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Novel mutations in *FZD4* and phenotype–genotype correlation in Chinese patients with familial exudative vitreoretinopathy

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Purpose: To identify novel mutations in the *frizzled 4* (*FZD4*) gene in patients with familial exudative vitreoretinopathy (FEVR) in southern China and to delineate the mutation-associated clinical manifestations.
Methods: Clinical data and genomic DNA were collected from 100 probands and their family members. The coding regions of *FZD4* were screened for mutations with PCR and Sanger sequencing. Cosegregation analysis was used to verify suspected variants, and clinical symptoms in the probands were analyzed.
Results: Fourteen causative heterozygous mutations in *FZD4* in 21 unrelated probands were noted, in 21.0% of the index patients (21/100). Four novel missense mutations (C45R, C45S, C53S, and C90R) and three novel deletion mutations (T326fsX356, G492fsX512, and S345_A351del) with a high possibility of pathogenicity were detected. None of these mutations were found in current online databases and 150 ethnically matched control subjects without retinopathy. The majority of the mutations in *FZD4* were identified in probands with retinal folds (15/21) and ectopic macula (5/21). No mutations in *FZD4* were found in probands with complete tractional retinal detachment in infancy or with mild asymptomatic FEVR in adulthood.
Conclusions: Seven novel mutations found in this study have broadened the spectrum of mutations in *FZD4* known to cause FEVR, providing a deeper understanding of this disease. The results show that mutations in *FZD4* are associated with the phenotypes of retinal folds or ectopic macula in FEVR but might not be associated with extreme severe bilateral FEVR during infancy, at least in southern Chinese patients.

Familial exudative vitreoretinopathy (FEVR, OMIM 133780) is a hereditary disorder that can cause severe visual impairment at a young age. The primary pathological process of FEVR involves a deficiency in the peripheral retina vascularization. By itself, this usually causes no clinical symptoms; the visual problems associated with FEVR mainly result from secondary complications due to retinal ischemia [1], such as vitreous hemorrhage, vitreoretinal traction with deformation of the posterior retina, retinal folding, and retinal detachment throughout childhood and adulthood. FEVR exhibits highly variable clinical features among patients from the same family, and there may even be asymmetric manifestation in one individual. Severely affected patients may be registered blind during their infancy, whereas mildly affected individuals may not even be aware of any symptoms, and in such cases, the disease is diagnosed only with fluorescein angiography (FFA).

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FEVR is genetically heterogeneous and is inherited in different modes. Autosomal dominant FEVR (adFEVR, OMIM 133780) is most common, and mutations in the *frizzled class receptor 4* (*FZD4*), *low-density lipoprotein receptor protein 5* (*LRP5;* Gene ID 4041, OMIM 603506), and *tetraspanin 12* (*TSPAN12;* Gene ID 23554, OMIM 613138) genes [2-4] are involved with adFEVR. In recent studies, the *zinc finger protein 408* (*ZNF408;* Gene ID 79797, OMIM 616454) and *KIF11 kinesin family member 11* (*KIF11;* Gene ID 3832, OMIM 148760) genes were suggested to be associated with adFEVR [5-7]. *LRP5* and *TSPAN12* have also been associated with autosomal recessive FEVR (arFEVR, OMIM 601813) [8,9]. Mutations in the *Norrie disease protein* (*NDP*; Gene ID 4693, OMIM 300658) gene are involved with X-linked recessive FEVR (OMIM 305390) [10]. The proteins encoded by the *FZD4*, *LRP5, TSPAN12*, and *NDP* genes are involved in the highly conserved Wnt/β-catenin and Norrin/β-catenin signaling pathways, which play an important role in eye development and angiogenesis [11-13].

The *FZD4* gene is located on chromosome 11q14.2 and encodes a seven-transmembrane protein of 537 amino acids, which acts as a coreceptor for the Wnt and Norrin ligands along with LRP5 [13]. To date, more than 50 mutations in

FZD4 have been described in FEVR (see Appendix 1). Pedigrees in previous studies exhibited high interfamilial variability, and genotype–phenotype correlations have not yet been established. To better understand the molecular characterization of mutations in *FZD4* in southern Chinese patients with FEVR, mutation screening of *FZD4* and analysis of clinical features were performed in 100 unrelated individuals and their families in the present study.

METHODS

Patients and pedigrees: Written informed consent in accordance with the guidelines of the Declaration of Helsinki was obtained from the participating individuals or their guardians before the clinical data and DNA samples were collected. All procedures were approved by the Internal Review Board of the Zhongshan Ophthalmic Center at Sun Yat-sen University. Diagnosis of FEVR was based on criteria previously described [14]. One hundred patients were recruited and had a final diagnosis of FEVR after comprehensive ophthalmic examinations, including slit-lamp, color photography, and fluorescein angiography. Seventy-six cases were confirmed with fluorescein angiography. Twenty-four patients under 2 years old were not available for FFA; therefore, the diagnosis of FEVR was based on typical fundus changes and a positive family history. Among the 24 children without FFA, 18 were diagnosed based on typical fundus change and family history (confirmed with FFA). Another six patients without a positive family history were diagnosed based on typical fundus change, including retinal folds (three children) or total tractional retinal detachment in infancy (three children). Patients with a gestational age of less than 38 weeks or with a neonatal birthweight of less than 2,000 g were excluded, to eliminate the possible presence of retinopathy of prematurity. Moreover, all possible cases of persistent fetal vasculature, microphthalmia, and unclassified chorioretinal dysplasia were ruled out. Data collected from medical records included gender, gestational age at birth, birthweight, age at diagnosis, family history, clinical presentation, and angiographic findings. Fundus examinations, color photography, and fluorescein angiography were performed for family members. One hundred fifty ethnically matched individuals without history of ocular diseases were recruited as controls. Comprehensive fundus examinations were performed, and no signs of retinal diseases were observed.

Genetic analysis: Tissue samples (blood or buccal swabs) were obtained from each participant for genomic DNA extraction using the standard protocols of the QIAGEN QIAamp DNA Blood Kit (Qiagen, Hilden, Germany) and the Epicenter Master Pure Complete DNA and RNA Purification Kit (Illumina, Madison, WI). For mutational analysis, the coding exons of *FZD4* and their adjacent intronic sequences were amplified with PCR (primers listed in Appendix 2). The annealing temperature was 59 °C. A total of 50 ng of DNA was amplified in a reaction volume of 20 µl. The amplification conditions were as follows: 95 °C for 30 s, annealing temperature for 30 s, and 72 °C for 1 min. The resulting amplicons were analyzed with Sanger sequencing. Direct sequencing was performed with an ABI BigDye Terminator Cycle Sequencing Kit using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The reference sequence of *FZD4* (GenBank NC_000011.9 for gDNA, NM_012193.2 for mRNA) was used for the identification of variants.

Assessment of missense mutations: Sequence alignments for novel *FZD4* missense mutations were performed using the online COBALT program. The following protein sequences were used: *Homo sapiens frizzled 4* (GenBank NP_036325), *Mus musculus frizzled 4* (GenBank NP_032081), *Bos taurus frizzled 4* (GenBank NP_001193198), *Rattus norvegicus frizzled 4* (GenBank NP_072145), *Gallus gallus frizzled 4* (GenBank NP_989430), *Xenopus laevis frizzled 4* (GenBank NP_001083922), *Danio rerio frizzled 4* (GenBank NP_001292398), *Homo sapiens frizzled 1* (GenBank NP_003496), *Homo sapiens frizzled 2* (GenBank NP_001457), *Homo sapiens frizzled 3* (GenBank NP_059108), *Homo sapiens frizzled 5* (GenBank NP_003459), *Homo sapiens frizzled 6* (GenBank NP_001158087), *Homo sapiens frizzled 7* (GenBank NP_003498), *Homo sapiens frizzled 8* (GenBank NP_114072), *Homo sapiens frizzled 9* (GenBank NP_003499), and *Homo sapiens frizzled 10* (GenBank NP_009128). Online protein prediction programs were used to estimate the pathogenicity of the missense changes (SIFT, PolyPhen-2, and Align-GVGD).

