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Cornea & External Disease

MicroRNA Profiling in the Aqueous Humor of Keratoconus Eyes

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Purpose: To identify differentially expressed (DE) microRNAs (miRNAs) in the aqueous humor (AH) of keratoconus (KC) eyes using next-generation sequencing and to explore whether DE miRNAs might play roles in KC pathophysiology.

Methods: The small RNAs in the AH of 15 KC eyes and 15 myopia eyes (the control group) were sequenced on an Illumina NovaSeq 6000 platform. Gene Oncology and Kyoto Encyclopedia of Genes and Genome enrichment analyses were performed. Receiver operating characteristic curves were used to identify potential KC biomarkers.

Results: We identified 204 miRNAs in the AH of the KC group and 200 in the AH of the control group. Fourteen miRNAs were differentially expressed between the two groups; four miRNAs were upregulated and 10 downregulated in KC AH. The possible pathways regulated by the DE miRNAs included antigen processing and presentation, endocytosis, mismatch repair, and Hippo signaling. The AH concentrations of miR-222-3p, miR-363-3p, and miR-423-5p exhibited areas under the curves of 1.

Conclusions: We profiled the DE miRNAs of the AH of KC eyes. These miRNAs may be associated with KC pathogenesis and could serve as KC biomarkers.

Translational Relevance: Data on aberrantly expressed miRNAs in KC combined with bioinformatics analyses suggest possible roles for specific miRNAs. The DE miRNAs may serve as diagnostic KC biomarkers.

Introduction

Keratoconus (KC) is characterized by bilateral and (usually) asymmetrical, conical corneal changes reflecting ectatic corneal involvement. KC usually commences in adolescence and progresses to the third or fourth decade of life, and it is one of the most common indications for keratoplasty in the developed world.[1] The global KC prevalence ranges between 0.2 and 4790 cases per 100,000 worldwide.[2,3] KC etiology and pathophysiology remain poorly understood, although there are several known risk factors.[4–6] As imaging technology improves, the incidence of KC will rise.[7]

MicroRNAs (miRNAs) are small noncoding RNAs involved in silencing or regulation of gene expression at the translational and post-transcriptional levels. miRNAs primarily reduce gene expression.[8] Some biofluid miRNAs serve as biomarkers of cancer, cardiovascular, and ocular diseases, as well as other disorders.[9–11] miRNAs have been found in ocular fluids (tears, aqueous humor [AH], and vitreous humor).[12–14]

Only a few studies have explored miRNA expression in the cornea of KC eyes[15–17]; the AH miRNAs of KC eyes have not been analyzed.

The principal KC morphological change commences at the posterior surface of the cornea.[18] Tellouck et al.[19] found that corneal posterior steepening served to measure the KC progression rate. The cornea receives nutrients via diffusion from the AH[20]; thus, it was important to explore miRNA changes in the KC AH. We collected AH from KC eyes and profiled the miRNAs via next-generation sequencing (NGS).

Materials and Methods

Patients

Patients and controls were recruited in the Shanghai Ninth People's Hospital. AH samples were obtained from 15 KC patients prior to corneal crosslinking (CXL) and from 15 myopic patients (controls) before intraocular lens surgery. Written informed consent was obtained from all participants. The Ethics Committee of the Shanghai Ninth People's Hospital approved the study. All relevant tenets of the Declaration of Helsinki were followed.

The KC inclusion criteria were (1) age > 14 years; (2) a clinical diagnosis of KC progression; (3) a thinnest corneal thickness > 400 µm before ultraviolet A irradiation (as recommended by the transepithelial CXL protocol); and (4) no history of any systemic or ocular disorder/condition (ocular surgery, trauma, or disease). The inclusion criteria for the control group were (1) age ≥ 18 years; (2) best-corrected visual acuity (BCVA) ≥ 20/25; and (3) no ocular disease (other than myopia), no severe systemic disease, and no history of internal eye surgery or eye trauma. All patients underwent preoperative ophthalmologic examinations, including measurement of BCVA, slit-lamp and fundus examinations, intraocular pressure measurement, and corneal evaluation using a Scheimpflug device (OCULUS Pentacam, OCULUS Optikgeräte GmbH, Wetzlar, Germany).

AH Samples and RNA Extraction

An anterior chamber puncture was performed under sterile conditions to collect 100 to 150 µL of AH from each eye prior to surgery. Cells and debris were removed by centrifugation (12,000g, 4°C, 20 minutes). The AHs from three random eyes (100 µL AH from each) were pooled prior to RNA extraction (300 µL AH/sample); thus, we obtained five KC samples and five control samples. Total RNAs were isolated using the TRIzol Reagent (Life Technologies, Carlsbad, CA).

miRNA Sequencing and Bioinformatics Analyses

RNA sequencing libraries were prepared as described previously.[14] Sequencing proceeded on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA) for 50 cycles. The fold differences in miRNA expression levels, *P* values (probabilities), and false discovery rates (*P* values corrected using the Benjamini–Hochberg method) between the two groups were calculated. Differentially expressed (DE) miRNAs were those with fold changes ≥ 1.5 and $P \leq 0.05$. Gene Ontology (GO; http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genome (KEGG) pathway enrichment analyses were used to identify the functions of DE miRNAs and the associated metabolic or signal transduction pathways.

Biomarker Assessment

We drew receiver operating characteristic (ROC) curves to assess whether DE miRNAs distinguished KC patients from controls. ROC analyses were performed with the aid of Prism 9 (GraphPad Software, San Diego, CA) using normalized miRNA expression levels.

miRNA Validation by Quantitative PCR

AH samples of another 18 patients with KC and 18 patients with myopia (control group) were obtained as described above. The AHs from three random eyes were pooled prior to RNA extraction; thus, we obtained six KC samples and six control samples. RNA was reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). Quantitative PCR (qPCR) was performed using a TaqMan MicroRNA Assay Kit (Thermo Fisher Scientific) specific for the selected miRNAs, in accordance with the manufacturer's instructions, and u6 was used as an internal reference (forward primer: CTCGCTTCGGCAGCACA; reverse primer: AACGCTTCACGAATTT GCGT) for the expression of miRNA. The $2^{-\Delta\Delta CT}$ values were calculated and compared between the two groups.

