

Refinement of the X-linked Nonsyndromic High-Grade Myopia Locus *MYP1* on Xq28 and Exclusion of 13 Known Positional Candidate Genes by Direct Sequencing

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PURPOSE. Myopia is a common vision problem affecting almost one third of the world's population. It can occur as an isolated genetic condition or be associated with other anomalies and/or syndromes. Seventeen myopia loci have been identified on various chromosomes; however, no specific gene mutations have yet been identified.

METHODS. Two large multigeneration Asian Indian pedigrees (UR006 and UR077) with isolated, nonsyndromic myopia were studied, in which the condition appeared to segregate as an X-linked recessive trait (*MYP1*; MIM 310460). The degree of myopia was variable in both families, ranging from -6 to -23 D (mean, -8.48 D) with the majority >7.0 D. To map the myopia locus in these families, polymorphic microsatellite markers covering the entire X chromosome were used in linkage analyses performed on 42 genomic DNA samples (13 affected and 29 normal) from both families.

RESULTS. Marker *DXYS154*, which is located within the pseudoautosomal region in distal Xq28 (PAR2; pseudoautosomal region 2), gave a combined maximum LOD score of 5.3 at $\theta = 0$ under an autosomal recessive model. Other markers in the region (near but not within the PAR2 region) that showed no recombination with the phenotype in both the families included *DXS1108*, *DXS8087*, and *F8I13*.

CONCLUSIONS. Observation of recombination in family UR006 refined the disease locus to a ~1.25-Mb region flanked by the proximal marker *DXS1073* and distal marker *DXYS154*. Mutation search in exons and splice junctions of candidate genes *CTAG2*, *GAB3*, *MPP1*, *F8Bver*, *FUNDC2*, *VBP1*, *RAB39B*, *CLIC2*, *TMLHE*, *SYBL*, *IL9R*, *SPRY3*, and *CXYorf1* did not detect a pathogenic or predisposing variant. (*Invest Ophthalmol Vis Sci.* 2011;52:6814-6819) DOI:10.1167/iov.10-6815

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Supported by funds from the Green Cross Blood Bank, Ahmedabad, India, and partially by funds from the University and Cantonal Hospital of Geneva. TLY lab is supported by funds from Research to Prevent Blindness, Inc., and NIH NEI R01 EY014685.

Submitted for publication November 1, 2010; revised November 24 and December 21, 2010, and January 17, 2011; accepted February 9, 2011.

Disclosure: U. Ratnamala, None; R. Lyle, None; R. Rawal, None; R. Singh, None; S. Vishnupriya, None; P. Himabindu, None; V. Rao, None; S. Aggarwal, None; P. Paluru, None; L. Bartoloni, None; T.L. Young, None; A. Paoloni-Giacobino, None; M.A. Morris, None; S.K. Nath, None; S.E. Antonarakis, None; U. Radhakrishna, None

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Myopia (MYP), generally known as near-sightedness, is the natural refractive state of having a focal point in front of the retina: Near objects are in focus, but distant objects appear out of focus. The condition is due to excessive elongation of the eyeball in the axial dimension.¹ It is generally categorized as mild (0-1.5 D), moderate (1.5-6.0 D), and high-grade (>6.0 D). Pathologic myopia (>8.0 D) is often associated with retinal disease, cataract, glaucoma, and choroidal neovascularization, and the risk of threats to vision can also occur in patients with moderate- and/or high-grade myopia.² There are a few factors that have been hypothesized as potential environmental risk factors for myopia, but to date, none have been conclusively proven, including night lighting,^{3,4} excessive nearwork,⁵ and certain occupations.⁶ The prevalence of myopia is often presumed to be much higher, in that exact population-based data are not available. In general, high-grade myopia has a prevalence of ~2.8% to 9.1%^{7,8}; however, it is specific to the age and sex of the individual ethnic population. The prevalence of X-linked myopia is uncertain, as there is a paucity of data due to a very low number of published reports of families with X-linked myopia.⁹⁻¹³

