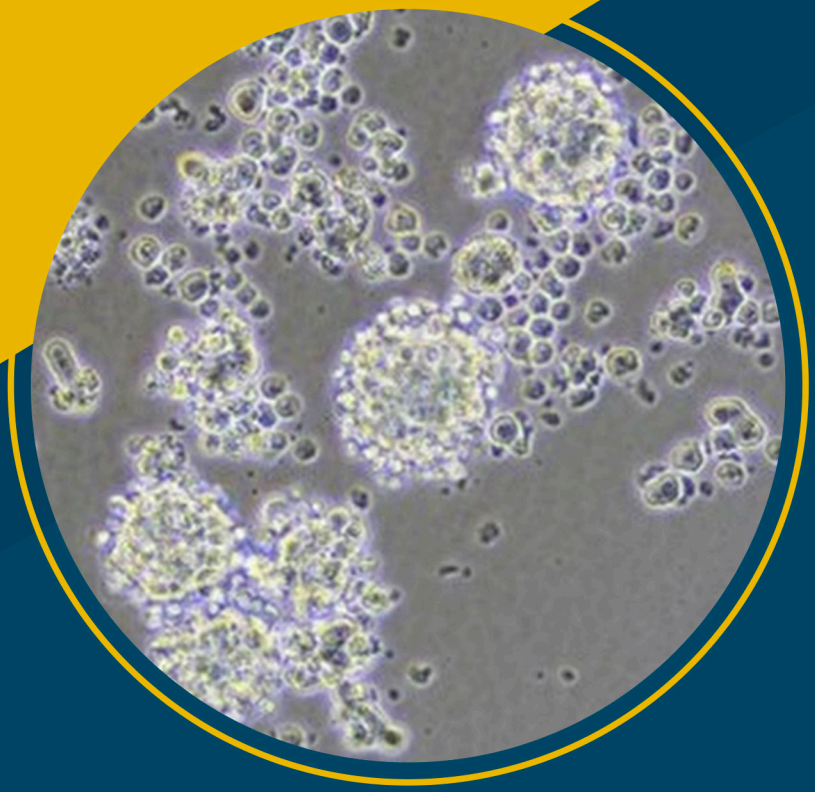
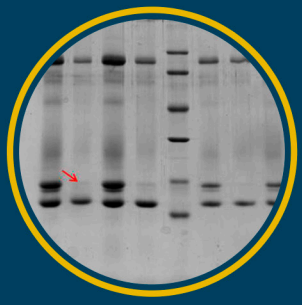
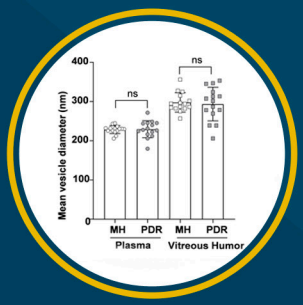
RESEARCH IN OPHTHALMIC SCIENCES

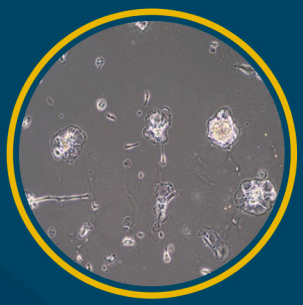
Aravind Medical Research Foundation

ANNUAL REPORT 2023 - 2024









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MISSION

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

RESEARCH IN OPHTHALMIC SCIENCES

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Annual Report 2023 - 2024

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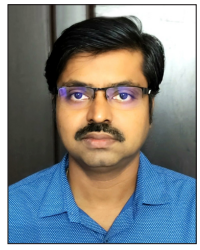

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FOREWORD

AMRF is continually advancing in the implementation of research projects and exploring new initiatives. Its innovative measures to enhance research are remarkable, supported by clinicians and scientists.

AMRF successfully organised the annual meeting of the Indian Eye Research Group (ARVO-India) on 29-30 July 2023, with the participation of 220 attendees. The programme included poster sessions, talks, and three award lectures, notably the Bireswar Chakrabarti Oration by Prof. Colin Willoughby from Ulster University, UK, the D Balasubramanian Oration by Dr. Subhabrata Chakrabarti from LV Prasad Eye Institute, Hyderabad, and the SS Badrinath Oration by Dr. V. Mohan from Dr. Mohan's Diabetes Specialities Centre, Chennai. This meeting served as a platform to showcase advancements in eye research in India, with numerous research projects demonstrating significant progress and an increasing number of clinicians engaging in research activities.

Numerous new and ongoing projects are progressing well, and those initiated during this year are expected to yield rewarding achievements. AMRF will continue to be actively involved in research, education and clinical translation.

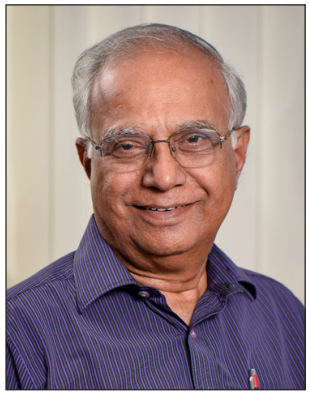
- Dr. P. Namperumalsamy
President, AMRF

INTRODUCTION

The progress detailed in the following pages of this year's annual report highlights the concerted efforts of our researchers towards achieving our goals. In addition to the ongoing projects, several new projects were initiated. Apart from research, AMRF members actively participated in various educational activities and awareness programmes, engaging college teachers and students through lectures at their institutions or special events organised at AMRF.

Furthermore, this year, AMRF hosted a significant Eye Research Conference, attracting 220 attendees to various scientific sessions. AMRF is grateful to several donors who have financially supported our research endeavors.

Prof. K. Dharmalingam
Director - Research



CONTENTS

MOLECULAR GENETICS

The genetic diversity in retinal dystrophies and optic neuropathies allows the utilization of next-generation sequencing (NGS) platforms to uncover the genetic factors associated with disease pathogenesis. Even though it is incurable, this irresistible approach offers accurate molecular diagnosis, thereby facilitating access to precise novel therapeutic interventions and enabling more personalized genetic counselling, to adapt an effective disease management protocol.

Currently, our lab is focused on characterizing the genetic factors underlying rare neural degenerative disorders in the human visual system, such as Juvenile X-linked retinoschisis (JXLR), Leber's hereditary optic neuropathy (LHON), and Leber's congenital amaurosis (LCA). Furthermore, our endeavour aims to construct a gene registry of the visual system specific to the South Indian ethnicity.

Investigation of nuclear genes involvement in a Mitochondrial Disorder: Leber's Hereditary Optic Neuropathy

Investigators : Dr. P. Sundaresan
Dr. S. Mahesh Kumar
Research scholar : C. Prakash
Funding Agency : SERB – CRG
(CRG/2022/000926)

Introduction

The study aims to explore the mito-nuclear genetic implications in a larger LHON cohort. This investigation involves a sequential characterization in

LHON probands, encompassing primary mitochondrial DNA (mtDNA) mutation analysis, whole mtDNA sequencing, and whole exome sequencing. It's worth noting that our earlier findings revealed a significant nuclear gene influence specifically evident in 50% of LHON probands in group II and 66.6% in group III, particularly in cases lacking primary mtDNA mutation. Currently, the study has been performed primary mtDNA mutation screening in 40 distinct samples, followed by whole mtDNA sequencing and whole exome sequencing. This comprehensive approach aims to unravel the molecular implications and understand the inheritance patterns among the affected probands.

Decoding the Unknown Genetic Etiology to ameliorate the molecular diagnosis of Leber's Congenital Amaurosis.

Investigators : Dr. P. Sundaresan,
Dr. S. Senthil kumari,
Dr. Rupa Anjanamurthy
Junior Research Fellow: S. Shiva Sankari
Funding Agency : Indian Council of Medical Research - Grant-in-aid Scheme
(R.11015/10/2023-GIA/HR)

Leber's Congenital Amaurosis (LCA), an early and severe form of inherited retinal dystrophy, constitutes 5% of all inherited retinopathies. It affects 1 in 30,000 to 81,000 live births. Globally, 29 genes have been implicated in LCA pathogenesis, accounting for 70% of diagnosed cases. Despite its rarity, our recent five years prospective investigation diagnosed 135 LCA cases, indicated a higher prevalence in South Indian cohort, due to common practice of consanguineous and endogamous marriages. Molecular diagnosis of 135 LCA cases using a targeted gene panel identified mutations in 21 known LCA candidate genes, resulting in a detection rate of 84%. However, the 16% of genetically unresolved cases will be further subjected to comprehensive phenotyping combined with whole exome sequencing to decode the potential genetic factors associated with LCA.

Furthermore, this study will recruit 75 new LCA cases to broaden the understanding of the genetic landscape in the South Indian ethnicity. With informed consent, blood samples and clinical data from 15 new LCA cases have been collected from the Department of Paediatric clinic, Aravind Eye Hospital, Madurai. This effort will provides a valuable insights into the genetic underpinnings of LCA within the South Indian population.

Investigating genotypic and phenotypic variations in X-linked retinoschisis: Insights from a cohort of South Indian patients

Investigators : Dr. P. Sundaresan,
Dr. Rupa Anjanamurthy
Research Scholar : Susmita Chowdhury

Funding Agency : Lady Tata Memorial Trust – Senior Research Fellowship

Introduction

Retinoschisis is a rare monogenic disorder typically characterized with intraretinal layer clefts. Ophthalmic investigation including fundus examination, optic coherence tomography (OCT) and electroretinography (ERG) revealed a distinct spoke wheel or cartwheel pattern in the macular region, histological alterations in the retinal layers and a decrease in b-wave amplitude, respectively. It's inherited in an X-linked pattern due to mutations in the RS1 gene. It encodes a 24kDa monomeric protein known as retinoschisin (RS1). This extra cellular protein plays a crucial role to uphold its intricate retinal cellular architecture. Therefore, a molecular analysis of the RS1 gene holds the potential to offer definitive validation for the diagnosis of X-linked retinoschisis (XLRS), thereby helping to differentiate it from overlapping clinical diagnoses. Currently, the study has performed RS1 genetic screening on twenty-two unique cases of XLRS within the South Indian population, focusing on the clinical implications of these cases, particularly in probands carrying RS1 genetic mutations.

Results

The clinical data from twenty-two South Indian XLRS patients, collected over seven years (2016-2023), revealed a median disease presentation age of 14.5 years (range: 4 to 34 years). The average visual acuity of the probands was 0.79 ± 0.39 logMar, with a range of 0.17 to 1.77. Fundus appearance of the XLRS probands showed a distinctive spoke wheel-like configuration, either with foveal or peripheral schisis [Fig. 1a, b]. Furthermore, OCT analysis showed 13.6% of probands with foveal schisis and 22.7% with foveal schisis extending to the macula (macular schisis). Additionally, 13.6% exhibited macular schisis extending to the peripheral retina, while 4.5% had peripheral schisis. Macular detachment was observed in one proband, and retinal detachment was evident in 18.1% of probands.

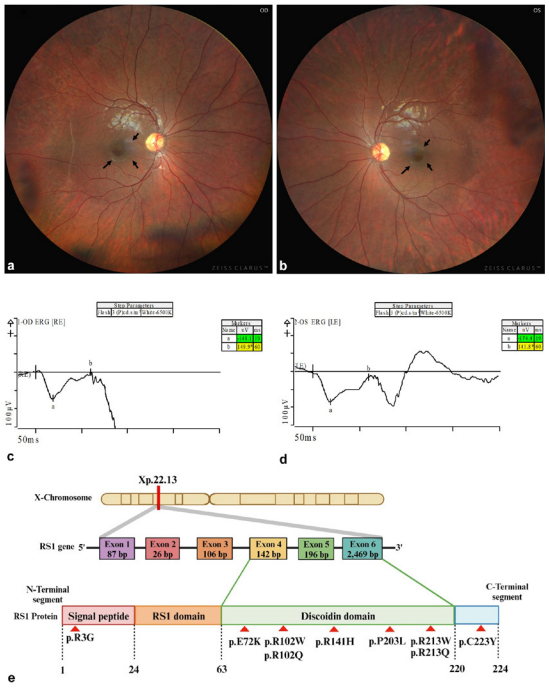


Figure 1: Genetic map and retinal imaging of individual with XLRS. (a, b). Fundus photograph of an XLRS patient indicating a spoke-wheel pattern at the macula due to foveal schisis in an 8-year-old boy carrying an RS1-gene c. G305A mutation. (c, d). Full-field electroretinogram (ff-ERG) images of the same proband showing Dark-adapted 10 ERG (strong flash ERG, combined response) of the patient showing a reduction in the "b" wave amplitudes with larger "a" wave suggestive of negative ERG in both eyes. (e). Genetic map of RS1 gene. It has an N-terminal signal peptide sequence, RS1 domain, Discoidin domain, and C-terminal segment. The genetic mutations and their respective locations are indicated in an inverted red triangle.

Conversely, 13.6% of the probands showed no signs of schisis formation, and one eye appeared nearly normal except for foveal atrophy. Eventually, 86% (n=19) of the probands in the study showed a complete reduction in the b-wave amplitude, along with a larger a-wave in both eyes during full field ERG [Fig. 1c, d].

Sanger sequencing analysis of the study probands revealed hemizygous mutations in the RS1 gene, including a novel missense variant in the signal peptide sequence (c.7C>G), and eight distinct mutations in the discoidin domain: c.214G>A, c.304 C>T, c.305G>A, c.422G>A, c.608 C>T, c.637C>T, c.638G>A, and c.668 G>A. The mutations identified in this study cohort as an inverted red triangle on the genetic map of the RS1 gene [Fig. 1e].

Additionally, genotype-phenotype correlation was conducted for mutations identified in the discoidin domain. Three probands carrying the c.305 G>A mutation displayed early onset symptoms in their first decade of life. Among them, two of these individuals displayed schisis affecting the outer nuclear layer (ONL), inner nuclear layer (INL), and inner plexiform layer (IPL) retinal layers, with foveal thicknesses measured at 650/560 µm and 505/306 µm, respectively [Fig. 2a, b]. While the third proband had schisis in the INL retinal layer, with a measured foveal thickness of 132/159 µm. Likewise, a proband with c.638 G>A mutations unveiled the separation of four retinal layers ONL, INL, IPL and ganglion cellular layer (GCL) with measured foveal thicknesses measuring 651/548 µm [Fig. 2c, d]. In contrast, a proband with a c.422 G>A mutation in exon 5 did not display schisis pattern but displayed defective ERG patterns and foveal atrophic changes measured thicknesses of 132/159 µm in their third decade of life [Fig. 2e, f].

Conclusion

In conclusion, our seven-year study of twenty-two XLRS patients in South India revealed diverse clinical presentations, genetic mutations, and genotype-phenotype correlations. Particularly noteworthy was the clustering of missense mutations within the RS1 discoidin domain and the identification of a novel mutation in the RS1 signal peptide sequences. However, the study's major limitation is its small sample size due to the rarity of the disorder. Nonetheless, these findings greatly contribute to our understanding of XLRS in this population, providing insights into its pathogenesis and enhancing diagnostic accuracy for affected individuals.

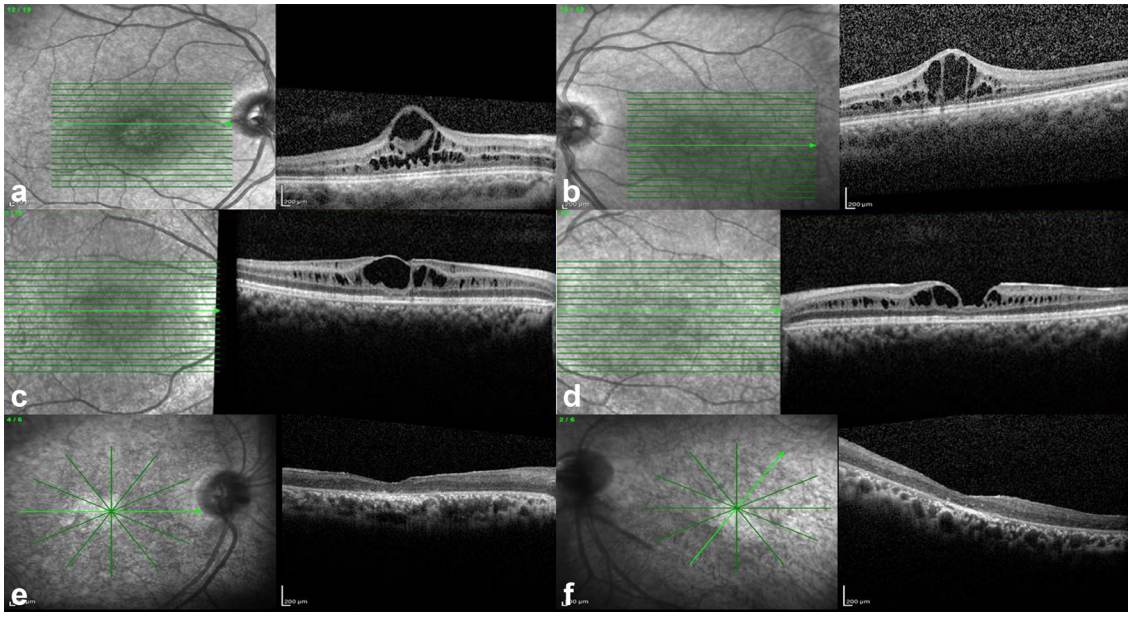


Figure 2: Distinct OCT findings of the probands harboring the RS1 mutation in the Discoidin domain are as follows: (a, b) Both eyes of a patient harboring c.305 G>A mutation had macular schisis involving ONL, INL, IPL, and cystoid macular edema with foveal thickness of 650 µm and 560 µm, respectively, and intact outer retinal layer. (c, d) Patient with c.638G>A mutation showed schisis involving ONL, OPL, INL, IPL and GCL with lamellar macular hole in the left eye with Foveal thickness of 651 µm and 548 µm. (e, f) The patient had foveal atrophy in both eyes with a foveal thickness of 132 µm and 159 µm harboring c.422G>A mutation

GENETICS OF OCULAR TUMORS

Ocular tumors often have diverse clinical presentations and are seen at all ages. The initial symptoms are often ignored as they are mostly innocuous. When it is presented at an advanced stage, the treatment options become limited. Patients may lose their vision, globe and even life depending on the severity of the disease. Hence an early detection of the tumor can save the vision and improve the quality of life. Our research projects on retinoblastoma and lymphoma are focused on the theme of early diagnosis, better prognosis and improved therapeutics. Our approaches span from Sanger sequencing to high throughput next generation sequencing methods to dissect out the genomic complexity of ocular tumors.

Genetic testing of Retinoblastoma

Investigators : Dr. A. Vanniarajan, Dr. Usha Kim, Prof. VR. Muthukkaruppan
Genetic Analyst : K. Murugan
Funding Agency : Aravind Eye Care System, Madurai

Introduction

Retinoblastoma (RB), the most common eye tumor in children, is caused by loss-of-function of the *RB1* gene. Genetic screening is crucial for managing RB and can impact important clinical decisions. This involves using different methods like Sanger sequencing, Multiplex ligation-dependent probe amplification, Real-time PCR, and Next-generation sequencing. Choosing the right methods speeds up the process and reduces the cost of screening.

Results

Genetic testing of *RB1* was performed in 57 new patients during the year. Out of 57, 36 were unilateral and 21 were bilateral RB. Among these 57 patients, 18 were older patients who were visiting the clinic for follow-up or referred from other centres. The disease was found to be either inherited from the parents or will be inheriting to the next generation owing to the germline mutations in 18 patients. In five patients, somatic mutations were identified in 5 tumors but not in blood cells indicate a low risk of RB in the next generation. Similarly, absence of mutations in rest of the blood samples imply the low or no risk of RB inheritance.

During the year, a 20-year-old female, who had her eye enucleated at age 2 (Unilateral Group E) presented after defaulted follow-up. The family chart indicated that her parents had consanguineous marriage and one of the siblings died of retinoblastoma. *RB1* screening showed germline splice mutation in exon 6. Familial mutational screening confirmed the mutation in another sibling who had regressed retinoblastoma in one eye. The mother tested negative for the mutation (Figure 1)

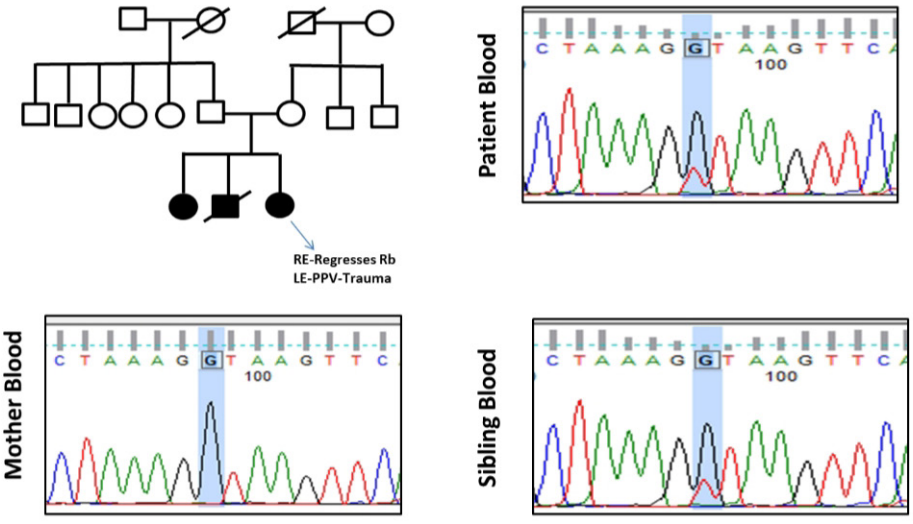


Figure 1: Splice mutation c.607+1G>T in Exon 6 identified in the proband and sibling but not in the mother

Conclusion

Genetic testing can thus precisely indicate the risk of retinoblastoma in the children and also the inheritance in family members.

Genomic Characterization of Kinome related genes in Retinoblastoma

Investigators : Dr. A. Vanniarajan, Dr. Usha Kim, Prof. VR. Muthukkaruppan
Research scholar : K. Jeyaprakash
Funding : Aravind Eye Foundation, USA

Introduction

Retinoblastoma (RB) is an aggressive intraocular pediatric cancer, characterized by frequent copy number alterations. Our study on nonresponsive tumors showed a higher gain of RTK genes especially *ALK*, which can serve as potential drug target. Therefore, the current study investigates the mRNA and protein expression of *ALK* and the effects of FDA-approved *ALK* inhibitors on RB cell growth.

Results

The transcript level analysis showed increased expression of *ALK* in RB tumors compared to

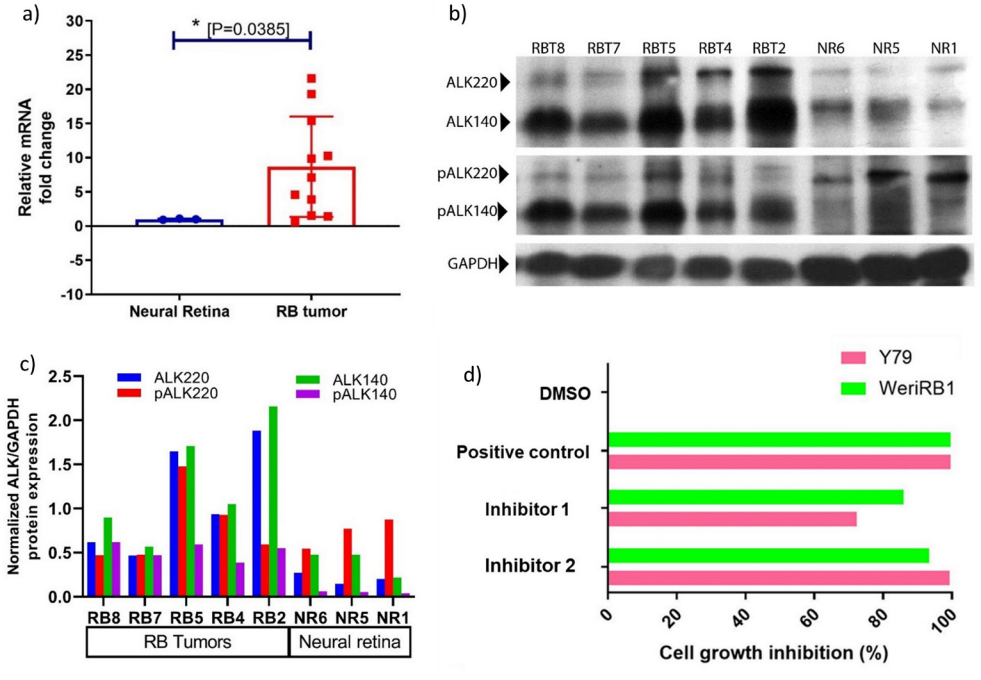


Figure 2: Analysis of ALK gene expression in RB tumors (a) Normalized mRNA expression of ALK (b) Western blot image of ALK and phosphor (p) ALK protein expression (c) Normalized ALK protein expression (d) Cell growth inhibition by ALK inhibitors

neural retina control. Western blot analysis found overexpression of total and phosphorylated *ALK* in RB tumors. With the consistent upregulation of *ALK* at both mRNA and protein levels in primary RB tumors, *ALK* inhibitors (*ALK* inhibitor1 and *ALK* inhibitor2) were used to evaluate their efficacy against RB cell growth in vitro. Both *ALK* inhibitors 1 and 2 showed effective growth inhibition in Y79 (72.34% and 99.35%) and WERI-RB1 cell lines (85.98% and 93.43%) respectively (Figure 2).

Conclusion

Increased *ALK* and phosphorylated *ALK* were observed in RB tumors. Concordantly, *ALK* inhibitors suppressed both RB cell lines (Y79 & WeriRB1) growth. Upon further validation, these inhibitors can serve as potential therapy for retinoblastoma.

Translational Genomics of Ocular Cancers

Investigators : Dr. A. Vanniarajan, Dr.Usha Kim, Dr.R.Shanthi, Dr. D. Bharanidharan, Prof. VR. Muthukkaruppan
Research scholar : K. Saraswathi
Funding Agency : Aravind Eye Foundation; Lady Tata Memorial Trust (Fellowship)

Introduction

B-cell non-Hodgkin lymphoma (B-NHL) is a notably diverse tumor, and the presence of identical clinical and histological characteristics does not consistently result in a similar prognosis. Consequently, there is a crucial need to establish precise predictions of disease subtypes, which serve as key prognostic indicators. Immunohistochemistry serves as the foremost technique for discerning subtypes through distinctive marker expression. Additionally, its confirmation is reinforced by subtype-specific chromosomal translocations. Thus, the study is aimed to delineate these B-NHL subtypes by employing Immunohistochemistry and Polymerase Chain Reaction techniques.

Results

Patients suspected with lymphoma underwent diagnostic incisional biopsy. Formalin fixed paraffin embedded sections of the tissues were stained with Hematoxylin & eosin. They were further analyzed immunohistochemically with markers CD5, CD10, BCL2, BCL6, CCND1, CD20, CD3, CD23 and CD79a. Lymphoma was confirmed with B Cell marker CD20. Follicular Lymphoma was diagnosed with consistent BCL2 positivity, along with BCL6 and CD10 expression. Mantle Cell Lymphoma was identified by CCND1 expression, which is a hallmark dysregulation. Other subtypes identified in the patients include Extramarginal Zone Lymphoma, Lymphoplasmacytic Lymphoma, and Small Lymphocytic Lymphoma (Figure 3).

Furthermore, specific fusion genes associated with Follicular Lymphoma (IGH/BCL2) and Mantle Cell Lymphoma (IGH/CCND1) were analyzed in B-NHL tumors. A fusion amplicon involving the Major Breakpoint Region of BCL2 and the JH region of the IGH gene was identified. Similarly, IGH/CCND1 translocation was also noted in these patients (Figure 4). Confirmation of the fusion was achieved through Sanger sequencing, aligning with both immunohistochemistry and polymerase chain reaction results.

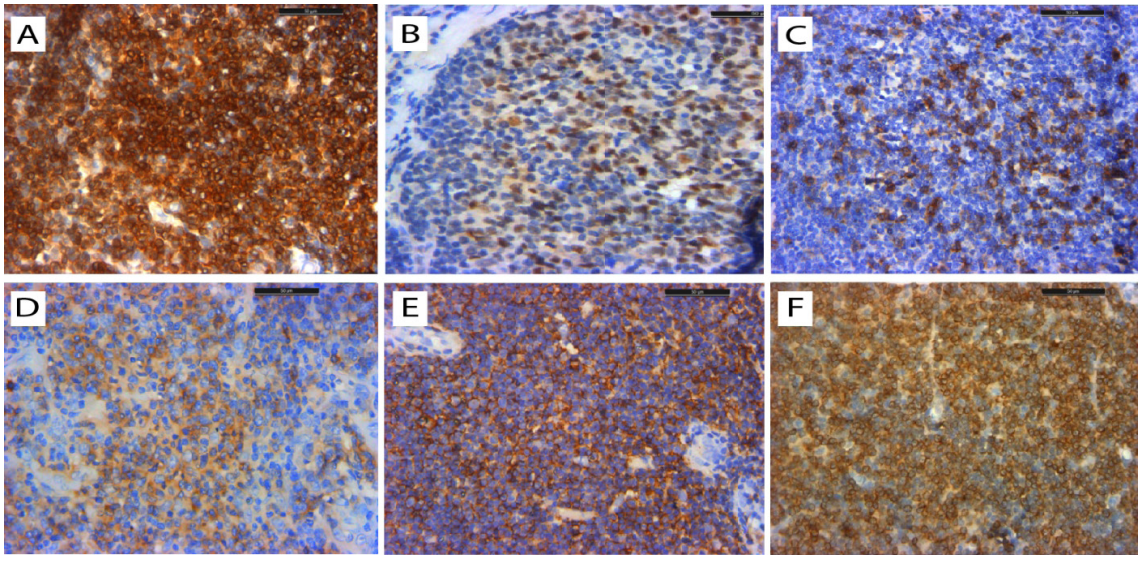


Figure 3: Representative Immunomarkers expression in B-NHL FFPE Sections (A-BCL2, B- BCL6, C-CD5, D-CD10, E-CD20, F-CCND1)

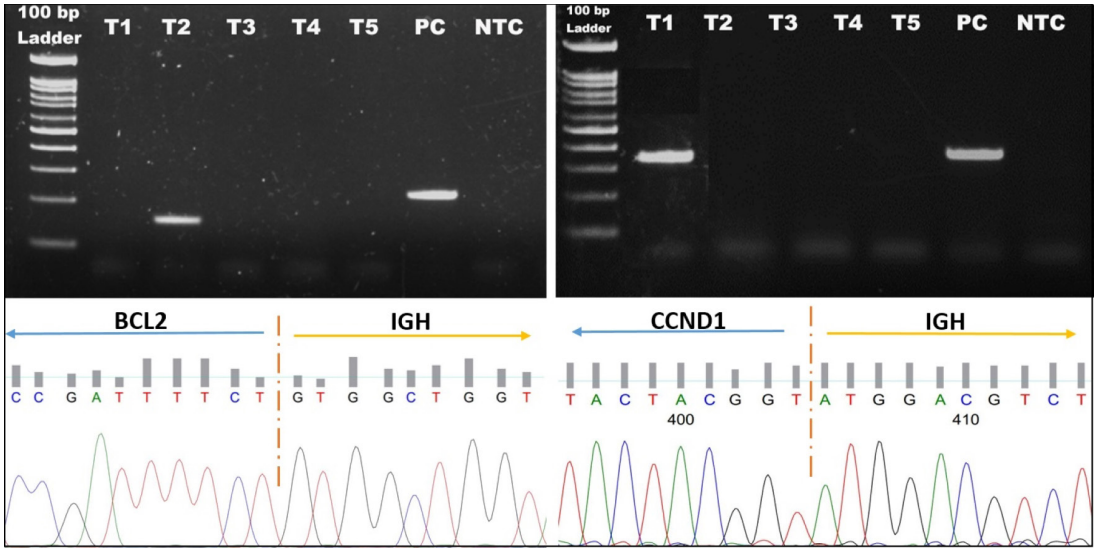


Figure 4: Translocation detection by PCR. Lane 1 100bp ladder, Lane T1-T5-Lymphoma samples. Lane PC-SUDHL cell line/REC 1 cell line translocation positive and NTC non template control

Conclusion

Subtyping of lymphoma with multiple markers was established. This will facilitate the understanding the variability in treatment response.

Molecular Characterization of Ocular Lymphoma for improved disease prognosis

Investigators : Dr. A. Vanniarajan, Dr. Usha Kim, Dr. R. Shanthi
Research scholar : K. Saraswathi
Funding Agency : Aravind Eye Foundation; Lady Tata Memorial Trust (Fellowship)

Purpose

Ocular adnexal B cell lymphoma (OABL) is the most common orbital malignancy affecting the middle-aged to elderly population. Studies have investigated genetic alterations such as mutations and chromosomal translocation contributing to OABL pathogenesis. Notably, the *MYD88* L265P mutation has been extensively studied in primary vitreoretinal lymphoma. Nevertheless, the presence of this specific hotspot mutation in OABL remains unknown. Therefore, this study aims to screen the *MYD88* L265P mutation in OABL tumors.

