Mutation spectrum of the FZD-4, TSPAN12 AND ZNF408 genes in Indian FEVR patients

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Abstract

Background: Mutations in candidate genes that encode for a ligand (NDP) and receptor complex (FZD4, LRP5 and TSPAN12) in the Norrin β-catenin signaling pathway are involved in the pathogenesis of familial exudative vitreoretinopathy (FEVR, MIM # 133780). Recently, a transcription factor (ZNF408) has also been implicated in FEVR. We had earlier characterized the variations in NDP among FEVR patients from India. The present study aimed at understanding the involvement of the remaining genes (FZD4, TSPAN12 and ZNF408) in the same cohort.

Methods: The DNA of 110 unrelated FEVR patients and 115 unaffected controls were screened for variations in the entire coding and untranslated regions of these 3 genes by resequencing. Segregation of the disease-associated variants was assessed in the family members of the probands. The effect of the observed missense changes were further analyzed by SIFT and PolyPhen-2 scores.

Results: The screening of FZD4, TSPAN12 and ZNF408 genes identified 11 different mutations in 15/110 FEVR probands. Of the 11 identified mutations, 6 mutations were novel. The detected missense mutations were mainly located in the domains which are functionally crucial for the formation of ligand-receptor complex and as they replaced evolutionarily highly conserved amino acids with a SIFT score < 0.005, they are predicted to be pathogenic. Additionally 2 novel and 16 reported single nucleotide polymorphisms (SNP) were also detected.

Conclusions: Our genetic screening revealed varying mutation frequencies in the FZD4 (8.0 %), TSPAN12 (5.4 %) and ZNF408 (2.7 %) genes among the FEVR patients, indicating their potential role in the disease pathogenesis. The observed mutations segregated with the disease phenotype and exhibited variable expressivity. The mutations in FZD4 and TSPAN12 were involved in autosomal dominant and autosomal recessive families and further validates the involvement of these gene in FEVR development.

Keywords: Retina, Abnormal angiogenesis, Mutation screening, Candidate genes
density lipoprotein receptor like protein 5 (LRP5; OMIM 603576) [1, 2, 6, 14–16, 19, 21], Tetraspanin-12 (TSPAN12; OMIM 613138) [1, 2, 4, 5, 22–25]. Zinc finger proline-408 (ZNF408) [1, 3] and Kinesin family member 11 (KIF11) [26, 27]. Mutations in these genes have resulted in autosomal dominant (FZD4, LRP5, TSPAN12, ZNF408 and KIF11) [3, 7, 8, 14–16, 24, 26, 27], autosomal recessive (LRP5, TSPAN12) [21, 23] and X-linked (NDP) FEVR [9, 10] and have accounted for ~50 % of all patients [1–3]. Among these, the autosomal dominant mode of inheritance has been widely reported. The proteins encoded by FEVR candidate genes (excluding ZNF408 and KIF11) have been shown to be involved in the formation of a signal transduction complex of Norrin-β catenin signaling pathway [28–32]. The knockout mouse models of these genes have exhibited phenotype resembling FEVR and have provided further evidence on the role of this signaling pathway in the formation, maturation and maintenance of intraretinal vasculature [28–31, 33–37]. Norrin, a protein encoded by NDP gene being similar to canonical-Wnt signaling pathway, acts as a ligand and interacts with FZD4-LRP5 receptor complex. This interaction further stabilizes the cytoplasmic β catenin molecules to induce the T-cell factor/lymphoid enhancer factor (TCF/LEF) mediated gene expression after its translocation into the nucleus [30–32]. TSPAN12 enhances the Norrin signaling by augmentation of FZD4 multimerization [28]. However, the roles of the ZNF408 and KIF11 genes in the developmental retinal angiogenesis and Norrin-β catenin signaling pathway is yet unknown.

The FZD4 is a 537 amino acid protein containing an N-terminal extracellular domain (composed of signal sequence, FZ domain and a linker region), 7 transmembrane hydrophobic helical domains, 3 extracellular, 3 intracellular loops and a C terminal cytoplasmic domain [38, 39]. The extracellular FZ domain is cysteine rich (CRD) and is essential for the recognition and binding to FZD4 specific ligands [40, 41]. The cytoplasmic C terminal region contains two highly conserved PDZ binding domains (postsynaptic density 95/disc-large/zona occludens-1: KTXXXW and ETVV) and play a crucial role in recruiting downstream regulator proteins to induce the signaling mechanism [39]. The TSPAN12 gene is located on chromosome 7q31 and encodes for a 305 amino acid transmembrane protein. Structurally, this protein contains four transmembrane domains connected by two extra cellular loops (ECL-1 and 2) and an intra cellular loop, and intracellular N and C terminals. The ECL-1 is small compared to the ECL-2 [4, 5, 22].

