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Whole exome sequencing revealed novel pathogenic variants in Vietnamese patients with FEVR

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Background: Familial exudative vitreoretinopathy (FEVR) is a rare inherited disorder marked by incomplete retinal vascularization associated with exudation, neovascularization, and tractional retinal detachment. FEVR is genetically heterogeneous and is caused by variants in six genes: *FZD4, LRP5, NDP, TSPAN12, ZNF408,* and *CTNNB1.* In addition, the phenotypic overlap between FEVR and other disorders has been reported in patients harboring variants in other genes, such as *KIF11, ATOH7*, and *RCBTB1.*

Purpose: To identify pathogenic variants in Vietnamese pediatric patients diagnosed with FEVR and to investigate the clinical findings in correlation with each causative gene.

Methods: A total of 20 probands underwent ocular examinations with fundoscopy (ophthalmoscopy) or fluorescein angiography. Genomic DNA was extracted from the peripheral blood of the probands and their family members. Multiplex ligation-dependent probe amplification (MLPA) was employed to detect copy number variants of FEVR-causing genes. Short variants were screened by whole-exome sequencing (WES) and then validated by Sanger sequencing.

Results: Fluorescein angiography showed retinal vascular anomalies in all patients. Other ocular abnormalities commonly found were strabismus, nystagmus, exudation, and retinal detachment. Genetic analysis identified 12 different variants in the *FZD4*, *NDP*, *KIF11,* and *ATOH7* genes among 20 probands. Four variants were novel, including FZD4 c.169G>C, p.(G57R); NDP c.175–3A>G, splicing; KIF11 c.2146C>T, p.(Q716*) and c.2511_2515del, p.(N838Kfs*17). All patients with the *KIF11* variant showed signs of microcephaly and intellectual disability. The patient with Norrie syndrome and their family members were found to have a deletion of exon 2 in the *NDP* gene.

Conclusions: This study sheds light on the genetic causes of ocular disorders with the clinical expression of FEVR in Vietnamese patients. WES was applied as a comprehensive tool to identify pathogenic variants in complex diseases, such as FEVR, and the detection rate of pathogenic mutations was up to 60%.

Familial exudative vitreoretinopathy (FEVR; OMIM 133780) is a rare inherited disorder of blood vessel development in the retina. The principal sign of FEVR is abnormal retinal angiogenesis, which causes incomplete peripheral retinal vascularization and poor vascular differentiation [1]. The clinical presentation of FEVR is extremely variable owing to the degree of retinal ischemia and varies from the absence of symptoms to a range of eye conditions, such as early-onset neovascularization, falciform retinal folds, exudation, and tractional retinal detachment. In severe cases, retinal complications can result in complete blindness. Other remarkable ocular findings include vitreous hemorrhage, secondary epiretinal membrane formation, and glaucoma. According to Gilmour's review, retinal detachment stands out as the most popular ocular feature found in FEVR patients, accompanied by asymmetry, which refers to the difference in expression and severity between the two eyes of the same individual [1].

Insights into the pathogenic basis of FEVR have emerged in the last few years. According to the Online Mendelian Inheritance in Man (OMIM) database, there are six different genes—*FDZ4* (11q14), *LRP5* (11q13.4), *NDP* (Xp11), *TSPAN12* (7q31), *ZNF408* (11p11), and *CTNNB1* (3p22)—responsible for FEVR (types 1, 2, 4, 5, 6, and 7, respectively). Another locus mapped to chromosomal region 11p13-p12 is also involved in FEVR (type 3), even though the candidate gene remains unknown. In addition to the well-known FEVR genes above, patients harboring the *KIF11*, *ATOH7*, *JAG1, DOCK6, ARHGAP31,* and *RCBTB1* variants have been reported to

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have phenotypes that overlap with those of FEVR [2-6]. The inheritance patterns of FEVR are heterogeneous and can follow an autosomal dominant, autosomal recessive, or X-linked recessive mode. Variants in the *NDP* gene can also cause Norrie disease, which is a rare, severe retinopathy. This disorder is primarily characterized by early-onset blindness and shares many ocular manifestations with FEVR [7].

In recent years, next-generation sequencing (NGS) technologies, particularly targeted NGS and whole-exome sequencing (WES), have been increasingly used to identify pathogenic variants of FEVR [8-16]. Such approaches enable the rapid and comprehensive detection of variants in genes of interest.

In this study, we employed various analytic methods, including WES, Sanger sequencing, and multiplex ligation-dependent probe amplification (MLPA), to identify the causative variants in Vietnamese patients diagnosed with FEVR. Once the candidate variant was identified in a proband, the patients' family members would undergo further genetic testing. We also investigated phenotypic expressivity in correlation with each causative gene.

