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Identification of Five Novel Variants in the *TSPAN12* Gene in Chinese Families With Familial Exudative Vitreoretinopathy

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Purpose: To report the novel causative variants in five Chinese families with familial exudative vitreoretinopathy (FEVR).

Methods: Five unrelated Chinese families diagnosed with FEVR were enrolled in this study. Ocular examinations and genetic analysis were performed on the probands and family members. Luciferase assay was performed to evaluate the variants' impacts on Norrin/β-catenin signaling activity.

Results: Five novel variants, including two frameshifts, c.518delA (p.Glu173Glyfs*42) and c.719delT (p.Leu240Profs*21), two missenses, c.482G>T (p.Gly161Val) and c. 614G>C (p. Gly205Ala), and one nonsense, c.375G>A (p.Trp125*), were identified in the *TSPAN12* gene in this study. All the variants were co-segregated within each family and were predicted as pathogenic in silico. The luciferase assay showed all variants lead to various degrees of compromised Norrin/β-catenin signaling activity.

Conclusions: Our study expanded the variant spectrum and provided information for the genetic testing of FEVR by showing five novel FEVR-associated pathogenic variants in *TSPAN12*.

Translational Relevance: Our study expanded the spectrum of FEVR-associated *TSPAN12* variants and further supported the inclusion of *TSPAN12* gene in the evaluation of cases concerning for FEVR.

Introduction

Familial exudative vitreoretinopathy (FEVR; OMIM 133780), first described by Criswick and Schepens[1] in 1969, is a rare inherited disorder with defective development of vasculature in the peripheral retina. The clinical features of FEVR vary widely, ranging from slight peripheral retinal vascular anomalies with no visual impairment to total blindness caused by severe retinal detachment.[2–6] As a genetic disease, FEVR can be inherited in an autosomal dominant, autosomal recessive, or X-linked pattern. Sixteen genes and 1 locus including *FZD4*,[7] *NDP*,[8] *LRP5*,[9] *TSPAN12*,[10] *ZNF408*,[11] *KIF11*,[12] *RCBTB1*,[13] *CTNNB1*,[14] *ILK*,[15] *JAG1*,[16] *ATOH7*,[17] *CTNNA1*,[18] *CTNND1*,[19] *LRP6*,[20] *DGL1*,[21] *TGFBR2*,[22] and *EVR3*[23] had been identified to be associated with FEVR. Most of them are related more or less to Norrin/β-catenin signaling pathway, which has been proven to be pivotal in vascular development. In the on-state, Wnt or Norrin binds to the FZD receptor and LRP co-receptor, which breaks the APC/AXIN/GSK3β complex, leading to the nuclear accumulation of β-catenin and starts the transcription of Wnt target genes such as *c-MYC, SOX9,* and *CD44*.[24] *TSPAN12* encodes tetraspanin 12 proteins, which cooperatively promotes the multimerization

of FZD4 and its associated proteins such as LRP5 to elicit physiological levels of signaling in this process.[10]

In this study, we identified five novel variants in the *TSPAN12* gene in five unrelated families. The pathogenicity of variants was assessed in silico. Co-segregation analysis was performed. Moreover, the dual-luciferase reporter assay was performed and revealed that all variants led to a compromised activity of Norrin/β-catenin signaling.

Patients and Methods

Patients

This study complied with the tenets of the Declaration of Helsinki and was approved by the institutional review board of Zhongshan Ophthalmic Center, Sun Yat-Sen University. Targeted exome sequencing or whole exome sequencing was performed on patients who were referred to Zhongshan Ophthalmic Center and diagnosed as FEVR from January 1, 2013, to July 31, 2021. The Sanger sequencing was performed on the family members.[25] Informed written consent was obtained from the patients or their legal guardians.

