Large Deletions of *TSPAN12* Cause Familial Exudative Vitreoretinopathy (FEVR)

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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 (MRC-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

at full term or premature birth with no evidence of retinopathy of prematurity, (2) a presence of peripheral retinal avascular area, (3) variable degree of nonperfusion, vascular leakage, or retinal neovascularization in fluorescein angiography.

These patients were previously screened and determined to be negative for *NDP*, *FZD4*, *LRP5*, and *TSPAN12* mutations. Additionally, these patients lacked large deletions and duplications of *NDP*, *FZD4*, and *LRP5*. Whole blood was collected from patients in EDTA blood collection tubes and the DNA was extracted using Gentra PureGene blood kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Experiments were performed according to the Declaration of Helsinki.

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 analyzed using an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) with the GeneMapper ID 3.7 software (Applied Biosystems). For each patient sample, PCR amplification was done in triplicate. The peak height ratio of each exon was calculated by dividing the mean peak heights of the *TSPAN12* gene with that of the reference genes, *HBB* and *B2M*. Afterward, normalized gene dosage was determined using the following equation:

$$\text{Gene dosage} = \frac{\text{Peak height of the target(patient)}}{\text{Peak height of the reference gene(patient)}} \div \frac{\text{Peak height of the target(control)}}{\text{Peak height of the reference gene(control)}}. \quad (1)$$

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 5′ 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 20× primers, TaqMan Probe mix (Applied Biosystems), 7 μL nuclease-free water, and 1 μL DNA template. To normalize the patient data, 20× 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 hexachlorofluorescein. 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

Table 1. Primer and Probe Sequences for Semiquantitative Multiplex PCR and ddPCR Assays Targeting *TSPAN12* Exons

Purpose	Targeted Region	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	MGB Probe (5′-3′)	Product Size, bp
Semiquantitative multiplex PCR	Exon 1	AGAGGACTTGGGAGGGACAG	AGACGCGTGGCCACTTAC		286
	Exon 2	GGTGAGATGTCCCGTGTTCT	AATGCTTAGCCATGCCCCTTT		269
	Exon 3	TTTGGTGCCTAGGACATGATT	GCTGAGGGGCAAAGTTACTTAAAA		331
	Exon 4	TGAGGCATCATGATTGAAAGA	TCACTGCTCCCCTAATCTTGTGA		342
	Exon 5	TGCCTCTGTTTTCTTGGTCA	TACCCAGTCTTGGGCAGTTC		294
	Exon 6	TGTGAATCTGTGTCTGTGTGTGA	GAAGAAAAGCAGGCCATGAA		349
	Exon 7	TCTGAGGCTGACTAGTGATTTTT	TTCCTGGTGGGCCTGTTT		150
	Exon 8	AACTGCAGGTGCTGAGGTTT	TTAAAGCTGTTTGCCATGGAT		235
ddPCR	Exon 3	TCCATCAGTGTGTTGGCAGT	CAGCAAGAAAGAATTCAATCAA	TGCTTGGATGAGGGACTACC	116
	Exon 4	TGGTTCATCCGGTCATGATT	CCATGCAAGAAGCAACAGAT	TTGTGGAACGGTGAAAAGAA	101

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,[4] 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

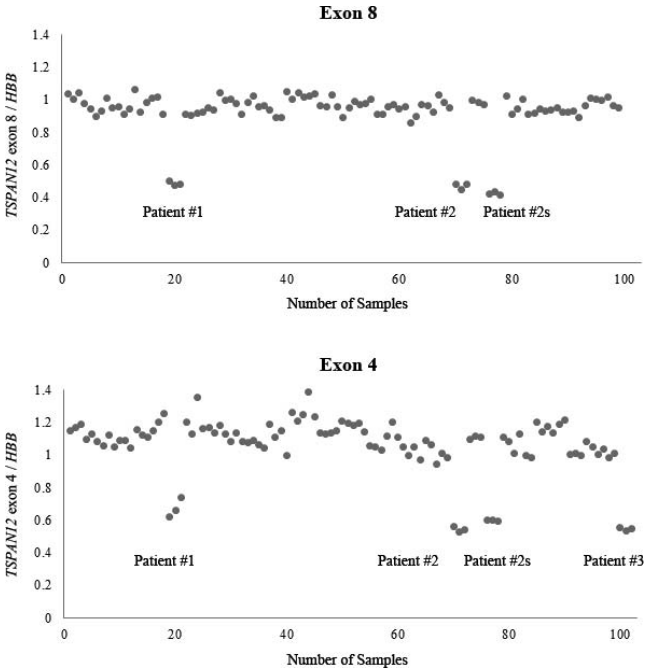

Figure 1. *TSPAN12* deletions in FEVR patients. Semiquantitative multiplex PCR was used to normalize the ratio of *TSPAN12* exons 4 and 8 (targets) to *HBB* genes (endogenous references). *Dots* denote normalized dosages of each patient's samples. All assays were done in triplicate. Patients #1, #2, and #2s possessed half the ratio in both exons. Patient #3 had a single deletion in exon 4.

