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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 Yang[2]

[1]Department of Ophthalmology, Xin Hua Hospital affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China; [2]Sichuan 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; [3]West 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 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

Correspondence to: Dr. Zhenglin Yang, Center for Human Molecular Biology & Genetics, Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, 32 The First Round Road 2 West, Chengdu, Sichuan 610072, China; Phone: 86-28-87393548; Fax: 86-28-87393596; email: zliny@yahoo.com

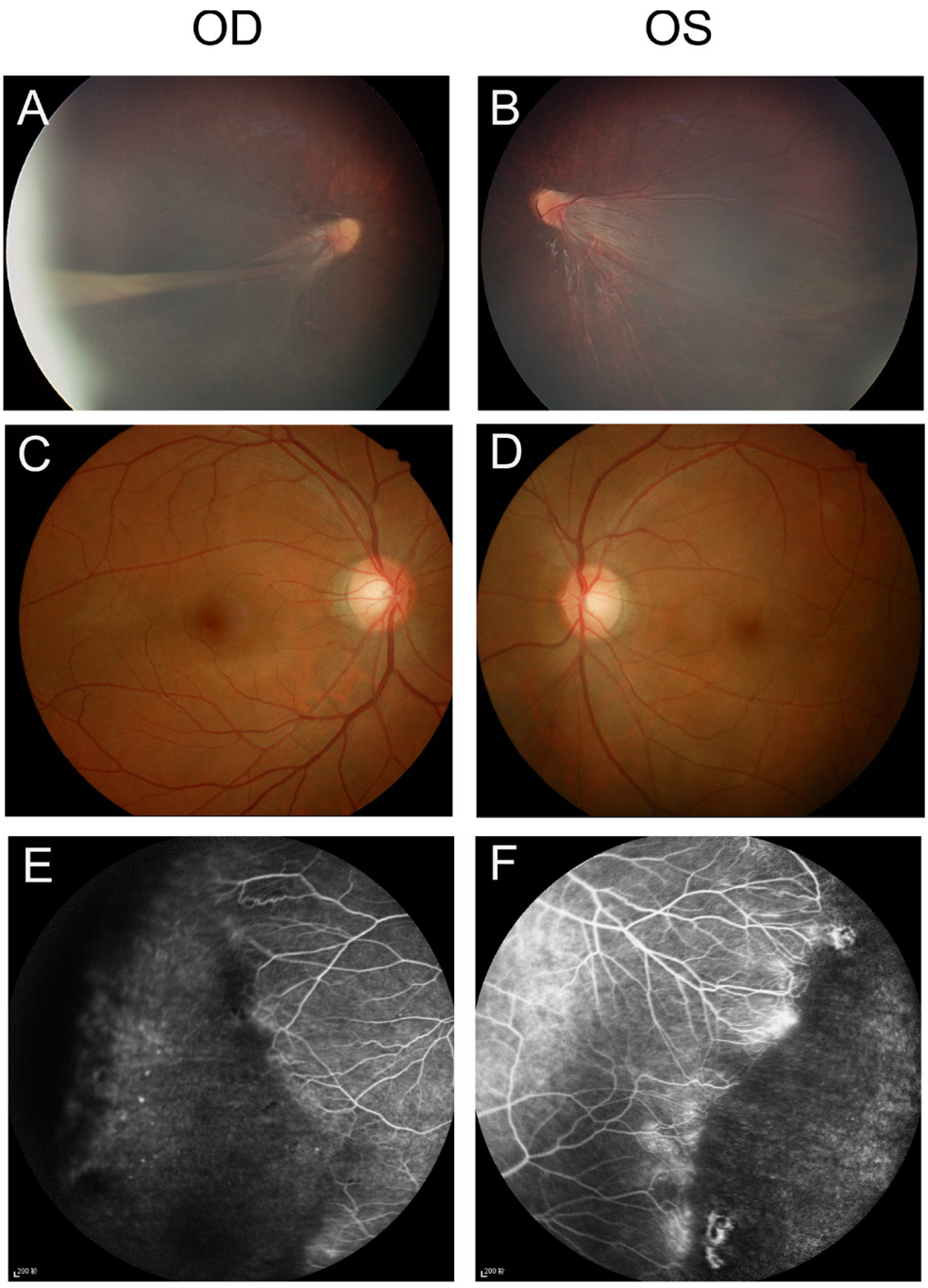

Figure 1. Fundus changes in the proband with the heterozygous c.1264G>A (p.A422T) mutation in Family 1. **A** and **B**: The fundus of Patient 1 showed a retinal fold of the right eye and dragged disc of the left eye. **C**-**F**: Fundus photos of the asymptomatic father. **C** and **D**: Fundus photos of the asymptomatic father showed normal posterior fundi. **E** and **F**: Fundus fluorescein angiography (FFA) showed that the father had nonperfusion areas in both eyes.

