H244R VSX1 Is Associated with Selective Cone ON Bipolar Cell Dysfunction and Macular Degeneration in a PPCD Family

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Purpose. To elucidate the retinal dysfunction and the molecular basis of posterior polymorphous corneal dystrophy (PPCD) associated with macular dystrophy, both inherited in a dominant manner through a three-generation family.

Methods. Ophthalmologic examinations including slit lamp examination, visual acuity tests, fundus visualization by scanning laser ophthalmoscopy, fluorescein angiography, color vision tests, electro-oculography, photopic and scotopic electroretinography (ERG) according to the International Society for Clinical Electrophysiology of Vision (ISCEV) protocols, and oscillatory potential (OP) recordings were conducted on affected family members. Corneal button from one affected patient was examined by transmission electron microscopy. All exons and intron-exon boundaries of the *VSX1* and the *COL8A2* genes were amplified by polymerase chain reaction and sequenced.

Results. The presence of endothelial cells that have epithelial-like features with multiple layers, desmosomal junctions, and microvillous projections supports the diagnosis of PPCD. Sequence analysis indicated that the H244R variant in the *VSX1* segregated with corneal and macular disease phenotypes in this family. Electrophysiologic studies indicated normal scotopic ERG findings, decreased amplitude of the photopic b-wave, photopic OP2 and OP3 barely recordable with a preserved OP4 amplitude, and variably decreased 30-Hz flicker amplitude.

Conclusions. The human *VSX1* is required for cone ON bipolar cell function but not for rod and cone OFF bipolar cells, giving a unique example of such a selective heritable retinal defect in humans. Furthermore, the authors provide the first clinical support for a new alternative role of *VSX1* in cone biology, probably similar to that proposed for its goldfish ortholog during retinal differentiation. (*Invest Ophthalmol Vis Sci.* 2006;47:48–54) DOI:10.1167/iovs.05-0479

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The human visual system homeobox gene *VSX1* is a paired-like gene containing a highly conserved domain, denoted as CVC because it was originally identified in mouse *Chx10*, goldfish *Vsx1* and *Caenorhabditis elegans Ceh-10* genes.[1–3] Although the role of this domain must be more defined, it is necessary for transcriptional regulation and efficient ubiquitination for the degradation of *Vsx1* by the 26S proteasome.[4] However, the CVC-domain of *Vsx1* is required for protein function given that mutations within the CVC domain of the *Ceh-10* gene are lethal because of defects in interneuron formation.[5] Numerous orthologs and homologs of these genes have now been isolated, and all have demonstrated a high expression level in the inner nuclear layer (INL) of developing or adult retinas, suggesting that homeobox/CVC proteins play a role in bipolar interneuron biology.[6–13] Understanding the role of such genes is a significant challenge because interneurons are thought to constitute most cells in the nervous system and relatively little is known concerning their functioning. The *Vsx1* gene was initially identified in goldfish, a species in which retinogenesis and retinal regeneration after injury is maintained throughout life, in contrast to mammals.[1] In adult goldfish retina, *Vsx1* expression is restricted to bipolar cells (BCs), suggesting that this gene stabilizes the differentiated state of these interneuron cells. However, in the immature retina, *Vsx1* is also expressed, transiently, in mitotically active cone, horizontal, and bipolar cell progenitors at the proliferative retinal margin before it is switched off in retinal cells other than BCs when differentiation begins.[1,12] Therefore, this specific BC expression results from a tightly controlled temporal and spatial downregulation of *Vsx1*. This restrictive expression pattern of *Vsx1* closely parallels that of the mouse *Chx10* gene, which is indispensable for retinal progenitor cell (RPC) proliferation during the early stages of retinogenesis and for differentiation of BCs.[2,11,14] Recent results provided by the engineering of *Vsx1*-null alleles in mice implicated *Vsx1* in retinal physiology.[15,16] Although these mice had normal eye appearance with typical corneal and retinal histology, retinal electrophysiological experiments indicated that *Vsx1* regulates the visual photopic pathway and that it is probably specifically required for the late differentiation of cone OFF-BC. Furthermore, among *Vsx1*-BCs, some are recoverin positive whereas others are recoverin negative, suggesting heterogeneity between BCs in which *Vsx1* exerts its function.[16] Finally, several lines of evidence support the view that *Vsx1* retinal expression is downregulated in a *Vsx1*-dependent manner, suggesting that *Vsx1* is required in a feedback loop to negatively regulate the generation of cone bipolar interneurons.[16]

