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病理性近視之基因體篩選(2/3)

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中文摘要

台灣現在的學童近視比率，在全世界僅次於新加坡，稱為「近視王國」也不為過。在近十年來的研究一直認為近視是由近距離長時間工作和不正確的過度使用眼睛，所造成睫狀肌不能放鬆和眼球增長的一種眼病變。但是這些環境因素實在不能完全解釋華人的高盛行率近視，由其在一些地區如台灣、香港、新加坡等地華人移民所構築的地區。這些地區的高度近視也是相當可觀。

高度近視併發症的嚴重均可導致失明，如：視網膜剝離、黃斑部變性、青光眼、白內障等。以往高度近視被認為和遺傳有相大的關係，在白人和華人的流行病學研究當中都證明了這點。在基因體的研究當中也認為和染色體 12q、18p 有關。此外所謂 nanophthalmos 和病理性近視的病理表現是完全相反，這個疾病也可以提供我們研究病理性近視的染色體研究基礎，而 nanophthalmos 和染色體 11 有相當的關連。由於台灣高度近視的病患也相當多，我們認為如果可從高度近視的基因體研究切入來探討近視的遺傳可能性，可能是一個相當有利的角度，目前全世界對這方面的研究也在激烈的競爭著，而近視是我們的國病之一，我們更有義務進行這個研究。

我們希望能從數個可能基因的單核苷酸多型性切入，以病例對照的方式來研究這些可能基因的是否在高度近視和正常人之間有差異。如果時間和經費許可，我們計劃以全基因體分析、連鎖分析、或連鎖不平衡分析來找出其他可能的近視有關基因。我們有下列三大目的：一是找出近視產生的可能遺傳因素，二如果知道產生近視的可能基因之後，我們期望能利用藥物或方法來抑制已產生近視的進行，三是找出近視併發症的危險因子及預防方法。近視在台灣的嚴重性就如同肝炎一樣，所以更需要更多人的投入研究，才能解決這方面的問題，我們希望我們的研究能解決台灣最嚴重的視力問題。

ABSTRACT

Myopia is common in the Taiwan, and its cost to society is high. A nationwide survey which is performed in 2000 to determine the prevalence and severity of myopia among schoolchildren in Taiwan and to compare these findings with the results of the last survey performed in 1995 showed that the myopia rate increased from 20% at 7 years, to 61% at 12 years, and 81% at 15 years. A myopic rate of 84% is found for schoolchildren aged 16 years through 18 years. The mean refractive index reached myopic status at the age of 8, and increased to -4.12 D in girls and -3.15 D in boys at the age of 18 years. The prevalence of high myopia (> -6.0 D) at the age of 18 years is 24% in girls and 18% in boys. The increase in axial length corresponded with the progression of myopia. The prevalence and severity of myopia in schoolchildren in Taiwan in 2000 increased compared to 1995, with the most severe increases occurring in younger age groups.

Pathologic myopia, which is a well-defined disease with severely increased axial length and equatorial diameter, is presumed to be inherited with a Mendelian rule. In Taiwan, the incidence of myopia, even high myopia, is extremely high. The socioeconomic cost of high incidence of myopia is a very serious problem in Taiwan. It is hard to explain why only the environmental factors induce the high incidence rate of myopia in this region. There must be existed some genetic factors in the development of myopia in this region. If we find the possible genes in myopia, we will be able to find the possible environmental stimuli which can influence the expression of the genetic components. As aforementioned, the Mendelian transmission of pathologic myopia in Chinese is confirmed by genetic epidemiological study. Therefore, we are very interested in the possible genetic factors, chromosome, in the role of development of pathologic myopia. Through the discovery of possible genetic components of pathologic myopia, we can further define these genes in the general myopia as the role of GLCA1 in juvenile glaucoma.

Linkage disequilibrium (LD) analysis, which effectively incorporates the effects of many past generations of recombination, has often been instrumental in the final phases of gene localization. These successes have fueled hopes that similar approaches will be effective in localizing genes underlying susceptibility to common, complex diseases.

