

Spectrum and Frequency of *FZD4* Mutations in Familial Exudative Vitreoretinopathy

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PURPOSE. Mutations in the frizzled-4 gene (*FZD4*) have recently been associated with autosomal dominant familial exudative vitreoretinopathy (FEVR) in families linking to the *EVRI* locus on the long arm of chromosome 11. The purpose of this study was to screen *FZD4* in a panel of 40 patients with FEVR to identify the types and location of mutations and to calculate what proportion of this heterogeneous condition is attributable to *FZD4* mutations.

METHODS. PCR products were generated from genomic DNA with primers designed to amplify the coding sequence of *FZD4*. The PCR products were screened for mutations by single-strand conformational polymorphism-heteroduplex analysis (SSCP-HA) and by direct sequencing.

RESULTS. In total, eight mutations were identified, seven of which were novel. Three were deletions (c957delG, c1498delA, and c1501-1502delCT), one was a nonsense mutation (Q505X), and four were missense mutations (G36D, M105T, M157V, and S497F).

CONCLUSIONS. Eight mutations have been identified in the *FZD4* gene in a cohort of 40 unrelated patients with FEVR. This result indicates that *FZD4* mutations are responsible for only 20% of FEVR index cases and suggests that the other FEVR loci may account for more cases than previously anticipated. (*Invest Ophthalmol Vis Sci.* 2004;45:2083–2090) DOI:10.1167/iov.03-1044

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Familial exudative vitreoretinopathy (FEVR) is a well-defined inherited disorder of retinal vessel development (Online Mendelian Inheritance in Man [OMIM] 133780).¹ The disorder was first described by Criswick and Schepens in 1969.² Since then, many more FEVR cases and families have been described, and, although its frequency within the population has never been calculated, the disorder is thought to be relatively rare. FEVR is reported to have a penetrance of 100%, but clinical features can be highly variable, even within the same family. Severely affected patients may be registered blind during the first decade of life, whereas mildly affected individuals may not even be aware of symptoms, and in them, the disease is only diagnosed by fluorescein angiography.³

The primary pathologic process in FEVR is believed to be a premature arrest of retinal angiogenesis-vasculogenesis or retinal vascular differentiation, leading to incomplete vascularization of the peripheral retina.⁴ This failure to vascularize the peripheral retina is the unifying feature in all affected individuals, but, by itself, usually causes no clinical symptoms. The visual problems and variable phenotype associated with FEVR result from secondary complications due to retinal ischemia.¹ Leaky abnormal vessels can develop at the edges of the retina and can cause subretinal exudation. Furthermore, the neovascularization can regress, leading to the formation of scar tissue that produces tractional forces on the retina. These features cause a reduction in visual acuity and in 20% of cases can lead to partial or total retinal detachment.⁴

FEVR is genetically heterogeneous, with X-linked,^{5,6} autosomal dominant,^{7,8} and autosomal recessive^{9,10} modes of inheritance described, autosomal dominant being the most common mode.^{11–13} To date, one X-linked and two autosomal dominant loci have been mapped: *EVRI* on 11q,¹⁴ *EVRI* on Xp,¹⁵ and *EVRI* on 11p.¹⁶

The gene underlying X-linked FEVR, *EVRI*, has been identified and shown to be the same gene as that mutated in Norrie disease (ND).¹⁷ ND is a rare, X-linked, recessive neurodevelopmental disorder characterized by congenital blindness, with a proportion of patients (30%) also having sensorineural deafness and mental disturbances.¹⁸ The ND gene encodes a secreted protein, NDP, containing a cystine knot motif.¹⁹ However, the function of NDP remains elusive, despite the creation of a mouse with targeted disruption of the gene.²⁰

The autosomal dominant *EVRI* gene has been mapped within a 14-cM interval on 11p12-p13, defined by markers GATA34E08 (telomeric) and D11S4102 (centromeric).¹⁶ To date, no other *EVRI* family has been reported, and the causative gene remains unknown.

The first FEVR locus to be described was *EVRI*, which was localized to 11q13-q23 in two large European families.^{14,21} Additional linkage studies mapped further families to this region,^{11–13,22} but none of these families was either informative or large enough to refine the disease interval accurately. However, Robitaille et al.²³ in 2002 mapped a large Canadian family to the *EVRI* locus, enabling them to place the disease gene within an interval of 1.55 Mb, which contained only two genes. Sequencing these genes in this family led to the identification

TABLE 1. Sequences of Oligonucleotide Primers Used in the Present Study

Exon	Forward Primer 5'→3'	Reverse Primer 5'→3'	Size (bp)
1	CTGCTACCCCGATGCTG	GGATGATCAACTTGGCATGG	396
2a	CTGGAAGCATTCAACTCAGC	TGGTTCCCACAGAGTGACA	367
2b	GTGCCCTTACCTCACAAAACC	CAGGATATCCTTCCCAGGCC	347
2c	CGGCCATCATATTCTCAG	CACAAGCCAGTCAGTTCATC	374
2d	CATCCCGCAGTGAAAACCA	GGAATCATCTGCAGAATACCC	358
2e	GTTCTGCAACGTGTGTGAT	CGGGGTCACCTAATTGTTG	407

