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VSX1: A gene for posterior polymorphous dystrophy and keratoconus

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We identified mutations in the *VSX1* homeobox gene for two distinct inherited corneal dystrophies; posterior polymorphous dystrophy (PPD) and keratoconus. One of the mutation (R166W) responsible for keratoconus altered the homeodomain and impaired DNA binding. Two other sequence changes (L159M and G160D) were associated with keratoconus and PPD, respectively, and involved a region adjacent to the homeodomain. The G160D substitution, and a fourth defect affecting the highly conserved CVC domain (P247R), occurred in a child with very severe PPD who required a corneal transplant at 3 months of age. In this family, relatives with the G160D change alone had mild to moderate PPD, while P247R alone caused no corneal abnormalities. However, with either the G160D or P247R mutation, electroretinography detected abnormal function of the inner retina, where *VSX1* is expressed. These data define the molecular basis of two important corneal dystrophies and reveal the importance of the CVC domain in the human retina.

INTRODUCTION

The cornea is an important transparent avascular tissue of the front part of the eye through which light enters the eye to be transmitted to the retina (1). Clear vision depends upon establishing corneal transparency in early development and maintaining it throughout adult life. Corneal dystrophy refers to an inherited, bilateral non-inflammatory corneal opacification (2). Posterior polymorphous dystrophy (PPD) is a well-established, slowly progressive hereditary disorder of the corneal endothelium (OMIM# 122000) that leads to a variable degree of visual impairment, usually in adulthood (3). A major gene for this condition was mapped to chromosome 20p11–q11 (4) and is known to have significant intrafamilial phenotypic variability (3,5). The phenotype can range from scattered vesicles that go unnoticed throughout life to a congenital hereditary endothelial dystrophy requiring a corneal graft in early childhood. PPD is usually inherited as an autosomal dominant trait (2). The hallmark pathological findings include the acquisition of epithelial features of the endothelial cells as well as fibroblastic transformation and degeneration of the endothelial cells of the cornea (6,7). These may also be associated with variable degrees of anomality of Descemet's membrane (7,8). The abnormal endothelial cells retain their ability to divide and may extend onto the trabecular meshwork to cause glaucoma in up to 40% of cases (3).

PPD has been associated with keratoconus in several reports (9–13). Keratoconus (OMIM# 148300) is a frequent corneal dystrophy with a reported incidence that varies from 50 to 230 per 100 000 (approximately 1/2000) (14). Characteristically, the cornea assumes a conical shape as a result of a progressive non-inflammatory thinning of the corneal stroma (14). The thinning of the cornea causes irregularity in its curvature (astigmatism) and corneal protrusion resulting in a variable degree of visual

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impairment. Keratoconus is a major indication for corneal transplantation in the Western world (15,16). Depending on the stage of the disease, every layer of the cornea may become involved in the pathological process. Although Descemet's membrane and endothelial cells may show minor changes, the major pathological defects lie in the anterior cornea, with compaction of the stroma and breaks in Bowman's membrane (17). Keratoconus is most often an isolated sporadic condition, despite multiple single reports of its coexistence with other disorders (14). Multifactorial and Mendelian inheritance have been documented, with cases of autosomal dominant and autosomal recessive transmission. The suspected genetic heterogeneity and phenotypic variability of keratoconus have been hurdles to the identification of the genes for this condition (14). Fuchs' dystrophy is another dystrophy of the posterior cornea (endothelial) that shares some similarities with PPD. It is characterized by a progressive decompensation of the endothelium associated with a variable degree of nodular thickening of Descemet's membrane (18), and the association of bilateral Fuchs' dystrophy with keratoconus has also been reported (19). Although the condition is most often sporadic, families with autosomal dominant Fuchs' corneal dystrophy are well documented (18).

Human VSX1 (20,21) is a member of the Vsx1 group of vertebrate paired-like homeodomain transcription factors. These transcription factors are distinguished by the presence of the CVC domain, a highly conserved region of unknown function, which lies C-terminal of the homeodomain. The vsx1 gene was first identified in goldfish (22), and orthologues have since been identified in zebrafish (23), chicken (24), bovine (20) and mouse (25,26). In situ hybridization studies on these species showed that vsx1/VSX1 is expressed in the outer tier of the inner nuclear layer of the retina, suggesting that it plays a role in the development of retinal bipolar interneurons. In humans, VSX1 mRNA has been detected in the inner nuclear layer of the retina (20), in embryonic craniofacial tissue (20) and adult cornea (21). In the mouse, vsx1 expression is first detectable by in situ hybridization at postnatal day 5 and is later restricted to cone bipolar cells in the adult mouse retina (25). VSX1 was localized to the 20p11–q11 region and its five exons are distributed across about 6.2 kb of coding sequence (20,21). Because of the ocular expression of VSX1 and its chromosomal localization, we selected it as a candidate gene for posterior polymorphous dystrophy.

We screened the coding sequence of VSX1 for mutations in the DNA of patients affected with PPD, KC, Fuchs' corneal dystrophy and/or glaucoma. We identified VSX1 mutations in samples of patients affected with PPD and/or keratoconus.

