

# Clinical and whole-exome sequencing findings in Individuals from Yunnan Province with Familial Exudative Vitreoretinopathy

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## Research

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# Abstract

**Background :** Familial exudative vitreoretinopathy (FEVR) is a rare inherited retinal disorder characterized by the failure of peripheral retinal vascularization at birth. FEVR can cause further pathological changes, such as neovascularization, exudation, haemorrhage, and retinal detachment, in turn. The molecular diagnosis enables a deeper understanding of this disease, so the differentiation of FEVR is important for the accuracy of genetic counselling. However, to date, only six genes have been identified as being responsible for this disease.

**Methods :** Without a known FEVR gene mutation, six families were enrolled in our study between 2016 and 2017 from the clinical practices of ophthalmologists. The referring physician made a diagnosis for FEVR probands, and clinical data and DNA were collected from each participant. Whole-exome sequencing was used to detect the mutations present in the probands. The raw reads were obtained from Illumina Miseq. Then, an in-house bioinformatics pipeline was performed to detect both single nucleotide variants (SNVs) and small insertions/deletions (InDels). The pathogenic mutations were identified with stringent criteria and were further confirmed by conventional methods and cosegregation in families.

**Results :** Using this approach, bioinformatic predictions showed that six mutations were found in our study: three mutations in the known genes of ZNF408, LRP5 and KIF11 and three mutations in the newly identified genes NR2E3, KRT3 and FOXL2. To test the hypothesis that cases of FEVR are caused by NR2E3, KRT3 and FOXL2, probands who were diagnosed as FEVR by a physician using wide-field fluorescein angiography were found to not have any mutations in any of the six known FEVR genes. Compared with previous reports, mutations in NR2E3, KRT3 and FOXL2 are believed to cause a broader spectrum of ocular disease. The NR2E3, KRT3 and FOXL2 genes likely play a role in retinal vascular development.

**Conclusions :** This report is the first to describe FEVR mutations in Yunnan province children with FEVR. This study would provide information on the genetic forms of the disease and direct counselling by analysing the genetic testing and genotype-phenotype interaction.

## Background

Familial exudative vitreoretinopathy (FEVR, OMIM 133780) is a rare hereditary disorder characterized by anomalous retinal vascularization, including a retinal avascular zone, exudate, macular traction, retinal neovascularization, traction or rhegmatogenous retinal detachment [1, 2] and affects the growth and development of blood vessels in the retina of the eye. FEVR is one of several paediatric vitreoretinopathies and can lead to visual impairment, including complete blindness in one or both eyes [3, 4]. Although rare, FEVR is a potentially preventable cause of vision loss in patients younger than 30 years of age [5]. In 1969, Criswick and Schepens first described this disease [6], while in 1976, Canny and Oliver were the first to confirm the clinical features through the use of fundus fluorescein angiography [7]. Wide-field fluorescein angiography has become the gold standard from the diagnostic standpoint in the

diagnosis and monitoring of FEVR [3]. However, FEVR has complicated and variable clinical manifestations, even between the individual eyes of a patient or among affected siblings in the same family, and its clinical manifestations differ greatly among patients [8-10]. Mild forms of FEVR can be asymptomatic and only exhibit peripheral retinal vascular abnormalities, such as venous telangiectasias, altered arterial tortuosity and a peripheral avascular zone [11]. Severe forms of FEVR differ greatly within the same family and are associated with subretinal and intraretinal haemorrhages, retinal neovascularization, retinal folds, exudates, and tractional retinal detachment [3, 12].

FEVR most often presents as visually significant in childhood, although it might progress at any age with sight-threatening manifestations [13]. In 1998, a 5-stage FEVR classification scheme was described by

Pendergast and Trese [14]. In Stage 1, the avascular periphery is notable, while Stage 2 occurs when neovascularization develops with the avascular periphery. Stage 3 is defined by macula-sparing retinal detachment and proceeds to Stage 4 with macula-involving retinal detachment. Stage 5 is complete retinal detachment, with the A versus B designations being for the absence versus the presence of exudate [13].

