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Epigenetic changes of the Klotho gene in age-related cataracts

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Abstract. – OBJECTIVE: To determine the mRNA and protein expression, and methylation levels of the Klotho in lens epithelial cells (LECs) of normal transparent lenses and age-related cataracts (ARCs), and to explore the role of epigenetic changes of the Klotho gene in regulating the development of ARCs.

PATIENTS AND METHODS: A total of 90 subjects were divided into three groups: a young adult group with normal transparent lenses aging from18 to30 years, a middle-aged group with ARC aging from 40 to 49 years, and an elderly group with ARC aging from 67 to 85 years. The LECs were collected through curvilinear capsulorhexis. The mRNA expression of the Klotho gene was determined using the reverse transcription polymerase chain reaction (RT-PCR). Protein expression of the Klotho gene in LECs was detected using immunohistochemical (IHC) staining. The methylation specific polymerase chain reaction (MSP) was used to detect the methylation level of the target gene.

RESULTS: Decreased mRNA expression of the Klotho gene was reversely correlated with age. IHC results showed that the Klotho was mainly expressed in the cell membrane and cytoplasm of LECs. It was strongly positive in the young adult group (100.0%), with even distribution; weakly positive in the middle-aged group (36.7%), with expression distributed 4-5 mm away from the center of the anterior lens capsule; and negative in the elderly group (0.0%). MSP results showed that the Klotho gene was highly methylated in the elderly group (93.3%) and weakly methylated (56.7%) in the middle-aged group, but barely methylated in the young adult group (3.3%).

CONCLUSIONS: Klotho were positively expressed in the LECs of normal individuals at the mRNA and protein level. Its promoter showed increased methylation as age increased, resulting in Klotho gene silencing as well as down-regulated expression or no expression of the Klotho protein. These epigenetic changes could affect the biological activities of LECs, which provided the basis for further studies of the association between the Klotho gene and ARC.

Key Words:
Age-related cataract, Lens epithelial cells, Klotho gene, Methylation.

Introduction

Age-related cataract (ARC), sometimes termed "senile cataract," is the most common type of cataract and typically occurs with aging. Clinically, it is mainly manifested as painless and progressive loss of vision, which can be classified into three types: nuclear, cortical, and posterior sub capsular, with cortical ARC being the most common type[1]. As the population ages and life span increase, the incidence of ARC has increased annually, accounting for 40.6% of all cases of low vision or blindness[2]. Currently, there is no medication that has been proven effective to

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treat or prevent ARC. The only available treatment is surgical removal of the cloudy lens. However, the monetary expenses and clinical complications of surgery can cause a large financial and emotional burden.

Klotho is a recently discovered anti-aging gene, which was first reported by Kuro et al[3], with its expression levels closely correlated with age. The Klotho protein can affect aging and age-related diseases by regulating the metabolisms of vitamin D, calcium, and phosphorus, as well as protecting the cardiovascular system and affecting cell structures and immune functions[3]. Additionally, Klotho knockout mice showed behavioral and pathophysiological changes (e.g., short lifespan, hearing loss, and clouding of the cornea) resembling human aging. Genetically based remedial treatment using the Klotho gene resolved these symptoms of premature aging[4]. In our previous studies, the biological activity of lens epithelial cells (LECs) decreased with age, and aging induced cataracts[5], suggesting that Klotho gene might play an important role in the development of ARC.

Epigenetic studies have characterized changes in inherited gene expression or cell phenotypes due to certain mechanisms[6]. These changes included DNA methylation, RNA interference, and histone modification. Studies have shown that dietary supplements and changes in environmental exposures could change the epigenetic features of gene expressions in mice, thus, affecting the coat color, body weight, and susceptibility to tumorigenesis[7]. When humans were exposed to different environmental conditions, no significant differences in the DNA content and genomic distribution of 5-methylcytosine were found between young twins, but significant differences were found between elderly twins[8].

Numerous epigenetic studies have been performed on ARCs. Zhou et al[9] reported that hypermethylation of the alpha-A crystallin (CRYAA) gene promoter, which used *in vitro* cultured LECs obtained from ARC patients, was associated with increased biological activities of LECs, which provided new epigenetic insights into the pathogenesis of cataracts. Abnormal methylation of the Klotho gene promoter has been found in patients with tumors, arteriosclerosis, and/or renal failure[10]. However, whether the Klotho gene is expressed in human lens epithelial cells and whether changes in its expression affect epigenetic traits remain unclear.