Several genome-wide linkage studies conducted using multiplex MYP families of different ethnic origins showed evidence of linkage at 17 loci for either high- or low-grade myopia (MYP) on Xq28 (*MYP1* [MIM 310460]), 18p11.31 (*MYP2* [MIM 160700]), 12q21-q23 (*MYP3* [MIM 603221]), 17q21-q22 (*MYP5* [MIM 608474]), 22q12 (*MYP6* [MIM 608908]), 11p13 (*MYP7* [MIM 609256]), 3q26 (*MYP8* [MIM 609257]), 4q12 (*MYP9* [MIM 609258]), 8p23 (*MYP10* [MIM 609259]), 4q22-q27 (*MYP11* [MIM

609994], 2q37.1 (*MYP12* [MIM 609995]), Xq23-q25 (*MYP13* [MIM 300613]), 1p36 (*MYP14* [MIM 610320]), 10q21.1 (*MYP15* [MIM 612717]), 5p15.33-p15.2 (*MYP16* [MIM 612554]), 7p15 (*MYP17* [MIM 608367]), and 14q22.1-q24.2 (*MYP18*; [MIM 613626]).^{9,10,14-25} In addition, three independent association and linkage studies of different populations have identified myopia loci at 11q24.1, 15q14, and 15q25, respectively.^{15,16 26-28} Increased incidence of gene polymorphism associated with pathologic myopia was reported recently^{16,17,29-31}; however, no specific gene mutations or pathogenic causative genomic variations have yet been identified for these loci. The majority of these studies were performed on autosomal dominant MYP families. X-linked myopia may occur as an isolated genetic anomaly or it may be associated with various other anomalies, such as night blindness,³²⁻³⁵ cone-rod dysfunction,³⁶ albinism,³⁷ Aland Island eye disease,^{38,39} and blue cone monochromacy,⁴⁰ combined with astigmatism, impaired vision, hypoplasia of the optic nerve heads, and deuteranopia (color blindness).^{9,12} Although most reports have associated X-linked myopia with other anomalies, a few have suggested that it is isolated.^{11,41,42}

We report *MYP1* locus homogeneity to the previously defined Xq28 region⁹ in two large multigeneration Asian Indian families with nonsyndromic high-grade myopia (UR006 and UR077), and the exclusion of potential candidate genes located in this region. Recombination in family UR006 refined the previously mapped *MYP1* disease locus to ~1.25 Mb flanked by the proximal marker *DXS1073* and distal marker *DXYS154*. Thirteen positional candidate genes were excluded by direct sequence analysis of known exons and splice junctions. The present study represents the first confirmation of locus homogeneity of the *MYP1* locus for nonsyndromic X-linked high-grade myopia without any associated anomalies to Xq28.

MATERIALS AND METHODS

Family UR006

The six-generation pedigree UR006 with isolated, nonsyndromic, high-grade myopia was from Gujarat, in western India. The original pedigree consisted of 60 persons with 10 affected individuals (Fig. 1A). The age at onset ranged from 4 to 6 years, and the degree of myopia among affected family members was variable, ranging from -6 to -23 D (mean, -9.53 D), with the majority >8.0 D.

Family UR077

This five-generation pedigree UR077 is from the southern state of India, Andhra Pradesh, and consists of 22 individuals, with 3 male members affected with myopia (Fig. 1B). The age at onset was between 4 and 12 years, and the degree of myopia was between -6.00 to -9.5 D (mean, 7.43).

Fifty-four individuals from both the pedigrees who joined in the study were followed up for several years by RS and VVR. To exclude those with a known syndromic form of myopia, such as Marfan syndrome, Stickler syndrome, or Knobloch syndrome, a detailed physical examination was performed and medical and ophthalmic histories were acquired; those with other known ocular or systemic diseases were also excluded. Detailed ophthalmic examinations were conducted by experienced ophthalmologists (RS and VVR) for each patient included in the study, including visual acuity, slit lamp, intraocular pressure, axial length, color vision testing, and funduscopy examination. Electroretinogram (ERG) testing was performed in selected affected individuals. In all patients, individual eye refraction was performed (RT5100; Nidek, Gamagori, Japan; Fig. 2). For the genotyping analysis, the minimal requirements of phenotype for a subject was considered to be high-grade myopia if the refraction error in the either eye was -6 D or below and a maximum age at onset was 4 to 12 years.

