Genotype-phenotype variations in five Spanish families with Norrie disease or X-linked FEVR

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Purpose: Norrie disease (OMIM 310600) is a rare X-linked disorder characterized by congenital blindness in males. Approximately 40 to 50% of the cases develop deafness and mental retardation. X-linked familial exudative vitreoretinopathy (XL-FEVR) is a hereditary ocular disorder characterized by a failure of peripheral retinal vascularization. Both X-linked disorders are due to mutations in the NDP gene, which encodes a 133 amino acid protein called Norrin, but autosomal recessive (AR) and autosomal dominant (AD) forms of FEVR have also been described. In this study, we report the molecular findings and the related phenotype in five Spanish families affected with Norrie disease or XL-FEVR due to mutations of the NDP gene.

Methods: The study was conducted in 45 subjects from five Spanish families. These families were clinically diagnosed with Norrie disease or similar conditions. The three exons of the NDP gene were analyzed by automatic DNA sequencing. Haplotype analyses were also performed.

Results: Two new nonsense mutations, apart from other mutations previously described in the NDP gene, were found in those patients affected with ND or X-linked FEVR.

Conclusions: An important genotype-phenotype variation was found in relation to the different mutations of the NDP gene. In fact, the same mutation may be responsible for different phenotypes. We speculate that there might be other molecular factors that interact in the retina with Norrin, which contribute to the resultant phenotypes.

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Norrie Disease (ND; OMIM 310600) is an X-linked recessive disorder characterized by a range of fibrous and vascular changes of the retina at birth. There is a clinical progression throughout childhood or adolescence that causes different degrees of visual impairment. The most severe retinal findings are grayish, yellow fibrovascular masses, referred to as “pseudogliomas” since they resemble tumors, which occur in the first few months of life resulting in total blindness. These masses cause an increasing fibrotic reaction and vascular changes in the retina, often resulting in vitreous hemorrhage. Fifty percent of the affected males also have mental retardation, and at least 40% of them develop a progressive sensorineural hearing loss that starts in early childhood [1,2].

Less severe findings are found in the X-linked familial exudative vitreoretinopathy (XL-FEVR). These include a fibrotic white stalk from the optic disk to the lens, persistent hyperplastic primary vitreous (PHPV), and peripheral retinal vascular anomalies. Affected males do not present sensorineural hearing loss nor mental retardation [3]. X-linked forms of FEVR have been associated with NDP mutations, but autosomal recessive (AR) and autosomal dominant (AD) forms have also been described. Indeed, there are two autosomal dominant genes for FEVR, Fzd4 and LRP5 [4].

The molecular basis of some cases of apparent retinopathy of prematurity (ROP) and Coats disease can be due to mutations of the NDP gene [5,6].

The NDP locus maps to chromosome Xp11.4, spans 28 kb and comprises three exons. However, only exons 2 and 3 of the NDP gene are translated. The 5' UTR region and the promoter have been characterized. Exon 1 has a CT repeat region in it, which may be involved in gene expression [7]. Exon 2 contains the first 58 codons of the open reading frame. Exon 3, which is the largest one, contains residues 59-133 of the open reading frame and a 917 bp untranslated 3' region. The NDP gene encodes a 133 amino acid protein called Norrin whose cysteine residues and their disulfide bonds play an important structural and functional role. Norrin is a member of the cystine knot growth factor family, and it may have a role in neuroectodermal cell-cell interaction, which is critical for retinal, central nervous system, and cochlear development. Up to now, about 70 mutations causing ND, 10 mutations producing XL-FEVR [8-12] and 5 changes said to be related to the ROP phenotype [6,13,14] have been described in NDP.

In about 85% of patients the clinical diagnosis can be confirmed through the detection of unique point mutations in the coding region of the NDP gene. Intragenic and submicroscopic deletions, including NDP and adjacent regions, have been identified in about 15% of patients [9,15,16]. In the study presented here, genotype-phenotype variations were identified through clinical and molecular study in patients affected with ND or XL-FEVR.
Figure 1. Pedigree of Family 1. Non-carrier woman (I:2) and her two sons, one of which is affected with ND (II:2). Haplotype analysis showed that each son had inherited a different X-chromosome from his mother.

Figure 2. Partial sequences from the mother and the affected son of Family 1. Top: The mother’s normal electropherogram. Bottom: The affected son’s sequence, presenting a “de novo” hemizygotic deletion of 5 nucleotides (TGTCG), which generated a premature stop codon and a truncated protein (V89fsX101 [654_658 del TGTCG]).

Figure 3. Pedigree of Family 2. In this family, the affected male (II:2) presented with microphthalmia, hearing loss, and low intelligence quotient.

Figure 4. Electropherogram showing normal and mutated sequences of Family 2. There is a hemizygotic C->A change at nucleotide 776. Tyrosine was substituted for a stop codon (Y120X [776 C->A]).
METHODS

The study was carried out in 45 Spanish individuals from five different families diagnosed of Norrie disease or similar conditions. The Hospital Ethics Committee approved the study and all patients participated under informed consent (following the Principles of the Declaration of Helsinki). The ophthalmic examination was performed and the diagnosis was established according to Warburg’s criteria [2].

