Electronic letter

De novo mutations in the 5' regulatory region of the Norrie disease gene in retinopathy of prematurity

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EDITOR—Retinopathy of prematurity (ROP) is the commonest cause of potentially preventable blindness in infants.[1 2] In developed countries this may reflect the increasing survival of ever more premature babies.[3] In emerging countries this may reflect the recent provision of neonatal care.[4 5] There is, however, evidence that a genetic component may be involved in the development of severe ROP. For example, the incidence of severe ROP is higher in white infants than in Afro-Caribbean infants in both the United Kingdom[6] and the United States.[7] There is also great variation in the incidence of severe ROP in different developed countries. In Israel, the incidence of threshold ROP (stage 3 plus or worse) in infants weighing >1000 g is 26%, but none progressed to severe visual loss.[8] A similar study in Denmark, however, reported that of 170 infants weighing <1500 g, threshold ROP developed in 26.5% and that 40% of these infants went on to stage 5 ROP despite intervention.[9]

Norrie disease is an X linked recessive disorder, which is characterised by bilateral retinal dysplasia with retinal detachment, resulting in congenital blindness.[10] In addition, approximately one third of patients develop sensorineural hearing loss and a variable spectrum of mental retardation.[11] The causative gene for Norrie disease is the *NDP* gene.[12 13] Recently, in a cohort of American patients, mutations in exon 3 of the Norrie's disease protein gene (*NDP*) have been associated with advanced ROP.[14] We undertook a study to determine whether *NDP* mutation may be a factor influencing disease severity in a cohort of patients undergoing investigation in the UK. Such an association would contribute to developing a better understanding of ROP pathophysiology and would provide paediatric ophthalmologists with an adjunctive diagnostic test for predicting likely progression of disease.

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Patients and methods

Local ethical committee approval for clinical and molecular genetic study was obtained before investigations were undertaken. Thirty-one premature babies who received treatment for ROP stage 3 plus or worse were studied (table 1). Control DNA was obtained from 26 premature babies (40 X chromosomes) who were screened for ROP, but who either did not develop ROP (16 babies) or whose ROP did not reach threshold and hence need treatment (10 babies).

Table 1 Clinical data of infants treated for ROP

Number of patients	31
Male	20
Female	11
Mean weight (range) (g)	816 (637–1560)
Gestational age (weeks)	24–30
Stage of ROP (number of eyes)	
Stage 3 threshold	40
Stage 4	6
Stage 5	16

MUTATION ANALYSIS

DNA was extracted from blood specimens of approximately 200 µl in EDTA using the Nucleon Genomic DNA extraction kit (Nucleon Biosciences). *NDP* covers 28 kb with three exons of 201, 380, and 1257 bp, respectively. Exon 1 is untranslated and is located in the 5' regulatory region of the gene.[15] The open reading frame begins part of the way through exon 2 and encodes a protein of 133 amino acids.[12 13 16] The three exons of *NDP* were analysed by polymerase chain reaction (PCR) amplification of genomic DNA, using intronic primer sequences that amplified the three exons of *NDP*.[17] PCR amplification was performed with 100 ng genomic DNA, 10 pmol of each primer, 1.5 mol/l betaine (Sigma, to reduce secondary structure), 0.5 nmol dNTP, and 0.5 U *Taq* polymerase (Biotaq) in a NH_4^+ buffer (Bioline) containing 1.5 mmol/l $MgCl_2$. Cycling parameters were 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Single band PCR products were confirmed by agarose gel electrophoresis and excess primers were removed through a Centricon tube (Millipore). Direct sequencing was undertaken using big dye terminators (PE Biosystems) and analysed on an ABI 373XL automated DNA sequencer. In DNA samples where sequence variations were detected, 85 healthy subjects (122 X chromosomes) were screened from the general population. Additionally, the sequence variations were verified with restriction enzyme

