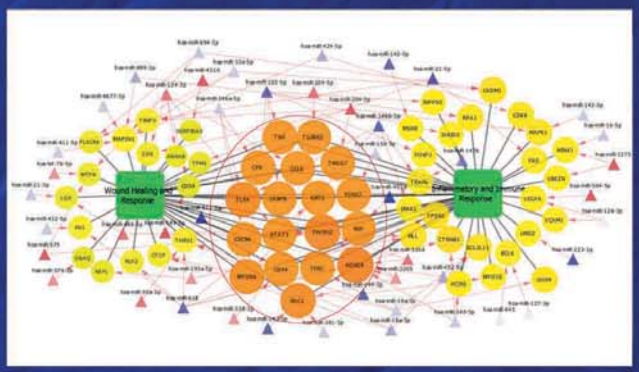
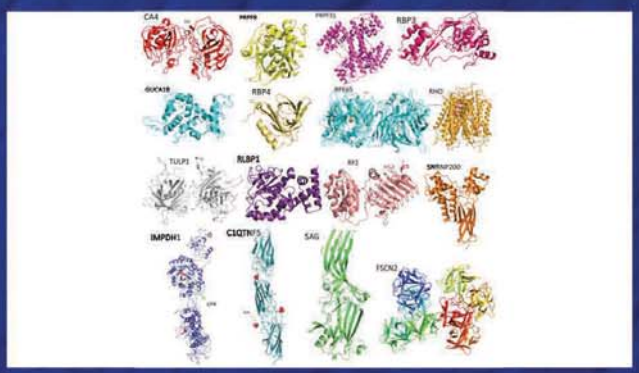
RESEARCH IN OPHTHALMIC SCIENCES

ARAVIND MEDICAL RESEARCH FOUNDATION





ANNUAL REPORT 2014-2015

Aravind Medical Research Foundation

Aravind Medical Research Foundation is recognized as
Scientific and Industrial Research Organization (SIRO) by
the Department of Scientific and Industrial Research (DSIR)

MISSION

To eliminate needless blindness by providing evidence through research and evolving methods to translate existing evidence and knowledge into effective action.

RESEARCH IN OPHTHALMIC SCIENCES

Dr. G. Venkataswamy Eye Research Institute

Annual Report 2014 - 2015

Aravind Medical Research Foundation

Much has been done, but much remains to be done… we look to the future with renewed strength to continue the mission of providing quality eye care and hope that some of what we have learned will be useful to other eye care workers around the world.

G. Venkataswamy

RESEARCH IN OPHTHALMIC SCIENCES

Dr. G. Venkataswamy Eye Research Institute

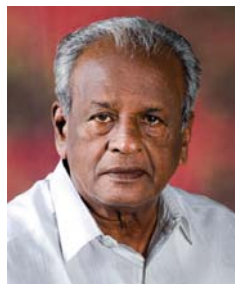
Annual Report 2014 - 2015

ARAVIND MEDICAL RESEARCH FOUNDATION

BOARD OF MANAGEMENT

Dr. P. Namperumalsamy, MS, FAMS

Dr. G. Natchiar, MS, DO



Er. G. Srinivasan, BE, MS

Mr. R.D. Thulasiraj, MBA

Dr. S.R. Krishnadas, DO., DNB

Dr. R. Kim, DO., DNB

Dr.N.Venkatesh Prajna DO., DNB., FRCophth

Dr. S. Aravind, MS, MBA

RESEARCH ADVISORY COMMITTEE

Chairman

Dr. P. Namperumalsamy
Chairman – Emeritus
Aravind Eye Care System
1, Anna Nagar, Madurai - 625 020

Member-Secretary

Prof. K. Dharmalingam
Director - Research
Aravind Medical Research Foundation
1, Anna Nagar, Madurai - 625020

Members

Prof. VR. Muthukkaruppan
Advisor-Research
Aravind Medical Research Foundation
1, Anna Nagar, Madurai -625020

Dr. C. Mohan Rao
Director
Centre for Cellular & Molecular Biology, Uppal Road,
Hyderabad 500 007

Dr. Kumaravel Somasundaram
Professor
Microbiology and Cell Biology
Indian Institute of Science, Bangalore - 560 012

Dr. R. Kim
Chief Medical Officer
Aravind Eye Care System
No.1, Anna Nagar, Madurai - 625020

Dr. N. Venkatesh Prajna
Chief-Medical Education
Aravind Eye Care System
1, Anna Nagar, Madurai -625020

Dr. D. Karunagaran
Professor & Head
Department of Biotechnology
Indian Institute of Technology (Madras), Chennai

Prof. Anuranjan Anand
Faculty
Human Genetic lab
Jawaharlal Nehru Centre for Advanced Scientific Research
Jakkur, Bangalore 560 064

INSTITUTIONAL ETHICS COMMITTEE (IEC)

Chairman

Ms. Shobhana Ramachandhran m.a
Managing Director
TVS Sri Chakra Ltd, Madurai

Member-Secretary

Dr. Lalitha Prajna Md Dnb
Chief Microbiologist
Aravind Eye Hospital, Madurai

Members

Dr. C. Srinivasan m.sc.,ph.d
UGC Emeritus Professor
New No. 2/249 (Old No 2/172)
7th Street, Kalvinagar, Rajambadi, Madurai- 625 021

Dr. T.S. Chandrasekaran Ms Do
Ophthalmologist,
No.6, N.M.R.Subbaraman Road,
Chokkikulam North, Madurai

Mr. M. Senthilkumar m.a., b.l
Advocate
Plot No.32, Sindanayalar Nagar
Senthamil Nagar, Karuppayoorani East, Madurai

Mr. R. Raja Govindasamy m.a., m.a (usa)
Former Principal, Thiagarajar College
169-1, 2nd Cross Street, I Main Road, Gomathipuram,
Madurai - 625020

Dr. S. Sabhesan Dpm Mnams Phd
Consultant – Psychiatrist, Apollo Specialty Hospitals
Lake view Road, K.K.Nagar, Madurai

Mr. ARM. Ganesh b.com llb
Advocate
D-3/4, First Cross Street, K.K.Nagar West, Madurai - 20

Dr. J.R. Vijayalakshmi md (Pharmacology)
Professor of Pharmacology
CSI College of Dental Sciences, Madurai

Prof. R. Venkataratnam m.a., phd
Senior Prof.of Sociology (Retired)
Madurai Kamaraj University, Madurai

Dr. A. Amirtha Mekhala bds mph mfdsrcps
Private Practice
208 Karpaga Nagar 8th Street, K.Pudur, Madurai - 625007

Dr. R. Sharmila dnb
Medical Officer, Glaucoma Clinic
Aravind Eye Hospital, Madurai - 625020

FACULTY

Prof. K. Dharmalingam
Director - Research &
DBT Distinguished Biotechnology
Research Professor

Prof. VR. Muthukkaruppan
Advisor-Research

Dr. P. Sundaresan
Senior Scientist
Molecular Genetics

Dr. C. Gowri Priya
Scientist, Immunology &
Stem Cell Biology

Dr. S. Senthilkumari
Scientist, Ocular Pharmacology

Dr. A. Vanniarajan
Scientist, Molecular Genetics

Dr. J. Jeya Maheshwari
Scientist, Proteomics

Dr. D. Bharanidharan
Scientist, Bioinformatics

Dr. M. Vidyarani
Scientist, Microbiology

CLINICIAN-SCIENTISTS

Dr. P. Vijayalakshmi, MS
Professor of Ophthalmology &
HOD - Paediatric Services

Dr. S.R. Krishnadas,
DO., DNB
Professor of Ophthalmology
& HR - Director

Dr. SR. Rathinam,
MNAMS, PH.D
Professor of Ophthalmology &
HOD - Uvea Services

Dr. Haripriya Aravind, MS
HOD - IOL & Cataract Services

Dr. R. Kim, DO., DNB
Professor of Ophthalmology &
Chief Medical Officer

Dr. N. Venkatesh Prajna
DO., DNB., FRCophth
Professor of Ophthalmology &
HOD - Cornea Services

Dr. Usha Kim, DO, DNB
Professor of Ophthalmology
& HOD - Orbit, Oculoplasty &
Ocular Oncology Services

Dr. Lalitha Prajna,
MD., DNB
HOD - Microbiology

Dr. R. Santhi,
MD (Pathology)
Pathologist

CONTENTS

FOREWORD

Aravind Medical Research Foundation is the research arm of Aravind Eye Care System (AECS). In consonance with the ideals of Aravind Eye Care System, the focus of AMRF is improving eye care through research and development, which will ultimately help millions of visually impaired people.

The faculty of AMRF works closely with clinician scientists of AECS and this collaboration has resulted in several unique discoveries in fundamental science and clinical practice. The institute is now exploring ways and means to facilitate this cross-talk among the basic science groups and clinicians. Since Aravind facilities are physically closely located, it is easy to improve the interaction. Encouraging post graduate medical students to take up time bound research projects at AMRF is another approach that will help creation of research oriented clinician pool.

Being a research organization, its financial support comes from AECS and government granting agencies. The organization also gets financial support through competitive grants. However, in order to take up really challenging long term projects, the organization needs additional support. To achieve this, it is approaching those who are interested in being partners in its endeavour to improve Indian eye care through innovative research.

Armed with the state-of-the-art infrastructure and a dedicated band of scientists, the institute is one of the preferred sites for ophthalmic research. Being a recognized centre for Ph.D programmes, it inculcates positive attitude among the younger generation towards research in ophthalmic science. It is appropriate time that extra efforts are taken to develop young scientists in every field, ophthalmic basic and clinical sciences where there is huge potential and opportunities for research.

Research is one of the priority areas in India that needs intense and active collaboration of various disciplines,expertise, technology, innovative techniques and financial support.

Dr. P. Namperumalsamy
President, AMRF

INTRODUCTION

Analysis of single nucleotide variations (SNV) allows the identification of mutations that are associated with a specific disease. Bioinformatics group developed methods to identify SNVs in selected eye diseases. They also developed pipelines for the analysis of exome sequence data and whole bacterial genome sequence data. This group collaborates with experimental scientists and this collaboration has resulted in several intergroup publications. The high performance computational facility helped in this effort.

Stem cell research has undertaken elaborate studies on the identification of corneal epithelial stem cells. They also optimized protocols for the expansion and maintenance of stem cells for use in translational applications. GMP facility and confocal microscopy facility helped in their studies.

Ocular microbiology group has several programmes on the analysis of *Pseudomonas* causing keratitis. They have undertaken whole genome sequencing and other approaches for the identification of virulence factors including secretion systems. Acanthamoeba keratitis is another area this group is actively working on. Studies on the immune pathogenesis of infectious uveitis have resulted in significant progress in the understanding of the disease.

Ocular pharmacology division focused on the analysis of the mechanism of VEGF secretion in cell lines in order to understand the mechanism of new vascularization in diabetic retinopathy. This group also studies the role of macular carotenoids in Age Related Macular Degeneration (AMD).

Genetics group focused mainly on the application of genome wide association studies in the identification of disease associated mutations in Indian populations. Primary Open Angle Glaucoma (POAG), Primary Angle Closure Glaucoma (PACG), Coloboma, Leber's Congenital Amaurosis (LCA) are some of the diseases. The analysis of retinoblastoma (RB) genetics led to some important finding that could be used in the early prediction of RB in children.

Proteomics group is carrying out research on various aspects of *A.flavus keratitis* to understand the disease mechanism with a focus on the host-pathogen interactions. Discovery phase studies are underway using infected tissues such as tear and cornea to identify prognostic biomarkers. These putative biomarkers will be taken for validation using the newly acquired Triple Quad Mass spectrometer. Another important disease this group is focusing on is Diabetic Retinopathy. Proteomics studies are carried out to identify biomarkers that can predict the subset of diabetes patients who most likely would develop microvascular complications in the retina.

Prof. K. Dharmalingam
Director - Research

MOLECULAR GENETICS

Currently, the department is working on molecular genetics of various eye diseases. Prevalence of these eye diseases (Cataract, Diabetic retinopathy, Glaucoma and retinal dystrophies) is high in Indian population. The department has given priority to study the mitochondrial genes involvement in the pathogenesis of Diabetic Retinopathy and Leber's Hereditary Optic Neuropathy (LHON). It has also taken steps to determine the levels of cytokines in the aqueous humour of Primary Angle closure Glaucoma patients. In addition, the role of recently reported candidate genes of Primary Open Angle Glaucoma is being studied to understand the pathogenesis. The department has demonstrated the usefulness of its new strategy for rapid and cost-effective genetic testing of retinoblastoma. Next Generation Sequencing analysis of retinoblastoma was extended to include both the single nucleotide variants and copy number variants.

Mitochondrial genes involvement in Leber's Hereditary Optic Neuropathy (LHON)

Investigators	:	Dr. P. Sundaresan
		Dr. S. Mahesh Kumar, Aravind Eye Hospital, Madurai
PhD scholar	:	Bibhuti Ballav Saikia
Funding agency	:	DST, CSIR (SRF)

Background and aim

Leber's Hereditary Optic Neuropathy (LHON) is the first reported mitochondrial disorder caused due to point mutations present in the mitochondrial genome. The three point mutations: m.3460G>A, m.11778G>A and m.14484T>C, affect complex I subunit of the Electron Transport Chain (ETC) in mitochondria called as primary mutations due to its high prevalence (95%) in the European population.

Apart from these primary mutations, there are other factors like haplogroup, where mitochondrial heterogeneity has been reported, but there were no clear evidences. LHON patients can be screened for the presence of three primary mutations along with other mtDNA variants by sequencing of whole mitochondrial genome. This study will help in finding the factors involve in the clinical expression of LHON.

In this study, seventy five LHON affected individuals and seventy five age matched controls have been recruited. Long PCR based strategy has been implemented to screen whole mitochondrial genome of patients as well as control individuals. The sequences were analysed against rCRS (revised Cambridge Reference Sequence). Three primary mutations: m.3460G>A, m.11778G>A and m.14484T>C were identified in LHON patients. These three primary mutations were not identified in the control individuals. Apart from these variations, some other mtDNA variants; haplogroup (fig) specific variants and some already reported disease associated variants have also been identified in both patients and control individuals.

The phylogenetic analysis of mtDNA based haplogrouping of the LHON patients revealed that they fell in different haplogroups.

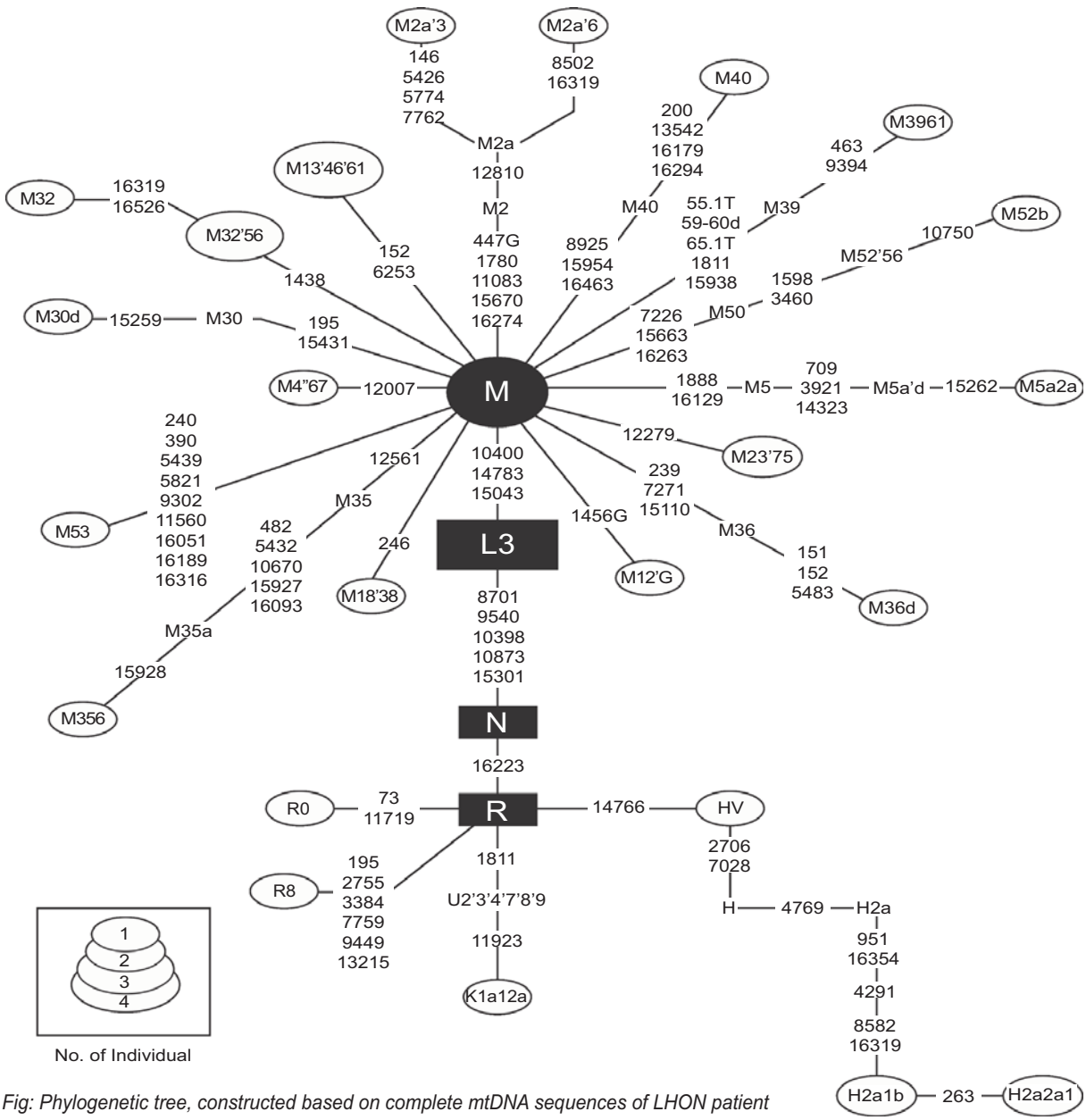


Fig: Phylogenetic tree, constructed based on complete mtDNA sequences of LHON patient

Implication of the project

The prevalence of primary mutations is very less in south Indian population compared to European population. The presence of other mtDNA variants may have influence on the pathophysiology of LHON. Influence of haplogroup in the clinical expression of LHON is inconclusive because of the less sample size. Further experiments are needed to confirm the influence of haplogroup in LHON patients.

Molecular genetic studies on Primary Angle Closure Glaucoma (PACG)in south Indian population

Investigators	:	Dr. P. Sundaresan, Dr. R. Krishnadas, Dr. George V. Puthuran, Aravind Eye Hospital, Madurai Dr. R. Venkatesh, Dr. Kavitha Srinivasan, Aravind Eye Hospital, Pondicherry Dr. Pradeep Ramulu, Johns Hopkins Hospital, USA Dr. Robert Wojciechowski, Johns Hopkins Bloomberg School of Public Health, Wilmer Eye Institute, Johns Hopkins School of Medicine, USA
Ph.D scholar	:	Roopam Duvesh
Funding agency	:	Aravind Eye Care System

Background and aim

Primary angle closure glaucoma (PACG) is a heterogeneous disorder, resulting from an appositional contact between peripheral iris and trabecular meshwork, making drainage angle occluded. This could lead to an increase in intra ocular pressure (IOP) and thus damage to the optic nerve and eventually blindness. It is less common than primary open angle glaucoma (POAG), though it accounts for nearly 50% of glaucoma-related blindness, especially in Asians.

PACG is a complex disease with several genetic, environmental, anatomical and physiological factors involved in its pathogenesis. Also, many studies have suggested a genetic basis for PACG. Previously,three SNP markers: rs11024102 in PLEKHA7, rs3753841 in COL11A1 and rs1015213 located intergenically between PCMTD1 and ST18genes were screened which have been reported with PACG. Genotyping of cases and control groups was performed by Taqman real time allelic discrimination assay and the association of each of these SNPs was determined in South Indian population. Significant genetic association was identified for rs1015213 (PCMTD1-ST18) in the PAC/PACG (p= 0.004) (Duvesh R et al, Invest OphthalmolVis Sci. 2013). Apart from this, SNP's (rs17576 & rs3918254) of Matrix metalloproteinase-9 (MMP9) gene are also being evaluated for association using PCR-RFLP and Sanger sequencing methods.

Many studies have reported altered aqueous humor concentrations of cytokines, chemokines and growth factors in various ocular diseases such as diabetic retinopathy, age-related macular degeneration (AMD) and glaucoma. But, very limited knowledge is present in the case of PACG. Therefore, the department is also interested in determining the aqueous cytokine expression levels of Primary Angle Closure Glaucoma patients (n=19) as compared to cataract controls (n=14). Aqueous humor samples were collected at the beginning of cataract and glaucoma surgery and were immediately stored at -80˚C until the analyses were performed. A multiplex bead immunoassay (Bio-Plex cytokine assay; Bio-Rad Laboratories) was used to determine the concentration of 27 cytokines, chemokines and growth factors (27-plex human panel). The plate was read by Bio-Plex array reader (Bio-Rad). Representation of standard curve showing values for standards and unknown samples is depicted in fig.1. Overall, there is significant increased concentration of IL-8 (37.3 ± 38.3; p=0.0013), Eotaxin (12.36 ± 5.0; p=0.00056) and MIP-1β (53.24 ± 27.4; p=0.0001) in cases when compared to control group (IL-8 11.6 ± 4.1; Eotaxin8.0 ± 1.0; MIP-1β 22.39 ± 8.7). Eotaxin and IL-8 is a chemokine with IL-8 is known to be involved

in pro-inflammatory response. Particularly, MIP-1β (Macrophage Inflammatory Protein-1β) is also a pro-inflammatory chemokine, showed more than two-fold increase in case group. Studies will be done to elucidate that how these factors could be involved in PACG pathogenesis. As we know that the aqueous turnover rate is very fast and it can influence the levels of aqueous cytokines. So, we also checked all these levels in different subgroups (i.e. Patients undergone Trabeculectomy vs. Phacotrabeculectomy& Patients with high IOP vs. Low IOP). Concurrently, all above mentioned three cytokines showed significance in respective subgroups ($p<0.05$).

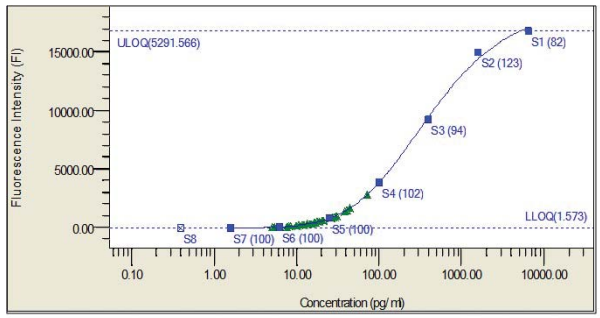


Fig. 1: **Standard curve representation**

Implication of the work

This study will help us to understand the involvement of genetic components in PACG susceptibility and determining and studying the role of aqueous cytokines levels in pathogenesis of angle closure glaucoma

Genetic and functional approaches to understand the pathogenicity of Primary Open Angle Glaucoma (POAG)

Investigators : Dr. P. Sundaresan, Dr. S.R. Krishnadas
Ph.D scholar : Mohd Hussain shah
Funding agency : Aravind Medical Research Foundation

Background

Primary open angle Glaucoma is a neurodegenerative disease characterized by the progressive loss of retinal ganglion cells, optic nerve degeneration and visual field loss, eventually blindness and is a complex and genetically heterogeneous disease. It is the second leading cause of irreversible blindness in the world. By the year 2020, it is estimated that approximately 80 million people will be affected worldwide.

The obstruction of the Trabecular meshwork (TM) – the aqueous humor outflow pathway - is the major cause of increase in IOP in open angle glaucoma. Many studies suggested an autosomal dominant inheritance with incomplete penetrance. However, the inheritance pattern of this disorder seems to be multifactorial resulting from the interaction of one or more genes and/or environmental stimuli. Although more than 26 genetic loci have been reported to be associated with disease, only 3 genes MYOC (myocilin), OPTN (optineurin), and WDR36 were linked to Primary open Angle Glaucoma (POAG). Recently TBK1 gene has been reported to be involved in NTG; Copy number variations (duplications) that encompass the TBK1 gene were recently shown to be associated with glaucoma through family-based studies. These data suggest that an extra copy of TBK1 leads to NTG and may be responsible for some fraction of sporadic-appearing NTG case.In addition, genome wide-association (GWASs) has allowed the identification of candidate genes such as, CAV1 /CAV2, which are expressed in the trabecular meshwork as well as in the retinal ganglion cells, TMCO1, CDKN2B-AS1, TMCO1 is also expressed in retinal ganglion cells and SIX1-SIX6 , SIX6 are highly expressed in developing eyes.

Aim of the study

The lab has screened some of the reported genes like myocilin, optinurin etc. Results suggested that these gene mutations appear to be less prevalent in primary open angle glaucoma due to heterogeneity

as well as complex pathways involved to understand the pathogenesis of POAG. Currently, the department in the process of screening recently identified known candidate genes by Genome Wide Association Study (GWAS) of POAG.

In addition, one large four generational POAG family with 364 participants is also being studied. Out of the 364 participants, 22 are POAG cases and 20 suspected.

Whole exome sequencing is being performed to identify the novel genes for POAG from patients lacking known gene mutations in Indian population and also In vitro gene expression studies of novel mutations identified from known genes / novel gene associated with Primary Open Angle Glaucoma.

Implication of the project

The study will be helpful in identifying mutations of known genes and increases the causative mutation spectrum of POAG. It is useful to provide genetic counselling and early diagnosis to affected families that will reduce the occurrence of the disease.

Identification of novel genes will provide new approaches to understand genetic pathways involved in POAG, in turn providing potential for novel treatment.

The genetic studies of complex diseases like POAG may provide information for future animal and clinical treatment trials.

Genetic analysis of Retinoblastoma

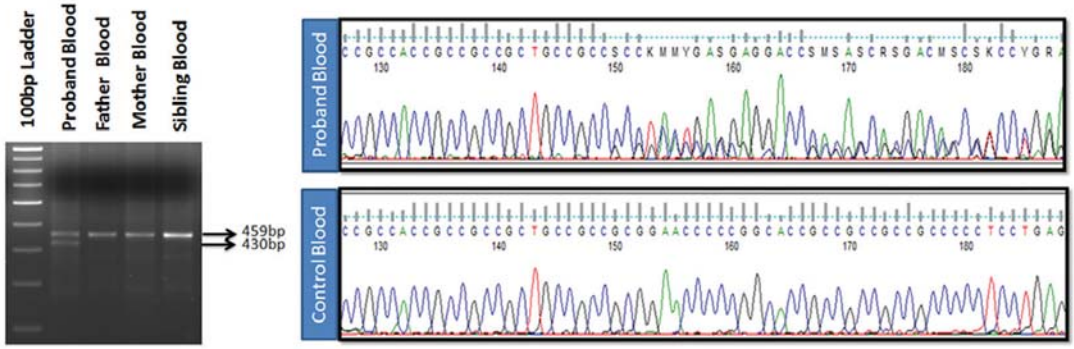
Investigators	:	Dr. A. Vanniarajan, Dr. Usha Kim, Dr. D. Bharanidharan, Prof. VR. Muthukkaruppan
Project fellow	:	Thirumalairaj Kannan and A. Aloysius Abraham
Funding agency	:	Aravind Eye Foundation (USA) and Aravind Medical Research Foundation (AMRF)

Retinoblastoma (RB) is the predominant form of intraocular tumour in children under 5 years of age. In most patients, it is diagnosed in advanced stages and left with little options for treatment. It may be lethal in some cases and vision may be lost in most patients. Nearly 90% of children are the first one in their family to develop retinoblastoma. In 10% of the cases, someone in the family – father, mother, sibling or a grandparent had retinoblastoma. RB is primarily initiated by biallelic inactivation of RB1 gene. Upon identification of mutations in proband, the risk can be predicted for siblings and offspring, thereby enhancing the management of the disease. Only individuals with mutations who are at risk of getting RB require further follow-up. This is cost-effective to the families especially when it eliminates unnecessary ophthalmic surveillance in those who do not have the mutations.

Optimisation of methods for RB1 screening

RB1 gene spans more than 180 kb on chromosome 13q14, which consists of 27 exons with no reported hotspots. The large size of this gene and its multiple dispersed exons increase the time and cost of the analysis. Further complexity is added by the wide spectrum of RB1 mutations that include point mutations, indels, large deletions and duplications. Methods such as karyotyping, Southern blot detect only the loss of RB1 at the chromosome level. Single-strand conformation polymorphism and denaturing high performance liquid chromatography have a low mutation detection rate for point mutations. Quantitative multiplex polymerase chain reaction (QM-PCR) and exon by exon sequencing to get an enhanced mutation detection rate, are time consuming, labour-intensive and expensive.

