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The *TGFβ1* gene codon 10 polymorphism contributes to the genetic predisposition to high myopia

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Purpose: Transforming growth factor-β (TGFβ), a multifunctional growth factor that plays a key role in the remodeling of scleral tissue, may be involved in the predisposition and pathophysiology of high myopia. Our aim was to examine the association between myopia and the polymorphisms within codon 10 of the TGFβ1 gene.
Methods: This was a case control study. The study group contained participants who had high myopia and a spherical equivalent greater than -6.00 D. The control group was composed of medical students whose spherical equivalent was less then -0.5 D. All volunteers in this study were over 16 years old and had never undergone ocular surgery. Genotyping was conducted by restriction fragment length polymorphism, and the results were compared between myopia patients and control subjects.
Results: The frequency of the CC genotype in TGFβ1 codon 10 differed significantly between patients in the high myopia group (n=201) and individuals in the control group (n=86; p<0.001). People with either the CT or TT genotype had a lower probability of having high myopia with a spherical equivalent greater than -6.00 D than those with the CC genotype. Furthermore, there was a higher frequency of the C allele in the high myopia group than with the control group (p<0.001, OR=1.83, CI=1.27-2.63).
Conclusions: The frequency of the CC homozygote in the high myopia group was much higher than in the control group, indicating people with the CC homozygote may be at a higher risk of developing high myopia. Varied expression of this gene may contribute to the genetic predisposition to high myopia in Chinese Taiwanese.

Simple myopia can be corrected with spectacles or contact lenses, whereas “high” (pathological) myopia is often associated with potentially blinding conditions such as retinal detachment, macular degeneration, and glaucoma. The prevalence of myopia varies by country and by ethnic group, reaching as high as 70-90% in some Asian populations, 30-40% in Europeans and Americans, and 10-20% in Africans [1]. According to epidemiological evidence, the incidence of myopia is increasing, especially in Asia. The occurrence of pathological myopia has been estimated to be 1-3% in population based studies [2]. In addition to the visual disability, there is a profound economic cost to society. Many studies have suggested that myopia is a complex disease with multiple causes, including the interaction of multiple genes with environmental stimuli [3]. Therefore, to understand myopia, it is necessary to apply the equation that genes plus the environment is equal to the outcome [3]. Evidence that supports the idea that myopia and refractive errors are in large part genetically determined comes from twin studies and studies of refractive errors in parents and their children [4,5]. A well conducted study showed that refractive errors are much more strongly correlated in monozygotic twins than in dizygotic twins [5].

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The pathologic changes in high myopia, such as macular degeneration, subretinal hemorrhage, and retinal detachment are assumed to be a result of mechanical stresses related to the excessively enlarging eye [6]. Excessive enlargement of the eye, predominantly in the axial dimension, results in the development of high myopia. Individuals with high degrees of myopia are at significantly increased risk of developing irreversible, sight-threatening pathology of the retina and choroid [7]. Studies of high myopia in animal models have demonstrated that the increase in eye size is facilitated by the outer coat of the eye (sclera). It has also been shown that active remodeling of the sclera plays a significant role in the enlargement of the ocular globe [8]. The sclera is a fibrous connective tissue consisting largely of heterologous collagen fibrils comprised mainly of type I collagen with small amounts of other fibrillar and fibril-associated collagens [9,10]. An important candidate in the search for factors involved in the scleral remodeling in myopia is transforming growth factor-β (TGFβ). TGFβ exhibits a distinct time-dependent alteration in expression levels that appears to be important for conferring organization and strength at wound sites [11]. TGFβ belongs to a family of polypeptides that display a broad range of multifunctional activities. TGFβ activates gene transcription and increases the synthesis and secretion of matrix proteins [12]. It decreases synthesis of proteolytic enzymes which degrade matrix proteins and increases the synthesis of protease inhibitors that block the activity of proteolytic enzymes [12]. TGFβ

also increases transcription, translation, and processing of cellular matrix proteins receptors [11]. TGFβ content and concentration reduce significantly in myopic eyes in the retinal pigmented epithelium (RPE), choroid, and the sclera [13]. TGFβ modulates the production of extracellular matrix (ECM). The turnover of ECM is the major mechanism of changing axial length [14]. Researchers have reported that TGFβ is a growth regulator of scleral chondrocytes and scleral fibroblasts [10,15]. Using the chick model of myopia, investigators demonstrated that fibrous growth factor 2 (FGF-2) inhibited the growth of myopic eyes. This effect was abolished, however, when TGFβ was co-administered [10]. Although TGFβ itself was not found to induce myopia, FGF-2 and TGFβ cooperatively were important candidates in control of eye growth [10]. In a study of tree shrews, TGFβ was found to work with other factors to influence eye size [16]. The afore mentioned evidence indicates that TGFβ plays an important role in the control of scleral remodeling during the development of high myopia [12,17].