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

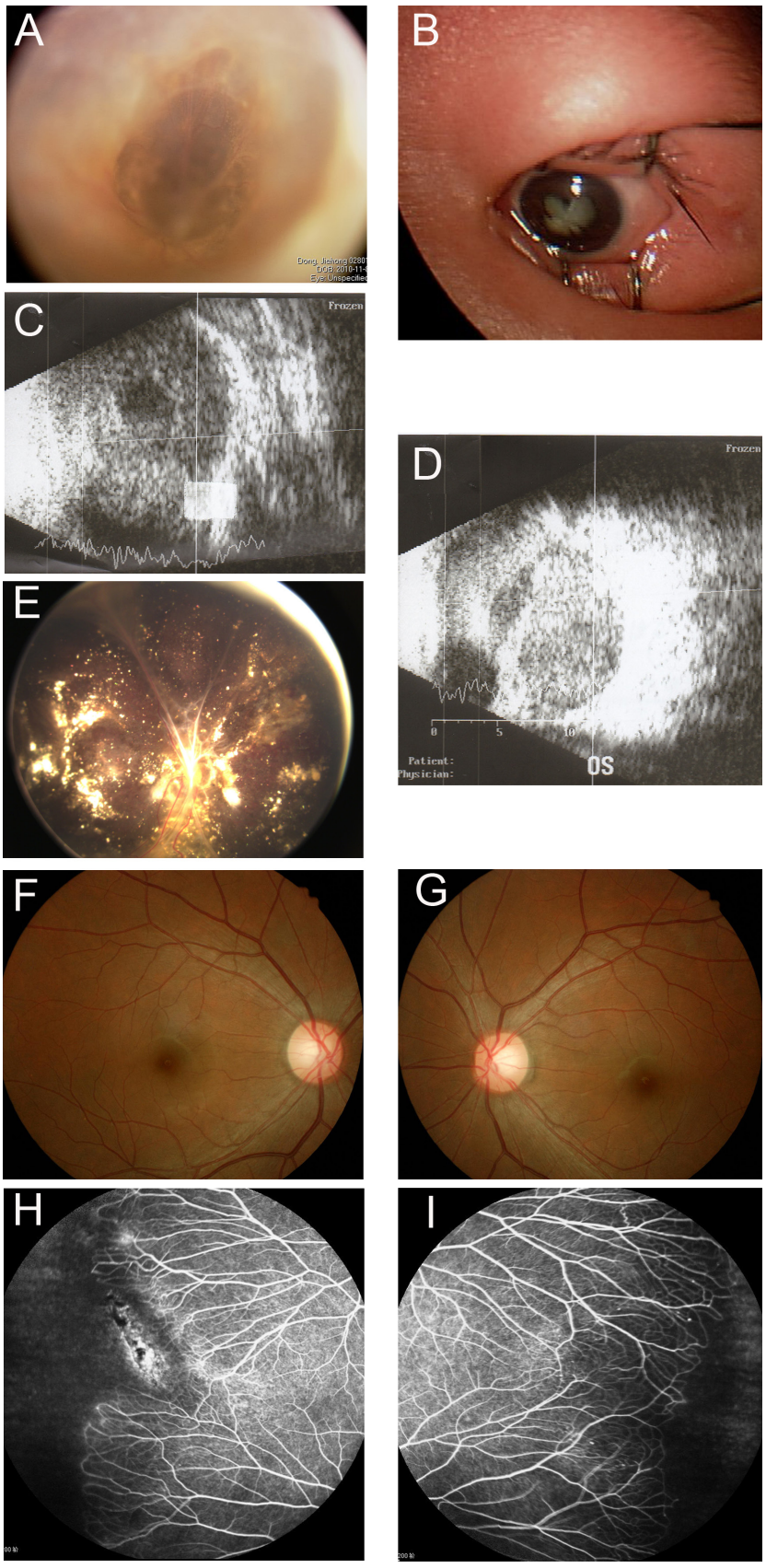

Figure 2. Fundus changes in the proband with the heterozygous c.1619T>C (p.L540P) mutation in Family 2. **A** and **B**: RetCam examination of both of Patient 2's eyes. The fundus of the left eye could not be viewed due to the opacity of the lens. **C** and **D**: Ultrasound B- scan of Patient 2. These 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.

