MicroRNAs as biomarkers in glaucoma and potential therapeutic targets

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Abstract

Glaucoma is a neurodegenerative disease in which optic nerve damage and visual field defects occur. It is a leading cause of irreversible blindness. Its pathogenesis is largely unknown although several risk factors have been identified, including increased intraocular pressure (IOP), age, race, family history, medical conditions, physical injuries to the eye, corticosteroid use, and other eye-related risk factors such as retinal detachment. The conventional pathway consists of the trabecular meshwork, uvea, and sclera. The disease in most cases is connected with raised IOP (>21 mm Hg), there are cases with low IOP values and continuous progression. The normal IOP range in healthy individuals is 8-18 mm Hg (Wang et al., 2018). The lowering of IOP of glaucoma patients is currently the only proven treatment strategy (Lusthaus and Goldberg, 2019) and which has an effect on topographic pressure and ophthalmic examination of the eyes should be investigated using suitable animal models of glaucoma.

Key Words: aqueous humor; biomarkers; blood plasma; blood serum; glaucoma; intraocular pressure; microRNA; peripheral blood mononuclear cells; tears; therapeutic targets

Introduction

Glaucoma is a neurodegenerative disease in which optic nerve damage and visual field defects occur. It is a leading cause of irreversible blindness (Jonas et al., 2017) affecting more than 60 million people worldwide and predicted to affect 112 million by 2040 (Tham et al., 2014). The number of undetected cases will also increase. Vision loss is due to loss of retinal ganglion cells (RGCs) and degeneration of the optic nerve, which has a pronounced effect on independent living and quality of life. Its pathogenesis is largely unknown although several risk factors have been identified, including increased intraocular pressure (IOP), age, race, family history, medical conditions, physical injuries to the eye, corticosteroid use, and other eye-related risk factors such as retinal detachment. The conventional pathway consists of the trabecular meshwork, uvea, and sclera. The disease in most cases is connected with raised IOP (>21 mm Hg), there are cases with low IOP values and continuous progression. The normal IOP range in healthy individuals is 8-18 mm Hg (Wang et al., 2018). The lowering of IOP of glaucoma patients is currently the only proven treatment strategy (Lusthaus and Goldberg, 2019), and which has an effect on topographic pressure and ophthalmic examination of the eyes should be investigated using suitable animal models of glaucoma.

Open-angle glaucoma is the most common form of the condition, accounting for ~90% of all cases of glaucoma (Healthline 2018), with primary open-angle glaucoma (POAG) and exfoliation glaucoma (XFG) being the most frequent types (Ritch, 1994; Gupta and Weinreb, 1997; Weinreb and Khaw, 2004). POAG is the most common form of glaucoma, appearing in two-thirds of all cases (Auckland Eye) and has the presence of the glaucomatous optic neuropathy without any identifiable secondary cause (Kwon et al., 2009; Liu and Allingham, 2011). Closed-angle glaucoma is much less common and comprises less than 20% of cases in the United States. Primary angle closure glaucoma (PACG) is the most severe stage of primary angle closure disease (Xu et al., 2018).

The term pseudoexfoliation (PFX) has been used by some groups to describe the accumulation of exfoliative material in different parts of the eye causing glaucomatous neurodegeneration and irreversible blindness (Ekström and Alm, 2008). Abnormal extracellular matrix remodeling together with the accumulation of protein aggregates in the ocular tissues gradually causes progressive fibrosis leading to trabecular meshwork dysfunction, obstruction to aqueous humor outflow, and raised IOP (Ekström and Alm, 2008; Aboobakar et al., 2017). Clinically, an eye with PFX disease may originally lack obvious PFX deposits (unmanifest disease). This progresses to manifest PFX with normal IOP; then the stage with raised IOP (ocular hypertension, OHT), and lastly to pseudoexfoliative glaucoma (PEXG) with irreversible optic nerve/visual field damage. PFX and PEXG are associated with changes in the peripheral blood, therefore making it possible to identify putative markers indicating progression to the next stage and identifying “eyes at risk” (Vessani et al., 2003; Aboobakar et al., 2017; Rao et al., 2020). With reference to the previous paragraph, PFX is equivalent to XFS (Mitchell et al., 1999) and PEXG to XFG (Álvarez et al., 2015). To be consistent herein in the manuscript, the terms XFS and XFG have been used.

