

# Mutation spectrum of the Norrie disease pseudoglioma (*NDP*) gene in Indian patients with FEVR

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**Purpose:** Mutations in the *Norrie disease pseudoglioma* (*NDP*; Xp11.3) gene have been involved in retinal blood vessel formation and neural differentiation and are implicated in familial exudative vitreoretinopathy (FEVR) cases. However, the role of the gene has not been explored in the Indian context. Thus, this study was designed to understand the involvement of *NDP* among Indian patients with FEVR.

**Methods:** The study cohort comprised 225 subjects, including unrelated patients with FEVR (n = 110) and ethnically matched healthy subjects (n = 115) recruited from a tertiary eye care center in India. The entire coding regions, intron–exon boundaries, along with the 5' and 3' untranslated regions of *NDP* were screened with resequencing following standard protocols. The spectrum of the observed variants was analyzed in conjunction with data available from other populations.

**Results:** Eight potentially pathogenic mutations (p.His4ArgfsX21, p.Asp23GlufsX9, p.Ile48ValfsX55, p.His50Asp, p.Ser57\*, p.Gly113Asp, p.Arg121Gln, and p.Cys126Arg, including five novel ones), were observed in the coding region of the *NDP* gene in ten unrelated FEVR probands (9%). The novel changes were not observed in the control subjects and were unavailable in the dbSNP, ESP5400, NIEHS95, and ExAC databases. All probands with *NDP* mutations exhibited classical features of the disease as observed among patients with FEVR worldwide.

**Conclusions:** This is perhaps the first study to demonstrate the involvement of *NDP* among patients with Indian FEVR that further expands its mutation spectrum. The data generated could have broad implications in genetic counseling, disease management, and early intervention for a better prognosis in FEVR.

Familial exudative vitreoretinopathy (FEVR) is a rare, inherited, bilateral eye disorder characterized largely by the avascular peripheral retina that leads to retinal ischemia. This may further progress to retinal neovascularization, subretinal exudation and hemorrhages, and partial or total retinal detachment that leads to complete blindness [1,2]. FEVR is a genetically heterogeneous disorder that exhibits variable clinical phenotypes across patients. The clinical heterogeneity is further compounded by the course of the disease that may vary between the two eyes of a patient and among the affected members of a family [1,2].

Thus far, mutations in five candidate genes, viz., *Norrie disease pseudoglioma* (*NDP*; Xp11.3; OMIM 300658), *Frizzled-4* (*FZD4*; 11q14.2; OMIM 604579), *low-density lipoprotein receptor related protein-5* (*LRP5*; 11q13.2; OMIM 603506), *tetraspanin-12* (*TSPAN12*; 7q31.31; OMIM 613138), and *Zinc finger protein-408* (*ZNF408*; 11p11.2; OMIM 616454) have been implicated in FEVR [3-29]. The mutations

in these genes were involved in autosomal dominant (*FZD4*, *LRP5*, *TSPAN12*, and *ZNF408*) [10-12,17,20-22,24,26], autosomal recessive (*LRP5* and *TSPAN12*) [15,16,23], and X-linked (*NDP*) forms of FEVR [3-9,30]. Overall, the mutations in these genes accounted for less than 50% of all FEVR cases worldwide suggesting the involvement of other unidentified genes in the disease pathogenesis [26,28].

The proteins encoded by the *NDP*, *FZD4*, *LRP5*, and *TSPAN12* genes are involved in the formation of a ligand-receptor complex that leads to the stabilization and nuclear translocation of the cytoplasmic  $\beta$ -catenin molecules and activation of T-cell factor (TCF)/lymphoid enhancer factor (LEF)-mediated gene expression, similar to the canonical Wnt/ $\beta$ -catenin pathway [31-37]. The *NDP* gene encodes for a 133 amino acid-containing protein, Norrin, that is secreted by the Müller glial cells of the retina [34]. Though Norrin is structurally divergent from the Wnt ligands, it specifically binds to the transmembrane *FZD4* receptor and *LRP5* coreceptor complex on the retinal vascular endothelial cells and induces the canonical Wnt/ $\beta$ -catenin pathway [31,33,34]. The *TSPAN12* protein enhances *FZD4* oligomerization through physical binding and thus, efficiently induces the signaling

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pathway [32,36]. This pathway plays a crucial role in hyaloid vessel regression, developmental retinal angiogenesis, and maintenance of retinal blood vessel integrity and blood-retinal barrier (BRB) property in adults [31-33,35,37-39]. This pathway also has roles in placental angiogenesis, rescuing oxygen-induced retinal vessel loss, maintenance of blood-brain barrier (BBB) property, and induction of neuroprotective growth factor expression [35,37,40-45]. In addition to its healthy physiologic roles, this pathway is involved in the angiogenesis of tumors in colorectal cancer [46]. A recent study also suggested the role of Norrin as a ligand for leucine-rich repeat containing G-protein coupled receptor-4 (LGR4) and bone morphogenesis protein (BMP) antagonist [47]. The most recently identified candidate gene in FEVR is *ZNF408*, and its role in the disease pathogenesis is not yet known [26].