To date, 6 genes have been identified that account for some cases of FEVR. These genes are *FZD4* (Frizzled 4 receptor), *LRP5* (low-density-lipoprotein receptor-related protein 5), *NDP* (Norrie disease protein), *TSPAN12* (tetraspanin-12), *ZNF408* (zinc finger protein 408), and *KIF11* (kinesin family member 11) [15-23]. Mutations in these gene have been implicated in abnormal signalling of known biological pathways and are inherited in autosomal dominant (AD), autosomal recessive (AR), and X-linked recessive fashions [3]. AD is the most common mode of inheritance and is associated with mutations in *FZD*, *LRP5* and *TSPAN12*. Mutations in *LRP5* are associated with AR inheritance, whereas mutations in *NDP* have caused X-linked recessive forms of the disease [24] [25, 26].

Though occurring almost 50 years ago, the first descriptions of FEVR were beautifully illustrated, and subsequently, the underlying pathophysiology, genetics, diagnosis, and management of the disease have been learned [3]. Only 40% to 50% of the patients with FEVR have had a corroborating genetic mutation identified, and the contribution of any given gene to the disease differs among study populations [27, 28].

However, the detailed mutational spectrum of these six genes was unknown in Chinese individuals [29]. Even genetic testing has not been carried out in Yunnan province, and there are few reports regarding mutations in the 25 ethnic minority groups in Yunnan. In the present study, genetic testing was performed on individuals with FEVR in Yunnan province to investigate the genetic causes of FEVR.

WES was used to sequence DNA from affected proband of FEVR pedigrees with no known causative FEVR gene mutations (*FZD4*, *LRP5*, *NDP*, *TSPAN12*, *ZNF408*, and *KIF11*). We identified three pedigrees

with a heterozygous *ZNF408*, *LRP5* and *KIF11* profile and three pedigrees with a heterozygous *NR2E3*, *KRT3* and *FOXL2* mutations. To test the hypothesis that more cases of FEVR are caused by

*NR2E3*, *KRT3* and *FOXL2* mutations, we screened for mutations in the *NR2E3*, *KRT3* and *FOXL2* genes in a cohort of FEVR probands who did not have a mutation in any of the known FEVR genes.

## Results

### Clinical findings

#### Patient 1

The mutation, *ZNF408*, p.G290S, identified in a proband, was associated with a FEVR phenotype. The family, which has one girl (the proband) and unaffected parents, was recruited into the FEVR project specifically because of the fundus appearance mimicking FEVR. The proband was a 13-day-old baby at the time of recruitment.

#### Patient 2

The mutation in *LRP5* involved in splicing, identified in a proband was associated with a stage 4-FEVR phenotype. The family, which has two girls (the proband, and an unaffected elder sister of proband) and unaffected parents, was recruited in the FEVR project specifically because of the stage 4- FEVR. The proband was a 3-year-old girl at the time of recruitment. She had been noted as have retinal abnormalities at age 3 years. She was born full-term with a birth weight of 3.2 kg. The elder sister was examined at age 5 but she is normal.

#### Patient 3

The mutation in *KIF11*, p.1423T, identified in proband was associated with a FEVR phenotype. The family, which has two boys (the proband and an unaffected elder brother) and unaffected parents, was recruited into the FEVR project specifically because of stage-1FEVR. The proband was an 8-month-old baby at the time of recruitment. The retinal abnormalities were first noted at age 14 days. He was born full-term with a birth weight of 2.9 kg. There was no family history of health problems compatible with a diagnosis of FEVR, and the mutation was found in his unaffected father and elder brother.

#### Patient 4

The mutations in *NR2E3*, p.H361R, identified in a proband were associated with a FEVR phenotype. The family, which has one boy (the proband) and an unaffected elder sister, as well as unaffected parents, was recruited into the FEVR project specifically because of stage-4 FEVR. The proband was a 3-year-old boy at the time of recruitment.

#### Patient5

The mutation, *KRT3*, p. R271H, identified in proband was associated with a FEVR phenotype. The family, which has one boy (the proband) and unaffected parents, was recruited in the FEVR project specifically because of stage-4FEVR. The proband was a 2-month-old boy at the time of recruitment.

## Patient 6

The *FOXL2* mutation, p.333-337del, was identified in a proband was associated with a FEVR phenotype. The family, which has one boy (the proband) and unaffected parents, was recruited in the FEVR project specifically because of stage-4 FEVR. The proband was an 11-month-old boy at the time of recruitment.

