Clinical Presentation and Genetic Correlation of Patients With Mutations Affecting the FZD4 Gene

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Objective: To correlate the ophthalmic findings of patients with pediatric vitreoretinopathies with mutations occurring in the FZD4 gene.

Methods: A total of 123 patients diagnosed with autosomal-dominant familial exudative vitreoretinopathy (AdFEVR) or retinopathy of prematurity (ROP) and 42 control patients were enrolled in the study. Diagnoses were based on retinal findings at each patient’s first examination or during ROP screening. Genomic DNA was isolated and polymerase chain reaction and direct sequencing of the FZD4 gene performed.

Results: FZD4 gene mutations were discovered in 13 of the 123 (10.6%) patients. Nine of the 63 patients with AdFEVR (14.3%) have mutations in the FZD4 gene. Four heterozygous mutations were identified: C117R, C181Y, P33S, and P168S. Four of the 60 patients with ROP (6.7%) have a double missense mutation P33S/P168S that was also found in the patients with FEVR. No other FZD4 mutations were found in the patients with ROP. Additionally, patients expressing the double mutation had clinical presentations that overlapped, making it difficult to assign a definitive diagnosis. None of the mutations found in the patients with FEVR or ROP were seen in the control chromosomes.

Conclusion: Mutations occurring in the FZD4 gene affect patients diagnosed with both FEVR and ROP. The clinical picture often overlaps and may require a detailed birth and family history for diagnosis. Genetic testing confirms inherited vitreoretinopathy and helps direct clinical management.

Clinical Relevance: Patients diagnosed with ROP may have a mutation in the FZD4 gene and display characteristics consistent with FEVR. Analysis of the FZD4 gene should be considered.

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(Wnt/Norrin) domain 7 Transmembrane domains Intracellular C-terminal domain


(Wnt/Norrin) Ligand-binding domain Canonical Wnt signaling motif PDZ-1, noncanonical, JNK activation motif

Figure 1. Diagram of frizzled-4 receptor (FZD4) amino acid sequence. AA indicates amino acid; JNK, c-Jun N-terminal kinase; SS, signal sequence.

β-Catenin Pathway

Planar Cell Polarity Pathway

Ca$^{2+}$ Pathway

Figure 2. Schematic of frizzled-4 receptor (FZD4)–dependent pathways. APC indicates antigen-presenting cell; β-cat, β-catechin; Ca$^{2+}$, calcium ion; CamK-II, calcium-calmodulin kinase 2; CREB, cAMP response element-binding protein; DAMM, death-associated molecule related to Mch2; G-proteins, guanine nucleotide-binding proteins; GSK, glycogen synthase kinase 3; JNK, c-Jun N-terminal kinase; LRP, low-density lipoprotein receptor–related protein; NFAT, nuclear factor of activated T cells; P, phosphorylated site; PKC, protein kinase C; ROCK2, Rho-associated, coiled-coil–containing protein kinase 2; TCF/LEF, transcription factor and lymphoid enhancer–binding factor.

Rac) is activated, which in turn activates an alternative signal transduction pathway. The planar cell polarity pathway mediates cytoskeletal organization and cell migration. In the Wnt-Ca$^{2+}$ pathway, FZD receptor activation stimulates an intracellular Ca$^{2+}$ release, activating calcium-calmodulin kinase 2 and protein kinase C. (Figure 2). This pathway is important in cell adhesion and cell movement during gastrulation.

In this study, we used direct sequencing to screen for mutations in the coding sequence of FZD4 in patients with FEVR and ROP. The possible implications of these sequence variations in relation to signal transduction and disease type will be discussed.

**METHOD**

**PATIENTS**

Patients were recruited to the study through a protocol approved by the internal review board at William Beaumont Hospital and consented to participation. They were diagnosed with FEVR or ROP based on fundus examination, family history, and
gestational age. Participants provided a blood sample from which genomic DNA was isolated from the leucocytes using the Purgene GenomicDNA Purification Kit (Qiagen, Valencia, California). When possible, genomic DNA from the relatives of patients expressing an $FZD4$ mutation were tested for sequence variations.

**SEQUENCING**

The coding sequence and flanking splice sites of $FZD4$ were amplified from 100 ng of genomic DNA using Herculase Hot-start PCR Master Mix (Stratagene, La Jolla, California). Four sets of primers were used (Table 1). Amplification conditions were as follows: 1 cycle at 98°C for 1 minute, 40 cycles of 30 seconds at 98°C, 30 seconds at 55°C, and 1 minute at 72°C, and a final extension for 10 minutes at 72°C. Amplified DNA was cleaned using the QIAquick Multiwell PCR Purification Kit (Qiagen). Sequencing reactions were performed using the Beckman Dye Terminator Cycle Sequenc Quick Start Kit (Beckman Coulter, Inc, Fullerton, California) and a Beckman CEQ 8000 autosequencer.