In both the pedigrees, only male members were affected and the degree of myopia increased with advance age. The affected subjects had high-grade myopia, and the carrier females had no functional or structural visual problems. No other associated visual anomalies, specifically color vision defect or night blindness, were present in any member of these pedigrees. No male-to-male transmission was observed in either family and none of the female members was affected, suggestive of an X-linked recessive mode of inheritance with full penetrance. The research adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all subjects who participated in the study, and their blood samples were collected for the genetic studies. Both are unrelated families of different geographic regions, live in urban areas, and are of higher socioeconomic condition than the Indian average. None of the affected individuals had a history of occupational exposures none were involved in any hazardous occupations.

Linkage Analysis

Genomic DNA was purified from peripheral blood samples with a DNA extraction kit (Genra Systems, Inc., Minneapolis, MN). In the present study, we used 42 samples including 13 affected and 29 normal individuals from both the families, numbered under each individual in the pedigrees (Fig. 1). Initially, we presumed that the phenotype in both the pedigrees (UR006 and UR077) would be allelic to the known *MYP1* locus on Xq28. To test this assumption, we performed linkage analysis with the 25 microsatellite markers previously used for genetic analysis of the candidate genomic regions, including the markers that covered the entire X-chromosome. These markers were selected from the Marshfield, Genethon, and CHLC collection (NIH/CEPH Collaborative Mapping Group, 1992).^{43,44} One oligonucleotide primer of each marker was labeled with γ [³²P]ATP, with T4 polynucleotide kinase. PCR was performed from 120 ng of genomic DNA in a total volume of 15- μ L mixture per reaction containing 0.4 pM of labeled forward primer, 2.6 pM of unlabeled reverse primer, 1.3 μ M of each dNTP, and 0.25 U *Taq* polymerase (Pharmacia, San Diego, CA). PCR products were separated by electrophoresis in a 6% denaturing urea/polyacrylamide gel. Genotyping was performed as previously described.⁴⁵

Family information and marker genotypes were stored in a pedigree program (Cyrillic 2.1; Cherwell Software, Colorado Spring, CO). Statistical analysis was performed on the basis of an X-linked recessive trait for X chromosome and recessive for PAR2 (pseudoautosomal region 2) with 100% penetrance. For all markers, the allele frequencies were kept equal, and the gene frequency was set to 0.00001. Maximum LOD scores (Z_{max}) were calculated for each marker by the use of the ILINK, MLINK, and LINKMAP programs of LINKAGE ver. 5.2⁴⁶ and FASTLINK ver. 3.0.⁴⁷ Two-point and multipoint analysis for both X-chromosome and PAR2 were performed separately as described.^{48,49}

Candidate Gene Eye Tissue Expression Studies

For reverse transcription-polymerase chain reaction (RT-PCR), total RNA from sclera, cornea, optic nerve, and retina was extracted from pooled human donor eyes (Pennsylvania Lions Eye Bank, Philadelphia) using an extraction reagent (TRIZol; Invitrogen, Carlsbad, CA). The eyes were treated by submersion in RNA stabilizer (RNAlater solution; Ambion, Inc., Austin, TX) within 2 to 12 hours postmortem. RT-PCR was performed using standard methods with random hexamers and reverse transcriptase (SuperScript II; Invitrogen). Subsequent gene-specific PCR for all tested candidate genes was performed using *Taq* polymerase (Platinum; Invitrogen) to validate gene expression in human ocular tissue. The resulting PCR amplicons were visualized on 2% agarose gels after electrophoresis and staining with ethidium bromide.

