Recessive Mutations in *TSPAN12* Cause Retinal Dysplasia and Severe Familial Exudative Vitreoretinopathy (FEVR)

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Purpose. Familial exudative vitreoretinopathy (FEVR) is an inherited disorder that disrupts the development of the retinal vasculature and can result in blindness. FEVR is genetically heterogeneous and mutations in four genes, *NDP*, *FZD4*, *LRP5*, and *TSPAN12*, encoding components of a novel ligand-receptor complex that activates the Norrin-β-catenin signaling pathway, account for approximately 50% of cases. We recently identified mutations in *TSPAN12* as a cause of dominant FEVR. The purpose of this study was to identify recessive *TSPAN12* mutations in FEVR patients.

Methods. Mutation screening was performed by directly sequencing PCR products generated from genomic DNA with primers designed to amplify the coding sequence of *TSPAN12*. Splicing defects were verified by reverse transcriptase PCR of leukocyte cDNA.

Results. *TSPAN12* screening in a large dominant FEVR family unexpectedly led to the identification of homozygous mutations in severely affected family members, whereas mildly affected family members were heterozygous. Further screening in a cohort of 10 retinal dysplasia/severe FEVR patients identified an additional three cases with recessive *TSPAN12* mutations. In all examined cases, single mutation carriers were mildly affected compared to patients harboring two *TSPAN12* mutations.

Conclusions. We report for the first time recessive mutations in *TSPAN12* and describe the first genetic cause for the clinical variation seen in FEVR families. Our data raise the possibility that patients with severe FEVR actually may harbor two mutant alleles, derived either from the same gene or potentially from other genes encoding components of the Norrin-β-catenin signaling pathway. (*Invest Ophthalmol Vis Sci.* 2012;53:2873–2879) DOI:10.1167/iovs.11-8629

Familial exudative vitreoretinopathy (FEVR) is an inherited blinding disorder caused by defects in the development of the retinal vasculature (MIM 133780). It was described first by Criswick and Schepens in 1969,[1] and has since become a well recognized and intensively studied condition. This is because FEVR, though rare, offers an opportunity to unravel the molecular pathway controlling retinal angiogenesis, a process that is involved in many major blinding disorders, including diabetic retinopathy, age-related macular degeneration and retinopathy of prematurity.[2]

FEVR is genetically heterogeneous and can be inherited as a dominant, recessive or X-linked trait, but dominant inheritance is by far the most common. Over the past 20 years four genes mutated in FEVR have been identified: *NDP* (MIM 300658, X-linked), *FZD4* (MIM 604579, dominant), *LRP5* (MIM 603506, dominant and recessive) and *TSPAN12* (MIM 613138, dominant).[3–8] Each of the encoded proteins is a component of the Norrin/β-catenin signaling pathway (also referred to as the Norrin/Frizzled-4 pathway).[2,9] In this pathway, the ligand Norrin binds to a receptor complex comprised of the receptor Frizzled-4, the co-receptor low-density lipoprotein receptor-related protein-5, and the auxiliary protein tetraspanin-12. In the absence of Norrin binding, signaling is not activated in a cell. This results in cytoplasmic β-catenin becoming phosphorylated and targeted for degradation through the ubiquitin-proteasome pathway. As a result, prospective target genes remain repressed. Signaling is activated by Norrin binding at the cell surface to the Frizzled-4, LRP5, and TSPAN12 receptor complex. This complex transduces a signal that inhibits the destruction of β-catenin, allowing its cytoplasmic levels to increase. Subsequently, β-catenin enters the nucleus where it interacts with the T-cell factor (TCF)/Lymphoid enhancing factor (LEF) family of transcription factors to turn on the expression of Norrin target genes.[2] This signaling pathway shares many similarities to the canonical Wnt/β-catenin pathway except that Norrin substitutes Wnt as the ligand and no tetraspanins have been linked to Wnt/β-catenin

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signaling.[9] Identifying new FEVR genes, therefore, provides an ideal opportunity to discover new components of this important signaling pathway. An additional FEVR gene (*EVR3*) has been mapped to chromosome 11p12-p13, but remains to be identified.[10] However, further genes are likely to exist as mutations in the known genes account for only approximately half of all FEVR patients.[11–17]