### *Genetic Analysis*

WES was initially performed on an affected probands (pedigree I- $\square$ ) to identify a shared disease-causing variant. The reads aligned to the human genome, mapped to the target region with a mean coverage between 99.8 and 99.9%, SNPs and indels are shown in Table 1. Between 11,631 and 11,844 were identified as SNPs from six affected individuals, whereas 833-918 indels were identified in the unaffected individuals. Analysis based on the pattern of inheritance identified common homozygous and heterozygous variants in six affected individuals (Table 1). Among the identified variants, the numbers of selected gene were displayed in Table 1 according to our mutational analysis. Further analysis based on expression profiles identified six candidate variants (Table 1). Among these, the four software packages predicted six of the missense variants to be damaging. After filtering for suspected sequencing artefacts and with a multiple allele frequency greater than 5%, the shared missense or nonsense variants identified were displayed in Table 2.

Sanger sequencing revealed the novel, homozygous missense variants identified in these probands (Fig1 family tree, and Table 3); p.G290S in the *ZNF408* gene, splicing in *LRP5*, p.I423T in *KIF11*, p.H361R in *NR2E3*, p.R271H in *KRT3*, p. 333\_337del in *FOXL2* segregating with disease in families 1,2,3,4,5,6, respectively (Fig. 2).

In the families (pedigree I- $\square$ ), the remaining heterozygous, *ZNF408*, *LRP5* and *KIF11* genes were selected as the candidates because heterozygous mutations in these genes has previously been reported as being causative for FEVR[22]. There were no other siblings (pedigree  $\square$ ) and no family history of a condition compatible with a diagnosis of FEVR. The mutation was absent father, mother, and mother in family I, $\square$ , and  $\square$ ,

respectively. In families (pedigree  $\square$ - $\square$ ), *NR2E3*, *KRT3* and *FOXL2* were selected as the candidate. The unaffected father (pedigree  $\square$ ), mother (pedigree  $\square$ ) and mother (pedigree  $\square$ ) presented with mutations.

Sanger sequencing identified additional novel heterozygous mutations in 6 probands (Figure 1 family tree).

Gene type, amino acid changes and nucleotide changes of all mutations are shown in Table 3, and the amino acid changes in family2 (*LRP5*) are in an acceptor splice site.

Additionally, these variants were absent in 60 normal control individuals.

## Discussion

In this study, a comprehensive molecular screen for mutations in six families with FEVR was performed with a WES approach. In these six families, the probands were found by fluorescein angiography, which is critical for the diagnosis and management in FEVR [30]. Several bioinformatics tools predicted a strong effect on the amino acid changes inducing exons 5, 6, 11, 8, 25 and 18, in families 1, 2, 3, 4, 5 and 6, respectively. These novel variants found in our patients were neither reported in the literature nor in public databases. To investigate the effects of mutation on the transcript, we analysed *ZNF408*, *LRP5*, *KIF11*, *NR2E*, *KRT3* and *FOXL2* DNA obtained from patient's whole blood. The potential regions were amplified by standard PCR. The nucleotide substitutions in these families caused the amino acid changes in exons. As a result, we were able to obtain accurate mutations in FEVR disease gene from these families (pedigree I-III) and identify putative pathogenic mutations for 50% of the patients from genes that are published in the literature. We were also able to find mutation in three genes, *NR2E3*, *KRT3* and *FOXL2*, in the families (pedigree III-VI).

