· Basic Research ·

Aberrant expression of *COL4A1* in age-related cataract and its effect on cell proliferation, apoptosis and gene expression changes

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Abstract
● **AIM:** To evaluate the regulation of the aberrant expression of *collagen type IV alpha 1 chain* (*COL4A1*) in the development of age-related cataract (ARC).
● **METHODS:** Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot analysis were employed to evaluate the expression of *COL4A1* in ARC patients and healthy controls. The proliferation, apoptosis, cell cycle and epithelial-mesenchymal transition (EMT) of human lens epithelial cell (HLE-B3) were further analyzed under the condition of *COL4A1* gene silence. Alteration of gene expression at mRNA level after knockdown *COL4A1* were also evaluated by qRT-PCR on HLE-B3 cells.
● **RESULTS:** The aberrant expression of *COL4A1* was identified a clinically associated with the ARC. Silencing of *COL4A1* promoted the apoptosis and inhibited the proliferation of HLE-B3 by blocking the cell cycle. Moreover, *COL4A1* gene silence didn't affect the cytoskeleton of HLE-B3 but down-regulated the *Collagen type IV Alpha 2 Chain* (*COL4A2*), *paired box 6* (*PAX6*), *procollagen-lysine 2-oxoglutarate 5-dioxygenases 1* (*PLOD1*) and *procollagen-lysine 2-oxoglutarate 5-dioxygenases 2* (*PLOD2*) expression levels in HLE-B3 cells. Silencing the *COL4A1* gene induced EMT of the HLE-B3 cells by promoting the *transforming growth factor beta* (*TGF-β*) expression.
● **CONCLUSION:** Silencing of *COL4A1* induces S-phase arrest, also inhibits the proliferation and enhance HLE-B3 apoptosis and EMT, and down-regulates the expression of *COL4A2*, *PAX6*, *PLOD1* and *PLOD2*. Thus, the expression alteration of *COL4A1* may play a critical role in the pathogenesis of ARC.
● **KEYWORDS:** age-related cataracts; *COL4A1*; human lens epithelial cell
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INTRODUCTION
Cataract, defined as the opacification of the crystalline lens in the eye, remains one of the most common causes of blindness and the leading cause of vision impairment world wide[1]. Cataracts typically are classified according to the pathogeny as age-related cataract (ARC), childhood cataract, and secondary cataracts[2]. Among these categories, ARC is the most common type in the elderly population, accounting for 50% in the 65-74 age group and more than 70% in over 75y group[3-4]. Therefore, the prevention and treatment of ARC is particularly important.

The exact pathogenesis of ARC still remains poorly understood. Previous explanations mostly focused on the influence of oxidative stress which causes denaturation and aggregation of lens proteins and triggers apoptosis of lens epithelial cells[5]. Nevertheless, it is generally accepted that aberrant DNA methylation plays an indispensable role in the development of ARC[6]. In previous study, we successfully identified that abnormal *collagen type IV alpha 1 chain* (*COL4A1*) gene methylation was clinically associated with ARC patients[7].

The *COL4A1*, as the extracellular matrix protein, which constitute the major component of nearly all basement membranes[8]. Mutation of *COL4A1* has been found to be

Table 1 shRNA sequence information

shRNA	Sequence information
shCOL4A1-1810-h-F	CCGGCCGGGTTCTGTAGGATTGAAATTCAAGAGATTTCAATCCTACAGAACCCGGTTTTTTGGTACC
shCOL4A1-1434-h-F	CCGGGCCAGGATTTATAGGCGAAATTTCAAGAGAATTTCGCCTATAAATCCTGGCTTTTTTGGTACC
shCOL4A1-1723-h-F	CCGGATTTCGACTTGCGGCTCAAAGTTCAAGAGACTTTGAGCCGCAAGTCGAAATTTTTTTGGTACC
shCOL4A1-1810-h-R	AATTGGTACCAAAAAACCGGGTTCTGTAGGATTGAAATCTCTTGAATTTCAATCCTACAGAACCCGG
shCOL4A1-1434-h-R	AATTGGTACCAAAAAAGCCAGGATTTATAGGCGAAATTCTCTTGAAATTTCGCCTATAAATCCTGGC
shCOL4A1-1723-h-R	AATTGGTACCAAAAAAATTTCGACTTGCGGCTCAAAGTCTCTTGAACTTTGAGCCGCAAGTCGAAAT

COL4A1: Collagen type IV alpha 1 chain.

involved in the pathogenesis of many diseases, including the kidney, eye, small-vessel disease[9-10]. Several single mutation sites of *COL4A1* have been identified in adult patients presenting with bubble cataract and lens abnormality[7]. Our previous findings also demonstrated that aberrant methylation expression of *COL4A1* linked with ARC, and represented as hypermethylation target (P=0.017, β=0.241)[7]. However, the role of *COL4A1* in the progression of ARC remains to be elucidated.

To study the possible effect and mechanism of *COL4A1* on the progression of ARC diseases, in this work, the expression level of *COL4A1* in anterior lens capsules of healthy human and ARC patients were detected. The proliferation, apoptosis, cell cycle, cytoskeleton and epithelial-mesenchymal transition (EMT) changes of human lens epithelial cells (HLE-B3) after knockdown of *COL4A1* was evaluated. Signaling pathways possibly involved in this process were also elucidated.

MATERIALS AND METHODS

Ethical Approval All experimental protocols were approved by the Ethics Committee of Xi'an Medical University (approval No.XYLS2021185). The informed consent was obtained from all subjects.

Reagents and Cells Materials, including fetal bovine serum, MEM (Gibco), trizol reagent (TAKARA), lipofectamine 2000 (Invitrogen), power SYBR Green PCR Master Mix (Thermo) and RT Master Mix (TAKARA) were purchased from the companies as indicated. Antibodies against tublin, β-actin and COL4A1 were purchased from CST. HLE-B3 cells were obtained from the cell bank of Chinese Academy of Sciences (Shanghai, China). ShRNA-COL4A1 was cloned by standard molecular biology techniques. Sequences of shRNA-COL4A1 were shown in Table 1.