In present study, we approach the myopia gene in various chromosomes through the modern technology. First, we would like to screen SNPs in several possible candidate genes in age-matched patients with pathological myopia and control group through a case control study for chromosome 11, 12, and 18. DNA sequencing of these genes will be performed to find out the possible polymorphisms/mutations in the

family with well-defined pedigrees. Finally, we will use the model of linkage analysis and genome wide scanning in certain families with pathological myopia.

INTRODUCTION

Pathogenesis of Myopia

Myopia is a complex disease involving multiple interacting genetic and environmental factors. Environmental factors, such as educational level, occupation, and individual income, have been associated with the prevalence of myopia.[1] Other personal factors, such as reading habits and use of computers, may also affect the progression to high myopia.[2;3] It is believed that excessive or sustained accommodation produces tension in the ciliary muscle and with it, a tension in the extraocular muscles of convergence.[4;5] Regarding the pathogenesis of myopia, heredity is thought to be the basic determinant of ocular refraction in previous textbook, but there are also numerous agents can produce both temporary and permanent myopias.[6] High myopia is also termed "pathological" myopia because of its potential complications. It is usually defined as a refraction error equal to or below -6 diopters (D) in each eye. It is caused by excessive axial elongation that primarily involves the ora-equatorial area and the posterior pole. Peripheral fundus changes and posterior staphyloma formation are ophthalmoscopic evidences of this process. Pathological myopia often accompanied by glaucoma, cataracts, macular degeneration, and retinal detachment, leading to blindness when the damage to the retina is extremely severe.

High myopia is especially common in Asia.[7;8] In Japan, pathologic or high myopia reportedly affects 6% to 18% of myopes and 1% to 2% of the general population.[6] Comparative prevalence rates from different countries show considerable variability, but confirm that myopia affects a significant proportion of the population in many countries.[6;8;9] Pathologic myopia occurs primarily because of increased axial length of the eye rather than corneal or lenticular conical changes. Population and family studies in Chinese have provided evidence for a genetic component to pathologic myopia.[10] The incidence of myopia among school children in Taiwan approaches 70%, and the prevalence of high myopia (> -6.0 D) at the age of 18 years is 24% in girls and 18% in boys, which suggest that this population is genetically susceptible to myopia.[11-13] Children of myopic parents are more likely to have myopia than are children of nonmyopic parents.[14;15] The ocular components (axial length, anterior chamber depth, and corneal curvature) and refractive errors of MZ twins are more closely aligned than are those of DZ twins.[16] **Therefore, it is possible to search a potential candidate gene for myopia through the genomic study of pathological (high) myopia either with a genetic linkage study or with a case control study.**

Present Condition of Genomic Study in Pathological Myopia

Although the inheritance of myopia is not clear at the present time, several genealogical studies have shown autosomal dominant or autosomal recessive modes of inheritance of pathological myopia could be found in some cases. Rare cases of sex linked transmission have also been observed. For example, a X-linked recessive form of myopia has been mapped and was designated the first myopia locus, MYP1 [17] Recently, several autosomal dominant loci have been mapped , MYP2, on chromosome 18p,[18-20] MYP3 on chromosome 12q,[20] MYP4 on chromosome 7q[21] and new locus maps to the long arm of chromosome17.[22]

The Importance of Localization of Pathologic Myopia Gene in Taiwan

As aforementioned, Chinese have provided evidence for a genetic component to pathologic myopia.[10] The incidence of myopia among school children in Taiwan approaches 70%, and the prevalence of high myopia (> -6.0 D) at the age of 18 years is 24% in girls and 18% in boys, which suggest that this population is genetically susceptible to myopia.[7;11;13] **The prevalence of high myopia in Chinese is different from the situation in Caucasian.** But in previous Study, Children of myopic parents are more likely to have myopia than are children of nonmyopic parents.[14;15] The ocular components (axial length, anterior chamber depth, and corneal curvature) and refractive errors of MZ twins are more closely aligned than are those of DZ twins.[16;23;24] These studies provides a strong clue for us to study the possible candidate gene for pathologic myopia, which itself can be used to trace the gene for myopia. Pathologic myopia does not relate to other ocular and systemic disease,[25] it can be a good candidate to approach the myopia gene. **In the present study, we will also use the model of genomewide microsatellite genotyping to study the possible candidate of pathological myopia. For a case control study, the candidate genes which we will sequence the chromosomal DNA will be lumican, decorin, laminin, pax6, and DSPG3 to find out the possible mutation in the family with well-defined pedigree.[26-28]**