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[3] 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

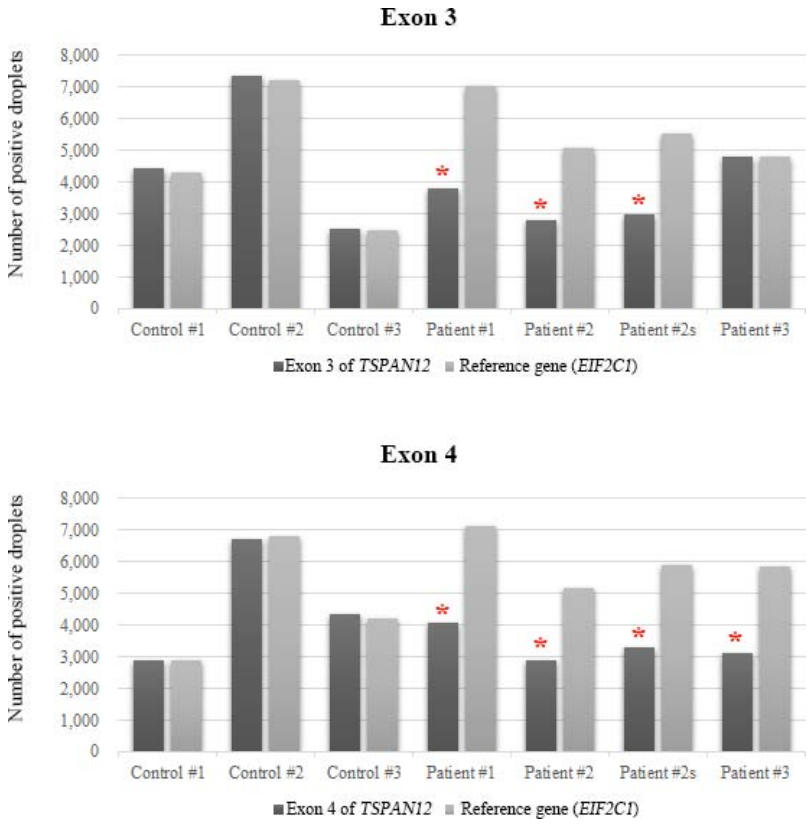

Figure 2. *TSPAN12* ddPCR analysis of FEVR patients. Data is from seven patient samples, including three normal controls and four patients with *TSPAN12* deletions. The y-axis represents the raw number of positive droplets from the target exon and the reference gene. *Asterisks* denote heterozygous deletion of the target, *TSPAN12*, as compared with the *EIF2C1* reference gene.

TSPAN12). Indeed, there were significant differences for either FEVR stage ($P = 0.021$, 1-way ANOVA) or visual acuity ($P = 0.019$, 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 *FZD4* mutation than in patients with the *LRP5* mutation ($P = 0.004$). There was no significant difference in FEVR stage or visual acuity between *FZD4*- and *TSPAN12*-mutation patient groups nor between *LRP5*- and *TSPAN12*-mutation patient groups.

Discussion

To our knowledge, there has not been a report on FEVR patients with large deletions of *TSPAN12*. There are several published reports highlighting patients with chromosome 7 deletions in the 7q31 region, but this is the first study to report FEVR patients genotypically confirmed for such pathogenic large deletions. In a previous report of a patient with a 7q31.2-q32.2[5] deletion, the patient had speech and language disorders due to the deletion of the *FOXP2* gene, which maps to 7q31.1 and is implicated in speech and language impairment. Because *TSPAN12* also resides in this deletion range, we expected the patient to have FEVR-related features. However, this study contained a limited description of "mottling of retinal pigmentation" for this patient. To note, a mild FEVR phenotype may have gone undetected because the patient had no visual impairment yet mottled retinal pigmentation. Another report of a patient confirmed with a 5.4-Mb deletion in the 7q31.31 region had congenital nystagmus due to bilateral persistent hyperplastic primary vitreous (PHPV), which phenotypically overlaps with FEVR.[6] However, this patient was determined to not have FEVR. He had dysmorphic facial features such as low set ears and developmental delays, which led to an autism spectrum disorder (ASD) diagnosis. Haploinsufficiency of *CADPS2* and *TSPAN12* genes in the 7q31.31 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,[6] 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.