Recently, Collin et al. [3] functionally characterized this new candidate gene ZNF408 in FEVR. A novel founder mutation was observed in two Dutch families and further screening of 132 patients revealed another mutation in a Japanese patient. The ZNF408 gene encodes for a 720 amino acid protein and predicted to contain ten zinc finger DNA binding domains. Based on the presence of zinc finger domains, this protein was suggested to be a transcription factor [3]. Functional analysis of mutant proteins indicated a dominant negative disease mechanism and subsequent knockdown of Znf408 in zebrafish, suggested its putative role in retinal vasculogenesis.

So far, 88 different mutations have been reported in FZD4 (n = 58), TSPAN12 (n = 27) and ZNF408 (n = 3) in patients with FEVR and other vitreo-retinal abnormalities. These genes have been quite well characterized in Caucasian, Chinese and Japanese FEVR patients. However, in the Indian context, there is only a single report from a relatively smaller cohort of FEVR patients on FZD4 gene screening [42] (Nallathambi J et al.). Thus, the present study was undertaken to explore the involvement of the FZD4, TSPAN12 and ZNF408 genes and to assess their mutation spectrum in a large cohort of Indian FEVR patients.

Methods
The study was approved by the Institutional Review Board (IRB) of L.V. Prasad Eye Institute, Hyderabad, India, (Ref no LEC06104) and adhered to tenets of the Declaration of Helsinki. We recruited 110 unrelated FEVR cases (34 familial and 76 sporadic) along with their family members after obtaining a written informed consent. All the patients had a history of full term birth. A senior ophthalmologist examined all the patients and diagnosed the disease based on indirect ophthalmoscopic examination, B-scan ultrasoundography and fundus fluorescein angiography in selective patients. As described previously, the diagnosis of FEVR was confirmed based on the presence of bilateral peripheral retinal avascularity along with or without any of the ocular features such as subretinal exudation, neovascularization, vitreoretinal traction, retinal folds, ectopic macula and partial or total retinal detachments [7, 12, 20]. The different stages of the disease were ascertained based on the Pendergast and Trese classification [12]. The diagnosis and staging of the disease was independently confirmed by another ophthalmologist through evaluation of the medical records and fundus photographs of the patients. Additionally, 115 ethnically matched normal subjects aged over 60 years and without any present and past history of retinal disorders nor signs or symptoms of FEVR or any other ocular or systemic conditions were enrolled as controls. They were generally subjects with senile cataract who were also evaluated for any other ocular complications through a comprehensive eye examination.

Mutation screening of the FZD4 and TSPAN12 genes
Genomic DNA was extracted from peripheral blood leukocytes using standard protocols of Phenol-Chloroform extraction method [43]. Mutation screening of entire coding and intron-exon boundaries of FZD4, TSPAN12 and ZNF408 genes were accomplished by polymerase
chain reaction (PCR) based amplification of these regions with 26 different sets of overlapping primers followed by resequencing on an automated DNA sequencer ABI 3130 XL (Applied Biosystems, Foster City, CA) using the Big Dye chemistry (version 3.1) following the manufacturer’s guidelines. The primers used for PCR and their optimum annealing temperatures are listed in Additional file 1: Table S1. The observed variations were further validated by resequencing. Samples of the available family members of the respective probands in the FEVR-affected families were also screened for assessing the segregation of the identified variations. In addition to the screening of the observed variants in the ethnically matched controls, their allele frequencies were also checked in the dbSNP [44], ESP5400 [45], NIEHS95 [46] and ExAC [47] databases. All the identified non-synonymous substitutions were assessed to predict their functional significance by computational prediction algorithms; SIFT [48], PolyPhen-2 [49], and Mutation Taster [50]. Multiple sequence alignments were carried out using Clustal W to determine the evolutionary conservation of wild type amino acid residues at the position of substitutions [51]. Evolutionarily non-conservative missense changes predicted as deleterious by at least two computational tools were considered as potentially pathogenic mutations. The remaining missense changes were categorized as variants of unknown significance.

