

Mutations in *LRP5*, *FZD4*, *TSPAN12*, *NDP*, *ZNF408*, or *KIF11* Genes Account for 38.7% of Chinese Patients With Familial Exudative Vitreoretinopathy

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PURPOSE. Familial exudative vitreoretinopathy (FEVR) is a severe hereditary retinal disorder characterized by defects in retinal vascular development. To date, six genes have been reported to be responsible for this disease, including *LRP5*, *FZD4*, *TSPAN12*, *NDP*, *ZNF408*, and *KIF11*. The purpose of our study was to investigate the genetic defects in Chinese patients with FEVR through mutational analyses of 31 pedigrees.

METHODS. Clinical data and peripheral blood were collected from 31 pedigrees with FEVR. All coding sequences and intron/exon junctions were amplified and sequenced comprehensively, followed by cosegregation testing to verify suspected variants in the family members. Finally, we assessed clinical relevance of the identified mutations, according to the standards and guidelines from the American College of Medical Genetics and Genomics.

RESULTS. Twelve index cases (12/31, 38.7%) were confirmed to harbor mutations in the known genes, including one previously reported mutation and 11 novel mutations. Among the detected mutations, *LRP5* accounted for the largest proportion with a mean mutation rate of 16.1% (5/31, 16.1%), followed by *NDP* (3/31, 9.7%), *FZD4* (2/31, 6.5%), *TSPAN12* (1/31, 3.2%), and *KIF11* (1/31, 3.2%). All the novel changes were predicted to be pathogenic by a series of bioinformatics analyses.

CONCLUSIONS. We comprehensively screened six known disease-causing genes in 31 pedigrees with FEVR and achieved a clear picture of the mutation spectrum in Chinese patients with FEVR, which highlights the importance and utility of clinical genetic diagnosis.

Keywords: familial exudative vitreoretinopathy, mutations, genetic diagnosis, genotype-phenotype correlations

Familial exudative vitreoretinopathy (FEVR) is a rare Mendelian monogenic disorder resulting in tractional retinal detachment and blindness in adolescents. The disease was first reported by Criswick and Schepens¹ in 1969. The primary pathologic process in FEVR is incomplete retinal vascular development on the temporal side of the peripheral retina, which is followed by neovascularization of the retina.^{2,3} New retinal blood vessels are prone to leakage and rupture, which result in retinal falciform fold, retinal traction with ectopic macula, intraretinal hemorrhage, exudation, followed by subtotal and total retinal detachment, total vision loss and blindness. The clinical manifestations of this disease differ greatly among patients even between the two eyes of the same patient and among affected siblings in the same family, from asymptomatic to complete retinal detachments with blindness.^{2–4} Many of these retinal abnormalities are similar to retinopathy of prematurity (ROP). However, the affected cases with FEVR, unlike patients with ROP, usually go through a normal gestational period and have no history of low birth weight and supplemental oxygen therapy.⁴ Additionally, detachments often do not occur in FEVR until the first or second decade of life. In fact, the retinal manifestations may appear to

remain stable when a patient reaches adulthood in this variable progressive disorder.⁴

FEVR is inherited as an autosomal dominant, an autosomal recessive, or an X-linked recessive trait. However, autosomal dominant inheritance due to haploinsufficiency is the most prominent form of the disease. To date, six genes have been reported to associate with FEVR, including *LRP5*, *FZD4*, *TSPAN12*, *NDP*, *ZNF408*, and *KIF11*.^{5–14} The proteins encoded by the first four genes are cooperative in the Wnt/Norrin signaling pathway, which plays a critical role in eye development and angiogenesis. As a result, mutations in these genes can lead to abnormal retinal vascular formation. A recent study¹⁵ has demonstrated that *Lrp5* knockout (*Lrp5*^{-/-}) mouse model of FEVR displays significantly delayed retinal vascular development and subsequent pathologic glomeruloid vessels. In addition, as a new candidate gene potentially related to FEVR, novel founder mutations in *ZNF408* have been reported in one Dutch family and a Japanese family. Subsequently, knockdown of *znf408* in zebrafish indicates that it plays a putative role in retinal vasculogenesis.¹¹ Mutations in *KIF11* are reported to be associated with microcephaly, chorioretinopathy, or mental retardation (MCLMR).¹⁶ Furthermore, *KIF11*



mutations have been recently identified in affected individuals with FEVR by Robitaille et al.¹² Besides, there is a phenotypic overlap between the two diseases caused by *KIF11* mutations. Other recent studies^{13,14} have also reported novel *KIF11* mutations in FEVR patients.