Exon 2 was screened in five overlapping segments designated 2a-2e.

of *FZD4* as the defective gene at the *EVR1* locus.²³ The *FZD4* mRNA is 7391 bp long and comprises two exons that encode the 537-amino-acid protein Frizzled-4.²⁴ Frizzled-4 is a member of the Frizzled family of seven-pass transmembrane receptors that bind Wnt proteins. The Wnt signaling pathway plays a major role in differentiation and patterning during embryogenesis and regulates cell proliferation in adult tissues.²⁵ In addition, several components of the Wnt signaling cascade are implicated in several human cancers.^{26,27}

In the original study only two *FZD4* mutations were reported, and both of these were deletions found at the C-terminal end of the protein.²³ In another recent study, four further FEVR mutations were identified in a screen of 24 FEVR probands, three missense changes, and a nonsense mutation.²⁸ The purpose of this study was to screen this gene in our patient cohort to determine further the spectrum and frequency of mutations in *FZD4* that cause FEVR.

METHODS

Subjects

Genomic DNA samples from 17 FEVR families and 23 single individuals with diagnosed FEVR were analyzed in this study. Clinical diagnosis was made based on the presence of retinal abnormalities on funduscopy deemed typical of FEVR. These included primarily an area of deficient peripheral retinal neovascularization, together with exudation and/or sequelae of retinal traction, such as macular ectopia, retinal folds, and retinal detachment. Fundus fluorescein angiography was performed in selected cases to confirm the diagnosis. Informed consent was obtained from all subjects tested, and the research adhered to the tenets of the Declaration of Helsinki. Ethical approval was obtained from the Leeds Teaching Hospitals Trust Research Ethics Committee and the Royal Victorian Eye and Ear Hospital Research and Ethics Committee. Control subjects were normal partners of patients attending the Yorkshire Regional Genetics Department.

Mutation Detection

The coding exons and flanking splice junctions of one affected individual from each family were amplified by PCR with the primers detailed in Table 1. The method used was the same as that described elsewhere.²⁹ Where the single-strand conformational polymorphism-heteroduplex analysis (SSCP-HA) showed a mobility shift, PCR amplification of the coding exon and flanking intronic sequence was performed to provide a template for sequencing. Reactions were performed in a 50- μ L volume with 50 ng of genomic DNA; 20 pmol of each primer; 200 μ M dATP, dCTP, dGTP and dTTP; 10 mM Tris-HCl (pH 8.3), 50 mM KCl; 1.5 mM MgCl₂; 0.01% gelatin and 1 unit *Taq* DNA polymerase (Invitrogen Ltd., Renfrew, UK). After the initial denaturation step at 96°C for 3 minutes, the samples were processed through 35 cycles of 94°C for 30 seconds, 60°C to 65°C for 30 seconds, and 72°C for 30 seconds. A final extension step was performed at 72°C for 10 minutes. Both the forward and reverse strands of the PCR products were directly sequenced on a sequencer (Long Read IR 4200; Li-Cor, Inc., Lincoln, NE) using the fluorescence-labeled primer cycle sequenc-

ing kit (Thermo Sequenase; Amersham Pharmacia Biotech, Amersham, UK). Sequencing reactions were set up according to the manufacturer's instructions using either the forward or reverse primers detailed in Table 1. The nomenclature used to describe the mutations conforms to the guidelines established by den Dunnen and Antonarakis.³⁰

Accession Numbers

Protein sequences for the alignment figure: human Frizzled-4 NP_036325, *Mus musculus* NP_032081, *Rattus norvegicus* NP_072145, *Gallus gallus* Q91A05, *Xenopus laevis* Q9PT62, *Danio rerio* NP_57122, human Frizzled-1 NP_003496, human Frizzled-2 NP_001457, human Frizzled-3 NP_059108, human Frizzled-5 NP_003459, human Frizzled-6 NP_003497, human Frizzled-7 NP_003498, human Frizzled-8 NP_114072 (amino acids 151-213 not shown in alignment), human Frizzled-9 NP_003499 and human Frizzled-10 NP_009128 (accession numbers from GenBank, <http://www.ncbi.nlm.nih.gov/Genbank>; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).

RESULTS

FEVR Patient Screening

To determine the types and frequencies of *FZD4* mutations we tested 40 unrelated individuals with a clinical diagnosis of FEVR. Oligonucleotide primers were designed to amplify both the coding exons and flanking splice junctions of *FZD4*, and these were analyzed by SSCP-HA and sequencing. Because of its large size, exon 2 was amplified in five overlapping segments (2a-2e). We identified mutations in 8 (20%) of 40 individuals with FEVR. Eight different mutations were found, of which seven are novel. The characteristics of these mutations are presented in Figures 1 and 2 and Table 2.

