Comparison of MicroRNA Expression in Aqueous Humor of Normal and Primary Open-Angle Glaucoma Patients Using PCR Arrays: A Pilot Study

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Purpose. MicroRNAs (miRNAs) are small, endogenous noncoding RNAs that have been detected in human aqueous humor (AH). Prior studies have pooled samples to obtain sufficient quantities for analysis or used next-generation sequencing. Here, we used PCR arrays with preamplification to identify and compare miRNAs from individual AH samples between patients with primary open-angle glaucoma (POAG) and normal controls.

Methods. AH was collected before cataract surgery from six stable, medically treated POAG patients and eight age-matched controls. Following reverse transcription and preamplification, individual patient samples were profiled on Taqman Low Density MicroRNA Array Cards. Differentially expressed miRNAs were stratified for fold changes larger than ± 2 and for significance of $P < 0.05$. Significant Kyoto Encyclopedia of Genes and Genomes pathways influenced by the differentially expressed miRNAs were identified using the predicted target module of the miRWalk 2.0 database.

Results. This approach detected 181 discrete miRNAs, which were consistently expressed across all samples of both experimental groups. Significant up-regulation of miR-518d and miR-143, and significant down-regulation of miR-660, was observed in the AH of POAG patients compared with controls. These miRNAs were predicted to reduce cell proliferation and extracellular matrix remodeling, endocytosis, Wnt signaling, ubiquitin-mediated proteolysis, and adherens junction function.

Conclusions. This pilot study demonstrates that miRNA expression within the AH of POAG patients differs from age-matched controls. AH miRNAs exhibit potential as biomarkers of POAG, which merits further investigation in a larger case-controlled study. This technique provides a cost-effective and sensitive approach to assay miRNAs in individual patient samples without the need for pooling.

Keywords: glaucoma, microRNA, aqueous humor, biomarker

Glaucoma is a leading cause of worldwide visual impairment, characterized by progressive loss of retinal ganglion cells (RGCs) and optic nerve damage that is often secondary to elevated intraocular pressure (IOP).[1–3] The physiologic regulation of IOP is determined by aqueous humor outflow resistance, to which the endothelium of Schlemm's canal and extracellular matrix (ECM) within the trabecular meshwork (TM) make significant contributions.[4] Due to its intimate relationship with these structures, aqueous humor (AH) has been proposed as a location where potential molecular biomarkers of aqueous outflow function with greater pathophysiologic relevance and specificity may be identified.[5] Identification of such biomarkers may help to characterize and stratify both the severity of outflow dysfunction and responses to treatment. Biomarkers of this type not only have the potential to underpin future research to identify novel therapeutic targets to modulate IOP but may also be used to help identify the phenotype of a specific individual's outflow facility and perhaps predict responses to therapeutic interventions.

MicroRNAs (miRNAs) are small (~18 to 22 nt), endogenous noncoding oligoribonucleotides that modulate the posttranscriptional regulation of gene expression in vivo[6] through recognition of specific sequences in target mRNAs. They act predominantly to reduce target gene expression.[7] In contrast to mRNAs, miRNAs show remarkable stability within biofluids such as plasma.[8] Preliminary reports also suggest that miRNAs may be identified within human AH samples[9–11] and more specifically within AH exosomes.[12] These observations support the hypothesis that extracellular miRNAs may facilitate cell-to-cell communication.[13] In the context of IOP regulation, this may represent intercellular feedback between mechanisms regulating AH secretion by the ciliary processes and its egress via outflow pathways. Several miRNAs have also been

implicated in aspects of both normal and pathologic TM physiology including cell contraction and ECM synthesis.[14]

The study of miRNA expression within AH using high-throughput screening techniques is restricted by the quantity of starting template required, with microarrays usually requiring upwards of 30 ng total RNA. This usually increases to 500 ng to perform traditional next-generation small RNA sequencing, although a recent report has described the use of adapter-driven amplification for this technology to be used.[11] Prior studies have pooled AH samples from multiple individuals[10,12] to increase the amount of total RNA or did not perform subsequent validation.[9] In this study, we used preamplification to increase RNA yield prior to performing PCR arrays and individual quantitative PCR (qPCR) validation of specific targets. Preamplification has been demonstrated to be sensitive and reliable without introducing bias in the analysis of low-input samples.[15] Using this cost-effective method, we performed a pilot study to test the hypothesis that miRNAs within AH are differentially expressed between patients with primary open-angle glaucoma (POAG) and normal controls.

