Overview of the Mutation Spectrum in Familial Exudative Vitreoretinopathy and Norrie Disease with Identification of 21 Novel Variants in FZD4, LRP5, and NDP

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ABSTRACT: Wnt signaling is a crucial component of the cell machinery orchestrating a series of physiological processes such as cell survival, proliferation, and migration. Among the plethora of roles that Wnt signaling plays, its canonical branch regulates eye organogenesis and angiogenesis. Mutations in the genes encoding the low density lipoprotein receptor protein 5 (LRP5) and frizzled 4 (FZD4), acting as coreceptors for Wnt ligands, cause familial exudative vitreoretinopathy (FEVR). Moreover, mutations in the gene encoding NDP, a ligand for these Wnt receptors, cause Norrie disease and FEVR. Both FEVR and Norrie disease share similar phenotypic characteristics, including abnormal vascularization of the peripheral retina and formation of fibrovascular masses in the eye that can lead to blindness. In this mutation update, we report 21 novel variants for FZD4, LRP5, and NDP, and discuss the putative functional consequences of missense mutations. In addition, we provide a comprehensive overview of all previously published variants in the aforementioned genes and summarize the phenotypic characteristics in mouse models carrying mutations in the orthologous genes. The increasing molecular understanding of Wnt signaling related to ocular development and blood supply, offers more tools for accurate disease diagnosis that may be important in the development of therapeutic interventions.


KEY WORDS: FZD4; LRP5; NDP; familial exudative vitreoretinopathy; FEVR; Norrie disease; ND

Introduction

Familial exudative vitreoretinopathy (FEVR; MIM# 133780) and Norrie disease (ND; MIM# 310600) are two inherited retinal disorders with highly overlapping ocular manifestations that are caused by alterations in the Wnt signaling network. FEVR and ND share an abnormal vascularization of the peripheral retina with the formation of retinal folds, retinal detachment, and in many cases the creation of a fibrovascular membrane located behind the lens [Laqua, 1980; Miyakubo et al., 1984; Shukla et al., 2003; van Nouhuys, 1982]. Genetic analyses have thus far identified three causative genes for the two disorders, that is, frizzled 4 (FZD4, located on 11q14.2; MIM# 604579), low-density lipoprotein 5 (LRP5, located on 11q13.2; MIM# 603506), and Norrin (NDP, located on Xp11.3; MIM# 300658). Those genes encode proteins involved in the evolutionary highly conserved Wnt signaling network that plays an important role in eye development and angiogenesis. FEVR is a genetically heterogeneous disease and is inherited in an autosomal dominant (adFEVR; MIM# 133780) [Criswick and Schepens, 1969; Feldman et al., 1983; Gow and Oliver, 1971; van Nouhuys, 1982, 1985], autosomal recessive (arFEVR; MIM# 601813) or an X-linked recessive mode (MIM# 305390) [Chen et al., 1993a; de Crecchio et al., 1998; laqua, 1980], with autosomal dominant being the most prominent mode of inheritance. Mutations in FZD4 and LRP5, which are located on 11q14.2 and 11q13.2, respectively, are associated with autosomal dominant FEVR [Robitaille et al., 2002; Toomes et al., 2004a]. In addition, one family has been found to link to the EVR3 locus on chromosome 11p12–p13 [Downey et al., 2001]. Mutations in LRP5 have also been associated with arFEVR [Jiao et al., 2004], whereas X-linked recessive FEVR is caused by mutations in the Norrie disease gene (NDP) located on Xp11.3 [Berger et al., 1992b; Chen et al., 1993a]. The pathologic features of the disease initiate from the abnormal retinal development due to the incomplete vascularization of the peripheral retina and/or retinal blood vessel differentiation [Canny and Oliver, 1976]. The

Additional Supporting Information may be found in the online version of this article.

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resulting avascular peripheral retinal zone [Miyakubo et al., 1982, 1984; Shukla et al., 2003; van Nouhuys, 1982] is the hallmark of the disease and in many mildly affected patients may be FEVR's only manifestation. In more severe cases, retinal complications such as the formation of holes and tears, neovascularization, exudation, vitreous haemorrhage, macular ectopia, falciform folds, and retinal detachment, can result in blindness.

Norrie disease (MIM# 310600) is a severe X-linked recessive form of congenital blindness, which in about one-third of the cases is accompanied by mental retardation and deafness [Bleeker-Wagemakers et al., 1985; Warburg, 1966]. A characteristic intraocular mass in the retina (pseudoglioma) can lead to microphthalmia [Johnston et al., 1982; Townes and Roca, 1973]. Other ocular findings include the development of cataract, alterations in the composition of the vitreous body [Warburg, 1975], vitreo-retinal hemorrhages, creation and deposition of retrolental fibrovascular tissue, retinal folding and detachment, accompanied by subretinal exudates [Enyedi et al., 1991; Polomeno et al., 1987], which are signs bearing high phenotypic overlap with FEVR [van Nouhuys, 1982]. ND is genetically homogeneous, and is caused by mutations in NDP [Berger et al., 1992b; Chen et al., 1993b].

In addition to causing FEVR, defects in LRP5 have also been associated with two groups of bone abnormalities. The first group includes osteoporosis–pseudoglioma syndrome (OPPG; MIM# 259770), a rare autosomal recessive condition that initiates during early childhood. The major symptoms of OPPG include visual loss due to several vitreo-retinal dysplasias and bone weakness that results in multiple fractures and deformities as the bone mass reduces. Carriers of OPPG-associated LRP5 mutations are often found to have reduced bone mass density. The second group of diseases comprises endosteal hyperostosis (MIM# 144750), osteosclerosis (MIM# 144750), osteopetrosis (MIM# 607634), van Buchem disease type 2 (VBCH2; MIM# 607636), and high-bone-mass trait (HBM; MIM# 601884). The aforementioned conditions are all characterized by a high-bone-mass phenotype and are inherited in autosomal dominant mode.

