Spectrum of Variants in 389 Chinese Probands With Familial Exudative Vitreoretinopathy

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Submitted: December 6, 2017
Accepted: September 24, 2018

Citation: Li J-K, Li Y, Zhang X, et al. Spectrum of variants in 389 Chinese probands with familial exudative vitreoretinopathy. *Invest Ophthalmol Vis Sci*. 2018;59:5368–5381. https://doi.org/10.1167/iovs.17-23541

Purpose. To identify potentially pathogenic variants (PPVs) in Chinese familial exudative vitreoretinopathy (FEVR) patients in *FZD4*, *LRP5*, *NDP*, *TSPAN12*, *ZNF408*, and *KIF11* genes.

Methods. Blood samples were collected from probands and their parent(s). Genomic DNA was analyzed by next-generation sequencing, and the sequence of selected variants were validated by Sanger sequencing. The potential pathogenicity of a variant was evaluated by in silico analysis and by cosegregation of the variant with disease. Each proband was subjected to comprehensive retinal examinations, and the severity of FEVR was individually graded for each eye. Whenever possible, fundus fluorescein angiography was obtained and analyzed for parent(s) of each proband. Variation in mutation expressivity was analyzed.

Results. Three hundred eighty-nine consecutive FEVR patients from 389 families participated in this study. About 74% of the probands were children younger than 7 years old. One hundred one PPVs, 49 variants with unknown significance (VUS), were identified, including 73 novel PPVs and 38 novel VUS. One hundred ten probands carried PPV (28.3%), and 51 probands carried VUS (13.1%). PPVs in *FZD4*, *LRP5*, *TSPAN12*, *NDP*, *ZNF408*, and *KIF11* were found in 8.48%, 9.00%, 5.91%, 4.63%, 0.77%, and 0.77% of the cohort, respectively. Probands carrying PPVs in *NDP* and *KIF11* had more severe FEVR in general than those carrying PPVs in other genes. Overall, variants in *LRP5* and *FZD4* showed more significant variation in phenotype than variants in *TSPAN12* and *NDP* genes.

Conclusions. Our study expanded the spectrum of PPVs associated with FEVR.

Keywords: familial exudative vitreoretinopathy (FEVR), PPVs, cosegregation

Familial exudative vitreoretinopathy (FEVR) is an inheritable disorder of retinal blood vessel development that is characterized by incomplete vascularization and poor differentiation in the retina.[1] FEVR was first described by Criswick and Schepens in 1969.[2] The clinical manifestations of the disease are complicated and variable.[3] Mild forms of the condition can be asymptomatic and only exhibit peripheral retinal vascular abnormalities, such as a peripheral avascular zone, venous telangiectasias, and altered arterial tortuosity. Severe forms of FEVR are associated with retinal neovascularization, subretinal and intraretinal hemorrhages, exudates, retinal folds, and tractional retinal detachment.

Variants in genes involved in Wnt/Norrin signaling pathway, *NDP* (OMIM 300658), *FZD4* (OMIM 604579), *LRP5* (OMIM 603576), and *TSPAN12* (OMIM 613138), were known to be causative of FEVR.[4] The roles of the Wnt pathway in ocular development and retinal vascular development were also demonstrated in animal models.[5,6] In addition, variants in *ZNF408* (OMIM 616465) and *KIF11* (OMIM 148760) were also associated with FEVR.[7,8] *ZNF408* encodes a zinc finger protein. It was required for zebrafish retinal vasculogenesis.[7] As a transcription factor, ZNF408 protein was recently reported to affect the expression of genes involved in vasculature development and tube formation in cultured cells.[9] However, so far, only a few *ZNF408* variants were found in FEVR patients.[10,11] *KIF11* encodes a kinesin protein that is required for spindle assembly during mitosis. It was known to cause a rare autosomal dominant inheritable disease called microcephaly with or without chorioretinopathy, lymphedema, or mental retardation ([MCLMR] OMIM 152950).[12] Recently, variants in *KIF11* were also found in FEVR patients with or without microcephaly or mental retardation, suggesting that it may be a causative gene for FEVR.[8,13,14] However, it remains unclear how *KIF11* is involved in the development of FEVR.

In this study, we screened for potentially pathogenic variants (PPVs) in the above six genes in 389 unrelated Chinese FEVR patients and explored the phenotypical characteristics associated with variants in each gene.

Methods

Ethical Declarations

This study was approved by the Institutional Review Board of Xin Hua Hospital, which is affiliated with Shanghai Jiao Tong University School of Medicine. All investigation was performed in accordance with the Declaration of Helsinki. Informed written consent was obtained from the adult patients or parents/guardians of the underage patients.

Clinical Examinations, Diagnosis, and Grading of FEVR

Each proband was subjected to the following ocular examinations: wide-field fundus photography using either a RetCam (Clarity Medical Systems, Pleasanton, CA, USA) or an Optos 200Tx (Optos, Inc., Marlborough, MA, USA) and indirect ophthalmoscopy with a 28D lens plus scleral depression when needed. The following clinical presentations were recorded: peripheral avascular zone, peripheral neovascularization, exudation, peripheral vascular tortuosity, tractional retinal detachment, falcifold retinal detachment, macular dragging, retrolental plaque, complete retinal detachment, lens opacity, and chorioretinopathy. For probands who carried variants in *KIF11*, we also examined for the existence of microcephaly and mental retardation.

The diagnosis of FEVR was based on the presence of at least one of the following retinal vascular developmental anomalies as previously described:[15] a lack of peripheral retinal vasculature with or without variable degrees of nonperfusion, vitreoretinal traction, subretinal exudation, retinal neovascularization occurring at any age, or total retinal detachment with fibrotic mass behind the lens. Patients with history of premature birth were excluded.

The severity of FEVR was graded according to the following criteria:[3,16] stage 1 FEVR had only retinal avasculature in the periphery; stage 2 had retinal neovascularization with or without exudate; stage 3 had extramacular retinal detachment with or without exudate; stage 4 had subtotal macular-involving retinal detachment with or without exudate; stage 5 had total retinal detachment. Each eye was graded separately, and the stage of the more affected eye was taken as the overall stage for a proband.

In addition, the biological parents of each proband were asked to have fundus fluorescein angiography (FFA) using an ophthalmic imaging platform (Spectralis HRA2; Heidelberg Engineering GmbH, Heidelberg, Germany), if they were available and agreeable. The results were used to evaluate if they had clinical presentations of FEVR and the stage of the disease.

Targeted Gene Capture and Next-Generation Sequencing (NGS)

Targeted gene capture and sequencing were performed as previously described (MyGenostics, Baltimore, MD, USA).[14] Briefly, peripheral blood was drawn from each proband and the proband's direct family members, and genomic DNA was extracted and fragmented. Illumina adapters were added to the DNA fragments, and the samples were size-selected for 350- to 400-bp products. This pool of DNA fragments was amplified by PCR and hybridized with DNA capture probes that were specifically designed for the targeted genes. The captured DNA fragments were eluted, amplified again, and subjected to NGS using a sequencing system (Illumina HiSeq 2000; Illumina, Inc., San Diego, CA, USA).

Data Analysis and the Criteria for Reporting and Classification of Variants

The sequenced reads were mapped to the UCSC hg19 (http://genome.ucsc.edu; provided in the public domain by University of California-Santa Cruz, Santa Cruz, CA, USA) human reference genome using the Burrows Wheeler Aligner (BWA) (http://bio-bwa.sourceforge.net/; provided in the public domain by SourceForge Media, La Jolla, CA, USA). Variants were calibrated with Genomic Analysis Toolkit (https://software.broadinstitute.org/gatk/; provided in the public domain by Broad Institute, Cambridge, MA, USA) and the MyGenostics database of 1000 samples.

For a variant that was not reported previously, it was first evaluated by its minor allele frequency (MAF). Any variant with MAF higher than 0.005 (for a potentially recessive variant) or 0.001 (for a potentially dominant variant) was regarded as a sequence polymorphism and would not be further analyzed. Those that passed the filter were then subjected to the following in silico analyses: PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/; provided in the public domain by Harvard University, Cambridge, MA, USA), Sorting Intolerant From Tolerant (http://sift-dna.org; provided by Bioinformatics Institute, Singapore), Mutation Taster (http://www.mutationtaster.org/; provided in the public domain by Berlin Institute of Health, Berlin, Germany), and GERP++ (http://mendel.stanford.edu/SidowLab/downloads/gerp/index.html; provided in the public domain by Stanford University, Stanford, CA, USA). A variant was reported if it was predicted to be conserved by GERP++ and pathogenic by at least one other algorithm, or if it was not conserved but predicted to be pathogenic by at least two other algorithms.

Finally, each in silico–predicted novel pathogenic variant was examined for genotype-phenotype cosegregation in the affected families. Since almost all families that participated in our studies were small, with parents and one child, we were only able to determine cosegregation of a variant based on the phenotype of the variant-carrying parent. If the variant-carrying parent showed signs of FEVR, then we classified the variant as a PPV. If the variant-carrying parent showed no signs of FEVR, or if he/she was not available for diagnosis, the variant was classified as variant of unknown significance (VUS). A de novo variant was also classified as VUS.

All previously identified pathogenic variants were reported in this study. However, since different studies had different criteria to define a pathogenic variant, these variants were reevaluated. If the MAF of a known variant was higher than 0.001 in any of the databases listed above, then it was reclassified as VUS or benign variant based on available data on genotype-phenotype cosegregation. If a known variant showed an MAF of less than 0.001, but no evidence of genotype-phenotype cosegregation in the literature or in this study was found, it was reclassified as a VUS. Otherwise, a known variant was classified as a PPV.

PCR and Sanger Sequencing Validation

Primer3 was used to design all of the PCR primers for the Sanger sequencing that was conducted to validate the PPVs. The average amplicon size was 400 bp. The DNA was sequenced on a genetic analyzer (ABI 3130XL; Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) and subsequently analyzed using Mutation Surveyor.

