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LABORATORY STUDY

A PAX6 gene polymorphism is associated with genetic predisposition to extreme myopia

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Abstract

Purpose **The PAX6 gene is a homeobox gene involved in oculogenesis, ocular growth, and form-deprivation myopia. Our aim was to determine whether *PAX6* polymorphism at position −12 of intron 9 (IVS9-12C to T, rs667773) is associated with high myopia in Chinese Taiwanese.**

Methods **This case–control study compared a study group ($n = 188$) with high myopia whose spherical equivalent was greater than −6.0 D with a control group ($n = 85$) whose spherical equivalent was less than −0.5 D. Genotyping of IVS9-12C to T was conducted by restriction fragment length polymorphism analysis, and results were compared for the two groups.**

Results **No significant difference in genotype and allelic frequency at this position between the study and control groups was detected. However, there was a significantly higher frequency of the CC genotype in extremely myopic (greater than −10 D) patients ($P < 0.001$, odds ratio (OR = 5.265), confidence interval (CI = 2.0342–13.626)). Furthermore, there was a higher frequency of the C allele in the extreme myopia group than in the control group ($P = 0.002$, OR = 3.73, CI = 1.57–8.81).**

Conclusions **The elevated frequency of the CC genotype within the extreme myopia group indicated that the CC genotype could act as a genetic marker, identifying patients predisposed to develop extreme myopia. Varied expression of this genotype may contribute to the genetic predisposition to high myopia in Chinese Taiwanese.**

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Introduction

Myopia, highly prevalent worldwide, is frequently observed in Asian populations, such as in Taiwan where the prevalence may exceed 65%;[1] thus, it has become a public health problem in modern society.[2,3] High myopia (> −6.0 D) can result in severe ocular morbidity, and extreme myopia (> 10.0 D) exhibits complications that potentially manifest in blindness, such as retinal detachment, macular degeneration, and glaucoma.[4] Early identification of patients predisposed to high or extreme myopia, particularly children, is important so that preventive measures can be taken.

The effects of environmental factors, such as increased near-work activity, on myopia have been studied and found to be critical in its development.[2,5–8] However, myopia is a complex disease involving not only environmental factors but multiple interacting genetic factors as well.[5–8] Understanding the genetics of myopia is challenging, as myopia is a multigenic condition involving several overlapping signaling pathways, each associated with a group of distinct genetic profiles. Genetic association studies are currently regarded as the most powerful approach to map the genes underlying such complex traits.[9] Single nucleotide polymorphisms (SNPs), the most abundant types of DNA sequence variation in the human genome, have proven to be effective and useful markers for analysing complex gene-associated diseases such as high myopia and for identifying patients predisposed to the disease.

Recently, polymorphisms in the transforming growth factor-β 1 and lumican genes were found to be associated with high myopia.[10,11] Transforming growth factor-β is important for

ocular growth, and lumican is one component of the scleral extracellular matrix. Because high myopia results from excessive enlargement or growth of the eye, and both transforming growth factor- β 1 and lumican are proteins involved in ocular growth, it follows then that polymorphisms of these genes involved in ocular growth could potentially be associated with high myopia. Thus, these genes may be good candidates for containing genetic clues that can be used to screen patients for predisposition to high and extreme myopia.

In addition to growth factors and extracellular matrix components, homeobox genes are also critical for ocular growth.[12–14] Homeobox genes control the activity of many genes involved in morphogenesis and are considered 'master' genes.[12] Among the homeobox genes, paired box (PAX) genes encode tissue-specific transcription factors; the PAX6 gene is involved in oculogenesis and ocular growth, and is associated with form-deprivation myopia in two chicken studies.[15,16] In addition, *PAX6* is located on chromosome 11p13, a possible locus for myopia (MYP7, OMIM 609256). Hence, due to its role in ocular growth, its expression in form-deprivation myopia, and its location at MYP7, we predicted that SNPs in *PAX6* might be associated with high myopia.

To investigate whether *PAX6* polymorphisms are correlated with high myopia in Taiwanese Chinese, *PAX6* polymorphisms in patients with high myopia (>-6.0 D) and in emmetropic volunteers were examined using PCR-restriction fragment length polymorphism to determine whether the distribution of *PAX6* polymorphisms differs between control subjects and patients with high myopia. Among the 93 polymorphisms of the PAX6 gene in the public SNP database (National Center for Biotechnology Information), only the polymorphism at position −12 of intron 9 (IVS9-12C to T, rs667773) has been associated with diseases, including aniridia, keratoconus, and glaucoma.[17–20] In addition, the results of *PAX6* sequencing in our volunteers revealed that not every reported polymorphism of *PAX6* existed in our population, and only IVS9-12C to T showed high allele frequency. Hence, we further evaluated the distribution of the IVS9-12C to T polymorphism in our two study populations.

