Aravind Medical Research Foundation

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

MISSION

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

RESEARCH IN OPHTHALMIC SCIENCES

Dr. G. Venkataswamy Eye Research Institute

Annual Report 2016 - 2017

Aravind Medical Research Foundation

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

G. Venkataswamy

RESEARCH IN OPHTHALMIC SCIENCES

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FOREWORD

Aravind Medical Research Foundation, the research arm of Aravind Eye care System (AECS), is a unique organization where the basic science researchers and clinicians interact on a daily basis to solve some of the problems facing the clinical practitioners not only in India but also in other countries. Aravind model of health care management is well respected and followed by developing countries as well. This model has done enormous service to people who cannot afford quality health care and has also led to the evolution of a research wing.

As in the past, this year also AECS pitched in to support our research efforts. Efforts were taken to reach out to many who are interested in joining hands with us to tackle some of the long and short -term problems. Of course, the Government is the major supporter of research since AMRF is a teaching and research institution. Our Ph.D students have done well after their training at AMRF and they are our goodwill ambassadors.

AMRF strives to do its best in basic research, and tries hard to translate some of the finding and at the same time spend time and effort in human resource development. In order to achieve all these, an organization has to move forward constantly. Towards this AMRF has recruited new faculty recently and they are now settled in to do their research by receiving grants from the Government and other sources

Dr. P. Namperumalsamy
President, AMRF

INTRODUCTION

Diseases of the eye may be the very first of the diseases that could benefit from gene therapy approaches. Clinical trials in gene therapies targeting eye diseases are in promising stages and the success of these efforts might bring to limelight the importance of these new approaches in translational medicine. Leber's Congenital Amaurosis, an early optic neuropathy leading to vision loss is the disorder targeted for gene therapy by various organizations. Type10 is one among the 13 types of this disease and this is targeted using CRISPR technology. A few other types are also being taken for clinical trials using other approaches. All these clinical trials are in various institutes in the US and Europe.

Ramping the research efforts and keeping the translational domain in focus is important for the sustained progress of any organization. In the absence of appropriate mechanisms in place for full-fledged translational effort, AMRF could focus on intensive cutting edge research in these areas that are of importance to Indian population.

Looking at the annual report it is clear that the investigators have done their best in their areas of expertise. I wish they invest still more effort to take some of their findings to the translational level and also publish in high impact journals.

Prof. K. Dharmalingam
Director - Research

MOLECULAR GENETICS

The department of molecular genetics has given importance to understand the molecular pathogenesis of some of the eye diseases especially Primary Open Angle Glaucoma (POAG) and has performed Next Generation Sequencing (NGS) for two large POAG families to identify the candidate gene responsible for glaucoma. Involvement of SIX6 gene and its regulatory elements with POAG was proved using in vitro and in vivo models. In addition, a significant association was determined with extra cellular matrix gene (*MMP9*) in South Indian Primary Angle Closure Glaucoma (PACG) patients. The department is also focusing on identification of putative genetic markers for diagnosis of inherited eye diseases and genetic counselling. Further,the department is keen to identify the spectrum of *CHST6* mutations in the Macular Corneal Dystrophy (MCD). Molecular genetics team also attempts to unlock the heritable locks involved in the pathogenesis of Pseudoexfoliation syndrome.

Genetic screening for CHST6 mutations of Macular Corneal Dystrophy (MCD) patients in Indian population

Investigators : Dr.P.Sundaresan, Dr.N.V.Prajna, Dr.V.Lumbini, Dr.K.Rohan Agashe
Ph.D Scholar : M. Durga
Funding Agency : Cornea clinic research grant - Mutt study

Introduction

Macular Corneal Dystrophy (MCD) is an inherited autosomal recessive disorder of Keratan Sulfate (KS) metabolism. It is caused by mutations in the carbohydrate sulfotransferase-6 (*CHST6*) gene, encoding corneal N-acetyl glucosamine-6-O-sulfotransferase (C-GlcNAc-6-ST) enzyme. The abnormal accumulation of glycosaminoglycan's (unsulfated Keratan Sulfates) in the stroma,

keratocytes, Descemets membrane, endothelial cells leading to severe visual impairment. The onset usually occurs in the first decade of life, starting with a fine superficial stromal haze in the central stroma and followed by an accumulation of irregular, focal, grey white deposits.

MCD is most prevalent in Iceland followed by Japan, India and Saudi Arabia. In South Indian population, the high prevalence of MCD is probably a result of high frequency of consanguineous marriages. At present, there is only a limited study conducted in the Indian population with regard to MCD genetics. Therefore, this study was undertaken to determine the spectrum of genetic variations in *CHST6* gene and understand its role in MCD pathogenesis. In this study, 55 families have been recruited (90 study subjects with and without MCD) who were screened for mutations in *CHST6* gene by Sanger sequencing.

Results & Conclusion

Several homozygous missense and deletion mutations in MCD patients and their siblings were observed. Interestingly, a complex frameshift mutation; c.581_586 delACCTACinsGGT (Fig.1) was identified in two MCD probands. The mutation c.581_586 delACCTACinsGGT is a 6 base pair deletion with 3 base pair insertion (p.Asn194_Arg196del insArgCys) resulting in a frameshift at asparagine-194 residue. Insilico analysis will be done to predict the effect of this mutation involved in the pathogenesis of MCD. In addition, future studies will be also focused on the histopathological analyses of MCD cornea.

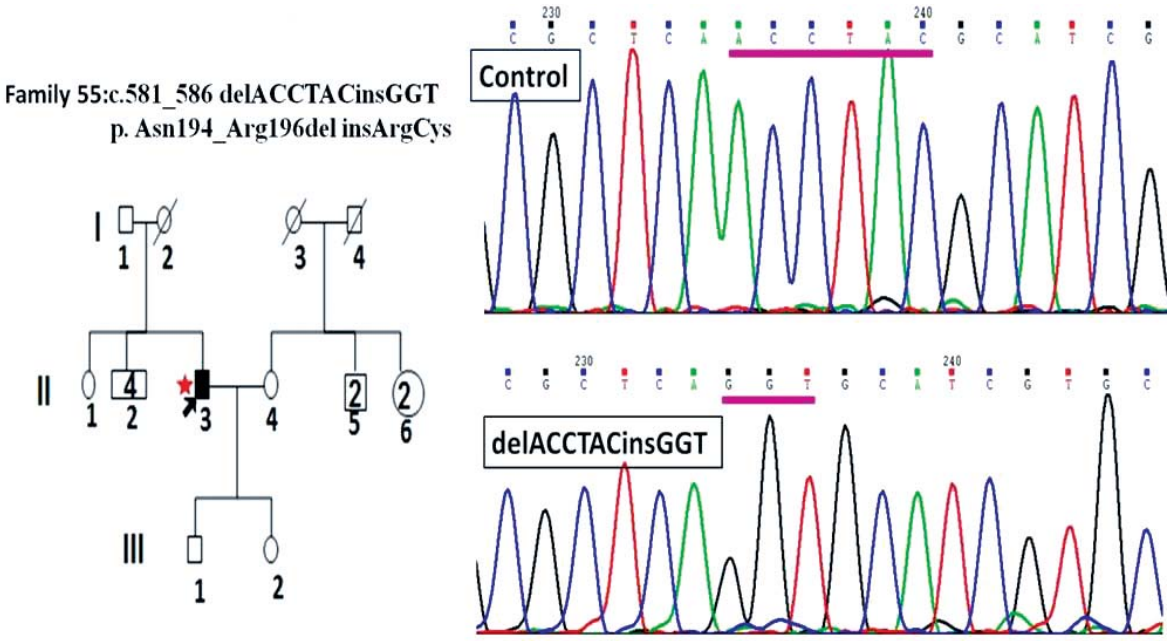

Figure: 1 Left panel shows the pedigree chart of MCD family: 55 and right panel shows the chromatogram that explains the presence of frameshift mutation; c.581_586 delACCTACinsGGT in proband but not in control.

Evaluating the association of candidate gene polymorphisms with Primary Angle Closure Glaucoma in South Indian population

Investigators : Dr.P. Sundaresan, Dr.R. Venkatesh, Dr.S. Kavitha, Dr.S.R. Krishnadas
Ph.D Scholar : Roopam Duvesh
Funding Agency : UGC fellowship & AMRF

Introduction

Primary Angle Closure Glaucoma (PACG) is a genetically heterogeneous disease of anterior chamber angle of the eye. It is a leading cause of bilateral blindness worldwide with highest prevalence in Asians. Shorter axial length is one of the features for PACG, which is determined by extracellular matrix (ECM) remodelling process. Several single nucleotide polymorphisms (SNPs) in candidate genes involved in remodelling mechanism have been studied in different ethnic groups. Matrix metalloproteinases (*MMPs*) are major enzymes involved in ECM remodelling during growth and development of various ocular tissues. In addition, Fibronectin type III domain containing 3B (*FNDC3B*), a glycoprotein of ECM has also been associated with intra-ocular pressure (IOP) and central corneal thickness (CCT) in glaucoma and thus might also have possible role in PACG pathogenesis. Therefore, present study aimed to investigate the genetic association of SNP markers rs17576 and rs3918254 of MMP9 and rs16856870 (*FNDC3B*) with PACG in South Indian population.

All the study subjects were recruited from Glaucoma Clinic, Aravind Eye Hospital, Madurai & Pondicherry. With informed consent, blood samples were collected from the study subjects followed by DNA extraction by salting out method (Miller *et al*., 1988). 200 PACG and 290 controls were genotyped for rs16856870 (*FNDC3B*) using Taqman allelic discrimination assay. Genotyping of 170 PACG cases and 263 age-, ethnic-matched controls was done for rs17576 and rs3918254 using PCR-Restriction Fragment Length Polymorphism (RFLP) and Sanger sequencing. For rs17576, association analysis was also done in primary angle closure subjects (PAC; N=140). Genetic association analysis was performed using PLINK v1.07 tool and STATA v11 software.

Results

In the present study, significant genetic association was observed for rs17576 (p=0.03; OR=1.4) in South Indian PACG subjects. However, no significant association was found for rs17576 in PAC group and rs3918254 (both p>0.05). Further, data were analysed for gender-specific associations. For rs17576, a significant association was identified in females (p=0.01; OR=1.53) in PACG group. However, no significant associations were observed in male subjects for rs17576 (both PACG and PAC group) and rs3918254. Additionally, significant association was also observed for rs16856870 (p=0.004; OR=2.4) in South Indian PACG patients.

Conclusion

This is the first study which showed a significant association of rs17576 and rs16856870 in PACG subjects from South India.

Identification of the risk variants of the Exfoliation syndrome by Genome-Wide Association Study (GWAS)

Investigators : Dr. P. Sundaresan, Dr. Haripriya Aravind
Collaborator : Dr. Chiea Chuen Khor
Ph.D Scholar : G. Prakadeeswari
Funding Agency : AEH-AMRF APEX project, Genome Institute of Singapore.

Introduction

Pseudoexfoliation Syndrome (PEXS) is a clinical condition first described by Lindberg in 1917. Being a late onset disorder, it may lead to complications like secondary open angle glaucoma. The clinical presentation includes deposition of white flaky abnormal fibrillar material and histologically present on the anterior eye segment comprising anterior lens capsule, lens zonules, iris, trabecular meshwork, cornea, ciliary body and the lamina cribrosa of the optic nerve. The prevalence of PEXS in Southern India is 3% - 6%.

Genome-wide association studies use high-throughput genotyping technologies to assay hundreds of thousands of single nucleotide polymorphisms (SNPs) and relate them to clinical condition and measurable traits.

The primary goal of performing a GWAS was to identify risk variants associated with exfoliation syndrome which will help in unbiased way of determining the new risk variants. GWAS was performed with 932 PEXS case and 449 control subjects for 6,93,045 SNPs (only autosome) through Illumina Infinium High Throughput Screening assay, followed by scanning with HiScanSQ system and iScan system. Uniform quality control filters were applied for both individual samples and SNP markers across PEXS cases-controls. From starting number of 932 PEXS cases and 449 controls, genotype data on 6,23,021 SNPs were available for 860 PEXS cases and 411 controls after stringent quality control filters. Downstream data analysis was performed with PLINK v1.07.

Results

A total of 37,086 SNPs were showing a significant association with PEXS cases (Figure: 1 & 2). Among these, 26 SNPs showed a genome-wide significant (P <5E-08) association with pseudoexfoliation syndrome (Table: 1) and 97 SNPs were showing suggestive significant (P<1E-04) association with PEXS (data not shown). All the 26 genome-wide significant SNPs were located at chromosome 15, the peak region that has a dominant association with pseudoexfoliation syndrome (Figure: 1).

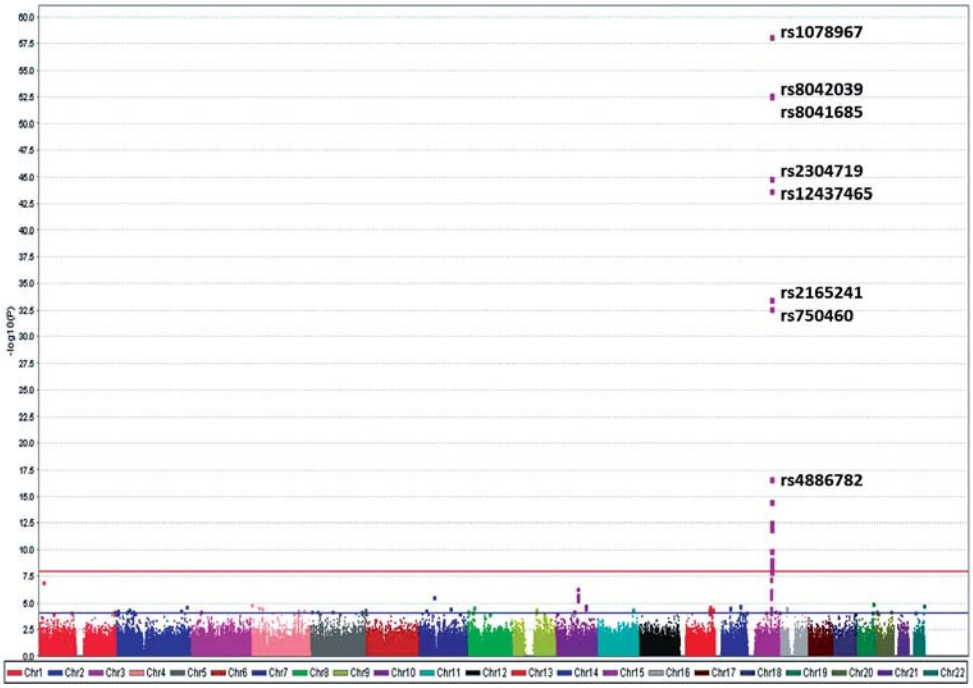

Figure: 1 Manhattan plot showing the Exfoliation syndrome cases vs control: p-values scale from 0 to 1E-60, with representation of the –log10 p-values (y-axis) of the associated SNPs plotted according to the chromosomal physical position (x-axis). The Blue line indicates the suggestive significant p-values (P < 1E-04) and red line indicates the genome-wide significant p-values (P < 5E-08).

Table: 1 Genome-wide significant SNPs (P<5E-08) associated with pseudoexfoliation syndrome.

S.No	CHR	SNP	CHISQ	P-value	OR	Gene
1	15	rs1078967	262	6.34E- 59	0.1275	*LOXL1*
2	15	rs8042039	236.9	1.87E- 53	0.1546	*LOXL1*
3	15	rs8041685	236.6	2.14E- 53	0.1547	*LOXL1*
4	15	rs2304719	201.3	1.07E- 45	0.2662	*LOXL1*
5	15	rs12437465	196	1.59E- 44	0.287	*LOXL1*
6	15	rs2165241	149	2.83E- 34	3.027	*LOXL1*
7	15	rs750460	145.5	1.68E- 33	2.994	*LOXL1*
8	15	rs4886782	72.26	1.89E- 17	2.55	*LOXL1*
9	15	rs13638	62.85	2.23E- 15	0.4085	*TBC1D21*
10	15	rs11072447	53.75	2.28E- 13	1.977	*TBC1D21*
11	15	rs11639300	52.14	5.17E- 13	1.959	*TBC1D21*
12	15	rs4886467	51.23	8.20E- 13	1.948	*TBC1D21*
13	15	rs4461027	41.5	1.18E- 10	1.771	*LOXL1- AS1*
14	15	rs1452389	38.17	6.49E- 10	0.5872	Intergenic variant
15	15	rs874955	36.99	1.19E- 09	0.5757	Intergenic variant
16	15	rs7174255	36.94	1.22E- 09	0.5814	Intergenic variant
17	15	rs1823718	36.69	1.38E- 09	1.739	Intergenic variant
18	15	rs896588	36.31	1.68E- 09	0.576	Intergenic variant
19	15	rs1478558	35.13	3.08E- 09	1.704	Intergenic variant
20	15	rs2415231	34.81	3.63E- 09	1.7	Intergenic variant
21	15	rs7173049	33.76	6.24E- 09	0.6048	*LOXL1*
22	15	rs4886776	33.63	6.65E- 09	0.5859	*LOXL1*
23	15	rs4272992	32.91	9.65E- 09	1.655	*TBC1D21*
24	15	rs876383	29.92	4.50E- 08	1.615	*PML*
25	15	rs4886717	29.07	6.99E- 08	1.596	*TBC1D21*
26	15	rs3784556	28.95	7.45E- 08	0.6042	*PML*

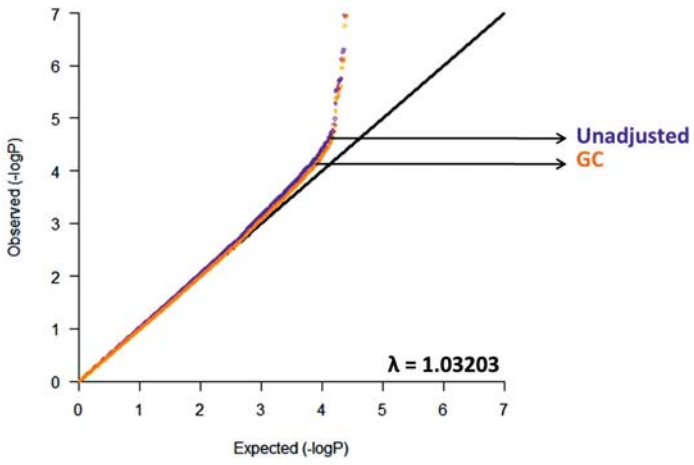

Figure: 2 Quantile – Quantile (QQ) plot showing the Exfoliation syndrome cases vs control: representation of the observed chi-square p-values plotted from minimum to maximum against their expected values. Purple dots are the uncorrected test statistics. Orange dots are the statistics corrected by the genomic control method (λ = 1.03602). Under the null hypothesis theory, in case of no association at any of the locus, the points will be expected to follow the black line (null distribution line). The deviation at the extreme tail end of the distribution suggests the true association of SNPs with the exfoliation syndrome.

Conclusion

In first stage GWAS on the South Indian Exfoliation syndrome patients, the strong association of the genes *LOXL1, LOXL1-AS1, TBC1D21* and *PML* as susceptible variants involved in the pathogenesis of PEXS at the locus 15q24.1 was reported.

Identification and characterization of variants and a novel 4bp deletion in the regulatory region of SIX6, a risk factor for Primary Open Angle Glaucoma

Investigators : Dr.P. Sundaresan, Dr.S.R. Krishnadas, Dr. Manju Pillai
Ph.D. Scholar : Mohd Hussain Shah
Collaborator : Prof. Paola Bovolenta, Madrid, Spain.
Funding Agency : ICMR Fellowship & AMRF

Introduction

Primary Open Angle Glaucoma (POAG) is the most common type of glaucoma. POAG affects approximately 60.5 million people worldwide and it is predicted to increase up to 79.6 million by 2020. POAG is a neurodegenerative disease, associated with loss of tissue in the rim of the optic disc, with a consequent increase in the size of the central portion of the optic disc, known as “cup”. These changes lead to retinal ganglion cell (RGC) loss, optic nerve degeneration and loss of vision. POAG is highly heterogeneous and associated with various genetic risk factors. Many of these factors have been identified through Genome-Wide Association Studies (GWAS) and include variants of genes with different functions such as *CAV1/CAV2, CDKN2B-AS1, TMCO1, ATOH7, GAS7, ABCA1, AFAP1, GMDS, PMM2, FNDC3B, TFGBR3, TXNRD2, ATXN2, FOXC1I* and *SIX1/SIX6*. However, the most consistent associations have been observed for the CDKN2B-AS1 region on chromosome 9p21, followed by that of the *SIX1/SIX6* locus. The gene encoding the transcription factor *SIX6* has been associated with primary open angle glaucoma (POAG) in different populations. Here, the team studied if there exists a genetic association between *SIX6* with POAG and/or it related quantitative endophenotypes in the south Indian population

Methods

The cohort of individuals analysed included 265 POAG and 265 age-matched controls from the south Indian population. All individuals received a comprehensive ocular examination before the collection of blood samples, to extract genomic DNA. PCR amplification, taqman based allelic discrimination assay, automated sequencing of the *SIX6* gene and its regulatory region were performed on the purified DNA samples. A novel 4 base pair deletion identified in the regulatory region was functionally tested using transgenesis in zebrafish and luciferase assay in HEK293 cell line.

Results

SIX6 gene and its regulatory region was sequenced and two known rare, two common variants in the *SIX6* gene and a novel 4bp deletion in an already characterized putative enhancer region were identified. In contrast to previous studies, the team could not establish a significant genetic association between the rs33912345 (Odds ratio (OR) =0.919, p=0.4) and rs10483727 SNPs at the *SIX1-SIX6* locus (Odds ratio (OR) =0.95, p=0.58) with POAG in south Indian patients. However, patients carrying either the rs33912345 ‘C’ or the rs10483727 “T” risk alleles presented a significant and dose-dependent reduction of the peripapillary retinal nerve fiber layer thickness (RNFL), which

was more evident in the superior and temporal-inferior quadrants, well in agreement with the knowledge that RNFL become thinner in glaucoma patients. It was also established that patients carrying two copies of the ‘‘C’’ or of the ‘‘T’’ risk allele presented a statistically significant increase of the vertical cup disc ratio (p=0.012; p=0.009). Furthermore, using transgenesis in zebrafish and luciferase assay, it was determined that the newly identified 4bp deletion in the enhancer region significantly reduces reporter expression in cells of the retinal ganglion and amacrine layers, where human *SIX6* is expressed, suggesting that SIX6 activity might be reduced in the retina of patients carrying this deletion.

Conclusion

The data provides additional support to the previously proposed implication of *SIX6* variants as POAG risk factors and further suggests that the reduced levels of *SIX6* expression might be implicated in POAG pathogenesis.

Targeted sequencing to identify the candidate gene in two south Indian POAG families

Investigators : Dr. P. Sundaresan, Dr. S.R. Krishnadas, Dr. D. Bharanidharan, Dr. Mohideen Abdul kader, Dr. R. Ramakrishnan
Ph.D. Scholar : Mohd Hussain Shah
Funding Agency : Indian Council of Medical Research (ICMR)

Introduction

Primary Open Angle Glaucoma (POAG) has a strong genetic component to its pathogenesis and is highly heterogeneous. Glaucoma is a complex disease, it may either be caused by the combined action of many genes or caused by mutations in a single gene such as Myocilin, Optinurein and *TBK1*. Candidate genes for glaucoma were discovered with studies of large pedigree with positive family history that have autosomal dominant inheritance of POAG . In the current study, two families have been recruited: a single large four generation south Indian family from kayalpattinam and another from Devakottai, Tamilnadu, India. 240 members from Kayalpattinam family and 51 from Devkottai family participated in this study. For the Next Generation Sequencing, 12 samples have been collected (7 POAG, 2 suspect and 3 controls) from kayalpattinam family and 4 samples (2 POAG, one suspect and one control) from Devakottai family. These 16 DNA samples were processed in Miseq (Illumina) to identify a novel candidate gene for POAG.

Methods

Before library preparation, all the sixteen samples were quantified by three methods: Gel electrophoresis followed by Nanodrop and Qubit dsDNA Assay kit.

Results

85 genes in 16 samples have been targeted based on the analysis of previous exome data. The Q30 scores, which denotes the quality of the run was above 95%. Obtained raw sequence reads from Miseq were analysed using bioinformatics pipeline as shown in Figure 1. Data was quality filtered using fastQC tool. The filtered reads were mapped to Hg19 reference sequence using Burrow-Wheeler Aligner. Resulting BAM files were locally realigned using Genome Analysis TK-3.1-1 (GATK) Indel-realigner tool to minimize the mismatches across the reads. GATK haplotype caller was performed to retrieve germline single nucleotide variants (SNVs) and small insertions/

deletions (InDels) with phred score 20 and minimum depth 5 from all the samples. All the SNVs and InDels were subjected to identify rare and potential variants. The rare variants were identified using ANNOVAR by filtering common variants with alternative allelic frequency higher than 1% based on 1000 Genomes project, dbSNP137 and ESP server. Of those, non-synonymous/synonymous SNVs, coding InDels, and intronic variants that were less than 10 bp beyond the canonical splice site junction were selected. The potential variants were identified using ClinVar, COSMIC and bioinformatics prediction tools. Detected variants were further manually assessed with the help of IGV viewer to avoid mapping errors. The variants of three genes were identified in Kayalpattinam family and two genes in Devakottai family.

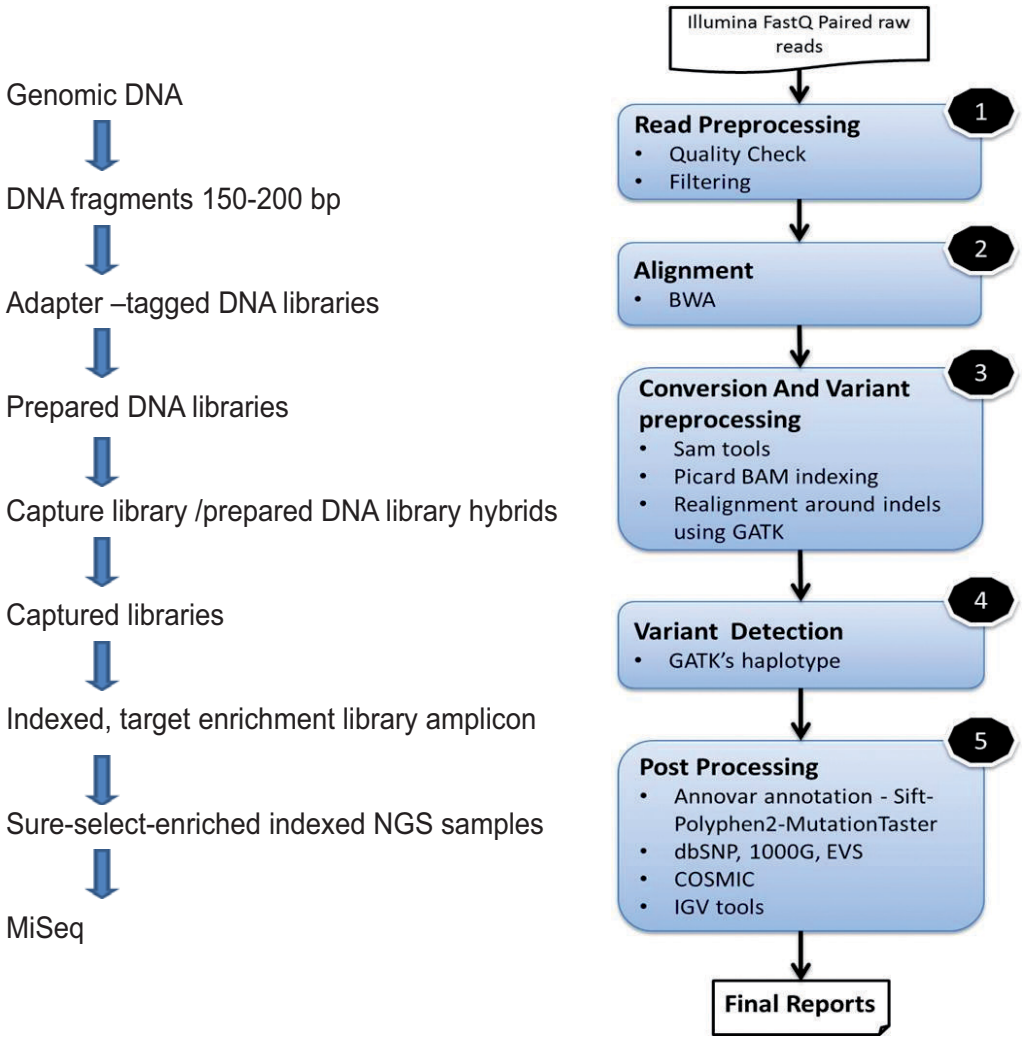

Figure.1 Following steps were performed to acquire genomic data from 16 samples

Conclusion

Based on the data on the exome seq of 16 samples, these variants in five genes will be further validated by Sanger sequencing in all the affected and unaffected members of two families and will be functionally characterized to prove their involvement in disease pathogenesis.

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 an intraocular childhood malignancy with highest incidence in India among all developing countries. Biallelic inactivation of *RB1* gene is known to be the first step in an avalanche of genetic events. Genetic testing plays a major role in risk prediction of siblings and offspring, thereby enhancing the management of the disease. A stepwise strategy has been adopted for the genetic testing of retinoblastoma using multiple methods such as Sanger sequencing, Multiplex Ligation dependent Probe Amplification (MLPA), and Methylation specific MLPA (MS-MLPA). Recently, Next Generation Sequencing methods using the Illumina Miseq platform were also utilized to understand the additional genetic changes other than *RB1*.

Results

Sanger Sequencing and MLPA

During the year, a total of 34 RB patients were screened for genetic alterations in *RB1* gene. Mutations were identified in 12 out of 14 bilateral patients in their blood samples and hence an increased chance of inheriting the mutation in their offspring was predicted. Among the 20 unilateral patients, tumor was accessible in 3 patients and two heterozygous mutations were identified in tumor but not in blood of 2 patients. In other 17 patients, blood samples were analysed but mutations were not identified implying that they may not have germline mutations and hence their siblings or offspring had very low or no risk of inheriting the pathogenic *RB1* mutation. Based on the test results, genetic counselling was also provided for two older unilateral patients who wanted to know the risk of the secondary malignancies and inheritance of RB to their offspring.

Next Generation Sequencing

Agilent Sureselect XT custom panel was designed and developed for 70 genes that included exonic regions of 24 RB related genes and hotspots of 46 cancer related genes. Libraries were prepared for 28 samples (16 tumor and 12 blood) and 2x150bp paired-end sequencing was performed in Illumina

Miseq platform using Miseq V2 kit. A sequencing depth of 250X was achieved with Q30 phred score of 96%. The in-house bioinformatics pipeline used for data analysis could detect both the single nucleotide variants (SNVs) and copy number variants (CNVs).

Some of the key findings of the analysis include:

i. Mosaicism

In the NGS run, the samples with known status of *RB1* mutations were included. All the *RB1* mutations detected by Sanger were concordant in NGS as well. Along with that, a mosaic mutation in a patient as shown in figure 1, which was present at 15 % level (56 out of 378) was detected, which was not detected by Sanger sequencing.