**RESULTS**

**MUTATION ANALYSIS**

$FZD4$ gene mutations were discovered in 13 of the 123 patients enrolled in this study. Nine of the 63 patients with AdFEVR (14.3%) were found to have mutations in the coding sequence of $FZD4$ (Table 2). Four heterozygous mutations were found: C117R, C181Y, Q505X, and P33S/P168S (eFigure; www.archophthalmol.com). In addition, 4 of the 60 patients with ROP (6.7%) had the double missense mutation P33S/P168S that was found in the patients with FEVR (Table 2). No other $FZD4$ mutations were found in the patients with ROP. None of the mutations found in the patients with FEVR and ROP were seen in the 84 control chromosomes.

Two novel missense mutations were found in patients with AdFEVR, C117R (2 patients) and C181Y. Both occur in 1 of the 13 conserved cysteine residues of $FZD4$ ligand–binding domain (Figure 1).

One patient with FEVR had a mutation resulting in early termination of protein translation, Q505X. This mutation has been reported in an Australian family.6 It is located immediately downstream from the highly conserved canonical Wnt activation motif (disheveled binding; KTXXXW) and causes premature termination prior to the planar cell polarity (c-Jun N-terminal kinase) pathway, PDZ1 binding motif (KTXV) (Figure 1). A double missense mutation, P33S/P168S, was found in patients with both FEVR (5 patients; 7.9%) and ROP (4 patients; 6.7%). The double mutation was previously re-
ported in a patient with AdFEVR.2 Both P33S and P168S have been independently reported in patients with AdFEVR6,7. AA33 is within the signal sequence and AA168 lies downstream of the cysteine-rich domain (CRD), Wnt-binding domain, and prior to the start of the 7-transmembrane domains (Figure 1). The P168S mutation has been reported in 1 control (of 400) but the P33S and double mutation have not been reported in control patients.

Several relatives of the patients with the double missense mutation P33S/P168S were screened and found to express the mutation as well. Asymptomatic family members demonstrated retinal peripheral avascularity but no abnormal extraretinal vessels or exudates (Figure 3). Interestingly, 1 patient with ROP had a fraternal twin with the mutation but no apparent manifestation of disease, although fluorescein angiography was not performed and retinal avascular zones could not be definitively assessed. The asymptomatic twin is fraternal, had minimal comorbidities, and had much better systemic health, suggesting a role for epigenetics in the severity of disease manifestation.

CLINICAL COURSE

The clinical course varied for the individual patients in which a mutation was identified. Patients presented between birth (up to 16 weeks premature) and 7 years of age, although all patients reported poor vision in at least 1 eye since birth. Four of the 13 patients had ROP, and 2 developed aggressive posterior ROP (APROP). Nine patients had phenotypes consistent with FEVR, although 2 of these patients were initially diagnosed with ROP. One patient had an initial diagnosis of persistent fetal vasculature syndrome due to a posterior lens plaque but surgical intervention demonstrated a temporal retinal fold with a posterior lens attachment consistent with FEVR. A single patient with the double mutation in FZD4 also expressed a cysteine mutation in the NDP gene, and demonstrated severe bilateral retinal dysgenesis. This patient was diagnosed with Norrie disease and excluded from this study.

All 4 infants with ROP with FZD4 mutations had the double mutation (Table 2), with various degrees of disease severity (Figure 4). Three of the 4 had poor outcomes. Two developed APROP (zone 1, stage 3, Plus) and developed bilateral stage 5 retinal detachments. One infant with classic (zone 2, stage 3, Plus) ROP progressed to bilateral stage 4b despite appropriate and timely laser ablation. One of the premature infants demonstrated peripheral avascular retina only and did not progress to neovascular changes. The infants with APROP were both products of multiple births, one with a surviving twin and the other a sole surviving triplet. The twins were fraternal but both have the FZD4 double mutation. The twins had very different postnatal courses; the twin with APROP had multiple comorbidities and poor health, whereas the asymptomatic twin had an uneventful course with no systemic maladies. The surviving triplet, similarly, had multiple comorbidities and poor systemic health. Both cases suggest a role for epigenetics in the development and severity of inherited diseases.

The patients with FEVR generally showed asymmetry between the eyes and ranged from stage 1 disease to stage 5 (Table 1 and Table 2; Figure 5). Two patients also had rhegmatogenous retinal detachments. All patients had a history of amblyopia or strabismus shortly after birth (Table 2).
The mutations found in this study involve residues that are located in distinct regions of the FZD4 protein. A novel mutation (C117R) was found in 2 patients. It is located in the extracellular Wnt-binding domain. In the wild-type protein, Cys117 forms a disulfide bond with Cys158 (in the CRD of FZD4).8 Presumably, disruption of this bond causes a change in the FZD4 conformation, therefore affecting its ability to bind ligands. Wu et al9 showed that similar cysteine mutations in the CRD of Norrin, a FZD4 ligand, resulted in the most severe phenotype.

The other novel mutation found in this study, C181Y, is located within the N-terminal extracellular domain of FZD4 as well. This cysteine residue is not known to form an intracellular disulfide bond but it is the 11th of 13 cysteine residues that are conserved in vertebrate and may be required for dimerization.10 Dann et al11 observed that other frizzled receptors can dimerize and proposed that this may be relevant to the mechanism of Wnt binding and signaling.