Haplotype Analysis

Haplotype analysis was performed to determine whether variants in *LRP5* and *FZD4* genes had a cofounder effect. Seventy-eight DNA samples from unrelated donors were used as controls. Single nucleotide polymorphisms (SNP) were selected from https://snpinfo.niehs.nih.gov/snpinfo/snptag.html (provided in the public domain by the National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA). For the *LRP5* gene, the following SNP markers were selected: rs312014 at Chr11. 68084962; rs312024 at Chr11. 68094431; rs634008 at Chr11. 68094741; rs312779 at Chr11. 68108676; rs11823032 at Chr11. 68145166; rs314773 at Chr11. 68149450; rs4930573 at Chr11. 68163456; rs583545 at Chr11. 68178635; rs3736228 at Chr11. 68201295. Collec-

Table 1. General Information of the Probands Involved in this Study

	Number of Probands (% in the Group)			
	Overall, $n = 389$	PPV Carriers, $n = 110$	VUS Carriers, $n = 51$	Negative, $n = 228$
Number of Probands				
Male	267 (68.6%)	84 (76.4%)	33 (64.7%)	150 (65.8%)
Female	122 (31.4%)	26 (23.6%)	18 (35.3%)	78 (34.2%)
Age structure				
0–3 y	200 (51.4%)	61 (55.5%)	28 (54.9%)	111 (48.7%)
4–6 y	89 (22.9%)	25 (22.7%)	15 (29.4%)	49 (21.5%)
7–10 y	58 (14.9%)	16 (14.5%)	2 (3.9%)	40 (17.5%)
11–17 y	28 (7.2%)	7 (6.4%)	4 (7.8%)	17 (7.5%)
≥18 y	14 (3.6%)	1 (0.9%)	2 (3.9%)	11 (4.8%)
Family history*				
Positive	166 (42.7%)	92 (83.6%)	11 (21.6%)	63 (27.6%)
Negative	148 (38.0%)	11 (10.0%)	29 (56.9%)	111 (48.7%)
Data missing	75 (19.3%)	8 (7.3%)	11 (21.6%)	54 (23.7%)

* A positive family history indicates that one or both parents was/were diagnosed with FEVR. A negative indicates that neither parent had FEVR. Data missing indicates that information for both parents was missing or that one parent was negative for FEVR and the information for the other parent was missing.

tively, a 116,333-bp region was covered (chromosome 11: 68,084,962–68,201,295). For the *FZD4* gene, the following SNP and/or short tandem repeat markers were selected: rs713065 at Chr11. 86657520; rs10898563 at Chr11. 86659213; rs3758657 at Chr11. 86668446; rs7925666 at Chr11. 86669535; and rs11234891 at Chr11. 86670842. Collectively, a 13,322-bp region was covered.

PCR amplification was performed using 50 ng genomic DNA under standard conditions in a total volume of 50 μL. Forward primers were labeled with fluorescein amidite. PCR products were sequenced using capillary electrophoresis in a genetic analyzer (ABI3130XL; Applied Biosystems). Data were analyzed using bioinformatics software (Haploview 4.2; Broad Institute, Cambridge, MA, USA). All SNPs had MAF > 5% and $r^2 > 0.8$.

Statistical Analysis

Statistical software (*SPSS* v. 21; IBM Corp., Armonk, NY, USA) was used for data analysis. χ^2 Analysis was performed to compare the differences between nominal variables, such as the distribution of gender, stage of FEVR, and existence of family history. A P value of 0.05 was accepted as statistically significant. For the comparison of the phenotypes caused by variants in different genes, an adjusted P value of 0.01 was accepted as statistically significant since there were six groups.

Results

Demographic Information of the Cohort and General Profile of FEVR

This study included 389 consecutive FEVR probands who came to our clinic from March 1, 2015, to August 31, 2016. They came from unrelated families, and all consented to this study. The general description of the cohort is in Table 1. The number of male probands (267) were about twice the number of the female probands (122). Almost 90% of the probands were younger than 11 years old. Only 14 probands were 18 years old and older. We obtained and analyzed 633 FFA images of parents from 320 families. One hundred fifty-five families had one parent who had signs of FEVR. In 11 families, both parents had FEVR. The detailed information about each proband and the family history of FEVR is provided in Supplementary Table S1.

We further divided the whole cohort into three groups based on the variants they carried: the PPV carriers, the VUS carriers, and negative, who did not carry any PPV or VUS identified in this study (Table 1). The male/female ratio and age structure were similar among three groups. Among probands who carried PPVs in different genes, there was also no significant differences in age structure and male/female ratio (data not shown). Overall, 42.7% of the probands had positive family history of FEVR. About 84% of the PPV carriers had positive family history of FEVR, which was consistent with the criteria we used to classify PPV in this study.

To better describe the phenotype of FEVR in this cohort, we separated all probands by age and counted the number of probands at each stage of FEVR. We found that the percentage of probands with stage 4 and 5 FEVR decreased progressively with age. At the age of 0 to 3 years, about 50% of the probands had stage 5 FEVR, 32% had stage 4 FEVR, and only about 10% had stage 1 and 2 FEVR combined. Among those 18 years and older, about 36% of the probands had stage 4 and 5 FEVR combined, and 21% had stage 1 and 2 FEVR combined. To simplify the analysis, we divided the cohort into two age groups: 1 to 6 years old and 7 years and older. The number of probands at five stages of FEVR in these two age groups was further separated by variants they carried, and the results are shown in Table 2. Overall, there was significant difference in FEVR profiles between the two age groups ($P < 0.001$, Pearson χ^2 analysis). The younger group had a higher percentage of stage 4 and 5 FEVR and lower percentage of stage 1 and 2 FEVR. However, within the same age group, the profiles were similar among the PPV carriers, VUS carriers, and negatives.

Overall Spectrum of Variants Identified

For the target captured sequences, the average coverage was 375.6 reads per base. About 98.5% of all bases had greater than ×10 coverage. Since FEVR is a rare disease with a predominantly dominant inheritance pattern, we took a strict filtering process as described in the Methods section to report a new variant. All previously reported variants identified in this study were also reanalyzed. Overall, we found 101 PPVs and 49 VUS, including 73 new PPVs and 38 new VUS (Table 3). Eleven previously reported PPVs were reclassified as VUS. Two previously reported variants were reclassified as benign

Table 2. Number of Probands at Five stages of FEVR Separated by Age and Variant Carried

	Number of Probands (% in the Group)							
	0–6 y				≥7 y			
	Overall, $n = 288$	PPV Carriers, $n = 85$	VUS Carriers, $n = 43$	Negative, $n = 160$	Overall, $n = 99$	PPV Carriers, $n = 23$	VUS Carriers, $n = 7$	Negative, $n = 68$
Stage 1	13 (4.51%)	2 (2.35%)	2 (4.65%)	9 (5.63%)	11 (11.11%)	2 (8.70%)	1 (12.5%)	8 (11.76%)
Stage 2	25 (8.68%)	12 (14.12%)	4 (9.30%)	9 (5.63%)	9 (9.09%)	2 (8.70%)	1 (12.5%)	6 (8.82%)
Stage 3	29 (10.07%)	8 (9.41%)	3 (6.98%)	18 (11.25%)	24 (24.24%)	5 (21.74%)	1 (12.5%)	18 (26.47%)
Stage 4	94 (32.64%)	30 (35.29%)	19 (44.19%)	45 (28.13%)	32 (32.32%)	8 (34.78%)	2 (25.0%)	22 (32.35%)
Stage 5	127 (44.10%)	33 (38.82%)	15 (34.88%)	79 (49.38%)	23 (23.23%)	6 (26.09%)	3 (37.5%)	14 (20.59%)

There were two probands without data on the stages of FEVR, one in each age group. Therefore, the total number of probands was 387 instead of 389. There was significant difference in FEVR profiles between the two age groups ($P < 0.001$, Pearson χ^2 analysis). However, within the same age group, the profiles were similar among the PPV carriers, VUS carriers, and the negatives.

variants. The references for known variants are listed in Supplementary Table S2.

A total of 110 probands (28.3%) in this cohort carried PPV, and 51 probands (13.1%) carried VUS. Seven probands carried two heterozygous PPVs in *LRP5*, one proband carried two heterozygous VUS in *LRP5*, and 11 probands carried two heterozygous variants in two different genes. No proband was homozygous for any of the variants found in this study. Two hundred twenty-eight probands (57.8%) were negative for PPV or VUS.

Variants in *FZD4*

We found nine previously reported variants and 22 new variants that were predicted to be pathogenic by in silico analysis. Fifteen novel variations were classified as PPV and seven were classified as VUS (Table 4). Most of the variants were single nucleotide changes and inherited. There were four de novo variants. Three were single nucleotide changes, and one was an insertion of seven nucleotides that caused frameshift of the downstream sequences. A total of 33 probands carried PPVs in *FZD4*, which accounted for 8.48% of the cohort. Thirteen probands carried VUS in *FZD4*, which accounted for 3.34% of the cohort.

The c.40_49del, p.P14fs variant was classified as VUS since the variant-carrying mother of the proband showed normal retinal vasculature by FFA. However, a c.39_49del, p.P14fsX57 variant was previously identified in a big Chinese family with complete genotype-phenotype cosegregation.[17] The proband who carried the c.701C>T, p.T234I variant had a brother who was also heterozygous for this variant. He also had clinical signs of FEVR.

With the exception of c.205C>T, p.H69Y and c.1589G>A, p.G530E, the rest of all previously reported variants found here had MAFs smaller than 0.001. They also exhibited phenotype-genotype cosegregation in the affected families in this study; therefore, they were classified as PPVs.

The c.205C>T, p.H69Y was identified by multiple groups in Japanese and Chinese FEVR patients.[17–19] MAF of this variant was 0.0037 in gnomAD_genome_EAS, but it was 0.000 in other ethnic groups. Previously reported data showed inconsistent cosegregation of the variant with the phenotype of FEVR in the affected families. Cosegregation was observed in two Chinese families.[17] In vitro studies showed that the p.H69Y-mutated FZD4 protein caused small yet significant decrease in Norrin binding.[20] In a recent publication, Kondo et al.[21] proposed that it is a "pathogenic risk allele" that may cause severe FEVR phenotype if an additional variant in *trans* is present. Here we found six probands who were heterozygous for this variant. Genotype-phenotype cosegregation was observed in one family. There was one proband who also carried another variant in *LRP5* (c.2237G>A). This proband inherited both variants from his mother, who had normal retinal vasculature. The variant-carrying parents of the other five probands all showed normal retinal vasculature. Collectively, our data did not support its role as a PPV. In an attempt to undercover the cause for such high allele frequency in the cohort, we performed a haplotype analysis on four consented probands, and the results suggested a cofounder effect (Table 5). Taking together evidence from these studies, we tentatively classified c.205C>T, p.H69Y as a VUS.

The c.1589G>A, p.G530E variant was also reported as potentially pathogenic.[22] This variation was identified in a patient with no family history. The MAF for this variant was 0.00185 for East Asian in the GNOMAD database, and it was 0.000 in other ethnic groups. It was classified as "likely benign" in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/25555963/, provided in the public domain by National Center for Biotechnology Information, Bethesda, MD, USA). We found six probands carrying this variant. They all inherited the variant from their respective parents. FFA images obtained from all four variant-carrying parents showed normal retinal vasculature. Haplotype analysis that was performed on four consented probands suggested a cofounder effect (Table 5). Collectively, we classified this as a benign sequence variant.