Despite the individual identifications of the causal genes in previously reported cases, few studies have attempted to screen for the whole panel of causative genes in a defined cohort.^{14,17,18} Additionally, genotype–phenotype correlation in FEVR has rarely been studied.^{14,19,20} Thus, the present study was carried out to fill in the knowledge gap by exploring the mutation spectrum and genotype–phenotype correlation in 31 pedigrees of Chinese FEVR patients.

METHODS

Clinical Examinations

This study was carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient, following the ethical approval from The Eye Hospital of Wenzhou Medical University and Kunming Maternal and Children's Hospital. We recruited 31 pedigrees with FEVR and 200 healthy controls. All individuals are native Chinese, of full-term birth and normal birth weight. Full ophthalmic examinations were conducted, including visual acuity measurement, slit-lamp examination, detailed fundus photography, and fundus fluorescein angiography (FFA). Each proband and his/her available family members were assessed by a panel of retinal specialists. The disease severities were further classified and assessed in accordance with the staging system reported by Pendergast et al.⁴

Comprehensive Mutational Screening

Peripheral blood samples were collected from the 31 pedigrees and preserved in -80°C before use. Genomic DNAs were extracted by using a whole blood DNA extraction kit (Simgen, Hangzhou, China) according to standard protocols. Polymerase chain reactions were performed by using specific primers targeting all open reading frames and the flanking intronic sequences for direct sequencing on genetic sequencer. After the initial denaturation step at 95°C for 5 minutes, the samples were subjected to 35 cycles of amplification at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds. A final elongation step was performed at 72°C for 5 minutes.

Pathogenicity Evaluation

To evaluate the pathogenicity of the identified mutations, we explored 1000 Genome database (1000G),²¹ Exome Aggregation Consortium (ExAC),^{22,23} and in-house data from previous studies. Potential deleteriousness was evaluated from the standards and guidelines of the American College of Medical Genetics and Genomics (ACMG)²⁴ and variant bioinformatics tools including SIFT, MutationTaster, Polyphen2 HDIV, Polyphen2 HVAR, Mutation Assessor, PhD-SNP, SNPs&GO, LR, PROVEAN, and RadialSVM. Detailed functional predictive scores were calculated by using Min-Max normalization.

Multiple Sequence Alignment

Amino acid sequences were obtained from National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/> protein; in the public domain), and multiple protein alignments were produced with the ClustalW2 (<http://www.ebi.ac.uk/clusterw2/>; in the public domain).²⁵ Sequence logos were generated on account of WebLogo3 ([\[threeplusone.com/\]\(http://threeplusone.com/\); in the public domain\).²⁶ The following sequences were used: NP_000257.1 \(*Homo sapiens*, NDP\), XP_016799622.1 \(*Pan troglodytes*, NDP\), NP_001102284.1 \(*Rattus norvegicus*, NDP\), NP_035013.1 \(*Mus musculus*, NDP\), XP_004283455.1 \(*Orcinus orca*, NDP\), NP_001265016.1 \(*Gallus gallus*, NDP\), and XP_009303083.1 \(*Danio rerio*, NDP\).](http://weblogo.</p>
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RESULTS

Clinical Manifestations

In this study, we recruited 31 pedigrees clinically diagnosed with FEVR. Among the probands, 13 were females and 18 were males. Among those, no one had a history of low birth weight or premature birth. Before being diagnosed with FEVR, most probands were found to exhibit other ocular abnormalities, such as strabismus, nystagmus, leukocoria, and the inability to follow moving objects. Fundus photography revealed typical falciform retinal folds and temporal dragging of optic disc and retinal detachment. FFA exhibited incomplete vascularization of the peripheral retina, such as a peripheral avascular zone, especially in patients with unnoticed mild phenotypes. Additionally, a 2-year-old proband (F28, II: 2) was found to have microcephalus, whose head circumference was only 44 cm, 2 standard deviations (SDs) below the mean. The clinical classification for each family is shown in Supplementary Table S1.

