MicroRNA Profiling in Aqueous Humor of Individual Human Eyes by Next-Generation Sequencing

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Purpose. Extracellular microRNAs (miRNAs) in aqueous humor were suggested to have a role in transcellular signaling and may serve as disease biomarkers. The authors adopted next-generation sequencing (NGS) techniques to further characterize the miRNA profile in single samples of 60 to 80 μL human aqueous humor.

Methods. Samples were obtained at the outset of cataract surgery in nine independent, otherwise healthy eyes. Four samples were used to extract RNA and generate sequencing libraries, followed by an adapter-driven amplification step, electrophoretic size selection, sequencing, and data analysis. Five samples were used for quantitative PCR (qPCR) validation of NGS results. Published NGS data on circulating miRNAs in blood were analyzed in comparison.

Results. One hundred fifty-eight miRNAs were consistently detected by NGS in all four samples; an additional 59 miRNAs were present in at least three samples. The aqueous humor miRNA profile shows some overlap with published NGS-derived inventories of circulating miRNAs in blood plasma with high prevalence of human miR-451a, -21, and -16. In contrast to blood, miR-184, -4448, -30a, -29a, -29c, -19a, -30d, -205, -24, -22, and -3074 were detected among the 20 most prevalent miRNAs in aqueous humor. Relative expression patterns of miR-451a, -202, and -144 suggested by NGS were confirmed by qPCR.

Conclusions. Our data illustrate the feasibility of miRNA analysis by NGS in small individual aqueous humor samples. Intraocular cells as well as blood plasma contribute to the extracellular aqueous humor miRNome. The data suggest possible roles of miRNA in intraocular cell adhesion and signaling by TGF-β and Wnt, which are important in intraocular pressure regulation and glaucoma.

Keywords: miRNA, aqueous humor, sequencing

MicroRNAs modulate gene expression on a posttranscriptional level by initiating target degradation or inhibiting translation.[1,2] MiRNAs act intracellularly but have also been detected in most body fluids, where they are preserved in microvesicles, exosomes, or bound to carrier proteins.[3,4] Distinct circulating miRNA signatures were proposed as serum biomarkers of cancer, diabetes, or ischemic cardiovascular diseases[5–7] and may evolve further to support diagnosis and therapeutic decisions.[8] Earlier observations indicate that extracellular miRNAs also have a role in transcellular signaling.[9] Vascular endothelial cells were shown to release miRNA-loaded vesicles to communicate with vascular smooth muscle cells,[10] and targeted delivery of endogenous miRNAs by high-density lipoproteins has been observed.[11]

In the eye, miRNAs are expressed in a tissue-specific fashion[12–14] and have essential roles in ocular development and retinal homeostasis.[15–19] Changes in miRNA expression were reported in a mouse model of retinal ischemia,[20] and miR-132 was recently suggested as a possible therapeutic target to prevent ocular neovascularization.[21] Trabecular meshwork cell contractility and extracellular matrix turnover are influenced by distinct miRNAs[22–24] that may therefore be relevant in glaucoma. Moreover, trabecular meshwork cells were shown to secrete exosomal vesicles containing miRNA, which may serve in transcellular communication.[25,26] Specific miRNA-dependent effects have also been reported in the lens and cornea.[27–29] Thus, miRNAs appear to have specific functions in the eye that are just beginning to be understood.

Extracellular miRNA has recently been detected in intraocular fluid by quantitative PCR (qPCR)[30,31] and microarray analysis.[32] A most recent study analyzed the exosomal miRNA fraction in pooled aqueous humor samples by small RNA sequencing.[26] Intraocular cells are an obvious source of extracellular miRNAs in aqueous humor. However, miRNAs may also derive from blood plasma, since plasma proteins and associated solutes enter the aqueous humor by diffusing from the ciliary body stroma to the iris root and into the anterior chamber.[33,34] Human aqueous humor can be safely obtained by microscope-guided anterior chamber puncture or during standard anterior chamber penetrating procedures such as

cataract surgery.[35] Aqueous humor analysis may be complicated by blood contamination due to small hemorrhages at the puncture site and by the fact that typically only up to 100 μL aqueous can be safely retrieved.