Deletions

Three different deletions were observed that were not detected in 200 normal control chromosomes. Mutation screening in a North American family known to be linked to the *EVR1* locus identified a 2-bp deletion in exon 2 (c1501-1502delCT). This mutation segregates with the disease and causes a frameshift resulting in 32 incorrect amino acids after codon 500 (NH₂-SHVAEVFQQIGEFWKGKERERKWLGEAWKRQ-COOH) followed by a premature termination at codon 533 (L501fsX533). This mutation is the same as one reported in the original study by Robitaille et al.²³ It is possible that both these mutations were found in different branches of the same family as both originated from the United States, but haplotype analysis must be performed to confirm this hypothesis.

Also in exon 2, we identified a 1-bp deletion, c1498delA, in a single British patient who had an affected parent that we were unable to examine. This mutation causes a frameshift resulting in the substitution of 12 amino acids after codon 499 (NH₂-LFTRGRSVPTDW-COOH) and a premature stop in codon 512 (T500fsX512).

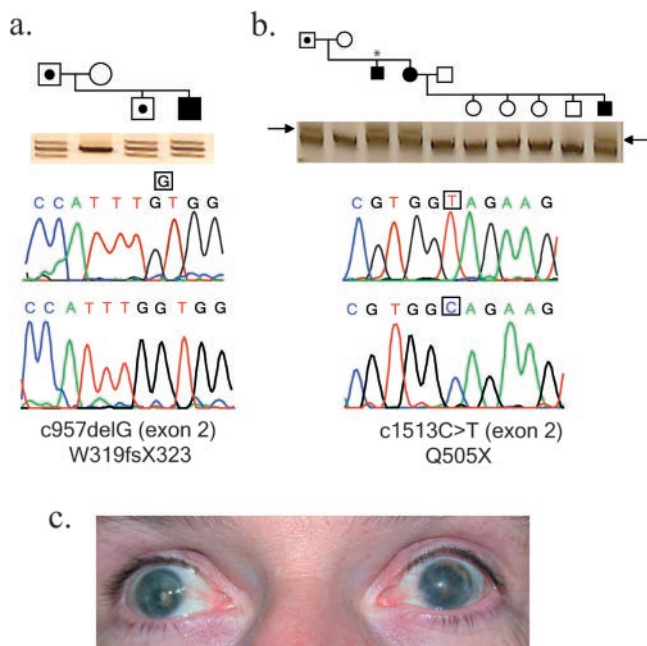


FIGURE 1. (a) *FZD4* mutation c957delG tracking in a family with asymptomatic individuals. The figure shows the HA result obtained using amplimers 2c and the sequence trace of the mutation containing allele and the corresponding wild-type allele. The symbols with dots represent asymptomatic individuals. (b) *FZD4* mutation c1513C→T tracking in a family. The figure shows the HA result obtained using amplimers 2e and the sequence trace of the mutation containing allele and the corresponding wild-type allele. The sequence traces shown in (a) and (b) were obtained from cloned PCR products and therefore only show one allele. (c) Photograph of the eyes of a severely affected member of the family in 1b (indicated by *) demonstrating leukocoria (a white mass behind the pupil due to a total retinal detachment).

The third deletion was identified in an Australian patient—another 1-bp deletion in exon 2, c957delG. This mutation causes a frameshift after codon 318 replacing four amino acids (W319C, W320G, V321L, and I322F), before introducing a premature stop at codon 323 (W319fsX323). This mutation was found in two asymptomatic family members: the proband's father and sibling (Fig. 1a). It is possible that the 6-year-old sibling is presymptomatic, but the father had undergone a full examination using slit lamp biomicroscopy and indirect ophthalmoscopy in conjunction with mydriatics, and no retinopathy was observed. Nevertheless, because neither of these individuals was examined by fluorescein angiography, the only accurate clinical method for diagnosing asymptomatic FEVR cases, their lack of symptoms is not unexpected.

Nonsense Mutations

One nonsense mutation was detected in an Australian family in exon 2, Q505X. This change resulted from a C→T transition in the first base of the codon (c1513C→T), the mutated C being part of a highly mutable CpG dinucleotide. The effect of this mutation would be a shortened protein of 504 amino acids instead of the 537 amino acids found in the wild-type protein. This mutation segregates with the disease in this family apart from in the patriarch, who was asymptomatic (Fig. 1b). The mutation was not found in 200 normal control chromosomes.

Missense Mutations

Four putative missense mutations were identified. In exon 1 we identified G36D (c107G→A) in a single North American patient. In exon 2, we identified three changes. M157V

(c469A→G) segregates with the disease in a large American family known to be linked to *EVRI* and M105T (c314T→C) and S497F (c1490C→T) were identified in two British patients, each with a family history of FEVR. None of these changes was identified in 400 ethnically matched normal chromosomes.