Mutations in the FEVR Cohort

We considered a variation as “pathogenic” by the following standards: (1) excluding variants identified in 1000G, ExAC, database of SNP (dbSNP), and 200 normal individuals; (2) excluding variants predicted to be benign by the standards and guidelines from ACMG and 11 bioinformatics tools; and (3) excluding incompatible variants by cosegregation testing. Ultimately, we identified 11 novel mutations and 1 reported mutation in 12 unrelated FEVR families (Fig. 1; Table; Supplementary Fig. S1). Furthermore, we also observed that some affected individuals harbored multiple variants in *LRP5* and *TSPAN12*. However, all these variants were considered as polymorphisms (Supplementary Fig. S2).

In family 15, the index case (F15: III: 3) harbored a nonsense mutation (c.345T>G, p.Y115*) in *TSPAN12*, which was a newly identified mutation. In family 9, a nonsense mutation (c.1384C>T, p.R462*) was discovered in *LRP5*. Deletions and insertions were uncovered in family 11 (*LRP5*, c.2817_2827+1del12bp, p.N940fs), family 21 (*NDP*, c.52_53ins32bp, p.S29fs), family 20 (*FZD4*, c.1282_1285del-GACA, p.D428fs), family 23 (*FZD4*, c.227delA, p.E76fs), and family 28 (*KIF11*, c.1830_1833delACAA, p.E610fs). Each of them was predicted to cause a frameshift mutation and a prematurely truncated protein. Of the five missense mutations, three occurred in *LRP5* gene (c.4205G>A, p.G1402D; c.2237G>C, p.R746P; c.2618A>T, p.K873M) and two in *NDP* gene (c.195C>G, p.C65W; c.127C>A, p.H43N) (Fig. 2A). Structural modeling of the NDP protein (Fig. 2B) demonstrated that both C65W and H43N were located in the first antiparallel β -sheet, which was required for receptor binding. Additionally, both mutated amino acids were highly conserved residues from human to zebrafish (Fig. 2C).

In accordance with the guidelines from ACMG, one missense variant was perceived as “pathogenic” and four missense variants were predicted to be “likely pathogenic”. The pathogenicity was further verified by 11 bioinformatics tools (Fig. 3; Supplementary Table S3). Taken together, we identified 12 mutations in different genes in 12 unrelated FEVR families and 11 of them were described for the first time.