The Q505X mutation falls immediately after the highly conserved Wnt signaling motif (KTXXXW). Presumably, this early termination results in haploinsufficiency due to nonsense-mediated mRNA decay. It is possible that a truncated protein is produced and terminated prior to the PDZ binding motif (ETXV) of FZD4 (c-Jun N-terminal kinase pathway) (Figures 1 and 2). Perhaps noncanonical signal transduction via the c-Jun N-terminal kinase pathway is necessary for normal angiogenesis and vascularization of the retina. Alternatively, the mutant allele may form an oligomer with the wild type, trapping both in the endoplasmic reticulum. Kaykas et al12 demonstrated that a similar FZD4 mutation (L501fsX533) resulted in this occurrence. Finally, Robitaille et al13 demonstrated that the L501fsX533 mutation decreased activation of the Wnt-Ca2+ pathway. It is therefore difficult to tell which intracellular Wnt pathway is disrupted by this mutation.

A double missense mutation, P33S/P168S, was found in 9 of the screened patients. The P33S mutation is in the extracellular Wnt-binding domain. In the wild-type protein, Cys117 forms a disulfide bond with Cys158 (in the CRD of FZD4).8 Presumably, disruption of this bond causes a change in the FZD4 conformation, therefore affecting its ability to bind ligands. Wu et al9 showed that similar cysteine mutations in the CRD of Norrin, a FZD4 ligand, resulted in the most severe phenotype.

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A double missense mutation, P33S/P168S, was found in 9 of the screened patients. The P33S mutation is in the signal sequence portion of the gene and could impair translocation of FZD4 to the plasma membrane. Additionally, proline is not a common amino acid and has the unique characteristic of rigidity that could be essential to the proper conformation of the protein. Another possibility is that this mutation affects a second transcript of the FZD4 gene, FZD4S. This transcript variant retains the intron between exon 1 and exon 2 and is 327 amino acids long. Currently, it is unclear whether FZD4S is an antagonist or agonist of FZD4 signaling.14,15

New blood vessel formation requires the coordination of endothelial cell division and the morphogenic movement of vessel expansion,16 and there is evidence for both canonical and noncanonical signal transduction in this process. For instance, LRP5 is known only to participate in canonical signal transduction. Because mutations in LRP5 have been associated with some cases of AdFEVR, it is likely that the canonical pathway plays a role in vascular eye development. Additionally, in vitro testing in STF cells has shown that mutations in either FZD4 or LRP5 result in decreased Wnt/β-catenin signal compared with wild type.3

There is also evidence to support the noncanonical transmission of the signal. Zeng et al16 demonstrated that perpendicular orientation of endothelial cell division is necessary for elongation of new vessels and suggests that planar cell polarity is required for this proper orientation. The FZD4 ligand, Wnt5a, has been shown to activate signal transduction by a planar cell polarity path-
way. Wnt\(_5a^{−/−}\) animals have a disrupted polarization of sensory hair cell stereocilia in the cochlea of the inner ear,\(^1\) similar to that seen in the FZD4\(^{−/−}\) mice, which also have enlarged and disorganized vessels in both the cochlea and retina.\(^3\)

Perhaps the FZD4 signal transduction, through both canonical and noncanonical pathways, is necessary for normal vessel development. One could envision upregulation of endothelial cell growth stimulated by canonical activated transcription followed by noncanonical stimulated cytoskeletal rearrangement and subsequent expansion of cells to form new vessels.

**CLINICAL MANIFESTATIONS**

This study highlights the complexity of categorizing genetic diseases. There is a clinical diagnosis and a genetic diagnosis, neither of which alone sufficiently describes the disease state. Often a diagnosis of ROP or FEVR is determined by family and birth histories in addition to clinical findings. This study indicates that the differentiation between these two diseases is not always distinct because the same FZD4 mutation was highly prevalent in both types of patients but not in controls.

Both ROP and FEVR share similar clinical phenotypes such as avascular peripheral retina, neovascular changes, vascular tortuosity and dilation, exudation, and vitreoretinal fibrosis. However, the presence of a peripheral avascular zone alone does not always lead to secondary complications in either disease. This is shown in this study by the fact that the parents of both types of patients and the twin with ROP who had the double-missense mutation were asymptomatic despite periph-eretal retinal avascularity (Figure 3). Presumably, many individuals with a peripheral avascular zone and a FZD4 mutation are never evaluated.

It has long been suspected that genetics plays a role in ROP, and a recent twin study\(^1\) concluded that there is a 70% genetic predisposition to development of ROP. Prior to this study, only 1 FZD4 mutation in a patient with ROP had been described.\(^2\) We suggest that the severity of retinal abnormalities is secondary to both genetic predisposition and epigenetics. It is our goal to investigate this possibility in further studies.

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**Additional Information:** The eFigure is available at http://www.archophthalmol.com.

**REFERENCES**