Cloning sequencing: The target fragments covering the mutation sites were amplified with PCR. The PCR products were gel purified by using the Universal DNA Purification Kit (TIANGEN, Beijing, China) and cloned into pMD19-T (Takara BIO, Shiga, Japan) according to the manufacturer's instructions. *Escherichia coli* DH5α was transformed with the resulting plasmids and cultured in Luria-Bertani (LB) agar plates with ampicillin at 37 °C for 12–14 h. A single colony was placed in 5 ml of liquid LB medium with ampicillin and grown overnight in the 37 °C incubator shaker for 12–14 h. The plasmids were isolated from the suspension with the TIANprep Mini Plasmid Kit (TIANGEN), and Sanger sequencing was used to confirm the mutant and wild-type alleles.

Statistics: All analyses were performed using SPSS software version 16.0. The independent samples Student *t* test was used for the normally distributed continuous variables, while the Kruskal–Wallis test was used for the non-normally distributed continuous variables. The Pearson chi-square test and Fisher's exact test were used for the categorical variables. A p value of less than 0.05 was considered statistically significant.

RESULTS

Fourteen heterozygous mutations in *FZD4*, including seven novel and seven known mutations, were identified in 21 of the 100 individuals with FEVR (Table 1). Among the 21 individuals with mutations in *FZD4*, 16 had a positive familial history of FEVR, and five were sporadic cases. Of the seven novel mutations, four were missense (C45R, C45S, C53S, and C90R), and three were deletion mutations (T326fsX356, G492fsX512, and S345_A351del; Figure 1).

Chromatograms of these novel mutations are shown in Figure 2. None of these novel mutations were detected in 150 ethnically matched control subjects. These variants were absent in current online databases of human sequence variants, including the 1000 Genomes Project, dbSNP, ExAC databases and the human gene mutation database (HGMD). All four novel missense changes affected evolutionarily conserved residues (Figure 3).

Deletion mutations: Three novel deletion mutations (T326fsX356, G492fsX512, and S345_A351del) were detected in exon 2 of *FZD4*. Mutant and wild-type alleles of these heterozygous deletion mutations were confirmed with cloning sequencing (Figure 2H–J). The T326fsX356 mutation resulted from a 4 bp heterozygous deletion of bases 975–978 (975_978delCACT). This mutation was identified in two unrelated individuals with a family history of FEVR following cosegregation analysis (Families 3 and 7). This mutation caused a frameshift resulting in 30 incorrect amino acids after codon 325 and a premature termination at codon 356. The proband in Family 3 was a 4-year-old boy with bilateral retinal folds. Peripheral avascularization with a scalloped border was observed in both eyes of his mutation-carrying grandfather, father, and elder sister, who were without any visual symptoms. The proband's affected paternal aunt, who exhibited a characteristic dragged disc in her right eye and retinal avascularization in both eyes, was not available for mutation analysis (Family 3, Figure 4; Table 2). The proband in Family 7 was a 14-year-old girl with tractional retinal detachment in her left eye. Peripheral retinal avascularization and remarkable retinal exudates were observed in her right eye. Her mother and brother, who had bilateral peripheral avascularization and retinal exudates but who were completely asymptomatic, carried the same mutation (Family 7, Figure 4; Table 2).

The second mutation resulted from a 1 bp heterozygous deletion of base 1475 (1475delG), which caused 20 incorrect amino acids after codon 491 and introduced a premature stop at codon 512 (G492fsX512). This mutation was found in a proband as sporadic FEVR (Family 25, Figure 4; Table 2). He was a 13-year-old boy who displayed retinal folding in the left eye with poor visual acuity (hand movement only) from infancy. His parents and sister underwent a full examination, and no retinopathy or mutation was observed.

A 21 bp heterozygous deletion (1034_1054delCTTATTTCCACATTGCAGCCT) was identified in an 11-year-old boy with asymmetric manifestation (Family 4, Figure 4; Table 2). This mutation caused an in-frame deletion of seven amino acids, from Ser345 to Ala351 (S345_A351del). The proband displayed tractional retinal detachment in his right eye, resulting in vision limited to light perception. His left eye suffered milder visual impairment due to an ectopic macula. His affected father and paternal uncle were clinically healthy, only with bilateral myopia, and FEVR was diagnosed only with fluorescein angiography. Moreover, the proband's mutation-carrying paternal aunt had undergone a full ophthalmic examination and showed no sign of retinopathy. Intriguingly, Y221H, a reported pathogenic mutation, was identified in the proband's unaffected mother and sister [15].

Missense mutations: A recurrent mutation, C45Y, previously reported in a patient from Southern California in the United States [16], was detected in a 6-year-old boy with retinal folding in his right eye and an ectopic macula in his left eye (Family 29, Figure 4; Table 2). His affected but asymptomatic father carried the same mutation. Furthermore, two novel mutations altering the same codon (C45R and C45S) were found in two of the southern Chinese pedigrees. The C45R mutation was detected in an 8-year-old boy with bilateral retinal folds. His father and elder brother carried the same mutation. Straightening and increased branching of peripheral vessels with bilateral avascular retina were noted on FFA of the proband's father, while ectopic macula (right eye) and vessel exudates (left eye) were identified in the proband's elder brother (Family 26, Figure 4; Table 2). The C45S mutation was detected in a 7-month-old infant girl with retinal folding in her right eye (Figure 5D). Her affected mother and asymptomatic elder sister carried the same mutation. The proband's mother showed retinal folding and a secondary cataract in her right eye with poor visual acuity since childhood (Family 43, Figure 4; Table 2).

Novel missense mutation C53S was identified in an 8-year-old boy with bilateral dragged discs. Peripheral retinal

Table 1. FZD4 variants identified in 100 probands with familial exudative vitreoretinopathy and 150 control subjects.

Family ID	Location	Allele status	cDNA Change	Protein Change	Type	Probands With FEVR (n=100)	Control Subjects (n=150)	SIFT	PolyPhen2	Align GVGD	Source
33	Exon1	Hetero	107G>A	G36D	Missense	1/100	0/150	0.03,D	Benign	Class C65	[17]
26	Exon1	Hetero	133T>C	C45R	Missense	1/100	0/150	0,D	Probably damaging	Class C65	Novel
43	Exon1	Hetero	133T>A	C45S	Missense	1/100	0/150	0,D	Possibly damaging	Class C65	Novel
29	Exon1	Hetero	134G>A	C45Y	Missense	1/100	0/150	0,D	Probably damaging	Class C65	[15]
12	Exon1	Hetero	158G>C	C53S	Missense	1/100	0/150	0,D	Probably damaging	Class C65	Novel
42	Exon1	Hetero	223G>A	A75T	Missense	1/100	0/150	0,D	Probably damaging	Class C55	-
30	Exon1	Hetero	268T>C	C90R	Missense	1/100	0/150	0,D	Probably damaging	Class C65	Novel
36,44	Exon2	Hetero	313A>G	M105V	Missense	2/100	0/150	0.02,D	Possibly damaging	Class C15	[14,18-21]
27	Exon2	Hetero	957G>A	W319X	Nonsense	1/100	0/150	NA	NA	NA	[21,22]
3,7	Exon2	Hetero	975_978delCACT	T326fsX356	Deletion	2/100	0/150	NA	NA	NA	Novel
4	Exon2	Hetero	1034_1054delCTTATTTCCACATT-GCAGCCT	S345_A351del	Deletion	1/100	0/150	NA	NA	NA	Novel
14,31,37,38,39,41	Exon2	Hetero	1282_1285delGACA	D428fsX429	Deletion	6/100	0/150	NA	NA	NA	[14,19,20]
25	Exon2	Hetero	1475delG	G492fsX512	Deletion	1/100	0/150	NA	NA	NA	Novel
28	Exon2	Hetero	1498delA	T500fsX512	Deletion	1/100	0/150	NA	NA	NA	[17]

Hetero: Heterozygous; NA: Not available; For SIFT, amino acids changes with probabilities <0.05 are predicted to be deleterious. (D: Deleterious. T: Tolerated); For Align GVGD, class C0 indicates that a change is unlikely to be pathogenic, while class C65 represents the highest likelihood of a change to be pathogenic.

