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MISSION

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

Dr. G. Venkataswamy
1918 - 2006

RESEARCH IN OPHTHALMIC SCIENCES

Dr. G. Venkataswamy Eye Research Institute

Annual Report 2018 - 2019

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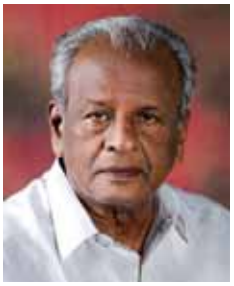

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FOREWORD

A new research programme supporting systematic analysis of Paediatric Eye Disease Genetics, with a focus on translational aspect, has been funded by the Department of Biotechnology, Govt. of India, this year. I hope clinicians and scientists working on this area will do their best to prove our ability to translate basic clinical research into clinical practice. Achieving this goal will fulfil the basic objective of Aravind Eye Care System namely delivering affordable health care to the needy people.

This year also we received support from our well-wishers and friends for our research efforts. However, AECS has been the major supporter of our research efforts and its help has allowed us to explore newer areas of research. Several of our Ph.D. students have completed their training at AMRF and many have gone abroad for further studies.

Our efforts in the areas of Genetics, Stem Cell Biology, Computational Biology, Proteomics are substantial. It is time to consolidate and make significant impact on eye research in the country and abroad. Closer group interaction among scientists and more involvement from clinicians will go a long way in this endeavor. In order to achieve excellence in all these areas, we hope to hire new faculty soon.

Dr. P. Namperumalsamy
President, AMRF

INTRODUCTION

Vision impairment including blindness is a major public health problem. It is estimated that approximately 1.3 billion people live with some form of vision impairment. As per the international classification, vision impairment is classified into distance and near presenting vision impairment groups. With regard to the distance vision impairment, 185.5 million people have mild vision impairment, 217 million have moderate to severe vision impairment and 36 million are blind, and in addition 826 million people live with near vision impairment (WHO: October 2018 data). India has one third of the blind people. Every effort is needed in India to achieve the Vision 2020 goal of 0.3% blind in the total population.

Major funding for the study of Paediatric Genetics was received this year from the Department of Biotechnology, Govt. of India to study the most common retinal disorders- Retinoblastoma, Leber Hereditary Optic Neuropathy, Leber Congenital Amaurosis, and X-linked Retinoschisis - which affect children. Understanding molecular mechanisms of these disorders will pave the way for translating the findings from this programme for early detection and possible therapeutic intervention. This new grant support adds strength to our research effort to enable early screening, diagnosis and prognosis of eye diseases through clinical research.

Microparticles are becoming important messengers that are involved in signalling and transport processes in normal and disease conditions. A project on the role of serum microparticles in Diabetic Retinopathy has been initiated at AMRF with financial support from the Department of Health Research, Govt. of India.

Continuing the research programs initiated earlier and the addition of these new areas of research will hopefully lead to some tangible outcome in terms of clinical application soon.

Prof. K. Dharmalingam
Director - Research

MOLECULAR GENETICS

The Department of Molecular genetics has been working on various eye diseases and performed targeted and whole exome sequencing to identify candidate genes in two large South Indian families with primary open angle glaucoma (POAG). Based on the data, panel of genetic variations was proposed which may be the candidate genes for these two families and will be functionally characterized to prove their involvement in disease pathogenesis. The team previously reported significant association of rs1015213 *(PCMTD1-ST18)* in primary angle closure glaucoma (PACG) subjects from South India. The current study also found siginficant association of this marker rs1015213 (P=0.05) in primary angle closure suspects (PACS), suggesting that this locus may confer susceptibility to a narrow angle configuration.

The team also established the localization of sulfated keratan sulfate in corneal tissue of macular corneal dystrophy (MCD) patients through histochemistry, Immunohistochemistry analysis which demonstrated the type IA MCD as a predominant phenotype in Indian population, followed by MCD type I and II. In addition, five genome-wide significant exfoliation syndrome (XFS)-associated loci [13q12 (*FLT1-POMP*), 11q23.3 (*TMEM136*), 6p21 (*AGPAT1*), 3p24 (*RBMS3*) and 5q23 (*SEMA6A*)] were identified which shows the association of new biological pathways for disease pathogenesis. Moreover, the differential *LOXL1-AS1* expression profile on XFS patients was observed and the prolonged induction of oxidative stress at higher concentration of H_2O_2 over B-3 human lens epithelial cells resulted with decreased expression of *LOXL1-AS1*. This suggests the involvement of the cellular stressors as one of the risk factors for the formation of exfoliation syndrome.

Immunophenotypes of Macular Corneal Dystrophy patients with CHST6 gene mutations

Investigators	:	Dr. P. Sundaresan Dr. N. V. Prajna
Ph.D Scholar	:	M. Durga
Funding Agency	:	Aravind Eye Care System, DST-INSPIRE, New Delhi

Introduction

Macular corneal dystrophy (MCD) is an inherited, autosomal recessive disorder of defective keratan sulfate (KS) metabolism. It is caused by mutations in the carbohydrate sulfotransferase 6 gene (CHST6) which is essential for the sulfation of keratan sulfate. Mutations in *CHST6* results in abnormal accumulation of unsulfated KS extracellularly in corneal stroma, Descemet's membrane, and intracellularly in keratocytes and endothelium. It is characterised by bilateral, progressive clouding of the corneal stroma with the presence of greyish-white, ill-defined opacities. The prevalence of MCD varies immensely in different parts of the world but in most of the populations, this condition is rare. Unlike the western world, MCD is the most common corneal stromal dystrophy in India. The disease is highly prevalent in South Indian population as reported; it could be due to high frequency of consanguineous marriages. MCD is subdivided into three immunophenotypes, type I, IA and II, based on the reactivity of serum and corneal tissue to an antibody that recognises sulfated keratan sulfate (KS). MCD type I is reported as a predominant immunophenotype in Indian patients

which is associated with missense mutations in the coding region of *CHST6*.

Understanding the genetic aspects and pathophysiological mechanism of MCD is very essential for the earlier accurate diagnosis, better treatments and preventive therapy. So far, there are limited studies related to *CHST6* mutations and immunophenotypes in MCD. Therefore, this study aimed to determine the immunophenotypes of MCD to correlate with *CHST6* mutations. We have recruited 60 MCD families, 39 unaffected relatives and 100 healthy volunteers without any corneal dystrophies for PCR and Sanger sequencing analysis. Additionally, 20 MCD corneal buttons were collected from 20 families during penetrating keratoplasty (PKP) and 10 cadaver corneal buttons (control) also received for histochemistry and immunohistochemistry. Cross-sections of the 10% formalin fixed, paraffin embedded corneal sections (4 μm) were used for the histochemical staining (Alcian blue, haematoxylin and eosin stain) and immunohistochemical staining (Monoclonal antibody [1/20/5-D-4]) for detecting the sulfated keratan sulfate level.

Results & Conclusion

MCD type I was identified in eight unrelated families associated with different missense mutations (p.R50C, p.S53L, p.R93H, p.S131P, p.R205Q) with the absence or very low level of AgKS (sulfated KS) in corneal tissue. MCD type IA was identified with the absence or low AgKS only in stromal keratocytes. This phenotype IA was observed in ten different patients associated with various frameshift mutations (p.V66VfsX3, p.Q182RfsX198, p.N194_R196delinsRC). In addition, we observed MCD type II in two patients allied with negative mutation with the presence of abnormal AgKS in stroma, Keratocytes, Descemet's membrane and endothelium. This study revealed MCD type IA also equally present in Indian population which is correlated with frameshift mutations. These results showed immunophenotype IA is not an uncommon phenotype in Indian population. Further immunohistochemical studies and ELISA will be performed to confirm the frequency of type IA of MCD patients.

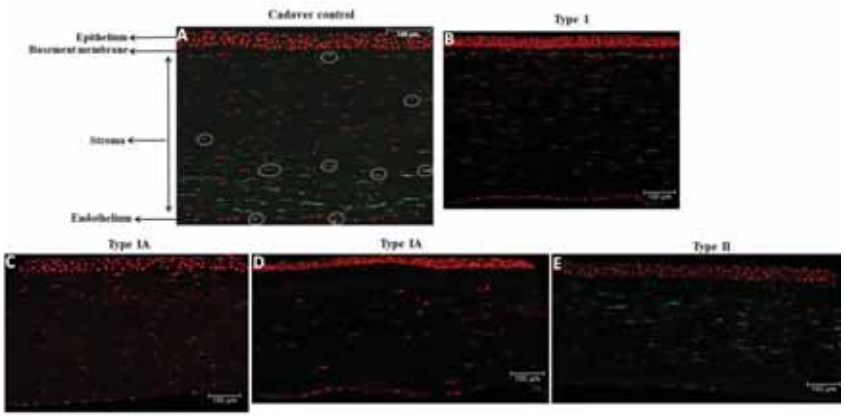


Figure 1. Confocal microscopic images of representative corneas show different immunophenotypes of MCD compared with control (FITC-green, PI-red). A: Control cornea shows AgKS expression in stroma, keratocytes, Descemet's membrane, endothelium. B: MCD cornea with the absence of detectable AgKS or very low level is shown in MCD type I (20X). B: Cornea shows the absence of detectable AgKS or present only in stromal keratocytes in MCD type IA (20X). C: A cornea is shown with AgKS detected throughout the stroma, keratocytes, Descemet's membrane and endothelium in MCD type II (20X).

Molecular genetics of ABCA4 gene in Stargardt disease

Investigators	:	Dr. P. Sundaresan, Dr. R. Kim, Dr. A. Rupa
PhD Scholar	:	R. Kadarkarai Raj
Funding Agency	:	Aravind Medical Research Foundation

Introduction

Stargardt disease (STGD1, MIM #248200) is an autosomal recessive disease and highly heterogeneous both clinically and genetically. The prevalence rate of this disease is 1 in 10,000 globally. The onset of this disease is generally in 1-2 decades of life and less frequently in adulthood. The clinical characteristics of this disease are as follows; bilateral central visual loss, macular atrophy and yellow-white flecks at the level of the retinal pigment epithelium (RPE) at the posterior pole. During visual process, a cyclic event happens inside the retina for the conversion of light signal into electric signal. The photons activated the opsin which contains 11-cis retinal chromopore that undergo photo isomerization to all-trans retinal. This process changes the confirmation of opsin and reduced to all tans retinol. For esterification, this all-tans retinol was transferred to adjacent RPE and converted into 11-cis-retinol by Lecithin retinol acyltransferase (LRAT). Before returning to the photoreceptors the 11-cis retinol was further oxidized into 11-cis retinal forms opsin. This visual cycle was regulated by ABCA4 gene, located in chromosome 1p13. This gene has 50 exons and encodes an ATP-binding cassette (ABC) transporter protein, expressed in the outer segment (OS) of rods which transports potentially toxic substances (all-transretinal aldehyde)

out of photoreceptor cells. If any defects in coding region of ABCA4 gene cause excess building of toxic substance-lipofuscin-in the photoreceptor cells that leads to progression of STGD1. Mutations in ABCA4 gene also associated with other inherited retinal dystrophies like Cone Dystrophy, 'Cone - Rod, 'Rod – Cone' Dystrophy (CRD) and autosomal recessive Retinitis Pigmentosa (arRP). However earlier studies revealed the mutation spectrum of ABCA4 gene is not conserved and it's likely to be sporadic across the exons in different ethnic population. In order to know the mutation spectrum in south Indian cohorts, our present study aims to explore the genetic characteristic of ABCA4 gene in STGD1 patients.

Results and Conclusion

Clinically confirmed STGD1 (n=15) patients were recruited from Retina clinic-Aravind Eye Hospital, Madurai. The coding regions and adjacent intronic sequences of ABCA4 gene were amplified by Polymerase Chain Reaction (PCR) followed by Sanger Sequencing. Sequencing results revealed 33.3% (5/15) was positive for ABCA4 gene mutations. In total, five potentially disease causing variants were found in ABCA4 gene, including three reported, one novel missense and a in/del variant. Among the disease causing variant, most frequent variant was in exon-14 (c.C1995A; p.Y665Ter) (13.3%; 2/15). The research findings may contribute to the improvement of clinical care, genetic diagnosis and counselling as well as disclose the genetic causes and pathogenesis of Stargardt disease.

Clinical and Molecular Diagnosis of Retinal Dystrophies

Investigators	:	Dr. Pankaja Dhoble, Dr. P Sundaresan, Dr. K.C Lavanya
PhD Scholar	:	R. Kadarkarai Raj
Funding Agency	:	Subroto Bagchi Grant

Introduction

Retina is a light-sensitive tissue (0.5mm) located at back of eye. The retina consists of two layer, anterior multi-layered (neuro retina) and posterior (pigmented monolayer). Anterior retinal layer composed 6 major neuronal cell types are rods, cones, bipolar, horizontal, amacrine and ganglion cells. Rod and cone cells are located in the outer nuclear layer. Cone cells are responsible for sharp and colour vision. It was densely populated at macula as well as throughout retina and rod cells are responsible for dim light (night). Inherited Retinal Dystrophies (IRDs) are clinically heterogeneous and characterized by degeneration of photoreceptor (Rod, Cone) and retinal pigment epithelium RPE cells. Recent reports shows that cerebral visual impairment, optic nerve hypoplasia, and inherited retinal disorders are the most common causes of childhood blindness in the high- and middle-income countries. More than 250 genes are involved in retinal degeneration as well as large number of genes associated with IRD is unknown. Identification of the molecular cause of disease in many patients remains challenging because of genetic and phenotypic heterogeneity in the symptoms of IRD. Currently available next-generation sequencing methodologies offer an opportunity to screen the entire genome, exome of all the genes responsible for IRD. Here we describe the phenotype of 3 pedigrees with recessive IRD and the identification of the underlying mutations by Clinical Exome Sequencing.

Results and Conclusion

Three unrelated families with non-syndromic autosomal recessive Retinal Dystrophy Patients (Stargardt [n=1], Best [n=1] and Usher syndrome [n=1]) from Retina clinic, Aravind Eye Hospital, Pondicherry. Ophthalmic evaluation including measurement of best-corrected visual acuity (BCVA), Full-field Electroretinography (ERG), Fundus Finding and OCT were done to all study subjects. Approximately 4-5 ml of peripheral blood was collected from probands and their family members. DNA was extracted by modified salting-out method. Target genes were enriched with the Agilent Sure Select kit (V3) panel and the sequencing was performed on Illumina HiSeq4000 (Illumina, San Diego, CA, USA). Once sequence was completed, Primary analysis of raw FASTQ files processed using open-source fastq-mcf command line tool followed by secondary analysis was trimmed FASTQ reads to a human genome reference sequence (hg19/ GRCh37) and finally, tertiary analysis was done using the pipeline to annotate variants through VariMAT - Variation and Mutation Annotation Toolkit. From ANNOVAR file, the candidate genes reported in literature as responsible for the retinal dystrophies were further filtered to identify the pathogenic variant. This clinical exome sequencing results revealed that the presence of pathogenic variants in genes of *ABCA4* (Ex-3; c.A217T; p.I73F, Ex-42; c.G5882A; p.G1961E), *BEST1* Ex-9 (c.C701T; p.A234V), *MYO7A* (Ex-43; c.5880_5882del; p.F1963del) could be associated with the retinal dystrophies of Stargardt, Best and Usher syndrome. This study will initiate to increase the screening of retinal dystrophies genes in large cohort through targeted genomic sequencing approach to know the candidate genes associated in South Indian population.

Genetics of Ocular Tumors

Eye Cancer is a debilitating disease, which threatens the sight, eye and life as well. Every year, about 10,000 new eye cancer cases are diagnosed in India, 70 - 80% in adults and 20 – 30% in children. About 1500 children are diagnosed with Retinoblastoma (RB) each year in India. The major challenges of these ocular tumors are (1) The disease course of these tumor is much diverse and remains as an enigma (2) Response to the chemotherapeutic drugs varies highly from one individual to another (3) Inspite of multimode treatments, tumors still recur at new sites or on same site with higher vigor (4) It is very difficult to assess the risk of tumor spreading from one organ to another. In order to address these challenges, detailed molecular analysis is required. New projects were initiated to understand the deregulated cancer pathways in Retinoblastoma and the molecular basis of lymphoma, lacrimal gland tumors and melanoma, which are more aggressive and deadly if not diagnosed and treated on time.

Genetic Testing of Retinoblastoma

Investigators	:	Dr. A.Vanniarajan, Dr.D. Bharanidharan, Dr. Usha Kim, Prof. VR. Muthukkaruppan
Project Fellow	:	A. Aloysius Abraham
Funding Agency	:	Aravind Eye Foundation, USA

Introduction

Retinoblastoma (RB) is a rare childhood embryonic tumor arising from the cone precursor cells. RB is responsible for 1% of childhood cancer death and 5% of childhood blindness. RB is primarily caused by biallelic inactivation of the *RB1* tumor suppressor gene. In order to evaluate the possible risk of germline *RB1* mutations in offspring and siblings, families with twins were analysed. From 200 retinoblastoma families screened for *RB1* mutations, three families with twins, each having only one twin affected by bilateral RB was identified and genetic analysis of *RB1* was performed in all three family members through Sanger Sequencing and Multiplex Ligation dependent Probe Amplification methods along with DNA profiling.

Results

All probands were female and the disease was confirmed by clinical and radiological investigations. Out of three families, family III had a positive family history. Families I and II had similar looking twins. In family I, twin A was presented at 15 months with OS asymmetric nystagmus and intermittent leukocoria in both eyes. Her twin sister (Twin B), examined as a part of sibling screening, was found to have a small suspicious lesion in her right eye. Mutational screening revealed deletion of exons 4, 5 6 in twin A. Parents and sibling blood samples did not show any deletion. In family II, twin A was 30 months and presented with bilateral RB having exophytic tumors with vitreous seeding. Sibling screening confirmed that twin B was normal. Sanger sequencing of twin A blood revealed a nonsense mutation (c.1399 C->T) in exon 15 of the *RB1* gene, leading to premature protein truncation. Twin B and father did not have this mutation but mother was found to have this same mutation.

DNA profiling was carried out, in families I and II having similar looking twins, for zygosity mapping using AmpFLSTR™ Identifiler® PCR Amplification Kit according to the manufacturer's instructions. Profiles of families I and II clearly demonstrated the

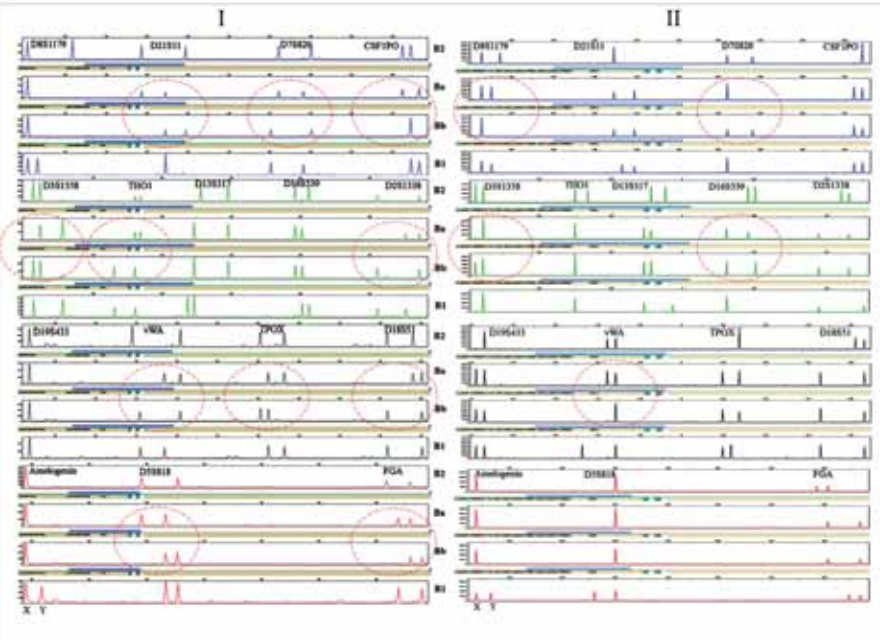


Figure 1: DNA profiling of two families demonstrating the discordance of retinoblastoma among twins. Ba-Twin A, Bb-Twin B, B1-Father, B2-Mother. Discordant loci are shown in dotted red circles.

discordance among the twins for many loci such as D7S820, D3S1350 and vWA [Fig. 1]. In family III, apart from the affected twin, there was an affected sibling, who was not her twin. The affected twin had group C in RE and group in E in the left eye which was enucleated as a part of treatment process. Co-segregation analysis in all the family members revealed 29bp deletion in exon-1 in all members of the family except the father, making them definitive carriers.

Conclusion

Combining high resolution techniques like DNA profiling and genetic testing of *RB1*, risk prediction for all members of affected families was made, thereby enhancing the quality of disease management. DNA profiling, a well validated technique which uses the microsatellites spread across the chromosome, is essential as it not only gives the biological identity of families and zygosity but also rules out the possibility of any sample switchovers.

Genetic Analysis of *RB1* in FFPE tumor samples

Investigators	:	Dr. A.Vanniarajan, Dr. Usha Kim, Dr. R. Shanthi, Prof. VR. Muthukkaruppan
Project fellow	:	K. Thirumalairaj
Funding Agency	:	Indian Council of Medical Research

Introduction

Tumor sample is most preferred for identification of the somatic mutation in Retinoblastoma. In advanced stage of tumors, enucleation will be carried out either with or without chemoreduction to avoid the extraocular spread of tumor. Primary enucleation is performed in most of the unilateral patients and secondary enucleation after chemoreduction is performed in bilateral patients. The tumor gets shrunken or becomes calcified due to the action of the chemotherapeutic drugs and viable tumor cells may not be available in the gross sectioning of the eye ball and hence tissue could not be accessible for analysis. Next choice of genetic analysis will be Formalin Fixed Paraffin Embedded (FFPE) tissues. Unlike the other tumors where FFPE sections had been frequently used for genetic studies, there are limited studies available for the genetic analysis of Retinoblastoma from the archival tissues. Thus, specific method needs to be developed for screening *RB1* mutation in FFPE sections in order to improve the mutation detection rate in Retinoblastoma cases.

Results

For the optimization of DNA isolation, three commercial kits were selected. Initially, 5µm thickness with three sections from non RB whole eye was used and genomic DNA was isolated as per the each kit protocol. Initially the quality of FFPE DNA was evaluated with three kits by checking the absorption maxima of isolated DNA through the 260/280 ratio. QIAmp DNA FFPE tissue kit provided poor quality DNA with 260/280 ratio of 2.03 indicating the higher RNA concentration. Crude isolation of Kappa express extract along with Kappa2G Robust Hot start ready mix (Kappa Biosystems, Wilmington, MA, USA) also yielded very low quality DNA with 260/280 ratio of 0.75 probably due to increased protein contamination and presence of other inhibitors. With

Table 1.Sample details and DNA yield of FFPE tumor samples

Sample ID	Laterality	Group	Age of Onset (Months)	Age at enucleation (Months)	Primary/ Secondary enucleation	DNA Concentration (µg)	DNA quality (260/280 ratio
FFPE U1	Unilateral	RE-E	42	42	Primary	1.6	1.72
FFPE B2	Bilateral	RE-E	6	9	Secondary	2.1	1.67
FFPE U3	Unilateral	RE-E	18	20	Primary	1.15	1.87

the ReliaPrep™ FFPE gDNA Mini prep isolation kit (Promega, Wisconsin, USA), good quality DNA was obtained with 260/280 of 1.85.

Since Promega kit gave good results, it was further used for DNA isolation of all FFPE tissues. With three sections of 5 µm, the yield was 0.68ug. For improving the quantity of DNA, number of sections was increased to 10 sections. The yield of DNA was 2.60 µg with 260/280 ratio of 1.82 and hence 10 sections of 5 µm was used for the FFPE tumor sections. FFPE sections were obtained from enucleated eyes of 2 unilateral and 1 bilateral patients. In bilateral patient FFPE B2, one eye was preserved and other eye enucleated. The DNA concentration of each FFPE tumor was given in the table 1.

Conclusion

Utility of FFPE will be very much useful to analyse *RB1* mutations in a large collection of tissue archives. Further optimization of the PCR and sequencing conditions are under progress which could be useful to know whether the FFPE tissues could be a suitable alternate for the genetic analysis.

Understanding the Molecular Basis of Chemoresistance in Retinoblastoma

Investigators : Dr. A. Vanniarajan, Dr. Usha Kim, Dr. R. Shanthi and Prof. V.R.Muthukkaruppan

Research Scholar : T.S. Balaji

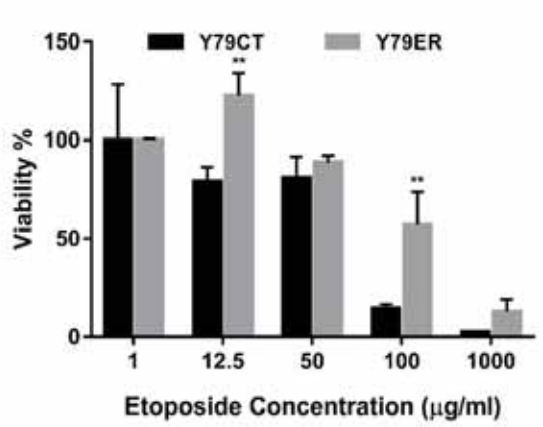
Funding Agency : Council of Scientific & Industrial Research (Fellowship)

Introduction

Retinoblastoma (RB) can be treated if diagnosed early, however, most RB cases in India are presented at late stages. At late stages, primary enucleation or enucleation after neoadjuvant chemotherapy is inevitable choice of treatment in consider with risk of life. In bilateral cases, efforts will be taken to preserve at least one of the eyes by treating with chemotherapy as main treatment modality. In such cases, chemoresistance is a main hindrance for the success of therapy. Identifying the key players involved in chemoresistance could help in development of new treatment strategy in these non-responding patients. In this study, involvement of ABC transporters in chemoresistance of RB tumors with a special focus on etoposide resistance was carried out using in vitro models.

Results

RB patients were categorized into 2 groups, Group I, n=17 with chemo-reduced patients treated with 2-6 vincristine, etoposide and carboplatin (VEC) cycle and Group II n=14 with non-responders treated with extensive VEC plus cyclosporin A (CSA). For in vitro study, etoposide resistance RB cell line (Y79ER) was established by stepwise drug concentration increment method. Y79 cells were considered as etoposide resistance when drug resistance index (DRI) reaches above 3 (figure 1).



IC50 concentration (ug/ml)		Drug Resistance index (DRI)
Y79CT	Y79ER	
64.5	502.8	7.8

Figure 1: Cytotoxicity assay for determining IC50 of etoposide in Y79 etoposide resistance cell line

Clinical resistance tumors (group II) were having high number of poorly differentiated (PD) tumors (85.7%, n=12) compared to chemoreduced RB (group I) (11.76%, n=3). Differentiation status was analysed in Y79ER using soft- agar colony formation assay. Y79ER had produced larger colony size in area (356 ± 87.52 µm) compared to its passage control (Y79CT24.18 ± 8.166 µm) (figure 2).

Gene expression of ABC transporters (ABCB1, ABCC1, ABCC3, ABCG2 and ABCE1) were analyzed, in which resistance RB tumors (group II) had significantly overexpressed ABCB1 and ABCC3, whereas Y79ER had significant overexpression of all transporters studied (figure 3)

Conclusion

Among the various suggested mechanisms of chemoresistance such as drug efflux pump (ABC transporters), presence of cancer stem cells (CSC), resistance of apoptotic pathway, the involvement of ABC transporters had been evaluated in etoposide resistance. Upon analysing the other factors, it is possible to derive the key players or common players that can initiate these mechanisms and could develop new strategies to combat chemoresistance.

Molecular Characterization of Tumor Progression in Retinoblastoma

Investigators : Dr. A. Vanniarajan, Dr. Usha Kim, Dr. D. Bharanidharan, Prof. VR. Muthukkaruppan
Research Scholar : T. Shanthini
Funding Agency : DST-INSPIRE Fellowship

Introduction

Cancer is a heterogeneous disease caused by multiple molecular mechanisms and eye cancer Retinoblastoma is not an exception which is caused

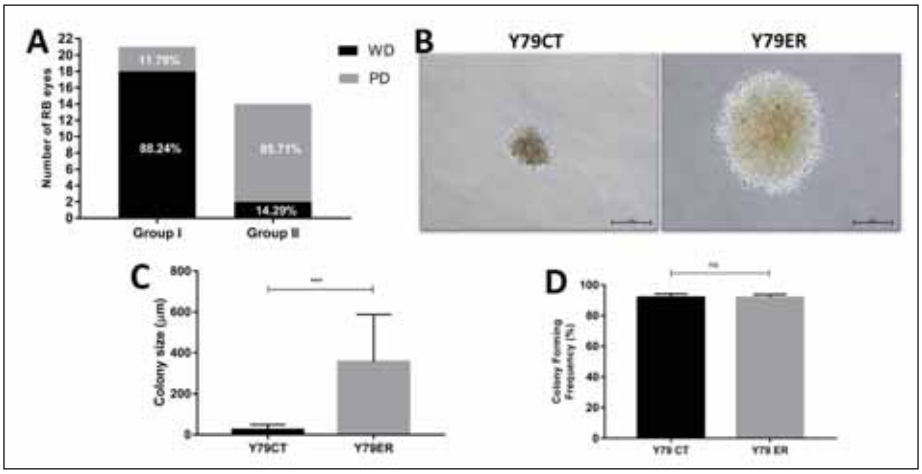


*Figure 2: Effects of therapeutic resistance in differentiation. A) Differentiation pattern in chemoresistance RB tumors, B) Colony forming assay, C) average area size and D) colony forming frequency (CFF). WD-Well differentiation, PD-Poorly differentiation, Magnification-20X, scalebar-500 µm. ***P<0.001*

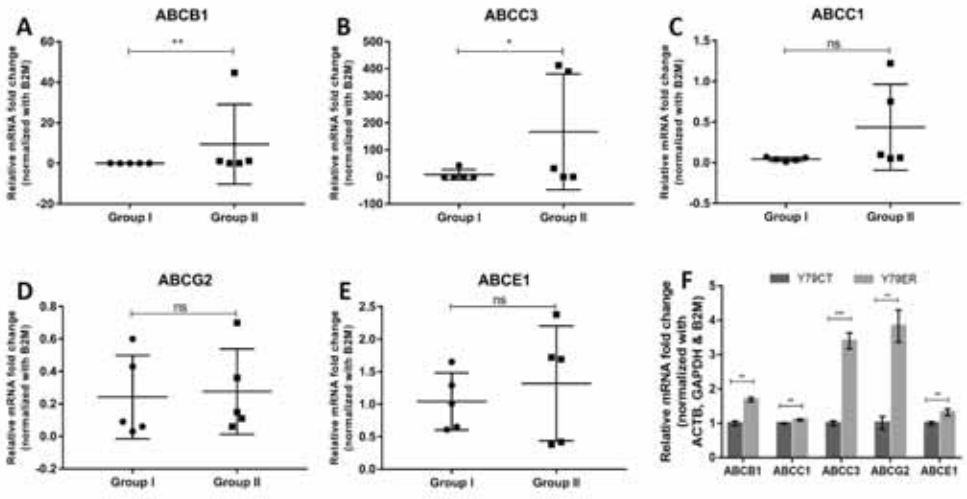


*Figure 3: Gene expression of ABC transporters in chemoresistance RB tumor and etoposide resistance cell line. *P<0.05, **P<0.01 and ***P<0.001*

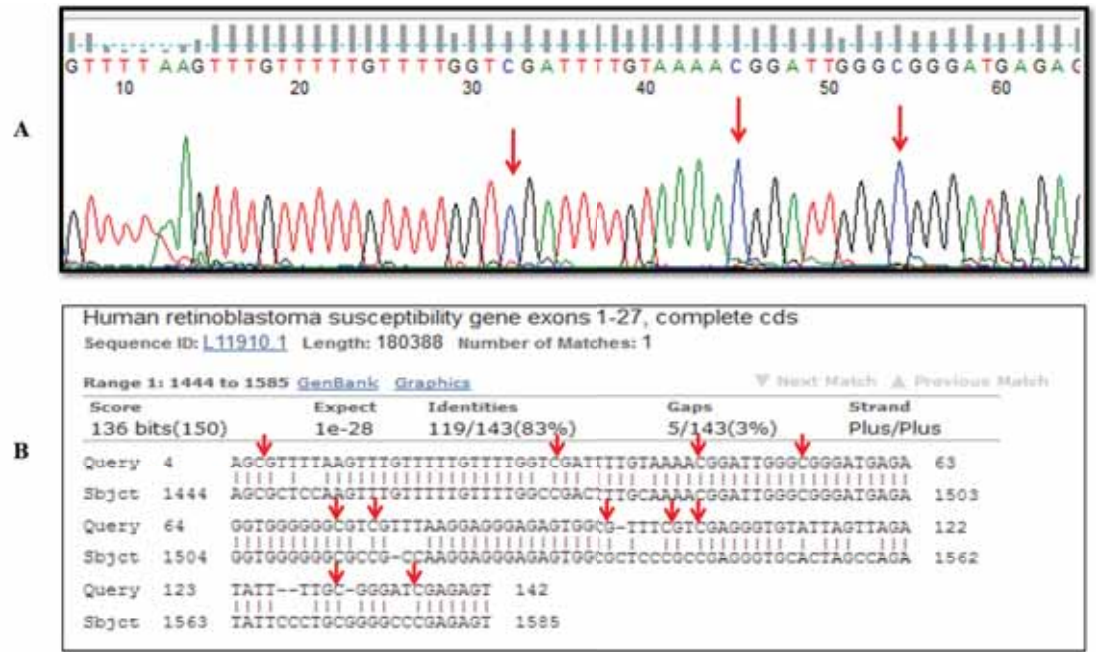


Fig 1 Bisulfite sequencing - complete methylation

A) Sequence of RB1 promoter with red arrow indicating methylated cytosine that remained unchanged after bisulfite conversion
B) BLAST analysis showing the complete methylation where all the CpGs were methylated

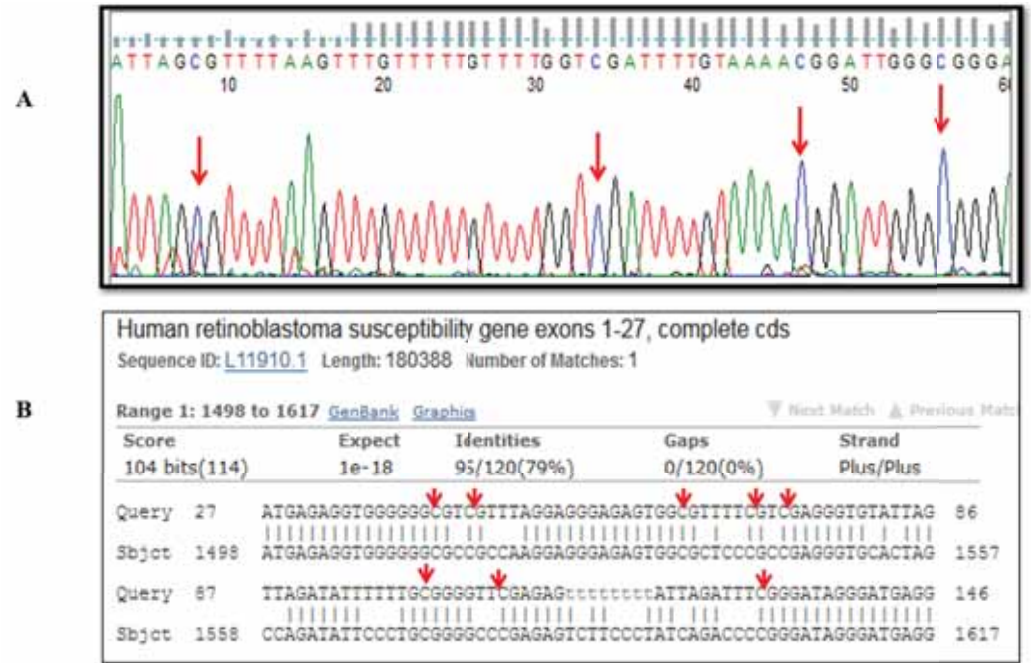


Fig 2 Bisulfite sequencing - Partial methylation

A) Sequence of RB1 promoter with red arrow indicating methylated cytosine that remained unchanged after bisulfite conversion
B) BLAST analysis showing partial methylation where only 8 of the 11CpGs were methylated

by genetic and epigenetic mechanism. It is well documented that promoter methylation of *RB1* could cause the inactivation of *RB1* and similar epigenetic changes in other genes could lead to the tumor progression in retinoblastoma. The present study looks at the epigenetic changes occurring at tumor initiation and progression.