The production and outflow of aqueous humor in the anterior segment of the eye maintains IOP levels. This clear fluid is responsible for maintaining the shape and optical properties of the eye while also providing nutrients and removing waste from the anterior segment tissues. Aqueous humor is secreted by the ciliary epithelium and exits the eye via two outflow pathways: the conventional pathway and the unconventional pathway (Coca-Prados and Escribano, 2007; Goel et al., 2010). The conventional pathway consists of the trabecular meshwork and Schlemm’s canal tissues and accounts for about 80% of the aqueous humor drainage in older adults (Goel et al., 2010). Decreased outflow through these tissues is the main contributor to the elevated IOP levels in glaucoma (Goel et al., 2010; Stamer and Azcot, 2012). With XFG, the abnormal fibrially material found in the anterior segment can accumulate along the conventional outflow pathway, leading to disorganization and degeneration of the trabecular meshwork and Schlemm’s canal and elevation of IOP (Schützer-Schrehardt and Naumann, 2006). Such elevated IOP levels are a major risk factor to RGC degeneration and, if left untreated, visual impairment.
To date, the early diagnosis of POAG remains unsatisfactory which aggravates the global burden of glaucoma. Although many linkage analyses and genome-wide association studies (GWAS) have attempted to unravel the genetics of glaucoma, their genetic associations were identified for less than 10% of all glaucoma cases (Shastry, 2013; Wang and Wiggs, 2014; Liu and Allingham, 2017). Therefore, other genetic factors such as microRNAs (miRNAs) are likely to be involved in the disease pathogenesis (Gonzalez et al., 2014; Jayaram et al., 2015; Kong et al., 2014). Among the miRNAs associated with RNA-binding proteins and contained within extracellular vesicles including exosomes (Dunmire et al., 2013; Tanaka et al., 2014; Dismuke et al., 2015; Wecker et al., 2016). Since extracellular vesicles released from the clinal body may contribute to outflow pathway signaling, miRNAs could play a role in the outflow pathway (Lerner et al., 2017). Identification of such biomarkers may help to characterize and stratify the severity of outflow dysfunction and responses to treatment. Also, biomarkers of this type may identify other potential targets to resegment IOP and be used as the phenotype of a specific individual’s outflow facility and perhaps predict responses to therapeutic intervention.

MicroRNAs (miRNAs) are single-stranded non-coding RNA molecules approximately 22 nucleotides long that recognize sequences in the 3’-untranslated regions (3’-UTR) of target mRNAs and either induce mRNA degradation (Barg et al., 2005) or inhibit their translation (He and Hannon, 2004; Meister, 2007). MiRNAs have been found to be dysregulated in a variety of diseases and disorders (Peplow et al., 2019), and may play important roles in the pathogenesis of POAG (Kong et al., 2014; Ran et al., 2015; Zhang et al., 2017; Molasy et al., 2017). For example, miR-155-3p and miR-24 are involved in gene regulation in trabecular meshwork cells (Luna et al., 2013). Moreover, the miRNA expression levels have been linked to maintaining the balance of the aqueous humor, the barrier in the trabecular meshwork, and the apoptosis of RGCs (Jayaram et al., 2015, 2017; Drewry et al., 2016). Several miRNAs (e.g., miR-29b, miR-200c, miR-204, and miR-24) are also reported as potential diagnostic biomarkers for therapeutic intervention for glaucoma in humans (Gonzalez et al., 2014; Molasy et al., 2017). Recent reviews have described the mechanism of miRNAs in POAG regarding elevated IOP and optic nerve damage (Wang et al., 2021) and the relationship between miRNA and the trabecular meshwork (Guo et al., 2017).

Only a very small volume of aqueous humor (~100 μL) can be collected at optical coherence tomography (e.g., cataract or glaucoma surgery). Blood plasma or serum is more suitable for the identification of possible biomarkers in the early detection of glaucoma and response to therapy, as it can be obtained via a minimally invasive procedure and can be collected at frequent intervals to monitor changes in the levels of biomarkers with disease progression and response to treatment. Recently, elevated serum antibodies against autoantigens in ocular tissues have been suggested as biomarkers for diagnosing glaucoma and distinguishing between normal-tension and high-tension glaucoma patients (Shin et al., 2021). In addition, autotaxin and transforming growth factor-β levels in aqueous humor were promising diagnostic biomarkers for distinguishing open-angle glaucoma subtypes (Igarashi et al., 2014). We chose to analyze recent literature on the expression levels of miRNAs measured in aqueous humor, blood samples, and tears in glaucoma which could serve as diagnostic biomarkers to distinguish from controls and between subtypes, monitor disease severity, and as potential therapeutic targets.

**MicroRNAs in Glaucoma**

We performed a PubMed search for original research articles published during January 2014–October 2021 on possible miRNA biomarkers of glaucoma compared to healthy controls (usually cataract patients) in aqueous humor, blood plasma, or tears. In total, we examined 15 articles to determine whether they could distinguish between various glaucoma subtypes. The steps involved in the review and its contents are shown (Figure 1). A total of 15 articles were found for this review. Of these, 8 had used aqueous humor, 3 aqueous humor and blood plasma, 1 blood serum, 1 tears, 1 peripheral blood mononuclear cells, and 1 GWAS data. The relevant findings in the research articles from the PubMed search are summarized as follows.

**Aqueous humor**

RNA sequencing was used by Seong et al. (2021) to analyze aqueous humor from 6 normal-tension glaucoma patients and 7 control subjects. Each glioclumic population was defined using one topologically distinct expression. Eighteen miRNAs were significantly upregulated compared to controls: let-7a-5p, let-7c-5p, let-7f-5p, miR-192-5p, miR-10a-5p, miR-10b-5p, miR-375, miR-143-3p. No significantly downregulated miRNAs were found. The results of RNA sequencing were verified using qPCR, which showed significantly increased let-7c-5p expression compared to controls.

