Mutation Spectrum of the LRP5, NDP, and TSPAN12 Genes in Chinese Patients With Familial Exudative Vitreoretinopathy

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Purpose. LRP5, NDP, and TSPAN12 are known to be associated with familial exudative vitreoretinopathy (FEVR). In this study, a comprehensive mutation screening for the three genes was performed in patients with a clinical diagnosis of FEVR in Han Chinese.

Methods. Genomic DNA and clinical data were collected from 100 probands and their family members. Sanger sequencing was performed to screen for LRP5, NDP, and TSPAN12 mutations and phenotype-genotype correlation was analyzed.

Results. There were 23 causative mutations identified in 23 unrelated probands (10/23 in LRP5, 8/23 in TSPAN12, and 5/23 in NDP). Apart from NDP mutations, only two LRP5 mutations inherited in an autosomal recessive manner. Among the 23 causative mutations, 13 were novel variants (4/10 in LRP5, 6/8 in TSPAN12, and 3/5 in NDP). According to the modified classification system, statistical significance was observed in the distribution of mutated genes (P = 0.049). None of the causative mutations was found in group I FEVR. Proband with LRP5 or NDP mutations were mainly categorized into group III and IV, TSPAN12 mutations were mainly observed in probands with group IV and V FEVR.

Conclusions. The detection rate for mutations in the three known genes was 23%. Mutations in LRP5 and TSPAN12 were more frequent, accounting for 10% and 8%, respectively. The NDP mutations were only identified in 6% in this cohort. There were 13 novel variants found, which provided a deeper understanding of this disease. Potential phenotype-genotype correlation was observed in the modified system. TSPAN12 mutations might lead to the most severe phenotype.

Keywords: familial exudative vitreoretinopathy, mutational analysis, phenotype-genotype correlation

Familial exudative vitreoretinopathy (FEVR) is a genetic disorder affecting the development of retinal vasculature. The primary feature in FEVR is referred to as a premature arrest of vascularization in the peripheral retina. The subsequent ischemia and hypoxia can induce neovascularization and variable secondary complications, including vascular leakage, fibrovascular proliferation, traction of the posterior pole structure, macular dragging, retinal fold, retinal tears, and partial or total retinal detachment. The severity of clinical manifestation ranges from the absence of visual impairment to total blindness.

FEVR is genetically heterogeneous and has been reported to have autosomal dominant (adFEVR; Mendelian Inheritance in Man [MIM# 135780]), autosomal recessive (arFEVR; MIM# 134780), and X-linked recessive (XL-FEVR; MIM# 305390) inheritances. To date, mutations in the FZD4 (MIM# 604579) have been shown to contribute to the dominant FEVR. The TSPAN12 (MIM# 613138) and LRP5 (MIM# 605306) mutations are believed to be inherited as dominant or recessive traits. while mutations in the NDP (MIM# 300658) gene have been found in X-linked FEVR. Moreover, mutations in the ZNF408 (MIM# 616468), KIF11, RCBTB1, and CTNNB1 (MIM# 617572) have been linked to FEVR patients in recent studies.

The proteins, encoded by the FZD4, LRP5, NDP, and TSPAN12 genes, are involved in the Wnt/Norrin-β-catenin signaling pathways that are critically required for retinal angiogenesis during eye development. As an atypical Wnt ligand, Norrin activates the canonical Wnt pathway through its specific binding to the Frizzled4 receptor and Lrp5/6 coreceptor, which is selectively enhanced by Tetraspanin. It produces a downstream signal that stabilizes the cytosolic β-catenin. The increasing β-catenin translocates into the nucleus and interacts with the lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factor, which induces the transcription of target genes.

In this study, sequence analysis of LRP5, NDP, and TSPAN12 was performed in 100 unrelated Chinese individuals with clinical diagnosis of FEVR and their families. A comprehensive mutation spectrum for LRP5, NDP, and TSPAN12 in China was determined.

