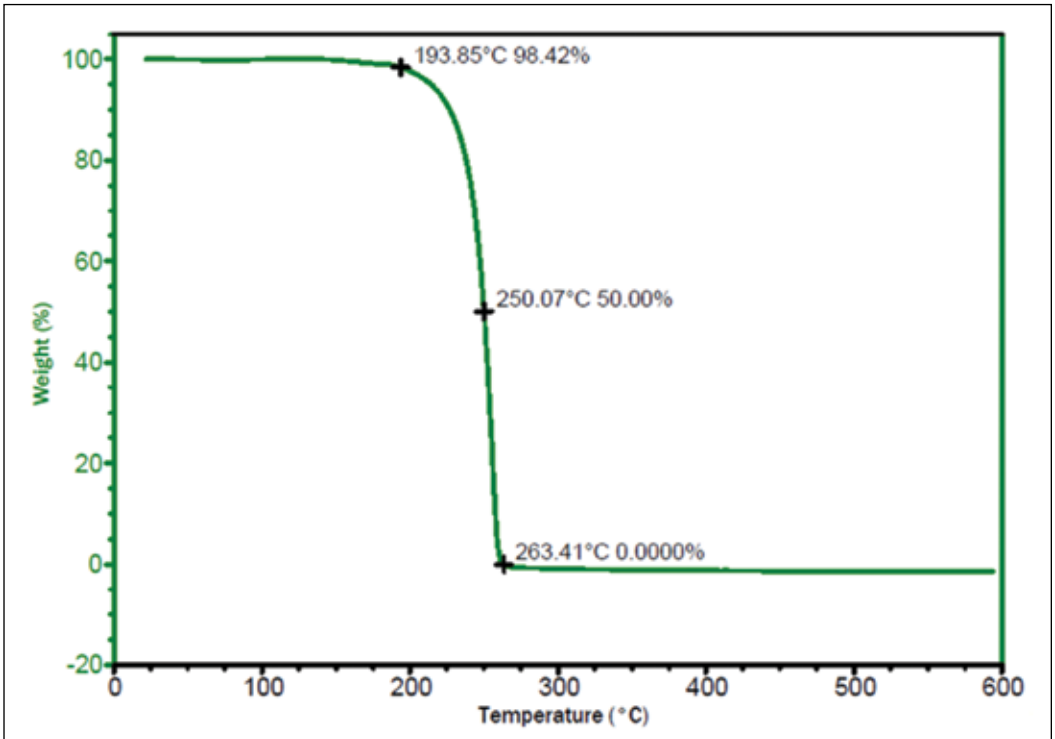

Figure 7: Thermogravimetric analysis of PHB produced by recombinant *E. coli*

PHB granules were visualised under a confocal microscope (Fig 6).

The polymer extracted from the cultured cells was confirmed as PHB through Fourier transform infrared spectroscopy (FTIR) and C^{13} Nuclear Magnetic Resonance (NMR) analyses. Average molecular weight of the polymer was analysed by Gel Permeation Chromatography (GPC) and was found to be 78947 Da with a polydispersity index of 1.81. Differential Scanning Calorimetry (DSC) analysis revealed the stability of the polymer between 0-150°C and the melting point of the polymer as 170°C. Thermal degradation study of the polymer was conducted using Thermogravimetric analysis (TGA) and was observed that thermal degradation starts at around 193°C and complete loss of original mass was occurred at 263°C (Fig 7). The PHB production level was found to be 42% of the dry cell mass with the recombinant bacterial system using glucose as carbon source. The results indicate that the PHB obtained from the heterologous expression system is of good quality and is comparable to wild strains. Further studies to optimise media and other parameters to increase the PHB production are progressing.

Molecular phylogenetic analysis of methanotrophic bacteria from the Kuttanad wetland ecosystem, Kerala

Kavitha, T and Hari Krishnan. K

Methane is one of the significant green house gases present in the atmosphere responsible for global warming. Wetlands are considered as the major natural source where the rate of production of methane is comparatively high. Kuttanad is the largest wetland in Kerala and a very few reports are available regarding the diversity of methanotrophs in this ecosystem. Methanotrophs are capable of utilizing methane and other one carbon compounds as the sole source of carbon and energy. Hence they aid in control of methane emission through biological oxidation along with other chemical oxidation processes. The genes coding for the enzymes methane monooxygenase and methanol dehydrogenase are generally used for the studies on the diversity of methanotrophs. During the study, metagenomic libraries were generated from the DNA isolated from the Kuttanad wetland soil, based on the primers designed for the amplification of the genes 16S rRNA, methanol dehydrogenase (*mxaF*) and methane monooxygenase (*pmoA*). The clones in the library were sequenced and subjected to homology search in the public database. The sequences from the 16S rRNA library revealed that the bacterial community belonged to the taxa *Acidobacteria*, *Chloroflexi, Firmicutes, Planctomycetes Proteobacteria* and *Verrucomicrobia.* Among them the methanotrophs constitute only 18% of the total population and were scattered among the prominent taxon *Proteobacteria* and the minor taxon *Verrucomicrobia.* (Fig.8). The taxon *Proteobacteria* dominate the wetland followed by *Acidobacteria*. The four classes of the group Proteobacteria, alpha, beta, gamma and delta Proteobacteria together constituted 50% of the population and represented by the genera *Methylobacter, Thiobacillus, Thiomonas, Leeia, Sphingomonas* and *Geobacter*.

The phylogenetic analysis of clones carrying *mxaF* revealed that majority of them belonged to the genus *Methylobacter.* The sequences from the clones carrying the gene *pmoA* revealed that 50% of them belonged to the genus *Methylomicrobium,* 40% belonged to *Methylocystis* and the remaining 10% designated as *uncultured bacterium.* A phylogentic tree was generated following the maximum

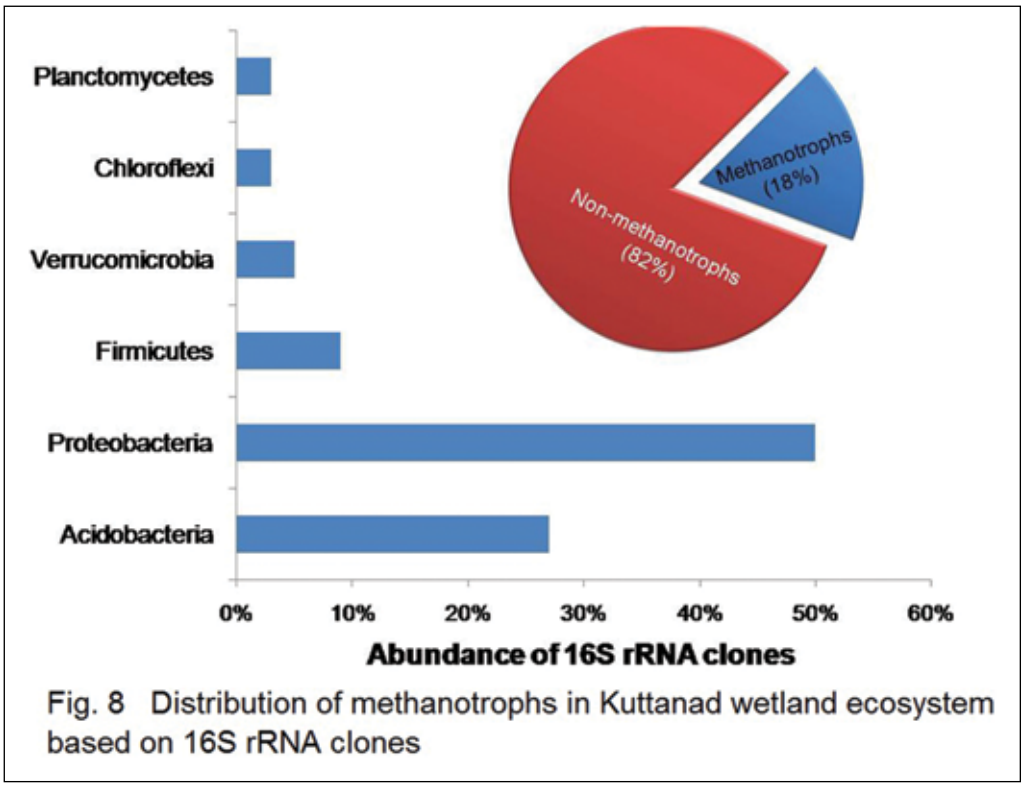

Fig. 8 Distribution of methanotrophs in Kuttanad wetland ecosystem based on 16S rRNA clones

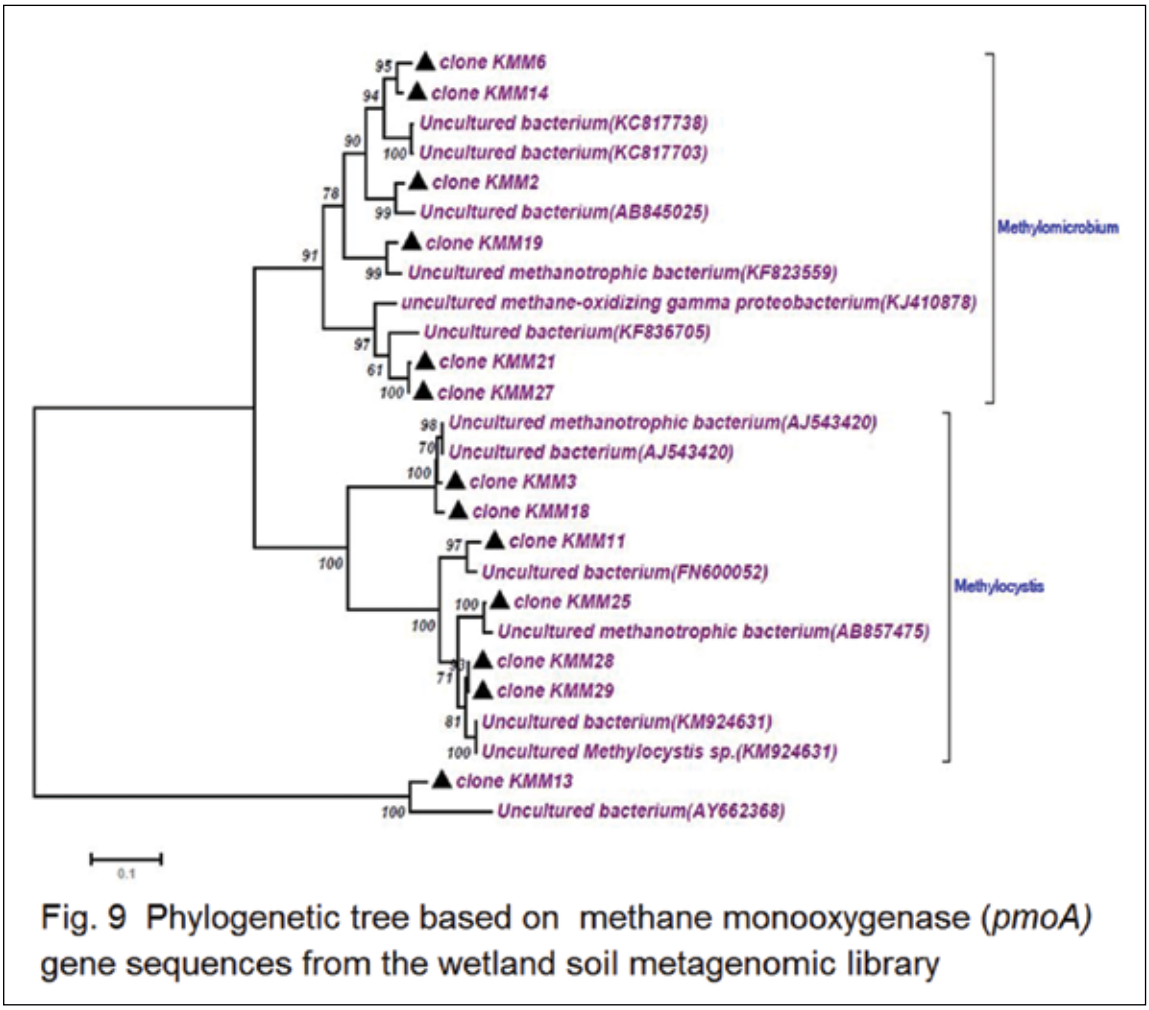

Fig. 9 Phylogenetic tree based on methane monooxygenase (*pmoA*) gene sequences from the wetland soil metagenomic library

likelihood method to analyse the diversity of *pmoA* gene in the wetland (Fig 9). Four methanol utilizing strains were isolated from this wetland through culture based methods. They were characterized by biochemical and molecular methods and identified as *Achromobacter spanius, Acinetobacter* sp., *Bacillus novalis* and *Methylobacterium radiotolerans.* The gene, *mxaF* was partially amplified from the genomic DNA of all these isolates and sequenced. It has been reported that methanotrophs can utilize methanol also, the first oxidized form of methane, as a carbon source. Further studies are progressing to elucidate the bioremediation potential of these microorganisms in wetland ecosystems.

Metagenomic analysis of gut microbiome from type 2 diabetic subjects in Kerala

Silju Juby and Hari Krishnan. K

The human gastrointestinal tract harbours a complex microbial ecosystem. Accumulating evidence shows that human gut microbiota plays intricate and key roles in our health and disease. Assessment and characterization of gut microbiota has become a major research area in human disease, including type 2 diabetes (T2D), the most prevalent endocrine disease worldwide. This study is a preliminary attempt to characterize the diversity of the human gut microbiome in type 2 diabetic population in Kerala. Faecal samples from male and female age matched subjects (T2D, non diabetic and prediabetic) were intended for the analysis and the studies were initiated with the samples collected from four individuals. Since majority of the gut bacteria are resistant to cultivation, we have followed the culture independent metagenomic approach for the study. Metagenomic DNA from faecal samples was isolated using commercially available isolation kits. The bacterial diversity in the samples were analysed by 16S rRNA gene sequencing. The 16S rRNA from the stool metagenomic DNA were amplified using universal primers for eubacteria and archaea and the amplicons were cloned and generated metagenomic libraries. The amplicons from the positive recombinants were subjected to Restriction Fragment Length Polymorphism (RFLP) analysis, the clones with similar banding patterns were grouped together and one representative clone from each group was sequenced, aligned, edited and compared with the public data base. The sequences showing maximum homology were taxonomically identified using online RDP (Ribosomal Database Project) classifier. One stool sample was analysed by qPCR using microbial identification qPCR array kit for metabolic disorders (QIAGEN, USA), which is a tool used for screening of bacteria from gut derived samples associated with obesity, T2D and metabolic syndrome. Altogether 64 species of bacteria were identified from the four samples through 16S rRNA clone library sequencing and qPCR array analysis. The study revealed that, the gut microbiota is dominated by 2 phyla, the gram-positive Firmicutes and the gram-negative Bacteroidetes. The other groups represented at subdominant levels are Actinobacteria, Proteobacteria and Verrucomicrobia (Figs. 10 & 11). The qPCR array analysis for sample 2 alone revealed 21 organisms and both the methods, 16S rRNA sequencing and qPCR together for sample-2 revealed the representation of 38 species in the sample.

The analysis of the remaining samples and the comparison on the gut bacterial diversity profile between T2D, non-Diabetic and prediabetic subjects will provide insights into the difference in the gut microbial ecology among them. Further processing of the remaining samples and their analysis are progressing.

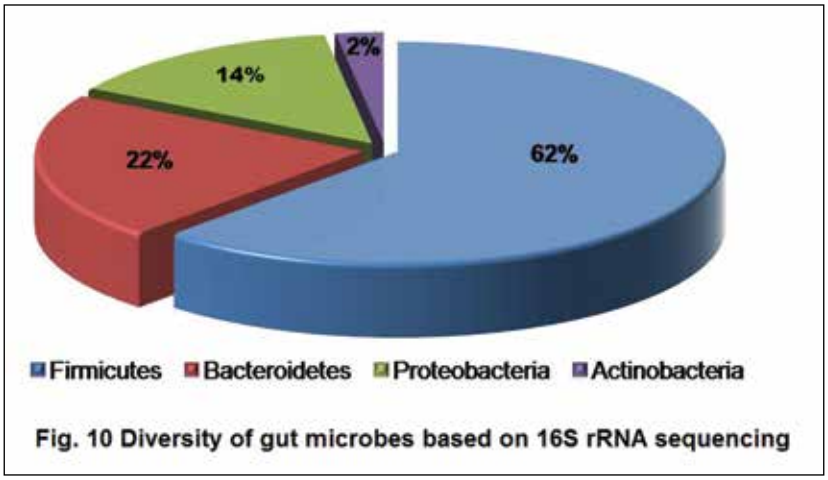

Fig. 10 Diversity of gut microbes based on 16S rRNA sequencing

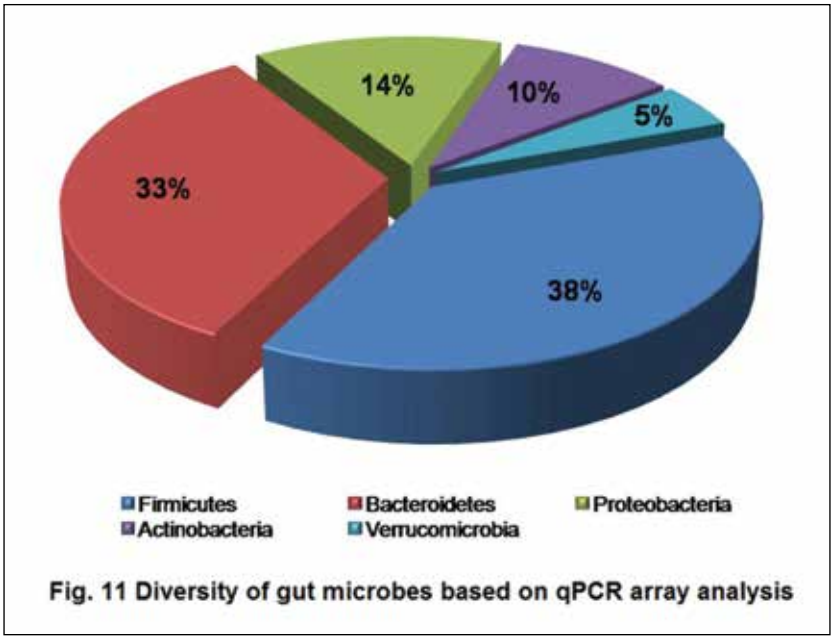

Fig. 11 Diversity of gut microbes based on qPCR array analysis

PUBLICATIONS

- *Arjun J.K, Aneesh B and Hari Krishnan K.* 2015. Sequencing and characterization of L-asparaginase (ansB) gene of *Bacillus megaterium* isolated from Western Ghats, Kerala, India " *Int.J.Curr.Microbiol.App.Sci. 4(6): 753-760*
- *Hari Krishnan K,* 2015. Application of metagenomics in agriculture. In: *Agriculture Bioinformatics (eds) Keshavchandran, R and Raji Radhakrishnan, S. Published by New India Publishing Agency, New Delhi. Pp 295 -318*

GENBANK SUBMISSIONS

- *Arjun J.K., Aneesh B., Kavitha.T. and Hari Krishnan, K.* Periplasmic L-asparaginase (ansB) gene, complete cds of *Bacillus megaterium* strain PHB29 (Acc. No. KP768443) and *Bacillus* sp. TVS 55 (Acc. No. KP720593)(2015)
- *Aneesh B. and Hari Krishnan K.* 16 S ribosomal RNA gene sequences of *Bacillus cereus* strain PHB3 (Acc. No. KP241851) (2015)
- *Kavitha T, Arjun J K, Aneesh B, Silju Juby and Hari Krishnan K.* 16 S ribosomal RNA gene sequences methanol oxidising bacteria from Kuttanad wetland (Acc. Nos. KT025237, KT025238, KT025239, KT025240, KT025241, KT025242, KT025243) (2015)

CONFERENCE PRESENTATIONS

- *Arjun J.K. and Harikrishnan K* (2014). Production and characterization of L-Asparaginase by *Stenotrophomonas rhizophila*, a rhizosphere associated bacteria. *55th An-*

nual conference of Association of Microbiology of India, National conference on Empowering Mankind with Microbial technologies, 12-14 November, 2014, TNAU, Coimbatore, India. (Poster presentation)

- *Aneesh B. and Harikrishnan K* (2014). Cloning, characterization and heterologous expression of κamylase gene from *Bacillus megaterium* isolated from Western Ghats, Kerala. *55th Annual conference of Association of Microbiology of India, National conference on Empowering Mankind with Microbial technologies, 12-14 November, 2014, TNAU, Coimbatore, India. (Poster presentation)*
- *Kavitha T. and Harikrishnan K* (2014). Diversity of methanogenic Archaea in Kuttanad wetland ecosystem, Kerala. *55th Annual conference of Association of Microbiology of India, National conference on Empowering Mankind with Microbial technologies, 12-14 November, 2014, TNAU, Coimbatore, India. (Poster presentation)*
- *Silju Juby and Harikrishnan K* (2014). Metagenomic analysis of human gut microbiome -a preliminary study. *55th Annual conference of Association of Microbiology of India, National conference on Empowering Mankind with Microbial technologies, 12-14 November, 2014, TNAU, Coimbatore, India. (Poster presentation)*

AWARDS, HONORS, ETC

- Hari Krishnan. K has been nominated as a member of the State Level Expert Appraisal Committee (SEAC), Kerala by the Ministry of Environment and Forest, Govt of India

EXTRA MURAL GRANTS

Title	Investigator(s)	Funding Agency	Duration
Studies on the distribution and diversity of methanotrophic microbial communities, mitigating methane emission in Kuttanad wetland ecosystem, Kerala	Hari Krishnan. K (PI) G.S. Vinod Kumar (Co-PI)	Department of Environment and Climate Change, Government of Kerala	2013 - 16
Metagenomic analysis of gut microbiome from type 2 diabetic subjects in Kerala - a preliminary study	Hari Krishnan. K (PI) Abdul Jaleel. K.A (Co-PI)	Kerala State Council for Science,Technology & Enironment	2014 - 17

NEUROBIOLOGY PROGRAM

MOLECULAR NEUROBIOLOGY LABORATORY

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Synaptic Calcium Signaling and associated Biochemical Mechanisms

Archana, G. M., Ramya R. Prabhu., Mathew John, Lakshmi, K., Mayadevi M., and Omkumar R.V.

Several higher order brain functions share common molecular mechanisms. The diversity in the functional outcome at the *in vivo* level arises from variations in the spatiotemporal patterns of these fundamental molecular events. Calcium signalling at synapses plays a major role in the mechanisms underlying learning and memory as well as neurodegeneration. While regulated calcium influx is necessary for synaptic plasticity, a cellular event underlying learning and memory, excessive calcium influx into synapses causes excitotoxicity that leads to neuronal death in many neurodegenerative diseases. Molecules like calcium/calmodulin dependent protein kinase II (CaMKII) and N-methyl-D-aspartate receptors (NMDAR) are primary players in synaptic calcium signalling and has key roles in synaptic plasticity and excitotoxicity. We have earlier reported that binding of the GluN2B subunit of NMDAR to the T-site of CaMKII causes allosteric modulation of its catalysis as well as makes it resistant to dephosphorylation by phosphatases. These regulatory events are consistent with the bistable switch model involving CaMKII and protein phosphatase 1 (PP1) proposed as a molecular mechanism for memory. Subsequently, we have found that CaMKIIN-a, a natural inhibitor of CaMKII that binds to the T-site, also inhibits dephosphorylation of CaMKII. We have also identified and studied a mutant of CaMKII in which GluN2B binding does not cause catalytic modulation or inhibition of dephosphorylation. This suggests that both the GluN2B induced effects in CaMKII described above, might be resulting from the same structural changes. We are working on elucidating the mechanism of the GluN2B-mediated modulation of CaMKII using a combination of structural bioinformatics and mutagenesis. We are also investigating the physiological significance of this regulation. In a collaborative programme we are attempting to do computational modelling of the biochemical functions of CaMKII under different conditions. Results of the modelling experiments will be validated with further wetlab experiments. Binding interaction between CaMKII and NMDAR is regulated by the phosphorylation status of the GluN2B subunit. We have monitored the phosphorylation status of GluN2B in primary cortical neurons subsequent to different types of stimuli. There were changes in the phosphorylation level of GluN2B. The changes in levels of phosphorylation were found to be mediated by protein phosphatase 1. We are currently investigating whether the calcium signal mediated alterations in GluN2B phosphorylation levels happen *in vivo*. Administration *in vivo*, of agonists and antagonists of various calcium channels followed by biochemical and histochemical analysis of brain tissue, is being undertaken for this purpose. We have also been studying calcium signalling in cerebellar granule (CG) cells. Our data show that depolarisation of CG cells in culture by KCl treatment leads to a proteome profile that differs with that of CG cells *in vivo*. It is likely that KCl induced depolarisation would lead to activation of voltage sensitive ion channels including calcium channels. Mechanistic insights obtained from this *in vitro* system would help in designing experiments to understand regulation of proteins that are differentially expressed *in vivo*.

Novel Techniques for the assay of Ca^{2+} channels

Arunkumar R. C., Manthosh Kumar, Soumya Paul, Mayadevi M., and Omkumar R. V.

Calcium channel inhibitors are widely used as drugs in many disease conditions arising from impaired function of the channels. Since many of the currently used drugs targeting calcium channels have undesirable side effects or insufficient efficacy, search for new drugs directed towards calcium

channels is widely pursued. Discovery of novel channel modulators is critically dependent on the availability of a suitable assay for the activity of the channel. All the existing methods for calcium channel activity assay are both technically challenging as welechnically advances as wel as expensiveassayitions. Since all this extracts is being studied for its neuroprotective actpeaniml as expensive. We had earlier developed a new assay for calcium channels based on end point measurement that is comparatively simpler as well as inexpensive. The assay involves transfecting HEK-293 cells with a pair of proteins, GFP-CaMKII and GluN2B sequence motif, that interact upon intracellular calcium release. Their interaction results in a characteristic fluorescence pattern that acts as the signal for calcium. We now have developed a stable clone of HEK-293 cells, which we termed as Calcium Sensor cell line, that constitutively expresses GFP-CaMKII and the GluN2B sequence motif. This further simplifies the assay method by eliminating the transfection step for introducing the calcium sensing proteins. The Calcium sensor cell line showed a sigmoidal relationship between the concentration of calcium added to the medium and the signal measured as the number of responding cells. Our experiments suggest that such quantitative relationship seems to arise from the heterogeneity among cells with respect to their calcium sensitivity. Calcium channels such as P2X2 receptor and TRPV1 receptor were successfully subjected to activity assays using this cell line. Further, the P2X2 channel was stably integrated into the calcium sensor cell line using a second antibiotic selection marker thereby generating a cell line that can be used as an exclusive P2X2 assay system without any further transfection step. Single cell derived clones of the calcium sensor cell line were generated. However, the clones still exhibited heterogeneity among cells in the expression level of GFP fluorescence.

Bioprospecting for Neuroprotectants

Mantosh Kumar,Soumya Paul, Arun kumar, R. C., Mayadevi, M., and Omkumar, R. V.

We have been involved in screening extracts from plants that are described to have medicinal value in traditional medicine. Their effects are being tested on cellular targets such as CaMKII, calcium channels, etc. for which assays have been developed in our lab. We have found that certain plant extracts as well as curcumin inhibit CaMKII activity. By screening for NMDAR inhibitory activity using the calcium sensing methodology developed in our laboratory we have found a plant extract that inhibits NMDAR ectopically expressed in HEK-293 cells. The extract also inhibits glutamate/NMDA induced excitotoxicity in primary cortical neurons. Currently this extract is being characterised for its neuroprotective activity in a rat excitotoxicity model using behavioural assays. Our data shows that administration of the plant extract to rats subjected to excitotoxic treatment ameliorates their impaired performance in Morris water maze test. The extract is also being subjected to subfractionation towards identifying the active compound/s.

PUBLICATIONS

- Ramasarma, T., Rao, A. V., Devi, M. M., Omkumar, R. V., Bhagyashree, K. S., Bhat, S. V. (2015) New insights of superoxide dismutase inhibition of pyrogallol autoxidation. ***Mol Cell Biochem.*** 400, p 277-85

CONFERENCE PRESENTATIONS

- John Mathew, Arunkumar, R.C, Mayadevi, M and Omkumar, R.V. Effect of tat CN21 peptide on CaMKII-GluN2B interaction in HEK-293 cells, Poster at the XXXII Annual Conference of Indian Academy of Neurosciences, November 1-3, 2014, at the National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore

- Mantosh Kumar, Paul S, Mayadevi M, and Omkumar RV. Effects of an NMDAR inhibitory plant extract on behavioural aspects of spatial learning and memory, Poster at the XXXII Annual Conference of Indian Academy of Neurosciences, November 1-3, 2014, at the National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore
- Lakshmi K., Archana G M., Ramya Prabhu R., Ani Das V., Mayadevi M and Omkumar R V. Characterisation of H282A mutant of a-CaMKII by pull-down assay, Poster at the XXXII Annual Conference of Indian Academy of Neurosciences, November 1-3, 2014, at the National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore

PATENTS

- Title of Invention: Assay for detection of transient intracellular Ca^{2+} Inventor's Names: Omkumar R. V., Rajeevkumar R., Mathew Steephan, Mayadevi M. and Suma Priya S. Granting Agency: Indian Patent Office Status: **Patent granted** No. 260367; Date of grant: 28/04/2014.
- Title of Invention: A method of detecting and quantifying the calcium conductingactivity of calcium channel proteins
- Inventor's Names: Omkumar, R. V., Mathew Steephan, Soumya Paul, Arunkumar R. C., Mayadevi M.
- Indian Patent Application No.: 2331/CHE/2013; Date of filing: 29-11-2014
- Title of Invention: A process for the preparation of plant extract that can inhibit a neuronal ligand-gated calcium channel
- Inventor's Names: Omkumar R. V., Soumya Paul and Mayadevi M.
- Indian Patent Application No.: 3616/CHE/2013, Date of filing: 16-02-2015

EXTRA MURAL FUNDING

Name of Grant	Funding Agency	Duration
Detailed state Model of CaMKII activation and autophosphorylation in the presence of NR2B and its behaviour in Epileptic conditions (As Co-investigator)	Kerala State Council for Science, Technology and Environment	2015-17

NEUROBIOLOGY PROGRAM
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Mayadevi received her M.Sc and PhD in Chemistry from the University of Kerala and worked at Case Westsrn Reserve University and Baylor Research Institute, USA, before joining RGCB in 1993.

Mechanism of dephosphorylation of CaMKII

Mayadevi M. and R. V. Omkumar

The activity dependent changes that causes the strengthening of the synapse leads to the formation of memory in the brain. Calcium/calmodulin dependent protein kinase II (CaMKII) is the neuronal enzyme that is considered as a memory molecule. CaMKII, autophosphorylated at T286, maintains calcium independent "on" state. Protein phosphatase I can dephosphorylate CaMKII. The kinase/ phosphatase system is considered as a molecular switch which models memory. The switch is influenced by the T-site binding proteins of CaMKII. GluN2B, the subunit of NMDAR and CaMKII inhibitor protein CaMKIINa can both bind to the T-site of CaMKII. We have investigated the functions, other than the inhibitory action of CaMKIINa. CaMKIINa inhibits dephosphorylation of CaMKII similar to GluN2B. Nevertheless, the mode of action of CaMKIINa appears different from that of GluN2B. CaMKIINa does not modulate the activity of CaMKII. A mutant of CaMKII which is not catalytically modulated by GluN2B does not show any change in the dephosphorylation rate. We propose that similar kind of GluN2B sensitive structural changes that takes place during catalysis may be happening during the dephosphorylation event.

PUBLICATIONS

- *T. Ramasarma, Aparna V. S. Rao, M. Maya Devi, R. V. Omkumar, K. S. Bhagyashree and S. V. Bhat.* (2015) New insights of superoxide dismutase inhibition of pyrogallol autoxidation *Mol Cell Biochem. 400(1-2):277-85*

NEUROBIOLOGY PROGRAM

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Evaluation of factors involved in axonal extension of retinal ganglion cells into brain visual centers

Abdul Rasheed VT, Lalitha S and Jackson James

MicroRNAs are a part of post-transcriptional regulatory mechanisms that control the gene expression and involves in the regulation of various cellular processes. Earlier studies from our lab have demonstrated the role of miR-23a and miR-374 in the regulation of *Brn3b* expression which is the major transcription factor for the fate specification of retinal ganglion cells (RGC) during early retinogenesis. These microRNAs are thought to have major role in the late retinal developmental stages also with respect to retinal axonal guidance. The process of retinal axonal guidance involves guiding the RGC axons from the peripheral retina to the optic disc (intra-retinal axonal guidance) to form the optic nerve, guiding towards the optic chiasm region where few retinal axons from both the eyes decide to cross, further guiding the axons towards the major brain visual centres of the brain, the lateral geniculate nucleus and the superior colliculus. This complex process involves various axonal guidance molecules and their expression in a specific gradient throughout the path where the retinal axons travel. To understand the specific factors those are unique and important for retinal histogenesis and axonal guidance, we have done a whole transcriptome comparison between E14 retina and E14 brain, the stage at which RGC differentiation and intra-retinal axonal guidance are in peak. We have classified differentially expressed genes based on specific functions like retina related, visual cortex related, neural progenitors related, eye-lens related, axonal pathfinding related (Fig.1A) etc., Among various axonal guidance factors analyzed from the microarray results, Robo-Slit family of proteins have a major role in the intra-retinal axonal guidance, especially *Robo1* and *Robo2*. *In silico* analysis shows that miR-23a has a single target site on *Robo1* and five sites on *Robo2*, whereas miR-374 has no sites on *Robo1* and a single site on *Robo2*. We have analysed the expression of *Robo1/Robo2* along with *Brn3b* at different retinal developmental stages but we couldn't find any significant correlation. Interestingly, the expression of miR-23a/miR-374 correlated well with the expression of *Robo1/Robo2*, (Fig.1B,C) indicates that these two microRNAs may have regulatory effects on *Robo1/Robo2* directly, rather than acting through *Brn3b*. Further *in vivo* studies need to be carried out to elucidate the role of these two microRNAs on various axonal guidance factors with respect to the retinal axonal guidance. In addition to the microRNAs we are also screening various transcription factors which could regulate the axonal guidance genes.

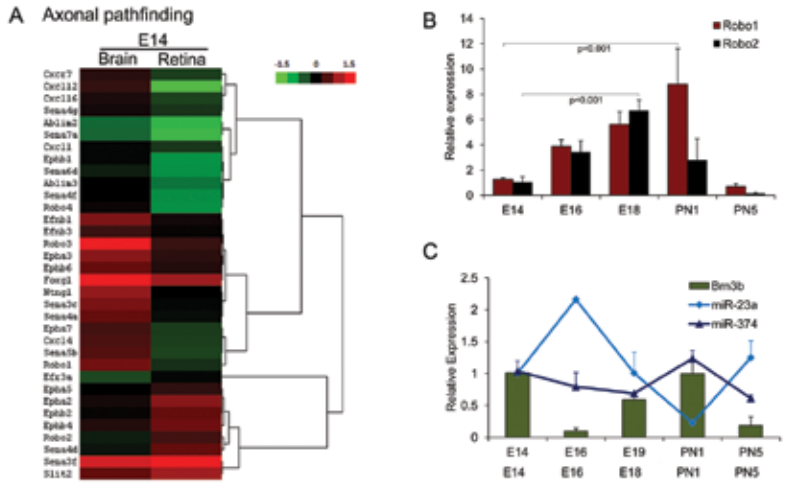

Figure-1. A. Heat map showing differentially expressed genes related to axonal pathfinding from the transcriptome analysis. **B.** Expression pattern of *Robo1* and *Robo2* at different retinal developmental stages. **C.** Temporal analysis of transcripts corresponding to *Brn3b*, miR-23a, miR-374 also exhibited differential expression pattern during retinal development.

Neural stem cells requires non-canonically activated Hes-1 for its maintenance and acquires canonical expression as they transit into a radial glial fate throughout the neocortical development

Dhanesh SB and Jackson James

***Collaborators**: Shubha Tole, TIFR, Mumbai; R V Omkumar, Molecular Neurobiology Laboratory, RGCB, Trivandrum; Santhosh Kumar Sankaran, Animal Research Facility, RGCB, Trivandrum*

Our laboratory had previously reported the presence of a novel Notch independent Hes-1(NIHes-1) expression in ES cell derived neural progenitors that is mediated through FGF2-JNK pathway (*Sanal et.al. J. Neurochem., 113, 2010, 807–818*). We also showed that these progenitors are present in the VZ of the developing neocortex along with Notch/CBF1 dependent Hes-1(NDHes-1) progenitors. In order to investigate whether these unique progenitors are directly differentiating into neuron and glia or are they transiting from NIHes-1 expressing state to NDHes-1 expressing state, we generated a dual reporter system, pCBFRE-mCherry-mtCBF1-EGFP (Fig: 2A) having separate expression cassettes to simultaneously monitor NDHes-1 progenitors which will be expressing mCherry and NIHes-1 expressing progenitors that will express EGFP. *In utero* electroporation with this dual construct in E14 brain and analysis after 1day post *in utero* electroporation (1DPI) revealed the existence of three distinct types of neural progenitors such as NIHes-1 expressing neural stem cells (EGFP^{+ve}, 21.19±5.38%), NDHes-1 progenitors (mCherry^{+ve}, 28.66±3.65%) and progenitors having both NIHes-1 and NDHes-1 expression (EGFP^{+ve} & mCherry^{+ve}, 50.13±9.03%, Fig.2B&C). To evaluate this further, we collected the embryonic brains that were *in utero* electroporated with the same construct after 2DPI. Interestingly, we observed an increase in the number of progenitors expressing both NIHes-1 and NDHes-1 (EGFP^{+ve} & mCherry^{+ve}, 86.33±1.96%). Whereas the number of NIHes-1 expressing neural stem cells and NDHes-1 expressing progenitors decreased decreased (EGFP^{+ve}, 6.29±0.94% and mCherry^{+ve}, 7.37±1.53%, Fig.2D&E). Therefore, from the above results it is clear that the NIHes-1 expressing neural stem cells do not readily migrate into the upper cortical layers but transit from NIHes-1 expressing state to NDHes-1 state. Further to investigate whether this transition is coupled with radial glial transformation by two days of electroporation, we electroporated both wild type Hes1-EGFP and mutated mtCBF1-Hes1-EGFP constructs at E14 and analyzed at E16 (2DPI). Observation of the sections showed that majority

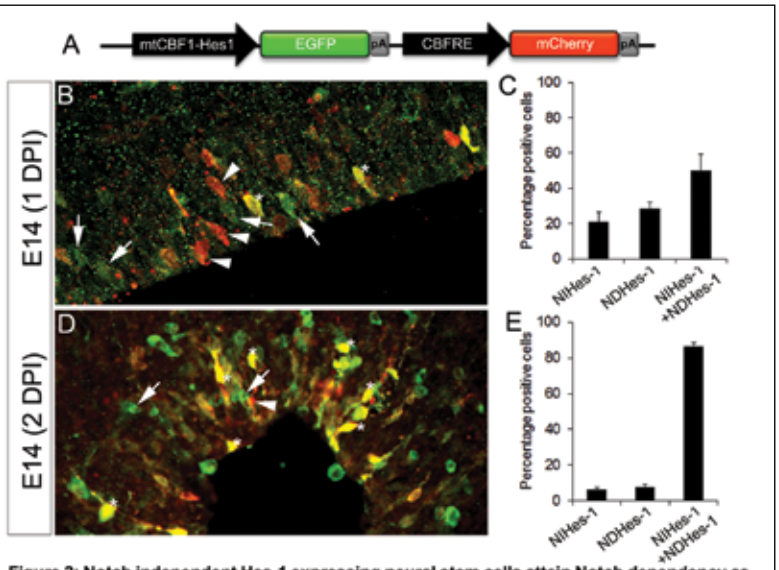

Figure 2: Notch independent Hes-1 expressing neural stem cells attain Notch dependency as development progress A) Schematic of dual fluorescent reporter construct, pCBFRE-mCherry-mtCBF1Hes1-EGFP. **B)** Confocal image of coronal cortical section of E15 brain that was *in utero* electroporated with the construct at E14 and analysed after 1DPI show EGFP+ve Notch independent Hes-1(NIHes-1) expressing progenitors (arrows), mCherry+ve Notch dependent Hes-1(NDHes-1) expressing cells (arrowheads) and yellow cells that express both or have transited from Notch independent to dependent state. **C)** Quantification of percentage of differentially distributed cells out of total transfected cells in the ventricular zone (VZ) of neocortex. **D)** Coronal cortical section of E16 brain that was *in utero* electroporated with same construct at E14 and analysed after 2DPI. **E)** Graph showing a significant increase in cells (NIHes-1+NDHes-1) that have transited from a NIHes-1 to NDHes-1 expressing state in the VZ.

of cell bodies of NDHes-1 expressing progenitors reflected typical morphological features of radial glial cells with short apical process that were in contact with inner lining of VZ and the basal process reaching the pial surface and had brighter EGFP expression. This was not the same with NIHes-1 expressing neural stem cells that had very short basal process with less intense EGFP expression compared to NDHes-1 expressing progenitors and these progenitors resides mostly in the apical surface near the ventricle despite very few cells migrated out of ventricular zone. Further in order to emphasis the fact that NIHes-1 expressing neural stem cells were not readily migrating into upper cortical layers we *in utero* electroporated the plasmid, pCAG-mCherry-mtCBF1-EGFP into the E14 embryonic brain and analysis at E15. Our results indicated that NIHes-1 expressing neural stem cells are not readily migrating into the SVZ but remain in the VZ. Whereas the progenitors expressing mCherry driven by CAG promoter readily migrates from the VZ to SVZ as development progresses.

Tlx3 induced by Pax6 is required for the restricted expression of Chrnα3 in developing cerebellum

Divya T S and Dr. Jackson James

***Collaborators**: ShubhaTole, TIFR, Mumbai*

Tlx3, a homeobox gene, is identified as a post-mitotic selector factor involved in glutamatergic neurogenesis and is found to be expressed in a subset of spinal neurons, brainstem and cerebellum. Previous studies conducted in our lab to identify the regulatory mechanism involving Tlx3 in neural fate specification have demonstrated that Hes-1, a Notch target gene act as a repressor of Tlx3 expression *(Indulekha et.al., Mol. Life. Sci, 2012, 69:611–627)*. Further studies to identify the upstream regulators of Tlx3 demonstrated that Pax6, a proneural gene which is found to have an evident role in glutamatergic neuron fate determination, positively regulate Tlx3 expression specifically in cerebellum. It was confirmed by analyzing the absence of Tlx3 expression in $Pax6^{-/-}$Sey mouse cerebellum. The major feature identified was the specificity in Tlx3 expression which is conferred to the granule neurons present in the posterior lobes of cerebellum. To identify the possible downstream targets activated by Tlx3 in posterior cerebellum, both anterior and posterior lobes were dissected out separately and microarray analysis was carried out (Fig. 3A). Analysis of the differentially expressed gene set (Fig. 3B) showed that nicotinic cholinergic receptor α3 subunit (Chrnα3) was up-regulated in posterior lobes which were further confirmed by RT PCR analysis of anterior and posterior cerebellum samples. Expression pattern analysis of Tlx3 and Chrnα3 proved that both these proteins are co-expressed in the granule neurons present in the posterior cerebellar lobes. The dependency of Chrnα3 on Pax6 and Tlx3 was confirmed using $Pax6^{-/-}$Sey cerebellum which demonstrated that in the absence of Pax6 both Tlx3 and Chrnα3 expression is abolished in cerebellum. This has led to the conclusion that Pax6 through Tlx3 regulates Chrnα3 expression in the granule neurons of posterior cerebellum. Since Pax6 and Chrnα3 are candidate genes involved in autism, a neurodevelopmental disorder, further studies has to be done to analyze the role of Pax6-Tlx3-Chrnα3 axis in development of autistic spectral disorders.

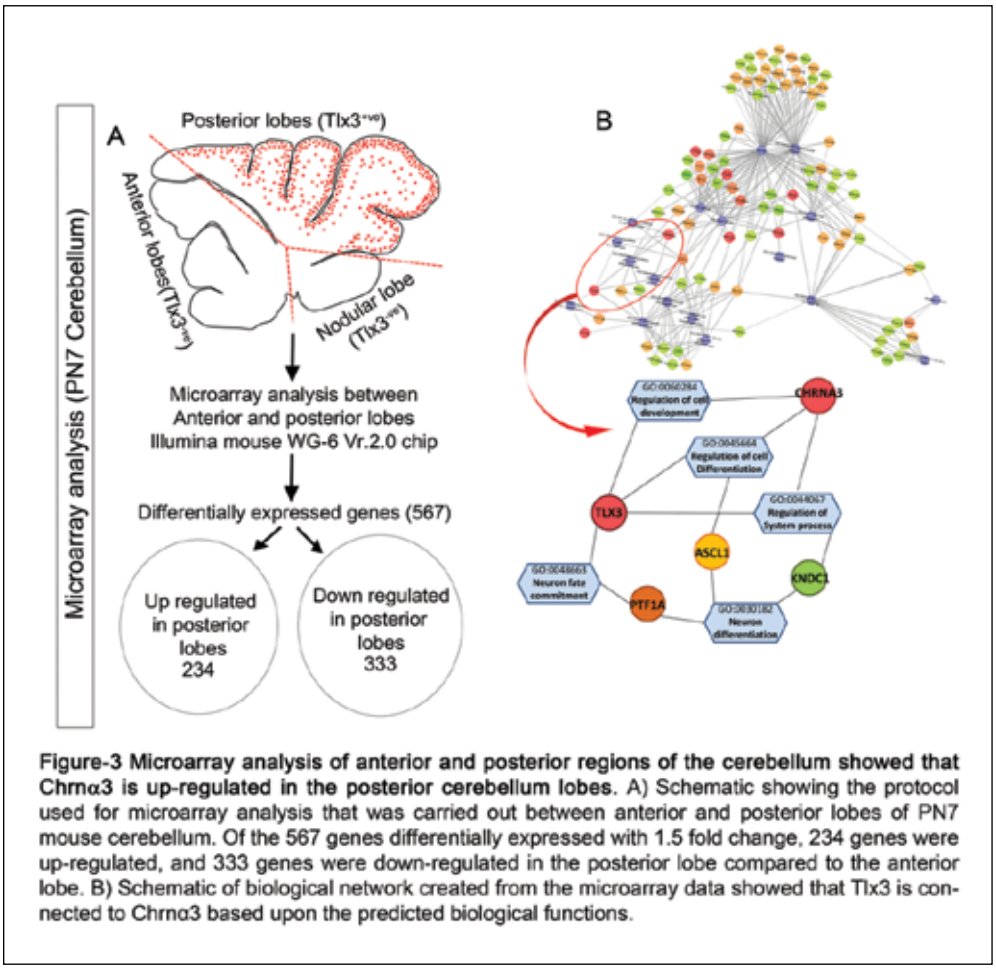

Figure-3 Microarray analysis of anterior and posterior regions of the cerebellum showed that Chrnα3 is up-regulated in the posterior cerebellum lobes. A) Schematic showing the protocol used for microarray analysis that was carried out between anterior and posterior lobes of PN7 mouse cerebellum. Of the 567 genes differentially expressed with 1.5 fold change, 234 genes were up-regulated, and 333 genes were down-regulated in the posterior lobe compared to the anterior lobe. B) Schematic of biological network created from the microarray data showed that Tlx3 is connected to Chrnα3 based upon the predicted biological functions.

Wnt5a mediates proliferation of cerebellar granule neurons during cerebellar development

Subashini C and Jackson James

Collaborator: Rejji Kuruvilla, Johns Hopkins University, Baltimore, USA

Though various studies have demonstrated the function of some of the Wnt ligands in cerebellar development, spatio-temporal expression pattern and function of several other important Wnt ligands such as Wnt5a is yet to be identified. Wnt5a, one of the major non-canonical Wnt ligand is known to play a pivotal role in development, especially in neurogenesis. It has been reported that Wnt5a promotes dopaminergic neurogenesis in midbrain and GABAergic neurogenesis in forebrain but its expression pattern and the role in the cerebellar development remains unclear. Our spatio-temporal expression pattern analysis revealed robust expression of Wnt5a during embryonic and postnatal stages. Thus, we next sought to determine the functional role exerted by Wnt5a during cerebellar development using Wnt5a conditional knockout mice (CKo) model. Histological analysis of mid sagittal sections of E14.5 Wnt5a CKo cerebellum showed reduction in the size of the cerebellum as compared to the wild type siblings. Likewise at early postnatal stages, PN1 and PN7 we continued to observe marked reduction in area of cerebellum in mutants

as compared to wild type siblings. Additionally we observed a notable reduction in the thickness of molecular layer (ML) and internal granular layer (IGL) in anterior and posterior lobes of cerebellum in mutants as compared to the wild type littermates but, we did not detect any significant difference in the external granular layer (EGL) thickness. These observations prompted us to decipher the role of Wnt5a in regulating GABAergic and glutamatergic neurogenesis. Mutants displayed a significant reduction in number of purkinje neurons, GABAergic interneurons, and some of the major glutamatergic neuronal subtypes highlighting the requirement of Wnt5a in precise generation of GABAergic and glutamatergic neuronal subtypes. Moreover, as we observed a significant reduction in number of purkinje neurons and in thickness of ML, where the purkinje neurons extend their axons we also analyzed the purkinje cell branching. Immunohistochemical analysis with calbindin and parvalbumin at PN7 indicated normal arrangement of purkinje neurons in the mutants (Fig. 4D-F) as compared to the wildtype counterparts (Fig. 4A-C). Even though these neurons were positioned rightly, they failed to develop elaborate dendritic branches unlike the wildtype counterparts and remained stunted (Fig. 4G). In addition, primary dendrites in mutants appeared thinner. Further to check if the dendritic arborisation defects persist, we analyzed the purkinje cell morphology at PN14 using immunohistochemical analysis. Mutants displayed poor dendritic branching and remained stunted as in PN7. As the purkinje cell differentiation in Wnt5a knockout is defective, we further wanted to check if the innervations on to purkinje neuron was affected in any way. Parallel fibers and climbing fibers innervate purkinje neurons and establish synaptic connections. Staining with vGlut2 and parvalbumin indicate intact synaptic terminals in both wildtype and mutants and showed no evident defects in innervations on to PC. Taken together our results suggest that Wnt5a is necessary for precise generation of GABAergic and glutamatergic neurons and for purkinje cell maturation.

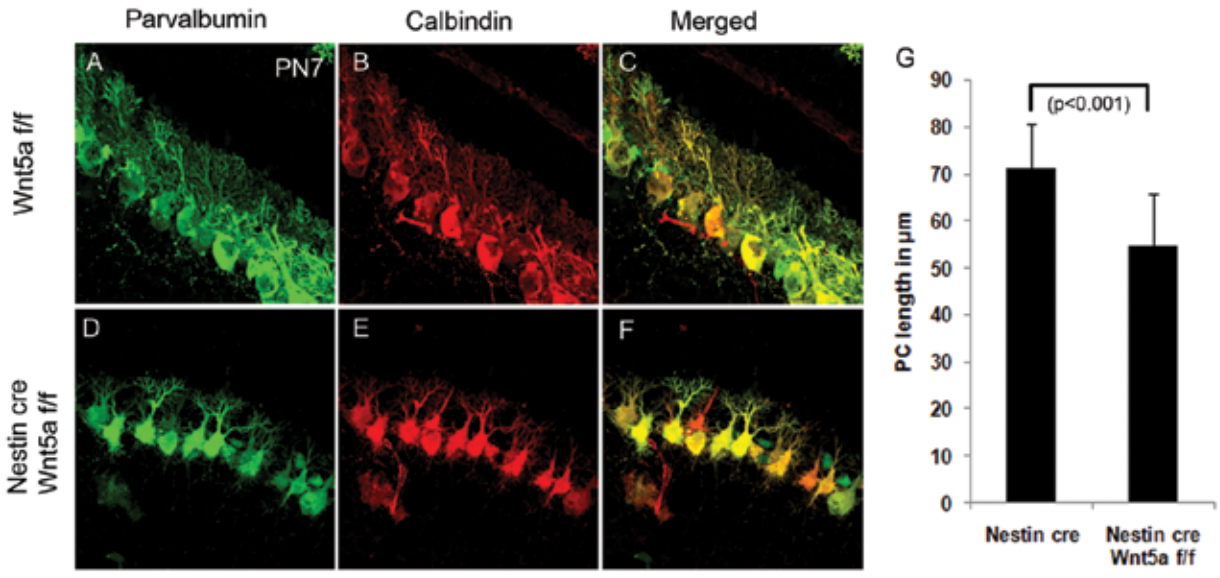

Figure 4: Purkinje cell dendritic branching defects at PN7 in Wnt5a conditional knockout mice. A-C) Immunostaining with calbindin and parvalbumin at PN7 reveal elaborate dendritic banching in wild-type while in Wnt5a conditional mutants D-F) purkinje cell dendrites remained shorter and poorly branched. G) Quantitation of purkinje cell length showed significant reduction in mutants as compared to wil-type control.

PUBLICATIONS

- *Vazhanthodi A Rasheed, Sreekumaran Sreekanth, Sivadasan B Dhanesh, Mundackal S Divya, Thulasi S Divya, Palakkottu K Akhila, Chandramohan Subashini, Krishnankutty Chandrika Sivakumar, Ani V Das & Jackson James.* Developmental wave of Brn3b expression leading to RGC fate specification is synergistically maintained by miR-23a and miR-374; *Developmental Neurobiology (2014)1155-1171.*
- *Nishit Srivatsava, Jackson James and KS Narayan.* Morphology and electrostatics play active role in neuronal differentiation processes on flexible conducting substrates; *Organogenesis (2014)10:1, 1-5.*

RESEARCH GRANTS

No.	Investigator(s)	Title	Funding Agency	Duration
1.	Dr. Jackson James (PI) Dr. RV Omkumar Dr. Santhosh Kumar SN	Characterization of Notch independent Hes-1 mediated maintenance andfate specification of neural progenitors	Department of Biotechnology, Government of India	2013-2016
2.	Dr. Jackson James (PI) Dr. RV Omkumar	Transcriptional regulation of Tlx3(Hox11L2) by Notch signaling and its involvement in excitatory Vs. inhibitory fate specification of neural progenitors	Department of Science & Technology, Government of India	2013-2016
3.	Dr. Sreekumar E (PI) Dr. Jackson James (Co-PI)	Characterization of Neurovirulence of Chikungunya virus in cellular and animal models	Department of Biotechnology, Government of India	2012-2015

NEUROBIOLOGY PROGRAM
NEURO-BIO-PHYSICS LABORATORY

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Rashmi obtained her PhD in Neuroscience from the National Brain Research Centre, Manesar, India. She did post doctoral training at Max Planck Intitute of Molecular Cell Biology and Genetics, Dresden, Germany, the Curie Intitute, Paris, France and Tufts University School of Medicine, Boston USA. She currently holds the Department of Biotechnology's Ramalingaswami Fellowship.

Research Fellows:

Sebastian John
Mohit Rastogi

Mechanotransduction through Lipid Rafts in homeostatic control of cell proliferation and tumorigenesis

Sebastian John and Rashmi Mishra

Cancers are driven by imbalances in biophysical forces such as osmotic, hydrostatic fluid pressure, shear and rigidity stresses mediated by both soluble and non-soluble factors. Cholesterol and sphingolipid enriched domains called lipid rafts exists as nanoscale assemblies on plasma membrane and harbor several pro and anti-tumoral signalling molecules that are dynamically modulated by precise lipid-protein interactions. Since the structural confirmation of these molecules play a critical role on their activity, it is indispensible to study their cross-talk with tumor biophysical environment and the net computation generated by these cells to survive in altered environment. We have focussed on this aspect in the proposed project with glioblastomas as tumor models and have found that surface clustering agents that are differentially regulated in various grades of gliomas, dramatically remodel lipid rafts associated glycoconjugates in a concentration dependent manner and generate varying thresholds of rigidity stresses that is sensed, mechanotransduced and interpreted by the cell to generate adaptive response to the shifts in mechanical environment. Selective perturbations of these surface remodelling principle modules is further found to switch malignant tumor phenotype into a benign. This work hence suggests novel druggable target for glioblastoma therapeutics.

Biophysical forces in stem cells dynamics

Sebastian John and Rashmi Mishra

The central goal of this research work is to decipher the mechanisms underlying how normal neural stem cells and different neuronal subtypes respond to altered osmotic and hemodynamic pressure, shear stress, rigidity forces and further exploring the link between signal transduction and physiological/ pathophysiological responses. A detailed analysis of our mechanoadaptive circuitries associated with stiffening and shear flow stress show several classes and isoforms of galectins, FOXO3A, CAMKIIA, CAMKIIB, redox and lipogenic genes as major players in homeostatic control of neural stem cell morphogenesis and associated axonal, migratory, proliferative vs. quiescent fate.

How neurons respond to biomechanical forces: Implications to brain functions and Neurodegeneration

Mohit Rastogi and Rashmi Mishra

Far less is known about mechanotransduction in neurons; hence we are taking careful and simple baby steps to discern this mechanism in the perspective of brain. So far, Glutamatergic and GABAergic neurons from different brain regions were isolated from mouse and were grown in stretchable microfluidic compartments. Tensile or stretch forces were applied by culturing cells on elastomeric PDMS stamps and stretching to various percentages from 5-60% on a cell stretcher

and further processing for morphological and biochemical analysis. We plan to now proceed for global proteomics on our stiffening forces regime to get a high resolution understanding of mechanoadaptive networks in two different neurotransmitter type neurons and how that impacts axonal functions and maintenance.

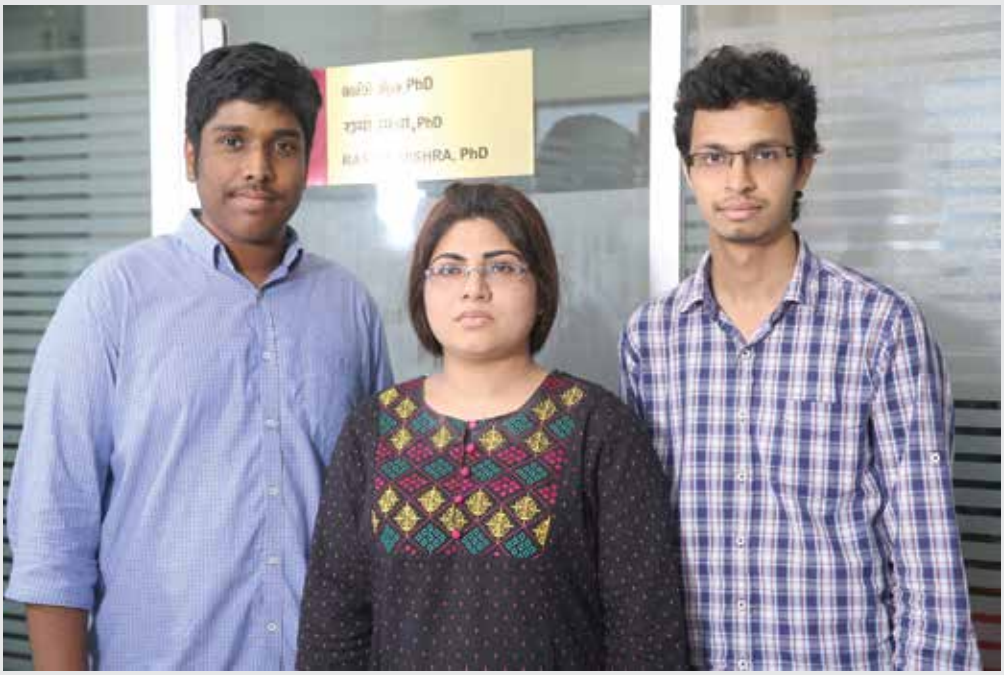
Role of Galectins in CNS morphogenesis, mechanotransduction and tumorigenesis

Mohit Rastogi and Rashmi Mishra

Galectins is a unique family of proteins that was initially discovered to play a role in axonal pathfinding however, their precise role in CNS morphogenesis still remain obscure. Galectins have been associated with various pathologies, most notably cancers, where they have been implicated in tumor immune escape, tumor angiogenesis and homo-heterotypic adhesion of tumor cells thereby mediating tumor metastasis. Hence, we are interested in the structure-function analysis of galectins and how galectins' mediated signaling cascades can be exploited in cancers therapeutics, regenerative medicine and developmental disorders. Last year we found a unique working model wherein galectin-glycosphigolipid form vectorial transport cargo couples in apical membrane biogenesis of neural stem cells. This year we find that mechanical forces play a crucial role in trafficking kinetics of this couple, actively and reversibly switching the neural stem cells from symmetrical division to asymmetrical neurogeneic fate. We are now deciphering the galectin-glycoconjugate synergism in enrichment of specific neurotransmitter type neurons and associated axon pathfinding principles that will further aid in our efforts towards neuro-regenerative medicine.