In these three families (pedigree I-III), there were newly occurring mutations, with most of the alleles identified having never been reported on before. Through previously reported work, it is known that a number of variants discovered in the genes *ZNF408*, *LRP5* and *KIF11* correlate with FEVR. Our study allowed us to obtain an accurate estimation of the number of patients carrying mutations in each FEVR gene. In our patient cohort, *ZNF408*, *LRP5*, and *KIF11*, which are the most frequently mutated, were found in families (pedigree I-III). In one Dutch family and a Japanese family, novel mutations in *ZNF408* have been reported. Subsequently, it has been shown to play a putative role in retinal vasculogenesis through the use of a knockdown of *znf408* in zebrafish [31]. That mutations in *LRP5* can lead to abnormal retinal vascular formation, delayed retinal vascular development and subsequent pathological glomeruloid vessels was demonstrated by the use of a *Lrp5* knockout (*Lrp5*<sup>-/-</sup>) mouse model of FEVR [32]. The *LRP5* gene has been shown to exhibit two inheritance patterns, AD and AR. Due to compound heterozygous genotypes consisting of missense mutations, AR of *LRP5* is most common [33]. AR inheritance patterns have been observed in our study. This proband carries compound heterozygous mutation, and *LRP5* mutations are found in the unaffected father, while only wild-type variants are present in the unaffected mother. Mutations in *KIF11* are associated with microcephaly, chorioretinopathy, or mental retardation [34]. Mutations of this gene have been recently identified in individuals affected with FEVR. Furthermore, there is a phenotypic overlap between the two diseases caused by *KIF11* mutations [35-37]. With this hypothesis, the mutations of *NR2E3*, *KRT3* and *FOXL2* had been found in three patients with FEVR. In 2000, the nuclear receptor subfamily 2, group E, member 3 gene (*NR2E3*) on chromosome 15q23, which encodes a ligand-dependent transcription factor, was found to play an important role in photoreceptor development and differentiation and is responsible for the enhanced S-cone syndrome (ESCS) and was identified [38, 39]. Subsequently, mutations in *NR2E3* were found in patients with Goldmann-Favre syndrome and clumped pigmentary retinal degeneration [40], autosomal recessive retinitis pigmentosa, and dominant retinitis pigmentosa [41]. These mutations of *NR2E3* gene expanded the phenotypic variations for people with retinal dystrophy. Keratins are a group of structural proteins in the epithelia of cornea [42]. In 1997, keratin 3 (*KRT3*) was first linked to mutations in Meesmann's corneal dystrophy [43]. The gene family of Fork head box (*Fox*), which named after

the *Drosophila melanogaster* fork head gene (*fkh*), is an evolutionarily ancient gene family[44]. The Forkhead Box L2 gene (*FOXL2*) which encodes the fork head transcription factor FOXL2 and plays an important role in the development of the eyelids and ovary, is the primary gene that underlies Blepharophimosis Syndrome[45, 46]. We found these mutations in these genes in families (pedigree 1-3), with FEVR. Further research needs to be carried out on these genes: *NR2E3*, *KRT3* and *FOXL2*.

To the best of our knowledge, the roles of *NR2E3*, *KRT3* and *FOXL2* were first reported in this study, and the expression patterns of *NR2E3*, *KRT3* and *FOXL2* in different cell types are still not fully defined. Interestingly, traces of *NR2E3*, *KRT3* and *FOXL2* transcript in whole blood samples were found to allow for the direct study of the effects of the mutations, making the in vitro model redundant for the mutation assay. Further research will clarify the roles of the *NR2E3*, *KRT3* and *FOXL2* genes in the development of retinal vessels.

## Conclusion

In summary, the use of the WES method in this study allows us to efficiently identify all of the most likely causative variants in disease-associated genes associated with FEVR. Mutations in these genes cause a broader spectrum of ocular disease than previously reported. The molecular diagnosis together with the clinical phenotype provides potentially useful information for the diagnosis of the disease, the prognosis for the patient, and guidance for genetic counseling. This information is particularly valuable, given the phenotypic variation found even within the same family.

## Methods

### *Participants and Clinical Data Collection*

This study was approved by the Ethics Committee of the Children's Hospital of Kunming Medical University, and written informed consent was obtained from either the participants or their guardians. No stipend was offered.

With the aim of identifying FEVR genes and describing the phenotypic spectrum of the disease, participants were recruited from six nonconsanguineous families by the Children's Hospital of Kunming Medical University (Kunming, China). In these families, children suffered from FEVR; however, the parents and other members of the family were normal. Additionally, our study enrolled 60 individuals of normal control aged between 6 and 35 years old, including 35 males and 25 females without associated hereditary diseases.

Clinical data were collected prospectively by the referring physician. These data included results from eye examinations and, whenever possible, ultrasonography and fundus photography. Parents and siblings at risk were also invited to participate and an eye examination was performed, including best-corrected

visual acuity, ocular alignment, slit lamp examination, dilated fundus examination and, in some cases, intravenous fluorescein angiography (IVFA).

The ethnicity of each participant was recorded by the referring physician who completed the study history questionnaire. This information was used in the analysis of the novel mutation screening.