FEVR is a notable example of a classic Mendelian single gene disorder in which there is extensive variation in disease severity among patients, even between members of the same family.[18] Severely affected patients often are registered blind during infancy and can present with a phenotype resembling retinal dysplasia. Conversely, mildly affected patients frequently have few or no visual problems and have just a small area of avascularity in their peripheral retina, which may be detected only by fluorescein angiography (FA).[18] As such, it is not uncommon for affected individuals to be unaware that they have the disorder until other more severely affected family members are diagnosed, prompting them to undergo invasive FA examination and/or molecular testing.

In humans, the superficial vascular network located within the vitreal surface of the retina is formed primarily by vasculogenesis, while the peripheral superficial vasculature and the two intraretinal capillary beds are formed by angiogenesis.[19] Given that peripheral retinal avascularity is the only defect observable in asymptomatic FEVR patients, the principal abnormality underlying this disorder is believed to be deficient retinal angiogenesis. Analyses of mouse models of FEVR support this theory as these mice also show an absence of the two intraretinal capillary beds.[20–27] Unfortunately, these deep capillary networks cannot be visualized routinely in patients, so it is not known whether they are abnormal in FEVR. The sight-threatening features seen in FEVR are thought to arise from the resulting retinal ischemia caused by the avascularity. They include the development of hyperpermeable blood vessels, neovascularization, vitreoretinal traction, retinal folds and retinal detachments.[18]

This study is the first report of recessive mutations in *TSPAN12,* and shows that patients with two mutant alleles have a severe form of FEVR or retinal dysplasia, whereas heterozygous family members have mild FEVR phenotypes.

Materials and Methods

Human Subjects

Clinical diagnosis was made based on the presence of retinal abnormalities on funduscopy deemed typical of FEVR or retinal dysplasia. These included primarily an area of deficient peripheral retinal neovascularization together with exudation and/or sequelae of retinal traction, such as macular ectopia, retinal folds and retinal detachment. Fundus FA was performed in selected cases to confirm the diagnosis. Informed consent was obtained from all subjects tested after explaining the nature and possible consequences of the study, and the research adhered to the tenets of the Declaration of Helsinki. Ethical approval was obtained from the Leeds Teaching Hospitals Trust Research Ethics Committee.

Mutation Detection and mRNA Analysis

The coding sequence and flanking splice sites of *TSPAN12* were PCR amplified and screened by direct sequencing as described previously.[7] Leukocyte RNA was extracted from whole blood using a QIAamp RNA blood extraction kit (Qiagen, Crawley, UK) according to the manufacturer's guidelines. cDNA was reverse-transcribed using a cDNA synthesis kit (BioLine, London, UK) using random hexamer primer mix. For RT-PCR reactions *TSPAN12* was amplified between exons 2 and 7 using the following primers: TSPAN12_RT_2F 5′-TCTGCGCTGCCTGCTCTACG-3′ and TSPAN12_RT_7R 5′-ACAGCAGGAATCTGGGGGCCA-3.′ The housekeeping gene, *β-actin* also was amplified as a positive control using the following primers: *β-actin*_RT_F 5′-CTGGGACGACATGGAGAAAA-3′ and *β-actin*_RT_R 5′-AAGGAAGGCTGGAAGAGTGC-3.′ The identity of alternatively spliced *TSPAN12* RT-PCR products was established by cloning and direct sequencing using standard procedures.

Autozygosity Mapping

Genome-wide SNP microarray analysis was performed on genomic DNA from individual IV:1 from Family TM via the Affymetrix Human SNP Array 6.0 (Affymetrix, High Wycombe, Buckinghamshire, UK) according to the manufacturer's instructions. Regions of homozygosity were identified via AutoSNPa software with NCBI build 36 (hg18).[28]

Protein Alignment

Multiple protein alignments were calculated using ClustalW (http://www.ebi.ac.uk/clustalw2/).[29] Protein sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov/protein). Accession numbers: Human O95859; Orangutan Q5R8B5; Mouse AAH68240; Rat Q569A2; Cattle Q29RH7; Horse XP_001502093; Dog XP_855095; Opossum XP_001364876; Chicken XP_416007; Zebrafinch XP_002192381; Platypus XP_001516347; Zebrafish NP_957446; Seasquirt Tspan12 XP_002123238.

