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ORIGINAL ARTICLE

A novel frameshift variant in the *TSPAN12* gene causes autosomal dominant FEVR

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Abstract

Background: Familial exudative vitreoretinopathy (FEVR) is an inherited blinding eye disease with abnormal retinal vascular development. We aim to broaden the variant spectrum of FEVR and provide a basis for molecular diagnosis and genetic consultation.

Methods: We recruited five FEVR patients from one large Chinese family. Whole-exome sequencing (WES) and Sanger sequencing were applied to sequence, analyze, and verify variants on genomic DNA samples. Immunocytochemistry, western blot, qPCR, and luciferase assay were performed to test the influence of the variant on the protein expression and activity of the Norrin/β-catenin pathway.

Results: We identified a novel heterozygous frameshift variant c.533dupC (p.D179Rfs*6) in Tetraspanin 12 (*TSPAN12*) gene that is related to FEVR. This variant caused degradation of the entire TSPAN12 protein, which failed to activate Norrin/β-catenin signaling, possibly causing FEVR.

Conclusion: Our study revealed a novel frameshift variant D179Rfs*6 in *TSPAN12* that is inherited in an autosomal dominant manner. We found that D179Rfs*6 caused a failure to activate Norrin/β-catenin signaling. This finding broadens the variant spectrum of *TSPAN12* and provides invaluable information for the molecular diagnosis of FEVR.

Li Peng and Erkuan Dai have contributed equally to this work.

KEYWORDS
FEVR, frameshift variant, Norrin/β-catenin pathway, *TSPAN12*

1 | INTRODUCTION

Familial exudative vitreoretinopathy (FEVR) was first described by Criswick and Schepens (1969). It is an inherited blinding eye disease with abnormal retinal vascular development. FEVR can be inherited in an autosomal recessive, autosomal dominant, or X-linked trait (Shafienia et al., 2020). In addition to genetic heterogeneity, FEVR also shows a high degree of clinical heterogeneity (Wang et al., 2021). The primary features of FEVR include retinal neovascularization, vitreous hemorrhage, vascular leakage, and retinal detachment (Tian et al., 2019). The left and right eyes of most patients are at different disease stages, and even within the same family, the illnesses may range from asymptomatic to complete blindness (Gilmour, 2015). The clinical and genetic heterogeneity greatly hinders the clinical diagnosis of FEVR.

Molecular diagnosis of FEVR based on whole-exome sequencing (WES) has been widely used to assist in clinical diagnosis. To date, 14 genes and one locus have been associated with FEVR, among them, eight are related to the Norrin/β-catenin signaling pathway (Chen et al., 1993; Li et al., 2021; Park & Yamamoto, 2019; Poulter et al., 2010; Tucci et al., 2014; Zhang et al., 2021; Zhu et al., 2021). However, these previously reported variants can only explain approximately 50% of FEVR cases, which led us to seek novel pathogenic variants (Rao et al., 2017). The Norrin/β-catenin pathway plays an important role in retinal vascular development (Chen et al., 1993; Park & Yamamoto, 2019; Poulter et al., 2010; Tucci et al., 2014; Yang et al., 2021; Zhang et al., 2021; Zhu et al., 2021). In this pathway, the extracellular ligand Norrin (encoded by *NDP*) binds to a receptor complex composed of Low-density lipoprotein receptor-related protein-5 (LRP5) and Frizzled-4 (FZD4) to inhibit the degradation of β-catenin, which activates the expression of downstream genes (Fei et al., 2015; Han et al., 2020; Panagiotou et al., 2017; Peng et al., 2022; Wang et al., 2021; Zhao et al., 2022). TSPAN12 plays a role in stabilizing ligand-receptor complex in Norrin/β-catenin signaling pathway (Ke et al., 2013; Panagiotou et al., 2017; Schatz & Khan, 2017; Xin et al., 2010). The *TSPAN12* gene (OMIM: 613138) is located on chromosome 7q31, which encodes Tetraspanin 12 protein, a member of the cell surface transmembrane four superfamily. Tetraspanin 12 interacts with other proteins through its four transmembrane domains, two extracellular loops, and one intracellular loop (Poulter et al., 2012). Pathogenic variants in *TSPAN12* are common causes of FEVR; thus far, at least 88 pathogenic variants in *TSPAN12* have been reported to cause FEVR (Sun et al., 2021).

