


Research Article

Explicating the Role of miR-638 in the Pathogenesis of Exfoliation Syndrome and Exfoliation Glaucoma

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Abstract

Introduction: Pseudoexfoliation syndrome (XFS) poses a significant risk for pseudoexfoliation glaucoma (XFG) by affecting intraocular pressure (IOP) and visual acuity. Previous studies have linked microRNAs (miRNAs) to the development of XFS and XFG. This research aims to explore the varying levels of miRNAs in the anterior lens capsule (ALC) of individuals with XFS, XFG, and those without the conditions.

Methods: Total RNA, including miRNA, was extracted from the ALC of XFS, XFG and cataract (CAT) patients. A miRNA microarray was performed and significantly expressed miRNAs were confirmed using real-time PCR. Furthermore, the expression levels of miR-638 and its target genes were validated using real-time PCR, and oxidative stress levels were evaluated using a lipid peroxidation (LPO) assay.

Results: Analysis of RNA extracted from ALC samples revealed the presence of 2550 miRNAs, with 44 showing distinct expression patterns among the groups. Further validation through qRT-PCR in 12 samples per group highlighted 8 clinically important miRNAs, with miR-638 significantly upregulated in XFG compared to both XFS and control groups ($p > 0.0001$). Investigation into potential targets of miR-638 identified SRY-Box transcription factor 2 (SOX2) as a key player, displaying marked downregulation in XFG compared to controls. Additionally, testing for lipid peroxidation function revealed heightened oxidative stress in samples from individuals with XFS and XFG.

Conclusion: These findings collectively suggest that the dysregulation of miR-638, its target gene SOX2, and the disrupted balance between oxidative and antioxidative processes may contribute significantly to the pathophysiology of XFG.

Keywords: Pseudoexfoliation, microRNA, anterior lens capsule, miR-638, SOX2

Introduction

Pseudoexfoliative syndrome (XFS, OMIM #177650) is a condition associated with aging that was initially discovered in 1917 by Lindberg [1]. It has a global prevalence, impacting approximately 60-70 million individuals worldwide, with varying rates among different ethnic groups and regions [2]. XFS results in the accumulation of abnormal fibrillar material and amyloid proteins in the eye tissues, particularly in the anterior segment along the aqueous humor outflow pathway [3, 4]. This accumulation obstructs outflow, leading to elevated intraocular pressure (IOP) [5] and is a primary cause of

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pseudoexfoliation glaucoma (XFG) [6]. Elevated IOP levels pose a significant risk of damage to retinal ganglion cells and subsequent vision loss if not treated promptly. Apart from intraocular pressure-related factors, oxidative damage plays a pivotal role in the progression of pseudoexfoliation [7, 8]. This condition is marked by increased levels of reactive oxygen species (ROS) within cells, leading to DNA damage. Studies have highlighted various ROS markers in serum and eye fluid samples of individuals with XFS, underscoring the importance of maintaining a balance between oxidants and antioxidants in disease development [9, 10].

MicroRNAs (miRNAs) have been associated with the development of XFS and XFG, primarily by regulating messenger RNA targets in the eye tissues [11-15]. Recent research has examined miRNA profiles in the anterior lens capsule using Next-generation Sequencing (NGS), revealing a link between miRNA expression and age in XFS [16, 17]. Lens epithelial cells have been identified as a primary source of pseudoexfoliating material, indicating their crucial role in disease progression [18]. This study aims to profile miRNAs in the anterior lens capsule of the Indian population to identify deregulated miRNAs and their potential impact on oxidative stress-induced lipid peroxidation, highlighting non-IOP factors in disease modulation.