METHODS

Subjects: This study recruited pediatric patients diagnosed with FEVR at the National Eye Hospital (VNEH) in Hanoi, Vietnam, and their family members. The patients underwent a clinical ocular examination, including fundoscopy (ophthalmoscopy) or fluorescein angiography. For patients under one year old, only fundoscopy was used to detect retinal vascular anomalies. Patients were diagnosed with FEVR once they met the clinical criteria, which were composed of three standards: the appearance of incomplete vascularisation of the peripheral retina and poor vascular differentiation (marked by peripheral retinal avascularity) in one eye or both eyes in ophthalmoscopy or fluorescein angiography; a full-term birth or a pre-term birth with retinopathy of prematurity (ROP)-unrelated disease progression; and variable levels of vitreoretinal traction, falciform, subretinal exudation, neovascularization, and retinal detachment. This study was approved by the Ethics Committee in Biomedical Research of the Institute of Genome Research, Vietnam Academy of Science and Technology. Written informed consent for the genetic testing was obtained from the parents or guardians of the patients. The peripheral blood samples (2 ml) of patients and their available family members were collected into ethylenediaminetetraacetic acid (EDTA)-containing tubes and stored at –20 °C.

DNA extraction: Genomic DNA was isolated from peripheral blood using the Exgene Blood SV Mini Kit (GeneAll Biotechnology Co. LTD, Seoul, Korea) following the manufacturer's formal protocol. The quality of the DNA sample was assessed using 0.8% agarose gel electrophoresis and spectrophotometry at 260 and 280 nm (BioSpectrometer basic; Eppendorf, Hamburg, Germany).

Whole exome sequencing: The DNA library was prepared using SureSelect V6-Post (Agilent Technologies, Santa Clara, CA) according to the manufacturer's guidelines. Quantification of the enriched library was conducted using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA). A bioanalyzer using a High Sensitivity DNA Chip (Agilent Technologies) was employed to check the library size distribution, with an expected size range from 200 bp to 400 bp. Paired-end sequencing was performed on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA) with paired reads of 150 bp. The mean exome coverage was more than 100X, and each target base had at least 20X coverage.

Data analysis and pathogenicity evaluation: The sequence reads were mapped to the hg19/GRCh37 human reference genome using the Burrows–Wheeler Aligner tool (BWA. v0.7.12). Picards were used to mark duplicates. GATK and Samtools were used to detect single nucleotide variants (SNVs) and short indels. To exclude false positives, all variants with depth reads lower than 10X were removed. Short indels in the repeat regions and within a 10 bp range from the start and end of the read were also removed. The remaining variants were then filtered from the 1000 Genome database (1000G) and the Exome Aggregation Consortium (ExAC). The variants were annotated using the ANNOVAR program.

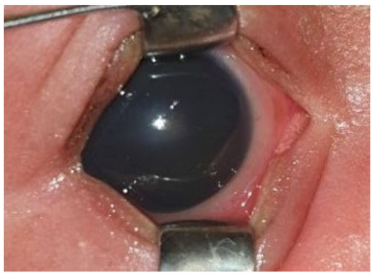
To identify the potential causative variants, we first screened the rare and novel variants in six known FEVR genes (*FDZ4, LRP5, NDP, TSPAN12, ZNF408*, and *CTNNB1*), followed by 231 retinal dystrophies-associated genes. Novel or rare missense variants that were not reported previously were analyzed using *in silico* tools, such as Sorting Intolerant From Tolerant (SIFT), PolyPhen-2, and MutationTaster, to anticipate the damaging effect on corresponding proteins. Amino acid conservation was checked using the MULTIZ alignment (UCSC). Furthermore, each pathogenic or likely pathogenic variant was examined for segregation in the family members. The pathogenicity of the variants was evaluated using the guidelines established by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP). The presence of candidate causative variants was further checked in the Human Gene Mutation Database (HGMD), LOVD Database, Genome Aggregation Database (gnomAD), and an in-house WES database of 535 Vietnamese without any FEVR phenotypes (VN WES DB).

Sanger sequencing validation: Specific primers were provided by PHUSA Biochem Company (Can Tho, Vietnam; Appendix 1). Polymerase chain reaction (PCR) was performed using a total reaction volume of 20 µl that included 10 ng genomic DNA, 1X NEB master mix (New England Biolabs, Ipswich, MA), 10 pmol of each primer, 1 µl DMSO (Bioworld, Dublin, Ohio (OH; 43017), and 16.5 µl deionized water. The thermal cycle comprised denaturation at 95°C for 5 min, followed

A

First visit	23 days	**Gender**	Male
Present age	20 months	**Chief complaint**	Lack of pursuit
		First examination	
		OD	**OS**
Visual acuity		Light perception	Light perception
Slit-lamp	**Corneal opacity**	+	-
	Corneal diameter	13 mm	12.5 mm
	Shallow Anterior chamber	+	+
	Pupil	Posterior synechiae	Posterior synechiae
	Cataract	+	+
Intraocular pressure (IOP)		19 (I-care) mm Hg	13 (I-care) mm Hg
Fundus		Invisible	Invisible
Ultrasound	**Axial length**	20.7 mm	14.8 mm
	Vitreo-Retina	▪ The density of vitreous opacities ▪ Difficult to distinguish the posterior membranes	▪ Thick membrane attached to the optic disc ▪ Total funnel configuration of retinal detachment (end-stage)
Electroretinogram		Unrecordable	Unrecordable

B



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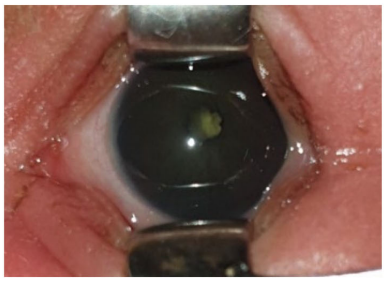