EXTRA MURAL FUNDINGS

S.No	Grant Title	Funding Agency	Duration
1	Rapid Grant for Young Investigator How Neurons Respond to Biomechanical Forces: Implications to Brain Functions and Neurodegeneration	Department of Biotechnology, Government of India	2013-2016
2	Ramalingaswami Fellowship Mechanotransduction through Caveolae: Lipid Rafts in homeostatic control of cell proliferation signaling and tumorigenesis	Department of Biotechnology, Government of India	2012-2017
3	Neuro Task Force Grant Mechanotransduction through Caveolae in Neural Stem Cell Niches: Role in Cell Signaling and Proliferation Control	Department of Biotechnology, Government of India	2013-2017


PhD
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NEUROBIOLOGY PROGRAM
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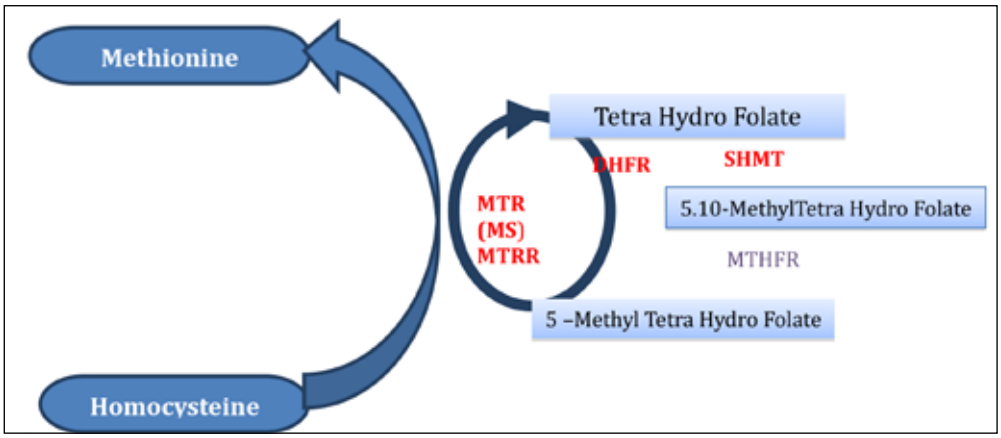
Genetics and Epigenetics in Schizophrenia

Saradalekshmi K R, Chandrasekaharan Nair, Priya M Allencheri, Indu V Nair and Moinak Banerjee

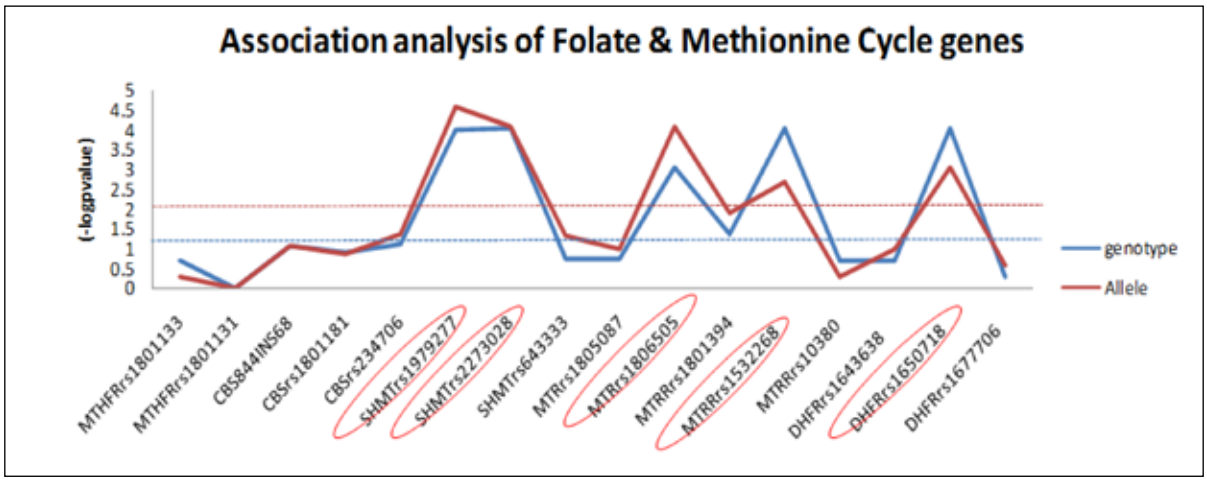
***Collaborators**: Nairs Hospital, Cochin and Mental Health Centre, Thiruvananthapuram*

The methylation maintenance machinery consists of DNA methyltransferases, Methyl CpG binding proteins and Ten eleven Translocases family of proteins. We analysed the association of 32 polymorphisms in these genes *DNMT1, DNMT3A, DNMT3B, DNMT3L, MECP2, MBD2 and TET2.* We report a novel association of *DNMT1* rs2114724 and rs2228611 with Schizophrenia in our population. We analysed the association of the variants with demographic factors such as Gender, Age of Onset, and family history. Gender analysis revealed that DNMT3B rs 2424932is associated with male patients. While analysing the effect on age of onset, we found that *DNMT3B* rs1569686 and *TET2* rs7661349 was associated with an early age of onset in our patients. *DNMLT3L* rs2070565 was significantly associated with age of onset in patients with a positive family history. Further we analysed the association of polymorphisms with drug response in the patients. We identified *MBD2* rs584330 to be associated with response in our patients. Since symptom presentation in Schizophrenia is heterogeneous, we looked at the symptoms which were predominant in our patient population and identified a set of symptoms which were both frequent and severe in our patients. Global DNA methylation levels in our patients were significantly higher compared to controls. When we analysed the expression of methylation maintenance genes in peripheral blood leukocytes we found that levels of *DNMT3B* and *TET2* were higher in patients but with marginal significance. Genotype based analysis of expression levels and methylation levels did not yield any significant observation.

However we see a positive correlation with DNMT1 expression and Global methylation. The folate and one carbon metabolism pathway is important in determining the methionine turnover and this will affect the process of DNA methylation as S- Adenosyl methionine is the substrate for methylation reactions. We analysed 18 polymorphisms in *MAT1A, MTHFR, MTR, MTRR, DHFR, SHMT* and *CBS* genes. Variant in genes in the folate pathway, *MTR, MTRR* and *SHMT* were significantly associated with the disease status in our study. We observe a significant increase in the level of homocysteine in plasma from the patients compared to Controls. Except for a marginal association with SHMT genotypes, none of the associated genotypes were significantly associated with homocysteine levels. However, genotypes and DNA methylation levels were comparable, suggesting that the associated SNPs have an effect on the methionine levels. Phylogenetic analysis of the genes based on allele frequencies in different population suggest that with respect to polymorphisms in both DNA methylation maintenance machinery and Folate and One carbon metabolism, our population is placed proximal to Mexican suggesting that populations living under similar environmental condition based on latitudinal effect are likely to evolve similarly indicating of an environmental phenotype.



GENE	SNPID	F Score
MTR	rs1806505	0
MTRR	rs1532268	0.941
SHMT1	rs1979277	0.868
	rs2273028	0.5
DHFR	rs1677706	0.101

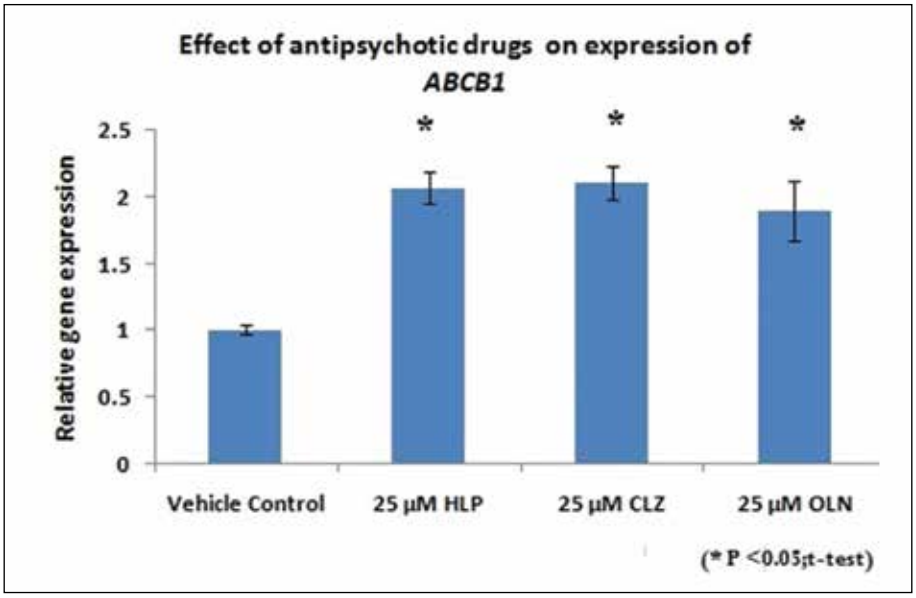


Evaluating pharmacoepigenomic response of antipsychotic drugs

B Swathy and Moinak Banerjee

Antipsychotic drugs are the most common form of treatment for Schizophrenia and related mental disorders. The patients receiving antipsychotic medications show a wide variability in drug response and drug induced side effects which could be attributed to genetic or non-genetic components influencing drug response. Pharmacogenetic studies have identified the potential involvement of polymorphic genes in treatment response and drug-induced adverse events in patients diagnosed with schizophrenia. Epigenetics offers another level of explanation for the drug response variability which cannot be accounted by gene polymorphisms. The present study involves assessing the epigenetic modifications induced by antipsychotic drugs in cell culture model. Our previous studies has shown that antipsychotic drugs can induce changes in global DNA methylation as well as the expression of epigenetic genes in a dosage and time dependent manner. Also the gene expression of various pharmacologically relevant genes including multi drug transporter, neurotransmitter receptors and transporters and drug metabolizing enzymes demonstrated antipsychotic drug specific modulation. Among these, *ABCB1* showed up regulation in gene expression with treatment of antipsychotic drugs including haloperidol (HLP), Clozapine (CLZ) and Olanzapine (OLN) in HepG2 cell line.(Fig. 1). To determine whether epigenetic mechanisms underlie alteration in *ABCB1* gene expression, various epigenetic parameters including DNA methylation, chromatin accessibility and microRNA expression were determined. Determination of the methylation status of CpG sites within the *ABCB1* promoter by bisulfite sequencing PCR has shown that methylation pattern was not altered by antipsychotic drugs (Fig

2). Chromatin accessibility assay was performed to determine the status of chromatin accessibility at *ABCB1* promoter region. *ABCB1* being a bona fide target of miR-27a, the antipsychotic drug specific alteration in miRNA expression was determined. Antipsychotic drugs induced down regulation of miR-27a expression level. (Figure 3) Thus the overexpression of *ABCB1* gene expression in response to antipsychotic drug treatment seems to be modulated by miR-27a.



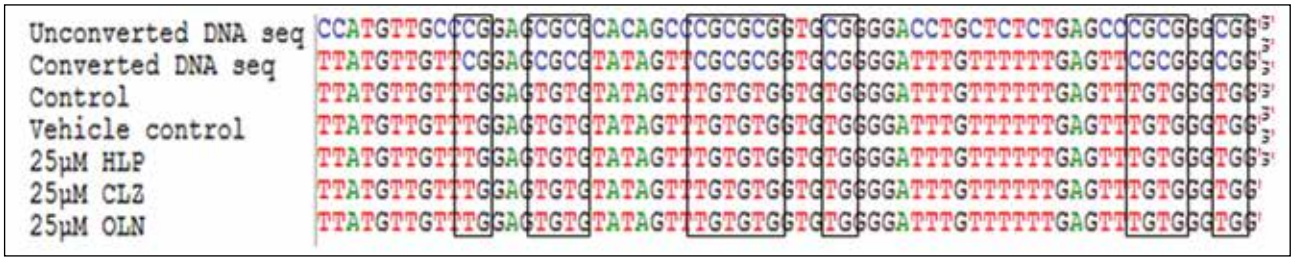
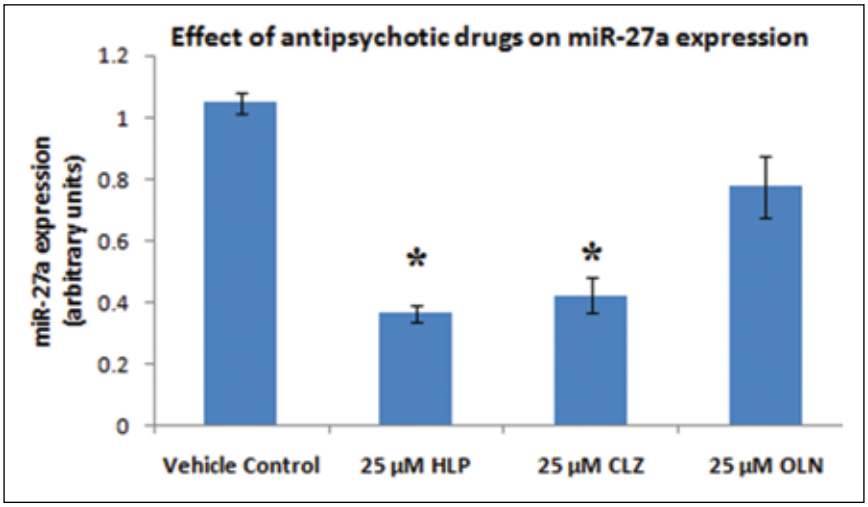


Figure 2: Bisulfite sequencing of ABCB1 promoter region.



Genetic and epigenetic control of Monoamine biosynthetic pathway in Autism phenotypes

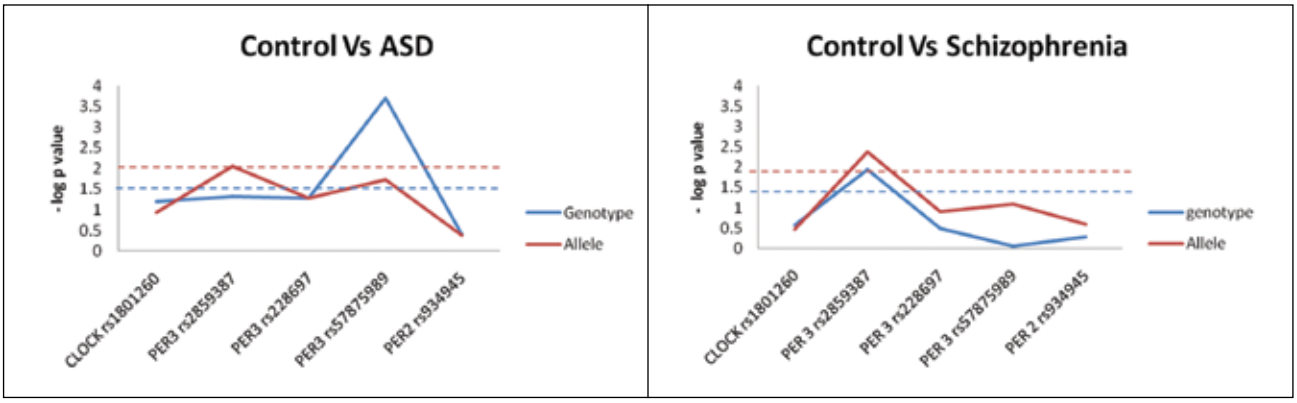
Ann Mary Alex, Dr. P.A Suresh, Moinak Banerjee

***Collaborators**: Institute for Communicative and Cognitive Neuro-Science, Shoranur.*

Autism Spectrum Disorder (ASD) and Schizophrenia are distinct disorders with unique characteristics, but they share similarities in many functional domains. There is an overlap in co-occurring abnormalities and clinical phenotypes between autism and schizophrenia. Symptoms of ASD can be seen from early childhood, while the onset of schizophrenia typically occurs in young adulthood. Sleep disorder has been identified as a co-morbid factor in ASD and schizophrenia with5 0%-80% of patients experiencing this feature. Sleep is an active process that is finely and reliably regulated by two parallel mechanisms– Circadian Regulation and Sleep Homeostasis. Sleep homeostasis is a regulatory mechanism based on prior duration of continuous sleep and wakefulness. The circadian system is a self sustained endogenous biological clock situated in the suprachiasmatic nucleus (SCN) in the hypothalamus which regulates the behavioral and physiological processes. The circadian rhythms are controlled by a set of clock genes organized in a system of interlocked transcriptional feedback loops. The core clock genes control sleep homeostasis also at the molecular level. Children with autism have severe problem sassociated with sleep onset and maintenance. Delayed sleep onset, impaired sleep continuity and increased time awake are common in Schizophrenia patients also. A hallmark characteristic of most circadian sleep disorders is the inability to sleep during desired times, with difficulty in initiating or maintaining sleep, early awakening and/or impaired alertness during waking hours. These data suggest a common pattern of sleep disturbance in both the neuropsychiatric disorders which might be the consequence of a circadian dysfuncion.The aim of this study is to understand the genes that influence these two major underlying phenotypes independently and how they influence the genetic network, in the development of autism and Schizophrenia from a population perspective. We screened the core clock components, *CLOCK* and *PER3* and *PER2* in ASD and Schizophrenia patients. Genotypic and allelic frequencies of the SNPs studied were analyzed to understand if there exists an association with the disease. *PER3* VNTR polymorphism (rs57875989) is a four or five repeats of 54-nucleotide sequence (18-amino acid). The longer repeat which has been linked to a deficit in executive function has been found to be associated with ASD in our population (pvalue-0.0182). The VNTR repeats contain clusters of predicted case in kinase-1ε phosphorylation sites. This may result in a disruption in the network of proteins controlling the circadian and homeostatic sleep regulation. However this association was not observed in Schizophrenia while rs2859387 was found to associated with both ASD and Schizophrenia in our population.

Functional Significance of studied SNPs

rs ID	Gene	Chromosome	Region	Alleles	FS score	Ensembl-NS	Ensembl-TR	miRNA (miRanda /Sanger)		
rs1801260	*CLOCK*	CHR 4	3 Prime Utr	T/C	0.398	Frame shift Coding	Regulatory Region	Y		
rs ID	**Gene**	**Chromosome**	**Region**	**Alleles [Amino acid change]**	**FS score**	**PolyPhen**	**SIFT**	**SNPeffect**	**LS-SNP**	**Splicing Regulation**
rs57875989	*PER 3*	CHR 1	Exon							
rs2640909	*PER 3*	CHR 1	Non Synonymous Coding	T/C [Met - Thr]	0.849	Possibly Damaging	Tolerated	Deleterious	Benign	Changed
rs2859387	*PER 3*	CHR 1	Synonymous Coding	G/A [Pro - Pro]						
rs228697	*PER 3*	CHR 1	Non Synonymous Coding	C/G [Pro - Ala]	0.377	Possibly Damaging	Tolerated	Deleterious	Benign	Changed
rs934945	*PER 2*	CHR 2	Non Synonymous Coding	G/A [Gly - Glu]	0.365	Benign	Tolerated	Benign	Benign	

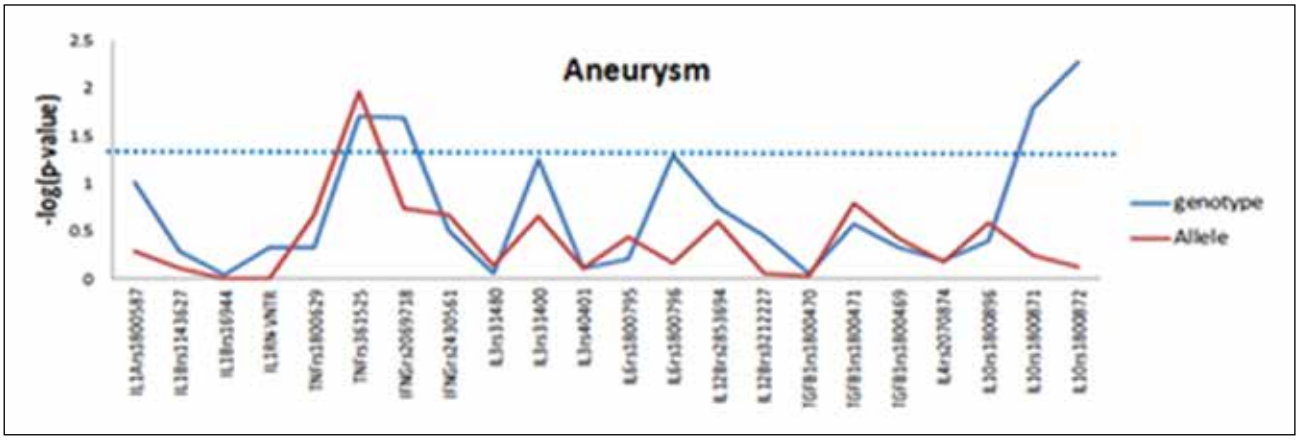


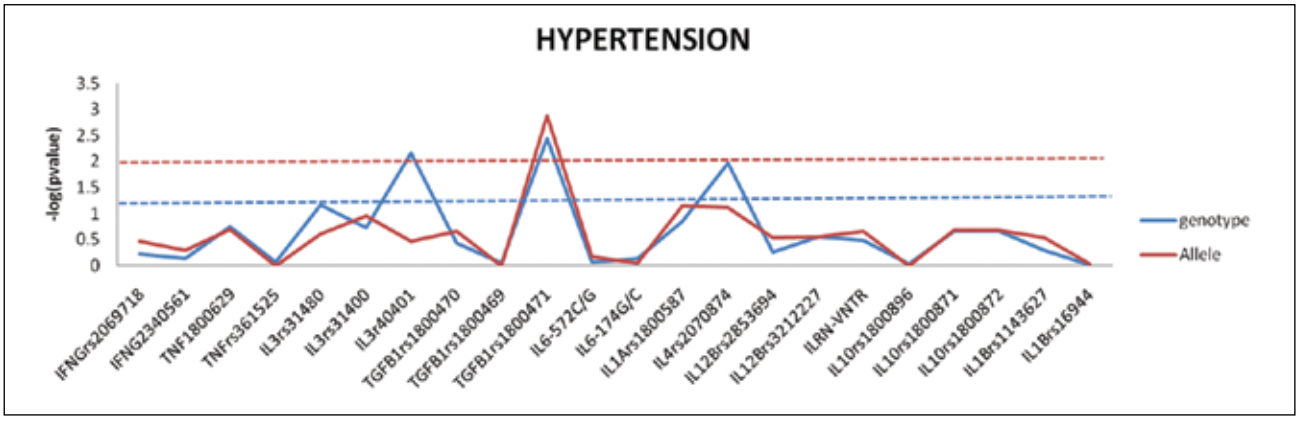
Cytokines in Development of Intracranial Aneurysm

Sanish Sathyan, Linda Koshy, HV Easwer, S*. Premkumar, Jacob P Alapatt**, Suresh Nair*, R.N. Bhattacharya* and Moinak Banerjee

Collaborators: **Sree Chitra Tirunal Institute of Medical Sciences & Technology, Thiruvanathapuram and **Calicut Medical College, Kozhikode*

The most common cause of subarachnoid hemorrhage (SAH) is rupture of intracranial aneurysm, which affects 2% of the population and accounts for 500000 hemorrhagic strokes annually in mid life (median age 50), often resulting in death or severe neurological impairment. Aneurysmal SAH is associated with mortality rate as high as 40% to 50% and genetic factors have shown to play an important role in SAH in conjunction with predisposing environmental factors. Environmental risk factors that associate with IA can result in modifying the effect of inherited genetic factors and thereby increase the susceptibility to SAH. In addition subsequent to aneurismal rupture the nature and quantum of inflammatory response might be critical for repair. Therefore, genetic liability to inflammatory response caused by polymorphisms in cytokine genes might be the common denominator for gene and environment in the development of aneurysm and complications associated with rupture. Functionally relevant polymorphism in the pro- and anti-inflammatory cytokine genes [IL-1 complex (IL1A, IL1B and IL1RN), TNFA, IFNG, IL3, IL6, IL12B, IL1RN, TGFB1, IL4 and IL10] were screened in radiologically confirmed IA patients and controls from genetically stratified Malayalam speaking Dravidian ethnic population of South India. Pro-inflammatory cytokines TNFA rs361525, IFNG rs2069718 and anti-inflammatory cytokine IL10 rs1800871 and rs1800872 were found to be significantly associated with IA, independent of epidemiological factors. TGFB1 rs1800469 polymorphism was observed to be associated with IA through co-modifying factors such as hypertension and gender. Functional prediction of all the associated SNPs of TNFA, IL10 and TGFB1 indicate their potential role in transcriptional regulation. Meta-analysis further reiterates that IL1 gene cluster and IL6 were not associated with IA. The study suggests that chronic exposure to inflammatory response mediated by genetic variants in pro-inflammatory cytokines TNFA and IFNG could be a primary event. While stochastic regulation of IL10 and TGFB1 response mediated by comorbid factors such as hypertension may augment the pathogenesis of IA through vascular matrix degradation.



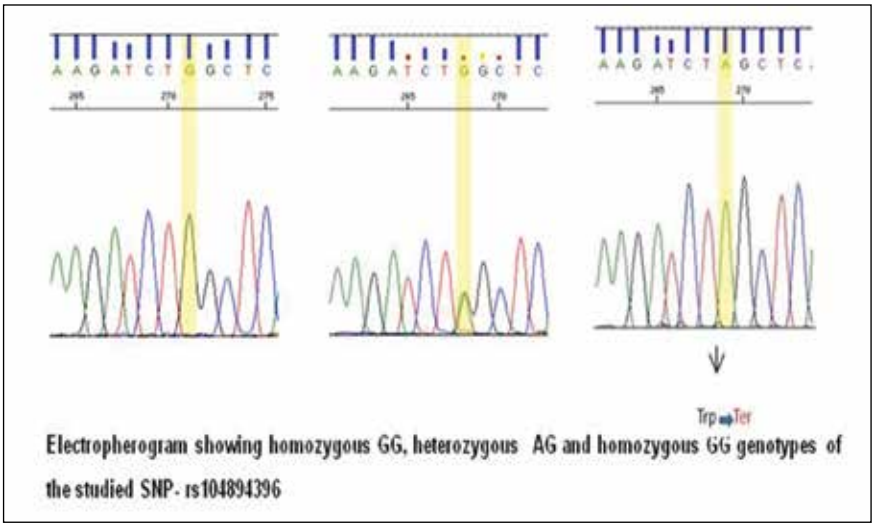


GJB2 Gene mutations in Autosomal recessive nonsyndromic neurosensory deafness in south Indian Population

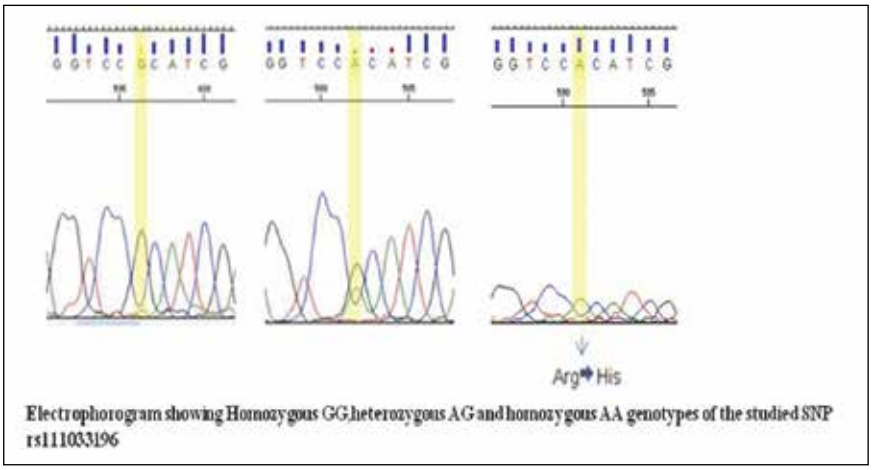
Maria Sebastian*, Praveena Davis, Padmaja Ramdas* and Moinak Banerjee

***Collaborators**: *National Institute of Speech and Hearing, Trivandrum.*

Autosomal recessive nonsyndromic neurosensory deafness also known as "DFNB" causes 20% of all childhood deafness and may have a carrier rate as high as 2.8%. Fifty to eighty percent of autosomal recessive congenital severe to profound hearing impairment results from mutations in a single gene, *GJB2*(DFNB1), that encodes the protein connexin 26(Cx26) is located on chromosome 13q11-12. The promoter, exonic and the 3'UTR region of GJB2 were screened. Of the total 227 variations reported in GJB2, 4 SNPs were found to be polymorphic in the population studied, others were found to be monomrphic. Of the 4 polymorphic SNPs, rs104894396 (W24X) which is nonsense mutation was found to have a higher frequency. A total of 21 families and 26 probands were found to have homozygous (AA) condition. This particular mutation results in an early truncation of protein (24 amino acids instead of 226 amino acids). When present in a homozygous state, no functional Cx26 monomers are present in cells. This in turn affects the potassium recycling to endolymph and thereby resulting in poor sound stimuli. rs111033196 (R127H), a misesnse mutation located in the cytoplasmic loop of Cx26 results in the formation of defective homotypic junctional channels. This variant found to be in a homozygous state in 6 individuals.

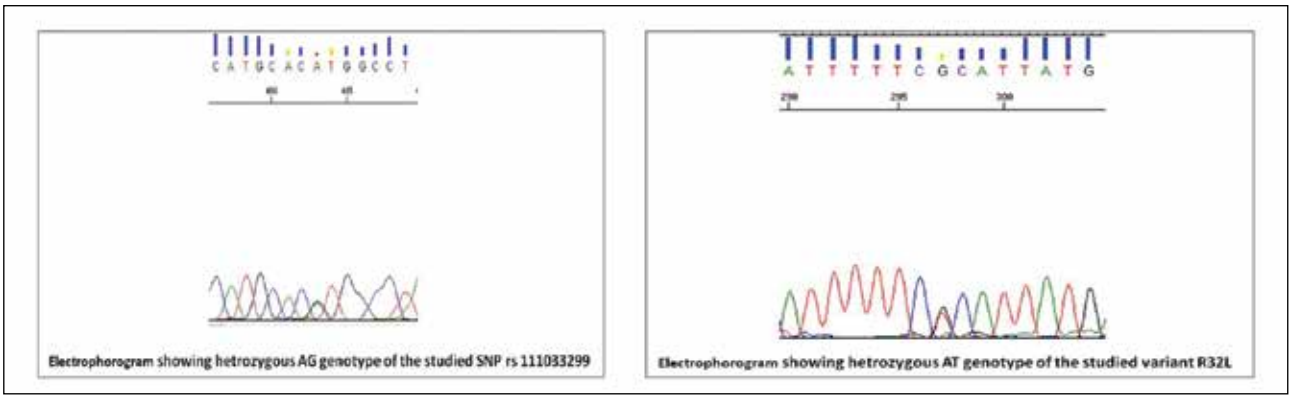


Electropherogram showing homozygous GG, heterozygous AG and homozygous GG genotypes of the studied SNP- rs104894396



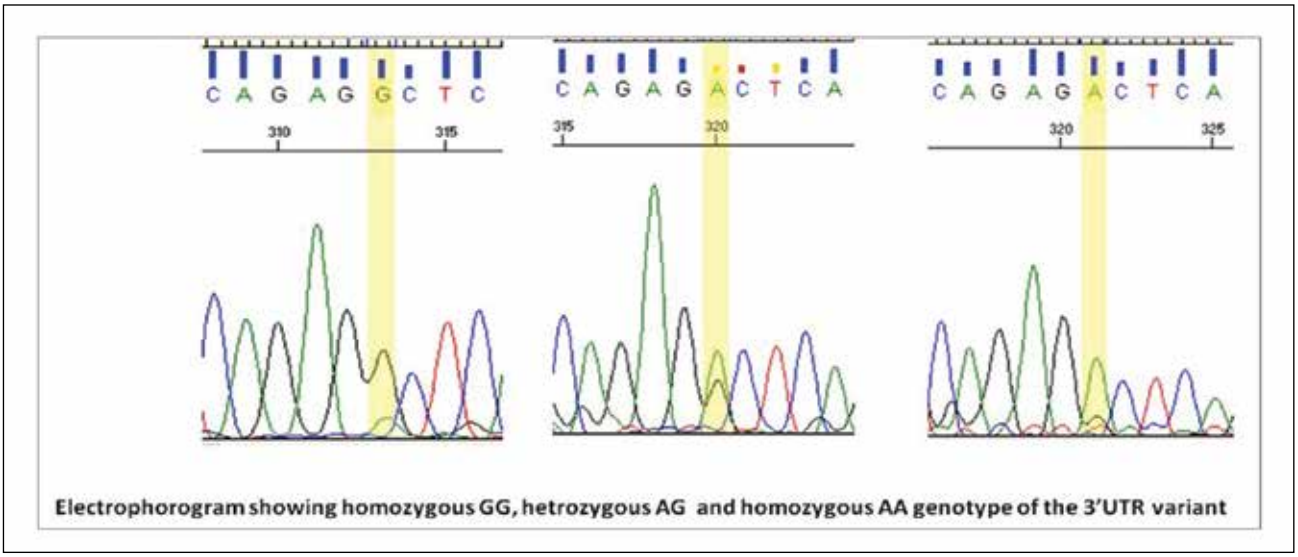
Electrophorogram showing Homozygous GG,heterozygous AG and homozygous AA genotypes of the studied SNP rs111033196

A rare variant rs 111033299 (V95M) which is also found in the cytoplasmic loop was found in 2 individuals in a heterozygous condition. This variant was reported to alter the permeation of Gap Junctions for molecules larger than simple ions. Another rare variant R32Llocated in the first transmembrane domain of GJB2 was also found in our study. 2 individuals showed heterozygous condition for the R32L variant. A novel 3'UTR variant was also observed in the GJB2 gene in the present study. In-silico prediction of this variant using different miRNA prediction tools suggest that this change can cause a differential binding of miRNA including hsa-miR-924, hsa-mir-501-5p,hsa-mir-1225-3p,hsa-mir-558 and hsa-mir-615-3p. Based on the observed polymorphic variants we are developing a scoring matrix to evaluate the significance of these variants with respect to Autosomal Recessive Non Syndromic Deafness. Homozygous mutant was provided the maximum score. The score for each variants were given based on their function and significance. This was then correlated with the audiological data.



Electrophorogram showing hetrozygous AG genotype of the studied SNP rs 111033299

Electrophorogram showing hetrozygous AT genotype of the studied variant R32L



Electrophorogram showing homozygous GG, hetrozygous AG and homozygous AA genotype of the 3'UTR variant

PUBLICATIONS

- *Sathyan, S., Koshy, L., Balan, S., Eashwer, H. V., Premkumar, S., Nair, S., Bhattacharya, R. N., Allapat, P. J., Banerjee, M.* (2014). Association of Versican (VCAN) gene polymorphism rs251124 and rs2287926 (G428D), with intracranial aneurysm. *Meta Gene 2, 651-660.*
- *Sharma, N., Joseph, R., Arun, R., Chandni, R., Srinivas, K. L., & Banerjee, M.* (2014). Cytokine gene polymorphism (interleukin-1beta +3954, Interleukin-6 [-597/-174] and tumor necrosis factor-alpha -308) in chronic periodontitis with and without type 2 diabetes mellitus. *Indian Journal of Dental Research, 25(3), 375-380.*
- *Jose, M, Banerjee, M, Mathew, A, Bharadwaj, T, Vijayan, N, & Thomas, S.* (2014). Pharmacogenetic evaluation of ABCB1, Cyp2C9, Cyp2C19and methylene tetrahydrofolate reductase polymorphisms in teratogenicity of anti-epileptic drugs in women with epilepsy. *The Annals of Indian Academy of Neurology, 17(3), 259-266*
- *Saradalekshmi, K. R., Neetha, N. V., Sathyan, S., Nair, I. V., Nair, C. M. & Banerjee, M.* (2014). DNA Methyl Transferase (DNMT) Gene Polymorphisms Could Be a Primary Event in Epigenetic Susceptibility to Schizophrenia. *PLOS ONE, 9, e98182.*
- *Paul, B., Saradalekshmi, K., Alex, A. M. & Banerjee, M.* (2014). Circadian rhythm of homocysteine is hCLOCK genotype dependent. *Molecular Biology Reports, 41, 3597-3602.*
- *Balan, S., Bharathan, S. P, Vellichirammal, N. N., Sathyan, S., Joseph, V., Radhakrishnan, K. and Banerjee, M.* (2014). Genetic association analysis of ATP binding cassette protein family reveals a novel association of ABCB1 genetic variants with epilepsy risk, but not with drug-resistance. *PLOS ONE 9(2): e89253*

AWARDS

- Best Poster award during Nutrigenomics meeting of International of Society of Nutrition in Gold Coast Australia. 2-5th May 2014 (Saradalekshmi KR)
- European Molecular Biology Organization travel award and Centre for International Co-operation in Science (CICS) travel award to attend EMBO workshop on Epigenetic Plasticity: implications in Neural (dys) function, Braga, Portugal, Oct 22-25,2014. (Swathy B)

CONFERENCES PRESENTATIONS

- In silico analysis of microRNA associated with antipsychotic drug response. 17th ADNAT Convention, Symposium on Genomics In Personalized Medicine and Public Health, Thiruvananthapuram, Jan 23-25, 2014.
- Genomic Characterization of IMR-32 from Two Different Cell banks. International Conference on Human Genetics & 39th Annual Meeting of Indian Society of Human genetics, Ahmedabad, Jan 22-25, 2014.
- Impact of variants in Folate and Methionine cycle genes on Homocysteine levels and DNA Methylation and their association with Schizophrenia in South Indian Population" 8th Congress of International Society for Nutrigenetics/Nutrigenomics at Gold Coast, Australia, May2-3, 2014
- Modulation of Epigenome by Antipsychotic Drugs in an Invitro System, EMBO workshop on Epigenetic Plasticity: implications in Neural (dys) function, Braga, Portugal, Oct 22-25,2014
- Epigenetic aspects of cellular response to antipsychotic drugs, XXXVIII All India Cell Biology Conference & International Symposium on "Cellular Response to Drugs",

CSIR-Central Drug Research Institute, Lucknow, UP, Dec 10-12, 2014.

- Role of clock gene variants in differential regulation of sleep homeostasis and circadian rhythm in Autism Spectrum Disorder and Schizophrenia. 15th International Meeting on Human Genome Variation and Complex Genome Analysis (HGV2014). Belfast, Ireland. 17 - 19 September 2014

PLANT DISEASE BIOLOGY & BIOTECHNOLOGY

PDDB LABORATORY - 1

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Structural and Biological Characterisation of an anti-hepatocellular carcinoma compound from *Glycosmis pentaphylla*

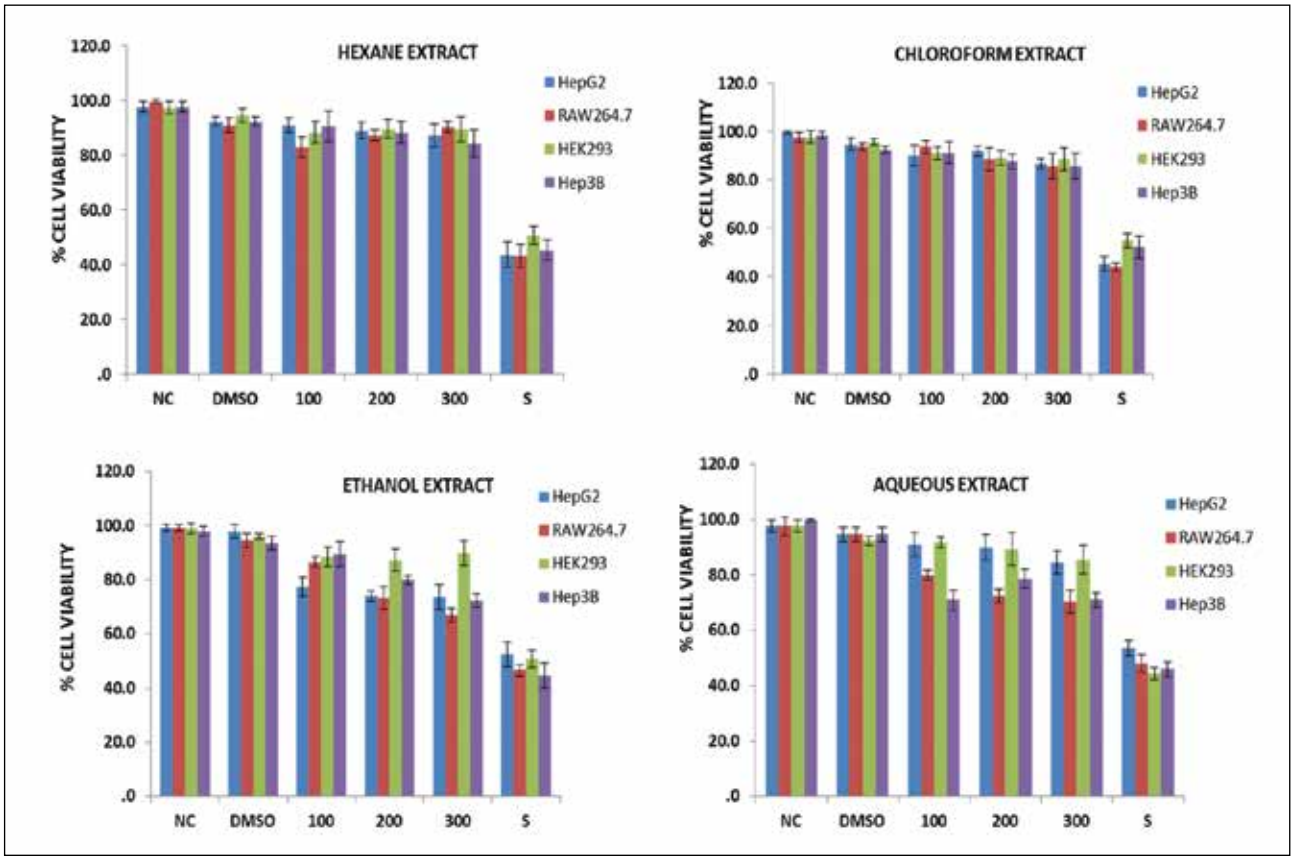
Sreejith P S and Asha VV

Hepatocellular carcinoma (HCC) is a major clinical problem because of its worldwide increasing incidence and poor survival rate. It is the fifth most common cancer and second leading cause of cancer related death in the world. It is estimated that more than 60% of the currently used chemotherapeutics have their origin from natural sources. Hence the search for new lead compounds from plant sources is a crucial element of modern pharmaceutical research. Glycosmis pentaphylla (Retz) Correa is commonly known as vananimbuka (Sanskrit) and paanal (Malayalam). It belongs to Rutaceae family. In Ayurveda *G. pentaphylla* is considered as an important option for curing fever, cough, rheumatism, anaemia, cancer and liver disorders. However a scientific validation regarding the active compounds, mechanism of action, safety and efficacy is not yet done. The cytotoxic and apoptosis inducing effect of the active extract and its fractions were estimated on Hep3 B, HepG2, HEK293, LX2 and RAW264.7 cell lines by MTT assay, morphological studies, Hoechst staining, DNA fragmentation, reverse transcription polymerase chain reaction and western blotting. The phytochemical profiling of the active extract was accomplished by different biochemical assays. Separation and purity determination of different fractions in alcohol extract was identified by TLC and HPLC methods. The pure compound was crystallized and structural identification done by spectroscopic methods (NMR, IR, LCMS etc.). Molecular docking, druggability and ADME prediction of the compound was confirmed using different in silico methods. Different biological properties (anti- inflammatory, anti-HBV, anti-oxidant, anti-angiogenic and anti-metastasis) of the isolated compound were identified by preliminary *in vitro* studies. *In vivo* acute toxicity of isolated compound and active extracts were done in Wistar rat. The preliminary investigation showed a selective anti-HCC activity of *G. pentaphylla* alcohol extract on HCC cell lines (Hep3 B and HepG2) without significant cytotoxicity on non-hepatocyte cell lines, HEK 293, LX2 and RAW 264.7. Morphological observation, Hoechst staining, DNA fragmentation, transcript and translational studies showed that the cytotoxicity of active extract is by regulating the apoptosis cascade. It induces apoptosis by modifying the ratio of expression of Bax and Bcl2 proteins time and dose dependently. Phytochemical screening of the active extract confirmed the presence of alkaloids, flavonoids, chalcones and sterol. Compound isolation and characterization was done by chromatography, phytochemical and spectroscopic techniques. Our studies showed that the alcohol extract of *G. pentaphylla* contain a modified form of chalcone which was responsible for its anti HCC activity. Preliminary studies related to anti-inflammatory, anti-metastasis, anti-fibrotic, anti-viral and anti-oxidant properties of the isolated compound were also done. Structure activity analysis with *in silico* methods showed that the isolated compound is a druggable candidate. Comparative docking study of compound with sorafenib showed a comparable binding efficacy of the compound on B RAF and mutated B RAF proteins. *In vivo* acute toxicity studies demonstrated that the treatments with alcohol extract and active compound did not cause any significant morphological, biochemical, histopathological and toxicological changes in rat. Works on further derivatization, chemical synthesis, anti HCC activity on *in vivo* xenograft models using the isolated active compound are ongoing.

Identification and characterisation of the active anti-inflammatory principle of *Tinospora cordifolia* (Thunb.) Miers.

Sheena Philip and Asha V V

Many cancers arise from sites of infection, chronic irritation and inflammation. Inflammation, a healing response of the body, is a double-edged sword. While it is typically recognized as a process for the remission of diseases, the persistence of inflammation may lead to various diseases including arthritis, atherosclerosis, and even cancer. The tumor microenvironment, composed of inflammatory cells, is an important participant in the neoplastic process, promoting proliferation, survival and migration. Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used anti-inflammatory drugs in the treatment of pain, fever and inflammation. However, the use of these drugs is associated with the occurrence of adverse digestive effects, particularly gastric erosions. Both the beneficial and detrimental effects of NSAIDS are due to the inhibition of prostaglandin synthesis through directly blocking cycloxygenase enzyme. Of the two isoforms of COX, COX-1 is the constitutively expressed form and is required for the maintenance of homeostasis – gastro-intestinal mucosa protection, inhibition of platelet aggregation, maintenance of renal blood flow-and the COX-2 isoform is induced only during the inflammatory processes. Thus selective COX-2 inhibition can not only control the inflammation but also limit the toxic side effects. Preclinical and clinical studies have established plant derived dietary substances are suitable candidates for

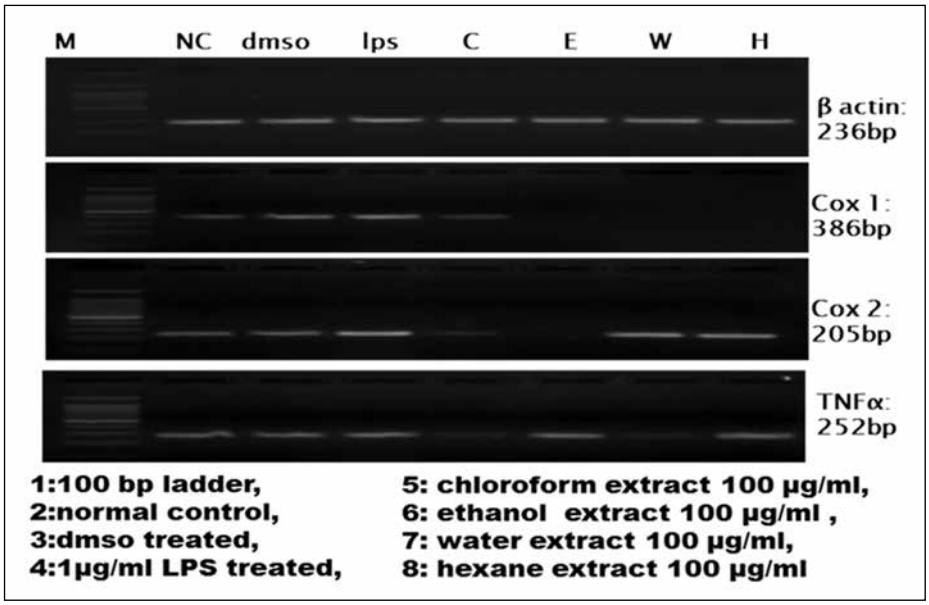


In vitro cytotoxicity assessment-MTT assay

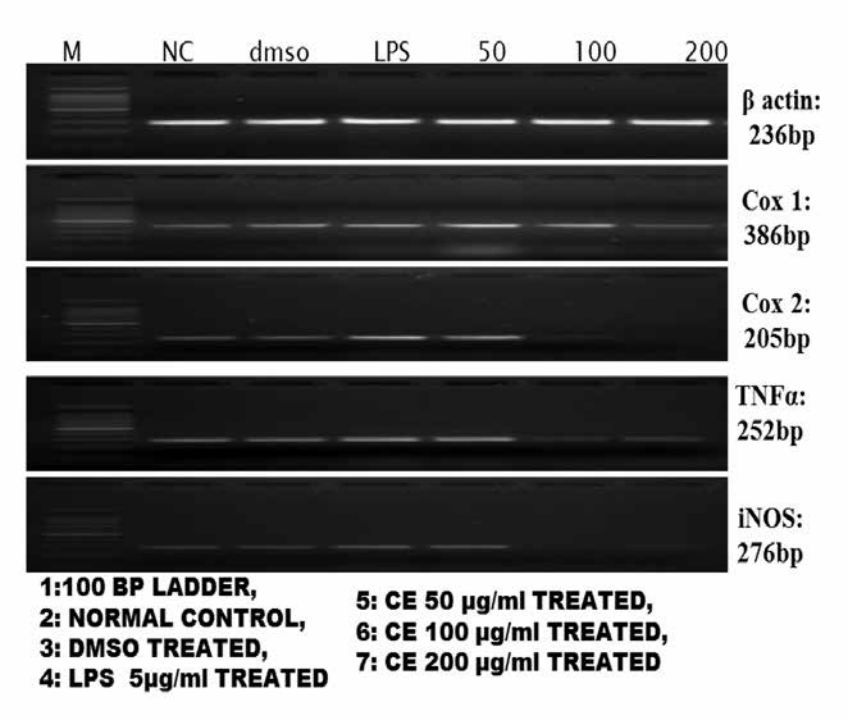
treating various types of inflammatory disorders due to their broad chemical diversity. The search for new lead compounds from plant sources is a crucial element of modern pharmaceutical research. *Tinospora cordifolia* (Thunb.) Miers (Guduchi) is a large, perennial, deciduous, climbing shrub of weak and fleshy stem found throughout India. It is widely used in folk and Ayurvedic systems of medicine. Potential medicinal properties reported include anti-diabetic, antipyretic, antispasmodic, anti-inflammatory, anti-arthritic, antioxidant, anti-allergic, anti-stress, antimalarial, hepato-protective, immuno-modulatory and anti-neoplastic activities. There are many studies suggesting the anti-inflammatory activity of *Tinospra cordifolia*- Eg. The plant exhibited anti-inflammatory effect in cotton pellet granuloma and formalin-induced arthritis model, and the mode of action resembles that of non-steroidal anti-inflammatory agent. However the molecular basis of action and the phyto-chemistry of the active extract have not yet been elucidated. Initially the *in vitro* cytotoxicity of four extracts of *Tinospra cordifolia* were analyzed on different cell lines. None of the extract exhibited any significant level of cytotoxicity.

Then in order to assess the anti-inflammatory activity of the different extracts (hexane, chloroform. ethyl alcohol and water extracts) of *Tinospra cordifolia,* RT-PCR analysis were carried out. Murine macrophage cell lines (RAW264.7) were treated with bacterial lipopolysaccharide to stimulate expression of inflammatory genes, which were then treated with each of the extract, followed by RNA isolation, RT-PCR and agarose gel electrophoresis. The results showed that the chloroform extract (CE) of this plant can selectively inhibit the expression of COX-2 gene along with TNF-κand iNOS genes. Also the activity of the CE was found to be dose dependent. The optimum activity was found to be associated with 100µg/ml CE.

The phytochemical analysis of CE revealed the presence of carbohydrates, cardiac glycosides, phenolic compounds, phyto-sterols like triterpene and resins. There exist various studies about the anti-inflammatory activity of triterpenoid derivatives. In order to identify and characterize the active moiety behind this activity in this chloroform extract of *Tinospora cordifolia,* we performed thin layer chromatographic analysis of the CE as a preliminary step. The solvent system used was Hexane: ethyl acetate: methanol in the ratio 4:0.2:1.4 and obtained 8 bands. Each band was then scraped out and analysed for anti-

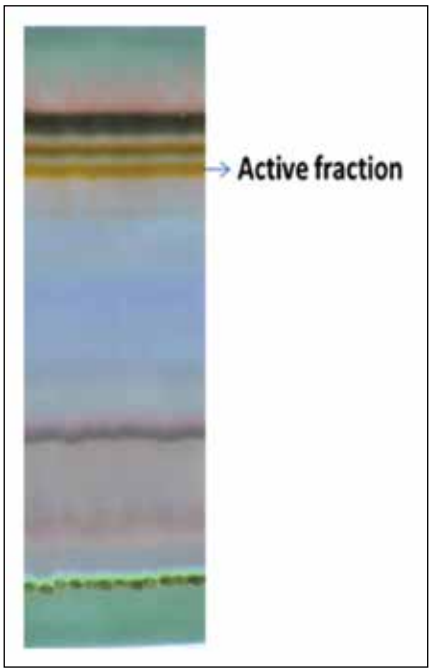


Selective inhibition of COX-2, TNF-α and iNOS genes by CE

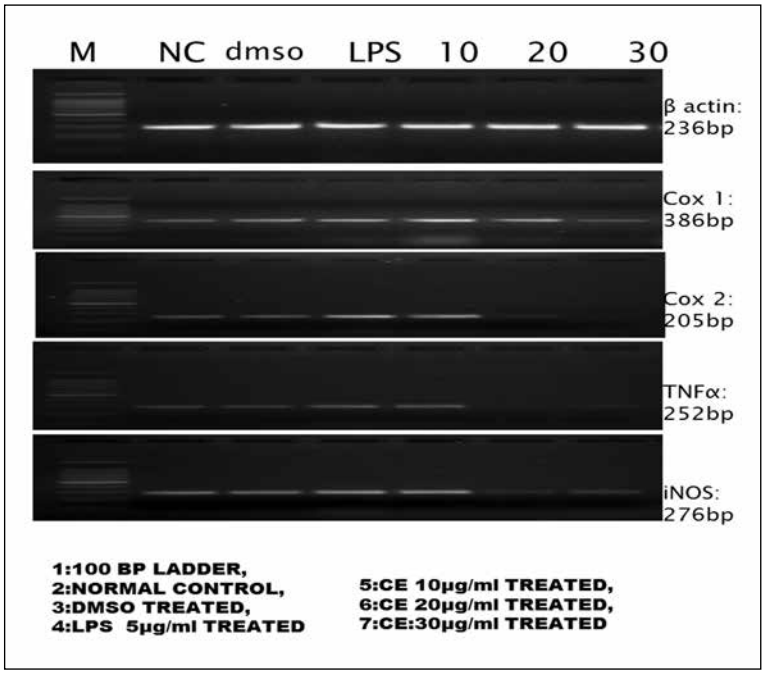


The anti -inflammatory activity of the CE is concentration dependent

inflammatory activity by RT-PCR analysis in RAW 264.7 cell lines pre incubated with bacterial lipopolysaccharide. Band-3 was then found to be associated with the selective inhibition of COX-2, TNF-α and iNOS genes when stimulated by bacterial lipopolysaccharide. The preliminary *in vitro* studies was successful in identification of the active fraction of the chloroform extract of *Tinosprora cordifolia*. However further isolation and characterization is underway.



Analytical thin layer chromatography pattern of CE

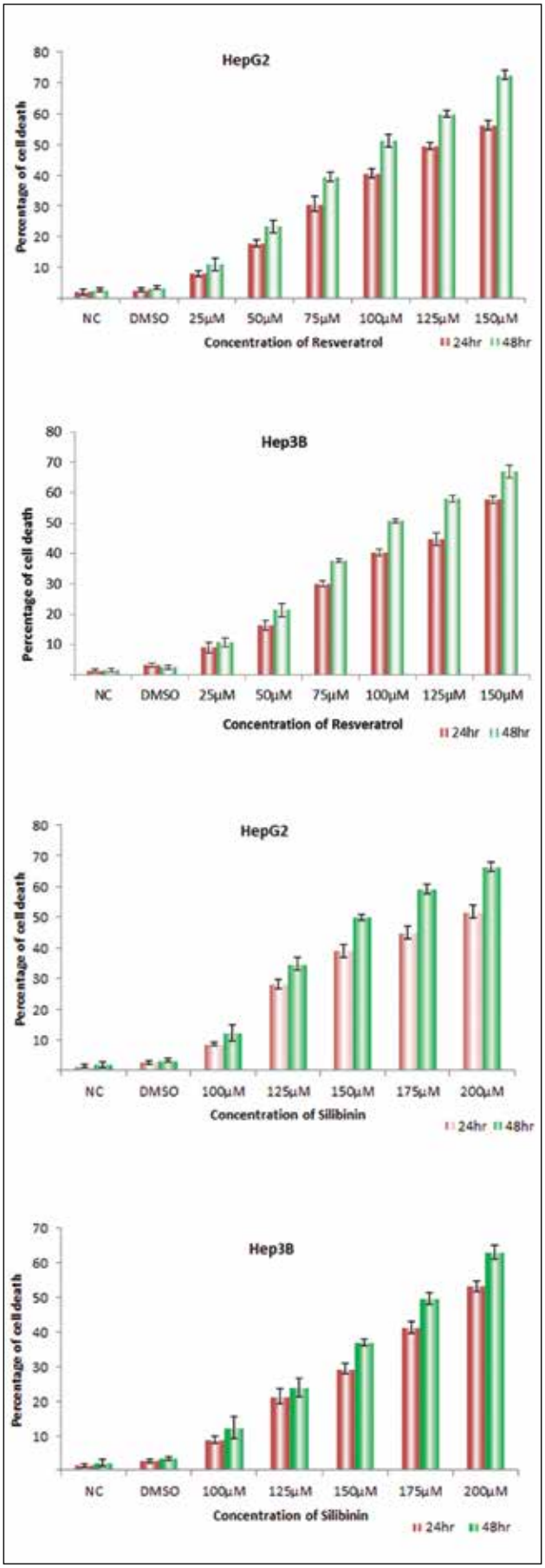


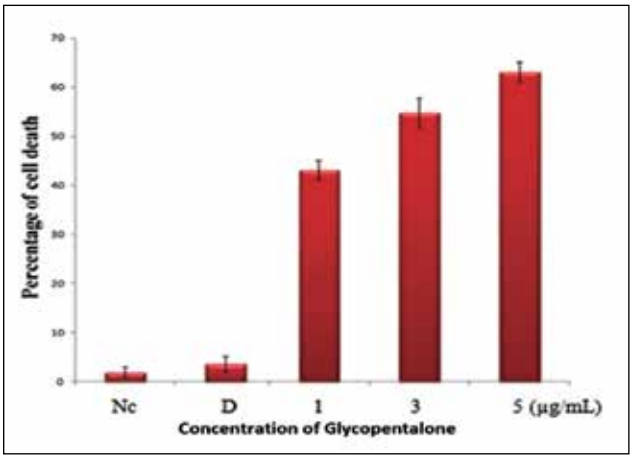
Selective inhibition of COX-2, TNF-α and iNOS genes by the ACTIVE FRACTION isolated by TLC analysis

Studies on the interplay of autophagy and apoptosis in the chemotherapy of Hepatocellular carcinoma using plant based anticancer lead molecules

Greeshma Tom and Asha V V

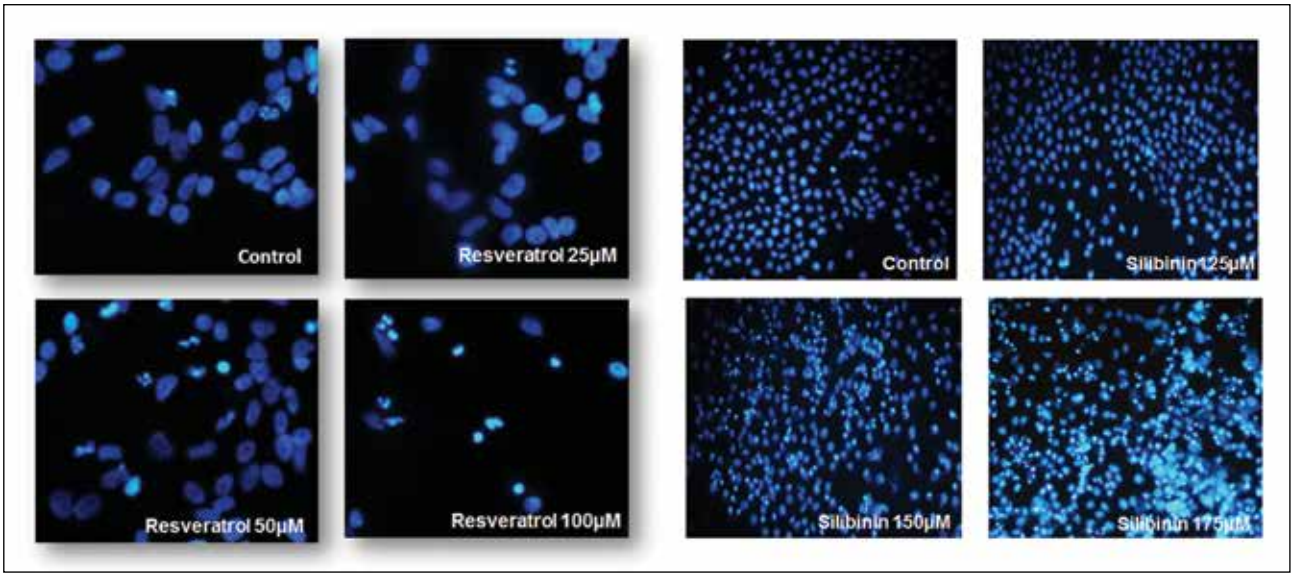
Plants have always served as an important source of novel pharmacologically active compounds; many blockbuster drugs being derived directly or indirectly from plants throughout the history. Majority of these plant derived lead molecules have proven anticancer activity. Apoptosis is the prominent mode of cell death employed by many of these agents to kill cancer cells. Recently, autophagy has emerged as a potential target in cancer chemotherapy, but its precise role being controversial. Certain chemotherapeutic agents induce autophagy as a cell death mechanism whereas others stimulate autophagy as a cell survival mechanism in response to therapeutic stress. The present study intends to deepen the understanding of the relationship between autophagy and apoptosis upon exposure of Hepatocellular carcinoma cells to selected anticancer lead molecules from plants. HCC, one of the most deadly malignancies worldwide, is significant because of its inherent resistance to chemotherapeutic drugs. For improving the survival of HCC patients, refined therapeutic regimens that potentiate the effects of chemotherapeutic drugs need to be fabricated. Improving the apoptosis inducing effects of plant compounds with combinatorial approaches can be an impressive alternative. Prospects of sensitizing HCC cells to chemotherapy by combining autophagy and apoptosis modulators are being investigated. The anticancer agents have been validated for their cytotoxic activity in HCC cell lines HepG2 and Hep3B using MTT assay. To determine whether the apoptotic pathway mediates cell death, Hoechst staining which affirmed the activation of apoptosis by these agents characterized chromatin compaction. Our studies demonstrated that the selected anticancer agents were able to induce autophagy together with apoptosis, dose and time dependently. Activation of autophagy in HCC cell lines was evidenced by fluorescence microscopic detection of autophagic vacuoles, formation of acidic vesicular organelles (AVOs), immunoblotting patterns for conversion of LC3-I to LC3-II, immunofluorescence analysis of recruitment of LC3-II to the autophagosomes. The signaling pathways involved in the activation of autophagy need to be traced out through studies at the levels of transcription and translation. Preliminary observation indicating antagonistic roles of autophagy and apoptosis in response to chemotherapy has been obtained. The existence of crosstalk between apoptosis and autophagy needs to be further analysed through gene knockout studies. Through the study, we propose that manipulating autophagy and apoptosis pathways concomitantly can be a promising therapeutic strategy to enhance the effects of chemotherapy and improve the clinical outcomes in HCC patients. To begin with, the effect of the selected compounds on cell viability was studied through MTT assay. The effect of the compounds on HCC cells was investigated over a wide concentration range for each compound. Hep3B and HepG2 cells were treated with 25-150µM resveratrol for 24 and 48 hours. Resveratrol reduced cell viability in a dose- and time-dependent manner. Following treatment with 150µM resveratrol for 24h, only 44% of HepG2 and 43% of Hep3B cells survived. After 48h treatment, survival was reduced to 28% and 33% for HepG2 and Hep3B cells respectively. IC50 values at 24h were 125µM for HepG2 and 150µM for Hep3B cells indicating that HepG2 cells were more sensitive to the cytotoxic effects of resveratrol. Similar studies were performed for silibinin and glycopentalone. Treatment with silibinin and glycopentalone resulted in marked growth inhibition of both HepG2 and Hep3B cells dose and time dependently. The IC50 values of silibinin against HepG2 and Hep3B cells were 200µM. Glycopentalone killed half of the cells at a concentration of 3µg/ml. Further studies were carried out exploiting the IC50 values obtained through MTT assay.