Results

A total of 48 RB patients were screened for deletion/ duplications and promoter methylation analysis of *RB1* gene using MS-MLPA and identified promoter methylation in one patient. In order to further validate, another method is also optimised. Bisulfite modification of DNA is the most commonly used, gold standard method for DNA methylation studies providing single nucleotide resolution. This technology is based on the chemical conversion of unmethylated cytosine to uracil. Methylated cytosines are protected from this conversion allowing to determine DNA methylation at the singe nucleotide level by sequencing.

The samples showing positive for methylation in MS-MLPA were taken for optimizing bisulfite sequencing. 2µg of starting material DNA was taken for bisulfite conversion. Bisulfite conversion was carried out using Zymo - EZ DNA methylation kit. After bisulfite conversion, bisulife PCR and direct sequencing was done. For PCR and sequencing, the primers were designed using bisulfite primer seeker tool that covers for the 11 CpG sites of the *RB1* promoter sequence.

The sample which shows the homozygous methylation in MS-MLPA also shows the complete methylation (Fig.1) upon sequencing in which 11 of the 11 CpG sites were methylated and remained as cytosine after bisulfite conversion. The sample having heterozygous methylation (Fig.2) shows the partial methylation in which only 8 of the 11CpGs were methylated.

Conclusion

Promoter methylation had expanded the spectrum of alterations in *RB1* gene. Further the confirmation of the methylation sites by bisulfite sequencing helped also to distinguish partial and complete methylation. In future, the promoter methylation for the panel of genes will be validated through bisulfite sequencing.

Identification and Validation of deregulated pathways in Retinoblastoma

Investigators : Dr. A. Vanniarajan, Dr. Usha Kim, Dr. R. Shanthi, Dr, D. Bharanidharan
Project Fellow : Anindita Rao
Funding Agency : DST-SERB

Introduction

Retinoblastoma (RB) is an embryonic malignant neoplasm of retinal origin and biallelic inactivation of *RB1* is the first step in tumorigenesis. Extensive research has shown that *RB1* mutations alone are insufficient for the tumor progression. There are additional genetic events that occur in the pre-neoplastic retinal cells after *RB1* inactivation for the complete tumor formation. This inactivation of the retinoblastoma tumor suppressor (pRb) in turn alters the expression of genes important for cell cycle progression, senescence, insulin signaling, JAK/STAT pathway, DNA damage and apoptosis. There may be processes that counterbalance the transcriptional changes that mark tumor development and it is important to identify and analyse the key genes involved in those deregulated pathways. This project is initiated in this year and the work flow is given as below in Figure 1.

Figure 1: Workflow for understanding the deregulated cancer pathways.

Results

Twelve datasets containing gene expression profiles by microarray of Retinoblastoma tumors were retrieved from online biological databases – GEO (NCBI) and ArrayExpress (EMBL). After normalizing the data and parameters across the datasets, computational analysis using R-Script will be performed to find genes commonly dysregulated across samples. Hub genes associated with Retinoblastoma will be identified based on co-expression network analysis with the help STRING database and CYTOSCAPE software. For validation, RNA from normal young adult neural retinal tissues of cadaver eyes was extracted. These samples serve as controls for validating the gene expression changes identified in RB tumors.

Conclusion

The gene expression data will be filtered out based on the abundance, significance and closeness to *RB1*. With this approach, it is possible to detect how the identified genes crosstalk with *RB1* and understand the deregulated pathways in Retinoblastoma for developing suitable therapies.

Molecular analysis of Ocular Cancers

Investigators : Dr. A.Vanniarajan, Dr. Usha Kim, Dr.R. Shanthi, Dr. D.Bharanidharan, Prof. VR. Muthukkaruppan
Project fellow : K. Saraswathi
Funding Agency : Aravind Eye Foundation, USA

Introduction

Ocular cancers are unique among the other diseases of the eye, threatening both vision and life. The most common cancers of eye include Carcinoma, Conjunctival neoplasia, Choroidal melanoma, Ocular adnexal lymphoma and Metastatic orbital tumors. Lymphoma is the neoplastic proliferation of lymphocytes at various stages of differentiation. It is broadly classified into Hodgkin and non-Hodgkin lymphoma (NHL). Most ocular lymphomas are NHL and are of B- cell origin (about 85%). Ocular lymphoma can either present in intraocular or ocular adnexal structure. Ocular adnexal lymphoma is the most common form of ophthalmic NHL, representing about 5-15% of all tumors present in the orbit, conjunctiva, lacrimal apparatus and eyelid. This new project was initiated in this year.

Results

As a first step towards understanding the prevalence and nature of lymphoma, clinical data was collected from 2012. Total number of lymphoma cases presented at AEH, Madurai from 2012 to 2018 was 73. The most common symptom noticed among ocular lymphoma patients was painless swelling, reported as proptosis. Patient turnover details are given in the below table 1.

Male predominance was noticed with 49 males over 24 females during the past 6 years. Unilateral presentation was more frequent than bilateral, accounting for about 90%. The age of presentation ranged from 30 to 80 years. Anatomically ocular adnexal lymphoma was found in lacrimal gland area, conjunctiva, upper and lower eyelids. Typing of lymphoma was done based on H & E staining followed by IHC with CD3, CD20, LCA antibodies. All these cases were of B-cell origin, classified based on shape and size of cells as small, medium, and large. Based on the pattern, they were classified as follicular, diffuse, anaplastic variant. Blood samples were collected from five patients that include new as well as follow-up patients along with their family. Blood DNA was isolated and quality checked. Tumor samples were collected for 4 patients and tumor tissue DNA was isolated for one sample and quality checked. The clinical picture and the tissue collected from lymphoma patient is shown in Fig 1.

Proptosis, swelling *Collected Tumor Tissue*

Figure1: Clinical picture showing the proptosis and tumor tissue collected from a Lymphoma patient

Conclusion

Similar analysis of carcinoma and melanoma will be performed like lymphoma. Genetic analysis involving translocation detection for these samples will be performed with the isolated DNA.

Year	Gender		Laterality		Eye		Total no. of cases
	Male	Female	Uni	Bi	RE	LE	
2012	4	2	5	1	3	1	6
2013	8	5	13	-	7	-	13
2014	6	6	11	1	4	1	12
2015	9	5	11	3	8	3	14
2016	6	3	8	1	5	1	9
2017	5	2	7	-	2	-	7
2018	11	1	11	1	6	1	12

Table 1: Clinical details of Lymphoma patients from 2012-18

Translational Genomics of Paediatric Eye Diseases

Investigators : Dr. P. Sundaresan, Dr. A. Vanniarajan, Dr. D Bharanidharan, Dr. VR. Muthukkaruppan, Dr. P. Vijayalakshmi, Dr. R. Kim, Dr. Usha Kim

Project Fellows : R.C. Vignesh, Gowri Poigaialwar, A.S.Sriee Viswarubhiny, Susmita Chowdhury, A.Aloysius Abraham K. Jeya Prakash, A. Mohamed Hameed Aslam, C. Prakash, K. Manoj kumar

Funding agency : Department of Biotechnology

Introduction

Visual impairment or blindness is a major public health problem affecting 285 million worldwide. Among them, 45 million of them are totally blind and 1.4 million are children under age 16. India ranks first among all countries accounting for 20% of total blind population. It is estimated that prevalence of childhood blindness in India is 0.8 per 1000 children in less than 16 years age group. Most of these children lose their vision before the age of 5, the crucial period in life when 75% of their learning is through sight.

The leading causes of childhood blindness in India include congenital anomalies and retinal dystrophies together contributing to 40%. Retina being the most affected site of blindness contributes about 29% of abnormalities in children. The most common retinal diseases in India include

Summary of work done:

Project	Project Title & Objectives	Work Done
COE LEAD	Translational Genomics of paediatric eye diseases Objectives: To create gene panel for paediatric ocular diseases with known molecular cause	- equipment purchase and installation - Staff recruitment and training completed - Proforma for patient details made - Patient identification done and analysis started.
COE PR-I	Molecular analysis of mitochondrial diseases with ophthalmic manifestations Objectives: To clinically evaluate the mitochondrial involvement in ocular diseases	- LHON Patients recruited - Primary mitochondrial mutations analysed - Methods for whole mtDNA sequencing established
COE PR-II	Epigenetic mechanisms underlying tumor progression in retinoblastoma Objectives: Identify patient samples with RB who required further molecular events for progression of RB	- Patients with and without RB1 mutations identified - Prepared RNA/DNA samples from representative patients - MS-MLPA and Bisulphite sequencing optimised for RB1 - Primers for a set of genes were designed
COE PR-III	Functional validation of novel candidate genes using alternate model Objectives: Selection and validation of candidate human disease gene from genomic data	- Recruitment done. - Establishment of the setup is planned and will be made based on recommendations of Monitoring Committee
COE R & D	Computational methods for whole exome/ genome sequencing of paediatric eye diseases Objectives: Evaluation of alignment, variant calling tools and designing analysis pipeline by integrating tools for eye-disease associated clinical exome sequencing data	- Benchmarking of variant calling pipelines performed with standard human exomes, simulated and exome data of eye diseases. - A comprehensive in-house whole exome analysis pipeline established.

Table 1: Objectives of each project with the achievable targets and work done during the 10 months duration

Retinoblastoma, Leber Hereditary Optic Neuropathy, Leber Congenital Amaurosis, X-linked Retinoschisis that are predominantly caused by one or few genes. Understanding the molecular mechanisms of paediatric eye diseases affecting retina and translating the findings for early detection and possible treatment is the major focus of this project.

Approaches

The workflow involves patient recruitment, collection of clinical and genetic data and analysis of the blood/ tumor samples of the patients as shown in Figure 1. The methods used include Sanger Sequencing, End Point PCR-RFLP, LCM and Bisulphite Sequencing.

Patient recruitment and Clinical Data:

Details of the patients recruited so far are given in the table 2. Blood samples were collected from patients and family members. Tumor samples were collected from RB patients undergoing surgical treatment.

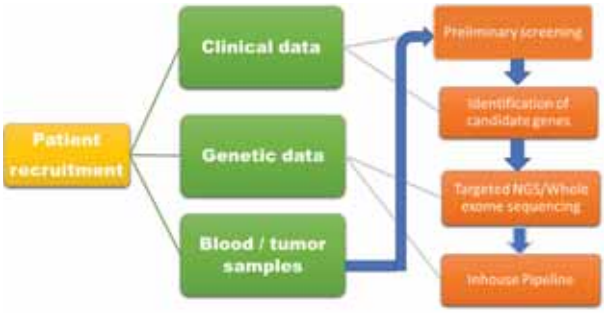


Fig 1: Approaches used to obtain the data and their analyses

Design of Targeted gene panel

Through the survey of literature and databases, a customised gene panel was designed with 24 genes of RB; 29 genes of LCA, 37 genes of LHON along with RS1 of JXLR as listed in Figure 2.

Molecular analysis of mitochondrial disease with ophthalmic manifestations

Based on the clinical phenotype and maternal inheritance pattern, 94 unrelated LHON patients

Diseases	No. of Probands	Male	Female	Family members	Retcam/ Fundus	OCT	MRI	ERG/ USG-B scan	Histopathology
Retinoblastoma (RB)	75	34	41	163	√	√	√	√	√
Leber Congenital Amaurosis (LCA)	61	23	38	137	√	√	NA	√	NA
Leber Hereditary Optic Neuropathy (LHON)	94	84	10	59	√	√	NA	√	NA
Juvenile X linked Retinoschisis (JXLR)	22	19	3	80	√	√	NA	√	NA

Table 2: Patients recruited and the relevant clinical details

RB			LCA			LHON				JXLR
RB1	OTX2	CCND1	AIPL1	IFT140	RPE65	MT-TF	MT-TW	MT-ATP8	MT-TL2	RS1
MYCN	SYK	BRCA2	ALMS1	IMPDH1	RPGRIP1	MT- RNR1	MT-TA	MT-ATP6	MT-ND5	
TP53	BAZ1A	BRCA1	CABP4	IQCB1	PRPH2	MT-TD	MT-TN	MT-CO3	MT-ND6	
BCOR	DDX1	CCNE1	CEP290	KCNJ13	SPATA7	MT-RNR2	MT-TC	MT-TG	MT-TE	
MDM4	CDH13	E2F1	CLUAP1	LCA5	TULP1	MT-TL1	MT-TY	MT-ND3	MT-CYB	
KIF14	CHFR	CHEK2	CRB1	LRAT	CNGA3	MT-ND1	MT-CO1	MT-TR	MT-TT	
DEK	GATA5		CRX	NMNAT1	MYO7A	MT-TI	MT-TS1	MT-ND4L	MT-TP	
E2F3	TP73		DTHD1	OTX2	CCT2	MT-TQ	MT-TD	MT-ND4		
CDH11	E2F2		GDF6	RD3	MERTK	MT-TM	MT-CO2	MT-TH		
			GUCY2D	RDH12		MT-ND2	MT-TK	MT-TS2		

Figure 2: Targeted gene panel for four retinal diseases with known molecular cause

Figure 3: Temporal optic nerve pallor and thinning or RFNL shown by fundus and OCT respectively in a LHON patient

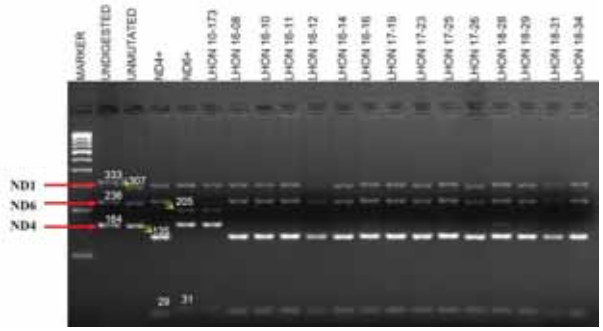


Figure 4: Multiplex PCR – RFLP indicating the Mt ND4 and Mt ND6 gene mutations

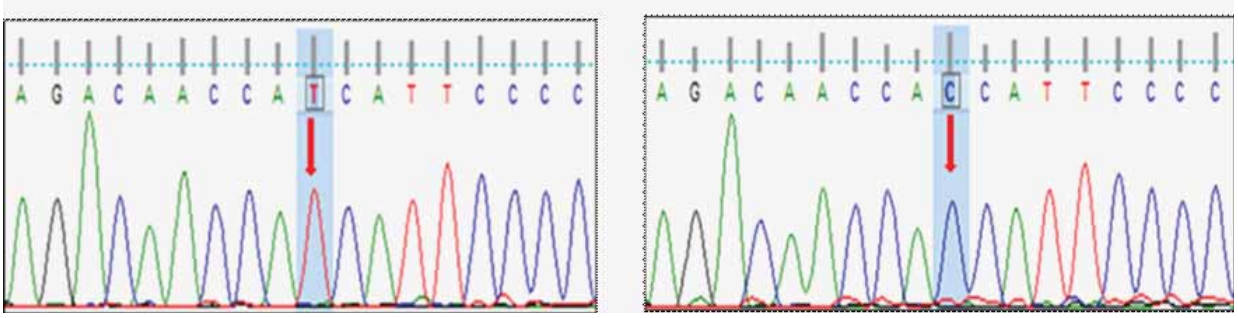


Figure 5: Sanger sequencing showing the mitochondrial ND6 mutation 14484T>C in LHON proband (right) compared to normal (left)

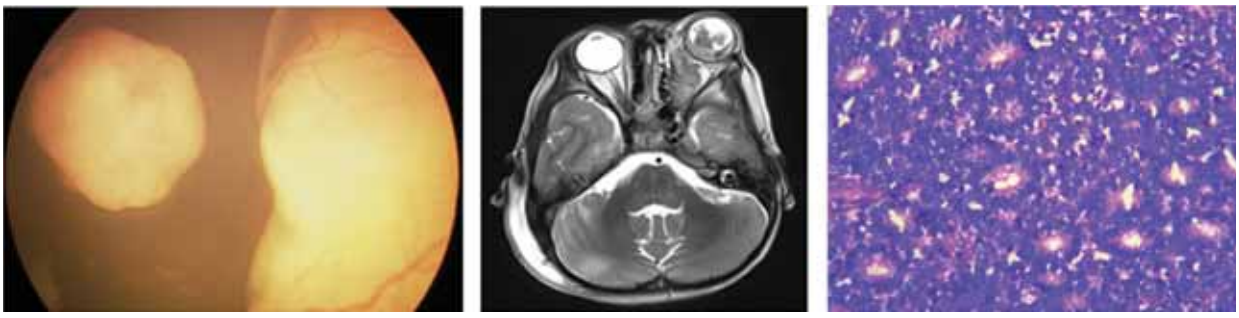


Figure 6: Retinoblastoma tumor shown by large mass in Retcam (left) and MRI images (Middle) and as rosettes in H & E staining (right)

were recruited for the study and 153 samples were collected from proband and family members. Clinical details of one patient are shown in Figure 3.

Multiplex PCR-RFLP, a simple and cost-effective approach was employed to detect all three primary mutations in the mitochondrial genome of LHON patients as shown in Figure 4. Using the Capillary electrophoresis instrument 3500 Genetic Analyzer purchased in this project, Sanger sequencing was done for validation (Figure 5).

Epigenetic Changes Underlying the Tumor Progression in Retinoblastoma

Clinical investigations of Retinoblastoma was done using multiple imaging and histopathological modalities. The representative images of tumor are shown in Figure 6. Laser capture microdissection (Figure 7) was performed to isolate the tumor cells

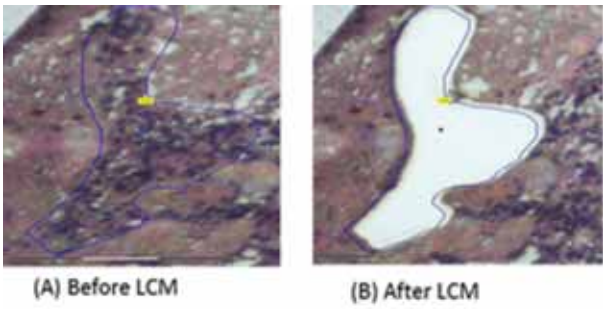


Figure 7: Laser Capture Microdissection performed to isolate RB tumor

alone and optimised the DNA isolation procedures. Bisulphite sequencing was optimised for *RB1* gene, that showed 11 CpG sites demonstrating the complete methylation. The results were also verified by MS-MLPA.

Whole exome sequencing (WES) and Data Analysis

WES performed in blood DNA samples of two Trios in each disease LCA, LHON and JXLR, and six tumor DNA samples of RB with Illumina HiSEQ4000 at Medgenome, Bangalore (Table 3). In-house pipeline was developed based on the benchmarking on well-known tools as shown in the workflow (Fig. 8). More than 50 pathogenic variants were identified in each disease. In LCA trios, two causative genes, IQCB1 and GUCY2D were identified in probands AS0041 and AS9101 respectively, Pattern of segregation was shown in family of AS0041 (Fig.9). *RB1* pathogenic mutations were identified in 85% of the tumor samples.

Table 3 : Details of patients samples sent for exome sequencing

Sample ID	Age (years)	Gender	Relation	Disease
S257	7	Male	Proband	Juvenile X linked retinoschisis
S837	35	Male	Father	Nil
S293	29	Female	Mother	Nil
S8161	11	Female	Proband	Juvenile X linked retinoschisis
S8162	39	Male	Father	Nil
S8163	36	Female	Mother	Nil
AS0041	3	Female	Proband	Leber's Congenital Amaurosis
AS0042	31	Male	Father	Nil
AS0043	26	Female	Mother	Nil
AS9101	4	Female	Proband	Leber's Congenital Amaurosis
AS9102	35	Male	Father	Nil
AS9103	31	Female	Mother	Nil
PG2251	18	Female	Proband	Leber's hereditary optic neuropathy
PG2252	41	Male	Father	Nil
PG2253	39	Female	Mother	Nil
PG7741	25	Male	Proband	Leber's hereditary optic neuropathy
PG7742	55	Male	Father	Nil
PG7743	41	Female	Mother	Nil
BR1	3	Male	Proband	Retinoblastoma
BR2	3	Female	Proband	Retinoblastoma
BR3	1	Female	Proband	Retinoblastoma
BR4	2	Male	Proband	Retinoblastoma
BR5	3	Female	Proband	Retinoblastoma
BR6	2	Female	Proband	Retinoblastoma

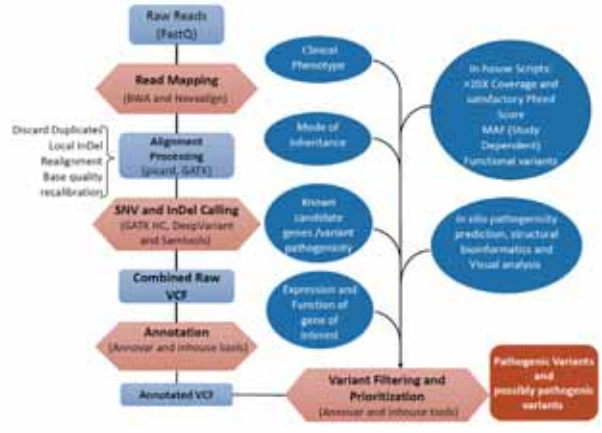


Figure 8: In house pipeline developed for whole exome sequencing analysis

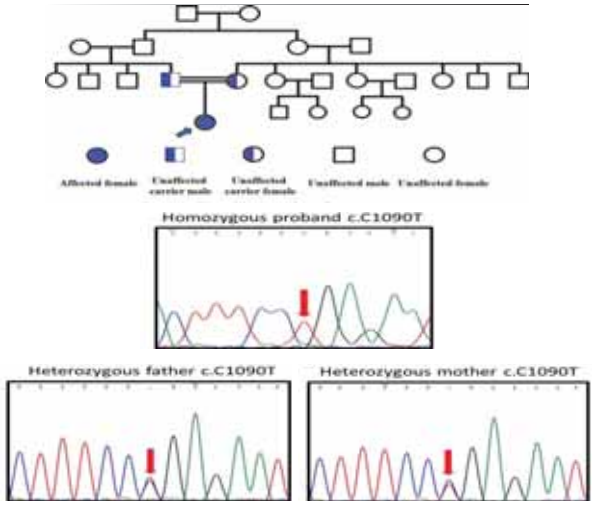


Figure 9: Pedigree and Segregation of IQCB1 mutation in family of AS0041

STEM CELL BIOLOGY

The research focus of this department is on the basic biology of adult stem cells of human eye. Studies on corneal epithelial stem cells led to the establishment of a specific method for their identification and quantification, and a two-step protocol to enrich the stem cells from 3-5% to 80%. Recent studies using such enriched stem cells have identified miRNAs that are specific to corneal epithelial stem cells. This year the functional role of two stem cell specific miRNAs – miR-143-3p and miR-150-5p have been analysed by transfection studies and by analysing the expression of their target genes reported to be associated with the maintenance of stemness. In addition, studies on the stem cells in human trabecular meshwork have also been initiated to understand its biology - identification, localization and content with ageing and in glaucoma.

Limbal miRNAs and their potential targets associated with the maintenance of stemness

Investigators	:	Dr. Gowri Priya Chidambaranathan
Co-Investigators	:	Dr. Bharanidharan Devarajan, Dr. VR. Muthukkaruppan, Dr. N. Venkatesh Prajna
Research Scholar	:	Lavanya Kalaimani
Funding	:	Department of Biotechnology

Introduction including background

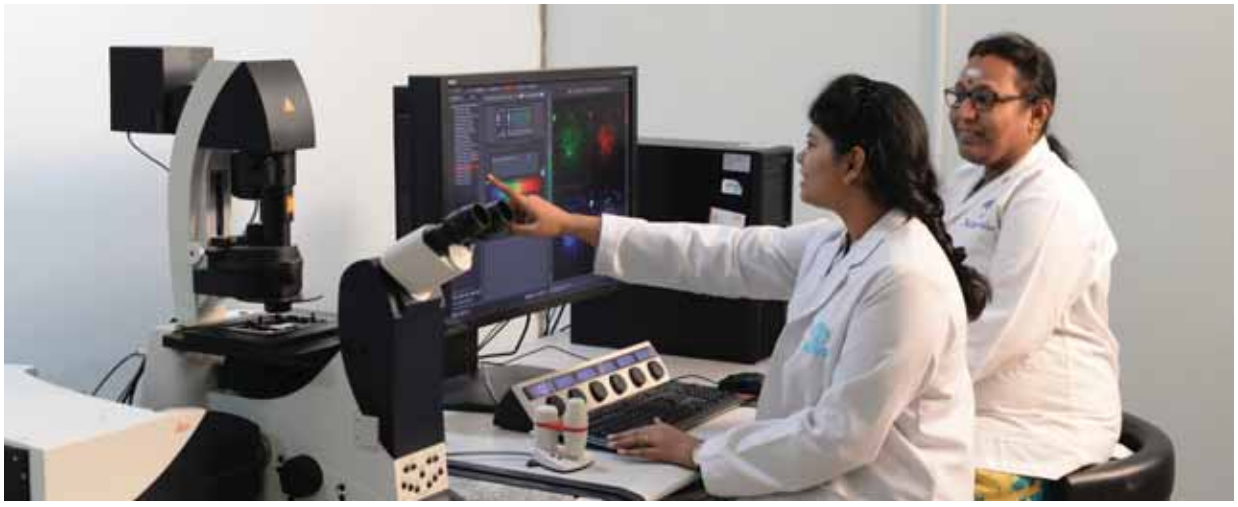
Cornea and the tear film comprise the anterior transparent window which acts as a physical barrier between the optical and external environment.

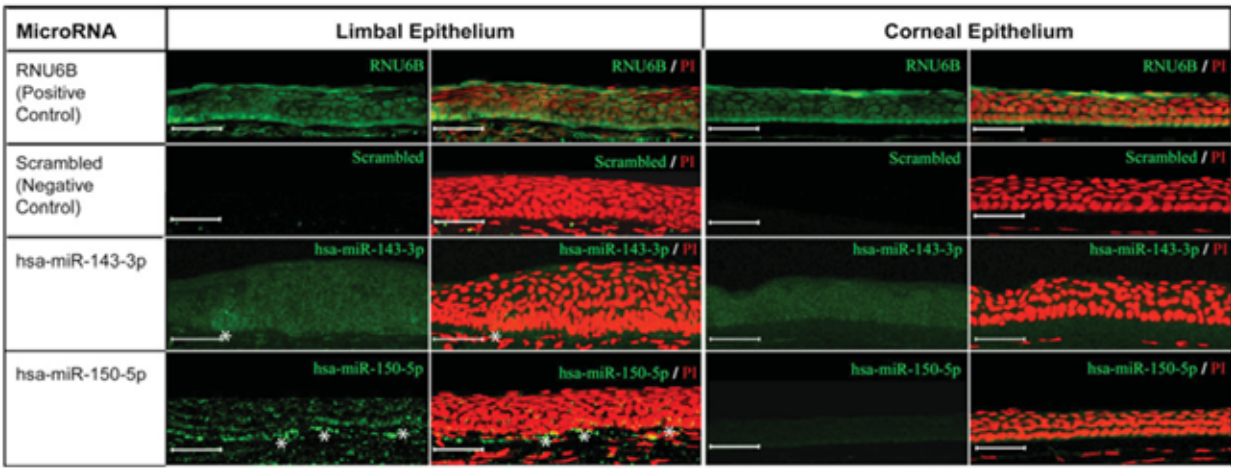
The corneal epithelium is the outer most layer of the cornea and is regenerated from the stem cells that are present in the adjacent region – limbus. These adult stem cells constitute 3-5% of the total limbal epithelium and are normally quiescent. They divide when there is a need and it is still not clear how the stem cells are primed to renew themselves or to differentiate. The objective of the study is to understand the molecular regulation of these corneal epithelial stem cells (CESCs) by microRNAs (miRNAs).

The current study is of significance since a highly enriched stem cell population (nearly 80%) from the 3 to 5% in native population is used for profiling the miRNAs in CESCs. Using the enriched CESCs, six microRNAs (hsa-miR-3168, hsa-miR-21-5p, hsa-miR-143-3p, hsa-miR-150-5p, hsa-miR-1910-5p and hsa-miR-10a-5p) were identified to be highly expressed in stem cells by small RNA sequencing. This data was validated by quantitative real time PCR (Q-RT PCR) and locked nucleic acid in-situ hybridization (LNA-ISH).

Results

Among the six miRNAs, hsa-miR-143-3p and hsa-miR-150-5p showed higher expression in CESCs in comparison to CCECs with fold change of 76.44±3.1 and 13.86±1.5 respectively by Q-RT PCR analysis. By LNA-ISH, the expression of hsa-miR-143-3p was identified to be restricted to clusters of cells in the limbal basal epithelium. Though the expression of hsa-miR-150-5p was found to be higher in limbal basal epithelial layer compared to other layers, its expression was highest in a few clusters of cells in limbal basal epithelium.





Locked nucleic acid in-situ hybridization of hsa-miR-143-3p and hsa-miR-150-5p. The expression of hsa-miR-143-3p (green) was restricted to clusters of cells in the limbal basal epithelium, while the expression of hsa-miR-150-5p (green) was evident in all layers of limbal epithelium however highly prominent in a few clusters in basal layer. The expression of both these miRNAs in corneal epithelium was undetectable. Nuclei were stained with propidium iodide (PI, red). Housekeeping RNU6B was detected in all layers of epithelium both in limbus and cornea, whereas no signal was detected when hybridized with scrambled sequence. Asterisks represent the positivity in clusters. Scale bar: 50µm

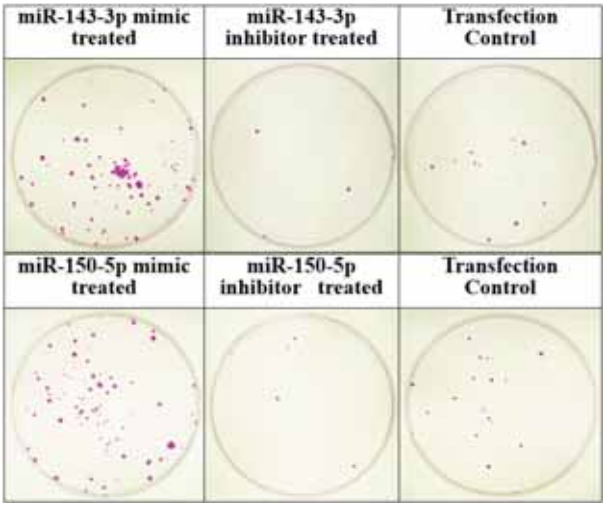
MiRNA transfection studies were carried out with these two miRNAs independently to analyse their potential regulatory role in the maintenance of stemness. The primary limbal epithelial cells were transfected with miRNA (hsa-miR-143-3p and hsa-miR-150-5p) mimics and inhibitors.

The hsa-miR-143-3p mimics treated cells showed increased percentage of colony forming cells (mean ± SD: 10.04 ± 0.45; P value < 0.0001) compared to that of control (3.33 ± 0.23) and hsa-miR-143-3p inhibitor treated cells (0.26 ± 0.08). In addition, the percentage of holoclones (7.65 ± 0.81; P value < 0.0001) was higher than the control (0.69 ± 0.69) and the inhibitor treated cells did not form any holoclone.

Similarly, the hsa-miR-150-5p mimics treated cells had higher colony forming efficiency (8.28 ± 0.33; P value < 0.0001) compared to that of control (1.8 ± 0.15) and hsa-miR-150-5p inhibitor treated cells (0.71 ± 0.10). A significant increase in the holoclone percentage (3.80 ± 0.84; P value < 0.0001) was observed in comparison to that of the control (1.11 ± 1.11) and the inhibitor treated cells produced no holoclone.

Thus, it was evident that higher expression of hsa-miR-143-3p and hsa-miR-150-5p increased the colony forming efficiency and supported holoclone formation. Since only stem cells are capable of producing holoclones, their increase is strongly correlated with maintenance of stemness.

