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Whole exome analysis identifies dominant *COL4A1* mutations in patients with complex ocular phenotypes involving microphthalmia

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Abstract

Anophthalmia/microphthalmia (A/M) is a developmental ocular malformation defined as complete absence or reduction in size of the eye. A/M is a heterogeneous disorder with numerous causative genes identified; however, about half the cases lack a molecular diagnosis. We undertook whole exome sequencing in an A/M family with two affected siblings, two unaffected siblings, and unaffected parents; the ocular phenotype was isolated with only mild developmental delay/learning difficulties reported and a normal brain MRI in the proband at 16 months. No pathogenic mutations were identified in 71 known A/M genes. Further analysis identified a shared heterozygous mutation in *COL4A1*, c.2317G>A, p.(Gly773Arg) that was not seen in the unaffected parents and siblings. Analysis of twenty-four unrelated A/M exomes identified a novel c.2122G>A, p.(Gly708Arg) mutation in an additional patient with unilateral microphthalmia, bilateral microcornea, glaucoma and Peters anomaly; the mutation was absent in the unaffected mother and the unaffected father was not available. Mutations in *COL4A1* have been linked to a spectrum of human disorders; the most consistent feature is cerebrovascular disease with variable ocular anomalies, kidney and muscle defects. This study expands the spectrum of *COL4A1* phenotypes and indicates screening in patients with A/M regardless of MRI findings or presumed inheritance pattern.

Keywords

microphthalmia; Peters anomaly; whole exome sequencing; *COL4A1*; small vessel disease; stroke

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Conflict of interest
The authors declare that they have no conflict of interest.

Introduction

Anophthalmia and microphthalmia (A/M) are defined as the complete absence or reduction in size of the eye, respectively. A/M has a combined incidence between 1-3.2 cases per 10,000 live births (1-3). These conditions are highly debilitating as approximately 10% of all pediatric visual impairment is due to A/M and one third of all patients with A/M have additional systemic features (1-3).

A/M is a genetically heterogeneous disorder that has been associated with mutations in over 70 genes. *SOX2*, *OTX2* and *FOXE3* represent the most common genetic factors, each accounting for approximately 10-20, 4-8 and 15% of dominant (*SOX2*, *OTX2*) and recessive (*FOXE3*) cases, respectively (4-8). Other causative genes include transcription factors (*CHX10*, *RAX*, *PAX6*, *PITX3*), major lens proteins (*CRYBA4*, *CRYAA*, *CRYBB2*), members of the bone morphogenic family (*BMP4*, *BMP7*, *GDF6*, *GDF3*) and many more. Despite this progress, currently at least half of A/M patients lack a molecular diagnosis (9).

Anophthalmia/microphthalmia (A/M) can be observed as part of various syndromes associated with specific genes (10). However, due to the extreme variable expressivity and complexity of these conditions, assignment of a specific diagnosis based on phenotypic features alone is often difficult. Whole exome sequencing has been successfully employed to identify causative mutations in genetically heterogeneous disorders including A/M; recently several new A/M genes have been discovered via whole exome sequencing, including *ALDH1A3* (11), *FNBP4* (12), *C12orf57* (13,14) and *ODZ3* (15). In some cases, whole exome sequencing has allowed for the expansion of phenotypes associated with genes previously reported to play a role in human disease (16).

Materials and Methods

Human patients

This human study was approved by the Institutional Review Board of the Children's Hospital of Wisconsin with written informed consent obtained from each participant and/or their legal representative, as appropriate. DNA was extracted from blood samples using standard protocols and analyzed for quantity/quality using the NanoDrop 1000 spectrophotometer (Thermo Fisher, USA).

