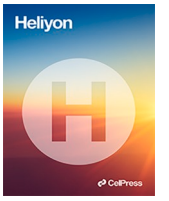
Heliyon 9 (2023) e20214

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Integrated analysis of microRNA expression in tears of Kazakh patients with climatic droplet keratopathy in Xinjiang, China

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ARTICLE INFO

Keywords:
microRNA
Tear
Climatic droplet keratopathy
Biomarker
Diagnosis
Tumor necrosis factor

ABSTRACT

Climatic droplet keratopathy (CDK) is a corneal diseases, which is characterized by increased oil-like deposits on the anterior elastic lamina and anterior stromal layer. Severe CDK can even cause blindness, with no specific available treatment. Besides. CDK is poorly understood in terms of its pathogenic mechanisms. Thus, to determine potential biomarkers for CDK, we analyzed the microRNA expression profile in tear samples from CDK patients and investigated their putative roles in the pathogenesis of CDK. Herein, miRNA sequencing and following bioinformatics analysis was performed to explore the roles of their target genes in CDK. A total of 67 differentially expressed miRNAs were identified, of which 25 were upregulated and 42 were downregulated. qPCR verification showed that among the up- and down-regulated miRNAs, expression of five and six, respectively, was most significantly different.The target genes of the differentially expressed miRNAs are involved in the FoxO signaling pathway, tumor necrosis factor (TNF) signaling pathway, and steroid hormone biosynthesis. Protein–protein interaction network analyses identified 20 hub genes, including *PTEN*, *GSK3B*, and *SMAD3*. In conclusion, the panel of differentially expressed miRNAs identified may have potential utility as early diagnostic biomarkers for CDK. Moreover, the TNF signaling pathway is a new potential target in CDK for the development of treatments.

1. Introduction

Climatic droplet keratopathy (CDK) is characterized by corneal opacity resulting from an increase in oil-like deposits on the anterior elastic lamina and anterior stromal layer [1]. CDK mostly occurs in developing countries such as Argentina [2], Western Rajasthan, India [3], and Southwest Ethiopia [4]. Additionally, CDK prevalence varies greatly between regions and ethnic groups, from 4.4% to 20%, according to literature published after 2000 [2–5]. The clinical manifestations of CDK in the early stage are usually

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https://doi.org/10.1016/j.heliyon.2023.e20214
Received 30 March 2023; Received in revised form 20 August 2023; Accepted 13 September 2023
Available online 15 September 2023

not obvious, whereas in later stages, severe CDK manifests as deposition covering the cornea in the form of bands, affecting vision and even causing blindness [1,6].

To date, no specific pharmacological treatment is available for CDK. As to surgical approaches, the superficial keratectomy along with transplantation of the amniotic membrane or cornea is the only way to treat the patients of severe CDK [7]. Therefore, it is important to elucidate the molecular mechanism underlying CDK occurrence and develop strategies for early diagnosis and effective treatment of patients with CDK. Epidemiological researchs have demonstrated that specific environmental elements, including temperature, humidity, exposure to ultraviolet rays [1], and genetic factors [8], exert a crucial effect on the onset and progression of CDK. Phosphoproteomic and proteomic analyses have indicated that formation of CDK droplets might be linked to oxidative stress damage and chronic inflammation [9–11]. It is challenging to collect corneal tissue from both CDK patients and healthy individuals. As a body fluid, tears are relatively easy to obtain as samples non-invasively, and tear-derived biomarkers of diseases could have great applications in the clinical setting. miRNAs are small, non-coding, single-stranded RNA molecules that regulate gene expression by either inhibiting miRNA function or by targeting mRNA for degradation [12,13]. miRNAs play important roles in cell development, differentiation, and repair [12,13]. In addition, they may contribute to human diseases, including corneal diseases such as keratoconus [14], keratitis and corneal angiogenesis [15,16]. Furthermore, miRNAs can be packaged in membranous sacs such as exosomes [17], which are released by secretory cells into various extracellular fluids and then regulate other target cells via body fluid circulation including human tears [18]. miRNAs in human tears are stable and can withstand repeated freeze–thaw cycles and enzymatic hydrolysis [19]. The expression of some miRNAs in patient tears has been reported to be differential including patients with Alzheimer's disease [20], cancer [21], Sjogren's syndrome [19] and glaucoma [22]. However, differential expression of miRNAs in tears of CDK patients and their potential functions therein remain unclear.

Thus, in the present study, we sequenced miRNAs from tears in patients with CDK and the healthy Kazakh population, and then conducted bioinformatics analysis to investigate the potential roles of differentially expressed miRNAs as well as the biological processes underlying CDK. We present miRNAs that can potentially be used as diagnostic biomarkers of CDK and contribute to understanding the complex mechanisms underlying the disease.

