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Investigation of MicroRNA Expression in Anterior Lens Capsules of Senile Cataract Patients and MicroRNA Differences According to the Cataract Type

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Purpose: We investigated the microRNAs (miRNAs) expression in the anterior lens capsules of patients with senile cataract and compared it to that in the anterior lens capsules of healthy controls. Moreover, we compared the differences in miRNAs expression according to the types of cataracts.

Methods: Individual lens epithelium samples were collected from 33 senile patients and 10 controls. The cataract patients were classified into cortical, nuclear, posterior and anterior subcapsular and mixed. The expression of 12 different miRNAs in lens epithelium was measured using real-time polymerase chain reaction and compared between the senile cataract patients and controls. The differences of miRNA levels according to cataract type were analyzed.

Results: The expression levels of let-7g-5p, miR-23a-3p, miR-23b-3p, and miR-125a-5p were significantly upregulated in patients with senile cataract when compared with those in the control group ($P < 0.05$). The expressions of let-7a-5p, let-7d-5p, miR-16-5p and miR-22-3p were significantly downregulated in the senile cataracts ($P < 0.05$). Let-7a-5p, let-7d-5p, let-7g-5p and mir-23b-3p had significant difference in expression between nuclear and anterior subcapsular cataracts.

Conclusions: The eight differentially expressed miRNAs may be involved in the pathogenesis of senile cataract, in particular, related to oxidative stress and autophagy.

Translational Relevance: We infer that several miRNAs in lens epithelial cells are promising candidate biomarkers of senile cataracts.

Introduction

Cataract, defined as the clouding of natural crystalline lens in the eye, is the most common cause of blindness worldwide and accounts for nearly 42% of all causes of blindness.[1,2] Multiple factors, such as aging, eye injury, diabetes mellitus, ultraviolet exposure, drug use, and other ocular diseases increase the risk for cataract.[3–5] However, the most common cause is aging. Due to the global expansion of aging population, senile cataract has become the leading cause of blindness. The only effective treatment available currently is the surgical removal of the affected lens and replacement with an artificial lens.[6,7] However, the cost of surgery and the need for highly trained

personnel to perform ocular surgery limit the restoration of vision in certain parts of the world. Hence, the prevention and noninvasive treatment of cataracts is imperative.

MicroRNAs (miRNAs) are a group of endogenous small noncoding RNAs, approximately 20 to 25 nucleotides in length, which regulate gene expression posttranscriptionally.[8,9] MiRNAs regulate mRNA degradation or translation by binding to a complementary sequence in the 3′-untranslated region of the target gene mRNA.[10] MiRNAs play a significant role in many cellular processes, including cell differentiation, proliferation, and apoptosis. Although most of the miRNA studies are related to cancers, accumulating evidence has demonstrated that miRNAs are also involved in the pathogenesis of ocular diseases, including cataract.[11–13]

Several studies have investigated microRNA expression in the anterior lens capsule of cataract patients. Chien et al.[14] found a positive correlation between high miR-34a levels and increased severity of lens opacity. Li et al.[15] found that miR-15a and miR-16-1 were expressed at a significantly higher level in the lens epithelial cells of patients with cataract compared with normal epithelial cells. Wu et al.[16] reported distinct miRNA profiles in the central epithelium of normal transparent lens and the lens in patients with age-related cataract. However, the miRNA study of cataract was limited by difficulties involved in obtaining normal control samples. In the previous studies, lens of postmortem eyes or lens capsules of mild cataracts were used as controls.[14,16,17]

We conducted a preliminary study using miScript miRNA polymerase chain reaction (PCR) Array Human miFinder (MIHS-001ZF-12; Qiagen, Valencia, CA, USA) to compare the miRNA expression in the lens epithelium of cataract patients and controls. In our preliminary study, miR-22-3p, miR-23b-3p, miR-26a-5p, and miR-125b-5p were abundantly expressed in the lens epithelium and the levels of let-7a-5p, let-7d-5p, let-7e-5p, miR-22-3p, miR-23a-3p, miR-23b-3p, miR-26a-5p, and miR-125a-5p were significantly different between the two groups. This study was carried out using miScript miRNA PCR Array Custom to analyze the aforementioned miRNAs and the miR-15a-5p, miR-16-5p, miR-34a-5p, which were significantly expressed in the previous study.