Whole Exome Sequencing and Data Analysis

Genomic DNA was submitted for whole exome sequencing by Perkin Elmer, Inc (Branford, CT); exome capture was performed with the Agilent Sure Select v4 + UTR and 100 base pair paired end sequencing was performed using the Illumina HiSeq 2000. The obtained data were aligned using the Burrows-Wheeler Aligner (BWA) and variants were called using the Genome Analysis Toolkit (GATK v2.10 or v2.20) analysis pipeline available through Perkin Elmer. The samples were analyzed for mutations in 71 genes previously associated with A/M or coloboma (Online Resource 1) and other ocular genes (NEIBank list of Human Eye Disease Genes at http://neibank.nei.nih.gov/index.shtml) using the SNP & Variation Suite (Golden Helix, Bozeman, MT); analysis of variants for their possible effect on protein function was performed within the SVS program by accessing data from dbNSFP, which

provides scores from multiple functional prediction programs (SIFT, Polyphen2, Mutation Taster, MutationAssessor, and FATHMM) as well as two conservation scores (GERP++ and PhyloP) (17). The observed variants were evaluated for their frequency in the general population using publicly available databases such as dbSNP (http://www.ncbi.nlm.nih.gov/snp), Exome Variant Server (NHLBI Exome Sequencing Project (ESP), Seattle, WA, (http://evs.gs.washington.edu/EVS/), and 1000 Genomes project (http://www.1000genomes.org/data).

Variant Confirmation

Primers flanking variant sites were designed and genomic DNA was amplified in probands and all available family members to confirm the variant and determine its inheritance. The following primers and conditions were utilized for the *COL4A1* mutations: *COL4A1*-1F 5'-TGGTTTGATAAAAATACTGACTTTGC-3', *COL4A1*-1R 5'-CCACGAGCTCTAGGCCTCTAA-3', *COL4A1*-2F 5'- AAGGCCTCCACAGTTGACC-3', *COL4A1*-2R 5'- TCATCAAAAACAAAAATCTGCTG-3'. PCR products were sequenced bidirectionally using Big Dye Terminator chemistry and ABI 3730XL sequencer (Applied Biosystems/Life Technologies, Carlsbad, CA, USA). Sequences were reviewed manually and using Mutation Surveyor (SoftGenetics, State College, PA).

Results and Discussion

A female Hispanic patient (Patient 1A, proband) was referred to the study with a diagnosis of bilateral microphthalmia, congenital cataracts (extracted in infancy), recurrent postsurgical pupillary membranes, mild developmental delay and normal brain MRI at 16 months of age (Figure 1). Her brother, Patient 1B, was similarly affected with bilateral congenital cataracts (extracted in infancy), microcornea, irregular pupils, glaucoma (diagnosed at age 8 years), and mild learning difficulties. Both parents and two additional siblings are unaffected (Figure 2).

The proband's genomic DNA was submitted first for whole exome sequencing. Mean coverage of the target region in Patient 1A was 67.74X with 79% of the region showing greater than 10X coverage. Screening in 71 genes associated with A/M and/or coloboma (Online Resource 1) identified 2 novel and 2 rare heterozygous variants in the proband (Table 1). Among the identified variants, a *STRA6* nonsense allele, (p.Gln20*), predicted to result in an early truncation of the encoded protein and a *CRYBA4* missense allele, (p.Arg25Trp), predicted to be damaging by 4 out of 5 functional effect predictor programs, appeared to be the most significant; neither allele has been previously reported in general populations and mutations in both *STRA6* and *CRYBA4* were reported in association with a dominant pattern of inheritance (18,19). All four variants were confirmed by Sanger sequencing in the proband and analyzed for co-segregation in other family members. None of the alleles co-segregated with the affected phenotype; specifically, both *STRA6* and *CRYBA4* alleles were absent in the affected sibling and present in one of the unaffected parents (Table 1).

