REPORT

High Myopia Caused by a Mutation in *LEPREL1*, Encoding Prolyl 3-Hydroxylase 2

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Autosomal-recessive high-grade axial myopia was diagnosed in Bedouin Israeli consanguineous kindred. Some affected individuals also had variable expressivity of early-onset cataracts, peripheral vitreo-retinal degeneration, and secondary sight loss due to severe retinal detachments. Through genome-wide linkage analysis, the disease-associated gene was mapped to ~1.7 Mb on chromosome 3q28 (the maximum LOD score was 11.5 at $\theta = 0$ for marker D3S1314). Sequencing of the entire coding regions and intron-exon boundaries of the six genes within the defined locus identified a single mutation (c.1523G>T) in exon 10 of *LEPREL1*, encoding prolyl 3-hydroxylase 2 (P3H2), a 2-oxoglutarate-dependent dioxygenase that hydroxylates collagens. The mutation affects a glycine that is conserved within P3H isozymes. Analysis of wild-type and p.Gly508Val (c.1523G>T) mutant recombinant P3H2 polypeptides expressed in insect cells showed that the mutation led to complete inactivation of P3H2.

Myopia is the most common human eye disorder and constitutes a significant public health concern.[1] Specifically, high myopia is a leading cause of visual impairment and blindness worldwide because of its associated ocular comorbidities of retinal detachment, macular degeneration, premature cataract, and glaucoma.[1,2] Childhood myopia is primarily due to growth in axial length of the eyeball; most cases are either congenital (usually high myopia) or juvenile (onset between 7 and 16 years of age). In general, the earlier the onset of myopia occurs, the greater the degree of progression.[1,2] Twin studies and heritability measures suggest a strong genetic component in myopia.[3,4] Myopia is mostly regarded as a multifactorial polygenic disorder.[1] Genome-wide association studies of the common presumably polygenic multifactorial myopia in large cohorts have recently identified association with genomic loci at chromosomes 13q12,[5] 15q14,[6] and 15q25,[7] as well as with genetic variants in *CTNND2* (MIM 604275) at 5p15.[8] There are more than 150 defined monogenic syndromes in which familial high myopia is one of the features.[1,3] These syndromes are associated with a range of other phenotypic changes, and most are associated with severe congenital myopia.[3] Some syndromes affect processes, such as retinal processing, that are likely to be important in the regulation of eye size or affect the properties of connective-tissue macromolecules such as collagen.[3] Pedigrees of apparently monogenic nonsyndromic hereditary myopia were previously described and were mostly found to be autosomal dominant; rarely, they were found to be X linked or recessive.[1,9] At least 17 susceptibility loci have been described for nonsyndromic monogenic myopia.[1,9] However, no clear causative gene mutations have been identified so far in these loci.

Large consanguineous Israeli Bedouin kindred presented with an apparently autosomal-recessive phenotype of nonsyndromic severe myopia with variable expressivity of cataract and vitreo-retinal degeneration (Figure 1). Thirteen affected and 32 unaffected individuals (M1–M45, Figure 1) of the kindred underwent complete ophthalmic examination, including slit-lamp biomicroscopy, indirect ophthalmoscopy, measurement of visual acuity and refractive errors, and examination of eye movements and ocular alignment. The study was approved by the Soroka Medical Center institutional review board and the Israeli Helsinki Committee for genetic studies, and informed consent was obtained from all individuals studied.

The affected individuals (seven males and six females, ages 7 to 65 years) were examined by a clinical geneticist and had no apparent dysmorphology. They all appeared normal at birth and presented with poor sight in childhood. In addition to myopia, many affected individuals developed cataracts and peripheral vitreo-retinal degeneration, and some had subluxated lenses. The onset of myopia usually preceded that of the cataracts and the retinal degeneration. Most affected individuals had high myopia between −5 and −18 diopters (Table 1), but two had mild myopia. Preoperative refractive error was not available in two patients and in one eye of other three patients. Table 1 shows the spherical equivalent of myopia, calculated for each eye as a sphere plus half of the cylinder of the refractive error. Mean refractive error was −11.3 diopters.

