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Variable reduction in Norrin signaling activity caused by novel mutations in *FZD4* identified in patients with familial exudative vitreoretinopathy

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Purpose: To identify novel mutations in *FZD4* and to investigate their pathogenicity in a cohort of Chinese patients with familial exudative vitreoretinopathy (FEVR).
Methods: Next-generation sequencing was performed in patients with a clinical diagnosis of FEVR. Wide-field angiography was performed in probands and family members if available. Clinical data were collected from patient charts. The effect of the mutations in *FZD4* on its biologic activity in the Norrin/β-catenin signaling pathway was analyzed with the luciferase reporter assay.
Results: Four novel mutations in *FZD4* (c.1188_1192del/p.F396fs, c.1220delC/p.A407Vfs*24, c.905G>A/p.C302Y, c.1325T>A/p.V442E) were identified in four unrelated families. The mutations were not detected in 200 healthy individuals. The variability of the ocular phenotypes was not only observed in the probands and parents harboring the same mutation but also between two eyes in one individual. All four novel mutations introduced reduction in luciferase activity. Compared with the wild-type, the FZD4 level of the four mutants also decreased variably.
Conclusions: Four novel mutations in *FZD4* were identified in Chinese patients with FEVR. No correlation in the reduced luciferase activity and the ocular phenotype was observed in this study. This study further emphasized the complexity of the FEVR-causing machinery.

Familial exudative vitreoretinopathy (FEVR) is a hereditary ocular disorder characterized by impaired development of the retinal vessels and various secondary complications, including retinal folds and retinal detachments [1]. The clinical phenotypes of FEVR vary from asymptomatic to complete blindness, even within the same family [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, X-linked) [5], *FZD4* (OMIM 604579, dominant) [6], *LRP5* (OMIM 603506, dominant and recessive) [7,8], *TSPAN12* (OMIM 613138, dominant and recessive) [9-11], and *ZNF408* (NCBI 79797, dominant) [12]. Recently, Robitaille et al. first identified mutations in *KIF11* (OMIM 148760) in patients with FEVR [13].

The gene *FZD4* encodes a member of the frizzled and smoothened superfamily of seven-transmembrane-domain cell-surface proteins that can function as receptors for wingless (Wnt) proteins [14]. In the best studied "canonical" Wnt signaling pathway, Wnt ligand exerts its activity through binding to the receptors of FZD4 and LRP5, leading to stabilization of intracellular β-catenin, which forms a complex with members of the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors and activates downstream target genes [15].

However, the pathogenic mechanism of FEVR is complicated. Until now, no clear genotype–phenotype correlation has been identified. In addition, the pathogenicity of missense mutations is not clear, and thus, genetic counseling cannot be provided. In this study, we identified four novel mutations in *FZD4* with next-generation sequencing in a cohort of 621 patients with FEVR. We performed the SuperTopFlash (STF) reporter assay to demonstrate these four novel mutations in *FZD4* induced variable reduction in the Norrin signaling activity. This study further emphasized the complexity of the FEVR-causing machinery.

METHODS

Participants and clinical data collection: The study was approved by the Ethics Committee of Xinhua Hospital and was performed in accordance with the tenets of the

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Declaration of Helsinki. Informed written consent was obtained from the parents or guardians of each participant because they were minor children. Between January 2010 and October 2017, 621 clinically diagnosed patients with FEVR were collected in our clinic. All participants were born full-term. Patients with a clinical diagnosis of 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 (if available) of the ora serrate using a Retcam under anesthesia or a Spectralis HRA2 (Heidelberg Engineering GmbH, Heidelberg, Germany) based on the patients' age. Additionally, wild-field fluorescein angiography was routinely performed in patients' direct family members, primarily the parents and siblings (if any) who could tolerate fluorescein sodium using the Spectralis HRA2 (Heidelberg Engineering GmbH) in the clinic when available. Optos imaging was performed in family members who could not tolerate fluorescein sodium.

Genetic testing: Next-generation sequencing (NGS) was performed with MyGenostics (Baltimore, MD). Briefly, peripheral blood was drawn from each proband and his or her direct family members, and the genomic DNA was extracted and fragmented. 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 ºC and processed within 24 h after blood drawn. Genomic DNA samples were extracted blood DNA extraction kit following manufacturer's instruction (Bioteke). Illumina adapters were added to the fragments, and the samples were size-selected for the 350 to 400 bp products. This pool of DNA fragments was amplified using PCR and allowed to hybridize with DNA capture probes that were specifically designed for the targeted genes. PCR working conditions are as following: initial denature temperature 95 ºC for 3 min, followed by 33 cycles of reaction: template denature at 95 ºC for 15 s, annealing for 15 s at 59 ºC and extension at 72 ºC for 20 s. A final step of 7 min reaction extension at 72 ºC was applied to fill in the gaps of PCR product. The captured DNA fragments were eluted, amplified again, and subjected to NGS using an Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA). A

