Novel mutations in the TSPAN12 gene in Chinese patients with familial exudative vitreoretinopathy

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Purpose: Familial exudative vitreoretinopathy (FEVR) is a group of inherited blinding eye diseases characterized by defects in the development of the retinal vessels. Recent studies have identified genetic variants in tetraspanin 12 (TSPAN12) as a cause of FEVR. The purpose of this study was to identify novel TSPAN12 mutations in Chinese patients with FEVR and to describe the associated phenotypes.

Methods: Mutation screening was performed by directly sequencing PCR products of genomic DNA with primers designed to amplify the seven coding exons and adjacent intronic regions of the FEVR-causing gene TSPAN12. Clinical phenotypes of the patients with TSPAN12 mutations were documented. Wild-type and mutant TSPAN12 proteins were assayed for the Norrin-β-catenin signaling pathway with luciferase reporter assays.

Results: Three novel heterozygous mutations in TSPAN12 were identified: c.566G>A (p.C189Y), c.177delC (p.Y59fsX67), and c.254T (p.T85M). All three mutations involved highly conserved residues and were not present in 200 normal individuals. Ocular phenotypes included increased ramification of the peripheral retinal vessels, a peripheral avascular zone, inferotemporal dragging of the optic disc and macula, and retinal folds. The probands showed relatively severe retinopathy, whereas the other family members were often asymptomatic. In SuperTopFlash (STF) cell line transfection studies, C189Y, Y59fsX67, and T85M mutants failed to induce luciferase reporter activity in response to Norrin.

Conclusions: We found three novel TSPAN12 mutations in Chinese patients with autosomal dominant FEVR, and suggest that TSPAN12 mutations cause FEVR. The phenotypes associated with the TSPAN12 mutations showed extensive variation in disease severity among members of the same family, which implied the complexity of FEVR mutations and phenotypes.

Familial exudative vitreoretinopathy (FEVR, OMIM 133780, 305390, 605750, 601813, 613310) is a hereditary disorder with defects in the development of retinal vasculature [1]. A wide range of clinical manifestations have been found in patients with FEVR. Severely affected patients suffer from blindness during infancy, and manifest retinal folds or detachments. In contrast, mildly affected patients usually have no visual problems and do not need to undergo fluorescein angiography unless a severely affected family member is diagnosed.

In the past two decades or so, four genes that cause FEVR have been identified: NDP (OMIM 300658, X-linked), LRP5 (OMIM 603506, dominant and recessive), FZD4 (OMIM 604579, dominant) [2-4], and TSPAN12 (OMIM 613138, dominant and recessive) [5-10]. All the proteins encoded by the FEVR genes have been found to participate in the Norrin-β-catenin signaling pathway [4-7]. In addition, another locus for autosomal dominant FEVR (adFEVR), EVR3, has been mapped to chromosomal locus 11p12-p13 [11]. In this study, three novel TSPAN12 mutations in patients with FEVR were identified, and we demonstrated that these three mutants failed to induce luciferase reporter activity in HEK293 transfection studies.

METHODS

Patients and clinical examinations: This study adhered to the ARVO statement on human subjects and was approved by the Institutional Review Board of the Xinhua Hospital affiliated with the Shanghai Jiao Tong University School of Medicine
The SuperTopFlash (STF) reporter, in mutations: Genomic DNA samples were extracted blood DNA extraction stored in 4 ºC and processed within 24 h after blood drawn. Beijing, China). Venous blood in EDTA vautainers was obtained from all subjects in EDTA Vacutainers. Detection of TSPAN12 of eye diseases were observed. Venous blood samples were further the diagnosis. In the 200 normal matched controls, fundus fluorescein angiography (FFA) was performed in selected cases to confirm the wild-type TSPAN12 cDNA by site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). LRP5, FZD4, and Norrin expression vectors (generously provided by Dr. Jeremy Nathans of Johns Hopkins University) have been described previously [12]. The recombinant plasmids containing TSPAN12-Myc-Flag fusion constructs were first verified with DNA sequencing, and then prepared for transfection using Qiagen plasmid Maxi preparation kit (Qiagen). Luciferase assays: The SuperTopFlash (STF) reporter, in which firefly luciferase was driven by seven lymphoid enhancer factor/T-cell factor (LEF/TCF) consensus binding sites, was a kind gift from Dr. Jeremy Nathans (John Hopkins University) have been described previously [12]. The recombinant plasmids containing TSPAN12-Myc-Flag fusion constructs were first verified with DNA sequencing, and then prepared for transfection using Qiagen plasmid Maxi preparation kit (Qiagen).