In order to facilitate causative gene identification, whole exome sequencing of genomic DNA of proband's affected sibling (Patient 1B) was performed. Mean coverage of the target

region in Patient 1B was 73X with 96.3% showing greater than 10X coverage. Analysis of shared exome variation considered both recessive and dominant modes of inheritance: 50 homozygous alleles in 39 genes, 248 possible compound heterozygous alleles in 38 genes, and 408 novel nonsynonymous heterozygous variants were identified. These variants were analyzed further to isolate shared alleles in 666 genes from the NEIBank list of Human Eye Disease Genes resulting in the identification of 17 shared heterozygous variants in 9 genes. A *COL4A1* mutation in exon 30, c.2317G>A, p.(Gly773Arg), emerged as the most likely pathogenic allele from this list. Additional analysis included whole exome sequencing of both unaffected parents and utilizing their WES data to identify de novo variants (under dominant model) or rare/novel heterozygous paternal and maternal alleles (under recessive model) shared by both affected children. This analysis resulted in the identification of the *COL4A1* allele as the only de novo mutation observed in both affected children (maternal and paternal samples had 30X and 60X coverage, respectively, for this region and showed normal sequence); no variants consistent with recessive pattern were identified. The *COL4A1* mutation was confirmed by Sanger sequencing (Figure 2) in both affected individuals and ruled out in blood samples from both unaffected parents, buccal sample from the unaffected mother, as well as blood samples from the two unaffected siblings; it is presumed that one parent has gonadal mosaicism for the *COL4A1* mutation. The *COL4A1* p.(Gly773Arg) mutation is expected to change a conserved glycine residue of the Gly-X-Y repeat of the triple helical domain into arginine; it is predicted to be damaging by SIFT, PolyPhen, MutationTaster, MutationAssessor, and FATHMM, has high nucleotide conservation scores (GERP++ =4.7 and PhyloP=2.328), and is not reported in dbSNP, 1000 Genomes, or EVS. Moreover, this mutation was previously reported in two siblings affected with bilateral cataracts, spastic quadriplegia or hemiplegia, and white matter changes (20).

Following the identification of the *COL4A1* mutation, Patients 1A and 1B were seen in clinic for follow-up. Both siblings demonstrated marked microcornea and moderate microphthalmia (Figure 1). A non-specific history of mild learning difficulties and occasional muscle cramps was reported for both siblings, however there were no reports of muscle weakness, kidney disease, or episodes of stroke. A subsequent brain MRI and MRA performed at 6 years of age in Patient 1A identified poorly defined T2/FLAIR hyperintensity in bilateral centrum semiovale (right more than left) and a few smaller T2 hyperintense foci in the cerebellar white matter (Figure 1). These findings are non-specific but compatible with the small vessel disease process seen with *COL4A1* mutations. There were no signs of intracranial hemorrhage or other focal brain parenchymal abnormalities and no evidence of porencephalic cyst. MR angiography of the brain was normal. Retrospective evaluation of the MRI from 16-months of age demonstrated mild T2/FLAIR hyperintensity in the centrum semiovale that was in part masked by the immature (incomplete) myelination on this study due to the patient's age. Other studies completed in Patient 1A, including serum CK level, renal and liver ultrasound, and urinalysis were all normal. Unfortunately, further clinical evaluation of Patient 1B was not possible.

In order to determine the contribution of *COL4A1* to A/M, whole exome data from twenty-four additional probands with A/M of unknown genetic etiology were analyzed for mutations in *COL4A1*. The mean coverage of the target region was 67.3 ± 17.3 with 94.9%

± 2.7% of bases showing >10X coverage. The mean coverage of *COL4A1* was ~40-50X. A novel heterozygous *COL4A1* mutation, c.2122G>A, p.(Gly708Arg) was identified in Patient 2, a 5-year-old female patient from India referred with a diagnosis of cataract, glaucoma, and retinal detachment in the left eye, microphthalmia in the right eye, and bilateral Peters anomaly with microcornea (Figure 3). There is no history of developmental delays and the patient has never had a brain MRI; the only non-ocular feature reported is clinodactyly. She is the first child born to her unaffected parents; family history reveals paternal aunts who were stillborn (twin pregnancy) but no history of ocular or cerebrovascular disease. The mother was found to have normal *COL4A1* alleles based on Sanger sequencing, the father was unavailable for screening. The c.2122G>A, p.(Gly708Arg) mutation is similarly expected to change a conserved glycine residue of the Gly-X-Y repeat of the triple helical domain into arginine; it is predicted to be damaging by SIFT, PolyPhen, MutationTaster, MutationAssessor, and FATHMM, shows high nucleotide position conservation scores (GERP++ =4.86; PhyloP=2.53) and is not reported in dbSNP, 1000 Genomes, or EVS.