There are three isoforms of TGFβ. The expression and roles of each isoform differs in myopia progression [18]. We selected TGFβ1 as the first candidate genetic polymorphisms to map myopia in Taiwanese Chinese. The *TGFβ1* gene is encoded on chromosome l9ql3.1-ql3.3 and contains seven exons [19]. After sequencing *TGFβ1* from our volunteers (Figure 1), we found that not every reported polymorphism of *TGFβ1* existed in our population. Among all of the polymorphisms, a polymorphism located in exon 1, codon 10 showed high allele frequency. This polymorphism leads to an amino acid change from leucine to proline. The polymorphism located at exon 1, codon 10 of *TGFβ1* [15] has been correlated with blood pressure change [20], bone mineral density [21], end-stage breast cancer [22], heart failure due to cardiomyopathy [23], and asthma [24]. In order to investigate whether the *TGFβ1* exon 1, codon 10 polymorphism is correlated with high myopia in Taiwanese Chinese, the polymorphism of high myopia patients (myopia in excess of 6.0 D) and volunteers in the control group were tested using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to reveal whether the frequency of *TGFβ1* differs between control subjects and patients with high myopia.

METHODS

Participants: Refractive error was measured in 3,000 volunteers. They were all ethnic Chinese living in different areas of Taiwan. None of them were related and all were native Taiwan citizens and all were medical school students. The participants enrolled in this study also met specific criteria: age 16-25 years and visual acuity with distance correct of 0.2 logMAR (20/32) or better. Refractive error was measured in diopters (D) and determined by the mean spherical equivalent (SE) of the two eyes of each individual after one drop of cycloplegic drug (1% mydricyle, Alcon, Berlin) had been given. Those with myopia greater than or equal to -6.00 D (both eyes) were included in the study group and individuals with myopia less than or equal to -0.5 D were collected as the control group. None of the participants had any known ocular disease, or an insult such as a history of retinopathy, prematurity, neonatal problems, or genetic disease and/or connective tissue disorders associated with myopia, such as Strickler or Marfan syndromes. Clinical examination included visual acuity, refraction error, slit lamp examination, ocular movements, intraocular pressure, and fundoscopy. Patients with organic eye disease, a history or evidence of intraocular surgery, history of cataract, glaucoma, retinal disorders, or laser treatment were excluded. Our study was reviewed by the ethics committee, and informed consent was obtained from all patients. A comprehensive ophthalmic examination and blood collection were performed. The study was performed according to the tenets of the Declaration of Helsinki for research involving human subjects. A total of 201 high myopia and 86 control subjects were enrolled from February to November, 2004. There was a male to female ratio of 1.8:1.0. As with all data collection procedures, autorefraction (Autorefractor/autokeratometer, ARK 700A; Topcon, Tokyo, Japan) was taken on both eyes by experienced optometrists who were trained and certified on study protocols. Refractive data, sphere(s), negative cylinder, and axis measurements were analyzed by calculating the SE refractive error.

Genotype determination: Genomic DNA was extracted from whole blood samples after a standard protocol of digestion by proteinase K and purification with phenol-chloroform. PCR reactions were carried out in a total volume of 25 μl, containing 50 ng genomic DNA, 2-6 pmole of each primer, 1 unit of *Taq* polymerase buffer (1.5 mM $MgCl_2$), and 0.25 units of AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA). The primer sequences are listed as follows: 5'-GCC TCC CCA CCA CAC CAG-3' (sense) and 5'-GCC GCA GCT TGG ACA GGA T-3' (antisense). A PCR product of 237 bp was generated. The amplification protocol was as follows: one cycle of denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s, and one final 5 min elongation cycle at 72 °C. Preventive contamination measures were taken by the inclusion of PCR reaction mixture without DNA (negative control) in every run of amplification. The PCR product was digested with 10 units of *Msp*A1I. Restriction digest conditions were those recommended by the enzyme manufacturer (New England Biolabs, Mississauga, Ontario, Canada). The DNA fragments were separated by horizontal electrophoresis on 6% agarose gels. Each gel was run for 60 min at 150 mA and refrigerated at 10 °C, stained with ethidium bromide, and photographed under ultraviolet lights. The "T" allele was 104 bp plus 92 bp plus 41 bp, and the "C" allele was 133 bp plus 92 bp plus 12 bp as shown in Figure 2. The small fragments (41 bp and 12 bp) were too small to be presented on the gel clearly.

