Associations of Toll-like receptor 3 gene single nucleotide polymorphisms with cataract

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Abstract. – OBJECTIVE: The aim of this study was to investigate the expression of Toll-like receptor 3 (TLR3) gene in the anterior capsule of cataract patients and to explore the correlations of its polymorphisms with the incidence of cataract.

PATIENTS AND METHODS: A total of 80 cataract patients (Cataract group) and 38 clear lenses (Control group) were selected as research subjects. Western blotting assay was used to detect the protein expression of TLR3 in Cataract group and Control group. Subsequently, single nucleotide polymorphisms (SNPs) rs128912, rs100321 and rs129103 in the promoter region of TLR3 gene were classified by conformation-difference gel electrophoresis. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to test the correlations of different gene polymorphisms with the protein expression of TLR3. Meanwhile, chi-square test was applied to check whether the distribution frequency of TLR3 genotypes conforms to the Hardy-Weinberg genetic equilibrium law. Furthermore, the associations of alleles and gene polymorphisms of TLR3 with the incidence of cataract were analyzed.

RESULTS: Western blotting results found that the protein expression level of TLR3 in the anterior capsule in Cataract group was significantly higher than that in Control group ($p<0.05$). Hardy-Weinberg genetic equilibrium analysis showed that three TLR3 gene polymorphisms conformed to the genetic equilibrium distribution ($p>0.05$). Subsequent results demonstrated that the protein expression level of TLR3 in cataract patients with genotype TT of TLR3 gene polymorphism rs128912 was remarkably higher than that in patients with genotypes AA and AT ($p<0.05$). Moreover, the results of genetic association analysis manifested that only gene polymorphism rs128912 and its alleles were associated with cataract ($p<0.05$). However, gene polymorphisms rs100321 and rs129103 and their alleles were not correlated with the incidence of cataract ($p>0.05$).

CONCLUSIONS: TLR3 was highly expressed in the anterior capsule of patients with cataract. In addition, rs128912 in the promoter region of TLR3 gene was correlated with the incidence of cataract, while rs100321 and rs129103 were not associated with the incidence of cataract.

Introduction

Cataract is one of the major causes of blindness in the world. With the rapid increase of population and aggravation of population aging, the incidence of cataract is increasing year by year[1]. The main clinical manifestations of cataract patients include lens opacity and painless vision loss with the aging of affected patients[2]. At present, phacoemulsification surgery is the primary clinical treatment for cataract. However, some postoperative adverse complications are still inevitable. In addition, the demand for cataract surgery is increasing as well, whose huge cost brings a serious economic burden to the country and society[3].

Cataract is a polygenic disease, and its occurrence and development are jointly regulated by environment and multiple genes[4]. Therefore, further elucidating the genetic mechanism of cataract is of great significance for the development of targeted therapy in the future.

Toll-like receptors (TLRs) are important components of early pathogen recognition and host defense systems in human. TLRs play a key role in protecting cells from damage and mediating tissue homeostasis[5]. As an important member of the TLR family, TLR3 acts as an endogenous receptor for danger-associated molecular patterns and tissue necrosis in the acute phase of inflammatory response[6]. Besides, TLR3 is a crucial receptor for recognizing viral pathogen-associated molecular patterns (PAMPs)[7]. Human TLR3 gene has been confirmed located on chromosome 4q35. Recently, a functional single nucleotide polymorphism (SNP) has been identified in human TLR3 gene Leu412Phe (TLR3 L412F, rs3775291), which affects the activation of intracellular NF-κB

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and IRF3[8]. Meanwhile, TLR3 L412F polymorphism inhibits macular degeneration by reducing TLR3-mediated apoptosis of retinal pigment epithelial cells[9,10]. However, the associations of other polymorphisms in the promoter region of TLR3 gene with the incidence of cataract have not been fully elucidated.

In the present study, polymorphisms rs128912, rs100321 and rs129103 in the promoter region of TLR3 gene were analyzed, and their distribution in cataract patients was detected. All our findings might help to provide certain references for further exploration of the genetic pathogenesis of cataract.