Here, we report on the adaptation of next-generation sequencing (NGS) techniques to allow for miRNome analysis in small, unpooled samples of human aqueous humor, carefully canceling out possible contamination sources. This approach avoids limitations of hybridization-based detection and lays the foundation for an open exploration of ocular fluids retrieved in different states of disease.

Methods

Aqueous Humor Samples

Sixty to 80 μL aqueous humor were collected at the outset of surgery from four female Caucasian patients, aged 46, 47, 72, and 78 years, undergoing routine cataract surgery in otherwise healthy eyes. The patients were informed and had provided written consent to the collection and scientific use of the specimen prior to the procedure. The study has been approved by the institutional review board and ethics committee of the Faculty of Medicine, Freiburg University and conducted in accordance with the tenets of the Declaration of Helsinki.

To collect aqueous humor at the outset of surgery, the ocular surface was rinsed with sterile solution (BSS; Alcon, Fort Worth, TX, USA) prior to anterior chamber puncture. The cornea was penetrated in an avascular peripheral area over a length of 1 mm with a paracentesis lancet, and contact with limbal or peripheral corneal vessels was avoided. Aqueous humor (0.06–0.08 mL) was drawn with a blunt cannula on a tuberculin syringe (Omnifix-F; B. Braun, Melsungen, Germany) without contact with intraocular structures. If any bleeding was observed, the sample was not used for further analysis. The needle was withdrawn, and the syringe was capped and transferred to a −80°C freezer in the vicinity of the operating room suite. For further sample preparation, the syringes were transferred to the lab on dry ice, thawed on ice, and aqueous humor was transferred to reaction tubes using filter-tipped pipettes to retrieve the full sample volume.

RNA Extraction

Aqueous humor samples were centrifuged successively (2000*g* for 20 minutes at 4°C and 10,000*g* for 30 minutes at 4°C) to remove cells and cell debris. Synthetic *Caenorhabditis elegans* miR-39 spike-in control was added to the supernatant, and RNA was extracted using an RNA purification kit (Quick-RNA MicroPrep; Zymo Research Corp., Irvine, CA, USA), according to the manufacturer's instructions.

Library Preparation and Sequencing

All extracted RNA from each individual patient was used to generate sequencing libraries with a GenXPro low-input small RNA library preparation kit (GenXPro GmbH, Frankfurt, Germany). Briefly, RNA was ligated with specific adapters (TrueQuant Adapters; GenXPro GmbH) to the 3′- and 5′-end, successively. Adapter-ligated libraries were reverse transcribed and amplified by PCR with sample-specific index primers. Amplified libraries were size-selected by polyacrylamide gel electrophoresis and validated with a DNA analysis cartridge (Agilent High Sensitivity DNA Kit; Agilent Technologies, Santa Clara, CA, USA) using a lab-on-chip processing system (Agilent Bioanalyzer 2100; Agilent Technologies). Individual libraries were pooled and sequenced in 50-bp reads on a sequencing system (HiSeq 2000; Illumina, Inc., San Diego, CA, USA).

Sequencing Data Analysis

The sequencing data are deposited at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), with Sequence Read Archive accession numbers SRX1035025, SRX1035026, SRX1035027, and SRX1035028. Raw sequencing data of every sample were independently preprocessed in omiRas after removal of adaptors and elimination of all duplicate reads originating from PCR amplification with a software tool (TrueQuant Software; GenXPro). OmiRas is an online tool for analysis of miRNAs derived from sequencing data of small RNAs.[36]

Next, the data were transferred to Galaxy, a web-based open source platform for bioinformatic calculations, in FASTA-format[37–39] and subsequently converted to FASTQ-format.[40] FASTQ data were filtered to exclude sequences shorter than 19 bases. Blast+ using the bastn-short algorithm was utilized to detect overlaps between sequencing data and mirBase data (release 21).[41–47] Only miRNAs annotated as mature in mirBase were included. Of these, miRNAs occurring in *Homo sapiens* were selected and deposited in an output file, which was further analyzed using GNU R and additional packages.[48–53]

A single data file of every sample was imported to GNU R and checked for plausibility of results. Reads per million were calculated on a per sample basis. Subsequently, data from all four samples were merged in a single file to facilitate comparison with serum data provided by Williams et al.[54] and aqueous humor data by Dunmire et al.[30] Since the degree of isomir differentiation varies among these studies, isomirs and closely related miRNAs were merged under their common stem names to allow for comparisons.