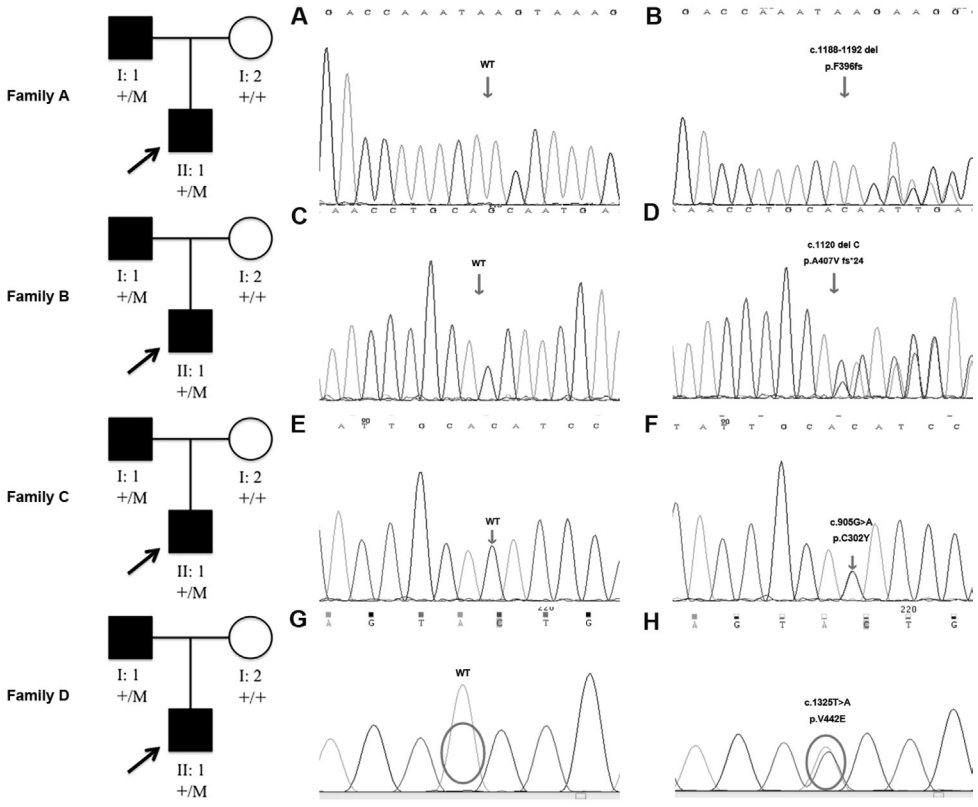

Figure 1. Chromatograms and pedigrees of four families with familial exudative vitreoretinopathy. **A**, **B**: In family A, the c.1188_1192del (p.F396fs) was identified in a 4-year-old boy and his affected father. **C**, **D**: In family B, we identified the c.1220delC (p.A407Vfs*24) mutation in a 3-year-old boy and his affected father. **E**, **F**: In family C, the c.905G>A (p.C302Y) mutation was identified in a 3-year-old boy and his affected father. **G**, **H**: In family D, we identified the c.1325T>A (p.V442E) mutation in a 2-year-old boy and his affected father. In the pedigrees, M sign represents a variant; WT represents a normal allele; arrows, probands; squares, males; circles, females; filled symbols, affected individuals; open symbols, unaffected individuals.