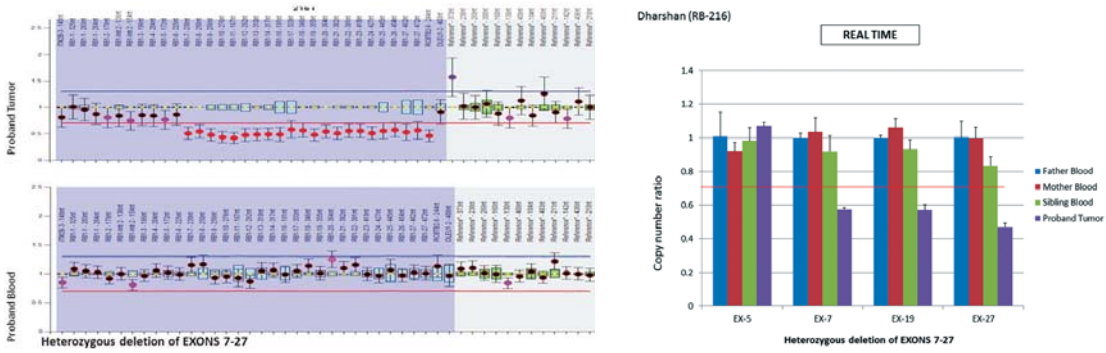
There are a few regions in RB1 such as exon 1 that contain high GC content which is difficult to amplify and sequence. A new primer set was synthesized for this exon and optimized using high fidelity



Deletion of 29bp (CCGCCGCTGCCGCCGCGGAACCCCCGGCA) in exon 1 was identified in proband blood and confirmed by Sanger sequencing

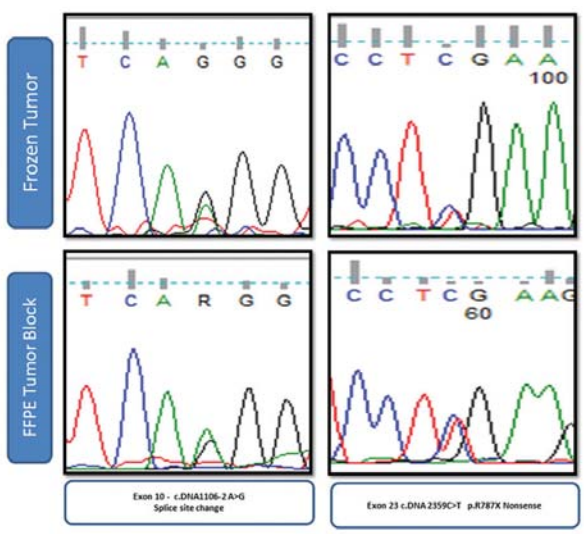
DNA polymerase Phusion (NEB, USA) by Touchdown PCR. The figure below shows the deletion of 29 bases in exon 1 by agarose gel electrophoresis and sequencing in a RB patient.

Multiplex ligation-dependent probe amplification (MLPA) was employed for detecting the deletions and duplications in RB1. This is a sensitive method that utilises the probes of all exons except 15 for deletion/ duplication analysis in a single step. The figure shows the deletion found in a RB patient following the MLPA analysis. The results were further validated using real time PCR.



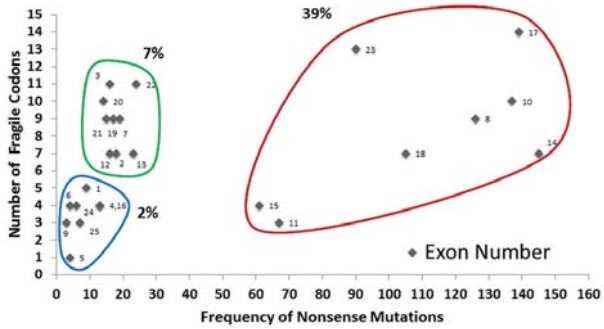
Deletion of exons 7-27 was detected by MLPA in proband tumor and confirmed by real time PCR

Enucleation is performed in most of the RB patients in order to avoid the tumor spread and save the life of the child. However, fresh tumor is accessible only in few cases for molecular analysis. When fresh tumor is not available, formalin fixed paraffin embedded tissue sections were used for DNA isolation, which remained as a challenging task earlier. Optimisation of the paraffin removal followed by the increased tissue digestion with the reagents provided in FFPE DNA isolation kit (Qiagen, USA) gave a good quality DNA for molecular analysis. The figure below shows the traceable amount of two mutations in fresh tumor and FFPE tumor from the same patient.



Two somatic RB1 mutations were identified in fresh tumor DNA of the patient which was also replicated in the DNA isolated from FFPE

Step wise strategy for RB1 mutational analysis



Frequency of nonsense mutations reported in RB1 gene. Each of the exons was plotted based on the frequency of nonsense mutations reported in RB1-Leiden Open Variation Database (X-axis) and the number of fragile codons that can become stop codon by a single base change (Y-axis). Percentage refers to the frequency of nonsense mutations in the above three cluster of exons, among pathogenic variants. There were no nonsense mutations reported in exons 26 and 27.

Currently, the genetic analysis of RB1 remains very expensive and it is carried out in very few labs in the world. The cost is not affordable to many patients, especially in developing countries like India. Therefore, a sensitive method of RB1 screening with reduced time and cost is needed. The department developed a new strategy based on the frequency of nonsense mutations and prioritized the exons for screening in a sequential manner. Nonsense mutations often occur in fragile codons wherein a single base change leads to stop codon. On looking for the fragile codons that frequently get mutated in RB1, it was found out CGA (Arg) fragile codon has higher frequency than other fragile codons. With this precursory look, the number of fragile codons present in each exon of RB1 versus nonsense mutations was analysed.

Accordingly, they were grouped into three clusters as shown in figure below. The cluster containing eight exons (8,10,11,14,15,17, 18 and 23) correspond to 39% of nonsense mutations among the pathogenic variants, thus forming the first step of the strategy. The remaining two clusters of exons with 7% and 2% of nonsense mutations form step III and IV respectively. As deletions and duplications reported in RB1 gene contribute 15-25% of all variants in RB patients, the MLPA analysis of all the exons (except 15) of RB1 was included in the strategy as step II.

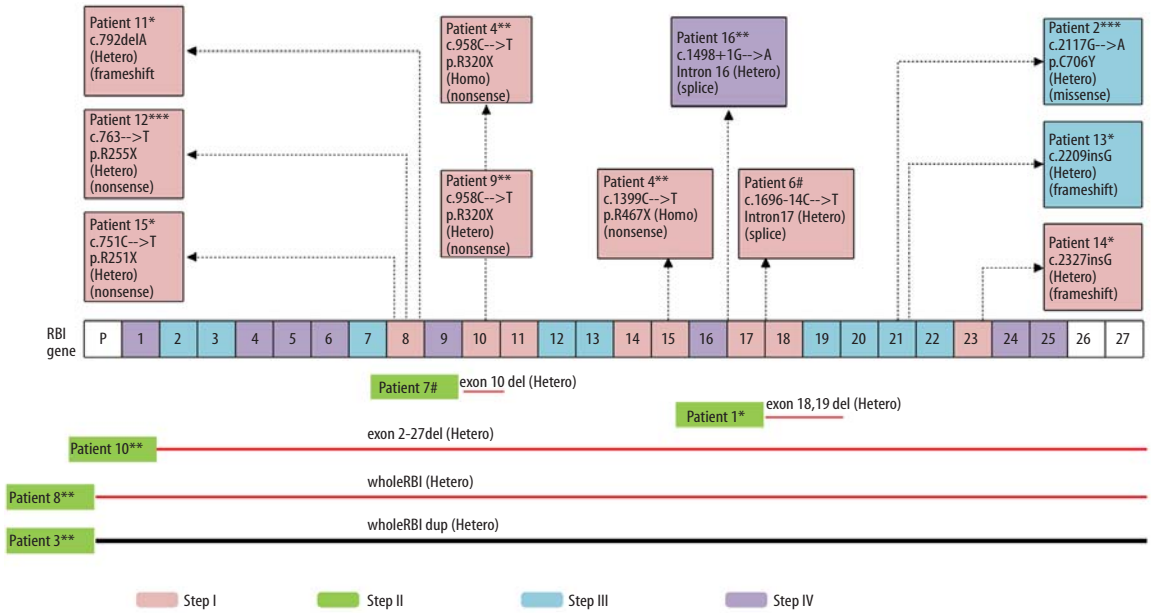
This newly developed screening strategy was validated in 21 RB patients. Mutations were identified in 16 out of 21 patients within 5 weeks. Exons 26 and 27 are not included in the strategy as no nonsense mutations were reported in the database and Indian retinoblastoma patients. The table below shows the order of exons used and also the mutations identified in each step.

Steps	Exons	Mutations identified in patients	Time taken for 21 patients (weeks)
I: Sanger	8, 10, 11, 14, 15, 17, 18, 23	8	2
II: MLPA	All exons except 15	5	1
III: Sanger	2, 3, 7, 12, 13, 19, 20, 21, 22	2	1
IV: Sanger	1, 4, 5, 6, 9, 16, 24, 25	1	1

Mutations were identified in 16 out of 21 patients within 5 weeks. Exons 26 and 27 are not included in our strategy as no nonsense mutations were reported in the database and Indian retinoblastoma patients

With this screening strategy, a mutation detection rate of 76% was achieved. Since the first step could identify mutations in about 50% of patients within two weeks, the screening results could be delivered to clinic faster than the conventional exon by exon screening of RB1. In this strategy, the number of samples for further analysis is sequentially reduced.

With the reduction in consumption of reagents and hands on time, genetic screening can be done with 1/3 of the cost compared to the analysis of all exons. This reduction in cost and time will be very much beneficial for the genetic counselling of patient families especially in developing countries like India.



** Mutation seen in patient's blood*
****Mutation seen in both patient's tumor and blood*

***Mutation seen only in patient's tumor*
#Mutation inherited from father

Spectrum of mutations identified in 16 patients using the strategy

Next Generation Sequencing (NGS)

The major limitations of Sanger sequencing are the extended time taken and limited data (2X) generated from the sequencing runs. Accurate identification of RB1 pathogenic variants within a short time is very important for diagnosis, confirmation, genetic counselling, risk assessment, and carrier screening of RB patients and their family members. Next Generation sequencing (NGS) has been found to be a time-efficient and accurate approach for the molecular diagnosis of simple to complex diseases including cancer. Due to this improved efficiency, NGS has been widely used as diagnostic tool for retinal dystrophies. Therefore to further reduce the cost and time, targeted next generation sequencing approach with in-house bioinformatics pipeline was used for the molecular diagnosis of RB.

Next generation sequencing is ideal for processing large samples in a single run. For validation of targeted NGS of RB1, 33 patient samples were included. A primer library was custom-designed to amplify 27 exons, exon/intron boundaries and promoter region of RB1gene using the Illumina Truseq custom Amplicon and Agilent Sure Select in-solution hybridization capture kits by the service provider (Scigenom, Kochi, India). Briefly, 2 µg of each genomic DNA was sheared into 100-500 bp fragments. Regions of interest were enriched using the above methods and libraries were prepared. The high sensitivity DNA chips were used in Agilent Bioanalyzer, to validate the enrichment process.

Quantitative PCR was used to measure the quantity of the library before sequencing. Captured libraries were sequenced in a multiplexed fashion on Miseq with paired end run to obtain 2x150 bp reads with at least 100X depth of coverage. The coding region with <20X depth of coverage was covered by Sanger sequencing. The data obtained through the targeted NGS is huge making the analysis cumbersome. Therefore, an in-house pipeline was developed for the analysis using various available tools specific for tumor and blood.

Somatic variants were detected in tumor samples of 7 out of 11 patients with unilateral RB and no family history as shown in table below. Homozygous variants were identified in 4 patients that include three nonsense variants and one novel frameshift variant. Two heterozygous variants were identified in other 3 patients. All the somatic variants and zygosity were confirmed by Sanger sequencing in patient tumor and blood samples.

Patient ID	cDNA Change	AAChange	Functional Consequence
RB8	c.940-2 A>G/ c.2359C>T	p.R787X	Altered splicing/Premature Protein Termination
RB9	c.380G>A /c.1363C>T	p.S127N /p.R455X	Altered splicing-Missense/ Premature Protein Termination
RB10	c.763C>T*	p.R255X*	Premature Protein Termination
RB12	c. 1072C>T*	p.R358X*	Premature Protein Termination
RB22	**c.1731-1732 Ins T***	**p.K577fs***	Frameshift
RB29	c.1654C>T*	p.R552X*	Premature Protein Termination
RB31	c.409 G>T /c.751 C>T	p.E137X /p.R251X	Premature Protein Termination

*RB1 Variants identified in tumor samples from patients with unilateral Retinoblastoma through targeted next-generation sequencing (NGS). Variants were confirmed as somatic events with no family history and no variants identified in family and patient blood samples through Sanger sequencing and targeted-NGS. Novel variants are marked in bold and homozygous variants are marked with**

Pathogenic variants were identified in blood samples of 15 patients, of which eight were novel and seven were previously reported as listed in the table given below. Surprisingly, all the reported pathogenic variants were found to be nonsense variants, resulting in premature protein termination. Five of them were shown to be de novo as only the patient had the mutation and not the family members. Two of them were inherited from one of their parents. The novel pathogenic variants either caused aberrant splicing or frameshift due to deletions.

Patient ID	cDNA Change	AAChange	Functional Consequence	Co-segregation
RB1	**c.-212_-195del**		Promoter Deletion	Heterozygous Father
RB2	c.1399C>T	p.R467X	Premature Protein Termination	Heterozygous Father
RB4	**c.265-9T>A**		Altered splicing	Heterozygous Mother and Sibling
RB11	**c.46_74del**	**p.16_25del**	Frameshift	Heterozygous Mother and all Siblings[a]
RB13	c.751C>T	p.R251X	Premature Protein Termination	De novo
RB14	**c.2520+4 A>G**		Altered splicing	Heterozygous Father
RB15	**c.2114_2117del**	**p.705_706del**	Frameshift	Heterozygous Father
RB16	c.1363C>T	p.R455X	Premature Protein Termination	De novo
RB17	**c.1960+2T>A**		Altered splicing	Heterozygous Father and Sibling
RB18	**c.38_66del**	**p.13_22del**	Frameshift	De novo
RB19	c.1399 C>T	p.R467X	Premature Protein Termination	Heterozygous Mother
RB24	**c.1961_1963del**	**p.654_655del**	Altered Splicing	De novo
RB25	c.1072C>T	p.R358X	Premature Protein Termination	De novo
RB26	c.521T>A	p.L174X	Premature Protein Termination	De novo
RB27	c.160G>T	p.E54X	Premature Protein Termination	De novo

RB1 Variants identified in blood samples from patients with bilateral/unilateral Retinoblastoma through targeted next-generation sequencing (NGS). Variants in boldface are novel. Co-segregation was analysed through Sanger sequencing.; [a]Variants were also confirmed through Targeted-NGS for one sibling with bilateral RB

ExomeCNV and Cn.MOPS were used to detect somatic and germline CNVs in tumor and blood samples respectively. All the pathogenic variants were further confirmed by conventional methods and co-segregation. Somatic events were re-confirmed by their absence in the same patient blood sample.

Patient ID	Chr. Start	Chr. End	CNV	logR	Exon	MLPA Confirmation
ExomeCNV						
RB27	48941631	48941739	**Deletion**	-5.4	10	Yes
RB40	48934153	49054207	Deletion	-1.1	7-27	Yes
Cn.MOPS						
RB3	48877913	49054207	Deletion	-1.0	1-27	Yes
RB5	48919217	48923159	Deletion	-1.0	4-6	Yes
RB7	49039135	49039247	Deletion	-1.0	22	No
RB7	49047497	49050980	Deletion	-5.5	24-25	Yes
RB8	48941630	4894768	Deletion	-1.2	10-12	Yes

Copy number variations (CNVs) in tumor/blood sample from patients with Retinoblastoma. Exome CNV used to identify somatic CNVs from tumor/blood pairs, while Cn.MOPS for germline CNVs from only blood samples. CNVs identified were confirmed and analysed for co-segregation using MLPA. Homozygous CNVs are marked in boldface.

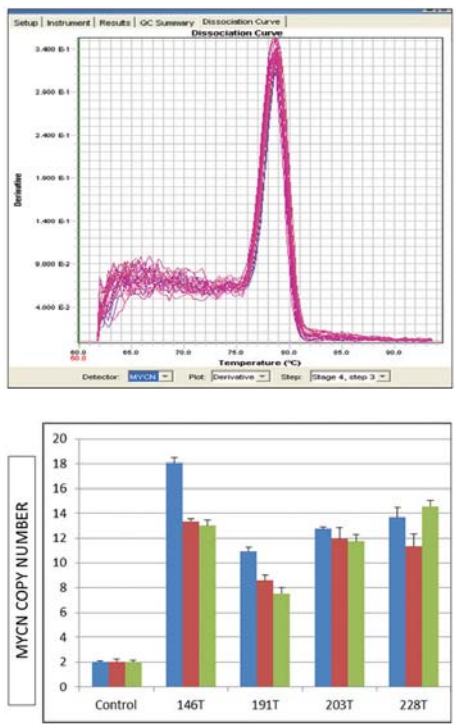
This approach using targeted NGS approach with bioinformatics pipeline could detect germline and somatic variants including novel pathogenic variants. This approach could also detect copy number variations (CNVs) along with the SNVs in RB1 gene. This comprehensive approach reduces the time and number of assays required for the pathogenic variants by conventional methods.

MYCN analysis

It has been recently postulated that RB may also be caused by genetic alterations other than RB1 mutations. Amplification of MYCN (v-myc avian myelocytomatosis viral-related oncogene, neuroblastoma-derived) gene, located on chromosome 2p24.3 was suggested to be involved in the pathogenesis of retinoblastoma, neuroblastoma and several other neuroectodermal cancers. Its role as an oncogene was supported by studies showing amplification occurred in advanced metastatic stages of neuroblastoma.

Analysis of MYCN copy number was carried out in tumor samples with no RB1mutations, using Sybr green chemistry by realtime PCR. The primer concentration and conditions were standardised for optimal amplification of MYCN as shown by the dissociation curve given below. The copy number analysis of MYCN was carried out by absolute quantification in 19 tumor samples that were selected randomly. Out of these, three samples had high copy number of MYCN (>10 copies) as given below and the results were further confirmed by repeating it three times.

The amplification of MYCN was observed in one sample where RB1 mutation was not identified. Hence it is now clear that more genes are involved in the pathogenesis of retinoblastoma.



Dissociation curve showing optimisation of MYCN primers and the amplification of MYCN with copies higher than 10 was observed in three tumors

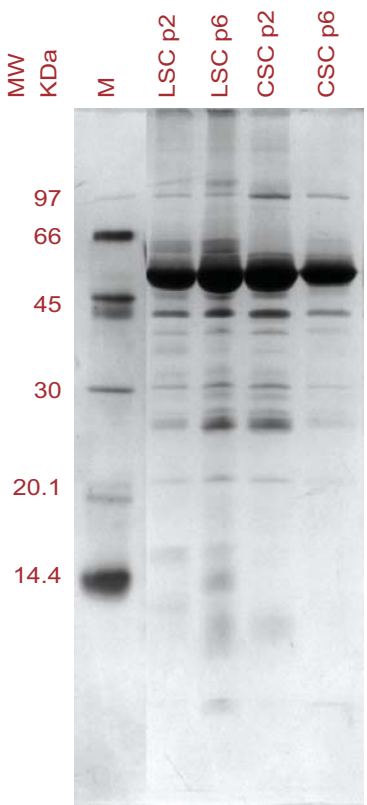
STEM CELL BIOLOGY

The main focus of research is on limbal epithelial stem cells that help in maintaining the corneal epithelial homeostasis. The department earlier established (i) a two parameter analysis (high p63 expression in cells with high nucleo-cytoplasmic ratio) as a specific method for their identification and quantification (since there are no single specific marker), (ii) a protocol for ex-vivo expansion of autologous limbal/ buccal epithelial stem cells and (iii) transplantation of such stem cell rich bioengineered epithelium in patients with limbal stem cell deficiency with 25% success. The current focus of research is to understand the components of limbal stromal niche and to assess the damage to limbal stroma as a result of injury, which will play an important role in developing better treatment modalities to patients with Limbal Stem Cell Deficiency (LSCD). In addition, the molecular mechanisms that govern the maintenance of these tissue specific, non-keratinized, adult stem cells are being analysed using an enriched stem cell population.

Studies on the characterization of limbal niche - their role in maintenance and *ex-vivo* expansion of human corneal epithelial stem cells

Principal investigator : Dr. Gowri Priya Chidambaranathan
Co-investigators : Prof. VR. Muthukkaruppan, Dr. N. Venkatesh Prajna
Senior research fellow : Saumi Mathews
Funding : Aravind Medical Research Foundation

Limbal epithelial stem cells (LESC) are maintained by intrinsic and extrinsic factors in their microenvironment or niche. It is constituted by limbal extracellular matrix (ECM), adjoining vasculature, mesenchymal stem cells (MSC) and stromal cells that provide various soluble growth and survival factors. MSCs in the anterior limbal stroma have been identified as unique clusters of cells, double positive



Secretome profile of limbal (LSC) and corneal (CSC) stromal cells at different passages (p).Gels were stained with colloidal coomassie brilliant blue. Lane 1: M- marker; Lane 2: LSC p2; Lane 3: LSC p6; Lane 4: CSC p2; Lane 5: CSC p6. Protein per lane -10 µg

for CD90 and CD105 subjacent to the basal limbal epithelium. They were absent in posterior limbal stroma and corneal stroma. Functional analysis proved that these limbal stromal MSCs are responsible for the regulation of stemness in limbal epithelium.

This study was carried out to understand the trophic signals released by limbal stromal cells expressing MSC markers that modulate the LESC behaviour. Limbal stromal cells (LSC) and corneal stromal cells (CSC) were cultured in Dulbecco's modified Eagle medium containing serum, and then maintained in serum-free medium for 24 hours. Culture supernatants were precipitated by trichloro acetic acid-acetone. A 90µg of protein was fractionated and subjected for LC-MS/MS using ORBITRAPVelos Pro LTQ mass spectrophotometer. Protein identification and classification were performed using Proteome Discoverer v1.4 and Uni-Prot Protein database respectively.

A total of 638 and 427 proteins with high peptide confidence were identified in the culture supernatant of LSCs and CSCs respectively. Among the identified proteins, 445 proteins were unique to LSCs. Further analysis of these unique proteins revealed that 25% of proteins had signal peptide, characteristic of classical secreted proteins, 23% of proteins devoid of signal peptide were known to be present in extracellular region and 52% neither exhibited a signal peptide nor had been previously described extracellularly. Majority of the proteins were involved in cell physiology and metabolic process (49%) while the remaining proteins take part in developmental process, signalling mechanisms and immune responses. Some of the unique proteins identified include pigment epithelial derived factor, stromal cell-derived factor 1, stem cell growth factor, platelet-derived growth factor-D, beta–nerve growth factor, agrin, laminin subunits which regulate LESCs.

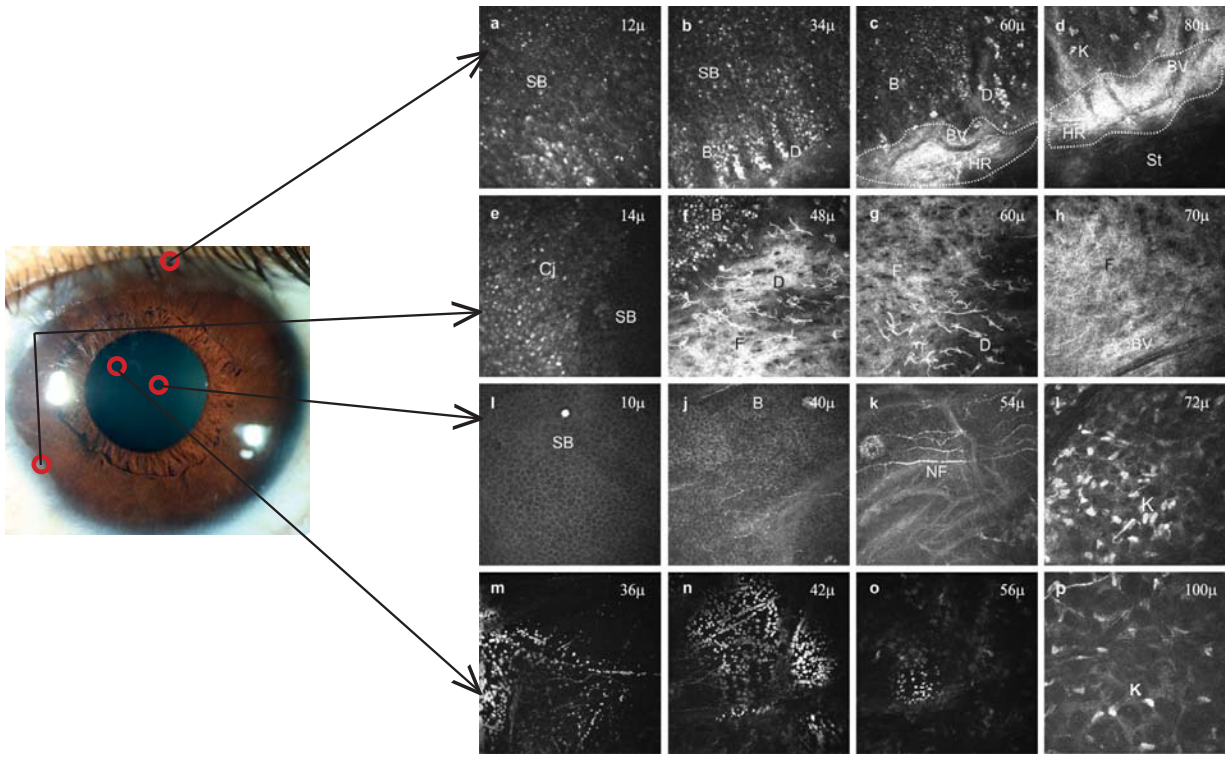
This is the first study to identify a large number of growth factors, extracellular matrix components and signalling molecules secreted by LS-MSCs which regulates stem cell self renewal and its pluripotency. Further studies are necessary to understand the interactions of the secreted factors and LESCs with reference to stem cell homeostasis.

A pilot study to analyse the limbal epithelial stem cell niche in limbal stem cell deficient patients

Principal investigator : Dr. Gowri Priya Chidambaranathan
Co-investigators : Dr. N. Venkatesh Prajna, Dr. VR. Muthukkaruppan
Senior research fellow : Saumi Mathews
Funding agency : AMRF-Aurolab Research Grant

With the advent of in *vivo* confocal microscope (IVCM), live imaging of the entire cornea and limbal epithelial architecture has been possible. The department has established the use of non-invasive IVCM to analyse the anterior limbal stroma beyond the basal epithelium for the first time in healthy subjects. Unique clusters of hyper reflective structures were identified in the anterior stroma subjacent to the basal epithelium which corresponds to MSCs - an important component of the limbal stromal niche. Having established the normal limbal stromal architecture, this study was carried out to understand the nature of limbal stroma in limbal stem cell deficient (LSCD) patients.

Both the normal and the affected limbus of 10 LSCD patients were scanned using Heidelberg Retina Tomograph III Rostock Corneal Module, after getting informed consent. The limbus was scanned from epithelium to deeper stroma at which structures could be resolved, while the central cornea was scanned from epithelium to endothelium.



In vivo confocal images of limbus (a-h) and cornea (i-p) of a patient with focal LSCD (area of scan indicated in red boxes). Normal limbus (a-d); affected limbus (e-h,)– normal suprabasal epithelial cells (e); presence of dendritic cells (f) in the subepithelium as well as in the anterior stroma (g); fibrotic appearance of deeper stroma with blood vessels (h); normal cornea (i-l); affected cornea (m-p) bright, circular lime deposits in the subepithelium (m,n) and anterior stroma (o) ; stroma with keratocytes (p). SB- suprabasal cells; B- basal cells; D- dendritic cells; HR- hyperreflective clusters; BV- blood vessels; BM-Bowman's membrane with nerve plexus; K – keratocytes; St- stroma.

In LSCD, the limbal and corneal epithelium was replaced by conjunctival epithelium, without palisades of Vogt. A large number of inflammatory cells and dendritic cells were identified along with blood vessels. It was not possible to identify the unique clusters of hyper reflective structures in these LSCD patients as in the anterior limbal stroma of healthy individuals. These hyper reflective structures were replaced by fibrotic tissue in the affected region of LSCD. However, limbal stroma appeared normal in the unaffected area in the focal LSCD. Subepithelial fibrosis was observed in the corneal stroma of LSCD patients except in the unaffected region of focal LSCD. Activated keratocytes (elongated) were observed in deeper stroma in five cases and in one they appeared as elongated needles and condensed cell bodies. Thin, sparsely distributed sub basal nerve fibres were observed only in the partial LSCD patients. But in some LSCD patients with sub epithelial fibrosis, normal corneal epithelial phenotype was observed even after several months of injury. Hence, further studies are essential to understand the nature of limbal stromal niche by analyzing the stromal biopsies from different grades of LSCD patients, which may have a major influence on the clinical outcome.

