

Identification of a novel mutation in *KIF11* with functional analysis in a cohort of 516 familial patients with exudative vitreoretinopathy

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Purpose: To identify a novel mutation in *KIF11* with clinical and functional analysis among 516 familial patients with exudative vitreoretinopathy (FEVR).

Methods: Next-generation sequencing was performed on 516 patients with FEVR between January 2015 and October 2017. Clinical data were collected from patient charts, including sex, age at presentation, visual acuity if available, axial length, stage, and systemic clinical findings. Protein and mRNA levels were detected with western blotting and real-time quantitative PCR, respectively. Mass spectrometry was used to analyze the interacting protein of *KIF11*.

Results: In total, 304 of 516 patients were identified with at least one mutation in FEVR causative genes. Mutations in *KIF11* were identified in 14.47% of all carriers. The novel mutation p. H718L accounted for the greatest proportion (12/44; 27.30%) among all mutations in *KIF11*. Fundus presentations in these 12 individuals varied from the avascular zone of the peripheral retina to total retinal detachment. The p. H718L mutation can reduce the proliferation of human retinal endothelial cells (HRECs) compared to the wild type. The mRNA level of vascular endothelial growth factor- α , transforming growth factor- α , metalloproteinase-1, and angiopoietin-like 4 were depressed in the *KIF11* (p. H718L) groups under hypoxia stimuli. Mass spectrometry results demonstrated that eukaryotic elongation factor 2 (EEF2) was an interacting protein of *KIF11* and that the p. H718L mutation can attenuate the binding activity.

Conclusions: Patients with the most frequent *KIF11* mutation p. H718L showed typical FEVR presentations in this cohort. The mutation in *KIF11* likely plays a role in the proliferation of HRECs, and the p. H718L mutation can reduce the proliferation.

Familial exudative vitreoretinopathy (FEVR; OMIM 133780) is a rare hereditary developmental disorder first described by Criswick and Schepens in 1969 [1]. The clinical manifestations of FEVR are complicated and variable, ranging from aberrant and incomplete vascularization of the peripheral retina to total retinal detachment [2-4]. To date, approximately 50% of the clinically identified patients with FEVR have been found to be associated with the following five causative genes: *NDP* (OMIM 300658) [5], *FZD4* (OMIM 604579) [6], *LRP5* (OMIM 603506) [7,8], *TSPAN12* (OMIM 613138) [9-11], and *ZNF408* (NCBI79797) [12].

Recently, mutations in *KIF11* were first identified in patients with FEVR by Robitaille et al. [13] and subsequently by Huan Hu [14] and Peiquan Zhao [15]. Mutations in *KIF11* were reported to be associated with microcephaly,

lymphedema, and chorioretinal dysplasia (MLCRD, OMIM 152950), as well as chorioretinal dysplasia, microcephaly, and mental retardation (CDMMR, OMIM 156950) [16-21]. Phenotypic overlap between FEVR and MLCRD and CDMMR varies across studies. In addition, the roles of *KIF11* in retinal vascular development are not clear yet.

In the present study, we identified 44 patients with FEVR with mutations in *KIF11* based on next-generation sequencing (NGS) of 516 patients clinically diagnosed with FEVR. The novel mutation in *KIF11* (c.2153A>T; p. H718L) made up the highest proportion and was found in 12 (27.30%) of 44 individuals. The clinical findings of these 12 individuals with this novel mutation is described. We also examined the effect of the p. H718L mutation on the proliferation of human retinal endothelial cells (HRECs) with an in vitro system.

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METHODS

Participants and clinical data collection: This study was approved by the Institutional Review Board of the Xinhua Hospital affiliated with the Shanghai Jiaotong University School of Medicine. The research followed the tenets of the Declaration of Helsinki, and informed written consent was obtained from the parents or guardians of each participant because they were all minor children.

In the clinic, patients with clinically diagnosed FEVR routinely underwent a complete ophthalmologic evaluation, including visual acuity measurement (if available), anterior segment examination, ultrasound examination, indirect ophthalmoscopy with a 28D lens, fundus examination using a Retcam (Clarity Medical Systems, Pleasanton, CA) or Optos 200Tx (Optos, Inc., Marlborough, MA) imaging device, and wide-field fluorescein angiography to the ora serrate using Retcam under anesthesia or Spectralis HRA2 (Heidelberg Engineering GmbH, Heidelberg, Germany) based on the patients' age. Additionally, wide-field fluorescein angiography was routinely performed on patients' direct family members, primarily parents and siblings (if any) who could tolerate fluorescein sodium using Spectralis HRA2 in the clinic when available. Optos imaging was performed on family members who could not tolerate fluorescein sodium. Data collected from the patients' charts included sex, gestational age at birth, birthweight, age at presentation, affected eye, family history, initial diagnosis, visual acuity if available, and fundus presentation of the affected eye. All participants were born full-term. The disease severities of all participants were further classified according to the staging system described by Ranchod et al. [22]. The eyes with FEVR were staged using the clinical staging criteria for FEVR described previously.

Genetic testing: Targeted gene capture and sequencing were performed by MyGenostics (Baltimore, MD).

Briefly, peripheral blood was drawn from each proband and his or her direct family members using a whole blood DNA extraction kit (BioTeke, Beijing, China). Venous blood in EDTA vacutainers was stored in 4 °C and processed within 24 h after blood drawn. Then the genomic DNA was extracted and fragmented. Illumina adapters were added to the fragments, and the samples were size-selected for the 350–400 bp products. This pool of DNA fragments was amplified using PCR and allowed to hybridize with DNA capture probes specifically designed for the targeted genes. The captured DNA fragments were eluted, amplified again, and subjected to NGS using an Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA). A custom Genetic Pediatric Retinal Diseases Panel based on targeted exome capture technology was used

and covered the following 21 genes: *ABCB6* (OMIM 605452), *GDF6* (OMIM 601147), *LRP5*, *RS1* (OMIM 312700), *SOX2* (OMIM 184429), *TENM3* (OMIM 610083), *VSX2* (OMIM 142993), *FZD4*, *IKBKKG* (OMIM 300248), *NDP*, *SALL2* (OMIM 602219), *STRA6* (OMIM 610745), *TSPAN12*, *YAPI* (OMIM 606608), *GDF3* (OMIM 610522), *KIF11* (OMIM 148760), *PAX6* (OMIM 607108), *SHH* (OMIM 600725), *TBX1* (OMIM 602054), *TUBA8* (OMIM 605742), *TUBA8*, and *ZNF408*.

