• Basic Research •

Association of IGF1R polymorphisms (rs1546713) with susceptibility to age-related cataract in a Han Chinese population

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Abstract

● **AIM:** To explore the susceptible association between the insulin-like growth factor-1 receptor (IGF1R) single nucleotide polymorphism (SNP) and age-related cataract (ARC), and investigate the underlying mechanisms in human lens epithelium (HLE) cells.

● **METHODS:** Totally 1190 unrelated participants, comprising 690 ARC patients and 500 healthy individuals in Han Chinese population were recruited and genotyped for target SNP. The χ^2-test was used to detect genotypic distribution between the patient and control groups and the logistic regression was performed to adjust the age and gender. Meanwhile, different biological experimental methods, such as cell counting kit 8 (CCK-8) assay, flow cytometry, quantitative real time polymerase chain reaction (Q-PCR) and Western blot, were used to detect cell viability, cell cycle progression and apoptosis in HLE cells or IGF1R knockdown HLE cells.

● **RESULTS:** The rs1546713 in IGF1R gene was identified (P=0.046, OR: 1.606, 95%CI: 1.245-2.071), which shown a significant relevance with ARC risk under the dominant model. The results demonstrated that IGF1R knockdown inhibited cell proliferation by inducing cell cycle arrested at S phase and promoting apoptosis. Mechanistically, the cell cycle blocked at S phase was linked with the alterations of cyclin A, cyclin B, cyclin E and P21. The pro-apoptosis function of IGF1R may related with stimulating the activation of Caspase-3 and altering the expression levels of apoptotic proteins, including Bcl-2, Bax and Caspase-3.

● **CONCLUSION:** This study first report that IGF1R polymorphisms may affect susceptibility to ARCs in Han Chinese population and provide new clues to understand the pathogenic mechanism of ARCs. Notably, IGF1R is likely a potential target for ARC prevention and treatment.

● **KEYWORDS:** insulin-like growth factor-1 receptor; age-related cataract; single nucleotide polymorphism
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INTRODUCTION

Cataracts are the leading cause of blindness[1-2], resulting in more than 50% of the vision loss worldwide, including 33.4% of sight-disabled people and 18.4% of people with varying degrees of visual impairments[3-4]. More than half of elderly people over 65 years old suffer from age-related cataracts (ARCs)—the most common cataract type[5]. Recently, genetic factor, especially single nucleotide polymorphisms (SNPs) becomes a popular topic, which plays a crucial role in the development of ARCs[6-7]. Consequently, many genes and SNPs related to ARCs have been identified[8]. Although genetic variation has become the focus of research, the definite pathogeny of ARCs is not completely clear.

The insulin-like growth factor-1 receptor (IGF1R) gene, a member of the tyrosine kinase receptor superfamily[9], is located at 15q26 (MIM:147370). This gene encodes the IGF1R protein, which is composed of two extracellular alpha subunits and two intracellular beta subunits[10]. It regulates numerous downstream signals and is usually involved in tumorigenesis, survival and metastasis by regulating the cell growth and apoptosis of tumour cells[11-13], but the effects of IGF1R on cataracts have not been investigated.

In this study, we first found an association between IGF1R SNP and ARCs in ARC patients and explored the impact of

IGF1R in human lens epithelium (HLE) cells. These data provide new information for understanding the pathogenic mechanism of ARCs, and notably, IGF1R is likely a potential therapeutic target for ARCs[4].

SUBJECTS AND METHODS

Ethical Approval This study strictly complied with the Declaration of Helsinki and was approved by the Ethics Committee of the Second Affiliated Hospital, Zhejiang University School of Medicine. Each participant received a full explanation of the purpose and procedures of the research and then signed an informed consent form.

Study Participants Totally 1190 participants, 690 ARC patients and 500 healthy controls, were recruited in this study. All of them were unrelated Han Chinese and recruited from their visit to the Eye Center, the Second Affiliated Hospital, Zhejiang University School of Medicine.

All subjects were undergone a full ophthalmologic examination firstly and diagnosed with ARC by the lens opacity classification system II (Locs II)[14]. The ARC patients were divided into four subgroups: cortical cataract (C), posterior subcapsular cataract (PSC), nuclear cataract (N) and mixed cataract (M), based on the range of the lens opacity. Exclusive criteria: patients with diabetes, glaucoma, trauma or other reasons resulting in secondary cataracts. The control subjects were healthy individuals whose lenses were transparent and without other diseases.