Benign Variants

In addition to the above mutations, a missense change that was not disease specific was detected, P168S (c502C→T). This change appears to be rare, with the serine allele only being found once in 200 white control individuals, a frequency of less than 0.3%. A polymorphism was also identified in the 3' untranslated region (UTR), 2 bp after the STOP codon, c1616g→t. The frequency of the t allele was calculated to be 2% in whites.

Phenotypic Characteristics in Patients with *FZD4* Mutations

To assess the range of clinical features associated with mutations in *FZD4* and to identify any specific characteristics that may be unique in patients with FEVR with mutations in this gene, a combination of clinical reexamination and/or inspection of existing clinical notes was undertaken in the patients with FEVR in whom we had identified *FZD4* mutations.

The large North American family with the c1501-1502delCT mutation consisted of 39 members within three generations in which FEVR was dominantly transmitted to 12 members. All affected patients manifested ophthalmic features within the first decade of life, and most were severely visually disabled by the second decade. The youngest affected individual was noted at 20 days of age (after a full-term, uncomplicated pregnancy) to have temporal dragging of the macula and optic nerve, widespread peripheral retinal avascularity, and extraretinal neovascularization. Older (untreated) members of this family had bilateral cicatrized tractional retinal detachments with subretinal cholesterol crystals and chronic intraretinal exudates. No asymptomatic carriers of this mutation were identified; however, we examined DNA from only 11 family members and only five of these had received a clinical diagnosis as unaffected; thus, the possibility of clinically normal mutation carriers cannot be ruled out.

A highly variable phenotype was noted in the Australian family with the Q505X mutation. Four mutation-carrying individuals were identified spanning three generations, but in only two of these had "Norrie disease/severe FEVR" originally been diagnosed. The proband's maternal uncle (Fig. 1b, top, individual marked with an asterisk) had a bilateral total retinal detachment by age 6, resulting in no light perception (Fig. 1c), and the proband, who was also male, was noted to have a temporal sector of retina with deficient vascularization associated with areas of white fibrotic preretinal tissue. However, his mother was initially reported to have only high myopia and myopia-associated visual deterioration, as she showed no signs of retinopathy, and his grandfather was clinically normal.

The third family studied, harboring the M157V mutation, also showed a wide spectrum of phenotypes. This U.S. family contained 17 members spanning four generations, nine of which were affected. However, in three of these affected individuals, FEVR was diagnosed only by fluorescein angiography, and they had no clinical problems, whereas other affected individuals had a more severe range of phenotypes, including macular folds and retinal detachments.

In the Australian patient with the c957delG mutation, the disorder was diagnosed at 8 months, with a peripheral retinal fold in the left eye associated with retinal traction. The left eye showed areas of preretinal fibrosis, and both eyes showed characteristic deficient vascularization of the peripheral retina.

