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Original Article

Association of TLR3 gene polymorphisms with age-related macular degeneration

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Abstract: Purpose: In this study, we selected rs3775291 and rs3775296 polymorphisms of from toll-like receptor 3 (TLR3) gene to investigate their association with the susceptibility of age-related macular degeneration (ARMD or AMD). Haplotypes of the two polymorphisms were also detected in AMD patients. Methods: A hospital-based multiple-center case-control study was designed. 110 AMD cases and 108 healthy controls were enrolled in this study. χ^2 test was applied to count and analyze the genotype and allele frequencies in case and control groups. The relationship between TLR3 polymorphisms and the susceptibility of AMD was presented by odds ratios (ORs) and 95% confidence intervals (CIs). Results: The genotype distributions of TLR3 rs3775291 and rs3775296 polymorphisms in control group were in accordance with Hardy-Weinberg equilibrium (HWE) ($P>0.05$). The distributions of rs3775291 polymorphism AG genotype in case and control groups had statistically significant differences ($P<0.05$), and AG genotype could increase the onset risk of AMD by 1.89 times when compared with GG genotype (OR=1.89, 95% CI=1.09-3.28). In addition, rs3775296 polymorphism TT genotype was related to the occurrence of AMD (OR=4.70, 95% CI=1.26-17.46), and T allele could also increase the risk of AMD (OR=1.64, 95% CI=1.06-2.55). Linkage disequilibrium (LD) and haplotype analysis suggested that rs3775291 and rs3775296 polymorphisms formed 4 haplotypes, and the distribution differences of one of them, namely T-A haplotype, between case and control groups were statistically significant ($P<0.05$). Conclusions: TLR3 polymorphisms can increase the onset risk of AMD.

Keywords: Age-related macular degeneration (AMD), toll-like receptor 3 (*TLR3*) gene, haplotypes

Introduction

Age-related macular degeneration (ARMD or AMD) is a serious irreversible blinding eye disease. It mainly implicates the multi-layer tissues of retinal pigment epithelium (RPE), photoreceptor cell layer and choroid [1]. Epidemiological investigation results showed that AMD usually occurs in people over the age of 50, and the lesions invade both eyes of some patients [2]. AMD remains the leading cause of blindness among the aged populations in America and other developed countries [3, 4]. There are about 50 million AMD patients all over the world and the morbidity is rapidly increasing along with the aging of the population. The older the age, the higher the incidence. About 6%~8% of the people over 75 years old in America have advanced macular degeneration that can lead to severe visual impairment [5, 6]. In China, the morbidity of AMD is approximately 5% in people over the age of 50, 8.8% in people aged 50~64 years, and 12% in people over 75 years old. AMD is gradually becoming one of the main eye diseases that can result in blindness [7].

Although most scientists believe that family history, heredity, smoking, dysimmunity, chronic photopathy, hyperopia, iris color, alimentary deficiency, obesity, chronic diseases and some other factors may have something to do with AMD, the specific pathogenesis of the disease is still cannot be determined at present [8]. There are two kinds of AMD: dry AMD and wet AMD, the latter of which does a greater harm to the eyesight. Various family studies indicated that AMD had a heritability as high as 71%, and it demonstrated that genetic factors played important roles in the occurrence of the disease [9, 10]. With the development of molecular biological technology, related researches on toll-like receptor 3 (*TLR3*) gene become a hot spot. *TLR3* gene widely express in the mammalian body, and has a close relationship with variety of signal transductions in the body [11, 12]. In the last few years, studies on the relationship between Toll-like receptors (*TLR*s) and AMD have been gradually increasing. The latest study conducted among Caucasian populations in Europe and America pointed out that *TLR3* rs3775291 single nucleotide polymorphism (SNP) was associated with the occurrence and features of AMD [13, 14].

This study genotyped *TLR3* rs3775291 and rs3775296 SNPs using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Relationship between the two SNPs and AMD, association between the haplotypes of the two SNPs and AMD were analyzed in this study too. We hoped that it would provide a scientific basis for the early prevention of AMD.

Materials and methods

Clinical information

According to the diagnostic criteria of AMD [15], we collected the clinical information of 110 diagnosed wet AMD patients (48 males and 62 females) who were sporadically hospitalized in the ophthalmology department of Qianxinanzhou People's Hospital. The cases were proved to have choroidal neovascularization (CNV) by fluorescence fundus angiography (FFA) and they were excluded the possibilities of having neovascularization diseases, high myopia and diabetic retinopathy, with a mean age of 65 years. 108 healthy persons in the same hospital volunteered to take part in the study. The healthy controls were 45 males and 63 females with a mean age of 60. Their eye examination results were normal, and they did not suffer from systemic blood diseases like hypertension and diabetes, and had no family history of AMD. The cases and controls were all Han population of China living in the local area for a certain time, and they were not connected with each other by blood. The subjects were fully informed the study contents and they agreed to sign informed consent.