Materials and Methods

Patient Selection and Acquisition of AH Samples

This study was reviewed and approved by the Institutional Review Board at Oregon Health & Science University and adhered to the tenets of the Declaration of Helsinki for research involving human subjects. After obtaining written informed consent, AH samples were collected from patients scheduled to undergo routine cataract surgery. Six patients with stable POAG controlled with topical medication alone and eight age-matched controls gave consent to participate in this pilot study. Approximately 100 to 150 μL AH was collected from each patient through a clear corneal paracentesis using a 30-gauge needle in the operating room at the beginning of surgery, prior to placement of the initial cataract incision. Sample collection was atraumatic in all cases, thereby eliminating the risk of contamination with blood or cellular debris. All AH samples were fully anonymized before immediate transfer to the research laboratories, where they were stored at −80°C prior to processing. Clinical data relating to each sample was extracted from the electronic patient record and collated in a fully anonymized manner. Data included age, sex, eye laterality, mean IOP (of last three clinic visits), topical and/or systemic medication used, and details of ocular and/or systemic comorbidities (if known).

Isolation and Assessment of miRNA

AH samples were thawed on ice prior to isolation using the *mir*Vana PARIS miRNA purification kit (Applied Biosystems, Thermo Fisher Scientific, Grand Island, NY, USA). The manufacturer's protocol was modified to include a second aqueous phase extraction during the phenol-chloroform purification step[16] and a final elution volume of 200 μL using sequential 100-μL aliquots on the same filter cartridge to minimize miRNA loss. This solution was further purified and concentrated using the RNA Clean & Concentrator-5 kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. A modified final elution step was also performed using sequential aliquots of 6 μL RNAse-free water on the same filter column to achieve a final elution volume of 24 μL per sample to maximize RNA yield. RNA quantitation was performed with the Agilent Bioanalyzer 2100 using the RNA 6000 Pico Chip on the eukaryotic Total RNA program (Agilent Technologies, Santa Clara, CA, USA).

Reverse Transcription and Preamplification

A fixed volume of 3.2 μL total RNA was used as input into individual reverse transcription (RT) reactions using the Taqman miRNA reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific). Each sample was reverse transcribed using primers specific for the A and B array cards, respectively, in separate 7.5-μL volumes containing 3.2 μL RNA, 0.8 μL Megaplex RT primers (10×) (Applied Biosystems, Thermo Fisher Scientific), 0.2 μL 100 mM dNTPs with dTTP, 1.5 μL Multiscribe Reverse Transcriptase (50 U/μL), 0.8 μL (10×) RT buffer, 0.1 μL RNAse inhibitor (20 U/μL), and 0.9 μL 25 mM $MgCl_2$ for 30 minutes at 16°C, 30 minutes at 42°C, and 5 minutes at 85°C in a thermal cycler (GeneAmp PCR System 9700; Thermo Fisher Scientific).

Preamplification was performed in 25-μL reactions (for A and B array cards, respectively) containing 2.5 μL RT product, 12.5 μL (2×) TaqMan PreAmp Master Mix, 2.5 μL Megaplex PreAmp primers (10×) (Applied Biosystems, Thermo Fisher Scientific), and 7.5 μL nuclease-free water according to the manufacturer's instructions. Samples were incubated for 10 minutes at 95°C, 2 minutes at 55°C, and 2 minutes at 72°C, followed by 12 cycles of 95°C for 15 seconds and 60°C for 4 minutes, with a final 10-minute incubation at 99.9°C in a thermal cycler. Tris-EDTA buffer (0.1×, 175μL) was added to each reaction to form the diluted preamplification product used for the subsequent steps.

miRNA Profiling Using TaqMan Low-Density PCR Arrays

miRNA profiling was performed for each sample using TaqMan Low-Density Human MicroRNA Arrays (TLDA,; Applied Biosystems, Thermo Fisher Scientific). Each TLDA card detects 384 probes including 377 human miRNAs, endogenous small RNA controls, and a negative control. Together, the two array cards (A and B Cards v3.0) can detect 754 mature miRNAs present in miRBase v20 (http://www.mirbase.org, in the public domain). The eight ports on each array card were loaded with 100 μL reaction mix made up of 2 μL diluted preamplification product, 50 μL (2×) TaqMan Universal Master Mix II, No Amperase UNG, and 48 μL nuclease-free water, before brief centrifugation and sealing. Each array card was processed individually on the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific) in the gene profiling core facility at Oregon Health & Science University (Portland, OR, USA).