FZD4, LRP5, and NDP are components of the Norrin-induced FZD4/LRP5/β-catenin signaling pathway. It has been demonstrated that Wnt signaling pathways are one of the main drivers for vascular development in the mammalian eye. Consequently, mutations in these three genes cause FEVR and ND that have an almost identical pathologic impact on intraretinal vasculature.

Recently a series of dedicated experiments revealed that a new gene, TSPAN12 (located on 7q31.31; MIM# 613138) plays a key role in Norrin-induced β-catenin signaling transduction and regulation (Fig. 1). TSPAN12 is a member of the tetraspanin family that share certain specific structural features that distinguishes them from other proteins that pass the membrane four times. The protein was postulated to act via a mechanism that enhances FZD4 multimerization and clustering by binding to Norrin multimers, a crucial step for induction of physiological levels of signaling [Junge et al., 2009].

Wnt Signaling and Eye Vasculature: Brief Overview

The Wnt signaling transduction network influences cell survival, differentiation, proliferation, and migration by a highly orchestrated transcriptional regulation of target genes. The network can be roughly categorized in two broad branches: the canonical or Wnt/β-catenin pathway and the noncanonical pathway, which includes the Wnt/calcium signaling and planar cell polarity pathways. Wnt genes encode a family of soluble, secreted cysteine-rich proteins that are often glycosylated and/or palmitoylated. Wnts can act as ligands for Frizzled receptors that, in concert with LRP5/6 coreceptors, form a ternary complex [Tamai et al., 2000] and subsequently initiate Wnt canonical signaling. Canonical Wnt signaling regulates the amount of β-catenin that reaches the nucleus via the action of Disheveled proteins that block its phosphorylation by GSK-3 [Li et al., 1999] and proteolytic degradation in the proteosome [Polakis, 2000]. Presence of β-catenin inside the nucleus promotes transcription of several genes implicated in different processes. Wnts are expressed in a wide variety of tissues, and the specific type of Wnt-Frizzled induced signaling is determined mostly by the nature of the different interactions and the locations in which these occur. FZD4 has been shown to act also in the noncanonical Wnt signaling pathway [Robitaille et al., 2002] by regulating components belonging to the Wnt/calcium signaling network.

Wnt signaling also plays an important role in eye organogenesis and angiogenesis. Beta-catenin regulation is present in critical steps of ophthalmogenesis such as anterior neural tissue definition from the remaining brain compartments, eye field formation, and fine definition of the neural retina [Fuhrmann, 2008]. In addition, ocular blood vessel formation during development initiates with the formation of a provisional blood vessel system in the extraretinal hyaloid region called the hyaloid vascular system (HVS). This system is responsible for the supply of nutrients to the early vitreal structure and the retina. When the intraretinal vasculature develops, the HVS recedes in the maturing eye.

Retinal angiogenesis is dependent on TSPAN12, FZD4, and LRP5 to initiate the β-catenin signaling cascade upon NDP binding (Fig. 1).

Mutations in FZD4

FZD4 is a member of the Frizzled gene family and encodes a protein of 537 amino acids. Similar to the other members of the Frizzled family it is composed of a seven pass transmembrane helix and an extracellular Frizzled (FZ) domain (Supp. Fig. S1).

To date, a total of 32 different mutations have been reported for FZD4 (Supp. Table S1). Ten mutations result in a premature stop codon, 21 are missense changes and one is an in-frame deletion of two amino acids (p.M493_W494del). No splice mutations have been reported for FZD4 and mutations are not clustering in a specific “hotspot.” All patients with FZD4 mutations are heterozygous carriers, except for a 5-month-old baby carrying a homozygous change (p.R417Q) for which both parents harboring the same mutation heterozygously exhibited mild phenotypes [Kondo et al., 2003], suggesting an autosomal dominant mode of inheritance. The effect of a few FZD4 nucleotide variants has been assayed functionally. Robitaille et al. [2002] measured the FZD4-dependent induction of β-catenin signaling upon activation of the receptor. They found that p.M493_494del and p.L501SfsX333 mutant proteins did not exert any detectable activity. Two additional studies, using Norrin dependent signaling reporter assays, showed the reduced activity of FZD4 mutants (p.M105V, p.M157V, p.W319X, and p.R417Q) ranging from ~30 to 95% of the wild-type levels [Qin et al., 2008; Xu et al., 2004].

In this study we report five novel variants in FZD4, one of which is located in exon 1 (p.E40Q) and the other four in exon 2 (p.C204Y, p.E286X, p.D428fsX2, and p.G525R). For these and
all novel mutations reported, the patients and methods are described in the online supporting Information section.) Because FZD4 contains only two exons, protein truncating mutations in exon 2 likely do not result in complete nonsense mediated decay (NMD) but rather in a nonfunctional protein.

The FZD4 proteins containing the two protein-truncating mutations p.E286X, p.D428SfsX2 (Table 1, Supp. Table S2) are predicted to lack the conserved KTXXXW domain and PDZ binding motif (Supp. Fig. S1). The absence of these elements probably abolishes the ability of FZD4 to induce the downstream β-catenin signaling pathway. The KTXXXW domain is located directly after the seventh transmembrane (TM) helix at the C-terminus, and is essential for generation of FZD4-mediated signal transduction [Umbhauer et al., 2000]. Moreover, a truncated protein (p.L501SfsX33) that contained the KTXXXW domain but lacked the PDZ binding motif was shown to be defective. The mutated protein was absent from the plasma membrane, probably due to impaired oligomerization of the wild-type protein with the mutant form, which lead to the retention of FZD4 in the endoplasmic reticulum [Kaykas et al., 2004].