In this study, we investigated the miRNA expression in the epithelial cells of transparent and cataractous lenses. We investigated the differences in miRNA levels according to the type of cataract.

Materials and Methods

Materials

Thirty-three samples of lens epithelium from senile cataract patients and 10 from normal subjects were collected at the Hanyang University Hospital. This study followed the tenets of the Declaration of Helsinki. The authors also received consent for research use with the approval of the Hanyang University Institutional Human Experimentation Committee (IRB number: HYUH 2018-10-013-003). All patients underwent a complete preoperative ophthalmologic examination. Only those who had no ocular disease other than age-related cataracts were included. Patients who had previously undergone ocular surgery or those with diabetes or rheumatic diseases were excluded from the study. The cataract patients were classified into cortical cataracts, nuclear cataracts (NC), and posterior and anterior subcapsular cataracts (ASC), and the remainder as mixed cataracts. Agreement for the assessment of cataract grading was witnessed by two individuals (one cataract specialist and one ophthalmologist). The control lens epithelium was obtained from patients with few cataracts undergoing clear lens extraction, using multifocal intraocular lenses for presbyopia correction. The mean best corrected visual acuity of the control group was less than 0.05 logMAR.

Sample Collection

Samples of lens epithelium were used with centered anterior capsules of lens measuring 5 to 6 mm in diameter. These were obtained via intact continuous curvilinear capsulorhexis, with care taken to avoid vascular contact or damage to the iris and other intraocular structures. All samples were carried in microtubes and immediately frozen at −80°C until further examination.

Total RNA Extraction, Complementary DNA (cDNA) Synthesis, and Real-Time PCR

All collected lens capsule samples were homogenized in QIazol reagent (Qiagen). Total RNA including small RNAs and miRNAs was isolated from the lens epithelial samples, using miScript Micro Kit (Qiagen) according to the manufacturer's instructions. RNA quality was confirmed by calculating the OD260/280 ratio using absorbances measured with a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA samples were stored at −80°C until cDNA

Table 1. Demographic and Clinical Characteristics of Cataract Patients and Normal Controls

	Cataract (n = 33)	Control (n = 10)	*P* Value
Age (mean ± SD)	67.25 ± 10.68	61.00 ± 4.18	0.061
Sex			
Female	15	9	
Male	18	1	
BCVA (logMAR, mean ± SD)	0.65 ± 0.47	0.05 ± 0.58	<0.001*
SE (mean ± SD)	−0.80 ± 3.01	0.53 ± 1.23	0.099

SD, standard deviation; BCVA, best corrected visual acuity; SE, spherical equivalent.
* $P < 0.05$.

reaction. The isolated RNAs were reverse-transcribed into cDNA using miScript II RT Kit (Qiagen). All reactions were performed as specified in the manufacturer's protocols.

The miRNA expression profiling was performed using a customized miScript miRNA PCR Array of selected miRNAs of interest. It included the following 12 miRNAs: let-7a-5p, let-7d-5p, let-7g-5p, miR-15a-5p, miR-16-5p, miR-22-3p, miR-23a-3p, miR-23b-3p, miR-26a-5p, miR-34a-5p, miR-125a-5p, miR-125b-5p. Quantitative real-time PCR reactions were carried out via SYBR Green-based RT-PCR with a Roche Light Cycler 480, following the manufacturer's protocol.