Results

MYD88 L265P mutation detection was carried out by both Allele-Specific Polymerase Chain Reaction (AS-PCR) and Sanger sequencing. A total of twenty histologically confirmed OABL tumor samples were included in the analysis, consisting of 12 (60%) males and 8 (40%) females. The average age of the participants was 61.1 years, ranging from 30 to 85 years. Among the samples, 13 were primary

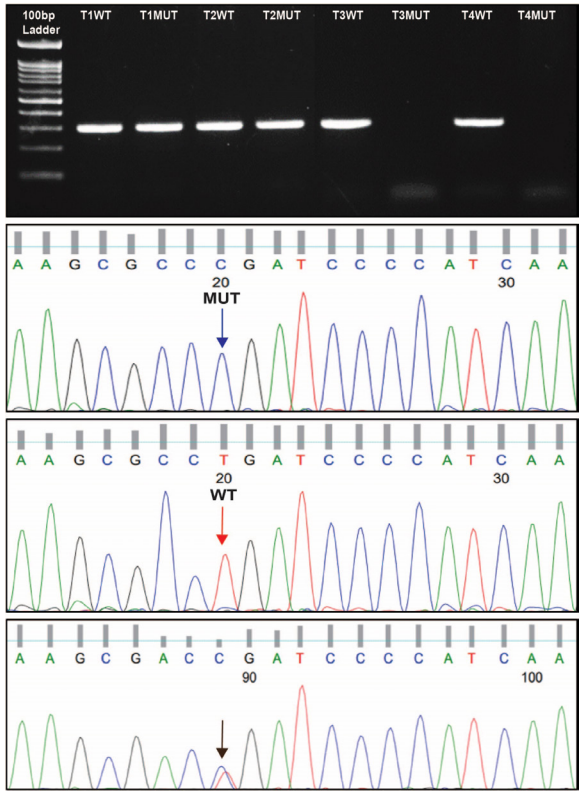


Figure 5: AS-PCR detected MYD88 L265P mutation detection in T1 & T2 and Sanger sequencing validation of AS-PCR confirms the presence of mutation (T>C). Heterozygous MYD88 L265P mutation was observed in the tumor sample. (WT-Wild Type, MUT-Mutant)

tumors, 7 were recurrent, and one exhibited bilateral presentation. The AS-PCR results revealed that two out of twenty OABL samples tested positive for the *MYD88* L265P mutation, while the remaining 18 exhibited wild-type alleles. Subsequent Sanger sequencing confirmed the presence of the mutation, specifically the substitution of leucine by proline at position 265, where T (wild type) was replaced by C (mutant) (Figure 5).

Conclusion

The *MYD88* L265P mutation in OABL was identified as infrequent, emphasizing the necessity for additional validation in a larger cohort.

Targeted Modulation of E2F3 and KIF14 pathway in Retinoblastoma refractory to existing chemotherapeutic drugs

Investigators : Dr. A. Vanniarajan, Dr. Usha Kim, Prof. K. Dharmalingam
Project Fellow : R. Sethu Nagarajan

Introduction

In retinoblastoma (RB), we found frequent copy number gains of cell cycle modulators such as *E2F3* and *KIF14*. Targeting these cell cycle modulators could be a competent treatment approach for retinoblastoma. Initially the target genes were pharmacologically inhibited using small molecule inhibitors (HLM006474, Ispinesib). The inhibition was confirmed at both RNA and protein level and functionally validated. In this study, we used gene knockdown strategy to specifically inhibit the *E2F3* and *KIF14*, and functionally validate them in RB cell lines.

Results

The western blot analysis showed upregulation of *E2F3* and *KIF14* in RB tumors. Transcript analysis of the siRNA treated cells showed the decreased expression of *E2F3* and *KIF14*. The growth kinetic assay showed delayed doubling time in silenced cells compared to normal cells confirming the reduction of tumor progression (Figure 6).

Conclusion

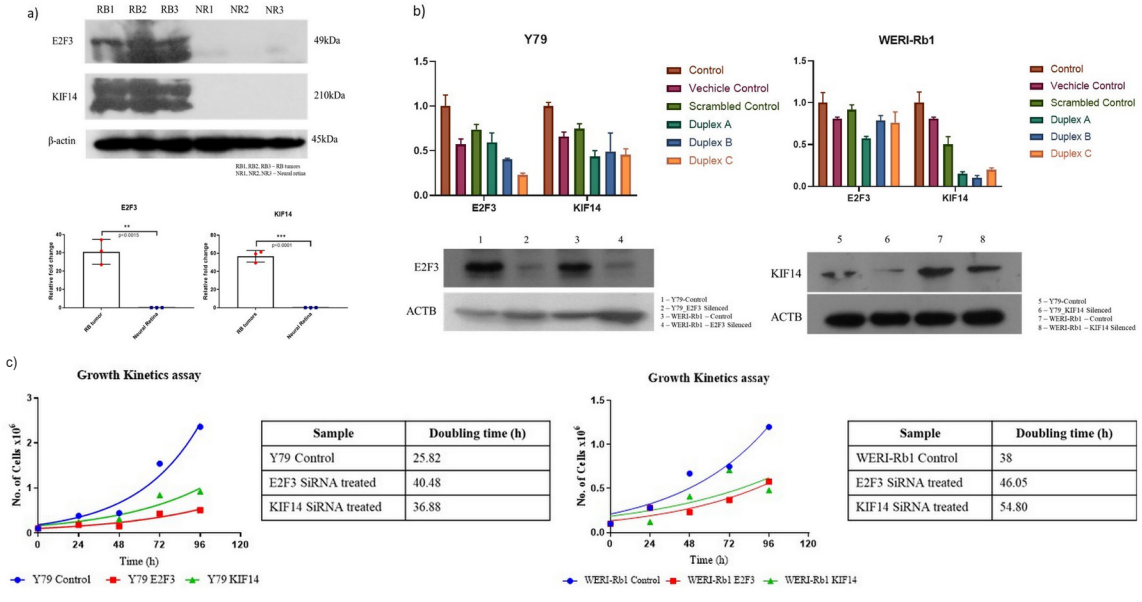
Reduction of tumor progression as indicated by delayed doubling time in silenced cells suggests *E2F3* and *KIF14* as potent therapeutic targets in retinoblastoma.

Elucidating the role of cancer stem cells in chemoresistant retinoblastoma and their therapeutic implications

Investigators : Dr. A.Vanniarajan, Dr. Usha Kim
Ph.D Scholar : R. Sethu Nagarajan
Funding Agency : ICMR – SRF (Fellowship)

Introduction

In Retinoblastoma, chemoresistance remains a major huddle in treating patients. There are several reasons behind the development of chemoresistance and one of the major factors is cancer stem cells. The current study is primarily focused on understanding the role



Sample	Doubling time (h)
Y79 Control	25.82
E2F3 SiRNA treated	40.48
KIF14 SiRNA treated	36.88

Sample	Doubling time (h)
WERI-Rb1 Control	38
E2F3 SiRNA treated	46.05
KIF14 SiRNA treated	54.80

Figure 6: Tumorigenic role of E2F3 and KIF14 in RB cells (a) Protein expression of E2F3 and KIF14 in RB tumors (b) Transcript and protein expression of siRNA treated RB cells (c) Growth kinetic assay on E2F3 and KIF14 silenced RB cells

of cancer stem cells and the signaling pathways that regulate the stemness in chemoresistant retinoblastoma using patient-derived primary cultures.

Results

Totally 16 samples (7 vitreous and 9 retinoblastoma tumors) were used for primary culture. Of which nine samples (1 vitreous and 8 tumors) were successfully cultured. The culture conditions were optimized using different media compositions and the primary cells remain viable in DMEM+F12 media with defined growth factors. The morphology of the primary cells was well-defined. The culture initially remained as a single cell suspension, and later grew into solidly packed spheroids (Fig.7a).

The *RB1* mutation in primary culture was analysed using Sanger sequencing and MLPA, which showed the presence of a similar *RB1* genotype and confirmed the origin of cultured primary cells from tumor cells. Further in-vitro cytotoxicity assay was performed and IC50 was determined against carboplatin. The IC50 varied from 15 to 200 µM and the mean IC50 (121.52 µM) was set as a cut-off to define carboplatin resistance (Fig. 7b). Based on the in-vitro drug responsiveness, five samples were identified as chemoresistant (above the mean IC50 cut-off). Clinically, all these cases showed high-risk pathological features with tumor infiltrations.

Conclusion

Primary culture of retinoblastoma tumors was established. The cytotoxicity assay using primary cultures showed different inhibitory effects (IC50) against carboplatin and was clinically correlated.

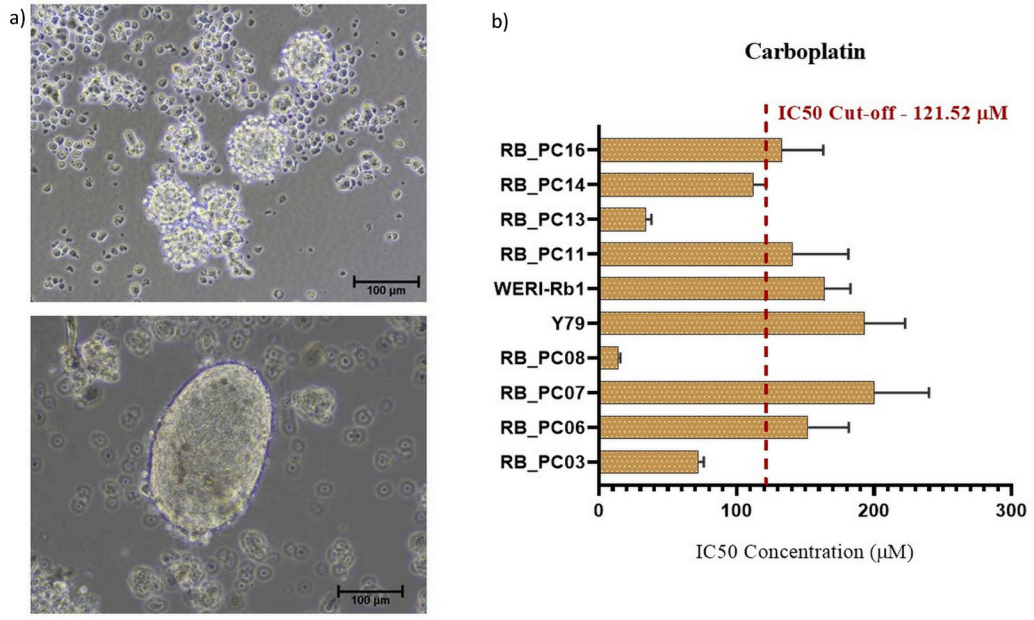


Figure 7 Primary culture establishment and In-vitro cytotoxicity assay (a) Morphology of RB patient-derived primary culture (b) IC50 of carboplatin in cultured RB primary cells. The dotted line represents the mean value of carboplatin IC50, used as a cut-off for resistant RB primary cells

IMMUNOLOGY AND STEM CELL BIOLOGY

Adult tissue resident stem cells are present in unique microenvironment in each tissue of the body and they maintain tissue homeostasis throughout life. The focus of research in the department of Stem Cell Biology is to understand the basic biology of the adult ocular stem cells and to develop better stem cell based therapies for several ocular conditions. The current focus of research is to understand the molecular regulation of these stem cells (human trabecular meshwork stem cells and retinal pigment epithelial stem cells), and to elucidate the changes with ageing/ in diseased condition (glaucoma and age-related macular degeneration). Studies are being carried out to identify the changes at the cellular level in age-related cataract. In addition, the role of small extracellular vesicles or exosomes as an alternate to stem cell based therapy for primary open angle glaucoma is also being explored.

Characterisation of adult human lens epithelial stem cells in the maintenance of tissue homeostasis throughout life and their functional status in cataractous lens

Investigator : Dr. Madhu Shekhar
Co-Investigators : Dr. Gowri Priya Chidambaranathan
Prof. VR. Muthukkaruppan,
Dr. Haripriya Aravind
Research Scholar : P. Saranya
Funding agency : Science and Engineering Research Board

The crystalline lens is entirely derived from a single cell type –the anterior lens epithelial cells. The epithelial cells are known to differentiate into lens fibres throughout life. The location of stem cells in the lens epithelium remains controversial. The objective of this study was to identify and characterize the lens epithelial stem cells, their role in maintaining tissue homeostasis and in the development of age related cataract. The four zones in the human anterior lens epithelium were demarcated based on the expression of specific markers (Cx-43 and crystallins). SOX-2^+ but Cx-43^- adult lens epithelial stem cells were confined to the central zone of the human anterior lens epithelium. Functionally, the label retaining cells (LRCs) expressing SOX2 were identified in both the central and equatorial zone explant cultures of normal lens. In cataractous lens, the label retaining $SOX2^+$ cells were restricted to the central zone and significantly reduced to 1.7±0.8% compared to normal lens. Further, the absence of SOX-2^+ cells in the cataractous lens indicated a probable association with cataract development. In continuation, studies were carried out in this year to characterize the lens epithelial stem cells in cataract donors (i) morphologically and (ii) functionally by sphere forming ability of cultured normal and cataractous donor tissues.

Results

Autophagy vacuole formation in the central zone cells of the cataractous human anterior lens epithelium: Confocal analysis of the unstained lens

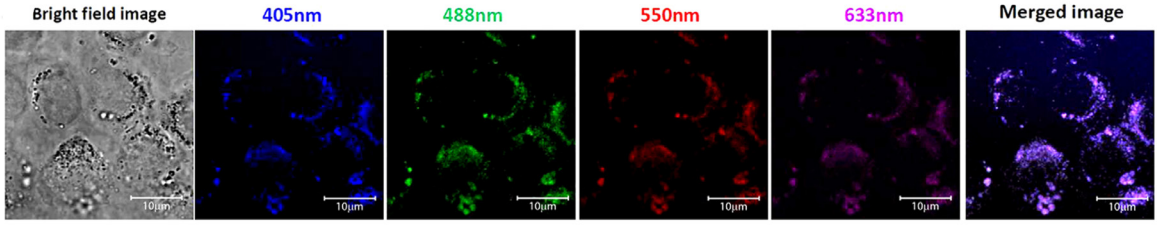


Figure 1: Cataractous human anterior lens epithelial whole mount (59 years old donor) was fixed with 4%PFA and analysed for autofluorescence (unstained sample) in all excitation wavelengths.

epithelial whole mount revealed the presence of vacuoles in the cytoplasm of cells (39.8±13.1%) only in the central zone of cataractous lens (Figure 1). Such vacuoles emitted autofluorescence in all excitation wavelengths (UV to far red range). Further transmission electron microscopic (TEM) analysis of the central zone confirmed the presence of vacuoles containing deposits (Figure 2). Comparison of these data with the previous reports revealed that the vacuoles might represent autophagy vacuoles consisting of lipofuscin deposits.

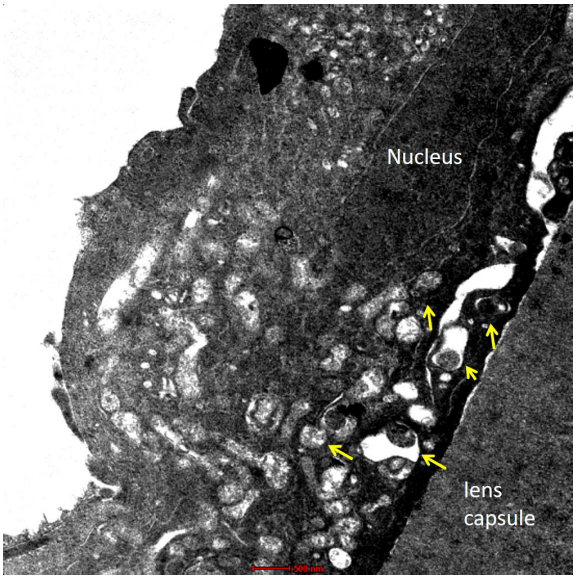


Figure 2: TEM analysis of cataractous human anterior lens epithelium (central zone, 72years old donor) revealed that membrane bound vesicles containing deposits were seen (yellow arrow indicates).

Characterization of lens spheres:

The explant cultures were established from three zones of normal (n=6) and cataractous (n = 3) human anterior lens epithelium. After 2 weeks, the cultured cells were trypsinized and 1×10^3 viable cells were seeded on ultra low attachment dishes (experimental as well as biological triplicates). Further, the cells were maintained in sphere medium containing DMEM/F12 (1:1) + Glutamax™, 2% B27, epidermal growth factor (20ng/ml), fibroblast growth factor (20 ng/ml), heparin (5 µg/ml), bovine serum albumin (0.1 mg/ml), L-glutamine (2.5 mM), Penicillin-Streptomycin (250µg/ml) and cultured for seven days to observe the sphere formation. The diameter of the sphere was measured using ImageJ and spheres with diameter >60µm were counted and the percentage was calculated.

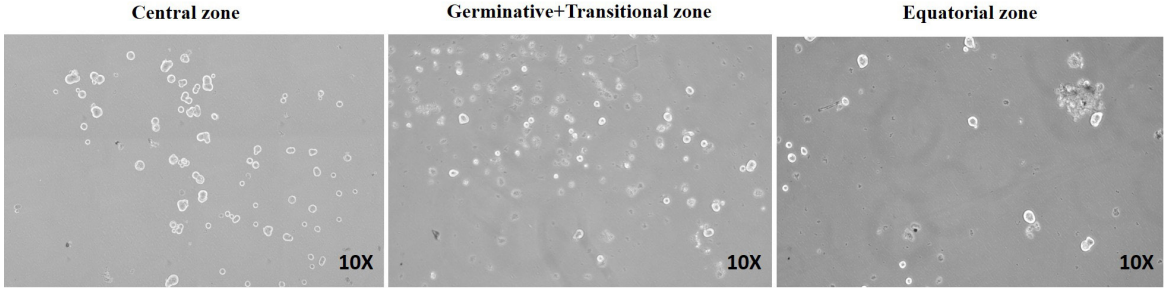


Figure 3: Represnetative phase contrast microscopic images of spheres from central, germinative + transitional zone and equatorial zone of cultured human anterior lens epithelium

After 7 days of culture, the cells from the central zone were identified to have a significantly higher percentage of spheres-1.68 ± 1.0% ($p = 0.007$) compared to other zones (germinative + transitional zones-0.20 ± 0.28%and equatorial zone-0.04 ± 0.08%, Figure 3). A significant reduction in the

Spheres formed/10^3 cells (spheres >60µm)			
Age (years)	Central zone (% of spheres)	Germinative+ Transitional Zone (% of spheres)	Equatorial zone (% of spheres)
Spheres established from normal lens epithelium			
18	1.3	0.6	0.2
20	1.1	0	0
21	3.1	0.4	0
41	2.4	0	0
54	0.5	0	0
Mean±SD	1.68±1.04	0.2±0.28	0.04±0.08
Spheres established from cataractous lens epithelium			
51	0.4	0	0
53	0.4		
60	0.2	0	0
Mean±SD	0.33±0.11	0	0

Table1: Percentage of spheres (>60µm) was analysed in normal (n = 5) and cataractous (n = 3) lens epithelial explant cultured cells. Central zone had higher percentage of sphere forming ability in normal lens, and that was significantly reduced in cataractous lens.

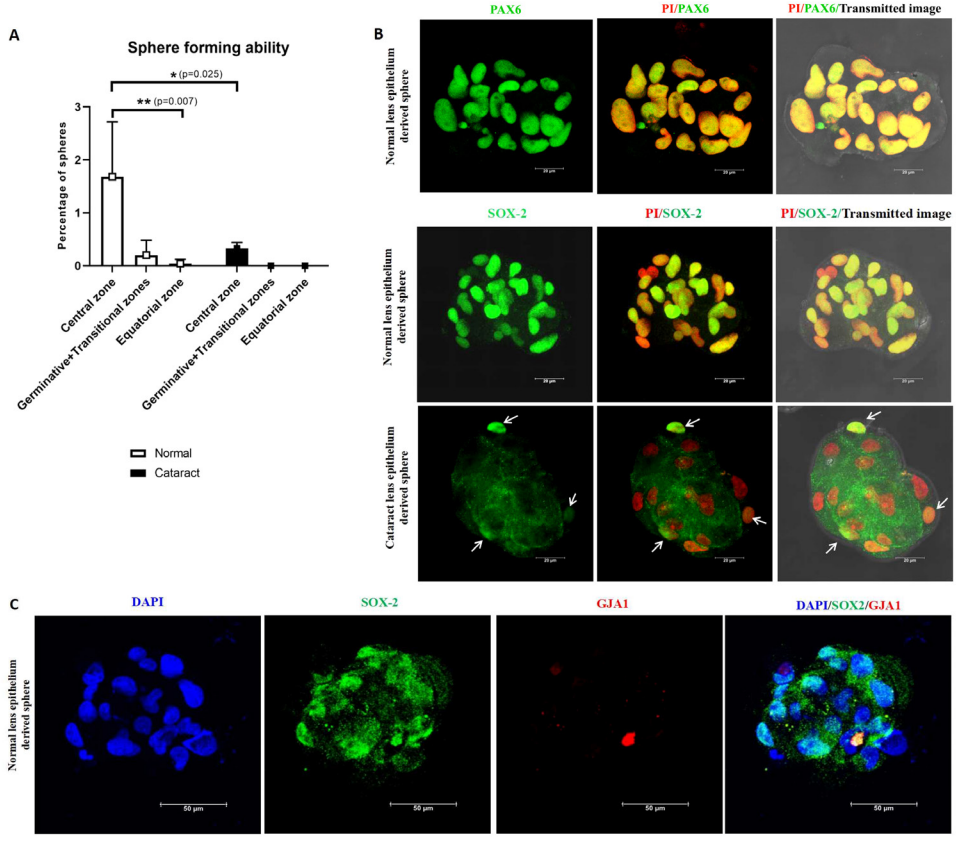


Figure 4: (A) The sphere forming ability of the cultured lens epithelial cells was analysed in both normal and cataract lens epithelium. The central zone alone had high sphere forming ability in normal lens epithelium, and a significant reduction was evident in cataract lens (p = 0.025; ** p = 0.007). (B) Characterization of the human lens spheres derived from cultured central zone epithelial cells. Representative confocal microscopic images of lens spheres immunostained for PAX6/SOX2 (green) counterstained with propidium iodide (PI-red). The expression of PAX6 was evident in all cells in the sphere, and SOX2 was observed in most of the cells in the normal lens epithelium-derived spheres, while only a few cells in the spheres derived from the cataractous lens expressed SOX2(arrows). Scale bar 20 um. (C) Representative confocal microscopic images of a sphere derived from central zone double immunostained for SOX2 (green), Cx-43 (red), counterstained with DAPI (blue). The SOX2 positive cells in the sphere were negative for Cx-43.*

percentage of sphere forming ability (0.33 ± 0.11%) (p = 0.025) was observed in the cultured central zone cells from the cataractous lens epithelium, and no spheres were observed with the cells from other zones (Figure 4A, Table 1).

Characterization of lens spheres (a minimum of three spheres from each culture) for the expression of the stem cell marker SOX2 revealed that the normal lens epithelium-derived spheres had SOX2 expression in almost all cells, whereas cataract lens epithelium-derived spheres had one or two cells with low expression (Figure 4B). Double immunostaining of spheres derived from normal central zone lens epithelial cells revealed the presence of SOX2 positive but Cx-43 negative cells, confirming that the putative adult lens epithelial stem cells were localised in the central zone (Figure 4C). Further, the expression of PAX6 by all cells in the sphere confirmed the neuroectodermal origin of these lens epithelial cells (Figure 4B).

Conclusion

- The higher sphere forming ability and the presence of $SOX2^{+}$ $PAX6^{+}Cx\text{-}43^{-}$ cells in these spheres confirmed that the adult lens epithelial stem cells were located only in the central zone.
- TEM analysis identified the presence of cytoplasmic vacuoles containing particles in the central zone of cataractous human anterior lens epithelium.
- The presence of autofluorescing vacuoles in the central zone of native tissue and the reduction in the percentage of sphere forming ability of cultured lens epithelial cells in the cataractous lens, highlights the cellular changes in the central zone of cataract lens epithelium.

Role of trabecular meshwork stem cell-derived extracellular vesicular miRNAs in human trabecular meshwork regeneration

Investigators : Dr. Gowri Priya Chidambaranathan
Co-Investigators : Dr. S. R. Krishnadas, Prof.VR. Muthukkaruppan, Prof.K.Dharmalingam
Research Scholar : R. Iswarya
Funding agency : Sun Pharma

Introduction including Background

Glaucoma is an optic neuropathy, one of the most common causes of irreversible blindness globally. Trabecular meshwork (TM), a porous tissue located in the irido-corneal angle, regulates intraocular pressure (IOP). Recent research has concentrated on developing cell-based therapeutics for glaucoma, taking into account the limitations of traditional pharmacological treatment and invasive surgery. Considering the difficulties in the maintenance and handling of stem cells *in vitro*, as an alternative, the efficacy of TM stem cell (TMSC) derived exosomes to enhance TM regeneration was evaluated in this study (as the exosomes mimic the nature of source cells). Previous findings from this laboratory revealed that TMSC exosomes have better wound healing and antioxidant properties. The primary goal of this study was to identify the protein cargo responsible for the increased functional efficacy of the TMSC exosomes. The exosomes from the TM and TMSC conditioned media were isolated by ultracentrifugation, characterized, and labelled with CM-Dil.

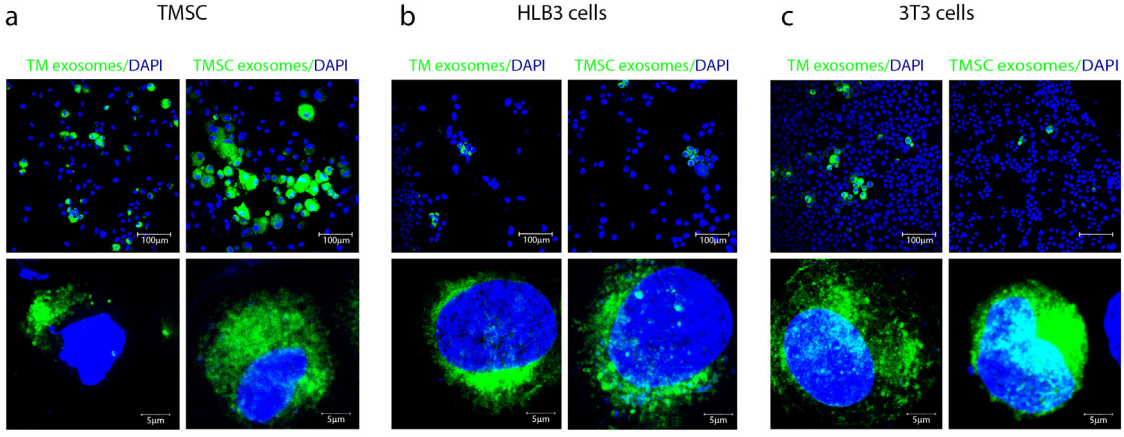


Figure 1: Cellular uptake of TM and TMSC exosomes (CM-Dil- green) by different cells- a- TM cells, b-HLEB3 cells, c-3T3 cells nuclear counter stained by DAPI (blue). Top panel lower magnification (20X), bottom panel- higher magnification (63X zoom6). Confocal analysis demonstrated the uptake of both TM and TMSC exosomes by all the tested cell types with highest degree of uptake by TM cells.

The labelled exosomes were treated with different cell types (human primary TM cells -TM Cells, mouse fibroblast cell line - 3T3, and human lens epithelial cell line - HLEB3) to determine the specificity of uptake. To further investigate the effect of exosomes on TM cell proliferation, exosome-treated cells were immunostained for Ki67, a proliferation marker. To decipher the protein cargo the exosomal proteins were separated by gel electrophoresis and subjected to mass spectrometry. The identified proteins were subjected to bioinformatics analysis to elucidate the differential protein expression between TM and TMSC exosomes. STRING analysis was carried out to identify the functional clusters involved in the regeneration and anti-oxidant effects in *in vitro* experiments.

Results

Uptake and Cellular specificity of the exosomes

Confocal imaging of cells incubated with CM-Dil labelled exosomes showed the presence of exosomes in the perinuclear region of the cells (Figure 1). Further analysis revealed that the degree of exosome uptake was higher in TM cells than HLEB3 and 3T3 cell lines (Table 1) indicating the uptake is not through simple diffusion. Further studies are essential to identify the mechanism/receptor involved in this specificity.

Effect of exosomes on cell proliferation:

Ki67 staining demonstrated that the TMSC exosomes increased the proliferation of the TM cells significantly to 23.7±4.6% (p=0.013) (Figure 2C) compared to control (12.62 ±4.37%) and TM exosomes (12.13 ±0.88%) (Figure 2a and b).

Cell type	TM Exosome uptake (%)	TMSC Exosome uptake (%)
TM cells	40.7±12.8	74.6±16.9
HLEB3 cell line	21.8±2.9	10.7±3.7
3T3 cell line	11.5±7.1	2.98±2.3

Table 1: Uptake of TM and TMSC exosomes by different cell types

Protein profiling of the exosomes by mass spectrometry

Proteomic analysis of exosomes identified with high confidence 1328 proteins in TMSC exosomes and 1248 in TM exosomes. 25.2% of the proteins were common between TM and TMSC exosomes while 36.8% proteins were only found in TMSC exosomes and 37.9% proteins only in TM exosomes (Figure 3).

Bioinformatic analysis showed distinct protein expression pattern between TM and TMSC exosomes (Figure 4). STRING analysis was carried out to identify the functional clusters involved in the regeneration and anti-oxidant effects in *in vitro* experiments. Differential expression analysis showed high abundance of wound healing and anti-oxidant proteins in TMSC exosomes compared to TM exosomes (Figure 5). Further validation is essential to confirm these findings.

Conclusion

Mass spectrometry analysis of the exosomes emphasized the higher abundance of anti-oxidant and regeneration associated proteins in TMSC exosomes compared to TM exosomes as demonstrated in the *in vitro* experiments. Thus, the "proof of concept" for developing a TMSC exosome based therapy for patients with primary open angle glaucoma was established.

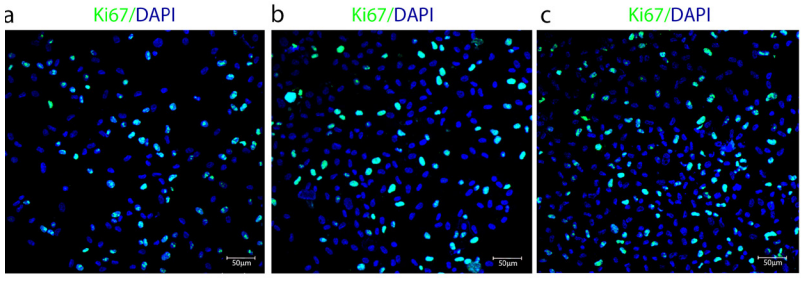


Figure 2: Effect of TM and TMSC exosomes in TM cell proliferation (Ki67 (green), nuclear counter stain DAPI - blue). Representative confocal images of TM cells a- untreated -control, b-TM exosome treated, c- TMSC exosome treated. Ki67 positivity was increased upon TMSC exosome treatment indicating that the TMSC exosomes promoted proliferation of the TM cells.

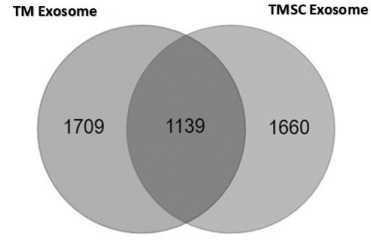


Figure3: Venn diagram representing comparison of TM and TMSC exosomal proteins.

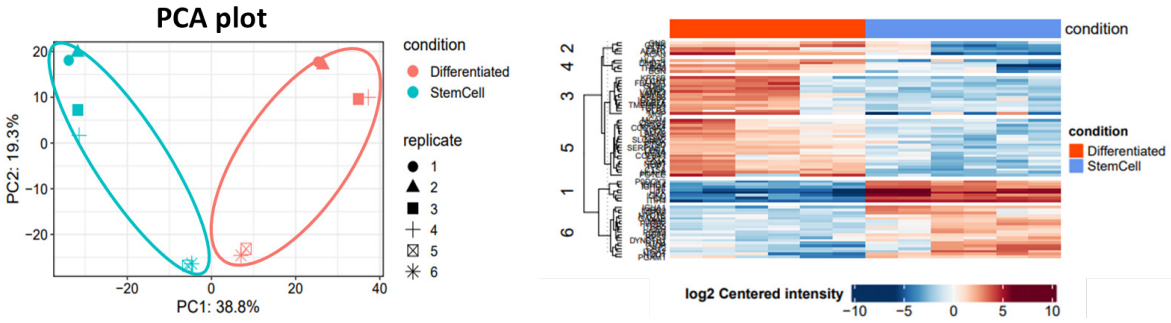


Figure 4: PCA plot and heat map showing distinct profile of TM vs TMSC exosomal proteins

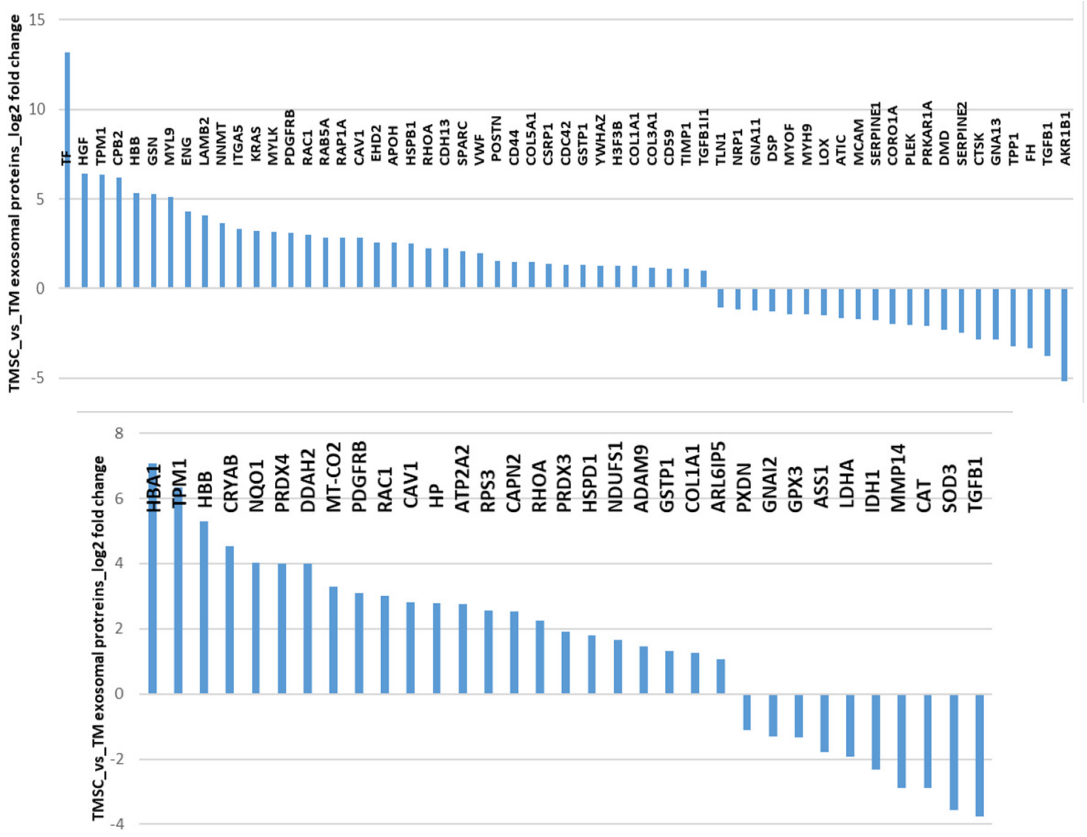


Figure 5: Expression of wound healing and anti-oxidant associated proteins in TMSC exosomes in comparison with TM exosomes

Molecular regulators associated with the maintenance of human trabecular meshwork stem cells in relation to their reduction in ageing and glaucoma

Investigators : Dr. Gowri Priya Chidambaranathan
Co-Investigators : Prof. VR. Muthukkaruppan
Dr. S. R. Krishnadas
Dr. D. Bharanidharan
Research Scholar : Sneha Nair
Funding Agency : Science and Engineering Research Board

Introduction including Background

Previous studies from this laboratory confirmed the presence of stem cells in the Schwalbe's line/ anterior non-filtering region of the meshwork. With ageing, a reduction in the TM cells was observed which was significantly associated with the loss of trabecular meshwork stem cells (TMSCs). Further, a drastic reduction in total TM cell count along with a significant loss of TMSCs was identified in glaucomatous donor eyes compared to age-matched controls. The molecular basis governing the loss of TMSCs with ageing and in glaucomatous condition remains poorly understood. Hence, the objective of

this study was to elucidate the molecular signature - transcriptome and miRNA profile of the filtering and the non-filtering region of the human TM to identify the molecular regulators of TMSCs. Regeneration of TM by residual TMSCs through the identified molecular factors will aid in the restoration of the normal tissue homeostasis.

