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CLINICAL STUDY

Association of tumour necrosis factor alpha −308 gene polymorphism with primary open-angle glaucoma in Chinese

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Abstract

Purpose **Genetic factors are known to play a role in the aetiology of glaucoma, and in particular the role of the immune system is highly suspected. In this study, we evaluated the association between tumour necrosis factor alpha −308 (TNF α −308) and primary open-angle glaucoma (POAG).**

Methods **A total of sixty POAG patients and 103 healthy volunteers as control group were enrolled in this case-controlled study. Furthermore, we used polymerase chain reaction based analysis to resolve the TNF α −308 polymorphism. Statistical analysis for the relative risk of TNF α −308 polymorphism was compared by the χ^2 test.**

Results **There were significant differences in the distribution of the polymorphism between the POAG patients and the control subjects ($P = 0.00016$; $P < 0.05$) and it was found that the A^{-308} allele occurred more frequently in POAG patients (odds ratio: 2.72; 95% confidence interval: 1.66–4.45).**

Conclusion **The results of our study concluded that the distribution of TNF α −308 was significantly higher in the POAG patients than in the control group. Therefore, the A^{-308} allele appears to be associated with POAG and, therefore, could be used as a genetic marker for disease mapping. POAG is a complex disease, and a single gene could not be responsible. Understanding the role of genetic polymorphisms, like TNF α, could be a prediction of the disease and useful for developing new treatments for POAG.**

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Introduction

Glaucoma is the secondary leading cause of blindness worldwide.[1] It is a heterogeneous ocular disease characterized by optic neuropathy and a progressive loss of the visual field.[2,3] Primary open-angle glaucoma (POAG) is the most common form of glaucoma[3–5] characterized by elevated intraocular pressure and an open anterior chamber angle.

Environmental factors do not seem to play a definitive role in glaucoma and therefore genetic influences are responsible for the aetiology of POAG. A family history of glaucoma is one of the major risk factors for the disease, as a substantial subset of cases of POAG is hereditary.[6–9] The relationships between glaucoma and genetic polymorphisms, although not traditionally perceived as being causally related, have been highly suspected recently.[10] The role of the immune system in glaucoma is likely one of surveillance, in which signal pathways of the immune system regulate cell death in response to conditions that stress retinal neurons in glaucoma. These might include mechanical stress from high intraocular pressure, ischaemia, and excessive excitatory amino acid.

Tissue constitutive expression of tumour necrosis factor receptor-1 (TNF-R1) in the vessels of the optic nerve heads has been demonstrated, although there was no positive labelling for tumour necrosis factor alpha (TNF α).[11] However, in the glaucomatous optic nerve heads, the expressions of both TNF α and TNF-R are apparently upregulated. The same

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researchers believe that TNF α contributes to the progression of optic nerve degeneration in glaucoma by both a direct effect on the axons of the retinal ganglion cells and by inducing NOS-2 in astrocytes.[11] Besides, glial cells have been found to secrete TNF α as well as other noxious agents, such as nitric oxide, after exposure to stress.[12] Furthermore, it has been noted that retinal ganglion cell apoptosis can be attenuated by neutralized antibodies against TNF α.[12] TNF α has been shown to be a component of astroglial activation in glaucomatous optic nerve heads. The expression of these proteins may play a role in the progression of glaucomatous optic neuropathy.[13]

Cytokines are molecules involved in signalling between cells during immune response, and TNF α is one such protein. An increasingly well-defined chain of protein–protein recognition events ties the binding of a cytokine at the cell surface, initiating a downstream signalling cascade thereby inducing the actions of diverse transcription factors inside the nucleus. TNF α activates members of the MAP kinese family, resulting in increased binding of the transcription factors AP-1, $NF_\kappa B$, and NFIL-6 to DNA.[14] The variation of transcriptive states results in a change in protein expression.

The TNF α gene encodes proteins that are pleiotropic in appearance. Recently, several genetic polymorphisms have been described in the human TNF α promoter.[15,16] Biallelic G to A polymorphism, 308 nucleotides upstream from the transcription initiation site in the TNF promoter, is associated with elevated TNF levels, disease susceptibilities, and poor prognosis in several diseases.[16] The existence of different TNF α alleles is related to different levels of TNF α. In this study, we tried to evaluate whether the TNF α 308 polymorphism is a useful marker for predicting the susceptibility of POAG.

