Novel Mutations in Norrie Disease Gene in Japanese Patients with Norrie Disease and Familial Exudative Vitreoretinopathy

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PURPOSE. To search for mutations in the Norrie disease gene (NDP) in Japanese patients with familial exudative vitreoretinopathy (FEVR) and Norrie disease (ND) and to delineate the mutation-associated clinical features.

METHODS. Direct sequencing after polymerase chain reaction of all exons of the NDP gene was performed on blood collected from 62 probands (31 familial and 31 simplex) with FEVR, from 3 probands with ND, and from some of their family members. The clinical symptoms and signs in the patients with mutations were assessed. X-inactivation in the female carriers was examined in three FEVR families by using leukocyte DNA.

RESULTS. Four novel mutations—118K, K54N, R115L, and IVS2-1G→A—and one reported mutation, R97P, in the NDP gene were identified in six families. The severity of vitreoretinopathy varied among these patients. Three probands with either K54N or R115L had typical features of FEVR, whereas the proband with R97P had those of ND. Families with IVS2-1G→A exhibited either ND or FEVR characteristics. A proband with 118K presented with significant phenotypic heterogeneity between the two eyes. In addition, affected female carriers in a family harboring the K54N mutation presented with different degrees of vascular abnormalities in the periphery of the retina. X-inactivation profiles indicated that the skewing was not significantly different between affected and unaffected women.

CONCLUSIONS. These observations indicate that mutations of the NDP gene can cause ND and 6% of FEVR cases in the Japanese population. The X-inactivation assay with leukocytes may not be predictive of the presence of a mutation in affected female carriers. (Invest Ophthalmol Vis Sci. 2007;48:1276–1282) DOI:10.1167/iovs.06-1042

Norrie disease (ND) is a rare, X-linked recessive disorder characterized by congenital blindness due to retrolental masses referred to as pseudogliomas.1 Mental retardation and hearing loss are also observed in approximately 25% of the cases.2 ND is genetically homogeneous and is caused by mutations in the NDP gene, which codes for the 133-amino-acid protein norrin.2,3 This protein does not have strong sequence identities with other known proteins, but sequence comparisons and modeling studies have predicted that it has a regular number and spacing around six half-cystine residues and some hydrophobic residues to form cystine knot domains.4 The tertiary structure of norrin has a strong resemblance to transforming growth factor-β.5 Although the exact function of the protein is unknown, it has been suggested to play a role in the development and regulation of the neuroectoderm.4,6

Familial exudative vitreoretinopathy (FEVR) is a genetically heterogeneous vitreoretinal disorder characterized by a deficiency in the vascular development of the peripheral retina leading to various secondary complications, including macular traction, retinal detachment, and retinal folds.7–9 The disease has different degrees of severity, ranging from the absence of visual symptoms to total blindness.10 FEVR has three inheritance patterns: autosomal dominant (adFEVR), autosomal recessive (arFEVR), and X-linked recessive (XFEVR).7,10–12 Ad- and arFEVR are known to result from defects in either the Wnt coreceptors frizzled-4 or low-density lipoprotein receptor-like protein 5 (LRP5) which activates the classic Wnt signaling pathway.13–16 This pathway plays a central role in retinal vascular development.17 XFEVR is also caused by mutant norrin,18 and norrin itself has been recently found to act as a ligand for frizzled-4.17 This would indicate a common molecular mechanism underlying both ND and FEVR.

A large number of mutations in the NDP gene have been described: translocation and inversion mutations, several deletion mutations, and more than 80 point mutations (for review, see Ref. 19).20 Different structural alterations in norrin may lead to different degrees of phenotypic severity.19 Deletion and truncation mutations in NDP cause ND without exception, whereas missense mutations cause either FEVR or ND.20 Mutations that do not disrupt any predicted disulfide bonds are more likely to express milder phenotypes.19,21–22 However, only a few experimental studies have addressed the functional relevance of missense mutations and their correlation with the severity.20 In addition, studies on whether the mutations can predict the severity of the disease have not been reported.24 Certain missense mutations are known to be associated with other retinal diseases such as retinopathy of prematurity and Coats disease.25–27

Thus, an establishment of a genotype-phenotype relationship can offer additional information that can lead to a more accurate prognosis, prenatal diagnosis, and genetic counseling. To the best of our knowledge, there are no reports regarding mutations in NDP in Japanese patients with FEVR. We therefore investigated the cohorts of Japanese patients with FEVR and ND by screening NDP and identified mutations in patients with FEVR and ND. We also observed female carriers exhibiting FEVR characteristics clustered in a single family, and we
examined a profile of the inactivation of the X chromosome in the female carriers by using leukocyte DNA.