Patients and methods

Participants

Study participants (1000 volunteers) were unrelated Chinese Taiwanese first-year medical students of 16–25 years of age. Volunteers had visual acuity with distance correction of 0.2 log MAR (20/32) or better. Refractive error was measured in dioptres (D) and determined by the mean spherical equivalent of both eyes of each individual after one drop of a cycloplegic drug (1% mydricyle, Alcon, Berlin, Germany) was administered. Individuals with myopia $\geqslant 6.00$ D (both eyes) were included in this study, with the control group comprised of individuals with myopia $\leqslant 0.5$ D. A total of 255 high myopia and 85 control subjects were enrolled from February 2004 to November 2004; the male to female ratio was 1.8 : 1.0. Our study was reviewed by the ethics committee of the China Medical University Hospital, Taichung, Taiwan, and informed consent was obtained from all patients.

A comprehensive ophthalmic examination and blood collection were performed. None of the participants previously or currently had ocular disease, ocular insult such as a history of retinopathy, prematurity, or neonatal problems, nor had any genetic disease and/or connective tissue disorder associated with myopia, such as Strickler or Marfan syndromes. Clinical examination included visual acuity, refractive error, slit lamp examination, ocular movements, intraocular pressure, and fundoscopy. All extreme myopia patients received the above examination, as well as corneal topography and axial length. Moreover, to lessen the possibility of accommodation influencing refraction results, autorefraction data refined by cycloplegic refraction was done. Patients with organic eye disease, a history or evidence of intraocular surgery, history of cataract, glaucoma, retinal disorders, or laser treatment were excluded. As with all data collection procedures, autorefraction (Autorefractor/autokeratometer, ARK 700A; Topcon, Tokyo, Japan) was performed on both eyes by experienced optometrists who were trained and certified with study protocols. Refractive data, sphere(s), negative cylinder, and axis measurements were analysed by calculating the spherical equivalent refractive error. The study was performed according to the tenets of the Declaration of Helsinki for research involving human subjects.

Genotype determination of the IVS9-12C to T SNP

The primer sequences were as follows: (F) 5′-TGG CAC AAT ATG GAA AAT CAA-3′ and (R) 5′-CGG AGC AAA CAG GTT TAA AGA-3′. PCR amplification was performed in a programable thermal cycle GeneAmp PCR System 2400 (Perkin Elmer, Waltham, Massachusetts, USA). Cycling conditions for PCR were as follows: one cycle at 95°C for 5 min, 35 cycles of 95°C for 30 s, 60°C for 40 s, and 72°C for 40 s, and one final extension cycle at 72°C for 10 min. The PCR product (681 bp product) was digested with 10 units of *Bcc*I following the conditions recommended by the manufacturer (New England Biolabs, Mississauga,

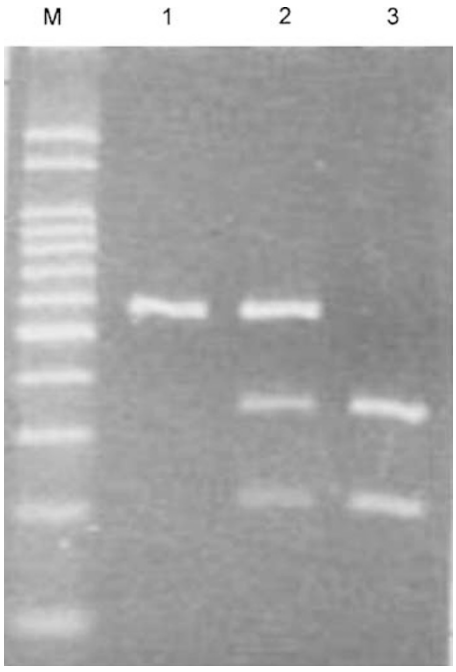

Figure 1 PCR-based restriction analysis of *PAX6* polymorphism at position −12 of intron 9 (IVS9-12C to T) using *Bcc*I restriction enzyme. Digested samples were analysed by electrophoresis on a 3% agarose gel. PCR of the polymorphic region resulted in an undigested fragment of genotype CC (681 bp; lane 1), a heterozygote with genotype CT (lane 2), and a digested fragment representing genotype TT (491 bp fragment and 190 bp fragment; lane 3). Lane M: 100 bp DNA ladder.

