An Essential Role of the Cysteine-rich Domain of FZD4 in Norrin/Wnt Signaling and Familial Exudative Vitreoretinopathy*

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The Wnt pathway plays important yet diverse roles in health and disease. Mutations in the Wnt receptor FZD4 gene have been confirmed to cause familial exudative vitreoretinopathy (FEVR). FEVR is characterized by incomplete vascularization of the peripheral retina, which can lead to vitreous bleeding, tractional retinal detachment, and blindness. We screened for mutations in the FZD4 gene in five families with FEVR and identified five mutations (C45Y, Y58C, W226X, C204R, and W496X), including three novel mutations (C45Y, Y58C, and W226X). In the retina, Norrin serves as a ligand and binds to FZD4 to activate the Wnt signaling pathway in normal angiogenesis and vascularization. The cysteine-rich domain (CRD) of FZD4 has been shown to play a critical role in Norrin-FZD4 binding. We investigated the effect of mutations in the FZD4 CRD in Norrin binding and signaling in vitro and in vivo. Wild-type and mutant FZD4 proteins were assayed for Norrin binding and Norrin-dependent activation of the canonical Wnt pathway by cell-surface and overlay binding assays and luciferase reporter assays. In HEK293 transfection studies, C45Y, Y58C, and C204R mutants did not bind to Norrin and failed to transduce FZD4-mediated Wnt/β-catenin signaling. In vivo studies using Xenopus embryos showed that these FZD4 mutations disrupt Norrin/β-catenin signaling as evidenced by decreased Siamois and Xnr3 expression. This study identified a new class of FZD4 gene mutations in human disease and demonstrates a critical role of the CRD in Norrin binding and activation of the β-catenin pathway.

Pathological growth of new blood vessels in the retinal vasculature (neovascularization) has been implicated in several human diseases, including familial exudative vitreoretinopathy (FEVR), retinopathy of prematurity, age-related macular degeneration, and diabetic retinopathy. Complications of neovascularization include bleeding, retinal detachment, and irreversible scarring of the retina. FEVR is a developmental disorder characterized by incomplete vascularization of the peripheral retina (1–3). Mutations in FZD4 (Frizzled 4) have been linked to autosomal dominant forms of FEVR (4). The primary effect of FZD4 mutations is caused by the premature arrest of retinal angiogenesis within the peripheral retina (5). Complications arising from incomplete retinal vascularization and subsequent retinal ischemia include development of hyperpermeable vessels, neovascularization, bleeding, and tractional retinal detachment. In severely affected patients with FZD4 mutations, blindness may occur before 10 years of age, whereas mildly affected individuals may not be aware of symptoms and are diagnosed only by fluorescein angiography.

Activation of the canonical Wnt pathway in the retina has been shown to be developmental stage-dependent and spatially modulated and is also important in retinal regeneration (6, 7). FZD4 is a member of the Frizzled family of seven-transmembrane Wnt-binding receptors. The N-terminal extracellular cysteine-rich domain (CRD), conserved among Frizzled family members, determines binding specificity for Wnt ligands. The seven-pass transmembrane region is followed by the intracellular Thr-X-Val PDZ-binding and Lys-Thr-X-X-Trp Dvl (Disheveled) association sites for Wnt/β-catenin canonical signaling.

Inherited mutations in FZD4, the coreceptor gene LRP5, or the FZD4 ligand Norrin have all been linked to FEVR (4, 8–12). It has also been shown that Norrin binding to FZD4 is highly specific for the FZD4-LRP5 receptor complex and plays a critical role in the normal development of retinal vas-

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3 The abbreviations used are: FEVR, familial exudative vitreoretinopathy; CRD, cysteine-rich domain; EYFP, enhanced YFP.
culature (13). However, little is known about the role of the CRD in retinal development and disease.

In this study, we identified five FZD4 mutations in five families with FEVR. We characterized the FZD4 mutations for altered cellular processing, plasma membrane targeting, interaction with the Norrin ligand, and the ability to activate the Wnt/ß-catenin pathway in vitro and in vivo.