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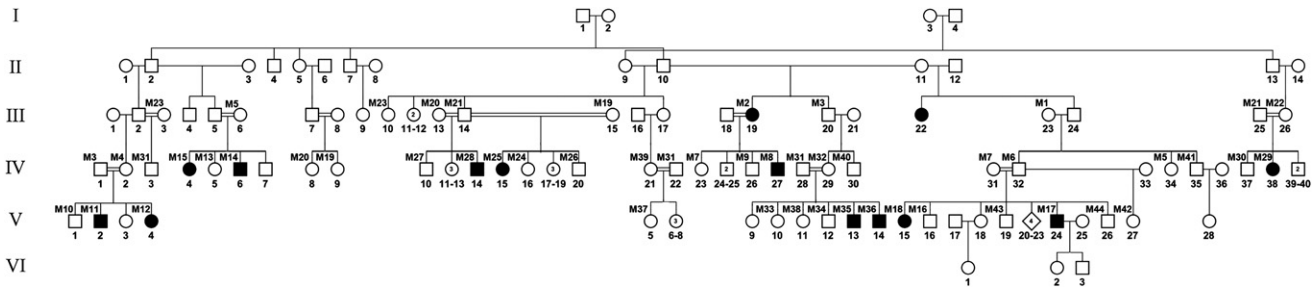

Figure 1. Pedigree of Affected Israeli-Bedouin Kindred
Solid and open symbols represent affected and unaffected individuals, respectively. M numbers denote individuals whose DNA samples were analyzed. Because of the complexity of the pedigree, some individuals (M3, M5, M7, M19, M20, M21, M23, and M31) are drawn twice, with the same M number.

All patients had axial myopia, as evidenced by an increased axial length of the eye (the length ranged between 25.1 and 30.5 mm; Table 1). Eleven patients developed cataracts that were significant enough to warrant surgery in one or both eyes, usually in the first or second decade of life. In three patients, subluxated lenses were detected and were associated with cataracts in two patients and lens coloboma in one of them. In an additional five patients, lens instability due to weak or partially missing lens zonules was found during cataract surgery. This caused surgical complications, including rupture of the lens capsule and decentration of the inserted intraocular lens, which required reoperation. In four patients, lens instability made it impossible to insert the intraocular lens at all, which left them aphakic. Peripheral vitreo-retinal degenerative changes were found in nine patients (Table 1), four of whom developed retinal tears (mostly dialyses) causing retinal detachment in one or both eyes. In three patients (23% of affected individuals), in spite of multiple surgical procedures, intractable retinal detachments led to blindness in one eye.

Assuming a founder effect, we studied 11 affected individuals and seven healthy individuals by performing a genome-wide linkage analysis with 400 fluorescently end-labeled polymorphic markers (ABI PRISM Linkage Mapping Set MD10 [Applied Biosystems]) as previously described.[10] PCR products were separated by electrophoresis on an ABI PRISM 377 DNA Sequencer (Applied Biosystems) and analyzed with Gene-Scan software (detailed results in Document S1). Fine-mapping was carried out using polymorphic markers as previously described.[10] Haplotypes were manually constructed and analyzed. Noninformative regions were excluded by the use of additional adjacent microsatellite markers. Analysis of the results with SUPERLINK software[11] yielded two loci with LOD scores above two: a LOD score of 4.1764 at marker D3S3530 and a LOD score of 3.04 at marker D7S484. Fine mapping at the chromosome 7 locus ruled out its association with the disease (Document S2). We performed fine mapping surrounding D3S3530 by using the following additional polymorphic markers to test DNA samples of the 45 available family members (13 affected and 32 unaffected): D3S1580, D3S3549, D3S2398, D3S_A, D3S1294, D3S1314, D3S1288, D3S2747, D3S3043, D3S_B, D3S2455, and D3S1601 (D3S_A and D3S_B are polymorphic markers designed with Tandem Repeats Finder; primer sequences are given in Document S3). As seen in Figure 2, an interval of homozygosity common to all affected individuals in the family was demonstrated between markers D3S3530 and D3S2455. Six affected individuals (M2, M14, M15, M17, M18, and M25) lacked homozygosity at D3S3530, setting the upper limit of the homozygosity interval at D3S3530 (Figure 2). Ten affected individuals (M2, M8, M14, M11, M12, M15, M17, M18, M29, and M25) presented with a recombination event at D3S2455, setting the lower limit of the homozygosity interval at D3S2455. The common haplotype indicated a minimal candidate interval of 1.7 Mbp (~4 cM) that contained six known or predicted genes. Linkage was demonstrated by two-point analysis with SUPERLINK,[11] which provided a LOD score of 11.5 at $\theta = 0$ for Marker D3S1314 (data available upon request).