Molecular signature of Corneal Epithelial Stem Cells (CESCs)

Principal investigator : Dr. Gowri Priya Chidambaranathan
Senior research fellow : M. K. Jhansi Rani
Funding agency : Department of Biotechnology

Till date, there is no single specific positive marker identified for Corneal Epithelial Stem Cells (CESCs) and hence there is no specific method for their isolation. One of the probable reasons for not identifying molecular mechanisms associated with the stemness might be the low SC content (maximum - 16%) in the population analysed. The department has now established a method to enrich the SC content up to 80% by isolating the limbal basal epithelial cells followed by Laser Capture Micro-dissection (LCM) of cells with N/C ratio > 0.7, whichis proposed to be a better choice to understand the molecular signature of these LESCs. Further it has been demonstrated that the ΔNp63α isoform is expressed at mRNA levels only in the 80% enriched stem cell population and not in the differentiated cells indicating the purity of the enrichment and a probable role for this isoform in the maintenance of stemness. Recently, the trail RNA sequencing of the enriched CESCs at Pico level RNA using smarter cDNA synthesis kit, Illumina has been successfully done and the final sequencing is in progress.

Since the expression of ΔNp63α was specific to CESCs, further studies are now being carried out to understand the associated molecular regulators like miRNAs. miR-203 has been demonstrated to control the differentiation of skin keratinocytes. Hence the role of miR-203 in maintenance of CESCs was analysed. miR-203 transfection suppressed holoclone formation by cultured limbal epithelial cells and also reduced the ΔNp63α expression. In contrast, antago miR-203 increased the ΔNp63α expression and also increased the colony forming efficiency. Further studies are essential to understand the signaling pathways associated with the molecular regulation of stemness in CESCs by miR-203.

PROTEOMICS

In the Department of Proteomics,the primary focus lies in understanding the pathology of eye disease at the protein level. This understanding is important to develop methods or markers that will allow early diagnosis as well as improved treatment. Focus is mainly on three important diseases, namely, Fungal Keratitis, Diabetic Retinopathy and Glaucoma. High-throughput proteomics approaches are employed to carry out large-scale comprehensive studies of specific proteomes that include information on protein abundances, their variations and modifications, their interacting partners and the networks they are involved in. A state-of-the-art Mass Spectrometry facility has been established that allows us to profile complex proteomes in the discovery phase studies where proteins that have the potential to be used as biomarkers are shorlisted. These proteins will be validated across a large number of samples during the validation phase. The proteomics work is further complemented by studies at the genome and transcriptome level. A cell culture facility has been established where cell lines are used as a model system to test hypothesis.

PROTEOMICS OF HOST IMMUNE RESPONSE TO FUNGAL INFECTION

1.1 Proteomics of tear of *A.flavus* Keratitis patients

Rationale of the study

Tear proteins play an important role in maintaining the ocular surface, and changes in tear protein components may reflect the changes in the health of the ocular surface. Proteome analysis can provide more insights about protein expression patterns, which are associated with various pathological conditions and the identification of tear proteins and their posttranslational modifications have the potential to reveal the mechanisms underlying fungal keratitis.

Objectives

1. Identification of tear proteins that are differentially regulated during fungal infection
2. To identify the tear proteins that are differentially glycosylated during *A.flavus* infection of the cornea

Results

Profiling of tear proteome

In-depth profiling of tear of normal persons as well as that collected from *A.flavus* infected keratitis patients was carried out using Orbitrap Mass Spectrometer. A total of 2575 and 1966 proteins were identified in the control and infected tear, respectively.

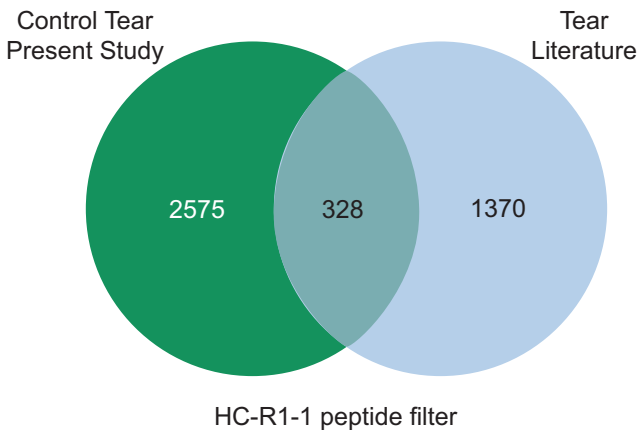


Fig 1.1. Comparison of tear proteome from this study with that of reported data

Zhou *et al.,*(2012) reported 1543 tear proteins, which is the largest number of proteins reported in tear so far. In a recent report, Semba *et al.,* (2013) have compiled the tear protein identifications using MS from literature and found that 4842 non-redundant proteins have been identified across the different compartments of the eye of which 1698 non-redundant proteins were reported only in tear film. In this study, much higher number of proteins have been identified compared to what has been reported in tear by other groups.

Comparison of tear proteins identified in this study with that reported previously by other groups (figure 1.1) shows that only 328 proteins identified in this study has been reported so far. The presence of a large number of unique proteins in this study could be attributed to the ethnic difference in the persons from whom tear was collected. This study represents the proteins identified in tear from south Indian population.

Comparative analysis of tear proteome

Tear proteins identified in tear of *A. flavus* infected patients was compared with control tear proteome. Proteins such as matrix metalloproteinase-9, ficolin-2, cathepsin G, CD5 antigen like protein, serum amyloid P component, lumican and complement proteins were identified in the tear from fungal keratitis patients, but not in tear from healthy individuals. Interestingly, most of complement cascade proteins were identified only in the tear of fungal keratitis patients, mainly isoform 2 of Ficolin-2 and isoform 2 of mannan-binding lectin serine protease 2, which activates the complement lectin pathway. Except complement protein C3b, rest of the complement proteins involved in alternative complement pathway were also identified. Proteins that regulate the complement activation, namely factor H and factor I was identified only in infected tear. Additionally, other proteins such as apolipoprotein, haptoglobin, serum albumin, alpha-1-antitrypsin, complement C3, cystatin, extracellular glycoproteins were found to be differentially regulated.

Proteases are associated with host defense mechanisms against pathogens and extracellular matrix remodeling during healing and wounding processes. To understand the role of proteases in the tear, proteins identified in control tear and infected tear were searched against the MEROPS (peptidase) database. This analysis revealed that proteases constitute 7-8% of the tear proteins while 3-3.6% were protease inhibitors. Protease and protease inhibitor have been shown to be in constant equilibrium in healthy tear. In this study, a greater percentage of proteases and protease inhibitors were identified in infected tear when compared to control. Some of the important proteases identified in infected tear

include cathepsin G, cathepsin S and Apolipoprotein A-II, Apolipoprotein E. The pathogenesis of corneal ulceration is probably due to the degradation of collagen and proteoglycan by destructive enzymes and action of these enzymes can inhibited by some protease inhibitors such as alpha 1 antitrypsin, alpha 2-macroglobin and alpha-1-antichymotrypsin. These protease inhibitors found in infected tear can be expected to play a protective role in minimizing tissue damage during fungal infection.

Glycosylation is the most widespread and complex form of protein post-translational modification and more than 50% of human proteins are known to be glycosylated. Changes in glycosylation pattern of proteins have been reported in other diseases such as cancer. To understand the role of glycosylation modification in fungal infection and its effect on protein function, glycoproteins were enriched from total tear proteins using conAlectin columns. Proteins in the conA enriched fraction as well as the glycoprotein depleted tear were identified by mass spectrometry. From the comparative analysis of the glycoproteins identified in the infected and control tear, it was evident that a subset of tear proteins were differentially glycosylated during fungal infection. This suggests that during infection there is an alteration in the post-translational modification of proteins in tears. Some of the proteins that exhibited differential glycosylation include matrix metalloproteinase-9, ficolin-2, cathepsin G, CD5 antigen like protein, serum amyloid P component, lumican and complement proteins.

Validation of some of these proteins for their use as biomarker for early diagnosis of fungal keratitis is in progress.

1.2 Expression of Zinc alpha 2-Glycoprotein (ZAG) in human tear during Fungal Keratitis

Rationale of the study

A previous study at the institute, (Ananthi *et al* (2013)) compared the proteome profile of tear from Fusarium keratitis patients with control tear and showed significant upregulation of six proteins in Fusarium infection. Five of these proteins were also found to be upregulated in the tear from A. flavus infected patients. Interestingly one protein, Zinc-alpha-2-glycoprotein found to be upregulated in Fusarium infection was downregulated A.flavus infection.

Zinc α-2-glycoproteinis a novel adipokine, similar to MHC class I antigen-presenting molecule. Downregulation of ZAG has been reported during macrophage-associated inflammation in adipose tissue. As Macrophage-associated inflammation is the primary host response in mycotic keratitis, this study aims at understanding the role of ZAG in A.flavus infection. The distinct pattern of expression in Fusarium and Aspergillus keratitis warrants further study.

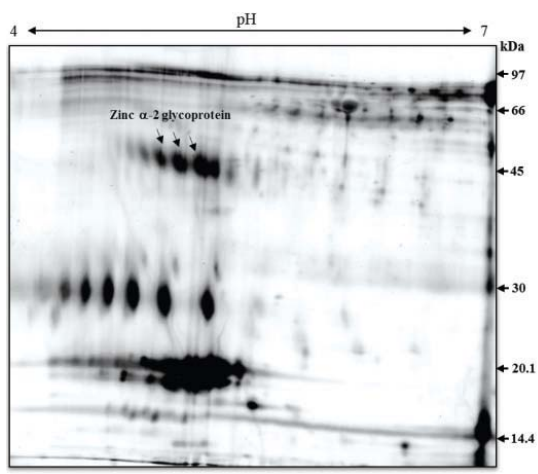


Fig 1.2.1. 2D Proteome profiles of normal control tear. Two-dimensional electrophoresis was performed using 30µg of tear protein in an 18 cm IPG strip of pH 4-7 in the first dimension followed by separation on a 12.5% SDS gels in the second dimension. The proteins were visualized by glutaraldehyde silver staining. Arrows indicate the proteoforms of ZAG.

Objectives

1. To decipher the role of ZAG in fungal infection.
2. To determine if ZAG levels in tear can be used as a diagnostic tool to differentiate A.flavus and Fusarium infection.

ZAG is one of the abundant proteins in tear and three different proteoforms were identified in

a 2D map (figure 1.2.1). The calculated molecular weight of the 298 amino acid protein is 34.2 kDa with a pI of 5.7. However, the protein migrates in the SDS-PAGE at around 45 kDa. This is probably due to the glycosylation of ZAG and four sites for glycosylation have already been reported earlier by other groups. Increase in the molecular weight of the ZAG proteoforms along with the shift towards the acidic side suggests that the acidic proteoform of ZAG might carry sialic acid in the glycan moiety.

To determine the regulation of ZAG during *A. flavus* infection of cornea, 2D proteome profile of tear from three different stages of infection (early, intermediate and late) was compared.

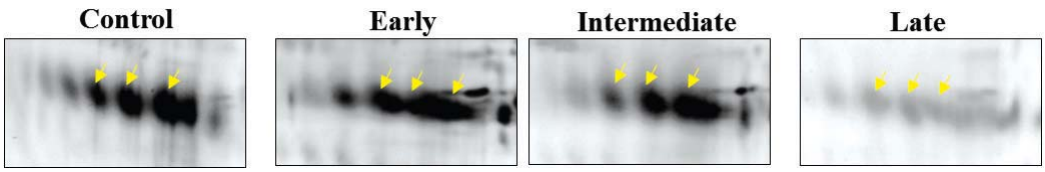


Fig 1.2.2. Comparison of ZAG levels in tear from different stages of A.flavus infection. Zoomed in view of the region corresponding to ZAG in the 2D proteome profile of tear collected from patients at different stages of infection.

From figure 1.2.2, it is evident that the levels of all the three proteoforms of ZAG decrease as A.flavus infection progresses. In the late stage of infection, only negligible levels of ZAG were found in the tear. To quantitate the extent of decrease in the ZAG levels, tear proteins were analyzed on a 2D-DIGE, and the abundance of ZAG across all the samples were determined (figure 1.2.3).

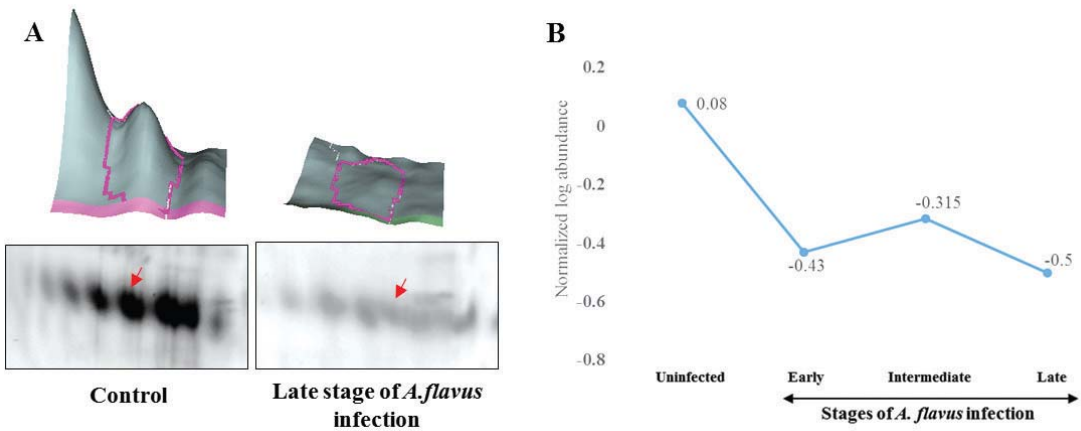


Fig 1.2.3. Comparison of ZAG levels at different stages of infection. Tear samples from patients at different stages of infection were analyzed by 2D-DIGE. DeCyder analysis was performed to quantify the levels of the protein spot. A.comparison of the 3D-view representing the level of ZAG proteoform (indicated by red arrow) in tear from control and late stage of infection. B. Plot of the abundance of ZAG across different stages of infection.

A 5.4 fold decrease in ZAG level was observed at the onset of *A. flavus* infection and as the infection progresses, there was a small increase in the intermediate stage, which decreases further in the late stage.

Decrease in ZAG levels during infection was further validated by western blot analysis. Figure 1.2.4 shows the western blot of tear samples across the different stages of infection and the ZAG levels were quantified by the densitometric analysis of the blot.

ZAG levels in the immunoblot represent the combined level of all the three proteoforms and the decrease in the total ZAG concentration followed a similar trend as observed in DIGE analysis.

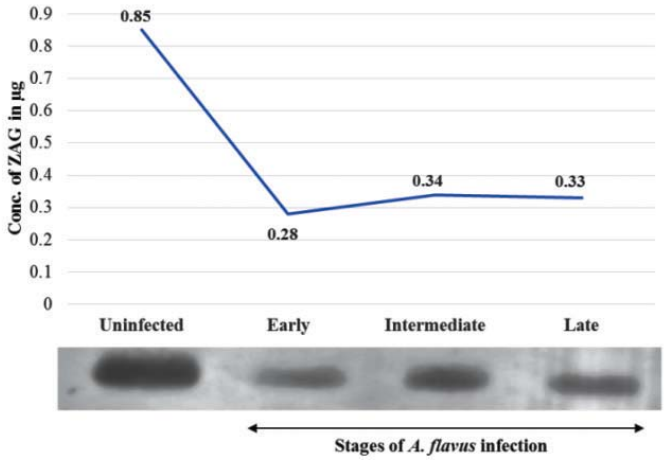


Fig 1.2.4. Validation of ZAG downregulation by western blot analysis. Two microgram of total tear was resolved by 1D SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-ZAG antibody. Densitometric analysis of the immunoblot was done using Image Quant software and the values are plotted against the different stages of infection.

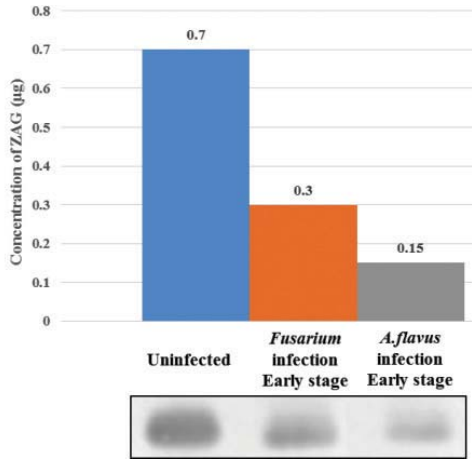


Fig 1.2.5. Comparison of ZAG levels in Fusarium and A.flavus keratitis. Two microgram of total tear was probed with anti-ZAG antibody. Densitometric analysis of the immunoblot was done and the values were plotted against the type of infection.

ZAG levels were compared between the A. flavus and Fusarium infection in the early stage and the results are shown in figure 1.2.5.

Decrease in ZAG levels were observed in both fungal infections. However, while only a two-fold decrease was observed in Fusarium infection, a 4.6 fold decrease was observed in A. flavus infection. In order to validate this infection specific difference in ZAG levels, further experiments are in progress to quantitate and validate ZAG across a large number of samples using ELISA.

1.3. Proteome analysis of fungal cell wall component (β-glucan) induced alterations in human corneal epithelial cells

Rationale of the study

Corneal epithelium is the outermost layer of the cornea which acts as the first line of defense against pathogens. Infection of the cornea by pathogenic fungus occurs when the corneal epithelium is breached. It is still not clear how these epithelial cells contribute to the initiation of the immune response when the fungi infects. Hence, to understand the role of this epithelial layer during fungal infection, a proteomics approach was adopted and SV40 immortalized human corneal epithelial cell line was chosen as a model for corneal epithelial cells to study the early events during pathogen recognition. Interaction between the pathogen and host cell is mediated through the cell wall components of the fungus primarily by β-glucan. In this study, β-glucan from S.cerevisiae (known as zymosan) was used to examine β-glucan induced modulation in HCE cells to understand how corneal epithelial cells recognize the fungal pathogens to initiate an immune response.

Objectives

1. Comparative proteome analysis of HCE cells with or without zymosan treatment
2. To quantify the differentially expressed HCE proteins in response to zymosan by 2D-DIGE
3. Identify the differentially expressed proteins as well as to profile the global changes in the proteome by Mass spectrometry.

Results

Protein profile of HCE cells treated with zymosan

To analyze the proteome wide changes in HCE cells in response to zymosan, total protein was prepared from HCE cells that were treated with two different concentrations of zymosan (100 µg/ml or 200 µg/ml). Figure 1.3.1 shows the comparison of the 2D proteome profile of HCE cells.

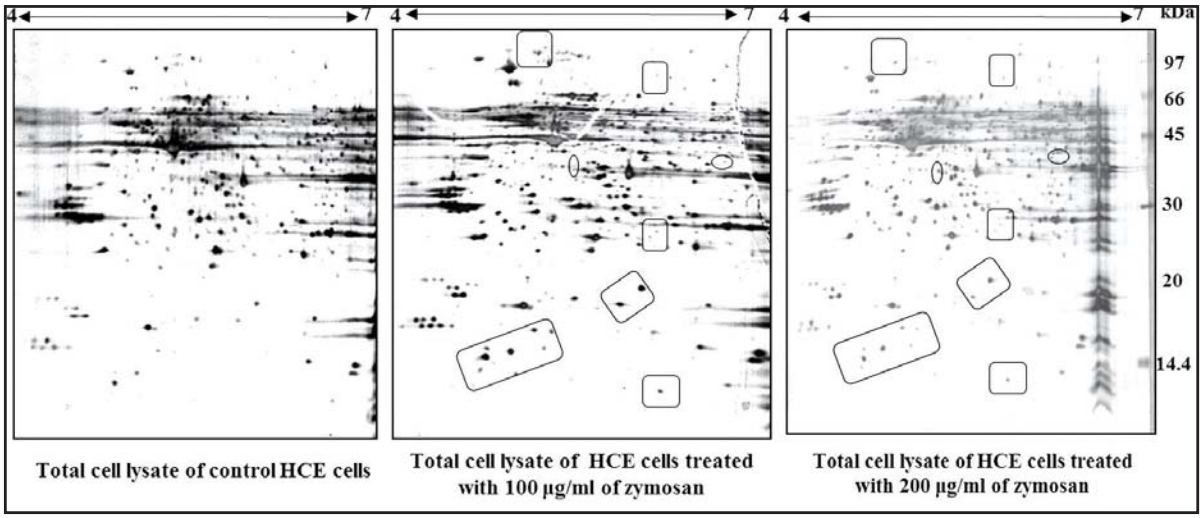


Fig1.3.1. Comparison of 2D proteome profile of HCE with and without zymosan treatment

Sixty microgram of total cell lysate was resolved in the first dimension in a 4-7 pH range IPG strip followed by separation on a 12.5% SDS-PAGE in the second dimension. Gels were stained with glutaraldehyde-silver. Rectangle and circle indicate upregulated and downregulated spots, respectively in zymosan treated sample when compared to control.

The overall proteome profile of HCE cells upon zymosan treatment appeared similar to that of the untreated cells. However, comparative quantitative analysis of the 2D gels using IMP7 identified 143 spots common between zymosan treated and untreated HCE proteome of which 11 spots showed difference in abundance. Among these, nine spots were found to be upregulated while two spots were down regulated (table 1.3.1).

Spote No	Fold change in HCE cells treated with Zymosan with reference to untreated cells		Avg. fold change	Regulation
	100ug/ml	200ug/ml		
2	1.9	1.9	1.9	Upregulation
12	1.7	1.95	1.8	Upregulation
94	1.8	1.7	1.75	Upregulation
102	1.9	1.7	1.8	Upregulation
112	1.6	1.8	1.7	Upregulation
134	1.9	1.9	1.9	Upregulation
135	1.95	1.5	1.72	Upregulation
140	2.1	1.5	1.8	Upregulation
142	2.4	2.6	2.5	Upregulation
41	-2.8	-2.4	-2.6	Downregulation
53	-2.6	-1.8	-2.2	Downregulation

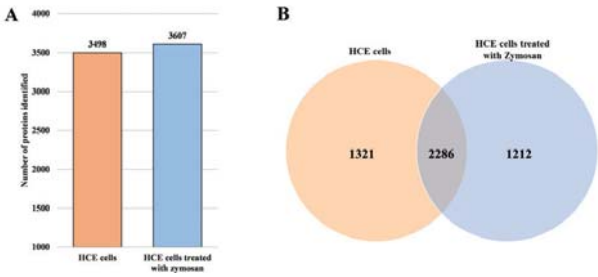


Fig 1.3.2: Mass spectrometry analysis of HCE cells. A. Comparison of proteins identified in HCE cells with and without zymosan treatment. B. Venn diagram depicting the number of proteins common between the treated and untreated HCE proteome.

In-depth proteome profiling of HCE cells by mass spectrometry

The number of proteins resolved and visualized in a 2D gel is limited. To overcome this limitation as well as to profile the proteome, HCE cell lysate was taken for identification by high resolution Mass Spectrometry. Protein identification details for the two samples and its comparison is shown in figure 1.3.2.

More than 3000 proteins were identified in zymosan treated and untreated HCE cells (figure 1.3.3A). Nearly one-third of the identified proteins identified in each case were unique to the condition (figure 1.3.4B). Many proteins have been previously reported to be induced upon zymosan treatment in different cell types. Two of these proteins, MyD88 and Phospholipase A2 activating protein were identified exclusively in zymosan treated HCE cells. The function of these proteins with reference to zymosan signaling is discussed below.

Myeloid differentiation primary response protein (MyD88)

MyD88 is an adapter protein that recruits IRAK to TLR, which is further activated by phosphorylation and associates with TRAF6 leading to the activation of NF-kB. In this study, the adapter protein MyD88 was identified only in zymosan treated HCE cells indicating that zymosan might activate TLR and recruit MyD88 to activate NF-kB resulting in the production of proinflammatory cytokines (figure 1.3.4A). In order to confirm the activation of this pathway in HCE cellsbyzymosan, translocation of NF-kB from cytoplasm to nucleus will be checked by immunoassays. In order to examine the role of other proteins in this pathways both phosphoproteomic and transcript anlaysis will be carried out.

Phospholipase A2 activating protein (PLAP)

It has been reported earlier that zymosan stimulates the production of leukotrienes in phagocytic cells such as macrophages, neutrophils, and non-phagocytic cells such as epithelial cells. Leukotriene is synthesized via arachidonic acid pathway and PLAP regulates phospholipase A2 activity as well aseicosanoid (leukotriene, prostaglandin and thromboxanes) synthesis (figure 1.3.4B). Leukotriene B4 is an important inflammatory mediator that attracts neutrophil at the site of infection and the infected cornea in fungal keratitis patients is characterized by extensive infiltration of neutrophils. In this study, PLAP was identified exclusively in zymosan treated HCE cells and it is likely that this protein might have a regulatory role in the production and secretion of leukotrienes from HCE cells which inturn may recruit neutrophils to the site of infection. Secretion of leukotriene B4 by HCE cellsupon zymosan treatment has to be confirmed by performing ELISA

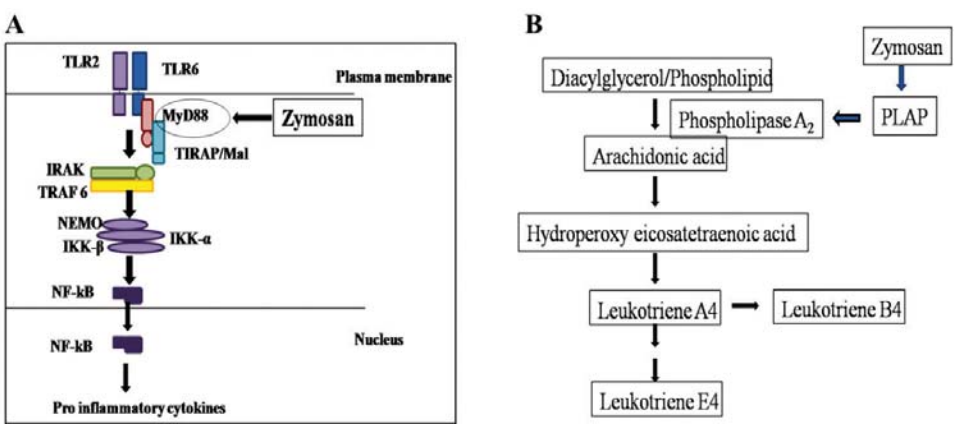


Fig 1.3.4. Probable mode of action of zymosan.

A. TLR2:TLR6 pathway activation.

B. Leukotriene biosynthetic pathway

1.4. In-depth proteome profiling of infected cornea from *Fusarium* Keratitis patients

Rationale of the study

Human cornea is a transparent, avascular, multi-layered tissue that facilitates the majority of light refraction in the optical apparatus of the eye. During fungal infection, the cornea undergoes progressive damage resulting in visual impairment and finally blindness. Damage to the cornea is not only because of the infecting fungus but primarily due to the host immune response against the pathogen. Analysing the proteome wide changes in the infected cornea would provide an insight into the mechanisms involved in host response to fungi.

Specific objectives

1. Proteome profiling of human cornea
2. Identification of infection specific proteins in Fusarium sp. infected cornea

Results

Deep proteome coverage was achieved for the corneal proteins using Orbitrap Mass Spectrometry. Figure 1.4.1 shows the comparison of the proteins identified.

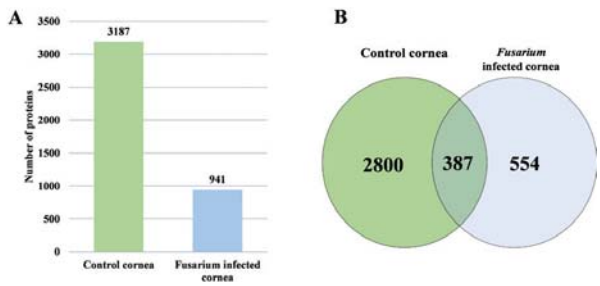


Fig 1.4.1.Corneal proteome profiling by Mass spectrometry. A. Comparison of proteins identified in uninfected and infected cornea. B. Venn diagram depicting the number of proteins that are common as well as unique to the infected and Fusarium infected cornea.

In infected cornea, only 941 proteins were identified which is three times lesser than the number of proteins identified in cadaver cornea (3187 proteins). This difference in total number of proteins identified could be attributed to the fact that infected corneal tissue had gone through pronounced tissue destruction (ulcerated cornea is usually excised at the late stage during penetrating keratoplasty). Comparative analysis revealed that only 387 proteins were common owing to the predominance of structural proteins in normal cornea while immune responsive proteins are the major proteins in infected cornea.