Quantitative PCR

Aqueous humor samples of five independent patients, obtained as above, were cleared of potential debris by centrifugation (16,000*g*, 5 minutes), a C. elegans miR-39 spike-in control was added to the supernatant, and small RNAs were extracted using an extraction kit (miRNeasy Plasma Kit; Qiagen, Hilden, Germany) following the manufacturer's instructions. Subsequently, the samples were reverse transcribed using a commercial kit (RT^2 Kit, Qiagen). Commercially available primers (miScript; Qiagen), as well as a qPCR kit (miScript qPCR Kit; Qiagen), were used to assess the presence of miR-451a, -202, and -144 following the manufacturer's instructions.

Results

MiRNAs Detected by NGS in Human Aqueous Humor

On average, 259,944 (±90,015) reads were obtained per sample. When restricted to sequences of 20 nucleotides and longer, 20,703 (±13,990) of the reads mapped to miRBASE entries, revealing 181 ± 107 miRNAs per sample. A core set of 158 miRNAs were detected in all four samples sequenced, an additional 59 in three of four samples, and 377 different miRNAs were observed in total (Fig. 1).

The most abundant miRNA detected in aqueous humor was miR-451a, followed by miR-184, miR-16-5p, and miR-4448. Reads of the 40 most abundant miRNAs found in all samples are depicted as reads per million in Figure 2. The absolute reads for 158 miRNAs detected in all four samples, ranked by median normalized abundance, are provided in Supplementary Table S1.

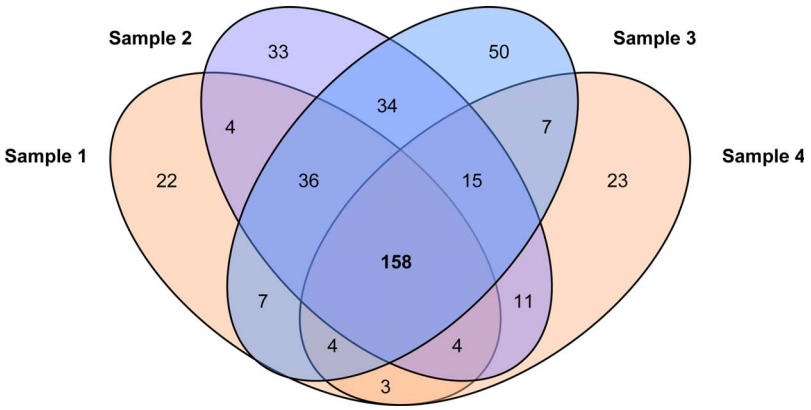

FIGURE 1. MicroRNA detection by NGS in four independent aqueous humor samples. All mature miRNAs detected by NGS with at least one miRbase-mapped read are compiled in a Venn diagram. One hundred fifty-eight miRNAs were found in all four samples, with an additional 59 miRNAs in three samples.

