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Original Article

Mutations in the *NDP* gene: contribution to Norrie disease, familial exudative vitreoretinopathy and retinopathy of prematurity

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Abstract

Background: To examine the contribution of mutations within the Norrie disease (*NDP*) gene to the clinically similar retinal diseases Norrie disease, X-linked familial exudative vitreoretinopathy (FEVR), Coat's disease and retinopathy of prematurity (ROP).

Methods: A dataset comprising 13 Norrie-FEVR, one Coat's disease, 31 ROP patients and 90 ex-premature babies of <32 weeks' gestation underwent an ophthalmologic examination and were screened for mutations within the *NDP* gene by direct DNA sequencing, denaturing high-performance liquid chromatography or gel electrophoresis. Controls were only screened using denaturing high-performance liquid chromatography and gel electrophoresis. Confirmation of mutations identified was obtained by DNA sequencing.

Results: Evidence for two novel mutations in the *NDP* gene was presented: Leu103Val in one FEVR patient and His43Arg in monozygotic twin Norrie disease patients. Furthermore, a previously described 14-bp deletion located in the 5′ unstranslated region of the *NDP* gene was detected in three cases of regressed ROP. A second heterozygotic 14-bp deletion was detected in an unaffected ex-premature girl. Only two of the 13 Norrie-FEVR index cases had the full features of Norrie disease with deafness and mental retardation.

Conclusion: Two novel mutations within the coding region of the *NDP* gene were found, one associated with a severe disease phenotypes of Norrie disease and the other with FEVR. A deletion within the non-coding region was associated with only mild-regressed ROP, despite the presence of low birthweight, prematurity and exposure to oxygen. In full-term children with retinal detachment only 15% appear to have the full features of Norrie disease and this is important for counselling parents on the possible long-term outcome.

Key words: Norrie disease, retinopathy of prematurity, X-linked familial exudative vitreoretinopathy.

Introduction

The Norrie disease protein (*NDP*) norrin is a member of a family of growth factors that are grouped together on the basis of a characteristic 'cysteine knot' motif. Norrin is predicted to comprise 133 amino acid residues, an N-terminal signal sequence and the cysteine knot motif, located at the carboxyl-terminal end.[1] Although the precise function of

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norrin is still unknown, there is recent evidence to suggest that the norrin protein is involved in the Frizzled-dependent signalling cascade involved in vascular development and maintenance of the inner ear and retina.[2] Greater than 70 point mutations, chromosomal rearrangements, frame-shift and splice site mutations in the *NDP* gene have been identified, the vast majority in patients diagnosed with Norrie disease (OMIM #310600).[3] Norrie disease is an X-linked disorder occurring in full-term infants. Findings may be restricted to ocular abnormalities including retinal dysgenesis, retinal detachment, corneal opacities and cataract, which occur early in childhood. In more severe cases these ocular features are also associated with deafness and mental retardation.[4] Evidence has also been presented for the existence of three additional allelic forms of Norrie disease. These diseases have similar ocular features to Norrie disease and include X-linked familial exudative vitreoretinopathy (FEVR; OMIM #305390),[1] Coat's disease (OMIM #300216)[5] and retinopathy of prematurity (ROP).

Familial exudative vitreoretinopathy exists as an autosomal dominant disease and an X-linked disorder. Like Norrie disease, FEVR occurs in full-term infants and is characterized by abnormal vascularization of the retina resulting in retinal traction, retinal folding and detachment. Unlike Norrie disease, FEVR progresses more slowly and retinal detachment may not occur until later in life. Several point mutations in the *NDP* gene have been reported to be associated with the X-linked form of this disease.[6] More recently mutations within the *LRP5* and *FZD4* genes have been reported to contribute to an autosomal dominant form of FEVR.[7]