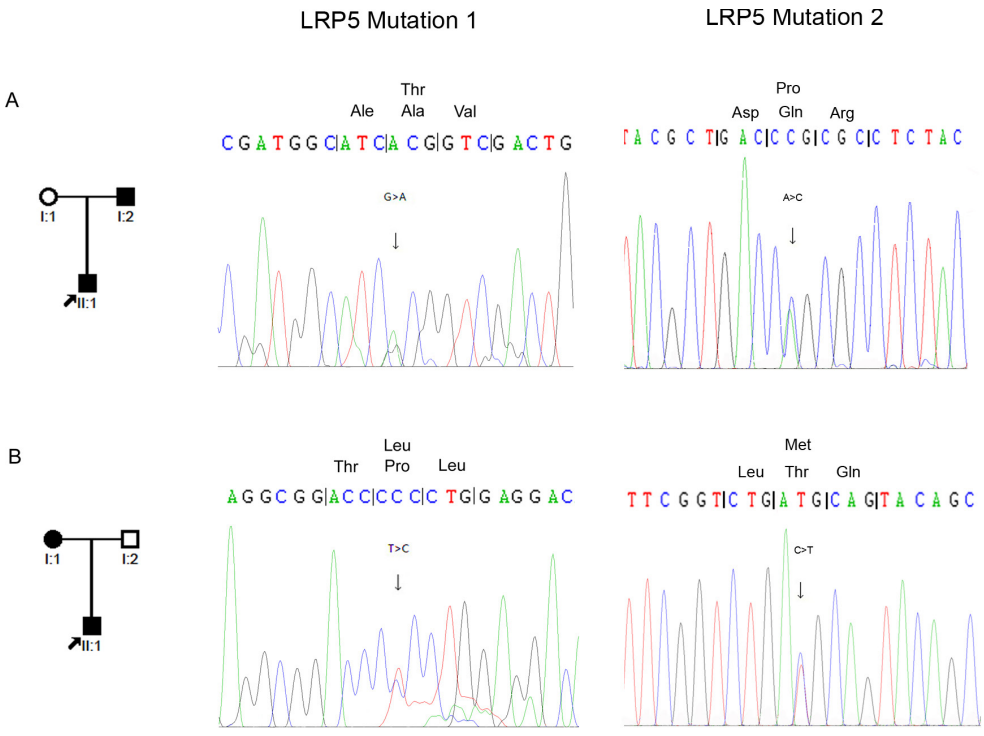

Figure 3. Pedigrees of families with autosomal dominant familial exudative vitreoretinopathy (FEVR) and the mutations identified in *LRP5*. **A**: In Family 1, Patient 1 had two *LRP5* mutations, c.1264G>A (p.A422T) from the affected father and c.2447A>C (p.Q816P) from unaffected mother. **B**: In Family 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.

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, including lysis, precipitation, wash and resuspension. Genomic DNA was isolated using the salt precipitation method. PCR primers were designed to include flanking

	A422T	L540P	Q816P	T852M
H.sapiens	PDGIAVDWVA	KRRTLLEDKLP	DYADQRLYW-T	PFGLTQYSDY
P.troglodytes	PDGIAVDWVA	KRRTLLEDKLP	DYADQRLYW-T	PFGLTQYSDY
C.lupus	PDGIAVDWVA	KRRTLLEDKLP	DYADQRLYW-T	PFGLTQYSDY
B.taurus	PDGIAVDWVA	KRRTLLEDKLP	DYADQRLYW-T	PFGLTQYSDY
M.musculus	PDGIAVDWVA	KRKTLLEDKLP	DYADQRLYW-T	PFGLTQYSDY
R.norvegicus	PDGIAVDWVA	KRQTLLEDKLP	DYADQRLYW-T	PFGLTQYSDY
G.gallus	PDGIAVDWVA	MRKTLLEDKLP	DYADQRLYW-T	PFGLTQYSDY
D.rerio	PDGIAVDWVA	NRMTILEDKLP	DYIDQRLYW-T	PFGLTQYRDY
D.melanogaster	PDGLALDWLA	GRRVVISDNLK	DQETRRLYWAT	PYAVSLYQDY
A.gambiae	PDGLAIDWLA	GRNTILSDNLP	DPDTRRLYWAR	PVALTIYQDW

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* (XP_003432463.1), *Bos taurus* (XP_002699451.1), *Macaca mulata* [*M. musculus*] (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.