Ontario, Canada). The DNA fragments were separated by horizontal electrophoresis on 3% agarose gels. Each gel was run for 20 min at 100 V, and photographed under ultraviolet lights. As shown in Figure 1, the 'T' allele was digested into two fragments, 491 bp plus 190 bp (lane 3), while the 'C' allele was represented by an undigested 681 bp fragment (lane 1). Direct DNA sequence of *PAX6* polymorphisms at position −12 of intron 9 (IVS9-12C to T, rs667773) is shown in Figure 2.

Statistical analysis

Genotypes were quantified in both study and control groups, with subsequent calculation of allele frequencies. Statistical analysis was undertaken using the χ^2 test and Fisher's exact test, and probability values (P) were calculated using the Minitab program. A value of $P<0.05$ was considered significant. Adherence to the Hardy–Weinberg equilibrium constant was tested using the χ^2 test with one degree of freedom.

Results

The genotype distributions for the *PAX6* IVS9-12C to T polymorphism identified in the patients and controls are

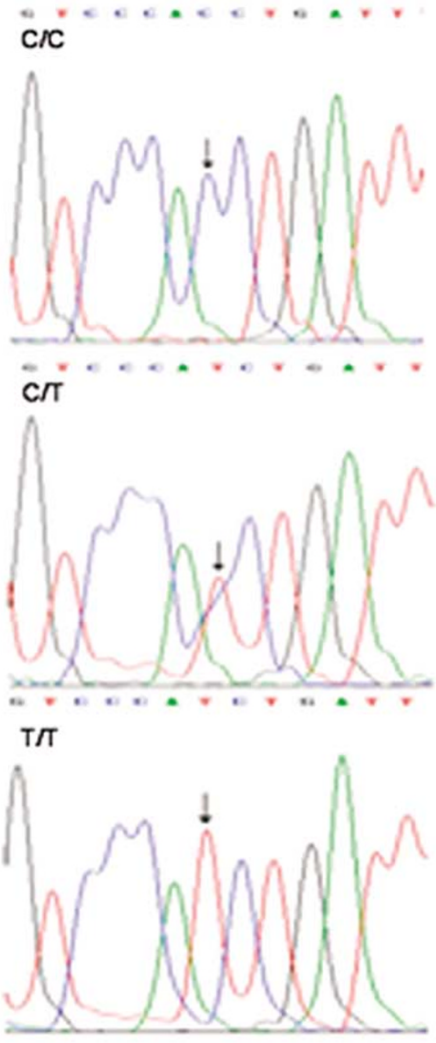

Figure 2 Direct sequence of *PAX6* polymorphisms at position −12 of intron 9 (IVS9-12C to T, rs667773). The arrow indicates the position of the C/C (upper), C/T (middle) and T/T (bottom) polymorphism.

shown in Table 1. No significant difference between the two groups in the distribution of genotype and allelic frequency was detected. Although the proportion of CC genotype and C allele is higher in the overall patient group, there was no difference between the CC genotype compared with CT and TT genotype (OR = 1.20, CI = 0.71–2.01), or for the C allele compared with the T allele (OR = 1.01, CI = 0.64–1.61).

After further stratification at −10.0 D, we identified a significant difference between extreme myopia (> −10.0 D) and the control group in the distribution of genotype ($P<0.001$) and allelic frequency ($P=0.002$) (Table 1). Subjects with the CC genotype have a higher risk to develop extreme myopia than those with either the CT or TT genotype (OR = 5.265, CI = 2.0342–13.626). Furthermore, we observed a higher frequency of the

Table 1 Genotype and allelic frequency of PAX6 IVS9-12C to T in the myopia study group and in the control group

	Genotype			P	*Allelic frequency*		P
	CC	*CT*	*TT*		*C*	*T*	
Control	56 (65.88%)	29 (34.12%)	0 (0%)		141 (82.94%)	29 (17.06%)	
Myopia (high and extreme)	178 (69.80%)	68 (26.67%)	9 (3.53%)	0.11	424 (83.14%)	86 (16.86%)	0.95
Extreme myopia	61 (91.04%)	5 (7.46%)	1 (1.49%)	<0.001	127 (94.78%)	7 (5.22%)	0.002

C allele in the extreme myopia group than in the control group (OR = 3.73, CI = 1.57–8.81).

