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Identification of novel FZD4 mutations in Indian patients with familial exudative vitreoretinopathy

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Purpose: To identify novel mutations in *FZD4* gene that cause familial exudative vitreoretinopathy (FEVR) in Indian patients.
Methods: The study was conducted on 75 subjects from 53 Indian families. These families were clinically diagnosed to have FEVR by fundus examination and fluorescein angiography. The candidate gene FZD4 was amplified from genomic DNA and PCR products were screened for mutations by single strand conformational polymorphism (PCR-SSCP), TA-cloning followed by bi-directional sequencing.
Results: For the *FZD4* exonic region, three mutations were identified, including two novel sequence variations (C204R, F82fsX135) and one reported (P33S) mutation. These sequence changes were not observed in 100 normal controls and clinically unaffected family members analyzed.
Conclusions: Mutations in *FZD4* were observed in 5.6% of the clinically diagnosed FEVR, in the studied Indian population. The identified genetic variations of *FZD4* could play a vital role in pathogenesis and provide greater insight in to the genotype/phenotypic functions of *FZD4* gene.

Familial exudative vitreoretinopathy (FEVR) is a well-characterized inherited disorder with variable retinal findings, including macular ectopia, retinal exudates, neovascularization, peripheral fibro-vascular mass, and falciform folds. The primary pathology in FEVR patients is an immature arrest of retinal vasculogenesis, caused by improper vascularization of peripheral retina. The secondary complications in FEVR patients are mainly due to retinal ischemia [1]. Exudation leaky abnormal vessels can reduce the visual acuity and lead to total or partial retinal detachment whereas early onset of the disease can be diagnosed only by molecular genetic analysis and fluorescein angiography [2]. The most common mode of inheritance is autosomal dominant and the chromosomal loci associated with FEVR map to 11q13-23 (*EVR1*; OMIM 133780), Xp11.4 (*EVR2*; OMIM 305390) and 11p13-12 (*EVR3*; OMIM 605750), *EVR4* was recently identified on chromosome 11q13 in a family originally reported as linking to the *EVR1* locus [3]. Mutations in the frizzled-4 gene (*FZD4*) have recently been associated with autosomal dominant FEVR in families linking to the *EVR1* locus on the long arm of chromosome 11 [2]. The *FZD4* mRNA consists of two exons and 7391 bp long codes for 537 amino acid protein frizzled-4 [4]. The binding of frizzled 4 protein, a member of seven-pass transmembrane receptors, to the wnt protein in the Wnt signaling pathway plays a major role in differentiation and cell proliferation [5].

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Genetic analysis of FEVR patients with different ethnic backgrounds has revealed several mutations in the *FZD4* gene [6]. However, there is no report of mutational analysis in Indian FEVR patients. Here we report two novel and one reported *FZD4* mutations in Indian FEVR patients with a predominantly adult-onset presentation.

METHODS

Clinical evaluation: In total, blood samples from seventy-five affected individuals in 53 FEVR families (their available unaffected family members) and 100 age-matched normal controls were collected. Clinical evaluation included refraction, visual acuity, intraocular pressure, slit lamp biomicroscopy, and in eyes with hazy media, ultrasonographic examinations. Fluorescein angiography was carried out on available affected individuals. A detailed history of prematurity/perinatal complications was elicited to rule out retinopathy of prematurity: no patient had any remarkable perinatal history. Presence of bilateral peripheral avascular zones on fluorescein angiography was the sine qua non for diagnosis of FEVR: observed in all the patients in this series. The other clinical features suggestive of FEVR observed in this series were: temporal dragging of macula (11 eyes), straightening, acute-angle branching and brush-like peripheral anastomoses (83 eyes) and active/fibrosed peripheral new vessels (7 eyes). All patients were examined at the Retina Clinic, Aravind Eye Hospital, Madurai, India. Clinical characteristics and surgical management of some of these patients have been reported previously [7]. The study followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all subjects involved in the study.

