New COL6A6 Variant Causes Autosomal Dominant Retinitis Pigmentosa in a Four-Generation Family

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PURPOSE. To report that variants in the gene for a large lamina basal component protein, COL6A6 (collagen type VI alpha 6 chain, Col6α6), linked to chromosome 3p22.1 causes retinitis pigmentosa (RP) in patients with autosomal dominant transmission (adRP).

METHODS. A positional-cloning approach, whole exome sequencing, and modeling were used. The proband and several affected family members have been phenotyped and followed for over 12 years.

RESULTS. A heterozygous missense variant, c.509C>G (p.Ser170Cys) in exon 2 of COL6A6 (comprised of 36 exons and 2236 amino acids), was observed in a four-generation family and is likely to cause the adRP phenotype. It was identified in 10 affected members. All affected family members had a distinct phenotype: late-onset rod cone dystrophy, with good retained visual acuity, until their late 70s. Immunohistochemistry of human retina showed a dot-like signal at the base of the inner segments of photoreceptors and outer plexiform layer (OPL). The structural modeling of the N7 domain of Col6α6 suggests that the mutant might result in the abnormal cellular localization of collagen VI or malformation of collagen fibers resulting in the loss of its unique filament structure.

CONCLUSIONS. COL6A6 is widely expressed in human tissues and evolutionary conserved. It is thought to interact with a range of extracellular matrix components. Our findings suggest that this form of RP has long-term useful central visual acuity and a mild progression, which are important considerations for patient counseling.

Keywords: COL6A6, collagen type VI alpha 6 chain, inherited retinal disease, retinitis pigmentosa, rod–cone dystrophy, genotype–phenotype correlation

Retinitis pigmentosa (RP) is a group of retinal diseases caused by primary or secondary death of photoreceptors and occurs in approximately 1 per 4000 individuals. Transmission may be autosomal or X-linked, dominant or recessive, and digenic inheritance or mitochondrial involvement has been reported in rare situations. Recent advances in technology, mainly the availability of whole exome sequencing (WES), have allowed the identification of a great number of genes. Those genes are mainly responsible for autosomal recessive forms of RP, as the mapping strategy is based on the identification of homozygous or compound heterozygous pathogenic or probable pathogenic variants. WES only rarely identifies allele dropouts (whole exon or gene deletions). Gain-of-function variants are also more complicated to evaluate and validate. In this report, we show that a dominant probable pathogenic variant in COL6A6 results in autosomal dominant retinitis pigmentosa (adRP).

MATERIALS AND METHODS

Clinical Sample Collection

The protocol of the study adhered to the tenets of the Declaration of Helsinki and was approved by the local ethics committee, Federal de la Sante Publique (DFSP 035.0003-48).

Subjects and Clinical Evaluation

The study was conducted in a four-generation Swiss/French family. Eighteen patients were affected with typical retinitis pigmentosa (Fig. 1). Some of them were admitted to the Jules-Gonin Eye Hospital in Lausanne and underwent a full ophthalmic and electrophysiological examination. Records sent by other treating ophthalmologists from France were reviewed when accessible. Subjects underwent color fundus
5.12 under the assumption of autosomal dominant inheritance with complete penetrance (new mutation rate of 0.001 as described in the 1000 Genomes or ExAC/gnomAD). The quality control criteria for this analysis were total pass-filter (PF) reads, 125,171,510; unique PF reads, 115,774,525; percent Q score of 30 (Q30) bases, 91.49%; mean target coverage depth, 189.5; and percent autosomal callability, 96.48%.

