Identification of two novel LRP5 mutations in families with familial exudative vitreoretinopathy

Ping Fei,1 Qi Zhang,1 Luling Huang,2 Yu Xu,1 Xiong Zhu,2 Zhengfu Tai,2 Bo Gong,2 Shi Ma,2 Quanyao Yao,3 Jing Li,1 Peiquan Zhao,1 Zhenglin Yang2

1Department of Ophthalmology, Xin Hua Hospital affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China; 2Sichuan Key Laboratory for Human Disease Gene Study, Institute of Laboratory Medicine, School of Medicine, University of Science and Technology of China & Sichuan Provincial People’s Hospital & Sichuan Provincial People’s Hospital, Sichuan, China; 3West high school, Salt Lake City, UT

Purpose: To investigate the clinical features and disease-causing mutations in two Chinese families with familial exudative vitreoretinopathy (FEVR).

Methods: Clinical data and genomic DNA were collected for patients with FEVR. The coding exons and adjacent intronic regions of FZD4, LRP5, TSPAN12, and NDP were amplified with PCR, and the resulting amplicons were analyzed with Sanger sequencing. Wild-type and mutant LRP5 proteins were assayed for the Norrin/β-catenin pathway by luciferase reporter assays.

Results: Two novel heterozygous mutations in the LRP5 gene were identified in two relatives—p.A422T and p.L540P. Typical FEVR fundus change and mild reduced bone mineral density (BMD) was found in the two patients and the affected parent. In the luciferase studies, both p.A422T and p.L540P mutants displayed a significant reduction of the luciferase activity in SuperTopFlash (STF) cells in response to Norrin (87% reduction for p.A422T and 97% reduction for p.L540P). Both patients had an additional LRP5 sequence change (p.Q816P in Patient 1 from the unaffected mother and p.T852M in Patient 2 verified as a new mutation). Luciferase assay showed no reduction for p.Q816P and 94.9% reduction for p.L540P). Both patients had an additional LRP5 sequence change (p.Q816P in Patient 1 from the unaffected mother and p.T852M in Patient 2 verified as a new mutation). Luciferase assay showed no reduction for p.Q816P and 94.9% reduction for the new mutation p.T852M, suggesting that p.Q816P may be not pathogenic and p.T852M may be pathogenic.

Conclusions: Our findings demonstrated two new novel LRP5 mutations in Chinese patients with FEVR and mild reduced BMD. They emphasize the complexity of FEVR mutations and phenotypes.

Familial exudative vitreoretinopathy (FEVR, OMIM 133780) is a hereditary disorder with abnormal retinal vascular development [1]. This disease is characterized by a premature arrest of the vascularization in the peripheral retina, which may result in features such as retinal neovascularization or tractional retinal detachment [2]. However, the clinical phenotype of FEVR varies widely from asymptomatic to complete blindness, even within the same family.

FEVR is inherited in an autosomal dominant manner in most cases [3-7]. It can also be inherited as an autosomal recessive [8] or X-linked disorder [9]. Mutations in FZD4 (Gene ID: 8322, OMIM 604579) [10-13], LRP5 (Gene ID: 4041, MIM 603506) [12,13], and TSPAN12 (gene ID 23554, MIM 613138) [14-16] can cause an autosomal dominant form of FEVR [1,3-7], and mutations in LRP5 [13] and NDP (Gene ID: 4693, MIM 300658) [9,12] may cause autosomal recessive and X-linked forms of FEVR, respectively. Recessive TSPAN12 mutations can also cause FEVR [17,18].

FZD4, LRP5, NDP, and TSPAN12 are components of the Wnt pathway and the Norrin/β-catenin pathway. The Wnt pathway and Norrin/β-catenin pathway play important and diverse roles in the physiological and pathological situations, such as cell survival, proliferation, migration and angiogenesis [19,20]. Activation of the canonical Wnt pathway or Norrin/β-catenin pathway has been shown to be important in eye organogenesis and angiogenesis [19-21].

LRP5 is a member of the low-density lipoprotein (LDL) receptor family and belongs to a subfamily consisting of its mammalian homolog LRP6 and the Drosophila protein Arrow [22]. LRP5 encodes single-pass transmembrane receptors that partner with members of the frizzled family of seven-pass transmembrane receptors to bind Wnt proteins or Norrin, forming a functional ligand-receptor complex that activates the canonical Wnt-β-catenin pathway or Norrin-β-catenin pathway [23-25]. Mutations in the Wnt and Norrin coreceptor gene LRP5 have been confirmed to cause FEVR [26].