In addition to the two protein-truncating mutations we also identified three novel missense changes. Variant p.E40Q was found in one sporadic patient (Table 1). The wild-type glutamic acid residue is located at the N-terminus, directly following the putative signal peptide at the FZ domain (Supp. Fig. S1). According to the secondary structure prediction [Bryson et al., 2005], this residue is located at a coiled domain on the surface of the protein where interactions might occur. The function of the FZ domain is not fully elucidated, although mutagenesis and crystal structure studies suggest that it may be important for interaction with other proteins such as Wnts or NDFP [Dann et al., 2001]. Glutamic acid is a negatively charged amino acid and is often involved in ionic interactions. The mutant glutamine has the same molecular structure except for the negatively charged oxygen. The pathogenic effect of this change is therefore likely due to the loss of ionic interactions of the FZ domain with other proteins or by itself. In addition, the same patient carries a splice site mutation c.4489–1G>A. To better assess the disease-causing potential of the two aforementioned changes, the affected mother was also included in the study. She was found to carry both variants, that is, FZD4 and LRP5 splice site mutation c.4489–1G>A, which does not exclude a digenic mode of inheritance. Notably, previous studies have also provided some evidence that FEVR may not exclusively rely on a monogenic model of inheritance. Shastry and Trese [2004] reported that a factor V change cosegregated with the FZD4 mutation in a patient with autosomal dominant FEVR.

The p.C204Y variant (Table 1, Supp. Table S2) is located in a stretch of residues that connects the FZ domain with the first TM domain of FZD4. Using the PHDacc-server [Rost, 1996] (accessible via “Project HOPE,” http://www.cmbi.ru.nl/hope/), secondary structure prediction indicates that the mutated cysteine is placed in a large coiled region, buried in the core of the protein preceding the first TM helix. Mutation of a cysteine residue, possibly employed to form a disulfide bond, into a tyrosine, a hydrophilic amino acid with a large bulky side chain, is expected to lead to destabilization or even unfolding of the domain. In family W06-237 we identified the p.G525R variant in FZD4 (Table 1, Supp. Table S2). This missense change affects an amino acid located in the intracellular C-terminal tail of FZD4, in a stretch of residues predicted to form a coil. This residue is partly exposed to the solvent and partly buried. The two following residues (tryptophan and valine) are predicted to be completely buried in the core of the protein and by being hydrophobic, they are probably important forming the core of the C-terminal domain. Glycine residues are known for their flexibility, as no other residue can make the same backbone angles. It seems possible that the residues preceding tryptophan and valine form a typical structure that is needed to locate the hydrophobic tryptophan and valine in their correct position. This is underlined by the fact that the glycine residue at position 525 is preceded by an asparagine (a residue often present in structural turns) and by another flexible glycine. The p.G525R variant changes the flexible glycine into a less flexible arginine, thereby disturbing the putative backbone structure that is needed for the positions of tryptophan and valine. This potentially could affect the normal function of KTXXXW and PDZ binding in the Wnt/β-catenin pathway, thereby impeding the action of FZD4 receptor complex. All the changed residues are highly conserved among several FZD4 orthologues (Supp. Fig. S2). We have summarized the clinical data of five FEVR patients with FZD4 variants in Supp. Table S2. Their visual acuity ranges from normal to complete blindness. Macular ectopia is frequently observed.

**Table 1. Novel FZD4 Sequence Variants That Are Likely to Cause Familial Exudative Vitreoretinopathy**

<table>
<thead>
<tr>
<th>Nucleotide variant</th>
<th>Effect</th>
<th>Exon</th>
<th>Occurrence in patients (probands)</th>
<th>Occurrence in control alleles</th>
<th>Segregation</th>
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<tr>
<td>c.856G&gt;T</td>
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<td>1/16</td>
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<tr>
<td>c.1282_1285del</td>
<td>p.D428SfsX2</td>
<td>2</td>
<td>1/16</td>
<td>0/100</td>
<td>Yes</td>
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<td>c.116G&gt;C</td>
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<td>1/16</td>
<td>0/100</td>
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<tr>
<td>c.611G&gt;A</td>
<td>p.C204Y</td>
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<td>1/16</td>
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<tr>
<td>c.1573G&gt;C</td>
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<td>2</td>
<td>1/16</td>
<td>0/100</td>
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</tbody>
</table>

Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (GenBank NM_012193.2), according to journal guidelines (www.hgvs.org/mutnomen). The translation initiation codon is codon 1 (GenBank NP_036325.2).

*This patient also carries a splice site mutation in LRP5 (c.4489–1G>A), see Table 2.
those segments are YWTD LDL-class B repeats, whereas the sixth does not contain the required YWTD motif to be recognized as a LDL-class B repeat. The first two propeller domains were suggested to be important for interaction with the Wnt/Frizzled complex [Mao et al., 2001]. The four propeller domains are followed by three LDL-class A repeats (also known as LDL-receptor like ligand binding domains), one TM domain, and a cytoplasmic region with one or multiple short signals for receptor internalization through coated pits.

**Figure 1.** Schematic representation of the activation of the Norrin/β-catenin canonical signaling pathway from TSPAN12/FZD4/LRP5 coreceptors upon activation by NDP. Mutations in the corresponding genes have been associated with impaired activation of the pathway and result in FEVR and Norrie disease, two conditions that not only share common molecular aetiologies but to a high extent show phenotypic overlap.