Normalization and Relative Quantification of Anterior Lens Capsule miRNA Expression

To eliminate the normalization issue associated with miRNA expression in the anterior lens capsules, depending on the absence of stable RNA, we used the global mean normalization method to normalize tissue miRNA expression data as described previously.[18] The global mean normalization of the miRNA quantitative real-time PCR reactions data was performed via The Gene Global Data Analysis Center (Qiagen). The relative expression of miRNAs was calculated using the comparative $2^{-\Delta\Delta Ct}$ method. Fold regulation (FR) was calculated using the equation $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct$ (target gene) – Ct (reference gene).[19,20]

Statistical Analysis

The data are presented as the means and standard deviation. Differences between the two groups were estimated with a two-tailed Mann-Whitney U-test. The differences in miRNA levels according to cataract type were analyzed using Kruskal-Wallis test. All analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). A P value < 0.05 was considered indicative of statistical significance.

Results

Clinical Demographics

In this study, 33 patients with cataract (mean age, 67.25 ± 10.68 years; range, 44–89 years) and 10 controls (mean age, 61.00 ± 4.18 years; range, 53–63 years) were included. There was no significant difference in age and spherical equivalent (SE) between patients in the cataract and the control groups ($P = 0.061$ and 0.099). Mean best corrected visual acuity was significantly worse in the cataract group than in the control group ($P \leq 0.001$). Table 1 shows demographic and clinical data of cataract patients and normal controls.

MicroRNA Expression in the Central Epithelium of Transparent and Cataractous Human Lenses

We identified 12 miRNAs (let-7a-5p, let-7d-5p, let-7g-5p, miR-15a-5p, miR-16-5p, miR-22-3p, miR-23a-3p, miR-23b-3p, miR-26a-5p, miR-34a-5p, miR-125a-5p, and miR-125b-5p). The results demonstrated that eight miRNAs were significantly different between the transparent and cataractous lens samples (Table 2). The expression levels of let-7g-5p (FR = 1.58, $P = 0.021$), miR-23a-3p (FR = 1.27, $P = 0.024$), miR-23b-3p (FR = 1.46, $P = 0.001$), and miR-125a-5p (FR = 2.32, $P = 0.004$) in cataractous samples were significantly higher than in the controls. Among the four miRNAs, only miR-125a-5p showed a fold regulation of 2 or higher. Expression levels of let-7a-5p (FR = −6.21, $P = 0.001$), let-7d-5p (FR = −5.77, $P = 0.002$), miR-16-5p (FR = −13.13, $P < 0.001$), and miR-22-3p (FR = −1.36, $P = 0.041$) in the cataractous samples were significantly lower than in controls. The miR-16-5p exhibited the greatest decrease in expression (Fig. 1).

Table 2. Expression of microRNAs in Patients With Senile Cataract Compared With Control Group

MicroRNA	Cataract ΔCt* (Mean ± SD)	Control ΔCt* (Mean ± SD)	Fold Regulation†	*P* Value
let-7a-5p	11.01 ± 2.69	8.38 ± 1.83	−6.21	0.001‡
let-7d-5p	−4.20 ± 2.73	−6.73 ± 1.87	−5.77	0.002‡
let-7g-5p	0.85 ± 1.09	1.51 ± 0.66	1.58	0.021‡
miR-15a-5p	0.09 ± 0.73	−0.15 ± 0.47	−1.18	0.204
miR-16-5p	3.95 ± 1.94	0.23 ± 1.85	−13.13	<0.001‡
miR-22-3p	4.12 ± 0.55	3.68 ± 0.80	−1.36	0.041‡
miR-23a-3p	0.95 ± 0.92	1.29 ± 0.47	1.27	0.024‡
miR-23b-3p	0.97 ± 0.37	1.51 ± 0.49	1.46	0.001‡
miR-26a-5p	5.60 ± 3.01	7.36 ± 1.08	3.37	0.118
miR-34a-5p	7.99 ± 1.71	7.61 ± 1.29	−1.31	0.386
miR-125a-5p	1.34 ± 1.18	2.56 ± 0.96	2.32	0.004‡
miR-125b-5p	2.29 ± 3.51	0.48 ± 0.61	−3.50	0.435

*Ct (gene of interested) – Ct (house-keeping gene).