**METHODS**

**Participants**

Sixty-two probands (31 familial and 31 simplex) with FEVR, 3 probands with ND, and some of their family members were examined at the Department of Ophthalmology at Fukuoka University or Osaka University. A signed informed consent was obtained from all subjects, and the procedures used conformed to the tenets of the Declaration of Helsinki. In addition, the experimental protocol was approved by the Ethics Review Board of Fukuoka University.

All the patients were Japanese and were born at term of normal weight. The diagnosis of FEVR was based on the presence of at least one of the typical clinical signs, (e.g., peripheral retinal avascularization with abnormal retinal vascular formation, severe retinal exudates, retinal neovascularization, peripheral fibrovascular mass, macular ectopia, retinal folds, retinal detachment, and vitreous hemorrhage). The diagnosis of ND was made by the presence of bilateral retinal detachment or retinal folds with retrolental fibrous tissue, blindness within the first year of life,²¹ presence or absence of mental retardation and hearing loss, and a family history of X-linked recessive inheritance.

**Clinical Examinations**

All patients had a comprehensive ophthalmic examination, which included Snellen visual acuity measurements, intraocular pressure, slit-lamp biomicroscopy, and ophthalmoscopy. Some of the patients were also examined by fluorescein angiography and/or ultrasonography. Bone mineral density (BMD) was evaluated in some adult cases by measuring the area of BMD of the lumbar spine with dual-energy x-ray absorptiometry (model QDR-4500A; Hologic Inc., Waltham, MA). For statistical analyses, the BMD value was converted to an SD score (z-score) appropriate for gender- and age-matched control subjects.

**Laboratory Studies**

DNA samples were extracted from peripheral blood with a DNA extraction kit (QiAamp; Qiagen, Valencia, CA). The primers that bracketed all exons of ND were used as 5'attGGACCTTTCCATTGACA-3' and 5'-gttATGTCGGTTGGAAGAGG-3' for exon 1; 5'-attGGATCCTAGGAGGTTAAGGCC-3' and 5'-gttCTAGGAAGGAATGCTCCTC-3' for exon 2; and 5'-attATGCCCCAGAGTAACCAAC-3' and 5'-gttCATCAGGAAGCCACACACAG-3' for exon 3. Tagged sequences of att or gtt were added at the 5' end for postlabeling purposes.²⁸

Polymerase chain reaction (PCR) and sequencing were performed. Annealing temperatures for PCR were 60°C for exon 1 and 65°C for exons 2 and 3. Other details of the procedures have been described.¹⁴,²⁸

An inactivation of the X chromosome was determined by polymorphic CAG repeats of the human androgen receptor gene that were amplified from peripheral blood DNA.²⁹ The methylation state of the two X chromosomes of each woman was determined by using methylation-sensitive restriction enzyme (HpaII, New England Biolabs, Beverly, MA). Original sequences of the primers were used but tagged with ‘att’ or ‘gtt’ at the 5’ end for fluorescein-labeling of the amplicons.²⁸ The labeled amplicons were separated by electrophoresis (model 310 sequencer, Applied Biosystems [ABI], Foster City, CA). A fragment size analysis was then performed (GeneScan software; ABI). Signals from the two chromosomes of each woman were adjusted before digestion with methylation-sensitive enzyme, and then a relative signal ratio of the affected chromosome was calculated after digestion.