In the present study, by harvesting specimens directly from the clinic, we examined the Klotho gene expressions and protein levels in LECs from a young adult group and from two ARC groups (a middle-aged group and an elderly group), and detected the methylation levels of the gene promoter. The results showed that the methylation level of the Klotho gene increased with age, resulting in low and even complete lack of expression of the Klotho gene and protein. Thus, epigenetic modifications of the Klotho gene may be involved in the development of ARC, and these findings may assist in the further studies to develop targeted therapies.

Patients and Methods

Specimen Collection

A total of 60 patients (60 diseased eyes), who underwent ARC surgeries for cortical cataracts from September 2013 to October 2014, were enrolled in this study. Patients with disease such as systemic conditions (e.g., diabetes mellitus, hypertension, and heart disease), or eye diseases (e.g., iridocyclitis, glaucoma, eye injuries, as well as chronic ocular radiation exposure) were excluded from the study. In addition, 30 healthy subjects were also enrolled. A total of 90 subjects were divided into three groups: a young adult group with normal transparent lenses (aging from 18-30 years; mean age of 25.00 years) (n=30, 18 male and 12 female; 30 eyes), a middle-aged group with ARC (aging from 40 to 49 years; mean age of 45.33 years) (n=30, 17 male and 13 female; 30 eyes), and an elderly group with ARC (aging from 67 to 85 years; mean age of 75.53 years) (n=30, 15 male and 15 female; 30 eyes). Before surgery, the gender and age of the patients as well as the degree and type of lens opacities were examined and recorded by the same physician. Phacoemulsification for cataract removal was performed by the same surgeon, involving routine curvilinear capsular-hexis (5.5 mm in diameter). The anterior lens capsule of normal young adults was obtained from the Eye Bank of the Henan Institute of Ophthalmology after corneal transplantation. Using the same method, transparent anterior lens capsules were used as the control group, with all the anterior lens capsules rinsed in phosphate-buffered saline (PBS) to remove blood, iris pigment, and intraocular tissues, including the lens cortex and vitreous body. All sampling procedures were approved by the

Medical Ethics Committee of the hospital and a statement with the human use procedures adhered to the tenets of the Declaration of Helsinki were requested. The obtained specimens were immediately stored in a −80°C freezer for further use.

Reagents and Instruments

The main reagents included a Trizol kit (Invitrogen, Carlsbad, CA, USA), E.Z.N.A. MicroElute Total RNA kit (Omega Bio-Tek, Norcross, GA, USA) a highcapacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), gDNA Eraser (Takara, Otsu, Shiga, Japan), a real-time fluorescence quantitative polymerase chain reaction (PCR) kit (SYBRP remixEx Taq PCR), a SYBR premix (Takara, Shiga, Japan), TaqMan probes (ABI Applied Biosystems, Foster City, CA, USA), and an EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA). The primary antibody (goat anti-human Klotho antibody) and the secondary antibody (donkey anti-goat antibody) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. The 1,3-dimethyl-5-acetyl-barbituric acid (DAB) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

The PCR equipment included the Applied Biosystems 7300 RealTime (Applied Biosystems, Foster City, CA, USA) PCR System and 2720 Thermal Cycler. A gel imaging analyzer (Fluor Chem Q; Alpha Innotech, San Leandro, CA, USA) was also employed.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The anterior lens capsule was quickly placed into an Eppendorf tube, rinsed with PBS. RNA was isolated from anterior lens capsule according to the E.Z.N.A. MicroElute Total RNA kit's instruction. The reverse transcription system was used to synthesize cDNA from RNA concentrations with gDNA Eraser, and the mixture was stored at –80°C.

After the human Klotho gene sequence was obtained from Gen-Bank (Klotho ID: 16591; Klotho mRNA: NM-013823.1), specific primers were designed using Primer (Premier software version 5.0, synthesized by Invitrogen, Carlsbad, CA, USA) involving the following sequences: sense, 5'-ACCTGGTGGCGCACAC-3'; anti-sense, 5'-TTGGCAAACCAACCTAG-TACA-3'; GAPDHsense, 5'-GAAGGTGAAG-GTCGGAGT-3', GAPDH anti-sense, 5'-GAA-GATGGTGATGGGATTTC-3'.