Results

miRNA profiling of filtering and non-filtering TM

miRNA profiling was carried out to identify the differentially expressed miRNAs in the filtering and non-filtering regions of human TM on Nanostring nCounter SPRINT. Analysis of differentially expressed miRNAs using nSolver identified 26 significantly upregulated miRNAs and three downregulated miRNAs in the non-filtering region of TM with fold change >±1.2, p-value ≤ 0.05. Among them, the top 10 significantly upregulated miRNAs and the three downregulated miRNAs are given below (Figure 1).

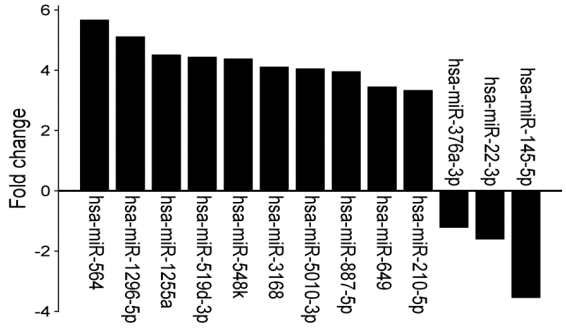


Figure 1. Differentially expressed miRNAs in non-filtering TM compared to filtering TM.

DE miRNA Pathway Analysis

Gene targets of differentially expressed miRNAs were identified using miRDIP 5.2, an online database of experimentally validated targets of miRNAs. The 4748 targets identified were grouped in to 133 KEGG pathways in DAVID. Fifteen pathways of interest with more than 20 target genes and p<0.05 were further analyzed. Among them pathways functionally associated with the maintenance of stemness included MAPK, Wnt, PI3K-AKT FOXO, Hippo, HIf-1 and Ras signalling (Figure 2). Additionally, other important pathways such as signalling pathways regulating pluripotency of stem cells and pathways related to cell-cell and cell-matrix junction (focal adhesion, tight junction and adherens junction) were enriched.

Conclusion

miRNAs specific to the non-filtering region of TM, where the stem cells are located were identified. The targets of the up regulated miRNAs were predicted to be associated with MAPK, Wnt, PI3K-AKT, Hippo, HIF and FOXO signalling pathways known to play important role in the maintenance of stemness. Further studies are required to validate their targets in relation to regulation of TM stem cells.

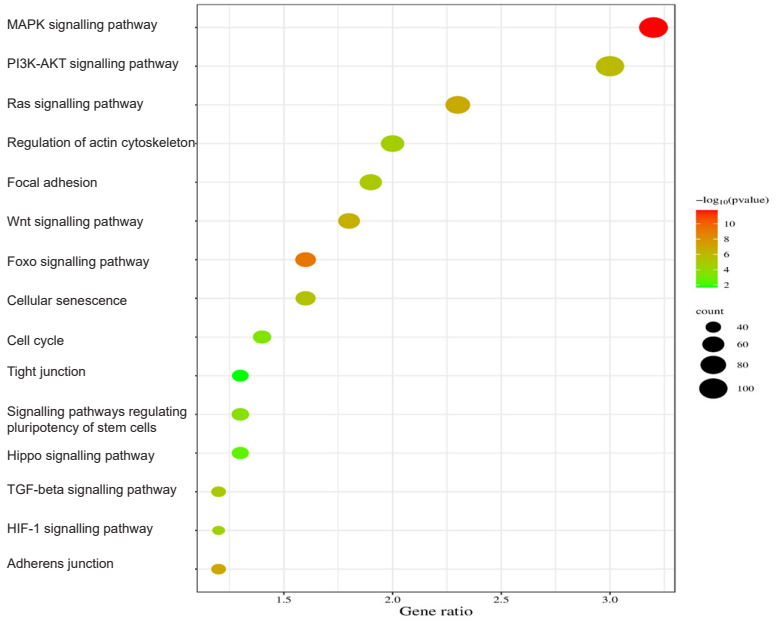


Figure 2: KEGG Analysis of upregulated miRNAs

PROTEOMICS

Some of the major ocular diseases that are investigated in the Proteomics department include diabetic retinopathy, fungal keratitis, keratoconus, pterygium, and glaucoma, all of which contribute a lion's share towards vision loss in the Indian population. We utilise a multi-omics approach to investigate the above diseases. The major techniques that are employed to study disease mechanisms, biomarker discovery and to develop therapeutic interventions are proteomics, transcriptomics, whole genome sequencing, whole genome methylation analysis, metabolomics and state-of-art cell and molecular biology techniques. The proteomics facility is equipped with two mass spectrometers to perform gel-based and non-gel based proteome analysis from ocular tissues, tear, blood and cells. Further, to study the role of nano-sized vesicles called extracellular vesicles in various ocular diseases, the proteomics department is equipped with ultracentrifuge and NanoParticle Tracking Analysis NS300 instruments. The outcomes of the basic research is translated as diagnostic or therapeutic strategies to improve disease management.

Proteomic and Metabolomic analysis of Microbial Keratitis Patient's tears to identify biomarkers (molecular signatures) of microbial keratitis corneal ulcer progression

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Dr. N. Venkatesh Prajna,
Dr. Lalitha Prajna,
Dr. Ninad Mudaraddi,
Dr. D.Bharanidharan.
Collaborator : Dr. Beth Mills
Research fellow : Karthik Alagarsamy
Funding : AEH

Background of the Study

Microbial keratitis (MK) is one of the most prevalent ocular emergencies. It is the world's fourth most common cause of blindness, with 1.5 to 2.0 million new cases each year in developing countries (Whitcher JP et al 1997, Chidambaram JD et al 2018). Deeper tear phenotyping will give the possible signatures of the disease. Tear fluid is composed of hundreds of proteins, lipids and metabolites. Proteomics studies undertaken on tears identified several potential biomarkers implicated in inflammation, immunity and oxidative stress in eye diseases. These previous studies from our lab also showed that clinical isolates of the corneal fungal pathogens are distinct in their protein profile compared to environmental isolates. Since metabolites are more closely related to the biological phenotype than proteins, they may also make appealing candidates for biomarker development, particularly in immunological illnesses, given the great diversity found in tears.

The development and technological advances of proteomics and metabolomics technologies offer an understanding of these complex mechanisms of

specific diseases. We might be able to establish the precise functions of these proteins and metabolites in the underlying pathophysiological processes and provide diagnostics biomarkers. The studies of differential expression of this complex in the biofluids such as tear require rapid, highly reproducible, and accurate quantification. These requirements can be satisfied by mass spectrometry-based analysis (Zhan X et al 2021).

Liquid chromatography-mass spectrometry (LC-MS/MS): LC-MS/MS is a high-throughput method that allows for the identification and quantification of thousands of proteins in a single sample. This method involves separating peptides based on charge in liquid chromatography and identifying peptides by mass using mass spectrometry. The advantage of this method is its high sensitivity, which allows for the detection of low-abundance proteins and metabolites.

In this study, we aimed to identify and quantify different classes of metabolites and proteins from the tear of a single patient collected during his multiple follow-up visits during the entire treatment course. The methyl tert-butyl ether (MTBE) method (Salem et al. 2017) was used for the extraction of protein and metabolite from the same aliquot of tear.

Results

The control tear sample was extracted using MTBE. Extracted protein concentration was estimated using BCA protein assay kit. 77.5 % of tear proteins were recovered from the whole tear after MTBE extraction.

1D SDS -PAGE profile of Neat tear Vs MTBE extracted Tear

The representative protein profiles of control tear resolved in 1D SDS-PAGE and stained with colloidal coomassie brilliant blue followed by silver nitrate stain depicted in Fig.1.B The 1D profile shows that distribution of the abundant proteins in the tear.

Proteome analysis of MTBE extracted Tear Proteins

A total of 498 proteins from shot gun and 471 proteins from Analytical gel were identified (Fig.1.C) and 311 proteins were identified using STrap method (Fig.1.D). Venn diagram comparing human tear proteomes from in gel (Shotgun and Analytical) and STrap method. 104 proteins are common in both methods were shown in Venn diagram (Fig.1.E). Control and infected tear (*Fusarium* sp) was subjected in to MTBE extraction and mass spectrometry was done in University of Edinburgh, UK. 278 proteins were identified in the control tear proteome, and 436 proteins were identified in the infected tear proteome. 134 proteins were common in both and 298 proteins were unique in Infected Tear, 92 proteins were unique in control tear were shown (Fig.1.F)

Gene enrichment Analysis

Functional classification of identified human tear proteins from this study was performed using

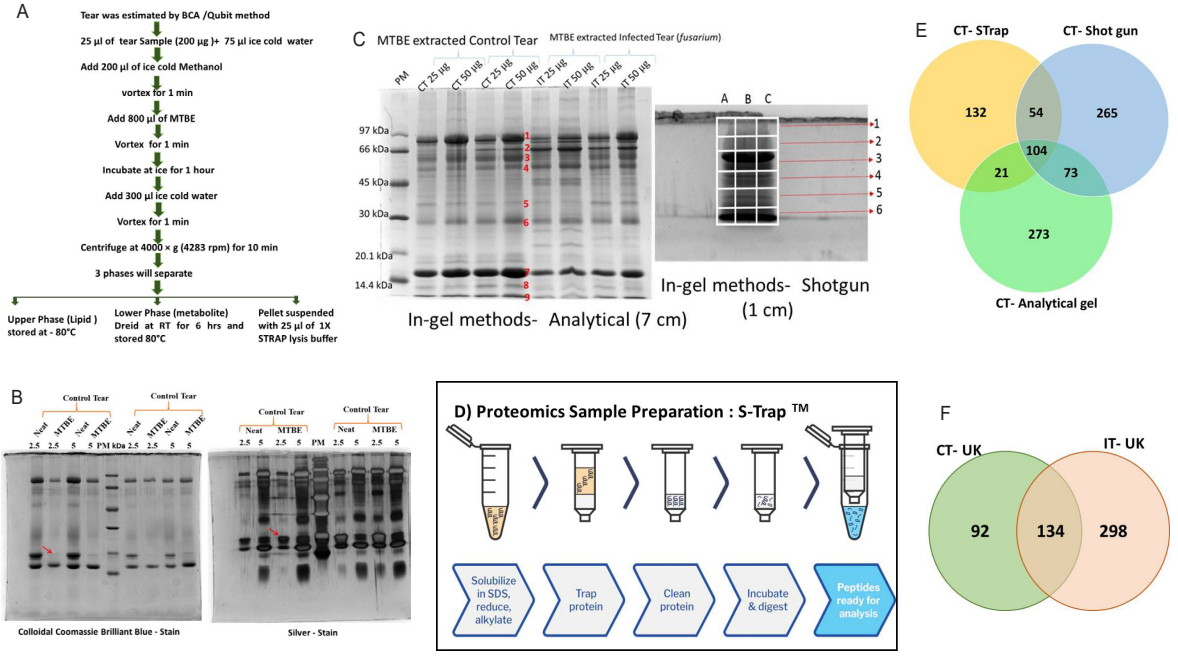


Fig. 1. Analysis of MTBE extracted tear proteome.

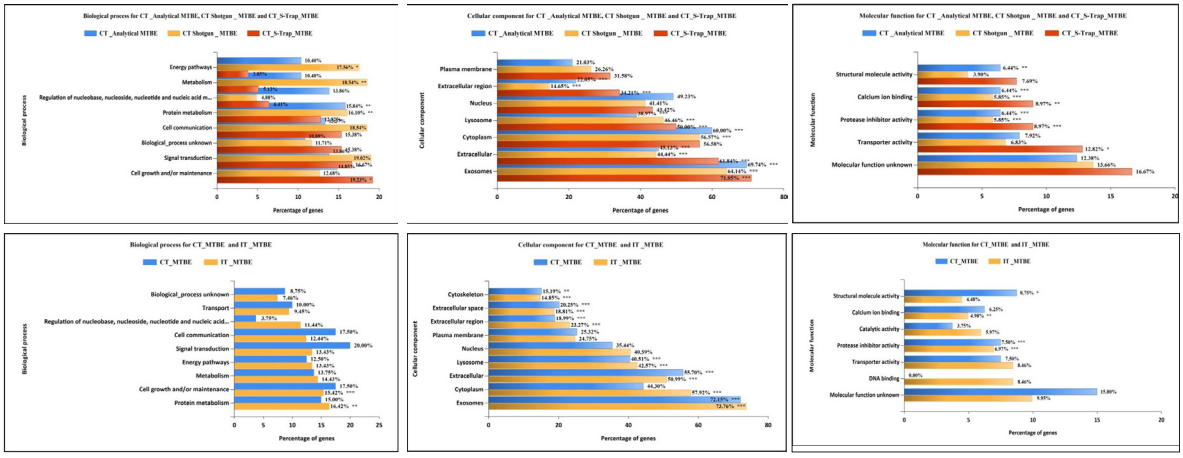


Fig. 2. Gene enrichment Analysis of MTBE extracted tear

Funrich 3.1.3 tool (Fig2). In all the categories, the percentage of the gene was slightly high in the STrap method when compared with the In-gel method. A classification based on biological processes revealed that these proteins are mainly involved in cellular communication, transport, cell growth, signal transduction, protein metabolism, immune response, and energy pathway. For the cellular component, the top six sub-categories are exosome, cytoplasm, plasma membrane, extracellular, cytoskeleton and lysosome. For molecular function, several major subcategories are calcium-binding activity, transporter activity, protease inhibitor activity and structural molecule activity.

Metabolomics analysis of MTBE extracted control and Infected Tear

Lower phase (metabolite phase) was transferred in to separate tube and dried at RT for 6 hours. A total of 121 metabolites were identified in control and 121 from infected tear metabolome.

Reference:

1. Whitcher JP, Srinivasan M. Corneal ulceration in the developing world – a silent epidemic. Br J Ophthalmol 1997;81:622-3.
2. Chidambaram JD, Venkatesh Prajna N, Srikanthi P, Lanjewar S, Shah M, Elakkiya S, et al. Epidemiology, risk factors, and clinical outcomes in severe microbial keratitis in South India. Ophthalmic Epidemiol 2018;25:297-305.
3. Zhan X, Li J, Guo Y, Golubnitschaja O. Mass spectrometry analysis of human tear fluid biomarkers specific for ocular and systemic diseases in the context of 3P medicine. EPMA J. 2021 Dec 3;12(4):449-475.
4. Salem M, Bernach M, Bajdzienko K, Giavalisco P. A Simple Fractionated Extraction Method for the Comprehensive Analysis of Metabolites, Lipids, and Proteins from a Single Sample. J Vis Exp. 2017 Jun 1;(124):55802.

Proteomics of fungal Extracellular Vesicles: A comparative analysis of a saprophyte and a clinical isolate of *Aspergillus flavus*

Investigators : Prof. K. Dharmalingam,
Dr. Venkatesh Prajna,
Dr. Lalitha Prajna,
Research fellow : Hariharan Gnanam

Introduction including background

The major causative agents of fungal keratitis are *Aspergillus flavus* and *Fusarium solani*. EVs are spherical bilayer lipid membrane enclosed vesicles produced and released by all living cells. Their role in fungal infection is not studied yet. The cargo of EVs is specific for the producer cell and altered by the physiological and environmental stress acting on the mother cell. They are signal transducers and modifiers of several cell functions and are capable of crossing all biological barriers and altering the physiological state of the recipient cells. The aim of this study is to comparison of the protein cargo of EVs from *Aspergillus flavus* saprophyte (ATCC 200026) and clinical isolate (MTCC 13369) and the functional role of the EV cargo in infection.

Results

EVs were isolated from the secretome of the fungal cultures grown on agar plates, as described previously from our lab. Secretomes were collected

and filtered with a cell strainer (40 µm), the filtered secretome were clarified by centrifugation at 11,900 x g for 15 min. The clarified secretome was centrifuged at 1,20,000 x g for 70 minutes at 4°C to pellet the EVs. EVs were suspended in PBS and used for further experiments

EV samples were diluted to 1:500 using filter sterilised PBS, and analysed using Nano sight NS 300 (Malvern, UK) to determine the size distribution and quantification of isolated EVs. The manufacturer's manual's recommended settings for the parameters were used. A total of three runs with fifteen captures (60 seconds/capture) were recorded. A representative figure is given below (Fig1 A). Based on the yield of EVs, we finalized the solid state fermentation method for EV preparation.

Protein profiling of Extracellular Vesicles

EV isolation was done in replicates using solid state fermentation. 50 µg of EV proteins were subjected to analysis for in-gel tryptic digestion and peptide extraction followed by peptide desalting clean-up using C18 columns and were analysed in a nano LC-Orbitrap mass spectrometer. Raw data were searched against the *Aspergillus flavus* proteome database. 1034 non-redundant proteins from ATCC-26 and 1318 non-redundant proteins were identified (Fig1B). Figure shows the Volcano plot of the distribution of the identified proteins (Fig1C). Gene enrichment analysis was done by using of Funrich software V3.1.3 for the proteins identified (Fig 2).

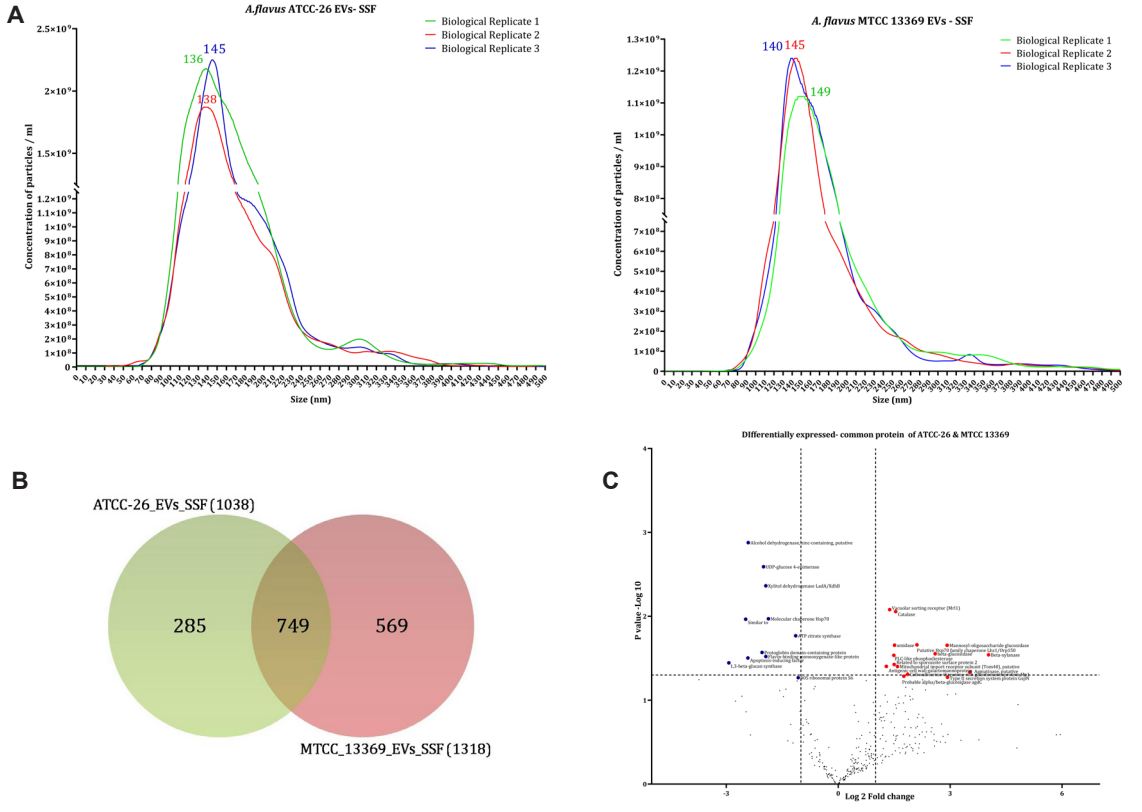


Fig.1. A. Particle size distribution: NTA data. B. Venn diagram of EVs from MTCC and ATCC isolates. D. Volcano plot showing.

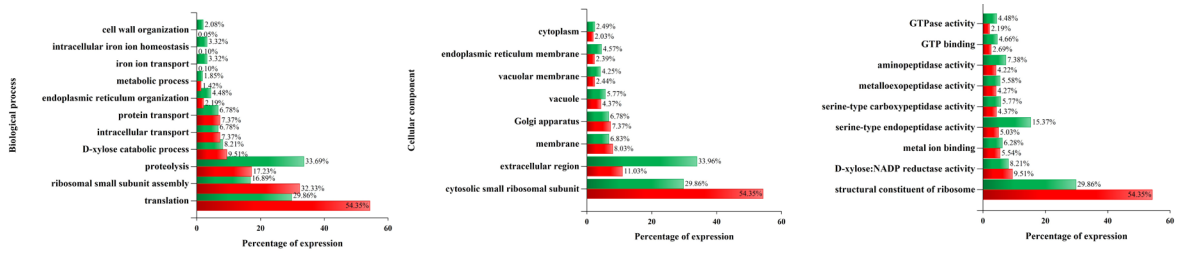


Fig.2. Gene enrichment Analysis of A. flavus Extracellular vesicles

Identification of Retinol Binding Protein 3 (RBP3) from vitreous and plasma extracellular vesicles as a predictive biomarker for diabetic retinopathy.

Investigators : Dr. Daipayan Banerjee, Dr. Bhavani S, Dr. Prithviraj Udaya, Aadhithiya T. Gr, Dr. K. Naresh Babu, Dr. R. Kim, Prof. K. Dharmalingam

Funding : Sun Pharmaceuticals

Introduction including background

Diabetic retinopathy (DR) is the most serious ocular complication of diabetes and affects approximately one-third of people suffering from diabetes. DR shows no symptoms in the early stages, and manifests only if one develops VTDR in the form of proliferative DR or diabetic macular edema(Jampol et al., 2020). Hence, identifying and validating robust biomarkers for both DR onset and its transition to advanced stages is crucial for enabling early intervention and preventing vision loss. Our earlier study using a large cohort of samples show that the serum protein cystatin C is a valid biomarker for early prediction and could be used along with HBA1C for targeted screening of diabetic patients for the early detection of DR(Gurudas et al., 2022). Thus, unravelling the complex mechanisms of DR requires pursuing innovative approaches for identifying a panel of predictive biomarkers.

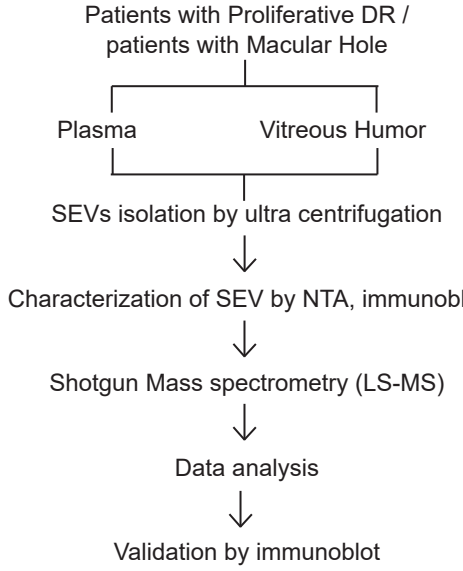
Extracellular vesicles (EVs) are lipid-bilayered non-dividing nano-vesicles that carry physiology-altering biological information in the form of regulatory nucleic acid, protein, lipids and metabolites. EVs lipid bilayer protects the protein and nucleic acid cargo from proteolytic cleavage and degradation while in circulation, thus, are excellent candidate for novel biomarker discovery. DR is a microvascular inflammatory disorder where dysregulation of angiogenesis plays a key role in vision loss (Abcouwer, 2013). Recent studies have started to unravel the role of extracellular vesicle-derived protein and miRNAs in DR pathogenesis. Thus, exploiting circulating EVs as source of DR biomarker and understanding the mechanism how EV cargo modulated target cell function in DR is critical to develop novel diagnostic and therapeutic tools. The overall aim of this study is to expand the panel of usable circulating protein biomarkers for early prediction of diabetic retinopathy. Specifically, we aimed to detect protein cargo of plasma and vitreous EVs in patients, specific for sight-threatening proliferative diabetic retinopathy and to identify vitreous EV proteins present in the circulation.

Results and conclusion

Workflow: The workflow for this study is shown in Figure 1a. A total of 36 patients were enrolled in the study, 18 patients with PDR and the comparative control group consisted of 18 patients with MH. The mean age of patients with MH was 61.7±10.4 years (8M:10F) and the mean age of patients with PDR was 53.2±7.2 years (12M:6F). A multi-pronged approach was employed to characterize the small extracellular vesicles (SEVs), utilizing Nanoparticle Tracking Analysis (NTA) for size distribution and concentration, immunoblotting for specific protein identification, and shotgun mass spectrometry (LC-MS) for a comprehensive protein profile analysis (Figure 1a).

Figure 1a

Overall Workflow



Nanoparticle Tracking Analysis:

The mean SEV diameter as obtained from NTA was 228.5±3.08 nm and 229.4±11.10 nm for plasma SEVs from patients with MH and PDR, respectively. The mean vesicle diameter for VH-SEVs from patients with MH and PDR was 297.6±16.77 nm and 293.3±17.01 nm, respectively (Figure 1b). While the mean vesicle diameter of plasma SEVs or VH-SEVs from the MH and PDR groups was not significantly different, however, we observed that the mean diameter of VH-SEVs was significantly larger compared to SEVs from the plasma ($p<0.0001$). The mean concentration of both plasma and VH-SEVs was significantly lower in PDR patients compared to MH patients ($p < 0.0001$). In addition, the mean concentration of plasma SEVs was significantly higher compared to VH SEVs regardless of disease group ($p < 0.0001$) (Figure 1c).

Figure: 1b

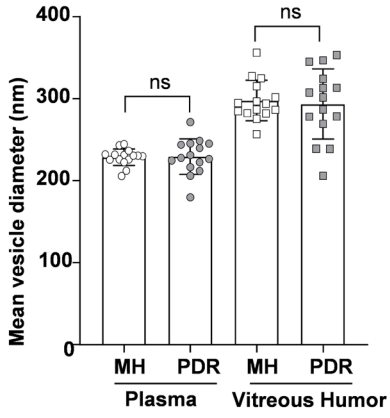
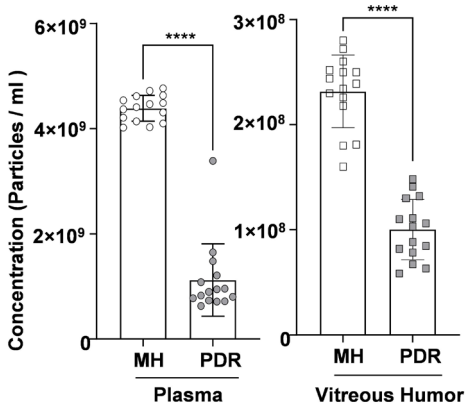


Figure: 1C



Mass spectrometric analysis:

Owing to the low yield of VH-SEVs from individual patients, pooled samples were used for proteomic analysis. The expression of canonical EV specific marker, tetraspanin CD63 (Figure 1d) was found in the SEV fraction of MH and PDR samples. In the plasma SEVs from patients with PDR and MH, we identified 126 and 97 proteins, respectively using shotgun mass spectrometry under the stringent conditions of identification used. Of these, 70 proteins were found only in plasma SEVs from PDR patients and 41 were found only in the MH patient group. Of the 56 common plasma SEV proteins 15 were upregulated and 5 were downregulated in the PDR patient group (log 2 fold change cutoff |1.4|). In the VH SEVs from patients with PDR and MH, we identified 152 and 104 proteins, respectively. Of these, 101 proteins were unique to the VH SEVs of PDR patients and 53 proteins were unique to the MH patient group. Of the 51 common VH SEV proteins, 17 were upregulated and 9 were downregulated in the PDR patient group (log 2 fold change cutoff |1.4|) (Figure 1e).

Identification and validation of SEV-RBP3 from VH and plasma

From the dysregulated plasma and VH SEV proteins identified in Table 1, we selected retinol-binding protein 3 (RBP3) for validation due to its critical role in retinal function and potential protective effect against DR. Photoreceptor-derived RBP3 serves as a vital retinoid transporter, shuttling vitamin A molecules between photoreceptors and the retinal pigment epithelium (RPE) as well as Müller cells. Using vitreous samples from diabetic patients with proliferative and non-proliferative diabetic retinopathy (PDR, NPDR), RBP3 is shown to be decreased in early stages of diabetic retinopathy (Balaiya et al., 2017; Garcia-Ramírez et al., 2009; Yokomizo et al., 2019). Increased expression of RBP3 in vitreous of patients with type I diabetes protected against the development of advanced DR (Yokomizo et al., 2019).

We used immunoblotting to confirm that RBP3 was significantly reduced in the neat vitreous of PDR patients compared with patients with macular hole (Figure 1f). Shotgun mass spectrometry identified

Figure 1d

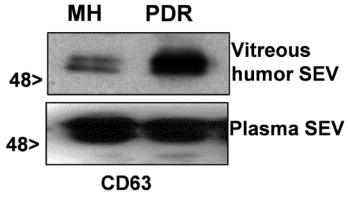
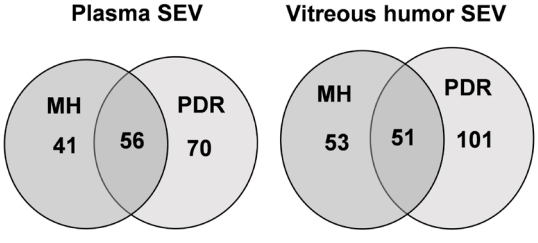


Figure 1e



RBP3 in the VH-SEVs and was lower in patients with PDR which was confirmed by immunoblotting using pooled VH-SEV proteins from patients with PDR and MH (Table 1 and Figure 1g). Although RBP3 is identified in neat VH, it is not detectable in neat plasma by immunoblot (Figure 1h). Along the same lines, Yokomizo et al had previously reported that the concentration of RBP3 in serum was 1000–5000 times lower than the concentration of RBP3 in vitreous using a highly sensitive ELISA (Yokomizo et al., 2019). Therefore, the predominant presence of RBP3 in the VH and its low detectability in circulation restricts its practical applicability in large-scale biomarker studies as VH sample collection involves invasive vitrectomy procedure. To test whether RBP3 could be detected in plasma SEVs, we isolated SEVs from plasma of individual patients with PDR and MH and performed immunoblot analysis for RBP3. Interestingly, we were able to detect RBP3 in plasma SEVs, and the levels were significantly lower in patients with PDR compared with patients with MH (Figure 1i), mirroring the results of neat VH and VH SEVs. Thus, we confirmed the presence of RBP3 in vitreous SEVs and successfully detected RBP3 in circulating SEVs present at low levels in patients with PDR compared to patients with MH.