†Log^2 fold change; positive values indicate elevated values, and negative values indicate reduced levels in cataract samples, respectively.

‡*P* value < 0.05.

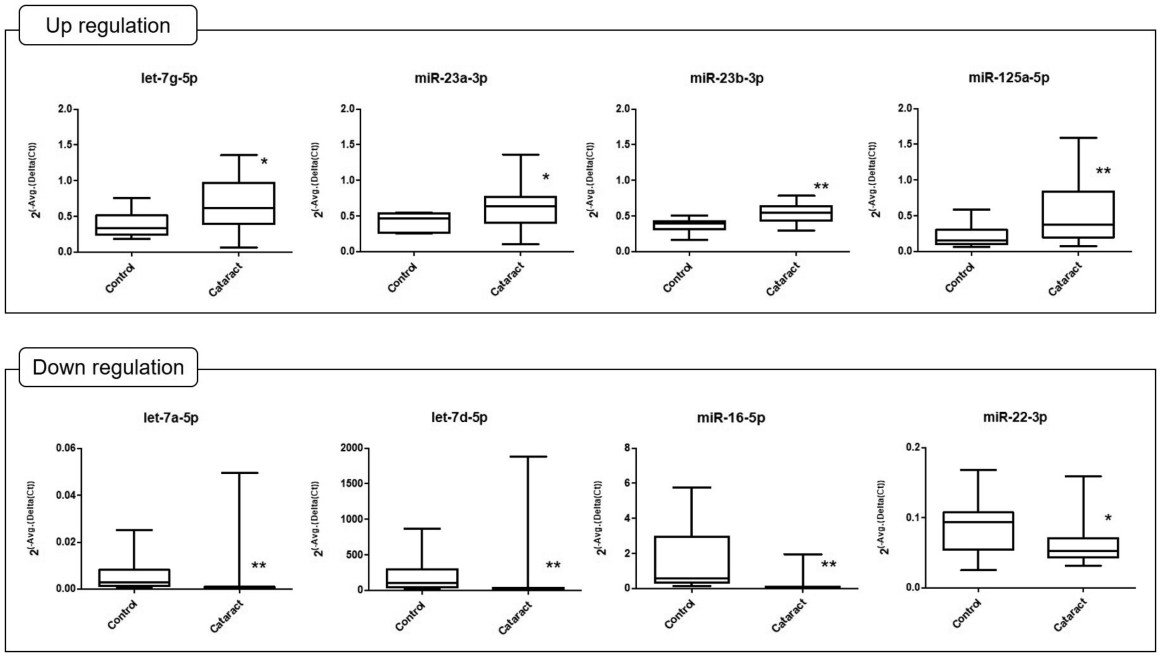

Figure 1. Expression of microRNAs with significant differences between the cataract patients and controls. Values on the y-axis are reported as $2^{(-Average(Delta(Ct))}$. * $P < 0.05$, ** $P < 0.01$ versus control.

Differential Expression of microRNAs According to Cataract Type

Lens epithelial cells were derived from 33 patients with age-related cataracts, including six with cortical cataracts, nine with NC, four with posterior subcapsular cataracts, and four with ASC. Ten others were of mixed cataract. No microRNAs showed statistically significant differences among the four cataract types using the Kruskal-Wallis test (Table 3). Comparison between the two types of cataracts using Mann-Whitney U-test revealed significant differences in the expression of let-7a-5p, let-7d-5p, let-7g-5p, and miR-23b-3p between NC and ASC groups ($P = 0.031$, 0.021, 0.021, and 0.031). There were no significant differences between the other two groups.