Haplotype The linkage disequilibrium blocks and the haplotype frequencies were tested *via* the Haploview software. The haplotype around the rs1546713 with D′ values >0.9.

Statistical Analysis The continuous variables of the subject characteristics were analysed by independent-sample *t*-test to detect the correlations between groups, and the discontinuous variables were analysed by χ^2 test. The χ^2 test was performed to compare the genotypic distributions between the case and control groups and to estimate odds ratios (ORs) and 95% confidence intervals (CIs) using version 11.0 SPSS software (USA) and also used to evaluate the Hardy-Weinberg equilibrium (HWE) of SNP using PLINK (v1.07). A logistic regression analysis was executed to adjust for age and sex. To cut down the rate of type I errors, a Bonferroni correction was conducted. In addition, the susceptible relevance between ARCs and SNPs was detected under diverse genetic models: additive, recessive and dominant. The *P* value <0.05 was considered statistically significant.

Cell Culture and Transfection The HLE cell line was obtained from the RIKEN Cell Bank (SRA01-04, Tsukuba, Japan) and were maintained in Dulbecco's modified Eagle's medium (DMEM, Corning, NY, USA) with 10% foetal bovine serum (FBS, Biological Industries, Israel) and a 1% penicillin-streptomycin solution (Gibco, Waltham, MA, USA) in a humidified incubator at 37°C. The cells were cultured in six-well plates and added a mixture of 7.5 μL Lipofectamine TM 3000 reagent (Invitrogen, Carlsbad, CA, USA) and 10 μL IGF1R siRNA (100 nmol/L) or NC siRNA until the cell density reached to 30%.

Table 1 Sequences of forward and reverse primers used in Q-PCR

RNA species	Primer pairs
IGF1R	Forward: 5'-GAGTGCTGTATGCCTCTGTGAAC-3′
	Reverse: 5'-CCTTGGCAACTCCTTCATAGAC-3′
Bcl-2	Forward: 5'-GCCACTTACCTGAATGACCACC-3′
	Reverse: 5'-AACCAGCGGTTGAAGCGTTCCT-3′
Bax	Forward: 5'-AGACACCTGAGCTGACCTTGGAG-3′
	Reverse: 5'-GTTGAAGTTGCCATCAGCAAACA-3′
CyclinA	Forward: 5'-TAGCTGCTCCAACAGTAAATCAG-3′
	Reverse: 5'-AGGTATGGGTCAGCATCTATCAA-3′
CyclinB	Forward: 5'-TGCAGCAGGAGCTTTTTGCT-3′
	Reverse: 5'-CCAGGTGCTGCATAACTGGAA-3′
CyclinE	Forward: 5'-ATCAGCACTTTCTTGAGCAACA-3′
	Reverse: 5'-TTGTGCCAAGTAAAAGGTCTCC-3′
P21	Forward: 5'-CCTGTCACTGTCTTGTACCCT-3′
	Reverse: 5'-GCGTTTGGAGTGGTAGAAATCT-3′

Q-PCR: Quantitative real time polymerase chain reaction.

Cell Proliferation Assay The alteration of cell growth rate was assessed by cell counting kit 8 (CCK-8) assay (Dojindo, Kyushu, Japan). The HLE cells were allowed to adhere to a 96-well plate at a concentration of 1×10^4 cells/mL overnight. The IGF1R siRNA groups and NC groups were transfected with IGF1R siRNA and NC for 24, 48, and 72h, respectively. Then CCK-8 reagent was added for 2h at 37°C. Finally, the cell proliferation was calculated based on the optical density (OD) value, which was tested by microplate reader at 450 nm.

Apoptosis Assay The cells were collected and resuspend softly in 1×binding buffer at a density of 1×10^6 cells/mL. Next, using Annexin V-FITC and PI (BD Pharmingen, San Diego, USA) stained the cells for 15min in the dark at room temperature. Subsequently, the apoptotic cells were tested *via* a CytoFLEX LX cytometer.

Cell Cycle Analysis The HLE cells were collected and suspended in 1 mL DNA staining solution and 5 μL permeabilization solution (CCS012, Multi Sciences, Hangzhou, China) and then incubated in the darkroom for 30min. Finally, the samples were analysed by a CytoFLEX LX cytometer.