Kosior-Jarecka et al. (2021) selected 22 miRNAs for analysis based on previous publications that showed them to be the most abundant in aqueous humor. The groups studied were 19 POAG, 14 XFG, 9 PACG, and 36 cataract patients as control. Using RT-PCR and microarray assay, 8 miRNAs were detected in at least 25% of the samples regardless of the glaucoma group. Three miRNAs were miR-1260b, miR-4634 and miR-4634 were detected in at least 20% of samples in all studied groups. The most frequently expressed miRNA in the studied panel was miR-1260b, which was detected in 19 POAG (100%), 13 XFG (92%), and 7 PACG (20%) significantly higher than that of any other miRNA. In comparison, miRNAs were detected in a minimum of three samples of two glaucoma subgroups and cataract group, enabling differential expression analysis. No significant differences were observed in the frequency of expression and level of expression for these miRNAs. However, there was a tendency for different expression levels of miR-184 and miR-672-3p. The downregulated miRNAs had AUC values between 89% and 90%, suggesting they might have potential as diagnostic biomarkers of POAG. The upregulated miRNAs had AUC values between 0.51 and 0.56 and did not distinguish glaucoma patients from controls. The combination miR-143-3p and miR-221-3p had AUC 0.96, specificity 89%, specificity 100%, identifying it as a good test to distinguish between POAG and controls.

Using small RNA sequencing, Hubens et al. (2021) examined samples from 9 POAG and 10 cataract patients. The POAG group included patients with early, moderate, advanced, and severe glaucoma (Milis et al., 2006), and most POAG patients were on at least two types of topical IOP lowering medication. 262 miRNAs were identified of which 62 were detected in at least 60% of the samples. Of these, 7 miRNAs were significantly differentially expressed, with 4 being upregulated miR-30a-3p, miR-143-3p, miR-211-3p, miR-221-3p, and 3 being downregulated miR-451a, miR-486-5p, miR-92a-3p in POAG. Of the expression levels of the 7 differentially expressed miRNAs, that of miR-143-3p correlated weakly with IOP. None of them correlated significantly with disease severity (mean deviation) or disease progression (mean deviation loss per year). By receiver operating curve (ROC) analysis, the upregulated miRNAs had AUC values between 0.77 (miR-211-3p) and 0.89 (miR-143-3p, sensitivity 89%, specificity 89%) suggesting they might have potential as diagnostic biomarkers of POAG. The downregulated miRNAs had AUC values between 0.51 and 0.56 and did not distinguish glaucoma patients from controls. The combination miR-143-3p and miR-221-3p had AUC 0.96, specificity 89%, specificity 100%, identifying it as a good test to distinguish between POAG and controls.

Using the limma package of the R software for statistical analysis to establish differentially expressed IncRNA and miRNA, Zhou et al. (2020) analyzed data from the Gene Expression Omnibus with 10 aqueous humor samples collected from POAG patients and 10 aqueous humor samples from cataract patients. POAG patients did not receive any glaucoma medication 4 months before surgery and had uncontrolled IOP. Both IncRNAs and miRNAs that were negatively correlated with certain common miRNAs were defined as candidate circRNA (competitive endogenous RNA) pairs. A total of 4130 differentially expressed RNA was identified. Eight miRNAs (miR-508 and p53 down-regulated) and 3089 were miRNA (2135 up- and 954 down-regulated). 9 miRNAs (miR-20b-5p, miR-761, miR-17-5p, miR-338-3p, miR-24-3p, miR-125b-3p, miR-3619-5p, miR-129-5p, and miR-27a) and 4 IncRNAs (RNAID27-AS1, AS1 F213898, OIP5-AS1, and SNA292P) were established as hub RNAs in the ceRNA network. Comparing the differences in the degree, closeness, and betweenness centrality among IncRNAs, miRNAs, and miRNAs that showed IncRNAs and miRNAs had a higher degree, closeness, and betweenness centrality than miRNAs, indicating that IncRNAs and miRNAs tended to be pivotal to the risk of POAG.

Two cohorts of patients were recruited by Hindle et al. (2019) with cohort 1 being Caucasian and consisting of 17 POAG and 11 cataract patients, while cohort 2 was Japanese and comprised 13 XFG, 3 XFG, and 22 cataract patients. In the initial miRNA selection analysis, 9 samples from cohort 1 were included. In the positive set analysis, all of cohort 2 plus additional 19 participants from cohort 1 were included. By RT-PCR, 73 miRNAs were detectable in every sample of aqueous humor. 6 of the 20 miRNAs shown to be elevated in the...
blood plasma of glaucoma and XFS patients (described in the section blood plasma) were also significantly increased in aqueous humor from the same subject: miR-637, miR-99b-3p, miR-4725-3p, miR-4724-5p, miR-4358, miR-446, miR-464, miR-3173-3p, miR-608, miR-4725-3p, miR-4448, miR-323b-5p, miR-4538, miR-3913-5p, miR-3159, miR-4663, miR-4767, miR-4724-5p, miR-1306-5p, miR-181b-5p, miR-433-3p. There were no interaction effects in two-way ANOVA comparison between disease and cohort factors for any of miRNAs were expressed solely in the POAG samples. The expression of miR-184, miR-486-5p, and miR-93-5p using qPCR agreed with the sequencing results.

