Evaluation of MMP2 as a candidate gene for high myopia

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Purpose: Matrix metalloproteinase 2 (MMP2) has been shown to be expressed in the human sclera, and is increased in the sclera of the eye with myopia induced by form deprivation in chicks when compared with the control eye. The purpose of this study was to examine the relationship between high myopia and MMP2 in a mainland Han Chinese population.

Methods: Four hundred unrelated patients with high myopia and 400 normal controls in a mainland Han Chinese population were studied. All the subjects were genotyped for 20 tag single nucleotide polymorphisms (SNPs) in MMP2 with the dye terminator-based SNPshot method. The distribution of the genotypes in the cases and controls was compared with a χ2 test. Screening for mutations in the coding regions and the adjacent intronic regions of MMP2 was performed in 200 patients with high myopia and 200 normal controls by direct sequencing.

Results: None of the 20 tested SNPs showed significant association with high myopia in this study. Seven variations were detected upon sequencing of the coding regions and the adjacent intronic regions of MMP2 in 200 subjects with high myopia and 200 normal controls. One novel variation, c.1287G>A (p.K429K), was detected in 79 of the 200 patients with high myopia (65 heterozygous and 14 homozygous) and in 84 of the 200 controls (67 heterozygous and 17 homozygous). The c.1810G>A mutation (p.Arg500His) was detected in three of the 200 patients with high myopia (65 heterozygous and 14 homozygous) and in 84 of the 200 controls (67 heterozygous and 17 homozygous). The c.1810G>A mutation (p.Arg500His) was detected in three of the 200 patients with high myopia but not in the controls. The five other variations, known as polymorphisms, were detected in the case and control groups.

Conclusions: We found no evidence that MMP2 is responsible for high myopia in these Han Chinese subjects and hence is unlikely to be important in the genetic predisposition to high myopia. Our results imply that MMP2 may not play a major role in high myopia in the Han Chinese population.

Myopia, the most common eye disease worldwide, is also the leading cause of visual impairment. Myopia can be classified as low, medium, or high myopia. High myopia is defined as having a spherical equivalent of less than or equal to −6.00 diopter sphere (DS) and an axial length longer than or equal to 26.0 mm [1]. High myopia is a complex disease associated with environmental and genetic factors. Environmental factors such as work at close range and prolonged reading are suggested to be involved in the progression of myopia [2]. Family studies have shown an increased risk of myopia in children with myopic parents, compared with those with no myopic parents [3], as well as a fourfold increased sibling risk [4]. However, although abundant evidence has demonstrated that genetic factors play an important role in the development of high myopia, the exact molecular basis of high myopia and the genes that cause a predisposition to this disorder are still unclear.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that degrade extracellular matrix proteins; more than 20 members of the MMP family have been identified in humans [5]. Among them, MMP-1[6], -2[6,7], -3[6], and -14[7] have been shown to be expressed in the human sclera and are potential participants in scleral remodeling. MMP2 is increased in the sclera of the eye with myopia induced by form deprivation in chicks when compared with the control eye [8-10]. Increased scleral MMP2 expression in form-deprivation myopia has been shown in tree shrews at the protein [11] and the messenger RNA (mRNA) levels [12,13] and in guinea pigs at the protein level [14]. An increased MMP2 transcript level has also been found in human scleral fibroblasts mechanically stretched in an in vitro system [15] and in lens-induced myopia in the tree shrew [16]. For high myopia, variations in the expression of the MMP genes in the sclera due to polymorphisms in the promoter regions can cause variations in scleral remodeling.
Refractive errors (Diopter, ±)
Age (Years)*

| Groups    | Number | Age (Years)* | Refractive errors (Diopter, ±) | Axial length (mm) |
|-----------|--------|--------------|--------------------------------|-------------------|
| Cases     | 400    | 33.3±10.7    | −9.97±3.14 (OD), −9.79±3.26 (OS) | 27.56±1.85 (OD), 27.85±1.78 (OS) |
| Controls  | 400    | 51.1±9.5     | −0.41±0.56 (OD),               | 23.37±0.71 (OD), 23.42±0.59 (OS) |

*The age when the cases and controls were recruited. ±: standard deviation; OD: right eye; OS: left eye; mm: millimeter.

activity, a key factor in axial elongation of the eye. The expression of many MMPs is regulated mainly at the transcription level, and SNPs in the promoter region of several MMP genes have been shown to be transcriptional regulators [17]. MMP2 has been shown to have functional SNPs in the promoter regions, such as rs243865 (MMP2 C-1306T) [18] and rs2285053 (MMP2 C-735T) [19]. In an Amish population, rs9928731 showed evidence of association with refractive phenotypes, located between the sixth and seventh exons of MMP2 [20]. However, no significant difference was detected in the distribution of the two SNPs (rs243865 and rs2285053) and the other 17 SNPs in MMP2 between high myopia cases and general-population controls in a Japanese population [21] and in a Hong Kong Han Chinese population [22], respectively.