Methods

Patients and Sample collection

Anterior lens capsules were obtained from patients undergoing elective cataract surgeries at the Glaucoma and Lens Clinic of the Advanced Eye Center, Post Graduate Institute of Medical Education and Research in Chandigarh, India. This research was authorized by the Institutional Ethics Committee of the Post Graduate Institute of Medical Education and Research (approval no. IEC-02/2015-156) and complied with the Declaration of Helsinki. Each patient provided informed consent after being briefed about the study. The participants were classified into three groups: Group 1 consisted of patients with XFS, Group 2 comprised XFG patients, and Group 3 (control group) included age and sex-matched individuals without XFS or glaucoma who underwent cataract surgery. Inclusion criteria for XFS and XFG patients were age 50 years or older with pseudoexfoliation changes, such as the presence of XFS material on the central disc, a clear annular zone, or XFS material flakes on the lens surface, iris, or corneal endothelium in either eye. Patients with XFG exhibited the above XFS characteristics along with certain additional criteria. Exclusion criteria encompassed conditions like trauma, lens dislocation, aphakic and pseudophakic eyes, diabetes, and hypertension. Throughout the cataract surgeries, anterior lens capsules from the specified groups were removed and promptly preserved in RNA later at -80°C for subsequent procedures.

RNA isolation, miRNA array and identification of altered miRNAs

Total RNA, including miRNA, was isolated from the ALC using the mirVana™ miRNA Isolation Kit (with phenol) according to the manufacturer's instructions. A human genome-wide miRNA array profiling was performed on miRNAs from the ALC of 2 patients from each group. 100 ng of total RNA was labelled with cyanine 3-pCp after dephosphorylation and denaturation. Subsequently, it was probed at 55°C in a hybridization oven with the Agilent Human miRNA Microarray V2.0, which has 62976 features and 2549 targets, for 20 hours. Subsequently, the slides were scanned using an Agilent Scanner (Agilent platform with scanner C model; Agilent Technologies, Inc.). Agilent Feature Extraction Software (Cernusco sul Naviglio, Milan, Italy) was used to analyze images. It generated the intensity value of the individual probe in a text file that was imported to Genespring for data analysis. The raw data was normalized and analyzed using GeneSpring GX software (zcomSilicon Genetics, Redwood City, CA, USA). The false discovery rate was set at 0.05 to obtain the selected miRNAs and each value was converted to log₂. The fold change between the groups was compared to the expression data generated. The differentially expressed miRNAs were compared using a t-test.

Real-time PCR (qRT-PCR) for miRNA and gene quantification

A total RNA fraction containing miRNAs was isolated from the ALCs of patients suffering from XFS, XFG, and control (12 patients from each group). The expression of selected miRNAs and genes was quantified by qRT-PCR and normalized to that of SNORD47 and β-actin, respectively. qRT-PCR was performed using SYBR chemistry. Fold change was calculated using the 2^{-ΔΔCt} method. The primers used are listed in Supplementary Tables 1 and 2.

Computational prediction for possible miR-638 target genes

The miRNA targets were identified *in silico* using miRTarBase (<http://mirtarbase.cuhk.edu.cn>), TarBase V.8 (<http://www.microrna.gr/tarbase>) and miRWalk 2.0 (<http://mirwalk.umm.uni-heidelberg.de/>) database tools. 'Seed' matches and the minimum free energy of interaction of potential target genes were examined at their 3'-UTR sequence using Diana-labs (MicroT-CDS) http://diana.imis.athena-nnovation.gr/DianaTools/index.php?r=microT_CDS/index.

Oxidative stress analysis

The LPO in ALC from 6 samples from each group was measured using the EZAssay TBARS Estimation Kit for Lipid Peroxidation (Hi-Media, Mumbai, India). The samples

were rinsed, resuspended in phosphate-buffered saline, and sonicated for 15-20 seconds. The supernatant was collected by centrifugation of the tissue homogenate at 5000 rpm for 5 min, to estimate LPO according to the manufacturer’s instructions.

Statistical analysis

GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA, USA) was used to perform statistical analyses. The comparison between different groups was carried out using one-way analysis of variance (ANOVA). Statistical significance between groups was assessed by the Mann–Whitney U test. To evaluate the correlation between LPO and miR-638 expression in ALC, Pearson's correlation coefficient was employed. A p-value of <0.05 indicated statistical significance.

