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# Exclusion of lumican and fibromodulin as candidate genes in MYP3 linked high grade myopia

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**Purpose:** The proteoglycans lumican and fibromodulin regulate collagen fibril assembly and show expression in ocular tissues. A recent mouse knockout study implicates lumican and fibromodulin as functional candidate genes for high myopia. Lumican maps within the chromosome 12q21-q23 autosomal dominant high grade myopia-3 (MYP3) interval, and fibromodulin maps to chromosome 1q32. We screened individuals for lumican and fibromodulin sequence alterations from the original MYP3 family, and from a second high grade myopia pedigree that showed suggestive linkage to both the MYP3 interval and to chromosome 1q32.

**Methods:** A total of 10 affected (average spherical refractive error was -16.13 D) and 5 unaffected individuals from the 2 families were screened by direct DNA sequencing. Six primer pairs spanning intron-exon boundaries and coding regions were designed for the 3-exon 1804 base pair (bp) lumican gene. Two primer pairs for the 2-exon 2863 bp fibromodulin gene were designed. Polymerase chain reaction products were sequenced and analyzed using standard fluorescent methods. Sequences were quality scored and aligned for polymorphic analysis.

**Results:** Direct DNA sequencing of lumican amplicons yielded the expected sequence with no evidence of polymorphism or pathologic mutation. Sequencing of fibromodulin amplicons revealed 6 polymorphisms, 1 of which was novel. One polymorphism was a silent mutation, and five were in the 3' untranslated region. No polymorphism segregated with high myopia.

**Conclusions:** Although null and double null *Lum* and *Fmod* mouse models have been developed for high myopia, our human cohort did not show affected status association with these genes. Sequencing of the human lumican and fibromodulin genes has excluded them as candidate genes for MYP3 associated high grade myopia.

Myopia is a highly prevalent, complex phenotype involving genetic and environmental factors. Myopia affects approximately 25% of the adult population of the United States [1-5] and is a significant public health problem, especially in Asian populations, as it is associated with increased risk for visual loss [1,6-10]. The development of methods for preventing the onset or limiting the progression of myopia would be of considerable importance.

Previously, we reported significant linkage of autosomal dominant high myopia of -6.00 D or greater to a locus at chromosome 12q21-23 in a large German/Italian family (the MYO10 pedigree) [11]. The maximum LOD score with two point linkage analysis in this pedigree was 3.85 at a recombination fraction of 0.0010, for markers D12S1706 and D12S327. Recombination events identified flanking markers D12S1684 and D12S1605, which defines a 30.1 cM interval. This locus was named the high grade myopia MYP3 locus by the Human Gene Nomenclature Committee (OMIM 603221).

The development of high myopia involves anterior-posterior enlargement of the eye, scleral thinning, and frequent detachment of the retina resulting from stress associated with excessive axial elongation. The sclera, the white tough outer covering of the eye, is a connective tissue that provides the structural framework for defining the shape and axial length of the eye. The extracellular matrix of the sclera contains collagen fibrils in close association with proteoglycans and glycoproteins [12,13]. Alterations in any of these extracellular matrix components are likely to lead to changes in scleral shape, which in turn could affect visual acuity, as the axial length of the eye is a major component in determining ocular refraction [14-16].

A recent mouse knockout study, implicated the proteoglycans lumican (LUM) and fibromodulin (FMOD) as functional candidate genes for high myopia [17]. The study focused on how the morphology and ultrastructure of the sclera is affected in *Lum*$^{-/-}$*Fmod*$^{-/-}$ double deficient mice. The results showed that mice deficient in Lum and Fmod manifest certain features of high myopia. These features include structural changes in collagen fibrils in the sclera, thinning of the sclera, retinal detachment, and increased ocular axial length compared with those features in wild type mice.