A NanoString assay was used in a discovery set of 12 POAG, 12 XFG and 11 cataract patients by Drewry et al. (2018). Two miRNAs were differentially expressed in POAG compared to control, miR-122-5p and miR-124-3p (upregulated). Five miRNAs were differentially expressed in XFG vs. control: miR-122-5p, miR-3144-3p, miR-320e, miR-630 (upregulated), miR-320a (downregulated). Two miRNAs were differentially expressed in XFG vs. cataract, miR-122-5p (upregulated), miR-320a (downregulated). The clustering separated the 3 sample types and identified 2 miRNAs among the top 20 plasma biomarkers, miR-210-3p (upregulated) and miR-451a (upregulated). Five miRNAs were differentially expressed in XFG vs. control: miR-122-5p, miR-3144-3p, miR-320e, miR-630 (upregulated), miR-320a (downregulated). Two miRNAs were differentially expressed in XFG vs. cataract, miR-122-5p (upregulated), miR-320a (downregulated). Three miRNAs were differentially expressed in XFG vs. POAG: miR-125b-5p (upregulated), miR-320a, miR-302d-3p (downregulated).

Jayaram et al. (2017a) analyzed samples from 6 POAG and 8 cataract patients using PCR. The POAG patients had a mean of 1.2 topical medications; 2 had dry AMD as ocular comorbidity and 1 had retinal detachment repair. None of the cataract patients were using topical medications; 1 had dry AMD as ocular comorbidity and 1 had retinal detachment. In comparison, no miRNA was significantly upregulated and miR-660 significantly downregulated in samples from all POAG samples compared with controls. Three miRNAs miR-135a, miR-9, and miR-128a were consistently expressed in controls but not detected in POAG patients. No miRNAs were expressed solely in the POAG samples.

Aqueous humor from 4 cataract Caucasian subjects was analyzed by Wecker et al. (2018). By qPCR, 3 miRNAs were identified in the samples, miR-451a, followed by miR-184, miR-16-5p, and miR-464. By qPCR, 3 miRNAs were analyzed in 5 independent aqueous humor samples. The ones chosen were miR-451a as the most abundant in NGS, miR-144 as of intermediate abundance, and miR-184 which was not detected by NGS in the samples but has been described as the most abundant miRNA in aqueous humor in pooled samples using qPCR (Dunnire et al., 2013). The mean Ct values obtained were 27.9, 32.7, and 37.5 for miR-451a, miR-144, and miR-202, respectively, thereby confirming the relative expression pattern obtained using NGS.

Buys et al. (2015) analyzed samples from 6 POAG patients of which 3 had peripheral visual field loss (PVFL) and 3 had early parasellar visual field loss (ePaVFL). POAG: miR-125b-5p (upregulated), miR-302d-3p (downregulated). Using a validation set of 17 POAG, 14 XFG and 10 cataract patients, 3 miRNAs were differentially expressed in POAG vs. control: miR-451a, miR-302d-3p (upregulated), miR-125b-5p (downregulated). Five miRNAs were differentially expressed in XFG vs. control: miR-122-5p, miR-3144-3p, miR-320a, miR-630 (upregulated), miR-320a (downregulated). Three miRNAs were differentially expressed in XFG vs. POAG: miR-125b-5p (upregulated), miR-320a, miR-302d-3p (downregulated).

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Comparing phenotypes of XFS (classical and radial pigmentary) with controls, miR-144-3p showed differential downregulation in pigmentary phenotype of XFS. In the validation stage by qPCR, the miRNAs related to fibrosis were validated in various disease stages which confirmed the role of miR-122-5p which was upregulated in XFG compared to XFS with OHT. A total of 19 other miRNAs were found to be related to fibrosis or TGF-β1 pathway and were significantly upregulated in later stages of XFG (miR-26a-5p, miR-101-3p, miR-124-2-3p, miR-138-3p, miR-195a-5p, miR-199a-3p, miR-223-3p, miR-1-3p, miR-200a-3p, miR-204-5p, miR-208a-3p, miR-215-5p, miR-338-5p, miR-449a, miR-449b-5p, miR-5011-5p, miR-661). MiR-19a-3p and miR-30a-5p related to proteoglycans were significantly upregulated in XFG compared to XFS with OHT.

in the study by Rao et al. (2020) it was not reported what the group sizes were for the discovery and validation stages or whether patients in the discovery stage were also included in the validation stage.

Tears
Raga-Cervera et al. (2021) collected reflex tears from the inferior meniscus of the eye without instilling anesthetics from 20 POAG and 22 OHT as controls (eyes with elevated IOP but not displaying optic disc damage or altered visual field). Using NGS, 95 miRNAs were identified as present in tears of POAG and OHT patients. Of these, 6 miRNAs were upregulated (miR-26b-5p, miR-27a-3p, miR-152-3p, miR-30e-5p, miR-125b-2-5p, miR-224-5p), and 2 miRNAs downregulated (miR-151a-3p, miR-1307-3p) in tears from POAG patients compared to controls. By ROC analysis, the AUC value of 4 of the 8 miRNAs (miR-26b-5p, miR-30e-5p, miR-151a-3p, miR-152-3p) was > 0.75, so they could be considered as fair tests to distinguish POAG from OHT patients.

The study by Raga-Cervera et al. (2021) should be considered as a pure discovery study as there was no validation cohort.