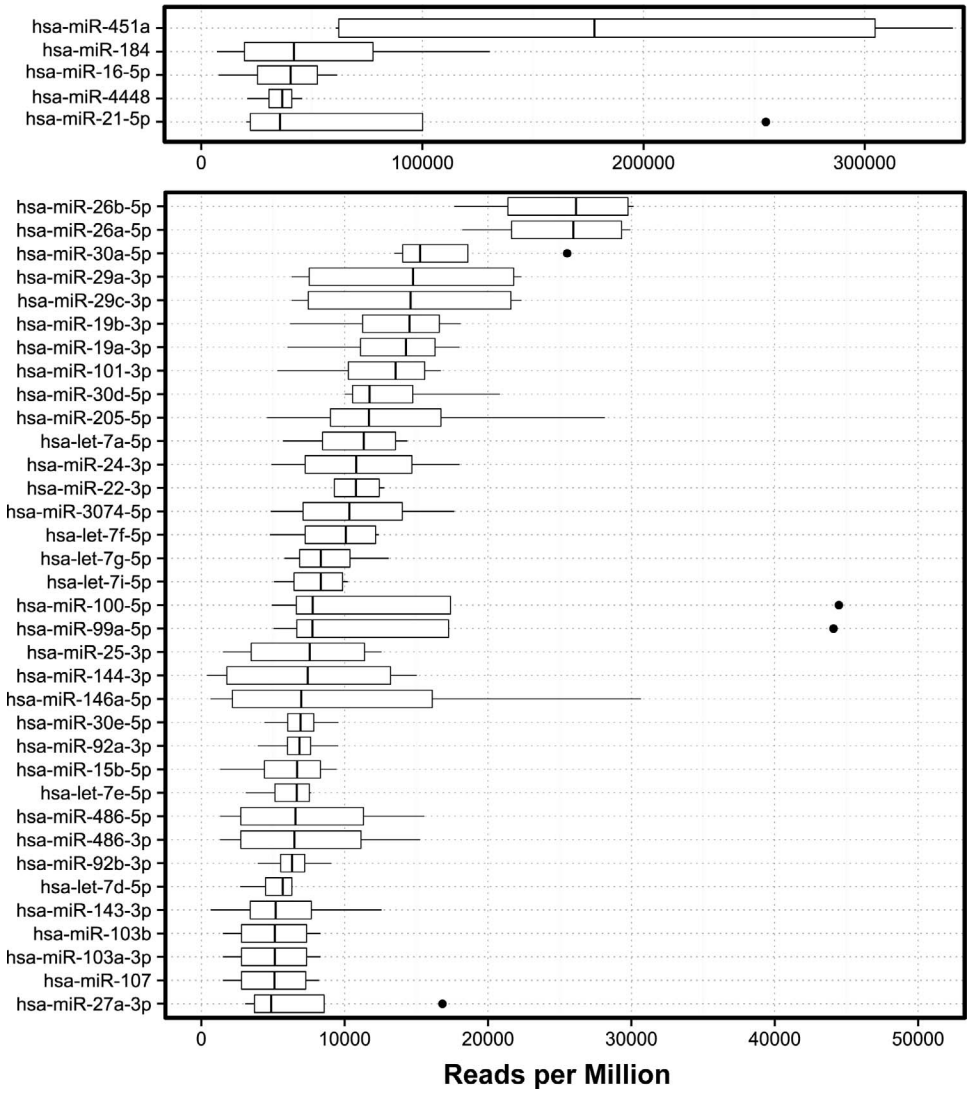

FIGURE 2. Frequency distribution of the 40 most abundant mature miRNAs in human aqueous humor as determined by NGS. Box plots indicate the prevalence of miRNAs in four aqueous humor samples. MicroRNAs are arranged by median read frequencies expressed in reads per million. The upper and lower borders of the boxes correspond to the first and third quartiles, while the whiskers reach to the lowest respectively highest value within 1.5 * interquartile range (IQR) (IQR = distance between first and third quartile). Data outside 1.5 * IQR are outliers depicted as *dots*.

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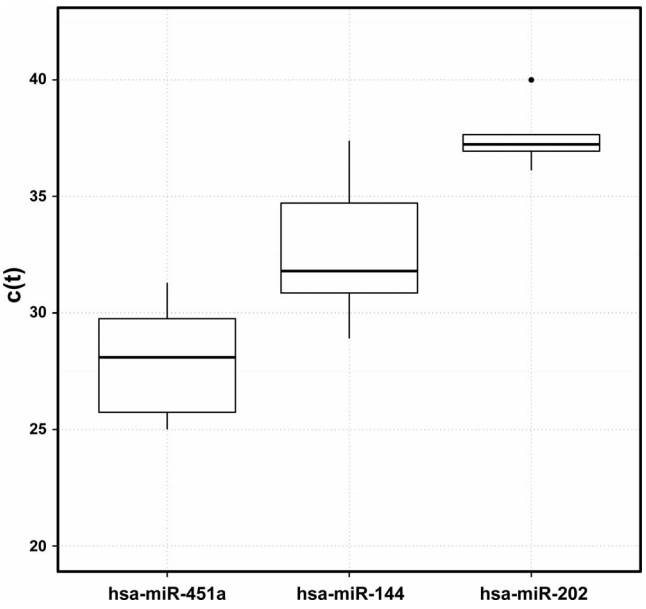