The molecular genetics of FEVR have been well characterized across different ethnic groups, including Caucasian, Chinese, and Japanese patients [3-30]. Although the mutation spectrum of the *NDP* gene in FEVR has been extensively documented in these populations, there are no such reports from India. Thus, we screened the *NDP* gene in patients from southern India, to understand the gene's contribution in FEVR.

## METHODS

*Enrollment and clinical evaluation of the subjects:* The study was approved by the Institutional Review Board of L.V. Prasad Eye Institute (LVPEI), Hyderabad, India, and adhered to the tenets of the Declaration of Helsinki. The 225 consecutive subjects, including 110 unrelated patients with FEVR and 115 healthy controls, were recruited at LVPEI from January 1, 2008, to December 31, 2009. The diagnosis of FEVR was based on indirect ophthalmoscopic examination, B-scan ultrasonography, and fundus fluorescein angiography (in selective patients). The clinical examination was performed by an ophthalmologist with specialization in the retina (SJ). The stages of the disease were classified based on the Pendergast and Trese classification [48]. All healthy subjects underwent a comprehensive ophthalmic examination including a dilated fundus examination with indirect ophthalmoscopy. Written informed consent was obtained from all adult participants and the guardians of minors before their enrollment in the study. All control subjects were ethnically matched and recruited from the same geographic region as the patients with FEVR. Blood samples from all subjects were collected in heparinized vacutainers and immediately stored in -20 °C deep freezers until further use.

*Clinical evaluation of the subjects:* The inclusion criteria for the patients with FEVR included the following criteria.

*Essential criteria:* The following essential criteria were used:

- i. Each FEVR patient born full term without any evidence of low birthweight, septicemia, apnea, or oxygen supplementation;
- ii. Bilateral presence of the disease with a healthy corneal diameter;
- iii. Vitreoretinal characteristics including a persistent peripheral avascular retina that was never vascularized (in eyes in which the peripheral retina was visualized); bilateral infantile or neonatal retinal detachment presenting as leukocoria associated with a clear lens and a retrolenticular membrane (pseudoglioma) where all other causes are excluded; variable extent of vitreous condensation, retinal new vessels, retinal angiomatous proliferation or exudation at the posterior edge of the avascular retina; retinal stretching and dragging or folds due to vitreous condensation and traction;
- iv. One eye has a persistent peripheral avascular retina;
- v. Vitreoretinal characteristics described above with the other eye having a pseudoglioma retinal detachment since birth or early infancy with a healthy corneal diameter; and
- vi. Rhegmatogenous retinal detachments associated with any of these described vitreoretinal characteristics.

*Additional criteria:* Additional features were investigated if a family member revealed any of these vitreoretinal signs. The family members were then clinically evaluated following their pedigree documentation. Systemic features, including seizures, mental retardation, hearing problems, developmental delays, etc., were recorded but did not constitute exclusion of patients with typical ocular findings.

*Mutation screening in the *NDP* gene:* Genomic DNA was extracted from blood leucocytes using standard Phenol-Chloroform extraction protocols [49]. The 28 kb *NDP* gene consists of three exons with a transcript length of 1.85 kb and an open reading frame of 399 bp (NM\_000266.3). The coding and non-coding exons along with the intron-exon boundaries were PCR amplified with seven overlapping sets of primers. The 5' untranslated region (UTR) and the coding regions of the gene were amplified by using three different sets of primers that have been previously described [50], while four sets of predesigned primers were used for the amplification of the 3' UTR (Table 1). Finally, these amplicons were screened with resequencing on an automated DNA sequencer ABI3130 XL (Applied Biosystems, Foster City, CA) using the Big Dye chemistry (version 3.1) and following the manufacturer's guidelines.

**Confirmation of the observed variants and bioinformatics analysis:** The observed variants in the *NDP* gene were further validated with resequencing by another investigator who was masked to the genotype of the subjects. The observed variants were also searched in the dbSNP [51], ESP5400 [52], NIEHS95 [53], and ExAC [54] databases and available literature for filtering out the known polymorphisms. Multiple sequence alignments were performed to determine the conservation of the wild-type amino acid residues at the sites harboring the missense changes using the ClustalW program with different protein orthologs retrieved from the NCBI database [55]. The functional effects of the identified missense changes were predicted using computational tools, Sorting Intolerant From Tolerant (SIFT) [56] and PolyPhen-2 [57]. The detailed protocols on the use of these programs are provided as a supplementary item (Appendix 1). The observed missense changes were considered pathogenic when they segregated with the disease phenotype, were absent in the ethnically matched healthy controls along with the exome databases and their wild-type protein residues were highly conserved, and were predicted to be damaging based on the bioinformatic analysis (SIFT and PolyPhen-2). The effect of these changes on mRNA splicing was further evaluated using the Human Splicing Finder analysis [58].