Analysis of the array data was performed using Expression-Suite Software v1.0.3 (Applied Biosystems, Thermo Fisher Scientific) to generate a threshold cycle (C_T) value and an AmpScore metric (an indicator of amplification quality in the linear phase) for individual reactions in each array card. Robust detection thresholds for this platform were adopted from prior published studies.[17] Individual assays were excluded from the analyses if $C_T \geq 36$ or were reported as "undetected" by the software, or if the AmpScore was <0.9, which indicates poor amplification quality.

Experimental samples were normalized to the endogenous control U6-snRNA to correct for any variation in the initial amounts of starting template. Comparison of glaucoma and control groups was performed using the comparative C_T ($\Delta\Delta C_T$) method.

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Table 1. Baseline Demographics and Characteristics of Participants

Subject ID	Disease Status	Age, y	Sex	Eye Laterality	Mean IOP, mm HG*	Topical Medication	Ocular Comorbidity	Total AH (RNA) ng/μL	Total RNA Yield per Sample, ng
1	Control	76	Female	Left	16	None	Horner syndrome	0.05	1.2
2	Control	69	Female	Right	12	None	None	0.1	2.4
3	Control	74	Male	Right	14	None	None	0.03	0.72
4	Control	63	Female	NA	NA	None	None	0.03	0.72
5	Control	74	Female	NA	NA	None	None	0.05	1.2
6	Control	68	Female	Right	13	None	Dry AMD	0.09	2.16
7	Control	59	Male	Right	13	None	RD repair	0.1	2.4
8	Control	72	Male	Right	8	None	Amblyopia	0.06	1.44
9	POAG	57	Female	Left	21	Travoprost	Fuch's Endothelial Dystrophy	0.1	2.4
10	POAG	79	Male	Left	NA	Dorzolamide/ timolol	None	0.03	0.72
11	POAG	NA	Male	Left	16	Dorzolamide/ timolol	Dry AMD	0.09	2.16
12	POAG	70	Female	Right	22	Timolol	Dry AMD	0.04	0.96
13	POAG	69	Male	Left	12	Timolol	RD repair	0.08	1.92
14	POAG	67	Male	NA	NA	NA	None	0.08	1.92

NA, data not available; RD, retinal detachment.
* Of the last three clinic visits.

Confirmation of Differentially Expressed miRNAs With Individual qPCR Assays

Individual qPCR reactions were performed using the diluted preamplification product to confirm the array findings. These were performed in 20-μL reactions for each sample (with six technical replicates) containing 1 μL (20×) of the respective individual TaqMan MicroRNA Assay, 0.2 μL diluted PreAmp product, 10 μL (2×) TaqMan Universal Master Mix II, No Amperase UNG, and 8.8 μL nuclease-free water by incubation in a thermal cycler (Chromo4; Bio-Rad, Hercules, CA, USA) at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Fluorescence data were analyzed using Opticon Monitor 3 software (Bio-Rad). A baseline was subtracted using the average-over-cycle range method (2 to 20 cycles) with a threshold set at 0.2. Differential miRNA expression between control and glaucomatous AH was calculated by relative quantification using the comparative C_T method with normalization performed using the endogenous control U6-snRNA, as with the array analysis. The results were expressed as the fold change in glaucomatous AH compared with AH from control eyes, representing the ratio of the mean normalized expression values of both groups. If this number (relative quantity) was <1, the (negative) reciprocal was reported (e.g., 0.5, or a decrease of 50% compared with the control, is reported as −2 fold change). Statistical analysis was performed by comparing the normalized expression of miRNAs in glaucomatous and control AH using an unpaired 2-tailed *t*-test with significance considered for values of $P < 0.05$.