In humans, *VSX1* missense mutations were unexpectedly found to be associated with dominant posterior polymorphous corneal dystrophy (PPCD) and keratoconus, two conditions known to affect solely corneal tissues.[17] Although patients with PPCD showed no clinical retinal phenotype, they probably had reduced scotopic activity, as suggested by electroretinography (ERG) data.[17] More recently, ocular and nonocular abnormalities, including severe craniofacial malformations,

central nervous system defects, and decreased auditory functions, were reported in a *VSX1* family, suggesting therefore that the phenotypic spectrum of *VSX1* appears broader than previously believed. Moreover, retinal electrophysiological experiments performed on these patients indicated, in addition to a cone bipolar pathway defect, a subclinical macular impairment in some patients with *VSX1*, though no evident macular phenotype was detected in the patients.[18]

This report describes, for the first time, a family with PPCD and severe macular dystrophy in which both traits were inherited in a dominant manner, highlighting a new alternative role of *VSX1* in cone photoreceptors. In addition, these patients showed an unusual postreceptoral retinal defect, providing a valuable resource for understanding the role of *VSX1* in specific retinal bipolar cell pathways.

Materials and Methods

Patients

Nine members of a family (II.1, III.1, III.2, III.3, IV.1, IV.2, IV.3, IV.4, IV.5) were examined and followed up at the Department of Ophthalmology of Hôtel-Dieu Hospital for a period of 30 years. Medical records were recently obtained for two other family members (III.5, III.6) from an ophthalmologist of their community. A standard ophthalmologic examination including measurement of visual acuity, Goldmann visual field testing, determination of intraocular pressure, gonioscopy, and slit lamp examination was performed. Endothelial specular microscopy was performed in four members (III.1, IV.1, IV.2, IV.4), and fluorescein angiography was performed in four other members (III.1, IV.1, IV.2, IV.3). Informed consent was obtained from all participating members of this family, in accordance with the guidelines established by the ethics committees of the Hôtel-Dieu Hospital and Cochin Hospital in Paris.

Histopathology

Corneal transplantation was performed on the proband (III.1), and his corneal button was examined by light and transmission electron microscopy using standard procedures.

Mutation Detection and Restriction Enzyme Analysis

Genomic DNA was extracted from peripheral blood leukocyte samples, in accordance with the tenets of the Declaration of Helsinki, and each exon with exon-intron junctions of the *VSX1* and *COL8A2* genes was amplified by polymerase chain reaction (PCR) using the appropriate forward and reverse sets of primers previously reported.[17,19] PCR products, purified and sequenced on both strands, were resolved on an automatic fluorometric DNA sequencer (ABI Prism 3100 Genetic Analyser; Applied Biosystems, Foster City, CA). Because the nucleotide substitution (A→G) abolishes a *Bts*I restriction site, PCR-amplified fragments from a control population of 100 persons (200 chromosomes in total) were digested with *Bts*I (Boehringer Mannheim, Mannheim, Germany), which recognizes the wild-type allele (5′-GCAG*T*-GNN-3′) but not the mutant (5′-GCAG*C*GNN-3′), after which electrophoresis was performed on a 2% agarose gel.

Psychological and Electrophysiological Tests

All three children of the fourth generation (IV.1, born in 1980; IV.2, born in 1982; IV.3, born in 1986) underwent three visual explorations. The father (III.1, 1952) underwent electro-oculography (EOG) and ERG recordings only because of his poor vision and severe photophobia. First, color vision was tested with saturated and desaturated color tests (D-15; Lanthony, Luneau, France). These tests were separately viewed by each eye under calibrated daylight (two fluorescent lamps; TLD 36W/95; Philips, Amsterdam, The Netherlands). Second, to obtain electrophysiological recordings, ERG and EOG were performed (Metrovision device; Lille, France; ERG corneal electrodes; Dencott, Paris France; EOG skin electrodes; Comepa, Bagnolet, France). They were conducted according to international ISCEV-EOG and ISCEV-ERG protocols.[20,21] Briefly, after 20-minute adaptation to dark with pupils fully dilated, the scotopic response (i.e., the response of the rod system) and the maximal combined response (i.e., the combined responses of the rod and cone systems) were recorded. Then, after a 10-minute adaptation to light and on a photopic background (30 cd/m^2), the oscillatory potentials (OPs) and the photopic responses, including the low (1 Hz) and the high temporal frequency responses (30 Hz) (respectively designed as the cone response and the flicker response) were recorded. The cone response represents that of the three cone systems, and the flicker response is limited to the response of the two major L and M cone systems. The EOG was first recorded and followed by the ERG recordings. Finally, eye fundi of the three patients were visualized by means of a scanning laser ophthalmoscope (SLO; personal prototype). The aspect of each SLO eye fundus was tape recorded together with the localization of the patient's preferred retinal locus (PRL). Comparison of its position with the patient's clinical visual acuity gives good indication of the functioning mode of the PRL.