COL4A1 has been associated with a diverse set of multi-systemic disorders. Initially, *COL4A1* was found to cause porencephaly (21). It has since been associated with cerebrovascular disease (brain small vessel disease, intracerebral hemorrhage, leukoencephalopathy) as well as nephropathy, muscle cramps (associated with elevated levels of creatine kinase) and ocular anomalies (22, 23). Ocular abnormalities associated with *COL4A1* mutations were examined by Coupry et. al. (2010) in 7 affected individuals from two unrelated families and most commonly included cataracts (7/7), iris anomalies (6/7), and microcornea (6/7); corneal opacities (2/7), retinal detachment (2/7), anterior synechiae (2/7), increased intraocular pressure (3/7) and myopia (3/7) were also observed but no microphthalmia (23). Careful evaluation of all published *COL4A1* mutation reports which presented ophthalmological findings revealed that microphthalmia was reported in 4 out of 97 cases with cerebrovascular disease (20,23-38); while no images or details were provided in these manuscripts, this supports our finding of *COL4A1* mutations in A/M.

COL4A1 is ubiquitously expressed in basement membranes, including in the cornea, lens and retina, and is important for their stability (39). *COL4A1* contains three domains: the N-terminal 7S domain, a triple-helix (collagenous) domain and a C-terminal non-collagenous (NC1) domain. COL4A1 and COL4A2 form a heterotrimer consisting of two α1 molecules and one α2 molecule. The collagenous domain contains interrupted Gly-X-Y repeats that are responsible for forming the heterotrimer. According to one recent review, approximately 80% of mutations affect the glycine of the Gly-X-Y repeat and disrupt triple helix formation (22). It has been suspected that the disruption of the triple helix leads to accumulation of the misfolded protein and results in ER stress (40).

The phenotypes observed in Patients 1A, 1B, and 2 in this paper are generally consistent with the ocular manifestations in patients previously reported with *COL4A1* mutations, but microphthalmia and Peters anomaly have not previously been recognized as features associated with *COL4A1* mutations. In addition, both families demonstrated a primarily ocular phenotype, with only mild learning difficulties observed in one family. Interestingly, a normal brain MRI was observed in Patient 1 at the initial exam at 16 months of age; while this patient went on to develop MRI abnormalities, it is important to note that a normal MRI

at a young age does not rule out *COL4A1* involvement and it may be worthwhile to consider repeating MRI as a child ages in patients with anterior segment disorders, especially in the presence of learning difficulties. Further analysis of *COL4A1* in patients with microphthalmia and/or anterior segment dysgenesis is needed to determine the frequency of mutations and penetrance of cerebrovascular disease in this population.