All the subjects were diagnosed using a comprehensive age-appropriate ophthalmic examination including best-corrected visual acuity, intraocular pressure, and refractive errors. The fundus photograph and fundus fluorescein angiography (FFA) were obtained using Heidelberg HRA Spectralis/HRA2 (Heidelberg Engineering, GmbH, Dossenheim, Germany), or wild field scanning laser ophthalmoscope (California FA; Optos, Dunfermline, UK), or RetCam imaging (RetCam; Clarity Medical Systems Inc., Pleasanton, CA, USA). The diagnosis of FEVR was made by characteristic clinical features such as peripheral retinal vascular anomalies, retinal folds, vitreoretinal traction, subretinal exudation, or retinal neovascularization occurring at any age. Patients with a disease tempo consistent with retinopathy of prematurity were excluded.[26,27]

Genetic Test

Whole-exome sequencing was performed in five probands and the identified variants were validated using Sanger sequencing within family members. Peripheral blood samples were extracted for genomic DNA isolation according to the standard protocols mentioned in our previous study.[28] The minor allele frequency was defined as less than 0.03% in the public databases Genome Aggregation Database (gnomAD, http://www.gnomad-sg.org).[29] The HGMD, dbSNP151, and gnomAD were also used to identify the reported pathogenic variants. The pathogenicity of missense variants was further estimated by using SIFT (http://sift.jcvi.org), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), Mutation Taster (http://www.mutationtaster.org), PROVEN (http://provean.jcvi.org/index.php) and CADD (https://cadd.gs.washington.edu) online algorithms and via evolutionary conservation analysis.

Dual-Luciferase Assays

To assess the activity of the mutant TSPAN12 proteins in the Norrin/β-catenin pathway, HEK293 cells stably harboring the Norrin/β-catenin reporter SuperTOPFlash (HEK293 STF cells, which was a gift from Dr. Jeremy Nathans of Johns Hopkins University, Baltimore, MD, USA)[30] was seeded onto a six-well plate and then co-transfected with the following expression plasmids: 400 ng NORRIN, 400 ng FZD4, 400 ng LRP5, and 400 ng pGL4.1-Renilla with 400 ng WT or mutant TSPAN12 or empty vectors using Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Trans-Detect Dual-Luciferase Reporter Assay System (Cat. no. FR201-01; TransGen Biotech Co., Beijing, China) was used to detect the luciferase activity of the transfected cells after 48h. Firefly luciferase activity was normalized to the co-expressed Renilla luciferase activity. Each assay was performed in triplicate simultaneously and repeated three times.[31]

Statistical Analysis

The results were analyzed using SPSS 22.0 for Windows (IBM Corporation, Armonk, NY, USA). The comparisons between multiple experimental groups were analyzed by one-way analysis of variance with Dunnett's multiple comparison tests. Statistical significance was exhibited with a 95% confidence interval, and $P < 0.05$ was considered as statistically significant.

Results

Our in-house data showed *TSPAN12* variants were identified in 43 probands among 524 patients by genetic testing (8.2%). Five families with novel variants were enrolled in this study, and all of them were Han Chinese. The clinical manifestations of five probands

Table 1. Causative Variants in the Five Families

Family Number	Nucleotide Change	Protein Change	Position	Type	Co-Segregation	Exon	SIFT	Poly-Phen 2	Mutation Taster	PROVEAN	CADD	Functional Significance	Source
1	c.375G>A	p.Trp125*	chr7:120450610	Nonsense	Paternal	6	—	—	Dc	—	39	Pathogenic	Novel
2	c.482G>T	p.Gly161Val	chr7:120446733	Missense	Paternal	7	Damaging	Probably Damaging	Dc	Deleterious	32	Pathogenic	Novel
3	c.518delA	p.Glu173Glyfs*42	chr7:120446697	Frameshift	Maternal	7	—	—	—	—	—	Pathogenic	Novel
4	c.614G>C	p.Gly205Ala	chr7:120428950	Missense	Paternal	8	Damaging	Probably Damaging	Dc	Deleterious	32	Pathogenic	Novel
5	c.719delT	p.Leu240Profs*21	chr7:120428845	Frameshift	Paternal	8	—	—	Dc	—	—	Pathogenic	Novel

are shown in Table 1. Five novel variants were identified. All the variants were validated using Sanger sequencing within family members (Fig. 1A) and predicted to be pathogenic in silico (Table 1), and conservation analysis of two missense variants was performed (Fig. 1B). The schematics of the *TSPAN12* gene with five novel variants and protein with domains and positions of the novel variants indicated are shown in Figure 2.[32]