Quantitative Real-time Polymerase Chain Reaction Analysis Using 1 mL of Trizol reagent to extracte the total RNA, and transcribing 500 ng of RNA into cDNA. Quantitative real time polymerase chain reaction (Q-PCR) was conducted with a ChamQ SYBR qPCR Master Mix kit (Q311-02, Vazyme, Nanjing, China) and 7500 Fast Real-time PCR System. The sequence of primers is summarizing in Table 1. The relative RNA levels were analysed using the $2^{-\Delta\Delta Ct}$ quantification method after normalization to β-actin.

Table 2 The characteristics of the participants included in this study

Group	*n*	Gender		Age (y)	
		Male (%)	Female (%)	Mean±SD	Range
Control	500	58.80	41.20	64.306±7.586	49-92
ARC	690	47.83	52.17	66.323±9.781	43-91
N	126	38.10	61.90	68.259±9.856	45-87
C	131	34.35	65.65	67.75±8.383	43-88
PSC	73	45.21	54.79	66.484±10.274	45-90
M	360	56.11	43.89	65.170±9.936	38-91

ARC: Age-related cataract; C: Cortical cataract; N: Nuclear cataract; PSC: Posterior subcapsular cataract; M: Multiple cataract.

Table 3 The bioinformatics characteristics of the target SNP

SNP	Minor allele	Call rate	MAF	Test for HWE (*P*)	Control (MAF)	ARC (MAF)
rs1546713	A	0.9974	0.367	0.516	0.365	0.369

Western Blot Assay The cells were collected and lysed for 30min. The protein was extracted and mixed with 1× SDS sample buffer . Equal quantity of samples were electrophoresed by a 12% SDS-PAGE gel and then transferred onto 0.22-μm PVDF membranes. Used 5% (w/v) non-fat milk in TBST as a blocking agent for two hours and then incubated the membranes with primary antibodies (9665T, anti-Caspase-3, 1:1000; 2872T, anti-Bcl-2, 1:1000; 5023T, anti-Bax, 1:1000 from CST; ab52866, anti-α-Tubulin, 1:1000 from Abcam) at 4℃ overnight. Then, with the appropriate secondary anti-bodies for one hour. The blots were detected by ECL chemiluminescence detection kit (Vazyme, Nanjing, China).

Caspase-3 Activity Assay The HLE cells and IGF1R knockdown HLE cells were lysed and extracted the supernatant. Then, Bradford Protein Assay was used to test the concentrations of sample. The proteins and Ac-DEVD-pNA (2 mmol/L, Beyotime Biotechnology) were mixed at a certain proportion and then incubated for 12h at 37℃. The OD value was detected by the absorbance at 405 nm. Finally, the result was calculated as the OD value/μg protein.

RESULTS

Characteristics of the Participants This study comprised 690 ARC patients and 500 control subjects. The age or sex between the two groups had no significant difference (P>0.05; Table 2).

Bioinformatics Characteristics of Target SNP The target SNP in IGF1R were genotyped (Table 3). The target SNP in this research were consistent with HWE.

Relevance Between the Target SNP and Risk of ARCs The IGF1R target SNP (rs1546713, pa=0.046, OR 1.606, 95%CI 1.245-2.071) showed a notable correlation with general type ARC susceptibility under the dominant model (Table 4).

The Haplotype Around rs1546713 The Haplotype around rs1546713 were inferred by Haploview software to test the linkage disequilibrium (LD) block structure. As shown in Table 5, the LD block located in IGF1R gene.

Table 4 The association between target SNP and ARC risk

Cataract type	Logistic regression	
	P/pa	OR (95%CI)
rs1546713		
Dominant		
General	2.69×10^{-4}/0.046	1.606 (1.245, 2.071)
C	-	-
N	-	-
PSC	-	-
M	-	-
Recessive		
General	-	-
C	-	-
N	-	-
PSC	-	-
M	-	-
Additive		
General	-	-
C	-	-
N	-	-
PSC	-	-
M	-	-

Table 5 The Haplotype around rs1546713 in a Han Chinese population

Haplotype	Frequency
CCTAT A CATGAGTAAGAACTCGGTCGCCCAAC	0.368
TTCCT G TGCATACGGAGGTCTTCCTGCTCAGC	0.281
TTCAG G TATATACGGAGGTCTGCCTATTTGGT	0.214
TTCCT G TGTATACGGAGGTCTTCCTGCTCAGC	0.067