Statistical analysis: Genotypes were obtained by direct counting with subsequent calculation of allele frequencies. Statistical analysis was undertaken using the χ^2 test and probability p values calculated using the Minitab program. A $p<0.05$ was considered significant. Adherence to the Hardy-Weinberg equilibrium constant was tested using the χ^2 test with one degree of freedom.

RESULTS

PCR-RFLP revealed (Figure 2) and gene sequencing (Figure 1) verified three distinct genotypes of *TGFβ1* codon 10 polymorphism: CC, CT, and TT. The genotype distributions for the *TGFβ1* codon 10 gene obtained from the patients and controls are shown in Table 1. The genotype frequency of 27.4%:57.7%:14.9% in the high myopia group and CC:CT:TT was 18.6%:45.3%:36.1% in the control group. The allelic frequency of C:T was 56.6%:43.8% in the high myopia group and 41.3%:58.7% in the control group. The genotype distribution of the *TGFβ1* codon 10 polymorphism showed statistical differences in the distribution of frequencies between high myopia patients and normal controls ($p<0.001$). People with the CC genotype have a higher probability than those with either the CT or TT genotype of having high myopia with a spherical equivalent over -6.00 D. There was also a significant difference between the two groups in the frequency of the CC and TT genotypes (OR=3.55, 95% CI=1.68-7.52). Furthermore, the distribution of the C allele was significantly higher in the high myopia group than in the control group ($p=0.001$; OR=1.83, 95% CI=1.27-2.63). The C allele and CC homozygote of *TGFβ1* codon 10 will cause patients to have a higher incidence of high myopia than the general population. We also calculated "power" to test the null hypothesis by SPSSR. Based on the genotype CC homozygote, there is a power of "100%" to yield a statistically significant result in this sample size.

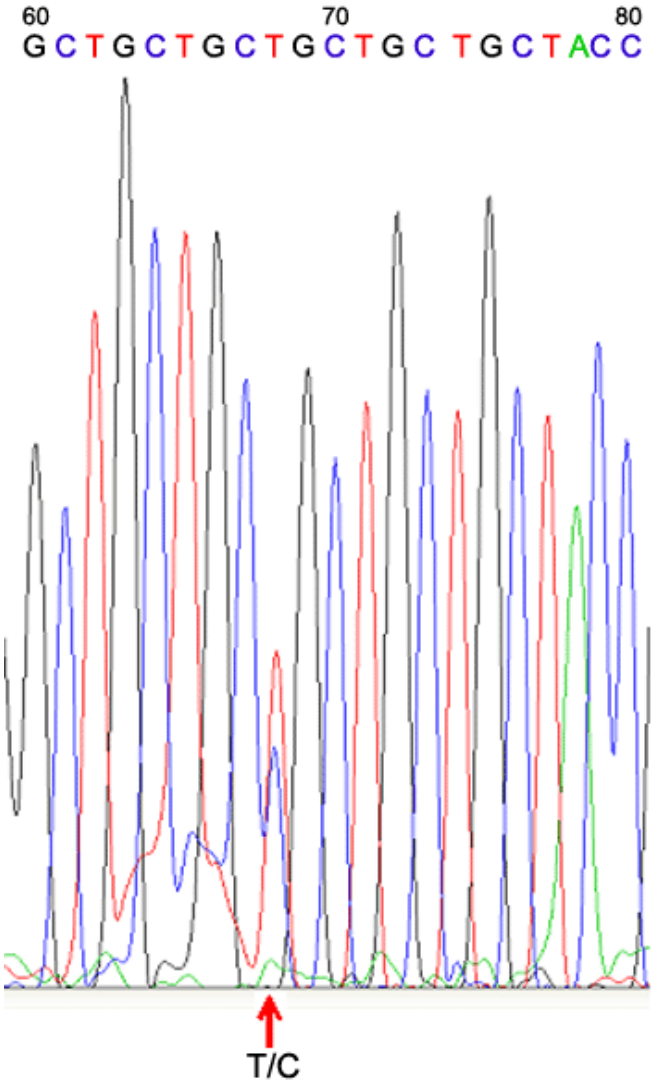

Figure 1. Sequence of transforming growth factorβ (TGFβ1) gene codon 10 polymorphism after digestION by *Msp*A1I. The arrow indicates the position of the TGFβ1 codon 10 (T/C) polymorphism; it is located at nucleotide 29, downstream from the ATG start codon.