Materials and methods

From May 2000 to July 2000 we recruited POAG patients from the Department of Ophthalmology at the China Medical College Hospital. All patients in this study received serial ophthalmic examinations, including IOP, visual acuity, automated perimetry, gonioscopy, optic disc examination, and retinal examination. Patients with ocular diseases other than POAG were excluded from our study. The volunteers in the control group were examined by the same ophthalmologist. The volunteers were healthy people collected when they attended their routine healthy examination. If there were any doubts regarding the patients' eye diseases, they were excluded from the study. Patients included in this study were POAG patients who were required to fulfil one criterion from both the visual field and the optic nerve categories.

Visual field criteria

(1) At least two abnormal visual field tests by Humphrey automated perimetry, as defined by computer-based objective criteria.
(2) The presence of one or more absolute defects in the central visual field 30°, with ophthalmologic interpretation as glaucomatous visual field loss.

Optic disc criteria (optic disc damage present in fundus photographs)

(1) Either a horizontal or vertical cup-to-disc ratio of 0.6 or more.
(2) The narrowest remaining neuroretinal rim was 20% or fewer disc diameters.

Ophthalmologic criteria

Patients with other possible causes for disc and field changes other than POAG were excluded.

This study was carried out with approval from the Human Study Committee of the China Medical College Hospital. Informed consent was obtained from all patients who participated in this study. The genomic DNA was prepared from peripheral blood using a Genomaker reagent kit (Blossom, Taiwan).

The loci of the TNF α gene were studied as previously described by Galbraith and Pendey.[15] It involved a G to A transition at position −308 in the 5′-flanking promoter region of the TNF α gene. The region was amplified by polymerase chain reaction (PCR) using a Gene Amp PCR kit (Perkin-Elmer, Norwak, CT, USA). The sequences of the primers were as follows: 5′-AGGCAATAGGTTTTGAGGGCCAT-3′ and 5′-ACACTCCCCATCCTCCCGGCT-3′. About 50 ng of genomic DNA was mixed with 20 pmol of each PCR primer in a total volume of 25 μl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.0 mM $MgCl_2$, as well as 0.2 mM of each deoxyribonucleotide triphosphate, and 1 unit of Amplitaq DNA polymerase (Perkin-Elmer). PCR was programmed as follows: 35 cycles at 95°C for 15 s followed by 35 cycles at 60°C for 30 s.

The polymorphism was analysed by PCR amplification followed by *Nco*I restriction analysis.[15] The products were analysed by electrophoresis on agarose gel and each allele was recognized according to its size. Restriction enzyme digestion was performed with *Nco*I and subsequent electrophoresis on a 5–8% polyacrylamide gel. The molecular analyses of patients and controls are performed in the same laboratory at the same time and the gels are inspected by investigators who are masked to the clinical phenotype of the individuals being studied.

Statistical analysis for the relative risk of TNF α in the control and POAG groups was compared by the χ^2 test. Results were considered statistically significant when the probability of findings occurring by chance was less than

5% ($P<0.05$). The allelic frequencies were rechecked by odds ratio with 95% confidence intervals (CI).

Results

A total of 103 healthy volunteers (55 males and 48 females) and 60 POAG patients (30 males and 30 females) were enrolled in this study. The volunteers ranged in age from 55 to 70 years (mean 50 years) and were free from ophthalmic diseases. The POAG patients ranged in age from 20 to 70 years (mean 55 years) and all were unrelated. Patients with ocular diseases other than POAG were excluded from our study. Patients were followed up between 2 and 8 years (mean 5 years). Ten of the patients had received trabeculectomy and two of the 10 patients received trabeculectomy twice from different sites. Of the POAG patients, 50 were prescribed antiglaucoma drugs. Each patient used 1.3 types of antiglaucomatous drugs on an average. Nine patients did not need drugs to control IOP after trabeculectomy.

Homozygote G^{-308} allele showed two fragments of 97 and 20 bp. Homozygote A^{-308} was undigested and resulted in a single band of 117 bp. Heterozygote A/G was detected by the presence of all three fragments (Figure 1). The frequencies of the genotypes in the control and POAG patient groups are shown in Table 1. Using the χ^2 test, we compared the distribution of the TNF α −308 polymorphism. There were significant differences between the two groups ($P=0.00015885$; $P<0.05$). The distribution in the control group revealed 6.8% A^{-308} allele homozygote, 29.1% heterozygote, and 64.1% G^{-308} allele homozygote. The genotype distribution in the POAG group revealed 31.7% A^{-308} allele homozygote, 21.7% heterozygote, and 46.6% G^{-308} homozygote. The allelic frequencies in the POAG group were 42.5% A and 57.5% G. The allelic frequencies in the control group were 21.4% A and 78.6% G. The odds ratio of the A^{-308} allele was 2.721 (95% CI: 1.66–4.45) in comparison with the A and G alleles in POAG patients (Table 2). The frequencies of allele A were significantly higher in the POAG group than in the control group. When we set the odds ratio of the heterozygote as '1', the odds ratio of allele A homozygote was determined to be 6.263 (95% CI: 2.521–15.559), and the odds ratio of allele G homozygote was 0.979 (95% CI: 0.424–2.257) (Table 1). The A/A homozygote was significantly higher in the POAG group.