FIGURE 3. Detection of selected miRNAs in aqueous humor by qPCR. Box plots provide the qPCR threshold cycle numbers obtained in five independent human aqueous humor samples for miR-451a, -144, and -202.

qPCR Verification of NGS Data

To reassess possible differences in miRNA abundance suggested by NGS, three miRNAs were analyzed by qPCR in five independent aqueous humor samples. We chose to study miR-451a as the most abundant in sequencing, miR-144 as of intermediate abundance, and miR-202, which was not consistently detected by NGS in our samples but has been described as the most abundant miRNA in aqueous humor detected in pooled aqueous humor samples in an earlier report using qPCR.[30] The mean c(t) values obtained were 27.9, 32.7, and 37.5 for miR-451a, miR-144, and miR-202, respectively (Fig. 3).

NGS-Derived MiRNA Profiles in Aqueous Humor and Reported MiRNA Profiles in Plasma

Ocular cells, whole blood, or blood plasma are possible sources of miRNAs in aqueous humor samples. To assess possible similarities to blood, which may also be a sample contaminant, we compared published data on circulating miRNA detected by NGS in blood[54] to our NGS data obtained in aqueous humor. Isomirs and closely related miRNAs had to be merged under their common names to allow for comparisons. This slightly changed aqueous humor miRNA rank orders as compared to isomer-specific ranking, but the general pattern was preserved. MiR-451a was the most abundant miRNA in both plasma and aqueous humor (Fig. 4). However, miR-486 ranked second in plasma[54] but was far less abundant in aqueous humor (Fig. 4). MiR-184 and miR-4448 were highly prevalent in aqueous humor but scarce or undetected in plasma, respectively. To enhance the assessment of possible specific differences in miRNA profile patterns, we also compared miRNA abundance ranks. Rank differences of the 20 most abundant aqueous humor miRNAs to their reported plasma ranks are provided in Figure 5. In aqueous humor, seven (miR-184, -4448, -205, -3074, -29, -100, -27) of the 20 most abundant miRNAs ranked more than 25 ranks higher than in plasma. Ranks of the generally rather abundant miRNA families miR-21, -16, -19, -144, and -let-7 were similar in both groups (Fig. 5), and a Wilcoxon rank-sum test rejected the null hypothesis of unrelatedness between the published serum data and our aqueous humor data ($P < 0.01$).

DISCUSSION

The small volume of aqueous humor samples typically available from human eyes has previously hampered NGS of aqueous humor. To overcome these limitations, we devised a distinct sample preparation workflow including an initial controlled adapter-driven global PCR-amplification step. This allowed sequencing of unpooled samples, using as little as 80 µL aqueous humor.

With this approach, 158 miRNAs were consistently found in all four samples, and a total of 217 miRNAs was common to three samples. These data are in line with recent observations indicating the presence of 110 or 158 distinct miRNAs in aqueous humor of cataract patients as assessed by qPCR or microarray screening, respectively.[30,32]