Results

Clinical Data

Clinical information for the 15 eyes from KC patients and 15 from controls is provided in Table 1. Age, BCVA, thinnest corneal thickness (TCT), corneal anterior flat keratometry (K1) data, steep keratometry

Table 1. Baseline Clinical Data for Eyes, the AHs of Which Were Subjected to NGS

Variable	Keratoconus Eyes	Myopia Eyes
Eyes/people, *n*	15/15	15/15
Age (y), mean ± SD	23.33 ± 3.81	20.93 ± 3.22
Sex (male/female), *n*	14/1*	8/7*
BCVA (logMAR), mean ± SD	0.45 ± 0.53**	0.03 ± 0.53**
TCT (mm), mean ± SD	456.93 ± 43.6**	544.6 ± 26.24**
K1, mean ± SD	46.49 ± 5.83**	42.07 ± 1.52**
K2, mean ± SD	51.25 ± 7.68**	43.61 ± 1.7**
PE, mean ± SD	58.07 ± 31.15**	5.87 ± 3.22**

Data were analyzed using SPSS 26.0. Student's *t*-test was used to compare the data for age, BCVA, TCT, K1, K2, and PE between the groups.

*$P < 0.05$.

**$P < 0.05$ (Pearson's χ^2 test was employed to compare the sex ratios).

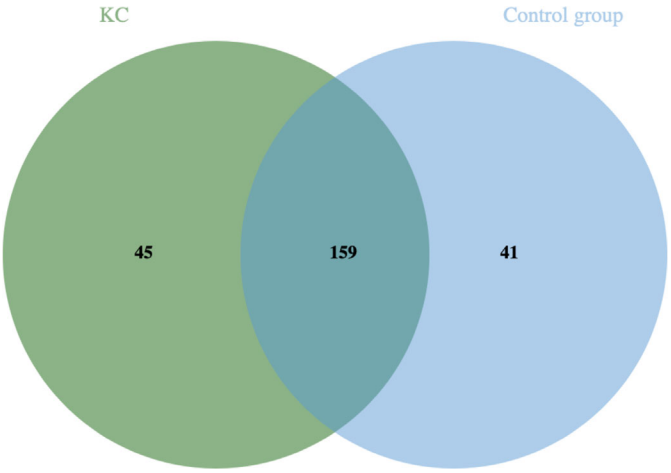

Figure 1. Venn diagram of miRNA detection via NGS (known and novel miRNAs) A total of 204 miRNAs were detected in KC eyes and 200 in control eyes. A total of 159 miRNAs overlapped.

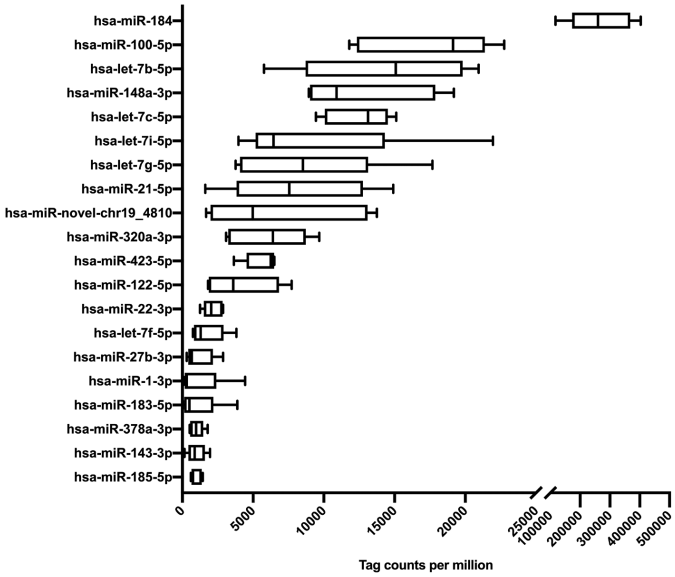

Figure 2. Frequency distributions of the 20 most abundant miRNAs in KC eyes as determined via NGS. The box plots indicate the prevalence of miRNAs in five aqueous humor samples. The miRNAs are arranged by the mean read frequencies (per million). The upper and lower borders of the boxes indicate the first and third quartiles, respectively; the whiskers show the lowest and highest values.

(K2) data, and posterior corneal elevation (PE) all differed significantly between the groups ($P < 0.05$).

miRNAs of KC AH

A total of 204 miRNAs were detected in KC AH and 200 in control AH; 159 miRNAs were expressed in both groups (Fig. 1). The miRNA expression profiles (top 20, from high to low) of the two groups are shown in Figures 2 and 3, respectively. Of the most highly expressed miRNAs, hsa-miR-184, hsa-miR-100-5p, hsa-let-7b/c/i/g/f-5p, hsa-miR-148a-3p, hsa-miR-21-5p, hsa-miR-320a-3p, hsa-miR-423-5p, hsa-miR-122-5p, hsa-miR-22-3p, hsa-miR-27b-3p, hsa-miR-143-3p, and hsa-miR-185-5P were found in both groups. The miR-184 level was highest in both groups.

DE miRNAs in Patients With KC

Fourteen miRNAs were DE between the two groups (fold change ≥ 1.5; $P < 0.05$); four miRNAs were significantly upregulated in the KC group and 10 significantly downregulated. Of the latter, one was novel (Table 2).

Predicted Target Genes of the DE miRNAs

The predicted target network of the four miRNAs (hsa-miR-7-5p, hsa-miR-193b-5p, has-miR-195-3p, and hsa-miR-199a-5p) upregulated in the AH of KC

Table 2. Mature miRNAs That Were (Significantly) Differentially Expressed in KC and Control Eyes Arranged by Fold- Change in Expression Levels

Mature miRNA	Sequence	Fold Change	*P*	Regulation
hsa-miR-195-3p	CCAAUAUUGGCUGUGCUGCUCC	101.6	0.047	Up
hsa-miR-199a-5p	CCCAGUGUUCAGACUACCUGUUC	19	0.047	Up
hsa-miR-193b-5p	CGGGGUUUUGAGGGCGAGAUGA	8.527778	0.038	Up
hsa-miR-7-5p	UGGAAGACUAGUGAUUUUGUUGUU	4.435115	0.046	Up
hsa-miR-28-3p	CACUAGAUUGUGAGCUCCUGGA	−6.17333	0.019	Down
hsa-miR-222-3p	AGCUACAUCUGGCUACUGGGU	−5.04348	0.042	Down
hsa-miR-363-3p	AAUUGCACGGUAUCCAUCUGUA	−4.66667	0.002	Down
hsa-miR-95-3p	UUCAACGGGUAUUUAUUGAGCA	−4.61538	0.003	Down
hsa-miR-181a-5p	AACAUUCAACGCUGUCGGUGAGU	−3.71186	0.020	Down
hsa-miR-novel-chrX_13407	AUGGAUUUUUGGAGCAGGGA	−2.06816	0.008	Down
hsa-miR-320a-3p	AAAAGCUGGGUUGAGAGGGCGA	−1.8392	0.023	Down
hsa-miR-22-3p	AAGCUGCCAGUUGAAGAACUGU	−1.80227	0.031	Down
hsa-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU	−1.69574	0.001	Down
hsa-miR-185-5p	UGGAGAGAAAGGCAGUUCCUGA	−1.58942	0.042	Down