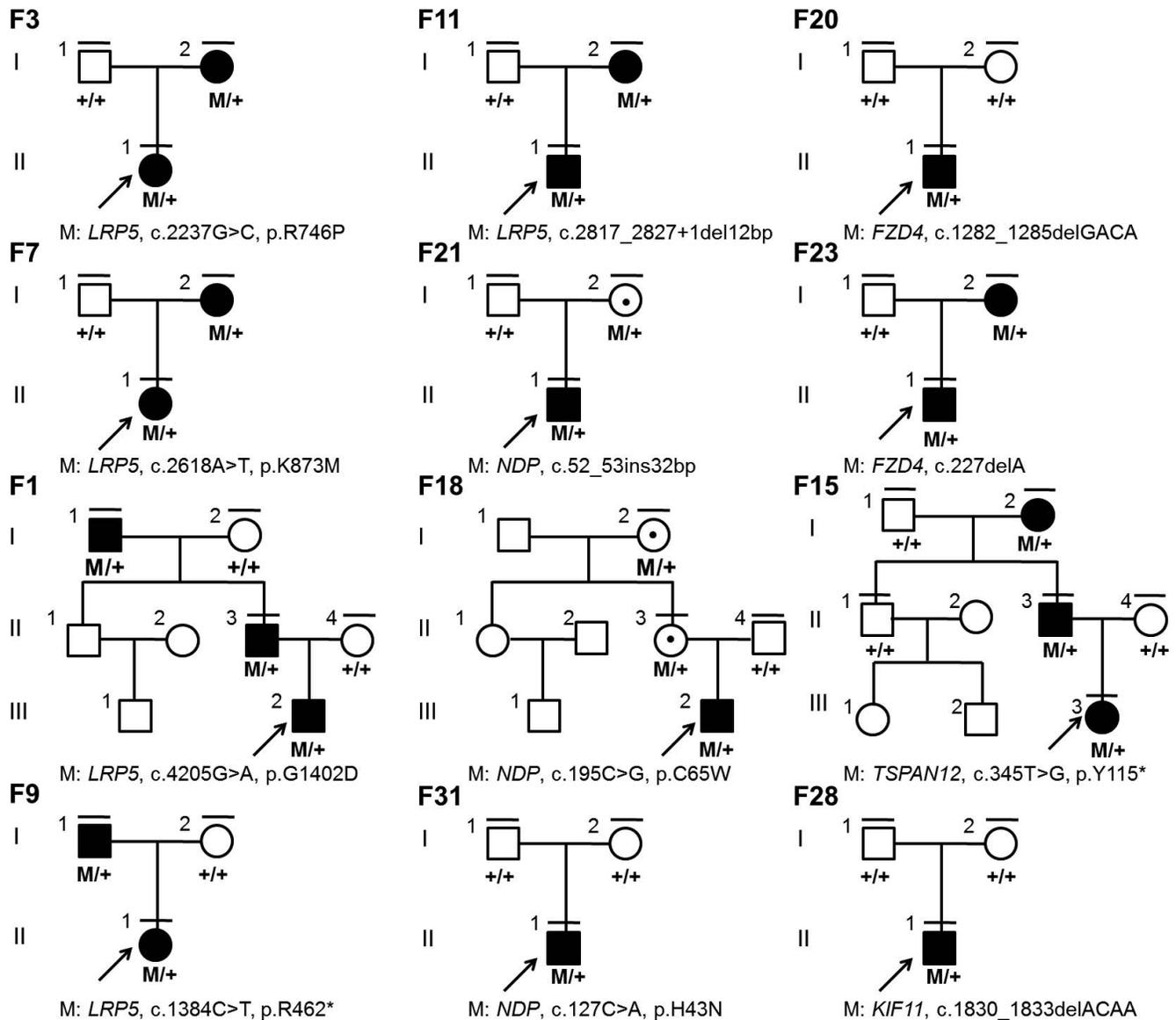


FIGURE 1. Pedigree information. In these pedigrees, the bars over the symbols denote the individuals recruited in the study; filled symbols indicate affected patients with FEVR, the unfilled symbols indicate unaffected individuals, and the dotted symbols denote carriers. M represents a variant, and + indicates normal allele.

TABLE. Identified Mutations in Five Genes of Twelve Families With FEVR

| Family | Gene | Type | Mutation | 1000G | ExAC | Reference | Cosegregation | ACMG |
|--------|----------------|------------|--------------------------------|-------|-------|-----------|---------------|-------------------|
| F1 | <i>LRP5</i> | Missense | c.4205G>A, p.G1402D | Novel | Novel | Novel | Paternal | Likely pathogenic |
| F3 | <i>LRP5</i> | Missense | c.2237G>C, p.R746P | Novel | Novel | Novel | Maternal | Likely pathogenic |
| F7 | <i>LRP5</i> | Missense | c.2618A>T, p.K873M | Novel | Novel | Novel | Maternal | Likely pathogenic |
| F9 | <i>LRP5</i> | Nonsense | c.1384C>T, p.R462* | Novel | Novel | Novel | Paternal | Pathogenic |
| F11 | <i>LRP5</i> | Frameshift | c.2817_2827+1del12bp, p.N940fs | Novel | Novel | Novel | Maternal | Pathogenic |
| F18 | <i>NDP</i> | Missense | c.195C>G, p.C65W | Novel | Novel | Novel | Maternal | Likely pathogenic |
| F31 | <i>NDP</i> | Missense | c.127C>A, p.H43N | Novel | Novel | Novel | De novo | Pathogenic |
| F21 | <i>NDP</i> | Frameshift | c.52_53ins32bp, p.S29fs | Novel | Novel | Novel | Maternal | Pathogenic |
| F20 | <i>FZD4</i> | Frameshift | c.1282_1285delGACA, p.D428fs | Novel | Novel | Reported | De novo | Pathogenic |
| F23 | <i>FZD4</i> | Frameshift | c.227delA, p.E76fs | Novel | Novel | Novel | Maternal | Pathogenic |
| F15 | <i>TSPAN12</i> | Nonsense | c.345T>G, p.Y115* | Novel | Novel | Novel | Paternal | Pathogenic |
| F28 | <i>KIF11</i> | Frameshift | c.1830_1833delACAA, p.E610fs | Novel | Novel | Novel | De novo | Pathogenic |