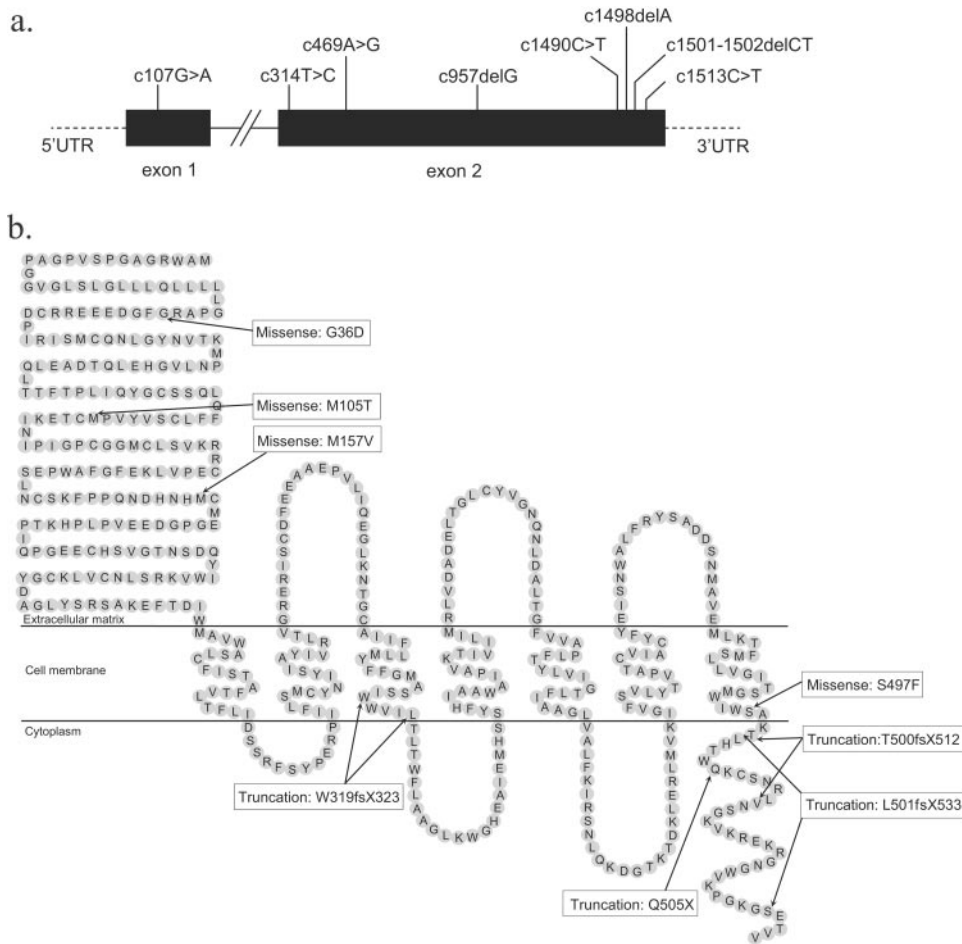


FIGURE 2. (a) Schematic diagram of the *FZD4* gene and (b) the Frizzled-4 protein showing the types and locations of the mutations. The locations of the transmembrane domains were derived from entry Q9ULV1 in GenBank.

Aged 4 at the time of this report, the patient had poor visual acuity (OD -6/48 and OS -2/60). On molecular testing, his brother and father were found to be carriers of this mutation, but both are emmetropic and appear clinically normal (Fig. 1a).

The British patient with the M105T mutation had a known family history of FEVR. Her sister was severely affected, resulting in partial blindness and her niece (the sister's child) was blind from a very young age, due to bilateral retinal detachment. The patient presented to the clinic as an emergency case

with a left macula-off rhegmatogenous retinal detachment which was successfully repaired. Further examination identified inadequate vascularization of the peripheral temporal retina. She currently has visual acuity of OD -6/9 and OS -6/12-1.

The British patient with the c1498delA mutation was noted to have very little vision (hand movements only) in the left eye. This eye was microphthalmic with evidence of posterior lenticonus with lens opacity, a degenerative vitreous with a band

TABLE 2. Summary of Mutations Found in *FZD4* to Date

Exon Amplifiers	cDNA Change	Protein Change	Family/Single	Family History	Freq.	Reference
1	c107G→A	G36D	Single	No	1	Present study
2(a)	c313A→G	M105V	Family	Yes	1	28
2(a)	c314T→C	M105T	Single	Yes	1	Present study
2(a)	c469A→G	M157V	Family	Yes	1	Present study
2(c)	c957delG	W319fsX323	Family	Yes	1	Present study
2(c)	c957G→A	W319X	Single	No	1	28
2(d)	c1250G→A	R417Q	Family	Yes	2	28
2(e)	c1463G→A	G488D	Family	Yes	1	28
2(e)	c1490C→T	S497F	Single	No	1	Present study
2(e)	c1479-1484delGTGGAT	M493-W494del	Family	Yes	1	23
2(e)	c1498delA	T500fsX512	Single	Yes	1	Present study
2(e)	c1501-1502delCT	L501fsX533	Family	Yes	2	Present study and 23
2(e)	c1513C→T	Q505X	Family	Yes	1	Present study

Amino acid and nucleotide numbering follows the cDNA sequence (Accession no. NM_012193), with nucleotide position 1 assigned to the first nucleotide of the ATG initiation codon in exon 1. Family indicates that the mutation segregated in other family members. Single indicates that the mutation was found in a single patient for whom no further family DNAs were available. Freq., frequency.

extending from a small pale optic disc head with extensive chorioretinal mottling and attenuated retinal vasculature. The right eye was highly myopic with some cortical lens opacities. The vitreous was degenerative with a small myopic optic disc and diffuse nonspecific pigmentary changes in the retina. The patient's father was mildly affected but able to drive. He had undergone childhood surgery for strabismus and had an amblyopic left eye. Detailed examination of his left eye was difficult because of lens opacities, but he had some linear circumferential vitreous opacities with areas of tractional retinal detachment and subretinal exudation.

The British patient with the S497F mutation was noted to have the classic features of FEVR. She had disc-dragging in both eyes and localized retinal elevation temporally in both eyes with hemorrhage and gliosis. Exudation was present temporal to the left fovea. Her mother had dragged discs, and temporal sectors of pigment change typical of FEVR.

Unfortunately, we were unable to obtain detailed clinical notes for the patient with the G36D mutation, and so the severity of this patient's phenotype is unknown. Notes state that the patient had no family history of FEVR but only the mother's DNA was tested for the mutation.

DISCUSSION

We screened 40 unrelated patients with FEVR to determine the types and frequencies of mutations in *FZD4* and identified eight different disease-causing mutations. Four of these mutations are predicted to result in truncation of the mRNA, with the proportion of the mRNA deleted ranging from 40% for the c957delG deletion to 1% for the c1501-1502delCT deletion. Although the most likely outcome for mutations resulting in premature termination codons (PTC) is haploinsufficiency caused by nonsense-mediated mRNA decay, this is not always the case if the PTC is not followed by a downstream intron.³¹ All the truncating mutations identified in *FZD4* are located within the large last exon, and it is therefore possible that these mutations result in the production of a truncated protein. Further experiments are needed to determine whether this is indeed the case.

