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Expressions of Senescence-Associated β-Galactosidase and Senescence Marker Protein-30 are Associated with Lens Epithelial Cell Apoptosis

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

B Dan Zhou
C Dan Yin
D Fang Xiao
ABCD Jie Hao

Eye Hospital, The First Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, P. R. China

Corresponding Author: Jie Hao, e-mail: haojiehj0815@163.com
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Background: To investigate associations of senescence marker protein-30 and senescence-associated β-galactosidase expression with lens epithelial cells apoptosis among Chinese age-related cataract patients.

Material/Methods: A total of 145 age-related cataract patients (69 cases with nuclear cataract in 91 eyes and 76 cases of cortical cataract with 102 eyes) were enrolled in our study. An annular tear of the central part of anterior lens capsules was performed for each patient. Immunohistochemical staining and real-time PCR were used to detect the protein and mRNA expression levels, and TUNEL was used to assess lens epithelial cells apoptosis. Comparisons of protein expression levels and lens epithelial cells apoptosis were made between the 2 groups.

Results: The results showed a higher protein expression level of senescence marker protein-30 in surrounding parts of the anterior lens capsule compared with the central part of the anterior lens capsule; however, the positive rate of senescence-associated β-galactosidase was remarkably higher in the central part than in the surrounding part. Compared with cortical cataract patients, nuclear cataract patients had elevated senescence marker protein-30 protein and mRNA expression levels, but had a decreased positive rate of senescence-associated β-galactosidase. TUNEL results showed that the lens epithelial cell apoptosis rate was higher in the central part of the anterior lens capsule than in the surrounding part in both groups. Within either central or surrounding area of anterior lens capsule, cortical cataract patients exhibited a significantly higher lens epithelial cell apoptosis rate in contrast with nuclear cataract patients.

Conclusions: Our study results suggest that senescence marker protein-30 and senescence-associated β-galactosidase expressions in both nuclear cataract and cortical cataract patients were associated with lens epithelial cells apoptosis.

MeSH Keywords: Apoptosis • Cataract • Epithelial Cells

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Background

The category of age-related cataract (ARC) includes nuclear, cortical, and posterior subcapsular cataract [1]. Nuclear cataracts is the most common subtype of cataracts, and accounts for approximately 60% of ARC cases, cortical cataract accounts for about 30% of cases of ARC, as well as posterior subcapsular cataract accounts for remaining 10% of cases [2]. Evidence shows that ARC is responsible for 59 million cases of visual impairment and 18 million cases of blindness worldwide [3]. The prevalence of ARC keeps rising, which may affect 30.1 million Americans by 2020, leading to the substantial increase in public health cost and social economic burden [4]. While the etiology of ARC remains unclear, previous studies suggested that various risk factors, including age, sex, social status, ultraviolet radiation, smoking, and diabetes mellitus can contribute to ARC [5,6]. Recently, widespread agreement on associations of ARC to oxidation and aging of the lens among researchers has been achieved [7,8]. Lens epithelial cells (LECs) are believed to protect the underlying fiber cells from injuries and maintain the transparency of the lens [9]. The LECs are reported to provide stabilization for intraocular lens designs in the capsular bag, thus the apoptosis of LECs may lead to the formation of posterior capsule opacification and even cataract [10,11]. It was demonstrated that all ARC patients had a remarkable increase in apoptotic epithelial cells compare to healthy people [12]. Evidence also indicated that senescence marker protein-30 (SMP-30) and senescence-associated β-galactosidase (SA-β-gal) expression progressively decline with age [13,14]. Therefore, the question of whether SMP30 and SA-β-gal are associated with LEC apoptosis in ARC poses a new direction for ARC investigation. SMP-30 is a 34-kDa protein whose expression decreases with aging. SMP-30, initially identified as a cytosolic marker of aging, is now considered as a novel anti-aging factor in various organs and its amino acid sequences are highly conserved among vertebrates [15,16]. SMP-30 widely exists in many tissues, including liver, kidney, brain, testis, lungs, adrenal gland, stomach, ovary, uterus, and epidermis [17]. Previous studies reported the protective effects of SMP-30 in cell injury in liver, lungs, and brain [18,19]. Evidence showed that SMP-30 may regulate the effect of Ca^{2+} on liver cell functions and also possess gluconolactonase activity; hence, it plays an important role in ascorbic acid biosynthesis in the liver [17]. It was recognized that SMP-30 plays a protective role in cell apoptosis and cell injuries, thus it is possible that the decrease of SMP-30 with aging may result in senescent frailty [20]. Consistent with our hypothesis, a positive association between accelerated senescence and cataract formation was identified in BubR1 hypomorphic mice and discard of p16Ink4a, which may significantly postpone the appearance of cataracts in mice [21,22]. Currently, the identification of senescence-specific biomarkers is rather scant and there is an urgent need for the identification of additional biomarkers for senescence cells both *in vitro* and *in vivo* [23]. Senescent cells have been shown to accumulate with aging at sites of age-related pathology [24]. SA-β-Gal was first discovered by Judith Campisi at pH 6 in dermal fibroblasts and epidermal keratinocytes and SA-β-gal were commonly used biomarkers of cellular senescence [25,26]. However, whether SA-β-gal is indicative of senescence needs further investigations [27]. Moreover, although great efforts were exerted to investigate the possible risk factors for cataract, the possible associations between SMP-30 and SA-β-gal expressions and LEC apoptosis in cataract and ARC are currently unclear [28,29]. Therefore, we carried out the present study with the aim to identify the association of SMP-30 expression level and SA-β-gal positive rate with LEC apoptosis in ARC.

