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Five novel copy number variations detected in patients with familial exudative vitreoretinopathy

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Purpose: Familial exudative vitreoretinopathy (FEVR) is an inherited retinal vascular disease genetically heterogeneous with multiple causative genes. The aim of this study is to report five novel copy number variation (CNV) regions in FEVR patients and to investigate the possible contributions of novel CNVs to FEVR.
Methods: In this study, 824 FEVR families were collected. All cases were performed using the targeted next generation sequencing (NGS) assay, and families with no definite pathogenic mutations in FEVR genes were screened for CNVs according to the NGS results. Droplet digital polymerase chain reaction (ddPCR) testing was introduced to validate the screened CNV regions. We also reviewed the clinical presentations of the probands and affected family members associated with the novel CNVs and conducted segregation analysis.
Results: Five CNVs in five patients were detected in this study: heterozygous deletions of kinesin family member 11 (*KIF11*) exons 2–4, KIF11 exon 11, KIF11 exons 1–10, tetraspanin-12 (*TSPAN12*) exons 1–3, and low-density lipoprotein receptor-related protein 5 (*LRP5*) exons 19–21. Among the five affected families, *TSPAN12* exons 1–3 heterozygous deletion and *LRP5* exons 19–21 heterozygous deletion originate from the mother and the father of the proband, respectively. No other family members manifested as FEVR except for the probands. The correlation between disease severity and CNV loci seems uncertain.
Conclusions: Five novel CNV loci in FEVR patients were uncovered in this study, including one maternally-inherited and one paternally-inherited CNV region. Though there is no evidence of co-segregation between these CNVs and FEVR, our findings suggest novel genetic risk factors for FEVR.

Familial exudative vitreoretinopathy (FEVR) is an inherited vitreoretinal disorder first described in 1969 [1]. As a retinal vascular disease, FEVR is characterized by avascular zones or incomplete vascularization of the peripheral retina, and the clinical presentations of FEVR vary in different people from mild peripheral avascularity to severe retinal detachment (RD) [2-4]. Generally, FEVR is associated with mutations in genes involved in the Wnt/Norrin signaling pathway, including genes encoding the low-density lipoprotein receptor-related protein 5 (*LRP5*), Norrie disease protein (*NDP*), tetraspanin-12 (*TSPAN12*), and the receptor frizzled-4 (*FZD4*). Mutations in zinc finger protein 408 (*ZNF408*) and kinesin family member 11 (*KIF11*) were also identified as genetic causes for FEVR via other mechanisms [5-9]. The mutations mentioned above can only explain the causation in approximately 40% to 50% of FEVR patients, and the remaining genetic causes are still unknown [10,11].

Copy number variation (CNV), a form of chromosome submicroscopic structural variation, is defined as the deletion or duplication of a DNA segment whose size is more than 1 kb [12,13]. Studies have suggested that CNVs contribute to many human disorders [14-16] through several mechanisms: altering gene dosage, changing the 3D architecture of the genome, forming chimeric genes, and so on [17-19]. Many novel CNVs have recently been found in ophthalmic diseases, including retinitis pigmentosa [20], inherited retinal degeneration [21,22], and esotropia [23]. Previous genetic studies on FEVR focused on mutations in nucleic acids known as single nucleotide variants (SNV), while the possible role of CNV in FEVR has rarely been investigated [24-26]. Here, we identified five novel CNV regions in FEVR patients, which may contribute to the diagnosis of FEVR.

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METHODS

Subjects: This study was approved by the Institutional Research Committee of Xinhua Hospital, affiliated to Shanghai Jiao Tong University School of Medicine, and conducted under the light of the Declaration of Helsinki. We collected 824 patients who were clinically diagnosed with FEVR in the ophthalmology department of Xinhua Hospital between April 2015 and July 2019. Patients with a history of premature birth and oxygen inhalation were excluded. Written

informed consent was obtained from the parents or guardians of the patients before the experiments were conducted.