**Figure 2.** Schematic representation of LRP5 and 3D modeling of LRP5 missense changes. A: Schematic overview of LRP5 and its different domains. B: Overview of the second β-propeller domain of LRP5 and a closeup of the p.E441K change in the second β-propeller domain. The side chain of the wild-type residue (glutamic acid) in position 441 is shown in green, whereas the mutant lysine is shown in red. Two neighboring arginines are colored blue. C: Overview of one of the EGF-like domains and the p.C1253F change. Pair of cysteines forming disulfide bridges are shown in yellow. One of these cysteines is mutated into a phenylalanine, depicted in red.

**Figure 3.** 3D modeling of NDP missense changes. A: Ribbon model of NDP. The protein is colored in gray, the cysteine side chains that participate in forming disulfide bonds and creating the critical knot motif are shown in yellow. The wild-type and mutant residues are depicted in green and red, respectively. B: Closeup of the p.C55R missense change. The wild-type cysteine creates a bond with the neighboring cysteine. The arginine residue at position 55 disrupts the disulfide bond. C: Closeup of the cysteine knot in NDP and missense change p.G67R. The cysteines forming the typical knot motif are indicated. NDP contains an additional cysteine bond that is shown in gray. Additionally, assumptions on the effect of p.G67E on the cysteine knot motif can be drawn from the same figure as the stereochemical structure of glutamic acid is very similar to arginine. D: Closeup of the p.F89L missense change. The side chains of phenylalanine and leucine are shown in green and red, respectively. E: Closeup of the p.S92P missense change. The mutation precedes one of the important bonding cysteines in the protein disturbing the creation of the disulfide bond and possibly affecting the local structure of NDP. F: Closeup of the missense change p.P98L. The change is located close to the last cysteine in the cysteine knot motif and to the unbound cysteine that is needed to form dimers. Conversion of proline to leucine will probably have an impact on the local structure of the protein averting its normal function.

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Whereas LRP5 mutations that are associated with FEVR are scattered throughout the gene, other mutations in LRP5 that seem to be clustered locally cause different phenotypes. Individuals suffering from a group of disorders demonstrating high bone mass and sclerosing bone dysplasias, show autosomal dominant missense variants clustered in exons 2 to 5 that encode the first "β-propeller" domain of LRP5. These changes are hypothesized to act in a dominant negative way. The mutant LRP5 proteins are impaired from binding to Wnt pathway antagonists, for example, SOST or DKK-1, because of defective receptor action or cell trafficking, thereby promoting abnormal growth in bone-forming cells [Boydien et al., 2002; Semenov and He, 2006; Zhang et al., 2004].

LRP5 variants have also been associated with OPPG, a rare autosomal recessive condition for which the vast majority of loss-of-function mutations identified are located in the second and third "β-propeller" domain and the following LDL repeats of LRP5. Of the FEVR-causing mutations, four mutations result in premature stop codons, whereas at least six missense changes have been associated with the autosomal dominant form of the disease. In addition, 11 missense variants (of which, three homozygous and two compound heterozygous) have been associated with autosomal recessive or sporadic FEVR cases. All these changes seem to result in a reduced or lost interaction with FZD4 and NDP and thus disturbing the normal Wnt signaling cascade that regulates retinal vessel formation.

Functional characterization of nucleotide changes in LRP5 has been performed by various groups. Gong et al. [2001] studied the effect of p.Q853X and p.E1270RfsX169 by transiently transfecting COS-7 cells with mammalian expression constructs containing either the wild-type or mutant LRP5 sequences. They demonstrated that even if the mutant proteins were synthesized they were not able to be secreted, due to impaired cell trafficking, adding evidence to the assumption that OPPG results from loss-of-function mutations. Boydien et al. [2002] studied the effects of p.G171V by performing in vitro and in vivo experiments measuring Wnt signaling output. They used a luciferase activity assay, which was responsive to LRP5 by cotransfection experiments in NIH3T3 cells and by measuring fibronectin levels in affected individuals with the high bone mass phenotype. The p.G171V variant did not influence normal Wnt signaling in the absence of Wnt antagonists. However, when such an antagonist (e.g., Dkk-1) was present, the mutant LRP5 construct was able to reduce its inhibitory action. Zhang et al. [2004] showed that the p.G171V mutation seemed to disrupt the Msd-mediated recruitment of LRP5 to the surface of osteoblasts, which occurs via the first β-propeller domain. They proposed that paracrine Dkk-1, which is produced by osteoclasts and binds to the third "β-propeller" domain of LRP5, has limited target availability, that is, minimal antagonizing potential, boosting autocrine Wnt action in the osteoblasts. Adding to the complexity of events that act synergistically for high bone mass phenotype, it was also postulated that p.G171V was able to directly reduce binding ability of a bone specific LRP5 antagonist, SOST, to the first β-propeller structure, potentially affecting bone mass regulation [Semenov and He, 2006]. Ai et al. [2005] designed a series of expression constructs containing OPGG-associated mutations (p.S356L, p.T390K, p.G404R, p.D434N, p.G520V, and p.G610R) and FEVR-associated mutations (p.T173M, p.R570Q, p.Y1168H, p.C1361G, and p.E1367K) along with wild-type LRP5 and transiently transfected HEK293T cells in an ex vivo reporter assay for Wnt and Norrin signal transduction. With the exception of p.S356L and p.G520V, OPPG-associated LRP5 mutations, which affected the first or second β-propeller domain, resulted in impaired subcellular trafficking of mutant LRP5. However, there was no clear-cut correlation between assay activity and phenotypes. When FEVR-associated mutations were tested in the same assay they showed variable results. The p.Y1168H missense variant that causes autosomal dominant FEVR almost completely abolished Wnt and Norrin signal transduction, whereas p.T173M had no significant impact. A similar effect was shown for two other mutations associated with autosomal recessive FEVR (p.R570Q and p.E1367K). Finally, it was demonstrated in concordance with all the previous experiments, that LRP5 mutants p.R444C and p.A522T exhibited a variable reduction in the Norrin-dependent Wnt signaling (45 and 26%, respectively) that correlated with the mild phenotype observed in the patients who carry these changes [Qin et al., 2008].