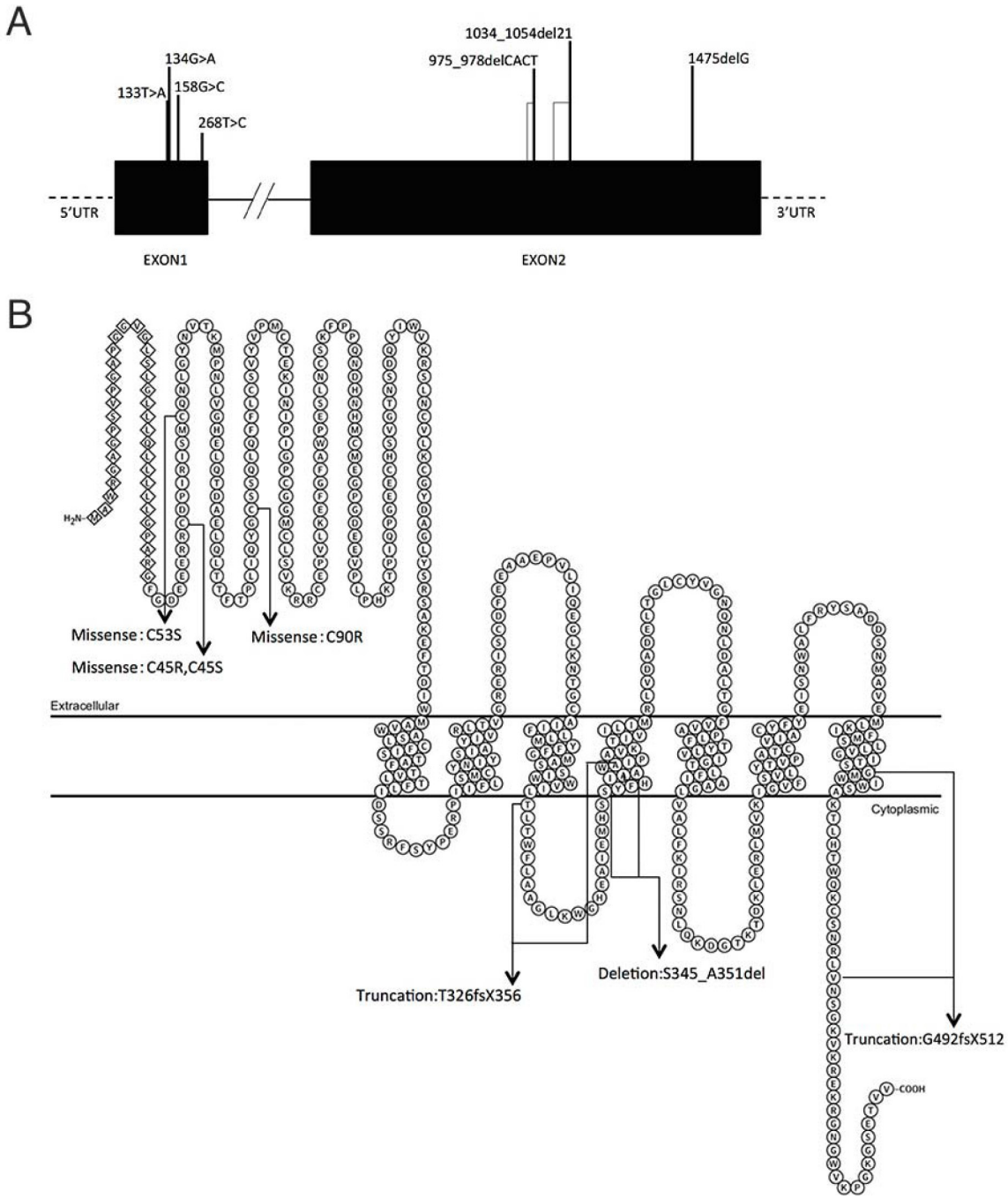

Figure 1. Types and locations of novel mutations. **A**: Schematic diagram of the *FZD4* gene. **B**: The Frizzled 4 protein shows the types and locations of the novel mutations. The locations of the transmembrane domain were derived from GenBank entry Q9ULV1. The first 36 amino acids (rhombus) were a Frizzled-4 signal peptide.

avascular zone and neovascularization with exudates were observed in both eyes. His mutation-carrying mother and sister had normal vision acuity, and FFA showed typical peripheral non-perfusion area in their eyes (Family 12, Figure 4; Table 2).

Mutation A75T was identified in a 26-year-old female patient. She had blurred vision in the right eye caused by vitreous hemorrhage from retinal neovascularization. Her left eye had normal visual acuity with typical peripheral retinal avascularization and neovascularization (Family 42, Figure 4; Table 2). Although the A75T variant has been reported as