However, the miRNA sequences we identified employing NGS technology differed significantly from the pattern obtained by qPCR screening of pooled aqueous humor samples reported by Dunmire et al.[30] Of the five most abundant miRNAs in aqueous humor based on qPCR screening (miR-202, -193b, -135a, -365, and -376a),[30] we detected only a single one by NGS in all our samples (miR-135a, ranked 110th). To further evaluate this issue, we studied the presence of three distinct miRNAs in five independent aqueous humor samples by qPCR. The resulting c(t) values obtained for miR-451a, -144, and -202 strongly support our NGS data (Fig. 3). Several reasons may account for these divergent findings. Outliers in single samples can strongly influence abundance ranks when samples are pooled as in the qPCR-based earlier study.[30] In this regard, the possibility of assessing distinct samples individually as provided by our workflow appears advantageous. Data robustness is further enhanced in our study as only sequences detected in all samples were considered. Interestingly, a significant variation in miRNA detection across different technology platforms, including qPCR techniques and microarray hybridization, has been reported by several groups, and technical constraints have been discussed.[55,56] Given the short length of mature miRNAs, accurate detection of isomers by hybridization-based systems such as qPCR and microarrays is challenging. This may strongly influence the perceived relative abundance of distinct miRNAs and lead to different results using qPCR, microarrays, or NGS. Furthermore, optimal hybridization conditions will rarely be identical for an array of different probes, and individual hybridization stringency is difficult to verify in a microarray system. In contrast, sequencing data analysis provides well-defined control of mapping length and mismatch allowance.

Ribonucleic acid release from contaminating cells that may rupture during sample freeze-thaw cycles is another valid concern. To address this issue, we considered small nucleolar RNAs (snoRNAs) and Y-RNAs in our aqueous humor data (data not shown) and NGS data of two human trabecular meshwork cell culture samples (own unpublished data) in comparison. Small nucleolar RNAs are abundant in cells and less prevalent in extracellular fluids, while, in contrast, small Y-RNAs were reported to be enriched in extracellular exosomes.[57] The snoRNA/miRNA ratio was 0.12 in aqueous humor, but 3.8 in the cellular samples. Furthermore, Y-RNAs were abundant in aqueous humor, but negligible in the cell culture samples. These observations strongly suggest that leakage of small RNAs from disrupted cells did not significantly contribute to the aqueous humor miRNA patterns we detected.