Results
The screening of the entire coding and flanking regions of FZD4, TSPAN12 and ZNF408 genes revealed a total of 11 potentially pathogenic mutations in 15 different FEVR probands (Table 1 and Fig. 1). Of these 11 mutations, six were novel. The novel mutations included a missense change (c.341 T > G; p.Ile114Ser), a single base pair insertion (c.1395_1396insT; p.Arg466Serfs*6) and a non-stop change (c.1613A > C; p.558SerCys729*2) in the FZD4 gene. Three novel mutations c.125 T > C (p.Val42Ala), c.479G > A (p.Cys160Tyr) and c.2145G > T (p.Glu715Asp) were also observed in the TSPAN12 and ZNF408, respectively (Table 1 and Fig. 1). In addition to these novel changes, two previously reported missense mutations; c.313A > G; [p.Met105Val] [1, 2, 16, 18, 20] and c.470 T > C; [p.Met157Thr] [2], two small base pair deletions; c.1282-1285delGACA [p.Asp428Serfs*2] [1, 2, 15, 16] and c.1286-1290delAGTTA [p.Lys429Argfs*28] [52] in the FZD4 gene; a rare variant in TSPAN12 gene; c.334G > A [p.Val112Ile] and two novel missense changes (c.130C > T [p.Pro44Ser] and c.694A > G [p.Met232Val]) in ZNF408 of unknown significance were also observed. None of these mutations were observed in the 115 normal controls. Apart from these mutations, three reported single nucleotide polymorphisms (SNPs); c.502C > T [rs61735303], c.576C > T [rs2011686860] and c.1078 A > G [rs530613772] were observed in FZD4 gene. Two intronic (IVS2 + 24C > G [rs545602315] and IVS6 + 19G > C) and five 3’ UTR single nucleotide substitutions (c.*39C > T [rs41622], c.*334A > T [rs545129654], c.*1010 T > G, c.*1140 T > A and c.*1243A > T [rs189221112]) were observed in the TSPAN12 gene. Likewise, two novel 5’ UTR variations (c.*-214_-210delGAATC and -111C > A) and six SNPs (c.402A > G [rs561320549], c.408C > A, c.576_587del12bp [rs1484055528], c.581_592del12bp [rs72461400], c.1971A > G [rs37653625] c.1850C > A [rs547169524]) were observed in ZNF408.

All the nucleotide changes observed in FZD4 gene were heterozygous. Excluding the c.313A > G (p.Met105Val) and c.1286-1290delAGTTA (p.Lys429Argfs*28) mutations, the remaining mutations were observed in one proband each. A missense p.Met105Val mutation was observed in three different probands and likewise a frameshift p.Lys429Argfs*28 mutation was observed in two different probands. Interestingly, a FEVR proband harbored two changes: c.1282-1285delGACA (p.Asp428Serfs*2) and c.1613A > C (p.558SerCys729*2) in the FZD4 gene.

In TSPAN12, two mutations c.125 T > C (p.Val42Ala) and c.334G > A (p.Val112Ile) were identified in one proband each, while the c.479G > A (p.Cys160Tyr) mutation was observed in four different probands, wherein, two of them harbored heterozygous changes and the remaining were homozygous. The p.Val112Ile mutation has also been identified in homozygous condition whereas, p.Val42Ala mutation was observed in heterozygous condition. The wild type valine residue at codon 42 was highly conserved across different species. However, this change was predicted to be non-pathogenic by PolyPhen-2 whereas, Mutation Taster and SIFT indicated for the harmful effect of this substitution.

Three heterozygous missense changes (p.Pro44Ser, p.Met232Val and p.Glu715Asp) identified in ZNF408, were observed in one proband each. Of the total nine missense changes identified in the present study, except p.Pro44Ser and p.Met232Val changes of ZNF408, the remaining were predicted as deleterious by at least two computational prediction tools used for in silico analysis. Furthermore, the wild type amino acids located at these missense changes were highly conserved across different species (Fig. 2). Thus, these missense changes were categorized as potentially pathogenic mutations, while the p.Pro44Ser and p.Met232Val changes of ZNF408 were variations of unknown significance.