Data analysis: The sequenced reads were mapped to the UCSC hg19 human reference genome using the Burrows Wheeler Aligner (BWA). Variants were detected with GATK and further annotated using the 1000 Genomes database, ESP6500, Single Nucleotide Polymorphism Database (dbSNP), and the company's own in-house database of 800 samples. The pathogenicity of the variant was assessed with the following databases: PolyPhen-2, Sorting Intolerant From Tolerant (SIFT), MutationTaster, and GERP+ +.

PCR and Sanger sequencing validation: Primer3 was used to design all of the PCR primers for the Sanger sequencing that was conducted to validate the potential pathogenic variants. The average amplicon size was 400 bp. The DNA was sequenced on the ABI 3130XL platform and then analyzed using Mutation Surveyor.

Plasmids and antibodies: The cDNA of *KIF11* was a gift from Han Jiahui Lab. Flag-*KIF11* was generated using standard cloning procedures (Vazyme Biotech Co., Ltd, Piscataway, NJ). Flag-*KIF11*-H718L was generated using site-directed mutagenesis (StrateGene, Palo Alto, CA). Antibody against FLAG M2 was purchased from Sigma (Saint Louis, MO), and Myc and actin from Cell Signaling Technology (Boston, MA).

HREC culture: The HRECs were isolated as previously described [23]. The cells were grown in a human endothelial medium supplemented with 10% fetal bovine serum (FBS), 5 µg/l β-ECGF, and 1% (v/v) penicillin–streptomycin at 37 °C in 5% CO₂ and 95% air atmosphere. Cells at passages 3 to 6 were used in all experiments.

Immunoprecipitation and immunoblotting: Cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4), 400 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, and a mixture of protease inhibitors, and cleared with centrifugation. The cleared cell lysates were incubated with 10 µl of anti-FLAG M2-agarose affinity gel (Sigma) for 2 h. After extensive washing, the beads were boiled at 100 °C for 10 min. Proteins were resolved with SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene

TABLE 1. THE PRIMERS FOR REAL-TIME RT-PCR WERE AS FOLLOWS.

Gene	Primer (5'-3')
18S	F: AGTCCCTGCCCTTTGTACACA R: CGATCCGAGGGCCTCACTA
KIF11	F: TCCCTTGGCTGGTATAATTCCA R: GTTACGGGGATCATCAAACATCT
Endothelial growth factor-a (VEGF-a)	F: AGGGCAGAATCAGCACGAAGT R: AGGGTCTCGATTGGATGGCA
Transforming growth factor-a (TGF-a)	F: AGGTCCGAAAACACTGTGAGT R: AGCAAGCGTTCTTCCCTTC
Metalloproteinases 1(MMP1)	F: CTTCTGCAATTCCGAACCTCGT R: ACGCTGGTATAAGGTGGTCTG
Angiopoietin-like 4(ANGPLT4)	F: GTCCACCGACCTCCCGTTA R: CCTCATGGTCTAGGTGCTTGT

difluoride (PVDF) membranes (Millipore, Darmstadt, Germany), followed by immunoblotting using corresponding antibodies according to the manufacturer's instructions. Immunoblots were analyzed using the LAS-4000 system (Fujifilm, Tokyo, Japan). The mass spectrometry method is described in the Appendix 1.

Stable cell lines: Flag-*KIF11* or Flag-*KIF11*-H718L lentiviral plasmids were cotransfected into human embryonic kidney (HEK) 293T cells with lentivirus packaging vectors with the calcium phosphate-DNA coprecipitation method. Viral supernatants were collected 48 h after transfection. HRECs were infected with the lentiviral supernatant in the presence of 8 µg/ml polybrene for 12 h. Cells were sorted for green fluorescent protein (GFP)-positive cells with flow cytometry 72 h after infection. Nineteen short tandem repeat (STR) loci plus the gender-determining locus, amelogenin, were amplified using the commercially available EX20 Kit from AGCU (Wuxi, China). The cell line sample was processed using the ABI Prism® 3500 Genetic Analyzer. Data were analyzed using GeneMapper® ID-X v1.2 software (Applied Biosystems, Suzhou, China). Appropriate positive and negative controls were run and confirmed for each sample submitted. The STR analyses are presented in Appendix 2.

RNA isolation and quantitative real-time PCR (qPCR): Total RNA was isolated from the cells by using TriPure isolation reagent (Roche, Basel, Switzerland). The reverse transcription PCR kit from Takara company of Japan was used under the following conditions: 37 °C, 15 min for reverse transcription reaction; 85 °C, 5 s for reverse transcriptase inactivation reaction; 4 °C, the end of reaction, the product was cDNA. For mRNA analysis, an aliquot containing 2 µg of total RNA was reverse-transcribed using the cDNA synthesis kit (Takara,

Kyoto, Japan). Real-time quantitative PCR was performed using SYBR Green PCR master mix (Applied Biosystems, Foster, CA) and detected with the ABI Prism 7500 sequence detection system (Applied Biosystems). The primers for qPCR are in Table 1.