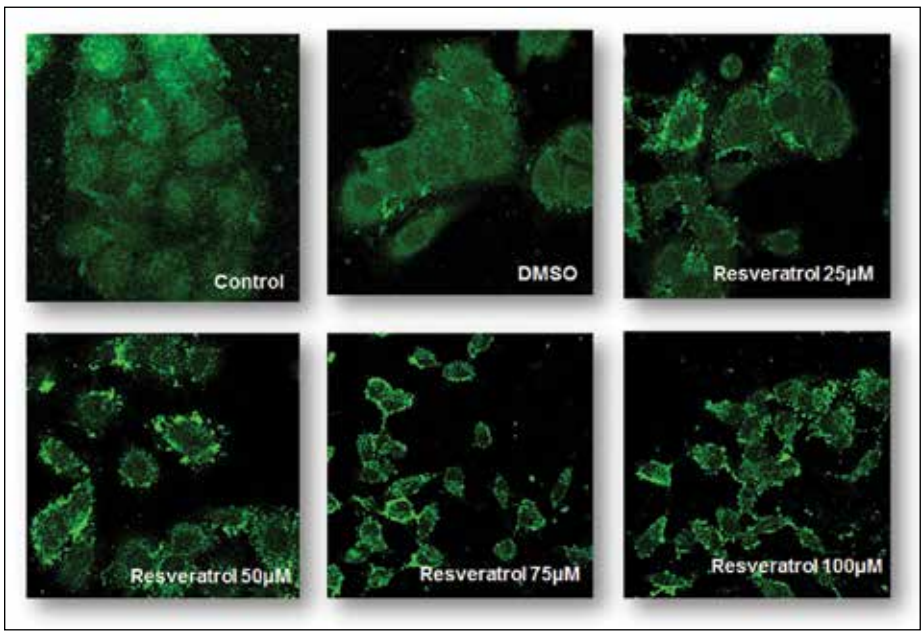
To ascertain whether the reduction in cell viability caused by the compounds is due to apoptosis, Hoechst staining was conducted to characterize chromatin compaction in the treated cells. At their IC50s, resveratrol and silibinin induced apoptotic cell death in HepG2 and Hep3B cells. The cellular membrane looked intact but the chromatin seemed condensed and aggregated at the periphery of the nucleus. Untreated and vehicle treated cells did not show such morphological changes.

Having verified that the selected compounds cause cytotoxicity through apoptosis, our next task was to see whether they trigger autophagy in HCC cells. Preliminary observation was taken from two staining procedures – Acridine Orange staining and Monodansylcadaverine staining. Acridine Orange is an acidotropic dye that accumulates in acidic spaces and fluoresces bright orange. After treatment with the compounds, the cells were stained with 1µg/ml acridine orange for 15min. The AVOs (Acidic Vesicular Organelles) appeared orange while the cytoplasm and nucleus appeared green. Resveratrol and Silibinin at their IC50s induced pronounced formation of orange AVOs in HepG2 cells. Next, vital staining of HepG2 cells grown on coverslips was performed with MDC dye, after treatment with increasing doses of resveratrol. Monodansylcadaverine is a selective fluorescent marker of autophagic vesicles that accumulates in acidic and lipid rich compartments. The dye shows diffuse staining in non-autophagic cells, but exhibits punctate vesicular staining when autophagy is induced. Following treatment with different concentrations of resveratrol for 24 hours, the cells exhibited higher intensity of

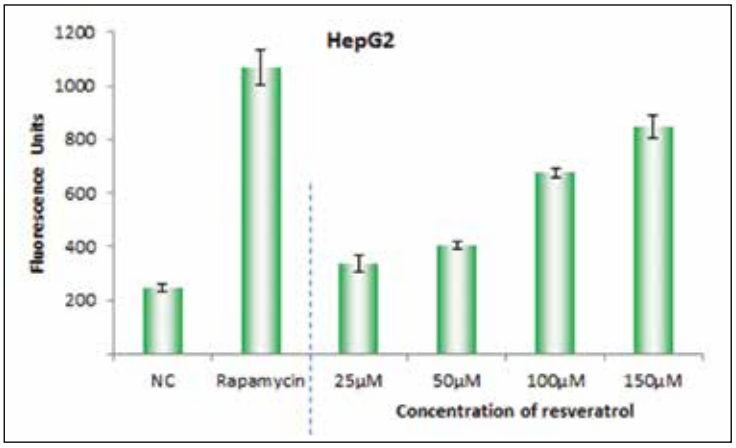


green punctate fluorescence. The quantification of green fluorescence was done by repeating the experiment in a 96well plate and taking the reading in a microplate reader at excitation and emission wavelengths of 463 and 534nm respectively.

The execution of autophagy involves a set of evolutionarily conserved gene products, known as the Atg proteins, that are required for the formation of the isolation membrane and the autophagosome (referred to as the "autophagic machinery"). In mammalian cells, most of the Atg proteins are observed on isolation membranes (e.g., ULK1/2, Atg13, FIP200, Atg101, Beclin 1, Atg14, LC3, Atg12, Atg16L1) but not on complete autophagosomes. To date, only microtubule-associated protein light chain 3 (LC3), a mammalian homolog of yeast Atg8, is known to exist on autophagosomes, and therefore, this protein serves as a widely used marker for autophagosomes. Among the four LC3 isoforms, LC3B is most widely used. Soon after synthesis, nascent LC3 is processed at its C terminus by Atg4 and becomes LC3-I, which has a glycine residue at the C-terminal end. LC3-I is subsequently conjugated with phosphatidylethanolamine (PE) to become LC3-II (LC3-PE) by a ubiquitination-like enzymatic reaction. In contrast to the cytoplasmic localization of LC3-I, LC3-II associates with both the outer and inner membranes of the autophagosome.

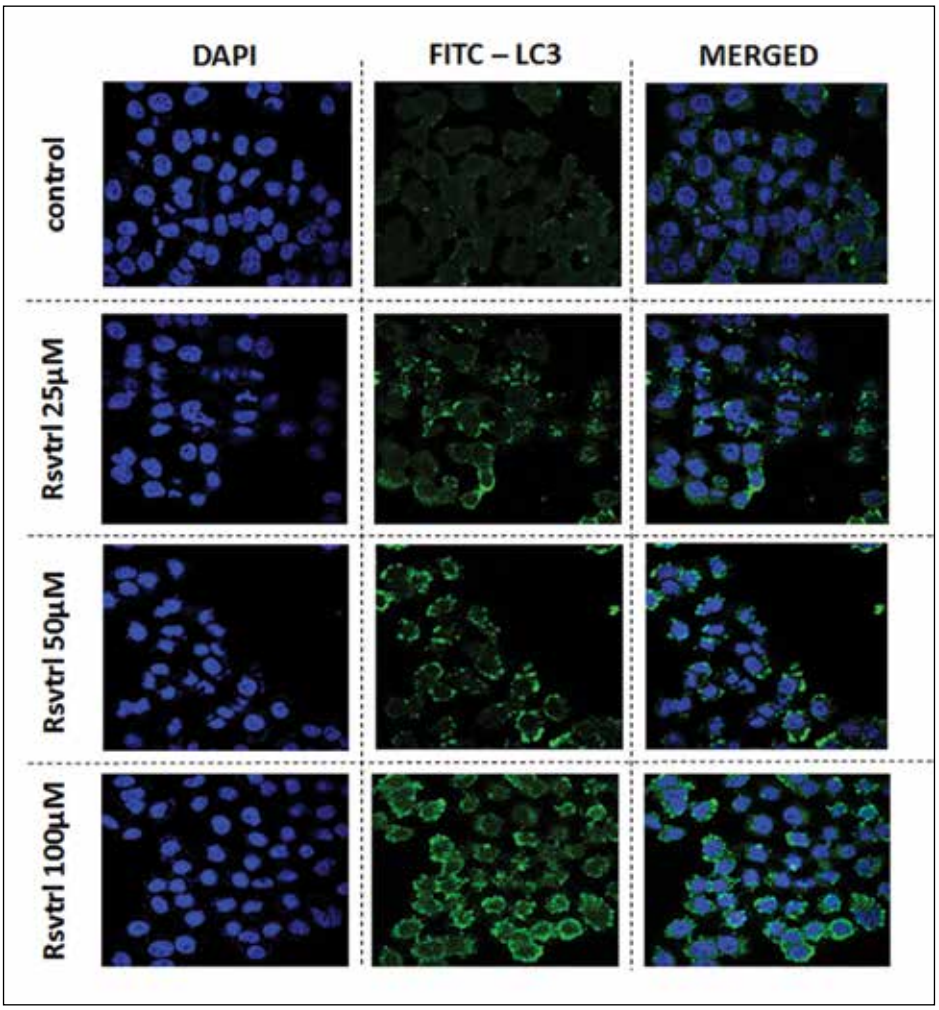


After fusion with the lysosome, LC3 on the outer membrane is cleaved off by Atg4 and LC3 on the inner membrane is degraded by lysosomal enzymes, resulting in very low LC3 content in the autolysosome. Thus, endogenous LC3 can be visualized by fluorescence microscopy either as a diffuse cytoplasmic pool or as punctate structures that primarily represent autophagosomes. As indicated in Figure, the control and DMSO-treated



cells revealed diffuse and weak LC3 punctate dots, whereas the resveratrol treated cells exhibited a cornucopia of green LC3 punctate dots in the cytoplasm. Increase in LC3 immunofluorescence was evident when concentration of resveratrol was increased; a similar pattern was observed when time of exposure to the drug was increased.

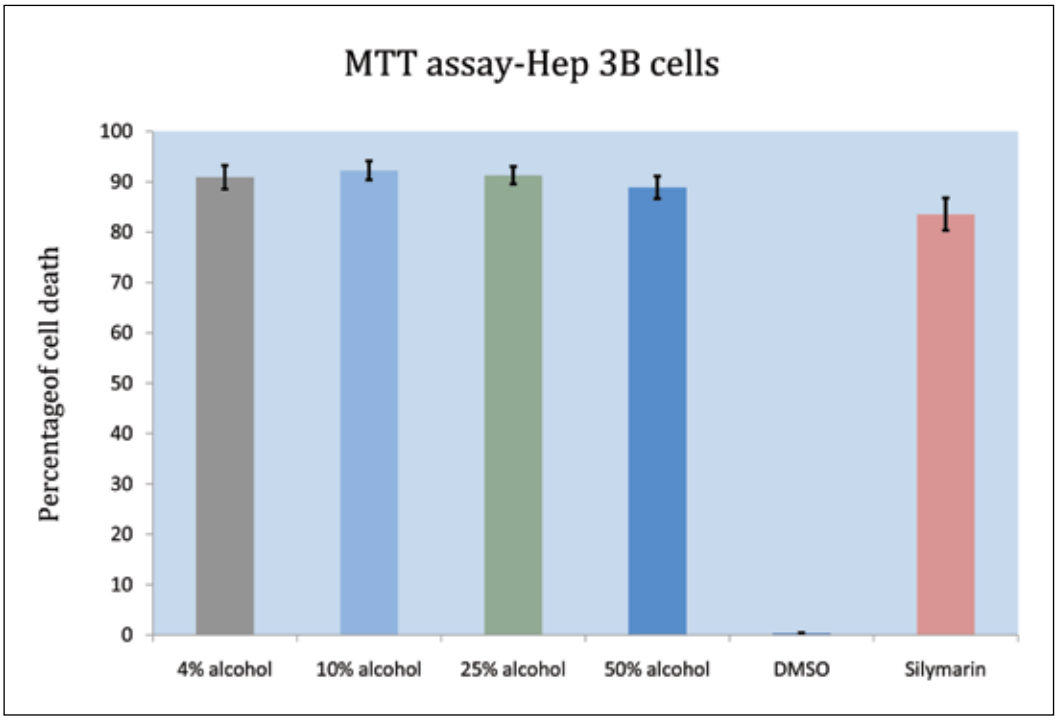
In addition to its utility in fluorescence microscopy assays, LC3 is also usefulin biochemical assays to assess autophagosome numbers. The conversion from endogenous LC3-I to LC3-II can be detected by immunoblotting with antibody against LC3. In Western blotting, LC3 is detected as two bands; cytosolic LC3-I and LC3-II, which is bound to PE in the autophagosome membrane. This makes the molecular weight of LC3-II (apparent MW is 14 kD) greater than LC3-I (apparent MW is 16 kD). However due to its hydrophobicity, LC3-II migrates faster in SDS-PAGE and therefore displays a lower apparent molecular weight. LC3-II was accumulated in resveratrol treated HepG2 cells, and the accumulation was more pronounced when the dose of resveratrol was increased. Beclin1 also showed a higher expression with increase in resveratrol concentration.



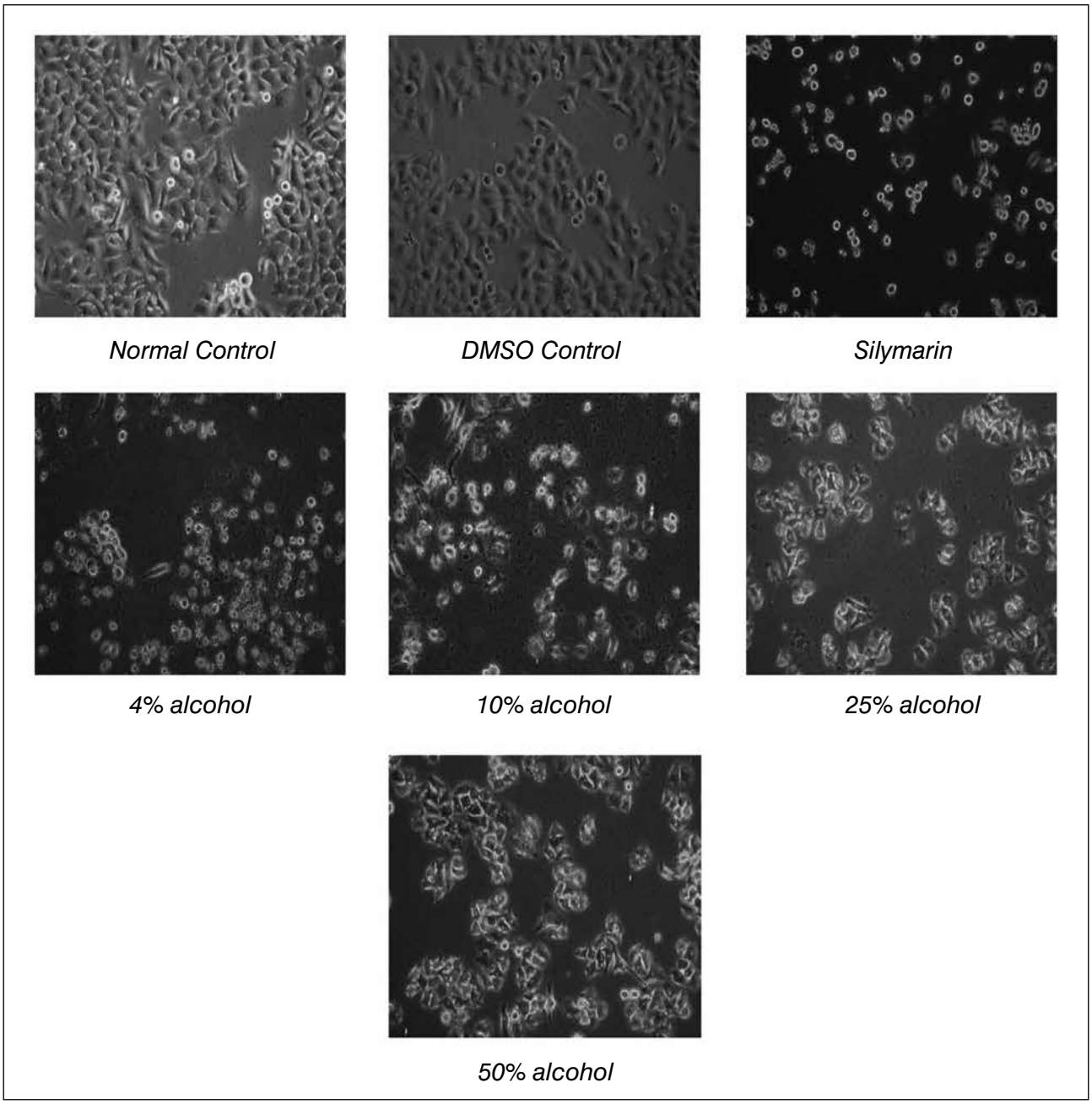
Identification of possible molecular mechanisms by which alcohol induces hepatotoxicity

Muneeb Hamza K H and Asha V V

According to the American Medical Association "alcoholism is an illness characterized by significant impairment that is directly associated with persistent and excessive use of alcohol. Impairment may involve physiological, psychological, or social dysfunction". Excess use of alcohol can have immediate and long term consequence on health and social life. About 76 million people are currently affected by alcoholic use disorders. According to Global Information System on Alcohol and Health (GISAH), about 2.5 million people are dying around the world due to harmful use of alcohol, mainly due to the liver injury caused by alcohol. Alcohol can induce cytotoxicity by different mechanisms. Alcohol induces cytochrome P4502E1 (CYP2E1) that in turn metabolizes and activates toxicologically important substrates such as ethanol, carbon tetrachloride, acetaminophen and N-nitrosodimethylamine and thereby produces more toxic substances. CYP2E1 dependent ethanol metabolism produces oxidative stress through the generation of reactive oxygen species (ROS), a possible mechanism by which alcohol induces hepatotoxicity. Pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) also play a significant role in onset of liver diseases. From the clinical studies with animal models of ethanol induced liver injury, enhanced circulation of TNF-α and other cytokines is observed in patients with alcohol liver disease (ALD). Identification of molecular mechanism behind alcohol induced liver damage simplifies the steps to develop herbal drugs against alcohol induced liver damage. Studies has been initiated to identify the possible mechanism(s) by which ethanol can induce liver injury. To begin with, assays were carried out to assess the possibility of apoptosis to cause liver injury. Different concentrations of alcohol were selected for the study. MTT assay showed that alcohol can inhibit the cell proliferation independent of concentrations (Fig 1).



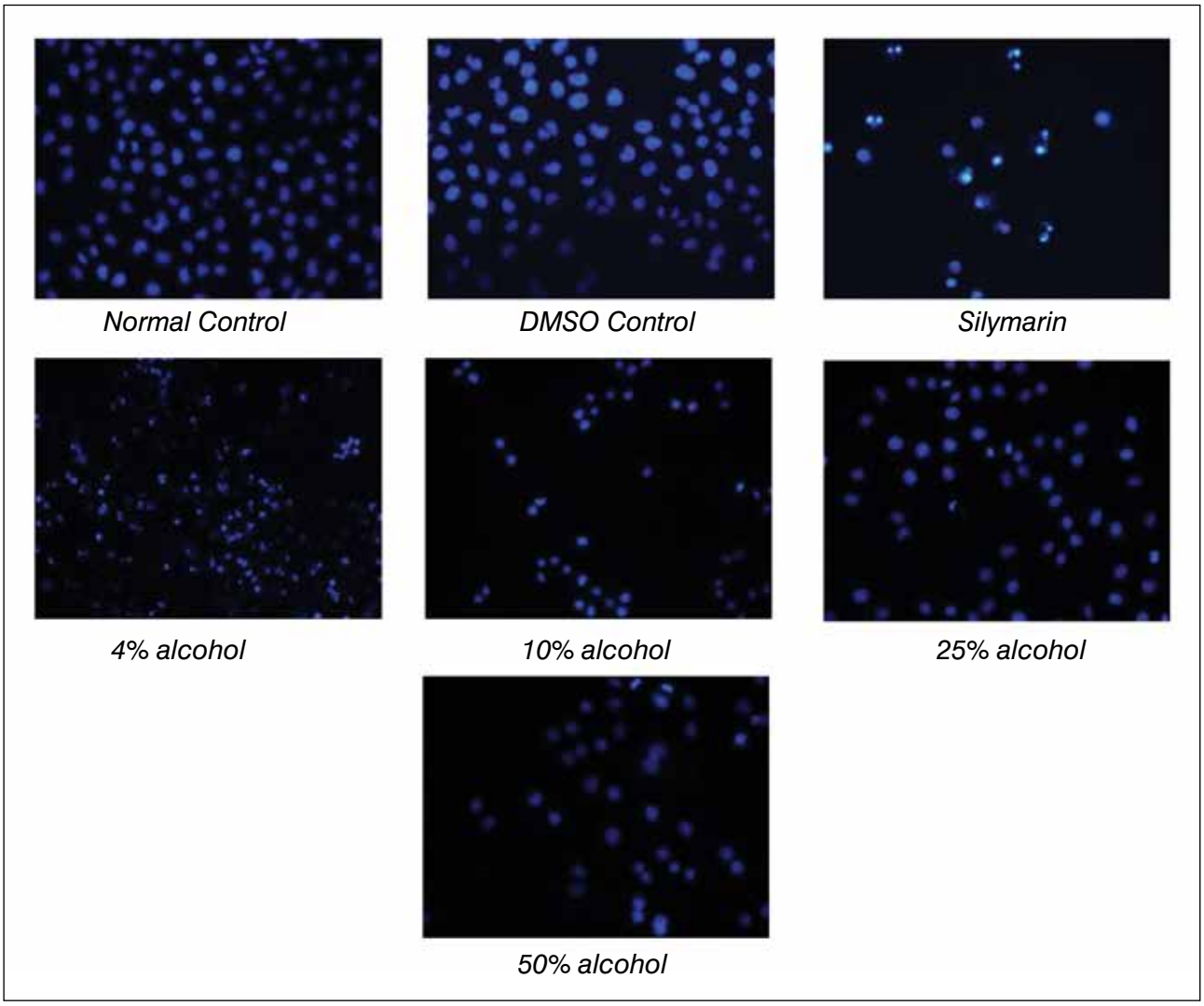
(Figure 1 showing the effect of different concentration of alcohol on cell viability)



(Figure 2 showing morphological changes on treatment with different concentrations of alcohol)

Cells treated with alcohol showed rough and distorted cell membrane compared to normal cells (Fig. 2). From Hoechst staining it has been evidenced that the chromatin condensation is observed only at 4% alcohol, which is an indication of apoptosis. Nucleus is found to be normal at high concentration of alcohol (Fig 3). Thus, which factors drive the cells to death at high concentration of alcohol is still a matter of controversy.

As a future plan, further investigations will be made to identify the exact mechanism of alcohol induced liver injury even when the concentration is increased. Once the mechanism is identified, attempt will be made to develop herbal drugs which can act against alcohol induced liver injury.



(Figure 3 showing effect of different concentration of alcohol on nuclear condensation)

PUBLICATIONS

- Suresh V., Sojan Jose, Krishna Radhika N., **Asha V. V.** (2014) Anti-HBV activity of the different extracts from Phyllanthus rheedei Wight in cell culture based assay systems. Journal of Ethnopharmacology *156, 309-315.*
- Sheeba M. S., Muneeb Hamza K.H., Krishna Radhika N. and **Asha V. V.** (2014) Molecular diversities among Cardiospermum halicacabum Linn. populations in Kerala assessed using RAPD markers. Annals of Phytomedicine *3(2): 87-92.*
- Krishnakumar K. A., Muneeb Hamza K.H. and Asha V. V. (2014) Antifibrotic activity of Phyllanthus maderaspatensis L. in Wistar rats. Annals of Phytomedicine *3(1): 68-79.*

CONFERENCE PRESENTATIONS

- Sreejith P. S and Asha V. V (2015). "Isolation and characterization of a novel chalcone derivative, Glycopentalone from Glycosmis pentaphylla (Retz.) Correa with potent anti-hepatocellular Carcinoma activity." International Symposium on Phytochemistry and Prof. Dr. A. Hisham Endowement Award Ceremony dated 25th April 2015 at Kerala State Science and Technology Museum, Thiruvananthapuram. (Poster Presentation).
- Greeshma Tom and Asha V. V (2015). "Studies on the interplay of autophagy and apoptosis in the chemotherapy of hepatocellular carcinoma using plant based anticancer lead molecules." International Symposium on Phytochemistry and Prof. Dr. A. Hisham Endowement Award Ceremony dated 25th April 2015 at Kerala State Science

and Technology Museum, Thiruvananthapuram. (Poster Presentation).

- Sheena Philip and Asha V. V. (2015). "Studies on the elucidation of the mechanism behind the anti-inflammatory activity of Tinospora cordifolia (Thunb) Miers." International Symposium on Phytochemistry and Prof. Dr. A. Hisham Endowement Award Ceremony dated 25th April 2015 at Kerala State Science and Technology Museum, Thiruvananthapuram. (Poster Presentation).

PLANT DISEASE BIOLOGY & BIOTECHNOLOGY

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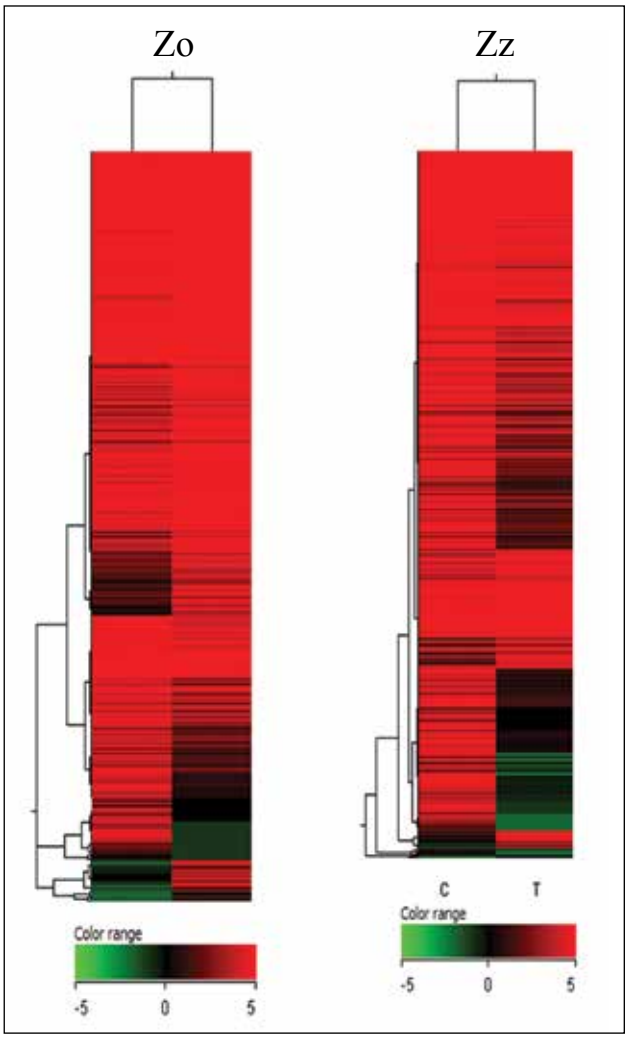
Vinitha M R
Neethu Mathew
Remya Madhu

Molecular basis of incompatible interactions in *Zingiber-Pythium* pathosystem.

Geethu Elizabath Thomas, Smini Varghese, Lesly Augustine, Remya Madhu and George Thomas

Relatively little is known about molecular basis of host resistance against necrotrophic oomycetes. *Pythium* is one of them and is capable of devastate crops once outbreak occurs. Source of resistance against this pathogen is scarce and so are control measures against their infection on plants. All cultivars of spice crop ginger *(Zingiber officinale)* in India are susceptible to soft rot disease caused by various *Pythium* spp. In an attempt to identify source of resistance against *Pythium,* we performed an extensive screening of wild congeners of ginger and identified *Pythium* resistance in *Zingiber zerumbet.* We performed comparative analysis between incompatible (resistant) *Z. zerumbet* – *Pythium* pathosystem and compatible (susceptible) ginger – *Pythium* pathosystem by employing several genomics tools and identified many genes with putative role in host response against *Pythium* infection. Although, these candidate gene approaches provided baseline information about the molecular reprogramming following *Pythium* infection, the relative contribution of each candidate gene in biological networks, which ultimately govern host defense, remained unclear. The recent advances in high throughput genomics technologies coupled with system biology approaches have rendered the elucidation of gene network associated with biological functions is largely feasible. Our long term goal is to elucidate the critical segment(s) in defense pathways that determine the incompatibility in *Z. zerumbet* against *Pythium* and to explore the possibility of translating the information in priming ginger against soft rot, as ginger is an obligate asexual and any breeding improvement using sexual hybridization is impossible.

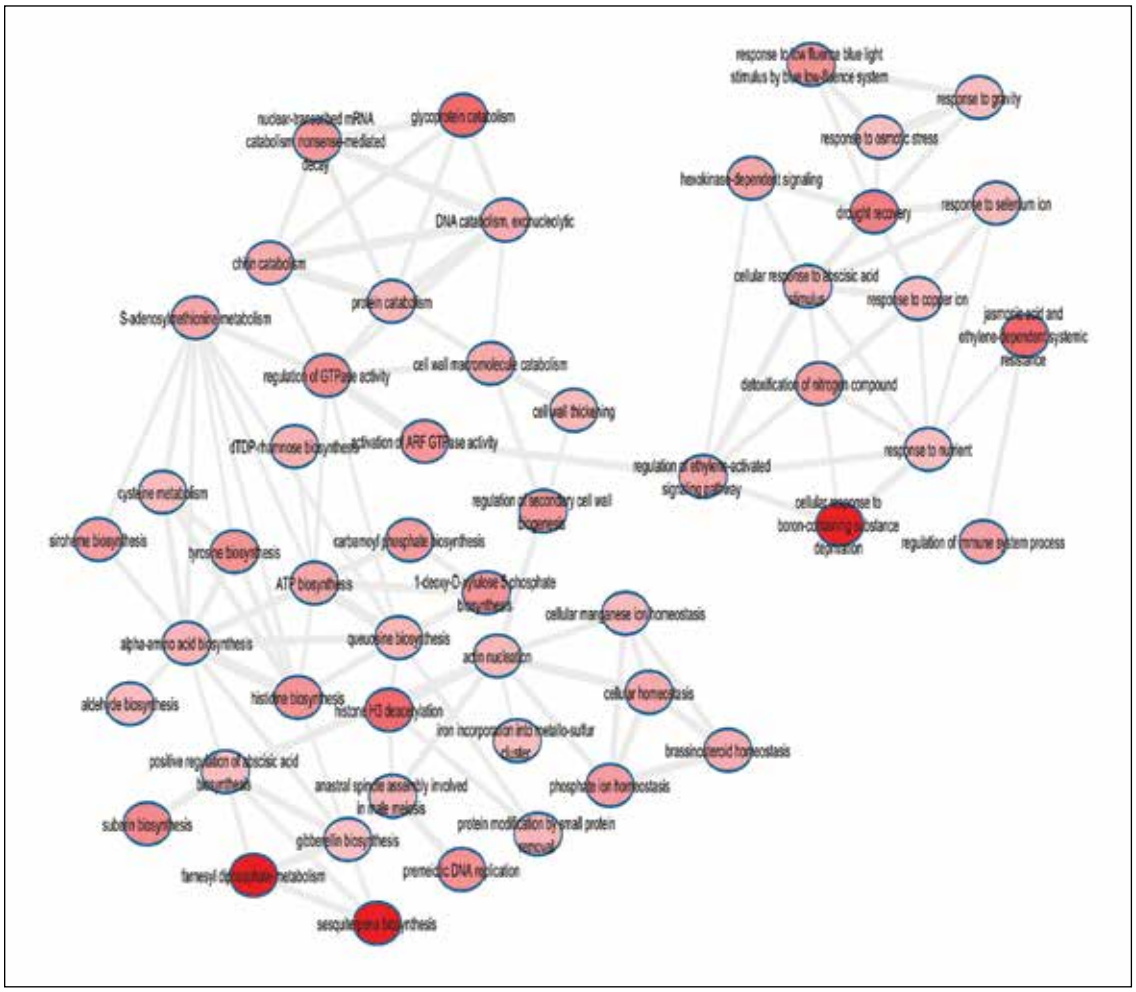


Hierarchical clustering of expression profiles of transcripts by comparing the digital gene expression between control (c) and 24h post inoculation (T) with Pythium in Z. officinale (Zo) and Z. zerumbet (Zz);

We developed four whole genome transcriptome libraries (RNA-Seq library) by sequencing the transcriptome in control and after *Pythium* inoculation in both ginger and *Pythium* resistant *Z. zerumbet*. The unigene sets were developed and digital gene expression was determined. Network of genes that are known to be associated with pathogen defense in other plant species were constructed using digital gene expression and relative role of different genes were examined. Transcripts of over 100 key genes identified in the networks were quantified by qPCR at different time periods following *Pythium* inoculation in both the species.

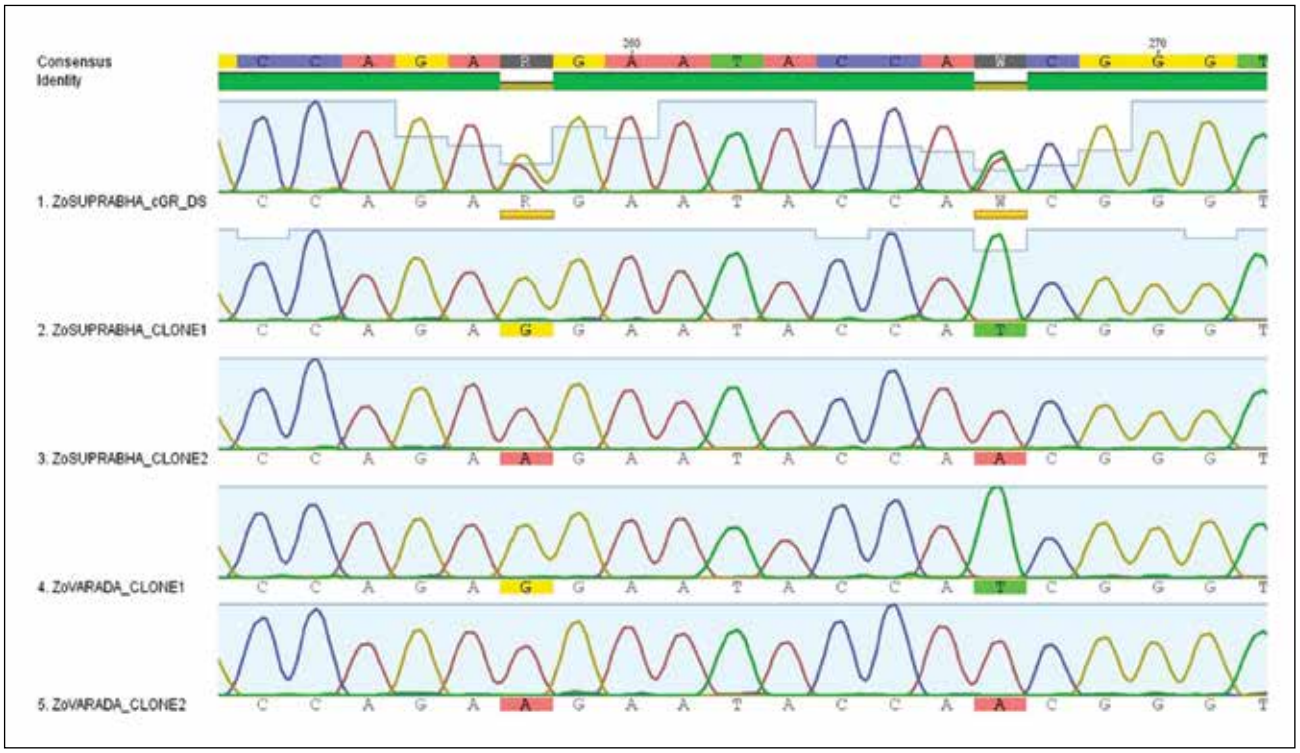
The interactive graph view of the enriched Gene Ontology (GO) of differentially reprogrammed molecular functions in *Z. zerumbet* after inoculating with *Pythium;* the bubble colour indicates the *p*-value and its significance in the plant-pathogen interaction.

The gene networks that were produced by integrating the digital gene expression and the qPCR datasets helped to fine tune the biological reprogramming in host against invading *Pythium* pathogen. Rigorous bioinformatics of data identified many segments in defense response pathways that are differentially modulated between compatible and incompatible pathosystems. Currently, we give much thrust to understand the basis of differential modulation of genes involved in reactive oxygen species (ROS) production, ethylene mediated signalling, hypersensitive response (HR) and the role of R- genes and phenylpropanoid pathway in determining incompatible reactions against *Pythium* in resistant *Zingiber zerumbet.* Sex is maintained in most species despite its two fold disadvantage of producing males, who themselves do not produce offspring. Of the numerous theories and models that hypothesize the mechanism of maintenance of sex, the trade-off between vegetative and sexual reproductive strategies, the eco-evolutionary feed backs of reproductive strategies and advantages of sexual reproduction, the Red Queen hypothesis and the Muller's ratchet are the major ones. These theories explain the two sides of the same coin. While Red Queen hypothesis postulate that pathogen maintain sex in hosts, because the sexual reproduction is essential for the host to generate genetic raw material to enter into evolutionary arms race with the invading pathogens, the Mullet ratchet postulates the extinction asexual due to the accumulation of deleterious mutations and excessive parasitism. Generation of genetic variability, development of



new allele combinations with adaptive fitness and purging out of deleterious mutation are the general advantages attributed to sexual reproduction over the asexual strategies. Evolutionary and ecological consequences of reproductive strategies of organisms attract evolutionary biologists and crop geneticists alike. Now we understand that anthropogenic disturbance to natural habitats and global warming negatively affect reproductive strategies of organisms and put biodiversity at risk. The relationship between reproductive strategy and genetic diversity is well documented. But the contribution of genetic diversity to the evolution of adaptive trait is not well understood.

Family Zingiberaceae constitute a valuable system to study the relationship between breeding system and the molecular genetics of disease resistance in plants. Cultivated ginger is obligatory asexual. Our earlier studies revealed seed multiplication in wild congeners *Z. cernuum and Z. neesanum* and mixed breeding system in *Z. zerumbet,* which comprises of certain populations with seed set while absolutely no seed set in others. Corroborating theoretical expectations, the intra-specific diversity in these four species corresponded with reproductive strategies. However, the response of these species to soft-rot pathogen *Pythium* mostly contrasted with the postulations of Red Queen hypothesis. We investigated the reason behind the contradiction to Red Queen hypothesis observed in *Zingiber* species in pathogen resistance by analyzing nucleotide diversity of 40 segments of 32 genes, covering altogether 20 kb, in *Z. zerumbet* populations and ginger cultivars. Varying levels of heterozygosity was evident in analyzed samples. The data suggest distinct difference in the genome architecture between resistant and susceptible accessions, and also the contribution of the past evolutionary history in the evolution of resistance. Comparative analysis of genome architecture, level of diversity and pathogen response between ginger and *Z. zerumbet* provided deep insight into the molecular genetics of pathogen response in hosts with contrasting breeding system.



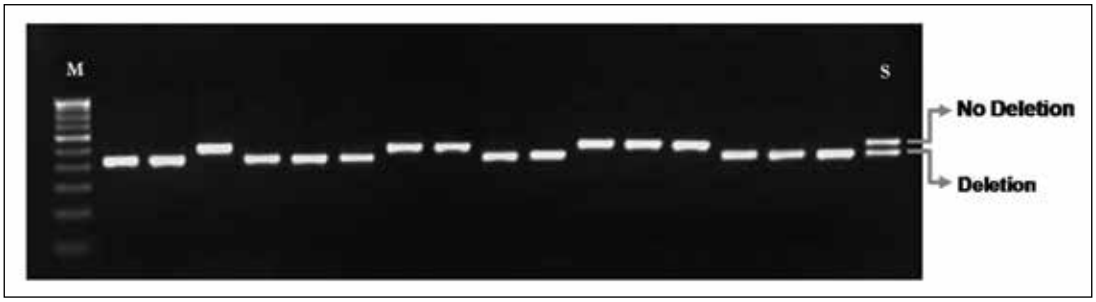
Cloning and sequencing of PCR amplified gene fragments reveals identical haplotype configuration in distinct ginger varieties

Microsatellite assisted genetic resources characterization and circumscription of the medicinal rice 'Njavara'

George Varghese, Vinitha M. R., Neethu Mathew and George Thomas.

The rice cultivar *Shashtika* is referred in several Sanskrit Ayurveda texts written in Northern part of India from late BC to eighteenth century AD. According to these texts, *shashtika* is a short duration cultivar (matures in 60 days), medicinally and nutritionally rich, best in *vrihi* type of rice and mitigates three *doshas*. The medicinal rice Njavara, which is traditionally considered endemic to South India, especially to the present day Kerala state is a *Shashtika*. This has aroused interest in us: how can *shashtika*, which is referred by North Indian authors, be endemic to Kerala?' and 'why is *Shashtika* not seen now a days in other parts of India?'.

Earlier we performed detailed analyses of over 900 rice samples including the *O. rufipogon*, the immediate progenitor of cultivate rice using many phylogenetic tools. The pattern of distribution of cultivars belonging to different ancestral groups was elucidated and different *O. rufipogon* populations that progenitored the cultivated rice populations were identified. Although, the analysis helped to gain deep insight into the origin and evolution of cultivated rice, including Njavara in India, little is understood about the contribution of past evolutionary history of *O. rufipogon* in deciding the present day genetic structure of cultivated rice. Geographic centre of origin of Njavara rice and its spread to Kerala is intertwined with the origin and evolution of rice in India. The nature of chloroplast genome - nuclear genome relationships can provide in depth knowledge about the past evolutionary history of a plant species. We determined the chloroplast genotypes in *O. rufipogon* and two distinct genotypes were identified, one closest to *japonica* ancestral group of rice and the other to *indica* and *aus* ancestral groups. Further analysis is underway by comparing the chloroplast and nuclear genomes.



Chloroplast genotype of Oryza rufipogon, the immediate progenitor of cultivated rice. The no-deletion genotype is phylogenetically close to japonica ancestral group of cultivated rice while the deletion genotype close to indica.

PUBLICATIONS

- Mahadevan C, Jaleel A, Deb L, Thomas G. and Sakuntala M. 2015. Development of an efficient virus induced gene silencing strategy in the non-model wild ginger-Zingiber zerumbet and investigation of associated proteome changes. PLoS ONE 10(4): e0124518. doi: 10.1371/journal.pone.0124518
- Aishwarya K, Vinitha M, Thomas G. and Sabu M. 2015. A new species of Boesenbergia and rediscovery of B. rotunda (Zingiberaceae) from India. Phytotaxa 197: (3): 186–196.

EXTRAMURAL FUNDING

Investigator	Title	Funding Agency	Duration
George Thomas Collaborator: V. G. Jayalekshmy, Kerala Agricultural University	Development of rice varieties for Kerala with pyramided genes for resistance to BLB by marker assisted selection	Department of Biotechnology (DBT)	2013 - 2018
George Thomas	De novo transcriptome sequencing microarray development and elucidation of Pythium responsive defense pathways in Zingiber zerumbet Smith	Council of Scientific and Industrial Research	2015 to 2017

PLANT DISEASE BIOLOGY & BIOTECHNOLOGY

PDDB LABORATORY – 3

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Soniya was awarded PhD from the Department of Botany, University of Kerala. Following this, she worked as a Research Associate at Central Tuber Crops Research Institute, Thiruvananthapuram. She was a INSA/DFG visiting Scientist in Max Planck Institute of Chemical Ecology, Jena, Germany.

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Characterization of stress responsive microRNAs and other small functional non-coding RNAs from black pepper *(Piper nigrum L.)*

Asha S, Sweda Sreekumar, Divya Kattupalli and E V Soniya

MiRNA guided gene regulation plays a crucial role in the plant defence against pathogens and abiotic factors. Our study focused to trace out stress responsive miRNAs and other functional small RNAs from black pepper *(Piper nigrum)*, widely known as 'King of spices'. The deep sequencing analysis of sRNAs revealed micro RNAs and other functional small RNAs from black pepper. The conserved miRNAs were validated by stem loop qRT PCR and their secondary structures were *in silico* predicted. We could also characterize black pepper specific novel miRNAs (Fig 1) and their corresponding targets were predicted. They include transcription factors and the genes involved in diverse physiological processes and plant pathogen interactions. MiRNA mediated cleavage was experimentally validated on their respective mRNA targets. The elucidation of microRNA and other small RNA mediated regulation of important genes in plant-pathogen interaction as well as stress signalling pathways are in progress.

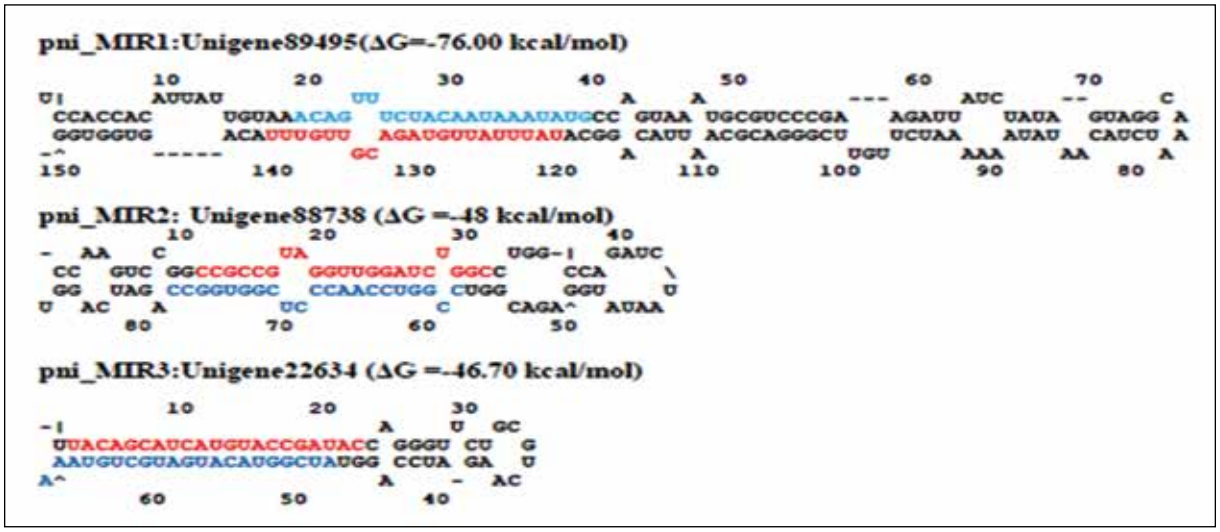

Fig.1. ***The secondary hairpin structures black pepper specific miRNAs predicted by MFOLD. Mature miRNA*** *(red colour) and miRNA* sequence (blue colour) are highlighted in the predicted precursors.*

Identification of novel forms of Type III polyketide synthases for exploiting its potential use in metabolic engineering

Aiswarya G, Mallika V and E V Soniya

Type III polyketide synthases are the largest family of multi-functional enzymes that produce an array of natural products with remarkable biological and pharmacological properties. Among the three different form of PKSs (type I, II and III), type III PKSs are structurally and mechanically distinct from the other PKSs and can be easily distinguished by their physical composition. The

Figure1: Purified fractions of MSD1 and MSD2

Figure2: EoCHS in HEPES + NaCl after concentration

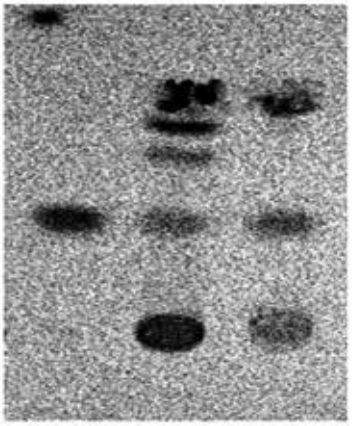

Figure3: Radio-TLC profile of MSD1 and MSD2 with P-coumaroyl CoA

enormous biochemical diversity is revealed by the isolation and characterization of the type III PKSs including chalcone synthase (CHS), 2-pyrone synthase, acridone synthase, quinolone synthase etc. The catalytic promiscuity of type III PKS make an excellent platform and ideal candidate for the production of unnatural natural products. *Emblica officinalis* Gaertn (Indian gooseberry), is a key medicinal plant used in unani and ayurvedic systems of medicine, due to the presence of flavonoids, tannins, alkaloids and other phenolic compounds. Type III Polyketide synthase is a key enzyme for the synthesis of these compounds. Our present study describes characterization of a novel type III PKS from *Emblica officinalis*. A cDNA, encoding chalcone synthase (*EoCHS1*) was for first time cloned and sequenced and its polymorphic nature was confirmed by southern hybridization. Recombinant EoCHS was successfully expressed in *E. coli* BL21 and purified. Radio labeled TLC resulted in several bands, which confirmed the wide range affinity of EoCHS to various substrates ranging from aliphatic to aromatic CoA's. The product resulted was characterized as naringenin which confirmed its chalcone synthase activity. EoCHS mutants (MSD1 and MSD2) were generated by site directed mutagenesis and over expressed and purified. The homology models of both mutants showed remarkable enlargement of the cavity volume when compared to wild type, which suggested that the mutants are capable of accommodating bulkier starter substrate. Radio-TLC with p-coumaroyl CoA resulted in products, which confirmed that both of the mutant proteins are functionally active. Further assay and product characterization need to be done. Kinetic studies of wild and mutants are going on for studying the optimum substrate concentration and other conditions.

Additionally, we already reported a novel type III PKS named QNS that produce acridone/quinolone in *Aegle marmelos* (Rutaceae) (Resmi et al., 2013; Resmi et al., 2014). Kinetic analysis indicated that the catalytic efficiency of QNS protein to accept larger acyl-CoA substrate is several fold higher than that for smaller substrates. Further, modelling and mutagenesis studies provide an insight into the structural mechanism for the enzyme that could be used to generate pharmaceutically important products. Currently, our research interests also include three dimensional structure of type III PKSs with their associated substrates, in order to find out the exact reaction mechanism behind the wide substrate affinity. The knowledge of accurate molecular structures is a prerequisite for rational drug design which will aid the development of effective therapeutic drugs.

PUBLICATIONS

- Resmi M S, Vivek P J, Soniya E V (2015). Over-expression of bael quinolone synthase in tobacco improves plant vigor under favorable conditions, drought, or salt stress. FEBS Letters. Jan 30; 589(3):332-41. doi: 10.1016/j. febslet. 2014.12.016. Epub 2014
- Tara G Menon and E V Soniya (2014. Isolation and characterization of salt-induced genes from Rhizophora apiculata Blume, a true mangrove by suppression subtractive hybridization. Current Science. August 25; 107 (4).

CONFERENCE PRESENTATIONS

- Aiswarya G, Mallika V and E.V Soniya. Functional characterization and kinetic studies of Chalcone synthase from Emblica officinalis Gaertn. 6th Annual Meeting of Proteomics Society, India and International Conference on proteomics from Discovery to Function held at the Indian Institute of Technology Bombay (7th-9th December 2014).
- Mallika V, Aiswarya G and E.V Soniya. "Insights in to the protein-small molecule interaction of Quinolone synthase from Aegle marmelos Corr." 6th Annual Meeting of Proteomics Society, India and International Conference on proteomics from Discovery to Function held at the Indian Institute of Technology Bombay (7th-9th December 2014)

EXTRA MURAL FUNDING

Title of Project	Funding agency	Duration
Identification and functional validation of Type III PKS involved in quinolone alkaloid biosynthesis in Aegle marmelos Corr.	Department of Science and Technology, Government of India	2012-2015
Cataloguing of micro RNAs and elucidation of its role in stress adaptation/response in black pepper	Department of Bio Technology, Government of India	2015-18

PLANT DISEASE BIOLOGY & BIOTECHNOLOGY

PDDB LABORATORY – 4

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Molecular mechanism of disease resistance in *Piper colubrinum*

Anu K, Gayathri G S and Manjula S

The study mainly focuses on cloning and functional validation of candidate defense genes from the resistant wild *Piper* sp-*P.colubrinum*. *Serine Threonine Protein Kinase (PcSTPK)* is one prominent member, which has vital role in pathogen defense as evidenced by gene silencing approaches. In order to further establish its role as a potential defense gene candidate, we attempted overexpression of *STPK* in susceptible model plants. Full-length *PcSTPK* sequence was deduced by aligning and assembling the sequences of 5' and 3' RACE PCR products of 2 kb and 500bp respectively. An open reading frame of 1.7 kb was amplified from *P. colubrinum* cDNA with added KpnI and Bam HI restriction sequences to facilitate cloning into the expression vector pCAMBIA1305.2 (Fig 1). Purified *PcSTPK* PCR products were cloned into pGEM-T easy vector and transformed into DH5α *E. coli* competent cells. pGEM-T:*PcSTPK* plasmids were isolated from positive colonies after confirmation by colony PCR. Colony PCR was perfomed using hygromycin gene specific primers. Both pCAMBIA1305.2 and pGEM-T:*PcSTPK* were digested using *KpnI* and *BamHI* restriction enzymes (Fig 2). Purified pCAMBIA1305.2 plasmid and *PcSTPK* were cloned and transformed to DH5α *E. coli* competent cells. Plasmids isolated from positive colonies were transformed into GV3103 *Agrobacterium* cells and used for overexpression studies. on of full length PcSTPK

To transiently overexpress PcSTPK, Agrobacterium strain harbouring binary vector were grown overnight in 5 mL LB medium containing antibiotics (50 µg/ml kanamycin, 25 µg/ml rifampicin) at 28°C in an orbital shaker maintained at 180 rpm. In the next day, 100µl of the initial culture was transferred to 50 mL LB medium with antibiotics and was incubated until it reaches an O.D600 of 0.8–1.0. The cells were pelleted and resuspended in infiltration media (10 mM MgCl2, 10 mM 2-(Nmorpholino) ethanesulfonic acid (MES; SIGMA), pH 5.7 and 200 lM acetosyringone (SIGMA), and incubated at room temperature for 3 h without shaking. pCAMBIA 1305.2 vector contains GUS plus gene as reporter gene (ORF interrupted with a catalase intron) and kanamycin as resistance marker for bacterial selection. To check the transient expression of PcSTPK in planta, the Agrobacterium cultures were infiltrated into Arabidopsis thaliana (var Col 0) leaves using a needless syringe. Three days post agroinfiltration Arabidopsis leaves were checked for transient expression of gene by GUS assay (Ref..). Blue coloration in the agroinfiltrated area confirmed GUS activity, which confirmed successful transient expression of PcSTPK (Fig 3).

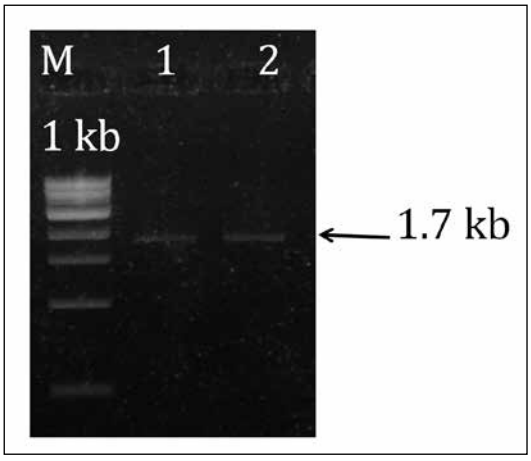

Fig 1: PCR amplification of full length *PcSTPK*

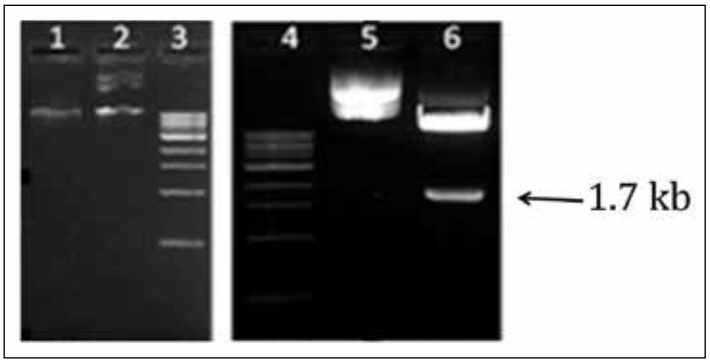

Fig 2: Restriction digestion of pCAMBIA 1305.2 & pGEMT *Pc-STPK* using *KpnI* & *BamHI* . Lane 1- *KpnI* and *BamHI* digested pCAMBIA1305.2 plasmid, Lane 2- Uncut pCAMBIA1305.2 vector, Lanes 3 & 4 -1kb ladder, Lane 5- Uncut pGEM-T:*PcSTPK* plasmid, Lanes 6- *KpnI* and *BamHI* digested pGEM-T:*PcSTPK* plasmid.

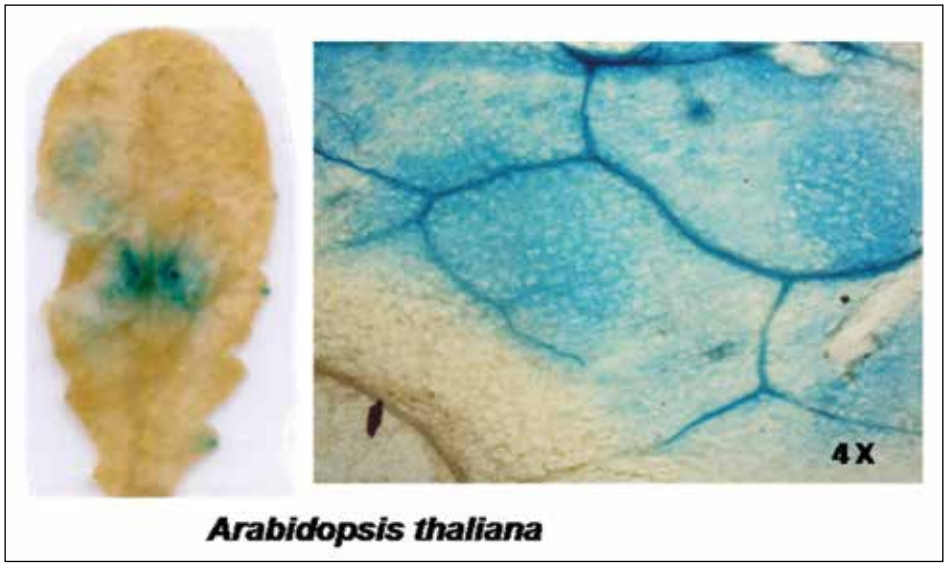

Fig 3: Transient expression of GUS in pCAMBIA:*PcSTPK* agroinfiltrated *Arabidopsis* leaf ; left panel- whole leaf after GUS staining; right panel- photomicrograph of leaf cells (x).

PcSTPK overexpression in *Nicotiana tabacum* and *N.benthamiana* and investigation of its pathogen defense role

Nicotiana tabacum and *Nicotiana benthamiana* model systems were selected to functionally validate the role of *PcSTPK* by transient over expression. The pCAMBIA:*PcSTPK* harbouring *Agrobacterium* were infiltrated into the lower surface of leaves with a needless syringe. One half of the leaf was infiltrated with empty vector harbouring *Agrobacterium* (control inoculation) and upper half was infiltrated with *PcSTPK* harbouring *Agrobacterium* (Fig.4 left panel and Fig.5 0h). After 3 days, *PcSTPK* infiltrated area appeared more green compared to empty vector infiltrated area. The leaf areas were challenged with pathogen. Virulent strains of *P. capsici* were used to

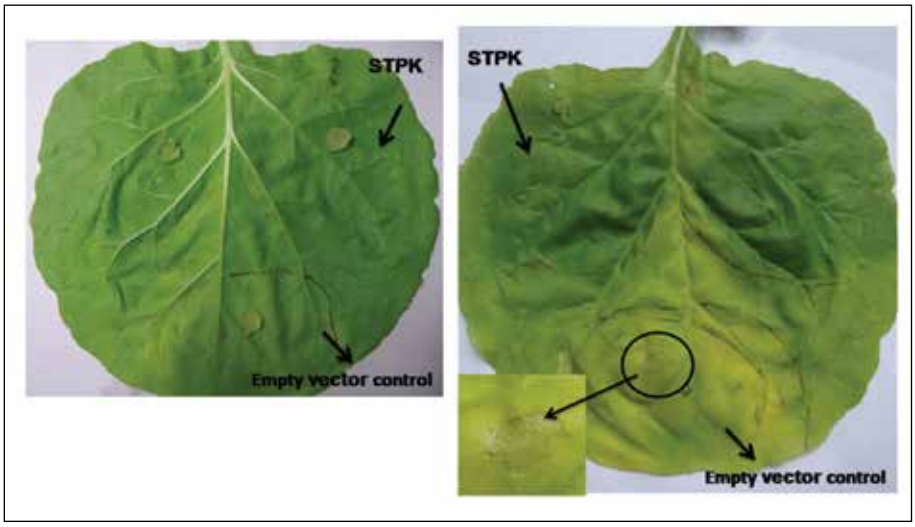

Fig 4 : *Nicotiana benthamiana* leaves overexpressing PcSTPK.