RESULTS

Mutational analysis

The sequence changes identified are summarized in Table 1.

Case 1 (Table 1 (G160D/+), Fig. 1A (II-2)) had a strong family history of PPD (Fig. 1A) where most of those affected had mild visual impairment (mean visual acuity 20/25, at an average age of 40 years). However, her daughter (Fig. 1A (III-1)), the proband, had corneal grafts at the age of 3 months because of severe bilateral corneal opacities due to autosomal dominant PPD (Fig. 1A). Microscopic examination of the corneal button taken at the time of her first corneal transplant confirmed the diagnosis of PPD (Fig. 1B and C). None of the family members had keratoconus or glaucoma. Our analysis revealed that the severely affected proband (Fig. 1A (III-1)), and the mildly affected relatives of maternal origin, shared a G160D mutation. G160 is not well conserved (Table 2), but it segregated with the affected status in this family and was bsent in 277 controls suggesting that an acidic residue at this position is detrimental. The proband also inherited a second change (P247R) from her father (Case 6, Table 1, Fig. 1A (II-1)), that was also absent in 196 control individuals. The carrier of the P247R change had a normal eye examination.

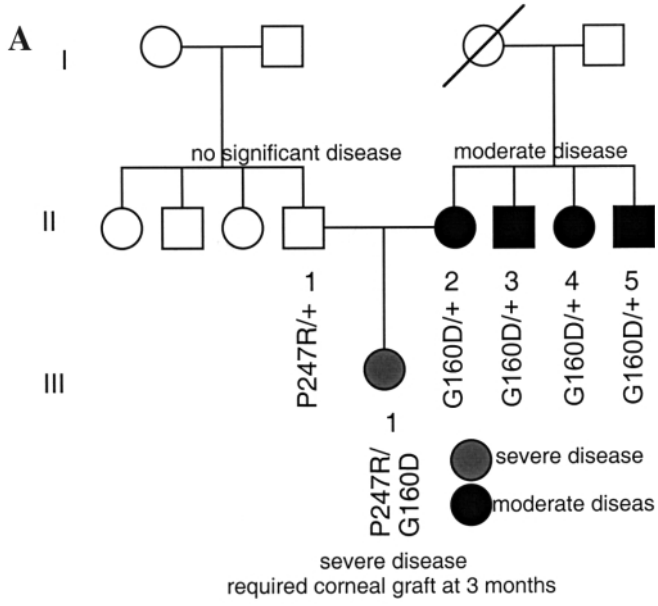
Knowing that VSX1 is expressed in the retinal inner nuclear layer (21–26) prompted us to investigate whether the G160D and/or P247R mutations caused any retinal dysfunction. Electroretinography performed on II-1 (P247R/+) and II-2 (G160D/+), the parents of III-1 (Fig. 1A), showed significant reductions of the rod-cone b/a ratio (Table 3, Fig. 2). These individuals has a normal retinal examination. It was not possible to perform an electroretinogram on the proband and the other family members were not available for testing. It was not possible to perform an electroretinogram on the proband and the other family members were not available for testing.

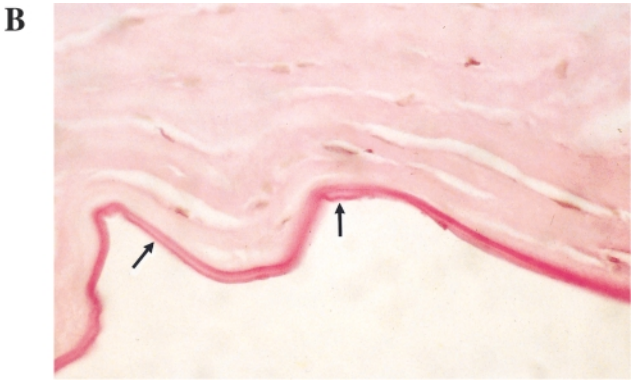
Case 2 was an isolated case of keratoconus with visual impairment for whom a corneal graft was required in adulthood. A VSX1 mutation (R166W; Table 1, Figs 3 and 4)

Table 1. Phenotype–genotype correlation of sequence changes identified

Case	Mutation	Phenotype	Familial disease? (no. of affected tested)	Control individuals tested (no. positive)
1	G160D[a]	PPD, ERG abnormal	Yes (5)	277 (0)
2	R166W	Keratoconus	No	277 (0)
3	L159 M	Keratoconus	Yes (4)	277 (0)
4	D144E	PPD and keratoconus	Yes (2)	277 (0[b])
5	H244R	Keratoconus	Yes (3)	277 (2)
6	P247R[a]	No clinical PPD or keratoconus, ERG abnormal	No	196 (0)

[a]Cases 1 and 6 are the mother and father of the proband of family A (Fig. 1A).
[b]The D144E was seen in one glaucoma patient (1/90).
PPD, posterior polymorphons dystrophy; ERG, electroretinogram.