Regulatory Effects of IGF1R Knockdown on Cell Proliferation and the Cell Cycle in HLE Cells IGF1R knockdown HLE cells were constructed by siRNA (transfected for 72h), and

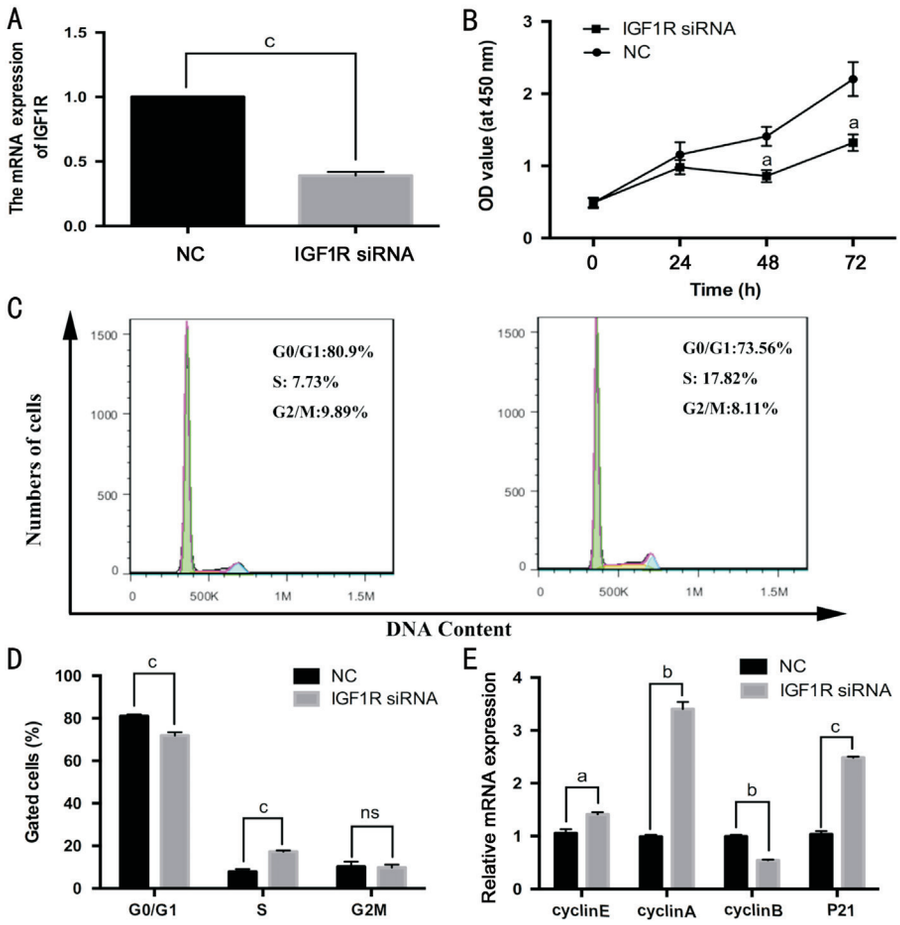

Figure 1 IGF1R-knockdown inhibits the cell proliferation and induced cell cycle arrested in HLE cells A: The silence efficiency of IGF1R siRNA; B: The alteration of cell proliferation after treatment for 24, 48, 72h, and a significant difference was observed after 48h; C: The cell cycle analysis in IGF1R knockdown HLE cells; D: Bar diagram of different cell phase in HLE cells; E: The mRNA expression of S-phase related moleculars. All data presented the means±SD from three independent experiments. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ indicted a significant difference, compared with the NC groups.

the silencing efficiency was confirmed *via* Q-PCR, with 63% decrease in mRNA expression level of IGF1R ($P<0.001$, Figure 1A). Next, to explore the effects of IGF1R silence on cell proliferation in HLE cells, the CCK-8 assay was used. As shown in Figure 1B, cell growth was suppressed by IGF1R knockdown compared to NC groups ($P<0.05$). In addition, to determine the mechanism of the anti-proliferation function of IGF1R knockdown, we detected the influence of IGF1R siRNA on cell cycle progression *via* flow cytometry analysis. As shown in Figure 1C, a lessened proportion of the G0/G1-phase cells were observed from 81.05%±0.68% to 71.85%±1.24%, and the proportion of G2/M-phase cells was slightly declined from 10.24%±1.85% to 9.78%±1.18% for the NC groups compared to the IGF1R groups. However, a remarkable accumulation of HLE cells in the S phase was noticed from 7.87%±1.04% (NC groups) to 17.28%±0.39% (IGF1R siRNA groups; $P<0.05$; Figure 1D), which indicated that IGF1R knockdown blocked the cell cycle at the S phase. Moreover, to further confirm the above results, we detected the expression of S phase-related molecules using Q-PCR. After IGF1R knockdown, the cyclin A, cyclin E and P21 mRNA expression level were risen, and the cyclin B mRNA expression level was declined ($P<0.05$; Figure 1E). These findings demonstrated that the knockdown of IGF1R inhibited cell proliferation by inducing S-phase arrest and altered S-phase-related molecules.

