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Targeted next-generation sequencing analysis identifies novel mutations in families with severe familial exudative vitreoretinopathy

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Purpose: Familial exudative vitreoretinopathy (FEVR) is a genetically and clinically heterogeneous disease, characterized by failure of vascular development of the peripheral retina. The symptoms of FEVR vary widely among patients in the same family, and even between the two eyes of a given patient. This study was designed to identify the genetic defect in a patient cohort of ten Chinese families with a definitive diagnosis of FEVR.
Methods: To identify the causative gene, next-generation sequencing (NGS)-based target capture sequencing was performed. Segregation analysis of the candidate variant was performed in additional family members by using Sanger sequencing and quantitative real-time PCR (QPCR).
Results: Of the cohort of ten FEVR families, six pathogenic variants were identified, including four novel and two known heterozygous mutations. Of the variants identified, four were missense variants, and two were novel heterozygous deletion mutations [*LRP5*, c.4053 DelC (p.Ile1351IlefsX88); *TSPAN12*, EX8Del]. The two novel heterozygous deletion mutations were not observed in the control subjects and could give rise to a relatively severe FEVR phenotype, which could be explained by the protein function prediction.
Conclusions: We identified two novel heterozygous deletion mutations [*LRP5*, c.4053 DelC (p.Ile1351IlefsX88); *TSPAN12*, EX8Del] using targeted NGS as a causative mutation for FEVR. These genetic deletion variations exhibit a severe form of FEVR, with tractional retinal detachments compared with other known point mutations. The data further enrich the mutation spectrum of FEVR and enhance our understanding of genotype–phenotype correlations to provide useful information for disease diagnosis, prognosis, and effective genetic counseling.

Familial exudative vitreoretinopathy (FEVR; OMIM 133780) is a rare genetic disorder characterized by abnormal development of retinal blood vessels or incomplete vascularization of the peripheral retina [1], first described by Criswick in 1969 [2]. The symptoms of FEVR vary widely among patients in the same family, and even between the two eyes of a given patient [3], with phenotypes ranging from the absence of visual symptoms to total blindness. Most symptomatic individuals with FEVR experience onset in early infancy, frequently manifesting retinal folds, tears, and detachments in the first decade of life [4]. These ocular anomalies are then followed by additional complications, such as peripheral retinal ischemia, retinal neovascularization and exudates, temporal dragging, vitreous bleeding, vitreoretinal traction, ectopia of the macula, and cataracts [5].

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FEVR is genetically heterogeneous, displaying various inheritance patterns depending on the genetic architecture involved [6]. To date, six candidate genes have been confirmed to be associated with the development of FEVER. The *frizzled-4* (*FZD4*; 11q14-q21; OMIM 604579) [7], *low density lipoprotein receptor like protein 5* (*LRP5*; 11q13.4; OMIM 603576) [8], and *tetraspanin-12* (*TSPAN12*; 7q31.31; OMIM 613,138) [9] genes have all been associated with autosomal dominant or autosomal recessive FEVR. Mutations in the *zinc finger protein-408* (*ZNF408*; 11p11.2; OMIM 616454) [10] and *kinesin family member 11* (*KIF11*; 10q23.33; OMIM 148760) [11,12] genes have exclusively been reported to have an autosomal dominant pattern of inheritance. In contrast, X-linked recessive FEVR can be caused by mutations in the *Norrie disease pseudoglioma* (*NDP*; Xp11.4-p11.3; OMIM 300658) [13] gene. However, it has been reported that the genetic effect attributable to the gene mutations above is

about 50% [10], indicating that additional risk loci for FEVR remain to be identified.

Recently, it has been reported that FEVR severity detected in patients with *TSPAN12* deletion mutation was not more severe than in a patient with *TSPAN12* point mutation [14], which is different from our traditional knowledge. To gain a more comprehensive mutation spectrum in FEVR patients and explore the severity of deletion mutation in FEVR, we custom designed a targeted gene region capture protocol, using next-generation sequencing next-generation sequencing (NGS) to identify genetic defects in a patient cohort of ten unrelated Chinese families afflicted with FEVR. Pathogenic mutations have been identified for six probands, and two novel heterozygous deletion mutations [*LRP5*, c.4053 DelC (p.Ile1351IlefsX88); *TSPAN12*, EX8Del] were identified to give rise to a relatively severe FEVR phenotype, which could be explained by the protein function prediction.