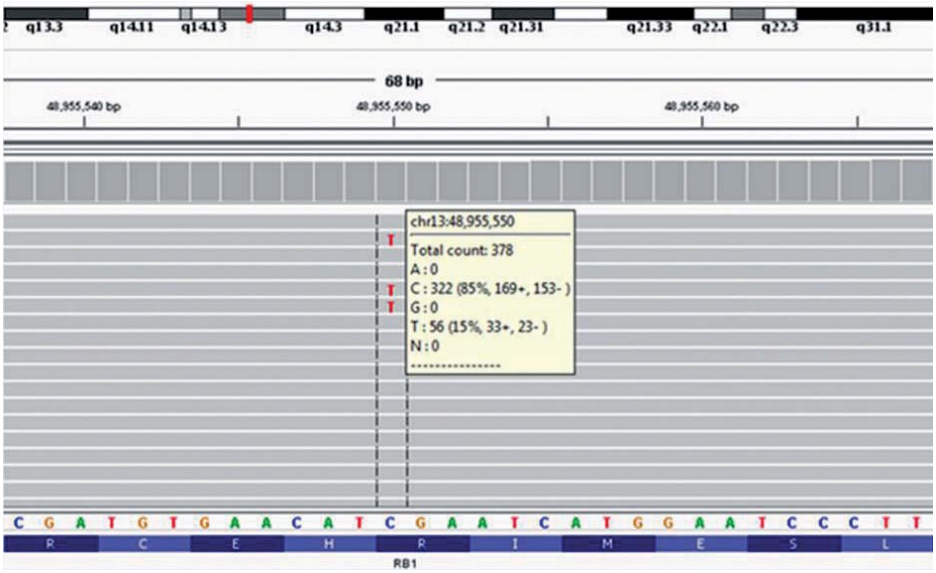

Fig 1: Snapshot of NGS reads by Integrated Genome Viewer (IGV) tool showing the mosaic mutation c.C1666T in exon 17 identified in a patient's blood

ii. Loss of Heterozygosity (LoH)

The identification of compound heterozygous mutations or a homozygous mutation of *RB1* gene in tumors would be sufficient to find out the causative mutations. However, it may not be possible to detect whether the homozygous nature is due to copy neutral LoH or hemizygosity unless both Sanger sequencing and MLPA are done (Figure 2).

iii. Genetic alterations in other RB and cancer related genes

With the custom panel and bioinformatics pipeline, it was possible to detect both the copy number changes and pathogenic variations in cancer related genes. Out of 16 tumors analysed with CNVkit,

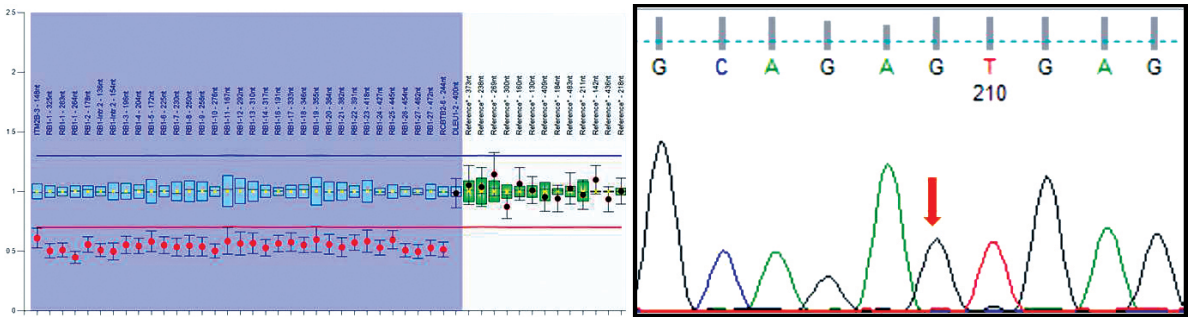

Fig 2: LoH in a patient identified by deletion of one copy of RB1 detected by MLPA leading to homozygous mutation (c.A2069G: p.N690S) detected by Sanger sequencing. Using NGS, it is possible to detect both the loss of RB1 and mutation in a single run.

frequent copy number gains were detected in KIF14 (9), MDM4 (10), MYCN (6), DDX1 (4), DEK (6), E2F3 (8), GATA5 (8) and recurrent loss were identified in TP53 (4), *RB1* (6), CDH11 (8) and CDH13 (8).

SNVs were found in WT1, ATM, FANCF, and BRCA2 that were predicted to have deleterious effect and have involvement in pathogenesis. In few patients without RB1 mutations, variants in tumor suppressor genes TP53, STK11 and cell cycle gene CHEK2 were detected which need further evaluation to elucidate their role in RB tumor formation.

Conclusion

Genetic testing of *RB1* gene has helped our adult RB patients to know the risk of getting secondary malignancies and inheriting the retinoblastoma to the offspring along with the management in paediatric patients. NGS methods had increased the sensitivity of the genetic testing through detection of mosaic mutations and improved the understanding on the mechanism of LoH in a single run. NGS had also paved the way for understanding the process of tumorigenesis though the analysis of other RB and cancer related genes.

Genetic and transcript analysis of Retinoblastoma

Investigators	:	Dr. A. Vanniarajan, Dr. Usha Kim, Dr. R. Shanthi Prof. VR. Muthukkaruppan
Project Fellow	:	Thirumalairaj Kannan
Funding agency	:	Indian Council of Medical Research

Introduction

Retinoblastoma is primarily caused by mutations in *RB1* gene, which spans more than 180 kb on chromosome 13q14, consisting of 27 exons. There is a wide spectrum of mutations ranging from point mutation to whole gene deletion. A sequential method of analysing the diverse mutations have been now adapted. In addition, this study is aimed at two other aspects:

1. Understanding the impact of mutations at the transcript level

It is important to know the effect of mutation at the transcript level in order to understand the pathogenesis of retinoblastoma. Genetic changes may either lead to altered splicing or differential expression of the transcripts. The sequencing of the transcript was done to check the alterations and real time PCR was done to check the gene expression.

2. Developing methods to identify the mutations from archival samples

Fresh or Flash-frozen sample is ideal for identifying the mutations in the tumor, however, it is not accessible either because of the small size of the tumor or calcified/regressed tumor due to treatment. The tissues are formalin-fixed paraffin embedded (FFPE) and subjected to pathological investigations. The FFPE tissues would be a great help in identifying the mutations in most of the cases.

Results

Altered transcripts and differential expression of *RB1*

For studying the transcriptional consequences, different type of mutations such as non-sense, splice variant, indels, missense, large deletion and duplications were analyzed. RNA was isolated from blood of one patient and tumor of other patients. The table 1 shows the effect of mutations observed at the transcript level.

Table 1: Transcriptional alterations caused by diverse mutations

Sample No	RNA isolated from	Mutation at DNA level	Exons	Types of Mutation	Transcriptional effect of RB1 mRNA
1	Blood	c.861+1 del G	8	Splice site	Exon Skipping
2	Tumor	c.2489+1G>C	23	Splice site	Intron Retention and Exon Skipping
3	Tumor	c.1214 InsG; c.2213delT	12; 22	Frameshift	Low expression of RB1
4	Tumor	Deletion of Exons 7-27	7-27	Deletion	Low expression of RB1
5	Tumor	Whole RB1 deletion	All exons	Deletion	Low Expression of RB1
6	Tumor	Whole RB1 duplication	All exons	Duplication	High Expression of RB1
7	Tumor	c. 1072C>T;p. R358X	11	Nonsense	Low expression of RB1
8	Tumor	c.2117G>A p.C706Y	21	Missense	Low expression of RB1

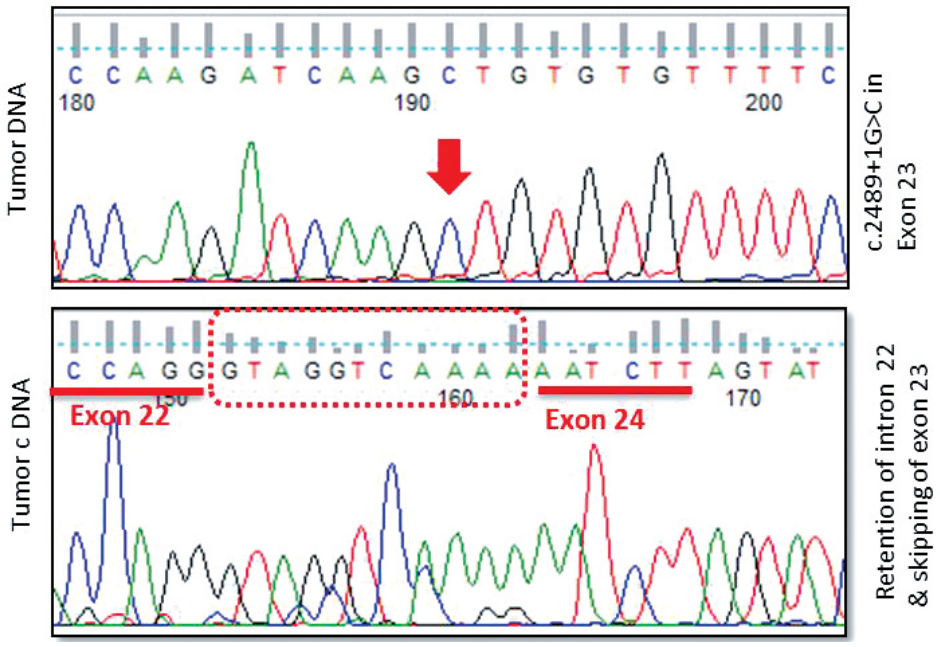

Fig 1: The tumor sample of patient 2 with splice mutation at DNA level showed the retention of intron 22 and skipping of exon 23 at the transcript level

Alternative splicing was identified in two patients with canonical splice mutations, one had exon skipping and other had both intron retention and exon skipping (Fig 1). In case of patients with frameshift, nonsense and missense mutations, there is no sequence alteration at the transcript level when compared to DNA but the reduced expression of *RB1* was detected. The *RB1* expression was reduced with whole *RB1* deletion and increased with duplication.

Genetic screening of *RB1* mutations from FFPE tissues

Although multiple methods are available for DNA extraction from FFPE tissues,, it is much limited for ocular tissues. The protocol for DNA isolation was optimized upon evaluating multiple kits and different procedures. As a proof of principle, first the mutations in the FFPE block where the mutation status was known from the frozen tissue were checked. Compound heterozygous mutations (Exon 10 & 23) were observed in both fresh and FFPE in a unilateral patient. Further, the tumor tissue of a

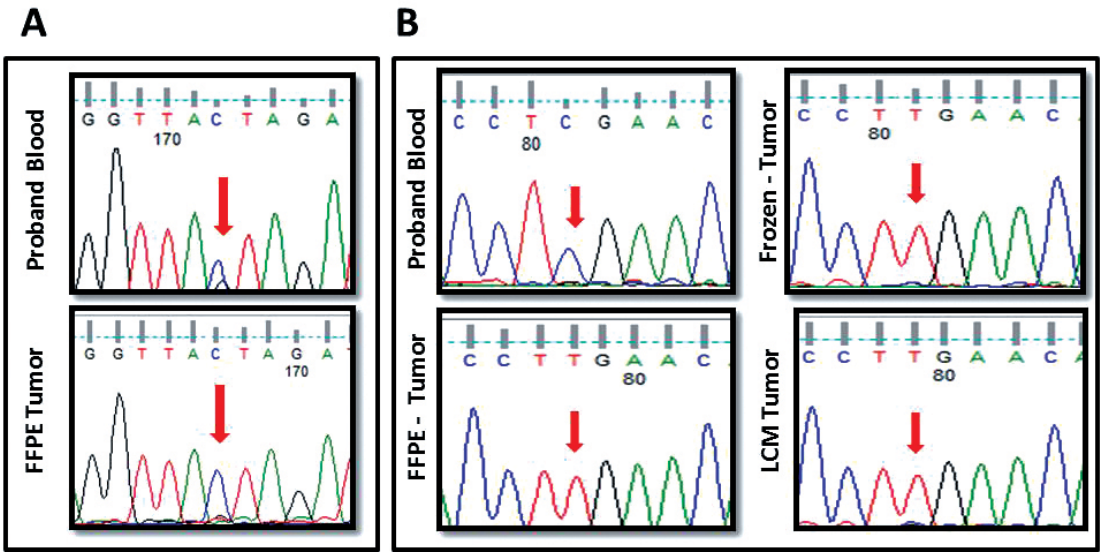

Figure2. A. A splice variant in exon 19 (c.1960+5 G>C) identified in blood (heterozygous) and FFPE tumor (homozygous) in a bilateral patient; B. Homozygous nonsense mutation (c.751C>T) identified in Frozen, FFPE and LCM captured tumor samples

bilateral patient was also analysed and found a homozygous mutation in exon 19, which was present in heterozygous state in blood (Fig 2A). Further, Lacer Capture Micro dissection (LCM) was utilised for isolating the tumor cells without normal cell contamination. The mutation analysis showed the same homozygous nonsense mutation in exon 8 in frozen, FFPE and LCM tissues but not in blood (Fig 2B).

Conclusion

Altered splicing and differential gene expression was observed at the transcript level corresponding to the mutations identified at DNA level, which has improved the understanding of their role in the inactivating the RB. Demonstration of the molecular analysis of FFPE along with LCM captured tumor samples would help in the analysis of the mutations in the samples where the fresh tumor is not accessible. Further isolation of normal retina, retinoma and retinoblastoma regions from the same patient using LCM would dissect out the molecular mechanism of retinoblastoma in detail.

Understanding the molecular basis of chemoresistance in retinoblastoma

Investigators : Dr. A. Vanniarajan, Dr. Usha Kim, Dr. R. Shanthi, Prof. VR. Muthukkaruppan
Project Fellow : T.S. Balaji
Funding : Council of Scientific and Industrial Research (Fellowship)

Introduction

Chemoresistance is clinically defined as lack of reduction in tumor size or relapse after initial response upon chemotherapy. Though different mechanisms have been reported in

chemoresistance of cancer, ABC transporter mediated chemotherapeutic drug efflux is widely suggested mechanism. PGP/ABCB1 and MRP1/ABCC1 are widely studied ABC transporters in many cancers including retinoblastoma. One of the objectives of the study is to analyse the role of ABC transporters in chemoresistance of retinoblastoma.

Results

Gene expression analysis of ABC Transporters

Out of 48 ABC transporters, 31 were reported to be expressed in Y79 cell line. Hence gene expression analysis was carried out using semiquantitative reverse transcriptase PCR with Y79 and neural retina samples. The primers were optimised for 17 transporters and real time PCR will give the relative quantity of these transporters in the cell line versus neural retina control.

Selection of patients for studying chemoresistance

The treatment regimens for retinoblastoma include 6 cycles of Vincristine, Etoposide & Carboplatin (VEC) at a suitable concentration. If there is no response or relapse after initial response, Cyclosporine A (modulator of PGP/ABCB1) is added to the normal regimen. For this study, 27 were selected out of 86 retinoblastoma patients, who underwent minimum 6 cycles of VEC and 2 cycles of Cyclosporine A. RNA was isolated from the tumors of 6 eyes enucleated after treatment failure with chemotherapy.

Gene expression of ABC Transporters in tumors with treatment failure

Real-time PCR analysis was done to study the gene expression of 3 ABC transporters (PGP/ABCB1, MRP1/ABCC1 & BCRP/ABCG2) in 6 tumor samples with treatment failure. One of these tumors showed upregulation of 2 ABC transporters (MRP1/ABCC1 & BCRP/ABCG2) and other tumor had increased expression of PGP/ABCB1 compared to neural retina with B2M as normalizing gene.

Conclusion

The preliminary results show that some of the ABC transporters have a potential role in the chemoresistance of retinoblastoma. Further analysis of these transporters and other genes would enhance knowledge on chemoresistance and might help to develop newer drugs.

Molecular characterization of tumor progression in retinoblastoma

Investigators : Dr. A. Vanniarajan, Dr. Usha Kim, Dr. D. Bharanidharan, Prof. VR. Muthukkaruppan

Research Scholar : T. Santhini

Funding agency : Department of Science and Technology (Inspire fellowship)

Retinoblastoma is the most common paediatric ocular tumor that acts as a good model for studying the tumor progression in many tumors. Inactivation of *RB1* has been an initiating factor of retinoblastoma. However, additional genetic changes are required for the tumor progression. Retinoblastoma tumorigenesis has been recently postulated to be driven by epigenetic mechanisms more than mere alterations of the genome. The major epigenetic changes like promoter methylation, histone modification and miRNA regulation were studied either individually or in a limited number of samples and there is no comprehensive study available in retinoblastoma tumors. Hence the aim of the study is to elucidate the epigenetic mechanisms underlying the tumor progression of retinoblastoma. Promoter methylation of *RB1* promoter has been identified in 3 retinoblastoma patients so far.

STEM CELL BIOLOGY

The Corneal Epithelial Stem Cell (CESC) derived mesenchymal stem cell transplant is the second most-common form of adult stem cell based therapy next to bone marrow transplant. These CESCs reside in the basal layer of the limbus at the corneo-scleral junction. A specific method to identify and quantify these CESCs has been established earlier by the team. These stem cells after ex vivo expansion in compliance with good manufacturing practice have been successfully used for corneal surface reconstruction in 25% patients with limbal stem cell deficiency at Aravind Eye Hospital, Madurai. One of the probable reasons for this “sub-optimal” success is the severity of the initial injury. Subsequently, the team demonstrated the presence of mesenchymal stem cells essential for the maintenance of CESCs in the anterior limbal stroma and the loss of these stromal stem cells along with limbal epithelium in patients with limbal stem deficient patients using in vivo confocal microscopy. The current focus is to understand the basic biology of these stem cells specifically the signaling pathways and molecular regulators associated with the maintenance of stemness using a 80% enriched CESCs and to assess the cellular cross talk with the neighboring cells to develop better treatment modalities for these patients.

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	:	Ms. Lavanya Kalaimani
Funding	:	Department of Biotechnology

Introduction

Corneal epithelium covers the anterior surface of the eye and its homeostasis is maintained by the corneal epithelial stem cells (CESCs) residing in the basal layer of limbus at the corneo-scleral junction. These stem cells constitute 3-5% of the total limbal epithelium. Since there is no exclusive

marker for their isolation, understanding the molecular mechanisms regulating the maintenance of stemness is still not clear. In this study, 80% enrichment of CESCs was obtained by i) differential enzymatic treatment to isolate the basal limbal epithelial cells followed by ii) laser capture micro dissection of cells with nucleus to cytoplasm ratio ≥ 0.7, using donor tissues obtained from Rotary Aravind International Eye Bank, Madurai.

Results

Total RNA was extracted from enriched CESCs and differentiated central corneal epithelial cells (CCECs). MicroRNA (miRNA) expression profiling was caried out using Illumina Nextseq 500 platform. Small RNA sequence data were aligned to *Homo sapiens* hg19 genome reference, allowing for one mismatch using bowtie1 aligner in the sRNA bench tool and mature miRNAs were annotated using miRBase (release 21). A total of 62 miRNAs were identified in CESCs and 611 miRNAs in CCECs. R tools were used to identify significantly differentially expressed miRNAs in CESCs. The top ten miRNAs upregulated in CESCs included, (i) hsa-miR-21-5p, hsa-miR-3168, hsa-miR-143-3p and hsa-miR-99b-5p with ≥10 fold change, (ii) stem cell proliferation and maintenance specific hsa-miR-21-5p, hsa-miR-191-5p, hsa-miR-26a-5p, hsa-miR-10a-5p, hsa-miR-99b-5p and (iii) novel, embryonic stem cell specific hsa-miR-3168 and hsa-miR-1910-5p. Target prediction of these ten miRNAs identified 2181 targets that were further annotated by DAVID. 33 KEGG pathways were significantly regulated ($p<0.05$;FDR<0.01), including signalling pathways that regulate pluripotency of stem cells, focal adhesions, toll like receptors, PI3-AKT pathway, MAPK pathway, cancer related pathways and neurotrophin signalling pathways that regulate cell migration, growth and proliferation. Of these, the 54 genes involved in signalling pathways regulating pluripotency of stem cells are of interest to explore further.

Conclusions

Comparative miRNA profiling data and target prediction analysis identified the candidate miRNAs that are to be explored further to understand the molecular mechanism governing the maintenance of stemness. Studies are now being carried out to confirm the sequence data using RT-PCR, Northern blotting and LNA *in situ* hybridization.

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miR-203 and its regulation of ΔNp63α expression in human corneal epithelial stem cells (CESCs)

Investigator	:	Dr. Gowri Priya Chidambaranathan
Co-Investigators	:	Dr. VR. Muthukkaruppan, Dr. N. Venkatesh Prajna
Research Scholar	:	Ms. M.K. Jhansi Rani
Funding	:	ICMR - SRF

Introduction

In skin keratinocytes, miR-203 has been reported to repress stemness by inhibiting ΔNp63α expression, an isoform of the nuclear transcription factor p63. The team has earlier demonstrated a unique mRNA expression of this isoform in enriched corneal epithelial stem cells (CESCs). Hence, this study aims to elucidate whether miR-203 has a similar influence on ΔNp63α isoform expression and in the maintenance of stemness in the non-keratinized corneal epithelium.

Results

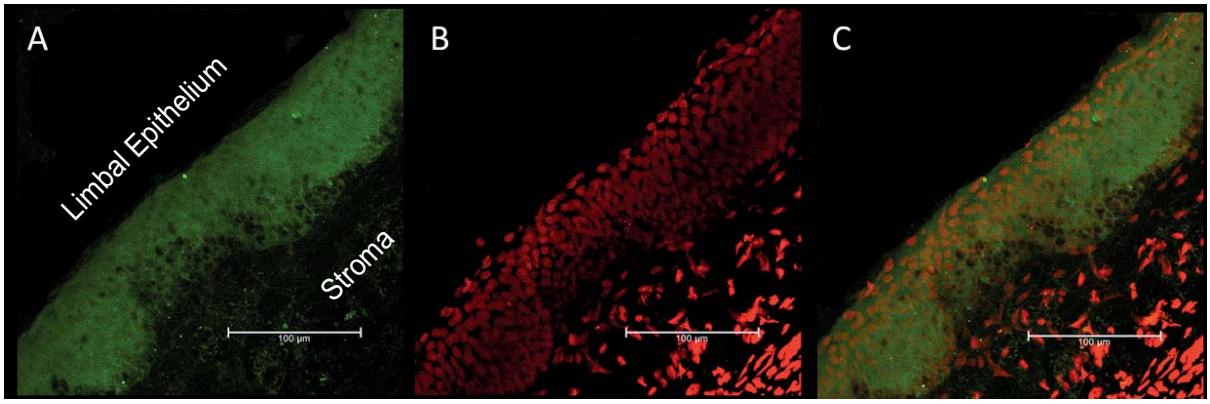
The team's previous report on transfection of the cultured limbal epithelial cells with miR-203 resulted in suppression of ΔNp63α expression by real time PCR and reduced colony forming

efficiency compared to the antagomiR transfected cells. In order to understand whether miR-203 has a direct role in differentiation, *in situ* hybridization was carried out in cryosections of limbal tissues from donor eyes.

Higher expression of miR-203 was observed in the suprabasal and superficial layers of the limbal epithelium. The expression was lower in basal epithelial cells and almost negative in some clusters, wherein the stem cells reside.

Conclusion

Taken together, these findings indicate that miR-203 by suppressing ΔNp63α expression is involved in the regulation of corneal epithelial differentiation. Further studies are essential to understand the signaling pathways associated with this molecular regulation.



Confocal microscopic images of a limbal cryosection after LNA in-situ hybridization. Confocal analysis identified higher expression of miR-203 in the suprabasal and superficial layers (green) while it was much reduced in the basal layer of the limbal epithelium (A); propidium iodide (red) counterstaining of cell nuclei (B) and their overlay (C).

Structural and functional integrity of corneal endothelium after storage in Cornisol, an indigenous intermediate stage corneal storage medium

Investigators : Dr. N. Venkatesh Prajna, Dr. Gowri Priya Chidambaranathan
Dr. Ganesh Gaikwad, Dr. Kishan A Prajapati
Research Scholar : Ms. Yogapriya Sundaresan

Introduction

Corneal blindness is the fourth (5.1%) leading cause of blindness after cataract, glaucoma and age related macular degeneration in world. Corneal transplantation stands to be the only option for restoration of vision in these patients. Due to acute shortage of donor eyes most of the cases go untreated. According to WHO, approximately 4 million people are blind due to corneal pathology. For better utilization, the healthy corneas procured from deceased donor are stored in a biochemically defined tissue culture medium. McCarey-Kaufman (MK) medium is short term storage medium which can preserve the corneal tissue only up to 4 days, while Eurobio Cornea Cold and organ culture medium have been used successfully for long term storage in Europe and Australasia but not globally. One of the most widely used intermediate term storage mediums is Optisol-GS and is traditionally considered as a gold standard intermediate one. But due to its high cost, Optisol-GS has become unaffordable to the third world.

In order to overcome this, Aurolab an integral part of Aravind Eye Care System manufactures Cornisol, an intermediate storage medium which is available at an affordable cost for the storage of

human donor corneas. Preliminary studies have established Cornisol to be equivalent to Optisol-GS by analysing the endothelial cell count and viability after storage. The corneas that have been stored in Cornisol are now regularly used for clinical application. For further validation, the current study was carried out to compare the functional status of the corneal endothelium by immunostaining of corneas stored in Cornisol and in Optisol-GS using a specific marker Na+/K+ ATPase which plays a significant role in maintaining the corneal homeostasis. In addition, the expression of markers for structural integrity like tight junction protein, ZO-1 and actin were also analysed.

Results

Fifteen optical grade corneal donor buttons (6 pairs; 3 individual) obtained from Rotary Aravind International Eye Bank were used for the study. The left eye of the paired sample was preserved in Cornisol and the right in Optisol-GS. The individual buttons were used for the baseline data. The corneas were assessed with slit lamp and specular microscope before and after storage time (7, 10 or 14 days). They were then immunostained for markers of structural integrity (ZO-1, Phalloidin) and functionality (Na+/K+ ATPase). The images were acquired using confocal microscope and analysed using ImageJ.

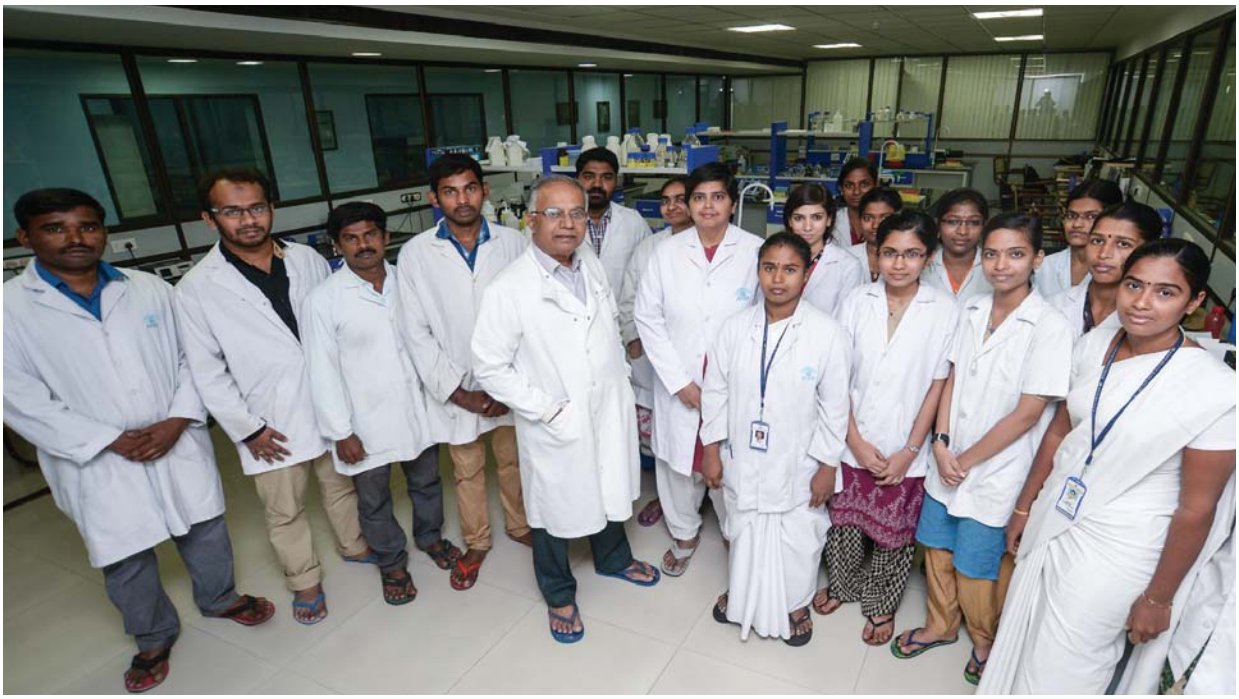
There was no difference in the clinical evaluation of the corneal layers between the two media. No marked variation was observed in the immunostaining data with reference to the storage period. Intact cellular integrity was identified in 91% (51%,98%) (Median (min, max)) of cells in Cornisol and 94% (38%,98%) cells in Optisol based on ZO-1 staining, comparable to the baseline data (87% (76%,97%)). Stress fibres were detected in 42.5% (1%,88%) cells in Cornisol stored corneas and in 55% (11%,94%) in Optisol when stained for actin cytoskeleton, which correlated with the presence of epithelial defect before storage and vacuolated endothelial cells after storage. No difference was observed between the two media based on the staining pattern for Na+/K+ ATPase.

Conclusion

Cornisol and Optisol-GS are equivalent in maintaining the structural integrity and functionality of the donor corneas.

PROTEOMICS

Research at the Department of Proteomics focuses on three ocular diseases, namely, Fungal Keratitis, Diabetic Retinopathy and Keratoconus. Using proteomics approaches, the department aims to understand the pathological mechanisms underlying these diseases and to identify biomarkers that will improve the management of the same. The state-of-the-art proteomics facility with two mass spectrometers has all the amenities to carry out gel-based and non-gel based proteomic analysis. Complementary approaches such as transcriptomics and genomics are adopted, wherever necessary to support the findings. The projects are funded by the Department of Biotechnology and Science and Engineering Research Board, Government of India. Research in Keratoconus and Fungal Keratitis is further strengthened through collaborations with the University of Liverpool, UK and Institut Pasteur, Paris, respectively.



Complement factor H levels in tear film as one of the predictors of clinical outcome in *Aspergillus flavus* keratitis

Investigators	:	Dr. J. Jeya Maheshwari, Prof. K. Dharmalingam
Clinician Scientists	:	Dr. N. Venkatesh Prajna, Dr. Lalitha Prajna
Team	:	K. Sandhya, Naveen Luke Demonte, Dr. Lakshey Dudeja, Dr. Ramya Seetham Raju
Funding	:	Department of Biotechnology, Govt. of India

Rationale of the study

Fungal infection of the cornea is one of the leading causes of blindness in tropical countries, particularly India. Corneal inflammation is one of the hallmark features of fungal ulcer. In addition to the fungal factors, an exaggerated inflammatory response causes host tissue damage. Through a tear proteomics study, the team identified that the complement pathways are central to the inflammatory response during *A. flavus* infection (Jeyalakshmi *et al*., 2016). In addition, neutrophil proteins and the coagulation cascade proteins cross-talk with the complement proteins to amplify the inflammatory response. Harboe *et al*., (2004) have shown that under certain experimental conditions, alternative pathway contributes to nearly 80% of the components of membrane attack complex, although the initiation occurs through classical or lectin pathway. During *A. flavus* infection, a seven-fold upregulation of complement factor H (CFH), the key negative regulator protein of the alternative pathway was observed in tear, suggesting that this pathway is probably responsible for the amplification effect (*Kandhavelu et al*., 2016).

Hence, the level of CFH in tear from *A. flavus* keratitis patients at different stages of infection was examined. The team further examined if CFH level can be used to predict the outcome of treatment in these patients. This model may be relevant in the clinical setting for better and more personalized management of the disease.

Results

Using anti-CFH antibody, the level of CFH protein in the tear from 51 *A. flavus* keratitis patients (Figure 1) was compared. The team observed variable levels of CFH protein across tear samples from keratitis patients, irrespective of the stage of infection. However, in control tear samples, CFH band was either not detected or detected at very low levels. Based on the densitometric analysis, CFH levels were calculated to be in the range from as low as <2 ng/µg to ≥30 ng/µg of total tear proteins.

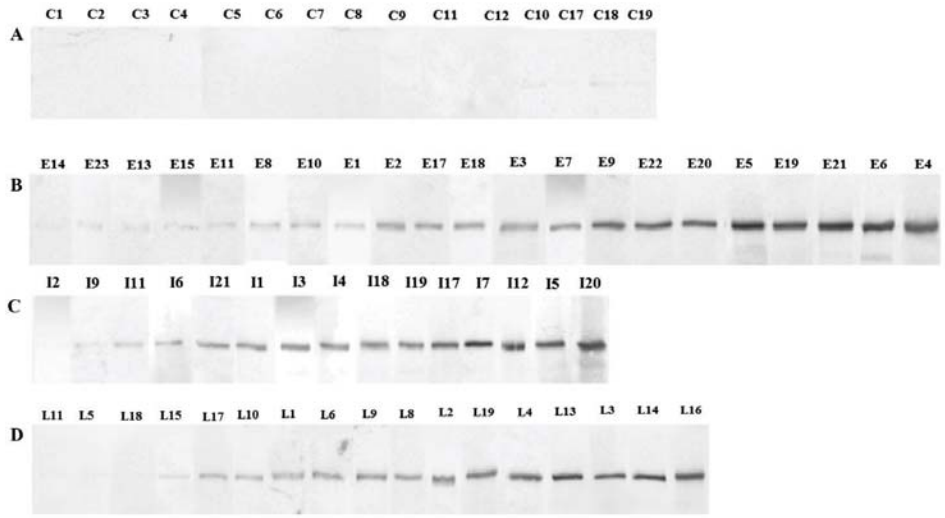


Figure 1. CFH level in tear from A. flavus keratitis patients at different stages of infection and healthy controls. Fifteen micrograms of total tear proteins were resolved on a 8% SDS-Polyacrylamide gel and taken for immunoblot analysis using anti-CFH antibody

Through a comprehensive statistical analysis, it was found that depth, duration of symptoms and CFH level cannot independently serve as predictors of treatment outcome in *A. flavus* keratitis patients. However, when these parameters were combined, the team was able to develop a good predictive model, which is shown in Figure 2. This model to predict the treatment outcome in *A. flavus* keratitis patients begins with an assessment of the CFH level in tear. If the CFH level is

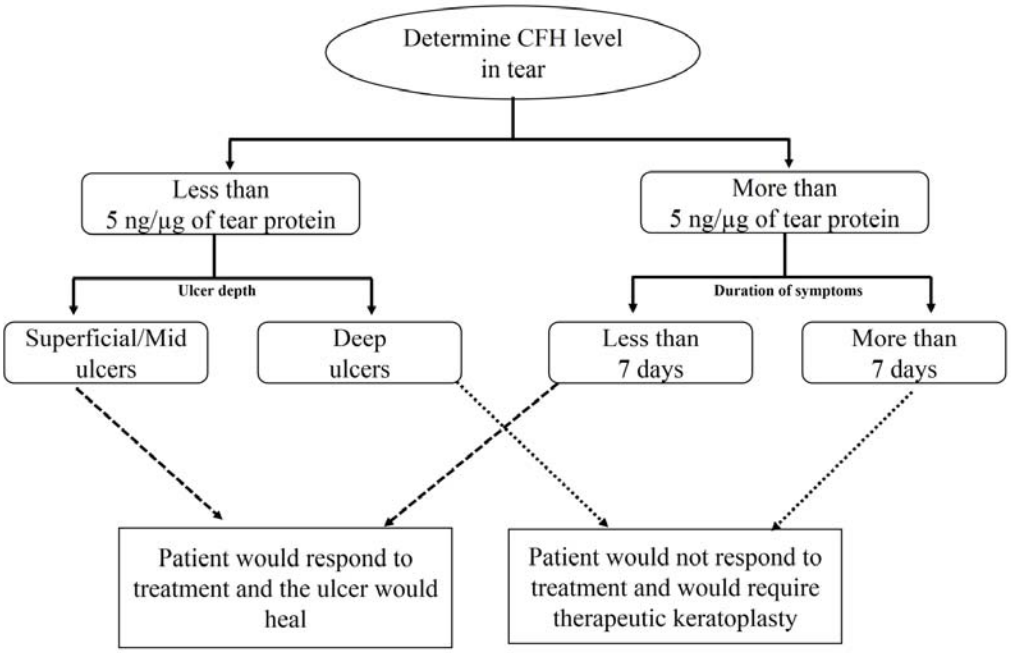


Figure 2. Model to predict the response of A.flavus keratitis patients to anti-fungal treatment

lower than the threshold value of 5 ng/µg of tear protein, depth is used as a predictor. On the other hand, if the CFH is more than 5 ng/µg of tear protein, duration of symptoms is used as a predictor. This model has predictive power with 86% sensitivity and 82% specificity. The area under the ROC curve is 0.82 that qualifies this model as good prediction system.