Material and Methods

Ethics statement

The study was approved by the Ethics Committee of the Eye Hospital, the First Affiliated Hospital of Harbin Medical University. Informed consents were obtained from all the involved subjects prior to the study. All procedures in this study followed the Declaration of Helsinki [30].

Clinical data collection

From March 2010 to October 2011, patients undergoing phacoemulsification surgery in the Eye Hospital of the First Affiliated Hospital of Harbin Medical University were considered as potentially eligible subjects for the current study. Patients with mature cataract, hypermature cataract, diabetes, or abnormal hepatorenal function were excluded. According to the lens opacity classification system III, there were 76 patients with 102 eyes of age-related cortical cataract (cortical cataract group) and 69 patients with 91 eyes of age-related nuclear cataract (nuclear cataract group) in this study [31]. The mean age of the nuclear cataract group was 65.88±4.83 years (range 58~79 years) and 65.97±4.35 years (range 60~80 years) in the cortical cataract group. No significant differences were found in age between the 2 groups (P=0.908). During the surgery, an annular tear (5~6 mm) of the central part of the anterior lens capsules was made with specimens from each group. Then specimens were randomly assigned into 2 copies by random number table method. The specimens in the first copy were mounted on a polylysine-coated glass with the cell side upward and transferred to ice boxes for TUNEL and immunohistochemical staining. And specimens in the second copy were randomly placed into ethylene diamine tetraacetic acid (EDTA) Eppendorf (EP) tubes and were then stored at –70°C for real-time polymerase chain reaction (PCR).

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Immunohistochemical staining

Streptavidin-peroxidase (SP) immunohistochemistry was used to examine expressions of SMP-30 and SA-β-gal in LECs. Antigen retrieval was undertaken at 95°C in sodium citrate buffer solution (0.01 mol/L) for 15 min. Then, the stretched preparations were incubated with rabbit polyclonal antibody against human (1: 150) overnight at 4°C (first antibody: Bioss, Beijing, China). Subsequently, secondary antibody was added in an SP Kit according to the manufacturer's directions (SP Kit: Bioss, Beijing, China). Subsequently, cells were colored by diaminobenzidine (DBA), counterstained with hematoxylin, and mounted in neutral balsam. Negative controls were treated in the same way, replacing the first antibody with phosphate-buffered saline (PBS). An image analyzer (Fuji Film) was utilized to record the positive cell counts and gray value intensity of the positive reaction for SMP-30 semi-quantitative analysis. Image gray scale referred to the degree of translucency in translucent medium containing 0~256 different grades. The lower grade indicated more intense positive reaction, and thus higher SMP-30 expression. Five high-power fields of each slice were randomly chosen to calculate the positive cell rate of SA-β-gal.