The conditions for the reverse transcription reaction were as follows: 20°C, 10 mins; 42°C, 60 mins to produce complementary DNA (cDNA) via reverse transcription. The reaction was terminated by heating at 70°C for 10 mins, and the product was stored in a −20°C freezer for further use. Agarose gel electrophoresis was used to characterize appropriate amounts of the RT-PCR products. If image analysis showed that the target lanes were clear and without any mixed samples, the primer design and synthesis were considered to be accurate, and fluorescence quantitative PCR was performed.

The real-time fluorescence quantitative PCR system was performed using TaqMan probes synthesized by ABI. The reaction system for the Klotho PCR gene reaction involved 3.0 μL of 10 × PCR buffer solution (Mg^{2+}free), comprised of 1.8 μL of 25 mm $MgCl_2$, 0.36 μL of 25 mm dNTP, 1.0 μL of 10 μm upstream primer, 1.0 μL of 10 μm downstream primer, and Taq enzyme (5 U/μL). Solubility curve analysis and agarose gel electrophoresis were performed to verify the amplification. The test was repeated for three times for each sample. The changes in the Klotho mRNA expression in different groups were calculated using the $2^{-\varnothing\varnothing Ct}$ method.

Immunohistochemical (IHC) Staining

The harvested anterior lens capsule was rinsed with PBS, and then smeared on a slide, dried, and stained using the avidin-biotin complex (ABC) method. The cells were treated with 1:200 goat anti-human Klotho antibody at room temperature overnight, and then treated with the secondary antibody (donkey anti-goat antibody, 1:50) for 2 hours, followed by treatment with diaminobenzidine (DAB) solution for 5 mins. The samples were then rinsed with PBS to terminate staining, and the cells were mounted onto slides after drying.

For each slide, five fields of view were randomly selected. The number of positively stained cells for every 50 cells was counted. The results were based on the percentage of positively stained cells among these 50 cells: 0, percentage of positively stained cells ≤5%; 1, percentage of positively stained cells >5% but ≤25%; 2, percentage of positively stained cells >25% but ≤50%; 3, percentage of positively stained cells >50% but ≤75%; and 4, percentage of positively stained cells >75% but ≤100%. The cell staining intensity was divided into 0 (negative), 1 (yellowish granules), 2 (brownish granules), and 3 (brown granules). A final score was calculated based on the staining intensi-

ty and the percentage of the positively stained cells. The scores were divided into 4 grades based on the product of these two figures: -, ≤4; +, ≤8; ++, <12; and +++, =12, among which "-" denoted negative Klotho expression, and "+", "++", and "+++" denoted positive results.

Methylation-Specific PCR

Methylation-specific polymerase chain reaction (MSP) was used to detect the level of Klotho methylation. DNA was extracted from the specimens from three groups. Then, 500 ng of DNA was treated with sulfite according to the instructions for the EZ DNA Methylation-Gold Kit[11]. With 1 μL of the treated DNA as the template, MSP was performed using TaqGold DNA polymerase. The reaction system was as following: pre-denaturation at 95°C for 10 mins, denaturation at 95°C for 30 secs, annealing at 58°C for 30 secs, and extension at 72°C for 30 secs. Amplification was used for 35 cycles, with the final step extended to 5mins at 72°C. The PCR product underwent 1.2% agarose gel electrophoresis and ethidiumbromide (EB) staining, and the product lanes were observed on a UV detector. The methylation and unmethylation primers were designed using Meth-Primer software (http://www.urogene.org/methprimer/index1.html). The Klotho methylation (M) primer sense was 5'-GTCGTCGTTGTAGTTCGTTATC-3', and the anti-sense was 5'-CAACAAACGC-CGATAATAACG-3'. The Klotho unmethylation (M) primer sense was 5'-TTGTTGTTGTTGTAC TTTGTTATT-3', and the anti-sense was 5'-CCAACAAACAC-CAATAATAAC-3'.

Statistical Analysis

Statistical analysis was performed using the SPSS software package (IBM SPSS 19.0, SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation. One-way ANOVA was used for intergroup comparisons, and the Welch test was used when the variances were not homogeneous. The count data were expressed using frequencies and percentages, and their intergroup comparisons were performed using Pearson's x^2-test or Fisher's exact test. The ranked data were expressed using percentages and average ranks, and their intergroup comparisons were performed using the Kruskal-Wallis test.