2. Material and METHODS

2.1. Tear samples collection

Tear samples were collected in Tacheng area of Xinjiang, as described in our previous epidemiology study [5]. A number of CDK patients were found in Kazak residents (Fig. 1a). Basal tear fluid was collected from the inferior tear meniscus of both eyes of both the CDK group and the healthy control (N-CDK) group using micropipettes (Shengzekanghua, China) (Fig. 1b).

Precautions were taken to ensure the corneal and conjunctival surfaces were not touched.Following short intervals and several

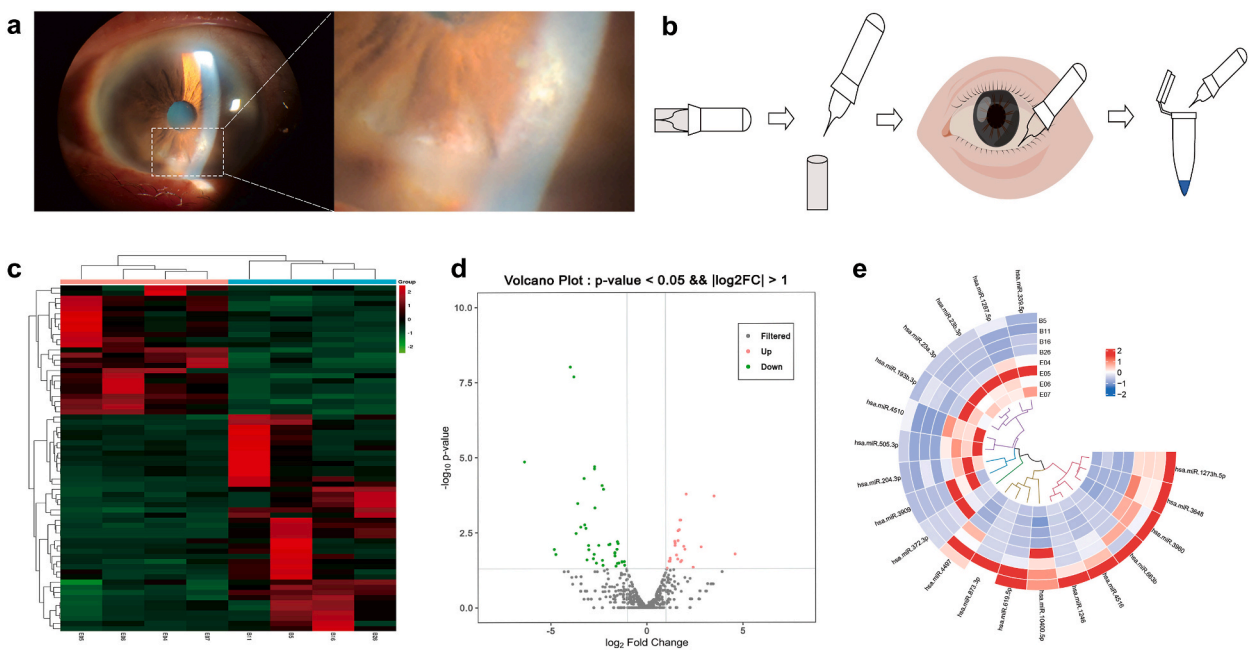

Fig. 1. Clinical findings, sample collection method, and microRNA (miRNA) sequencing results. (a) Slit lamp biomicroscopy: subepithelial oil-like deposits at the inferior corneal limbus in a climatic droplet keratopathy (CDK) patient in Xinjiang. Inset shows the locally enlarged plot. (b) Tear sample collection using micropipettes. (c) Heatmap of miRNA expression profiles between the CDK and the N-CDK groups. (d) Volcano plot showing relative expression of miRNAs in the CDK and N-CDK groups. (e) Heatmap showing top 10 up and downregulated miRNAs.

blinks, tear samples were gathered until a volume of approximately 10 μl was achieved. For participants with insufficient tears, tear samples were collected using the same method, but only after the instillation of 10 μl of distilled water. The gathered tear samples were then stored in cryo-tubes and maintained at −80 °C until subsequent analysis. This research adhered to the principles of the Declaration of Helsinki. All the samples were collected with informed consent. An ethical approval was obtained from the Bioethical Committee of the Ninth Division Hospital of the Xinjiang Production and Construction Corporation.

2.2. miRNA extraction from tear and sequencing

From the 8 tear samples (4 CDK vs. 4 N-CDK), total RNA was extracted using the EZB-RN2-serum RNA purification kit (EZBioscience, USA) following the manufacturer's guidelines. The RNA's purity and concentration were assessed using the NanoDrop 2000 (Thermo Fisher Scientific, USA). The acceptable range for RNA was an absorbance ratio (A260/A280) between 1.5 and 2.1. Small RNA sequencing was conducted by KangChen Biotech (Shanghai, China) using the Illumina Small RNA Sequencing platform (Illumina, USA).