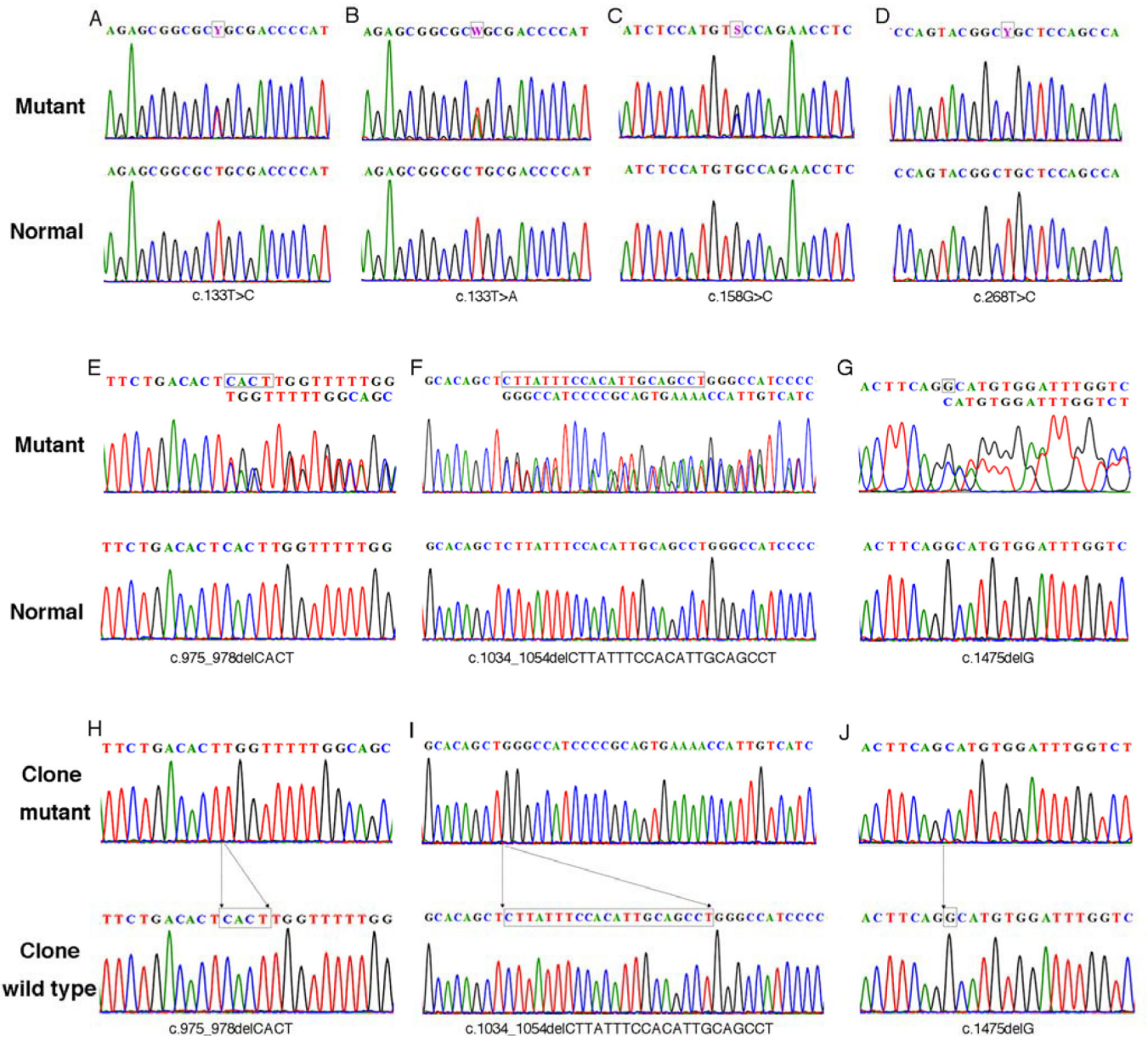

Figure 2. Sequence chromatograms of novel mutations in *FZD4*. **A**: Heterozygous mutation c.133T>C. **B**: Heterozygous mutation c.133T>A. **C**: Heterozygous mutation c.158G>C. **D**: Heterozygous mutation c.268T>C. **E**: Heterozygous mutation c.975_978delCACT. **F**: Heterozygous mutation c.1034_1054delCTTATTTCCACATTGCAGCCT. **G**: Heterozygous mutation c.1475delG. **H**: Cloning sequencing demonstrated the heterozygous mutation c.975_978delCACT. **I**: Cloning sequencing demonstrated the heterozygous mutation c.1034_1054delCTTATTTCCACATTGCAGCCT. **J**: Cloning sequencing demonstrated the heterozygous mutation c.1475delG.

SNV (rs764357846) in NCBI, the minor allele frequency of this change was 0.01273% in the East Asian population (data from the ExAC database). Moreover, in the in silico analysis, all three programs predicted the variant to be deleterious. Thus, we considered it a rare disease-causing variant.

Another novel missense mutation, C90R, was detected in a 3-year-old female proband with retinal folding in her right eye (Family 30, Figure 4; Table 2). Her mutation-carrying mother was clinically normal, but peripheral retinal avascularization with vascular tortuosity in both eyes was observed with FFA. Her healthy father did not carry this mutation.

The four novel missense mutations (C45R, C45S, C53S, and C90R) were detected in exon 1 of *FZD4*. The cysteines at codons 45, 53, and 90 are located in the N-terminal extracellular cysteine-rich domain (CRD), which acts as the binding site for Wnt or Norrin [11,17]. These amino acids are highly

conserved across all members of the Frizzled protein family, as well as in the FZD4 protein in other vertebrate species. These mutations were predicted to be pathogenic in the in silico analysis (Table 1).

Other variants: Variants without strong evidence of pathogenicity were considered as being of unknown significance. The Y221H variant was identified in a singleton case, and the proband's healthy son did not carry the mutation (Appendix 3). This variant was previously reported as a pathogenic mutation in a Chinese singleton case [15]. However, the Y221H variant was identified in two unaffected members from the pedigree that harbored the S345_A351del mutation (Family 4, Figure 4). Y221H was not found in the control group of this study. The protein prediction programs SIFT and Align-GVGD predicted this variant to be deleterious, while PolyPhen-2 predicted it to be benign (Appendix 4). Biological assays are needed to determine the mutation's pathogenicity. A novel nonsynonymous variant, I437T, was identified in a 43-year-old female patient with a positive familial history of FEVR, but this change did not segregate with the disorder in her family (Appendix 3). Codon 437 is located in the sixth transmembrane domain of FZD4. The I437T variant changed a hydrophobic isoleucine residue into a hydrophilic threonine residue. It was predicted to be deleterious by bioinformatics analyses (Appendix 4) and was not found in the control subjects and current online databases. The patient's right eye showed an ectopic macula and atrophic retinal holes, and visual acuity was limited to finger counting. Both eyes showed typical deficient vascularization and exudates of the peripheral retina. Fluorescein angiography confirmed that her