The ocular features of Norrie disease and FEVR also resemble those of Coat's disease (although the retinal detachment in Coat's disease is usually unilateral), which has been associated with a mutation within the *NDP* gene.[5] Retinal changes observed in Norrie disease and FEVR are similar to those observed in cases of severe ROP. However, ROP occurs in premature infants, most commonly those of less than 32 weeks gestation and those with low birthweight (<1250 g) treated with supplemental oxygen. Most infants with ROP undergo spontaneous regression.[8] In others, however, ablative surgical treatment is required to decrease the risk of progression to macular folds, macular drag and/or retinal detachment and consequent permanent visual impairment. It is not clear why some patients undergo spontaneous regression whereas others progress. Although close monitoring of supplemental oxygen levels is essential, there is circumstantial evidence that there may be an additional underlying genetic basis for ROP based on observed racial differences in ROP incidence and severity.[6] Furthermore, it has been suggested that gene expression analysis is likely to be important.[9] Recently, there has been conflicting evidence as to whether the *NDP* gene plays a role in severe ROP. Polymorphisms within the *NDP* gene have been reported in a small minority of patients with severe ROP,[6,10] and it has been postulated that around 3% of severe ROP cases may be attributed to sequence changes within the *NDP* gene. Other groups, while identifying a similar prevalence of *NDP* gene polymorphisms in their populations, failed to find evidence for any association of these mutations with ROP.[11–13] Insertion and deletion mutations within the CT repeat region of exon 1 of *NDP* gene are reported to be associated with three cases of Norrie disease and three cases of severe ROP.[10,14,15] Racial differences between population datasets may be one reason for the inconsistency in these results.[11] It has been suggested that rather than causing the disease, sequence variations within this gene impact disease severity.[15] The role of the *NDP* gene in this group of retinal diseases remains unclear. We therefore investigated mutations of the *NDP* gene in a group of patients diagnosed with Norrie disease, FEVR, Coat's disease or ROP, who were predominantly of Northern European ancestry.

METHOD

Ascertainment and clinical examination

This study was approved by the Royal Children's Hospital, Melbourne, Australia and the University of Tasmania, Australia Human Research Ethics Committees. Thirteen Norrie-FEVR subjects and a single Coat's disease patient were identified on the basis of hospital records from the Royal Children's Hospital, Victoria and the Royal Hobart Hospital, Tasmania, and diagnosis was confirmed by one of five ophthalmologists (DAM, JEC, SMC, ST, HM). Of the 13 index cases diagnosed with Norrie-FEVR, there were two sporadic female cases and eight sporadic male cases. Of the three familial cases there were: one pair of monozygotic twins with no other affected relatives, one child with an affected maternal uncle (although this would appear to be an X-linked pedigree, subsequent analysis identified an FZD4 Q505X – 1513C>T exon 2 that causes autosomal dominant FEVR),[16] another index case had an affected maternal cousin and grandfather. In addition to these 13 index cases there was an affected father and son family, who clearly excluded X-linked inheritance and were not screened for the Norrie disease gene. Another of the sporadic cases was found to have an FZD4 W319fsX323 c957delG exon 2 mutation[16] and another sporadic case was found to have a mutation in LRP5 C1361G (c4081TrG), exon 19, that also causes autosomal dominant FEVR.[7]

Retinopathy of prematurity subjects were selected using the following criteria: gestational age ≤ 34 weeks, birthweight ≤ 1500 g and stage 2, 3, 4 or 5 ROP confirmed by ophthalmic examination. Control subjects were ex-premature babies selected on the basis of gestational age < 32 weeks and normal ophthalmic examination to exclude the presence of any ocular abnormality associated with Norrie disease, FEVR or ROP. Informed consent was obtained from patients or their parents.

In order to minimize the influences of prematurity that might affect phenotypic expression of *NDP* mutations in ROP, we identified ex-premature controls with similar gestational age, birthweight and oxygen supplementation as ROP cases.