This study is a pilot study that would be extended further to refine the model to improve its predictive power, particularly the sensitivity. The current mode of treatment involves topical application of anti-fungal drugs with a combination of systemic antifungals (wherever indicated). The patient is monitored for the response which can be favorable if the ulcer begins to heal or the ulcer can worsen in spite of the best possible medical management. Corneal transplantation is generally done in patients who do not respond to medical management and in cases of perforation of the cornea. Keratoplasty is resorted to if keratitis worsens on medical management. This model developed in this study would be useful in identifying those patients who would not respond to treatment and can be advised keratoplasty at the earliest. Early therapeutic keratoplasty can eradicate infection and ensure a better long-term visual outcome in such cases.

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Characterization of Aspergillus flavus exoproteome during early stages of propagation Aspergillus flavus keratitis

Investigators : Dr. J. Jeya Maheshwari, Prof. K. Dharmalingam, Dr. Lalitha Prajna
Team : M.Kanmani, Lakshmi Prabha
Funding : Indo-French Centre for the Promotion of Advanced Research-CEFIPRA

Rationale of the study

Fungus secretes a wide range of biological macromolecules such as enzymes, toxins, effector molecules, secondary compounds. In pathogenic fungi, many of the secreted proteins are usually the main effectors that mediate interactions between the host and fungus. The composition of the exoproteome is a signature of each specific fungus and is dependent on the host and the environment it encounters (Girard *et al*., 2013).

During the infection of the cornea, the fungal spores gain access into the cornea, presumably through epithelial trauma. The early events that subsequently lead to a full blown infection are still not clear. To understand this, we are characterizing the extracellular proteins of *A. flavus* conidia in the early stage of germination. The secreted proteins of the germinating conidia are probably the earliest fungal factors that help the pathogen to invade the host. Thus, these proteins are potential virulence factors as they help in establishing an infection as well as in combating the host response to infection.

Results

Analysis of the secretome of the early germinating conidia is relatively an unexplored area due to the inherent difficulties in exoprotein preparation, particularly due to the presence of heterogeneous and dilute population of proteins. Hence, various parameters for the exoprotein preparation were optimised. Spores were inoculated in the minimal medium (Czapak Dox liquid broth) and incubated at 30°C for six hours with shaking (150 rpm). The supernatant containing the extracellular proteins was concentrated by ultrafiltration using a 10 kDa cut-off filter. The proteins were resolved in a 12.5% SDS-PAGE and visualized by staining with coomassie or silver.

A time course analysis was first performed for a duration during which the spore swells, germinates (6 h), forms hyphae (12 h) that result in dense mycelium (24 h) (Figure 1).

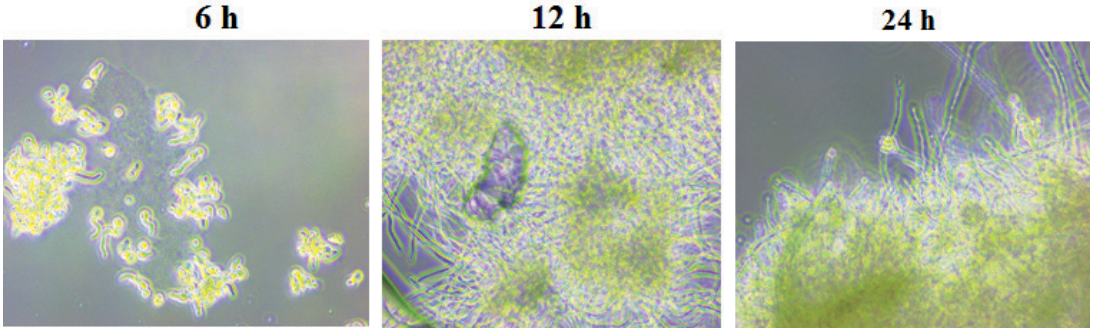

Figure 1. Different stages of growth of A. flavus. Spores grown at 30 °C in Czapak Dox broth were observed under the phase contrast microscope at three different time points.

Using the optimised method of exoprotein preparation, the exoproteins of 6, 12 and 24-hour culture were compared as shown in Figure 2A. It is evident that some proteins (shown in black arrows) are seen exclusively in the early time point that is when the spores are in the germination stage. On the other hand, there were many proteins (indicated in red) that were seen only in the later stages of growth (hyphal and mycelial forms).

As the early stage proteins are potential virulence determinants of *A. flavus*, the exoprotein profile of a reference strain was examined with that of two clinical strains (Figure 2B). Although many proteins were found to be common to the three strains, some proteins were either unique or differentially regulated in each of the *A. flavus* strains examined (indicated by red arrows).

Current efforts are to obtain a comprehensive in-depth identification of proteins that are secreted by germinating conidia as well as to identify the differentially expressed proteins in each of the *A. flavus* strains. This would be done by analyzing the exoproteins using a bottom-up mass spectrometry-based approach. The identification and characterization of these early stage conidial proteins would throw light on the pathogenic mechanisms underlying *A. flavus* infection of the cornea.

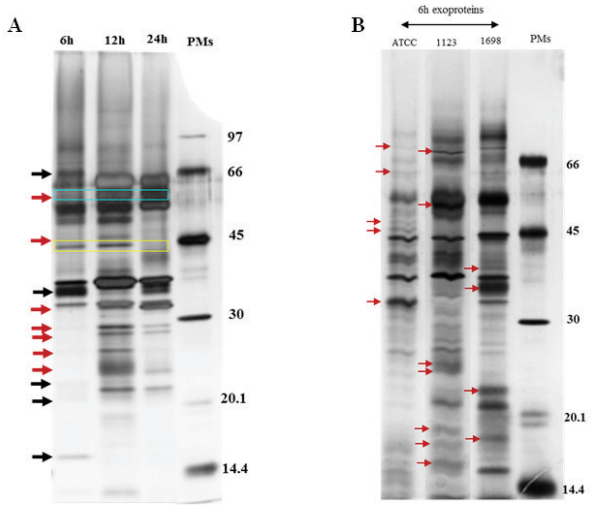

Figure 2. Analysis of variation in exoprotein profile across different time points and strains. A.Time course analysis of the changes in the exoprotein profile at different stages of growth. B. Comparison of exoprotein profile across the reference strain (ATCC) and two corneal isolates (CI1123 and CI1698).

References

1. *Girard, V., Dieryckx, C., Job, C. & Job, D. (2013). Proteomics 13, 597-608.*

Predictive Biomarkers for Diabetic Retinopathy among diabetic patients and stage-specific biomarkers for NPDR and PDR

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Clinician Scientists	:	Dr. Kim, Dr. Bhanu Pangtey
Team	:	Naveen Luke Demonte, Roopesh Pai, Sharmila, Anjhu V. Nair, Dr. Haemoglobin, Dr. Ashish Kamble, Dr. Piyush Kohli
Funding	:	Mindtree Grant

Rationale of the study

Diabetic Retinopathy (DR) is the result of vascular changes in the retinal circulation seen in a subset of diabetic individuals. In 2002, DR accounted for about 5% of world blindness, representing almost 5 million blind and has been listed as one of the priority eye diseases by WHO. A study by Namperumalsamy and group (2009) in the Theni district revealed that 22% of diabetes patients have diabetic retinopathy and 10% amongst them developed a severe form of the disease. Only a subset of diabetes patients develop DR and a subset of these DR patients progress rapidly to proliferative diabetic retinopathy (PDR). Therefore, the aim of the on-going project is to identify predictive and prognostic biomarkers for DR that can

1. predict the subgroup of diabetics who are at high risk of developing DR
2. monitor the progression of DR

I. Identification of Serum biomarkers

Using a comprehensive bottom-up approach (Figure 1A), 57 proteins (Figure 1B) were found to be differentially regulated in the serum of PDR patients in comparison to that of diabetics.

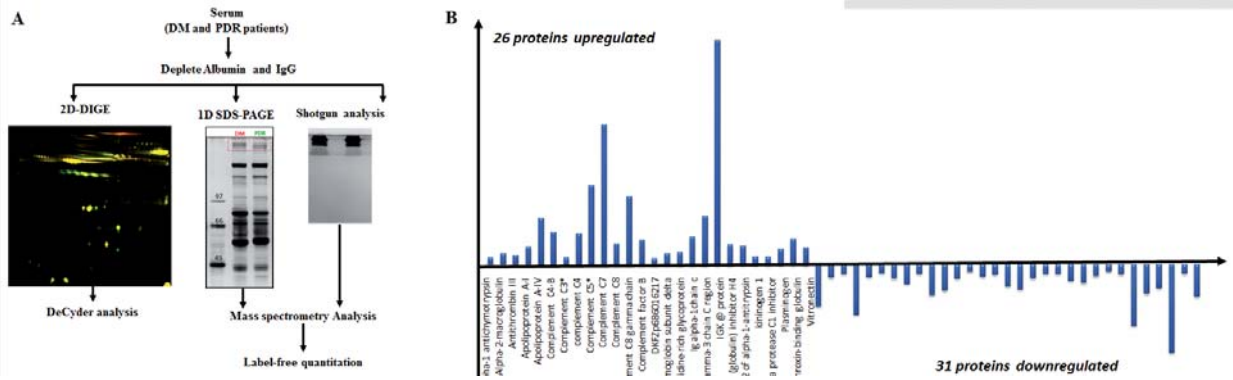

Figure 1. Discovery phase study for identification of serum biomarkers. A. Workflow followed in the bottom-up approach for identification of differentially expressed proteins in PDR patients. B. Differential expression of 57 proteins identified using the workflow shown in figure 1A.

Analysis of complement activation in DR

Complement system not only produces pro-inflammatory molecules such as C3a but is also involved in the active regulation of inflammatory response. Complement protein C3 is the converging point of all the three complement pathways. In the discovery phase experiments, C3 was identified to be both upregulated and downregulated. On careful analysis, it was found that the 110 kDa C3b form was 7-fold downregulated while the processed form at 41 kDa, C3dg was 1.6 fold upregulated in PDR when compared to DM patient serum. Differential levels of C3b (and C3dg) can be considered as an indication of the activation of fluid-phase complement system. To examine the activation of complement system in patients representing different stages of disease progression, immunoblot analysis was performed with anti-C3dg antibody for albumin depleted serum from four different stages of disease: Healthy controls (non-diabetic), type 2 diabetics, non-proliferative DR and proliferative DR. Figure 2A shows the variation in the levels of C3dg (at 20 µg of total serum proteins) and Figure 2B shows the difference in the expression of the 110 kDa C3b levels (at 5 µg of total serum proteins). It can be observed that the level of both C3b and C3dg are variable in control, DM and NPDR patients. However, both these forms are present in most of the PDR samples analyzed suggesting the presence of an activated inflammatory response in PDR.

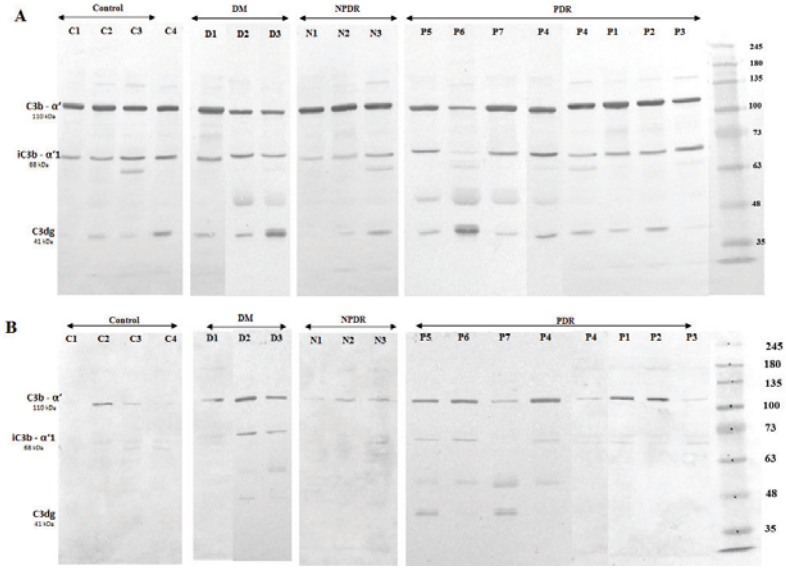

Figure 2. Analysis of complement activation at different stages of DR. Levels of C3b and its processed forms were analyzed by immunoblot analysis using monoclonal anti-C3dg antibody in albumin depleted serum at two different serum concentrations, 20 µg (A) and 5 µg (B).

Densitometry analysis was done to compare the difference in the level of each of the three bands across the samples from different categories. This analysis revealed that the total C3 levels are higher in DM patients which decrease in the NPDR stage and increases in the PDR condition (Figure 3A). Figure 3B shows the relative proportion of C3b and its two processed forms, C3b-α'1 and C3dg across the four sample categories. Interestingly, in NPDR although the total C3 is less, the proportion of C3b is much higher than the processed forms when compared to the other categories.

Currently fluid phase activation of complement through detection of different forms of C3 in a larger sample set is being examined.

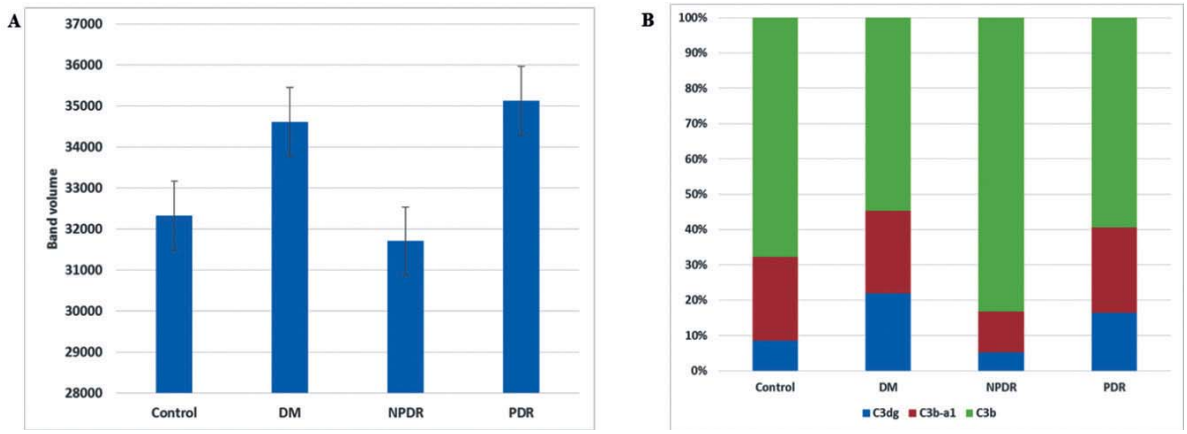

Figure 3. C3 level across different stages of DR. Based on the densitometry analysis of the blot shown in Figure 2A, the comparison of the total C3 level (A) and the relative proportion of C3b and its processed forms (B) across the four different sample categories is shown.

II. Microparticles as a source of biomarkers

In serum, proteins are present in a vast dynamic range spanning 12 orders of magnitude. The presence of high and moderate abundant proteins limits the ability to detect changes in the proteins present at low and very low levels. To overcome this problem and to identify proteins that might reflect DR-specific changes, a sub-proteome approach was adopted, where the proteome of circulating microparticles in blood was analysed. An elaborate comparison of the proteome of microparticles from serum as well as from plasma was carried out. This comparative analysis revealed that although the majority of the proteins were identified in serum and plasma microparticles, additional proteins were identified in serum microparticle proteome. The serum microparticle proteome of DM and PDR patients was examined then. Proteins in the serum microparticles from DM and PDR patients were identified by mass spectrometry and compared. When the proteins identified in these microparticles were taken for GO enrichment analysis, it was found that 53 proteins were already annotated to be present in extracellular exosomes (Figure 4). The others were either not annotated as exosome proteins or were carryover contaminating proteins from serum.

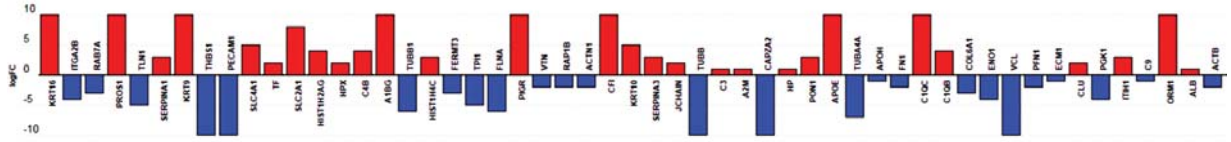

Figure 4. The GO annotation term "extracellular exosome" (GO:0070062) includes 70 proteins, of which 53 were found in our dataset. The proteins are ranked based on the significance of their measured fold change. Upregulated proteins are shown in red, downregulated genes are shown in blue.

Comparative analysis of DM and PDR microparticle proteome revealed that 50 proteins were upregulated (≥ 2-fold) in PDR microparticles while 53 proteins were downregulated (≤ 2-fold). Figure 5 is a volcano plot for 67 proteins with statistically significant fold change.

Proteins upregulated in the microparticles in PDR (Figure 6) include complement proteins as well as anti-inflammatory proteins indicative of a systemic inflammatory response.

(Figure 7) shows the fold change of the downregulated proteins that could, in turn, be categorized into various classes such as

1. Proteins involved in regulation of actin cytoskeleton
2. Microtubule network and gap junction proteins
3. Focal adhesion complex proteins
4. Signaling pathway proteins
   a. Rap1 signalling
   b. PI3K-Akt signalling

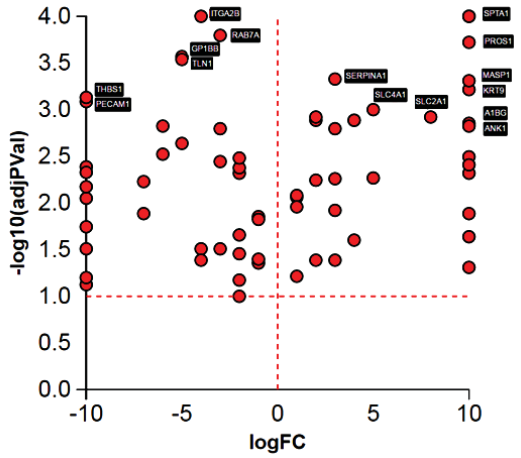

Figure 5. Volcano plot: All differentially expressed proteins are represented in terms of their measured expression change (x-axis) and the significance of the change (y-axis). The significance is represented in terms of negative log (base 10) of the p-value. Dotted lines represent the threshold used to select the differentially expressed proteins

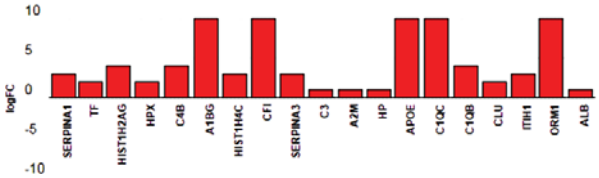

Figure 6. Expression bar plot showing the proteins upregulated in PDR microparticles.

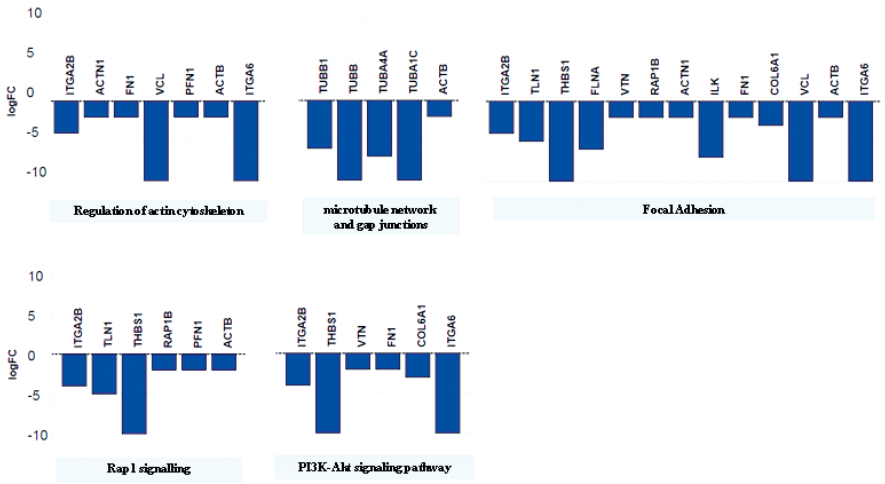

Figure 7. Expression bar plot shows the downregulated proteins of different functional categories. The proteins are ranked based on the significance of their measured fold-change.

The proteins found to be downregulated in PDR microparticles are known to interact with each other and are involved in the regulation of multiple processes such as coagulation, cell adhesion, migration, angiogenesis (Figure 8).

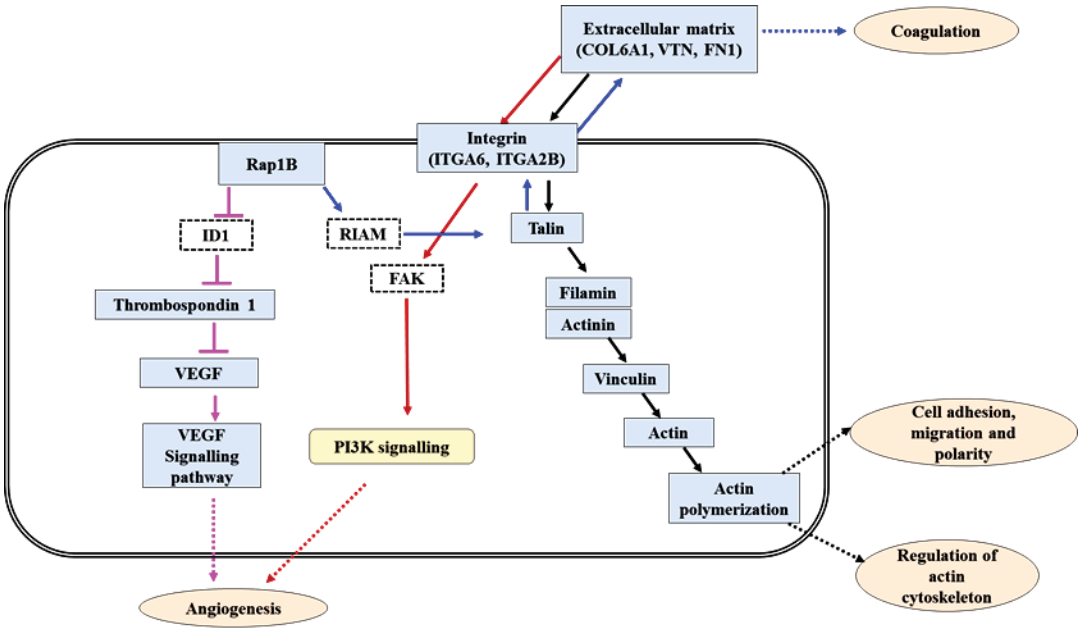

Figure 8. Schematic representation of the interaction between proteins found to be downregulated in PDR microparticles.

The functional relevance of these proteins and the effect of downregulation with special reference to DR is being currently examined. Selected proteins will further be validated in a larger number of samples.

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1. *Namperumalsamy, P., Kim, R., Vignesh, T. P., Nithya, N., Royes, J., Gijo, T., Thulasiraj, R. D. & Vijayakumar, V. (2009). Br J Ophthalmol 93, 429-434.*

PROTEOMICS OF HOST IMMUNE RESPONSE TO FUNGAL INFECTION

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Clinician Scientists : Dr.N.Venkatesh Prajna, Dr. Lalitha Prajna
Team Members : K.Sandhya, S.Mohammed Razeeth, Naveen Luke Demonte, Dr.Ramya Seetham Raju, Dr.Lakshay Dudeja
Funding : Programme support for research on human Mycotic keratitis, Department of Biotechnology, Government of India.

1.1 Proteomics of mycotic Keratitis patient tear

Aspergillus flavus and *Fusarium sp*. are primary causative agents of keratitis that results in corneal tissue damage leading to vision loss particularly in individuals from the tropical parts of the world. Proteins in the tear film collected from control and keratitis patients were profiled and compared. A total of 1873 proteins from control and 1400 proteins from patient tear were identified by mass spectrometry. While 847 proteins were found to be glycosylated in the patient tear, only 726 were glycosylated in control tear. And, some of the tear proteins showed alterations in their glycosylation pattern after infection. Complement system proteins, proteins specific for neutrophil extracellular traps and proteins involved in would healing were found only in the patient tear. The presence of these innate immune system proteins in the tear film of patients supports the previous data indicating the involvement of neutrophil and complement pathways in antifungal defense. High levels of wound healing proteins in keratitis patient tear implied activation of tissue repair during infection.

The early appearance of the host defense proteins and wound healing response indicates that tear proteins could be used as an early marker system for monitoring the progression of pathogenesis. Identification of negative regulators of the above defense pathways in keratitis tear indicates an intricate balance of pro and anti-defense mechanisms operating in fungal infection of the eye.

Publications from this work

1. Jeyalakshmi Kandhavelu, Naveen Luke Demonte, Venkatesh Prajna Namperumalsamy, Lalitha Prajna, Chitra Thangavel, Jeya Maheshwari Jayapal, Dharmalingam Kuppamuthu "*Aspergillus flavus* induced alterations in tear protein profile reveal pathogen induced host response to fungal infection" J Proteomics. 2017 Jan 30.
2. Jeyalakshmi Kandhavelu, Naveen Luke Demonte, Venkatesh Prajna Namperumalsamy, Lalitha Prajna, Chitra Thangavel, Jeya Maheshwari Jayapal, Dharmalingam Kuppamuthu "Data set of *Aspergillus flavus* induced alterations in tear proteome: Understanding the pathogen-induced host response to fungal infection" Data in Brief 9(2016):888–894.

1.2. Role of complement control Factor H in fungal keratitis

Complement is the major humoral component of the innate immune system, likely to have a role in antifungal defense in ocular infections. Mass spectrometry of glycosylated protein fraction of tear proteins revealed that most of the complement proteins were found in patient tear and not in control tear.

Interestingly, inhibitors of C3 convertase, factor H (CFH) and factor I (FI) along with other negative regulators of complement pathway, vitronectin and clusterin were also found in the patient tear. These data show that the complement pathways could be activated upon fungal infection. (Jeyalakshmi *et al*., 2017) and their regulation is intricate.

1.2.1. Demonstration of Factor H in *A.flavus* and *Fusarium* keratitis patient tear

To confirm the presence of CFH in the tear film of keratitis patients observed in tear proteome, tear proteins were separated by SDS-PAGE and analyzed by Western blotting. CFH could be detected in the tear fractions as a 180-kDa protein by an anti-CFH antibody (FIG.1.2.1).

1.2.2. Absence of Factor H in uninfected fellow eye from *A.flavus* keratitis patients

CFH was observed only in the tear from infected eye whereas CFH was not detectable in the normal fellow eye of the same individual indicating that CFH is elicited only in response to the fungal infection.

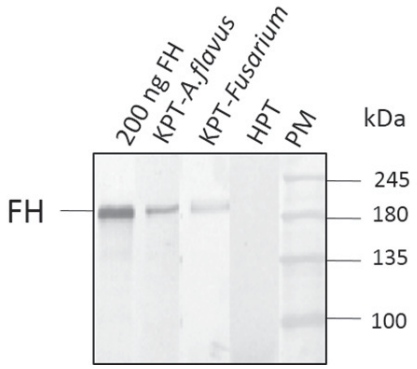


FIG: 1.2.1 Immunoblot analysis of keratitis patient tear (KPT) and healthy person tear (HPT). 200ng purified FH (1) was used as control.

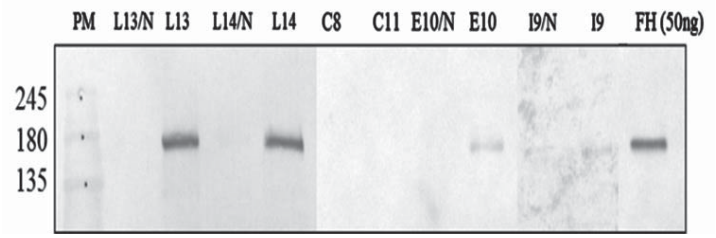


Fig 1.2.2: Western blot analysis of CFH for tear collected from the infected eye and the fellow eye without infection. 15 µg of total tear protein from Aspergillus flavus infected keratitis eye and from normal uninfected fellow eye were resolved on 1D SDS-PAGE (8%), transferred onto nitrocellulose membrane and probed against anti-CFH antibody (SC 33156).

L13 and L14 -tear collected from late stage *A.flavus* infection.

L13/N and L14/N -tear collected from the uninfected fellow eye of the same patient respectively.

C-tear collected from healthy individuals.

E10- indicates tear collected from early stage *A.flavus* infection.

E10/N- tear collected from the uninfected fellow eye of the same patient.

I9- tear collected from intermediate stage *A.flavus* infection.

I9/N- indicates tear collected from the uninfected fellow eye of the same patient.

1.2.3. CFH is down regulated upon treatment

Level of CFH was compared during the infection and after the completion of treatment. Results show that the levels of CFH were decreased when the intensity of the infection is reduced due to treatment indicating that the complemet control is reversible (fig.1.2.3).

E22- tear collected from early stage *A.flavus* keratitis infection.

E22/FO- tear collected from the same patient who has undertaken antifungal treatment.

I17- tear collected from intermediate stage *A.flavus* keratitis patient.

I17/FO- tear collected from the same patient who has underwent TPK surgery (Therapeutic penetrating keratoplasty).

1.2.4. CFH appears early in infection

Expression levels of CFH was compared (Fig.1.2.4) for tear samples collected on three consecutive days of early infection (< 7 days of infection) and the results show the appearance of CFH very early in infection.

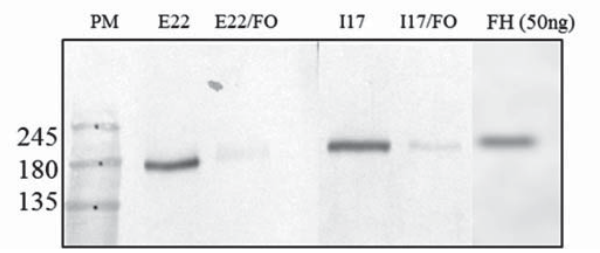


Fig 1.2.3: Western blot analysis of CFH before and after treatment

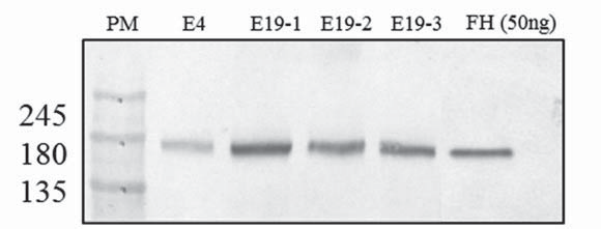


Fig 1.2.4: Western blot analysis of CFH. Tear collected from the keratitis patients starting from day one.

E-1- tear collected on the first day of visit to hospital from a patient.

E-2- tear collected from same patient on the second day.