2.3. Reverse transcription and real-time quantitative polymerase chain reaction

To verify the changes in miRNA expression detected using miRNA-seq, 20 miRNAs were selected according to their expression levels in each group (fold change value > 1.5, $p < 0.05$). Of these, 10 each were the most significantly downregulated and upregulated. cDNAs were synthesized from total RNA extracted from tear samples using the EZB-miRT4-microRNA Reverse Transcription Kit (EZBioscience, USA). According to the manufacturer's instructions, quantitative polymerase chain reaction (qPCR) was performed with diluted cDNAs using an EZ-Probe qPCR Master Mix for microRNA (EZBioscience, USA) on the CFX96 real-time PCR system (BioRad, USA). U6 small nuclear RNA (snRNA) was used as the control. The primers were designed using the Primer NCBI website and the reverse primers were universal for miRNA (Table 1).

qPCR was carried out in a 20 μl mixture, comprising 0.4 μl of probe, 0.8 μl of forward primer, 0.8 μl of Universal 3′ qPCR Primer, 8 μl of cDNA, and 10 μl of qPCR Master Mix for miRNA. The reaction began with a denaturation step at 95 °C for 5 min, then proceeded through 40 amplification cycles: 95 °C for 10 s, 60 °C for 30 s, and a return to 95 °C for 10 s. Three copies of each PCR reaction were performed. The relative expression levels of each miRNA was calculated using the $2^{-\Delta\Delta CT}$ method.

2.4. In silico gene expression analysis

In order to identify the potential biological functions of the miRNAs in the tear, miRDB [23], miRwalk [24], TargetScan [25], and RNA22 were used to predict target genes of the verified miRNAs with significantly expressed difference [26]. Furthermore, gene Ontology (GO) functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) [27,28] signal pathway enrichment analyses were performed on the predicted target genes. Only genes that overlapped among the four databases were considered. The central target gene for each miRNA was selected from the STRING database [29]. The protein-protein interaction (PPI) network was analyzed using Cytoscape (v3.9.0) based on the results from STRING. Using the CytoHubba plugin, the connectivity of each node was assessed, and key genes were recognized as the top 20 genes exhibiting the highest connectivity.

Table 1
Primers for the real-time quantitative PCR (RT-qPCR) assay.

Target	F	Primer Sequence (5' →3')	Up/Down
hsa-miR-3909	F	GTGTCCTCTAGGGCCTGCA	Up
hsa-miR-372-3p	F	GAAGTGCTGCGACATTTGAG	Up
hsa-miR-204-3p	F	GCTGGGAAGGCAAAGGGA	Up
hsa-miR-505-3p	F	CGTCAACACTTGCTGGTTTC	Up
hsa-miR-23a-3p	F	GGGTCACATTGCCAGGGAT	Up
hsa-miR-1287-5p	F	GTTGCTGGATCAGTGGTTCG	Up
hsa-miR-193b-3p	F	ACCTGGCCCTCAAAGTCC	Up
hsa-miR-4510	F	GGTGAGGGAGTAGGATGTATGG	Up
hsa-miR-23b-3p	F	CACATTGCCAGGGATTACC	Up
hsa-miR-339-5p	F	TCGTATTCCCTGTCCTCCAG	Up
hsa-miR-3648-1	F	ATAAGCCGCGGGGATCG	Down
hsa-miR-873-3p	F	TGGCGGAGACTGATGAGTTC	Down
hsa-miR-663b	F	AACCGGCCGTGCCTGA	Down
hsa-miR-619-5p	F	GCTGGGATTACAGGCATGA	Down
hsa-miR-4516	F	GCTGGGAGGTCAAGGCTG	Down
hsa-miR-1273h-5p	F	GCTGGGAGGTCAAGGCTG	Down
hsa-miR-1246	F	GAATGGAGTTTTGGAGCAGG	Down
hsa-miR-3960	F	GGCGTCGGCGGAGGC	Down
hsa-miR-4497	F	TAATCTTCGGGACGGCTGG	Down
hsa-miR-10400-5p	F	AATAATGCGGCGGCGGCTCT	Down

2.5. Statistical analysis

Quantitative variables were represented as mean and standard deviation (mean ± SD). Qualitative variables were presented in absolute numbers (n) and percentages (%). The Kolmogorov – Smirnov test was employed to determine the normal distribution of all variables. For data that followed a normal distribution, a two-tailed Student's t-test was utilized. A threshold of $p < 0.05$ was established for statistical significance. Data processing was conducted using SPSS 26.0 (IBM/SPSS, USA) or GraphPad Prism 9 (GraphPad Software, USA).

3. Results

3.1. Clinical characteristics of CDK and N-CDK patients

In the present study, tear samples were collected from 30 patients with CDK (mean age: 68.79 ± 7.44 years; range: 55–85 years) and 30 N-CDK controls (mean age: 70.03 ± 6.16 years; range: 57–87 years). Of the CDK patients, only grade 3 patients were included. No significant differences were observed in the distribution of age and sex between the CDK patients and controls. To increase comparability and reliability, 4 CDK and N-CDK samples each were selected for miRNA sequencing after matching for age and sex (Table 2). The remaining samples were used for subsequent qPCR validation of miRNAs.