Bioinformatics Analysis
The obtained data were aligned using the human reference data hg19. Exome data were analyzed using the SNP & Variation Suite (Golden Helix, Bozeman, MT, USA) to identify variants that are shared between the affected individuals and then prioritized based on their absence/rarity in the general population, as reported in the publicly available databases dbSNP (http://www.ncbi.nlm.nih.gov/snp), 1000 Genomes (http://www.1000genomes.org), and gnomAD (https://gnomad.broadinstitute.org). The impact of missense variants on protein function was evaluated using Polymorphism Phenotyping v2 (PolyPhen-2),\textsuperscript{3} PROVEAN,\textsuperscript{4} and SIFT.\textsuperscript{5} Only genes expressed in the eye with heterozygous variants were retained. We excluded synonymous variants and variants with a minor allele frequency higher than 0.01 as described in the 1000 Genomes or ExAC/gnomAD databases, as well as variants located in the non-coding sequence. Candidate variants were validated in affected individuals and in controls by conventional Sanger sequencing.

Variant Analysis
Primers flanking the exons, introns, splice junction, and 5′ and 3′ untranslated regions (UTRs) were designed using Primer3 Input 0.4.0 for the candidate gene RHO evaluated by linkage analysis, and also primers flanking exon 2 of the candidate gene COL6A6 (encoding collagen alpha-6(VI) chain [col6a6])\textsuperscript{6} identified via exome sequencing. Each 20-μL PCR amplification reaction contained 10 μL of FastStart PCR Master Mix (Roche), 10 pmol of each primer (Eurogentec, Liège, Belgium), and 20 ng of genomic DNA. The
DNA was denatured at 95°C for 5 minutes prior to 35 cycles of amplification of 1 minute at 94°C, 1 minute at 58°C, 1 minute at 72°C, and a final extension step at 72°C for 10 minutes using a GeneAmp 9700 thermal cycler (Applied Biosystems, Waltham, MA, USA). The PCR products were resolved in 1% agarose gel stained with ethidium bromide. The amplified fragments were purified and sequenced in the forward direction using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and loaded onto an ABI Prism 3100 genetic analyzer (Applied Biosystems). The data were analyzed using ABI Prism Navigator software.

**Immunohistochemistry**

Sections (4 μm) were cut from paraffin-embedded, formalin-fixed tissues from eyes enucleated for uveal melanoma retrieved from the archives of the pathology laboratory at Jules-Gonin Eye Hospital (local ethics committee authorization 340/15). After blocking endogenous peroxidase by 4% hydrogen peroxide for 10 minutes and antigenic unmasking at pH 6, the sections were incubated for 60 minutes with anti-COL6A6 antibody (HPA045239, 1/1000 dilution; Atlas Antibodies, Stockholm, Sweden). A streptavidin/biotin detection method with 3,3'-diaminobenzidine tetrachloride was used for signal detection (Dako EnVision+ Dual-Link System–HRP; Agilent Technologies, Santa Clara, CA, USA).

**Structural Modeling of N7 Domain of col6α6**

The online PHYRE2 server was used to generate the homology-based structural model of col6α6 N7 domain. The model (residues 25–217) was generated with high confidence based on the p.Arg1061Gln mutant structure of col6α3 (PDB ID, 4HK). The figure was generated using Pymol.