Regarding the proportion of each gene's contribution to FEVR in the Korean population,[3] pathogenic genetic anomalies were detected in 21 patients among our original 51 patient cohort, with three more patients added to this current study due to detecting large *TSPAN12* deletions. This resulted in 41.2% of patients being genetically confirmed for FEVR. *FZD4* mutations still accounted for the largest proportion (13/51 patients, 25.5%), followed by *LRP5* (4/51 patients, 7.8%), and *TSPAN12* mutations (4/51 patients, 7.8%). In our cohort, the number of FEVR patients with *TSPAN12* mutations was the same as those patients with *LRP5* mutations. This was probably due to less *LRP5* variants detected than expected. In another study, which considered the disease-associated variants of FEVR-related genes in a single cohort,[7] 92 FEVR patients were screened for five genes associated with FEVR using next-generation sequencing (NGS). *LRP5* was the most frequently mutated gene (19.6% of patients) in this cohort, followed by

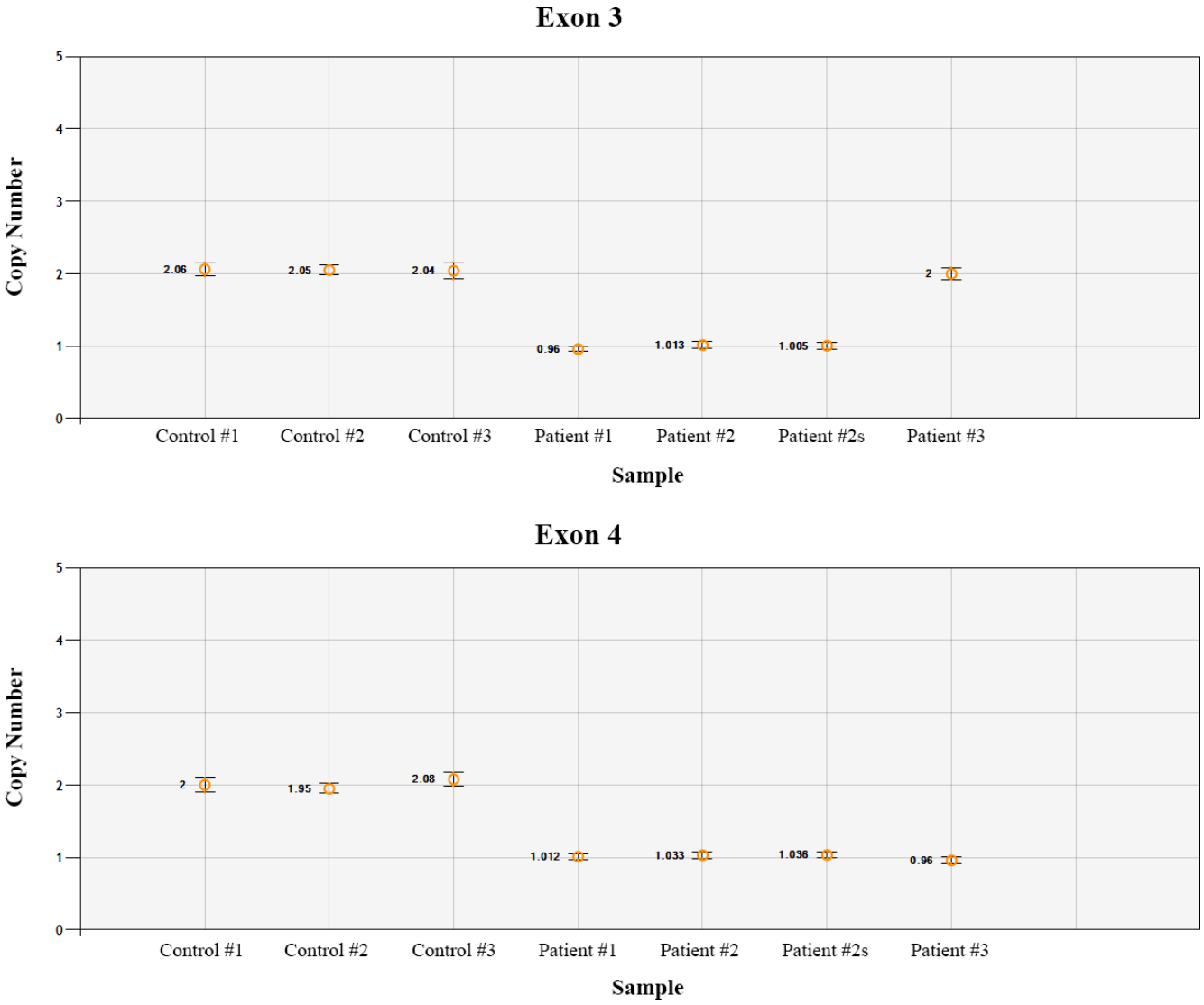

FIGURE 3. Determination of *TSPAN12* large deletions by ddPCR. Deletion of exon 3 was detected in Patients #1, #2, and #2s. Deletion of exon 4 was detected in Patients #1, #2, #2s, and #3.