**EXPERIMENTAL PROCEDURES**

**Patient Screening**—Study approval was obtained from the Institutional Review Boards of the West China Hospital of Sichuan University and the University of California San Diego, and informed consent was obtained from all participants. Ophthalmic examination was conducted in five Caucasian kindreds with FEVR. Two-hundred normal controls were collected from the same regions as the families being studied. Genomic DNA was extracted from blood samples of members of those kindreds. Each of the two exons of the FZD4 gene was amplified by PCR and sequenced using an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

**Construction of Expression Plasmids**—Wild-type FZD4 cDNA was amplified using forward primer 5′-cccaagcttggtgccggatcccg-3′ and reverse primer 5′-cgggatccggattaccaagcttgggt-3′. The gene encoding wild-type FZD4 was subcloned in-frame into the Clontech pEYFP-N1 vector (BD Biosciences) with the N-terminal enhanced YFP (EYFP) fusion protein using HindIII and BamHI sites. This vector utilizes a CMV promoter and expresses EYFP following transfection protein using HindIII and BamHI sites. This vector utilizes a CMV promoter and expresses EYFP following transfection protein using HindIII and BamHI sites.

**Western Blotting**—Western blotting was performed as described previously using EYFP and ß-actin monoclonal antibodies (15).

**RESULTS**

**Identification of Novel FZD4 Mutations**—DNA sequence analysis identified five mutations, including 444G→A (C45Y), 479A→G (Y58C), 984G→A (W226X), 916T→C (C204R), and 1794G→A (W496X), in the five FEVR families, respectively (Fig. 1). Three of the five mutations were novel. All mutations cosegregated with the disease phenotype of the respective families and were absent in 200 normal controls.

**Defective Norrin Binding in FZD4 Mutants**—C45Y, Y58C, and C204R mutations are located in the CRD of FZD4. However, little is known about the role of the CRD in retinal development and disease.

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**Luciferase Assays**—The SuperTopFlash (STF) construct (generously provided by Dr. Randall Moon) contains a firefly luciferase reporter driven by seven LEF/TCF consensus binding sites. This reporter plasmid was stably transfected into HEK293 cells as reported previously (13) to generate the STF cell line. The STF cells were cotransfected with 50 ng of Norrin, 50 ng of FZD4 (wild-type or mutant), 60 ng of LRP5, and 1 ng of Renilla luciferase in a 24-well plate using FuGENE 6 (Roche Applied Science). The transfected cells were washed with PBS twice after 48 h of transfection and assayed using the Promega Dua-Luciferase assay reagents. The firefly luciferase activity was normalized to the coexpressed Renilla luciferase activity. Each assay was repeated in triplicate at the same time.

**Embryo Manipulations and RT-PCR**—Wild-type or mutant pEYFP-N1/hFZD4 was digested with XhoI and XbaI and then subcloned into the pCS2 + vector. Capped RNAs were transcribed in vitro from linearized plasmids with SP6 RNA polymerase according to the manufacturer’s protocol (Ambion, Austin, TX). Capped RNAs were injected into Xenopus embryos at the two-cell stage as described (16). Animal caps were dissected at stage 8 and cultured until stage 10.5. RNA from Xenopus embryo was prepared with TRIzol (Invitrogen), and RT-PCR was performed as described (16).

**CRD of FZD4 in Norrin/Wnt Signaling and FEVR**

**Confocal Microscopy**—Fluorescent images were captured by LSM 510 confocal microscope (Zeiss, Thornwood, NY).

**Overlay Assay**—The HEK293 cells were transfected with wild-type or mutant FZD4-EYFP constructs. The transfected cells were collected after 24 h, and the lysates of transfected cells were run on 8% polyacrylamide gel. The protein on the gel was then transferred to a PVDF membrane. The membrane with transferred FZD4-EYFP protein was blocked by 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 and incubated with AP-3Myc-mNorrin conditional medium overnight. The binding of Norrin was detected by anti-Myc mAb.

**Western Blotting**—Western blotting was performed as described previously using EYFP and ß-actin monoclonal antibodies (15).
responsive firefly luciferase reporter. All five FZD4 mutants failed to induce the luciferase reporter activity in STF cells in response to Norrin (Fig. 2D), consistent with the notion that defective Norrin/FZD4 signaling underlies FEVR.