Subjects The study subjects were randomly divided into 3 experimental groups and 3 control groups. The inclusion criteria of experimental groups were: ARC patients over 50y of age, cortical ARC with LOCS III score of nuclear color (NC) ≥4, regardless of diabetes, uveitis, glaucoma and other factors. The control groups were selected as age matched anterior lens capsule without cataract in Eye Bank of Shaanxi Province, China. Patient's sex, age, classification of cortical, eye examination, and systemic disease were recorded, and the pictures of anterior segment were captured from each patient.

Generation of siCOL4A1-HLE-B3 Stable Cell Line The HLE-B3 (2.5×10^6) cells were transfected with pMD2.G (1 μg) and psPAX2 (1 μg) together with pLKO.1-Puro-control plasmid (4 μg) or pLKO.1-Puro-shCOL4A1-1810, pLKO.1-Puro-shCOL4A1-1434, pLKO.1-Puro-shCOL4A1-1723 plasmid with lipo2000, respectively. After 6h, cell media were removed and new medium were added, then incubated for another 48h. The medium with lentivirus was filtered with 0.45 μm and concentrated with 5×PEG-8000 buffer. After that the lentivirus was added to allocated cells, then the cells were cultured with medium containing puromycin (1 μg/mL) for 5d before further assays.

Cell Proliferation Assay Four wells of siCOL4A1-HLE-B3 stable cells (1×10^4) were seeded into 96-well plates, and another four wells of HLE-B3 cells were simultaneously incubated as normal controls. Totally 10 μL of CCK8 reagent (HY-K0301, Dojindo, USA) was added into the each of the dishes for 4h at 37℃ at 1, 2, 3, and 4d, respectively. The results were measured by a microplate reader (Infinite M100 PRO, TECAN, Switzerland) at a wavelength of 450 nm.

Cell Cycle Analysis HLE-B3 cells (2×10^5) were collected at 24h or 48h and fixed with 70% ethanol at 4℃ for 12h. Then, using cold phosphate buffered solution (PBS) to wash indicated cells for 3 times and centrifuged after which the supernatant was discarded. In addition, propidium iodide (PI) dye (5 μL/sample) was prepared for staining and cell cycle distribution was analyzed using FACScan flow cytometer (CytoFLEX, Beckman Coulter, USA).

Cell Apoptosis Assay HLE-B3 cells (2×10^5) were collected at 24h or 48h. Using cold PBS to wash indicated cells for 3 times and the cells were resuspended in 1×binding buffer. Annexin-V- fluorescein isothiocyanate (FITC; 5 μL/sample/PI (5 μL/sample) dye was incubated with cells for 15min, then, the cells in binding buffer for analysis the cell apoptosis using FACScan flow cytometer.

Immunofluorescence Inoculate the HLE-B3 cells in a 12-well plate for one day, then the cells were fixed with 4% paraformaldehyde in room temperature and permeated with

0.5% Triton X-100 for 30min, and then moistened with PBS for 2-3 times. The cells were blocked with 10% sheep serum for 1h, then the first antibody [anti-tublin (1:500), anti-COL4 (1:100)] was incubated overnight at 4℃. After washed for 3 times, the second antibody [donkey anti-mouse IgG (H+L) (1:1000), goat anti-mouse IgG (H+L) (1:500)] was incubated for 1-2h at room temperature and protected from light. DAPI staining was next performed and the images were captured by fluorescence microscope within zoom 400× areas (TCS SP5, Leica, USA).

RNA Extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction Total RNA (10×10^6 cells or 50-100 mg tissues) was extracted by Trizol reagent and the mRNA levels of *COL4A2*, *PAX6*, *PLOD1, PLOD2, LMX1B, Fibronectin, α-SMA, Laminin, TGF-β, E-cadherin, N-cadherin* and *Glyceraldehyde 3 phosphate dehydrogenase* (*GAPDH*) were analyzed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). After that, 1.5 μg of extracted RNA was reverse transcribed to cDNA. SYBR Green-mediated RT-PCR amplification and real time fluorescence detection were performed with ABI 7900HT FAST q-PCR detector (7900HT FAST, Applied Biosystems Inc, USA). The data was presented as the relative abundance of normalized mRNA relative to GAPDH. qRT-PCR primer sequences were listed in Table 2.

Western Blot Totally 50 mg tissues were homogenate in an ice bath with cold NP40 lysis buffer (NP40 buffer with phosphatase and protease inhibitor cocktail), the concentrations of protein were measured by the Bradford assay. After that, the proteins (40 μg) were subjected to SDS-PAGE analysis. Then the protein was transferred to a nitrocellulose membrane and blocked 5% BSA in TBST for 1h at 37℃, then, the first antibody (anti-COL4A1, 1:1000; anti-E-cadherin, 1:1000; anti-N-cadherin, 1:1000; anti-β-actin, 1:2000) was incubated overnight at 4℃. Cold PBS was applied to wash membrane for 3 times before and after the second antibody [rabbit anti-mouse polyclonal antibody IgG labeled by horseradish peroxidase (HRP), 1:2000)] incubation (1-2h). The nitrocellulose membrane was incubated with electrochemiluminescence for 1min. The results of β-actin (an internal reference), COL4A1, E-cadherin, and N-cadherin expressions were analyzed by chemiluminescence imaging system (4600, Tanon, China).

Statistical Analysis All results were statistically analyzed using Prism software (GraphPad Software, San Diego, USA). Experiments were repeated at least three times and the results are presented as the means±standard deviation (SD). The significance of statistical differences between two groups was calculated by unpaired Student's *t*-test (significant for $P<0.05$, and very significant for $P<0.01$, $P<0.001$).

RESULTS

Expression of COL4A1 in Anterior Lens Capsules Tissues of Healthy Human and ARC Patients To study the possible effect of aberrant expression of COL4A1 on the propagation of ARC diseases, we first checked the expression level of *COL4A1* in anterior lens capsules in healthy human beings and ARC patients. Total RNA from the control and patient were extracted and the mRNA levels of *COL4A1* along with *GAPDH* were analyzed. Figure 1A showed the relative abundance of the normalized mRNA relative to control *GAPDH* obtained from qRT-PCR with the designed primers (Table 2). Each patient group contains 12 number of samples. mRNA level of *COL4A1* in ARC group dropped to 33.4% of the healthy group ($P<0.001$). Similar trend was observed in protein expression level that was determined by western blot (Figure 1B), about 54% of lower in the ARC group ($P<0.01$). These results together showed that the expression level of COL4A1 was significantly lower in ARC group compared to the healthy group.