## **WES and variant analysis**

According to the principles of the Declaration of Helsinki, two-millilitre peripheral blood samples were collected from the probands, their parents, and siblings in tubes containing 0.2 M EDTA. Genomic DNA extraction was performed on these samples using standard protocols. Briefly, DNA was extracted from the venous blood of each subject with the QIAamp DNA blood extraction kit (TIANGEN, Beijing, China). Following extraction, 3 micrograms of genomic DNA was fragmented by Covaris 32, and the 3' end of each DNA fragment was A-tailed according to the manufacturer's protocol (MyGenostics, Inc., Beijing, China). Then, Illumina adapters were ligated to these fragments. To obtain a 350-400 base-pair product, all samples were checked with Nanodrop 2000 or Qubit systems to determine if they represented a captured library.

Each qualified captured library was loaded on an Illumina MiSeq 2000 sequencing platform, and the sequences were determined to ensure that each sample met the desired average sequencing coverage.

## **Mutation analysis**

Raw image files were processed for base calling and raw data generation with Bcl2Fastq software (Bcl2Fastq 2.18.0.12, Illumina, Inc.). In addition, low-quality variations were filtered out to achieve a quality score  $\geq 20$ . Then, the clean and high-quality reads were aligned to the reference human genome (UCSC hg19, <http://genome.ucsc.edu/>) using Short Oligonucleotide Analysis Package (SOAP) aligner software (SOAP2.21, [soap.genomics.org.cn/soapsnp.html](http://soap.genomics.org.cn/soapsnp.html)).

The polymerase chain reaction (PCR) duplicates were removed by using the Picard programme [47] [48]. The single nucleotide polymorphisms (SNPs) were determined by using the SOAP programme [49]. The reads were realigned using Burrows-Wheeler Aligner (BWA) software 0.7.15, and the deletions and insertions (indels) were detected with Genome Analysis Toolkit software 3.7. In addition, the identified indel SNPs were annotated with the Exome-assistant programme (<http://122.228.158.106/exomeassistant>). The non-synonymous variants were evaluated to determine their pathogenicity by four algorithms, namely, PolyPhen (<http://genetics.bwh.harvard.edu/pph2/>), Protein Analysis Through Evolutionary Relationships (PANTHER, [www.pantherdb.org](http://www.pantherdb.org)), Sorting Intolerant from Tolerant (SIFT, <http://sift.jcvi.org/>) and Pathogenic Mutation Prediction (Pmut, <http://mmb.pcb.ub.es/PMut/>).

## Mutation validation

In these families, Sanger sequencing and PCR (polymerase chain reaction) with an ABI3500 sequencer were used to confirm potential causative variants. Regions sequenced included the 5 coding exons of the *ZNF408* gene, the 6 coding exons of the *LRP5* gene, the 11 exons of the *KIF11* gene (including the 5' untranslated and promoter regions), the 8 coding exons of the *NR2E3* gene, the 2 coding exons of the *KRT3* gene, the 1 coding exons of the *FOXL2* gene. The sites of variation were identified in order to compare the DNA sequences using the corresponding GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) reference sequences. Table 1 displays the sequences of forward and reverse primers. Thermocycling conditions were as follows: an initial denaturation of 95°C for 10 min, 35 cycles of denaturation at 94°C for 30 sec., annealing at 64°C for 30 sec., extension at 72°C for 45 sec. and a final extension of 72°C for 5 min. The sequences of forward and reverse primers are in Table 4.

## Availability Of Data And Materials

The data sets used and analysed during this study are available from the corresponding author on reasonable request.

## Abbreviations

FEVR: Familial exudative vitreoretinopathy

SNVs: single nucleotide variants

InDels: small insertions/deletions

*FZD4*: Frizzled 4 receptor

*LRP5*: low-density-lipoprotein receptor-related protein 5

*NDP*: Norrie disease protein

*TSPAN12*: tetraspanin-12

*ZNF408*: zinc finger protein 408

*KIF11*: kinesin family member 11

AD: autosomal dominant

AR: autosomal recessive

*NR2E3*: nuclear receptor subfamily 2, group E, member 3 gene

*KRT3*: keratin 3

*FOXL2*: Forkhead Box L2 gene

IVFA: intravenous fluorescein angiography

SOAP: Short Oligonucleotide Analysis Package

PCR: polymerase chain reaction

SNPs: single nucleotide polymorphisms

SIFT: Sorting Intolerant from Tolerant

## **Declarations**

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## **Author information**

### **Author notes**

Zhen Zhang, Yi-shuang Xiao, Hong-chao Jiang should be considered joint first author.