To understand the significance of the proteins present exclusively in infected cornea, 537 infection specific proteins were analyzed using Reactome v.49. Among these, 183 proteins were assigned to pathways (figure 1.4.3). It is clear that immune responsive proteins and signal transducing proteins encompasses the major portion of infection specific proteins reflecting their involvement in inflammatory cascades. Table 1.4.1 shows a list of proteins exclusive to infected cornea that have been previously reported to be involved in the clearance of other fungal pathogens.

S.No.	Protein	Pathway involved	Reported roles
1.	Integrin alpha M (CR3)	Rap 1 signalling	Promotion of *Calbicans* clearance
2.	Integrin beta (CR3)	Rap 1 signalling	Promotion of *Calbicans* clearance
3.	Protein S100-A8	TLR 4 signalling	Regulation of fungal killing in Fusarium keratitis
4.	Protein S100-A9	TLR 4 signalling	Regulation of fungal killing in Fusarium keratitis
5.	Neutrophil defensin 1	Fe gamma R mediated phagocytosis	Promotion of Aspergillus sp. clearance
6.	Beta defensin 112	Fe gamma R mediated phagocytosis	Promotion of Aspergillus sp. clearance
7.	Arachidonate 5-lipoxygenase activating protein	Leukotriene biosynthesis	Mediators of inflammation in respiratory diseases

Table 1.4.1. List of infection specific proteins involved in host immune response

Some of the proteins mentioned are present in both the cornea and tear from infected patients. Interestingly, some proteins such as metalloproteinase inhibitor 1 (TIMP-1), grancalcin, stromelysin, eosinophil cationic protein, Ras related C3 botulinum toxic substrate and an isoform of heat shock protein 70 were found only in Fusarium infected cornea but not in Fusarium infected tear (Ananthi et al, 2013).Though tear film is claimed to mirror the host response being in close proximity with cornea, additional clues can be obtained about the host response from the corneal proteome studies.

One of the important proteins identified exclusively in infected cornea is Metalloproteinase inhibitor 1 (TIMP-1). TIMP's are known to inhibit the activities of all known matrix metalloproteinases (MMPs) and play a key role in maintaining the balance between extracellular matrix (ECM) deposition and degradation in different physiological processes. Fungi are known to produce MMPs for its adherence and nutrient acquisition. Identification of TIMP as an infection specific protein suggests a role for TIMP-1 on fungal MMPs to facilitate fungal clearance. Additionally, TIMP can also act on host MMPs thereby controlling tissue destruction.

The role of other infected tissue specific proteins has to be explored to understand the events behind corneal ulceration.

2. PROTEOMICS OF FUNGAL PATHOGEN

2.1. Identification of virulence associated exoproteins of *A. flavus* causing Fungal Keratitis

Rationale of the study

Fungi secrete numerous proteins and metabolites that help to fend off competitive organisms, alter host signaling processes, act as virulence factors in pathogenic processes and also influence and regulate the fungal growth in the new environment. Exoproteins are the primary fungal components involved in host-fungus interactions. Hence, in this study, *A.flavus* was grown at two different temperatures (30°C and 37°C) to two different time points (24h and 40h) and exoproteins examined in each condition. Although exoproteins were examined at 30°C at 40h, 37°C was chosen to reflect the growth of the fungus at corneal temperature, which is around 36°C. Similarly, 24h time point was selected to identify the exoproteins that would be elaborated in the early time points of infection. For comparative exoproteome analysis, two strains of *A.flavus* were selected - ATCC 26, a saprophyte and a corneal isolate (CI1123).

Specific objectives

1. Profiling of exoproteome of *A.flavus* grown at 30°C and 37°C
2. Identify corneal isolate (CI) specific proteins that might play a role in virulence

Results

The two strains of *A.flavus* (ATCC26 and CI1123) were grown at 30°C or 37°C using solid state fermentation technique. Figure 2.1.1 shows the comparison of the cultures grown at 37°C with that of 30°C.

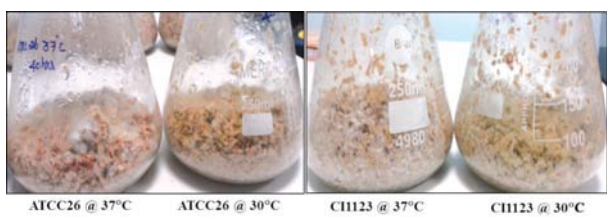


Fig 2.1.1. Solid state Fermentation culture of A.flavus strains. Spore suspension of 1 x108 spores/10 gm of wheat bran was inoculated and incubated at 30°C or 37 °C for 40h.

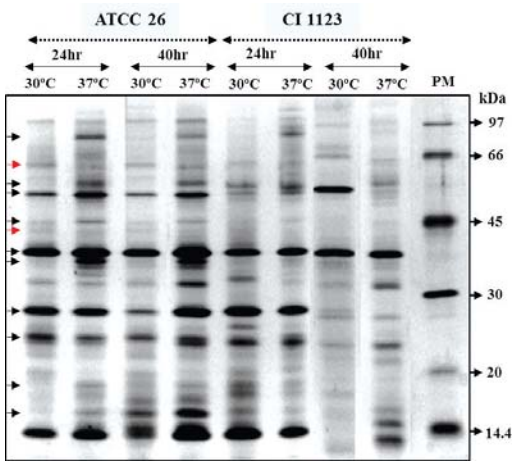


Fig 2.1.2.Comparison of exoprotein profiles of ATCC 26 and corneal isolate 1123. A. flavus strains were grown in SSF at two different temperatures and exoproteins prepared from cultures grown to 24h or 40h time points. Exoproteins (3 µg) were resolved on an 11% polyacrylamide gel and stained with glutaraldehyde silver. Black and red arrow indicates proteins that are upregulated at 37°C and 30°C, respectively.

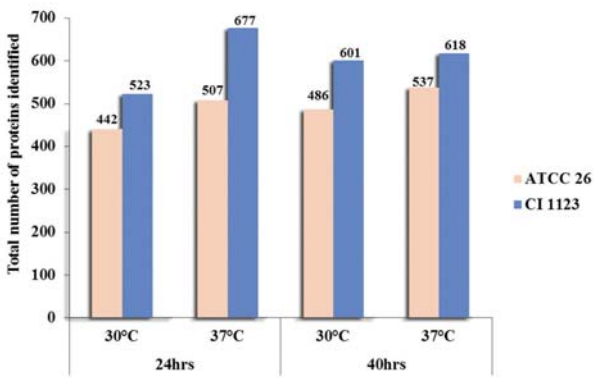


Fig 2.1.3. Comparison of the exoproteins from the two strains of A.flavus. Exoproteins were prefractionated by 1D SDS-PAGE and processed for identification by Orbitrap MS. Identified exoproteins were filtered with high peptide confidence and rank 1 peptide.

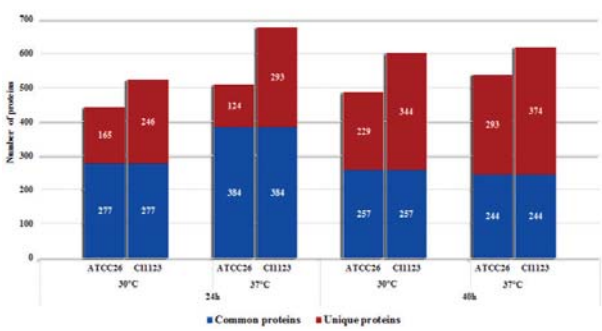


Fig. 2.1.4. Comparison of the number of proteins that is unique to the saprophyte and corneal isolate at different growth conditions. Blue portion of the bar indicates proteins that are common between the two strains while the red portion shows the difference in the unique proteins in each strain.

At 37°C, after 40 h of growth in wheat bran, there was no spore formation in both saprophyte and CI, whereas spores were visible (appearance of greenish yellow color) in cultures grown at 30°C. This observation suggests that higher temperature affects spore formation of *A. flavus* strains.

Exoproteins were isolated from cultures grown at two temperatures for 24h or 40h. Protein profiles of the isolated exoproteins were compared on a 1D SDS PAGE (Figure 2.1.2).

Protein profile of the exoproteins of *A.flavus* grown at 30°C was distinctly different from that at 37°C. Many proteins were present in higher levels in one of the two temperatures. To determine the differentially expressed proteins, exoproteome profiling was done for all the exoprotein samples using Orbitrap Mass Spectrometer. Details of the protein identification and the comparison are shown in figure 2.1.3.

One striking observation from figure 2.1.4 is that the corneal isolate grown at 37 °C for 24h has more than twice the number of unique proteins when compared to that of ATCC under similar conditions. This suggest that at the early time point of infection (24h) in the cornea (with a temperature of 37 °C), the corneal isolate elaborates more proteins that might help in its adaptation as well as invasion of the host.

Proteins made within the cell adopt different pathways to be exported out of the cell. Proteins secreted by the classical pathway are characterized by the presence of a N-terminal signal peptide.

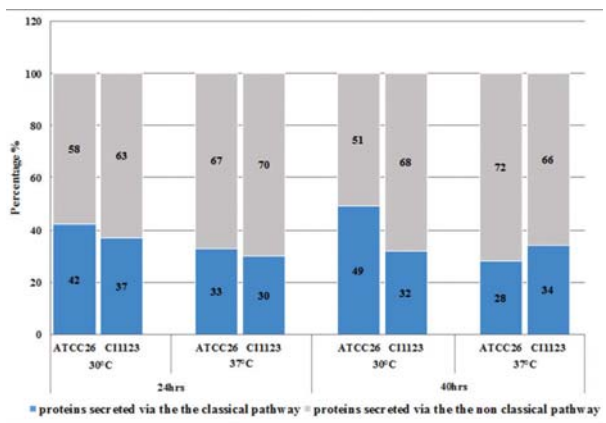


Fig. 2.1.5. Comparison of the exoprotein secretion pathways. Blue portion of the bar indicates proteins secreted by classical pathway and gray portion shows the number of proteins adopting non-classical pathway.

Organisms also employ additional non-classical pathways to export proteins lacking a signal peptide. To identify those proteins secreted by *A. flavus* strains via the non-classical pathways, exoproteins identified were compared against a list of *A. flavus* proteins predicted to be secreted via classical pathway and the comparison is shown in figure 2.1.5.

Corneal isolates at 37 °C has slightly higher percentage of protein secreted via the non-classical pathway at 24h. Alkaline protease, extracellular metalloproteinase and mycelial catalase are proteins with N-terminal signal peptide that are most abundant (high PSMs) under all growth conditions. As the corneal isolate secretes more proteins by the non-classical pathway, work is in progress to determine the role of these proteins with reference to infection of the cornea.

2.2. Characterization of multiple proteoforms of alkaline protease secreted by *A. flavus*

Rationale of the study

Alkaline protease is one of most abundant exoproteins of *A. flavus.* Selvam *et al.,* (2015) has shown the existence of this alkaline protease (Alk) in 24 different proteoforms. This study aims at characterizing the modifications at both the protein and transcript level that results in multiple proteoforms.

Purification of Alk from *A.flavus* exoproteins

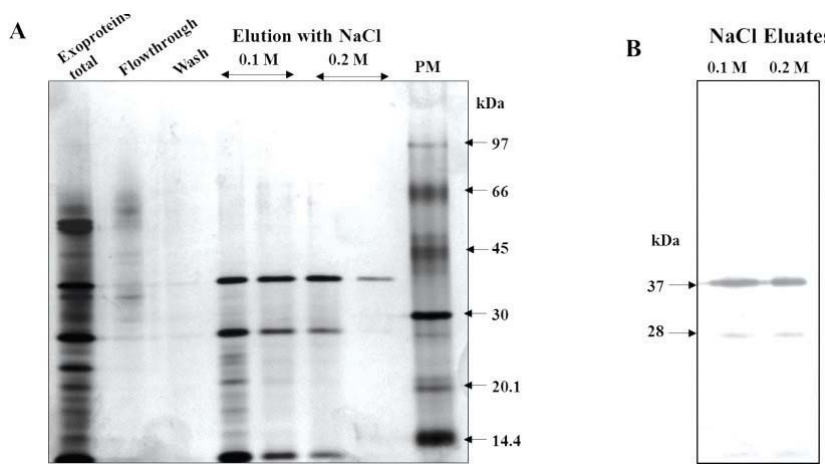


Fig 2.2.1. Purification of alkaline protease. A. Alkaline protease in exoproteins of A. flavus was bound to a cation exchange column and eluted using two different concentrations of NaCl. Ten microliter of each fraction obtained during purification was checked on a 12.5% SDS-PAGel. B. Immunoblot analysis of the purified protein.

Exoproteins were prepared from a corneal isolate M662 and purification was performed using cation exchange column (figure 2.2.1A). Using this column, it was possible to purify a protein that migrates at around 37 kDa in a 1D SDS-polyacrylamide gel. An additional 28 kDa band was also seen in all the eluates. To confirm that the purified protein was alkaline protease, these proteins were probed with anti-alkaline protease antibody. Presence of two bands corresponding to 37 kDa and 28 kDa in the immunoblot (figure 2.2.1B) confirms that the two proteins in the cation column eluates are indeed alkaline protease. Further confirmation was done by identification using mass spectrometry (Table 2.2.1). Both immunoblot and mass spectrometry analysis confirmed both the 37 kDa and 28 kDa bands as alkaline protease.

M. wt of band excised	Accession number	Protein description	Coverage (%)	Unique peptides	# of PSMs
37 kDa	B8N106	Alkaline protcase	28.29	7	7392
28 kDa	B8N106	Alkaline protcase	17.62	5	2887

Table 2.2.1. Identification of Alk by Mass Spectrometry

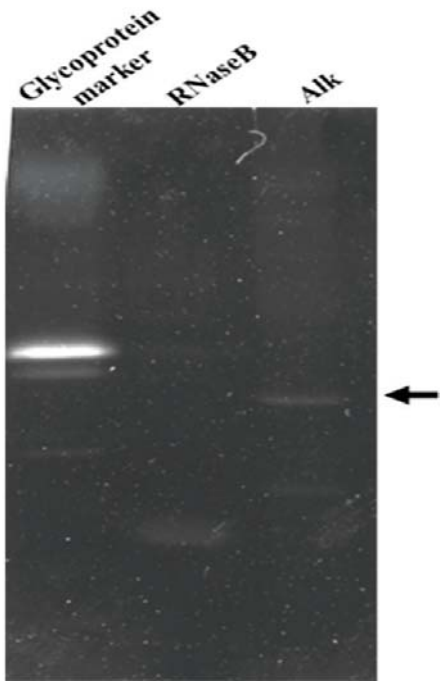


Fig 2.2.2. Glycoprotein staining of Alk. Two microgram of purified Alk was resolved on a 12.5% SDS-polyacrylamide gel and stained with Pro-Q Emerald glycoprotein stain

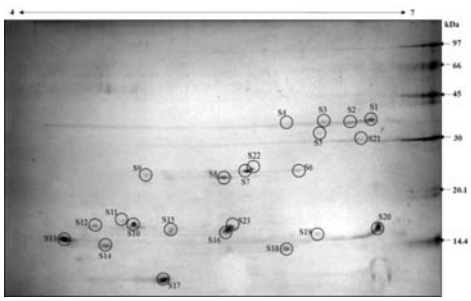


Fig 2.2.3. Multiple proteoforms of Alk. Twenty microgram of purified Alk was resolved on a 4-7 pH range IPG strip in the first dimension followed by separation on a 12.5% SDS-PAGE in the second dimension. The different proteoforms were visualized after staining with colloidal commassie stain.

Analysis of post-translational modification of Alk

Alk has 401 amino acids of which 1-19 amino acids correspond to the signal peptide followed by propeptide (20-126aa). The calculated molecular weight of the secreted form of Alk after removal of the signal and propeptide is 28 kDa (277 aa). However, in addition to the 28 kDa protein, a 37 kDa protein was consistently observed in all the experiments and this 37 kDa was also the most abundant form of Alk. A 11 kDa shift in the molecular mass indicates the presence of a post-translational modification such as glycosylation. To analyze if the 37 kDaprotein is a glycosylated form of 28 kDa, the purified protein was resolved on a 1D SDS-PAgel and stained with glycoprotein staining reagent. Figure 2.2.2 shows that the 37 kDaAlkband is stained by the glycoprotein stain indicating that it is a glycoprotein.

Multiple proteoforms of Alk

Purified Alk was resolved by 2D SDS-PAGE (fig 2.2.3) and twenty three different protein spots spanning the pH of 4-7 and molecular mass of 10-45 kDa were observed. All the spots were excised and identified by mass spectrometry.

Out of the 23 spots analyzed, 19 spots were identified as alkaline protease. Protein spots with a molecular mass lower than 28 kDa were also identified as Alk suggesting either they are the truncated forms of Alk or proteins resulting from differential splicing. Alkaline protease gene has four exons and three introns (figure 2.2.4). As intron retention is common in fungal transcripts, the possibility of intron retention resulting in the lower molecular mass proteoforms was explored. For this analysis, MS raw data for the 23 spots was searched against an in-house database that includes the translated product of all possible combinations of introns retained in the transcript. Interestingly, many protein spots identified the translational product of a transcript that retained intron 2. Retention of intron 2 will result in a short form of Alk (393 aa) in which the first 256 aa are identical to Alkprotein (figure 2.2.4).

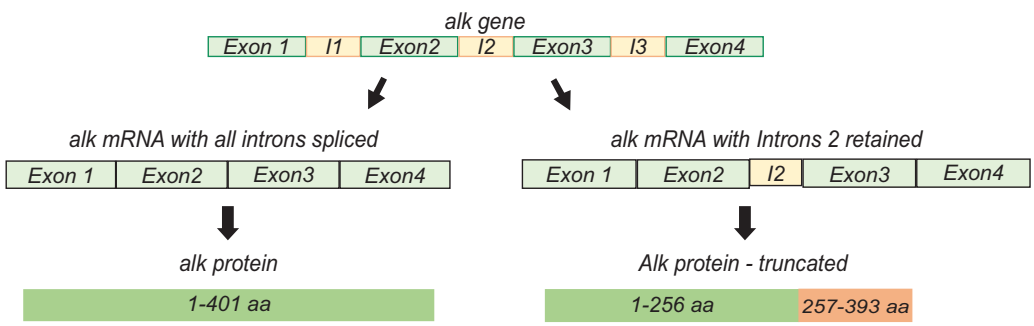


Fig 2.2.4. Alkaline protease gene architechture along with the transcription and translational product

Fig 2.2.5.Identification of normal and truncated form of Alk by PEAKS analysis. Sequence coverage of A. normal Alk that is the product of mature transcript with all the introns spliced out; B. truncated Alk that results from the translation of the transcript with intron 2 retained. Dotted box indicates the peptide identified in the C-terminus region of the two proteins that are different in sequence

The C-terminus 137 amino acids results from the frameshift of the reading frame due to retention of intron 2. Two peptides from this C-terminus region (299-305 aa and 319-327 aa shown in figure 2.2.5) was detected by MS in 11 Alk spots.

Further analysis is in progress to validate this observation and characterize Alk and its proteoforms.

SPONSORED PROGRAMME

Predictive biomarkers for diabetic retinopathy among diabetic patients and stage specific biomarkers for NPDR and PDR

Team leader	:	Prof. K. Dharmalingam
Co-team leader	:	Dr. Kim
Investigators	:	Dr. J. Jeya Maheshwari, Dr. S. Senthil Kumari
Team members	:	Roopesh R. Pai, R. Sharmila, KRP. Niranjana, Naveen Luke, A. Divya, Sandhya. K
Funding	:	Mindtree
Duration	:	November 2014 - 2017

Rationale of the programme

Diabetic Retinopathy (DR) is one of the micro vascular complications of diabetes that leads to loss of vision in majority of affected individuals. DR is the second leading cause of blindness due to retinal damage and this disease contributes to an overall 4.8% blindness across the globe. In India, approximately 11.4 million T2DM patients will develop DR in 2025. It is reported that 75% of DM patients will develop DR after 15 years of diabetes. Therefore, identifying early biomarkers that will allow prediction of development of DR among DM patients will be of enormous help in dealing with this condition. Towards this, this study has been undertaken. This report highlights the progress made in the last 3 months in some aspects of the proposed study.

1. Identification of stage-specific serum/ plasma markers using high-throughput proteomics approaches using 2D DIGE and mass spectrometry.
2. Validation of the putative biomarkers identified in phase one of this study as well as examining the markers already reported across a large number of samples using high throughput quantitative methods (Bioplex and MRM assays).

PROJECT 1. In-depth profiling of DM vs PDR: serum proteomics, DIGE and mass spectrometry

Objectives

1. In-depth profiling of serum proteome in DM and PDR conditions
2. Identification and quantitation of differentially regulated proteins between the two conditions
3. Characterization of PDR specific post-translational modifications of proteins

Results

Serum was collected from diabetes patients who do not show any signs of DR (DM) and from Proliferative Diabetic Retinopathy (PDR) patients. The top two abundant proteins in serum, albumin and IgG, were first depleted before performing any proteome analysis. Figure 1.1 shows the comparison of the protein profile of the albumin and IgG depleted serum on a 1D SDS-PAGel. Five protein bands (B1, B2, B3, B6, B7) that showed difference in abundance was excised out and identified using Mass Spectrometer. The details of the identification is given in table 1. Two bands, B4 and B5, showed a considerable shift in the PDR sample. This increase in molecular weight of these two proteins with reference to the DM sample suggests that post-translational modification could be altered in PDR although the levels are similar.

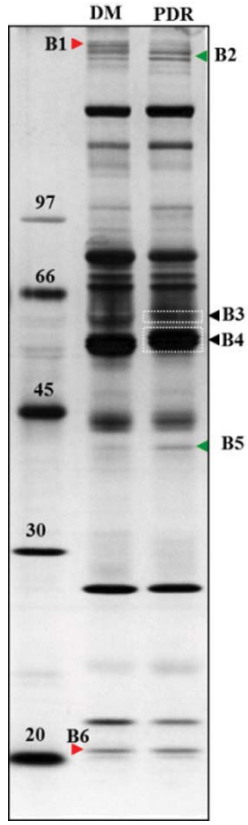


Fig 1.1. Comparison of the protein profile of DM and PDR serum proteins. Serum proteins after depletion of albumin and IgG were resolved on a 1D SDS-Polyacrylamide gel and visualised by silver staining. Arrows indicate the proteins that were excised out for identification by mass spectrometry.

DM vs PDR	BAND #	Description	DM Σ# PSMs	PDR Σ# PSMs	Mr (kDa)
Up regulated in DM	BAND 1	Apolipoprotein B (Including Ag(X) antigen)	1795	1753	515.2
		Apolipoprotein B variant (Fragment)	607	737	183.5
		Alpha-2-macroglobulin	289	317	163.2
		Complement C3	76	25	187.0
		Apolipoprotein(a)	51	5	501.0
		von Willebrand factor	10	9	298.2
		Hornerin	5	4	282.2
Up regulated in PDR	BAND 2	cDNA FLJ75416, highly similar to Homo sapiens complement factor H (CFH), mRNA	63	48	138.9
		Inter-alpha-trypsin inhibitor heavy chain H2	28	49	105.2
		Isoform 2 of Inter-alpha-trypsin inhibitor heavy chain H3	25	33	99.3

		cDNA FLJ78071, highly similar to Human MHC class III complement component C6 mRNA	18	13	104.6
		cDNA FLJ58441, highly similar to Attractin	4	2	129.7
Decrease in mobility (Increase in molecular mass)	BAND 3	Keratin, type I cytoskeletal 9	250	65	62.0
		Keratin, type I cytoskeletal 16	94	9	51.2
		Alpha-1-antitrypsin	106	52	46.7
		cDNA FLJ53075, highly similar to Kininogen-1	83	63	46.5
		Putative uncharacterized protein DKFZp686K04218 (Fragment)	149	177	51.6
		cDNA FLJ14473 fis, clone MAMMA1001080, highly similar to Homo sapiens SNC73 protein (SNC73) mRNA	304	396	53.1
		Alpha-1-antichymotrypsin	112	178	47.6
		Keratin, type I cytoskeletal 14	49	15	51.5
		Antithrombin-III	127	62	52.6
		cDNA FLJ41552 fis, clone COLON2004478, highly similar to Protein Tro alpha1 H,myeloma	298	387	53.3
		Putative uncharacterized protein DKFZp686M08189	241	286	52.2
		Putative uncharacterized protein DKFZp686K18196 (Fragment)	293	383	56.4
		cDNA, FLJ93695, highly similar to Homo sapiens serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4 (SERPINA4), mRNA	32	14	48.5
		Keratin, type I cytoskeletal 10	111	50	58.8
		cDNA FLJ51361, highly similar to Keratin, type II cytoskeletal 6A	95	26	55.8
		Keratin, type II cytoskeletal 2 epidermal	73	51	65.4
		Hemopexin	61	94	51.6
		cDNA FLJ53950, highly similar to Angiotensinogen	96	69	51.0
		Ig mu chain C region	37	24	49.3
		Keratin, type II cytoskeletal 5	85	28	62.3
		Putative uncharacterized protein DKFZp686O16217 (Fragment)	166	214	54.1
		Thyroxine-binding globulin	23	7	46.3
		IGH@ protein	20	16	51.1

		Putative uncharacterized protein DKFZp686C02220 (Fragment)	82	89	54.1
		Putative uncharacterized protein DKFZp686G11190	21	17	52.0
		Putative uncharacterized protein DKFZp686O01196	20	15	52.6
		Phosphatidylcholine-sterol acyltransferase	15	10	49.5
		cDNA, FLJ94361, highly similar to Homo sapiens serine (or cysteine) proteinase inhibitor, clade A(alpha-1 antiproteinase, antitrypsin), member 6 (SERPINA6), mRNA	23	21	45.1
		LBP protein	24	25	52.9
		Carboxypeptidase B2	9	5	48.4
		cDNA, FLJ93914, highly similar to Homo sapiens histidine-rich glycoprotein (HRG), mRNA	9	8	59.5
		Vitronectin	17	20	54.3
		Haptoglobin	7	17	45.2
		Heparin cofactor 2	10	9	57.0
Decrease in mobility (Increase in molecular mass)	BAND 4	Alpha-1-antitrypsin	1246	1108	46.7
		Vitamin D-binding protein	181	161	52.9
		Keratin, type I cytoskeletal 9	72	21	62.0
		Keratin, type I cytoskeletal 10	85	38	58.8
		cDNA, FLJ93695, highly similar to Homo sapiens serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4 (SERPINA4), mRNA	27	15	48.5
		Hemopexin	40	21	51.6
		Antithrombin-III	64	134	52.6
		Keratin, type II cytoskeletal 2 epidermal	52	25	65.4
		cDNA FLJ41552 fis, clone COLON2004478, highly similar to protein tro alpha1 H, myeloma	52	61	53.3
		cDNA FLJ53075, highly similar to Kininogen-1	21	37	46.5
		Keratin, type II cytoskeletal 5	30	10	62.3
		Keratin, type I cytoskeletal 14	19	3	51.5
		Ig mu chain C region	15	14	49.3
		Alpha-1-antichymotrypsin	11	16	47.6
		cDNA, FLJ94361, highly similar to Homo sapiens serine (or cysteine) proteinase inhibitor, clade A(alpha-1 antiproteinase, antitrypsin), member 6 (SERPINA6), mRNA	16	21	45.1

		cDNA FLJ51361, highly similar to Keratin, type II cytoskeletal 6A	20	8	55.8
		Heparin cofactor 2	14	10	57.0
		Coagulation factor X	13	4	54.7
		Putative uncharacterized protein DKFZp686O16217 (Fragment)	18	26	54.1
		Keratin, type I cytoskeletal 15	11	4	49.2
Up regulated in PDR	BAND 5	Haptoglobin-related protein	234	168	39.0
		Ig alpha-1 chain C region	23	43	37.6
		Alpha-2-HS-glycoprotein	23	35	39.3
		Zinc-alpha-2-glycoprotein	21	30	34.2
		FN1 protein (Fragment)	8	26	30.1
		cDNA FLJ57644, highly similar to Serum paraoxonase/arylesterase 1 (EC 3.1.1.2)	12	21	39.7
		Protein AMBP	25	17	39.0
		Apolipoprotein A-I	22	14	30.8
		cDNA FLJ54228, highly similar to Leucine-rich alpha-2-glycoprotein	1	10	36.5
		Heavy chain of factor I (Fragment)	4	8	35.9
		Serum paraoxonase/lactonase 3	3	4	30.7
		Coagulation factor XII (Hageman factor)	1	4	32.2
		Isoform 2 of Keratin, type II cytoskeletal 73	3	3	42.0
		Isoform 2 of SUN domain-containing protein 3	1	2	30.7
Up regulated in PDR	BAND 6	Haptoglobin	246	426	45.2
		Keratin 1	151	98	66.0
		Keratin, type I cytoskeletal 10	159	42	58.8
		Keratin, type I cytoskeletal 9	11	24	62.0
		Keratin, type II cytoskeletal 2 epidermal	143	23	65.4
		Full-length cDNA clone CS0DD006YL02 of Neuroblastoma of Homo sapiens (human)	6	4	41.2

It was possible to observe clear difference in levels for these five protein bands even in a 1D gel. However, since at least 50 proteins were identified in each band with high confidence, it was difficult to determine which of these proteins contributed to the difference in levels observed in the gel. Based on the number of PSMs, the amount of proteins has been calculated. Hence, to determine the protein that is differentially regulated in PDR and to quantitate the levels, the depleted serum was analysed by 2D-DIGE.