Table 2 Primer sequences information

Primer	Sequences (5'-3')
GAPDH-hF	TGACAACTTTGGTATCGTGGAAGG
GAPDH-hR	AGGCAGGGATGATGTTCTGGAGAG
COL4A1-hF	GGGATGCTGTTGAAAGGTGAA
COL4A1-hR	GGTGGTCCGGTAAATCCTGG
LMX1B-hF	CGGACTGCGCCAAGATGTT
LMX1B-hR	TTGACTCGCATCAGGAAGCG
PLOD1-hF	GGTCATTCTCTTCGCAGACAG
PLOD1-hR	CCACCGGATACTTGGTCTCCA
PLOD2-hF	CATGGACACAGGATAATGGCTG
PLOD2-hR	AGGGGTTGGTTGCTCAATAAAAA
PAX6-hF	AACGATAACATACCAAGCGTGT
PAX6-hR	GGTCTGCCCGTTCAACATC
Fibronectin-hF	GTGTCCTCCTTCCATCTTC
Fibronectin-hR	CAGACTGTCGGTACTCACG
α-SMA-hF	CCACTGCTGCTTCCTCTTC
α-SMA-hR	CGCCGACTCCATTCCAAT
Laminin-hF	CATCGTTCCACGGGTGTATTA
Laminin-hR	GCATGTGTCCAGCTCTACTT
TGF-β-hF	CAGCAACAATTCCTGGCGATACCT
TGF-β-hR	CGCTAAGGCGAAAGCCCTCAAT
E-cadherin -hF	TTATGATTCTCTGCTCGTGT
E-cadherin -hR	ATAGTCCTGGTCTTTGTCTG
N-cadherin -hF	ATAGTCCTGGTCTTTGTCTG
N-cadherin -hR	AGCGTTCCTGTTCCACTCA

GAPDH: *Glyceraldehyde 3 phosphate dehydrogenase; COL4A1*: *Collagen type IV alpha 1 chain; LMX1B*: *LIM homeobox transcription factor 1-beta; PLOD1*: *Procollagen-lysine 2-oxoglutarate 5-dioxygenases 1; PLOD2*: *Procollagen-lysine 2-oxoglutarate 5-dioxygenases 2; PAX6*: *Paired box 6; α-SMA*: *Alpha-smooth muscle actin; TGF-β*: *Transforming growth factor beta; E-cadherin*: *Epithelial cadherin; N-cadherin*: *Neural cadherin;* F: Forward; R: Reverse.

Generation of siCOL4A1-1434-HLE-B3 Stable Cell Line The decreased expression level of COL4A1 in ARC patients suggested that lowering expression of COL4A1 might be a revenue to promote ARC progress. We chose HLE-B3 cells to test this hypothesis. The shRNAs with different sequences of *COL4A1* were transfected into HLE-B3, and gene silencing efficiency was quantitatively analyzed by qRT-PCR. As shown in Figure 2A, compare with negative control (NC), both siCOL4A1-1810 and siCOL4A1-1434 are able to significantly reduce the mRNA expression of *COL4A1* (P<0.01). The knockdown efficiencies were 76% and 77%, respectively. Thus, siCOL4A1-1434 was selected for further study.

siCOL4A1 Inhibited HLE-B3 Cells Proliferation and Enhanced Apoptosis To detect the influence of *COL4A1* knockdown on the proliferation and apoptosis, we knocked down *COL4A1* with siCOL4A1-1434 in HLE-B3 cells. Cell viability was detected by cell counting kit-8 (CCK-8) assay kit. As shown in Figure 2B, we found that COL4A1 shRNA-1434 significantly inhibited the growth rate of HLE-B3 cells from day the 3rd day after culture (P<0.01; Figure 2B). Further experiments Annexin V/PI double staining indicated that no apparent apoptosis rate was detected in each group after 24h transfection, while the difference was observed after 48h transfection (P<0.01). Compared with NC, the rate of apoptosis was dramatically increased in siCOL4A1-1434 group treated HLE-B3 cells after 48h incubation (Figure 3). Taken together, our results solidly proved that silencing the *COL4A1* suppressed HLE-B3 cells proliferation and caused cell apoptosis.

siCOL4A1 Led to a Significant Increase of the Percentage of S-phase Cells In order to detect the influence of COL4A1 knockdown on the cell cycle of HLE-B3 cells, PI single staining was conducted to evaluate the effect of *COL4A1* on cell cycle distribution. As shown in Figure 4, no obvious changes of cell percentages were observed in G1-phases of two groups. After 24h or 48h of transfection, the cell percentage in the S-phase increased significantly in the siCOL4A1-1434 group compared with NC group (P<0.001).

Change of Tublin, COL4 and Extracellular Matrixes Distribution in HLE-B3 To explore whether knockdown of *COL4A1* has an effect on the morphology of cytoskeleton, the distribution of tublin was detected by confocal microscopy. Results revealed that knockdown of *COL4A1*, compared with NC group, did not change the expression and distribution of tublin (Figure 5A). In addition, in order to explore whether knockdown of *COL4A1* has an effect on the extracellular matrix, the distribution of COL4 was staining and examined by confocal microscopy. As shown in Figure 5B, compared with NC group, knockdown of *COL4A1* decreased the expression and distribution of COL4. Knockdown of *COL4A1*

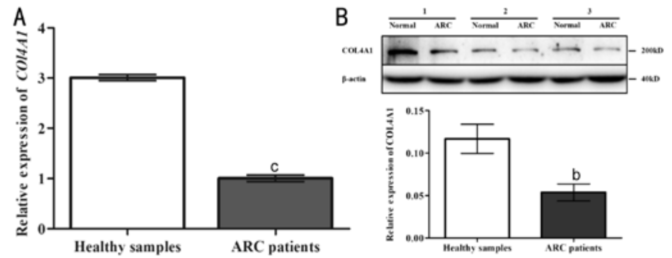

Figure 1 Expression of *COL4A1* in healthy human and age-related cataract patients A: qRT-PCR results confirmed the mRNA expression of *COL4A1* in the age-related cataract patients were significantly less than the healthy human. ^{c}P<0.001 *vs* the healthy human group. (n=12/group). B: Western blot analysis the expression of COL4A1 and β-actin in the indicated samples. ^{b}P<0.01. The data are presented as the mean±SD. ARC: Age-related cataract. *COL4A1*: Collagen type IV alpha 1 chain.