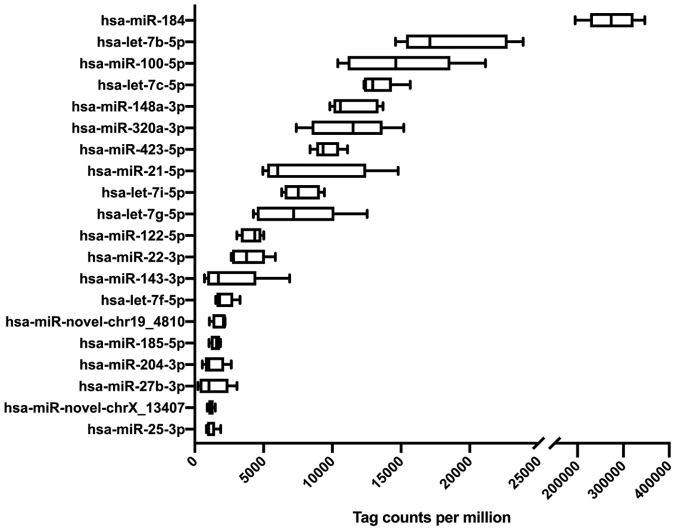

Figure 3. Frequency distributions of the 20 most abundant miRNAs in myopia eyes as determined via NGS. The box plots indicate the prevalence of miRNAs in five aqueous humor samples. The miRNAs are arranged by the mean read frequencies (per million). The upper and lower borders of the boxes indicate the first and third quartiles, respectively; the whiskers show the lowest and highest values.

eyes is shown in Figure 4. The predicted target network of the top five downregulated miRNAs (ranked by fold change) (hsa-miR-28-3p, hsa-miR-222-3p, hsa-miR-363-3p, hsa-miR-95-3p, and hsa-miR-181a-5p) in the AH of KC eyes is shown in Figure 5.

Bioinformatics

The GO enrichment scores of the DE miRNAs in KC AH are shown in Figure 6, including biological process cell component and molecular function effects of the DE miRNAs. The KEGG pathways affected by the four upregulated miRNAs are shown in Table 3 and Figure 7. The top 10 KEGG pathways (ranked by enrichment score) affected by all 10 downregulated miRNAs are shown in Table 4 and Figure 8.

AH DE miRNAs As Biomarkers

To evaluate whether DE miRNAs of AH could serve as diagnostic biomarkers, ROC curves were created for all 14 DE miRNAs (Fig. 9). All upregulated miRNAs evidenced area under the ROC curve (AUC) values of 0.8 to 0.98; for hsa-miR-195-3p and hsa-miR-7-5p, the *P* values were <0.05. Nine downregulated DE miRNAs exhibited AUC values between 0.84 and 1. All *P* values were <0.05, except that of hsa-miR-22-3p.

miRNA Validation

Clinical information for the 18 eyes from KC patients and 18 from controls is shown in Table 5. When miR-195-3p, miR-222-3p, and miR-181a-5p were analyzed using qPCR to verify the results of RNA sequencing from the two groups, the expression of miR-195-3p increased significantly in the AH of the KC group compared with the control group, and miR-222-3p and miR-181a-5p showed decreased expression in the KC group compared with the control group, in accordance with the sequencing results (Fig. 10).

Discussion

To the best of our knowledge, this is the first study to explore AH miRNA expression in KC eyes.

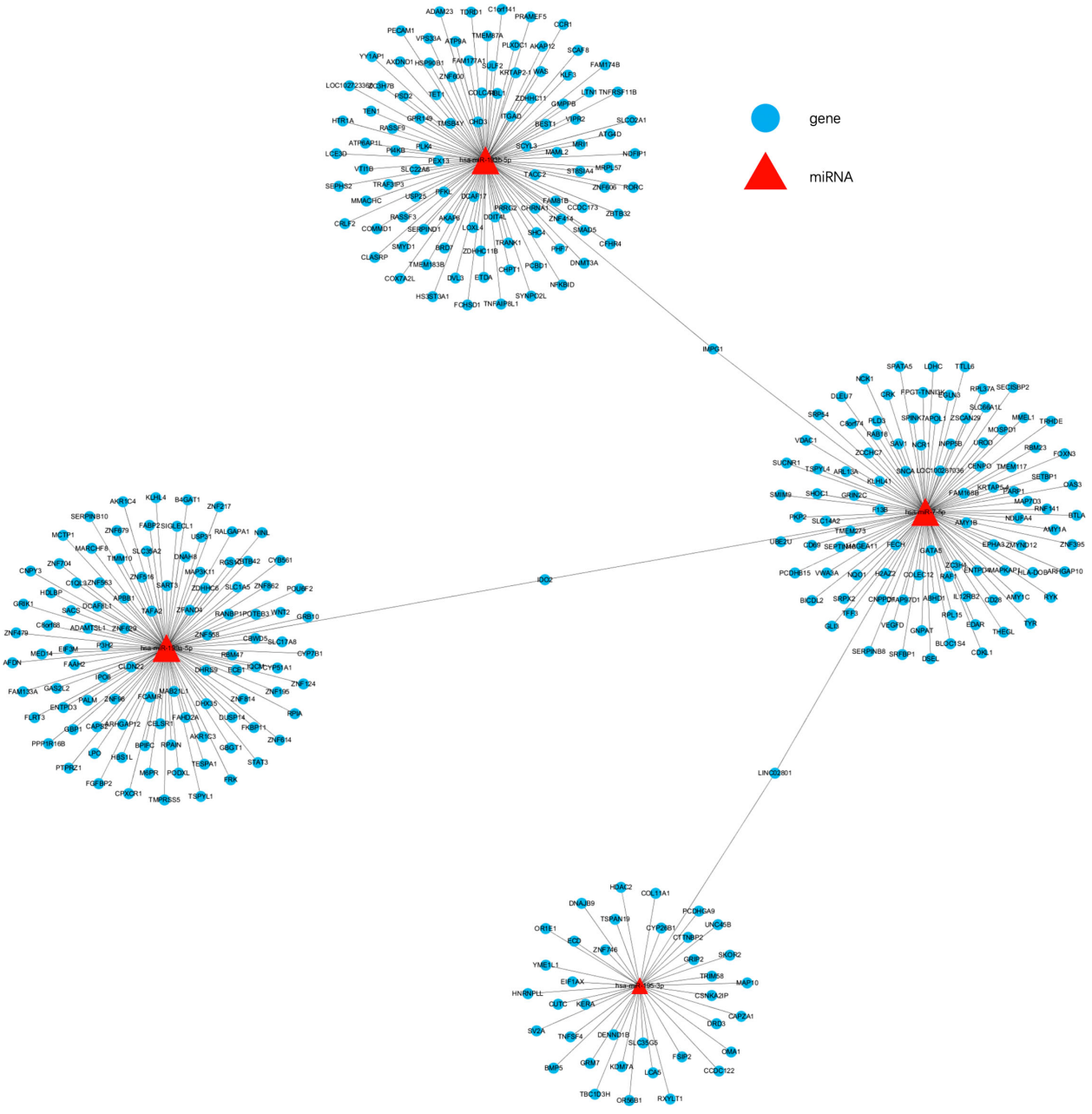

Figure 4. The predicted target network of upregulated miRNAs in the AH of KC eyes.