### **Contributions**

Zhen Zhang, Yi-shuang Xiao and Hong-chao Jiang conceived and designed the experiments; Zhen Zhang, Ru Shen and Jing Ma performed the experiments; Zhen Zhang, Yi-shuang Xiao, Jing Ma, Hong-chao Jiang, Xiao-hong Yang and LiTang analysed the data; Yi-shuang Xiao, recruited patients and collected clinical information. Yi-shuang Xiao and Huai-yu Gu contributed to accumulation and interpretation of clinical data. Wen-Ji He and Zhen Zhang coordinated the project. All authors read and approved the final manuscript.

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### **Ethics approval and consent to participate**

This study was approved by the ethics committee of the Children's Hospital of Kunming Medical University. All the patients and their related family members accepting the genetic testing assigned a consent form.

### **Consent for publication**

The authors give their consent for publication of this manuscript.

### **Competing interests**

The authors declare that they have no competing interests.

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## Tables

Table 1 Part of Data of Analysis

No.	Reads (M)	Read length (bp)	Reads aligned to the human genome	Reads mapped to the target region	Mean coverage	SNPs		Indels,	
						Non-synonymous	Splice sites	Coding sequence	Splice sites
1	68.4	148	10155.88	10196.76	99.92%	10959	773	622	296
2	92.0	149	13700.82	13752.12	99.87%	10887	828	598	235
3	96.1	149	14283.23	14337.44	99.88%	11027	817	625	249
4	74.6	149	11098.47	11137.29	99.86%	10840	791	660	230
5	113.5	148	16803.20	16881.81	99.84%	10980	816	628	249
6	113.2	148	16729.23	16790.3	99.87%	10983	826	630	236

Table 2 The filtering for suspected sequencing artifacts

No.	Unknown	Synonymous	Nonsynonymous	Stopgain	Splicing	Nonframeshift	Frameshift
1	11	-	259	5	20	15	10
2	17	-	355	13	19	20	29
3	5	-	265	4	20	8	9
4	4	232	241	4	15	17	8
5	9	-	253	5	20	25	11
6	9	2	226	5	19	15	6

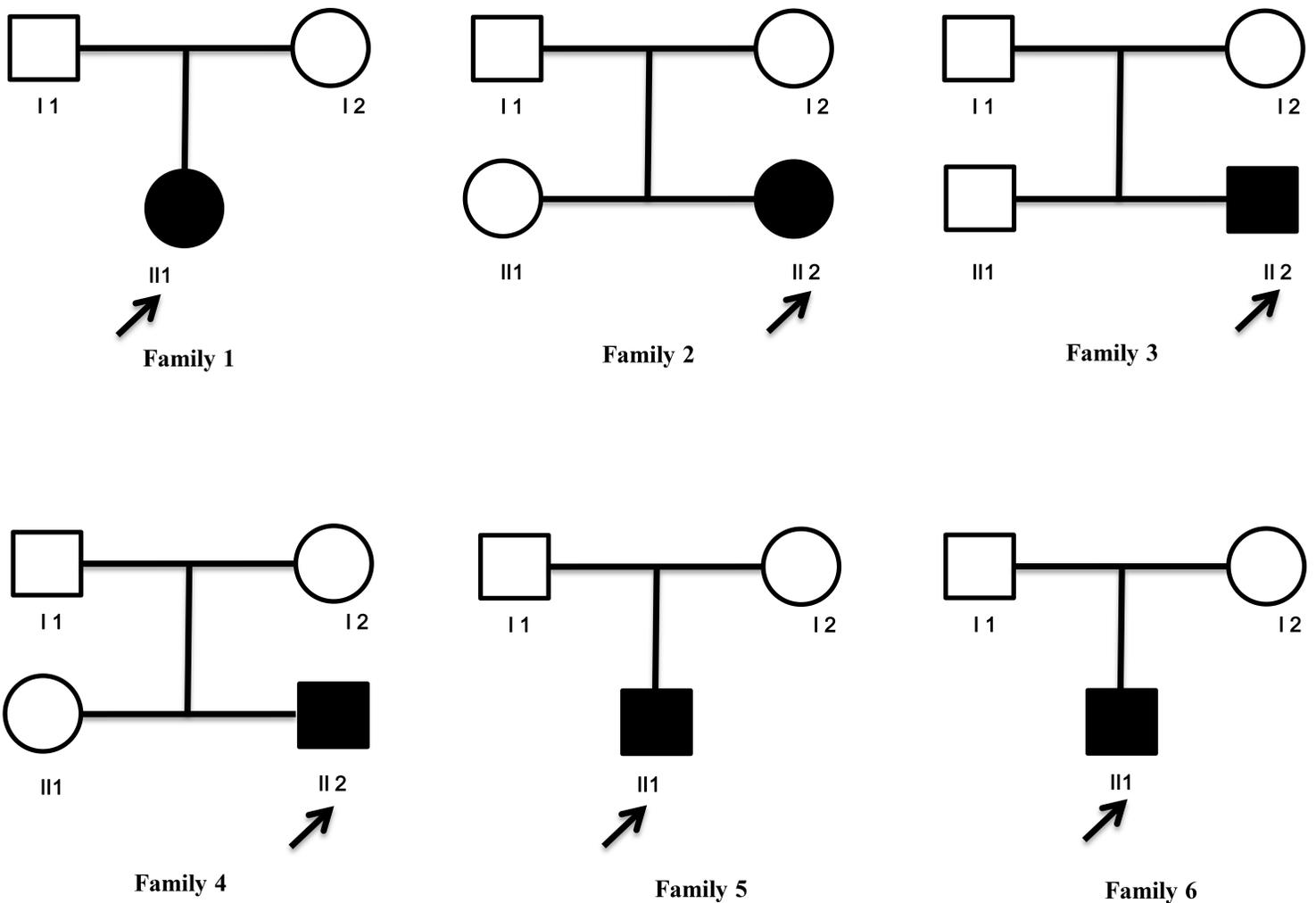
Table 3 Gene mutations of family 1 -6.

