Report

Autosomal Recessive Familial Exudative Vitreoretinopathy Is Associated with Mutations in *LRP5*

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Familial exudative vitreoretinopathy (FEVR) is a hereditary eye disorder that affects both the retina and vitreous body. Autosomal recessive FEVR was diagnosed in multiple individuals from three consanguineous families of European descent. A candidate-locus–directed genome scan shows linkage to the region on chromosome 11q flanked by markers *D11S905* and *D11S1314*. The maximum LOD score of 3.6 at $\theta = 0$ is obtained with marker *D11S987*. Haplotype analysis confirms that the critical region is the 22-cM (311-Mb) interval flanked by markers *D11S905* and *D11S1314*. This region contains *LRP5* but not *FZD4*; mutations in both of these genes cause autosomal dominant FEVR. Sequencing of *LRP5* shows, in all three families, homozygous mutations R570Q, R752G, and E1367K. This suggests that mutations in this gene can cause autosomal recessive as well as autosomal dominant FEVR.

Familial exudative vitreoretinopathy (FEVR [MIM 133780]) is a rare childhood hereditary eye disorder that affects both the retina and the vitreous body (Criswick and Schepens 1969). It is clinically similar to advanced retinopathy of prematurity, but the affected individuals do not have a history of premature birth and oxygen therapy. The condition is characterized by the premature arrest of the vascularization of the peripheral retina, leading to incomplete vascularization of the peripheral retina. This then results in the development of hyperpermeable blood vessels, neovascularization, and, hence, retinal folds, retinal exudates, and tractional retinal detachment in 20% of affected individuals. The disorder is bilateral and has a high degree of penetrance and a variable expressivity (Shastry and Hiraoka 2000). Minimally affected individuals do not show any visual symp-

toms. FEVR tends to be a slowly progressive disorder, with retinal detachment often not occurring until after the 1st decade of life.

FEVR is inherited in autosomal dominant (adFEVR [MIM 133780]), X-linked (MIM 305390), and autosomal recessive (arFEVR [MIM 601813]) fashions, although the autosomal dominant form is the most common. X-linked FEVR has been mapped to Xp11.4 and has been associated with mutations in the Norrie disease gene (*ND*), although genetic heterogeneity has been suggested by the failure to identify mutations in some patients (Chen et al. 1993; Fullwood et al. 1993; Shastry et al. 1995, 1997*a*, 1997*b*). adFEVR was initially mapped to two loci: *EVR1* on chromosome 11q13-q23 (Li et al. 1992; Muller et al. 1994; Price et al. 1996; Shastry et al. 2000; Kondo et al. 2001) and *EVR3* on chromosome 11p13-p12 (Downey et al. 2001).

Whereas the *EVR3* locus has not been associated with mutations in any specific gene, the more common *EVR1* locus has been associated with mutations in two linked genes: the Wnt-receptor frizzled-4 gene (*FZD4*) and the low-density lipoprotein receptor–related protein 5 gene (*LRP5*) (Robitaille et al. 2002; Toomes et al. 2004). Both FZD4 and LRP5 are involved in the Wnt signaling pathway, combining to bind Wnt proteins and to form a

functional ligand-receptor complex (Pinson et al. 2000). It is interesting that norrin, the product of *ND,* is also a specific high-affinity ligand for FZD4 in concert with an LRP coreceptor, activating the Wnt signaling pathway (Xu et al. 2004). Mutations associated with Norrie disease decrease Wnt signaling to 20%–80% of control levels.

LRP5 is a member of the low-density lipoprotein receptor (LDLR) family, with four EGF precursor spacer domains—each comprising five YWTD repeats and one EGF repeat that together form a β-propeller structure—followed by three LDLR ligand-binding domain motifs, a single transmembrane domain, and a cytoplasmic tail (Hey et al. 1998). In addition to the interactions of LRP5 with FZD4 and Wnt, the intracellular domain of LRP5 binds Axin. This binding is facilitated by Wnt and is a part of Wnt signaling (Mao et al. 2001). LRP5 mutants lacking the extracellular domains bind Axin constitutively, inducing lymphoid enhancer-binding factor 1 (LEF1) activation by destabilizing Axin and stabilizing β-catenin. In animal models, LRP5 has been shown to be essential for bone accrual, eye development, and me-