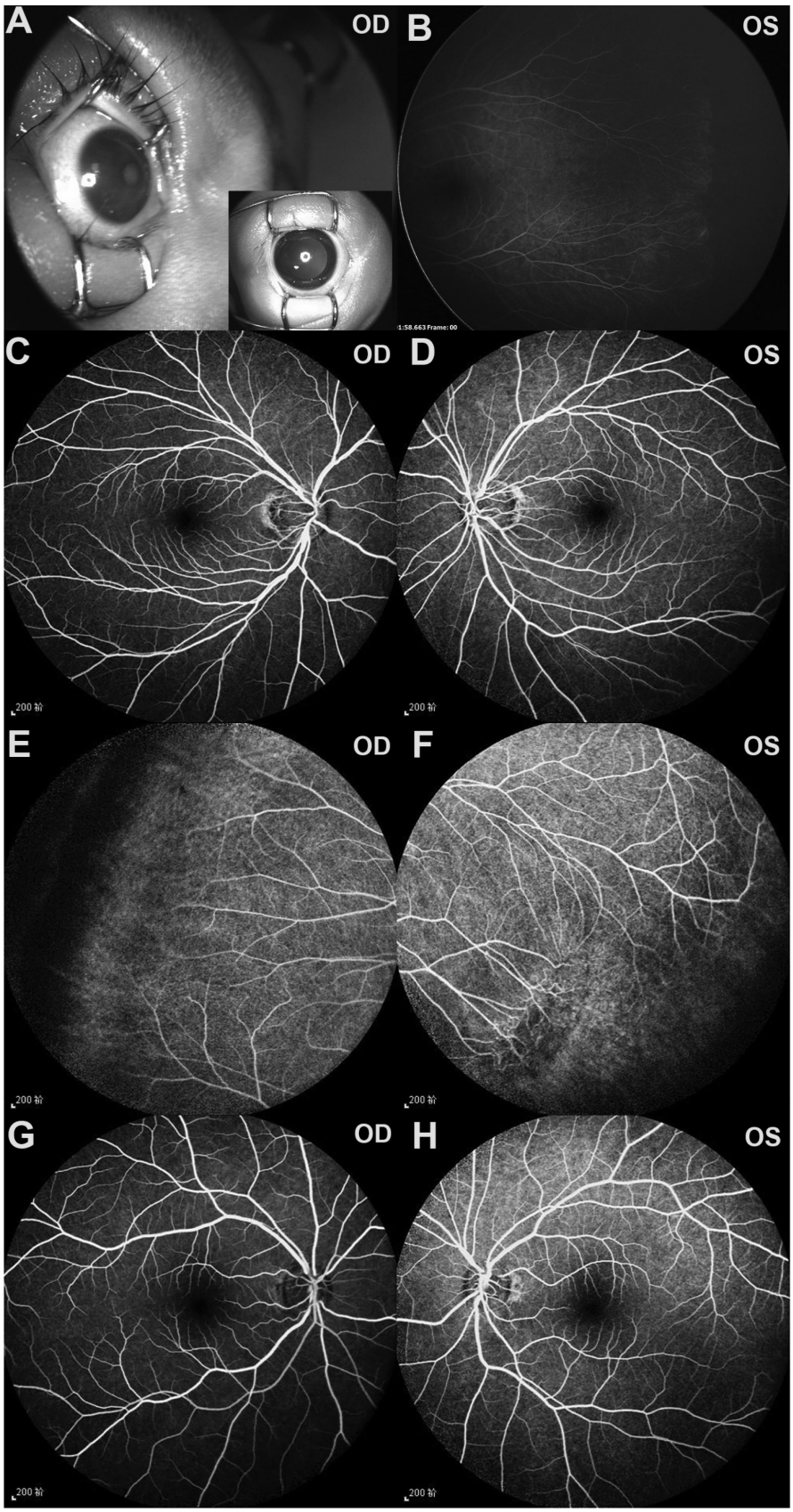

Figure 2. Fundus and angiographic images of family B with familiar exudative vitreoretinopathy. **A**, **B**: Color photographs and an angiographic image of the proband show a disappeared anterior chamber in the right eye and aberrant vessels with an avascular area in the peripheral retina of the left eye. A control picture of a normal anterior chamber from another individual is provided as reference (bottom right corner in **A**). **C**–**F**: His mutation-carrying father is asymptomatic with a peripheral avascular area and abnormal vessels in the left eye. **G**, **H**: His mother has normal retinal vasculature demonstrated with angiography.