Diagnosis: The FEVR diagnosis was made based on medical recordings and ophthalmic examinations, including slit-lamp biomicroscopy, B-scan ultrasound, RetCam III (Clarity Medical Systems, Pleasanton, CA), Optos 200Tx (Optos Inc., Marlborough, MA), and fundus fluorescein angiography (FFA). According to the examinations, patients presenting with at least one of the following typical clinical features were diagnosed as having FEVR: RD, retinal folds, vitreous hemorrhage, retinal neovascularization, peripheral avascular zones, severe subretinal exudates, or vitreoretinal dragging with macular ectopia [27]. All probands and their family members were examined and staged for FEVR, as described previously (Appendix 1) [28,29].

CNV screening and validation: Blood samples of probands and their family members were collected and used to extract genomic DNA using Gentra PureGene blood kits (Qiagen, Valencia, CA). The retinal disease panel used in this study involved 463 targeted genes (Appendix 2), and all enrolled cases underwent panel-based targeted next generation sequencing (NGS) assay. The standard Illumina libraries were prepared using a DNA Sample Prep Reagent Set (MyGenostics Inc., Beijing, China), and a GenCap capture kit (MyGenostics Inc., Beijing, China) was used to capture the regions containing the 463 targeted genes. Extracted DNA was quantified using Qubit 2.0 (Thermo Fisher Scientific, Waltham, MA) and sheared into DNA fragments via Diangenode Bioruptor® Plus. Captured fragments were removed from the solution using streptavidin-coated magnetic beads (Dynabeads® MyOne™ Streptavidin T1, Thermo Fisher Scientific) and subsequently eluted. DNA fragments were enriched and sequenced on an Illumina HiSeq X ten sequencer for paired-reading of 150 bp. After interpreting the results of the Illumina HiSeq X ten sequencer into reads, a Burrows-Wheeler Aligner (BWA; ver. 0.7.11) was used for alignment between the clean reads and the human reference genome (hg19) [30]. Raw variants were analyzed via the genome analysis toolkit (GATK) Haplotype Caller and annotated with ANNOVAR software (http://annovar.openbioinformatics.org/en/latest/) according to the following databases: 1000 Genome Project, Exome Sequencing Project, Exome Sequencing Project, and the Human Gene Mutation Database (HGMD) [31]. Patients with no definite pathogenic mutations in FEVR genes were screened for potential CNVs by CapCNV analysis through CNVkit software, and the pathogenicity of the CNVs was classified into five categories, “pathogenic,” “likely pathogenic,” “uncertain significance,” “likely benign,” and “benign,” according to the American College of Medical Genetics and Genomics (ACMG) guidelines [32,33].

Then, ddPCR was performed to validate the CNV regions in the probands according to a previous study [34]. The genomic DNA was digested using an enzymatic digestion mixture at 37 °C for 1 h, followed by inactivation at 65 °C for 15 min. After DNA digestion, template DNA at a concentration of 40 ng/μL was used for ddPCR analysis performed on a QX100 system (BioRad Laboratories, Inc., Shanghai, China). PCR amplifications were conducted three times for each sample, with an optimized PCR thermal profile. Primers were designed by Primer Premier 5.0, and the sequences of primers are displayed below (Appendix 3). QuantaSoft v.1.2.10.0 software (BioRad Laboratories, Inc., Shanghai, China) was used to analyze the results.

Statistical analysis: The clinical characteristics and ophthalmological findings of the probands and affected family members were recorded for genotype–phenotype correlation analysis. Descriptive statistics were presented as median and range, statistical data were analyzed by the statistical analysis system (version 9.4), and a p value less than 0.05 was accepted as statistically significant.

RESULTS

In this study, 824 unrelated FEVR families were enrolled, and 406 families without definite pathogenic mutations in FEVR genes were screened for CNVs. Five novel CNV loci were confirmed in five probands, one female and four males; the median age at diagnosis was 5 years (range: 4 months to 7 years of age). The demographic and clinical data are summarized in Table 1. The age at diagnosis was significantly different between inherited CNV probands and de novo CNV probands ($p = 0.0056 < 0.05$).