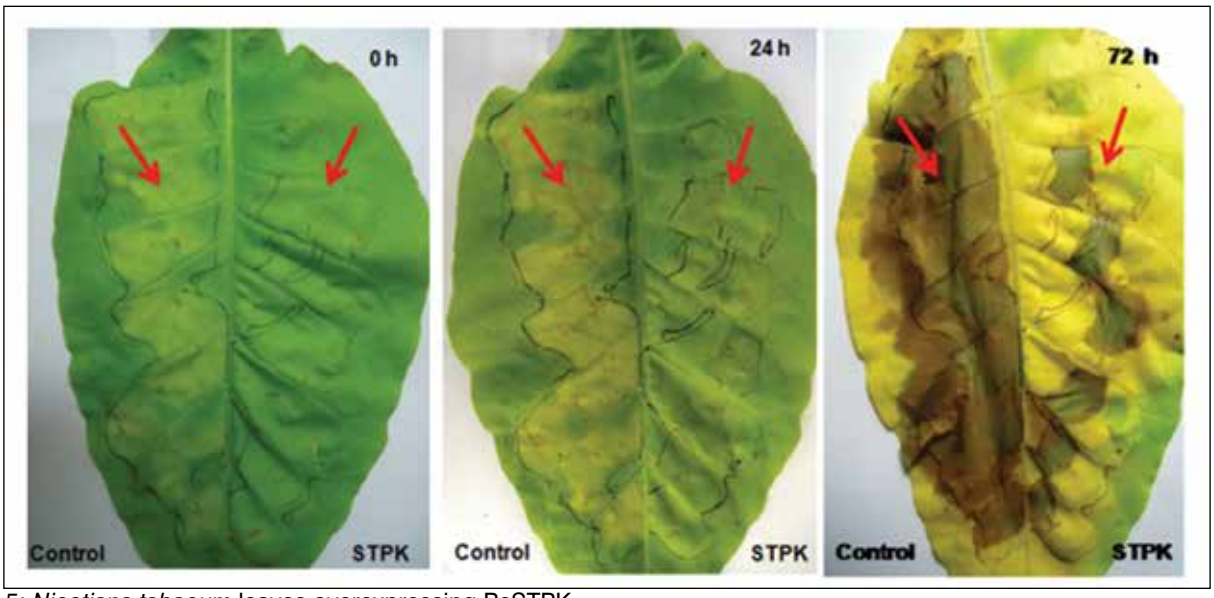

Fig 5: *Nicotiana tabacum* leaves overexpressing PcSTPK

infect leaves of *Nicotiana benthamiana* which is a known host for *P.capsici* and the bacterial pathogen *Pseudomonas syringae* were used to infect *Nicotiana tabacum*, which is not a natural host to *P.capsici*. Pathogen challenged leaf areas were monitored every 12 hours. In *Nicotiana benthamiana* leaves challenged with *Phytophtora capsici,* the disease symptoms (necrotic lesions) appeared by 24 hours in empty vector infected area where as *PcSTPK* over expressed area did not show any symptom and remained healthy (Fig 4 right panel). Similarly, *PcSTPK* overexpression *Nicotiana tabacum* leaf showed delayed onset of disease symptoms uoto 72h by *Pseudomonas syringae* (Fig 5). The results of transient overexpression studies support the defense role of *PcSTPK*.

The *PcSTPK* protein isolation and the generation of *PcSTPK* overexpressing stable transgenic lines of *Arabidopsis thaliana* are in progress.

Molecular analysis of Pathogen Associated Molecular Patterns (PAMP) - triggered immunity in *Piper nigrum – Phytophthora capsici phytopathosystem.*

Chidambareswaren. M and Manjula. S

Plant immunity is an interconnected pathway involving transcriptome and proteome changes. Understanding the molecular protein components especially those that are differentially expressed during host-pathogen interaction have recently been explored in diverse omics platforms. Black pepper (*Piper nigrum* L), a major spice of Kerala has significantly been affected by an oomycete hemibiotrophic pathogen, *Phytophthora capsici.* Identification and characterization of innate immune responsive components of black pepper remains a major challenge towards development of crop improvement and crop protection strategies. Our initial efforts to understand the transcript level response of black pepper genes against known defense elicitors suggest that early innate immune responses in black pepper are in consensus with known model systems like *Arabidopsis thaliana*. Consequently, *Somatic embryogenesis receptor-like kinase 3* (*PnSERK3*) and *Mitogen activated*

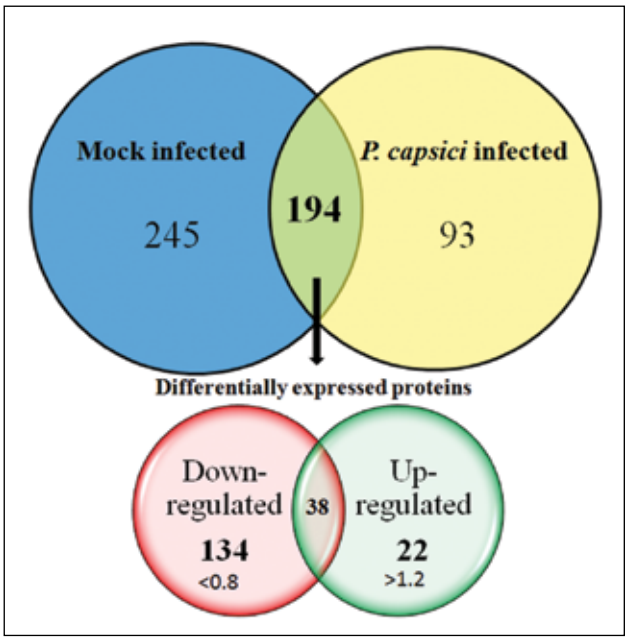

Figure-1.Venn diagram showing the representation of proteins identified in the leaf proteome of black pepper. Based on PLGS ratio between control vs. test, 194 proteins were found to be differentially expressed of which 134 proteins were differentially down-regulated and 22 proteins were up-regulated when challenged with *P. capsici* at 24hpi.

protein kinase 8 (*PnMAPK8*) revealed significant up-regulation during the elicitor treatment at various early time points. We then attempted to study the proteome changes associated with early immune responses of black pepper against *P. capsici* infection at three time points, 6hpi, 12hpi and 24hpi against uninfected leaf control proteins using a nanoscale ultraperformance liquid chromatography–coupled to a Quadrupole-Time of Flight mass spectrometer (SYNAPT-G2- HDMS^E, Waters Corporation). The Protein Lynx Global Server (PLGS 2.5.3) (Waters Corp., Manchester, U.K.) was used to process the continuum LC–MS data. We have for the first time developed a transcriptome assisted label-free quantitative proteomics to identify novel molecular components regulated during early host immune response of black pepper leaves when challenged with *P. capsici.* Analysis of the proteomic data resulted in identification of 532 novel proteins, which were completely annotated in the present study. Of these, 194 proteins were differentially regulated between control and test protein dataset, 245 proteins were expressed in control protein dataset and 93 were uniquely identified in test protein dataset (Fig.1).

Comprehensive functional annotation of the identified protein components was carried out using BLAST2GO tool which lead to identifying of novel proteins and genes from black pepper. Major differential expression took place in a number of pathways underlying the response of black pepper to *P. capsici* infection such as photosynthesis, protein processing in endoplasmic reticulum, carbon metabolism, oxidative phosphorylation and endocytosis. Some of the significantly down-regulated protein components have roles

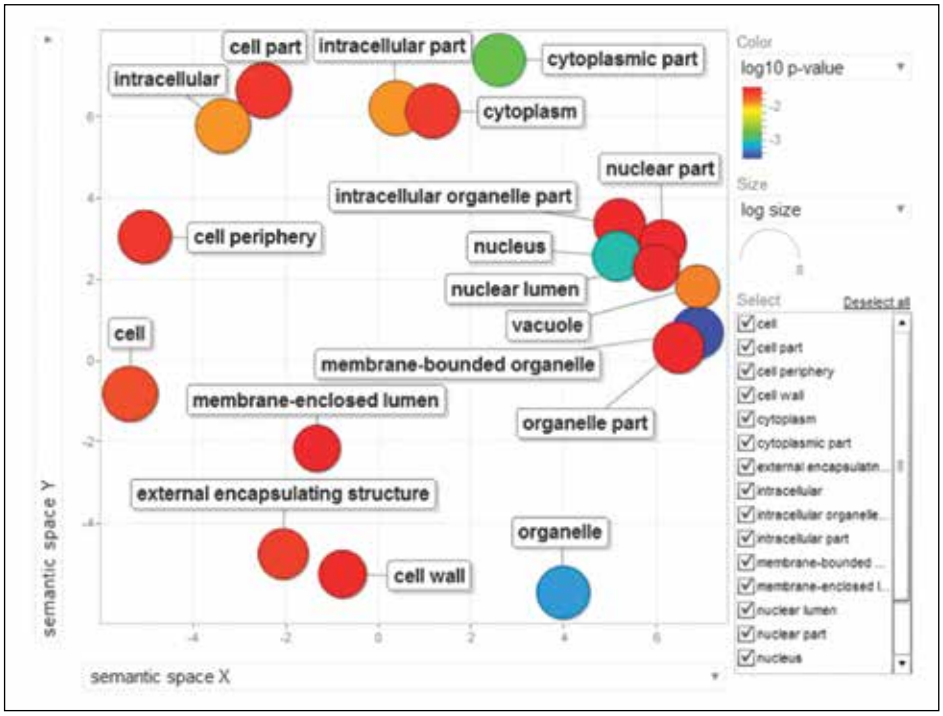

Figure-2. Cellular component distribution of differentially down-regulated proteins based on p-value and uniqueness of GO terms.

in carbohydrate metabolic process, response to stress, single-organism transport, transmembrane transport, response to stimulus and single-organism localization, as identified by Revigo tool (Figs.2-4).

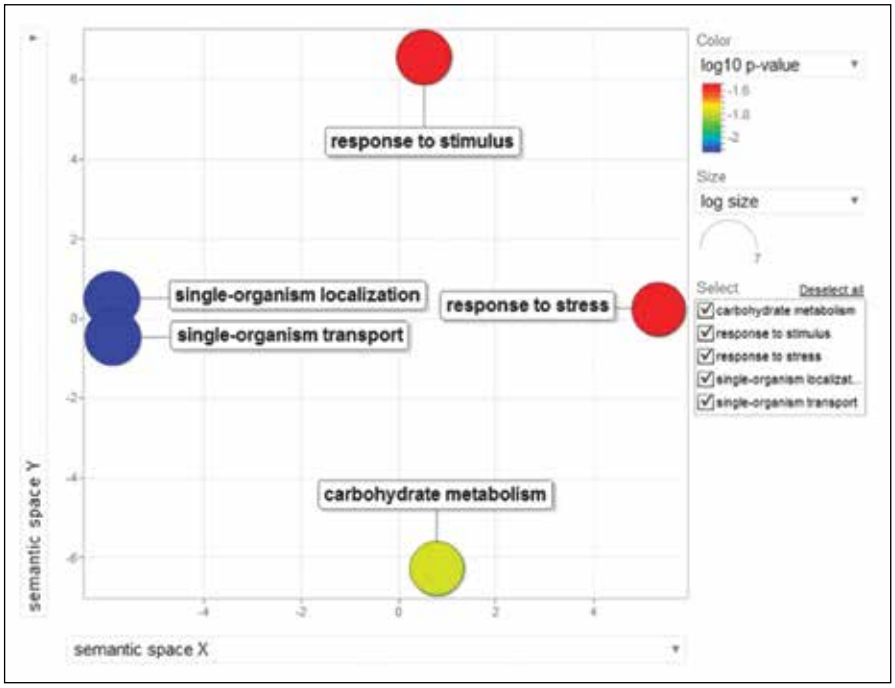

Figure-3. Biological process distribution of differentially down-regulated proteins based on p-value and uniqueness of GO terms.

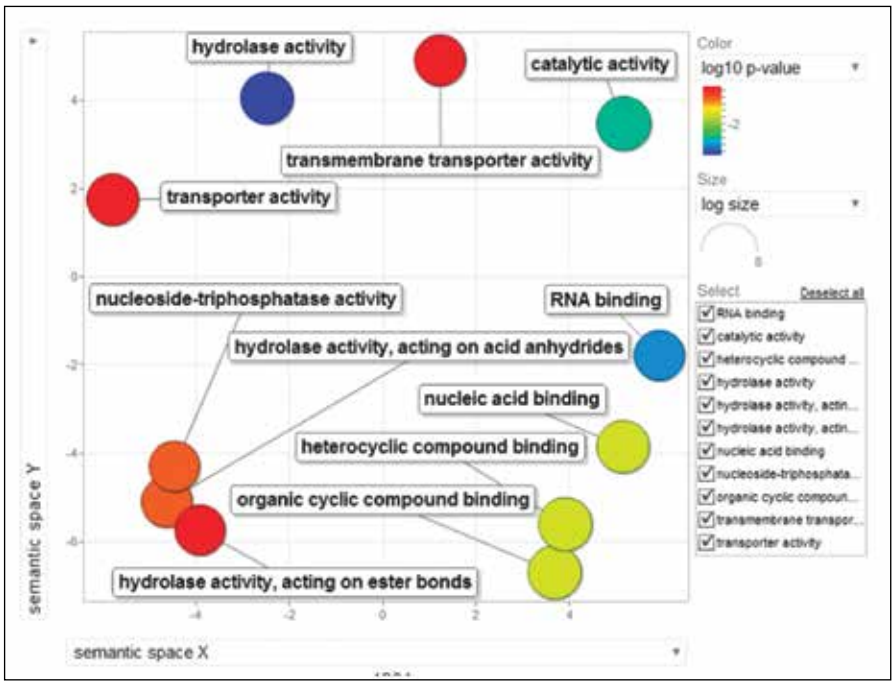

Figure-4. Molecular function distribution of differentially down-regulated proteins based on p-value and uniqueness of GO terms.

Protein–protein interaction network for the differentially expressed proteins was derived from the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database v9.1 containing predicted and known physical and functional association between protein molecules based on the evidences retrieved from seven different evidences. One hundred and ninety four protein sequences were initially mapped to STRING protein database of *Arabidopsis thaliana*. Protein interactions retrieved from STRING analysis only with high confidence score (≥0.9) were retained in the network. STRING analysis revealed predicted protein–protein interaction among proteins especially involved in the Photosynthesis, metabolic pathways, carbon fixation in photosynthetic organism, photosynthesis-antenna protein, protein processing in endoplasmic reticulum, carbon metabolism, glycoxylate and dicarboxylate metabolism, oxidative phosphorylation, micobial metabolism in diverse environment, endocytosis and spliceosome (Fig.5).

The quantitative proteomics dataset was further validated using Real Time qPCR analysis. The expression of ten black pepper genes that were differentially up-regulated or down-regulated during late necrotrophic growth, i.e. 24hpi are in consensus with our proteomic results (Fig.6).

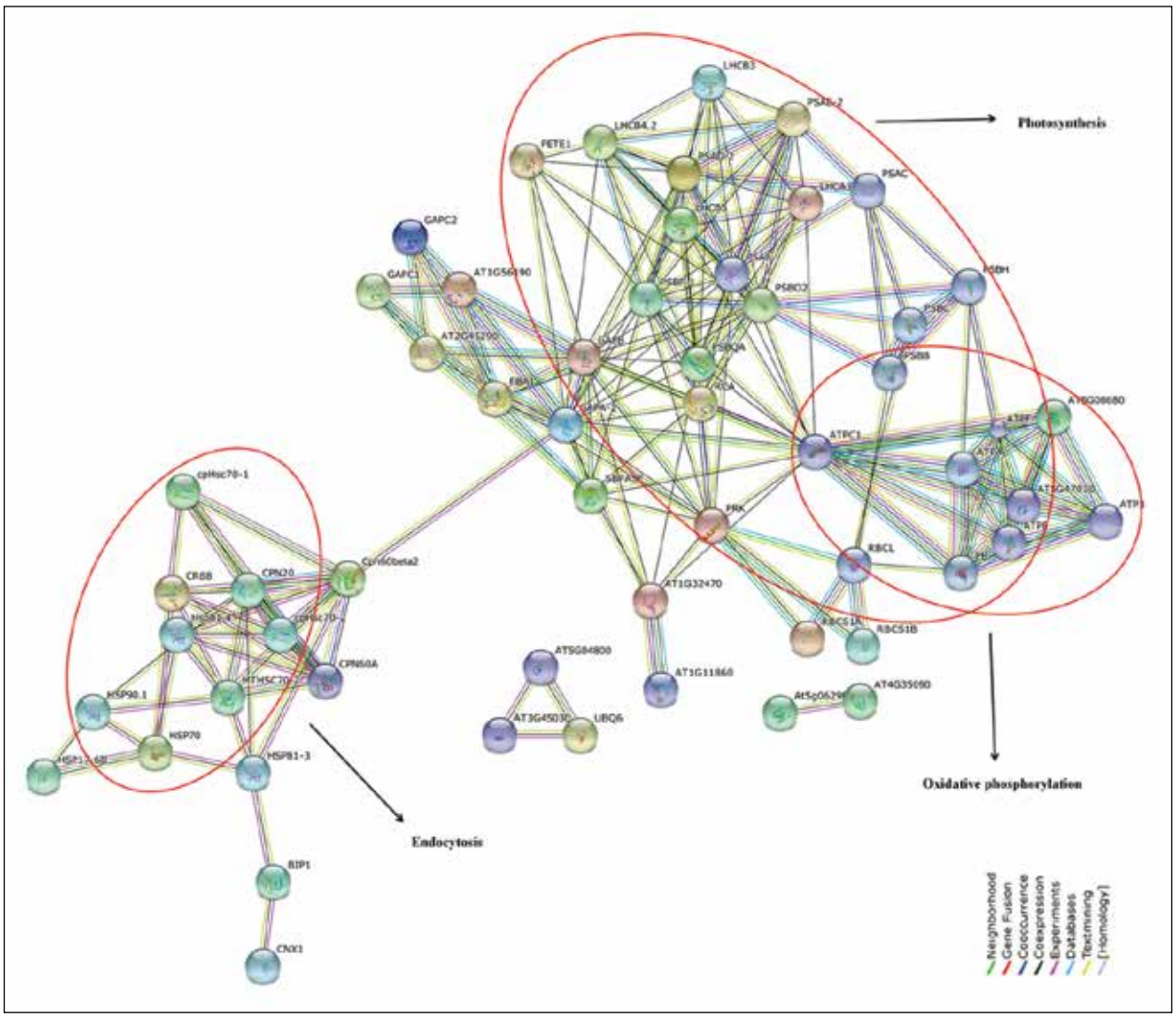

Figure-5. Protein-protein interaction network of the differentially expressed proteins mapped against *Arabidopsis thaliana*. The most enriched pathway involved in differential down-regulation has been highlighted in red circles.

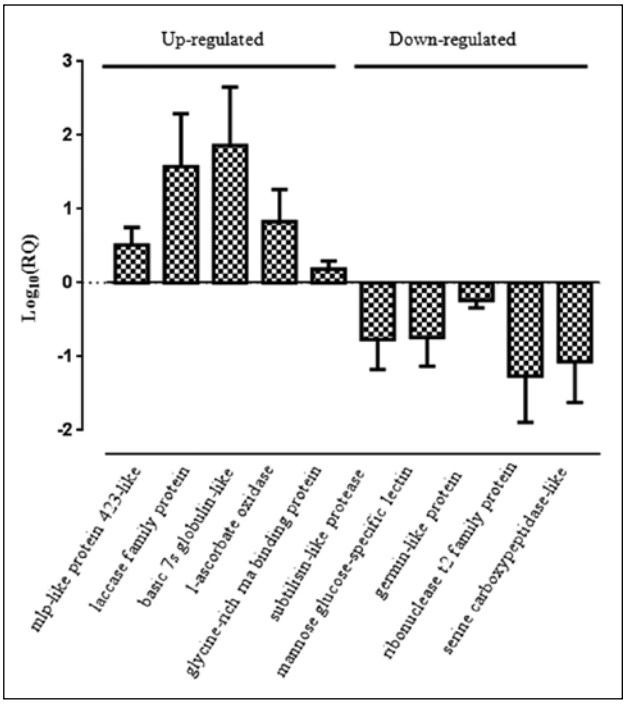

Figure-6. Real-time qPCR analysis of selected transcripts differentially expressed during *P. capsici* infection on black pepper leaves.

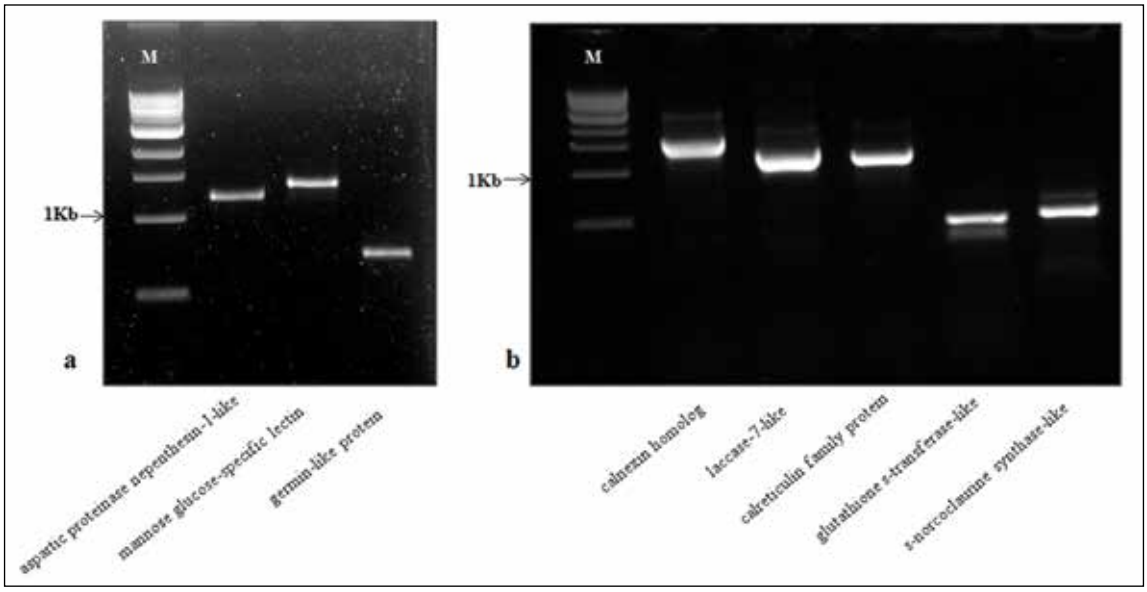

Figure-7. PCR amplification of some genes selected for full length cloning and characterization. M-1Kbp ladder (NEB)

Our present strategy also helps identification, cloning and characterization of the ORFs associated with each identified peptide/protein and to search these identified ORFs, using BLAST. We have successfully cloned nine of these genes with a diverse size ranging from 500bp to 1600bp using our approach (Fig.7).

In conclusion, our current study provides a high-throughput tool in black pepper, which will significantly enhance the knowledge base required to design strategies for crop protection in the otherwise unknown non-model plant, black pepper.

PUBLICATIONS

- Nair N R., Chidambareswaren M. & Manjula, S. (2014). Enhanced heterologous expression of biologically active human granulocyte colony stimulating factor in transgenic tobacco BY-2 cells by localization to endoplasmic reticulum. Molecular Biotechnology, 56(9), 849-862.
- Mahadevan C., Jaleel A., Deb, L., Thomas, G. & Sakuntala M. (2015). Development of an efficient virus induced gene silencing strategy in the non-model wild ginger-Zingiber zerumbet and investigation of associated proteome changes. PLoS One. 2015; 10(4): e0124518.
- Anu Krishnan, Chidambareswaren Mahadevan, Tomson Mani, and Manjula Sakuntala. 2015."Virus-induced gene silencing (VIGS) for elucidation of pathogen defense role of serine/threonine protein kinase in the non-model plant Piper colubrinum Link." Plant Cell, Tissue and Organ Culture: 1-15.
- Anu K, Jessymol K K, Chidambareswaren M, Gayathri GS and Manjula S. "Down-regulation of osmotin (PR5) gene by virus-induced gene silencing (VIGS) leads to susceptibility of resistant Piper colubrinum Link. to the oomycete pathogen Phytophthora capsici Leonian." Indian Journal of Experimental Biology 53 (2015): 329-334. (Cover Page Article)
- Anu K, Chidambareswaren M, Gayathri GS and Manjula S. "Cloning and sequence characterization of a partial Piper colubrinum phytoene desaturase (PcPDS) gene homologue for virus-induced gene silencing studies." Biotechnological Research 1 (2015): 113-117.

CONFERENCE PRESENTATIONS

- K Anu, M Chidambareswaren and S Manjula. "Virus-induced gene silencing as a reverse genetics approach for functional genomics in Piper colubrinum." International Conference on Biosciences- State of the art advancements, Kottayam, Kerala, Sep 11-12 2014. (Oral Presentation).
- Anu K, Jessymol K K, Chidambareswaren M, Gayathri

G S and Manjula S. "Down-regulation of osmotin (PR5) gene by virus-induced gene silencing (VIGS) leads to susceptibility of resistant Piper colubrinum Link. to the oomycete pathogen Phytophthora capsici Leonian." International Conference on Emerging Trends in Biotechnology, Jawaharlal Nehru University, New Delhi, Nov 6-9, 2014. (Oral Presentation).

MOLECULAR REPRODUCTION
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Scientist G

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Pradeep Kumar was awarded PhD in Life Sciences from Devi Ahilya Unviersity, Indore in the year 1988 for his Biophysical Studies of Sperm Membranes. He joined the Faculty of Life Sciences of the same university in the year 1989. He worked as a Fellow-in-residence at the Centre for Biomedical Research at the Rockefeller University, New York, NY; visiting faculty in University of Virginia, Charlottesville, VA and University of Florida, Gainesville, FL. He joined Rajiv Gandhi Centre for Biotechnology in the year 2004.

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Indu S

Project Personnel

Anil Kumar TR

Program #1: Germline Stem Cell division and differentiation
Germline stem cell isolation, enrichment and differentiation in vitro

Indu S and Pradeep G Kumar

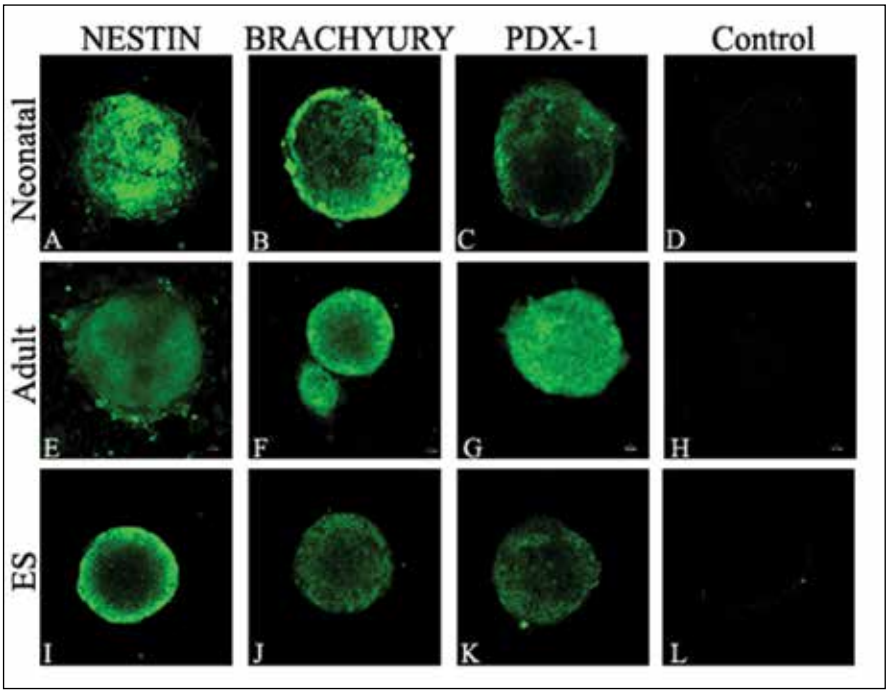

Figure 1. Expression of NESTIN (A and E), BRACHYURY (B and F) and PDX-1 (C and G) in germline stem cell (GS) embryoid bodies (EBs) derived from neonatal and adult mice testicular cells 10 days after removal of LIF and GDNF. X 20.Expression of germ layer markers NESTIN (I), BRACHYURY (J) and PDX-1 (K) in ES derived EBs 10 days after removal of LIF are shown as positive control. X 10.Embryoid body probed with secondary antibody alone as negative control (D, H and L).

We had previously demonstrated that the spermatogonial stem cells (SSC) harvested from the testis of the neonatal Swiss albino mouse formed colonies *in vitro* expressing several pluripotency markers. As a continuation of this study, we checked the colony forming and differentiation potential of SSCs from the testes of adult Swiss albino mouse. SSCs were isolated by differential plating on gelatin and maintained in Shinohara medium containing LIF and GDNF. Adult mouse testicular SSCs also formed colonies and upon induction of differentiation formed embryoid bodies (EBs). These EBs were analyzed for the expression of all the three germ layer makers, viz., Nestin, Brachyury and PDX-1. SSC-derived EBs were positive for nestin, Brachyury and PDX-1 indicating the presence of ectoderm, mesoderm and endoderm in them. However, the time required for the formation of stem cell colonies in adult mouse testicular cultures were more than that was required in neonatal mouse testicular cultures. We initiated investigations to determine whether the aging process affects spermatogonial stem cells similar to other somatic cells of the body. SSCs differ from adult somatic stem cells in maintaining the integrity of their genome which has to be transmitted to the progeny. DNA double strand breaks (DSBs) represent the greatest threat to genomic integrity. SSCs possess an efficient mechanism to counteract the deleterious effect of DSBs by repairing the damaged sites on DNA. We hypothesize that SSCs maintain uncompromised DSB repair activity by the expression and activities of DNA-PK, Ep-CAM, ATM and c-Abl. We have evaluated the expression of markers like integrin α6, Ep-CAM and c-Abl in the testes of 14 day old mice testes and 90 day old mice testes. The expression of integrin α6 (SSC marker) was higher in 14 day old mice testes compared to that of 90 day old mice testes. The expression of DSB repair markers Ep-CAM and c-Abl were found to be significantly higher in 90 day old mice testes indicating robust DSB repair activity going on in these cells.

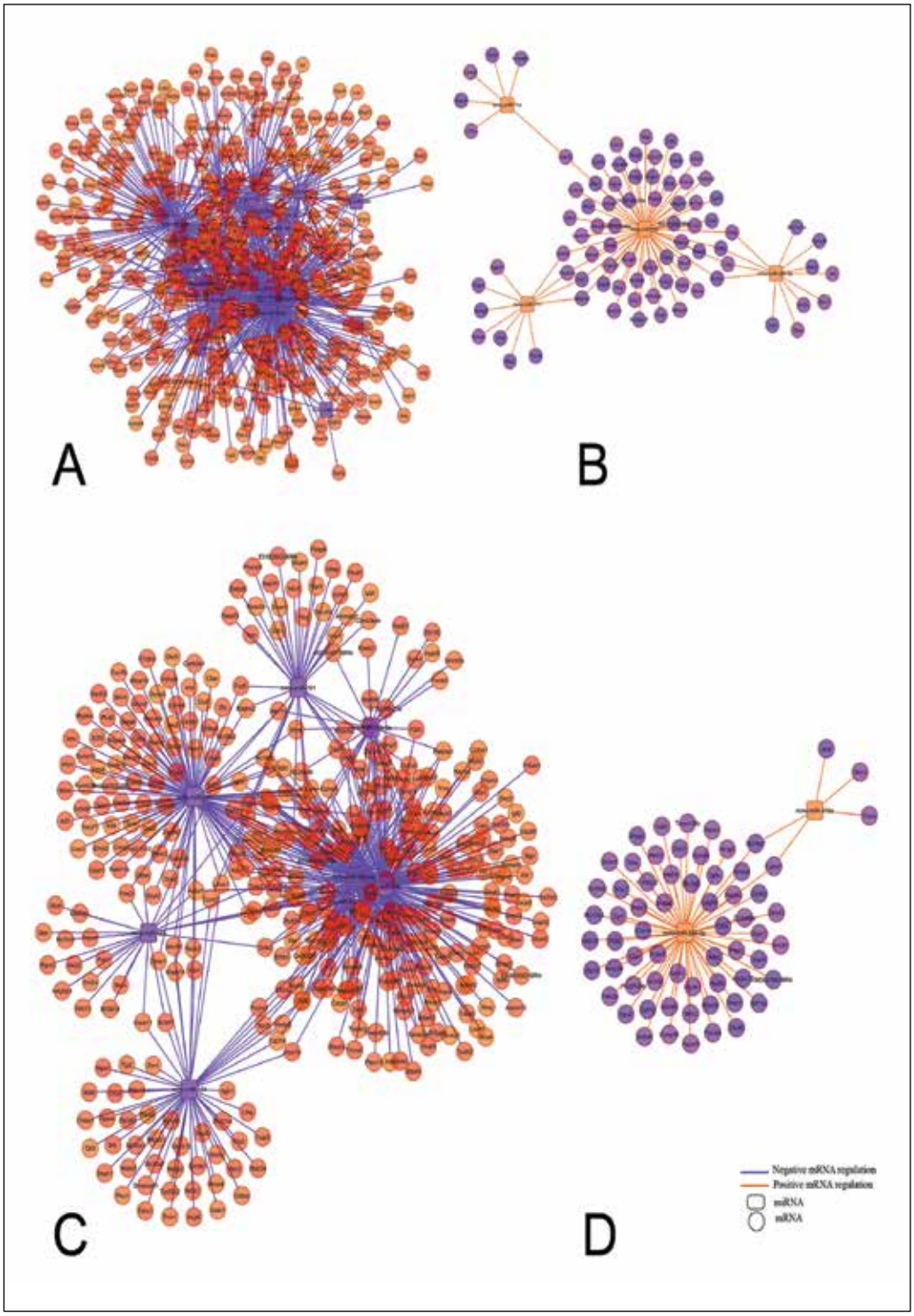

Figure 2. Networks of differentially expressed microRNAs and target genes. Networks generated based on statistically significant interactions (fold change ≥ 2.0), miRNAs are represented by boxes, while target mRNAs are shown as spheres. Orange color represents downregulation and purple color denotes upregulation. (A) Upregulation of 19 miRNAs were associated with downregulation of 228 target genes as the testis progressed from P8 to P16 period; (B) Downregulation of four microRNAs (miR-290-3p, miR-711, miR-762 and miR-714) was linked with the upregulation of 81 target genes during the progression of testis from P8 to P16 period; (C) Upregulation of 8 microRNAs (mmu-miR-191, mmu-miR-375, mmu-miR-34b-5p, mmu-miR-34c, mmu-miR-34b-3p, mmu-miR-124, mmu-miR-449a, mmu-miR-425) was linked with downregulation of 389 target genes during P16 to P24 transition and (D) 2 miRNAs (mmu-miR-376a & mmu-miR-335-5p) were downregulated, which could be linked with the upregulation of 64 target genes during P16 to P 24 transition. The networks were visualized using Cytoscape v.2.8.3.

miRNA-mRNA networks during the initiation of 1st wave of spermatogenesis in mouse testis

Sreesha Sree and Pradeep G Kumar

MicroRNA profiling of neonatal (P8), adolescent (P16) and adult (P24) testes yielded a set of 67 miRNAs exhibiting differential expression with the onset of spermatogenesis. This included 25 miRNAs which were upregulated from P8 to P16 and 10 miRNAs which were upregulated from P16 to P24. Four miRNAs (miR-34b-5p, -34c, -34c* & -449a) were common in these two categories of upregulated miRNAs. The remaining differentially displayed sets of miRNAs appeared as unique sets changing their levels in two distinct waves. On the other hand, 6 miRNAs (miR-290-3p, -711, -714, -762, -21* & -770-3p) were downregulated during P8 to P16 transition, while 2 miRNAs (miR-335-5p, -376a) were downregulated during P16 to P24 transition. A comparison of P8 testis with P24 testis yielded 40 upregulated and 27 downregulated miRNAs. Expression level changes of a few of these miRNAs were validated by quantitative real time PCR. A whole transcriptome microarray of total testicular cDNA from P8, P16 and P24 mice was performed. Of the 21,615 transcripts, 8,226 had a fold change, Fc ≥ 1.5. Microarray expression pattern of few of these mRNAs were validated by RT-PCR, selecting three downregulated (*Cdh11*, *Notch1* and *Fbxl20*) and three upregulated (*Sycp1 & Sycp3* & *Ldhc1*) genes from the list of differentially displayed genes (Fig. 5). RT-PCR analysis confirmed the microarray profile of these mRNAs between the three periods. We consolidated the two microarray datasets by assigning qualifying attributes (fold change ≥ 2.0 for miRNAs, fold change ≥ 1.5 for target mRNAs, p-value ≤ 0.05 for the differences in their levels and the expected inverse relation in miRNA-mRNA levels) and generated miRNA-mRNA networks. This comprised of a network of upregulated miRNAs linked with downregulated mRNAs and a network of down-regulated miRNAs linked with upregulated mRNAs in the successive stages of testicular development. The networks were validated by manipulation of the levels of mmu-miR-34c and mmu-miR-290-3p in *ex vivo* cultures of neonatal and adult mouse testes respectively. In the first set, miR-34c mimics were introduced and expression levels of five targets (*Celsr1*, *Nav1*, *Met*, *Itga6 and Rhoq)* were analyzed. Of these, *Celsr1*, *Nav1*, *Met* and *Itga6* are common targets of miRNAs 34c, 34b-5p & -34a. In addition, *Nav1*, *Met* and *Rhoq* are predicted targets of other miRNAs like miRs -449a, -201, -471, -204& miR-741in the network. Upregulation of miR-34c did not exercise any statistically significant downregulation in the levels of expression of any of these genes, presumably because of the fact that targets of miR-4c were targets of a wide array of miRNAs. MiR-290-3p formed the network of downregulated miRNAs from P8 to P16 along with miRs -711, -762 &-714. We introduced miR-290-3p mimic and evaluated transcript levels of five genes including three targets of miR-290-3p (*Strbp*, *Lrat* and *Gramd1c)* and two of mmu-miR-711 (*Ttc14* and *Trim44)*. *Strbp* is a common target of miRs-290-3p & miR-762. A≥2-fold down-regulation in *Strbp*, *Lrat*, *Ttc14*, *Trim44* and *Gramd1c* levels (Fig. 1B) was observed. As *Strbp*, *Lrat* and *Gramd1c* were mainly predicted targets of 290-3p in the networks and also showed approximately 2 .5 fold change in the microarray analysis, an effect of elevation of miR-290-3p on these targets was as predicted, but, down-regulation in levels of targets of miR-711 (*Ttc14* and *Trim4 4)* was unexpected, and could be off-target effects. However both *Ttc14* and *Trim4 4* were predicted as targets of mmu-miR-290-3p by the Diana-microT algorithm and thus possibly both are targets of miR-290-3p in mouse testis. Taken together, the inefficiency of miR-34c and the efficiency

of miR-290-3p mimics appear to demonstrate the functional redundancy of miR-34c and the functional dominance of miR-290-3p in regulation of respective predicted targets in our experimental set upindicating the relative weakness and strength of individual players in a regulatory network and thereby support our network analysis. Further studies involving manipulation of individual or combination of miRNAs would illustrate the role of miRNA-dependent gene expression regulation in mammalian spermatogenesis.

Proteome profiling in mouse testis during initiation of spermatogenesis

We generated proteomic profiles of mouse testis before (15 days old) and after (3 months old) the establishment of spermatogenesis. 192 proteins or variant forms were identified to be differentially displayed. 27% of these proteins were expressed uniquely in the mature mouse testis, 11% uniquely in the immature mouse testis, and 62% were expressed in both the age groups. The proteins showing changes in expression were subjected to Panther analysis, which revealed that chaperones and proteins involved in apoptotic processes were up-regulated in mature testis. Proteins belonging to nucleic acid binding class, integrin signalling pathways and gonadotropin releasing hormone pathways were found to be down-regulated in mature testis as compared to 15 days old moue testis. The relevance of these proteins in the context of spermatogenesis is being evaluated.

Proteome profiling during busulfan-induced germ cell depletion and subsequent re-establishment of spermatogenesis in mouse testis

Nomesh Yadu and Pradeep G Kumar

1, 4-methanedioldimethanesulfonate (busulfan) is a bifunctional alkylating agent used as chemotherapeutic drug. Busulfan's therapeutic usage is mainly due to its effect on hematopoietic cells. Additionally, other tissues are affected resulting in side effects, which may include liver toxicity, teratogenicity, immune function alterations and sterility. Busulfan is considered carcinogenic in humans. Toxicity of Busulfan to germ cells is well established. Human recipients of busulfan suffer from temporary or permanent sterility. Many of the male patients regain fertility few years after busulfan treatment; whereas some of them remain infertile. Female patients exposed to therapeutic levels of busulfan never regained fertility. In murine models, busulfan administration causes selective depletion of germ cells, leaving the somatic cells alive and fully functional. This property of busulfan has been exploited in the germ cell transplantation assay, where busulfan is used to deplete germ cells from the recipient testis. Being a bifunctional alkylating agent, busulfan can cause DNA alkylation resulting in DNA-DNA and/or DNA-protein crosslinks. These cross-links may trigger DNA damage responses and result in apoptosis or cell death via the p53 pathway. However, studies have not supported this mode of action. In fact, the mechanisms involved in toxicity to germ cells due to busulfan are not fully understood.

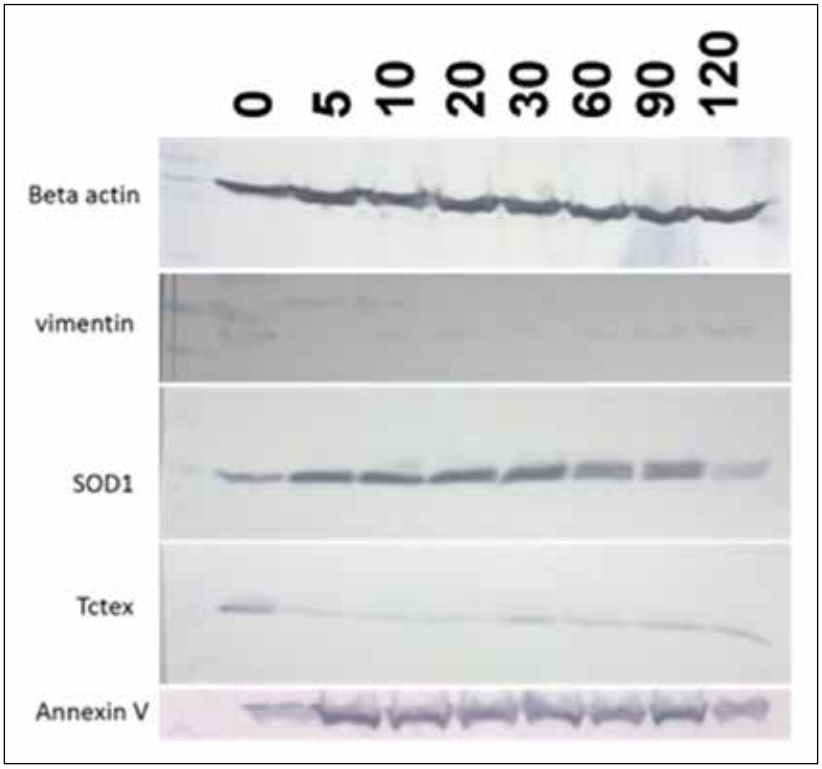

Figure 3. Western blot analysis of vimentin, SOD1, TCTEX and Annexin V in mouse testis on days 0-120 after busulfan treatment

With an aim to reveal the genetic changes associated with busulfan induced germ cell toxicity, a global proteomic approach was adopted. Mature mice were given single intraperitoneal injections of busulfan (40 mg/kg body weight). Testes from treated mice were isolated at various time points after busulfan treatment. Time points were Day 5, day 10, day 20, day, 30, day 60, day 90, day 120. Untreated mature mouse testis served as controls. Up to day 30 there was gradual loss of germ cells. Semeniferous tubules remained free of germ cells up to day 60. From Day 90 and Day 120 testis sections revealed regeneration of germ cells in some seminiferous tubules. Total protein was isolated from the treated and control testes in Rapigest- ammonium bicarbonate buffer. Subsequently, the protein samples were analyzed by mass spectrometry and a proteomic profile of treated and untreated samples was generated. The relative protein expression levels were determined for comparison between control and treated samples. As compared to the control, 36 proteins were found to be upregulated in all the samples from day 5 to Day 30. 18 proteins were found to be down regulated in the same duration. 75 proteins were upregulated from Day 60 to Day 120. And 79 proteins were down regulated. Groups of proteins which exhibited changes in expression levels were subjected to Panther analysis. Panther analysis revealed that the expression levels of nucleic acid binding proteins and Oxidoreductases had changed in response to busulfan treatment. Expression levels of for proteins, viz, Vimentin, Superoxide dismutase1, tctex and annexin V were validated by western blotting. This study indicates that testis responds to busulfan by upregulating the free radical machinery as evidenced by upregulation of SOD and oxidoreductase class of proteins.

Epigentic regulation of gene expression during the onset of spermatogenesis

We investigated the change in the pattern of histone modification, H3K4Me3 at three critical time points during the first wave of spermatogenesis using ChIP seq with input DNA (Non ChIP'd genomic DNA) as the control. Following standardization of the protocol using RNA polII, chromatin immmunoprecipitation was carried out in day 8, day 16 and day 24 mouse testes using H3K4Me3 antibody. The immunoprecipitation was confirmed by checking for the enrichment of ĸactin promoter in the pulldown samples using PCR. The pulldown samples showed enrichment as compared to the negative and the input sample. The samples were given for next generation sequencing. Quality check of the raw reads was performed using SeqQC v2.2. Raw reads were processed to filter adapters, B-blocks and Low complexity regions. Processed reads were considered for further analysis. Filtered high quality reads were aligned to reference genome (mm10) using Bowtie-0.12.8 alignment tool in consideration of all the chromosomes. IP and INPUT samples with sufficient reads aligned to the reference genome were used for peak detection using HOMER v3.12 software with fold change cutoff of 4 for enrichment. A number of quality control analyses have been carried out for each of the samples namely tag count distribution, auto correction analysis and sequence bias analysis. A total of 16289, 16455 and 19562 H3K4Me3 peaks with variable widths were obtained for 8 day, 16 day and 24 day mouse testes which were further annotated. The peaks obtained were characterized into seven classes namely promoter transcription start site (TSS), 5' UTR, 3'UTR, Exon, intron, intergenic regions and noncoding RNA. It was found that in 8 day old mouse testis H3K4me3 marks were mostly evenly distributed with 30% marks in promoter region, 29% in introns, 14% in intergenic region, 14% in exons and 10 in % UTRs. 8d (only spermatogonia) and 24d (spermatcytes and spermatids) testis showed a very similar distribution of h3k4me3 at the intergenic regions as well as protein coding regions. However in 16 day old testis H3K4me3 marks were highly enriched in the intronic region (38%) and intergenic region (55%), together amounting to 93% of the H3K4Me3 peaks. Consequently, H3K4Me3 marks at the promoter and exon were reduced to 3% and 2% respectively. Thus 8 day to 16 day transition was accompanied by a dramatic decline in the h3K4me3 marks at the promoter and the 5' UTR regions and a corresponding increase in the h3k4Me3 marks at the intergenic and the intronic regions. Majority of the cells (around 44%) on day 8 are in the pachytene stage of meiosis. We also observed a dramatic decrease in H3K4trimethylation at X chromosome on day 16.

Identification and functional analysis of critical molecules associated with spermatogenic disorders

TAR DNA binding protein 43 (TDP-43)

Divya Saro Varghese and Pradeep G Kumar

TDP-43 (TAR DNA binding Protein) is a nuclear protein that contains two RNA Recognition Motifs (RRM), and a glycine-rich C-terminal domain, characteristic of the heterogeneous nuclear ribonucleoprotein (hnRNP) superfamily. Loss of function of TAR DNA binding Protein (TDP-43) has been implicated in neurodegenerative disorders in human beings and in animal models. TDP-43 has also been shown to act as a cis-acting transcriptional repressor of acrosome vesicle (*Acrv*)

gene in mouse. We evaluated the expression profile of TDP-43 transcript and protein in the germ cells from 11 fertile and 98 subfertile men to verify its potential association with poor seminograms. The expression profile of TDP-43 was characterized in the immature germ cells and spermatozoa from semen from fertile and subfertile males using Reverse Transcriptase PCR, Western blotting and immunofluorescence. PCR using a combination 717F and 1269R yielded products corresponding to exon 6 of *TARDBP* in all the normozoospermic controls. Among the infertile men, 10, 9 and 13 subjects out of 23 astheno-, 25 oligo- and 20 teratozoospermic cases were positive for *TARDBP*, with the remaining being negative. For each assay, PCR negativity was overruled by loading the positive control, PCR product of normal samples that amplified the transcript, on the same gel. β *ACTIN* and *GAPDH* were used as loading controls (Figure 5).

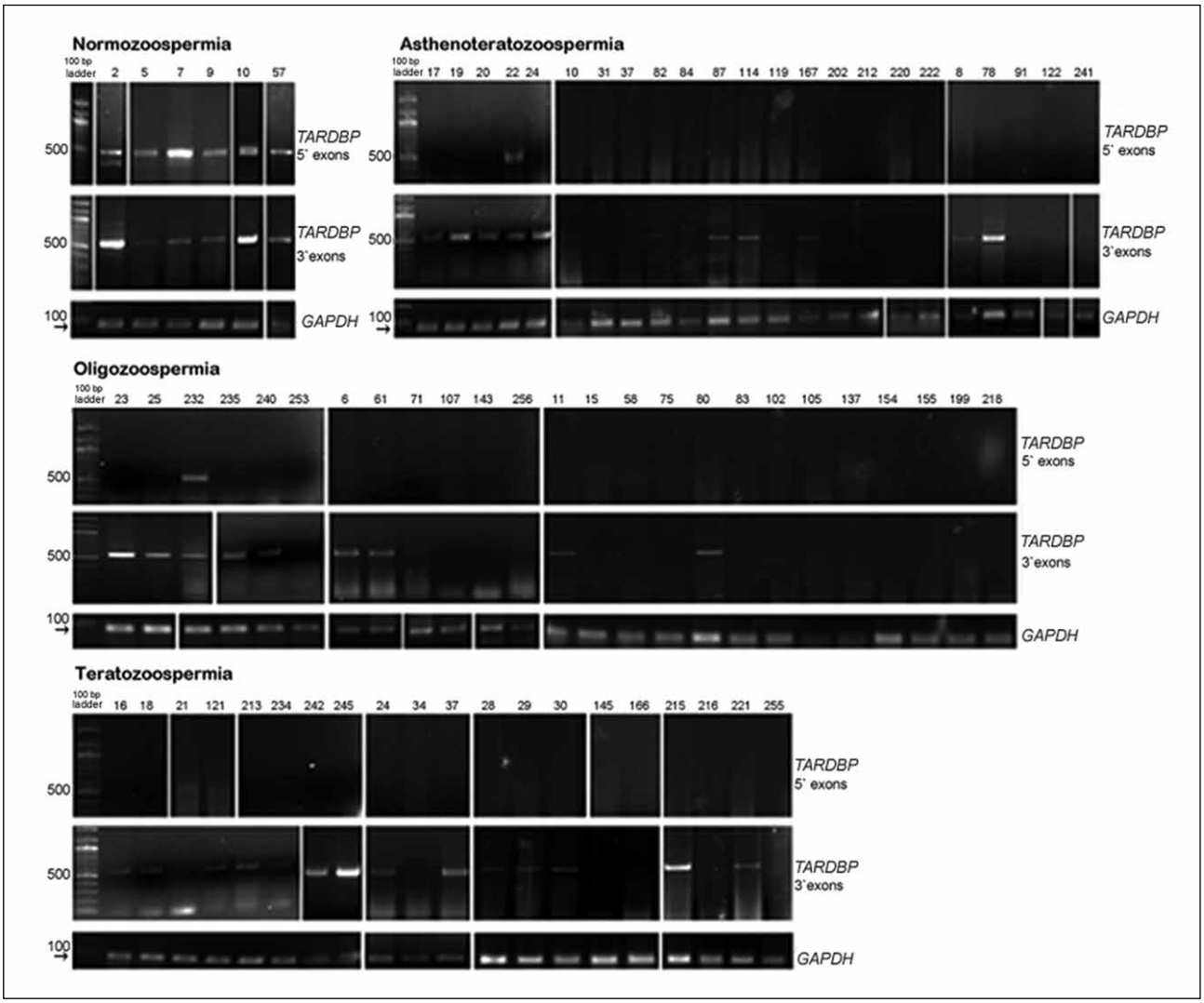

Figure 5. RT-PCR analysis of TARDBP expression in the germ cells from fertile and infertile men

Expression profiling at the protein level was evaluated using Western blot analysis. Protein lysates from the spermatozoa of fertile human males had detectable levels of expression of TDP-43. Western blot analysis detected negligible to very low levels of the 43 kDa protein in astheno-, oligo- and teratozoospermic conditions of infertility even in those cases in which PCR yielded products. Immunofluorescence with anti-TARDBP-1 antibody was carried out to understand the localization of the protein or its truncated fragments in fertile and subfertile spermatozoa. TDP-43 localized to the head of spermatozoa in normozoospermic individuals with minimal expression in the mid-piece or tail. A similar localization pattern was observed in asthenozoospermic conditions though the expression was lesser in these cases. While abnormal localization of TDP-43 was manifested in head and tail in oligozoospermic conditions, severely reduced protein levels were expressed ectopically in the mid-piece and tail segment in teratozoospermic cases. We conclude that the deficiency in the expression of TDP-43 is associated with defective spermatogenesis and male infertility. We propose that TDP-43 could be used as a marker of male factor infertility.We generated full-length TDP-43-EGFP (pEGFPN1-TDP43$_{FL}$) and a deletion construct of TDP-43 (pZsYC1-TDP43$_{(1-58)}$) lacking the NLS. Co-transfection studies indicated dimerization of TDP-43 using the N-terminal domains and the translocation of the complex into the nucleus. While TDP-43$_{FL}$ localized to the nucleus, TDP-43$_{1-58}$ was confined to the cytoplasm as it lacked the NLS. However, upon cotransfection of GC-1 cells with both pEGFPN1-TDP43$_{FL}$ and pZsYC1-TDP43$_{(1-58)}$, the latter also was translocated to the nucleus implicating the self-dimerization of TDP-43 using its N-terminal domain.

T-Complex Testis-Expressed 1 (TCTEX-1)

Indu S, Jeeva SE and Pradeep G Kumar

TCTEX1 (T-Complex Testis-Expressed Protein 1) is a dynein light chain protein encoded by *Dynlt1b* or *Tctex1* gene in the t complex region of mouse chromosome 17. *DYNLT1* has been linked with male germ cell development and function in the mouse and the fly. Though defects in the expression of this gene are associated with male sterility in both these models, there has been no study examining its association with spermatogenic defects in human males. We evaluated the levels of *DYNLT1* and its expression product in the germ cells of fertile human males and males suffering from spermatogenic defects. We screened fertile (N=14), asthenozoospermic (N=15), oligozoospermic (N=20) and teratozoospermic (N=23) males using PCR and western blot analysis. Semiquantitative PCR indicated either undetectable or significantly lower levels of expression of *DYNLT1* in the germ cells from several patients from across the three infertility syndrome groups, when compared with that of fertile controls. DYNLT1 was localized on head, mid-piece and tail segments of spermatozoa from fertile males. Spermatozoa from infertile males presented either a total absence of DYNLT1 or its absence in the tail region. Majority of the infertile individuals showed negligible levels of localization of DYNLT1 on the spermatozoa.

A recent study has indicated the progenitor specific expression of TCTEX1 in the adult brain and associated with stemness in the neural progenitors. Based on the above context, we hypothesised that Tctex-1 might regulate stemness in germ cell progenitors in the adult testes and the inadequate quantity of germ cell progenitors may be the root cause of infertility in animals and humans. To evaluate the functional significance of the molecule in relation to stemness, spermatogenesis and feritility, we have successfully generated pEGFPN1-Tctex1 over expression and pEGFPC1-Tctex1

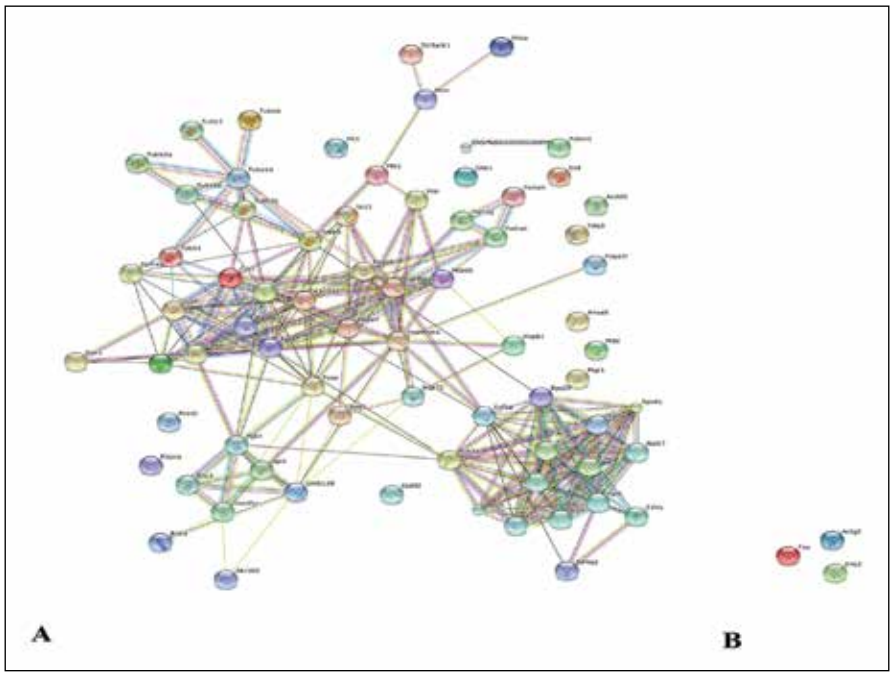

Figure 6. . STRING analysis of upregulated (A) and down regulated (B) with respect to TCTEX1 over expression.

silencing constructs. The over expression and silencing constructs were transiently transfected into GC1spermatogonial cell line and after twenty four hour post transfection, the cells were harvested and lysed for RNA and protein preparation.

The mass spec analysis showed 121 differentially expressed proteins with respect to TCTEX1 overexpression and among them 112 were up regulated and 9 were down regulated. The mass spec identified differentially expressed proteins were subjected to PANTHER and STRING analyses. PANTHER analysis categorised the up regulated proteins into seventeen categories according to protein class. The major proportion of the upregulated proteins were categorised under chaperones followed by nucleic acid binding proteins and cytoskeletal proteins. Likewise, the down regulated proteins were classified into three

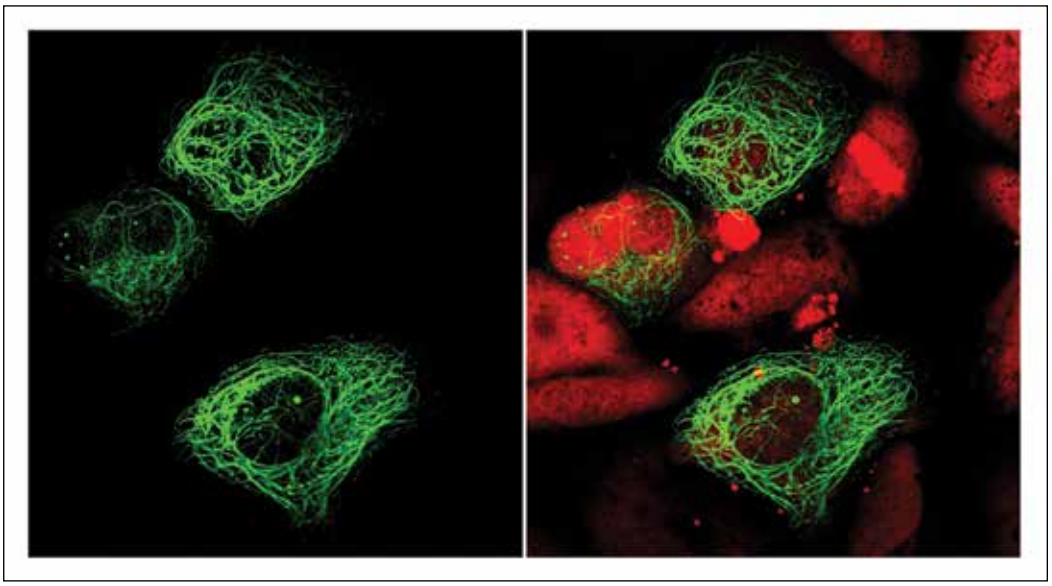

Figure 7. Expression of AIRE-EGFP in GC1-spg cells

categories nucleic acid binding proteins, lyases and cytoskeletal proteins in equal proportion. The STRING prediction analysis showed four clusters of interacting protein sets like ribosomal protein group, cytoskeletal proteins group, chaperones and metabolic proteins among the upregulated protein set and there were no interacting clusters in case of the down regulated protein set. The transcriptome change were under evaluation, showed the up regulation of some of the major stem cell markers like c-Myc, Nanog, Lin28 with respect to *Tctex1* over expression. The expression profiling of remaining other stemness markers and proteome changes with respect to *Tctex1* over expression are under validation. The sub-cellular localization, transcriptome and global proteome change due to *Tctex1* silencing in GC1spermatogonial cell line will be evaluated.