In this study, we identified a novel frameshift variant c.533dupC: (p. D179Rfs*6) in *TSPAN12* from a large Chinese family. Using luciferase reporter assay, western blot analysis, quantitative real-time PCR, and immunocytochemistry, we found that this variant could cause degradation of the TSPAN12 protein, which was consequently insufficient to activate Norrin/β-catenin signaling and downstream genes expression both in HEK293T cells and human umbilical vein endothelial cells (HUVECs). In conclusion, this research reports a novel autosomal dominant variant in *TSPAN12* and provides insights into the mechanism by which this variant causes FEVR. These findings broaden the variant spectrum of *TSPAN12*, offering invaluable data for the molecular diagnosis of FEVR.

2 | MATERIALS AND METHODS

2.1 | Subjects and clinical assessments

The patients of the family were recruited from Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine. We have given written informed consents to all participants and obtained consent. This study was approved by the Ethical Oversight Committee of Sichuan Provincial People's Hospital and Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine. All experiments were carried out according to the tenets of the Declaration of Helsinki. The diagnostic criterion of FEVR was performed as previously described (He et al., 2022). Briefly, the diagnosis of FEVR was evaluated using slit-lamp biomicroscopy, fundus photography, type-B ultrasonic imaging, or fundus fluorescein angiography (FFA) by an ophthalmologist with the following criteria: (1) The proband was born at full-term and without history of oxygen inhalation; (2) the presence of retinal avascular area; and (3) vascular leakage or retinal neovascularization.

2.2 | DNA extraction and whole-exome sequencing (WES)

A whole blood DNA extraction kit (#U9221, TianGen) was used to extract DNA from peripheral blood of the

participants. Then, we measured DNA concentration with an ultraviolet spectrophotometer NanoDrop ND-2000C (Thermo Fisher Scientific). Then, we refer to the previous method to perform WES analysis on the samples (Asano et al., 2021). The captured library was sequenced on Illumina HiSeq 2500 (Illumina). NextGene V2.3.4 software was used to analyze the output sequencing data. Variants were filtered using a public database that includes the ExAC, 1000G, gnomAD database, and 1000 in-house non-FEVR controls.

2.3 | Variant validation

We used Sanger sequencing to confirm mutations. Polymerase chain reaction (PCR) primers were designed by Primer3 Input (SourceForge.Net) and synthesized by Qingke Biotechnology. The primers used to amplify genomic DNA fragments were as follows: F: 5′-CACTGACTGGTTGGAAATGACA, R: 5′-ACTGAGATCTTCCTGGTGGG. Finally, the DNA samples were analyzed by Sanger sequencing using the ABI 3730 automatic sequencer (Applied Biosystems) according to the manufacturer's instructions.

2.4 | Construction of expression plasmids

N-terminal FLAG-tagged wild-type (WT) *TSPAN12* (NM_012338.4) expression plasmid was purchased from Youbio Biotechnology. We used a Q5®Site-Directed Mutagenesis Kit (New England Biolabs) to construct the TSPAN12-c.533dupC expression plasmid according to the manufacturer's instructions.

2.5 | Luciferase assays

In order to investigate the effect of the mutant TSPAN12 protein on the Norrin/β-catenin pathway, the HEK293STF cells were seeded onto a 24-well plate with 80% confluence and then each well was co-transfected with the following expression plasmids mix: 100 ng NORRIN, 100 ng LRP5, 100 ng FZD4, and 100 ng pGL4.1-Reilla with 200 ng TSPAN12 (wild-type, variant, or empty vectors) using Lipofectamine™ 3000 Transfection Reagent (Cat# L3000001, Invitrogen). After 48 h, the transfected cells were subjected to luciferase activity assays using the *TransDetect*® Double-Luciferase Reporter Assay Kit (Cat# FR201-01, TransGen Biotech).