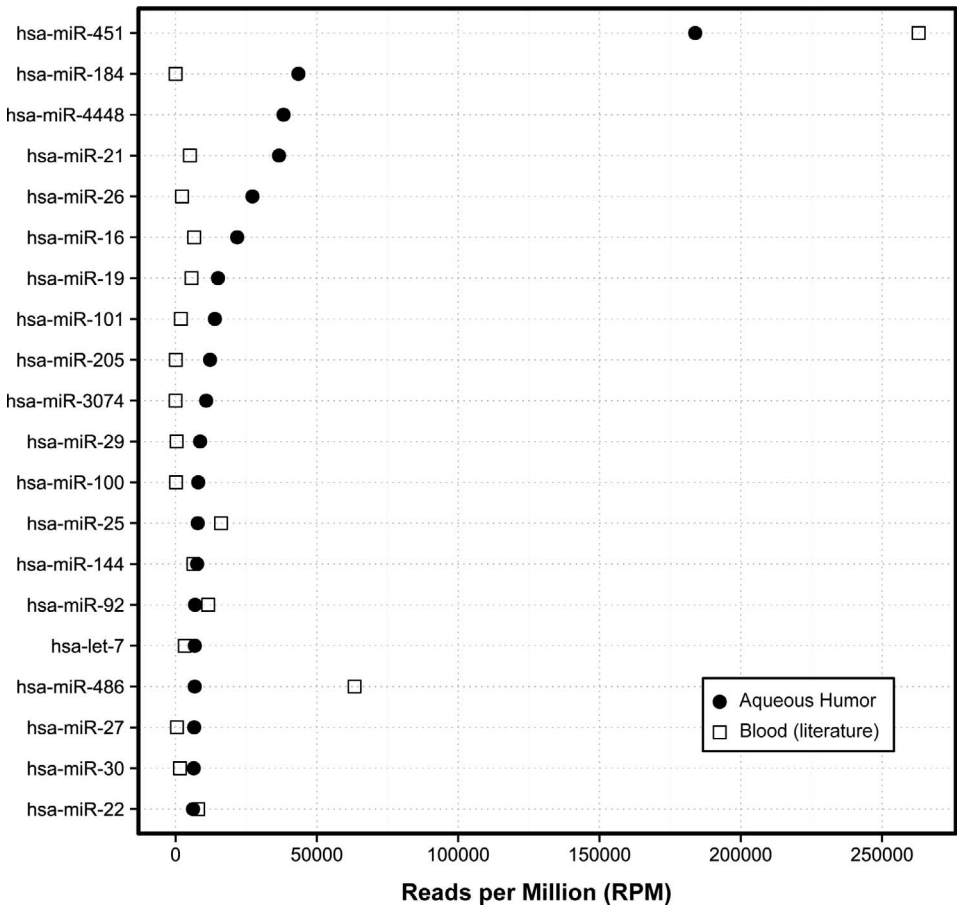

FIGURE 4. Circulating miRNA abundance in aqueous humor and in blood. The median read frequencies of the top 20 miRNAs in four human aqueous humor samples and the respective reads in a published NGS analysis[54] of 10 human blood samples are plotted for comparison. MiR-4448 was not reported in the study of blood samples.[54]

In our study, miR-451a was the most prevalent miRNA in aqueous humor. MiR-451 is essential in erythropoiesis and protects against oxidant stress by enhancing FoxO3 signaling.[58] Intriguingly, enhanced levels of antioxidants are present in aqueous humor[59] indicating a specific need for oxidative stress reduction in the anterior eye segment. Ascorbic acid in aqueous humor was shown to prevent UV radiation–induced tissue damage to the lens and other tissues in the path of light.[60,61] Along these lines, miR-451 may also have a role in ocular antioxidant homeostasis.

Earlier studies using NGS detection characterized miR-451 as the most prevalent circulating miRNA in plasma and serum.[5,54] Since aqueous humor is mainly derived from active transport of water and solutes across the blood–eye barrier in the ciliary body epithelium as well as by protein diffusion through the iris root,[34] some overlap of detected extracellular miRNAs in plasma and aqueous is expected. However, a blood contamination of aqueous humor during sample withdrawal is a possible confounder and needs to be considered.

We therefore compared our aqueous humor data to published findings on circulating miRNAs in blood samples (Figs. 4, 5).[54] Many miRNAs highly prevalent in blood, such as miR-451, -21, -16, -26, -25, and -92, are also found in aqueous humor, albeit in very different relative quantities (Figs. 4, 5). The low abundance of miR-486, which is the second most frequent in plasma, and the very low abundance of miR-106b, among the top 10 in blood, in aqueous humor also argue against a significant blood contamination of our aqueous humor samples. Furthermore, miR-184, -4448, and -205 were highly prevalent in aqueous humor, but typically were very low ranking or undetected in plasma samples.[5,54] In earlier studies miR-184 and miR-205 were characterized as strongly expressed in the anterior segment of the eye.[12,14] Mutations in the MIR-184 gene were shown to disturb target site competition of miR-184 and -205 and are associated with familial keratoconus and early cataract in humans.[27] These data further support our findings of a significant prevalence of miR-184 and miR-205 in aqueous humor.

Other miRNAs prominent in aqueous humor have been reported to modulate TGF-β and Wnt signaling, which are important in intraocular pressure control and glaucoma[62]: miR-4448[63] and the miR-99/100 polycistrons[64] target Smad proteins essential in TGF-β receptor signaling. A gene enrichment analysis for predicted targets of the 15 most prevalent miRNAs in aqueous humor using DIANA software[65] revealed a list of several important pathways, such as the PI3K-AKT survival pathway, focal adhesion-dependent cell-matrix interactions, the mTOR pathway, and the TGF-β and Wnt signaling pathways (Table).

In summary, our data strongly suggest that human aqueous humor contains a set of "background miRNAs" derived from plasma, which is augmented by additional miRNAs derived from intraocular cells. Some of these, such