On the basis of data from the UCSC Genome Browser, only six genes were located within the identified interval. Their coding sequences and exon-intron boundaries were amplified from genomic DNA with PCR primers designed with the Primer3 program; all primer sequences are given in Document S3). PCR products were subject to agarose-gel electrophoresis and gel extraction (QIAGEN), followed by sequencing with either the forward or reverse primer on an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Results were analyzed with ChromasPro software, and the obtained DNA sequences were compared with published sequences (BLAST software). No mutations were found in the coding regions or intron-exon boundaries of five genes within the 3q28 locus. As shown in Figures 3A–3C, sequence analysis of *LEPREL1* (RefSeq number NM_018192.3; MIM 610341) revealed that exon 10 contained a c.1523G>T mutation, which led to the replacement of glycine at position 508 by valine (p.Gly508Val; c.1523G>T) in the encoded protein, LEPREL1 (Leprecan-Like 1, termed also P3H2). It should be noted that *LEPREL1* was suggested by our S2G software[12] to be the top candidate gene of the six genes within the locus.

Table 1. Clinical Data of Affected Individuals

Number	Sex	Age (y)	Myopia (diopters)[a]		Axial length (mm)		Cataract		Cataract surgery age (y)		Vitreo-Retinal Changes	Comments
			OD	OS	OD	OS	OD	OS	OD	OS		
M14	M	17	8.00	5.00	27.7	26. 3	yes	yes	9	13	OU vitreous opacities OD retinal dialysis and detachment OS retinal tears	OU rupture of lens capsule in cataract surgery, aphakia OD glaucoma, and blindness
M15	F	23	1.00	NA	25.1	25.5	yes	yes	23	20	OD pigmented retinal scar	
M35	M	13	14.50	14.50	28.9	28.8	yes	yes	9	8	OD vitreous opacities, OS lattice degeneration	OU zonulolysis and phacodonesis seen in cataract surgery, aphakia
M11	M	12	8.00	11.00	NA	NA	no	no	no	no	no	OU subluxated lenses
M12	F	17	11.50	13.50	NA	NA	no	yes	no	17	no	
M17	M	28	1.25	NA	NA	NA	yes	yes	no	13	OD retinal tears and detachment OS retinal dialysis and total detachment	OS blind
M18	F	25	NA	NA	NA	NA	yes	yes	20	15	OU fine vitreous opacities	OU rupture of lens capsule in cataract surgery, OS subluxated IOL
M29	F	7	12.00	10.50	NA	NA	no	no	no	no	no	
M2	F	65	NA	NA	NA	NA	yes	yes	41	46	OD lattice degeneration, OS retinal dialysis & detachment	OD aphakia OS subluxated IOL
M25	F	24	NA	11.00	26.0	27.3	yes	yes	7	no	OD retinal dialysis and detachment, OS vitreous opacities	OD blind OS subluxated lens, lens coloboma
M28	M	13	16.00	18.00	28.4	27.6	yes	yes	7	7	no	OU subluxated lenses, aphakia, OS rupture of lens capsule in cataract surgery
M36	M	16	15.00	17.00	30.5	29.5	yes	yes	no	12	OU lattice degeneration and vitreous veils	OS reoperation for subluxated IOL due to rupture of lens capsule
M8	M	29	13.00	14.00	>26.5	>26.5	yes	yes	8	7	OU vitreous opacities	deafness