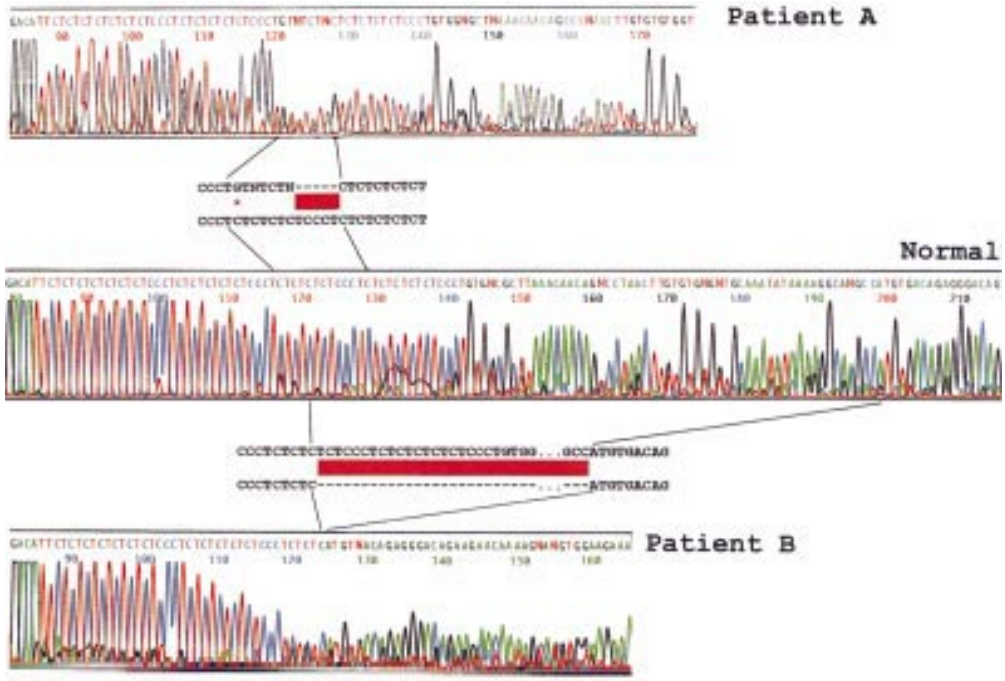

Figure 1 Variation in CT repeat element in exon 1 of NDP associated with severe ROP. Patient A, nucleotide sequence showing a 5 bp deletion (nucleotides +33 to +37) and a C to G transversion at nucleotide +26. Normal, DNA sequence of NDP from father of patient A. Patient B, nucleotide sequence from severe ROP twin with a 71 bp deletion (nucleotides +31 to +102). Red bars indicate deleted regions. Nucleotide numbering based on Allen et al.*[17]

digest of exon 1 PCR products, visualised by agarose gel electrophoresis.

X INACTIVATION STATUS

The X chromosome inactivation pattern was determined in peripheral blood lymphocytes by analysis of a polymorphic trinucleotide repeat in the first exon of the androgen receptor gene,[17] which is very close to two methylation sensitive *Hpa*II sites. DNA (0.2 µg) samples were digested overnight with the methylation sensitive enzyme *Hpa*II. The digested sample and a separate undigested DNA sample from each patient were amplified with primers surrounding the CAG repeat including the *Hpa*II sites. The forward primer was end labelled with $^{32}\gamma$-dATP using T4 polynucleotide kinase (Gibco BRL). PCR products were separated on standard 6% denaturing polyacrylamide sequencing gels containing 8 mol/l urea and visualised by autoradiography.

Results

Two babies with very severe ROP (stage 4/5 in both eyes) had deletions (one 5 bp and the other 71 bp) of the CT repeat sequence in exon 1 of *NDP* (fig 1). Patient A was female and had been born at 26 weeks' gestation, weighed 780 g, and had required ventilation for five weeks. Patient A had undergone bilateral retinal laser therapy for stage 3 threshold ROP at 34 weeks. The patient was followed up at three monthly intervals. At 17 months of age, bilateral cataract was documented. A total, fixed, retinal detachment (stage 5) was identified in the left eye. An inferior, subtotal, tractional, retinal detachment was documented for the right eye and the patient underwent right vitreolensectomy resulting in flattening of the retina and retention of sufficient vision to follow moving objects. No systemic disease was documented. Analysis of a DNA sample from patient A showed a 5 bp deletion (nucleotides +33 to +37) and a C to G transversion at nucleotide +26 (fig 1). We determined that both alterations were on the same *NDP* allele by sequencing five independent clones of the exon 1 PCR product (data not shown). The deletion resulted in the creation of an *Acc*I restriction site, where the mutant allele was cut into two fragments of 216 bp and 136 bp compared to the wild type 352 bp allele (fig 2). Parental alleles were not cut by this enzyme, indicating that the mutation had arisen de novo.