Mutation screening and sequence analysis: Genomic DNA was isolated by salt precipitation method from peripheral blood of the study subjects. For mutational analysis, the entire coding exons of *FZD4* and their flanking splice junctions were amplified by PCR using the primer reported elsewhere [6]. SSCP analysis was carried out in 10% non-denaturing polyacrylamide gel. PCR products that showed altered mobility on SSCP were gel-eluted and purified using Perfectprep gel clean up kit (Eppendorf, Hamburg, Germany). They were sequenced bidirectionally on an Applied Biosystems (ABI) model 3730 automated sequencer at Microsynth (Balgach, Switzerland). The direct sequencing of the PCR products gives superimposed peaks due to the presence of both normal and mutant alleles (heterozygous). In this case, the PCR products were cloned into the pGEM-T vector (Promega, Madison, WI) and sequenced bi-directionally using universal T7, SP6 primers. Nucleotide sequences were analyzed using pairwise BLAST [8] to examine if there were any changes from the normal sequence available in the database.

RESULTS

In the present study, we analyzed 75 samples with a clinical diagnosis of FEVR for mutations in the *FZD4* gene. Three mutations were found in three patients, which two of these were missense with familial (Pro33Ser, Cys204Arg). A novel compound frame shift mutation (F82fsX135) was identified in one patient. The clinical features of the affected individuals are summarized in Table 1.

Family 1: In this family, two-affected individuals had the typical FEVR phenotype (Figure 1A,B) and mutation screening revealed a reported heterozygous missense mutation, c.97C>T was identified in IV: 3 and III: 6. The fundus of the patient IV: 3 showed peripheral lattice degeneration, atrophic holes were seen in both eyes with straightening of vessels. The right eye had macular ectopia and fluorescein angiogram shows the bilateral peripheral avascular zones in both eyes. His father (III: 6) also had classical features of FEVR. This mutation (CCG to TCG codon change) in exon 1, that led to the replacement of proline at position 33 by Serine (Figure 2B,C). The wild-type proline residue was evolutionary conserved among Human, Rat and Mouse, but not other species such as Chick and Xenla (Figure 3). In this family, this mutation was cosegregated with the disease phenotype, and was not observed in any of the unaffected family members or 100 normal controls.

Family 2: The clinical features of typical FEVR were associated with affected individuals of this family. The right eye of the patient (III: 8) showed a large temporal peripheral

Table 1. FEVR patients: *FZD4* mutations and associated clinical characteristics

Family	Affected Individuals	Family history	Age (yrs)/sex	Amino acid change	BCVA		PAZ	Macular ectopia	Retinal holes	Retinal detachment	NVE/ FPE	VH
					RE	LE						
1	IV:3	+	18/M	P33S	6/6	6/6	BE	RE	BE	-	-	-
	III:6	+	46/M	P33S	6/6	6/6	BE	-	LE	-	-	-
2	III:8	+	13/M	C204R	6/6	1/60	BE	LE	BE	LE	BE	LE
	III:7	+	22/M	C204R	6/6	PL	RE	-	LE	LE	-	-
	III:5	+	28/M	C204R	6/6	6/6	BE	-	-	-	-	-
	II:5	+	55/M	C204R	6/6	PL	RE	-	LE	LE	-	-
	III:12	+	34/M	C204R	6/6	6/6	BE	-	BE	-	-	-
3	II:1	-	53/M	F82fsX135	6/6	6/12	BE	BE	-	-	LE	-

This table summarizes the clinical data and phenotypes of subjects with *FZD4* mutations. No patient had retinal exudates or falciform folds. Right eye (RE), left eye (LE) and Both eye (BE) are abbreviated in the table. BCVA refers to best-corrected visual acuity, PAZ denotes peripheral avascular zone. The terms of NVE and FPE indicate and peripheral new vessels and fibrous proliferations, respectively, VH designates vitreous hemorrhage. In the best-corrected visual acuity column, PL refers to perception of light. The symbols + and - represent present and absent, respectively.