Results

Patient profile

A total of 60 individuals, with 20 participants in each group, were included in this research project. Two miRNA samples from every group underwent miRNA array analysis, while 12 samples were analyzed using qRT-PCR to validate the findings. Additionally, six samples from each group were utilized for the lipid peroxidation (LPO) test. The average age of the participants was 63.05±9.12 years, with the control group at 68.50±9.09 years, XFS group at 69.15±8.24 years. There were no significant differences in age between the groups (p=0.0643). Similarly, there were no significant gender variations among the groups (p=0.2314) (Table 1).

miRNA microarray analysis

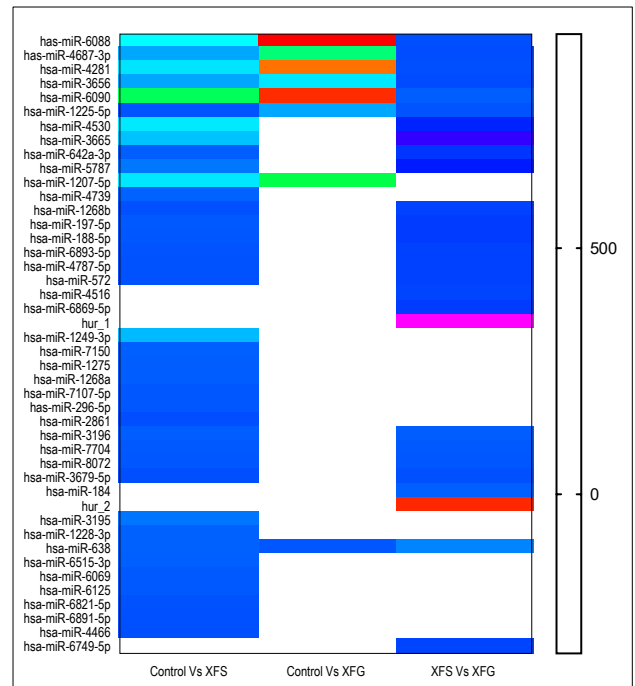
The current research project carried out an extensive miRNA microarray analysis, identifying a total of 2,550 miRNAs across three distinct study groups. Out of these 2,550 miRNAs, only 44 were found to have varying levels of expression (shown in Fig.1), indicating differing expression levels in at least one group when compared to others. Specifically, 16 miRNAs showed significant

variances between the control group and XFS groups, while six miRNAs exhibited significant differences between the XFS and XFG groups (with a fold change greater than 2 and a p-value of less than or equal to 0.05). There were no unique miRNAs significantly differing between the control and XFG groups. Moreover, seven miRNAs—namely miR-3656, miR-4687-3p, miR-6088, miR-4281, miR-1225-5p, miR-6090, and miR-638—showed significant differences in expression levels across all study groups when compared against each other (shown in Fig. 2).

Validation of miRNA microarray profile by qRT-PCR

In order to validate our miRNA microarray data and assess the expression of clinically significant miRNAs, we selected 8 miRNAs for qRT-PCR analysis using 12 patient samples per group. Out of these, miR-6515-3p, miR-4687-3p, and miR-638 displayed consistent expression patterns matching the microarray data. Particularly, compared to the control group, these three miRNAs exhibited a notable increase in expression in both XFS and XFG groups, with p-values of ≤0.001 for miR-6515-3p and ≤0.0001 for miR-4687-3p and miR-638 as depicted in Figure 3. Furthermore, miR-6088, miR-6749-5p, miR-6869, miR-184, and miR-4516 were also validated by qRT-PCR. Nevertheless, their expression profiles did not align with our miRNA microarray data. Detailed expression profiles for these miRNAs can be found in Supplementary Figure 1

Figure 1: Differential expression analysis of miRNA microarray data from ALC of Control, XFS and XFG group