Effects of IGF1R on apoptosis in HLE cells To determine whether IGF1R is associated with ARCs by affecting the apoptosis process, we conducted an Annexin V-FITC/PI staining assay. In comparison with the control, IGF1R siRNA groups had remarkably increased apoptosis, with 9.13% and 22.24% apoptotic cell rates, respectively ($P<0.05$; Figure 2A-2B). To further verify the authenticity of the experimental data, we separately measured the expression of apoptosis-related mRNAs and proteins through Q-PCR and Western blot assays. As shown in Figure 2C and 2D, the analysis indicated that Caspase-3 and Bax expression levels were distinctly increased in the IGF1R siRNA groups, expect for the decreased expression of the Bcl-2 ($P<0.05$). The Q-PCR results indicated similar results, that the mRNA level of Bax was significantly increased, but the level of Bcl-2

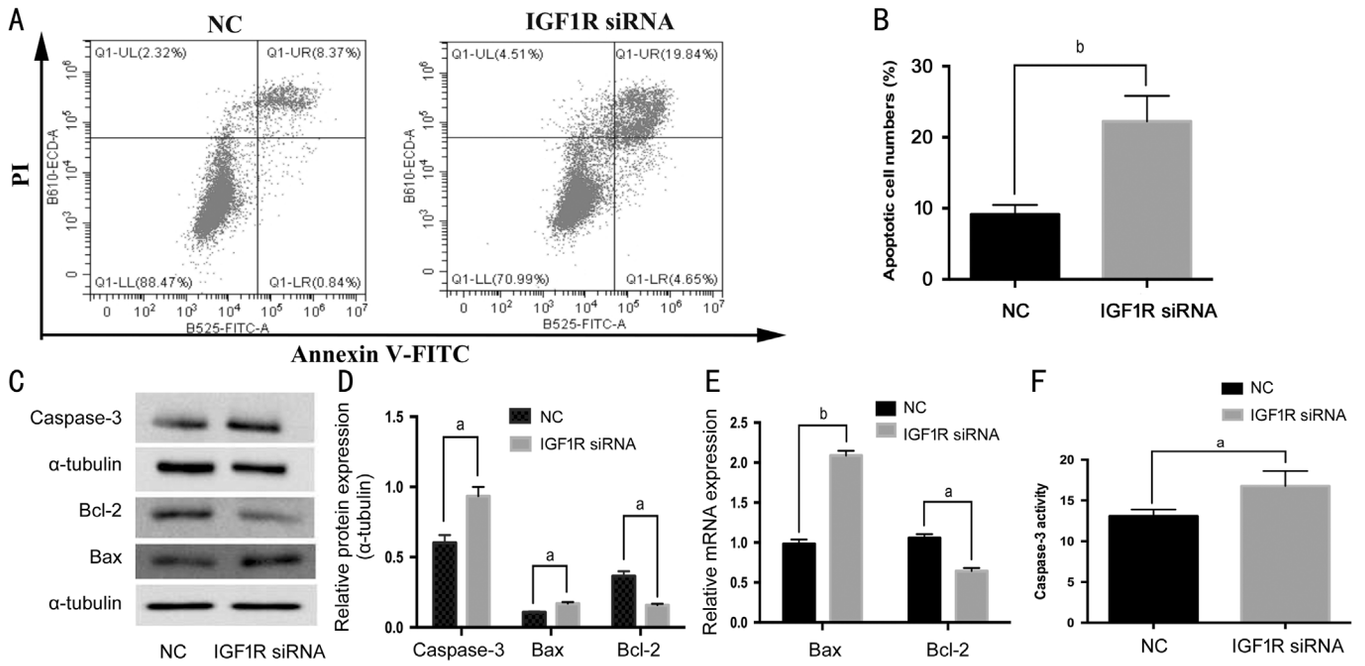

Figure 2 IGF1R-knockdown induced apoptosis and altered the apoptosis-related molecule expression and Caspase-3 activities in HLE cells A: The apoptotic events after transfected with siRNA; B: The apoptotic proportion of IGF1R knockdown HLE cells; C: The apoptosis-related protein expression level; D: The bar diagram of expression levels of apoptosis-related protein; E: The Bax and Bcl-2 mRNA expression levels; F: The Caspase-3 activities. All data are shown as means±SD from three independent experiments. [a]P<0.05, [b]P<0.01 indicated a significant difference, compared with the NC groups.