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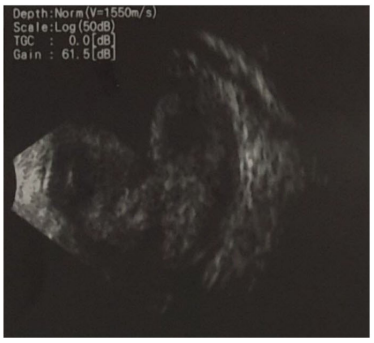

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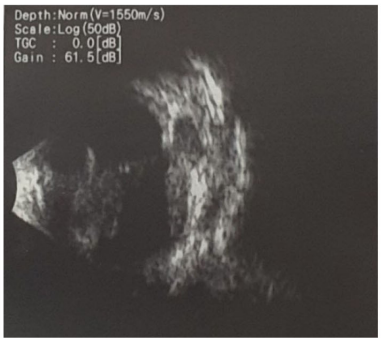

Figure 1. Clinical features of patient EVR-20 with Norrie disease. **A**: Summary of ocular findings. **B**: Anterior segment of the right eye with cataract, corneal clouding, and posterior synechiae. **C**: Anterior segment of the left eye with cataract and posterior synechiae. **D**: Ocular ultrasound of the right eye shows the density of vitreous opacities distributed almost throughout the vitreous body. **E:** Ocular ultrasound of the left eye shows a thick membrane attached to the optic disc and the funnel configuration of retinal detachment. **OD** (oculus dexter): right eye; **OS** (oculus sinister): left eye; **mm**: millimeters; **mm Hg**: millimeters of mercury; +: yes; **-**: no.

Table 1. Genetic findings in patients diagnosed with FEVR.

Patient ID	Gene	cDNA change	Amino acid change	Zygosity	Effect	Reference	Heredity	Clinical significance
EVR-05	*FZD4*	c.1282_1285del	p.(D428Sfs*1)	Het	Frameshift	rs80358295	*De novo*	Pathogenic [a b]
EVR-14	*FZD4*	c.313A>G	p.(M105V)	Het	Missense	rs80358284	Paternal	Pathogenic [a b]
EVR-18	*FZD4*	c.169G>C	p.(G57R)	Het	Missense	Novel	*De novo*	Likely pathogenic [c]
EVR-10	*NDP*	c.112C>T	p.(R38C)	Hemi	Missense	rs758550101	Maternal	Pathogenic [a b]
EVR-15	*NDP*	c.131A>G	p.(Y44C)	Hemi	Missense	rs104894870	Maternal	Pathogenic [c]
EVR-19	*NDP*	c.175-3A>G	Splicing	Hemi	Splicing	Novel	Maternal	Likely pathogenic [c]
EVR-20	*NDP*	Exon 2 deletion		Hemi	Large deletion	reported	Maternal	Pathogenic
EVR-07	*KIF11*	c.2146C>T	p.(Q716*)	Het	Nonsense	Novel	*De novo*	Likely pathogenic [c]
EVR-08	*KIF11*	c.388-1G>C	Splicing	Het	Splicing	rs1844462368	Maternal	Likely pathogenic [b]
EVR-11	*KIF11*	c.2511_2515del	p.(N838Kfs*17)	Het	Frameshift	Novel	*De novo*	Likely pathogenic [c]
EVR-06	*ATOH7*	c.145G>T	p.(E49*)	Hom	Nonsense	rs560230254	Paternal & Maternal	Likely pathogenic [c]
EVR-16	*ATOH7*	c.145G>T	p.(E49*)	Hom	Nonsense	rs560230254	Unknown	Likely pathogenic [c]

De novo Likely pathogenic [c] Het: heterozygosity; hemi: hemizygosity; hom: homozygosity. Reference sequences: *ATOH7* (NM_145178), *FZD4* (NM_012193), *KIF11* (NM_004523), *LRP5* (NM_002335), *NDP* (NM_000266). [a]: Clinical classification according to LOVD; [b]: Clinical classification according to ClinVar (NCBI); [c]: clinical classification based on ACMG/AMP guidelines.