METHODS

Clinical evaluation: The present study involved ten probands and their family members, from ten unrelated families (n=41, male=19, female =22, age= 38.74±21.87 years old) as well as 256 normal control volunteers (male=123, female=133, age= 35.55±23.99 years old) between July 2015 and August 2016 at the Eye & Ent Hospital of FUDAN University, who come from the South-East of China. Patients were diagnosed with FEVR by clinical diagnostic criteria based on an ophthalmic examination, including best-corrected visual acuity testing, color vision (the Ishihara color plate), slit-lamp biomicroscopy, tonometry (Humphrey VisualField Analyzer, Carl Zeiss Inc., Jena, Germany), dilated fundus examination, spectral domain optical coherence tomography (SD-OCT, Spectralis HRA + OCT, Heidelberg Engineering Inc., Heidelberg, Germany), and fundus fluorescein angiography (FFA) using Retcam (Clarity Medical Systems, Inc., Pleasanton, CA). Blood samples were collected from peripheral blood in EDTA blood collection tubes and stored in 4 °C before further analysis. Genomic DNA was extracted from whole-blood samples using a Gentra Puregene Blood kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. All families' participants in this study signed informed consent and were involved in publication; all procedures were performed in accordance with the tenets of the Declaration of Helsinki and were approved by the Ethics Committee of the Eye & ENT Hospital of Fudan University.

Target capture and next-generation sequencing: Total genomic DNA was extracted by using a blood DNA extraction kit according to the standard protocol. The genomic DNA sample of the proband in each family was subjected to analysis using panel-based NGS.

A gene capture panel was designed to encompass the exons and untranslated (UTR) region of 790 genes related to eye disease. The capture probes were custom designed and produced by BGI (Shenzhen, China). Before the study, we tested the sensitivity of our method using one YH sample (a Han Chinese, a representative of Asian individual), by sequence capture performed on a BGISEQ-500 sequencer at different times. Briefly, all samples had an average depth of more than 400X, and the coverage of the target region was around 99.9% by using BGISEQ-500 (Figure 1). Exon deletion was identified by copy number variation (CNV) detection using a statistical algorithm in the workflow as was reported in 2014 [15].

Bioinformatics analysis: We aligned sequence data to the human reference genome (UCSC hg 19) with the Burrows-Wheeler aligner version 0.7.10 (BWA-MEM) [16], performed variant calling using Genome Analysis Toolkit version 3.3, and completed functional annotation of the variants using Annovar [17] and SnpEff [18]. Then the variants identified through this pipeline were further filtered to eliminate benign variants with minor allele frequency (MAF) >0.1% in the 1000 Genomes data set [19], the Single Nucleotide Polymorphism (dbSNP) [20], Exome Aggregation Consortium (ExAC) [21], and ESP6500 [22] databases, and internal data. Finally, the variant prioritizations were performed combining total depth, quality score, MAF, potential deleterious effect, and the existence of mutation reports in common databases, such as the Human Gene Mutation Database (HGMD) [23], the Retinal Information Network (RetNet) [24], ClinVar [25], and Online Mendelian Inheritance in Man (OMIM) [26].

Mutation validation: The NGS-panel sequencing and data analysis called the different heterozygous genetic variants in each respective proband family. Sanger sequencing was used to identify the point deletion variant. To confirm the large deletion, quantitative real-time (QPCR) was performed on the sample. PCR primers were designed with Primer3, the sequences were as follows: (family A) 5′-GGT GGG TGG AGA CTG TAC TA-3′ and 5′-ACT GAG GCA GAC TCT GTA GC-3′; (family B) 5′-CCC TGA TCA GCT TAA GAC AAT G-3′ and 5′-TCA GAA AAC TTT CAA TAT TGG TGA C-3′. The first step in the PCR is performed at 95 °C for 5 min, then followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55°C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 7 min. The first stage of QPCR is performed at 95 °C for 2 min, the second stage includes 30 cycles of 95 °C for 15 s, 58 °C for 30 s, 72 °C for 30 s, the third stage is the melt curve stage.