mRNA was reduced after IGF1R knockdown (P<0.05; Figure 2E). Additionally, as shown in Figure 2F, we also found that Caspase-3 activity increased after treatment with IGF1R siRNA.

The above results revealed that IGF1R knockdown promoted apoptosis in HLE cells. Moreover, the proportion of S phase-cells was notably risen, which indicated that the effects of IGF1R knockdown on inducing cell apoptosis were possibly related to the arrested cell cycle progression in the S phase.

DISCUSSION

IGF1R, a type 2 tyrosine kinase transmembrane receptor[15], consists of two α and two β subunits and has two major ligands, IGF1 and IGF2. Once activated, the α subunits induce tyrosine autophosphorylation of the β subunits and then activate and regulate various downstream signalling pathways within cells[16]. IGF1R is expressed in numerous cell types and is crucial for controlling proliferation during growth progression. Homozygous IGF1R-/- mice die at birth and exhibit a serious growth defect, reaching only 45% of the normal size[17-18]. A study of zebrafish embryos stressing the importance of IGF1R in the eyes suggested that IGF1R is indispensable for lens development[19]. Other studies shown that the IGF1R expressed in lens epithelial and fiber cells of adult chicken, which in charge of phosphorylating substrate when the hormones binding[20]. Besides, the expression of the IGF1R gene in the human lens varies by age, even in the human embryo stage, and is slightly decreased at 65y and older. IGF1R seems to be age-related, in accordance with the decreased proliferative capacity of HLE cells in elderly persons[21]. These findings may provide new insights into the effects of IGF1R in lens.

As mentioned above, IGF1R is essential for lens development, but no previous study has linked IGF1R polymorphisms with the risk of ARCs. In the current study, we first identified rs1546713 in the IGF1R gene, which is significantly relevant to an altered risk of ARCs under the dominant model in the Han Chinese population. Generally, variants in lens proteins result in rapid and direct protein aggregation and usually lead to cataract formation. Conversely, genetic mutations merely increase susceptibility to environmental hazards and tend to cause ARC through the accelerated accumulation of damage to lens proteins[22]. Thus, the identification of genetic variants for ARC risk is becoming increasingly important. Finding potentially risky genes and their signalling pathways will provide therapeutic targets for the prevention of ARC and improve the efficiency of screening populations with incipient lens opacity; these targets could even help to directly identify susceptible individuals through genetic testing to start therapeutic measures as early as possible[23]. Additionally, to explore its mechanisms, we investigated the impact of IGF1R on cell proliferation, cell cycle and apoptosis in HLE cells, which resulted in new information for understanding the pathogeny of ARC.