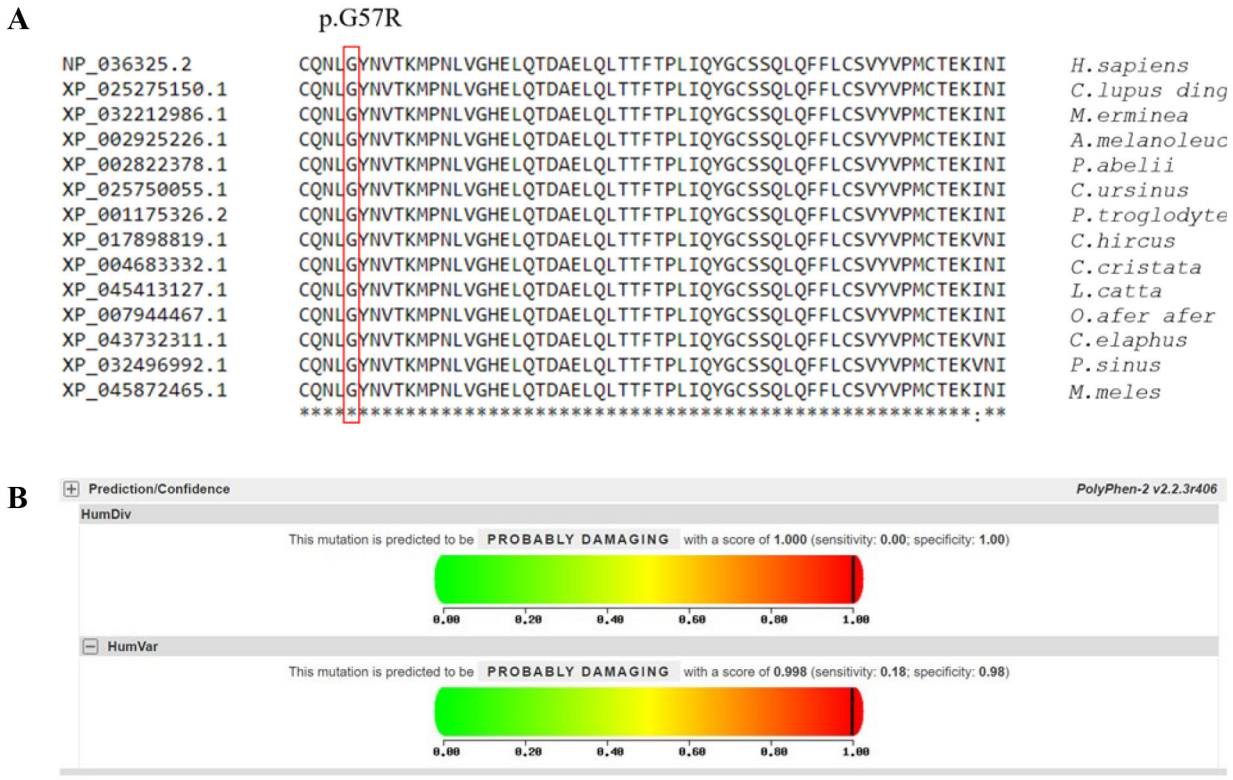

Figure 2. Amino acid conservation (**A**) and PolyPhen-2 functional prediction (**B**) of the novel variant c.G169C (p.G57R) in the *FZD4* gene. The amino acid residue p.G57R, which is highly conserved, is indicated in a red box. Reference sequence for FZD4 protein: *H. sapiens* (NP_036325.2), *C. lupus dingo* (XP_025275150.1), *M. erminea* (XP_032212986.1), *A. melanoleuca* (XP_002925226.1), *P. abelii* (XP_002822378.1), *C. ursinus* (XP_025750055.1), *P. troglodytes* (XP_001175326.2), *C. hircus* (XP_017898819.1), *C. cristata* (XP_004683332.1), *L. catta* (XP_045413127.1), *O. afer* (XP_007944467.1), *C. elaphus* (XP_043732311.1), *P. sinus* (XP_032496992.1), and *M. meles* (XP_045872465.1).

by 40 cycles of 95°C for 15 s, 58°C for 30 s, 68°C for 20 s, and a final extension at 68°C for 5 min. The PCR products were then purified using a MultiScreen-PCR 96 Filter Plate (Merck-Millipore, Burlington, MA) and subsequently bi-direction sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Kit Version 3.1 (Applied BioSystem, Waltham, MA) on an ABI genetic analyzer 3500 (Applied Biosystems, Waltham, MA).

Multiplex ligation-dependent probe amplification: MLPA was performed according to the manufacturer's standard protocol using the SALSA MLPA Probemix P285-LRP5 (MRC-Holland, Amsterdam, the Netherlands). This assay can detect deletions/duplications in the *FZD4*, *LRP5*, *NDP,* and *DKK1* genes. The data were analyzed using Coffalyser software (MRC Holland). Genomic DNA samples (50 ng) were denatured at 98°C for 5 min. Hybridization was performed at 60°C for 16 h, followed by ligation at 54°C for 15 min and amplification of the ligated probes. Capillary electrophoresis of the PCR products was performed using a 3500 Genetic Analyzer (Applied Biosystem, Foster City, CA). Coffalyser. Net software was used to analyze the data; relative probe values below 0.7 and above 1.3 indicated the borders for loss and gain, respectively.

RESULTS

Clinical features of patients: A total of 20 pediatric patients from unrelated families were enrolled in this study (Appendix 2). Their median age at diagnosis was 4 months (ranging from 14 days to 11 months), and the ratio of males to females was 15:5. All were born at full term and had no history of oxygen therapy treatment. Fluorescein angiography or the indirect ophthalmoscope were used to detect retinal vascular anomalies in 100% of the patients, while strabismus was found in 95% (19/20). Retinal detachment, exudation, nystagmus, and asymmetry were found in 45%, 35%, 30%, and 20% of the patients, respectively. Other ocular characteristics, such as cataract, microphthalmia, band keratopathy, corneal clouding, posterior synechiae, megalocornea, retinal fibrosis,

retinal degeneration, and proliferative vitreoretinopathy, were identified in several patients. Notably, four patients showed signs of microcephaly, and one had hydrocephalus.