tabolism of cholesterol and glucose (Kato et al. 2002; Fujino et al. 2003). In humans, LRP5 is expressed by osteoblasts and affects bone mass (Gong et al. 2001), with specific *LRP5* alleles associated with osteoporosis (Mizuguchi et al. 2004). Conversely, mutations in the amino-terminal part of LRP5 have been associated with a variety of conditions with increased bone density that are diagnosed as endosteal hyperostosis, Van Buchem disease, and osteopetrosis (Van Wesenbeeck et al. 2003). In addition to adFEVR, mutations in *LRP5* have been associated with osteoporosis-pseudoglioma syndrome (OPPG [MIM 259770]), characterized by abnormal retinal vascularization often resulting in vitreal changes and pseudoglioma (Gong et al. 2001). Thus, mutations in *LRP5* can result in either dominant or recessive disease affecting bone density, retinal vascularization, or both. It is notable that a number of individuals diagnosed with adFEVR and shown by Toomes et al. (2004) to have *LRP5* mutations also had low bone density or a history of fractures.

DNA samples were collected from three consanguineous families of European descent (families 68001,
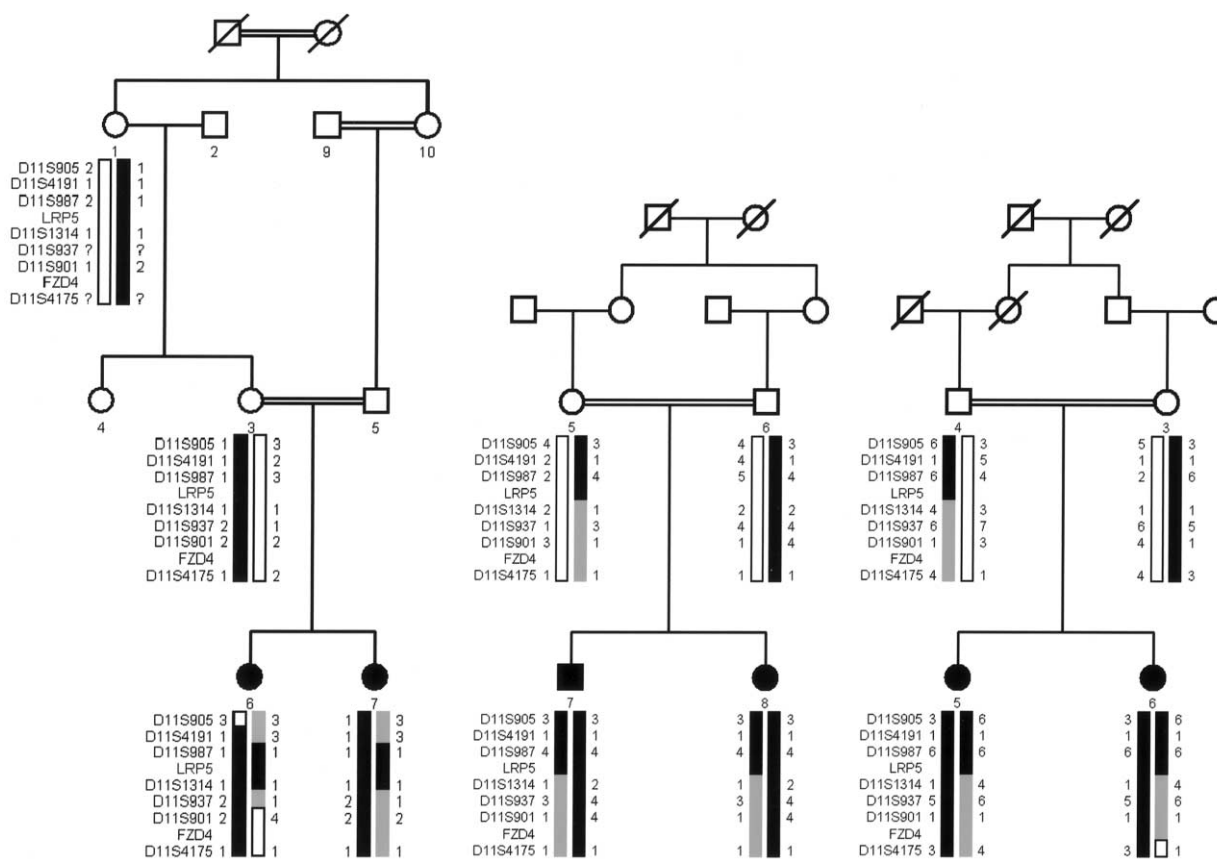

Figure 1   Pedigree of families 68001, 68002, and 68003. The blackened boxes correspond to affected haplotypes with alleles that cosegregate with the disease and that are homozygous in affected individuals.