Mutation screening

Buccal mucosa swabs were collected from individuals meeting the above selection criteria. Genomic DNA was isolated using Puregene DNA isolation kits (Gentra Systems Inc., Minneapolis, MN, USA). In a small number of cases blood samples were obtained and DNA was isolated using a Nucleon genomic DNA extraction kit (Amersham International, Buckinghamshire, UK). Primer pairs, as published by Berger *et al.*[17] and Schuback *et al.*,[14] were used to amplify the *NDP* gene. The three exons of the *NDP* gene were screened in all affected individuals by direct automated DNA sequencing using ABI PRISM BigDye Terminator Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 310 DNA Analyser (Applied Biosystems). Identified mutations were confirmed by bidirectional sequencing.

Denaturing high-performance liquid chromatography (DHPLC) was used to screen controls for the two novel point mutations in exon 1 and exon 2 identified through DNA sequencing of cases. Polymerase chain reaction products were analysed on a helix column using a Varian DHPLC Analyser (Varian, CA, USA) using the manufacturer's protocols. Point mutations were detectable by DHPLC in positive controls.

Denaturing polyacrylamide gel electrophoresis (PAGE) was used to screen controls for the deletion identified in exon 1 of the *NDP* gene. Any identified gel shifts were confirmed by DNA sequencing.

Results

Affected sample group

The 31 ROP-affected patients (20 male and 11 female) ranged in birthweight from 625 g to 1500 g, 23–34 weeks gestation (median 26 weeks), and oxygen therapy was confirmed by review of medical records in all cases. One female patient with stage 3 ROP was born at 34 weeks gestation weighing 1450 g and one male patient with stage 4 ROP was born at 29 weeks gestation weighing 1500 g. All other ROP cases and controls were born <32 weeks and <1250 g. Eleven of the ROP patients had severe ROP worse than stage 3, and the remainder were stage 2 or 3, which regressed. There were 13 index cases diagnosed with Norrie-FEVR. Only two index cases (the monozygotic male twins and one sporadic male case) had the additional Norrie disease features of deafness and possible mental retardation. At the time of writing subjects ranged in age from 6 to 57 years for ROP (including a member of Hugh Ryan and Kate Campbell's original retrolental fibroplasia [RLF] cohort) and 6–58 years for Norrie-FEVR (including a full-term child that was among the same RLF cohort).[18]

Control group

A total of 90 premature control babies (50 male and 40 female) were recruited and examined, including index cases from seven sets of dizygotic twins and one set of triplets. The birthweight range for control subjects was 515–1978 g. The gestational age ranged from 24 to 32 weeks (median 28 weeks). Oxygen therapy was confirmed from hospital records in 50 of these cases. Hospital records and ophthalmic examination revealed no ocular abnormalities in 37 cases. The remainder had some degree of non-retinal ocular abnormality, such as myopia or strabismus, but no indication of Norrie disease, FEVR, Coat's disease or ROP.

Point mutations of the *NDP* gene

A novel missense mutation, c536a>g, resulting in a His43Arg transition in the coding region of exon 2 was detected in twin boys affected with Norrie disease (Fig. 1). The twins were born at 38 weeks' gestation. Clinical examination at 5 years of age revealed no response to light, presence of the oculo-digital sign, bilateral total retinal detachment and hearing loss necessitating hearing aids. This mutation was not detected by DNA sequencing in any of the other patients affected with Norrie-FEVR, Coat's disease or ROP. Screening of controls by DHPLC did not reveal this mutation.

A novel missense mutation, c715c>g, resulting in Leu103Val amino acid change was detected in a patient diagnosed with FEVR (Fig. 2). Family members were not available for screening; however, a positive family history was noted. This missense mutation was not detected in any of the other Norrie disease, FEVR or ROP patients by DNA sequencing. DHPLC screening did not reveal the mutation in any of the controls.