**Construction of expression plasmids:** The cDNA encoding wild-type TSPAN12 (Origene, Rockville, MD) was subcloned in-frame into the pCMV-entryMyc-Flag vector (Origene) using SgfI and MluI sites. All mutations were introduced into the wild-type TSPAN12 cDNA by site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). LRP5, FZD4, and Norrin expression vectors (generously provided by Dr. Jeremy Nathans of Johns Hopkins University) have been described previously [12]. The recombinant plasmids containing TSPAN12-Myc-Flag fusion constructs were first verified with DNA sequencing, and then prepared for transfection using Qiagen plasmid Maxi preparation kit (Qiagen).

**Luciferase assays:** The SuperTopFlash (STF) reporter, in which firefly luciferase was driven by seven lymphoid enhancer factor/T-cell factor (LEF/TCF) consensus binding sites, was a kind gift from Dr. Jeremy Nathans (John Hopkins University, Baltimore, MD). This reporter plasmid was stably transfected into HEK 293 cells as reported previously to generate the STF cell line. In 24-well plates, 160 K STF cells/well were transfected with 800 ng DNA and 1.5 µl Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad,

| Exon | Forward primer (5’>3’) | Reverse primer (5’>3’) | Amplicon length (bp) |
|------|------------------------|------------------------|---------------------|
| 2    | GGTGAGATGTCGGCTTGGTCA  | TCAAGGGCATTTAAGAAGGTC  | 340                 |
| 3    | AATCCCTGAGTTAAGGAGAAT  | AGGCCACCACTTAAGGAGAT   | 314                 |
| 4    | TGCTATGCTTGGGTCATT     | AAACGAAAGCGTCCCTTCTTT  | 331                 |
| 5    | TGCCCTGTGTTTCTGGTCA    | TTCACCTCTGACATGATTT    | 367                 |
| 6    | CGAGTGATGGTGGTACG      | GAAGAAAGGAGGCTGCGGAA   | 393                 |
| 7    | TTTCTGTTTCTGAGGCTGA    | TTCTCTGTGCTTCCCCATA    | 333                 |
| 8    | ACAGATTTGTGCTTTCA      | GCTTAGGTGTTATTTTAGGC   | 505                 |

Primer used for amplification and sequence analysis of human TSPAN12.
CA). The DNA mix contained 200 ng of Norrin, 200 ng of FZD4, 200 ng of LRP5, 100 ng of pSV-β-galactosidase control vector and 100 ng of TSPAN12 plasmid (wild-type or mutation). Forty-eight hours after transfection, the cells were harvested and washed twice with PBS (1X; 120 mM NaCl, 20 mM KCl, 10 mM NaPO₄, 5 mM KPO₄, pH 7.4), and the luciferase activities were measured with a Dual-Luciferase Assay Kit (Promega) according to the manufacturer’s instructions. Reporter activity was normalized to the coexpressed β-galactosidase activity in each well. Each test was performed in triplicate. The reporter assay was repeated three times, and a representative result was obtained.

Expression of TSPAN12 in Cos 7 cells: Cos 7 cells (American Type Culture Collection [ATCC], Manassas, VA) were cultured in DMEM (ATCC, Manassas, VA) with high glucose (HyClone) supplemented with 10% fetal bovine serum and 1% (vol/vol) penicillin/streptomycin at 37 °C in a 5% CO₂.

Figure 1. Chromatograms and pedigrees of three families with familial exudative vitreoretinopathy. Three novel mutations were identified in TSPAN12. A: In Family A, the affected mother and son had the c.566G>A (p.C189Y) mutation. C: In Family B, the patient and her affected mother had the c.177delC (p.Y59fsX67) mutation. E: In Family C, the affected mother and son had the c.C254T (p.T85M) mutation. The columns from left to right display the pedigree and the sequence chromatograms of these patients (A, C, E) and the normal controls (B, D, F). Arrows indicate the positions of the altered nucleotides.
atmosphere. Cells were seeded in six-well plates (Corning Inc., Corning, NY) and transfected at 50% confluency with 1 μg of wild-type and mutant pCMV6-entry-TSPAN12 or empty vector using Lipofectimine 2000 (Invitrogen) according to the manufacturer’s instructions. After 48 h, the cells were washed with PBS, and fixed with 4% paraformaldehyde (PFA) for 15 min. Anti-Flag antibody was used to detect TSPAN12 expression using standard immunostaining method.

RESULTS

Identification of novel TSPAN12 mutations and phenotypes:

We identified three proband patients with FEVR in our clinic. To identify causative genes for FEVR, we sequenced all the exons and the flanking intronic sequences for four known FEVR genes: FZD4, LRP5, Norrin, and TSPAN12. Three novel mutations in the coding sequence of the TSPAN12 gene were found in these families, including c.566G>A (p.C189Y), c.177delC (Y59fsX67), and c.C254T (p.T85M; Figure 1).