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Table 1. All sequence variants of known FEVR disease causing genes in patients with familial exudative vitreoretinopathy.

Patient	Gene (chromo ome)	Chromosome start and end (hg19)	referred base	altered base	homo-zygous/ heterozygous	region	change	Nucleotide variant	Effect	dbSNP132
Patient 1	*FZD4* (chr11)	86657520	A	G	het	UTR3				rs713065
		86658244	CAAA	-	het	UTR3				rs34325935
		86658245	CAAACAAA	-	het	UTR3				
		86660449	A	-	het	UTR3				
		86660612	C	G	hom	UTR3				rs4944641
		86660886	G	A	hom	UTR3				rs3802892
		68131167	A	T	hom	intron				rs10791978
		68154032	G	A	het	exon 6	nonsyn-onym-ous SNV	c.1264G>A	p.A422T	
		68171013	T	C	hom	exon 8	synonymous SNV	c.1647T>C	p.F549F	rs545382
		68174122	G	A	hom	exon 9	synonymous SNV	c.1932G>A	p.E644E	rs2277268
	LRP5 (chr11)	68177614	T	C	hom	intron				rs4988322
		68179032	A	C	het	exon 11	nonsyn-onym-ous SNV	c.2447A>C	p.Q816P	
		68179125	C	T	hom	intron				rs2242339
		68192690	G	A	het	exon 15	synonymous SNV	c.3357G>A	p.V1119V	rs556442
		68204445	C	T	het	exon 19	synonymous SNV		p.D1363D	rs3736229
		68206173	T	C	het	intron				rs901824
	TSPAN12 (chr7)	120428607	G	A	hom	UTR3				rs41622
		120428799	C	A	hom	exon 8	synonymous SNV	c.765G>T	p.P255P	rs41623
		120450658	G	A	hom	intron				rs7805733
		120450678	-	CA	hom	intron				rs112555207

Patient	Gene (chromo ome)	Chromosome start and end (hg19)	referred base	altered base	homo-zygous/ heterozygous	region	change	Nucleotide variant	Effect	dbSNP132
		86657520	A	G	het	UTR3				rs713065
		86658244	CAAA	-	het	UTR3				rs34325935
	FZD4 (chr11)	86660449	A	-	het	UTR3				
		86660612	C	G	hom	UTR3				rs4944641
		86660886	G	A	het	UTR3				rs3802892
		68131167	A	T	hom	intron				rs10791978
		68154032	G	A	het	exon 6	nonsyn-onym-ous SNV	c.1264G>A	p.A422T	
		68171013	T	C	hom	exon 8	synonymous SNV	c.1647T>C	p.F549F	rs545382
Father of Patient 1	*LRP5* (chr11)	68174122	G	A	het	exon 9	synonymous SNV	c.1932G>A	p.E644E	rs2277268
		68177319	A	-	het	intron				
		68177614	T	C	het	intron				rs4988322
		68179125	C	T	het	intron				rs2242339
		68179166	A	G	hom	intron				rs689179
		68192690	G	A	hom	exon 15	synonymous SNV	c.3357G>A	p.V1119V	rs556442
		120428607	G	A	hom	UTR3				rs41622
		120428799	C	A	hom	exon 8	synonymous SNV	c.765G>T	p.P255P	rs41623
	TSPAN12 (chr7)	120450658	G	A	hom	intron				rs7805733
		120450678	-	CA	het	intron				rs112555207
		120450710	GT	-	het	intron				

Patient	Gene (chromo ome)	Chromosome start and end (hg19)	referred base	altered base	homo-zygous/ 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
	LRP5 (chr11)	68170985	T	C	het	exon 8	nonsyn-onym-ous SNV	c.1619T>C	p.L540P	
		68171013	T	C	hom	exon 8	synonymous SNV	c.1647T>C	p.F549F	rs545382
		68179166	A	G	hom	intron				rs689179
		68181208	C	T	het	exon 12	nonsyn-onym-ous SNV	c.2555C>T	p.T852M	
		68192690	G	A	hom	exon 15	synonymous SNV	c.3357G>A	p.V1119V	rs556442
	TSPAN12 (chr7)	120428607	G	A	hom	UTR3				rs41622
		120428799	C	A	hom	exon 8	synonymous SNV	c.765G>T	p.P255P	rs41623
		120450658	G	A	hom	intron				rs7805733
		120450679	CA	-	hom	intron				
		120498053	-	A	het	UTR5				