E-3 -indicates tear collected from same patient on the third day.

1.2.5. C3 and other product in patient tear

Another central component of complement cascade is C3 protein which forms ultimately C3 convertase. Western blot analysis showed the appearance of C3 only in patient tear and not in control tear.

1.2.6. Demonstration of CFH and C3 binding to spores

In order to act as antifungal defense, complement complex (MAC) should form on the spores. To determine whether spores of *A.flavus* binds CFH, conidia of strain CI 1123 were incubated in purified CFH. After extensive washing, the bound proteins were eluted. The wash and elute fractions were separated by SDS-PAGE and analyzed by Western blotting. CFH was detected in the eluate

fractions as a 180-kDa protein. The presence of CFH in the eluate and not in the wash fractions (data not shown) demonstrates that CFH conidia binds to the spore surface. Similar experiments with C3 also showed binding of C3 to the spores. This and other experiments showed that the complement components can assemble on the infecting spore surface and possibly have a role in immune defense.

1.2.7.Activation of alternative pathway in the tear fluid Mycotic keratitis

To demonstrate the functional alternative pathway of complement cascade in Mycotic keratitis, Tear samples from *Aspergillus flavus* keratitis patients were incubated with rabbit RBCs and checked for the lysis, which directly correlates with the activation of MAC as the cell surface.

Enhanced hemolysis of rabbit erythrocytes was observed in tears collected from Fusarium keratitis (10-15%) patients compared to the healthy individuals (4-5%) indicating activation of alternative complement pathway for patients with mycotic keratitis

Isolation and characterization of Rodlet layer and morphological property of *A. flavus*

Investigators : Prof.K.Dharmalingam, Dr.A.Rabbind Singh, Dr.J.JeyaMaheshwari
Clinician Scientists : Dr.N.Venkatesh Prajna, Dr. Lalitha Prajna
Team Members : S.Mohammed Razeeth, Naveen, Dr.Ramya, Dr.Lakshay Dudeja
Funding : CEFIPRA

Introduction

Mycotic keratitis is a leading cause of blindness in tropical countries. Airborne spores (conidia) of these filamentous fungi express a surface protein that confers hydrophobicity (hydrophobin) and covers cell wall. Conidia are coated by a hydrophobic rodlet layer composed of regularly arranged RodA hydrophobins, which are covalently bound to cell wall polysaccharides by GPI anchor proteins. Surface conidia mask recognition by immune system and hence prevent immune response.

Rodlet layer isolation

Dry *Aspergillus flavus* spores were incubated with 100% formic acid at different time points 1, 2, 3, 6 and 72 hours at 4°C to extract the conidial surface proteins. The extract was dried by sparging nitrogen gas. The dried protein mixture obtained was resuspended in 1X SDS sample buffer and boiled for 5 min. Proteins were separated in SDS PAGE and stained with coomassie (FIG.1).

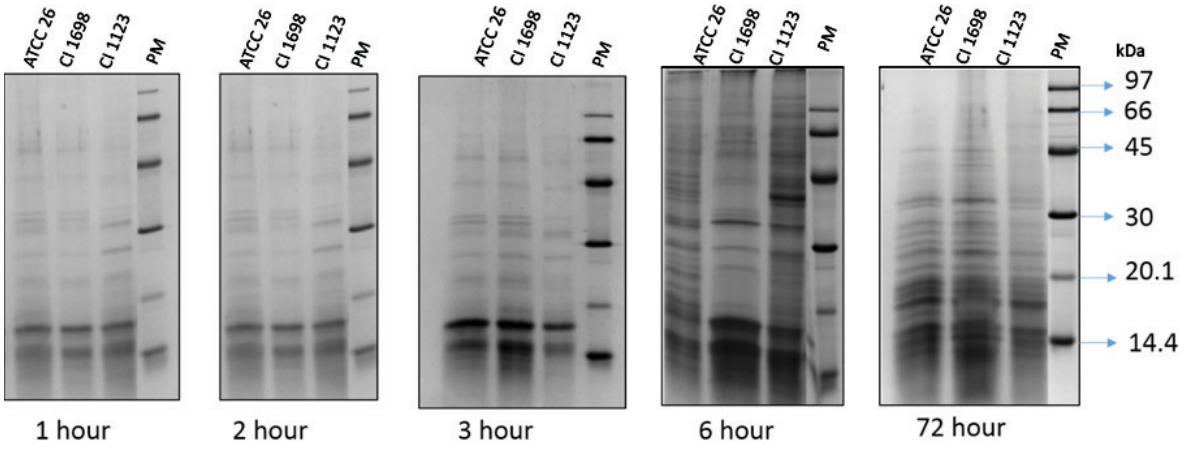


FIG. 1. Analysis of formic acid soluble material from extract of A.flavus conidia.10µl of extract was loaded in SDS-PAGE (%16) gel were stained with commasie brilliant blue

MS analysis of Rodlet layer

Upon analyzing the protein extracted from different time points. One hour formic acid extraction seemed to be sufficient to extract the conidial surface proteins. Protein extracted from one hour formic acid treatment was taken for MS shot gun analysis. One hour formic acid extract of three *A. flavus* isolates ATCC- 200026 saprophyte and two other clinical isolates CI-1698 (Healed case) and CI-1123(Surgery case) were chosen for MS sample preparation. Proteins were digested using enzymes Trypsin and Glu-C to obtain higher peptide fragment. Dried peptides were suspended in 10 µl of 0.1% FA and analyzed in a nanoLC-Orbitrap Velos Pro mass spectrometer. Raw data file was analyzed using proteome discover. (FIG.2)

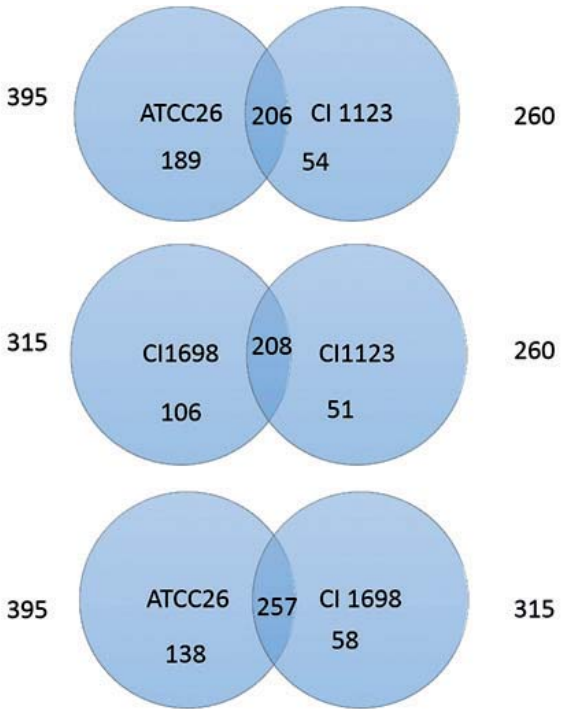


Fig. 2 MS analysis of surface protein of A.flavus conidia

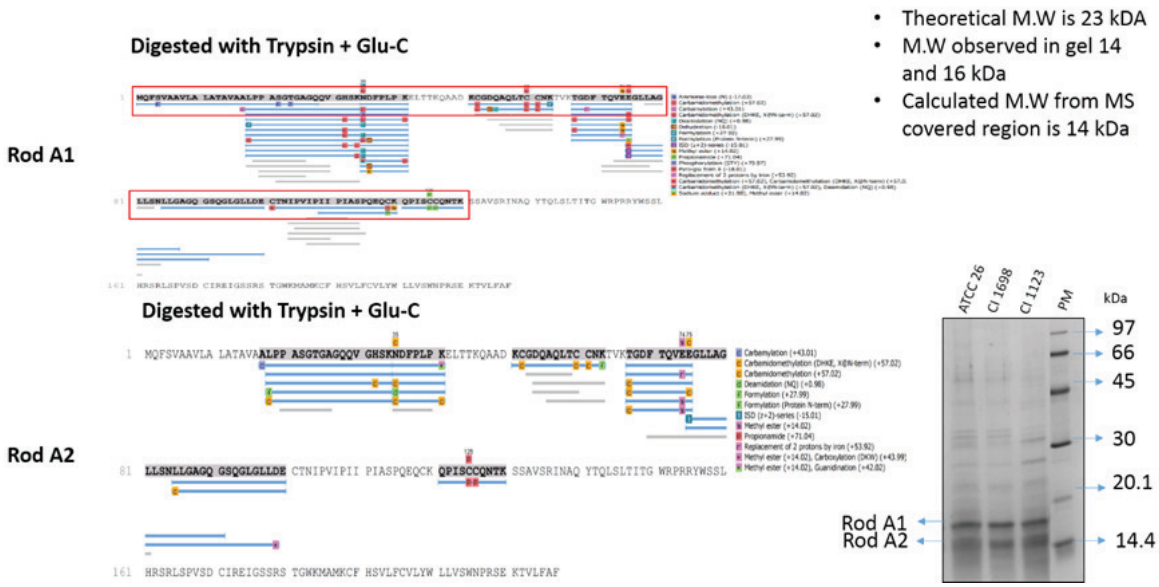
MS analysis shows identification of rodlet layer RodA and also protein associated with cell wall component such as beta-glucan family proteins, Allergenic proteins like Allergenic cerato-platanin, allergen Asp and Extracellular cell wall glucanase Crf1/allergen Asp.

Table 1. List of cell wall associated protein identified from MS analysis

Uniprot id	Name	ATCC 26	CI 1698	CI 1123
B8N2S0	ABC multidrug transporter	+	-	-
B8N1C2	MFSmultidrug transporter	+	+	-
B8NQL4	Molecular chaperone and allergenMod-E/Hsp90/Hsp	+	+	+
B8MZ47	Extracellular cell wall glucanase Crf1/allergen	+	+	+
B8NHG5	Allergenic cerato-platanin Asp F13	+	+	+
B8N2H3	Enolase/allergen Asp F 22	+	+	+
B8NTJ8	Conidial hydrophobin RodA/RolA	+	+	+
B8NAF9	Antigenic cell wall galactomannoprotein	+	-	-
B8NA16	GPI-anchored cell wall organization protein	+	+	+
B8N327	GPI anchored protein	+	+	+

Identification of two intense band near 14 kDa

Coomassie stained 1D-SDS PAGE shows two intense bands near 14kDa. Both these bands were digested using Trypsin and GLU-C followed by MS/MS analysis. Raw files were analyzed using Peak'S 7.0 software. MS/MS analysis results identified the two bands as Rod A. Presented below are the peptides (highlighted in yellow) picked up by MS/MS analysis.



Analysis of Rod A and Rod B transcript by PCR

Rodlet layer is formed by proteins RodA and RodB. NGS transcriptomics data shows expression variation between ATCC-26 and clinical isolates. To confirm this variation in expression levels of RodA and RodB, RNA was isolated from fungi grown at 30°C and 37°C for 24 and 40 hours. Extracted RNA was converted into cDNA and RT PCR was carried out.

RodA gene of *A. flavus* is of 706 bp having two exons and an intron of 52 bps. RodA transcript was amplified from cDNA using primer E1F (exon 1 forward) and E2R (exon 2 reverse) expected amplicon length being 199bp. The result of PCR shows two bands with one in expected size range of 199bp and another one of 250bps. This observation indicates a possibility of an intron retention alternate spliced form. The intensity of alternate spliced form was higher at 37°C when compared to 30°C at 24 hour. Native form with only exonic region is very low in 24 hours. In case of 40 hours cultures, alternate spliced form was seen at a very minimum level.

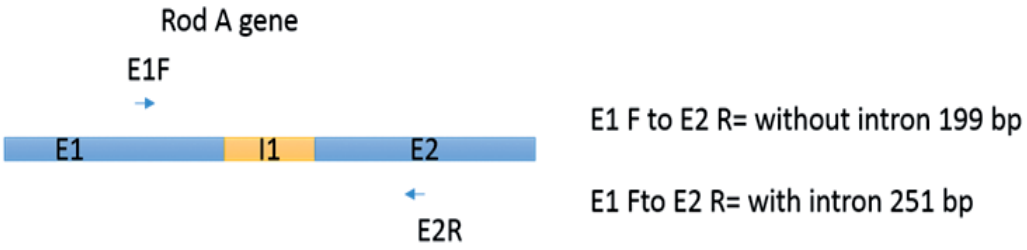


Fig. 3 Gene architecture of RodA gene

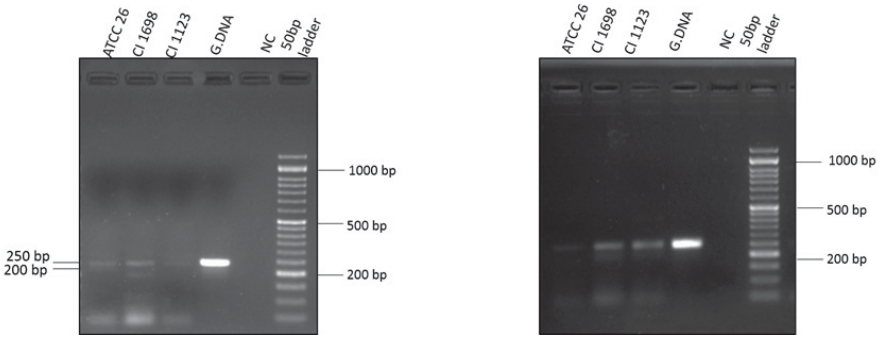


FIG 4, RT-PCR was carried out using total RNA extracted from A.flavus grown at 30°C and 37°C for 24 hours. FIG 4A @ 30°C Rod A, FIG 4b- @ 37°CRod A. lane 1- genomic DNA, lane 2- ATCC 26, lane 3- CI 1698, lane 4-CI 1123 , Lane 5- 50bp DNA ladder. Amplification using primers E1 F to E2 R

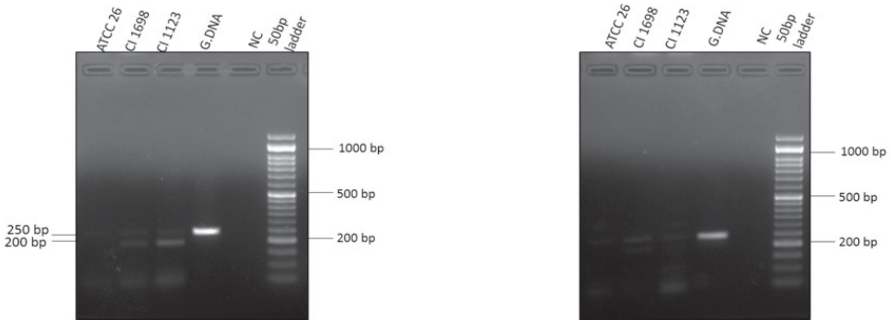


FIG 5, RT-PCR was carried out using total RNA extracted from A.flavus grown at 30°C and 37°C for 24 hours. FIG 5A @ 30°C Rod A, FIG 5b- @ 37°C Rod A. lane 1- genomic DNA, lane 2- ATCC 26, lane 3- CI 1698, lane 4-CI 1123 , Lane 5- 50bp DNA ladder. Amplification using primers E1 F and E2 R

RodB gene of *A. flavus* is of 847 bps having three exons and two introns. RodB transcript was amplified from cDNA using primers E1F and E1R and the expected length of the amplicon being 200 bps. The results of the PCR product revealed a single band of the expected length. The levels of expression was nearly same in the cultures grown at 30°C and 37°C in both 24 and 40 hours but expression in ATCC 26 was barely visible in 37°C grown mycelium.

Gene architecture of RodB gene

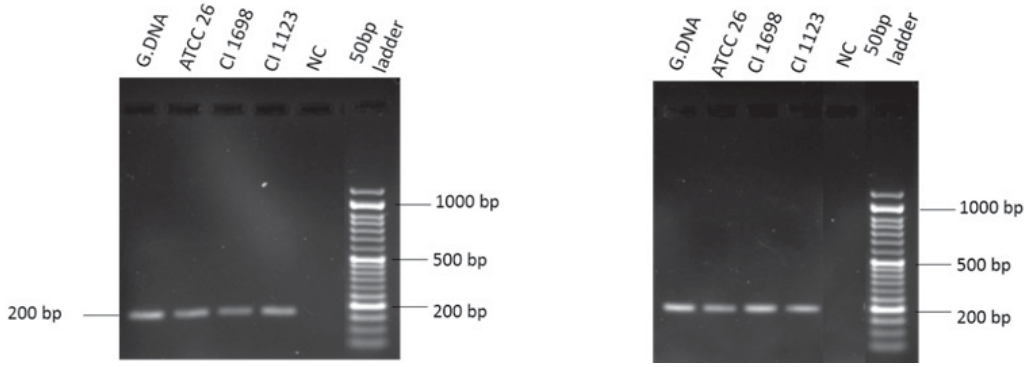


Fig: 6a *Fig: 6b*

FIG 6, RT-PCR was carried out using total RNA extracted from A.flavus grown at 30°C and 37°C at 24 hours. FIG 6a @ 30°C Rod B, FIG 6b- @ 37°C. Amplification using primers E1 F and E1 R

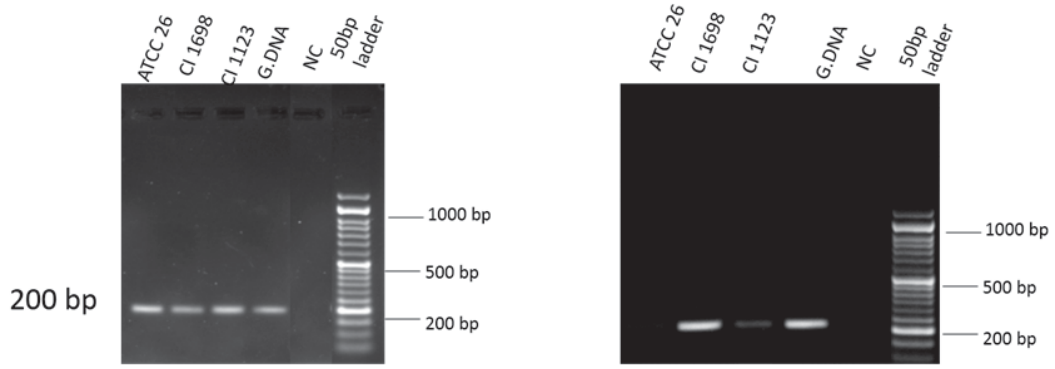
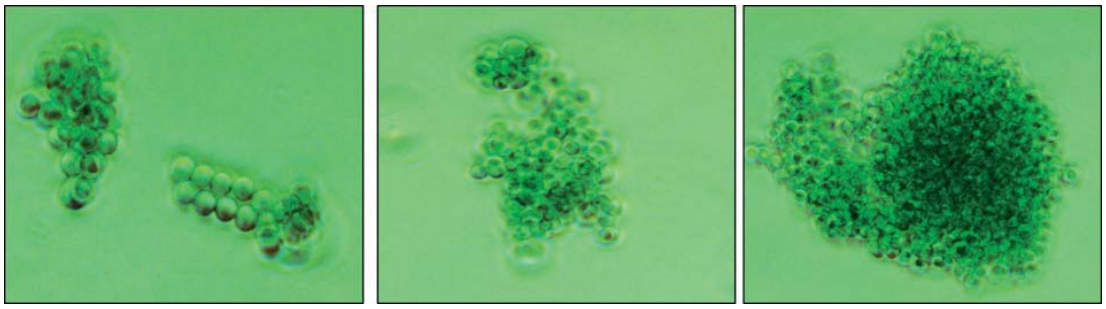


Fig: 7a *Fig: 7b*

FIG 7, RT-PCR was carried out using total RNA extracted from A.flavus grown at 37°C/24 and 40 hours. FIG 7a @ 30°C Rod B, FIG 7b- @ 37°C. Amplification using primers E1 F and E1 R

Spore clumping assay

Difference in spore clumping property between ATCC 26 and clinical isolates was examined. *A.flavus* spores were allowed to swell in CZB for 3hours and examined in a phase contrast microscope at 40X magnification. ATCC 26 saprophyte spores show minimal level of clumping but the clinical isolates CI 1123 and CI 1698 shows maximum spore clumping. FIG.7 clearly shows that spore clumping varies between ATCC 26 and Clinical isolates. There is no direct relationship between spore clumping and virulence property.



ATCC 26 *CI 1698* *CI 1123*

FIG 7 A. flavus spores were allowed to swell in CZB for 3 hours and spores examined under phase contrast microscope 40X magnification

1.3 Human Corneal Epithelial cell line as a model system to study fungal infection

Investigators	:	Prof. K. Dharmalingam, Dr. O.G. Ramprasad
Clinician Scientists	:	Dr. N. Venkatesh Prajna, Dr. Lalitha Prajna
Team members	:	A. Divya, S. Mohammed Razeeth, Naveen Luke Demonte
Funding	:	Programme Support for Research on Human Mycotic Keratitis, Department of Biotechnology, Government of India.

1.3.1 *A.flavus* infection induced changes in the proteome of Human Corneal Epithelial cell line (RCB2280)

Human Corneal Epithelial cells were stimulated with live and heat inactivated *Aspergillus flavus* spores and germinated conidia and the changes were examined using fluorescence microscopy and mass spectrometry.

1.3.2 Interaction of *A.flavus* conidia and hyphae with HCE cells

HCE cells were infected with *A.flavus* conidia and hyphae for 16hrs in DMEM:F12 serum free medium and the interaction was assessed by phase contrast microscopy. When conidia were added to the cells, they settled onto the cells within 30min, and swollen by 3hrs (Figure 1.3.2.1 B). It started to germinate after 6hrs (Figure 1.3.2.1 C) and formed hyphal mat at 9hrs (Figure 1.3.2.1 D). After 12hrs hyphal mat had completely covered HCE cells (Figure 1.3.2.1 E). There is also significant difference in the control and infected HCE nucleus. Shrinking of nucleus in some cells was observed by 9hrs and it was more at 12hrs.

Since the interaction with the host is mediated by the exposure of β-glucan during conidial germination, germinated hyphae was also used for infecting the HCE cells. These hyphae rapidly elongated so that a hyphal mat was formed by 6hrs (Figure 1.3.2.1 H). After 9hrs HCE cells were completely covered by hyphal mat (Figure 1.3.2.1 I). Shrinking of nuclei was observed by 6hrs and it increases with incubation time.

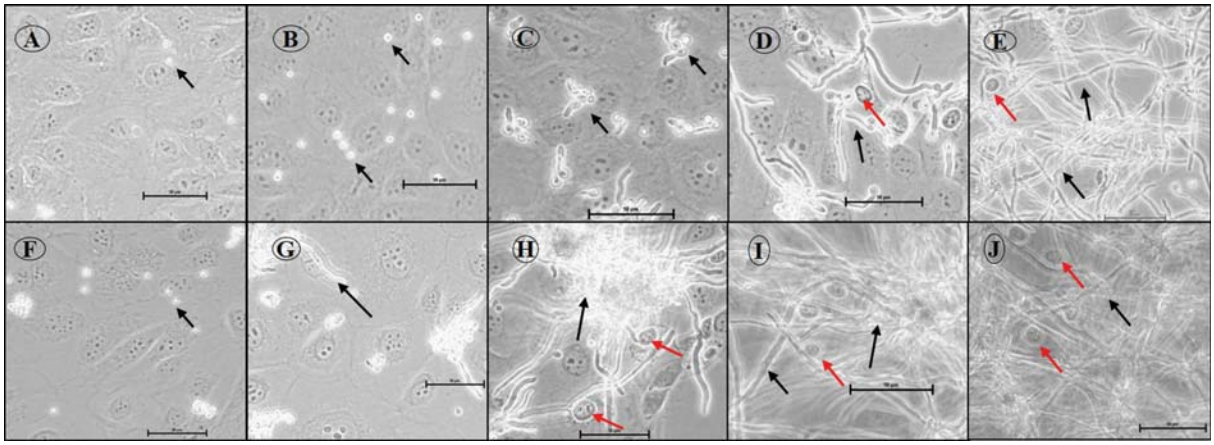


Figure 1.3.2.1: Morphological examination of HCE cells infected with A.flavus: Photomicrographs of Human Corneal Epithelial monolayer infected with A.flavus ATCC200026 conidia (A-E) and hyphae (F-J) after 0hr (A,F), 3hrs (B,G), 6hrs (C,H), 9hrs (D,I) and 12hrs (E,J) taken at 400X magnification. Black arrows indicate the organisms & red arrow indicates shrinked nucleus.

1.3.3 Phagocytosis and actin rearrangement assays

HCE cells($1x10^5$) were cultured in 18x18mm coverslip and infected with FITC labelled *A.flavus* conidia with m.o.i of 1:10 for various time point (0-2hrs). Following infection, cells were washed with 1X PBS, fixed with 4% PFA, permeabilized with 0.1% triton x-100 for 10 mins and cells were incubated with TRITC-phalloidin (1:40 in 1X PBS) for 40min to stain cellular actin filaments. Coverslip was washed with PBS, mounted using vectashield mounting medium containing DAPI and viewed under Leica LAS AF confocal microscope.

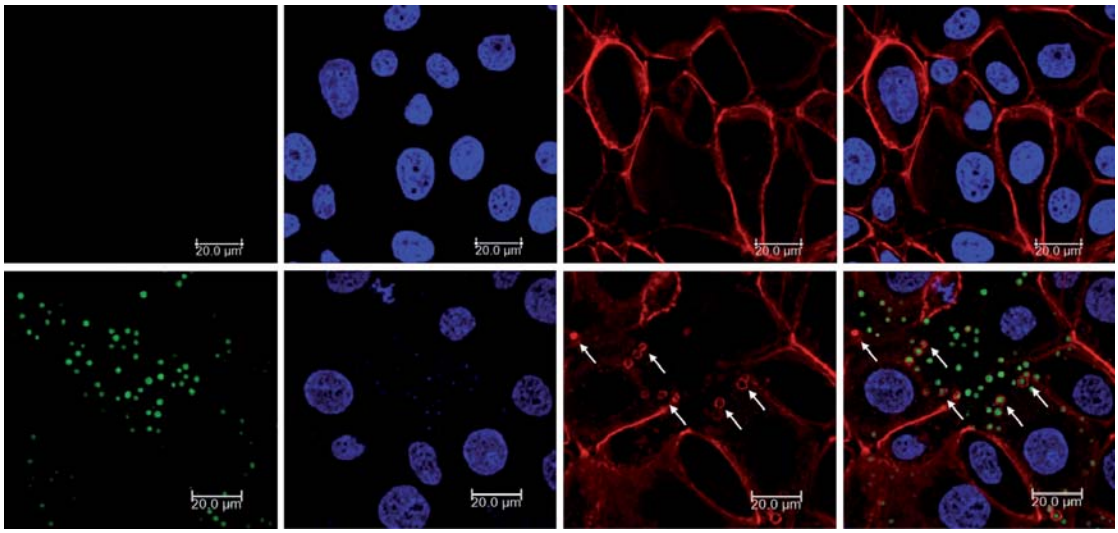


Figure 1.3.3.1: Confocal images of actin rearrangement after internalization of A.flavus conidia by HCE cells. A) Uninfected HCE B) HCE infected with FITC-labelled A.flavus ATCC200026 conidia for 15min at 37ºC. The images correspond to middle section of the cells by z-stacking. From left to right, panels show fluorescence image of green channel (FITC labelled conidia), fluorescence image of blue channel (DAPI), fluorescence image of red channel (F actin staining with TRITC-phalloidin) and merged overlay of all images. White arrow indicates actin ring surrounding the engulfed conidia.

HCE cells started to engulf *A.flavus* conidia within 15min and the engulfed conidia was found inside the vacuoles coated with polymerized actin (Figure 1.3.3.1). Although HCE is a nonprofessional phagocyte, it engulfed conidia within 15min showing they are as efficient as professional phagocyte RAW 264.7 (data not shown).

1.3.4 Comparative proteomics of HCE

HCE soluble proteome of uninfected and conidia/hyphae infected were compared by 1D gradient gel electrophoresis (Figure 1.3.4.1). Densitometric analysis was carried out using Image QuantTL software to select the differentially expressed proteins upon infection. There is no visible difference in the profile of HCE infected with A.flavus conidia/hyphae, however minor changes could have been missed.

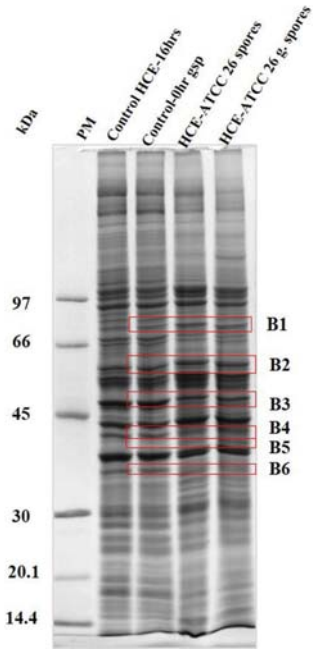


Figure 1.3.4.1: 1D gradient SDS-PAGE profile of HCE cells soluble proteins. 20µg of uninfected and A.flavus conidia/hyphae infected HCE soluble proteins were fractionated in 8-16% gradient gel electrophoresis and stained with colloidal-coomassie G250. Red box-Differentially expressed proteins.

Table 1.3.4.1 Number of proteins identified from the six bands

# Band	Number of proteins identified (Non redundant)		
	Control-HCE	HCE-ATCC26 spores	HCE-ATCC26 germinated spores
1	167	146	158
2	167	138	146
3	158	136	168
4	219	168	170
5	239	184	191
6	166	158	155

Six bands represented in the figure were taken for mass spectrometry identification and quantitation as a first step.

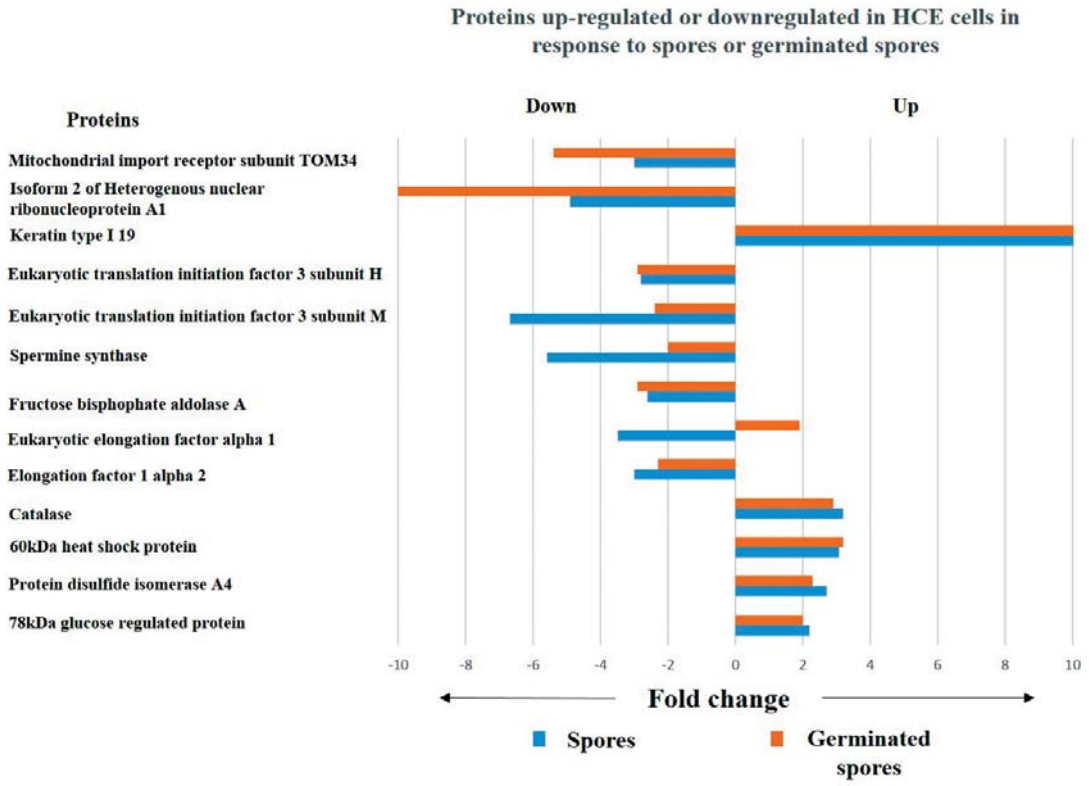


Figure 1.3.4.2 Proteins up-regulated or down regulated in HCE cells in response to spores or germinated spores

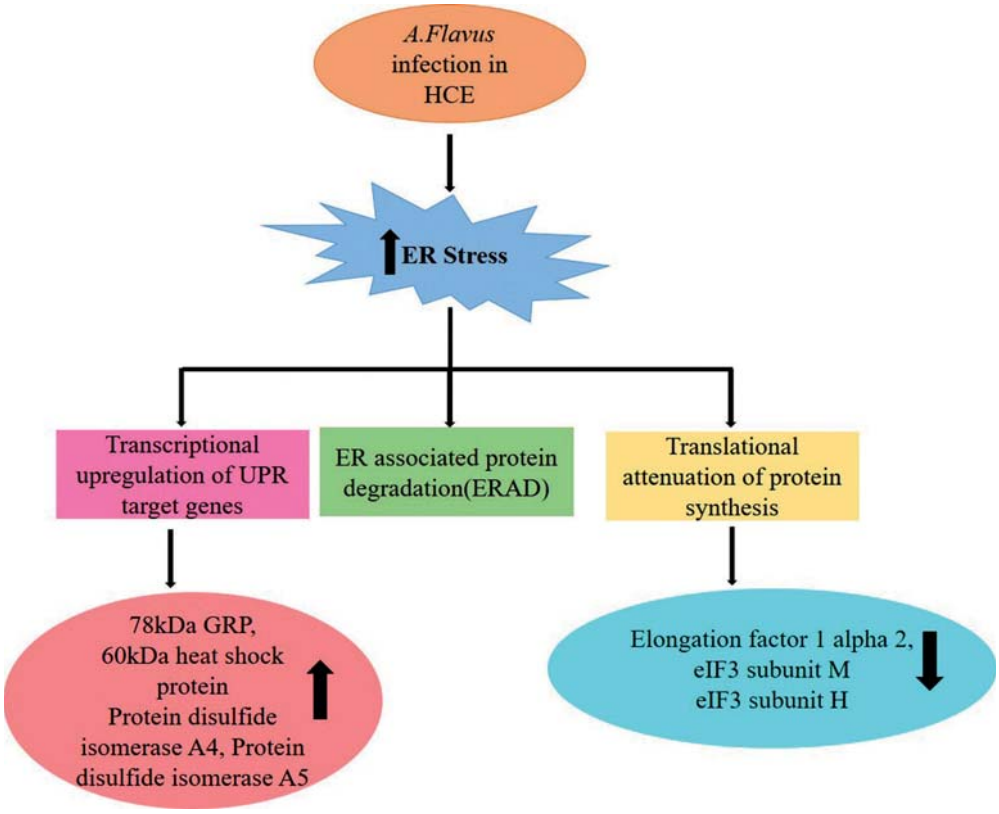


Figure1.3.4.3: Proposed model of A.flavus infection induced changes in HCE

Unfolded protein response (UPR) is a cellular homeostatic response to endoplasmic reticulum (ER) stress. Newly synthesized transmembrane and secretory proteins are folded and post-translationally modified within the endoplasmic reticulum (ER). Accumulation of unfolded proteins within the ER due to hypoxia, Ca2+ perturbation, and reactive oxygen species (ROS), causes a condition termed ER stress. To overcome ER stress and maintain ER function, cells activate the unfolded protein response (UPR) pathway. This signaling results in the reduction of protein amount entering into the ER by translational inhibition, enhancement of protein folding by transcriptional upregulation of ER chaperones, and degradation of misfolded proteins through ER-associated degradation (ERAD). If ER stress is prolonged and severe, UPR induces apoptosis. Several studies highlighted that UPR has implicated in many bacterial and viral infections, this is the first study exploring UPR and ER stress in corneal epithelial cell infected with fungus. The limited dataset shows *A.flavus* infection induced UPR specific marker Grp78 in HCE. In addition fungal infection promotes increased expression of many chaperone proteins and at the same time decreasing the proteins involved in the metabolic process in the host. So UPR pathway might have been activated in HCE cells upon fungal infection. Based on this data a model (Fig 1.3.4.3) is proposed.