Serum sample from DM and PDR was labelled with cy3 and cy5 dyes. These were mixed and resolved by 2D PAGE and scanned. Figure 1.2 shows the comparison of the 2D profile of Cy3 and Cy5 labelled samples as well as the overlay of these images.

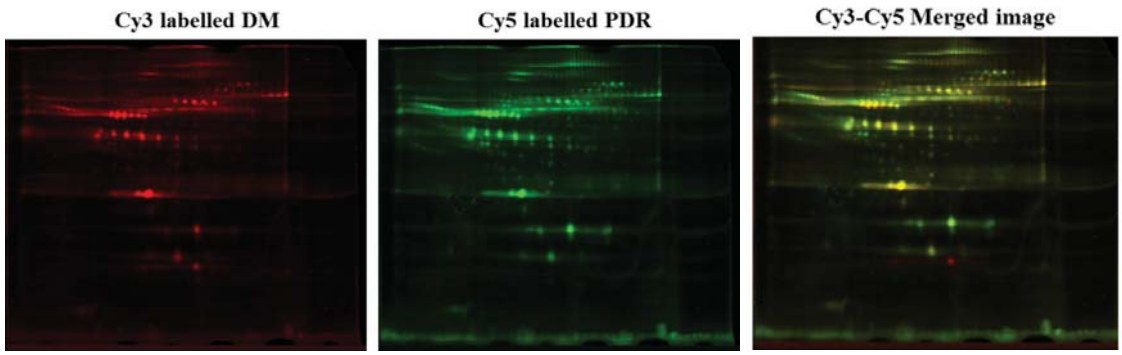


Fig 1.2. Comparative serum proteome analysis by 2D-DIGE. Albumin and IgG depleted serum samples from DM and PDR patients were labelled with Cy3 and Cy5, respectively and resolved by 2D PAGE. Gels were scanned and the scanned image was overlaid to identify the differentially expressed proteins.

Many proteins showed significant upregulation (seen as green) or downregulation (seen as orange-red spots) in the PDR sample. Study is in progress to quantify the difference in the expression level as well as characterize the post translational modifications. The type of glycans also is of significance when defining the biomarkers as shown in this study.

Project 2: Bioplex analysis of serum markers specific for DM (No DR) and PDR and further analysis of markers in various stages of DR

Objective

- To quantitate the levels of serum biomarkers- Ceruloplasmin (CP), α-1-Acid Glycoprotein (AGP) and Serum Amyloid P in serum samples of DM (No DR), NPDR, PDR and healthy controls using Bioplex assay.

Methods

In the present study, a panel of serum protein markers that are reported previously in DR patients was evaluated by Bioplex assay in patients with DM (No DR) (n=10), NPDR (n=10), PDR (n=10) and healthy controls (n=8). The diagnosis of probable DM(No DR) and NPDR were made by AEH clinicians, according to the blood glucose levels (WHO criteria) and the presence of micro aneurysms, dot or blot haemorrhages, venous abnormalities, intra retinal micro vascular abnormalities, cotton wool spots and absence of neovascularization respectively whereas PDR includes neovascularization in addition to all the above alterations. The blood samples were collected after receiving informed consents and used in accordance with the Declaration of Helsinki.

Immunoassay-Luminex measurement

The proteins which are listed in Human Neurological Disorders Magnetic Bead Panel 2(Milliplex MAP kit-HND2MAG-39K; 96-well plate format; EMD Millipore) were quantified as per the manufacturer's instruction using Luminex 200 (BioRad, USA)

Serum protein concentration in each sample was estimated using Lowry's method in 96-well plate method. The serum samples were diluted to give a final dilution of 1:25,000 as recommended in the protocol. Each assay well was first rinsed with assay buffer before sample loading. 25µl of assay buffers was added to 25µl standards, controls and samples followed by 25µl of beads to bring the total volume in each well to 75µl. The assay plates were incubated at 40C. The beads in the plates were washed twice with 200µl wash buffer and incubated for 1h with 25µl of biotinylated detection antibody. 25µl of fluorescently labelled reporter (streptavidin-Phycoerythrin) was added to each well and incubated for 30min. Finally the assay plate was washed twice with 200µl wash buffer and the beads were suspended in 100µl of sheath fluid. Then fluorescent intensities of the beads were analyzed using in-built software.

Results and discussion

There were no significant differences between the groups with regard to age or sex. The concentrations of serum ceruloplasmin (CP), α-1-Acid Glycoprotein (AGP) and Serum Amyloid P (SAP) were examined and the results are given below.

Ceruloplasmin (CP)

According to study conducted by Kim et al., 2013, AGP, increase in plasma of early grade NPDR compared to DM (No DR) was found using mass spectrometry analysis. In Rema *et al.,* 1996 study, the serum levels of AGP estimated by single radial immune diffusion assay, were not significantly different either in the group with background or proliferative diabetic retinopathy as compared to the controls or the diabetic patients without retinopathy (DM).

In this study, significant decrease in mean serum concentration of AGP was found in DM patients (1754.02±1253 µg/ml) as compared to healthy controls (3259.16±1538.6µg/ml). However, the AGP concentration was found to increase in NPDR and PDR as compared to patients with DM.

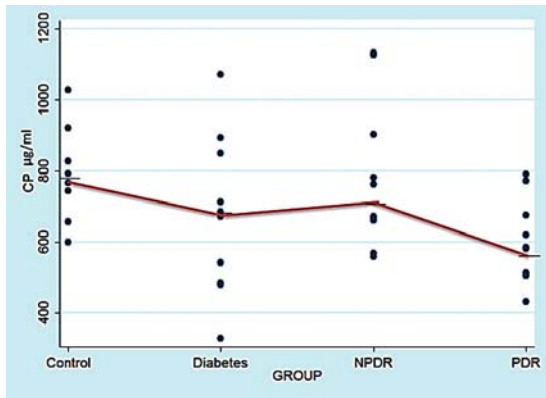


Fig 2.2. The cluster plot describes the concentration of AGP (µg/ml) in serum samples from healthy controls, DM (No DR), NPDR and PDR. Each point represents a single measurement; Horizontal bar represents medians

α-1-Acid Glycoprotein (AGP)

According to study conducted by Kim et al., 2013, AGP, increase in plasma of early grade NPDR compared to DM (No DR) was found using mass spectrometry analysis. In Rema et al., 1996 study, the serum levels of AGP estimated by single radial immune diffusion assay, were not significantly different either in the group with background or proliferative diabetic retinopathy as compared to the controls or the diabetic patients without retinopathy (DM).

In this study, significant decrease in mean serum concentration of AGP was found in DM patients (1754.02±1253 µg/ml) as compared to healthy controls (3259.16±1538.6µg/ml). However, the AGP concentration was found to increase in NPDR and PDR as compared to patients with DM.

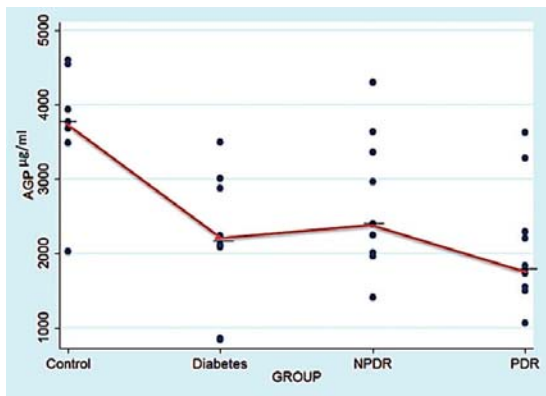


Fig 2.2. The cluster plot describes the concentration of AGP (µg/ml) in serum samples from healthy controls, DM (No DR), NPDR and PDR. Each point represents a single measurement; Horizontal bar represents medians

Serum Amyloid P (SAP)

In a study conducted by Nelson *et al.,* 1991, normal reference interval for serum amyloid P component (SAP) concentration in the serum was established in 500 healthy adult individuals (274 women, 226 men), by electroimmunoassay. The SAP (mean±SD) concentration was significantly lower in women (24±8 µg/ml), than men (32±7 µg/ml) ($P < 0.001$). The values observed in the present study sample is given in the table below.

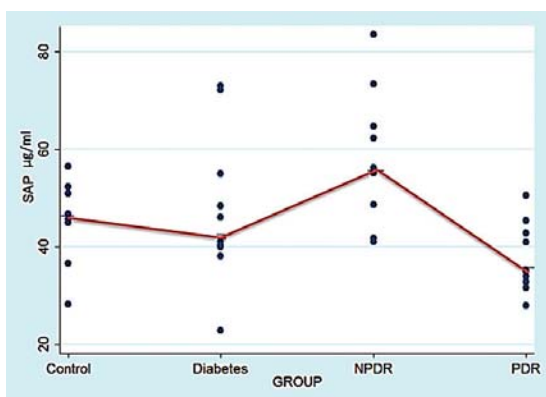


Fig 2.3. The cluster plot describes the concentration of SAP (µg/ml) in serum samples from healthy controls, DM (No DR), NPDR and PDR. Each point represents a single measurement; Horizontal bar represents medians

S.No	Gender	Age	Sex	Mean Protein conc (µg/ml)	SAP concentration (µg/ml)
	Female				
1	F1	48	F	51.2	28.20
2	F2	48	F	57.6	46.72
3	F3	50	F	59.3	52.35
4	F4	55	F	56.0	45.74
5	F5	64	F	75.3	44.96
	Male				
6	M1	62	M	58.9	56.56
7	M2	45	M	54.9	50.93
8	M3	55	M	51.2	36.65

Even though there were no significant differences between the groups with regard to sex, significant changes were found in SAP levels among groups. Especially SAP concentration is strikingly higher in NPDR as compared to all the other three study groups.

Summary

The team has successfully estimated the previously reported serum markers in the study patients using Bioplex assay. Further studies are underway to quantify other reported putative serum biomarkers for DR patients.

References

- Kyunggon Kim, sng Jin Kim, Dohyun Han, Jonghwa Jin, Jiyoung Yu, Kyong Soo Park, Hyeong Gon Yu and Youngsoo Kim. Verification of multi markers for detection of early stage of Diabetic Retinopathy using multiple reaction monitoring. Journal of Proteome 2013; 12:1078-1089.
- Nelson SR, Tennent GA, Sethi D, Gower PE, Ballardie FW, Amatayakul-Chantler S, et al. Serum amyloid P component in chronic renal failure and dialysis. Clin Chim Acta 1991; 200: 191–200.
- Rema M, Mohan V, Snehalatha C. Acute phase serum proteins in diabetic retinopathy. Indian journal of Ophthalmology 1996; 44(2) 83—85.
- Sarkar A, Dash S, Barik B K, Muttigi M S, Kedage V, Shetty J K and M Prakash. Copper and ceruloplasmin levels in relation to total thiols and GST in Type 2 diabetes mellitus patients. Indian journal of clinical biochemistry 2010; 25 (1) 74-76.

OCULAR PHARMACOLOGY

The main research focus of the department is to understand the molecular mechanism(s) involved in the pathogenesis and to identify newer or modified therapeutic drug targets for ocular diseases such as diabetic retinopathy, age-related macular degeneration and glaucoma.

The department is currently engaged in bringing out newer IOP lowering drugs using a newly established ex vivo model system called human organ culture anterior segment (HOCAS) system. Unlike the currently available anti-glaucoma drugs, the newly developed class of drugs act on the cytoskeleton assembly of the target tissue, the trabecular meshwork (TM) which is getting affected during glaucoma. This would be a potential class of drugs for the clinical management of glaucoma with better IOP control.

Effect of SB77, a Rhokinase inhibitor on human aqueous outflow facility

Investigators	:	Dr. S. Senthilkumari, Dr. SR. Krishnadas
International Collaborators	:	B'Ann Gabelt and Prof. Paul L.Kaufman, Department of Ophthalmology and visual sciences, University of Wisconsin, Madison, USA
Funding Source	:	Aravind Eye Foundation (AEF), New York

Background and aims

Glaucoma is the second leading cause of blindness in India and world. Primary open angle glaucoma (POAG) is the most predominant form of glaucoma the world over, accounting for 74% of those affected. Over the past 60 years, it has been implicated that TM dysfunction is observed in patients with POAG. Currently available drugs do not act on the affected tissue, the trabecular meshwork. Drugs that modulate the aqueous outflow by acting on the TM will be more beneficial in reducing IOP effectively. Development of new candidate drugs that alter trabecular outflow of aqueous and regulate and reduce IOP is a possible therapeutic alternative for management of glaucoma and prevention of blindness.

Human Organ Culture Anterior Segment (HOCAS) system serves as an *ex vivo* model system to study the drugs/investigational agents that have the potential to alter the conventional outflow facility by reducing resistance in TM. The present study was designed to establish HOCAS and to study the effect of SB77, a potent Rho kinase inhibitor (RKI) on outflow facility.

Methods

Inclusion Criteria

- Donor eyes with no history of ocular diseases and cancer, Both gender & age-group: 50–95 yrs, Enucleation time not exceeding 5 hours

Exclusion Criteria

- Donor eyes with history of ocular diseases, Enucleation time more than 5 hours

The entire globes of human donor eyes not suitable for corneal transplantation were obtained from Rotary Aravind International Eye Bank of Aravind Eye Hospital and were handled in accordance with the Declaration of Helsinki. Baseline outflow facility (OF) was measured in paired eyes. One eye of each pair was exchanged with either 2μM Lat B (actin microfilament disrupting drug, increases Ctrab in monkeys) or 10μM SB77 followed by continued perfusion with the medium containing the test drug. Contralateral eyes received the vehicle. The intraocular pressure (IOP) was monitored for 24 hours post treatment; the eyes were then fixed by perfusion using 4% paraformaldehyde and the aqueous outflow tissues were assessed using light microscopy.

Results

The mean (±SD) donor age was 45.4.6±14.5 years and the elapsed time between enucleation and perfusion of the anterior segment was 24.0±14.3 hours. 2μM Lat B increased OF by 70% ($p<0.1$) as compared to baseline and corrected for contralateral control (N=4) while 10μM SB77 increased OF by 35% (N=6) in 24 hours ($p<0.05$).

Drug	Outflow Facility (μl/min/mmHg)		OF Ratio	P value
	Treated	Control		
2μM Lat B (N=4)				
BL	0.21 ± 0.07	0.21 ± 0.02	0.96 ± 0.30	-
Rx-3hrs/BL	1.71 ± 0.44	1.16 ± 0.15	1.46 ± 0.24 *	P<0.1
Rx-12hrs/BL	1.86 ± 0.63	1.07 ± 0.11	1.72 ± 0.42 *	P<0.1
Rx-24hrs/BL	1.81 ± 0.68	1.04 ± 0.34	1.70 ± 0.48 *	P<0.1
10μM SB77 (N=6)				
BL	0.25±0.05	0.31±0.15	0.98 ±0.42	-
Rx-3hrs/BL	0.29± 0.08	0.31 ±0.13	1.05 ±0.38	P<0.8
Rx-12hrs/BL	1.30± 0.21	1.10± 0.13	1.21 ±0.29 *	P<0.1
Rx-24hrs/BL	1.43± 0.27	1.06 ±0.14	1.36 ±0.32 *	P<0.05

** Statistical significance; Two-tailed paired t-test*

Summary

SB77 at the studied concentration was effective in enhancing OF in HOCAS and thus may be a potential clinical candidate for the management of glaucoma therapy.

Characterization of primary human trabecular meshwork cells

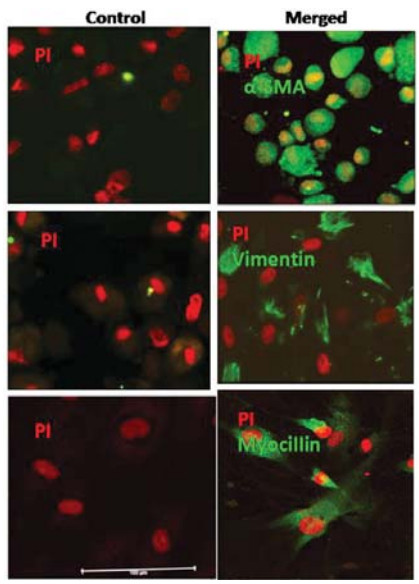
Investigator : Dr. S. Senthilkumari
Project fellow : M. Uthaya Lakshmi
Funding source : Aravind Eye Foundation (AEF), New York

Background and aims

The trabecular meshwork (TM) accounts for most aqueous outflow resistance in the anterior chamber of the human eye. It is believed that the interplay between the endothelial-like cells of this tissue (trabecular cells) and the surrounding extracellular matrix are responsible for maintaining the resistance necessary for preservation of the aqueous outflow pathway. The trabecular cells are phagocytic cells, actively removing debris such as pigment granules, erythrocytes, and pseudoexfoliation material from the aqueous outflow system. They are also involved in the production and turnover of extracellular matrix. Studies of trabecular cell function using animal systems in *vivo* have been difficult, and it is possible that human trabecular cells may have different properties from cells which line the outflow pathway in animals. However, establishing these cells in culture system will help in understanding the molecular mechanisms involved in POAG development and drug response. Therefore, the objective of the present study was to establish human trabecular meshwork cell culture and to characterize them with suitable markers for further studies.

Methods

The TM explant culture was established successfully from the post-mortem donor eyes as the method described by Polansky et al (1979). The human TM cells were characterized with selected cytoskeletal (alpha smooth muscle actin), transmembrane (Vimentin) and dexamethasone induced myocillin protein using immunocytochemistry with appropriate primary antibodies.



Characterisation of Trabecular Meshwork cells

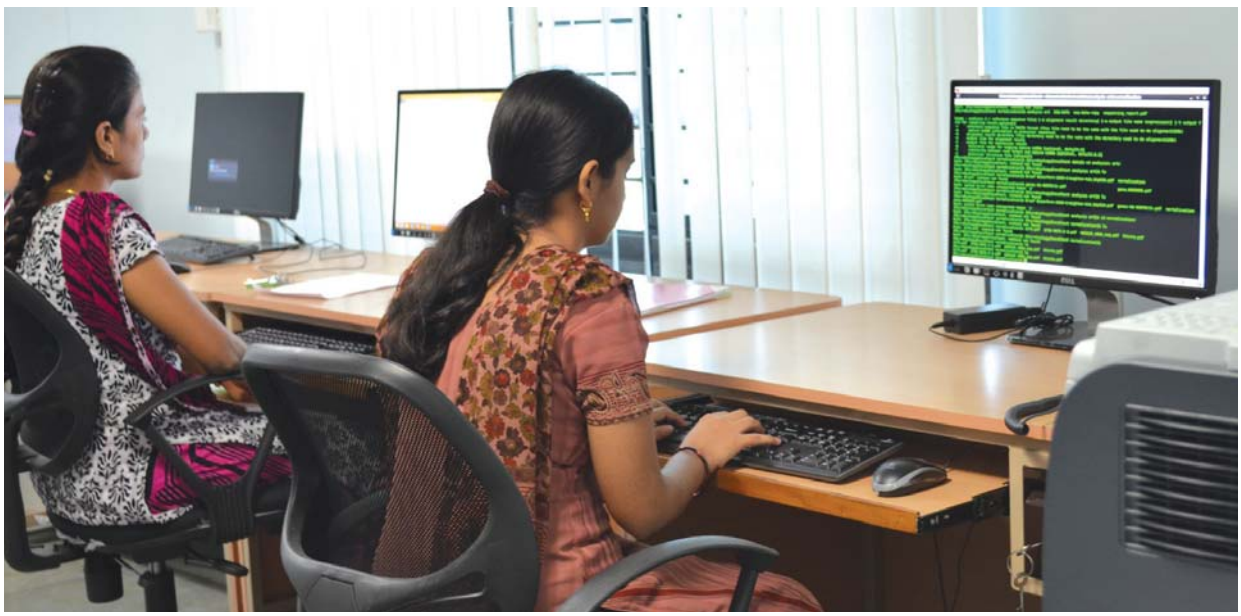
HTM cells were plated; both subconfluent and confluent cells were examined. They were fixed with cold 4% buffered paraformaldehyde for 15 minutes, blocked with Avidin-biotin blocking solution for 10 minutes and incubated overnight with primary antibody at 25°C (Primary antibody-vimentin, α-SMA, myocilin (Dilution -1:100), antibody-1µl, 0.1% triton X 100-1µl, 5% BSA-98µl). For the secondary antibody, cells were incubated with biotinylated secondary antibody (Goat anti-rabbit IgG & Goat anti-mouse IgG biotinylated (Santacruz, USA) for 1 hour at 25o C. Then tertiary antibody streptavidin incubated with Streptavidin-FITC (1:1000 in PBS) for 1 hour in dark, and incubated with RNase for 30 minutes at 37°C. Finally cells were incubated with propidium iodide (1:1000 in RNase solution) for 10 minutes and mounted with vectashield (Vector Laboratories Burlingame, CA, USA). The expression of different markers was observed under fluorescence or confocal microscope. For myocilin detection, an additional complement of cells was grown to confluence in growth factor supplemented media. They were then given media with 10% serum and treated with 100nM dexamethasone (DEX) for 7-10days to induce greater expression.

Summary

The adult TM cells were successfully isolated, cultured and characterized for its future use in understanding the cytoskeletal remodeling in response to actin-cytoskeletal drugs which would help us in studying the molecular mechanism involved in maintaining homeostasis of intraocular pressure and aqueous humor outflow pathway by these cells.

BIOINFORMATICS

Research activities at Bioinformatics Department are directed towards two major areas, i) Structural Bioinformatics. This uses structural bioinformatics approach to the analysis of Single Nucleotide Variants (SNVs) and prediction of its association with retinal dystrophies. This analytical approach will help create a platform to understand the pathogenesis of all other genetic eye diseases that are common in India. ii)Next-generating sequencing(NGS) Data analysis: The NGS technologies enable scientists to analyse millions of DNA sequences in a single run. The hereby produced gigabytes of raw data need to be further analysed in order to gain biological meaningful results. Tools are developed and applied for the analysis for NGS sequencing data, from processing of raw data and mapping of reads to downstream statistical and bioinformatics analysis of the data. Listed below are the current ongoing projects in the above areas:



Structure and sequence - based bioinformatics approach to the analysis of non-synonymous single Nucleotide Variants (nsSNVs) and prediction of its association with Retinitis Pigmentosa

Investigator : Dr. D. Bharanidharan
Project fellows : K. Manoj Kumar, P. Logambiga
Funding Source : Aravind Medical Research Foundation

Retinitis pigmentosa (RP) is a heterogeneous group of inherited diseases characterized by progressive degeneration of the mid peripheral retina, leading to night blindness, visual field constriction, abnormal or nonrecordable electro retinogram (ERG)and eventual loss of vision. It is one of the leading causes of blindness in adults. Over 90 forms of RP have been identified. RP can be syndromic or nonsyndromic and can be inherited in an autosomal dominant, recessive, or X-linked manner as well as in digenic, mitochondrial, or simplex patterns. Most patients with RP are isolated or periodic with no known affected

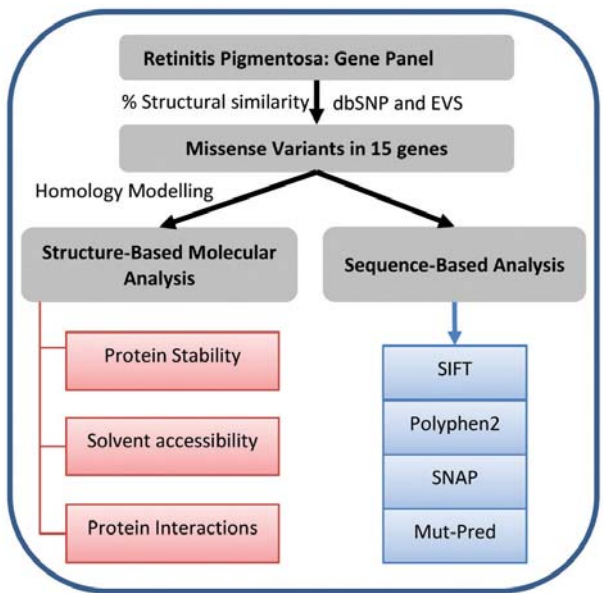
relatives, although some of these may have inheritances that are autosomal or X-linked recessive or dominant with incomplete penetrate. Despite having similar characteristics, there is a wide spectrum of clinical and genetic heterogeneity between the different modes of inheritance. Thus, the genetics of RP is complicated and clear genotype-phenotype correlations are not yet possible.

The mutations within over 144 genes have been listed as associated with RP. Most of them are missense variants and genetic, mostly in a dominant manner. Missense variants or Non-synonymous single nucleotide variants (nsSNVs) in the coding region of the proteins have become intense, that affect the structure and function of proteins by giving rise to amino acid substitutions. The effect of nsSNVs on the structure and function of proteins is of critical importance to understand the molecular mechanisms of the disease and to clarify the association between missense variants and disease phenotype. Here,bioinformatics approach was used to readily evaluate the impact of genetic variation on the structure and function of a gene product at the molecular level and predict the molecular mechanisms of the underlying disease.

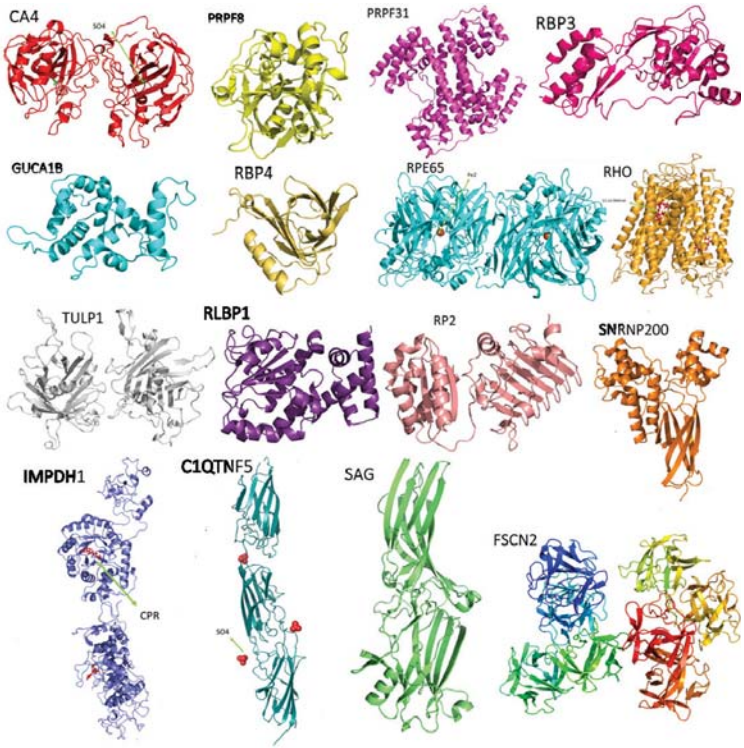
Workflow

All genes associated with RP were collected. Genes with high sequence similarity (50% against Protein Data Bank (PDB)) or have experimental protein structure was selected for further analysis as shown in the box. For genes with unknown protein, structure was modelled using Maestro software by applying comparative homology. The mutant proteins were modelled using wild type model protein as a template. The final model quality is assessed before proceeding with analysis. Structure – based analysis using wild type and mutated protein structure was used to predict the molecular causes of RP and their disease severity were predicted with the integration of sequence-based analysis.

Structure-based analysis



Protein wild and mutant structure was used to analyse protein stability, solvent accessibility and protein interactions. Protein stability is analysed by calculating the potential energy difference between wild type and mutant protein using Gromacs, SDM and Popmusic. Mutations which resulted in a model with a higher energy than the wildtype model are likely to be disease-causing. Therefore, a straightforward approach was taken for classifying a mutation as disease-causing or non-disease-causing depending on its energy difference: a mutation is disease-causing, if its energy difference is positive and if it belongs to the ten mutations with the highest energy difference. Solvent accessibility is the ratio between the solvent accessible surface area of a residue in a three dimensional structure and in an extended tri-peptide conformation. Machine learning-based programme such as Naccess and ASA View is used to predict the solvent accessibility of the amino acid residue. Protein Interaction is predicted using various features such as protein 3D structure, sequence and physicochemical properties. The interaction between proteins and other molecules such as small molecule, DNA and RNA is fundamental to all protein function. MetaPPI, MetaDBsite, MetaPocket are the different servers used to predict the protein binding site and the energetics analysis predicts the effect of amino acid substitution on protein interaction.