Patients and Methods

Patients

A total of 80 cataract patients treated in our hospital from January 2017 to July 2019 were selected as research subjects, including 41 males and 39 females aged (68.55±5.87) years old. 4 mL of venous blood was drawn, added with sodium citrate for anticoagulation and frozen in a refrigerator at -20°C for use. Meanwhile, clear lenses were collected from 38 patients with an age of (57.13±2.19) years old who died of accidents or suffered from lens dislocation as Control group. The obtained capsules were immediately refrigerated in liquid nitrogen and finally stored in a refrigerator at -80°C. This investigation was approved by the Ethics Committee of of Binzhou Medical University Hospital. Informed consent was obtained from all subjects before the study.

Western Blotting

Fresh frozen capsule tissues of each group of patients were first taken out from the -80°C refrigerator, preliminarily cut into pieces by scissors and fully ground in a grinder. After ultrasonication, the lysis buffer was centrifuged, and the supernatant was aspirated and sub-packaged into Eppendorf (EP) tubes. Protein concentration was determined through the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) and ultraviolet spectrophotometric assay, and all protein samples were maintained at a constant volume and equal concentration. Next, the proteins were sub-packaged and preserved in a refrigerator at -80°C. Subsequently, total proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After incubation with primary antibody against TLR3 at 4°C overnight, the membranes were incubated with goat anti-rabbit secondary antibody in the dark for 1 h. Immunoreactive protein bands were finally scanned and quantified using an Odyssey membrane scanner (Seattle, WA, USA).

Deoxyribonucleic Acid (DNA) Extraction

A total of 4 mL of EDTA-anticoagulated blood was collected to extract genomic DNA according to the instructions of DNA extraction kit (Guge Bio-Technology Co., Ltd., Wuhan, China). Subsequently, 2 μL of extracted DNA was used to measure the quality in 1.5% agarose gel electrophoresis. Meanwhile, its concentration was detected by virtue of an ultraviolet spectrophotometer.

Polymerase Chain Reaction (PCR) Amplification

Primers of TLR3 gene polymorphisms rs128912, rs100321 and rs129103 were designed for amplification (Table I). PCR was performed in a 20 μL system (including 2.0 μL of DNA

Table I. Primer sequences and product sizes in this research.

Polymorphism	Primer sequence (5'-3')	Product (bp)
rs128912	Forward: TCGATCCGATGTAGATGCT Reverse: ACGTGTAGTCGATGTCGTA	188
rs100321	Forward: CAGCTACGCTAGATCGATC Reverse: CAGCATGATAGCTAGCTGCA	105
rs129103	Forward: ACGATCGATCGTAGTGTAGC Reverse: ACGTAGCTGACTACGATCGT	215
TLR3	Forward: ACGATCGATCGTAGTGTAGC Reverse: ACGTAGCTGACTACGATCGT	156
GAPDH	Forward: ATTGCTAGGATCGTTTACCA Reverse: TGTAGTCGTAGTCGTGTAGT	129

Table II. Probe sequences of ligase reaction and product sizes of different TLR3 gene polymorphisms.

Polymorphism	Probe	Probe sequence (5'-3')
Frs128912	rs128912 rs128912-A rs128912-T	P-ACGGTAGTCGTAGTTTTTTTTTTTTTTTTTTTT-FAM TTTTTTTTTTCCCGTAGTCCCCATTTTTTTTTAT TTTTTTTTTTTTTTCAGCTACGACCGTTTTTTTTTAAA
rs100321	rs100321 rs100321-C rs100321-T	P-AGCACACGTGTCAGCTTTTTTTTTTTTTTTTT-FAM TTTTTTTTTTTTTTTTTTTTACACACGTAGCTAGTCG TTTTTTTTTTTTTTTTTTTTTTTCAGCTAGTAGATGCTA
rs129103	rs129103 rs129103-C rs129103-G	P-TAGCTGCTAGCTCCTTTTTTTTTTTTTTTTTTTT-FAM TTTTTTTTTTTTTTTTTTTTTTTTCACACGTAGATCG TTTTTTTTTTTTTTTTTTTTTTTTTCACGTAGAGTGTGA

template, 10.0 μL of 2× MIX, 0.4 μL of forward primers, 0.4 μL of reverse primers and 7.2 μL of ddH$_2$O) under the following amplification conditions: 95°C for 120 s, 94°C for 30 s, 57°C for 90 s and 72°C for 60 s for 35 cycles in total, followed by extension at 72°C for 10 min. Next, the amplification of gene fragments was examined by means of agarose gel electrophoresis.