Two other variants were also previously reported and found in multiple probands in our cohort: c.313A>G (five probands)[18,23,24] and c.1282_1285del (seven probands).[25] For

Table 3. Overall Spectrum of Variants Found in This Study

Gene Name	*FZD4*	*LRP5*	*TSPAN12*	*NDP*	*KIF11*	*ZNF408*
NCBI access number	NM_012193	NM_002335	NM_012338	NM_000266	NM_001184751	NM_004523
PPV, *n*, total/new	22/15	33/24	22/18	18/12	3/1	3/3
VUS, *n*, total/new	8/7	17/12	6/6	0/0	13/8	5/5
*Total probands with PPV, *n* (%)	33 (8.48%)	35 (9.00%)	23 (5.91%)	18 (4.63%)	3 (0.77%)	3 (0.77%)
*Total probands with VUS, *n* (%)	13 (3.34%)	26 (6.68%)	8 (2.06%)	0 (0.00%)	13 (3.34%)	5 (1.29%)

* The percentage in parentheses is the percentage in the whole cohort.

Table 4. Variants Identified in *FDZ4*

Feature	Effect	Variants	SIFT	PolyP_2	M_T	GERP++	Heredity	Stage	Sample ID	Seg	Path
New	Missense	c.134G>C, p.C45S	0.001/D	0.687/Ps	1/Dc	4.27/Cons	Father	3 (2/3)	C160926C00701	Unkn	VUS
New	Frameshift	c.158delG, p.C53fs	NA	NA	NA	NA	Mother	4 (4/4)	C150713C02201	Yes	PPV
New	Missense	c.182C>T, p.T61I	0.005/D	0.967/Pb	1/Dc	4.8/Cons	de novo	5 (5/4)	C151224C01201	Nappl	VUS
New	Frameshift	c.204delG, p.H69Tfs*11	NA	NA	NA	NA	Father	5 (5/5)	C160729C00501	Unkn	VUS
New	Missense	c.217_234del, p.73_78del	NA	NA	NA	NA	Father	4 (4/4)	C160809C07501	Yes	PPV
							Mother	5 (5/0)	C160413C00601	Yes	
New	Missense	c.224C>G, p.A75G	0.001/D	0.999/Pb	1/Dc	4.98/Cons	Mother	4 (4/1)	C151224C01101	Yes	PPV
New	Missense	c.341T>A, p.I114N	0/D	0.989/Pb	1/Dc	5.82/Cons	de novo	3 (3/3)	C160406C01601	Nappl	VUS
New	Missense	c.382T>C, p.C128R	0.0/D	0.999/Pb	1/Dc	5.82/Cons	Mother	3 (1/3)	C160809C01901	Yes	PPV
New	Missense	c.478G>C, p.E160Q	0.346/T	0.254/Bn	1/Dc	5.82/Cons	Mother	4 (4/3)	C160621C07901	No	VUS
New	Frameshift	c.621_642del, p.D207Efs*26	NA	NA	NA	NA	Father	2 (2/2)	C160826C01301[a]	Yes	PPV
New	Missense	c.701C>T, p.T234I	NA	1/Pb	1/Dc	5.54/Cons	Mother	5 (1/5)	C150827C01001[b]	Yes	PPV
New	Frameshift	c.747dupC, p.Y250fs	NA	NA	NA	NA	Mother	5 (1/5)	C150616C01001	Yes	PPV
New	Missense	c.905G>A, p.C302Y	0/D	1/Pb	1/Dc	5.59/Cons	Father	4 (1/4)	C160517C00501	Yes	PPV
New	Frameshift	c.917_936del, p.F306fs	NA	NA	NA	NA	Father	5 (2/5)	C160524C00101	Yes	PPV
New	Frameshift	c.936dupT, p.G313fs	NA	NA	NA	NA	Father	5 (5/5)	C160325C00101	Yes	PPV
New	Stopgain	c.981G>A, p.W327X	NA	NA	1/Dc	5.59/Cons	Father	1 (1/1)	C160926C00501	Yes	PPV
New	Frameshift	c.1010dupA, p.H337fs	NA	NA	NA	NA	Mother	4 (4/4)	C150702C01201	Yes	PPV
New	Frameshift	c.1188_1192del, p.F396fs	NA	NA	NA	NA	Father	5 (5/0)	C160527C00701	Yes	PPV
New	Missense	c.1310T>C, p.I437T	0.0/D	0.997/Pb	1/Dc	5.77/Cons	Father	2 (2/2)	C160620C00701	Yes	PPV
New	Missense	c.1325T>A, p.V442E	NA	1/Pb	1/Dc	5.77/Cons	Father	4 (4/4)	C150914C04401	Yes	PPV
New	Frameshift	c.1504_1505insTGTTGGA, p.H502fs	NA	NA	NA	NA	de novo	5 (5/4)	C160505C00601	Nappl	VUS
New	Frameshift	c.40_49del, p.P14fs	NA	NA	NA	NA	Mother	4 (4/0)	C160201C00901	No	VUS
Known	Missense	c.205C>T, p.H69Y	0.007/D	0.183/B	1/Dc	4.8/Cons	Father	5 (5/0)	C160513C00301	Yes	VUS
							Father	2 (2/2)	C160517C03501	No	
							Mother	4 (1/4)	C160728C04101	No	
							Father	2 (2/0)	C151029C03901	No	
							Mother	2 (2/2)	C151201C02501	No	
							Mother	4 (4/2)	C160324C03601[c]	No	
Known	Missense	c.313A>G, p.M105V	0.858/T	0.53/Pd	1/Dc	5.82/Cons	Mother	4 (3/4)	C160104C04601	Yes	PPV
							Mother	4 (2/4)	2015-S07652	Yes	
							Mother	4 (4/0)	C150722C02601	Yes	
							Father	4 (4/0)	C151204C00101	Yes	
							Mother	5 (2/5)	C160201C00801[d]	Yes	
Known	Missense	c.317G>C, p.C106S	0/D	1/Pb	1/Dc	5.82/Cons	Mother	5 (2/5)	C160229C01601	Yes	PPV
Known	Stopgain	c.400G>T, p.E134X	NA	NA	1/Dc	5.82/Cons	Mother	2 (2/2)	C160829C05101	Yes	PPV
Known	Missense	c.542G>A, p.C181Y	0.022/D	0.33/Bn	1/Dc	5.82/Cons	de novo	4 (4/2)	C160331C00101	Nappl	PPV
Known	Missense	c.757C>T, p.R253C	0/D	1/Pb	1/Dc	5.69/Cons	Mother	5 (5/4)	C151112C01501[e]	Yes	PPV
Known	Frameshift	c.1282_1285del, p.D428fs	NA	NA	NA	NA	Father	4 (4/1)	C160826C00601	Yes	PPV
							Father	2 (2/2)	C160219C00601	Yes	
							Father	5 (1/5)	C160322C01501	Yes	
							Mother	4 (0/4)	C150731C00501	Yes	
							Father	5 (5/0)	C160428C00101	Unkn	
							Mother	5 (1/5)	C160414C00601	Yes	
							Father	4 (4/4)	C160718C04701[f]	Yes	
Known	Stopgain	c.1488G>A, p.W496X	NA	NA	1/Dc	6.06/Cons	Mother	5 (5/5)	C160122C00801	Yes	PPV
Known	Missense	c.1589G>A, p.G530E	0.013/D	0.078/B	1/Dc	4.14/Cons	Mother	4 (0/4)	C151127C00201	Unkn	Benign
							Mother	4 (4/4)	C160728C04201	No	
							Mother	5 (5/5)	C160513C01201	Unkn	
							Father	2 (1/2)	C151012C01001	No	
							Father	5 (5/3)	C160321C03001[g]	No	
							Mother	4 (4/4)	C151012C00801	No	

Variants that fit the criteria as described in Methods are listed here. PolyP_2, PolyPhen_2 database; Stage, FEVR stage of the proband in the format of overall stage (right eye/left eye); Seg, status of phenotype-genotype cosegregation in the family; Nappl, de novo variants are not applicable for the analysis; Path, potential pathogenicity of the variant; Unkn, unknown due to the lack of data.

a: This proband was also heterozygous for a PPV in *TSPAN12* (c.826G>A). He inherited both variants from his father, who had stage 2 (2/2) FEVR.

b: This proband had a brother who was also heterozygous for this variant and had clinical signs of FEVR.

c: This proband was also heterozygous for a VUS in *LRP5* (c.2237G>A). He inherited both variants from his mother, who had no signs of FEVR.

d: This proband was also heterozygous for a VUS in *LRP5* (c.3922G>A). He inherited both variants from his mother, who had stage 2 (2/2) FEVR.

e: This proband was also heterozygous for a VUS in *LRP5* (c.3361A>G). She inherited the PPV in *FZD* from her father and the VUS in *LRP5* from her mother. Both parents had stage 1 (1/1) FEVR.

f: This proband was also heterozygous for a VUS in *LRP5* gene (c.3361A>G). He inherited both variants from his father, who had stage 3 (3/2) FEVR.

g: This proband was also heterozygous for c.2092-4C>T in *LRP5*. He inherited both variants came from his father, who had no signs of FEVR.

Table 5. *FZD4* Gene Haplotypes in Probands Carrying Variants as Listed

		Alleles					
Patient ID	**Variant**	**1**	**2**	**c.313**	**3**	**4**	**5**
C151204C00101	c.313A>G	A	A	C	T	C	A
C160104C04601		A	A	C	C	C	G
		Alleles					
		1	**2**	**c.205**	**3**	**4**	**5**
C151029C03901	c.205C>T	A	A	A	T	C	A
C160728C04101		A	A	A	T	C	A
C160513C00301		A	A	A	T	C	A
C160517C03501		A	A	A	T	C	A
		Alleles					
		1	**2**	**c.1589**	**3**	**4**	**5**
C151012C00801	c.1589G>A	G	G	T	T	C	G
C151127C00201		G	G	T	T	C	G
C160728C04201		G	G	T	T	C	G
C160513C01201		G	G	T	T	C	G
		Alleles					
		1	**2**	**c.1282_1285del**	**3**	**4**	**5**
C150731C00501	c.1282_1285del	G	A	D	T	T	G
C160414C00601		A	A	D	C	C	G
C160322C01501		A	A	D	C	C	G
C160826C00601		G	A	D	T	T	G
C160428C00101		G	A	D	T	T	G

The haplotype of the copy that carried the indicated variant was determined. The alleles were as follows: 1: rs713065; 2: rs10898563; 3: rs3758657; 4: rs7925666; and 5: rs11234891. For chromosomal localization of each SNP, please refer to the Methods. The variants were placed according to their chromosomal localization relative to the SNPs: c.313 at Chr11. 86663485; c.205 at Chr11. 86665923; c.1589 at Chr11. 86662209; c. 1282_1285del at Chr11. 86662516. The c.1282_1285del was represented by letter D.

each variant, genotype-phenotype cosegregation was observed in families carrying the variant. MAF was not available for c.313A>G in the GNOMAD database and was 0.000 for c.1282_1285del. Therefore, they were PPVs. Haplotype analysis was also performed among the variant carriers; however, it was not conclusive whether a cofounder effect existed (Table 5).