Table 1: List of SEV proteins from plasma and VH

Source of SEV	Uniprot ID	Protein name	Function	log2 ratio (FC)	Status
Plasma	HRG_HUMAN	Histidine-rich glycoprotein	Regulates angiogenesis	1.8	Up
	HEMO_HUMAN	Hemopexin	Promotes angiogenesis	1.8	
	VTNC_HUMAN	Vitronectin	Maintains vascular homeostasis	1.8	
	HORN_HUMAN	Hornerin	Epidermal antimicrobial barrier	-2.6	Down
	CD5L_HUMAN	CD5 antigen-like	Autophagy inducer	-1.7	
	Q5VY30_HUMAN	Galectin-3-binding protein	Negative regulation of NF-κB signaling	-1.4	
	AACT_HUMAN	Alpha-1-antichymotrypsin	Regulates inflammation	4/12 (peptide/PSM)	Unique
	ANT3_HUMAN	Antithrombin-III	Key enzyme in coagulation cascade	4/18 (peptide/PSM)	
	APOD_HUMAN	Apolipoprotein D	Oxidative stress and inflammation	2/3(peptide/PSM)	
Vitreous humor	AACT_HUMAN	Alpha-1-antichymotrypsin	Regulates inflammation	3.4	Up
	ANT3_HUMAN	Antithrombin-III	Key enzyme in coagulation cascade	3.1	
	GELS_HUMAN	Isoform 2 of Gelsolin	Apoptosis inhibitor	1.5	
	I3L425_HUMAN	Pigment epithelium-derived factor	Retinal neuroprotection	-4.5	Down
	RET3_HUMAN	Retinol-binding protein 3	Maintenance of photoreceptor	-2.8	
	Q5VY30_HUMAN	Galectin-3-binding protein	Negative regulation of NF-κB signaling	-2.2	
	SPB3_HUMAN	Isoform 2 of Serpin B3	Induces hypoxia	3/9 (peptide/PSM)	Unique
	H0Y7Z1_HUMAN	Fibronectin (Fragment)	Thickening of endothelial basement membrane	6/23(peptide/PSM)	
	PLAK_HUMAN	Junction plakoglobin	Promotes cells proliferation	4/12(peptide/PSM)	

Figure: 1g

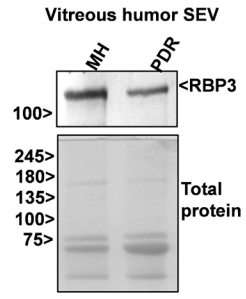


Figure: 1f

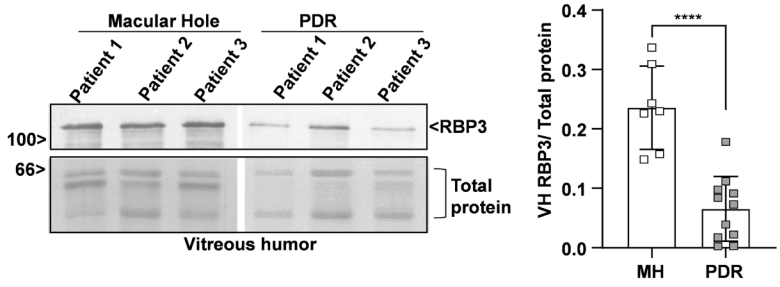


Figure: 1h

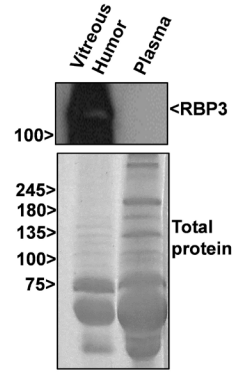
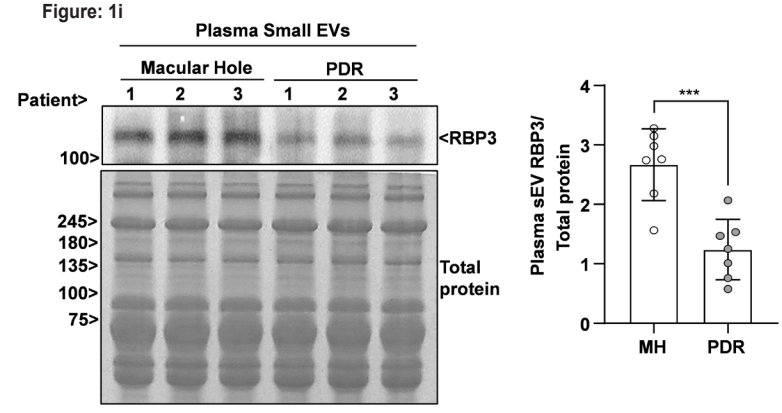


Figure: 1i



Conclusion

Identifying RBP3 in circulatory SEVs, not only sheds light on its potential as a biomarker but also reveals other EV protein cargo as potential key players in DR pathogenesis. These findings present exciting prospects for validation and mechanistic exploration, laying the groundwork for the development of innovative therapeutic strategies in the management of diabetic retinopathy.

Decipher the role of epigenetic modifications in regulating gene expression in pterygium pathogenesis.

Investigators : Dr. Daipayan Banerjee,
Research Scholar : Mathan Loganathan
Funding : SERB-SRG

Introduction including background

Pterygium stands out as a prevalent and progressive eye disease affecting the conjunctiva, with a substantial impact on the quality of life and visual capabilities of individuals, particularly those with a low socioeconomic status engaged in outdoor work. This condition manifests as a wing-shaped fibrovascular overgrowth on the nasal side of the eye, gradually advancing towards the cornea. In southern India, the incidence rate is notably high at 25.2 per 100 person-years, contributing to around 4% of corneal blindness cases. Ultraviolet (UV) exposure emerges as a crucial factor in the onset of this disease, although its complete etiology remains unclear. Despite a significant prevalence rate of 12%, there is currently no pharmaceutical intervention capable of halting the progression of pterygium. Surgical removal remains the sole treatment option available, and there are no prognostic tools for predicting disease progression or recurrence. The rationale for investigating this disease further lies in recognizing the pivotal role of dysregulated epigenetic modifications in its pathogenesis. While chronic exposure to sunlight has been linked to epigenetic alterations in skin, contributing to conditions such as skin cancer, the exploration of how epigenetic changes regulate gene expression in pterygium remains largely uncharted territory, despite the constant ocular exposure to sunlight in affected individuals. We hypothesize that aberrant epigenetic modifications regulate gene expression in pterygium pathogenesis. Here, we decipher the role of epigenetic modifications in regulating gene expression in pterygium pathogenesis, specifically, genome wide methylation analysis was performed using Illumina Infinium MethylationEPIC v2.0 beadchip array.

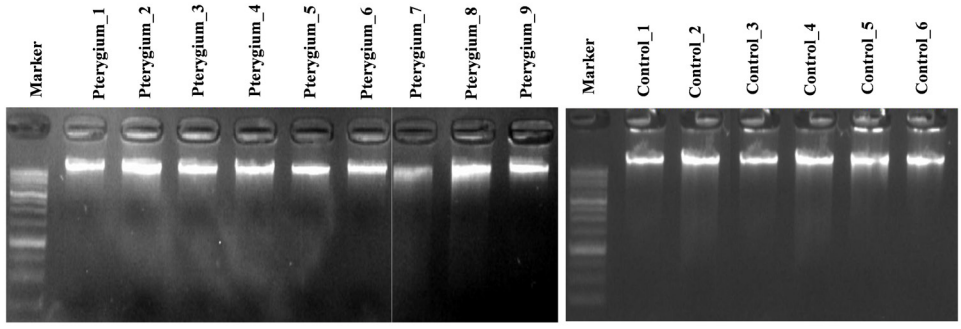


Figure 2a: QC Report OF DNA Sample from Conjunctival Tissue in Gel Electrophoresis

Results and Conclusion

Sample collection: Samples were collected from 9 Patients (4M/5F) with pterygium and 6 patients (3M/3F) with cataract (Table 2). The average age for the patients with pterygium is 53.4 ± 5.01 and the patient with cataract is 57.67 ± 4.46 years. The two-tailed P value is 0.133. By conventional criteria, this difference is considered to be not statistically significant. The intact band on the gel shows that DNA is permissible for methylation analysis (Figure 2a).

Infinium MethylationEPIC v2.0 beadchip array: Analysis of overall signal median value of methylated and unmethylated shows the median signal value of the methylation site in the sample (log 2), and the ordinate shows the median signal value of the non-methylation site in the sample (log 2). All samples are represented as scattered points. There were no outliers in the pterygium and control samples (Figure 2b).

The boxplot shows the distribution of normalized DNA methylation level (β values) between pterygium and control groups in Figure 2c.

S.no	Sample	Age	Sex
1	Pterygium_1	54	M
2	Pterygium_2	53	F
3	Pterygium_3	47	F
4	Pterygium_4	55	F
5	Pterygium_5	61	F
6	Pterygium_6	57	M
7	Pterygium_7	58	F
8	Pterygium_8	52	M
9	Pterygium_9	44	M
10	Cataract_1	61	M
11	Cataract_2	53	F
12	Cataract_3	60	M
13	Cataract_4	52	F
14	Cataract_5	57	F
15	Cataract_6	63	M

Table 2: Sample Details

Figure 2b: Log median intensity of methylated and unmethylated channel

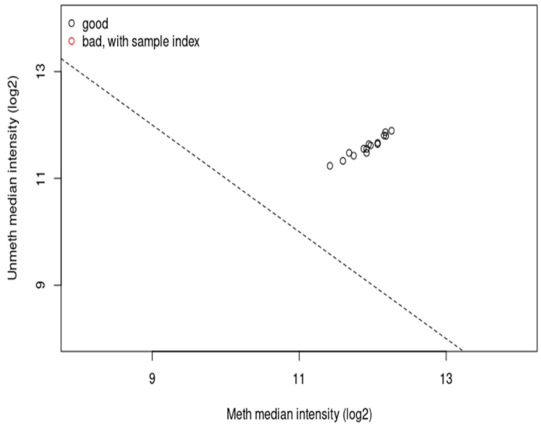
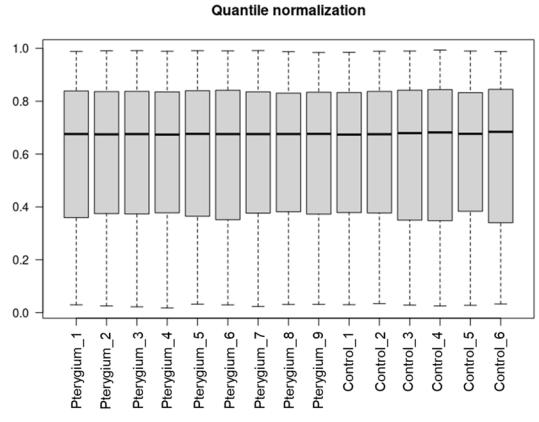


Figure 2c: Boxplot



We used the scatter volcano plot of differentially methylated probe (DMP) in the pterygium group compared with control group where blue dot represents the hypomethylated probes and red dot represents hypermethylated probes. The distribution of CpG were sorted by absolute value of Δβ>0.1 and P<0.01. In this group, we identified 116 hypomethylated probes and 283 hyper methylated probes in the conjunctival tissue from patients with pterygium compared with control group (Figure 2d).

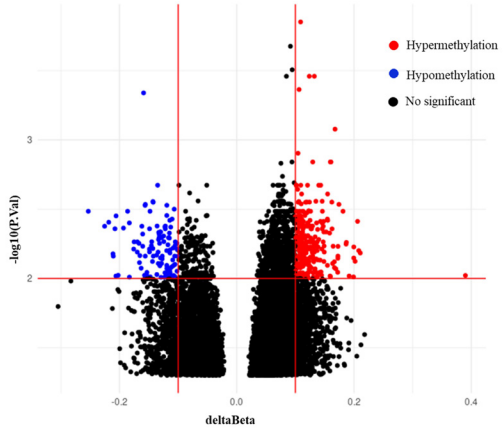


Figure 2d: Volcano plot for DMP in the pterygium group compared with control group

Patient samples were also ordered by hierarchical clustering, and a heat map was produced to allow the visualization of the hypermethylated region and hypomethylated region associated with pterygium progression. The heat map shows all the different methylation sites in the individual control and pterygium samples where the dark color denotes the hypermethylation site, and the light color denotes the hypomethylation site (Figure 2e).

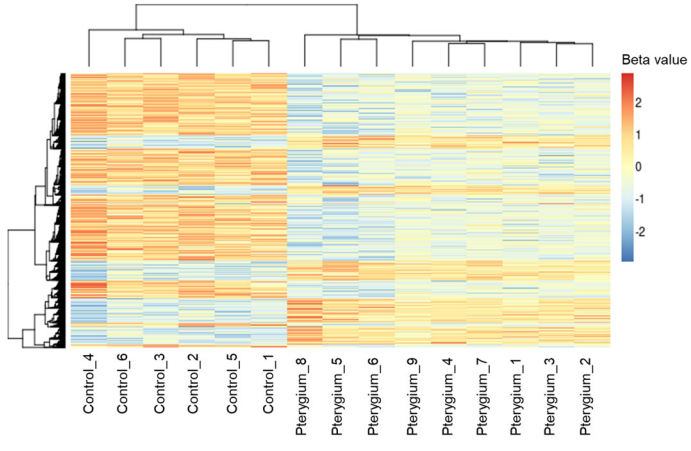


Figure 2e: Heatmap for differentially methylated probes in pterygium and control

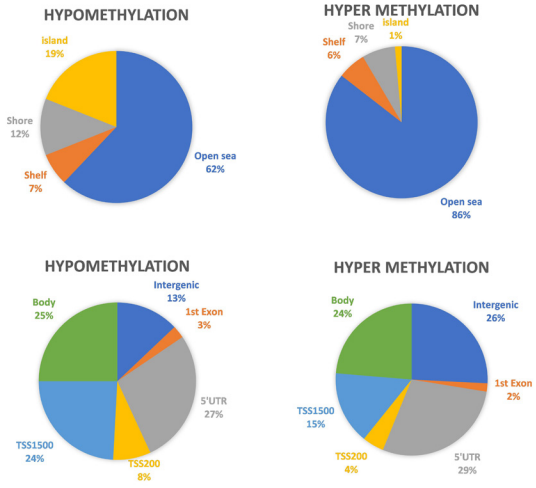


Figure 2f: Distribution of DMP in and around CpG island and gene structural elements in hypermethylation and hypomethylation site

The distribution of differentially methylated probe in and around CpG island and the gene structural elements in the hypermethylation and hypomethylation sites are shown in Figure 2f.

To discern pathways that are differentially methylated genes in pterygium conjunctival tissue compared to control conjunctival tissue, Gene Ontology (GO) functional enrichment analysis of the differentially methylated genes was conducted using the DAVID online server (Figure 2g).

Finally, the CpG probe IDs with the genes for the top hypermethylated and hypomethylated sites are listed in Table 3 and Table 4.

This is the first study to shed light into the changes in methylation status that occur in the conjunctival tissue in a ocular surface disorder pterygium. This will help in understanding the role of epigenetic changes in pterygium pathogenesis.

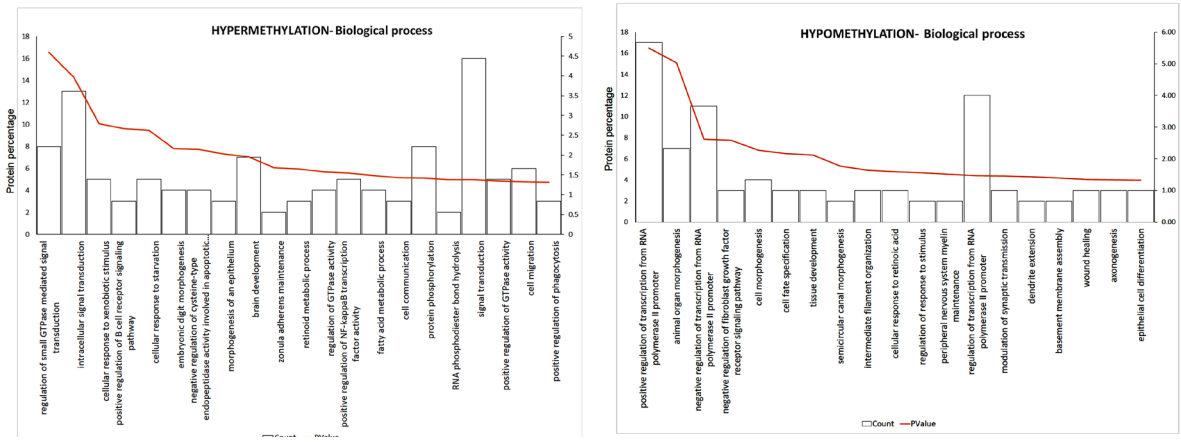


Figure 2g: Gene Ontology (GO) functional enrichment analysis

Table 3: Top Hypermethylated Genes

Gene ID	Gene name	delta Beta	Feature	Biological function
NTNG1	Nertin G1	-0.20034	Body	Promotes tumorigenesis
TSTD1	Thiosulfate:glutathione sulfurtransferase	-0.15859	TSS1500	Cancer cell proliferation
NFATC4	Nuclear factor of activated T-cells, cytoplasmic 4	-0.1544	TSS1500	Increased the cell proliferation, colony formation, migration, invasion
PRDM5	PR domain zinc finger protein 5	-0.13204	5'UTR	Growth suppressive activities
LPIN2	Phosphatidate phosphatase LPIN2	-0.12152	5'UTR	Suppress NF-κB signaling pathway affect cellular apoptosis
TNS1	Tensin 1	-0.11663	5'UTR	Involved in fibrillar adhesion formation, Essential for myofibroblast differentiation
MEIS2	Myeloid ecotropic insertion site 2	-0.11207	5'UTR	Essential for cell survival and proliferation
GLIS1	Gli-similar	-0.11029	5'UTR	Mesenchymal-epithelial transition
PLEC	Plectin	-0.10886	5'UTR	Regulator of Cancer Cell Survival and Proliferation
MYB	myeloblastosis proto-oncogene	-0.10761	TSS1500	Promotes tumourigenesis

Table 4: Top Hypomethylated Genes

Gene ID	Gene name	delta Beta	Feature	Biological function
MARK4	microtubule affinity regulating kinase 4	0.17638	Body	Promote proliferation and migration of cancer cell
LGMN	legumain	0.150833	5'UTR	Tumor invasuin, proliferation and angiogenesis
CDH4	cadherin 4	0.150421	5'UTR	Tumor supressor gene
HEG1	heart development protein with EGF like domains 1	0.141691	5'UTR	Invasion, metastasis and EMT
VAV2	vav guanine nucleotide exchange factor 2	0.134122	5'UTR	Promote proliferation of cancer cell
STK24	serine/threonine kinase 24	0.131498	5'UTR	Promotes tumourigenesis
ARHGEF10L	Rho guanine nucleotide exchange factor 10 like	0.118833	TSS1500	Tumorigenesis and EMT
RASSF3	Ras association domain family member 3	0.111114	Body	Antitumor effect
TIAM2	TIAM Rac1 associated GEF 2	0.105337	Body	Promote EMT
PREX1	phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1	0.102088	5'UTR	Promote cancer metastasis

Understanding the mechanism of action of a novel chemical cross-linker designed to treat keratoconus

Investigators : Dr.O.G.Ramprasad, Prof. K. Dharmalingam and Dr. Venkatesh Prajna
Research fellow : Adhithya Subramanian,
Clinical fellow : Dr. Pooja Deepak Andhare
Funding : ICMR

Introduction including background

Corneal collagen crosslinking has become a vital method to treat keratoconus (KC), a progressive, bilateral corneal ectatic disorder affecting the young population usually in their second and third decades of life. The diseased cornea becomes cone shaped leading to astigmatism. The conventional photochemical crosslinking involving ultraviolet-A radiation and riboflavin introduced by Wollensak et al. in 2003 is one FDA-approved technique which is known to halt mild to moderate KC progression. While moderate KC can be treated with the conventional cross-linking method, severe forms of the disease requires corneal transplantation. Keratocyte apoptosis, mild corneal haze, infectious keratitis and delayed epithelial healing are some of the risks associated with the conventional cross-linking procedure. UV-A penetration in thin KC corneas <400 μm also results in endothelial cell loss.

Our collaborators at the University of Liverpool, UK have developed a novel, PBS soluble, eye-drop based chemical cross-linker (CXL), consisting of EDCI/NHS [1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide] mediated chemistry and a suberic acid spacer that has the potential to be developed as an alternative form of treatment for mild to moderate keratoconus. It can 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. We, at Aravind Medical Research Foundation and Aurolab have established the proof-of concept in human corneas, wherein treatment of the diseased keratoconus cornea for 15 minutes at 37°C with the novel chemical cross-linker is able to increase the stiffness of the weak keratoconus cornea by cross-linking collagen molecules. The cross-linker also does not cause cytotoxicity or morphological changes to the corneal cell layers. The details about the investigation are available in the previous progress reports as well as in the publication (Haneef et al., 2021).

Purpose of the study

The mechanism of action of the chemical cross-linker in stiffening the cornea is not clearly understood. This study aimed to understand the effect of the novel chemical cross-linker in the expression of

select matrix metalloproteases (MMPs)- MMP-1, 2, 3 and 9 under in vitro conditions and in the epithelial and stromal layers of KC patient tissues. Some of the proteins affected by the cross-linker in the keratoconus cornea are also described. The difference in the arrangement of epithelial cells in the corneal epithelium and the stromal cells in the corneal stroma necessitated the separate analysis of the activity of matrix modulating enzymes and the differential protein expression affected by the application of the CXL in these layers. The effect of CXL in the collagen fibre assembly of the keratoconus stroma and its effect in stiffening of the cornea are reported.

Results

Evaluation of the stiffening of the KC cornea by the cross-linker

The CXL significantly stiffened the KC cornea at 0.2 M concentration and at the diluted 0.02 M concentration (8.82 and 6.01 fold increase respectively compared to the normal keratoconus cornea) (Figure 1 A). The increase in stiffness in KC cornea was comparable to the control cadaver cornea. Further dilutions of the cross-linker did not stiffen the cornea as 0.02 M CXL. UV-riboflavin treatment significantly increased the stiffness of the KC corneas (>2.61 folds) compared to the non-cross-linked KC corneas. The increase in stiffness due to the chemical cross-linker was considerably greater when compared to the UV-riboflavin induced increase in stiffness.

Most of the epithelial cells of the KC cornea were viable and negligible cell death was seen after 0.02 M CXL treatment as visualized by the live dead staining (Figure 1 B). We evaluated the safety of corneal endothelium using trypan blue and alizarin red stain after treating cadaver cornea with 0.2 M and 0.02 M cross-linker solution. Tight junctions of the corneal endothelium remained intact with negligible cytotoxicity to the endothelial cells. There was no compromise on the human corneal endothelial integrity and viability.

Effect of CXL on the KC stromal collagen fibril assembly

The collagen fibril arrangement in the central corneal stroma was assessed by transmission electron microscopy (Figure 2 A). The collagen lamellae and the fibrils were densely packed in the control cadaver stroma, while in KC corneal stroma, the dense packing was no longer present. The interfibrillar spacing was more in the KC stroma with disorganized collagen fibril assembly. The CXL treatment reduced the inter-fibrillar spacing and generated an organized and packed collagen fibril assembly comparable to the native state. The collagen fibril density per unit area in the central corneal stroma assessed by transmission electron micrographs (Figure 2 B) was analyzed as an indicator of cross-linking. The number of collagen fibrils per unit area was lower in KC corneal stroma compared to cadaver control but significantly increased after CXL treatment. The mean fibril diameter in KC corneal stroma was lower compared to the cadaver control stroma. After cross-linker treatment the collagen fibril diameter significantly increased (>1.29 folds) and was comparable to the control fibril diameter values

Figure 1: Stiffening of KC cornea after CXL treatment

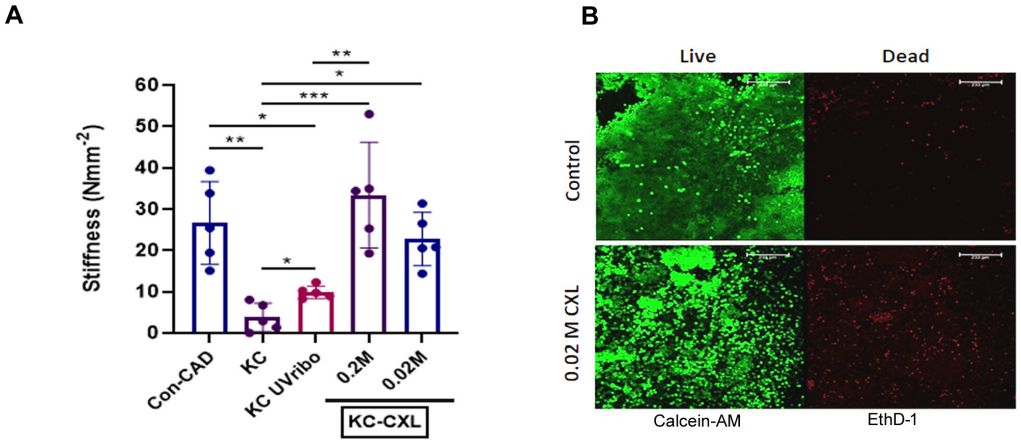


*Figure legend: (A) Changes in the stiffness of KC cornea after CXL treatment at various concentrations. n = 5 corneas were used for each category in control cadaver (Con-CAD), keratoconus (KC), conventional UV-riboflavin treated KC cornea (KC Uvribo) and cross-linker treated keratoconus cornea (KC-CXL). Data in mean ± SD; **P<0.05 for the indicated conditions (by 2-way ANOVA). (B) Representative live dead staining images of KC corneal epithelium treated with 0.02 M CXL. Calcein-AM fluorescence represents live cells while Ethidium homodimer-1 (EthD-1) fluorescence represents the dead cells. Bars represent 233 μm.*

Figure 2: Organization of collagen fibrils in the stroma

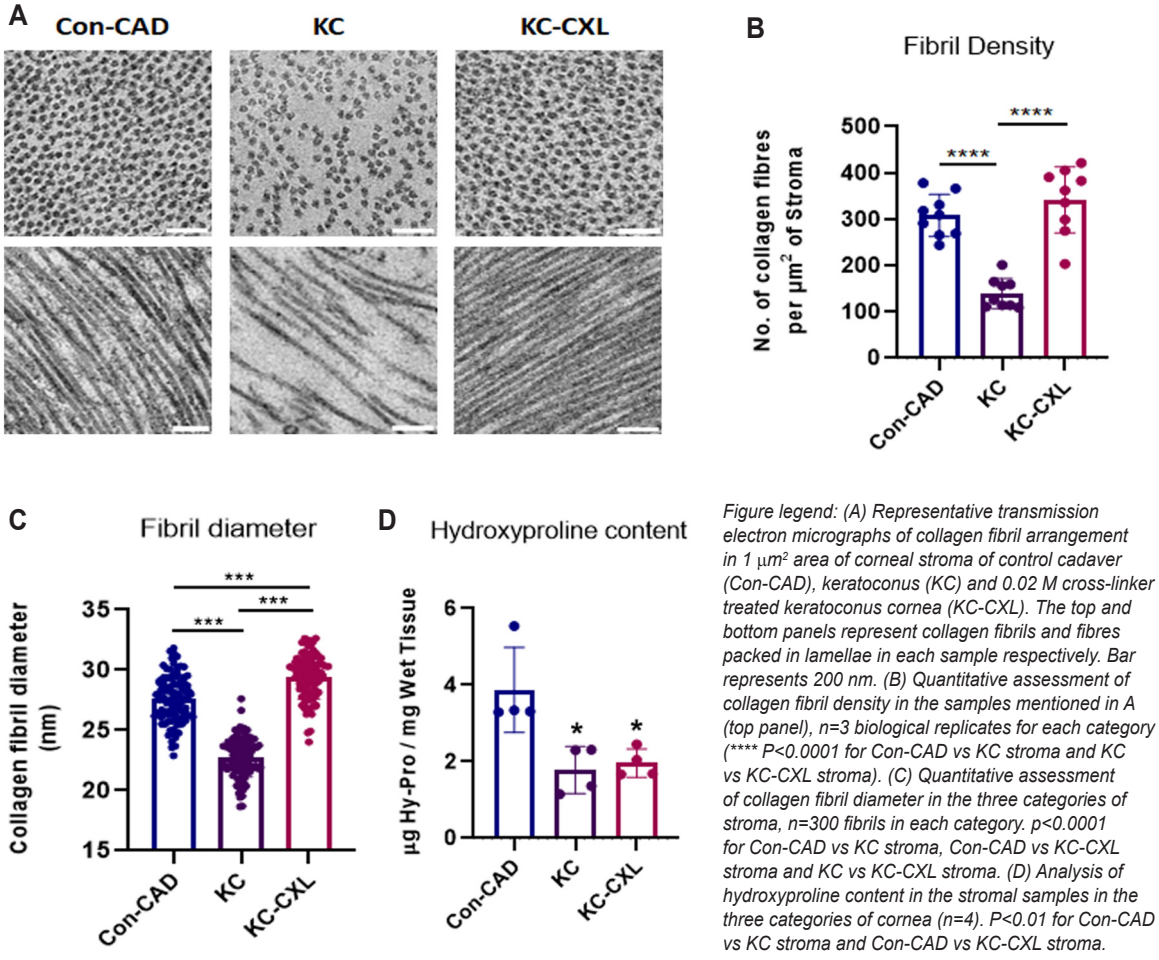


*Figure legend: (A) Representative transmission electron micrographs of collagen fibril arrangement in 1 µm² area of corneal stroma of control cadaver (Con-CAD), keratoconus (KC) and 0.02 M cross-linker treated keratoconus cornea (KC-CXL). The top and bottom panels represent collagen fibrils and fibres packed in lamellae in each sample respectively. Bar represents 200 nm. (B) Quantitative assessment of collagen fibril density in the samples mentioned in A (top panel), n=3 biological replicates for each category (**** P<0.0001 for Con-CAD vs KC stroma and KC vs KC-CXL stroma). (C) Quantitative assessment of collagen fibril diameter in the three categories of stroma, n=300 fibrils in each category. p<0.0001 for Con-CAD vs KC stroma, Con-CAD vs KC-CXL stroma and KC vs KC-CXL stroma. (D) Analysis of hydroxyproline content in the stromal samples in the three categories of cornea (n=4). P<0.01 for Con-CAD vs KC stroma and Con-CAD vs KC-CXL stroma.*

(Figure 2 C). The measured hydroxyproline content, an indicator of total collagen, decreased in KC corneal stroma and remained at the same levels after CXL treatment indicating that CXL functioned mostly on the re-assembly of collagen fibrils (Figure 2 D).

CXL decreases the secretion of MMPs from HCE cells and KC fibroblast cultures

Previously we reported that the activity of MMPs- 2 and 9 are reduced in the epithelial and stromal layers of the KC cornea after treatment with the CXL. Now, we have analyzed the effect of CXL on the secretion of MMPs from HCE cells and fibroblasts derived from KC cornea in vitro. Induction of inflammatory conditions in HCE cells akin to keratoconus disease condition was stimulated in HCE cells treated with 10 ng/ml TNF-α

The control HCE cells had lesser amounts of secreted and cytosolic MMP-9, while TNF-α induced a 20-fold increase in the production of MMP-9 compared to the control by 24 hours (Figure-3A). After 6 or 24 hours of CXL treatment, the secretion of MMP-9 from TNF-α supplemented HCE was significantly reduced with no effect at the transcript level (Figures 3A and B). The extent of reduction of cytosolic MMP-9 was lesser compared to the secreted levels. The cytosolic levels of MMP-9 indicated that the CXL was potent enough to stop the secretion of MMP-9, making it localized to the cytoplasm. Secretion of MMPs-1, 2 and 3 from KC fibroblasts highly declined after CXL treatment. In KC fibroblasts, secreted MMP-1 and MMP-2 levels were more than the control, while there was no significant difference in secreted MMP-3 levels. The presence of MMP-9 was negligible to be detected in the conditioned media of KC fibroblasts.

Figure 3: Analysis of secreted and cytosolic MMP-9 and mRNA levels from human corneal epithelial cell line (HCE)

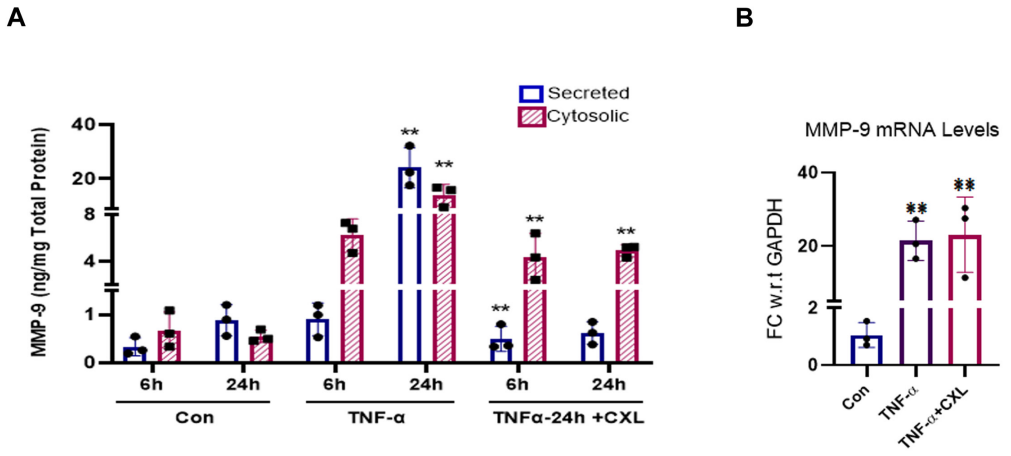


*Figure legend: (A) Secreted and cytosolic MMP-9 protein levels from TNF-α supplemented human corneal epithelial cell line before and after cross-linker at 6h and 24h post treatment. Data in mean ± SD. Secreted MMP-9 levels are significantly different for the indicated groups compared to the control. ** P<0.01 by 2-way ANOVA. (B) Relative expression of MMP-9 mRNA in untreated (Con), 10ng/ml TNF-α treated and 10ng/ml TNF-α + 0.002 M CXL treated cells 6h post treatment. ** P<0.05 for Con vs. TNF-α and for Con vs. TNF-α + CXL. n=3 for all the experiments.*

Comparative protein profiles of cross-linked vs non-cross-linked KC cornea:

Identification of proteins cross-linked by the novel chemical cross-linker becomes important in order to have an idea of the biochemical and molecular changes occurring in the cross-linked cornea. We have identified the proteins cross-linked in the human KC corneal epithelium and human KC corneal stroma through mass spectrometry.

The protein profile of corneal epithelium and stroma of human KC cornea before and after cross-linker treatment is shown in Figure 4. We did observe some prominent protein bands in the profile of KC human corneal epithelium between 66 to 30 kDa region. After cross linker (CXL) treatment, the intensity of the protein bands increased in the epithelium of human KC cornea. In KC human corneal stroma and CXL treated stroma, protein band smear was observed above 100 kDa corresponding to the high molecular weight collagen. Many protein bands were found to have medium molecular weight which ranged from ~50kDa to ~70kDa.