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,[8–10] 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

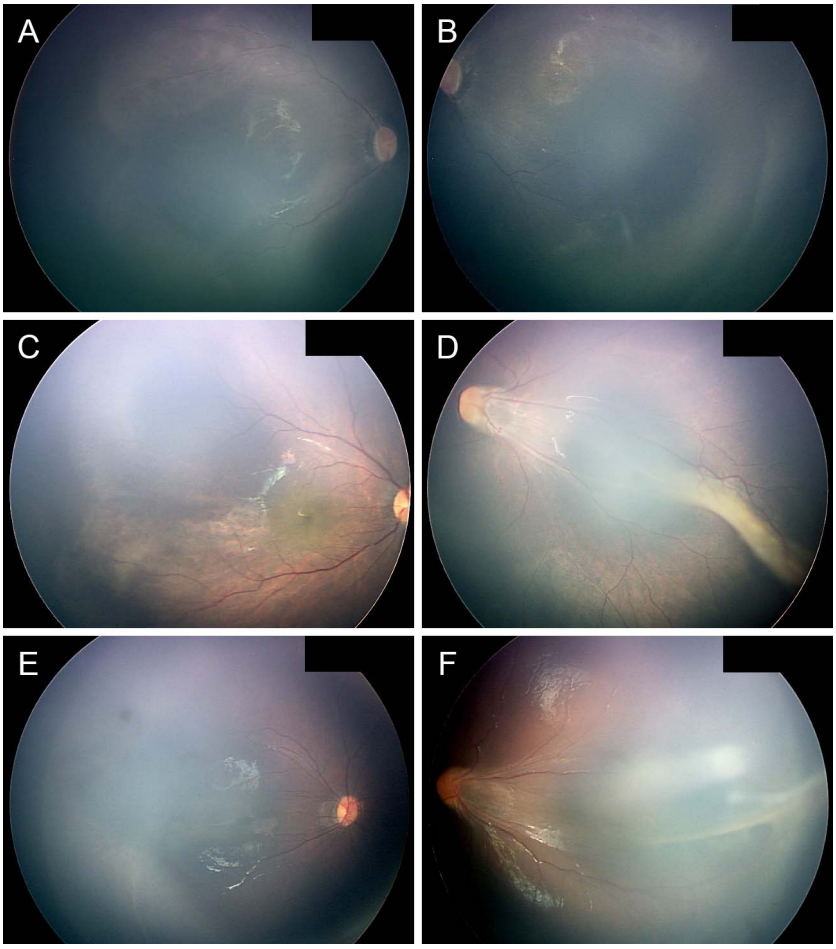

Figure 4. Color fundus photographs of patients with large *TSPAN12* deletion. (**A**, **B**) A 4-month-old boy (patient #1 in table 2) with whole-gene deletion of *TSPAN12*. Peripheral avascular area with incomplete vascular development in the right eye (**A**) and the left eye (**B**). (**C**, **D**) A 4-month-old girl (patient #2 in Table 2) with whole-gene deletion of *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 *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.

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.[11–13] The use of ddPCR in our study efficiently enhanced our resolution of *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 *TSPAN12* large deletions more frequently than *TSPAN12* point mutations among our cohort of FEVR patients. Large deletion/duplication analyses of *TSPAN12* should be taken into consideration and evaluated in routine genetic workups for FEVR patients as gene dosage abnormal-

Table 2. Clinical Characteristics of Patients With *TSPAN12* Pathogenic Variants or Large Deletions

		At Diagnosis			At Last Follow-Up		
ID	Sex	Age	Stage[14] (OD/OS)	Retinal Folds or Macular Dragging (OD/OS)	Refractive Error (Spherical Equivalent) (OD/OS)	Visual Acuity (OD/OS)	Genetic Abnormality
#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)

M, male; F, female; NA, not assessed; F&F, fix and follow.
* The patient with *TSPAN12* point mutation from the previous study is included in this table and labeled as Patient #4.

ities and copy number variation of FEVR-related genes may be more prevalent than originally suspected.

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