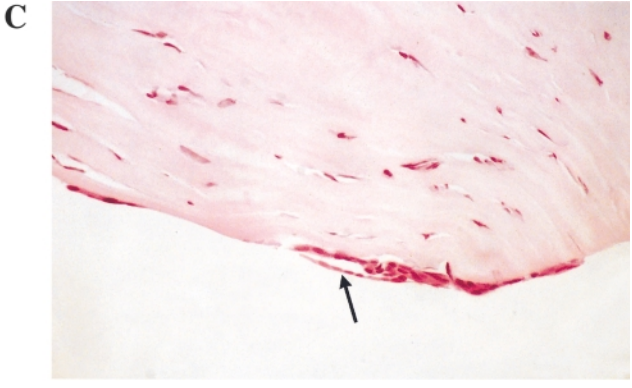


Figure 1. Molecular and histopathological characterization of Case 1 (Table 1) affected with PPD. (A) Pedigree Family A, where two different VSX1 changes (G160D, P247R) are identified. Participants with identifiers were examined by slit lamp biomicroscopy. Filled symbols reflect an affected status, clear symbols reflect an unaffected status. (B) Histopathology of the corneal button of case 1 taken at the time of her first corneal graft shows the characteristic bilaminar appearance of Descemet's membrane (PAS reagent; ×650). (C) Area of multilayering of corneal endothelium with vesicle (arrow). An adjacent area of Descemet's membrane is devoid of endothelial cell nuclei. (Hematoxylin & eosin; ×400).

was found to change the highly conserved third amino acid in the homeodomain (Table 2). To investigate the effect of the homeodomain mutation R166W on DNA binding, we performed an electrophoretic mobility shift assay (EMSA) using a probe (P3) that is known to bind CHX10 (K. Dorval et al., unpublished data). In vitro translated (i.v.t.) VSX1 interacted with the P3 element, albeit at reduced affinity relative to CHX10 (Fig. 4A, lanes 2 and 5). Binding was specific, since no complex was observed with reticulolysate (Fig. 4A, lane 11), and the shifted complex (arrow) was competed efficiently by unlabelled wild type competitor probe (Fig. 4A, lanes 3 and 6), but was unaffected when critical bases in the P3 site were mutated (Fig. 4A, lanes 4 and 7). Notably, R166W also interacted with the P3 probe in a specific fashion (Fig. 4A, lanes 8–10), but its affinity relative to wild-type VSX1 was reduced more than 2-fold (Fig. 4A, lanes 5 and 8). Equal amounts of i.v.t. protein were used in the binding assay (Fig. 4B). We also performed a titration experiment to examine the interaction of wild-type and mutant VSX1 with the P3 probe (Fig. 4C), using a range of protein amounts (Fig. 4D). This experiment confirmed that wild-type VSX1 binds DNA with higher affinity than does R166W (Fig. 4C).

Case 3 had autosomal dominant keratoconus (Figs 5 and 6) and was found to have a different VSX1 change (L159M; Table 1). As with the adjacent glycine mutated in case 1 (G160D), L159 is not highly conserved (Table 2). However, L159M segregated with the disease phenotype in three other affected family members available (Table 1) and was absent in 277 controls, suggesting that it is a disease-causing alteration. Furthermore, nowhere in the evolution of the documented homologous sequences is there a methionine or an acidic amino acid at positions L159 or G160.

Two other VSX1 changes were observed (D144E and H244R), which may or may not be disease-causing. Case 4 (D144E/+; Table 1, Fig. 5) had phenotypic characteristics of both PPD and keratoconus. The D144E VSX1 alteration segregated with the disease phenotype in the one similarly affected relative available. Although this alteration was not seen in the control population studied, it was observed in one glaucoma patient (1/90) who had a normal cornea. Case 5 (H244R/+) had keratoconus and the mutation co-segregated with the disease in two other family members (Table 1, Fig. 5). H244 lies in the CVC domain and is 100% conserved from flies to humans (Table 2). However, this change was also detected in 2 controls (n = 277).

In addition to these changes, five VSX1 polymorphic changes were identified (see Supplementary Material). These were not specific to any disease phenotype.

VSX1 expression

To determine whether VSX1 is expressed in the human cornea, we performed RT–PCR on RNA isolated from adult cornea. VSX1 was not detected in corneal cDNA obtained from two different adult donors after 35 cycles of PCR (Fig. 7, lane 6 and data not shown) in contrast to the positive control gene, PAX6 (27). Furthermore, VSX1 was not expressed in the lens (Fig. 7, lane 5) but was present in the retina (Fig. 7, lane 4). These observations are similar to the RT–PCR results obtained in the adult mouse, where Vsx1 expression was detected in the retina