68002, and 68003) (fig. 1) with 14 individuals, of whom 6 are affected. Informed consent was obtained from each individual studied. This study has been approved by the ethics review board of William Beaumont Hospital (Royal Oak, MI) and the institutional review board of the National Eye Institute and is consistent with the tenets of the Declaration of Helsinki. Patients were examined using indirect ophthalmoscopy and fluorescein angiography. Visual acuity was assessed in all patients.

Clinical descriptions of these families have been published elsewhere by Shastry and Trese (1997) (family 68001) and by de Crecchio et al. (1998) (families 68002 and 68003). In brief, the two affected sisters in family 68001 also have an affected paternal uncle (a product, along with five unaffected siblings, of the consanguineous mating of individuals 9 and 10, as shown in fig. 1). A fourth affected individual in this family is an affected nephew of individual 9, who is himself the product of a consanguineous mating (fig. 1) (see Shastry and Trese [1997]). Although the affected nephew—the son of an unaffected brother of individual 9—is not the product of a known consanguineous mating, the family belongs to an isolated Amish community, and it seems likely that his parents are distantly related. Both sisters were diagnosed at 3 years of age, at which time they manifested typical signs of FEVR, including large retinal folds, peripheral traction and exudates, and an avascular demarcation line near the equator and vitreous detachments. Unaffected relatives, including the parents, were examined and showed no funduscopic abnormalities (fig. 2) (see Shastry and Trese [1997]). Neither of the affected sisters, both 10 years old at the time of the study, has a history of bone fractures.

The affected brother and sister in family 68002 (family 2 in de Crecchio et al. 1998) were diagnosed with retinal detachments at ages 6 years and 8 years, respectively, and also showed other typical signs of FEVR. They showed no systemic symptoms, including fractures and traumatic injuries, by ages 12 years and 10 years, respectively. No additional family members are known to be affected, including seven maternal and seven paternal

aunts and uncles. The affected sisters in family 68003 (family 1 in de Crecchio et al. 1998) were diagnosed at ages 5 years and 7 years and have shown typical signs of FEVR that required multiple procedures, including photocoagulation, cryopexy, and vitrectomy. They show no signs of systemic disease, including fractures, at ages 29 years and 31 years. Consanguinity in this family was disclosed after publication of the article by de Crecchio et al. (1998). No additional family members are known to be affected, including five paternal and four maternal aunts and uncles. In families 68002 and 68003, unaffected relatives, including both pairs of parents, underwent full ophthalmological examinations that included fluorescein angiography and showed no abnormalities, including no vascular tortuosity. Both the presence of consanguinity and the normal results of examination of the unaffected parents in each family strongly support autosomal recessive inheritance in these families. In addition, although we are unable to address bone density specifically, the absence of fractures in the six affected individuals in these three families, with an age range of 10–31 years, would be highly atypical for patients with OPPG.

For linkage analysis, DNA was extracted directly from blood by standard phenolchloroform protocols (Smith et al. 1989). A candidate-oriented genomewide scan was begun with 382 fluorescently labeled microsatellite markers (ABI Linkage Mapping Set MD-10) with the use of only a subset of samples, to conserve the limited amounts of DNA available from some individuals. Multiplexed PCR was performed as described elsewhere (Jiao et al. 2000). Two-point linkage analyses were performed using the FASTLINK implementation of the MLINK program of the LINKAGE program package (Lathrop and Lalouel 1984; Cottingham et al. 1993). For screening, equal allele frequencies were used for all markers. For fine mapping on chromosome 11, the allele frequencies were calculated using 15 control individuals (30 chromosomes) of European ancestry.

Evidence of significant linkage in the genomewide scan was first observed with markers *D11S987* and *D11S4191,* which gave maximum LOD scores of 3.3 at $\theta = 0$ and 2.43 at $\theta = 0$, respectively. For fine mapping using markers taken from the Genethon database, additional individuals were added to the families, estimated allele frequencies were used, and the maximum LOD score for *D11S987* increased to 3.6 at $\theta = 0$, whereas, for *D11S4191,* the LOD score decreased to −1.1 at $\theta = 0$, with a LOD score of 1.1 at $\theta = 0.1$ (table 1). By use of LOD scores $\leqslant -2$ to exclude linkage in these families, arFEVR mapped to a 21.7-cM (311-Mb) interval flanked by *D11S905* and *D11S1314.* Markers *D11S901* and *D11S4175,* which flank *FZD4,* both show obligate recombinations.