Discussion
In the present study, several novel mutations (missense, non-stop and insertion) were detected in the coding regions of FZD4, TSPAN12 and ZNF408 genes among the unrelated FEVR probands. None of these nucleotide changes were observed in 115 ethnically matched controls
and were not present in the dbSNP [44], ESP5400 [45], NIEHS95 [46] and ExAC [47] databases. The probands harboring the mutations in these genes exhibited severe disease phenotype with neovascularization, falciform retinal folds, exudative or tractional retinal detachments and severe visual impairment. Some of these mutations were also observed in the family members of the probands who exhibited a highly variable phenotype based on indirect ophthalmoscopic examination. The phenotype of the family members ranged from normal retinal vascularization or very mild avascularization to neovascularization, partial and/or total retinal detachments with or without exudation. Since, some of the asymptomatic family members with the identified mutations were unavailable for fluorescein fundus angiography, we could not rule out the possibility of missing very mild retinal vascular changes. The patients identified with FZD4 or TSPAN12 or ZNF408 mutations exhibited classical clinical features of FEVR, but failed to exhibit any genotype-phenotype correlation.

### Table 1: Mutations observed in FZD4, TSPAN12 and ZNF408 genes in FEVR patients

| Family number | Gene/Exon change | Protein change | In silico analysis | Occurrence in Patients | Novel/Reported | Functional significance |
|---------------|------------------|---------------|-------------------|-----------------------|---------------|------------------------|
| 1             | FZD4/2           | c.341T>G      | p.Ile114Ser       | Damaging (0.00)       | 1/110         | Novel                  |
| 2             | FZD4/2           | c.1395_1396insT | p.Arg466Ser fs*6  | -                     | 1/110         | Novel                  |
| 3             | FZD4/2           | c.1613A>C     | p.*538Serext*2     | -                     | 1/110         | Novel                  |
| 4             | FZD4/2           | c.1282_1285delAGTTA | p.Lys429Arg fs*28 | -                     | 2/110         | Pathogenic             |
| 5             | FZD4/2           | c.470T>C      | p.Met157Thr       | Damaging (0.039)      | 1/110         | Novel                  |
| 6             | ZNF408/5         | c.694A>G      | p.Met232Val       | Tolerated (0.508)     | 1/110         | Novel                  |
| 7             | FZD4/2           | c.313A>G      | p.Met105Val       | Tolerated (0.858)     | 3/110         | Pathogenic             |
| 8             | TSPAN12/3        | c.125T>C      | p.Val42Ala        | Damaging (0.023)      | 1/110         | Novel                  |
| 9             | TSPAN12/7        | c.479G>A      | p.Cys160Tyr       | Damaging (0.000)      | 4/110         | Pathogenic             |
| 10            | TSPAN12/5        | c.334G>A      | p.Val112Ile       | Tolerated (0.338)     | 1/110         | Reported [47]          |
| 11            | ZNF408/2         | c.130C>T      | p.Pro44Ser        | Tolerated (0.054)     | 1/110         | Novel                  |
| 12            | ZNF408/5         | c.2145G>T     | p.Glu715Asp       | Damaging (0.003)      | 1/110         | Novel                  |

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a NCBI Reference Sequences for FZD4, TSPAN12 and ZNF408 mRNA are NM_012193.2, NM_012338.3 and NM_024741.2 respectively. b NCBI Reference Sequences for FZD4, TSPAN12 and ZNF408 proteins are NM_036325.2, NP_036470.1 and NP_079017.1 respectively. The effect of missense changes were predicted using SIFT, PolyPhen-2 and Mutation Taster online prediction tools. In SIFT, the scores less than 0.05 were considered as damaging. In PolyPhen-2, the scores less than 0.5 were considered as disease causing.
FEVR (Fig. 1; family 2; II:1) that segregated in all the affected family members. The proband and her female siblings (II:1 and II:3) with this frameshift mutation developed bilateral vitreo retinal traction and total retinal detachment at their infancy while their mother (I:2) who was a carrier for this mutation, had avascularized peripheral retina (Fig. 3). This is the third insertion mutation to be observed in the FZD4 gene so far.