Results

Klotho Gene Expression in the Lens Epithelium

The expression of the Klotho gene in transparent lens epithelium was calculated using the 2^{-Ct} method, which showed that the relative expression levels of the Klotho gene were significantly different among the three groups ($p<0.05$) (Figure 1, Table I).

Klotho Protein Expression in LECs

Klotho protein was homogeneously expressed in the cytoplasm and cell membrane in the young adult group (Figure 2a). In the middle-aged group, Klotho protein was weakly expressed in the center of the anterior lens capsule. Color development with DAB was uneven, and the cells

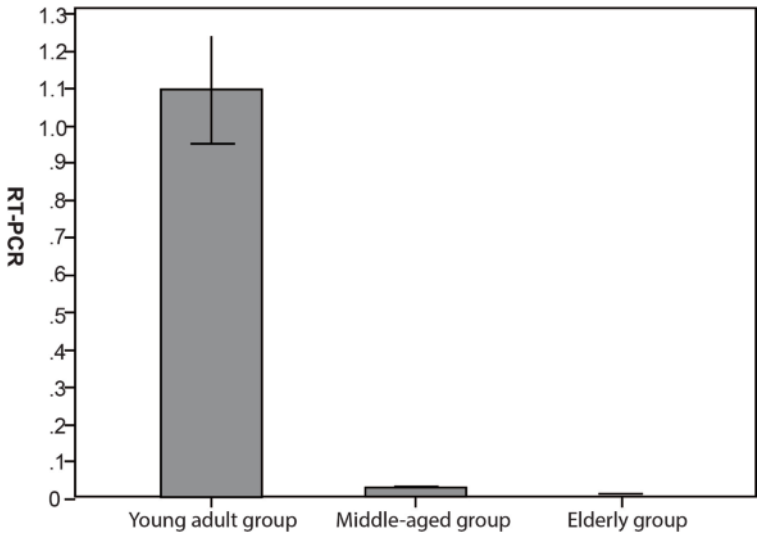

Figure 1. Expression of the Klotho gene in the lens epithelium. ***A,*** Young adult group, ***B,*** middle-aged group, ***C,*** elderly group.

Table I. Relationship between DNA methylation and Klotho protein expression in the three groups

Groups	Young adult group (n=30)	Middle-aged group (n=30)	The expressions were negative in the elderly group. (n=30)	F/x²	P
Age (mean±SD)	25.00 ± 3.97	45.33 ± 3.15	75.53 ± 5.76	784.885	< 0.001*
Gender (male) n (%)	13(43.3%)	11(36.7%)	12(40.0%)	0.278	0.87
mRNA(mean±SD)	1.097 ± 0.140	0.023 ± 0.004	0.004 ± 0.002	1119.45	< 0.001*
IHC positive, n(%)	30(100.0%)	11(36.7%)	0(0.0%)	-	< 0.001#
MSP positive, n(%)	1(3.3%)	17(56.7%)	28(93.3%)	49.18	< 0.001

*The Welch test was used because the variances were not homogeneous; #Fisher's exact test was used for intergroup comparisons.

had varied morphologies, showing pseudopod-like protrusions (Figures 2b, d and e). Klotho protein expression was undetectable in the elderly group (Figure 2c). The differences among these three groups were significant ($p<0.05$) (Table I).

Klotho Gene Methylation Levels

The percentages of Klotho gene methylation were 3.3% (1/30), 56.7% (17/30), and 93.3% (28/30) in the young adult group, middle-aged group, and elderly group, respectively. The percentage of Klotho gene methylation was significantly higher in the middle-aged group and elderly group than that in the young adult group ($p<0.05$) (Figures 3 and 4, Table I).

Correlation of Klotho Gene Methylation with Klotho Gene Expression and Age

The methylation level was relatively low in the young adult group and high in the elderly group, and comparisons among these three groups showed statistical significance ($p<0.05$) (Table I).