Gene	Exon	Amino Acid Changes	ID	Nucleotide Changes	Gene Type	Inheritance mode
<i>ZNF408</i>	5	p.G290S	chr11-46726142	c.868G>A	het	AD
<i>LRP5</i>	6	splicing	chr11-68153782	c.1016-2A>T	het	AR
<i>KIF11</i>	11	p.I423T	chr10-94388615	c.1268T>C	het	AD
<i>NR2E3</i>	8	p.H361R	chr15-72106440	c.1082A>G	het	AR
<i>KRT3</i>	2	p.R271H	chr12-53187949	c.812G>A	het	AD
<i>FOXL2</i>	1	p.333_337del	chr3-138664553-138664568	c.997_1011del	het	AD

Table 4. PCR primers for amplification

Gene	Exon	Forward primers	Reverse primers
<i>ZNF408</i>	5	TAAGTTCCCAACCCAGGACC	TGTACCACCTGGTGCTCTTTG
<i>LRP5</i>	6	ACCAGCCTTTGCAAGGAGAG	CGTGTCGGTCCAGTAGAGGT
<i>KIF11</i>	11	ATTGAGGGCAAGGGAAAAAT	GATCACGCTATTGCACTCCA
<i>NR2E3</i>	8	GCCACTCCTGTTGACTGTG	TGAACTGAGACCCCTTGTGCTG
<i>KRT3</i>	2	TTGCTGGAATTGAGGATTGTG	TGAATACTTCCATTTCATGCCC
<i>FOXL2</i>	1	TACCTGCAGTCTGGCTTCCT	CAGAGGGTGTGAGGTCAGG