LUM maps within the chromosome 12q21-q23 MYP3 interval [18], and FMOD maps to chromosome 1q32 [19]. LUM and FMOD are members of the small leucine rich proteoglycan (SLRP) gene family [20]. The core proteins of these proteoglycans are structurally related, consisting of a central region composed of leucine rich repeats flanked by

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disulfide bonded terminal domains. LUM is a keratan sulfate proteoglycan present in large quantities in the corneal stroma and in interstitial collagenous matrices of the heart, aorta, skeletal muscle, skin, and intervertebral discs [21,22]. FMOD exhibits a wide tissue distribution, with the highest abundance observed in articular cartilage, tendon, and ligament [23]. It has been suggested that FMOD participates in the assembly of the extracellular matrix by virtue of its ability to interact with type I and type II collagen fibrils and to inhibit fibrillogenesis in vitro [7,24]. Recent microarray studies in our laboratory confirm the expression of LUM and FMOD in human sclera [25].

We sought to determine if the LUM and FMOD genes are causally related to MYP3 associated high myopia by direct DNA sequencing of these genes. We screened subjects from the original MYP3 family (MYO-10 [11]), and from Pedigree-2 (a family that we report here shows suggestive linkage to the MYP3 locus and to a locus at chromosome 1q32).

## METHODS

*Patients:* The study protocol was approved by the Children's Hospital of Philadelphia Institutional Review Board on Human Subjects Research, and adhered to the tenets of the Declaration of Helsinki. Probands and affected subject representatives of the MYO10 family [11] and Pedigree-2 were studied (Table 1). Both pedigrees displayed an autosomal dominant transmitted form of high myopia. Clinical details regarding the MYO10 pedigree were published previously [11], and some of the clinical characteristics of these subjects previously reported in [11] are reproduced here as a convenience to

**Table 1. Subjects used in this study**

| | Refractive Error | |
|---|---|---|
| Subject | Right eye | Left eye |
| MYO10 Pedigree | | |
| 3 | -7.50 + 1.50 x 28° | -6.50 + 1.00 x 148° |
| 4 | +8.00 | +9.25 |
| 5 | -14.00 + 0.75 x 35° | -13.25 |
| 6 | -3.50 + 0.50 x 65° | -4.00 + 1.00 x 65° |
| 7 | -13.00 | -14.00 |
| 15 | -12.00 | -13.00 |
| 20 | -13.00 | -15.00 |
| 23 | -3.50 + 0.25 x 100° | -4.00 + 0.50 x 80° |
| 25 | Plano | Plano |
| Control | Plano | Plano |
| Pedigree-2 | | |
| 6 | -16.50 + 3.00 x 17° | -16.25 + 3.50 x 24° |
| 7 | -32.00 | -32.00 |
| 9 | -28.00 | -25.00 |
| 11 | -23.75 + 4.00 x 155° | -20.00 + 2.25 x 35° |
| 12 | +1.25 | +0.50 |
| 14 | Plano | Plano |
| Control | Plano | Plano |

A list of all subject DNA samples used for gene mutation screening from the MYP3 mapping study [11] and from Pedigree-2 of the present study. For the convenience of the reader, subject numbers and refractive errors from MYO10 family members are the same as Table 1 of reference [11]. The affected individuals for the MYP3 pedigree are subjects 3, 5, 7, 15, 20, and 23; subjects 6, 7, 9, and 11 are affected in Pedigree-2. The refractive errors of all control participants are provided. Subject 23, an 8 year old, should have a hyperopic refractive error. This patient is therefore affected. Subject 13 from Pedigree-2 was not used in mutation screening for economic reasons.