## RESULTS

Among the 110 clinically diagnosed and unrelated probands with FEVR (67 men and 43 women), 34 (30.91%) had a family history of the disease. Eight different mutations were observed in the coding regions of ten probands with FEVR (9.09%, 95% confidence interval [CI], 7.04–19.17%; Figure 1, Figure 2, and Table 2). Consanguinity was observed in three families with FEVR who harbor *NDP* mutations. Five mutations were novel, including three missense changes (p.His50Asp, p.Gly113Asp, and p.Cys126Arg) and two small base pair frame shift deletions (p.Asp23GlufsX9 and p.Ile48ValfsX55). The

p.His50Asp mutation was observed in three familial FEVR cases while the p.Gly113Asp, p.Cys126Arg, and p.Arg121Gln changes were observed in three sporadic cases (Figure 1). Although the p.His50Asp change segregated with the disease phenotype in all affected family members of the probands, some of the female carriers were found to be healthy in indirect ophthalmoscopic examination but were unavailable for further evaluation with fundus fluorescein angiography.

Additionally, three previously reported mutations comprising a deletion (p.His4ArgfsX21) [18], a nonsense change (p.Ser57\*) [59], and a recurrent missense change (p.Arg121Gln) [9,17,30] were observed in three sporadic FEVR cases. These mutations were not observed in the 115 ethnically matched controls and were not reported earlier in the dbSNP [51], ESP5400 [52], NIEHS95 [53], and ExAC [54] databases. Apart from these potential mutations, a previously reported 14 bp deletion -409\_-395del114bp [6,7] and a single nucleotide polymorphism (SNP; rs45501198) in the 5' UTR were observed in a family with three affected siblings. Further, two novel variations in the 3' UTR, c.\*522T>C and c.\*974C>G, were observed in two patients with disease stages 3B (sporadic) and 5 (familial), respectively (Appendix 1). However, these changes were non-pathogenic and did not appear to cosegregate with FEVR.

The wild-type residues of the three novel missense changes identified in this study were highly conserved across different species and were predicted to be pathogenic based on bioinformatic analysis (Figure 3 and Table 2). However, no significant effect of these mutations was observed on the potential splice sites or splicing-regulatory elements. As *NDP* is an X-linked gene, mutations were predominantly observed in the male probands in the hemizygous condition, and some of the female carriers exhibited the disease phenotype (Figure 1). In the present study, the three deletions (p.His4ArgfsX21, p.Asp23GlufsX9, and p.Ile48ValfsX55) and a reported truncating mutation (p.Ser57\*) were found in exon 2. All three

TABLE 1. PRIMERS FOR AMPLIFICATION OF THE 3' UTR OF *NDP*.

| Primer      | Primer sequence             | Amplicon size | Annealing temperature (°C) | MgCl <sub>2</sub> concentration (mM) |
|-------------|-----------------------------|---------------|----------------------------|--------------------------------------|
| NDP-3UTR-1F | 5'-CCAGACTTCCAAGCTGAAGG-3'  | 352           | 58                         | 1.5                                  |
| NDP-3UTR-1R | 5'-ACCAAACACTGACAGCCTGA-3'  |               |                            |                                      |
| NDP-3UTR-2F | 5'-TTGGCTCTCAATGCTGTTTG-3'  | 499           | 58                         | 1.5                                  |
| NDP-3UTR-2R | 5'-GCTGTCAAGAGTTCCAGCATC-3' |               |                            |                                      |
| NDP-3UTR-3F | 5'-CAGCCAGCGAACTGACATTA-3'  | 297           | 54                         | 1.5                                  |
| NDP-3UTR-3R | 5'-TTAGAGAATGATGCCCCGTGA-3' |               |                            |                                      |
| NDP-3UTR-4F | 5'-GCATGCAAATTAGACAACCAA-3' | 458           | 58                         | 1.5                                  |
| NDP-3UTR-4R | 5'-AGGAGATGCTCAAGCACTAGC-3' |               |                            |                                      |

deletions led to a frame shift and the formation of premature termination codons. In the case of p.His4ArgfsX21 (c.11\_12delAT) and Asp23GlufsX9 (c.69delC) deletions, the premature termination codons were formed in exon 2, which is followed by exon 3 and was therefore suggestive of nonsense-mediated mRNA decay [60]. The p.Ile48ValfsX55 (c.142\_145delATCA) deletion led to the formation of a premature termination codon in exon 3 that could result either in nonsense-mediated mRNA decay or in the formation of a truncated protein [60].

*Phenotypes of the patients with NDP gene mutations:* All ten probands who harbor *NDP* gene mutations presented with typical clinical features of FEVR characterized by avascularized peripheral retina, nystagmus, retinal hemorrhages, exudation, vitreous traction, various degrees of ectopic macula, falciform retinal folds, closed funnel retinal folds with or without partial or total retinal detachments and retrolental membranes, and no other systemic features. Additional clinical details of these probands and their affected family members are provided in Appendix 1 and Figure 4.

