Large Deletions of TSPAN12 Cause Familial Exudative Vitreoretinopathy (FEVR)

Soo Hyun Seo,1 Man Jin Kim,2 Sung Wook Park,3 Jeong Hun Kim,4 Young Suk Yu,4 Ji Yun Song,2 Sung Im Cho,2 Joo Hyun Ahn,5 Yeon Hee Oh,5 Jee-Soo Lee;2 Seungjun Lee,6 Moon-Woo Seong,2 Sung Sup Park,2,5 and Ji Yeon Kim5

1Department of Laboratory Medicine, Seoul National University Boramae Medical Center, Seoul National University College of Medicine, Seoul, Korea
2Department of Laboratory Medicine, Seoul National University Hospital, Seoul National University College of Medicine, Seoul, Korea
3Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul, Korea
4Department of Ophthalmology, Seoul National University Hospital, Seoul National University College of Medicine, Seoul, Korea
5Biomedical Research Institute, Seoul National University Hospital, Seoul, Korea
6Department of Laboratory Medicine, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul, Korea

Correspondence: Ji Yeon Kim, Biomedical Research Institute, Seoul National University Hospital, 101 Dachak-ro, Jongno-gu, Seoul 110-744, Korea; jkimmd@snu.ac.kr.

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PURPOSE. Familial exudative vitreoretinopathy (FEVR) is a rare, hereditary visual disorder. The gene TSPAN12 is associated with autosomal dominant inheritance of FEVR. The prevalence and impact of large deletions/duplications of TSPAN12 on FEVR patients is unknown. To glean better insight of TSPAN12 on FEVR pathology, herein, we describe three FEVR patients with TSPAN12 deletions.

METHODS. Thirty-three Korean FEVR patients, who previously screened negative for TSPAN12 mutations, mutations in other FEVR-associated genes such as NDP, FZD4, LRP5, and large deletions and duplications of NDP, FZD4, and LRP5, were selected for TSPAN12 large deletion and duplication analyses. Semiquantitative multiplex PCR for TSPAN12 gene dosage analyses were performed, followed by droplet digital PCR (ddPCR) for validation.

RESULTS. Among the 33 patients, three patients were confirmed to carry large TSPAN12 deletions. Two of them had whole-gene deletions of TSPAN12, and the other patient possessed a deletion of TSPAN12 in exon 4. FEVR severity detected in these patients was not more severe than in a patient with TSPAN12 point mutation.

CONCLUSIONS. Regarding previously reported proportions of FEVR-associated genes contributing to the disorder’s autosomal dominant inheritance pattern in Korea, we determined that patients with TSPAN12 large deletions were more common than patients with single nucleotide variants in TSPAN12. Evaluating TSPAN12 large deletions and duplications should be considered in FEVR screening and diagnosis as well as in routine genetic workups for FEVR patients.

Keywords: familial exudative vitreoretinopathy, TSPAN12, large deletions, droplet digital PCR

Familial exudative vitreoretinopathy (FEVR) is a rare, genetically heterogeneous disorder that impairs vision and causes retinal detachment. Autosomal dominant inheritance is the most common form of FEVR and is known to be associated with the FZD4, LRP5, and TSPAN12 genes. Recently, the ZNF408 and KIF11 genes were also implicated in FEVR.1,2 In a previous report by our group, mutational studies regarding FZD4, LRP5, and TSPAN12 were carried out in 51 unrelated FEVR patients lacking NDP mutations. Among them, mutations with high probabilities of being pathogenic were detected in 18 patients.3 FZD4 mutations accounted for the largest proportion of autosomal dominant FEVR cases (13/18 patients, 72.2%), followed by LRP5 (4/18 patients, 22.2%), and TSPAN12 (1/18 patients, 5.6%) mutations. In rest of the 33 patients, no FZD4, LRP5, and TSPAN12 mutations were detected.