RESULTS

Patient characteristics: A total of ten probands and their family members were recruited to participate in this study based on the diagnosis of FEVR in one or more member. Three patients in family A (five members) and four patients in family B (six members) were identified as having a clinical diagnosis of severe FEVR. The proband in family A is a 25-year-old woman. She has had a progressive decrease in visual acuity, experiencing blindness at age 2–3 years old. Her right eye was enucleated at age 4. The ophthalmic examination (left eye) showed the intraocular pressure (IOP) was 11 mmHg. The pupil was occluded by pupillary membrane; thus, the fundus could not be observed. The B-scan ultrasonographic showed an unsmooth peripheral wall of the eyeball and persistent fetal vasculature with the hyaloid artery (Figure 2A). After vitrectomy, the color photos demonstrate prominent subretinal exudate, mild subretinal hemorrhage, and laser scars in the periphery (Figure 2B). The proband in family B is a 34-year-old woman. She has had a progressive decrease in visual acuity since birth. In the fundus were retinal vascular abnormalities, expansion, and a falciform retinal fold with tractional hole formation in the inferotemporal quadrant (Figure 2C). SD-OCT detected retinal detachment and hyperreflective epiretinal membrane-like appearance in the inner surface of the retina (Figure 2D). The FFA test showed various vascular branches of the posterior pole retina, the temporal peripheral retinal showed nonperfusion zones along with abnormal new blood vessels and fluorescence leakage (Figure 2E). The fundus examination was consistent with the diagnosis of FEVR. An ophthalmological examination of the other family members confirmed that they exhibited mild symptoms of FEVR, and the FFA test showed the peripheral avascular area, the most typical feature in FEVR (Figure 3). Clinical information of the probands in the ten families is shown in Table 1.

Mutation identification: Through sequencing and bioinformatics analysis, six pathogenic variants were identified, including four novel and two known heterozygous mutations. Of the variants identified, two novel heterozygous deletion mutations [*LRP5*, c.4053 DelC (p.Ile1351IlefsX88); *TSPAN12*, EX8Del] were identified in the proband families A and B, respectively (Figure 4). Sanger sequencing was used to

