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ARTICLE

Novel mutations in *PXDN* cause microphthalmia and anterior segment dysgenesis

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We used exome sequencing to study a non-consanguineous family with two children who had anterior segment dysgenesis, sclerocornea, microphthalmia, hypotonia and developmental delays. Sanger sequencing verified two Peroxidasin (*PXDN*) mutations in both sibs—a maternally inherited, nonsense mutation, c.1021C>T predicting p.(Arg341*), and a paternally inherited, 23-basepair deletion causing a frameshift and premature protein truncation, c.2375_2397del23, predicting p.(Leu792His*fs67). We re-examined exome data from 20 other patients with structural eye defects and identified two additional *PXDN* mutations in a sporadic male with bilateral microphthalmia, cataracts and anterior segment dysgenesis—a maternally inherited, frameshift mutation, c.1192delT, predicting p.(Tyr398Thr*fs**40) and a paternally inherited, missense substitution that was predicted to be deleterious, c.947 A>C, predicting p.(Gln316Pro). Mutations in *PXDN* were previously reported in three families with congenital cataracts, microcornea, sclerocornea and developmental glaucoma. The gene is expressed in corneal epithelium and is secreted into the extracellular matrix. Defective peroxidasin has been shown to impair sulfilimine bond formation in collagen IV, a constituent of the basement membrane, implying that the eye defects result because of loss of basement membrane integrity in the developing eye. Our finding of a broader phenotype than previously appreciated for *PXDN* mutations is typical for exome-sequencing studies, which have proven to be highly effective for mutation detection in patients with atypical presentations. We conclude that *PXDN* sequencing should be considered in microphthalmia with anterior segment dysgenesis.**
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INTRODUCTION

Anophthalmia (absent eyes), microphthalmia (small eyes) and coloboma (defective closure of the optic fissure) are important structural birth defects because of the medical significance of severely reduced vision. Mutations in several genes, including *SOX2*, *ALDH1A3*, *STRA6*, *OTX2*, *RAX*, *BMP4*, *VSX2*, *GDF6*, *SIX6*, *PAX6*, *VAX1* and *SMOC1* can cause anophthalmia and microphthalmia (A/M), but there is substantial genetic heterogeneity and >50% of affected individuals do not receive a molecular genetic diagnosis for their birth defect after testing of the currently known causative genes.[1–8] A/M can occur as an isolated malformation (simplex A/M) or can be combined with additional ocular abnormalities affecting anterior or posterior segments of the eye (complex A/M). Complex A/M often includes anterior segment dysgenesis, an eye defect constituting maldevelopment of the anterior structures behind the cornea and in front of the lens, including the iris, ciliary body and trabecular meshwork. Anterior segment dysgenesis is important to recognize because it is frequently associated with cataracts and predisposes to increased ocular pressure and developmental glaucoma in 50% of cases.[9] *FOXC1* and *PITX2* disruptions, either mutations or deletions, have been estimated to account for 6–40% of patients with anterior segment dysgenesis,[10–11] but are frequently associated with systemic findings, such as dental and umbilical anomalies in patients with *PITX2* haploinsufficiency and cardiac and hearing deficits in patients with *FOXC1* haploinsufficiency.[11] Mutations in *FOXE3*, *PITX3*, *PAX6*, *B3GALTL*, *MAF*, *COL4A1*, *CYP1B1* and *SH3PXD2B* have also been described in patients with anterior segment dysgenesis,[9,12–19] but there is considerable genetic heterogeneity similar to A/M and many genes remain unidentified. Next-generation sequencing has proven to be an outstandingly successful technology for new gene identification,[20] and we present our results for two families, a sibship with complex microphthalmia and anterior segment dysgenesis and a sporadic male patient with unilateral microphthalmia anterior segment dysgenesis. In both families, exome sequencing identified *PXDN* mutations as a cause for their eye defects.