Recessive LRP5 mutations are also known to underlie osteoporosis-pseudoglioma syndrome (OPPG, OMIM 259770), a disorder characterized by extremely low bone mass and congenital or infancy onset blindness [27]. Similarly, reduced bone mass has been reported in the heterozygous
mutation carriers in OPPG families and in FEVR patients with dominant and recessive LRP5 mutations [13,28,29].

In this study, we identified two novel LRP5 mutations in patients with FEVR. Mild reduced bone mineral density (BMD) was also revealed in members with LRP5 mutation in both of the families. We further confirmed that the two mutants failed to induce luciferase reporter activity in response to Norrin in a HEK293 cell line transfected with a luciferase reporter, which implicated the mutants to be pathologic.

METHODS

Patients and clinics: Study approval was obtained from the institutional review boards of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital and Xin Hua Hospital affiliated to Shanghai Jiao Tong University.
Figure 2. Fundus changes in the proband with the heterozygous c.1619T>C (p.L540P) mutation in Family 2. A and B: RetCam examination of both of Patient 2’s eyes. The fundus of the left eye could not be viewed due to the opacity of the lens. C and D: Ultrasound B-scan of Patient 2. These photos demonstrate leukocoria and severe retinal detachment of both eyes. The left eye is more severely affected than the right eye. Posterior synechia is evident in the left eye. E: Fundus of Patient 2’s right eye at the last follow-up after lensectomy and vitrectomy. The retina was mostly attached. F-I: Fundus photos and fundus fluorescein angiography (FFA) of the asymptomatic mother with c.1619T>C in Family 2. F and G: Fundus photos show normal posterior fundi. H and I: FFA shows that the mother has peripheral nonperfusion areas, increased ramification, and shunts of the peripheral retinal vessels in both eyes.
School of Medicine, China and informed consent was obtained from all the participants. The study adhered to the tenets of the Declaration of Helsinki. Ophthalmic examinations including the fundus photography or ultrasound examination were conducted in all patients with FEVR. Fundus fluorescein angiography (FFA) was performed in parents to confirm the family history. In the 300 normal matched controls, all individuals underwent an eye examination and no signs of eye diseases were observed. The family members in which the \( LRP5 \) mutation was detected underwent standard dual-energy X-ray absorptiometry (DEXA) bone scanning to measure BMD using the GE Lunar Prodigy DEXA densitometer. T scores (number of standard deviations from the mean derived from healthy young sex-matched adults) are the usual format for expressing BMD in adults. For children, Z scores (standard deviations from the mean derived from an age, sex, and racially matched population) are generated by imaging the spine.

**Mutation screening:** Peripheral blood was collected in EDTA tubes from patients and parents with FEVR and normal control subjects and was preserved in -20 °C prior to use. Genomic DNA was isolated using the salt precipitation method. PCR primers were designed to include flanking sequences. Genomic DNA was isolated using the salt precipitation method. PCR primers were designed to include flanking sequences.

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**Figure 3.** Pedigrees of families with autosomal dominant familial exudative vitreoretinopathy (FEVR) and the mutations identified in \( LRP5 \). **A:** In Family 1, Patient 1 had two \( LRP5 \) mutations, c.1264G>A (p.A422T) from the affected father and c.2447A>C (p.Q816P) from unaffected mother. **B:** In Family 2, Patient 2 also had two \( LRP5 \) mutations; c.1619T>C (p.L540P) was from the affected mother, but c.2555C>T (p.T852M) was not detected in either parent. The columns from left to right display the pedigree and the sequence chromatograms for these patients. **Squares**, male; **circles**, female; **closed**, affected; **open**, unaffected.