We also calculated 'power' for a test of the null hypothesis by SPSS®. There is a power of 55% to yield a statistically significant result in this sample size. An age-adjusted analysis for the A allele also revealed a significant difference between the two groups. This meant that age did not have an influence on the result.

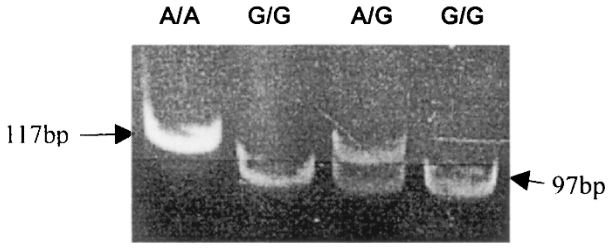

Figure 1 Genotypes of TNF α −308 panel shown on a 3% agarose gel stained with ethidium bromide after PCR amplification. Homozygote G^{-308} allele has two fragments: 97 and 20 bp (lines 2 and 4). Homozygote A^{-308} allele is an undigested single band: 117 bp (line 1). Heterozygote A/G includes all three fragments (line 3).

Discussion

Wax[10] brought out that glaucomatous optic neuropathy may occur directly by autoantibodies or indirectly by way of a 'mimicry' autoimmune response to a sensitizing antigen.[10] The immune system acts as an arbiter to help determine whether a neuronal cell confronting stress will ultimately survive or be sacrificed to injury.

The A^{-308} allele of the TNF α promoter affects the binding of transcription factors and increases transcription promoter activity, which may further alter the TNF production, the immune response, and susceptibility to certain autoimmune, infectious, and malignant diseases.[17] Besides, the A^{-308} allele may inhibit repressors of transcription.[18] The TNF α promoter polymorphism may also be functionally silent or have selectable effects only when there is linkage with selectable HLA alleles.[18,19] The presence of a G to A polymorphism at position 308 of the TNF α promoter gene could increase transcription six- to seven-fold.[20] It is obvious that the variable polymorphisms that lie within the promoter region of the TNF α locus make it a likely candidate for playing a regulatory role in disease.

In our study, owing to the frequency of the −308A allele noted in POAG patients, we have to consider the possibility that a functional distinction between A^{-328} and G^{-308} becomes prominent upon a physiologic TNF-inducing stimulus that is specific for POAG. Such a stimulus may be different from the standard *in vitro* TNF-inducing agents, and variations in the genes coding for TNF have been found to affect the production of relevant cytokines.[11] However, we cannot exclude A^{-308} allele as a genetic marker for other polymorphisms that have functional implications for TNF gene expression. Consequently, A^{-308} may influence the susceptibility of POAG by way of immune effects. It may be involved in the formation of the disease, through a complex pathway,[15] such as a change from signal transduction

Table 1 Distribution of TNF α genotype among glaucoma patients and healthy control subjects

	A/A (%)	*A/G (%)*	*G/G (%)*	P *value*
POAG	19 (31.7)	13 (21.7)	28 (46.6)	0.0015885
Control	7 (6.8)	30 (29.1)	66 (64.1)	
Total	26 (16.0)	43 (26.4)	94 (57.7)	
Odds ratio	6.263	1	0.979	
(95% CI)	(2.521, 15.5569)		(10.424, 2.257)	

Table 2 Allelic frequencies in healthy subjects and POAG patients

	A (%)	*G (%)*
POAG	42.5	57.5
Control group	21.4	78.6

Note: Odds ratio of allele A is 2.721 of POAG group (95% CI: 1.66–4.45).

between cells and then change the function of transformation.

In this study we noted that allele A of TNF α −308 polymorphism is a useful marker for predicting the susceptibility of Chinese POAG patients. These results would support a mechanism involving the immune response in glaucomatous damage, and this may provide a novel therapeutic target for neuroprotection in the treatment of glaucomatous optic neuropathy. An understanding of the genetic and immune role in POAG is an important way to design new treatment for glaucoma.

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