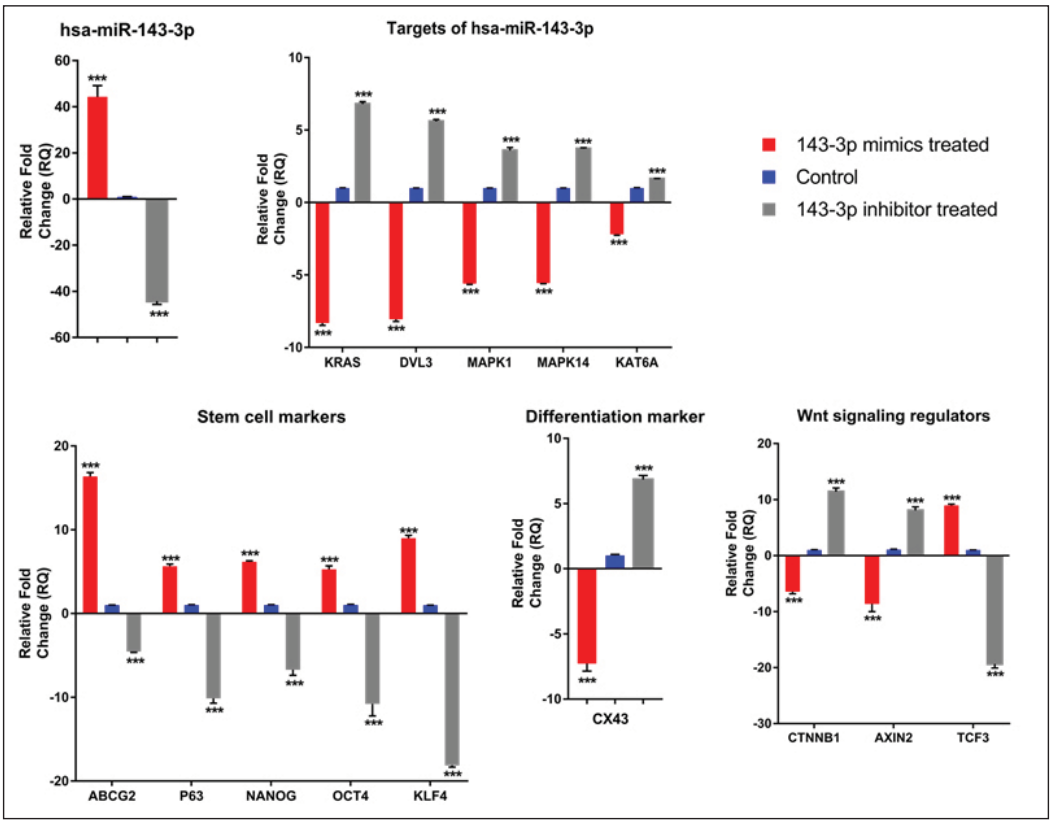
The mimics treated group showed increased expression of putative stem cell marker genes - ABCG2, Nanog, Oct4, Klf4 and ΔNp63, reduced expression of corneal differentiation marker - connexin 43 and their predicted targets by Q-RT PCR analysis. In this mimics treated group, Wnt signalling was found to be down regulated on the basis of down regulation of genes involved in Wnt signalling - β catenin and Axin2 along with the up regulation of TCF3 gene, a known inhibitor of Wnt signalling.



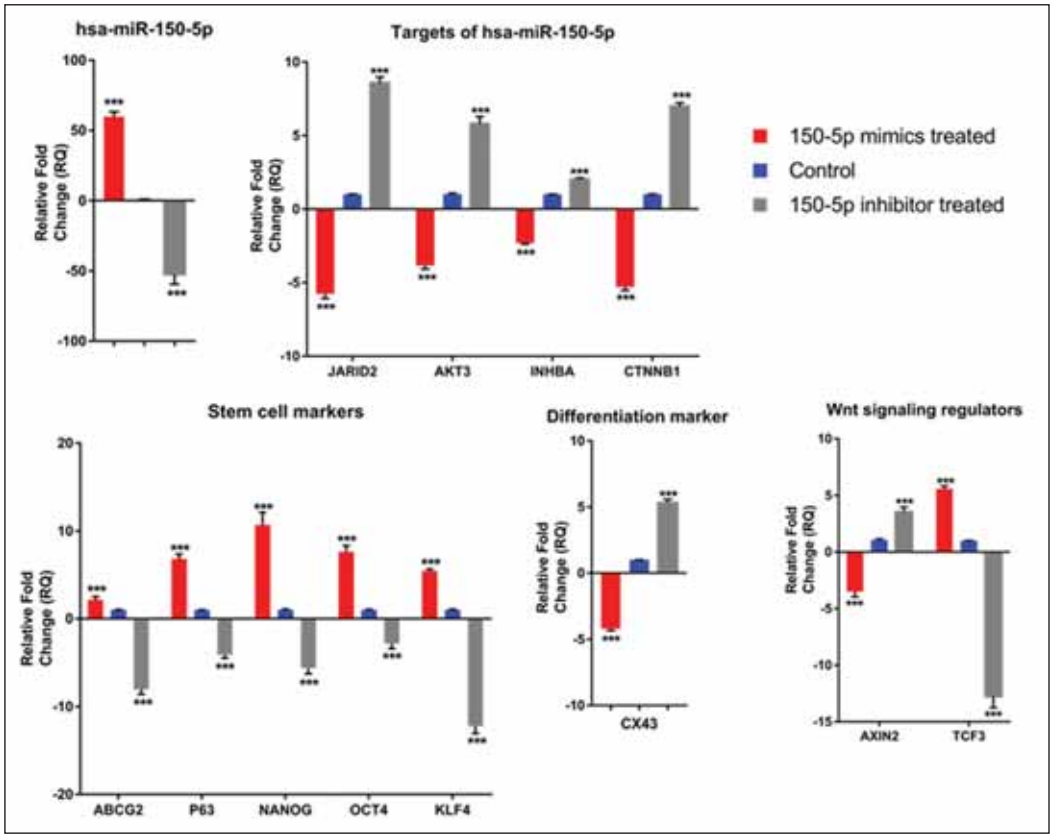
Rhodamine B stained limbal epithelial cell colonies, 12 days after transfection with miRNA mimics and inhibitors. The colony forming efficiency was higher in miR-143-3p and miR-150-5p mimic treated cells compared to the inhibitor treated and control cells.

Conclusion

The functional analysis at the transcriptional level confirmed that hsa-miR-143-3p and hsa-miR-150-5p were associated with the regulation of stemness of CESCs by inhibiting Wnt signalling pathway. Further analysis at the translational level is essential to prove their regulatory potential and to understand their mechanism of action in Wnt signalling pathway.



Expression profile of hsa-miR-143-3p, its predicted targets, stem cell markers, differentiation marker and Wnt signalling regulators upon transfection with hsa-miR-143-3p mimic and inhibitor



Expression profile of hsa-miR-150-5p, its predicted targets, stem cell markers, differentiation marker and Wnt signalling regulators upon transfection with hsa-miR-150-5p mimic and inhibitor

Characterization and Functional Evaluation of Trabecular Meshwork Stem Cells in Glaucoma Pathogenesis

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Funding : Science and Engineering Research Board

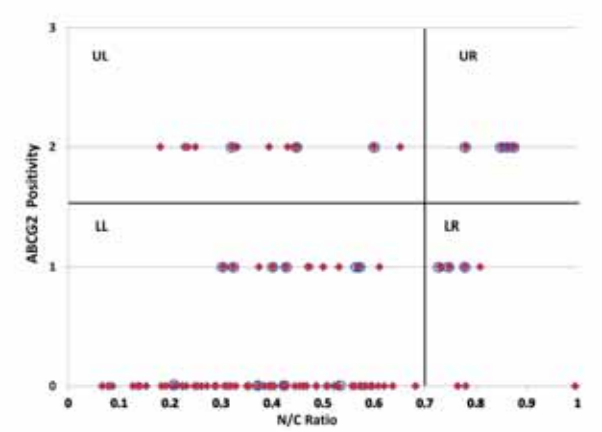
Introduction including Background

Glaucoma is a neurodegenerative disease characterized by progressive loss of retinal ganglion cells leading to optic nerve damage resulting in peripheral vision loss. Primary open angle glaucoma (POAG) is a predominant form (74%) of glaucoma. Elevated intraocular pressure (IOP) due to increased resistance in aqueous humor (AH) outflow is the major risk factor for POAG. Earlier reports have demonstrated the age-related reduction in the trabecular meshwork (TM) cells and this decrease was more pronounced in glaucomatous condition. Along with this anatomical change, deposition of extracellular material and appearance of cross-linked actin network in the TM are responsible for the increase in the outflow resistance and hence leading to elevated IOP. There are growing evidences suggesting the presence of stem cells in the anterior non- filtering region of the human TM. Recent studies on cultured human TM stem cells (TMSCs) demonstrated its ability to home and integrate to the TM in a normal, non-glaucomatous animal model. Further studies using iPSC-derived TM cells revealed that the transplanted cells activated the endogenous stem cells to proliferate and regenerate the TM in a glaucomatous animal model. But till date no reports are available on the role of the TMSCs in maintaining the tissue homeostasis. Hence, this study aims to identify, quantify and characterize the native and cultured TMSCs in normal and glaucomatous human eyes to understand the basic biology of these adult tissue resident stem cells during ageing and glaucomatous condition.

Results

The cytosmears of native human TM cells of age group <30 years, 30-60 years and >60 years (n=5 pairs/ age group) obtained from Rotary Aravind International Eye Bank) were double immunostained for the universal stem cell marker ABCG2 and the neural crest derived stem cells marker p75. Images for 100 consecutive cells were acquired using Leica SP8 confocal microscope and were analysed for two parameters: marker expression and nucleus to cytoplasmic ratio (N/C ratio).

Two-parameter analysis of native TM cells revealed 21.0±1% (Mean±SD) of cells in younger donors (<30 years) to have high ABCG2 expression and high N/C ratio, a feature of adult stem cells. This percentage

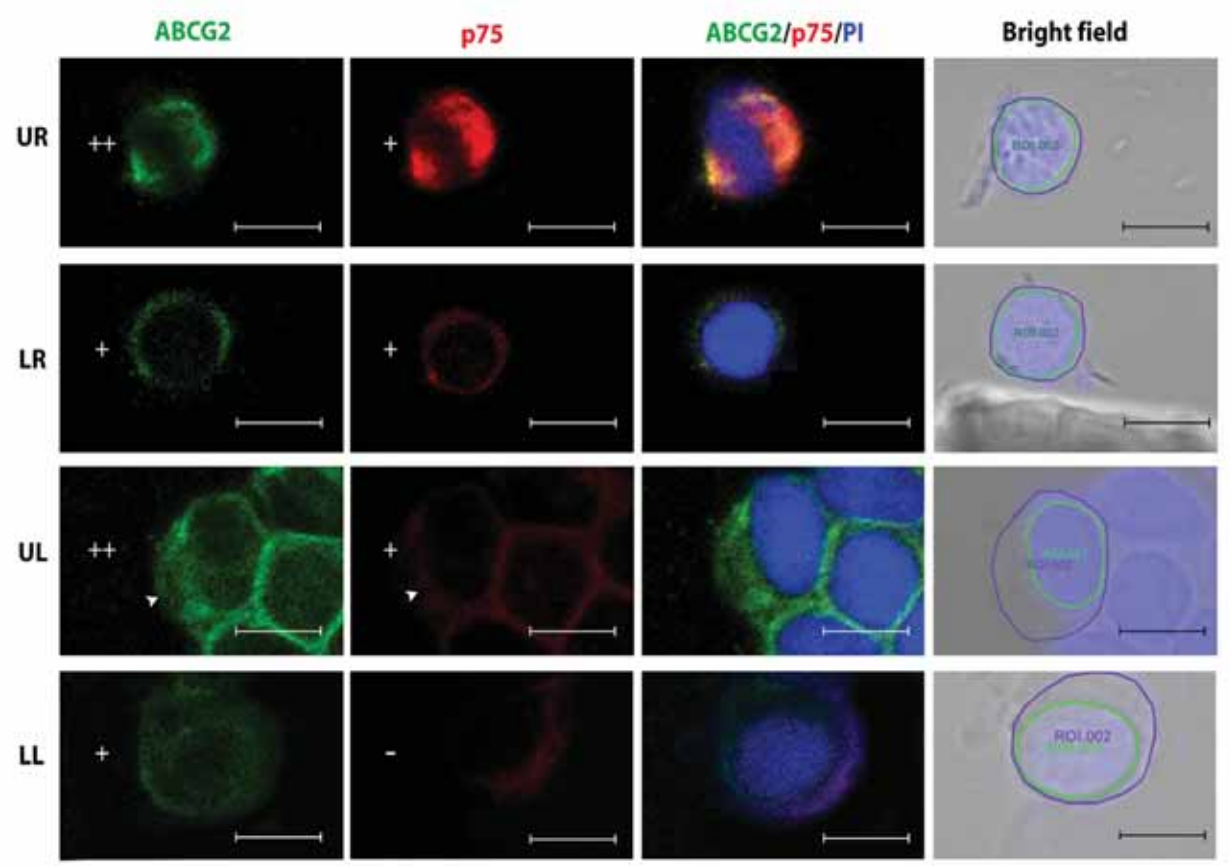


Representative scatter plot with two parameters (ABCG2 positivity versus N/C ratio) indicating that the stem cells in the upper right (UR) quadrant were strongly positive for ABCG2 and had high N/C ratio. UL: upper left, LL: lower left; LR: lower right. Each red diamond represents a cell. Dark blue circle denotes that the cell was p75 positive. Cells with no circle were negative for p75. All the cells in the UR quadrant were positive for p75.

decreased significantly to 12.6 ± 6.6% in middle (30-60 years) and 4±3.5% in older age group (>60 years) (rho=-0.92 and p=<0.001). The putative stem cells with high ABCG2 positivity and high N/C ratio were also positive for p75.

Age group	Percentage of cells (mean± SD)			
	LL	UL	LR	UR
<30 years	29.75 ± 10.2	43.0 ±1 2.4	5.75 ± 4.6	21.0 ±1.4
30-60 years	37.0 ± 20.5	44.2 ± 19.8	6.8 ± 5.8	12.6 ± 6.6
>60 years	65.0 ± 18.2	23.8 ± 13.6	7.4 ± 4.7	4 ± 3.5

Distribution of cells in the four quadrants of the scatter plot in three different age groups. The UR cells with high ABCG2 expression and high N/C ratio were designated as stem cells. UR: upper right: UL: upper left, LL: lower left; LR: lower right.



Representative confocal images of isolated TM cell cytosmears immunostained for ABCG2 (FITC-green) and p75 (Alexa 633-red) counterstained with propidium iodide (PI-blue). The cells with mean pixel intensity 188±24 (Mean ±SD) were graded high positive (++), 125±42 positive (+) and 53±28 as negative for ABCG2. The expression of p75 was graded either positive (+) or negative (-). Nuclear and cytoplasmic areas were measured by marking the region of interest (ROI) around each cell on the bright field image. The cells in the upper right (UR, first row) quadrant of the scatter plot were highly positive for ABCG2 with high N/C ratio and these were designated as stem cells. These UR cells also expressed p75. Lower right (LR, second row) quadrant cells had high N/C ratio but low ABCG2 expression. The white arrow head indicates a cell in the upper left (UL, third row) quadrant having high ABCG2 expression but low N/C ratio and lower left (LL, fourth row) quadrant cells had low ABCG2 expression and low N/C ratio. Scale bar 10 µm.

TMSCs in glaucomatous donor eyes: Anterior segments were dissected from known glaucomatous (n=4) and age-matched normal (n=6) human donor eyes into four quadrants. A minimum of three sections from all quadrants were double immunostained for ABCG2 and p75. Quantification of the total TM cellularity (Mean ± SD) of normal donor eyes were calculated to be 57.08± 3.96 cells/section. But in glaucomatous donor eyes the TM cellularity significantly (p=0.049) reduced to 45.33 ±11.81. In contrast, no significant (p=0.65) reduction in TMSC content was observed in glaucomatous donors 0.16±0.18 cells/section in comparison to normal donors 2.99±4.64.

Conclusion

Two-parameter analysis is a specific method for the identification of TMSCs. Quantification of the stem cell content revealed no significant difference between glaucomatous and age-matched controls. As the glaucomatous donors were above 80 years of age, further analysis using younger TM is essential to understand the role of TMSCs in the development of glaucoma.

PROTEOMICS

Research at the Department of Proteomics focuses on ocular diseases like Fungal Keratitis, Diabetic Retinopathy, Pseudo-exfoliation syndrome and Keratoconus. Proteomic, genomic, transcriptomic and cell biological approaches are employed to understand the pathological mechanisms underlying the above diseases and their management. Using these approaches, we also aim to discover predictive biomarkers for the progression of diseases like Fungal Keratitis and Diabetic Retinopathy which can have additional value for patients in the clinic. Funding for the above research activities come from national agencies, collaborative international projects and from private sources.

Corneal Innate Immune Response to Fungal Infections

Interaction of Human Corneal Epithelial cells and *A.flavus*

Investigators : Prof. K. Dharmalingam,
Dr. J. Jeya Maheshwari,
Dr. N. Venkatesh Prajna,
Dr. Lalitha Prajna

Team Member : A. Divya

Background

Fungal pathogens are global threat to humans, animals and plants. *A.flavus* and *Fusarium solani* are the main corneal pathogens. Immune response against the fungal invasion is crucial to protect the host which includes innate as well as adaptive response. Phagocytosis is a fundamental process of innate immunity that destroys the pathogen after engulfment. Professional phagocytes such as neutrophils have been implicated as the first line of defense against fungi. Cornea is an avascular tissue in which Human Corneal Epithelium, a five to seven layers of stratified squamous epithelial sheet, encounters invading pathogens first. This study is designed to examine the interaction and the outcome of fungal and corneal cell interaction.

Magnetic labelling of *A.flavus* conidia:

The protocol for isolation of conidia-containing phagolysosomes by magnetic separation is shown in fig.1. *A. flavus* conidia were labelled with 10 mg/mL EZ link sulfo-NHS-LC biotin in 50 mM Na2CO3 and incubated for 2 h at 4°C on a rotator. After washing

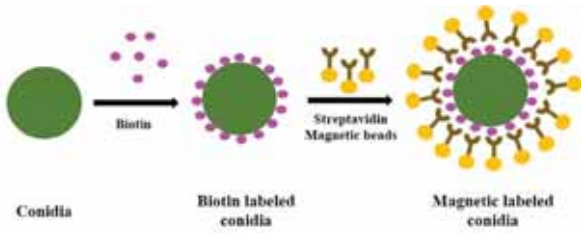


Figure 1: Schematic illustration of magnetic labelling of A.flavus conidia. (Modified from, Schimdt et al., Proteomics 2018; 17(6): 1084-1096)

in 1X PBS containing 100mM glycine, 50 µL of the streptavidin-coupled magnetic beads were added in labelling buffer (PBS with 2 mM EDTA) to the spore suspension and incubated for 15 min at 4°C on a rotator. After incubation labelled conidia were

separated from unlabeled spores under magnetic field. The efficiency of labelling were checked and found to be 95%.

Infection of RAW264.7 cells and Corneal epithelial cells with labelled spores

Infection was performed in six well plates. Briefly 80-90% confluent monolayer cultures were infected with magnetic labelled conidia at an MOI of two for RAW264.7 cells and MOI of ten for HCE cells (Cell line & Primary). The mix was incubated for 2hrs at 37ºC, 5% CO2 incubator. The morphology was assessed under inverted phase contrast microscopy.

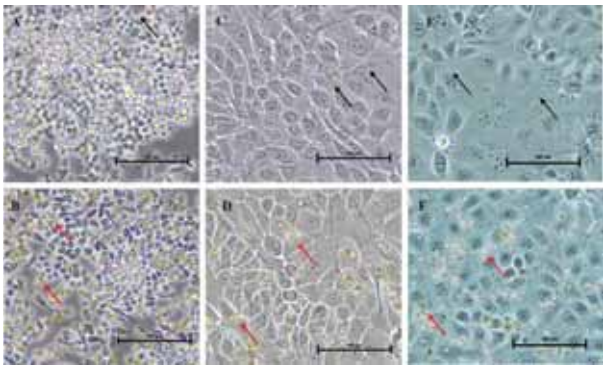


Figure 2: Inverted phase contrast microscopic images of control and infected cells: (A,B) control and infected RAW264.7 cells; (C,D) Control and infected primary HCE cells; (E,F) Control and infected HCE cell line. Black arrow indicates streptavidin magnetic beads and red arrow indicates magnetic labelled A.flavus conidia.

After incubation the cells were washed with PBS, scratched off and collected in homogenization buffer (3 mM imidazole, 250 mM sucrose, pH 7.4 and proteinase inhibitors. Cell lysis was achieved by passing the cell suspension 60 times through a needle (27G) and was monitored (fig2.). To isolate the phagosomal fraction the lysate was kept under Quadro MACS separator. Phagosomes with magnetically labelled conidia were retained on the tube by magnetic field and proteins were directly eluted with 1X sample buffer (2 % (w/v) SDS, 60 mM Tris pH-6.8, 10 % (v/v) glycerol, 25 mM DTT) by heating at 98 ºC. Proteins of phagosomal and non phagosomal fractions were checked in 1D SDS-PAGE.

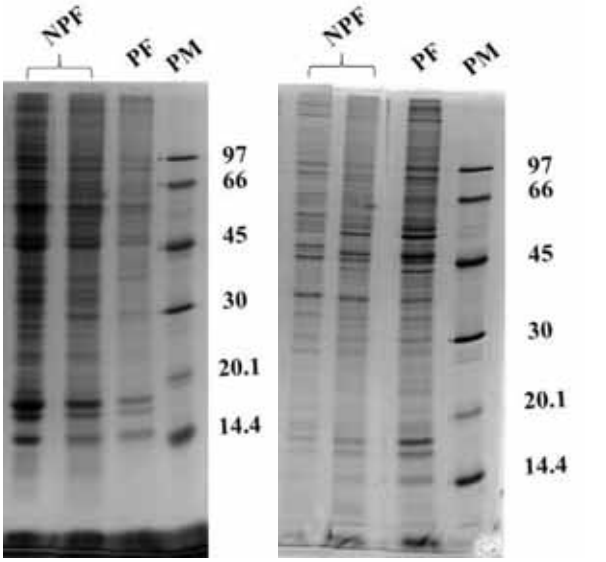


Figure 3; 1D SDS-PAGE profile of phagosome and non phagosomal fractions: (a) 1D protein profile of phagosomal and non phagosomal fractions of RAW624.7 cells; (b) 1D protein profile of phagosomal and non phagosomal fractions of HCE cell line; NPF- Non phagosomal fraction (includes soluble and insoluble fractions separately); PF- Phagosomal fraction.

Figure 3 shows the protein profile of phagosome and non phagosomal fractions. Phagosomal protein is a subcellular fraction from the total cell lysate but the protein profile of phagosomal fraction was found to be similar to the profile of total cell proteins.

1.3.4. Identification of phagosome proteins:

Phagosomal proteins were separated using 10% polyacrylamide gel. Electrophoresis was stopped after the migration of the dye up to a cm in the separating gel. Stained bands were cut into four pieces, reduced, alkylated and in gel digested with trypsin. Samples were anaysed in Orbitrap Velos pro Mass spectrometer and proteins were identified using Proteome Discoverer 1.4 software. Total proteins identified were represented in figure 4(b).

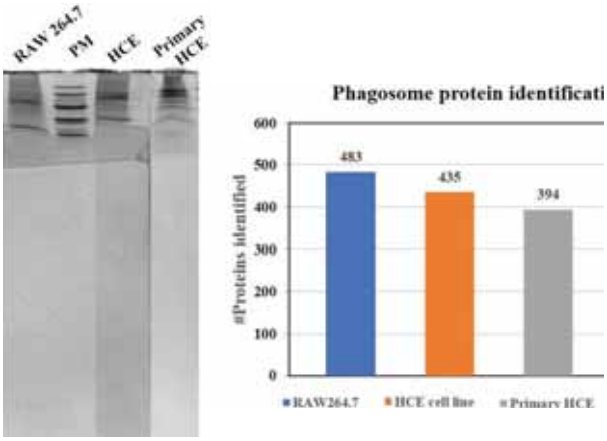


Figure.4: Protein identification of phagosomal fraction: (a)1D shot gun SDS-PAGE profile of phagosome and non phagosomal fractions. NPF- Non phagosomal fraction (includes soluble and insoluble fractions separately); PF- Phagosomal fraction. (b) Number of proteins identified from phagosomal fractions of RAW264.7 cells/HCE cells.

Phagosomal proteins identified in RAW264.7 cells were more compared to HCE cells. Identified phagosomal proteins were searched against Kegg pathway to check the number of proteins identified with respect to phagocytosis and the representation was given below (figure 5).

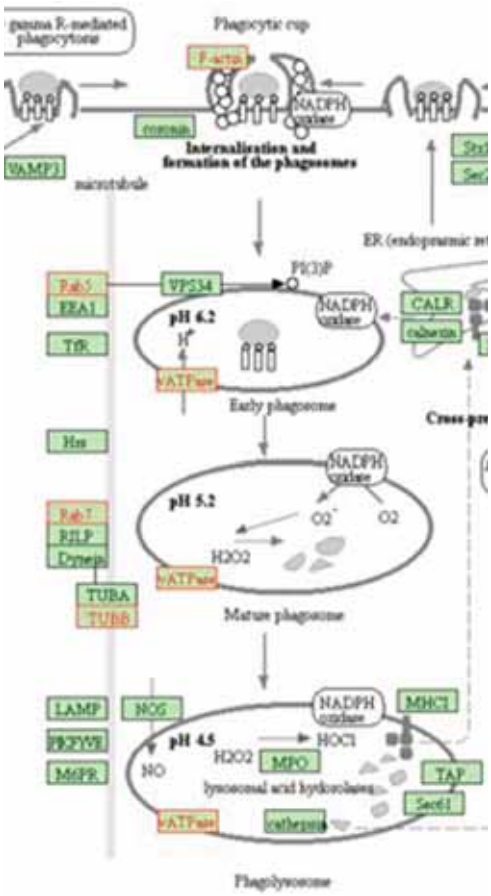


Figure 5: Identified phagosomal proteins of Primary HCE cells searched in Kegg pathway. Proteins involved in the phagosome pathway are green shaded. Orange highlighted are identified proteins from our data.

Figure 5 indicates that one of the markers for early and mature phagosomes including vATPase, which catalysis the pH in each stage of phagocytic process were identified. But proteins involved in phagolysosome (e.g. LAMP) could not be detected.

Conclusion

Fungal spores could be phagocytosed by corneal epithelial cells. Further, the preliminary data indicate that the engulfed spores may not be degraded since the fusion of phagosomes fails to fuse with lysosomes.

Role of alternative complement pathway in antifungal immunity

Investigators : Prof.K.Dharmalingam, Dr.J. Jeya Maheshwari, Dr.N. Venkatesh Prajna, Dr. Lalitha Prajna

Team Members : K.Sandhya

Introduction

Comparative tear proteome analysis of mycotic keratitis patients showed that all the three complement pathway proteins were present only in the patient tear but not in control tear. (K. Jeyalakshmi, N.L. Demonte, N.V. Prajna, P. Lalitha, T. Chitra, J.J. Maheshwari, K. Dharmalingam, *Aspergillus flavus* induced alterations in tear protein profile reveal pathogen-induced host response to fungal infection, J. Proteom. 152, 13–22 (2016). In our previous report we demonstrated functional integrity of alternative complement pathway in fungal keratitis tear by hemolytic assay. Interestingly up regulation of the central negative regulator of alternative pathway namely CFH in *A.flavus* & *Fusarium keratitis* tear samples indicates the complement mediated immune response is tightly regulated.

Work done during this period

We analyzed the expression of other Complement central components such as complement factor B and Complement C3 in *fusarium* keratitis patients using immunoblot analysis. We have already shown there is a 10 fold up regulation of Factor B and 48 fold up regulation of Complement C3 in *A.flavus* keratitis tear using mass spectrometry analysis. (K.Jeyalakshmi *et al*., 2016)

Up regulation of Complement Factor B in Fusarium keratitis patients

Factor B is a single-chain protein that contains five distinct domains. Upon activation of the alternative complement pathway, factor B (85 kDa) is cleaved by factor D into a Ba fragment (~25kDa) and a Bb fragment (~60kDa). Factor B is primarily produced in the liver, but studies have also shown that it is produced in tears, monocytes, macrophages etc.

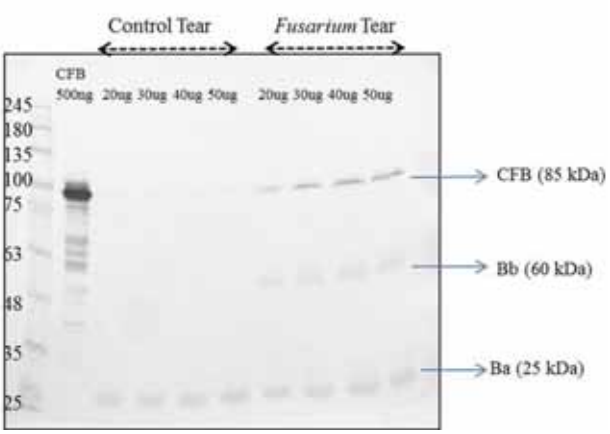


Fig .2A: Western blot analysis of CFB for control and early stage fusarium keratitis tear sample.

Demonstration of cleaved fragments of CFB in Fusarium keratitis tears

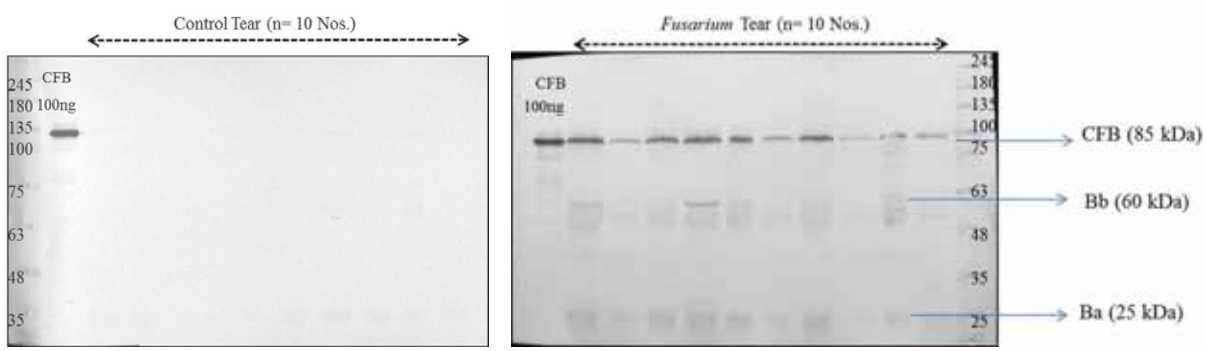


Fig .2B: Western blot analysis of CFB for control and early stage fusarium keratitis tear sample.

30ug of total tear protein from early stage of Fusarium keratitis patients (Duration of symptoms less than 7 days) and control tears were resolved on 1D SDS-PAGE (8%), transferred onto nitrocellulose membrane and probed against anti-CFB antibody.

Up regulation of CFB during keratitis infection was confirmed with higher number of samples (n= 10 Nos). Only partial cleavage of CFB protein was observed during infection which is evidenced by the presence of some amount of total CFB protein at 85 kDa.

Upregulation of Complement C3 in Fusarium keratitis patients

Complement C3 plays a central role in the activation of the complement system. The molecular weight of C3 is 187 kDa with four N-glycosylation sites, Cleavage of C3 with the help of Factor I (FI) and Factor H leads to formation of C3 alpha chain (115kDa), iC3b(68kDa), and C3dg(40kDa).

Total tear proteins from early stage of Fusarium keratitis patient (Duration of symptoms less than 7 days) and control tear were resolved on 1D SDS-PAGE (8%), transferred onto nitrocellulose membrane and probed against anti-C3 antibody (Fig 3)

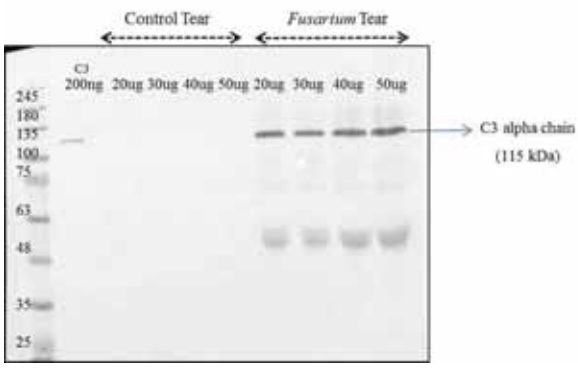


Fig .3: Western blot analysis of C3 for control and early stage fusarium keratitis tear sample.

Demonstration of cleaved fragments of C3 in Fusarium keratitis tears

20ug of total tear protein from early stage of Fusarium keratitis patients (Duration of symptoms less than 7 days) and control tears were resolved on 1D SDS-PAGE (8%), transferred onto nitrocellulose membrane and probed against anti-C3 antibody.

Cleaved fragments of C3 protein (C3 alpha, iC3b, C3dg) were observed in the tears collected from the keratitis patients.

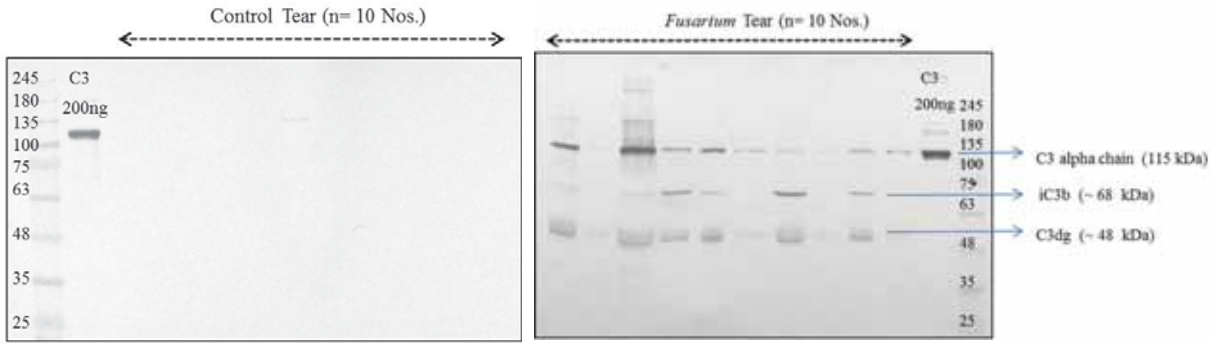


Fig .4: Western blot analysis of C3 for control and early stage fusarium keratitis tear sample.