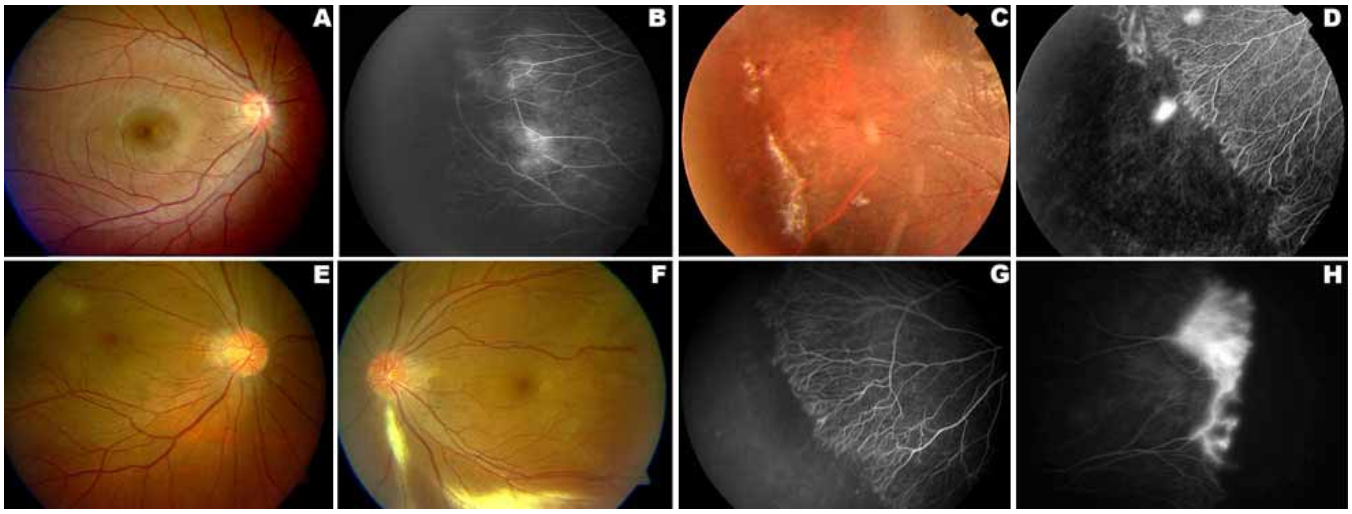

Figure 1. Fundus photography and fluorescein angiography of the FEVR patients with *FZD4* gene mutations. **A**,**B**: The fundus picture of patient IV: 3 from family one, which shows macular ectopia of the right eye; the angiogram demonstrates the peripheral avascular zone. **C**,**D**: In the second family, ophthalmic examination of a patient (III: 7) shows the peripheral avascular zone with fibrous proliferation in the right eye; angiogram reveals neovascularization with peripheral avascular zone. **E**-**H**: Ophthalmoscopic appearance of the patient (II: 1) from third family, demonstrates macular ectopia in both eyes with medullated nerve fibers in the left eye. The angiogram shows the peripheral avascular zones in both eyes with leaking fibrovascular proliferation in the left eye.