Patient	Gene (chromo ome)	Chromosome start and end (hg19)	referred base	altered base	homo-zygous/ heterozygous	region	change	Nucleotide variant	Effect	dbSNP132
Mother of Patient 2	*FZD4* (chr11)	86660886	G	A	het	UTR3				rs3802892
		68131167	A	T	hom	intron				rs10791978
	LRP5 (chr11)	68170985	T	C	het	exon 8	nonsyn-onym-ous SNV	c.1619T>C	p.L540P	
		68171013	T	C	hom	exon 8	synonymous SNV	c.1647T>C	p.F549F	rs545382
		68179166	A	G	hom	intron				rs689179
		68192690	G	A	hom	exon 15	synonymous SNV	c.3357G>A	p.V1119V	rs556442
	TSPAN12 (chr7)	120428607	G	A	hom	UTR3				rs41622
		120428799	C	A	hom	exon 8	synonymous SNV	c.765G>T	p.P255P	rs41623
		120450658	G	A	hom	intron				rs7805733
		120450679	CA	-	het	intron				
		120450680	CACA	-	het	intron				
		120498053	-	A	het	UTR5				

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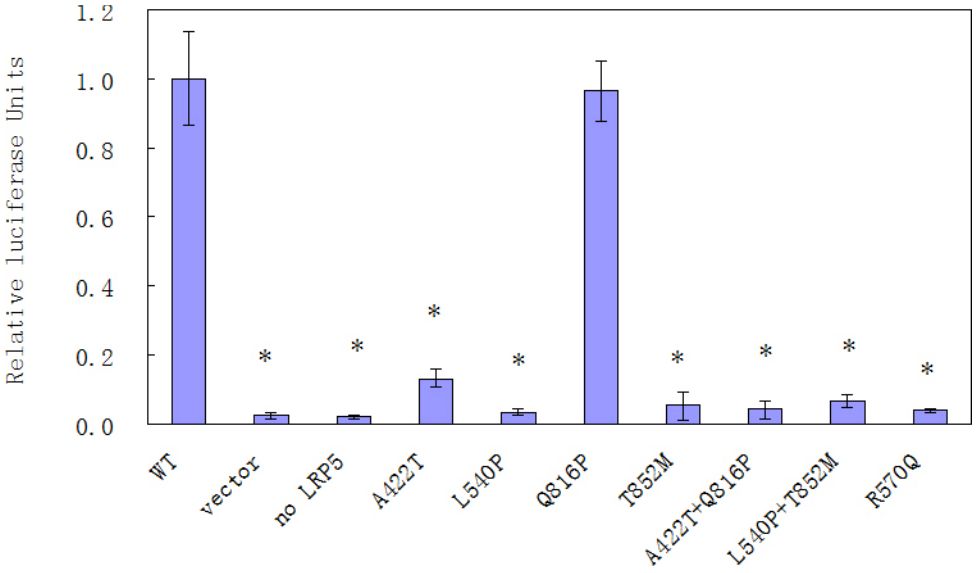

Figure 5. Failure of mutant LRP5 in activation of the Norrin/β-catenin pathway. SuperTopFlash (STF) cells cotransfected with LRP5-pRK5 and FZD4 constructs were treated with Norrin and assayed for luciferase reporter activity. Each assay was performed in triplicate at the same time and repeated three 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 ($p<0.05$).

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.

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Table 2. *LRP5* sequence variants in patients with familial exudative vitreoretinopathy from previous reports.

Nucleotide variant	Effect	Exon	Occurrence in patients (probands)	Occurrence in control alleles	Disease	Reference
Truncating changes						
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.4119dupC	p.L1374QfsX176	20	1/32	0/400	FEVR	[28]
c.891–892delTC	p.Arg298Leu fsX2	5	1/49	0/96	FEVR	[37]
Missense changes						
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	This study
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.Aspl363Asn	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 p.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 addition to causing FEVR, defects in *LRP5* have also been associated with bone abnormalities [13,26,28,29,31,35,36], which were also found in our patients. Although they showed no signs of reduced bone mass clinically, the reduced BMD suggested osteopenia of these patients and their affected parents. This result added further evidence supporting the claim that bone density abnormalities are a consequence of *LRP5* mutations. Therefore, we should inquire into the history of bone defects in those patients who were diagnosed as FEVR. BMD examination should be performed if possible, especially for those in which *LRP5* mutation is detected.

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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