Biological Interpretation of Differentially Expressed miRNAs

Specific genomic loci of the identified differentially expressed miRNAs and loci associated with IOP were identified through database searches (http://www.ncbi.nlm.nih.gov/gene; http://www.ncbi.nlm.nih.gov/pubmed, in the public domain). The predicted gene targets of the differentially expressed miRNAs were determined using four freely available target prediction algorithms within the predicted target module of the curated online database miRWalk 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/index.html, in the public domain)[18]: (1) miRWalk 2.0, (2) RNAhybrid, (3) miRanda, and (4) Targetscan 6.2, using our previously described approach.[19] Genes were only accepted as potential targets of miRNAs when predicted by at least two of the four algorithms. This module was then used to identify significant KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways influenced by the predicted gene targets of the differentially expressed miRNAs. The comparison of predicted gene targets to those within a known pathway uses a hypergeometric distribution of overlapping genes utilizing the whole human genome as background. This generates a significance value (*P* value), which is subsequently corrected using a false discovery rate analogue to correct for multiple testing (*q* value). Based on the assumption that the dominant action of miRNAs is to decrease target mRNA translation or levels,[7] pathways associated with up-regulated miRNAs would be expected to show reduced expression and those associated with down-regulated miRNAs would be expected to show enhanced expression.

Results

Demographics and Baseline Characteristics of Patients

The baseline characteristics of the patient groups studied are summarized in Table 1. Subjects in the control ($n = 8$) and POAG ($n = 6$) groups were aged 69.4 ± 5.9 (mean ± SD) and 68.6 ± 6.3 years, respectively. The mean IOPs of the control and POAG groups were 12.7 ± 2.7 and 17.8 ± 4.6 mm Hg, respectively. POAG patients were using a mean of 1.2 topical medications.

RNA Isolation From Aqueous Humor Samples

Total RNA concentrations obtained from the bioanalyzer analysis following isolation and calculated yield obtained from each AH sample are shown in Table 1. The total yield of RNA was 1.53 ± 0.70 ng (mean ± SD) isolated from control subjects and 1.68 ± 0.68 isolated from AH of patients with

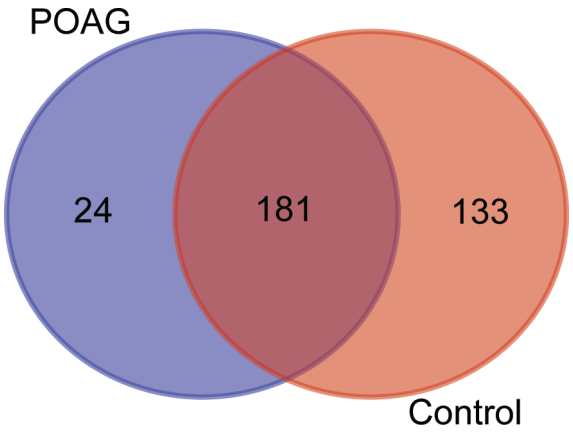

FIGURE 1. Venn diagram illustrating miRNAs detected across all POAG ($n = 6$) and control ($n = 8$) samples, with the intersection representing miRNAs common to both experimental groups.

POAG. The yields did not differ significantly between experimental groups (unpaired 2-tailed *t*-test; $P = 0.70$).

Differential miRNA Expression in AH From Glaucoma Patients Using TaqMan Low-Density PCR Arrays and Individual qPCR Confirmation

A total of 205 mature miRNAs were detected across all six AH samples from patients with POAG and a total of 314 miRNAs were detected across all eight control samples. Of these, 181 miRNAs were common to both groups, whereas 24 miRNAs were unique to POAG samples and 133 miRNAs were unique to control AH (Fig. 1; Supplementary Table S1). The 338 miRNAs that were detected are illustrated on the volcano plot (Fig. 2). Three miRNAs, miR-518d, miR-143, and miR-660, showed significant differential expression in AH from POAG patients following stringent analysis of the normalized array data (Table 2). miR-518d and miR-143 were significantly up-regulated, with miR-660 significantly down-regulated in AH from all POAG subjects compared with controls. To verify the direction and magnitude of change detected in the PCR arrays that contain $n = 1$ probe per miRNA, individual qPCR reactions ($n = 6$) for each miRNA were performed, and similar results were obtained (Table 2). In terms of relative expression, miR-143 and miR-518d were within the top 40% of miRNAs expressed according to their mean C_t values in control samples. In POAG subjects, miR-143 rose to the top 30% of miRNAs, and miR-518d rose to the top 10% of miRNAs expressed. miR-660 was within the top 50% of miRNAs in control samples in terms of relative expression. However, following down-regulation in POAG samples, it was found within the lower 10% of all expressed miRNAs. Three miRNAs, miR-135a, miR-9, and miR-128a, were consistently expressed in control AH but were not detected in AH from POAG patients.