Autoimmune Regulator (AIRE)

Bhagya KP, Karthika Radhakrishnan and Pradeep G Kumar

Previous studies have pointed towards a possible role of AIRE in regulating germ cell apoptosis suggesting that AIRE may be important during normal spermatogenesis. Homozygous Aire deficient mice were shown to reproduce only occasionally indicating that AIRE might also impact fertilization and embryo development. We have evaluated how AIRE alters the cellular proteome of GC1 cell line, a germ cell derived cell line. Autoimmune Regulator (*AIRE*) is a gene associated with a rare autosomal recessive autoimmune disease, Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). AIRE has been extensively studied in the thymic epithelial cells where it has been shown to play an important role in maintaining self tolerance through regulating the expression of tissue specific antigens. Testis is the most predominant extra-thymic location where a heavy expression of AIRE is reported. However, its functional role in the testis is not understood. Homozygous *Aire*- deficient mice were shown to reproduce only occasionally suggesting that AIRE may be important during normal spermatogenesis. Previous studies have pointed towards a possible role of AIRE in regulating germ cell apoptosis. In this report we have evaluated how AIRE alters the cellular proteome of GC1 cell line, a germ cell derived cell line. High efficiency capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to analyze proteins extracted from Aire over expressing GC1 cells. Peptide identification was performed using PLGS (Protein Lynx Global Server) software. A total of 384 proteins were found to be differentially expressed out of which 99 proteins were up regulated and 285 proteins were down regulated. Functional analysis of the differentially expressed proteins showed increased levels of various nucleic acid binding proteins and transcription factors and a decreased level of various cytoskeletal and structural proteins in the Aire over expressing cells as compared to the empty vector transfected controls. Further, we could show a corresponding increase in the transcripts for the many of the up regulated proteins shortlisted from our proteomics data. Intriguingly, no such corresponding decrease in the mRNA levels could be detected for down regulated set of genes. However, the upregulated set of proteins in GC1-spg cells over-expressing AIRE would set the foundation in building up the functional role of AIRE in germ cell biology.

Protocadherin 11Y (PCDH11Y)

Anilkumar TR and Pradeep G Kumar

Protocadherin Y- linked 11 (PCDH11Y), a differentially displayed molecule in a fertile vs infertile proteome analysis done in our laboratory, is a member of the cadherin super family with established roles in cell adhesion and canonical Wnt signaling pathway by interacting with beta catenin(Chen X *et al.* 2002). PCDH11Y is encoded by Yp11.2 loci and exists in three isoforms A, B and C and is also reported to be a retinoic acid responsive gene, up-regulated by retinoic acid (RA) signaling, an essential pathway for germ cell differentiation and initiation of meiosis in spermatogenesis. Although there are various reports on PCDH11Y being implicated in neuronal cell differentiation and proliferation, there have been no attempts till date to study its role in relation to spermatogenic differentiation and its association with sub fertility which may occur due to a defective spermatogenesis. In this study we characterized a shorter variant of *PCDH11Y* (NM_032973), designated as variant B, in human spermatozoa by RT- PCR using variant specific primers. A comparative analysis of the expression of PCDH11Y in spermatozoa from fertile and infertile men carried out by RT-PCR, western blotting and immunofluorescence analysis indicated the expression of the gene in 13 fertile men, recruited as normal control, at the transcript and protein levels while much of the infertile men indicated a defect at the transcript and protein levels with the oligozoospermic individuals showed a severe defect for the gene. Immunolocalization of PCDH11Y on spermatozoa from fertile men showed a prominent localization on its head and mid piece region while spermatozoa from infertile subjects showed a quantitative reduction in the localization with the teratozoospermic sperms with head defects showed an abnormal localization in which the absence of the molecule was noted on the head and found localized more towards the tail and midpiece. PCDH11Y has a close homologue PCDH11X in animal model (*Mus musculus* Protocadherin) and is an ideal candidate for the functional studies in the model organism and mouse derived spermatogonia cell lines. RT- PCR analysis of the expression of *Pcdh11X* in different age group mice testes showed an up-regulation in the meiotic and post meiotic stages when compared to the premeiotic stages and shows an increasing trend down the line of spermatogenic differentiation. Spermatogonia cell line treated with retinoic acid, an inducer for spermatogenic differentiation showed an up regulation of *Pcdh11X,* indicating its possible involvement in spermatogenic differentiation. PCDH11X was knocked down in spermatogonia cell line using shRNA technology which could be a useful tool in establishing its functional interactome and possible signalling pathways involved

Nephrycystin 1 (NPHP1)

Devi AN, Anilkumar TR and Pradeep G Kumar

NPHP1, the gene that encodes the protein Nephrocystin-1 has been identified to be mutated in Juvenile Nephronophthisis, an autosomal recessive cystic kidney disorder which is the most frequent genetic cause of end stage renal disease in children and young adults. Nphp1 targeted mutant mice studies have shown that it did not express renal manifestations of nephronophthisis; instead male mice were infertile with oligoteratozoospermia signifying the crucial role of Nphp1 in relation

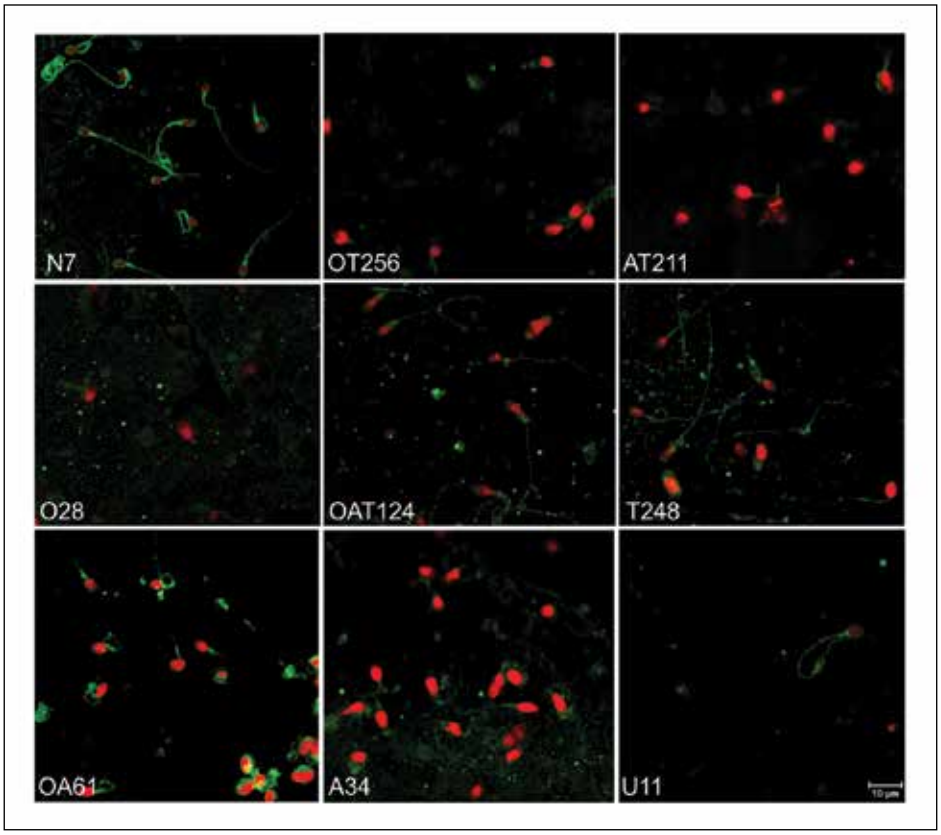

Figure 8. Expression of NPHP1 (green channel) in fertile (N7) and infertile (remaining images) men.

to murine spermatogenesis. Whether an aberrant expression of NPHP1 in testis might lead to spermatogenic defects in human and ultimately male factor infertility is a possibility that have not been investigated so far. In this regard, characterization of NPHP1 in spermatozoa from fertile and infertile males was carried out by employing RT-PCR, western blotting and immunofluorescence analysis. In all the 23 normozoospermic samples we screened, NPHP1 was significantly expressed at the target message and protein level and also prominent localization pattern of NPHP1 was observed at the head, midpiece and tail segments of spermatozoa. Conversely, in majority of the 103 infertile samples we screened, aberrant pattern of NPHP1 expression was detected at the transcript and protein level and abnormal localization pattern of expression was observed in spermatozoa. Anomalies detected in infertile cases when compared with the normozoospermic controls points to the indispensable role of NPHP1 in relation to spermatogenesis. Thus, besides the decisive association with juvenile nephronophthisis, our study provides the first direct evidence that NPHP1 is associated with male factor infertility and also could be a possible biomarker for the assessment of male fertility status. Expression profiling during the 1st wave of spermatogenesis in mouse testis detected Nephrocystin at all stages of male germ cell development. Further, we have successfully generated the pEGFP-SH3 domain construct of Nephrocystin and were transiently transfected in GC-1spermatogonial cell line. Sub-cellular localization evaluated by confocal microscopy revealed the prominent expression in cytoplasm and nuclear lamina. The SH3 domain of nephrocystin might regulates protein trafficking events at sites of anchoring junction at the Sertoli-spermatid junction which is related with the signalling events essential for post-meiotic germ cell development and maturation. In addition, Nephrocystin is reported to be a docking protein in regulating the actin cytoskeleton at sites of epithelial cell–cell adhesion. The possible role of Nephrocystin in relation to the Wnt-κ catenin signalling pathways in the context of spermatogenesis is being evaluated.

Cyclin M1

Soumya A, Indu S, Anilkumar TR, Devi AN, Irfan Khan and Pradeep G Kumar

Cyclin M1 (CNNM1) functions as a copper storage protein in neuronal cells. We report that *Cnnm1* is expressed in mouse testis and brain, and has a coding sequence of 1761 bp that encodes a 584 amino acid protein with a molecular weight of 66 kDa. *Cnnm1* is expressed in the testes of mice from neonatal to adult stages with relatively higher levels in neonates. CNNM1 expression appeared in two distinct waves in adult testis, with the 1st wave restricted to early spermatogonial cells and the second wave predominantly in the post-meiotic germ cells. Silencing of *Cnnm1* in GC-1-spg cells resulted in a significant reduction in the number of cells in G1 phase with concomitant increase in the numbers of cells in both S and G2/M phases. Spermatogonial stem cells (SSCs) expressed *Cnnm1*, and their differentiation into embryoid bodies *in vitro* resulted in the loss of *Cnnm1* expression. Further, retinoic acid (RA) downregualted the expression of *Cnnm1* in GC1-spg cells. We conclude that CNNM1 is associated with stemness and self-renewal, and its downregulation triggers differentiation in spermatogonial cells in mouse.

PUBLICATION

- *Radha A, Sreesha S, Faisal K, Kumar PG, Oommen OV, Akbarsha MA* (2014) Antigenic homogeneity of male Müllerian gland (MG) secretory proteins of a caecilian amphibian with secretory proteins of the mammalian prostate gland and seminal vesicles: evidence for role of the caecilian MG as a male accessory reproductive gland. *Zoology (DOI: 10.1016/j.zool.2014.03.005)*
- *Sreesha S, Radhakrishnan K, Indu S and Kumar PG* (2014) Dramatic changes in 67 microRNAs during Initiation of first wave of spermatogenesis in *Mus musculus* testis: Global regulatory insights generated by microRNA-mRNA network analysis. *Biol. Reprod. 91 (3) 69, 1-11. (doi: 10.1095/biolreprod.114.119305).*
- *Varghese DS, Chandran U, Soumya A, Pillai S, Jayakrishnan K, Reddi PP and Kumar PG* (2014) Aberrant expression of TAR DNA binding protein-43 is associated with spermatogenic disorders in human males. *Reprod. Fertil. Dev. (DOI: 10.1071/RD14090).*
- *Devi AN, Kumar ATR, Pillai SM, Jayakrishnan K and Kumar PG* (2015) Expression profiles of NPHP1 in the germ cells in the semen of men with male factor infertility. *Andrology (DOI: 10.1111/andr12062)*

PhDs submitted

- *Divya Saro Varghese* (2015) Functional analysis of TDP-43 in relation to spermatogenesis
- *Sreesha Sree* (2015) Role of microRNAs in germ cell differentiation and fertilization.
- *Bhagya KP* (2015) Functional evaluation of AIRE in mammalian testis

INVITED LECTURES

- *Kumar PG* (2014) Where to go? *Inspire Internship Science Camp, St. Xaviers Catholic College of Engineering, Chunkankadai, Nagercoil, Tamilnadu, 14-18 July, 2014.*
- *Kumar PG* (2015) Where to go? *Inspire Internship Science Camp, SN College, Kannur, Kerala, 23-28 December, 2014*
- *Bhagya KP, Radhakrishnan K, Yadu N and Kumar PG* (2015) There is AIRE in the testis. What is its business there? *Labhsetwar Award lecture at the 25th Annual Meeting of Indian Society for the Study of Reproduction and Fertility and International Conference on Reproductive Health, National Institute for Research in Reproductive Health, Parel, Mumbai, 14-17 February, 2015.*

POSTER PRESENTATIONS

- *Indu S and Kumar PG* (2014) Testicular Stem Cells from Adult Mouse are Pluripotent. *Keystone Symposia on Molecular and Cellular Biology, held at Resort at Squaw Creek, Olympic Valley, California, USA, April 6-11, 2014.*
- *Shoeb M and Kumar PG* (2015) Individual SNARE Proteins Associated with Membrane Raft Microdomains but SNARE Complex Formation Occurs in NonraftMembraneDomains. *25th Annual Meeting of Indian Society for the Study of Reproduction and Fertility and International Conference on Reproductive Health, National Institute for Research in Reproductive Health, Parel, Mumbai, 14-17 February, 2015.*
- *Indu S and Kumar PG* (2015) Defects in Dynein Light Chain 1 (dynlt1) Expression may Lead to Male Factor Infertility. *25th Annual Meeting of Indian Society for the Study of Reproduction and Fertility and International Conference on Reproductive Health, National Institute for Research in Reproductive Health, Parel, Mumbai, 14-17 February, 2015.*
- *AnilKumar TR, Oommen OV and Kumar PG* (2015) Expression Profiling and Functional Characterization of Protocadherin y -Linked 11 in Relation to Spermatogenesis. *25th Annual Meeting of Indian Society for the Study of Reproduction and Fertility and International Conference on Reproductive Health, National Institute for Research in Reproductive Health, Parel, Mumbai, 14-17 February, 2015 (won the best poster award)*
- *Radhakrishnan K and Kumar PG* (2015) Histone Methylation and Spermatogenesis. *25th Annual Meeting of Indian Society for the Study of Reproduction and Fertility and International Conference on Reproductive Health, National Institute for Research in Reproductive Health, Parel, Mumbai, 14-17 February, 2015.*
- *Soumya A and Kumar PG* (2015) Effect of Raft Destabilization on Sperm - Zonapellucida Interaction. *25th Annual Meeting of Indian Society for the Study of Reproduction and Fertility and International Conference on Reproductive Health, National Institute for Research in Reproductive Health, Parel, Mumbai, 14-17 February, 2015.*
- *Jeeva SE and Kumar PG* (2015) Association between Stemness and TCTEX1 Expression in TesticularGerm cells from Adult Mouse Testis. *25th Annual Meeting of Indian Society for the Study of Reproduction and Fertility and International Conference on Reproductive Health, National Institute for Research in Reproductive Health, Parel, Mumbai, 14-17 February, 2015.*

HONORS AND AWARDS

- *Labhsetwar Award 2015* of the Indian Society for the Study of Reproduction and Fertility (ISSRF)
- *Best Poster Award to Anilkumar TR* at the 25th Annual Meeting of the Indian Society for the Study of Reproduction and Fertility and International Conference on Reproductive Health, National Institute for Research in Reproductive Health, Parel, Mumbai, 14-17 February, 2015.

EXTRA-MURAL FUNDING

No.	Investigator(s)	Title	Funding Agency	Duration
1	Pradeep Kumar G	Molecular evaluation of interactions between sperm membrane rafts and zona pellucida proteins	Department of Biotechnology, Government of India	2011-2015
2	Pradeep Kumar G	Association between stemness and TCTEX1 expression in testicular germ cells from adult mouse testis	Board of Research in Nuclear Sciences	2011-2015
3	Pradeep Kumar G	Role of CLP-1 in cell cycle regulation in spermatogenic cells	Department of Science & Technology, Government of India	2013-2016
4	Pradeep Kumar G	Evaluation of cellular aging and genome stability in spermatogonial stem cells	Council of Scientific and Industrial Resaerch	2014-2017

MOLECULAR REPRODUCTION
LABORATORY - 2

Malini Laloraya

Scientist F

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Malini Laloraya received her PhD from Devi Ahilya Vishwavidyalya, Indore. She was a Fellow in Residence at a Center for Biomedical Research, Population Council, Rockefeller University, New York and was a Visiting Faculty at University of Virginia, VA and University of Florida, Gainesville, FL, USA. She joined Rajiv Gandhi Centre for Biotechnology in the year 2004.

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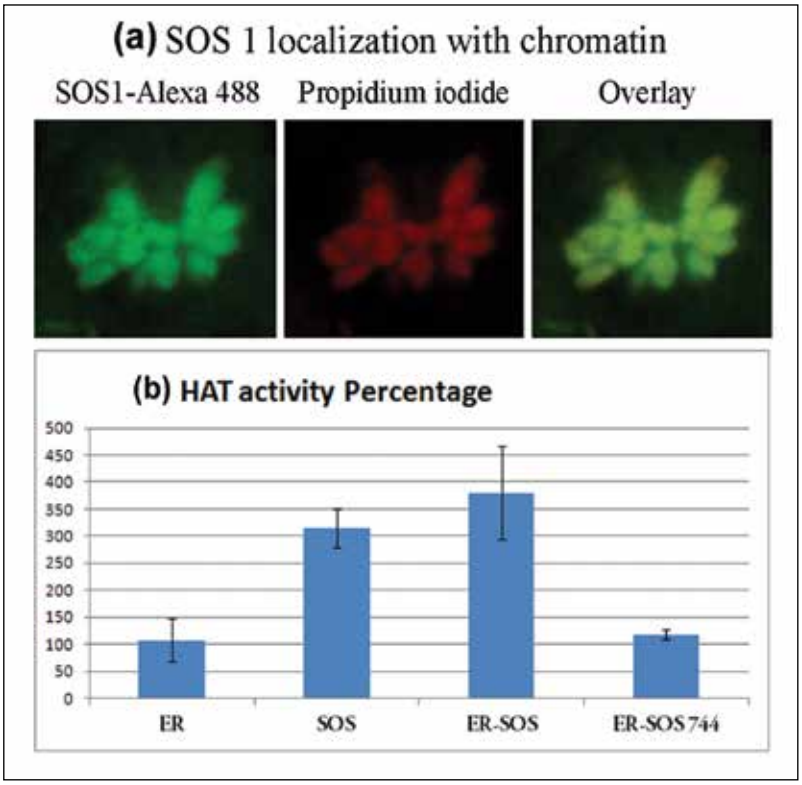
Sora S

Son of sevenless1 is a Histone Acetyl Transferase (HAT).

Renjini AP, Anand G and Malini Laloraya

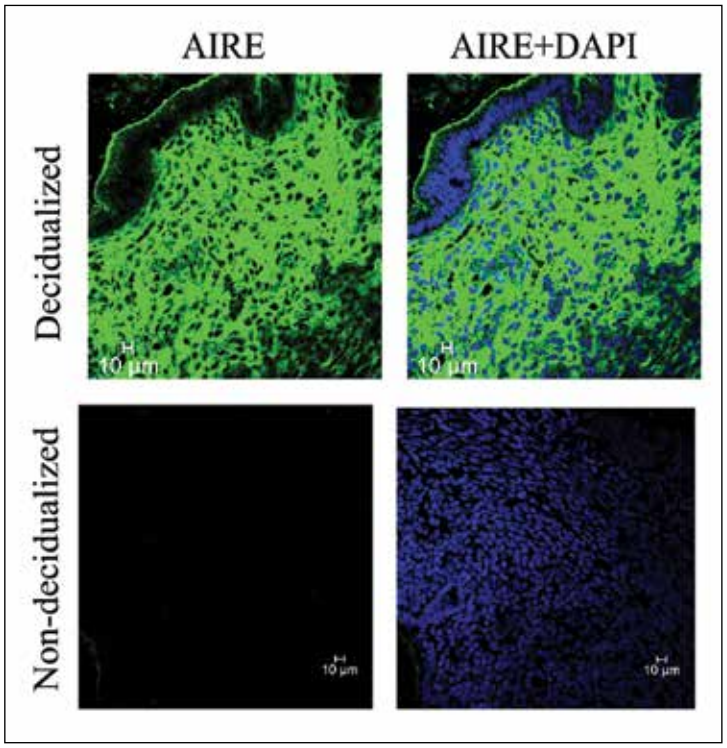
Embryo implantation is the result of supreme interaction between the uterus and the embryo, the process controlled by several biomolecules acting in harmony under the influence of hormones. Son of sevenless (SOS), the Guanine nucleotide exchange factor (GEF) of Ras, is a critical linker between RTK signaling and its downstream effector, the small GTPase, RAS. The accepted model of SOS function is the RasGEF activity and recruitment of SOS to the plasma membrane via formation of SOS- Grb2 complex is the basic mechanism ultimately responsible for activation of the mature, membrane-bound Ras proteins. The SOS GEFs can also be involved in RAC activation, in proliferation of human renal cell carcinoma cells and components of the SOS-RAS-RAF-MAPK pathway are downregulated during differentiation of hESC. The identity of SOS signaling in the embryo implantation scenario comes from the evidence that targeted disruption of both alleles of mouse Sos1, which encodes a Ras-specific exchange factor, conferred mid-gestational embryonic lethality. In this study, we demonstrate that the classically known cytoplasmic molecule, SOS1 is nuclear during window of implantation which is specifically restricted to implantation sites, suggesting its importance in implantation and that SOS1 expression and subcellular distribution would be regulated by estrogen. Classically, proteins (>45 kDa) that translocate from cytosol to nucleus possess nuclear localization signals (NLS) and with the aid of cargo proteins viz., karyopherins (importins) they translocate into nucleus. Our computational analysis had identified the presence of simple bipartite karyophilic clusters of arginines and lysines similar to nuclear localization signals (NLS) in nucleus-targeted protein signaling and SOS1 is targeted to nucleus via importin α. Co transfection experiments show estrogen regulated nuclear migration of SOS1 and mutation in the NLS region caused disruption of its nuclear entry. The concept of nuclear entry of SOS1 by associating with a classical karyopherin importin α was further validated by presence of positive bands for SOS1 and importin α in immunoblotting experiments in SOS1 co-immunoprecipitaes of SOS1 transfected cells. Finally, we also show that SOS1 localizes with chromatin (Fig.1a), associates with Histones H1, H2A, H2B and H4 and shows Histone acetyl transferase activity measured by HAT activity colorimetric assay kit. In vitro HAT assay prove SOS1 to acetylate Histone H4. SOS1 has acetyl CoA binding sites which when mutated reduced its HAT activity confirming the importance of the motif itself and the HAT function (Fig. 1b).



Autoimmune regulator, AIRE - a key molecule in Implantation

Soumya V and Malini Laloraya

Proper embryo implantation is crucial for successful pregnancy. Implantation failure, which is presently the major obstacle in human fertility, is mainly attributed to the failure of acquisition of uterine receptivity. The transition into a receptive uterus includes both cellular and molecular changes. Embryonic implantation in the uterus involves apposition, adhesion and finally invasion of the embryo. The uterus during this time-point develops a unique immune environment during pregnancy Autoimmune regulator (AIRE) is a new candidate molecule in this network. It was identified in uterus as an interacting partner of Estrogen receptor α and Dock180 in our previous laboratory studies. Autoimmune regulator (AIRE) is the master regulator of autoimmunity. It was first identified as a mutated protein patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (also called Autoimmune polyendocrine syndrome type 1 (APS-1), a serious autoimmune disorder. Even though some other roles are suggested, it is mainly a transcription factor, which is able to transcribe nearly fifty tissue specific antigens in thymus. AIRE has a crucial role in negative selection of immune cells. Extrathymic expression of AIRE is also reported, including in the reproductive organs such as ovary and testis.Even though role in immune system is widely studied, the significance of extrathymic AIRE is a less explored area. Some significant studies are carried out in testis, but nothing is known about the role of intrinsic AIRE in female reproductive system. In our lab we have amplified and sequenced Aire from uterus. The uterine Aire shows homology with the standard Aire NM_009646. In vivo silencing approach reveals its significance in uterus as pregnancy is abolished. Since AIRE is known to bind to target gene promoters thereby affecting gene expression of target genes viz., Bmp2, Bmp4 and Ins1 and Ins2, genes, which are focal to decidual reprogramming during early pregnancy, we studied the impact of Aire silencing on key decidualizing markers. Our earlier report qRT-PCR using candidate markers of decidualization revealed that Hoxa10, Igfbp5, Bmp2 & Bmp 4 are down regulated in Aire silenced uteri. Immunohistochemistry and western blotting of HOXA10 confirmed the impact on decidualization. Studies using an in vivo decidualization model reveal that AIRE expression is high in deciduoma (Fig. 2), compared to other regions of the decidualized uterus and also with the non-decidualized control. This hints at the possible role of AIRE in decidualization, the process in which the endometrium undergoes extensive changes in morphology and expression and secretion patterns to support the implanting blastocyst and thus vital for maintenance of pregnancy.



Hyporesponsiveness to IL2 signaling contributes to low Tregs in Polycystic Ovarian Syndrome (PCOS)

Meera Krishna B, Annu Joseph, Anand G. Subramaniam, Sathy M. Pillai* and Malini Laloraya

Collaborator - SAMAD IVF Hospitals

PCOS is the most common endocrinopathy among adolescent women and is the leading cause of anovulatory infertility. PCOS is classified as a metabolic syndrome characterized by oligo/anovulation, hyperandrogenemia, insulin resistance and accompanying hyperinsulinemia. The endocrine and metabolic defects contribute to infertility, endometrial disorders leading to implantation failure in PCOS. It is also associated with an adverse risk profile for developing pregnancy complications, diabetes, CVDs and endometrial cancer. Thus PCOS lies at the crossroads of women's health, and elucidating its roots would positively impact the reproductive and long-term health of all women. Even though prevalence of PCOS is worldwide, the pathophysiology remains poorly understood, and treatment largely empirical. Identification of the complex regulatory networks under any disease condition is imperative to understand its pathology. In the earlier reports we have shown the differential expression of integrating miRNA and mRNA in peripheral blood of PCOS patients. The miRNA expression profiling reported earlier led to the identification of major enriched pathways including leukocyte transendothelial migration, phosphatidylinositol signaling system, GnRH signaling pathway, regulation of actin cytoskeleton, MAPK signaling pathway and autoimmunity pathways. Our transcriptome profiling identified differentially expressed genes (DEGs) involved in angiogenesis, oxidative stress, androgen signaling, insulin signaling, TGF β signaling, BMP signaling, BCR and TCR signaling Toll like receptor signaling etc. A network was created with Cytoscape V 8, a PCOS-specific miRNA-TF regulatory network was made by compiling miRNAs, its target genes and known human transcription factors (TFs) from the profiling data. According to the earlier network created, along with the traditional hormonal pathways, the involvement of novel autoimmune networks, cytoskeletal and angiogenic pathways in PCOS, was postulated. In this year we focused our attention the autoimmune network aspect. Autoimmune mechanisms are also regulated by regulatory T (CD4+CD25+ CD127-) cells (Tregs). So the implications from

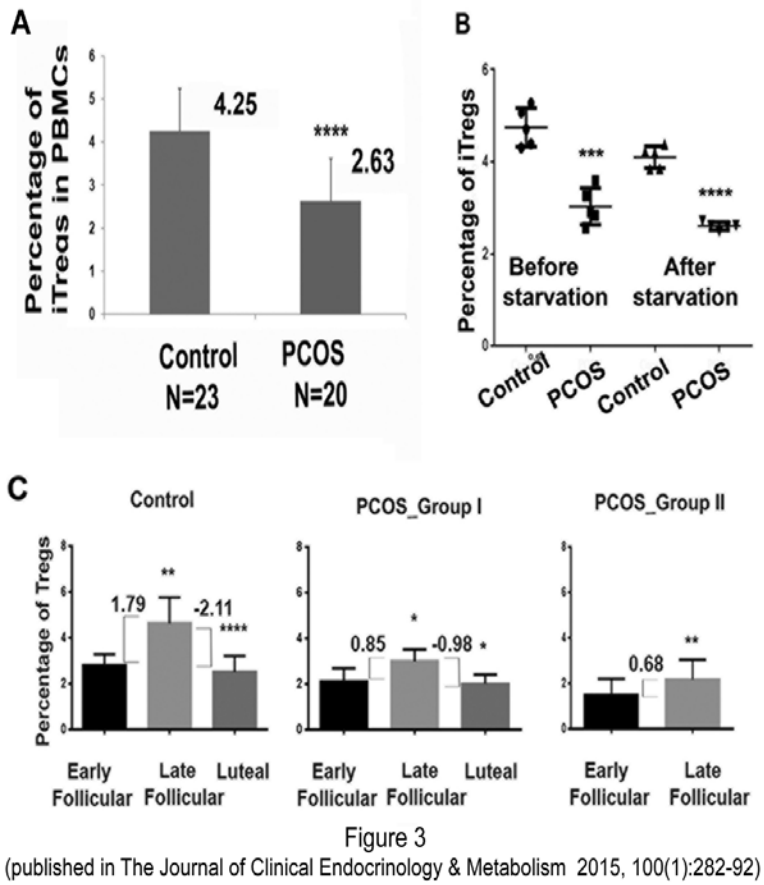


Figure 3

(published in The Journal of Clinical Endocrinology & Metabolism 2015, 100(1):282-92)

our miRNA-mRNA expression profiling drove us to investigate proportionate difference if any, occurring in the number of Tregs in the peripheral blood of PCOS patients. Through FACS analysis during the follicular phase, we found a significant reduction in the number of CD4+CD25+ CD127- TREGs in PCOS patients when compared with control women (Figure 3a). In order to address the hypothesis that low tregs are inherent to PCOS, serum starvation experiments showed that reduction in Tregs is consistent with earlier observations even after serum starvation (Fig. 3b). Follicular phase expansion of Tregs during menstrual cycle has been reported earlier. Since we observe a decline in Tregs at early follicular phase, we planned experiments to assess whether the follicular expansion of Tregs is affected in PCOS women or not. We found a significant decline in the Treg expansion is impaired in PCOS women as there was only a marginal increase of Tregs from early to late follicular phase ; PCOS group I (0.8479% ± 0.321) and Group II PCOS (0.679%±0.185) women compared to control women (1.795%±0.436). The expansion was more

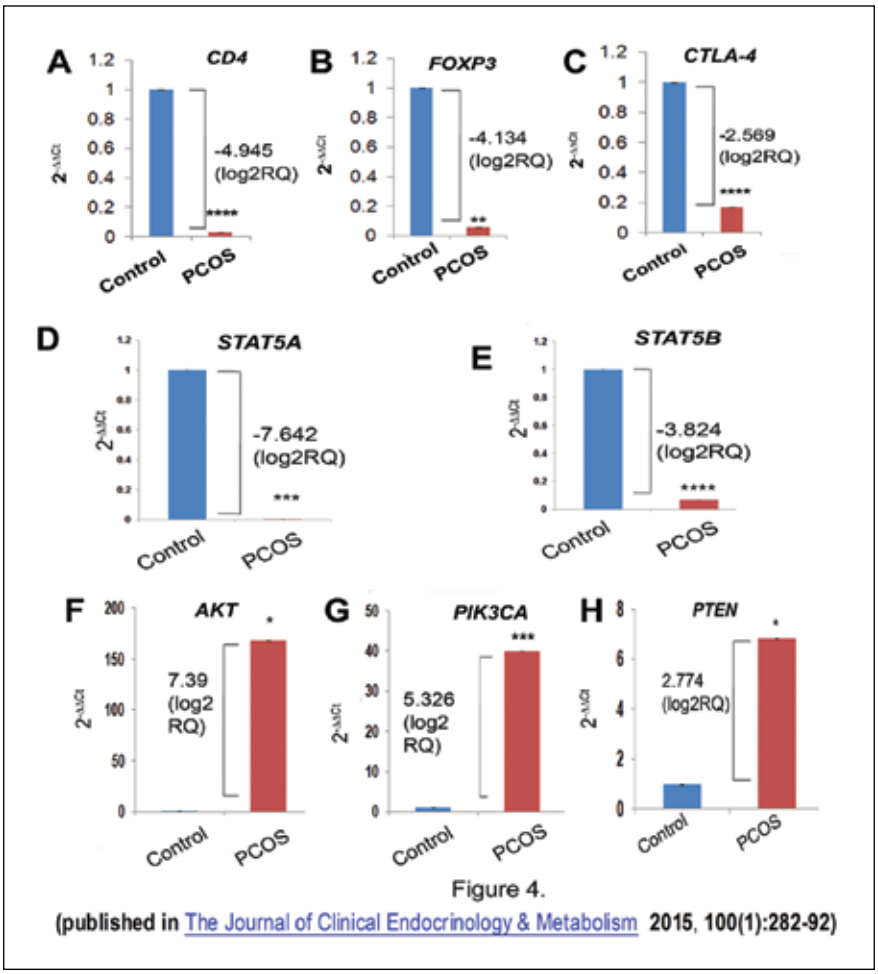


Figure 4.

(published in The Journal of Clinical Endocrinology & Metabolism 2015, 100(1):282-92)

worsened in the group with chronic oligo ovulation (Group_II PCOS) compared to the group with mild oligo ovulation (Group_I PCOS). The Tregs showed a decrease of 2.116 %±0.26 (Control) and 0.98% ±0.28 (PCOS_Group I) from late follicular to luteal phase (Fig. 3c).

Expression of classical Treg markers *CD4, FOXP3* and *CTLA4* was significantly down in PCOS group (Fig, 4 A-C). To look into the pathways or factors regulating Treg generation we explored the factors regulating Treg generation. IL2 signaling can regulate FOXP3 expression and Treg generation via STAT5 and AKT/PIK3. The real time analysis showed under-expression of *STAT* transcripts *STAT5A* (fold change= 7.642, P

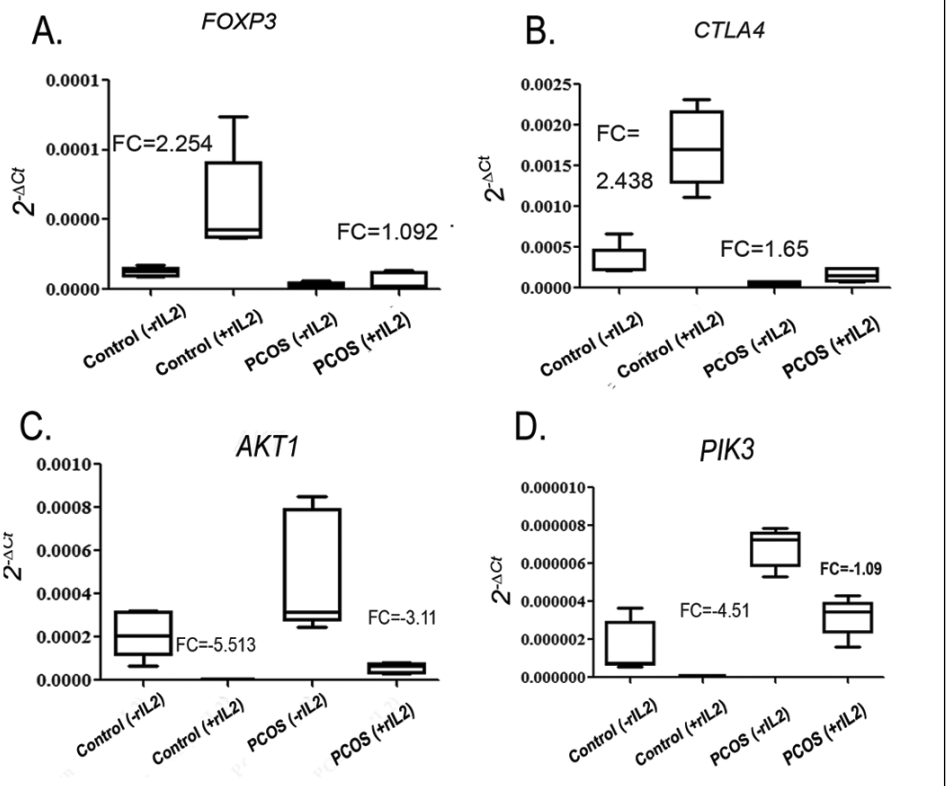


Figure 5

(published in The Journal of Clinical Endocrinology & Metabolism 2015, 100(1):282-92)

value<0.0004) and *STAT5B* (fold change= 3.824, P value<0.0001) (Figure 4 D-E). PTEN acts as a negative regulator of AKT/PIK3 axis. The analysis revealed that the *AKT/PIK3* mRNAs were over expressed with *AKT (*fold change =7.39, p<0.05), *PIK3CA* (fold change = 5.326, p<0.0002) and *PTEN* (fold change = 2.775, p<0.05) (Fig. 4 F-H).

Studies on impact of IL2 stimulation on Treg generation revealed that supplementation of exogenous IL2 signal could bring down the AKT/PIK3 mRNA and protein levels in PCOS and control women. The activated AKT also was reduced under IL2 stimulation. But IL2 stimulation did not affect the STAT5A/B mRNA levels in either PCOS or controls. Although IL2 stimulation could increase the expression of *FOXP3/ CTLA4* mRNA levels in control by more than 2 fold, no increase in patients was seen (Fig.5 A-B). The results were further confirmed with western blot analysis. Western blot also showed failure of phosphorylation of STAT5 in PCOS patients in comparison to controls on IL2 stimulation. Thus, the IL2 signal was insufficient to increase FOXP3 presumably due to failed STAT5 phosphorylation. Thus our study reveals that diminished Tregs in PCOS are a consequence of IL2 hyporesponsiveness which is unable to activate STAT5b and thus leads to reduced FOXP3 expression. Even though IL2 signaling could not improve FOXP3 levels, it could reduce AKT/PIK3 levels, the negative arm. IL2 based therapeutic strategies can thus be tested in PCOS as they might improve Tregs by suppressing the AKT/PIK3 arm. Since an efficient Treg population is a prerequisite for maintaining statics of peripheral tolerance and autoimmunity, our study implicates an autoimmune aetiology in the pathogenesis of PCOS. Our study predicts Tregs as the major 'cues of induction' of the aforesaid complications. This underlines their potentiality as predictive markers for pregnancy complications and metabolic anomalies in PCOS. The study reiterates on the implementation of Treg-based intervention in management of PCOS.

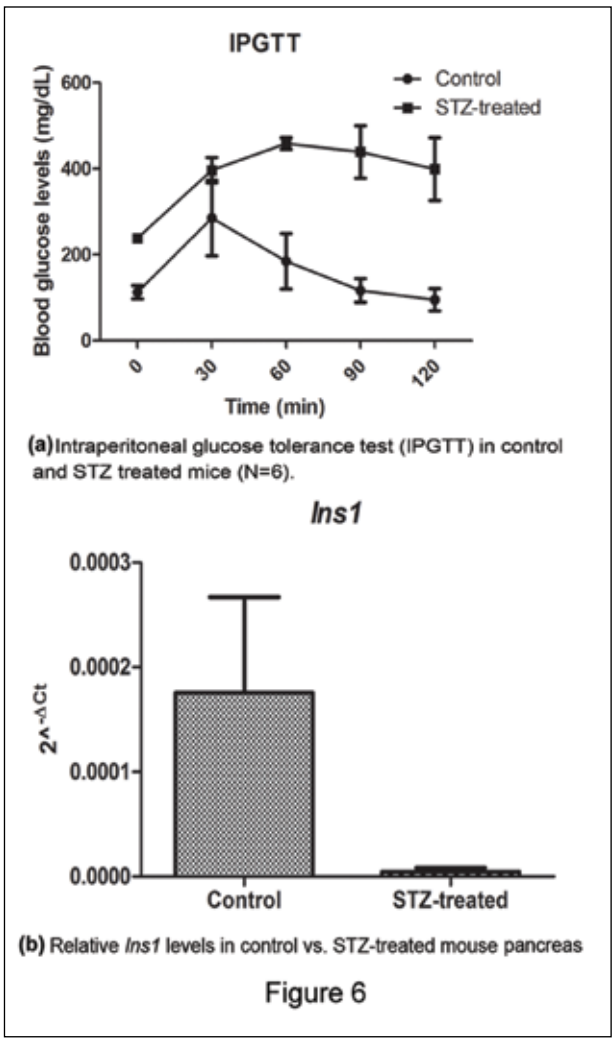


Figure 6

Analyzing the differential proteomic profile in normal and streptozotocin treated diabetic mouse model

Annu Joseph and Malini Laloraya

Type 1 diabetes is caused by the autoimmune destruction of beta cells in the islets of Langerhans in pancreas. Significant inroads have been made regarding the understanding of type 1 diabetes (T1D); however the core mechanisms involved is still not fully addressed. Several animal models have been developed to understand the systemic and metabolic complications related to T1D; multiple low dose streptozotocin induced diabetes model because of its high reproducibility is one of the most widely used. Streptozotocin invokes T-cell mediated autoimmune responses among other actions leading to the selective destruction of pancreatic islets. The experimental group of animals were given 50mg/kg streptozotocin drug for 5 consecutive days to induce beta cell damage. The animals were considered to be *hyperglycemic* if the blood glucose levels were >300mg/dL and sacrificed. Intraperitoneal glucose tolerance test in control mice showed a decrease in blood glucose

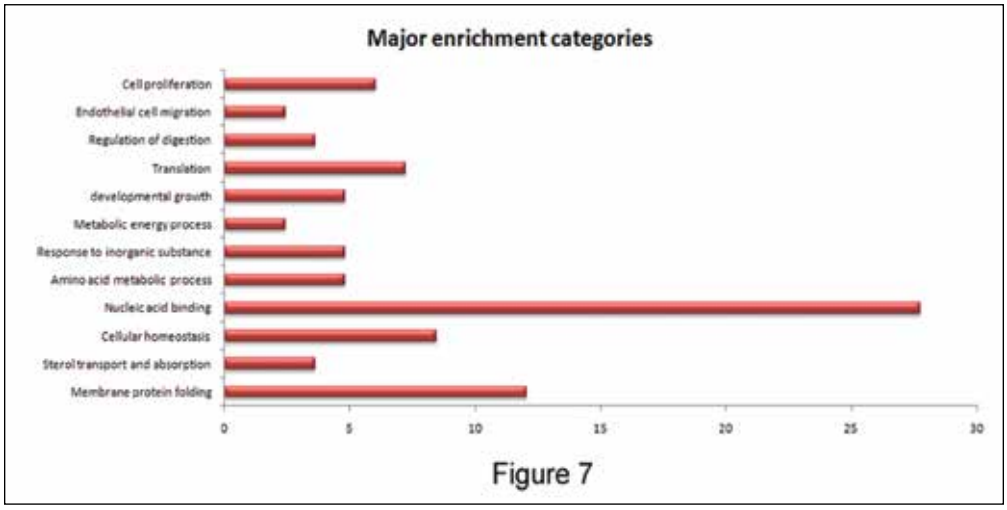


Figure 7

levels during the 2 hour period while the glucose levels remained persistently high (around 400 mg/dL) in STZ-treated mice showing the impaired glucose metabolism in the diabetic animals (Figure 5a). *Ins1* showed a fold change of -5.64, indicating the loss of insulin-producing beta cells (Fig. 5b). In order to understand the islet-specific molecular mechanisms leading to T1D, we compared the proteome profile of pancreas in a multiple low dose streptozotocin induced diabetes mouse model *vs.* control by employing label-free ion mobility UPLC-ESI-QTOF-MS^{E}. The data generated was processed and analyzed using the software ProteinLynx Global Server (PLGS) v2.5.3 with ADH as internal control. Functional enrichment analysis using DAVID 6.7 revealed highest enrichment with protein folding. Pancreatic beta cells contain highly developed network of endoplasmic reticulum and related vesicles as a mechanism for effective insulin secretion. Among various factors projected to be responsible for beta cell death in T1D, ER stress followed by ER dysfunction is considered to be a leading candidate (Fig. 7). Our preliminary data illustrates that the various proteins including chaperones involved in protein folding machinery show significant deviation from the normal levels (Fig. 8). This indicates that chaperone-assisted protein folding mechanism is impaired leading to accumulation of unfolded or misfolded proteins eventually ending in beta cell death in T1D.

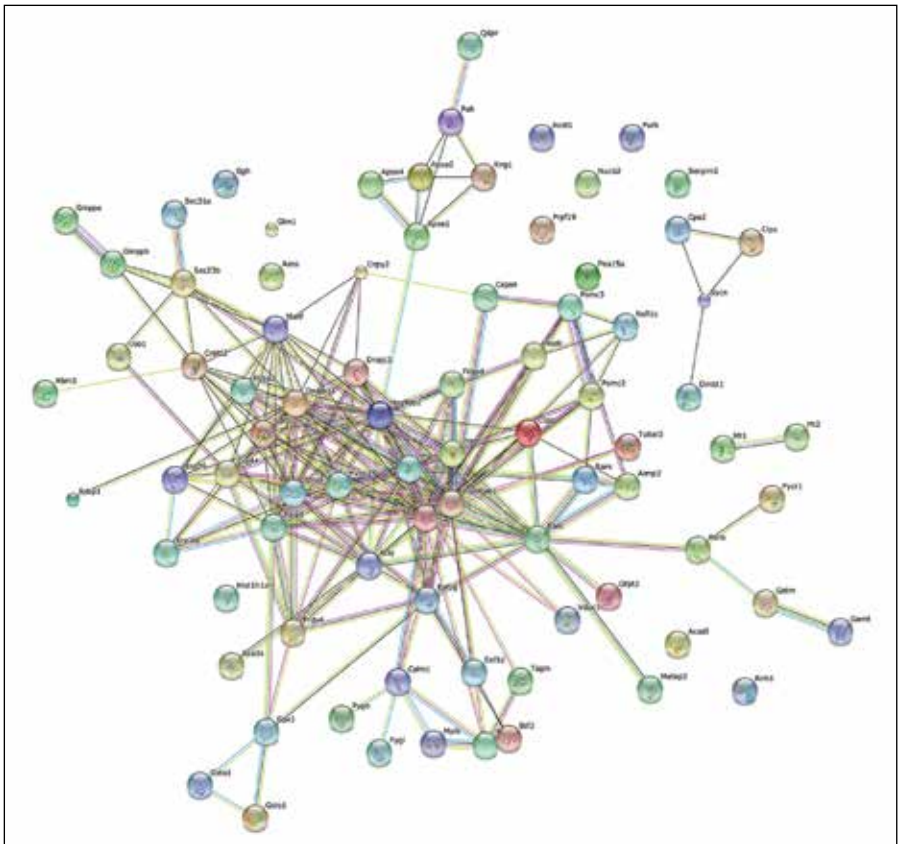


Figure 8

PUBLICATIONS

- *A. P. Renjini, Shiny Titus, Prashanth Narayan, Megha Murali, Rajesh Kumar Jha, and Malini Laloraya* STAT3 and Mcl-1 unite to cause mesenchymal epithelial transition. *Journal of Cell Sciences. 2014, 127(Pt 8): 1738-50.*
- *Meera B Krishna 1, Annu Joseph1, Anand G. Subramaniam 1, Arundhati Gupta1, Sathy M. Pillai2, and Malini Laloraya1.* Reduced Tregs in Peripheral Blood of PCOS Patients - a Consequence of Aberrant Il2 Signaling. *Journal of Clinical Endocrinology and Metabolism 2015;100(1):282-92.*

CONFERENCE PRESENTATIONS

- Talk entitled "EMT/MET switching - a cellular paradigm crucial for successful pregnancy" at *25th Silver Jubilee – Annual Meeting along with an International Conference on Reproductive Health (ISSRF-2015), February 14-17, 2015, NIRRH, Mumbai.*
- Talk entitled "PROTEIN INTERACTOMICS IN EMBRYOIMPLANTATION" AT "INTERNATIONAL CONGRESS ON EMBRYO IMPANTATION AND PREGNANCY: INTRICACIES AND STRATEGIES FOR ITS SUCCESS, March 9-11, 2015 at *National Institute of Immunology, New Delhi, INDIA*

POSTER PRESENTATIONS

- *Annu Joseph and Malini Laloraya** Pancreatic protein expression profiling reveals dysregulated protein folding machinery in Type 1 diabetes. Presented at the *Keystone Symposium on Emerging Concepts and Targets in Islet Biology under Keystone Symposia on Cellular and Molecular Biology 6th – 11th April 2014 at Keystone, Colorado, USA.*

EXTRA-MURAL FUNDING

Sl. No.	Investigator	Title	Funding Agency	Duration
1.	Dr. Malini Laloraya(PI)	Mechanism of STAT5B in pancreatic beta cell proliferation/sustenance and its significance in diabetes.	Board of Research in Nuclear Sciences	2015 to 2018.

TROPICAL DISEASE BIOLOGY
MYCOBACTERIUM RESEARCH GROUP – 1

Sathish Mundayoor

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Sathish Mundayoor obtained his PhD from All India Institute of Medical Science, New Delhi, did Post Doctoral training at Forshungsinstitut Borstel, Germany and then at Washington University in St Louis, Missouri. He was also a visiting Scientist at Centers for Disease Control, Atlanta, USA before joining RGCB in 1995.

PhD Students:

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Annapoorna K
Retnakumar R J

Junior Research Fellow:

Vishnu A

Manager (Technical Services)

Laiza K Paul

Mycobacterium tuberculosis, the causative agent for Tuberculosis persists in macrophages, even though macrophages are normally programmed to kill these and other invading pathogens. The infection of the macrophage and the survival of the bacteria in this milieu cause major alterations in the macrophages and our laboratory has been studying these changes.

Downregulation of Macrophage surface receptors by Mycobacteria

Retnakumar R J, Vishnu A and Satish Mundayoor

When *Mycobacterium tuberculosis* or the vaccine strain, *M. bovis* BCG infects macropahges, it has been shown to affect the surface receptors of macrophages. Alterations in Complement Receptor, Fc receptor and Mannosyl Fucosyl Receptor have been documented earlier. We have previously shown that Rv 2404c, a Probable GTP-binding Protein (LepA) from *Mycobacterium tuberculosis* showed downregulation of Scavenger receptor A in THP1 cells by. Similar genes are also present in the vaccine strain M bovis BCG. We cloned homologues of the genes that we had screened in *Mycobacterium tuberculosis* H37Rv from BCG and transformed these constructs into *Mycobacterium smegmatis,* a non-pathgoenic fast growing strain of mycobacteria. These recombinant *Mycobacterium smegmatis* were used to infect THP1 cells and the Scavenger receptor was visualized by immunofluorescent staining. *M smegmatis* carrying BCG 2420c, a homolog of Rv 2404c (a Probable GTP-binding Protein LepA) showed significant reduction in Scavenger receptor expression at 48 hrs. On the other hand, BCG1935c, a homolog of Rv1936c, another conserved hypothetical protein did not show any downregulation and acted as a control. The property and mode of functioning of these genes are being investigated.

Live Mycobacteria downregulate cytoskeletal proteins of macrophages

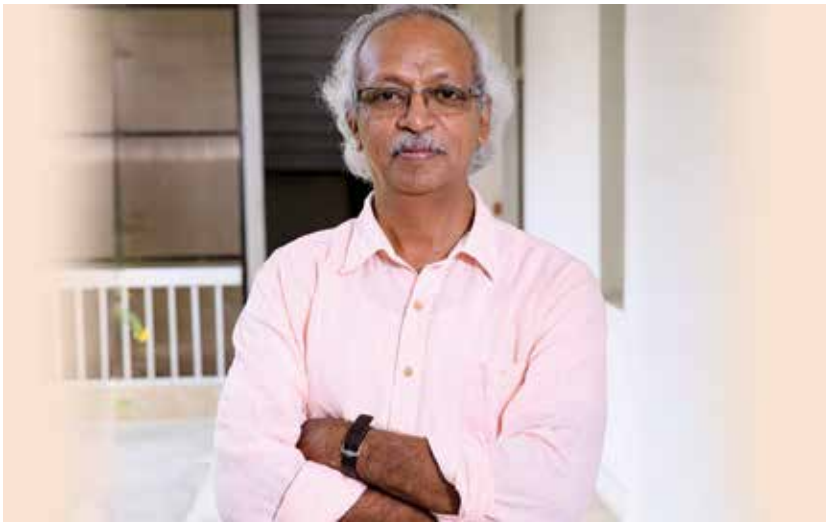
Mahesh PP and Satish Mundayoor

Mycobacterium tuberculosis, persists primarily in macrophages after infection and manipulates the host defence pathways in its favor. Our 2D gel electrophoresis results show that vimentin, an intermediate filament protein, is down-regulated in macrophages infected with live *Mycobacterium tuberculosis*, H37Rv when compared to macrophages infected with heat killed H37Rv. The down-regulation was confirmed by Western blot and quantitative RT-PCR. Besides, the expression of vimentin in avirulent strain, H37Ra- infected macrophages was similar to the expression in heat-killed H37Rv-infected macrophages. The down-regulation of vimentin by H37Rv suggests that live mycobacteria express substances that modulate expression systems of macrophages. Treatment of heat- killed H37Rv infected macrophages with PDTC, an inhibitor of NF-κB; NAC, a ROS scavenger and DPI, an inhibitor of both NADPH oxidase and NOS resulted in steep reduction in the expression of vimentin. These results imply that down-regulation of vimentin by *Mycobacterium tuberculosis* may be due to its inherent anti-inflammatory effect on macrophages. Conversely, the incubation of macrophages with anti-vimentin antibody increased the ROS production and decreased the survival of H37Rv. We also showed that the pattern of phosphorylation of vimentin in macrophages by PKA/PKC is different from monocytes, emphasizing a role for vimentin phosphorylation in macrophage differentiation.

EXTRA MURAL GRANTS

Sl. No	Investigators	Title of Project	Funding agency	Duration
1	Sathish Mundayoor and R Ajay Kumar	Identification of Mycobacterial Genes Involved in Downregulation of Macrophage Receptors, viz Complement, Fcg and Mannose Receptors	Kerala State Committee for Science, Technology and Environment	2014-2017

TROPICAL DISEASE BIOLOGY
MYCOBACTERIUM RESEARCH GROUP - 2



R. Ajay Kumar

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Ajay Kumar received his PhD in Microbiology from Madurai Kamaraj University, Madurai and did postdoctoral training at Indian Institute of Science, Bangalore, Sri Chitra Thirunal Institute for Medical Sciences Technology, Trivandrum, and University of Massachusetts Medical School, Worcester, USA

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Research Associate

VipinGopinath

Junior Research Fellows

Akhil Raj
Balaji M

Tuberculosis (TB) kills 1.4 million people a year. *Mycobacterium tuberculosis,* the causative agent, is an intracellular pathogenic bacterium and resides in professional phagocytes. After infection, a strong protective immune response of the body limits bacterial growth, but often it fails to completely eradicate the bacilli from host. This results in latent infection, and the bacteria exist in a dormant state, where it is non-replicative and less active but can become reactivated under favorable conditions. In order to prevent these dormant bacteria from causing 'reactivation TB', they must be prevented from getting reactivated. This can be achieved only by therapeutic strategies targeted specifically against dormant or reactivated bacteria. Towards this end, it is essential to identify proteins that play crucial roles during dormancy and reactivation. Such proteins can be novel targets for therapeutic intervention to prevent reactivation of latent TB.

Profiling the proteome of M. tuberculosis during dormancy and reactivation.

Vipin Gopinath, Sajith R, Akhil Raj P, Sathish Mundayoor, Abdul Jaleel and R. Ajay Kumar.

Tuberculosis still remains a major global health crisis. The main obstacle in eradicating this disease is the ability of this pathogen to remain dormant in macrophages, and to get reactivated later under immuno-compromised conditions. The physiology of hypoxic nonreplicating *M. tuberculosis* is well studied using many in vitro dormancy models. However, the physiological changes that take place during the shift from dormancy to reactivation (aerobic growth) have rarely been subjected to detailed investigation. In this study, we developed an in vitro reactivation system by re-aerating the virulent laboratory strain of *M. tuberculosis* that was made dormant employing Wayne's dormancy model, and compared the proteome profiles of dormant and reactivated bacteria using label-

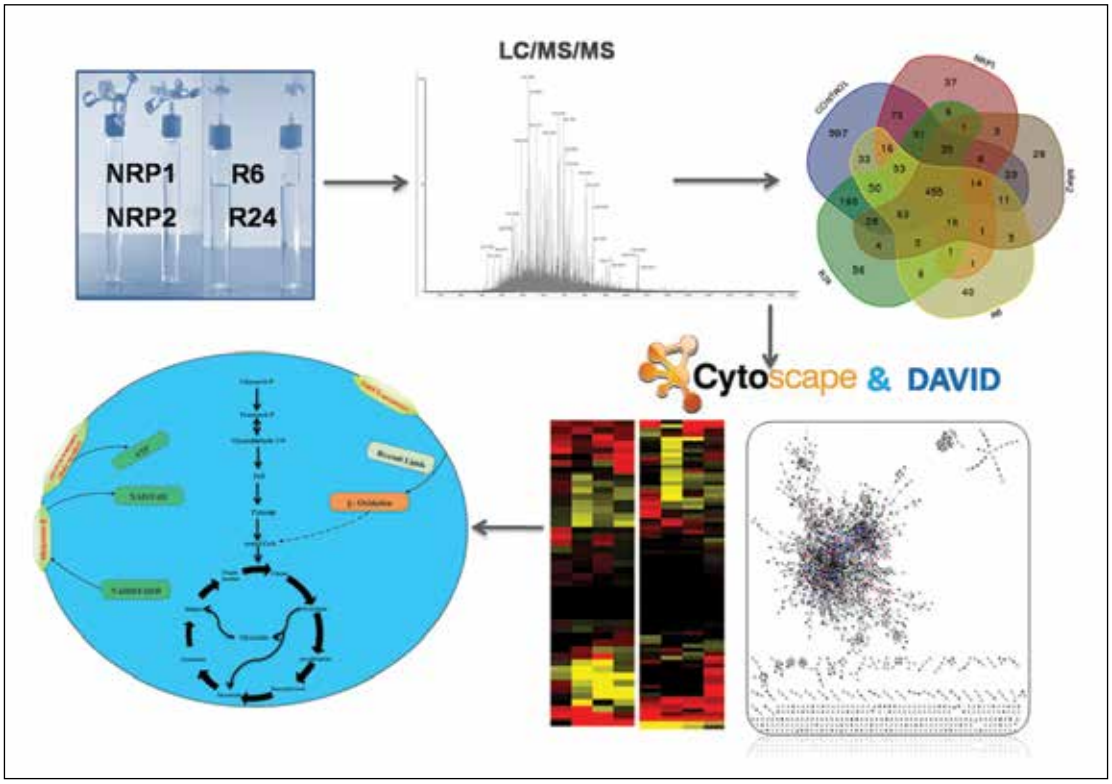

Fig 1. Graphical representation of the quantitative proteomic analysis of dormant and reactivated M. tuberculosis.

free one-dimensional LC-MS-MS analysis. The proteome of dormant bacteria was analyzed at nonreplicating persistent stage 1 (NRP1) and stage 2 (NRP2), while that of reactivated bacteria was analyzed at 6 and 24 hours post re-aeration. Proteome of normoxially grown bacteria served as the reference. Three biological replicates of each stage, with three technical replicates of each, were analyzed. In total, 1,871 proteins comprising 47% of the *M. tuberculosis* proteome were identified, and many of them were observed to be expressed differentially or uniquely during dormancy and reactivation. The number of proteins detected at different stages of dormancy (764 at NRP1, 691

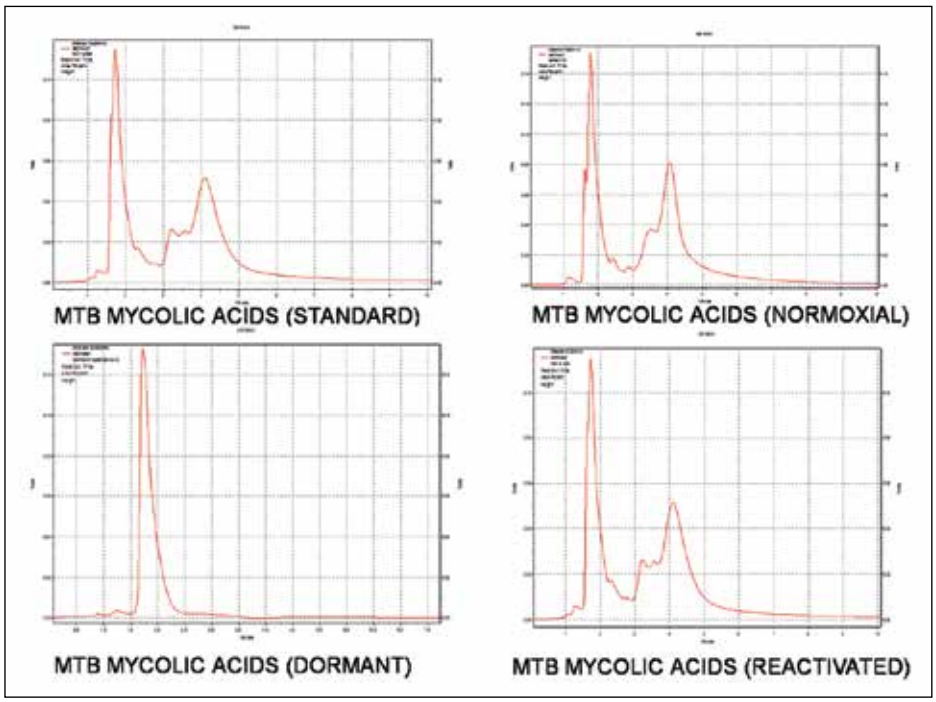

Fig. 2. HPLC profiles of Mycolic acids isolated from control, dormant and reactivated MTB.