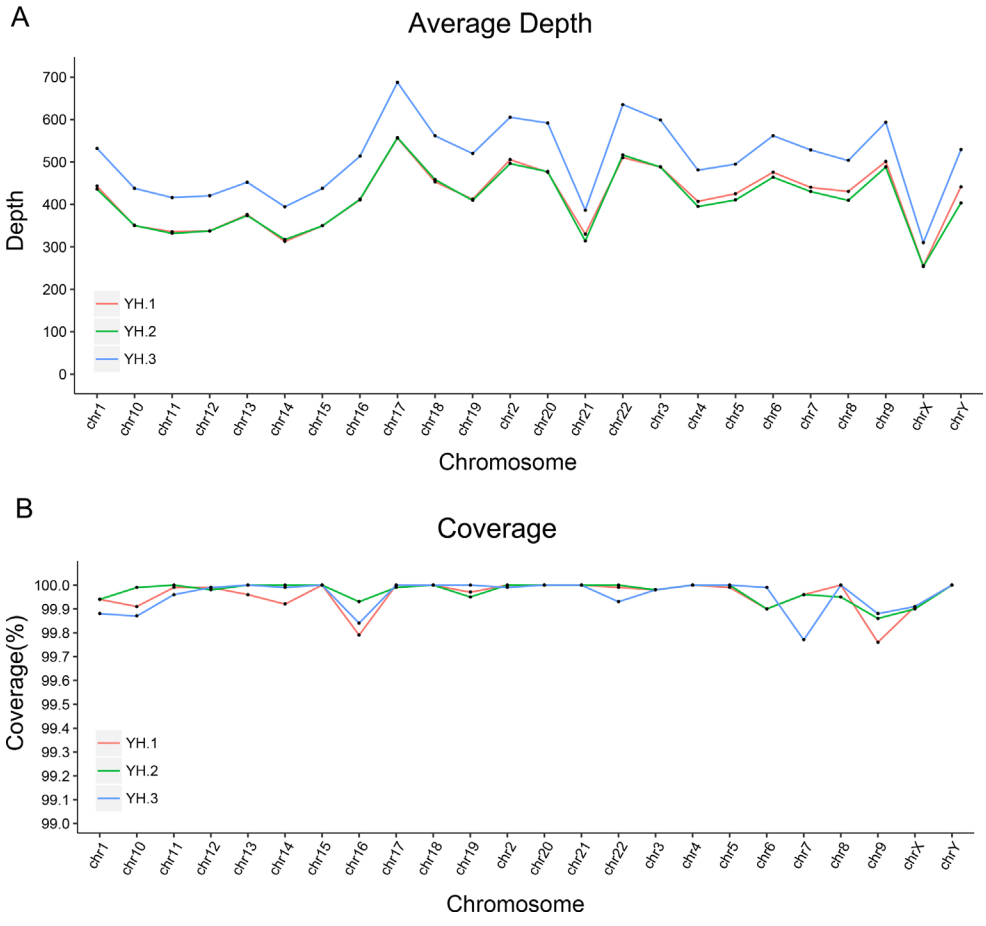

Figure 1. The evaluation of capture panel by YH sample in different sequencing times (YH.1, YH.2, YH.3). **A**: The average depth of the target regions on different chromosomes. **B**: The coverage (%) of target regions on different chromosomes.

further identify the variation (which would result in a frame-shift and a premature-termination codon, p.Ile1351IlefsX88) in members of family A [I:2 (the mother of the proband, affected), II:1 (the proband), III:1 (the son of the proband, affected)]. QPCR was performed on members of family B [II:1 (the proband), II:2 (the sister of the proband, unaffected), II:3 (the sister of the proband, affected), and II:4 (the brother of the proband, affected)]. The results showing the mutations in each family cosegregated with phenotype (shown in Figure 5).

Multiple orthologous sequence alignment (MSA) revealed that 1,351 codon isoleucine of *LRP5* and its subsequent sequences were highly conserved amino acids across different species (Figure 6A). This suggests that any mutation at those codons may lead to a deleterious effect.

The TSPAN12 protein is a member of the tetraspanin family, of which there are 33 members in humans [27]. TSPAN12 contains four transmembrane domains linking three loops: a small extracellular loop (EC1), a large extracellular loop (EC2), and a tiny inner loop (Figure 6B). Within the EC2, tetraspanins have a conserved CCG motif and two other cysteine residues, which are crucial for forming disulfide bonds and protein folding [28]. Exon 8 of the *TSPAN12* gene encodes 101 amino acids from position 205 to 305. The first amino acid is glycine, which is located in the front of the cysteine residue on the EC2. Therefore, the deletion of exon 8 of *TSPAN12* could disrupt the folding of this domain and then abolish the protein function.

DISCUSSION

FEVR is a rare hereditary ophthalmic disease with obvious genetic and phenotypic heterogeneity. Because of this heterogeneity, the relationship between the causative gene and the clinical characteristics is unclear. To our knowledge, there are many reports of pathogenic point variations associated with FEVR but comparatively few reports of deletion mutations. In addition, few reports include measurements of FEVR severity in patients with different types of genetic variants. In this study, we mainly focused on two FEVR families with deletion variants and describe their genotype–phenotype correlations. Two novel pathogenic deletion variants in two different genes (*LRP5*, c.4053DelC (p.Ile1351IlefsX88)