Results

Corneal and Retinal Phenotypes

The family reported here is a nonconsanguineous kindred from Poland that includes eight PPCD-affected members over three generations (Fig. 1A). The affected male (III.1), who was the most affected member of the family, was first referred to the Department of Ophthalmology of Hôtel-Dieu Hospital at the age of 25 years to undergo corneal transplantation. Slit lamp examination showed numerous corneal endothelial vesicular lesions often grouped in clusters and surrounded by a gray halo, diffuse thickening of the Descemet's membrane with bandlike lesions, and islands of abnormal endothelial cells with multilamellar pattern. Specular microscopy revealed endothelial changes with pleomorphism, polymegathism, and endothelial cell loss (500/mm^2), suggestive of severe endothelial damage (data not shown). Optical, scanning, and transmission electron microscopy analysis of the corneal button from this proband indicated granular deposits in the posterior stroma, thickened Descemet's membrane composed of three layers with a normal anterior fetal zone, an intermediate anarchic zone composed of fibrils, and long and short collagen fibers, and an irregular adult zone with some long period collagen fibers (data not shown). This layer was lined posteriorly by degenerate overlapping endothelial cells that have epithelial-like features with multiple layers, desmosomal junctions, and microvillous projections (Fig. 1B). These abnormal findings suggest that corneal endothelial cells have degenerated and have been progressively replaced by pseudoepithelial cells producing collagen fibers and fibrils.

The proband of this family (III.1) also exhibited bilateral macular dystrophy that progressively worsened over a period of 30 years. In early examinations, the proband experienced severely decreased visual acuity and photophobia, whereas nocturnal and peripheral visions were unaffected. At that time, his best-corrected visual acuity was 0.2 in the right eye and 0.1 in the left eye. His visual field showed a bilateral central scotoma. At ophthalmoscopy, the fundi showed asymmetric macular lesions with white polymorphous flecks, sparing the fovea. Fluorescein angiography revealed bilateral parafoveal hyperfluorescent well-circumscribed lesions, with central hypofluorescence, giving the aspect of "bull's-eye " lesions (Fig. 2A). At the present time, the proband's visual acuity is limited to counting fingers,