METHODS

Subjects

This project will comprise 300 cases during 2002-2005, at the Department of Ophthalmology, National Taiwan University Hospital according to a unified ascertainment protocol. They are unrelated Chinese subjects with high myopia $\leqq$ -6.00D. The diagnosis of myopia is determined by the refractive error. Anisometropic individuals, with a refractive error of $\leqq$-6.00 D for one eye and $\leqq$-6.00 D for the

other eye, with at least a 2-D difference between the two eyes, are considered unaffected. Individuals are excluded if there is known ocular disease or insult that could predispose to myopia, such as retinopathy of prematurity or early-age media opacification, or if they had a known genetic disease associated with myopia, such as Stickler or Marfan syndrome. And three hundred unrelated control subjects are recruited from patients who attended the Hospital for conditions other than high myopia. They and their family members do not have eye diseases except senile cataract, floaters, and itchy eyes. The control subjects are non- or mildly myopic, with refractive errors ≧-1.00D of either eyes. All patients and controls involved in this study are similar in social background and are from the local ethnic Hans Chinese population, with no ethnic sub-division. For linkage analysis, well-defined families as mentioned above with complete characterization of all members in three generations will be enrolled for genome-wide scanning and linkage study.

Affection status is difficult to determine for a common complex quantitative trait such as myopia. Despite the fact that refraction depends on corneal curvature, lens power, and axial length (measurements difficult to obtain in most of our participants), historically in the literature pathologic myopia has been defined as a refractive error less than -6.00 D. The degree and progression of myopia has environmental influences as well. An affection status of -6.00 D degrees of myopia is chosen to select for the more severe phenotypic form of this disorder, under the assumption that this will disproportionately represent a genetic etiology. This is further supported by the low frequency of pathologic myopia represented in the general population.[25]

Anisometropic individuals are classified as "unaffected" to insure that the phenotype/genotype is as specific as possible. Anisometropia may be secondary to a nongenetic event, such as inadvertent occlusion of one eye or unilateral ptosis [29] or retinal hemorrhage during early childhood [30]. There may not be residual structural changes observable during a routine screening of an adult.

An ophthalmology examination is performed by one of the authors (Y.F.Shih) at the time of sample collection, for most participants, and for at least one member of each family. For family members who are not locally available, examination records are obtained from the individual's ophthalmologist. The ophthalmologic evaluation included retinoscopy, a slit-lamp evaluation of the anterior segment, measurement of intraocular pressure, and a fundus examination, with special notation on the health and degree of cupping of the optic nerve head. Cycloplegic retinoscopy is performed in children age 10 years. In most instances, at least one affected adult from each family also underwent axial-length measurements of their eyes and keratometry measurements of their corneas. Most participants declined to have these measurements taken. Venous blood is collected after informed consent is obtained.

This study is approved by National Taiwan University Hospital

Mutation Aanalysis

PCR amplication

Genomic DNA was extracted from 3 mL of whole blood with a kit (PureGene, Gentra systems, Minneapolis, MN). Polymerase chain reaction primers that would amplify each exon of the candidate gene from genomic DNA templates were designed from published sequence information. PCR for the DNA fragments provided was performed in a total volume of 25 mL containing 50 ng of genomic DNA, 0.12 mM of each primer, 100 mM dNTPs, 0.5 units of AmpliTaq Gold^{TM} enzyme (PE Applied Biosystems, Foster City, CA), and 2.5 mL of GeneAmp 10X buffer II (10 mM Tris-HCl, pH = 8.3, 50 mM KCl), in 2 mM $MgCl_2$ as provided by the manufacturer. Amplification was performed in a thermal cycler (model 9700; ABI Prisms).