Patient B was a male, non-identical twin who had also been born at 26 weeks' gestation, weighed 800 g, and had required ventilation for five weeks. At 6 months of age, a total, fixed, retinal detachment (ROP stage 5) with microphthalmos was found in the right eye. Peripheral vascular attenuation (ROP stage 3) was seen in the left eye. Bilateral, high myopia (−5.0 DS right, −6.5 DS left) and right divergent squint was also found. The patient was prescribed spectacles, and after squint surgery at 5 years of age had a visual acuity of 6/9 left, no light perception right. Patient B had also required bilateral inguinal hernia repair and cardiac surgery for a septal defect. DNA analysis in patient B showed a 71 bp deletion from nucleotides +31 to +102, inclusive (fig 1). This deletion was easily detectable by agarose gel electrophoresis as a 281 bp allele compared to the 352 bp wild type allele (fig 2). However, it also resulted in the loss of an *Sph*I restriction site that was present in the parental alleles, indicating a de novo mutation.

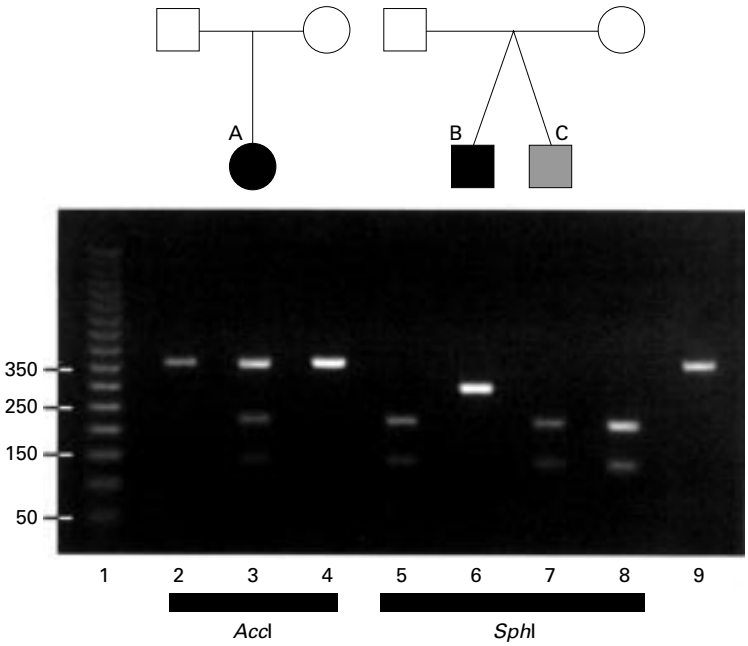

Figure 2 Restriction digests confirming de novo deletions. Solid symbols indicate severe ROP patients, grey symbol indicates mild ROP patient, open symbols indicate unaffected subjects. Patients B and C were dizygotic twins. DNA in each lane of the gel is from subjects depicted above and was either digested with AccI or SphI. Extra bands present in patient A (lane 3) are absent from parental DNA (lanes 2 and 4), indicating gain of AccI site. Patient B (lane 6) had a 281 bp band compared to the parental 352 bp band (lane 9) indicating a deletion, which did not cut with SphI, unlike the parents (lanes 5 and 8) and twin brother (patient C, lane 7). Lane 1, 50 bp DNA ladder (Promega).

Patient C, the twin brother of patient B, at 6 months of age, was also myopic (−6.0 DS right, −5.5 DS left) with bilateral retinal signs of ROP stage 2. Visual acuity was good and with appropriate myopic correction was recorded as 6/6 right and left at 5 years of age. This sib had also had inguinal hernia and cardiac septal defects requiring surgery. DNA from this mildly affected sib was sequenced and did not show the exon 1 deletion found in his brother. Additionally, restriction analysis indicated the presence of a *Sph*I site, confirming the parental genotype.

DNA of the parents of the children investigated was also examined and no exon 1 deletions were found. The two exon 1 deletions we identified were not found in the control DNA from 26 premature babies (40 X chromosomes) or in 85 normal subjects (122 chromosomes). No sequence abnormality (mutation or polymorphism) was found in exons 2 or 3 or in these exon splice sites in any of the patients studied.

The X chromosome inactivation pattern was determined in the female patient's family and from 14 female control premature babies by evaluating the pattern of methylation of the androgen receptor (AR) gene. The nearby *Hpa*II sites are methylated on the inactive X chromosome, but not on the active X chromosome. Thus, after digestion with *Hpa*II, a PCR product is only derived from the inactive X chromosome. From the father's DNA, the AR allele was not amplified after *Hpa*II digestion, because it is unmethylated and cleaved (fig 3, lane 2). Both the patient and her mother were heterozygous at the AR locus, as shown by the presence of two different sized bands after PCR amplification (fig 3, lanes 3 and 5, respectively). In the patient, the lower band represents the paternally derived allele, whereas the upper band is inherited from her mother. We observed extremely skewed X inactivation in the patient as only the paternal allele was amplified (fig 3, lane 4). Random inactivation would have resulted in both bands being amplified after *Hpa*II digestion. Presumably, the exon 1 deletion and base change we identified arose de novo on the active maternal X chromosome. Unexpectedly, her healthy mother also had skewed inactivation, but of the X chromosome that she did not pass on to her daughter. This is not a result of cytogenetic abnormalities (for example, Turner syndrome) because both the patient and her mother had a normal karyotype (data not shown). In the 14 female control premature babies, we only observed a random pattern of X inactivation.