In situ cell apoptosis detection

Apoptosis was determined with a TUNEL kit (Boster, Wuhan, China) according to manufacturer's protocol. A negative control was employed by replacing TUNEL reaction mixture with marking fluid without DNA terminal transferase. After counterstaining with hematoxylin, specimens were observed for cell apoptosis. With the use of a light microscope (Leica DM LB, Germany), apoptosis cells presented with yellow solid precipitations in the nucleus and normal cells presented with blue solid precipitations. Apoptosis rates in both groups were recorded and analyzed. LEC apoptosis number was counted by calculating apoptosis rates. The apoptosis rates were calculated by randomly choosing 3 fields in each stretched preparations under a microscope and 100 cells were counted in each field to calculate apoptosis rates.

Real-time PCR

A total of 70 mg cyst membrane and 1 ml Trizol reagent were added and then ground in a grinder for 20~30 min and then transferred to a sterilized EP tube. Subsequently, 250 μl methenyl trichloride was added, blended, and maintained for 5 min. After centrifuging at 13 000 g for 10 min at 4°C, the solution was transferred to another sterilized EP tube, mixed with the same volume of dimethyl carbinol, and kept in a refrigerator at 4°C for 15 min. After centrifugation at 13 000 g for 10 min at 4°C, the liquid in the top of the tube was removed and the white precipitants that remained in the bottom of the tube were RNA. Then, 1 ml of 75% volume fraction of ethyl alcohol was added to the tube, followed by centrifugation at 7500 g for 5 min at 4°C, discard of the supernatant, centrifugation, aspiration of the liquid, and dissolving in deionized water. Oligo (dT) (1 μl), RNA (3 μl), dNTP (1 μl), and sterilized deionized water were mixed in nuclease-free an EP tube to 12 μl. Then the tube was heated for 5 min at 65°C followed by rapid cooling in ice water. After 4 μl of 5×Buffer, 2 μl of 01 mol/L DTT, and 1 μl of RNase were added, the tube was incubated at 37°C for 2 min. After 1 μ of M-MLV reverse transcriptase was blended at room temperature, the tube was incubated at 37°C for 50 min and heated at 70°C for 15 min. The primers used for RT-PCR were: forward: 5'-AGGAAGTGTCCAACTCTCTGCT-3'; reverse: 5'-TCTTGTCGTTATCCACCGTG-3'. The fragment after PCR amplification was 225 bp. The human β-actin was treated as internal control with primers: forward: 5'-GTCCACCGCAAATGCTTCTA-3'; reverse: 5'-TGCTGTCACCTYCACCGTTC-3'. The fragment for β-actin after PCR amplification was 190 bp. The amplification was conducted with PE9600 PCR amplifier (ABI, USA). The PCR reaction was conducted with a total volume of 25 μl with 2.0 μl of 5 mmol/L primers, 12.5 μl of SYBR Green mix, 8.0 μl of ddH_2O, 2.5 μl of 10-times diluted cDNA. The condition for PCR amplification: pre-denaturing at 95°C for 1 min, total 40 cycles of denaturing at 95°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 45 s, followed by extension for 5 min. PCR amplifier automatically analyzed the Ct value in each specimen. The amplification rate=$2^{-\Delta\Delta Ct}$ and $\Delta\Delta Ct=\Delta CT_{experimental}-\Delta CT_{control}$.