MATERIALS AND METHODS

Clinical reports

In the first family, the older male sibling (family 1, patient (P); Figure 1a) had bilateral microphthalmia and sclerocornea with iridocorneal dysgenesis and glaucoma. The right eye was more severely affected than the left eye. He had persistent hypotonia that was first diagnosed in the neonatal period and had experienced mild gross motor and fine motor delays. He rolled over at 5 months, sat at 9 months and walked at 26 months of age. He required occupational therapy for his motor delays. He spoke in phrases at 12 months of age and was educated in a mainstream classroom with support for visual impairment. His younger sister (family 1, sibling (S); Figure 1b) had bilateral

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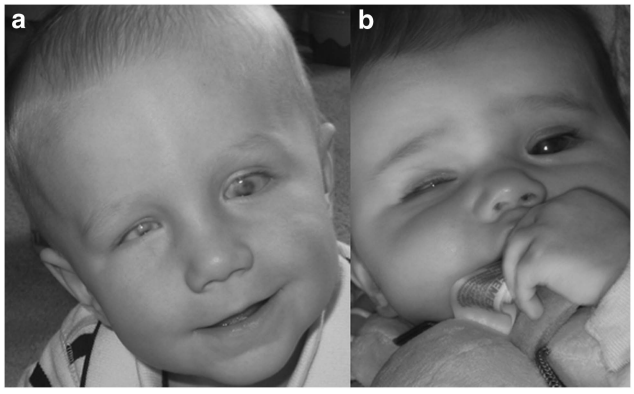


Figure 1 (**a**) Frontal view of the male sibling with *PXDN* mutations c.1021C>T predicting p.(Arg341*) and c.2375_2397del23, predicting p.(Leu792His*fs**67), showing bilateral scleral opacities. (**b**) Frontal view of the female sibling with *PXDN* mutations c.1021C>T predicting p.Arg341* and c.2375_2397del23, predicting p.(Leu792His*fs**67), showing bilateral scleral opacities and right microphthalmia.

microphthalmia, sclerocornea and anterior segment dysgenesis. She rolled over at 6 months and cruised at 18 months of age. She did not have phrases at 2 years of age and received speech therapy. She has had hypotonia and required occupational therapy for delays in fine motor skills and tactile defensiveness. An evaluation by early intervention services at 24 months of age determined her development to be compatible with 12 months chronological age. There was no other history of eye defects in the family and no known consanguinity. Ethnicity was Caucasian.

The second patient was a Caucasian male with bilateral anterior segment dysgenesis and left microphthalmia diagnosed in the newborn period. He had a moderate sized cataract in his left eye and a small cataract in his right eye and underwent left lensectomy and pupilloplasty at 11 days of age. His development was normal at 3 years and 4 months of age and a magnetic resonance imaging scan of the brain was reported as unremarkable. His maternal half brother had amblyopia and a small nodule on the helix of his right ear. Both parents were healthy with no history of eye defects. *PITX2* and *FOXC1* sequencing returned normal, and array comparative genomic hybridization (180K, Agilent, Santa Clara, CA, USA) as a clinical test was unrevealing. There was no known consanguinity.