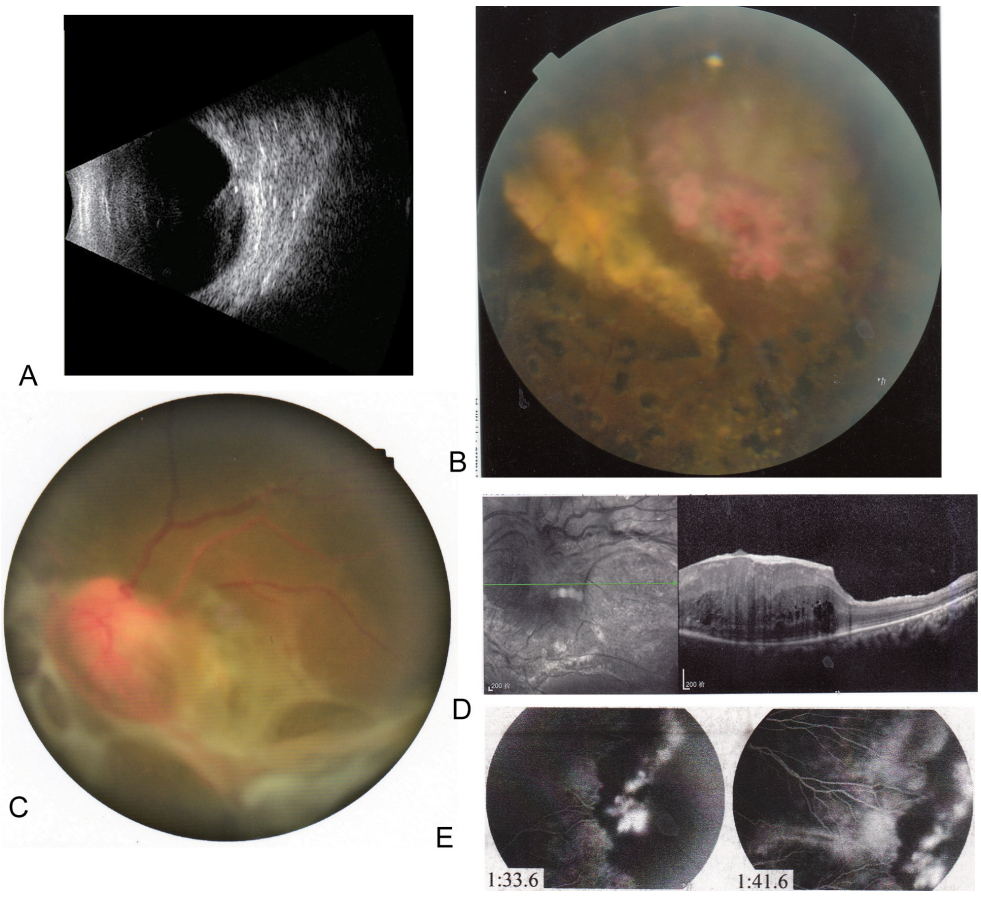

Figure 2. Clinical examinations of patients with severe FEVR. **A**: B-scan ultrasonographic of the proband in family A reveals an irregular peripheral wall of the eyeball and persistent fetal vasculature with the hyaloid artery. **B**: Fundus image of the proband in family A. **C**: The same for family B. **D**: Spectral domain optical coherence tomography (SD-OCT) detected retinal detachment and hyperreflective epiretinal membrane-like appearance in the inner surface of the retina. **E**: Fundus fluorescein angiography (FFA) shows the various vascular branches of the posterior pole retina. The temporal peripheral retinal shows nonperfusion zones along with abnormal new blood vessels and fluorescence leakage.