3.2. Tear miRNA expression profiles of CDK and N-CDK patients

The high-throughput sequencing identified 815 miRNAs in the tear samples of the CDK and N-CDK groups. Under the criteria of fold change >1.5 and $p < 0.05$, 25 genes were up-regulated and 42 were down-regulated in the CDK group compared with their expression in the N-CDK group. The differences in the miRNA expression profiles between the two groups were shown as a heat map (Fig. 1c) and volcano plot (Fig. 1d). Table 3 details the top 20 miRNAs identified in the sequencing, comprising the top 10 upregulated and top 10 downregulated miRNAs, as also depicted in a heat map (Fig. 1e).

3.3. Differential miRNA expression in tears of CDK and N-CDK patients

To validate the differentially expressed miRNAs detected using miRNA sequencing, the top 10 most upregulated and down-regulated miRNAs in the tear samples were selected and examined using qPCR. We observed general consistency between the qPCR and high-throughput sequencing results. Among the top 10 upregulated miRNAs, five (hsa-miR-204-3p, hsa-miR-23a-3p, hsa-miR-1287-5p, hsa-miR-4510, and hsa-miR-23b-3p) showed similar expression trends and significant differences. Among the top 10 downregulated miRNAs, six (hsa-miR-3648, hsa-miR-873-3p, hsa-miR-1273h-5p, hsa-miR-3960, hsa-miR-4497, and hsa-miR-10400-5p) showed similar expression trends and significant differences (Fig. 2 a and b). To re-evaluate these inconsistent results, the up- and down-regulated miRNAs were re-ranked by the $\log_2$ fold change as determined by qPCR (Fig. 2 c and d).

3.4. Bioinformatic analysis of miRNAs based on qPCR results

3.4.1. miRNA target gene prediction

To explore the possible roles of the differentially expressed miRNAs, target genes of the top five up- and down-regulated validated miRNAs were predicted using the databases mentioned earlier. For highlighting the relationship between the four databases' results, two Venn diagrams were created, in which the expression of 367 overlapping genes for the upregulated miRNAs and 217 overlapping genes for the downregulated miRNAs in the CDK group compared with that in the N-CDK group (Fig. 3a and b).

3.4.2. GO and KEGG enrichment

To further accentuate the differential expression of the miRNAs, GO and KEGG functional enrichment analyses were conducted on the predicted mRNA targets of both the up- and down-regulated miRNAs.

GO function annotations of the predicted target genes of the upregulated miRNAs were shown in Fig. 3c. The main molecular functions (MFs) enriched were transcription factor activity (GO: 0000983) and extracellular matrix constituent conferring elasticity

Table 2
Clinical characteristics of CDK patients and healthy volunteers.

Group	ID	Age	Gender	Grade
CDK (TEST)	E4	66	F	3
	E5	65	F	3
	E6	68	F	3
	E7	73	F	3
N-CDK (CONTROL)	B5	66	F	0
	B11	65	F	0
	B16	68	F	0
	B26	70	F	0

Table 3
Differentially expressed miRNA in the group with CDK as compared to the normal group.

miRNA UP TOP 10	log2 Fold Change	P	miRNA DOWN TOP 10	log2 Fold Change	P
hsa-miR-3909	4.602108993	0.016450645	hsa-miR-3648	−6.353658628	0.000
hsa-miR-372-3p	3.509445568	0.000193768	hsa-miR-873-3p	−4.81186273	0.011
hsa-miR-204-3p	2.835597965	0.009530045	hsa-miR-663b	−4.734005718	0.017
hsa-miR-505-3p	2.417568047	0.045382081	hsa-miR-619-5p	−3.96047097	0.000
hsa-miR-23a-3p	2.05945711	0.000166985	hsa-miR-4516	−3.781513897	0.000
hsa-miR-1287-5p	1.990250318	0.01141166	hsa-miR-1273h-5p	−3.679141791	0.003
hsa-miR-193b-3p	1.92949632	0.009132733	hsa-miR-1246	−3.59221367	0.000
hsa-miR-4510	1.81685041	0.02696017	hsa-miR-3960	−3.434028052	0.002
hsa-miR-23b-3p	1.768297536	0.001220189	hsa-miR-4497	−3.254671131	0.000
hsa-miR-339-5p	1.76420364	0.029702882	hsa-miR-10400-5p	−3.22729385	0.002