7-AAD viability staining: The cell pellets were resuspended in 0.5 ml of Cell Staining Buffer, to which 5 µl of 7-amino-actinomycin D (7-AAD) per million cells was added, and incubated for 5–10 min in the dark before analysis. Evaluated on a FACSCanto flow cytometer (Becton Dickinson, Franklin Lake, NJ). Data were further analyzed using FlowJo software (Tree Star, Alpharetta, GA).

Statistical analysis: All data shown represent the results obtained from triplicate independent experiments and are expressed as mean ± standard deviation (SD) values. Statistical analyses were performed with a two-tailed unpaired Student *t* test. A *p* value of less than 0.05 was considered statistically significant.

Data availability: The data sets generated or analyzed during the present study are available from the corresponding author on reasonable request.

RESULTS

Cohort description and clinical date of the 12 probands with c.2153A>T (p. H718L) mutation in KIF11: NGS was performed on 516 patients with a clinical diagnosis of FEVR. Among them, 12 participants with the c.2153A>T (p. H718L) mutation in *KIF11* were enrolled. The rate of this novel mutation was highest (27.30%) in the 44 probands with mutations in *KIF11* among all 304 carriers of FEVR causative genes (*LRP5*, *FZD4*, *NDP*, *TSPAN12*, *ZNF408*, and *KIF11*) in the present Chinese cohort. This mutation involves a highly

evolutionarily conserved residue. Demographic and clinical data are summarized in Table 2. The pedigrees of the 12 unrelated families and chromatograms are shown in Figure 1.

Visual acuity was not available in nine probands because of noncooperation. The visual acuity of the remaining three individuals varied from 0.6 to light perception. Fundus presentations varied significantly, ranging from the avascular zone of the peripheral retina to total retinal detachment. The representative images are shown in Figure 2. Interestingly, three individuals presented as entirely unilateral FEVR (Figure 3). For the remaining nine individuals, the expression of clinical features was symmetric in five individuals and asymmetric in four individuals. Further, microcephaly with mild learning difficulties was identified in two patients, while lymphedema was not present in any of the patients.

For proband 12, we detected the *LRP5* mutation c.121C>T (p. R41W) in his affected father and the *LRP5* mutation c.1480C>T (p. R494W) in his affected mother. The mutation in *KIF11* p. H718L was also identified in his affected mother and affected sister. In total, proband 12 carried three mutations, one mutation in *KIF11* and two mutations in *LRP5*. During the fundus examination, an avascular zone and exudation on temporal side were observed in the right eye. The left eye exhibited total retinal detachment with a retrolenticular fibrotic mass and a shallow anterior chamber. Unfortunately, falciform retinal detachment developed in the right eye during follow-up (Figure 4). The 12 family members who carried the *KIF11* mutation p. H718L and the one family member who carried the *LRP5* mutation p. R41W presented as FEVR stage 1 as indicated with fluorescein fundus angiography (FFA) examination (Figure 5).

Pathogenicity evaluation with bioinformatics tools: The pathogenicity of the variant c.2153A>T (p. H718L) is possibly damaging (PolyPhen-2), tolerable (SIFT), disease causing (MutationTaster), and conserved (GERP+ +).

KIF11 (wild-type) and KIF11 (p. H718L) were stably overexpressed in HRECs: Retinal endothelial cells form the inner vascular lining and have an important role in retinal vascular development. To address the key role of this site, stable cell lines of HRECs overexpressing human *KIF11* (wild-type, WT) and *KIF11* (p. H718L) mutation proteins were established, demonstrated at the protein and mRNA levels with western blot assay using anti-FLAG antibody and real time q-PCR, respectively (Figure 6A,B).

The p. H718L mutation in KIF11 can reduce the proliferation but not affect apoptosis of HRECs: After being cultured in a human endothelial medium for 3 days, the number of HRECs was counted with the Countess II Automated Cell

Counter (Thermo Fisher, Waltham, MA). The cell number of the vector group increased from 1×10^4 to 3.53×10^4 , that for the *KIF11* (WT) group increased from 1×10^4 to 4.63×10^4 , and that for the *KIF11* (p. H718L) group increased from 1×10^4 to 3.97×10^4 . The cell number between *KIF11* (WT) and *KIF11* (p. H718L) had a statistically significant difference ($p=0.032589$; Figure 6C). Thus, the p. H718L mutation in *KIF11* may affect the proliferation or apoptosis of HRECs. To ascertain the effect on apoptosis, apoptosis between the *KIF11* (WT) group and the *KIF11* (p. H718L) group was compared. HREC stable cell lines were stained with 7-AAD. However, there was no difference in the percentage of 7-AAD-positive HRECs between the *KIF11* (WT) and *KIF11* (p. H718L) groups. The results demonstrated that the p. H718L mutation in *KIF11* reduced the proliferation but did not affect apoptosis of the HRECs (Figure 6D).

The mRNA levels of VEGF- α , TGF- α , MMP1, and ANGPT4 were depressed in the KIF11 (p. H718L) group under hypoxia stimuli: It has been reported that *KIF11* can be induced by VEGF- α , and *KIF11* blockade inhibits proliferation of endothelial cells and tumor cell lines [24]. As known, hypoxia is a potent stimulator of VEGF expression. Exposure to hypoxia may impose an additive effect on the expression of VEGF in retinal endothelial cells [25]. Thus, we cultured stable cell lines in hypoxia conditions (1%) and detected the mRNA level of several angiogenic factors, including VEGF- α , TGF- α , MMP1, and ANGPT4. After 24 h, HREC stable cell lines vector, *KIF11* (WT), and *KIF11* (p. H718L) with or without hypoxia treatment, were collected, RNA extracted, and quantitative real-time PCR performed to observe the mRNA level of these angiogenic genes (Figure 7A). The results revealed that overexpression of *KIF11* (WT) upregulated the expression of these angiogenic genes significantly upon hypoxia stimuli. However, overexpression of *KIF11* (p. H718L) only slightly increased the expression of these angiogenic genes in HRECs when treated with hypoxia. As shown in Figure 7A, the depressed mRNA level of these angiogenic genes in HRECs, caused by the c.2153A>T (p. H718L) mutation in *KIF11*, were 20.7% ($p=0.041628$) for VEGF- α , 52.0% ($p=0.009351$) for TGF- α , 48.3% ($p=0.013752$) for MMP1, and 23.1% ($p=0.044253$) for ANGPT4, respectively, when treated with hypoxia. These results illustrate that the p. H718L mutation in *KIF11* may reduce the proliferation of HRECs through the depressed expression of angiogenic genes.