Missense Mutations Analysis

The predicted biological effects of the missense mutations identified were assayed using a variety of bioinformatic tools. Blosum62 scores were obtained using a Blosum62 substitution matrix.[30] PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/) and Align GVGD (http://agvgd.iarc.fr/) scores were calculated using TSPAN12 protein sequence NP_036470.[31–33] Sorting Intolerant from Tolerant (SIFT, http://sift.jcvi.org/www/SIFT_enst_submit.html) scores were calculated using ENSEMBL transcript ID ENSP00000222747.[34]

Results

Previously, we described a large Mexican family (Family HMH) that appeared to segregate FEVR in an autosomal dominant fashion (Fig. 1). Three of the dominant FEVR loci, *EVR1* (*FZD4*), *EVR3* (gene unidentified) and *EVR4* (*LRP5*), had been excluded in this family by linkage analysis.[35] Subsequently, a whole genome linkage search using 700 microsatellite markers failed to map the mutated gene.[36] Following the identification of *TSPAN12* as a new dominant FEVR gene,[7,8] genomic DNA from an affected family member was screened and a putative missense mutation was identified in exon 6, c.413A > G (p.Y138C, Fig. 2). When segregation of this variant was checked in the family, three affected individuals were found to be homozygous (III:1, III:3, and V:7), two had no mutation (II:3 and V:2) and the remaining affected members were heterozygous (Fig. 1). While these data normally would exclude causation for this DNA change, given the difficulty encountered in trying to map the mutated gene in this family and that of assigning phenotype in FEVR in general, we investigated it further. The missense change is a nonconservative substitution of a tyrosine to a cysteine, which was predicted to be pathogenic by Blosum62, SIFT, Polyphen2 and AlignGVGD analysis (Table 1). Furthermore, the amino acid mutated is highly conserved in orthologues (Fig. 3) and the mutation was excluded from 500 control chromosomes (100 Hispanic and 400 Caucasian). Moreover, the phenotype of the three patients with the homozygous mutations was very severe when compared to the heterozygous mutation carriers. Homozygotes presented in early childhood with tractional