The remaining mutations identified were substitutions altering amino acids. It is more difficult to determine with absolute certainty whether these mutations are disease causing, although the identification of a second point mutation affecting amino acid 105 in an FEVR family adds strength to the proposed pathogenic nature of the M105T mutation.²⁸ The definition of a polymorphism is any genetic variant where the frequency of the rarer allele is greater than 1%. We excluded each of these changes in 400 normal ethnically matched control chromosomes, indicating with over 95% confidence that the frequency of these changes is less than 1%, providing supportive data that these are disease-causing mutations, rather than rare polymorphisms.³² Furthermore, we checked each of the amino acids changed for conservation within Frizzled-4 proteins from other species and also within other members of the human Frizzled protein family (Fig. 3). All the missense changes are nonconservative substitutions, with the exception of M157V, but the valine residue at this position was not seen in any of the homologues (Fig. 2). All the residues changed are well conserved among the orthologues with the exception of zebrafish Frizzled-4, which has different amino acids for three of the four mutations. The conservation of the changed amino acids within the paralogues is less convincing, with only the serine at codon 497 being highly conserved (Fig. 3).

Frizzled-4 contains a signal peptide in its N terminus, directing it to the plasma membrane, which is cleaved off after its translocation through the endoplasmic reticulum membrane.

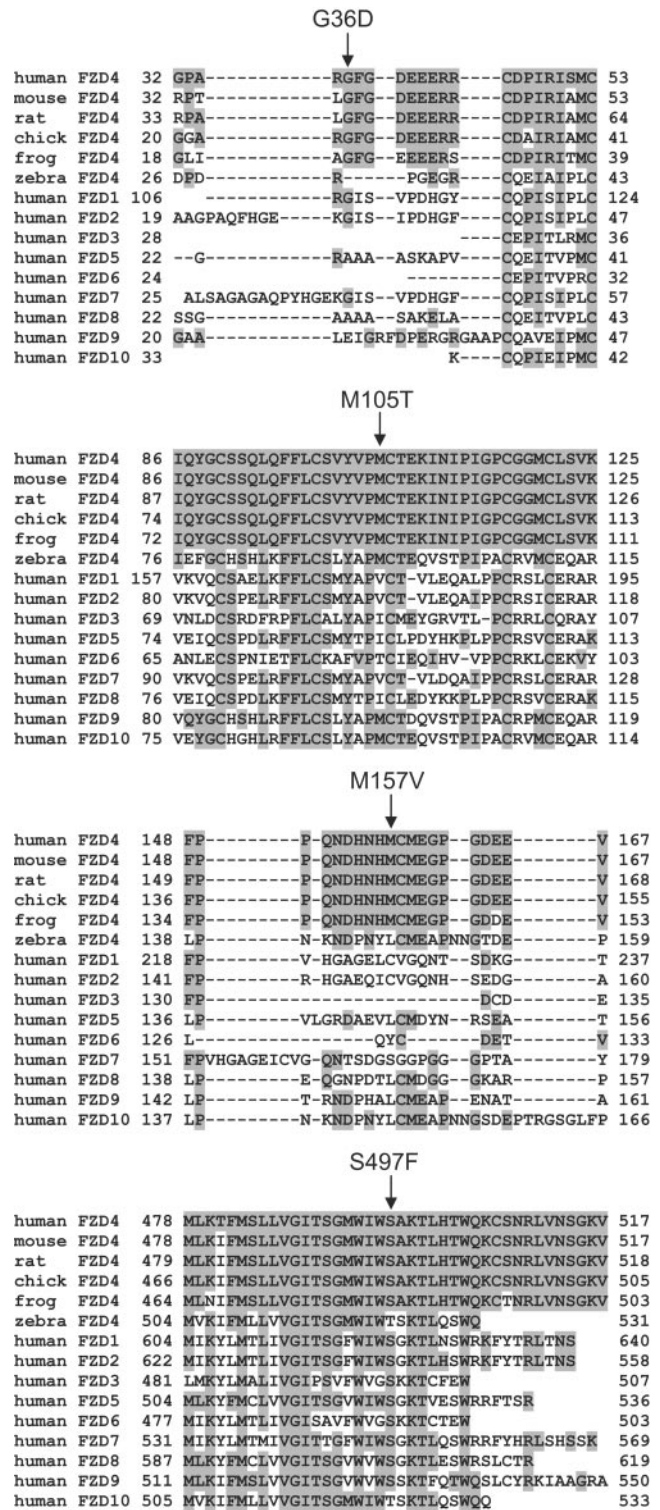


FIGURE 3. Protein sequence alignment of human Frizzled-4 with homologues from human and other species: mouse, rat, chick, frog, and zebrafish Frizzled-4 proteins and the human Frizzled family of proteins. Only 20 amino acid residues surrounding each mutation are shown. Conserved amino acid residues are highlighted. The positions of the missense mutations G36D, M105T, M157V, and S497F are indicated.