Methods

Patients

Written informed consent in accordance with the guidelines of the Declaration of Helsinki was obtained from the participating individuals or their guardians before the clinical data and DNA
samples were collected. All procedures were approved by the internal review board of the Zhongshan Ophthalmic Center, Sun Yat-sen University. There were 100 probands recruited and had a final diagnosis of FEVR. Patients with a gestational age of less than 38 weeks or a neonatal birth weight of less than 2000 g were excluded, to eliminate the possible presence of retinopathy of prematurity. The clinical diagnosis of FEVR was described in a previous study and EZDI mutations were found in 21/100 individuals.15 There were 70 ethnically matched individuals without history of ocular diseases recruited as controls. Comprehensive fundus examinations were performed, and no signs of retinal diseases were observed.

**Genetic Analysis**

Tissue samples (blood or buccal swabs) were obtained from each participant for genomic DNA extraction using the standard protocols of the QIAGEN QIAamp DNA Blood Kit (Qiagen, Hilden, Germany) and the Epicenter Master Pure Complete DNA and RNA Purification Kit (Illumina, Madison, WI, USA). For mutational analysis, the coding exons of LRPS, NDP, TSPAN12, and their flanking intronic sequences were amplified with PCR. The resulting amplicons were analyzed with Sanger sequencing. Direct sequencing was performed with an ABI BigDye Terminator Cycle Sequencing Kit using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The reference sequences of LRPS (GenBank NG_015855.1 for gDNA, NM_001291902.1 for mRNA), NDP (GenBank NG_009852.1 for gDNA, NM_000266.3 for mRNA), and TSPAN12 (GenBank NG_025203.1 for gDNA, NM_012538.3 for mRNA) were used for the identification of variants.

**In Silico Analyses**

Protein alignments for novel missense mutations were performed using the online COBALT program. Four online algorithms were used to estimate the pathogenicity of the missense changes, including SIFT, PolyPhen-2, Align GVGD, and Mutation Taster. For splice site mutations, another four algorithms were used to predict their potential outcome, including Human Splicing Finder, NNSPLICE, FSPlice, and NetGene2. For secondary structural analysis of mutant protein, PsiPred v.3.3 was used.

**Cloning Sequencing**

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

**Statistics**

All analyses were performed using SPSS software version 16.0 (IBM Corp., Armonk, NY, USA). The independent samples t-test was used for normally distributed continuous variables, while the Kruskal–Wallis test was used for the nonnormally distributed continuous variables. A P value of less than 0.05 was considered statistically significant.

**Results**

In this study, 23 potentially pathogenic mutations were identified in 23 unrelated FEVR probands, including one patient harboring compound heterozygous mutations in LRPS (c.[1058G>A];[922A>C]) and two unrelated individuals carried the same reported LRPS mutation (c.4488+2T>G). Apart from NDP mutations, only two LRPS mutations inherited as an autosomal recessive trait. The remaining nucleotide changes in LRPS and TSPAN12 were heterozygous and inherited as autosomal dominant pattern (Table 1). Of the 23 probands with LRPS, NDP, and TSPAN12 mutations, nine had a family history of FEVR and 14 were isolated cases (Fig. 1).

Among these 23 variants, 13 of them were novel (4/10 in LRPS, 6/8 in TSPAN12, and 5/5 in NDP). None of these novel mutations was detected in 70 ethnically matched control individuals. These variants were not found in current online databases of human sequence variants, including the Ensembl, dbSNP ExAC databases, gnomeAD, and The Human Gene Mutation Database (HGMD). Chromatograms of these novel variants and clone sequencing of heterozygous insertion and deletion mutations are shown in Figure 2.

Apart from these diseases causing mutations, 20 synonymous variants in LRPS and TSPAN12 were excluded from this study (data not shown). Six known variants in LRPS were considered as benign because the minor allele frequency was higher than 0.1% in the East Asian population (Supplementary Table S1). Besides that, three variants were considered as uncertain significance (Supplementary Table S2). Novel variant c.286+1G>T was located in the splice acceptor site of exon 4 in TSPAN12. It was predicted to damage the natural acceptor splice site by three algorithms (NNSPLICE, FSPlice, and NetGene2). The 4-year-old male proband showed a retinal fold in his right eye, however, the same variant was found in his healthy father. The c.1512G>T (p.[W504C]) variant in LRPS previously identified in an osteoporosis-pseudoglioma syndrome (OPPG) family with compound heterozygous mutations (p.[W504C];[W478R]) and members with a single p.(W504C) variants only exhibited low bone mineral density.16 The heterozygous p.(W504C) was detected in our index patient with an ectopic macula and her healthy mother. Additionally, the splice site variant c.3637+4C>T (rs746451473), located in the splice donor site of exon 16 of LRPS, was found in a sporadic male patient. Three of four algorithms considered it as benign.