Discussion

The *PAX6* gene (MIM 607108) is a member of the paired-domain Pax family and encodes a transcriptional regulator involved in oculogenesis and body development. *PAX6* regulates the tissue-specific expression of diverse molecules, including transcription factors, cell adhesion molecules, hormones, and structural proteins, all of which play a part in ocular development.[21] For example, during the early stages of eye development, *PAX6* induces the differentiation of progenitor cells in the retina into neurons, as well as promote the expression of crystallins in lens epithelial cells.[22] *PAX6* is located on human chromosome 11p13, and mutations in this gene lead to a variety of hereditary ocular malformations of the anterior and posterior segment, including aniridia,[17] coloboma of the iris,[23] keratitis,[24] congenital cataracts,[25] Peter's anomaly,[26] and optic nerve defects.[27] In addition to expression during development, *PAX6* is also widely expressed in the adult eye.[16] Hence, the *PAX6* gene may play a maintenance role in the adult eye, as well as in ocular growth related to myopia.[16,22] Bhat *et al*[16] and Zhong *et al*[28] found elevated *pax-6* expression in optical defocus-induced myopia and form-deprivation myopia in chicken and rhesus monkeys. Hammond *et al*[29] identified a strong linkage for refractive error in the vicinity of the *PAX6* gene in dizygotic twins, but no definite association between common *PAX6* variants and myopia was demonstrated in their study. All these three reports suggest that *PAX6* may play a critical role in the development of myopia in humans; however, no direct and definite evidence has yet been found.

In this study, we identified a *PAX6* gene polymorphism in our population at position −12 of intron 9 (IVS9-12C to T) that was associated with extreme myopia. *PAX6* IVS9-12C to T has previously been reported to be associated with aniridia in Indian and Caucasian populations.[18,19,30] However, to our knowledge, this polymorphism has not been implicated in myopia. Our results provide the first evidence that *PAX6* functions in the development of myopia, not only in chickens and monkeys, but also in humans. Although Hammond *et al*[29] also found a strong linkage for refractive error in the vicinity of *PAX6*, no association between myopia and *PAX6* polymorphisms was found. In their study, they selected five highly informative SNP markers in *PAX6*, referred to as 'tagging SNPs', typed the tagging SNPs in the clinical samples, and used the quantitative transmission/disequilibrium test to check linkage and association. Their failure to find a phenotypic association with a common SNP in the *PAX6* gene could have occurred for two reasons: first, the variant(s) were within the gene and were missed by the tagging SNPs; second, the variant(s) were located outside the *PAX6* gene. The tagging SNPs used in the study were rs3026401, rs662702, and rs1506 in the 3′-UTR, rs2239789 in intron 8 and rs628224 in intron 7. In our study, although the genotype distribution of rs667773 in intron 9 was not statistically different between the high myopia and control groups, when we examined extreme myopia (>10.0 D) patients within the total high myopia group, our data revealed that the distribution of genotype and allelic frequency was significantly different, and demonstrated an association of this polymorphism with extreme myopia. Hence, their failure to find an association between *PAX6* and myopia was most likely due to the less severe degree of myopia in their study group.

There are 93 SNPs in the *PAX6* gene; however, most of them were not found in our population. To reduce trial and error time, we sequenced exon 1 to exon 13 of the *PAX6* gene in a genomic DNA mix of 5 different people and 10 duplicates, for a total 50 PCRs. The only polymorphism we identified was rs66773. The polymorphism is within intron 9 and does not result in change of an amino acid; however, it changes the splice acceptor region of intron 9. Thus, the C to T substitution leads to disruption of *PAX6* expression due to premature termination, causing loss of activity at one allele.[31] Further investigations of *PAX6* protein translation and function are required to understand the molecular function that the *PAX6* IVS9-12C to T polymorphism plays in ocular development and/or myopia.

Many studies have suggested that myopia is a complex disease with multiple causes, including the interaction of

multiple genes with environmental stimuli.[32] One major hurdle in myopia research is the uncertainty surrounding the contribution of environmental influences and genetic factors in the equation. For example, environmental influences on members of a given family may account for the observed familial clustering, which is usually regarded as evidence of phenomena with a genetic basis. Therefore, to decrease the confusion from multiple gene interactions and gene/environment interactions confounding the results, we focused on the association of mapped SNPs and myopia in this study. Moreover, the emphasis on prolonged near visual tasks is an important environmental influence in myopia in Taiwan: individuals with higher education have a higher prevalence of myopia than people in the general population. First-year medical students in the same medical school are thus good candidates for the disease (myopia) and control groups, as the bias of environmental influence is decreased. Hence, to avoid the selection bias, we first selected students in the same college of the same school, who were previously performing similar near visual tasks. Second, we tested whether the overall incidence of strabismus in our 1000 volunteers was relevant with that observed in the general population of Taiwanese. Among the 1000 volunteers, there were 14 students with strabismus history or manifest strabismus, who were subsequently excluded. The prevalence of strabismus was 1.4% in the volunteer group, which was close to the reports in general population of Taiwanese.[33]

In conclusion, we observed that the frequency of the CC homozygote of *PAX6* IVS9-12C to T polymorphism was much higher in the extreme myopic group than in the control group. People who have the CC genotype may be at greater risk for developing extreme myopia.

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