Variants in *LRP5*

We found 14 previously reported pathogenic variants and 36 novel variants that were predicted to be pathogenic by in silico analysis in *LRP5* (Table 6). Twenty-four novel variants were classified as PPV, and 12 were classified as VUS based on cosegregation evidence. A total of 35 probands in the cohort carried PPVs in *LRP5*, which accounted for 9.00% of the cohort. Twenty-six probands carried VUS, which accounted for 6.68% of the cohort. There were seven probands who carried two heterozygous variants in *LRP5* gene, six probands who carried one variant in *LRP5*, and another one in one of the other genes examined here.

Among the known variants found in this cohort, we reclassified the following variants as VUS: c.518C>T, p.T173M; c.1265C>T, p.A422V; c.3361A>G, p.N1121D; c.1378G>A, p.E460K; c.4517C>T, p.T1506M; and c.433C>T, p.L145F.

The c.518C>T, p.T173M variant was first identified in a British woman with no description of family history.[26] The MAF for this variant in the GNOMAD genome database was 0.003 for East Asian, 0.000 for European, 0.001 for Latino and other. We found one proband in our cohort who was heterozygous for this variant. He was also heterozygous for *LRP5* c.3361A>G. He inherited both variants from his father, who had no signs of FEVR. Due to the lack of evidence on genotype-phenotype cosegregation, we classified this variant as VUS.

The c.1265C>T, p.A422V was first reported in a large family pedigree.[22] However, two heterozygous variants in *LRP5* coexisted in the affected family members, which made it impossible to determine the potential causation by a single variant. Another variant at the adjacent nucleotide was also identified in a FEVR patient (c.1264G>A, p.A422T) with no family history.[22] In our cohort, the variant-carrying parent showed no signs of FEVR. Collectively, we classified this variant as VUS.

The c.3361A>G, p.N1121D variant was first identified in a Japanese FEVR patient, and cosegregation of the variant with phenotypes of FEVR was confirmed in the family.[24] Later it was found in a Chinese FEVR patient with no information about his biological parents.[27] In this study, we found 10 probands who were heterozygous for c.3361A>G. Two out of eight heterozygous variant-carrying parents had signs of FEVR. The MAF for this variant was 0.008 in gnomAD_genome_EAS and 0.000 for other ethnic groups. We performed a haplotype analysis on available samples, and the results suggested a likely cofounder effect among carriers (Table 7). Based on the above information, we tentatively classified this variant as VUS.

The c.1378G>A, p.E460K variant was previously identified in a male proband who had compressed vertebrae and was blind at the age of 3 months.[28] However, no family history was reported. The proband who was heterozygous for this variant in our study (C151225C01901) also carried a variant c.1870C>T, p.R624W. Since we had no information about the family history, we tentatively classified this variant as VUS.

The c.1870C>T, p.R624W variant was also previously reported.[11] Functional analysis showed that the expression of the p.R624W-mutated LRP5 protein caused decreased ligand binding. Pedigree analysis suggested an autosomal recessive inheritance pattern. The heterozygous carriers of the variant were asymptomatic. In our cohort, we found two probands who were heterozygous for the variant. In one affected family, the affected mother who was heterozygous for this variant showed grade 2 FEVR, suggesting an autosomal dominant feature of the variant. Unfortunately, we did not have family history on the other affected proband (C151225C01901). While it was clearly a PPV, more data were needed to determine if it was a dominant or recessive variant.

The c.4517C>T, p.T1506M was previously reported as potentially pathogenic based on in silico analysis without information on family history.[22] In this study, the heterozygous variant-carrying father of the proband showed no abnormalities in retinal vasculature. Therefore, we classified this variant as VUS.

The c.433C>T, p.L145F was previously found in a family with four heterozygous carriers: a mother and three children.[24] All manifested with clinical signs of FEVR. Although we did not find signs of FEVR in the variant-carrying father of the proband, we classified this variant as PPV.

A common sequence polymorphism, c.266A>G, p.Q89R, was also found in nine probands. This variant was identified in both Japanese and Chinese FEVR patients.[24] The MAF for this variant was 0.1012 in East Asians, 0.004 in Europeans, 0.046 in Latinos, and 0.0016 in Africans. In vitro functional analysis

Table 6. Variants Identified in *LRP5* Gene

Feature	Effect	Variant	SIFT	PolyP_2	M_T	GERP++	Heredity	Sample ID	Stage	Seg	Path
New	Missense	c.121C>T, p.R41W	0.18/T	1/Pb	1/Dc	3.31/Cons	Mother	C150713C00801[a1]	5 (2/5)	Yes	PPV
New	Missense	c.485A>C, p.H162P	0.093/T	0.157/Bn	1/Dc	3.61/Cons	Father	C160613C02101	4 (3/4)	Yes	PPV
New	Missense	c.503C>G, p.T168R	0/D	1/Pb	1/Dc	3.56/Cons	Father	C160602C03801	5 (5/4)	Yes	PPV
New	Splicing	c.687-3C>A, splicing	NA	NA	NA	NA	Father	C160819C01301	3 (0/3)	Yes	PPV
New	Missense	c.821T>G, p.I274S	0.001/D	0.396/Bn	1/Dc	3.78/Cons	Father	C160122C00701[b1]	5 (5/1)	Yes	PPV
New	Missense	c.871C>T, p.R291W	0/D	1/Pb	1/Dc	1.35/NC	Father	C150910C03001[c1]	3 (2/3)	Yes	PPV
New	Missense	c.1123G>A, p.A375T	0.05/D	0.746/Ps	1/Dc	3.81/Cons	Mother	2015-S07405	1 (1/1)	Yes	PPV
							Father	C160622C02201	2 (2/2)	Yes	
New	Missense	c.1193G>A, p.R398H	0.01/D	1/Pb	1/Dc	3.81/Cons	Father	C150824C05501	3 (3/0)	Yes	PPV
New	Missense	c.1349G>A, p.R450H	0.001/D	0.903/Ps	1/Dc	3.02/Cons	Father	C160805C01401[d1]	3 (3/3)	No	VUS
New	Stopgain	c.1434G>A, p.W478X	NA	NA	1/Dc	3.3/Cons	Father	C160229C04701	3 (3/1)	Yes	PPV
New	Missense	c.1436G>T, p.G479V	0.001/D	0.999/Pb	1/Dc	3.3/Cons	Father	C160826C02101[e1]	4 (4/1)	Yes	PPV
New	Missense	c.1480C>T, p.R494W	0/D	1/Pb	1/Dc	2.33/Cons	Father	C150713C00801[a2]	5 (2/5)	Yes	PPV
New	Missense	c.1801G>A, p.G601R	0.042/D	0.993/Pb	1/Dc	4.13/Cons	Father	C160820C01101	4 (4/1)	Yes	PPV
New	Missense	c.1969A>G, p.R657G	0.032/D	0.305/Bn	1/Dc	0.193/NC	Father	C160125C00801	5 (0/5)	Yes	PPV
New	Frameshift	c.2050_2051insTGTC, p.V684fs	NA	NA	NA	NA	Father	C160304C00301	2 (2/2)	Yes	PPV
New	Splicing	c.2092-4C>T, splicing	NA	NA	NA	NA	Father	C160321C03001[f]	5 (5/3)	No	VUS
New	Missense	c.2101C>T, p.R701C	0.001/D	1/Pb	1/Dc	4.53/Cons	Mother	C160826C02101[e2]	4 (4/1)	Unkn	VUS
New	Missense	c.2237G>A, p.R746Q	0.008/D	0.934/Pb	1/Dc	4.53/Cons	Mother	C160324C03601[g]	4 (4/2)	No	VUS
New	Missense	c.2255G>C, p.R752P	0.009/D	0.999/Pb	1/Dc	4.53/Cons	Mother	C160805C01401[d2]	3 (3/3)	Yes	PPV
New	Missense	c.2263C>T, p.L755F	0.003/D	0.999/Pb	1/Dc	3.61/Cons	Mother	C160315C00501	4 (4/4)	Yes	PPV
New	Missense	c.2377G>A, p.G793R	0.0/D	1/Pb	1/Dc	3.55/Cons	Mother	C160613C02201[h1]	4 (4/1)	No	VUS
New	Missense	c.2434G>A, p.D812N	0/D	1/Pb	1/Dc	4.63/Cons	Mother	C151015C02301	4 (4/4)	Yes	PPV
New	Missense	c.2447A>C, p.Q816P	0.005/D	0.79/Ps	1/Dc	3.5/Cons	Mother	C160122C00101	2 (2/2)	Yes	PPV
New	Missense	c.2872C>T, p.R958W	0/D	1/Pb	1/Dc	4.36/Cons	Mother	C160129C01201	4 (0/4)	No	VUS
New	Missense	c.3122C>T, p.T1041M	0/D	1/Pb	1/Dc	4.65/Cons	Father	C151102C00901*	5 (5/5)	Yes	PPV
New	Missense	c.3392C>T, p.A1131V	0.01/D	0.64/Ps	1/Dc	4.93/Cons	Mother	C150804C03101	4 (1/4)	Yes	PPV
New	Missense	c.3536G>A, p.R1179H	0/D	1/Pb	1/Dc	3.72/Cons	Father	C151002C00301[k1]	4 (4/2)	No	VUS
New	Missense	c.3922G>A, p.G1308S	0.027/D	0.967/Pb	1/Dc	4.39/Cons	Mother	C160201C00801[l]	2 (2/5)	Yes	PPV
New	Missense	c.3977G>A, p.R1326H	0.057/T	0.341/Bn	1/Dc	3.87/Cons	Mother	C151112C01801	5 (0/5)	No	VUS
New	Missense	c.4027T>G, p.C1343G	0/D	1/Pb	1/Dc	4.37/Cons	Mother	C150804C02801	5 (1/5)	Yes	PPV
New	Missense	c.4268C>T, p.P1423L	0.11/T	0.296/Bn	1/Dc	4.53/Cons	Father	C160613C02201[h2]	4 (4/1)	No	VUS
New	Splicing	c.4348+3A>C, splicing	NA	NA	NA	NA	Mother	2015-S07769	4 (1/4)	Yes	PPV
New	Missense	c.4457C>T, p.S1486L	NA	NA	1/Dc	4.76/Cons	NA	C150824C03101	4 (4/4)	Unkn	VUS
New	Missense	c.4484C>T, p.P1495L	NA	NA	NA	NA	Mother	C160711C01501	4 (4/1)	No	VUS
New	Frameshift	c.4618_4619insCGACGCCCTGCAGCAC, p.T1540fs	NA	NA	NA	NA	Mother	C160524C00301	5 (1/5)	Yes	PPV
New	Missense	c.4643G>T, pC1548F	0/D	0.396/Bn	1/Dc	3.78/Cons	Mother	C160122C00701[b2]	5 (5/1)	No	VUS
Known	Missense	c.433C>T, p.L145F	0.002/D	0.989/Pb	1/Dc	3.71/Cons	Father	C160809C05801	3 (1/3)	No	PPV
Known	Missense	c.518C>T, p.T173M	0.108/T	0.75/Ps	1/Dc	1.67/NC	Father	C160520C00801[o1]	5 (5/5)	No	VUS
Known	Missense	c.1145C>T, p.P382L	0/D	0.998/Pb	1/Dc	3.81/Cons	Mother	C150924C03401	5 (1/5)	Yes	PPV
Known	Missense	c.1265C>T, p.A422V	0/D	1/Pb	1/Dc	3.85/Cons	Mother	C150910C03001[c2]	3 (2/3)	No	VUS
Known	Missense	c.1270G>T, p.D424Y	0/D	0.998/Pb	1/Dc	3.85/Cons	Father	C160201C00401	3 (3/1)	Yes	PPV
Known	Missense	c.1321G>A, p.E441K	0.005/D	0.975/Pb	1/Dc	2.99/Cons	Mother	C160602C03601	3 (3/0)	Yes	PPV
Known	Missense	c.1378G>A, p.E460K	0.004/D	0.934/Pb	1/Dc	3.94/Cons	Father	C151225C01901[p1]	NA	Unkn	VUS
Known	Missense	c.1708C>T, p.R570W	0/D	0.999/Pb	1/Dc	2/Cons	Mother	C151002C00301[k2]	4 (4/2)	Unkn	PPV
Known	Missense	c.1870C>T, p.R624W	0.04/D	0.975/Pb	0.9/Poly	1.75/NC	Mother	C150713C00501	3 (2/3)	Yes	PPV
							Mother	C151225C01901[p2]	NA	Unkn	
Known	Missense	c.3361A>G, p.N1121D	0.17/T	0.415/Bn	1/Dc	4.93/Cons	Father	2015-S07772	4 (1/4)	Unkn	VUS
							NA	2015-S07204	5 (2/5)	Unkn	
							Mother	C160317C00901	1 (1/1)	No	
							Father	C160331C03201	1 (1/1)	No	
							Father	C160428C00401	5 (0/5)	No	
							Father	C160802C02401	1 (1/1)	No	
							Father	C160520C00801[o2]	5 (5/5)	No	
							Father	C160718C04701[u]	4 (3/4)	Yes	
							Mother	C160722C02501[q]	5 (5/5)	No	
							Father	C151112C01501[r]	5 (5/4)	Yes	
Known	Missense	c.4049T>A, p.L1350H	0.12/T	0.949/Ps	0.997/Dc	3.24/Cons	Mother	2015-S07338[t]	4 (4/4)	Yes	PPV
Known	Splicing	c.4112-2A>G, splicing	NA	NA	NA	4.5/Cons	Mother	2015-S07468	3 (2/3)	Yes	PPV