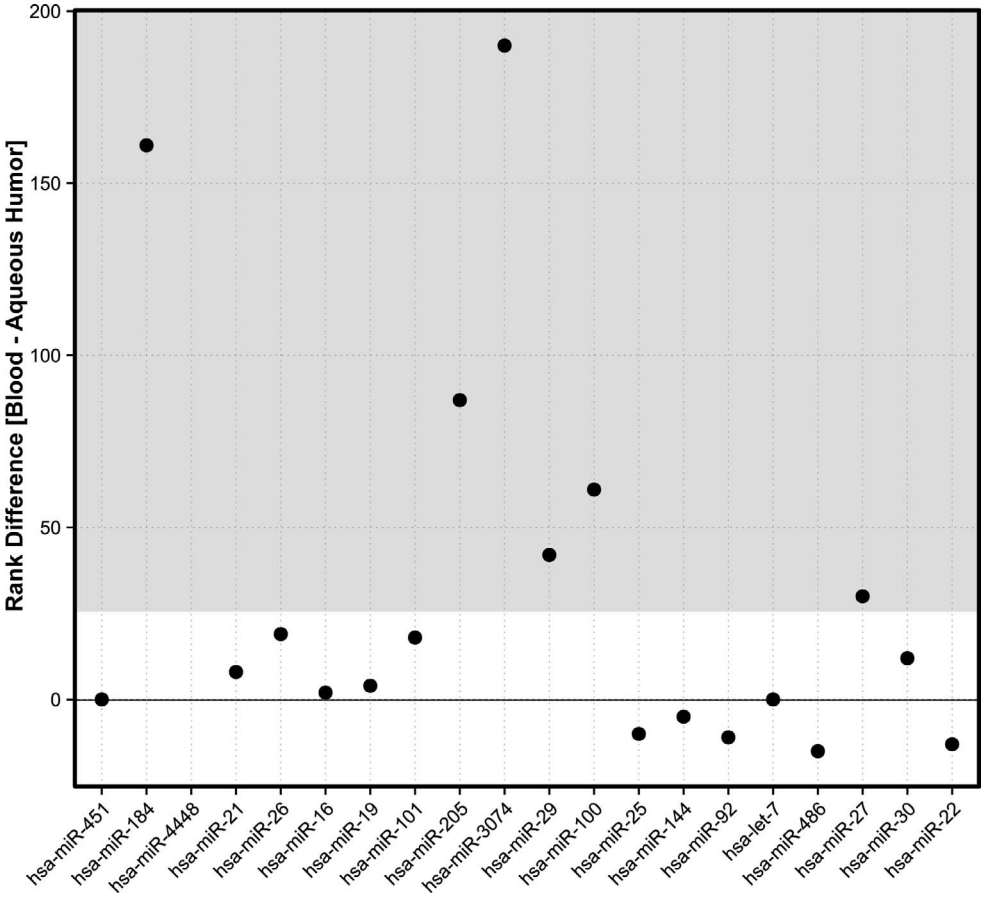

Figure 5. Blood-aqueous ranking comparison of the top 20 miRNA families in aqueous humor. MicroRNAs were ranked by read frequencies in aqueous and blood (published data[54]). The rank numbers in aqueous humor were subtracted from the ranks in blood[54] to obtain rank differences. The shaded area denotes miRNAs ranking more than 25 ranks lower in aqueous humor than in blood, suggesting a higher relative abundance in aqueous. Differences below zero indicate a lower rank in blood than in aqueous humor.

as miR-184, -4448 and -205, are not significantly present in plasma and thus appear eye-specific. Others, such as miR-26, -29, and -99/100, are also found in plasma but in significantly lower relative quantities, indicating additional intraocular sources. Thus, a characterization of the aqueous humor total miRNA profile is possible by NGS in unpooled single aqueous humor samples. This approach may likely allow monitoring ocular disease processes and improve pathophysiological understanding.

Table. Gene Enrichment Analysis (DIANA[65]) of Predicted Targets for the 15 Most Abundant miRNAs in Human Aqueous Humor

KEGG Pathway	*P* Value	No. Genes	No. miRNAs
PI3K-Akt signaling pathway	7.62E-26	125	15
Focal adhesion	1.53E-25	83	14
mTOR signaling pathway	9.16E-21	33	13
Colorectal cancer	7.66E-19	32	13
Pathways in cancer	2.40E-18	121	14
Adherens junction	2.97E-16	38	12
TGF-βsignaling pathway	3.54E-16	35	13
Wnt signaling pathway	3.77E-16	64	14
Prostate cancer	1.13E-15	39	12
Ubiquitin-mediated proteolysis	2.60E-13	54	13

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