Exome sequencing

Ethical approval for the study was obtained from the Committee on Human Research at the University of California, San Francisco. After obtaining written, informed consent, venous bloods were obtained and DNA was extracted from all three affected individuals and both sets of parents. Exome sequencing was performed as previously described.[7,21] Briefly, libraries were prepared using biotinylated DNA oligonucleotides (SeqCap EZ Human Exome Library v3.0; Roche Nimblegen, Madison, WI, USA) and sequencing was performed on a HiSeq2000 (Illumina, San Diego, CA, USA) for paired-end 100 cycles. Sequencing reads were aligned to the hg19 reference genome using the Burrows–Wheeler Alignment tool (BWAv0.5.9) using the default parameters that allow two mismatches. Indexing, realignment and duplicate removal were performed using Picard and Samtools, and variants were subsequently called using the Genome Analysis Toolkit v 1.3-21-gcb284ee. We then utilized wAnnovar (http://wannovar.usc.edu/) with default parameters to analyze the sequence variants.[22,23] We favored autosomal recessive inheritance because the first family had an affected male and female sibling pair, although we considered all possible modes of inheritance to analyze the data. Novel sequence variants were assessed for deleterious consequences using Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/) and Mutation Taster (http://mutationtaster.org/MutationTaster/).[24] Selected novel sequence variants were verified using Sanger sequencing. After verification of the two *PXDN* mutations in the first sibship, we re-examined our existing exome sequencing data from 20 patients with developmental eye defects for *PXDN* sequence alterations, and detected two predicted mutations in the sporadic male case that were subsequently verified by Sanger sequencing. We also used Sanger sequencing to interrogate *PXDN* for mutations in 13 additional patients with anophthalmia or microphthalmia who were selected because of abnormal anterior segment findings, including sclerocornea, microcornea or cataract (Supplementary Table S1). These patients had not undergone exome sequencing. The 13 additional A/M cases that we sequenced were ascertained by the population-based surveillance program—California Birth Defects Monitoring Program.[25] These A/M cases were linked to their newborn bloodspots, which represented the source of DNA. All samples were obtained with the approval form the State of California Health and Welfare Agency for the Protection of Human Subjects.

RESULTS

Coverage for each of the exomes is shown in Supplementary Table S2 and the filtering strategy is summarized in Supplementary Table S3. In the oldest sib from the first family, exome sequencing revealed 21 616 sequence variants, whereas the second sib had 21 995 sequence variants. After removing sequence variants in the database of single-nucleotide polymorphisms (http://www.ncbi.nlm.nih.gov/projects/SNP/) and variants in unknown genes, variants in 262 genes were shared by both sibs. Of these genes, 72 had synonymous variants and were not further considered. Genes (138) with only one shared variant were also not further considered. Of the remaining genes, 11 had exactly two novel sequence variants and four of these 11 genes were expressed in the eye (NEIBank; http://neibank.nei.nih.gov/index.shtml; data not shown). From the four genes, only *PXDN* was known to be involved in developmental eye defects.[26] The predicted mutations in *PXDN* (mRNA reference sequence NM_012293.1, Chromosome reference number NC_000002.11, National Center for Biotechnology Information Gene, http://www.ncbi.nlm.nih.gov/gene; Ensembl genomic reference ENSG00000130508 and transcript ENST00000252804; http://www.ensembl.org/index.html) in the first family were verified by Sanger sequencing: a maternally inherited, nonsense mutation: c.1021C>T, predicting p.(Arg341*) and a paternally inherited, intragenic deletion resulting in a frameshift and premature protein truncation, c.2375_2397del23, predicting p.(Leu792His*fs**67) (Figures 2a,b and Figure 3a). Re-examination of our existing exome data from 20 other families identified two additional *PXDN* mutations in a sporadic male patient, which were also confirmed with Sanger sequencing—a paternally inherited, missense mutation, c.947A>C, predicting p.(Gln316Pro), together with a maternally inherited, frameshift mutation, c.1192delT, predicting p.(Tyr398Thr*fs**40) (Figures 2c,d and Figure 3a). The missense mutation p.(Gln316Pro) was predicted to be probably damaging with Polyphen-2 (score 0.961; sensitivity 0.78 with specificity 0.95) and to be disease causing by Mutation Taster ($P=0.999$). It is likely that the pathogenic effect of the mutation results from the substitution of the amino acid, as the glutamine residue was conserved in chimp, mouse, chicken and cat (Figure 3b). Neither mutation was present in the Exome Variant Server database (http://evs.gs.washington.edu/EVS/). Of the four mutations that we identified, three were novel, whereas p.(Arg341*) has been previousIy published.[26] In the other individuals with A/M selected because of additional anterior segment abnormalities, we did not find two pathogenic sequence alterations in any patient. Mutations have been submitted to the Leiden Open Variation Database (http://databases.lovd.nl/shared/genes/PXDN).