**RESULTS**

**Mutation Analysis**

The DNAs from 62 FEVR and three ND patients were analyzed for mutations in the three exons and flanking exon-intron boundaries of the NDP gene. Five different mutations were identified in four (three simplex and one familial) FEVR patients and two patients with ND (Table 1). The three novel mutations were a T→A transition, c.53 T→A in exon 2, resulting in an isoleucine-to-lysine change at codon 18 (I18K) in family 1; a G→C transition, c.162G→C, in exon 2, resulting in a lysine-to-asparagine change at codon 54 (K54N) in the patients with FEVR in families 2 and 3; and a G→T transversion, c.344G→T, in exon 3, resulting in an arginine-to-leucine change at codon 115 (R115L) in a patient with simplex FEVR in family 4; and a G→A transversion, IVS2-1G→A at the acceptor site of exon 3 in a patient with ND in family 6. One previously reported mutation, a G→C transition, c.290G→C, in exon 3, resulting in a nonconserved arginine-to-proline change at codon 97 (R97P), was found in a patient with ND in family 5. All probands were men, and therefore these changes were interpreted as hemizygous. These sequence changes were not found in 180 unrelated and unaffected individuals (all were females) in the Japanese population.

When multiple sequence alignment of human norrin was performed against available species sequences, codons 118, K54, R97, and R115 were conserved in rats and mice (data not shown). K54N, R97P, and R115L were nonconserved mutations and were located within the cysteine knot domain. I18K was a nonconserved mutation and was located within the N-terminal signal sequence. The precise effect of the splicing mutation was not determined because the expression of norrin is confined to retinal and brain tissues,²,²³ and no such RNA was available from the patients. However, the cystic acceptor splice sites at 17- and 125-bp downstream of the original sites were deduced by a computational analysis (GeneSplicer, http://www.ncbi.nlm.nih.gov/Software/GeneSplicer/), provided by public domain by The University of Maryland Center for Bioinformatics and Computational Biology, College Park, MD. Both alternative splicings would lead to a 17- and 125-bp deletion at the beginning of exon 3, resulting in a frameshift after codons 64 and 100, respectively, followed by an elongated terminal.

Samples from family members were available for all cases, and a cosegregation of these sequence changes was searched for after sequencing. All changes cosegregated as the X-linked recessive form of the disease in all families except for family 3. In this family, the mother and three sisters were heterozygous for K54N. They presented with very different degrees of vascular abnormality, and the mother and one sister had a diagnosis of ND. Because the maternal grandmother and grandfather did not carry K54N, a de novo mutation was considered to be present in the mother.

**Phenotypes of Patients with FEVR**

**I18K.** Patient III-1 in family 1 (Fig. 1) was a 6-month-old boy. He was born at full term of normal weight. When he was examined at 4 months of age, a retrolental mass with total retinal detachment was found in the right eye. The typical features of FEVR, such as peripheral avascularization temporal to the macula and neovascularization of the retinal vessels, were observed in the left eye where laser photoacoagulation had been performed (Fig. 2A). No family member had FEVR, but the mother was heterozygous for the I18K mutation.

**K54N.** Patient III-1 in family 2 (Fig. 1) was a 6-year-old boy who had bilateral retinal folds resembling persistent hyperplastic primary vitreous (PHPV). This condition progressed to retinal detachment and macular traction with temporal avascularization in the left eye (Fig. 2B). He had undergone vitreous surgery in the left eye, but the retina could not be reattached. His corrected visual acuities were 0.5 OD and hand motion OS. No family member had FEVR, but the mother was heterozygous for the K54N mutation.
TABLE 1. Mutations in the NDP Gene and the Associated Clinical Findings