Abbreviations are as follows: OD, right eye; OS, left eye; OU, both eyes; F, female; M, male; NA, data not available; and IOL, intraocular lens.
[a] Spherical equivalent of myopia is shown, calculated for each eye as a sphere plus half of the cylinder of the refractive error.

To test for the mutation in a larger cohort, we amplified genomic DNA from 45 individuals of the pedigree and from 200 unrelated controls by using the following PCR primers: 5′-TGGAATAAAATGGGGATGGT-3′ and 5′-TCTG CCATTTACAGAAGCCTTA-3′. We analyzed PCR products by denaturing high-performance liquid chromatography (DHPLC, WAVE Nucleic Acid Fragment Analysis System, Transgenomics). Analysis of all 45 DNA samples of the kindred was compatible with association of the mutation with the disease phenotype, implying full penetrance with variable expressivity. The mutation was not found in any of 400 chromosomes from ethnically matched controls tested by DHPLC. It should be noted that the glycine at position 508 of LEPREL1 is conserved throughout evolution (Figure 3D). As seen in Figure 3E, the p.Gly508Val mutation is predicted to alter the three-dimensional structure of the prolyl-hydroxylase encoded by *LEPREL1* (NNpredict).[13]

To test for functional consequences of the *LEPREL1* mutation, we analyzed wild-type and p.Gly508Val mutant recombinant human P3H2 polypeptides expressed in insect cells. The P3H2 p.Gly508Val mutation was introduced into the pVL1392-P3H2 plasmid[14] with the QuickChangeXL site-directed mutagenesis kit (Stratagene). The recombinant vector was cotransfected into *Spodoptera frugiperda* (Sf9) insect cells with modified *Autographa californica* nuclear polyhedrosis virus DNA (BaculoGold, PharMingen) by calcium phosphate transfection, and the recombinant viruses were amplified.[15] Monolayer cultures of Sf9 cells in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum (HyClone) were infected with the recombinant viruses coding for wild-type or