Examination of the haplotypes cosegregating with

Table 1

LOD Scores of Markers on Chromosome 11q

	Position		LOD Score at $\theta =$						
Markers	cM	Mb	0	.01	.05	.1	.2	.3	.4
D11S905	51.95	409.38	−∞	−2.2	−.4	.1	.3	.2	0
D11S4191	60.09	597.75	−1.1	.6	1	1.1	.8	.5	.2
D11S987	67.48	...	3.6	3.5	3.1	2.7	1.8	1	.4
D11S1314	73.64	720.49	−4.7	−2	−.8	−.3	0	0	0
D11S937	79.98	775.8	−7.8	−2.9	−1	−.4	0	0	0
D11S901	85.48	815.71	−∞	−.4	.6	.8	.8	.5	.2
D11S4175	91.47	899.4	−∞	−.8	.3	.5	.5	.3	.1

arFEVR in the three families confirms and extends the linkage results. In family 68001, a recombination event between marker *D11S905* and the arFEVR locus in individual 6 provides the centromeric boundary to the linked region. In addition, lack of homozygosity for markers *D11S905* and *D11S4191* in both affected individuals suggests that the arFEVR locus might lie distal to *D11S4191* (fig. 1). In family 68002, lack of homozygosity in marker *D11S1314* suggests that the arFEVR locus lies proximal to that marker. Similarly, in family 68003, lack of homozygosity in markers *D11S905* and *D11S1314* suggests that arFEVR lies in the same interval suggested by the two-point linkage analysis: the linked

region between *D11S905* and *D11S1314* (fig. 1). This critical interval contains *LRP5*, in which mutations have recently been shown to cause adFEVR. *FZD4*, the other gene on chromosome 11q implicated in adFEVR and also part of the Wnt signaling pathway, lies between markers *D11S901* and *D11S4175* and is excluded from the linked region by an obligate recombination event in individual 7 of family 68001 and by lack of homozygosity in all families.