Five novel CNVs were detected: Among the 406 probands with no definite pathogenic mutations in FEVR genes, eight different CNV regions were screened in eight unrelated patients by NGS testing, including one duplication and seven heterozygous deletions. *KIF11* accounted for three CNV loci (3/8, 37.5%), followed by *TSPAN12* (2/8, 25%), *LRP5* (1/8, 12.5%), *FZD4* (1/8, 12.5%) and *COL11A1* (1/8, 12.5%). Among them, heterozygous deletion of exon 1 of *FZD4* and the whole gene of *TSPAN12* have been reported previously [25,26]. There were two CNV regions involved in the whole gene, one is *TSPAN12* heterozygous deletion, and the other is the duplication of *COL11A1*. Detailed information about the testing results is listed in Table 2. Notably, mutations were detected in two patients. Two heterozygous mutations were detected in patient No. 7, which are located at chr10–58232699, involving *KIF11*, and chr17–58232699, involving *CA4*, both resulting in

Table 1. The demographic data and clinical manifestations.

Patient NO. / Gender	Age at diagnosis	CNV	Ocular Manifestations	Stage
1/M	6 year	*KIF11*; exon2–4 Het Del	OD: dragged-disc OS: dragged-disc	OD: 3A OS: 3A
2/F	7 year	*KIF11*; exon1–10 Het Del	OD: retinal fold OS: dragged-disc, RPE atrophy	OD: 4B OS: 3A
3/M	5 year	*KIF11*; exon11 Het Del	OD: posterior synechia of the iris, retinal fold OS: shallow AC, pupil occlusion, total RD	OD: 4B OS: 5B
4/M	9 months	*TSPAN12*; exon1–3 Het Del	OD: retinal fold OS: dragged-disc	OD: 3A OS: 4A
5/M	4 months	*LRP5*; exon19–21 Het Del	OD: corneal opacity, shallow AC, total RD OS: peripheral retinal nonperfusion, neovascularization	OD: 5B OS: 1B

M=Male; F=Female; CNV=Copy number variation; Het Del=Heterozygous deletion; OD=right eye; OS=left eye; AC=anterior chamber; RD=retinal detachment; RPE=retinal pigment epithelium

DNA and protein changes. Both were predicted as mutations of uncertain pathogenicity, according to the ACMG. Patient No. 3 had a hemizygous mutation located at ChrX-43808330, containing the *NDP* gene, leading to a DNA mutation whose pathogenicity is uncertain based on the ACMG.

A ddPCR assay was performed in 24 cases from the eight families described above. Following the ddPCR assay, five of the eight CNV regions harboring FEVR-associated genes were validated in seven cases, five probands and two of their family members: *KIF11* exons 2–4 heterozygous deletion, *TSPAN12* exons 1–3 heterozygous deletion, *LRP5* exons 19–21 heterozygous deletion, *KIF11* exon 11 heterozygous deletion, and *KIF11* exons 1–10 heterozygous deletion. The results of the ddPCR are shown in Figure 1. According to validations of CNVs in family members, *TSPAN12* exons 1–3 heterozygous deletion was confirmed to be maternally inherited, and *LRP5* exons 19–21 heterozygous deletion was paternally inherited, while three CNVs in *KIF11* were de novo. The results are shown in Figure 2.

Phenotypes of FEVR patients and affected family members: The clinical manifestations of the five probands are summarized in Table 1, and fundus photography is shown in Figure 3. *KIF11* exons 2–4 heterozygous deletion was detected in a 6-year-old boy (patient No. 1). The fundus showed vascular and macular dragging from the optic disc to the temporal retina in bilateral eyes, and apparent macular ectopia was found in the left eye (Figure 3A). A 7-year-old girl (patient No. 2) was identified as carrying a *KIF11* exons 1–10 heterozygous deletion; fundus photography revealed a retinal fold extending to the temporal retina, with little exudates around the fold and mild vascular abnormalities in the right eye. Her left eye showed mild dragging from the optic disc to the temporal retina and was accompanied by retinal pigment epithelium atrophy (Figure 3B). One novel CNV of the *KIF11* exon 11 heterozygous deletion was found in a 5-year-old boy (patient No. 3). He presented with posterior synechia of the iris and a falciform retinal fold in the right eye. In his left eye, the fundus was invisible with a flat anterior chamber and pupil occlusion; B-scan ultrasound indicated total retinal detachment (Figure 3C). The parents of these three probands underwent FFA examination, and none of them showed FEVR features.