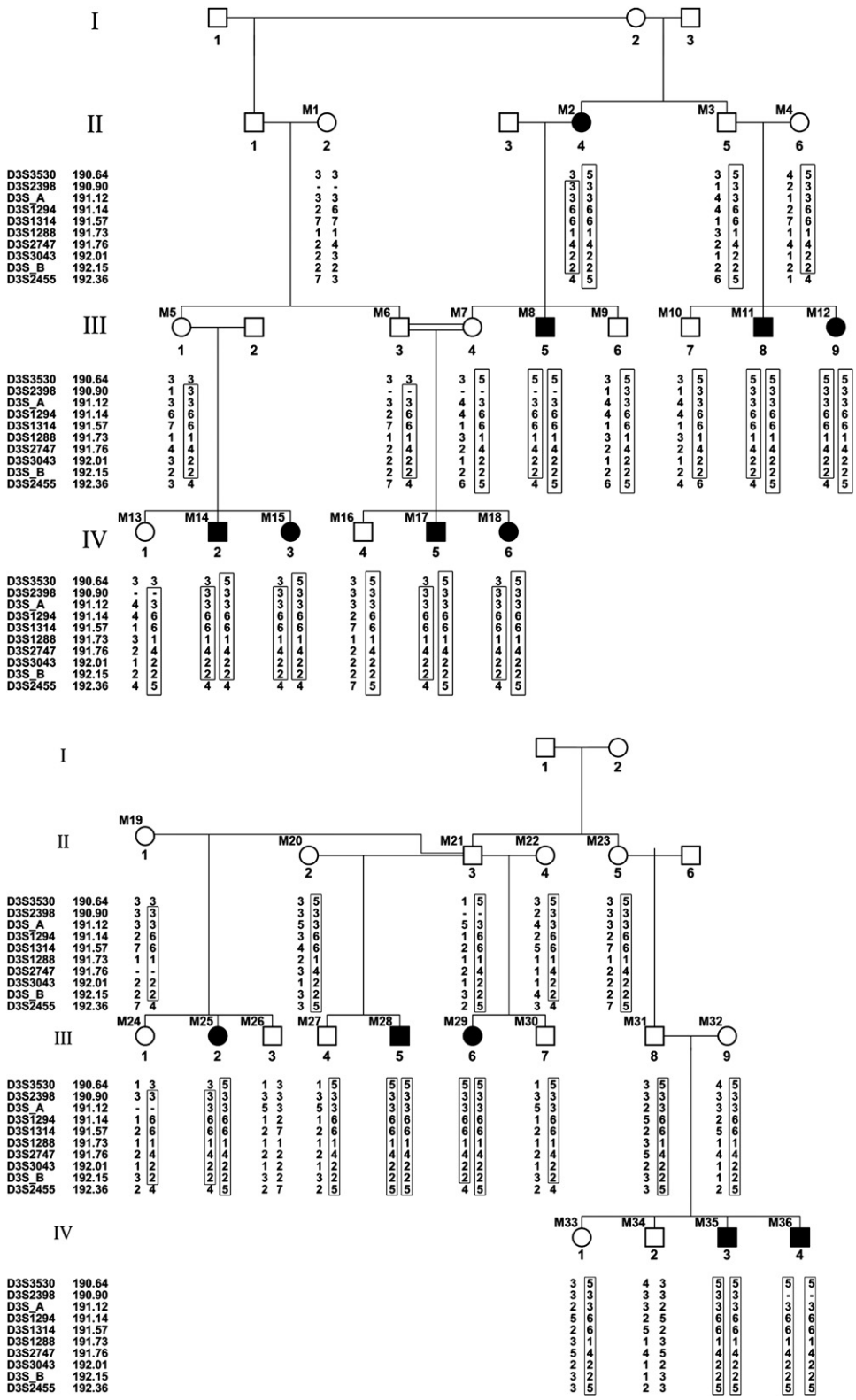

Figure 2. Defining the Disease-Associated Locus

A partial pedigree of the large Israeli-Bedouin kindred is shown. The disease-associated haplotype is boxed. The minimal homozygosity locus associated with the disease is defined between the markers D3S3530 and D3S2455. Individuals are given the same M numbers as in Figure 1 and Table 1.