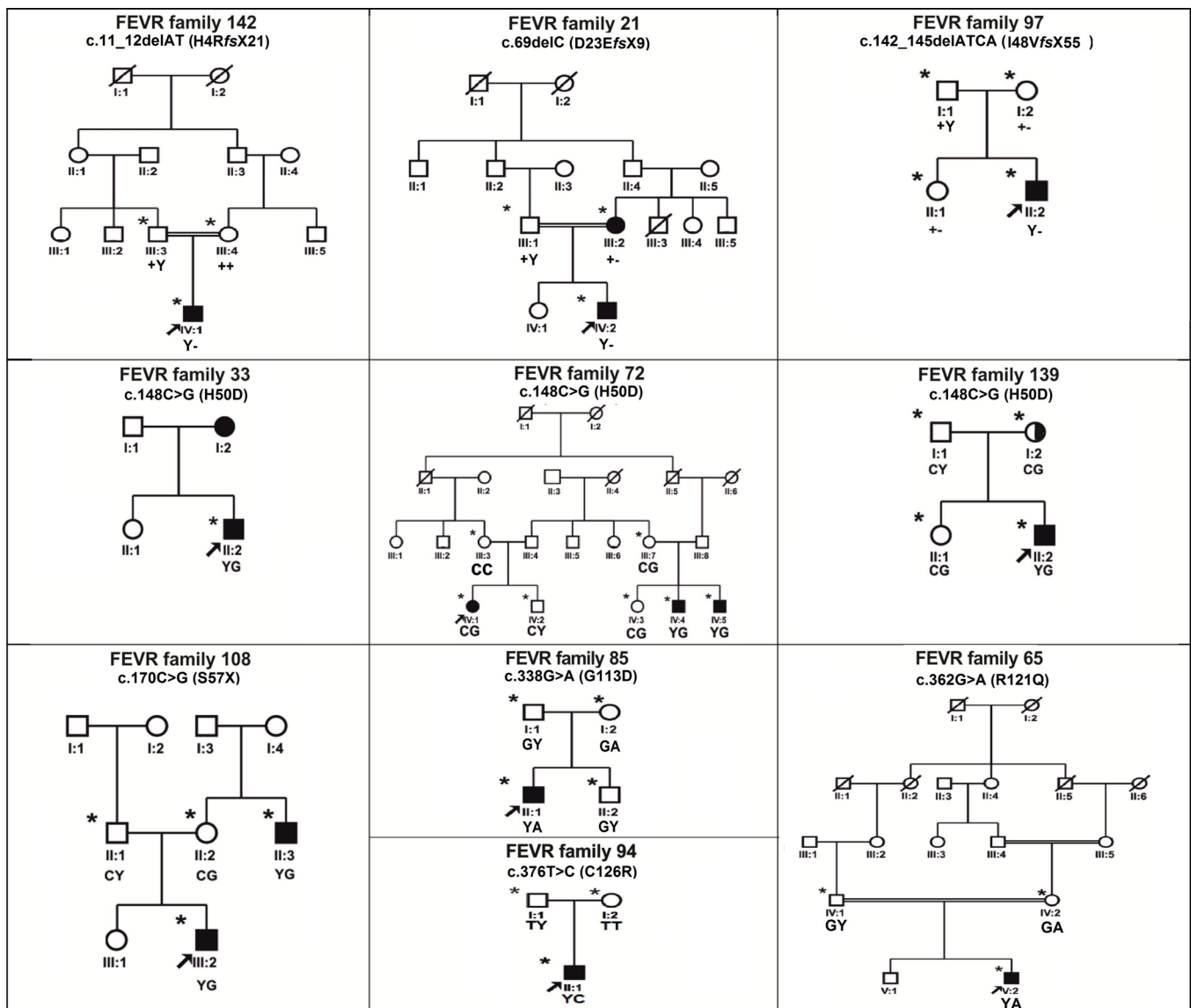


Figure 1. Pedigrees of the FEVR families with *NDP* gene mutations. Completely and partially shaded symbols represent severe stages and milder stages of the disease, respectively. Open symbols represent unaffected individuals. Y indicates the Y chromosome. - indicates the presence of deletion, and + indicates the wild-type allele. An asterisk (\*) over the pedigree symbol represents an individual screened for *NDP* gene variations.

Almost all probands harboring *NDP* mutations presented with a bilaterally severe form of FEVR at <1 year of age. Only the proband (FEVR family 85) with the Gly113Asp mutation had an atypical presentation with a bilateral avascular peripheral retina with vitreous condensation along with straightening of the arcades and was diagnosed at 24 years of age. The disease phenotype was also observed in the women who harbor the heterozygous p.His50Asp and p.Asp23GlufsX9 mutations (Figure 1) but with a lesser degree of severity compared to the male probands. The female carriers who harbored the heterozygous p.Ile48ValfsX55, p.Ser57\*, p.Gly113Asp, and p.Arg121Gln changes were phenotypically normal on indirect ophthalmoscopic examination (Figure 1). The p.His4ArgfsX21 and p.Cys126Arg mutations were detected only in the probands with bilateral total retinal detachment condition. These two changes were suggestive of de novo origin as they were not detected in the probands' parents.

## DISCUSSION

The Norrin-FZD4 signaling pathway is a variant of the canonical Wnt signaling pathway and plays a crucial role in the development of retinal angiogenesis [2]. It is presumed that alterations in highly conserved amino acids of genes involved in this pathway might affect either the structure of the ligand (Norrin) or the proteins involved in the formation of the receptor complex (FZD4, LRP5, and TSPAN12) and their localizations. These alterations might either inactivate or alter the pathways resulting in the inhibition of abnormal vascular development. Mice that lack *NDP*, *FZD4*, *LRP5*, and *TSPAN12* [31-33,39,61] have further demonstrated the role of these genes in capillary maturation and signaling mechanisms that are involved in retinal angiogenesis and normal retinal development.