In this study, thus, we sought to evaluate MMP2 as a candidate gene for high myopia. We examined the relationship between high myopia in a mainland Han Chinese population composed of 400 subjects with high myopia and 400 normal controls. All the coding regions were sequenced to screen novel variants in MMP2.

**METHODS**

**Subjects:** In total, 400 patients with high myopia and 400 matched normal controls were recruited from Sichuan Academy of Medical Sciences & Sichuan Provincial People’s Hospital. Clinical information about the cases and controls is listed in Table 1. This study was approved by the Institutional Review Boards of the Sichuan Academy of Medical Sciences & Sichuan Provincial People’s Hospital. Written informed consent was obtained from all subjects before the studies, and the subjects underwent an extensive, standardized examination by ophthalmologists, including visual acuity testing, a detailed clinical examination, optical coherence tomography, and ocular imaging before genetic testing. Refractive error and the radius of corneal curvature in the horizontal and vertical meridian were measured using an autorefractor (KR8800, Topcon, Tokyo, Japan). Subjects with syndromic disorders or systemic diseases that could lead to myopia were excluded. High myopia is defined by a spherical equivalent of less than or equal to −6.00 diopter sphere (DS) and an axial length longer than or equal to 26.0 mm in affected patients’ eyes. For the controls, the criteria were a spherical equivalent from −1.0 to +1.0 DS, an axial length less than or equal to 24.0 mm, and no evidence of disease in either eye.

**Single nucleotide polymorphism selection and genotyping:** We selected 20 tag SNPs, including rs243865, rs2285053, and so on in MMP2, to be genotyped in the mainland Han Chinese population (400 patients with high myopia and 400 normal controls). Venous blood was drawn from cubital veins of each subject and collected in an EDTA tube. The blood samples were preserved at −80 °C before genomic DNA extraction. Genomic DNA was extracted from the blood by serial phenol/chloroform extraction and ethanol precipitation. SNP genotyping was performed with the dye terminator-based SNaPshot method (Applied Biosystems, Foster City, CA). SNP analysis was performed on the ABI 3130 Genetic Analyzer (Applied Biosystems). In brief, the PCRs (10 μl final volume) contained 50 ng of genomic DNA, 1 μl of each primer (10 pmol/μl), 1 μl of 10 buffer (Takara Bio Inc., Shiga, Japan), 0.8 μl of deoxyribonucleotide triphosphates (2 mmol/l; Takara Bio Inc.), 0.4 μl MgCl₂ (2.5 mmol/l; Takara Bio Inc.), and 0.1 μl of ExTaq polymerase (5 U/μl; Takara Bio Inc.). The product was then processed according to the ABI SNaPshot protocol.

**Mutation analysis:** Screening for mutations in MMP2 was initially performed in 200 patients with high myopia and 200 matched normal controls. Amplified PCR products of all the coding exons and adjacent introns (the sequences of all primers used in this study are summarized in Table 2) were purified with spin columns (QIAquick, Qiagen, Valencia, CA) and sequenced directly (BigDye Terminators Sequencing Kit; Applied Biosystems) in both directions with an automated genetic analysis system (3130; ABI).

**Statistical analysis:** Hardy–Weinberg equilibrium (HWE) for each SNP polymorphism was tested with the χ² test. P values of the SNPs were calculated using an additive model. The unadjusted odds ratios (ORs) of the alleles and genotypes between the cases and controls were estimated with the χ² test. All statistical analyses were performed using the software SPSS 15.0 (SPSS Inc., Chicago, IL). Genetic power was
calculated by using the software PS: Power and Sample Size Calculation (PS version 3.0.43) [23].