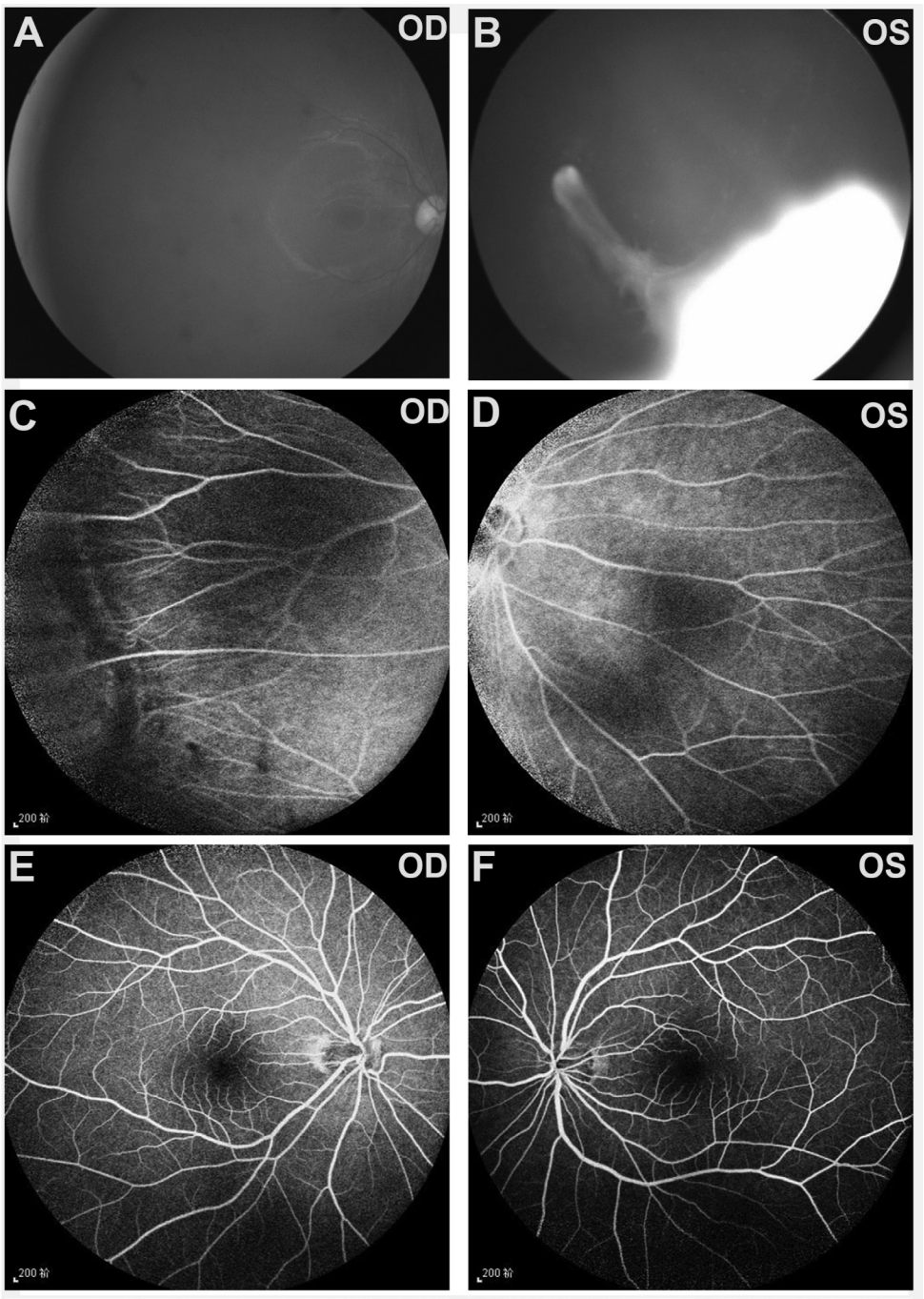

Figure 3. Fundus and angiographic images of family B with familiar exudative vitreoretinopathy. **A**, **B**: Fundus photographs of the proband show the peripheral avascular area in the right eye and falciform retinal fold in the left eye. Angiography was not performed for the proband because of his parents' unwillingness. **C**, **D**: The angiographic images of his affected father show straightened vessels and an avascular area in the peripheral retina. **E**, **F**: His mother has normal retinal vasculature demonstrated with angiography.

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* (OMIM 603506), *RS1* (OMIM 312700), *SOX2* (OMIM 184429), *TENM3* (OMIM 610083), *VSX2* (OMIM 142993), *FZD4* (OMIM 604579), *IKBKG* (OMIM 300248), *NDP*(OMIM 300658), *SALL2* (OMIM 602219), *STRA6* (OMIM 610745), *TSPAN12* (OMIM 613138), *YAP1* (OMIM 606608), *GDF3* (OMIM 610522), *KIF11* (OMIM 148760), *PAX6* (OMIM 607108), *SHH* (OMIM 600725), *TBX1* (OMIM 602054), *TUBA8* (OMIM 605742), and *ZNF408* (NCBI 79797). The pipeline that was used to filter the data and to generate the final result is shown in the supplemental material.

Plasmids: LRP5, *FZD4*, and *Norrin* plasmids were generously provided by Dr. Jeremy Nathans of Johns Hopkins University