In family 1, the proband was a five-year-old boy with nystagmus and poor vision at the first presentation. The c.375G>C (p.Trp125*) variant in TSPAN12 was detected in the patient and his asymptomatic father. The ectopic macula in the right eye and avascular zone in the left eye was noted in the proband, but only vascular anomaly in the peripheral retina was detected in his father (Figs. 3A–D). In family 2, the proband was a 12-year-old boy with sudden unilateral painless loss of vision in the right eye. Retinal detachment caused by FEVR was diagnosed, and the c.482C>T (p.Gly161Val) was detected in the patient and his father. The temporal mid-peripheral vitreoretinal interface abnormality (TEMPVIA) was detected in the left eye of the proband. The fundus examination of his father showed peripheral vascular anomalies such as late-phase leakage, increased vascular number, and straightening of vessels on FFA (Figs. 3E–H). In family 3, the proband was a 40-year-old woman who presented because the abnormal fundus was found during her routine physical examination. However, her younger daughter was diagnosed with vitreous hemorrhage and retinal neovascularization in Guangzhou Women and Children's Hospital when first born. She refused the examination on her daughter because she thought it was repetitive. She was asymptomatic, but the c.518delA p(Glu173Glyfs*42) was detected in the patient and her younger daughter. The avascular zone and peripheral vascular anomaly were noted in both eyes (Figs. 4A–D). In family 4, the proband was a four-year-old boy who presented with low vision of the left eye. The c.614G>C (p.Gly205Ala) was detected in the patient and his father. The TEMPVIA and avascular zone were observed in both eyes in the proband. The fundus of the asymptomatic father showed retinal leakage, avascular zone, and increase or straightening of vessels (Figs. 4E–L). In family 5, the proband was a five-year-old boy who presented with nystagmus and low vision. The fundus photograph demonstrated retinal folds, ectopic macula, and vascular anomalies in the peripheral retina in both eyes. The c.719delT (p.Leu240Profs*21) was detected in the proband and his father. As an asymptomatic family member, only mild vascular abnormality was detected (Figs. 4M–T).