A novel non-stop change (c.1613A > C; p.538Serext*2) along with a previously reported 4 base pair deletion (c.1282-1285delGACA; p.Asp428Serfs*2) [1, 2, 15, 16] was observed in a familial case in a compound heterozygous condition. This change resulted in disruption of proper termination and addition of two extra amino acids to the wild type protein. A severe disease phenotype with bilateral total retinal detachments was observed in the proband (II:3) and her female sibling (II:1) that might have resulted from the synergistic effect of both the mutated alleles. The father (I:1) and mother (I:2) of these siblings were carriers for the mutated alleles c.1613A > C and c.1282-1285delGACA, respectively. The parents of these siblings harboring either of the mutated alleles were normal or exhibited avascularized peripheral retina. The non-stop mutation has been identified for the first time in

**Fig. 1** Pedigrees of the FEVR families with nucleotide changes in FZD4 and TSPAN12 genes. Completely shaded symbols represent severe stages of the disease. Open symbols represent unaffected individuals. Asterisk (*) over the pedigree symbols represent the individuals screened for nucleotide changes in FZD4 and TSPAN12 genes. - indicates the presence of deletion/insertion and + indicates the wild type allele. The identified nucleotide change is represented above each pedigree.
**FZD4** gene and predicted to result in addition of two extra amino acids to the C-terminal cytoplasmic domain of FZD4 protein. Heterozygous compound mutations have previously been reported in **FZD4** gene and could explain the inter and intra familial variability of disease phenotype [18, 20]. The c.1282-1285delGACA change led to an open reading frame alteration at codon 428 and formation of a new stop codon at position 430.

The third frameshift heterozygous alteration c.1286-1290delAGTTA (p.Lys429Argfs*28) was observed in two unrelated probands in families 4 and 5 (Fig. 1; family 4; II:2 and family 5; II:1). Both the probands developed total retinal detachment in one eye during early childhood and the affected eyes developed subtotal retinal detachment excluding the fovea. The mother (I:2) of the proband 5 who was a carrier for this deletion exhibited peripheral retinal detachment.

![Multiple sequence alignment showing the conservation of wild type residues in respect to identified missense changes in FZD4 protein, TSPAN12, and ZNF408 across various species. Except p.M232V of ZNF408 remaining all the missense mutations were highly conserved across different species. Reference sequences of the FZD4 orthologues: Homo sapiens; AAR23924.1, Macaca mulatta; NP_001253804.1, Rattus norvegicus; NP_072145.1, Mus musculus; NP_032081.3, Gallus gallus; NP_989430.1, Xenopus laevis; NP_001083922.1, Canis lupus familiaris; XP_005633439.1, Equus caballus; XP_001489854.1, Elephas maximus; XP_006895316.1, Alligator mississippiensis; XP_006261662.1, Bos taurus; NP_001193198.1, Sus scrofa; XP_005667267.1. Reference sequences of the ZNF408 orthologues: Homo sapiens; NP_079017.1, Macaca mulatta; XP_001112101.1, Felis catus; XP_003993266.1, Canis lupus familiaris; XP_003639743.3, Equus caballus; XP_004908321.1, Pan troglodytes; JAA35243.1 Bos taurus; NP_001180086.1, Callithrix jacchus; XP_009006283.1, Condylura cristata; XP_004683622.1, Sus scrofa; XP_013849819.1, Elephas maximus; XP_006895777.1.](image)
avascularization while the proband’s father (I:1) despite having a single copy of the mutant allele did not exhibit any retinal vascular abnormality upon indirect ophthalmoscopy examination. This deletion led to frameshift at codon 429 along with the formation of a premature stop codon.

All the three frameshift mutations (p.Arg466Serfs*6, p.Asp428Serfs*2 and p.Lys429Argfs*28) were predicted to result in the formation of a truncated protein with a loss of 13 to 20% of the wild type FZD4 protein. Due to the presence of only two exons in FZD4, the frameshift mutations observed in the second exon may result in the formation of truncated protein rather than nonsense mediated mRNA decay [56]. The resulting truncated proteins affected the conserved KTXXXW domain and PDZ binding motif, essential for interaction with downstream regulator proteins. In the absence of these motifs, FZD4 would likely fail to induce the signaling pathway and consequently lead to haploinsufficiency. Alternatively, the truncated protein may oligomerize with wild type protein and become trapped in the endoplasmic reticulum as reported in the p.Lys501fs*533 mutation producing a dominant negative effect [57, 58].