DISCUSSION

There were stronger correlations than would be expected by chance in the study of the correlation between refractive error in parents and siblings [25]. Genetic studies of families with a history of pathological myopia have uncovered two polymorphisms and two separate loci for high myopia (chromosome 18p and 12q), indicating an autosomal dominant predisposition for the development of pathological myopia [26,27]. However, these genes cannot be solely responsible fully for the development of myopia in different ethnic groups with wide variability of the prevalence of myopia [27,28]. The difficulty here is the uncertainty surrounding environmental influences and genetic factors in the equation. Ideally, one set of genetic factors will interact with one set of environmental influences to produce identical outcomes, but it is unknown whether this is always the case. Therefore, to lessen the problem of multiple gene interactions and gene-environment interaction confounding the results, we mapped myopia with single nucleotide polymorphisms (SNPs) in this study. Individuals with higher education have a higher prevalence of myopia than people in the general population; therefore students in medical school are useful candidates for the control group in this study. The control group decreased the bias of environmental influence.

There are three highly conserved TGFβ isoforms found in mammals: TGFβ1, TGFβ2, and TGFβ3. The former two

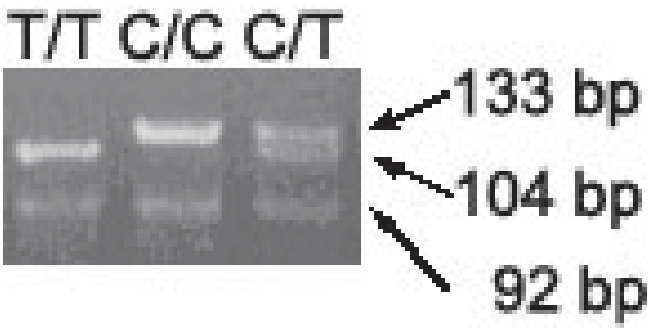

Figure 2. PCR based restriction analysis of transforming growth factorβ (TGFβ1) codon 10 polymorphism with *Msp*A1I cutting shown on 6% agarose electrophoresis. PCR of the polymorphic region resulted in a digestible fragment in lane 1 (104 bp plus 92 bp), an undigestible fragment in lane 2 (133 bp plus 92 bp), and a heterozygote in lane 3.

are profibrotic and inhibit matrix metalloproteinase (MMP) activity. In contrast, TGFβ3 plays an antifibrotic role [18]. It has been proposed that the level of TGFβ2 continues to decrease in form deprived myopia mammalians, whereas the decrease of TGFβ1 was constant between the duration of form deprived myopia. These evidences seem to emphasize the importance of TGFβ2 over TGFβ1. Nevertheless, in vitro experiments, using primary scleral fibroblasts demonstrated the capacity of all isoforms for increasing collagen production in a dose-dependent manner. The regulated roles of all three TGFβ isoforms will not be overemphasized. In this study, we scanned some known polymorphisms in *TGFβ1*; other polymorphisms in *TGFβ2* and *TGFβ3* also need to be examined in order to fully understand the role TGFβ plays in high myopia.

In the present study, we examined the hypothesis that polymorphisms within *TGFβ1* may influence the susceptibility of Taiwanse Chinese to high myopia. In *TGFβ1* codon 10, the frequency of the CC genotype was higher in the high myopia group than in the TT and CT genotype; there was also a significantly higher frequency of the C allele in high myopia patients. The polymorphisms in *TGFβ1* codon 10 studied here reveal an association with an increased risk of having high myopia in Taiwanese Chinese patients when compared with the control group. *TGFβ1* codon 10 polymorphism of *TGFβ1* is a suitable genetic marker of predisposition of developing of high myopia in Taiwanese Chinese. Because the control group had fewer participants than the high myopia group, we calculated "power" to test the null hypothesis by SPSS®. Based on the genotype CC homozygote, there is a power of "100%" to yield a statistically significant result in this sample size. Consequently, the sample size in this study was enough to represent the high myopia group in Taiwanese Chinese.