EEF2 is an interacting protein of KIF11 and the p. H718L mutation affects this interaction: An immunoprecipitation (IP-Flag) experiment was performed on HREC stable cell lines to enrich *KIF11* and was subsequently followed by mass spectrometry analysis. In total, we identified 79

TABLE 2. DEMOGRAPHIC AND CLINICAL DATA OF TWELVE FEVR PATIENTS WITH THE NOVEL *KIF11* c.2153A>T (p. H718L) MUTATION.

Proband No.	Gender	Age at presentation	Visual acuity		Axial length(mm)		Stage		Systemic clinical findings
			OD	OS	OD	OS	OD	OS	
1	F	2y	NA	NA	21	20.8	4A	4A	Normal
2	F	5m	NA	NA	16.7	15.9	5B	5B	Normal
3	M	8y	0.3	0.5	20.8	21.5	1A	1B	Microcephaly (-2D); Mild learning difficulties
4	F	1m	NA	NA	19.4	15.9	Normal	5B	Normal
5	M	2m	NA	NA	15.1	15.6	4A	4A	Microcephaly (-2D); Mild learning difficulties
6	M	9m	NA	NA	19.6	21.6	4B	1A	Normal
7	M	6y	1	FC/20cm	21.1	20.5	Normal	4A	Normal
8	M	4y	LP	0.6	17.7	20.1	4A	1	Normal
9	M	1y	NA	NA	16.6	20.9	5b	Normal	Normal
10	M	5m	NA	NA	17.8	18.1	1	1	Normal
11	M	4m	NA	NA	17.3	15.2	1	5B	Normal
12	M	5m	NA	NA	18.2	15.7	1B	5B	Normal

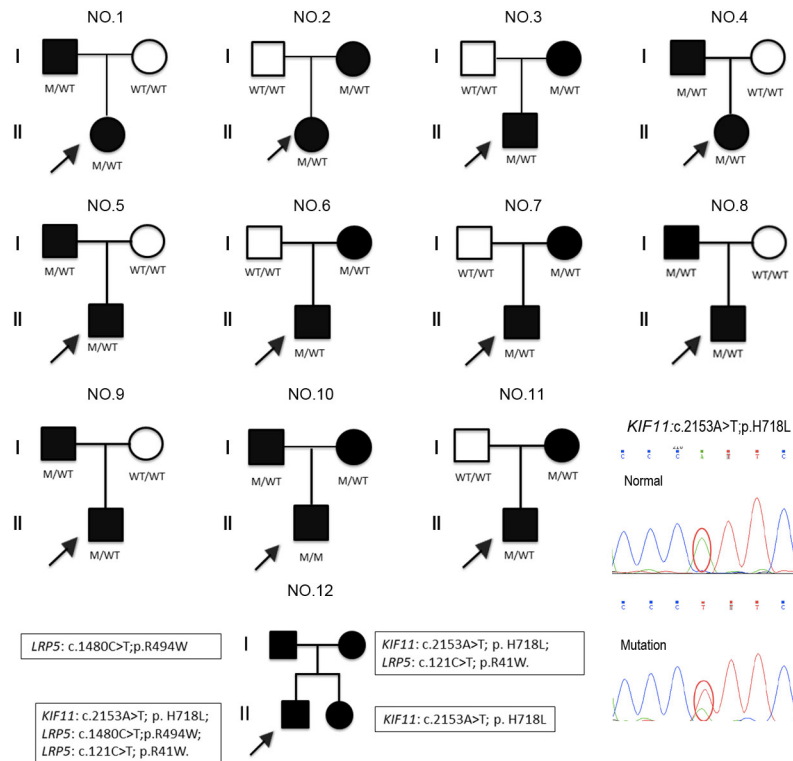


Figure 1. Pedigrees and chromatograms of the *KIF11* mutation c.2153A>T (p. H718L). In the pedigrees, M sign represents a variant; wild type (WT) represents a normal allele; arrows, probands; squares, males; circles, girls; filled symbols, affected individuals; open symbols, unaffected individuals. In the chromatograms, the variation is marked with a red circle. In pedigree 12, I-1 carries one mutation in *LRP5*, and II-2 carries one mutation in *KIF11* and a different mutation in *LRP5*. The proband carries two different mutations in *LRP5* and one mutation in *KIF11*.

proteins interacting with wild type or mutant *KIF11* through mass spectrometry. Ten interacting proteins were chosen according to the difference of interactive ability between the two groups and are listed in Appendix 3. Among them, the interactive ability of three proteins has a statistically significant difference between wild-type and mutant *KIF11* (Appendix 3). The protein–protein interaction network was added as Appendix 4 and Appendix 5. As the most significant difference and the core position in the interaction network, we picked *EEF2* for further research (Figure 7B,C). *EEF2* is a protein that is encoded by the *EEF2* (ID 1938, OMIM 130610) gene in humans. This protein is an essential factor for protein synthesis [26,27]. It promotes the GTP-dependent translocation of the ribosome [27]. To confirm these data, we cotransfected Flag-*KIF11* and Flag-*KIF11*-H718L with Myc-*EEF2*, respectively. The immunoprecipitation experiment was performed to check the specificity of the interaction between *KIF11* and *EEF2*. We detected the binding of *KIF11* (WT) and *KIF11* (p. H718L) to *EEF2*; the binding activity was attenuated in the *KIF11* (p. H718L) group (Figure 7B,C).