Statistical analysis

Data analysis was conducted with SPSS17.0 software (SPSS17.0; SPSS Inc., Chicago, IL). Data are presented with mean±standard deviation (mean ±SD) and the Shapiro-Wilk test confirmed normal distribution of data. The differences between the 2 groups were tested with the independent-samples *t* test. *P* value less than 0.05 was considered as statistically significant.

Results

SMP-30 mRNA expression in LECs

As demonstrated in Figure 1, using unclear cataract as a reference, the SMP-30 mRNA amplification rate was 1.00 in the nuclear cataract group and 0.49 in the cortical cataract group, indicating that SMP-30 mRNA expression levels in the cortical cataract group were significantly lower compared with the nuclear cataract group ($P<0.05$).

SMP-30 and SA-β-gal protein expressions in LECs

As shown in Figure 2A, the immunohistochemistry stained SMP-30 was brown yellow, mainly in cytoplasm of LECs while the cytoblast of the LECs presented with Mazarin after stained with

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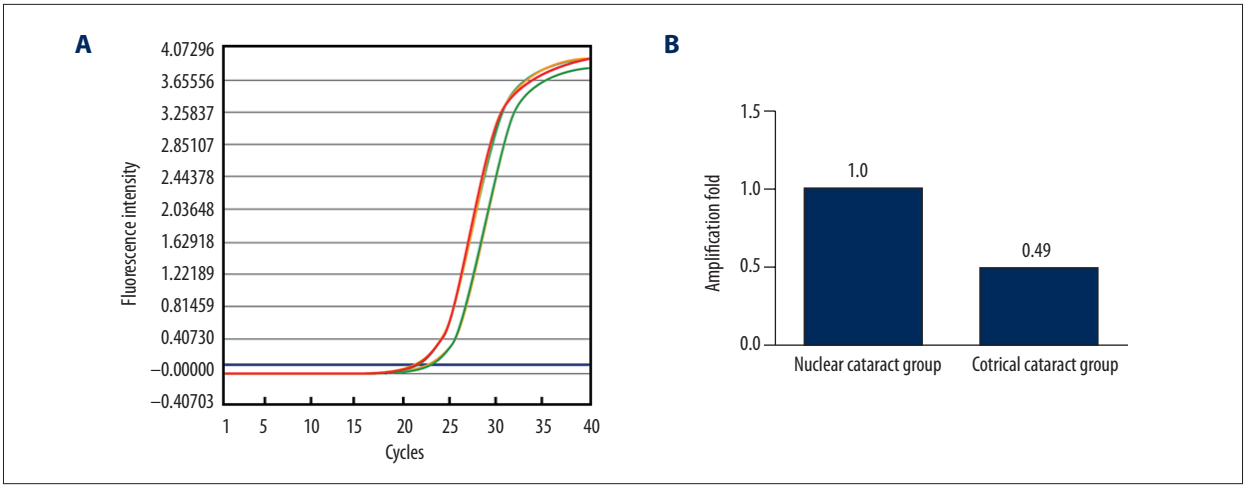

Figure 1. SMP30 mRNA expression by real-time PCR (**A**, real-time-PCR amplification curve; **B**, amplification rate in unclear cataract group was significantly lower than in the cortical cataract group; SMP-30 – senescence marker protein-30; PCR – polymerase chain reaction).