The mutations reported in this study are likely to represent mosaic (first family) and de novo or incompletely penetrant (second family) changes. Gonadal mosaicism has not yet been observed in association with *COL4A1* mutations but has been reported in other dominant genes associated with A/M (9) and in other pedigrees which suggested recessive inheritance (41). Our identification of dominant *COL4A1* mutations in two families with apparently recessive or isolated inheritance patterns highlights the importance of considering all modes of inheritance when searching for causative disease alleles in small pedigrees.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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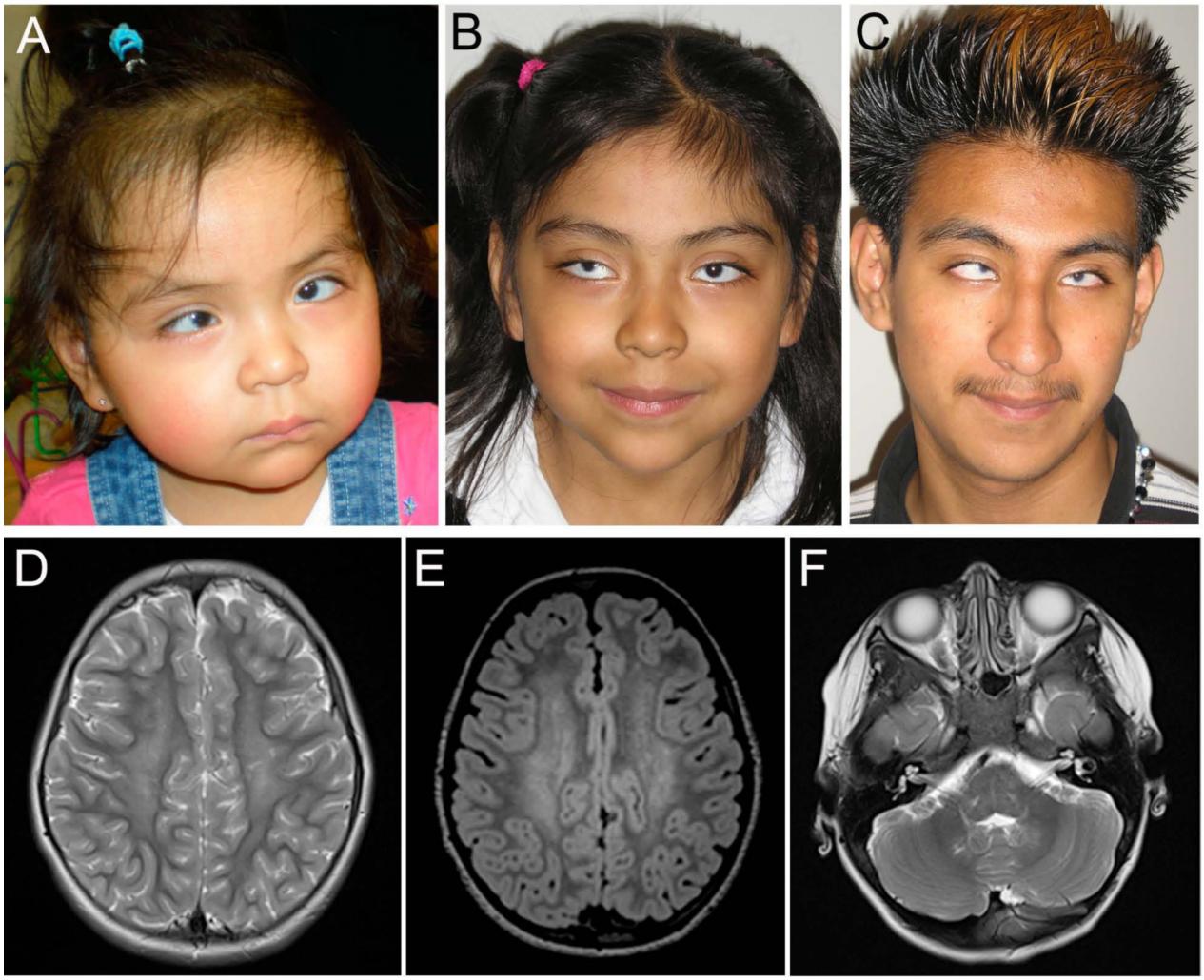

Figure 1. Clinical features of Patients 1A and 1B

Photographs of Patient 1A at the age of 16 months (A) and 6 years (B) and Patient 1B at 17 years (C). Brain MRI images of Patient 1A at the age of 6 years: axial T2 weighted (D) and FLAIR image (E) of the brain showing ill defined hyperintensity in the bilateral centrum semiovale; axial T2 weighted image at the level of the 4th ventricle with a few small, ill defined, T2 hyperintense foci seen in the cerebellar deep white matter (F).