**Mutations Detected in LRPS**

Three novel heterozygous missense mutations were found. The c.1318A>T (p.[I440F]) was found in a 9-month-old girl with retinal fold in both eyes, her mother and elder sister carried the same mutation and were diagnosed as FEVR. The isoleucine at codon 440 was conserved among various species (Fig. 3A) and three online programs predicted this change as pathogenic. The c.1942G>A (p.[V648I]) mutation was detected in a 19-year-old male with asymmetric manifestation. FEVR associated rhegmatogenous retinal detachment was observed in his right eye while an ectopic macula was found in the left eye. His healthy mother did not carry the same mutation. P.(V648I) was predicted as pathogenic variant by SIFT and Mutation Taster.

The valine was highly conserved (Fig. 3B) and was replaced by isoleucine. The c.2738G>T (p.[C913F]) was identified in a 4-year-old girl with a retinal fold in the right eye and macular degeneration. The mutation was detected in 21/100 individuals.15 There were 70 ethnically matched individuals without history of ocular diseases recruited as controls. Comprehensive fundus examinations were performed, and no signs of retinal diseases were observed.

The c.1512G>T was located in the splice acceptor site of exon 4 in TSPAN12. It was predicted to damage the natural acceptor splice site by three algorithms (NNSPLICE, FSPlice, and NetGene2). The 4-year-old male proband showed a retinal fold in his right eye, however, the same variant was found in his healthy father. The c.1512G>T (p.[W504C]) variant in LRPS previously identified in an osteoporosis-pseudoglioma syndrome (OPPG) family with compound heterozygous mutations (p.[W504C];[W478R]) and members with a single p.(W504C) variants only exhibited low bone mineral density.16 The heterozygous p.(W504C) was detected in our index patient with an ectopic macula and her healthy mother. Additionally, the splice site variant c.3637+4C>T (rs746451473), located in the splice donor site of exon 16 of LRPS, was found in a sporadic male patient. Three of four algorithms considered it as benign.

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The hydrophilic cysteine was replaced by hydrophobic phenylalanine. The cysteine at codon 913 was found in a 16-year-old boy with a retinal fold in the left eye. The hydrophilic cysteine was highly conserved and reported in patients with OPPG. No skeletal problem was reported in this family. The novel c.92-2A>T mutation located in the splice acceptor site caused a deletion of the following exon. Four algorithms predicted the loss of natural splice site.

Two recurrent mutations were found in our study. The splice site mutation c.4488+2T>G [5] was found in two unrelated probands with retinal folds and segregated among their families. The c.4087G>A (p.[D1363H]) mutation previously reported in a Chinese family, was identified in a 3-year-old boy with falciform retinal fold in the right eye. His healthy parents did not carry the same mutation.

### Mutations Detected in TSPAN12

Three novel frameshift mutations were found. The single base pair insertion c.438_439insT caused a frameshifting resulting in 11 incorrect amino acids after codon 146 and a premature termination at codon 158 (p.[R1578M]). The proband was a 1-month-old girl with total retinal detachment and massive vitreous proliferation in her left eye and macular ectopic in her right eye. Her father with bilateral retinal folds carried the same

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### Table 1. Causative Mutations Identified in 100 Probands With Familial Exudative Vitreoretinopathy