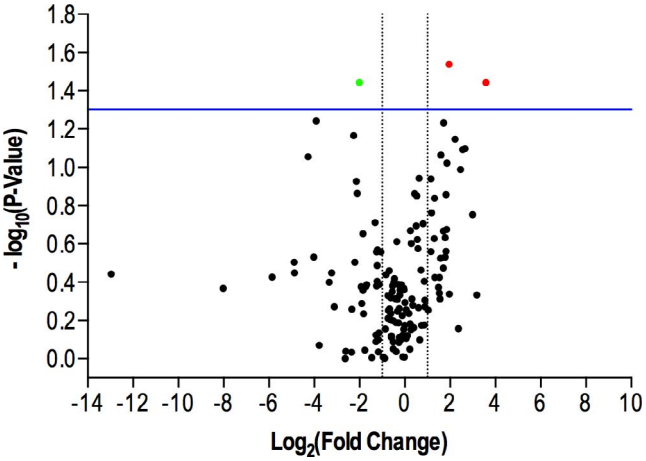

FIGURE 2. Volcano plot of the pairwise comparison of microRNA expression in aqueous humor from subjects with glaucoma versus unaffected controls. Up-regulated microRNAs are shown to the right of the plot (*red*) and were only selected if they passed the thresholds of $P < 0.05$ (*horizontal blue line*) and fold change >2 (*right vertical dotted line*). Accordingly, down-regulated microRNAs are shown to the left of the plot (*green*) and were only selected if they passed the thresholds of $P < 0.05$ (*horizontal blue line*) and fold change <−2 (*left vertical dotted line*).

Conversely, there were no miRNAs that were expressed solely in the POAG AH samples.

Biological Interpretation of Differentially Expressed miRNAs

miRNA expression has been suggested to occur in a coordinated manner with that of the host gene mRNA.[20] The miR-143 gene is located on the long arm of chromosome 5 (gene ID: 406935; Chr 5q32: 149,428,918-149,429,023). Two linkage loci associated with glaucoma and IOP (*GLC1G*, *GLC1M*) involving the region 5q21-32 have been extensively described by several groups,[21–23] with a recent study reporting an association between IOP and copy number variation at this locus.[24] The gene encoding miR-518d is located on the long arm of chromosome 19 (gene ID: 574489; Chr 19q13.42: 53,734,877-53,734,963). Although no glaucoma-related loci have yet been identified on the long arm of this chromosome, a locus associated with IOP was identified on the short arm (Chr 19p3.2: 9,804,797-9,805,040) within the Beaver Dam Eye Study population.[25] The miR-660 gene is located on the short arm of the X chromosome (gene ID: 724030; Chr Xp11.23: 50,013,241-50,013,337) with no currently identified IOP-related loci in close proximity.

The leading KEGG pathways, which include predicted gene targets of each of the differentially expressed miRNAs, are detailed in Tables 3 and 4. Due to the inhibitory action of miRNAs,[7] reduced activity of pathways associated with cancer

TABLE 2. Differentially Expressed MicroRNAs in the Aqueous Humor of Glaucoma Patients Compared With Controls

			Array Card		Individual qPCR	
miRBase ID	Accession Number	Assay ID	Fold-Change Glaucoma Versus Control	*P* Value	Fold-Change Glaucoma Versus Control (95% CI)	*P* Value
hsa-miR-518d-3p	MIMAT0002864	001159	11.94	0.036	8.67 (3.16 to 23.77)	0.010
hsa-miR-143-3p	MIMAT0000435	002249	3.87	0.029	2.46 (1.29 to 6.70)	0.021
hsa-miR-660-5p	MIMAT0003338	001515	−4.02	0.036	−3.67 (−2.15 to −5.18)	0.013

Table 3. Significant KEGG Pathways Potentially Influenced by Up-Regulated MicroRNAs in Aqueous Humor From Glaucoma Patients (i.e., Anticipate Down-Regulation of Targets/Pathways)