DISCUSSION

In this study, we detected a novel *KIF11* c.2153A>T (p. H718L) mutation in 12 probands with FEVR. We further verified the effect of this mutation on the proliferation of HRECs and analyzed the interacting protein of *KIF11* with mass spectrometry. To our knowledge, this study is the largest cohort study that has screened for mutations in *KIF11* in patients with FEVR.

It has been reported that ocular phenotypes vary across studies, including unclassified chorioretinal dysplasia, retinal folds, microphthalmia, myopic, and hypermetropic astigmatism, in patients with MLCRD or CDMMR syndrome [16,18,19,28]. In Ostergaard's study, chorioretinopathy was found in only five of 19 (26.3%) individuals with MLCRD and in five of eight (62.5%) individuals with CDMMR. One individual with CDMMR presented as retinal detachment in the right eye and peripheral retinal atrophy in the left eye [16].

Similar to ocular phenotypes, systemic anomalies vary across studies. In Ostergaard's study, microcephaly was

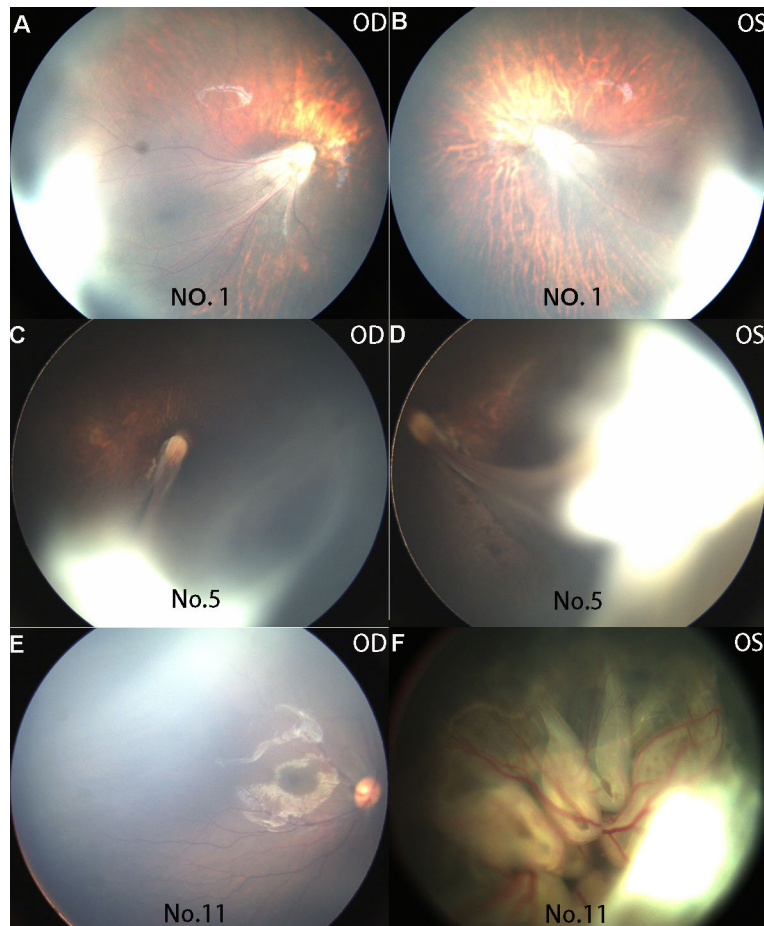


Figure 2. Representative fundus presentations of patients with the *KIF11* mutation c.2153A>T (p. H718L). Ocular phenotypes included temporal disc dragging (A, B), knife-like retinal fold (C, D), aberrant peripheral retinal vessels (E), and total retinal detachment (F). The patient number is marked at the bottom of each fundus photo. OD and OS represent the right and the left eye, respectively.

identified in all patients, and lymphedema was observed in 13 of 19 (68.4%) patients with MLCRD [16]. Robitaille et al. reported that mutations in *KIF11* were detected in four patients, all of whom showed typical signs of FEVR, two with microcephaly and none with mental retardation or lymphedema. In Huan Hu's study, mutations in *KIF11* were identified in seven patients with FEVR, including four patients with microcephaly and one with mental retardation [14]. In our previous study, three patients with microcephaly and one with mild mental retardation were identified out of seven patients with FEVR [15]. We identified two probands with microcephaly and mild learning difficulties in 12 patients with FEVR with the p. H718L mutation.

Variable expressivity of this mutation in *KIF11* (c.2153A>T; p. H718L) was also observed in the present study. The ocular presentations ranged from aberrant peripheral vascularization to total retinal detachment. In our previous study, we first described the clinical and genetic features of familial exudative vitreoretinopathy with only unilateral abnormalities in the Chinese cohort [29]. In this study, we

show for the first time that patients with mutations in *KIF11* may also present as entirely unilateral FEVR. Interestingly, retinal vascularization of three eyes of three patients was perfectly normal demonstrated with wide-field angiography. The mechanisms of variable ocular and systemic phenotypes of patients with mutations in *KIF11* are not yet clear. The present findings support the conceptualization that the combination of microcephaly, lymphedema, and chorio-retinopathy constitutes a single autosomal dominant genetic entity with variable expression. However, the question of why some mutations in *KIF11* cause broader defects that manifest as MLCRD or CDMMR while others cause ocular phenotypes identical to FEVR remains. This alone warrants further studies to investigate the roles of *KIF11* in central nervous system and retinal vascular development.

To date, only two previous studies reported that progression of chorioretinal atrophy may occur in patients with mutations in *KIF11* [30,31]. In this study, progression was observed in two probands, of whom one developed from retinal fold to exudative retinal detachment, and the other developed from

intraretinal exudation to retinal fold during the observation period. These observations verify that progression may also occur in patients with FEVR with mutations in *KIF11*.