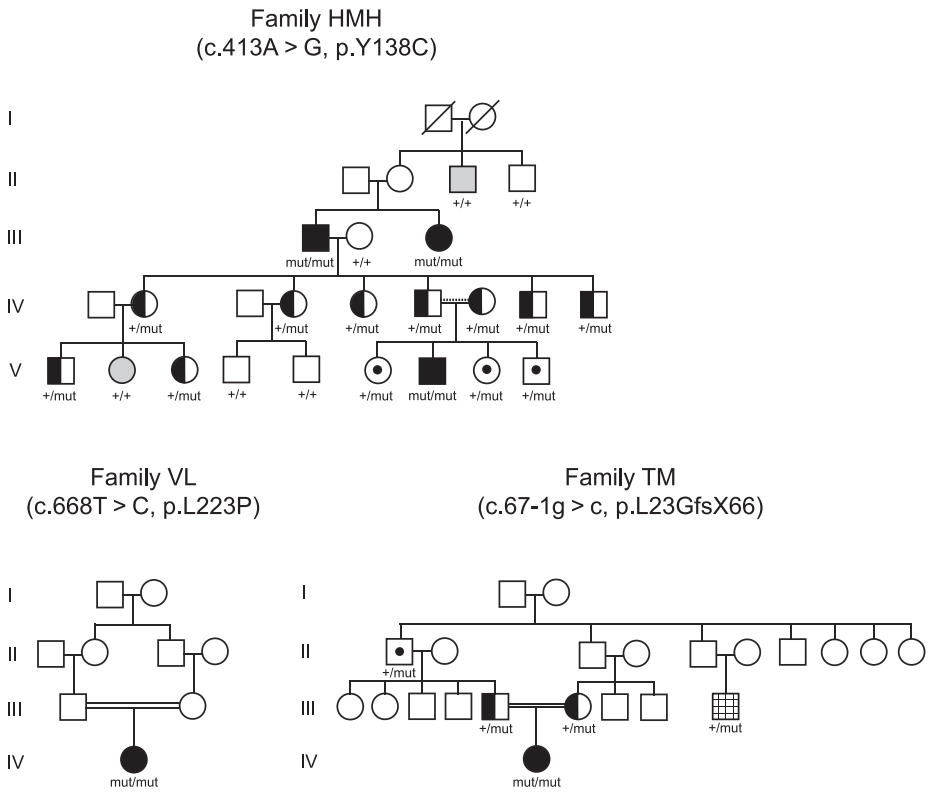

Figure 1. Pedigrees of FEVR families reported in this study. Severely affected FEVR patients (*blackened*), mild FEVR patients (*partially blackened*), phenocopy patients (*grey shading*), unilateral PFV patient (*hatched*), asymptomatic heterozygous mutation carriers (*dot*). Clinical descriptions of patients IV:7, V:6, V:8, and V:9 from Family HMH were not included in the original report of this family.[35] Recent examination showed that IV:7 had peripheral retina avascularity, but V:6, V:8, and V:9 showed no signs of FEVR upon indirect ophthalmoscopy. However, these children were not analyzed by FA, so subtle defects may have been missed and they may be too young to have had visible signs of the disease, such as exudates. The mutation genotypes for all tested family members are shown below each individual. The precise consanguineous relationship between individuals IV:6 and IV:7 from Family HMH is not known, but both individuals are from the same village in Mexico and believe their grandparents may be distantly related.

retinal detachments, whereas the heterozygous patients presented only with retinal exudates or peripheral avascularity.[35] The two affected patients without *TSPAN12* mutations were examined in their homes during a field trip to Mexico. Mydriatic examination of both individuals showed retinal exudate was present, but there were no other features of FEVR.[35] If the Y138C mutation is disease causing, then both of these individuals would be regarded as cases of misdiagnoses or phenocopies, or alternatively they could harbor mutations in other FEVR genes.

Intrigued by this finding, we screened a panel of 10 severe FEVR/retinal dysplasia patients without mutations in the known FEVR genes. A further three patients with recessive *TSPAN12* mutations were identified, confirming our earlier finding that carrying two mutant alleles of this gene results in severe FEVR/retinal dysplasia (Fig. 2).

A homozygous mutation c.668T > C (p.L223P) was identified in a female infant whose Pakistani parents were first cousins (Family VL in Fig. 1). This patient presented as a neonate and examination under anesthesia (EUA) revealed bilateral retinal folds across the posterior pole (Supplementary Fig. 1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8629/-/DCSupplemental). She was born post-term (birth weight 2.82 kg) and was under the care of the neonatologists for cystic fibrosis at the time of presentation. By 9 months of age she had deteriorated, and an ocular EUA documented bilateral shallow anterior chambers and posterior synechiae; a B scan ultrasound documented funnel retinal detachments. Unfortunately no examination of the parents was undertaken. This nonconservative missense change was almost fully conserved among orthologues (Fig. 3), was predicted to be pathogenic (Table 1) and was excluded from 500 control chromosomes (200 Asian and 300 Caucasian).

A homozygous mutation (c.67-1g > c) was identified in the splice acceptor site of exon 3 in an Indian female patient whose parents were first cousins (Family TM in Fig. 1). SNP microarray analysis had been performed on this patient and *TSPAN12* was shown to lie in an autozygous region of ~ 18 Mb. Analysis of parental leukocyte RNA showed that this mutation resulted in the deletion of exon 3, causing a frameshift followed by premature termination, p.L23GfsX66 (Fig. 2). The patient presented as a neonate with very poor vision and roving eye movements. Fundus examination revealed bilateral retinal folds, which remained stable through to the age of 6 years, when her latest examination documented a best corrected acuity of 1.5 LogMAR in each eye with myopia and astigmatism. The parents of the proband, although asymptomatic with normal visual function, had definite avascularity of the anterior retina bilaterally (Supplementary Fig. 2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8629/-/DCSupplemental). One 19-year-old male cousin of the parents had unilateral congenital visual loss (perception of light only) secondary to persistent hyperplastic vitreous (PHPV). The better-seeing right eye had 0.18 LogMAR acuity with a −10D myopic correction. He was found to be heterozygous for the c.67-1g > c variant. Detailed examination of the anterior retina of the left non-seeing eye has not been undertaken.