**RESULTS**

The index individual in family 1 (IV.10) was a 67-year-old female who had suffered from night blindness since she was 15 years old. When she first came to the Jules-Gonin Eye Hospital, visual acuity (VA) was 20/20 in both eyes, corrected with −3.50 D = −3.25/2° in the right eye and −1.5D = −2.75/165° in the left eye. Seven years later, her VA declined to 20/35 partly due to bilateral early lens sclerosis. Fundus examination showed some bone spicules at the mid-periphery with no macula abnormalities (Figs. 2A, 2B). Rod-specific ERGs were undetectable, whereas photopic ERGs were severely reduced. Peripheral visual field constriction via an I-4e isopter, as measured by Goldman kinetic perimetry, was within 10°, whereas it was 75° horizontally with a V-4e isopter. There was a relative scotoma at the mid-periphery. OCT did not show any macular edema. The outer nuclear layer, ellipsoid zone, and external limiting membrane (ELM) were well preserved at the level of the fovea and then disappeared outside fovea (Figs. 2E, 2F). Autofluorescence imaging showed a decrease or lack of autofluorescence around the optic nerve and alongside retinal temporal arcades (Figs. 2C, 2D, 3A, 3B). Over the 12-year follow-up, the AF images showed an increase of hypofluorescent areas near the optic nerve and along and beyond the arcades. Over the same period, there was mild constriction of the hyperautofluorescent ring (Figs. 3C, 3D). The proband’s affected brother (IV.9) also suffered from night blindness, since his early 20s. When he was 26 years old, he tried to become a train driver but never received authorization due to visual field constriction. Currently 68 years of age, he has a best-corrected visual acuity of 10/20 in the right eye and 15/20 in the left eye. He had bilateral macular edema, which improved following treatment (anti-vascular endothelial growth factor or dexamethasone implant intravitreal injections). Both patients were from a non-consanguineous family originating from Switzerland, with 18 affected individuals. The age of onset of symptoms within family members started in their mid-teens and early 20s, with night blindness and peripheral visual field constriction. The mother of the proband (III.3) lost her central vision when she was 80 years old. Her father (II.1) was blind when he died at the age of 75 years. One unaffected brother of the proband (IV.7) had been assessed by a private ophthalmologist in France when he was 58 years old. He underwent a complete ophthalmological and fundus examination and visual field evaluation, with results within normal limits. Currently 71 years old, he is still driving. No family member reported having a myopathy, atopic dermatitis, or any other major health condition.

**Combined Genetic Mapping and Whole Exome Sequencing**

In this family, we performed genome-wide linkage analysis and found a maximum LOD score of 1.51 at marker D3S5606. Ten of the affected patients who agreed to genetic testing were studied (Fig. 1; patients III.1, III.2, III.3, III.6, III.8, IV.3, IV.9, IV.10, IV.15, and IV.21). Haplotype analysis showed that affected individuals in the family shared a common haplotype delimited by markers D3S1589 and D3S1292, a region of approximately 5.7 Mb at 3q21 (Fig. 1). As this interval contained the RHO gene, all of its five exons, complete introns, 5′-UTR (600 bp upstream of the start codon ATG), and 3′-UTR (600 bp downstream of the stop codon TAA) were directly sequenced in two affected individuals, and none of them had variation in this gene. Multiplex ligation-dependent probe amplification analysis did not show any deletion of duplication. We used a combination of linkage studies and WES to elucidate the disease-causing variant. WES was performed in three affected individuals (Fig. 1; patients III.1, III.8, and IV.10). Comparison of the whole exome data for the first two patients identified 484 genes expressed in the eye and non-synonymous variants that were novel or rare (<1%). Three genes located within or close to the linked region (PLXNA1, COL6A6, and TOPBP1) showed heterozygous variants and were tested for variant co-segregation in the family. None of the variants present in the selected genes segregated in affected individuals except for the heterozygated missense variant c.509C>G (p.Ser170Cys) in exon 2 of COL6A6 (ENST00000358511.10) which was present in all 10 affected members. WES of the third patient confirmed the absence of pathogenic or probable pathogenic variant in RHO. This variant is novel and is predicted to be damaging by the PolyPhen-2 tool, with a score of 0.999; deleterious by PROVEAN, with a score of −3.370; and influencing protein function by SIFT, with a score of 0.01. This new variant was absent from non-affected family members tested (Fig. 1; patients IV.7, IV.8, and IV.12) and had not been described in gnomAD as of November 27, 2021.