To address the function of the 718-site in *KIF11* in retinal vascular development, we established an in vitro system by overexpressing *KIF11* (WT) and *KIF11* (p. H718L) in HREC. In the present study, the proliferation of HRECs decreased in the *KIF11* (p. H718L) group, and the 7-AAD staining showed that the frequency of apoptosis was comparable between the two groups. The findings thus suggest that the p. H718L mutation in *KIF11* can reduce proliferation without affecting apoptosis.

KIF11 encodes for Eg5, otherwise known as KIF11, which is a motor protein part of the kinesin family and required for mitotic progression [16,32]. In the present study, we identified elongation factor 2 (EEF2) was an interacting protein of KIF11 with mass spectrometry, and the p. H718L mutation affected their interaction. A 2011 study by Saunders et al. demonstrated *KIF11* was essential for polypeptide synthesis and ribosomes associate with microtubules through KIF11 [33]. In this study, we further discovered KIF11 can interact with EEF2, and the p. H718L mutation in *KIF11* affects this interaction. EEF2 catalyzes the GTP-dependent

ribosomal translocation step during translation elongation and finally, affects the translation of the protein. In the present study, we detected that the expression of several angiogenic factors (VEGF- α , TGF- α , MMP1, and ANGPT4) was lower in the *KIF11* (p. H718L) group. We speculated that the attenuated interaction between KIF11 and EEF2 caused by the p. H718L mutation in *KIF11* directly or indirectly led to lower expression of these angiogenic factors in the *KIF11* (p. H718L) group, and thus, affected the proliferation of HRECs.

In conclusion, the probands harboring the *KIF11* mutation c.2153A>T (p. H718L) showed typical FEVR presentation. We have shown the variable expression of the phenotype in these carriers. We demonstrated the p. H718L mutation in *KIF11* reduced the proliferation of HRECs by depressing the expression of several angiogenic factors. Further, we discovered this mutation inhibited the interaction between KIF11 and EEF2, and EEF2 was essential for protein synthesis. Further study is warranted to elucidate the mechanisms of the interaction of KIF11 and EEF2 involved in retinal vascular development.

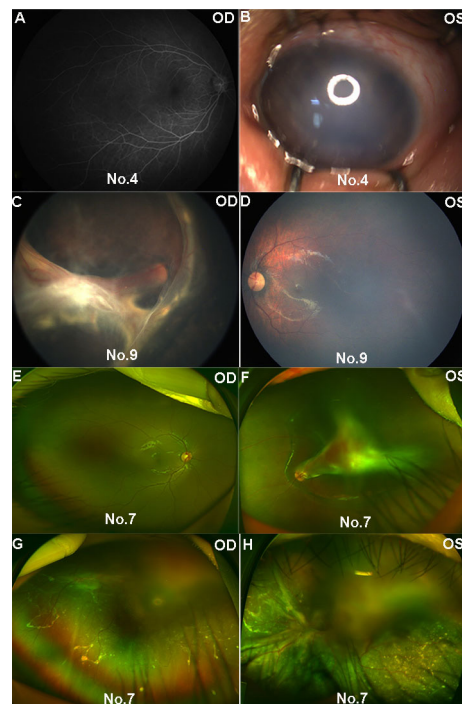


Figure 3. Three probands with the *KIF11* mutation c.2153A>T (p. H718L) present as entirely unilateral familial exudative vitreoretinopathy. **A, B**: The right eye is healthy, and the left eye presents as total retinal detachment with shallow anterior chamber in a 1-month old girl. **C, D**: Proband 4: The right eye of a 1-year old boy shows retinal fold with proliferation and exudation. The left eye is normal. **E, F**: Proband 9: The fundus images of a 7-year-old boy show that the right eye is normal, and a falciform retinal fold is observed in the left eye. **G, H**: Proband 7: During follow-up, the

retinal fold progressed to exudative retinal detachment gradually in the left eye of Proband 7. OD and OS represent the right and left eye, respectively.