2.6 | Quantitative real-time PCR and western blot

HEK293T cells were co-transfected with the following expression plasmids mix: 1000 ng NORRIN, 1000 ng LRP5, and 1000 ng FZD4 with 2000 ng TSPAN12 (wild-type, variant, or empty vector) using Lipofectamine™ 3000 Transfection Reagent (Invitrogen). HUVECs were purchased from Lonza, which were co-transfected with the following expression plasmid mix: 700 ng NORRIN, 700 ng LRP5, and 700 ng FZD4 with 700 ng TSPAN12 (wild-type, variant, or empty vector) using PromoFectin-HUVEC cell Transfection Reagent (Promocell).

After 48 h, 1/3 of cells were used for RNA extraction by TrizolUP (Cat#ET101-01, TransGen Biotech) according to the manufacturer's protocol, and the rest of the cells were lysed in 1×RIPA buffer containing protease inhibitor (Cat#HY-K0010, MedChemExpress) and sonicated for three times. The cDNA synthesized with TranScript All-in-one First-Strand cDNA Synthesis SuperMix (Cat#AT341, TransGen Biotech) was used for quantitative real-time PCR (qPCR) on a 7500 Real-Time PCR System (Applied Biosystems) with PerfectStartTM Green qPCR SuperMix (+Dye II) (Cat#AQ602, TransGen Biotech). The primers for qPCR are as follows: TSPAN12-F: 5′-AGTTTCTGCTTGGATGAGGGA, TSPAN12-R: 5′GCAACAGATTTCTTTTCACCGT; GAPDH-F: 5′-CCATGGGTGGAATCATATTGGA,GAPDH-R:5′-TCAACGGATTTGGTCGTATTGG.

For western blot analysis, equal amounts of protein (20 μg) were loaded to a 12% polyacrylamide gel and transferred to NC membranes (EMD Millipore HATF00010, MA) for immunoblotting. The antibodies used for western blot are as follows: rabbit anti-CTNNB1 (1:1000 dilution; CAT# 8480; Cell Signaling Technology), rabbit anti-p-GSK3β-ser-9 (1:1000 dilution; CAT# 12456; Cell Signaling Technology), rabbit anti-GAPDH (1:1000 dilution; CAT# 10494-1-AP; Proteintech), rabbit anti-GSK3β (1:1000 dilution; CAT# 22104-1-AP; Proteintech), rabbit anti-Cyclin D1 (1:1000 dilution; CAT# MA5-14512; Invitrogen), rabbit anti-c-Myc (1:1000 dilution; CAT# T55150; Abmart), and anti-rabbit IgG, HRP-linked Antibody (1:10000 dilution; CAT# 7074; Cell Signaling Technology).

2.7 | Immunocytochemistry

HEK293T cells were seeded on 5 μg/mL human fibronectin protein (Thermo Fisher Scientific) coated slides in 24-well plates (Corning) with 80% confluence and then each well was transfected with 200 ng wild-type, variant

TSPAN12 or empty vectors using Lipofectamine™ 3000 Transfection Reagent (Invitrogen). After 48 h, the slides in 24-well plates were fixed in 4% PFA in Phosphate buffered saline (PBS) (Sigma-Aldrich) at room temperature for 15 min, then rinsed with PBS for three times (5 min/time) and blocked in PBS containing 5% FBS (Invitrogen) and 0.2% Triton X-100 for 30 min at room temperature, followed by incubation with primary antibodies at 4°C overnight. The following primary antibodies were diluted in blocking buffer: Monoclonal ANTI-FLAG® M2 antibody produced in mouse (1:100 dilution; CAT# F3165; Sigma-Aldrich). Then, the slides were washed three times with PBS and labeled with goat anti-mouse IgG Secondary Antibody for 1–4 h, following with Alexa Fluor Plus 488 secondary antibody (1:300 dilution; CAT# A32723; Invitrogen) and DAPI (1:500 dilution; CAT# 4083; Cell Signaling Technology). Images were captured on an LSM 800 confocal scanning microscope (Zeiss,).