<table>
<thead>
<tr>
<th>Family</th>
<th>DNA Change</th>
<th>ID*/Sex/Age</th>
<th>Allele Status</th>
<th>Visual Acuity (Refration)</th>
<th>Vitreoretinal Findings</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>c.53T→A in exon 2 (118K)</td>
<td>III-1/M/6 mo</td>
<td>Hemizygous</td>
<td>RE: LP (nc) LE: follow a moving object</td>
<td>RE: RLF, total RD, flat anterior chamber LE: Avascular retina, retinal neovascularization</td>
<td>PHC (LE)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II-1/F/27 y</td>
<td>Heterozygous</td>
<td>RE: 1.0 LE: 1.0</td>
<td>BE: Normal</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>c.162G→C in exon 2 (K54N)</td>
<td>III-1/M/6y</td>
<td>Hemizygous</td>
<td>RE: 0.5 (+0.5 D) LE: HM (nc)</td>
<td>BE: Avascular retina, fibrovascular proliferation, dragged macula, persistent hyaloids remnant</td>
<td>Lx Vx (LE)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II-1/F/39 y</td>
<td>Heterozygous</td>
<td>RE: 1.5 (nc) LE: 1.5 (nc)</td>
<td>BE: Normal</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>c.162G→C in exon 2 (K54N)</td>
<td>III-3/M/5 y</td>
<td>Hemizygous</td>
<td>RE: 0.3 (~3.5 D) LE: 1.0 (~8.0 D)</td>
<td>BE: Avascular retina, fibrovascular proliferation, persistent hyaloids remnant</td>
<td>PHC, encircling (BE)</td>
</tr>
<tr>
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<td></td>
<td>II-1/F/42 y</td>
<td>Heterozygous</td>
<td>RE: 1.5 (~4.0 D) LE: 1.5 (~4.0 D)</td>
<td>BE: Avascular retina, tortuosity, vitreous degeneration, retinal holes</td>
<td>PHC (BE)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II-4/F/13 y</td>
<td>Heterozygous</td>
<td>RE: 1.5 (nc) LE: 1.5 (nc)</td>
<td>BE: Avascular retina, tortuosity, vitreous degeneration, retinal holes</td>
<td>PHC (BE)</td>
</tr>
<tr>
<td>4</td>
<td>c.344G→T in exon 3 (R97P)</td>
<td>III-1/M/21 y</td>
<td>Hemizygous</td>
<td>RE: 0.5 (~16.0 D) LE: 0.02 (~18.0 D)</td>
<td>BE: Avascular retina, tortuosity, dragged macula, fibrous tissue temporal</td>
<td>BMD −1.7; AL 30 mm, PHC, encircling (BE), Vx (LE)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II-1/F/44 y</td>
<td>Heterozygous</td>
<td>RE: 1.5 (nc) LE: 1.5 (nc)</td>
<td>BE: Normal</td>
<td>BMD −0.7</td>
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<tr>
<td>5</td>
<td>c.290G→C in exon 3 (R97P)</td>
<td>III-1/M/1 y</td>
<td>Hemizygous</td>
<td>RE: LP (nc) LE: LP (nc)</td>
<td>BE: RLF, total RD, flat anterior chamber, corneal opacity</td>
<td>Lx (BE)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II-1/F/25 y</td>
<td>Heterozygous</td>
<td>RE: 1.2 (nc) LE: 1.2 (nc)</td>
<td>BE: Normal</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>IVS2-1G→A (unknown)</td>
<td>III-1/M/1 y</td>
<td>Hemizygous</td>
<td>RE: LP (nc) LE: LP (nc)</td>
<td>BE: RLF, total RD, flat anterior chamber, corneal opacity</td>
<td>Lx (BE)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II-1/F/27 y</td>
<td>Heterozygous</td>
<td>RE: 2.0 (~1.25 D) LE: 2.0 (nc)</td>
<td>BE: Normal</td>
<td>BMD 0.4</td>
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</tbody>
</table>

RE, right eye; LE, left eye; BE, both eyes; BMD, z-score of bone mineral density; RLF, retrolental fibroplasia; RD, retinal detachment; PHC, photocoagulation; Vx, vitrectomy; Lx, lensectomy; AL, axial length.

* Identifications refer to Figure 1B.

Patient III-3 of family 3 was a 5-year-old boy who had FEVR with retinal vascular tortuosity and avascularization of the peripheral temporal retina. The changes in the posterior vitreous and retina resembled those of the proband of family 2 (Fig. 2C). Prophylactic retinal photocoagulation and an encircling buckle were placed on both eyes. The mother and one sister had peripheral retinal vascular abnormalities compatible with FEVR (Figs. 3A, 3B), and his other two sisters had a milder form of vascular tortuosity (Figs. 3C, 3D).