Patient No. 4, a 9-month-old boy with a heterozygous deletion of *TSPAN12* exons 1–3, presented with vitreoretinal traction in both eyes, which resulted in a radial retinal fold in the right eye and a dragged disc with ectopic macular in the left eye (Figure 3D). His mother was confirmed to carry the same CNV but with no clinical phenotypes of FEVR, according to fundus examinations. A heterozygous CNV of *LRP5* exons 19–21 deletion was found in a 4-month-old boy (patient No. 5). Corneal opacity, shallow anterior chamber, and total retinal detachment were present in his right eye, according to RetCam and B-scan ultrasound examinations. Compared to the right eye, his left eye was milder, with peripheral nonperfusion area and neovascularization in the retina (Figure 3E). His father, who carried the same CNV, showed no features of FEVR on fundus examinations.

DISCUSSION

FEVR is a genetic ophthalmic disease with various inheritance modes and phenotypes. Up to now, at least eight genes have been confirmed as FEVR-associated, disease-causing genes: *FZD4*, *TSPAN12*, *NDP*, *LRP5*, *ZNF408*, *KIF11*, *CTNNB1*, and *JAG1* [9,35-42]. Most studies focus on the point mutations in these genes leading to FEVR, while few studies investigate the role of CNV, a pattern of DNA structural

Table 2. NGS assay and ddPCR results.

Patient NO.	NGS							ddPCR			
	Gene	Locus	Type	Inheritance Mode	Novel CNV	SNV	pathogenicity of SNV	Gene	Locus	Type	Inheritance
1	*KIF11*	exon2–4	Het Del	AD	Yes	-	-	*KIF11*	exon2–4	Het Del	De novo
2	*KIF11*	exon1–10	Het Del	AD	Yes	-	-	*KIF11*	exon1–10	Het Del	De novo
3	*KIF11*	exon11	Het Del	AD	Yes	NDP, c.*715T>C (-)	Uncertain	*KIF11*	exon11	Het Del	De novo
4	*TSPAN12*	exon1–3	Het Del	AD	Yes	-	-	*TSPAN12*	exon1–3	Het Del	Maternal
5	*LRP5*	exon19–21	Het Del	AD/AR	Yes	-	-	*LRP5*	exon19–21	Het Del	Paternal
6	*TSPAN12*	whole	complete Het Del	AD	No	-	-	-	-	-	-
7	*COL11A1*	whole	Dup	AD/AR	Yes	KIF11, c.1924C>G (p.P642A) CA4, c.83A>G (p.Q28R)	Uncertain	-	-	-	-
8	*FZD4*	exon1	Het Del	AD	No	-	-	-	-	-	-

NGS=Next Generation Sequencing; ddPCR=Droplet Digital Polymerase Chain Reaction; Het Del=Heterozygous deletion; Dup=Duplication; AD=Autosomal Dominant; AR=Autosomal Recessive; SNV=Single Nucleotide Variant