**Figure 4.** Protein alignment of 10 (\( LRP5 \) orthologs demonstrates conservation in the regions with mutations. The 10 orthologs are from the following 10 species: \( Homo sapiens \) (NP_002326.2), \( Pan troglodytes \) (XP_508605.3), \( Canis lupus familiaris \) (NP_032539.2), \( Rattus norvegicus \) (NP_001099791.2), \( Gallus gallus \) (NP_001012915.1), \( Danio rerio \) (NP_001170929.1), \( Drosophila melanogaster \) (NP_524737.2), and \( Anopheles gambiae \) (XP_320740.4). The regions with the four missense mutations are highly conserved. A422T stands for Ala422Thr, L540P stands for Leu540Pro, Q816P stands for Gln816Pro, and T852M stands for Thr852Met.
| Patient  | Gene (chromosome) | Chromosome start and end (hg19) | referred base | altered base | homozygous/heterozygous | region | change | Nucleotide variant | Effect | dbSNP132 |
|----------|------------------|---------------------------------|-------------|-------------|-------------------------|--------|--------|-------------------|--------|-----------|
| FZD4 (chr11) |  |  |  |  |  |  |  |  |  |  |
| Patient 1 | LRP5 (chr11) |  |  |  |  |  |  |  |  |  |
| TSPAN12 (chr7) |  |  |  |  |  |  |  |  |  |  |
| 86657520 | FZD4 (chr11) | 86658244 | CAAA | - | het | UTR3 | rs713065 |  |  |  |
| 86658245 | FZD4 (chr11) | 86660449 | A | - | het | UTR3 | rs34325935 |  |  |  |
| 86660612 | FZD4 (chr11) | 86660886 | G | A | hom | UTR3 | rs4944641 |  |  |  |
| 68154032 | FZD4 (chr11) | TSPAN12 (chr7) | 68154032 | G | A | het | UTR3 | rs10791978 |  |  |  |
| 68171013 | FZD4 (chr11) | 68174122 | G | A | hom | UTR3 | rs3802892 |  |  |  |
| 68177614 | FZD4 (chr11) | 68179032 | A | C | het | UTR3 | rs8718372 |  |  |  |
| 68179125 | FZD4 (chr11) | 68192690 | G | A | hom | UTR3 | rs901824 |  |  |  |
| 68204445 | FZD4 (chr11) | 68206173 | T | C | hom | UTR3 | rs41622 |  |  |  |
| 120428607 | FZD4 (chr11) | 120428799 | C | A | hom | UTR3 | rs416223 |  |  |  |
| 120450658 | FZD4 (chr11) | 120450678 | - | CA | hom | UTR3 | rs7805733 |  |  |  |
| Patient       | Gene (chromosome) | Chromosome start and end (hg19) | referred base | altered base | homozygous/heterozygous | region | change       | Nucleotide variant | Effect | dbSNP132 |
|---------------|-------------------|---------------------------------|--------------|-------------|-------------------------|--------|-------------|-------------------|--------|----------|
|               |                    |                                 |              |             |                         |        |             |                   |        |          |
|               | **FZD4** (chr11)   |                                 |              |             |                         |        |             |                   |        |          |
|               | **Father of Patient 1** |                        |              |             |                         |        |             |                   |        |          |
|               | **LRP5** (chr11)   |                                 |              |             |                         |        |             |                   |        |          |
|               | **TSPAN12** (chr7) |                                 |              |             |                         |        |             |                   |        |          |
|               |                    |                                 |              |             |                         |        |             |                   |        |          |
| Patient 1     |                   |                                 |              |             |                         |        |             |                   |        |          |
|               | **FZD4** (chr11)   |                                 |              |             |                         |        |             |                   |        |          |
| 86657520      |                   | A                               |             | G           | het                     | UTR3   | c.1264G>A   | p.A422T          |        | rs713065 |
| 86658244      |                   | AAA                             |             | -           | het                     | UTR3   | c.1647T>C   | p.F549F          |        | rs494461 |
| 86660449      |                   | A                               |             | -           | het                     | UTR3   | c.1932G>A   | p.E644E          |        | rs3472935|
| 86660612      |                   | C                               |             | G           | hom                     | UTR3   | c.3357G>A   | p.V1119V         |        | rs3802892|
| 86660886      |                   | G                               |             | A           | hom                     | intron | c.765G>T    | p.P255P          |        | rs11255207|
| 68131167      |                   | A                               |             | T           | hom                     | intron |             |                   |        | rs10791978|
| 68154032      |                   | G                               |             | A           | het                     | exon 6 | nonsynonymous SNV | c.1264G>A | p.A422T |          |
| 68171013      |                   | T                               |             | C           | hom                     | exon 8 | synonymous SNV | c.1647T>C | p.F549F | rs545382 |
| 68174122      |                   | G                               |             | A           | het                     | exon 9 | synonymous SNV | c.1932G>A | p.E644E | rs2277268|
| 68177319      |                   | A                               |             | -           | het                     | intron |             |                   |        | rs4988322 |
| 68177614      |                   | T                               |             | C           | hom                     | intron |             |                   |        | rs2242339 |
| 68179125      |                   | C                               |             | T           | hom                     | intron |             |                   |        | rs689179  |
| 68179166      |                   | A                               |             | G           | hom                     | intron |             |                   |        | rs556442  |
| 68192690      |                   | G                               |             | A           | hom                     | exon 15| synonymous SNV | c.3357G>A | p.V1119V | rs41622  |
| 120428607     |                   | G                               |             | A           | hom                     | UTR3   |             |                   |        | rs41623  |