We obtained a comprehensive miRNA profile and identified putative roles of DE miRNAs via NGS and bioinformatics analyses. A total of 204 miRNAs were detected in the AH of KC eyes and 200 in the AH of control eyes. Fourteen miRNAs were differentially expressed between the two groups. The possible pathways regulated by such miRNAs include antigen processing and presentation, endocytosis, mismatch repair, and Hippo signaling.

Corneal PE is important when diagnosing ectasia and screening candidates for refractive surgery. Increased PE sensitively indicates early KC.[21] Corneal tissue is nourished principally by the AH; translational and post-transcriptional effects of miRNAs have been reported in other corneal diseases.[22] Because the AH bathes the posterior surface of the cornea, the detection of DE miRNAs in the AH of KC eyes is important.

Previous miRNA Studies on KC

Drewry et al.[16] found that miR-184 was the most abundant miRNA in corneal tissue. Mutations in miR-184 have been studied in patients with KC.[23–25]

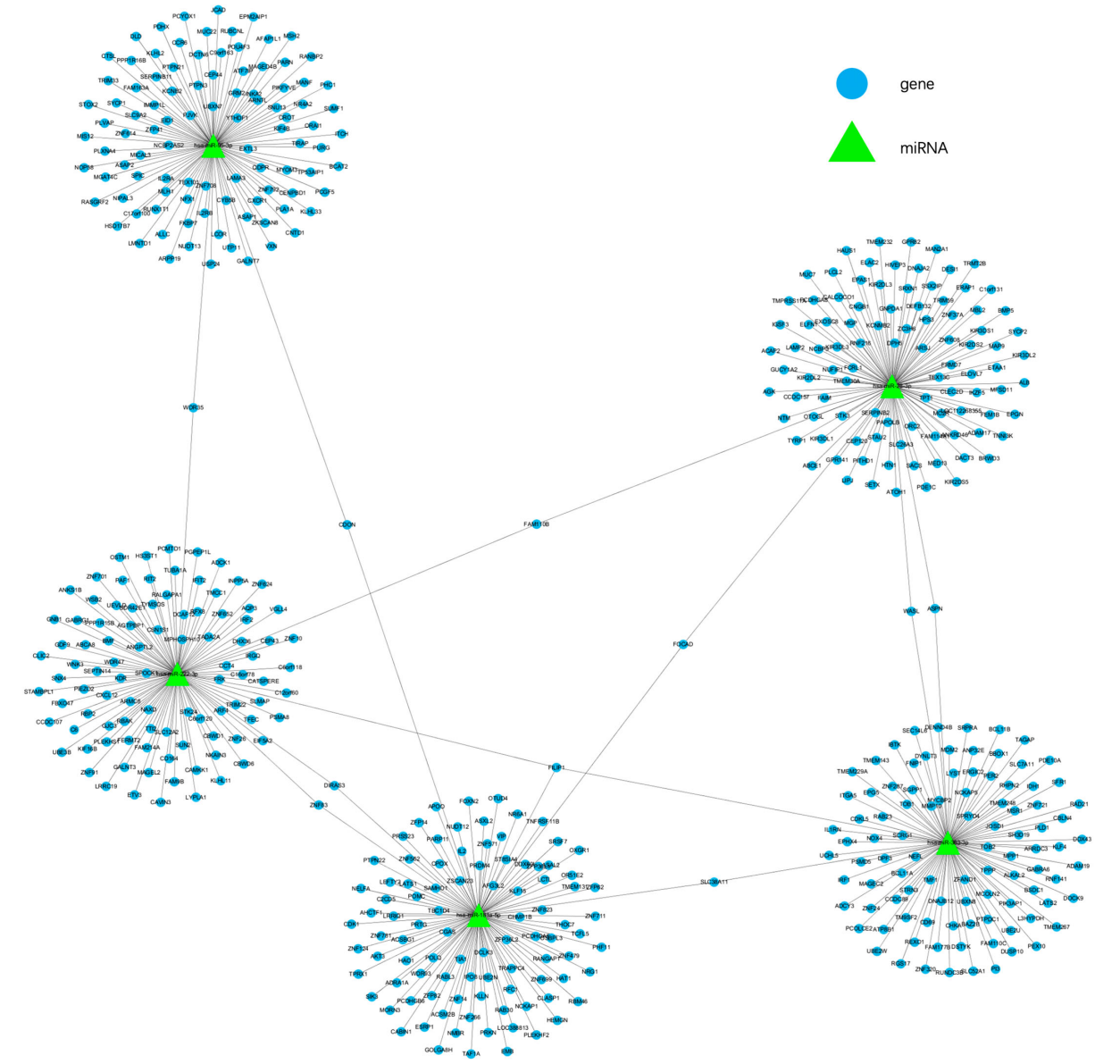

Figure 5. The predicted target network of downregulated miRNAs (top five by fold change in expression) in the AH of KC eyes.

However, Abu-Amero et al.[26] concluded that mutations in the seed region of miR-184 were more likely when KC was associated with other ocular abnormalities. miR-184 was the most abundant miRNA in both of our groups (268,489 reads per million in KC eyes and 274,575.6 in control eyes); this miRNA is highly enriched in eye tissues. Several studies have sought DE miRNAs of KC patients.[15–17] Drewry et al.[16] reported that miR-143-3p, miR-182-5p, and miR-92a-3p targeted genes were associated with KC. We found that miR-143-3p was among the top 20 miRNAs in both groups; however, the levels of the three miRNAs did not differ significantly between KC and control eyes. A miRNA microarray study compared the miRNAs of the corneal epithelium of 27 KC eyes to those of 26 control eyes, and 12 miRNAs were significantly downregulated in KC.[15] The predicted target genes participated in cell junction, division, and motor activities, as well as syndecan signaling. KC corneal epithelium (obtained during transplant surgeries) revealed changes in the levels of six miRNAs, whereas KC corneal epithelium obtained via impression cytology exhibited changes in the expression levels of four miRNAs (miR-151a-3p, miR-195-5p, miR-185-5p, and miR-194-5p). We also found that miR-185a-5p was downregulated in KC eyes. Tian