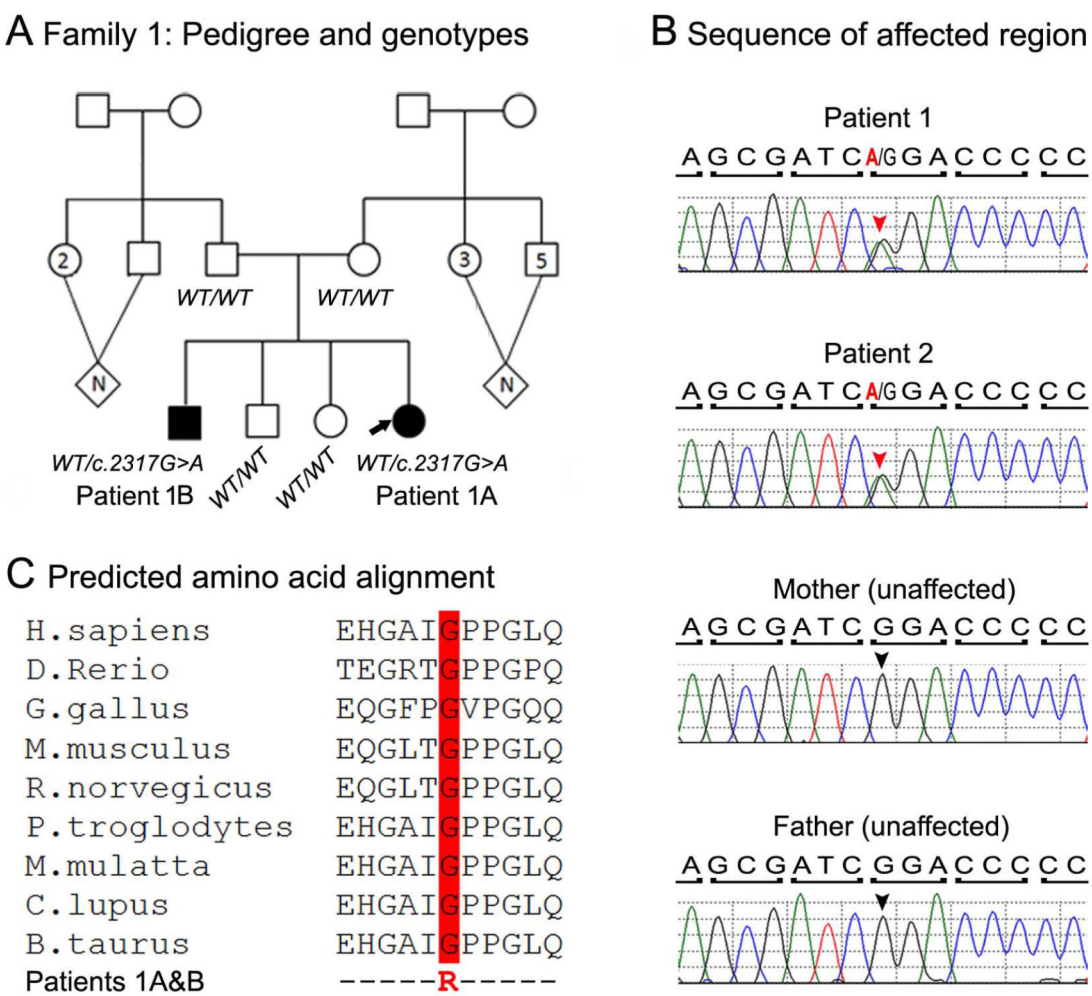

Figure 2. The *COL4A1* mutation identified in Family 1

Pedigree and genotypes (A), DNA sequence chromatograms for the affected siblings and unaffected parents (B) and protein alignment for the identified missense mutation (C) are shown.