MicroRNA	Pathway	Genes Predicted as Targets	Genes in Pathway	*P* Value	Adjusted *q* Value
hsa-miR-143-3p	Pathways in cancer	310	330	0.00001	0.00221
hsa-miR-143-3p	Chemokine signaling pathway	181	189	0.00002	0.00433
hsa-miR-143-3p	Endocytosis	177	187	0.00026	0.04994
hsa-miR-518d-3p	Pathways in cancer	269	330	0.00000	0.00000
hsa-miR-518d-3p	Wnt signaling pathway	127	152	0.00000	0.00066
hsa-miR-518d-3p	Ubiquitin-mediated proteolysis	113	134	0.00000	0.00091
hsa-miR-518d-3p	Adherens junction	67	76	0.00002	0.00384

(e.g., cell proliferation and ECM remodeling) may be attributable to upregulation of both miR-143 and miR-518d. Specific to the individual miRNAs, up-regulation of miR-143 within the AH of glaucoma patients may be associated with reduced endocytosis and up-regulated miR518d associated with reduced Wnt signaling, ubiquitin-mediated proteolysis, and cell adhesion. Conversely, the observed down-regulation of miR-660 may counter-balance these effects by acting to enhance activity in these pathways. The three miRNAs expressed solely in control AH (miR-135a, miR-9, and miR-128a) were also predicted to influence the same range of pathways as described above. The absence of these miRNAs in POAG samples may prove to be of importance in future studies to determine the miRNA signature of POAG patients.

Discussion

This pilot study identified numerous miRNAs in AH from individual normal and glaucomatous patients using preamplification and low-density PCR arrays. Prior studies have been performed using either arrays or RNA sequencing of individual or pooled AH samples. The first attempt to characterize AH miRNAs within individual samples from various patient phenotypes, including those with glaucoma, was performed using a microarray platform[9] and identified 165 unique miRNAs within eyes with cataract alone. The reported miRNAs differ from other published reports[10–12] and with our findings (Fig. 3; Supplementary Table S2). Furthermore, more than 80% of the miRNAs identified were less well characterized, with sequentially allocated miRBase IDs greater than 1000 (e.g., hsa-miR-1587). Later studies used pooled human AH samples, which allowed consistent detection of miRNAs by miRNA PCR arrays and small RNA sequencing (Supplementary Table S2).[10,12] In pooled AH samples from patients with cataract alone, 110 discrete mature miRNAs were identified,[10] 55% of which were detected in comparable samples in our study. Dismuke et al.[12] identified 11 mature miRNAs using small RNA sequencing of pooled samples of AH exosomes. Half of these were also identified by Dunmire et al.,[10] whereas there was 91% (10 of 11) similarity with our current study. Our AH samples likely included both vesicular (i.e., exosomal) and vesicle-free miRNAs, suggesting that the majority of abundant miRNAs within AH may be of exosomal origin. Only one miRNA, miR-184, was detected in all five studies. Because our approach detected miRNAs with a high degree of similarity to studies using next-generation sequencing, which is the "gold standard," our preamplification and PCR array approach appears to be a sensitive and cost-effective technique to analyze individual AH samples without the need for pooling. This approach has also detected miRNAs within cerebrospinal fluid, an acellular biofluid with many similarities to AH, to develop potential biomarkers for Alzheimer's disease.[17] Our approach may therefore be an effective method to identify miRNAs in other biological fluids.

The origin of miRNAs detected within AH is currently unknown. AH is produced by active secretion of water across the blood–aqueous barrier in the nonpigmented ciliary epithelium with small amounts of plasma-derived proteins thought to diffuse across the iris root.[26] As expected, there is an overlap of the miRNAs that are detected within plasma and AH.[11] Our data support this observation, because we detected, in AH from both groups, 6 of the 10 most abundant miRNAs detected in human plasma.[27] Exosomal miRNAs may be predominantly derived from these anatomic sites or other anterior segment structures and have been hypothesized to be a potential mode of communication between aqueous inflow and outflow tissues that would serve to maintain IOP homeostasis.[12] The physiologic direction of AH flow within the eye will lead to both nonvesicular and vesicular (exosomal) miRNAs coming into contact with cells within the aqueous outflow pathways, where they may be biologically active.