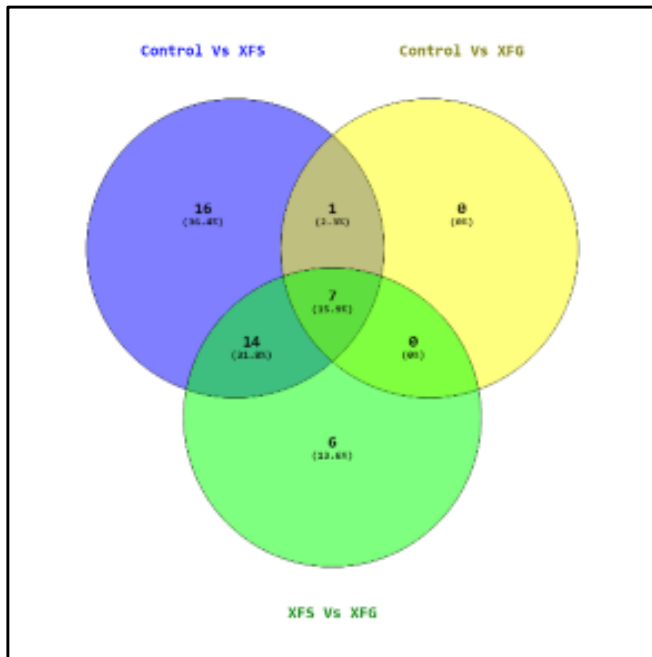
Expression levels are significantly different between XFS (pseudoexfoliation syndrome), XFG (pseudoexfoliation glaucoma) and control (Fold change (FC) ≥ 2 and p ≤ 0.05, t-test).

Table 1: Demographic data of patients enrolled in different groups

		Controls	XFS	XFG	Total	p-Value
Age	N	20	20	20	60	0.0643 ^a
	Mean (S.D.)	63.05 (9.122)	68.50 (9.093)	69.15 (8.242)		
	Median	63	69.5	71.5		
Gender, n%						0.2314 ^b
	Female	9 (45%)	6 (30%)	4 (20%)	19 (31.6)	
	Male	11 (55%)	14 (70%)	16 (60%)	41 (68.4)	

XFS= Pseudoexfoliation syndrome; XFG= Pseudoexfoliation Glaucoma. a= ANOVA b= Chi-Square Test

Figure 2: Venn diagram showing the common and unique microRNAs expressed in anterior lens capsule of control, XFS and XFG samples



XFS= Pseudoexfoliation syndrome; XFG= Pseudoexfoliation Glaucoma

Identification and selection of miR-638 targets for validation

Potential targets of miR-638 were discovered through the utilization of online bioinformatics tools such as miRTarBase, TarBase v.8, and miRWalk 2.0. Notably, miRTarBase and TarBase identified 53 targets linked to miR-638, whereas miRWalk pinpointed 52 targets. Among these, the transcription factors SP2 and SOX2 (SRY-Box Transcription Factor 2) were consistently identified as common targets across all three tools. Detailed analysis with Diana microT-CDS revealed the miR-638 binding site within the 3'UTR region of the SOX2 gene, upheld by a 0.5 threshold (shown in Fig.4.). Subsequently, the top seven targets identified by miRTarBase, which included SOX2, alongside SP2, OSCP1, PLD1, CDK2, STARD10, and TP53INP2, were chosen for validation using real-time PCR. Extensive information regarding the targets detected through bioinformatic methods can be accessed in Supplementary Tables 3–5.

Gene Expression analysis of target genes

Real-time qPCR analysis was conducted, revealing a decrease in the gene expression of SOX2 in XFG and XFS in comparison to control samples. The difference in SOX2 expression between the XFG and control groups was found to be highly significant ($p < 0.001$) (shown in Fig. 5). Conversely, the variation in expression between the XFS group and the control group did not achieve statistical significance. Furthermore, the study involved the evaluation

of STARD10, TP53INP2, CDK2, PLD1, OSCP1, and SP2 gene expression within the respective study groups, with the results detailed in Supplementary Figure 2. In addition, an examination of the cellular components, molecular functions, and biological processes' classification, along with Kegg pathway enrichment analysis, was carried out. The detailed outcomes of these analyses can be found in Supplementary Figure 3.