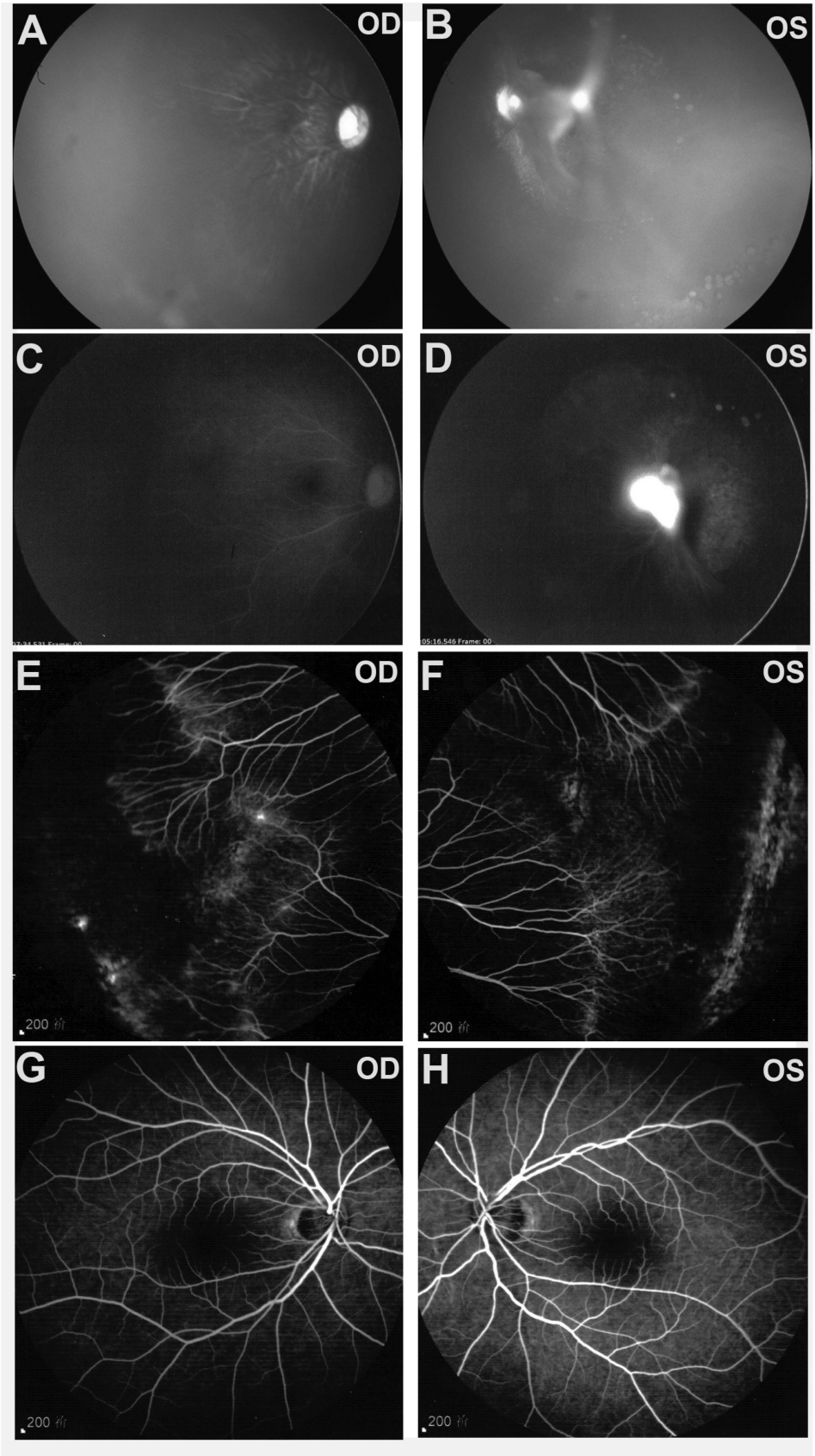

Figure 4. Fundus and angiographic images of family C with familiar exudative vitreoretinopathy. **A**–**D**: Fundus examination and angiography reveal extremely asymmetric ocular phenotypes of the proband. The right fundus shows the peripheral avascular area and abnormal vessels. However, the left fundus manifests as similar to Norrie disease with choroidal atrophy. **E**, **F**: Retinal vascular anomalous formation and an area of the avascular retina are observed in his affected father. **G**, **H**: His mother has normal retinal vasculature demonstrated with angiography.