3 | RESULTS

3.1 | Clinical evaluation

The proband (III-1) was diagnosed with FEVR during newborn screening. Fundus photograph showed falciform retinal fold in the left eye and peripheral avascular zone in the right eye of the proband (Figure 1a). The proband's father, paternal grandfather, paternal aunt, and female cousin were also diagnosed with FEVR. The fluorescein fundus angiography of the paternal grandfather (I-1) and female cousin (III-2) showed avascularity of the bilateral peripheral retina (Figure 1a).

3.2 | Identification of causative variants

We used WES to identify the causative variants in this FEVR family. As a result, a novel heterozygous variant c.533dupC (p.D179Rfs*6) in the *TSPAN12* gene was identified. This variant was absent in the ExAC, 1000G, gnomAD database, and 1000 in-house database. The variant was predicted to cause truncation of the TSPAN12 protein and possibly result in activation of the nonsense-mediated mRNA decay (NMD). As expected, the affected father, paternal grandfather, paternal aunt, and female cousin of the proband were all heterozygous carriers of this variant, while the other unaffected family members did not carry this variant, which was confirmed by Sanger sequencing analysis. Thus, the variant was proved to be co-segregated with the disease phenotype in the family (Figure 1b).

3.3 | Defective Norrin/β-Catenin signaling

To assess the impacts of variant on protein expression, we performed western blot and qPCR analysis

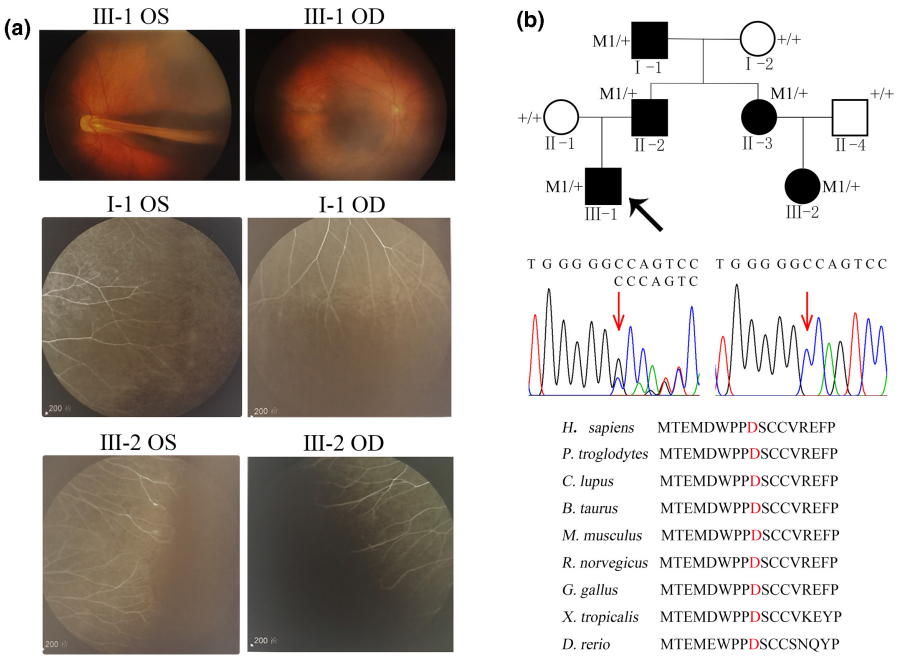

FIGURE 1 TSPAN12 variant in family with FEVR and clinical examinations. (a) Fundus photography of the proband (III-1), fundus fluorescein angiography of the paternal grandfather and female cousin (I-1 and III-2) in this family. (b) FEVR pedigree, Sanger sequencing analysis, and conservative analysis. Patients are denoted in black. Black arrow indicates the proband. Red arrows indicate the changed nucleotides

on HEK293T cells transfected with wild-type, variant TSPAN12, or vector plasmids. The results indicated that the plasmid carrying this variant encodes truncated and degraded protein (approximately 21 KDa) with minimal signal (Figure 2a), whereas the mRNA level of variant TSPAN12 was comparable to that of wild-type protein (Figure 2b), indicating that the mutant TSPAN12 protein was unstable and underwent posttranscription degradation. Consistently, the results of immunocytochemistry showed that wild-type TSPAN12 was mainly expressed in the cytoplasm and cell membranes of HEK293T cells, whereas the D179Rfs*6 variant could not be detected (Figure 2c).