Genome-wide scan
From the recent RWAS in glaucoma endophenotypes provided by the International Glaucoma Genetics Consortium was used by Ghanbari et al. (2017) to examine the association of miRNA-related genetic variants with POAG endophenotypes. The association was examined of 411 miRNA variants (in 332 miRNA genes) with IOP, VCDR (vertical cup-to-disc ratio), cup area and disc area. Two miRNA variants passed the Bonferroni-corrected significance threshold of 1.22 × 10^{-7} (0.05/411). Genetic variants in the miR-612 precursor and in the miR-4707 seed region were significantly associated with VCDR and cup area. The variant in miR-612 has been previously demonstrated to increase miR-612 expression (Kim et al., 2012). While the variant in miR-4707 does not affect miRNA expression itself, it does bind the miHDL of one of its glaucoma-associated target genes, CARD10.

Those miRNAs found to have altered expression in aqueous humor, blood plasma, blood serum, peripheral blood mononuclear cells, and tears in glaucoma and its subtypes are summarized in Tables 1 and 2.

Discussion
Primary open-angle glaucoma is the most common form of glaucoma, develops slowly and usually without any symptoms. Many people are unaware that they have this condition until they have significant vision loss, with peripheral vision being initially affected but which may advance to central vision loss. If patients have this condition until they have significant vision loss, with peripheral vision being initially affected but which may advance to central vision loss. If patients

Recent research studies have indicated miRNAs as diagnostic and prognostic advantages to have a blood-based screening test for glaucoma that could be used when specialized eye examination facilities are unavailable. Several studies have attempted to identify biomarkers of glaucoma in aqueous humor but collecting such fluid from patients is associated with high risk and cannot be performed regularly. Also, there is possible contamination with blood or tears during its collection.

These results of the study by Raga-Cervera et al. (2021) suggest that miRNAs can be used as potential biomarkers in the diagnosis of glaucoma and its subtypes. However, the results need to be validated in a larger-scale study to confirm their diagnostic accuracy.

In conclusion, miRNAs can be potential biomarkers for the diagnosis of glaucoma and its subtypes. Further research is needed to validate these results in a larger population and to understand the mechanisms behind the dysregulation of these miRNAs in glaucoma.

Several important limitations were identified in these recent studies. (i) Many had used very small-sized groups with some being as limited as 3 or 6 in number (Table 3). Only one study had performed a sample size calculation and used group sizes of 20 POAG and 22 OHT patients (Raga-Cervera et al., 2021). (ii) Heterogeneity of the glaucoma phenotypes evaluated was evident in some studies, e.g., the POAG group included patients with early, moderate, advanced, and severe glaucoma (Hubens et al., 2021), or the experimental group comprised POAG patients and controls (Kosior-Jarecka et al., 2021). (iii) Marked differences in age and gender of groups were seen in some studies, e.g., XFG patients were considerably older than earlier forms of XFS or controls (Rao et al., 2020). Glaucoma patients comprised 6 males and 4 females, whereas the controls comprised 10 males and 10 females (Li et al., 2019). (iv) Differences in IOP levels and the use of topical medication by glaucoma patients were reported e.g., in one study POAG patients were on at least two types of topical IOP lowering medication (Hubens et al., 2021), while in another study POAG patients did not receive any glaucoma medication for 4 months before surgery (Zhou et al., 2020) or were naive to oculocutaneous medical therapy (Rao et al., 2020). (Table 3). It would be helpful to categorize the patients into normal-tension glaucoma and high-tension glaucoma groups. (v) Patients in some of the studies included e.g., Caucasian and Japanese patients (Rao et al., 2020). (vi) In some studies patients had significant comorbidities such as dry AMD (Jayaram et al., 2017) while in other studies comorbid conditions had been excluded. (vii) Inclusion and exclusion criteria were not reported in several of the studies (e.g., Jayaram et al., 2017; Zhou et al., 2020). (viii) Many of the studies are best regarded as discovery studies as no validation cohorts were included (see Baumbach et al., 2020). (ix) Different analytical methods have been used including next-generation sequencing and PCR, and the findings were not always in agreement (Liu et al., 2019). Validation of data should be performed using PCR. (x) Normalization of miRNA data was not included in one of the studies (Zhou et al., 2020). (xi) ROC analysis to indicate which miRNAs are good or fair tests to distinguish glaucoma patients from controls or separate between different glaucoma subtypes was only performed in five of the studies (Tanaka et al., 2014; Hinde et al., 2019; Liu et al., 2019; Hubens et al., 2021; Raga-Cervera et al., 2021). Some of these limitations had been indicated previously (Jayaram et al., 2017b; Li and Wang, 2017).