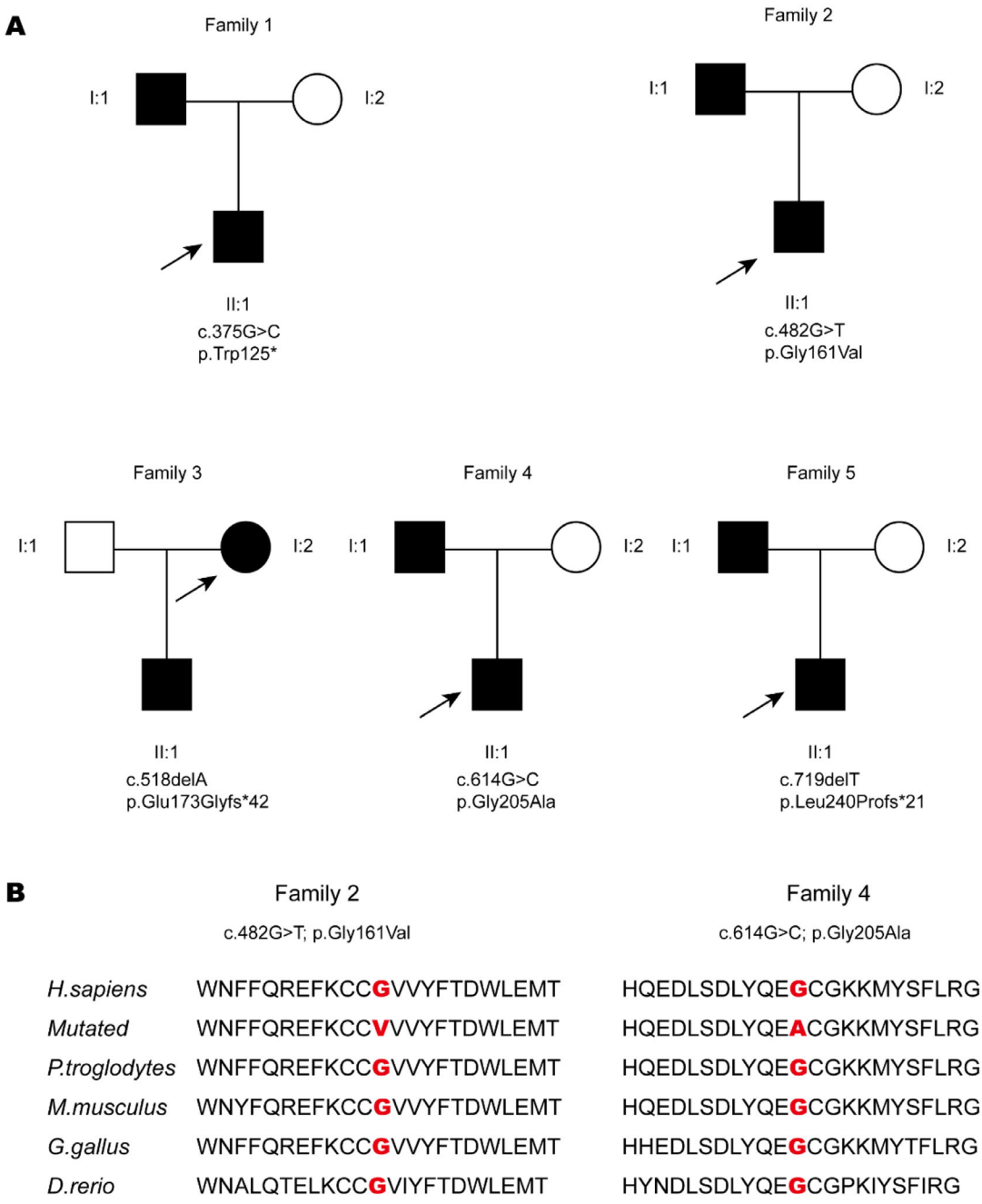

Figure 1. Five FEVR family pedigrees and the conservation analysis of two missense variants. Five FEVR family pedigrees. Patients are denoted in *black* and the probands are indicated with *black arrows* (**A**). The conservation analysis of two missense variants, c.482C>T and c.614G>C (**B**).

The detailed clinical manifestation is shown in Table 2.

To investigate the effects of the TSPAN12 variants on Norrin/ β-catenin signaling, the dual-luciferase reporter assay in HEK293 STF cells was performed. The value of Firefly/Renilla showed the signaling activity of all five variants was remarkably decreased compared with the wild-type protein (Fig. 5).

Discussion

The function of TSPAN12 in retinal angiogenesis, especially in Norrin/ β-catenin signaling pathway, has been studied recently. Junge et al.[10] detected Tspan12 expression in the vasculature. The *TSPAN12* mutant mice revealed distinct vascular defects similar to FZD4