Identification of cross-linked proteins in human KC corneal epithelium and stroma:

A total of 475 proteins were identified from cross-linker treated KC human corneal epithelium. The top 20 abundant proteins from the 0.2 M cross-linker treated human KC corneal epithelium are listed in Table 1. Many structural proteins were identified after cross-linking. The top abundant proteins identified were cytoskeletal proteins such as various keratins, tubulin and actin etc. Corneal epithelial specific keratin, K3/K12 were identified. Around 345 proteins were identified from cross-linker treated KC human corneal stroma. The top abundant proteins from the human corneal stroma are listed in Table 2. From the crosslinker treated human corneal stromal protein list,

Figure 4: Profile of laemmli extracted epithelium and stroma of Control, KC and CXL-KC cornea

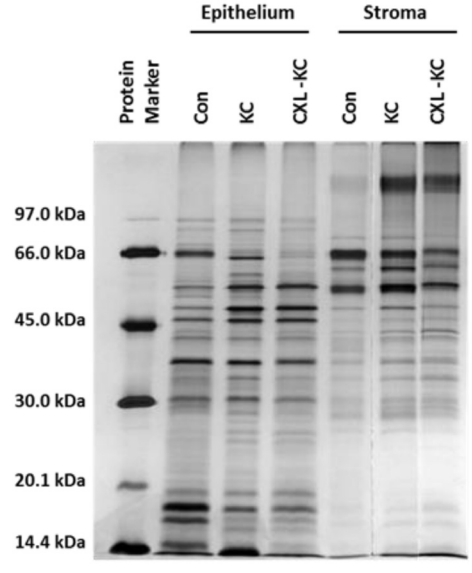


Figure legend: 1D protein profile of human corneal epithelial proteins and stromal proteins from cadaver, keratoconus (KC) and CXL treated KC cornea. 5µg of epithelial or stromal proteins were fractionated on 7cm, 12.5% 1D SDS-PAGE and visualised with silver staining protocol

Table 1: Top abundant proteins identified from CXL treated KC corneal epithelium

Accession	Description	ΣCoverage	Σ# PSMs
P13647	Keratin, type II cytoskeletal 5	54.24	8011
Q99456	Keratin, type I cytoskeletal 12	62.15	5020
P12035	Keratin, type II cytoskeletal 3	55.10	4285
H0YI76	Keratin, type II cytoskeletal 5 (Fragment)	63.68	2975
P30838	Aldehyde dehydrogenase, dimeric NADP-preferring	45.25	2663
P02538	Keratin, type II cytoskeletal 6A	45.39	2146
P13646	Keratin, type I cytoskeletal 13	46.29	1267
P19012	Keratin, type I cytoskeletal 15	46.49	1165
P60709	Actin, cytoplasmic 1	45.33	1159
P68032	Actin, alpha cardiac muscle 1	31.30	1074
P63261	Actin, cytoplasmic 2	45.33	1050
P02533	Keratin, type I cytoskeletal 14	48.31	1040
P13645	Keratin, type I cytoskeletal 10	29.45	1035
P08727	Keratin, type I cytoskeletal 19	59.00	1013
P06733	Alpha-enolase	48.16	961
P14618	Pyruvate kinase PKM	57.25	823
P14618-2	Isoform M1 of Pyruvate kinase PKM	54.99	791
Q9BQE3	Tubulin alpha-1C chain	38.08	772
P00352	Retinal dehydrogenase 1	58.48	736
P68371	Tubulin beta-4B chain	47.19	693

Table 2: Top abundant proteins identified from CXL treated KC corneal stroma

Accession	**Description**	**ΣCoverage**	**Σ# PSMs**
D6RGG3	Collagen alpha-1(XII) chain	37.26	3697
P51884	Lumican	43.20	3175
P12111-2	Isoform 2 of Collagen alpha-3(VI) chain	27.90	3089
P07585	Decorin	50.97	2215
Q15582	Transforming growth factor-beta-induced protein ig-h3	40.12	1752
P02768	Serum albumin	40.72	1482
P21810	Biglycan	36.41	1146
P35527	Keratin, type I cytoskeletal 9	37.72	1128
P13645	Keratin, type I cytoskeletal 10	37.16	987
P30838	Aldehyde dehydrogenase, dimeric NADP-preferring	30.46	984
O60938	Keratocan	36.36	981
P07585-2	Isoform B of Decorin	48.40	812
A0A087X0S5	Collagen alpha-1(VI) chain	22.03	644
P01857	Ig gamma-1 chain C region	45.76	560
H0Y5N9	Collagen alpha-1(XII) chain (Fragment)	22.22	550
P51888	Prolargin	36.13	367
P02538	Keratin, type II cytoskeletal 6A	22.70	299
P12110	Collagen alpha-2(VI) chain	12.27	286
A0A075B6N8	Ig gamma-3 chain C region (Fragment)	23.87	261
P01859	Ig gamma-2 chain C region	21.78	261
P08670	Vimentin	34.33	223
P35443	Thrombospondin-4	19.35	219

we identified collagen type VI and type XII as a major stromal collagen protein. In addition to collagens, proteoglycans such as keratocan, lumican, biglycan and decorin were also identified as abundant proteins in cross-linker treated human stroma.

Conclusions:

We have deciphered the reduction in the activity and secretion of select metalloproteases as one of the mechanisms by which the chemical cross-linker can act on the corneal tissue to halt the degradation of the matrix in the keratoconus cornea. The reduction in the activity of MMPs-2 and 9 and cathepsin G after CXL treatment was evident in the epithelial layers and stromal layers in the KC corneal tissues. In the in vitro cellular systems, a similar phenomenon was recapitulated by the decrease in the activity and secretion of MMP9 from HCE cells and of MMP2 from KC fibroblasts. Additionally, CXL could reduce the secretion of MMPs-1 and 3 from KC fibroblasts. Along with the above factors, CXL also brought together the disorganized collagen fibres in KC stroma leading to the increase in stiffness in keratoconus corneas. Additionally, the CXL cross-links structural proteins in the KC corneal epithelium and proteoglycans and various isoforms of collagen to bring about the stiffening of the cornea. This CXL is now proven to have clinically relevant effects on stiffening the cornea, reorganizing collagen fibre assembly and inactivating MMPs.

Mechanotransduction in the homeostasis of retinal pigmented epithelium: implications in age related macular degeneration.

Investigators : Dr. Siddharth Narendran
Funding agency : SERB
Research Scholar : S. Karvannan

Introduction including background

Age-related macular degeneration (AMD) refers to progressive degeneration of the macula, the photoreceptor dense central retina which is necessary for fine visual acuity. AMD is classified into atrophic and neovascular forms. Geographic Atrophy (GA), the untreatable advanced form of atrophic AMD is responsible for 20% of all blindness due to AMD and is characterized by degeneration of the retinal pigmented epithelium (RPE). GA is characterized initially by sub-RPE deposits that accumulate between the RPE and the Bruch's membrane (BM). The last three decades of AMD research have been primarily focused on identifying the biochemical components of these sub-RPE deposits and targeting individual components of these deposits has been the predominant treatment strategy. However, these strategies thus far, have been a futile endeavor as evidenced by multiple clinical trials. Despite being the pathological hallmarks of GA and AMD, the effect of the mechanical changes caused by these deposits on RPE homeostasis has not been studied. Mechanotransduction, a phenomenon governing the fates and functions of biological systems by mechanical forces, has been found to occur in all corners of the biological realm with an extensive and diverse repertoire of mechanisms. In exciting, new preliminary studies, we observed a role for mechanotransduction in RPE homeostasis and degeneration in GA. Hence, although inflammation is considered to be the primary process by which RPE cell death occurs in GA, we hypothesize that while RPE degeneration is perpetuated by inflammatory mediators, it is initiated by mechanical factors.

Results and Conclusions:

The macular Bruch's membrane is significantly stiffer in AMD donor eyes compared to normal donors:

The biomechanical properties of the BrM across different stages of AMD were investigated using AFM. Our findings revealed a decrease in BrM stiffness during early AMD, whereas GA was associated with a significant increase in stiffness (Figure 1). Spatial analyses revealed that these significant differences were primarily confined to the sub macular BrM, with no such significant variations in the peripheral BrM Regression analysis, after adjusting for age and gender, identified GA as the sole significant factor associated with a stiffer BrM.

Figure: 1 : Distribution of young's modulus values across the samples

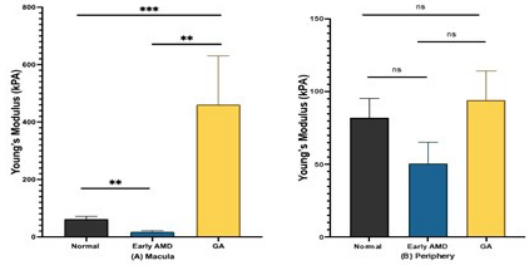
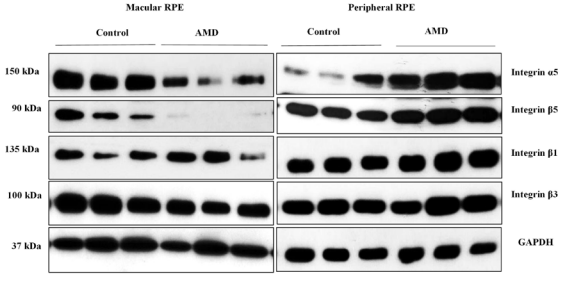


Figure 2 : Integrin protein profiling in normal and AMD tissues reveals differential expression



ii) Integrin alpha 5 and beta 5 levels are reduced in macular AMD tissues compared to those in healthy donor tissues

Integrin protein profiling was conducted on samples from both macular and peripheral regions of both AMD and healthy donor tissues. Immunoblot analysis revealed a significant decrease in integrin alpha 5 and beta 5 levels in macular AMD tissues compared to healthy macular tissues (Figure 2). On the contrary, the peripheral samples demonstrated a contrasting expression pattern, warranting comprehensive investigation through diverse molecular assays on a larger scale of samples.

iii) Exploring the regulation of cell adhesion as a therapeutic approach to reduce inflammation and halt RPE degeneration

Induced pluripotent RPE cells were cultured and maintained under sterile conditions. The adhesive capacity of these cells was evaluated on substrates with varying stiffness. Initially, adhesion was observed to be favorable on plates with stiffness levels of 16 and 64 kPa, consistent with our atomic force microscopy (AFM) data. However, after treatment with ATORC-K (a drug), adhesion significantly improved across all substrate stiffness plates (Figure 3).

Subsequently, RT-PCR was conducted to assess the expression levels of YAP and TAZ genes in iRPE cells cultured on plates with different stiffness levels following treatment with ATORC-K. It was observed that YAP and TAZ expression was notably elevated in cells cultured on 16 kPa stiffness plates after treatment, compared to the control condition (Figure 4).

iv) ATORC-K enhances the adhesive capacity of RPE on the macular Bruch's membrane (BrM).

Through the utilization of Scanning Electron Microscopy (SEM) imaging, Figure 5 presented clear visual evidence demonstrating that ATORC-K treatment led to increased adhesion of RPE cells compared to the control group. This observation was further substantiated by quantitative analysis using ImageJ software, as depicted in Figure 6, where the percentage of area covered by RPE cells was measured and plotted. The results revealed a notable enhancement in RPE cell attachment in the presence of ATORC-K, indicating its efficacy in promoting adhesion on the depithelialized macular BrM. These findings suggest the potential utility of ATORC-K as a means to improve cellular adhesion in retinal tissue, holding implications for various ocular pathologies and potential therapeutic strategies.

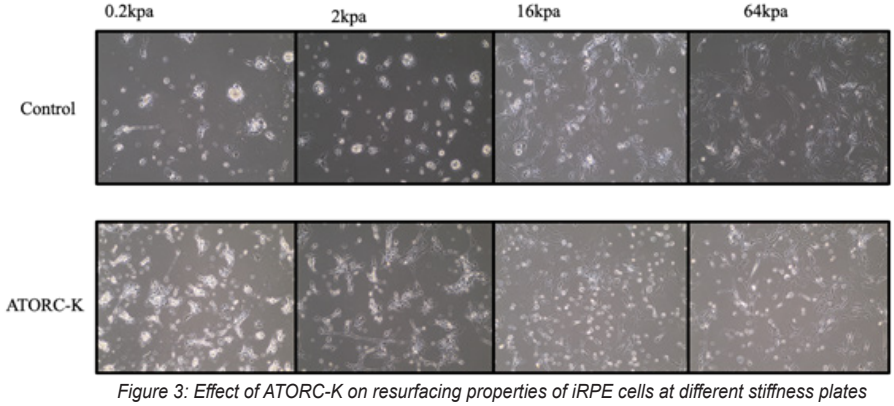


Figure 3: Effect of ATORC-K on resurfacing properties of iRPE cells at different stiffness plates

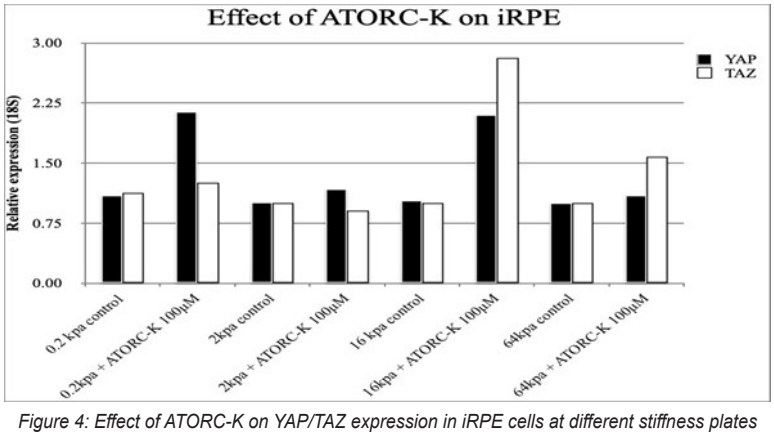
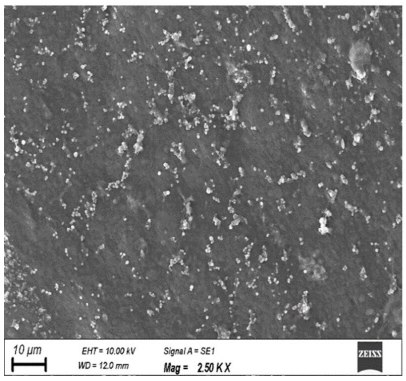
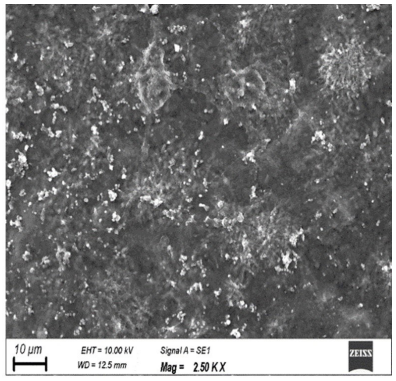


Figure 4: Effect of ATORC-K on YAP/TAZ expression in iRPE cells at different stiffness plates

Figure 5: Scanning electron microscopy analysis





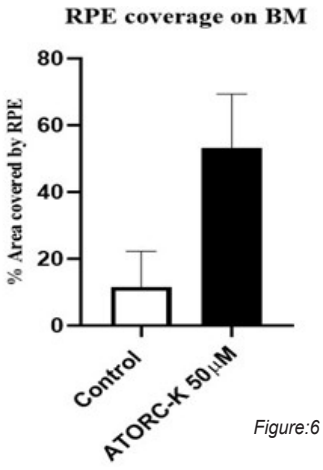


Figure:6

Deciphering the Role of CXCL-14: Understanding its Impact on Diabetic Retinopathy Progression

Investigators : Dr. Siddharth Narendran
Funding agency : Pending
Research Scholars: S. Karvannan & G.R. Elakkiya Nandhini

Introduction including background

Diabetic retinopathy, a debilitating complication of diabetes mellitus, remains a significant global health concern. It is characterized by progressive damage to the blood vessels in the retina, ultimately leading to vision impairment or loss. Despite advances in diabetes management, the molecular intricacies contributing to diabetic retinopathy's progression are not fully elucidated. In the context of proliferative diabetic retinopathy (PDR), where timely intervention is crucial, the search for reliable biomarkers gains urgency, especially in resource-limited settings. The subtle systemic inflammation observed in diabetes implicates various pro-inflammatory factors, including CXCL-14, a chemokine associated with obesity-induced insulin resistance and inflammation. Despite conflicting findings regarding its role in neoplasia-associated angiogenesis, understanding CXCL-14's specific involvement in diabetic retinopathy remains incomplete. Our study aims to elucidate its impact on disease progression, offering insights into potential therapeutic targets. This research contributes to the growing understanding of diabetic retinopathy, with implications for both diagnostics and therapeutic strategies aimed at mitigating its devastating effects.

Results and Conclusion

CXCL-14 expression varies significantly across samples from control (No DM), DM, NPDR, and PDR patients, suggesting modulation across these conditions

The plots depict CXCL-14 expression across plasma (Figure 1), vitreous (Figure 2), samples from different patient categories. ELISA assays were conducted to assess the concentration of CXCL-14 in various biological samples. Interestingly, the results revealed distinct patterns across different sample types. In plasma samples, CXCL-14 levels were notably elevated in patients with proliferative diabetic retinopathy (PDR) compared to those with non-proliferative diabetic retinopathy (NPDR), individuals with diabetes mellitus (DM), and healthy

Figure 1: CXCL-14 levels were higher in PDR samples

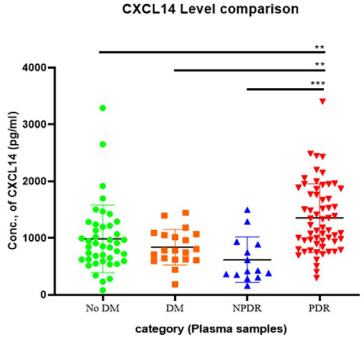


Figure 2: CXCL-14 levels was less in PDR samples

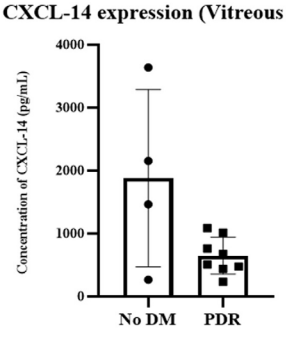


Figure:3

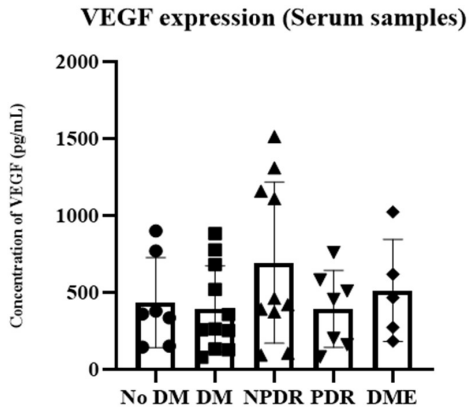
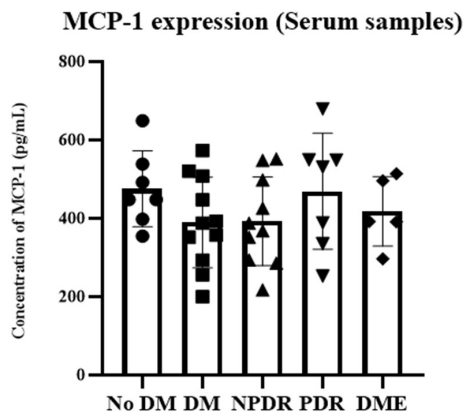


Figure:4



controls. Conversely, in vitreous samples, CXCL-14 concentrations were found to be higher in healthy controls than in PDR patients. These findings shed light on the differential expression of CXCL-14 in various biological samples across different stages of diabetic retinopathy. Further validation with larger sample sizes and diverse molecular assays is warranted.

Investigating the protein expression of angiogenic factor VEGF and MCP-1 across serum samples from various patient categories revealed distinct findings. VEGF exhibited elevated expression levels in NPDR and DME (Diabetic Macular Edema) patients (Figure 3), while MCP-1 displayed reduced expression across all diseased samples compared to control samples (Figure 4). Further validation in larger sample sizes is essential to strengthen the reliability of our findings.

Additional research is required to elucidate the involvement of CXCL14 in PDR, including its interactions in processes such as proliferation, infiltration, and immune responses. These investigations aim to provide insights into disease progression, diagnosis, and treatment strategies.

Clinical Validation of Novel Diagnostic Approaches for Improved Detection of Endophthalmitis

Investigator : Dr. Siddharth Narendran,
Dr. Venkatesh Prajna,
Dr. Lalitha Prajna
Funding agency : Velux Stiftung Foundation
Research Scholar : D. Hanithraj

Introduction including background:

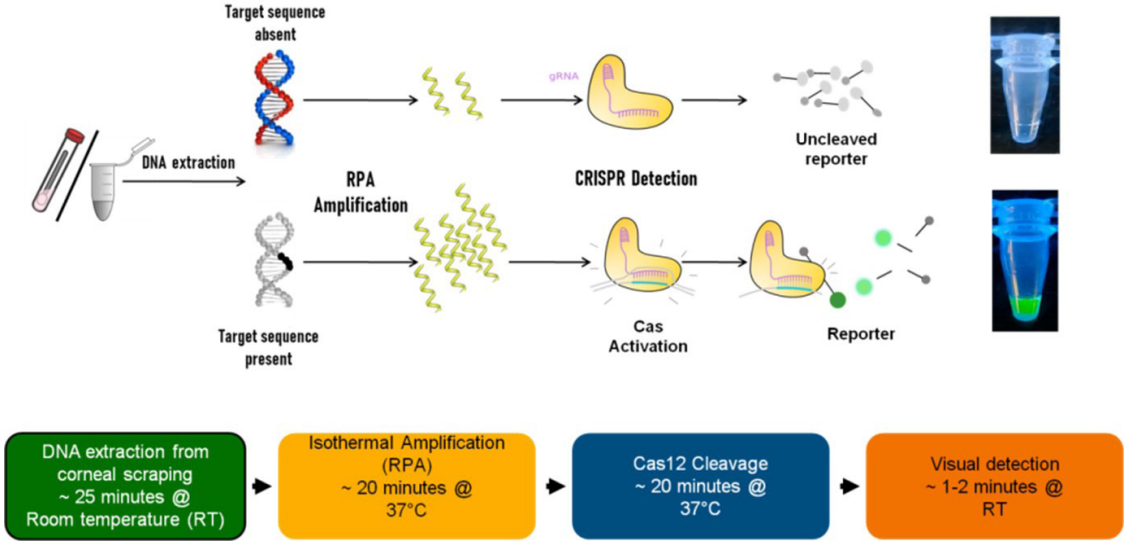
Endophthalmitis is a severe and potentially sight-threatening infection that affects the interior structures of the eye, including the aqueous and vitreous humor, retina, and surrounding tissues. It can occur as a result of various sources, including post-operative complications after intraocular surgery, penetrating ocular trauma, or secondary to systemic infections. Endophthalmitis is a potentially blinding intraocular infection that poses significant challenges in diagnosis, treatment, and prevention. The fungal endophthalmitis cases were categorized into 46.9% postoperative cases (predominantly after cataract surgery), 35.6% traumatic cases, and 17.5% endogenous cases.

The fungal species identified included 39.0% Aspergillus, with a higher prevalence observed in the central, east, and south zones. Candida accounted for 15.1% of cases and showed a higher prevalence in the west zone. Fusarium accounted for 15.9% of cases, with a higher prevalence in the north and west zones. Diagnosis of endophthalmitis relies on a combination of clinical examination, microbiological cultures, molecular techniques, and imaging modalities. However, these approaches have inherent limitations. Clinical examination alone may lack sensitivity and specificity, leading to diagnostic uncertainty. Microbiological cultures are time-consuming and may yield negative results due to prior antibiotic use or low microbial load. Molecular techniques, such as polymerase chain reaction, offer improved sensitivity but are costly and require specialized equipment and expertise. Imaging modalities provide valuable information, but their availability and interpretation can be challenging, particularly in resource-limited settings. Despite advancements in therapy, delays in diagnosis and inadequate penetration of drugs into the ocular tissues remain challenges. Preventing this condition entirely remains elusive due to the intrinsic risk associated with intraocular procedures and the

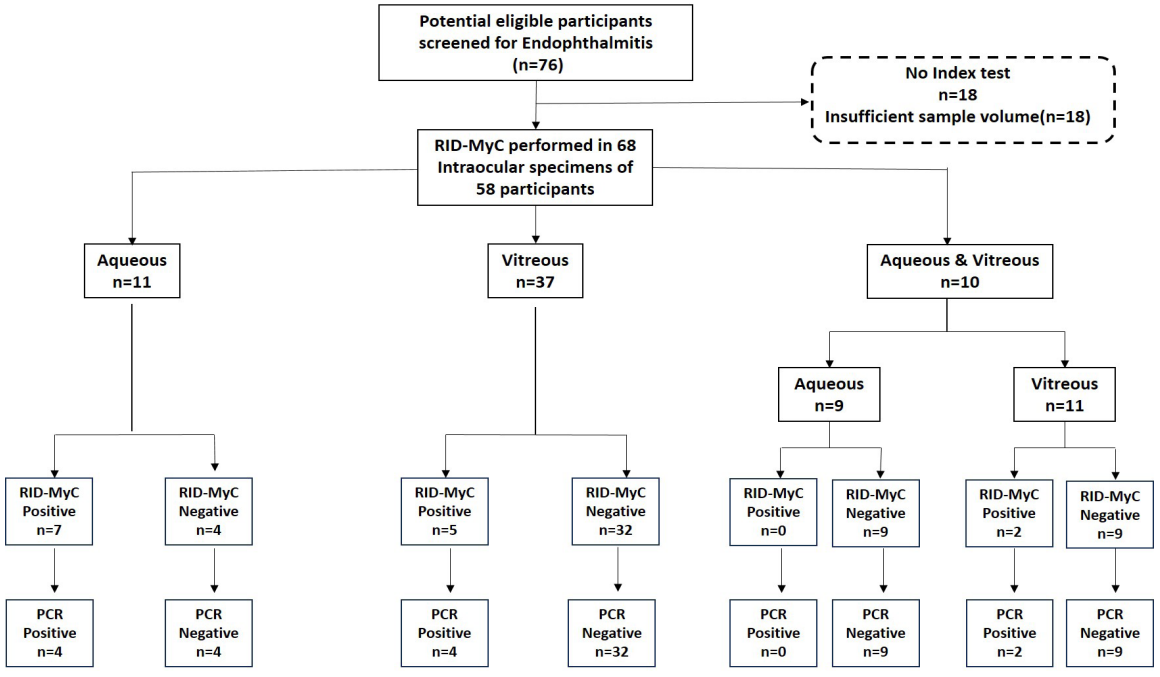
diverse nature of causative microorganisms. While significant progress has been made in understanding and managing endophthalmitis, several limitations persist. Improvements are needed in diagnostic accuracy, including the development of rapid, sensitive, and cost-effective point of care diagnostic tools. Microbial Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (CRISPR-Cas) adaptive immune systems contain programmable endonucleases with distinctive enzymatic properties that can be leveraged for the detection of microbial nucleic acids. Recent studies have highlighted the potential of these CRISPR-based nucleic acid detection methods as rapid and highly sensitive diagnostic modalities to detect pathogenic bacteria and viruses. However, the utility of these CRISPR-based diagnostic methods to diagnose fungal infections and their role as a potential diagnostic platform for ophthalmic infections remains to be elucidated. Here, we describe the development of a rapid, ultrasensitive easy-to-implement CRISPR–Cas12a-based tool, Rapid Identification of Mycoses using CRISPR (RID-MyC), for the detection of fungal nucleic acids. We have also validated our method using contrived reference samples and clinical samples from patients with suspected microbial endophthalmitis.

Results and Conclusion

Figure 1 illustrates the workflow of the RID-MyC assay for clinical specimens. The RID-MyC diagnostic platform combines Recombinase Polymerase Amplification (RPA) and CRISPR/Cas12a detection. Eighty-Six intra ocular fluids such as vitreous and aqueous from 76 consecutive patients with presumed microbial endophthalmitis presenting to our tertiary eye care facility was collected. Among the 76, 18 were excluded due insufficient volume. Of the 68 samples 11 aqueous 37 vitreous and 10 both aqueous and vitreous. Between RID-MyC and reference standard (Microscopy and Culture), 64 concordant (10 positive and 54 negative) and 4 discordant (4 RID-MyC positive but reference standard negative) results were observed (Figure 2). This definition of positive results was used as the gold standard to calculate and compare the test performances (sensitivity, specificity, PPV and NPV) between RID-MyC with reference standard (Table 1). The sensitivity, specificity, PPV and NPV of RID-MyC were 100%, 93.10%, 71.43 % and 100% respectively.



Schematic representation of the RID-MyC assay



The flowchart illustrates the specifics of the samples employed in the study, following the assessment of inclusion and exclusion criteria, RID-MyC analysis were conducted on 68 samples. Furthermore, the outcomes of RID-MyC were compared with those obtained using the PCR technique.

Performance of the RID-MyC and PCR assay for the diagnosis of fungal endophthalmitis

Results (n=68)	*Reference Standard**		*Performance of Assay*			
	Positive	*Negative*	*%Sensitivity*	*%Specificity*	*%PPV*	*% NPV*
Positive	*10*	*4*	*100.00%*	*93.10%*	*71.43%*	*100.00%*
Negative	*0*	*54*	*(69.15% to 100.00%)*	*(83.27% to 98.09%)*	*(41.90% to 91.61%)*	*(93.40% to 100.00%)*

**Reference Standard - Panfungal PCR*
Values within brackets in performance parameters indicate 95% Confidence Interval.
Abbreviations: PCR – Polymerase Chain Reaction, RID-MyC – Rapid Identification of Mycoses using CRISPR, CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats, PPV – Positive Predictive Value, NPV – Negative Predictive Value

OCULAR PHARMACOLOGY

The main research focus of the Department of Ocular Pharmacology is to understand the molecular mechanism(s) involved in the pathogenesis of glaucoma and to develop therapeutic targets for its management including glucocorticoid (GC) induced ocular hypertension (OHT)/glaucoma. The funding support from Welcome-DBT/India Alliance enabled the research team of the department to investigate the role of microRNA in the regulation of GC signaling and to develop miRNA based therapeutics for GC-induced glaucoma. Currently, our department is engaged in developing Human Relaxin (RLX2) as a potent anti-fibrotic therapeutic agent for the management of fibrosis associated with glaucoma on TGFβ2-induced elevated IOP in an ex-vivo model using Human Organ Cultured Anterior Segment (HOCAS) system.

Role of microRNA in regulating glucocorticoid receptor signaling in steroid-induced ocular hypertension/glaucoma

Investigators Details : Dr. S. Senthilkumari, Department of Ocular Pharmacology

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Dr. D. Bharanidharan, Department of Bio-informatics
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International Collaborator: Prof. Colin Willoughby, Faculty of Life & Health Sciences, University of Ulster, Northern Ireland, UK

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- Prof. VR. Muthukkaruppan, Research-Advisor, Department of Immunology and Stem Cell Biology, AMRF, Madurai
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Funding Agency : The Wellcome Trust-DBT/India Alliance (Intermediate Fellowship 2016)

Profiling of differentially expressed miRNAs in human trabecular meshwork tissues from steroid responder and non-responder eyes using NanoString technology

Introduction

MicroRNAs (miRNAs) belong to a class of small non-coding RNAs that regulate complementary mRNA translation in eukaryotic organisms (Guo et

al., 2017). They were found to have a critical role in human aqueous humor production, absorption, maintenance of TM homeostasis, regulation of retinal ganglion cells apoptosis and glaucoma (Hindle et al., 2019; Guo et al.,2017; Lu et al.,2018). It is known to be one of the epigenetic drivers in glaucoma which strongly suggests their involvement in the occurrence and development of glaucoma by regulating several biological processes of glaucoma-associated genes (Martinez and Peplow, 2022). Several miRNAs were identified in glaucoma affected tissues/fluids such as aqueous humor, tears, TM and retina of patients with glaucoma and animal models (Lu et al.,2018; Tanaka et a.,2014; Jayaram et a.,2017;Hubens et al.,2021; Kosior-Jarecka et al.,2021). However, the miRNAs that govern the differential steroid responsiveness is poorly studied.

In our previous study, we have identified an unique miRNA signature between GC-responder and non-responder HTM cultured cells using small RNA sequencing. In addition, we have identified several molecular regulators for steroid non-responsiveness using integrated analysis of miRNA-mRNA pair. However, the miRNAs pertained to differential steroid responsiveness in native TM tissue is not known. Therefore in the present study, the profiling of differentially expressed miRNAs in HTM tissues from GC-R and GC-NR eyes using nanostring technology was investigated.

For NanoString miRNA expression assay, total RNA from FFPE blocks after HOCAS experiments was isolated using Trizol reagent as per manufacturers' instructions with some modifications. TM tissues from each GC-R (n=4), GC-NR (n=5) and vehicle control (n=2) were subjected to RNA extraction after DEX or 0.1% EtOH treatment for 7 days. The miRNA quantity was measured using Qubit miRNA assay kit (Invitrogen) and the quality was assessed on Agilent 2100 bio analyzer. 100ng RNA was used to measure the expression of 800 pre-selected human miRNAs using the nCounter Human v3 miRNA expression assay kit from NanoString Technologies (Seattle, WA, USA), as per the manufacturer's instructions. After specific sample preparation and overnight hybridization, digital readouts of the relative miRNA abundance were obtained, translating to miRNA expression. The assay was outsourced to a commercial service provider, Theracues Innovations Pvt Ltd, Bangalore, India.

Differentially Expressed MiRNAs

The total number of DEMIRs identified in group #1 and group #2 were 246 and 68 respectively. There were 182 DEMIRs were found to be common (Group#3) between Group 1 and 2. The unique DEMIRs identified in GC-responder and non-responder eyes were found to be 64 (Group #4) and 4 (Group #5) respectively . The list of DEMIRS identified in group #3-#5 are represented in Table 1.