In this study, we report two new missense (p.E441K, p.C1253F), one new nonsense variant (p.W993X), and one novel splice site (c.4489–1G>A) change. The p.E441K variant has been identified in one sporadic FEVR patient (Table 2, Supp. Table S2). It is located in the second "β-propeller" domain of the protein, at an evolutionary highly conserved position (Supp. Fig. S3). As our structure prediction model (Fig. 2B) points out, the negatively charged and hydrophilic glutamic acid creates ionic interactions with two positively charged arginine residues in its close proximity (residues indicated in blue), thereby stabilizing and formulating the local structure of the whole domain. Introduction of an additional positive residue such as lysine is likely to disturb the ionic interactions, potentially destabilizing the whole domain structure and therefore impeding its normal function. This could have an effect on its potential interaction with NDP, which is also supported by the aforementioned functional analysis of p.R444C. More interestingly a different mutation, p.D434N, which was also characterized by decreased Wnt signaling potential, was associated

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<th>Table 2. Novel LRP5 Sequence Variants That Are Likely to Cause Familial Exudative Vitreoretinopathy</th>
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<td><strong>Nucleotide variant</strong></td>
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Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (GenBank NM_002335.2), according to journal guidelines (www.hgvs.org/mutnomen). The translation initiation codon is codon 1 (GenBank NP_002326.2).

This patient also carries a second missense variant in FZD4 p.E40Q (c.118G>C), see Table 1.
with OPPG. The second variant, p.C1253F, is located in the EGF-like domain following the third "β-propeller" module of LRP5 (Fig. 2C), and also affects a well-conserved residue (Supp. Fig. S3). The role of this domain is unclear. The third "β-propeller" structure and the three LDL-class A repeats play a role in binding of ligands such as Dkk-1. The EGF-like domains in general are stabilized by three disulfide bridges (Fig. 2C). The introduction of a phenylalanine at the position of cysteine residue is predicted to disturb one of the existing disulfide bridges. Moreover, phenylalanine has a much bulkier side chain when compared with cysteine and might exert steric hindrance effects. Interestingly, the same person carrying the p.C1253F variant was found to carry a second LRP5 missense variant, p.G610R, previously reported to be associated with FEVR (in a compound heterozygous mode) and with OPPG (Supp. Table S3 and Supp. Table S4). The p.G610R variant, when present heterozygously, was shown in at least one case to result in low bone mass density. The father of the proband was found to carry the p.C1253F variant, whereas the mother was carrying the p.G610R. Based on the clinical picture of this family (Supp. Table S2) and mutation segregation data an autosomal recessive mode of inheritance can be suggested, although autosomal dominant inheritance cannot be totally excluded.

Furthermore, a nonsense change (p.W993X) was found in one sporadic patient with FEVR that results in a premature stop codon in exon 13 likely causing NMD and thus leading to LRP5 haploinsufficiency (Table 2, Supp. Table S2). Finally, we identified a splice-acceptor mutation, c.4489–1G>A, in the same person that harbors the FZD4 p.E40Q variant. The effect of this mutation is predicted to be skipping of exon 22 and the subsequent in frame deletion of 20 amino acids. No RNA was available to test the effect of the splice site change. We summarized the clinical data for three patients with LRP5 variants in Supp. Table S2.

**LRP5 Polymorphisms**

Many groups have tried to find associations between LRP5 polymorphisms and osteoporosis, low bone mass index, hypercholesterolemia, obesity, and increased body mass index. The nonsynonymous p.A1330V (rs3766228:C>T) SNP in exon 18 has been in the center of attention in a large number of studies. This polymorphism was linked with low bone mass determination at the lumbar spine, femoral neck and total hip [Koay et al., 2004]. Polymorphisms and osteoporosis, low bone mass index, hypercholesterolemia, obesity, and increased body mass index. The three LDL-class A repeats play a role in binding of ligands such as Dkk-1. The EGF-like domains in general are stabilized by three disulfide bridges (Fig. 2C). The introduction of a phenylalanine at the position of cysteine residue is predicted to disturb one of the existing disulfide bridges. Moreover, phenylalanine has a much bulkier side chain when compared with cysteine and might exert steric hindrance effects. Interestingly, the same person carrying the p.C1253F variant was found to carry a second LRP5 missense variant, p.G610R, previously reported to be associated with FEVR (in a compound heterozygous mode) and with OPPG (Supp. Table S3 and Supp. Table S4). The p.G610R variant, when present heterozygously, was shown in at least one case to result in low bone mass density. The father of the proband was found to carry the p.C1253F variant, whereas the mother was carrying the p.G610R. Based on the clinical picture of this family (Supp. Table S2) and mutation segregation data an autosomal recessive mode of inheritance can be suggested, although autosomal dominant inheritance cannot be totally excluded.

Furthermore, a nonsense change (p.W993X) was found in one sporadic patient with FEVR that results in a premature stop codon in exon 13 likely causing NMD and thus leading to LRP5 haploinsufficiency (Table 2, Supp. Table S2). Finally, we identified a splice-acceptor mutation, c.4489–1G>A, in the same person that harbors the FZD4 p.E40Q variant. The effect of this mutation is predicted to be skipping of exon 22 and the subsequent in frame deletion of 20 amino acids. No RNA was available to test the effect of the splice site change. We summarized the clinical data for three patients with LRP5 variants in Supp. Table S2.