| 120428799     |                   | C                               |             | A           | hom                     | exon 8 | synonymous SNV | c.765G>T | p.P255P | rs7805733|
| 120450658     |                   | G                               |             | A           | hom                     | intron |             |                   |        |          |
| 120450678     |                   | -                               |             | CA          | het                     | intron |             |                   |        | rs112555207|
| 120450710     |                   | GT                              |             | -           | het                     | intron |             |                   |        |          |
| Patient | Gene (chromosome) | Chromosome start and end (hg19) | referred base | altered base | homozygous/heterozygous | region | change | Nucleotide variant | Effect | dbSNP132 |
|---------|------------------|---------------------------------|--------------|-------------|------------------------|--------|--------|-------------------|--------|----------|
| Patient 2 | FZD4 (chr11)    | 86660449                        | A            | -           | het                    | UTR3   |         |                   |        |          |
|         |                  | 86660612                        | C            | G           | hom                    | UTR3   |         |                   |        | rs4944641 |
|         |                  | 68131167                        | A            | T           | hom                    | intron |         |                   |        | rs10791978 |
|         |                  | 68170985                        | T            | C           | het                    | exon 8 |         | nonsynonymous SNV | c.1619T>C | p.L540P   |
|         |                  | 68171013                        | T            | C           | hom                    | exon 8 |         | synonymous SNV    | c.1647T>C | p.F549F   |
|         | LRP5 (chr11)    | 68179166                        | A            | G           | hom                    | intron |         |                   |        | rs545382  |
|         |                  | 68181208                        | C            | T           | het                    | exon 12|         | nonsynonymous SNV | c.2555C>T | p.T852M   |
|         |                  | 68192690                        | G            | A           | hom                    | exon 15|         | synonymous SNV    | c.3357G>A | p.V1119V  |
|         |                  | 120428607                       | G            | A           | hom                    | UTR3   |         |                   |        | rs41622   |
|         |                  | 120428799                       | C            | A           | hom                    | exon 8 |         | synonymous SNV    | c.765G>T  | p.P255P   |
|         | TSPAN12 (chr7)  | 120450658                       | G            | A           | hom                    | intron |         |                   |        | rs7805733 |
|         |                  | 120450679                       | CA           | -           | hom                    | intron |         |                   |        |          |
|         |                  | 120498053                       | -            | A           | het                    | UTR5   |         |                   |        |          |
| Patient | Gene (chromosome) | Chromosome start and end (hg19) | referred base | altered base | homozygous/heterozygous | region | change | Nucleotide variant | Effect | dbSNP132 |
|---------|------------------|---------------------------------|---------------|--------------|-------------------------|--------|--------|-----------------|--------|----------|
|         | FZD4 (chr11)     | 86660886                         | G             | A            | heterozygous            | UTR3   |        |                 |        | rs3802892 |
|         |                  | 68131167                         | A             | T            | homozygous              | intron |        |                 |        | rs10791978 |
|         |                  | 68170985                         | T             | C            | heterozygous            | exon 8 | nonsynonymous SNV | c.1619T>C | p.L540P |
|         | LRP5 (chr11)     | 68171013                         | T             | C            | homozygous              | exon 8 | synonymous SNV | c.1647T>C | p.F549F |
|         |                  | 68179166                         | A             | G            | homozygous              | intron |        |                 |        | rs689179 |
| Mother of Patient 2 |         | 68192690                         | G             | A            | homozygous              | exon 15 | synonymous SNV | c.3357G>A | p.V1119V |
|         | TSPAN12 (chr7)   | 120428607                        | G             | A            | homozygous              | UTR3   |        |                 |        | rs41622 |
|         |                  | 120428799                        | C             | A            | homozygous              | exon 8 | synonymous SNV | c.765G>T | p.P255P |
|         |                  | 120450658                        | G             | A            | homozygous              | intron |        |                 |        | rs7805733 |
|         |                  | 120450679                        | CA            | -            | heterozygous            | intron |        |                 |        |          |
|         |                  | 120450680                        | CACA          | -            | heterozygous            | intron |        |                 |        |          |
|         |                  | 120498053                        | -             | A            | heterozygous            | UTR5   |        |                 |        |          |
intronic sequences of each exon of the four genes (FZD4, LRP5, TSPAN12, and NDP) known to be responsible for FEVR (Supplementary Table). The exons of the four genes were analyzed via the direct sequencing of PCR products. Amplified products were purified using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) and sequenced with forward and reverse primers by the BigDye® Terminator v3.1 Cycle Sequencing Kit (ABI Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Amplified products were purified using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) and sequenced with forward and reverse primers by the BigDye® Terminator v3.1 Cycle Sequencing Kit (ABI Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions (After the template and the primers were set, prepare the BigDye premix reactions for 96-Well plate. Then perform the cycle sequencing on the system, purify the extension products and analyze the samples on a sequencer).