KEGG pathway analysis identified potential pathways that may be impacted by altered miRNA expression. An individual miRNA may influence multiple targets and specific targets may be influenced by multiple miRNAs, but with differing hierarchical effects. The observed increased expression of both miR-143 and miR-518d is predicted to reduce ECM remodeling, which would result in increased outflow resistance and elevated IOP.[28] Increased expression of miR-143 is

Table 4. Significant KEGG Pathways Potentially Influenced by Down-Regulated MicroRNAs in Aqueous Humor From Glaucoma Patients (i.e., Anticipate Up-Regulation of Targets/Pathways)

MicroRNA	Pathway	Genes Predicted as Targets	Genes in Pathway	*P* Value	Adjusted *q* Value
hsa-miR-660-5p	Pathways in cancer	192	330	0.00000	0.00000
hsa-miR-660-5p	Wnt signaling pathway	92	152	0.00000	0.00038
hsa-miR-660-5p	MAPK signaling pathway	150	272	0.00000	0.00085
hsa-miR-660-5p	Calcium signaling pathway	103	178	0.00001	0.00169
hsa-miR-660-5p	Ubiquitin-mediated proteolysis	80	134	0.00002	0.00349
hsa-miR-660-5p	Endocytosis	106	187	0.00002	0.00429

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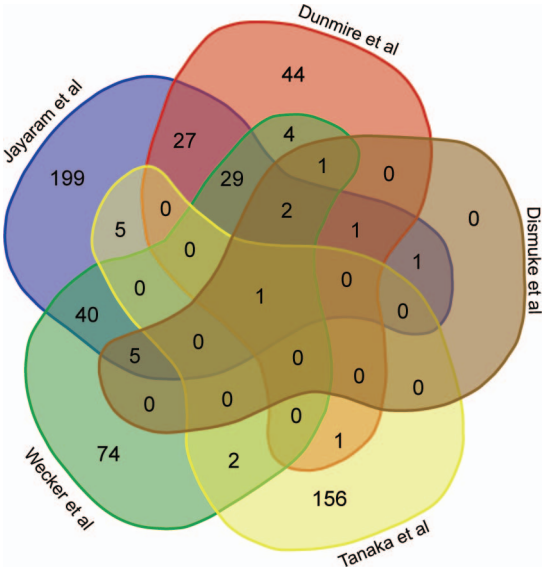

Figure 3. Venn diagram visually illustrating the overlap in detection of individual miRNAs detected in AH from the control group (cataract only) in this study, with comparable "cataract only" groups from the four other currently published reports.[9–12]

predicted to reduce endocytic pathway function, which may alter the uptake of essential molecules and contribute to the degenerative changes associated with glaucomatous TM.[4] The observed increased expression of miR-518d is predicted to reduce activity of ubiquitin-mediated proteolytic pathways such as the autophagy pathway, which work to degrade and clear proteins from the outflow channels.[29,30] miR-518d is also predicted to reduce activity in the Wnt signaling pathway. The canonical Wnt pathway has been shown to contribute to IOP regulation in human TM.[31] However, the inhibitory effects of these up-regulated miRNAs may be counterbalanced by the down-regulation of miR-660, which may restrict the extent of any biological shift. Although these types of informatics analyses guide research direction, it is important to emphasize that the in vivo biological impact of altered miRNA expression within AH cannot be accurately determined without tissue-specific gene expression data. Although logistically complex, future studies involving patients undergoing glaucoma surgery may offer an opportunity to directly integrate biological activity within the AH and TM of the same individual.

This exploratory study is limited by the relatively small sample size but highlights the potential for further research in this area. The impact of topical medications to lower intraocular pressure on miRNA expression within AH has yet to be determined. However, the inflammatory cytokine profile of AH from POAG patients on topical therapy is almost identical to cataract controls,[32] so it is possible that topical therapies may not be a major confounding factor. Future larger studies would benefit from stratifying participants by use of topical glaucoma medications, as well as glaucoma phenotype, stage, stability of disease, and diurnal intraocular pressure stability, to improve the specificity and generalizability of AH miRNAs as potential biomarkers of glaucoma.

In conclusion, miRNA expression within the AH of individual patients with POAG differs from that of age-matched cataract controls. These preliminary observations demonstrate that miRNAs within AH may be potentially useful as biomarkers of POAG and merit further investigation in a larger case-controlled study. Our low-density PCR arrays can detect miRNAs consistent with those identified by small RNA sequencing and provide a cost-effective approach to assay individual AH samples without the need for pooling.

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