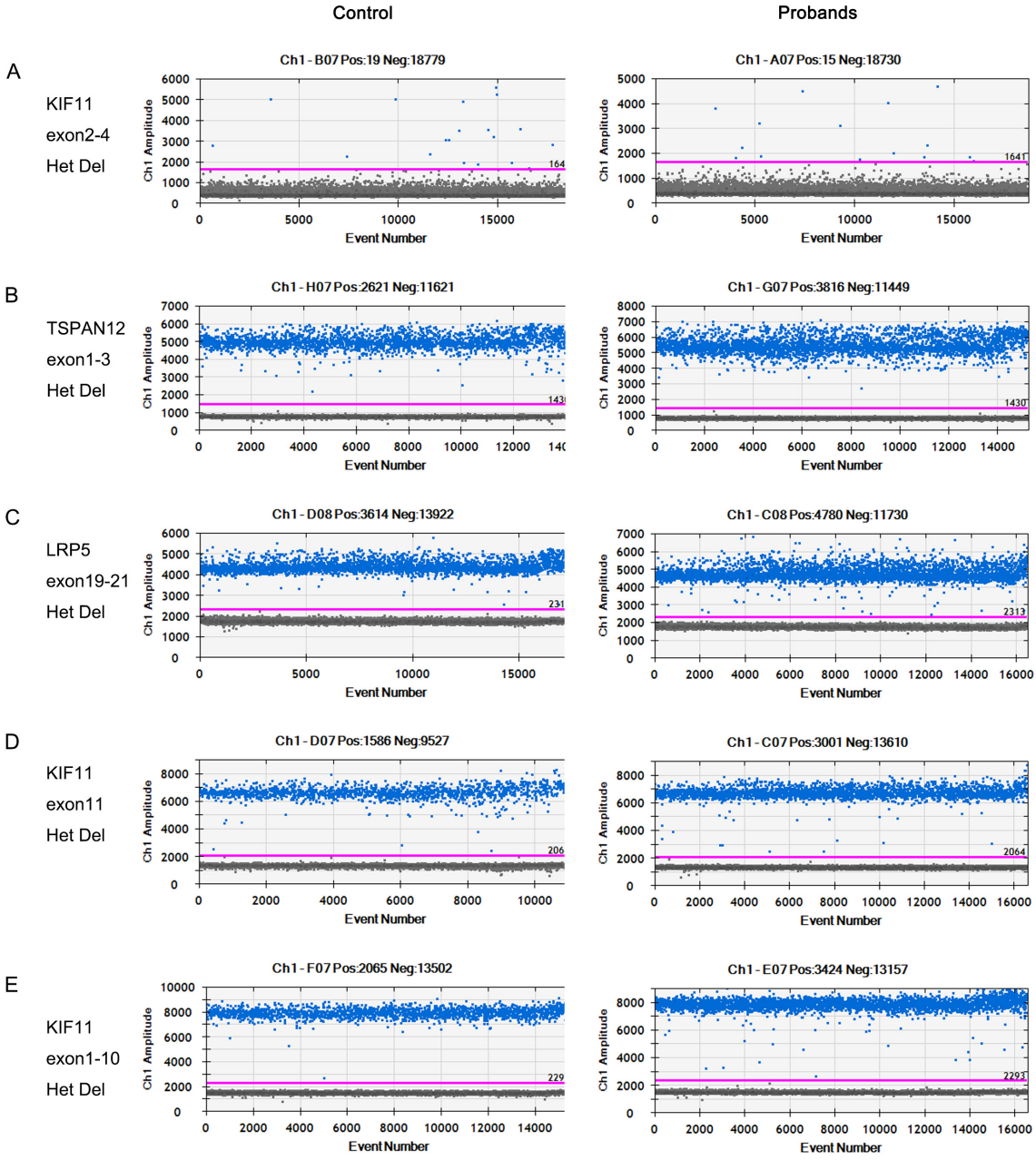

Figure 1. Results of ddPCR assay. One-dimensional scatter plot for healthy control (left) and probands (right) of five CNVs. The pink line is a manually set threshold, and gray points indicate template DNA negative droplets, while blue points represent template DNA positive droplets. A. *KIF11* exons 2–4; B. *TSPAN12* exons 1–3; C. *LRP5* exons 19–21; D. *KIF11* exon 11; E. *KIF11* exons 1–10.

variants, in FEVR [24-26]. CNVs are deletions or duplications of DNA segments that can influence at least five times more variable base pairs than SNVs and lead to a greater impact on disease phenotypes [43].

Herein, we detected five novel CNV regions in five out of 406 FEVR patients without definite pathogenic mutations in FEVR genes. Three of them affect the exons of *KIF11*, including exons 2–4, exon 11, and exons 1–10. Previous studies demonstrated that patients with mutations in *KIF11* present with microcephaly, mental retardation, lymphedema, and retinopathy with lacunar chorioretinal atrophic lesions [44], while other studies indicated that whether FEVR is

associated with the syndrome caused by *KIF11* mutations remains uncertain [45,46]. In our study, no patients harboring CNVs corresponding to *KIF11* manifested as microcephaly, mental retardation, or lymphedema. A novel heterozygous CNV of *LRP5* exons 19–21 deletion was detected in patient No. 5. Mutations in *LRP5* often contribute to orthopedic diseases, such as osteoporosis or high bone mass [47,48]. However, the proband and his father, who harbored CNV in *LRP5,* showed normal bone mineral density and had no orthopedic history. Studies have reported that clinical presentations in patients with large deletions are not more severe than those in a patient with point mutation [26]. Therefore, we suggest that absent or undiagnosed complications of the extraocular system may be partially due to the different patterns of mutations.