Peripheral blood samples were taken and DNA was extracted using a standardized protocol. The 3 exons of the NDP gene, including intron-exon junctions were amplified by PCR with primers previously described [17]. These fragments were electrophoresed in a 3% agarose gel and purified using a DNA extraction kit (QIAquick Gel Extraction Kit, Qiagen, Hilden, Germany). The sequencing reaction was performed using the 4 dye terminator cycle sequencing ready reaction kit (DNA Sequencing Kit, Applied Biosystems, Foster City, CA). The samples were sequenced and analyzed in an automatic genetic analyzer (ABI PRISM 3100 Genetic Analyzer). Haplotype analyses were performed using two [CA]n microsatellite markers flanking the NDP gene (Cen-DXS8080-NDP-MAOB-Tel), and these fragments were analyzed in the same instrument (ABI PRISM 3100 Genetic Analyzer). For several patients, five additional microsatellite markers were amplified (Cen-DXS1003-NDP-DXS7-DXS8012-DXS1068-DXS8090-TEL) in order to determine whether they share a common haplotype.

Figure 5. Pedigree of Family 3. Family 3 has one affected male with XL-FEVR (I:1) and his three daughters (II:1, II:3, II:5). Haplotype analysis confirmed that these three women were obligate carriers of the disease. One carrier of the disease (III:3) and two non-carriers (III:1, III:2) were identified in the third generation of this family.

Figure 6. Normal and mutated sequences from Family 3. Sequence analysis demonstrated that the proband (I:1) carried a G->A change at nucleotide 778, which generated an arginine to glutamine substitution at codon 121 (R121Q [778 G->A]).
RESULTS

Three individuals were studied in Family 1: the affected patient, his brother, and their mother. The affected child presented unilateral microphthalmia, cataracts, sensorineural hearing loss, and mental retardation. Haplotype analyses showed that each son had inherited a different X-chromosome from his mother (Figure 1). The sequencing analysis revealed that the affected son had a "de novo" hemizygotic mutation consisting of a 5 nucleotide deletion in exon 3, which generated a premature stop codon and a truncated protein (V89fsX101 [654_658 del TGTCG]; Figure 2).

In Family 2, the proband was a male presenting microphthalmia, cataracts, phthisis bulbi, corneal opacification, hearing loss and low IQ. After sequence analysis, we could ascertain that he carried a hemizygotic C->A change at nucleotide 776 in exon 3, which also generated a stop codon (Y120X [776 C->A]) and a truncated protein (Figure 3, Figure 4).

In Family 3, one affected man with XL-FEVR (congenital blindness, phthisis bulbi) and his three daughters were studied (Figure 5). Sequence analyses demonstrated that he carried a G->A change at nucleotide 778 in exon 3. This generated an amino acid change at codon 121 where arginine was replaced with glutamine (R121Q [778 G->A]; Figure 6) [18]. Haplotype analyses confirmed that these three women (II:1, II:3, II:5) were obligate carriers of the disease. In the third generation of this family, one carrier of the disease (III:3) and two non-carriers (III:1, III:2) were additionally identified.

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Figure 7. Simplified pedigree and haplotype analysis for Family 4. An affected man (IV:1) with a diagnosis of XL-FEVR and his two affected nephews were studied. One nephew presented with PHPV (V:5) and the other boy was diagnosed with XL-FEVR (V:6). Affected chromosomes (in black) show the haplotypes determined by microsatellite markers DXS8080 (183 bp) and MAOB (174 bp).

Figure 8. Normal and mutated sequences from Family 4. The mutated sequence (right) shows a C->T change at nucleotide 529, where arginine is replaced by cysteine at codon 38 (R38C [529 C->T]). This mutation occurred at a CG dinucleotide.
Three members of Family 4 were studied: A male was diagnosed with XL-FEVR suffering only from ophthalmic signs without sensorineural hearing loss or mental retardation. One of his nephews presented with PHPV who was blind since he was 5-year-old. Another nephew was diagnosed with XL-FEVR when he was 2-year-old, with conserved vision in his right eye through the age of 14 (Figure 7). By direct sequencing, in all three cases a C->T change at nucleotide 529 in exon 2 of the NDP gene was identified, where arginine was replaced by cysteine at codon 38 (R38C [529 C->T]; Figure 8). This mutation occurred at a CG dinucleotide, which is considered a mutation hot spot of the genome [19,20].

Family 5 is the largest one (27 members) that was studied in this series, with affected individuals presenting X-linked exudative vitreoretinopathy without deafness or mental retardation. An arginine to cysteine substitution at codon 38 was found in all affected members. However, the same R38C mutation was identified in a 60-year-old man (III:1) who had not developed any symptoms. Another blood sample was taken from this subject in order to confirm this mutation and the same result was obtained. Once the haplotype analyses were performed, it was found that Families 4 and 5 share the same haplotype, so identity by descent cannot be ruled out (Figure 9). In order to confirm this fact, five additional microsatellite markers, spanning over 10 cM, were amplified in affected members from both families. This molecular study showed that the common piece of DNA, which was inherited in both families, comprises 10.3 cM between the DXS8090 and DXS8080 markers (Figure 10).