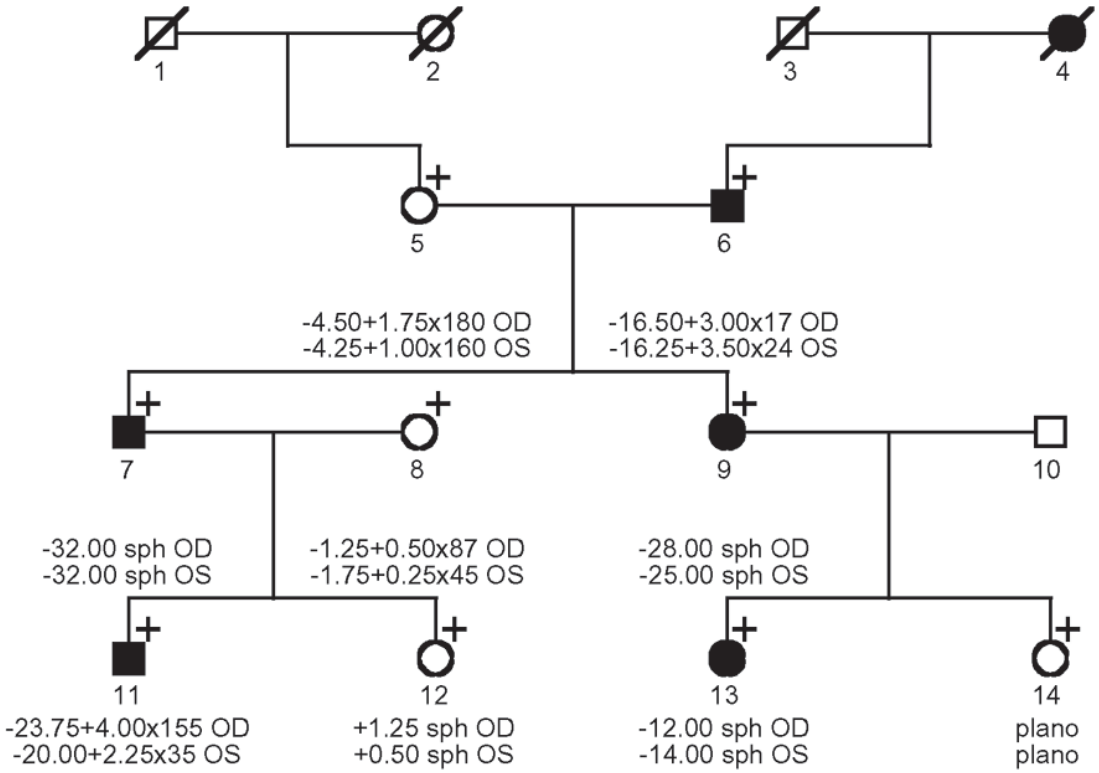


Figure 1. Pedigree-2, with familial high myopia. Pedigree-2 representing familial high myopia with 5 affected, 2 marry-ins, and 2 unaffected subjects and their refractive errors. Circles and squares denote females and males, respectively; blackened symbols denote affected subjects; a diagonal line through a symbol denotes a deceased subject; a plus sign indicates genotyped subjects.

the reader. Controls were obtained from family marry-ins, nonmyopic family members, and an unrelated subject. Figure 1 displays the family and member number of each individual in Pedigree-2 with refractive error. The criteria for selection included a history of onset of myopia before age 12 years in otherwise healthy affected subjects (parents and offspring), myopia of -6.00 D or higher, and two or more generations affected. The diagnosis of myopia was determined by the refractive error. Participants had no known ocular disease or insult that could predispose to myopia, such as a history of retinopathy of prematurity, neonatal problems, a known genetic disease, or connective tissue disorder associated with myopia, such as Stickler or Marfan syndromes.

Total genomic DNA was extracted from 10-15 ml of venous blood from all participants after informed consent was obtained. DNA was purified from lymphocyte pellets according to standard procedures using the PUREGENE kit (Gentra Systems Inc., Minneapolis, MN).