RESULTS

Clinical data: Eight hundred unrelated subjects were included in the study. The cohort consisted of 400 cases and 400 controls. For subjects with high myopia, the spherical refractive errors of the right and left eyes were –9.97±3.14 D and –9.79±3.26 D, respectively; the axial lengths of the right and left eyes were 27.56±1.85 mm and 27.85±1.78 mm, respectively (Table 1).

Single nucleotide polymorphism analysis: In total, 20 SNPs were genotyped in HWE (p>0.05) for MMP2, including 16 in intronic regions, one in exon 4, and three upstream of the 5′ region (Table 3). After association analysis of these SNPs, none of the 20 tested SNPs showed significant association with high myopia in the mainland Han Chinese population (400 patients with high myopia and 400 matched normal controls) (Table 3). In the previous study [20], rs9928731 in the MMP2 gene showed evidence of association with refractive phenotypes (p=0.00026) in Amish families. Therefore, we calculated the power of rs9928731 for detecting moderate/low ORs in the range of 1.2–1.8 based on our sample size. The power values ranged from 42.4% to 100% (42.4%, 72.0%, 90.4%, 97.60%, 99.50%, 99.90%, and 100.00% for OR=1.2, 1.3, 1.4, 1.5, 1.6, 1.7, and 1.8, respectively). The data suggested sufficient power to reject the null hypothesis of no association between rs9928731 and high myopia. Thus, the genotyping results indicated that there were no significant differences in the SNPs between the patients with high myopia and the controls.

Mutation analysis: Complete sequencing of the coding regions and the adjacent intronic regions of MMP2 in the 200 subjects with high myopia and 200 normal controls identified seven variations (Table 4). One novel variation, c.1287G>A (p.K429K), was detected in 79 of the 200 patients with high myopia (65 heterozygous and 14 homozygous) and in 84 of the 200 controls (67 heterozygous and 17 homozygous). This variation would not affect the encoded amino acid. The six other variations were known polymorphisms, including three

| Exon | Primer sequence(5′-3′) | Product size (bp) | Annealing temperature (°C) |
|------|------------------------|------------------|---------------------------|
| 1    | F: GTACTGTGCCATCCTAAT  | 445              | 56                        |
|      | R: CTGTCTGACTTCTTTTCT   |                  |                           |
| 2    | F: CACACACACGAGCACA     | 614              | 62                        |
|      | R: CCATATTGGGAGCAGCAG   |                  |                           |
| 3 and 4 | F: TTTCAGGGTCTAGTGTCACGG | 677          | 62                        |
|      | R: GCACCTATGGGTCATACG   |                  |                           |
| 5    | F: GAGGAGGAGCTCTTTACCA  | 463              | 62                        |
|      | R: GGATGTCATTCCGACAGAG  |                  |                           |
| 6    | F: AGCGCTATGTCATTGCTT   | 378              | 62                        |
|      | R: CTGGGTAGTTGGTGCTCT   |                  |                           |
| 7    | F: ACAAGAAGACTTTTGCGAC  | 595              | 59                        |
|      | R: TTCGGATAGGGAAGATTTA  |                  |                           |
| 8    | F: AGAGGACTGATTTGGTGAT  | 404              | 62                        |
|      | R: GGACAGAGCAGAGGGAG    |                  |                           |
| 9    | F: CAGGGGAGGAGATGTTTCC  | 561              | 61                        |
|      | R: AATGCTATCTGATTTGGG   |                  |                           |
| 10   | F: TGACTTCTAAAGCCCTCTG  | 375              | 59                        |
|      | R: AACTGTCGCTGCTTCTAC   |                  |                           |
| 11   | F: AAGGGCTAGTGCCAGTTTCC | 414              | 59                        |
|      | R: CAAGGAGGAGGAGGTCAGG  |                  |                           |
| 12   | F: TGGGCCTAGAGCTTCCCTC  | 309              | 59                        |
|      | R: TGTATGAGGAGCAGTGGA   |                  |                           |
missense variations (c.1026T>C, c.1810G>A, and c.2172G>C) and three synonymous variations (c.1460T>C, c.1691G>C, and c.2117C>T; Table 4). All except c.1810G>A ($\text{rs28730814}$) were detected in the patients with high myopia and the control groups. The c.1810G>A mutation (p. Arg500His) was detected in only three of the 200 patients with high myopia (three heterozygous), but not in the 200 controls. In this heterozygous variation, an arginine is replaced by a histidine in the encoded protein. No variation was identified in exons 1, 2, 3, 5, 10, and 12 of $\text{MMP2}$.