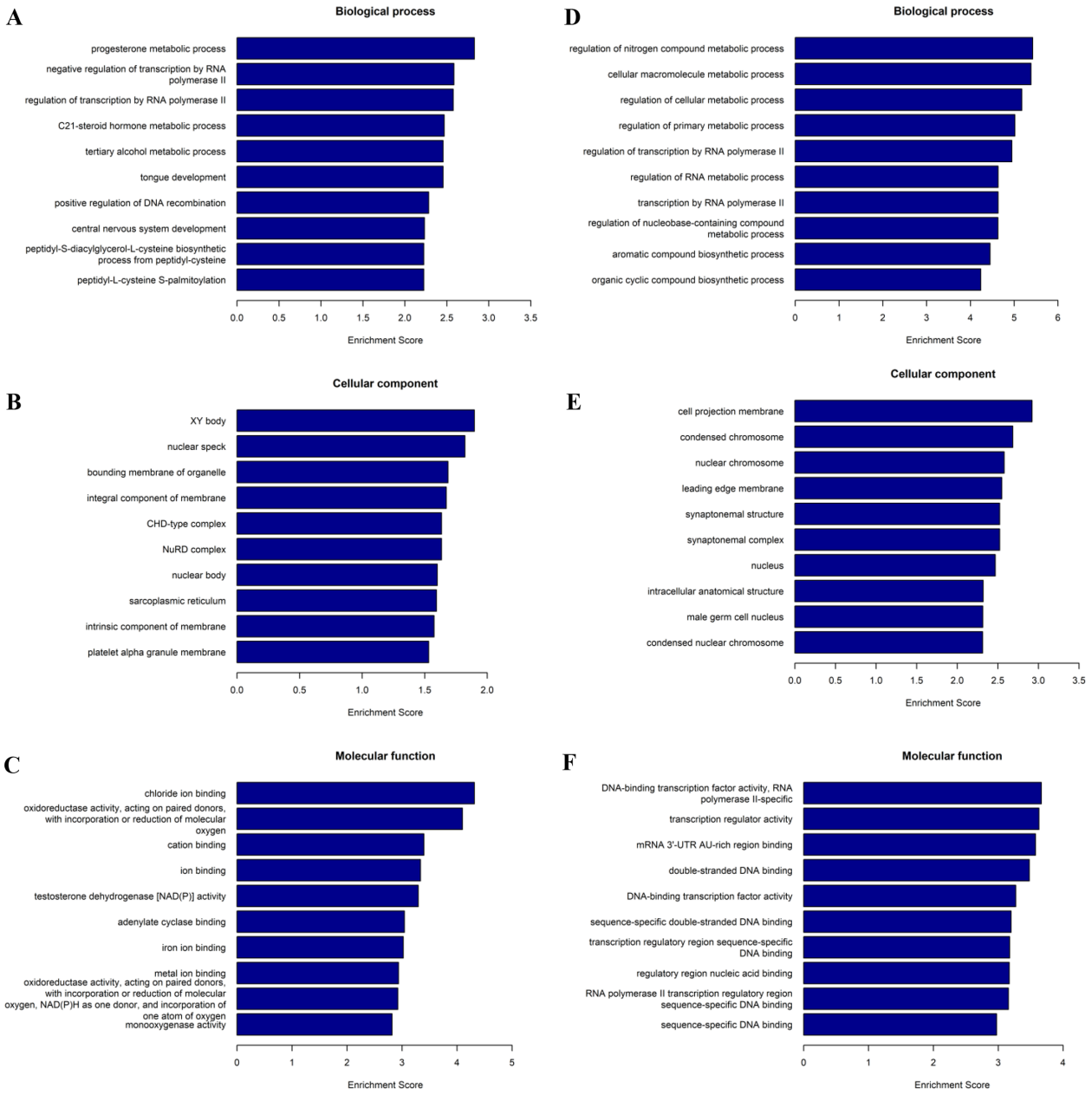

Figure 6. Enrichment scores of GO analysis of differentially expressed miRNAs in the AH of KC eyes. (**A**) Biological process effects of the upregulated miRNAs. (**B**) Cell component effects of the upregulated miRNAs. (**C**) Molecular function effects of the upregulated miRNAs. (**D**) Biological process effects of the downregulated miRNAs. (**E**) Cell component effects of the downregulated miRNAs. (**F**) Molecular function effects of the downregulated miRNAs.

Table 3. KEGG Analysis of the Effects of Upregulated miRNAs in the AH of KC Eyes (Fisher $P < 0.05$)

KEGG Pathways	Fisher *P*	Enrichment Score	Target Genes	Pathway Genes
Starch and sucrose metabolism	0.02374533	1.624422	3	36
Inflammatory bowel disease	0.02560582	1.591661	4	65
Central carbon metabolism in cancer	0.03248465	1.488322	4	70
Primary bile acid biosynthesis	0.03398241	1.468746	2	17
Chronic myeloid leukemia	0.0420392	1.376346	4	76
Carbohydrate digestion and absorption	0.04706991	1.327257	3	47
Herpes simplex virus 1 infection	0.04815081	1.317396	14	498