Table 3. Comparison of MicroRNA Expression in the Anterior Lens Capsule According to Cataract Type

MiRNA	CO ΔCt (Mean ± SD)	NC ΔCt (Mean ± SD)	PSC ΔCt (Mean ± SD)	ASC ΔCt (Mean ± SD)	*P* Value*	*P* Value† ASC vs. CO	*P* Value† ASC vs. NC	*P* Value† ASC vs. PSC
let-7a-5p	11.37 ± 3.25	11.62 ± 1.09	9.38 ± 4.70	7.72 ± 3.15	0.179	0.083	0.031‡	0.386
let-7d-5p	−3.96 ± 3.28	−3.49 ± 1.32	−5.85 ± 4.75	−7.55 ± 3.04	0.186	0.083	0.021‡	0.564
let-7g-5p	0.75 ± 1.13	0.58 ± 0.60	1.53 ± 1.71	2.27 ± 1.31	0.184	0.083	0.021‡	0.564
miR-15a-5p	−0.38 ± 0.90	−0.07 ± 0.81	0.32 ± 0.61	−0.07 ± 0.78	0.579	0.564	0.877	0.386
miR-16-5p	4.83 ± 2.48	4.76 ± 2.07	1.72 ± 2.44	3.43 ± 0.77	0.082	0.248	0.064	0.386
miR-22-3p	3.81 ± 0.74	3.99 ± 0.48	4.48 ± 0.06	3.87 ± 0.83	0.186	0.773	1.000	0.043
miR-23a-3p	0.94 ± 1.14	0.75 ± 0.44	1.54 ± 1.74	1.82 ± 1.03	0.497	0.149	0.090	1.000
miR-23b-3p	0.84 ± 0.55	0.72 ± 0.29	1.05 ± 0.16	1.20 ± 0.40	0.143	0.248	0.031‡	0.564
miR-26a-5p	7.24 ± 1.62	6.55 ± 3.71	6.04 ± 1.19	6.90 ± 2.99	0.796	0.564	0.643	0.773
miR-34a-5p	7.67 ± 1.70	9.02 ± 1.10	6.59 ± 1.71	7.09 ± 2.43	0.113	0.564	0.280	0.773
miR-125a-5p	1.82 ± 1.50	1.51 ± 1.18	1.41 ± 1.24	2.33 ± 0.62	0.428	0.386	0.165	0.083
miR-125b-5p	0.08 ± 0.51	2.25 ± 3.85	1.41 ± 1.67	0.82 ± 0.79	0.409	0.191	0.877	0.564

Ct (gene of interested) – Ct (house-keeping gene).
CO, cortical cataract; PSC, posterior subcapsular cataract.
*Comparison of four groups using Kruskal-Wallis one-way analysis of variance
†Comparison of each two groups using Mann–Whitney U-test
‡$P < 0.05$.

Discussion

In this study, we investigated the expression of 12 miRNAs in senile cataract patients and control subjects. Twelve miRNAs consisted of miRNAs, which were abundantly expressed in the lens epithelial cells or showed significant differences in expression between the two groups in our preliminary study, and miRNAs, which were significantly expressed in the previous study. We found eight miRNAs with significantly different expression in the anterior lens capsule of cataract patients compared with the controls. The expression of let-7g-5p, miR-23a-3p, miR-23b-3p and miR-125a-5p was increased, and the expression of let-7a-5p, let-7d-5p, miR-16-5p and miR-22-3p was decreased in the anterior lens capsules of the cataract patients compared with those in the controls. In this study, we used an efficient bioinformatics approach to systematically analyze the regulatory role of differentially expressed miRNAs. Using this method, we identified senile cataract-specific targets and predicted pathways (Fig. 2).