**DISCUSSION**

In this molecular study, 4 different mutations of the NDP gene associated with two different phenotypes, Norrie disease and X-linked familial exudative vitreoretinopathy were found. ND is an X-linked congenital retinal dysplasia that, in most cases, presents with blindness at birth. XL-FEVR is a developmental disorder characterized by incomplete vascularization of the peripheral retina. X-linked forms of FEVR have been associated with NDP mutations, however autosomal recessive (AR) and autosomal dominant (AD) forms have also been described [21]. Indeed, two AD-FEVR loci have been identified by positional cloning and correspond to the frizzled-4 (Fzd4) gene [22] and LRP5 [4], which both map to 11q13-23. Recently, Nathans et al. [23] have observed that both Norrin and Fzd4 act as a high affinity ligand-receptor pair. Norrin induces Fzd4 and LRP dependent activation of the classical Wnt pathway. This signalling system plays an essential role in the vascular development in the retina, inner ear, and cerebellum. This central conclusion was based on the similarity in vascular phenotypes caused by Norrin and Fzd4 mutations in both human and mice.

With this molecular screening of the NDP gene, two novel nonsense mutations: “de novo” V89fsX101 and Y120X were identified. These mutations produce a stop codon so a trun-
cated protein is generated. This truncated protein is associated with a more severe phenotype where ocular symptoms, sensorineural hearing loss, and mental retardation of different genotypes.

Figure 10. Simplified pedigrees for Families 4 and 5. Simplified pedigrees from Family 4 (top) and Family 5 (bottom), respectively. Haplotype analysis with more microsatellite markers (Cen-DXS1003-NDP-DXS7-DXS8012-DXS1068-DXS8090-Tel) confirmed that Families 4 and 5 share the same haplotype (drawn in black). Individuals with no marker numbers were not available. There are markers in the families that deviate from segregation (Family 4, individual IV:1, marker DXS8090; Families 4, and 5, marker DXS1003). This analysis showed the inherited common piece of DNA (black haplotype) and also that proximal and distal markers were not linked.
degrees are found. Indeed, as described in the literature, nonsense mutations have only been associated with the ND phenotype [24,25] although there are several examples where missense mutations are also responsible for ND (HGMD).

The missense mutations R121Q and R38C found in the patients reported here, only cause loss of vision and are associated with a milder form of the disease. There are a few examples of XL-FEVR with reported mutations in NDG; however, all of them are missense mutations [8-12]. The R121Q mutation found in patients from Family 3 was previously described in other families [8], including another Spanish family [18]. Both families share haplotypes, suggesting that our patients may belong to another branch of the same family (described by Fuentes et al. [18]). The arginine to cysteine substitution was found in two of the Spanish families analyzed (Families 4 and 5), which also share haplotypes, so identity by descent can be suspected. This mutation has been previously described in a French family by Royer and coworkers [26], where they found this alteration only in an affected child but not in a panel of 75 Caucasian control chromosomes. This finding together with our two families, suggests the possibility of a founder effect in the Southern European population. Cosegregation of the mutation within the families and its absence in control population [26], supports that this change (R38C) is a pathogenic mutation rather than a polymorphism.

All mutations identified in our study lie within the cystine knot domain, which spans from codon 32 to codon 133 and is thought to have a major role in neurological interactions. From all of the mutations affecting the NDP gene, most of them have been described at this domain [21], such as R121W (777 C>T) and R121G (777 C>G) associated with XL-FEVR and ND, respectively [8,27]. Since a few mutations have been described at this particular codon, it is tempting to assume that the Arginine at position 121 has an important role. The severity of phenotypes associated with mutations at the 121 codon may depend on the type of substitutions.

There is variable expressivity among patients from both families with the R38C mutation. In Family 4 (R38C), the boy affected with PHPV became blind at the age of 5 while his brother presented XL-FEVR and retains good vision in his right eye. This is a clear example of the same mutation in the same family producing two different phenotypes. This fact has been reported for many other hereditary diseases, including ophthalmic disorders [28-30]. Furthermore, this mutation produces an ND phenotype in the French family [26]. However, a member of Family 5 presenting with the arginine to cysteine substitution has not developed any symptoms of XL-FEVR. In this patient, we speculate incomplete penetrance for XL-FEVR, not described before in the literature. Expression studies of Norrin could help to test this hypothesis.

Numerous examples of variability of expression in affected males related to cognitive function and the onset or severity of hearing loss suggest that modifying genes or other factors have a significant though undefined role in the modulation of the disease phenotype [18]. Now that it is known that the Norrin-Fzd4 pair activates the classical Wnt pathway [23], we suspect that there are more aggressive mutations (such as nonsense mutations) that considerably alter the protein structure (even more at the cystine knot domain) and completely disrupt this acceptor-receptor binding and the Wnt pathway, resulting in a more serious defect in retinal vascularization.

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