We next investigated the effect of these five FZD4 mutants on canonical β-catenin signaling in vivo in Xenopus embryos. It is well established that Wnt/β-catenin signaling induces the expression of downstream target genes such as siamois and...
We injected mRNAs encoding Norrin plus one of the five FZD4 mutants into Xenopus embryos. Norrin plus wild-type FZD4, but neither Norrin nor FZD4 alone, activated the expression of Siamois or Xnr3 (Fig. 3A), indicating that xnr3 (17). We injected mRNAs encoding Norrin plus one of the five FZD4 mutants into Xenopus embryos. Norrin plus wild-type FZD4, but neither Norrin nor FZD4 alone, activated the expression of Siamois or Xnr3 (Fig. 3A), indicating that Norrin/FZD4 signaling is sufficient to activate β-catenin-dependent gene expression in embryos. In sharp contrast, none of the FZD4 mutants were able to mediate Norrin induction of these β-catenin target genes.
DISCUSSION

Human FEVR displays genetic heterogeneity characteristics. Mutations in the NDP gene (which encodes Norrin) cause Norrie disease and cross-linked FEVR (8), whereas heterozygous mutations in FZD4, LRP5, and TSPAN12 (transmembrane 4 superfamily member 12), which is a component of the Norrin-FZD4 complex, can cause autosomal dominant FEVR in humans (4, 9–11). FEVR serves as an excellent model to study Wnt signaling in human diseases, given the easy accessibility of the eye structure and importance of vision. Here, we have described the genetic and functional studies of several novel FEVR mutations associated with FZD4. Among the five mutations described in this study (Fig. 3B), a C204Y mutation was identified previously (18), and C45Y, Y58C, and C204R occur within or near the highly conserved CRD. C45Y eliminated the first conserved cysteine in the CRD proposed to form a disulfide bridge, with the fourth conserved cysteine residue at position 99 of the CRD. The Y58C mutation introduced an additional cysteine residue between the conserved second and third cysteines. We propose that both mutations affect correct protein folding of the CRD and, consequently, ligand binding. FZD4 binding to Norrin is disrupted by the C204R mutation, suggesting that the CRD may be beyond the previously predicted region (i.e. the 114-amino acid region extending from the first to the tenth conserved CRD cysteine) (19) or that Norrin binding to FZD4 requires the CRD plus additional residues C-terminal to the CRD.

Loss of protein processing or correct localization to the cell membrane observed in this study can be explained by loss of a correctly folded FZD4 protein. Consistent with these results, this would also impact Norrin binding and activation of the Wnt/β-catenin pathway. Decreased expression of Siamois and Xnr3 upon Norrin stimulation provides functional confirmation of the mutation’s ability to disrupt the Wnt pathway. Although the effects of the mutant protein can be explained by this model, it is important to note that these FZD4 mutations cause autosomal dominant FEVR in humans. This is yet another example of how, when mutated, Wnt signaling players can have an effect on pathophysiologic states ranging from FEVR to colon cancer to diabetes. The autosomal dominant feature of FZD4 mutations could be due either to haplo-insufficiency or to a dominant-negative effect, as findings have suggested previously (20).

Recently, Smallwood et al. (19) demonstrated that Norrin binds specifically to the CRD of FZD4 but not to CRDs of the other 14 mammalian Frizzled and secreted Frizzled-related proteins, further indicating that the FZD4 CRD is a critical part of FZD4 binding to Norrin. Among the four FEVR genes identified so far, FZD4 plays a crucial role in vascular development in the retina. Norrin does not bind directly to LRP5 without FZD4. Furthermore, Norrin multimers and TSPAN12 cooperatively promote multimerization of FZD4 and its associated proteins to elicit physiological levels of Wnt signaling (10, 13, 21). The subsequent dominant effects of the mutant proteins on the wild-type protein of FZD4 will require further assessment. Future studies will further delineate the role of mutant FZD4 in the Wnt pathway and subsequent effects on retinal vascular development. Understanding the modulation of the Wnt pathway is an exciting area of study and may reveal new insights into disease pathogenesis and new areas for drug discovery.

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