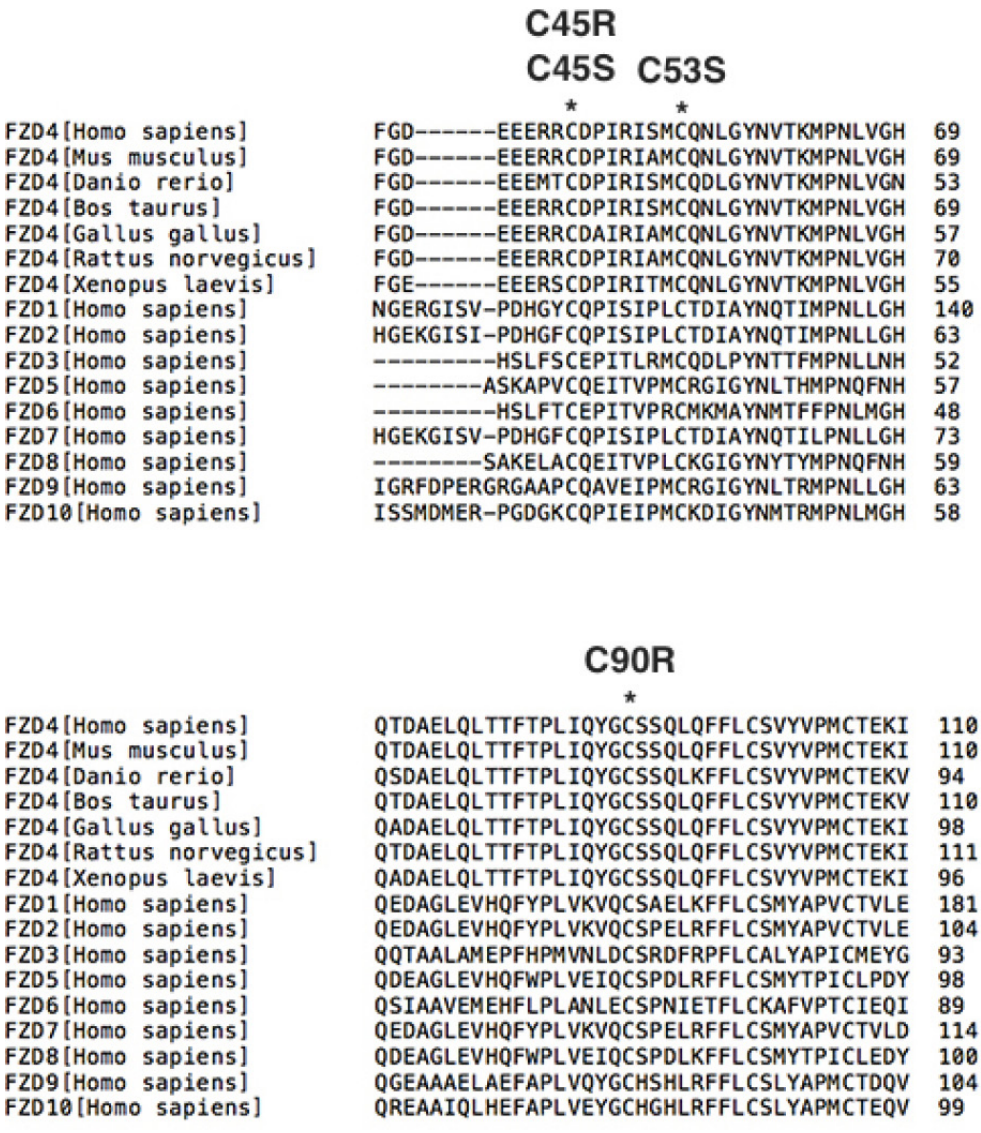

Figure 3. Protein sequence alignment of human Frizzled 4 with homologs from human and other species: mouse, zebrafish, cow, chicken, rat, and frog Frizzled 4 proteins and the human Frizzled family proteins. The novel mutations C45R, C45S, C53S, and C90R are indicated.

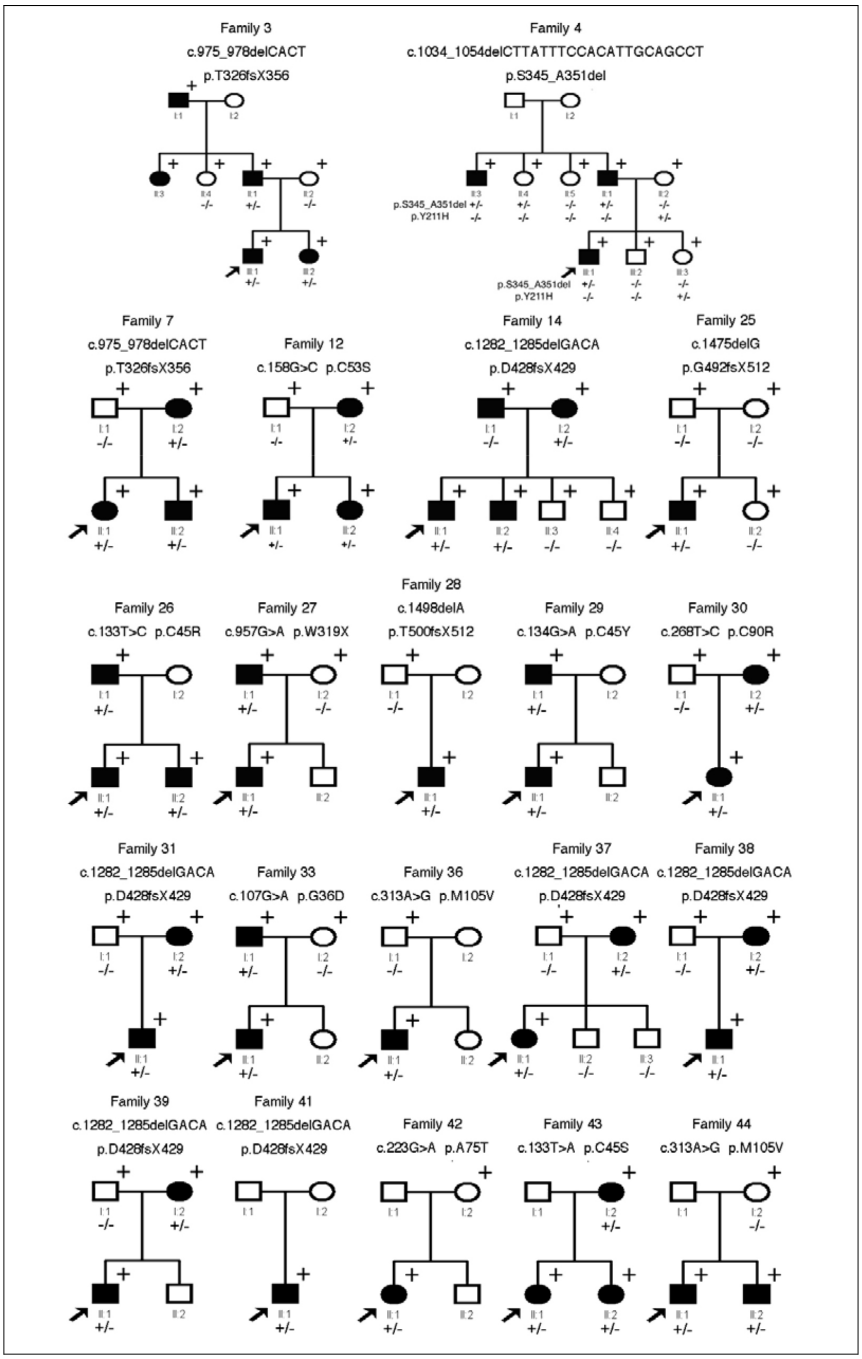

Figure 4. Schematic pedigrees of the families with mutations in *FZD4*. Arrows indicate proband; open symbols: clinically unaffected; solid symbols: clinically affected; +: clinically evaluated; +/−: heterozygous with familial mutation; −/−, wild-type. For the mutations in *FZD4*, the nomenclature is based on GenBank NM_012193.2.

mother also had FEVR, with bilateral retinal avascularization and exudates; however, after DNA sequencing, her mother did not harbor the same variants (I437T). Thus, we considered it less likely to be pathogenic mutation.

In addition to these mutations, we identified five previously reported disease-causing mutations: G36D [18], M105V [15,19-22], W319X [22,23], D428fsX429 [15,20,21], and T500fsX512 [18]. The G36D missense mutation was identified in an 8-year-old boy with retinal folding in his right eye (Family 33, Figure 4; Table 2). The W319X nonsense mutation was detected in a 15-year-old boy with an ectopic macula in his right eye (Family 27, Figure 4; Table 2). The T500fsX512

Table 2. Mutations in FZD4 and associated clinical findings.