Table 2. Summarized sequences of VSX1 functional homologues

Amino acid No:	144	159	160	166		244	247
Mutant amino acid:	**E**	**M**	**D**	**W**	**//**	**R**	**R**
Human RINX	SDE**D**SQSEDRNDLKASPT**L**-**G**KRKKR**R**HRTV	//	MVR**H**CI**P**LPD				
Bovine VSX1	.....P....S.R....AP-S.....	//	.....				
Chickn Chx10–1	.....L.G.ASE..MPASQ-I.....	//	....S.....				
Goldfish VSX1	..D.CL.G....G.N.----Q.....	//	....S.....				
Human Chx10a	DS..VS.S..KMS.SALNQTK.....I	//	....S.....				
Bovine Chx10	DS..VS.S..KMS.SALNQTK.....I	//	....S.....				
Mouse Chx10	DS..VS.S..KMS.SALNQTK.....I	//	....S.....				
Chicken Chx10	DS..VS.S..KMS.S.LNQSK.....I	//	....S.....				
Goldfish Vsx2	DS..VS.SE.KIS.S.LSQSK.....I	//	....S.....				
Zebrafish Alx	−SD.VS.SE.KMS.S.LSQSK.....I	//	....S.....				

Sequence alignments were performed using the programs CLUSTAL and SIM (http://www.expasy.ch/tools/sim-prot.html).

but not in corneal or lens tissue (25), but contrast with previous RT–PCR findings in which VSX1 expression was detected in human adult corneal tissue (21).

DISCUSSION

In this study, we have identified a common molecular basis for keratoconus and PPD – two conditions that are distinct, both histopathologically and clinically, since they involve different layers of the cornea. Our findings suggest that in some cases, these two conditions are allelic variants and that there is further genetic heterogeneity for both diseases. Few families are large enough to show significant linkage to the chromosome 20q11 locus, which complicates the assessment of genetic heterogeneity. If all the alterations listed in Table 1 are disease-causing, then VSX1 would play a role in at least 9% (2/22) of PPD cases and 4.7% (3/63) of keratoconus. These numbers may increase once the VSX1 promoter and regulatory regions have been screened for sequence changes. Our mutational analysis does not support the hypothesis that VSX1 is involved in the pathogenesis of Fuchs' corneal dystrophy, since no VSX1 mutations were identified in this patient group.

After single-strand conformation polymorphism (SSCP) analysis and direct sequencing of the VSX1 coding sequence in the original family (4), no disease-causing mutation was identified. It is possible that the disease in this family results from a mutation in a regulatory element not yet characterized or a large deletion. Several elements conserved between the human and mouse VSX1/vsx1 genes have been identified, and shown to direct correct expression of a reporter gene to the mouse retina (R. Chow and R.R. McInnes unpublished). We cannot rule out that a second tightly linked gene may be involved. Further analysis of this family is underway.

Table 3. Summary of abnormal electroretinography responses

Case	Rod–cone b/a ratio (OD/OS)	V_{max}
II-1 (P247R)	1.65/1.60	340
II-2 (G160D)	1.70/1.72	372
Normal range[a]	1.85–2.63	400–696

[a]Normal range is established on the testing of 45 age-matched controls with no significant refractive error.

Our data are the first to assign a molecular cause for keratoconus and unambiguously diagnosed PPD. There is a precedent for histopathologically distinct corneal dystrophies to share a common genetic background. Mutations of the βig-h3 gene have been associated with Reis–Bückler corneal dystrophy, which involves the anterior Bowman's membrane as well as different subtypes of stromal dystrophies of the cornea (28). More recently, mutations in COL8A2 were identified in patients with Fuchs' corneal dystrophy and one family suggested to have PPD (29).

We identified at least four (L159M/+, G160D/+, R166W/+, and P247R/+), and possibly two other (D144E/+ and a H244R/+), disease-causing mutations in VSX1. In family A (Fig. 1A), the proband had two mutations (G160D/P247R). The G160D change lies just N-terminal of the homeodomain, whereas the P247R change involves the CVC domain. The combination of these two sequence changes in different alleles may account for the very severe phenotype observed, which required a corneal transplant at 3 months of age. P247R represents a non-conservative alteration of an amino acid that has been conserved from flies to humans (Table 2, Fig. 3). The abnormal electroretinography recordings of the carriers of P247R and G160D suggest a deficit in scotopic b-wave (ON system) activity typical of abnormal bipolar cell function. Whether these changes will be progressive or not requires additional follow-up. As recent studies demonstrated expression of vsx1 to be specific to cone bipolar cells in mouse retina (25), our data support the argument that P247R, although not associated with a clinically abnormal cornea, is of pathogenic significance and demonstrate a previously unrecognized association between PPD and retinal dysfunction. Mutations in the CVC domain in the nematode ceh-10 homeobox gene are lethal owing to defects in neuronal migration (30). Our data are the first to demonstrate that the CVC domain is important for human retinal function.

G160D, and the amino acid changes L159M (Case 3) and R166W (Case 2), all lie near or within the positively charged VSX1 nuclear localization signal (Fig. 3) (31). This region interacts with Ubc9, an enzyme that conjugates the ubiquitin-like protein called SUMO-1. Ubc9 does not sumoylate zebrafishVSX1, but the interaction is essential for nuclear localization of VSX1 (31). It will be interesting, therefore, to determine whether L159M, G160D and/or R166W affect binding to Ubc9 and/or nuclear localization of VSX1.