**Construction of expression plasmids:** The gene encoding wild-type LRP5 (Origene, Rockville, MD) was subcloned in-frame into the pRK5 vector (BD Bioscience, San Jose, CA) using the XbaI and HindIII sites. FZD4 and Norrin cDNAs fused to reporter sequences/genes (generously provided by Dr. Jeremy Nathans) have been described previously [19,30]. LRP5 mutations were introduced into the wild-type LRP5 cDNA by primer-mediated PCR mutagenesis using the QuikChange® Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA). The recombinant plasmids containing LRP5-pRK-5 fusion constructs were verified by direct DNA sequencing and then amplified and purified for transfection (Tiangen, Biotech, Beijing, China).

**Luciferase assays:** The SuperTopFlash (STF) construct (generously provided by Dr. Amir Rattner and Dr. Jeremy Nathans of Johns Hopkins University) contains a firefly luciferase reporter driven by seven lymphoid enhancer factor/T cell factor proteins (LEF/TCF) consensus binding sites. LEF/TCFs mediate Wnt signals in the nucleus by recruiting β-catenin to Wnt target genes. This reporter plasmid was stably transfected into HEK293 cells as reported previously to generate the STF cell line [19]. The STF cells were cotransfected with 200 ng of Norrin, 200 ng of FZD4, 240 ng of LRP5 (wild type or mutant), and 100 ng of pSV-β-galactosidase Control Vector (Promega, Madison, WI) in a 24-well plate using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, Carlsbad, CA). The transfected cells were washed with PBS twice after 48 h of transfection and assayed using the Promega Luciferase reporter assay system. The firefly luciferase activity was normalized to the coexpressed β-galactosidase activity. Each assay was performed in triplicate at the same time and repeated three times.