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 fellow	:	Ranjani
Funding	:	SERB-Early career research grant (2016-2019) and the Mindtree grant.

Background

MicroRNAs (miRNAs) are a novel group of non-coding small RNA molecules with marked tissue specificity that post-transcriptionally control gene expression and are implicated in a large variety of physiological and pathophysiological processes (Ha and Kim, 2014). MicroRNAs have been detected in various body fluids including serum. Levels of miRNAs in the serum of humans have been shown to be stable, reproducible, consistent amongst healthy individuals but show changes during pathophysiology, allowing them to be of potential value as clinical biomarkers of diseases including cancers and metabolic disorders (Gilad *et al*., 2008).

Rationale

The role of serum microRNAs in the progression of DM to NPDR and NPDR to PDR in humans is largely unexplored. Therefore, the rationale of this study is to understand the regulatory function of microRNAs in the progression of microvascular complications among diabetic patients and also at the exploration of validating miRNAs as biomarkers.

Objectives

The objective of this study is to reveal disease specific miRNAs from serum of PDR, NPDR and DM patients and compare it with serum samples of control healthy patients. Further, identification of potential molecular targets of differentially expressed miRNAs and their regulatory networks towards the understanding of disease pathogenesis will be analyzed using computational tools. The differentially expressed miRNAs will be validated 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 methods utilized for the preliminary studies of the project involve miRNA isolation from serum samples and analysis of select miRNAs in purified RNA samples using quantitative real-time PCR. Isolation of total small RNA including microRNAs from 200µl of the serum samples of DM, PDR patients, NPDR patients (mild, moderate, severe) and control healthy patients was done using Qiagen miRNA serum/plasma kit. The miRNA yield in each sample was analyzed by Qubit miRNA fluorometric assay (Fig.1). Accordingly, micro RNA was found in serum samples of all the conditions. The DM, PDR and NPDR serum samples were having more miRNA than the normal control samples. A synthetic *C. elegans* miR 39 was used as a positive control for Qubit quantitation.

Quantitative real-time PCR amplification of specific miRNAs were done in all the serum samples. *C.elegans* miR-39 spike-in control was used for the normalization process during the relative quantification of the fold changes in the miRNA expression levels. Preliminary results indicate that miR-21 is upregulated by 2.63 folds in PDR patients and 3.82 folds in DM patients compared to control patients. miR-21 targets the angiogenesis process by activating AKT and ERK1/2 signalling pathways, thereby enhancing HIF-1α and VEGF expression which promotes neovascularization. Another miR-1179 didn't show any significant change in the levels of DM and PDR patients. This is significant because

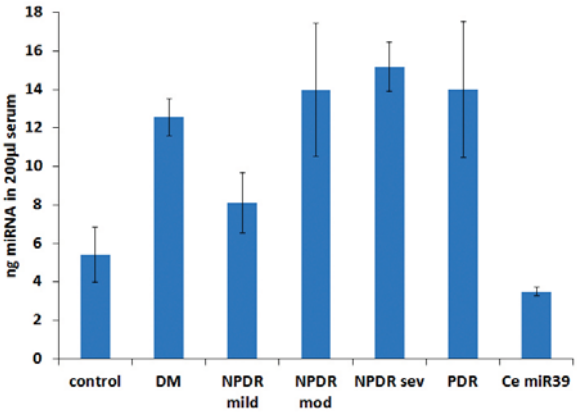


Fig.1: miRNA levels in serum samples of various category of DR, DM and control patients (n=5 from each condition). A synthetic C.elegans micro-RNA, miR-39 served as a positive control for quantification.

miR-1179 has been shown to be upregulated in Chinese population (Cell Physiol. Biochem, 2014, 34: 1733-1740). But, in patients of south Indian population there seems to be no changes in the expression levels of miR-1179.

Studies in progress: Subsequent studies will include the next generation sequencing of the miRNAs, their target prediction and regulatory networks using computational tools, their validation and functional analysis. Studies to analyze the changes in levels of miR-181c, miR-184, miR-200b, miR-31 and miR-125b will be expedited.

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

Investigators : Prof. Rachel Williams[1], Dr. N. Venkatesh Prajna[2], Dr. O.G. Ramprasad[3], Dr. Atikah Haneef[1], Prof. K. Dharmalingam[3], Prof. Colin Willoughby[1], Dr. Naveen Radhakrishnan[2], Dr.Kishan Prajapati[2], Mrs. Karpagam[4] and Dr. Kannan[4]

Project fellow : Ms. Jessica Judith Nunes

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2. Aravind Eye Hospital, Madurai.
3. Aravind Medical Research Foundation, Madurai
4. Aurolab, Madurai

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

Introduction

Keratoconus is one of the major bilateral corneal dystrophies 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. In the keratoconic corneal epithelium, the basal layer normally degenerates leading to the dissolution of Bowman's layer. Scarring, thinning and bulging of the cornea occurs in the diseased eye. Disruptions in collagen mechanical properties, significant loss of ECM, defective collagen cross-linking activity can lead to keratoconus (Rabinowitz, 1998; Kenney *et al*., 2012). The current treatment for mild keratoconus patients envisages the use of rigid gas permeable lenses and conventional corneal crosslinking. These treatments serve to slow and, in some cases, halt the progression of the disease by increasing collagen fibril linkages within the cornea, thereby preventing extreme curvature. 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.

Purpose of the study

This study aimed to develop a novel chemical cross-linker using EDCI/NHS [1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide] mediated chemistry and a suberic acid 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 the 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. The novel chemical cross-linker was used to analyse the cytotoxic effects and morphology of the corneal cells, its effect on the mechanical strength of the cornea and the depth of penetration into the cornea.

Results with porcine corneas

The cytotoxicity was assessed at 15 minutes and 24 hours post drug treatment using a resazurin assay on human corneal cell lines (HCE-T, HCEC-12) and primary porcine corneal epithelial and stromal cells. It was found that there was no cytotoxicity to the cell lines, however the metabolic activity of the sub-confluent primary cells initially decreased but recovered by 26% and 35% for stromal and epithelial cells respectively after 24 hours. The metabolic activity of fully confluent primary porcine epithelial and stromal cells was not affected significantly by the drug treatment. Histological analysis (H & E staining) showed that the epithelium and endothelium remained intact post drug treatment. The mechanical properties of corneal samples were measured and an increase in tensile strength of the cornea of 85% was determined after a 15 minute drug treatment.

Results with human corneas

The normal cadaver corneas obtained from the eye-bank at Aravind and the keratoconic corneas obtained from patients undergoing deep anterior lamellar keratoplasty (DALK) procedure were used for analysis.

The novel cross-linker showed negligible cytotoxicity to the normal as well as keratoconic corneal stromal cells harvested from the respective cornea. The change in metabolic activity measured by the resazurin assay on keratoconic stromal cells is shown in Fig.1. With regard to the epithelial cells, the novel drug reduced the metabolic activity of the cells only to an extent of 10% measured over a period of two weeks. The drug treatment induced only around 15%-20% cell death in epithelial and stromal cells derived from corneal explants as analysed by Calcein/EthD-1 fluorescent staining.

Drug penetration into the normal cadaver cornea was analysed by treating the cornea with NHS-FITC using an ex-vivo corneal model. The drug penetrated into the corneal stroma to an approximate depth of about 200µm after crossing the epithelial layers (Fig.2). The tensile strength analysis of the novel drug treated normal cadaver and keratoconic corneas were carried out at Aurolab. A 74% increase in tensile strength of treated keratoconic cornea was observed when compared to untreated keratoconic cornea. There was no significant increase in the tensile strength of the novel drug treated normal cadaver cornea (Fig.3) when compared with the untreated controls.

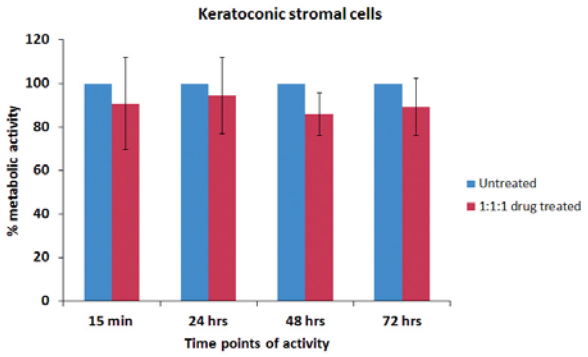


Fig.1: Resazurin fluorescence assay to assess the cytotoxic effect of novel drug on the treated and untreated keratoconic corneal stromal cells harvested by collagenase treatment. The values are normalized to the untreated cell number at each time point.

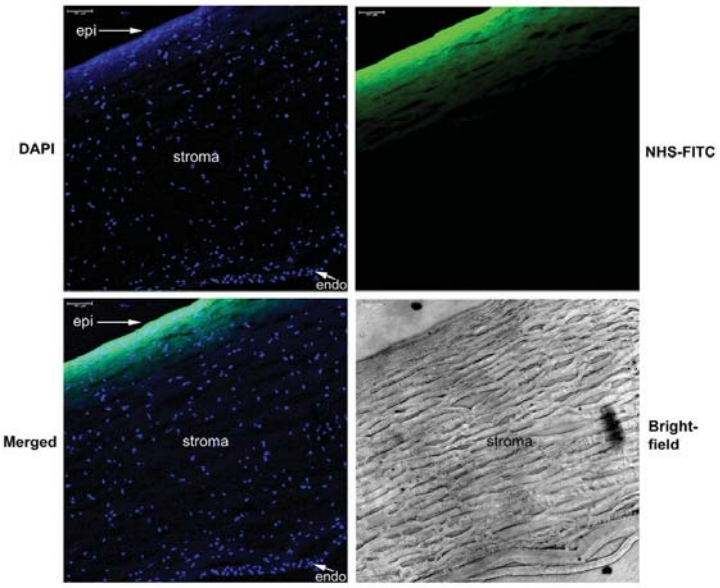


Fig.2: Cryosections of a normal cadaver cornea labelled with NHS-FITC showing the extent of penetration of the fluorescent conjugated NHS. DAPI counterstain reveals the nuclei of the cells in the epithelium (epi), stroma and the endothelium (endo). Scale bar, 100 µm.

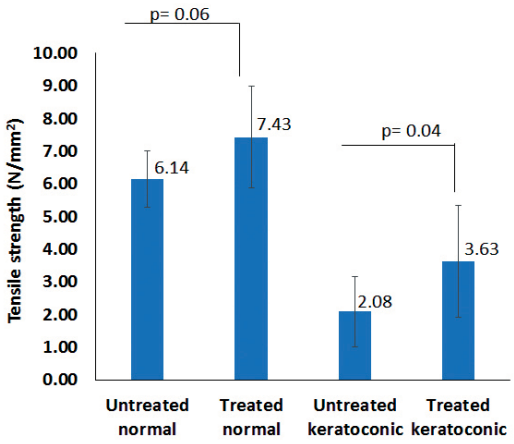


Fig.3: Tensile strength analysis of normal cadaver corneas (n=5) and patient keratoconic corneas (n=5) before and after treatment with 1:1:1 ratio of novel cross-linker.

Conclusions

The novel chemical cross-linker increased the tensile strength of the human keratoconic cornea without causing any significant cytotoxicity to the constituent cells of the corneal layers. The extent and the type of molecules involved in the cross-linking in the stromal layers is being analyzed by proteomic analysis. Thus, the novel chemical cross-linker is an alternative therapeutic approach to conventional corneal cross-linking for keratoconus.

OCULAR PHARMACOLOGY

The current research focus of the department of ocular pharmacology is to understand the molecular signalling in the trabecular meshwork especially Rho A /ROCK signalling in the pathogenesis of glaucoma and to develop Rho kinase inhibitor as a potential new candidate to reduce intraocular pressure using *ex vivo* Human Organ Cultured Anterior Segment (HOCAS) model.

Studies on assessment of macular pigment *in vivo* in both normal healthy individuals and in patients with early age-related macular degeneration (AMD) are also undertaken with the objective to provide an biological indicator for the risk of developing AMD due to the deficiency of macular pigment in our population.

Studying the role of Rho-A ROCK signaling in conventional outflow pathway using Human Organ culture anterior segment (HOCAS), an implication in Glaucoma therapy

Investigators : Dr. S. SenthilKumari, Dr. Gowripriya, Dr. SR.Krishnadas,
Research Scholar : S. Ashwin Balaji
Funding Source : Science and Engineering Research Board (SERB) (2015-18)

Introduction

Glaucoma is a neurodegenerative disease characterized by progressive damage of retinal ganglion cells resulting in optic nerve damage leading to irreversible blindness. Primary open angle glaucoma

(POAG) most common type of glaucoma is associated with elevated intraocular pressure (IOP). The elevated IOP is due to impaired aqueous humour outflow through the damaged conventional outflow pathway which includes Trabecular Meshwork (TM) and Schlemm's canal (SC).

Aqueous humour secretion is not a static process which involve, a series of physiologically dynamic processes responsible for aqueous humour circulation and the generation of intraocular pressure. In addition to diurnal variations, blood pulsations with each heartbeat transmit waves that create transient changes in IOP of 1-4 mmHg in magnitude (Ocular pulse). In *in vivo* condition, this kind of biomechanical stress plays an important role in tissue remodelling, affecting cell and tissue behaviour. However, the exact mechanism by which the trabecular meshwork tissues sense and respond to different types of mechanical stimuli such as elevated IOP, circadian rhythm, ocular pulse and shear flow is not well understood.

Previously it was reported that, cyclic oscillations as a result of pulsatile blood flow decrease trabecular outflow facility by 30% in perfused human and porcine anterior segments and Rho kinase inhibitors have a potential role in decreasing such resistance in TM in culture. Therefore, the proposed study aims to investigate the effect of cyclic IOP on conventional outflow using human anterior segment perfusion system and the involvement of RhoA –ROCK signaling in altering the contractility of intact TM.

In order to examine the effect of cyclic biomechanical stress on Outflow Facility (OF) (inflow rate/pressure), the experiments were performed on a physiologically relevant model, the anterior segment perfusion model (HOCAS), which uses post-mortem human eyes. In this system, the anterior segments were perfused with nutrient medium at a constant flow rate of 2.5 µl / min until to get a stable baseline within a physiological range. After a baseline stabilization (1-2 d), IOP pulsations were introduced with a peak-to-peak magnitude of approximately 2.7 mm Hg at a frequency of 1 Hz using pulsatile blood pump connected in tandem to syringe pump as by the method described previously (Ramos and Stamer , 2008). Peak-to-peak pressure was monitored and manually adjusted until the desired magnitude was obtained. The increase in IOP was monitored continuously using Labpro software. One eye of each pair received pulsation for 8 hours, while the contralateral eye received a steady perfusion (FR: 2.5 µl / min). The tissue viability of experimental and control anterior segments was analyzed post perfusion by histology.

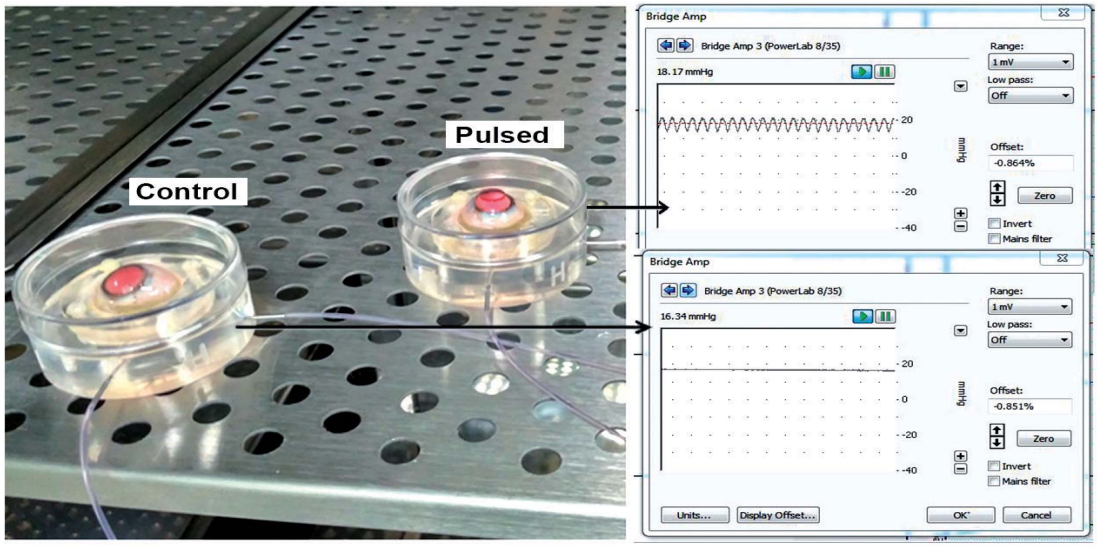
Results

Paired eyes from donors of a mean ± SD age of 64.14 ± 16.3 years were used for this study. The mean elapsed time between death and culture was 29.83 ± 8.42 hours and the elapsed time between death and enucleation was within 5 hours (4.2 ± 1.05 hours). The characteristics of human donor eyes used for HOCAS are given in the table below.

Donor Code	Age / Sex	COD	TOD-TOE (Hours)	TOD-TOR (Hours)
OCH-16-24	75 / M	Cardiac Arrest	3.5	29
OCH-16-23	40 / M	Cerebral Injury	4	22
OCH-16-26	60 / M	Cardiac Arrest	4	30
OCH-16-29	85 / F	Respiratory Arrest	3	25
OCH-16-27	72 / M	Cardiac Arrest	6	46
OCH-17-01	53 / F	Cancer	5	27

COD - Cause of Death; TOD- Time of Death; TOE – Time of enucleation.

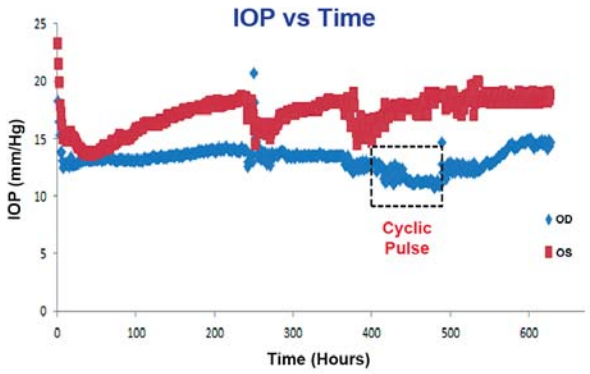
A representative picture showing human anterior segments in culture receiving cyclic IOP is given in the figure below:



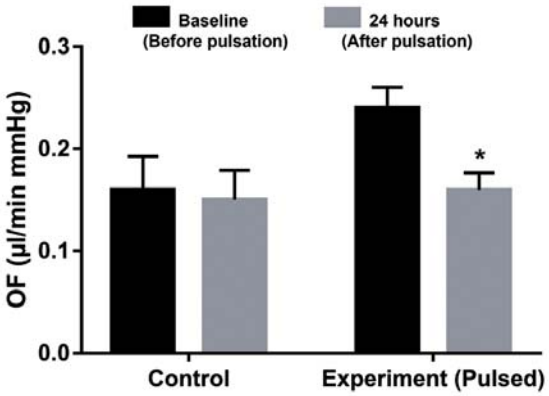
Representative Images showing perfusion cultured human anterior segments and the IOP chart showing the introduction of cyclic pressure oscillations in one eye and the fellow eye underwent a steady perfusion.

In this study, the experimental eyes (eyes received cyclic pulsation) showed a significant decrease in OF (0.18 µL / min / mmHg) from its own baseline (0.24 µL / min / mm Hg) (P=0.04, at 24 hours, whereas control showed no significant difference in OF (0.13 µL / min / mm Hg) to its own baseline (0.15 µL / min / mm Hg) (P=0.1855).

The change in OF in experimental eyes was normalized to respective controls and expressed as %OFR (Experiment / Control). Human anterior segments showed an average decrease in outflow facility (–19.79% ± 4.93%) in response to IOP oscillations which is given the following graph.



Representative IOP vs Time graph of an anterior segment, where one eye received a cyclic IOP and the fellow eye underwent steady state perfusion



Graph showing the effect of cyclic oscillations on OF

A significant decrease in OF (from baseline) was observed in eyes received cyclic pulsations as compared to control. (*p<0.05 Paired t-test BL vs 24 hours, N=6 Pairs).

Effect of Cyclic IOP on OF in human eyes;

Donor Code	Change in OF from Baseline		ΔOFR	% Facility Change
	Control	Experiment	(Expt/Ctrl)	
OCH-16-24	0.94	0.88	0.95	-5.44
OCH-16-23	1.07	0.64	0.6	-39.92
OCH-16-26	0.95	0.84	0.88	-11.62
OCH-16-29	0.8	0.68	0.85	-14.92
OCH-16-27	0.77	0.58	0.76	-24.23
OCH-17-01	0.85	0.65	0.77	-22.66
				-19.79 ± 4.93

OFR – Outflow facility Ratio

Conclusion

The present study revealed that, cyclic oscillations caused a significant reduction in percentage change in outflow facility in human eyes as compared to control eyes. Further studies are underway to investigate the involvement of Rho A/ROCK signalling in mediating OF resistance (increase in IOP) upon cyclic oscillations and the effect of rho kinase inhibitor in reducing such resistance in human eyes.

Indian Macular Carotenoid Research - A feasibility Study

Investigators : Dr. S. Senthilkumari, Dr. Anand Rajendran
Research Scholar : Ms. Madhavi Latha Yellchuri
Funding : Indian Council of Medical Research, India

Introduction

Xanthophylls especially lutein (L) and zeaxanthin (Z) were reported to accumulate in the macular region of the human retina, where oxidative damage of blue light is prevented by their absorption (Handelman and others 1992; Britton, 1995; Snodderly, 1995). The unique distribution, localization and high levels of both carotenoids within the macula lutea as well as their physicochemical properties make them suitable candidates for photo protection (Schalch, 1992). It is reported that, the ratio of Z to L was found to be 2.4:1 within 0.25 mm of the fovea and the ratio reverses to 1:2 to 1:3 in the periphery of the retina (Bone *et al*., 1988).

Human cannot synthesize carotenoids de novo and therefore they must be obtained from dietary consumption of coloured fruits and vegetables. Such has been reported to lower the incidence of many diseases including age-related macular degeneration (AMD) (Landrum and Bone, 2001; Krinsky *et al*., 2003; Trumbo and Ellwood, 2006). Several epidemiological studies and randomized clinical trials (RCTs) including Age-related Eye Disease (AREDS) study reinforce the importance of dietary consumption of carotenoids (L, Z and meso Z) in order to protect against the prevention of visual loss from AMD. It has also been shown that low plasma L and Z concentrations or dietary intake are associated with low macular pigment density and increased risk of AMD (Mares-Perlman *et al*., 1995). Though the prevalence of AMD in India is comparable with the western countries, the knowledge of association between macular pigment and AMD in Indian population is very limited. Therefore, in the present study, the mean Macular Pigment Optical Density (MPOD) was elucidated using MPS II in patients with early AMD.

Result

Based on the inclusion and exclusion criteria of the study, a total of 50 AMD patients were recruited and out of which MPOD data is available for 18 patients (N=25 eyes). The characteristics of early AMD patients recruited for the study is given in the table below.

Sl. No	Subject ID	Age	Sex	Clinical Feature	Best Corrected Visual Acuity	Systemic complications
1	IND-A002	61	F	RE-Dry AMD	6/18	Diabetic
				LE-Dry AMD	6/12p	
2	IND-A003	49	M	RE-Dry AMD	6/6	NIL
				LE-Dry AMD	6/6	
3	IND-A004	59	M	LE-Dry AMD	6/18	NIL
4	IND-A005	74	M	RE- DRYAMD	6/18	Diabetic
				LE-Dry AMD	6/9P	
5	IND-A006	69	M	RE-Dry AMD	6/12p	Diabetic
				LE-Dry AMD	6/60	
6	IND-A007	58	M	RE-Dry AMD	6/9p	Diabetic, Hypertensive
7	IND-A008	73	F	RE-Dry AMD	6/12	NIL
				LE - Dry AMD	6/12	
8	IND-A012	79	F	RE-Dry AMD	6/12p	Diabetic
				LE - Dry AMD	6/9p	
9	IND-A014	69	M	LE-ARMD	6/6	Hypertensive
10	IND-A015	62	M	RE-Dry AMD	6/12	NIL
				LE-Dry AMD	6/12	
11	IND-A016	69	M	RE-Dry AMD	6/36	NIL
				LE-Dry AMD	6/36p	
12	IND-A017	77	M	RE-Dry AMD	6/18	NIL
				LE-Dry AMD	6/36	
13	IND-A018	74	M	RE- Dry AMD	6/60p	Diabetic, Hypertensive
14	IND-A019	59	F	RE-Dry AMD	6/9	NIL
15	IND-A020	65	M	RE Dry AMD	6/9p	NIL
				LE-Dry AMD	6/24	
16	IND-A021	61	M	RE Dry AMD	6/12	Hypertensive
17	IND-A022	63	F	LE DRY AMD	6/9	Hypertensive
18	IND-A023	79	M	RE-Dry AMD	6/18p	Diabetic

The representative fundus and OCT images showing the presence of drusen in early AMD is given below.

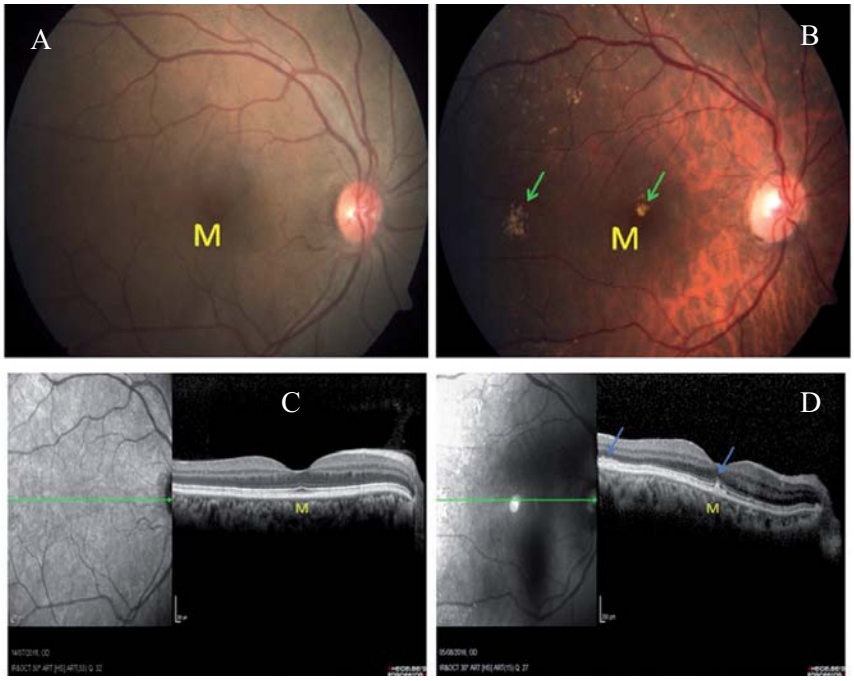


Fig 1: Representative fundus and OCT images of RE from healthy volunteer (IND # 131) (A,C) and patient diagnosed with early AMD (B,D)(IND-A 008; Table; serial no.6)

M- Macula. Green and blue arrows indicate the presence of drusen in the macula of AMD patient in fundus and OCT images respectively.

MPOD Measurement in AMD eyes

A total of 50 AMD patients have been recruited out of which 18 have completed MPOD tests (Ocular examination). The complete data is available for only 9 patients (MPOD, Ocular, Biochemical, OCT, Fundus etc.). The mean (±SD) MPOD in patient group was found to be 0.34 ± 0.19 and found no correlation between age and MPOD (r = -0.23; p = 0.24).

The average MPOD values of eyes with early AMD was found to be significantly very low as compared to healthy control (MPOD in AMD eyes : 0.34±0.19 (n=25) ; MPOD in healthy control : 0.47±0.14 (n= 229 ; p<0.0002). The mean MPOD of AMD and Control is given the figure below.

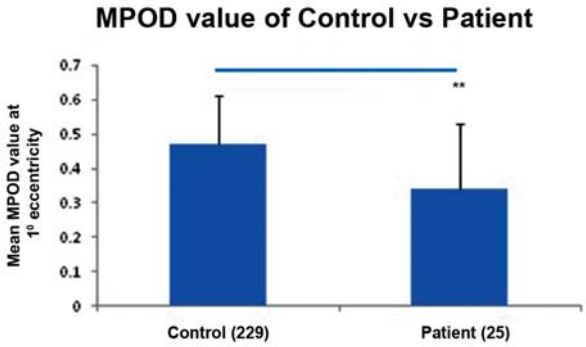


*Figure showing the comparison of mean MPOD value between control and patient group. The mean MPOD value of AMD patients were significantly low as compared to healthy control (** p<0.0002))*

MPOD Vs Lipid profile and Visual acuity of Patient group

The correlation between the MPOD and lipid profile may reflect the AMD pathogenesis. In the further analysis the association of lipid profile and visual acuity with MPOD was checked. Unfortunately the team could not the find any significance association of MPOD with lipid profile and visual acuity. The results are listed in the table below:

MPOD	n	r value	P- value
Triglycerides	9	0.43	0.39
HDL	9	-0.08	0.86
LDL	9	-0.05	0.92
VLDL	9	0.44	0.38
Cholesterol	9	0.095	0.85
Log Mar VA	25	-0.11	0.61

Conclusion

Early AMD patients showed significantly low MPOD as compared to healthy volunteers ($p<0.0006$). This study adding further evidence that low macular pigment density is one of the risk factors in AMD pathogenesis and supplementation of dietary carotenoids may be beneficial in delaying the progression of AMD in our population. Further studies are underway to elucidate the protective role of macular carotenoids in protection against the risk of developing AMD.