Three missense mutations p.Met105Val, p.Ile114Ser and p.Met157Thr identified in the present study in FZD4 were located in the extracellular cysteine rich domain (CRD) of the gene. The wild type amino acid residues located at the 105, 114 and 157 codon positions were experimentally found to be crucial for ligand binding during Norrin signal transduction [30, 40, 41]. A novel heterozygous mutation, c.341 T > G (p.Ile114Ser) was identified in a female proband (II:3) of family 3 (Fig. 1). The proband was noticed to have bilateral nystagmus with exudative retinal detachment including her
macula at 8 months of age. The mother of the proband (I:2) also harbored this change but was found to be clinically normal under indirect ophthalmoscopic examination (Fig. 3). Although, the p.Ile114Ser was identified in the present study, Robitaille et al., [53] reported a p.Ile114Thr mutation in a familial FEVR case with bilateral retinal folds at 2 months of age. The isoleucine residue at 114 codon position of FZD4 was highly conserved across multiple vertebrate species (Fig. 2). Replacement of hydrophobic isoleucine with a hydrophilic serine residue at codon 114 was predicted to have damaging effect on protein function by three algorithms in insilico analysis (Table 1). These findings provide evidence for the possible involvement of p.Ile114Ser mutation in disease pathogenesis.

Both the p.Met105Val and p.Met157Thr mutations have been previously identified in the Caucasian and Asian populations [1, 2, 16, 18, 20]. In the present study, we have identified the replacement of methionine by threonine at 157 codon position in a sporadic case (Fig. 1; family 6; I:2) with total retinal detachment in both eyes of the proband. This patient was examined at the age of 35 years with roaming movements in the right eye and bilateral ectopic macula towards the temporal side. Her two sons (II:3 and II:4) were also examined and found to be clinically normal and did not harbor the observed change. In addition to this mutation, a missense change (c.694A > G; p.Met232Val) in ZNF408 of unknown significance was also detected in the proband and one of her son without any observable retinal non perfusion, indicating that the p.Met157Thr was mainly responsible for the disease phenotype in this family. The methionine at 157 codon position precedes a cysteine (codon 158), which is involved in the formation of a disulfide bond crucial for structural maintenance of CRD [39]. Based on this information, we speculate that the altered solvent interactions of hydrophilic threonine instead of hydrophobic methionine in M157T mutation may interfere with the disulfide bridge formation by following cysteine. Thus, the structural alteration caused by this mutation may interfere with the ligand interaction or FZD4 homodimer formation and could result in the pathogenicity.

The recurrent heterozygous mutation c.313A > G (p.Met105Val) was observed in 3 different sporadic FEVR probands (Fig. 1; families 7, 8 and 9) with advanced stages of FEVR. All the probands having this mutation developed falciform folds and tractional retinal detachment either in one or both eyes within a few months of their birth. Among the three families, two of the mothers (Families 7 and 9; I:2) and a sibling of the proband 9 (II:1) failed to show severe retinal abnormalities despite of harboring a single copy of mutant allele. However, the mother of the proband 8 (I:2) showed peripheral retinal avascularization.

Notably, like the M157T, the M105V mutations also affected a cysteine residue at codon 106 that plays a crucial role in CRD structure formation via disulfide bridge formation with another cysteine at codon 45. Replacement of the highly conserved methionine at codon 105 with a valine residue led to severe impairment of FZD4 signaling (Fig. 2) [30]. This information strongly suggested the pathogenic nature of this missense mutation.

Mutations detected in the TSPAN12 gene

So far, 27 different mutations in TSPAN12 have been reported in FEVR patients [1, 2, 4, 5, 22–25, 59]. These mutations included missense, nonsense, small base pair deletions, insertions and splice site mutations. A novel, heterozygous c.125 T > C (p.Val42Ala) missense mutation was identified in a female proband (IV: 2) of a consanguineous FEVR family 10 (Fig. 1). The proband developed bilateral nystagmus along with attached retrolental membranes to the posterior capsule and funnel shaped retinal folds at 4 months of age. This mutation was also observed in the maternal uncle (III: 3) and aunt (III: 6) of the proband who was reported to be blind since birth. However, both the parents of the proband harboring this heterozygous change were clinically normal. Another novel missense mutation c.479G > A (p.Cys160Tyr) was identified in probands of four unrelated FEVR families (Fig. 1; families 11 to 14). In two probands (IV: 2) of consanguineous FEVR families (11 and 12), this mutation was found in a homozygous condition and showed autosomal recessive inheritance. The probands of the other two FEVR families (13 and 14) were heterozygous for this change. The two probands having homozygous mutation exhibited total retinal detachments with a funnel shaped detached retinas within a few months after birth. The probands with heterozygous mutation progressively developed exudative or tractional retinal detachments with fibrovascular proliferations during the first or second decades of their life (Fig. 4). The other members of these families harboring this mutation showed variable clinical expressivity that ranged from very mild peripheral retinal non perfusion to exudation, falciform retinal folds and total retinal detachments. The three prediction tools used for insilico analysis indicated the damaging effect of this non-conservative substitution on protein function.