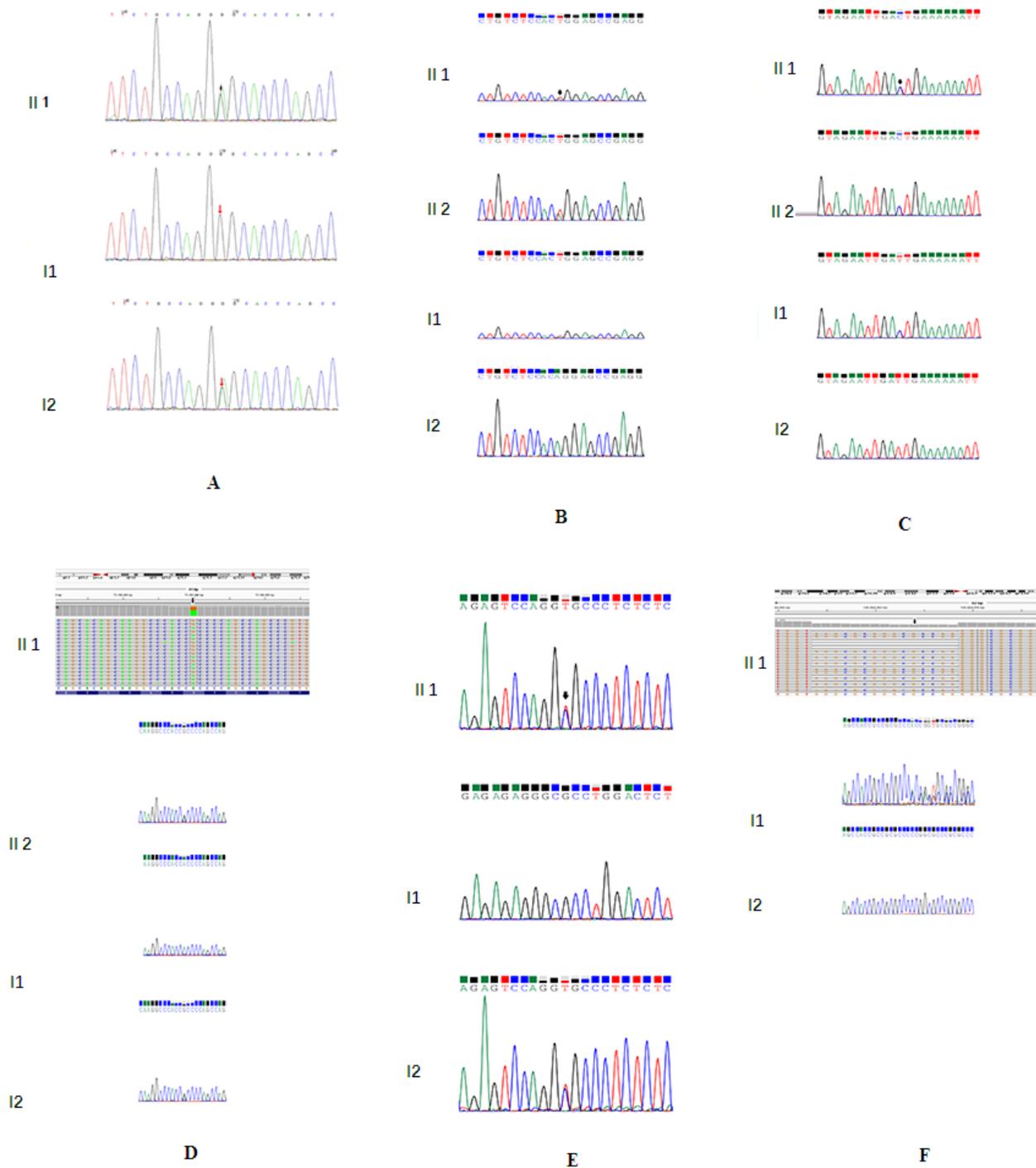
## Figures



**Figure 1**

Pedigrees of family with FEVR. Unaffected subjects are denoted as blank while affected subjects are represented with darkened symbols. The arrow indicates the proband. In all families, I 1 are the

unaffected father and I 2 are the unaffected mother; In families 1, 5 and 6, II 1 are the proband; In families 2, 3 and 4, II 1 are the unaffected sibling while II 2 are the proband.



**Figure 2**

Partial electropherograms of the genomic region covering the genes: (A) The heterozygous ZNF408 c.868G>A variant of the proband in family 1; (B) The heterozygous LRP5 c.1016-2A>T variant of the proband in family 2; (C) The heterozygous KIF11 c.1268T>C variant of the proband in family 3; (D) The heterozygous NR2E3 c.1082A>G variant of the proband in family 4; (E) The heterozygous KRT3 c.812G>A

variant of the proband in family 5; (F) The heterozygous FOXL2 c.997\_1011del variant of the proband in family 6. Arrows denote the mutations. II 1 the proband; II 2 unaffected sibling; I 1 the unaffected father; I 2 the unaffected mother