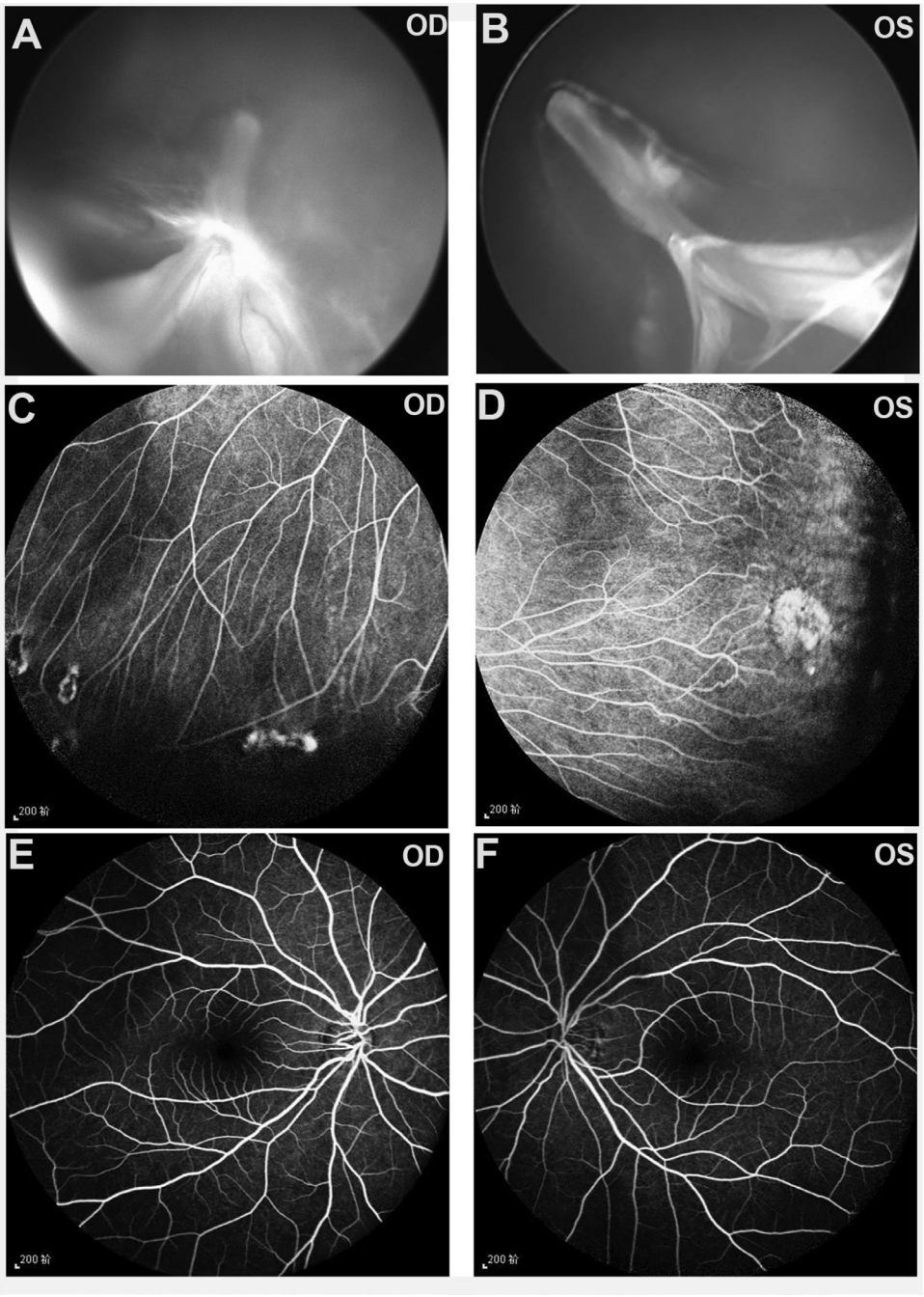

Figure 5. Fundus and angiographic images of family D with familiar exudative vitreoretinopathy. **A**, **B**: Fundus photographs of the proband show fibrovascular tissue with retinal folds involving the macula in two eyes. **C**, **D**: For his affected father, angiography reveals aberrant vessels and avascular area in the peripheral retina. **E**, **F**: His mother has normal retinal vasculature demonstrated with angiography.

(Baltimore, MD). All mutant plasmids were generated using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). The expression plasmids containing the FLAG tag were first verified with DNA sequencing, and then the expression was checked with transfection using a Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA).

Luciferase assays: The STF reporter, in which firefly luciferase was driven by seven LEF/TCF consensus binding sites, was kindly gifted by Dr. Jeremy Nathans. The reporter plasmid was stably infected into human embryonic kidney (HEK) 293 cells (short tandem repeat analysis shown in the Supplementary Data) as previously reported to generate the STF cell line [16]. In 24-well plates, STF cells were transfected with 700 ng DNA with 1.1 µl Lipofectamine 2000 Transfection Reagent (Invitrogen). The DNA mix contained 200 ng of Norrin, 200 ng of LRP5, 100 ng of pSV-β-galactosidase control vector, and 200 ng of FZD4 (wild-type or mutation).

Forty-eight hours after transfection, the cells were harvested and washed twice with PBS (1X; 120 mM NaCl, 20 mM KCl, 10 mM $NaPO_4$, 5 mM KPO_4, pH 7.4). Luciferase activities were measured with a dual-luciferase assay kit (Promega, Madison, WI). Reporter activity was normalized to the coexpressed β-galactosidase activity in each well. Each test was performed in triplicate. The reporter assay was repeated three times, and a representative result was obtained.

Cell culture: HEK 293 cells were cultured in Dullbecco's modified essential medium (DMEM, ATCC, Manassas, VA; Hyclone; Appendix 1) supplemented with 10% fetal bovine serum (Invitrogen) and 1% antibiotics (penicillin/streptomycin; Invitrogen).

Immunoblotting and antibodies: The transfected 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. Cleared cell lysates were boiled at 100 °C for 10 min. Proteins were resolved with SDS–polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene fluoride (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) according to the manufacturer's instructions. Antibodies against FLAG M2 were purchased from Sigma (Saint Louis, MO) and actin from Cell Signaling Technology (Beverly, MA).

Statistical analysis: Statistical analyses were performed with a two-tailed unpaired Student *t* test. All data shown represent the results obtained from triplicate independent experiments with a standard error of the mean (SEM; mean ± SD). P values of less than 0.05 were considered statistically significant.