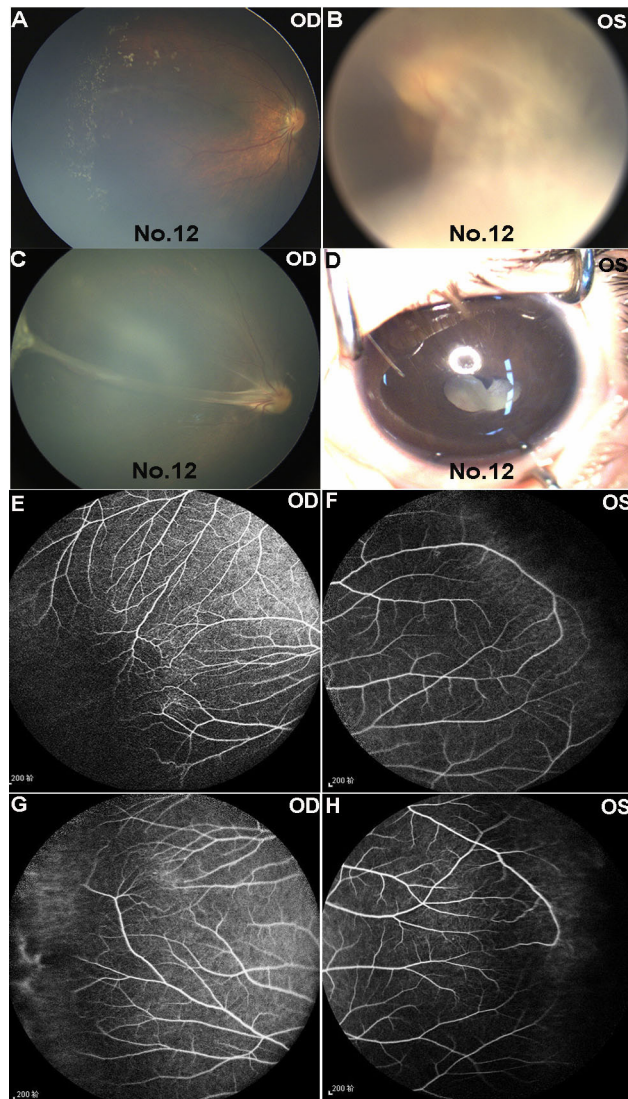


Figure 4. Fundus images of proband 12 (*LRP5* mutation p. R494W, *LRP5* mutation p. R41W, and *KIF11* mutation p. H718L). **A**: Avascular zone and exudation are observed in the right eye. **B**: The left eye exhibits total retinal detachment with a retrolenticular fibrotic mass. **C**, **D**: During follow-up, falciform retinal detachment developed in the right eye, and the left eye remained total retinal detachment. OD and OS represent the right and left eye, respectively. **E**, **F**: Peripheral aberrant vascularization and avascular zone are observed in the proband's affected father (*LRP5* mutation p. R41W, *KIF11* mutation p. H718L). **G**, **H**: The affected mother presents as avascular zone and peripheral aberrant vascularization (*LRP5* mutation p. R494W).

APPENDIX 1. THE METHOD OF MASS SPECTROMETRY.

To access the data, click or select the words "[Appendix 1.](#)"

APPENDIX 2. STR ANALYSIS.

To access the data, click or select the words "[Appendix 2.](#)"

APPENDIX 3. THE TEN PROTEINS CHOSEN ACCORDING TO DIFFERENCE OF INTERACTIVE ABILITY BETWEEN TWO GROUPS.

To access the data, click or select the words "[Appendix 3.](#)"

APPENDIX 4. THE PPI NETWORK GENERATED BY STRING OF THE 10 SELECTED INTERACTIVE PROTEINS.

To access the data, click or select the words "[Appendix 4.](#)"

APPENDIX 5. THE PPI NETWORK GENERATED BY STRING OF THE TOTAL 79 IDENTIFIED PROTEINS INTERACTING WITH WIDE TYPE OR MUTANT KIF11 THROUGH MASS SPECTROMETRY.

To access the data, click or select the words "[Appendix 5.](#)"

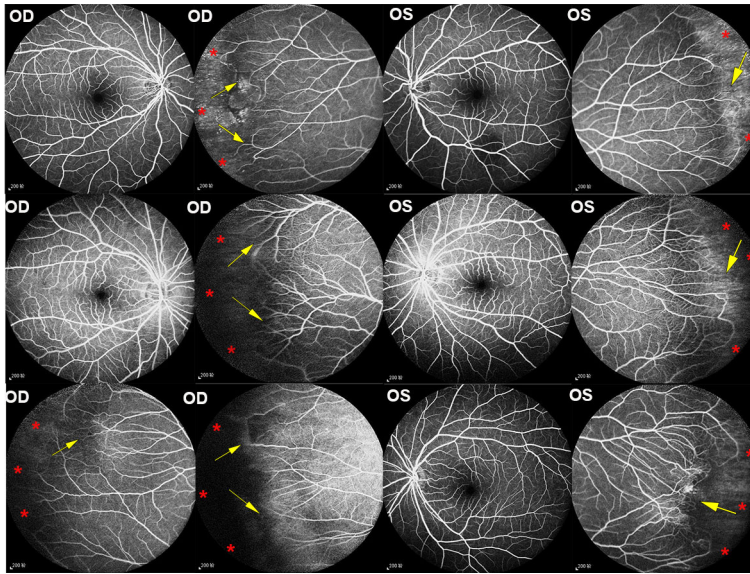


Figure 5. Fluorescein angiography images of probands' parents who carry the *KIF11* mutation p. H718L. Due to the normal posterior retinal vessels, their visual acuity was completely normal. However, the avascular area (red asterisk) and peripheral aberrant vascularization (yellow arrow) were detected after the fluorescein angiography examination. OD and OS represent the right and left eye, respectively.

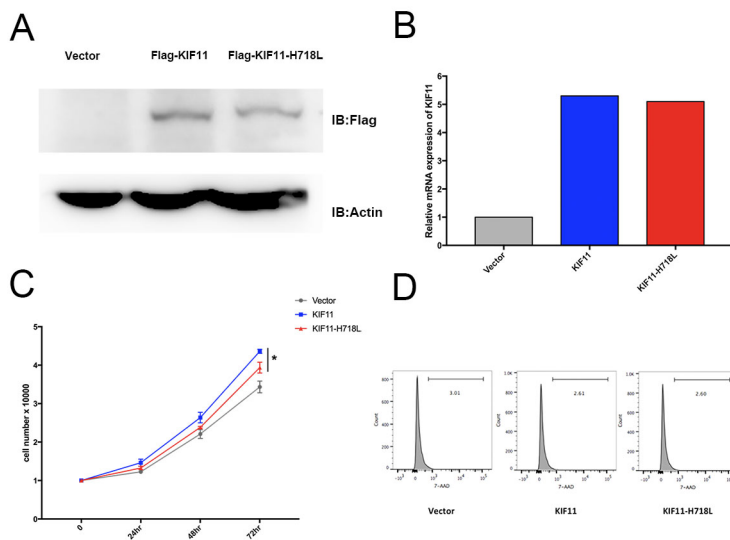


Figure 6. The p. H718L mutation in *KIF11* can reduce the proliferation but not affect apoptosis of HRECs. **A, B:** Stable cell lines of human retinal epithelial cells (HRECs) overexpressing human *KIF11* (wild-type) and *KIF11* (p. H718L) mutant proteins are established and demonstrated at the protein and mRNA levels with western blot assay using anti-FLAG antibody and real time quantitative real-time PCR (q-PCR), respectively. HREC stable cell lines were cultured in a human endothelial medium for 3 days. HREC numbers were counted with the Countess II Automated Cell Counter (Thermo Fisher). **C:**

The cell number of the vector group increased from 1×10^4 to 3.53×10^4 (gray line), *KIF11* (wild-type) group went from 1×10^4 to 4.63×10^4 (blue line), and *KIF11* (p. H718L) group increased from 1×10^4 to 3.97×10^4 (red line). The cell number between the *KIF11* (wild-type) and *KIF11* (p. H718L) groups showed a statistically significant difference ($p=0.032589$). **D:** HREC stable cell lines are stained with 7-amino-actinomycin D (7-AAD). There is no difference in the percentage of 7-AAD-positive HRECs between the *KIF11* (wild-type) and *KIF11* (p. H718L) groups.