The location of this cleavage site is predicted to be between amino acids 36 and 37 by two signal peptide prediction programs, PSORT (Predication of Protein Sorting Signals and Localization Sites in Amino Acid Sequences; <http://psort>).

nibb.ac.jp/ coded by Kenta Nakai, Human Genome Center, Institute for Medical Science, University of Tokyo, Japan) and SignalP (<http://www.cbs.dtu.dk/services/SignalP-2.0/>) provided in the public domain by the Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark). This is the precise location of the G36D mutation, and it is likely that this change affects the cleavage of the mature protein.

Two of the missense changes (M105T and M157V) are located within the extracellular cysteine-rich domain (CRD) of Frizzled-4. All Frizzled receptors have an extracellular CRD with 10 invariant cysteines, and this is reported to be the binding site for the Wnt proteins.³³ Although the sequence similarity of the CRD differs significantly between Frizzled receptors (ranging from 30% to 50% overall identity; Fig. 3),³⁴ they adopt the same three-dimensional crystal structure because of the conserved cysteines.³⁵ The remaining variability between the 10 human Frizzled receptor CRDs is thought to underlie their different Wnt binding specificities. This may explain why the mutated residues in the CRD are highly conserved among orthologues but less so between the other human Frizzled receptors (Fig. 3).

More detailed studies have been performed to determine which regions within the CRD are critical for Wnt binding.³⁵ In vitro binding assays, which exploit the high binding affinity of *Xenopus* Wnt8 to the CRDs of *Drosophila* Frizzled-2 and murine Frizzled-8, allowed Dann et al.³⁵ to mutate different regions of the CRDs and to determine what effect the mutations had on Wnt binding. Although none of the mutations created were the same as those found in this study, Dann et al. created a map highlighting the specific segments of the CRD surface important for Wnt binding. Given the homologous structure and function of the Frizzled CRD, it is assumed that all Frizzled CRDs bind Wnt proteins in a conserved fashion.³⁵ We therefore used the Cn3D 4.1 software available from the National Center for Biotechnology Information (NCBI; Bethesda, MD) to align the protein sequence of the Frizzled-4 CRD with the crystal structure of mouse Frizzled-8 CRD (1IJY-A) (<http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml>). This analysis showed that both the M105T and M157V mutations are likely to be located in the region of the CRD surface most strongly implicated in Wnt binding by Dann et al.

Furthermore, while determining the crystal structure for the CRD of Frizzled proteins, Dann et al.³⁵ found evidence that mouse Frizzled-8 and secreted Frizzled-related protein 3 form homodimers, and they mapped the dimer interface surface. Although this initial observation was only tentative, a further study has confirmed that *Xenopus* Frizzled-3 forms homodimers when overexpressed in *Xenopus* embryos.³⁶ However, dimerization is not a characteristic of all Frizzled receptors as the same study showed that *Xenopus* Frizzled-7 remains monomeric.³⁶ Whether Frizzled-4 undergoes dimerization has not been investigated and remains undetermined, but the M157V mutation is located within Frizzled-4's putative dimer interface surface raising the possibility that it may interfere with Wnt binding by hindering CRD dimerization.³⁵

The S497F mutation occurs at a highly conserved residue located at the beginning of the C-terminal cytoplasmic tail, directly adjacent to the conserved Lys-Thr-X-X-Trp motif which is required for the activation of the Wnt/ β -catenin pathway and for membrane relocalization and phosphorylation of Dishevelled.³⁴ It is therefore likely that this residue is of functional importance. Although the pathogenic effects of all these missense mutations have not been proven, these data suggest that they are likely to be pathogenic.

There are now a total of 13 different mutations reported to cause FEVR (Table 2), including four deletions, seven missense and two nonsense mutations. The location of these mutations

suggests that they result in loss of function of Frizzled-4. Seven of these mutations are predicted to alter the C-terminal cytoplasmic tail of Frizzled-4 which is crucial in all three of the signaling pathways that Frizzled receptors use to transduce their signal; the canonical Wnt/ β -catenin pathway,³⁷ the Wnt/ Ca^{2+} pathway³⁸ and the planar cell polarity (JUN) pathway³⁹ (Fig. 2b). Similarly, four of the missense mutations are in functional sites in the extracellular domain, three located within the CRD and one located at the signal peptide cleavage site.

Only one mutation was found in the N terminus of Frizzled-4 (G36D). Apart from its effect on Frizzled-4, this mutation could also alter a putative second transcript reported to be encoded by *FZD4* designated *SFZD4*.⁴⁰ This splice variant retains the intron between exon 1 and 2 of *FZD4* and is reported to encode a soluble polypeptide of only 125 amino acid residues due to the presence of a stop codon within the retained intron. The first 98 amino acids are identical with Frizzled-4 and encode the first half of the CRD but the last 27 residues are unique.⁴⁰ A family of five secreted Frizzled-related proteins (SFRP1-SFRP5) have been described; they contain the full CRD and act as Wnt antagonists but are encoded by distinct genes and are not splice variants of Frizzled receptor genes.⁴¹ Sagara et al.⁴⁰ performed a number of *Xenopus* embryo injections with *SFZD4* and suggested that it functions as a positive regulator of the Wnt signaling pathway. However, this variant has only been described in a single study and although the authors checked that the transcript was not due to genomic contamination of their cDNA, they did not rule out the possibility of contaminating heterogeneous nuclear RNA. Furthermore, no similar splice variants have been identified for the remaining nine Frizzled proteins. As the expression of *SFZD4* is not supported by other experimental evidence, its existence is still tentative and further studies are required.