The third missense mutation c.334G > A (p.Val112Ile) was identified in homozygous condition in a male proband of FEVR family 15. This patient was first examined in our hospital at 24 years of age and found to have total retinal detachment with falciform retinal folds in both eyes with bilateral nystagmus at the initial examination (Fig. 4). The mother and a male sibling of the proband, who also harbored this mutation in heterozygous state, were asymptomatic with mild pigmentary changes in the retina. This change was previously reported in
heterozygous condition as a rare variant by Exome Aggregation Consortium with the frequency of minor allele to be 0.0001 [47].

Another novel missense change, p.Cys160Tyr was prevalent (3.6 % of the FEVR probands) in the present study. This mutation was observed in the probands and their affected family members of four unrelated FEVR families (Fig. 1; families 11 to 14). Two severely affected probands of the FEVR families (11 and 12) had homozygous p.Cys160Tyr mutation while the remaining probands of two FEVR families (13 and 14) and their affected relatives were heterozygous. Compared to probands with the homozygous mutations, the patients with the heterozygous allele showed a slower progression of the disease or relatively less severe ocular phenotype, suggesting the recessive inheritance pattern of the disease and effect of allelic dosage on disease severity. The previously described FEVR patients with homozygous mutations in the candidate genes had severely reduced protein expression that was shown to be responsible for severity and rapid progression of the disease [21, 23, 60].

The p.Val42Ala mutation was located in the small extracellular loop while, p.Val112Ile and p.Cys160Tyr mutations were located in the large extracellular loop of the protein. The wild type cysteine as seen for p.Cys160Tyr mutation, is located at the second position in the highly conserved CCG signature motif of extracellular loop 2 (ECL2) of the TSPAN12 protein (Fig. 2). This cysteine residue was predicted to be involved in the formation of a disulfide bridge with another cysteine residue located at 181 position of TSPAN12. The ECL2 was suggested to be involved in protein-protein interactions with targeted proteins in the cell membrane [61]. Therefore, the disrupted structure of ECL2 due to p.Cys160Tyr

![Fundus photographs of the FEVR patients with novel nucleotide changes identified in the TSPAN12 gene.](image-url)
mutation could probably interfere in TSPAN12 interaction with FZD4 protein, thereby affecting the Norrin-FZD4 signaling.

**Mutations detected in the ZNF408 gene**

A novel, heterozygous, c.130C > T (p.Pro44Ser) change was observed in a male proband (II: 2) of FEVR family 16 (Fig. 5). The proband had bilateral total retinal detachments with retrolental membranes at 2 months of age. The father of the proband (I: 2) was found to be normal both phenotypically and genotypically while the mother (I: 1) remained unavailable for genetic analysis. All the three algorithms predicted it to be of benign nature. The second novel heterozygous c.2145G > T (p.Glu715Asp) change was observed in a sporadic proband of FEVR family 17. The proband exhibited total retinal detachment in the right eye and subtotal retinal detachment including macula in the left eye at one year of age. The mother of the proband (I: 2) was found to be clinically normal without this variation, while the father (I: 1) was unavailable for genetic analysis. A previously reported T617N change located in tenth zinc finger domain of the protein was also observed in a family, which could potentially interfere with DNA interaction. However, the presence of this change in the unaffected mother of the proband and in a control individual is suggestive for a less or non pathogenic nature of this variation. In family 6, the proband had M232V missense change in the ZNF408 gene and also a heterozygous M157T mutation in FZD4 gene. The heterozygous M232V change was also observed in one of the two unaffected sons of the FEVR proband (Fig. 5) however, but they lacked the M157T mutation of FZD4. The data thus suggested that M157T mutation was mainly responsible for the ocular phenotype in the proband. Further structural and functional studies are required to understand the effect of these missense changes on ZNF408 protein and its downstream interactions.