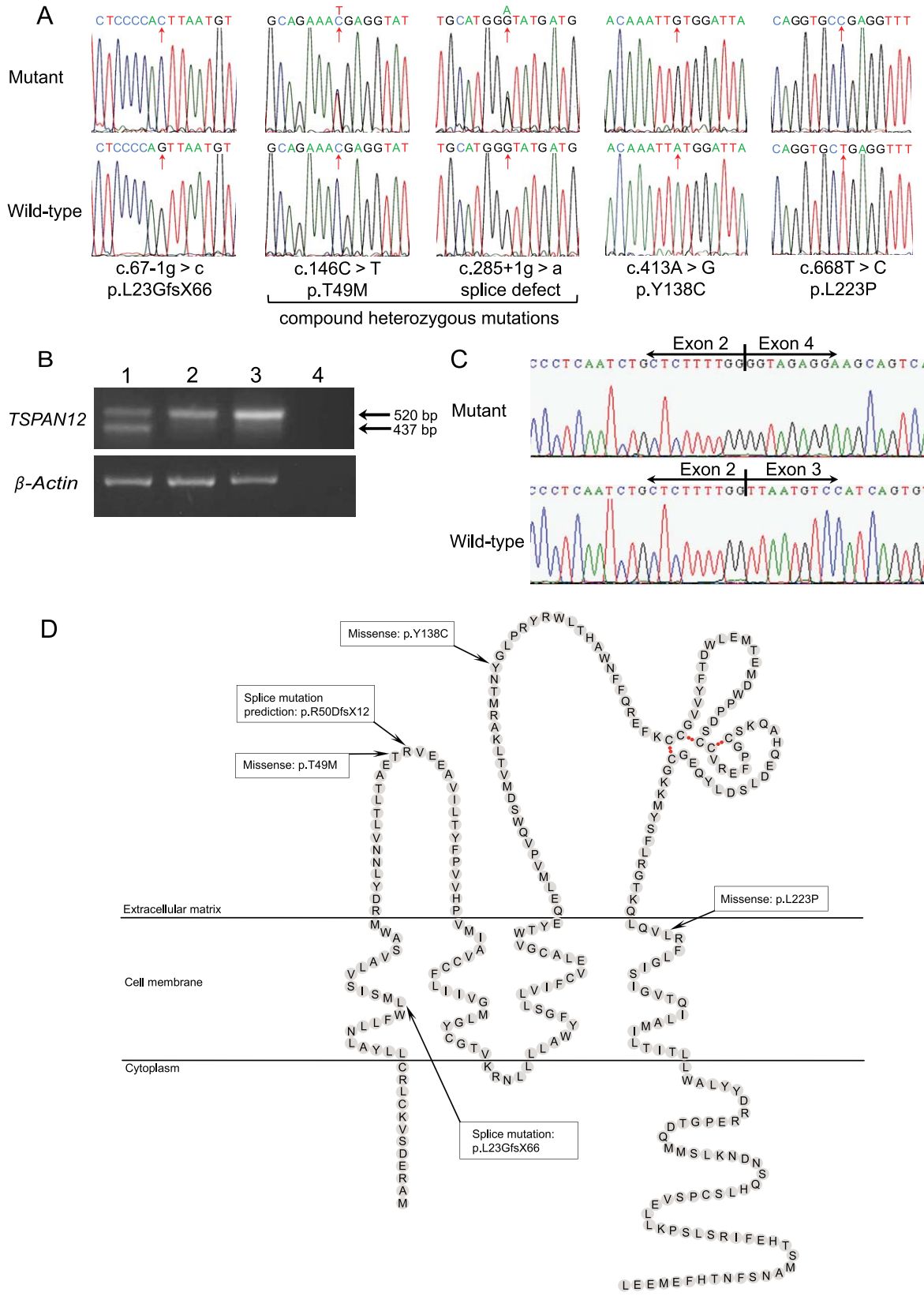

Figure 2. Recessive mutations identified in TSPAN12. (**A**) Sequence traces of the five mutations identified and the corresponding wild-type alleles. (**B**) RT-PCR amplification of *TSPAN12* cDNA. Lane 1, Leukocyte cDNA from parent (heterozygous for c.67-1g > c, RNA was unavailable for child IV:1). Lane 2, Leukocyte cDNA from control individual. Lane 3, Retinal cDNA from control individual. Lane 4, No cDNA template. (**C**) Sequence trace obtained from each *TSPAN12* cDNA PCR product shown in Lane 1 (**B**). The splice mutation c.67-1g > c causes exon 3 to be deleted, resulting in a frameshift and a premature termination codon (p.L23GfsX66). (**D**) Schematic diagram of the TSPAN12 protein showing the location of the mutations within the protein domains. The locations of the transmembrane domains were obtained from Kovalenko et al. 2005.[39] The intramolecular disulfide bonds crucial for the correct folding of the large extracellular loop are indicated by *red dots*. As we were unable to assess the actual consequence of the splicing mutation c.285+1g > a, only the predicted protein outcome is shown.

Table 1. Summary of Bioinformatic Analysis Undertaken to Predict the Pathogenic Nature of the Missense Mutations

Mutation	PolyPhen2	SIFT	Blosum62*	AlignGVGD
p.Y138C	Probably damaging (score 0.977)	Damaging (score 0.01)	Score −2	Most likely damaging (score 193.72)
p.L223P	Probably damaging (score 0.996)	Damaging (score 0)	Score −3	Most likely damaging (score 97.78)
p.T49M	Probably damaging (score 1.000)	Tolerated (score 0.11)	Score −1	Most likely damaging (score 81.04)

* Blosum62 scores range from +3 to −3 and negative scores are more likely to be damaging substitutions.

Two heterozygous mutations were identified in the final patient. The first was a missense change in exon 3 (c.146C > T, p.T49M) and the second was a splice donor mutation in exon 4 (c.285+1g > a, Fig. 2). Unfortunately, an RNA sample was not available for this patient so the precise effect is unknown, but the most common outcome for mutations in a splice donor site is deletion of the preceding exon. This would result in a frameshift and premature termination codon, p.R50DfsX12. The non-conservative missense change was 100% conserved among orthologues (Fig. 3), was predicted to be pathogenic in 3 of the 4 bioinformatic analyses undertaken (Table 1) and was excluded from 500 control chromosomes (340 African and 160 Caucasian). The patient is a 23-year-old female originating from Nigeria, where she had been diagnosed with