Sequence-Based Analysis

Four different prediction programmes, SNAP, SIFT, PolyPhen2 and Mut-Pred were used. To avoid false positives, the variants were selected from the intersection at least three programs.

16 genes were selected for analysis based on the 50% of structure similarity with PDB. More than 1155 nsSNVs from dbSNP and EVS server were culled for these genes and further analysed based on their structure and sequence. Comparative homology modelling was applied to get the model of all 16 (shown in Figure). Structure-based analysis of all patient-specific mutations reported in RHO gene, affects the protein stability or protein binding with RP phenotype.

However, the mutations from Congenital Stationary Night Blindness (CSNB) phenotypic peoples affect only the retinal binding site. These findings have important implications for the disease phenotypes. Currently, all missense mutations are being analysed with sequence and structure – based analytical approach to predict their molecular causes and association with RP and other phenotypes.

Implication of the data

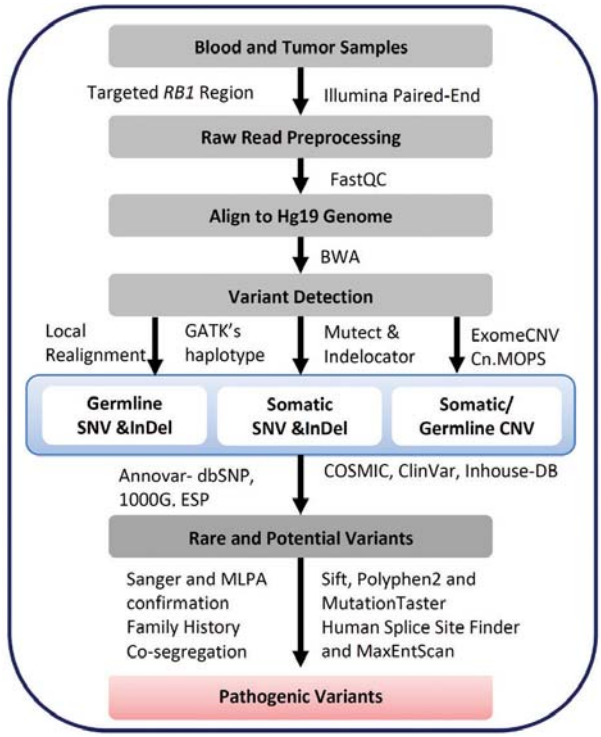
The study combined a structure-based approach with sequence-based method; and it was able to better predict the deleterious missense mutations with high accuracy from neutral. The analytical strategy described above would be helpful to understand the molecular mechanisms of diseases and its association with disease phenotype.

In-house bio-informatics pipeline to identify pathogenic variants of Retinoblastoma (RB)

Investigators : Dr. D. Bharanidharan, Dr. A. Vanniarajan, Dr. Usha Kim, Prof. VR. Muthukkaruppan
Project fellow : P. Logambiga
Funding source : Aravind Medical Research Foundation

Accurate identification of RB1 pathogenic variants in a short time is very important for diagnosis, confirmation, genetic counselling, risk assessment, and carrier screening of Retinoblastoma (RB) patients and their family members. However, genetic analysis of heterogeneous spectrum of variants in RB1 gene is no trivial task and essentially requires comprehensive approach. Target enrichment followed by next-generation sequencing offers a time-efficient and accurate approach for the molecular diagnosis of many eye diseases. However, identifying pathogenic variants is challenging as current bottleneck in next-generation sequencing shifted from sequencing to data analysis. Here, our purpose is to develop an in-house bioinformatics pipeline to identify heterogeneous spectrum of RB1 gene variants including SNVs, InDels and CNVs for the molecular diagnosis of RB.

Overview of in-house pipeline



The in-house pipeline integrates various programmes to detect both single nucleotide variants (SNVs) and small insertions/deletions (InDels) and to distinguish between somatic and germline mutations as shown in the box. Patients DNA from blood and tumor samples were sequenced using Illumina platform. Obtained paired-end raw reads were quality filtered using fast QC tool. The filtered reads were mapped to Hg19 reference sequence using Burrow-Wheeler Aligner (BWA version 0.7.5a-r405). Resulting BAM files were locally realigned using GenomeAnalysisTK-3.1-1 (GATK) Indel-realigner tool to minimize the mismatches across the reads. GATK haplotype caller was performed to retrieve germline single nucleotide variants (SNVs) and small insertions/deletions (InDels) with phred score 20 and minimum depth 5 from all the samples. MuTect-1.1.4 and GATK Indelocator tools were used to identify somatic SNVs and InDels from the tumor samples with blood matched control respectively. Wherever matched blood sample is not available, the blood sample with similar coverage was used. All the SNVs and InDels were subjected to identify rare and potential variants. The rare variants were identified using ANNOVAR by filtering common variants with alternative allelic frequency higher than 1% based on1000 Genomes project, dbSNP135 and ESP. Of those, non-synonymous/ synonymous SNVs, coding InDels, and intronic variants that were less than 10 bp beyond the canonical splice site junction were selected. The potential variants were identified using ClinVar, COSMIC and In-house (reported pathogenic variants) databases. The detection of germline SNVs and Indels was fully automated. Detected variants were further manually assessed with the help of IGV-2.3.25 viewer to avoid mapping errors. In order to identify pathogenic variants, the following criteria was used. i) known pathogenic variants; ii) if not, variants that could give rise to premature protein termination, frameshift, canonical splice site alterations and large exonic deletions; iii) nonsynonymous SNVs if Sift, Polyphen2 and MutationTaster all suggested pathogenic, and iv) splice variants selected from both Human splice site finder and MaxEntScan.Patients with no pathogenic variants were further analysed for copy number variations (CNVs). ExomeCNV was used to detect somatic CNVs from the tumor/blood pairs as described above,whereas, Cn.MOPS was used to detect the germline CNVs in the blood samples. Log Ratios (LogR) ≥ ±1 were set for Deletion/Duplication analysis in both the tools; median LogR score were used for Cn.MOPS. All the pathogenic variants were further confirmed by conventional methods and co-segregation. Somatic events were re-confirmed by their absence in the same patient blood sample.

Pathogenic variants in thirty-three patient samples were analysed. An array of pathogenic variants including SNVs, InDels and CNVs were detected in 28 patients. Among the variants detected, 63% were germline and 37% were somatic. Interestingly, nine novel pathogenic variants (33%) were also detected in the study. Thus, this approach, achieving a diagnostic rate of 85%, proved to be efficient for the molecular diagnosis of RB.

Implication of the data

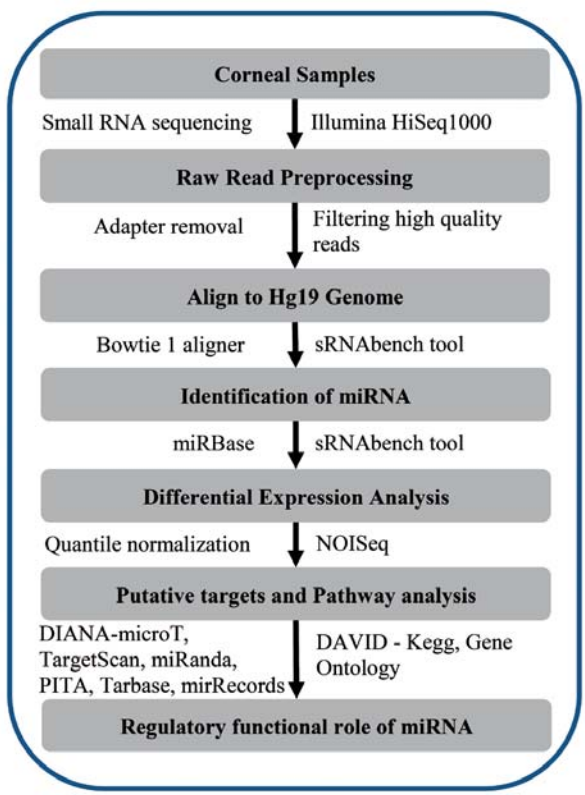
It was reported for the first time that this approach could detect germline and somatic variants including novel pathogenic variants in the RB1 gene. It was demonstrated that this approach could detect copy number variations (CNVs) in RB1 gene. This comprehensive approach reduces the time and number of assays required for the pathogenic variants by conventional methods. This approach is sensitive and efficient for RB1 screening. Further studies are necessary for the establishment of this approach in terms of cost-effectiveness.

Target gene prediction and functional analysis of differentially expressed MicroRNAs in fungal keratitis

Investigators : Dr. D. Bharanidharan, Dr. M. Vidyarani, Dr.P. Lalitha
Research associate : B. Hemadevi
Funding source : Aravind Medical Research Foundation

MicroRNAs (miRNAs) are small, stable non-coding RNA molecules with regulatory function and marked tissue specificity that post-transcriptionally regulate gene expression, and they can be involved in regulation of pathogenesis of fungal keratitis. The differential expression of miRNAs during the pathogenesis of fungal keratitis is expected to provide the insight into their roles in regulating the immune response to avoid excessive inflammation and to allow for corneal wound healing. Here, the regulatory functions of miRNAs in the pathogenesis of fungal keratitis from miRNA expression profiles were studied and to predict their function, the target genes were predicted, and then the functions were analysed via bioinformatics. This study was based on the miRNAs expression profile in human cornea from fungal keratitis patients that were obtained by Illumina deep-sequencing method.

Overview of analysis pipeline



Small RNA deep-sequencing and functional analysis were performed on pooled corneal sample of five fungal keratitis patients infected with *Aspergillus flavus* compared to pooled normal from three cadaver cornea as shown in the box. Small-RNA sequencing was performed on total RNA of infected and control corneas using illumina HiSeq1000 platform. Raw data obtained in the FASTQ format were quality filtered and adapter trimmed using our perl script, allowing no mismatches for adapter identification. The sequencing data were further aligned to Homo sapiens hg19 genome reference allowing for one mismatch using bowtie1 aligner in the sRNAbench tool. Next, this tool identified the validated miRNAs from the miRBase (release 18). For the prediction of novel miRNAs, machine learning algorithm in sRNAbench tool was used allowing no mismatches with other default parameters. miRBase was used for the miRNA current naming conversion where we followed the -3p or -5p nomenclature. For differential expression analysis, miRNAs with same isoMIRs were considered together and less represented miRNAs (0.01% of the total reads)

were eliminated from analysis. Further, the analysis was performed using NOISeq R package on the quantile normalized reads (Bioconductor 2.14 with R version 3.1.0). The method of NOISeq applied was NOISeqsim, which assumes no replicates for the experimental conditions. The variable expression of miRNAs between patients and controls was considered significant when the fold change was >2 and the probability score was>0.9.

DIANA-microT, TargetScan, miRanda, and PITA were used for the target gene prediction of differentially expressed miRNAs using default parameters. The targets that represent the intersection of at least 3 algorithms were selected to avoid false positives. Together with these targets, the experimentally validated targets available in the Tarbase and mirRecords database were included. The target genes were further filtered as putative target genes based on the corneal proteome database that was created from reported human corneal proteins under normal and any corneal diseases. In order to predict the functions of these miRNAs in the regulation of disease pathogenesis, the functional analysis of putative target genes was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID).The putative target genes were grouped into functional categories using GO term and KEGG pathways. P-value <0.05 and false discovery rate (FDR) <0.05 were used as a threshold to select significant GO categories and KEGG pathways. Interactions between putative target genes with GO categories of significant differentially expressed miRNAs were used to construct miRNA-regulated protein interaction networks. Then, Cytoscape (Version 3.2.0) was applied tovisualize the networks.

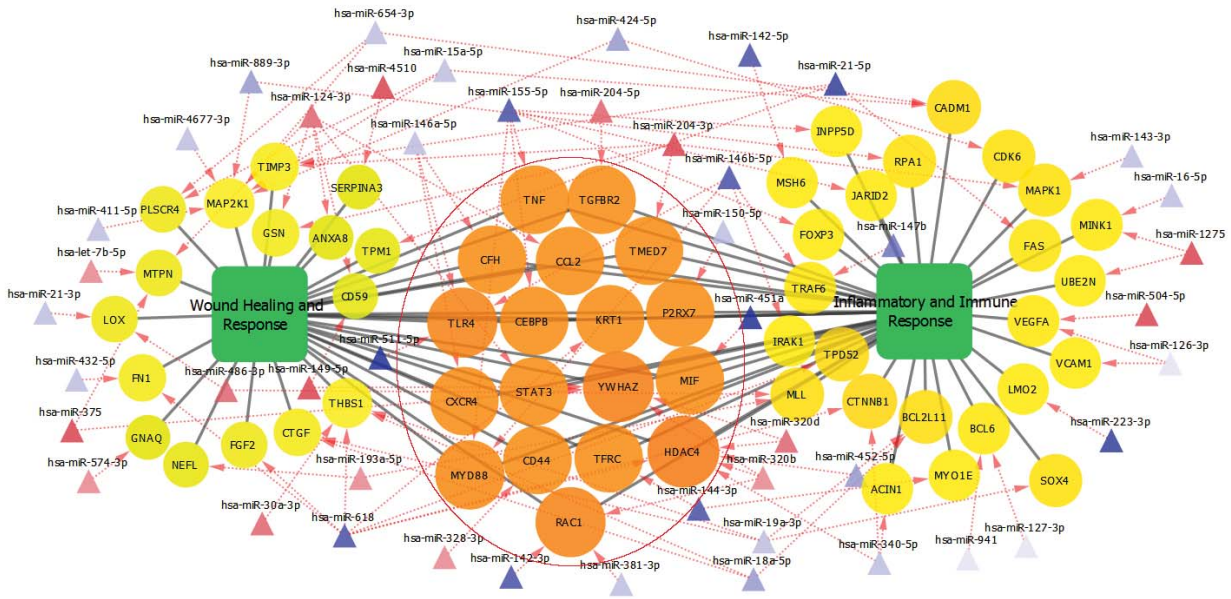
A total of 75 miRNAs greater than >2 fold were significantly differentially expressed in pooled keratitis corneas compared to normal: 43 miRNAs were up regulated and 32 were down regulated. More than 590 target genes were predicted for 75 miRNAs. Combining KEGG analysis and GO term with target prediction for these miRNAs, the regulatory functions of miRNAs were predicted. Following table shows the highly regulated pathways, generally involved in immune responses, cell migration, adhesion, and cell proliferation and wound healing.

KEGG Pathway Name	Gene Counts	P-Value	FDR
hsa04510:Focal adhesion	34	3.76 x 10-10	4.43 x 10-7
hsa04620:Toll-like receptor signaling pathway	23	1.76 x 10-9	2.07 x 10-6
hsa05200:Pathways in cancer	43	3.77 x 10-9	4.43 x 10-6
hsa05212:Pancreatic cancer	18	3.74 x 10-8	4.40 x 10-5
hsa05130:Pathogenic Escherichia coli infection	16	5.10 x 10-8	6.00 x 10-5
hsa04810:Regulation of actin cytoskeleton	30	4.19 x 10-7	4.93 x 10-4
hsa04722:Neurotrophin signaling pathway	21	1.81 x 10-6	0.002
hsa04670:Leukocyte transendothelial migration	20	3.40 x 10-6	0.004
hsa04666:Fc gamma R-mediated phagocytosis	17	1.15 x 10-5	0.013
hsa04520:Adherens junction	15	1.66 x 10-5	0.019

Functional annotation with GO terms showed that more than 46 significant differentially expressed miRNAs were regulating inflammatory and immune responses, and wound healing and responses. As shown in the figure 1, miRNA with high differential expression regulates the genes involved in wound inflammation (red circle).

Upregulated has-miR-21-5p, has-miR-223-3p, hsa-miR-142-5p, hsa-miR-155-5p, has-miR-511-5p were found to highly regulate inflammatory and immune responses, regulating Toll like receptor signalling pathway. Hsa-miR-451a with an increased expression (more than 8 fold, darkest blue colour in Figure 1) in keratitis may have a role in wound healing by targeting Macrophage Migration Inhibitory Factor (MIF), which is reported as a direct target of has-miR-451a.

Figure 1. miRNA - regulated target gene network for the functional GO terms of inflammatory and immune response, and wound healing and response. The target genes were annotated with closeness centrality (marked with red circle) between GO terms using Cytoscape 3.2.0, reported to be involved in wound inflammation during the pathogenesis of fungal keratitis. The intensity of triangle colour corresponds to the level of fold change. Red colour: down regulated; Blue colour: up regulated miRNAs in Fungal keratitis.



Implication of the study

Several miRNAs with high expression in fungal keratitis corneas point towards their role in regulation of pathogenesis. The combination of putative gene target prediction and pathway analysis for significant differentially expressed miRNAs allowed to understand their role in the regulation of pathogenesis. This study provides first report of miRNAs role in fungal keratitis and support further studies for deeper understanding of molecular mechanisms of gene expression by specific miRNA, which would help design new therapeutic strategies.

MICROBIOLOGY

The department of ocular microbiology focuses on the characterization of ocular pathogens and the host-pathogen interactions in the context of ocular infections. The virulence, persistence and antibiotic resistance mechanisms of clinical ocular isolates are probed using advanced technologies including whole genome sequencing. Intracellular survival of pathogens and the host response to infection are studied using *invitro* cell culture models, whereas the actual disease pathogenesis is explored using ocular tissue samples *exvivo.* A recent analysis of the corneal microRNA expression profile in fungal keratitis gave an insight on the regulatory role of small RNAs in corneal inflammation and wound healing. Ocular cytokine and chemokine profiles are being determined in uveitis patients with a view to identify potential biomarkers and to understand the disease pathogenesis. Since ocular infections remain a major cause of vision loss in our country, advanced therapeutic strategies need to be developed with an understanding of the underlying molecular events and regulatory networks. The research done at the ocular microbiology department aims to address these important aspects of disease by probing the host-pathogen interactions both at the cellular and molecular level.

Characterization of the virulence determinants of *Pseudomonas aeruginosa* causing ocular infections

Principal investigator : Dr. Vidyarani Mohankumar
Co-investigator : Dr. LalithaPrajna
Research scholar : J. Lakshmi Priya
Funding agency : Aravind Medical Research Foundation

Bacterial corneal infection is an important cause of keratitis, among which *Pseudomonas aeruginosa* (64%, Lalitha *et al.,* 2014), is the predominant causative agent. *Pseudomonas* keratitis is a serious ocular infection which could lead to corneal scarring and severe visual disability. The pathogenesis caused by *P. aeruginosa* is due to the production of several cell-associated, extracellular virulence factors and the secretion systems like Type III secretion system (T3SS). Our earlier studies indicate that

the invasive strains carrying exoS gene are predominant in ocular infection. The purpose of this study is to determine the expression of bacterial proteases in ocular *P. aeruginosa* isolates and to study the intracellular survival and clearance of *P. aeruginosa* strains in human corneal epithelial cells (HCET).

Analysis of protease gene expression in ocular *P. aeruginosa* isolates

The proteases secreted by the clinical isolates of *P. aeruginosa* mediate host cell death, degradation of stromal collagen, cleavage of host cell surface molecules, or induction of inflammatory response. To study the gene expression of Elastase B (lasB), Alkaline protease (apr), Protease IV and *Pseudomonas aeruginosa* small protease (PASP), 35 *P. aeruginosa* isolates from keratitis patients were initially screened for the presence of these protease genes by conventional PCR. The genes coding for apr, lasB, PASP and protease IV were present in all the isolates tested. Among the 35 isolates, fourteen were selected based on the T3SS genotypes and final clinical outcome for further analysis by Real time PCR. Reference strains PAO1 and PA14 were used as controls. The relative expression levels of the protease genes differed considerably between the isolates at the mRNA level. We found that invasive strains (exoS) & T3SS negative strains had increased Protease 4 production compared to cytotoxic strains. The expression level of protease IV was inversely proportional to the extent of cytotoxicity exhibited by these isolates in HCET cells. Such a difference at the level of gene expression was not appreciable with other proteases.

Intracellular survival of *P. aeruginosa* in human corneal epithelial cells

Although *P. aeruginosa* is generally considered as an extracellular bacterium, it has been shown to invade and replicate inside many cell types. Intracellular bacteria can be cleared by the traditional phagolysosome fusion or by additional mechanisms like autophagy and/LC3 associated phagocytosis. To study if *P. aeruginosa* induces autophagy in corneal epithelial cells, LC3-GFP transfected HCET cells were infected with *P. aeruginosa* isolates and autophagy was monitored after 1h of infection, using a Leica TCS SP8 confocal microscope. EBSS treated (amino acid starvation) HCET cells were used as positive control for autophagy. To study the intracellular survival and replication of *P. aeruginosa,* HCET cells were infected with strains belonging to different T3SS genotypes (Invasive (ST) / Negative (N), two isolates / group)in the presence of EBSS or inhibitors like 3 – methyl adenine (3MA; 3mM in EBSS) or Chloroquine (CQ; 20μM, in complete medium (CM)). After 3h, the extracellular bacteria were killed with gentamycin and the cells were incubated for another three hours to allow for intracellular bacterial replication. Diluted cell lysates were plated on to MacConkey agar, and the colonies were counted after an overnight incubation at 37°C. The experiments were repeated thrice in triplicates.

The HCET cells upon infection with *P. aeruginosa* showed an increased LC3 punctation, which was comparable to the EBSS treated cells (Fig 1). Since LC3 is a classical marker for autophagosome formation, these results indicate that *P. aeruginosa* induces autophagy in corneal epithelial cells. When the intracellular survival of different T3SS strains was compared, the bacterial load was relatively higher in T3SS negative strains when the cells were pretreated with the autophagy inhibitors, 3MA/CQ (Fig 2).The invasive (ST) strains did not show such a significant difference upon induction /inhibition of autophagy.

Implication of the data

The differential expression of proteases between cytotoxic and invasive *P. aeruginosa* strains may have an implication in determining the pathogenicity of the isolates. Autophagy induced by *P. aeruginosa* may differentially regulate the intracellular survival and clearance of strains with different T3SS genotypes, and it may represent an important host protective mechanism during human corneal infections.

Publication

- Jeganathan Lakshmi Priya, Lalitha Prajna, Vidyarani Mohankumar “Genotypic and phenotypic characterization of *Pseudomonas aeruginosa* isolates from post-cataract endophthalmitis patients” Microbial Pathogenesis.2015; 78: 67e73.

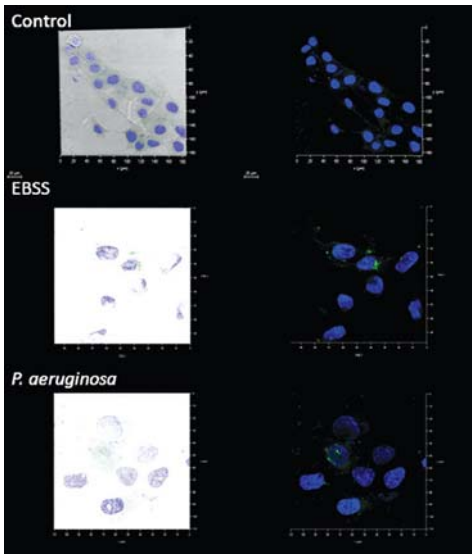


Fig 1: Formation of LC3-GFP puncta in P. aeruginosa infected and EBSS treated HCET cells

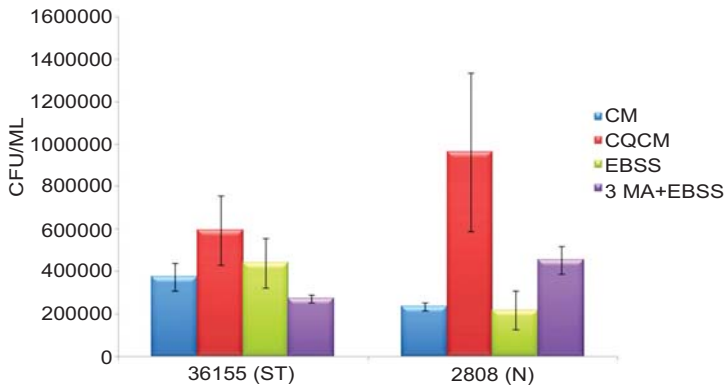


Fig 2: Intracellular survival of P. aeruginosa upon activation / inhibition of autophagy

Human corneal microRNA expression profile in Fungal Keratitis

Investigators : Dr. Lalitha Prajna, Dr. D. Bharanidharan, Dr. M. Vidyarani
Research-associate : Dr. B. Hemadevi
Funding agency : Aravind Medical Research Foundation

MicroRNAs (miRNAs) are small, stable non-coding RNA molecules with regulatory function and marked tissue specificity that post-transcriptionally regulate gene expression, however their role in fungal keratitis remain unknown. Fungal keratitis is an infectious disease of the cornea that is characterized by inflammation, often with stromal infiltration by leukocytes, and is considered an ophthalmic emergency requiring immediate attention. Patients with fungal keratitis continue to lose vision and suffer ocular discomfort while natamycin, which is still the drug of choice for antifungal treatment is ineffective during late stages of the disease. Therapeutic keratoplasty performed for fungal keratitis is known to have a poorer prognosis than similar surgeries done for bacterial keratitis. Our purpose was to identify the miRNAs in human cornea from fungal keratitis patients and understand their key role in regulation of pathogenesis.

Corneal samples from normal cadaver (n=3) and fungal keratitis (n=5) patients were pooled separately and total RNA was extracted using mirVana miRNA isolation kit. Deep sequencing was done using IlluminaHiSeq1000 platform to identify miRNA profile. Selective miRNAs were validated by real-time RT-PCR (Q-PCR) with normal cadaver (n=6) and fungal keratitis (n=6) corneas. We identified seventy five differentially expressed miRNAs in fungal keratitis corneas compared to controls. Among them 43 were up-regulated and 32 were down-regulated. MiR-21-5p, miR-223-3p, miR-146b-5p, miR-155-5p, miR-511-5p were found to be involved in inflammatory and immune responses, regulating toll like receptor signaling pathways, which is of particular interest. MiR-451a with an increased expression in keratitis may have a role in wound healing by targeting Macrophage Migration Inhibitory Factor (MIF). One novel miRNA was also detected in human cornea.

Implication of the data

MicroRNAs with an altered expression in fungal keratitis corneas may have role in the regulation of disease pathogenesis. Further insights in understanding the role of miRNAs in wound healing and inflammation may help design new therapeutic strategies.

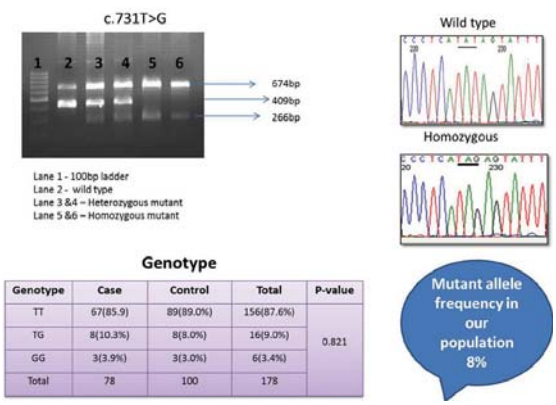
Elucidating the association of TLR4 and Dectin-1 gene single nucleotide polymorphisms in microbial keratitis

Principal investigator : Dr. P. Sundaresan
Co-investigator : Dr. LalithaPrajna
Research-associate : Dr. B. Hemadevi
Funding agency : AMRF (Submitted to ICMR)

Microbial keratitis is a leading cause of ocular morbidity and blindness worldwide, especially in developing countries such as India. In South India, approximately half of all infectious corneal ulcers are of fungal etiology. Polymorphisms in pathogen recognition receptors (PRRs), mainly TLRs and Dectins are associated with fungal infections. We intended to identify the association of genetic variations with disease progression and clinical outcome in corneal ulcer patients by screening single nucleotide polymorphisms in the PRRs, TLR4 and Dectin-1. Peripheral blood was collected from 78 culture proven fungal keratitis patients as well as from 100 healthy volunteers. Genomic DNA was isolated using salting out method. The Y238X polymorphism was screened using Bi-PASA (Bidirectional-PCR amplification of specific alleles) technique, which is a simple and rapid method for detecting the zygosity of known mutations in a single PCR reaction. To cross check the Bi-PASA PCR result, Bi-Directional sequencing was also employed. TLR4, rs10983755 polymorphism was screened using PCR-RFLP. The 304bp PCR products were restriction digested using 2 U of restriction enzyme HpyCHIV. Stata 11.0 version was used to predict the statistical association of genotypes with the phenotype. Screening of dectin-1 c.731T>G (Y238X) revealed the presence of mutant genotypes (TG-10.3% & GG-3.9%) in fungal keratitis patients. However both the mutant genotypes were also present in unaffected controls (TG-8% & GG 3%) (Fig1). TLR4, rs10983755 polymorphism screening revealed that mutant genotypes were GA-16.7% & AA-15.4% in cases and in controls GA-13% & AA-13% (Fig 2). The mutant allele frequencies in Indian population are 8% & 21% for Dectin-1 Y23X and TLR4, rs10983755 polymorphisms respectively.