Discussion

The cells used in the present study were directly harvested from human specimens, which avoided the complications of *in vitro* culture. For the first time, we found that the Klotho gene was expressed in the LECs of normal individuals, and its mRNA and protein expressions decreased with age. The Klotho gene was closely related to aging and has been shown to be an inhibitory factor in this process. Klotho knockout mice during early age showed various changes (e.g., short lifespan, movement disorders, atherosclerosis, and osteoporosis) resembling human aging. Furthermore, genetically based remedial treatments using the Klotho gene resolved these symptoms of premature aging[4]. However, lens opacity (which is a manifestation of aging) after Klotho gene depletion and expression of the Klotho gene in human eyes have not been previously reported. The Klotho protein exists in the membrane form or in the cytoplasm. The secreted Klotho protein regulated multiple signaling pathways, including the calcium channel TRPV5[12]. In the FGF23 pathway, it bounded to basic fibroblast growth factor receptors (bFGFR) 1-4 to form a nitric oxide (NO) receptor, thus, increasing NO synthesis[13]. In addition, by inhibiting the Wnt pathway, it suppressed cell apoptosis[14]. However, the relationship between the Klotho gene and LECs remains unclear. Our present study examined the expression of Klotho in the LECs of normal subjects, suggesting that Klotho may affect the activities of LECs, and its abnormal expression may induce cataracts.

Cytologically, ARC is caused by the senescence and apoptosis of LECs. The LECs are tightly attached to the monolayer cells on the inner surface of the anterior capsule and loosely connected with the lens fibers at the inferior side. The LECs have two major functions in the lens: 1) to continuously differentiate into lens fiber cells; and b) to form the lens capsule[16]. The lens cells have definite regions involving a central region comprised of tightly aligned monolayer cells, under which there are continuously lined vesicle membranes. Previous studies have reported that the vesicle membranes contained type IV collagen and lamin, which were similar to human lens capsule basement membrane. Thus, the spatial relationship with LECs, and/or the high density proliferation of these cells are key factors for

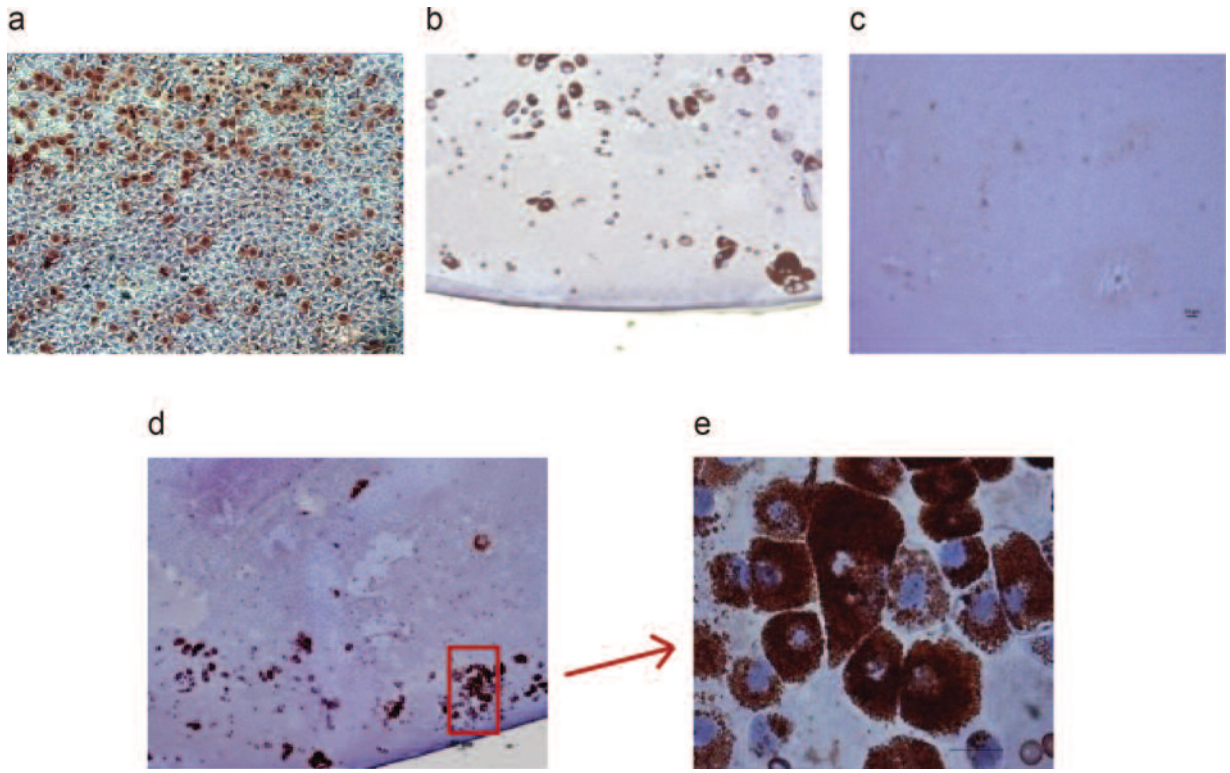

Figure 2. ***A,*** positive Klotho protein expression in LECs from the young adult group; ***B,*** weakly positive Klotho protein expression in LECs from the middle-aged group (×40); ***C,*** negative Klotho protein expressin in LECs from the elderly group (×20); ***D,*** weakly positive Klotho protein expression in LECs from the middle-aged group (×20); ***E,*** weakly positive Klotho protein expression in LECs from the middle-aged group (×100).