In our previous report, large deletions/duplications in NDP, FZD4, and LRP5 had been screened via multiplex ligation-dependent probe amplification (MLPA) using the SALSA P285-C1 LRP5-NDP-FZD4 (MRG-Holland, Amsterdam, Netherlands). Because TSPAN12 was not included, we looked for large deletions/duplications of TSPAN12 in the patients with no mutation detected to determine the genetic cause of FEVR. Gene dosage analysis by semiquantitative multiplex PCR and droplet digital PCR (ddPCR) were performed. In this study, we discovered and confirmed three cases of FEVR due to TSPAN12 large deletions.

METHODS

Subjects

Among the 51 FEVR patients from our previous study, 33 patients were selected for large deletion/duplication analyses of TSPAN12. These patients were diagnosed with FEVR between January 2008 and December 2012 at the Seoul National University Children’s Hospital (Seoul, Korea). The diagnostic criteria for FEVR composed of three of the following: (1) birth...
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Gene Dosage Analysis by Semiquantitative Multiplex PCR

Gene dosage of TSPAN12 was assessed by semiquantitative multiplex PCR to detect deletions or duplications. Eight exons of the TSPAN12 gene were amplified with the HBB and B2M genes as endogenous references. Primers were designed for all coding exons, and all forward primers were labeled with 6-fluorescein amidite (6-FAM) (Table 1). Exons 2, 5, and 8 were amplified in the first tube and exons 1, 3, 4, 6, and 7 in the second tube. After 18 cycles of PCR, the products were amplified in the first tube and exons 1, 3, 4, 6, and 7 in the second tube. After 18 cycles of PCR, the products were analyzed using an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). For each patient sample, PCR amplification was done in triplicate. The peak height ratio of the gene with that of the reference genes, HBB and B2M. Afterward, normalized gene dosage was determined using the following equation:

\[
\text{Gene dosage} = \frac{\text{Peak height of the target(patient)}}{\text{Peak height of the reference gene(patient)}} \div \frac{\text{Peak height of the target(control)}}{\text{Peak height of the reference gene(control)}}.
\]

FEVR patients with normalized values between 0.8 and 1.2 were determined to have two copies of an exon, while patients with normalized values between 0.3 and 0.7 only possessed one copy of an exon.

Droplet Digital PCR (ddPCR)

To confirm the TSPAN12 large deletions detected in semiquantitative multiplex PCR, ddPCR was performed. Primers and 6-FAM dye-labeled probes for TSPAN12 exons 3 and 4 were designed (Table 1), and copy number variation of TSPAN12 was evaluated using the QX200 Droplet Digital PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Digital PCR master mix solutions (20 µL) were prepared from ddPCR 2X Master mix (Bio-Rad Laboratories, Inc.), and ddPCR reactions also consisted of 0.5 µL 20X primers, TaqMan Probe mix (Applied Biosystems), 7 µL nuclease-free water, and 1 µL DNA template. To normalize the patient data, 20X reference assays for the human eukaryotic initiation factor 2C1 (EIF2C1) gene were used and served as endogenous controls, with the probes labeled at the 5' end with hexachloro-fluorescein. All probes were labeled with Black Hole Quencher-1 at their 3' ends. Each reaction mixture was then loaded into an 8-channel DG8 droplet generator cartridge (Bio-Rad Laboratories, Inc.). After loading 70 µL of droplet generation oil, emulsified droplets were created using the QX-200 Droplet Generator (Bio-Rad Laboratories, Inc.). Forty
microliters of droplet emulsions were then transferred to a 96-well PCR plate using an Eppendorf Xplorer 8-channel electronic pipette (Eppendorf AG, Hamburg, Germany). The loaded 96-well PCR plate was sealed with foil sheets in the PX1 PCR Plate Sealer (Bio-Rad Laboratories, Inc.). Polymerase chain reaction cycling was performed in a DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad Laboratories, Inc.) using the following conditions: denaturation at 95°C for 10 minutes, and 50 cycles of 94°C for 30 seconds, and 54°C for 1 minute (exon 3), and 50°C for 1 minute (exon 4), with the ramp rate set to 2°C/s. After the PCR reactions were complete, fluorescence of each thermally cycled droplet was measured using the QX200 Droplet Reader (Bio-Rad Laboratories, Inc.). The data were visualized and analyzed using the QuantaSoft software v1.7.4 (Bio-Rad Laboratories, Inc.). Thresholds for positive reactions were manually determined based on results from the no template control (NTC) wells.