Table 6. Continued

Feature	Effect	Variant	SIFT	PolyP_2	M_T	GERP++	Heredity	Sample ID	Stage	Seg	Path
Known	Missense	c.4517C>T, p.T1506M	0/D	0.999/Pb	1/Dc	4.53/Cons	Father	C160126C01201[s]	4 (1/4)	No	VUS
Known	Stopgain	c.4600C>T, p.R1534X	NA	NA	NA	2.74/Cons	Father	C160622C05201	3 (2/3)	Yes	PPV

The meaning of the abbreviations used here are the same as in Table 4.

a1, a2: These two variants were identified in one proband. The c.121C>T variant came from his mother, and the c.1480C>T variant came from his father. The mother had stage 1(1/1) and the father had stage 2(2/1) FEVR.

b1, b2: These two variants were identified in one proband. The c.821T>G variant was inherited from her father, who had stage 1 (1/1) FEVR, and the other variant was inherited from her mother, who had no signs of FEVR.

c1, c2: These two variants were identified in one proband. The c.871C>T was inherited from her father, who had stage 2 (2/1) FEVR. The other variant were inherited from his mother, who had no signs of FEVR.

d1, d2: These two variants were identified in one proband. The c.1349G>A variant was inherited from his father, who had no signs of FEVR. The c.2255G>C variant was inherited from his mother, who had stage 1 (1/1) FEVR.

e1, e2: These two variants were identified in one proband. The c.1436G>T was inherited from her father, who had stage 1 (1/1) FEVR. The other variant was inherited from her mother, whose information was unavailable.

f: This proband was also heterozygous for a variant in *FZD4* (c.1589G>A). He inherited both variants from his father, who showed no signs of FEVR.

g: This proband was also heterozygous for a variant in *FZD4*. Both variants were inherited from her mother, who had no signs of FEVR.

h1, h2: These two variants were found in one proband. One was inherited from his father and the other one from his mother. Neither parents had signs of FEVR.

k1, k2: These two variants were identified in one proband. The c.3536G>A variant was inherited from her father, who had no signs of FEVR, and the c.1708C>T variant was inherited from her mother, whose information was unavailable.

l: This proband also carried a variant in *FZD4* (c.313A>G). Both variants were inherited from her mother, who had stage 2(2/2) FEVR.

o1, o2: This proband carried two variants. Both were inherited from his father, who had no signs of FEVR.

p1, p2: This proband carried two variants that were from his mother and father, respectively. However, we had no further information regarding the proband and his family members.

q: This proband also carried a de novo point variant in *NDP* gene. The c.3361A>G variant was inherited from his mother, who had no signs of FEVR.

r: This proband also carried a variant in *FZD4*. The c.3361A>G variant was inherited from her father, who had stage 1(1/1) FEVR.

s: This proband also carried a variant in *TSPAN12* gene. The c.4517C>T variant was inherited from her father, who had no signs of FEVR.

t: This proband also carried a variant in *TSPAN12*. Both variants were inherited from her mother, who had stage 2 (2/2) FEVR.

u: This proband also carried a variant in *FZD4* gene. Both variants were inherited from her father, who has stage 3 (3/2) FEVR.

[*] : This proband had a sister who did not carry the variant. She showed no signs of FEVR.

Table 7. *LRP5* Gene Haplotypes in Probands Carrying Variants as Listed

		Alleles									
Patient ID	**Variant**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**c.3361**	**9**
C151112C01501	c.3361A>G	C	A	T	G	G	A	C	G	G	C
C160317C00901		C	A	T	G	G	C	C	G	G	C
C160520C00801		G	G	C	G	G	C	C	G	G	C
C160718C04701		C	A	T	G	G	C	C	G	G	C
C160722C02501		C	A	T	G	G	C	C	G	G	C
C160802C02401		C	A	T	G	G	A	C	G	G	C
C160428C00401		C	A	T	G	G	A	C	G	G	C
C160331C03201		C	A	T	G	G	A	C	G	G	C

		Alleles								
		1	**2**	**3**	**4**	**c.266**	**5**	**6**	**7**	**8**	**9**
C150918C01701	c.266A>G	C	A	T	G	G	G	A	G	G	C
C150924C03401		C	A	T	G	G	G	A	G	A	T
C151012C01001		C	A	T	G	G	G	C	C	G	C
C151015C02001		C	A	T	G	G	G	A	G	G	C
C151020C01701		C	A	T	G	G	G	A	G	G	C
C151022C01701		C	A	T	G	G	G	A	G	G	C

		Alleles									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**c.1870**	**8**	**9**
C150713C00501	c.1870C>T	C	A	T	G	G	C	C	T	A	T
C151225C01901		C	A	T	G	G	C	C	T	A	T

The haplotype of the copy that carried the indicated variants was determined. The alleles were as follows: 1: rs312014 at Chr11. 68084962; 2: rs312024 at Chr11. 68094431; 3: rs634008 at Chr11. 68094741; 4: rs312779 at Chr11. 68108676; 5: rs11823032 at Chr11. 68145166; 6: rs314773 at Chr11. 68149450; 7: rs4930573 at Chr11. 68163456, 8: rs583545 at Chr11. 68178635; 9: rs3736228 at Chr11. 68201295. The variants were placed according to their localization at the chromosome: c.266 at Chr11. 68115489; c.3361 at Chr11. 968192694; and c.1870 at Chr11. 68174060.

Table 8. Variants Identified in *TSPAN12* Gene

Feature	Effect	Variant	SIFT	PolyPhen_2	M_T	GERP++	Heredity	Sample ID	Stage	Seg	Path
New	Missense	c.1A>G, p.M1V	0/D	0.993/Pb	1/Dc	4.95/Cons	Mother	C150820C05401	4 (2/4)	Yes	PPV
New	Missense	c.50T>G, p.L17R	0/D	0.998/Pb	1/Dc	4.95/Cons	Mother	C150909C00601	4 (3/4)	Unkn	VUS
New	Splicing	c.67-2A>G, Splicing	NA	NA	1/Dc	4.49/Cons	Mother	C160729C04801	4 (4/4)	Unkn	VUS
New	Frameshift	c.176_179del, p.Y59fs	NA	NA	NA	NA	Father	C160718C01101[a]	4 (4/4)	Yes	PPV
New	Missense	c.194C>T, p.P65L	0.41/T	0.386/Bn	1/Dc	5.99/Cons	Mother	C150731C00601	2 (2/2)	Yes	PPV
								2015-S07338[b]	4 (4/4)	Yes	
New	Missense	c.233G>A, p.G78E	0.002/D	0.928/Pb	0.999/Dc	5.99/Cons	Father	C160812C03501	2 (2/2)	Yes	PPV
							Father	C151016C01401	4 (4/4)	Yes	
New	Missense	c.242G>A, p.G81E	0/D	1/Pb	1/Dc	5.99/Cons	Mother	2015-S07406	4 (4/4)	Yes	PPV
New	Stopgain	c.345T>G, p.Y115X	NA	NA	1/Dc	0.371/NC	De novo	C160331C02801	3 (3/3)	NAppl	VUS
New	Stopgain	c.349C>T, p.Q117X	NA	NA	1/Dc	5.44/Cons	Father	C151218C00501	4 (4/4)	Yes	PPV
New	Splicing	c.360+2T>C, Splicing	NA	NA	1/Dc	5.44/Cons	Mother	C160620C00801	4 (4/4)	Yes	PPV
New	Missense	c.392T>C, p.L131S	0.161/T	0.127/Bn	0.999/Dc	5.89/Cons	Mother	C160222C04401	2 (1/2)	Yes	PPV
New	Frameshift	c.467_468del, p.E156fs	NA	NA	NA	NA	Mother	C151112C01901	4 (4/4)	Unkn	VUS
New	Frameshift	c.471_478del, p.F157fs	NA	NA	NA	NA	Mother	C160627C03301	4 (4/4)	Yes	PPV
New	Missense	c.475T>G, p.C159G	0/D	1/Pb	1/Dc	5.83/Cons	NA	2015-S07402	5 (5/4)	Unkn	VUS
New	Frameshift	c.519_520del, p.E173Dfs*11	NA	NA	NA	NA	Mother	C160805C00701	1 (1/0)	Yes	PPV
New	Stopgain	c.527G>A, p.W176X	NA	NA	1/Dc	5.93/Cons	Mother	C160714C07501	4 (4/3)	Yes	PPV
New	Missense	c.535G>T, p.D179Y	0.001/D	0.978/Pb	1/Dc	5.93/Cons	Mother	C160126C01201[c]	4 (1/4)	Yes	PPV
New	Stopgain	c.583C>T, p.Q195X	NA	NA	1/Dc	5/Cons	Father	C160429C01501	4 (4/4)	Yes	PPV
New	Splicing	c.469-1G>A, Splicing	NA	NA	1/Dc	5.83/Cons	De novo	C150813C03301	4 (4/4)	NAppl	VUS
New	Missense	c.713T>C, p.M238T	0.001/D	0.99/Pb	1/Dc	5.68/Cons	Father	C160520C01601	4 (4/4)	Yes	PPV
New	Missense	c.826G>A, p.V276I	0.071/T	0.018/Bn	0.845/Dc	5.68/Cons	Father	C160826C01301[d]	2 (1/2)	Yes	PPV
New	Frameshift	c.833_842del, p.L278Qfs*25	NA	NA	NA	NA	Mother	C160819C02901	4 (4/4)	Yes	PPV
New	Deletion	Exon 1–3 deletion	NA	NA	NA	NA	De novo	C160923C01001	4 (4/3)	NAppl	PPV
New	Deletion	One copy missing	NA	NA	NA	NA	De novo	C160809C01001	2 (2/2)	NAppl	PPV
Known	Splicing	c.149+3A>G	NA	NA	NA	NA	Mother	C160905C05001	4 (4/4)	Yes	PPV
							Mother	2015-S07650	2 (2/2)	Unkn	
Known	Missense	c.479G>A, p.C160Y	0/D	1/Pb	1/Dc	5.83/Cons	Father	C160310C03101	4 (3/4)	Yes	PPV
Known	Missense	c.542G>T, p.C181F	0/D	1/Pb	1/Dc	5.93/Cons	Mother	C150909C04301	2 (2/1)	Yes	PPV
Known	Splicing	c.612+2T>A, Splicing	NA	NA	1/Dc	5.93/Cons	Mother	C160613C02301	5 (5/5)	Yes	PPV