RESULTS

Novel mutations in FZD4 and phenotypes: In this study, four novel mutations in *FZD4* (c.1188_1192del/p.F396fs, c.1220delC/p.A407Vfs*24, c.905G>A/p.C302Y, c.1325T>A/p.V442E) in the coding sequence were identified in four unrelated families. The Sorting Intolerant From Tolerant (SIFT) prediction and PolyPhen2 prediction of the two mutations (c.905G>A and c.1325T>A) are "damaging" and "probably damaging." In accordance with the guidelines from the American College of Medical Genetics and Genomics (ACMG) [17], the mutation (c.1188_1192del/p.F396fs) was perceived as "likely pathogenetic," and the mutation (c.1220delC/p.A407Vfs*24) was perceived as "pathogenetic." These two missense mutations occurred at residues highly conserved across species (see the supplemental material). All mutations cosegregated with the disease phenotype of these four families (Figure 1) and were not detected in 200 healthy individuals. In this cohort, other mutations or polymorphisms, including c.757 C>T, c.542 G>A, c.313 A>G, c.400 G>T, c.1589 G>A, c.341 T>G, c.205 C>T, c.678 G>A,

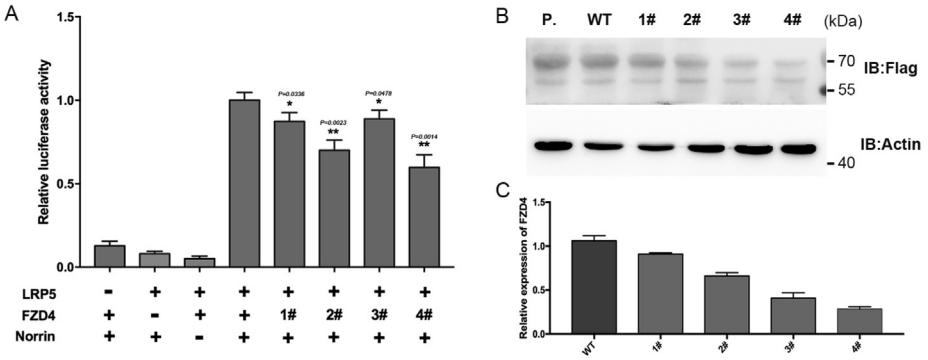

Figure 6. Analysis of mutations in *FZD4* on its biological activity in the Norrin/β-catenin signaling pathway. **A**: SuperTopFlash (STF) cells/well were cotransfected with 700 ng DNA (200 ng of Norrin, 200 ng of LRP5, 100 ng of pSV-β-galactosidase control vector, and 200 ng of FZD4 [wild-type or mutation]) and 1.1 µl Lipofectamine 2000 transfection reagent. Forty-eight hours after transfection, the cells were harvested and washed twice with PBS. Luciferase activities were measured with a dual-luciferase assay kit. Reporter activity was normalized to the coexpressed β-galactosidase activity in each well. The positive control (LRP+/FZD4+/Norrin+) was normalized to 1. Each test was performed in triplicate. The reporter assay was repeated three times, and a representative result was obtained. Statistical analyses were performed with a two-tailed unpaired Student t test. **B**: Human embryonic kidney (HEK) 293 cells were transfected with 600 ng FLAG-FZD4 WT or mutant plasmids, respectively. Forty-eight hours post-transfection, cell lysates were immunoblotted (IB) with FLAG or actin. P, positive control. **C**: Protein quantification: the gray value of FLAG-FZD4/actin and normalized wild-type to 1 (1#:c.1188_1192del/p.F396fs; 2#: c.1220delC/p.A407Vfs; 3#: c.905G>A/p.C302Y; 4#: c.1325T>A/p.V442E).

c.1282_1286del, c.40_49del, c.1488 G>A, and c.1463 G>A, have been reported [10,18-26].

In family A, the carrier of the c.1188_1192del (p.F396fs) mutation was a 4-year-old boy. His right eye presented as total retinal detachment and a disappeared anterior chamber. However, only a peripheral avascular area was observed in his left eye (Figure 2A,B). His mutation-carrying father was asymptomatic with a peripheral avascular area and abnormal vessels in the left eye (Figure 2C–F). His mother had normal fundi (Figure 2F,H).

In family B, we identified the c.1220delC (p.A407Vfs*24) mutation in a 3-year-old boy and his affected father. The ocular presentation of the proband was extremely asymmetric. He exhibited a peripheral avascular area in the right eye and a falciform retinal fold in the left eye (Figure 3A,B). Angiography was not performed for the proband because of his parents' unwillingness. His affected father had straightened vessels in the peripheral retina (Figure 3C,D). His mother had normal fundi (Figure 3E,F).

In family C, the c.905G>A (p.C302Y) mutation was identified in a 3-year-old boy and his affected father. The ocular presentation was also extremely asymmetric in this proband. The right fundus showed an avascular area and abnormal vessels in the peripheral retina. However, the left fundus manifested as similar to Norrie disease with choroidal atrophy (Figure 4A–D). Typical FEVR fundus changes were observed in his affected father (Figure 4E,F). His mother exhibited healthy retinal vasculature (Figure 4G,H).