In vitro Evaluation of Transamniotic Membrane Release Kinetics of Bevacizumab

Investigators : Dr. S. Senthil kumari, Dr.N. Venkatesh Prajna
Research Scholar : Ms. Madhavi Latha Yelchuri
Clinical Fellows : Dr. Nilam Gohil, Dr. Hitha Sara Sajeev
Funding Source : AEH - MUTT study grant

Introduction

Human cornea is a transparent, avascular, watch glass-like structure. Although the normal cornea does not contain blood vessels, factors derived from blood play important roles in corneal metabolism and wound healing.

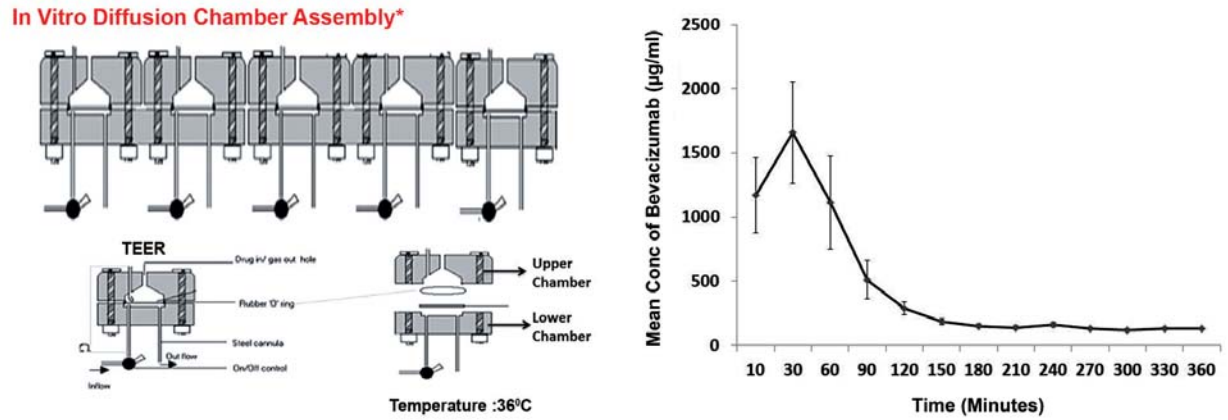
Corneal ulceration is a severe condition that may result from a variety of pathologic ocular surface conditions. Neovascularization in the cornea results from inflammation associated with various conditions such as trauma, microbial infection, or alkali burns or limbal stem cell deficiency can thus impair vision. In such condition, transplantation of human amniotic membrane (HAM) is a widely accepted technique for the reconstruction of corneal surface (Mayer *et al*., 2013).It not only impairs the vision but also poses a major risk factor for corneal graft rejection after transplantation and post-operative corneal graft survival (Chang *et al*., 2001).

HAM has a unique combination of properties, including the facilitation of migration of epithelial cells, the reinforcement of basal cellular adhesion, reduction of inflammation and scarring, inhibition of fibroblasts and the encouragement of epithelial differentiation (Touhami *et al*.,2002; Solomon *et al*.,2001 and Lee *et al*.,2000). HAM has been shown to be useful in reducing corneal neovascularisation (Kim and Tseng, 1995). Mayer *et al* (2013) demonstrated the utility of HAM as a depot carrier for the slow release of bevacizumab and found that effective VEGF blockade was observed upto 1 week in a static in-vitro system. However, anti-VEGF release kinetics in a dynamic model system is limited. This would reflect the physiological system in releasing drugs from the drug impregnated HAM. Therefore, in the present study, the transaminotic release kinetics of bevacizumab was carried out using *in vitro* corneal diffusion chamber. Anti-VEGF impregnated HMA may be an attractive strategy to overcome corneal graft rejection in the clinical set up.

Results

HAM buttons (12 mm) after soaking into 1% Bevacizumab for 3 hours, the buttons were mounted into *in vitro* diffusion chamber (Picture given below).

The release kinetics of bevacizumab (1% w / v) was studied for a period of 6 hours at 36°C. The amount of drug released at each time interval was quantified using size-exclusion high performance liquid chromatography as described by Paula *et al* (2013). The bevacizumab was found to be stable upto the study period. The release kinetics of bevacizumab from drug soaked HAM is given in the graph below.



The amount bevacizumab released at 10 minutes was found to be 1169.58 ±718.26 µg / ml and 1657.47 ± 791.33 µg / ml at 30 minutes. After 30 minutes, decreasing trend was observed as the time progressed. The cumulative amount of drug released was found to be 5869.79 µg / ml upto 6 h (58%). After 6 hours of release kinetics, the amount of drug released was found to be below the limit of detection by HPLC. Future studies with different soaking period would be necessary to identify an ideal soaking time for extended release kinetics of bevacizumab.

Conclusion

The present study investigated the feasibility of using full length anti-VEGF impregnated HAM as a drug reservoir for the sustained release of drugs in an *in vitro* dynamic system. Bevacizumab was found to be stable upto the study period. Three hours of drug soaking yielded about 58% drug release from HAM upto 6 hours. Further studies with low molecular weight anti-VEGF are warranted to investigate the influence of molecular size of the drug on release kinetics.

Evaluating the drug reservoir function of Human Amniotic Membrane (HAM) using Cefazolin – an in vitro study

Investigators : Dr. S. Senthil kumari, Dr. Venkatesh Prajna,
Research Scholar : Ms. Madhavi Latha Yelchuri
Clinical Fellows : Dr. Nilam Gohil, Dr. Hitha Sara Sajeev,
Funding : AEH-MUTT study grant

Introduction

Human amniotic membrane (HAM) is the innermost layer of the placenta. Its inhibitory action on fibroblasts and lipopolysaccharide induced upregulation of interleukins contributes to its antiscarring and anti-inflammatory properties (Lee *et al*., 2000; Solomon *et al*., 2001). Apart from producing various growth factors, it also acts as a transplanted basement membrane and thus serves as a substrate for epithelialisation. Its transparency, lack of immunogenicity and antiangiogenic properties make it a suitable biological material for the ocular surface reconstruction surgeries, as a carrier for ex vivo expansion of limbal epithelial cells, glaucoma surgeries, scleral melts and perforations (Azuara-Blanco *et al*. 1999; Dua *et al*., 2004,Malhotra and Jain, 2014). All such surgeries demand the use of topical antibiotics or anti-inflammatory drugs for better clinical outcome and prognosis.

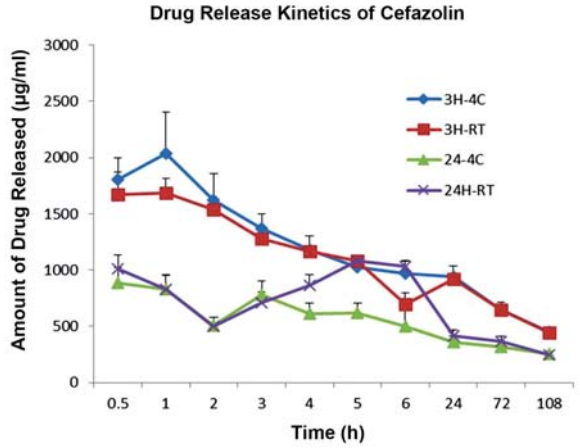
Kim *et al* (2001) have studied the aqueous levels of ofloxacin in rabbit eyes after AM transplantation. Barequet IS (2008) has also proved AM transplantation as a useful adjunctive treatment to cefazolin after staphylococcus aureus keratitis in rat model. The permeability behaviour

of HAM has been demonstrated in an in vitro model system with Netilmycin (Menccuci *et al*., 2006); ofloxacin (Resch *et al*., 2011) and bevacizumab (Mayer *et al*., 2013) ,thus proving that HAM can act as a drug delivery tool for the release of various drugs. But, the knowledge about the release kinetics of commonly used ocular drugs from HAM is insufficient leading to its limited clinical application. So, the purpose of the present study is to evaluate the reservoir function of HAM and to assess the release kinetics of anti-bacterial agent – cefazolin as a model drug.

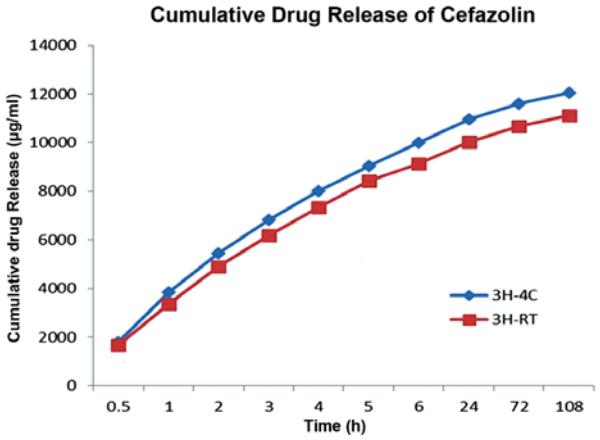
Results

Cefazolin, an antibacterial that is widely used in the treatment of bacterial keratitis due to gram positive organisms has been selected. The release kinetics of topical cefazolin (5%) after incubating HAM (12 mm punch) with cefazolin for 3 h (Group I) and 24 hours (Group II) has been evaluated at two different temperatures (4°C and at RT (25 ±2°C).

The amount of cefazolin released at different time intervals in both the groups were estimated by high performance liquid chromatography (HPLC) and the result is represented in the following graph:



Graph showing the release kinetics of 5% cefazolin after incubating HAM with cefazolin for 3 h and 24 h each at 4 ° C and at RT (25 ±2° C).



Cumulative drug release Kinetics of cefazolin incubated for 3 h at 4°C and RT

Cefazolin sodium was found to be stable at 4°C as reported earlier (Rojanaranta *et al* 2010, Mustafa Kemal *et al* 1999) as well as at RT upto the study period. No significant difference in the release kinetics of cefazolin was found with 3 hours and 24 hours incubation time periods. Three hour drug exposure showed significant increase in drug entrapment into HAM as compared to 24 hours. The cumulative amount of drug released after 3 h drug exposure at 4°C and RT was found to be 12,042 µg/ml and 11,126 µg /ml respectively (Figure above). There was significant increase in drug entrapment at 4 °C compared to RT (24 % Vs 11 %; $p < 0.005$).

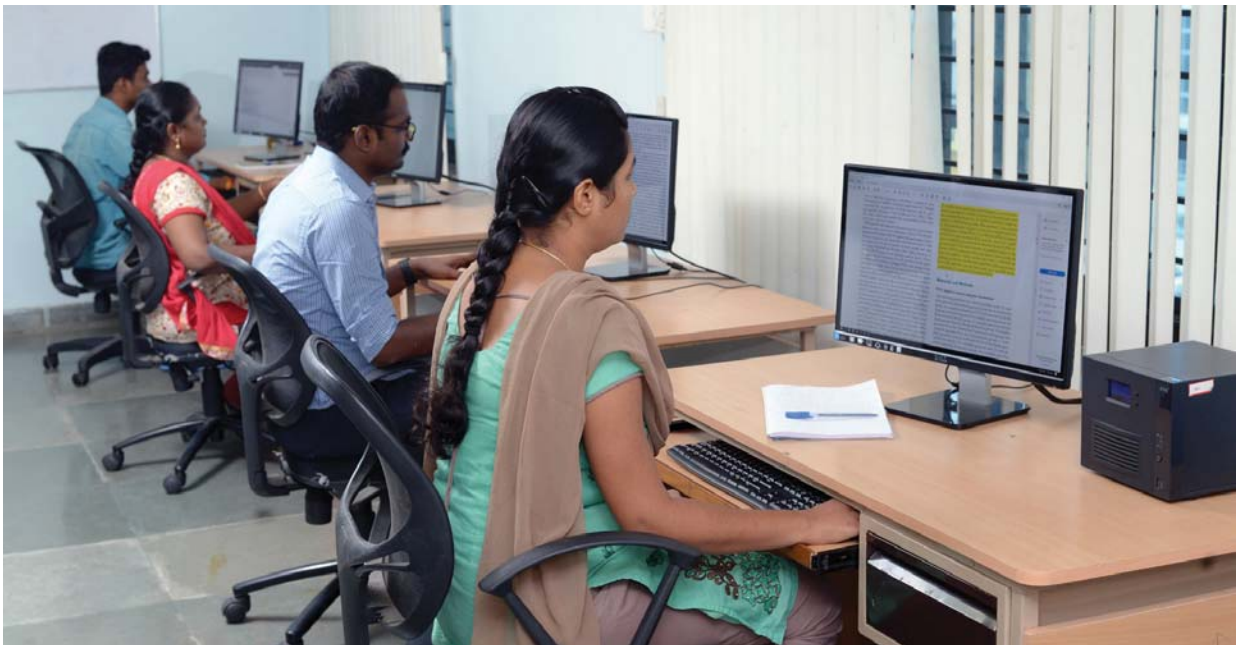
However, this findings further warrants the necessity to evaluate its suitability in a dynamic in vitro / in vivo system.

Conclusion

Entrapment efficiency of cefazolin was found to be higher in comparison to other drugs. This might be because the 5% cefazolin solution was used for incubation and molecular properties that may contribute to this difference. Drug impregnated HAM may be used to achieve sustained delivery of anti-bacterial for the better clinical outcome.

BIOINFORMATICS

The department primarily focuses on developing bioinformatics methods of omics analysis and noncoding RNAs for eye diseases. The availability of huge omics data from genome projects and high-throughput technology (next generation sequencing and microarray) has brought a great challenge to understand the complexity of biological process and disease mechanism in eye research. Bioinformatics facility has reliable infrastructure and framework comprised of LINUX and Windows based servers and desktop workstations, which allow to integrate those data and study them at systems level. It further provides to customize data analysis tailored to the needs of individual research projects across all the research groups. Current research foci are development of machine-learning approaches for variant prioritization, network approaches and multi-omics data integration for microRNA and their potential targets in eye diseases. In addition, the comparative genome analysis of ocular multidrug resistant pathogens is used to understand and identify resistance mechanisms.



Clinical exome analysis pipeline for eye disease next-generation sequencing panel

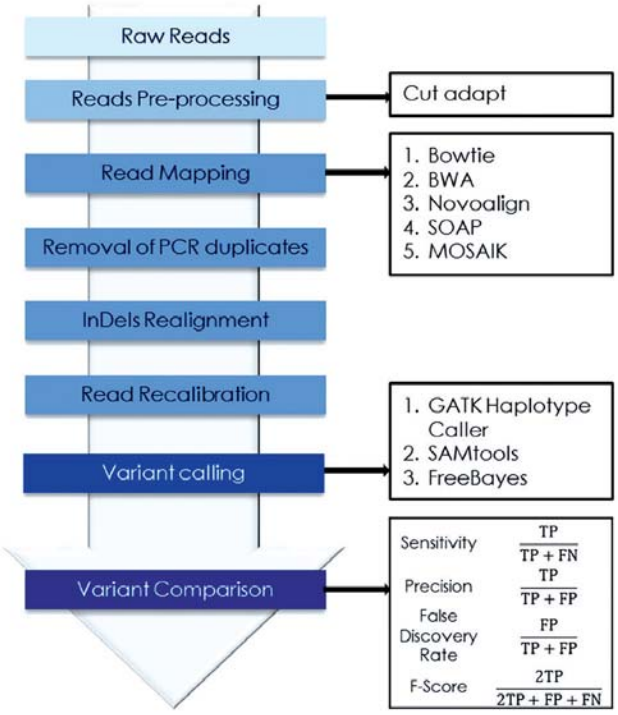
Investigator : Dr. D. Bharanidharan
Research Scholar : K. Manojkumar
Funding : DST-SERB

Introduction

The exome sequencing studies primarily aim at the discovery of single nucleotide variants (SNVs), and small insertions and small deletions (INDELs) of coding region that is about 85% of mutations among all the genetic variations. Exome sequencing method has been widely used to elucidate the genetic causes of many eye diseases, starting from single gene disorders and moving on to more complex genetic eye disorders, including complex traits and cancer. Although the exome sequencing has demonstrated identifying clinical variants, bioinformatics challenges are being

faced as the current bottleneck in exome/genome methods shifted from sequence generation to data management and analysis. In order to identify potential clinical variants, each step of the analysis workflow needs to be carefully considered, and specific tools need to be used for complete analysis in this clinical setup. Moreover, based on the diversity and the lack of standards for NGS analysis, many different tools and data formats were introduced, posing a problem when combining different methods to conclude the analysis and obtain meaningful clinical variants. To overcome these challenges, a comprehensive analysis pipeline is the possible solution with the selection of adequate tools, applying appropriate parameters, and combining them. For the pipeline development and reliable results, the accuracy of sequence alignment, consensus calling and variant detection is of paramount importance. Thus, we aim to assess the performance of widely used tools for the standard and eye-disease related human exome data.

Results and Conclusions



In order to develop streamlined pipeline for SNVs and INDELs separately for eye diseases, benchmarking was done with 5 alignment tools and 3 variant callers as shown in the overall workflow, on two whole exome data. First, the team used HapMap/1000 CEU female, NA12878 whole human exome data as a reference data since it has highly accurate and well characterized set of genome-wide reference material of NA 12878, including BED and VCF file of high-quality sequence regions and variant calls respectively, developed through the Genome in a Bottle Consortium (GIAB), the National Institute of Standards and Technology (NIST). Moreover, the GIAB call sets were built from the integration of eleven NA12878 whole human genome data sets and three exome data sets, generated across five sequencing platforms to eliminate bias from any single platform. Therefore, the NA12878 can be used for producing and comparing the analytical performance aligners and callers. In comparison, a simulated data has been generated using Hg19 human genome for benchmarking. Next, the team used one normal Indian human exome and one eye-disease associated clinical exome data, where dbSNP build 146 was used as comparison. All the evaluation work for SNVs and InDels was performed separately.

The comparison of SNVs and InDels of NA12878 whole exome data against NIST reference call set showed different performance between aligners and callers as previously described. By combining both Precision and sensitivity (F-Score, Figure 1), it was found that BWA_Samtools and Novoalign_Samtools performed well for SNVs. On the other hand, Novoalign_GATK and BWA_GATK performed well for InDels. These results surmise that BWA and Novoalign are best aligners compared to others for both SNVs and InDels, wherein both would be used for better results. In contrast, Samtools and GATK could be used separately for SNVs and InDels for both the aligners and could be merged. Therefore, ensemble of variants from Samtools and GATK for SNVs and InDels respectively would help us to identify most true positives. Similarly, the team did perform the

benchmarking on whole exome data of Normal Indian and human exome data from patient with Leber congenital amaurosis (LCA), which was published by Marni *et al*. in Nat Genet. 2012 44(9): 1040–1045.

The benchmarking of pipelines on simulated data showed similar performance for both SNVs and InDels (Table 1 and 2)

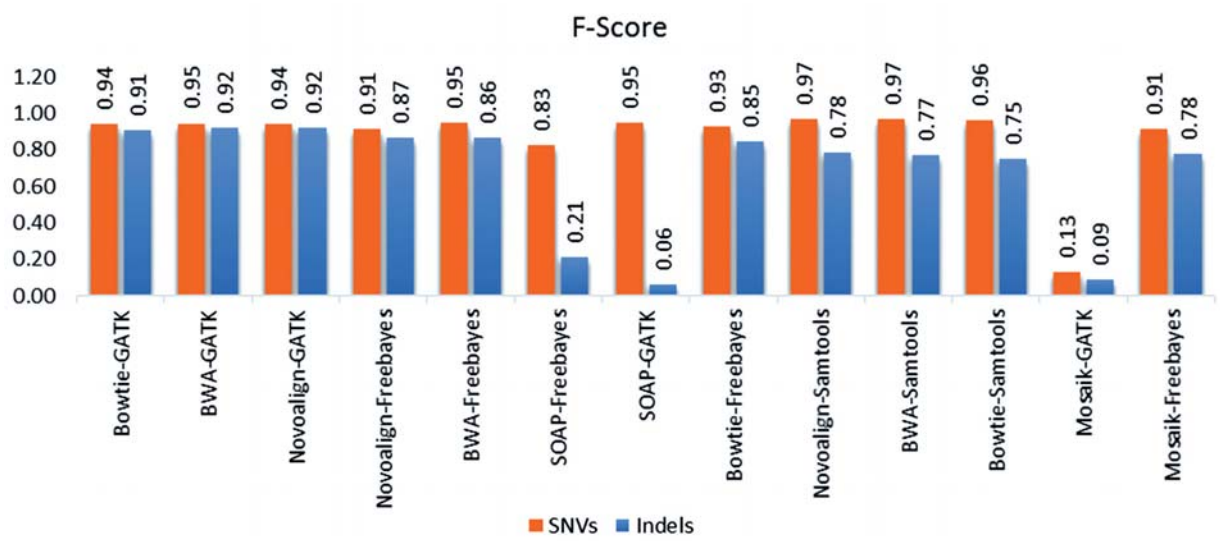


Figure 1. F-score of different pipelines for SNVs and InDels using NA12878 genome data

Table 1. The pipeline results for SNVs using simulated data

Pipeline	TP	FP	FN	Precision	Sensitivity	F-Score	FDR
Bowtie-Freebayes	22110	1405	1576	0.94	0.93	0.93	0.06
Bowtie-GATK	22231	967	1455	0.95	0.93	0.94	0.04
Bowtie-Samtools	22840	553	846	0.97	0.96	0.97	0.02
BWA-Freebayes	22108	431	978	0.98	0.95	0.96	0.02
BWA-GATK	22630	1043	1275	0.95	0.94	0.95	0.04
BWA-Samtools	22859	455	827	0.98	0.96	0.97	0.02
Mosaik-Freebayes	22096	1340	1590	0.94	0.93	0.93	0.06
Mosaik-GATK	22175	901	1511	0.96	0.93	0.94	0.04
Mosaik-Samtools	-	-	-	-	-	-	-
Novoalign-Freebayes	22069	1253	1617	0.94	0.93	0.93	0.05
Novoalign-GATK	22811	975	856	0.95	0.96	0.96	0.04
Novoalign-Samtools	22911	329	775	0.98	0.96	0.97	0.01
SOAP-Freebayes	22161	1256	1525	0.94	0.93	0.94	0.05
SOAP-GATK	22406	1020	1280	0.95	0.94	0.95	0.04
SOAP-Samtools	-	-	-	-	-	-	-

Table 2. The pipeline results for InDels using simulated data

Pipeline	TP	FP	FN	Precision	Sensitivity	F-Score	FDR
Bowtie-Freebayes	1070	1063	188	0.50	0.85	0.63	0.50
Bowtie-GATK	1037	95	221	0.91	0.82	0.86	0.08
Bowtie-Samtools	878	419	380	0.67	0.69	0.68	0.32
BWA-Freebayes	1078	1047	180	0.50	0.85	0.63	0.49
BWA-GATK	1120	108	138	0.91	0.91	0.90	0.08
BWA-Samtools	918	438	340	0.67	0.73	0.70	0.32
Mosaik-Freebayes	1067	1203	191	0.47	0.84	0.60	0.53
Mosaik-GATK	1086	103	172	0.91	0.86	0.88	0.09
Mosaik-Samtools	-	-	-	-	-	-	-
Novoalign-Freebayes	1090	1043	168	0.51	0.86	0.64	0.49
Novoalign-GATK	1118	104	140	0.91	0.88	0.90	0.09
Novoalign-Samtools	924	504	334	0.64	0.73	0.68	0.35
SOAP-Freebayes	-	-	-	-	-	-	-
SOAP-GATK	1104	102	154	0.91	0.87	0.89	0.08
SOAP-Samtools	-	-	-	-	-	-	-

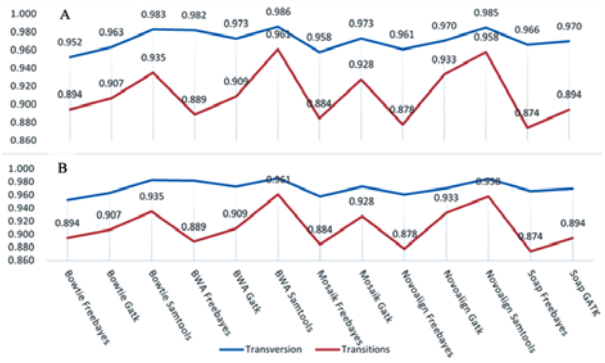
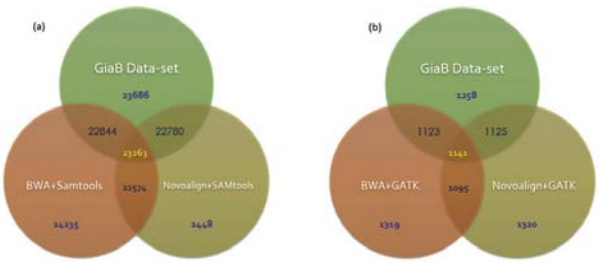


Figure 2 (A) Pipeline results for Transitions and Transversions of SNVs using (A) GiaB data and (B) simulated data



TP of GiaB data set's SNVs (a) and InDels (b) with pipelines.

In agreement with other previous comparison, BWA and Novoalign are the best aligners compared to others for both SNVs and InDels, wherein both would be used for better results. In contrast, Samtools and GATK could be used separately for SNVs and Indels for both the aligners and could be merged.

In conclusion, different aligners and variant callers should be used for SNVs and InDels separately for any exome data, even for the patient with eye diseases. In progress, top two pipelines (as presented in the Venn diagram) are merged to produce ensemble of SNVs and InDels separately. Further, machine learning approaches are being developed to filter true positives to achieve the maximum the sensitivity.

Comparative genomics of *Methicillin-Resistant Staphylococcus aureus (MRSA)* and *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
Funding : AEH

Background

Microbial keratitis due to either fungus or bacteria is a major cause of blindness in India. The bacteria usually responsible for ocular infection are *Pseudomonas aeruginosa*, methicillin-resistant *staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae*, same has been reported at Aravind Eye hospital, Madurai. Majority of times in spite of adequate medical management the ulcer does not heal and may require a corneal transplant. An array of virulence factors contributes 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, clinical isolates of *Pseudomonas* often exhibit multiple resistances to antibiotics. However, P.aeruginosa's incredible metabolic versatility is generally attributed to genomic diversity. During infection, it adapts through loss-of function mutation in virulence genes, which is essential for the onset of infection.

On the other hand, MRSA clones have been increasingly reported in Indian communities. Studies have shown an increase in the pervasiveness of ocular MRSA infections. The increasing of divergence between same bacterial strains is determined by their additional chromosomal substances such as genomic islands, plasmids and blocks of DNA inserted at various loci in the chromosome. Still, bacteria that did not carry one or more of these regions or resistance mechanisms is proposed as an adaptive process, but exact role played by these elements is not known. Hence, to efficiently control bacterial infections, it is mandatory to understand the strain diversity and evolution. This study focuses comparative genomics of ocular isolates of MRSA and *P. aeruginosa* exhibit different clinical outcomes.

Results and Conclusion

Five *P. aeruginosa* ocular isolates with different antimicrobial susceptibility and clinical outcomes were selected for sequencing (Table 1). The whole genome sequencing was performed by Illumina Nextseq platform with paired-end method at Genotypic, Bangalore. Raw were adapter trimmed and quality filtered for better assembly. The processed reads were assembled using CLC Genomics workbench version 8. Genes were annotated by RASTtk and PROKKA. The assembly and annotation statistics were shown in Table 2.

Of the isolates AMRF-BK4, BK5 and BK6 cultured from patients with non-responders, BK6 showed resistance to all drugs consistent with MIC that consists of 7.1 mb genome size. BK4 and BK5 susceptible for many drugs, still showed resistant to many drugs (data not shown). Moreover, In Table 3. the Prophages Pseudo_Pf1, Aeromo_phiO18P in BK4 and Escher_vB_EcoM_ECO1230-10 in BK5 were not present in the BK2 and BK3. The intact prophage Pf1 and Escher_vB_EcoM_ ECO1230-10 has been shown to contribute bacterial short term evolution and virulence. In order to study the evolution of treatment non-responders isolates and virulence factors and genetic determinants, further comparisons were made. The isolates shared several virulence factors, however, several virulence factors were not common and some of them unique. Still, comparative pathogenomics of mutations present in the major virulence factors of isolates respect to PA01 strain is being processed to correlate the clinical outcome.

Table 1. The MIC results of five P. aeruginosa ocular isolates from keratitis patients with different clinical outcome.

Isolates		AMRF-BK3	AMRF-BK2	AMRF-BK4	AMRF-BK5	AMRF-BK6
Susceptibility and MIC (mg/L)	Ticarcillin / Clavulanic acid	R ≥128	R ≥128	R ≥128	R ≥128	R ≥128
	Piperacillin / Tazobactam	R ≥128	R ≥128	R ≥128	R ≥128	R ≥128
	Ceftazidime	R ≥64	R ≥64	R ≥64	R ≥64	R ≥64
	Cefepime	R ≥64	R ≥64	R ≥64	R ≥64	R ≥64
	Doripenem	R ≥8	S 1	I 4	S 1	R ≥8
	Imipenem	R≥16	S 2	S 2	S 2	R ≥16
	Meropenem	R 4	S	S	S	I 4
	Amikacin	R >64	S 4	S 4	S 4	R 16
	Gentamicin	R >16	S 2	S 2	S 2	R> 16
	Ciprofloxacin	R >4	S <0.25	S 0.5	S <0.25	S 0.5
	Levofloxacin	R >8	S 1	S 1	S 1	S 1
	Minocycline	R ≥16	R ≥16	R ≥16	R ≥16	R ≥16
	Tigecycline	R ≥8	R ≥8	R ≥8	R ≥8	R ≥8
	Colistin	S 1	S 2	S 2	S 2	S 2
	Trimethoprim / Sulfamethoxazole	R ≥320	R ≥320	R ≥320	R ≥320	R ≥320
	Moxifloxacin	R ≥64	S 1	S 2	S 1	S 2
	Gatifloxacin	R ≥64	S 0.5	S 1	S 0.5	S 2
	Tobramycin	R≥64	S 1	S 1	S 1	R≥64
	Type[a]	MDR	Sus	Sus	Sus	MDR
	Clinical Outcome[b]	**Healed**	**Healed**	**TPK**	**TPK**	**TPK**

[a] MDR (Multi-drug Resistance) showing resistance to many antibiotics; Sus(Susceptible) showing sensitivity to many antibiotics; [b] TPK, Therapeutic Penetrating Keratoplasty

Table 2. Assembly and annotation statistics of five P. aeruginosa isolates

ISOLATES	SIZE (MB)	GC CONTENT	SCAF FOLDS	MAX CONTIG LENGTH	MIN CONTIG LENGTH	N50	CDS	TRNAS
AMRF-BK2	6.3	66%	45	703333	506	370564	6037	61
AMRF-BK3	7.1	66%	129	602407	510	232253	6722	60
AMRF-BK4	6.3	66%	68	877303	509	350169	6055	61
AMRF-BK5	6.5	64%	69	700326	509	347365	6141	60
AMRF-BK6	7.1	65%	113	594831	504	191913	7009	65

Table 3. The prophage regions predicted using PHAST for P. aeruginosa isolates

Isolates	PROPHAGE Name	Length (Kb)	No of ORFs	GC (%)
AMRF -BK2	Pseudo_B3	42.3	50	63.5
	Pseudo_PPpW_3	20.1	25	64.9
	Pseudo_phi297	51.2	65	63.4
AMRF -BK3	Pseudo_phiCTX	17.9	25	65.4
	Pseudo_vB_PaeS_PM105	33.9	34	64.2
	Pseudo_vB_PaeS_PM105	18.2	30	62.3
AMRF -BK4	Pseudo_Pf1	33.6	45	64.9
	Aeromo_phiO18P	24.2	31	66.0
	Pseudo_phiCTX	17.8	25	65.4
AMRF -BK5	Escher_vB_EcoM_ECO1230-10	17.9	25	65.4
	Pseudo_phiCTX	48.2	45	66.2
AMRF -BK6	Pseudo_F10	43.9	63	60.8
	Pseudo_YMC11/02/R656	30.2	36	64.5
	Pseudo_JD024	28.3	39	64.6
	Pseudo_F10	60.6	70	62.8

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. The key features of the pathogenic bacteria that drives non-responsive treatment is yet to be determined.

A Bioinformatics approach to understand the molecular mechanisms of different retinal dystrophies caused by non-synonymous single nucleotide variants (nsSNVs) in the RLBP1 gene

Investigator : Dr. D. Bharanidharan
Research Scholar : K. Manoj Kumar
Funding : AMRF

Introduction

Retinal dystrophy (RD) is a heterogeneous group of hereditary diseases caused by loss of photoreceptor function and contributes significantly to the etiology of blindness globally but especially in the industrialized world. The retinaldehyde-binding protein (CRALBP) gene mutations cause autosomal recessive retinal dystrophies, retinitis pigmentosa (RP), Bothnia dystrophy (BD), retinitis punctata albescens (RPA), fundus albipunctatus (FA) and Newfoundland rod-cone dystrophy (NFRCD). The RLBP1 gene encodes a cellular retinaldehyde binding protein (CRALBP) that isomerizes all-trans- to 11-cisretinol during the regeneration of rhodopsin. In human retina, RLBP1 is expressed in the retinal pigment epithelium (RPE), photoreceptors, Muller and ganglion cells. All of the retinal degenerations belong to the group of rod-cone dystrophies, characterized by early-onset night blindness, loss of peripheral vision and later in advanced stages of disease also loss of central vision, often leading to complete blindness as the disease progresses. The majority of affected individuals carry the RLBP1 mutations in a homozygous state, although compound heterozygotes have also been described.