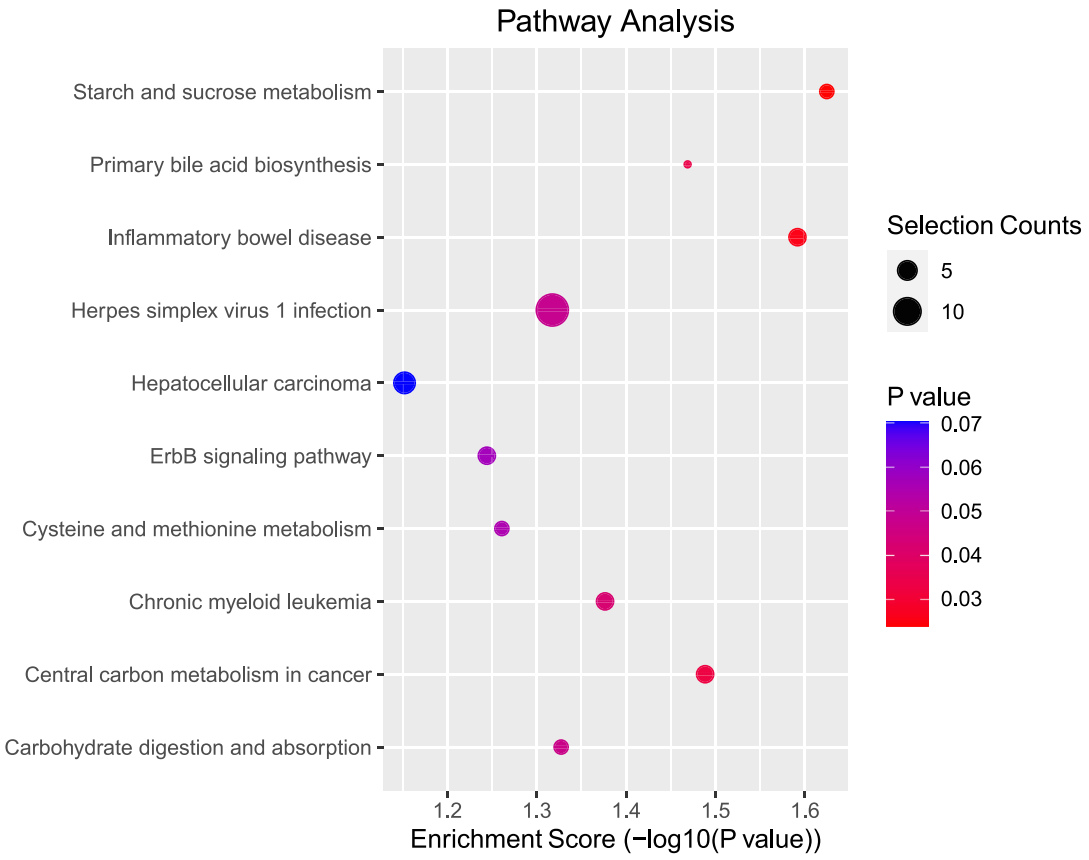

Figure 7. KEGG enrichment results of the effects of upregulated miRNAs in the AH of KC eyes.

Table 4. KEGG Analysis of the Effects of Downregulated miRNAs in the AH of KC Eyes (Fisher $P < 0.05$) for the Top 10 Pathways

KEGG Pathways	Fisher *P*	Enrichment Score	Target Genes	Pathway Genes
Herpes simplex virus 1 infection	0.000332395	3.478345	25	498
Graft-versus-host disease	0.000449054	3.347702	6	42
Antigen processing and presentation	0.000482423	3.316572	8	77
Endocytosis	0.007479979	2.1261	13	252
Mismatch repair	0.01688034	1.772619	3	23
Morphine addiction	0.02176659	1.66221	6	91
Salivary secretion	0.02394232	1.620834	6	93
Hippo signaling pathway, multiple species	0.03137622	1.503399	3	29
Viral protein interaction with cytokine and cytokine receptor	0.03269157	1.485564	6	100
Natural killer cell–mediated cytotoxicity	0.03780442	1.422457	7	131

et al.[17] investigated the post-transcriptional regulation of miRNAs and long noncoding RNAs (lncRNAs) in KC patients. Forty miRNAs were differentially expressed (29 upregulated and 11 downregulated) when KC and myopic control samples were compared. It was suggested that that six mRNAs, five miRNAs (miR-301a, miR-181a, miR-222, miR-98, and miR-128), nine lncRNAs, and the X-inactive specific transcript (XIST)–miR-181a–COL4A1 axis were involved in KC-associated RNA regulation.[17] We also found that miR-181a-5p and miR-222-3p were significantly downregulated in the KC group.

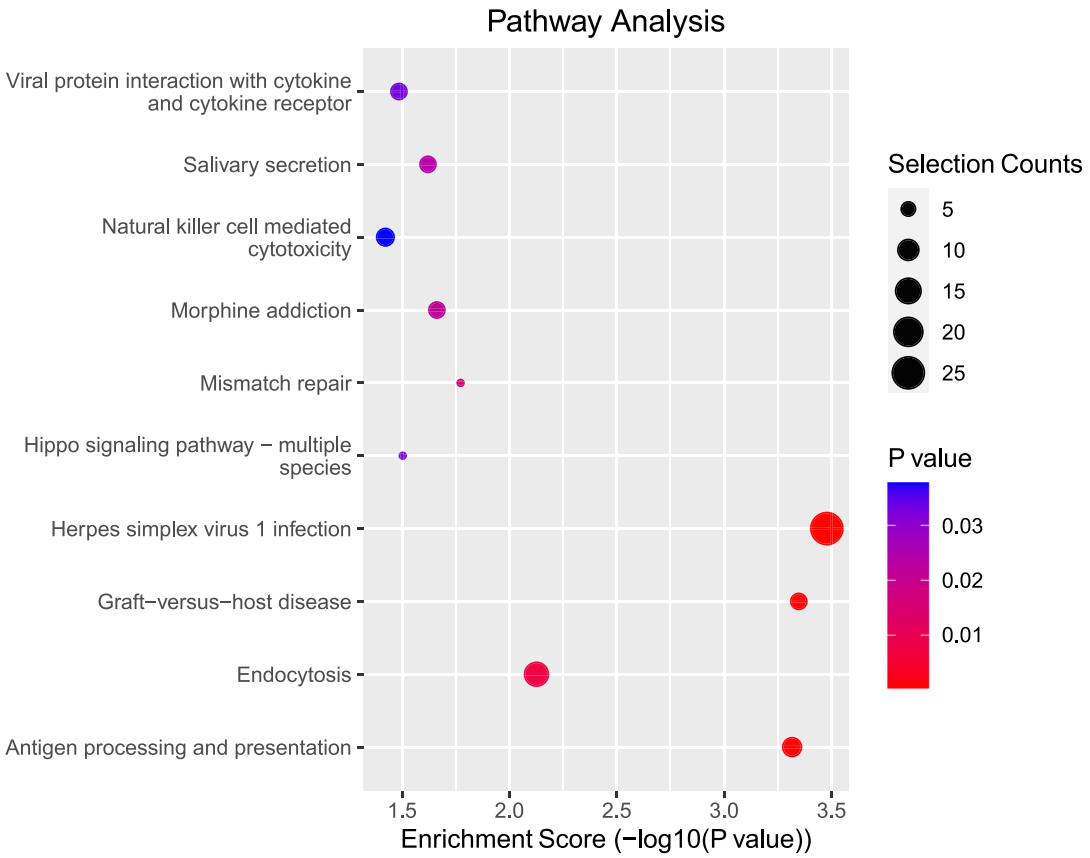

Figure 8. KEGG enrichment results of the effects of downregulated miRNAs in the AH of KC eyes.

The three studies cited above sought miRNAs in corneal tissue or epithelium. We explored DE miRNAs in the AH of KC eyes and revealed new information on translational and post-transcriptional regulation.