The meaning and the abbreviations used here are the same as in Table 4.

a: This proband was also heterozygous for a variant in the *KIF11* gene. She inherited both variants from her father, who had stage 2 (2/2) FEVR.

b: This proband was also heterozygous for a variant in the *LRP5* gene. She inherited both variants from her mother, who had stage 2 (2/2) FEVR.

c: This proband was also heterozygous for a variant in *LRP5* gene, which was inherited from his father. The *TSPAN12* variant was inherited from his mother, who has stage 1 (1/1) FEVR.

d: This proband also carried a variant in *FZD4*. Both variants came from his father, who had stage 2 (2/2) FEVR.

revealed a small and insignificant decrease in Norrin binding in cells expressing the mutant protein.[20] We performed haplotype analysis, and the results suggested a likely cofounder effect among the variant carriers in our study (Table 7). Three of the variant-carrying parents from three families showed clinical signs of FEVR. It was possible that other mechanisms existed in these families.

Variants in *TSPAN12*

We found four previously reported pathogenic variants and 24 new variants that were predicted to be pathogenic by in silico analysis in *TSPAN12* (Table 8). Eighteen new variants were classified as PPVs and six were classified as VUS. All known variants were PPVs according to our standards. Overall, 23 probands carried PPVs in *TSPAN12* (5.91% of the cohort), eight probands carried VUS (2.06% of the cohort). Four probands were heterozygous for one variant in *TSPAN12* and another variant in another gene.

One of the novel PPVs, c.233G>A, p.G78E, was found in two probands. One proband had stage 2 FEVR in both eyes, and the other proband had stage 4 FEVR in both eyes. Each proband inherited the variant from his/her father who also showed signs of FEVR. Another novel PPV, c.194C>T, p.P65L, was also found in two probands with different stages of FEVR. The variant-carrying parent of each proband also had FEVR.

We found two probands who lost large fragments of the *TSPAN12* gene. One lost exons 1 to 3 in one copy of the gene, and he had stage 4 FEVR in the right eye and stage 3 FEVR in the left eye. The other proband lost an entire copy of the gene, and he had stage 2 FEVR in both eyes. Large deletions of *TSPAN12* gene were also reported by other groups.[29] The affected probands did not show severe FEVR, which was similar to what we observed here.

Variants in *NDP*

We found six previously reported pathogenic variants and 12 novel variants that were predicted to be pathogenic by in silico analysis in *NDP* from 18 male probands (Table 9). None of the probands in our cohort showed hearing loss at the time of the diagnosis of FEVR. However, 15 out of 20 of the *NDP* variant carriers in our study were younger than 4 years old. We could not rule out the possibility that they would develop Norrie disease when they grew older. There was one de novo mutation of a single nucleotide change, c.109C>T, p.R37X, that caused premature termination of protein translation. One proband lost the entire gene. The rest of the variants were all inherited. Since *NDP* is an X-linked gene, all variants were classified as PPV. MAFs for all known variants were smaller than 0.001.

We found six mothers of six *NDP* PPV carriers with clinical signs of FEVR. They were heterozygous for the following variants: c.134T>G, p.V45G; c.188C>T, p.A63V; c.279delT, p.C93fs; c.181C>A, p.L61I; c.196G>T, p.E66X; and c.343C>T, p.R115X. The manifestation of FEVR in heterozygous female carries suggested the dominant effect of these variants.

Table 9. Variants Identified in *NDP* Gene

Feature	Effect	Variant	SIFT	PolyP_2	M-T	GERP++	Heredity	Sample ID	Stage	Seg	Path
New	Missense	c.22G>C, p.A8P	0/D	0.908/Ps	1/Dc	5.81/Cons	Mother	C160201C00701	NA	Unkn	PPV
New	Missense	c.134T>G, p.V45G	0/D	0.999/Pb	1/Dc	5.98/Cons	Mother	2015-S07278	5 (5/5)	Yes	PPV
New	Missense	c.164G>T, p.C55F	0/D	0.995/Pb	1/Dc	5.98/Cons	Mother	C160122C00601	5 (5/5)	Unkn	PPV
New	Missense	c.173A>G, p.K58R	0.029/D	0.989/Pb	1/Dc	5.15/Cons	Mother	C160711C01201	5 (5/5)	No	PPV
New	Missense	c.188C>T, p.A63V	0.011/D	0.992/Pb	1/Dc	5.96/Cons	Mother	C160321C03601	5 (5/5)	Yes	PPV
New	Missense	c.258G>T, p.K86N	0.008/D	0.995/Pb	1/Dc	5.1/Cons	Mother	C160301C04301	5 (5/4)	Unkn	PPV
New	Frameshift	c.279delT, p.C93fs	NA	NA	NA	NA	Mother	C151127C00501	5 (5/5)	Yes	PPV
New	Missense	c.285C>G, p.C95W	0/D	0.999/Pb	1/Dc	5.96/Cons	Mother	C151015C02401	5 (5/5)	No	PPV
New	Frameshift	c.291dupG, p.P98fs	NA	NA	NA	NA	Mother	C151127C00401	5 (5/5)	Unkn	PPV
New	Missense	c.308T>A, p.L103Q	0.0/D	0.998/Pb	1/Dc	5.96/Cons	Mother	C160620C00901	5 (5/1)	Unkn	PPV
New	Missense	c.313G>C, p.A105P	0.0/D	0.998/Pb	1/Dc	5.81/Cons	Mother	C160714C07301	5 (5/4)	No	PPV
New	Deletion	Whole gene deletion	NA	NA	NA	NA	De novo	C160812C02901	5 (5/5)	NAppl	PPV
Known	Stopgain	c.109C>T, p.R37X	NA	NA	1/Dc	4.93/Cons	De novo	C160722C02501	5 (5/5)	NAppl	PPV
Known	Missense	c.112C>T, p.R38C	0/D	0.82/Ps	1/Dc	5.81/Cons	Mother	C160826C02001	5 (5/4)	No	PPV
Known	Missense	c.164G>A, p.C55Y	0/D	0.995/Pb	1/Dc	5.98/Cons	Mother	C151104C01601	5 (5/5)	No	PPV
Known	Missense	c.181C>A, p.L61I	0.026/D	0.987/Pb	1/Dc	5.96/Cons	Mother	C160513C00201[a]	5 (5/5)	Yes	PPV
Known	Stopgain	c.196G>T, p.E66X	NA	NA	1/Dc	5.96/Cons	Mother	C160711C03401	5 (5/5)	Yes	PPV
Known	Stopgain	c.343C>T, p.R115X	1/T	NA	1/Dc	4.01/Cons	Mother	C151015C01701	5 (5/5)	Yes	PPV

The meaning and the abbreviations used here are the same as in Table 4.

a: This proband also carried a variant in *ZNF408 c.1963_1966del*. The variant in *NDP* was inherited from his mother, who had stage 1(1/1) FEVR.

Dominant effect of *NDP* variants were also reported by other groups.[25,30,31]

Many of the new variants in *NDP* identified in this study led to changes of amino acids that were known to cause X-linked FEVR or Norrie disease when mutated, although the exact nucleotide changes were not reported previously.[25] For example, changes at Val45: c.134T>A, p.V45E and c.133G>A, p.V45M; changes at Cys55: c.163T>C, p.C55R; changes at Lys61: c.188C>T, p.L61F; changes at K58: c.174G>T, p.K58N; changes at Cys95: c.283T>C, p.C95R, c.284G>T, p.C95F, and c.285C>A, p.C95X; changes at Leu103: c.307C>G, p.L103V; and changes at Ala 105: c.313G>A, p.A105T. These findings suggested the functional importance of the corresponding amino acids.

Variants in *ZNF408*

We found one previously reported variant (c.353G>A, p.S118N) and eight novel variants that were predicted to be pathogenic by in silico analysis in *ZNF408* in eight probands (Table 10). Three of the novel variants showed phenotype-genotype cosegregation, and they were classified as PPV. The rest were classified as VUS.

The c.353G>A, p.S118N variant was previously found in a Japanese proband with no clear cosegregation (as p.S126N using NM_024741 for *ZNF408*). The mutated protein showed normal nuclear localization in cells.[7] MAF for this variant was 0.0019 in the gnomAD_genome_EAS database. In our study, the proband (C160709C01001) who carried this variant was also heterozygous for c.1493G>A, p.R498H. He inherited the c.1493G>A from his mother, who had signs of FEVR. He inherited the c.353G>A from his father, who was heterozygous for the variant but showed no signs of FEVR. Due to the lack of evidence suggesting the pathogenicity of the variant, we tentatively classified it as a benign variant.