DISCUSSION

Mutations causing *PXDN* loss of function have previously been described in three families who had congenital cataract-microcornea

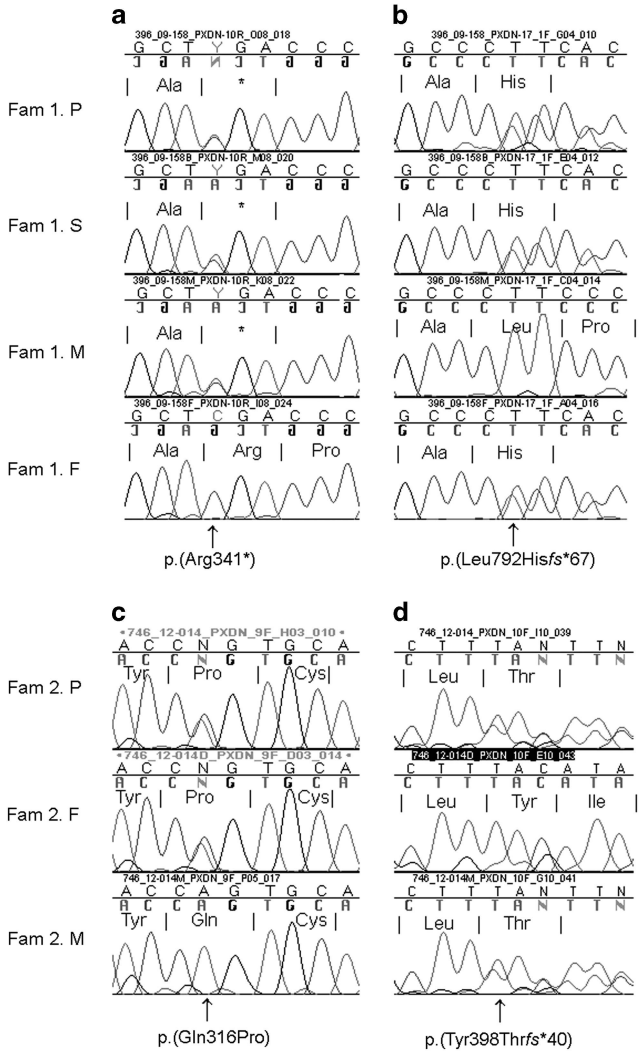


Figure 2 (**a**) Chromatograms showing c.1021C>T predicting p.(Arg341*) in *PXDN* in the patient (P) of the first family, sister of the patient (S) and mother of the patient (M). The father of the patient (F) does not have the mutation. (**b**) Chromatograms showing the start of mutation c.2375_2397del23, predicting p.(Leu792His*fs**67) in *PXDN* in the patient (P), sister of the patient (S) and father of the patient (F). The mother of the patient (M) does not have the mutation. (**c**) Chromatograms showing c.947A>C, predicting p.(Gln316Pro) in *PXDN* in the patient (P) of the second family and the father of the patient (F). The mother of the patient (M) does not have the mutation. (**d**) Chromatograms showing c.1192delT, predicting p.(Tyr398Thr*fs**40) in *PXDN* in the patient (P) of the second family and the mother of the patient (M). The father of the patient (F) does not have the mutation.

with corneal opacity (CCMCO; Table 1).[26] Defining phenotypic features for these families were microcornea and corneal opacification, with varying degrees of intrafamilial expression, but not microphthalmia. A fourth family with *PXDN* loss of function had developmental glaucoma, buphthalmos and extensive corneal opacification.[26] No patient had neurological abnormalities as seen in our first family. In all reported families, unaffected carriers have had no ocular abnormalities.