Sequencing

Genomic DNA from two affected and two normal individuals were PCR-amplified for regions covering the entire coding sequence and splice

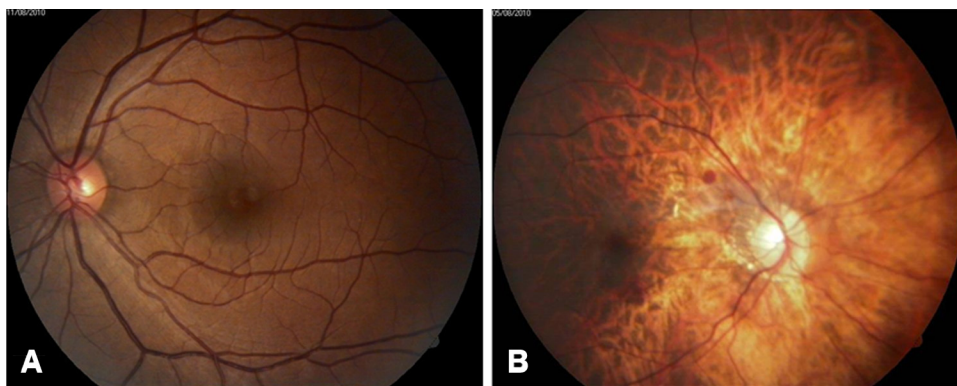


FIGURE 2. Fundus photographs of individuals from family UR006: (A) unaffected individual (6253) with normal fundus and (B) affected individual (6247) with high-grade myopia (-12.5 D) with tessellation pattern.

(BigDye Terminators Sequencing Kit; Applied Biosystems, Inc. [ABI], Foster City, CA) in both directions with an automated genetic analysis system (3100; ABI) and were analyzed with a commercial software program package (Sequencer 4.1; Gene Codes, Ann Arbor, MI).

Electronic Database Information

The following sites were accessed during the research for this study: The UCSC Human Genome Browser (<http://genome.cse.ucsc.edu/>, University of California Santa Cruz); MERLIN (<http://www.sph.umich.edu/csg/abecasis/Merlin/>, the University of Michigan, Ann Arbor, MI); National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>, National Institutes of Health [NIH], Bethesda, MD); and Online Mendelian Inheritance in Man (OMIM) (<http://www.ncbi.nlm.nih.gov/Omim/>, NCBI, NIH).

RESULTS

All myopia subjects included in the analysis had high-grade myopia in one or both the eyes (>6.0 D) without any associated anomalies, including color vision defect. All affected subjects developed high-grade myopia before they were of school age. None of the unrelated individuals in this family was observed with any form of myopia. The results of two-point linkage analyses of myopia families UR006 and UR077 and polymorphic microsatellite markers covering the Xq28 genomic region at various recombination fractions are shown in Table 1. A maximum combined two-point LOD score of 5.3 was obtained for marker *DXYS154*, which is located in the pseudoautosomal region of distal Xq28 (PAR2) at recombination fraction theta = 0. Other markers in the

TABLE 1. Combined Two-Point LOD Scores for MYP1 and Xq28 Markers for Families UR006 and UR077