**Clinical Manifestation According to Genetic Mutations/Anomalies**

Clinical characteristics used to determine FEVR staging, mainly angiographic findings and visual acuity at last follow-up visit, were analyzed.

**Statistical Analysis**

For the statistical analysis, visual acuity was converted from Snellen value to logMAR value. Statistical analyses were performed using SPSS software version 21.0 (SPSS, Inc., Chicago, IL, USA). P values less than 0.05 were considered to be statistically significant.

**RESULTS**

**Semiquantitative Multiplex PCR Detected Large TSPAN12 Deletions**

Semiquantitative multiplex PCR for gene dosage analyses of TSPAN12 were performed on 33 patients, and among them, three patients were detected to carry large deletions of TSPAN12. Two patients (Patients #1 and #2) were heterozygous for the TSPAN12 deletion, and another patient was found to have a deletion in exon 4 (Patient #3) (Fig. 1). Additionally, a symptomatic brother of Patient #2 (Patient #2s) was also tested and found to be positive for this deletion.

**ddPCR Validated TSPAN12 Deletions in FEVR Patients**

To validate the large deletions detected in semiquantitative multiplex PCR, we designed primer and probe sets for TSPAN12 exons 3 and 4 and performed a ddPCR assay. In the three patients with whole-gene deletions, Patients #1, #2, and #2s, exons 3 and 4 positive droplets were approximately half the number of the reference gene positive droplets (Fig. 2), demonstrating that these patients possessed heterozygous deletions as per measured TSPAN12 copy numbers (Fig. 3). For Patient #3, only exon 4 positive droplet counts were approximately half of the reference gene positive droplets.

**Clinical Manifestations of TSPAN12 Deletions in FEVR Patients**

Patient #1 was born with a cleft lip and low set, dysmorphic ears. He showed developmental delays with FEVR-features associated in both eyes (Fig. 4). He also suffered from systolic congestive heart failure. Karyotype analysis showed a deletion of chromosome 7, del(7)(q31.3q33). Considering that the location of TSPAN12 is 7q31.31, we suspected that whole-gene deletion was the pathogenic cause for FEVR in this patient. The patient died at 20 months of age. Patient #2, who also had a whole-gene deletion, had a brother, Patient #2s, who shared the same FEVR diagnosis. Patient #2 was originally found to carry a rare TSPAN12 variant, c.484G>A, which seemed to be homozygous via sequencing analysis. This variant was not detected in her brother, which implied that it was not the cause of their shared symptoms and highlighted the possibility of a large deletion in this exon. Gene dosage analysis confirmed such a deletion, demonstrating that the c.484G>A variant detected was actually in a hemizygous state for Patient #2. Both siblings did not show any other systemic symptoms. Lastly, Patient #3, with an exon 4 deletion, presented with esotropia of his left eye when he was 3-months old. Fundoscopy revealed dragging of the optic disc with macular ectopia in the left eye, confirming that the esotropia was secondary to retinopathy.

Upon comparing a patient with a TSPAN12 point mutation (Patient #4) and our patients with large TSPAN12 deletions, patients with the large deletions displayed relatively milder phenotypes (Table 2). However, it is important to note that data were limited as only one patient carrying a point mutation was available in this study.

**Genotypes and Phenotypes of FEVR**

Patients #1, #2, and #3, along with Patient #2s, were added to the total pool of genetically confirmed FEVR patients described in our previous study to assess the scope of FEVR phenotypes. Visual acuity and FEVR stage at last follow-up were analyzed to see whether there were significant differences according to the genes involved (FZD4, LRP5, or...
Indeed, there were significant differences for either FEVR stage \( (P = 0.021, \text{1-way ANOVA}) \) or visual acuity \( (P = 0.019, \text{1-way ANOVA}) \) at last follow-up according to the particular gene involved. However, post-hoc analyses using the Tukey test showed that, respectively, FEVR stage was significantly lower \( (P = 0.015) \) and visual acuity significantly higher in patients with the \( \text{FZD4} \) mutation than in patients with the \( \text{LRP5} \) mutation \( (P = 0.004) \). There was no significant difference in FEVR stage or visual acuity between \( \text{FZD4} \)- and \( \text{TSPAN12} \)-mutation patient groups nor between \( \text{LRP5} \)- and \( \text{TSPAN12} \)-mutation patient groups.