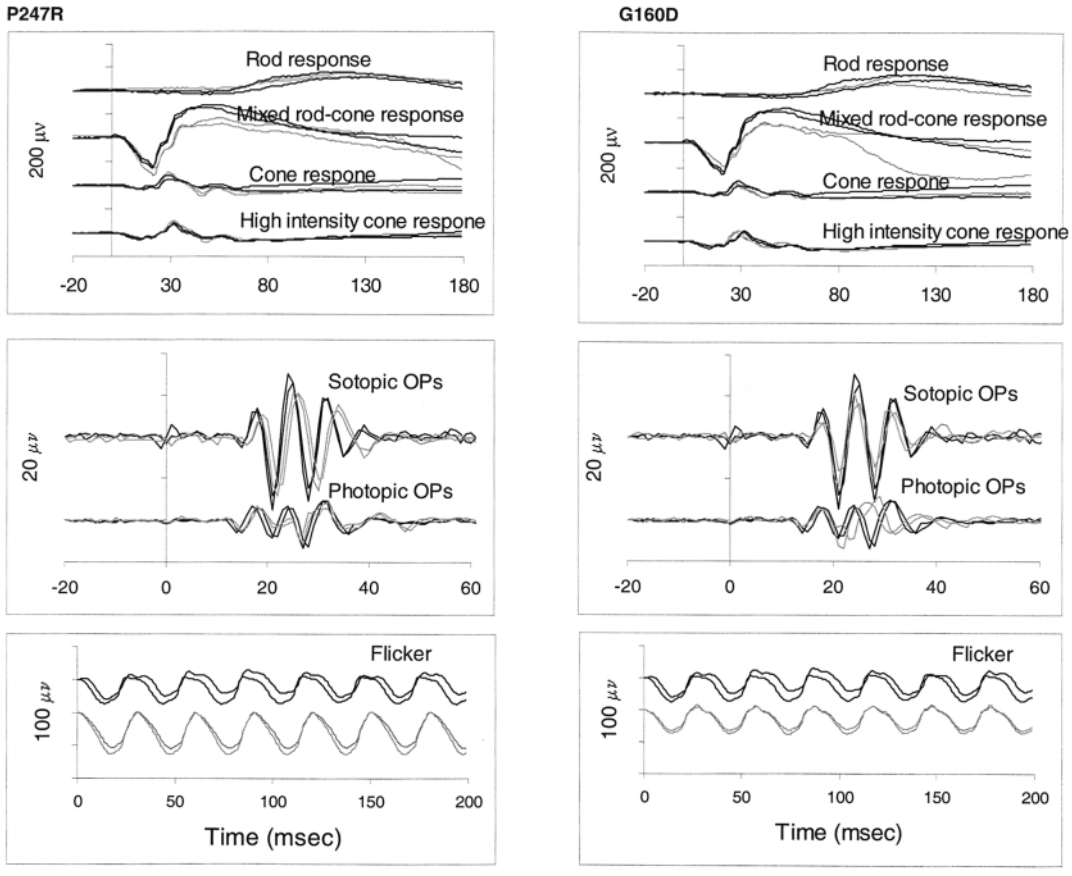


Figure 2. Electroretinography (ERG) of case II-1 (P247R) and II-2 (G160D) of Family A. Average responses for a control (black line) and the two cases II-1 (P247R, left) and II-2 (G160D, right). The two traces for control and subjects represent left and right eye responses. From top to bottom, recordings are shown for the rod response (standard flash, attenuated 2.6 log-units), maximal response (standard flash), cone response (standard flash), higher intensity cone response, scotopic and photopic oscillatory potentials and flicker response. Time is shown in milliseconds and amplitude in microvolts (µV). The µV value represents the distance between the y-axis tick marks. Positive electrical signals are in the upward direction. The y-axis crosses the ERG trace at stimulus onset. The baseline data from the control and case ERGs are arbitrarily matched.

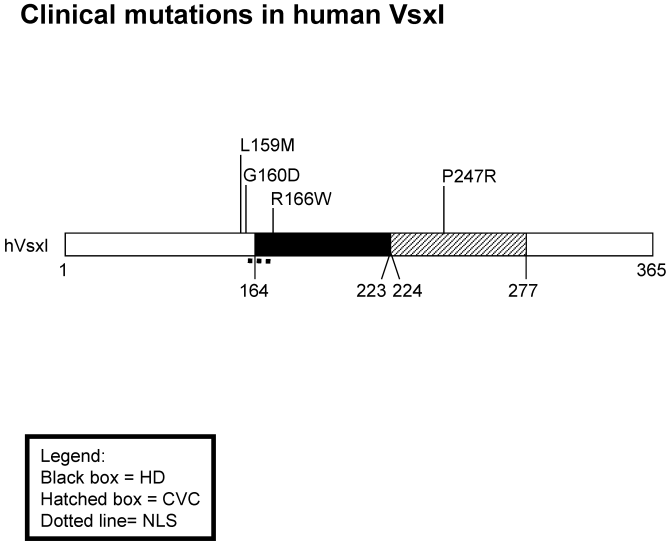


Figure 3. Ideogram of human VSX1 with position of sequence changes identified.