A luciferase reporter assay was performed to verify the effect of the variant on the Norrin/β-catenin signaling. To this end, TSPAN12 plasmids (wild-type, variant, or vector) were co-transfected with NDP/FZD4/LRP5 plasmids in HEK293STF cells. The variant revealed at least 50% loss of wild-type activity, which was comparable to the vector plasmid (Figure 3a). We then conducted a western blot analysis on HEK293T cells transfected with wild-type, variant, or vector plasmids to investigate the effect of the variant on the protein expression and downstream components of the Norrin/β-catenin signaling pathway. As expected, overexpression of the wild-type TSPAN12 significantly promoted phosphorylation of GSK3β-ser-9, which inhibited degradation of the β-catenin and activated expression of the downstream target genes Cyclin D1 and c-Myc (Figure 3b–f). However, the D179Rfs*6 variant failed to activate phosphorylation of GSK3β-ser-9, which was unable to prevent β-catenin from degradation, leading to compromised Wnt signaling activity, comparable to that caused by the vector plasmid. Given that defective endothelial Norrin/β-catenin signaling has been reported as a main cause of vascular defect in the pathogenesis of FEVR, we further used HUVECs to test whether the variant *TSPAN12* could cause defective endothelial Norrin/β-catenin signaling. As expected, the results were consistent with those for HEK293T cells (Figure 3g–k).

4 | DISCUSSION

The important role of *TSPAN12* in retinal angiogenesis was discovered in 2009. Pathogenic variants of this gene have been verified to cause vascular defects and affect neural cells through association with Norrin/β-catenin rather than Wnt/β-catenin signaling (Junge et al., 2009). The formation of microaneurysms and delayed vitreous vascular degeneration has been reported in *Tspan12* knockout mice (Junge et al., 2009; Tang et al., 2017). In 2010, a study revealed that mutations in *TSPAN12* cause autosomal dominant FEVR (Poulter et al., 2010). In the Norrin/β-catenin signaling, TSPAN12 serves as a specific co-receptor of Norrin that amplifies selectivity and signaling activity of ligand FZD4 (Lai et al., 2017). The formation of Norrin/FZD4/LRP5/TSPAN12 quaternary complex leads to phosphorylation of GSK3β and disruption of β-catenin "destruction complex" that consists of Axin, APC, GSK3β, and CK1, resulting in nuclear accumulation of β-catenin that facilitates binding of β-catenin to transcription factor (Doumpas