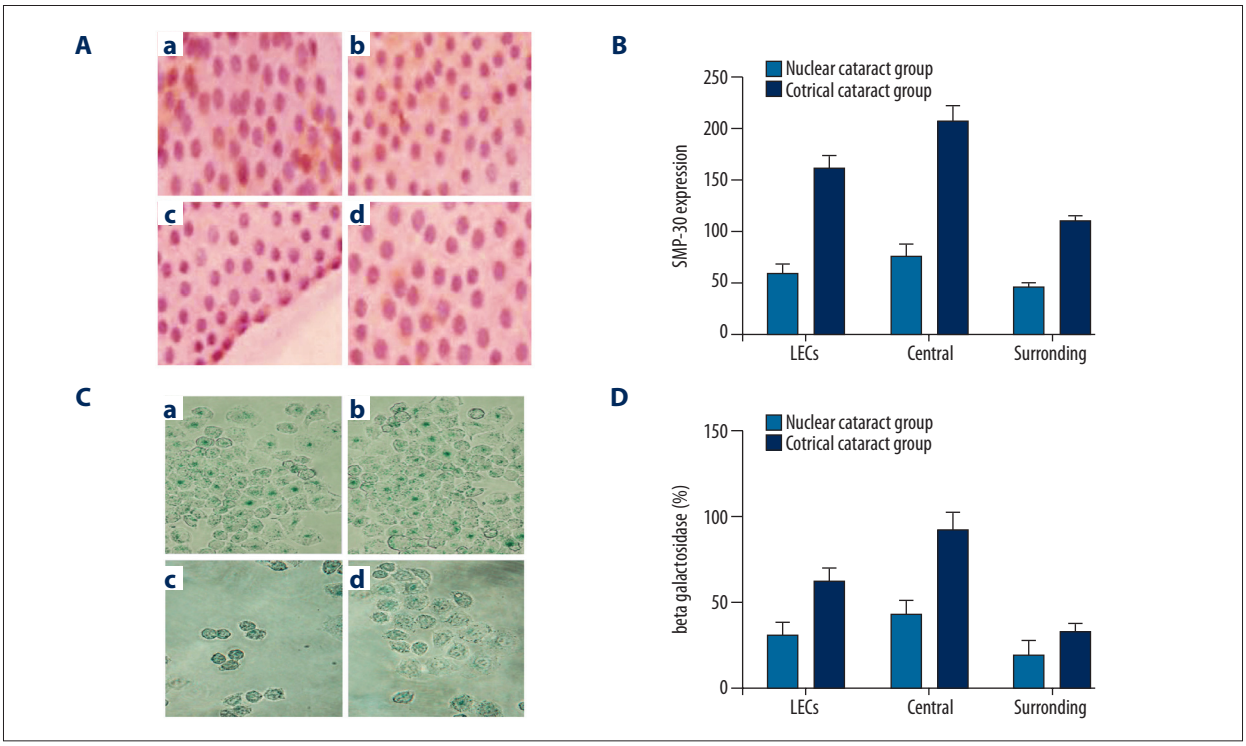

Figure 2. SMP30 and SA-β-gal expressions in lens epithelial cells (**A**) (**a**, SMP-30 expression in the surrounding part of the anterior lens capsules in nuclear cataract patients (DAB ×200); **b**, SMP-30 expression in the central part of the anterior lens capsules in nuclear cataract patients (DAB ×200); **c**, SMP-30 expression in the surrounding part of the anterior lens capsules among cortical cataract patients (DAB × 200); **d**, SMP-30 expression in the central part of the anterior lens capsules among cortical cataract patients (DAB × 200); (**B**) Histogram for SMP-30 protein expression; (**C**) Protein expression of SA-β-gal (**a**, SA-β-gal expression in the surrounding part of the anterior lens capsules in nuclear cataract patients (DAB ×200); **b**, SA-β-gal expression in the central part of the anterior lens capsules in nuclear cataract patients (DAB ×200); **c**, SA-β-gal expression in the surrounding part of the anterior lens capsules among cortical cataract patients (DAB ×200); **d**, SA-β-gal expression in the central part of the anterior lens capsules among cortical cataract patients (DAB ×200)); (**D**) histogram for SA-β-gal positive rate; SMP-30 – senescence marker protein-30; SA-β-gal – senescence-associated β-galactosidase; DAB – diaminobenzidine).