![Figure 5. Failure of mutant LRP5 in activation of the Norrin/β-catenin pathway. SuperTopFlash (STF) cells cotransfected with LRP5-pRK5 and FZD4 constructs were treated with Norrin and assayed for luciferase reporter activity. Each assay was performed in triplicate at the same time and repeated three times. The results are an average of three measurements. Both of the novel LRP5 mutants failed to induce the luciferase reporter activity in STF cells in response to Norrin (87% reduction for p.A422T, 97% reduction for p.L540P). The additional LRP5 mutation in Patient 1 (p.Q816P) from the unaffected mother had similar luciferase reporter activity compared with the wild type, while the second mutation of Patient 2 (p.T852M), which was not detected in either parent, exhibited a 94.9% reduction. The luciferase intensities of the two combinations (p.A422T and p.Q816P, p.L540P and p.T852M) decreased significantly compared to the wild type, but no significant differences were observed compared with the mere mutation (p.A422T or p.L540P; p>0.05). The mutant p.R570Q (positive control) exhibited a 96% reduction of its wild-type activity, which was similar to the results of a previous report. Asterisks indicate significant differences between the mutant and wild type as judged by the Student two-tailed t test (p<0.05).](image-url)
| Nucleotide variant | Effect | Exon | Occurrence in patients (proband) | Occurrence in control alleles | Disease | Reference |
|--------------------|--------|------|--------------------------------|-----------------------------|---------|-----------|
| c.803_812del       | p.G269RfsX4 | 4    | 1/56                           | 0/362                       | arFEVR  | [9]       |
| c.2978G>A          | p.W993X | 13   | 1/8                            | 0/100                       | FEVR    | [10]      |
| c.3804delA         | p.E1270RfsX169 | 18   | 1/32                           | 0/400,                      | FEVR,   | [27, 28] |
| c.419dupC          | p.L1374QfsX176 | 20   | 1/32                           | 0/400                       | FEVR    | [28]      |
| c.891–892delTC     | p.Arg2981LeufsX2 | 5    | 1.49                           | 0.96                        | FEVR    | [37]      |
| c.433C>T           | p.L145F | 2    | 1/56                           | 0/362                       | FEVR    | [9]       |
| c.518C>T           | p.T173M | 3    | 1/32                           | 0/400                       | FEVR    | [28]      |
| c.1264G>A          | p.A422T | 6    | 1/71                           | 0/300                       | FEVR    | [9]       |
| c.1321G>A          | p.E441K | 6    | 1/8                            | 0/100                       | FEVR    | [10]      |
| c.1330C>T          | p.R444C | 6    | 1/56                           | 0/362                       | FEVR    | [9]       |
| c.1532A>C          | p.D511A | 7    | 0.05                           | 0/80                        | FEVR    | [38]      |
| c.1564G>A          | p.A522T | 7    | 1/56                           | 0/362                       | FEVR    | [9]       |
| c.1604C>T          | p.T535M | 8    | 1/56                           | 0/362                       | arFEVR  | [9]       |
| c.1619T>C          | p.L540P | 8    | 1/71                           | 0/300                       | FEVR    | This study |
| c.1648G>A          | p.G550R | 8    | 1/1                            | 0/120                       | arFEVR  | [29]      |
| c.1709G>A          | p.R570Q | 8    | 1/3                            | 0/200                       | arFEVR  | [26]      |
| c.1828G>A          | p.G610R | 9    | 1/56                           | 0/362                       | arFEVR  | [9]       |
| c.1850T>G          | p.F617C | 9    | 1/56                           | 0/362                       | arFEVR  | [9]       |
| c.2302C>G          | p.R752G | 19   | 1/3                            | 0/200                       | arFEVR  | [26]      |
| c.2392A>G          | p.T798A | 11   | 1/56                           | 0/362                       | FEVR    | [9]       |
| c.2413C>T          | p.R805W | 11   | 1/20                           | 0/80                        | FEVR    | [38]      |
| c.3361A>G          | p.N1121D | 15   | 1/56                           | 0/362                       | FEVR    | [9]       |
| c.3502T>C          | p.Y1168H | 16   | 1/32                           | 0/400                       | FEVR    | [28]      |
| c.3758G>T          | p.C1253F | 17   | 1/8                            | 0/100                       | FEVR    | [10]      |
| c.4081T>G          | p.C1361G | 19   | 1/32                           | 0/400                       | FEVR    | [28]      |
| c.4147G>A          | p.E1367K | 20   | 1/3                            | 0/200                       | arFEVR  | [26]      |
| c.2484C>G          | p.Ile828Met | 11   | 1/49                           | 0.96                        | FEVR    | [37]      |
| c.2626G>A          | p.Gly876Ser | 12   | 1/49                           | 0.96                        | FEVR    | [37]      |
| c.4025G>A          | p.Arg1342Gln | 19  | 1/49                           | 0.96                        | FEVR    | [37]      |
| c.4087G>A          | p.Asp1363Asn | 19  | 1/49                           | 0.96                        | FEVR    | [37]      |
| Nucleotide variant | Effect     | Exon | Occurrence in patients (probands) | Occurrence in control alleles | Disease | Reference |
|-------------------|------------|------|----------------------------------|-----------------------------|---------|-----------|
| Splice-site changes |            |      |                                  |                             |         |           |
| c.4488+2T>G       | Splice defect | 21   | 1/32                             | 0/400                       | FEVR    | [28]      |
| c.4489–1G>A       | Splice defect | 22   | 1/8                              | 0/100                       | FEVR    | [10]      |
RESULTS