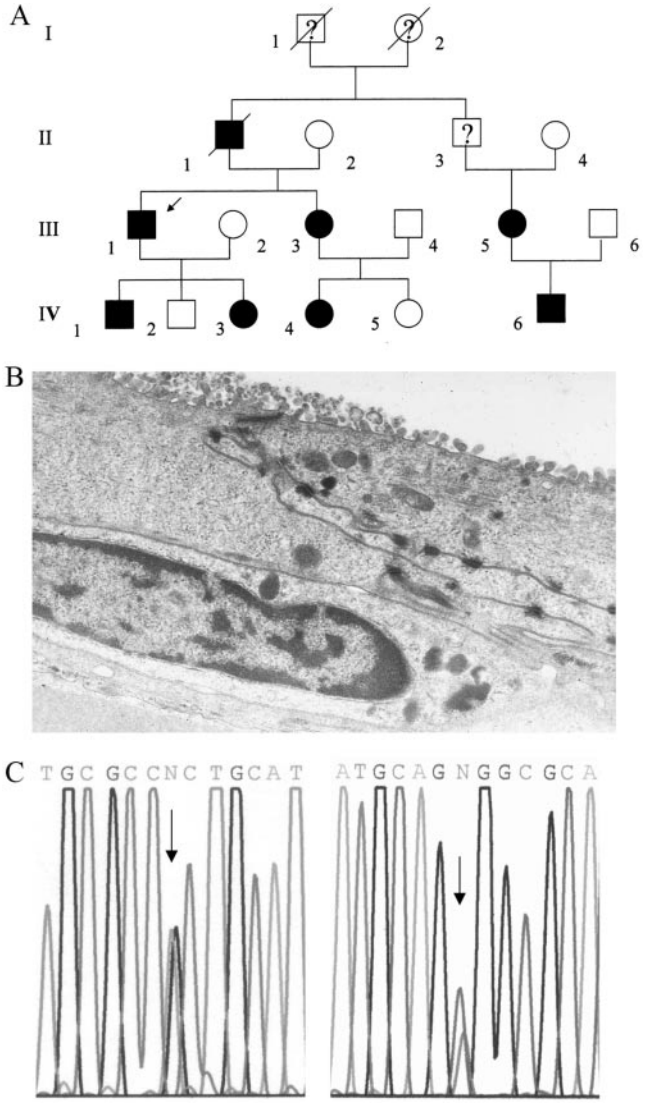

FIGURE 1. Pedigree of the family with the H244R mutation identified in the *VSX1* gene. (**A**) Affected family members are drawn as *closed circles* (females) and *closed squares* (males), and unaffected family members are represented by *open symbols*. (*Arrow*) Proband. (**B**) Transmission electron microscopy of the corneal button from the proband revealed stratified pseudoepithelial cells with microvilli and desmosomal attachments (final magnification, ×7520). (**C**) Electrophoregram depicting the H244R mutation in exon 2 of the *VSX1* gene from the proband's DNA. (*Arrowheads*) Reverse and forward partial mutant sequences showing the A→G change.

with preserved peripheral and nocturnal vision. Fluorescein angiography has demonstrated the progression of macular degeneration, which now also involves the fovea, giving a "beaten-metal " appearance (Fig. 2B). Two of the proband's children (IV.1, IV.3) were also diagnosed with PPCD and macular dystrophy at 11 and 4 years of age, respectively, whereas another child (IV.2) was not clinically affected. The proband's father (II.1), who died 25 years ago, was known to have a moderate form of PPCD, with subtle eye fundus abnormalities. Four other family members were recently diagnosed with mild PPCD (III.3, III.5, IV.4, IV.6). These patients also had decreased visual acuity, severe photophobia, and mild atrophic macular lesions observed at funduscopy, demonstrating that corneal and retinal traits were inherited in a dominant manner in this family.

Mutation Detection

It has been previously reported that missense mutations in *VSX1* or in the gene encoding the alpha2 chain of type VIII collagen (COL8A2) caused autosomal dominant PPCD.[17,19] Genetic screening of these two genes in the proband's DNA revealed a heterozygous nucleotide variation in the *VSX1* gene, corresponding to an A→G transition, which predicted the change of histidine to arginine at amino acid position 244 in *VSX1* (Fig. 1C). Sequence analysis indicated that this H244R variant was absent from the proband's clinically unaffected child (IV.2), whereas both clinically affected children (IV.1, IV.3) carried this mutation in the heterozygous state. This variant was also present in affected family members (III.3, IV.4) and absent in the unaffected (IV.5) patient, thus demonstrating that this *VSX1* variant segregated with both disease phenotypes in this family. The H244R *VSX1* variant has previously been identified in a familial case of keratoconus and in two apparently asymptomatic heterozygous persons.[17] Restriction analysis from our control population showed that the H244R allele was present in one of the 200 chromosomes studied (data not shown). The number of control chromosomes tested for this variant from PPCD or keratoconus studies now available is 1004, and only three control chromosomes have been found positive.[17] Therefore, the frequency of this variant is estimated at 0.30% instead of the 0.70% initially published. In addition, *VSX1* has also been screened in 161 pedigrees affected with various anterior segment abnormalities by Semina and colleagues,[22] and no mutations were found. All these data indicate that the H244R variant is not widespread and that it cannot be considered a common variant. PPCD is a slowly progressive corneal disorder that leads to a variable degree of visual impairment in adulthood, but usually this condition goes unnoticed throughout life and affected persons are diagnosed by chance, explaining why most PPCD cases are considered sporadic. Therefore, we cannot rule out the possibility that the positive controls could have mild, undiagnosed keratoconus, or PPCD, or even subclinical retinal dysfunction.