Ligase Detection Reaction

Upstream and downstream probes applied in the reaction were designed and synthesized by BGI. After modification by 5' terminal phosphorylation, all the upstream probes were prepared into a probe mixture at a concentration of 12.5 pmol/μL. Then, the ligase detection reaction was conducted using a system (3.05 μL) composed of 0.05 μL of ligases, 1 μL of buffer solution, 1 μL of PCR products and 1 μL of probe mixture under the following PCR amplification conditions: 95°C for 120 s, 94°C for 15 s and 50°C for 25 s, for a total of 30 cycles. Next, the concentration was measured using an ultraviolet spectrophotometer. BGI was finally entrusted to sequence the target gene and analyze its segments. All data were analyzed using GeneMapper (Table II).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for all statistical analysis. Enumeration data were presented as frequency and percentage, and measurement data were expressed by mean ± standard deviation. Genotype frequency was calculated and examined *via* Hardy-Weinberg equation. Chi-square test was used for examination and multiple comparisons of enumeration data, and *t*-test and ANOVA were adopted for measurement data. One-way ANOVA test was followed by Post-Hoc Test (Least Significant Difference). $p < 0.05$ was considered statistically significant.

Results

Expression of TLR3 in the Anterior Capsule of Cataract Patients

The results of Western blotting (Figure 1) indicated that the protein expression level of TLR3 was significantly up-regulated in the anterior capsule of cataract patients when compared with healthy anterior capsule ($p < 0.05$). This suggested that TLR3 might have potential correlations with the occurrence and development of cataract.
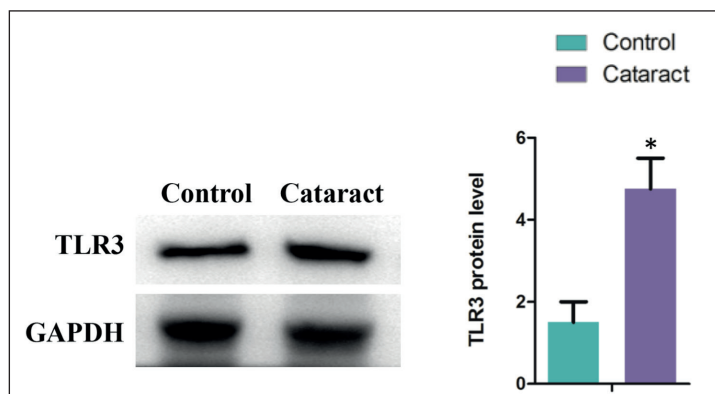

Figure 1. Expression of TLR3 in the anterior capsule of cataract patients. Control: Control group, Cataract: Cataract group, *p<0.05: a statistical difference *vs*. Control group.
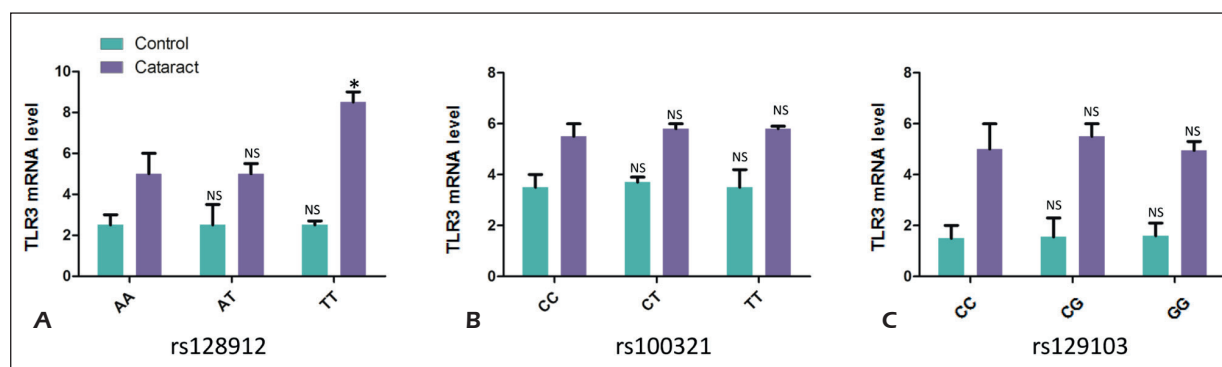
Figure 2. Correlations of TLR3 gene polymorphisms rs128912, rs100321 and rs129103 with mRNA expression of TLR3. Control: Control group, Cataract: Cataract group, **A**, *p<0.05: a statistical difference *vs.* genotype AA within Control group or Cataract group, no significance (NS): no statistical difference. **B**, *p*>0.05: no statistical difference *vs.* genotype CC within Control group or Cataract group, NS: no statistical difference. **C**, *p*>0.05: no statistical difference *vs.* genotype CC within Control group or Cataract group, NS: no statistical difference.