Conclusion

Cleavage of C3b is an indication for production of enzymatically active CFB, necessary for the cleavage of C3 and eventually formation of C3 convertase. The observation of cleavage products of C3 such as c3dg shows the destructive function of C3 convertase by CFI and CFH. In total there is a clear intricate regulation of complement cascade in tear film. Disruption of this will lead to fungal clearance and also possibly host damage.

Mycelial Exoproteome Analysis of saprophyte and corneal isolates of *A. flavus* grown at two temperatures

Investigators : Dr. J. Jeya Maheshwari, Prof. K. Dharmalingam
Clinician scientists: Dr. N. Venkatesh Prajna, Dr. Lalitha Prajna
Team member : Aswani Krishna

Rationale of the study

A. flavus keratitis is a difficult infection to treat and nearly 40% of the patients are refractory to treatment with antifungals. Our studies on different corneal isolates of *A. flavus* using the insect model, *Galleria mellonela* suggested an inter-species variation among these isolates. To understand the factors that contribute to the difference in virulence, we examined the secreted proteins of the fungus. Exoproteins are the major effectors in the host-fungal infection and has an important role in establishment of the infection in the cornea.

Objectives

For this study, we examined the exoproteins identified from a reference strain (ATCC26), an isolate from a patient who responded to treatment (CI1698) and one isolate from a patient who did not respond to treatment (CI1123). Exoproteins were prepared from the *A. flavus* strains grown on wheat bran for 24 hours at two temperatures, 30°C and 37°C. The proteins identified using a mass spectrometry-based proteomics approach were used for the extended computational analysis to understand the functional relevance of the secreted proteins with special reference to infection.

Results:

Mycelial Exoproteins of *A. flavus* strains

Exoproteins were prepared from different *A. flavus* strains using solid-state fermentation method grown at 30°C or 37°C for 24 hours. Proteins were identified from 80 µg of exoproteins after fractionation on a 1D SDS-PAGE followed by in-gel tryptic digestion and the number of proteins identified is compared in Figure 1.

Among the three *A. flavus* strains, highest number of proteins were identified in the exoproteome of the surgery case isolate, CI1123 and lowest in the healed case isolate, CI1698. This trend was observed at both the growth temperatures. Both CI1123 and ATCC secreted more proteins at 37°C than 30°C. While interestingly, CI1698 secreted lesser proteins at higher temperature. Temperature influences the number of proteins secreted by ATCC and CI1123 with an increase by 23 % at 37°C when compared to 30°C, while a 6% decrease was observed in 1698.

Mode of protein secretion: classical versus non-classical pathways

Out of the total non-redundant list of 509 proteins across all the three *A. flavus* strains, 49% were secreted through the classical pathway requiring the ER signal peptide while 51 % of the proteins were secreted through non-classical pathways. Interestingly, while ~42 % of the classically secreted proteins are common between the three strains, only 9 % are common among the strains in the non-

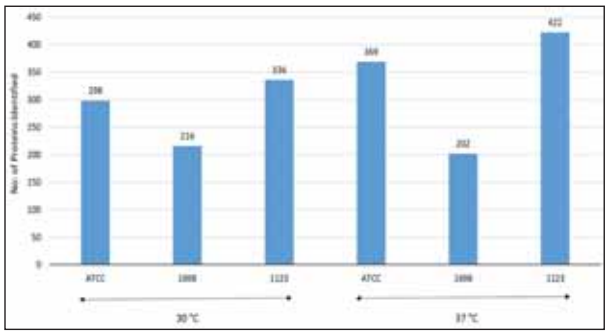


Figure1. Comparison of the exoproteins identified across the A. flavus strains grown at two growth temperatures.

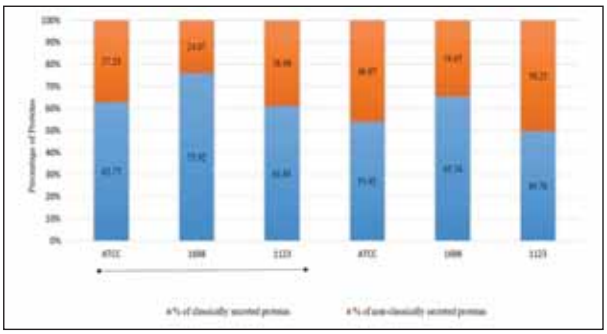


Figure2. Comparison of percentage of proteins secreted through classical and non-classical pathway across the A. flavus strains at two-growth temperature

classical protein category. The healed case isolate, CI1698 behaves differently when compared to the other two strains. Proteins secreted through the non-classical pathways significantly contribute to the increase in number of proteins at 37 °C in ATCC and CI1123. In the case of 1698, there was a decrease in the number of classically secreted proteins with a moderate increase in non-classically secreted proteins.

Major functional categories in secreted proteins in *A. flavus*

A non-redundant list of 509 proteins was analysed using multiple gene enrichment tools such as gProfiler, DAVID as well as curated manually based on function. Majority of the 509 proteins could be grouped into major categories as shown in Table 1 and their relevance to the fungus is discussed below.

1. Plant polysaccharide degrading enzymes: The *A. flavus* strains were grown using solid-state fermentation methods in a wheat bran medium for exoprotein preparation. The major components of wheat bran include cellulose, xylan, lignin, galactan and fractans. Nearly 30% of the abundance is contributed by the carbohydrate-degrading enzymes that potentially can act on the different components of wheat bran. Plant cell wall degrading enzymes helps fungi in nutrient acquisition during growth. CI1698 strain has the highest percentage of carbohydrate degrading enzymes at both 30°C and 37°C.
2. Fungal cell wall degrading enzymes: Nearly 2% of the total exoprotein abundance represent enzymes that act on polysaccharides in the fungal cell wall. These proteins might have an important role in fungal cell wall restructuring, an important process that occurs during fungal growth. This category includes enzymes acting primarily on β-glucan, chitinases and mannan residues.
3. Enzymes involved in nutrient acquisition: Another major category that constitute nearly 25% of total exoprotein abundance was found to have role in nutrient acquisition. This category includes proteases, phosphatases and lipases. Nutrient acquisition is an important mechanism when fungus encounters a new growth environment and plays a crucial role during host pathogen interaction. The fungus reprograms to secrete enzymes that allow it to acquire nutrition from the resources available in the immediate microenvironment. Therefore, these enzymes are a pre-requisite for fungal colonization and fitness. Proteases are the major subcategory in this class of proteins and are also the abundant ones. CI1123 strain, the virulent corneal isolate has higher abundance of proteins involved in nutrient acquisition at both 30°C and 37°C when compared to the other two strains.
4. Proteins involved in reactive oxygen species response: Pathogen recognition by the host rapidly triggers an oxidative burst as a part of its defense response and reactive oxygen species (ROS) has a major role in clearing the invading pathogen. Fungus responds to this stress by producing enzymes such as SOD, catalase and thioredoxin reductase that can neutralize the ROS stress. Nearly 2-5 % of exoprotein abundance belongs to this category and CI1698 at both 30°C and 37°C shows highest abundance in ROS proteins.

Temperature induced changes in exoprotein

In order to see the temperature-induced changes of these categories across the corneal isolate strains CI1698 and CI1123, proteins differentially expressed at 30°C and 37°C were analysed. Differentially expressed proteins at both 30°C and 37°C with a 2-fold difference with respect to the reference strain ATCC26 considered in each functional category of the

Table1. Percentage of proteins grouped into various functional categories

Major functional categories of A. flavus exoproteins (values indicate % abundance with respect to the abundance of all the proteins identified in the respective condition)						
	A30	Y30	C30	A37	Y37	C37
Plant polysaccharide degrading enzymes	40.29	46.17	37.84	45.97	51.10	36.64
Fungal cell wall polysaccharide degrading enzymes	2.30	2.84	3.93	2.89	2.27	3.03
Nutrient acquisition proteins	37.65	37.44	40.24	25.15	25.78	33.47
Stress response proteins (ROS)	3.38	3.50	2.65	4.99	5.20	4.49
Uncategorized	16.23	9.87	16.09	20.49	13.77	22.50

proteins discussed earlier. Out of 509 non-redundant list of proteins, 389 proteins found to be common across all the three strains at 30 °C, of which 4.3 % of proteins were up regulated and 6.4 % down regulated in both the corneal isolates. Further, 3.3 % proteins were found to be exclusively up regulated in strain CI1123 while 1.2 % CI1698. Similarly, at 37 °C, 483 proteins were found to be common among the three strains, of which 1.6 % of proteins were up regulated and 7% down regulated in both the corneal isolates. While 1.2 % and 8.2% of proteins were exclusively up regulated in CI1698 and CI1123, respectively.

In-vitro analysis of cytokines secreted by neutrophils infected with pathogenic *Aspergilli, A. flavus* and *A. fumigatus*

Investigators : Dr. Lalitha Prajna, Dr. J. Jeya Maheshwari, Prof. K. Dharmalingam
Clinician Scientists: Dr. N. Venkatesh Prajna
Team member : V. Lakshmi Prabha
Funding : Indo-French centre for the promotion of advanced research (CEFIPRA)

Rationale of the study

Neutrophils are the predominant cell type infiltrating the lungs and cornea upon infection. More than 90% of the infiltrating cells in the keratitis cornea are neutrophils (*Karthikeyan et al*., 2011). Neutrophils inhibit the germination of *A. fumigatus* conidia *in vivo* and *in vitro* (*Gazendam et al*., 2016). They are important for fungal clearance but also contribute to tissue destruction by release of proteolytic enzymes and reactive oxygen and nitrogen species. Neutrophils produce a wide array of inflammatory mediators, the cytokines, which are involved in both innate and acquired immunity. The neutrophil-derived cytokines during infectious diseases may play a critical role in determining the outcome of the infections.

Objectives

To identify the regulatory mechanisms of neutrophil dependent protection against two pathogenic *Aspergilli*, the involvement of cytokines was evaluated after neutrophils were stimulated with *A. flavus* or *A. fumigatus* spores. We quantified the level of multiple cytokines secreted by the neutrophils in response to two spore morphotypes, dormant and swollen spores. The time when host phagocytes are anticipated to directly engage with the *Aspergillus* conidia is postulated to be between 2 – 6 hours, which also corresponds to the time for the dormant conidia to swell and the swollen conidia to develop into germlings.

Results

Neutrophils were isolated from the peripheral blood of healthy donors using Easy Sep Human Neutrophil isolation kit and found to be 99% pure, viable and in resting state. Neutrophils were infected with dormant or swollen conidia of *A. flavus* corneal isolate (CI 1698) or *A. fumigatus* lung isolate (DAL) at multiplicity of infection of 5. Comparison with the spore controls indicates that neutrophils inhibit the germination of the spores of both Aspergilli (Figure 1).

At various time points during infection, the supernatant was collected and the levels of IL-8, TNF-α, IL-6 and IL-17 were quantified by sandwich ELISA. The absorbance was converted to concentration (picogram/ml) and compared.

IL-8 secretion by neutrophils upon stimulation with *Aspergilli* spore morphotypes

IL-8 is a chemotactic factor that attracts neutrophils, basophils, and T-cells, and is also involved in neutrophil activation. IL-8 secretion by neutrophils was compared between the two spore morphotypes

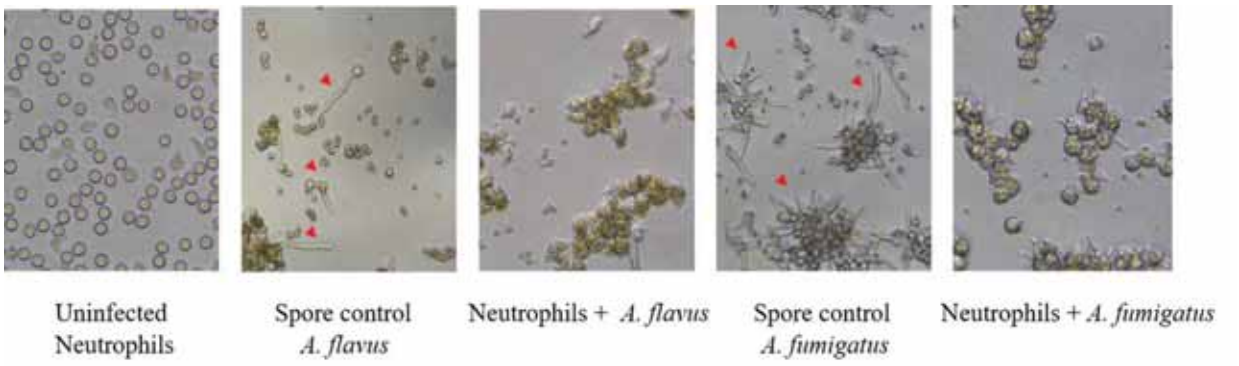


Figure 1: Representative images of Neutrophils infected with Aspergillus swollen spores at 5h during infection. The red arrows indicate germinated spores in spore controls that were not observed during infection.

of each *Aspergilli* as shown in Figure 2. In both pathogens, the swollen spores stimulated a significantly higher secretion of IL-8 when compared to dormant spores. The difference in IL-8 secretion between dormant and swollen spore stimulation was more pronounced in *A. flavus* when compared to *A. fumigatus.*

Comparison of IL-8 secretion by neutrophils upon stimulation with *A. flavus* or *A. fumigatus* showed that *A. fumigatus* spores are more immunostimulatory than *A. flavus* spores and the difference was significant at all-time points for the dormant spores (Figure 3).

TNF-alpha secretion by neutrophils

TNF-α is a cell signalling protein and a pro-inflammatory cytokine that has an important role to play in inflammation. Hence, we examined the secretion of TNF-α by neutrophils in response to both pathogenic *Aspergilli.* Low levels of this cytokine was secreted and was not in the quantifiable range of the assay and hence, the comparison was done using absorbance values. Only neutrophils stimulated with *A. flavus* showed detectable levels of TNF-α beyond the basal secretion of neutrophils and the level increased with time of infection (Figure 4).

Secretion of IL-6

Neutrophils are capable of secreting IL-6, a cytokine that is involved in the regulation of immune response and its secretion by neutrophils was examined upon *Aspergilli* infection. Similar to TNF-α, only A. flavus stimulated neutrophils to secrete IL-6 and higher level of IL-6 was observed when the spores are swollen (Figure 5).

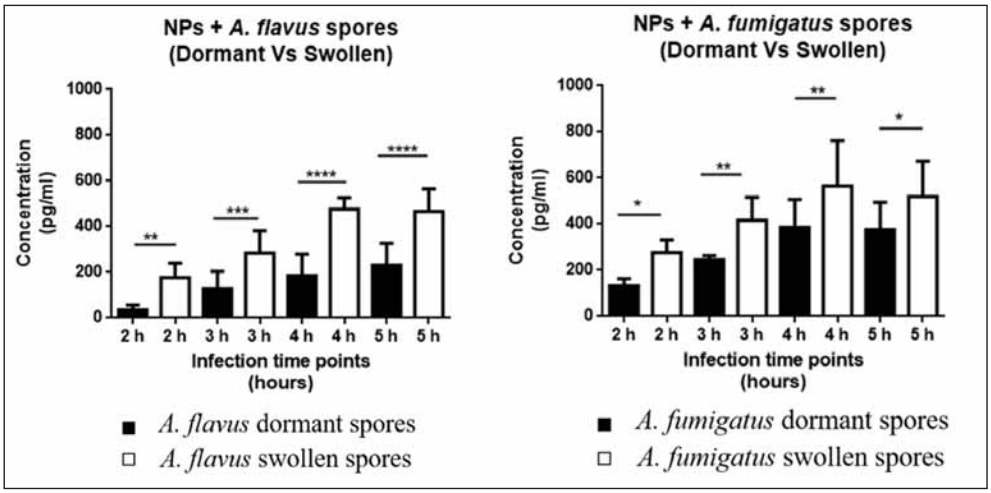


Figure2. Comparison of IL-8 secretion by neutrophils in response to spore morphotypes. Results are median secretion (pg/ml) of at least 3 independent experiments and triplicates in ELISA. Significance: ∗ p < 0.05, ∗∗p < 0.01, ∗∗∗ p < 0.001 and ∗∗∗∗p < 0.000.

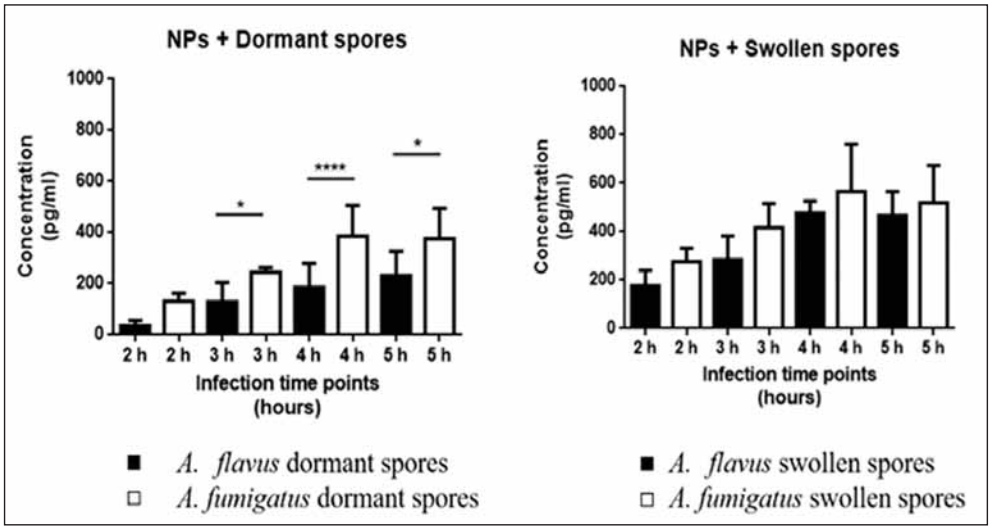


Figure 3: IL-8 secretion by neutrophils upon stimulation with A. flavus or A. fumigatus spores. Results are median secretion (pg/ml) of at least 3 independent experiments and triplicates in ELISA. Significance: ∗ p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001 and ∗∗∗∗p < 0.000.

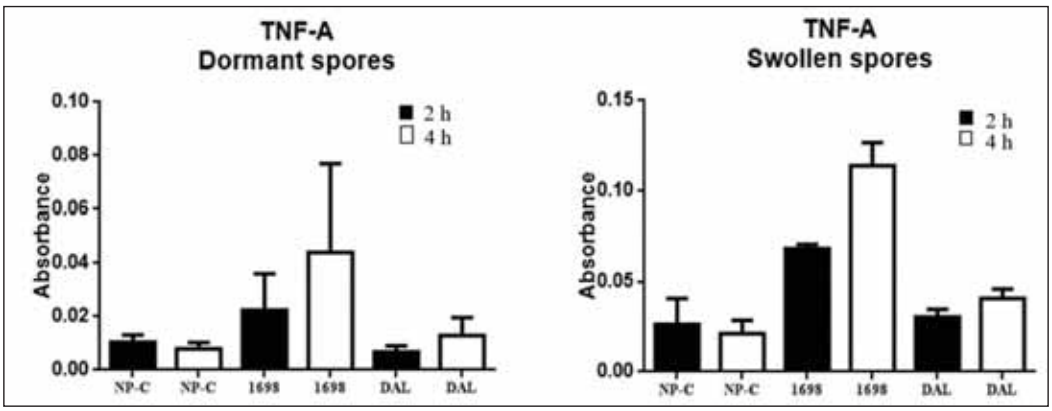
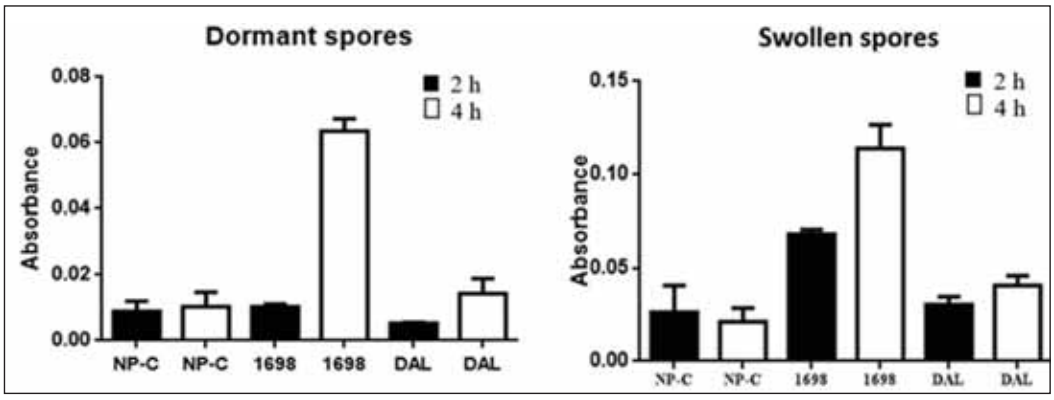


Figure 4: TNF-α secretion by neutrophils upon stimulation with Aspergillus spores for 2h and 4h. Results are mean with SD of absorbance of at least 2 independent experiments and duplicates in ELISA.



FFigure 5: IL-6 secretion was measured by ELISA upon stimulation of neutrophils with Aspergillus spores for 2h and 4h. Results are mean with SD of absorbance of at least 2 independent experiments and duplicates in ELISA.

We also found that neutrophils do not secrete IL-17 upon stimulation with either *A. flavus* or *A. fumigatus*.

Among the cytokines examined, IL-8 is the only cytokine secreted in abundance by neutrophil in response to *Aspergillus* infection. Although detected at lower levels, both TNF-α and IL-6 were secreted by neutrophils only when stimulated with *A. flavus*. The complex interplay between cytokines, chemokines and the cells of the immune system needs to be elucidated in the setting of *Aspergillus* infection.

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Analysis of NPDR and PDR Stage specific differential expression of Haptoglobin

Investigators : Dr. J. Jeya Maheshwari, Prof. K. Dharmalingam
Clinician Scientists: Dr. Kim, Dr. Piyush Kohli
Team members : P.Vignesh

Rationale of the study

Haptoglobin is an acute phase protein that reduces the hemoglobin mediated oxidative damage and helps to recover heme iron during its clearance in liver. Haptoglobin proteins consists of two β and two α chains. The alpha chain can be one of the three types of α chains that in turn determines the Hp phenotype, a combination of α-forms (α1f – 9 kDa, α1s-9 kDa and α2-16 kDa) resulting in Hp 1-1, Hp 1-2 or Hp 2-2 functional protein. Each of the α-chain in turn exists as 3-4 proteoforms as observed on a 2D gel. Differential regulation of the α-chain isoforms of Hp has been reported in head and neck cancer as well as systemic sclerosis. Our earlier discovery phase studies in serum indicated that Hp protein level as well as the α-chain isoforms were

differentially expressed in PDR patients. To validate this observation, in this study, we examined the level of Hp and that of the Hp α2-chain isoforms in a larger set of samples.

Analysis of Haptoglobin Alpha 2 total Level

The phenotype of Hp was initially determined in all the samples to be analysed. South Indian population has 90% Hp 2-2 phenotype and remaining 10% are Hp 2-1 phenotype and we also found similar distribution in the samples we examined. We selected only those patients who were Hp 2-2 phenotype for all further analysis. Two microgram of neat serum protein was taken for immunoblot analysis using anti-Hp antibody and the level of α2 band in the blot was quantified using densitometry in 30 samples, 10 samples in each of the three categories, DM, NPDR and PDR. Figure 1: show that the level of Hp-α2 chain was not significantly altered in the three conditions examined.

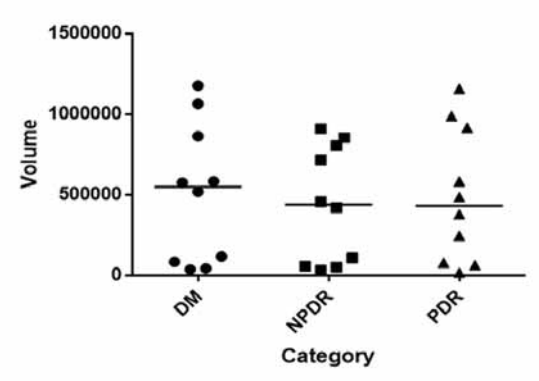


Figure1. Analysis of Hp α2 level across different stages of DR.

Analysis Alpha 2 chain proteoforms

2D-DIGE was carried out for the quantitation of the individual Hp α2 proteoforms in three individual samples in the NPDR and PDR category, and was compared against the pooled DM serum reference. Three major Hp α2 proteoforms have been reported in serum and are labelled as 2L, 2M and 2R based on their migration in the 2D gel (Figure 2).

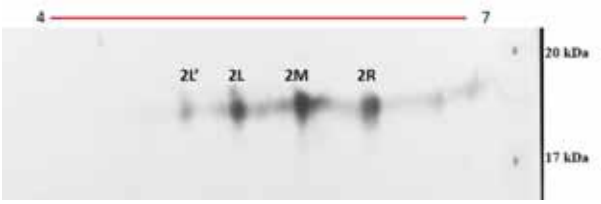


Figure2. Proteoforms of Hp α2 chain

2D-DIGE analysis of these spots across the NPDR and PDR samples show that the level of 2L and 2M spots was higher in PDR compared to NPDR while the proteoform 2R showed decreased expression. The total Hp-α2 level (2L+2M+2R) showed an increase in PDR compared to NPDR.

The variation in the total level of Hp α2 chain as well as that of α2M and α2R proteoforms was distinct based on 2D DIGE analysis. However, the statistical significance can be determined only after examining more number of samples.

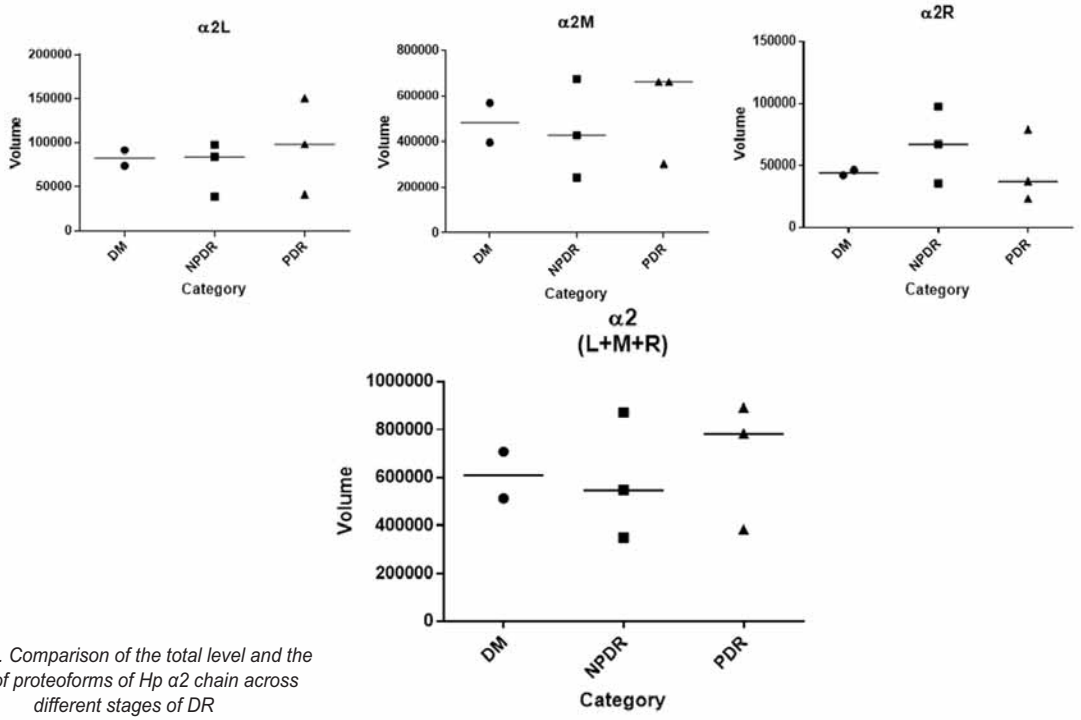


Figure3. Comparison of the total level and the level of proteoforms of Hp α2 chain across different stages of DR

Quantitative analysis of Complement factor B proteoforms in Diabetic Retinopathy

Investigators : Prof. K. Dharmalingam
Dr. J. Jeya Maheshwari
Dr. Kim
Team members : V. Nivetha

Rationale

Previously, we found differential regulation of 57proteins, including CFB, in the serum of PDR patients when compared to DM patients. Further, DIGE analysis of PDR and DM serum shows differential expression of CFB proteoforms. Therefore, further experiment was carried out to quantitate CFB proteoforms using 2D western analysis.

2D gel analysis of albumin depleted serum and plasma sample

CFB proteoforms were separated in albumin depleted serum and plasma samples in 2D PAGE and the CFB spots were taken for mass spec analysis (Fig 1). Mass spectrometry data showed the presence of six proteoforms in the serum and plasma.

2D western blot analysis of CFB in serum sample

In order to confirm the identity of the proteoforms, 2D western was done using anti-CFB antibodies. Data in fig 1.2 show the six proteoforms of CFB. Further experiments are in progress to determine the quantitative difference of proteoforms in DM and DR cases.

Conclusion

Quantitation of CFB proteoforms in DR and DM samples is in progress.

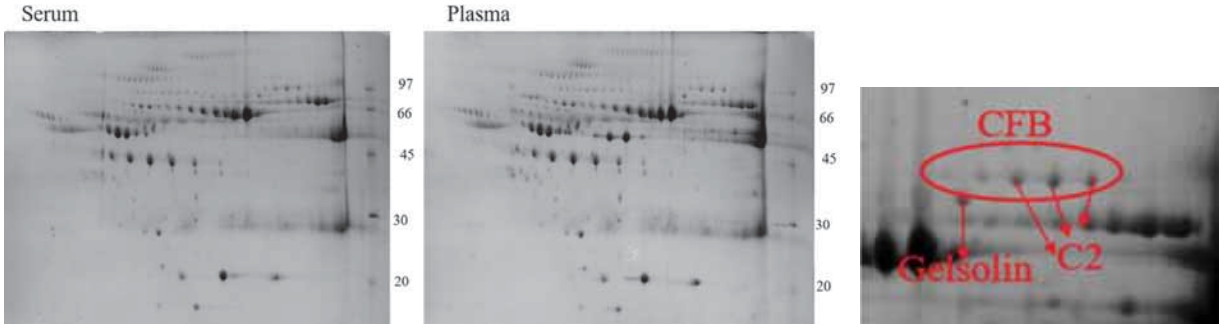


Fig 1. 2D PAGE analysis of serum and plasma sample

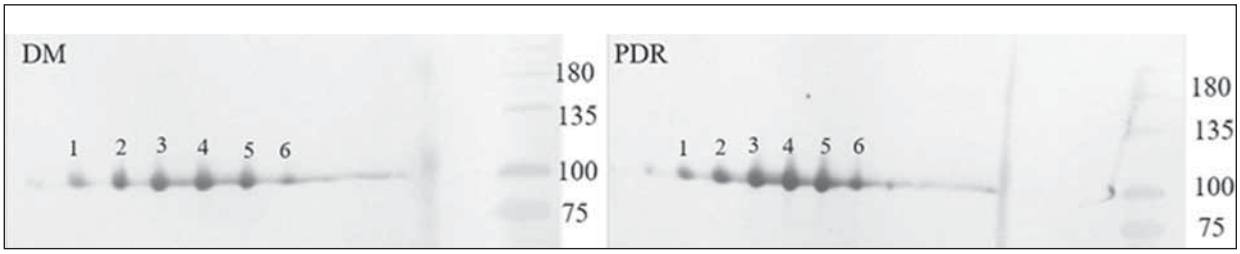


Fig 2. 2D western blot analysis of CFB proteoforms.

Prospective Multicenter discovery and validation of diagnostic circulating and urinary biomarkers and development of sensor(s) to detect sight threatening Diabetic Retinopathy

Investigators : Prof. K. Dharmalingam,
Dr. J. Jeya Maheshwari
Clinician Scientists: Dr. Kim, Dr. Piyush Kohli,
Dr. Sagnik Sen
Team members : P.Vignesh, V. Nivetha,
K.S.Guhan

This is a bi-national collaborative project on Diabetic Retinopathy with multiple work packages. Work package 3 involves the biomarker and biosensor study in UK and India involving three centers, Moorfields Hospital in UK, Aravind Medical Research foundation, Madurai, Vision Research Foundation, Chennai and Sastra University, Tanjore. This component of the work package involves the validation of 12 selected serum biomarkers for diabetic retinopathy in a larger patient cohort from both India and UK. Sensors will then be developed for the validated panel of biomarkers that will be tested in the lab first and then in the field.

Functional analysis of circulating microRNAs and their regulatory role in Diabetic retinopathy

Investigators : Dr. O.G. Ramprasad, Prof. K. Dharmalingam, Dr. D. Bharanidharan, Dr. Kim Ramasamy

Project fellows : R. Karthikeyan (from July 2018 onwards) and Evangeline Ann Daniel (till June 2018)

Funding : SERB-Early career research award.

Introduction including background

The role of serum microRNAs in the progression of Diabetes mellitus (DM) to Non-proliferative diabetic retinopathy (NPDR) and NPDR to Proliferative DR in humans is largely unexplored. Therefore, the rationale of this study is to identify the microRNAs and understand their regulatory function in the progression of microvascular complications among diabetic patients and also at the exploration of validating miRNAs as predictive biomarkers. The major objectives of the study are:

- To reveal disease specific miRNAs from serum of PDR, NPDR and DM patients and compare it with serum samples of control healthy subjects.
- To identify potential molecular targets of differentially expressed miRNAs and their regulatory networks towards the understanding of disease pathogenesis.
- Validation of differentially expressed miRNAs for their functional implication using human retinal endothelial cell line grown under different glucose conditions and tissue biopsies from patients.

Results and Conclusion

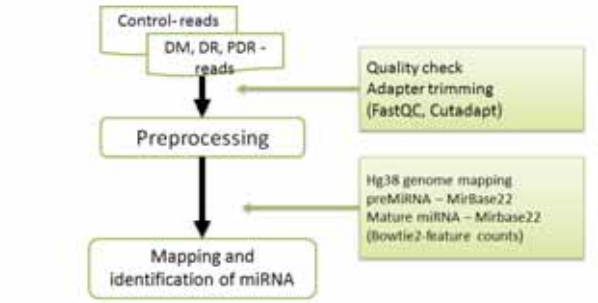
The main approaches in the current study involved (i) whole miRNA profiling from serum samples of healthy control, DM, NPDR and PDR subjects using Next generation sequencing (NGS) and (ii) analysis of select miRNAs in purified RNA samples using quantitative real-time PCR. The results from NGS experiments is a continuation of the previous year report; the experiments have been performed with more number of samples.