For mutation screening of *LRP5*, coding exons and adjacent intronic sequences were amplified from genomic DNA of two affected patients and one unaffected individual. The reference genomic sequence is available
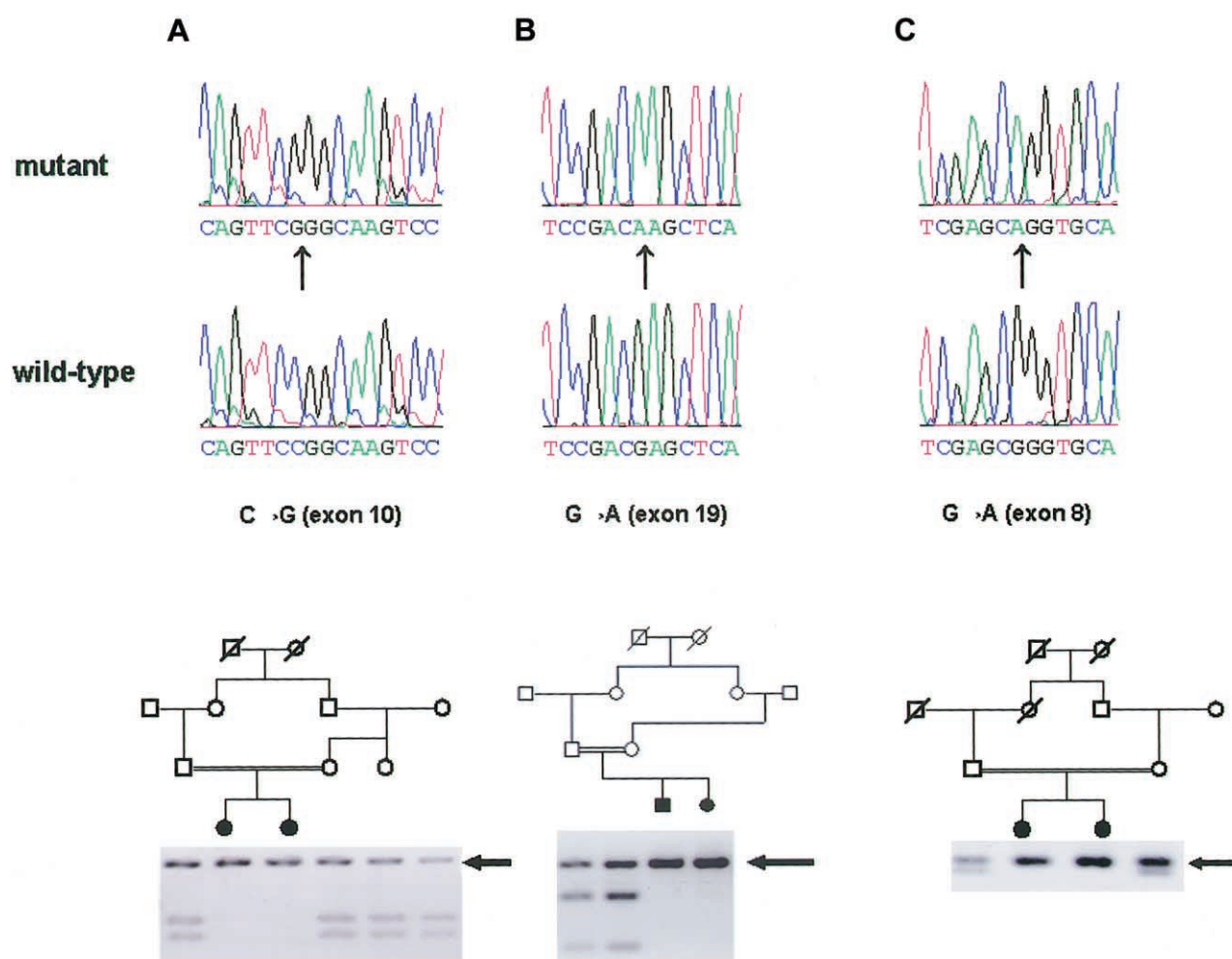

Figure 2   Mutation screening of *LRP5* in families; sequencing traces and restriction digests of amplified exons. *A*, Family 68001, showing a 2302C→G sequence change in exon 10 that eliminates an *Msp*I site, so that affected siblings show a single 386-bp fragment and heterozygotes also show digested 212-bp and 174-bp fragments. The maternal aunt and grandmother are carriers, having both alleles. *B*, Family 68002, showing a 4147G→A sequence change in exon 19 that eliminates a *Sac*I site, so that affected individuals show a single 269-bp fragment and heterozygotes also show digested 182-bp and 87-bp fragments. *C*, Family 68003, showing a 1757G→A sequence change in exon 8 that eliminates an *Aci*I recognition site, so that affected individuals show a single 252-bp fragment and heterozygotes also show a 179-bp fragment (the remaining 73-bp fragment was not seen on the gel). Mutant and control (wild-type) sequence traces are shown above the restriction-enzyme-digestion products of exons 10, 19, and 8.

from NCBI (accession number NM_002335). PCR amplification of 23 exons by use of primers listed in table 1 of Toomes et al. (2004) was performed in 20-μl reactions involving 2.5 U of *Taq* Gold (ABI), 10 × 2 μl of Buffer Gold *Taq*, 1.9 mM of $MgCl_2$, 0.25 mM of dNTP, 0.25 μM of primers, and 40 ng of genomic DNA. PCR cycling consisted of an initial 10-min denaturation step at 95°C for 5 min; 32 cycles at 94°C for 40 s, 55°C for 30 s, and 72°C for 30 s; and a final elongation step at 72°C for 5 min. PCR products were purified using either a Qiagen PCR purification kit or an Edge Biosystem QuickStepTm2 PCR purification kit. The PCR template was sequenced using ABI Dye Terminator, version 1, in a final reaction of 10 μl. Products of the sequencing reactions were purified using the Edge Biosystem Performa TM DTR gel-filtration system and were run on a 3100 ABI genetic analyzer. Sequences were aligned using the Seqman program of the DNASTAR program package.