Table 1. Primer sequences of rs3775291 and rs3775296 polymorphisms

Locus	Forward/Reverse	Primer sequence
rs3775291 A/G	Forward	5'-TATTCCAGGCATAAAAAG-3'
	Reverse	5'-TATTGCTTTTTATGCCTG-3'
rs3775296 C/T	Forward	5'-CATTTGAAAGCCATCTGC-3'
	Reverse	5'-ATAGCAGATGGCTTTCAA-3'

Sample collection process

4 ml fasting elbow venous blood of each participant was collected after 12 hours fasting. We injected the blood samples into anti-coagulative tubes and preserved the samples in refrigerator at -20°C until to use.

PCR-RFLP process and genotype determination

We applied polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method to detect the genotypes of *TLR3* gene rs3775291 and rs3775296 polymorphisms. Primer sequences used for amplification are listed in **Table 1**. The PCR process was occurred in a total of 30 μL systems, which consisted of 10× Buffer 3 μL, dNTPs 2.4 μL (final concentration 0.2 mM), forward and reverse primers 0.9 μL each (final concentration 0.3 μM), Taq DNA polymerase 0.24 μL (final concentration 0.04 mM), DNA template 2 μL and ddH_2O 20.56 μL. PCR reaction conditions were: at first, 94°C initial denaturation for 5 min; then 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s; finally 72°C extension for 10 min.

Restriction enzyme Taq I (TthHB8I) was used to digest the above PCR products, with a recognition sequence as T↓CGA. The total enzymatic system was 20 μL, including 10× Taq I Basal Buffer 2 μL, 10 u/μL Taq I 1 μL, 0.1% BSA 2 μL, PCR products 10 μL and dd H_2O 5 μL. Such enzymatic system was mixed well, centrifuged instantaneously, and then incubated in a water bath at 65°C for 30 min. Digested products were put in 2% agarose gel (containing 0.5 g/mL ethidium bromide) for electrophoresis. The results were determined using gel imaging system.

Statistical analysis

The data were processed by SPSS18.0 statistical software. Hardy-Weinberg equilibrium (HWE) was used to detect the representativeness of the subjects. Linkage disequilibrium

Table 2. Genotype and allele distributions of *TLR3* rs3775291 and rs3775296 polymorphisms

Genotype/Allele	Case (n=110)	Control (n=108)	χ^2	P	OR (95% CI)
rs3775291					
GG	42/38.2	58/53.7	-	-	1.00
AG	63/57.3	46/42.6	5.21	0.03	1.89 (1.09-3.28)
AA	5/4.5	4/3.7	0.62	0.50	1.73 (0.44-6.82)
G	147/66.8	162/75	-	-	1.00
A	73/33.2	54/25	3.53	0.07	1.49 (0.98-2.26)
rs3775296					
GG	57/51.8	67/62	-	-	1.00
GT	41/37.3	38/35.2	0.68	0.47	1.27 (0.72-2.23)
TT	12/10.9	3/2.8	6.20	0.02	4.70 (1.26-17.49)
G	155/70.5	172/79.6	-	-	1.00
T	65/29.5	44/20.4	4.89	0.03	1.64 (1.06-2.55)

Table 3. Linkage disequilibrium and haplotype analysis on rs3775291 and rs3775296 polymorphisms

Haplotype Locus1-Locus2	Case 2 (n=220)	Control 2 (n=216)	χ^2	P	OR (95% CI)
G-A	56/25.5	50/23.1	-	-	1.00
G-T	99/45	122/56.5	1.86	0.19	0.73 (0.46-1.15)
T-A	17/7.7	4/1.9	5.67	0.03	3.80 (1.20-12.03)
T-G	48/21.8	40/18.5	0.06	0.89	1.07 (0.61-1.89)

Notes: locus1, rs3775291; locus2, rs3775296.

and the haplotypes between *TLR3* gene polymorphisms were assessed by Haploview. Each difference between case and control groups was assessed by χ^2 test. Odds ratios (ORs) with 95% confidence intervals (CIs) were used to calculate the relationship between *TLR3* polymorphisms and AMD susceptibility. When $P<0.05$, differences between groups were considered with statistical significance.

Results

HWE detection

We inspected the HWE examination of the genotypes of *TLR3* rs3775291 and rs3775296 polymorphisms in case and control groups. Then we found that the genotype distributions in control group met the genetic equilibrium ($P>0.05$), which suggested that the controls had representativeness.