Clinical evaluation: The clinical information of the two patients is given below. Patient 1 was a 10-year-old boy who presented with poor vision and nystagmus in both eyes. The visual acuities were 2/200 in his right eye and 20/320 in his left eye, respectively. RetCam examination showed retinal fold of the right eye and dragged disc of the left eye. Both eyes had peripheral non-perfusion areas. The patient has been followed up for 2 years and the retinal folds remained stable (Figure 1A, B). His father had a good vision of 20/20 in both eyes, while FFA showed nonperfusion areas of both eyes (Figure 1C-F). Patient 2 was a 4-month-old male infant when first referred to our clinics. He was found to have leukocoria of both eyes by the parents. Ophthalmological examinations showed bilateral retinal detachment. The left eye was more severe than the right eye. Lensectomy and vitrectomy were performed in the right eye. The left eye’s condition was too severe and it was unlikely to benefit from surgery; thus, it remained untreated. The retina was mostly attached in the right eye during 2 years of follow-up. Visual acuity measurement was difficult in this patient due to his young age. However, the patient was capable of walking without aid at the last follow-up (Figure 2A-E). The mother had normal eyesight, while FFA showed nonperfusion areas, increased ramification, and shunts of the peripheral retinal vessels in both eyes (Figure 2F-I).

Neither the patients nor the parents showed any clinical sign of bone abnormality. However, DEXA analysis showed mild reduced BMD in the two patients and the affected father of Patient 1 and the affected mother of Patient 2. In Family 1, the Z score of Patient 1 was −1.8 and the T score of his affected father was also −1.8. In Family 2, the Z score of Patient 1 was −2.0 and the T score of his affected mother was −1.5. The unaffected mother of Patient 1 and father of Patient 2 had normal BMD. Normal range (−1.0 to 1.0), osteopenia (−1.0 to −2.5), osteoporosis (≤−2.5). T scores (or Z scores) of −1.0 to 1.0 are considered as normal range. Scores of −1.0 to −2.5 indicate osteopenia. Scores less than −2.5 indicate osteoporosis.

Identification of novel LRP5 mutations: DNA sequence analysis identified two novel mutations in LRP5, including c.1264G>A (p.A422T) in Patient 1 and his affected father and c.1619T>C (p.L540P) in Patient 2 and his affected mother (Figure 3). Both of the mutations cosegregated with the disease phenotype of the respective families and were absent in 300 normal controls. The two mutations involve a highly evolutionarily conserved residue (Figure 4).

All the sequence variants of known FEVR-causing genes detected in the affected members of Family 1 and Family 2 are listed in Table 1. In addition to p.A422T, an additional LRP5 sequence change (c.2447A>C, p.Q816P) from the unaffected mother was detected in Patient 1. Moreover, beyond p.L540P, Patient 2 had an additional LRP5 sequence change (c.2555C>T, p.T852M); this was not detected in either parent, suggesting that it was a new mutation in Patient 2 (Figure 3). The two sequence changes also involved evolutionarily conserved residues (Figure 4).

We also detected novel variants in the untranslated regions (UTRs), specifically a deletion in the 3′ UTR of the FZD4 gene in Family 1,2 and an insertion in the 5′ UTR of the TSPAN12 gene in Family 2 (Table 1). We searched the 3′ UTR mutation of the FZD4 gene in miRanda and Microcosm and we did not find any site change of miRNA binding. We also searched in TRRD and did not find any binding site of the transcript factor in either the wild-type and the mutant 5′ UTR area of the TSPAN12 gene. No NDP mutations were detected in either family.

Defective luciferase reporter activity mediated by mutant LRP5 protein: Under physiological conditions, a complex of Norrin, FZD4, and LRP5 activates Norrin/β-catenin signaling, which can be demonstrated using a Norrin responsive firefly luciferase reporter. Both of the novel LRP5 mutants displayed a significant reduction of the luciferase reporter activity in STF cells in response to Norrin (87% reduction for p.A422T and 97% reduction for p.L540P). We constructed one previously reported LRP5 mutation (p.R570Q) as a control. It lost 96% of its wild-type activity, which was similar to the results of a previous report (Figure 5) [31]. To further investigate the two additional LRP5 mutations (p.Q816P and p.T852M), we also carried out luciferase assay. The mutation of Q816P had similar luciferase reporter activity compared with the wild type, while the mutation of T852M exhibited a significant reduction (94.9%). As for the luciferase activity of the two combinations of mutations (p.A422T and p.Q816P, p.L540P and p.T852M), the intensities decreased significantly compared to the wild type, but no significant differences were noticed compared with the single mutation of p.A422T or p.L540P.