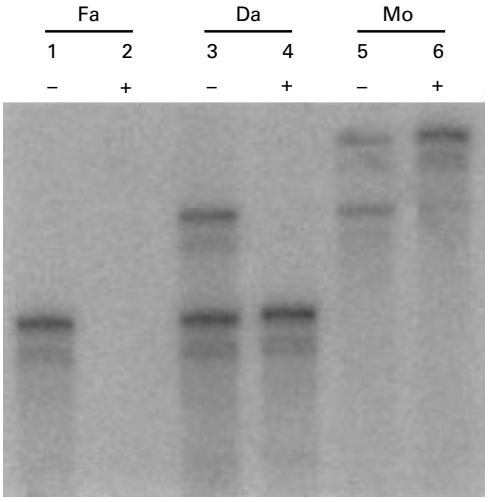

Figure 3 X inactivation analysis in female ROP patient and her parents. Amplification of AR locus from father's DNA (Fa, lanes 1 and 2), DNA from daughter with ROP (Da, lanes 3 and 4), and mother's DNA (Mo, lanes 5 and 6). DNA that was digested with HpaII before amplification is labelled with a plus (+) sign, DNA not predigested labelled with a minus (−) sign.

Discussion

Mutations in *NPD* coding sequence (mainly in exon 3, some in exons 1 and 2) have been causally associated with Norrie disease in a number of studies.[12 18 19] *NPD* mutation has also been found in a minority of cases of X linked familial exudative vitreoretinopathy (FEVR).[20] Some clinical similarities between these Norrie related phenotypes and ROP led researchers to consider *NPD* as a candidate gene predisposing to ROP. Despite lack of evidence to suggest that ROP is inherited as a conventional mendelian trait, racial differences in frequency and severity of ROP suggest that genetic factors might play a role. In support of this, most recently, two different mutations in *NDP* exon 3 (R121W and L108P) and a single base pair mutation in the 3' region of exon 3 have been found in a small number (5-6%) of

severe ROP patients.[14 21] It was suggested that *NDP* mutation made ROP worse rather than caused the disease per se. The R121W mutation had been previously reported as a cause of Norrie disease,[20] but the L108P mutation had not been associated with any phenotype. In the study presented here, two cases of severe retinopathy in premature babies (patients A and B) were found to have CT repeat deletions in exon 1 of the *NDP* gene. Systemic features of Norrie disease including deafness and mental retardation were not found in these children. This, plus the early onset and severity of retinopathy, led us to conclude that both cases represent severe ROP rather than examples of Norrie disease or *NDP* related FEVR. The exon 1 deletions reported here were not found in a cohort of premature babies including cases of mild ROP. In addition, these base changes were not seen in a large cohort of normal adults. It is particularly significant that the twin of one patient with a CT deletion and severe ROP had mild ROP but no CT deletion.

One of the exon 1 deletions we identified was in a female patient. An X linked gene might seem an unlikely candidate for ROP since the condition does not have a sex bias. In general, in most cases of X linked disease, females are normal. However, there are a growing number of examples of X linked disease that do result in a disease phenotype in some female carriers.[22] In ocular disease, examples include X linked ocular albinism and retinitis pigmentosa.[23] Of particular note, the female carriers of *NDP* mutations in X linked FEVR do show clinical symptoms,[24] suggesting that they exhibit retinal mosaicism or they are manifesting heterozygotes owing to non-random or skewed X inactivation.[25] Furthermore, four cases of manifesting female carriers of Norrie disease have also been reported.[26–29]