Recently, the crystal structure of Norrin and its structural basis for FZD4 receptor interaction was elucidated [36,62,63].

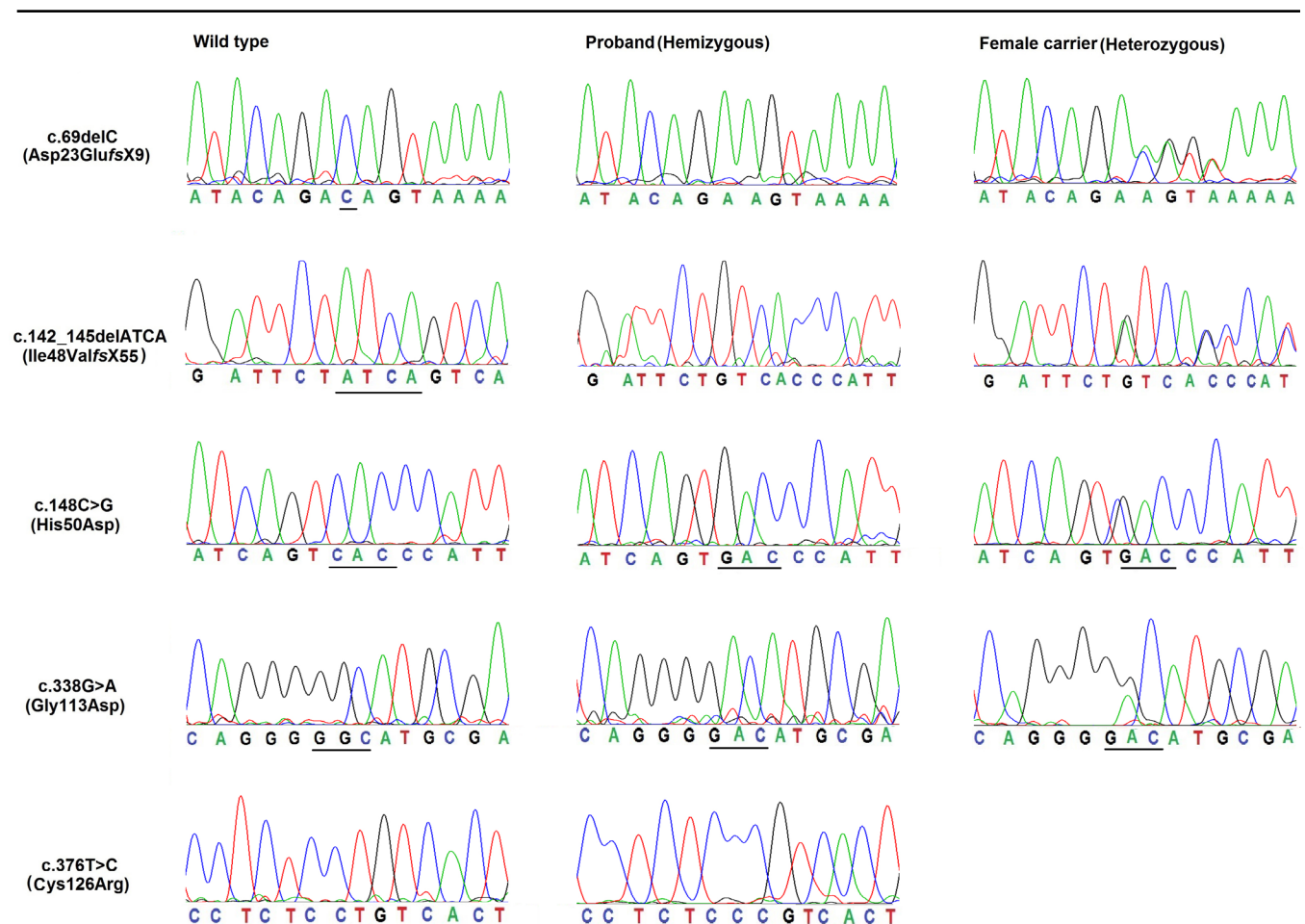


Figure 2. Electropherograms of the five novel nucleotide changes identified in the *NDP* gene. The cDNA positions of the nucleotide changes and the amino acid changes are shown at the left side of the picture. Wild-type and altered electropherograms are shown at the right side of each corresponding nucleotide change.