Analysis Results of TLR3 Gene Polymorphisms rs128912, rs100321 and rs129103

After cleavage by restriction enzyme BstU I in both Control group and Cataract group, 2 alleles (A and T) and 3 genotypes (AA, AT and TT) of TLR3 gene polymorphism rs128912, 2 alleles (C and T) and 3 genotypes (CC, CT and TT) of rs100321, and 2 alleles (G and C) and 3 genotypes (GG, GC and CC) of rs129103 were obtained.

Correlations of TLR3 Gene Polymorphisms rs128912, rs100321 and rs129103 with Messenger Ribonucleic Acid (mRNA) Expression of TLR3

The mRNA expression of TLR3 in the peripheral blood of Control group and Cataract group was shown in Figure 2. It was manifested that there was an apparent correlation between TLR3 gene polymorphism rs128912 and the mRNA expression of TLR3 (*p*<0.05). In Cataract group, patients carrying genotype TT exhibited a significantly higher mRNA expression level of TLR3 than those carrying genotypes AA and AT (*p*<0.05). However, there were no significant relations of TLR3 gene polymorphisms rs100321 and rs129103 with the mRNA expression of TLR3 (*p*>0.05).

Hardy-Weinberg Equilibrium Test Results

Hardy-Weinberg equation was applied to examine the linkage disequilibrium test results of different TLR3 gene polymorphisms. As shown in Table III, r^2<0.33 was detected among the polymorphisms in each group. Therefore, it was considered that these polymorphisms were in agreement with the equilibrium test.

Correlations of TLR3 Gene Polymorphisms With Cataract

Based on the genotype frequencies of gene polymorphisms in the two groups of participants (Table IV), polymorphism rs128912 was significantly associated with the onset of cataract (*p*<0.05). However, polymorphisms rs100321 and rs129103 were not notably correlated with the onset of cataract (*p*>0.05).

Correlations of Alleles of TLR3 Gene Polymorphisms With Cataract

Based on the allele frequencies of gene polymorphisms in the two groups of participants (Table V), the alleles of polymorphism rs128912 was evidently associated with the onset of cataract (*p*<0.05). However, there was no remarkable correlation between polymorphisms rs100321 and rs129103 with the onset of cataract (*p*>0.05).

Table III. Results of linkage disequilibrium test of TLR3 gene polymorphisms.

Polymorphism	r^2		
	rs128912	rs100321	rs129103
rs128912	–	0.002	0.051
rs100321	0.002	–	0.301
rs129103	0.051	0.301	–

Table IV. Distribution of different genotypes of TLR3 gene polymorphisms in cataract patients.

Group	rs128912			rs100321			rs129103		
	AA	AT	TT	CC	CT	TT	CC	CG	GG
Cataract (n=80)	7.50%	30.00%	62.50%	22.50%	55.00%	22.50%	23.75%	51.25%	25.00%
Control (n=38)	23.68%	50.0%	26.31%	26.31%	44.74%	28.95%	31.58%	36.84%	31.58%
C^2	3.234			0.459			1.723		
p	0.002			0.326			0.081		

Table V. Distribution of alleles of TLR3 gene polymorphisms in cataract patients.