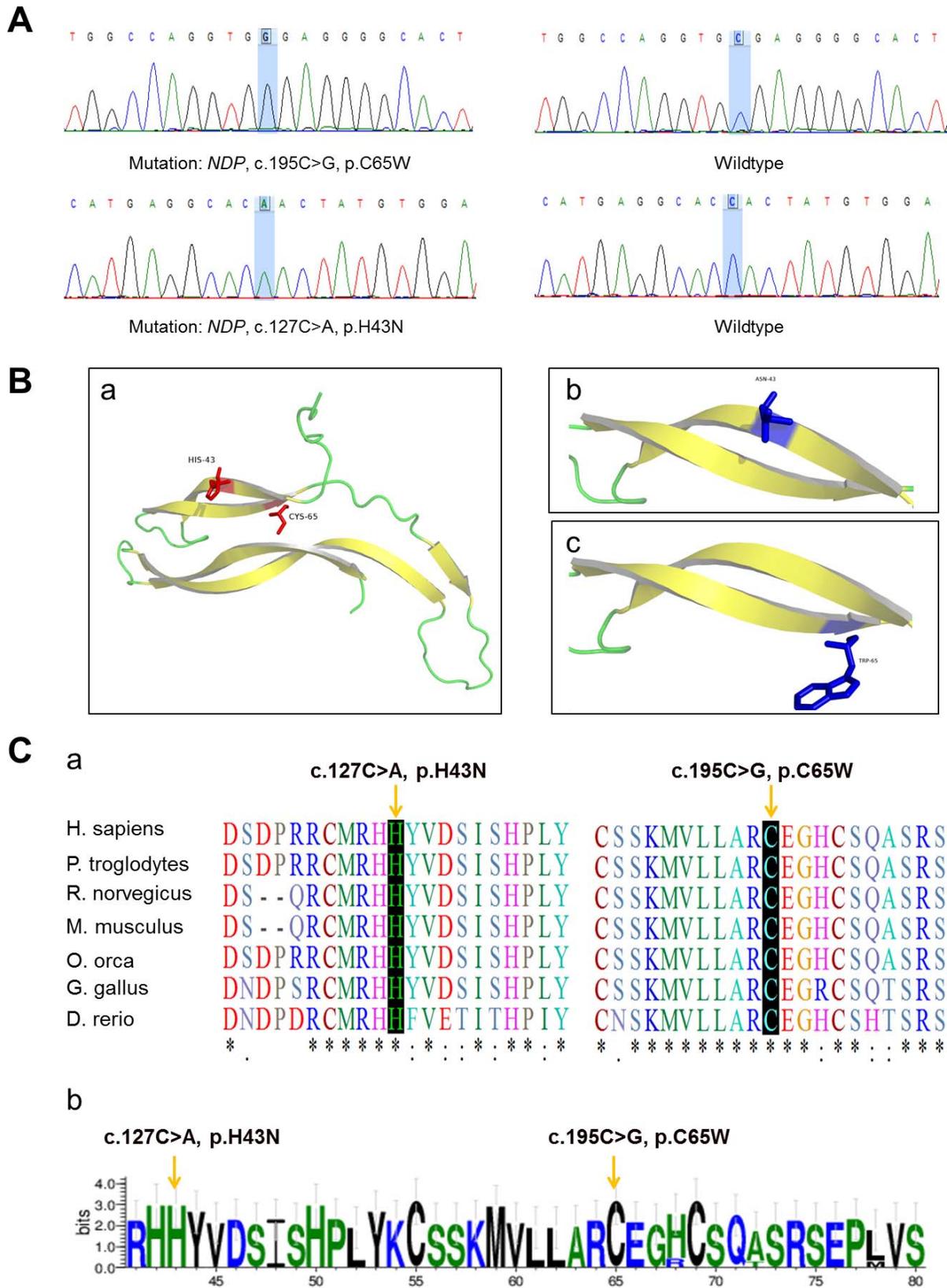


FIGURE 2. Mutational analysis of two novel mutations identified in *NDP* gene. **(A)** Chromatograms of the missense mutation c.195C>G (p.C65W) and c.127C>A (p.H43N) in the *NDP* gene. **(B)** Structural 3D modeling of *NDP* missense changes. Wild-type **(a)** and mutant residues **(b, c)** were indicated in *red* and *blue*, respectively. **(C)** Conservative amino acid analysis of *NDP* polypeptides from human to zebrafish, based on ClustalW2 **(a)** and WebLogo3 **(b)**. The affected amino acid residues were *underlined*.