R115L. Patient III-1 in family 4 was a 21-year-old man who had a fibrovascular mass adjacent to the terminals of the retinal avascularization bilaterally. At his first visit to our hospital at age 10 years, his best corrected visual acuity was 1.0 with high myopia in both eyes. Because of the severe vitreoretinal traction from the fibrovascular mass temporal to the macula, prophylactic retinal surgery with scleral buckling and laser photocoagulation was performed on both eyes at the age of 16 years. Later, a total retinal detachment developed in the left eye (Fig. 2D). Despite repeated surgeries, the retina remained detached, and the silicone oil remained in the eye. The ocular examination of the mother and sibling revealed no retinal changes.

Phenotype of Patients with ND

R97P. Patient III-1 in family 5 was a 1-year-old boy. He was born at full term of normal weight. Bilateral leukocoria was noted at 2 months of age. An examination showed a flat anterior chamber and a retrolental mass with total retinal detachment in both eyes. ND was strongly suspected (Fig. 2E). Lensectomy was performed to create and preserve the anterior chamber. Vision was restricted to light perception in both eyes, and he could not follow a moving finger with either eye. He did not have developmental or mental retardation, and his hearing was normal. Two maternal uncles have been blind since they were neonates, and one of them is mentally retarded.

IVS2-1G→A. Patient III-1 in family 6 was a 1-year-old boy. He was referred to our hospital at 5 months of age, and ND was suspected because of retrolental fibrous tissue and retinal de-
attachment with a flat anterior chamber in both eyes. The buphthalmia progressed in the left eye (Fig. 2F), and lensectomy was performed to preserve the anterior chamber in both eyes. Light sensation was preserved, but he could not follow a moving object. Although both parents did not have any ocular abnormalities, his maternal nephew had a diagnosis of FEVR because of bilateral retinal folds at 1 year of age.

X-Inactivation Assay

Three women in the FEVR family with the K54N mutation were analyzed for X-inactivation. The relative signal ratio of both chromosomes of the affected mother in family 3 was not obviously skewed at 37%:63%. The two sisters carrying heterozygous K54N mutation (III-1: unaffected and III-4: affected) were homozygous. We tried other polymorphic markers for a PCR-based X-inactivation assay.30 Despite the variable conditions for amplification tested, no product was obtained, and the status of X inactivation could not be determined. Two other unaffected female carriers in families 2 and 4 were also analyzed. As these cases were simplex, there was no possibility of determining which of the X chromosomes might be carrying the mutations. The relative signal ratios were 16%:84% in family 2 and 27%:73% in family 4.

In conclusion, no significant difference of skewing was observed between affected and unaffected women.

DISCUSSION

We identified five mutations in the NDP gene in six families. One was a novel splicing mutation, and the other four were missense changes. Three of these missense mutations were novel, and they were located within the cystine knot domain or N-terminal signal sequence. All the mutations segregated with the disease and were not found in normal individuals from the Japanese population. These findings suggest that these mutations are pathogenic. Our data indicate that NDP mutations cause 6% (4/62) of FEVR in the Japanese population.

Two patients with novel mutations, K54N and R115L, had the typical findings of FEVR. Of note, two unrelated probands with the K54N mutation had a failure in the regression of the hyaloid vasculature. This finding is consistent with those in an animal model with a NDP mutation.31 Thus far, the FEVR-causing mutations are confined to residues 41-58 and 121-126, as well as a residue 110.32–36 From the predicted secondary structure, these regions are within the main component of the cystine knot domain: first beta strand with subsequent loop for residues 41-58, and the fourth beta strand for residues 121-126.5 The cysteine residue 110 is directly involved in the dimer-forming disulfide bond.5 Mutations in these regions may act by disrupting the folding of the protein or by directly interfering with norrin-receptor interactions.5,37 K54N and R115L matched with such a predisposition, and the mutational spectrum was expanded. Although the functional relevance of each mutation should be explored in more detail, a predisposition in a particular region may be involved in the pathogenesis of FEVR.

Contrary to K54N and R115L, the novel mutation I18K had a different feature. This mutation was located within the N-terminal signal sequence. Signal sequences play a role in the
insertion of secretory proteins into the membrane. The characteristic feature of signal sequences is a hydrophobic core region that is flanked on its C-terminal side by a polar region with a net positive charge. A substitution of the nonpolar amino acid isoleucine for the basic amino acid lysine would alter the physicochemical properties of the putative signal sequence significantly and may prevent an efficient extracellular transport of norrin. So far, only the ND-like phenotype is known to be associated with mutations within the putative signal sequence, whereas the FEVR-phenotype is not.