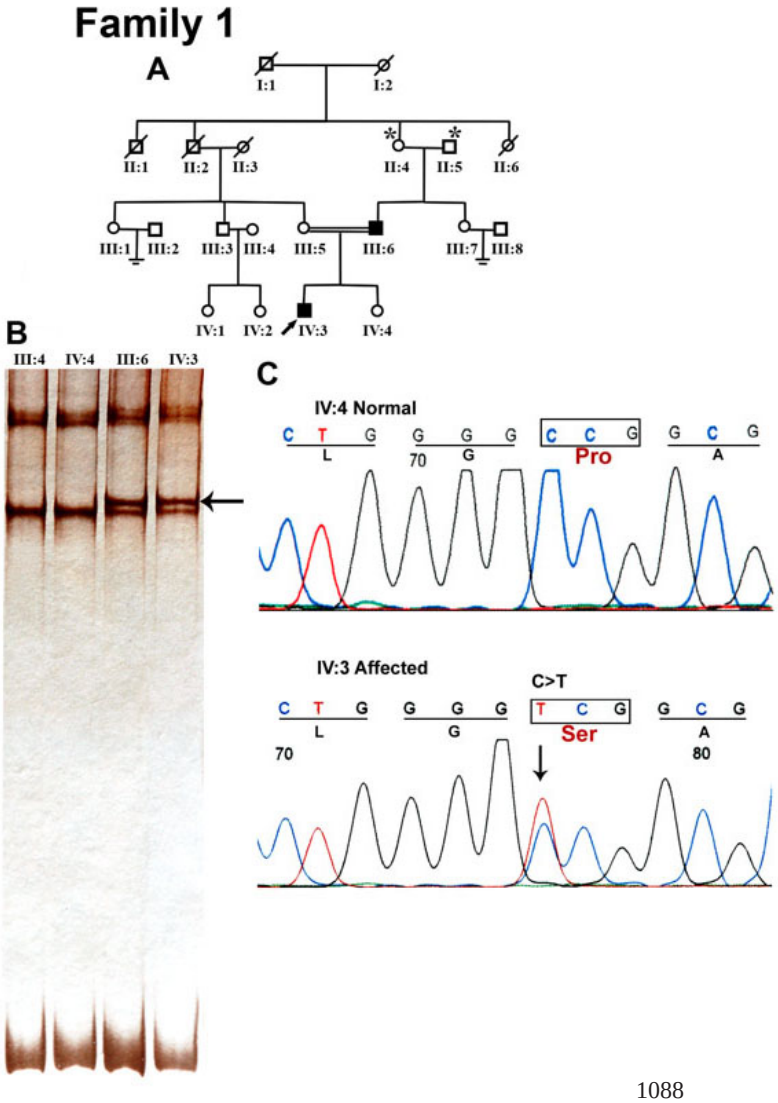

Figure 2. Novel *FZD4* mutation in family 1. **A**: Pedigree of the study family. The asterisk denotes the individuals whose phenotype-genotype is not known. **B**: Silver stained SSCP gel for exon 1 shows the mobility shift of the PCR products (arrow). **C**: Comparison of DNA sequences of the affected individual IV: 3 (top) to a normal IV: 4 (bottom). The patient IV: 3 DNA revealed a heterozygous C-to-T transition (boxed) in exon 1 of *FZD4*, resulting in a proline to Serine change (P33S).

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                                                          P33
                                                          *
Q9ULV1|FZD4_HUMAN   MAWRGAGPSVPGAPGGVGLSLGLLLQLLLLLG-PARGFGDEEERRCDPIRISMCQNLGYN
Q9QZH0|FZD4_RAT     MAWQGTGPSVRGMPGGVRLRLGLLLLQLLLLQRPALGFGDEEERRCDPIRIAMCQNLGYN
Q61088|FZD4_MOUSE   MAWPGTGPSSRGAPGGVGLRLGLLLQFLLLLR-PTLGFGDEEERRCDPIRIAMCQNLGYN
Q9IA05|FZD4_CHICK   ------------MERRGGGGRMLALLLAGLLGGARGFGDEEERRCDAIRIAMCQNLGYN
Q9PT62|FZD4_XENLA   ---------------MGARSLTLLYLLCCLVVGLIAGFGEEEERSCDPIRITMCQNLGYN
                                                  C204
                                                  *
Q9ULV1|FZD4_HUMAN   ECHSVGTNSDQYIWVKRSLNCVLKCGYDAGLYSRSAKEFTDIWMAVWASLCFISTAFTVL
Q9QZH0|FZD4_RAT     ECHSVGTNSDQYIWVKRSLNCVLKCGYDAGLYSRSAKEFTDIWMAVWASLCFISTTFTVL
Q61088|FZD4_MOUSE   ECHSVGSNSDQYIWVKRSLNCVLKCGYDAGLYSRSAKEFTDIWMAVWASLCFISTTFTVL
Q9IA05|FZD4_CHICK   ECHSMGSNSDQYIWVKRNLDCVLKCGYDAGLYSRSAKEFTDIWMAVWASLCFISTAFTVL
Q9PT62|FZD4_XENLA   DCNSFGPNSDQYTWVKRSMNCVLKCGYDSGLYNRLSKEFTDIWMAVWASLCFISTAFTVL
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Figure 3. Conservation analysis of human *FZD4* protein with other species. Amino acid sequence alignment of the human Frizzled-4 protein (amino acids from 1-59, 180-239) with other species. The Pro 33 and Cys 204 are shown in red.