Top10 DEMIRs in Group #3

miRNA	Group #1(R vs Vehicle)		Group #2 (NR vs Vehicle)	
	Log FC	p-Value	Log FC	p-Value
hsa-miR-4531	3.60	0.00	3.23	0.07
hsa-miR-548m	3.58	0.00	2.02	0.26
hsa-miR-4443	3.60	0.00	3.54	0.02
hsa-miR-518b	3.57	0.01	3.48	0.05
hsa-miR-92a-3p	4.02	0.01	2.40	0.22
hsa-miR-499b-5p	3.01	0.01	2.58	0.23
hsa-miR-485-3p	3.29	0.01	3.07	0.10
hsa-miR-376c-5p	2.87	0.01	2.62	0.19
hsa-miR-128-1-5p	3.87	0.01	3.51	0.04
hsa-miR-219a-5p	3.07	0.01	3.04	0.13

Top10 DEMIRs in Group #4

miRNA	R vs Vehicle	
	Log FC	p-Value
hsa-miR-3161	3.10	0.00
hsa-miR-487b-3p	3.23	0.00
hsa-miR-671-3p	3.02	0.00
hsa-miR-576-5p	3.05	0.00
hsa-miR-4755-5p	3.24	0.00
hsa-miR-206	3.34	0.00
hsa-miR-141-3p	2.98	0.00
hsa-miR-3131	3.16	0.00
hsa-miR-3158-3p	4.07	0.01
hsa-miR-1287-3p	3.18	0.01

DEMIRs in Group #5

miRNA	NR vs Vehicle	
	Log FC	p-Value
hsa-miR-151a-3p	2.10	0.25
hsa-miR-548n	2.16	0.38
hsa-miR-573	2.60	0.24
hsa-miR-516a-3p+hsa-miR-516b-3p	2.64	0.22

Table 1 shows the top 10 DEMIRs in Group#3 - #4 (ranked by the smallest P-value) and the DEMIRs in Group#5 with their LogFC>2 or <-2 and p value <0.5.

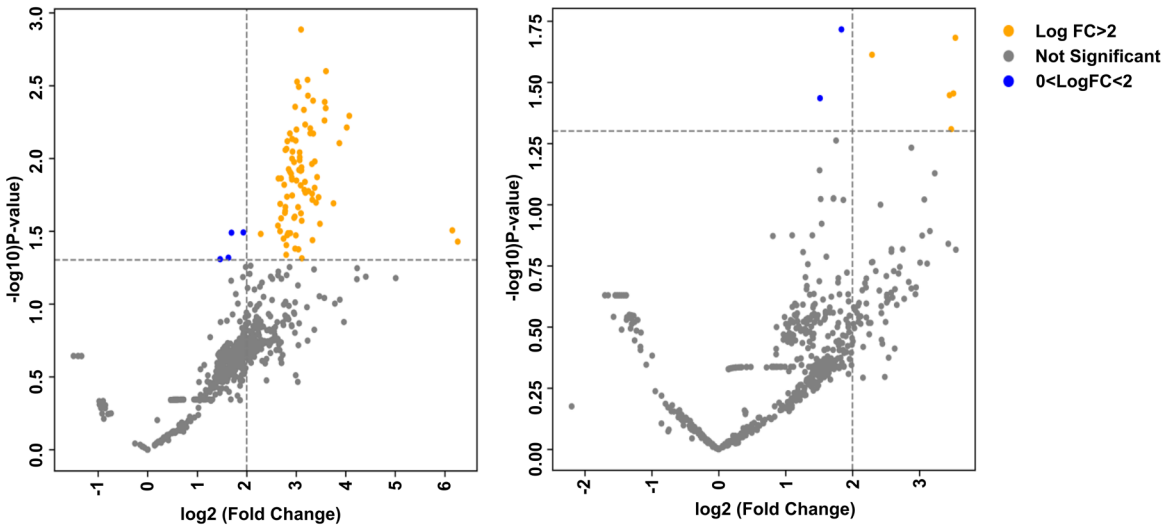


Figure 1. Volcano plot showing the distribution of DEMIRs from Group#1 - #2. The log2(Fold Change) and -log 10(P-value) of the DEMIRs from Group#1 - #2 are shown in volcano plot. Significant p-value <0.05. Orange: DEMIRs with LogFC>2 and p-value <0.05. Blue: DEMIRs with 0<LogFC<2 and p-value <0.05.

Pathway Enrichment

The pathway enrichment prediction of the DEMIRs in Group#1 - #5 was performed using the DIANA-miRPath v3.0. A total of 64 significant pathways were found in Group#3 (p-value<0.05). The top 15 significant pathways in Group#3 are shown in Table 2 (based on the smallest p-value). There were 243 significant pathways found in Group#4 and 7 significant pathways found in Group#5 (Table 2).

Top 15 predicted KEGG pathways in Group #3

KEGG pathway	p-value	#genes	#miRNAs
TGF-beta signaling pathway	0	53	12
Long-term potentiation	0	42	14
Axon guidance	0	69	16
Wnt signaling pathway	0	88	17
Endometrial cancer	0	33	18
Focal adhesion	0	119	19
Pathways in cancer	0	165	19
ErbB signaling pathway	0	55	20
mTOR signaling pathway	0	34	20
Transcriptional misregulation in cancer	0	95	20
Prostate cancer	0	55	21
Ubiquitin mediated proteolysis	0	87	24
PI3K-Akt signaling pathway	0	183	24
Neurotrophin signaling pathway	0	79	25
Glioma	6.66E-16	38	17

Top15 predicted KEGG pathways in Group #4

KEGG pathway	p-value	#genes	#miRNAs
Systemic lupus erythematosus	1.19E-56	28	4
Alcoholism	1.22E-50	48	6
Glycosaminoglycan biosynthesis - chondroitin sulfate	3.34E-40	9	17
Prion diseases	5.96E-40	10	8
ECM-receptor interaction	2.83E-35	36	20
Fatty acid biosynthesis	8.42E-35	2	5
Drug metabolism - cytochrome P450	2.27E-33	3	6
Wnt signaling pathway	2.37E-20	110	65
TGF-beta signaling pathway	1.33E-16	69	59
Ubiquitin mediated proteolysis	1.33E-16	98	52
Biosynthesis of unsaturated fatty acids	4.86E-14	9	4
Circadian rhythm	2.27E-13	24	24
Pathways in cancer	8.92E-13	220	53
Glycosaminoglycan biosynthesis - heparan sulfate / heparin	2.25E-12	17	25
Metabolism of xenobiotics by cytochrome P450	4.95E-12	5	3

Predicted KEGG pathways in Group #5

KEGG pathway	p-value	#genes	#miRNAs
Prion diseases	4.77E-18	6	2
ErbB signaling pathway	0.00633087	20	4
Proteoglycans in cancer	0.02411967	35	4
TGF-beta signaling pathway	0.044330992	15	2
Adherens junction	0.044330992	17	3
Glutamatergic synapse	0.044330992	21	3
Long-term depression	0.044330992	14	3

Table 2 shows predicted KEGG pathways in Group#3 - #5 with their p-value, the numbers of involved DEMIRs (#miRNAs) and target mRNAs (#genes). P-value<0.05 was considered to be statistically significant.

Prediction of DEMIRs target genes

A) Prediction of DEMIRs target genes in silico analysis- 'Target MRNA List 1'

As the miRNA function by regulating the gene expression, the prediction of miRNA target genes is essential for evaluating the miRNA functions. The prediction of DEMIRs target genes was performed using the IPA software. We found 6952 target genes (359 genes were experimentally validated) in Group #3, 14270 target genes (1369 genes were experimentally validated) in Group #4, and 291 target genes (2 genes were experimentally validated) in Group #5.

B) Prediction of DEMIRs target genes that negatively correlated with DEMIRs in experimental analysis – 'Target MRNA List 2'

To further analyse the DEMIRs target genes that negatively correlated with DEMIRs, we combined the Nanostring miRNA data with our previously generated mRNA library. The DEMIRs target genes that negatively correlated with DEMIRs were analysed using the IPA software. We found 13 mRNAs paired with 18 DEMIRs in Group #3, 324 mRNAs paired with 125 DEMIRs in Group #4, and 9 mRNAs paired with 2 DEMIRs in Group #. The interaction networks of the DEMIRs from Group #3, #4, and #5 show in the Figure 1a-1c.

Comparison of the DEMIRs from the miRNA-seq and Nanostring

The DEMIRs from the Group #1-#5 in the Nanostring were compared with the Group #1-#5 from the miRNA-seq of the cultured TM cells with/without GC treatment that we published before. Briefly, both hsa-miR-675-5p and hsa-miR-483-5p exhibited increased levels in the TM tissue and cultured TM cells that responded to GC compared to untreated controls in Group #1, as revealed by Nanostring and miRNA-seq

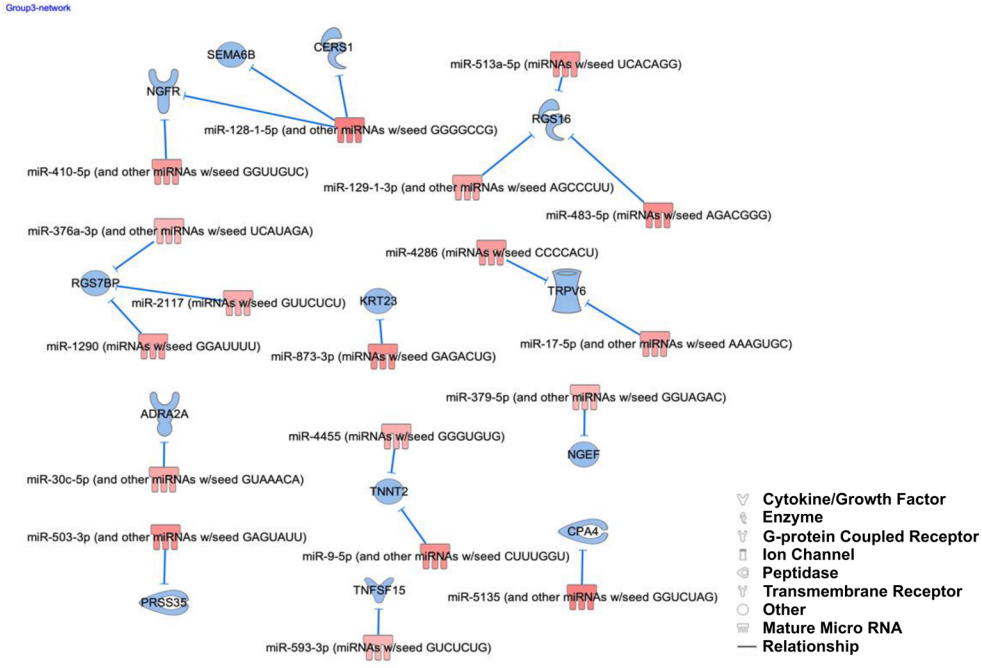


Figure 2a. Interaction networks of the DEMIRs from Group #3, (absolute LogFC>2 and P<0.5) and their negatively corelated target mRNAs (absolute FC>2 and P<0.05). Orange: upregulated miRNAs/mRNAs. Blue: downregulated miRNAs/mRNAs.

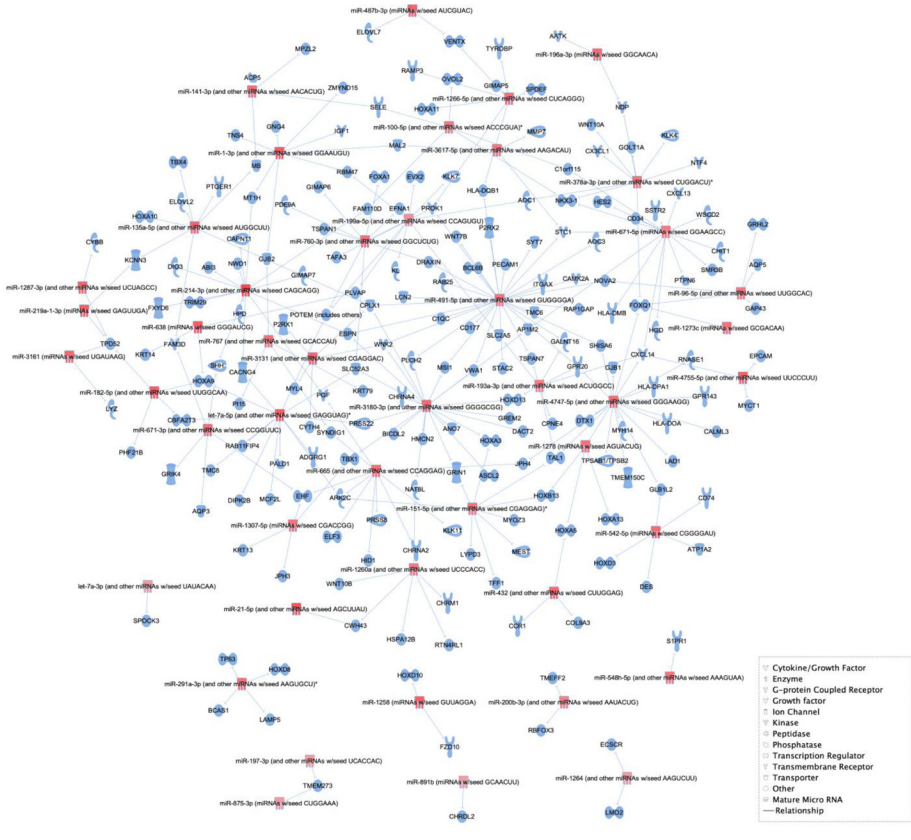


Figure 2b. Interaction networks of the DEMIRs from Group #4, (absolute LogFC>2 and P<0.5) and their negatively corelated target mRNAs (absolute FC>2 and P<0.05). Orange: upregulated miRNAs/mRNAs. Blue: downregulated miRNAs/mRNAs.

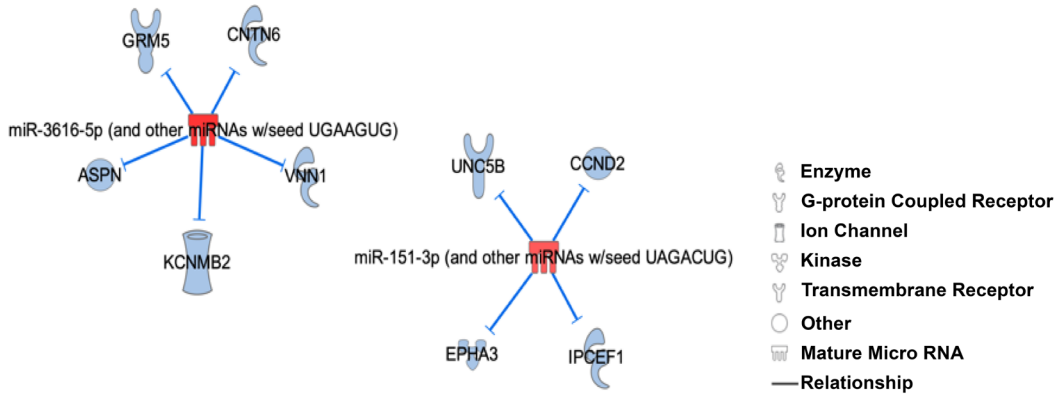


Figure 2c. Interaction networks of the DEMIRs from Group #5, (absolute LogFC>2 and P<0.5) and their negatively corelated target mRNAs (absolute FC>2 and P<0.05). Orange: upregulated miRNAs/mRNAs. Blue: downregulated miRNAs/mRNAs.

analyses. In Group #2, hsa-miR-1246, hsa-miR-3195, hsa-miR-483-5p, and hsa-miR-675-5p were identified as DEMIRs that increased in the TM tissue and cells that did not respond to GC. In Group #3, both hsa-miR-675-5p and hsa-miR-483-5p exhibited increased expression in both GC-responded and GC-non-responded TM tissue and cells, based on two datasets. No overlapping DEMIRs were identified in Group #4 and #5 through Nanostring and miRNA-seq analyses.

Evaluation of IOP lowering property and Anti-fibrotic property of Relaxin on TGFβ2-induced Elevated IOP Ex vivo model of glaucoma using Human Organ Cultured Anterior Segment (HOCAS)

Investigators : Dr. S. Senthilkumari
Co-Investigators : Dr. R. Sharmila, Glaucoma Clinic, Aravind Eye Hospital, Madurai
Junior Research Fellow: Suman
Funding Agency : Science & Engineering Research Board - Core Research Grant [2023-26]

Introduction

Relaxin (RXL2) is a heterodimeric peptide hormone with known vasodilatory and anti-fibrotic activity (Chen et a., 2020). This hormone was first identified by Frederick Hisaw in guinea pig model of pregnancy and parturition (Fevold Hisaw & Meyer, 1920; Hisaw, 1926). RXL2 hormone was found to loosen pelvic ligaments to facilitate parturition by reducing the density of collagen bundles and relaxing the collagen fibers (Wikinson et al., 2005; Chen et al., 2020). However, the IOP lowering property of relaxin was not completely understood. Very recently the Relaxin receptor Relaxin/Insulin-LikeFamily Peptide Receptor 1 (RXFP1) expression was demonstrated in sections of the anterior segment of the eye and found it in inner uveal, corneoscleral and cribriform meshwork and Schlemm's canal endothelium (Zloto et al.,2020) where as in another study relaxin gene knockout (Rln-/-) mice showed higher IOP, thicker corneas, larger endothelial cells, and lower endothelial cell density (Hampel et al.,2020) and it is also found RXFP1 mRNA expression in the retina, cornea, sclera, and choroid, while RXFP2 was only in the cornea.

Considering the distribution of relaxin and its receptors, it is hypothesized that relaxin may have an active role in the regulation of outflow facility and IOP in human eye. Therefore, in the present study it is proposed to study the IOP lowering property of relaxin (serelaxin, recombinant relaxin) on TGFβ2 induced elevated IOP ex vivo model of glaucoma using HOCAS and also to study the mechanism by which relaxin mediates anti-fibrotic activity in fibrosis induced by TGFβ2.

Expression of RXL2 and RXFP1 in Human TM Tissue by Immunofluorescence Analysis

As a first step towards the above objective, the expression of RXL2 and its receptor RXFP1 in TM was investigated in non-glaucomatous and glaucomatous donor eyes. The characteristics of donor eyes used for this study is given in Table.1.

The glaucomatous and non-glaucomatous donor eyeballs were collected for this study and the anterior segment was dissected and fixed with 4% Paraformaldehyde. These fixed tissues were dissected into four equal quadrants, dehydrated and embedded into paraffin blocks. The tissues were sectioned by microtome (Leica Biosystems) into 5µm thin sections onto poly-L-Lysine coated slides and rehydrated with descending concentrations of alcohol. The blocking of free antigenic sites was performed

Table1: Characteristics of Human Donor Eyes used for this study

S.No	RA No.	Age	Sex	Cause Of Death	Date and Time of Death	Experimental Eye
Non-Glaucomatous Human Donor Eyes						
1	1485	45	M	RTA	18.12.2023 at 01.15 pm	OD
						OS
2	1521	70	M	CVA	24.12.2023 at 06.00 am	OD
						OS
3	1523	72	M	CVA	23.12.2023 at 09.45 pm	OS
4	1526	65	F	Heart Disease	24.12.2023 at 08.30 pm	OD
5	94	80	M	CVA	13.01.2024 at 10.35 am	OD
Glaucomatous Eyes						
1	718	88	M	Respiratory Disorder	02.09.2016 at 12.05 am	OS
						OD
2	525	88	M	Respiratory Disorder	2016	OD
						OS
3	231	66	M	Respiratory Disorder with the history of Diabetes	04/03/2015 at 06.10 am	OD
4	151	75	F	Heart Attack	09/02/2016 at 09.15 pm	OS

with the help of Avidin-Biotin kit (Invitrogen) for 20 minutes each and 5% bovine serum albumin for 30 minutess to prevent non-specific binding. The sections were incubated overnight at 4ºC with rabbit recombinant monoclonal anti-Relaxin 2 primary antibody (1:500 dilution; Abcam) and rabbit polyclonal anti-RXFP1 primary antibody (1:100 dilution; Invitrogen) separately to allow binding to target proteins. The following day tissues sections were incubated with goat anti-rabbit IgG-B secondary antibody (1:500 dilution; Santa Cruz Biotechnology) for 2 hours. Then, the tissue sections were incubated with Streptavidin, DylightTM 594 (1:500 dilution; Vector Laboratories) for 1 hour. Later, all the tissue sections were fixed with Vectashield® Antifade mounting medium with DAPI (Vector Laboratories). Images were acquired using confocal microscopy.

Confocal image analysis revealed that the expression of RXFP1 and RXL2 were found to be higher in non-glaucomatous/normal TM tissues as compared to glaucomatous TM tissues. Further investigation in this line is in progress.

Having investigated the expression of RXL2 and RXFP1 receptor in the human anterior segment of the eyes from both non-glaucomatous and glaucomatous, the TGFβ2-induced elevated IOP model using HOCAS is in progress.

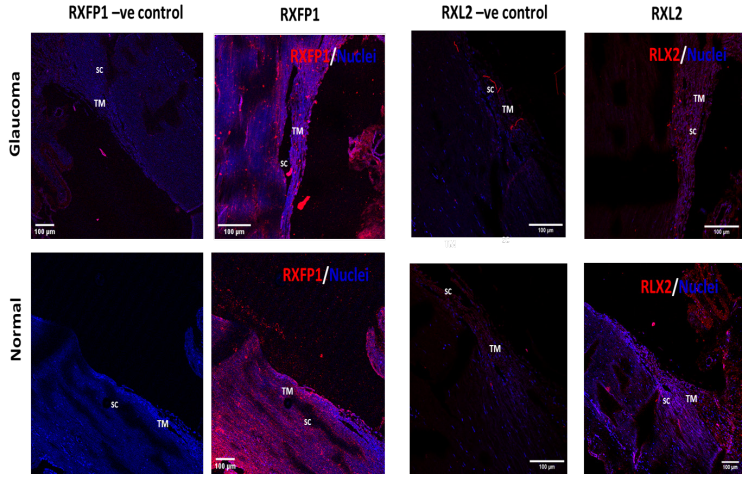


Figure 1: Confocal Images showing the expression of RXFP1 and RXL2 in both normal and glaucomatous TM tissues

BIOINFORMATICS

The primary goal is to develop and provide bioinformatics methods to analyze the omics data generated by next-generation sequencing methods. This includes i) developing data analysis pipelines for whole genome/ exome genome data analysis to identify eye-disease-specific pathogenic variants ii) transcriptomic analysis to detect the altered gene expression and transcripts and its role in the disease pathogenesis, iii) ocular microbiome analysis in fungal keratitis

A computational framework for the identification of eye-disease pathogenic variants from whole Exome/Genome sequencing : eyeVarP

Investigator : Dr. D. Bharanidharan
Research Scholar : K. Manojkumar

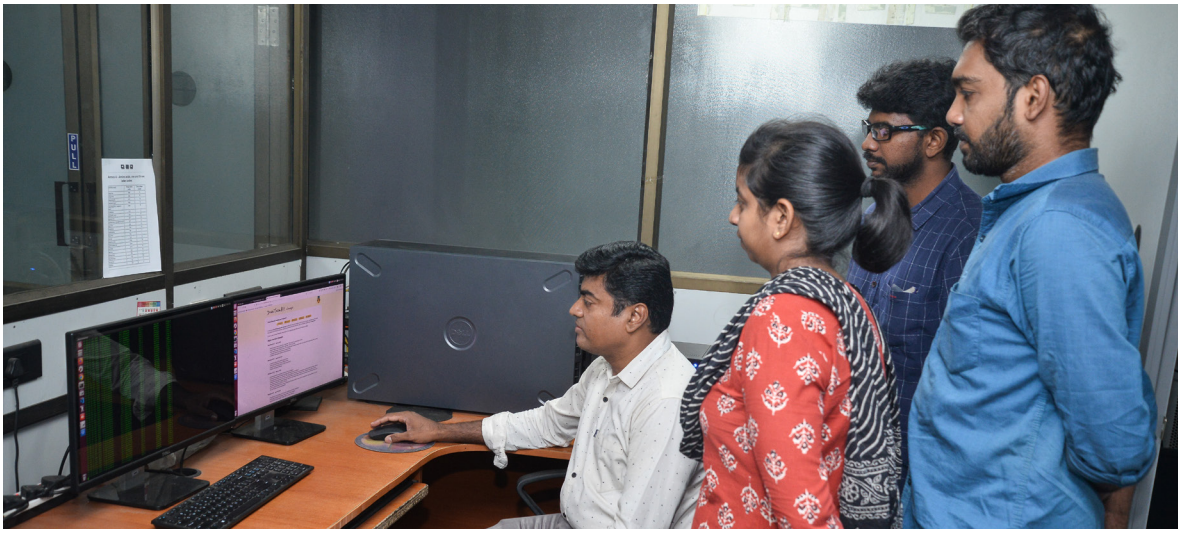
Background

Identifying pathogenic variants in eye genomic research is challenging. disease or novel genes. To this end, we developed eyeVarP, 1. a computational framework for the identification of eye-disease specific pathogenic variants from whole Exome/ Genome sequencing by using VarP and eyeVarP machine learning tools. This framework is automated to analyze the raw data to final prioritization of pathogenic variants including single nucleotide variants (SNVs) and small insertion and deletion (InDels) with interpretable pathogenicity score. Here, we provide the recent updates and power of eyeVarP and its utility in the identification of pathogenic variants specific to eye disorder.

Results and conclusions

We updated the eyeVarP pipeline with recent Annovar database and made available at github online link: https://github.com/bharani-lab/WES-pipelines/. The VarP tool in the eyeVarP pipeline also updated with newly trained machine learning model to filter and predict the pathogenicity of both SNVs and InDels.

To host the eyeVarP at AMRF and made it available online, in-house automated graphical user interface was developed using, the following technologies and programming libraries. HTML, CSS, Bootstrap, and VueJS: This JavaScript library was used to develop the front-end part of the tools, which provide create interactive charts and plots for visualizing the prioritized pathogenic variants. Mongo DB was used for computational analyses and data storage. All the backend data was connected using docker for running the entire pipeline. The final fully automated pipeline and the server will be hosted in any cloud server and the scripts will be made public on the GitHub repository soon.



NGS ID	Genes	Mutations	Types of mutation	Mutation ID	VarP	eyeVarP	SDM (ΔΔG)
NGS123	ERBB4	c.C271T/ p.L91F	Missense	COSV105027066	46	0.98	-1.69
NGS131	ERBB4	c.C3836A/ p.A1279E	Missense	Not reported	52	1	-1.84
NGS164	EGFR	c.A1196T/ p.K399M	Missense	Not reported	41	1	1.22

Utility of eyeVarP on retinoblastoma (RB) tumor samples:

RB tumor is mostly initiated by biallelic inactivation of RB1 gene. Here, we applied eyeVarP to detect other gene mutations in RB patient's tumor samples who were negative for RB1 gene mutations. eyeVarP clearly prioritized three mutations with high score in three RB patients as shown in the Table 1.

In this update, we have demonstrated that the eyeVarP method correctly predicts the eye-cancer-specific pathogenic variants. eyeVarP is consistently faster than other tools. eyeVarP is the only dockerized tool that can run on all three platforms (Windows, Linux, and Mac).

2. Identification and Analysis of Alternative Transcripts in Retinoblastoma Progression.

Investigators : Dr. D. Bharanidharan.
Funding : ICMR-SRF
Research Scholar : Mohamed Hameed Aslam A.

Background

Retinoblastoma (RB) tumor progression, besides RB1 inactivation, requires several factors including genomic instability, copy number variation, altered gene expression, and alternative splicing. However, alternative splicing events role in RB progression using transcriptomic data is not studies. Thus, we aim to elucidate the role of alternative transcripts in RB progression. Building upon our previous work, where transcriptome data of RB was analyzed through meta-transcriptome analysis, we developed a robust bioinformatics pipeline for analyzing alternative transcripts and differential gene expression. Through network analysis, we explored the correlation between differential gene expression (DGE) and alternative splicing events (DAS), revealing the activation of cell-proliferative pathways associated with drug response and patient survival. Functional networks constructed from enriched pathways highlighted the upregulation of genes linked to tumour progression. Notably, fatty acid metabolism pathways showed predominantly downregulated alternative splicing events, particularly focusing on ENO2, a glycolysis enzyme implicated in cancer progression. Protein-protein interaction analysis suggested a potential role for ENO2 alternative splicing in RB progression, through its interaction with insulin growth factors. Further, we have explored hub genes which will allow researchers to prioritize candidates for subsequent experimental validation and provides valuable insights into the underlying molecular mechanisms that govern complex traits of RB.

Figure : A

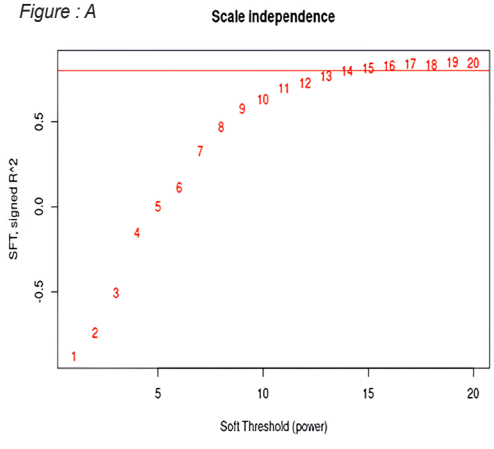


Figure: B

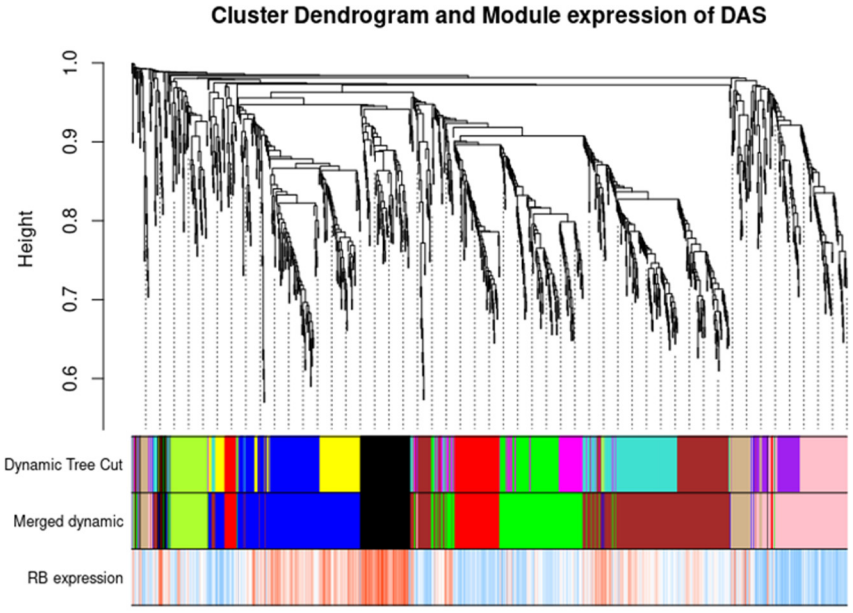


Figure 2.1: The Construction of the WGCNA co-expression network using Differential Alternative Splicing (DAS) genes. A) The scale-free fitting index and average connectivity of DAS genes are presented across different soft thresholding powers (β), with the red line indicating the correlation coefficient. B) Hierarchical cluster analysis is depicted, revealing the co-expression modules identified by WGCNA.

Results and Conclusions

We used the WGCNA (Weighted Gene Co-expression Network Analysis) method to identify hub genes from co-expression patterns among differential alternative splicing or transcript genes across the control retina and RB tumour.

We determined the soft threshold power as 19 to establish a scale-free network, based on the square of the correlation coefficient and mean connectivity (Figure 2.1A). The co-expression gene network was built using Pearson correlation among DAS genes. Subsequently, co-expressed DAS genes were clustered into modules using the dynamic tree-cut method. Modules sharing high similarities were merged, resulting in the identification of nine distinct gene modules. RB tumour expression served as the weight for our analysis (Figure 2.1B). Among modules, we selected the black module, which exhibited a high correlation with RB tumour expression. We then identified 42 candidate genes with high significance related to RB tumour within the black module. We conducted a Venn analysis on the candidate genes from both the black module identified through WGCNA and the Differentially Expressed Genes (DEG). This analysis revealed 10 common genes: CCNB1, CENPV, HMGB2, JPT1, LLR1, NASP, NT5DC2, RACGAP1, RXRG, and STMN1, which were designated as hub genes (Figure 2.2A).

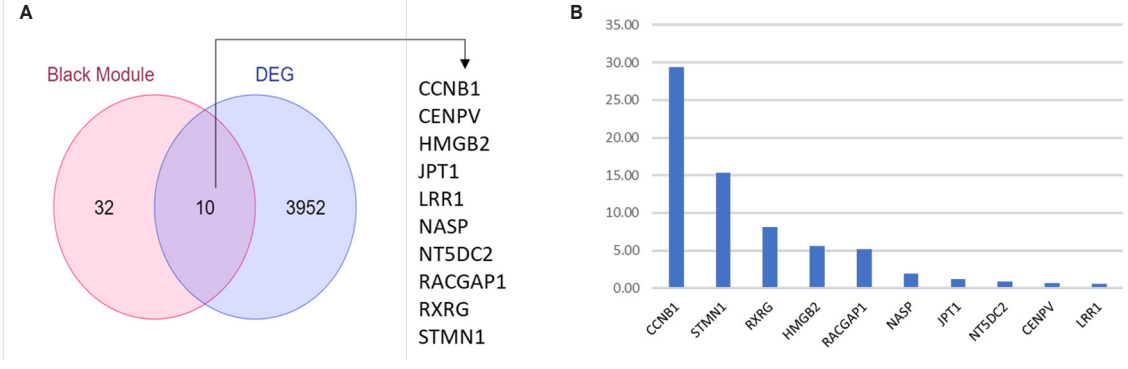


Figure 2.2 A)Venn analysis between candidate genes from the WGCNA black module and DEG. B) Correlation between hub gene and retinoblastoma.

The VarElect tool was used to analyse the correlation between hub genes and retinoblastoma and cancer phenotype (Figure 2.2B). we found that CCNB1, STMN1, RXRG, HMGB2, and RACGAP1—were associated with retinoblastoma, with CCNB1 exhibiting the highest correlation score. Aberrant splicing in CCNB1 causes tumour progression in gastric cancer but has not yet been reported in retinoblastoma. Based on our functional enrichment analysis, the hub genes HMGB2, JPT1, NASP, RACGAP1, and STMN1 are enriched in pathways related to RB progression, including the E2F target and G2M checkpoint pathways. Additionally, it's noteworthy that the hub gene NT5DC2 displays all alternative splicing events such as A3SS, A5SS, MXE, SE, and RI suggesting its potential involvement in RB progression.

Altogether, we show alternate transcripts of hub genes and their role RB progression, which warrants further experimental validation.