Family No.	Patient No./Sex/Age	Sequence Change	BCVA (OD; OS)	Highest stage	Clinical findings (OD)	Clinical findings (OS)
3	I:1/M/52Y	T326fsX356	0.4; 0.9	2	AZ, NV, SV	AZ, NV, SV
	II:1/M/29Y	T326fsX356	0.05; 1.2	2	AZ, NV, IPV, SV	AZ, NV, IPV, SV
	III:1/M/4Y	T326fsX356	NA	4	FRF	FRF
	III:2/F/6Y	T326fsX356	0.63; 0. 32	1	AZ, IPV, SV	AZ, IPV, SV
7	I:2/F/42Y	T326fsX356	NA	1	AZ, SV, IPV, VE	AZ, SV, IPV, VE
	II:1/F/14Y	T326fsX356	0.3; FC	5	MD, AZ, NV, VE	TRD
	II:2/M/9Y	T326fsX356	1.0; 1.0	2	AZ, NV, VE, IPV, SV	AZ, NV, VE, IPV, SV
4	II:1/M/41Y	S345_A351del	1.0; 1.0	1	AZ, IPV, SV	AZ, IPV, SV
	II:3/M/45Y	S345_A351del	0.9; 1.0	1	AZ, IPV, SV	AZ, IPV, SV
	II:4/F/44Y	S345_A351del	1.0; 1.0	NA	Normal	Normal
	III:1/M/11Y	S345_A351del	LP; 0.3	5	TRD	MD, AZ, IPV, SV
12	I:2/F/30Y	C53S	1.0; 1.0	1	AZ, IPV, SV	AZ, IPV, SV
	II:1/M/8Y	C53S	1.2; 0.6	1	AZ, IPV, SV	MD, AZ, IPV, SV
	II:2/F/15Y	C53S	1.5; 1.5	1	AZ, IPV, SV	AZ, IPV, SV
25	II:1/M/13Y	G492fsX512	1.0; HM	4	AZ, NV, IPV, SV, VE	FRF
26	I:1/M/37Y	C45R	1.0; 1.0	1	AZ, IPV, SV	AZ, IPV, SV
	II:1/M/8Y	C45R	0.1; FC	4	FRF	FRF
	II:2/M/12Y	C45R	0.1; 1.0	2	MD, AZ, IPV, SV	AZ, IPV, SV, NV, VE
27	I:1/M/38Y	W319X	1.0; 1.0	1	AZ, IPV	AZ, IPV
	II:1/M/15Y	W319X	FC; 1.0	1	MD, AZ, IPV	AZ, IPV
28	II:1/M/12Y	T500Lfs	0.04; 0.9	4	FRF	AZ, IPV, NV, VE
29	I:1/M/40Y	C45Y	1.0; 1.0	1	AZ, IPV, SV	AZ, IPV, SV
	II:1/M/6Y	C45Y	FC; 0.2	4B	FRF	MD, AZ, NV, VE, IPV, SV
30	I:2/F/35Y	C90R	1.0; 1.0	1	AZ, IPV, SV	AZ, IPV, SV
	II:1/F/3Y	C90R	NA	4	FRF	MD, AZ, IPV, SV
33	I:1/M/30Y	G36D	NLP; 0.04	5	TRD	FRF, SC
	II:1/M/8Y	G36D	0.03; 0.2	4	FRF	AZ, IPV
31	I:2/F/30Y	D428fsX429	0.8; FC	4	AZ, IPV, NV, VE	FRF
	II:1/M/5Y	D428fsX429	0.6; 0.1	2	AZ, IPV, SV	MD, AZ, IPV, SV, NV, VE
37	I:2/F/24Y	D428fsX429	0.4; 0.6	1	AZ, IPV, SV	AZ, IPV, SV
	II:1/F/2M	D428fsX429	NA	4	FRF	FRF
38	I:2/F/30Y	D428fsX429	NA	1	AZ, IPV, SV	AZ, IPV, SV

Family No.	Patient No./Sex/Age	Sequence Change	BCVA (OD; OS)	Highest stage	Clinical findings (OD)	Clinical findings (OS)
	II:1/M/2Y	D428fsX429	NA	4	FRF	FRF
39	I:2/F/26Y	D428fsX429	NA	1	AZ, IPV, SV	MD, AZ, IPV, SV
	II:1/M/4Y	D428fsX429	NA	4	AZ, IPV, SV	FRF
41	II:1/M/6M	D428fsX429	NA	4	AZ, SV	FRF
14	I:1/M/40Y	No	1.5; 1.5	1	AZ, IPV, SV	AZ, IPV, SV
	I:2/F/38Y	D428fsX429	1.2; 1.5	1	AZ, IPV, SV	AZ, IPV, SV
	II:1/F/15Y	D428fsX429	FC; 1.0	4	FRF	AZ, IPV, SV, NV, VE, VH
	II:2/M/9Y	D428fsX429	HM; HM	5	TRD, SC	TRD
42	II:1/F/26Y	A75T	0.2; 1.0	2	VH, AZ, IPV, SV, NV, VE	AZ, IPV, SV, NV
43	I:2/F/32Y	C45S	NA	4	FRF, SC	AZ, IPV, SV
	II:1/F/7M	C45S	NA	4	FRF	AZ, IPV, SV
	Ii:2/F/8Y	C45S	0.8; 0.8	1	AZ, IPV, SV	AZ, IPV, SV
36	II:1/M/10Y	M105V	O.4; 1.0	2	MD, AZ, SV, IPV, NV	AZ, IPV, SV, NV, VE
44	II:1/M/10Y	M105V	FC; 1.0	1	MD, AZ, SV	AZ, IPV, SV
	II:2/M/8Y	M105V	0.1; 1.0	1	MD, AZ, SV	MD, AZ, SV

LP: Light perception; HM: Hand movement; FC: Finger count; OD: Right eye; OS: Left eye; NA: Not available; BCVA: Best corrected visual acuity. TRD: Tractional retinal detachment; NV: Neovascularization; AZ: Avascular zone; IPV: Increasing of peripheral vessels; MD: Macular dragging; VE: Vessels exudates; FRF: Falciform retinal fold; SV: Straightening of vessels; SC: Secondary cataract

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deletion mutation was found in a 12-year-old boy with retinal folding in his right eye (Family 28, Figure 4; Figure 5B; Table 2). Two probands harbored the same M105V mutation (Families 36 and 44, Figure 4; Table 2), and six unrelated families carried the same D428fsX429 mutation (Families 14, 31, 37, 38, 39, and 41; Figure 4; Table 2). M105V was previously reported among ethnically diverse probands with variable symptoms and is considered a FEVR-causing mutation with high prevalence [15,19-22].

Ocular findings in family members: Overall, 100 probands and their family members were included in this study. In 64 pedigrees, screening in both parents was performed with fundus examination and FFA. The FEVR phenotype, mild or moderate, was identified in 53 families; however, no FEVR-associated retinopathies were identified in 11 families (data not shown).

Other first- or second-degree relatives were also checked if available. A total of 75 individuals from 53 families were diagnosed with FEVR. Importantly, among the 75 relatives, 69 (69/75) were clinically asymptomatic. An avascular zone and other peripheral retinopathy were observed only with careful ophthalmic examination and confirmed with FFA. These 69 individuals were not aware of the existence of this disease. Only 13 had visual dysfunction (data not shown).

Phenotype–genotype correlations based on Trese's staging system: The staging system of FEVR was recently updated by Trese's group [14], based on the widely used staging system Trese addressed in 1998 [24]. In this study, 100 probands were staged according to the Trese system based on the highest stage of disease in either eye. Thus, patients with macula-uninvolving folds were identified as stage 3, those with macula-involving folds were identified as stage 4, and patients with ectopic macula were sub-grouped in stage 1 or 2, depending on the existence of neovascularization. Patients with active vitreous hemorrhage secondary to retinal neovascularization were identified as stage 2 (Table 3). As a result, mutations in *FZD4* were seen in six patients with mild FEVR (stages 1–2) and in 15 patients with severe FEVR (stages 4 and 5). No distinct phenotype–genotype correlation was observed.