Implication of the data

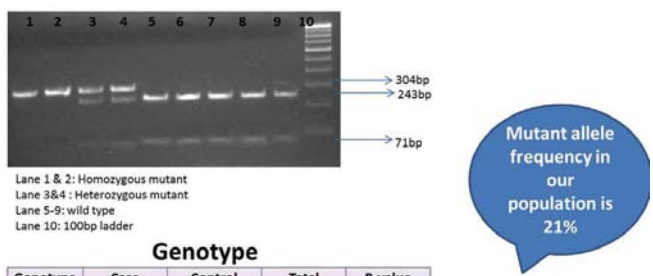
To the best of our knowledge this is the first study analyzing Dectin-1, TLR4 polymorphisms in Indian patients with fungal keratitis. In the present study there is no statistically significant association of either Y238X or rs10983755 polymorphism with the disease. The possibility of pathogenic changes being within all the exons, promoter, intronic or untranslated non coding regions of dectin-1 and TLR4 genes is currently being analyzed.



Genotype

Genotype	Case	Control	Total	P-value
TT	67(85.9)	89(89.0%)	156(87.6%)	0.821
TG	8(10.3%)	8(8.0%)	16(9.0%)	
GG	3(3.9%)	3(3.0%)	6(3.4%)	
Total	78	100	178	

Fig. 1: Dectin-1 Y238X polymorphism analysis using Bi-PASA



Genotype

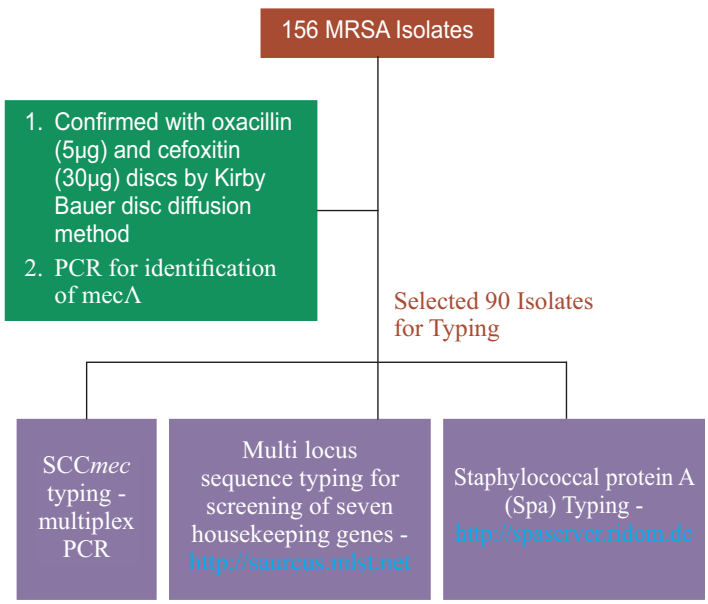
Genotype	Case	Control	Total	P-value
GG	53 (68%)	74 (74%)	127 (77%)	0.668
GA	13 (16.7%)	13 (13%)	26 (14.6%)	
AA	12 (15.4%)	13 (13%)	25 (14%)	
Total	78	100	178	

Fig. 2: RFLP analysis of TLR4 rs10983755 polymorphism

Genotypic characterization and analysis of virulence factors in methicillin resistant *Staphylococcus aureus* causing ocular infections

Principal Investigator : Dr. LalithaPrajna
Co-Investigator : Dr. A. Vanniarajan
Research Scholar : Nithya. V
Funding agency : ICMR

Methicillin resistant *Staphylococcus aureus (MRSA)* is an important cause of vision threatening ocular infections. MRSA that colonize healthy individuals and cause infections under favorable circumstances are considered as community acquired (CA). Community associated methicillin resistant Staphylococcus aureus (CA-MRSA) carry staphylococcal chromosome cassette mec (SCCmec) types IV and V, whereas hospital associated MRSA (HA-MRSA) carry SCCmec types I, II, and III. Most of the CA-MRSA strains harbor the gene coding for Panton-valentine leukocidine (PVL), which is known to be a marker for CA-MRSA. Although there are reports in the USA and Europe stating increasing prevalence, there are not many reports in India with MRSA ocular infections and their molecular epidemiology. We also reported that community acquired MRSA strains are predominantly involved with diverse clonal types. This study contributes for better understanding of MSSA to MRSA transition and their genotypic characterization.



During the period of seven years (January 2007 to December 2013), 866 isolates of *S. aureus* were collected from ocular infections. Out of these, 156 (18%) were confirmed as methicillin resistant. The percentage of MRSA infections increased from 9% to 26% (p= 0.00007) over the seven year period. Molecular characterization was done for 90 MRSA isolates that were randomly selected from the 156 ocular isolates. The majority of the isolates (63%) belonged to SCCmec type V followed by SCCmec type IV (29%). Five isolates (6%) could not be typed because the banding pattern did not match with the reference strain for the five major SCCmec types. Only two isolates were hospital associated, belonging to SCCmec type III. MLST was performed for all the 90 isolates and 61% percentage of the isolates belonged to ST772 of SCCmec type V, followed by ST22 and ST1037 of SCCmectype IV accounting for 22%. Remaining isolates belonged to ST30, ST672, ST2066, ST2124, ST8, and ST121. Most of the isolates fitted in agr type II (76%) followed by agr type I (13%) and agr type III(9%). One isolate had agr type IV with ST121and 80 strains (90%) harbored PVLgene.

Whole genome analysis of MRSA

Six MRSA isolates were selected for whole genome sequencing (WGS) based on the sequence type and clinical details. WGS was done by Illumina NEXTFlex paired end sequencing with 50X coverage.

S.No	Age/ Gender	Diagnosis	SCCmec Type	PVL	Spa Type	Sequence Type	Agr Type	Clinical Outcome
1	29/M	Lacrimal abscess	V	+ve	t657	ST772	Type II	Healed
2	71/M	Moorens Ulcer	V	-ve	t657	ST772	Type II	Not healed
3	42/M	Corneal ulcer	V	+ve	t657	ST772	Type II	TPK
4	36/M	Orbital infection	-	+ve	t1598	ST2066	Type III	DCR done
5	17/F	Corneal ulcer	-	+ve	t1598	ST2066	Type III	TPK
6	17/F	Suture Granuloma	IV	+ve	t852	ST22	Type I	Healed

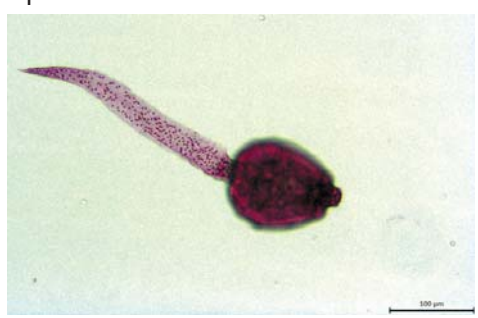
Raw data assembly was done using CLCbio workbench (ver 7.1.2) and annotation was performed with RAST server. We obtained around 2600 to 2700 ORFs for each isolate. The main purpose of this analysis is to predict the antibiotic resistant genes and phages in the epidemic clone ST772 in comparison with the other two types.

Etiology and immunopathogenesis of subconjunctival and anterior chamber granulomatous uveitis in children

Investigator : Dr. S.R Rathinam
Dr. Veena Tandon, North Eastern Hill University, Shillong
Research scholar : Lalan Kumar Arya
Funding agency : Indian Council of Medical Research

Paediatric parasitic ocular inflammation is one of the common clinical conditions in South India. Histopathological analysis and molecular methods confirmed it as trematode infection (Am Academy of Ophthalmology, 2001 and Am J Ophthalmology 2002 & Archives of ophthalmology 2012). To find out the source of infection, we analysed nearly 1500 snails from three different district of Tamilnadu from February – August 2014. Three different species of snails were identified as Indoplanorbisexustus, Paludomustranschauricus (Gmelin) and Thiarascabra based on shell morphology, by the National Zoological Survey of India. We found that among three species of snails,Indoplanorbisexustus and Thiarascabrareleased cercaria in the laboratory when provided with natural environmental conditions. Further using PCR and molecular sequencing, these trematodeswere confirmed at the species level as *Acanthostomumburminis & Isthimiophorahortensis.*

Trematode cercariae Trematode Species



Acanthostomumburminis

Isthimiophorahortensis

Cytokine and chemokine profile in aqueous humour of patients with trematode induced granulomatous uveitis

Cytokines and chemokines were measured in aqueous humour specimens obtained from patients using Bio-Plex bead based multiplex assay (17 plex kit). We included nine patients with trematode induced granulomatous uveitis presenting at Aravind Eye Hospital along with eight patients with endogenous uveitis and nine patients with no ocular pathology other than cataracts.

The trematode uveitis group showed significantly high concentration of pro inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α, & IL-12) and chemokines (MIP-1α, MIP-1β, IP-10 & RANTES) in comparison to other endogenous uveitis and cataract control groups. Kruskal Wallis analysis revealed significant differences in aqueous cytokine and chemokine concentrations among the trematode uveitis patients, endogenous uveitis patients, and the cataract controls.

The levels of type1 cytokines IL-2, interferon [IFN]- γ and IL-12, and type 2 cytokines IL-4,IL-5, IL-10, and IL-13 were detected in the aqueous humor samples. Levels of all type 1 cytokines, except IL-12, and all the type 2 cytokines except IL-4 were significantly higher in ocular trematode infection than endogenous uveitis and cataract controls. The increased levels of IL-5, IL-13 & RANTES were more specific for ocular trematode infection.

Implication of the project

The identification of biomarkers in the aqueous humor can potentially improve the diagnosis in conditions that may otherwise be considered as idiopathic uveitis. Ocular cytokine profile may, thereby, not only contribute to a closer understanding of the pathophysiological characteristics underlying uveitis but also provide guidance for the detection of new treatment targets.

CORE RESEARCH FACILITIES

MASS SPECTROMETRY FACILITY

A Core Mass Spectrometry facility is available at Aravind Medical Research Foundation with state-of-the-art high throughput Mass Spectrometers. This facility was established primarily to support proteomics research on different eye diseases. However, this facility also provides mass spectrometry services for proteomics research from other institutes and universities. It is equipped with two high performance mass spectrometers, both connected to Ultra high pressure nanoLC system.

1. Easy-nLC 1000

- Fully integrated nano-LC system that works upto 1000 bar (15000 psi.)
- Narrow column ID to increase analyte and improve detection sensitivity
- Seamless integration with state-of-the-art mass spectrometers

Service areas

- Identification of proteins and peptides in complex samples
- Detection of low-abundance peptides and proteins
- Label-free quantification of proteins
- Characterization of post-translational modifications of proteins
- Accurate quantitation of target proteins in complex mixtures
- High throughput screening of large number of samples

2. OrbitrapVelosProTMHybrid Ion Trap-OrbitrapMass Spectrometer

OrbitrapVelosProTMcombines a Thermo Scientific™ Orbitrap™ mass analyser and Velos Pro ion trap technology to deliver high resolution, speed, sensitivity, and flexibility.

- Has a resolving power up to 100,000 FWHM and mass accuracy better than 1ppm
- Fast scanning and parallel MS and MSn analysis
- Complementary fragmentation techniques—CID, HCD, and ETD—with the high mass resolution and mass accuracy
- Multiple activation types and Data Dependent Decision Tree capabilities for high-confidence peptide identifications
- Parallel acquisition capabilities to enable high-throughput sequencing
- High-resolution accurate-mass (HR/AM) capabilities for identification of unexpected PTMs

3. TSQ Quantum Ultra™ triple Quadrupole Mass Spectrometer

This MS allows Multiple Reaction Monitoring, the principal and powerful method for quantitative measurement of target proteins.

Features

- Ion Source with HESI-II probe
- HyperQuad™ Mass Filter to reduce noise and increase sensitivity
- Quantitation-enhanced data-dependent MS/MS (QED-MS/MS) for simultaneous compound confirmation and quantification
- High-resolution selected reaction monitoring (H-SRM) for quantification of many compounds in a single run
- Extended Mass Range for Large-Molecule Analysis

4. Data analysis

A dedicated computational facility for the analysis of Orbitrap MS generated high throughput data is also available along with additional support from AMRF Biocomputing Centre.

This facility includes

- One server and two workstations to handle computationally intensive workloads.
- Two dedicated network storage devices available for storing raw as well as analysed MS data.

5. Software

- Proteome Discoverer 1.4
- PEAKS studio 7
- PINPOINT 1.4
- MASCOT 2.4

Service description

Proteomics Service offered	**What is performed in the facility**
In-solution digestion	In-solution protein digest Zip-tip purification Nano-LC-MS/MS analysis Database search and report of results
In-gel digestion	In-gel protein digest Extraction of peptides Zip-tip purification Nano-LC-MS/MS analysis Database search and report of results
Peptide sequencing and protein identification	Zip-tip purification Nano-LC-MS/MS analysis Database search and report of results
Custom database searches with MASCOT and SEQUEST	Create a custom database with your specific protein sequences to be used for protein ID or modification searches

De novo peptide sequencing using PEAKS studio	De novo peptide identification for proteins from species whose genomes are not available
Label-free quantitation	LFQ based on spectral counting and intensity
Search for additional, custom or non-standard PTMs	Searches performed using user specified modifications

Data processing, results and storage

At the end of the analysis, the user will be provided with the instrument generated RAW data (.raw format), analysed data (.msf and .xlsx format) along with the sample analysis report (.pdf). Depending on the size, data will be sent to the user by email or copied onto a CD or DVD against a minimal fee. All the data will be archived into the facility's network attached storage and stored for three months.

Proteomics Service Offered	**Select one or more of these Services**
In-solution digestion	☐
In-gel digestion	☐
Peptide sequencing and protein identification	☐
Custom database searches with MASCOT and SEQUEST	☐
De novo peptide sequencing using PEAKS studio	☐
Label-free quantitation	☐
Search for additional, custom or non-standard PTMs	☐

AMRF BIOCOMPUTING CENTRE (ABC)

The Biocomputing center (ABC) has been established at Aravind Medical Research Foundation in 2014. ABC is a multidisciplinary research environment that supports collaboration to facilitate discoveries in eye-related research. It provides a core computational facility to support interdisciplinary and computational research by developing and maintain computing facilities including data storage, database development and maintenance, algorithm development, analysis of software tools, hardware support. Also it provides customized data analysis tailored to the needs of individual research projects across all the research groups and extend this service to others on mutually acceptable terms. In addition, it helps train manpower by way of workshops and short training courses.

Services

Data storage

- Providing network storage, data management and handling to all research groups such as Imaging, Microarray, NGS and Mass spectrometry

Method development

- Providing various computational environments to develop software and pipelines for Bioinformatics projects

Web- based database

- Managing, storing and querying data is an integral part of research. The way data is stored and accessed often defines the efficiency of the research process. ABC provides facility to develop customised tools, storage and databases, particularly in web settings for basic research on the core's computing resources.

Data analysis

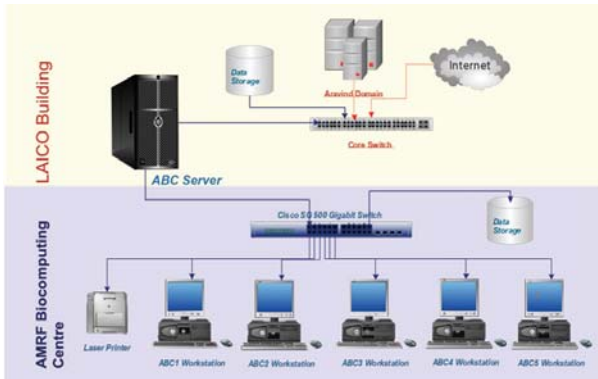
- Data analysis is highly specific to a particular project and data type. ABC provides data analysis that requires computers with powerful processing capabilities along with high memory requirements from high-through put technologies.

Training

- Hands-on training on Mass Spec data analysis was provided during clinical proteomics workshop

Activities

- Developing a structure and sequence-based bioinformatics method to the analysis of Single Nucleotide Variants (SNVs) and prediction of its association with genetic eye diseases.
- Developing an exome analysis software pipeline to detect and filter clinical variants for genetic eye disorders using Next-Generation Sequencing clinical data
- Comparative bacterial genome analysis to find genome wide differences between ocular isolates and to find mutations and genes associated with drug resistance mechanism using whole genome short-gun sequencing projects



- Transcriptome analysis of predicting target genes associated with the maintenance of stemness using next-generation RNA sequencing (RNA-seq) data.
- In-house bioinformatics pipeline to identify pathogenic variants of Retinoblastoma (RB)
- Target gene prediction and functional analysis of differentially expressed MicroRNAs in Fungal Keratitis
- RNA-seq analysis of saprophyte and corneal isolates of *A.flavus* at two different growth temperatures

Architecture

ABC has a powerful distributed computing platform equipped with HP DL580R07 (E7) CTO Server, five Intel i7-3370 3.5GHz workstations and Two Iomega 12TB storage devices. ABC provides Microsoft Windows, Linux-based programming environment and high speed internet (upto 10Mbps).

Data in public repository for open access

- *Pseudomonas aeruginosa* BK1, whole genome shotgun sequencing project, (2014) Lakshmi Priya, J. Logambiga P. Sivakumar, N. Lalitha, P. Vidyarani, M and Bharanidharan, D. GenBankAccession Number: JBTQ00000000.
- Staphylococcus aureus subsp. aureus AMRF2, whole genome shotgun sequencing project, (2014) Nithya, V. Logambiga P. Sivakumar, N. Lalitha, P. Vidyarani, M and Bharanidharan, D. GenBankAccession Number: JASM00000000
- Staphylococcus aureus subsp. aureus AMRF2, whole genome shotgun sequencing project, (2014) Nithya, V. Logambiga P. Sivakumar, N. Lalitha, P. Vidyarani, M and Bharanidharan, D. GenBankAccession Number: AZTC00000000.
- Human Corneal microRNA Expression Profile in Fungal Keratitis, Small Non-coding RNA deep-sequencing.(2014)Lalitha, P and Bharanidharan, D, GEO Accession Number: GSE64843.

- Exoproteome of Aspergillus flavus corneal isolates and saprophytes, (2015) Selvam RM, Nithya R, Devi PN, Shree RS, Nila MV, Demonte NL, Thangavel C, Maheshwari JJ, Lalitha P, Prajna NV, Dharmalingam K,ProteomeXchange ID: PXD001296

Future directions

- Integrated solutions with commercial applications (Ilumina, CLCbio)
- Keeping up to date with new developments in bio computing: applications and algorithm/software updates, especially in next generation sequencing
- Full implementation of next generation sequencing pipelines

CONFOCAL LASER SCANNING MICROSCOPY FACILITY (LEICA TCS SP8)

Leica TCS SP8 confocal laser scanning microscope is an inverted microscope designed for optical imaging with optimal photon efficiency and high speed; facilitating optical sectioning. All optical components are matched towards increasing optical resolution using point illumination and a spatial pinhole to eliminate out of focus light in specimens by preserving fluorescence photons for image contrast and to improve cell viability in live cell imaging. Backing up this sensitive detection are a high speed scanning system with up to 428 frames per second, large field of view of field number 22 and accelerated Z-stacking by a novel mode for the SuperZ galvanometer called Galvoflow. This microscope is equipped with 4 laser ports namely UV/405, laser blue 488nm, laser green 552nm and laser red 638nm. In addition to PMT, the Leica HyD has been integrated into Leica TCS SP8 system. With its high quantum efficiency, low noise and large dynamic range, the Leica HyD is the most versatile detector in the Leica TCS SP8 confocal platform. It synergizes perfectly with our filter-free spectral detection system and the acousto-optical beam splitter (AOBS) in the gapless light detection with maximum photon efficiency. This makes the Leica TCS SP8 ideally suited for quantitative measurements and all-purpose imaging.

Applications

Stem Cell Biology

- Two parameter analysis (high p63 expression in cells with high nuclear-cytoplasmic ratio) for limbal epithelial stem cell identification
- Characterization of limbal stromal niche by expression analysis for various markers in comparison to corneal stromal cells
- 3D construction of cornea and limbal tissues immunostained for the niche cell specific markers

Proteomics

- Receptor-lig and interaction between zymosan and phagocytic/non phagocytic cells

Microbiology

- Host cell (human corneal epithelial cell) autophagy in response to bacterial infection.

Ocular pharmacology

- Characterization of Human Trabecular Meshwork Cells by expression analysis.
- Evaluating the role of lutein (L) &Zeaxanthin (Z) in inhibiting the accumulation of A2E in ARPE-19 cell line using autophagy markers.
- Expression analysis of ALR and VEGF in ARPE-19 cells challenged with different glucose concentration under normoxia and hypoxia.

TRAINING OF PROJECT STUDENTS

- Ms. Keerthana Ragavi, II Year B.Tech. Genetic Engg. Student from SRM University, Chennai
- M. Balaji, M.Sc, Biotechnology student from Alagappa University,
- Ms. Ankita Kotnala, PhD Scholar, Dept. of Pharmacology, AIIMS, New Delhi
- Mr. Mr.Shirish Dongre, Ph D Scholar, DIPSAR, New Delhi
- S. Ijaz Ahmed from PSG College of Arts and Sciences, Coimbatore
- Ms. Raagavi Ravi, B.Tech - Biotechnology student from Kalasalingam University
- M. Manikanda Prabu, final year B.Tech – Biotechnology student from PSR Engineering College, Sivakasi
- Mr. V. Vignesh, M. Tech. student from Bharathidasan University

CONFERENCES ATTENDED

Annual conference of US ARVO 2014

Orlando, Florida, May 4-8

Dr. P. Sundaresan

- *Involvement of collagen – related genes and its influence on Keratoconus and Primary Open Angle Glaucoma patients in India*

Dr. Gowri Priya Chidambaranathan

- *Live imaging of limbal niche in Limbal Stem Cell deficiency patients*

Dr. S. Senthil Kumari

- *Ascorbate concentrations and polymorphisms in sodium transporter vitamin C genes in human plasma, aqueous humor and lens nucleus in an ascorbate depleted setting*

Bibhuti Ballav Saikia

- *Long PCR based analysis of whole Mitochondrial Genome of LHON patients*

21st Annual meeting of Indian Eye Research Group (IERG) - ARVO - India Chapter

LV Prasad Eye Institute, Hyderabad, July 26-27, 2014

Dr. P. Sundaresan, Chaired the session on Retina, Glaucoma and Oncology. Dr. K. Jeyalakshmi and R.Nithya received travel grants to attend the meeting.

Oral presentations

Dr. A. Vanniarajan

- *A new strategy for rapid screening of RB1 in Retinoblastoma patients*

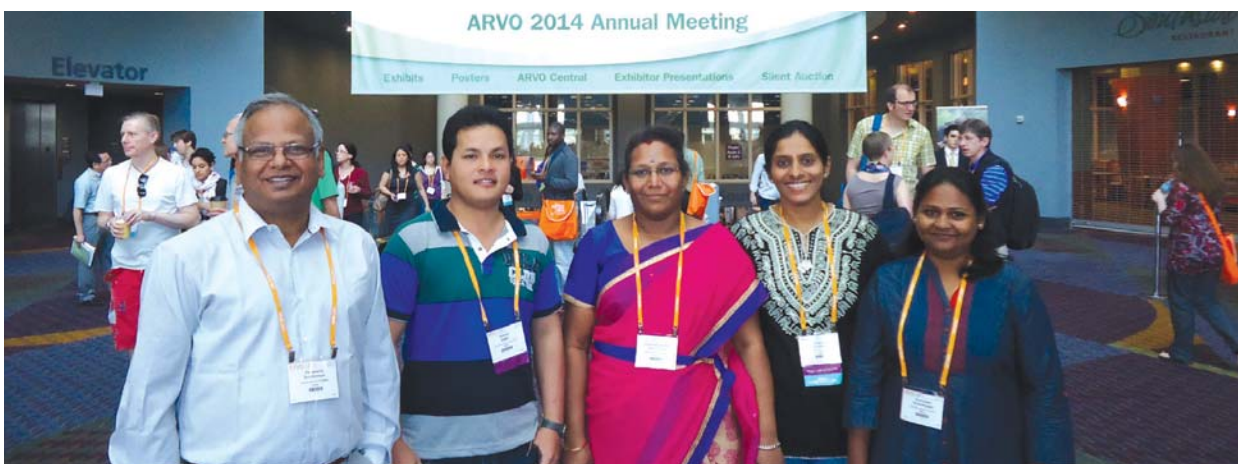
R. Sharmila

- *Alleviation of glucose-induced toxicity by epalrestat in ARPE-19 cells under Hypoxia*

Dr. K. Jeyalakshmi

- *Glycoproteomics of tear from Mycotic Keratitis patients*

Dr. P. Sundaresan, Mr. Bibhuti, Dr. C. Gowripriya, Dr. Sharmila and Dr. Senthil Kumari at the US ARVO meeting



AMRF team at the Annual Meeting of IERG

Dr. S. Senthil Kumari

- *Aqueous humor outflow facility response to SB772077B (SB77), a Rho Kinase inhibitor in Human Organ Cultured Anterior Segments (HOCAS)*

R. Nithya

- *Identification and comparison of Exoproteins of Asperigillus flavus causing Keratitis*

Dr. D. Bharanidharan

- *Next generation sequence analysis pipeline for the molecular characterization of RB1 gene in Retinoblastoma patients*

Poster Presentation

A. Aloysius

- *Discordance of retinoblastoma demonstrated by DNA profiling*

Conference on Applying Next-Generation Sequencing

The Capitol, Bangalore, September 9

Dr. P. Sundaresan

- *Recent findings from NGS studies in eye diseases*

4th Annual Conference of the Society for Mitochondrial Research and Medicine (SMRM)

Manipal University, Manipal, Karnataka, December 8 - 9

Apart from chairing a session, Dr. P. Sundaresan delivered a talk on *Establishment of Retinal Mitoscriptome gene expression signature for Diabetic Retinopathy using human cadaver eye.*

Brainstorming meeting and workshop on Proteomics: Present and Future

Centre for Cellular and Molecular Biology, Hyderabad, November 22- December 1

Prof. K. Dharmalingam

- *Quantitative Proteomics - methods and applications*

6th Annual meeting of Proteomics Society, India (PSI) and INDO-US Science and Technology Forum (IUSSTF) Workshop

IIT – Mumbai, December 6 - 11

Prof. K. Dharmalingam, *Proteomics from discovery to function* – International Proteomics Conference.

Dr. P. Sundaresan with the Executive Committee, SMRM

Prof. K. Dharmalingam delivering lecture at Sankara Nethralaya, Chennai

Dr. P. Sundaresan with Dr. Gerald Schultz from Loma Linda School of Medicine, US during the AIOS meet at Delhi

Science Academies' Lecture workshop from knowing Biology to solving problems

PSG college of Technology, Coimbatore, January 5 - 6

Prof. K. Dharmalingam

- *Proteomics and diseases*

Symposium on Sanger to Next Generation Sequencing – The Genomics Era

Organized by Vision Research Foundation, Sankara Nethralaya, Chennai, January 30 - 31

Prof. K. Dharmalingam

- *Understanding eye diseases*

Dr. P. Sundaresan

- *Identification of genes/mutations causing eye diseases using next generation sequencing*

73rd annual Conference of All India Ophthalmic Society (AIOS)

New Delhi, February 5 - 8

Dr. P. Sundaresan

- *Genetics of Glaucoma*

Asian Eye Genetic Consortium (AEGC) Meeting

New Delhi, February 4

Dr. P. Sundaresan attended the meeting and presented his views at the consortium.

Asia-ARVO-2015

Yokohama, Japan, February 16 - 20

Dr. P. Sundaresan

- *Human retinal mitoscriptome gene expression signature for diabetic retinopathy using cadaver eyes.*

During the meeting, he got an opportunity to interact with Dr. Aung Tin, Executive Director, Singapore Eye Research Institute and Dr. CC Khor, Genome Institute of Singapore.