In Family A, we identified a missense mutation in exon 7, c.566G>A, segregating with the disease. This mutation led to the replacement of a cysteine by a tyrosine at codon 189 (p.C189Y; Figure 1), which involved a highly evolutionarily conserved residue (Figure 2A) from *Homo sapiens* to *Danio rerio* (NP_957446). A: The residue of the missense mutation p.C189Y is highly conserved. B: The residue of the missense mutation p.T85M is also highly conserved.

We identified one base pair deletion in exon 4, c.177delC, in the proband of Family B and her asymptomatic mother. This mutation resulted in a frameshift followed by premature termination at codon 67 (p.Tyr59fsX67; Figure 1), which also involved highly evolutionarily conserved residues (Figure 2C) from *Homo sapiens* to *Danio rerio*. The proband was a 2-year-old girl with a complaint of esotropia. Fundus examination showed typical falciform retinal folds across the fovea in both eyes (Figure 4). Her father had normal fundi, but her mother had typical fundus changes in FFA examination, including increased ramification of the peripheral retinal vessels and an avascular zone in the peripheral retina, though she had normal visual acuity of 1.0 in both eyes and had no vision complaints (Figure 4).

In Family C, a missense mutation c.C254T (p.T85M) segregating with the disease was identified (Figure 1). This mutation led to the change in a threonine residue to a methionine at codon 85 (p.T85M), which is extremely conserved from *Homo sapiens* to *Danio rerio*. (NP_957446) and *Danio rerio* (NP_957446). A: The residue of the missense mutation p.C189Y is highly conserved. B: The residue of the missense mutation p.T85M is also highly conserved.

In Family A, a missense mutation had normal vision but with areas of avascular zone and abnormal vessels in the peripheral retina (Figure 3).

We identified one base pair deletion in exon 4, c.177delC, in the proband of Family B and her asymptomatic mother. This mutation resulted in a frameshift followed by premature termination at codon 67 (p.Tyr59fsX67; Figure 1), which also involved highly evolutionarily conserved residues (Figure 2C) from *Homo sapiens* to *Danio rerio*. The proband was a 2-year-old girl with a complaint of esotropia. Fundus examination showed typical falciform retinal folds across the fovea in both eyes (Figure 4). Her father had normal fundi, but her mother had typical fundus changes in FFA examination, including increased ramification of the peripheral retinal vessels and an avascular zone in the peripheral retina, though she had normal visual acuity of 1.0 in both eyes and had no vision complaints (Figure 4).

In Family C, a missense mutation c.C254T (p.T85M) segregating with the disease was identified (Figure 1). This mutation led to the change in a threonine residue to a methionine at codon 85 (p.T85M), which is extremely conserved from *Homo sapiens* to *Danio rerio*. (NP_957446) and *Danio rerio* (NP_957446). A: The residue of the missense mutation p.C189Y is highly conserved. B: The residue of the missense mutation p.T85M is also highly conserved.
Figure 3. Fundus photographs of Family A with familial exudative vitreoretinopathy. **A** and **B**: Fundus photographs of the proband from Family A (individual II:1 in Figure 1), showing a retinal fold and a dragged macula. **C** and **D**: The unaffected father without the mutation has normal fundi. **E** and **F**: Fundus photographs of the asymptomatic mother with the c.566G>A mutation show normal posterior fundi. **G** and **H**: The mother has areas of avascularity and abnormal vessels in the peripheral retina.
Figure 4. Fundus photographs of Family B with familial exudative vitreoretinopathy. **A** and **B**: Fundus photographs of the proband from Family B (individual II:1 in Figure 1), showing the retinal vessels drawn up in a retinal fold that is obscuring the macula. **C** and **D**: The unaffected father has normal fundi. **E** and **F**: Fundus photographs of the asymptomatic mother with the c.177delC mutation show normal posterior fundi. **G** and **H**: The mother has increased vessel branching in the equatorial area and an avascular zone on the peripheral retina.
Figure 5. Fundus photographs of Family C with familial exudative vitreoretinopathy. A and B: Fundus photographs of the proband from Family C (individual II:1 in Figure 1), showing a retinal fold and a dragged macula. C and D: The mother has areas of avascularity and abnormal vessels in the peripheral retina.