Biological Functions of DE miRNAs

It remains unknown whether the AH DE miRNAs contribute to KC pathology. We sought biological functions using bioinformatics approaches. We also searched the literature. We found that miR-195-3p, miR-199a-5p, miR-193b-5p, and miR-7-5p were upregulated in KC eyes. The expression level of miR-195-3p was 101.6-fold greater in KC than control AH, and this miRNA exhibited the highest DE. Zhang et al.[27] found that oxidative stress induced upregulation of miR-195 in the diabetic retina and suggested that miR-195 played a critical role in oxidative stress-induced, retinal endothelial cell injury targeting mitofusin-2 in diabetic rats. Oxidative stress injury to corneal stromal cells was found to play an important role in KC development in the rabbit.[28] The relationship between oxidative stress and miR-195 in KC should be further studied. Liu et al.[29] found that miR-199a might serve as a therapeutic target; this miRNA directly regulates the action of the specific protein 1 involved in the epithelial-to-mesenchymal transition of diabetic cataracts. Retinal miR-199a was downregulated during the oxygen-/retinopathy-induced vessel degeneration phase of rats.[30] miR-199a significantly repressed hypoxia-inducible factor 1-alpha (HIF-1α) and vascular endothelial growth factor (VEGF) activities in adult retinal pigment epithelial cells.[31] VEGF RNA levels were significantly decreased in KC eyes.[32] We found that miR-199 was significantly upregulated in KC AH, perhaps explaining the VEGF reduction. Few studies have explored the relationship between miR-193b and ocular disease. Dunmire et al.[33] reported that miR-193b was one of the top five miRNAs in the AH of cataract patients. In subjects with liver fibrosis, overexpression of miR-193a/b significantly repressed *COL1A1* and alpha-smooth muscle actin (α-SMA) expression.[34] We found that miR-193b was significantly overexpressed in the AH of KC eyes; any effect on corneal *COL1A1* expression would be interesting. In a rat model of diabetic retinopathy, overexpression of miR-7 decreased endothelial cell activity and the levels of phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt).[35] Because AH bathes the corneal endothelium, further work should explore

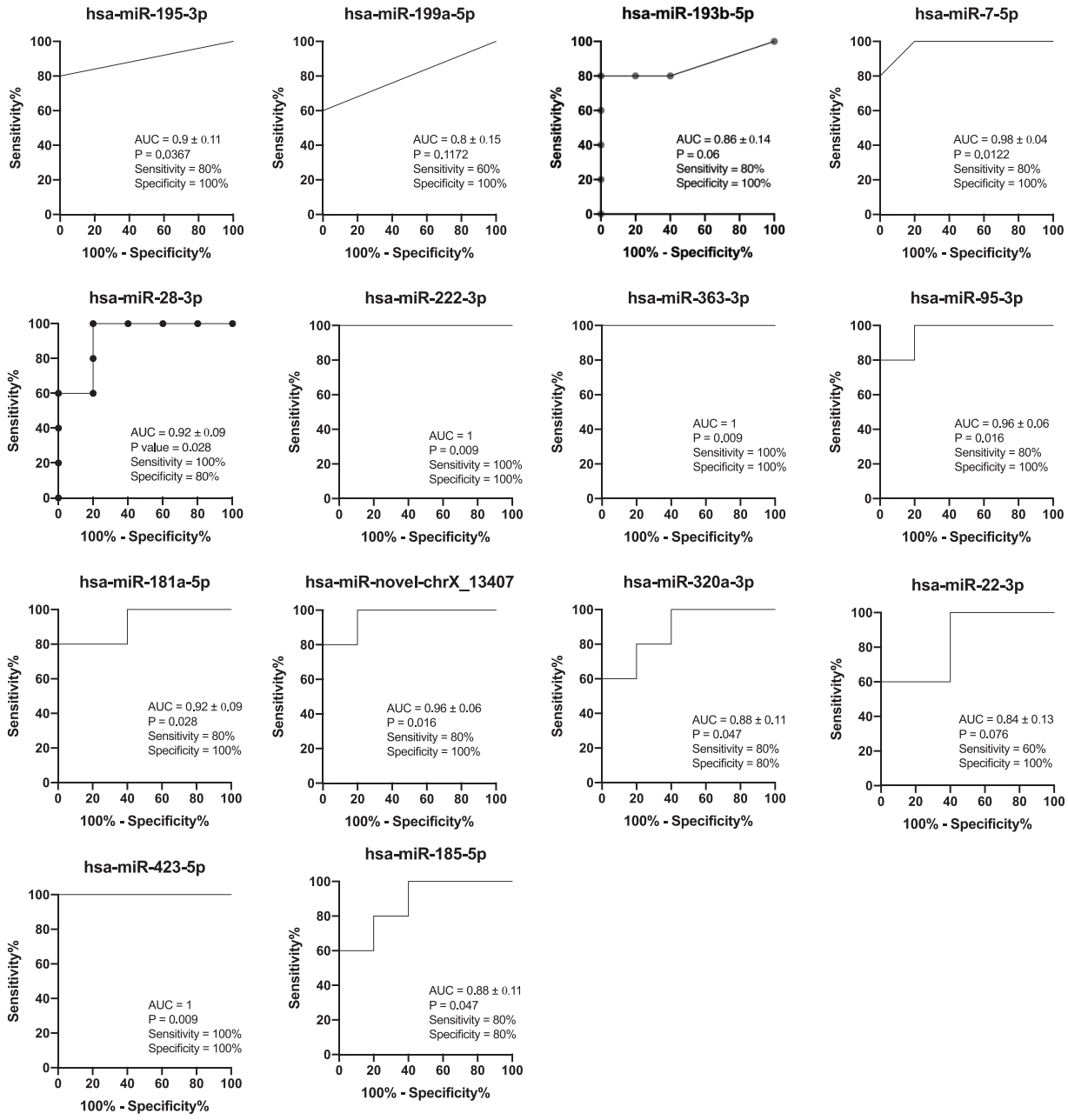

Figure 9. AUCs derived via ROC analysis of all 14 differentially expressed miRNAs (four upregulated and 10 downregulated).

whether miR-7 in AH influences the activity of corneal endothelial cells.