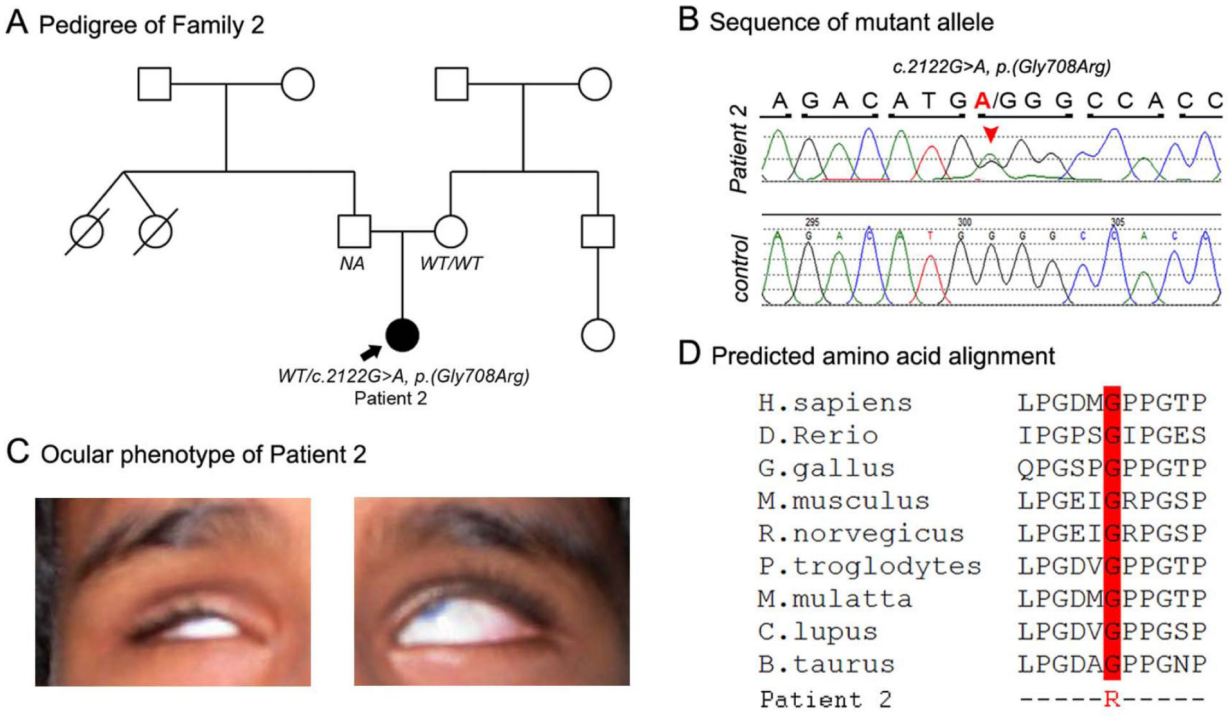

Figure 3. The *COL4A1* mutation identified in Family 2

Pedigree and genotypes (A), DNA sequence chromatogram for the affected child (B), photograph of the ocular phenotype (C) and protein alignment for the identified missense mutation (D) are presented.

Table 1

Summary of variants in A/M genes identified by whole exome sequencing in Patient 1A

Gene[a]	Position (nt)	Position (a.a.)	Functional Effect Predictions					PhyloP	GERP	EVS allele freq.	Segregation analysis
			SIFT	PolyPhen	Mutation Taster	Mutation Assessor	FATHMM				
STRA6	c.58C>T	p.Gln20*	NA	NA	NA	NA	NA	0.46	0.35	0/12,990	not present in affected sib and present in unaffected parent
CRYBA4	c.73C>T	p.Arg25Trp	Damaging	Probably Damaging	Disease Causing	Predicted Functional (High)	Tolerated	2.29	0.48	0/13,006	not present in affected sib and present in unaffected parent
FOXE3	c.601G>A	p.Val201Met	Tolerated	Benign	Polymorphism	Predicted Non-Functional (Neutral)	Damaging	1.75	0.31	0/11,318[b]	present in affected sib but also seen in unaffected parent
FREM1	c.335A>T	p.Asn112Ile	Tolerated	Benign	Polymorphism	Predicted Non-Functional (Low)	Tolerated	−5.73	−0.97	28/12,418	present in affected sib but also seen in unaffected parent

[a] GenBank numbers for *FOXE3* (NM_012186), *STRA6* (NM_001199040), *FREM1* (NM_001177704) and *CRYBA4* (NM_001886)

[b] Previously reported as a Hispanic variant (Reis et al. 2010)