**Immunofluorescence Analysis**

Type VI collagen is a subgroup of unconventional and network-forming collagens. It is ubiquitously expressed in...
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Figure 2. Fundus color, AF, and OCT of both eyes of the proband. (A, B) Fundus color showing bone spikes and signs of retinal atrophy nasal to the optic nerve and alongside arcades. (C, D) Hypoautofluorescence around the optic nerve, alongside arcades and patches of hypofluorescence nasal to the optic nerve. (E, F) OCT did not show any macular edema. The outer nuclear layer, ellipsoid zone, and ELM are well preserved at the level of the fovea and then disappear outside the fovea.

humans and plays important roles in tissue repair, neuron protection, injury response, and corneal development. Pathogenic or probable pathogenic variants in collagen VI compartments are reported to cause myopathies, including Bethlem myopathy. We assessed the expression of col6α6 by immunohistochemistry in healthy human retinas from enucleated eyes. Although there was a diffuse staining in the retina including some nuclei, a dot-like signal could clearly be identified at the base of the inner segments of photoreceptors and in the outer plexiform layer (Fig. 4A).

Collagen VI has a unique molecular structure that forms beaded filaments. The generally understood chain composition of collagen VI is α1(VI)α2(VI)α3(VI) heterotrimer helix; yet, the α3(VI) can potentially be substituted by α6(VI) coded by the COL6A6 gene. The α6(VI) consists of seven N-terminal von Willebrand factor A (VWA) domains (also referred to as N7–N1 domains) followed by a collagenous domain, two C-terminal VWA domains (also referred to as C1 and C2 domains), and a unique domain also known as C3 domain (Fig. 5A). The interaction among the collagenous domains of α1(VI), α2(VI), and α6(VI) drives the heterotrimer helix chain formation of collagen VI (Fig. 5B), which is necessary for secretion of the chain. When the chains are made, they assemble into a dimer and then form tetramers (Fig. 5C). Upon secretion and deposition of these tetramers in the extracellular matrix, the non-collagenous domains (i.e., N7–N1 and C1–C3 domains) contribute to the formation of beaded filaments (supramolecular aggregations) through metal ion–dependent adhesion and/or conserved hydrophobic moieties (Fig. 5D).

The heterozygous missense variant (c.509 C>G; p.Ser170Cys) in COL6A6 is located in the N7 domain. Structural modeling of the mutant col6α6 N7 domain demonstrates that the variant is located at the β4–α5 loop...
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FIGURE 3. AF imaging over 12 years of follow-up. (A, B) There is a decrease of AF around the optic nerve and alongside retinal temporal arcades. (C, D) Over the years, the AF images showed an increase of hypofluorescent areas around the optic nerves, along and beyond the arcades. Over the same period, there was a very mild constriction of the hyperautofluorescent ring.

FIGURE 4. Immunohistochemistry of COL6A6 expression in human retina. (A) COL6A6 expression could be diffusely identified in the retina with marked expression in the nerve fiber layers (black arrows) and a dot-like expression in the outer plexiform layer (downward white arrows), as well as in the inner segments of photoreceptors close to the ELM (upward white arrows). (B) Matched negative retinal control (magnification 252×).
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**FIGURE 5.** Collagen α6(VI) domain topology and structural modeling highlight the potential defects of the patient mutant in the collagen-formation dynamics. The patient variant (p.Ser170Cys) is highlighted in magenta throughout the figure. (A) Domain topology of collagen α6(VI). The N7 domain (the first VWA domain) that includes our patient variant is shown in the cyan box. Other VWA domains (N6–N1 and C1–C2) are shown in the gray boxes. The collagenous domain is shown in the green box. The unique domain (C3) is shown in the yellow box. (B) Illustration of the α1(VI)α2(VI)α6(VI) chain assembly in an intracellular space. The collagenous domains from each collagen VI protein form a heterotrimer helix resulting in a chain monomer. The clusters of N domains are represented as a cyan–gray circle, and the C domain cluster is represented by the gray circle. (C) Representation of the collagen VI chain multimerization in an intracellular space. Two monomer chains form a dimer, then two dimers form a tetramer. After tetramer formation, it will be secreted to an extracellular space. (D) Representation of collagen VI filament formation at an extracellular region. The non-collagenous domains at the end of each tetramer join to form a bead-like structure resulting in a beaded filament. (E) The homology-based structural model of the collagen α6(VI) p.Ser170Cys mutant N7 domain. It was modeled from the VWA structure of collagen α3(VI) (PDB ID, 4IHK). The mutant cysteine was exposed to solvent. This suggests that the mutant cysteine may be freely available to form disulfide bonds with the cysteines of itself, other collagen building blocks, or non-specific proteins. This might significantly affect the dynamics of collagen filament formation and result in abnormal collagen VI filament structures.
of the domain and is solvent exposed (Fig. 5E). The serine-to-cysteine change at the loop region is unlikely to disrupt protein stability, but the surface-exposed cysteine can form nonspecific disulfide interactions with freely available cysteines in other proteins. Thus, this additional cysteine in the mutant may significantly disrupt chain assembly dynamics and/or the supramolecular aggregation dynamics of collagen VI. These may result in the abnormal cellular localization of collagen VI compartments or malformation of collagen fibers, resulting in the loss of its unique beaded filament structure. Additionally, it may affect the accessibility of the collagen-binding proteins and lead to abnormal cellular signaling events.