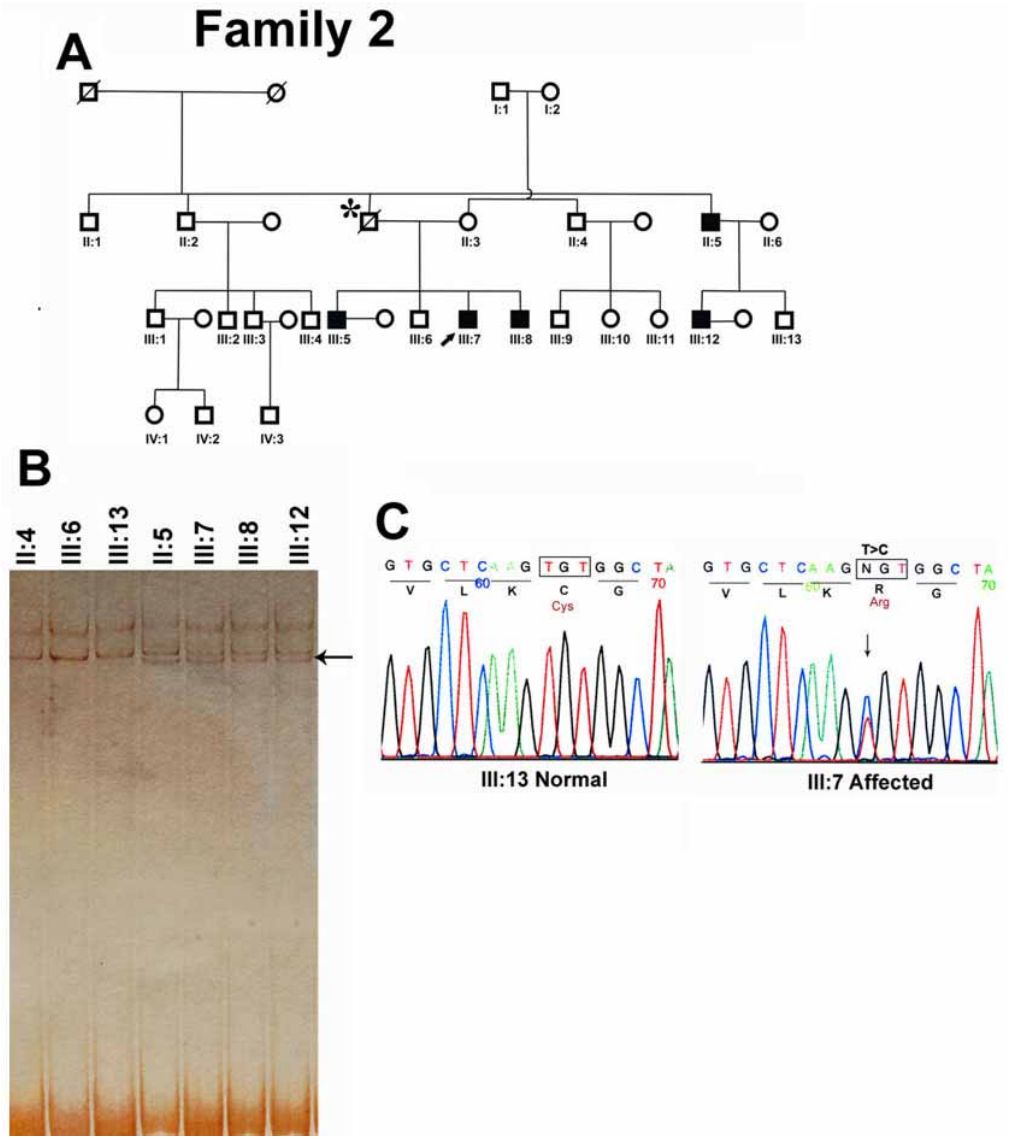

Figure 4. Mutation analysis of *FZD4* gene in family 2. **A**: Pedigree of the FEVR family. The phenotype-genotype is not known for the individual marked with asterisk. **B**: The exon 2 PCR product of *FZD4* showed an extra band (arrow) on the 10% polyacrylamide gel. **C**: Sequencing chromatogram of the normal III: 13(left) and affected III: 7(right) DNA revealed the T>C heterozygous change marked by arrowhead that replaces aminoacid Cysteine by Arginine. (C204R).