(i) Differential expression profile of select miRNAs obtained from next generation sequencing experiments:

Isolation of total small RNA including microRNAs from the serum samples of DM (Diabetes Mellitus), PDR (Proliferative diabetic retinopathy) patients, NPDR (Non- Proliferative diabetic retinopathy) patients and control healthy subjects was continued with a better yield of 250ng to 300ng from 200µl of each serum sample. The actual miRNA yield out of the total small RNA population in each sample was around 25-30ng as analysed by Qubit miRNA fluorometric assay. The miRNA obtained from serum samples of the four conditions were reverse transcribed and cDNA library were obtained. 12 moles of each of the four normalized cDNA library samples were loaded into the flow-cell of the next generation sequencer. The cDNA library samples from control, DM, NPDR and PDR yielded 2.7Gb of data in total after the NGS run. Around 250-300 miRNAs were identified from the serum of each condition as determined by the bioinformatics analysis outlined in Fig.1. The quality of the run was good as determined by the Phred score which was around 90% (Fig.2).

The top 10 abundant microRNAs identified across the DM and PDR serum samples are represented in Figure 3. Specifically miR-320a had a higher raw read count in PDR condition and had a 5-fold upregulation.

Figure 1: Computational workflow for Small-RNA seq data



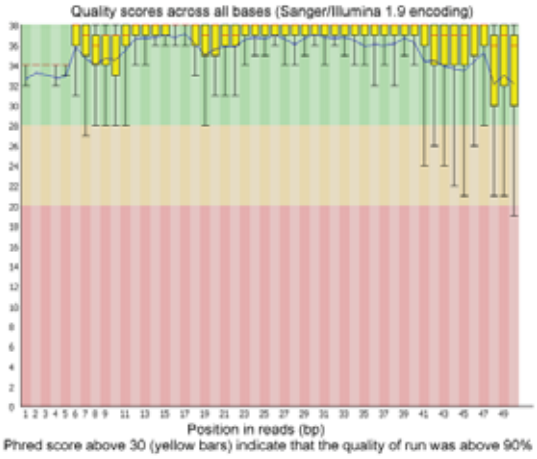


Fig. 2: Representative picture for the data quality across Control, DM, NPDR and PDR samples

The best predicted target genes for each significant differentially expressed miRNA were identified using four different algorithms. The targets that represent the intersection of at least 3 algorithms were selected to avoid false positives. The selected targets were compared with the experimentally validated targets available in the miRNA public databases.

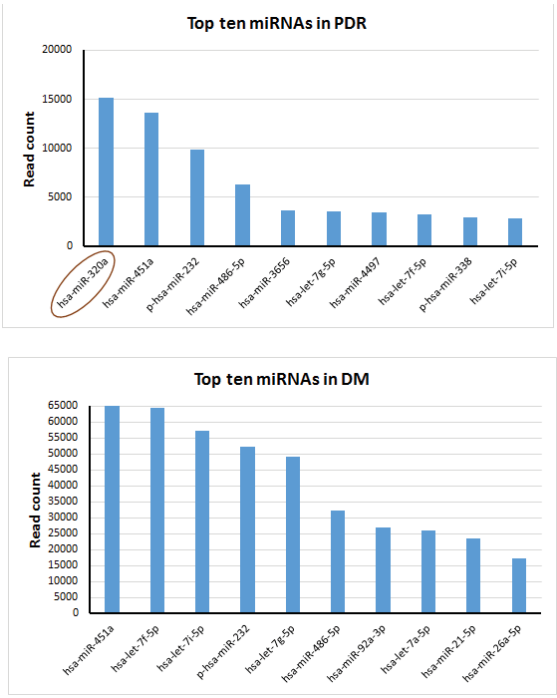


Fig. 3: List of 10 most abundant miRNAs expressed across serum samples of different conditions

Table 1: Gene target prediction for the differentially expressed miRNAs

Name of miRNA	Gene targets	Function
miR181a	ADCY1 (adenylyl cyclase), VEGF	Neoangiogenesis regulation
miR 320a	TSP-1 (thrombospondin1)	Angiogenesis inhibitor
miR 27b	TSP-1	Angiogenesis inhibitor
miR423-5p	FAM-3A, ATG7	Promotion of autophagy

(ii) Validation of differentially expressed microRNAs through quantitative Real-time PCR

The differentially expressed miRNAs obtained through next generation sequencing in the serum samples were validated using Real-time PCR using 5 different sets of serum samples. The results are shown in Fig. 4.

miR181a targets ADCY1 mRNA that is known to regulate neoangiogenesis process. Specifically miR181a represses VEGF pathway in retinal endothelial cells. The validation of miR181a in serum samples through q-PCR has shown that miR181a is highly expressed in DM with very less expression in PDR serum (Table 2). Since miR181a negatively regulates VEGF, less of miR181a in PDR correlates with more of VEGF in PDR leading to angiogenesis.

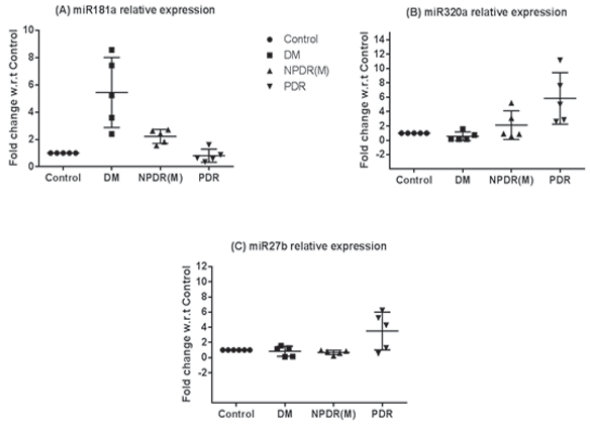


Figure 4: Fold changes in expression of miR181a, miR320a and miR27b with respect to control as validated by qPCR

Name of miRNA	Fold change in expression relative to control		
	DM	NPDR(M)	PDR
miR 181a	5.500	2.180	0.7920
miR 320a	0.5620	2.122	5.834
miR 27b	0.8322	0.6848	3.500
	Expression in control was kept as 1		

Table 2: Mean value of the fold changes in miRNA expression in Figure 4

miR320a and miR27b target thrombospondin-1 (TSP-I) which is a well-known inhibitor of angiogenesis. In both NGS and validation experiments, the relative expression of miR 320a and miR 27b increases to a great extent in PDR (Table 2) meaning it downregulatesTSP-I which leads to the progression of angiogenesis conditions in PDR. miR320a expression increases to more than 2-folds in NPDR (M) condition also suggesting that the patient with a moderate form of non-proliferative diabetic retinopathy might progress to PDR depending on the miR320a expression levels in serum in these stages. These are significant findings which are being analysed in more samples. Previously, it was reported that miR-21 was up regulated by 2.8 folds in PDR serum, 2.2 folds in DM serum and 1.9 folds in NPDR serum compared to control serum. miR-21 targets the angiogenesis process by activating AKT and ERK1/2 signalling pathways.

Analysis of VEGF levels in serum:

Circulating VEGF protein levels are affected by the differential expression of many miRNAs including miR181a, miR27b and miR21 reported in our study. VEGF levels were measured in serum using a specific sandwich ELISA kit. Accordingly, in a set of 3 samples, the level of VEGF in control was 222.2 ± 23.2 pg/ml, in DM was 99.09 ± 13.7 pg/ml, in NPDR was 281.21 ± 15.6 pg/ml and in PDR was 336.66 ± 25.7 pg/ml. The results reveal a clear trend of increased VEGF levels in PDR serum. Experiments are being repeated with more number of samples.

Subsequent studies will analyse the differential expression of other miRNAs in the four conditions, their target prediction and regulatory molecules affected using computational tools, their validation using real-time PCR and functional analysis using retinal endothelial cells.

New Observations

Next generation sequencing analysis has identified a novel microRNAs miR181a-5p, miR 27b and miR320a up regulated in the serum of PDR patients arising from Type 2 diabetes conditions. These observations have never been reported in the earlier literature for Type 2 diabetes patients, but reported only for patients with Type 1 diabetes in Caucasian and Chinese populations (Zampetaki et al., 2016). Our observations are significant concerning our Indian population because majority of the patients are affected with Type 2 diabetes.

Reference:

1. Zampetaki et al., Diabetes 2016;65: 216–227 | DOI: 10.2337/db15-0389

Analysis of human lens proteins for identification of cross-reacting antibodies in the serum of Leptospiral uveitis patients

Investigators : Dr. J. Jeya Maheshwari, Prof. K. Dharmalingam
Clinician scientist : Dr. SR. Rathinam
Team member : Irene Daniel

Rationale of the study:

Ocular manifestations of Leptospirosis are noted long after the systemic infection is cleared. One or both eyes can be affected with acute, non-granulomatous, diffuse uveitis. One of the complications in these patients is the sudden onset of cataract that remains stable or progress only slightly in majority of the patients once the inflammation is controlled (Tasman. W. *et al*., 1994). Parma and group (1992) demonstrated that inoculation of the horses with a pool of leptospiral antigens results in the development of antibodies that cross-react with the lens proteins. These leptospiral antigens specific antibodies that recognized the lens proteins is proposed to be probable trigger for cataract formation. Subsequently, Verma and group (Verma *et al*., 2010) identified that two leptospiral proteins, LruA and LruB, share immuno-relevant epitopes with eye proteins that contribute to the immunopathogenesis of Leptospira-associated recurrent uveitis.

Objectives

The studies by Parma group (1992) and Verma group (2010) were carried out in an equine system and the factors responsible for cataract formation in humans have not been explored. In this study, we examined if similar antigen mimicry exists in leptospiral uveitis patients that is responsible for cataract formation in these patients.

Results

Water soluble and urea soluble proteins were extracted from normal, senile cataract and lepto cataract lens and the proteins were resolved on a gradient 1D SDS-PAGE and compared (Figure 1). A major fraction of both water and urea soluble lens proteins were in the molecular weight range of 20-30 kDa and must be the crystallins, which are the abundant protein type in the lens.

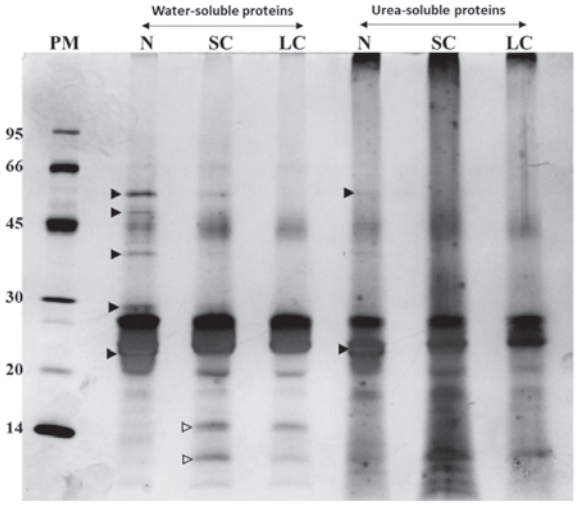


Figure 1. Comparison of the proteins from normal and cataract lens. Five microgram of each fraction was resolved on a 12-20% gradient SDS-PAGE and the proteins were visualized after staining with silver.

To examine if autoantibodies to lens proteins are present in circulation, the water and urea soluble lens proteins were probed for cross-reactivity using the serum from leptospiral uveitis patients. Figure 2 shows that the patient serum reacted with the water-soluble proteins from all three types of lens – normal, senile cataract and lepto cataract lens with greater

signal with that from normal lens when compared to both the cataract lens. On the other hand, the urea soluble proteins did not show significant cross-reaction similar to that of the water-soluble proteins. Hence, further experiments were carried out using the water-soluble lens proteins only.

To identify the specific lens proteins that cross-react with the circulating antibodies, the water-soluble proteins from the normal, senile cataract and lepto cataract lens were resolved on a 1D SDS-PAGE and taken for immuno blot analysis using the patient serum (Figure 3). It is evident that the antibodies in the patient serum recognizes two lens proteins, one is a small molecular weight protein migrating at approximately 18 kDa (A1) while the other one corresponds to protein of rough 58 kDa (A2). The signal intensity for both the bands were high in the normal lens and faint in both the cataract lens. This suggests that the protein(s) cross-reacting with the serum antibodies are significantly reduced in the cataract lens.

The proteins in the bands A1 and A2 were identified by on-membrane tryptic digestion followed by mass spectrometry. From Figure 3, it is evident that the level of the cross-reacting protein(s) in the lepto lens sample is lower than that of the normal lens. Hence, the abundance of each of the proteins identified was compared between the normal and lepto cataract samples to identify the protein that shows a decrease in abundance in the cataract lens. In band A1, three proteins – α-crystallin B, α-crystallin A2 and β-crystallin B2, abundant in normal lens was significantly reduced in the A1 band from lepto cataract lens. In the case of A2 band, retinal dehydrogenase 1 was the only protein that was abundant in normal lens but identified at extremely low level in lepto cataract lens. All other proteins identified in each of the A1 and A2 bands did not show significant difference in the abundance between normal and lepto cataract lens.

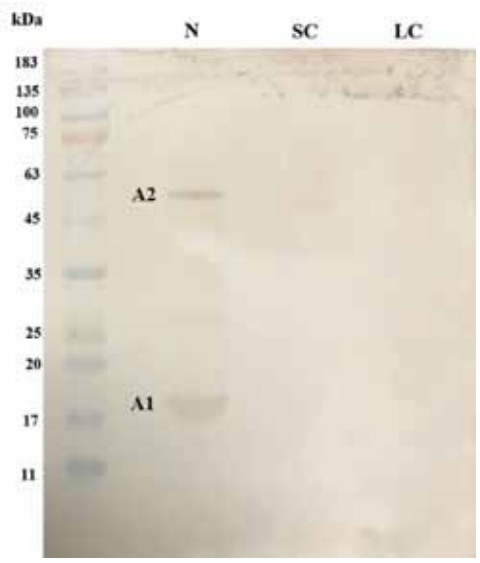


Figure3. Lens proteins cross react with patient serum. Water soluble proteins of the normal (N), senile cataract (SC) and lepto cataract (LC) lens were resolved on a 10% SDS-PAGE and taken for immunoblot analysis with the leptospiral uveitis patient's serum as the primary antibody. A1 and A2 indicates the bands that show strong signal in the blot.

We hypothesized that if the autoantibodies against the lens proteins are specific to the leptospiral infection, the bacterial antigen against which the antibodies are raised must share homology to these lens proteins. So, we computationally analyzed if any of these four lens proteins has homologs in Leptospira. None of the three lens crystallins had homologs in the leptospiral proteins. Interestingly, the retinal dehydrogenase protein shared a significant homology with that of the aldehyde dehydrogenase of many Leptospiral serovars/strains.

If these antibodies enter the aqueous humor and interact with the protein in the lens to inactivate their function, these proteins should be in a location that is accessible for the antibodies. If any of these proteins

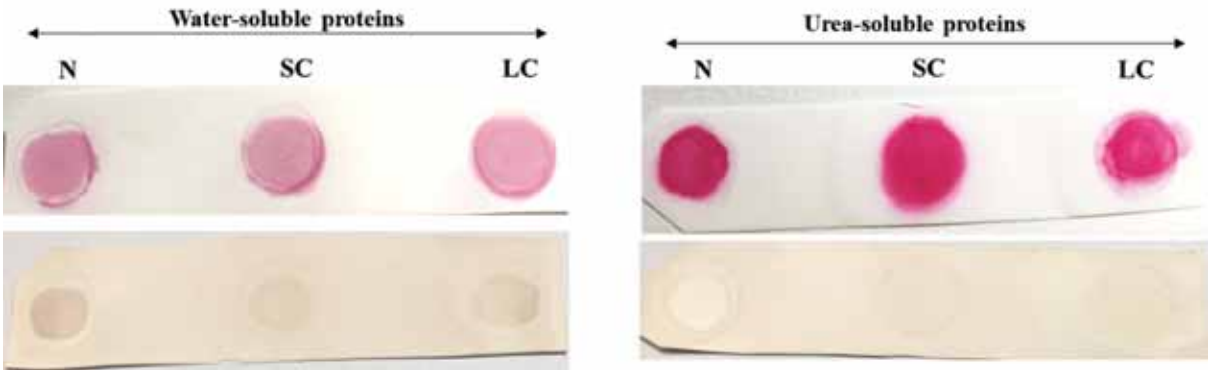


Figure 2. Hundred microgram of water soluble and urea soluble proteins from normal (N), senile cataract (SC) and lepto cataract (LC) lens were spotted on a nitrocellulose membrane and probed with serum from leptospiral uveitis patients as the source of primary antibody. The top panel shows the proteins stained with Ponceau S before the immunoblot analysis and the bottom panel is the immuno blot of the same membrane.

are located in the lens capsule, the interaction with the antibodies can impair the protein's function. Ronci and group (Ronci *et al.*, 2011) using a MALDI-MS imaging technique reported the proteins present in the human lens capsule. Of the four proteins identified in this study, except β-crystallin B2, all the other three proteins were identified in the lens capsule.

Based on the homology and location, retinal dehydrogenase 1 appears to be the ideal candidate for antigen mimicry. If the leptospiral aldehyde dehydrogenase acts as an antigen, the antibodies raised against this protein can cross-react with the retinal dehydrogenase 1 on the lens capsule, provided the antibodies enter the aqueous humor. Further, antibodies against one or more of the three crystallins, were also present. Antibodies against αB-crystallin or β-crystallin B2 are present in the patient serum but it is not evident whether they are leptospiral infection specific or not.

Both αB-crystallin and ALDHA1 does play a crucial role in maintaining lens transparency. If the antibodies interact with these proteins located in the lens capsule, it could potentially impede the functional capabilities of these lens proteins and could possibly initiate cataractogenesis.

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Novel chemical cross-linking of the cornea for the treatment of keratoconus

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Funding : Engineering and Physical Sciences Research Council, UK and Aurolab, India

Introduction

Keratoconus is one of the major bilateral corneal ectasias affecting the working or the young population in the age-group of 25-35 years. It is characterized by the thinning of the cornea followed by the formation of cone shaped cornea leading to defective vision in the form of severe astigmatism. Decrease in the mechanical strength of the collagen fibrils in the cornea, significant loss of ECM, defective collagen cross-linking activity lead to keratoconus (Rabinowitz, 1998; Kenney et al., 2012).The conventional crosslinking protocol involves removal of the central corneal epithelium, application of riboflavin and the illumination of the affected eye with UV-A light (370 nm, 3 mW/cm2) for 30 minutes. But, the removal of the epithelium is painful and the risk of infection is increased.

This study developed a novel chemical cross-linker using EDCI/NHS [1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide] mediated chemistry and a suberic acid (SA) spacer to cause corneal cross-linking without removing the corneal epithelium, or the use of UV-A irradiation, therefore avoiding the pain associated with the conventional crosslinking treatment of keratoconus and the risk of infection.

Results

The cross-linker was prepared as a solution containing all the three components and the optimum concentration of 1:1:1 molar ratio of EDCI:NHS:Suberic acid formulation was standardized at Liverpool. A treatment time of 15 minutes with the 1:1:1 molar ratios of the cross-linker was also optimised at Liverpool for treating porcine corneas as well as the cell layers harvested from the corneas. The same treatment conditions were employed for treating the human corneas and the harvested corneal cells at AMRF.

In our earlier report (2017-18), the team had extensively reported the effect of two different concentrations of the cross-linker (full and 1/8th) on the cytotoxicity, changes in the morphology of the corneal layers, changes to the tensile strength and stiffness of the cornea derived from the cadaver cornea. A pseudo-clinical treatment procedure for the application of the cross-linker to the corneal surface of the cadaver cornea was employed. Accordingly, the novel chemical cross-linker at the full or 1/8th concentration did not induce apoptosis or any gross morphological changes in the corneal layers of the cadaver cornea. The full concentration cross-linker penetrated upto 700 μm into the corneal stroma whereas the 1/8th diluted cross-linker penetrated upto 200 μm into the stroma to induce a 2.12 fold increase in stiffness of the cornea compared to the PBS control.

As a further continuation of previous years' experiments, the cytotoxicity of the cross-linker at the full and 1/8th concentrations were checked on the keratoconus corneas, phenotypic analysis of cells grown out from the cross-linker treated corneas were done using corneal cell layer specific markers and identification of cross-linked proteins in the porcine and human corneas were done by mass spectrometry. The results are discussed below:

(i) TUNEL assay on keratoconus corneas

The keratoconus corneas were exposed to different concentrations of the cross-linker on the epithelial side alone, cryo-sections were taken and the TUNEL assay mixture was applied to the cryo-sections to assess the apoptosis. The results are represented in Fig. 1.

The 1/8th concentration of the cross-linker did not induce apoptosis in the keratoconus corneal cell layers whereas the full concentration cross-linker did induce a bit of cell death in the keratoconus epithelium suggesting that higher concentration of the cross-linker is cytotoxic to the keratoconus corneas.

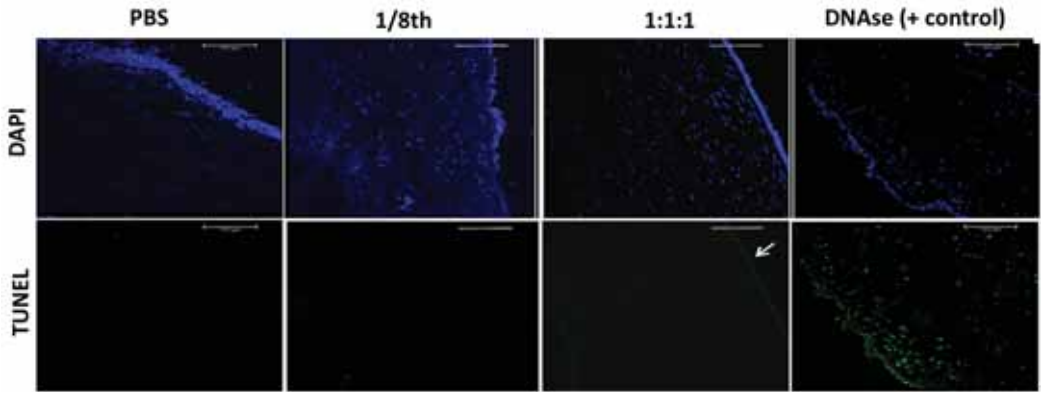
(ii) Assessing of phenotype of cells from corneas treated with the cross-linker

Cadaver corneas were treated with both the concentrations of the cross-linker using the pseudo-clinical model and cells were harvested from different layers of the corneas. Cells from limboscleral explants and stromal fibroblasts were proliferating and expressed the specific markers namely cytokeratin-3 (CK3) and vimentin. Similarly, the cells from endothelium expressed corneal –endothelial specific protein and cells from central corneal epithelium expressed CK3. The endothelial cells and the central corneal epithelial cell did not proliferate though being fully differentiated (Fig. 2). The results clearly demonstrated that cross-linker at either concentration did not affect the phenotype of cells of the corneal layers.

(iii) Identification of cross-linked proteins in porcine and human corneal stroma using mass spectrometry

The cross-linker treated cadaver corneas were separated from the cadaver globes, the individual corneal layers were separated and proteins were isolated from individual corneal layers. The isolated proteins were electrophoresed in a SDS-PAGE and trypsin digested to get the peptides. The peptides were analysed by mass spectrometry. Some of the proteins identified from porcine and human corneal stroma are tabulated in Table 1. In both the cases, matrix protein collagen of different subunits and proteoglycans lumican, decorin and keratocan represent the cross-linked proteins. These proteins are important components of stroma involved in maintaining the biomechanical rigidity of the stromal matrix.

Figure 1: TUNEL assay on keratoconus corneas after cross-linker treatment



The cross-linker at 1/8th concentration did not induce apoptosis in the corneal cell layers, whereas at full concentration, faint apoptotic signal was observed at the epithelial layer (arrow).

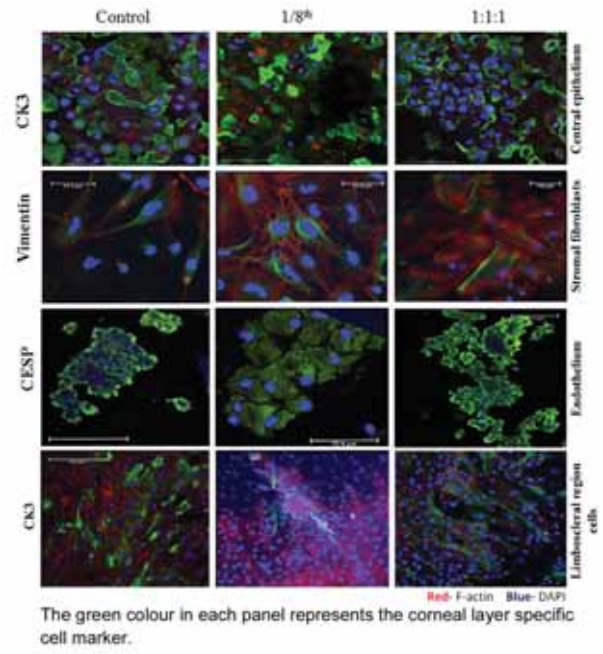


Figure 2: Immunostaining of the cells from different corneal layers before and after cross-linker treatment

A comparison of the percentage of cross-linked peptides in porcine and human stroma (Table 1C) indicates that only 5% of the peptides are cross-linked used the EDCI-NHS-Suberic acid combination. Majority of the crosslinking happened through EDCI and NHS. Even though only 5% of the cross-linked peptides were mediated through EDCI-NHS-Suberic acid combination, we think that it provides enough flexibility for the tissue to keep the stromal and epithelial cells in their normal physiological conditions.

Sample	Types of XL	Porcine	Human
Corneal Epithelium	EDCI-NHS	74	41
	EDCI-NHS-SA	7	5
	Single	19	54
Stroma	EDCI-NHS	72	44
	EDCI-NHS-SA	9	5
	Single	19	51

Table 1C. Percentage of cross linked peptides- a comparison between porcine and human corneal layers, XL indicates cross-linker.

Conclusions

The novel chemical cross-linker at 1/8th concentration increased the tensile strength and stiffness of the human keratoconic cornea as well as cadaver cornea without causing any significant cytotoxicity, change in phenotype of the constituent cells of the corneal layers or change in the gross morphology of the corneal layers and without causing apoptosis to the constituent cells of the corneal layers. Hence, the novel chemical cross-linker at 1/8th concentration is good for further formulation, packaging, sterility testing and further trials.

Accession	Description	Σ Coverage	Σ# Unique Pepticles	Σ# PSMs	Σ# AAs	MW (KDa)
13LUR7	Collagen alpha - 3 (VI) chain	35.17	79	3355	3170	342.2
F1SQ09	Lumican	44.28	11	1894	341	38.8
Q9XSD9	Decorin	33.22	9	847	360	39.9
F1SQ08	Keratocan	32.01	9	639	353	40.6
I3LQ84	Collagen type VI alpha 2 Chain	28.21	21	638	989	106.4

Table 1A.List of some important cross-linked proteins identified in porcine corneal stroma

Accession	Description	Σcoverage	Σ# Unique Peptides	Σ# PSMs	Σ# AAs	MW [kDa]
P12111	Collagen alpha-3(VI) chain	30.82	84	4423	3177	343.5
P51884	Lumican	40.24	13	2299	338	38.4
D6RGG3	Collagen alpha-1(XII) chain	34.49	65	2261	3062	333
P07585	Decorin	43.18	13	1660	359	39.7
P21810	Biglycan	36.14	10	1144	368	41.6
O60938	Keratocan	32.1	11	826	352	40.5

Table 1B. List of some important cross-linked proteins identified in human corneal stroma

OCULAR PHARMACOLOGY

The main research focus of the Department of Ocular Pharmacology is to understand the molecular mechanism(s) involved in the pathogenesis and to develop therapeutic targets for the management of glucocorticoid (GC) induced ocular hypertension (OHT)/glaucoma. The funding support from Wellcome-DBT/India Alliance enabled the research team of the department to investigate the role of microRNA in the regulation of GC signaling and to develop miRNA based therapeutics for GC-induced glaucoma.

HOCAS Characterized GC-responsive/ non-responsive Primary Human Trabecular Meshwork (HTM) Cell Strain to Study the Pathogenesis of Steroid-induced Glaucoma

Investigators	:	Dr. S. Senthilkumari, Dr. C. Gowripriya, Dr.Bharanidharan Dr. R. Sharmila
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Research Associate	:	R. Haribalaganesh
Junior Research Fellow:		K. Kathirvel
Research Assistant	:	T. Madhu Mithra (AMRF supported)
Funding Source	:	Wellcome-DBT/India Alliance - Intermediate Fellowship (2017-2022).

Introduction

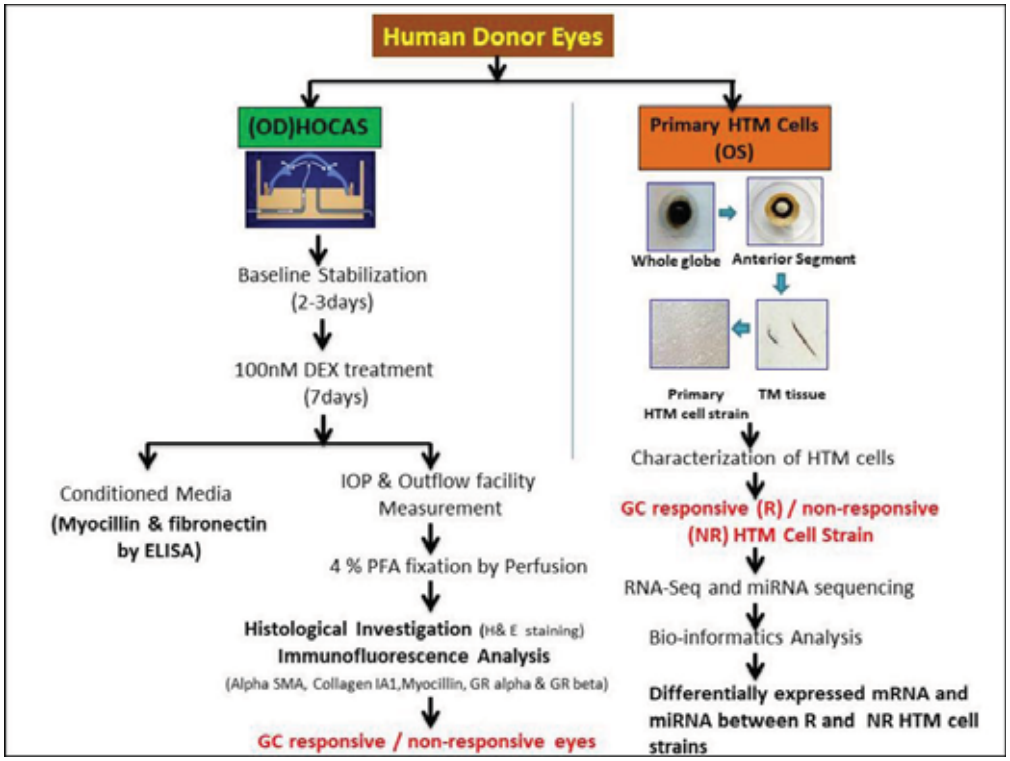
Glucocorticoid (GC)–induced glaucoma (GIG) is a sub-type of secondary glaucoma and the usage of GC either topical or systemic administration may induce ocular hypertension (OHT) in susceptible individuals (steroid responders), some of whom develop optic neuropathy / glaucoma even after steroid withdrawal *(*Mao *et al.* 2011). The clinical presentation of GIG shares similarities with POAG. In both conditions, the elevated intraocular pressure (IOP) is the major risk factor.

GCs affect the TM by increasing its stiffness, causing cytoskeletal rearrangement, inducing excessive extracellular matrix deposition, and altering cell adhesion. These alterations may contribute to IOP elevation and glaucoma pathogenesis.

Several *in vivo* and *in vitro* models are reported to study the pathogenesis of GIG. Perfused human anterior segment has long been used as an *ex-vivo* model in GIG research. The responder rate of perfusion cultured non-glaucomatous human eyes is very close to the observations in human subjects (Clark *et al*., 1995). However the yield of RNA from TM tissues after HOCAS is insufficient for RNA sequencing. Therefore, the aim of the present study is to establish the primary HTM cells from eyes with known GC responsive eyes. HOCAS is used to assess the responsiveness of human eyes to dexamethasone (GC) treatment. This will enable us to identify the differentially expressed genes between GC responders and non-responders.

Work Plan

The details of the study plan is given in the schematic diagram (Figure 1).



Results

HOCAS was established by the method as described previously (Johnson & Tschumper, *1987*). One eye from each pair was used to establish HOCAS and the other eye was used to establish GC responsive or non-responsive human TM cell strain.

Characterization of GC-Responsive/Non-Responsive Eyes using HOCAS

The characteristics of human donor eyes used to establish SI-OHT model using HOCAS is summarized in Table 1. The elapsed time between enucleation and culture is 34.02 ± 16.4 h. Out of 27 eyes used for HOCAS, data of only 20 eyes were included for the study and 7 eyes were discarded due to abnormal baseline pressure.

Out of 20 eyes received 100 nM DEX treatment, a significant IOP elevation was seen in 7/20 eyes (Mean ±SD - mΔIOP: 11.51±6.4; range: 5.27-23.13 mmHg; 35% responders). The remaining eyes were graded as GC- non-responsive eyes (n=13/20) (Mean ±SD - mΔIOP: 0.62±0.43; range: 0.2-4.89 mmHg; 65% non-responders). A representative data of DEX- responder and DEX-Non responder is shown in Figure 2.

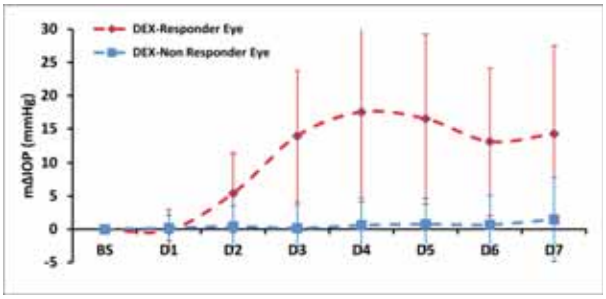


Figure 2: A representative intraocular pressure (IOP) graph of the anterior segment receiving 100nM DEX treatment. Out of 20 eyes received 100nM DEX treatment, 7 eyes showed elevated IOP over the period of treatment.