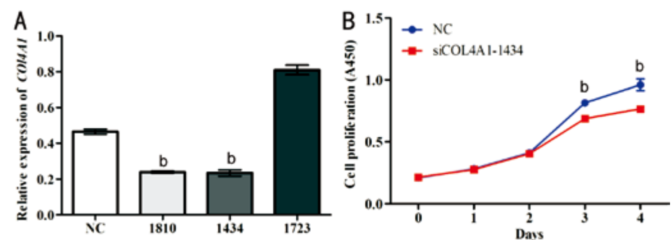

Figure 2 Generation siCOL4A1 stable cell line and evaluation proliferation A: The different gene site of shRNA-COL4A1 were transfected into HLE-B3, and gene silencing efficiency were quantitatively analyzed by qRT-PCR. shCOL4A1-1434 shown the best knockdown efficiency and the expression of *COL4A1* reduced to 0.235 (NC group: 0.465, shCOL4A1-1810 group: 0.240, shCOL4A1-1723: 0.810, respectively). ^{b}P<0.01 *vs* the negative control group. B: Cell viability was obviously inhibited after silencing *COL4A1*. The normal HLE-B3 and siCOL4A1- HLE-B3 cells were cultured then cell viability was detected by cell counting kit-8 (CCK-8) assay kit for indicated time points. The proliferation of HLE-B3 cells were suppressed from 3d after incubation. ^{b}P<0.01 *vs* the control cells group. NC: Negative control.

also inhibited the expression of other extracellular matrixes including fibronectin, α-SMA, and laminin (Figure 5C).

siCOL4A1 Promoted Epithelial-Mesenchymal Transition Process of HLE-B3 Cells To detect the influence of *COL4A1* knockdown on the EMT process, we knocked down *COL4A1* with siCOL4A1-1434 in HLE-B3 cells. Cell markers of EMT process were detected by qRT-PCR and Western blot. As shown in Figure 6, we found that COL4A1 shRNA-1434 significantly inhibited the expression of E-cadherin and promoted the expression of N-cadherin on both mRNA (Figure 6A) and protein (Figure 6B, 6C) level. Taken together, our results solidly proved that silencing the *COL4A1* stimulated the EMT process of HLE-B3 cells.

Expression of *PLOD2*, *PLOD1*, *PAX6*, *COL4A2* and *TGF-β* in HLE-B3 To further investigated the regulation

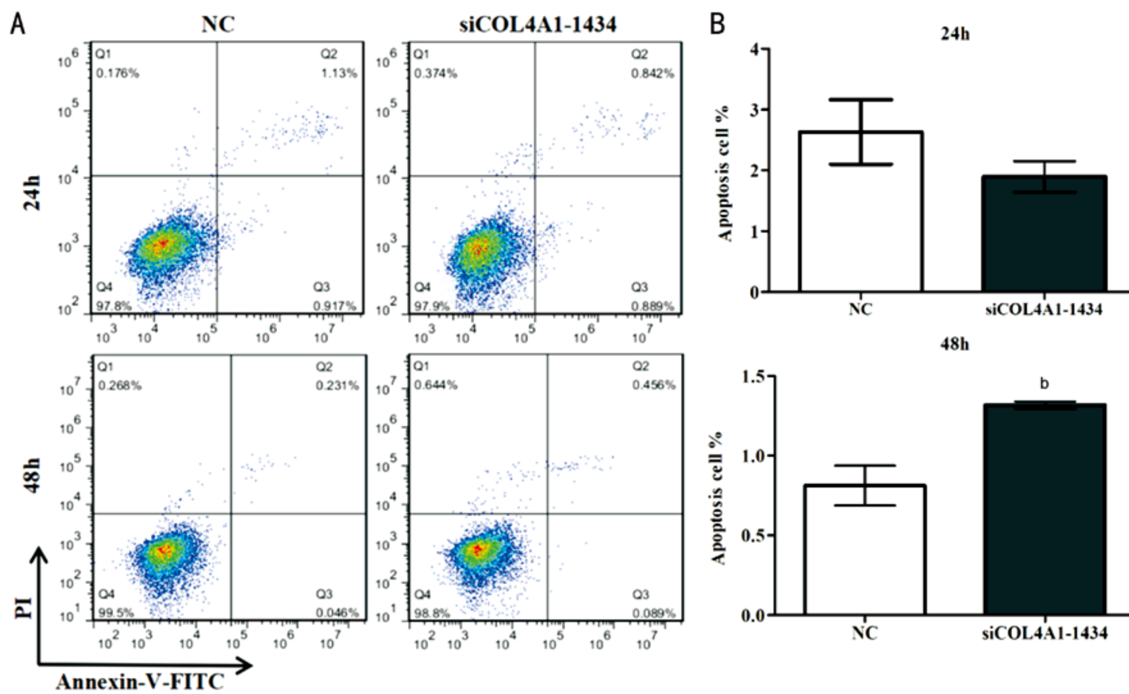

Figure 3 siCOL4A1 leaded to cell apoptosis A: Results of flow cytometry showed that HLE-B3 cells were treated with siCOL4A1 for 24 or 48h. B: the apoptosis rate was significantly increased after 48h treatment. [b]*P*<0.01 *vs* the control cells group. NC: Negative control. HLE-B: Human lens epithelial cell; *COL4A1*: Collagen type IV alpha 1 chain.