### Table 3. SNP Genotyping of the MMP2 Gene in 400 High Myopia and 400 Control Subjects

| SNPs       | Position (bp) | Allele* | Frequency of reference allele | P value | OR (95% CI) |
|------------|---------------|---------|-------------------------------|---------|-------------|
|            |               |         | Cases | Controls |           |             |
| rs11643630 | 54,067,960    | G/T     | 0.473 | 0.48     | 0.763 | 0.97 (0.80–1.18) |
| rs243865   | 54,069,307    | C/T     | 0.869 | 0.879    | 0.547 | 0.91 (0.70–1.23) |
| rs2285053  | 54,069,878    | C/T     | 0.704 | 0.681    | 0.329 | 1.11 (0.90–1.37) |
| rs1477017  | 54,074,663    | G/A     | 0.275 | 0.3      | 0.269 | 0.89 (0.71–1.10) |
| rs865094   | 54,074,733    | G/A     | 0.323 | 0.334    | 0.632 | 0.95 (0.77–1.17) |
| rs11076101 | 54,075,759    | C/T     | 0.866 | 0.86     | 0.716 | 1.15 (0.85–1.55) |
| rs17301608 | 54,076,111    | C/T     | 0.644 | 0.664    | 0.4   | 0.92 (0.74–1.12) |
| rs11646643 | 54,076,378    | G/A     | 0.131 | 0.116    | 0.362 | 1.15 (0.85–1.55) |
| rs1132896  | 54,077,036    | G/C     | 0.869 | 0.879    | 0.37  | 0.87 (0.65–1.17) |
| rs2241146  | 54,079,735    | G/A     | 0.778 | 0.773    | 0.811 | 1.03 (0.82–1.30) |
| rs9928731  | 54,080,512    | C/T     | 0.574 | 0.604    | 0.223 | 0.88 (0.72–1.08) |
| rs12599775 | 54,081,283    | C/G     | 0.13  | 0.119    | 0.495 | 1.11 (0.82–1.49) |
| rs243847   | 54,081,499    | C/T     | 0.416 | 0.409    | 0.76  | 1.03 (0.84–1.26) |
| rs243845   | 54,083,988    | G/A     | 0.709 | 0.684    | 0.277 | 1.12 (0.90–1.39) |
| rs243843   | 54,084,799    | G/A     | 0.431 | 0.449    | 0.481 | 0.93 (0.76–1.13) |
| rs183112   | 54,085,183    | G/A     | 0.734 | 0.756    | 0.302 | 0.89 (0.71–1.11) |
| rs1992116  | 54,085,392    | G/A     | 0.711 | 0.734    | 0.315 | 0.89 (0.72–1.11) |
| rs1639960  | 54,090,771    | G/A     | 0.28  | 0.268    | 0.575 | 1.23 (0.98–1.54) |
| rs243835   | 54,094,123    | C/T     | 0.389 | 0.378    | 0.643 | 1.04 (0.86–1.28) |
| rs1861320  | 54,098,541    | G/T     | 0.74  | 0.765    | 0.247 | 0.87 (0.70–1.10) |

* The alleles are named with reference to the sense/anti-sense strand of the respective gene.

### Table 4. MMP2 Variants Detected in 200 High Myopia and 200 Control Subjects by Direct Sequencing of All the Exons

| Location | Position (bp) | SNP ID | Allele* | Residue Change | Genotype Counts † | Allelic p |
|----------|---------------|--------|---------|----------------|-------------------|-----------|
|          |               |        |         |                | Cases | Controls |           |             |
| Exon 4   | 54,077,073    | rs11542001 | T/C | F239L | 0/1/199 | 0/3/197 | 0.32 |
| Exon 6   | 54,081,206    | rs243849 | T/C | D383D | 72/44/84 | 67/42/91 | 0.39 |
| Exon 7   | 54,083,320    | novel  | G/A | K429K | 14/65/121 | 17/67/116 | 0.51 |
| Exon 8   | 54,084,614    | rs2287074 | G/A | T460T | 32/67/101 | 28/60/112 | 0.25 |
| Exon 9   | 54,088,365    | rs28730814 | G/A | R500H | 0/3/197 | 0/0/200 | - |
| Exon 11  | 54,094,228    | rs10775332 | C/T | F602F | 13/60/127 | 11/63/126 | 0.93 |
| Exon 11  | 54,094,283    | rs16955280 | G/C | V621L | 0/2/198 | 0/1/199 | 0.56 |