Four of the 20 patients had a family history in which at least one family member had visual problems. Patient EVR-06 had an elder brother with highly similar eye conditions. For patient EVR-08, their maternal grandfather and uncle had early-onset glaucoma and primary myopia, respectively. The maternal grandfather of patient EVR-15 had nystagmus. In family F20, 11 males in four generations of his maternal family had severe vision loss. Both eyes of patient EVR-20 presented with a shallow anterior chamber, posterior synechiae, and total cataract. The intraocular pressure (IOP) values were 19 mmHg OD and 13 mmHg OS, which indicated a difference of 6 mmHg in IOP between the two eyes. Moreover, the right eye had megalocornea and corneal clouding. Ultrasound of the right eye showed the density of vitreous opacities distributed almost throughout the entire vitreous body, and it was difficult to distinguish posterior membranes. Meanwhile, the left eye showed a thick membrane attached to the optic disc, and the funnel configuration of retinal detachment in this eye was accompanied by end-stage retinal detachment. Due to his severe ocular phenotypes and family history, patient EVR-20 was suspected of having Norrie disease (Figure 1).

Pathogenic variants identified by whole-exome and Sanger sequencing: Genomic DNA of 20 probands was first used for WES analysis, and then novel and rare variants found in genes responsible for FEVR were evaluated for pathogenicity. Eleven probands were identified as carrying pathogenic or likely pathogenic variants, accounting for 55% of all subjects (Table 1). There were nine heterozygous or hemizygous

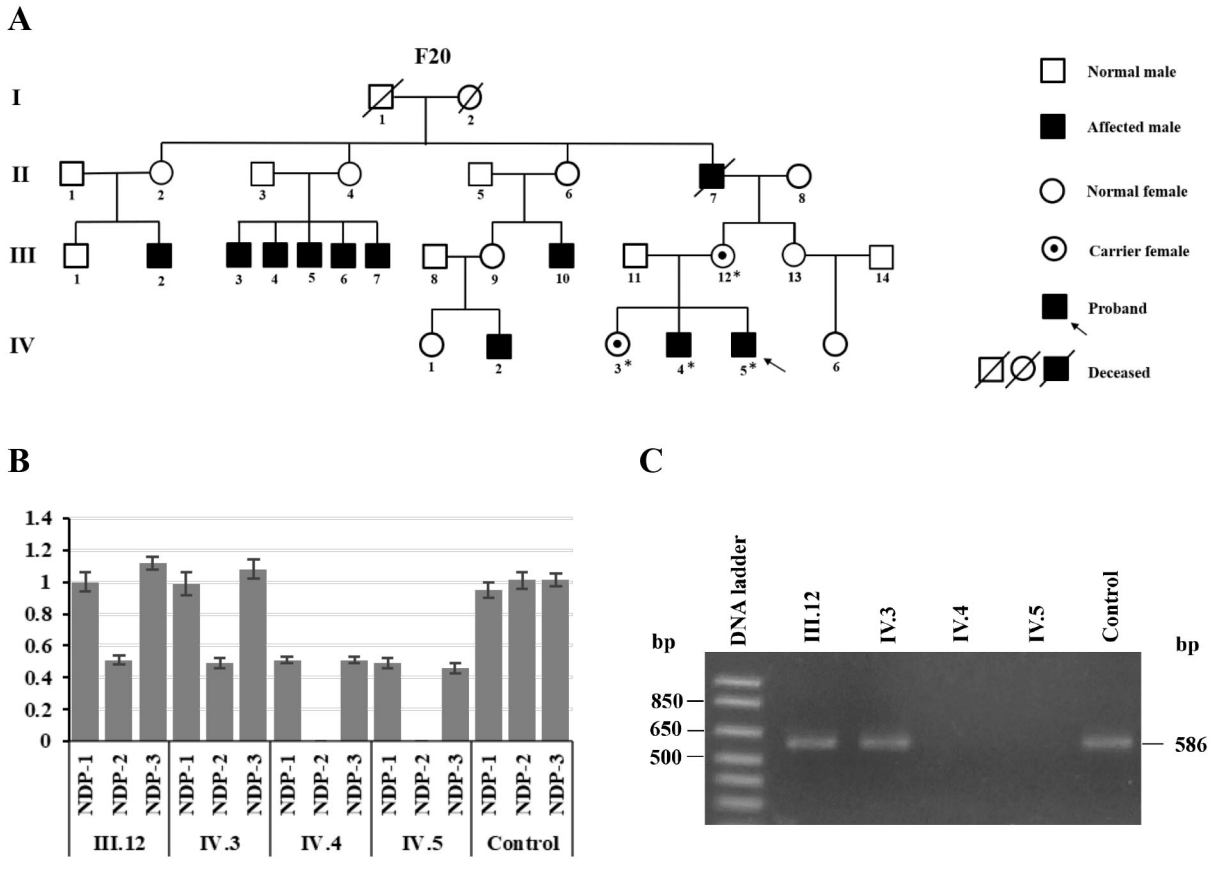

Figure 3. NDP-exon 2 deletion in the patient EVR-20. **A**: The pedigree chart of the family EVR-20 showed that 11 out of 19 males were affected. **B**: MLPA analysis of the proband (IV.5), the unaffected mother (III.12), the unaffected older sister (IV.3) and the affected older brother (IV.4). A complete deletion of NDP exon 2 was detected in the proband and his affected older brother. C: Amplification products of NDP exon 2 on ethidium bromide-stained agarose gel. Positive amplification of NDP exon 2 was shown in the unaffected mother (III.12) and older sister (IV.3) and the control. Meanwhile, negative amplification of NDP exon 2 was shown in the proband (IV.5) and his affected older brother (IV.4). *: patients who underwent genetic testing.

variants in *FZD4* (3 patients), *NDP* (3 patients), and *KIF11* (3 patients), as well as 1 homozygous variant in *ATOH7* (2 patients). The mutational types included 2 frameshift, 3 nonsense, 4 missense, and 2 splicing variants. Four of the 11 variants were novel and were not found in the in-house control group of the VN WES DB.