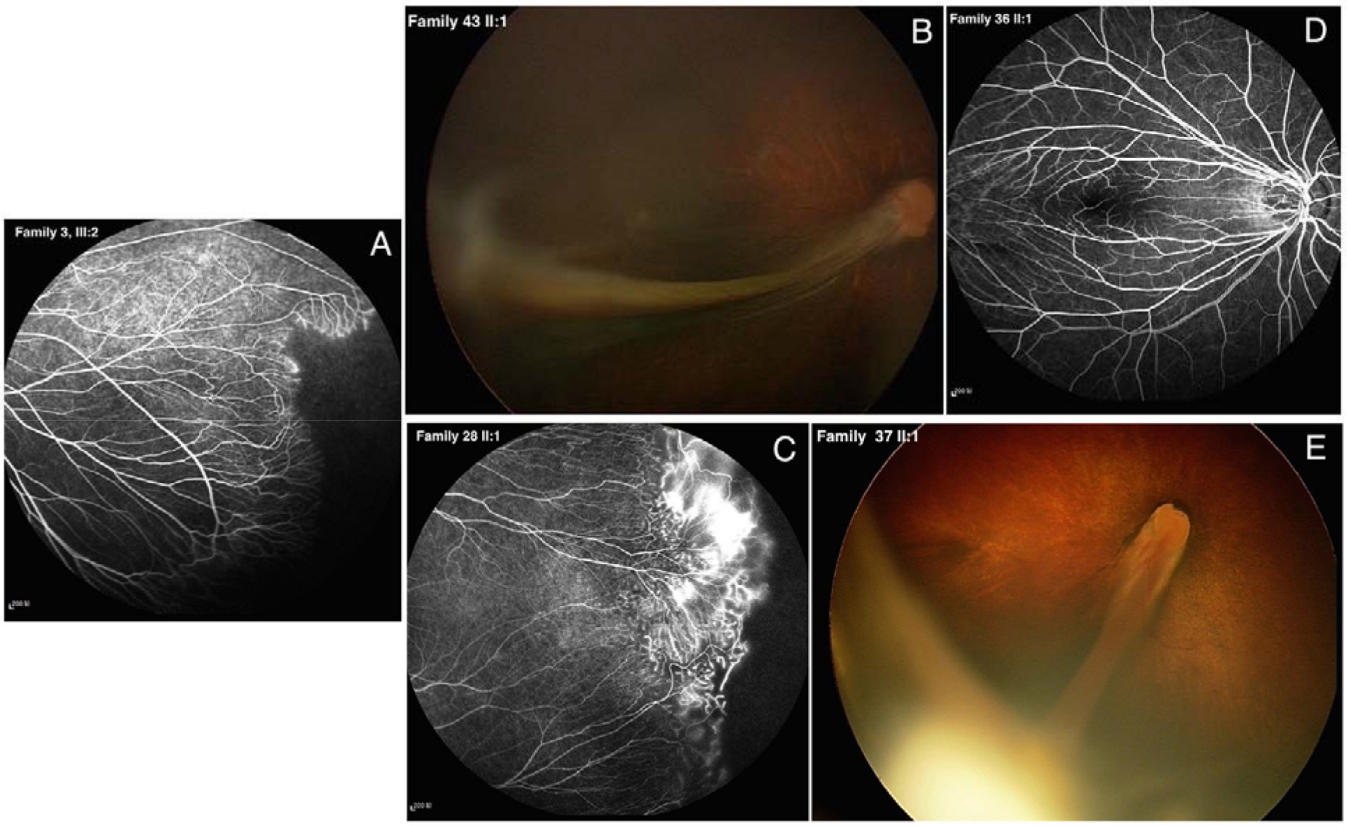

Figure 5. Fundus photography and fluorescein angiography of the FEVR patients with *FZD4* mutations. The patient ID is indicated on the top left of each picture. **A**: Peripheral avascular zone. **B**: Falciform retinal fold. **C**: Peripheral avascular zone and neovascularization. **D**: Ectopic macular. **E**: Falciform retinal fold.

Table 3. Phenotype-genotype analysis in traditional staging system.

Stage*		N.	Age at diagnosis	Gender M(M%)	N. of pathogenic *FZD4* mutations
	1	18(18.0%)	23.9±20.2	13(72.2%)	2
	2	14(14.0%)	29.6±16.8	8 (57.1%)	4
	3	4 (4.0%)	11.7±14.8	2 (50.0%)	0
	4	43(43.0%)	5.5±5.2	37(86.0%)	13
	5	21(21.0%)	3.6±4.9	17(81.0%)	2
P **value**		-	<0.001†	0.127‡	-
Total		100**(100%)**	12.1±**15.3**	77**(77.0%)**	21

N.: Number; M: Male * Patients were staged in the basis of the highest stage of FEVR in either eye. † p<0.001 in comparison with each subgroups using Kruskal–Wallis test; ‡ p=0.219 in comparison with each subgroups using chi –square test.

Phenotype–genotype correlations based on modified classification system: To delineate the possible genotype–phenotype correlation, all probands were categorized into four subgroups according to the new classification system, which was modified from the criteria described by Pendergast et al. [24] and Kashani et al. [14]. In this new classification system, not only the severity of the avascular area and its complications but also the age of onset was considered. Group I included individuals with asymptomatic FEVR confirmed only with FFA with bilateral peripheral retinopathy in adulthood (mild). Group II included patients with active vitreous hemorrhage due to retinal neovascularization or rhegmatogenous retinal detachment secondary to peripheral retinal degeneration in childhood or adulthood (moderate). Group III included patients with retinal folds or an ectopic macula in at least one eye, which typically occurred in infancy (called severe). Group IV included patients with FEVR with severe tractional total retinal detachment in at least one eye in infancy (extremely severe; Table 4).

In this study, with this new classification system, significant statistical differences were observed among the four groups in average age at diagnosis (p<0.001) and gender ratio (p=0.012). The more severe the phenotype, the younger the average age at diagnosis, and a higher prevalence of male probands was observed. Group IV had the youngest average age at diagnosis (2.0±2.3, range from 1 month to 7 years old), mostly in the first year of the life. However, the mean age at diagnosis for the probands in Group I was 44.0±15.1 years old (range from 12 years old to 61 years old); 82.3% of the probands in Group IV were male, while 40% in Group I. Most of the cases in Group I were registered as other diseases (uveitis in 1 case, central retinal venous occlusion in two cases, diabetic retinopathy in one case, age related macular degeneration in one case, macular hole in one case, macular edema in one case, and pathologic myopia in three cases) but were eventually confirmed with FFA (Table 4).

Table 4. Phenotype-genotype analysis in modified classification system.

Group	Phenotypes	N.	Age at diagnosis	Gender M(M%)	N. of pathogenic *FZD4* mutations
I**: Mild**	Bilateral asymptomatic but confirmed by FFA	10(10.0%)	44.0±15.1	4(40.0%)	0
II**: Moderate**	Vitreous hemorrhage due to RNV*	4(4.0%)	25.3±12.3	2(50.0%)	1
III**: Severe**	Retinal folding or ectopic macula*	69(69.0%)	9.2±10.3	57(82.6%)	20
IV**: Extreme severe**	Total tractional RD in infancy*	17(17.0%)	2.0±2.3	14(82.3%)	0
P **value**	-	-	<0.001Δ	0.012§	-
	Total	100**(100%)**	12.1±**15.3**	77**(77.0%)**	21

RD: Retinal detachment; RNV: retinal neovascularization N.: Number; M: Male * Seen in at least one eye; Δ p<0.001 in comparison with each subgroups using Kruskal–Wallis test; § p=0.012 in comparison with each subgroups using chi –square test.

Interestingly, 20 of 21 patients with disease-causing mutations in *FZD4* were categorized in Group III FEVR (15 retinal folds and five with an ectopic macula). For the 24 children without FFA (13 cases in Group III, 11 cases in Group IV), causative mutations in *FZD4* were identified in three children with severe FEVR (Group III). Only one patient with a mutation in *FZD4* was categorized in Group II due to vitreous hemorrhage secondary to retinal neovascularization. No mutations in *FZD4* were found in ten patients in Group I or in 17 patients in Group IV (Table 4).