Ten miRNAs were downregulated in KC eyes; miR-222-3p, miR-181a-5p, and miR-185-5p were earlier shown to be downregulated in KC,[15–17] but not the others. miR-222-3p knockdown increased matrix metalloproteinase 2 (MMP-2) and MMP-9 expression in the human trophoblast cell line HTR8/SVneo.[36] KC stromal cells overexpressed MMP-2.[37] It is essential to explore the relationship between miR-222-3p and MMP-2 in KC. Also, miR-181a-5p reduced retinoblastoma cell proliferation, migration, and invasion but enhanced apoptosis.[38] miR-181a-5p/endocan regulated retinal angiogenesis via the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway.[39] The levels of miR-185-5p were significantly higher in the vitreous of patients with proliferative diabetic retinopathy than in non-diabetic controls,[40] but this miRNA was significantly downregulated in the AH of patients with diabetic macular edema.[41] miR-22-3p was significantly downregulated in the lens epithelia of senile cataracts,[42] and miR-22-3p

Table 5. Baseline Clinical Data for Eyes, the AHs of Which Were Subjected to qPCR

Information	Keratoconus Subjects	Myopia Subjects
Eyes/people, *n*	18/18	18/18
Age (y), mean ± SD	23.73 ± 4.80	20.88 ± 3.18
Sex (male/female), *n*	15/3	11/7
BCVA (logMAR), mean ± SD	0.48 ± 0.33*	0.03 ± 0.06*
TCT (mm), mean ± SD	466.00 ± 49.14*	543.05 ± 26.28*
K1, mean ± SD	47.99 ± 6.48*	41.85 ± 1.12*
K2, mean ± SD	52.62 ± 6.66*	43.45 ± 1.18*
PE, mean ± SD	50.40 ± 37.66*	4.61 ± 1.85*

Data were analyzed using SPSS 26.0. Student's *t*-test was used to compare data for age, BCVA, TCT, K1, K2, and PE between the groups.

*$P < 0.05$ (Pearson's χ^2 test was employed to compare the sex ratios).

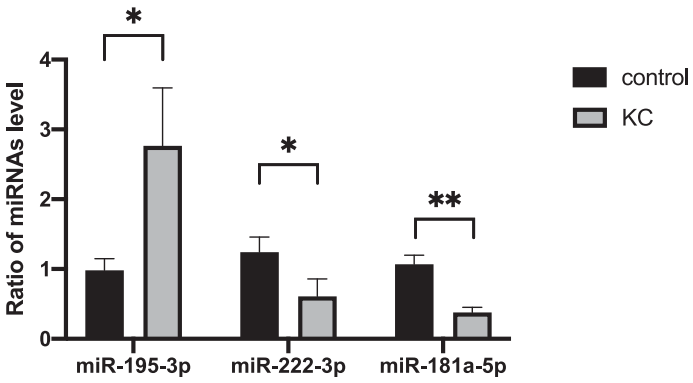

Figure 10. Quantitative PCR results for the ratio of miRNA expression in human AH. The *y*-axis represents mean $2^{-\Delta\Delta CT}$; *error bars* indicate standard deviations.

inhibition upregulated the expression of NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3), caspase-1, and interleukin-1β (IL-1β) in retinal pigment epithelial cells.[43] This shows that miR-22-3p action is linked to inflammation-induced injury. KC epithelium expresses elevated levels of inflammatory factors including IL-6 and tumor necrosis factor-α (TNF-α),[44] although KC is considered to be a non-inflammatory condition. Downregulation of miR-22-3p in KC may increase inflammatory injury. miR-423-5p is highly expressed in patients with proliferative diabetic retinopathy[45] but was downregulated in our study; any function for miR-423-5p in KC remains unknown. The possible ocular functions of miR-28-3p, miR-363-3p, miR-95-3p, and miR-320a-3p (especially miR-novel-chrX_13407) remain unclear, and further studies are required.

GO enrichment scores illustrated the molecular function, cell component and biological process of the DE miRNAs in KC AH. mRNA 3′ untranslated region (UTR)- and adenylate and uridylate (AU)-rich region binding, transcription regulatory region sequence-specific DNA binding, and DNA-binding transcription factor activity, RNA polymerase II-specific, are enriched biological processes in DE miRNAs in KC AH. It has been reported that miRNAs functioning is not limited to only post-transcriptional repression of target genes.[46] Determining the exact functions of DE miRNAs in KC AH requires further studies. KEGG bioinformatics analysis showed that possible pathways regulated by DE miRNAs include antigen processing and presentation, endocytosis, mismatch repair, and Hippo signaling. Chaerkady et al.[47] performed a highly sensitive mass spectrometry analysis of the epithelium and stroma from KC and normal donor corneas and reported that the levels of endocytosis-related proteins increased markedly in KC keratocytes. The Hippo signaling pathway is a major regulator of tissue growth and organ size.[48] The transcriptional regulator Yes-associated protein (YAP) is the main effector of the Hippo signaling pathway. In cultured retinal Müller cells, YAP inhibition suppressed transforming growth factor beta-1 (TGF-β1)-stimulated myofibroblast formation and extracellular matrix (ECM) production, whereas YAP activation augmented TGF-β1–independent fibroblast differentiation and ECM synthesis.[49] Also, two studies have reported that Hippo signaling may play a role in KC.[50,51] Further work is required to illustrate the relationship between KEGG bioinformatics analysis and KC.

DE miRNAs As Diagnostic Biomarkers

We explored whether DE miRNAs in AH might serve as diagnostic KC biomarkers. We drew ROC curves and calculated sensitivities and specificities. Most DE miRNAs reliably and specifically distinguished KC patients from controls (Fig. 9). The sensitivities and specificities of miR-222-3p, miR-363-3p, and miR-423-5p were all 100%. Although such results

are promising, our sample size was small; a future study with more patients is needed to confirm that DE miRNAs may serve as diagnostic KC biomarkers.

Our work had certain limitations, including the small sample size and pooling of samples. After pooling samples randomly, correlation analyses between miRNA expression and the index of KC progression (such as corneal curvature and PE) cannot be performed. Furthermore, grouping AH samples from KC patients at different stages and comparing the DE miRNAs between groups could further explore whether DE miRNA is related to KC progression. Also, most myopia control eyes evidenced high myopia, not emmetropia. However, the PE did not differ significantly between the high myopia and low-to-moderate myopia groups.[52,53] The number of DE miRNAs was low, with one reason being that the samples were derived from CXL patients, most of whom were at the early stages of KC, not severe. As shown in Table 1, the gender ratio differed significantly between the two groups, but the male sex is independently associated with KC progression.[54] The use of DE miRNAs for routine diagnostic purposes is limited because AH must be collected during (invasive) surgery. This may change. For example, Raman spectroscopy can analyze AH in situ, although further optimization is required.[55] AH could serve as an investigational material to identify DE miRNAs in KC early.

Conclusions

We used NGS to derive a comprehensive miRNA profile of the AH of KC eyes. It would greatly aid patients with KC if early accurate diagnoses were possible. Some KC epigenetic markers have been identified, but more information is required. To identify biomarkers of early and progressive corneal diseases, simple AH sampling methods are needed. Data on aberrantly expressed miRNAs in KC combined with bioinformatics analyses suggest possible roles for specific miRNAs, but further studies are required.

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