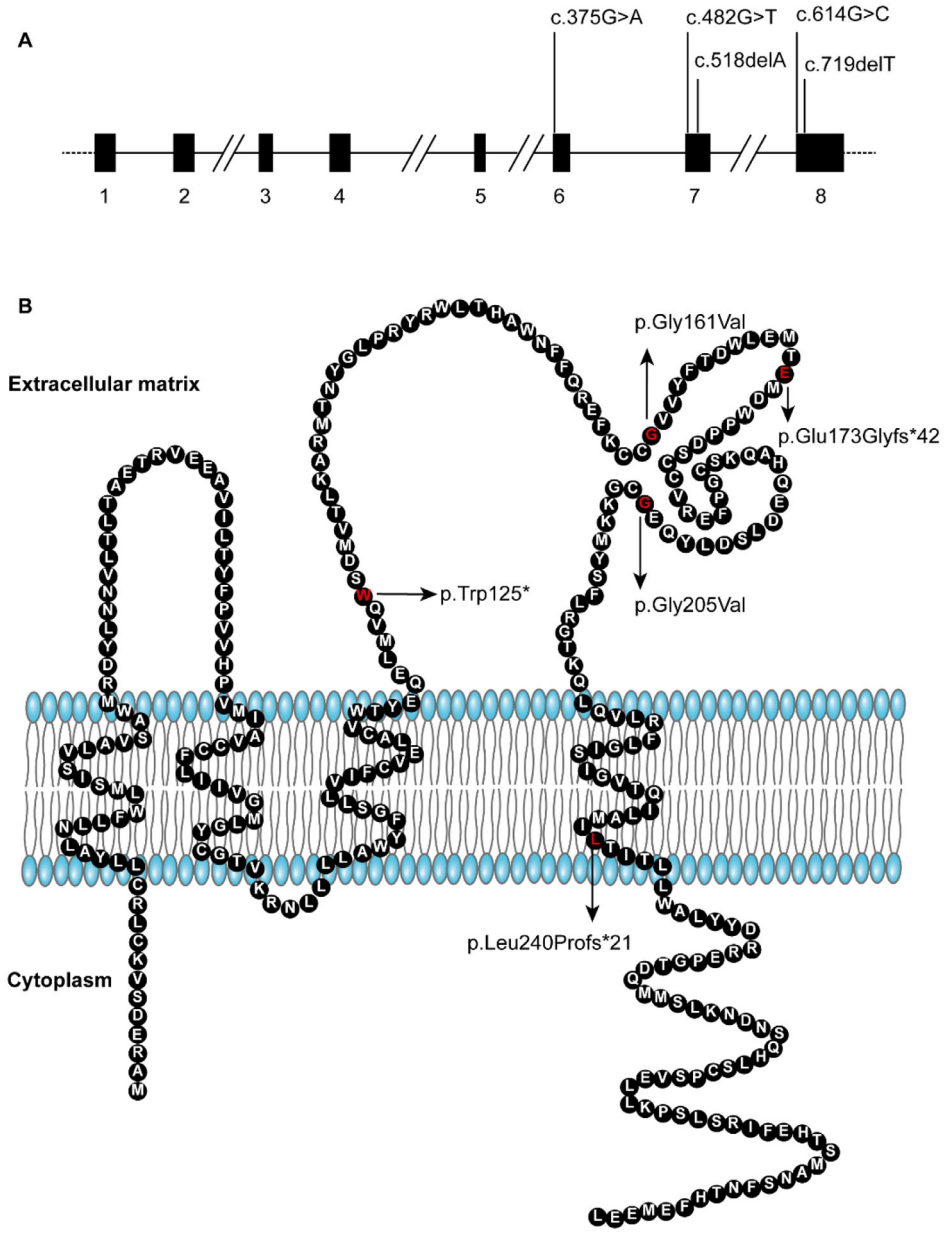

Figure 2. (**A**) Schematic of TSPAN12 gene and five novel variants in this study. (**B**) Schematic diagram of TSPAN12 protein with domains and the positions of five novel variants in this study.

and Norrin mutant mice, including microaneurisms extending from the NFL toward the inner nuclear layer, aberrant formation of retinal vascular fenestrations, delayed hyaloid vessel regression, and lack of vertical sprouts.[10] The TSPAN12 protein is formed by four domains, two extracellular loops, and one intracellular loop. Lai et al.[33] found that the large extracellular loop is required for enhancing NDP-induced Wnt signaling; moreover, they also confirmed 10 missense variants that impaired signaling activity. In this study, we reported that the percentage of FEVR with TSPAN12 variants was 8.2% in a cohort of 534 FEVR patients and five novel variants: c.375G>C, c.482C>T, c.518delA, c.614G>C, and c.719delT. Four of them are located in the large extracellular loop, and one is located in the fourth transmembrane domain. According to the analysis in silico, all of them were predicted as pathogenic.