For the *FZD4* gene (Appendix 3), two known pathogenic variants c.1282_1285del p.(D428Sfs*2) and c.313A>G, p.(M105V), were found in the probands EVR-05 and EVR-14, respectively. A novel variant c.169G>C, which resulted in an amino acid substitution p.(G57R), was identified in the proband of patient EVR-18. This variant was located in a highly conserved region of the FZD4 protein among species and was predicted to be a damaging variant by the SIFT and PolyPhen-2 tools (Figure 2). It was a de novo variant due to its absence in the proband's parents. According to ACMG/AMP, it should be a moderate pathogenic variant.

Regarding the *NDP* gene located in the X chromosome (Appendix 3), three variants were detected in three male probands in the hemizygous form. Two missense variants—c.112C>T, p.(R38C) in the proband of patient EVR-10 and c.131A>G, p.(Y44C) in the proband of patient EVR-15—were previously reported as pathogenic. A novel variant c.175–3A>G in the proband of patient EVR-19 was predicted to cause a splice site alteration. The unaffected mother of EVR-19 carried a heterozygous variant of *NDP* c.175–3A>G.

For the *KIF11* gene (Appendix 3), a previously reported splicing mutation c.388–1G>C was found in patient EVR-08. Two novel variants c.2146C>T, p.(Q716*) and c.2511_2515del, p.(N838Kfs*17) were identified in patients EVR-07 and EVR-11, respectively. Both are de novo and null variants and can be classified as pathogenic variants.

For the *ATOH7* gene (Appendix 3), the pathogenic variant c.145G>T, p.(E49*) was detected in the two probands patients EVR-06 and EVR-16 in the homozygous state. According to the OMIM database, the *ATOH7* gene is responsible for the persistent hyperplastic primary vitreous condition that follows the autosomal recessive model. Sanger sequencing identified a heterozygous variant c.145G>T, p.(E49*) in both unaffected parents of EVR-06. Unfortunately, the samples of EVR-16's parents were not available for genetic testing.

Pathogenic deletion variant identified by multiplex ligation-dependent probe amplification: Since nine of the 20 patients had not been identified as having causative variants by WES, their DNA samples were used for MLPA analysis to determine whether they had a gross deletion/insertion in the *FZD4*, *LRP5,* and *NDP* genes. The results showed that only patient EVR-20 had a hemizygous deletion of exon 2 of the *NDP* (Figure 3B). The genetic examination of his family members showed that his affected brother also had a hemizygous *NDP* exon 2 deletion, while his unaffected mother and sister carried a heterozygous deletion (Figure 3B). The validation of this variant by the PCR method indicated a complete absence of *NDP* exon 2 in the proband EVR-20 and his affected brother (Figure 3C).

Spectrum of pathogenic variants and their correlation with clinical phenotypes: In the current study, 12 pathogenic variants were identified in four genes, including *FZD4*, *NDP, KIF11,* and *ATOH7.* Among them, *NDP* variants made up the largest proportion (20%), followed closely by *FZD4* (15%), *KIF11* (15%), and *ATOH7* (10%; Figure 4). No significant

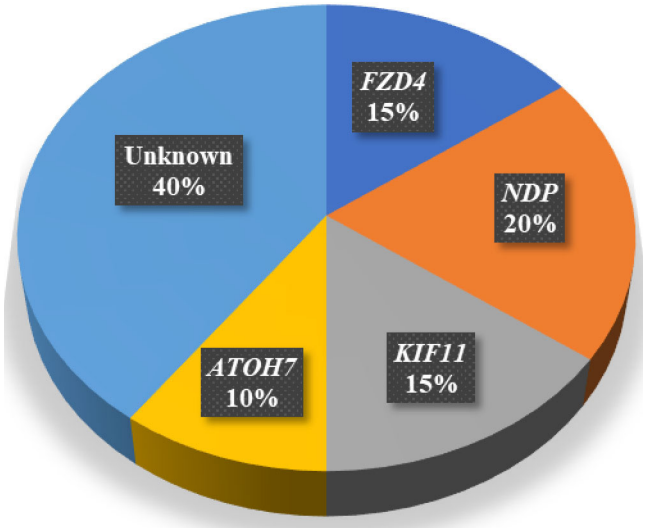

Figure 4. Spectrum of pathogenic mutations in probands.

Table 2. Genotype-phenotype correlation of probands.