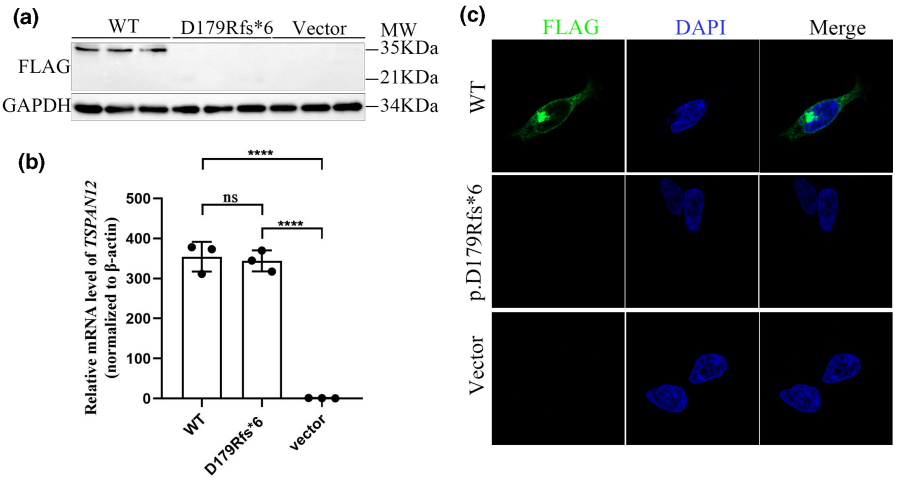

FIGURE 2 The variant cause truncation and degradation of TSPAN12. (a) Western blot analysis of HEK293T cells transfected with FLAG-tagged TSPAN12 (WT or variant) or vector showed the truncation and degradation of mutant TSPAN12 protein. (b) The qPCR analysis showed the comparable mRNA level of mutant TSPAN12 to that of wild-type. (c) The HEK293T cells were co-stained with FLAG (green) and DAPI (blue), and the results showed that wild-type TSPAN12 mainly expressed in cytoplasm and membrane, whereas no signal of mutant TSPAN12 was detected