**NDP Mutations**

The NDP gene encodes Norrin, a small secreted and cysteine rich protein that consists of 133 amino acids with weak homology to the transforming growth factor (TGF)-β family of ligands [Berger et al., 1992a; Chen et al., 1992; Meindl et al., 1992; Meitinger et al., 1993]. Norrin consists of two major parts: a signal peptide at the amino-terminus of the protein that directs its localization and a region containing a typical motif of six cysteines forming a cysteine-knot that provides the structural conformation required for receptor binding and subsequent signal transduction [Meitinger et al., 1993]. Our 3D model of NDP agrees with structures previously described [Vitt et al., 2001] and contains two antiparallel β-sheets and one α-helical structure (Fig. 3A).

Although Norrin is not structurally related to the Wnt family of proteins, it resembles their function by binding with high affinity to the receptor Frizzled-4, requiring LDL receptor-related protein (LRP) as a coreceptor and by activating the FZD4/LRP5/β-catenin signaling pathway Wnt signaling pathway [Xu et al., 2004]. One of the multiple routes that this pathway is involved in, is the transcriptional control and regulation of genes that are under the influence of TCF/Lef binding sites. NDP, together with FZD4 and LRP5, effectively plays a role in the development of the cells in the retinal neural epithelium by setting off the retreat of the HVS and in parallel by patterning the external and deep retinal vasculature, which is necessary for retinal function [Luhmann et al., 2005].

Ninety-five nucleotide variants have been reported for NDP (Supp. Table S5). Twenty-four mutations result in the introduction of a premature stop codon, 58 are missense changes, six changes affect splice sites and for seven variants their effect is different or unknown. Most of the mutations result in Norrie disease and a smaller percentage in X-linked FEVR. Five NDP changes (p.R41S, p.Y44X, p.C96W, p.L108F, and p.R121W) have been suggested to be associated with persistent fetal vasculature syndrome, Coats disease, and retinopathy of prematurity (ROP). These distinct clinical entities share some common pathological features such as abnormal retinal blood vessel growth that may result in scarring and retinal detachment.

Two groups have performed functional assays for NDP variants. Xu et al. [2004], using a cotransfection signaling assay in STF cells in parallel with immunoblotting, assessed total protein production, secretion efficiency to the extracellular matrix and potency for signaling transduction. They analysed 18 NDP mutations associated with FEVR or ROP. All missense mutants but one (p.L13R), showed no production or secretion efficiency aberrations; all but one (p.K58N) resulted in a 20 to 80% decreased Wnt signaling potential. Moreover, Qin et al. [2008], after testing two groups of NDP missense variants associated with either FEVR (p.R41K, p.K54N, p.R115L, and p.R121W) or Norrie disease (p.K58N, p.A63D, p.R97P, and p.R121W), in a similar way showed 17 to 96% reduced signaling potential. Only p.K58N, in agreement with the previous study, resulted in increased signaling ability. Interestingly, the authors suggested that mutants p.K34N, p.K58N, and p.R115L disturb binding of Norrin with other factors possibly related to Norrin’s physiological function. Finally, no mutant was observed to have any impact on the integrity of the protein.

Here, we report 12 novel nucleotide variants in NDP associated with Norrie disease or X-linked FEVR. These include six missense variants (p.C55R, p.G67E, p.G67R, p.F89L, p.S92P, and p.P98L), one nonsense (p.S111X), three frameshift (p.I4RfsX21, p.S99fsX4, and p.Y44MfsX60), and two splice site mutations (c.–208G>T, c.–2085G>A). The missense change p.C55R influences a cysteine residue located on the “top” of one of the antiparallel β-sheets forming a disulphide bridge with a cysteine on the top of the other β-sheet (Fig. 3B). The exact function of this bond is unclear, but it could be speculated that it might be necessary to stabilize the conformation of the two β-sheet domains that contain hydrophobic residues that can interact with another NDP monomer. Without the disulfide bridges the two sheets will have more freedom to move, thereby making it difficult to form the correct structure for dimerization. Moreover, this...
disulfide bridge and amino acid location was shown to be evolutionary conserved (Supp. Fig. S4) [Meitinger et al., 1993].

The glycine residue at position 67 is highly conserved among many NDP orthologues (Supp. Fig. S4) and the p.G67E and p.G67R variants change a small and flexible glycine into either a glutamic acid (negatively charged) or into an arginine (positively charged). Glycine is “required” at this position for the protein to adopt a typical backbone conformation crucial for formation of the cysteine knot (Fig. 3C). No other residue can make the correct backbone angles, and therefore, mutation of the glycine will disturb cysteine knot formation and the structure of the protein.

The missense change p.F89L is located in a highly conserved domain (Supp. Fig. S4) that adopts a z-helical structure. This amino acid is predicted to be one of the residues that participate in the dimerization of NDP [Meitinger et al., 1993]. The hydrophobic phenylalanine interacts with other hydrophobic residues on the other monomer. Leucine is also hydrophobic, but has a smaller sidechain (Fig. 3D). It seems possible that this leucine could make the same type of interactions, but because of altered stereo-specificity the dimer formation could be less strong and stable.

The missense change p.S92P affects a serine that precedes a charged). Glycine is “required” at this position for the protein to adopt a typical backbone conformation crucial for formation of the cysteine knot (Fig. 3C). No other residue can make the correct backbone angles, and therefore, mutation of the glycine will disturb cysteine knot formation and the structure of the protein.

The four novel protein truncating mutations likely result in a premature truncated protein [Kato et al., 2002]. These insertion of an IRES–LacZ–Neomycin cassette and thus resulting in a premature truncated protein [Kato et al., 2002]. These

Animal Models

Several mouse models have been made that either have no or partially functional Fzd4, Lrp5, and Ndph (Norrie disease pseudoglioma mouse homologue), to shed more light on the biochemical properties of these proteins and understand the pathology of the associated diseases.