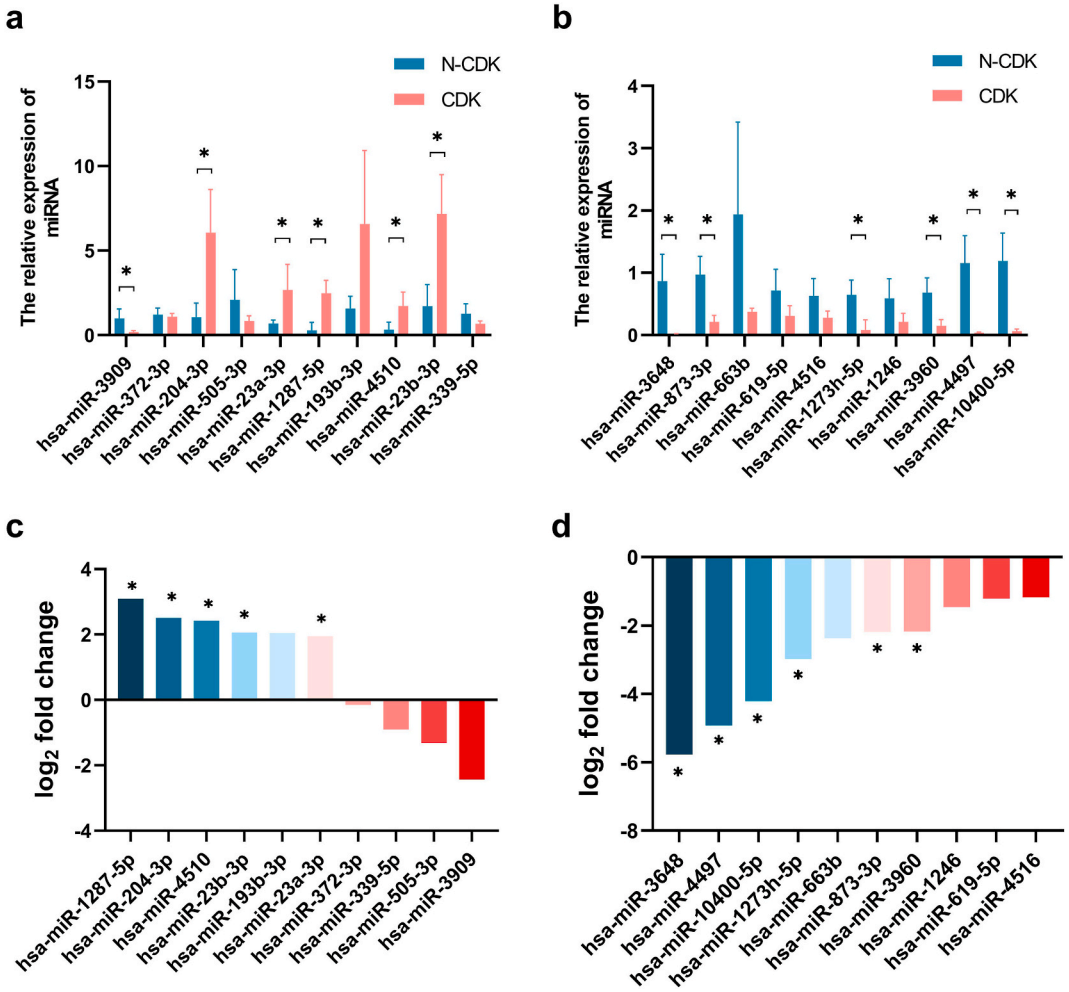

Fig. 2. Polymerase chain reaction (PCR) validation of microRNA (miRNA) sequence results. (*P < 0.05) (a) Quantitative (q)PCR verification of top 10 upregulated miRNAs. (b) qPCR verification of top 10 downregulated miRNAs. (c) Top 10 upregulated miRNAs ranked by their $\log_2$ fold change. (d) miRNA reranked by the $\log_2$ fold change in top 10 downregulated miRNA.

(GO: 0030023); the main biological processes (BPs) were the Wnt signaling pathway (GO: 0007223) and cellular response to cytokine stimulus (GO: 0071345); the main cellular components (CCs) were L-type voltage-gated calcium channel complex (GO: 1990454) and voltage-gated calcium channel complex (GO: 0005891). The GO functional annotations of the predicted target genes of the down-regulated miRNAs were shown in Fig. 3d. The main MF was monooxygenase activity (GO: 0004497); the main BP was positively regulated by receptor-mediated endocytosis (GO: 0048260) and nuclear-transcribed mRNA catabolic process (GO: 0000288); and the