Patient ID	Variants			Retinal vascular anomalies	Strabismus	Exudation	Retinal detachment	Nystagmus	Cataract	Asymmetry	Others
	Gene	cDNA change	Amino acid change								
EVR-05	*FZD4*	c.1282_1285del	p.(D428Sfs*1)	+	+	-	+	+	-	-	-
EVR-14	*FZD4*	c.313A>G	p.(M105V)	+	+	-	-	+	+	-	-
EVR-18	*FZD4*	c.169G>C	p.(G57R)	+	+	+	-	-	-	-	-
EVR-10	*NDP*	c.112C>T	p.(R38C)	+	+	+	-	-	-	+	-
EVR-15	*NDP*	c.131A>G	p.(Y44C)	+	-	-	+	+	-	+	band keratopathy, microphthalmia (left eye)
EVR-19	*NDP*	c.175-3A>G	Splicing	+	+	-	-	-	-	-	-
EVR-20	*NDP*	Exon 2 deletion		+	+	-	+	-	+	-	-
EVR-07	*KIF11*	c.2146C>T	p.(Q716*)	+	+	-	-	+	-	-	retinal fibrosis
EVR-08	*KIF11*	c.388-1G>C	Splicing	+	+	-	+	-	+	-	-
EVR-11	*KIF11*	c.2511_2515del	p.(N838Kfs*17)	+	+	-	-	-	+	-	microphthalmia
EVR-06	*ATOH7*	c.145G>T	p.(E49*)	+	+	+	-	-	-	-	-
EVR-16	*ATOH7*	c.145G>T	p.(E49*)	+	+	-	+	-	-	-	-

variants were detected in the remaining FEVR-causing genes (*LRP5*, *TSPAN12*, *ZNF408,* and *CTNNB1*). Unfortunately, there were still 8 probands in whom genetic causes were not detected.

All 12 patients positive for variants had retinal vascular anomalies, and 11 presented with strabismus. Approximately 33% (1/3) of patients with a FZD4 variant /a NDP variant and 50% (1/2) with an *ATOH7* variant had exudation. Retinal detachment was detected in about 33% (1/3) of patients with *FZD4* variants, 50% (2/4) with *NDP*, 33% (1/3) with *KIF11*, and 50% (1/2) with *ATOH7*. Nystagmus and cataracts were not present in the patients with *ATOH7* variants. Asymmetry was found only in patients with the *NDP* variant, with 50% of them showing this feature. All three patients carrying the *KIF11* variants presented with signs of microcephaly, and one patient even had microphthalmia in the left eye (Table 2, Figure 5).

DISCUSSION

Recently, genetic studies of various ocular diseases have been conducted effectively in Vietnam [17,18]. This study is the first report on genetic mutations in Vietnamese children with FEVR. Due to the genetic heterogeneity of FEVR, we employed multiple methods, such as WES, Sanger sequencing, MLPA, and PCR, and we ultimately detected pathogenic mutations in 12 out of 20 patients, resulting in a detection rate of 60%. Compared to recent studies, our detection rate was quite similar to that of a Chinese cohort (65%) and relatively higher than that of some Korean (35.3%) and American (48.9%) cohorts [13,19,20].

Among previously reported FEVR-associated genes, our study identified pathogenic or likely pathogenic variants, mainly in the *FZD4*, *NDP,* and *KIF11* genes, suggesting that variants in these genes are possibly common causes of FEVR in Vietnamese patients. Interestingly, variants found in the *NDP* gene were the most frequent (20%), while the number of variants of both the *FZD4* and *KIF11* genes contributed to an equal proportion (15%). More specifically, the frequency of *FZD4* mutations in our study was quite close to other studies on Chinese (6.45%–21.35%), American (15%), and Canadian (18%) patients [13,20,21]. In contrast, those of the *NDP* and *KIF11* genes were significantly higher than the corresponding figures for the Chinese (4.11%–9.68% and 1.61%–6.74%, respectively) and American (7% for *NDP*) cohorts [13,20]. Accordingly, the *NDP* and *KIF11* variants might be more frequent in Vietnamese populations than in other populations. Notably, no significant pathogenic variants in the *LRP5* gene were revealed in this study. However, *LRP5* stood out as one of the most frequently mutated genes in previous studies, including in the Chinese, Korean, and American cohorts, with a wide range of phenotypes ranging from mild to severe in patients [13,19,20]. In this study, the number of probands was relatively small, and most were diagnosed at the late