As the R661W mutation associated with keratoconus in Case 2 affects the highly conserved homeodomain, we investigated its effect on DNA binding. Wilson et al. (32) suggested that an alanine substitution of this arginine residue does not impair DNA binding by Prd homodimers. The results from our EMSA show that substitution of a more bulky tryptophan residue at this position does impair DNA binding. Therefore, the combination of genetic and functional evidence provides strong support that this mutation is pathogenic.

Case 4 (D144E/+) had phenotypic characteristic of both PPD and keratoconus. The associated amino acid change D144E was also observed in one glaucoma patient who had a normal corneal examination. Although the charge of the mutant amino acid is unchanged, the aspartic acid at position 144 is highly conserved (Table 2). Whether this change has functional implications and could predispose to glaucoma – extending the range of phenotypes associated with VSX1 – warrants further investigation. Three potential interpretations of this finding are that: (i) this mutation causes glaucoma in a small fraction of cases, (ii) this glaucoma patient has mild undiagnosed keratoconus or PPD, or (iii) this variation is a relatively rare non-disease-causing polymorphism. Functional studies are required to better understand the changes observed.

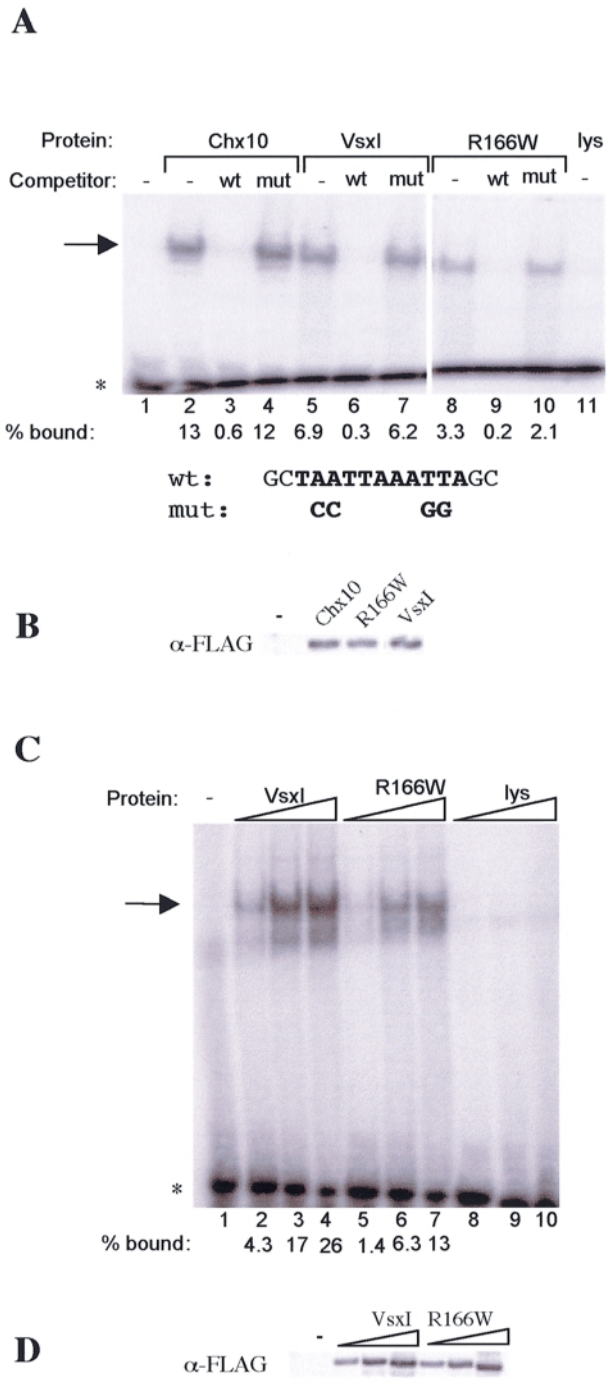


Figure 4. R166W impairs homeodomain function. (A) ^{32}P end-labeled P3 probe was incubated with no protein (lane 1), equal amounts of in vitro translated Chx10 (lanes 2–4), wild-type Vsx1 (lanes 5–7), R166W (lane 8–10), or reticulocyte lysate (lane 11) in the presence of no competitor (lanes 2, 5, 8), or a 100 × molar excess of wild-type P3 (lanes 3, 6, 9), or mutated competitor (lanes 4, 7, 10). The sequence of the P3 wild-type and mutated core sequences are shown below. The percentage of probe present in the bound complex (arrow) was calculated relative to total (bound plus free *), and is indicated below the gel. Lys, unprogrammed reticulocyte lysate. (B) Anti-FLAG western blot showing the amount of i.v.t. Chx10, Vsx1 and R166W used in (A). The first lane is unprogrammed reticulocyte lysate. (C) Titration of wild-type VsxI (lanes 2–4) versus mutant R166W (lanes 5–7) or unprogrammed lysate (lanes 8–10) on a ^{32}P end-labeled P3 probe. Lane 1 is free probe. (D) Anti-FLAG western blot showing the amount of i.v.t. Vsx1 and R166W used in (C).