Psychological and Electrophysiological Tests

We performed, on four family members (IV.1, IV.2, IV.3, III.1), a series of visual tests to define the level of the retinal defects and to quantify their extent. Color vision results showed a protan axis of confusion for each eye with the saturated and desaturated tests for patient IV.1. For patient IV.2, color vision data were normal. For patient IV.3, results of the saturated test were normal for both eyes, and those of the desaturated test showed many lines of confusion without any defined axis, similar to what is recorded in advanced macular dystrophies (data not shown). Then, the proband's children eye fundi were visualized by means of a SLO. The aspect of each SLO eye fundus was tape recorded, together with the localization of the patient's preferred retinal locus (PRL). Comparison of its position with the patient's clinical visual acuity gives good indications of the functioning mode of the PRL. The SLO eye fundi of patient IV.1 showed a round inhomogeneous macular area of approximately 10°, centered on the fovea. His visual acuity was 0.6, and his PRL were located inside the pathologic area, indicating that his small foveal areas are still functioning despite the dystrophic aspect of the macula (Fig. 2C). In contrast, the SLO eye fundi of patient IV.3 showed that her PRL was located outside the macular lesions, on the upper part of the atrophic areas, with a visual acuity of 0.4, suggesting functional retinal reorganization (Fig. 2D). EOG results of the four patients (III.1, IV.1, IV.2, IV.3) were normal, indicating that the function of the retinal pigment epithelium was normal and excluding this site as a possible origin of the macular dystrophy (data not shown). All ERG responses from IV.2, the proband's

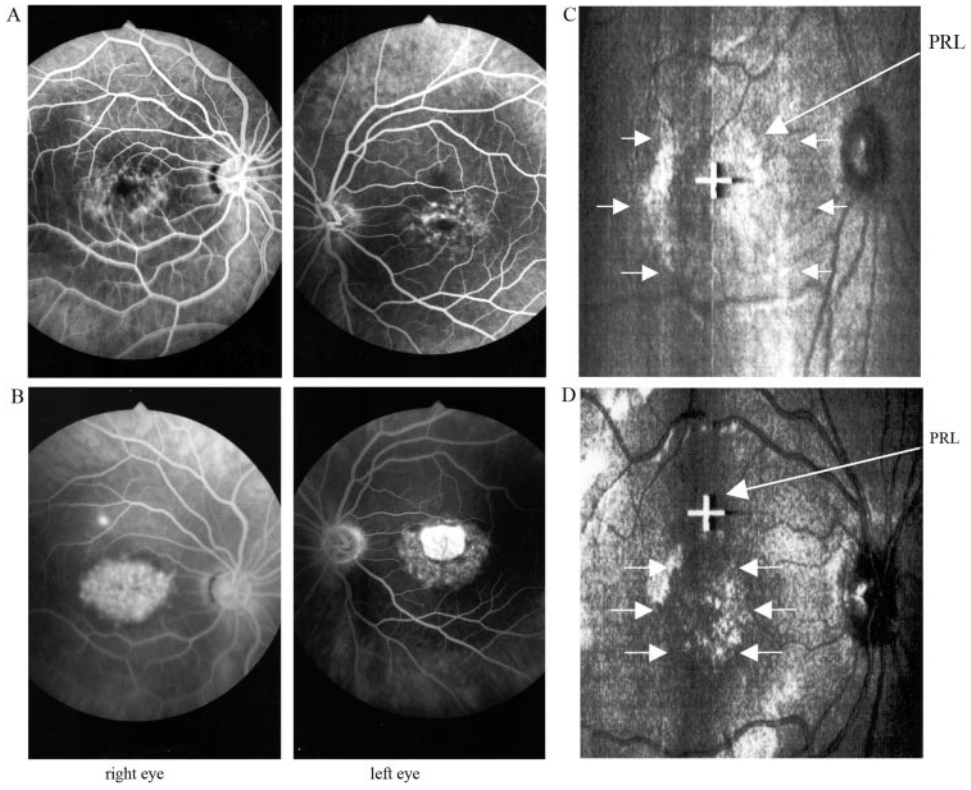

FIGURE 2. Aspects of patients' eye fundi. (**A**) Fluorescein angiography images from the proband (III.1) aged 25. (**B**) Fluorescein angiography images from the proband (III.1) aged 45. (**C**) SLO of proband's child (IV.1) showing the PRL inside the macular lesion. (**D**) SLO of proband's child (IV.3) showing that PRL is located at the upper part of the macular lesion. (*Arrows*) Limits of the macular lesion.

clinically unaffected son, showed normal amplitudes and implicit times (Figs. 3A, 3B, 4). Thus these normal responses negate any subclinical retinal dysfunction, which is in accordance with the absence of the H244R mutation in this family member. Implicit times of all the scotopic and photopic responses from affected persons (III.1, IV.1, IV.3) ranged within normal values. After dark adaptation, these patients had normal rod b-wave and mixed a- and b-wave amplitudes, indicating the absence of global dysfunctioning of the scotopic pathway (data not shown). Clinically, these patients had normal nocturnal vision and normal peripheral visual field vision, attesting that the global functioning of rod ON-BC was unaffected. After light