bilateral congenital cataracts. No family members were available for examination. Her parents have no history of visual problems, but her sister is reported to have poor vision. The patient's care within the United Kingdom began at age 2 when she was documented as having a phthisical right eye with chronic endophthalmitis due to a perforation, and nystagmus, which prevented any retinal examination of the left eye. Unfortunately, she failed to attend an EUA and was lost to follow up. She was next examined in the United Kingdom at age 22, when it appeared she had received ophthalmic care within the United States in the intervening years, and had undergone a left pars plana vitrectomy with Endolaser. She believed the working diagnosis during her care in the United States had been persistent hyperplastic primary vitreous. At this stage, she had no perception of light in the left aphakic eye, which demonstrated corneal opacity and an elevated intraocular pressure of 34 mmHg. Funduscopy suggested a large retinal fold traversing the posterior pole between the optic disc and temporal retinal periphery.

Discussion

We, along with Nikopoulos et al., recently identified *TSPAN12* as a new autosomal dominant FEVR gene and showed that it accounted for ~ 10% of FEVR cases.[7,8] Following this discovery, we screened *TSPAN12* in a large family segregating FEVR with an autosomal dominant pattern of inheritance, which previously had been excluded by linkage analysis from the known FEVR genes and in whom a whole genome linkage search had failed to identify a locus.[35,36] We identified a very convincing putative missense mutation (p.Y138C), but the variant did not segregate as expected in the family. The three severely affected family members were homozygous for the mutation, whereas the majority of the remaining affected family members who had a mild form of FEVR were heterozygous for this change. Of concern, two affected family members were found not to harbor the mutation, indicating that these cases may be misdiagnoses or phenocopies. Although unexpected, single cases of phenocopies have been identified previously in FEVR families harboring *FZD4* and *LRP5* mutations (unpublished results). While intriguing, these data did not provide conclusive evidence that recessive mutations in *TSPAN12* caused FEVR. However, screening of additional patients with severe FEVR or retinal dysplasia led to the identification of mutations in three further unrelated patients, allowing us to show convincingly that recessive *TSPAN12* mutations cause FEVR.