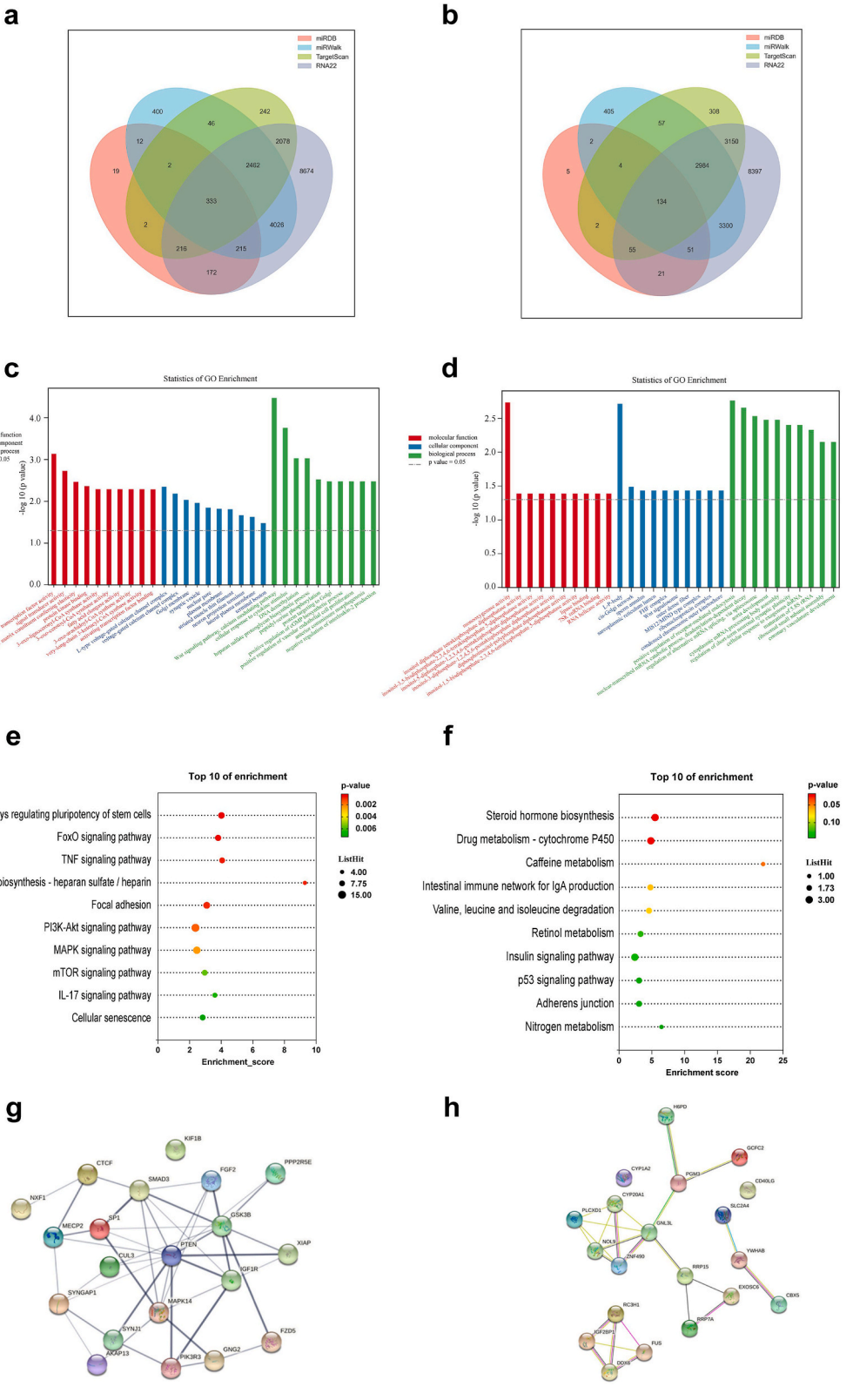

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Fig. 3. In silico analysis of differentially expressed microRNAs (miRNAs). (a) Venn diagram of predicted target genes of top five upregulated miRNAs. (b) Venn diagram of predicted target genes of top five downregulated miRNAs. (c) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for predicted target genes of upregulated miRNAs. (d) KEGG pathway analysis for predicted target genes of downregulated miRNAs. (e) Gene Ontology (GO) analysis for predicted target genes of upregulated miRNAs. (f) Gene Ontology (GO) analysis for predicted target genes of downregulated miRNAs. (g) Top 20 hub genes in protein–protein interaction (PPI) network of predicted target genes of upregulated miRNAs. (h) Top 20 hub genes in PPI network of predicted target genes of downregulated miRNAs.

main CCs were P-body (GO: 0000932), cis-Golgi network (GO: 0005801), and Wnt signalosome (GO: 1990909).

Regarding KEGG pathway enrichment, the predicted target genes of the upregulated miRNAs were mainly enriched in the FoxO (*PDPK1; PIK3R3; FBXO32; RBL2; SMAD3; TGFBR2; MAPK14; IGF1R; PTEN*) and tumor necrosis factor (TNF) (*PIK3R3; TNFAIP3; FAS; TAB3; CASP10; MAPK14; IL18R1; CASP7*) signaling pathways, and those of the downregulated miRNAs were mainly enriched in steroid hormone biosynthesis (*LRTOMT; SULT1E1; CYP1A2*) and caffeine metabolism (*CYP1A2*) (Fig. 3 e,and f). Nine target genes specific to the miRNAs were enriched in the TNF pathway (Fig. 4). Additionally, the affected target genes in the signaling pathways were detailed in the supplementary tables (Table S1 and Table S2).