Direct sequencing analysis for SNPs in Laminin, Decorin, Lumican, Pax6, and DSPG3

Samples that displayed variant elution peaks were selected for confirmatory direct sequencing. The corresponding amplicon was amplified once more from genomic DNA and the PCR product was purified using Gel / PCR DNA Fragment Extraction Kit (Geneaid). The PCR products were sequenced in both directions, using the same primers as in the PCR amplification. Sequencing was performed with BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Inc.) on an ABI Prism™ 3100 DNA sequencer according to standard conditions recommended by the manufacturer.

Statistical Analysis

Either the $\chi 2$ tests or the Fisher exact test was used to compare the allele frequencies of sequence alterations in patients and control subjects. SNPs with $P < 0.20$ were selected for the logistic regression analysis. In contrast to univariate analysis, which considers only one SNP at a time, logistic regression analysis is a multivariate analysis method that deals with all selected SNPs as a whole in the same model. All variables were considered to be categorical in this analysis. The dependent variable was disease status (patient, 1; control subject, 0), and the independent variables were SNPs (homozygote, 2; heterozygote, 1; wild type, 0). The logistic regression model was optimized by using a backward approach. The significance of interactions between SNPs was estimated by adding corresponding interaction items in the model. An optimal model was established when all the independent variables (SNPs) were significant ($P < 0.05$) in the model. Allelic frequencies of all detected SNPs were also assessed for Hardy-Weinberg equilibrium. Statistical analyses were

performed on computer (SPSS software, ver. 10.1; SSPS Science, Chicago, IL).[31]

RESULTS and DISCUSSION

In our previously study, we investigated the relationship between the polymorphisms of several candidate genes (decorin, lumican, DSPG3, and PAH) and high myopia through a case control study. We collect eighty-seven unrelated Chinese subjects with high myopia of -6.00 D or less in both eyes at the National Taiwan University Hospital (NTUH). Twenty-four unrelated control subjects were also recruited from patients who attended the hospital for conditions other than high myopia. The ages of both groups were restricted from 20 to 45 years old. The control subjects had no or mild myopia, with refractive errors of -1.00 D or less in either eye. After approved by IRB at NTUH and informed consents were collected, all study subjects received complete ocular examinations, and blood samples were collected for DNA extraction. DHPLC and direct DNA sequence were performed to check the possible SNPs in these four candidate genes. Then, χ^2 test was conducted to investigate the genotypic and allelic distribution between high myopic groups and control groups.

For 4 decorin (rs1803344，rs3138268，rs2070985，rs2070984), 7 lumican (rs1802763, rs1802743, rs2300588, rs3741835, rs3741834, rs3759223, rs3759222), 2 DSPGs (rs1135866 and rs1920748), 2 PAH (rs1042503 and rs3828474) and 4 pax6 (rs2071754, rs644242, rs2071164, rs1894620) SNPs, we used a case control study and χ^2 test to identify the relationship of these SNPs and pathological myopia. Unfortunately, there was no statistical difference of the allelic ($p \leqq 0.2$) or genotypic ($p=1$) distribution between myopic groups and control group.

However, according to the results of single nucleotide polymorphism we selected, sequence alternation really exists in both cases and controls. Although, the preliminary study showed that there is no allelic or genotypic association between the polymorphisms we selected and high myopia. We reason these results with several possibilities. First, the sample sizes are not large enough to reveal the possible statistical significance between the myopic and control groups. Therefore, collection of enough number of blood samples is the most important goal of the project in the following study. It is generally believed that the complete human sequence will reveal at least a million SNPs in nonrepetitive sequences of coding regions, including introns and promoters. So large-scale genotyping is crucial to define the association between the polymorphisms/mutation and diseases. Further more, SNPs rs3828474 (from C to T) was found in our study, but it is not found in the database of N.C.B.I. (C→G). This is a very interesting finding in our study. We suspect the SNP may be unique for Taiwanese people. In order to prove that, we need to collect more samples and

compare it with other databases

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