Of the three novel non-synonymous variations observed in the recently identified ZNF408 gene, the p.Pro44Ser and p.Met232Val changes were predicted to be harmless by the three algorithms used in *in silico* analysis and were considered as variations of unknown significance, while an evolutionarily non-conservative substitution p.Glu715Asp was predicted to be pathogenic (Fig. 2). None of these changes were seen in the controls (Table 1).

Interestingly, none of the variations in ZNF408 gene in our Indian cohort were observed in the Caucasian and Asian FEVR patients indicating the allelic heterogeneity of this condition. In summary, the present study provides an independent validation of the involvement of ZNF408 in FEVR from an ethnically diverse population from India. This also expands the mutation spectrum of ZNF408 in the pathogenesis of FEVR that would need to be further supplemented with diverse data from other populations.

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**Fig. 5 Pedigrees of the FEVR families with nucleotide changes in ZNF408 gene.** Completely shaded symbols represent severe stages of the disease. Open symbols represent unaffected individuals. Asterisk (*) over the pedigree symbols represent the individuals screened for nucleotide changes in ZNF408 gene. - indicates the presence of deletion/insertion and + indicates the wild type allele. The identified nucleotide change is represented above each pedigree.
Conclusion
In summary, the mutation frequencies of the 3 candidate genes among the Indian FEVR patients were comparable to previous reports in FZD4 (3.5–40 %) [1, 2, 7, 8, 14–20, 42], and TSPAN12 (3.5–10 %) [1, 2, 4, 5, 22–25] genes in different populations. In previous reports the frequency of FEVR patients with ZNF408 mutations varied from 0 to 1.8 % in different populations [1–3]. The frequency of patients with FZD4 mutations in our cohort was relatively higher when compared to a previous study by Nallathambi et al., in another cohort of Indian FEVR patients [42]. This indicates the broad spectrum of FZD4 mutations in FEVR pathogenesis among the Indian patients. Other than the common ocular features of FEVR, we did not recognize any distinguishable features specific to the cases harboring FZD4, TSPAN12 and ZNF408 mutations in these patients for undertaking genotype-phenotype correlation. The novel mutations identified in the present study further broadened the mutation spectrum in FEVR patients, which would facilitate better genetic counseling and diagnostics to treat these patients before the development of severe visual complications.

Ethics (and consent to participate)
The study was approved by the Institutional Review Board (IRB) of L.V. Prasad Eye Institute, Hyderabad, (Ref no LEC06104) India, and adhered to tenets of the Declaration of Helsinki. A written informed consent for research purposes was obtained from each subject prior to their participation in the study. In case of minors, the consent for participation in the study was obtained from either of the parents.

Consent to publish
Not applicable as the study does not identify the subjects and no personal information identifying the subject included in the manuscript.

Availability of data and materials
The data pertaining the study would be available from the corresponding author and principal investigator of the study upon request for any research use.

Additional file

Additional file 1: Primer and Amplification Details for the Three Candidate genes. (DOCX 17 KB)

Abbreviations
CRD: cysteine rich domain; DNA: Deoxyribonucleic Acid; ECL: extra cellular loops; ESP5400: Exome Sequencing Project 5400; ExAC: Exome aggregation consortium; FEVR: Familial Exudative Retinopathy; FZD4: Frizzled 4; IRB: Institutional Review Board; KIF11: Kinesin family member; LRP5: Low density lipoprotein receptor like protein 5; NDP: Norrie disease pseudoglioma; PCR: Polymerase Chain reaction; PolyPhen-2: Polymorphism Phenotyping Version 2; SIFT: Sorting Intolerant from Tolerant; TCF/LEF: T-cell factor/lymphoid enhancer factor; TSPAN12: Tetraspanin-12; ZNF408: Zinc finger protein-408.

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Authors’ contributions
All authors conceived of and designed the experimental protocol. IK and SC contributed to the study design, analyzed the data and performed critical revision of the manuscript for important intellectual content. SJ performed the eye examinations and clinical correlations of the data. GRM and SH collected the samples and performed the experiments. GRM and IK wrote the paper. All authors have read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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