Gene disruption in murine Fzd4 using a Fzd4–lacZ knock-in reporter allele assessed by X-gal staining provided in-depth analysis of its expression. During littermate development, Fzd4 seemed to be transiently present in a wide variety of tissues. In mature Fzd4+/− mice, expression was observed in the Purkinje cells of the cerebellum, in the retina, in the inner hair cells in the organ of Corti, and in the sensory epithelium of the otolithic organs [Wang et al., 2001]. Moreover, a prominent Fzd4 expression throughout development was also demonstrated in the ovary, with high prevalence in the corpus luteum [Hsieh et al., 2002]. Fzd4+/− mice are viable, although they are subject to high mortality rates (~50%) and impaired growth, beginning in the second postnatal week [Wang et al., 2001]. The Fzd4−/− mice also displayed progressive hearing loss and an inoperative swollen esophagus around P8 [Wang et al., 2001]. Xu et al. [2004] demonstrated that young adult mice show compromised intraretinal vasculature. Problematic blood supply of the retina especially in the nerve fiber layer (NFL) and outer plexiform layer (OPL) was noted. No significant differences in anatomic and/or phenotypic characteristics were noticed between Fzd4+/− and Fzd4−/− mice.

Similar to Fzd4, Lrp5 was found to be expressed in many tissues in the mouse. However, its expression was most abundant in central nervous system neurons, the islets of Langerhans, liver, osteoblasts, and ocular macrophages [Figureo et al., 2000; Kato et al., 2002]. In an effort to replicate the phenotypic manifestation of OPPG, Lrp5−/− mice were generated by disrupting exon 6 of the gene with the insertion of an IRES–lacZ–Neomycin cassette and thus resulting in a prematurely truncated protein [Kato et al., 2002]. These Lrp5−/− mice

Table 3. Novel NDP Sequence Variants That Are Likely to Cause Norrie Disease

<table>
<thead>
<tr>
<th>Nucleotide variant</th>
<th>Effect</th>
<th>Exon</th>
<th>Occurrence in patients (probands)</th>
<th>Occurrence in control alleles</th>
<th>Segregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.11_12del</td>
<td>p.H4REx21</td>
<td>2</td>
<td>1/46</td>
<td>0/100</td>
<td>Simplex</td>
</tr>
<tr>
<td>c.25_40del</td>
<td>p.S9P6X4</td>
<td>2</td>
<td>1/46</td>
<td>0/100</td>
<td>Simplex</td>
</tr>
<tr>
<td>c.129delC</td>
<td>p.Y44Mx60</td>
<td>2</td>
<td>1/46</td>
<td>0/100</td>
<td>Simplex</td>
</tr>
<tr>
<td>c.323C&gt;A</td>
<td>p.S11X</td>
<td>3</td>
<td>1/46</td>
<td>0/100</td>
<td>Simplex</td>
</tr>
<tr>
<td>c.163T&gt;C</td>
<td>p.C55R</td>
<td>2</td>
<td>1/46</td>
<td>0/100</td>
<td>Simplex</td>
</tr>
<tr>
<td>c.199G&gt;A</td>
<td>p.G67R</td>
<td>3</td>
<td>1/46</td>
<td>0/100</td>
<td>Simplex</td>
</tr>
<tr>
<td>c.200G&gt;A</td>
<td>p.G67E</td>
<td>3</td>
<td>1/46</td>
<td>0/100</td>
<td>Simplex</td>
</tr>
<tr>
<td>c.267C&gt;A</td>
<td>p.F89L</td>
<td>3</td>
<td>1/46</td>
<td>0/100</td>
<td>Simplex</td>
</tr>
<tr>
<td>c.274T&gt;C</td>
<td>p.S92P</td>
<td>3</td>
<td>1/46</td>
<td>0/100</td>
<td>Simplex</td>
</tr>
<tr>
<td>c.293C&gt;T</td>
<td>p.P98L</td>
<td>3</td>
<td>1/46</td>
<td>0/100</td>
<td>Simplex</td>
</tr>
<tr>
<td>c.-208+2T&gt;G</td>
<td>Splice defect</td>
<td>1</td>
<td>1/46</td>
<td>0/100</td>
<td>Simplex</td>
</tr>
<tr>
<td>c.-208+5G&gt;A</td>
<td>Splice defect</td>
<td>1</td>
<td>1/46</td>
<td>0/100</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (GenBank NM_000266.3), according to journal guidelines (www.hgvs.org/mutnomen). The translation initiation codon is codon 1 (GenBank NP_000257.1).
were viable and fertile; however, a fraction of them died prematurely because of bone fractures. The mice displayed low bone mass phenotype from 2 weeks and decreased bone volume and mineral content of the ECM surrounding osteoblasts. In 70% of Lrp5<sup>+/−</sup> mice hyaloid vessels regression was delayed at 6 months of age. This was postulated to result from failed macrophage-mediated apoptosis. Lrp5<sup>−/−</sup> mice displayed the same bone phenotype to a lesser extent and had no eye-related abnormalities. Moreover, Fujino et al. [2003], generated Lrp5<sup>−/−</sup> mice by disrupting a ligand binding repeat in exon 18. All mice appeared normal until the sixth month of their life when parietal bone weakness was noted in 70% of Lrp5<sup>−/−</sup> of the female population. The Lrp5<sup>−/−</sup> mice also displayed impaired chylomicron clearance, hepatic uptake, and glucose-induced insulin secretion. The authors did not report on eye-related abnormalities. Two other studies [Akhter et al., 2004; Babij et al., 2003] described transgenic mice with the p.G171V mutation, which is associated with the human high bone mass phenotype. The LRP5<sub>G171V</sub> mice showed greater bone mass, density, structure, and strength. Finally, Xia et al. [2008] identified a ENU-induced recessive mouse mutant line that bears many similarities with the eye pathology that FEVR shows in humans. The mutant r18 mouse carries a frameshift mutation that causes a premature stop at codon 1596 and the replacement of the 39 most C-terminal amino acids by 20 different ones. In Lrp5<sup>−/− c/t</sup> mice, the retina appeared hypopigmented and the retinal arteries weak. Moreover, early in their growth, the mice developed retinal hemorrhage. The retinal vasculature was disorganized, immature, and leaky, with less and smaller blood vessels. Meagre retinal blood supply was postulated to be a consequence of defective capillary lumen formation.