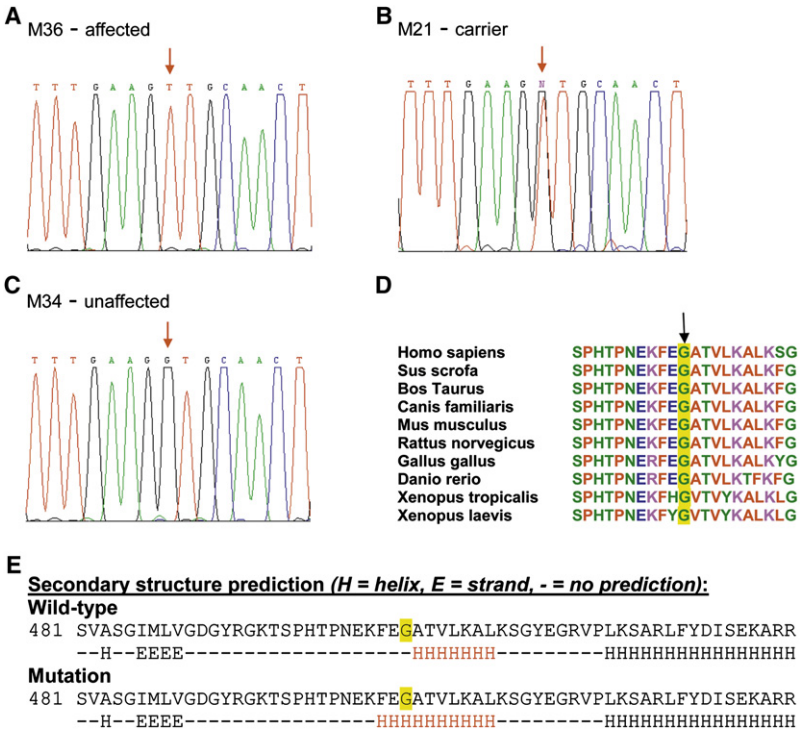

Figure 3. Analysis of the c.1523G>T (p.Gly508Val) Mutation in Exon 10 of *LEPREL1*
Sequence analysis is shown for an affected individual (A), an obligatory carrier (B), and an unaffected individual (C). The glycine at position 508 of LEPREL1 is conserved throughout evolution (D). (E) Changes in secondary structure as a result of the valine-to-glycine substitution.

mutant P3H2 at a multiplicity of 5 and a cell density of 5 × 10^6 per 100 mm plate. The cells were harvested 72 hr after infection, washed with a solution of 0.15 M NaCl and 0.02 M phosphate (pH 7.4; PBS), and homogenized in a solution of 0.1 M NaCl, 0.1 M glycine, 2 mM $CaCl_2$, 10 μM dithiothreitol, 0.1% Triton X-100, 0.01 M Tris (pH 7.8), and the complete EDTA-free protease inhibitor cocktail (Roche Diagnostics), and centrifuged at 13,000 x g for 20 min. The remaining pellets were solubilized in 1% SDS, and aliquots of both fractions were analyzed by 15% SDS-PAGE under reducing conditions followed by Coomassie Blue staining or ECL-immunoblotting (Amersham Biosciences) with a polyclonal antibody against P3H2[14] at a 1:500 dilution. Equal amounts of soluble wild-type and mutant P3H2 polypeptides were found in the Triton X-100-soluble fractions (not shown) that were assayed for P3H activity by a method based on the hydroxylation-coupled decarboxylation of 2-oxo-[1-^{14}C] glutarate[16] with differing amounts of a peptide substrate $(Gly\text{-}Pro\text{-}4Hyp)_5$, (Innovagen). In additional experiments, the peptide substrate was replaced by the synthetic peptides Ser-Lys-Gly-Glu-Gln-Gly-Phe-Met-Gly-Pro-4Hyp-Gly-Pro-Gln-Gly-Gln-4Hyp-Gly-Leu-4Hyp-Gly or Pro-Thr-Gly-Pro-Arg-Gly-Phe-Pro-Gly-Pro-4Hyp-Gly-Pro-Asp-Gly-Ley-4Hyp-Gly-Ser-Met-Gly, corresponding to two known prolyl 3-hydroxylation sites in the collagen IV α1 chain[17] or by Leu-Asn-Gly-Leu-4Hyp-Gly-Pro-Ile-Gly-Pro-4Hyp-Gly-Pro-Arg-Gly-Arg-Thr-Gly-Asp-Ala-Gly, corresponding to the only prolyl 3-hydroxylation site in the collagen I α1 chain at position 986 of the triple helix.[18] Triton X-100 extracts of insect cells expressing human collagen prolyl 4-hydroxylase isoenzyme II (C-P4H-II)[19] were used as negative controls. A large amount of P3H activity was obtained in the P3H2 sample when the synthetic peptide $(Gly\text{-}Pro\text{-}4Hyp)_5$ was used as the substrate, whereas the activity of the p.Gly508Val mutant P3H2 never exceeded the background values (Table 2). Also, the mutant P3H2 polypeptide did not show any activity when synthetic peptides representing the known prolyl 3-hydroxylation sites in human type I and type IV collagen polypeptides were used as substrates (data not shown).