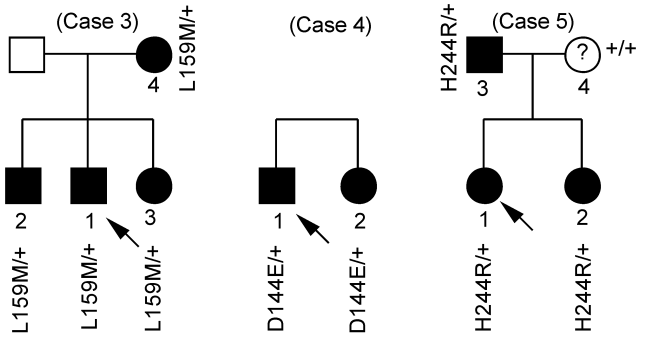


Figure 5. Pedigrees of cases 3, 4 and 5. Participants with identifiers were examined by slit lamp biomicroscopy and tested molecularly. Filled symbols reflect an affected status, clear symbols reflect an unaffected status. The proband of each pedigree is designated by an arrow. The clinical status of case 5–4 is unknown. + designates-wild-type.

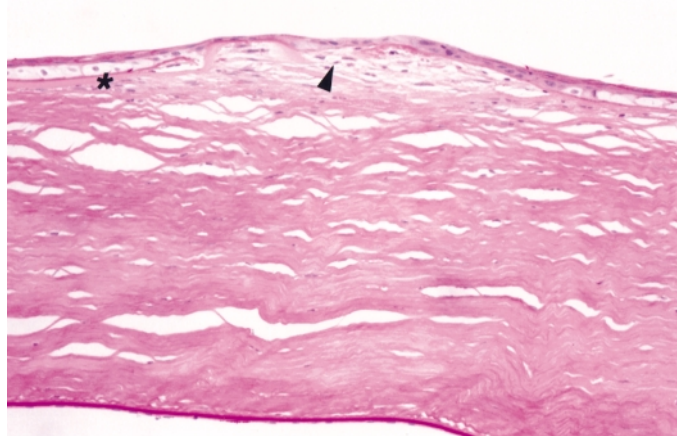


Figure 6. Histopathology of corneal button of Case 3 (L159 M), Table 1. Corneal button of a patient affected with keratoconus before corneal transplantation (PAS; × 400). The cornea is thinner centrally, with focal breaks (arrowhead) in Bowman's layer (*) and degenerative pannus.

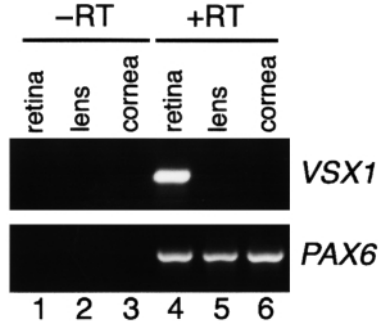


Figure 7. VSX1 expression in adult cornea. VSX1 expression in the adult eye (87 years old) is limited to the neuroretina. RT–PCR analysis of adult human retina, lens and cornea RNA. Lanes 1–3 contain cDNA made without reverse transcriptase. Lanes 4–6 contain cDNA made with reverse transcriptase; cDNA fragments were amplifed using intron-spanning primers for VSX1 (top row) and PAX6 (bottom row).

At this point it is unclear whether the H244/+ genotype of case 5 is disease causing or not, as it was observed in 0.7% of controls. We cannot rule out that this variation is a relatively rare non-disease-causing polymorphism, that it may be associated with undiagnosed disease or that it may represent a hypomorphic mutation. Functional studies are required to better understand the changes observed. As we have shown the functional importance of mutations in the CVC domain, we await further in vitro functional analyses and study of the murine homologue of the human VSX1 to clarify the implication of these sequence changes.

Our inability to detect VSX1 in the adult cornea (Fig. 7), despite the presence of corneal changes in patients with VSX1 mutations, raises several possibilities. The first is that VSX1 is normally expressed in the developing cornea but not in the adult cornea. A thorough examination of vsx1 expression in the developing cornea has not been documented in other species at this time. However, low levels of vsx1 expression have been detected by RT–PCR in the embryonic and early postnatal mouse eye prior to postnatal day 5, when vsx1 expression is first observed in the retina by in situ hybridization (25). This may reflect an early expression in the cornea. A detailed expression study is underway to determine if and when vsx1/VSX1 is expressed during corneal development. A second possibility is that VSX1 is required in a non-corneal cell type for the production of a signaling molecule(s) that is essential for normal corneal development and/or maintenance. Although the possibility that such a signal(s) originates from VSX1-expressing bipolar cells cannot be ruled out, we are also examining the possibility that VSX1 may be expressed in additional non-corneal ocular tissues.