In the present study, we found that in IGF1R-knockdown HLE cells, the cell growth rate was remarkably decreased. These findings supported that IGF1R promoted cell proliferation in HLE cells, and Kato *et al*[24] also reported the pro-proliferation function of IGF1R in prostate cancer cells. Moreover, cell cycle progression is one of the immediate cause of affecting cell proliferation[25]. Therefore, to explore the mechanism of the anti-proliferation effects induced by IGF1R siRNA, we detected the process of HLE cell cycle. As shown in the data, a higher proportion of HLE cells was obviously arrested at the S phase in the IGF1R siRNA groups compared to that in the NC groups. Although our results differ from some studies that the blockage of IGF1R by IGF1R knockdown or the addition of picropodophyllin (an IGF1R-specific inhibitor) resulted in cell cycle arrest at the G2/M phase. However, Sell *et al*[26] found that IGF1R affected each stage of the cell cycle by testing the distributions between wild-type and IGF1R knockdown mouse embryo fibroblasts. Thus, the different effects of IGF1R on cell cycle progression are likely attributed to different cell types. Furthermore, cell cycle progression is regulated by cyclins[27]. Therefore, we examined S phase-related molecules using Q-PCR. The expression level of cyclin A mRNA, which is essential for S phase initiation and DNA synthesis[28-29], were increased. In addition, the high level of cyclin A upon S phase arrest appears to be associated with DNA damage. Several studies suggested that the cyclin A level decreased when the cell cycle arrested at the S phase, but Preya *et al*[30] noted that α-terthienylmethanol remarkably promoted cyclin A expression when S phase arrest was induced. Similarly, we also found that IGF1R knockdown blocked the cell cycle at the S phase and simultaneously increased cyclin A expression in HLE cells[31]. Cyclin E, responsible for the transition from G0/G1 to S phase, was also increased. Simultaneously, the lower expression of cyclin B mRNA was in response to the fact that HLE cells that could not enter the G2/M phase easily[28,32]. $P21^{WAF1/CIP1}$, a CDK inhibitor (CKI), and $P21^{WAF1/CIP}$-overexpression may induce cell cycle arrest at any phase. A 2.5-fold increase was observed after transfection with IGF1R siRNA. Thus, the above data suggested that IGF1R silencing inhibited cell proliferation by inducing the blockage of the cell cycle at the S phase and affecting S phase-related molecule expression.

The cell cycle and apoptosis are two major mechanisms regulating cell proliferation. Interestingly, when the cell cycle arrests at specific checkpoints, apoptotic events occur[33-34]. It has been reported that some anti-cancer drugs act mainly by blocking the cell cycle at the G0/G1, S or G2/M phase to cause apoptosis. There are no data focusing on how IGF1R is related to apoptosis in HLE cells, but apoptosis is a normal physiological phenomenon and is responsible for cataractogenesis. Several pieces of clinical evidence have revealed that the apoptotic HLE cells of ARC patients are remarkably higher than those of healthy people[35]. It is widely accepted that the accumulation of damage from UV, oxidative stress and toxic agents triggers apoptosis in HLE cells[36]. Due to HLE cell death *via* apoptosis, cell growth will slow, leading to lower cell density and a thinner lens[37]. In addition, the breakdown of the lens epithelial cell barrier, which acts as an umbrella to protect the underlying fibre cells from impairment, will contribute to deficiencies in defence systems against stimuli and interfere with the integrity and transparency of fibre cells, thus resulting in lens opacification. These studies demonstrated that the apoptosis of lens epithelial cells is a common cellular basis for the formation of non-congenital cataracts[37], and there may be a potential therapeutic strategy for ARCs. In the present study, we detected apoptosis *via* an Annexin V-FITC kit in HLE cells. A significantly higher proportion of apoptotic cells was observed after transfection with IGF1R siRNA compared with that in the NC groups. These results accord with the previous data presented that the activation of IGF1R is able to protect glioblastoma cancer cells from apoptosis[11]. To explore its molecular mechanism, we detected apoptosis-related molecules *via* Western blot and Q-PCR assays. Caspase-3, which is a key executor of cell dismantling that is essential for the development of apoptotic bodies[38] and positively correlates with apoptotic levels, was distinctly increased in the IGF1R siRNA group. Bax, one of the most important proapoptotic genes, mediates the mitochondrial outer membrane (MOM) permeabilization, resulting in the release of proapoptotic factors[39-40], is also upregulated. However, a less Bcl-2 expression, which is an anti-apoptotic gene that directly inhibits pro-apoptotic proteins and blocks upstream pro-apoptotic signals[39], was observed in HLE cells after transfection with IGF1R siRNA. Consistent with the Western blot results, the mRNA expression levels of Bax and Bcl-2 were enhanced and suppressed, respectively. Moreover, Caspase-3 activity was also upregulated. These observations suggested that IGF1R knockdown promoted apoptosis by regulating apoptosis-related molecules and altering Caspase-3 activity.

In conclusion, our study first identified the relationship of rs1546713 in IGF1R with ARCs susceptibility in a Han Chinese population. Additionally, to better understand the underlying mechanism of its effects on ARC occurrence, we found that IGF1R knockdown inhibited HLE cell growth through blocked the progression of cell cycle in the S phase and induced the apoptosis in HLE cells, most likely due to the regulation of cell cycle- and apoptosis-related molecules. These results may provide a new basis for IGF1R transmembrane cell signalling in HLE cells for ARC formation. As a result, IGF1R may be a promising target for the treatment of ARCs.

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