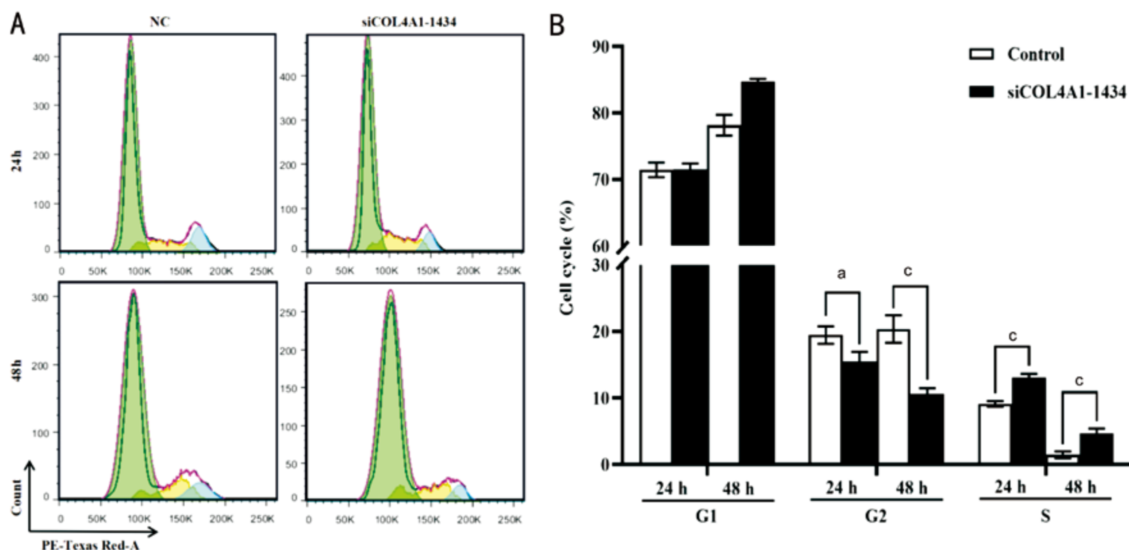

Figure 4 siCOL4A1 induced cell cycle S-phase arrest in HLE-B3 cells A: Flow analysis was used to analysis cell population under different stages of G1, G2 and S phase after cultured in 24 or 48h. B: siCOL4A1 induced significantly S-phase arrest after incubated 24 or 48h. [c]*P*<0.001 *vs* the control cells group. HLE-B: Human lens epithelial cell; *COL4A1*: Collagen type IV alpha 1 chain.

mechanism of *COL4A1* in ARC progression, we then explored the alteration of expression for these five genes at mRNA level through qRT-PCR on HLE-B3 cells after knockdown of *COL4A1*. The results indicated that after silencing *COL4A1* gene, the mRNA expression of *COL4A2*, *PAX6*, *PLOD1* and *PLOD2* were significantly inhibited while mRNA expression of *TGF-β* was significantly promoted compare with NC (*P*<0.01; Figure 7).

DISCUSSION

Globally, cataract is still the main causes of visual impairment and blindness[11]. Especially, ARC is the most common clinical pathological type of cataract[12-13]. Aberrant DNA methylation was considered to be the main cause of ARC and other eye diseases[7]. In previous study, we successfully identified that abnormal *COL4A1* gene methylation was clinically associated with ARC patients[7]. The *COL4A1* mutations have been shown to cause multi-system disorders in patients, including small-vessel brain disease, variably associated with eye defects and systemic findings[14]. Moreover, *COL4A1* has been already investigated in infants with congenital cataract[15-16]. However,

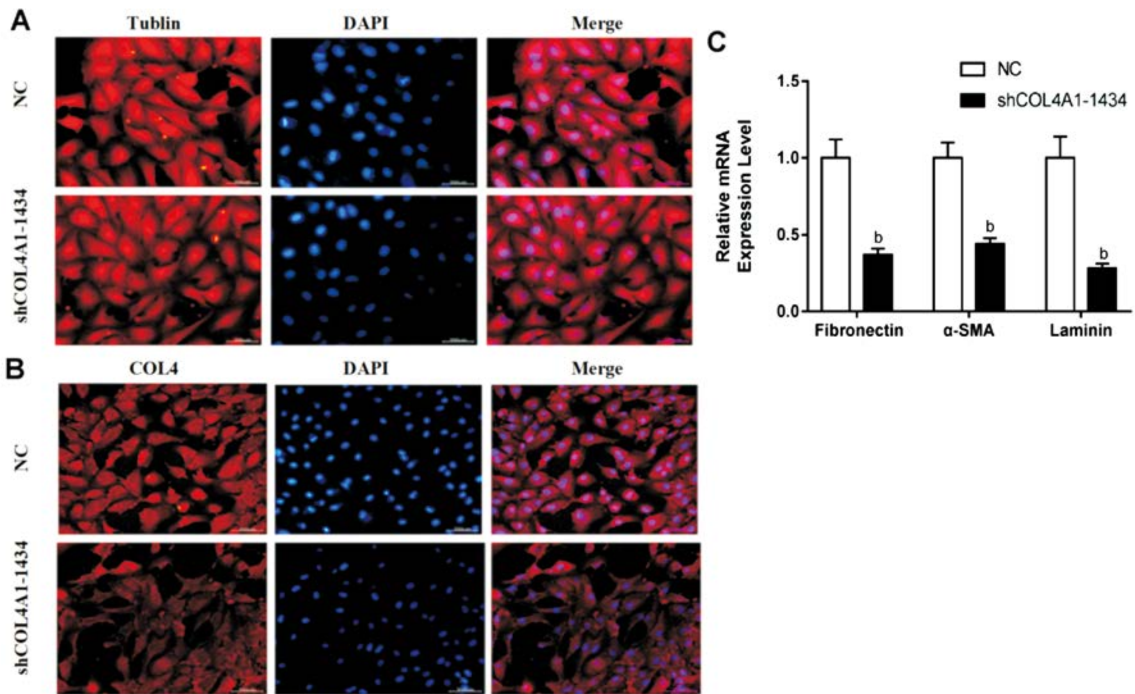

Figure 5 Morphological observation of cytoskeleton and extracellular matrix by confocal microscope Tubulin (A) and COL4 (B), as the protein markers of cytoskeleton and extracellular matrix, respectively, shown red color and the nucleus shown blue color. Images shown the expression and distribution of tubulin were unchanged and the expression and distribution of COL4 were decreased after HLE-B3 treated with siCOL4A1 (zoom: 400×). C: mRNA expression level of other extracellular matrixes including fibronectin, α-SMA and laminin. [b]P<0.01 *vs* the control cells group. NC: Negative control.