*Pedigree-2 Clinical Characteristics:* Pedigree-2 has 9 participating members, 5 of whom were affected. The average spherical refractive error for affected individuals was -22.00 D, ranging from -12.00 D to -32.00 D (Figure 1). Syndromic myopia linkage was excluded by using intragenic or flanking microsatellite polymorphic markers for Stickler syndrome type 1(12q13.1-q13.3), type 2 (6p21.3-p22.3), and type 2B (1p21); Marfan syndrome (15q15-q21.1); Ehlers-Danlos syndrome type 4 (17q21-q22); and juvenile glaucoma (1q21-q31).

*Genotyping Pedigree-2:* A genome wide linkage mapping study was performed on Pedigree-2, which showed suggestive linkage to the MYP3 locus and to an interval of chromosome 1q32. DNA analysis was performed as described elsewhere, with multiplexed primer pairs and fluorescent detection techniques initially using LI-COR DNA 4000 infrared sequencer (LI-COR, Lincoln, Nebraska) [26]. Confirmation genotyping was performed using an automated DNA sequencer

**Table 2. LUM and FMOD gene primers designed for mutation screening**

| Exon | Size | Position on contig | Primer Sequence 5'-3' | Primer |
|---|---|---|---|---|
| Contig NT_019546 | | | | |
| 1 | 710 | 14987011 | TTCTTTGCTCCAGTATTAGGGTCC | LUM1F |
| | | 14987721 | AAAAATGTTCTCACAGTGAGCTTCC | LUM1R |
| 2A | 919 | 14983585 | TTAATTGTTCCTTATCTGGATAC | LUM2AF |
| | | 14984504 | TGCAGCTTACTCATAACAAG | LUM2AR |
| 2B | 787 | 14984369 | GAAGCTCAAGTCAAGGTATTCG | LUM2BF |
| | | 14985156 | CTAACCTGAAAGCTGCTCACTAC | LUM2BR |
| 2C | 592 | 14984146 | TTTTAAGCTTGTTATAGGACAGA | LUM2CF |
| | | 14984738 | GCCTCCTGGAATCAAGTAT | LUM2CR |
| 3A | 1262 | 14979221 | TCACATAGCAGCTTCTTATCTA | LUM3AF |
| | | 14980483 | TCTTGATCTACCATGATTACTCT | LUM3AR |
| 3B | 547 | 14979222 | CACATAGCAGCTTCTTATCTA | LUM3BF |
| | | 14979769 | AAATACATATGGATGTTACTCTC | LUM3BR |
| Contig NT_004671 | | | | |
| 1 | 1579 | 14671023 | CATTTCCCAGCTGTCCCCCTACATCG | FMOD1F |
| | | 14672579 | TCACCCCAAATAGCCATGGTCCG | FMOD1R |
| 2 | 2611 | 14664068 | GAGTTGTAACATTTGCAACATGCTTGGTAC | FMOD2F |
| | | 14666650 | CTAATCTCACTGTGGCTTCAGGTTCGTAA | FMOD2R |
| 2-Nested | | 14666058 | CTGTGCTGGGCTGGTCGT | FMOD2(589F) |
| 2-Nested | | 14665613 | AATGGGCCAAACACACCT | FMOD(1034F) |
| 2-Nested | | 14665126 | TAACCCACCTGCCCTTTG | FMOD(1521F) |

Lumican (LUM) and Fibromodulin (FMOD) gene polymerase chain reaction primer pairs designed for mutation screening. Six primer pairs were designed for LUM gene (3 Exons). Two primer pairs and three nested primers were designed for FMOD gene (2 Exons). The forward and reverse primers are identified by an “F” and “R” suffix, respectively.