DISCUSSION

In our study, we detected two novel heterozygous LRP5 mutations in two families with FEVR that were not detected in 300 normal individuals. Thus far, 32 different mutations of LRP5 have been reported to relate to FEVR, including five premature stop codons, 25 missense changes, and two changes that affect splicing (Table 2).

LRP5 encodes single-pass transmembrane receptors that partner with members of the frizzled family of seven-pass
transmembrane receptors to bind Wnt proteins or Norrin, forming a functional ligand-receptor complex that activates the canonical Wnt-β-catenin pathway or Norrin-β-catenin pathway [23-25]. 

LRP5 consists of four extracellular domains, each of which is composed of six segments. Those segments form a β-propeller structure that is followed by an epidermal growth factor (EGF)-like domain. Five of those segments are YWTD LDL-class B repeats, whereas the sixth does not contain the required YWTD motif to be recognized as a LDL-class B repeat. The first two propeller domains were suggested to be important for interaction with the Wnt or Norrin/Frizzled complex [32]. Both of the novel missense mutations (p.A422T and p.L540P) are located in the second “β-propeller” domain of the protein, at an evolutionarily highly conserved position. Functional analysis was performed using luciferase reporter assay. The significant defective Norrin-β-catenin signaling with the p.A422T and p.L540P mutation in LRP5 underlies FEVR. This is consistent with the previously reported mutation p.R570Q, which was also located in the second β-propeller domain [31]. The difference of the decreased intensity of the two mutants (87% of p.A422T versus 97% of p.L540P) may due to the location and importance of this amino acid in the β-propeller domain. 

FEVR can exhibit variable phenotypes among patients from the same family, or even between the two eyes of one individual [1]. In our study, the two patients manifested more severe symptoms than the affected father or mother. Considering our gene screening results, besides p.A422T, an additional LRP5 mutation of p.Q816P from his unaffected mother was detected in Patient 1 and Patient 2 had an additional new LRP5 mutation of p.T852M that was not found in either of his parents. Previously, Qin et al. [13] reported a case with combined mutations of LRP5 (p.F617C and p.T535M), which were from the affected mother and the unaffected father, respectively. The affected mother was completely asymptomatic but had retinal avascularization with tortuosity of the retinal vessels in her right eye. She also had mildly reduced bone density. The father showed neither retinal change nor reduced bone mass. Kondo et al. [33] reported a FEVR patient exhibited a double sequence change in FZD4 (p.G488D from the affected mother and p.H69Y from the unaffected father). The proband with both p.G488D and p.H69Y presented with a more severe phenotype than the mother who carried a single p.G488D mutation. Our cases are similar to these reports. The two sequence changes (p.Q816P and p.T852M) were located in the third β-propeller domain of the protein, which is less important than the first two β-propeller domains. The additional LRP5 mutation of Q816P in Patient 1 had similar luciferase reporter activity compared with the wild type, which implies that the mutation (p.Q816P) may not be pathogenic. However, the mutation of p.T852M in LRP5 showed a significant decrease in luciferase activity. This implies the second new mutation of Patient 2 (p.T852M) may be pathogenic. Previously, Qin et al. [34] reported a combination of R444C in LRP5 and R417Q in FZD4 displayed a sharp decline compared with the single mutation. Our results showed no significant differences between the single mutation and the combination of the mutations. However, the exact pathogenicity of the sequence changes and whether the additional combination of the mutations will lead to the severe phenotype needs further study.

In conclusion, two novel heterozygous mutations (p.A422T and p.L540P) in LRP5 were verified in two families, and further confirmed by luciferase activity assay. The results provide additional evidence that mutations in LRP5 cause FEVR. The complexity of genotype–phenotype correlation needs to be further studied.

APPENDIX 1. PRIMERS USED FOR PCR AMPLIFICATION AND SEQUENCING OF FZD4, NDP, TSPAN12, AND LRP5.

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

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