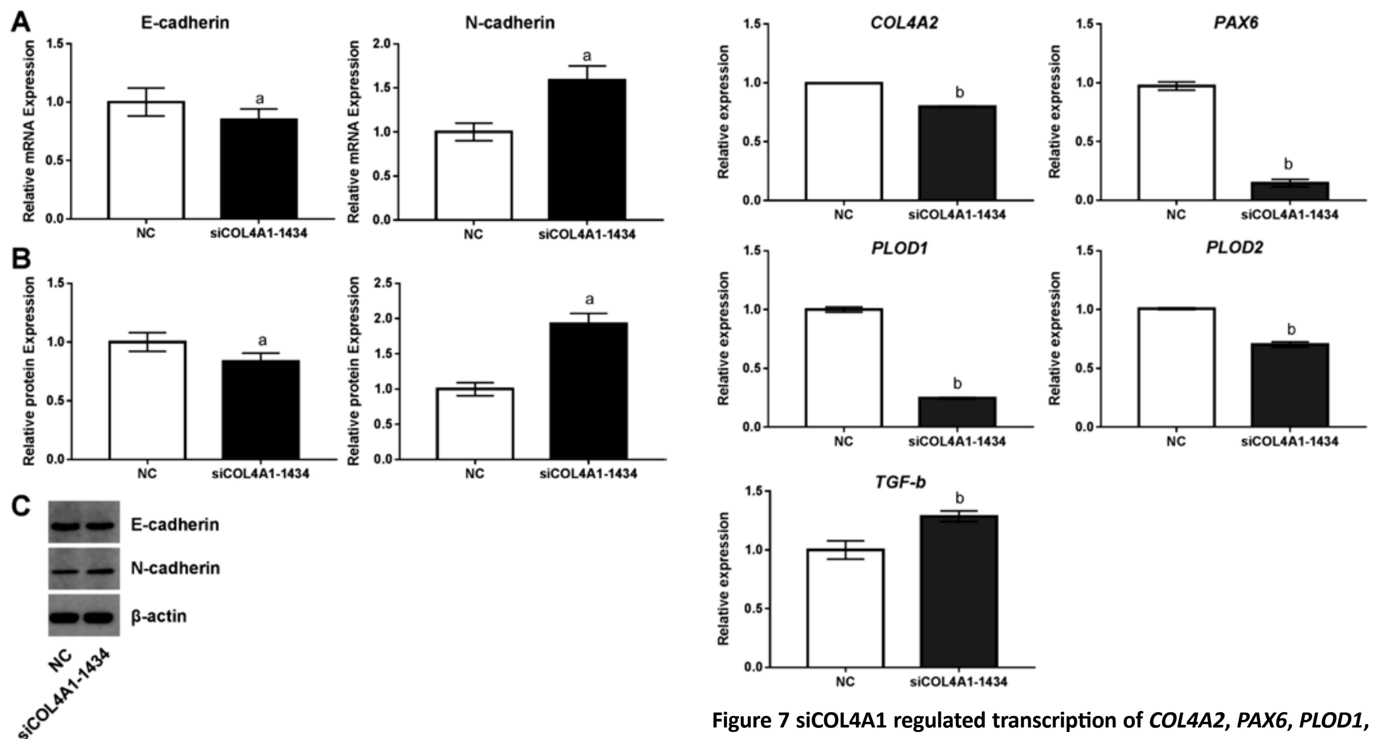

Figure 6 Influence of siCOL4A1 silencing on EMT process of HLE-B3 cells A: The mRNA expression level of E-cadherin and N-cadherin were quantitatively analyzed by qRT-PCR. [a]P<0.05 *vs* the negative control group. B: The protein expression level of E-cadherin and N-cadherin were analyzed by Western blot. [a]P<0.05 *vs* the negative control group. C: Results of Western blot detection. NC: Negative control.

Figure 7 siCOL4A1 regulated transcription of *COL4A2*, *PAX6*, *PLOD1*, *PLOD2 and TGF-β* mRNA was extracted from HLE-B3 or HLE-B3 treated with siCOL4A1 and expression level of the indicated genes was quantified through qPT-PCR. [b]P<0.01 *vs* the vector group. NC: Negative control.

the exquisite regulation of *COL4A1* gene functions within ARC remains unclear.

The growth inhibition and apoptosis of HLE-B3 cells is observed in various types of cataract and may initiate the ARC development[17]. Apoptosis of lens epithelial cells was a common cellular foundation for the cataract formation in human beings and animals[18]. We observed that silencing the *COL4A1* gene could induce apoptosis and inhibit the proliferation of the HLE-B3 cells by blocking the cell cycle to S phase, which clearly indicated that silent *COL4A1* gene was associated with the ARC progresses. A previous study found that *COL4A1* or *COL4A2* genes might participate in the occurrence of coronary artery disease through transforming growth factor beta/Smad3 (TGF-β/Smad3) signaling pathway[19]. In addition, Shirai *et al*[20] found that TGF-β/Smad3 signaling pathway is involved in the anterior subcapsular cataract. Thus, we suspected that *COL4A1* might induce apoptosis and inhibit the proliferation of the HLE-B3 cells *via* TGF-β/Smad3 signaling pathway. However, hypothesis needs to be verified by further experiments.

The cytoskeleton is a complex network consisting of three major filament systems: microfilaments, microtubules and intermediate filaments[21]. Cytoskeleton is most well-known for its importance to the lens[22]. Ultraviolet radiation, aging, hypoxia, and other stimuli could cause excess apoptosis of lens epithelial cells, thus causing cytoskeletal degradation, accumulation of crystal protein, and ultimately cataract[23]. Previous study proved that ARC associated with oxidative stress could trigger the degradation of cytoskeletal proteins in HLE-B3 cells[24]. However, our results showed that silencing of the *COL4A1* gene had no effect on the morphology of cytoskeleton, indicating *COL4A1* gene might cause ARC through alternative mechanisms. *COL4A1* is an extracellular matrix protein of basement membranes, which is essential for basement membrane stability[25]. Our results uncovered that knockdown of *COL4A1* decreased the expression and distribution of COL4 and other extracellular matrixes including fibronectin, α-SMA and laminin, indicating that silent *COL4A1* increased the degradation of extracellular matrix. EMT of lens epithelial cells is one of the important mechanisms of posterior capsular opacification (PCO) after cataract operation, which has attracted more and more attention of experts in recent years. Currently various signaling pathways are known to be involved in the occurrence of EMT in lens epithelial cells, such as TGF-β/Smad pathway, Jagged-1/Notch pathway, MAPK/ERK pathway, Wnt/β-catenin pathway, PI3K/AKT/mTOR pathway and so on. TGF-β/Smad is most important one in all the pathways. Our results suggested that silencing the *COL4A1* gene could induce EMT of the HLE-B3 cells by promoting the TGF-β expression, which may indicate a new pathogenesis mechanism of ARC.