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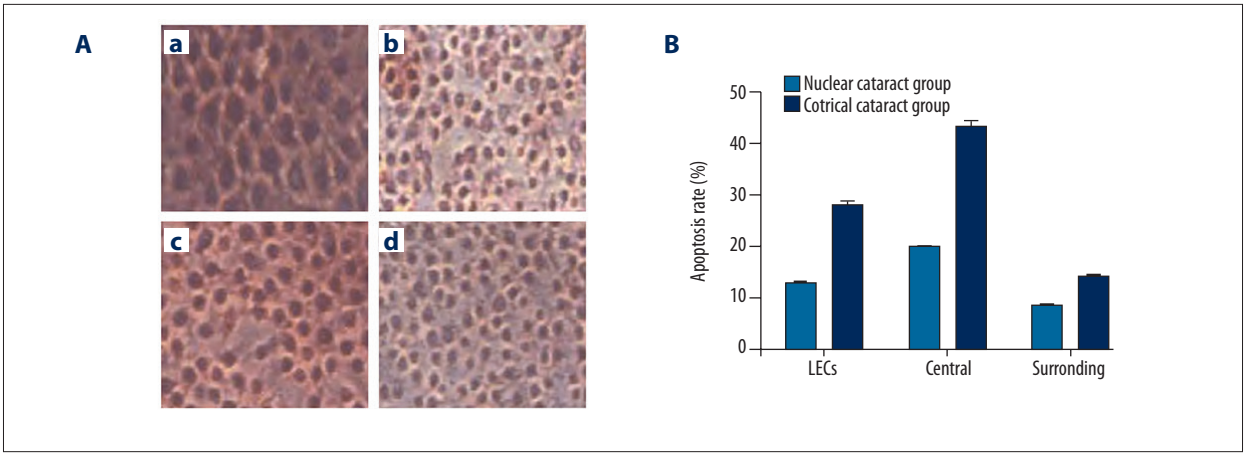

Figure 3. Comparisons of apoptotic lens epithelial cells between the unclear cataract and cortical cataract (**A**) (**a**, TUNEL-negative apoptotic lens epithelial cells (DAB ×200); **b**, TUNEL presented lens epithelial cells apoptosis within the surrounding area of the anterior lens capsule in cortical cataract (yellow-stained nuclei) (DAB ×200); **c**, lens epithelial cells apoptosis within the central area of the anterior lens capsule in cortical cataract (DAB ×200); **d**, lens epithelial cells apoptosis within the central area of the anterior lens capsule in nuclear cataract (DAB ×200); (**B**) Histogram for apoptotic lens epithelial cells in unclear cataract and cortical cataract; DAB – diaminobenzidine).

SA-β-gal (Figure 2C). Immunohistochemical results showed SMP-30 expression level in LECs of the cortical cataract group was lower than that in the nuclear cataract group (59.78±8.52 *vs.* 161.05±14.26, t=52.45, *P*<0.001), while SA-β-gal expression in LECs in the cortical cataract group (62.18±8.26 *vs.* 31.85±7.02, t=23.89, *P*<0.001), but not the nuclear cataract group, was stronger (Table 1) (Figure 2B, 2D). Table 2 shows that SMP-30 in the central part of the anterior lens capsules was expressed at a lower level than in the surrounding part of the anterior lens capsules in both groups (nuclear cataract group: t=22.84, *P*<0.001; cortical cataract group: t=52.71, *P*<0.001). Compared with the cortical cataract group, in LECs, SMP-30 expression both in the central and surrounding part of the anterior lens capsules of the nuclear cataract group was obviously higher (both *P*<0.05). The expression of SA-β-gal in the central part of the anterior lens capsules was evidently higher than in the surrounding part of both groups (nuclear cataract group: t=18.55, *P*<0.001; cortical cataract group: t=45.02, *P*<0.001). The expression of SA-β-gal in the cortical cataract group was obviously higher in LECs both in the central and surrounding part of the anterior lens capsules, compared with the nuclear cataract group (both *P*<0.05).