In our research, luciferase assay was performed to assess the effect on Norrin/β-catenin signaling with mutant *TSPAN12*, which demonstrated that all five variants impaired the function of the Norrin/β-catenin pathway. It should be noted that the c.614G>C led to a mild decrease in signaling activity. The relative proband presented mild fundus features, which matches the luciferase assay. A reasonable explanation is that the

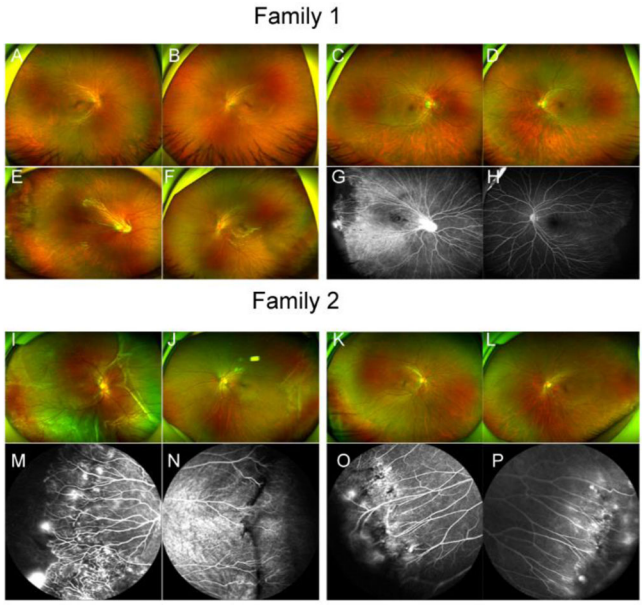

Figure 3. The fundus photographs of family 1 (**A–H**) and 2 (**I–P**). The fundus images of the patient's father showed an increase and straightening of vessels in the peripheral retina (**A**, **B**). The patient's mother's fundus images are normal (**C**, **D**). The patient's fundus and FFA images showed an ectopic macula and anomaly in the peripheral retina including leakage and incomplete vascular development (**E–H**). The fundus photographs of the proband showed retinal detachment and TEMPVIA in the right eye and left eye, respectively (**I**, **J**). The fundus photographs of the patient's father showed an increase and straightening of vessels in the peripheral retina (**K**, **L**). The FFA of the patient showed leakage in the right eye and anomaly vascular development (**M**, **N**). The FFA of the patient's father showed retinal terminal vascular leakage (**O**, **P**).