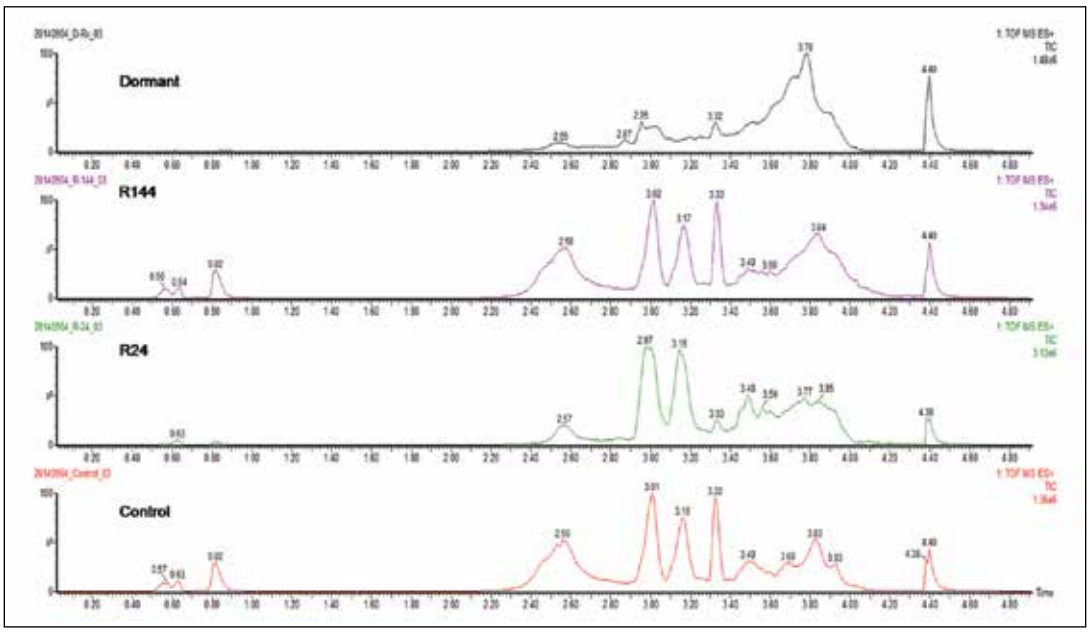

Fig 3. LC-MS/MS profiles of mycolic acids isolated from control MTB, 24 hr after reactivation, 144 hr after re-aeration, dormant and normoxially grown MTB.

at NRP2) and reactivation (768 at R6 and 983 at R24) was very low compared to that of the control (1663). The number of unique proteins identified during normoxia, NRP1, NRP2, R6 and R24 were 597, 66, 56, 73 and 94, respectively. We analyzed various biological functions during these conditions. Fluctuations in the relative quantities of proteins involved in various metabolic processes such as energy metabolism, amino acid metabolism, lipid biosynthesis, lipid biodegradation, DNA replication, repair, transcription, protein synthesis etc. were observed during dormancy and reactivation. The overall scheme of the study is shown in Fig 1.

Proteins that are upregulated or uniquely expressed during reactivation from dormancy offer to be attractive targets for therapeutic intervention to prevent reactivation tuberculosis. As we observed significantly low levels of proteins involved in the biosynthesis of mycolic acid and high levels of proteins associated with degradation of lipids during dormancy, we analysed the mycolic acids at these stages. An HPLC analysis of mycolic

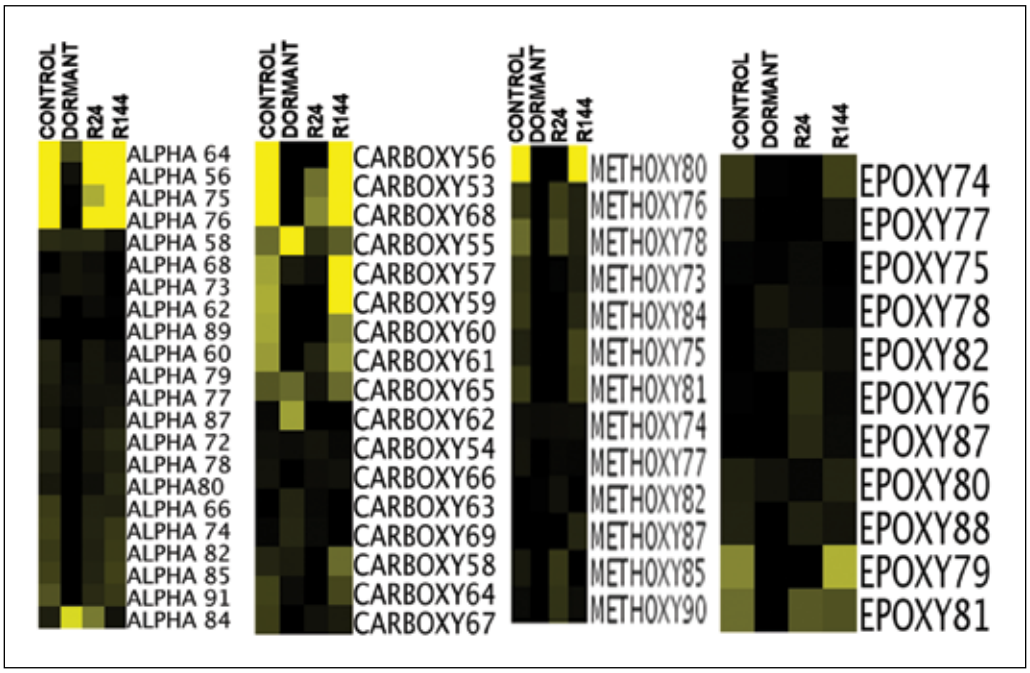

Fig 4. Heat map of alpha, carboxy, methoxy and epoxy groups of mycolic acid grown under normoxia, dormancy, 24 hr and 144 hr after reactivation.

acids isolated from normally grown, dormant and reactivated *M. tuberculosis* revealed interesting patterns (Fig. 2). The mycolic acids were further analyzed using MALDI-TOF and LC-MS/MS (Fig. 3).

We found considerable differences in the profiles of mycolic acid during dormancy and different stages of reactivation. While keto derivatives of mycolic acids were completely missing, a significant decrease in the alpha, carboxy, methoxy and epoxy derivatives was observed during dormancy. Interestingly, when dormant *M. tuberculosis* was subjected to re-aeration, the mycolic acid profile became similar to that of normoxially grown control (Fig. 4).

Host-Pathogen interactions:

Gene expression in eukaryotes is regulated by events that selectively expose or hide regulatory sequences of genes. These events are mainly orchestrated by chromatin remodeling and chromatin modifying factors. Since microbial infections cause changes in the host physiology, we are interested in how *M. tuberculosis* infection brings about changes in host gene expression.

M. tuberculosis engages host HDAC1 to suppress the expression of host immune genes.

We have observed that infection of macrophages with *M.tuberculosis* leads to a significant decrease in total histone H3 acetylation and a concomitant increase in HDAC1 expression in the macrophages at 24 hrs. We proposed that enhancement of HDAC1 expression may be a strategy employed by *M. tuberculosis* to silence the host defense gene expression by deacetylating histones at the regulatory DNA sequences to facilitate its survival inside the host. We found that infection with live virulent *M. tuberculosis* causes downregulation of expression of two key genes (*STAT4* and *IL-12*, Figs. 5 & 6) whose products are very important for eliciting Th1 responses in the host. And we have demonstrated that this is the result of recruitment of HDAC1 to the promoter regions of these two genes. Th1 response is crucial for eliminating intracellular pathogens such as *M. tuberculosis*. Identification of *M. tuberculosis* factors that are responsible for the recruitment of HDAC1 to the host promoters could be novel targets for drug intervention for the treatment of tuberculosis.

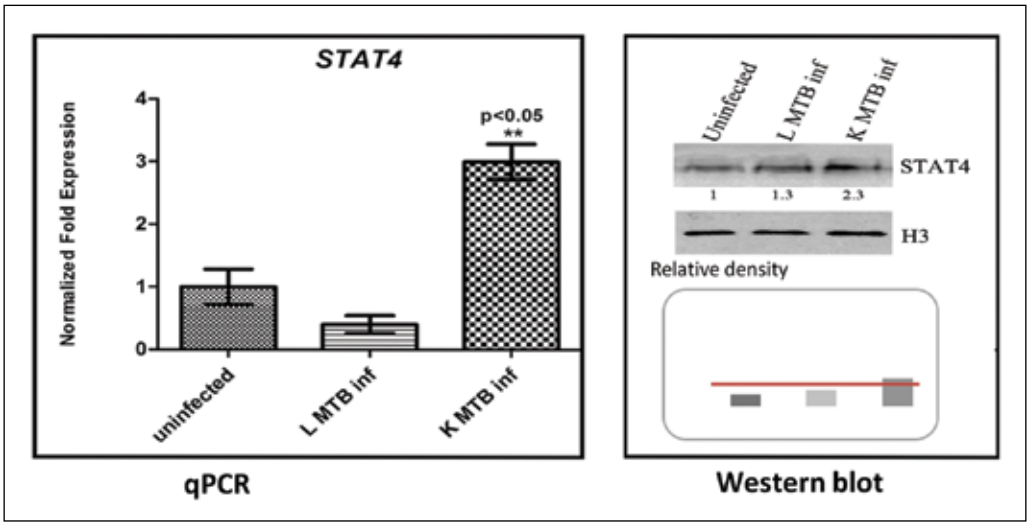

Fig. 5. Downregualtion of STAT4 in macrophages infected with live M. tuberculosis.

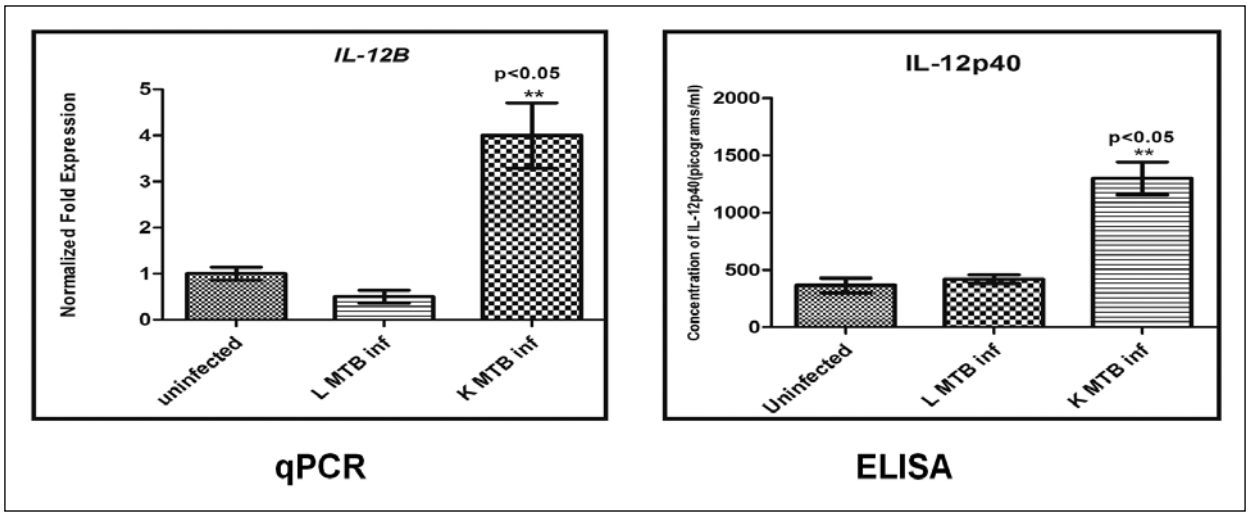

Fig. 5. Downregualtion of IL-12 in macrophages infected with live M. tuberculosis.

A novel histone acetyl transferase from *M. tuberculosis*.

We identified a novel chromatin-binding protein from *M. tuberculosis*. It was discovered by analyzing the proteins attached to the chromatin of macrophages infected with *M. tuberculosis* H37Rv. The gene for this protein was amplified from *M. tuberculosis* H37Rv, cloned, expressed and the protein was purified to homogeneity. Bioinformatics predictions followed by a number of biochemical studies revealed that this protein (Rv3423.1) is a histone acetyl transferase (MT-HAT). The target of its activity was found to be histone H3 and acetylation occurs at amino acid residues K9/K14. Anacardic acid inhibited its activity. Expression of *Rv3423.1* by intracellular bacteria was found to be elevated at 24 hrs post infection. Rv3423.1 expression was shown to enhance the intracellular growth of recombinant *M. smegmatis*. Since *M. tuberculosis* is exposed to different stresses inside a macrophage, we checked the expression level of *Rv3423.1* under different in vitro stress conditions. The gene was upregulated under elevated temperature and under nutrient starvation (Fig. 6), conditions that are believed to prevail in granuloma.

To identify the host genes regulated by MT-HAT, we performed a ChIP-sequencing, which indicated that it binds to nine promoter regions with significant peak scores. Following *M. tuberculosis* infection, expression of these 9 host genes in macrophages was analysed using qPCR. Significant up-regulation of eight genes after 24 hours post infection was observed (Fig.7).

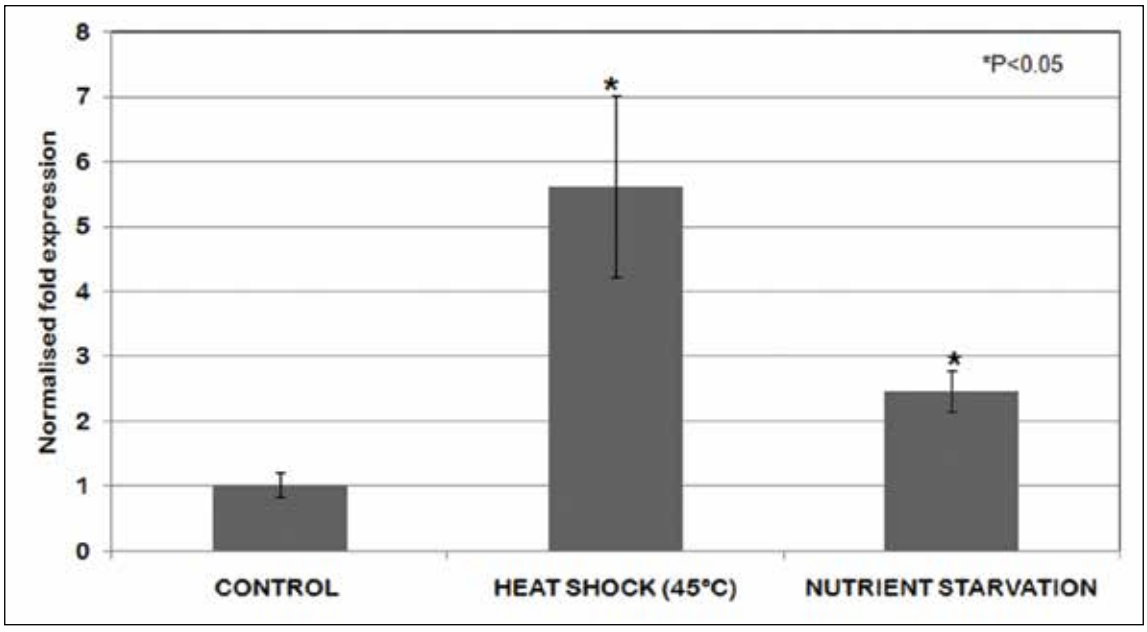

Figure 6: Expression of Rv3423.1 under different stress conditions

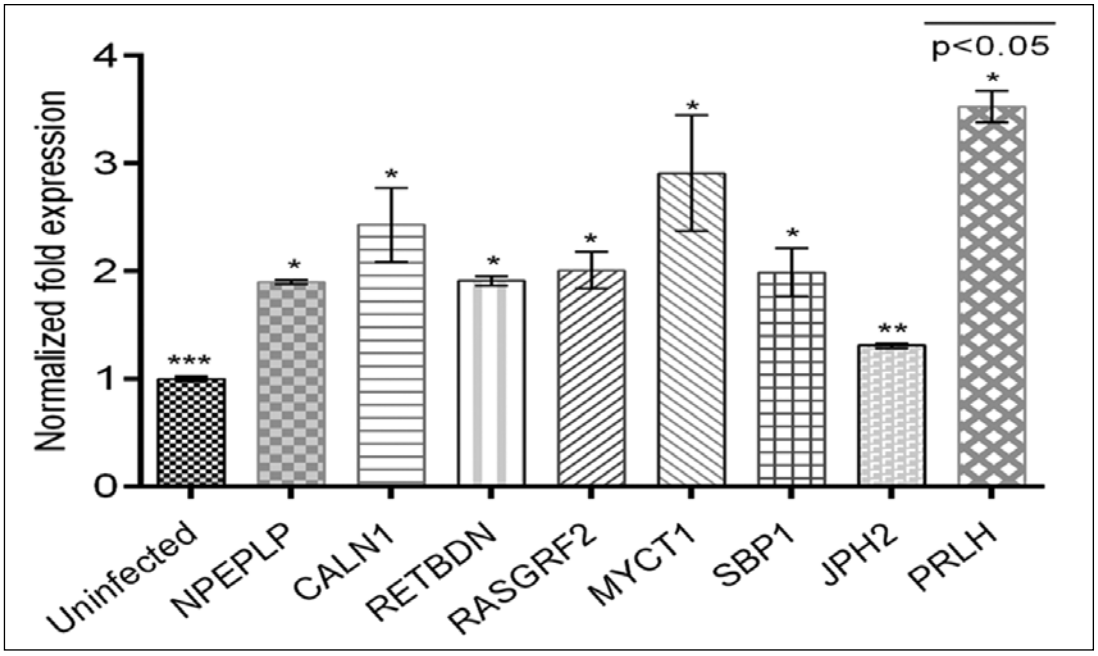

Figure 7: qPCR of genes identified using ChIP- sequencing at 24hr after infecting Macrophage with M. tuberculosis.

PUBLICATIONS

- *Gopinath V, Raghunandanan S, Gomez RL, Jose L, Surendran A, Ramachandran R, Pushparajan AR, Mundayoor S, Jaleel A, Kumar RA.* Profiling the proteome of *Mycobacterium tuberculosis* during dormancy and reactivation. *Molecular Cell Proteomics. (2015 DOI:10.1074/mcp.M115.051151).*

SYMPOSIUM PRESENTATIONS

- Recruitment of HDAC1 onto the promoters of host defence genes as a survival strategy of intracellular Mycobacterium tuberculosis. *Aneesh Chandran1, Cecil Antony2, K. Natarajan2 and R. Ajay Kumar1*. International conference on Host-Pathogen interactions. National Institute of Animal Biotechnology, Hyderabad. July 12-15, 2014.*
- Auto-regulation of a MerR family protein from Mycobacterium tuberculosis. *Roshna Lawrence Gomez, Leny Jose, Sathish Mundayoor and R. AjayKumar. International Conference on Biosciences:state - of - the-art advancements. Organised by: Society for Educational and Scientific Research (SESR). Venue :Lakesong Resort, Kumarakom. Date : Sept 11-12,2014*
- Mycobacterium tuberculosis infection induces HDAC1 expression and hypoacetylation of histone H3 in macrophages to abrogate Th1 responses. *Aneesh Chandran1*, Cecil Antony2, Sathish Mundayoor1, K. Natarajan2 and R. Ajay Kumar1. International Conference on Biosciences: state-of-the-art advancements Organized by: Society for Educational and Scientific Research (SESR) Venue: Lakesong Resort, Kumarakom, India. Date: Sep 11-12,2014.*
- Quantitative proteomic study reveals significant differences in the physiologic status of dormant and reactivated M. tuberculosis. Authors: *Vipin Gopinath*1, Sajith R*1, Roshna Lawrence Gomez1, Leny Jose1, Arun Surendran2, Ranjit Ramachandran1, Sathish Mundayoor1, Abdul Jaleel2 and R Ajay Kumar#1. Title of the symposium- Biosciences: State of the art – Advancements. Venue: Lakesong Resort Kumarakom. Date: September 11-12, 2014.*

BEST POSTER AWARD

- Recruitment of HDAC1 onto the promoters of host defence genes as a survival strategy of intracellular *Mycobacterium tuberculosis.* *Aneesh Chandran1, Cecil Antony2, K. Natarajan2 and R. Ajay Kumar1*. International conference on Host-Pathogen interactions. National Institute of Animal Biotechnology, Hyderabad. July 12-15, 2014.*

EXTRA MURAL FUNDING

Sl. No	Investigators	Title of Project	Funding agency	Duration
1	R. Ajay Kumar (PI) Abdul Jaleel Sathish Mundayoor	Identification of transcriptional regulators expressed in Mycobacterium tuberculosis during reactivation from dormancy in vitro, and identification of their target sequences	Department of Biotechnology, Government of India	2012-2015
2	R. Ajay Kumar (PI) Sabu Thomas	Isolation and characterization of antimycobacterial molecules from Actinomycetes	Council for Scientific & Industrial Research	2013-2016

TROPICAL DISEASE BIOLOGY
MOLECULAR VIROLOGY LABORATORY

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E. Sreekumar is a post-graduate in Veterinary Immunology, and has a PhD in Biotechnology from University of Kerala. He joined RGCB in 2004.

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Sneha Singh
Sreeja R Nair

Project Fellow

Vadivel Murugan

Technical Personnel

Unnikrishnan V R

Our research focuses on two major mosquito-borne viral infections-chikungunya and dengue. Our goals are to find ways to alleviate neurovirulence-associated morbidity in chikungunya virus infections and shock syndrome-associated mortality in dengue. To achieve this, we address specific issues in each of these diseases. In dengue we focus on the endothelial cellular mechanisms of enhanced permeability leading to vascular leakage. In chikungunya, the effort is to understand the molecular mechanisms of neurovirulence of the virus.

Elucidation of the cellular mechanisms of Vascular Permeability in Dengue

Anupriya M.G, Sneha Singh and E.Sreekumar

Increased vascular permeability is a hall mark of dengue shock syndrome and in most cases it is transient and self-limiting. However, excessive leakage can lead to severe and irreversible shock and mortality. Even though the exact mechanisms resulting in the enhanced vascular permeability are not clearly understood, there are two possible causes proposed (**Fig.1**). The first method could be the induction of mediators from dengue virus infected immune cells such as macrophages and liver cells. A second possibility is the direct infection of the microvascular endothelial cells during viremia, and resultant molecular alterations in signaling pathways that maintains integrity of endothelial cell junctions. These pathways are important especially in regulating the paracellular transport across the inter-endothelial cell junctions, which is a major route for fluid leakage from vascular lumen to the perivascular tissue.

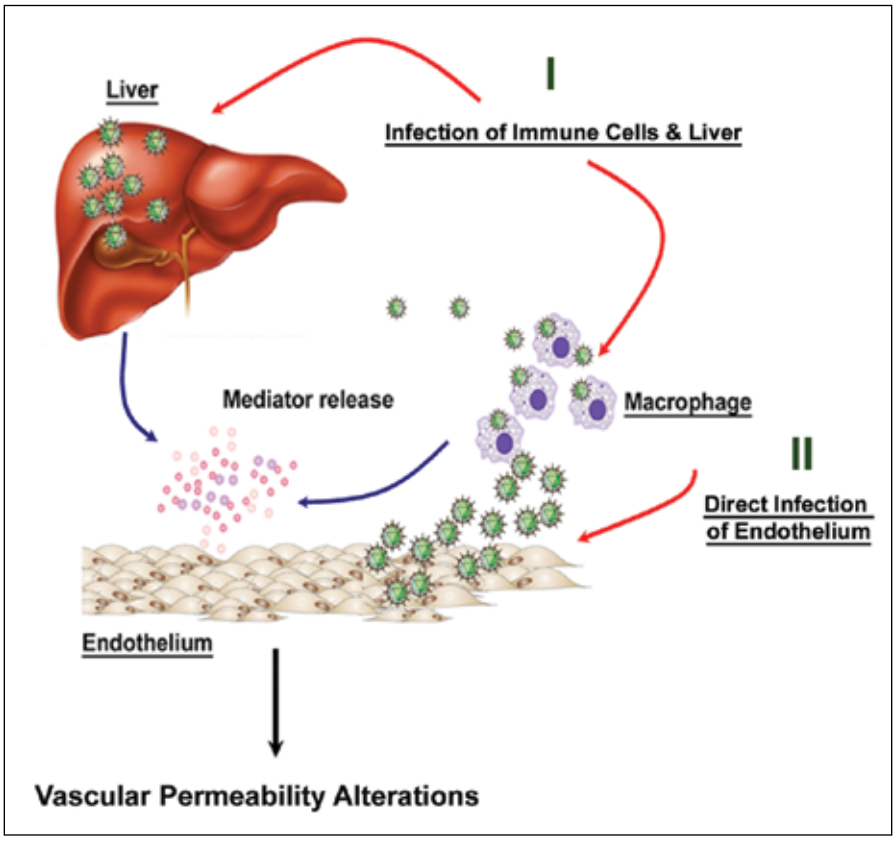

Fig.1 Two proposed causes for enhanced vascular permeability in Dengue

To explore further, from several clinical strains of Dengue, we identified the DENV-2 strain RGCB880/2010 that infects human microvascular endothelial cells (HMEC) and causes transient, reversible permeability changes as observed in clinical cases of dengue (**Fig.2**). An enhanced FITC-dextran permeability was observed across the cultured endothelial cell monolayer at 24h post-infection (p.i.), which lasted till 48h post-infection, and then decreased. At molecular level, there was a generalized activation of the endothelial cells along with an increase in the level of *the* mRNA transcripts of several adhesion molecules and endothelial cell receptors post-infection (p.i.). We could correlate the modulation of a few of them at the protein level with the onset of leakage at 24h p.i. Further, a label-free proteomics done by multi-dimensional liquid chromatography – tandem mass spectrometry in DENV-2 infected HMEC cells identified 210 proteins which were consistently modulated and were common to the window period of vascular leakage (between 24h and 48h). The major key molecules identified were those involved in cell adhesion, host-virus interaction and immune response. Further studies are going on to validate the role of these proteins, in modulating the vascular permeability.

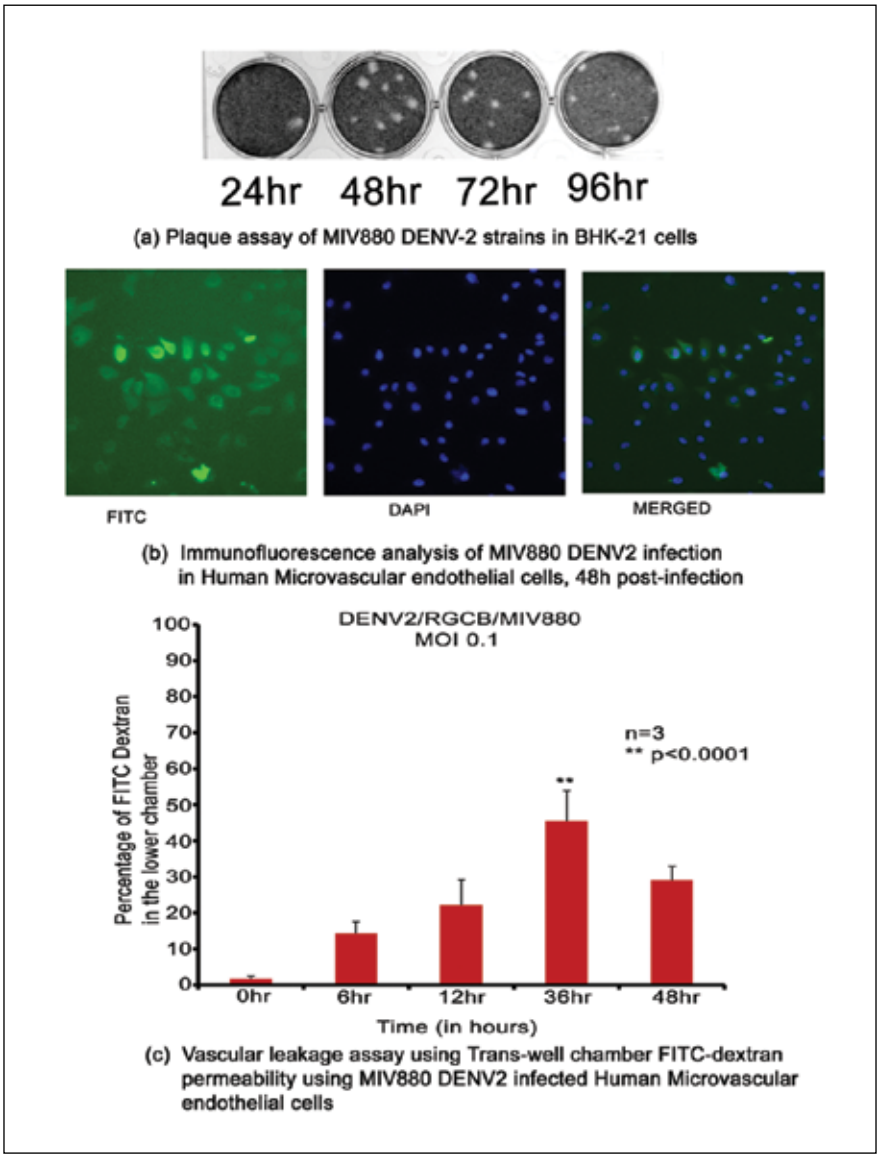

Fig.2 Enhanced trans-endothelial cell permeability in Dengue virus infected human microvascular endothelial cells

Understanding Host-virus Interactions in Chikungunya virus infection

Rachy Abraham, Prashant Mudaliar, Sreeja R. Nair Vadivel Murugan and E.Sreekumar

Our second project focus on neurovirulence of Chikungunya virus, a new clinical feature witnessed in recent outbreaks. The disease is generally an acute, febrile disease in the tropics leading to chronic debilitating arthralgia and occasional hepatic and neural complications. We use epithelial and astrocytic cell lines to understand the cell biology of CHIKV infection. Using high throughput proteomics approach of CHIKV infected HEK293 epithelial cells and U87-MG astrocytic cells we short-listed a common set of 50 proteins by comparing our results and results of earlier CHIKV proteomics studies that can be implicated in cellular pathology of CHIKV infection. One among them, Nucleophosmin, a nuclear chaperone, was found to form cytoplasmic aggregates in infected cells (**Fig.3**). Modulation of a few astrocyte-specific proteins was also identified. While characterizing clinical isolates of CHIKV, we identified strains harbouring novel mutations differing in cellular infectivity and plaque morphology. We plan to use a reverse genetics approach to generate new viral strains having targeted mutations in an isogenic background. Using these strains, we would further investigate the role of mutations in specific viral proteins in modulating the host-cell proteins identified from our proteomics study.

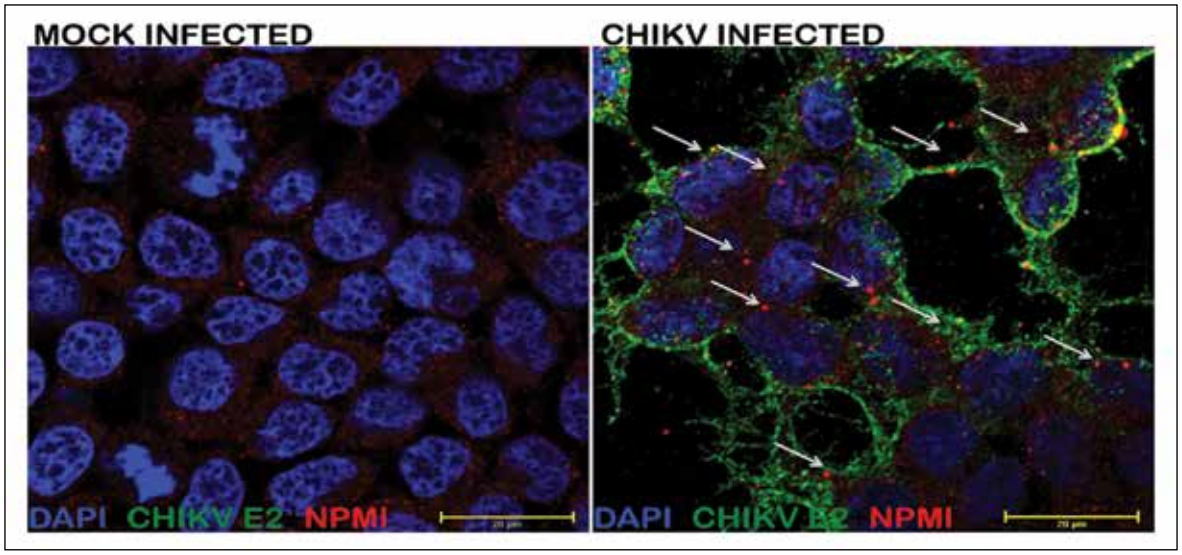

Fig.3 Formation of cytoplasmic granules of nucleophosmin in CHIKV infected HEK293 cells

PUBLICATIONS

- *Vijesh S Kuttiatt, Sanughosh Kalpathodi, Sindhu T Gangadharan, Lalitha Kailas, Easwaran Sreekumar, Suja M Sukumaran, Radhakrishnan R Nair* (**2014**) Detection of Measles Virus Genotype B3 in India**.** *Emerging Infectious Diseases 20, 1764-1765*
- *Konstantin A. Tsetsarkin, Rubing Chen, Ruimei Yun, Shannan L. Rossi, Kenneth S. Plante, Mathilde Guerbois, Naomi Forrester, Guey Chuen Perng, Easwaran Sreekumar, Grace Leal, Jing Huang, Suchetana Mukhopadhyay, Scott C. Weaver* (**2014**) Multi-peaked adaptive landscape for chikungunya virus evolution predicts continued fitness optimization in *Aedes albopictus* mosquitoes. *Nature Communications 5:4084 DOI: 10.1038/ncomms5084*
- *K Krithiga, Divakaran N Nair, E Sreekumar, Mammen J Abraham, Ajith Jacob George, CB Manomohan* (**2014**) Isolation and molecular confirmation of extra-intestinal pathogenic *Escherichia coli* (ExPEC) from domestic pigs. *Indian Journal of Veterinary Pathology 38 (2), 85-87*

- *Sheeba PM, Jose R, Sreekumar E, Vimalraj AN, Kurian S, Bai JTR.* A Comparative Study of Dengue Syndromes in a Tertiary Care Centre (**2014**) *Acad Medical Journal of India 2:60–66*

CONFERENCES PRESENTATIONS

- *Sneha Singh, Birendra Prasad Gupta, Anoop Manakkadan, KD Manandhar, E Sreekumar.* Molecular and evolutionary analysis of dengue virus serotype 2 in Nepal during 2013 outbreak. *12th International Conference on Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases (MEEGID), 11-13 December, 2014, Bangkok, Thailand.*
- *Rachy Abraham, Abdul Jaleel and E Sreekumar.* "Dissection of Host-virus Interactions in Chikungunya Using Expression Proteomics Analysis" at *International Union of Microbiological Societies Congresses held at Montreal, Canada, 27th June –August 1st 2014*
- *Rachy Abraham, Kirsten Kulcsar, Victoria K Baxter, Ashley Nelson, Diane E Griffin.*" Characterization of The Antiviral and Immune Responses to Fatal Alphavirus Encephalomyelitis in Susceptible C57Bl/6 and Resistant BALB/cByJ Mice" at *114th General Meeting of the American Society for Microbiology held at Boston , Massachusetts, USA:17-20th May 2014.* (Work done by the first author as part of the Fulbright Fellowship at Johns Hopkins Bloomberg School of Public Health, Baltimore)
- *M. Anoop, M.G. Anupriya, Sneha Singh, K.C. Sivakumar, E. Sreekumar* Genetic diversity of dengue virus serotype-2 Genotype IV (Cosmopolitan) strains in South India during 2008-2012. *12th International Conference on Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases (MEEGID), 11-13 December, 2014, Bangkok, Thailand.*
- *Rachy Abraham and E Sreekumar.*"Comparison of Chikungunya virus E1 and E2 envelope proteins and their antibodies with respect to immunogenicity and efficiency in virus detection" at *27th Kerala Science Congress held at Alappuzha, Kerala, India, 27-29th January 2015*
- *M G Anupriya, Anoop Manakkadan, Raji R P, Lalitha Kailas and E Sreekumar.* «Development of an ELISA system for serotype-specific detection of anti-dengue IgM antibodies in serum samples" at *27th Kerala Science Congress held at Alappuzha, Kerala, India, 27-29th January 2015*
- *Sneha Singh, Anoop Manakkadan, E Sreekumar.* Generation of auto-cleavable reporter-tagged expression system for functional studies of dengue viral proteins. at *27th Kerala Science Congress held at Alappuzha, Kerala, India , 27-29th January 2015*

RESEARCH GRANTS

No.	Title	Investigator(s)	Funding Agency	Duration
1.	Characterization of Neurovirulence of Chikungunya virus in cellular and animal models	E.Sreekumar & Jackson James	Department of Biotechnology, Government of India	2012 -2015
2	Elucidation of the role of endothelial cell signaling pathways in vascular permeability modulation in Dengue virus infection	E. Sreekumar & T.R.Santhosh Kumar	Indian Council of Medical Research	2013 -2016

TROPICAL DISEASE BIOLOGY
VIRAL DISEASE BIOLOGY LABORATORY - 1






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Developing Models to Forecast Outbreak of Dengue Virus in Kerala: Creating a freely accessible web resource

Reshmi G and M. Radhakrishna Pillai

During emerging epidemics of infectious diseases, it is vital to have up-to-date information on epidemic trends such as case incidence, distribution, eco-climatic condition in the epidemic area because health care facilities, hospitals and intensive care units often have limited capacity to deliver appropriate care. Prepared in the context of previous dengue and other viral fever outbreaks in Kerala, this study planned to develop, evaluate and execution of a forecasting model for infectious disease outbreaks. Our study aims for designing the deterministic models to understand the dynamics of vector borne diseases particularly dengue that may then serve as template for developing a variety of model for other infectious diseases outbreak forecasting. We collected dengue cases incidence data from the Directorate of Health Services, Government of Kerala for the year of 2009 to 2014. During this period, the state of Kerala documented several dengue outbreaks. We collected meteorological data and vector density data for the same period as well as remote sensing data from corresponding government authorities. Year wise analysis was carried out to find out main confounding factors that show positive correlation with the dengue incidence using both univariate and multivariate regression models. Using these models we tried to extract relationships between dengue incidence in different parts of Trivandrum district and meteorological, climatic, vector densities and socio-economical data from Kerala.

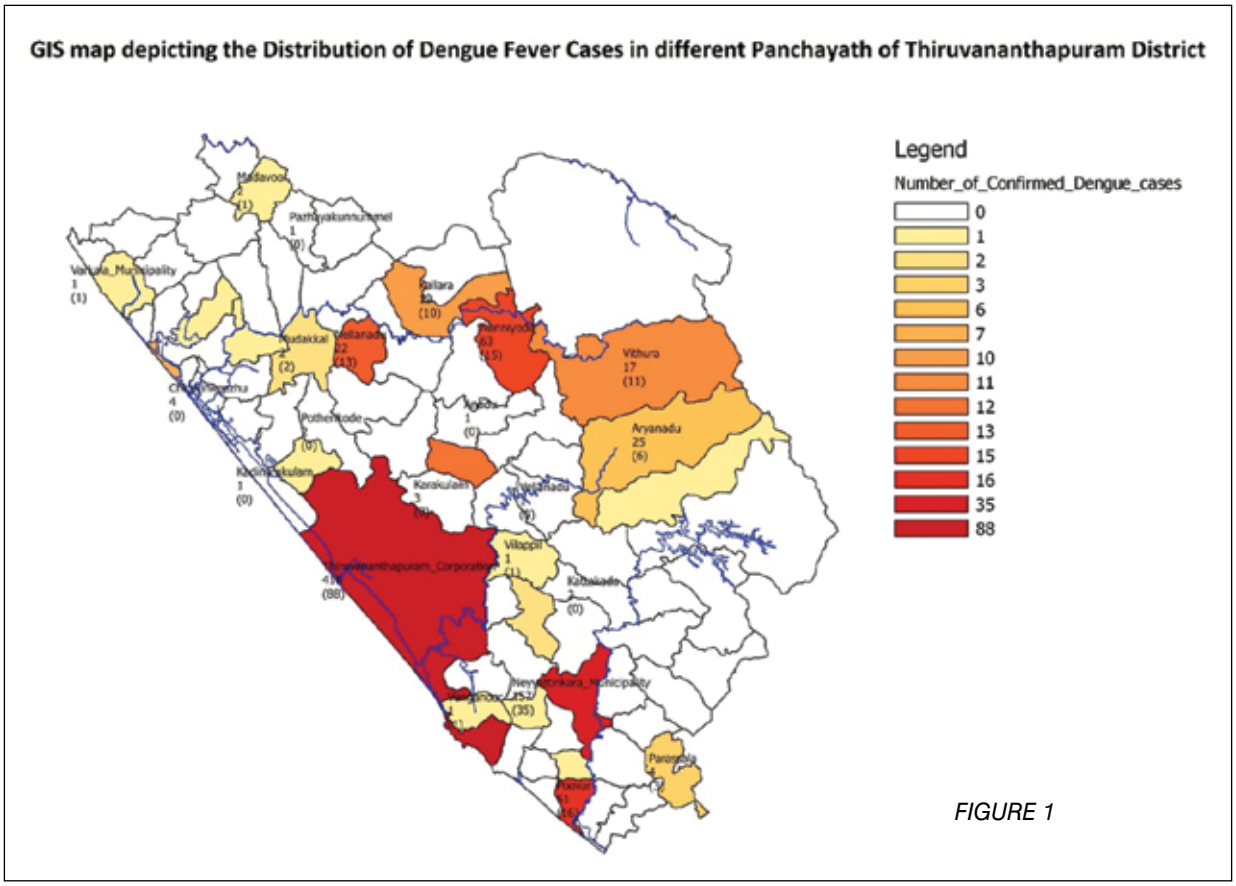


FIGURE 1

Most of the panchayath (local administration unit) areas were affected by Dengue during the period of 2012. Thiruvananthapuram Corporation area was severely affected and accounts for >40% of the total cases followed by Neyyattinkara,(16%), Nellanadu (7%) and Nenniyode (6%) (Figure1). We also analyzed the month wise incidence of dengue cases in Thiruvananthapuram District in 2012 and tried to find out impact of different climatic parameters with disease incidence rate. The pattern of emergence of dengue cases was seen to have one small peak in the month of February and maximum cases in the month of the June. The sudden increase in the dengue cases was observed with the start of rain and increases in humidity (Figure 2). Onset of southwest monsoon was noted in Kerala from mid June to September and North East Monsoon from October to November during 2012. Climate is therefore an important factor for dengue fever outbreaks.

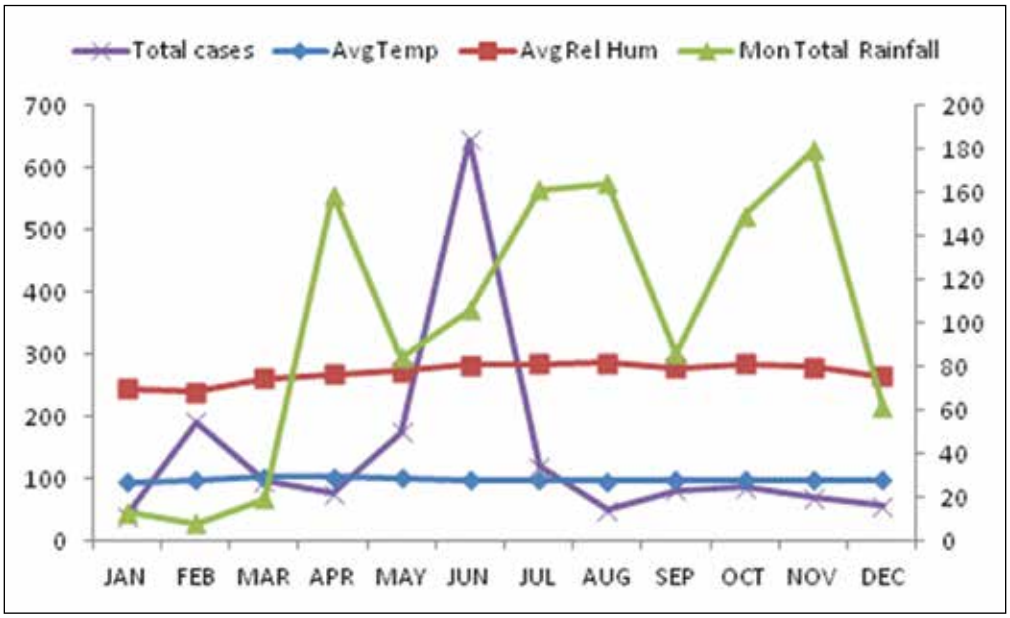
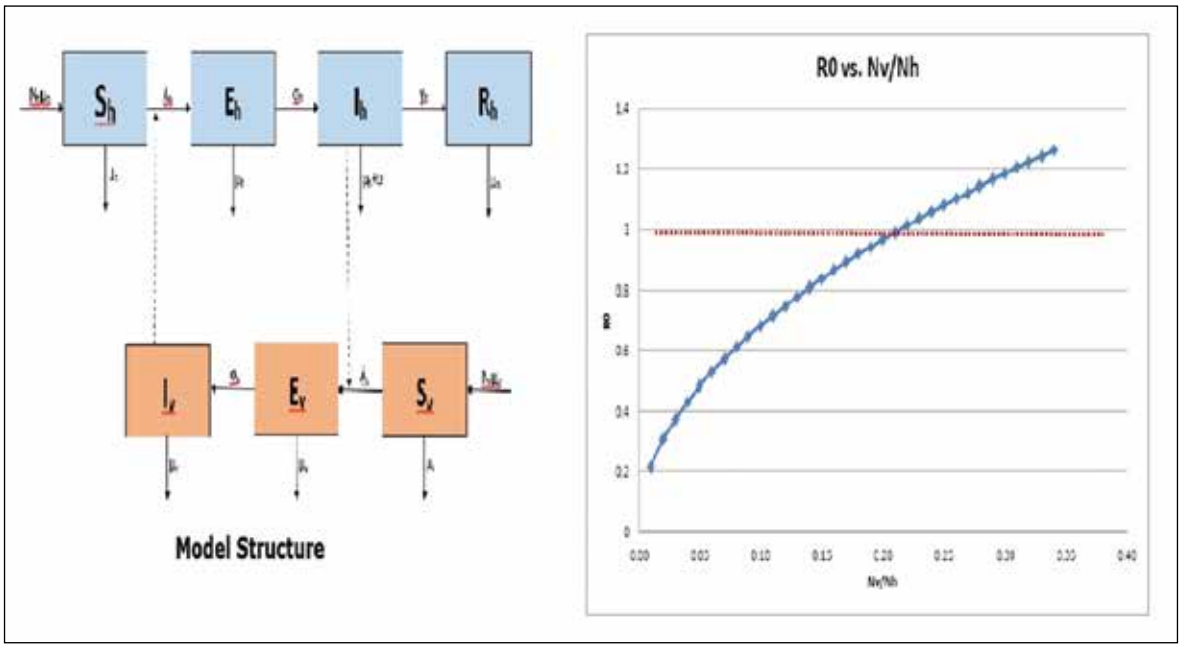


Figure 2: Relation between climatic parameters and Month wise Distribution of Dengue incidents for the year of 2012 in Thiruvanthapuram District

We propose an SEIR model for the human populations and an SEI model for the vector (female mosquitoes Aedesaegypti) to describe the transmission dynamics of a four-strain model with both primary and secondary dengue infections. In order to accomplish this, we propose and obtained an analytic solution of a system of coupled differential equations generated with definite parameters we selected. SEIR model is a mathematical model to analyse the simulation of the spreading of one serotype of dengue virus between host and vector. The model is based on the Susceptible, Exposed, Infected, and Removed (SEIR) of infectious disease epidemiology, which was adopted. SEIR model obtained two vectors, human population and Population vector. Human population are divided into four groups, including people that have the potential to get infected by dengue virus, people who show exposure of virus infection, people who were actually infected and people who have recovered from disease. Population vector or mosquitoes are divided into three groups of mosquitoes that potentially infected by dengue virus, mosquitoes are exposed to infection and mosquitoes that were infected with dengue virus. Result of the SEIR model indicates that infection to human is based on this data. This study is on the way of progression and we believe this to be the most complete analytical analysis of the transmission of dengue.



SEIR-SEI model of the transmission dynamics of Dengue infection

Manipulation of the Cell death machinery by West Nile Virus (WNV) and its variants

Pradip Fulmali and M. Radhakrishna Pillai

In our previous experiments with Indian WNV strains infection in different cell line, we showed differential replication pattern that in turn had an impact on cell death. We therefore tried to corroborate our finding in an *in vivo* murine model. In murine model replication kinetics, mice infected by intraperotonial route of infection with high dose of WNV had a high mortality rate of 99% ($p > 0.01$). However, mice inoculated with low dose rarely showed any clinical signs of disease and exhibited reduced mortality ($p < 0.01$) (Fig. 1).

To explore the possible reasons for reduced mortality with low dose, mice were dissected and tissues harvested and processed for viral load determination by plaque assay. On 6th day post infection, mice infected with high dose had a significant viral load in blood as compared to low dose of WNV. At this time point, animals infected with high dose exhibited the presence of virus in both spleen and brain whereas no virus was detected in spleen and brain of low dose WNV infected mice. Low-dose of WNV showed very low infectious titer in blood and spleen. However, as infection progressed, low-dose of WNV viral loads increased slowly over days 4 and 7 post infection. WNV has evolved specific strategies to avoid or attenuate innate and adoptive immune response. We therefore checked cytokine levels and found that 3–4 fold high levels of TNF-κ and IL-1κ correlated with increased mortality in mice inoculated with high-dose of WNV whereas delayed mortality was observed with low-dose of WNV (Fig 2a and 2b). To determine effects of the WNV infectious dose on the virus replication potential and process of cell death, we compared

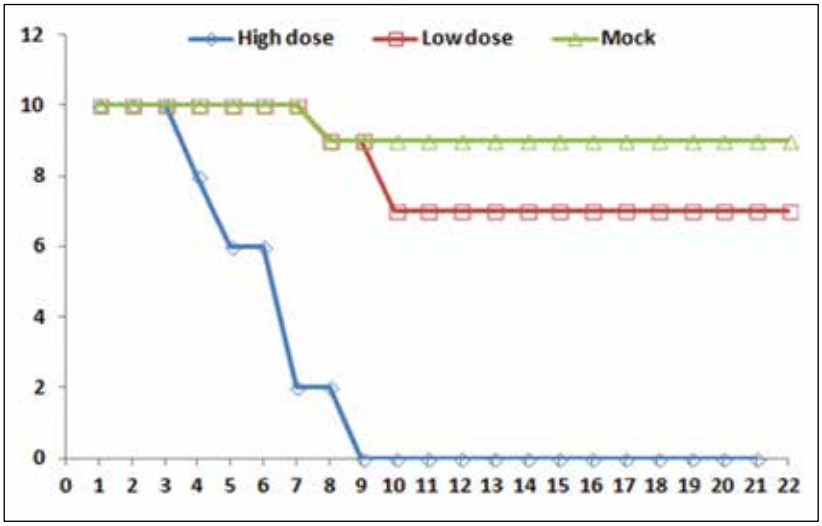


Fig 1: Survival data of mice infected with high dose (105 PFU), Low dose (102 PFU) of WNV (804994) strain and mock infected. Three-week-old Swiss mice were inoculated through i.p routes. Mice were observed daily for 21 days. The survival curves were constructed using data from three independent experiments (n=10 mice per experiment).

the replication of high-dose and low-dose of WNV in the MEF cells. It appears noteworthy that the virus load can also determine the kinetics of infection. MEF cells that were infected with a high-dose (10 moi) can accelerate infection and produce a much higher load of infectious progeny, whereas a low-dose (0.1 moi) results in a delay in kinetics, with a lower load of infectious progeny. Recent studies also suggest that necrosis can also proceed in a well-regulated manner, which is frequently defined as programmed necrosis. From this perspective, we presumed that WNV could trigger programmed cell death switching from apoptosis to programmed necrosis (necroptosis) in a dose dependent manner. We found that MEF cells that were infected with low-dose WNV mostly undergoes apoptotic cell death as observed by cleavage of PARP into the 85 kDa fragment was observed in infected cells whereas no PARP cleavage was noted in high dose infection. HMGB1 protein is confined predominantly to the nuclear fraction of mock-infected cells. However, HMGB1 protein is detected in the extracellular fraction of MEF cells infected with WN virus at a high infectious dose by 24 hrs. whereas no HMGB1 activity was observed in low dose. There was a significant increase in caspase-8 activity was observed in low dose infected MEF cell but caspases-8 inhibition was observed in MEF cell infected with high dose (Fig. 3a). Programmed necrosis is defined as necrosis highly regulated by RIP1 and RIP3. The latter is essential and a crucial component in the initiation of programmed necrosis, but has no role in apoptosis. To investigate how different dose of WNV infection specifically regulate necroptosis, the expression of Rip1 and Rip3 kinases was investigated. In MEF cell infected with high-dose (10 moi) showed increased expression of Rip1 kinase and also significant increase in the expression of Rip3 kinase. In the MEF cells infected with low-dose (0.1 moi) only RIP-1 expression was observed (Fig 3b). Cyclophilin D (CypD) is a vital protein involved in mitochondrial permeability transition and critical regulator in HNK-induced programmed necrotic cell death. We observed significantly increased level of CypD in cell infected with high-dose WNV compared to low-dose WNV (Fig 3b).

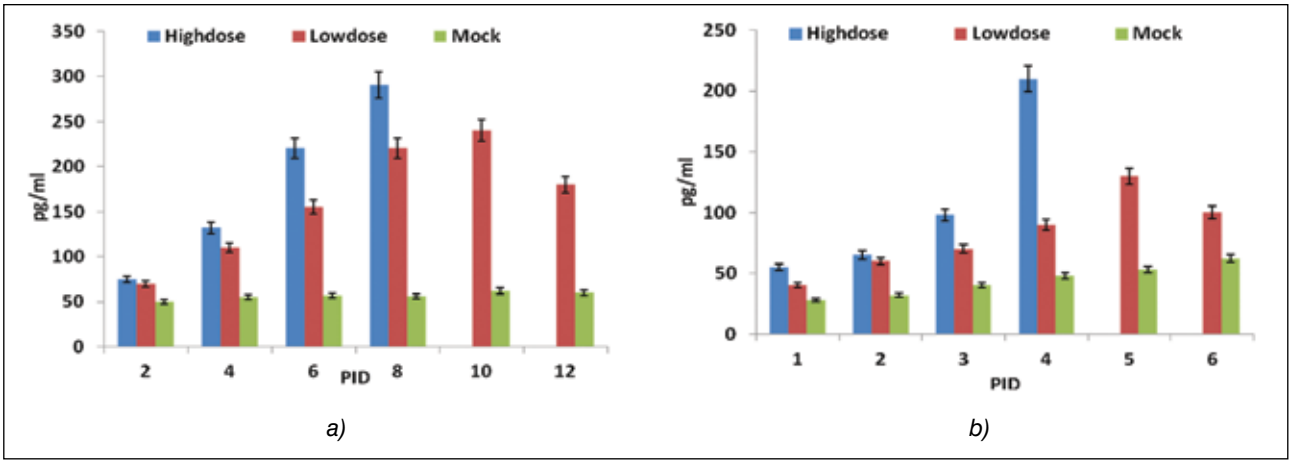


Fig 2: Induction of (a) TNF-α and (b) IL 1 β in the swera of mice infected with WNV high-dose, Low-dose and mock. Each dataset represents sera from a pool of mice tested in duplicate per time point. The error bars indicate the standard errors.

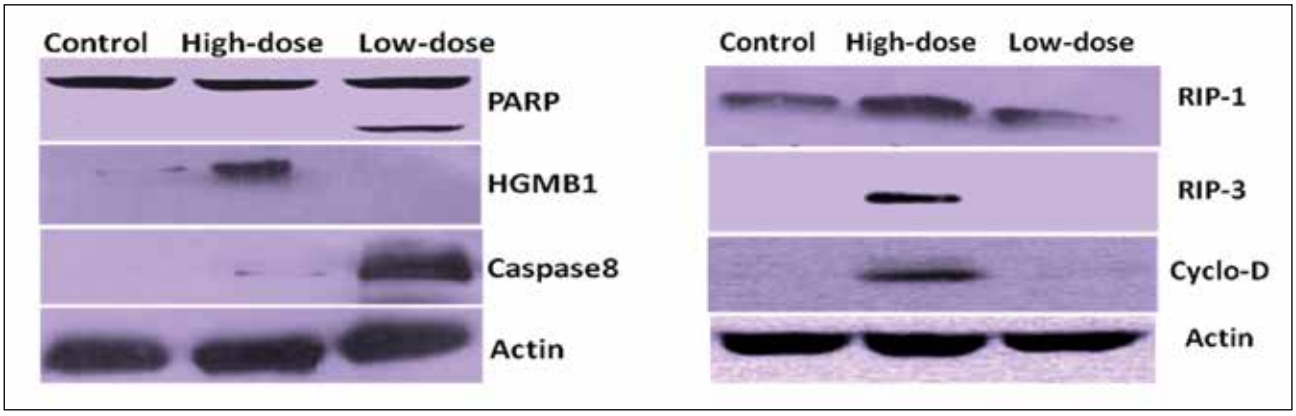


Fig 3: Blots showing the apoptosis and necroptosis regulating protein in MEF cell infected with high-dose (10moi) and low-dose (0.1moi)

Systems Biology Assessment of Influenza A/pH1N1 vaccination in an Indian cohort

Sara Jones, Joshy Jacob and M. Radhakrishna Pillai

Vaccination against influenza continues to be a well-established and promising strategy for protection against influenza and the resulting influenza-related complications. Indian companies have already developed and marketed several influenza vaccines and more are under development. Unfortunately, vaccine use is still sporadic and infrequent in the Indian subcontinent., especially among those most at risk. More than 90% of influenza-related annual deaths occur in adults who are older than 50 years of age. In order to develop more efficient approaches for protection against influenza in the elderly, immunosenescence and vaccine-induced immune responses require greater comprehension,

including understanding immune response dynamics and correlates of protection following immunization, as well as the interrelationships and dependencies among various immune response variables that determine immune function. Previous reports from our collaborators suggest the importance of age and specific markers of immunosenescence (e.g., CD28 expression on T cells, the expression levels of the peripheral white blood cell telomerase TERT, Th1/Th2 cytokine disbalance, etc.) for diminished vaccine-induced immune responses in older and elderly individuals. However, age and immunosenescence have not been systematically studied with regard to influenza vaccination in humans, besides their influence on the magnitude and kinetics of various humoral immune responses remains poorly understood. Our broad objective is to develop innovative immune profile signatures that explain and predict inter-individual variations in immune responses to influenza A/H1N1 vaccines. In this study we tried to understand the baseline reactivity of antibody responses if any to the pandemic flu (A/California/04/09) among the overall population of Kerala State. We selected 167 individuals in the age groups between 10 and >70 years and carried out hemagglutination inhibition assay (HI) on the donor serum (Fig: 1a). The reactivity of humans aged >50 to pH1N1 2009 were found to be below the cut off titer of <160 (Fig: 1b). This baseline study is highly critical as it not only helps primary standardization for the proposed second part of the study which involves H1N1 vaccination followed by immunological readout but also provides an overall rough demography of the prevalence and exposure to H1N1 among the general populace of Kerala.

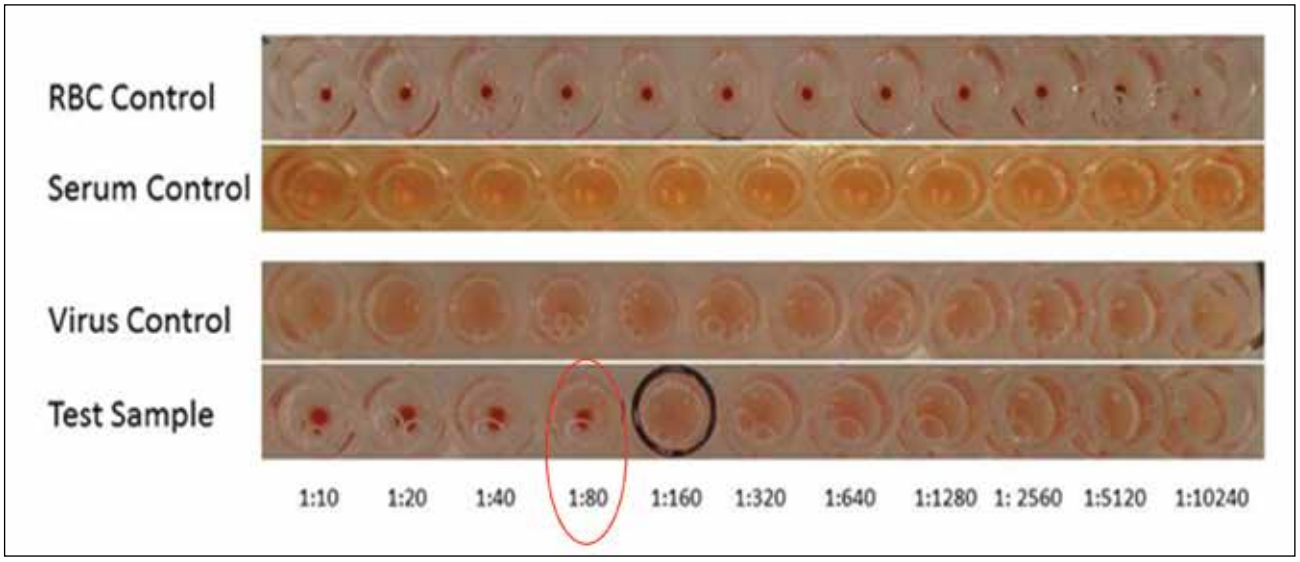


Fig: 1a, Representative figure showing hemagglutination inhibition assay. The neutralization titer is determined by identifying the last well in which the RBCs form a button and were hemagglutination does not occur. The reciprocal value of this corresponding dilution is the hemagglutination titer.