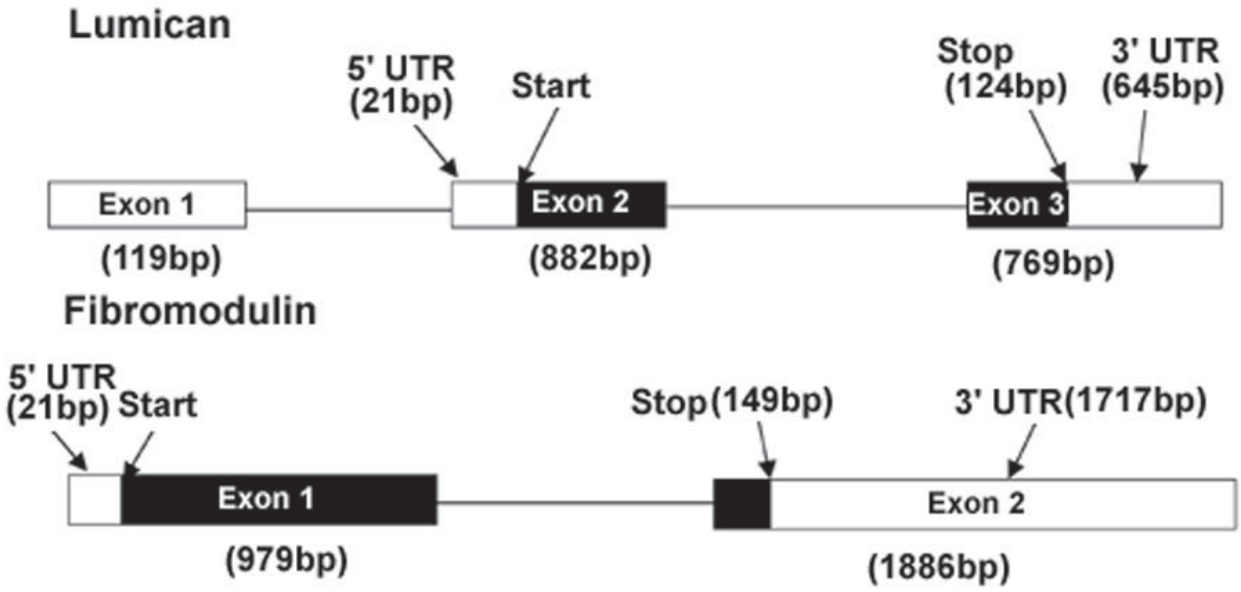


Figure 2. Exonic structure of lumican and fibromodulin genes. Exonic structure of the lumican gene on NT_019546 and the fibromodulin gene on NT_15004671.15. The exons are represented as boxes, the coding region is shown in black, and the noncoding region is in white. Arrows point to untranslated regions (3' or 5’UTRs), initiation codons (Start) and stop codons.

(Prism 377; Applied Biosystems, Inc., Foster City, CA). For fine mapping, additional markers were selected from the Applied Biosystems, Inc. HD-5 microsatellite marker set (Applied Biosystems Inc., Foster City, CA). Analysis of the genotype data was performed by parametric and nonparametric methods using the program GENEHUNTER 2.1 [27].

*DNA Amplification and Mutation Screening:* The genomic structures of the LUM and FMOD genes, as reported in MapViewer (build 34, 2003) of the reference human genome sequence are outlined in Figure 2. The genomic structures of the LUM and FMOD genes comprise 3 exons spanning 8.1 kb and 2 exons spanning 7.7 kb, respectively. The mature 1804 base pair (bp) LUM mRNA encodes a protein of 339 amino acids, and that of the 2863 bp FMOD mRNA encodes 337 amino acids.

A total of 10 affected and 5 unaffected individuals from the 2 families were screened by direct DNA sequencing. Six oligonucleotide primer pairs were designed to amplify the exonic sequences with 50-200 bp extensions beyond the intron-exon boundary for LUM, and 2 were designed to completely sequence FMOD (Table 2). Exon 1 of the FMOD gene was amplified with primers spanning 1579 bp, and exon 2 was amplified with primers spanning 2611 bp. Sequencing of exon 2 was accomplished with three nested sequencing primers at bp positions 589, 1034, and 1521 within the amplicon.