Deletions within the 5′ untranslated region of the *NDP* gene

A 14-bp deletion (nucleotides +3 to +16) was detected in the CT repeat region of exon 1 in one ROP-affected male patient (Figs 3,4). This patient, born at 25 weeks' gestation, weighing 950 g, had received oxygen therapy for 3 months and

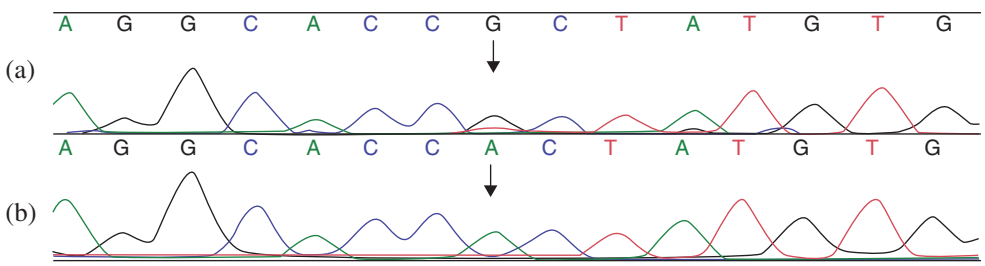

Figure 1. Electropherogram showing c536a>g mutation in exon 2 of the *NDP* gene: (a) Arrow indicates A to G mutation in Norrie disease-affected twin 1, and (b) wild-type DNA sequence.

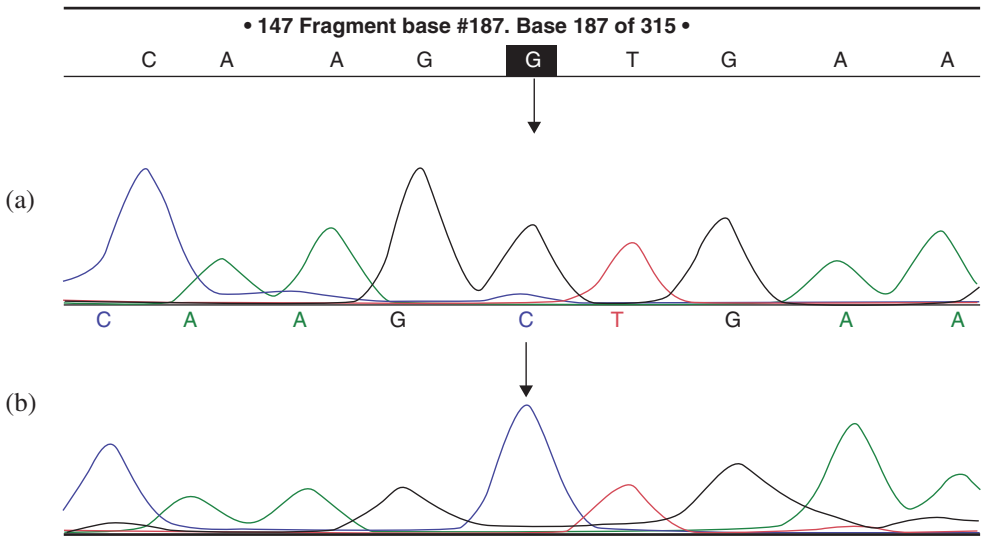

Figure 2. Electropherogram showing c715c>g mutation in exon 3 of the *NDP* gene: (a) Arrow indicates C to G mutation in FEVR-affected patient, and (b) wild-type DNA sequence.