In family D, we identified the c.1325T>A (p.V442E) mutation in a 2-year-old boy and his affected father. Fibrovascular tissue with retinal folds involving the macula was present in both eyes of the proband (Figure 5A,B). For his affected father, angiography revealed aberrant vessels and an avascular area in the peripheral retina (Figure 5C,D). His mother exhibited healthy retinal vasculature (Figure 5E,F).

Luciferase reporter assay: To verify the effect of the mutations in *FZD4* on its biologic activity in the Norrin/β-catenin signaling pathway, we constructed the corresponding *FZD4* mutant plasmids (c.1188_1192del, c.1220delC, c.905G>A, c.1325T>A) and analyzed the function of these mutant FZD4 proteins with a triplicate luciferase reporter assay. In the STF cells, we cotransfected *LRP5/Norrin/FZD4* or other mutant FZD4 plasmids, respectively. After transfection, we checked the mRNA levels of the wild-type *FZD4* and the four mutations, and we found the mRNA levels were comparable (Supplementary figure). We compared the luciferase activity to wild-type *FZD4*, and all four mutations in *FZD4* induced variable reductions (17% for p.F396fs, 32% for p.A407Vfs, 11% for p.C302Y, and 39% for p.V442E) in the Wnt signaling (Figure 6A). These results suggested that these four mutations were pathogenic as they attenuated the activity of the Norrin/β-catenin signaling pathway. Next, we checked the protein level of the four mutations in *FZD4* in the HEK 293 cells. We found that these mutations affected the stability of *FZD4* (Figure 6B). Compared with the wild-type, the protein levels of the four mutants decreased by 10%, 37%, 56%, and 63%, respectively (Figure 6C).

DISCUSSION

In the present study, we identified four novel mutations in *FZD4* in four families among 621 patients with FEVR but not in 200 healthy individuals. The typical FEVR phenotype was observed in all carriers. These results support the variable expressivity of FEVR, not only between family members but also between two eyes of an individual. These results indicate the complicated mechanism of FEVR.

FEVR is genetically heterogeneous and manifests a great variability of phenotypes. It has been reported that unrelated patients harboring the same mutation manifest the variable phenotype [27]. In the present study, three of the four probands exhibited extremely asymmetric phenotypes. One eye manifested mild fundus changes; however, the other eye exhibited advanced FEVR presentation, including total retinal detachment with a disappeared anterior chamber, similar to Norrie disease, and a facifold fold. For the four families, the parents harboring the same mutation were all asymptomatic with mild fundus changes. They were diagnosed with FEVR until angiography was performed. These results indicate that the prevalence rate of FEVR may be higher than expected in China. Thus, epidemiological investigation is suggested to investigate the prevalence rate in the so-called healthy population. Until now, the variability has remained unexplained. Further studies are needed to investigate the effects of the epigenetic effects or the presence of modifying genes in the variable phenotypes observed in unrelated patients harboring the same mutation or between two eyes of one individual.

LRP5 and FZD4, a coreceptor pair involved in the canonical Wnt signaling pathway, were identified with a positional screening approach [6,8]. Mutations in *FZD4* can cause autosomal dominant FEVR [7,8,28]. In the present study, we identified four novel mutations in four unrelated families. The mutations were located at the transmembrane (p.F396fs; p.A407Vfs; p.V442E) or the topological domain (p.C302Y) of FZD4. Typical phenotypes were observed in all carries. The complex genotype–phenotype correlation in FEVR remains unknown. Hayashi et al. found that a nonsense mutation in *FZD4* completely abolished its signaling activity,

while single missense mutations in *LRP5* and *FZD4* caused a moderate level of reduction (ranging from 26% to 48%, 36% on average), and a double missense mutation in both genes caused a severe reduction in activity (71%). They observed the reduction in activity correlated roughly with the clinical phenotypes [27]. In the present study, the four novel mutations in *FZD4* caused variable reductions in the Wnt signaling pathway, ranging from 11% to 39%. However, no correlation between the reduced luciferase activity and the clinical phenotype was observed in this study. The protein levels of the three mutations (p.F396fs; p.A407Vfs; p.V442E) correlated roughly with Wnt signaling activity. Interestingly, the Wnt signaling activity of the mutation p.C302Y was higher than that of the mutations p.F396fs and p.A407Vfs, but with a lower protein level. These results indicate that there might be other regulatory mechanisms and further demonstrated the complex disease-causing machinery of FEVR.

In summary, we identified four novel mutations in *FZD4* in four unrelated families. Variability in clinical phenotypes was observed not only in the probands and parents harboring the same mutation but also between two eyes in the probands. These data further emphasize the complexity of FEVR-causing machinery. Future studies are necessary to investigate the disease-causing machinery. In addition, we suggest an epidemiological study to find asymptomatic patients with FEVR in the population and promote eugenics.

APPENDIX 1. STR ANALYSIS

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

ACKNOWLEDGMENTS

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