In family 68001, exon 10 of the *LRP5* gene shows a c2302C→G transversion resulting in an R752G mutation in the third YWTD domain of the protein. The 2302C→G sequence change eliminates an *Msp*I site, so that amplification using the sequencing primers and digestion with *Msp*I yields 212-bp and 174-bp fragments in control alleles but yields a 386-bp fragment in mutant alleles (fig. 2*A*). This nonconservative change occurs in an arginine residue evolutionarily conserved, from human to *Drosophila,* in LRP4, LRP5, and LRP6, when sequences are aligned using the Clustal W algorithm as implemented in the MegAlign program (DNASTAR). In family 68002, exon 19 shows a c4147G→A transition resulting in a E1367K change in the third LDLR ligand-binding domain of the protein. The 4147G→A sequence change eliminates a *Sac*I site, so that amplification with the exon 19 sequencing primers, followed by digestion with *Sac*I, yields 182-bp and 87-bp fragments from control alleles but yields a single 269-bp fragment from the mutant allele (fig. 2*B*). In the protein, this change from a negatively to a positively charged amino acid occurs at a glutamate residue evolutionarily conserved, from human to *Drosophila,* in LRP5 and LRP6 but not in LRP4. Finally, affected individuals in family 68003 show a c1757G→A transition in exon 8, resulting in a R570Q substitution in the second YWTD domain of the protein. This is the same residue which, when mutated to tryptophan (R570W) results in the osteoporosis-pseudoglioma syndrome. Although bone-density measurements are not available for these affected siblings, they have had no fractures by ages 29 years and 31 years, which would be distinctly unusual for OPPG, and their ophthalmic course is consistent with FEVR rather than OPPG, which often presents with blindness in the neonatal period (Beighton et al. 1985; De Paepe et al. 1993).

The 1757G→A sequence change eliminates an *Aci*I recognition site, so that PCR amplification with the distal exon 8 sequencing primers followed by digestion with *Aci*I yields 73-bp and 179-bp fragments in control individuals but yields a single 252-bp fragment from the mutant allele (fig. 2*C*). In the protein, this substitution of a polar for a charged amino acid changes an arginine residue evolutionarily conserved, from human to *Drosophila,* in LRP5 and LRP6 proteins but not in LRP4 proteins from human and mouse. None of these changes were seen in 100 control individuals (200 chromosomes) of European ethnic extraction, when these individuals were tested using PCR amplification followed by digestion with the appropriate restriction endonuclease.

The combination of linkage and mutation analysis provides strong evidence that mutations in *LRP5* can cause arFEVR. Thus, mutations in *LRP5* can result in a variety of clinical presentations and can affect bone density and retinal vascularization. Mutations that truncate the protein or cause a frameshift early in the protein—in either the EGF or LDLR repeat domains—have been described in individuals with OPPG (Gong et al. 2001). Truncation or frameshift mutations more distal in the protein have been described in patients with adFEVR (although one family had associated osteoporosis) (Toomes et al. 2004). This was true in one mutation that occurred before the transmembrane domain, although, in this mutation, 52 novel amino acids of uncertain effect were encoded. Missense mutations in *LRP5* can also cause a variety of clinical presentations. A set of moderate to conservative mutations (e.g., G171V, G171R, A214T, A214V, A242T, and T253I) in the first EGF spacer domain have been shown to cause disorders of increased bone mass (Boyden et al. 2002; Little et al. 2002; Van Wesenbeeck et al. 2003). Other missense mutations in the EGF repeat domains or LDLR ligand-binding domains have been found in individuals with OPPG (Gong et al. 2001) or adFEVR (Toomes et al. 2004). Here, we describe three additional missense mutations in the second and third EGF repeat and in the third LDLR ligand-binding domain that are associated with arFEVR. Although one would suspect that these mutations cause milder decreases in function of the LRP5 protein than the functional decrease associated with adFEVR, the proof of this must await functional studies of the mutant proteins. There also remains the possibility that the effects of mutations in *LRP5* might vary with the genetic background, especially with regard to other genes in the Wnt signaling pathway. Finally, it seems likely that just as adFEVR can be caused by mutations in more than one member of the Wnt signaling pathway, arFEVR might show similar locus heterogeneity, which this study does not address.

Acknowledgments

We thank the members of the three families, for their participation in this study.

Electronic-Database Information

The accession number and URLs for data presented herein are as follows:

Genethon, http://www.genethon.fr/php/index_us.php
NCBI, http://www.ncbi.nlm.nih.gov/ (for *LRP5* reference genome sequence [accession number NM_002335])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for FEVR and OPPG)

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