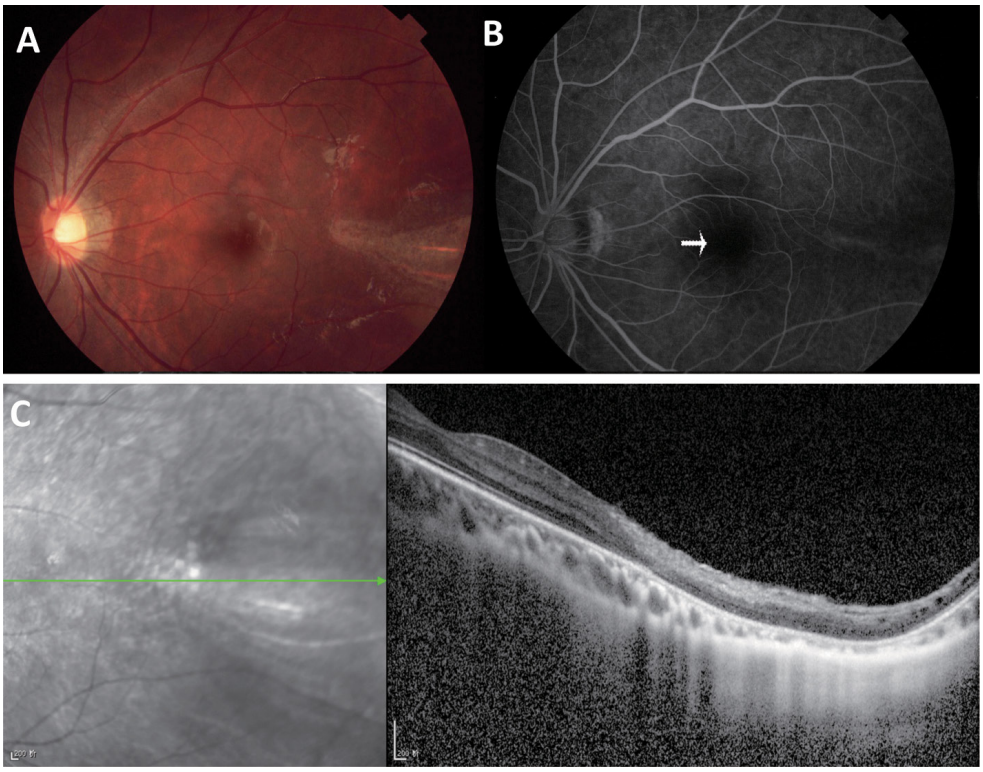

Figure 3. Clinical examinations of the patients with mild FEVR. **A**: Fundus image of the proband with mild familial exudative vitreoretinopathy (FEVR). **B**: Fundus fluorescein angiography (FFA) shows the vessels in the peripheral retina walking as broom-like, and the temporal peripheral retinal shows capillary nonperfusion zones. **C**: Spectral domain optical coherence tomography (SD-OCT) detected retinal dystrophy and a hyperreflective epiretinal membrane-like appearance in the inner surface of the retina.

and *TSPAN12*, EX8Del) were detected using targeted NGS. These two mutations are not recorded in all public mutations databases (dbSNP, EXAC, and ESP6500). The *LRP5* gene, encoding a transmembrane low-density lipoprotein receptor, is a coreceptor involved in the canonical Wnt and/or Norrin/b-catenin pathway signaling network which plays a vital role in vascular development [29,30]. *LRP5* mutations are reported to contribute 12–25% of FEVR. In this study, two out of ten families have pathogenic genetic variants in *LRP5,* a proportion which is consistent with past studies [29,31]. A novel pathogenic heterozygous frame-shift variant in *LRP*5, c.4053 DelC was detected in the affected patients from family A but

Table 1. Clinical information of the probands in the ten families.

Probands ID	Age/sex	BCVA (R/L)	Retinal vascular abnormalities	Retinal detachment	Retina fold	Retinal neovascularization	Fluorescence leakage	Nonperfusion zones	Mutation
1	25/female	*/NLP	-/+	-/+	-/+	-/+	-/+	-/+	LRP5; c.4053 delC
2	34/female	HM/0.15	+/+	-/+	-/+	+/+	+/+	+/+	TSPAN12; EX8DEL
3	18/female	0.8/0.4	+/+	–/–	-/+	–/–	+/+	-/+	LRP5; c.746_747 delCT
4	34/female	0.6/0.4	+/+	–/–	–/–	-/+	+/+	+/+	TSPAN12;c.806A>T
5	34/male	0.6/0.6	+/+	–/–	–/–	–/–	+/–	+/+	-
6	15/male	0.6/0.6	+/+	–/–	–/–	–/–	+/+	+/+	-
7	62/male	0.5/0.4	+/+	–/–	–/–	–/–	-/+	+/+	-
8	20/male	0.6/0.8	+/+	–/–	–/–	–/–	+/–	+/+	-
9	1/female	*	+/+	–/–	–/–	–/–	*	*	FZD4; c.313 A>G
10	2/male	*	+/+	–/–	–/–	–/–	*	*	FZD4; c.313 A>G

*Not obtained. NLP: no light perception. HM: hand motion.

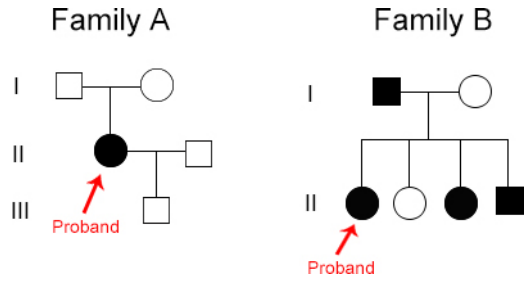

Figure 4. Pedigrees of the families with FEVR who participated in this study.

not in the 256 in-house controls. Furthermore, it has been reported that the transcript would be degraded if the stop codon was located >50–54 bp away from the beginning of the final exon [32]. *LRP5* has 23 exons, and c.4053 DelC, which located on exon 19, is more than 54 bp from the beginning of exon 23. Accordingly, as a new stop codon, c.4053 DelC would lead to degradation of *LRP5* mRNA. Based on the type of mutation and clinical features, it was presumed that the heterozygous frame-shift variation in *LRP5*, c.4053 DelC is causative of FEVR.