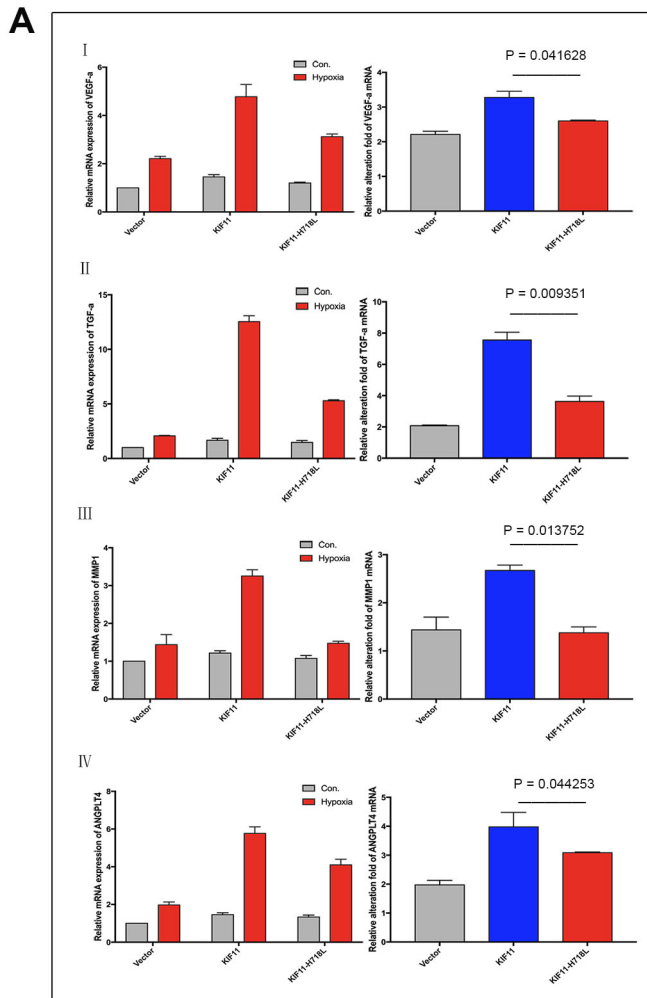
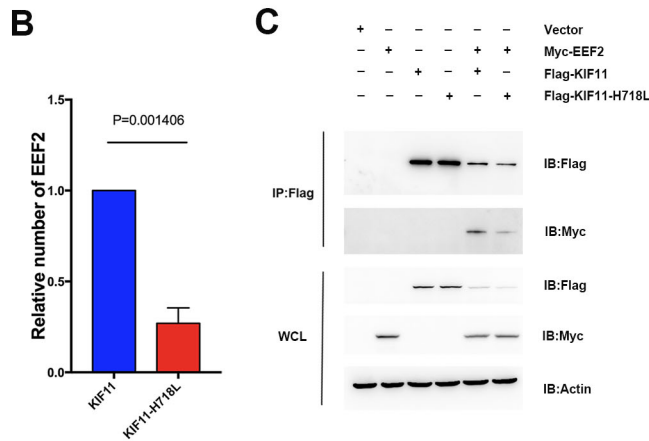


Figure 7. The mRNA level changes of angiogenic factors and mass spectrometry results. **A:** In the left of the graph, the red bar and the gray bar represent the mRNA level of vascular endothelial growth factor- α (VEGF- α , I), transforming growth factor- α (TGF- α , II), metalloproteinases 1 (MMP1, III), and angiotensin-like 4 (ANGPT4, IV) with and without hypoxia treatment, respectively. Overexpression of *KIF11* (wild-type) upregulated the expression of these angiogenic genes significantly upon hypoxia stimuli. However, overexpression of *KIF11* (p. H718L) increased the expression of these angiogenic genes only slightly in HRECs when treated with hypoxia. The relative alteration folds of these angiogenic factors are shown in the right of the graph. The depressed mRNA level of these angiogenic genes in HRECs, caused by the *KIF11* mutation p. H718L, were 20.7% for VEGF- α , 52.0% for TGF- α , 48.3% for MMP1, and 23.1% for ANGPT4, when treated with hypoxia. **B:** HREC stable cell lines vector, *KIF11* (wild-type), and *KIF11* (p. H718L) underwent immunoprecipitation (IP-Flag) to enrich *KIF11*, followed by mass spectrometry proteomic analysis. As a result, we identified that the eukaryotic elongation factor 2 (EEF2) was an interacting protein of *KIF11*. **C:** 293T cells were cotransfected with Flag-*KIF11*, Flag-*KIF11*-H718L, and Myc-*EEF2* as indicated. Flag-*KIF11* and Flag-*KIF11*-H718L proteins were pulled down by FLAG M2 beads from these cell lysates, respectively. Whole Cell lysate (WCL) was immunoblotted with anti-FLAG and anti-Myc antibody.



We detected the binding of *KIF11* (WT) and *KIF11* (p. H718L) to EEF2; however, the binding activity was attenuated in the *KIF11* (p. H718L) group.

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