Apoptosis in LECs

In the TUNEL assay for apoptosis analysis, TUNEL-positive nuclei in apoptotic LECs (brown-yellow) were observed in specimens from both nuclear cataract and cortical cataract patients (Figure 3). The apoptosis rate in the nuclear cataract group was significantly lower than in the cortical cataract group (13.68±0.19 *vs.* 28.15±0.79, t=124.4, *P*<0.001) (Table 2). In the anterior lens capsule with an increased apoptosis rate, there was an obvious difference between apoptosis of LECs in the central part and the surrounding area (nuclear cataract group: t=183.8, *P*<0.001; cortical cataract group: t=249.9, *P*<0.001). Within the central and surrounding areas of the anterior lens capsule, specimens of cortical cataract patients exhibited a significantly higher LEC apoptosis rate in contrast to specimens of nuclear cataract patients (both *P*<0.05)

Discussion

Cataract is a major worldwide public health issue and a main cause of blindness, especially among elderly people. In this study, we examined and compared SMP-30 and SA-β-gal expression levels and LEC apoptosis rate among patients with cortical cataract and nuclear cataract. The results show that SMP-30 and SA-β-gal expression levels might be associated with LEC apoptosis, which plays a more important role in the development and progression of both cortical cataract and nuclear cataract.

During Ca^{2+}-pumping activity in the plasma membrane of HepG2 and LLCPK1 cells, SMP-30 regulates Ca^{2+} kinetics through modulating the activity Ca^{2+}-binding proteins, showing that SMP30 can rescue cells from apoptotic death induced by the high intracellular Ca^{2+} level [32]. Previous studies have suggested that SMP30 can directly bind to Ca^{2+} pump, stimulate its activity, and play a multifunctional role in cell regulation, such as intracellular Ca^{2+} homeostasis maintenance, signal transduction suppression, protein synthesis, nuclear function, cell proliferation, and apoptosis in various types of cells and tissues [33,34]. As an important intracellular Ca^{2+} regulator, the changes in SMP-30

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Table 1. Comparison of apoptosis and SMP-30 and SA-β-gal expression between nuclear cataract and cortical cataract ($\bar{\chi}$±SD).

Group	n	Apoptosis rate (%)	SMP-30 expression (gray value)	SA-β-gal (%)
Nuclear cataract group	69	13.68±0.19	59.78±8.52	31.85±7.02
Cortical cataract group	76	28.15±0.79	161.05±14.26	62.18±8.26
t	–	154.8	52.45	23.89
P	–	<0.001	<0.001	<0.001

SD – standard deviation; SMP-30 – senescence marker protein-30; SA-beta-gal – senescence-associated beta-galactosidase; an independent sample *t* test was used for statistical comparisons.

Table 2. Comparison of apoptosis, SMP-30 and SA-β-gal expression in different areas of the anterior lens capsule ($\bar{\chi}$±SD).

		Central	Surrounding	t	*P*
Apoptosis rate (%)	Nuclear cataract	20.16±0.23	8.78±0.46	183.8	<0.001
	Cortical cataract	43.24±0.88*	14.09±0.51*	249.9	<0.001
SMP-30 expression(gray value)	Nuclear cataract	77.21±10.65	46.18±3.74	22.84	<0.001
	Cortical cataract	207.41±15.23*	109.54±5.48*	52.71	<0.001
SA-β-gal (%)	Nuclear cataract	44.37±6.89	19.32±8.85	18.55	<0.001
	Cortical cataract	91.79±10.21*	32.52±5.24*	45.02	<0.001

SD – standard deviation; SMP-30 – senescence marker protein-30; SA-beta-gal – senescence-associated beta-galactosidase; an independent sample *t* test was used for statistical comparisons; * compared with nuclear cataract, $P<0.05$.