**DISCUSSION**

To our knowledge, there has not been a report on FEVR patients with large deletions of \( \text{TSPAN12} \). Indeed, there were significant differences for either FEVR stage \( (P = 0.021, \text{1-way ANOVA}) \) or visual acuity \( (P = 0.019, \text{1-way ANOVA}) \) at last follow-up according to the particular gene involved. However, post-hoc analyses using the Tukey test showed that, respectively, FEVR stage was significantly lower \( (P = 0.015) \) and visual acuity significantly higher in patients with the \( \text{FZD4} \) mutation than in patients with the \( \text{LRP5} \) mutation \( (P = 0.004) \). There was no significant difference in FEVR stage or visual acuity between \( \text{FZD4} \)- and \( \text{TSPAN12} \)-mutation patient groups nor between \( \text{LRP5} \)- and \( \text{TSPAN12} \)-mutation patient groups.

Regarding the proportion of each gene’s contribution to FEVR in the Korean population, \( \text{TSPAN12} \) mutations still accounted for the largest proportion \( (13/51 \text{ patients, } 25.5\%) \) in this cohort, with the next largest being \( \text{LRP5} \) \( (4/51 \text{ patients, } 7.8\%) \), followed by \( \text{FZD4} \) \( (4/51 \text{ patients, } 7.8\%) \) and \( \text{FOXP2} \) \( (1/51 \text{ patients, } 2\%) \). In our cohort, the number of FEVR patients with \( \text{TSPAN12} \) deletions was the same as those patients with \( \text{LRP5} \) mutations. This was probably due to less \( \text{LRP5} \) variants detected than expected.

In another study, which considered the disease-associated variants of FEVR-related genes in a single cohort, \( \text{LRP5} \) was the most frequently mutated gene \( (19.6\% \text{ of patients}) \) in this cohort, followed by hyperplastic primary vitreous (PHPV), which phenotypically overlaps with FEVR. However, this patient was determined to not have FEVR. He had dysmorphic facial features such as low set ears and developmental delays, which lead to an autism spectrum disorder (ASD) diagnosis. Haploinsufficiency of \( \text{CADPS2} \) and \( \text{TSPAN12} \) genes in the 7q51.31 region has been shown to contribute to ASD and PHPV, respectively. The patient in our study with the 7q31.3-q33 deletion shared similar phenotypes with this previously reported case, such as dysmorphic facial features and developmental delays. However, ASD-related features were not significant in our patient because he suffered from more complicated symptoms such as heart failure and did not survive long enough to fully dissect the scope of his symptoms.
FZD4 (15.2%), TSPAN12 (8.7%), NDP (6.5%), and ZNF408 (1.1%). Though this study was different from our study in that minor portion of the patients with NDP mutation were included and large deletion/duplication of the related genes were not analyzed, the proportion of TSPAN12 variants being around 8% was almost the same as our cohort. However, the proportions of FZD4 and LRP5 variants were respectively higher and lower in our cohort than in the cohort of the abovementioned study. Interestingly, in our study, the proportion of large deletions in TSPAN12 was higher than that of point mutations, which had not been observed in other FEVR-related genes. Among FZD4-mutation positive patients reported by our group, only one patient was confirmed for a whole-gene deletion while 12 had single nucleotide variants or small deletions. Even in cases with positive NDP mutations tested by our group, large deletion cases were less frequently detected than point mutations (data not shown). It would be interesting to find out whether this high contribution of large deletions in TSPAN12 is also observed in other populations as well.