Treatment with miRNA mimics (agonists) or inhibitors (antagonists) may be a way to increase or lower the expression of selected miRNAs in glaucoma patients and slow the progression of the disease. In preclinical studies, IOP was reduced after intravitreal injection of miRNA (Zhou et al., 2020). Moreover, miR-450 increased MyD88, a myeloid transcription factor, and may influence the contractile component of the trabecular meshwork and the outflow of aqueous humor (Izotti et al., 2015). It was also shown that miR-96 affected the survival and apoptosis of retinal ganglion cells through interaction with caspase-2 (Wang and Li, 2014). Downregulation of miR-100 mediated miR-96 affected the survival and apoptosis of rat RGCs through interaction with caspase-2 (Wang and Li, 2014). Downregulation of miR-100 mediated
**Table 1 | Alterations of miRNA expression in aqueous humor in glaucoma and its subtypes**

| Author          | Analysis method         | Comparison, and number of subjects | Altered miRNA expression                                      |
|------------------|-------------------------|------------------------------------|----------------------------------------------------------------|
| Seong et al., 2021 | RNA sequencing          | NTG 6 vs. control 7                | Upregulated: miR-192-5p, 10a-5p, -10b-5p, -375, -143-3p, -7a-5p, -7c-5p, -7f-5p |
| Kosior-Jarecka et al., 2021 | RT-PCR/microarray       | POAG 19 vs. control 36             | Tended to be downregulated: miR-6515-3p |
| Kosior-Jarecka et al., 2021 | RT-PCR/microarray       | XFG 14 vs. control 36              | Tended to be downregulated: miR-1260b |
| Kosior-Jarecka et al., 2021 | RT-PCR/microarray       | PAGC 9 vs. control 36              | Tended to be downregulated: miR-1260b |
| Hubens et al., 2021 | Small RNA sequencing    | POAG 9 vs. control 10              | Upregulated: miR-30a-3p, -143-3p, -211-5p, -221-3p |
| Zhou et al., 2020 | Gene Expression Omnibus data | POAG 10 vs. control 10          | Hub miRNAs in ceRNA network: miR-20b-5p, -761, -17-5p, -338-3p, -24-3p, -125b-5p, -3619-5p, -129-5p, 27a |
| Hindle et al., 2019 | Real-time PCR            | POAG 17 vs. control 11            | Upregulated: miR-637, -9b-3p, -4725-3p, -4724-5p, -4358, -433-3p |
| Hindle et al., 2019 | Real-time PCR            | XFS+XFG 16 vs. control 22         | Upregulated: miR-637, -9b-3p, -4725-3p, -4724-5p, -4358, -433-3p |
| Liu et al., 2018  | NGS                      | POAG 6 vs. control 6              | Upregulated: miR-205-3p, -200b-3p, -136-3p, -488-3p, -200a-3p, -139-5p, -369-5p, -206, -501-3p, 30c-2-3p, 543-16-5p |
| Liu et al., 2018  | NGS                      | S-POAG 3 vs. M-POAG 3             | Upregulated: miR-205-3p, -206, -16-5p, -501-3p, -409-3p, -200a-3p, -200b-3p, -382-5p, -543, -136-3p, -30c-2-3p, -139-5p, -340-5p, -488-3p, -202-5p, -369-5p |
| Drewry et al., 2018 | PCR                      | POAG 17 vs. control 10            | Upregulated: miR-451a, -302d-3p |
| Drewry et al., 2018 | PCR                      | XFG 14 vs. control 10             | Downregulated: miR-125b-5p |
| Drewry et al., 2018 | PCR                      | XFG 14 vs. POAG 17                | Downregulated: miR-320a |
| Jayaram et al., 2017 | PCR                      | POAG 6 vs. control 8              | Downregulated: miR-518d, -143 |
| Buys et al., 2015  | Qiagen MIHS-3218Z array  | PVFL 3 vs. control 3              | Upregulated: miR-4738-3p |
| Buys et al., 2015  | Qiagen MIHS-3218Z array  | PVFL 3 vs. control 3              | Upregulated: miR-4740-3p |
| Buys et al., 2015  | Qiagen MIHS-3218Z array  | ePaVFL 3 vs. control 3            | Downregulated: miR-4740-3p |
| Buys et al., 2015  | CMHS-02263 array         | PVFL 3 vs. control 3              | Downregulated: miR-4740-3p |
| Tanaka et al., 2014 | Microarray               | POAG+XCG+XFG 10 vs. control 10    | Downregulated: miR-4848, -6515-3p, -3663-3p, -4433-3p, -6717-5p, -4725-3p, -1202, -3197 |
| Hubens et al., 2021 | Small RNA sequencing    | POAG 9 vs. control 10             | No significant differences in miRNA expression |
| Hindle et al., 2019 | Real-time PCR            | POAG 17 vs. control 11            | Upregulated: miR-4667-5p, -9b-3p, -637, -4490, -1253, -3190-3p, -3173-3p, -608, -4725-3p, -4444, -323b-5p, -4538, -3913-3p, -3159, -4663, -4767, -4724-5p, -1306-5p, -181b-5p, -433-3p |
| Hindle et al., 2019 | Real-time PCR            | XFS+XFG 16 vs. control 22         | Downregulated: miR-4724-3p, -4358-3p, -4724-5p, -433-3p |
| Buys et al., 2015  | Qiagen MIHS-3218Z array  | ePaVFL 3 vs. control 3            | Downregulated: miR-122-5p, -320e, -3144-3p, -630 |
| Buys et al., 2015  | Qiagen MIHS-3218Z array  | ePaVFL 3 vs. control 3            | Upregulated: miR-451a, -4469, -760 |
| Buys et al., 2015  | Qiagen MIHS-3218Z array  | ePaVFL 3 vs. control 3            | Downregulated: miR-4798-3p, -940 |