Group	rs128912		rs100321		rs129103	
	A	T	C	T	C	G
Cataract (n=80)	22.50%	77.50%	50.00%	50.00%	49.38%	50.62%
Control (n=38)	48.68%	51.32%	48.68%	51.32%	50.00%	50.00%
C^2	1.432		0.782		0.644	
p	0.000		0.114		0.412	

Discussion

SNP refers to the mutation of a single base on the genomic DNA sequence, with a mutation rate of at least 1% in the population[11]. This single base difference can be divided into different types, including transversion, insertion and deletion. Multiple studies have shown that there may be at least 15 million SNPs in human populations, with potential base mutations in every 300-600 bp on average. These SNPs may serve as potential genetic markers and key sites that directly affect genetic susceptibility of diseases[12,13]. With the completion of human genome project in recent years, how to exploit the traits of complex polygenic hereditary diseases by means of known SNP information of human genomes has become a hot topic in the field of biology.

Cataract is one of the common causes of blindness in the world. The incidence and prevalence rates of cataract are constantly increasing along with the worsening of population aging. Epidemiological investigations have indicated that cataract has become the leading cause of blindness in China[14]. Meanwhile, the prevalence rate of cataract rises prominently with age, whose incidence rate in women is significantly higher than that in men. According to opacity location and morphology, cataracts can be divided into four subtypes, including cortical opacity, posterior subcapsular opacity, nuclear opacity and mixed opacity[15]. As a typical polygenic disease, cataract is not only related to the age of onset of patients, but also close-ly associated with the change of genetic factors. Moreover, the incidence rate and clinical characteristics of cataract patients from different ethnic groups and under varying genetic backgrounds are different to some extent[16].

The TLR family, a category of conserved pattern recognition receptors, is an endogenous receptor in the body to defend against the infection of external pathogenic microorganisms. It can recognize different PAMPs on the surface of external pathogenic microorganisms. TLRs can mediate signal transduction by binding to specific ligands, thereby promoting the release of pro-inflammatory cytokines by host cells[17]. At present, 13 types of TLR genes (TLR1-13) have been found in mammalian genome research, among which TLR1 to TLR10 exist in human body. Meanwhile, each member of TLRs has a corresponding ligand[18]. TLR3 gene is located on chromosome 4q35, with a length of 3029 bp. It mainly consists of transcriptional products of two different lengths (4.0 kb mRNA and 6.0 kb mRNA). The TLR3 gene contains 5 exons and encodes 504 amino acids in total. Furthermore, TLR3 belongs to type I transmembrane protein, and is composed of extracellular domain, transmembrane domain and intracellular domain[19]. Previous studies have shown that there is a direct correlation between TLR3 gene polymorphisms and encephalitis B in Indians, showing no effect on the mortality rate[20]. Age-related macular degeneration (AMD) is a common degenerative disease in the elderly population, which can lead to distor-

tion of central vision in the early stage and even vision loss in the advanced stage. The occurrence of AMD is closely related to the inflammatory response. TLR3 recognizes viral double-stranded RNAs and induces the apoptosis of infected cells. TLR3 gene polymorphism rs3775291 is able to induce the death of retinal pigment epithelial cells by inhibiting double-stranded RNAs, possessing potential clinical significance[9]. In addition, TLR3 is up-regulated in the process of human choroidal neovascularization, suggesting that TLR3 plays an important role in ophthalmic diseases[21]. In this study, it was found that the protein expression of TLR3 in the anterior capsule of cataract patients increased significantly. Subsequently, the correlations of SNPs rs128912, rs100321 and rs129103 in the promoter region of TLR3 gene with the incidence of cataract were analyzed. The results showed that the TLR3 gene rs128912 polymorphism was remarkably correlated with the mRNA expression of TLR3 gene. Specifically, the mRNA expression level of TLR3 in cataract patients with genotype TT was significantly higher than that of cataract patients with genotypes AA and AT. Besides, only the polymorphism rs128912 and its alleles were associated with the onset of cataract.

Conclusions

In summary, this research revealed for the first time that TLR3 gene polymorphism rs128912 has a potential correlation with the occurrence of cataract. The novelty of this study was that TLR3 gene polymorphism rs128912 can be used as a genetic marker for risk assessment of cataract in elderly patients.

Conflict of Interests

The authors declare that they have no conflict of interest.

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