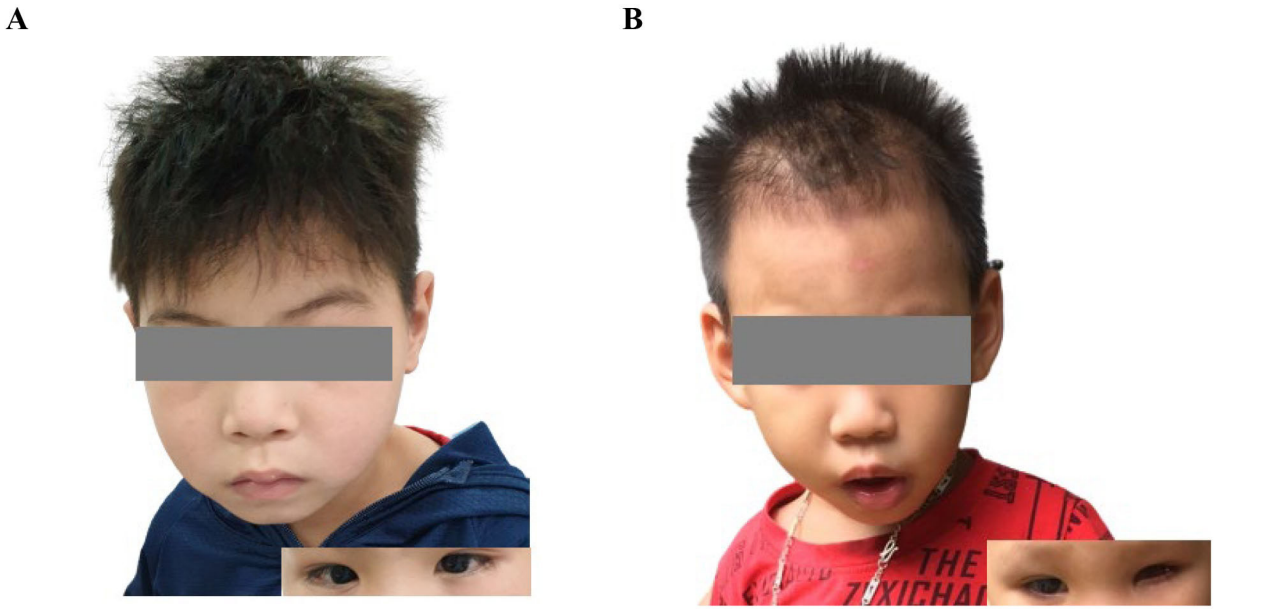

Figure 5. Facial expressions of patient EVR-08 (**A**) with *KIF11* c.388–1G>C and patient EVR-11 (**B**) with *KIF11* c.2511_2515del. Both had signs of microcephaly. Patient EVR-11 had microphthalmia in the left eye.

stages, presenting with severe phenotypes. This may explain the differences in the genetic etiology of our patients.

No significant variants were identified in 42% (8/20) of the index patients, which calls for expanded studies of other retinopathy-associated genes. Furthermore, other analyses to detect copy number variants of FEVR-related genes or locus 11p13-p12 should be performed in further studies.

Variable expressivity of ocular and extraocular phenotypes: Apart from eye abnormalities, Hu et al. highlighted a specific correlation between genotype and systemic phenotype for *KIF11*, in which patients with *KIF11* variants had a considerably higher possibility of microcephaly, lymphedema, and intellectual disability [22]. Likewise, we found that all three patients harboring the *KIF11* variants in this study displayed microcephaly, suggesting that this feature might be particular to patients with the *KIF11* variant (Figure 5).

Regarding the patient with Norrie disease, it is noteworthy that 11 out of 19 males in his family presented with ocular manifestations. This is the first time we have found such a large number of patients in a Vietnamese family with Norrie disease. It is apparent that copy number analysis and direct sequencing of the *NDP* gene may prove highly helpful in quickly identifying the genetic causes of *NDP*-related retinopathies, particularly Norrie disease.

Even though genetic tests indicated the same *NDP* exon 2 deletion as in previous studies, the clinical phenotypes were widely variable [23,24]. The proband in our study exhibited multiple ophthalmic abnormalities, including megalocornea, corneal clouding in the right eye, total cataract, posterior synechiae, and total tractional retinal detachment when he was just 23 days old, which was the time of detection (Figure 1). Notably, patients with Norrie disease in previous studies often showed microphthalmos [25,26], whereas our patient's right eye exhibited megalocornea. We assumed that his right eye had possibly developed secondary glaucoma due to pupillary block and a close anterior chamber angle. In addition, he was likely to have intellectual disabilities. At present, he is nearly 20 months old and still shows no symptoms of hearing loss; however, it remains unknown whether a future event will occur.

Intrafamilial variable expressivity: Clear evidence of intrafamilial variability and incomplete penetrance was observed in this study. A good illustration of this evidence is the intrafamilial occurrence of non-syndromic to syndromic manifestation in family F8 with the *KIF11* variant. Notwithstanding being heterozygous for the same *KIF11* c.388–1G>C variant, patient EVR-08 exhibited a phenotype with retinal detachment, while his mother did not show any symptoms. Notably, his uncle and grandfather also had several subtle ocular conditions; however, their DNA was not available for genetic testing. Another remarkable case was family F14 with the pathogenic variant *FZD4* c.313A>G, p.(M105V), in which the proband showed a range of eye conditions (retinal vascular anomalies, strabismus, nystagmus, and cataract), while the carrier's father's eyes were completely normal. The pattern was entirely different from previous observations, with all family members positive for such variants being affected [27,28].

Conclusion: In summary, this study was successful in using comprehensive WES analysis together with other methods to identify variants in the *FZD4*, *NDP*, *KIF11*, and *ATOH7* genes that caused *FEVR* phenotypes in Vietnamese patients. Our findings provide useful information for the clinical management and genetic counseling of this disease, especially in asymptomatic cases.

APPENDIX 1. LIST OF PRIMERS USED FOR VALIDATION OF VARIANTS DETECTED BY WES.

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

APPENDIX 2. CLINICAL FEATURES OF FEVR PATIENTS.

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

APPENDIX 3. PEDIGREES AND SEQUENCE CHROMATOGRAMS.

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

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