Genotype and allele frequency distributions of TLR3 polymorphisms

The distributions and comparisons results of genotypes and alleles in *TLR3* polymorphisms between case and control groups were given in **Table 2**. As we could see, for rs3775291 polymorphism, AG genotype was more frequently in case group than that in control group, and the distribution difference was statistically significant ($P<0.05$). Besides, AG genotype increased the onset risk of AMD when compared with GG genotype (OR=1.89, 95% CI=1.09-3.28). In rs3775296 polymorphism, TT genotype had a higher frequency in case group than that in control group and the distribution difference between groups was obviously ($P<0.05$). TT genotype could also increase the incidence of AMD (OR=4.70, 95% CI=1.26-17.49). In the mean time, T allele of rs3775296 significantly associated with the AMD susceptibility (P=0.03, OR=1.64, 95% CI=1.06-2.55).

Linkage disequilibrium and haplotype analysis on TLR3 polymorphisms

Analysis of linkage disequilibrium was performed by Haploview online software, then we discovered that there existed a high linkage disequilibrium between rs3775291 and rs3775296 polymorphisms of *TLR3*. The two polymorphisms could form four kinds of haplotypes: G-A, G-G, T-A and T-G. Haplotype analysis results were shown in **Table 3**. It could be observed that the distributions of T-A haplotype in case and control groups were significantly different ($P<0.05$), and T-A could increase the risk of AMD when compared with G-A haplotype ($\chi^2=5.67$, P=0.03, OR=3.80, 95% CI=1.20-12.03). Additionally, the distributions of G-A, G-G and T-G haplotypes in case and control groups were not statistically significant ($P>0.05$). Thus we concluded that T-A was a susceptible haplotype to AMD.

Discussion

In recent years, the incidence of AMD in middle and old aged people has been increasing year by year all over the world. AMD is the most common reason for irreversible loss of vision and it has been the main cause of blindness in people over 50 years old in the developed countries [16, 17]. In America, over 8 million people have suffered from the disease [3]. China has developed into an aging stage, and the vision problems of the elderly, especially age-related eye diseases have gradually become a social concern. Researches carried out in different regions showed that the incidence of AMD in China was about 3%-15.5%, which seriously affected the life quality of the elderly and increased the economic burden of the society [18].

Related studies about *TLR3* polymorphisms and eye diseases at present all have confirmed the existence of a close relationship between them, and *TLR3* can promote the occurrence and development of AMD [19-21]. *TLRs* play important roles in the human body. It can identify a variety of pathogenic modes. *TLR3* gene locates in human chromosome 4q35 region, which included 5 exons and 4 introns. The rs3775291 polymorphism of *TLR3* results in the 412th encoding amino acid mutating from leucine into phenylalanine. In the year 2009, Cho et al. undertook a study about the correlation between *TLR3* rs3775291 polymorphism and *TLR4* rs4986790 polymorphism and AMD, but they failed to find an obvious positive result [22]. The next year, Sng et al. compared the frequency distributions of genotypes and alleles of *TLR3* rs3775291 polymorphism in 246 Singaporean Chinese people who had exudative AMD. They found that the correlation between *TLR3* rs3775291 polymorphism and the onset of AMD among Singaporean Chinese people was not significant [23]. However, Maloney et al. indicated that TLR3 protein might be a receptor of the human wet AMD lesions [24]. Zhu et al. discovered that the mRNA and protein expressions of *TLR2* and *TLR3* from the peripheral blood mononuclear cells (PBMCs) were higher in case group than in control group, and they inferred that *TLR3* might be related to the pathogenesis of wet AMD [25].

The present study ascertained that *TLR3* polymorphisms (rs3775291 and rs3775296) were associated with AMD. As the detecting results indicated, rs3775291 polymorphism AG genotype was highly distributed in the case group and it was a susceptible factor to AMD; rs3775296 polymorphism TT genotype and T allele respectively increased 4.7 times and 1.64 times of the onset risk of AMD. The result suggested a significant association of rs3775296 polymorphism with the incidence of AMD in Chinese Han population. Furthermore, linkage disequilibrium and haplotype analysis found that the frequencies of T-A haplotype of the two SNPs of *TLR3* gene in case and control groups were significantly different. T-A haplotype had 3.80 times increased risk of developing AMD, which manifested that T-A was a susceptible haplotype for AMD.

Because of the racial differences, the research results among different races are not consistent with each other. Genetic influences of *TLR3* gene polymorphisms on AMD are quite different between Chinese people and white people, while the pathogenesis has not been fully understood. Even so, we have suggested that *TLR3* polymorphisms might associate with the susceptibility of wet AMD in China. With the development of research and application of molecular biology, we hope that the further studies will provide certain directive significance for the exploration of the pathogenesis and treatment of AMD.

Disclosure of conflict of interest

None.

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