The *TGFβ1* genotype and allelic frequencies were in Hardy-Weinberg equilibrium in control subjects ($p>0.05$) but the genotype found in the high myopia group was not in Hardy-Weinberg equilibrium in control subjects ($p<0.05$). It is possible that the result was just an artifact of population stratification. This hesitancy can be resolved by the following explanation. It may be that the SNPs were fresh in our study population and had not yet reached Hardy-Weinberg equilibrium. Furthermore, the Hardy-Weinberg equilibrium principle is calculated for an autosomal recessive trait disease (when a mutant allele leads to a severe disorder in the homozygous state which is present in a population with an undetectable heterozygous state) [29]; that is, only the homozygous state contributes to the attention as a result of illness. Moreover, this principle is valid for a single gene disease [30]. Therefore, the simple formula ($P+Q=1$) can be applied to ($P^2+2PQ+Q^2=1$) to calculate the carrier frequencies and for counseling the risk of a disease. In this study, we used genetic polymorphisms to test the association of a gene with a commonly seen and multifactorial disease instead of single gene disease. Because the nucleotide polymorphism is not strong enough to resulti n a lethal disease, this allele will not eventually disappear or reach a frequency equilibrium. That is, selective disadvantage for individuals with this allele will not occur. Furthermore, ethnic difference does exit. The literature has reported polymorphisms from Caucasians such as *TGFβ1* (-800) [31], *THSR* (D36H) [32], and collagen-related gene (*COL3A1* gene exon 32 and *COL1A1* intron 1) [33] which have been noted in Caucasians were not found in Taiwanese Chinese in our previous study. Therefore, we believed that this distribution existed in our ethnic group and was not produce by population stratification and thus would not affect the validity of our results. Nevertheless, replication of this study in a separate group of subjects is important to verify the role of the *TGFβ1* gene codon 10 gene polymorphism in myopia.

Table 1.

TGFβ1 (CCG>CTG)	Myopia patients (%)	Controls (%)	p value
Genotype			
CC	55 (27.4)	16 (18.6)	<0.001
CT	116 (57.7)	39 (45.3)	
TT	30 (14.9)	31 (36.1)	
Allelic frequency			
Allele C	226 (56.6)	71 (41.3)	0.001
Allele T	176 (43.8)	101 (58.7)	

A χ^2 test was used to compare the distribution of genotype and alleles of *TGFβ1* codon 10 polymorphism in healthy (86 persons) and POAG (201 persons) patients.

A correlation between *TGFβ1* codon 10 gene polymorphism and blood pressure change had been reported [20]. A recent study from Turkey revealed that hypermetropia correlated with hypertension [34]. This association may be bridged by TGFβ1 and is worthy of further investigation.

Complex diseases such as diabetes, cancer, asthma, and arthritis are probably due to subtle changes in multiple genes caused by environmental and lifestyle factors. Investigating the genetics of common and complex disorders such as myopia remains one of the great challenges in human genetics. Myopia is considered to be a complex and multigenic condition involving several overlapping signaling pathways, each one mediated by a group of distinct genetic profiles. Therefore, studying the genetic polymorphisms of myopia-related genes can further clarify the relationship between genetics and myopia. The association between myopia and various genetic markers has helped increase our knowledge of prevention and treatment of myopia. The relationship between TGFβ and scleral remodeling during the development of myopia is evident [13,35]. Researchers had reported that six SNPs correlated with high myopia in Hong Kong [36]. Our study adds support to the idea that the *TGFβ* gene contributes to the pathogenesis of myopia. It had been noted that protein expression levels of TGFβ is downregulated in patients with high myopia [17]. Further investigations of the protein translated by the gene are required to identify the role *TGFβ* codon 10 polymorphism plays in myopia. Future work is needed to identify other regions on the *TGFβ1* gene and other genes of *TGFβ* isoforms. The ultimate aim is to determine how these regions

relate to each other to collectively affect the expression of TGFβ1 and its activity. Genes further up- and downstream of *TGFβ1* also need to be investigated, as it is likely that a number of genes will form the genetic background in individuals with myopia, upon which environmental factors will act, to give rise to myopia. In the future, we also need to replicate this analysis in a separate population group, such as a nonstudent population, and to analyze the genotypes of the parents of the highly myopic subjects in order to elude the possibility of bias in this study. We also should analyze the data from the 3,000 subjects across the spectrum of refractive error in order to understand the relationship between *TGFβ1* and the severity of myopia.

In conclusion, we observed that the frequency of the CC homozygote was much higher in the high myopia group than in the control group. People who have the CC homozygote may be at greater risk for developing high myopia. Therefore, we conclude that *TGFβ1* codon 10 polymorphism is associated with high myopia and is a candidate genetic marker of the disease.

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