DISCUSSION

Mutation screening of genes known to cause FEVR would provide invaluable information for diagnosis and genetic counseling, especially for patients without a family history of the disorder or with atypical presentations. To our knowledge, the present study used one of the largest cohorts of patients with FEVR, with 100 families. Fourteen causative heterozygous mutations in *FZD4* in 21 unrelated probands were noted, accounting for 21.0% of the index patients (21/100). The percentage of FEVR caused by mutations in *FZD4* in this study is slightly higher when compared to data from Western countries, such as 20.0% (8/40) in the United Kingdom [18], 14.1–14.3% (13/92 and 9/63) in the United States [16,25], and 17.6% (12/68) in Canada [21]. However, data from Asian populations revealed a more varied range. For example, a comprehensive molecular screening of FEVR-associated genes in the Korean population indicated mutations in *FZD4* accounted for 25.5% (13/51) of FEVR cases [20]. In northern China, Jia et al. reported an even higher rate of 31.3% (15/48) [19]. However, in eastern China, Fei et al. recently reported only 4.9% (3/61) of families with FEVR in Han Chinese had causative mutations in *FZD4* [26]. Thus, the current study provides new evidence of the high prevalence of mutations in *FZD4* in FEVR in East Asia. One of the reasons for the differences is a possible ethnic difference in FEVR genotypes between Asian and Western populations. Another possible reason is that the diagnostic methods varied among the studies. Among the 100 pedigrees in the current study, diagnosis in the majority of the probands and family members was confirmed with FFA, which is considered the golden standard in the diagnosis of FEVR.

In this study, the novel in-frame deletion mutation S345_A351del was identified in one pedigree (Family 4, Figure 4). In this family, all affected relatives and one healthy family member harbored the same mutation, which suggested nonpenetrance in mutations in *FZD4*. Nonpenetrance was also observed in previously reported FEVR pedigrees with mutations in *FZD4* and other known genes [3,5,23]. Moreover, two novel disease-causing mutations were predicted to result in truncation of the *FZD4* mRNA (T326fsX356 and G492fsX512). Both mutations were located in the large exon 2 of *FZD4*, which encodes 442 amino acids. The proportions of the mRNA truncated were 33.8% for the T326fsX356 variant and 4.8% for the G492fsX512 variant, respectively. The novel missense mutations found in this study—C45R, C45S, C53S, and C90R—are located in the highly conserved cysteine-rich domain of *frizzled 4*. According to a previous study, Cys45 forms a disulfide bond with Cys106, Cys53 forms a disulfide bond with Cys99, and Cys90 forms a disulfide bond with Cys128 [27]. The substitutional residues damage these disulfide bonds and may affect the function of the cysteine-rich domain, which is critical for ligand binding.

Intriguingly, a recurrent mutation, D428fsX429, was identified in six unrelated probands. All individuals were from separate areas in southern China. There was no evidence of consanguinity. Among the six families, cosegregation analysis revealed that all affected individuals carried the same mutation, while unaffected individuals did not. As D428fsX429 was frequently detected in this study, we checked the occurrence in the control subjects, and none carried the mutation. This mutation was previously reported in a Caucasian female singleton case [21], a Chinese female [15,20], and two Korean patients [15,20]. The minor allele frequency of this change was far lower than 0.05% (0.001498%) according to the ExAC database, and it was considered pathogenic in the NCBI ClinVar database. Thus, we consider D428fsX429 a disease-causing mutation in the six families. All probands with the D428fsX429, in previous studies and the present study, showed retinal folds or ectopic macula as the initial symptoms.

In this study, probands showed highly variable clinical features. By using the traditional staging system, six probands with causative mutations in *FZD4* were classified with mild FEVR (stages 1–2, two cases in stage 1 and four cases in stage 2) while 15 of 21 probands with causative mutations were classified as severe FEVR (stages 3–5, 13 cases in stage 4 and two cases in stage 5). No obvious phenotype–genotype correlation was observed, which is consistent with previous reports [19,21]. However, with this modified system, in 100 unrelated probands, we found that mutations in *FZD4* were not found in Group I FEVR (mild cases, manifested with bilateral asymptomatic retinopathy) or Group IV FEVR (extreme severe cases, manifested with chorioretinal dysplasia in infancy). Interestingly, most mutations in *FZD4* (95.2%, 20/21) were identified in the probands in Group III FEVR (severe cases, manifested with retinal folding or ectopic macula); only one mutation in *FZD4* was noted in a proband with Group

II FEVR (moderate cases, manifested with vitreous hemorrhage or rhegmatogenous retinal detachment; Table 4). These results showed that mutation in *FZD4* is most likely one of the genetic etiology of retinal folds or ectopic macula due to FEVR. It is hypothesized that mutations in *FZD4* exert only moderate effects on retinal vascularization delay. Patients who carried the mutations were less likely to present with blindness immediately after birth. The underlying molecular mechanisms should be investigated in future studies.

In summary, this study offers insights into the type, distribution, and frequency of FEVR-causing mutations in *FZD4*. The discovery of seven novel mutations with potential pathogenicity has broadened the spectrum of mutations in *FZD4* underlying FEVR. Further discovery of novel causative genes may contribute to our understanding of the molecular mechanisms of retinal angiogenesis, which is crucial for FEVR and other retinal vascular diseases. It will also enable the accurate diagnosis of asymptomatic individuals and better genetic counseling of patients. Our emerging understanding of FZD signaling offers hope that pharmaceutical agents that selectively target this pathway will be developed to treat phenotypic characteristics of these diseases in the future.

APPENDIX 1. *FZD4* MUTATIONS ASSOCIATED WITH FAMILIAL EXUDATIVE VITREORETINOPATHY

To access the data, click or select the words "Appendix 1."

APPENDIX 2. SEQUENCE OF OLIGONUCLEOTIDE PRIMERS USED IN THE THIS STUDY

To access the data, click or select the words "Appendix 2." Exon 2 was screened in two overlapping segments designated 2A-2B

APPENDIX 3. PEDIGREE.

To access the data, click or select the words "Appendix 3." (A) Schematic pedigrees of the Family 35 with Y211H mutation. (B) Schematic pedigrees of the Family 34 with I437T mutation. Arrows indicate proband; open symbols: clinically unaffected; solid symbols: clinically affected; +: clinically evaluated; +/−: heterozygous with familial mutation; −/−, wild type. For the FZD4 mutations, the nomenclature is based on GenBank NM_012193.2. (C) Sequence chromatograms of heterozygous mutation c.1330T>C. (D) Protein sequence alignment of human Frizzled-4 with homologues from human and other species: mouse, zebrafish, cow, chicken, rat and frog Frizzled-4 proteins and the human Frizzled family proteins. The novel mutation I437T was indicated.

APPENDIX 4. *FZD4* VARIANTS WITH UNKNOWN SIGNIFICANCE IDENTIFIED IN 100 PROBANDS WITH FAMILIAL EXUDATIVE VITREORETINOPATHY

To access the data, click or select the words "Appendix 4." Hetero: Heterozygous; NA: Not available; * The Family 4 harbored both Y211H and S345_A351del mutations, and Y211H mutation was also found in a singleton. Family ID Location Allele status cDNA Change Protein Change Type Probands With FEVR (n=100) Control Subjects (n=150) SIFT PolyPhen2 Align GVGD Source 4,35 Eon2 Hetero 631T>C Y211H* Missense 1/100 0/150 0,D Benign Class C65 [12]. 34 Exon2 Hetero 1310T>C I437T Missense 1/100 0/150 0,D Probably damaging Class C65 Novel For SIFT, amino acids changes with probabilities <0.05 are predicted to be deleterious. (D: Deleterious. T: Tolerated); For Align GVGD, class C0 indicates that a change is unlikely to be pathogenic, while class C65 represents the highest likelihood of a change to be pathogenic.

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