DEX-induced MYOC and Fibronectin levels in HOCAS

The differential protein expression levels of myocillin (MYOC) and Fibronectin (FN) upon DEX treatment by ELISA was investigated in cell soup collected during perfusion at different time intervals. MYOC is a secreted glycoprotein and its physiological function is unknown in TM and other ocular tissues (Stone *et al., 1997;* Ngyune *et al., 1998*). MYOC expression can be inducible in TM cells with DEX treatment and it is one of the markers for TM cell identification (Clark *et al., 2000*). FN is an ECM protein and increased accumulation by DEX is reported previously.

Table 1: Details of Human Donor Eyes Used for the Study

Code	Age	Sex	Cause of Death	Time B/W Death & Enu cleation (h)	Time B/W Enucleation & Culture (h)	Experi ment Eye	HOCAS/ Culture	Treat ment (100nM)	Remarks
OCHD18-27	75	F	CVA	1.5	9.5	OD	HOCAS	DEX	Single eye Data included
OCHD18-28	65	F	CVA	1	39.5	OD	HOCAS	DEX	Single eye Data included
OCHD18-29	76	F	Natural	0.75	30.5	OD	HOCAS	DEX	Data included
						OS	Cell culture		TM Culture established
OCHD18-30	75	F	RD	1.5	53.25	OD	HOCAS	DEX	Data included
						OS	Cell culture	-	Discarded due to Contamination
OCHD18-31	83	M	Heart Disease	1.5	52	OD	HOCAS	DEX	Data included
						OS	Cell culture	-	Discarded due to no cell growth
OCHD18-32	79	M	RD	1.33	51.85	OD	HOCAS	DEX	Data included
						OS	Cell culture	-	Discarded due to no cell growth
OCHD18-34	78	M	CVA	2.5	5.5	OD	HOCAS	DEX	Data included
						OS	Cell culture	-	Discarded due to no cell growth
OCHD18-35	80	M	RD	2	52.25	OD	HOCAS	DEX	Data included
						OS	Cell culture	-	Discarded due to no cell growth
OCHD18-36	55	F	RD	1.67	47.42	OD	HOCAS	DEX	Data included
						OS	Cell culture	-	Discarded due to no cell growth
OCHD18-37	53	M	RTA	4.25	26	OD	HOCAS	DEX	Data included
						OS	Cell culture	-	Discarded due to no cell growth
OCHD18-38	68	M	CVA	2.83	39.83	OD	HOCAS	DEX	Data included
						OS	Cell culture	-	TM Culture established
OCHD18-44	83	F	Cardio Respiratory Arrest	2.5	20.75	OD	HOCAS	DEX	Data included
						OS	Cell culture	-	Discarded due to no cell growth
OCHD18-46	68	M	Heart Disease	2	37.5	OD	HOCAS	DEX	Data included
						OS	Cell culture	-	TM Culture established
OCHD18-47	78	M	Cardiac Arrest	1.58	20	OD	HOCAS	DEX	Data included
						OS	Cell culture	-	TM Culture established
OCHD18-48	71	F	Cardio Respiratory Arrest	5.33	17.42	OD	HOCAS	DEX	Data included
						OS	Cell culture	-	TM Culture established

OCHD18-49	48	M	Heart Disease	2.5	30.67	OD	HOCAS	DEX	Data included
						OS	Cell culture	-	TM Culture established
OCHD18-52	55	F	Vascular accident	2.75	67.92	OD	HOCAS	DEX	Data included
						OS	Cell culture	-	TM Culture established (On going)
OCHD18-53	67	M	Type 2 Diabetes	4.33	27	OD	HOCAS	DEX	Data included
						OS	Cell culture	-	TM Culture established (On going)
OCHD18-56	82	M	CVA	4	23	OD	HOCAS	DEX	Data included
						OS	Cell culture	-	TM Culture established (On going)
OCHD18-57	80	F	Respiratory disease	3	28.50	OD	HOCAS	DEX	Data included
						OS	Cell culture	-	TM Culture established (On going)

Mean (± SD) donor age: 70.95 ± 10.82 years; Elapsed time between death and enucleation: 2.44 ± 1.23 h; Elapsed time between enucleation and culture: 34.02 ± 16.38h.

Therefore, in the present study, the levels of secreted MYOC & FN upon DEX treatment were investigated.

The results indicated that secreted MYOC and FN level in conditioned medium were down-regulated after DEX treatment in both responder and non-responder eyes (Figure 3A and 3B).

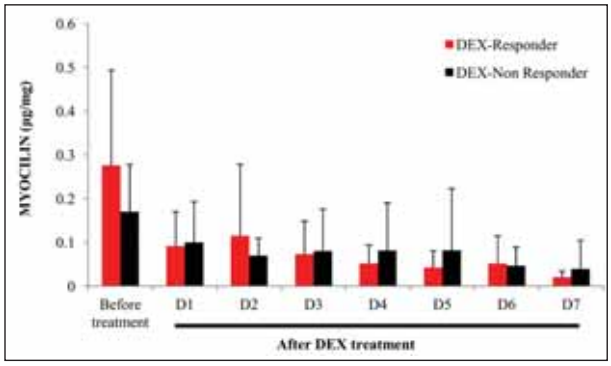


Figure3A. Levels of secreted myocillin in HOCAS Conditioned media. The levels in the conditioned media were assayed by Sandwich ELISA after total protein estimation.

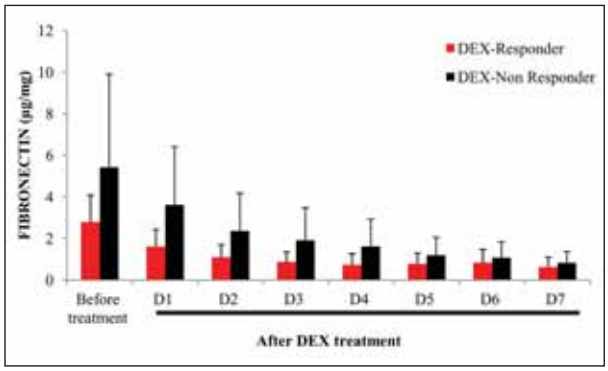


Figure 3B. Levels of secreted fibronectin in HOCAS Conditioned media. The levels in the conditioned media were assayed by Sandwich ELISA after total protein estimation. Effect of DEX on Actin and ECM (COL IA, MYOC and FN) in HOCAS-TM Tissue

The effect of DEX on smooth muscle actin (α-SMA) and ECM is shown in Figure 4. DEX treatment not only elevates IOP but also increases actin staining and ECM in TM region of the DEX-responsive eyes as compared to non-responsive eyes.

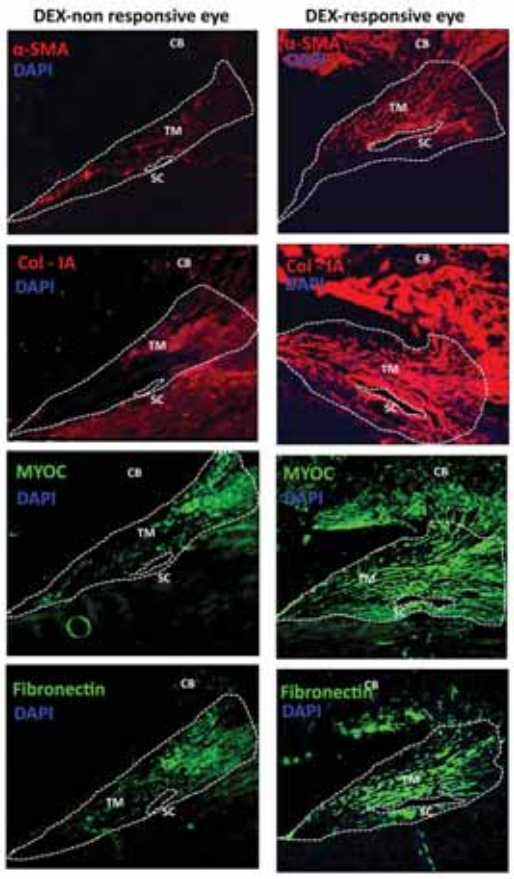


Figure 4: DEX increases α-SMA, COL1A (ECM), Myocillin and Fibronectin in TM as compared to their non-responder eyes respectively. SC-Schlemm's Cananl; TM – Trabecular meshwork; CB – Ciliary Body

Establishment of HOCAS-characterized GC-res and non-responsive Primary HTM Cells

Out of 20 HOCAS characterized eyes, successful HTM cell strains were established from 9 donor eyes and 1 cell strain from glaucoma suspect eye (based on posterior segment histological evaluation). The expression profile of TM markers in HOCAS characterized non-responsive and glaucoma suspect HTM cell strain is given in figure 5.

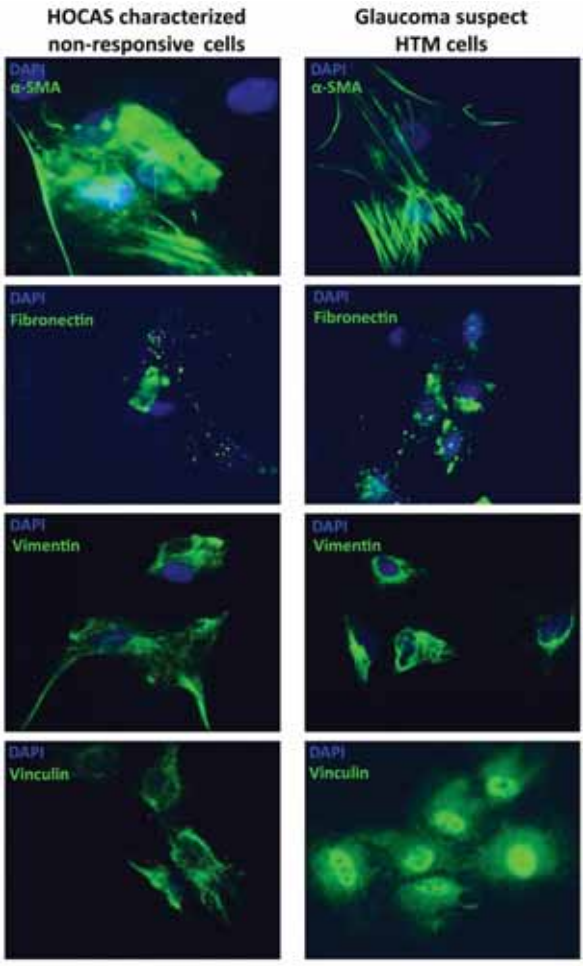


Figure 5: Expression of TM markers in HOCAS characterized non-responsive HTM cell strain and glaucoma suspect.

Conclusion

Primary HTM cells with known GC responsiveness was established using HOCAS model. Further studies are underway with these HTM cells to investigate the differentially expressed mRNAs and miRNAs in response to steroid treatment.

Integrated Analysis of mRNA and miRNA Expression Profiles in Primary Human Trabecular Meshwork (HTM) Cells after Steroid Treatment

Investigators : Dr.S.Senthilkumari, Dr. D. Bharanidharan.
Research Scholar : K. Kathirvel
Funding : Wellcome-DBT/India Alliance –Intermediate Fellowship (2017-2022)

Introduction

GC receptor signalling regulates microRNA expression. MicroRNAs (miRNAs) modulate gene expression at the posttranscriptional level and regulate many cellular functions (Yang and Wang, 2011). Recent studies demonstrated both in vitro and in glaucomatous aqueous humor and trabecular meshwork release extracellular miRNAs which can regulate the ECM, TM contractility and senescence (Luna *et al. 2009;* Villarreal *et al. 2011 and* Tanaka *et al. 2014*). However, no studies have been reported related to the role of miRNAs in steroid –induced glaucoma. There is evidence that steroid treated ocular fibroblasts demonstrate differential expression of small non-coding RNAs (miRNAs) (Liu *et al. 2011*). The predicted miRNA target genes include pathogenesis-relevant genes/pathways, such as ECM remodeling, wound healing, cell cycle regulation; these however were not experimentally validated. Therefore, the key goal of the present study is to understand the role of miRNAs and their target genes in the regulation of glucocorticoid receptor (GR) signalling in the TM and to develop new miRNA therapeutics for the treatment of steroid – induced OHT/ glaucoma.

Results

Primary Human Trabecular Meshwork cell line (PHTM17-14) was established from 89 year-old normal female donor eye, collected from Aravind eye hospital, Madurai. The cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 15% fetal bovine serum, 100 ug/mL streptomycin sulfate and 100 U/mL penicillin and maintained in a humidified 5% CO2 environment at 37°C. The 3rd passage primary cells at >85 percent confluence were treated with 100nM Dexamethasone and 0.1%EtoH for 7 days in T75

flask. Characterization of cells as steroid responder and non-responder were carried out by GR-α&GR-β localisation observed by immunohistochemistry and GR-α/GR-β expression ratio by qRT-PCR. Total RNA was isolated from treated TM cells by optimized TRiZol method. RNA quality and quantity were assessed by using TapeStation(Agilent) and Qubit 3.0, respectively. Additionally, the Quality of RNA was observed by ratio of 28S and 18S ribosomal bands (0.8 % agarose gel electrophoresis) (Figure 1).

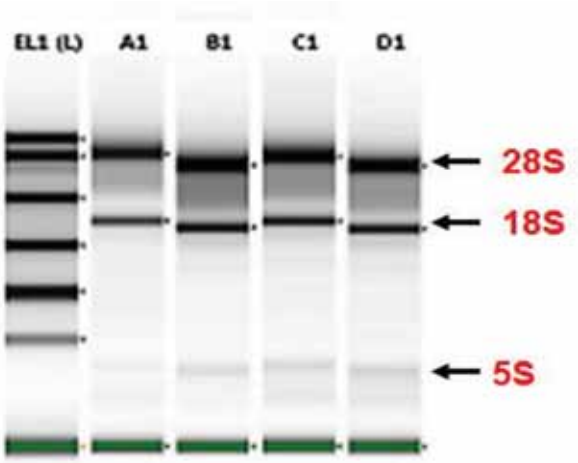


Figure 1. Gel electrophoresis profile of RNA purity and integrity by 28S and 18S ribosomal bands. 0.1EtoH (A1and C1) ; 100nM Dex (B1and D1)

More than one million treated cells were taken for RNA isolation. The RNA concentration of 0.1%EtoH and 100nM Dexamethasone samples was 2.0ug/µl and 2.1ug/µl for miRNA-sequencing, 3.7ug/µl and 2.7ug/µl for mRNA-sequencing, respectively. In brief, 1ug of total RNA was used to enrich miRNA and mRNA using NEB smallRNA and mRNA Isolation Kit, respectively. The miRNA library and transcriptome library was prepared using NEBNext smallRNA library prep set and NEB ultraII RNA library prep kit, respectively. Sequencing was done using Illumina Next Seq 500 paired end technology. The final enriched library is purified and quantified by Qubit and the size is analyzed by Bio analyzer. All samples had a RIN value of more than 9 (Table 1).

Totally, 32.6 and 23.7 Million reads were generated from 0.1%EtoH and 100nM Dexamethasone samples in miRNA-sequencing. Similarly, 44.2 and 37.9 million reads were generated from 0.1%EtoH and 100nM Dexamethasone samples in mRNA-sequencing, respectively. The quality of raw reads was assessed by FastQC toolkit and adapter sequences were removed using bbduk.sh shell script from bbmap short read aligner. Pre-aligned QC reports shows the quality score of forward and reverse reads were ≥ 30 in mRNA and miRNA datasets. The raw reads were mapped with Human

Sample Details		Tape station QC Details		QuBit Quantification Details			QC Status	
S.No	Sample ID	Conc. (pg/µl)	RIN	Conc.(pg/µl)	Vol. of RNA Available (µl)	Total RNA Amount Available (ng)	Pass/Fail	Remarks
1(A1)	0.1 EtOH-1	268	9.1	3760	7.4	27824	Pass	-
2(C1)	0.1 EtOH-2	364	9.1	2000	6.8	13600	Pass	-
3(B1)	100 DEX-1	361	9.1	2700	6	16200	Pass	-
4(D1)	100 DEX-2	453	9.2	2180	6.1	13298	Pass	-

Table 1. RNA Samples QC report. A1(0.1 % EtoH) and B1(100nm DEX) for mRNA-sequencing. C1(0.1 % EtoH) and D1 (100nm DEX) for miRNA-sequencing.

reference genome assembly (GRCh38) using STAR and HISAT2 version 2.1.0 for miRNA and mRNA, respectively. Nearly 42.2 million (95.6%) reads from 0.1%EtoH and 35.8 million (94.7%) reads from 100nM Dexamethasone, were aligned with reference genome GRCh38 in mRNA-sequencing. Similarly, 98.82% of reads from 0.1%EtoH and 97.98% reads from 100nM Dexamethasone, were aligned with reference genome in miRNA-sequencing. The detailed informations of genomic characteristics are shown in Table 2.

The miNRA and mRNA abundance in read counts were estimated using Feature counts. miRNAs with < 9 read counts and Genes with <49 read count were excluded for further analysis. The read counts were normalized by Quantile and TMM for miRNA and mRNA, respectively (Figure 2).

	miRNA		mRNA			
	0.1%EtoH	100nmDex	0.1%EtoH_ R1	0.1%EtoH_R2	100nmDex_R1	100nmDex_R2
Total no. of bases	2447537100	1776376125	3311876100	3311876100	2842040850	2842040850
Total no of Reads	32633828	23685015	22079174	22079174	18946939	18946939
% bases >=Q20	95.83	95.06	98.36	96.42	98.17	96.47
% bases >=Q30	92.14	91.31	95.77	91.70	95.48	91.97
Average read length	75	75	150	150	150	150
Data GB	2.4	1.8	6.6		5.7	
Data Million Reads	32.6	23.7	44.2		37.9	
Alignment rate	98.82	97.98	95.76%		94.70%	
# of features	1332	1229	16144		16142	

Table 2: Sequence and Alignment Statistics of miRNA and mRNA Sequence data

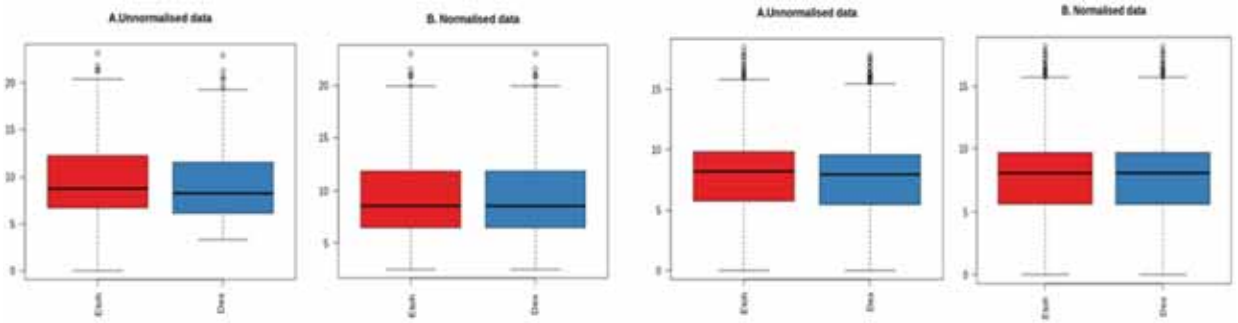


Figure 2. Quantile normalization. miRNA(A) and mRNA(B) normalization.

The Differential miRNA and mRNA Expression analysis was performed using NOISeq in Rscript. miRNAs (Figure 3) and Genes (Figure 4) were considered as differentially expressed if the absolute fold change (log2) value was >2, and the FDR value < 0.05. The binding target genes of differentially expressed miRNAs were predicted using miRWalk V3 with customized parameters. Pathways associated with differentially expressed genes were analyzed using GSEApre-ranked module in GenePattern against KEGG database.

In average, 16145 genes had read counts of >49 from both 0.1%EtoH and 100nM Dex treated cells, but only 8900 genes were dysregulated in Dex treated cells compared to control with significant P value. Out of 8900 genes, 33 were up-regulated and 7 were down-regulated with absolute fold change (log2) of >2, and FDR < 0.05 (Table 4).

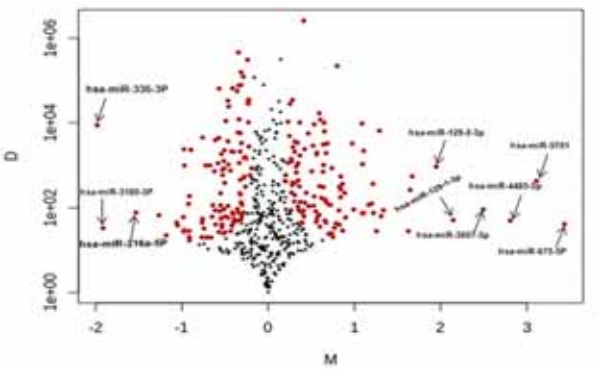


Figure 3: Volcano plot of differentially expressed miRNAs. The fold of change (M) and Difference (D) of the dysregulated miRNAs and mRNAs in Dex treated cells compared with vehicle control are shown in volcano plots. Significant FDR value <0.05, only taken for consideration.

miRNA	logFC	D	P Value
hsa-miR-675-5p	3.44	39.76979888	0.752427184
hsa-miR-5701	3.11	427.4262278	0.925048544
hsa-miR-4485-5p	2.81	48.61042661	0.773300971
hsa-miR-3607-3p	2.5	90.1174853	0.835436893
hsa-miR-129-1-3p	2.15	49.86827633	0.772524272
hsa-miR-129-2-3p	1.95	929.3886884	0.94184466
hsa-miR-31-3p	1.67	544.5826681	0.923300971
hsa-miR-6842-3p	1.65	262.3115057	0.897864078
hsa-miR-302d-3p	1.63	27.00129327	0.698058252
hsa-miR-216a-5p	-1.53	75.78049042	0.805145631
hsa-miR-3180-5p	-1.91	32.10405152	0.722621359
hsa-miR-335-3p	-1.98	8799.120396	0.982718447

Table 3. Dysregulated miRNAs. >1.5 fold change with significant P value. D - Difference.

In miRNA sequencing, we found 9 miRNAs were up-regulated and 3 miRNAs were down-regulated out of 255 miRNAs(read count >9), with absolute fold change (log2) of >1.5, and FDR < 0.05(Table 3).

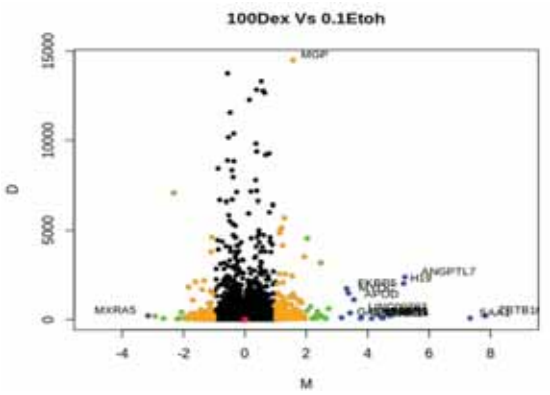


Figure 4: Volcano plot of differentially expressed mRNAs. The fold of change (M) and Difference (D) of the dysregulated miRNAs and mRNAs in Dex treated cells compared with vehicle control are shown in volcano plots. Significant FDR value <0.05, only taken for consideration.

Pathway analysis based on differentially expressed genes revealed that, 12 pathways were significantly deregulated and may play important role in GC response (Figure 5).

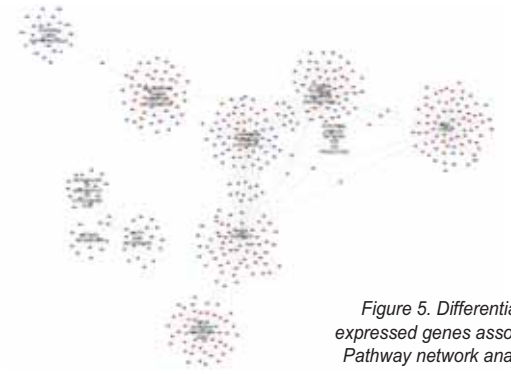


Figure 5. Differentially expressed genes associated Pathway network analysis.

Up-Regulated Genes			
Gene ID	Log FC	D	P Value
ZBTB16	7.82	206.5	1.0
SAA1	7.34	73.5	0.9
ANGPTL7	5.21	2377.6	1.0
H19	5.17	2018.5	1.0
LSP1	4.73	210.2	1.0
HIF3A	4.52	100.2	1.0
RGCC	4.48	78.0	1.0
TIMP4	4.42	149.3	1.0
P2RY14	4.13	60.3	0.9
CPM	3.79	117.7	1.0
STOX1	3.77	69.5	0.9
APOD	3.55	1116.9	1.0
LINC00702	3.43	366.2	1.0
MYOC	3.37	1473.1	1.0
FKBP5	3.32	1736.5	1.0
GALNT15	3.15	100.8	1.0
SPARCL1	2.73	613.7	1.0
CELF4	2.64	52.8	0.9
FAM107A	2.59	101.2	1.0
CXCL2	2.52	173.0	1.0
ABRA	2.47	212.6	1.0
ADH1B	2.47	3181.8	1.0
FMO2	2.46	201.2	1.0
CXCL8	2.37	450.2	1.0
TMEM178A	2.36	242.6	1.0
STEAP4	2.35	82.5	1.0
SCN3A	2.32	189.6	1.0
ARHGEF26	2.29	185.2	1.0
FP236383.4	2.24	44.4	0.9
TNFRSF1B	2.19	58.5	0.9
LINC00968	2.13	719.9	1.0
CEBPD	2.03	4538.2	1.0
TNFRSF21	2.03	487.1	1.0
Down-Regulated Genes			
Gene ID	Log FC	D	P Value
ADRA2A	-2.06	403.8	1.0
KIF20A	-2.12	74.0	0.9
IGSF10	-2.2	35.8	0.9
AQP1	-2.31	7038.8	1.0
CASC5	-2.65	63.8	0.9
MKI67	-2.92	181.0	1.0
MXRA5	-3.15	208.7	1.0

Table 4. UP/Down-Regulated genes. <2 fold change with significant P value. D - Difference

Among 12 pathways, Retinal metabolism, Drug metabolism cytochrome P450, Fatty acid metabolism, systemic lupus erythematosus, steroid hormone biosysnthesis, chemokine signalling pathways were up-regulated and cell adhesion, Axon guidance, intestinal immune network for IgA production, neuroactive ligand receptor interaction pathways were down-regulated (Table 6).

We found, the target genes of the highly dysregulated miRNAs are already dysregulated in transcriptome analysis (Figure 6).

Conclusion:

Many reported glaucoma associated genes were identified in our study, like SAA1, SAA2 and ANGPLT7. As reported by many studies, cell adhesion has altered after the GC treatment. The

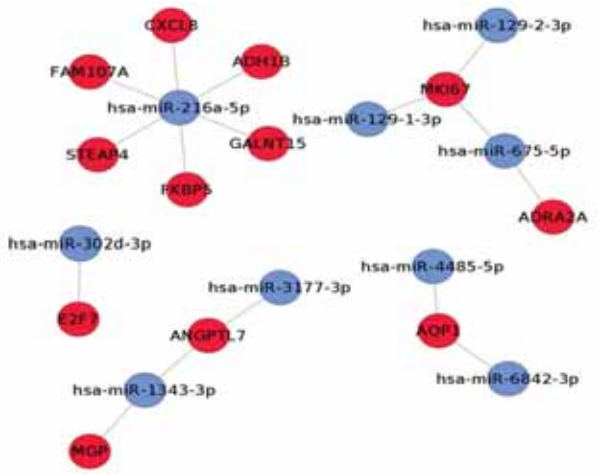


Figure 6: Differentially expressed miRNAs with their binding target genes which are already dysregulated in mRNA-sequencing. miRNAs (Blue), mRNAs(Red).

Pathway	#of Genes	NES	P value
Retinol Metabolism	13	1.79	0.0095
Drug Metabolism cytochrome_P450	18	1.66	0.0226
Fatty acid metabolism	22	1.57	0.0347
Systemic lupus erythematosus	26	1.52	0.0381
Steroid hormone biosynthesis	12	1.52	0.0563
Chemokine signaling pathway	87	1.46	0.0213
Cytokine-cytokine receptor interaction	76	1.36	0.0451
Cell adhesion molecules cams	53	-1.91	0.0022
Axon guidance	79	-1.82	0
Intestinal Immune network for IgA Production	5	-1.51	0.0345
Cell cycle	81	-1.46	0.0253
Neuroactive ligand receptor interaction	57	-1.43	0.04

Table 6. Dysregulated pathways enriched by DEGs. NES - Fold Change

binding target genes of few dysregulated miRNAs are identified as differentially expressed in our transcriptome study. These results are indicating that the GC treatment is affecting the miRNA and mRNA expressions in HTM cells. Further studies are underway with more number of GC responsive and non-responsive HTM cell strains to understand the role of miRNA in the pathogenesis of GC-OHT/GIG.

BIOINFORMATICS

The department is dedicated to understanding the complexity of biological processes and disease mechanisms in eye research. The department research uses high-throughput techniques such as next-generation sequencing and third-generation sequencing methods, and meta data from public resources. We have reliable infrastructure and framework comprising of LINUX and Windows based servers and desktop workstations, which allow us to integrate high through put data and study them at systems level. The department is highly interdisciplinary, at the interface of Biology, computational biology and Informatics. It also focuses on non-coding RNA expression and their regulatory role in eye diseases by integrating data from NGS and public, in close collaboration with wet lab scientists. It further provides to customize data analysis tailored to the needs of individual research projects across all the research groups.

Clinical Exome/Genome analysis pipeline for eye disease gene panel

Investigator : Dr. D. Bharanidharan
Co-investigators : Dr. P. Sundaresan, Dr. A. Vanniarajan
Research Scholar : K. Manojkumar
Funding : DBT-COE

Background

Recent advances in genomic technologies, particularly next generation sequencing (NGS) methods, have brought a paradigm shift in discovering eye disease-associated genetic variants from linkage and genome-wide association studies to NGS-based genome/exome studies. Whole genome sequencing (WGS) remains prohibitively expensive for most applications and requires concurrent development of bioinformatics approaches to expeditiously analyze the large data sets, whole exome sequencing (WES) is now made as a viable approach to uncover unknown etiology with a limited number of probands with eye disease. WES, focuses on only the protein-coding sequence of human genome, is become a powerful tool with many advantages in the research setting, and moreover is now being implemented into the clinical diagnostic arena. Spurred by NGS technologies, new efficient and well-designed bioinformatics tools emerged which are addressing different tasks in the downstream analysis of NGS data. Since combining these tools into an analysis pipeline greatly facilitates the interpretation of NGS results, an exome/genome sequencing pipeline is developed in this project that connects all necessary analysis steps into a unified application in the clinical settings for eye disease panel. The pipeline will support input data generated by the NGS Illumina platforms, handles correct execution of all integrated tools. It performs quality statistics on raw and processed reads, allows users to trim and filter sequence reads, and aligns the processed reads to a reference eye disease gene panel. The pipeline will deal single nucleotide variants (SNVs) and short InDels separately to improve the performance and to detect true positives. The integration of well-established tools and newly developed promising algorithms into a unified solution eases the analysis of next-generation exome/genome sequencing data.

Nevertheless, the identification of pathogenic variants amongst thousands to millions of genomic variants is a major challenge, and requires new strategies for variant filtering for diseases panel. Pathogenic variant prioritization using simple heuristic filtering approaches and functional implications of variants miss the true positives. The study was aimed to develop stringent filtering method and machine learning methods to prioritize pathogenic variants for eye diseases. We further wanted to focus the prioritization methods specific for Mendelian and complex eye diseases separately.

Results

Previously human whole exome data analysis pipeline was established based on our bench-marking results of GiaB (NA12878) using five different aligners and four variant callers were executed in all pairwise combinations (20 pipelines). Then the variant calling capabilities of the pipelines was examined by comparing their output with truth variant set for NA12878 exome. We reported several performance metrics with respect to F-score aimed to build an extensive benchmark for studying the performance of pipelines with up-to date well-known tools in detecting SNVs and InDels. Recently, the performance of pipelines was compared with new addition of GiaB call sets NA24385 and NA24632 and updated version of tools. Therefore, we repeated the benchmarking with the different performance matrices (Figure 1).

Based on the recent benchmarking, two separate modular pipelines (Figure 2) were developed using unix shell scripts and GUI was written using HTML. The merged pipelines would provide all necessary outputs as shown in the figure 1. The pipeline involves several steps to produce high-quality alignment files, and to predict definite variants. Initially, the quality of the raw reads obtained from SRA will be checked by FastQC and the low-quality reads, adapter contaminates will be trimmed by

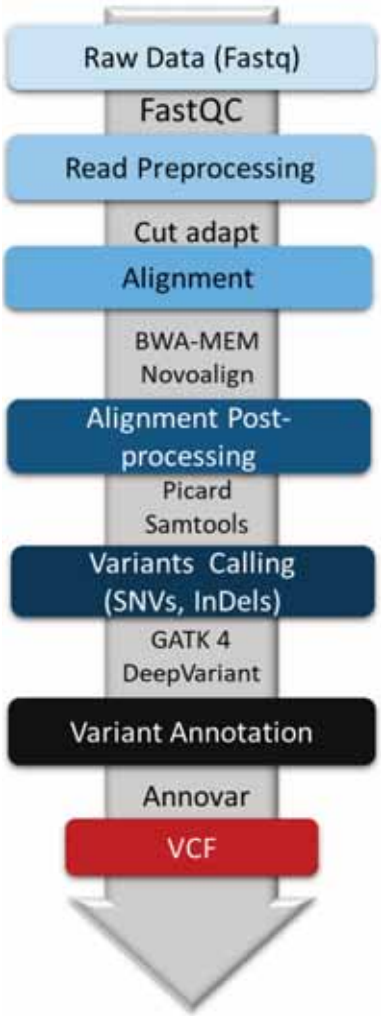


Figure 2. Exome/Genome Data Analysis Pipeline

Cutadapt. Alignment of the reads with the reference genome will be performed using BWA and Novoalign, PCR duplicates will be removed using PiCard Tools. This will be followed by read recalibration, based on target capture region , for error detection towards obtaining the base quality. Finally, SNVs and InDels

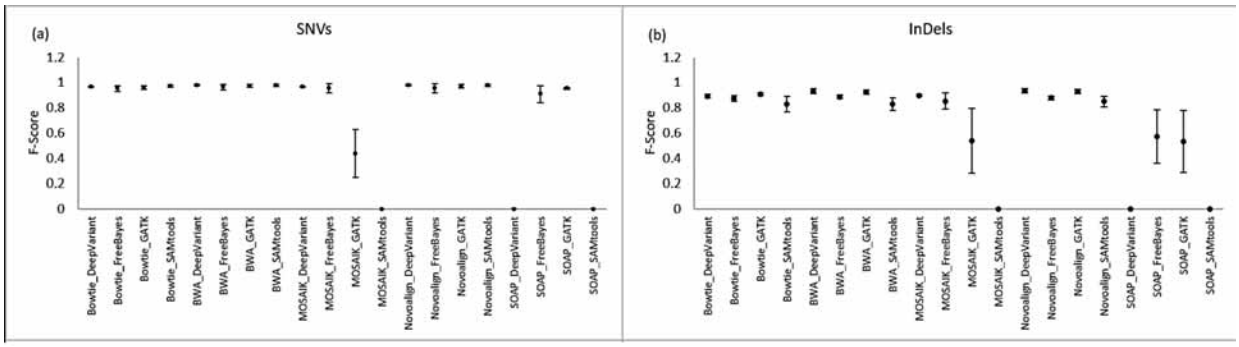


Figure1. Performance comparison of pipelines with respect to F-score on NA12878, NA24385, and NA24631. DeepVariant performed best invariably with all data sets. However, the aligners BWA and Novoalign both performed equally well on the new datasets compared with NA12878. The values and the error bars represent the average and standard deviation respectively of F-score obtained from all 3 datasets. Performance comparison of pipelines in SNVs and InDels detection for GRCh38 (a, b).

will be identified by DeepVariant and GATK. Annovar will be used to annotate the variants.