**DISCUSSION**

In this report, we describe a four-generation family with adRP, likely due to a c.509C>G (p.Ser170Cys) heterozygous missense variant in COL6A6 that segregated in all affected members. Our family has a very mild rod–cone retinal dystrophy, with slow progression, and they have retained central VA until their mid-70s, with the eldest members losing his VA in his late 70s. In a previous report, a COL6A6 variant was also detected in an adRP pedigree; however, that variant was linked to deletion of exon 5 in RHO, the COL6A6 variant, and deletion segregating in all affected members. That family also showed mild progression of rod–cone dystrophy.

**COL6A6** (OMIM 131873) is located on chromosome 3q22.1 and contains 36 exons. It encodes a large protein that is a component of the basal lamina of epithelial cells. In human normal tissue, COL6A6 is mainly expressed in lung, fat, heart, spleen, and skeletal and cardiac muscle. Its expression in the retina has been reported by de Sousa Dias et al., who used PCR amplification of a fragment from retinal cDNA containing juxtaposing sequences of exons 2 and 3.

Our results demonstrated a dot-like expression of COL6A6 in the inner segments of the photoreceptors and in the outer plexiform layer, as well as a diffuse staining in the retina. These findings are in line with single-cell RNA expression from the human protein atlas where COL6A6 has been mainly identified in rod photoreceptors and bipolar cells (https://www.proteinatlas.org/ENSG00000206384-COL6A6/celltype/eye). COL6A6 has been identified only recently, together with COL6A4 and COL6A5, at the same 3p22.1 locus and is evolutionarily conserved. It encodes the α6(VI) chain, containing a 336-amino acid triple helix flanked by seven N-terminal VWA-like domains. Collagen VI interacts with a range of extracellular matrix components. Due to its wide expression, COL6A6 is suspected to be involved in early-onset atopic dermatitis, as well as collagen VI–related myopathies. COL6A6 is also suspected of playing a causative role in ossification of the posterior longitudinal ligament of the thoracic spine in Chinese populations.

Other collagen genes have been widely described as being causative of syndromic RP. **COL2A1** is well known in Stickler syndrome (SS; MIM 108300), a progressive hereditary arthro-ophthalmopathy of collagen connective tissue with an associated RP phenotype recently reported. Retinal perivascular hyperpigmentation and retinal perivascular degeneration have also been described as a consistent finding in patients with SS and variants in the **COL2A1** gene.

Variants in others collagen genes have been reported to cause several subtypes of SS (**COL9A1**, **COL9A2**, and **COL9A3**) with variable retinal phenotypes, ranging from progressive chorioretinal degeneration, lattice degeneration, and retinal detachment to normal. More studies are needed to confirm these preliminary findings. This will enable a better understanding of the function of **COL6A6**, which may be deemed to be part of the panel of adRP genes.

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