<table>
<thead>
<tr>
<th>Family ID</th>
<th>Gene</th>
<th>Allele</th>
<th>cDNA Change</th>
<th>Protein Change</th>
<th>SIFT</th>
<th>PolyPhen2</th>
<th>Align GVGD</th>
<th>Mutation Taster</th>
<th>Source</th>
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<td>NDP</td>
<td>Hemi</td>
<td>c.196G&gt;A</td>
<td>p.(I66K)</td>
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<td>Disease causing</td>
<td>Reported</td>
</tr>
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<td>57</td>
<td>NDP</td>
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<td>Class C65</td>
<td>Disease causing</td>
<td>Novel</td>
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<td>p.(H94L)</td>
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<td>Novel</td>
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<td>NDP</td>
<td>Hemi</td>
<td>c.562G&gt;A</td>
<td>p.(R121Q)</td>
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<td>Reported</td>
</tr>
<tr>
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<td>p.(G151D)</td>
<td>0, D</td>
<td>Probably damaging</td>
<td>Class C35</td>
<td>Disease causing</td>
<td>Reported</td>
</tr>
<tr>
<td>61*</td>
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<td>p.(R353Q)</td>
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<td>Disease causing</td>
<td>Novel</td>
</tr>
<tr>
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<td>Benign</td>
<td>Class C65</td>
<td>Disease causing</td>
<td>Novel</td>
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<tr>
<td>82</td>
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<td>c.1318A&gt;T</td>
<td>p.(I440F)</td>
<td>0, D</td>
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<td>Novel</td>
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<tr>
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<td>c.1582G&gt;A</td>
<td>p.(E528K)</td>
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<tr>
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<td>p.(C913F)</td>
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<td>Disease causing</td>
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<td>p.(T1578M)</td>
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<td>Class C65</td>
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<td>Novel</td>
</tr>
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<td>p.(MIT)</td>
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<td>Class C65</td>
<td>Disease causing</td>
<td>Novel</td>
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<td>c.464G&gt;C</td>
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<td>Disease causing</td>
<td>Novel</td>
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<td>TSPAN12</td>
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<td>c.438_439insT</td>
<td>p.(T147Yfs*12)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Novel</td>
</tr>
<tr>
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<td>TSPAN12</td>
<td>Hetero</td>
<td>c.655delC</td>
<td>p.(Q219Nfs*5)</td>
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<td>NA</td>
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<tr>
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<td>TSPAN12</td>
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<td>p.(3066ex3c*35)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Novel</td>
</tr>
</tbody>
</table>

+5delTAABAA

WE, without effect; LNSS, loss of the natural splice site; NPS, new predicted splice site; Hetero, heterozygous; Hom, homozygous; Hemi, hemizygous; NA, not available; D, deleterious; T, tolerated.

For Align GVGD, class C0 indicates that a change is unlikely to be pathogenic, while class C65 represents the highest likelihood of a change to be pathogenic. For SIFT, amino acids changes with probabilities >0.05 are predicted to be deleterious.

* One patient harbored compound heterozygous mutations in LRP5.
† Two unrelated probands carried the same mutation.
FIGURE 1. Schematic pedigrees of the families with causative mutations. Arrows indicate proband; open symbols, clinically unaffected; solid symbols, clinically affected; +, clinically evaluated; +/-, homozygous mutation; +/-, heterozygous mutation; --/--, wild type.
novel mutation. A 1-bp deletion c.655delC was identified in a singleton. This novel mutation caused a frameshift resulting in four incorrect amino acids after codon 218, followed by premature termination at codon 223 (p.[Q219Nfs*5]). The female patient developed total retinal detachment in her left eye during early childhood, which resulted in no light perception. She visited our clinic at the age of 44 with decreased vision in the right eye, and a retinal fold with remarkable temporal exudates was observed. Her brother and father were reported to have the congenital cataract from childhood.