TABLE 2. MUTATIONS OBSERVED IN *NDP* GENE IN FEVR PATIENTS.

| Location in <i>NDP</i> gene | cDNA change <sup>#</sup> (Protein change) | Computational analysis |                           | Reported/ Novel    | Stage of FEVR | Proband No | Mutation frequency: Patients (n=110)/ Controls (n=115) |
|-----------------------------|---|------------------------|---------------------------|--------------------|---------------|------------|--|
|                             |   | SIFT (score)           | PolyPhen2 (score)         |                    |               |            |  |
| Exon 2                      | c.11_12delAT (p.His4ArgfsX21)             | -                      | -                         | Reported [18]      | OU:5          | 142 FR     | 0.009/0.00   |
|                             | c.69delC (p.Asp23GlufsX9)                 | -                      | -                         | Novel              | OD:4B OS: 5   | 21 FR      | 0.009/0.00   |
|                             | c.142_145del ATCA (p.Ile48ValfsX55)       | -                      | -                         | Novel              | OU:5          | 97 FR      | 0.009/0.00   |
|                             |   | -                      | -                         | Probably damaging  | OU:4B         | 33 FR      |  |
|                             | -   | -                      | Damaging 0 (0.991)        | Novel              | OU:4A         | 72 FR      |  |
|                             | c.148C>G (p.His50Aasp)                    | -                      | -                         | Novel              | OD:3B         |            |  |
| Exon 2                      | c.170C>G (p.Ser57*)                       | -                      | -                         | Reported [59]      | OS:5          | 139 FR     | 0.027/0.00   |
|                             | -   | -                      | Probably damaging         | OU:5               | 108 FR        |            |  |
|                             | -   | -                      | Damaging 0 (1.000)        | Novel              | OU:2B         | 85 FR      | 0.009/0.00   |
| Exon 3                      | c.362G>A (p.Arg121Gln)                    | Damaging -0.01         | Probably damaging (0.998) | Reported [9,17,30] | OU:5          | 65 FR      | 0.009/0.00   |
|                             | -   | -                      | Probably damaging         | Novel              | OU:5          | 94 FR      | 0.009/0.00   |
|                             | c.376T>C (p.Cys126Arg)                    | Damaging 0 (0.997)     | Probably damaging (0.997) | Novel              | OU:5          | 94 FR      | 0.009/0.00   |

<sup>#</sup>NCBI Reference Sequence: [NM\\_000266.3](#). To predict the pathogenic effect of the missense mutations, Sorting Intolerance from Tolerance (SIFT), PolyPhen-2 computational programs were used [56,57]. OU: both eyes, OD: right eye, OS: left eye.

|                       | H50D |   |   | G113D |   |   | C126R |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|-----------------------|------|---|---|-------|---|---|-------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Homo sapiens          | D    | S | I | S     | H | P | L     | Y | K | L | R | C | S | G | G | M | R | L | T | R | Y | I | L | S | C | H | C | E | E |
| Macaca mulatta        | D    | S | I | S     | H | P | L     | Y | K | L | R | C | S | G | G | M | R | L | T | R | Y | I | L | S | C | H | C | E | E |
| Bos taurus            | D    | S | I | S     | H | P | L     | Y | K | L | R | C | S | G | G | M | R | L | T | R | Y | I | L | S | C | H | C | E | E |
| Python bivittatus     | D    | S | I | S     | H | P | L     | Y | K | L | R | C | S | G | G | M | R | L | T | R | Y | I | L | S | C | H | C | E | E |
| Gallus gallus         | D    | S | I | S     | H | P | L     | Y | K | L | R | C | S | G | G | M | R | L | T | R | Y | I | L | S | C | H | C | E | E |
| Mus musculus          | D    | S | I | S     | H | P | L     | Y | K | L | R | C | S | G | G | M | R | L | T | R | Y | I | L | S | C | H | C | E | E |
| Rattus norvegicus     | D    | S | I | S     | H | P | L     | Y | K | L | R | C | S | G | G | M | R | L | T | R | Y | I | L | S | C | H | C | E | E |
| Felis catus           | D    | S | I | S     | H | P | L     | Y | K | L | R | C | S | G | G | M | R | L | T | R | Y | I | L | S | C | H | C | E | E |
| Sus scrofa            | D    | S | I | S     | H | P | L     | Y | K | L | R | C | S | G | G | M | R | L | T | R | Y | I | L | S | C | H | C | E | E |
| Lipotes vexillifer    | D    | S | I | S     | H | P | L     | Y | K | L | R | C | S | G | G | M | R | L | T | R | Y | I | L | S | C | H | C | E | E |
| Erinaceus europaeus   | D    | S | I | S     | H | P | L     | Y | K | L | R | C | S | G | G | M | R | L | T | R | Y | I | L | S | C | H | C | E | E |
| Monodelphis domestica | D    | S | I | S     | H | P | L     | Y | K | L | R | C | S | G | G | M | R | L | T | R | Y | I | L | S | C | H | C | E | E |
| Pteropus alecto       | D    | S | I | S     | H | P | L     | Y | K | L | R | C | S | G | G | M | R | L | T | R | Y | I | L | S | C | L | C | E | E |
| Poecilia formosa      | E    | T | I | T     | H | P | I     | Y | K | L | R | C | A | G | G | T | R | I | T | R | Y | I | L | A | C | N | C | E | E |
| Xenopus laevis        | D    | S | I | N     | H | P | Q     | H | K | L | R | C | S | G | G | R | R | M | T | R | Y | I | L | S | C | H | C | E | E |