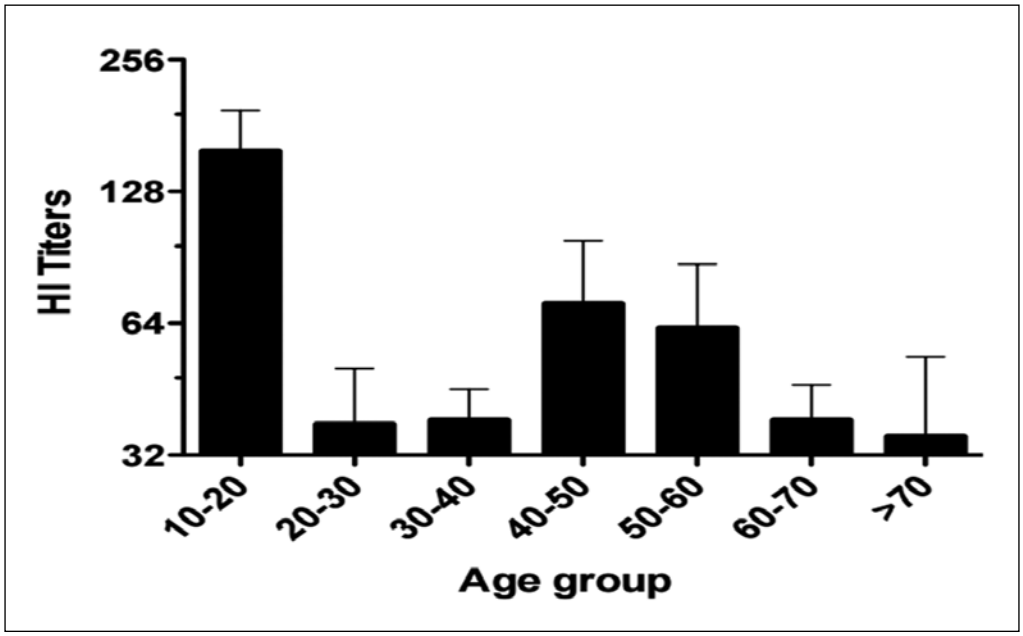


Fig: 1b, HI titer of 167 Kerala sera samples against 2009 pandemic H1N1 A/California/04/09 by age group

Genetic Characterization of Measles Viral Strains Circulating in Kerala

Vijesh Sreedhar and M. Radhakrishna Pillai

Defining the epidemiologic trend of a disease in a geographical region is an essential component of effective disease control. Though measles pose significant public health challenge in India, there is paucity of systematic studies. Molecular epidemiological investigations are necessary to identify the prevalent viral strains in a region and to delineate the presence of endemic strains from the imported ones. We initiated this study to understand the clinical and molecular characteristics of measles in the state of Kerala. We screened clinical specimens from children admitted with suspicion of measles in a tertiary care hospital using an in-house RT-PCR assay for measles RNA detection and/or a commercial measles IgM ELISA. Virus isolation was carried out using Vero-SLAM cell lines. Induction of infection in cell culture was detected by cytopathic effect (syncytium formation) or positive immunofluorescence assay for measles antigens. Viral genotyping was done based on nucleoprotein gene sequences of the measles virus as per standard WHO protocol. We analyzed clinical specimens from 92 children during March 2012 to November 2012. Seventy-two were confirmed as measles cases by RT-PCR assay and/or IgM ELISA. Mean age of the children was 71.8 months (range: 21 days to 12 years). Thirty-five (48.6%) were below the eligible age (9 months) of measles vaccination. Seventeen children had history of receiving at least a single dose of measles containing vaccine. Pneumonia was noted as the major clinical complication. Phylogenetic analysis of the sequences obtained from the clinical specimens revealed measles virus genotype B3 and D8 as the predominant circulating strains. Detailed data analysis is in progress. Changing epidemiological profile of measles with increased incidence in young infants is noted. High index of suspicion is necessary for differentiation of such cases from other rash illnesses. RT-PCR is especially useful in early diagnosis. Measles viral strain B3 is not known to be endemic in India and needs further studies to define its spread and evolution in the country.

PUBLICATIONS

- *Kuttiatt VS, Kalpathodi S, Gangadharan ST, et al.,* Detection of measles virus genotype B3 in India. *Emerging Infectious Diseases, 2014 Oct; 20(10): 1764-6*
- *V C Dhanya, P J Sara, Dharmaselan Sanjai, Fetle Amar, P M Deepa, G R Santosh, V T Jisa and M R Pillai.* Demographic and Clinical Characteristics of Pandemic Influenza A (H1N1) 2009 Outbreak in Kerala, Southern India. *British Microbiology Research Journal 4(10): 142-153, 2014*
- *Zinia T. Nujum, Achu Thomas, K. Vijayakumar, Radhakrishnan R. Nair, Pillai MR, P S Indu, Syam Sundar, Soumya Gopakumar, Devi Mohan, T. K. Sudheeshkumar.* Comparative performance of the probable case definitions of dengue by WHO (2009) and the WHO-SEAR expert group (2011) *Pathogens and Global Health 2014, 108, Issue 2, pp. 103-110*

RESEARCH GRANTS

Sl. No	Title of the project	Funding Agency	Duration
1	Systems Biology Assessment of Influenza A / pH1N1 Vaccination in an Indian Cohort.	Joint Program between Rajiv Gandhi Centre for Biotechnology and Mayo Clinic, USA under US-India (Bilateral Collaborative Research Grant on Human Immune Phenotyping and Infectious Disease), National Institutes of Health, USA and Department of Biotechnology, Government of India.	2012-2016
2	Establishment of a National Virology Network Laboratory.	Indian Council of Medical Research Government of India.	2011-2016

TROPICAL DISEASE BIOLOGY
VIRAL DISEASE BIOLOGY LABORATORY - 2

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John B. Johnson obtained his Ph.D. from the University of Pune, in pursuant to his research carried out at the National Centre for Cell Science, Pune. He trained as a Post Doctoral Fellow at Wake Forest Health Science, Winston Salem, North Carolina USA and continued as a Research Instructor at the same institution until he joined RGCB in 2014. He is also a recipient of the Department of Biotechnology's Ramalingaswamy Re-entry Fellowship.

Rhabdovirus components and complement factors in virus assembly, pathogenesis, neurovirulence and modified viral vectors.

Among the RNA viruses, rhabdoviruses are an interesting group of neurotropic viruses known to cause considerable infection in both humans and animals. The most prominent members include the well-known rabies virus and Chandipura virus, both of which are human pathogens known to cause significant morbidity and mortality. The host innate immune system of which the complement system forms an integral part is a potent barrier that these viruses should overcome to successfully disseminate to the central nervous system and cause pathogenesis. The complement system is considered as the first line of host defence against pathogens and they lack memory unlike the adaptive arm of the immune system. Thus activation of complement system can cause undue damage to the host which is prevented by a tightly regulated counter mechanism called the regulators of complement. These regulators are in essence yet another group of either soluble or membrane associated proteins that can actively inactivate important complement proteins or accelerate the disassembly of complement activation specific enzymes. Not much is known about the nature or mechanism of interaction of rhabdoviruses with the complement system. Since they are successful pathogens it could be hypothesized that these viruses have active mechanisms to modulate complement to prevent neutralization. How an RNA virus with limited genome is capable of doing it is poorly understood? Our lab will address these critical questions using vesicular stomatitis virus (VSV) as a model system, as it an established and well-studied prototypic rhabdovirus. The long-term goal is to extend our studies to the more potent human pathogens such as rabies and chandipura viruses.

PUBLICATIONS

- *Ganguly T, Johnson JB, Kock ND, Parks GD, Deora R.* (2014) The Bordetella pertussis Bps polysaccharide enhances lung colonization by conferring protection from complement-mediated killing. *Cell Microbiology 16(7):1105-18.*
- *Mayer AE, Johnson JB,* Parks GD. (2014) The neutralizing capacity of antibodies elicited by parainfluenza virus infection of African Green Monkeys is dependent on complement. *Virology. 460-461:23-33 doi: 10.1016/j.virol.2014.05.004. Epub 2014 May 29.*
- *Johnson JB, Borisevich V, Rockx B, Parks GD* (2014) A novel Factor I activity in Nipah virus inhibits human complement pathways through cleavage of C3b. *Journal of Virology. 2014 Oct 29. pii: JVI.02427-14. [Epub ahead of print] PMID:25355897*

TROPICAL DISEASE BIOLOGY
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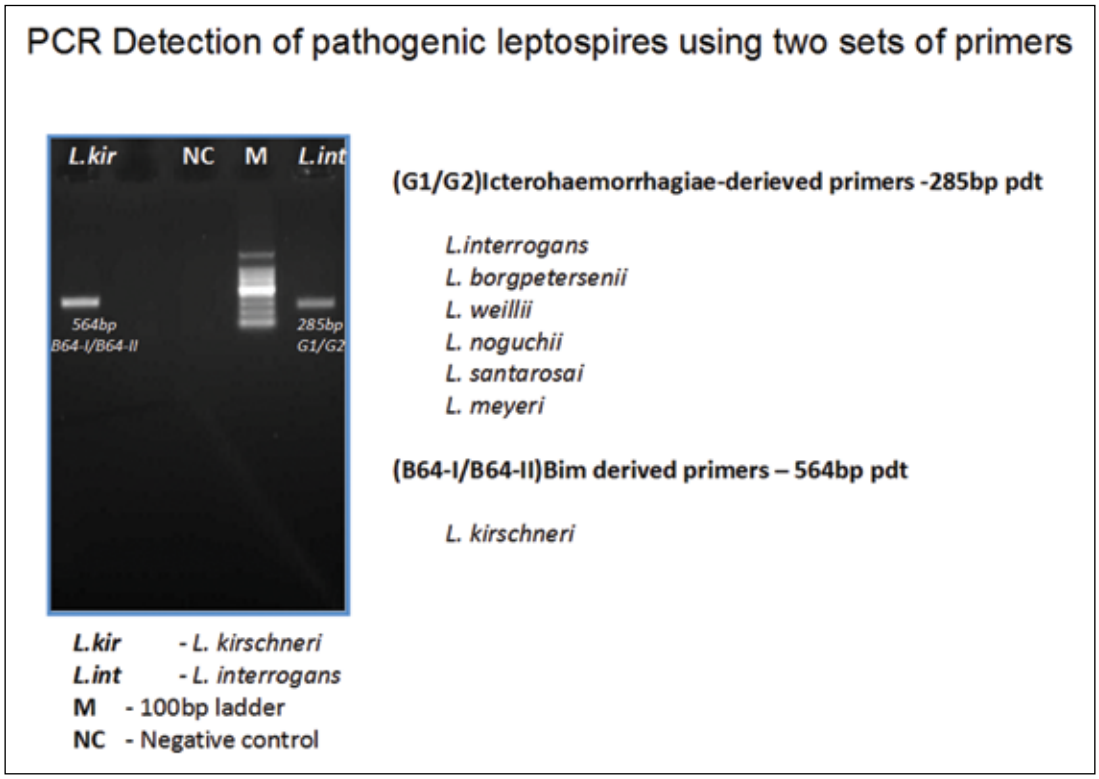
Epidemiology of Leptospirosis.

Leptospirosis is a zoonosis of worldwide distribution, endemic mainly in countries with humid subtropical or tropical climates with epidemic potential. It is one of the neglected zoonotic diseases and is a significant public health concern because of its global distribution, the risk of epidemics and the potential high case-fatality rates when left untreated. Kerala is endemic for this disease with reported case fatality rate ranging from 5.73% to 16.85%. Leptospirosis has been identified as a disease requiring priority attention in ten states including Kerala during the 12th five-year plan period by the Working Group on Disease Burden of communicable diseases of Planning Commission (NITI Aayog). The gold-standard test for Leptospira diagnosis in man and animals, the Micro-Agglutination Test (MAT) was established in our laboratory. For this purpose, a new set of 12 Reference strains of Leptospira was received from WHO collaborating Centre for Diagnosis, Research, Reference and Training in Leptospirosis, Regional Medical Research Centre (RMRC-ICMR), Port Blair, Andamans. Twenty serum samples of sick persons diagnosed with Leptospirosis, admitted to the General Hospital, Thiruvananthapuram were procured from the State Public Health and Clinical Laboratory, Thiruvananthapuram. Sixty percent of the samples gave positive results to MAT test for multiple serogroups (Australis and Autumnalis). The potential for using animal sero-survey with MAT as a proxy indicator for effectiveness of control measures is being explored. Ten serum samples of healthy elephants are to be tested for evidence of past leptospiral infection. The samples would be provided by Kerala State Animal Husbandry Department from domesticated elephants. Molecular diagnostic tests for Leptospirosis were established using culture of Reference strains. PCR based diagnosis using two sets of primers (G1 / G2 & B 64 – I / B 64 – II) was standardized in the laboratory.



The work on adapting this method for use in clinical settings is progressing. Thirteen human samples of patients who had acute renal failure requiring dialysis suspected to be of leptospiral origin has been received from Nephrology Department of Medical College, Trivandrum. Dried Blood Smear on Filter paper is also being tried for PCR based diagnosis. It is hoped that this method could be a major step in providing better diagnostic services in India where sample transportation in cold chain is difficult.

PUBLICATIONS

- *Iype Joseph.* Middle east respiratory syndrome corona virus (MERS CoV): The next steps. *Journal of Public Health Policy, advance online publication, 26 March 2015; doi:10.1057/jphp.2015.9*
- *Akhilandeswarre Dharmaprakash, Ramamurthy Thandavarayan, Iype Joseph & Sabu Thomas* Development of broad-spectrum antibiofilm drugs: strategies and challenges. *Future Microbiol. (2015) 10(6), 1035–1048*

TROPICAL DISEASE BIOLOGY
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Junior Research Fellow

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Studies on methylation of tRNA in human malarial parasites: A new epigenetic signal in *P. falciparum*

Gayathri G and Arumugam Rajavelu

Epigenetic modification in the human malarial parasite genome is gaining lot of attention. The parasite also encodes for many epigenetic enzymes including histone and C-5 methyltransferases. The DNA methylation in human malarial apicomplexan parasites is still under debate, but whole genome sequencing of *Plasmodium falciparum* shows that the parasites contains the putative C5 methyltransferases, which carries all the motif necessary for the DNA methylation activity as well as tRNA specificity. Recently, it has been reported that this enzyme shows minimal DNA methylation activity in the parasites but this is still not well characterized. To understand further on DNA methylation status in the human malarial parasite, we started to model and analyze the putative C5_Mtases protein sequence from *P. falciparum* with the DNA and tRNA methyltransferases from other organisms. Modeling results showed that Pf_C5 Mtases contains the classical co-factor binding (SAM binding) pocket. We also found the tRNA methyltransferase specific CFT motif (fig-1). The phylogenetic analysis of *P. falciparum* methyltransferase suggested that Pf enzyme clustered in the line of Arabidopsis and Geobacter organisms. It is known that Geobacter tRNA Mtases acts on totally different tRNA substrates than previous known substrates, which strongly favor our hypothesis that Pf enzymes might prefer different tRNA substrates. Nevertheless this will be validated with proper biochemical experiments with parasite enzymes.

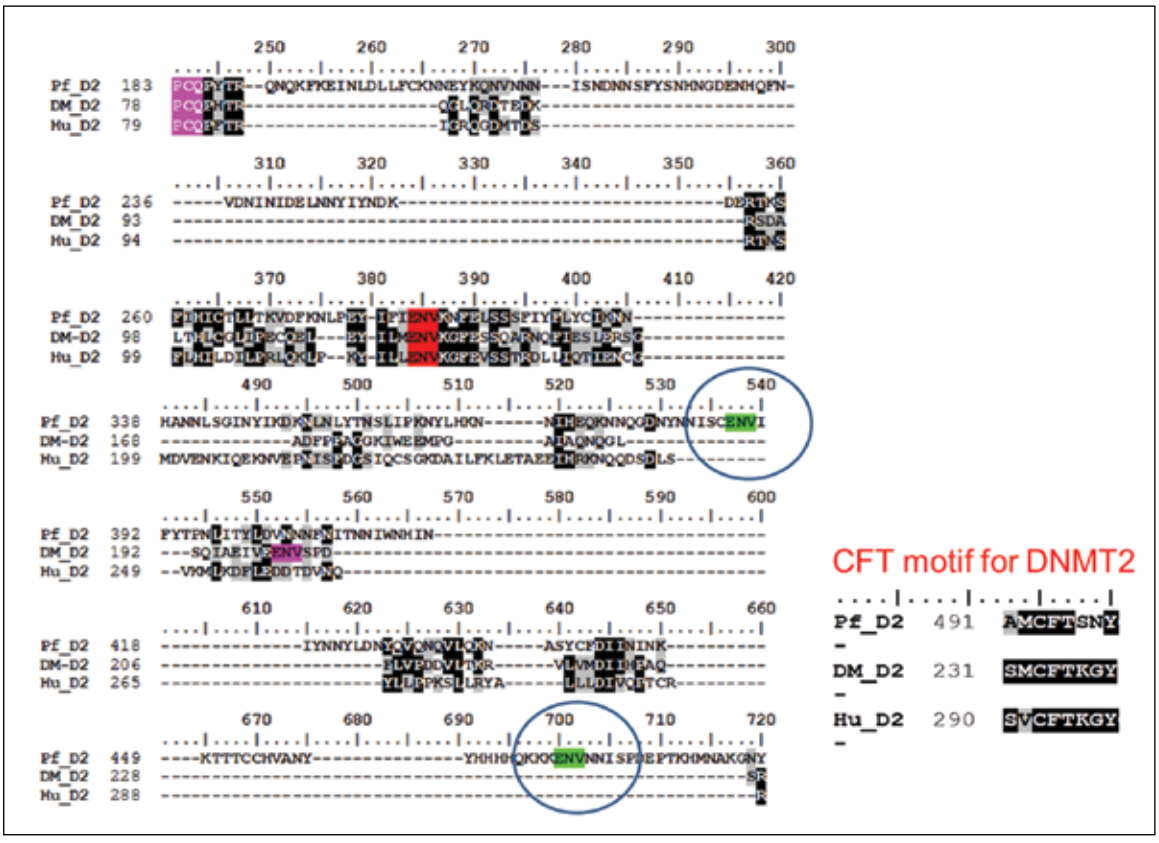

Fig 1. Comparative sequence analysis of tRNA methyltransferase from human and Drosophila, all three organisms conserved with C-5 mtase motifs and CFT motif.

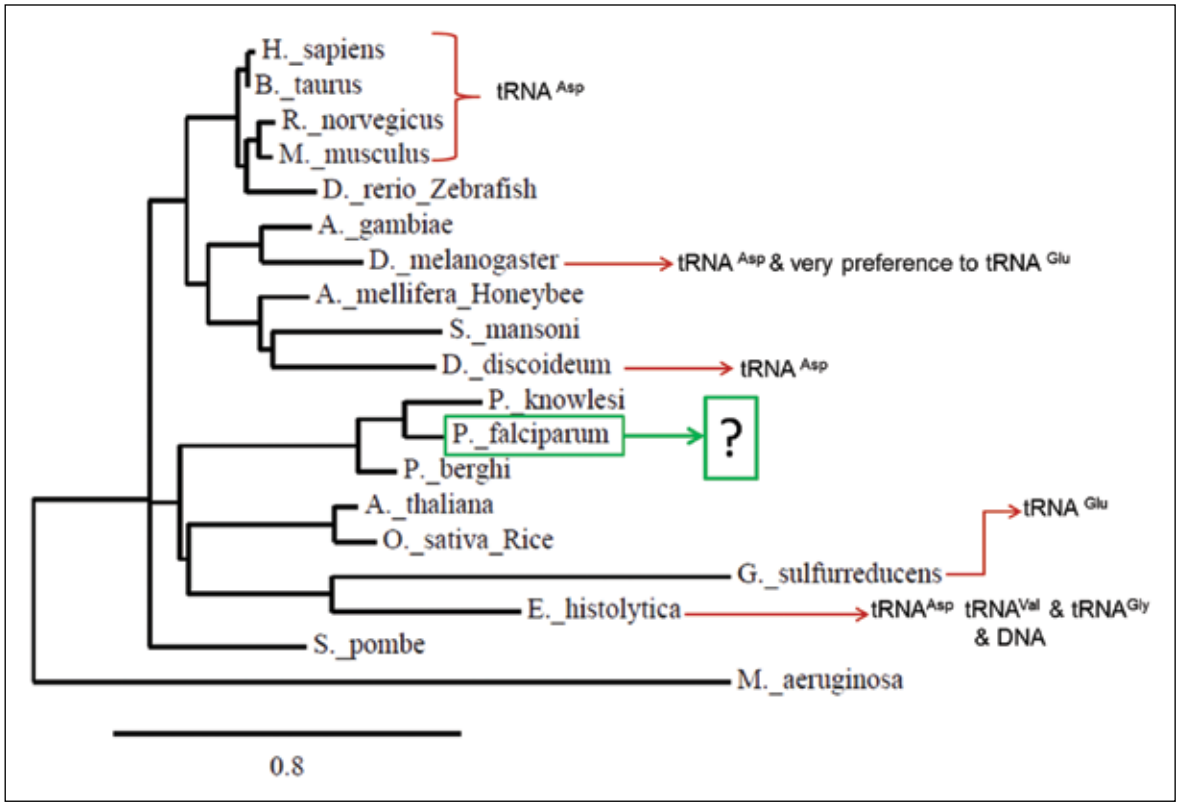

Fig 2. The phylogenetic analysis tRNA methyltransferases, the P. falciparum tRNA methyltransferase clustered in the line of Plants and bacteria.

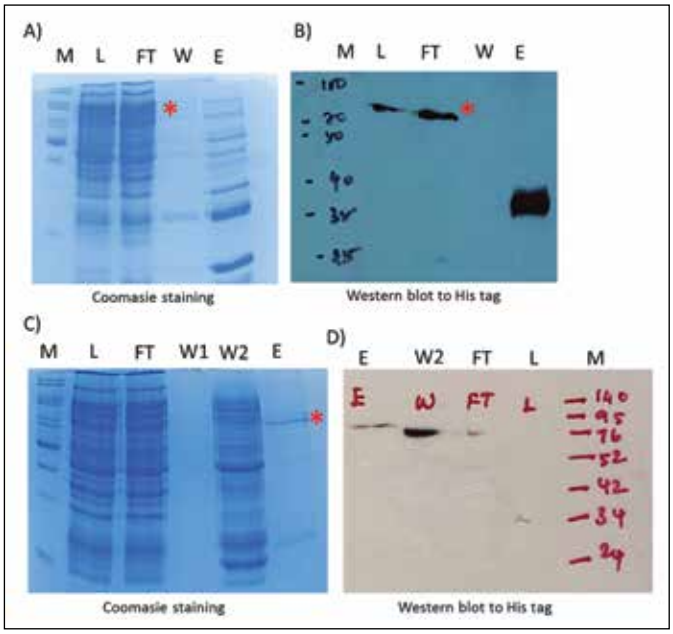

Fig 3. Expression and purification of recombinant Pf-tRNA methyltransferase, A) Purification profile of full length Pf_tRNA mtase stained with coomasie BB. B) Western blot to full-length protein shows that proteins do not bind with Ni-NTA resin. C). Purification profile of Pf_NΔtRNA mtase stained with coomasie BB. D) Western blot to Pf_NΔtRNA mtase, the protein binds with resin, though we see proteins in wash 2 but we achieved good quality of purification.

Next to understand about the C5-methyltransferases of *P. falciparum*, we synthesized cDNA from RNA isolated from trophozoite stage of the Plasmodium falciparum and cloned full length and truncated C5-methltransferase into pET28a (+) vector. The cloned genes were expressed and purified as recombinant protein from bacteria. Purity of the proteins were analyzed by coomasie staining and western blotting to detect the specific protein (Fig 3).

We performed biochemical analysis of the recombinant proteins at *in vitro*. Since Pf_C5 mtase enzyme consist motifs specific for both DNA and tRNA methyltransferases, we tested the activity of this protein on both DNA and tRNA substrates. Preliminary results showed that putative C5-methyltransferase of *P. falciparum* shows poor activity on the DNA substrates in comparison to human DNA methyltransferase (Fig 4). The preliminary results indicated that the Pf_Dnmt2 enzymes might act on the tRNA

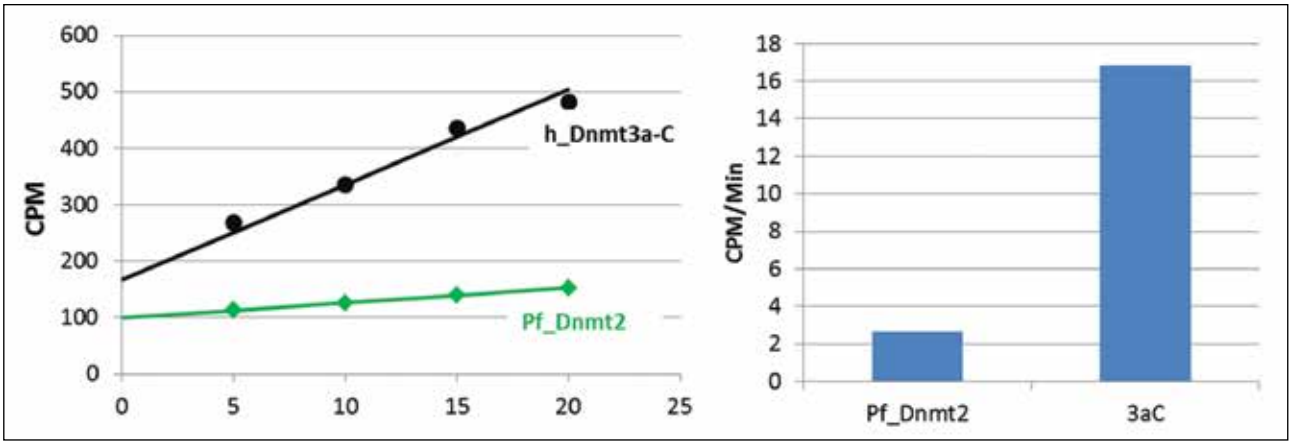

Fig 4. The kinetic assay with Pf_Dnmt2 and h-Dnmt3a enzymes on DNA substrates, Pf enzymes shows poor activity on the DNA substrates.

substrates, nevertheless this needs to be validated further in parasite level by performing RNA bisulfite sequencing and further the functional role of tRNA methylation will be established.

Identification and characterization of unique histone lysine methyl marks in the genome of human malarial parasite *P. falciparum.*

Gayathri G and Arumugam Rajavelu

The human malarial parasite's chromatin is very dynamic and most of chromosomes are in euchromatic state. To understand further parasite chromatin states at various stages of parasite, we planned to identify the unique histone modifications on histones of parasites. We will further characterize various histone lysine methyltransferases of the *P. falciparum.* Several putative histone methyltransferases are annotated (PlasmoDB) in *P. falciparum* genome, suggesting that malarial parasites encodes for *de novo* histone lysine methyltransferases. It has been shown through proteomics analysis for the presence of various histone lysine methylation marks in *P. falciparum*, which proves the Pf_HKMTs are active in malarial parasites and might play a significant role in differential gene expression and chromatin dynamics. We have prepared the sequence alignment for *P. falciparum* putative SET domains, which clearly shows that Pf HKMT SET domain carries all the motifs necessary for the lysine methyltransferase activity including the signature motifs. The annotated *P. falciparum* SET domains are similar to H3K4 methyltransferases family, H3K9 methyltransferases family and H3K36 methyltransferases family. This shows that parasite carries both gene activation marks and gene suppressor marks on the nucleosomes and needs to be validated for the role of these post translational epigenetic modifications in chromatin dynamics at different stages of parasites. To characterize the putative Pf_H3K9 and H3K36 methyltransferases on histone peptide array, we cloned the SET domain of these proteins. The cDNA was synthesized using *P. falciparum* RNA isolated from trophozoite stage of the parasite with oligo dT primers by standard procedures. The PCR fragment for the putative SET domains for H3K9 and H3K36 methyltransferases were generated and cloned into a pGEX-6P2 vector using BamH1 and Xho1 sites to express as GST-tagged recombinant proteins in bacteria cells. We confirmed the clones

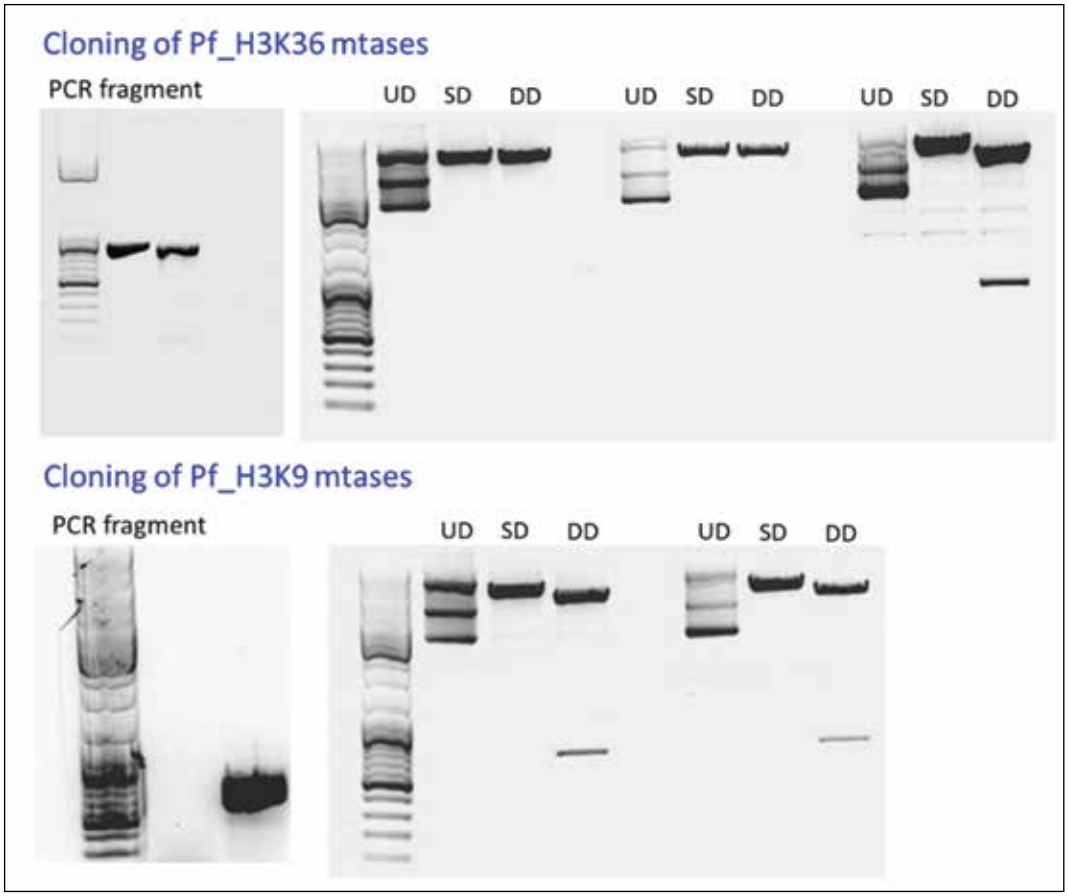

Fig 1. The SET domains specific for Pf_H3K36 and Pf_H3K9 methyltransferases from P. falciparum were cloned into pGEX6P2 vector. The clones were confirmed by restriction digestion and by DNA sequencing.

by DNA sequencing, which shows the correct reading frame and absence of secondary mutations in the clones (Fig 1)

Expression of putative H3K9 and H3K36 methyltransferases were carried out in BL21 E. coli cells and successfully purified using GST sepharose beads. The purity of the two recombinant SET domains were analyzed by SDS-PAGE gel electrophoresis and stained with coomasie brilliant blue and the proteins were confirmed by western blot with anti-GST antibodies (Fig 2).

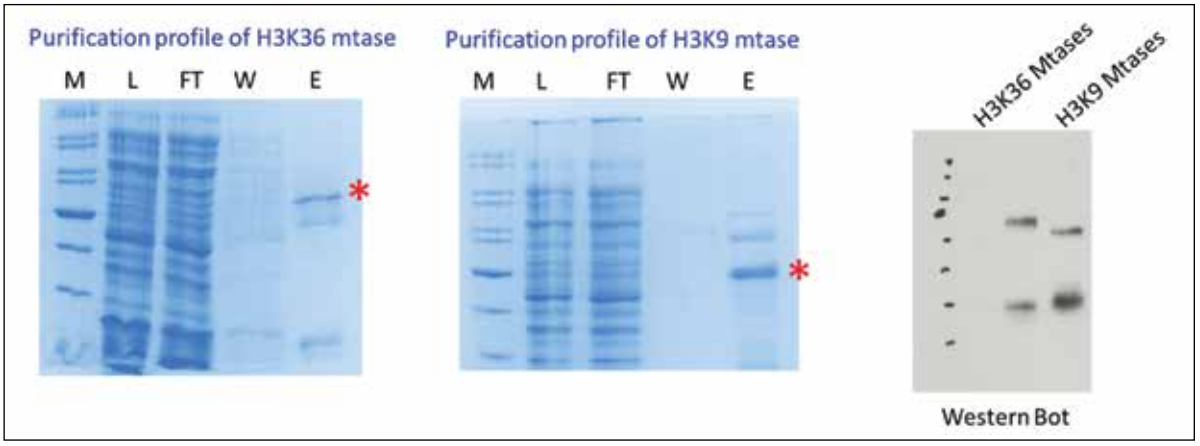

Fig 2. Expression and purification of SET domains specific for Pf_H3K36 and Pf_H3K9 methyltransferases as recombinant proteins, purity was confirmed by SDS-PAGE gel electrophoresis and red colour marks indicates the desired size of the GST coupled SET domains. WB was carried out to confirm the SET domains with anti-GST antibodies.

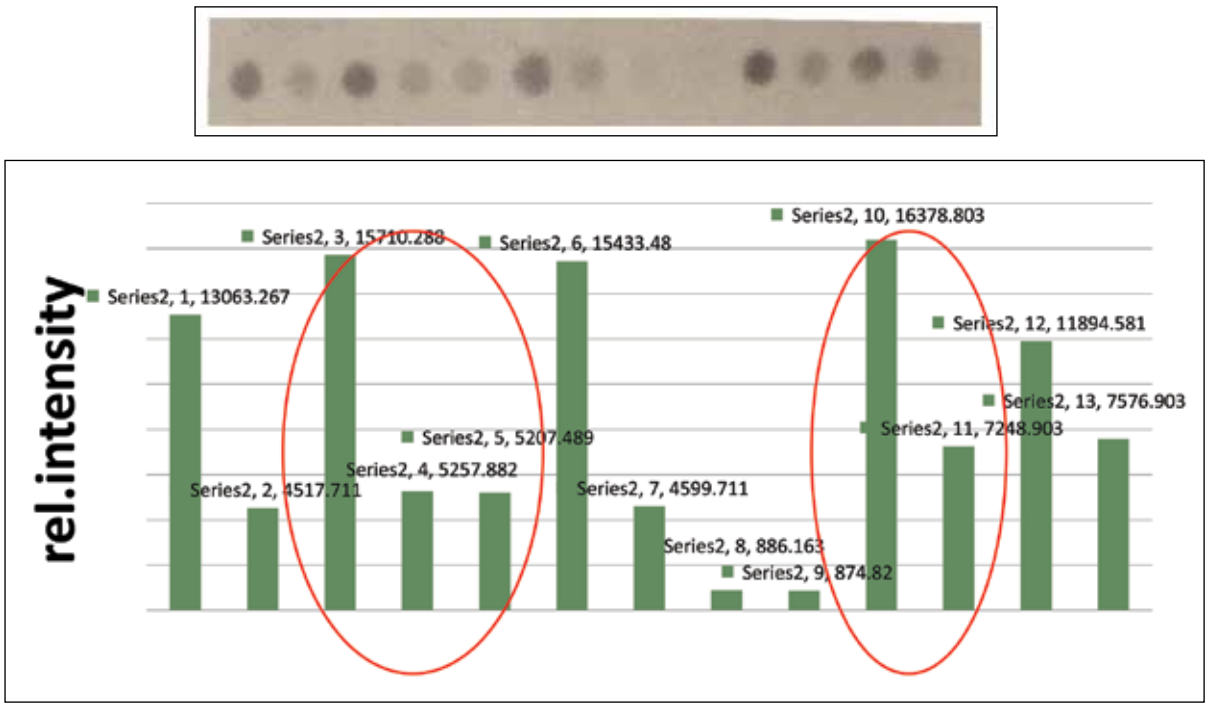

Fig 3. Peptide array methylation with H3K36 methyltransferases, the Pf enzyme shows predominant activity on the H3K14 site as well as H3K36 positions and introduction of mutant alanine at the these positions leads to the loss of activity.

We used SET domains of the purified enzymes to study the specificity and to identify the novel lysine substrates using peptide array approach. The preliminary study shows that parasite's H3K36 specific methyltransferases acts towards on its cognate substrates and also acts on H3K14 sites (Fig 3). Which is unique site in the malarial parasite and role of this modification will be validated further in the parasite level. Characterization of unique marks will provide the detailed information about dynamicity of P. falciparum chromatin.

TROPICAL DISEASE BIOLOGY
CHOLERA RESEARCH LABORATORY

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Molecular Characterization of Environmental *Vibrio parahaemolyticus* and Study of its Pathogenicity

Divya M.P and Sabu Thomas

***Collaborator**: T. Ramamurthy, National Institute of Cholera and Enteric Diseases, Kolkata*

Vibrio parahaemolyticus is a Gram-negative bacterium of the family *Vibrionaceae* that inhabits marine and estuarine waters. It is the leading cause of human acute gastroenteritis following the consumption of raw seafood. The organism possesses a repertoire of virulence factors including two hemolysins (TDH, TRH), Type III secretion systems (T3SS) and adhesins. Generally all the clinical strains harbor the virulence factors while only 1-10% of environmental isolates possess them. The purpose of this study was to investigate genetic diversity and pathogenicity of *V.parahaemolyticus* isolates from environmental sources and understand the distinction between clinical and environmental isolates of this dynamic pathogen. Environmental samples of marine and estuarine water, plankton and assorted seafood were collected during 2011-2013 from the coastal areas of Kerala, India for the isolation of *V.parahaemolyticus*. Samples were processed according to published guidelines and presumptive colonies on TCBS identified by PCR amplification of species specific *toxR* and *tlh* genes. Strains were tested for *tdh*, *trh* and pandemicity assessed by GS- PCR. Distribution of T3SS (T3SS1, T3SS2α and T3SSβ) effectors and apparatus genes in *tdh*$^+$ and/or *trh*$^+$ strains was studied by various PCRs. These isolates were serotyped and genetic lineage was determined by pulsed field gel electrophoresis. The maximum occurrence of *V.parahaemolyticus*

		T3SS2α genes							T3SS2β genes					
Strain	SeroGp	VPA 1321	VPA 1327	VPA 1336	VPA 1339	VPA 1346	VPA 1362	VPA 1370	vscC2	vop82	vscS2	vopC	VPA 1346 homo	VPA 1370 homo
H1	O1:K17	+	+	-	+	+	+	+	-	-	-	-	-	-
H2	OUT:KUT	+	-	+	-	-	-	+	-	-	-	-	-	-
H3	O5:KUT	+	-	+	+	+	+	-	-	-	-	-	-	-
H4	O5:K17	+	-	+	-	+	-	+	-	-	-	-	-	-
H5	O1:K19	+	+	-	-	-	-	-	-	-	-	-	-	-
H6	O1:K25	+	+	+	-	-	-	-	-	-	-	-	-	-
H7	O1:K25	+	+	+	-	-	-	-	-	-	-	-	-	-
H8	01:K23	+	-	-	+	-	+	+	-	-	-	-	-	-
H9	O1:K25	+	-	-	+	-	+	+	-	-	-	-	-	-
H10	O5:K17	+	-	+	+	+	+	-	-	-	-	-	-	-
H11	O1:K25	+	-	+	+	-	-	-	-	-	-	-	-	-
H12	O10:K24	+	-	+	-	-	+	+	-	-	-	-	-	-
H13	O2:KUT	+	-	+	-	-	+	-	-	-	-	-	-	-
H14	O4:K29	+	-	+	+	-	+	+	-	-	-	-	-	-
H15	O5:K17	+	-	-	-	-	+	-	-	-	-	-	-	-
H16	O1:KUT	+	-	-	-	-	+	+	-	-	-	-	-	-
H17	O5:K20	+	+	-	-	-	+	-	-	-	-	-	-	-
H18	O5:K20	+	-	-	-	+	+	-	-	-	-	-	-	-
H19	O4:K42	+	-	-	-	+	+	+	-	-	-	-	-	-
C12	O3:KUT	-	-	-	-	-	-	-	+	+	+	+	+	+
C13	O3:KUT	-	-	-	-	-	-	-	+	+	+	+	+	+
K23	O4:K36	-	-	-	-	-	-	-	+	+	+	+	+	+

tdh$^+$*trh*$^-$ | *trh*$^+$*tdh*$^-$

Fig.1. Distribution of T3SS genes in V.parahaemolyticus isolated from sea-food

was reported from sea-food (53.9%). Twentytwo strains isolated from sea-food were found to be toxigenic, which is surprisingly high. 19 isolates possessed *tdh* (*tdh*$^{+}$*trh*$^{-}$) while 3 had *trh* (*tdh*$^{-}$*trh*$^{+}$). All the *trh*$^{+}$ strains and 18 *tdh*$^{+}$ strains had *toxRS/* new gene, a marker for pandemicity. T3SS1 was conserved in all the toxigenic isolates. The genes tested for T3SS2α (that co-localizes with *tdh*) include those that encode proteins targeting actin cytoskeleton (VPA1357, VPA1370), modulate activity of eukaryotic cell signalling (VPA1321, VPA1327, VPA1336, VPA1346) and T3SS apparatus proteins (VPA1362, VPA1339). The *tdh*$^{+}$*trh*$^{-}$ strains showed variation in presence of T3SS2α as follows. VPA1370 could be detected in 9 strains, VPA1339 in 7 strains, VPA1362 in 13, VPA1327 in 5, VPA1321 and VPA1346 in 15 and 6 isolates respectively. 10 strains harbored VPA1336, which is responsible for producing enterotoxicity. T3SS2β (part of a different lineage of T3SS2), and homologues of VPA1346 and VPA1370 were detected in the 3 *tdh*$^{-}$*trh*$^{+}$ isolates (Fig.1). New serovars (O1:K19, O1:K23, O10:K24, 02:KUT, O4:K29, O5:K20, O4:K42, O4:K36) contained pandemic marker. DNA fingerprinting PFGE of NotI-restricted DNA produced varying patterns for 15 *V.parahaemolyticus* strains and 60% similarity to a clinical O3:K6 strain (Fig. 2). The strains identified in this study may be potentially pathogenic to humans and have a public health concern.

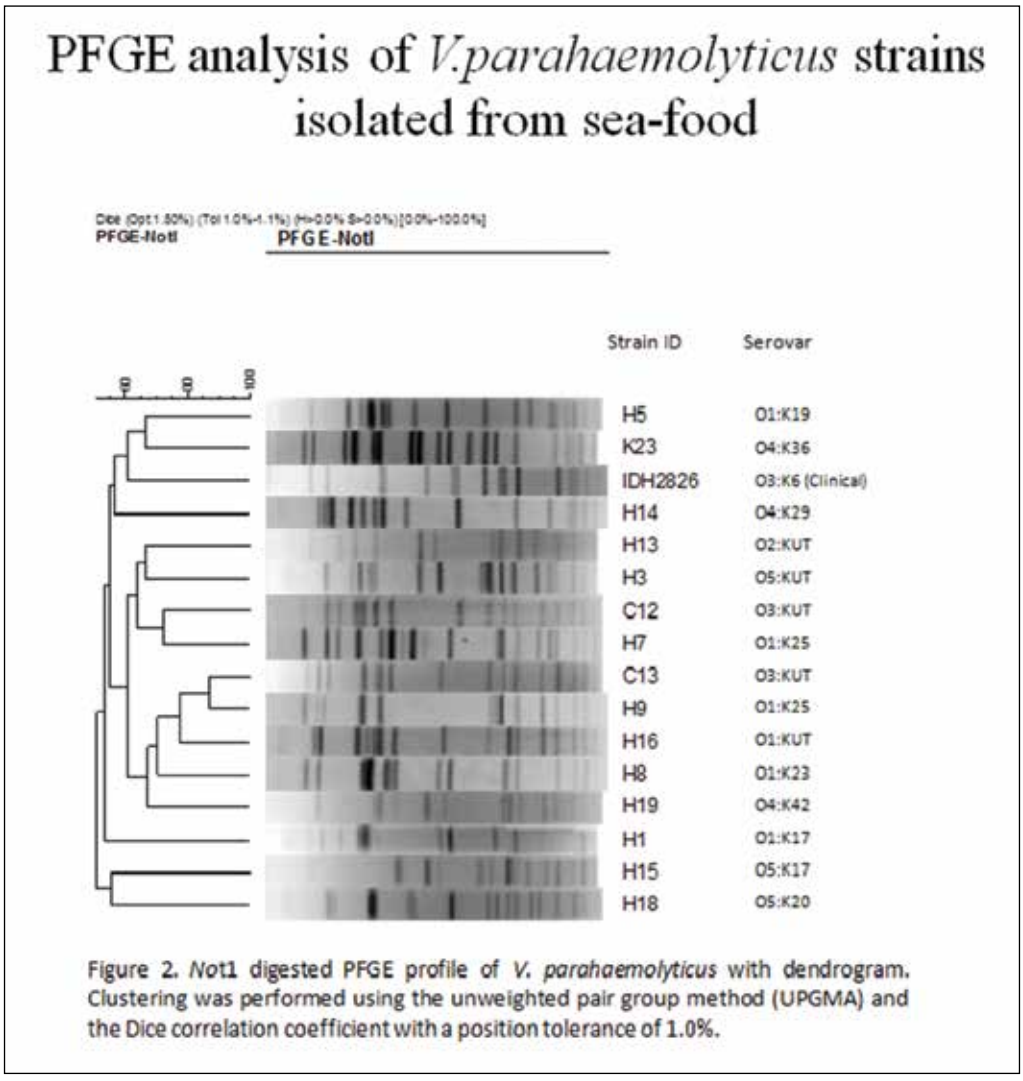


Fig.2. PFGE dendrogram generated by BioNumerics software, showing the relationship of fingerprints for 15 V. parahaemolyticus isolates.

Comparative transcriptome and proteome analysis of *Vibrio parahaemolyticus* and *V. cholerae* to identify potential biofilm inhibiting targets.

Akhilandeswarre D and Sabu Thomas

***Collaborator**: R. Sowdhamini , National Centre for Biological Sciences, Bangalore*

Vibrio cholerae and *V. parahaemolyticus* are two important human pathogens of Vibrionaceae family. The biofilm mode of life exhibited by these pathogens allows them to be highly resistant to antimicrobial agents and host immune responses. The present study is focused on identifying and understanding the conserved genes involved in the various stages of biofilm formation to identify conserved biofilm inhibiting target common to both the species employing omic approaches. Based on the biofilm formation and quantitation results, *V. parahaemolyticus* SC192 strain was selected and the transcriptome and proteome were analyzed at 4 hour log phase planktonic stage, 12 hour stationary phase planktonic stage and 24 hour biofilm stage of the strain grown in enriched medium. For transcriptome analysis, RNA was extracted from the harvested cells in three different conditions and analysed employing RNA sequencing method. From the comparative transcriptome analysis, 212 genes were downregulated and 503 genes were upregulated in the biofilm stage when compared to two different planktonic stages. Analyzing the proteome by employing Liquid chromatography coupled to tandem mass spectrometry validated the transcriptome analysis. The first step was to develop a non-gel based protocol to attain maximum proteome coverage in biofilm stage employing LC-MS analysis. Two protocols were standardized that differ in their detergent concentration and cell lysis methods for extracting the total proteome of planktonic and biofilm stages. The profiling method identified 45.5% of the total proteome of *V. parahaemolyticus* RIMD 2210633 reference genome, which is the largest proteome coverage obtained till date in *V. parahaemolyticus*. In continuation, the standardized protocol is used to extract the total proteome for quantitative proteomic analysis. The analysis revealed 52 down-regulated proteins and 214 up-regulated proteins in the biofilm stage compared to both the planktonic stages. On comparison of the transcriptomic and proteomic data, it was found that 54 genes were up-regulated and 43 genes were down-regulated in both mRNA and protein level (Table 1). Of the 54 up-regulated genes, 48 genes were found to be unique to the biofilm stage. The results from this integrated omics approach is further validated by DAVID to understand the pathways and functions that are differentially regulated at both mRNA and protein level during the biofilm stage. In the functional perspective, genes involved in transportation and carbohydrate metabolism were found to be specific to the biofilm stage.

Conditions	18h.B Vs 4h.P	18h.B Vs 12h.P	Only in 4h.P	Only in 12h.P	Both 4h.P & 12h.P	Total
Down-regulated RNA	738	425	526	213	212	951
Up-regulated RNA	839	658	336	155	503	994
Down-regulated proteins	73	84	21	32	52	105
Up-regulated proteins	76	103	29	56	47	132

Table 1: Results of Integrated Omics [Transcriptomics Vs Proteomics] Approach (B-Biofilm stage; P- Planktonic stage)

Novel Biofilm Inhibitors against *Vibrio cholerae* from selected herbal extracts

Diana Jose and Sabu Thomas

***Collaborators**: A.K.Goel, Defense Research & Development Establishment, Gwalior, Dr. R. Ajay Kumar, RGCB*

Vibrio cholerae is the causative agent of the water-borne disease cholera that still threatens a large proportion of world's population. Role of biofilm formation in *V. cholerae* pathogenesis is well established as it provides the bacterium with enhanced tolerance to antimicrobial agents and transforms it into a hyper infectious form. From the knowledge in Ayurveda, the ancient traditional medicinal system of India, four medicinal plants with anti-diarrhoeal properties were selected to test for their anti-biofilm activity against *V. cholerae*. Methanol extracts of these selected plants (*Centella asiatica, Elephantopus scaber*,

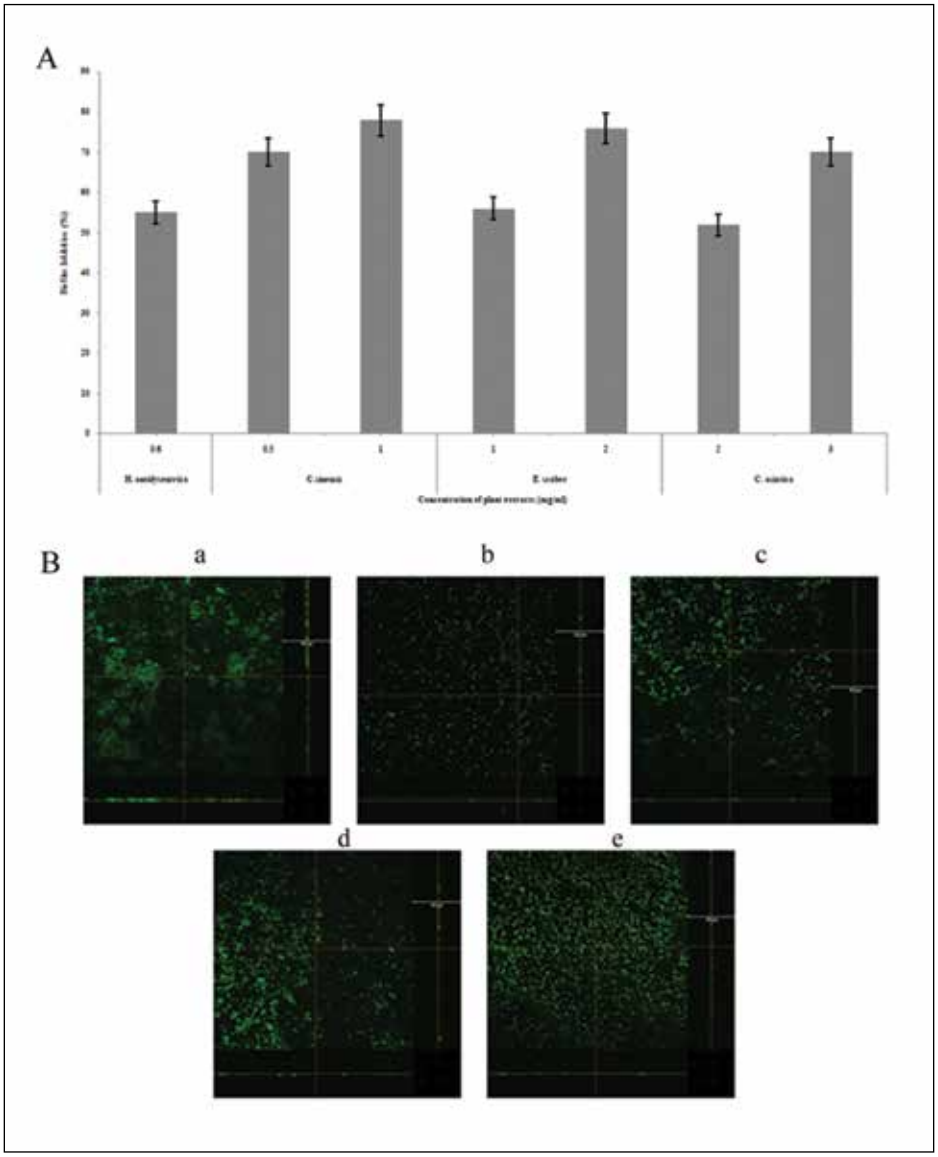


Fig.3. A) Graph showing percentage inhibition of biofilm by extracts of H. antidysentrica, C. sinensis, E. scaber and C. asiatica at different concentration. B) CLSM images of Air-liquid coverslip assay. a) V. cholerae biofilm (positive control), b-e) Biofilm treated with extract of E. scaber (1mg/ml), C. sinensis (1mg/ml), C. asiatica (3mg/ml) and H. antidysenterica (0.6 mg/ml)

Camellia sinensis and *Holerrhena antidysenterica*) were prepared to check the anti-biofilm activity against *V. cholerae* using crystal violet biofilm assay. Air-liquid interface coverslip assay was performed using confocal laser scanning microscopy (CLSM) to confirm the anti-biofilm activity. Viability of the treated bacterial sample was examined by MTT assay. Further, a combination of herbal extracts that showed significant anti-biofilm activity was made to develop a herbal formulation against *V. cholerae* biofilm. In this study, biofilm assay revealed that each of *C. asiatica, E. scaber*, *C. sinensis* (Green tea) and *H. antidysenterica* could significantly inhibit biofilm formation in *V. cholerae* O1. All the extracts showed biofilm inhibition of approximately 75%, 76%, 78% and 55% at concentrations 3, 2, 1, 0.6 mg/ml, respectively (Fig.3). Promising anti-biofilm activity was observed when a combination of *E. scaber* and *C. sinensis* (ESC) was used. This combination showed ~89% inhibition at 1.5 mg/ml concentration (Fig.4). The herbal extracts were thermo-stable at a wide temperature range of 40-100°C. MTT assay revealed that plant extracts did not affect the viability of the bacteria. This combination of herbal extracts can be used against *V. cholerae* infection.

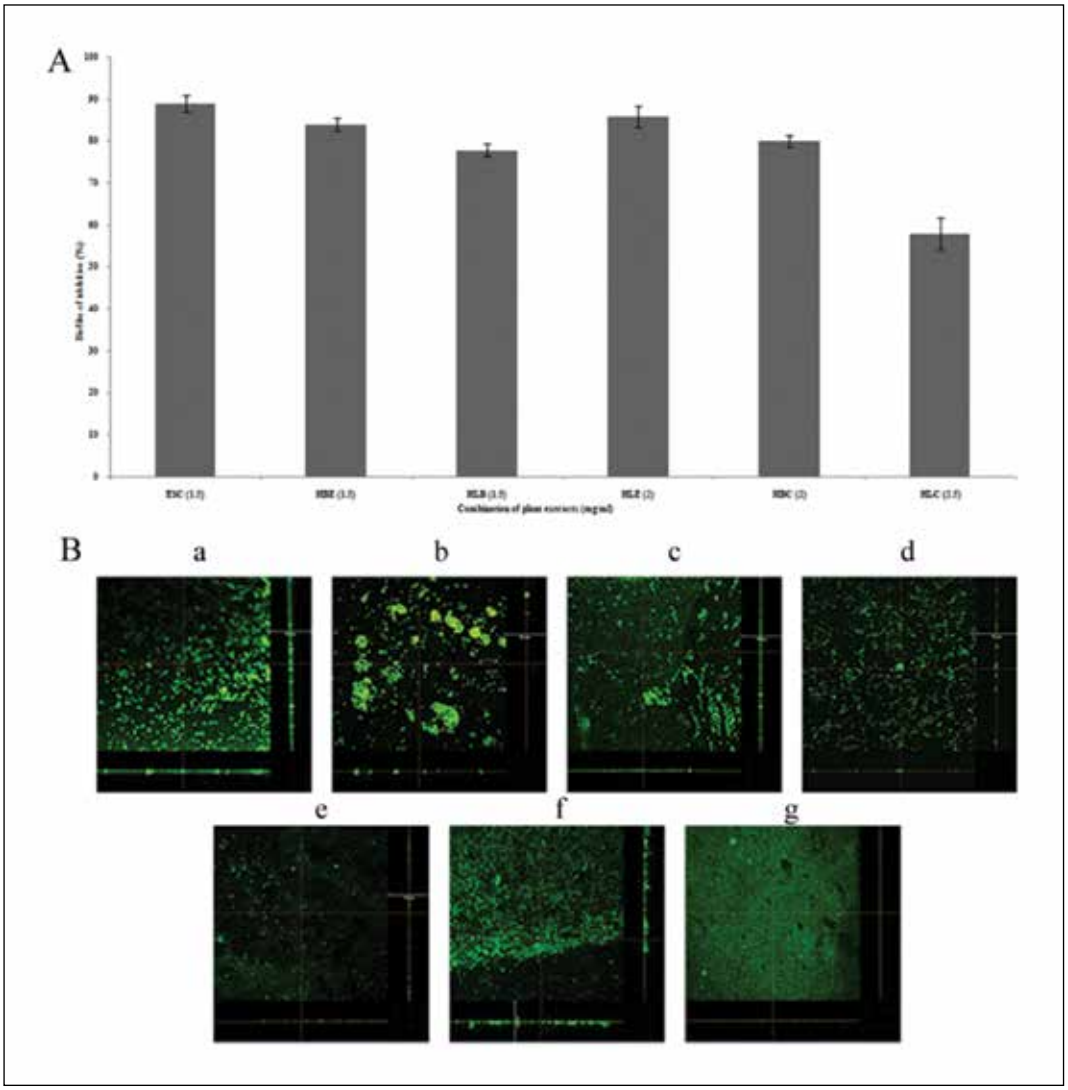


Fig.4. A) Graph showing percentage inhibition of biofilm by combinations of herbal extracts at different concentration. B) Air-liquid coverslip assay. a) V. cholerae biofilm (positive control), b-g) Biofilm treated with combination of herbal extracts of E. scaber and C. sinensis (ESC) combination, H. antidysentrica bark and E. scaber (HBE), H. antidysentrica leaf and E. scaber (HLE), H. antidysentrica bark and C. sinensis (HBC), H. antidysentrica leaf and H. antidysentrica bark (HLB) and H. antidysentrica leaf and C. sinensis (HLC) at 1.5 mg/ml.

Characterization of Polymicrobial Communities and its Biofilm Matrix Associated Components in Chronic Diabetic Ulcer

Karthika S and Sabu Thomas

Collaborators: *Dr. Harrison, S.K. Hospital, Trivandrum, Dr. Joby John, Medical College Hospital, Trivandrum*

Chronic wound biofilm is composed of diverse polymicrobial communities and pose an intractable problem for wound healing in chronic infections. The intrinsic resistance of the pathogenic biofilm to an array of antimicrobial agents and host defense mechanisms makes the chronic infections hard to treat. Identifying the diverse polymicrobial communities becomes a problem as the routine identification methods employed by the microbiology laboratories are not sufficient to determine the whole bacterial populations present in the wound samples. It is crucial to understand the diversity and ecology of microbiota in chronic wound biofilms as diabetes and related wound infections are on a rise in India. This study focuses on identifying the biofilm associated proteins and gene expression analysis of various biofilm stages of predominant wound pathogens. The present study centering the architecture of bacterial biofilms and the role of differentially expressed proteins in biofilm mode of life will enable the development of next generation therapeutics which will focus on dispersal of mature bacterial biofilms in non-healing chronic infections.Chronic diabetic ulcer samples (sample size, n=100) were collected from clinical settings by swabbing and debridement method. All the relevant clinical factors including the duration of diabetes, presence of neuropathy and glycemic level were noted which in turn will help to correlate the microbial infections and other factors associated with delayed wound healing. The microbial diversity analysis by 16S rRNA gene sequencing revealed that *Pseudomonas* sp., *Proteus* sp., *Enterococcus* sp. and *Staphylococcus* sp. were the predominant genera associated with the wound infections. Majority of the infections are polymicrobial in nature and 25 different bacterial genera were consistently identified. All the predominant bacterial isolates are good biofilm formers and most of them are multidrug resistant. The Metagenomic analysis to interpret the uncultured microbial community associated with the wound biopsy specimens are in progress. The presence of bacterial biofilm in chronic diabetic ulcer was confirmed by Fluorescent *in situ* Hybridization (Fig.5) and Scanning Electron Microscopy. The proposed study will enhance our knowledge concerning the nature and architecture of polymicrobial biofilm in chronic wound infections, which will aid in developing new diagnostic and treatment strategies for treating non healing chronic wound infections.