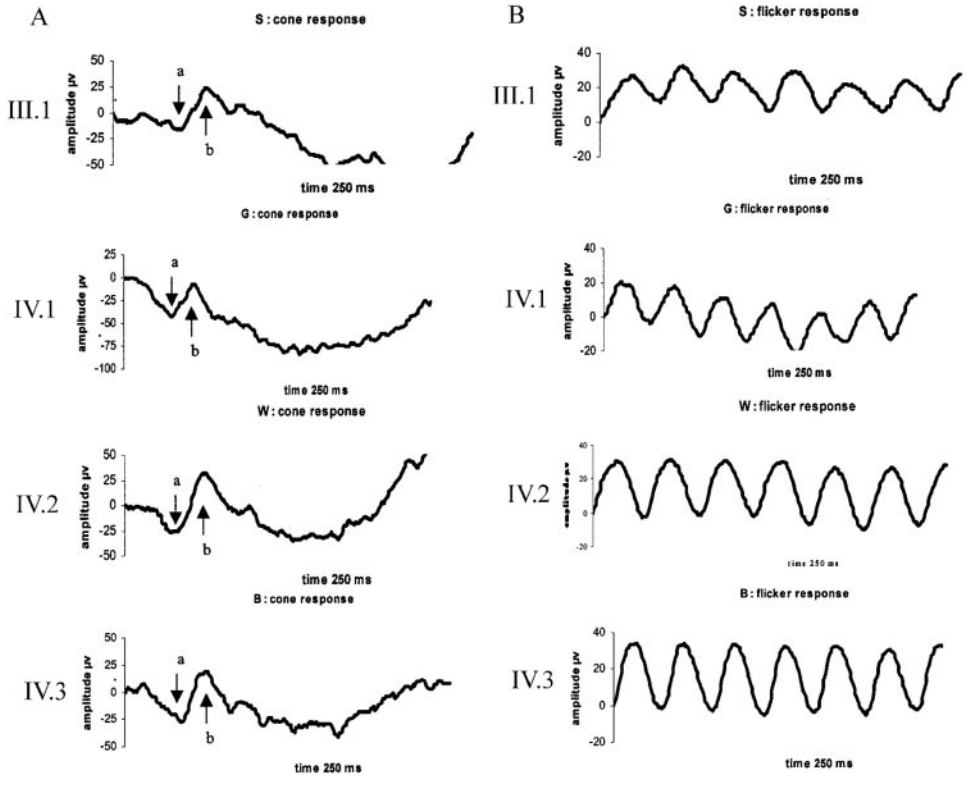

FIGURE 3. ERG recordings. (**A**) Photopic electroretinograms after adaptation to light. Proband's unaffected child (IV.2) showed normal b-wave amplitude, whereas cone b-wave amplitude was decreased in the affected family members (III.1, IV.1, IV.3). (**B**) 30-Hz flicker electroretinogram recorded from the same family members.