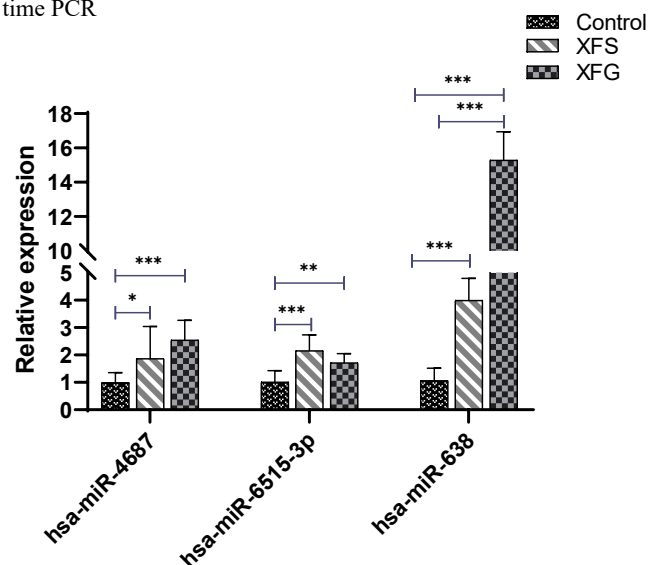
Lipid peroxidation analysis

The analysis of oxidative stress was conducted through Lipid Peroxidation (LPO) testing. The results indicated a significant increase in LPO levels among XFS and XFG samples compared to the control groups ($p = 0.0126$ and $p < 0.001$, respectively). However, the difference in LPO levels between XFS and XFG was not as prominent ($p = 0.0154$), implying that factors beyond oxidative stress may contribute to the progression from XFS to XFG. Additionally, a substantial positive relationship was noted between LPO and miR-638 expression in both XFS and XFG samples ($r^2 = 0.5866$; $p = 0.0037$; shown in Fig. 6). This suggests a potential correlation between lipid peroxidation and miR-638 expression in the development of XFS and XFG.

Discussion

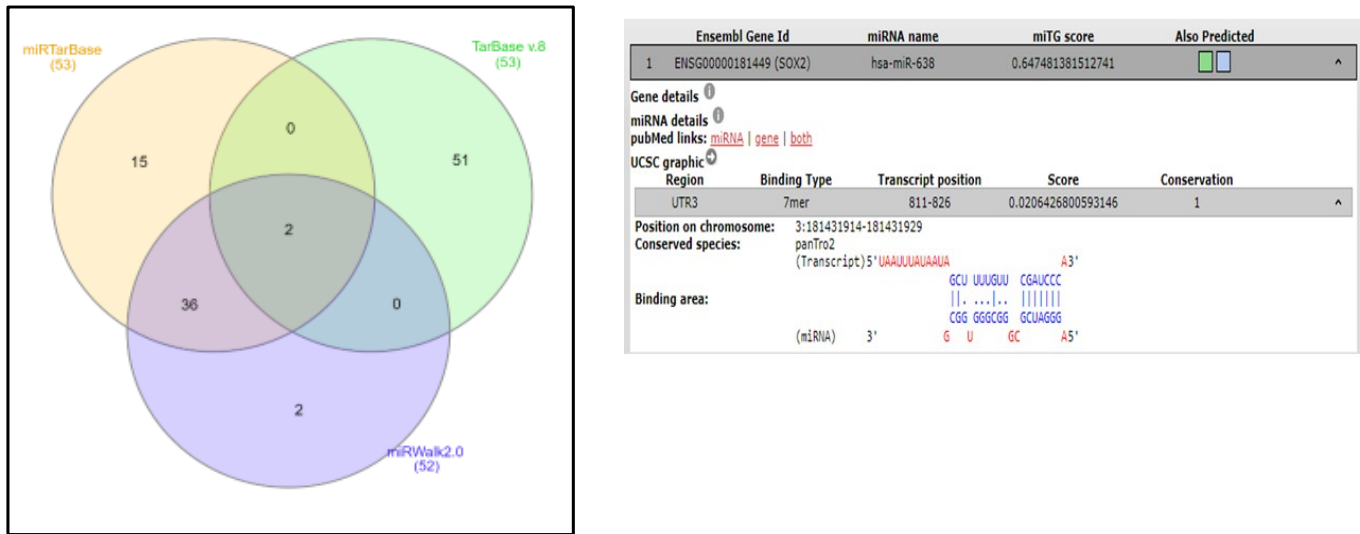
XFS is a condition related to aging that is defined as an abundance of extracellular matrix, particularly in the tissues of the eye [19, 20]. Patients with XFS face an increased risk of transitioning from ocular hypertension to glaucoma, damage to the optic disc and a higher incidence of blindness[5]. miRNAs play a crucial role in regulating gene expression,