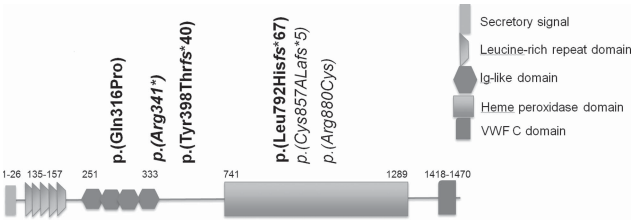
The human *PXDN* gene has 23 exons and encodes a 1479-amino-acid protein. At embryonic day (E) 18.5, *Pxdn* is expressed in the corneal endothelium and in adult murine eyes, and *Pxdn* is present in the corneal and lens epithelium.[26] *PXDN* was weakly expressed in normal brain tissue and was upregulated in the microvasculature and glioma endothelial cells of primary glial and metastatic brain tumors.[27] Expression has also been demonstrated in several other tumor types including melanoma, breast, ovarian, colon and renal cell cancer.[28,29] *PXDN* encodes peroxidasin, a protein that is located in the endoplasmic reticulum and secreted into the extracellular space. It contains a peroxidase domain common to other mammalian peroxidases, for example, myeloperoxidase and thyroid peroxidase, and other motifs typical for extracellular proteins, including an amino-terminal secretory signal sequence and ligand-binding domains comprising leucine-rich repeat regions, C2-type immunoglobulin-like motifs and a von Willebrand factor type-C domain.[26] In the mutations identified to date, p.(Gln316Pro) affects a residue in an immunoglobulin-like domain, and this glutamine residue is highly conserved (Figure 3). The stop mutation p.(Arg341*) and frameshift mutation p.(Tyr398Thr*fs**40) are not present in named protein domains, but both mutations are predicted to cause nonsense-mediated decay. The remaining mutations, p.(Leu792His*fs**67), p.(Cys857Ala*fs**5) and p.(Arg880Cys), occur at residues in the peroxidase domain, and the first two mutations predict nonsense-mediated decay (Figure 3a). At present, there is no phenotype–genotype correlation, and although the first sibship in this report had two truncating mutations, patients with homozygosity for other truncating mutations have not had similar additional findings of microphthalmia or developmental delays.[26]

Peroxidasins are involved in hydrogen peroxidase metabolism, and peroxidasin has been shown to catalyze the formation of a sulfilimine bond between apposed lysine and methionine residues in the C-terminal interface of collagen IV, the predominant constituent of the basement membrane.[30] Loss of peroxidasin resulted in defective basement membrane integrity in *Drosophila melanogaster* and *Caenorhabditis elegans* mutants, and it is most likely that the *PXDN* mutations in human patients compromise the integrity of the basement membrane of the lens and cornea by disrupting collagen IV formation.[30] Mutations resulting in haploinsufficiency for collagen IV have been shown to cause anterior segment defects in mice and in humans, including cataracts, corneal opacities, iris defects, iridocorneal adhesions and buphthalmos,[31–33] similar to the eye malformations seen in patients with *PXDN* loss of function.

In the first family with two siblings, both were found to have developmental delays and hypotonia, although these manifestations have not been described in other reported patients and were not present in the second patient in this report. *PXDN* is weakly expressed in the brain and it is plausible that collagen IV and basement membrane formation could also be disrupted in the brain in patients with loss of *PXDN* function. Haploinsufficiency for collagen IV caused type I porencephaly and intracranial hemorrhage in human patients and cortical lamination defects in mice that can be associated with developmental differences.[33,34] However, it is still speculative as to whether the *PXDN* mutations are responsible for the hypotonia and delays in this family, although we were unable to find any other mutations to explain this aspect of their phenotype (data not shown). A duplication involving the entire *PXDN* gene was present in two male half-siblings with autism and inherited from their mother, who had mosaicism for the duplication.[35] However, the duplication also disrupted *MYT1L*, a gene expressed in the brain and linked with schizophrenia and depression,[36] and aberrant MYTL function was presumed to be responsible for the autism diagnosis in these children.