Figure 6. Luciferase assays with the SuperTopFlash cell line transfected with the indicated plasmids. SuperTopFlash (STF) cells/well were transfected with 800 ng DNA (200 ng of Norrin, 200 ng of FZD4, 200 ng of LRP5, 100 ng of pSV-β-galactosidase control vector, and 100 ng of TSPAN12 plasmid [wild-type or mutation]) and 1.5 μl Lipofectamine 2000 transfection reagent. Forty-eight hours after transfection, the cells were harvested and washed twice with PBS. Luciferase activities were measured with a dual-luciferase assay kit. Reporter activity was normalized to the coexpressed β-galactosidase activity in each well. Each test was performed in triplicate. The reporter assay was repeated three times, and a representative result was obtained.
Figure 7. Western blot analysis by SDS-PAGE of the TSPAN12 mutants. Total protein (10 μg) isolated from cell lysates from luciferase assays was mixed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and subjected to SDS-PAGE and western blot analysis using anti-Flag antibody to detect TSPAN12 and FZD4 expression. Beta-actin was used as the loading control. The expression level of TSPAN12 C189Y was compatible with that of the wild-type. However, the T85M and Y59fs mutant proteins were not stable.

Figure 8. Immunofluorescence staining of the TSPAN12 C189Y mutant. Cos 7 cells were transfected either with human wild-type or mutant TSPAN12 cloned into the pCMV6-entry vector, or empty vector. Cells were washed with PBS after 48 h and fixed with 4% PFA for 15 min. Mouse monoclonal anti-Flag antibody and Alexa Fluor 594 goat anti-mouse immunoglobulin (IgG) secondary antibody were used to detect TSPAN12 expression with the standard immunostaining method. Red channel, TSPAN12; blue channel, 4',6-diamidino-2-phenylindole (DAPI) for nuclei staining.
Defective Norrin signaling mediated by mutant TSPAN12 proteins in vitro: To determine the effect of the TSPAN12 mutants on its biologic activity in Norrin/β-catenin signaling, we performed luciferase assays in STF cells using wild-type and three mutant TSPAN12 cDNAs, c.177delC, c.566G>A, and c.C254T were introduced into TSPAN12 with the site-direct mutagenesis method. Our in vitro analyses suggested that compared to wild-type TSPAN12, all three TSPAN12 mutants failed to induce luciferase reporter activity in STF cells in response to Norrin (Figure 6). Western blot analysis indicated that the T85M and Y59fsX67 mutant proteins were not stable (Figure 7). The C189Y mutant expression level was compatible to that of the wild-type (Figure 7 and Figure 8). The results suggest that the three TSPAN12 mutations identified in our study are pathogenic, and are consistent with the notion that defective Norrin/TSPAN12 signaling underlies FEVR.

**DISCUSSION**

In our study, three novel TSPAN12 mutations were detected in three families with FEVR, but not in 200 normal individuals. The clinical signs and symptoms showed variations among the patients with TSPAN12 mutations, from mild avascular peripheral retina with retinal degeneration to severe bilateral retinal folds. Our data suggest variable expressivity of FEVR: The probands manifested relatively severe retinopathy, whereas the other family members had normal vision and were often asymptomatic, as had been reported in cases carrying mutations in FZD4 or LRP5 [4,13-15]. Our results are consistent with the data reported by Kashani et al. [16]. These family members might develop advanced FEVR later on, which could deteriorate to vision loss. Therefore, it is necessary to screen immediate family members of the
patients with FEVR with retina angiography and clinical treatment.

The location of three novel TSPAN12 mutations identified in our study reveals their pathogenic nature (Figure 9). The c.177delC mutation in exon 4 caused a frameshift with a premature termination at codon 67, resulting in truncated proteins that might not be synthesized because of nonsense-mediated decay of the messenger ribonucleic acid. The highly conserved cysteine residue at position 189 among tetraspan family members plays a role in the formation of disulfide bridges, and is located in the large extracellular loop between the third and fourth transmembrane region. This extracellular loop is known to harbor most of the protein–protein interaction sites described for tetraspanins [17]. Thus, the substitution of a tyrosine residue for the cysteine at position 189 disrupts one of these interactions and, therefore, may have significant functional consequences, such as the interaction with the FZD4/LRP5/Norrin protein complex. Furthermore, the 189th cysteine is next to another TSPAN12 mutation G188R, a known FEVR mutation [6]. T85M is likely to bring about the change in the second transmembrane domain, as the other three known FEVR mutations (L101H, C105R, A237P) in the transmembrane domain do.

In addition, in our STF cell line transfection studies, the defective luciferase reporter activity mediated by the mutant TSPAN12 proteins revealed that Y59fsX67, C189Y, and T85M mutations in TSPAN12 led to a decrease in Norrin/FZD4/LRP5 signaling, which further suggested these three mutations were pathogenic. In conclusion, our study has identified three novel TSPAN12 mutations causing autosomal dominant FEVR. The findings provide additional evidence that mutations in TSPAN12 are causative in patients with FEVR, and TSPAN12 is crucial for the development of the retinal vessels.

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