Variants in *KIF11*

We found nine novel variants that were predicted to be pathogenic by in silico analysis and seven previously reported variants (Table 11). Unlike other FEVR-causing genes, *KIF11* is prone to de novo mutagenesis.[8,12,13,32,33] In this cohort, there were 13 de novo variants and three inherited ones. One of the novel variant, c.613C>T, p.H205Y, was inherited, and the variant-carrying father also had clinical signs of FEVR. The rest of the novel variants were all de novo changes. There were two probands who had large deletions of the gene: a 5-month-old female proband who lost one copy of the gene and a 5-year-old male proband who lost exons 2 to 4 of one copy. Both probands had microcephaly. The 5-year-old proband also

Table 10. Variants Identified in *ZNF408* Gene

Feature	Effect	Variant	SIFT	PolyP_2	M-T	GERP++	Heredity	Sample ID	Stage	Seg	Path
New	Missense	c.734G>A, p.G245D	0.002/D	0.046/Bn	1/Dc	2.11/Cons	Father	C160503C02901	5 (5/5)	Yes	PPV
New	Frameshift	c.1083delG, p.K361fs	NA	NA	NA	NA	Father	C151218C00401	5 (0/5)	No	VUS
New	Missense	*c.1261G>A, p.V421M	0.08/T	0.995/Pb	1/Dc	5.57/Cons	Mother	C160517C00701	3 (3/3)	Unkn	VUS
New	Missense	c.1472C>T, p.P491L	0.057/T	0.968/Pb	1/Dc	5.68/Cons	Father	C160622C02001	4 (4/4)	No	VUS
New	Missense	c.1493G>A, p.R498H	0.016/D	0.989/Pb	0.997/Dc	4.61/Cons	Mother	C160709C01001[a1]	4 (4/0)	Yes	PPV
New	Missense	c.1733G>A, p.R578H	0/D	0.685/Ps	0.708/Dc	2.31/Cons	Mother	C150702C00501	4 (1/4)	Yes	PPV
New	Frameshift	c.1963_1966del, p.R655fs	NA	NA	NA	NA	Father	C160513C00201[b]	5 (5/5)	No	VUS
New	Missense	c.2125G>A, p.E709K	0/D	0.202/Bn	0.822/Dc	4.27/Cons	Father	C160428C01001	4 (4/0)	No	VUS
Known	Missense	c.353G>A, p.S118N	0/D	0.261/Bn	1/Poly	4.07/Cons	Father	C160709C01001[a2]	4 (4/0)	No	Benign

The meanings of the abbreviations used here are the same as in Table 4.

* : This variant had an MAF of 0.001 in the gnomAD_exome_EAS but not in the gnomAD_genome_EAS database.

a1, a2: This proband carried two variants in *ZF408* gene. The c.1493G>A was from his mother who had a stage 1 (1/1) FEVR. The c.353G>A variant was from his father who had no signs of FEVR.

b: This proband also carried a variant in the *NDP* gene.

Table 11. Variants Identified in *KIF11* Gene

Feature	Effect	Variant	SIFT	PolyP_2	M-T	GERP++	Heredity	Stage	Sample ID	Seg	Path
New	Splicing	c.308+1G>A, splicing	NA	NA	1/Dc	5.94/Cons	De novo	5 (5/5)	C160503C01401	Nappl	VUS
New	Missense	c.413G>A, p.R138H	0.0/D	1.0/Pb	1/Dc	5.94/Cons	De novo	4 (4/3)	C160613C02001	Nappl	VUS
New	Missense	c.613C>T, p.H205Y	0.002/D	0.992/Pb	1/Dc	5.94/Cons	Father	4 (4/3)	C160718C01101[a]	Yes	PPV
New	Frameshift	c.648_649del, p.K216fs	NA	NA	NA	NA	De novo	5 (5/5)	C160311C00301[b]	Nappl	VUS
New	Frameshift	c.1437_1438del, p.E479fs	NA	NA	NA	NA	De novo	5 (5/5)	C151104C01701	Nappl	VUS
New	Frameshift	c.2123_2130del, p.T708fs	NA	NA	NA	NA	De novo	5 (5/5)	C160414C00301[c]	Nappl	VUS
New	Frameshift	c.2433_2437del,p.I811fs	NA	NA	NA	NA	De novo	5 (5/5)	C160705C02701	Nappl	VUS
New	Deletion	Exon 2–4 missing	NA	NA	NA	NA	De novo	2 (2/0)	C160802C00701[d]	Nappl	VUS
New	Deletion	One copy missing	NA	NA	NA	NA	De novo	4 (4/4)	C150701C03501[e]	Nappl	VUS
Known	Missense	c.511C>G, p.L171V*	0/D	1/Pb	1/Dc	5.54/Cons	De novo	4 (3/4)	*2015-S07577[f]	Nappl	VUS
Known	Splicing	c.790–2A>C, splicing*	NA	NA	1/Dc	5.14/Cons	De novo	4 (4/2)	*C150821C01401[g]	Nappl	VUS
Known	Frameshift	c.1030dupT, p.S348Efs*8	NA	NA	NA	NA	Mother	4 (4/4)	*C160829C03301	Yes	PPV
Known	Stopgain	c.1573C>T, p.Q525X*	0.8/T	NA	1/Dc	2.72/Nc	De novo	5 (4/5)	*2015-S07884[h]	Nappl	VUS
Known	Stopgain	c.2524C>T, p.Q842X*	0.44/T	NA	1/Dc	5.28/Cons	De novo	5 (5/5)	*2015-S07345[i]	Nappl	VUS
Known	Stopgain	c.2807C>G, p.S936X*	0.22/T	NA	1/Dc	4.08/Cons	De novo	4 (4/4)	*C151020C02401	Nappl	VUS
Known	Frameshift	c.2949delG, p.L983fs*	NA	NA	NA	NA	Father	4 (4/4)	*C150820C05001	Yes	PPV

The meaning and the abbreviations used here were the same as in Table 4.
* : These probands and the variants they carried were previously reported by our group (see text for details).
a: This proband carried two variants, and the other one was in *TSPAN12*; both variants came from her father, who had 2 (2/2) FEVR.
b: This proband showed microcephaly (–2 SDs) and mild mental retardation.
c: This proband had microcephaly (–2 SDs).
d: This proband had microcephaly (–2 SDs) and mental retardation.
e: This proband had microcephaly (–2 SDs).
f: This proband had microcephaly (–6 SDs).
g: This proband had microcephaly (–2 SDs).
h: The left eye of this proband was micro-ophthalmic.
i: This proband had microcephaly (–2 SDs) and mild mental retardation. The rest of the probands in this table had no MCLMR-related symptoms.

showed signs of mental retardation. However, the 5-year-old boy had stage 2 FEVR in the left eye and a normal right eye. He had the mildest FEVR among the rest of *KIF11* variant carriers in this cohort.

Among eight reported variants, seven were previously reported by our group: c.511C>G, p.L171V; c.790-2A>C; c.1573C>T, p.Q525X; c.2524C>T, p.Q842X; c.2807C>G, p.S936X; and c.2949delG, p.L983fs.[14] The probands who carried these variants were included in this study because they were diagnosed and treated within the time period of this study. These variants were classified as VUS in this study because they were all de novo changes.

The c.1030dupT, p.S348Efs*8 variant was previously identified in a boy with MCLMR.[34] This boy had unspecified retinopathy, and the variant was inherited from his mother who had microcephaly and mild learning problems, but no ophthalmologic abnormalities. In this study, the female proband showed no signs of MCLMR. She inherited the variant from her mother, who showed no retinal problems. We classified this variant as PPV.

In total, eight probands in this cohort had different degrees of microcephaly, and three of them also had mild mental retardation. There were eight probands who showed no signs of MCLMR. Details of each affected probands are denoted in Table 11.

Association Between Genes and the Severity of FEVR

There were studies that suggested that the severity of FEVR caused by variants in different genes was different.[35,36] Here, we analyzed this phenomena by separating PPV carriers by genes and counted the number of probands at each stage of FEVR (Fig. 1). Probands carrying compound variants were excluded. *KIF11* and *ZNF408* were not analyzed due to small numbers of probands carrying PPVs in these two genes. We found that 100% of the probands carrying PPVs in *NDP* had stage 5 FEVR. On the other hand, none of the probands carrying PPVs in *TSPAN12* had stage 5 FEVR. About 86.2% and 51.6% of the probands carrying PPVs in *FZD4* and *LRP5* had stage 4 and 5 FEVR combined, respectively. The profiles of FEVR of all PPV carriers, the negatives, the *FZD4*-PPV carriers, and the *LRP5*-PPV carriers were not significantly different among each other ($P > 0.05$, χ^2 analysis). However, the profiles of FEVR of *NDP* PPV carriers and *TSAPN12* PPV carriers were significantly different from the rest of the groups and between each other ($P < 0.01$, χ^2 analysis). The results suggested that the most pathogenic FEVR gene in causing FEVR was *NDP*, and the least pathogenic gene was *TSPAN12* in our cohort.

Another characteristic of FEVR is that the severity of the condition could be different between contralateral eyes (referred to as asymmetry hereafter). In an attempt to analyze this phenomena, we calculated the differences in grades between contralateral eyes for each proband and compared the results among probands carrying PPVs in different genes. We arbitrarily defined three levels of asymmetry: low, medium, and high. A low asymmetry referred to a difference of one grade or none between contralateral eyes (for example, a proband with grade 5 FEVR in both eyes or a proband with one grade 4 eye and another grade 3 eye). A medium asymmetry referred to a difference of two or three grades. A high asymmetry referred to a difference of four or five grades. The results were shown in Figure 2. Overall, 52.5% of the probands in the entire cohort showed low asymmetry, 25.3% showed medium asymmetry, and 22.2% showed high asymmetry. Among PPV carriers, 59.6% showed low asymmetry, 21.2% showed medium asymmetry, and 19.2% showed high asymmetry. In the negative group, 45% showed low asymmetry, 28.5% showed medium asymmetry, and 26.8% showed high asymmetry. The percentage of low asymmetry was 94.7%, 86.7%, 45.2%, and 37.9% for probands carrying PPVs in *TSPAN12*, *NDP*, *LRP5*, and *FZD4*, respectively. The percentage of high asymmetry was 0%, 13.3%, 16.1%, and