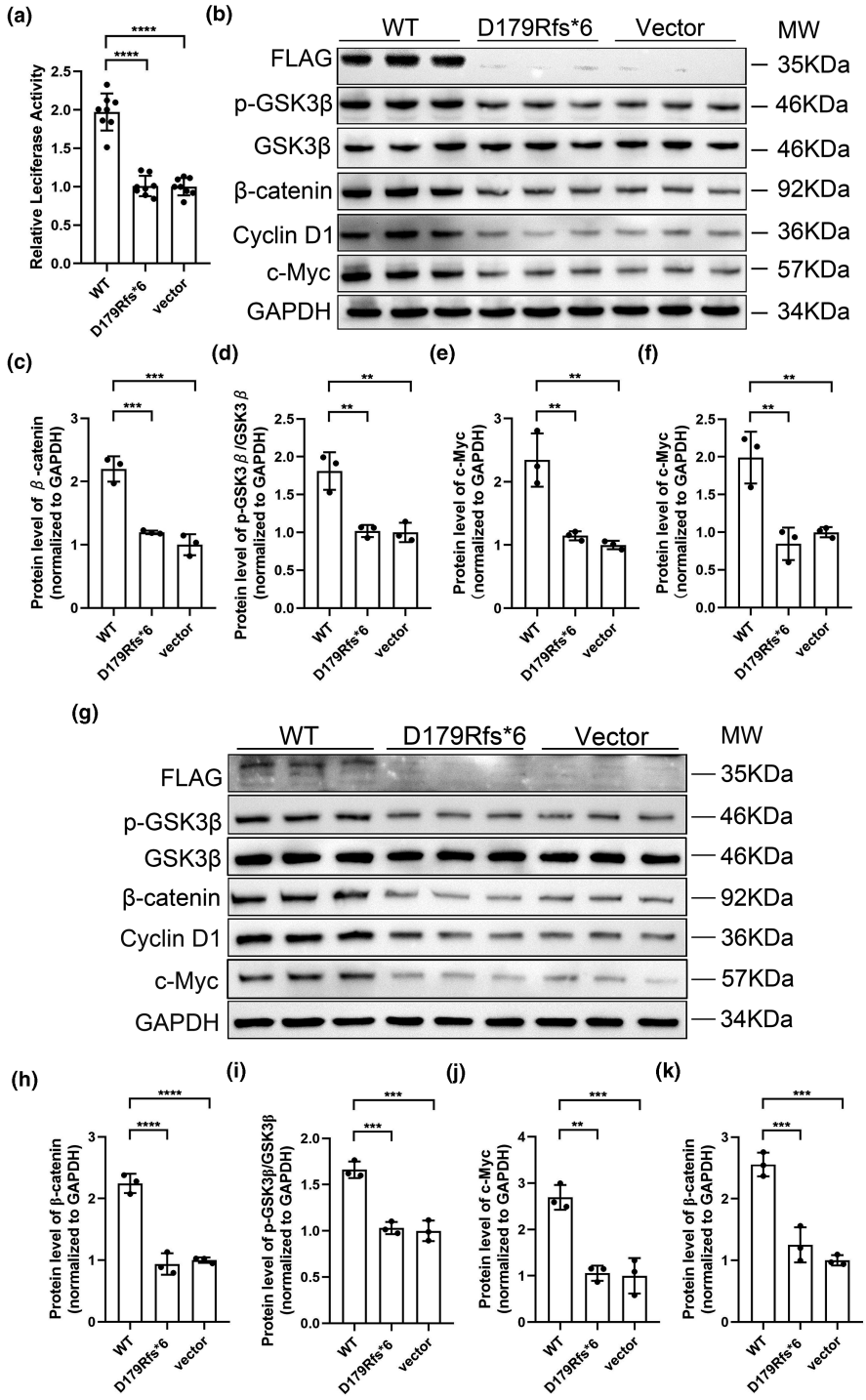

FIGURE 3 Variant in TSPAN12 causes defective Norrin/β-Catenin Signaling. (a) Luciferase reporter assay on HEK293 STF cells. The activity of vector was normalized as 1. Error bars, SD. *p*-Values were calculated from multiple comparisons in one-way ANOVA with Tukey's multiple comparisons test ($n = 8$); ****, $p < .0001$. (b–f) Western blot and statistical analysis of relative c-Myc, Cyclin D1, GSK3β, GSK3β-ser-9, and β-catenin protein levels in HEK293T cells co-transfected with FLAGtagged TSPAN12 (WT, variant, or vector) and NDP/LRP5/FZD4 plasmids. (g–k) Western blot and statistical analysis of relative c-Myc, Cyclin D1, GSK3β, GSK3β-ser-9, and β-catenin protein levels in HUVECs cotransfected with FLAG-tagged TSPAN12 (WT, variant, or vector) and NDP/LRP5/FZD4 plasmids

et al., 2019). Defective Norrin/β-catenin signaling activity was reported to cause decreased expression of tight junction protein Claudin-5 and increase of PLVAP, an EC-specific marker of high permeability, which might contribute to the phenotype of vessel leakage in FEVR (Wang et al., 2018; Yang et al., 2021). Till now, the known variants can explain only approximately 50% FEVR cases, and 58 variants in *FZD4*, 37 in *NDP*, 97 in *LRP5*, and 63 in *TSPAN12* were identified in Chinses FEVR cases.

D179 is in the large extracellular loop of TSPAN12, which was reported to be responsible for FZD4 or Norrin binding (Lai et al., 2017). The D179Rfs*6 variant was predicted to produce truncation and degradation of TSPAN12 using online tools, and this was verified by immunocytochemistry and western blot analysis. Therefore, the lack of TSPAN12 might disrupt the formation of ligand-receptor signaling complexes in the Norrin/β-catenin signaling pathway, which could eventually cause a decrease in phosphorylation of GSK3β

and promote β-catenin degradation, leading to compromised expression of targeted genes such as Cyclin D1 and c-Myc. This was confirmed not only by a luciferase reporter assay in HEK293STF cells, but also by western blot analysis in HEK293T cells and HUVECs. Nevertheless, although we report in this study a large family with five affected members, we lack complete clinical information for further evaluation of genotype–phenotype correlation analysis to uncover specific phenotypes of this variant.

In summary, this study provides a comprehensive understanding of the effect of D179Rfs*6 variant in *TSPAN12* on Norrin/β-catenin signaling and its association with FEVR. Variant screening of known genes would provide invaluable information for molecular diagnosis and genetic counseling, especially for patients without a family history or with atypical presentations. Further mechanistic analysis of novel variants in known genes could explain some mild clinical findings and contribute to our understanding of the molecular mechanisms of retinal angiogenesis, which plays a crucial role in FEVR and other retinal vascular diseases.

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CONFLICT OF INTEREST

The author(s) declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Li Peng conducted the Sanger sequencing and luciferase experiments. Rulian Zhao and Yunqi He performed the construction of plasmids. Erkuan Dai, Haodong Xiao, and Peiquan Zhao recruited FEVR cases. Li Peng, Erkuan Dai, Shujin Li, and Mu Yang wrote the draft. Zhenglin Yang edited the manuscript. Peiquan Zhao conceived the research.

ETHICS STATEMENT

All participants signed for written informed consent. This study was approved by the Institutional Review Boards of Sichuan Provincial People's Hospital.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the article.

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