Figure 3: Quantitative real-time PCR validation of microRNA expression in anterior lens capsule. Relative expression of microRNAs in control, XFS and XFG samples was done by real – time PCR



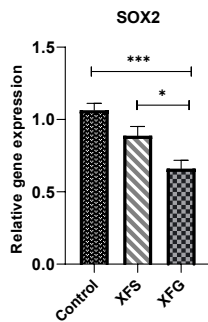
XFS= Pseudoexfoliation syndrome; XFG= Pseudoexfoliation Glaucoma. n=12 * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$

Figure 4: miR-638 target selection by using bioinformatic tools.



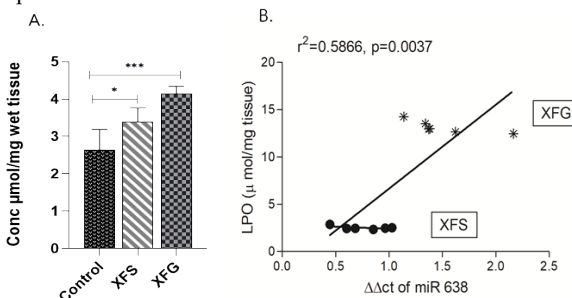
a. Venn diagram showing miRNAs targets detected by miRTarBase, Tarbase and miRWalk. Two targets viz. SOX2 and SP2 were common find in tools used. b. Predicted binding site of miR-638 in the 3'UTR of SOX2 were predicted with Diana microT-CDS available at http://diana.imis.athenainnovation.gr/DianaTools/index.php?r=microT_CDS/.

Figure 5: Quantitative real-time PCR validation of SOX2 expression in anterior lens capsule by real – time PCR.



XFS= Pseudoexfoliation syndrome; XFG= Pseudoexfoliation Glaucoma. n=12
 *=p<0.05; ***= p<0.001

Figure 6: Oxidative stress measurement in anterior lens capsule by lipid peroxidation



A. Oxidative stress measured as lipid peroxidation (LPO) in anterior lens capsule of Control, XFS and XFG samples. B. Positive relation between miR-638 expression and LPO in XFS and XFG (p = 0.0037; r = 0.7659; r² = 0.5866). Data was analysed by one-way ANOVA; R² was calculated to evaluate the correlation.

XFS= Pseudoexfoliation syndrome; XFG= Pseudoexfoliation Glaucoma. n=6
 *=p<0.05; **=p<0.01; ***=p<0.001

with their examination in various ocular tissues emphasizing their involvement in eye development, maintenance of internal balance, and illnesses[12]. In this study, miRNA microarray analysis was conducted on the anterior lens capsule from XFS and XFG tissues. Out of 2550 miRNAs identified, only 44 miRNAs displayed differing expressions among the three groups of samples. Through qPCR validation, a notable upregulation of miR-638 was observed in XFS and XFG compared to the controls, reinforcing the data from the miRNA array. The expression of miR-638 is evolutionarily preserved in both human and non-human primates, with its implications for the eye largely unexplored[21, 22]. Studies have suggested that miR-638 may influence gene expression networks linked to oxidative stress response and aging in lung tissue affected by emphysema[23]. Given the crucial role of oxidative stress in the development of XFS and glaucoma, the increased expression of miR-638 in these conditions may contribute to heightened oxidative stress responses in pseudoexfoliation, thereby playing a role in disease advancement[24].