OCULAR MICROBIOLOGY

The main focus is the diagnosis and prognosis of ocular infections and understanding their molecular mechanisms. The lab develops new molecular methods or identify molecular biomarkers such as miRNAs for the diagnosis of no growth or unidentified organisms in addition to routine diagnosis using microbiological, biochemical and molecular methods. The department uses genomic and transcriptomic approaches, and invitro cell culture and exvivo model system to understand the pathogenesis of ocular pathogens. .

Diagnostic markers for Intra-Ocular Tuberculosis

Investigators : Dr. D. Bharanidharan,
Dr. S. R. Rathinam,
Dr. Lalitha Prajna
Research Scholar : C. Swathi
Funding : DBT, ICMR-SRF

Background

Ocular tuberculosis (OTB) can occur as a primary exogenous infection of the eye or a secondary infection as a direct extension from surrounding tissues or the patient's sputum contamination. Due to the paucibacillary nature of OTB diagnosis, TB uveitis is differentially diagnosed. Moreover, if TB uveitis is not treated early with anti-tuberculosis therapy (ATT) leads to vision loss. Thus, early OTB diagnosis is imperative for the management of the disease. MicroRNA (miRNA) expression analysis has been shown to provide them as biomarkers in several eye diseases and has a regulatory role in pathogenesis. We have identified four miRNAs and three miRNAs as potential markers in aqueous and vitreous humor respectively. To confirm their diagnostic value, we have used using receiver operating characteristic (ROC) curve analysis. we also aim to detect miRNA expression signatures in the serum of IOTB, which will help to diagnose IOTB minimally invasively.

Results and Conclusions

To assess the statistical relevance of the biomarkers, a ROC curve was constructed using ΔCt, and the AUC (Area Under the ROC Curve) was determined. The AUC values obtained for the upregulated hsa-miR-150-5p, hsa-miR-21-5p, and hsa-miR-26b-5p were significant, with AUC values of 0.715 (95% confidence interval [CI]: 0.526 to 0.904, P - 0.017), 0.789 (95% CI: 0.609 to 0.969, P - 0.013), and 0.738 (95% CI: 0.556 to 0.920, P - 0.038), respectively. However, the combination of hsa-miR-21-5p and

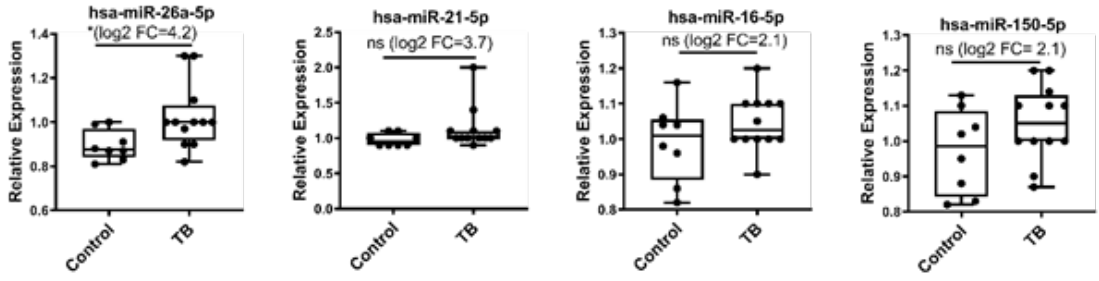


*Fig. 1. The bar graphs were plotted for miRNAs in the serum of IOTB patients. The expression levels of selected miRNAs were validated by RT-qPCR from IOTB serum patients (N=12) and noninfectious uveitis controls (N=8). Median values are shown by horizontal lines using the MannWhitney test. *P<0.05.*

hsa-miR-26b-5p showed better AUC=0.797 than individual miRNAs, suggesting a potential biomarker for IOTB.

To evaluate miRNA diagnostic value in blood, we selected potential miRNAs based on metadata analysis and ocular fluids miRNA profiling for validation in the serum of IOTB. The serum of patients (n=12) with intraocular tuberculosis (IOTB) was compared to noninfectious uveitis (n=8); the log2 FC of IOTB-specific miRNAs, hsa-miR-150-5p (log2 FC = 2.1), hsa-miR-21-5p (log2FC = 3.7), hsa-miR-16-5p (log2FC = 2.1), and hsa-miR-26a-5p (log2FC = 4.2, p - 0.01) were found to be upregulated (Figure 1).Specifically, hsa-miR-26a-5p was significantly upregulated in the serum of IOTB, which was earlier reported in active TB patients (Sinigaglia, Peta et al. 2020).This suggests that patient stratification into primary or secondary OTB is required for further serum biomarker analysis in a large cohort. However, the serum miRNA biomarker specific to primary OTB is still questionable.

2. Role of Human corneal miRNAs in the onset and severity of Fungal Keratitis

Investigators	:	Dr. D. Bharanidharan (PI), Dr. K. Dharmalingam, Dr. Venkatesh Prajna, Dr. Lalitha Prajna
Clinical Coordinator	:	Dr. Juhi Dhokne,
Research Scholar	:	Shreya Dinesh, Gayathri.M
Funding	:	ICMR
Purpose	:	To understand the role of dysregulated human corneal miRNAs in the onset and severity of Fungal keratitis

Background

Fungal keratitis, predominately due to either *Aspergillus flavus* or *Fusarium solani*, is more prevalent in India. We aim to find the human corneal miRNAs that could identify the onset and severity of disease early on since more than 40% of the patients are refractory to anti-fungal treatment. We have identified potential miRNAs based on their fold change, abundance and the functional significance in fungal keratitis pathogenesis. Further, we show pathways such as PI3/Akt signaling pathway, phagosome pathway, wnt signaling pathway involved in the pathogenesis of the disease. Also, we have performed all the small-RNA sequencing and bioinformatics analysis to identify miRNA dysregulation in response to *Aspergillus flavus* and *Fusarium sp*. separately. We have further validated these miRNAs using qPCR.

Results and Conclusion

In addition to pathways and GO analysis using DAVID and g:Profiler, we performed Gene set enrichment analysis (GSEA) and functional regulatory network was built on between miRNAs, target genes and enriched pathways. All the analysis was carried out for *Fusarium sp*. and *Aspergillus flavus* separately. As shown in Figure 2.1, the functional regulatory network for *Aspergillus flavus* Keratitis identified hsa-miR-27b-5p targeting CXCL10 and IL1B, hsa-let-7e-5p targeting TNFRSF1B, IL6 and EDN1, hsa-miR-495-3p and hsa-miR-30a-3p targeting IL1B, which are involved in TNF signaling and IL17 signaling, reported to play a role in fungal keratitis pathogenesis (Boomiraj H, et .al, 2015). hsa-miR-30a-3p targeting GREM1 is involved in TGF-B signaling, reported to have a regulatory role in corneal wound healing (Ljubimov AV et al. 2017).

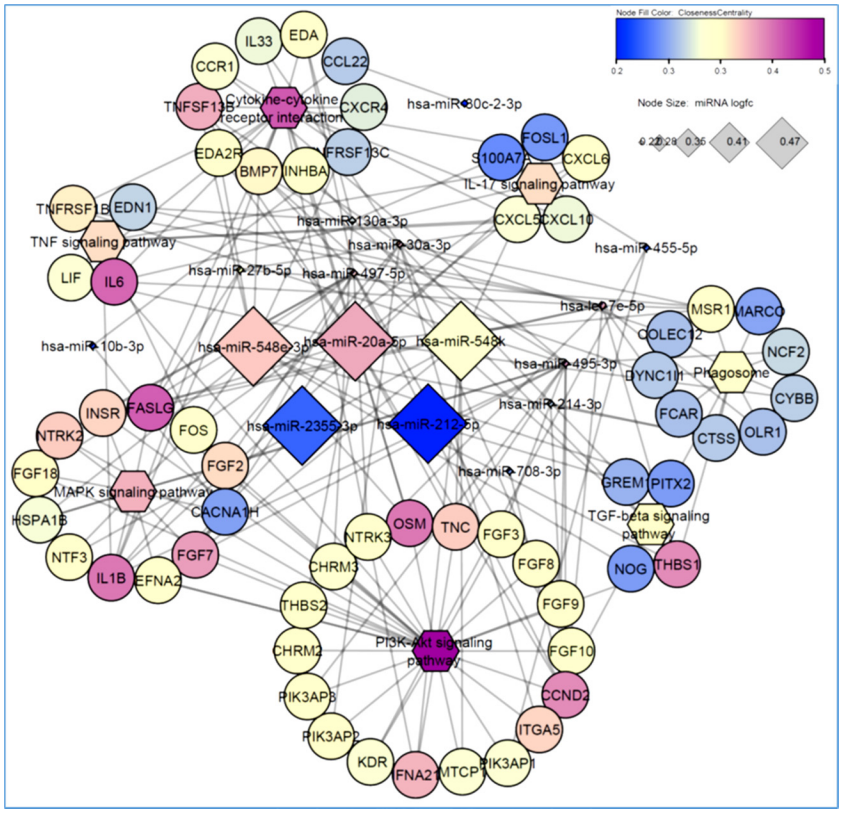


Figure 2.1. miRNA-target Functional Network involved in A. flavus Keratitis

In the case of *Fusarium sp*. Keratitis (figure 2.2), hsa-miR-16-5p, hsa-miR-19a-3p, hsa-miR-19b-3p, hsa-miR-328-3p targeting FGF7, HSPA1B, PDGFD and IL1B respectively are involved in MAPK signaling was upregulated, which has been reported to involve in the pathogenesis of fungal keratitis (Xia Hua et.al., 2017). hsa-miR-338-3p targeting THBS1, regulates p53 signaling was also upregulated and is reported to influence host inflammatory response (Wang WY, et al., 2020).

For the initial optimization and trials, qPCR validation was carried out in *Aspergillus flavus* (n=5) and *Fusarium sp*. infected keratitis samples (n=10). Foldchange and Relative expression was calculated for each miRNA in each disease group separately and the significance of expression was calculated and plotted using GraphPad prism. Furthermore, we selected targets - IL1B, CXCR4, CXCL5, CXCL8, IL6, WNT10A, IRAK3, MNDA, MMP8, and FGF7 based on functional network analysis for further qPCR validation.

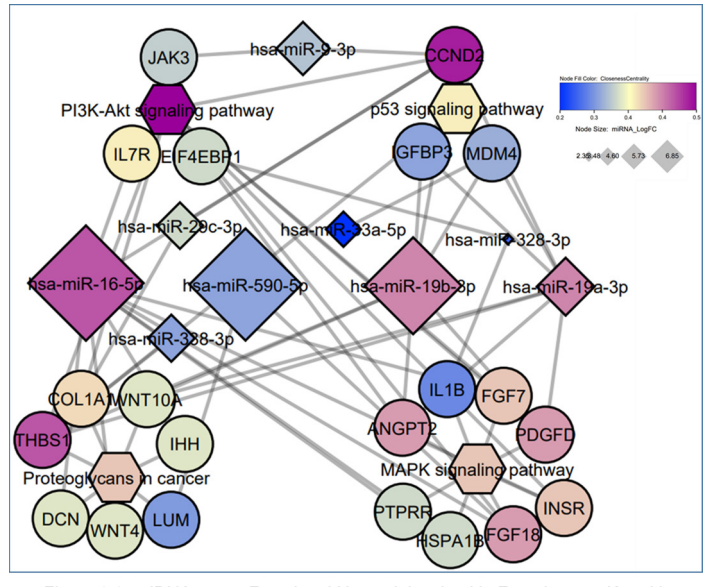


Figure 2.2. miRNA-target Functional Network involved in Fusarium sp. Keratitis qPCR Validation of Putative miRNA biomarkers

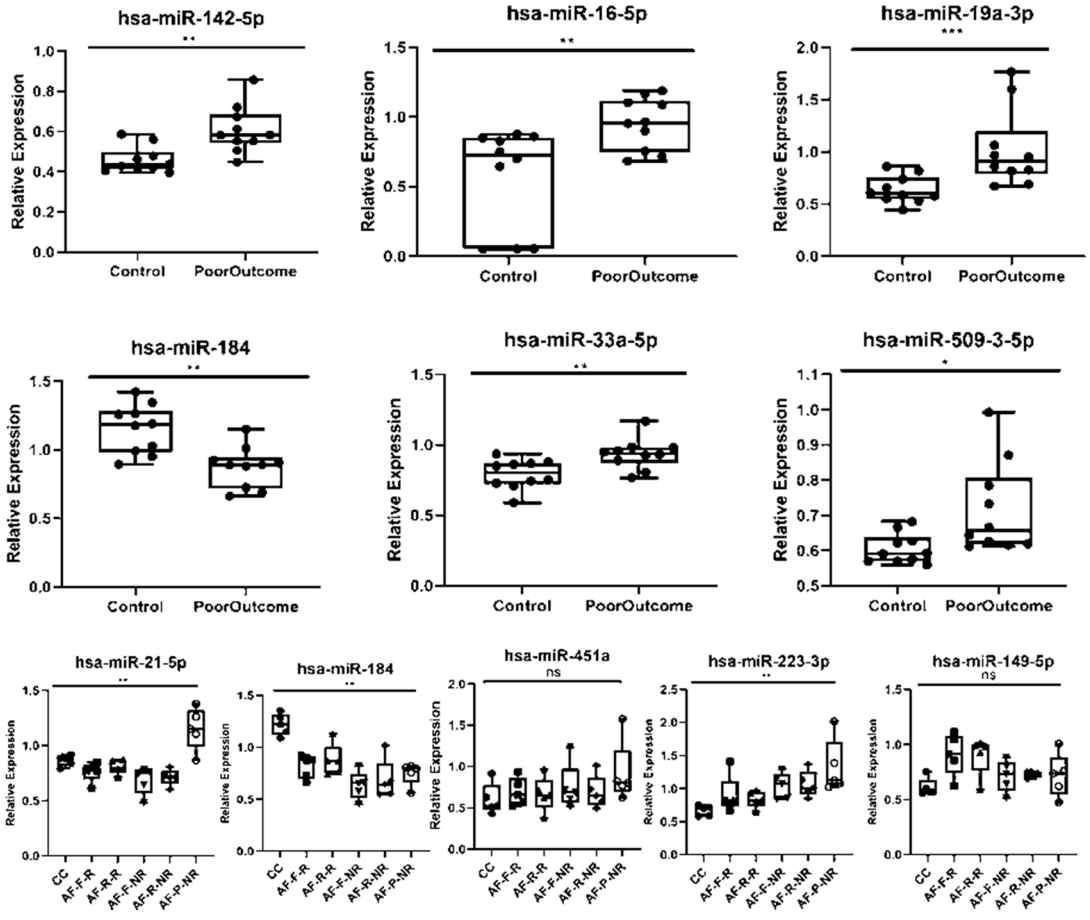


Figure 2.3: Relation expression of miRNAs that were concordant with NGS expression analysis

CC- Cadaver Cornea, FS - Fusarium sp., AF- Aspergillus flavus, F-R - At First Presentation Responder, R-R - At Review Responder. F-NR - At First Presentation Non-Responder; R-NR - At Review Non-Responder, P-NR - Poor Outcome Non-Responder

qPCR validation showed significant expression of hsa-miR-223-3p, hsa-miR-21-5p and hsa-miR-451a and showed concordant foldchange with NGS data plays important role in *Aspergillus flavus* keratitis pathogenesis as reported earlier (Boomiraj H, et .al, 2015).

The validation in small subset of *Fusarium* infected samples showed the significant upregulation of three *Fusarium* specific miRNAs hsa-miR-33a-5p, hsa-miR-16-5p and hsa-miR-19a-3p and two Keratitis specific miRNAs hsa-miR-142-5p and hsa-miR-509-3-5p, which was concordant with small-RNA sequencing data analysis. hsa-miR-184, the fungal specific miRNA, showed significant downregulation in both *Aspergillus flavus* and *Fusarium* sp. groups.

Altogether, several miRNAs are identified that have pivotal role in the pathogenesis of fungal keratitis and also specific miRNAs that are dysregulated in response to fungal species (*RB1 flavus* and *Fusarium* sp), suggesting potential biomarkers. Specifically, hsa-miR-223-3p, and hsa-miR-33a-5p, hsa-miR-16-5p and hsa-miR-19a-3p are potential biomarkers *Aspergillus flavus* and *Fusarium* sp respectively. Further miRNAs and target gene expression is being carried out in large sample size to confirm their diagnostic and prognostic value.

3. Expression profiling of human corneal miRNAs and their role in *Pseudomonas aeruginosa* keratitis

Investigators : Dr. D. Bharanidharan (PI), Dr. Venkatesh Prajna, Dr. Lalitha Prajna
Research Scholar : R. Praveenkumar
Funding : DBT-BET

In this project we aim to profile the expression of miRNAs in *Pseudomonas aeruginosa* (PA) keratitis patients and study their role in disease progression. Earlier, we had identified human corneal miRNAs comprehensive profile in PA keratitis patients using small-RNA sequencing. Further, we had identified differential expression of miRNAs in PA keratitis corneal tissues compared to cadaver controls and their implication in disease progression through bioinformatic functional analysis. Following this, we have profiled miRNAs from corneal swabs from the ulcer of patients who were prospectively known to have good outcome upon treatment. Samples are collected at presentation (before treatment) and at review (5-7 days after treatment). Subsequently, the beneficiary miRNAs that are associated with good outcome are identified and their therapeutic potential are explored.

Results and Conclusions

MiRNAs were profiled from cadaver corneas (n=3), poor outcome corneas (n=3), corneal swabs at presentation (n=4) from good outcome patients and corneal swabs at review (n=4) from good outcome patients by small-RNA NGS sequencing. Differential expression analysis was carried out between the expressed miRNA in control vs good outcome presentation using the edgeR platform with filtering criteria FDR adjusted p-value < 0.05 and Fold change > 1.5. A total of 395 miRNAs were found to be differentially regulated, out of which 212 were upregulated and 183 were downregulated (Table 1).

SNo	miRNA	logFC	log CPM	FDR
1	hsa-miR-223-3p	11.86	16.77	2.45×10^{-63}
2	hsa-miR-6821-5p	10.92	3.80	1.63×10^{-44}
3	hsa-miR-642a-5p	10.48	3.37	1.73×10^{-40}
4	hsa-miR-548e-5p	10.07	2.98	5.85×10^{-37}
5	hsa-miR-6859-5p	9.95	2.86	6.41×10^{-36}
6	hsa-miR-381-3p	-9.38	8.24	5.65×10^{-50}
7	hsa-miR-24-1-5p	-9.62	5.64	2.19×10^{-34}
8	hsa-miR-139-5p	-9.67	7.15	2.20×10^{-43}
9	hsa-miR-214-5p	-9.80	5.81	1.28×10^{-36}
10	hsa-miR-10b-5p	-12.46	10.34	2.33×10^{-65}

Table 1: List of top 10 differentially expressed miRNAs in good outcome samples compared to control

i) Beneficiary miRNAs associated with good outcome:

From the list of differentially expressed miRNAs, those associated with good outcome either upregulated or downregulated were filtered by comparing their expression with control and poor outcome. The criteria for filtering were Foldchange <-1.5 or >1.5 and CPM higher than 1000. Upon filtering, the top 13 upregulated and 15 downregulated miRNAs associated with good outcome were shortlisted as beneficiary miRNAs (Fig. 3.1a and 3.1b).

Figure 3.1 a

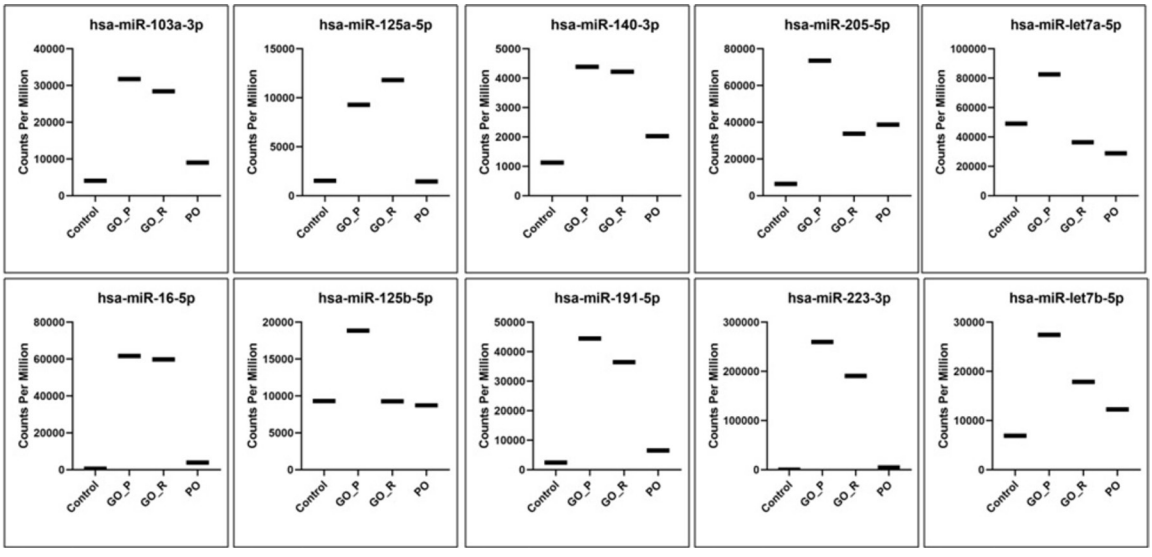


Figure 3.1 b

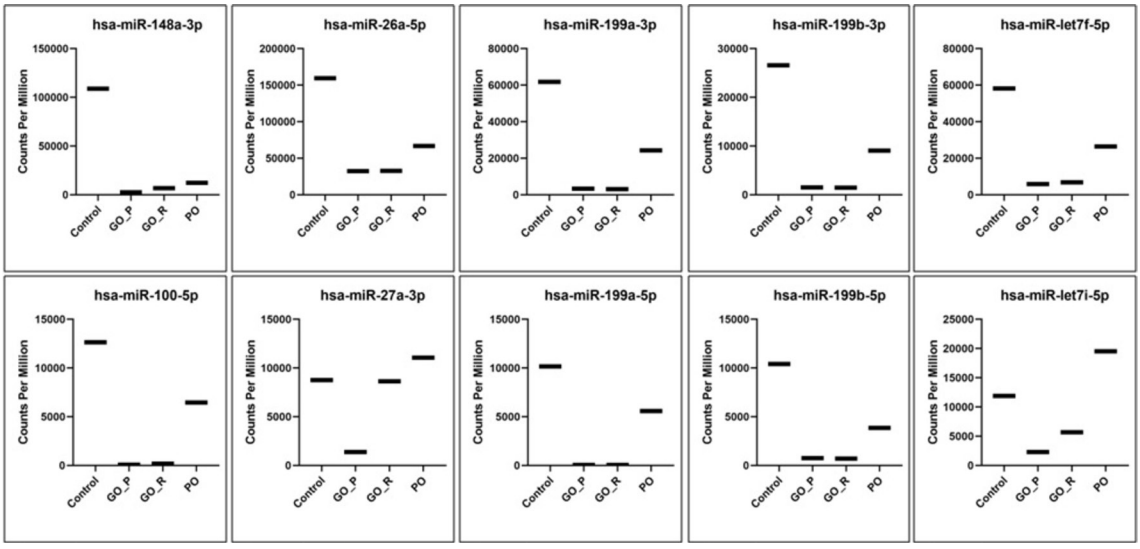


Figure 3.1 a and 3.1 b: Expression of selected miRNAs either upregulated (top) or downregulated (bottom) in samples from good outcome patients when compared to control and poor outcome (GO_P – Good outcome swab collected at presentation; GO_R – Good outcome swab collected at review; PO – Poor outcome corneal buttons)

ii) miRNA with therapeutic potential and implication in disease progression:

The expression pattern of each shortlisted miRNA was analyzed to select the potential miRNAs relevant for therapeutic potential. Notably, miR-125a-5p, miR-140-3p, miR-16-5p were consistently expressed during healing (both at presentation and review). These miRNA may play essential role in wound healing process and its noteworthy to study their potential as therapeutic molecules. Recent research has shown miR-125a-5p to have anti-inflammatory property and it can induce Treg cells differentiation (Zhan et al., 2023). Also, miR-16-5p was found to promote wound healing and re-epithelialization in skin ulcers by activating cell migration and survival through p38-MAPK pathway (Yan et al., 2020).

Similarly, among the downregulated miRNAs all the Mir-199 family namely, miR-199a-3p, miR-199a-5p, miR-199b-3p and miR-199b-5p were consistently downregulated in good outcome patients and they displayed similar expression pattern. Downregulation of Mir-199 family might play an essential role in disease progression and healing. Our finding is consistent with the previous study on miR-199 in zebra fish model of *Pseudomonas aeruginosa* lung infection; where it was shown that downregulation of miR-199 leads to better bacterial clearance and survival of the fish (Gagne, Eliot Rivers, 2016). Functional analysis of the above discussed miRNAs will throw more light on the disease progression of Pseudomonas aeruginosa keratitis and provide novel therapeutic approaches.

REGIONAL RESEARCH CENTRES (RRC)

AMRF applies fundamental science to clinical problems to eliminate the needless blindness specifically focusing on Indian patients. In this endeavor, the scientists of AMRF have strong interactions with clinician scientists from Pondicherry, Coimbatore, Tirunelveli, Chennai and Madurai. One of the successful projects in this area is Identification of families with myoclilin mutations causing JOAG in multiple members of the same family. The examination of the EMR data of families with clinical JOAG in multiple members has found 10% mutation in families. Similar approaches are being carried out starting from the clinician scientists in other areas such as Viral keratitis, Keratoconus. We also explore constantly other areas of collaborative research focusing mainly on clinically relevant problems.

RRC, ARAVIND - COIMBATORE

Screening of Family Members of Juvenile Open Angle Glaucoma (JOAG) Patients for Myocilin Gene Mutations

Investigators : Prof. K. Dharmalingam, Dr. S.R. Krishnadas, Dr. Indira Durai (PI), Dr. Ganesh V. Raman, Dr. A. Vanniarajan, Dr. D. Bharanidharan
Technician : V. Saravanan
Funding : SunPharma

Background

Myocilin mutations are associated with 2% to 4% of adult-onset POAG (Fingert 2011), and 10% of JOAG (Gupta et al. 2017; Sharma and Grover 2021; Wang et al. 2019). Unlike adult-onset POAG, JOAG patients with myocilin variants were reported to have high intraocular pressure (IOP) at early-onset (usually <40 years) with severe optic nerve damage, which, if left untreated, results in severe visual impairment (Gupta et al. 2018). Moreover, glaucoma is usually asymptomatic in its early stages, patients present to the hospital in the advanced stage with significant vision loss (Sharma and Grover 2021). Thus, screening myocilin mutations will prevent vision loss in a high-risk group, however, it is unfeasible at the population level. Because it is estimated that 0.33 % of Indians would get Myocilin mutations (Selvan et al. 2022) and thus screening the population is cost-intensive (Khawaja and Viswanathan 2018). However, screening Myocilin mutations among JOAG patients of the family help detect the disease early. In addition, by identifying individuals who carry myocilin mutations, it is possible to implement early diagnosis and preventative measures to reduce the progression of the disease (Gupta et al. 2017; Sharma and Grover 2021; Wang et al. 2019).

S.No.	Families	Genomic Position (NC_000001.11)	cDNA position (NM_000261.2)	Protein change (NP_000252.1)
1	J89 family	g.171636341C>T	c.1099G>A	G367R G [GGA] > R [AGA] SNP: rs74315334
2	J85 family	g.171636000G>C	c.1440C>G	N480K N [AAC] > K [AAG] SNP: rs74315332
3	J51 family	g.171636347A>C	c.1093T>G	Y365S Y [TAC] > K [GAC]
4	J59 family	g.171636352G>A	c.1088C>T	A363G A [GCT] > G [GTT]

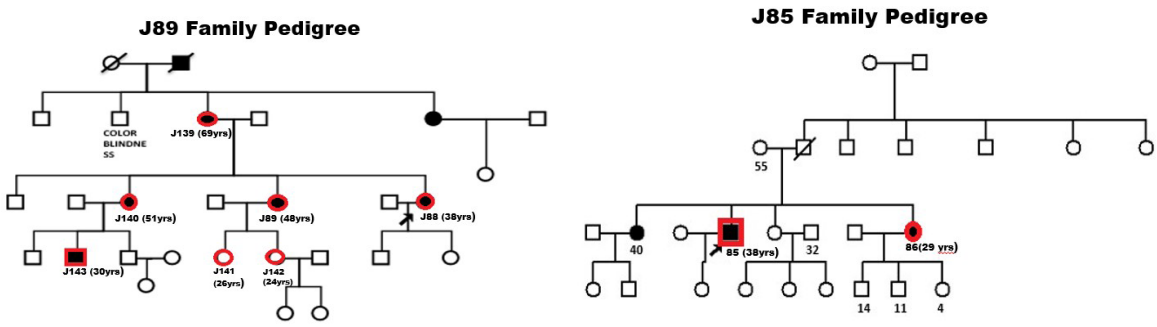


Figure 1: The pedigree chart of two families with JOAG patients having myocilin mutations. Individuals outlined with red border carries myocilin mutation G367R in J89 family and N480K in J85 family

In J89 family, we further extended our screening in the five family members. We detected the G367R mutation in all of them, of which, three are JOAG and two are normal young individuals (J141; 26 yrs and J142; 24 yrs). The G367R have been reported earlier in our cohort (Kanagavalli et al. 2007) and the in vitro studies show G367R is disease causing mutation in dominant manner (Kanagavalli et al. 2007; Zhou et al. 2022). Reports further reveals that G367R induced aberrant interactions between myocilin and chaperones (Grp94 and CRYAB), impairing the cellular degradation mechanism and exacerbating the accumulation of myocilin in the ER, which further resulted in ER stress, apoptosis and oxidative stress. Thus, the likelihood of these two young individuals developing JOAG is very high and requires routine followup.

In J85 family, we identified N480K mutation in proband and his sister who have JOAG. N480K has been shown to be a moderately stable mutation that produces moderately insoluble protein and causes cellular effects, and overall has an intermediate severity with onset ranging from 10 to 75 years (Burns et al. 2011; Hewitt et al. 2008; Wang et al. 2019). However, the amino acid asparagine at 480 position contributes significantly to stability of the Myocilin structure and further trimethylamine N-oxide treatment failed to stabilize the N480K mutation, suggesting that this mutation may cause severe glaucoma phenotype (mean IOP of 37±12.4) and early age of diagnosis (25.4±3.1) (Burns et al. 2011; Hewitt et al. 2008; Wang et al. 2019). This study observed that mean age at diagnosis was 39.29 ±17.1 years and mean IOP was 20.57 ±10.4 mmHg in POAG cases. Our earlier study has shown that N480K alteration is characterized by very high intraocular pressures and younger age of disease presentation (Kader et al. 2023).

The two mutations detected in J51 and J59 families are novel mutation and they were predicted to disease-causing mutations by VarP tool. However, further screening in the families and in vitro studies are warranted to confirm their role in JOAG.

Al together, this study reveals that MYOC mutation is primarily responsible for JOAG in four families, emphasizing the importance of screening other member of the family at a younger age for early treatment.

RRC, ARAVIND - PONDICHERRY

Title: Screening of Juvenile Open angle Glaucoma (JOAG) patients and their family members for Myocilin Gene Mutation

Investigators : Dr. Swati Upadhyaya,
Dr. S. Kavitha, Dr. R. Venkatesh,
Dr. D, Bharanidharan,
Dr. A. Vanniarajan,
Prof. K. Dharmalingam
Technician : Mr. V. Saravanan, Mr. G Joseph

Introduction

Juvenile open-angle glaucoma (JOAG) is a rare subset of glaucoma with early onset age ranging from three to forty years. It is an autosomal dominant disorder with high penetrance. Mutations in Myocilin gene are tightly linked with JOAG in about 3 - 36% of affected individuals across various populations. It is evident from the available reports that familial screening of the patients with JOAG will help in identifying the patients early and manage the disease with suitable medications.

Results

During the last year, we collected 25 blood samples from 24 JOAG patients and One JOAG suspect. We extracted the DNA from these samples and checked the DNA quality and quantity. Sanger Sequencing for Exon 3 of the myocilin gene in 6 families did not show any mutations. Screening of Exon-1 and 2 identified a

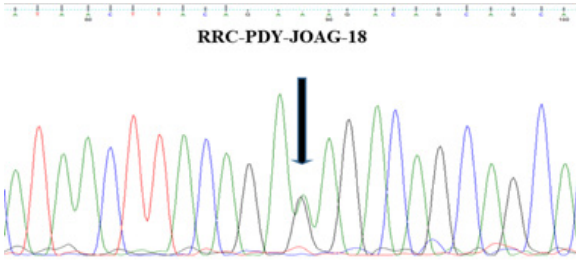


Figure 1: Myocilin variant R76K identified in three members of a same family with glaucoma

variant rs2234926 (c.287G>A, p.R76K) in one JOAG family. Father (40 Years - JOAG) and elder Son (14 Years - JOAG suspect) and younger son (12 Years – JOAG) have the variant (Figure 1).

Conclusion

Screening of the glaucoma and checking the myocilin gene mutations is now established at RRC, Pondicherry.

Title: Analysis of tear protein profile in Recurrent Herpes simplex viral keratitis

Investigators : Dr. Josephine Christy,
Dr. Aditi Parab,
Dr. A. Vanniarajan,
Prof. K. Dharmalingam
Research Scholar : Karthik Alagarsamy

Introduction

Diagnosis and management of viral ocular diseases remains as a challenge. Herpes simplex viral (HSV) infection can present as epithelial, stromal or endothelial keratitis affecting cornea. Host response to HPV infections vary largely among the patients and recurrences are not similar in all patients. Hence the present study is aimed to analyse the changes in tear film proteins of susceptible individuals, which can help to customise treatment modalities.

Results

Patients presenting with a recurrent episode of active HSV keratitis with at least 3 months of quiescence and belonging to any one of the following categories such as Epithelial/ dendritic ulcer, Geographic ulcer, Immune stromal keratitis with active corneal edema and Endothelitis/Disciform keratitis were included. Tear samples from 10 patients were collected and processed for the proteomic studies

Conclusion

Analysis of the proteomic profiles among the various groups of patients will be helpful to understand their host response to HSV.