† The genotype counts are presented as homozygote/heterozygote/wild-type.
DISCUSSION
Identifying the genes responsible for non-syndromic high myopia is very important but will be very difficult, although several loci for high myopia have been mapped [24-43]. However, no convincing causal genes have yet been identified at these loci. Differential MMP2 expression has been implicated in scleral remodeling in experimental myopia studies in tree shrews [11-13,16] and chicks [8]. In these form-deprivation animal models, myopic eyes show increased MMP2 mRNA expression compared with that in normally developing eyes, leading to increased collagen degradation and active sclera remodeling. A similar mechanism may be involved in common forms of heritable human refractive error. In this study, thus, we sought to determine whether MMP2 is associated with high myopia in a mainland Han Chinese population. First, we used a case-control study approach to examine the relationship between high myopia and the tag SNPs of MMP2 in a mainland Han Chinese population. Then all the coding regions were sequenced to screen novel variants in MMP2.

In this study, 20 SNPs were genotyped for MMP2, and none showed significant association with high myopia in the mainland Han Chinese population. The known variation, rs9928731, was previously reported to be associated with Amish patients with high myopia [20], suggesting that this variation is more likely to be a susceptibility polymorphism of high myopia. However, this variation was not replicated in our study. When the coding regions and the adjacent intronic regions of MMP2 in the 200 subjects with high myopia and the 200 normal controls were completely sequenced, one novel variation and six known variations were detected. All except c.1810G>A (rs28730814) were found in the patients with high myopia and the control groups.

Therefore, it is impossible to confirm or deny the susceptibility of the MMP2 gene with high myopia based on the current evidence, especially because of our limited understanding of complex diseases. To our knowledge, several studies have been conducted to investigate the association of MMP2 polymorphisms and refractive error phenotypes [18-22]. Nakanishi [21] detected no significant difference in the distribution of two SNPs (rs243865 and rs2285053) between high myopia cases and general-population controls. The researchers did not find statistically significant associations with these SNPs in a full analysis of 216 cases and 474 controls. A second study, which comprised 55 Amish and 63 Ashkenazi Jewish families including 358 Amish and 535 Ashkenazi Jewish subjects, analyzed four tag SNPs of MMP2 [20]. The study showed one SNP (rs9928731) was statistically associated with refractive phenotypes in the Amish subjects but not in the Ashkenazi Jewish subjects. The results suggested that the MMP2 gene was involved in refractive variation in the Amish population. Finally, a separate case-controlled study composed of 656 patients with high myopia and 654 controls demonstrated that there was no significant association with the 17 polymorphisms of MMP2 and high myopia in Southern Chinese subjects in Hong Kong [22]. Taken together, genetic and/or environmental heterogeneity most likely contributes to these differences in association results between ethnic groups.

As the weak linkage disequilibrium (LD) between common tag SNPs and rare casual variants, this indirect approach has low power in detecting association with rare variants. Rare variants could be identified by sequencing good candidate genes or even the whole genome for a very large number of samples [44]. However, when the role of the rare variants in high myopia was explored with DNA sequence analysis for the exons of MMP2 in small numbers of patients with high myopia, no fruitful results were found. We detected seven variations. The genotyping of these variations except c.1810G>A (rs28730814) were similar between the patients and controls (Table 4), suggesting that these variations are more likely to be polymorphisms and implying that this gene does not carry common sequence variants that are capable of influencing its function and/or regulation in the relevant ocular tissue. Additional studies for mutation screening are necessary to evaluate the role of the MMP2 gene in the genetic susceptibility to high myopia. In addition, the contribution of behavioral and environmental effects on high myopia should be considered.

In conclusion, we genotyped 20 SNPs at the MMP2 gene in a Han Chinese group composed of 400 patients with high myopia and 400 controls. None of the SNPs showed significant association with high myopia (p>0.05), and no novel variation causing high myopia was detected with direct sequencing in MMP2. Our results thus failed to identify MMP2 as a significant risk factor for high myopia in a mainland Han Chinese population. Therefore, the role of MMP2 in controlling refractive development requires further study and refinement in animal models and human genetic epidemiological studies.

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