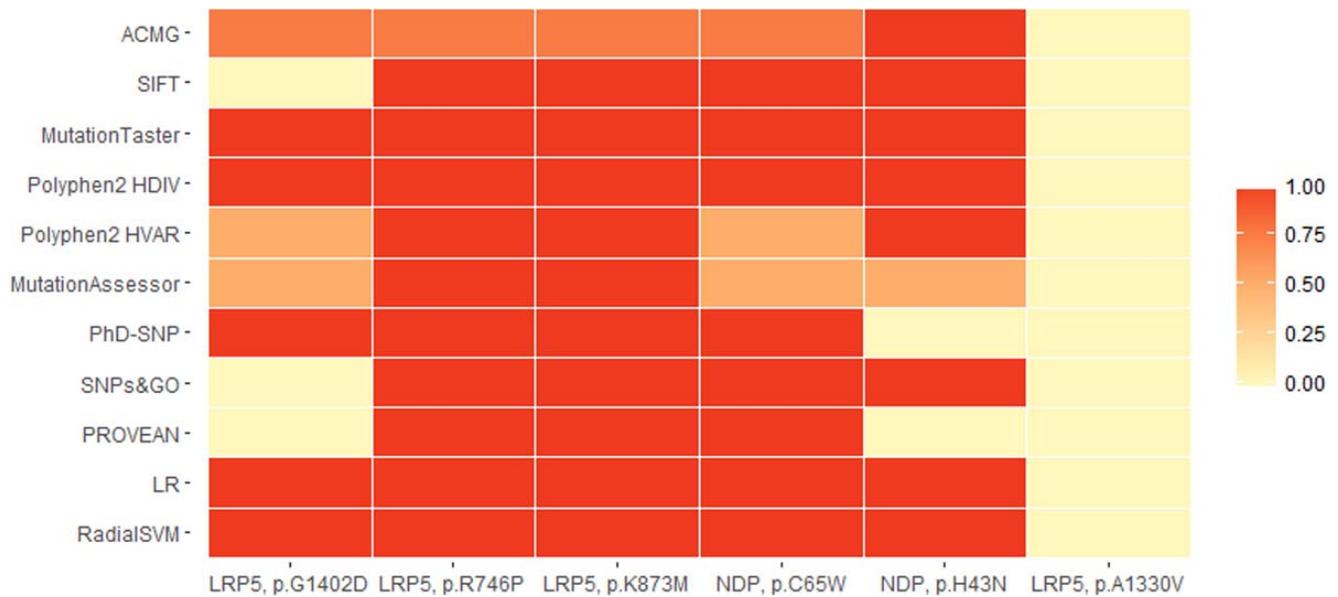
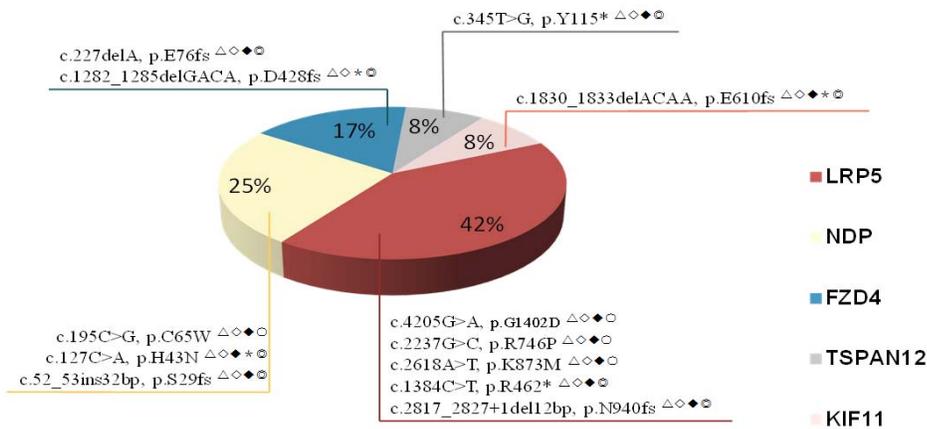


FIGURE 3. Comparative pathogenic analysis of the five missense mutations. The average of detailed functional predictive scores were calculated and shown in the heatmap after Min-Max normalization, based on the standards and guidelines by American College of Medical Genetics and Genomics and a series of bioinformatics tools. *Deeper colors* indicate higher pathogenicity. The *x-axis* represented various variants, while the *y-axis* depicted diverse pathogenic analysis. The variant in the *bottom right* (LRP5, p.A1330V) was a nonpathogenic polymorphism, which was used as a negative control.