The main Menu of In-house pipeline. The Analyze menu has items such as Raw data, Reference genome, Quality of the reads and Annotation. The final annotated file will be given as .CSV file as the output also with the .VCF (variant calling file). In addition, the user can also download the automated script for analyzing the raw reads (Fig3). Similarly performance assessment of Whole Genome Data is still in process.

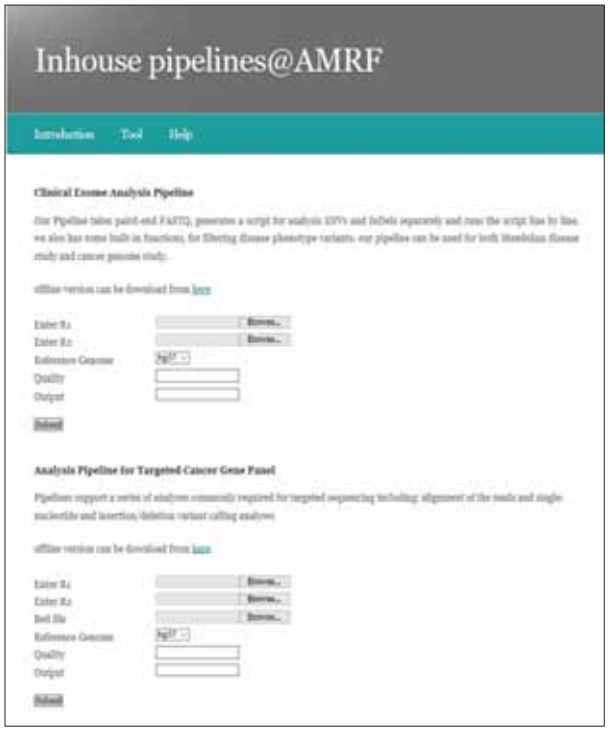


Figure 3. (Screen-shot of In-house pipeline at the host institute)

Heuristic variant filtering

In order to filter potential variants for prioritization, we have developed heuristic variant filtering scripts, which may differ based on the individual patients and laboratories. In common, variants with high minor allele frequency (MAF) equal to or greater than 1% in public databases will be removed. Several public databases include the 1000 Genomes Project (http://www.1000genomes.org/), ESP6500 (http://evs.gs.washington.edu/EVS/), dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), ExAC (http://exac.broadinstitute.org/) and GenomeAD (http://gnomad.broadinstitute.org) are now serve as a valuable resource for MAF estimations. MAF cutoff around 1% and 0.5% or less may be used for rare recessive disorders and a dominant or X-linked disorder respectively. Next, the remaining variants are filtered using the functional impact of the variant. The truncating variants (stop gain/loss, start loss, or frameshift), missense variants, canonical splice-site variants are considered first as protein-function altering variants, followed by silent and in-frame indels affecting protein-coding regions. Missense variants are further prioritized by their functional impact on the protein using in silico tools such as SIFT (http://sift.jcvi.org/) and Polyphen2 (http://genetics.bwh.harvard.edu/pph2/), and others. This prioritized set are further stratified whether those are present in the same gene, those are predicted to be the most deleterious, and those are residing in the genes that are implicated in the patient's phenotype. Subsequent variant analysis is differed in Trio sequencing compare to patient sequencing alone, where in mode of inheritance is applied. For example, homozygous or compound heterozygous variants will be retained for recessive mode of inheritance whereas heterozygous variants for dominant mode of inheritance. For de novo variants, Trio sequencing is highly recommended. On an average 1-2 de novo variants appears in a typical exome, which tend to be more deleterious than inherited variants, and are difficult to deduce from trio sequencing.

Variant Prioritization for eye disease gene panel

A support vector machine (SVM) learning method (Fig.4) was used to prioritize the variants based the eye diseases gene panel training set. The model was appropriately trained using manually curated missense genetic variant datasets for eye diseases gene panel consists of 528 genes, composed of 4381 pathogenic non-synonymous variations coming from HGMD and of 5694 neutral variations coming from 1000 Genomes Project. With following attributes SVM was trained using e1071 library in R. The SVM was

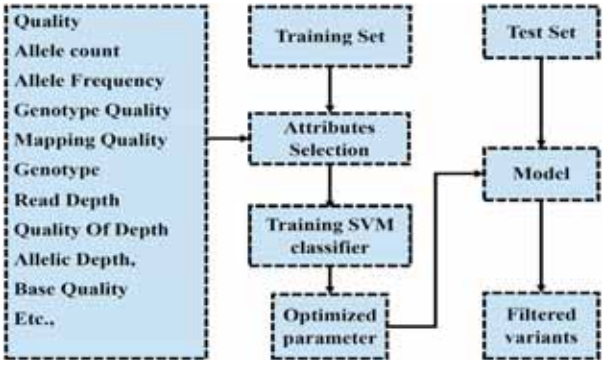


Figure 4. Schematic representation of SVM model

trained for SNVs and InDels separately.

Test Set: The exome data from patient with Leber congenital amaurosis (LCA) (SRR776598) was downloaded from SRA. The variants were detected through our automated pipeline. After heuristic filtering the SVM score was calculated to prioritize the variants. The top score variant of the gene NMNAT1

was identified, which was reported earlier as a LCA gene in the family Marni et al. Nat Genet, 44(9): 1040–1045.2012.

Webpage link to download the automated modular pipeline:

https://github.com/bharani-lab/WES-pipelines/

An *in silico* approach for identification of aberrant metabolic pathways in retinal angiogenesis towards early diagnosis and development of personalized medicine

Investigator : Dr. D. Bharanidharan
Postdoctoral Fellow : Dr. S. Umadevi

Funding : National Post-Doctoral Fellowship (NPDF) by Science & Engineering Research Board (PDF/2016/001448/LS)

Introduction

Retinal angiogenesis is found to be the leading cause of blindness in numerous clinical conditions such as diabetic retinopathy, retinal vein occlusion, age related macular dystrophy, macular edema and retinopathy of prematurity. Currently, retinal laser photocoagulation is the most effective treatment. However it destroys post mitotic retinal neurons and permanently affects visual function. Moreover, insufficient efficiency and resistance to VEGF-signaling blockade strategies (the prime target of antiangiogenic drug development) limit the overall success, necessitating the development of alternative mechanistically distinct strategies. Here, the hypothesis is that neovascularization in angiogenesis develops in individuals with unique metabolic switch. Hence, the analysis of altered metabolic pathways in individual patient may help to understand the disease status and could be an attractive customized therapeutic targets. The simplest way to identify individual's aberrance is to compare normal and disease sample data from the same individual. But it is often unavailable in clinical situation, especially at the time of treatment resistance in advanced stage of disease. To achieve this, the present work uses the concept of quantifying aberrance of individual sample's pathway by comparing with accumulated normal samples of other patients. To achieve this, the present work uses the concept of quantifying aberrance pathway of the individual sample by comparing with accumulated normal samples from other experiments. This work majorly focus to analyze the gene expression data obtained from microarray and RNA-Seq on series of steps, which consists of data processing, gene-level statistics, individualized pathway aberrance score (iPAS). As the preliminary of this meta-analysis of retinal angiogenesis diseases pathways, microarray gene expression data was analyzed and iPAS was calculated.

The Gene Set Enrichment Analysis (GSEA) was done using GESA preranked module of GenePattern tool. The pathways with p-value≤0.05 is the dysregulated pathway and normalized enrichment score (NES) based on fold change of genes also be considered for the analysis. In addition to GSEA, the pathway aberrance score was calculated using 4 different methods namely average z-score, Fisher's exact method, Euclidean distance and Mahalanobis distance. Further, the individualized pathway score (iPAS, using GSEA) of each sample provided dysregulated pathways and the clustering analysis identified the unique molecular aberrance.

Results and Conclusion

The gene expression level was evaluated in retinal angiogenesis and control using microarray data. Also the dysregulated pathways were identified based on the differential expression of certain genes in case samples. For this purpose, the processing of raw data has been done as stated in methodology. After normalization all the samples have the same distribution. The outlier samples were identified by PCA were excluded from the study. Removal of outlier of the samples formed the clear clustering of case-control samples.

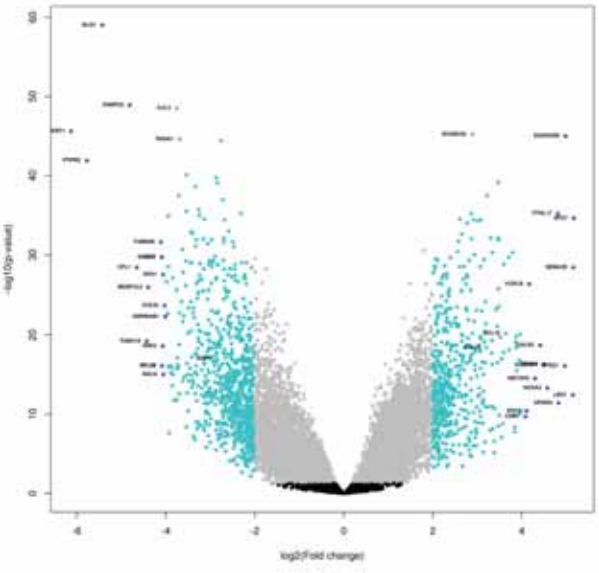


Fig.1 Volcano plot of gene expression of retinal angiogenesis samples

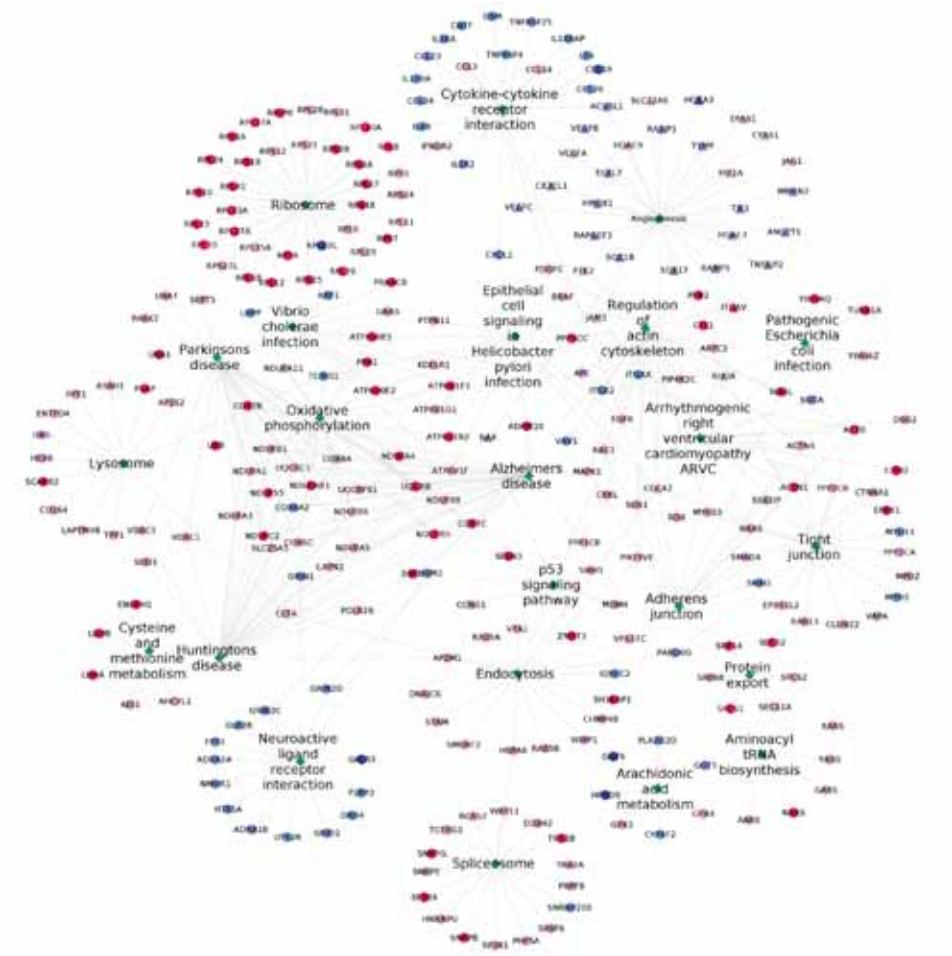


Fig : 2 Network of differntially expressed genes and dystregulated pathways in retinal angiogenesis

The gene expression profile between case and control samples is illustrated in figure 1,which represents the fold change of the genes in case samples. Seven genes FTHL17, SOX3, GALR3, CDR1, TPSG1, SPG7, LBX1 are highly expressing and 12 genes PTPRO, SH3KBP1, GLO1, PABPC3, CFL1, MORF4L2, LDHA, TUBA1A, FAM98B, RPS3A, RPL39, HUWE1 are down regulated with fold change≥4.

The gene set enrichment analysis reveals that, totally 22 pathways were dysregulated in case samples, namely 1.adherens junction, 2. alzheimers disease, 3. aminoacyl tRNA biosynthesis, 4. arachidonic acid metabolism, 5. arrhythmogenic right ventricular cardiomyopathy ARVC, 6. cysteine and methionine metabolism, 7. cytokine cytokine receptor interaction, 8. Endocytosis, 9. epithelial cell signaling in helicobacter pylori infection, 10. huntingtons disease, 11. Lysosome, 12. neuroactive ligand receptor interaction, 13. oxidative phosphorylation, 14. p53 signaling pathway, 15. parkinsons disease, 16. pathogenic escherichia coli infection, 17. protein export, 18. regulation of actin cytoskeleton, 19. ribosome, 20. spliceosome, 21. tight junction and 22. vibrio cholerae infection. The network of the DEGs and the associated dysregulated pathway is illustrated in figure 2. The different enrichment scores were calculated for the pathways associated with identified DEGs (figure 3). None of the pair of scoring methods provided the pathways with same ranking order. But each score is sensitive enough to identify clinically meaningful pathway pattern, and further study has to be done to set the threshold of the scores to detect the characteristics of the retinal angiogenesis.

The normalized enrichment score (NES) of the dysregulated pathways were calculated and the highly dysregulated is neuroactive ligand receptor interaction pathway. This pathway is part of the G protein coupled receptor signaling pathway and the dysregulation of this pathway to enhance tumor growth and promote angiogenesis. It was observed that, 20 significant pathways (except of the pathways cysteine and methionine metabolism and arrhythmogenic right ventricular cardiomyopathy arvc in the above list) are found both in analysis with

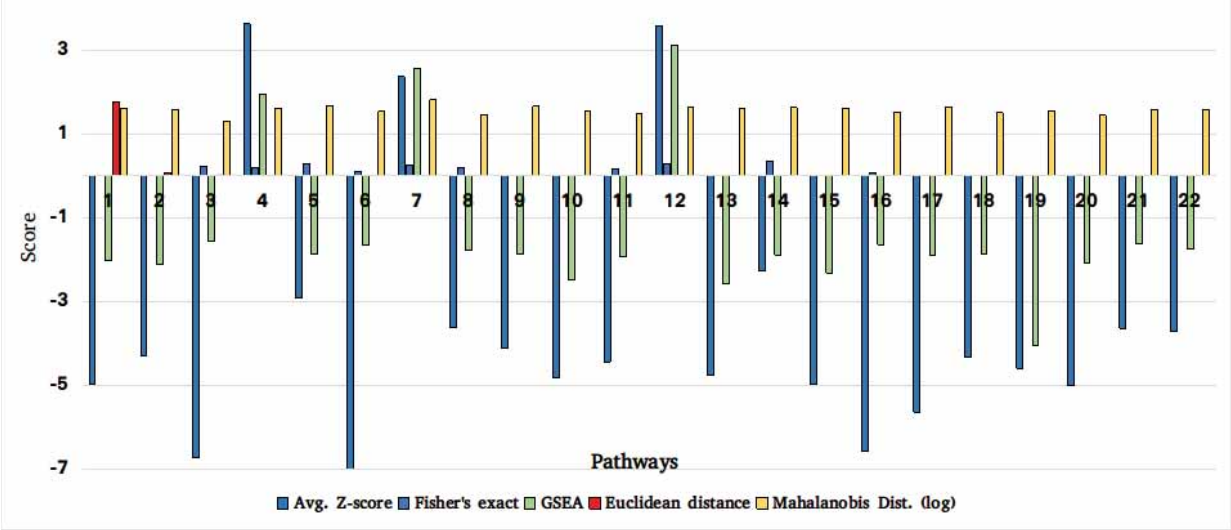


Fig.3 Aberrance scores of the dysregulated pathways in retinal angiogenesis

all the case samples and found in minimum of 10 samples in individual case analysis.

In summary, this meta-analysis highlighting the potential targets and altered metabolic pathways. In this cross platform meta-analysis, we have observed the samples of the same platform forms a cluster. This bias has not been nullified even after the normalization of the data. After the removal of outlier, the samples have been clustered into case, retina control and RPE/C control samples. Also the variation of gene expression in tissue specificity was observed.

Comparative genomics of *Pseudomonas aeruginosa* ocular isolates from keratitis patients with different clinical outcomes

Investigators : Dr. D. Bharanidharan,
Dr. M. Vidyarani,
Dr. Lalitha Prajna
Research Scholar : K. Kathirvel, T. Kannan
Funding : Aravind Eye Hospital

Background

Pseudomonas aeruginosa is a gram negative bacterium capable of causing a variety of life-threatening human infections. The metabolically versatile *P. aeruginosa* is an opportunistic pathogen of plants, animals, and humans and is ubiquitously distributed in soil and aquatic habitats[1]. Since most of the strains are highly virulent, *P. aeruginosa* ulcers are generally more difficult to treat and result in worse visual outcomes. In developing countries, ocular trauma remains a major risk factor for encountering ocular *P. aeruginosa* infections, whereas in the developed countries, it is frequently associated with contact lens wear. Since the bacterium is a common contaminant of moist surfaces such as a contact lens case, the exposure of the ocular surface to this bacterium can easily occur and bacterial adhesion is further aided by the hypoxia and trauma caused due to contact lens wear[2]. An array of virulence factors contribute to the pathogenicity of *P. aeruginosa*. Cell associated structures, including flagella, pili, fimbriae, and endotoxin (lipopolysaccharide), as well as extracellular products, such as proteases and exotoxins are associated with virulence, invasiveness, and colonization. Also, the clinical isolates of Pseudomonas often exhibit multiple resistance to antibiotics[3]. Multidrug resistance (MDR) is often related to the presence of specific efflux pumps and porins in *P. aeruginosa* strains[4]. Hence, to efficiently control *P. aeruginosa* infections, it is mandatory to understand the intrinsic and extrinsic virulence mechanisms of this bacterium. To better understand the infection, genome-wide identification of genetic features responsible for multiple virulence and multidrug-resistant mechanisms in keratitis *P.aeruginosa* isolates that impact on the patient outcomes is important. It is reported that, the analysis of five *P.aeruginosa* isolates from keratitis patients with different clinical outcome, wherein, the patients were grouped based on the corneal healing and who underwent surgery. All five isolates in the patient groups were differed in their antimicrobial susceptible profiles and the presence of T3SS virulence factors.

Results and Conclusion

Five *P. aeruginosa* ocular isolates (BK2-6) with different antimicrobial susceptibility and clinical outcomes were selected for sequencing. Two isolates (BK2 and BK6) were initially recovered from the

corneal scrapings of patients who subsequently responded to treatment and presented with a healed ulcer during follow-up. The other three isolates (BK3, BK4 and BK5) were grown from corneal buttons obtained during surgery from patients who did not respond to treatment. The isolates BK3 and BK6 were MDR strains. The whole genome sequence of the five isolates were compared and reported earlier. These genomes were compared with published genomes of *P. aeruginosa* ocular isolates[5]. A maximum-likelihood tree based on the core SNPs using Parsnp version 1.2 in Harvest Suite was plotted (Figure. 1A). The *Pseudomonas aeruginosa* complete genomes PAO1 and PA14 also included in phylogeny. The phylogenetic tree showed that the MDR strains BK3 and BK6 are closely related to the previously sequenced Indian *P. aeruginosa* keratitis strains (PA31, PA32, PA33, PA35, PA37 and PA82) while drug susceptible strains BK2, BK4 and BK5 are closely related to Australian strains (PA17, PA40, PA149, PA157 and PA175)[6]. Although the MDR isolates were closely related to each other in genome level, but presented with high degree of difference in clinical outcome, as similar as drug susceptible isolates.

genes were checked according to the Clusters of Orthologous Groups (COG) and it was identified that core genes were significantly enriched to class E (Amino acid transport and metabolism) and class T (Signal transduction mechanisms). Similarly, unique genes were enriched to class L (Replication, recombination and repair), class M (Cell wall/membrane/envelope biogenesis), class I (Lipid transport and metabolism) and class S (Function unknown). In comparison of susceptibility profile, MDR strains contains more genes in the classes of V (Defense mechanism), S (Function unknown), L (Replication, recombination and repair), K (Transcription) and U (Intracellular trafficking, secretion, and vesicular transport) than drug susceptible strains.

Totally, twenty one antibiotic resistance genes were commonly found in all five ocular strains, responsible for Aminoglycosides, Beta-lactam, Fosfomycin, Phenicol, Tetracyclin, Trimethoporim, Sulphonamide resistance. However, each genomes carry their unique resistance genes. In comparison of TypeIII secretion systems, MDR strains carries the exoU system and exoS in non-MDR strains.

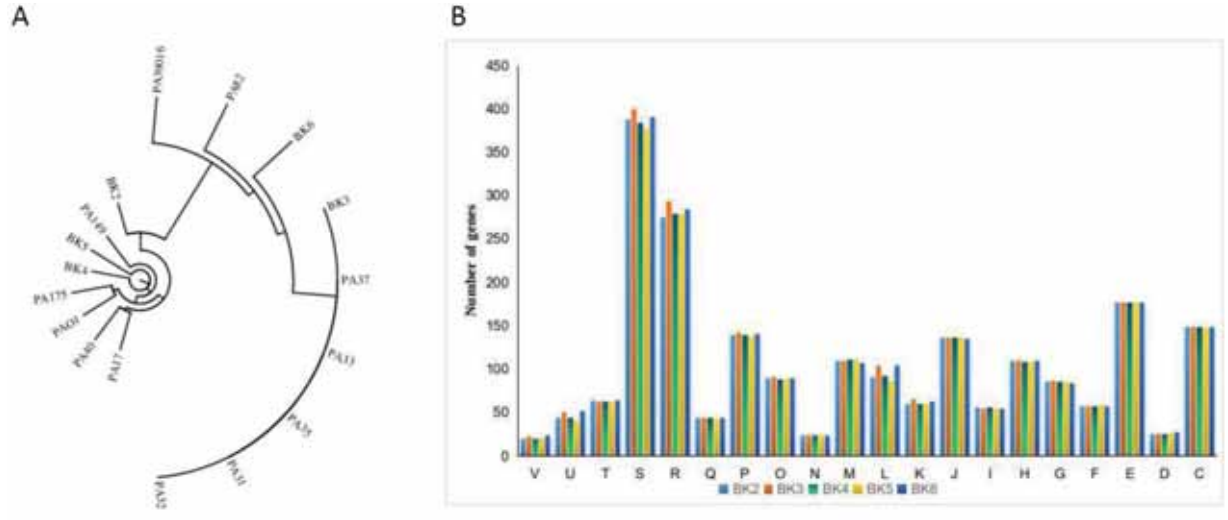


Figure 3.1. (A)Phylogenetic tree of P. aeruginosa isolates. (B)The distribution of genes according to the COG classification

The gene contents of all five P.aeruginosa ocular genomes were compared with PAO1 (Figure.1B), using Pan-Genome Analysis Pipeline (PGAP) to predict the core, accessory and unique genes which shared among five isolates. The size of the pangenome was 7530 genes, and there are 5106 genes predicted as core genes shared among five isolates. The two MDR strains BK3 and BK6 had more unique genes comparing with other isolates. Many of these unique genes are hypothetical and replication, recombination related genes. Further the core and unique

In conclusion, this study combined clinical data and whole genome analysis of ocular isolates of *P. aeruginosa* to link genetic basis of bacteria with different outcome of the patients. Though, the correlation between clinical outcome and genetic features of genomes were inconclusive, the isolates prefers either ExoU or ExoS for the infection, both the phenotype. Further, comparison of all the reported isolates from keratitis patients would help to identify the genetic features associated with virulence and drug resistance mechanisms.

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OCULAR MICROBIOLOGY

The department focuses on cellular and molecular basis of ocular infectious and inflammatory diseases that pose a major challenge to the community with a high morbidity rate. The thrust area of research is the elucidation of bacterial virulence and drug tolerance mechanisms, host-pathogen interactions and the genetic dissection of drug resistance in ocular pathogens. Experimental approaches to study host pathogen interactions involve *invitro* cell culture models and *exvivo* analysis of ocular tissue samples. Since inflammation is a leading cause of ocular morbidity and blindness worldwide, studies investigating the functional and molecular aspects of ocular inflammation could find applications in clinical management.

A study on Genotyping and phylogenetic analysis of newly emerging pathogen *Pythium insidiosum* causing corneal ulcer

Investigator details : Dr. Lalitha Prajna
Research Associate : Dr.R.Siva Ganesa Karthikeyan
Project Fellow : A.Selva Pandiyan

Introduction

P. insidiosum is the newly emerging ocular pathogens, which was reported previously in human systemic infection mainly in Southeast Asia and Australia. Pythium is classified in the Phylum Straminipila, Class-Oomycetes, Order-Pythialesand, Family-Pythiaceae. Majority of the *Pythium sp* belongs to the Oomcetes which are known to cause infection in plants among which *P. insidiosum* can cause a serious life threatening infectious condition in human and animals (horses, dogs, catsandcattles) known as pythiosis. Pythiosis is highly endemic in tropical and subtropical areas, majority of the human pythiosis are reported in Thailand. Pythiosis are very difficult to treat and surgical treatment involving the removal of infected organ is the ultimate option to prevent spreading of the infection. Corneal infections caused by *P. insidiosum* are very rare and majority of the reports have so far been primarily from Thailand. In recent decades newer cases of *P. insidiosum* keratitis had been reported in several countries, including Thailand, India, Australia, Haidi, New Zealand, Israel, Malaysia, Canada, and China. *P. insidiosum* infection diagnosis mainly relies on culture based identification, but it is difficult to grow and very time consuming. The molecular based diagnosis including PCR and sequencing are widely employed for the species identification and also to study molecular phylogenetic relationship. Cytochrome oxidase II (COXII) gene codes for the enzyme involved in the electron transport chain in mitochondria, it was found to be more variable than nuclear DNA and have conserved region which is less ambiguous compared to rDNA.The information about the variation in the clades causing keratitis is not well understood. The study focuses on the molecular identification of the *Pythium sp* using ITS regions and the clade identification by COXII gene sequencing

Results and conclusion

This was a study on genotyping of *Pythium insidiosum* in human keratitis, conducted at Aravind eye Hospital, Madurai, during the period from April 2018 to December 2018. Through the microbiological laboratory reports, fifteen cases of *Pythium sp* isolated from keratitis patients were selected. DNA was extracted followed by PCR and sequencing of the ITSand COX II gene. The Phylogentic tree was constructed by Neighbor-Joining method using MEGA X software for our clinical isolates as well as the reference sequences downloaded from Genbank database.

During the follow up, seven patients underwent therapeutic penetrating keratoplasty (TPK) and in three patients there was a reinfection of the corneal graft resulting in failure of the graft. There was no significant increase in the visual acuity observed in any of the patients. One patient did not respond to the medical treatment and the infection was very severe, resulting in the evisceration of the infected eye. Based on the rDNA and mtDNA sequences (ITS region and COX II, respectively) the Pythium insidiosum were classified into clade^{ATH}, clade^{BTH}, clade^{CTH}, outgroup (other species) and non typeable (NT). None of the ocular isolatesbelonged to the Clade^{ATH}. Five of the Pythium isolates were placed in BTH clade, eight in CTH clades, and two of the strains were non typeable. The two non typeable strains formed a separate cluster, indicating they maybe a genetically divergent group which is not reported previously. In conclusion, Clade B and Clade C were the predominant clades causing human keratitis; there were two non- type able lineage which needs further molecular evaluation.

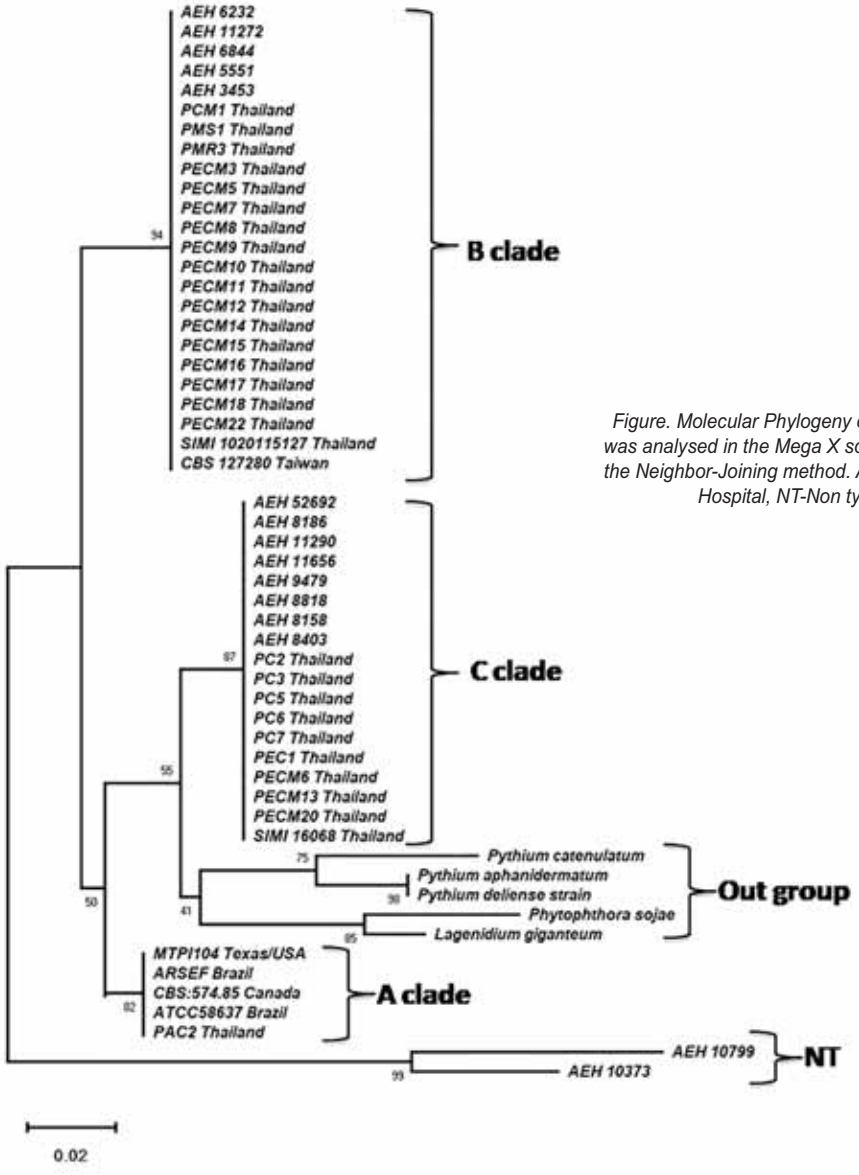


Figure. Molecular Phylogeny of the COXII gene was analysed in the Mega X software by applying the Neighbor-Joining method. AEH – Aravind Eye Hospital, NT-Non typeable.

Complete genome sequencing of Methicillin-resistant Staphylococcus aureus of ocular isolates using Oxford Nanopore Technology

Investigators : Dr. D. Bharanidharan, Dr. Lalitha Prajna

Research Associate : Rajapandian Siva Ganesa Karthikeyan

Project Fellow : O. Rudhra

Funding : AEH

Background

Methicillin-resistant Staphylococcus aureus (MRSA) is one of the most common causes of Bacterial keratitis infection, causing significant morbidity and mortality in developing countries like India. Thus, MRSA ocular infections are diseases of emerging importance, which need our attention for best and effective therapeutics. Identification of its genetic determinants of pathogenesis in ocular infections is hindered by the unavailability of its complete genome sequence. Several studies reported that Oxford Nanopore Technology (ONT) sequencing is a valuable tool for high-throughput complete bacterial genome sequencing. This study was carried out to identify the clinical characteristics of MRSA like antimicrobial resistant pattern, pathogenicity, host susceptibility and genomic variation among MRSA isolates which have been procured from patients with severe bacterial ocular infections using whole genome sequencing. Here, the results of a de novo assembly using Oxford nanopore long reads, complemented with Illumina short reads for polishing and comparative study of clinically significant antibiotic-resistant MRSA Keratitis isolates, were reported.