3.4.3. PPI analysis

To elucidate the interactions among hub genes, a PPI network for the predicted target genes of both upregulated and downregulated miRNAs was constructed using the STRING database and visualized with Cytoscape software. The upregulated network included 243 nodes and 429 edges, whereas the downregulated network included 62 nodes and 65 edges. The top 20 genes, both up- and down-regulated in the PPI, were illustrated in Fig. 3g and h. Based on the degree ranking by the CytoHubba plugin, *PTEN*, *SMAD3*, *GSK3B*, *FGF2*, and *MECP2* were identified as the top five hub genes in the upregulated PPI network. Meanwhile, in the downregulated PPI network, *GNL3L*, *DDX6*, *NOL9*, *PLCXD1*, and *YWHAB* were determined as the top five hub genes.

4. Discussion

CDK is a rare ocular disease of unknown etiology that can affect eyesight and even cause blindness. It occurs mainly in remote areas

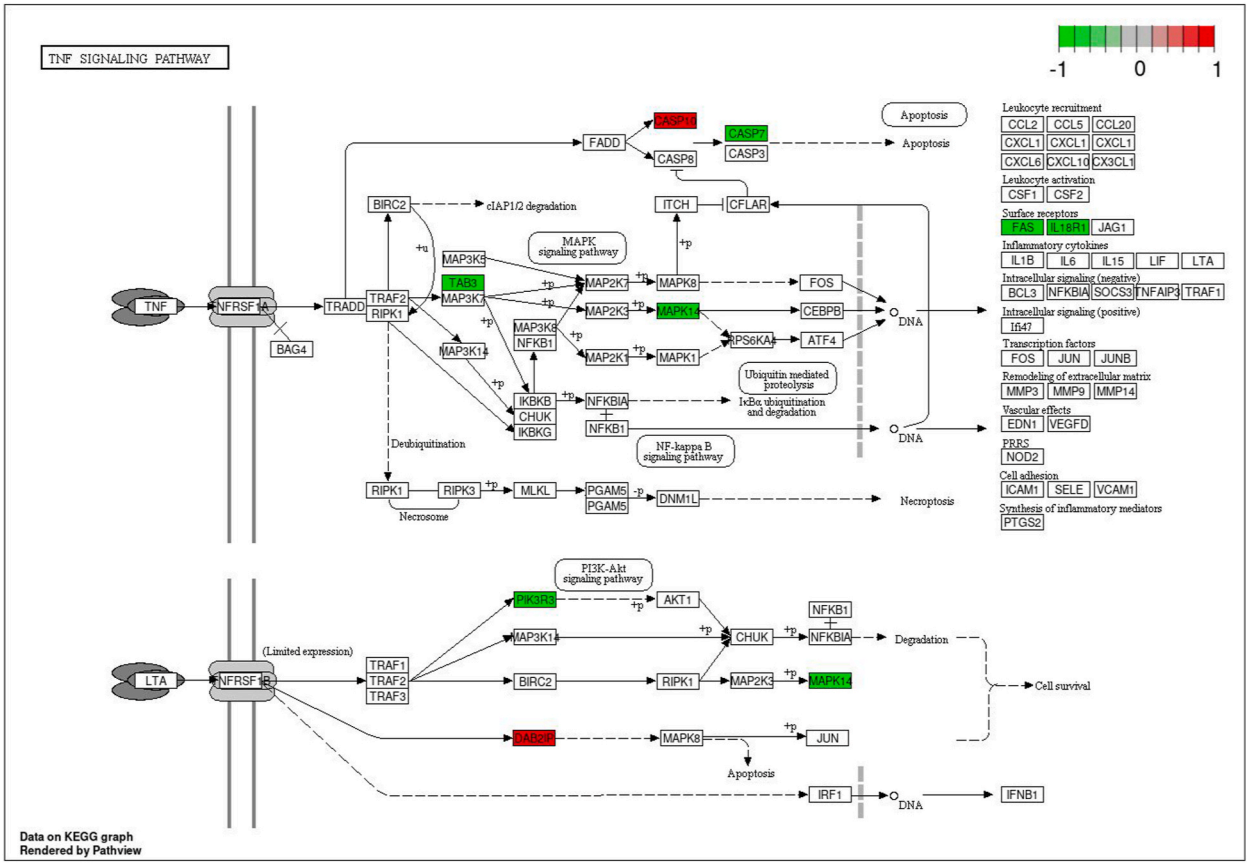

Fig. 4. Representation of the KEGG TNF Signaling Pathway, showing up-regulated (red boxes) and down-regulated (green boxes) genes.

and often in developing countries [1]. Owing to poor medical and economic conditions, patients are often diagnosed in the middle or late stage. Although there have been some studies on the differential expression components in tears of patients with CDK [30,31], no study has been conducted on the effects of the miRNAs in the tears on CDK, and little is known about their roles in CDK. To the best of our knowledge, this study is the first to use miRNA sequencing and qPCR to investigate miRNA expression in CDK tear samples. We assessed the biological functions of the differentially expressed miRNAs that we identified, using bioinformatic approaches, to explore the pathogenic mechanisms underlying CDK.