Figure 3. Multiple sequence alignment of various orthologs of the Norrin protein at the positions of the novel missense changes (p.H50D, p.G113D, and p.C126R) demonstrates the evolutionarily conserved nature of the wild-type residues. The amino acid sequences of the Norrin orthologs (*Homo sapiens*, NP\_000257.1; *Macaca mulatta*, NP\_001253901.1; *Bos taurus*, NP\_001039555.1; *Python bivittatus*, XP\_007434018.1; *Gallus gallus*, NP\_001265016.1; *Mus musculus*, NP\_035013.1; *Rattus norvegicus*, NP\_001102284.1; *Felis catus*, XP\_004000470.1; *Sus scrofa*, NP\_001106528.1; *Lipotes vexillifer*, XP\_007457029.1; *Erinaceus europaeus*, XP\_007528285.1; *Monodelphis domestica*, XP\_007493375.1; *Pteropus alecto*, XP\_006917969.1; *Poecilia formosa*, XP\_007571466.1; *Xenopus laevis*, NP\_001154869.1) were retrieved from the NCBI database.

*catus*, XP\_004000470.1; *Sus scrofa*, NP\_001106528.1; *Lipotes vexillifer*, XP\_007457029.1; *Erinaceus europaeus*, XP\_007528285.1; *Monodelphis domestica*, XP\_007493375.1; *Pteropus alecto*, XP\_006917969.1; *Poecilia formosa*, XP\_007571466.1; *Xenopus laevis*, NP\_001154869.1) were retrieved from the NCBI database.

It was found that Norrin exists as a homodimer in the solution and contains different binding surfaces for FZD4, LRP5, and the extracellular matrix [62,63]. Each Norrin monomer has an L-shaped structure and comprises three  $\beta$ -hairpins ( $\beta$ 1– $\beta$ 2,  $\beta$ 3– $\beta$ 4, and  $\beta$ 5– $\beta$ 6, a  $\beta$ 7 strand) and four intramolecular disulfide bridges (C39–C96, C65–C126, C69–C128, and C55–C110) [36,62,63]. These disulfide bridges are crucial for maintenance of the cysteine knot domain of the Norrin [36,63].

In the present study, the three novel missense changes (p.His50Asp, p.Gly113Asp, and p.Cys126Arg) were located in the highly conserved cysteine knot domain of the protein. These changes were predicted to be pathogenic by SIFT and PolyPhen-2. Further, these mutations were located in the regions of Norrin that are crucial for its structural maintenance and for receptor binding and segregated in all affected family members (Figure 1). This evidence suggests the pathogenic nature of these variations. Additionally, the three pathogenic deletions observed in this study led to frame shift and premature termination that could either result in nonsense-mediated mRNA decay or in the formation of a truncated protein thus affecting its function.

The *NDP* gene mutations contributed to 9% (10/110) of all the cases (Table 2) suggesting its involvement in Indian patients with FEVR. These findings were in accordance with the previously published reports on FEVR from other populations [5,7,8,17,28]. Similarly, X-linked gene mutations were also observed in some of the women with the disease phenotype, as observed earlier [64,65]. The healthy

phenotype observed in some of the female carriers could be due to the presence of the mutated allele on the inactivated X-chromosome, although this requires further functional validation.

Some of the earlier reports indicated the presence of frame shifts, truncated mutations, and mutations in the structurally crucial cysteine residues of *NDP* mainly in the case of patients with Norrie disease [7]. In this study, we observed two novel (p.Asp23GlufsX9 and p.Ile48ValfsX55) mutations, a reported frame shift (p.His4ArgfsX21) mutation, and a missense change at cysteine 126 position (p.Cys126Arg) in four unrelated patients with FEVR. However, none of the typical clinical features of Norrie disease, such as microphthalmia, corneal opacifications, hearing loss, and mental retardation, were observed in these patients. Recently, a similar finding of two novel truncating *NDP* mutations in three probands with FEVR further supported the involvement of pathogenic truncating mutations in this disease [28]. Based on the recently published clinical studies, we avoided distinguishing between Norrie disease and FEVR as two separate entities due to their overlapping clinical features and genotypes [6,7].

In the future, we will verify our novel missense mutations in silico with new promising approaches, including, among others, “combined annotation dependent depletion” [66] and “mutation significance cutoff” [67]. These methods improve upon SIFT and Polyphen-2 for variant interpretation. We plan to test the consequences of our new missense mutations in functional assays to verify that they are causative.