Results

Whole genome sequencing for five Staphylococcus aureus strains were performed using Oxford Nanopore MinIONsequencing according to the manufacturer's instructions in Genotypic technology, Bangalore. Table 1 presents comparisons of different types of assembly for MRSA genome. These data showed clearly that overall, Canu-Pilon provided consistently good quality assemblies both in terms of assembly statistics (N50, number of contigs) and assembly accuracy with respect to the reference sequence.The remaining MRSA genome was de novo assembled through the assembly pipeline Canu v0.1.8 (Koren et al., 2017), the resulting assembly was polished using Pilon (v1.20) (Walker et al., 2014). Mauve has been applied to align five MRSA genomes and to determine organization of genomes, gene duplication and inversion or translocation (Fig 1). Genotypic characteristics of MRSA isolates were tested by multilocus sequence typing using consensus sequences from each sample for seven housekeeping genes (arc, aroE, glp, gmk, pta, tpi,

Assembler	CPU hours	N50	Lrgest Contig	Total Contigs	Misassemblies
Raw					
Canu	48 h	79104	2830243	1	67
Unicycler	24 h	81174	2816660	1	68
Pilon polished 5x					
Canu	4 h	79570	2846786	1	67
Unicycler	4 h	81222	2816631	1	67
Nanopolish	28 h	90641	2841181	1	67
Hybrid assembly					
unicycler	24 h	81214	2773482	3	66
Spades	16 h	81214	988163	38	65
Pilon polished 5x					
Unicycler	4 h	81214	2773483	2	66
Spades	7 h	81213	988172	38	63
Illumina only assembly					
Spade	8h	73076	544277	187	42
unicycler	12h	73051	342104	38	42

Table 1: Summary of de novo and hybrid assembly results

Samples	No of contigs	Total l ength	Largest contig size	Genome Size (Mb)	GC content	No. of coding sequ ence	N50	No.of Contigs (with PEGs)	t RNAs	viru-lence gene
MRSA 1	1	283698 bp	28309	2.8	32.8	2719	79104	1	80	69
MRSA 2	1	290740 bp	287828	2.8	33	6613	94556	1	76	216
MRSA 3	7	302821 bp	252628	2.5	32.9	3125	2542191	6	80	68
MRSA 4	4	288381 bp	281688	2.8	33	6529	2816889	3	77	227
MRSA 5	1	289669 bp	288939	2.8	32.9	6836	2889309	4	71	222

Table 2: Overview of five MRSA genome annotation results

Sample name	MLST	virulence factor	Resistance gene
Mrsa 1	ST772	scn, hlgA, hlgB, hlgC,sec,sec3,sen,seo	aac(6')-aph(2''),aph(3')-III,blaZ,mph(C),dfrG,ant(6)-Ia,mecA,msr(A)
Mrsa 2	ST2066	hlgA,hlgB,hlgC,lukD,lukE,lukF-PV,luKS-PV,seb,sak	aac(6')-aph(2''),mecA
Mrsa 3	ST772	scn,hlgA,hlgB, hlgC,luKF-PV,lukS-PV,sec,sec3,sen,s	aac(6')-aph(2''),aph(3')-III,blaZ,mph(C),dfrG,mecA,msr(A),ant(6)-Ia
Mrsa 4	ST2066	hlgA,lukF-PV,luKS-PV,seb,sak,scn	Not found
MRSA5		hlgA,hlgB,hlgC,lukD,lukE,seb,sak,scn,aur,spIA,spIB	aac(6')-aph(2''),blaZ,mph(C),dfrG,mecA

Table 3: Multilocus sequence typing, Antimicrobial resistance gene and virulence factor

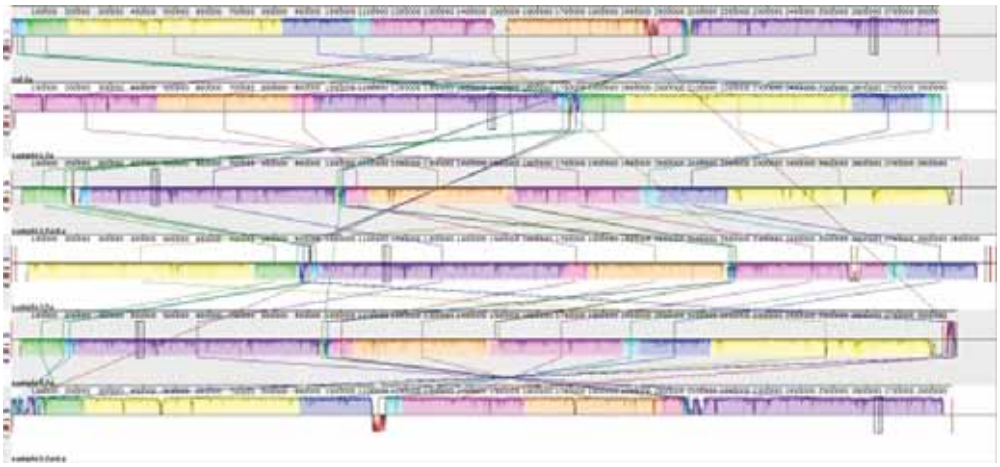


Fig1: A pictorial representation of five MRSA genome rearrangement generated by the Mauve viewer

yqiL) using standard programs available at (https://cge.cbs.dtu.dk/services/MLST/) to assign sequence types (ST). In silico multilocus sequence typing revealed 2 different sequence types ST 772 and ST 2066 among the four MRSA genome. MLST for sample five has yet to be confirmed. Four out of the five isolates possessed the mecA gene, thus classified as MRSA Additionally, some of the mecA positive isolates were found to harbor Panton-Valentine Leukocidin (pvl) virulence gene. Other antimicrobial and virulence genes possessed by all isolates are further described in table3.

Conclusion

Taken together, our data indicate that such long read sequencing data can be used to affordably sequence and assemble gigabase-sized MRSA genome. The complete genome determination using MinION reads complemented with illumina reads identified a significant number of genes encoding for pathogenesis. The availability of the complete genome of MRSA will facilitate comparative genomic analysis of ocular MRSA species, including studies of its virulence mechanisms and the development of treatment strategies for severe keratitis.

CONFERENCES ATTENDED

4th Asia-Pacific Glaucoma Congress (APGC)

Bussan, South Korea, 13-15 April 2018.

Dr. P. Sundaresan delivered a talk on Current trends in molecular genetics of Primary Open Angle Glaucoma (POAG) and Primary Angle Closure Glaucoma (PACG). After the meeting, he visited Department of Ophthalmology, Seoul National University (SNU), Seoul where he delivered a talk on Genetics of Diabetic Retinopathy and met Dr. Hyeong Gon Yu, Prof. Ki Ho Park and Dr. Sung Wook Park.

In addition, Dr.P.Sundaresan visited SNU Bundang Hospital and delivered a talk on Mitoscriptome Gene Expression Signature for Diabetic Retinopathy Using Cadaver Eyes

Annual meeting of Association for Research in Vision and Opthalmology (ARVO) 2018

Honolulu, Hawaii, USA, April 29 - May 3

Prof. K. Dharmalingam, Dr.P. Sundaresan, Dr. S. Senthilkumari, Dr. J.Jeya Maheshwari, S. Yogapriya from AMRF and Dr. Ashok Vardhan from AEH attended the meeting.

Poster presentations:

- Targeted sequencing to identify the candidate gene in two south Indian POAG families - Dr. P. Sundaresan Enhanced aqueous outflow facility in human eyes by sb772077b, a new Rho kinase inhibitor – Dr. S. Senthilkumari
- Age related cell loss in Human Trabecular Meshwork is associated with loss of stem-like cells in the Schwalbe's line region - S.Yogapriya
- Analysis of fluid phase complement system activation in diabetic retinopathy - Dr.J.Jeya Maheshwari
- How many Cataract surgeries are needed in a given population? - Dr.Ashok Vardhan

Ms.Yogapriya visited labs in Center for Disease control- Atlanta, Stony Brook University and New York University- New York.

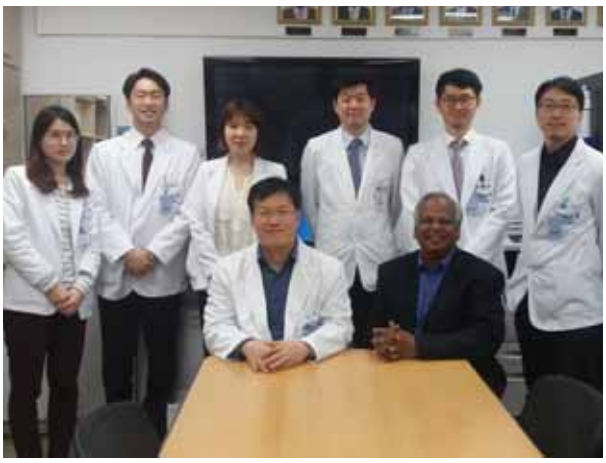
25th ARVO-India – Indian Eye research Group meeting

Hyderabad, July 27-29, 2018

Faculty members and research scholars from AMRF actively participated in the Silver Jubilee meeting of the Indian Eye Research Group (IERG) and ARVO India chapter held at Hyderabad.

Dr. P. Namperumalsamy was honoured at inaugural ceremony and he delivered a talk about his association with IERG over the past 25 years. Prof. K. Dharmalingam chaired Prof. D. Balasubramanian Oration lecture. Dr. P. Sundaresan chaired the session on Molecular mechanisms in eye diseases. Dr. Lalitha Prajna chaired the session on combating ocular infections. The following faculty members gave lectures during the pre-conference workshop on themes related to their area of research: Dr. C. Gowripriya on ocular stem cells and regenerative medicine, Dr. S. Senthilkumari on cell culture models in ocular pharmacology research, Dr. D.Bharanidharan on microRNAs and ocular infections, Dr. Lalitha Prajna on proteomics approach to understand mycotic keratitis and R. D. Thulasiraj on IOL initiatives in India.

Dr. P. Sundaresan visited Seoul National University, Seoul



Dr. Jeya Maheswari, Dr. K. Dharmalingam, Dr. Senthil Kumari, Dr. P. Sundaresan, S. Yogapriya at ARVO meeting, Honolulu, Hawali

Poster presentations

1. P.Saranya : Presence of Stem cells in the central region of the human anterior lens epithelium
2. S.Yogapriya : Age related reduction of stem cells in native human trabecular meshwork cells
3. K.Lavanya : Association of hsa-mir-143-3p in the regulation of cornea epithelial stem cells
4. T.Shanthini : RB1 promoter methylation analysis in Retinoblastoma patients
5. V.Lakshmi Prabha : Comparative analysis of the exoproteome of Aspergillus flavus spores during the early stages of growth: saprophyte versus corneal isolate
6. R. Kadarkarai Raj : Molecular genetics of abca4 gene in stargardt Disease
7. T.Madhumithra : Differential expression of genes related to steroid responsiveness in human trabecular meshwork cells
8. Dr.S.Uma Devi : Metadata analysis towards the identification of dysregulated metabolic pathways in neovascular age related macular degeneration
9. Dr. O.G. Ramprasad : Exploration of a novel chemical cross-linker For the treatment of Keratoconus

S. Yogapriya and K Lavanya, Stem cell Biology lab were selected for the best poster award. V. Lakshmi Prabha, V.Nivetha and T.Madhumithra won second prize in the quiz competition. K.Lavanya, P.Saranya, T.Shanthini, R.Kadarkarai Raj, S.Umadevi and T.Madhumithra were selected for travel grants.

28th Annual conference of Glaucoma Society of India

Thiruvananthapuram, September 14-16, 2018

- Dr. Roopam : Update on genetics of angle closure glaucoma

Dr. P. Sundaresan and Ms. Roopam at 28th Annual conference of Glaucoma Society of India, Thiruvanthapuram.

- Dr. P.Sundaresan : Current trends in molecular genetics of Primary Open Angle Glaucoma and Normal Tension Glaucoma.

Genomics- From Disease Prevention to Treatment Population, Medicine and Society

Haifa, Israel, 2-5 October 2018

Dr.P.Sundaresan delivered an invited talk on Molecular and prenatal diagnosis for some of the inherited eye diseases and gene discoveries and interacted with many scientists, collaborators and genetic counsellors.

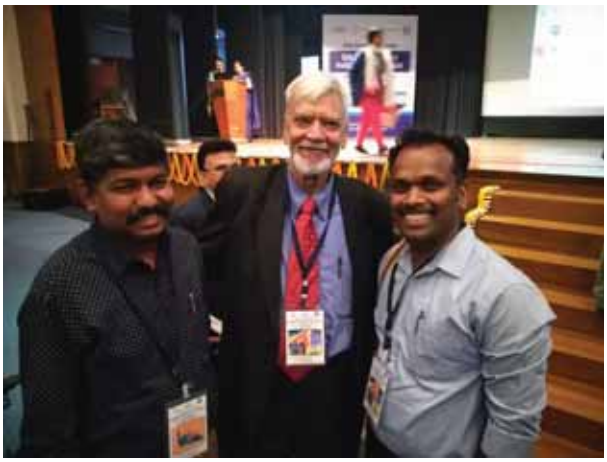
7th annual conference of the Society of Mitochondrial Research and Medicine (SMRM) - 2018

Lucknow, November 28-30, 2018

P. Gowri from the Department of Genetics, AMRF attended the conference and presented her research work as a poster titled Leber's Hereditary Optic Neuropathy: Relative frequency of primary mutations in South Indian population.

AMRF Team at IERG meeting, Hyderabad

Ms. P. Gowri at SMRM meeting, Lucknow



Dr. Vanniarajan at Indian Society of Human Genetics meeting, Kolkatta

Workshop on 'Analysis of Next Generation Sequencing Data"

Institute of Bioinformatics and Applied Biotechnology, Bangalore, November 26 -30, 2018

Ms. A.S. Sriee Viswarubhiny, Research Scholar, Department of Genetics, AMRF attended the workshop on 'Analysis of Next Generation Sequencing Data' organized by EMBL-Europian Bioinformatics Institute-IBAB.

25th Indian Society of Chemists and Biologists (ISCB) International conference

Lucknow, January 12-14, 2019

Dr. O.G.Ramprasad, Scientist, AMRF participated in the conference organized by the scientists of Central Drug Research Institute, Lucknow, held between 12th and 14th January, 2019. He gave an oral presentation about his research work entitled, "Exploration of a novel chemical cross-linker for the treatment of keratoconus".

Annual Meeting of Indian Society of Human Genetics 2019

Kolkatta, January 30- Feb 1, 2019

Dr. A. Vanniarajan, Scientist, AMRF attended the meeting and interacted with both national and international delegates. He also visited the Core Genomics Facility at National Institute of BioMedical Genomics, Kolkata.

Aqua-Terr Golden Jubilee International conference on Genome Biology and Host Defence: Bacteria to Mammals

Madurai Kamaraj University, February 27 to March 1, 2019

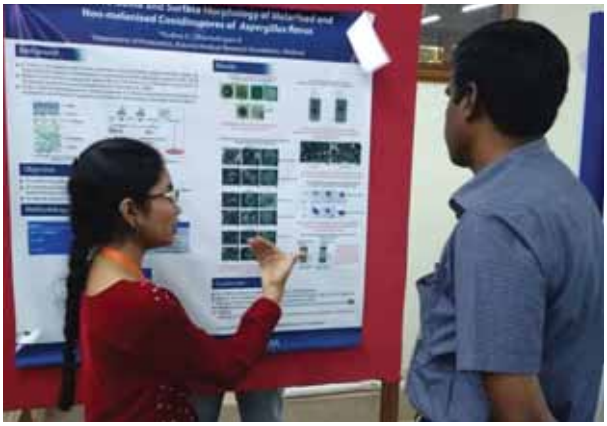
In the meeting Prof.VR.Muthukkaruppan introduced Max D.Cooper, Department of Pathology and Laboratory Medicine, University school of Medicine; Emory University, Atlanta, USA. Prof.K.Dharmalingam and Dr.P.Sundaresan were invited to deliver a talk in the session of "Genome Biology and Proteomics of Eye Diseases". Two research scholars were presented their work as poster presentation. Mr.Jeyaprakash, Ms.Swathi and O.G. Ramprasad were attended the meeting.

- Prof.K.Dharmalingam : Proteoforms in Eye Diseases
- Dr. P.Sundaresan : Update on the Molecular Genetics of Eye Diseases

Poster presentations:

- R.Karthikeyan : Expression profiling of serum miRNA in Diabetic Retinopathy patients
- O.Ruthra : Surface proteome and surface morphology of melanised and non-melanised conidiophores of Aspergillus flavus

O.Ruthra at Madurai Kamaraj University meeting



CONFERENCES / WORKSHOPS CONDUCTED

16th Research Advisory Committee Meeting

March 24, 2018

During the meeting, faculty members of AMRF presented their work and received feedback. Research scholars presented their findings as posters and got an opportunity to interact with the committee members. Best poster was selected for Prof.VR.Muthukkaruppan Endowment award for the year 2018.

Workshop on "Vision Science to Patient Care- A Translational Approach"

AMRF, 25th August, 2018

The workshop was organised on the occasion of observing Dr. V's birth centenary.

Dr. Kumaramanickavel - The importance of academic research in private R&D institutions and ophthalmic genetic counselling.

Dr. Banusri Velpandian - An overview about the various funding schemes available in BIRAC, DBT, India for the basic science researchers.

Dr. Velpandian - The innovations in basic research with excellent examples and provided the various aspects of intellectual property rights in innovations.

Prof. VR. Mutukkaruppan - The art of carrying out basic research in private institutions.

Dr. P. Sundaresan - The importance of networking in basic research and skills.

Dr. Bharanidharan - The importance of statistics in biomedical research.

Poster session during RAC meeting

Dr. Rathinam - The importance of storage and transportation of the aqueous tap for the timely diagnosis and treatment of ocular infections.

Dr.Lalitha- The importance and intricacies involved in collection of aqueous humor, corneal scrapings and tears from infected patients for basic research.

Dr. Piyush Kohli - The gaps between clinicians and scientists in sample collection for basic research and further insisted the importance of understanding each other's need with good communication.

Dr. Vijayalakshmi - The ways of collecting trabecular meshwork and iris tissue samples for proteomics research.

16th Research Advisory Committee Meeting

Participants of the workshop on "Vision Science to Patient Care - A Translational Approach"

Participants of AMRF - Dartmouth Education and Research Conference

AMRF – Dartmouth Education and Research Conference

AMRF, November 28-29, 2018

The two-day conference was organised by Prof. K. Dharmalingam, Director-Research, and coordinated by Dr. D. Bharanidharan, Scientist-Bioinformatics, AMRF and Ms. Dawn E. Carey, Partner Relationship Manager, The Dartmouth Institute for Health Policy and Clinical Practice.

Students from Dartmouth College and AMRF research scholars presented research proposals on various eye diseases. Research work at AMRF was presented as posters. An update on the research programmes at AMRF and the facilities available were explained to Dartmouth during the lab visits.

Conference on "Advances in Clinical Genomics"

AMRF, 9th February 2019

The conference was conducted as part of the DBT Centre of Excellence programme on Translational Genomics of Paediatric Eye Diseases. AMRF scientists - Dr. P. Sundaresan, Dr. D. Bharanidharan and Dr. A.Vanniarajan organised the conference.

The conference included talks given by speakers from leading institutes in Bangalore and Chennai along with five speakers from AMRF and AEH. A wide spectrum of topics including gene discovery, gene therapy, data analysis, functional validation, clinical management was discussed in detail. A total of 60 participants including faculty, research scholars and post graduate students from different institutions attended the meeting.

- Dr. Rajani Battu, Aster CMI Hospital, Bangalore
  Inherited Retinal Diseases
- Dr. Usha Kim, AEH, Madurai
  Thrust for Quest
- Dr. P. Sundaresan, AMRF, Madurai
  Discovery of Genes causing Blindness
- Dr. Kshitish K Acharya, Institute of Bioinformatics and Applied Biotechnology, Bangalore
  Integrated approaches for efficient biomarker identification

Participants of Conference on "Advances in Clinical Genomics"

- Dr. Vamsi Veeramachaneni,Strand Life Sciences, Bangalore
  Strandomics: a platform for clinical genomics data interpretation and reporting
- Dr. A.Vanniarajan, AMRF, Madurai
  Looking beyond RB1: Comprehensive Molecular Analysis of Retinoblastoma
- Dr. S. Kirankumar, SRM Institute of Science and Technology, Chennai
  A Zebrafish model for the characterization of VHL disease
- Dr. O.G. Ram Prasad, AMRF, Madurai
  Genome wide profiling of miRNA and their functional analysis in Diabetic Retinopathy
- Dr. D. Bharanidharan, AMRF, Madurai
  Variant Prioritization in Mendelian and complex eye diseases from whole exome sequencing

Award

Ph.D awarded

Mr.Bibhuti Ballav Saikia, Department of Molecular genetics.

Thesis : Mitochondrial Genes Involvement in Leber's Hereditary Optic Neuropathy (LHON).

Guide : Dr.P.Sundaresan.

Ms.Roopam, Department of Molecular genetics.

Thesis : Molecular Genetics and Cytokine Profiling in South Indian Patients with Primary Angle Closure Glaucoma.

Guide : Dr.P.Sundaresan.

Ms. V.Nithya, Department of Microbiology

Thesis : Genotypic characterization and analysis of virulence factors in Methicillin resistant Staphylococcus aureus causing ocular infections

Guide : Dr.SR.Rathinam.

Vocational Excellence Award

Dr.P.Sundaresan received Vocational Excellence Award from Rotary Club of Madurai on 8th June 2018.

Dr. P. Sundaresan receiving Vocational Excellence Award

Annual Meeting of the Indian Eye Research Group

Hyderabad, July 27-29, 2018

Best Poster Award

K.Lavanya, Department of Stem Cell Biology
- Association of hsa-mir-143-3p in the regulation of corneal epithelial stem cells

S. Yogapriya, Department of Stem Cell Biology
- Age related reduction of stem cells in native human trabecular meshwork cells

K. Lavanya receiving best poster award from Prof. Justine Smith

S. Yogapriya receiving best poster award from Prof. Justine Smith

Second Prize in Iquest

V.Nivetha, V. Lakshmi Prabha & T. Madhumithra won second prize in quiz programme

Received trophy from Prof.Neeru Gupta, University of Toronto, Canada

Prof. VR.Muthukkaruppan Endowment Award

Students and colleagues of Prof.VR.Muthukkaruppan, Advisor, Aravind Medical Research Foundation created an Endowment in his name in 2014 out of which an award will be given to the best researcher at the Institute every year. The award is given based on the scientific merit of abstracts and presentation by the research scholars. This award carries a certificate and cash prize of Rs.25,000/-

Prof. VR.Muthukkaruppan Endowment award 2018 was given to Ms. K.Lavanya, Department of Stem Cell Biology for her outstanding research work on Molecular regulators associated with the maintenance of corneal epithelial stem cells.

GUEST LECTURES DELIVERED BY VISITING SCIENTISTS

Dr. Satish Kumar, Chief Scientist & Group Leader, Centre for Cellular and Molecular Biology, Hyderabad.

Topic: Mouse Wdr13 gene: One mutation, multiple phenotypes.

5th June 2018

Prof. Patric Turowski, Professor of Vascular Signalling, Faculty of Brain Sciences, UCL Institute of Ophthalmology London.

Topic: Mechanism of retinal leakage in the retina.

22nd January 2019

Dr. Gopalakrishna Ramaswamy, Founder and CEO, Theracues Innovations Pvt Ltd., Bangalore

Topic: Application of nanoString technologies for translational research; theraCUES perspectives

30th January 2019

Dr. Venkatachalam Udhayakumar
Senior Biomedical Research Scientist, Malaria Branch, CDC, Atlanta, USA

Topic: Application of a multiplex bead assay for multi disease surveillance and malaria elimination

26th February 2019

Dr.Thuy Doan, Assistant Professor of Ophthalmology, (Uveitis specialist, and uses genomic technologies for pathogen discovery), F I Proctor Foundation, UCSF, USA.

Topic: Next generation sequencing for Infectious diseases.

11th October 2018.

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- *"SB772077B, A New Rho Kinase Inhibitor Enhances Aqueous Humour Outflow Facility in Human Eyes"*

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- *"Identification of the proteoforms of surface localized Rod A of Aspergillus flavus and determination of the mechanism of proteoform generation"*

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Ravindran RD, Sundaresan P, Tiruvengada Krishnan, Praveen Vashist, Maraini G, Saravanan V, Usha Chakravarthy, Liam Smeeth, Dorothea Nitsch, Ian S Young, Astrid E Fletche

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Sara SH, Prajna NV, Senthilkumari S.

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Kannan Thirumalmuthu, Bharanidharan Devarajan, Lalitha Prajna and Vidyarani Mohankumar.

- *Mechanisms of Fluoroquinolone and Aminoglycoside Resistance in Keratitis Associated Pseudomonas aeruginosa*

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Aloysius Abraham, Kannan Thirumalairaj, Namrata Gaikwad, Veerappan Muthukkaruppan, Alia G.Reddy, Kumarasamy Thangaraj, Usha kim, Ayyasamy Vanniarajan

- *" Retinoblastoma discordance in families with twins"*

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Book Chapters

- *Dharmalingam K. Immunology of the eye. In: Prajna NV, editor. Peyman's principles & practice of ophthalmology. 2nd ed. 2 Vols. New Delhi: Jaypee Brothers Medical Publishers; 2019. p. 163-174*
- *Dharmaraj S, Verma A, Sundaresan P, Kannabiran C. Leber congenital amaurosis in Asia. In: Prakash G, Iwata T, editors. Advances in vision research: genetic eye research in Asia and the Pacific. Vol II. Singapore: Springer Nature; 2019. p. 191-231.*
- *Usha Kim, Thirumalairaj K, Aloysius Abraham, Shanthi R, Bharanidharan D, Muthukkaruppa VR, Vanniarajan A. Genetics of retinoblastoma: basic research and clinical applications. In: Prakash G, Iwata T, editors. Advances in vision research: genetic eye research in Asia and the Pacific. Vol II. Singapore: Springer Nature; 2019. p. 313-321.*
- *Prakadeeswari G, Haripriya A, Banushree R, Sundaresan P. Genetics of exfoliation syndrome in Asians. In: Prakash G, Iwata T, editors. Advances in vision research: genetic eye research in Asia and the Pacific. Vol II. Singapore: Springer Nature; 2019. p. 381-391.*
- *Kim R, Krishnadas R, Dharmalingam K, Jeya Maheshwari J. Proteomics of neurodegenerative disorders of the eye. In: Prakash G, Iwata T, editors. Advances in vision research: genetic eye research in Asia and the Pacific. Vol II. Singapore: Springer Nature; 2019. p. 393-402.*
- *Bharanidharan D, Vanniarajan A, Sundaresan P. Genomic approaches to eye diseases: an Asian perspective. In: Prakash G, Iwata T, editors. Advances in vision research: genetic eye research in Asia and the Pacific. Vol II. Singapore: Springer Nature; 2019. p. 403-415.*

ONGOING RESEARCH PROJECTS

No	Projects	Funded by	Investigators	Research Scholar
PROTEOMICS				
1.	Pathogenic Aspergillus: Interaction with Innate Immune cells (Indo-French Collaborative project)	CEFIPRA	Dr. Lalitha Prajna Dr.J.Jeya Maheshwari Dr. K.Dharmalingam Dr. Rabbind Singh	V. Lakshmi Prabha
2.	Study on “Human mycotic keratitis”	AMRF & AEH	Dr. N.Venkatesh Prajna Dr. K. Dharmalingam Dr. Lalitha Prajna Dr. Chitra Thangavel Dr. J.Jeya Maheshwari Dr. O.G.Ramprasad Dr.Rabbind Singh	A. Divya K. Sandhya Aswani Krishna
3.	Prospective Multicenter discovery and validation of diagnostic circulating and urinary biomarkers and development of sensor(s) to detect sight threatening diabetic retinopathy - Biomarker and Biosensor study in UK and India (Indo-UK collaborative project)	Research Councils UK	Dr. K. Dharmalingam Dr. Kim Dr. J.Jeya Maheshwari	V. Nivetha Guhan Ramaraj
4.	Proteome profiling of serum microparticles in diabetes and diabetic retinopathy patients: Towards identification and validation of predictive biomarkers	Department of Health Research (DHR)	Dr. J.Jeya Maheshwari Dr. K.Dharmalingam Dr. R.Kim	P. Vignesh
5.	Functional analysis of circulating microRNAs and their regulatory role in Diabetic Retinopathy	SERB	Dr. O.G.Ramprasad Dr. K.Dharmalingam Dr. Kim Ramasamy Dr. Bharanidharan	Evangeline Ann Daniel R. Karthikeyan
6.	Novel chemical cross-linking of the cornea for the treatment of keratoconus	EPSRC, UK and Aurolab	Dr. Venkatesh Prajna Prof. Rachel Williams Dr. O.G. Ramprasad Dr. Atikah Haneef Prof. K. Dharmalingam Prof. Colin Willoughby Dr. Naveen Radhakrishnan Karpagam Kannan	T.R. Divya A. Divya
MOLECULAR GENETICS				
7.	Molecular genetics of macular corneal dystrophy (MCD) in Indian population	DST INSPIRE Fellowship	Dr. P.Sundaresan Dr. N.Venkatesh Prajna	M. Durga
8.	Genetics of Retinal Dystrophies	Aravind Eye care system	Dr. P.Sundaresan, Dr. Pankaja Dhoble Dr. K.C.Lavanya	R. Kadarkarai Raj

9.	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
10.	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 Prof. VR.Muthukkaruppan Dr. D.Bharanidharan	A.Aloysius Abraham
11.	Understanding the molecular mechanisms of chemoresistance in retinoblastoma	CSIR-NET	Dr. A.Vanniarajan	T.S.Balaji
12.	Molecular characterization of tumor progression in retinoblastoma	DST INSPIRE Fellowship	Dr. A.Vanniarajan	T.Shanthini
13.	Identification and validation of deregulated cancer pathways in retinoblastoma	SERB	Dr. A. Vanniarajan Dr. Usha Kim Dr. D. Bharanidharan Dr. R. Shanthi	Anindita Rao
14.	Translational Genomics of Ocular Cancers	Aravind Eye Foundation	Dr. Usha Kim Dr. A. Vanniarajan Dr. D. Bharanidharan Dr. R. Shanthi Dr. VR. Muthukkaruppan	K.Saraswathi
15.	COE LEAD: Translational Genomics of paediatric eye diseases	DBT	Dr. P. Sundaresan Dr. A. Vanniarajan Dr. D. Bharanidharan Dr. P. Vijayalakshmi Dr. R. Kim Dr. Usha Kim	Dr. R.C. Vignesh Gowri Poigaialwar A.S.Sriee Viswarubhiny Susmita Chowdhury
16.	COE PR-I: Molecular analysis of mitochondrial diseases with ophthalmic manifestations	DBT	Dr. P. Sundaresan Dr. A. Vanniarajan Dr. P. Vijayalakshmi	A. Aloysius Abraham
17.	COE PR-II: Epigenetic mechanisms underlying tumor progression in retinoblastoma	DBT	Dr. A. Vanniarajan Dr. D. Bharanidharan Dr. VR. Muthukkaruppan Dr. Usha Kim	K. Jeya Prakash A. Mohamed Hameed Aslam
18.	COE PR-III: Functional validation of novel candidate genes using alternate model	DBT	Dr. P. Sundaresan Dr. A. Vanniarajan	C. Prakash
19.	COE R&D: Computational methods for whole exome/genome sequencing of paediatric eye diseases	DBT	Dr. D. Bharanidharan Dr. P. Sundaresan Dr. A. Vanniarajan	K. Manoj kumar
IMMUNOLOGY AND STEM CELL BIOLOGY				
20.	Limbal miRNAs and their potential targets associated with the maintenance of stemness	DBT	Dr .C. Gowri Priya Dr. Venkatesh Prajna Prof. VR.Muthukkaruppan Dr. D. Bharanidharan	K. Lavanya (CSIR-SRF from May 2018)

21.	Characterization and Functional Evaluation of Trabecular Meshwork Stem Cells in Glaucoma Pathogenesis	SERB	Dr. C. Gowri Priya Dr. S. Senthilkumari Dr. Neethu Mohan Dr. SR.Krishnadas Prof. VR. Muthukkaruppan	S. Yogapriya (Lady Tata Memorial Fellow) R. Iswarya
OCULAR PHARMACOLOGY				
22.	Role of miRNA in the regulation of Glucocorticoid Receptor (GR) signalling and Development of New therapeutics for Steroid-induced glaucoma	Wellcome-DBT /India Alliance Intermediate Fellowship (2017-2022)	Dr. S. Senthilkumari Dr. C. Gowri Priya Dr. D. Bharanidharan Dr. R. Sharmila	Dr. R.Haribalganesh K. Kathirvel T. Madhu Mithrar
BIOINFORMATICS				
23.	Diagnostic Markers for Ocular Tuberculosis	DBT	Dr. D.Bharanidharan Dr. SR.Rathinam Dr. Lalitha Prajna Dr. M. Vidyarani	Swathi Chadalawada
24.	Computational Methods For Whole Exome/Genome Sequencing Of Paediatric Eye Diseases	DBT-COE, R&D	Dr. D.Bharanidharan Dr. A.Vanniarajan Dr. P.Sundaresan	K. Manojkumar
25.	Comparative genomics of Methicillin-Resistant Staphylococcus aureus (MRSA) and Pseudomonas aeruginosa ocular isolates from keratitis patients with different clinical outcomes	AEH	Dr. D.Bharanidharan Dr. M. Vidyarani Dr. Lalitha Prajna	K. Kathirvel O.Rudhra
26.	An in-silico approach for identification of aberrant metabolic pathways in retinal angiogenesis towards early diagnosis and development of personalized medicine	SERB-NPDF	Dr. D.Bharanidharan	Dr. S. Umadevi
MICROBIOLOGY				
27.	Genotyping and phylogenetic analysis of Pythium insidiosum causing human corneal ulcer	AEH	Dr. Lalitha Prajna	A. Selva Pandiyan
28.	Identification and evaluation of genetic relationship based on Multilocus Sequence Analysis in Nocardia sp causing human ocular infection	AEH	Dr. Lalitha Prajna	A. Selva Pandiyan
29.	Genotyping and Phylogenetic analysis of Acanthamoeba sp using the 18S Ribosomal DNA region (JDP 1/2) from Acanthamoeba Keratitis patients	AEH	Dr. Lalitha Prajna	A. Selva Pandiyan