The pathogenic nature of the mutations identified in this study is compelling. The homozygous c.67-1g > c splicing

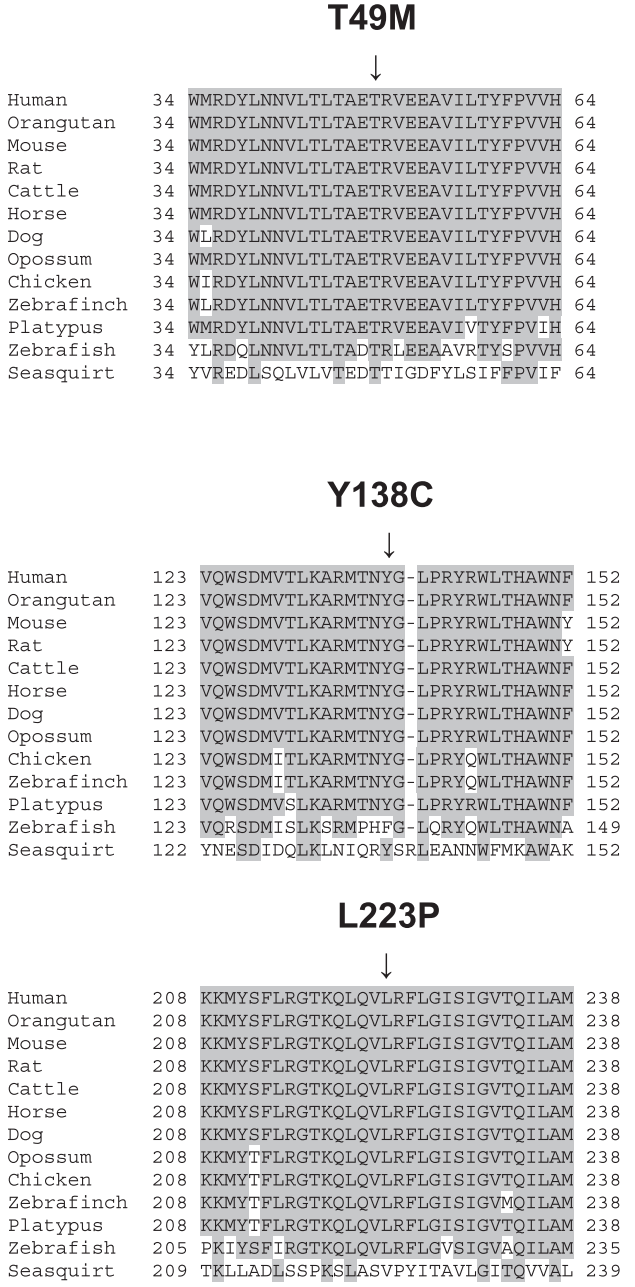

Figure 3. Protein sequence alignment of human TSPAN12 with its orthologues. Only 30 amino acid residues surrounding each mutation are shown. Conserved amino acid residues are shaded. The positions of the missense mutations p.T49M, p.Y138C, and p.L223P are indicated.

mutation was shown to cause skipping of exon 3 resulting in a premature termination codon (p.L23GfsX66). Although RNA could not be checked to determine the outcome of the other splicing mutation c.285+1g > a, it alters the first base of intron 4 and, therefore, will abolish the splice site. All the missense mutations were non-conservative substitutions that altered highly conserved amino acids and were predicted to be pathogenic. They also were excluded from ethnically matched control chromosomes.