The TSPAN12 protein is a member of the tetraspanin family and is required for Norrin-induced β-catenin signaling transduction and regulation [33]. It accounts for 3% to 10% of the mutation frequencies of FEVR [31]. Recent research has reported that deletions in exon 4 of *TSPAN12* can result

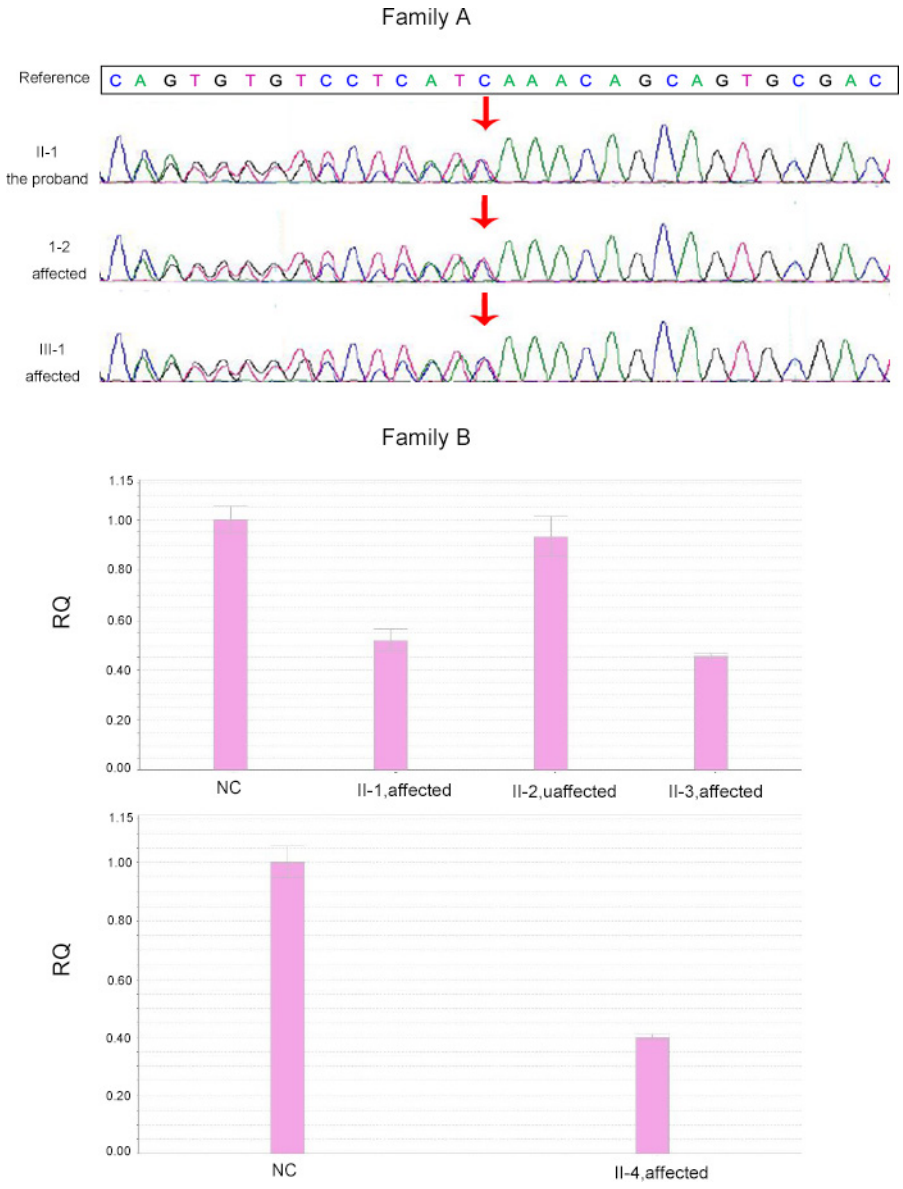

Figure 5. Identification of the mutations in two families. Family A: A heterozygous deletion *LRP5*, c.4053 DelC. Family B: A heterozygous deletion *TSPAN12*, EX8Del.

in FEVR, and large *TSPAN12* deletions were more common than single nucleotide variants [14]. In the current study, large *TSPAN12* deletions in exon 8 were detected in the patients from family B and were cosegregated with the expected phenotype within the families, though further investigation is required to evaluate the functional significance of this sequence change.