can be functionally critical to $Ca2^+$ homeostasis during aging. Therefore, during the aging process, the decrease of SMP-30 levels may expose cells to abnormal $Ca2^+$ fluctuations, causing cell death. With respect to a cytosolic role, $Ca2^+$-SMP-30 binding facilitates $Ca2^+$ transportation to the extracellular milieu, resulting in a lowered intracellular $Ca2^+$ concentration, while the inefficient $Ca2^+$ regulation making the aged body become more vulnerable to cellular dysfunction, thereby contributing to age-related functional deficits [35]. Inflammation-induced LEC damage, UV light, and hyperglycemia are thought to contribute to perturbation of lens homeostasis [9]. The homeostasis and transparency of the lens was maintained by crystallins, a group of soluble proteins produced by the lens fiber cells. Accumulated LEC damage may lead to death by apoptosis, which was considered an early event of cataract genesis, thus the LEC apoptosis may be a potential cause of cataract formation [36]. Consistent with our results, Kim et al. reported that the LEC apoptosis levels increase in cataract patients with DM and with or without DR [37]. Evidence suggested that SMP-30 is related to hepatocyte proliferation and tumorigenesis and is possibly related to colon cancer tumorigenesis because it can be observed in mucosa of colonic polyps [38,39]. Moreover, a study also demonstrated that the expression of SMP-30 in patients with cataract was significant elevated compared with healthy controls; therefore, an increased expression level of SMP-30 may be indicative for LEC damage [40].

Our results demonstrate that the positive rate of SA-β-gal in cortical cataract patients was elevated compared with that in nuclear cataract patients, while the apoptosis rate in cortical cataract patients was also increased compared to nuclear cataract patients, suggesting that the positive rate of SA-β-gal may be positively associated with the LEC apoptosis in cataract patients. In cultured cells, SA-β-gal activities have been observed in senescent human fibroblasts, skin, liver, muscle, and endothelial cells [41]; however, our study found SA-β-gal activity in the LECs. In agreement with our results, a previous study also showed that senescence proceeds with either an increased percentage of SA-β-gal-positive cells or increased enzyme activity in human peritoneal mesothelial cells [42]. Senescent cells, including SA-β-gal, may be conducive to many stimuli, including telomere shortening, DNA damage, and oxidative stress, among which only 2 pathways were involved – the p53 pathway and the pRB pathway [21]. Usually, the tumor suppressor protein p53 is constitutively targeted at proteasome-mediated degradation. However, once it encounters mitogenic stress or DNA damage, functional p53 is able to activate the cyclin-dependent kinase inhibitor p21, which stops the cell cycle and induces cell apoptosis [43]. It is widely agreed that critically shortened leukocyte telomeres provoke cellular senescence and apoptosis, impairing the function and viability of a cell, which can also be considered as a biomarker for many age-related diseases [44,45]. Moreover, the association between telomere

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lengths and lens transparency has also been revealed [46]. Therefore, based on the above information, it is reasonable to look for an association between the positive rate of SA-β-gal and LEC apoptosis in cataract patients.

Our results also revealed that nuclear cataract patients have higher expression levels of SMP-30 and SMP-30 mRNA compared with cortical cataract patients, indicating the relationship between SMP-30 expression level and ARC. There are 3 major types in ARC, including cortical cataract, nuclear cataract, and posterior subcapsular [47]. Compared with the other 2 cataract types, cortical cataract is more likely to be associated with secondary angle-closure glaucoma that is caused by lens expansion and could cause critical damage to the entire eye; however, nuclear cataract generally develops slowly and usually induces a myopic shift [48]. Song et al. reported a weaker association between cortical cataract and lower survival rate compared with nuclear cataract, which is partly in agreement with our results [49]. The TUNEL and immunohistochemical staining revealed that the LECs apoptosis in the central part of the anterior lens capsules was more severe than in the surrounding part. Based on its definition and features, evidence suggested that selective removal of senescent cells or delaying their accumulation may be very effective in preventing its effects in various tissues [50].

Conclusions

This study provides convincing evidence that LECs apoptosis may be associated with SMP-30 expression levels as well as SA-b-Gal-positive rate in both cortical and nuclear cataract patients, thereby offering a new direction for future cataract treatment by regulating SMP-30 and SA-b-Gal expressions in LECs. Our results may be limited by the small sample size, so well-designed studies with large sample sizes are needed to further validate and confirm our findings.

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Competing interests

The authors declare that there are no conflicts of interest.

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