A novel stop loss mutation, c.916_918+3delTAAAAA, was identified in a 4-year-old boy with dragged macular in his right eye. His mutation-carrying mother manifested bilateral esotropia with characteristic peripheral avascular retina. The 6-bp deletion removed the termination codon (TAA) at the end of exon 8 and the extra three bases in the following intron (AAA). It was predicted to abolish the original stop codon 306 (*306E)

**FIGURE 2.** Sequence chromatograms of novel mutations. (A–D) Novel heterozygous mutations in LRP5. (E, F) Novel hemizygous mutations in NDP. (H–M) Novel heterozygous mutations in TSPAN12, heterozygous frameshift mutations were confirmed by cloning sequencing (K–M).
and resulted in an elongated protein with 34 additional amino acids at the C-terminus (p.*306Eext*35). To establish whether the stop loss mutation could affect the structure of Tetraspanin12, PsiPred v.3.3 was used to analyze the secondary structure of wild-type and mutant protein. According to the prediction, two extra α-helices and one new β-sheet would be generated, hypothetically, the additional amino acids would alter the structure of Tetraspanin 12 (Fig. 4).

Two novel splice acceptor mutations were found. The c.150-1G>A mutation, located in the splice acceptor site of exon 4, was identified in a 10-year-old boy with bilateral retinal folds and his asymptomatic father. Four algorithms revealed the creation of a novel splice site, which would result in a one nucleotide deletion in the mRNA, and thereby would lead to a shift in the open reading frame and protein truncation. The proband showed asymmetric manifestation with worse visual acuity in his right eye due to retinal fold involving the macula. The second splice acceptor mutation, c.469-1G>A, was identified at the end of intron 6. All four online tools predicted the loss of natural splice site. The proband was a 5-year-old boy with bilateral retinal folds. His healthy parents did not carry the same mutation.

The novel c.2T>C (p.[M1T]) was identified in a 17-year-old male patient. The proband was noticed to have FEVR associated rhegmatogenous retinal detachment in his right eye, which pars plana vitrectomy was performed. Falciform retinal fold was observed in his left eye. His asymptomatic father exhibited with typical FEVR features of bilateral peripheral avascularization; however, no DNA sample was available for mutational analysis. The methionine at codon 1 was highly conserved from human to other spices (Fig. 3D), and this mutation led to the replacement of a hydrophobic residue by a hydrophilic threonine residue. This change was predicted to be deleterious in all four online programs.

The missense mutation c.464G>C (p.[R155T]), was found in a patient with asymmetric manifestation. This 28-year-old male patient suffered total blindness in the right eye with retinal detachment during early childhood, while his left eye was asymptomatic. Peripheral retinal capillary nonperfusion was observed by fluorescein angiography. P.[R155T] was never
reported as the causative mutation in FEVR and the minor allele frequency of this change was lower than 0.1%

A recurrent mutation c.285+1G>A, located in the splice donor site of intron 5, was identified in a full-term infant, and cosegregated among his family. The boy visited our clinic 1 week after birth and fundus screening showed bilateral tractional retinal detachment with pronounced peripheral retinal exudation and hemorrhage. After the intravitreal injection of Ranibizumab in both eyes, the subretinal exudate and hemorrhage were absorbed, however a falciform retinal fold was presented in the left eye. This mutation was previously reported in a Nigerian female with autosomal recessive FEVR, who carried two heterozygous TSPAN12 mutations, c.146C>T; c.285+1G>A.3

**Mutations Detected in NDP**

Two novel missense mutations were detected. The c.203A>C (p.[H68P]) mutation was found in a 35-year-old male with ectopic macular in the right eye. The hydrophilic histidine at codon 68 was conserved across majority of the species (Fig. 3E). This mutation changed the hydrophobic alkaline histidine into the hydrophobic proline residue. No family member was available for fundus examinations and molecular analysis. The c.281A>T (p.[H94L]) was identified in a 3-month-old male infant with bilateral retinal fold. The characteristic features of FEVR, including peripheral avascular zone and retinal exudates were observed in both eyes. No family member had complained about ocular symptoms and his healthy mother was heterozygous for this mutation. The histidine at codon 94 was highly conserved from human to various species (Fig. 3F), and this mutation led to the replacement of a hydrophilic alkaline residue by a hydrophobic leucine residue. Both p.[H68P] and p.[H94L] were predicted to be deleterious by two algorithms (Align GVGD and Mutation Taster).