The distinctions between FA, RP, BD, RPA and NFRCD is poorly defined and is likely to depend on the effect of the mutation on the protein structure and function. Number of patients sharing the same c.700C>T (p.R234W) homozygous mutation were reported from northern Sweden and

represent the group of BD. In contrast, splice-junction mutations that are likely to give rise to a null allele, tend to lead to a more severe phenotype, for NFRCD. Allelic mutations of the RLBP1 gene also include missense and indel mutations resulting in frameshift and truncation of the respective polypeptide, or undergoing nonsense-mediated decay, thereby further extending the clinical spectrum of disease. Hence, a detailed underlying molecular mechanism is needed to understand this complexity of correlation between variants and disease phenotype. Here, bioinformatics approaches are used to readily evaluate the impact of genetic variation on the structure/function of a gene product at the molecular level and predict the molecular mechanisms underlying disease.

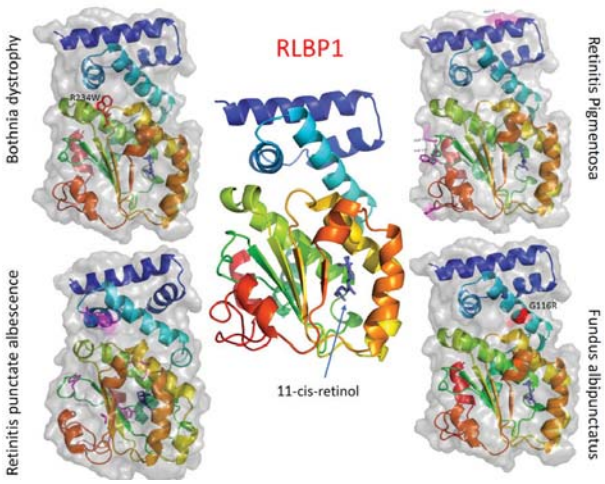
Results

The full RLBP1 protein structure was modelled using the PDB template using Modeller. The modelled structure was then used for mutant structures. Based on the literature search, there were 12 sSNVs of RLBP1 gene reported in patients with four recessive retinal dystrophies BD, FA, RP and RPA. The wild and mutant structure were used to analyze protein stability, solvent accessibility and protein interactions. MetaPPI, MetaDBsite, MetaPocket are the different servers used to predict the protein binding site and the energetics analysis predicts the effect of amino acid substitution on protein interaction. The sequence conservation based tools was used to predict the variants effects. The results were shown in the following table:

nsSNVs	Zygosity	Segregation	Patient's Phenotype	Polyphen	SIFT	SNAP	SDM	Solvent Accessibility	Energy	Active Site	Ligand Binding
R234W	Homo	Yes	BD	PD	D	D	IS	NC	R	-	A
G116R	Hetro	Yes	FA	PD	T	N	IS	NC	I	-	A
S149F	Hetro	NA	RP	PD	T	D	IS	I	R	A	-
A72P	Hetro	NA	RP	B	D	N	RS	I	R	-	-
T292M	Hetro	NA	RP	PD	T	N	IS	I	R	-	-
R151Q	homo	Yes	RP	PD	T	D	RS	I	R	A	-
R103W	Hetro	yes	RPA	PD	D	D	IS	R	R	-	-
G146D	Hetro	yes	RPA	PD	D	D	RS	NC	R	-	-
R151W	Hetro	Yes	RPA	PD	D	D	RS	I	R	A	A
I201T	Hetro	Yes	RPA	PD	D	D	RS	NC	R	-	A
M226K	Hetro	Yes	RPA	PD	D	D	RS	NC	R	-	-
G298D	homo	Yes	RPA	PD	D	D	RS	NC	HR	-	A

PD-Probably damaging; B-benign; D-Deleterious; N-neutral; IS-Increased protein stability; RS-reduced protein stability <0.5; NC-No change; I-Increased; R-reduced; A-affected

The R234W identified in BD patient affects protein stability and buried inside, while the G116R from FA patient affects protein stability with changing increased energy since it is located on the secondary structure. The nsSNVs identified from RPA patients were in the proximity of 11-cis-retinol binding (Figure) and thereby affecting the function of the protein. Whereas, all variants from RP patients were located on the surface of the protein that could affect the surface potential and solvent accessibility. Nevertheless, though the R103W in RPA showed far away from the 11-cis-retinol



binding site and reduced stability, the variants with R234W might cause the RPA as compound heterozygote. Similarly, homozygote R151Q causes the RP in one patient whereas heterozygous R151W causes RPA in another patient as compound effect.

Conclusion

The molecular causes of RP and RPA could be decoupled. RPA may arise through disruption of 11-cis-retinaol interactions, while RP is triggered by variants which disrupt interactions with other proteins or disrupting the solvent accessibility. The variants disrupting protein stability may cause BD and FA. These findings have important implications for retinal dystrophies and additionally, predicting effects of genetic variation will be critical as required for next-generation sequencing methods accelerates. The analytical strategy described above would be helpful to understand the molecular mechanisms of other genetic diseases and its association with phenotype.

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.

Analysis of bacterial persistence mechanisms in recalcitrant ocular *Pseudomonas aeruginosa* infections

Principal Investigator : Dr. Vidyarani Mohankumar
Funding Agency : SERB
Project Fellow : R. Sangeetha

Background

Pseudomonas aeruginosa keratitis could lead to corneal scarring and severe visual disability. Bacterial persistence due to inefficient clearance of bacteria from the host or drug tolerant nature of the bacterium can lead to treatment failure. The aim of this study is to identify the bacterial persistence mechanisms in *P. aeruginosa* isolates obtained from keratitis patients.

Results and discussion

Induction of host cell autophagy by *P. aeruginosa*

P. aeruginosa has been shown to invade and replicate inside human corneal epithelial cells (HCET). Intracellular bacteria can be cleared by traditional phagolysosome fusion or by additional mechanisms like autophagy. This research aims to study the role of autophagy in limiting intracellular bacterial infection in HCET cells *invitro*. Earlier the team has shown the induction of autophagy upon *P. aeruginosa* infection by LC3 punctation in HCET cells using confocal microscopy. In the current work, western blot analysis of infected cells was done to determine the conversion of LC3-I to LC3-II which helps in the quantitative assessment of autophagy.

During autophagy, LC3-I is converted to LC3-II through addition of phosphatidyl ethanolamine. LC3-II is present on isolation membranes and autophagosomes and much less on autolysosomes. Despite increased molecular weight due to lipidation, LC3-II migrates faster than LC3-I in the gel due to its extreme hydrophobicity. LC3-I is detected at a molecular mass around 18 kDa and LC3-II at 16 kDa. The presence of LC3 in autophagosomes and the conversion of LC3-I to LC3-II have been used as indicators of autophagy. The amount of LC3-II is closely correlated with the number of autophagosomes, serving as a good indicator of autophagosome formation.

HCET cells were treated with EBSS to induce starvation, and chloroquine to inhibit lysosomal degradation of LC3-II. The cells were infected with *P. aeruginosa* at two different time points and cell lysates were collected. Proteins from infected HCET cells were separated by SDS-PAGE and transferred onto 0.2 µm PVDF (polyvinylidene fluoride) membranes. Membranes were incubated overnight with rabbit anti-LC3 and anti-actin primary antibodies followed by anti-rabbit secondary antibodies conjugated to HRP. The protein bands were visualized using enhanced chemiluminescence (ECL) reagent. HCET cells infected with ocular *P. aeruginosa* isolates showed increased conversion of LC3-I to LC3-II at 4hours and the ratio was determined by densitometric quantification (Fig.1).

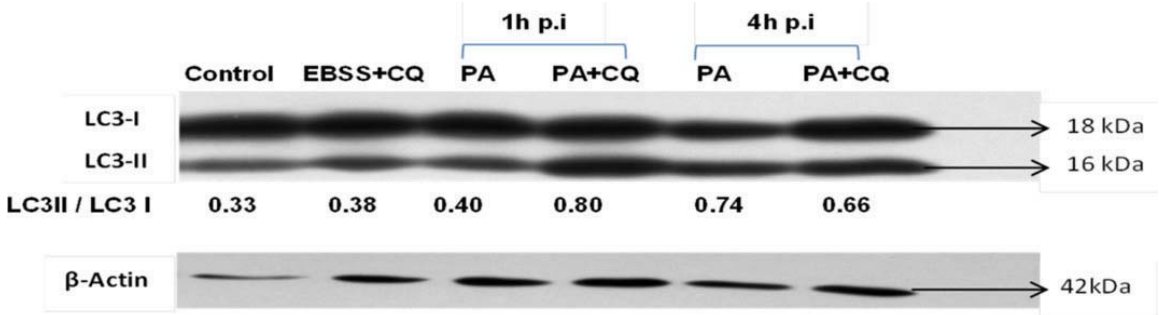


Fig.1. Western blot image showing increased conversion of LC3-I to LC3-II upon P. aeruginosa infection in HCET cells; Corresponding levels of actin are shown in the lower panel.

Quantitative comparison of persister formation

The persister fraction that survives after antibiotic exposure was quantified in *P. aeruginosa* isolates obtained from treatment responders and non-responders. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of five standard antibiotics was determined and 10X MIC used to isolate persisters.

The overnight culture was diluted in fresh LB to an OD_{600} of 0.2, which was then split into two flasks. To one flask desired concentration of antibiotic was added to yield persisters and to other flask the same volume of solvent used to dissolve antibiotic was added to yield total cells. The cultures were returned to the 37℃ shaker for 4 hours. Then the cells were washed thrice with medium (with antibiotics for persisters) to isolate unlysed persister cells and total cells. The cells were plated to quantify viable counts (Table1).

Table1. Quantitative comparison of persisters among P. aeruginosa isolates obtained from treatment responders and non-responders. TPK- Therapeutic Penetrating Keratoplasty; Hld – Healed ulcer

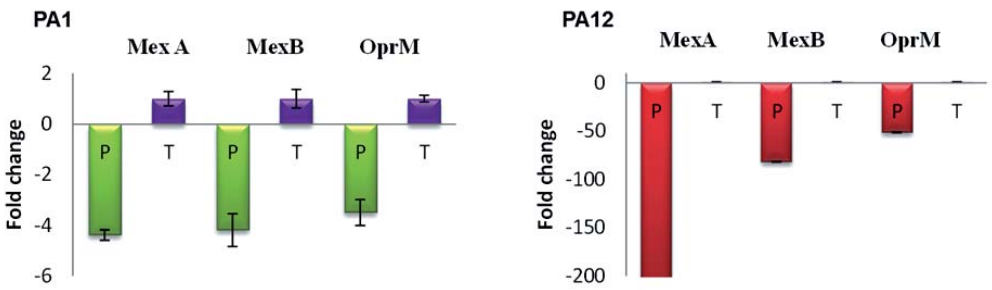
Isolates	Initial inoculum	Solvent (Moxi, Genta, Ami, Tobra)	Solvent (Gati)	Moxiflox	Gatiflox	Gentamicin	Amikacin	Tobramycin
PA1 (TPK)	1.26×10	2.66×10^{8}	5.01×10^{8}	6.8×10^{4}	8.2×10^{4}	2.3×10^{4}	4.7×10^{4}	1.9×10^{4}
PA2 (TPK)	1.5×10^{8}	4.16×10^{8}	2.04×10^{8}	4.7×10^{4}	4.8×10^{4}	4.9×10^{4}	8.3×10^{4}	7.0×10^{4}
PA3 (TPK)	2.92×10^{8}	1.7×10^{8}	4.21×10^{8}	0	0	0	2.98×10^{4}	0
PA4 (TPK)	2.51×10^{8}	1.21×10^{8}	4.95×10^{8}	0	0	0	9.43×10^{4}	4×10^{2}
PA5 (TPK)	3.26×10^{8}	1.69×10^{8}	1.99×10^{8}	0	0	0	1.2×10^{3}	0
PA6 (TPK)	3.58×10^{8}	1.81×10^{8}	1.99×10^{8}	0	0	1×10^{2}	2.74×10^{4}	0
PA7 (TPK)	3.91×10^{8}	3.65×10^{8}	3.82×10^{8}	3×10^{2}	1.2×10^{3}	7×10^{6}	1.8×10^{8}	2.7×10^{7}
PA8 (TPK)	2.12×10^{8}	3.76×10^{8}	3.82×10^{8}	1.7×10^{3}	3.8×10^{3}	6.12×10^{4}	8.43×10^{4}	9.01×10^{4}
PA9 (Hld)	1.79×10^{8}	4.74×10^{8}	2.01×10^{8}	1.8×10^{7}	$1.3x10^{7}$	7×10^{6}	3.5×10^{8}	6.7×10^{7}
PA10 (Hld)	3.82×10^{8}	4.96×10^{8}	2.91×10^{8}	9.2×10^{3}	8×10^{2}	1.9×10^{7}	9.8×10^{7}	4.8×10^{7}
PA11 (Hld)	4.2×10^{7}	2.67×10^{8}	3.23×10^{8}	2×10^{3}	3×10^{3}	5×10^{3}	1×10^{3}	5×10^{3}
PA12 (Hld)	2.4×10^{8}	1.78×10^{8}	1.76×10^{8}	1.7×10^{7}	8×10^{6}	7×10^{6}	3×10^{7}	1.2×10^{7}

Except for isolates PA3, PA4, PA5 and PA6 that showed increased sensitivity to fluoroquinolones and aminoglycosides, all other isolates had a considerable fraction of drug tolerant cells that survived antibiotic treatment.

Analysis of transcriptional regulation in persister cells

Enhanced efflux activity has been shown to facilitate drug tolerance in dormant *E.coli* cells. To verify this phenomenon in the isolates, relative expression levels of MexAB- OprM efflux pump genes were analyzed in persister cells with total cells as control. Real time PCR was done with rplu as housekeeping gene. The persisters had lower expression of efflux pump genes compared to total cells, suggesting maintenance of dormancy in these cells (Fig 2).

Similarly expression of virulence genes like protease 4 and exotoxin A were also compared between persister and total cells of two isolates. Protease 4 expression was significantly downregulated in the persister fraction of both isolates, whereas that of exotoxin A was downregulated in one isolate (Fig 2).



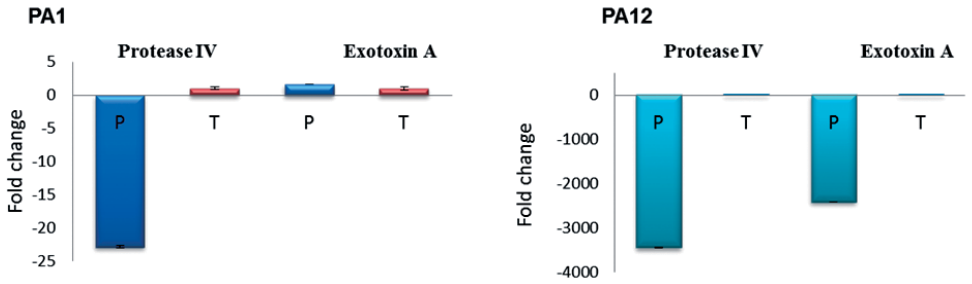


*Fig.2. Relative expression levels of MexAB-OprM efflux pump genes (A) and virulence genes (B) in persister and total cells of two P. aeruginosa isolates PA1 and PA12. * P- Antibiotic treated persister cells; T- Untreated total cells*

Induction of host cell autophagy by persisters

Since autophagy has been shown to be involved in intracellular clearance of *P. aeruginosa*, this mechanism was studied in the clearance of persister cells. HCET cells were infected with persister and total population of a *P. aeruginosa* isolate (MOI 10) for 4h and LC3 immunoblotting was done. LC3-I to LC3-II conversion was relatively decreased in persisters indicating reduced induction of autophagy by persisters (Fig.3).

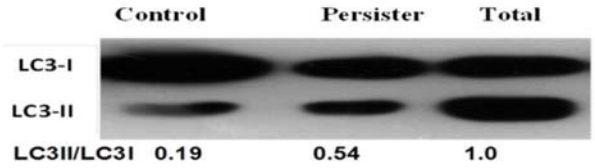


Fig.3. Western blot image showing decreased conversion of LC3-I to LC3-II upon P. aeruginosa infection in HCET cells infected with persisters compared to total bacterial cells.

Conclusion

Persisters form a fraction of drug sensitive isolates obtained from both treatment responders and non-responders. They remain in a quiescent state by downregulating virulence gene expression and upon infection they induce minimal host response to facilitate their intracellular survival. Complete eradication of these persisters may be possible by enhancing host defense mechanisms along with appropriate antibiotic treatment.

Functional characterization of drug resistance mechanisms in corneal isolates of *Pseudomonas aeruginosa*

Investigators : Dr. Vidyarani Mohankumar, Dr. Bharanidharan Devarajan, Dr. Lalitha Prajna
Funding Agency : AMRF
Project Fellow : T. Kannan

Background

Bacterial keratitis caused by *P. aeruginosa* poses challenges to treatment upon development of drug resistance through mechanisms like mutational alteration of target protein, enzymatic inactivation of the drug, acquisition of resistance genes from other species, decreased membrane permeability, loss of outer membrane porins and increased drug efflux. Multidrug resistant (MDR) *P. aeruginosa* often exhibits more than one of the aforementioned resistance mechanisms. This study was carried out to functionally characterize the drug resistance mechanisms in five *P. aeruginosa* isolates selected based on clinical outcomes and antimicrobial susceptibility pattern.

Results and discussion

Among the five isolates, three were obtained from corneal buttons of patients who underwent TPK surgery, and two from corneal scrapings of ulcers that later healed. Both groups included one MDR strain.

Antimicrobial susceptibility testing & Next Generation sequencing

The Minimum Inhibitory Concentration (MIC) was determined using micro broth dilution method and VITEK (Table1). Whole genome sequencing of *P. aeruginosa* isolates was done in an Illumina platform. MDR isolate BK6 was resistant to all antibiotics tested except colistin, and BK3 was resistant to more than three classes of antibiotics. Comparative whole genome analysis of all five isolates with PAO1 as a reference strain, revealed various known and novel mutations in genes encoding efflux pumps, β-lactamases, outer membrane porins and other resistance associated proteins.

Real time quantitative PCR

To compare the expression level of resistance associated genes, real time PCR was done using the 7900 HT Fast Real-Time PCR system. With rplu as housekeeping gene, the expression levels were calculated relative to PAO1. MDR isolate BK6 had two fold increased expression levels of nalC, nalD and ampC genes, whereas in BK3, all the genes except nalC and ampC were highly down regulated. The transcript levels of porin (oprD), ampC and efflux pump (nalC, nalD, mexA, mexB, oprM, mexR) genes in the drug sensitive isolates were relatively same as that of PAO1 (Fig.1).

Ethidium Bromide agar cartwheel assay

The efflux activity of P. aeruginosa isolates was determined using Ethidium bromide (EtBr) agar based screening method in the presence or absence of an efflux pump inhibitor (EPI)

Antibiotics	*P. aeruginosa* isolates				
	BK 2	BK 3	BK 4	BK 5	BK 6
Ticarcillin / Clavulanic acid	R ≥128	R ≥128	R ≥128	R ≥128	R ≥128
Piperacillin / Tazobactam	R ≥128	R ≥128	R ≥128	R ≥128	R ≥128
Cefepime	R ≥64	R ≥64	R ≥64	R ≥64	R ≥64
Doripenem	S 1	R ≥8	I 4	S 1	R ≥8
Imipenem	S 2	R ≥16	S 2	S 2	R≥16
Meropenem	S	I 4	S	S	R 4
Amikacin	S 4	R 16	S 4	S 4	R >64
Gentamicin	S 2	R> 16	S 2	S 2	R >16
Ciprofloxacin	S <0.25	S 0.5	S 0.5	S <0.25	R >4
Levofloxacin	S 1	S 1	S 1	S 1	R >8
Minocycline	R ≥16	R ≥16	R ≥16	R ≥16	R ≥16
Tigecycline	R ≥8	R ≥8	R ≥8	R ≥8	R ≥8
Colistin	S 2	S 2	S 2	S 2	S 1
Trimethoprim / Sulfamethoxazole	R ≥320	R ≥320	R ≥320	R ≥320	R ≥320
Moxifloxacin	S 1	S 2	S 2	S 1	R ≥64
Gatifloxacin	S 0.5	S 2	S 1	S 0.5	R ≥64
Tobramycin	S 1	R≥64	S 1	S 1	R≥64

Table.1 MIC was determined using broth dilution method and Vitek. Values are represented in µg/ml. R – Resistant, I – Intermediate, S – Sensitive.

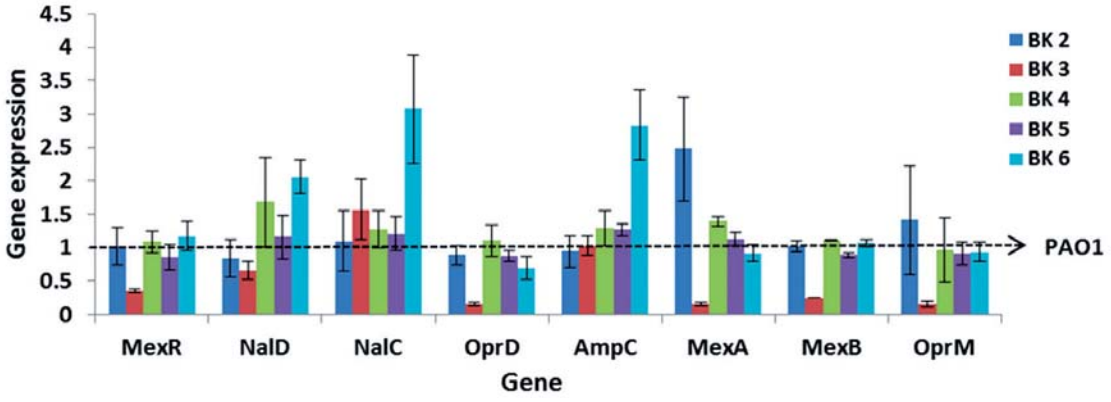


Fig.1. Expression levels of efflux pump, ampC and porin genes in P. aeruginosa isolates relative to the reference strain PAO1.

Carbonyl cyanide m-chlorophenyl hydrazone (CCCP). In general, an increased efflux or decreased accumulation of EtBr will present as reduced fluorescence in the bacterial population. The MDR isolates showed high fluorescence similar to the drug sensitive ATCC strain. Isolates BK 2 and BK 5 showed intermediate fluorescence and BK 4 showed very less fluorescence. Efflux pump inhibitor CCCP did not have much effect on the results (Fig.2).

MIC reversal assay

Drug resistance caused by increased drug efflux could be reverted by the addition of an EPI along with antibiotics. To test this possibility, an EPI based assay was performed, where the MIC of gentamicin and moxifloxacin were tested in the presence and absence of CCCP for both the MDR isolates. Although the MIC values for gentamicin were slightly lower in the presence of CCCP, the EPI could not resensitize either of the bacteria, suggesting presence of additional resistance mechanisms (Table 2).

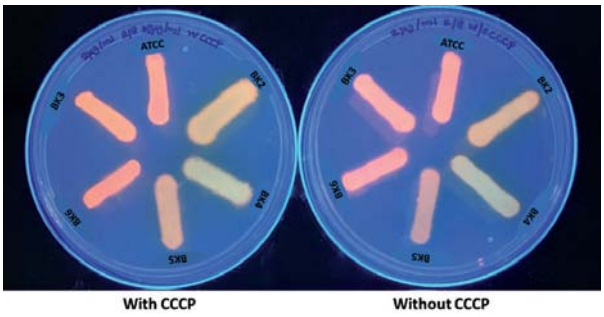


Fig.2. Cart Wheel assay: Ethidium bromide and CCCP were added at concentrations of 2 µg/ml and 15 µg/ml respectively. Test isolates were swabbed in a cartwheel pattern and incubated overnight.

Isolates	Gentamicin (µg/ml)		Moxifloxacin (µg/ml)	
CCCP	-	+	-	+
BK 6	>512	512	256	256
BK 3	>512	256	2	2

Table.2 MIC was determined using broth dilution method, in the presence and absence of CCCP at a concentration of 25µg/ml. The MIC of CCCP was 100µg/ml.

Real time fluorescence measurement

Cartwheel screening assay gives endpoint results and the kinetics of accumulation or efflux cannot be distinguished. To determine the real time accumulation and efflux of EtBr over time, fluorescence was measured in an automated multimode reader at defined time intervals at an excitation wavelength of 518nm and emission of 605nm.

Accumulation assay

Conditions that result in maximum accumulation of Ethidium Bromide (incubation at 25°C) inside the bacteria were employed. EtBr and CCCP were added to the bacteria suspended in PBS and

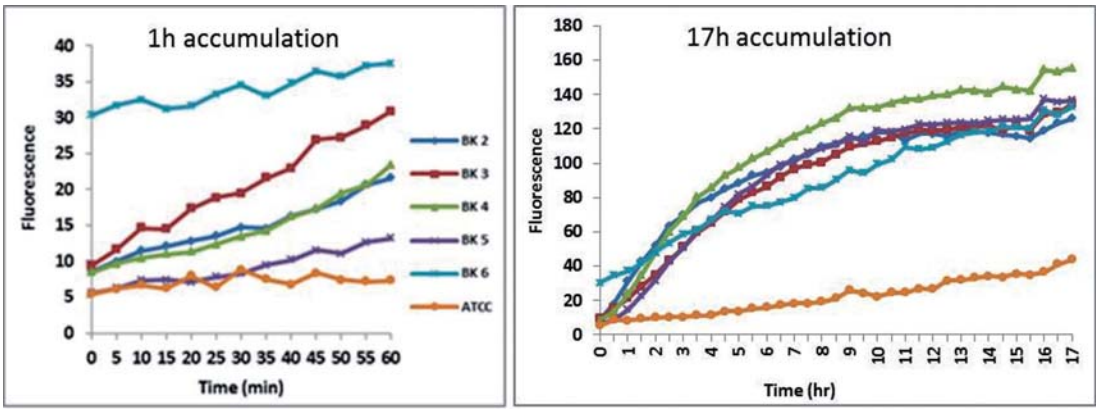


Fig.3. Fluorescence produced by accumulation of EtBr (2 µg/ml) inside P. aeruginosa in the presence of CCCP at a concentration of 15 µg/ml at 25°C.

the fluorescence was measured at various time intervals initially for one hour followed by 16 hour incubation. At one hour incubation period, both the MDR isolates displayed increased accumulation of fluorescence than all other isolates, whereas lowest accumulation was seen in the ATCC strain. During 17 hours incubation, the accumulation gradually increased in all the isolates and started to reach a steady state after 10 hours of incubation in all isolates except BK 6 (Fig.3).

Efflux assay

Efflux of EtBr was measured at maximum (with glucose and without CCCP) and minimum efflux (without glucose and presence of CCCP) conditions. After 1h accumulation at 25°C, bacterial cells were washed with PBS and the fluorescence was measured at 37°C for 30min. Increased efflux activity was seen only in the MDR isolate BK6 (Fig.4).

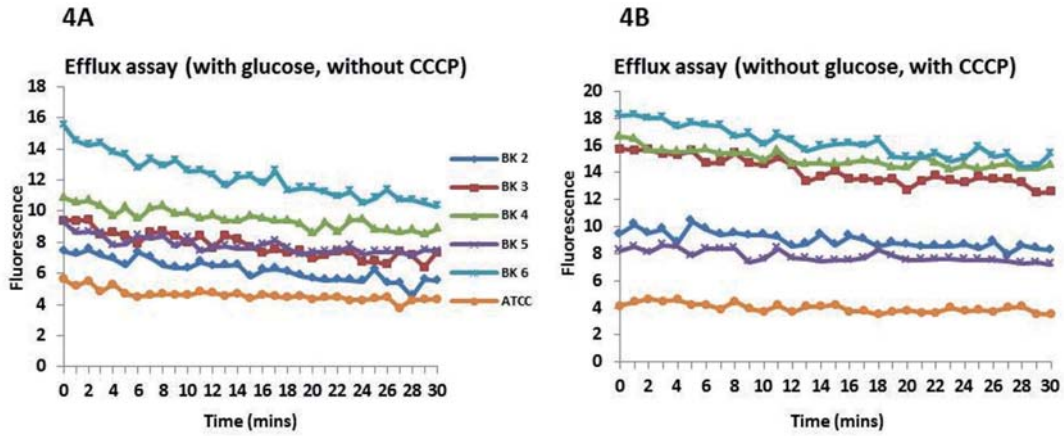


Fig.4. (A) Efflux of EtBr from P. aeruginosa isolates at 37°C in the presence of glucose and absence of CCCP. (B) Efflux of EtBr from P. aeruginosa isolates at 37°C in the absence of glucose and presence of CCCP.

Disk antagonism test

To determine the production of ampC β-lactamase, a disk test was performed with cefoxitin (FOX), a substrate for ampC along with ceftazidime (CAZ) and cefotaxime (CTX), which were placed over the agar at equal distances from FOX disk. Blunting of ceftazidime and cefotaxime zone of inhibition adjacent to the cefoxitin disk is considered positive for ampC β-lactamase production. Also, reduced susceptibility to all three disks is also considered positive for ampC production. All the tested isolates showed blunting of zone of inhibition, and BK6 was resistant to all three drugs indicating that all five isolates were ampC β-lactamase producers (Fig.5).

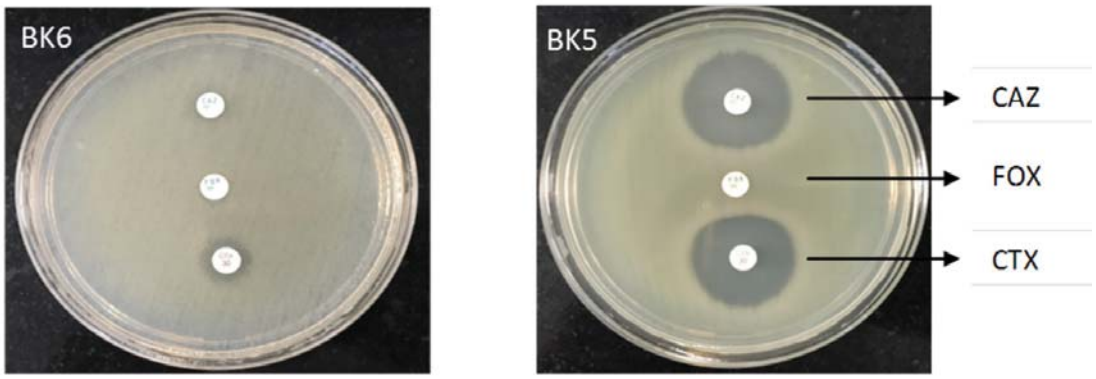


Fig.5. Disk antagonism test to detect ampC β-lactamase. CAZ – Ceftazidime, FOX – Cefoxitin, CTX – Cefotaxime.

Modified Hodge test

The production of Metallo Beta Lactamase (MBL) enzyme was determined by the modified Hodge test. A 0.5 McFarland culture of *E.coli* ATCC 25922 diluted 1 in 10 was spread on Mueller Hinton agar (MHA) plates and Imipenem, a substrate of MBL (10µg), was placed at the centre. The test isolates were swabbed from the edge of the disk to the periphery of the plate. The clover-leaf type of indentation at the intersection of tested organism and *E.coli* within the zone of inhibition is considered as a positive result. The tested isolate BK6 was found to be a MBL producer (Fig.6).

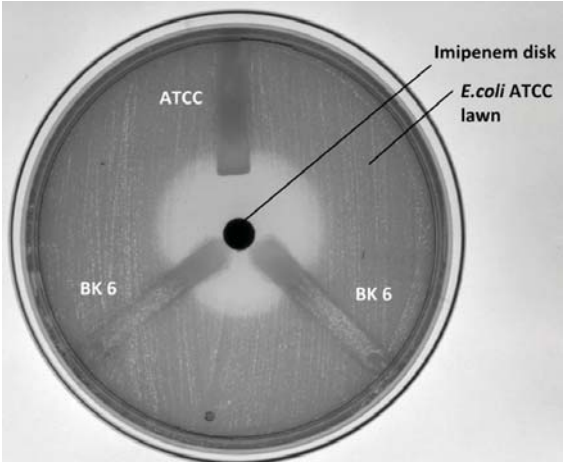


Fig.6. Modified Hodge test

Imipenem EDTA combined disk test

As a confirmatory test for MBL, imipenem EDTA combined disk test was performed based on the inhibitory effect of EDTA on metalloenzymes. Two imipenem disks were placed on the surface of the MHA containing the lawn of test isolates and 10 µl of 0.5M EDTA was added to one of the disks. After overnight incubation, an increase of >7mm in the zone of inhibition of imipenem+EDTA disk than the imipenem disk alone is considered positive for MBL. The MDR strain BK6 alone was found to be positive for MBL showing a significant increase in inhibition of > 34mm (Fig.7).