Genetic factors are considered to play an important role in the ARC formation[7]. To date, genetic studies have identified several gene variants associated with crystallin mutation[26]. *COL4A2*, which encodes the type IV α2 collagen chain and shared a common locus at 13q34 with *COL4A1*[27]. Considering the similar genomic organization between *COL4A1* and *COL4A2* (abnormalities of collagen IV), *COL4A2* mutations also may be involved in a broad spectrum of disorders, including myopathy, glaucoma and small vessel diseases[28]. Previous study found that mice with *COL4A2* point mutations developed cataracts, abnormalities of the lens[29]. Procollagen-lysine 2-oxoglutarate 5-dioxygenases (*PLOD*) plays an important role in the lysyl hydroxylation of collagen[30]. Three *PLOD* isoforms have been identified in human genome, namely *PLOD1*, *2* and *3*, which share high homology in protein sequences[31]. Mutations in *PLOD1* or *PLOD2* are linked with Ehlers-Danlos syndrome type VI in patients[32], who present ocular similarities to Axenfeld-Rieger anomaly/Axenfeld-Rieger syndrome, particularly glaucoma and microcornea[10,33]. *PAX6* gene is reported to influence development of the central nervous system and also has been known as a key regulator of eye development[34]. Pathogenic mutations of *PAX6* also have been demonstrated to involve in the congenital cataracts, aniridia, anophthalmia and other multiple ocular defects in human[35]. *PAX6* and *COL4A1* were identified as ocular anterior segment disorders (ASDs) genes[36]. We found that *PLOD2*, *PLOD1*, and *COL4A2* were regulated by *COL4A1*, and *PAX6* was regulated by *LMX1B*[37]. Moreover, Bongers *et al*[38] reported that *LMX1B* was regulated by *COL4A1*. Interestingly, our study proved that silencing the *COL4A1* gene down-regulated the expression level of *COL4A2*, *PAX6*, *PLOD1* and *PLOD2*, which indicating that *COL4A1* probably involved in the pathogenic process of ARC *via* regulating *COL4A2*, *PAX6*, *PLOD1* and *PLOD2*.

Thus far, the relationship between aberrantly expressed *COL4A1* gene and occurrence of ARC still remains unknown. Our study indicated that aberrant expression of *COL4A1* could cause HLE-B3 cells S-phase arrest, and further inhibited the proliferation and enhanced HLE-B3 apoptosis. This process didn't influence the cytoskeleton but down-regulated the expression of some genes related to ARC, such as *COL4A2*, *PAX6*, *PLOD1* and *PLOD2*. Furthermore, silencing the *COL4A1* gene could induce EMT of the HLE-B3 cells by promoting the TGF-β expression. However, the precise regulation mechanisms underlying the *COL4A1* gene was still not well elucidated. Further studies are required to investigate the main signaling pathway that involves in *COL4A1* gene regulation and also the effect of aberrant *COL4A1* on inducing cataract *in vivo*.

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REFERENCES

1 Yoshizaki M, Ramke J, Zhang JH, Aghaji A, Furtado JM, Burn H, Gichuhi S, Dean WH, Congdon N, Burton MJ, Buchan J. How can we improve the quality of cataract services for all? A global scoping review. *Clin Exp Ophthalmol* 2021;49(7):672-685.

2 Liu YC, Wilkins M, Kim T, Malyugin B, Mehta JS. Cataracts. *Lancet* 2017;390(10094):600-612.

3 Ağaoğlu NB, Varol N, Yıldız SH, Karaosmanoğlu C, Duman R, Özdemir Erdoğan M, Solak M. Relationship between sirt1 gene expression level and disease in age-related cataract cases. *Turk J Med Sci* 2019;49(4):1068-1072.

4 Lim JC, Umapathy A, Donaldson PJ. Tools to fight the cataract epidemic: a review of experimental animal models that mimic age related nuclear cataract. *Exp Eye Res* 2016;145:432-443.

5 Lim JC, Grey AC, Zahraei A, Donaldson PJ. Age-dependent changes in glutathione metabolism pathways in the lens: new insights into therapeutic strategies to prevent cataract formation-a review. *Clin Exp Ophthalmol* 2020;48(8):1031-1042.

6 Wang Y, Guan HJ. The role of DNA methylation in lens development and cataract formation. *Cell Mol Neurobiol* 2017;37(6):979-984.

7 Wang L, Li P, Guo X. Screening of methylation genes in age-related cataract. *Int J Ophthalmol* 2018;11(7):1102-1107.

8 Sato Y, Shibasaki J, Aida N, Hiiragi K, Kimura Y, Akahira-Azuma M, Enomoto Y, Tsurusaki Y, Kurosawa K. Novel *COL4A1* mutation in a fetus with early prenatal onset of schizencephaly. *Hum Genome Var* 2018;5:4.

9 Kuuluvainen L, Mönkäre S, Kokkonen H, Zhao F, Verkkoniemi-Ahola A, Schleutker J, Hakonen AH, Hartikainen P, Pöyhönen M, Myllykangas L. *COL4A1* and *COL4A2* duplication causes cerebral small vessel disease with recurrent early onset ischemic strokes. *Stroke* 2021;52(10):e624-e625.

10 Scoppettuolo P, Ligot N, Wermenbol V, Van Bogaert P, Naeije G. p.Gly743Val mutation in COL4A1 is responsible for familial porencephaly and severe hypermetropia. *Front Neurol* 2020;11:827.

11 Abdolalizadeh P, Ghasemi Falavarjani K. Correlation between global prevalence of vision impairment and depressive disorders. *Eur J Ophthalmol* 2022;32(6):3227-3236.

12 Yang M, Zhang JF, Su S, Qin B, Kang LH, Zhu RR, Guan HJ. Allelic interaction effects of DNA damage and repair genes on the predisposition to age-related cataract. *PLoS One* 2018;13(4):e0184478.

13 Chen X, Zhou DY, Shen J, Wu YB, Sun QZ, Dong JM, Yu JC. Prevalence and risk factors on age-related cataract and surgery in adults over 50y old in Binhu District, Wuxi, China. *Int J Ophthalmol* 2020;13(3):445-451.

14 Mao M, Kiss M, Ou Y, Gould DB. Genetic dissection of anterior segment dysgenesis caused by a *Col4a1* mutation. *Dis Models Mech* 2017;10(4):475-485.