It has been widely reported that the lens is exposed to oxidative stress throughout its life and oxidative damage is a major cause of cataract formation.[21,22] Current studies also suggest that with increasing age, the accumulation of oxidized lens components and the decreased capacity of repair mechanisms result in increased levels of reactive oxygen species (ROS)[23,24] and lens epithelial cell death, promoting cataractogenesis.[22,25] Under normal conditions, the lens uses multiple physiological defense strategies to scavenge and maintain ROS at low levels to protect the lens from the toxic effects of oxidative damage.[26,27] However, the protective systems decrease with age, and long-term exposure to oxidative stress increases the risk of cumulative oxidative damage and cataract formation.[21]

Several miRNAs with significant differences in expression in this study were associated with oxidative stress. Overexpression of miR-23 induced ROS generation more than twofold and sensitized cardiomyocytes to H_2O_2 treatment.[28] Overexpression of miR-23a~27a~24-2 cluster increased ROS production and induced caspase-dependent and -independent apoptosis in human embryonic kidney cells.[29] Cao et al.[30] reported that miR-15a/16 deficiency resulted in lung epithelial cell apoptosis in response to hyperoxia, by modulating both intrinsic and extrinsic apoptosis.

During lens development, lens epithelial cells grow and differentiate into elongated fiber cells. The differentiating lens fiber cells lose their organelles to produce an organelle-free zone, thus contributing to lens transparency.[31] Currently, multiple studies have shown that autophagy plays an essential role in the development of organelle-free zone of the lens.[32] Autophagy is a kind of cellular degradation pathway that maintains cellular homeostasis via degradation of intracellular proteins, lipids, and organelles in response to various environmental conditions.[33] The accumulation of dysfunctional mitochondria that produce ROS occurs in various types of autophagy-deficient cells.[34,35] The housekeeping role of autophagy may be particularly important in the slowly differentiating fiber cells of