Three different algorithms highlighted the SP2 and SOX2 genes as potential targets of miR-638. Target validations conducted through mirTarBase revealed a key interaction of miRNAs with target genes, supporting this finding. The transcription factor SOX2 is vital for maintaining undifferentiated embryonic stem cells' pluripotency and is pivotal throughout the eye development stages[25]. The study demonstrated significantly diminished levels of SOX2 and heightened expression of miR-638 in the anterior lens capsule of XFG patients in comparison to controls. The observed link between reduced SOX2 expression and aging across various tissues in mice and humans further underscores this association. Furthermore, Lipid Peroxidation (LPO) was

assessed to monitor oxidative stress levels in the samples. The degradation of lipids by reactive oxygen species leads to LPO, producing lipid peroxides due to lipid breakdown. Evidence indicates disrupted oxidative-antioxidative balance in XFS and XFG patients, with reduced levels of antioxidative enzyme content, ascorbic acid, glutathione, and trace elements in the aqueous humor[26, 27]. Studies have shown that oxidative stress promotes miR-638 upregulation through radiation-induced Dynamin2 (Dnm2) transcription[28]. This study reaffirmed that oxidative stress induces an increase in miR-638 expression in the anterior lens capsule of XFS and XFG patients in comparison to controls. Additionally, a positive correlation was established between LPO and miR-638 expression in XFS and XFG, suggesting that miR-638 may be involved in oxidative stress-induced XFG progression.

Strengths and Limitations

This research presents a microRNA profile with differential expression in the anterior lens capsule of XFS and XFG samples within the Indian population. Past studies have largely concentrated on the significance of aqueous humor in pseudoexfoliation. In this study, patients with hypertension and diabetes were excluded to eliminate the previously observed impact of these conditions on lipid peroxidation[29, 30]. Nevertheless, there are limitations to this current study. Initially, the sample size for the array analysis was limited (2 samples per group). The study could have been more robust with a larger number of samples for the microRNA array. Secondly, the functional relevance of predicted miRNA-mRNA target pairs was not experimentally validated through a reporter assay. Despite this, the miRNA microarray findings were confirmed through real-time PCR, and their functionality was assessed by examining their impact on the gene expression of target genes.

Conclusion

The latest data indicates that the varied expression of miRNA could be fundamental in the pathophysiology of XFG. In particular, the interaction between miR-638 and its potential target SOX2 gene may contribute to XFG by disturbing the balance between oxidation and antioxidation. Nevertheless, further investigations involving larger groups of patients and more experimental data are necessary to validate the initial findings of this study.

Statements

Statement of Ethics

Study approval statement: This research was reviewed and approved by the Institutional Ethics Committee of the Post Graduate Institute of Medical Education and Research (approval no. IEC-02/2015-156) and complied with the Declaration of Helsinki.

Consent to participate statement: Each patient provided written informed consent was obtained from each patient who participated in the study after being briefed about the study.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

A.K. did all the experiments, analysed all the data and compiled the results, wrote the manuscript, plotted graphs and made figures; M.K. checked and commented on the manuscript; J.R., F.T. and P.C.G. did cataract surgeries and provided anterior lens capsule for experimentation; G.K. checked and edited the manuscript, N.S. reviewed the manuscript; S.S.P supervised the research and reviewed the manuscript.

Data Availability Statement

Data is available upon request by contacting the corresponding author at (kheraalka@yahoo.com).

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Please follow supplementary file here