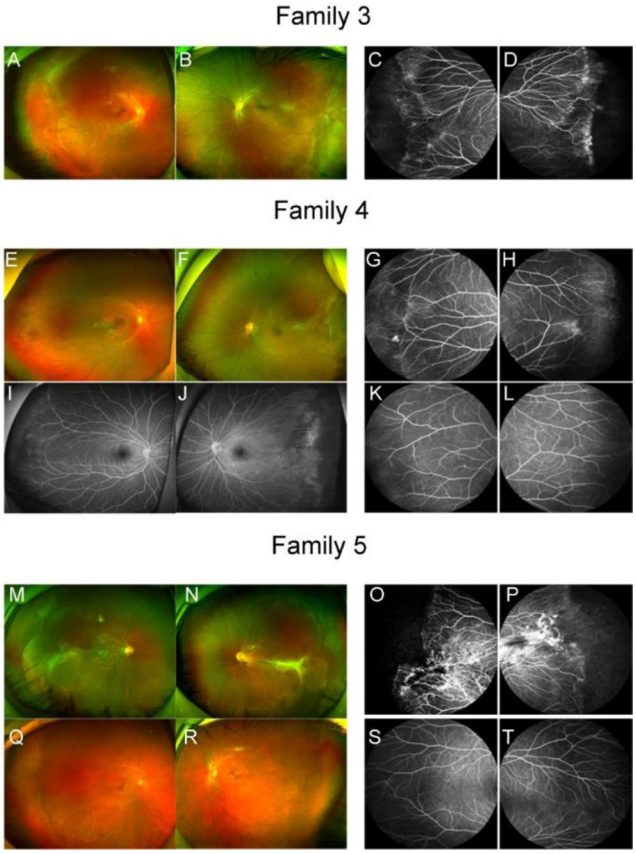

Figure 4. The fundus photographs of family 3 (**A–D**), 4 (**E–L**), and 5 (**M–T**). The fundus images and FFA of proband 3 showed abnormality of peripheral retinal vascular development including incompleteness, increase and straightening of vessels, and vascular leakage (**A–D**). The fundus images (**E**, **F**) and FFA (**I**, **J**) of proband 4 demonstrated abnormality of peripheral retinal vascular development and TEMPVIA in the right eye and left eye, respectively. His father's FFA revealed a vascular anomaly in the peripheral retina (**G**, **H**) and his mother's FFA was normal (**K**, **L**). The fundus photographs and FFA demonstrated TEMPVIA, ectopic macula, and retinal folds in proband 5 in two eyes, respectively (**M–P**). The peripheral vascular anomaly was observed in the asymptomatic father (**Q–T**).

variant causes a relatively small influence as a missense mutation. The c.375G>C variant causes severe impairment of Norrin/β-catenin signaling. The proband with this mutation presented as a severe type with the ectopic macula and low vision. However, the mild features in the left eye indicated the unknown mechanism of clinical variables between different eyes. The c.719delT, located in the last transmembrane domain, still decreased the activity of signaling significantly. By now, the function of the four transmembrane domains of TSPAN12 is still unknown. Our results showed the function of the last transmembrane domain is still important, and further study is needed. In addition, the results also showed that *TSPAN12* variants with variable phenotypes have full penetrance. It is important to perform fluorescein angiography in seemingly unaffected relatives to identify any peripheral retinal vascular abnormalities.

Because no phenotypic differences were found between FEVR patients with truncating variants and those with missense mutation, a proposed mechanism of *TSPAN12* variants was haploinsufficiency.[32] In this study, the frameshift variant c.518del is located in exon 7 of 8, which should be anticipated to result in nonsense-mediated mRNA decay along with the nonsense variant c.375G>A. In contrast, the c.719del located in exon 8 would likely escape nonsense-mediated mRNA decay and instead produce a truncated protein.[34] However, these variants caused a similar severity of phenotype as missense variants. These results further support the viewpoint that haploinsufficiency is the mechanism of *TSPAN12*-related FEVR.

In this study, our results showed that five novel variants of *TSPAN12* damaged the development of retinal vascularization by inhibiting Norrin/β-catenin signaling and led to FEVR. The study of the mutants in