A



B



FIGURE 4. Mutation spectrum in Chinese patients with FEVR. (A) The mutation spectrum indicates the proportion of different causal genes observed in the Chinese FEVR cohort. Each *colored box* represents different genes. △ represents “novel” in 1000G; ◇ represents “novel” in ExAC; ◆ and ★ represent “novel” and “reported” in-house data from previous studies, respectively; and ○ represents “pathogenic” and “likely pathogenic,” respectively. In accordance with the guidelines from ACMG. (B) Each *colored box* represents a mutation type: *pink*, missense; *red*, nonsense; *blue*, frameshift.

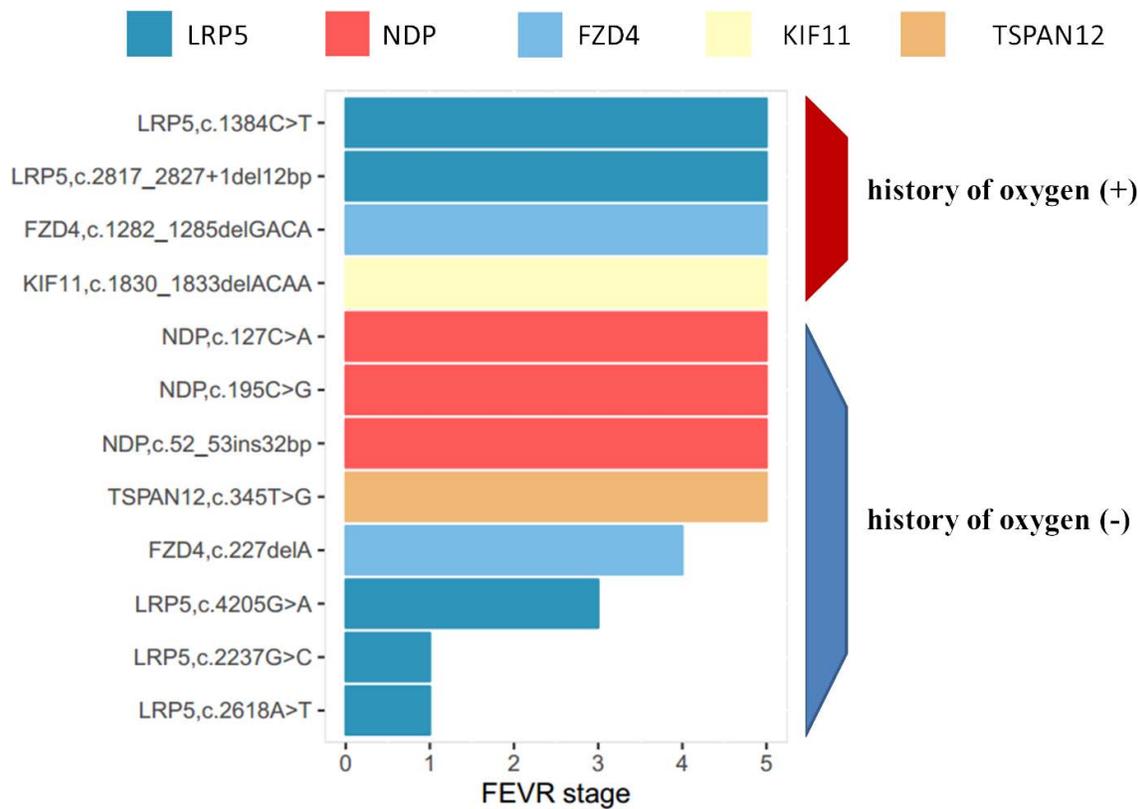


FIGURE 5. Genotype-phenotype correlations. The phenotypic severity of each proband is classified into five stages, which is indicated in boxes of different size. Each colored box represents mutations in different causal genes.