Ocular manifestations of the ten eyes studied were diversified from peripheral retinal nonperfusion to total retinal detachment. All eyes were staged for FEVR. Except for one eye that was classified as a stage of 1B, the remaining eyes were all staged from 3A to 5B, indicating a severe fundus condition. Previous studies have demonstrated that the severity of FEVR is associated with the gene loci in patients with SNVs [11,49]. In this study, there was no significant relationship between the severity of FEVR and gene loci in the five probands, which may be due to the different mutation types and the limited population in our study. The contralateral eyes of patient No. 5 were staged for 5B and 1B, respectively, and the asymmetric ocular presentation was described as a characteristic of FEVR previously [50].

According to ddPCR verification results, three CNVs corresponding to *KIF11* are de novo variations, and the

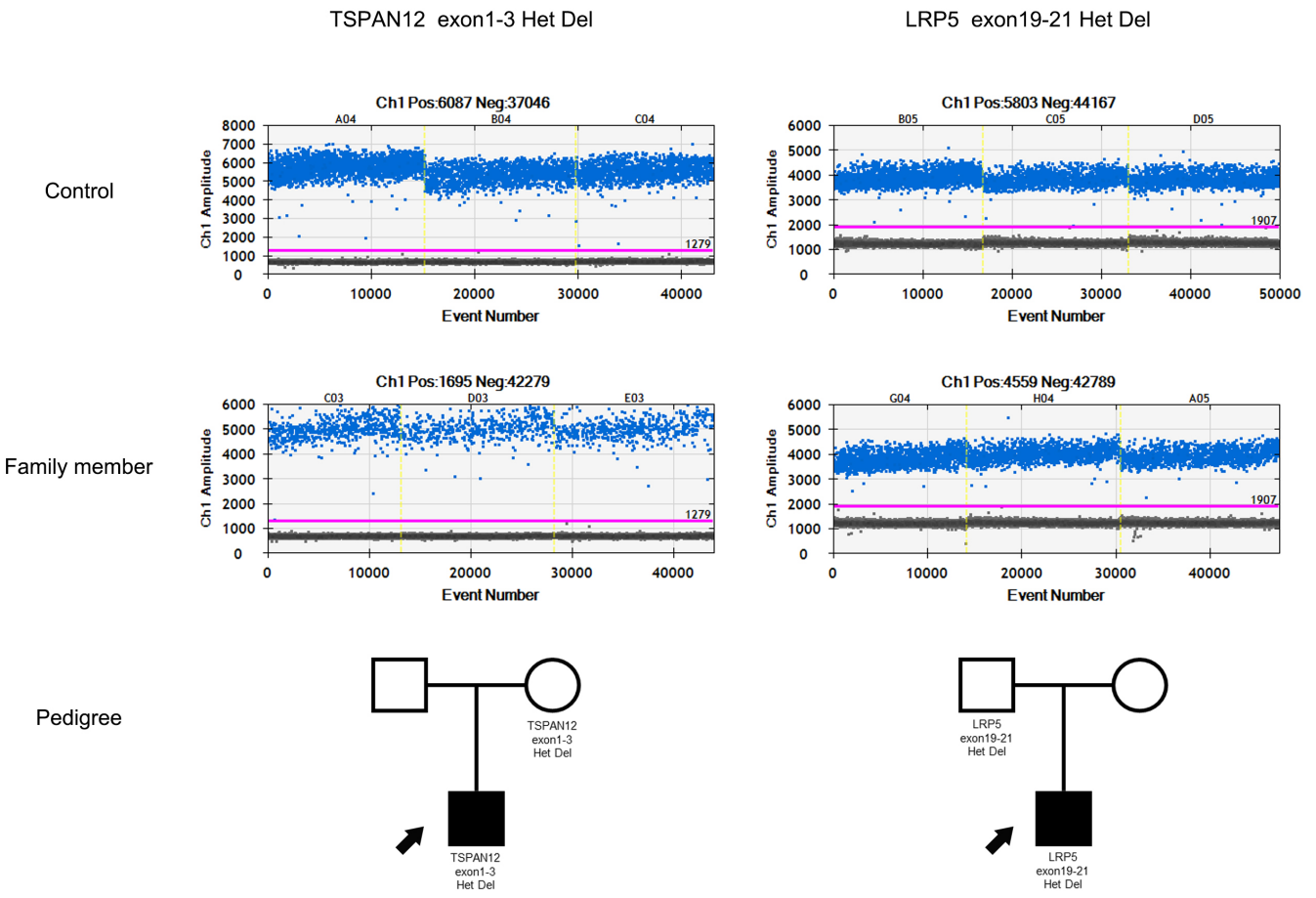

Figure 2. The ddPCR results of affected family members and pedigrees of families with inherited CNVs. Squares represent men, circles indicate women; black and white symbols represent affected and unaffected individuals, respectively; arrows refer to probands. The left column shows the ddPCR results of the mother carrying CNV of *TSPAN12* exons 1–3 heterozygous deletion and the pedigree of the family. The right column reveals the ddPCR result of the father harboring *LRP5* exons 19–21 heterozygous deletion and the pedigree.

heterozygous deletion of *TSPAN12* exons 1–3 detected in patient No. 4 originated from the mother; the heterozygous deletion of *LRP5* exons 19–21 identified in patient No. 5 is inherited from the father. Notably, among the inherited-CNV families, neither the mother harboring a *TSPAN12* exons 1–3 heterozygous