LEPREL1 is localized to the ER and the Golgi network.[20] The 708 amino acid protein contains a signal sequence, four tetratricopeptide repeats (TPRs), a leucine zipper, a P loop, and a C-terminal region that contains the catalytically critical residues that are required for binding of Fe^{2+} and 2-oxoglutarate and are shared with the collagen-modifying lysyl hydroxylases and prolyl 4-hydroxylases, as well as other 2-oxoglutarate-dependent dioxygenases and a C-terminal KDEL ER-retention motif.[19,20] The mutation results in a glycine-to-valine substitution at amino acid 508 within the C-terminal region.[19] The mutated glycine is a highly conserved residue within the P3H family (Figure 3D), suggesting its importance: the p.Gly508Val variant is predicted (via PolyPhen algorithms) to be probably damaging with a position-specific independent counts (PSIC) score difference of 2.423; moreover, substitution of glycine with valine leads to putative changes in the secondary structure of the protein (Figure 3E).[13] Accordingly, the p.Gly508Val mutation led to complete inactivation of recombinant P3H2 polypeptide expressed in insect cells, both when analyzed with a $(Gly\text{-}Pro\text{-}4Hyp)_5$ peptide substrate that fulfills the substrate requirements of P3H and when analyzed with synthetic peptides representing known prolyl 3-hydroxylation sites in human type I and type IV collagen polypeptides.

The large kindred presented here is distinctive in its recessive heredity of myopia. Interestingly, a study of juvenile cataract with myopia in a Chinese family demonstrated linkage to a locus between D3S3606 and D3S1262, approximately 3 Mbp from the region highlighted in our kindred;[21] however, the genomic loci in the two kindreds do not overlap. In contrast with that of the Bedouin kindred, the phenotype in the Chinese family was dominantly inherited, manifested as cataracts with likely

Table 2. P3H Activities of Triton X-100-Soluble Fractions of Insect Cells Expressing Wild-Type P3H2, p.Gly508Val Mutant P3H2, or C-P4H-II with the peptide (Pro-4Hyp-Gly)$_5$ as the Substrate

Enzyme	Peptide Concentration (μM)	Activity (dpm/100 μl[a])
P3H2	50	4570 ± 320
	100	8170 ± 610
P3H2Gly508Val	50	<100
	100	<100
C-P4H-II[b]	100	<100
	100	<100

[a] The values are means ± SD from at least three independent experiments.
[b] The Triton X-100-soluble fraction of insect cells expressing C-P4H-II was used as a negative control.

secondary myopia that appeared in only some of the affected individuals, and was found to be associated with a heterozygous mutation in *BFSP2* (MIM 603212), a gene known to be mutated in dominant cataracts.[21]

The homozygous *LEPREL1* mutation identified in affected individuals of the Bedouin kindred was associated with full penetrance and variable expressivity of the disease. The main clinical phenotype was severe myopia. However, cataracts, vitreo-retinal degeneration, and subluxated lenses were also found in some patients. These led to severe ocular morbidity and a high rate of surgical complications, which resulted in blindness in 23% of our patients. We clearly demonstrate that the myopia is due to increased axial eye length, although one cannot rule out the possibility that changes in the lens might also contribute to the myopia phenotype. Although the cataract and vitreo-retinal degeneration might be related to the myopia, it is plausible that the myopia, cataracts, vitreo-retinal degeneration, and lens subluxation are all independent consequences of the *LEPREL1* mutation.