Workshop on Microbial Genomics and Proteomics

Alagappa University, Karaikudi, February 18

Prof. K. Dharmalingam

- *Proteome diversity*

Dr. P. Sundaresan at AEGC meeting as part of AIOS meet

Dr. P. Sundaresan with Prof. Colin E Willoughby at University of Liverpool, UK

Workshop on Emerging Trends in Biotechnology

Srimad Andavan Arts and Science College, Trichy, February 19

Prof. K. Dharmalingam

- *Genome to proteome - Origin and expansion of protein diversity*

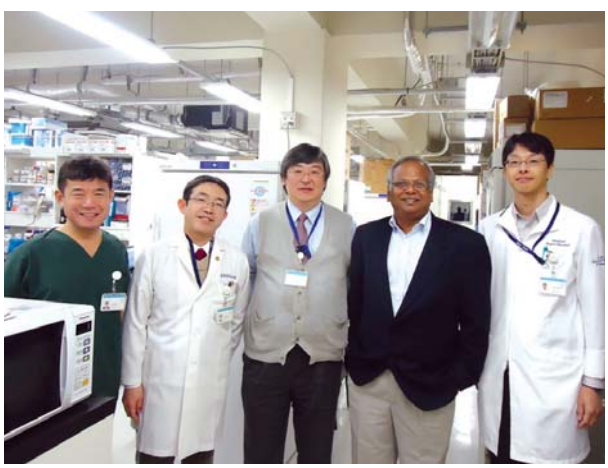
Professional Visits Abroad

Visit to University of Liverpool

UK, May 11 - 20, 2014

Dr. P. Sundaresan visited Department of Eye and Vision Science, Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool, UK and discussed with Prof. Colin E Willoughby and Dr. Sajjad Ahmad on future collaborative projects on genetics of glaucoma and cornea. He also visited Centre for Genomic Research (CGR) facilities at the University of Liverpool to access sequencing and array technologies related to genomics and human genetics. In addition, he also met Dr. Astrid Fletcher at London School of Hygiene and Tropical Medicine and discussed the INDEYE project.

Dr. P. Sundaresan with Prof. Tomoki Todo at University of Tokyo, Tokyo



Visit to Tokyo Medical Centre and University of Tokyo

Tokyo, Japan, February 16 - 20

Dr. P. Sundaresan visited Dr. Takeshi Iwata laboratory at the Division of Molecular and Cellular biology, National Institute of Sensory Organs, Tokyo Medical Centre, Tokyo. He also visited Prof. Tomoki Todo laboratory at the Department of Surgical Neuro-Oncology, Institute of Medical Science, University of Tokyo, Tokyo, Japan and gave guest lectures on HSV-1 gene therapy and Molecular genetics of Eye diseases in Indian Population.

Awards/Fellowships/Membership/Honors/ Student research fellowship/Conference prizes

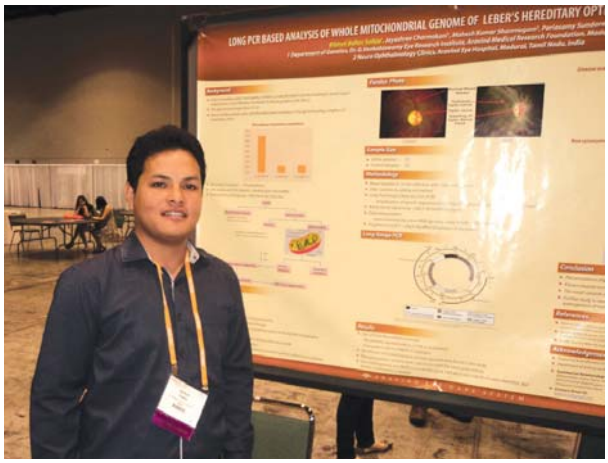
PhD Awarded

K. Renugadevi,was awarded PhD in Biomedical Sciences by Madurai Kamaraj University for her thesis “Molecular Genetics and Functional Analysis of Albinism Patients in India”. She carried out her study under the guidance of Dr. P. Sundaresan.

R. Sivaganesa Karthikeyan was awarded PhD in Biomedical Sciences by Madurai Kamaraj University for his thesis “Characterization of the Immunopathogenic Mechanism in Human Mycotic Keratitis”. He carried out his study under the guidance of Dr. C. Gowripriya.

Mr. Sivaganesa Karthikeyan defending his Ph.D thesis

Ms. K. Renugadevi receiving her Ph.D degree from His Excellency Dr. K. Rosaiah, Governor of Tamil Nadu



Mr. Bibuti at the US ARVO meeting, Orlando, USA

Travel fellowship (US ARVO 2014)

Bibhuti Ballav Saikia received ARVO Foundation Developing Country Eye Researcher (AFER) Travel Fellowship to attend US ARVO meeting, at Orlando, Florida, USA.

Prof.VR.Muthukkaruppan Endowment Award - 2014

Students and Colleagues of Prof. VR. Muthukkaruppan created an Endowment in 2014 out of which an award will be given to the best researcher at Aravind Medical Research Foundation every year.

The inaugural Prof. VR. Muthukkaruppan Endowment award was presented to Mr. Mohammed Razeeth, Project Fellow, Proteomics Department for his outstanding research on Generating genome wide deletions in *A.flavus* – To understand *A.flavus* pathogenic mechanism on 20th September, 2014. The award is given based on the scientific merit of abstracts submitted by the research scholars of Aravind Medical Research Foundation. The award carries certificate and a cash prize of Rs.25,000/-.

Mr. Mohammed Razeeth receiving Prof. VR. Muthukkaruppan Endowment Award from Dr. P. Namperumalsamy

CONFERENCES / WORKSHOPS CONDUCTED

Workshop on Clinical Proteomics: Methods and applications

October 10 - 11, 2014

This workshop conducted as part of October Summit aimed at introducing young research students to the state-of-the-art proteomics technologies and its applications, primarily in the area of clinical research. The workshop was inaugurated by Dr. Mohd. Aslam, Adviser, Department of Biotechnology, Govt. of India. Fifteen-participants from different universities and institutes, took part in the workshop. Speakers from various premier institutions such as CCMB, IGIB, IISc, IOB as well as companies offering proteomics services (Sandor Life Sciences, Agilent Technologies) shared their research experience and expertise with the participants. The participants were provided hands-on training in data analysis and interpretation of high-throughput proteomics data. This workshop helped the participants understand various methodologies available to generate data as well as to analyze and understand such complex data. This workshop was sponsored by the Department of Biotechnology, Government of India, ThermoFisher Scientific India and PonmaniGlasschem Agencies, Madurai.

Invited speakers

Dr. Inderjeet Kaur, LVPEI, Hyderabad

- *Proteomics of Retinopathy of Prematurity*

Dr. Deepalakshmi, IISc, Bangalore

- *Mass spectrometers as an analytical tool for clinical proteomics*

Mr. Santhosh Renuse, ThermoFisher Scientific

- *TMT-based multiplexed quantitative Proteomics using Orbitrap Mass Spectrometry*

Dr. Ravi Krovvidi, Agilent Technologies Inc.,

- *Profiling of cell surface Proteome and Metabolome from an in-vitro model system using agilent automation and LC/MS platforms*

Dr. S. Karutha Pandian, Alagappa University, Karaikudi

- *Analysing the effect of quorum sensing and biofilm inhibitors on human Pathogens at Proteome level - Gel based Proteomic approach*

Dr. Shantanu Sengupta, IGIB,New Delhi

- *Quest towards identifying potential markers for Coronary Artery disease*

Dr. Suman Thakur, CCMB, Hyderabad

- *Quantitative high throughput Proteomics has potential to improve human health*

Participants to the workshop on Clinical Proteomics

Dr. Mandar Kulkarni, Sandor Life Sciences Pvt Ltd., Hyderabad

- *The use of LC-MS/MS for rapid detection of carbapenem resistant bacterial infections*

Dr. Karthi Sivaraman, H3Me, Chennai

- *Transcriptomics and proteomics in characterization of infectious diseases*

Dr. Kumaravel Somasundaram, Indian Institute of Science, Bangalore

- *Proteomic approaches to study glioma-stroma interaction*

Dr. N.Venkatesh Prajna, Aravind Eye Hospital, Madurai

- *Proteomics of fungal pathogens*

Dr. SR. Krishnadas, Aravind Eye Hospital, Madurai

- *Proteomics of Glaucoma*

Dr. Kim, Aravind Eye Hospital, Madurai

- *Proteomics of Diabetic Retinopathy*

Research Advisory Committee Meetings

The 12th Research Advisory Committee meeting of Aravind Medical Research Foundation was held on 19th April 2014 at Dr. G. Venkataswamy Eye Research Institute. Research scholars presented their findings as posters and got an opportunity to interact with the committee members.

The 13th Research Advisory Committee meeting was held on February 21, 2015. The meeting started with the inauguration of poster session by Dr. Mohan Rao. Close to thirty posters were displayed. Experts analyzed the posters and three were selected. Ms. Jhansi Rani Kasinathan won the first prize. Dr. Aalia Rasool Sufi and Dr. M. Soundaram received the second prize. Mr. S. Mohammed Razeeth bagged the third prize. Prof. VR. Muthukkaruppan Endowment award will be presented to the first prize winner. The poster session was followed by a brief presentation on the research activities at AMRF. The faculty members presented their work and received feedbacks.

Members at the Research Advisory Committee meeting

GUEST LECTURES DELIVERED BY VISITING SCIENTISTS

Dr. G. Kumaramanickavel, Director-Research, Narayana Nethralaya, Bangalore visited AMRF on 14th March 2014 and gave a lecture on *"Gene Mapping in Ophthalmic Diseases - From linkage to association studies"*

Dr. Indranil Biswas, Professor, Director of Graduate studies, University of Kansas, Medical Center, Kansas city, Kansas, USA visited AMRF on 13th May 2014 and gave a lecture on *"Streptococcal pathogenesis: How one regulator determines the disease outcome".*

Dr. Aravindh Babu, Scientist, Translational Research platform for veterinary Biologicals, Tamilnadu Veterinary and Animal Sciences Universtiy, Madhavaram, Chennai visited AMRF on 12th July 2014 and gave a lecture on *"Development and evaluation of vector based mucosal vaccines for foot-and – mouth disease virus".*

Dr. Jayanthi Pande, Associate professor, Department of Chemistry, University at Albany, State University of Newyork, Albany, USA visited AMRF on 30th July 2014 and gave a lecture on *"Protein-Protein interactions in cataract and implications for glaucoma".*

Dr. Solomon F.D.Paul, Professor and Head, Department of Human Genetics, Sri Ramachandra University, Porur, Chennai visited AMRF on 3rd September 2014 and gave a lecture on *Radiation biodosimetry.*

Dr. T. Ramasami, Former Secretary, Department of Science and Technology (Govt. of India) visited AMRF on 18th October 2014 and gave a lecture on *"Paradigms for National Health Research".*

Dr. Velpandi Ayyavoo, Professor, Assistant Chair & Director of IDM Graduate Programs, Department of Infectious diseases & Microbiology, University of Pittsburgh/GSPH, Pittsburgh, USA visited AMRF on 7th January 2015 and gave a lecture on *"Systems biology approaches towards.*

Mr. Emanuel Nazareth, Stem cell Bioengineering lab, University of Toronto, USA visited AMRF on 21st January 2015 and gave a lecture on *"High throughput screening of human pluripotent stem cells in engineered niches".*

Ms. Pamela C. Sieving, Former Biomedical Librarian/informationist, National Institutes of Health Library Bethesda, Maryland and **Ms.Bette Anton,** Library Emerita University of California, Berkeley visited AMRF on 8th January 2015 and gave lectures on *"Assessing the impact of Research, A few thoughts on open access"*

Dr. Carl Sheridan, Senior lecturer, Department of Eye and Vision Science Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool, UK visited AMRF on 28th January 2015 and gave a lecture on *"Progenitor cells for corneal endothelium and TM Cells".*

Professor. Colin Willoughby, Professor of Molecular Ophthalmology, Institute of Ageing and Chronic Disease, University of Liverpool, Honorary Consultant Ophthalmic Surgeon, St. Paul's Eye Unit, Royal Liverpool University Hospital, Liverpool visited AMRF on 2nd February 2015 and gave lecture on *"Developing Molecular Therapies for Aniridia Related Keratopathy"*

Dr. David Chang, Clinical Professor, Department of Ophthalmology, University of California, San Francisco visited AMRF on 14th February 2015. Prof.K.Dharmalingam gave a brief presentation about the AMRF activities and research facilities.

Mr. Subroto Bagchi, Chairman, Mindtree Ltd, Bangalore and Mrs. Susmita Bagchi, Director, White Swan Foundation, Bangalore Visit AMRF

Mr. Subroto Bagchi and Mrs. Susmita Bagchi visited AMRF on August 1 and held discussions with the senior team on the various research activities being carried out at AMRF. The guests then visited the laboratories. The project “Diabetic Retinopathy Early Prediction among Diabetic patients” is supported by a generous grant from Mr. Bagchi.

Dr. A.P.J. Abdul Kalam, Hon’ble Former President of India Visits AMRF

Dr. A.P.J Abdul Kalam, Former President of India made a visit to Aravind on May 31 mainly to have a talk with the research team. His visit is essentially to motivate young researchers to pursue quality research and to apply modern research findings to help solve the problems of people with vision defect. Dr. Kalam delivered a talk on “Bench to Bedside: Taking research to clinical practice”.

PUBLICATIONS 2014-15

Nithya Velusamy, Logambiga Prakash. Neelamegam Sivakumar, Aju Antony, Lalitha Prajna, Vidyarani Mohankumar, Bharanidharan Devarajan (2014)

- *Draft genome sequences of staphylococcus aureus AMRF1 (ST22) and AMRF2 (ST672): Ocular methicillin resistant isolates*

Genome Announc 2(2):e00168-14

Lakshmipriya Jeganathan, Logambiga Prakash, Neelamegam Sivakumar, Aju Antony, Sami Alqarawi, Lalitha Prajna, Bharanidharan Devarajan, Vidyarani Mohankumar (2014)

- *Draft Genome Sequence of an Invasive Multi drug resistant strain Pseudomonas aeruginosa BK1, isolated from a keratitis patient*

Genome Announc 2(2):e00153-14

Asai-Coakwell M, March L, Dai XH, Duval M, Lopez I, French CR, Famulski J, De Baere E, Francis PJ, Sundaresan P, Sauvé Y, Koenekoop RK, Berry FB, Allison WT, Waskiewicz AJ, Lehmann OJ.

- *Contribution of growth differentiation factor 6 dependent cell survival to early-onset retinal dystrophies*

Hum Mol Genet. 2013; 1-11

Dubey SK, Hejtmancik JF, Krishnadas SR, Sharmila R, Haripriya A, Sundaresan P

- *LysylOxidase-Like 1 Gene in the Reversal of Promoter Risk Allele in Pseudoexfoliation Syndrome*

JAMA Ophthalmol. 2014;132(8):949-55.

George J. Manayath,Prasanthi Namburi,Sundaresan Periasamy,Jeevan A. Kale,Venkatapathy Narendran,Anuradha Ganesh

- *A novel mutation in the NR2E3 gene associated with Goldmann-Favre syndrome and vasoproliferative tumor of the retina*

Molecular Vision 2014; 20:724-731

Srinivasan Senthilkumari, Badri Talwar, Kuppamuthu Dharmalingam, Ravilla D Ravindran, Ramamurthy Jayanthi, Perisamy Sundaresan, Charu Saravanan, Ian S Young, Alan D Dangour, Astrid Fletcher

- *Polymorphisms in sodium-dependent vitamin C transporter genes and plasma, aqueous and lens ascorbate concentrations in an ascorbate depleted setting*

Exp.Eye Res.2014;124:24-30

Sivaganesa Karthikeyan R, Vareechon C , Prajna NV , Dharmalingam, K Pearlman E, and Lalitha P

- *IL-17 expressionin peripheral blood neutrophils from fungal keratitis patients and healthy cohorts in south India*

Journal of Infect Dis. 2014 Jul 7. E pub ahead of print.

Renugadevi K, John Asnet Mary, Vijayalakshmi P, Suresh Seshadri, Sujatha Jagadeesh, Beena Suresh, Sheela Nampoothiri, Rajaiah Shenbagarathai, Sankaran Krishnaswamy and Sundaresan P

- *Molecular Genetic Testing for Carrier - Prenatal Diagnosis and Computational Analysis of Oculocutaneous Albinism Type I*

Journal of Genetic Disorders & Genetic Reports 2014, 3:2

Murugeswari P, Shukla D, Kim R, Namperumalsamy P, Stitt AW, Muthukkaruppan V,

- *Angiogenic potential of vitreous from proliferative diabetic retinopathy and Eales' disease patients*

PLoS One.2014 Oct 13;9(10):e10755.

Sundaresan P, Simpson DA, Sambare C, Duffy S, Lechner J, Dastane A, Dervan EW, Vallabh N, Chelerkar V, Deshpande M, O'Brien C, McKnight A J, Willoughby CE.

- *Whole-mitochondrial genome sequencing in primary open-angle glaucoma using massively parallel sequencing identifies novel and known pathogenic variants*

Genet Med. 2014 Sep 18

Neethirajan G, Krishnadas SR, Vijayalakshmi P, Shashikanth Shetty, Sundaresan P

- *PAX6 mRNA Transcript Analysis in Various Ocular/Non-Ocular Tissues*

Adv Ophthalmol Vis Syst 2014, 1(4): 00026

Lakshmi Priya Jeganathan, Lalitha Prajna, Vidyarani Mohankumar.

- *Genotypic and Phenotypic Characterization of Pseudomonas aeruginosa isolates from post-cataract endophthalmitis patients*

Microbial Pathogenesis 2015; 78;67-73

Selvam RM, Nithya R, Devi PN, Shree RS, Nila MV, Demonte NL, Thangavel C,Maheshwari JJ, Lalitha P, Prajna NV, Dharmalingam K.

- *Exoproteome of Aspergillusflavus corneal isolates and saprophytes: Identification of proteoforms of an oversecreted alkaline protease*

J Proteomics. 2015;115:23-35

Selvam RM, Nithya R, Devi PN, Shree RS, Nila MV, Demonte NL, Thangavel C,Maheshwari JJ, Lalitha P, Prajna NV, Dharmalingam K.

- *Data set for the mass spectrometry based exoproteome analysis of Aspergillus flavus isolates*

Data in Brief. 2015; 2: 42-47

Sushil Kumar Dubey, Mahalakshmi, Perumalsamy Vijayalakshmi, Periasamy Sundaresan
- *Mutational analysis and genotype-phenotype correlations in sporadic and familial aniridia patients from southern India.*

MolecularVision2015;21:88-97

Tin Aung et al.,
- *A common variant mapping to CACNA1A is associated with susceptibility to Exfoliation syndrome*

NATURE GENETICS 2015 (E Pub ahead of Print)

Sushil K. Dubey, James F. Hejtmancik, Subbaiah R. Krishnadas, Rajendrababu Sharmila, Aravind Haripriya, Periasamy Sundaresan
- *Evaluation of Genetic Polymorphisms in Clusterin and Tumor Necrosis Factor-Alpha Genes in South Indian Individuals with Pseudoexfoliation Syndrome*

Current Eye Research Early Online, 1–7, 2015

Saumi Mathews, Jaya Devi Chidambaram, Shruti Lanjewar, Jeena Mascarenhas, Namperumalsamy Venkatesh Prajna, Veerappan Muthukkaruppan, Gowri Priya Chidambaranathan
- *In vivo confocal microscopic analysis of normal human anterior limbal stroma*

Cornea 2015;34:464–470

Candidates registered for PhD under Madurai Kamaraj University, Madurai and Alagappa University, Karaikudi

No	Year	Name	Title of Thesis	Guide
1.	2012	Bibhuti Ballav Saikia	Mitochondrial genes involvement in Leber's Hereditary Optic Neuropathy	Dr.P.Sundaresan
2.	2012	V.Nithya	Genotypic characterization and analysis of virulence factors in Methicillin resistant Staphylococcus aureus causing ocular infections	Dr.SR.Rathinam
3.	2012	M.K.Jhansi Rani	Molecular signature of Human limbal epithelial stem cells	Dr.C.Gowripriya
4.	2012	*Roopam	Molecular Genetic studies of Primary Angle Closure Glaucoma in South Indian population	Dr.P.Sundaresan
5.	2012	*G.Ramesh Kumar	Detection, Identification and molecular characterization of extended – spectrum-B-Lactamases (ESBLS), AMPC-B-Lactamases and Metallo-B lactamases (MBLS) - mediated resistance among gram-negative bacterial isolates recovered from ocular infections treated at tertiary eye care referred centre in South India	Dr.SR.Rathinam
6.	2013	J.Lakshmipriya	Characterization of the virulence determinants of *Pseudomonas aeruginosa* causing keratitis	Dr.M.Vidyarani
7.	2014	*A.Aloysius Abraham	Identification of modifier genes involved in tumorigenesis of Retinoblastoma	Dr.A.Vanniarajan
8.	2014	K.Thirumalairaj	Characterization of Genetic and Transcriptional alterations in Retinoblstoma	Dr.A.Vanniarajan
9.	2014	Mohd Hussain Shah	Genetic and functional approaches to understand the pathogenicity of Primary Open Angle Glaucoma (POAG)	Dr.P.Sundaresan
10.	2014	M.Durga	Molecular Genetics of Macular Corneal Dystrophy (MCD) in Indian population	Dr.P.Sundaresan
11.	2014	G.Prakadeeswari	Molecular analyses of various risk factors involved in Pseudoexfoliation Syndrome	Dr.P.Sundaresan

**PhD Registered in Alagappa University*

PhD Thesis submitted to Madurai Kamaraj University

No	Year	Name	Title of Thesis	Guide
1.	November 2013	Anshuman Verma	Molecular studies of Leber Congenital Amaurosis in Indian population	Dr. P. Sundaresan
2.	March 2014	G. Gowthaman	Investigating the role of Nuclear, Mitochondrial genome and Micro RNA in the pathogenesis of Diabetic Retinopathy	Dr. P.Sundaresan
3.	June 2014	Prasanthi Namburi	Identification of Genetic variants in genes associated with Primary Open Angle Glaucoma in Indian population	Dr. P. Sundaresan
4.	April 2015	Lalan Kumar Arya	Etiology and Immunopathogenesis of subconjunctival and anterior chamber granulomatous uveitis in children of South India	Dr. SR. Rathinam
5.	April 2015	Saumi Mathews	Studies on the characterization of Limbal Niche – their role in maintenance and Ex vivo expansion of human corneal epithelial stem cells	Dr. C. Gowripriya

Completion of PhD viva-voce examination

No	Viva voce date	Name	Title of Thesis	Guide
1.	31st March 2015	Sushil kumar Dubey	Identification and characterization of mutations in candidate genes involved in major congenital globe anomalies	Dr. P. Sundaresan

ONGOING RESEARCH PROJECTS

No	Projects	Funded by	Investigators	Research Scholar
MICROBIOLOGY				
1.	Etiology and Immunopathogenesis of Trematode induced Uveitis in children of South India	ICMR	Dr.SR.Rathinam Dr.Lalitha Prajna Dr.Veena Tandon	Lalan Kumar Arya
2.	Epidemiology, pathogenomics, and system biology of A. flavus infections in India - an integrative approach	DBT	Dr.Lalitha Prajna	J.Lakshmipriya
3.	Characterization of the virulence determinants of *Pseudomonas aeruginosa* causing ocular infections using genomic and proteomic approaches	AMRF	Dr.M.Vidyarani	J.Lakshmipriya
4.	Genotypic characterization and analysis of virulence factors in Methicilin resistant staphylococcus aureus (MRSA) causing ocular infections in South India	AMRF	Dr.Lalitha Prajna	V.Nithya
5.	Microbiological clearance time and sensitivity assay for Acanthamoeba keratitis	AMRF	Dr.Lalitha Prajna Dr.M.Vidyarani	Madhu Srinivasan B. Hemadevi
6.	Elucidating the role of microRNAs in microbial keratitis	CSIR-Research Associate (From Oct 2014)	Dr. Lalitha Prajna	Dr.B.Hemadevi
PROTEOMICS				
7.	Quantitative Proteomics of host pathogen ineraction in human Aspergillus Keratitis	DBT	Dr.N.Venkatesh Prajna Dr.K.Dharmalingam Dr.Lalitha Prajna Dr.J.Jeya Maheshwari	K.R.P.Niranjana R.Nithya Naveen Luke Demonte
8.	CoE – Human Mycotic Keratitis	DBT	Dr.N.Venkatesh Prajna Dr.K.Dharmalingam Dr.Lalitha Prajna Dr.J.Jeya Maheshwari	Project Fellows: S.Mohammed Razeeth M.Nivedhitha A.Dhivya Research Associates: Dr.Partho Chattoraj (Till 15.7.2014) Dr.B.Hemadevi (Till 30th Sep 2014) Dr.Jeyalakshmi Kandhavel (Till 31st August 2014)

9.	Proteomics and peptidomics of human infectious diseases and biomarker discovery	DBT-Distinguished Biotechnology professorship award	Dr. K. Dharmalingam	Suresh Palanivel (Till 22nd Sep 2014) C. Arulmozhi (Till Dec.2014) K.Sandhya
10.	Predictive biomarkers for diabetic retinopathy among diabetics and stage specific biomarkers for NPDR and PDR.	Bagchi grant	Dr. K. Dharmalingam Dr.R.Kim Dr.J.Jeya Maheshwari Dr.S.Senthilkumari	E.King Solomon (Till 15th Dec 2014) Roopesh R.Pai R.Sharmila KRP. Niranjana Naveen Luke Demonte A.Divya K. Sandhya
11.	Mycotic Ulcer Treatment Trial	AEH	Dr.K.Dharmalingam Dr.N.Venkatesh Prajna	K.Divya Manjari (Till Apl.2015)
MOLECULAR GENETICS				
12.	Molecular genetics of Albinism in the Indian population	AMRF Post-Doctoral Fellow	Dr.P.Sundaresan Dr.P.Vijayalakshmi	K.Renugadevi
13.	Mitochondrial genes involvement in Leber's Hereditary Optic Neuropathy (LHON)	DST	Dr.P.Sundaresan Dr.Mahesh Kumar	Bibhuti Saikia
14.	Molecular genetics studies of Primary Angle closure Glaucoma (PACG) in South Indian Population	UGC Fellowship	Dr.P.Sundaresan	Roopam Duvesh
15.	A Genetic component to the INDEYE study of cataract and age related macular degeneration in India	Wellcome Trust, UK	Dr.P.Sundaresan Dr.R.D.Ravindran	
16.	Genetic and functional approaches to understand the pathogenicity of primary open Angle Glaucoma (POAG)	AMRF	Dr.P.Sundaresan	Moh'd Husain Shah
17.	Genetic and transcript analysis of RB1 gene in South Indian Retinoblastoma Patients	ICMR	Dr.A.Vanniarajan Dr.Usha Kim Dr.R.Santhi	K.Thirumalai raj
18.	Establishing the genetic testing centre for childhood ocular cancer (retinoblastoma) in Aravind Medical Research Foundation	Aravind Eye Foundation	Dr.A.Vanniarajan Dr.Usha Kim Dr.R.Santhi	A. Aloysius Abraham
19.	ABCB6 and ABTB2 genes screening for ocular anomalies	AEH	Dr.P.Sundaresan	G.Prakadeeswari
20.	Molecular genetics of macular corneal dystrophy (MCD) in Indian population	DST INSPIRE Fellowship	Dr.P.Sundaresan Dr.N.Venkatesh Prajna	M.Durga

IMMUNOLOGY AND STEM CELL BIOLOGY				
21.	Translational research to generate Corneal/ Buccal Epithelial stem cells with GMP compliance for corneal surface and socket reconstruction	AMRF	Dr.C.Gowri Priya Dr.N.Venkatesh Prajna Dr.VR.Muthukkaruppan	Saumi Mathews
22.	Enrichment of human limbal epithelial stem cells to understand the stem cell to understand the stem cell related properties by whole genome analysis and p63 isoform expression profile	ICMR	Dr.C.Gowri priya, Dr.VR.Muthukkaruppan	M.K.Jhansirani
OCULAR PHARMACOLOGY				
23.	Evaluating the Role of Macular Carotenoids in the accumulation of A2E, A fluorophore in the pathogenesis of Age related Macular Degeneration	DST	Dr.S.Senthilkumari	
24.	Role of Aldose Reductase in Retinal pigment epithelium- An understanding towards the pathogenesis of Diabetic Retinopathy	DBT	Dr.S.Senthilkumari	R.Sharmila
25.	Human Organ Culture Anterior Segment (HOCAS) for Trabecular Meshwork	Aravind Eye Foundation	Dr.S.Senthilkumari	M.Uthayalakshmi (Till Nov 2014) E. Esther Jebarani (Till Dec.2014) T.Preethi Meena (Till Apl.2015)
BIO INFORMATICS				
26.	1. Comprehensive Exome analysis pipeline using clinical next-generation sequencing data 2. Understanding the Molecular Mechanisms of Indian Genetic Eye Diseases: A Structure-Based Bioinformatics Approach and Database Development	AMRF	Dr.D.Bharanidharan	P.Logambiga (Till Sep 2014)

DR. G. VENKATASWAMY EYE RESEARCH INSTITUTE

Aravind Medical Research Foundation

1, Anna Nagar, Madurai 625 020, Tamilnadu, India.
Phone: (0452) 435 6550

www.aravind.org

ARAVIND EYE CARE SYSTEM