deletion nor the father carrying an *LRP5* exons 19–21 heterozygous deletion had typical clinical manifestations of FEVR, according to fundus photography results, which may be consistent with the fact that the severity of FEVR varies among family members [51]. A previous study suggested that, except for the pathogenic variant, additional disease modifiers can influence penetrance [52]. DNA methylation may act as an epigenetic modification that contributes to incomplete penetrance in patients with known mutations [53,54]. Herein, the phenomenon of inconsistency between genotype and phenotype may be explained as incomplete penetrance, which is associated with many other mechanisms, such as DNA methylation. Further studies should be conducted to confirm this detailed mechanism. In addition, the significant difference ($p = 0.0056 < 0.05$) between the diagnostic age of the probands with inherited CNVs and that of the probands with de novo CNVs implied that the inherited CNVs may predispose patients to earlier occurrences of FEVR.

In our study, patient No. 3, who harbored a de novo CNV of *KIF11* exon 11 deletion, was found to have a hemizygous mutation corresponding to the *NDP* gene, which the ACMG predicted to have unknown pathogenicity. Whether the CNV or the SNV is the causal variation of FEVR is inconclusive. This mutation was detected in patient No. 3's mother, who showed no FEVR clinical features, contradicting the hypothesis that SNV causes the FEVR phenotype. A study demonstrated that the impact on the phenotype triggered by CNV is generally considered stronger than SNV [26]. Additionally, all five CNVs detected in this study were classified as "pathogenic," according to ACMG guidelines. Taken together, our results support the idea that the deletion of *KIF11* exon 11 is