Polymerase chain reactions were performed on 150 ng genomic DNA using AmpliTaq Gold® DNA Polymerase according to standard methods. Amplified products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. Amplicons were purified using QIAquick purification columns (Qiagen, Inc., Valencia, CA) and were sequenced using BigDye™ Terminator version 3.1 on an ABI 3700® Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA). Chromatograms were trimmed for quality, and aligned using Sequencher™ (Gene Codes, Inc., Ann Arbor, MI). Resulting contigs were compared between normal and affected individual DNA sample readings. Novel single nucleotide polymorphisms (SNP)s were submitted to the publicly available dbSNP database (NCBI/SNP).

**Table 3. Two point linkage analysis of Pedigree-2 using polymorphic microsatiellites markers at chromosomes 12q23-24 and 1q23-32**

| Marker | LOD score | NPL score | p value | Information |
|---|---|---|---|---|
| D12S346 | 0.336437 | 1.976623 | 0.0625 | 0.574837 |
| D12S78 | 0.406907 | 2.038583 | 0.0625 | 0.740833 |
| D12S1613 | 0.030702 | 0.855281 | 0.0625 | 0.462905 |
| D12S1583 | 1.406333 | 2.038583 | 0.0625 | 0.745644 |
| D12S1646 | -0.001336 | 0.151861 | 0.28125 | 0.218872 |
| D12S79 | 1.406333 | 2.038583 | 0.0625 | 0.734998 |
| D12S1718 | -0.000996 | 0 | 0.28125 | 0.10817 |
| D12S86 | 1.405871 | 1.914664 | 0.0625 | 0.8 |
| D12S324 | 1.405871 | 1.914664 | 0.0625 | 0.8 |
| D12S1659 | 0.002513 | 0 | 0.28125 | 0.2 |
| D12S1723 | 1.115712 | 0.801826 | 0.0625 | 0.527807 |
| D1S2726 | -0.267785 | -0.300887 | 0.5 | 0.574837 |
| D1S252 | 0.445379 | 0.238118 | 0.25 | 0.35 |
| D1S484 | 1.406333 | 2.038583 | 0.0625 | 0.727807 |
| D1S2878 | 1.328281 | 1.731621 | 0.0625 | 0.674837 |
| D1S196 | 0.288437 | 0.595295 | 0.125 | 0.318872 |
| D1S218 | -1.470127 | 0.196812 | 0.25 | 0.8 |
| D1S238 | -2.449207 | -0.32559 | 0.5625 | 0.7 |

A total of 11 markers at 12q23-24 and 7 markers for 1q23-32 were used for fine point genotyping. The list displays the polymorphic microsatellite markers used for genotyping, LOD scores at theta max, nonparametric linkage (NPL) scores (model free analysis with 0.9 penetrance, 0.10 phenocopy rate), p values, and informative marker values (marker heterozygosity). The red color denotes the highest LOD scores for both chromosomal intervals.

## RESULTS

Pedigree-2 genotyping analyses gave maximum LOD scores of 1.41 for each of the markers (D1S484, D12S1583, and D12S79). Thus, pedigree-2 displayed evidence of suggestive linkage on chromosome 1q23-32 and within the MYP3 locus (Table 3).

Mutation analysis by direct sequencing showed no polymorphisms for LUM. Six exonic polymorphisms were found for FMOD (Table 4). Of these, 5 were in the 3' untranslated region (UTR), and 1 polymorphism at mRNA position 14666200 was a synonymous substitution in the FMOD protein sequence. Five polymorphisms corresponded with previously reported SNPs in public databases. One polymorphism at mRNA position 14665831 was novel, and has been submitted to the dbSNP database. None of the sequence variants cosegregated with the affected phenotype.