15 Khan AO. col4a1 mutations in two infants with congenital cataracts and porencephaly: an ophthalmologic perspective. *J AAPOS* 2019;23(6):362.

16 Rechsteiner D, Issler L, Koller S, Lang E, Bähr L, Feil S, Rüegger CM, Kottke R, Toelle SP, Zweifel N, Steindl K, Joset P, Zweier M, Suter AA, Gogoll L, Haas C, Berger W, Gerth-Kahlert C. Genetic analysis in a Swiss cohort of bilateral congenital cataract. *JAMA Ophthalmol* 2021;139(7):691-700.

17 Huang JN, Yu WS, He Q, He XY, Yang M, Chen W, Han W. Autophagy facilitates age-related cell apoptosis-a new insight from senile cataract. *Cell Death Dis* 2022;13(1):37.

18 Su D, Hu S, Guan L, Wu X, Shi C, Yang X, Ma X. Down-regulation of GJA3 is associated with lens epithelial cell apoptosis and age-related cataract. *Biochem Biophys Res Commun* 2017;484(1):159-164.

19 Turner AW, Nikpay M, Silva A, Lau P, Martinuk A, Linseman TA, Soubeyrand S, McPherson R. Functional interaction between COL4A1/COL4A2 and SMAD3 risk loci for coronary artery disease. *Atherosclerosis* 2015;242(2):543-552.

20 Shirai K, Saika S, Tanaka T, Okada Y, Flanders KC, Ooshima A, Ohnishi Y. A new model of anterior subcapsular cataract: involvement of TGFbeta/Smad signaling. *Mol Vis* 2006;12:681-691.

21 Akhmanova A, Kapitein LC. Mechanisms of microtubule organization in differentiated animal cells. *Nat Rev Mol Cell Biol* 2022;23(8):541-558.

22 Karnam S, Maddala R, Stiber JA, Rao PV. Drebrin, an actin-binding protein, is required for lens morphogenesis and growth. *Dev Dyn* 2021;250(11):1600-1617.

23 Cheng TY, Xu MD, Qin B, Wu J, Tu YY, Kang LH, Wang Y, Guan HJ. lncRNA H19 contributes to oxidative damage repair in the early age-related cataract by regulating miR-29a/TDG axis. *J Cell Mol Med* 2019;23(9):6131-6139.

24 Ganatra DA, Johar KSR, Parmar TJ, Patel AR, Rajkumar S, Arora AI, Kayastha FB, Pal AK, Gajjar DU, Vasavada AR. Estrogen mediated protection of cytoskeleton against oxidative stress. *Indian J Med Res* 2013;137(1):117-124.

25 Okano S, Shimada S, Tanaka R, Okayama A, Kajihama A, Suzuki N, Nakau K, Takahashi S, Matsumoto N, Saitsu H, Tanboon J, Nishino I, Azuma H. Life-threatening muscle complications of COL4A1-related disorder. *Brain Dev* 2020;42(1):93-97.

26 Shiels A, Hejtmancik JF. Mutations and mechanisms in congenital and age-related cataracts. *Exp Eye Res* 2017;156:95-102.

27 Zagaglia S, Selch C, Nisevic JR, *et al*. Neurologic phenotypes associated with *COL4A1/2* mutations: expanding the spectrum of disease. *Neurology* 2018;91(22):e2078-e2088.

28 Kuo DS, Labelle-Dumais C, Gould DB. COL4A1 and COL4A2 mutations and disease: insights into pathogenic mechanisms and potential therapeutic targets. *Hum Mol Genet* 2012;21(R1):R97-R110.

29 Favor J, Gloeckner CJ, Janik D, Klempt M, Neuhäuser-Klaus A, Pretsch W, Schmahl W, Quintanilla-Fend L. Type IV procollagen missense mutations associated with defects of the eye, vascular stability, the brain, kidney function and embryonic or postnatal viability in the mouse, Mus musculus: an extension of the Col4a1 allelic series and the identification of the first two Col4a2 mutant alleles. *Genetics* 2007;175(2):725-736.

30 Qi YF, Xu R. Roles of PLODs in collagen synthesis and cancer progression. *Front Cell Dev Biol* 2018;6:66.

31 Xu WH, Xu Y, Wang J, Tian X, Wu JL, Wan FN, Wang HK, Qu YY, Zhang HL, Ye DW. Procollagen-lysine, 2-oxoglutarate 5-dioxygenases 1, 2, and 3 are potential prognostic indicators in patients with clear cell renal cell carcinoma. *Aging* 2019;11(16):6503-6521.

32 Heikkinen J, Toppinen T, Yeowell H, Krieg T, Steinmann B, Kivirikko KI, Myllylä R. Duplication of seven exons in the lysyl hydroxylase gene is associated with longer forms of a repetitive sequence within the gene and is a common cause for the type VI variant of Ehlers-Danlos syndrome. *Am J Hum Genet* 1997;60(1):48-56.

33 Hjalt TA, Amendt BA, Murray JC. PITX2 regulates procollagen lysyl hydroxylase (PLOD) gene expression: implications for the pathology of Rieger syndrome. *J Cell Biol* 2001;152(3):545-552.

34 Ochi S, Manabe S, Kikkawa T, Osumi N. Thirty years' history since the discovery of Pax6: from central nervous system development to neurodevelopmental disorders. *Int J Mol Sci* 2022;23(11):6115.

35 Functional role of *pax6* during eye and nervous system development in the annelid *Capitella teleta*. *Dev Biol* 2019;456(1):86-103.

36 Ma AL, Yousoof S, Grigg JR, *et al*. Revealing hidden genetic diagnoses in the ocular anterior segment disorders. *Genet Med* 2020;22(10):1623-1632.

37 Szklarczyk D, Kirsch R, Koutrouli M, Nastou K, Mehryary F, Hachilif R, Gable AL, Fang T, Doncheva NT, Pyysalo S, Bork P, Jensen LJ, von Mering C. The STRING database in 2023: protein-protein association networks and functional enrichment analyses for any sequenced genome of interest. *Nucleic Acids Res* 2023;51(D1):D638-D646.

38 Bongers EF, Knoers NM. From gene to disease; the nail-patella syndrome and the LMX1B gene. *Ned Tijdschr Geneeskd* 2003;147(2):67-69.