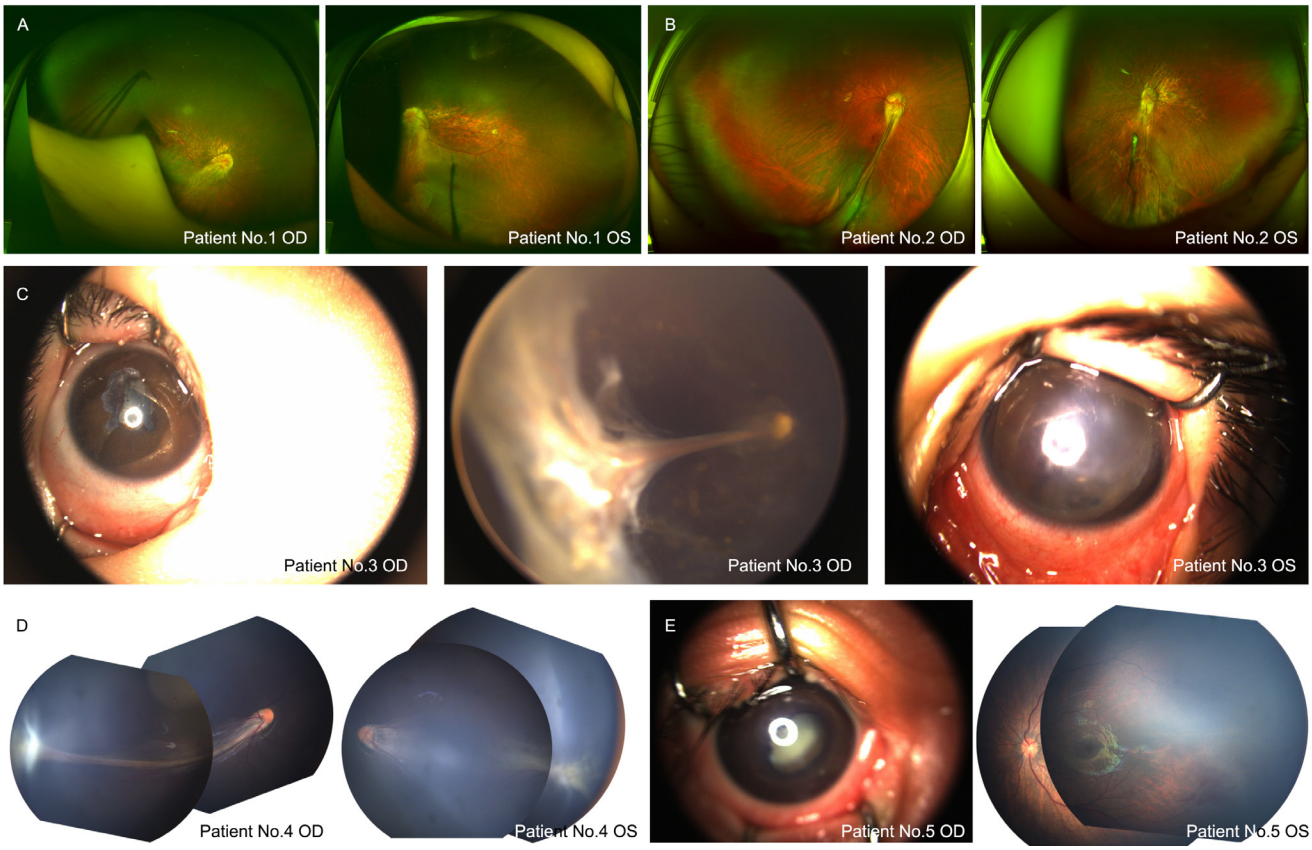

Figure 3. Ocular manifestations of five FEVR patients with CNVs. OD: right eye; OS: left eye. A. Dragged disc in bilateral eyes (patient No. 1); B. retinal fold in the right eye and dragged disc in the left eye (patient No. 2); C. deformed pupil and falciform retinal fold in the right eye, and pupil occlusion of the left eye (patient No. 3); D. bilateral retinal fold (patient No. 4); E. corneal opacity and shallow anterior chamber in the right eye, peripheral nonperfusion area and neovascularization of the retina in the left eye (patient No. 5).

likely to be a causal variation in the clinical manifestations of FEVR.

Our study has several limitations. First, we did not validate the expression quantity of these novel CNVs in normal controls, which reduced our ability to evaluate the significance of the novel CNVs. Second, the fact that the cases were diagnosed and referred to the ophthalmology department of a tertiary health care center may represent referral bias. Nevertheless, we collected a population of 824 FEVR families and detected five novel CNVs.

In conclusion, CNV is known to be a genetic risk factor for many diseases. Herein, we uncovered five novel CNVs in FEVR patients and hypothesized that they are genetic risk factors for the occurrence of FEVR. To investigate their determined significance, more experiments are warranted.

APPENDIX 1. STAGE DEFINITION OF FEVR.

To access the data, click or select the words "Appendix 1."

APPENDIX 2. 463 TARGETED GENES TESTED BY NGS.

To access the data, click or select the words "Appendix 2."

APPENDIX 3. SEQUENCES OF PRIMERS AND PROBES USED FOR DDPCR ASSAY, GAPDH IS SERVED AS INTERNAL REFERENCE.

To access the data, click or select the words "Appendix 3."

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