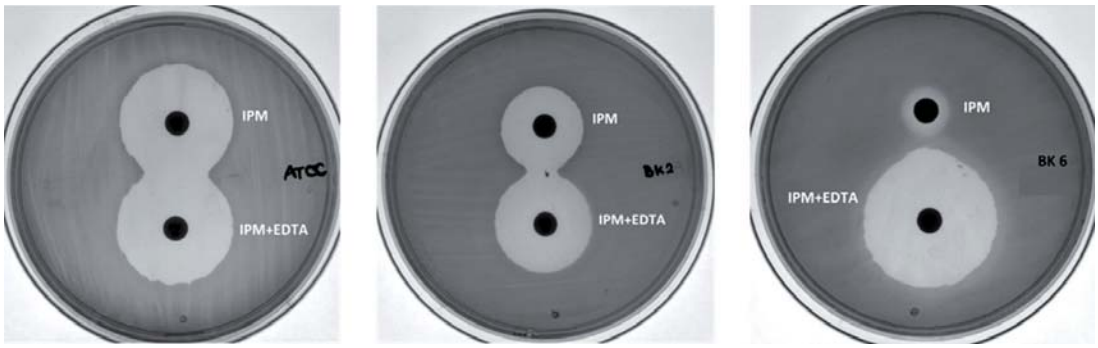


Fig.7. Imipenem EDTA combined disk test

Conclusion

Among the five ocular isolates of *P. aeruginosa*, MDR strain BK6 was shown to exhibit multiple mechanisms of resistance like increased efflux and production of ampC and metallo β-lactamases. However, increased efflux is probably not a major mechanism of resistance in this isolate, since the efflux pump inhibitor could not resensitize the bacteria. Relative gene expression of transcriptional regulators nalC and nalD was higher in the MDR strain BK6, which may have a repressive effect on the expression of MexAB-OprM efflux pumps. All other isolates were found to be producers of ampC β-lactamase.

CONFERENCES ATTENDED

Annual Meeting of Association for Research in Vision and Ophthalmology (ARVO) 2016

Seattle, Washington, USA, May 1-5

Prof.K.Dharmalingam, Director-Research, Dr.P.Sundaresan, Senior Scientist and Mr.Naveen, Proteomics Department attended the meeting. Mr.Naveen poster presentations "Antifungal defence proteins induced in tear fluid of mycotic keratitis patients – A quantitative proteomics study" (Authors: Kuppamuthu Dharmalingam, Kandhavelu Jeyalakshmi, Demonte Naveen L, Thangavel Chitra, Jayapal Jeya Maheshwari, Prajna Lalitha, Namperumalsamy Venkatesh Prajna). Dr.P.Sundaresan Identification of SIX6 gene variants in Indian Primary Open Angle Glaucoma patients. He interacted with eminent scientists and collaborators and discussed the various on-going projects and prospects of future collaborations.He also participated in the Asian Eye Genetics Consortium meeting.

23rd Annual Meeting of Indian Eye Research Group (IERG) – ARVO India Chapter

LV Prasad Eye Institute, Hyderabad, July 30-31, 2016

Prof. K. Dharmalingam received Bireswar Chakrabarti Oration Award 2016 and gave a talk on “Proteomics of eye diseases”. He also conducted an hour long session on the topic, “Proteomics for Clinicians”.

Dr. Gowripriya

- *Molecular signature of highly enriched corneal epithelial stem cells*

Dr. S. Senthil kumari:

- *Attenuation of Lipofuscin bisretinoids (A2E) accumulation by macular carotenoids in Human Retinal pigment epithelial cells in vitro*

Prof. K. Dharmalingam with the AMRF team at IERG meeting

Aswin Balaji

- *Effects of SB772077B on rock expression in human trabecular meshwork cells*

T S Balaji

- *Assessing the role of RB1 mutations in chemoresistance of retinoblastoma*

A. Divya

- *Aspergillus flavus infection induced changes in the proteome of human corneal epithelial cell line (Best poster in Basic Science category).*

Ms.Yogapriya, Ms. A. Divya, Mr. Mohd. Razeeth, Mr. Balaji and Ms. Y. Madhavi Latha received travel fellowship to attend the meeting.

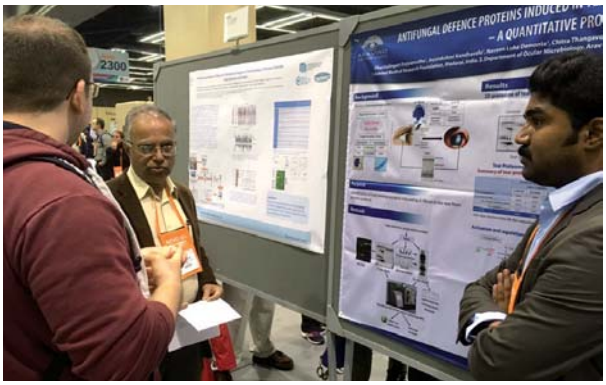
The AMRF team won third prize in the quiz programme.

ASIA-ARVO Conference

Brisbane, Australia, 04 - 08 February 2017

Dr. Ramprasad and Dr. Aloysius Abraham attended the ASIA-ARVO 2017 conference at Brisbane, Australia. Oral presentations on the collaborative work with Univ. of Liverpool on “Development of a novel chemical cross-linker for the treatment of keratoconus” were given by

Prof. K. Dharmalingam and Mr. Naveen during poster presentation at US-ARVO



Dr. Ramprasad with Prof. Traian Chirila, Prof. Rachel Williams, Dr. Atikah Haneef and Dr. Shuko Suzuki at Queensland Eye Institute, Brisbane, Australia

Mr. Aloysius Abraham with Dr. Sandra Staffieri, Retinoblastoma Care Co-ordinator at Royal Children's Hospital, Melbourne.

Dr. P. Sundaresan with the delegates at the 6th Annual Conference of SMRM.

one of our collaborators, Dr. Atikah Haneef from the University of Liverpool, UK and it was well received with good critical comments from the panel of scientists and the audience. Aloysius Abraham presented a poster on the "Effective and Comprehensive Genetic Analysis of Retinoblastoma by Next Generation Sequencing". After the conference, he gave an invited talk on "Efficient genetic testing of Retinoblastoma in South Indian population" at Centre for Eye Research, Australia and visited Peter MacCallam Cancer Centre, Melbourne. Dr. Ramprasad also visited the lab of Prof. Traian Chirila, Chief Scientist at the Queensland Eye Institute (QEI) to gain knowledge related to the use of silk proteins as biomaterials in ophthalmic tissue engineering.

6th Annual Conference of the Society for Mitochondrial Research and Medicine (SMRM), Jawaharlal Nehru University, New Delhi

Feb. 10-11, 2017

Dr. P. Sundaresan

- *Mitoscriptome analysis to understand Diabetic Retinopathy*

Professional Visits abroad

Visit to Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, Chengdu, Sichuan, China

April 20-27

Dr.P.Sundaresan delivered an invited talk at the Eye Care Meeting of Sichuan Province and the 13th Sino-American Academic Symposium of Ophthalmology entitled "Molecular genetics of Inherited eye diseases and gene discovery through Next Generation Sequencing" on April 22. He gave a guest lecture at the Sichuan Provincial Key Laboratory for Human Disease Gene Study. In addition, he interacted with research students and his collaborators especially Dr. Zhenglin Yang, Dr. Xianjun Zhu at Sichuan Provincial People's Hospital, Chengdu and discussed the on going and future collaborative projects.

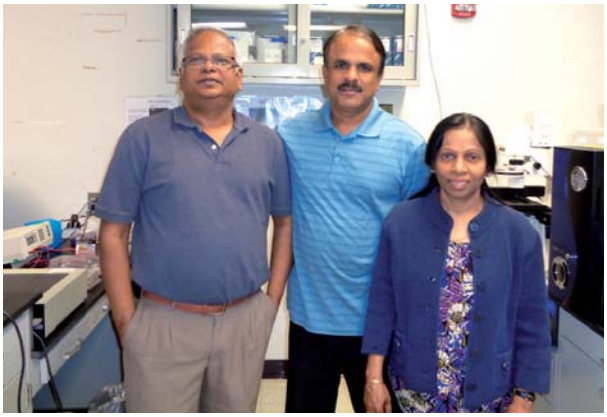
Visit to Stony Brook University, Stony Brook, New York

May 6-9, 2016

Dr.P.Sundaresan visited the Department of Physiology and Biochemistry, Stony Brook University, Stony Brook,

Dr. P. Sundaresan at 13th Sino American Academic Symposium of Ophthalmology, Sichuan, China

Dr. P. Sundaresan with Dr. Varadaraj and Dr. Sindhu Kumari at Stony Brook University, Stony Brook, New York



New York and met Drs. K.Varadaraj and Sindhu Kumari and held discussions on cataract genesis in mouse and humans.

Visit to Israel under Indian National Science Academy (INSA) Exchange Programme

May 13-28, 2016

Dr.P.Sundaresan visited the Department of Ophthalmology, Hadassah Hebrew University Medical Centre, Jerusalem, Israel. He gave a talk and met ophthalmologists and researchers including Prof. Pe'er, Dr. Yahalom, Prof. Chowers, Prof.Banin, Prof. Dror Sharon and Dr. Anat Blumenfeld. Dr.P.Sundaresan discussed future potential collaborations.

Dr. P. Sundaresan at the Dept. of Ophthalmology, Hadassah Hebrew Univerisity, Medical Centre, Jerusalem

Visit to Institute of Ageing and Chronic Disease, University of Liverpool, UK

July 11-August 31, 2016

Dr. O.G. Ramprasad visited the labs of Prof. Rachel Williams and Prof. Colin Willoughby at the Department of Eye and Vision Science, Institute of Ageing and Chronic Disease, University of Liverpool, UK as part of an exchange programme under the ongoing collaborative research project on "Novel chemical cross-linking of the cornea for the treatment of keratoconus". The visit was primarily aimed to assess the biological effects of the novel chemical cross-linker on the cells harvested from different layers of porcine cornea. Various biological assays were optimized utilizing the porcine corneas, which would be replicated later to test the effect of the novel drug in the human corneas at AMRF. Dr. Ramprasad worked with Dr. Atikah Haneef, a post-doctoral fellow and Prof. Rachel Williams at the University of Liverpool.

In continuation to this visit, Dr. Atikah Haneef visited AMRF from September 28, 2016 to November 18 2016. She worked on various experiments to analyze the effects of the novel cross-linker on human cadaver and keratoconic corneas.

Prof. Colin Willoughby, Dr. Ramprasad, Prof. Rachel Williams and Dr. Atikah Haneef during a meeting at the University of Liverpool.

Visit to Dartmouth College and Medical Centre, Hanover, USA

October 26 – November 7

Dr. D. Bharanidharan was invited to Dartmouth college and Dartmouth-Hitchcock Medical Centre as part of Aravind-Dartmouth research exchange programme initiated by the interaction between Dr. N. Venkatesh and Dr. Michael E. Zegans, Section Chief Ophthalmology and Professor of Surgery, Dartmouth College and Medical Centre, Hanover, USA. He spent three weeks and had the opportunity to learn advanced proteomics and other molecular techniques in the labs of Dr. Scott Gerber and Dr. Robert Cramer. He worked on bioinformatics projects to analyze next-generation sequencing datasets that describe microbial communities and microbial gene expression in clinical samples. He interacted with Dr. Michael E. Zegans and his bio-class students. He also discussed with other scientists on future collaborative projects.

Awards

Bireswar Chakrabarti Oration Award 2016

Prof. K. Dharmalingam received "Bireswar Chakrabarti Oration Award 2016" and gave a talk on "Proteomics

Dr. Bharanidharan with Dr. Micheal Zegans and Ms. Dawn Carey, Dartmouth College, Hanover, USA.

Prof. K. Dharmalingam receiving the Bireswar Chakrabarti Oration Award from Dr. G.N.Rao

Ms. Prakadeeswari receiving award for best oral presentation

of eye diseases" at the 23rd Annual Meeting of Indian Eye Research Group (IERG) held at at LV Prasad Eye Institute, Hyderabad on July 30-31, 2016.

Best Poster Award

Ms. A. Divya, Proteomics Department received Best Poster Award for the presentation on "*Aspergillus flavus* infection induced changes in the proteome of Human Corneal Epithelial cell line" at the 23rd Annual Meeting of Indian Eye Research Group (IERG) meeting at LV Prasad Eye Institute, Hyderabad on July 30-31, 2016.

Award for Best Oral presentation

Ms. G. Prakadeeswari, Junior Research Fellow, Department of Molecular Genetics received the award for her presentation on "Association Study on homocysteine gene polymorphisms in South Indian individuals with Pseudoexfoliation Syndrome" at the 5th National Conference on Emerging Trends and New Challenges in Biotechnology- Advances in Free Radicals and Antioxidants on conducted by PG and Research Centre in Biotechnology, MGR College, Hosur from February 2-3, 2017.

Ph.D Awarded

Mr.G.Gowthaman, Department of Molecular Genetics defended his Ph.D thesis entitled "Investigating the role of Nuclear, Mitochondrial Genome and microRNA in the pathogenesis of Diabetic Retinopathy" on 1st June. He carried out his studies under the guidance of Dr.P.Sundaresan.

Ms.Saumi Mathews, Department of Stem cell Biology defended her Ph.D thesis entitled "Studies on the characterization of limbal niche- their role in maintenance and ex vivo expansion of human corneal epithelial stem cells" on October 21st. She carried out her studies under the guidance of Dr.C.Gowri Priya

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 poster presentation by the research scholars. The award carries a certificate and cash prize of Rs.25,000/-.

Mr. Gowthaman defending his Ph.D thesis

Ms. Saumi Mathews defending her Ph.D thesis

Mr. Aloysius Abraham receiving Prof. VR. Muthukkaruppan Endowment Award from Dr. P. Namperumalsamy

Dr. P. Sundaresan receiving the award from the Honorable President of India

Prof.VR.Muthukkaruppan Endowment award 2016 given to Mr. Aloysius Abraham, Department of Molecular Genetics for his outstanding research work on "Effective and Comprehensive genetic screening for Retinoblastoma patients". The selection committee included Dr. Sundar, Professor and Head, Department of Biotechnology, Kalasalingam University,

Dr. S. Thiyagarajan, Faculty Scientist, Institute of Bioinformatics and Applied Biotechnology (IBAB) Bangalore and Dr. D. Karunagaran, Professor & Head, Department of Biotechnology, IIT, Chennai.

Hari Om Ashram Alembic Research Award

Dr. Sundaresan, Senior Scientist and Head of the Department of Molecular Genetics, Aravind Medical Research Foundation received the Medical Council of India- Hari Om Ashram Alembic Research Award for 2010 from the Honorable President at Rashtrapati Bhawan, New Delhi on 28th March 2017.

Training of project students

- Mr. K. Rajkumar, MSc Microbiology student from Bharathidasan University
- A. Kalaivani, M.Sc., Human Genetics and Molecular Biology, Bharathiar University, Coimbatore
- P. Karthikeyan, M.Sc., Biotechnology, Alagappa University, Karaikudi
- N. Narmatha, B.Tech Biotechnology, Mepco Schlenk Engineering College, Sivakasi
- K. Jenepha, B.Tech Biotechnology, Mepco Schlenk Engineering College, Sivakasi
- M. Kanmani, M.Sc. Biotechnology, Alagappa University, Karaikudi

NEW FACILITIES

Dr. P. Namperumalsamy inaugurating the Cancer Genetic Testing Facility

Inauguration of Cancer Genetic Testing Centre

Retinoblastoma is the most common intra-ocular tumour of children. Unfortunately half of these are heritable. Aravind has been treating retinoblastoma cases and found very good results in those who presented early. Lot of work has been done at Aravind's research institute on the gene responsible for retinoblastoma. A lot of mutations have been identified and cost effective methodology has been established to predict this disease in the siblings, offspring as well as secondary tumours in the same patient, results of which have been published. Now a cancer genetic testing lab has been established at Aravind - Madurai, thanks to the generous support of Allene Reuss Memorial Trust, the So-hum Foundation and Mr. Shyam Prakash Gupta. Dr. P. Namperumalsamy inaugurated the centre. The facility extends its services to patients beyond Aravind Eye Care System, at an affordable cost.

CONFERENCES / WORKSHOPS CONDUCTED

14th Research Advisory Committee Meeting

May 27-28

The 14th Research Advisory Committee meeting of Aravind Medical Research Foundation was held in the last week of May. During the meeting, faculty members of AMRF presented their work and received feedback. The poster session held on May 27 was inaugurated by Dr. D. Karunagaran, Professor & Head, Department of Biotechnology, IIT, Chennai. Along with him two special invitees - Dr. Sundar, Professor & Head, Department of Biotechnology, Kalasalingam University and Dr. S. Thiyagarajan, Faculty Scientist, Institute of Bioinformatics and Applied Biotechnology (IBAB) Bangalore were also assessed the posters which were 24 in number. Best poster was selected for Prof. VR. Muthukkaruppan Endowment Award for the year 2015.

Members at the Research Advisory Committee Meeting

One day conference on "Approaches to understand Modern Biology through Research"

October 7

As part of October Summit 2016, AMRF organized a one day conference to motivate post graduate students (Life Sciences) towards research. A total of fifty seven participants from eight colleges attended the conference. The programme started with an introduction by Dr. P. Namperumalsamy, followed by a series of presentations by the faculty from AMRF, Mepco Schlenk Engineering College, Kalasalingam University, The American College and Lady Doak College. A total of eight models explaining various scientific themes were prepared by AMRF research scholars and technicians, which were well received by the participants. The interactive sessions were excellent.

AMRF – Dartmouth Education and Research Conference

December 2-3

Aravind Medical Research Foundation conducted a two day Education and Research Conference in collaboration with Dartmouth College which has been initiated by the interaction between Dr. N. Venkatesh Prajna, Cornea Chief and Dr. Michael E. Zegans, Section Chief Ophthalmology and Professor of Surgery, Dartmouth College and Medical Center, Hanover, USA. The conference was organized 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. A booklet entitled "AMRF – Dartmouth Education and Research Conference" covering the proceedings of the conference was also brought out.

Ten students and a research scholar from Dartmouth presented research proposals on various

Participants to the conference on Approaches to understand Modern Biology through Research

Participants of the Dartmouth Education & Research Conference

Participants of the workshop on Clinical Proteomics

eye diseases. Research work at AMRF was presented through lectures and posters. Details of the core research facilities and on-going research projects were presented and demonstrated to Dartmouth during the Lab visits. This conference would pave the way for future collaborative research projects and long term associations between the two institutions.

Workshop on Basics of Tissue Culture

December 26-30

A five day workshop was organized at Aravind Medical Research Foundation, sponsored by Department of Biotechnology, New Delhi. The workshop was inaugurated by Dr. P. Namperumalsamy. Prof. K. Dharmalingam gave an introduction of research activities at AMRF. The manual for the workshop was released by Er. G. Srinivasan, Director-Finance, AECS and the first copy was received by Prof. K. Dharmalingam.

A total of 20 participants were selected for the workshop which included 6 post graduate students, 12 research scholars and 6 faculty from nearby colleges/ universities including The American College, Lady Doak College, Thiagarajar College, Kongunad College of Arts and Science, Kamaraj College of Engineering and Technology, Mepco Schlenk Engineering College, Bannari Amman Institute of Technology, Madurai Kamaraj University, Bharathidasan University and Anna University (Trichy). The workshop included lectures and laboratory sessions. Certificates for the participants were distributed by Prof. VR. Muthukkaruppan.

Participants of the workshop on Basics of Tissue Culture

Workshop on Clinical Proteomics and Proteomics Day Celebration

March 18

Aravind Medical Research Foundation hosted this annual event in commemoration of the formation of the Proteomics Society of India. Prof.K. Dharmalingam, Director-AMRF introduced the event to the participants and highlighted the role of Society in spreading education for research in Proteomics. Eminent scientists including Prof. Kumaravel Somasundaram from IISc, Dr.Karunagaran from IIT-Madras, Prof.Anuranjan Anand from Jawaharlal Nehru Centre for Advanced Scientific Research, Prof.Balamurugan from Alagappa University and, Dr.Subbulakshmi from Pondicherry University were invited to give lectures. AMRF faculty also addressed the audience. This one-day seminar was supported by Proteomics Society of India.

GUEST LECTURES DELIVERED BY VISITING SCIENTISTS

Dr. Kanury V.S.Rao, National Chair, Translational Health Science and Technology Institute (THSTI), Faridabad and Head, Drug Discovery Research Centre Topic: “Exploiting Systems-Based Approaches for Drug Target Discovery”, May 23

Dr. S. Thiyagarajan, Faculty Scientist, Institute of Bioinformatics and Applied Biotechnology (IBAB), Bangalore Topic: “The Paralogue Story of ArsR in Agrobacterium tumefaciens 5A”, May 27.

Prof.Tassignon Marie Jose, Chief and Head of Dept of Ophthalmology, Immediate Past Medical Director, University hospital, Antwerp, Belgium Topic: “Stem Cell Research”, June 22.

Dr.V.L.Ramprasad, Chief Operating Officer, MedGenome Labs Private Ltd. Bangalore Topic: "Application of Genomic Knowledge in Current Medical Practice, including Ophthalmology”, July 2.

Dr. K.Varadaraj, Research Associate Professor, Department of Physiology & Biophysics, Stony Brook University Medical Centre, Stony Brook New York Topic: “Role of Aquaporins in lens transparency, refractive index gradient and biomechanics”, September 6

Dr. C. Subbulakshmi, UGC-Assistant Professor, Department of Biochemistry and Molecular Biology, Pondicherry University, Topic: “Protein-Protein and Protein-RNA interaction studies in the vitreous humor of Diabetic Retinopathy patients to analyze the functional network of novel somatic PIWI-like proteins and PIWI-interacting RNAs (piRNA)”, September 12

Dr.Elavazhagan Murugan, Adjunct Instructor, Duke-NUS Medical School, Singapore Eye Research Institute, Singapore Topic: “pH Induced Conformational Transitions in the Transforming Growth Factor β-Induced Protein (*TGFβIp*) Associated Corneal Dystrophy Mutants”, December 28.

Drs. Tin Aung and Jay Wei from Singapore Eye Research Institute (SERI) and Dr. CC Khor from Genome Institute of Singapore visited Aravind on April 7. They interacted with the senior team at Aravind, faculty of both Aurolab and AMRF and clinic heads.. Dr. Tin Aung delivered a lecture on “Update on Angle closure glaucoma” The purpose of their visit was to expand collaboration and partnership between Aravind and SNEC/SERI.

PUBLICATIONS 2016-17

Yanan Di, Lulin Huang, Periasamy Sundaresan, Shujin Li, Ramasamy Kim, Bibhuti Ballav Saikia, Chao Qu, Xiong Zhu, Yu Zhou, Zhilin Jiang, Lin Zhang, Ying Lin, Dingding Zhang, Yuanfen Li, Houbin Zhang, Yibing Yin, Fang Lu, Xianjun Zhu & Zhenglin Yang

- *Whole-exome Sequencing Analysis Identifies Mutations in the EYS Gene in Retinitis Pigmentosa in the Indian Population*

Scientific Reports | 6:19432 | DOI: 10.1038/srep19432

Chiea Chuen Khor *et al*.,

- *Genome-wide association study identifies five new susceptibility loci for primary angle closure glaucoma*

Nature Genetics (published online 4 April 2016)

Karthikeyan Arcot Sadagopan, Renukadevi Kathirvel, Rosanne B. Keep, P. Sundaresan, Hui Huang, Arndt Rolfs, Kannan Parthiban & P. Vijayalakshmi

- *Cutaneous freckling: Possible new clinical marker for the diagnosis of Hermansky-Pudlak syndrome in Indian Asian patients with oculocutaneous albinism*

Ophthalmic Genet. 2016 May 13:1-3.

Zhang L, Yang Y, Li S, Tai Z, Huang L, Liu Y, Zhu X, Di Y, Qu C, Jiang Z, Li Y, Zhang G, Kim R, Sundaresan P, Yang Z, Zhu X.

- *Whole Exome Sequencing Analysis Identifies Mutations in LRP5 in Indian Families with Familial Exudative Vitreoretinopathy*

Genet Test Mol Biomarkers. 2016 May 26. [Epub ahead of print]

Mohideen Abdul Kader, Prasanthi Namburi, Sarika Ramugade, R. Ramakrishnan, Subbiah R. Krishnadas, Ben R. Roos, Sundaresan Periasamy, Alan L. Robin & John H. Fingert

- *Clinical and genetic characterization of a large primary open angle glaucoma pedigree*

Ophthalmic Genetics 2016

Yin Yang, Yeming Yang, Lulin Huang, Yaru Zhai, Jie Li, Zhilin Jiang, Bo Gong, Hao Fang, Ramasamy Kim , Zhenglin Yang, Periasamy Sundaresan, Xianjun Zhu & Yu Zhou

- *Whole exome sequencing identified novel CRB1 mutations in Chinese and Indian populations with autosomal recessive retinitis pigmentosa*

Scientific Reports | 6:33681 | DOI: 10.1038/srep33681

Jeyalakshmi Kandhavelu, Naveen Luke Demonte, Venkatesh Prajna Namperumalsamy, Lalitha Prajna, Chitra Thangavel, Jeya Maheshwari Jayapal, Dharmalingam Kuppamuthu

- *Aspergillus flavus induced alterations in tear protein profile reveal pathogen induced host response to fungal infection*

Journal of Proteomics 2016 (accepted 19th Oct 2016)

Jeyalakshmi Kandhavelu, Naveen Luke Demonte, Venkatesh Prajna Namperumalsamy, Lalitha Prajna, Chitra Thangavel, Jeya Maheshwari Jayapal, Dharmalingam Kuppamuthu

- *Data set of Aspergillus flavus induced alterations in tear proteome: Understanding the pathogen-induced host response to fungal infection*

Data in Brief 9(2016):888–894.

Bibhuti BS, Sushil Kumar Dubey, Mahesh Kumar S, Sundaresan P

- *Whole mitochondrial genome analysis in South Indian patients with Leber's hereditary optic neuropathy*

Journal of Mitochondrion 2016

Senthilkumari S, Sharmila R, Gowripriya C, Vanniarajan A.

- *Epalrestat, an Aldose Reductase Inhibitor Prevents Glucose-Induced Toxicity in Human Retinal Pigment Epithelial Cells In Vitro*

J Ocul Pharmacol Ther. (2016 E pub Nov 11)

Jhansi Kasinathan , Prajna NV, Muthukkarupan VR, Gowri Priya C

- *A novel method for a high enrichment of human corneal epithelial stem cells for genomic analysis*

Journal of Microsc Res Tech. 2016 Nov. (EPub ahead of Print)

ONGOING RESEARCH PROJECTS

No	Projects	Funded by	Investigators	Research Scholar
MICROBIOLOGY				
1.	Analysis of bacterial persistence mechanisms in recalcitrant ocular *Pseudomonas aeruginosa* infections	SERB	Dr.M.Vidyarani	R. Sangeetha
2.	Functional characterization of drug resistance mechanisms in corneal isolates of *Pseudomonas aeruginosa*	AMRF	Dr. M. Vidyarani	T. Kannan
3.	Regulatory role of human microRNAs in microbial keratitis	AMRF	Dr. M. Vidyarani Dr. D. Bharanidharan Dr. Lalitha Prajna	K. Kathirvel
PROTEOMICS				
4.	CoE – Human Mycotic Keratitis	DBT	Dr.N.Venkatesh Prajna Dr.K,Dharmalingam Dr.Lalitha Prajna Dr.J.Jeya Maheshwari Dr.O.G.Ramprasad Dr.Rabbind Singh	S.Mohammed Razeeth A. Dhivya K.R.P. Niranjana Naveen Luke Demonte C. Sathya Priya S. Nithyalakshmi B. Muthukumar R.V.Angela Asir O.Ruthra
5.	Predictive biomarkers for diabetic retinopathy among diabetics and stage specific biomarkers for NPDR and PDR.	Bagchi grant	Dr.K.Dharmalingam Dr.R.Kim Dr.J.Jeya Maheshwari Dr.O.G.Ramprasad	Roopesh R.Pai R. Sharmila Naveen Luke Demonte A. Divya K. Sandhya Ranjani Anjhu Nair
6.	Pathogenic Aspergillus interaction with Innate Immune cells	CEFIPRA	Dr.Lalitha Prajna Dr.Rabbind Singh Dr.J.Jeya Maheshwari Dr.K.Dharmalingam	P.M.Vaishali M. Kanmani V. Lakshmi Prabha
7.	Functional analysis of circulating microRNAs and their regulatory role in Diabetic Retinopathy	SERB	Dr.O.G.Ramprasad Dr.K.Dharmalingam	S. Ranjani

8.	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, Dr. Kishan Prajapati, Mrs. Karpagam Mr. Kannan	Jessica Judith Nunes
9.	Biomarker identification for accelerated ageing of eye in primary open angle glaucoma (POAG) and age related macular degeneration (AMD).	SERB National Post Doctoral Fellow	Dr. K. Karuppasamy	
10.	Comparative genomics of *Aspergillus flavus* clinical isolates	DBT - RA fellowship	Dr. C. Sathyapriya	
MOLECULAR GENETICS				
11.	Molecular genetics of Albinism in the Indian population	AMRF Post-Doctoral Fellow	Dr. P. Sundaresan Dr. P. Vijayalakshmi	K. Renugadevi
12.	Molecular genetics studies of Primary Angle Closure Glaucoma (PACG) in South Indian Population	UGC Fellowship	Dr. P. Sundaresan Dr. S.R. Krishnadas Dr. R.Venkatesh Dr. S. Kavitha	Roopam Duvesh
13.	Genetic and functional approaches to understand the pathogenicity of Primary Open Angle Glaucoma (POAG)	ICMR-SRF	Dr. P. Sundaresan Dr. S.R. Krishnadas	Moh'd Husain Shah
14.	Genetic and transcript analysis of RB1 gene in South Indian Retinoblastoma Patients	Aravind Eye Foundation	Dr. A.Vanniarajan Dr. Usha Kim Dr. R.Santhi Prof.VR.Muthukkaruppan Dr. D.Bharanidharan	K. Thirumalai Raj
15.	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

16.	Understanding the molecular mechanisms of chemoresistance in retinoblastoma	CSIR-NET JRF	Dr. A.Vanniarajan	T. S. Balaji
17.	Molecular characterization of tumor progression in retinoblastoma	DST-Inspire Fellowship	Dr. A.Vanniarajan	T. Shanthini
18.	Genetic evaluation of genes involved in homocysteine metabolism and hyperhomocysteinemia with Pseudoexfoliation syndrome in South Indian population	AEH	Dr.P.Sundaresan Dr. A. Haripriya	G.Prakadeeswari
19.	Molecular genetics of macular corneal dystrophy (MCD) in Indian population	DST INSPIRE Fellowship	Dr.P.Sundaresan Dr.N.Venkatesh Prajna	M.Durga
20.	Whole-exome Sequencing Analysis Identifies Mutations in the FAM161A and EYS Gene in Retinitis Pigmentosa in Indian Population	CSIR-SRF	Dr.P.Sundaresan Dr.Kim	Bibbuti Ballav Saikia
IMMUNOLOGY AND STEM CELL BIOLOGY				
21.	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
22.	Molecular signature of Corneal Epithelial Stem Cells (CESCs))	ICMR-SRF	Dr.C.Gowri Priya Dr.Venkatesh Prajna Prof.VR.Muthukkaruppan	M. K. Jhansirani
23.	Structural and functional integrity of corneal endothelium after storage in Cornisol, an indigenous intermediate stage corneal storage medium	AEH	Dr. N. Venkatesh Prajna Dr.C.Gowri Priya	S. Yogapriya
OCULAR PHARMACOLOGY				
24.	Studying the Role of Rho A – Rock Signalling in conventional outflow pathway using Human Organ Culture Anterior Segment (HOCAS)	SERB	Dr.S.Senthilkumari Dr.SR.Krishnadas Dr.C.Gowri Priya	S. Ashwin Balaji

25.	Indian Macular Carotenoids Research (INDMACARE) – A Feasibility study	ICMR	Dr.S.Senthilkumari Dr.Anand Rajendran	Yelchuri Madhavi Latha
26.	Is Human Amniotic Membrane (HAM) a Suitable Reservoir System for the Release of Drugs in Ocular use?	AEH	Dr.S.Senthilkumari Dr.Venkatesh Prajna	Yelchuri Madhavi Latha
BIOINFORMATICS				
27.	Clinical exome analysis pipeline for eye disease next-generation sequencing panel	SERB	Dr. D.Bharanidharan	K Manojkumar
28.	Comparative genome analysis to identify genomic variants and genes associated with drug resistance in Methicillin-Resistant Staphylococcus aureus (MRSA) and Pseudomonas aeruginosa ocular strains	AEH	Dr. D.Bharanidharan Dr. M. Vidyarani Dr. Lalitha Prajna	K. Kathirvel
29.	Structure and Sequence - Based Bioinformatics Approach to the Analysis of Non-synonymous Single Nucleotide Variants (nsSNVs) and Prediction of its Association with Retianl Diseases	AMRF	Dr. D. Bharanidharan	K. Manojkumar