Clinically, the proband with I18K had a diagnosis of FEVR because of the typical retinal avascularization in the left eye. However, the fellow eye exhibited a different severity with an infantile total retinal detachment. It is possible that mutations in the signal sequence cause FEVR as well as ND.

The recurring mutation, R97P, exhibited the phenotype of ND, and this mutation was found in a family with a history of infantile blindness and mental retardation which is consistent with the previous report of ND. Codon 97 is adjacent to one of the half-cystine residues (C96) and participates in forming the disulfide bonds. Thus, R97P may interfere with the disulfide bonding. The splicing mutation caused a more variable phenotype. The proband presented with the typical features of ND, whereas a maternal nephew had a diagnosis of FEVR.

Despite the fact that ND and FEVR are viewed as distinct diseases, the phenotypic spectrum of each is diverse, and they share several common features, such as microphthalmia, cataracts, retinal folds, and PHPV-like findings. Therefore, Allen et al. recently advocated that NDP mutation-associated diseases should be called ND because the term FEVR does not adequately convey the potential spectrum of the severity of the disease. It has been shown that the K58N mutation, which is very similar to the K54N mutation found in this study, can lead to either FEVR or ND. Intrafamilial variability of the ocular phenotype has also been described, and the involvement of additional (epigenetic or genetic) factors was suggested. These findings, as well as the splicing mutation in this study, imply the limited prognostic value of genetic testing. Clinicians should to be aware that the phenotypic variability in ND/FEVR often renders the initial clinical diagnosis inappropriate, and genetic counseling can be compromised.

Although it is rare, both ND and XFEVR can occur in women. Our report is the first to demonstrate that the affected female carriers exhibiting FEVR were clustered in a single family. Indeed, the disease in this family was initially regarded as being dominantly inherited. Compared with the retinal findings of the male proband, the phenotypes of these carriers were much milder, ranging from subnormal levels with minimum tortuosity of the retinal vessels to a typical form of peripheral retinal degeneration.

In X-linked recessive disorders, heterozygous women may be affected because they have the X chromosome that does not carry the mutation as the inactive one in most of their cells. This process is called skewing of X inactivation.

**FIGURE 2.** Fundus photographs and fluorescein angiograms of patients. (A) Fluorescein angiogram of the left eye of the proband of family 1 (III-1), showing neovascularization and a severe retinal avascularization typical of FEVR. (B) Fundus image of the right eye of the proband of family 2 (III-1), showing a dragged macula with persistent hyaloid remnant. (C) Right eye of the proband of family 3 (III-3) showing a finding similar to that shown in (B). (D) Fundus image of the left eye of the proband of family 4 (III-1) showing a total retinal detachment. (E) External photograph of the right eye of the proband of family 5 (III-1) showing retrolental fibroplasia. (F) External photograph of the left eye of the proband of family 6 (III-1) showing a flat anterior chamber with corneal opacity progressing to buphthalmia.
mutations in affected female carriers have been attributed to this mechanism.46–49 We therefore analyzed whether the X-inactivation of the affected women was skewed in favor of the phenotype. By comparing other unaffected female carriers, X-inactivation in the affected mother was not apparently skewed while those in the affected sisters could not be determined. Shastry et al.49 tested a female patient with ND carrying the NDP mutation and found a discordance of X-inactivation in favor of the affected chromosome, which was attributed to a presumably different expression pattern in the leukocytes from that in retinal tissue. Our results may show that the X-inactivation assay of leukocytes does not provide enough information to predict the phenotype in female carriers. The clustering of the affected female patients within a family was not easily attributable to independent events of skewed X-inactivation.52 Further investigations are necessary to understand why FEVR and ND developed in the affected female patients.

In summary, we identified five mutations in the NDP gene that resulted in FEVR or ND. At present, results of genetic testing should be used with caution when offering a prognosis or counseling. Further studies to identify the phenotype–genotype correlations as well as exploring the functional relevance of each mutation will help us to understand the mechanism of these disorders and the clinical variability.

Acknowledgments

The authors thank the patients and their families.

References