Mutation Spectrum

In this study, 12 patients (12/31, 38.7%) were successfully identified with genetic mutations. Among them, mutations in *LRP5* (5/31, 16.1%) accounted for 42% of patients with defined mutations, indicating that *LRP5* was the most frequent gene mutated in Chinese FEVR patients. The second leading mutation was located in *NDP*, which corresponded to 25% of patients with defined mutations, whereas mutations in *FZD4*, *TSPAN12*, and *KIF11* accounted for the rest of the 33% of patients (Fig. 4). These results indicated that genetic factors were responsible for ~61.3% of patients with as yet unknown gene(s) that remain to be identified. Among the six known genes screened in this study, *LRP5* and *NDP* were the predominant genes (67%), while no pathogenic mutations were identified in *ZNF408*. Through the comprehensive screening, we revealed the mutation spectrum of the six implicated genes in Chinese FEVR patients.

Genotype-Phenotype Correlations

To investigate the relationship between causative genes and FEVR symptoms, we also recruited other affected individuals (apart from the probands) to carry out genotype-phenotype analyses in the 12 families with disease-causing mutations (Supplementary Table S4). We noticed that the affected individuals harboring the *LRP5* mutations showed broader phenotypic spectra that varied from stage 2 to stage 5. However, all patients carrying *NDP* mutations and truncating mutations were correlated with severe phenotypes (stage 4 or above) (Fig. 5). Our results revealed that patients with history of short-term oxygen therapy also showed more severe phenotypes (stage 4 or above). We, therefore, were tempted to hypothesize that short-term oxygen therapy after birth

might be a major contributor to genetic causality of FEVR. In addition, owing to relatively small sample size, our findings warrant further investigation.

DISCUSSION

FEVR, a clinically heterogeneous disorder causing tractional retinal detachment at a very young age, is characterized by avascularity in the peripheral retina and compensatory retinal neovascularization. To date, six causative genes have been identified as related to FEVR, including low-density-lipoprotein receptor-related protein 5 (*LRP5*), frizzled-4 (*FZD4*), tetraspanin-12 (*TSPAN12*), Norrie disease protein (*NDP*), zinc finger protein 408 (*ZNF408*), and kinesin family member 11 (*KIF11*). Previous studies have shown that approximately 35% to 50% of FEVR patients are genetically diagnosed by screening the six known genes. However, the detailed mutation spectrum of each gene was unknown in Chinese individuals.^{14,17,18} In the present study, we comprehensively screened all known genes in 31 pedigrees and uncovered 38.7% of unrelated FEVR patients harboring mutations in these genes. In addition, we, for the first time, revealed the detailed mutation spectrum in Chinese patients with FEVR.

Among the 31 families, 12 index ones were identified with genetic predispositions (38.7%), including five *LRP5* mutations (5/31, 16.1%), three *NDP* mutations (3/31, 9.7%), two *FZD4* mutations (2/31, 6.5%), one *TSPAN12* mutation (1/31, 3.2%), and one *KIF11* mutation (1/31, 3.2%). Except for the *NDP* mutations, which resulted in X-chromosome-linked FEVR, mutations in the other five genes cosegregated with the disease in an autosomal dominant manner. It should be noted that among the mutations identified in this study, most were described for the first time in this study.

From the clinical examinations of fundus photography and fundus fluorescein angiography, we noticed that retinal folds and detachments were common in severely affected patients, while mildly affected patients displayed avascularity in their peripheral retina. We thus attempted to elucidate whether a genotype-phenotype correlation is present in FEVR. We found that patients with mutations in the *LRP5* gene displayed broader phenotypic spectra, from asymptomatic to complete retinal detachments, while in those with *NDP* mutations, the clinical phenotypes were found to be rather serious. Additionally, relevance between history of oxygen uptake and severe FEVR phenotypes were observed in our study. We found that history of oxygen uptake is probably a significant factor that leads to deterioration of FEVR. As a novel gene related to FEVR, *KIF11* mutations were reported to be associated with MCLMR. Recently, mutations in *KIF11* and their associations with FEVR were reported. Besides, there were phenotypic overlaps between these two diseases that are caused by *KIF11* mutations. In addition, we identified one novel truncating mutation in this study (c.1830_1833delACAA, p.E610fs), which broadened the current mutation frequency in this gene.

In conclusion, we performed a comprehensive screening in Chinese patients with FEVR and uncovered the mutation spectrum as well as the complexity of genotype-phenotype correlation, thus extending the current understanding of genetic predispositions in FEVR. However, we found that the detection rate of mutations found in the known genes did not exceed 50% in this study. In light of these results, we suspect that other unidentified genes are likely to be responsible for FEVR as well, which warrant further genetic studies.

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