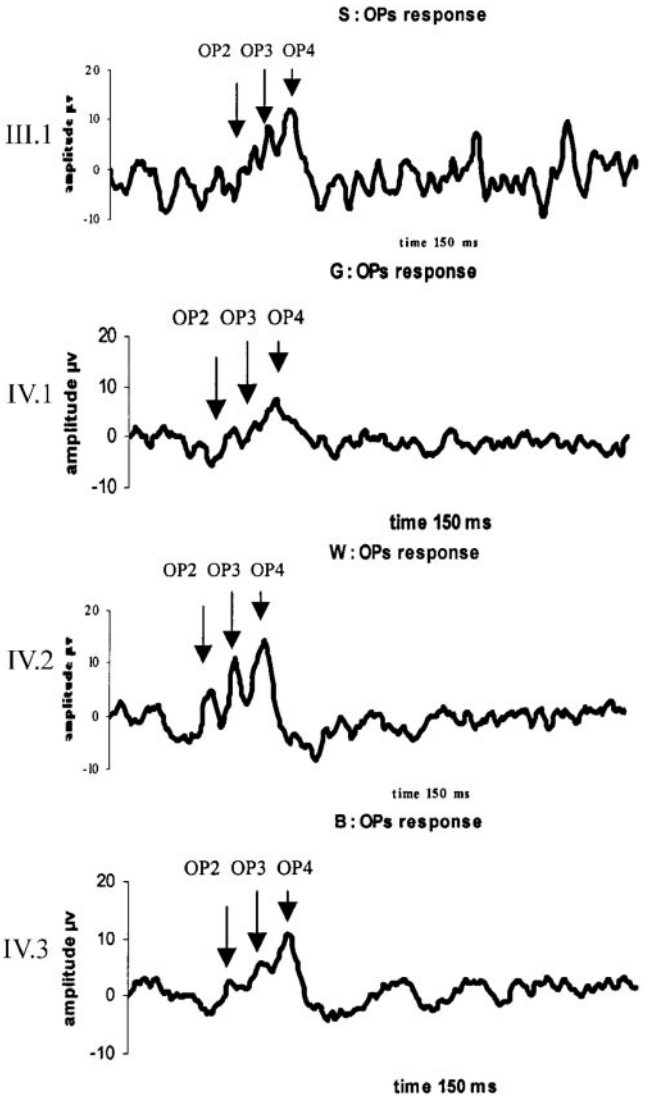

FIGURE 4. Cone-mediated OPs. In affected patients (III.1, IV.1, IV.3), OP2 and OP3 are markedly diminished to below the normal range, whereas OP4 amplitude is preserved. Normal OP pattern is shown in the unaffected child in this family (IV.2).

adaptation, for each of these patients, the two first photopic OPs (OP2 and OP3) were barely recordable, whereas the third one (OP4) had normal amplitude values (Fig. 4). Furthermore, for these patients cone a-wave amplitudes were normal, whereas cone b-waves amplitudes were moderately or severely decreased (Fig. 3A). Flicker amplitudes were within normal range in patient IV.3, and they were decreased and severely decreased in patients IV.1 and III.1, respectively (Fig. 3B). Together, these results suggest a defect in the visual signal transmission to the ON bipolar cells in the cone visual pathway only and a preserved global function of photoreceptors, outside the macula.[23,24]

DISCUSSION

The H244R variant is of special interest because H244 is 100% conserved from flies to humans, and it is located in the functionally important CVC domain, which is essential, with the HD domain, for the repressive transcriptional action of *VSX1*.[25] In this report, H244R is associated with PPCD, macular dystrophy, and cone ON bipolar cell dysfunction, whereas the same variant has previously been identified in a familial case of keratoconus, highlighting that, despite the common molecular genetic etiology, the ocular phenotype in each family is variable.[17] Similarly, in a recent study, the P247R and G160D alleles of *VSX1* were associated with keratoconus, whereas the same *VSX1* variants in the series published by Héon et al.[26] were found to cosegregate with PPCD, inner retinal dysfunction, or both. Two genes are implicated in PPCD, but each of them accounts for the disease in only a small fraction of cases, indicating that other genes are probably involved. Although *VSX1* allelic heterogeneity could explain in part the phenotypic variability observed in unrelated patients, it remains difficult to understand how persons with the same mutation can have different phenotypes and can even be asymptomatic carriers. However, this is common in a number of heritable forms of visual and hearing impairment, and it has been proposed that modifier genes or environmental interactions may obscure phenotype–genotype relationships by interacting in the same biologic pathway as the disease gene.[27] The availability of a second unrelated family, reported here, in which the H244R variant perfectly cosegregates with the disease phenotype, which is different from that previously described, reinforces the idea that H244R is a causative mutation and indicates that genetic modifiers might interfere with the pathologic effects of *VSX1* variants by enhancing the phenotype or by reducing, or even suppressing, the effect of the mutant allele to the extent that it completely restores the normal condition. A noteworthy example of such a situation is provided by the spontaneous null allele for the CVC-homeobox *Chx10* gene, closely related to *Vsx1*, which gives rise to an ocular retardation phenotype (*or*^{J}) in mice that can be partially restored depending on the genetic background.[14,28]