MATERIALS AND METHODS

Clinical assessment

The project was approved by the Toronto Hospital Human Subjects Review Committee, The Hospital for Sick Children Research Ethics Board and the University of Iowa Hospital and Clinics Human Subjects Review Committee. After informed consent, all participants were questioned about their personal medical history and a family tree was drawn. All participants had a comprehensive eye examination and gave a blood sample (20 ml) for DNA extraction (33). In selected cases, keratometry, corneal topography and electroretinography were performed. Electroretinograms (ERGs) (LKC Technologies, Gaithersburg, MD) were recorded according to International Society of Clinical Electrophysiology of Vision (ISCEV) standards (34) and Neuroscan Inc. recording equipment and software. In addition, we recorded ERGs to a predetermined series of stimulus intensities to record the b-wave amplitude/log intensity (V/log I) curve. V_{max} is the maximum response (μV) derived from the V/log I function. This reflects the maximum scotopic b-wave response as calculated by Naka Rushton, reflecting the activity of the ON system. ERG responses were considered abnormal when the implicit times of responses were delayed beyond the upper range of age-matched control data (mean plus 2 SD) or when the amplitude of response was diminished below the lower range of age-matched control data (mean minus 2 SD).

Histopathology

A corneal graft was performed on Case 1 with the G160D change and affected with PPD, as well as on Case 3 affected with keratoconus. The corneal buttons were examined by light and electron microscopy.

Mutational analysis of VSX1

VSX1 has 5 exons, of 698, 89, 124, 181 and 352 nucleotides in length. The coding sequence was screened for mutations by a combination of SSCP (35) and direct sequencing (see Supplementary Material). Abnormal amplicons were directly sequenced from genomic DNA. All SSCP gels were independently scored by a minimum of two experienced investigators. Methods of PCR amplification were described previously (36). Amplimers showing a band shift were re-amplifed and sequenced bidirectionally using a ABI377 automated sequencer and dye-terminator chemistry or were purified using a QIAquick PCR Purification Kit (Qiagen, Mississauga) according to the manufacturer's protocol. The column-purified amplicon was then sequenced on a MicroGene Blaster automated DNA sequencing unit [Visible Genetic Inc. (VGI), Toronto] using Cy5.5-labeled M13 universal or M13 reverse primers and the Thermo Sequenase Cycle Sequencing Core Kit (US 79610, VGI) as described previously (36). Gene-specific primers tailed with M13 universal primer (5′gtaaaacgacggccagt 3′) or M13 reverse primer (5′cacaggaaacagctatgac 3′) were used.

This was performed on four groups of patients: 22 patients with PPD, 63 with keratoconus, 90 with Fuchs' corneal dystrophy and 90 with open angle glaucoma. The patients tested were from the Toronto area and from Iowa City, the majority of whom were Caucasian. The control population included samples from both sites in balanced proportions. All sequence changes observed were screened for in 277 control individuals, except for P247R, which was screened for in 196 control individuals.

EMSA

FLAG-tagged CHX10 and VSXI proteins were in vitro translated using rabbit reticulocyte lysates (Promega) and quantified by α-FLAG (Sigma) western. 200 ng of double-stranded P3 probe was end-labeled with γ-^{32}P and polynucleotide kinase. The probe was purified using Stratagene NucTrap columns and 40 000 cpm were used per reaction. The i.v.t. protein was incubated with labeled probe for 15 minutes at 26–28°C in a final volume of 15 µl (25 mM Tris pH 8.0, 0.5 mM EDTA, 10 glycerol, 0.05 Triton, 88 mM KCl, 150 ng/µl poly-dI–dC, 1 mM DTT and 12.5 ng/µl salmon sperm DNA). Samples were resolved on a 4.5 non-denaturing acrylamide gel using 0.25 × TBE running buffer. Gels were dried and exposed on a phosphoimager screen (Bio-Rad).

RT–PCR

Human eye tissue from an 87-year-old male donor and a 68-year-old male donor were obtained from the Eye Bank of Canada. Total RNA was prepared using Trizol (Gibco) according to the manufacturer's protocol. To prepare cDNA, 0.5 µg of total RNA was reverse-transcribed with random

hexamers using Superscript-II reverse transcriptase (Gibco-BRL). cDNA was amplified for 35 cycles with a 60°C annealing temperature. The following intron-spanning primers were used: VSX1, agactccgtgctcaactcc/tctcatgtggctcccaccttc; PAX6, aactccatcagttccaacgg/tgtgtgtctgcatatgtggg.

Accession numbers

The VSX1 reference sequence was accession no. AF176797. PCR primers were designed from clone RP5-1025A1 (accession no. AL080312). The gene is in reverse in the clone (region 48421–52021).

SUPPLEMENTARY MATERIAL

For supplementary material, please refer on HMG Online.

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