We have shown that our patient has extremely skewed paternal X inactivation, with her maternal X chromosome being the active one and thus carrying the de novo mutations. Unexpectedly, her mother had skewing of the X chromosome not passed on to her daughter. The reason for these observations is unclear; however, approximately 7% of the normal female population under the age of 25 years and 16% of women over the age of 60 show severely skewed X inactivation,[30] suggesting that the skewing could have occurred by chance. The de novo mutations coupled with the skewed X inactivation is convincing evidence for the molecular mechanism explaining the disease phenotype in the female patient. Examples of X linked disorders where female carriers with a skewed pattern of X chromosome inactivation show the disease phenotype include Duchenne muscular dystrophy,[31] Wiskott-Aldrich syndrome,[32] Lesch-Nyhan syndrome,[33] haemophilia B,[34] and Hunter syndrome.[35] Our data, along with these reports, suggest that a skewed pattern of X chromosome inactivation increases the risk of X linked diseases in females. The mother was only 45 years of age when investigated, so skewing attributed to old age in her is an unlikely explanation. Recent evidence suggests that the inactivation process itself is genetically determined and can be inherited as a mendelian trait.[36 37] Other family members were not available to investigate this possibility.

In order to assess the importance of the observations reported in this study, functional consequences need to be established. Previously reported in vitro studies support the concept that abnormalities in exon 1 of *NDP* lead to abnormalities of Norrin expression.[15] *NDP* deletion constructs have been cloned into reporter vectors to remove the entire exon 1 CT repeat sequence. This resulted in loss of reporter gene expression above basal levels.[15] This suggests that this region of *NDP* is vital in Norrin expression and further suggests that the deletions we report here have profound functional consequences for *NDP* expression level or alternatively stability and translation of mRNA transcripts. In further support of the hypothesis that the CT repeat region is important in *NDP* expression, CT repeat insertions in exon 1 have been shown to cause three cases of Norrie disease[15 19] and have been linked to severe ROP.[38]

It is of interest that only a small proportion of UK infants with severe ROP had *NDP* abnormalities. This is comparable with the results of screening in the USA.[14] Studies in other countries, however, have not found a similar association between *NDP* mutation and severe ROP.[39] Mutation in other genes or epigenetic factors could be responsible for enhanced severity of ROP in these other cases. The variable prevalence of these factors in different populations may explain the variation in severity of ROP in different countries.

Norrin (*NDP* gene product) shares homology with a protein domain termed a cysteine knot, which is a component of a variety of cysteine rich neurotrophins, such as transforming growth factor and nerve growth factor.[40] It also appears to have a role in cellular adhesion.[18] It therefore may play an important role in providing signals for developing neuronal and retinal connections and for angiogenesis in the developing retina.[41 42] Observational studies on an 11 week old fetus with Norrie disease are consistent with this primary role in vascular proliferation.[43] Retinal vascular maturation is not complete until about 32-34 weeks' gestation,[28] leaving the retina vulnerable to environmental factors such as exposure to oxygen free radicals. Thus, a premature infant born before 32 weeks carrying an abnormality of the NDP protein may be at an enhanced risk of developing the vascular defects associated with ROP if exposed to high levels of oxygen.[44] Studies focusing attention on these molecular mechanisms may therefore highlight new avenues of study into developing effective treatments for these diseases.

From our observations we hypothesise that if a deletion is present in exon 1 of *NDP* then an infant born prematurely will develop severe ROP. Presumably, offspring of the patients with exon 1 deletions will also be at risk of severe ROP if they inherit these mutations and are

- The aim of the study was to determine whether mutations in the Norrie disease protein gene (*NDP*) are associated with severe retinopathy of prematurity (ROP), that is, stage 3 plus disease or worse.
- Detailed clinical assessments were undertaken by experienced paediatric ophthalmologists. Direct sequencing of all three exons and splice sites of *NDP* was undertaken on DNA samples from 31 premature babies with stage 3 plus disease or worse and 26 control premature babies.
- Two of 31 babies (6.5%) with very severe ROP (both with stage 4/5 ROP in both eyes) were found to have novel CT repeat deletions in exon 1 of the *NDP* gene. These abnormalities are localised within the promoter region of the gene. These promoter sequence deletions were not found in either parent of these babies or in the twin (with mild ROP) of one of these children. Additionally, these deletions were not found in the control group or the general population sample, and no sequence abnormality was identified in coding exons 2 or 3 or in splice sites of the *NDP* gene.
- This study supports the suggestion that abnormalities in *NDP* are associated with severe ROP and particularly highlight mutation in the *NDP* promoter sequence as one factor that may adversely influence outcome in babies with ROP. The identification of *NDP* promoter sequence mutations could have significant bearing on the timing of indirect laser or cryotherapy treatment for eyes approaching threshold disease.

premature. Of immediate value, a molecular test (*NPD* mutation screen) might be a useful adjuvant in identifying those premature babies who will go on to develop sight threatening complications of ROP.

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