Numerous studies on OPs after pharmacologic synaptic blockages or in various human or mouse model retinal diseases in which the ON-BC pathway is compromised, with preservation of the OFF counterpart, have led to the concept that OP2 and OP3 are associated with the ON retinal pathway but that OP4 reflects the OFF pathway.[23] The ERG b-wave is elicited when glutamate release from photoreceptors is suppressed in response to light stimulation. In darkness, glutamate, continuously released by photoreceptors, hyperpolarizes ON-BC through the mGluR6-Go-protein complex, which is believed to be exclusively expressed by ON-BC.[24,29] In response to light stimulation, the cation channels that had been closed after activation of the mGluR6-Go-protein complex in darkness reopen, thereby depolarizing ON-BC.[29–31] Thus, absence or reduction of the b-wave amplitude is considered to reflect ON-BC dysfunctioning after light increment. The mGluR6- or Go-deficient mice fail to produce scotopic and photopic b-waves because of the absence of synaptic transmission from rods and cones to the ON bipolar cell pathway, resulting in the absence of depolarization of their ON-BC, mimicking darkness responses.[32,33] In the same way, the metabotropic glutamate agonist 2-amino-4-phosphonobutric acid (APB) selectively blocks the mGluR6 receptor on ON-BC and hyperpolarizes these cells by antagonizing their responses to light. APB produces ERG responses similar to those observed in mGluR6- or Go-deficient mice and induces a loss of OP2 and OP3 with a preserved OP4 amplitude.[24,34,35] Therefore, by analogy with all these models, we predict that our patients have a defective ON-BC pathway, leaving the OFF-bipolar circuitry relatively intact. Thus this study illustrates a selective dominant heritable cone ON-BC dysfunction that spares the OFF-BC circuitry and the scotopic pathway. These results are unusual because, as has been pointed out by Nelson and Connaughton,[36] the human disease processes or animal models that target the ON-BC pathway usually impair both

the rod and the cone ON-BC and are always accompanied by a loss of nocturnal vision. The selective retinal defect present in this family could be explained by the restricted expression pattern of the *Vsx1* gene, which is found in ON-BC and which has established input with cone photoreceptors only.[6,15,16] The electrophysiological results presented here differ from the published data on *Vsx1* null mice and on *VSX1* patients previously reported with cone OFF bipolar cell dysfunction and an abnormal scotopic bipolar cell pathway, respectively.[16,17] The explanation for this discrepancy is not clear and might be multifactorial. This discrepancy may reflect species differences or, alternatively, could depend on the nature of the *Vsx1* mutation itself, which could have distinct molecular pathogenic mechanisms. Considering the existence of potential modifier genes for *VSX1*, a third possibility is that the genetic background of each person harboring a *VSX1* missense mutation might contribute highly to the variable inner retinal dysfunction.

To date, this is the first clinical report describing maculopathy associated with PPCD in patients with *VSX1*, highlighting a possible new alternative role of *VSX1* in cone photoreceptors. Although the eye fundi of patients with *VSX1* have been normal, subclinical macular impairment is suspected in some patients.[18] The assumption that *VSX1* could play a role in cone biology is further reinforced by the fact that *VSX1* transcript is found in the human WERI retinoblastoma cell line, which expresses cone-specific genes, and not in the Y-79 retinoblastoma cell line, which is rod-specific, leaving the possibility that *VSX1* could play a role in the differentiation or maturation of cone photoreceptors.[8] Also supportive is that *VSX1* is capable of binding in vitro to the core of the locus control region, which controls the expression of the red/green visual pigments indispensable for the maturation and viability of cone photoreceptors.[8] However, disease genes identified thus far as being implicated in inherited macular dystrophies have been shown to encode proteins that are expressed either in the photoreceptors or in the retinal pigment epithelium in which they exert specific roles. Recently, it has been shown that the human ABCR transcript, thought to be present in rods only, is in fact also expressed by foveal and peripheral cones, as has been found for its frog ortholog.[37] *Vsx1* is absent in mature photoreceptors in all the species studied; however, in goldfish, *Vsx1* is weakly expressed in a subset of undifferentiated proliferating neuroepithelial cells of the presumptive neural retina before it is repressed in retinal cells other than BCs when differentiation begins.[1,12] Because *VSX1* functions as a transcriptional repressor and is repressed in a *VSX1*-dependent manner, one would predict that some specific mutations, notably those located in the CVC-domain of *VSX1*, could lead to aberrant or persistent expression of *VSX1* in some retinal cells in which this gene is normally downregulated. Consequently, direct target genes of *VSX1* would be upregulated, and a downregulator(s) of *VSX1* could be temporally or spatially incorrectly activated, resulting in retinal defects. At present, this genetic pathologic mechanism is speculative, and the maculopathy observed in our patients remains unexplained. Further insight into the pathophysiology of *VSX1* requires the development of a knock-in mouse carrying the H244R allele in the orthologous murine *Vsx1* gene.

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