development of the lens capsule. In the equatorial and pre-equatorial regions, the LECs constantly undergo mitosis and differentiate into lens fibers, which exert pressure on the central region and form several optical plane structures, as viewed with the slit lamp. The LECs in the central region of the anterior lens capsule are regular cuboidal epithelial cells and will not undergo mitosis. This LEC layer is the most active metabolic site in the lens. The LECs utilize glucose and oxygen to produce energy to maintain the transport of carbohydrates and amino acids into the lens, and to provide the energy for synthesizing proteins required by lens fibers in the equatorial region. Thus, it plays key roles in the growth, differentiation, and injury repair of the lens[17,18]. Injury or age-related changes of LECs may, therefore, affect the physiological functions of lens fibers and cause lens opacities.

Whether the Klotho gene is expressed in human LECs and its epigenetic changes affect the apoptosis of LECs and formation of cataracts, need to be addressed in experiments using specimens directly harvested from human anterior lens capsules. In our present study, high methylation levels of Klotho gene were found in elderly patients with ARC, which result in the silencing of target gene expression and the lack of expres-

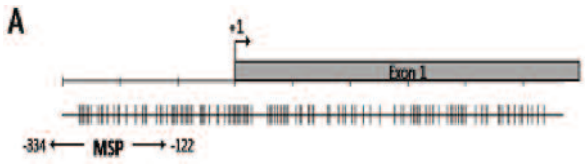

Figure 3. The methylation primers information of Klotho gene.

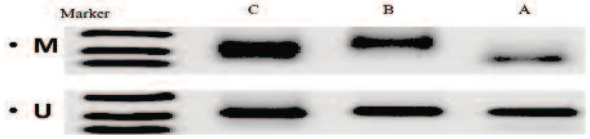

Figure 4. Methylation status of Klotho promoters. (M), methylated; (U), unmethylated.

sion of functional proteins. Notably, in the middle-aged group, Klotho protein expression was found at the near-central region of the LECs, but negative in the central region. With increasing age, the morphologies of LECs changed, showing pseudopod-like changes, along with non-homogenous positive reactions. To the best of our knowledge, there has not been any study comparing the expression of the same protein between the central and peripheral regions of LECs. In addition, our findings are consistent with our speculation that LECs in the near-central area have genetic functions, while cells in the central region lack of these functions. It was also found that the Klotho gene and its protein expression were correlated with the aging of LECs.

DNA methylation is a reversible epigenetic phenomenon[19]. It can affect gene expression, which can be reversed by using methylation inhibitors. Studies on CpG island hypermethylation can be used to screen and identify new genes, facilitating the development of gene therapy for cataract. Thus, in our future studies, we will further elucidate this mechanism, using *in vitro* cell culture and DNA demethylating drug-based interventions.

Because the lens epithelium was composed of monolayer cells, the sampling range was limited to an area of 5.5 mm × 5.5 mm to ensure the safety of surgical procedures. However, the specimens needed to be divided into three equal parts for RT-PCR, protein IHC tests, and gene methylation level measurements. As a result, there was no remaining tissue for further laboratory tests such as Western blotting, calculation of cell apoptosis rates, and detection of relevant ion levels. Our group consulted with Dr. He Shi-kun from the Doheny Eye Institute at the University of Southern California/Keck School of Medicine, who suggested that *in vitro* cell culture might be a solution. Accordingly, we collected different human LEC lines from different sources for *in vitro* cultures, which will be used in our future studies.

Conclusions

The pathogenesis of ARC is a complex process involving multiple factors. The Klotho gene was involved in the occurrence and development of cataract, and its epigenetic change is one of the causes of senile cataracts. The results of the present study elucidated the involvements of aging and epigenetic regulation of Klotho gene in pathogenic mechanisms of ARC, which will provide the impetus for future studies in these areas.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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