The phenotype of FEVR has long been assumed to be isomorphic to the genes associated with it. Toomes et al. identified that patients with two variant alleles in *TSPAN12* have a much more severe phenotype than those with a single variant [34]. The *LRP5* and *TSPAN12* genes usually result in a more severe phenotype [30,34]. *FZD4* variants seem to result in a milder phenotype than variants in *LRP5* [35]. In this study, all affected members harboring *LRP5*, c.4053 DelC (p.Ile1351IlefsX88), or *TSPAN12*, EX8Del have a severe phenotype, including tractional retinal detachments, whereas the majority of the remaining affected family members with characterized genetic variants presented a milder phenotype, restrained to retinal exudates and peripheral avascularity. These data raise the possibility that patients with heterozygous mutations in *LRP5*, c.4053 DelC (p.Ile1351IlefsX88), or *TSPAN12*, EX8Del have a severe form of FEVR. Due to the limited number of subjects, analyzing a larger population of clinically diagnosed patients actually might provide hints for suggesting the precise genotype–phenotype correlation.

This study is the first replication that a heterozygous frame-shift variant in *LRP5*, c.4053 DelC or deletions within exon 8 of *TSPAN12* can result in FEVR, and these genetic variations exhibit an exceptionally severe form of FEVR with tractional retinal detachments. The present study provides useful information for disease diagnosis, prognosis, and genetic counseling. Further analyses of these two novel, presumably causative genetic variants and the correlations between the genotype and the phenotype associated may contribute to a better understanding of the pathophysiological consequences of FEVR, as well as possible prophylactic treatments for retinal detachment in individuals with this genetic predisposition.

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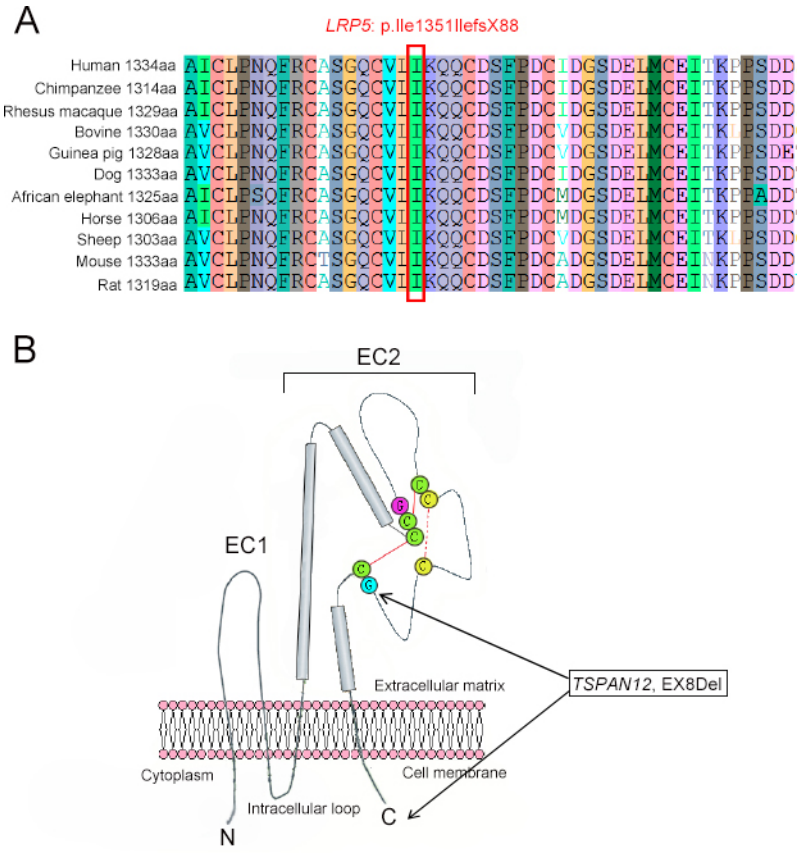

Figure 6. Overview of mutations in *LRP5* and *TSPAN12*. **A**: Protein sequence alignment of human *LRP5* with its orthologs in family A. **B**: The location of the mutation on the TSPAN12 protein domains (structural features of tetraspanins were obtained from Hemler et al. (2005)). Tetraspanins contain four transmembrane domains, a small extracellular loop (EC1), a large extracellular loop (EC2), and a tiny inner loop. Most protein–protein interaction sites and monoclonal antibody epitopes map to EC2. All tetraspanins contain a CCG motif and two other conserved cysteines (green), which are arranged to form two intramolecular disulfide bonds (red lines). Many tetraspanins contain two additional cysteines (yellow), which form another intramolecular disulfide bond (dotted red line).

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