Table 2 | Alterations of miRNA expression in blood plasma, blood serum, tears, PBMCs in glaucoma and its subtypes, and data from genome-wide scan

| Author          | Analysis method         | Comparison, and number of subjects | Altered miRNA expression                                      |
|------------------|-------------------------|------------------------------------|----------------------------------------------------------------|
| Hubens et al., 2021 | Small RNA sequencing    | POAG 9 vs. control 10             | No significant differences in miRNA expression |
| Hindle et al., 2019 | Real-time PCR            | POAG 17 vs. control 11            | Upregulated: miR-4667-5p, -9b-3p, -637, -4490, -1253, -3190-3p, -3173-3p, -608, -4725-3p, -4444, -323b-5p, -4538, -3913-3p, -3159, -4663, -4767, -4724-5p, -1306-5p, -181b-5p, -433-3p |
| Buys et al., 2015  | Qiagen MIHS-3218Z array  | XFS + XFG 16 vs. control 22       | Downregulated: miR-122-5p, -320e, -3144-3p, -630 |
| Buys et al., 2015  | Qiagen MIHS-3218Z array  | ePaVFL 3 vs. control 3            | Downregulated: miR-4724-3p, -4358-3p, -4724-5p, -433-3p |
| Liu et al., 2019  | RT-PCR                  | POAG 33 vs. control 33            | Upregulated: miR-210-3p |
| Rao et al., 2020  | PCR                      | XFG 11 vs. XFS with OHT 11        | Upregulated: miR-124-3p, -424-5p, -122-5p, -30c-5p, -96-5p, -32-5p, -142-5p, -9-5p, -143-3p, -302a-3p, -302b-3p, -223-3p, -19a-3p, -30a-5p |
| Rao et al., 2020  | PCR                      | Pigmentary XFS NA vs. classical XFS NA | Downregulated: miR-144-3p |
| Raga-Cervera et al., 2021 | NGS                   | POAG 20 vs. OHT 22               | Upregulated: miR-26b-5p, -27a-3p, -152-3p, -30e-5p, -125b-2-5p, -224-5p |
| Ghanbari et al., 2017 | Data from GWAS in glaucoma endophenotypes provided by International Glaucoma Genetics Consortium | No significant differences in miRNA expression |

miPaVFL: Primary open-angle glaucoma with early paracentral visual field loss; M-POAG: moderate primary open-angle glaucoma; NGS: next generation sequencing; NTG: normal tension glaucoma; PAGC: primary angle closure glaucoma; PCR: polymerase chain reaction; POAG: primary open-angle glaucoma; PVFL: primary open-angle glaucoma with peripheral visual field loss; S-POAG: severe primary open-angle glaucoma; XFG: exfoliation glaucoma; XFS: exfoliation syndrome. All the listed miRNAs are human miRNAs (hsa-miRs).
Table 3 | Number of subjects in glaucoma and control groups, intraocular pressure and use of topical medication by glaucoma patients

| Author | Intraocular pressure (mmHg) | Use of topical medication by glaucoma patients |
|--------|-----------------------------|-----------------------------------------------|
|        | Glaucoma group | Control group |                               |
| Seong et al., 2021 | 6 NTG | 7 control | Each NTG patient using 1 medication |
|         | 14.8±1.8 | 15.4±2.3 |                               |
| Raga-Cervera et al., 2021 | 22 POAG | 22 OHT | NA |
|         | 16.0±0.6 | 18.2±0.8 |                               |
| Kosior-Jarecka et al., 2021 | 19 POAG | 36 cataract | NA |
|         | 34.2±13.8 | 18.3±2.8 |                               |
|         | 14 POXG | 24.5±7.5 |                               |
|         | 9 PACG | 35.6±9.7 |                               |
| Hubens et al., 2021 | 9 POAG | 10 cataract | Most were on at least 2 types of medication |
|         | 15.2±1.5 | 16.3±1.1 |                               |
| Zhou et al., 2020 | 10 POAG | 10 cataract | No medication |
|         | 27.8±2.1 | 18.8±0.7 | 4 mon before surgery |
| Hao et al., 2020 | 17 PXF | 11 cataract | Without treatment |
|         | 18±5.6 | 12±1.6 |                               |
|         | 11 PXF with OHT | 20±5 |                               |
|         | 11 PXF with PEKG | 28±4.8 |                               |
| Liu et al., 2019 | 33 POAG | 33 controls | Using a mean of 1.3 medications |
|         | 17.4±4.7 | 14.1±3.7 |                               |
| Hindle et al., 2019 | 17 POAG | 11 control | Without treatment |
|         | 23.8 | 19.4 |                               |
|         | 13 XFS+3 XFG | 22 cataract |                               |
| Liu et al., 2018 | 3 M-POAG | 6 cataract | Using a mean of 2.5 medications |
|         | 20.7±5.5 | 13.3±3.9 |                               |
|         | 3 S-POAG | 19.3±7.5 |                               |
| Drewry et al., 2018 | 17 POAG | 10 cataract | NA |
|         | 18.0±0.8 | NA |                               |
|         | 14 XFG | 17.0±6.8 | NA |
|         | 23.3±2.2 | NA |                               |
| Jayaram et al., 2017 | 6 POAG | 8 cataract | Using a mean of 1.2 medications |
|         | 17.8±4.6 | 12.7±2.7 |                               |
| Wecker et al., 2016 | Not included | 4 control | NA |
|         | NA | NA |                               |
| Buys et al., 2015 | 3 PVFL | 3 cataract | NA |
|         | NA | NA |                               |
|         | 3 ePaVFL | NA |                               |
|         | 3 PVFL | 4 control | NA |
|         | NA | NA |                               |
|         | 3 ePaVFL | 3 control | NA |
| Tanaka et al., 2014 | 7 POAG+2 PEXG+1 PACG | 10 control | NA |
|         | 20.5 | 12.7 |                               |