Additionally, the phenotypes of the patients with large deletions were no more severe than those of a patient with a point mutation, although large deletions are generally considered more harmful than point mutations. Though not statistically significant, the patient with a TSPAN12 point mutation (Patient #4) showed more severe symptoms in both eyes as compared with our patients with large TSPAN12 deletions in this study. Upon reviewing other previously reported cases, there were patients with TSPAN12 missense mutations who possessed high stage FEVR with retinal detachments. Nevertheless, it is hard to conclude whether these TSPAN12 missense mutations showed dominant negative effects due to limited description of the fundus examination results. The difference in phenotypes according to the mutation type should be further considered in more large-scale studies.

To detect these large deletions/duplications of TSPAN12, we performed a semiquantitative multiplex PCR along with a ddPCR assay, which is known for its improved sensitivity and resolution. Traditional methods for large deletion detection, such as real-time quantitative PCR (qPCR) or MLPA, have several limitations. Quantitative PCR is known for its imprecise
quantitation and needs of standardized materials, while designing and optimizing a new panel for MLPA can be labor intensive and time consuming. The use and application of ddPCR in large deletion detection and copy number variation analyses have been reported in several studies.\textsuperscript{11–13} The use of ddPCR in our study efficiently enhanced our resolution of \textit{TSPAN12} dynamics in FEVR patients, and the use of such an assay may be a viable choice in better guiding FEVR diagnosis and estimating prevalence.

In conclusion, we detected \textit{TSPAN12} large deletions more frequently than \textit{TSPAN12} point mutations among our cohort of FEVR patients. Large deletion/duplication analyses of \textit{TSPAN12} should be taken into consideration and evaluated in routine genetic workups for FEVR patients as gene dosage abnormal-

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Color fundus photographs of patients with large \textit{TSPAN12} deletion. (A, B) A 4-month-old boy (patient \#1 in table 2) with whole-gene deletion of \textit{TSPAN12}. Peripheral avascular area with incomplete vascular development in the right eye (A) and the left eye (B). (C, D) A 4-month-old girl (patient \#2 in Table 2) with whole-gene deletion of \textit{TSPAN12}. (C) Peripheral avascular area with incomplete vascular development in the right eye (D). Fibrovascular stalk to inferotemporal direction is concomitant with macular ectopia in the left eye. (E, F) A 20-month-old boy (patient \#3 in Table 2) with exon 4 deletion in \textit{TSPAN12} gene. (E) Extraretinal fibrovascular membrane is present along with temporal avascular border in the right eye. (F) Fibrovascular stalk to temporal direction is concomitant with extramacular retinal detachment in peripheral retina of the left eye.}
\end{figure}

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
ID & Sex & Age & Stage\textsuperscript{14} (OD/OS) & Retinal Folds or Macular Draggging (OD/OS) & Refractive Error (Spherical Equivalent) (OD/OS) & Visual Acuity (OD/OS) & Genetic Abnormality \\
\hline
\#1 & M & 4 m & 1A/2B & --/-- & NA/NA & NA/NA & Whole-gene deletion (del(7)(q31.3q33)) \\
\#2 & F & 4 m & 1A/3B & --/+ & 0.00/−3.50 & 0.50/0.01 & Whole-gene deletion \\
\#2s & M & 2 m & 1A/1A & --/-- & −0.50/−0.50 & F&F good/good & Whole-gene deletion \\
\#3 & M & 20 m & 1A/3B & --/+ & 0.25/0.75 & 0.30/0.02 & Exon 4 deletion \\
\#4* & M & 4 m & 3B/3B & ++/+ & −6.50/−5.50 & 0.06/0.06 & c.56T>G (p.Leu19Arg) \\
\hline
\end{tabular}
\caption{Clinical Characteristics of Patients With \textit{TSPAN12} Pathogenic Variants or Large Deletions}
\end{table}

M, male; F, female; NA, not assessed; F&F, fix and follow.
* The patient with \textit{TSPAN12} point mutation from the previous study is included in this table and labeled as Patient \#4.
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ities and copy number variation of FEVR-related genes may be more prevalent than originally suspected.

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