Ndph mutant mice replicate most of the symptoms associated with Norrie disease and partially that of FEVR, both in terms of eye/retinal symptomatology and audiological defects. The mutant mice display retrolental structures in the vitreous body and a perturbed ganglion cell layer along with the formation of pseudogliomas in the posterior vitreal chamber [Berger et al., 1996; Richter et al., 1998]. All the different cell layers of the retina were reduced along with the photoreceptors, but specific segments of the retina such as the OPL could completely disappear [Berger et al., 1996; Lenzner et al., 2002]. Moreover, the Ndph knockout mice developed severe structural irregularities of the retinal vascular system formation. The hyaloid blood vessels delayed to regress [Luhmann et al., 2005; Ohlmann et al., 2005; Richter et al., 1998], and both superficial and intraretinal vasculature was severely impaired because a large number of blood vessels or capillaries was defective or even absent. The lack of normal blood supply that nourishes the different retinal cell layers results in local hypoxic conditions, especially in the inner retina. This was associated with increased expression of vasodilatation indicators such as eNOS and VEGF, and increased permeability of retinal blood vessels [Kaur et al., 2006; Luhmann et al., 2005]. Defects in the cochlear stria vasularis of Ndph<sup>+/−</sup> animals resulted in progressive hearing loss, leading to deafness, similar to the human phenotype [Rehm et al., 2002]. Adding to that, more light was shed on the biochemical role of NDP by overexpressing it transgenically in the eyes of Ndph<sup>+/−</sup> mice under the control of a lens-specific promoter [Ohlmann et al., 2005]. Ectopically expressed Norrin surprisingly restored the normal retinal vasculature, while respecting the normal local architecture of the blood vessels. Recently, the fact that vascular changes occur in the cerebellum of hemizygous Ndph<sup>+/−</sup> animals was noted as the first evidence of a brain phenotype in mice that could be correlated with the mental retardation seen in one-third of human Norrie disease patients [Luhmann et al., 2008]. Finally, Schafer et al. [2009] provided further evidence about the multidimensional role of NDP during retinal angiogenesis. They postulated that the function of NDP may not only be limited to transcriptional regulation of β-catenin target genes that, for example, regulate blood vessel formation and integrity, but also play a role in the transcription of other targets like Pitx1 [Carson-Walter et al., 2005; Stan et al., 1999]. The latter would expand Norrin’s functional spectrum, which might provide us with an insight into the phenotypic heterogeneity observed even in individuals of the same family suffering from FEVR or ND. The parallel contribution of other genetic determinants could affect the severity of disease symptoms by putatively altering target gene expression, something that potentially could be explored as a diagnostic or prognostic tool regarding the phenotypic severity of patients.

Clinical, Diagnostic, Biological Relevance, and Future Prospects

With the present study on Fzd4, Lrp5, and Ndph, we have attempted to provide a comprehensive summary of mutations that have been reported to affect these genes, with a focus on FEVR and Norrie disease. The three proteins cooperate in the Wnt/β-catenin and/or Norrin/β-catenin signaling pathway, by regulating angiogenesis and maintaining blood vessel integrity in the retina. Patients suffering from FEVR and to a lesser degree, Norrie disease, are often difficult to diagnose. Nonpenetrance in FEVR can be observed in up to 25% of mutation carriers [Boonstra et al., 2009; Riveiro-Alvarez et al., 2005] and genotype-phenotype correlations are often not accurate enough. Wu et al. [2007] have suggested that NDP mutations affecting cysteine residues that play a firm role in the formation of the cysteine knot motif resulted in severe retinal dysgenesis and were diagnosed as Norrie disease. Nucleotide variants that affected noncysteine residues were associated with FEVR-like phenotypes. Furthermore, it was also postulated that mutations in Lrp5 found in patients with FEVR or OPPG can result both in ocular and bone abnormalities, classifying both diseases under the same phenotypic spectrum [Qin et al., 2005]. The relatively small number of 28 exons and 32 amplicons that need to be analyzed for theses three genes renders Sanger sequence analysis cost effective. Molecular testing is important for diagnostic and prognostic purposes in individuals suspected to suffer from FEVR or ND, especially when coupled with a detailed family history. At least 40% of patients with FEVR do not carry mutations in Fzd4 or Lrp5 [Boonstra et al., 2009; Qin et al., 2005; Toomes et al., 2004a], so we are pursuing the identification of additional FEVR genes. Recently, in a cohort of eleven Dutch families that were tested negative for mutations in the ORF of Fzd4, Lrp5, and Ndph, respectively; we identified Tspan12 as a gene causative for autosomal dominant FEVR [Nikopoulos et al., 2010]. The gene was mutated in 5 out of the 11 Dutch families tested, rendering it a relative frequent cause of other genetic determinants could affect the severity of disease symptoms by putatively altering target gene expression, something that potentially could be explored as a diagnostic or prognostic tool regarding the phenotypic severity of patients.

Acknowledgments

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