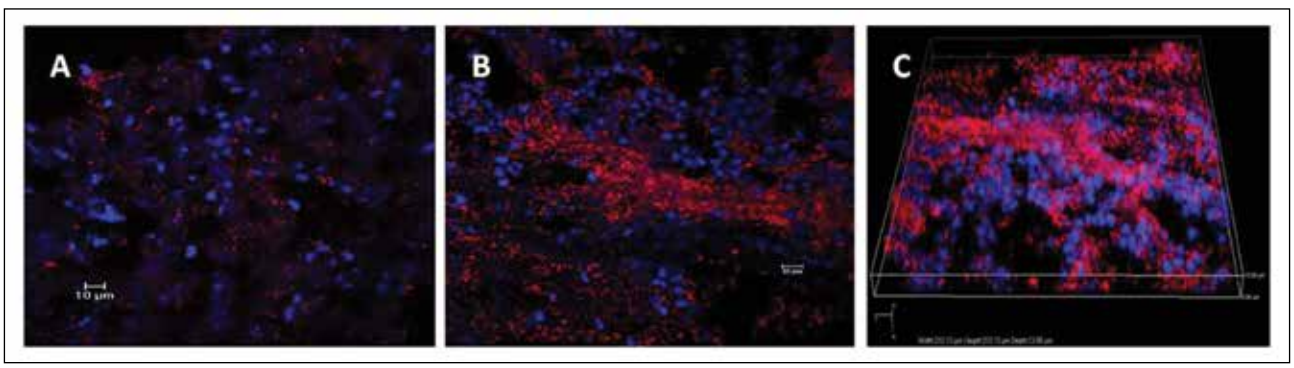


Fig.5. Fluorescent in situ Hybridization shows bacterial cells (red - cy3 labelled universal 16SrRNA bacterial probe EUB338) attached to the host wound debridement tissue (blue -DAPI). A) Acute wound, B) Chronic wound with bacterial biofilm, C) Isometric view showing biofilm clusters attached to the chronic wound host tissue.

Whole Genome Sequence Analysis of *Pseudomonas psychrophila* MTCC 12324, isolated from the Arctic fjord

Wilson Peter Abraham and Sabu Thomas

The polar Arctic region is an unexplored area of the earth, and this environment possesses microorganisms that permanently thrive at temperature at and around the freezing point of water. In the present investigation, a Gram-negative rod-shaped bacterium, *Pseudomonas psychrophila* MTCC 12324, was isolated from fjord water in the Arctic having a temperature of 2°C. The test organism was identified as *P. psychrophila* MTCC 12324 by sequencing its 16S rRNA gene (Accession no. JX271040.1). The bacterium grows at a temperature range between 4°C and 30°C, with optimal growth at a temperature between 20 to 25°C. Growth pattern of this organism indicates that it falls in the category of being facultative psychrophile. The genome of *P. psychrophila* MTCC 12324 was sequenced using an Illumina HiSeq 2000 and an Illumina MiSeq sequencing system. The reads were assembled into 150 large contigs (>2,330 bp) using ABySS, SPAdes, Velvet, and SOAP*denovo*2. The contig*N*50 was approximately 57.3 kb, and the largest contig assembled was approximately 235.3 kb. The draft sequence consists of 5,269,174 bases, with a mean G+C content of 57.52%. A total of 5,036 coding sequences and its gene ontology (Fig.6) were predicted and using Glimmer and RAST genome analysis tool. 51 structural RNAs were also identified from the contigs using tRNAscan-SE online tool. Draft genome sequence of *P.psychrophila* MTCC12324 is submitted in NCBI Genbank (Accession no. LBHT00000000- NCBI Biosample ID - SAMN03406680 & Bioproject ID - PRJNA278112). Several genes pertaining to cold adaptation, such as fatty acid desaturase, polynucleotide phosphorylase and peptidyl-prolyl*cis-trans* isomerase have been identified from the sequenced genome of *P. psychrophila* MTCC 12324. Genomic information acquired through whole-genome sequencing will shed light on the complicated cold adaptation mechanisms of bacteria, which are very diverse in nature.

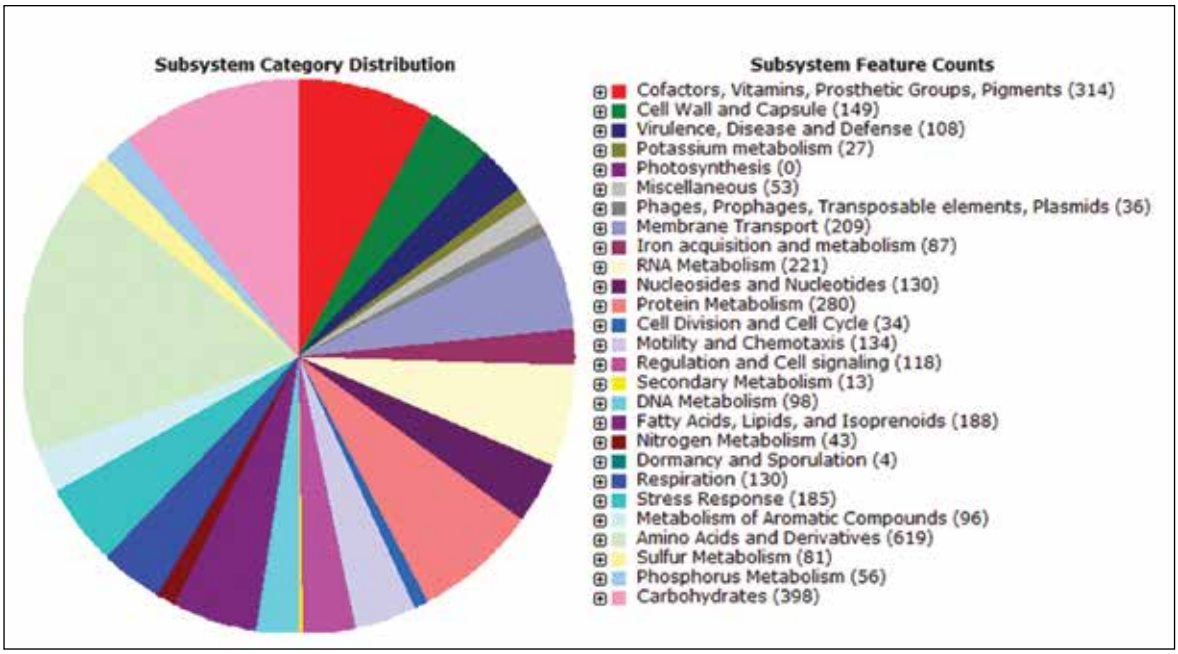


Fig.6. Gene ontology of the predicted genes from Pseudomonas psychrophila MTCC 12324 using RAST online genome analysis tool. Number in brackets indicates the number of genes/proteins involved in a biological function.

PUBLICATIONS

Primary publications from Laboratory

- *Dharmaprakash Akhilandeswarre, Mutt Eshita, Jaleel Abdul, Ramanathan Sowdhamini and Sabu Thomas* (2014). Proteome profile of pandemic *Vibrio parahaemolyticus* SC192 strain during planktonic and biofilm condition. ***Biofouling:*** *The Journal of Bioadhesion and Biofilm Research. 30(6), 729-739.*
- *Divya M P, Sivakumar K C, Sarada Devi K L, Remadevi S and Sabu Thomas* (2014). Novel multiple mutations in topoisomerase gene of Haitian variant *Vibrio cholerae* O1, *Antimicrobial Agents and Chemotherapy. 58(8): 4982-3.*
- *Karthika S, Reshma MJ, Wilson Peter Abraham, Anita Das R, Sarma US, Hari KrishnanK and Sabu Thomas.*2014. Characterisation and Evaluation of phenol degrading Bacillus spp.for enhancing the softness of coir fiber. *Journal of Natural Fibres (In Press).*

Publications with Collaborators

- *Vinoj G, Vaseeharan B, Thomas S, Spiers AJ, Shanthi S.*2014. Quorum-quenching activity of the AHL-lactonase from *Bacillus licheniformis* DAHB1 inhibits vibrio biofilm formation in vitro and reduces shrimp intestinal colonisation and mortality. *Mar Biotechnol (NY). 16(6): 707-15.*
- *Ballav S, Kerkar S, Thomas S, Augustine N.* 2015.Halophilic and halotolerant actinomycetes from a marine saltern of Goa, India producing anti-bacterial metabolites. *J Biosci Bioeng. 119(3): 323-30.*

CONFERENCE PRESENTATIONS

- *Karthika S, Joby John, Sabu Thomas.* Characterization of bacterial population associated with chronic wound infections and its biofilm formation. *International Symposium on New Perspectives in Modern Biotechnology, Pondicherry, India, March 2015. (Best Poster Award).*

INVITED LECTURE

- *Sabu Thomas,* 2014. **Trends in Biotechnology Towards Environment and Bio-Conservation**. *National Seminar on Recent Trends in Biosciences- Challenges and Future Prospects,13-15 March, Organised by Marthoma College of Science & Technology,Ayur, Sponsored by KSCSTE, Govt. of Kerala.*
- *Sabu Thomas,* 2014. **Environmental Biotechnology: Trends and Future Perspectives**. *National Seminar on Translational Biotechnology for a Better Tomorrow, 3-4 September, Organised by St.Peter's College, Kolenchery, Sponsored by UGC.*

EXTRAMURAL GRANTS

Title	Investigators	Funding Agency	Duration
Novel biofilm inhibitors against Vibrio cholerae from selected plants: isolation and characterisation	Dr.Sabu Thomas (PI) Dr. R. Ajay Kumar (Co-I) Dr. A.K. Goel (Co-I)	Defense Research & Development Establishment, Government of India	2012-14
Isolation and characterization of antimycobacterial molecules from Actinomycetes	Dr. R. Ajay Kumar (PI) Dr. Sabu Thomas (Co-PI)	Council for Scientific & Industrial Research-OSDD	2012-15

LABORATORY MEDICINE & MOLECULAR DIAGNOSTICS

Radhakrishnan R. Nair

Scientist E I

radhakrishnan@rgcb.in

Radhakrishnan received his PhD from the University of Kerala working at the Regional Cancer Centre, Thiruvananthapuram. He subsequently worked in faculty positions at various Medical Colleges including Manipal College of Medical Sciences, Oman University Medical College (Affiliated to the West Virginia University, USA), Sparten University, New York and the Atlantic University, New York. He joined RGCB in October 2011.

Laboratory Personnel

Sita Dayakar, PhD	Program Scientist
Sanjai D, MSc (MLT)	Senior Manager (Technical)
Kannan TR MSc	Laboratory Technician
Karthika V	Receptionist
Jayalakshmi S MSc	Senior Research Fellow
Vinitha P.T MSc, DMLT	Laboratory Technician
Sreeja S BSc MLT	Laboratory Technician
Akhila Suresh, MSc	Project Assistant
SreejaS,MSc	Research Assistant
Sumaja S	House Keeping

Laboratory Medicine & Molecular Diagnostics (LMMD) was initiated as a special purpose vehicle to meet advanced diagnostic demands of the state. Since February 2015, LMMD operates out of the Bio-innovation Center of RGCB. The facility translates the expertise and infrastructure available in RGCB to the community for an impact on both public health as well as individual patient health. LMMD now performs 39 viral, 3 bacterial, 4 cancer markers and pan of cardiology parameters and currently is arguably the only facility in India performing these many parameters under one roof. The facility has included tests used to identify emerging infections, antibiotic resistance, exposure to toxic substances as well as detection of chemical and biological threats. Another advance is personalized medicine approaches that can tailor medical treatments to individual patient needs, which is being utilized in the pharmaco-genomic test done for optimizing drug dosage. Cancer marker studies on patients facilitate selective anti-cancer therapy, diagnostic aid and prognostic markers. Currently JAK2 gene point mutation EGFR gene BRCA 1 & 2 and PGDFRA gene studies are offered. Cardio vascular disease biomarkers are routinely done in the division covering, analysis of MYH, TPT, MBP genes in hypertrophic cardiomyopathy, ACE (Ins/Del) polymorphism analysis, SCN5A gene in Brugada syndrome, ACTA2 gene in Thoracic Aortic Aneurysms and Aortic Diseases. Additionally, LMNA, MYH7, TNNT2, TPM1, ACTC1 gene study in Dilated Cardiomyopathy and KCNQ1, KCNH2, SCN5A, KCNE1 genes in long Q-T syndrome is also done. LMMD has trained 89 MD residents in Medicine, Microbiology, Biochemistry, Blood Banking and Pathology. In addition 51 MSc Microbiology/Biotechnology students were also trained. LMMD offers a unique Biotechnology Skill Development Program with 5 students admitted this year. RGCB has signed MOUs with the collegiate hospital of Government Medical College, Kerala Institute of Medical Sciences (KIMS) and PRS Hospital to provide regular molecular diagnostic facilities. In addition LMMD receives samples from Cosmopolitan Hospital, SK Hospital and public health centers of the Department of Health Services.

During 2014-15, LMMD performance increased in the infectious disease-testing segment. A total number of 5017 patients sample were tested. Among these, 1188 tested positive in any of the existing 42 infectious diseases parameters currently performed. This data clearly indicates infectious diseases are a major contributor for mortality and morbidity among Kerala population. We have identified and reported Scrub typhus as an emerging infection in Kerala. We also identified relative role of Cytomegaloviruses infection leading to high morbidity in renal transplant patients. In 2014-15, we conducted feasibility studies for oncology parameters and scientific collaborations initiated in oncology as well as post-transplant monitoring. LMMD also participates with the Office of Technology Ventures of RGCB in providing consultancy services for start-up companies and established corporates alike. Viral penetration studies in female condom studies were done for Hindustan Lifecare Limited and Bactericidal property testing for Eco Health products Chennai. LMMD performed 5017 PCR and 2564 ELISA based testing during 2014 January to May 2015 depicted in Figure1a, b and 2.

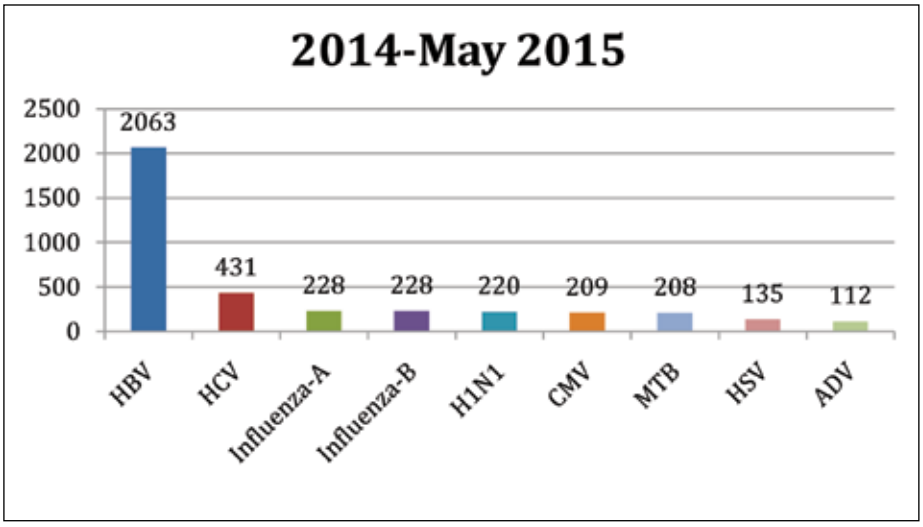


Fig: 1a PCR based testing during 2014-May 2015

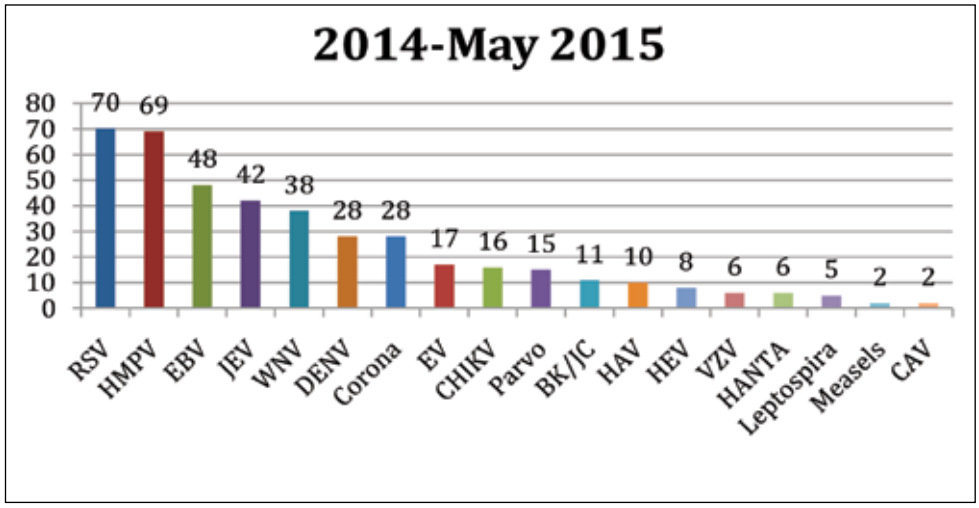


Fig: 1b

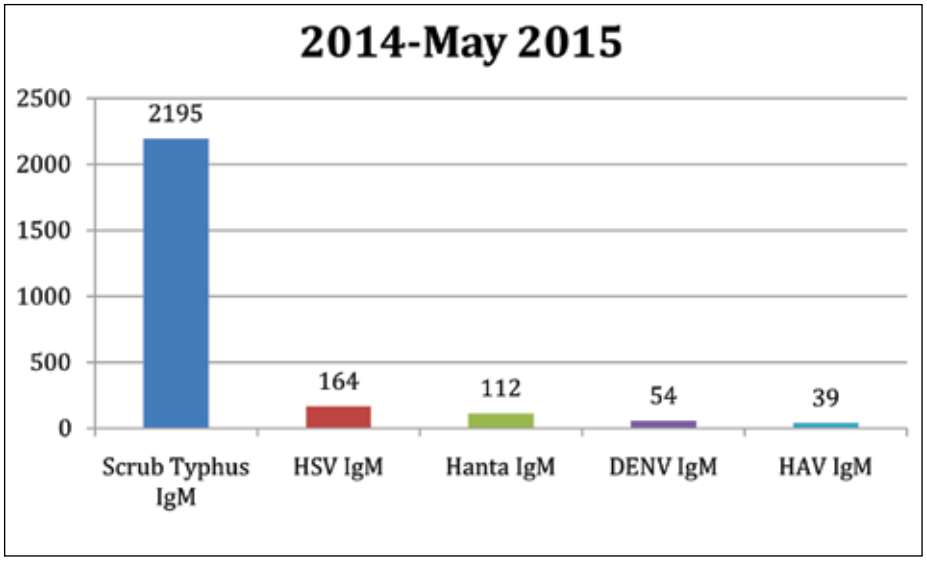


Fig:2 ELISA based Testing during 2014-May 2015

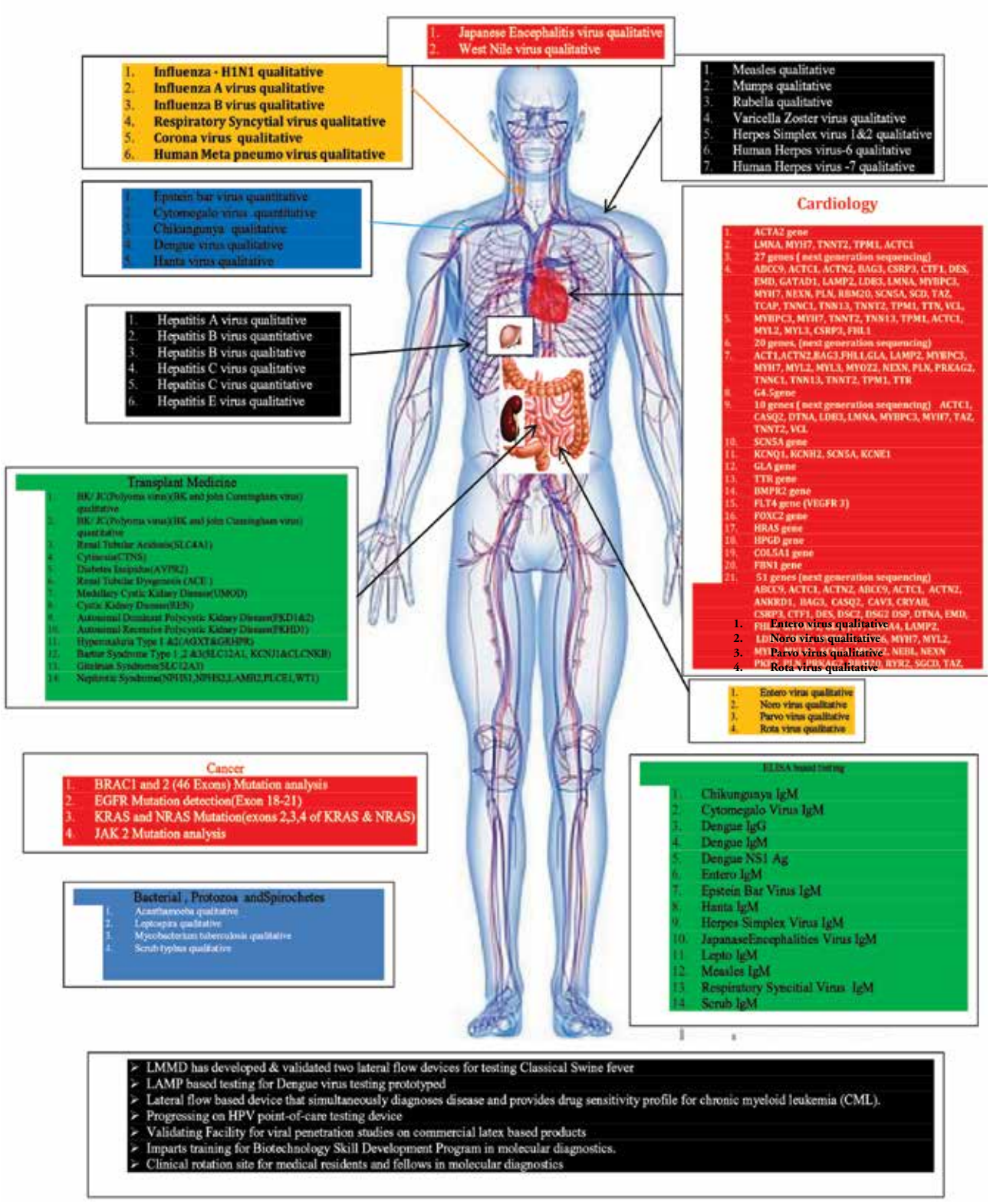
APRIL 2014 TO MARCH 2015

Sl No.	Name of Test	Method	Total tests done	Positive cases
1	Dengue IgM	ELISA	53	26
2	HSV 1/2 IgM	ELISA	114	25
3	Hanta IgM	ELISA	77	0
4	Scrub IgM	ELISA	1658	296
5	HAV IgM	ELISA	39	15
Sl No.	**Name of the Test**	**Method**	**Tests done**	**Positive cases**
1	HEV Qualitative	PCR	8	0
2	Measles Qualitative	PCR	0	0
3	Coxsackie Qualitative	PCR	1	0
4	Hanta virus Qualitative	PCR	3	0
5	Scrub Typhus Qualitative	PCR	0	0
6	HBV Qualitative	PCR	0	0
7	HBV Quantitative	qPCR	1562	658
8	HCV Qualitative	PCR	0	0
9	HCV Quantitative	qPCR	302	31
10	BK JC Qualitative	PCR	5	0
11	BK Quantitative	qPCR	9	0
12	JC Quantitative	qPCR	6	0
13	Dengue Qualitative	PCR	24	1
14	Chikun Qualitative	PCR	7	1
15	HSV Qualitative	Real Time PCR	90	2
16	WestNile Qualitative	Real Time PCR	30	0
17	Enterovirus Qualitative	PCR	10	0
18	Leptospira Qualitative	PCR	4	1
19	CMV Qualitative	PCR	0	0
20	CMV Quantitative	qPCR	150	61
21	EBV Qualitative	PCR	0	0
22	EBV Quantitative	qPCR	37	8
23	JEV Qualitative	PCR	0	0
24	TB Qualitative	Real Time PCR	146	5
25	HAV Qualitative	PCR	9	0
26	Parvovirus Qualitative	Real Time PCR	10	4
27	H1N1 Qualitative	Real Time PCR	163	28
28	Influenza A virus Qualitative	Real Time PCR	164	21

29	Influenza B virus Qualitative	Real Time PCR	164	2
30	Parainfluenza virus 1 Qualitative	PCR	0	0
31	Parainfluenza virus 2 Qualitative	PCR	0	0
32	Parainfluenza virus 3 Qualitative	PCR	0	0
33	RSV Qualitative	Real Time PCR	43	0
34	Human Meta pneumo virus Qualitative	Real Time PCR	42	0
35	Rota virus Qualitative	PCR	0	0
36	Noro virus Qualitative	PCR	0	0
37	Adeno virus Qualitative	Real Time PCR	60	3
38	Acanthamoeba Qualitative	PCR	0	0
39	Mumps Qualitative	PCR	0	0
40	Varicella Zoster Virus Qualitative	PCR	5	0
41	Rubella Qualitative	PCR	0	0
42	HHV-6 Qualitative	PCR	0	0
43	HHV-7 Qualitative	PCR	0	0
44	HHV -8 Qualitative	PCR	0	0
45	Corona Qualitative	PCR	22	0
			5017	1188

LMMD is a self-sustaining service facility and the increase in income from 2012 to 2015 is represented below.

Overview of Molecular Diagnostics Molecular Diagnosis tests in viral, bacterial, cancer, cardiovascular and pharmacogenomics by qRTPCR/qPCR/in-house PCR, PCR Sequencing and next generation sequencing



PUBLICATIONS

- *Seetha Dayakar, Iravathy K. Goud, Heera Pillai, Viji Remadevi,Sanjai Dharmaseelan, Radhakrishnan R. Nair , M. Radhakrishna Pillai* (2015) Molecular Diagnosis of Chikungunya virus (CHIKV) and Dengue virus (DENV) and its concomitant circulation in South Indian population. *Virology Reports, 2015.*
- *Suman Omana Soman, Govindan Vijayaraghavan, Ramesh Natarajan, Radhkrishnan Nair, Heera Pillai, Kartha CC.* Hypertensive Hypertrophic Cardiomyopathy - Is it a Part of Systemic Hypertension or Genetic Abnormality? *American journal of Cardiology 2015, Vol: 115(1) Pp S139.*
- *Ramakrishnan Lakshmy, Radhakrishna Pillai Madhavan, and Nair Radhakrishnan R.* Dengue Vaccine Development: Strategies and Challenges. *Viral Immunology. 2015, 28(2): 76-84.*
- *G.K. Mini, Mark Nichter, Radhakrishnan R. Nair, K.R. Thankappan.* Confirmation of self-reported non-smoking status by salivary cotinine among diabetes patients in Kerala, India. *Clinical Epidemiology and Global Health, Volume 3, Issue 1, April 2015, Pages 44–46*
- *Nair SS, Sarasamma S, Gracious N, George J, Anish TS, Radhakrishnan R.* Polymorphism of the CYP3A5 gene and its effect on tacrolimus blood level. *Experimental and Clinical Transplantion. 2015 Apr; 13 Suppl 1:197-200.*
- *Vijesh S. Kuttiatt, Sanughosh Kalpathodi, Sindhu T. Gangadharan, Lalitha Kailas, Easwaran Sreekumar, Suja M. Sukumaran, and Radhakrishnan R. Nair.* Detection of Measles Virus Genotype B3, India. *Emerging Infectious Diseases Vol. 20, No. 10, October 2014*
- *Zinia T Nujum, Achu Thomas, K Vijayakumar, Radhakrishnan R Nair, M Radhakrishna Pillai, P S Indu, Syam Sundar, Soumya Gopakumar, Devi Mohan, and T K Sudheeshkumar* Comparative performance of the probable case definitions of dengue by WHO (2009) and the WHO-SEAR expert group (2011). *Pathogens Global Health. 2014 Mar; 108(2): 103–110*

GENEBANK ACCESSION

Made available on Dec 31, 2014

- 2Denguesequences.sqn LLMD-231/RGCB DEN4/2012 KJ938501
- 2Denguesequences.sqn LLMD-239/RGCB DEN4/2012 KJ938502
- 2Denguesequences.sqn LLMD-237/RGCB DEN4/2012 KJ938503
- 2Denguesequences.sqn LLMD-233/RGCB DEN4/2012 KJ938504
- 2Denguesequences.sqn LLMD-238/RGCB DEN4/2012 KJ938505
- 2Denguesequences.sqn LLMD-230/RGCB DEN4/2012 KJ938506
- 2Denguesequences.sqn LLMD-236/RGCB DEN4/2012 KJ938507

REGIONAL FACILITY FOR DNA FINGERPRINTING (RFDF)

Chief Scientific Officer

Dr. E V Soniya

Scientific Officer

Dr. Harikrishnan K

Case Registrant

Ambili S Nair

DNA Examiner

Suresh Kumar U

Laboratory Technician

Ratheesh R V

Research Assistant

Kannan P

RFDF offers DNA fingerprinting services to legal bodies, crime investigating and law enforcing agencies. The samples analysed at RFDF relates to maternity/paternity disputes, crime, rape incidents and cases involving man missing. CO1-based molecular identification and DNA barcoding of fauna especially for species identification in wildlife forensics is yet another service offered by RFDF. Other services offered by this facility include DNA fingerprinting of plants and animals in case-by-case manner using RAPD, AFLP or microsatellite markers and DNA barcoding of animals using CO1 gene and plants using matK and rbcL. The facility also offers hands on training on DNA fingerprinting and DNA barcoding techniques. Details about various DNA fingerprinting/ barcoding services and training programmes are provided in our website.

In 2014-2015 we analysed more than 130 samples related to identification, maternity/paternity and relationship disputes forwarded by courts from different districts of Kerala and Kerala Women's Commission. In addition we have received more than 164 samples related to animal poaching forwarded from various forest range offices through court. Nine candidates were given training in DNA fingerprinting/barcoding during this period.

Animal poaching is one of the major threats to the animals in wild. It is imperative to punish the offenders to prevent illegal poaching. Samples confiscated by forest officers in Kerala Forest Department are forwarded to our lab for identification of species, so as to enable them to charge the case and punish the offenders. DNA Barcoding helps to identify animals even from minute or cooked samples. But the exact identification of species from the Western Ghats region of Kerala, which is one of the hottest biodiversity hotspots, is often difficult or not possible due to the lack of reference sequences in databases. In collaboration with Zoological Gardens, Department of Museum & Zoos, Thiruvananthapuram we are in the process of developing a DNA Barcode database of captive animals in Thiruvananthapuram Zoo, which will be useful in wildlife forensics to provide evidence to the legal bodies to punish the offenders in poaching cases and thus aiding in the conservation of endangered and endemic animals. We have collected blood/muscle samples from captive animals in Thiruvananthapuram Zoo, India. Samples included many endemic and threatened species present in the Western Ghats and many local as well as migratory birds. DNA was isolated from the samples and COI as well as Cytochrome B genes were amplified and sequenced using universal primers. A preliminary data generated was presented at the 6th International Barcode of Life Conference held at University of Guelph, Canada from August 18, 2015 to August 21, 2015.

CONFERENCE ABSTRACTS

- *Suresh Kumar U, Ratheesh R V, Jacob Alexander and E V Soniya* (2015) Development of DNA Barcode Database of captive animals in Thiruvananthapuram Zoo, Kerala, India. Scientific abstracts from the 6th International Barcode of Life Conference. *Genome,* 58(5): 239. DOI: 10.1139/gen-2015-0087.

INSTRUMENTATION ENGINEERING

Shaj Upendran
Senior General Manager

Staff Members

Rajasekharan K
Rahul C S Nair
Sajan I X
S Rajeev
S Ajith Kumar
K Manoj Kumar
V Prem Kumar
V Shaji
A S Vijayakumar

Rajiv Gandhi Centre for Biotechnology houses a large number of molecular biology and biotechnological research instruments. The Instrumentation Engineering Division is responsible for the installation, maintenance and repair of sophisticated research instruments in RGCB as well as the maintenance of Central instrumentation facility. The Division also maintains a well-equipped engineering workshop with facilities required for the repair and calibration of the sophisticated instruments as its part. Repair up to the PCB level is done here reducing the downtime and repair costs. By attending to many essential repair works of instruments, dependence on expensive maintenance contracts with dealers has been reduced. The instrumentation Division also carries out design, modification, and fabrication of research instruments. During the year, problems of DNA sequencer (96 capillary), Nucleic Acid Extraction System, White Laser Confocal Laser Scanning Microscope, In vivo Animal Imager, Liquid Nitrogen Plant, FACS ARIA Flow Cytometer, Confocal Laser Scanning Microscope, Spectrophotometers, Ultra Centrifuges, High speed Centrifuges, Table Top Centrifuges, Gel Documentation systems, Transmission Electron Microscope, Upright and Inverted Microscopes, PCR machines, Electronic balances, Speed Vac Concentrator, CO2 Incubators, HPLC, Freeze Dryers, Microplate Washer etc., have been repaired successfully. The Instrumentation Division also maintains the Centralized Instrumentation facilities, Computers, PC based security surveillance system, Biometrics Time Attendance recorders, Conferencing facilities, Communication systems, Liquid Nitrogen Plant, Incinerator, Auditoriums, Convention Centre etc. It also carries out the supervision of 11KV electrical substation, 340 ton AC plant, 630 KVA, 750 KVA & 1010KVA DG sets. The in house engineering department also carries out all minor electrical works required by the scientific staff. The Engineering division has been successfully completed the installations of new instruments in the new Bio-Innnovation Center located in the KINFA Film and Video Park. This includes the installation of instruments, electrical work, air-conditioning works, etc.

Centralized Instrumentation Facility

In addition to the basic facilities available in all the research laboratories, we have a centralized core facility equipped with several minor and major equipments to cater the requirements of our research personnel. The following are the facilities available in the core facility:

Spectroscopy

- MALDI TOF/TOF Mass Spectrometer - Bruker
- Bench top MALDI TOF Mass Spectrometer – Shimadzu
- Spectrophotometer - Perkin elmer, Thermo, Labomed
- Luminescence Spectrometer - Perkin Elmer
- Multimode Plate Reader - Tecan
- FTIR Spectrometer - Thermo

Genomics and Proteomics

- Next Generation Sequencing Systems (NGS): Ion Proton Torrent System and Ion Proton Personal Genomic Machine – Life Technologies
- Gene Chip Scanner – Affymatrix
- DNA sequencer (96 capillary)- Applied Biosystems (ABI)
- Genetic Analyzer (Single capillary) - Applied Biosystems (ABI)
- DNA sequencer (48 capillary)- Applied Biosystems (ABI)
- High Definition Mass Spectrometer (HDMS) -Waters Corporation
- Protein sequencer – Shimadzhu
- Protein Interaction Analyzer by SPR - Biorad
- Real Time PCR- Applied Biosystems, Biorad, Cepheid.
- Automated Peptide Synthesizer - Applied Biosystems
- Amino acid analyzer - Shimadzhu
- Nucleic Acid Extraction System - Precision System Science, Beckman
- Fully Automatic Nucleic Acid Extraction System - BioMerieux

Separation and Purification

- Pulse Field Electrophoresis - Biorad

- Ultra Centrifuges - Beckman Coulter
- High speed Centrifuges - Sorvall, Hittachi, and Kuboto.
- Table Top Centrifuges - Hareaus, Zigma, Jouan, Eppendorff.
- Protien purification system - Biorad
- HPLC – Shimadzhu, Waters Corporation
- UPLC – Waters Coroporation
- nanoLC - Bruker
- Gas chromatograph - Shimadzhu
- Automated Flash Chromatograph - Biotage

Imaging

- Phospher Imager - Biorad
- Multi Imager - Biorad
- Gel Documentation systems - Biorad, UVP, Syngene.
- Confocal Laser Scanninmg Microscope – Leica Microsystems, Nikon
- Super Continnum White Laser Confocal Laser Scanning Microscope - Leica Microsystems
- Stuctured Illumination Microscope with Confocal Laser Scanning System - Nikon
- Spinning Disc Confocal Microscope - BD Biosciences
- Transmission Electron Microscope - Jeol
- Upright and Inverted Microscopes - Leica, Zeiss, Nikon, Olympus
- Bench top High Throughput Bioimager - BD Biosciences
- Invivo Animal Imager – Xenogen
- Ultra Sound Scanner - Phillips

Flow Cytometry

- FACS ARIA Flow Cytometer with sorter - BD Biosciences
- FACS ARIA II Flow Cytometer - BD Biosciences
- FACS ARIA III Flow Cytometer - BD Biosciences

Others

- Fully Automated Liquid Handling System – Beckman Coulter
- Liquid Scintillation Counter - Wallac
- Ultra Microtome - Leica
- Submicron size Analyser - Beckman Coulter
- Robotic Spotter for MALDI TOF/TOF plates
- Non-Invasive Blood Pr. Monitor with ECG for animal facility – Iworks/IITC
- Sample preparation System for NGS, Ion OneTouch – Life Technologies
- Eletrophysiology Setup
- Liquid Nitrogen Plant - Sterling
- Incinerator - Thermax

IT MANAGEMENT

Shaj Upendran
Rajasekharan K
Durga Prasad C
Amal V
Renadeep CS Nair

RGCB's comprehensive infrastructure includes 7 Servers, more than 300 Desktops and Laptops, Network Printers etc. and houses advanced computing network with constant upgradation in a bid to provide the students and staff with state-of-the art facilities. The Institute has been connected through the National Knowledge Network, which provides 1Gbps leased line with multiple redundant backups. The highly distributed computing environment at RGCB uses sophisticated computer simulation to solve problems for Staff and Research Scholars. It is managed and actively supported by the experienced engineers in the IT Department. IT department is also responsible for maintaining and administrating RGCB Website and Mail Servers. IT Department provides technical support to Staff and students within the Institute on LINUX, WINDOWS platforms and also provides software development for research groups.Internet facilities are provided throughout the campus through 1 Gbps and 10 Mbps leased lines from NKN and BSNL respectively. RGCB has invested in a high-speed Fibre Optic Backbone with high-end security for networking across the campus. Wireless connectivity is provided at strategic locations to provide Internet access to the faculty.

ANIMAL RESEARCH FACILITY

Veterinarians

Santhosh Sankaran, BVSc

R. Rajagopal, MVSc

Supporting staff

Vinod. V M
G Vinod
K. Y. Anwar
Pradeep Kumar S
Rajeev R V
Alex A Anto
Dileep R K

Laboratory animals play an important role in biomedical research as they serve as the most befitting models for many of the human and animal diseases. RGCB has a compact and well-organized Animal Research Facility (ARF). The RGCB ARF has separate breeding and experimental rooms for rats and mice, procedure & surgery rooms, store room and one room exclusively for rabbits. All mice rooms are equipped with Individually Ventilated Caging System (IVC). We maintain colonies of several valuable and imported strains including immune compromised, transgenic, and knockout animals. ARF also caters the various surgical needs of the Investigators by providing the necessary expertise and with its rich array of surgical equipment viz., Isoflurane Inhalant Anesthesia Machine, Small Animal Ventilator, Non-invasive Blood Pressure Monitoring Apparatus (NIBP) etc and procedure room is equipped with class II level Biosafety cabinet. During the past years we have successfully standardized various procedures for Thoracotomy, Aortic ligation and Jugular vein cannulation in rats etc. ARF has an in vivo imager (Caliper, USA), which can be used for the fluorescent and bioluminescent imaging of small animals. Our facility is registered with the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA) and an Institutional Animal Ethics Committee ensures that the research that uses animals as the study models, are carried out humanely. All projects that request the use of animals from the Animal Research Facility must first obtain approval from this committee before the animals can be used. ARF conducts a mandatory Training program for PhD students/research personnel encompassing all aspects of laboratory animal science viz., basic handling and physiology of animals, animal ethics, animal welfare, bio-statistics, animal genetics, alternatives to animal experimentation, laboratory animal surgery and practicals on animal handling, animal behavior and essential surgical skills. Construction of another animal research facility at the RGCB Bio Innovation Center is ongoing and expected to be fully functional by the end of year 2015.

DISTRIBUTED INFORMATION SUB-CENTRE

Coordinator

Dr. Sathish Mundayoor

Technical Officer

Sivakumar KC

The Distributed Information Sub-Centre at RGCB, setup and funded by DBT, GOI under the National Bioinformatics Network program started functioning from May 2002 with a view to catering the needs of the scientific community and to augmenting their research activities through information inputs. The main function of the Centre is to act as a member of Bioinformatics Network System for providing information to the interested users on topics pertaining to the relevant areas of Biotechnology specially genomics and proteomics.

OBJECTIVES

To provide a national bio-information network designed to bridge the inter disciplinary gaps in biotechnology information and to establish link among scientists in organizations involved in R&D and manufacturing activities in biotechnology

To build up information resources, prepare databases on biotechnology and to develop relevant information handling tools and techniques

To evolve and implement program on education of users and training of information scientists responsible for handling of biotechnology information and its applications to biotechnology research and development.

To develop, support and enhance public information resources for biotechnology e.g. Gene banks, molecular biology data and related research information resources.

INFRASTRUCTURE

To keep pace with the development in the bioinformatics field, impetus is given to set up the necessary computational infrastructure and resources for the research community. The Centre has access to online CDAC Tera-scale Supercomputing Facility (CTSF) on Param Padma Super Computer situated at CDAC-Bangalore. The center has upgraded its computational resources by setting up high performance computing facility. 12 Intel Core-i7 Desktop PC's, 3 Dell PowerEdge T300 Workstations and 5 Dell Vostro 400 PC. The molecular modeling package Accelrys Discovery Studio 2.0 purchased on DBT grant is used for a molecular modeling, docking and simulation studies. Apart from commercial package Discovery Studio2.0, the centre have popular Open bioinformatics softwares such as, EMBOSS, Autodock, WHATIF, MODELLER ClustalW, Phylip, Cn3D, Rasmol, HMMR, MEGA4, XMGRACE, GROMACS, FTDOCK, PyMol, Jackal, Patchdock, Z-dock etc.

PUBLICATIONS

Publications generated from the use of this facility

- *Divya M, Sivakumar KC, Devi KS, Remadevi S, Thomas S:* Novel Multiple Mutations in the Topoisomerase Gene of Haitian Variant Vibrio cholerae O1. *Antimicrobial agents and chemotherapy 2014, 58(8):4982-4983.*
- *Rasheed VA, Sreekanth S, Dhanesh SB, Divya MS, Divya TS, Akhila PK, Subashini C, Chandrika Sivakumar KC, Das AV, James J:* Developmental wave of Brn3b expression leading to RGC fate specification is synergistically maintained by miR-23a and miR-374. *Developmental Neurobiology 2014, 74(12):1155-1171.*
- *Thomas NE, Thamkachy R, Sivakumar KC, Sreedevi KJ, Louis XL, Thomas SA, Kumar R, Rajasekharan KN, Cassimeris L, Sengupta S:* Reversible Action of Diaminothiazoles in Cancer Cells Is Implicated by the Induction of a Fast Conformational Change of Tubulin and Suppression of Microtubule Dynamics. *Molecular cancer therapeutics 2014, 13(1):179-189.*
- *Augustine N, Goel A, Sivakumar KC, Kumar RA, Thomas S:* Resveratrol–a potential inhibitor of biofilm formation in Vibrio cholerae. *Phytomedicine 2014, 21(3):286-289.*

LIBRARY AND INFORMATION SERVICES

Librarian
Lathika.K

Technical Assistant
Meera N. V

Library Assistant
Gopakumar G

RGCB Library maintained its distinction as the responsibility of advanced most sought after books and journals on Life Science its special emphasis on Biotechnology during the year under review as well. Progress and development achieved was quite evident in the qualitative and quantitative improvements made available in the infrastructure facility and other convenience additionally provided to cater to the emerging needs of the every increasing number of the users. The Library has a collection of more than 7950 documents. The library subscribes a good number of reputed journals mostly in the electronic version. In addition, being a member of DeLCON, Delnet, Biomed Central etc. RGCB library can access a wide range of online journals, e- books etc. Apart from the books on life science, the library also has a good collection of books on protocols, standards, manuals, PhD theses, conference proceedings, scientific general reading books etc. There is also a wide collection of audio-visual materials. The library has a separate newspaper reading section. 18 popular magazines and 10 daily newspapers are made available. Membership in the Department of Biotechnology's e- Library Consortium (DeLCON) facilitated access to 926 e- journals of twenty international publishers. These publishers included American Association for Advancement of Science (AAAS), American Association for Cancer Research (AACR), American Chemical Society (ACS), Annual Reviews, American Society for Biochemistry and Molecular Biology, American Society For Microbiology, Cold Spring Harbor Laboratory Press, Taylor and Francis, Lippincott William and Wilkins (LWW), Marry Ann Liebert, Nature Publication Group, Oxford University Press (OUP), Springer, Society for General Microbiology, Wiley-Blackwell, Elsevier Science (Science Direct), American Society of Plant Biologist, American Association of Immunologists, etc. The DeLCON has tremendously contributed in enhancing the level of usage of e- journals particularly those of Springer and Nature publishing Group. This was also due to the regular monitoring and user awareness and promotional programs under the initiative of the library. Updating of in house databases of books, periodical, bound volumes of journals, PhD these, reports, conference proceedings etc. was carried out and uplinked to the website. Database of PhD theses and peer –reviewed publications of RGCB covering the period 2013-2014 was updated. A Catalogue of library documents was made available in Online Public Access Catalogue (OPAC).

AFFILIATION IN NATIONAL & INTERNATIONAL BODIES

- ***Biomed Central*** – *BMC*:- RGCB continued to be a member of BMC and this enabled the publication of three articles during the period.
- ***DeLCON***: - DBT's electronic library consortia provided access to e- journals, e- books and databases.
- ***DELNET***: - Developing Library Network facilitated document delivery services and sharing of e-resources on account of RGCB's membership with delnet.
- ***Turnitin:*** - Anti- plagiarism internet based device was made available in the library to facilitate quick and effective checks to all research works in a fraction of time necessary to scan a few suspects' papers using search engine.

LIBRARY SERVICES

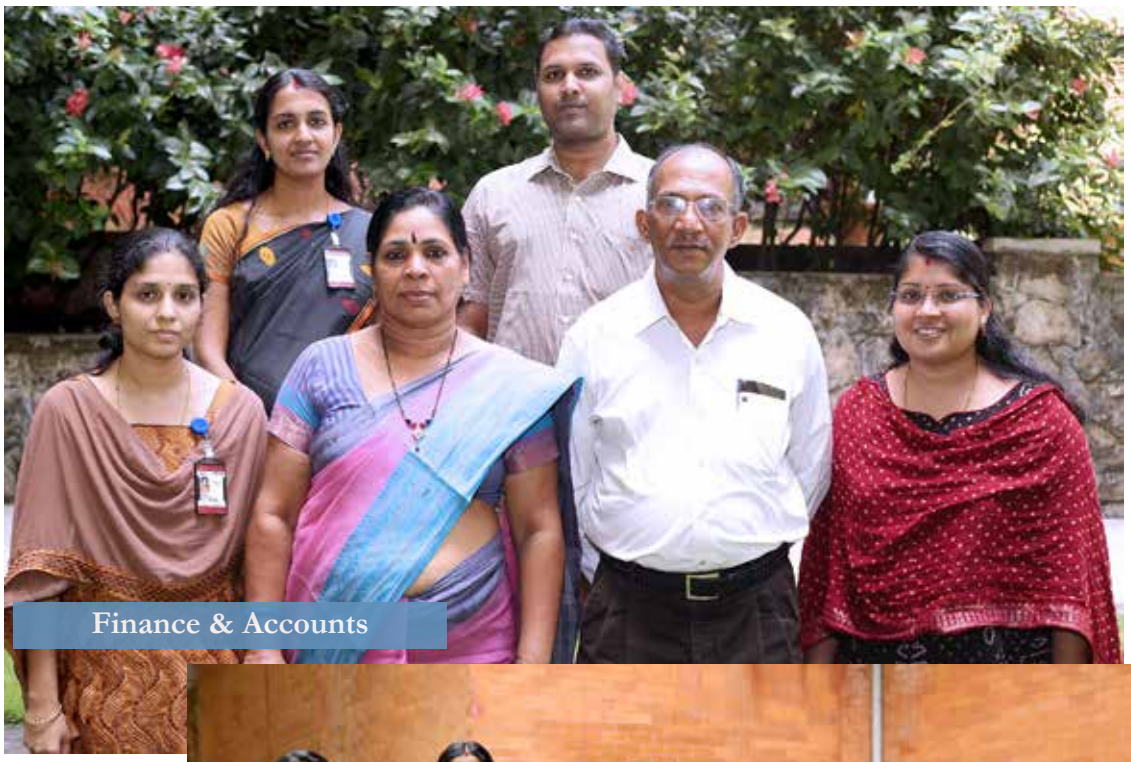
- ***Current Awareness Services:*** - initiated included forthcoming events, daily news, headlines and recent additions on books and periodicals through intranet and display board.
- ***Literature search/ Electronic document delivery:*** - was carried out by responding immediately such request, this being very frequently relied upon by the scientific community.
- ***Selective Dissemination of Information-SDI***: - was used regularly to alerts the users with the latest information available in their on chosen field of study.
- ***Reference and Referral services***: - as usually continued to be a frequently sought after service from the library.
- ***Media clipping***: - from newspapers, journals, magazines, periodicals, web resources etc. were conveniently categorized and displayed in the library for the benefits of users.
- ***Reprographic/printing services***: - provided by the library proved itself to be yet another most sought after service. One color digital photocopier/printer added to reprographic section of the library.

RGCB ADMINISTRATION

The RGCB Administration continued to support institute management and development following its motto of ART [Accountability – Responsibility – Transparency]. Officers in the administration and finance have the unenviable task of balancing the demands of scientists who understandably seek extension of the realm of academic freedom into interpretation and enforcement of government mandated procedures. The establishment of the BioInnovation Centre at the Akkulam campus continued to give concern to the Administration. After all tender procedures RGCB identified a Government firm to award the work, but the proposal had to dropped on recommendations of the Building Committee which wanted more options than the two bids that were received. The Administration followed up the work with vigor and the project has been is re-tendered. As in previous years all recruitment and departmental promotion cases were completed in time, as earlier. The RGCB Vigilance and Security Group continued its surveillance procedures. Security system was improved with inclusion of better monitoring and surveillance modern gadgets. Physical security measures were revamped and made more proactive. The Vigilance week 2014 was observed in the right earnest where the employees were impressed upon the relevance of vigilance in the day-to-day activities of the Centre. The group took up a comprehensive safety audit of the entire campus and plugged loop holes. RGCB took up Prime Minister's "Swatch Bharat Mission" in right earnest and all employees voluntarily participated in the cleanliness drive and gave the estate a fresh face-lift. All statutory meetings of the RGCB Society General Body, Finance Committee, Governing Council and Scientific Advisory Council were promptly convened. The transit campus at the Film and Video Park Campus, Kazhakuttom was made fully operational and the Honorable Union Minister of Science and Technology, Dr. Harsh Vardhan dedicated it to the Nation. The Finance and Accounts Division continued to play its vital role and in spite of severe budgetary constraints and cuts helped RGCB accomplish its goals. The Finance Division is justifiably proud, that in spite of the wide gap between the funds available and the projected expenditure, it could meet all essential targets. The Division introduced user-friendly software and better accounting systems so that cheque generation and financial control could be effectively monitored. The Project Management Division continued its valuable support in project monitoring activities. All statutory audits were completed and no major audit paragraphs were raised. The Purchase and Stores wings also gave impressive outputs during the year. The Purchase introduced new pre-vigilance checks on all high-value purchases. All formalities for introducing e-procurement were completed. The Estate Management Division also had a commendable performance during the year. The problems faced by non-availability of parking of employees' vehicles inside the campus were solved to a great extent by identifying additional spaces. The existing parking slots were also given face-lift. Functioning of the cafeteria was thoroughly reviewed to keep up better services and hygiene. All obsolete and unserviceable items were identified and disposed off as per procedures. A new Guest House was set up at Mudavanmughal near RGCB to meet growing demands. Other welfare facilities such as medical benefits for both regular employees and project employees were also reviewed and strengthened.

Director's office

Administration



Finance & Accounts

Stores & Purchase

Transport Group

Project Management Division

Technical Support
BIO-IMAGING FACILITY

RGCB PERMANENT STAFF LIST AS ON 30.06.2015

SCIENTIFIC STAFF

1 Dr. M. Radhakrishna Pillai
Director

2 Dr. C.C. Kartha
Professor of Eminence

3 Dr. Sathish Mundayoor
Scientist G

4 Dr. Pradeep Kumar G.
Scientist G

5 Dr. R.V. Omkumar
Scientist F

6 Dr. Malini Laloraya
Scientist F

7 Dr. Moinak Banerjee
Scientist F

8 Dr. Santhoshkumar K.
Scientist F

9 Dr. Ruby John A.
Scientist F

10 Dr. George Thomas
Scientist F

11 Dr. Ajaykumar R.
Scientist E II

12 Dr. V.V. Asha
Scientist E II

13 Dr. Soniya E.V.
Scientist E II

14 Dr. Suparna Sengupta
Scientist E II

15 Dr. T.R. Santhoshkumar
Scientist E II

16 Dr. Priya Srinivas
Scientist E II

17 Dr. S. Asha Nair
Scientist E II

18 Dr. Abdul Jaleel K.
Scientist E II

19 Dr. Jackson James
Scientist E II

20 Dr. Sabu Thomas
Scientist E II

21 Dr. Radhakrishnan R.
Scientist E I

22 Dr. Sanil George
Scientist E I

23 Dr. Sreeja S.
Scientist E I

24 Dr. Manjula S.
Scientist E I

25 Dr. E. Sreekumar
Scientist E I

26 Dr. G.S. Vinodkumar
Scientist E I

27 Dr. Harikrishnan K.
Scientist E I

28 Dr. Mayadevi M.
Scientist C

29 Dr. Rashmi Mishra
Scientist C

30 Dr. Harikumar K.B.
Scientist C

31 Dr. Debasree Dutta
Scientist C

32 Dr. Laishram Rakesh Singh
Scientist C

33 Dr. John Bernet Johnson
Scientist C

34 Dr. S. Rajakumari
Scientist C

TECHNICAL STAFF

1 S. Mohanan Nair
Chief General Manager

2 Shaj Upendran
Sr. General Manager (Instrumentation)

3 R. Jayachandran Nair
Deputy General manager

4 Jiji V.
Sr. Manager (Technical Services)

5 George Varghese
Sr. Manager (Technical Services)

6 Sanjai D.
Sr. Manager (Technical Services)

7 Lathika K.
Librarian

8 Manoj P.
Manager (Technical Services)

9 Rajasekharan K.
Manager (Technical Services)

10 Dr. S. Santhoshkumar
Veterinarian & Animal House in-charge

11 M. Saravanakumar
Manager (Technical Services)

12 Indu Ramachandran
Manager (Technical Services)

13 Laiza Paul
Manager (Technical Services)

14 Sudha B. Nair
Manager (Technical Services)

15 Bindu Asokan
Manager (Technical Services)

16 Ciji Varghese
Manager (Technical Services)

17 Ambili S. Nair
Manager (Technical Services)

18 Rahul C. S. Nair
Senior Technical Officer

19 Sajan I.X.
Senior Technical Officer

20 Ajithkumar S.
Deputy Engineer (Electrical)

21 C. Durga Prasad
Deputy Engineer

22 Lekshmi R.
Technical Officer

23 K.C. Sivakumar
Technical Officer

24 Arun Surendran
Technical Officer

25 Rintu T Varghese
Technical Assistant

26 G. Johny
Technical Assistant

27 G. Sheela
Technical Assistant

28 Unnikrishnan V. R.
Technical Assistant

29 Antony K. P.
Technical Assistant

30 Edwin S.
Technical Assistant

31 Velthai G.
echnical Assistant

32 Rajeev S.
Technical Assistant

33 Biju S. Nair
Technical Assistant

34 S. Santhosh
Technical Assistant

35 Meera N. V.
Library Assistant

36 Aswanikumar S.
Technical Assistant

37 Prakash R.
Laboratory Technician

38 Kannan T.R.
Laboratory Technician

39 Amal V.
Technical Assistant

40 Dileep Kumar R.
Technical Assistant

41 Reena Prasad
Technical Assistant

42 Gopakumar G.
Helper

43 Venugopalan J.
Helper

44 K.A. Vinod Lal
Helper

45 Jayanandan J.
Helper

46 Sumaja V.
Helper/Lab Helper

ADMINISTRATION

1 K.M. Nair
Chief Controller

2 Dr. Ashok R.
Registrar Grade II

3 M.Babu
Finance Officer

4 Jeevan Chacko
Chief Manager (Purchase)

5 K.K. Jayasree
Accounts Officer

6 Suthakumari S.
Administrative Officer

7 V. K. Reghukumar
Senior Manager (Security & Vigilance)

8 Jayakrishnan N.
Senior Manager (Purchase)

9 R. Kumar
Manager (Accounts & Audit)

10 Jayalekshmi U. S.
Sr. PS to Director

11 Priya R.
Sr. PS to Director

12 Asha R. Nair
Asst. Administrative Officer

13 O.Girijakumari
Private Secretary

14 Preetha J.
Section Officer

15 Subash K.
Office Assistant

16 Anilkumar R.
UDC

17 Harikumar S.
Driver

18 Vijayakumar S.
Driver

19 T. Wilson
Senior Attendant

20 Chandrika Devi B.
Senior Attendant

21 Thapasi Muthu Nadar C.
Attendant

22 Usha B.
` Attendant Grade

23 Vinodkumar S. R.
Attendant Grade

24 Thankamany R.
Attendant Grade

25 Manukumar V. M.
Driver

6th World Ayurveda Congress & AROGYA Expo – 2014

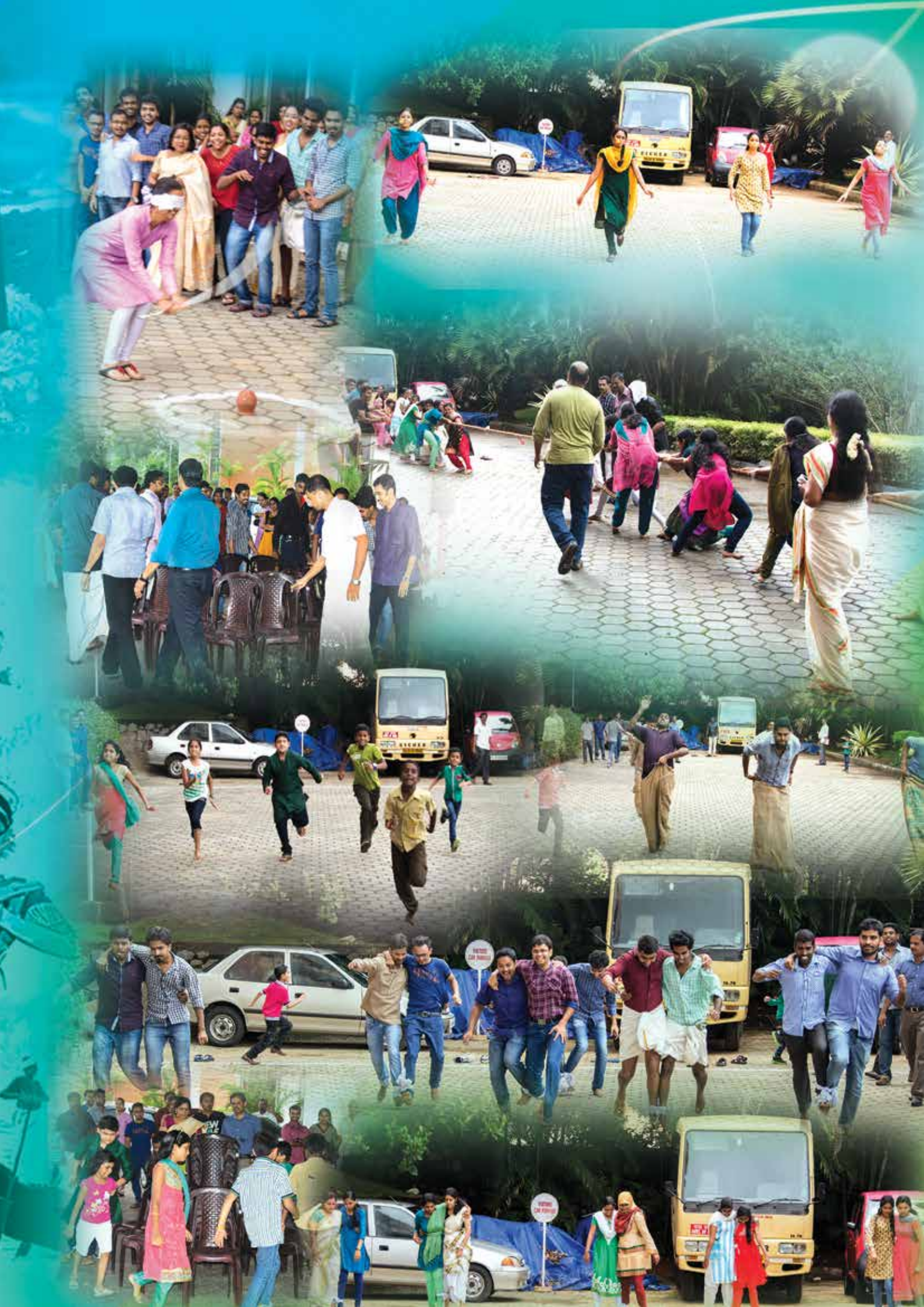
General body 2014

General Body 2014

Next Generation Sequencing Data Analysis Workshop – 2014

RGCB
Srinivasa Ramanujan
Institute MOU
for Joint Research Program

Onam Celebrations



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