The association of the *LEPREL1* mutation with ocular abnormalities is in line with previous findings: LEPREL1 is found in a wide variety of tissues, including the eye;[22] specifically, it was demonstrated in the iris, lens, trabecular meshwork, and retinal pericyte cells (NEIBank). Recent protein-localization studies of the P3H isoenzymes in mouse demonstrated that P3H2 appears to be coordinately expressed with and therefore recognizes basement membrane collagens such as type IV collagen.[23] Furthermore, it was suggested that other basement-membrane-associated collagens may be targets of this enzyme as well.[23] It is noteworthy that collagen type IV is found in various ocular structures, including the lens, iris, retina, retinal pigment epithelium, and trabecular meshwork (NEIBank). Interestingly, P3H2 is expressed in the developing lens capsule of mouse embryos (E11–E15).[23] Because the lens capsule is a basement membrane whose main component is type IV collagen, these findings further suggest that defective collagen hydroxylation as a result of P3H2 inactivation might cause abnormality of the lens capsule, possibly leading to the development of cataracts and possibly contributing to the myopia phenotype by affecting distensibility of the lens capsule. An abnormally weak lens capsule can also account for a capsular rupture during cataract surgery; such a rupture occurred in many of our patients. Because type IV collagen has been demonstrated in the ciliary zonules of the eye (suspensory ligament of the lens),[24] its abnormality may cause a defective zonular structure. Weakening of the zonules probably underlies lens instability, as evidenced by spontaneous lens subluxation in some of our patients, as well as pathological zonulolysis during cataract surgery in others.

Another basement membrane in the eye is the inner limiting membrane (ILM) of the retina. It has been shown that ILM is important for proper retinal development and in the early regulation of eye size: ILM disruption resulted in marked eye enlargement in chick embryos, implicating that defective ILM might lead to congenital high myopia.[25] Inactivation of P3H2 as a result of the *LEPREL1* mutation in our patients might cause a weakened retinal ILM and thus lead to excessive eye growth and development of myopia. Likewise, a defective retinal basement membrane harbors greater risk for retinal tears and dialyses, which can lead to retinal detachments such as those observed in some of our patients. This predisposition, in addition to complicated cataract surgery, might have further aggravated the prognosis of retinal detachments in our patients.

It is worth noting that defects in collagen hydroxylation have been previously demonstrated to cause phenotypes similar to those caused by collagen mutations: autosomal-recessive variants of osteogenesis imperfecta, a disease known to be caused by dominant collagen mutations, can be caused by defects in collagen prolyl 3- hydroxylation.[18,26,27] Development of myopia has been shown to be associated with decreased rates of collagen synthesis in the sclera.[28] Moreover, various clinical syndromes such as Stickler syndrome (MIM 108300), Marshall syndrome (MIM 154780), and Weissenbacher-Zweymuller syndrome (MIM 277610), in which myopia is a prominent feature, are caused by defects in collagens.[3,4] Thus, whereas mutations in collagen genes cause autosomal-dominant myopia syndromes, defects in collagen hydroxylation might cause autosomal-recessive myopia. It of course yet remains to be tested whether P3H2 modifies the collagens known to act in those processes. Our findings demonstrate a significant role of *LEPREL1* in the molecular pathways of ocular growth, as well as in the development of myopia and cataracts. Further studies will need to explore whether *LEPREL1* mutations or polymorphisms are associated with ocular abnormalities in a wider population. An improved understanding of the molecular basis of pathological myopia might allow early diagnosis and treatment and thus prevent irreversible visual loss, as developed in our patients.

Supplemental Data

Supplemental Data include tables showing the results of the genome-wide scan, the results of a scan excluding chromosome 7, and the DNA sequences of the primers used. They can be found with this article online at http://www.cell.com/AJHG/.

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Web Resources

The accession number and URLs for data presented herein are as follows:

BLAST, http://www.ncbi.nlm.nih.gov/blast/
GenBank, http://www.ncbi.nlm.nih.gov/Genbank/
NEIBank, http://neibank.nei.nih.gov/cgi-bin/runSearch.cgi/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org
PolyPhen, http://genetics.bwh.harvard.edu/pph2/
Primer3, http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/
SUPERLINK, http://bioinfo.cs.technion.ac.il/superlink/
Tandem Repeats Finder, http://tandem.bu.edu/trf/trf.html/
UCSC Genome Browser, http://www.genome.ucsc.edu/

Accession Numbers

[accession number NM_018192])

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