avascular zone with lattices containing multiple atrophic holes. Straightening of the vessels was also noted with excessive terminal branching and telangiectasia (Figure 1C,D). In the left eye, a large long standing tractional retinal detachment was observed. He underwent laser barrage photocoagulation in the right eye; no intervention was done for the left eye. His brother (III: 7) had a phthisical left eye. His right eye had peripheral avascular zone with straightening of vessels. Fundus examination of patient III: 5 revealed a temporal avascular zone with straightening of vessels in both eyes. Affected individuals II: 5, III: 12 also demonstrated similar features of FEVR. SSCP and bi-directional sequencing analysis of patient (III: 7) showed a heterozygous c.DNA.916 T>C mutation, TGT (Cys 204) to CGT (Arg 204) in exon 2 (Figure 4B,C). In this four-generation family, mutation was co-segregated all affected individuals with disease phenotype. The mutation was not present in 100 normal controls and the unaffected family members. Amino acid conservation analysis revealed that the wild type Cysteine 204 was highly conserved among the FZD4 orthologs (Figure 3).

Family 3: In patient II: 1, the plasmid based clones were sequenced, revealing deletion with insertion of bases at position 244 in codon 82 (c.244_251del8ins27), which resulted in a novel frame shift mutation in *FZD4* gene (Figure 5B,C). Both eyes showed macular ectopia, straightening of vessels with increased terminal branching and angiographically evident peripheral avascular zone. The left eye additionally had fibrovascular fronds in the temporal periphery, with medullated nerve fibers in the posterior pole being a coincidental feature. The phenotypic expression of FEVR is shown in Figure 1E-H. We were unable to examine the siblings (III: 1, III: 2, and III: 3). This frameshift resulting in the substitution of