Table 2. The Ocular Manifestations of Five Families

Families	Family Number	Age	Sex	Status	Symptoms at Presentation	BCVA (log MAR)		Fundus	
						OD	OS	OD	OS
1	I:1	34	M	A	Asymptomatic carrier	0.1	0.1	Vascular leakage of the peripheral retina	Vascular leakage of the peripheral retina
	I:2	33	F	UA	Normal subject	0	0	Normal	Normal
	II:1*	5	M	A	Nystagmus, poor vision	1.5	0.2	Ectopic macula, incomplete development of the peripheral retinal vascular	Incomplete development of the peripheral retinal vascular
2	I:1	40	M	A	Asymptomatic carrier	0	0	Vascular leakage of the peripheral retina; increase or straightening of vessels	Vascular leakage of the peripheral retina; increase or straightening of vessels
	I:2	40	F	UA	Normal subject	0	0	Normal	Normal
	II:1*	12	F	A	Unilateral painless loss of vision	1.7	0.1	Retinal detachment	Incomplete development of the peripheral retinal vascular
3	I:1	40	M	UA	Normal subject	0	0	Normal	Normal
	I:2*	40	F	A	Asymptomatic carrier	0	0	Vascular leakage of the peripheral retina; increase or straightening of vessels	Vascular leakage of the peripheral retina; increase or straightening of vessels
	II:1	0	F	A	Vitreous hemorrhage	NA	NA	Vitreous hemorrhage	Retinal neovascularization
4	I:1	40	M	A	Asymptomatic carrier	0	0	Vascular leakage of the peripheral retina; increase or straightening of vessels	Vascular leakage of the peripheral retina; increase or straightening of vessels
	I:2	40	F	UA	Normal subject	0	0	Normal	Normal
	II:1*	4	M	A	Low vision	0.1	0.7	Incomplete development of the peripheral retinal vascular, vascular leakage of the peripheral retina	Temporal mild-peripheral vitreoretinal interface abnormality
5	I:1	33	M	A	Asymptomatic carrier	0	0	Increase or straightening of vessels	Increase or straightening of vessels
	I:2	32	F	UA	Normal subject	0	0	Normal	Normal
	II:1*	5	M	A	Nystagmus, poor vision	0.6	1	Ectopic macula	Retinal folds

BCVA, best-corrected visual acuity; OD, oculus dexter; OS, oculus sinister; M, male; F, female; A, affected; UA, unaffected; NA, none available.

*Proband.

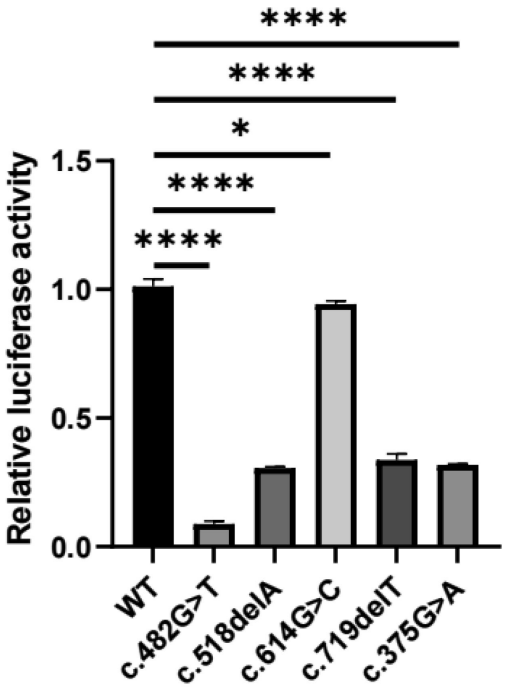

Figure 5. The effects of TSPAN12 variants on the Norrin/β-catenin signaling activity. *Error bars*: square deviation. *P* values were calculated from multiple comparisons in one-way analysis of variance with Tukey's multiple comparisons test (n = 3). The experiment was repeated three times. *$P < 0.05$; ****$P < 0.0001$.

the *TSPAN12* gene and families with FEVR expanded the knowledge of this disease and could be in favor of further clinical and genetic diagnosis.

Several limitations should be considered in this study. First, owing to the limited number of patients, the genotype-phenotype relationship is difficult to explore. Second, although we successfully detected the prominently reduced Norrin/β-catenin signaling activity induced by mutant *TSPAN12* in vitro, the underlying mechanism from these mutations still required further investigation, especially for the transmembrane domain.

Conclusion

Our study expanded the variant spectrum of *TSPAN12* involved in the Norrin/β-catenin signal pathway and provided information for the genetic testing of patients with FEVR.

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