In our study, 815 miRNAs were found in the tear samples using miRNA sequencing. Among these miRNAs, 25 were up- and 42 were down-regulated in the CDK group compared with their expression in the N-CDK group. Next, the top 20 up- and down-regulating miRNAs were selected for verification using qPCR. We acknowledge that the level of agreement showed inconsistency to some extent between qPCR and sequencing results. The results might have been caused by various factors, such as technical differences, the complexity of miRNA expression analysis in low-expression, and individual variations in clinical samples.

Furthermore, qPCR verification showed that among the upregulated miRNAs, expression of five (hsa-miR-204-3p, hsa-miR-23a-3p, hsa-miR-1287-5p, hsa-miR-4510, and hsa-miR-23b-3p) was significantly different, and among the downregulated miRNAs, that of six (hsa-miR-3648, hsa-miR-873-3p, hsa-miR-1273h-5p, hsa-miR-3960, hsa-miR-4497, and hsa-miR-10400-5p) was significantly different. These 11 miRNAs could be potential biomarkers for early screening and diagnosis of CDK.

In order to determine whether these miRNAs were associated with CDK occurrence and development, we searched the literature that have been published.hsa-miR-204 has been closely related to corneal wound healing [32]. Typically, miR-204 is highly expressed in normal corneal epithelial cells to suppress corneal neovascularization by suppressing the function of human microvascular endothelial cells [33]. In addition, miR-204 downregulation contributes to corneal healing by promoting cell proliferation and migration in human corneal epithelial cells (HCECs) [32]. In CDK patients, the expression of miR-204 levels are high in tears, and this may inhibit corneal epithelial repair after injury, causing chronic tissue damage. For the other miRNAs, no study on the human cornea or HCEC is available. However, some other studies have shown that hsa-miR-1287-5p also inhibits epithelial cell migration [34], hsa-miR-3648 promotes cell proliferation [35,36], and hsa-miR-3960 promotes cell differentiation [37]. Further studies are needed to explore the function of these miRNAs in CDK. Specific miRNAs could be selected for further study of their functional regulation and protective effects against ultraviolet-induced photodamage in HCEC.

To further elucidate the underlying functions of these verified miRNAs, we performed GO and KEGG pathway enrichment analyses. The TNF signaling pathway was significantly enriched in patients with CDK in our study. Evidence suggests that the TNF signaling pathway promotes epithelial cell survival and contributes to HCEC transdifferentiation [38,39]. Levels of TNF-α, MMP-2, and MMP-9 are higher in tears of CDK patients than in those of control individuals [30,31]. These previous studies speculated that chemokines in tears are responsible for CDK droplet formation. TNF-α can regulate corneal epithelial repair and promote MMP-2 and MMP-9 secretion [40–42]. From this standpoint, the TNF signaling pathway can be considered an important participant in the pathogenesis of CDK. As for PPI network and module analysis, previous studies have discovered that *PTEN* and *SMAD3* were involved in the corneal repair, which may participate in the occurrence of CDK [43–45]. Further studies are needed to elucidate the function of other hub genes in corneal endothelial cells.

Nevertheless, this study has limitations. First, the sample size for miRNA sequencing was small and did not fully represent the expression profiles of all patients. Secondly, this study only verified the expression levels of miRNAs in tear samples. However, it is hard to sample corneal tissue from CDK patients and people is challenging. In future studies, we will furtherly compare the expression of miRNAs in corneal epithelial tissue of patients with CDK and normal controls.

5. Conclusion

Our study illustrated the miRNA expression profile in CDK tears using a sequencing approach. Five up- and six down-regulated miRNAs were found to be involved in the pathogenesis of CDK. The TNF signaling pathway may play an important role in CDK. Thus, this study furthers our understanding of the pathogenic mechanisms underlying CDK and may aid the development of new treatments for climatic droplet keratopathy based on the potential miRNA biomarkers we identified.

Author contribution statement

Zhixiang Hua: Wrote the paper; Performed the experiments; Conceived and designed the experiments; Analyzed and interpreted the data. </p>

Xiaoyan Han: Wrote the paper; Conceived and designed the experiments; Analyzed and interpreted the data. </p>

Guoqing Li, Li Lv, Nuerguli Jianimuhan, Dongmei Ma, Lei Cai, Fangyuan Hu: Contributed reagents, materials, analysis tools or data. </p>

Jin Yang: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Revised the paper. </p>.

Data availability statement

Data associated with this study has been deposited at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE208377.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This research received financial backing from the Xinjiang Production and Construction Corps Financial Technology Plan Project (Grant 2020CB030). Our gratitude extends to KEGG (https://www.genome.jp/kegg/), GO (http://geneontology.org/), miRDB (http://www.mirdb.org/), miRwalk (http://mirwalk.umm.uni-heidelberg.de/), TargetScan (https://www.targetscan.org/), RNA22 (https://cm.jefferson.edu/rna22/), and STING (https://string-db.org/) programs for generously making their data available for analysis. Furthermore, our gratitude extends to Figdraw (https://www.figdraw.com) for generously offering a selection of valuable eye images for our use.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20214.

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