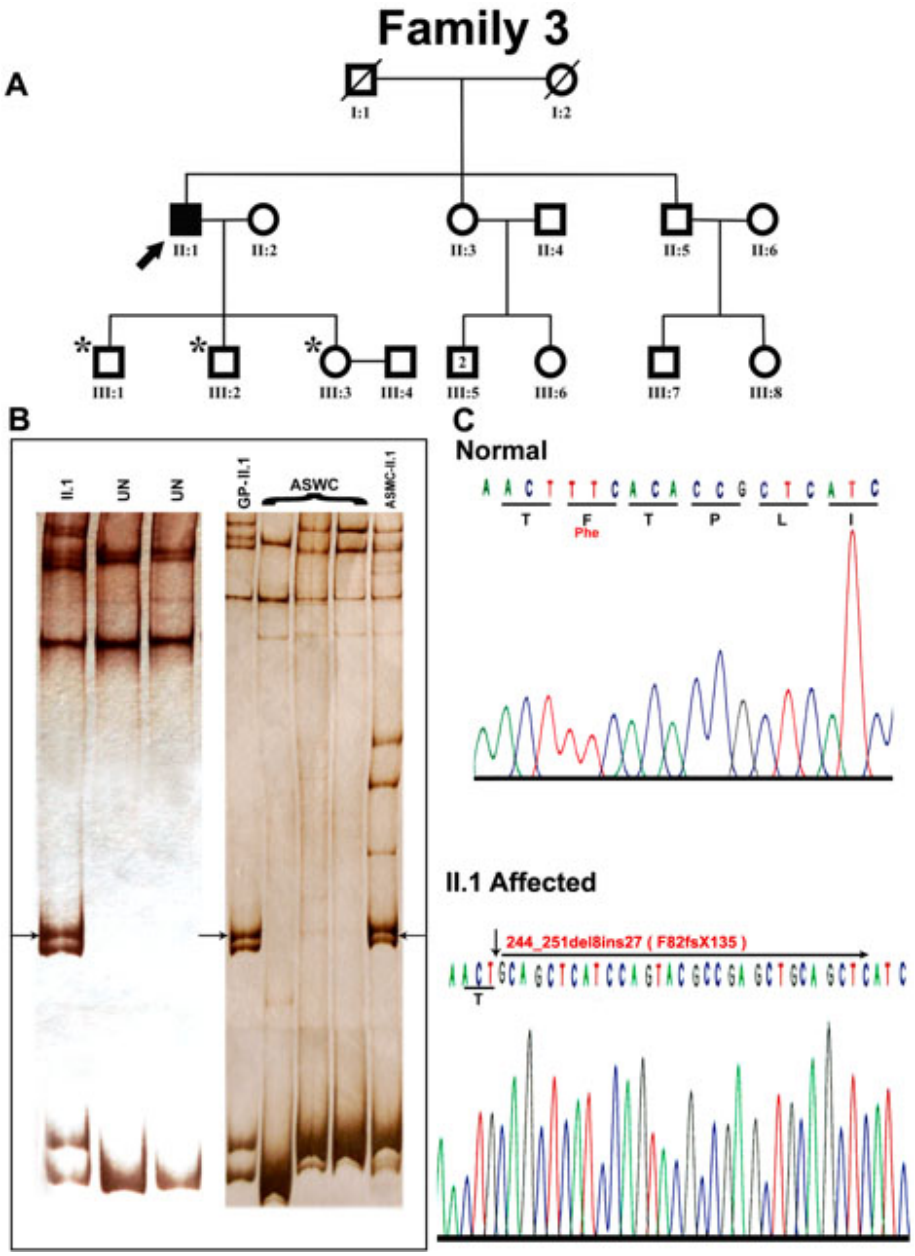

Figure 5. Novel *FZD4* frameshift mutation in family 3. **A**: Pedigree of family. The asterisk denotes the individuals whose phenotype-genotype is not known. **B**: SSCP gel shows the aberrant band shift (arrow) of patient (II: 1) exon 1 PCR product, unrelated normal controls indicate by UN. GP-II: 1 denotes patient II: 1 genomic DNA used in PCR. ASMC II: 1 refers to allele specific mutant PCR clone shown by arrow, three lanes under ASWC designate allele specific wild type PCR clones were identified in 10% non-denaturing polyacrylamide gel **C**: Partial DNA sequence of *FZD4* exon one from a normal control (top) and from the mutant allele subcloned into the pGEM-T vector (bottom), demonstrated that the patient (II: 1) carried a (F82fsX135) frame shift change at nucleotide c.244_251del8ins27, which generated a premature stop codon and a truncated protein.

53 amino acids after codon 81 (NH_2- AAH PVR RAA AHP VRL LQP AAV LPL FCL CAN VHR EDQ HPH WPM RRH VSF SQE TLX-COOH) and a premature stop in codon 135 (F82fsX135) is likely to be non-functional properties of *FZD4*.

In addition, we found a non-pathogenic reported polymorphism at position c.1616g>t in the 3' untranslated region (UTR) of Indian FEVR patients and controls with 2.6% frequency.

DISCUSSION

Mutations of the *FZD4* gene are known to cause many cases of inherited as well as sporadic FEVR in various ethnic backgrounds (summarized in Table 2). It is a cell surface receptor of Wnt signaling, which activate the Wnt canonical pathway involving β-catenin stabilization and TCF-dependent gene transcription appears to be activated in neural retina, ciliary margin and other ocular structures [9]. The *FZD4* is clearly vital for the normal development of the intra retinal vasculature in mice and human beings. FEVR was found to be associated with heterozygosity for apparent loss-of-function [10]. We screened 53 unrelated families with FEVR for mutations in the *FZD4* gene. Two patients with familial FEVR and one with sporadic FEVR had a total of three mutations in the *FZD4* gene, two of which were missense mutations and the other a compound (insertion with deletion) frameshift mutation.