Markers	Family	Recombination Fractions							Physical Position (Mb)
		0	0.01	0.05	0.10	0.20	0.30	0.40	
<i>GATA31E08</i>	UR006	-∞	-2.87	-1.55	-1.04	-0.59	-0.35	-0.17	140.062
	UR077	-∞	-0.84	-0.20	-0.02	0.16	0.16	0.10	
	Combined	-∞	-3.71	-1.75	-1.02	-0.43	-0.19	-0.07	
<i>DXS1193</i>	UR006	-∞	0.15	0.69	0.80	0.70	0.49	0.25	148.187
	UR077	1.70	1.71	1.59	1.44	1.11	0.76	0.39	
	Combined	-∞	1.86	1.66	2.24	1.81	1.25	0.64	
<i>DXS8011</i>	UR006	-∞	2.15	2.60	2.57	2.16	1.54	0.79	149.617
	UR077	1.30	1.23	1.14	1.03	0.80	0.56	0.29	
	Combined	-∞	3.38	3.74	3.60	2.96	2.10	1.08	
<i>DXS8061</i>	UR006	-∞	1.56	2.04	2.06	1.75	1.25	0.63	151.772
	UR077	2.10	2.03	1.89	1.71	1.33	0.92	0.47	
	Combined	-∞	3.59	3.93	3.77	3.08	2.17	1.10	
<i>DXS15</i>	UR006	-∞	2.15	2.60	2.57	2.16	1.54	0.79	152.128
	UR077	1.70	1.71	1.59	1.44	1.11	0.76	0.38	
	Combined	-∞	3.86	4.19	4.01	3.27	2.30	1.17	
<i>DXS1177</i>	UR006	-∞	2.15	2.60	2.57	2.16	1.54	0.79	152.285
	UR077	2.00	1.98	1.84	1.67	1.29	0.88	0.45	
	Combined	-∞	4.13	4.44	4.24	3.45	2.42	1.24	
<i>DXS1073</i>	UR006	-∞	1.38	1.87	1.89	1.60	1.13	0.58	152.547
	UR077	2.00	2.01	1.87	1.69	1.31	0.90	0.46	
	Combined	-∞	3.39	3.74	3.58	2.91	2.03	1.04	
<i>F8i13</i>	UR006	2.22	2.18	2.00	1.78	1.34	0.91	0.46	153.482
	UR077	0.92	0.91	0.84	0.76	0.58	0.40	0.20	
	Combined	3.14	3.09	2.84	2.54	1.92	1.31	0.66	
<i>DXS8087</i>	UR006	1.15	1.12	1.01	0.87	0.61	0.38	0.18	153.8
	UR077	1.07	1.05	0.97	0.86	0.65	0.42	0.19	
	Combined	2.22	2.17	1.98	1.73	1.26	0.80	0.37	
<i>DXS1108</i>	UR006	1.29	1.26	1.14	0.99	0.69	0.42	0.19	154.515
	UR077	0.92	0.91	0.84	0.76	0.58	0.40	0.20	
	Combined	2.21	2.17	1.98	1.75	1.27	0.82	0.39	
<i>DXYS154</i>	UR006	3.99	3.91	3.59	3.19	2.33	1.42	0.57	154.731
	UR077	1.31	1.27	1.13	0.96	0.62	0.33	0.11	
	Combined	5.30	5.18	4.72	4.15	2.95	1.75	0.68	

region (near but not within PAR2) that showed no recombination with the phenotype included *DXS1108*, *DXS8087*, and *F8i13*. However, recombination was observed between the phenotype and markers *GATA31E08*, *DXS1193*, *DXS8011*, *DXS8061*, *DXS15*, *DXS1177*, and *DXS1073* for family UR006. Other X chromosome markers that showed negative LOD scores are not shown. RT-PCR results confirmed expression of all genes tested in human ocular tissue (Fig. 3). Sequence analysis of known exons and splice junctions of 13 positional candidate genes did not yield any mutations in the exons and intron-exon boundaries for the affected and normal individuals.

DISCUSSION

Myopia is a heterogeneous disorder and has a multifactorial etiology in which both genetics and environment play important roles. Seventeen loci⁵⁰ with evidence of linkage for either high- or low-grade myopia have been identified on various chromosomes.^{9,10,13-23,50,51} However, to date no convincing candidate gene has been found to be mutated in myopia. In the only known X-linked *MYPI* family, affected males and homozygous females exhibited myopia and color vision defect.⁹ The same family was revisited by Young et al.¹⁵ to include additional subjects and

genealogic data using polymorphic microsatellite markers. All affected males had high-grade myopia with protanopia and mild cone dysfunction on electroretinogram testing, whereas carrier females had normal findings in functional and structural ophthalmic evaluations. Zhang et al.¹⁰ mapped another X-linked myopia locus (*MYPI3*) to the Xq23-q25 region. In both studies,^{10,15} all affected individuals had associated anomalies; however, in the present two families (UR006 and UR077), all female carriers were normal, and no affected male members had any associated anomalies, including color vision defect. Linkage analysis showed that the disease locus maps to the previously identified region on Xq28⁹; however, haplotype analysis (Fig. 1A) revealed informative recombination events in the affected individual III-42 (6256) of family UR006 reduced the previously defined genomic interval of 6.8 cM at Xq27.3-Xq28¹³ to ~1.4 cM, by flanking the proximal marker at *DXS1073* and the distal boundary at *DXYS154*. Marker *DXYS154* gave the highest combined LOD score at $\theta = 0$; however, no distal markers are available. Although families UR006 and UR077 are of Indian origin, they belong to different ethnic groups. Haplotype analysis from the Xq28-linked region of affected individuals did not find extensive common haplotypes shared between these two families, yet a few common identical alleles were observed for selected markers. The *MYPI3* locus was excluded by linkage and haplotype analysis for both the families.