Our finding of a broader phenotype involving microphthalmia in patients with *PXDN* loss of function is characteristic for exome

a



b

H. sapiens	Q	N	T	Q	E	T	D	Q	G	I	Y	**Q**	C	M	A	K	N	V	A	G	E	V	K
Mutation	*	*	*	*	*	*	*	*	*	*	*	**P**	*	*	*	*	*	*	*	*	*	*	*
P. troglodytes	*	*	*	*	*	*	*	*	*	*	*	**Q**	*	*	*	*	*	*	*	*	*	*	*
M. musculus	-	-	-	*	*	A	*	E	*	V	*	**Q**	*	*	*	*	*	*	*	*	*	A	*
F. catus	*	*	*	*	*	*	*	*	*	*	*	**Q**	*	*	*	*	*	A	*	*	Q	*	*
G. gallus	*	*	*	*	*	*	*	*	*	*	*	**Q**	*	*	*	*	*	*	*	*	*	*	*

Figure 3 (**a**) Diagram of peroxidasin showing domain location of *PXDN* mutations described to date. Adapted from reference;[26] not drawn to scale. Figures above domains represent amino-acid numbers. The mutations in bold were described in this paper and the mutations in italics were described in Khan *et al*.[26] (**b**) Conservation of amino-acid residues at p.Gln(Q)316, the site of c.947A>C and p.(Gln316Pro). The glutamine residue is conserved in all species with this residue.

Table 1 Phenotypic features associated with *PXDN* mutations

Family	*Microcornea*	*Corneal opacity*	*Iridocorneal adhesion*	*Cataract*	*Microphthalmia*	*Hypotonia/delays*	*Mutation(s)*
MEP60[a]							
V-1	+	p>c[d]	+	+	—	−/−	p.(Cys857Ala*fs**5) HZ[e]
V-7	+	p>c	+	+	—	−/−	p.(Cys857Ala*fs**5) HZ
V-2	+	p>c	+	+	—	−/−	p.(Cys857Ala*fs**5) HZ
MEP59[a]							
IV-3	+	p	—	+	u	−/−	p.(Arg880Cys) HZ
IV-1	+	p	—	+	u	−/−	p.(Arg880Cys) HZ
IV-2	+	p	—	+	u	−/−	p.(Arg880Cys) HZ
IV-4	+	p	—	+	u	−/−	p.(Arg880Cys) HZ
CA2[a]							
IV-3	Large	+	u	u	—	−/−	p.(Arg341*) HZ
IV-8	Large	+	u	u	—	−/−	p.(Arg341*) HZ
IV-10	+	+	u	u	—	−/−	p.(Arg341*) HZ
IV-11	Large	+	u	u	—	−/−	p.(Arg341*) HZ
This paper							
Fam. 1, P[b]	u[c]	+	—	—	+	+/+	p.(Arg341*)/p.(Leu792His*fs**67)
Fam. 1, S[b]	u	+	—	—	+	+/+	p.(Arg341*)/p.(Leu792His*fs**67)
Fam. 2, P[b]	—	—	—	+	+	−/−	p.(Gln316Pro)/p.(Tyr398Thr*fs**67)

[a]MEP60, MEP59 and CA-2 are families that were previously described in reference.[26]
[b]Fam. 1, P = family 1, patient; Fam.1, S = family 1, sibling; Fam. 2, P = family 2, patient; + indicates present; − indicates absent.
[c]u = undetermined.
[d]p = peripheral; c = central.
[e]HZ = homozygous.

sequencing, which has proven to be highly effective for mutation detection in atypical presentations. We conclude that *PXDN* mutations should be considered in microphthalmia with anterior segment dysgenesis in addition to CCMCO.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)