RRC, ARAVIND - TIRUNELVELI

Genetic biomarkers for preclinical prediction for the onset of Keratoconus among Down's syndrome patients from Southern India

Investigators : Dr. V. Anitha,
Dr. R. Meenakshi,
Dr. O. G. Ramprasad,
Dr. A. Vanniarajan,
Prof. K. Dharmalingam

Introduction

Down Syndrome (DS) Children have many developmental issues related to vision. There is an increased incidence (10-300 times) of Keratoconus (KC) with DS in other ethnicities. However, the association of KC with DS in Indian population.is not known. KC exists in DS due to the unidentified genetic relationship between them, which needs to be explored.

Results

Totally 38 patients with Down's syndrome has been screened at the AEH, Tirunelveli. Karyotypically, they had three copies of chromosome 21. The patients were recruited through schools for intellectual disability and through District early intervention center, Tirunelveli Medical College, Tirunelveli. Mean age of the patients was: 12.05 years The 38 DS patients were screened tomographically with Pentacam for Keratoconus, we found 18 DS patients with tomographic diagnosis of KC, requiring treatment with conventional collagen crosslinking procedure.

Conclusion

Clinical studies helped to identify the DS patients with KC. Further molecular analysis will identify the genetic association between DS and KC.

CONFERENCES / MEETINGS

Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO) 2023

New Orleans, La., 23-27 April 2023

Faculty members and Research Scholars from Aravind Medical Research Foundation (AMRF) took part in the annual meeting of ARVO 2023, held at Ernest N. Morial Convention Center, New Orleans, Louisiana, United States of America. Saranya received the DBT Travel Grant, while Dr. C. Gowri Priya received a partial travel grant from CSIR-HRDG to attend the meeting. The following members presented papers and posters during the event:

- Prof. K. Dharmalingam
  Defining the host response to ocular pathogens using transcriptomics and proteomics (Paper Presentation)
- Dr. Gowri Priya Chidambaranathan
  Decline in the function of adult human retinal pigment epithelial stem cells with ageing (Paper Presentation)
- P. Saranya
  Decline in the adult human lens epithelial stem cell function with ageing and in cataract (Paper Presentation)
- K. Saraswathi
  Genomic and transcriptomic profiling identifies aberrant signalling pathways in ocular B cell lymphoma (Paper Presentation)
- A. Vanniarajan
  Distinct epigenomic alterations in retinoblastoma (Poster Presentation)
- R. Iswarya
  Human trabecular meshwork stem cells derived exosomes enhanced wound healing and antioxidant property: an attempt towards a cell-free therapy for glaucoma (Poster Presentation)

69th Annual conference of Physiologists and Pharmacologists of India - APPICON 2023

West Bengal, 1st December 2023

Dr. Senthilkumari Srinivasan delivered an invited talk at the scientific symposium on the topic, “Innovations and translations in ocular therapeutics: Innovative Ex-vivo model of human organ-cultured anterior segment (HOCAS) for screening of drugs for glaucoma”, during the annual conference organised by Departments of Physiology & Pharmacology, AIIMS, Kalyani.

Prime Summit 2023

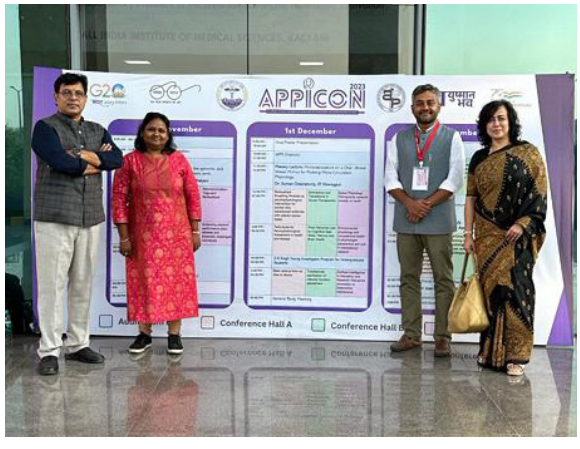
Narayana Nethralaya, Bangalore, 8th -10th December 2023

Faculty members and Research scholars from AMRF participated in the “Prime Summit 2023,” organised by Narayana Nethralaya Foundation, Bangalore. The summit focused on the theme of “Progress in Regenerative & Investigational Medicine in Eye”. The event commenced with a workshop on the first day, centred around “Immunology and Gene Therapy” followed by a two-day conference on “Cell and Gene Therapy”.

Dr. Swagata Ghosh won a prize in the quiz conducted during the workshop. At the conference, Prof. Dharmalingam, and Dr. Sundaresan served as moderators of panel discussions on “Initiating and

AMRF team members at ARVO meeting, USA

Dr. S. Senthilkumari at APPICON meeting, West Bengal



L.Mathan explaining a poster during the Prime Summit meeting, Bangalore

Dr. S. Senthil Kumari at Swamy Vivekananda College of Pharmacy, Namakkal

sustaining research career in India" and "Identifying clinical problems and organising research strategies relevant to India". The following faculty members and research scholars delivered talks and presented posters during the event.

- Dr. Vanniarajan: Retinoblastoma Genetics to Therapeutics (Invited talk)
- Dr. Swagata Ghosh: Regulation of inflammatory response in corneal infections: The dualism of MIF signalling (Invited talk)
- Prakash C: Does Leber's hereditary optic neuropathy inherit only maternally? (Poster Presentation)
- Aadhithiya T: Gr Identification of Retinol binding protein 3 (RBP3) from vitreous and plasma extracellular vesicles as a predictive biomarker for diabetic retinopathy (Poster Presentation)
- Mathan L: Genome-wide DNA methylation analysis reveals epigenetic dysregulation in pterygium (Poster Presentation)
- Sriee Viswarubhiny: Functional analysis of GUCY2D: An intrinsic cause of Leber's congenital amaurosis (Poster Presentation)

7th National Conference on "Emerging Infectious Diseases and Novel Drug Development (EID-NDD 2023)

Namakal, 11th December 2023

Dr. S. Senthilkumari delivered an invited talk in the National Conference organised by Swamy Vivekananda College of Pharmacy, Namakkal on the topic, *"Human Amniotic membrane (HAM) as a drug reservoir for ocular Anti-microbial agents: An implication in ocular infections".*

National Conference on Frontiers in Life Science Research NCFLSR 24

American College, Madurai, 23rd January 2024

Faculty members and research scholars from AMRF participated in the "National conference on Frontiers in Life Science Research NCFLSR 24 organised by The American College, Madurai. During the

R. Iswarya receiving the best poster award at NCFLSR 24 meeting, Madurai

R.Sethu Nagarajan receiving the best poster award at NCFLSR 24 meeting, Madurai

conference, Prof. K. Dharmalingam delivered an invited talk on Extracellular vesicles: Signaling to epigenetic regulation. The following research scholars presented posters during the event.

- R.Iswarya: Protein profiling of human trabecular meshwork stem cell derived exosomes by mass spectrometry (Best poster award)
- R.Sethu Nagarajan: Evaluation of GD2 Synthase as a prognostic biomarker in retinoblastoma (Best poster award)
- G.Hariharan: Isolation and characterisation of the extracellular vesicles from Aspergillus flavus clinical isolate and a saprophyte
- R.Praveen Kumar: Human corneal mirnas and their role in pseudomonas aeruginosa keratitis
- T.Gr. Aadhithiya: Deciphering the proteome signature of plasma extracellular vesicles from proliferative diabetic retinopathy patients

CONFERENCES CONDUCTED

AMRF-UoE Cornea Research Conference

AMRF, 14-15 June 2023

AMRF conducted a two-day Cornea Research Conference in collaboration with the University of Edinburgh (UoE). This conference was initiated through the interaction between Dr. N. Venkatesh Prajna, Chief, Cornea & Refractive Surgery Services, Aravind-Madurai , Prof. Kev Dhaliwal and Dr. Beth Mills, Centre for Inflammation Research, Institute for Regeneration and Repair, the University of Edinburgh, UK. The conference was led by Prof. K. Dharmalingam, and coordinated by Dr. D. Bharanidharan.

During the conference, scientists from UoE introduced a new technology called “FluoroPi” aimed at establishing and developing a cost-effective device for diagnosing the causative microbe of microbial keratitis in patients. Further, clinicians, scientists, and research scholars engaged in brainstorming the current challenges and solutions in microbial keratitis. Dr. Bharanidharan also provided an overview of the core research facilities and ongoing research projects of AMRF. Moreover, sharing of the research activities between the two institutions served as a remarkable initiative, laying foundation for on-going and future collaborative projects, as well as fostering long-term associations between them.

A total of 14 papers were presented by UoE, Aravind Eye Hospital, and AMRF team over the course of two days, covering various aspects of corneal research. Additionally, 16 posters were presented and Hariharan G’s poster titled, Isolation and characterisation of the extracellular vesicles from Aspergillus flavus clinical isolate received the Favourite poster award.

Below is the list of papers presented:

- Prof. K. Dharmalingam: Comparatives proteomics of conidial surface proteome and early exoproteome of *A.flavus* and *A.fumigatus*
- Dr. Lalitha Prajna: Microbial keratitis: Current clinical diagnostics, pitfalls, and wish-list
- Dr. N. Venkatesh Prajna: Microbial keratitis: Current treatments, patient prognosis and state of the art (trials)
- Dr. Stuart Dickson: Seeing is believing: Microscopic molecular imaging. The distal lung as a window to the eye
- Dr. Syam Mohan & Dr. Kay Russell: Development & evaluation of FluoroPi: A frugal microscope for microbial keratitis diagnosis with SmartProbes
- Dr. Beth Mills: AspLFD: Point of care diagnosis of Aspergillus causing fungal keratitis
- Dr. N. Siddharth: Development and clinical evaluation of A CRISPR/Cas12a based diagnostic platform for ocular fungal infections
- Dr. Leonie Fingerhut – Approaches to 3D cell culture model of microbial keratitis

Participants of the AMRF-UoE Cornea Research Conference

- Dr. Sheelagh Duncan – Antimicrobial photodynamic therapy: A new approach for treating microbial keratitis
- Dr. Gowri Priya C: MiRNAs regulating human limbal epithelial stem cells
- D. Bharanidharan: MiRNAs role in fungal keratitis
- Dr. OG. Ramprasad: Exploring the mechanism of action of a novel chemical crosslinker designed to treat keratoconus
- Dr. Daipayan Banerjee: Comparative transcriptomic and proteomic analysis to identify key genes and pathways in pterygium in the Indian population
- Dr. Anshupa Patnaik: Proteomics in fungal keratitis: A clinician's perspective

Symposium on "Innovations in Eye Health: Technology to Translation"

AMRF, 28th July

A one-day symposium on Innovations in Eye Health: Technology to Translation was conducted with support from SERB, DBT, and CSIR. There were 108 participants from various research institutions and universities across the country. The symposium featured fifteen talks by scientists in translational research, covering five key areas.

Gene Editing/Cell Therapy for Retinal Diseases:

- Dr. Arkasubhra Ghosh - AAV vector transgene engineering for therapeutic applications
- Dr. Sowmya Parameswaran - Unveiling hidden horizons: Addressing subtle barriers in retinal pigment epithelial cell transplantation
- Dr. Indumathi Mariappan - Targeted genome editing: Creating disease models and developing tailored therapies for IEDs

Immunopathology of Ocular Disorders:

- Dr. SR. Rathinam - Translational research in uveitis
- Dr. Nallathambi Jeyabalan - Role of autophagy and its modulation in the pathogenesis of pterygium: Therapeutic approach and applications
- Dr. Swaminathan Sethu - Ocular immune cell diversity in health and disease

Novel Approaches for Treatment of CornealInfections

- Dr. Thirumurthy Velpandian - Innovative concepts to widen the scope of antimicrobials for corneal infections and drug resistance
- Dr. Sanhita Roy - Peptides as alternative therapeutics for corneal infections
- Dr. Vivek Singh - Hydrogel and 3D printing in corneal wound healing

Exosomes in Eye Health and Disease

- Dr. Swathi Kaliki - Serum extracellular vesicles in retinoblastoma: What do we know so far?
- Dr. Daipayan Banerjee - Extracellular vesicles in diabetic retinopathy: A comparative proteomic analysis of plasma and vitreous humor derived Evs
- Dr. Gowri Priya - Trabecular meshwork stem cells derived exosomes enhanced proliferation and anti-oxidant potential of trabecular meshwork cells
- Dr. Nirmal Jayabalan - Nanomicelles: A solution-driven platform technology to treat corneal infection

Genetic Diagnosis and Therapy for Inherited Eye Diseases

- Dr. Inderjeet Kaur - Predictive testing for retinopathy of prematurity
- Dr. G. Kumaramanickavel - Paradigm shift in genetic counselling in the Post-NGS era

Participants of the symposium on Innovations in Eye Health: Technology to Translation

29th Annual Meeting of Indian Eye Research Group

AMRF, 29-30 July

The 29th annual meeting of the Indian Eye Research Group - ARVO India Chapter was held at AMRF, Madurai, with the theme of “Innovations in Eye Health: Technology to Translation”.

The meeting featured seven scientific sessions covering various ocular segments, such as the retina, cornea, orbit, and diseases like diabetic retinopathy, glaucoma, keratoconus, and ocular infections. Many talks focused on translational research, including gene therapy and stem cell therapy for retinal diseases and corneal dystrophies. The significance of artificial intelligence in diagnosing ocular disease was discussed in detail. There was a special discussion on Emerging areas of Indian Eye Research: Future perspective that helped establish goals for future research.

The event saw participation from 220 individuals, including scientists from academia and industry, young researchers, clinical fellows, and optometrists from across the country. There were six invited talks, 18 free papers, 123 posters, and two industry talks. Two best presentation awards were given in both posters and free paper categories. Additionally, travel support was provided to eleven young trainees/ clinical fellows. The popular quiz programme, I-Quest, was conducted, and prizes were given to the winner and runner-up. Three oration lectures were presented by eminent scientists in frontier areas of research:

- Prof. Colin Willoughby, Professor of Ophthalmology, Ulster University, UK, delivered the Bireswar Chakrabarti Oration titled *Reverse Engineering Glaucoma: A path to improving glaucoma treatment.*
- Dr. Subhabrata Chakrabarti, Associate Director, LV Prasad Eye Institute, Hyderabad delivered the D. Balasubramanian Oration titled *Molecular dissections of a blinding disease in children through evolving omics in the sands of time*.
- Dr. V. Mohan, Chairman & Chief of Diabetology, Dr. Mohan’s Diabetes Specialities Centre, Chennai gave the Dr. S.S. Badrinath Oration on *Making India diabetes complications free: Hype or reality?*

The meeting also included a special talk on Vaigai – The cradle of Tamil civilisation and a cultural programme. The event was organised by AMRF under the aegis of ARVO with support from Aurolab, Spinco Biotech, Mankind Pharma, Sun Pharma, Thermo Scientific, Zivira Labs, Lific, Biokart, Theracues, Fortune Biosciences, Synergy Scientific, National Scientific, Ileco and Spinco Analytica.

Glimpses of ARVO-India meeting

Workshop on Single-Cell Omics

AMRF, 12-13 October

AMRF organised a two-day workshop to commemorate the 105th birth anniversary of the founder, as a part of Scientific Social Responsibilities under an on-going SERB project (CRG/2020/00528). Dr. Ishwariya Venkatesh, Senior Scientist, Centre for Cellular and Molecular Biology (CCMB), Hyderabad, Dr. K. Manojkumar, Senior Research Associate, CCMB, Hyderabad, Dr. Gopalakrishna Ramaswamy, Founder & CEO, TheraCUES Pvt. Ltd., Bangalore, and Prof. K. Dharmalingam delivered the invited talks. Along with them, Dr. C. Gowri Priya, Dr. D. Bharanidharan and Dr. Daipayan Banerjee; as well as research scholars, including R. Iswarya, A. Karthik, G. Hariharan, M. Gayathri, Shreya Dinesh, R. Praveenkumar, T. Gr. Aadhithiya, L. Mathan, and M. Kanmani, served as resource persons for the laboratory modules. The participants were updated on the recent advances in single-cell transcriptomics, spatial transcriptomics, extracellular vesicles, proteomics, and data analysis. A total of 22 faculty members from colleges and universities participated in the workshop.

Participants of the workshop on Single-Cell Omics

Research Facility Training Programme on Genetic Testing Techniques

AMRF, 29th February

As an integral component of SERB-LHON project, AMRF organised a one-day research facility training programme on Genetic testing techniques as part of the scientific social responsibility programme (SERB-SSR). The programme consisted of two modules: the first covering DNA isolation and quantitation, and the second focusing on Sanger sequencing. Dr. P. Sundaresan, the Principal Investigator and Convenor of the programme, elucidated the fundamentals of ophthalmic genetics and its application in molecular diagnosis. In his concluding remarks, Prof. K. Dharmalingam emphasised the significance of epigenetics and its implications on human diseases. Thereafter, certificates were distributed to all the participants by Prof. K. Dharmalingam. The programme saw active participation from 30 students representing 14 different colleges in the vicinity of Madurai, spanning across 8 distinct academic departments.

Participants of the research facility training programme on Genetic Testing Techniques

AWARDS

Ph.D awarded by Alagappa University

P.Gowri
Department of Genetics
Thesis: Molecular genetics and functional studies of mitochondrial genes associated with Leber's hereditary optic neuropathy
Guide: Dr.P.Sundaresan

K. Jeyaprakash
Department of Molecular Genetics
Thesis: Genomic characterisation of Kinome related genes in retinoblastoma
Guide:Dr.A.Vanniarajan

Ph.D awarded by SASTRA University

K. Kathirvel
Department of Microbiology and Bioinformatics
Thesis: Characterisation of antimicrobial resistance and virulence in ocular Pseudomonas aeruginosa and methicillin-resistant Staphylococcus aureus strains through comparative genomics approach
Guide: Dr.D.Bharanidharan

K. Manojkumar
Department of Microbiology and Bioinformatics
Thesis: Identification of pathogenic variants associated with genetic eye diseases in whole exome and genome data
Guide: Dr.D.Bharanidharan

Prof. VR. Muthukkaruppan Endowment award 2023

In 2014, students and colleagues of Prof. VR. Muthukkaruppan established an endowment in his name. From this endowment fund, an annual award is bestowed upon the best researcher of the institute, selected based on the scientific merit of their research abstracts and presentations. This award includes a certificate and cash prize.

Iswarya, Department of Immunology and Stem Cell Biology, won the award for her outstanding research work on "Protein profiling of human trabecular meshwork stem cell derived exosomes by mass spectrometry".

R. Iswarya receiving Prof.VR. Muthukkaruppan Endowment award

GUEST LECTURE

Dr. Paul Pown Raj, Research Scientist, Nationwide Children's Hospital, Institute for Genomic Medicine, Columbus, Ohio, USA

Topic : "Role of Alx genes in frontonasal and ocular development"

6th July 2023

Prof. Kannan Rangaramanujam, Professor of Ophthalmology, Co-Director, Center for Nanomedicine, Wilmer Eye Institute, Johns Hopkins School of Medicine, Baltimore, MD 212877

Topic: "Development and translation of targeted systemic nanomedicines for ocular disorders".

18th July 2023

Prof. Kulandaiappan Varadaraj, Professor, Department of Physiology & Biophysics Health Sciences Center, School of Medicine, Stony Brook University, USA

Topic : "Lens Aquaporins (AQPs) and Glutathione Peroxidase (GPX1) are critical for lens transparency and Homeostasis".

19th October 2023

PUBLICATIONS 2023 - 2024

Kumar Jeyaprakash, Kannan Thirumalairaj, Usha Kim, Veerappan Muthukkaruppan, Ayyasamy Vanniarajan

- *"RB1 transcript analysis detects novel splicing aberration in Retinoblastoma"*

Pediatr Blood Cancer. 2023; e30290.

Prakash Chermakani, Periasamy Sundaresan

- *"Traboulsi Syndrome: A Rare Eye Disease and its Genetic Association"*

TNOA J Ophthalmic Sci Res 2023; 61:41-5

Manojkumar Kumaran, Bharanidharan Devarajan

- *"EyeVarp: a computational framework for the identification of pathogenic variants specific to eye disease"*

Genetics in Medicine April 2023

Kuppan Gokulakrishnan, Joyappa Nikhil, Biju Viswanath, Chinnasamy Thirumoorthy Sandhya Narasimhan, Bharanidharan Devarajan, Ebin Joseph, Arul Kevin Daniel David, Sapna Sharma, Kavitha Vasudevan, Vanteemar S. Sreeraj, Bharath Holla, Venkataram Shivakumar, Monojit Debnath, Ganesan Venkatasubramanian , Shivarama Varambally

- *"Comparison of gut microbiome profile in patients with schizophrenia and healthy controls - A plausible non-invasive biomarker?"*

Journal of Psychiatric Research 2023; 162: 140-149

Kadarkarai Raj Rajendran, Prakash Chermakani, Rupa Anjanamurthy, Clayton Fernando Rencilin, Periasamy Sundaresan

- *"Association of ABCA4 Gene Variants in Patients with Autosomal Recessive Cone-Rod Dystrophy and Retinitis Pigmentosa Cohorts from South India"*

Cytology and Genetics, 2023, Vol. 57, No. 3, pp. 258–267

Lakshmi Prabha Venugopalan, Vishukumar Aimanianda, Venkatesh Prajna Namperumalsamy, Lalitha Prajna, Dharmalingam Kuppamuthu, Jeya Maheshwari Jayapal

- *"Comparative proteome analysis identifies species-specific signature proteins in Aspergillus pathogens"*

Applied Microbiology and Biotechnology 2023;107, 4025–4040

Sagnik Sen, Prithviraj Udaya, Jayapal Jeya Maheshwari, Piyush Kohli, Haemoglobin Parida, Naresh Babu Kannan, Kim Ramasamy, Kuppamuthu Dharmalingam

- *"Comparative proteomics of proliferative diabetic retinopathy in people with Type 2 diabetes highlights the role of inflammation, visual transduction, and extracellular matrix pathways"*

Indian J Ophthalmol 2023;71:3069-79

Sagnik Sen, Prithviraj Udaya, Jayapal J Maheshwari, Kim Ramasamy, Naresh B Kannan, Kuppamuthu Dharmalingam

- *"Profiling of idiopathic macular hole vitreous proteome identifies the role of extracellular matrix remodelling, epithelial–mesenchymal transformation and unfolded protein-response pathways"*

Indian J Ophthalmol 2023;71:2027-40.

Vignesh Elamurugan, Toshit varshney, Naresh Babu K, Siddharth Narendran, Hanithraj Deivarajan, Karvannan Sevugamurthi

- *"Crabtree Effect as a parallel pathogenetic pathway in Diabetic Retinopathy"*

Medical Hypotheses August 2023

Mohideen Abdul Kader, Bharanidharan Devarajan, Saravanan Vijayan, Rengappa Ramakrishnan, Periasamy Sundaresan, Mohammed Sithiq Uduman, Subbiah R Krishnadas, Dharmalingam Kuppamuthu

- *"Myocilin mutation N480K leads to early onset Juvenile and adult-onset Primary Open Angle Glaucoma in six generations of a family"*

Journal of Glaucoma 2023

Swagata Ghosh, A.H.Humera Khathun, G.S.Athulya, P.Vignesh, L.Mathan, Ninad Muddaraddi, Siddharth Narendran, Prajna Lalitha, N.Venkatesh Prajna

- *" Host cell-type and pathogen-specific immunomodulatory functions of macrophage migration inhibitory factor (MIF) in infectious keratitis"*

Experimental Eye Research Sep 2023

Prakash Chermakani, Poigaialwar Gowri, Shanmugam Mahesh Kumar

- *"Exploring Mito-Nuclear Genetic Factors In Leber's Hereditary Optic Neuropathy: Insights From Comprehensive Profiling Of Unique Cases"*

EXCLI Journal 2023;22:1077-1091

Kandasamy Kathirvel, Xiaochen Fan, Ravinarayanan Haribalaganesh, Devarajan Bharanidharan, Rajendrababu Sharmila, Ramasamy Krishnadas, Veerappan R Muthukkaruppan, Colin E. Willoughby and Srinivasan Senthilkumari

- *"Small RNA Sequencing Reveals a Distinct MicroRNA Signature between Glucocorticoid Responder and Glucocorticoid Non-Responder Primary Human Trabecular Meshwork Cells after Dexamethasone Treatment"*

Genes 2023, 14, 2012

Swathi Chadalawada, SR.Rathinam, Prajna Lalitha, Naresh Babu Kannan, Bharanidharan Devarajan

- *" Detection of microRNAS expression signatures in vitreous humor of intraocular tuberculosis human trabecular meshwork cells after Dexamethasone treatment"*

Molecular Biology Reports Oct 2023

Almar Neiteler , Anwar A. Palakkan, Kevin M. Gallagher, James A. Ross

- *"Oxidative stress and docosahexaenoic acid injury lead to increased necroptosis and ferroptosis in retinal pigment epithelium"*

Scientific Reports | (2023) 13:21143

Pandi Saranya, Madhu Shekhar, Aravind Haripriya, Muthukkaruppan V, Gowri Priya C

- *"Towards the identification and characterization of putative adult human lens epithelial stem cells"*

Cells Dec 2023

Taiyab A, Belahlou Y, Wong V, Saranya P, Madhu Shekhar, Gowri Priya C, West-Mays J

- *" Understanding the role of Yes-Associated Protein (YAP) signaling in the transformation of Lens Epithelial Cells (EMT) and Fibrosis"*

Biomolecules Dec 2023

Rathinam, S. R., Kohila, G. J., Gowri, P. C., & Balagiri, K. S.

- *Leptospiral uveitis - "Transition 'from epidemic to endemic form" difficulties in laboratory confirmations.*

Indian journal of ophthalmology 2023; 71(8), 3031–3038.

Rathinam, S. R., Vedhanayagi, R., Radhika, M., Balamurugan, M. S., Balagiri, K., Priya, C. G., & Kohila, G. J.

- *Why do Doctors Miss the Diagnosis of Leptospiral Uveitis? Emergence of New Serovars and Challenges in Diagnosis.*

Ocular immunology and inflammation 2023; 1–6.

ONGOING RESEARCH PROJECTS

No	Projects	Funded by	Investigators	Research Scholar
PROTEOMICS				
1.	Whole genome sequence analysis of selected family members of the Kadaladi family and identification of markers for the early detection of JOAG using tear extracellular vesicles	Sun Pharma 2022-2023	Prof.K.Dharmalingam Dr.P.Sundaresan Dr.D.Bharanidharan Dr.SR.Krishnadas Dr.Mohideen Abdul Kader	Karthik A
2.	Proteomic and Metabolomic analysis of Microbial Keratitis Patients tears to identify biomarkers (molecular signatures) of corneal ulcer progression	INDO-UK 01.03.2023-31.03.2024	Dr. N. Venkatesh Prajna Prof. K. Dharmalingam	G.Hariharan
3.	A comparative proteomic analysis of plasma and vitreous humor derived small Extracellular Vesicles (SEVs) from Proliferative Diabetic Retinopathy (PDR) patients.	Sun Pharma March 2023-May 2024	Dr. Daipayan Banerjee Dr. Bhavani S Dr. K. Naresh Babu Dr. R. Kim Prof. K. Dharmalingam	Aadithiya T Gr
4.	Deciphering predictive and preventative methods in the progression of pterygium using multiomics approaches	SERB 1st November 2022-31st October 2024	Dr. Daipayan Banerjee	L.Mathan
5.	Understanding the mechanism of action of a novel chemical cross-linker designed to treat keratoconus	ICMR 2020 – May 2023	Dr.O.G.Ramprasad Prof. K. Dharmalingam Dr. N.Venkatesh Prajna Dr. Naveen Radhakrishnan	G. Adhithya Subramanian
MICROBIOLOGY				
6.	Development and validation of a non-invasive point-of-care diagnostic tool for fungal keratitis	VELUX STIFTUNG 01.08.2021-31.07.2025	Dr.N.Venkatesh Prajna Dr. Lalitha Prajna Dr.K. Dharmalingam Dr.Thulasiraj Ravilla Dr.N. Siddharth	Hanithraj D. Padmapriya S Hari Vignesh Kanmani
7.	Mechanotransduction in retinal pigmented epithelium homeostasis and degeneration: Implications in age-related macular degeneration	SERB Dec 2021-Dec 2023	Dr. Siddharth Narendran	S. Karvannan
8.	Role of Human Corneal MiRNAs in the onset and severity of Fungal Keratitis	ICMR Nov.2021-Nov 2024	Dr. D. Bharanidharan Dr. K. Dharmalingam Dr. N. Venkatesh Prajna Dr. Lalitha Prajna	Gayathri M
9.	Dysregulated human Corneal miRNAs and their role in disease progression	ICMR-SRF 2022-2025	Dr. D. Bharanidharan	Shreya Dinesh

MOLECULAR GENETICS				
10..	Molecular Characterization of Leber's Congenital Amaurosis in South Indian Cohort	Lady Tata Memorial Trust 02.01.2021 – 01.01.2026	Dr. P. Sundaresan	A.S. Sriee Viswarubhiny
11.	Investigating the Crosstalk between Nuclear and Mitochondrial Genome in Patients with Leber's Hereditary Optic Neuropathy	ICMR-SRF 01.03.2021 – 28.02.2024	Dr. P. Sundaresan	C. Prakash
12.	Molecular Genetics of Juvenile X-linked Retinoschisis in South Indian Population	Lady Tata Memorial Trust 01.08.2019 – 31.07.2023	Dr.P.Sundaresan	Susmita Chowdhury
13.	Investigation of nuclear genes involvement in a Mitochondrial Disorder: Leber's Hereditary Optic Neuropathy	SERB 18.08.2023 – 17.08.2026	Dr. P. Sundaresan Dr. S. Mahesh Kumar	-
14.	Decoding the Unknown Genetic Etiology to Ameliorate the Molecular Diagnosis of Leber's Congenital Amaurosis	DHR-GIA, ICMR 06.11.2023 – 05.11.2026	Dr.P.Sundaresan, Dr. S. Senthilkumari Dr. Rupa Anjanamurth	S. Shiva Shankari
15.	Translational Genomics of Ocular Cancers	Aravind Eye Foundation	Dr. Usha Kim Dr. A. Vanniarajan Dr. D. Bharanidharan Dr. R. Shanthi Dr. VR. Muthukkaruppan	K. Jeyaprakash
16.	Molecular characterization of ocular lymphoma for improved disease prognosis	Lady Tata Memorial Trust 01.08.2020 – 31.07.2025	Dr.A. Vanniarajan	K. Saraswathi
17.	Elucidating the role of cancer stem cells in chemoresistant retinoblastoma and their therapeutic implications	ICMR-SRF 2022-2025	Dr. A. Vanniarajan	R. Sethu Nagarajan
IMMUNOLOGY AND STEM CELL BIOLOGY				
18.	Characterization of adult human lens epithelial stem cells in the maintenance of tissue homeostasis throughout life and their functional status in cataractous lens	SERB 29.05.2019 – 30.11.2022 Lady Tata Memorial Trust – SRF 01.08.2019 – 31.07.2023	Dr. Madhu Shekhar Dr. Gowri Priya Chidambaranathan Dr.Haripriya Aravind Prof. VR. Muthukkaruppan	P. Saranya
19.	Identification and Characterization of adult human retinal pigment epithelial stem cells	Part time PhD from 17.03.2022	Dr. Gowri Priya Chidambaranathan	A.Waseema

20.	Adult stem cell derived extracellular vesicular miRNAs for trabecular meshwork regeneration in glaucoma	Sun Pharma UGC-SRF 03.06.2020 – 02.06.2025	Dr. Gowri Priya Chidambaranathan Dr. S. R. Krishnadas Prof. VR. Muthukkaruppan	R. Iswarya
21.	Molecular regulators associated with the maintenance of human trabecular meshwork stem cells in relation to their reduction in ageing and glaucoma	SERB Oct.2021 – Oct.2024	Dr. Gowri Priya Chidambaranathan Dr. D. Bharanidharan Dr. S. R. Krishnadas Dr. VR. Muthukkaruppan	Sneha Nair
22.	Molecular characterization of human retinal pigment epithelial stem cells and their role in age related macular degeneration	ICMR Dec 2022-Dec 2025	Dr. C.Gowri Priya Dr. D.Bharanidharan Dr. R.Kim Prof. VR.Muthukkaruppan Dr.Siddharth Narendran	R.Kanthimathi
23.	Molecular regulation of adult human lens epithelial stem cells: changes with aging and cataract	ICMR-JRF Oct 2023- Oct 2025	Dr. C. Gowri Priya	P. Thushmitha
24.	A scalable system for the production of human induced pluripotent stem cell derived functional retinal pigmented epithelial cells	SERB 04.10.2023 – 03.10.2026	Dr.P. Anwar Azad	S.Gopika
OCULAR PHARMACOLOGY				
25.	Evaluation of IOP lowering property and Anti-fibrotic property of Relaxin on TGFβ2-induced Elevated IOP Ex vivo model of glaucoma using Human Organ Cultured Anterior Segment (HOCAS)	SERB-CRG grant (2023-2026)	Dr. S. Senthilkumari Dr. R. Sharmila	Suman R
26.	'Relaxin' the pressure in glaucoma	Fight for Sight PhD Studentship 2022	Prof. Colin Willoughby (UK) Dr. S. Senthilkumari Dr Kazuhiro Yamamoto (UK)	-
27.	Role of vitamin D3, dopamine and serotonin levels in myopia development and progression	AEH, Madurai 02-Aug-2023 - 31-Jul-2024	Dr. Sahithya Dr. Elackiya Dr. P. Vijayalakshmi Dr. S. Senthilkumari	
BIOINFORMATICS				
28.	Expression profiling of human corneal miRNAs and their role in Pseudomonas aeruginosa induced keratitis	DBT-BET (Fellowship) 2022-2027	Dr. D. Bharanidharan	R.Praveen kumar
29.	Identification and Analysis of Alternative Transcripts in Retinoblastoma Progression	ICMR-SRF 2022-2025	Dr. D. Bharanidharan	Mohd Hameed Aslam

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.

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