The novel c.334delIG mutation was found in a 6-month-old boy, who exhibited bilateral tractional retinal detachment. The single base pair deletion was predicted to cause a frameshift after codon 112 and generate a prolonged protein at the C-terminal (p.[G113afs149]). His elder brother with tractional retinal detachment in both eyes carried the same mutation. His mother carried the same mutation in the heterozygous state and exhibited typical FEVR manifestation of avascularization in peripheral retina. Normally, female carrier did not show typical fundus changes of FEVR. Similar cases were reported previously in Japanese patients.13,14 The phenotype of affected female carrier was much milder than the male proband and affected male patient in the same family.

The recurrent missense mutation p.(E66K) (c.196G>A),20 was identified in a 19-year-old male proband with macular dragging in the left eye. His healthy sister carried the same mutation in a heterozygous pattern. The c.362G>A (p.[R121Q]) was previously described as the pathogenic change in patients with XL-FEVR or Norrie disease.21-23 It was found in a 1-year-old boy with retinal fold in the left eye. His 9-year-old brother carried the same mutation, showed total tractional retinal detachment in the left eye with no perception of light and retinal folds in the right eye. Their healthy mother was heterozygous for this mutation.

**Clinical Finding in Patients With Causative Mutations**

There were 100 unrelated Chinese individuals with the clinical diagnosis of FEVR enrolled in this study. Potentially pathogenic mutations were found in 44 probands, including 10 patients with LRP5 mutations, 8 with TSPAN12, 5 with NDP mutations, and 21 patients with causative FZD4 mutations in our previous study.15 There were 44 probands with causative mutations staged according to the Trese’s staging system.24 In Trese’s system, 35 (35/44) patients with causative mutations in these four genes were staged into severe FEVR (stage 3–5), including 15 (15/21) in FZD4, 10 (10/10) in LRP5, 7 (7/8) with TSPAN12, and 3 (3/5) with NDP mutations. However, statistical significance was not established in this classification system (P = 0.411) (Table 2).

After analyzing the clinical features of the 100 probands, a modified approach based on the Trese’s staging system was addressed. Probands with asymptomatic FEVR, in whom the diagnosis of FEVR was only confirmed by fundus fluorescein angiography, were categorized into group I. Patients with vitreous hemorrhage due to retinal neovascularization were categorized into group II. Patients staged into group I or II visited our clinic for the first time mainly in adolescent or adulthood. Patients with an ectopic macula were categorized into group III, while patients with retinal folding were categorized into group IV. Patients with severe total tractional retinal detachment at least in one eye were categorized into group V.

Combined with FZD4 mutations in these probands we reported before, most of the probands (86.4%, 38/44) with pathogenic variants were categorized into group III and IV, including 9 cases in group III and 29 in group IV. None of the FZD4, LRP5, TSPAN12, and NDP mutation was found in probands with group I FEVR, and only one proband was categorized into group II (1/21 in FZD4 mutations). Five patients were staged into group V (5/44). Among the five probands, three of them carried the TSPAN12 mutation (3/5) and one of them harbored the NDP variants (1/5), while only one patient with compound heterozygous mutation in LRP5 exhibited extremely severe FEVR (1/5). According to the modified system, statistical significance was observed in the distribution of four genes (P = 0.049) (Table 3).

**DISCUSSION**

After screening the coding and flanking regions of LRP5, NDP, and TSPAN12 for mutations, 23 pathogenic mutations were found in 23 unrelated FEVR probands, considering our previously study that 14 causative mutations in FZD4 was found in 21 unrelated individuals.15,17 37 causative mutations in FZD4, LRP5, NDP, and TSPAN12 were identified in 44 unrelated probands. The detective rate of four known genes was 44% (44/100). FZD4 mutations accounted for the largest proportion (21/44), followed by LRP5 mutations (10/44), TSPAN12 mutations (8/44), and NDP mutations (5/44) (Fig. 5).

This study was one of the largest cohorts of FEVR in Chinese populations and provided a comprehensive mutation-
genes would provide invaluable information for diagnosis and genetic counseling, especially for patients without family history or with atypical presentations. Further analysis of novel causative genes may explain some mild clinical findings such as group I FEVR patients, and definitely will contribute to our understanding of the molecular mechanisms of retinal angiogenesis, which is crucial for FEVR and other retinal vascular disease.

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References