This 1.25-Mb linked genomic region on Xq28 contains multiple candidate genes for high-grade myopia, including *CTAG2*, *GAB3*, *MPP1*, *F8Bver*, *FUNDC2*, *VBP1*, *RAB39B*, *CLIC2*, and *TMLHE*, proximal to PAR2, and *SYBL*, *IL9R*, and *SPRY3CXYorf1* in PAR2. Sequence analysis indicated that none of these genes is involved in the pathogenesis of the *MYPI* phenotype in both the pedigrees. The absence of pathogenic polymorphic variants in the coding regions of these genes indicates that the mutation may lie in a regulatory region of one of these candidate genes, in another gene, or in another functional element (conserved or not) within the *MYPI* region. It is also possible that the mutation is a duplication or a deletion within this region. However, a deletion of the coding sequence of genes outside PAR2 is excluded, because we successfully amplified all sequences in the males. There are genes in the PAR2 region that are only expressed from the X-chromosome; thus, they behave as X-linked recessive. *HSPRY3* and *SYBL1* are both inactive on Y and are subject to X inactivation in humans.⁵² In contrast, *IL9R* and *CXYorf1* are expressed on Y and are not subject to X inactivation.^{53,54} Identification of the mutant gene would potentially provide significant insights into the molecular mechanisms underlying the etiology of nonsyndromic myopia.

Acknowledgments

The authors thank all members of families UR006 and UR077 for their participation and donation of blood samples.

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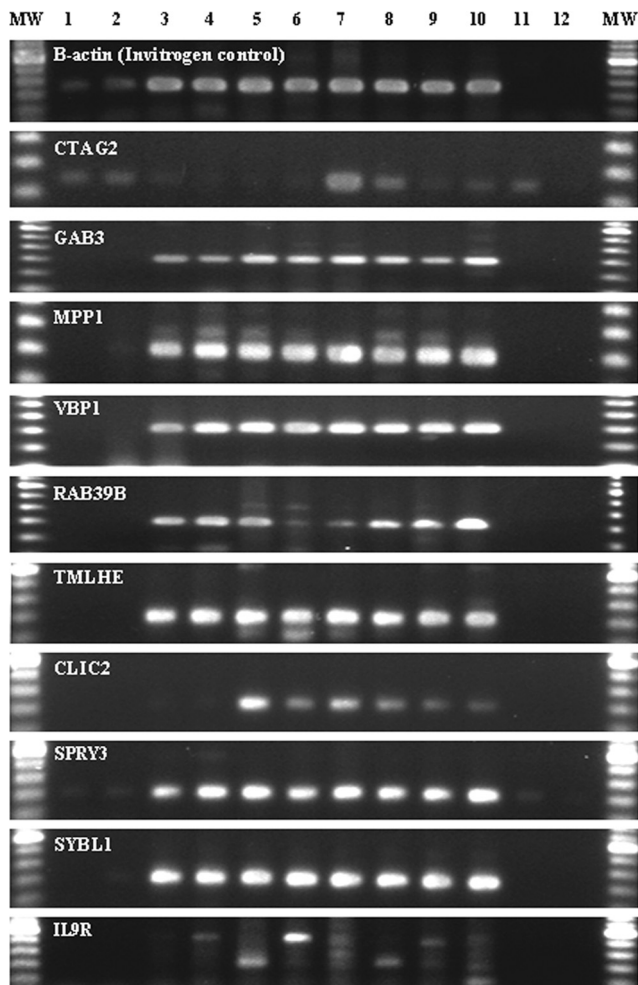


FIGURE 3. Gene-specific PCR for some tested candidate genes by reverse transcription-polymerase chain reaction. RT-PCR results: lane 1: sclera; lane 2: cornea; lane 3: optic nerve; lane 4: retina; lane 5: lung; lane 6: skeletal muscle; lane 7: heart; lane 8: trachea; lane 9: kidney (all Clontech, Inc., Temecula, CA); lane 10: brain (Ambion); lane 11: cRNA; lane 12: no RT (H₂O).

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