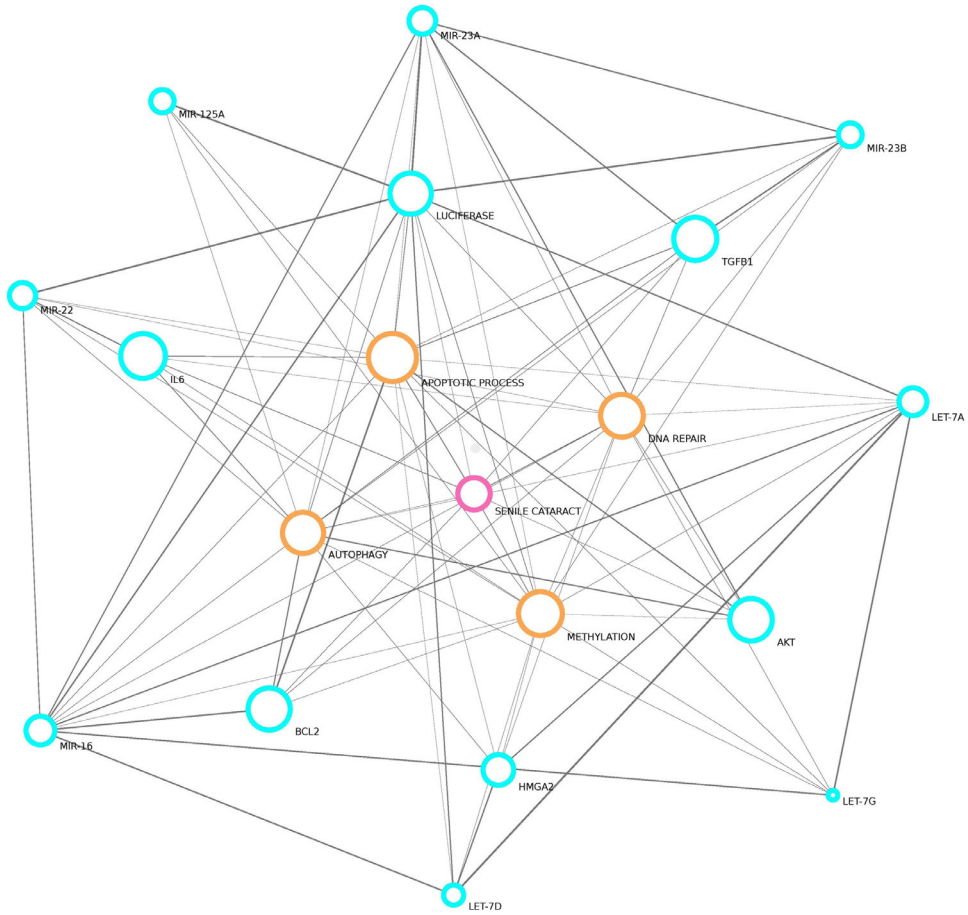

Figure 2. Network of the miRNAs and their target genes related with the pathogenesis of senile cataract using bioinformatic analysis.

adult lens. Costello et al.[36] demonstrated the presence of autophagic vesicles in embryonic and adult lens via electron microscopy and confocal microscopy.

Most miRNAs with significant differences in expression reported in this study were associated with autophagy. Four miRNAs overexpressed in cataract patients were known to inhibit autophagy.[37–40] The miR-23b directly targeted the 3′-untranslated region of autophagy-related gene 12 mRNA to suppress the activation of neuronal autophagy.[41] A few miRNAs with decreased expression in cataract patients are known to promote autophagy. MiR-22 inhibits apoptosis and promotes autophagy of human ovarian cancer cells via suppression of the Notch signaling pathway.[42] Huang et al.[43] demonstrated that miR-15a and miR-16 are potent inducers of autophagy.

In previous studies, it was reported that multiple miRNAs were closely related to the pathogenesis of cataract. Chien et al.[14] found a positive correlation between high miR-34a levels and severity of lens opacity. Qin et al.[44] showed that miR-125b was downregulated in the anterior lens capsules of patients with age-related cataract compared with normal anterior lens capsule. Li et al.[15] reported the expression of hsa-miR-15a-5p, hsa-miR-15a-3p, and hsa-miR-16-1-5p rose in lens epithelial cells of age-related cataract.

In this study, the expression of miR-34a, miR-125b, and miR-15a-5p, which were significant in previous studies, was not significantly different between cataract patients and controls. The discrepancies were presumably due to the differences in the characteristics of the control group and the severity of cataract in the experimental group. Previous studies selected postpartum tissues or samples obtained from mild cataract patients as controls and the clinical features of the control group were unclear. Asians often have myopia, with a strong desire for near vision and for life without glasses. To meet these needs, clear lens extraction using a multifocal intraocular lens is widely performed for the correction of presbyopia in South Korea. Patients without cataract but with a desire to undergo cataract surgery for presbyopia correction are increasing in number. Therefore we used anterior lens capsules from

patients without cataract as a control. This study did not include patients with very severe cataract, and therefore the severity of cataract may be milder than in previous studies.

Our study had several limitations. First, the research was limited by the small sample size of the control group. Although we aimed to include additional control samples, we could not identify an adequate number of patients without cataract. Second, the classification of cataracts is subjective and the correlation between the severity of cataract and the level of microRNA expression could not be investigated. In addition, this study did not establish the target gene or pathway of the miRNAs expressed significantly. In future studies, we intend to identify reliable cataract-related miRNAs in a large cohort of patients and predict their target genes regulated by the differentially expressed miRNAs.

Conclusions

In conclusion, we have identified eight miRNAs in lens anterior capsule, promising candidate biomarkers of senile cataract. Although a small cohort, miRNAs that show differences between NC and ASC may provide clues to different pathogenesis depending on the types of cataract. Through bioinformatics, it has been inferred that significant miRNAs are related to mechanisms such as apoptosis and autophagy. Further exploration of the predicted pathways and target genes can provide insight into the pathophysiology of senile cataract and may identify novel therapeutic targets.

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