The location of two of the missense mutations identified in this study also points to their pathogenic nature. Like all 33 members of the human tetraspanin superfamily, TSPAN12 contains four transmembrane domains linking a small extracellular loop (SEL), a large extracellular loop (LEL) and a tiny inner loop (Fig. 2D). Within the LEL, tetraspanins have a signature CCG motif plus additional cysteine residues, which form disulfide bonds and are crucial for protein folding.[37] The p.Y138C mutation is located within the LEL where an additional cysteine residue could disrupt the folding of this domain and, therefore, abolish protein function. Similarly, the p.L223P mutation is located in the fourth transmembrane domain and it is likely that the proline substitution will destabilize the helical structure of this domain. Indeed, a similar mutation (p.A237P) in the same transmembrane domain has been reported previously in dominant families with FEVR.[8] Furthermore, the transmembrane and LEL domains of tetraspanins are believed to be the major sites for protein-protein interactions (including tetraspanin homo- and heterodimerization), so even a subtle amino acid change may have significant functional consequences.[38,39] The remaining missense mutation, p.T49M, is located in the SEL. Little is known about the function of this domain but this same mutation has been reported previously in a heterozygous state in a sporadic Chinese FEVR patient with retinal folds, and the threonine is conserved fully within orthologues (Fig. 3).[40]

This study suggests that the FEVR phenotype is sensitive to the dosage of TSPAN12. Patients with two mutated alleles have a much more severe phenotype than those with one. This is the first time this phenomenon has been reported for FEVR. Although dominant and recessive mutations in *LRP5* have been reported in FEVR, there is no obvious dosage effect.[5,6] Carrier parents of recessive FEVR patients with *LRP5* mutations show no signs of eye disease, although reduced bone mass density has been reported.[6,17,41] However, as only a handful of recessive *LRP5*-FEVR families have been reported, and given the difficulty in detecting the mild phenotype, it is possible that this and other genetic forms of FEVR also are sensitive to dosage effects. Interestingly, a case report provides evidence for this possibility. Kondo et al. described a single patient with bilateral retinal detachment harboring a homozygous missense mutation in *FZD4*, p.R417Q.[42] This patient's heterozygous parents displayed straightening of the retinal vessels but not the peripheral avascular zone usually diagnostic of mild FEVR.

Given the wide phenotypic variability seen in dominant FEVR families, it is interesting to speculate whether the severe cases can be accounted for by multiple mutant alleles in FEVR genes either as a second allele of the documented gene, as seen in *TSPAN12,* or within a different gene. Some of these additional alleles could be documented polymorphisms but, nevertheless, may modify the signaling pathway. Indeed, many missense polymorphisms in *LRP5* have been shown to affect protein function as measured using the cell-based Topflash reporter assay.[43,44] More convincingly, these polymorphisms have been shown to influence bone mineral density in the general population.[45] Given that the proteins known to be mutated in FEVR to date are all components of a ligand-receptor complex, it is easy to envisage that subtle amino acid differences may increase or decrease the strength of the interactions within this complex, and cell-based signaling assays suggest this is the case.[25,27] Furthermore, anecdotal evidence exists for digenic inheritance in FEVR. Qin et al. identified an *FZD4* mutation (p.R417Q) and an *LRP5* mutation (p.R444C) co-segregating in a dominant family.[13] All affected family members carried both mutations, so an additive effect on the disease phenotype was not evident. However, a separate dominant FEVR family with this same *FZD4* mutation was found to have a milder phenotype. Similarly, Nikopoulos et al. found an *LRP5* mutation (c.4489-1g > a) and an *FZD4* mutation (p.E40Q) in an affected parent and child.[17] Again, family members harboring single mutations were not available, so no conclusions could be made. However, given the results of this study, it would be interesting to search for second mutant alleles in *TSPAN12*, and in other FEVR genes, in severe FEVR cases with known dominant FEVR mutations.

In summary, we have shown that patients with mutations in both alleles of *TSPAN12* have severe FEVR or retinal dysplasia. These mutations appear to show a milder phenotype in heterozygous mutation carriers, indicating that the FEVR phenotype is sensitive to the number of *TSPAN12* mutations. To our knowledge, this is the first time a genetic cause for the phenotypic variation observed in FEVR families has been proven and raises the intriguing possibility that severe FEVR is not a classic Mendelian single gene disorder, but may result from the additive effect of two or more mutant alleles in one or more genes.

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