## DISCUSSION

Nonsyndromic myopia is a common, complex disorder that is likely to result from alterations of multiple genetic factors. Indeed, several loci have been mapped for nonsyndromic high myopia. An X-linked recessive form of myopia has been mapped and was designated the first myopia locus, MYP1 [28]. We have also studied several medium to large multigenerational families with AD high myopia and found significant linkage at chromosomes 18p11.31 (MYP2) and 17q 21-23 (MYP5) [26,29].

**Table 4. Observed sequence polymorphisms in the fibromodulin (FMOD) gene**

| Subject-Pedigree | 14672146 wt=G/G rs7543148 | 14666200 wt=G/G rs3738022 | 14665831 wt=G/G novel | 14664950 wt=C/C rs4605 | 14664864 wt=C/C rs7208 | 14664736 wt=A/A rs2886220 |
|---|---|---|---|---|---|---|
| 12-2 | | A/A | G/G | C/C | C/C | A/A |
| 14-2 | | G/A | G/G | G/C | C/T | A/G |
| 11-2 | A/A | A/A | G/G | C/C | C/C | A/A |
| 7-2 | | A/A | G/G | C/C | C/C | A/A |
| 5-2 | | A/A | G/G | C/C | C/C | A/A |
| 9-2 | | G/A | G/G | G/C | C/T | A/G |
| 6-MYO10 | | G/A | G/G | G/C | C/T | A/G |
| 4-MYO10 | A/A | G/A | G/G | G/G | C/C | A/A |
| 25-MYO10 | A/G | | G/G | G/G | C/C | A/A |
| 5-MYO10 | A/A | G/A | G/G | G/C | C/T | A/G |
| 3-MYO10 | | G/A | | G/C | C/T | A/G |
| 23-MYO10 | A/G | G/A | G/G | G/G | C/T | A/G |
| 20-MYO10 | | | G/G | G/C | C/T | A/G |
| 15-MYO10 | | A/A | G/G | G/C | C/C | A/A |
| 7-MYO10 | | G/G | G/G | G/G | T/T | G/G |
| Control | A/G | A/A | G/A | G/C | C/C | A/A |

Each column represents a separate sequence variant with base pair position on the scaffold sequence. Wt illustrates wild type sequence published on scaffold NT_04671.15. Rs represents the reference cluster single nucleotide polymorphism (SNP) identification. The red color denotes affected subjects.

Several relevant candidate genes that map to the MYP3 locus are members of the small interstitial proteoglycan family of proteins (Dermatan sulfate proteoglycan [DSPG3], keratocan, Lumican, and Decorin) that are expressed in the extracellular matrix of various tissues. To date, chromosome 1q32 is not associated with a known myopia susceptibility locus. Despite animal studies of *Lum*$^{-/-}$*Fmod*$^{-/-}$ double null mutant mice mimicking pathologic human high myopia, the present mutation analysis of the encoded LUM and FMOD genes did not identify associated sequence alterations in two high myopia pedigrees. We conclude that LUM and FMOD are not the disease genes in these two families (Pedigree-2 and the MYO-10 family).

Another consideration is the possibility of false positive results when studying knockout mice due to the "hitchhiker" effect [30,31]. When dealing with complex traits such as eye size, the interpretation of the effects of gene inactivation in knockout mice relies on phenotype comparison of wild type compared to heterozygous animals. However, the segment of the chromosome that carries the knockout gene may also carry large numbers of adjacent altered genes (hitchhiking genes) that may influence the phenotype. It is possible that any of these alleles could exacerbate or neutralize the phenotypic effects of the knockout mouse. Thus the differences observed between mutant and control mice may be due to genetic differences directly related to the linked background gene, and not necessarily due to the null mutation.

We continue with our efforts to identify the gene(s) responsible for this myopia phenotype by reducing the critical region through recruitment and analysis of new families before conducting further candidate gene analysis. We have previously screened all four proteoglycans, which mapped within this interval, (DSPG3, Keratocan, Decorin, and Lumican) and found no sequence variants that segregated with the affection status in MYO-10 family [32].

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