We identified *FZD4* mutations in 20% of our index patients (8/40). Similarly, the original study by Robitaille et al.²³ identified two mutations in five autosomal dominant FEVR families and the study by Kondo et al.²⁸ identified five mutations in a panel of 24 probands screened. Detection rates of 20% to 40% were unexpected as *EVRI* was always thought to be the major autosomal dominant FEVR locus, based on the results of a series of linkage studies that report *EVRI* linked pedigrees.^{11-14,22} It is possible to speculate that this low detection figure may be due to a proportion of our patients' harboring mutations within the ND gene or in an unidentified recessive FEVR gene, especially in those patients without a family history. However, we found only *FZD4* mutations in 6 (35%) of our 17 families, each of which had a clear pattern of autosomal dominant inheritance. Furthermore, of the 21 single patients in whom we failed to find an *FZD4* mutation, 7 were females and 10 of the males had already tested negative for ND gene mutations, leaving only 4 males for whom a diagnosis of X-linked FEVR could not be ruled out. We anticipate that a few mutations may have been missed by the mutation detection method used in this study. Similarly, large rearrangements or deletions within the gene have not been examined and the promoters, introns, and the 5'- and 3'UTRs have also not been screened. However, it is more likely that the low mutation detection rate obtained in all three studies is due to autosomal dominant FEVR's being more heterogeneous than previously thought.

Confirmation of this theory has recently been provided by the discovery of a new autosomal dominant locus adjacent to *FZD4*.⁴² By performing high-resolution genotyping in a large Asian family originally linked to the *EVRI* locus by Price et al.,²² we were able to genetically exclude *FZD4* as the mutated gene in this family and consequently identified a third autosomal dominant FEVR locus, designated *EV4*.⁴² This new locus

is positioned only 10-cM centromeric to *FZD4* and spans a 15-cM interval flanked by the markers D11S1368 and D11S937.⁴² The occurrence of two FEVR loci within such close proximity provides a likely explanation as to why Kondo et al.²⁸ could find only *FZD4* mutations in 3 of their 11 families reportedly showing linkage to *EVR1*. Similarly, it is now possible to speculate that the reason *EVR1* was always reported as the major FEVR locus was because there were in fact two neighboring loci in this chromosomal region. However, the original linkage studies reporting *EVR1* linked families were performed before the identification of *EVR3*, and, as a result, many laboratories performed their analysis on the assumption that autosomal dominant FEVR was homogeneous. By reanalyzing the available data from these studies it is now evident that no one major FEVR locus exists.⁴² In light of this reappraisal, the identification of *EVR3*¹⁶ and *EVR4*⁴² and evidence for further locus heterogeneity,⁴³ the low frequency of *FZD4* mutations identified in this and other studies^{23,28} suggests that the remaining patients most likely harbor changes in other, as yet unidentified, FEVR genes.

The clinical features present in most of our *FZD4* patients with FEVR fit well with classic descriptions of FEVR¹ and with the phenotypes seen in families linking to other FEVR loci.^{16,42-43} The one exception to this is a patient with the c1498delA mutation. Along with features typical of FEVR, this individual also has characteristics suggestive of additional disease; however, these features were not seen in a second family member who has features consistent with an FEVR diagnosis. As a consequence, we were not able to identify any distinguishing features specific to *FZD4*-FEVR, nor were we able to see any genotype-phenotype correlation between the different mutations observed, although this was not unexpected as widely varying phenotypes are often seen within the same family. It is interesting to speculate why these patients with FEVR have defects only in the development of the retinal vasculature when *FZD4* is widely expressed throughout the body.²⁴ This may indicate redundancy of Frizzled-4 function in other tissues, most likely as a result of the other nine human Frizzled genes compensating for the loss. However, it may also be that the development of the retinal vasculature is the only process limited by the amount of Frizzled-4 protein available and as a result is the only tissue affected by the Frizzled-4 haploinsufficiency observed in FEVR.

This study offers insights into the type, distribution, and frequency of *FZD4* mutations that cause FEVR. Continued identification of mutations will allow accurate diagnosis of asymptomatic individuals, avoiding in some cases the need for fluorescein angiography (which is not without complications) and will permit better genetic counseling of patients. This study also provides evidence that autosomal dominant FEVR is more heterogeneous than previously thought and strongly suggests that further loci remain to be identified.

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