In the first family, the patient IV: 3 and his father III: 6 had a c.96C>T mutation associated with the clinical features of peripheral avascularity and retinal atrophic holes. However, proline 33 amino acid residue was located in the N-terminal region of the frizzled 4-signal peptide, which directs the protein into plasma membrane predicted by SignalP 3.0. Previously P33S mutation was identified in the retinopathy phenotype Table 2. This missense change Pro33Ser may alter the translocation of frizzled 4 protein and affects the retinal vascularization that leads to FEVR. Another novel missense mutation was observed in the second family, all affected individuals had a c.DNA.916 T>C change (C204R). The proband and his affected family members showed the classical phenotypic expression of FEVR. In addition, the pathogenic nature of this mutation also comes from cysteine, which is conserved at amino acid position 204 in different *FZD4* orthologs. This is consistent with previously identified mutation studies in a Canadian family [11]. Prior studies suggest that the non-conservative substitution due to missense mutation occurring within the CRD region is critical for Wnt bindings and resulted in FEVR phenotype [10,12]. Our study is also consistent with the effect of mutation, which might exploit the high Wnt binding affinity and could express in the pathogenic activity. The altered Frizzled proteins may undergo dimerization due to missense mutation (C204R) located within the Frizzled putative dimer hindering [12]. Analysis of amino acid mutation stability for P33S and C204R mutations identified in this study using the Amino Acid Mutation Stability Prediction Server Mupro 1.0 (AAMSPSM) suggests a decrease in the stability of FZD4 protein structure.

Table 2. Summary of mutations in human *FZD4*

Mutation	Ethnic groups	Reference
P33S	Indian/American	Present study, [16]
G36D	North American	[6]
H69Y	Japanese	[17]
M105V	Japanese	[18]
M105T	British	[6]
M157V	American	[6]
P168S	European	[6]
C181R	Japanese	[17]
C204R	Indian	Present study
M342V	Japanese	[19]
R417Q	Japanese	[18]
G488D	Japanese	[18]
S497F	British	[6]
W319X	Japanese	[18]
Q505X	Australian	[6]
F82fsX135	Indian	Present study
W319fsX323	Australian	[6]
M493-W494del	Canadian	[11]
T500fsX512	British	[6]
L501fsX533	North American, Canadian	[6,11]

Shown are *FZD4* gene mutations that have been identified in present and other studies.

Interestingly, we found a compound frame shift mutation (F82fsX135) in FEVR patient II: 1 in family 3. The disease status is not known for the unaffected siblings of this family since we could not obtain the clinical and genetic information to reveal whether this frame shift resulted in sporadic or familial FEVR. This case suggests that genetic counseling is important, especially for asymptomatic individuals. This view is endorsed in a report of an Australian patient with FEVR (W319fsX323) and his two asymptomatic family members [6]. The frameshift mutation in patient II: 1 showed that the pathogenic effects appear because of loss of function of *FZD4*, due to premature truncation of amino acids. This leads to FEVR with the presence of peripheral avascular zone, macular ectopia in both eyes and peripheral neovascularization in the left eye. This truncating mutation (F82fsX135) occurs in the amino terminal region of the frizzled 4 protein predicted to be a haploinsufficiency of nonsense-mediated mRNA decay [13]. Therefore, *FZD4* mRNAs that contain truncating mutations would be degraded and would not be translated. Due to relatively older age of presentation and the observed disease severity was relatively mild and asymmetric, we were unable to correlate the genotype-phenotype for the mutations identified in this study. We identified the *FZD4* mutation in 3 (5.6%) of 53 individuals with clinically diagnosed FEVR, This finding demonstrated that the frequency of *FZD4* mutations associated with FEVR is low, requiring the elucidation of other candidate genes in remaining patients and their family members. Mutations in *LRP5* (OMIM 603506) have also been reported to be associated with FEVR [14] and mutations in *NDP* (OMIM 305390) have been reported to cause X-linked FEVR [15]. Identifying new mutations in *FZD4* contributes valuable information towards carrier detection for specific mutations and genetic counseling.

In summary, we add one novel missense mutation and a frameshift mutation, to the existing spectrum of *FZD4* muta-

tion observed in Indian patients with the characteristic phenotype of FEVR, and we confirm that *FZD4* is important in the maintenance of retinal vascularization.

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