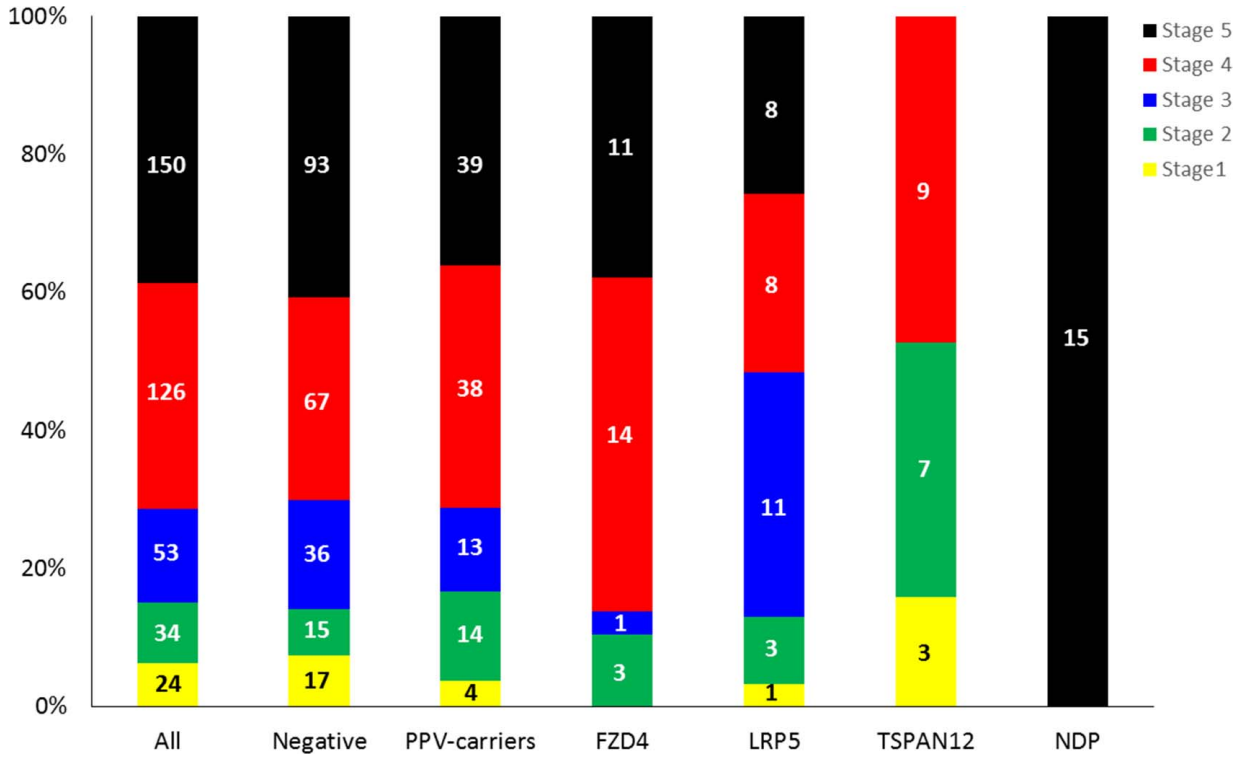

Figure 1. The percentage of probands at five different stages of FEVR in different groups. "All" refers to the entire cohort of 387 probands. Two probands were not included because we did not have data on the stages of the condition for them. The definitions for "negative" and "PPV carriers" are the same as in Table 1. Probands who carried compound variants were excluded from the individual gene group to simplify the comparison. The number of probands at each stage in each group is embedded in the respective color blocks.

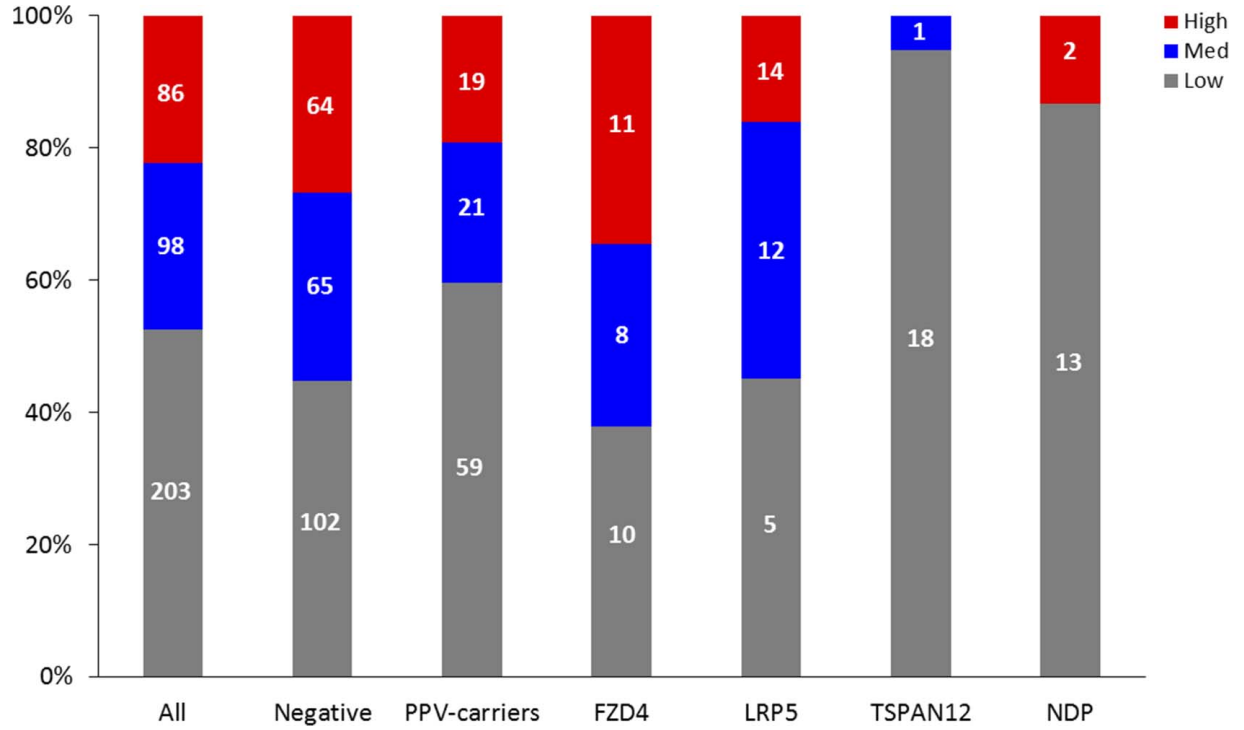

Figure 2. The percentage of FEVR patients with different degrees of asymmetry between contralateral eyes. The definition for each group is the same as for Figure 1. The number of probands at each FEVR stage is embedded in the respective color blocks.

Investigative Ophthalmology & Visual Science

34.5% for probands carrying PPVs in the above genes in the same order, respectively. The results suggested that PPVs in *NDP* and *TSPAN12* tended to cause less difference in disease severity between contralateral eyes than PPVs in *FZD4* and *LRP5*.

Discussion

This is probably the largest cohort of FEVR patients collected and screened for genetic variants by a single clinic.[10,11,22,25,30,35,37–43] Following criteria that combined in silico analysis and the evidence for genotype-phenotype cosegregation, we identified 73 new PPVs, 38 new VUS, and reevaluated 13 previously reported variants. PPVs and VUS in the six genes accounted for 28.3% and 13.1% of the probands in this cohort, respectively. The percentage of PPV carriers was lower than most studies published so far, probably due to the strict definition we used. About 27.0% of probands carried PPVs in genes involved in the Wnt/Norrin pathway. Only 0.77% of the probands carried PPVs in *ZNF408* or *KIF11*.

We found several variants in *FZD4* and *LRP5* genes that were carried by multiple families in our cohort. Some of the variants were benign or of unknown significance, and they all had high MAF, specifically among East Asians (gnomAD_genome_EAS). Haplotype analysis suggested a likely cofounder effect for these variants in our cohort. Our study also identified two mutational "hotspots" in *FZD4*: c.313A>G and c.1282_1285del. Both variants were reported by several groups.[18,22,25,42] There were five probands carrying c.313A>G and seven probands carrying c.1282_1285del, which accounted for 1.28% and 1.80% of the cohort. We were not able to conclude whether there was a cofounder effect among the carriers. In another study from Korea,[35] haplotype analysis was also performed on five carriers of c.313A>G, and the results indicated that it was unlikely to have derived from a common founder.

The size of the cohort and the number of PPVs identified in this study enabled us to analyze features of FEVR associated with variants in different genes. Our results suggested that FEVR caused by variants in *NDP* and *TSPAN12* tended to be more uniform between contralateral eyes than that caused by variants in *LRP5* and *FZD4*. In addition, patients with PPVs in *NDP* exhibited more severe FEVR in general than did patients who carried PPVs in *FZD4*, *LRP5*, and *TSPAN12*. Overall, probands carrying PPVs in *TSPAN12* had the mildest FEVR. This result was contrary to a recent study, also on Han Chinese, that showed that *TSPAN12* mutations were associated with more advanced FEVR.[31] Age of the probands could be a fact in causing the discrepancy. Young FEVR patients tend to have more severe FEVR than do adult patients. Furthermore, since FEVR is a progressive disease, the severity of FEVR may change with age in a proband, leading to different grading of the disease.

ZNF408-associated FEVR represented a different mechanism causing the disease other than Wnt/Norrie pathway.[9] However, only a few pathogenic *ZNF408* variants have been identified so far. Here we found three probands carrying PPVs in *ZNF408*, which accounted for 0.77% of the cohort. In a previous study of 31 Chinese pedigrees, no pathogenic variant in *ZNF408* was found.[43] Another study on Indian FEVR patients reported one pathogenic variant that would be classified as VUS according to the criteria we used here.[10] Although PPVs in *ZNF408* were scarce in FEVR patients, further studies focusing on the downstream target genes of *ZNF408* may lead to the finding of new elements involved in retinal vasculature development.

KIF11 is also a newly recognized FEVR-causing gene.[8] So far, reports from different groups have shown that variants in *KIF11* accounted for no more than 5% of the FEVR probands.[8,13,14,43] In this cohort, only 0.77% of the probands carried PPV in *KIF11*. This was because all de novo variants were classified as VUS due to the lack of evidence in phenotype-genotype cosegregation. Our study corroborated previous findings that *KIF11* was prone to de novo mutagenesis, and it could cause FEVR without MCLMR.[8,12,13,32,33] We also noticed that most of the probands carrying PPV or VUS in *KIF11* had advanced FEVR in both eyes, suggesting strong pathogenicity of the gene. Future studies are needed to reveal the involvement of *KIF11* in the development of FEVR at the molecular level.

In summary, FEVR is a rare but potentially blinding genetic disorder. So far, about 40% of FEVR patients could be attributed to mutations in the above six genes. Clearly, there are additional genes and molecular mechanisms that contribute to the development of the condition. One obstacle that hindered research on FEVR is the small size of patient pools. It is probably time to initiate an international study to combine all FEVR patient sources in order to have a major breakthrough in the genetic studies of the disease.

Acknowledgments

The authors thank Xu Minjie and Cao Ting from Amplicon-Gene Co. Ltd. for haplotype analysis.

Supported by grants from the National Natural Science Foundation of China: 81371063 (JL) and 81470642 (PZ).

Disclosure: **J.-K. Li**, None; **Y. Li**, None; **X. Zhang**, None; **C.-L. Chen**, None; **Y.-Q. Rao**, None; **P. Fei**, None; **Q. Zhang**, None; **P. Zhao**, None; **J. Li**, None

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