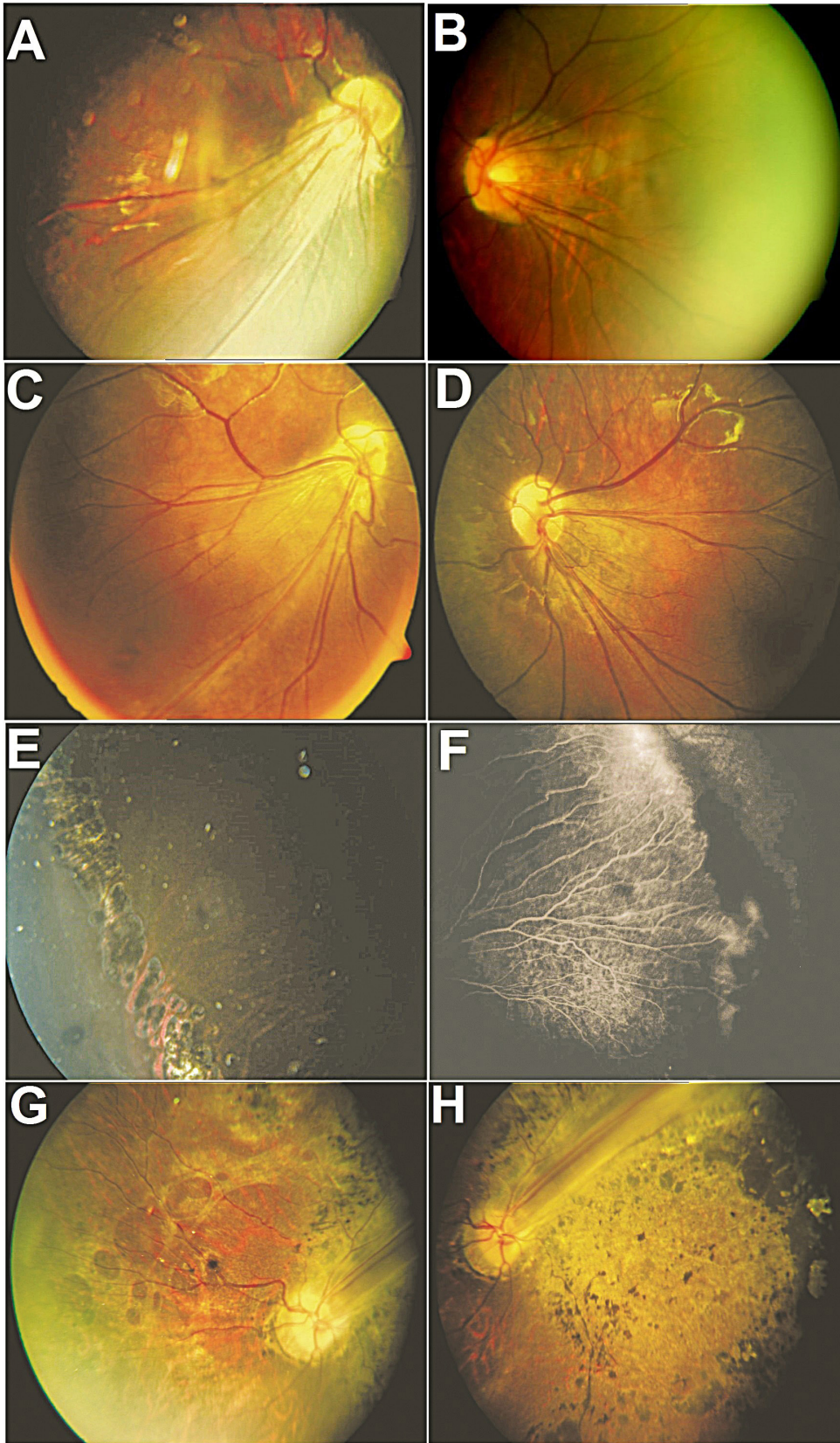


Figure 4. Fundus photographs and fluorescein angiogram pictures of the patients with FEVR with novel changes (p.H50D, p.G113D, and p.D23EfsX9) identified in the *NDP* gene. **A**: Patient ID: family 33-II:2 (p.H50D); the right eye of the patient shows straightening of the blood vessels and macular dragging toward the inferotemporal area due to fibrovascular traction. **B**: Patient ID: family 33-I:2 (p.H50D; affected mother of the proband); the left eye of the patient shows vitreoretinal traction with macular dragging. **C**, **D**: Patient ID: family 72-IV:1 (p.H50D); the right (**C**) and left (**D**) eyes show dragging and vitreoretinal traction with an ectopic macula. **E**: Patient ID: family 139-II:2 (p.H50D); the right eye of the patient shows an avascular peripheral retina with neovascularization and laser scars after the treatment. **F**: Patient ID: family 85-II:1 (p.G113D); fundus fluorescein angiogram of the left eye shows an avascular peripheral retina, straightening of the blood vessels, and dye leakage at the avascular and neovascular junction. **G**, **H**: Patient ID: family 21-III:2 (p.D23EfsX9; affected mother of the proband); the right (**G**) and left (**H**) eyes of the patient show pigmentation and vitreoretinal traction with a dragged macula.



In summary, to the best of our knowledge, this is perhaps the first study providing a mutation spectrum of the *NDP* gene in Indian patients with FEVR. The five novel mutations identified in this study further broaden the allelic heterogeneity of *NDP* and provide interesting insights into the clinical manifestations of this disease. These data would be valuable for genetic counseling that could further aid in proper management and early intervention before the development of severe visual complications in patients with FEVR.

## APPENDIX 1. CLINICAL FEATURES OF PATIENTS HARBORING MUTATIONS/ VARIATION IN THE *NDP* GENE

To access the table, click or select the words “Appendix 1.”

### ACKNOWLEDGMENTS

**Grant Support:** Supported in part by a grant from the Program Support grant by Department of Biotechnology to IK.

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13:109-10. [PMID: 26820543].

Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 16 May 2016. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.