| ePaVFL: Primary open-angle glaucoma with early paracentral visual field loss; M-POAG: moderate primary open-angle glaucoma; NA: not available; NTG: normal tension glaucoma; OHT: ocular hypertension; PACG: primary angle closure glaucoma; PEXG: pseudoxefoliation glaucoma; POAG: primary open-angle glaucoma; PVFL: primary open-angle glaucoma with peripheral visual field loss; PXF: pseudoxefoliation; S-POAG: severe primary open-angle glaucoma; XFG: exfoliation glaucoma; XFS: exfoliation syndrome.

been shown to target SMAD4 and SFRP1 (Kosior-Jarecka et al., 2021), which are involved in the outflow regulatory mechanisms in the anterior chamber. Another possible target is miR-143-3p which was upregulated in three of the studies (Jayaram et al., 2017; Hubens et al., 2021; Seong et al., 2021). MiR-143-3p, as part of the miR-143/miR-145 cluster, is important for the regulation of outflow capacity of the trabecular meshwork (Li et al., 2017). MiR-143-3p is located on the long arm of chromosome 5 (5q32), a locus associated with increased risk for developing POAG (Pang et al., 2006). Two linkage loci associated with glaucoma and IOP (GLC1G, GLC1M) involving the region 5q21-32 have been described (Monemi et al., 2005; Kramer et al., 2006; Pang et al., 2006), and an association was found between IOP and copy number variation at this locus (Nag et al., 2013). Also, miR-125b-5p could be a possible target as it was shown to be a hub miRNA in the ceRNA network (Zhou et al., 2020) and pivotal to the risk of glaucoma. It was downregulated in the aqueous humor of POAG patients compared to controls but upregulated for XFG compared to POAG patients (Drewry et al., 2018) and in tears of POAG patients (Raga-Cervera et al., 2021). Gene targets for miR-125b-5p in POAG include AKT1, ATXN1 (Huang et al., 2008; Han et al., 2011), BAK1, BCL2, BCL2L2 (Nickells et al., 2008) which are involved in RGC survival. A search of the ClinicalTrials.gov website (U.S. National Library of Medicine) and EU Clinical Trials Register did not show any clinical trials that were in progress or recruiting to test miRNA therapeutics in glaucoma patients.

A number of recent genetic studies of glaucoma have been performed. Genes associated with increased IOP or POAG risk included ABCA1, APOE, ARHGEF12, ATXN2, CAV1, CDKN2B-AS1, FOXC1, GA57, GMD5, S6K1/S6K6, TMCO1, and TXNRD2. However, variations in risk and genetic factors based on ethnic and geographic differences were found. While unified molecular pathways accounting for POAG pathogenesis remain undefined, inflammation and senescence likely play important roles. There are similar ethnic and geographic complexities in PACG, but several genes have been associated with this disorder, including MMP9, HGF, HSP70, MPRF, and eNOS. Genes implicated in XFG included LOK1, CNCNA1A, POMP, TMEM136, AGPAT1, RBMS3, and SEMA6A (Zuckerman et al., 2021). Many common variants and associated endophenotypes have been discovered in POAG through GWAS, particularly in ethnically diverse cohorts. Although the functional significance of these common variants is unknown, these advances have increased heritability estimates and helped create polygenic risk scores. In contrast, few variants have been identified in XFS/XFG, hampering efforts to examine endophenotypes, create satisfactory animal models, and establish a firm genetic basis (Tan and Pasquale, 2021).

In conclusion, some progress has been made in identifying miRNAs that have altered expression in POAG patients and their various subtypes. However, there are a large number of limitations and confounding factors in many of these studies that make the comparison of results difficult or unreliable. Future studies are warranted to obtain miRNA expression data for aqueous humor and blood samples of glaucomatous patients in the early stages of the disease, which is usually without any symptoms and can only be detected by ophthalmic examination, so that biomarkers can be identified and treatment started. These studies should be designed to reduce the number of limitations so that they are more compatible. In addition, suitable animal models of glaucoma should be used to test whether modifying the levels of specific miRNAs in aqueous humor or tears has a beneficial effect on IOP and ophthalmic examination of the eyes. Animal models (spontaneous and induced) used to study POAG have included monkeys, dogs, mice, rats, and rabbits (Bouhenni et al., 2012). Laser photoacoagulation has been used to develop experimental glaucoma in the rhesus monkey (Burgoyne, 2015). Recently, the development of POAG-like features was reported in a rhesus macaque colony from Southern China (Pasquale et al., 2021) and provides a unique opportunity to test novel therapeutic strategies for the disease. Performing studies in monkeys that spontaneously develop glaucoma-like features would raise fewer ethical concerns than using animals subjected to laser photoacoagulation.

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