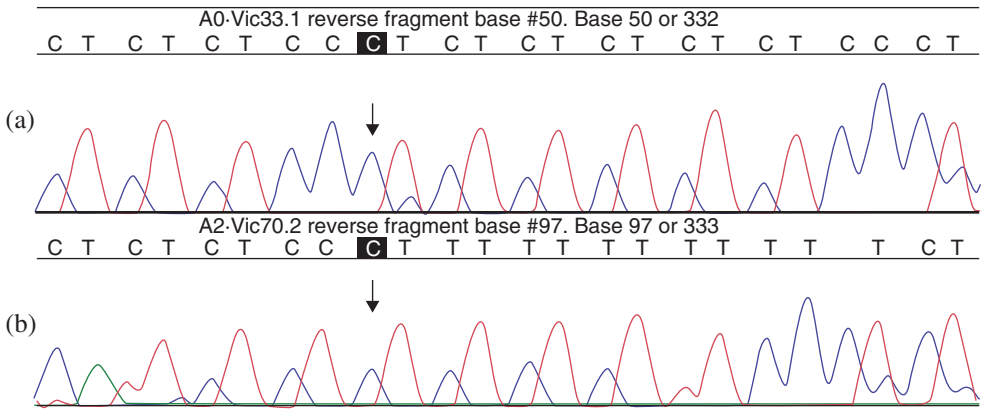

Figure 3. Electropherogram showing 14-bp deletion in CT repeat region of exon 1 of the *NDP* gene. (a) Wild-type DNA sequence, and (b) deletion observed in ROP-affected twin 1.

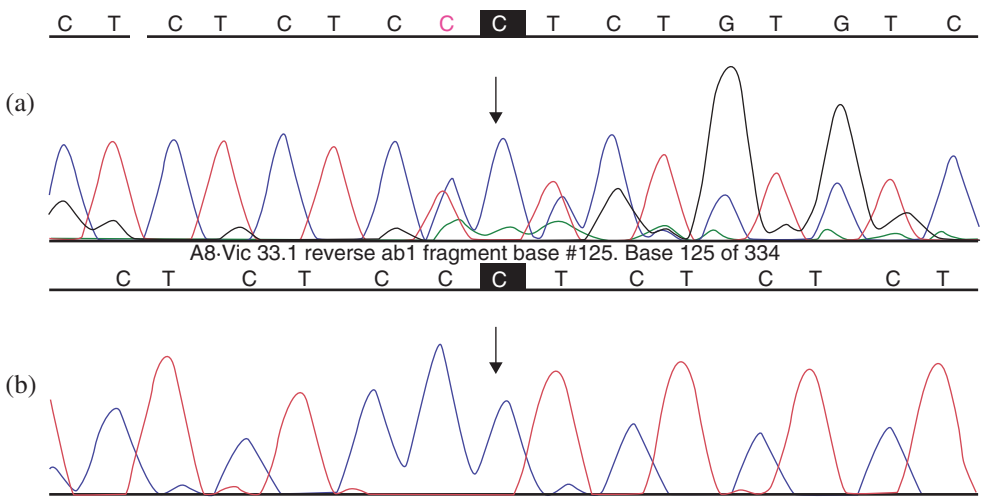

Figure 4. Electropherogram showing 14-bp deletion in CT repeat of exon 1 of the *NDP* gene. (a) Deletion in unaffected control patient, and (b) wild-type DNA sequence.

was diagnosed with stage 2 ROP that had regressed spontaneously. Subsequent ophthalmic examination at 18 months revealed mild hypermetropia. Examination of family members revealed that his mother, who displayed no ocular symptoms, was heterozygous for this deletion. Examination of remaining affected patients and controls by PAGE, and confirmation by DNA sequencing revealed this deletion was also present in twin boys born at 26 weeks' gestation, of birth-

weights 871 g and 740 g, respectively. Both boys had stage 3 ROP, which regressed spontaneously. At 5 years of age, both boys had normal vision.

A heterozygous 14-bp deletion (nucleotides +27 to +40) was also detected in the CT repeat region in a premature female control. This subject was born at 29 weeks' gestation, weighing 1195 g and had received oxygen therapy. Ophthalmic examination revealed no ocular abnormalities and a follow-up eye examination was normal. Family members were not available for screening.

Screening of the remaining Norrie/FEVR patients, the single Coat's disease patient and the remaining severe and regressed ROP patients revealed no detectable *NDP* gene mutations.

Discussion

In this study we have avoided trying to define and distinguish between Norrie disease and FEVR. One could argue that all X-linked detachments and all cases with mutations in the Norrie disease gene have Norrie disease, and all autosomal dominant pedigrees and all cases with mutations in FZD4 or LRP5 have FEVR. Norrie himself only described the ocular features, although Warburg recognized that 20 of 35 cases had moderate to sever mental retardation and 25% have deafness.[19] In Australia, paediatric ophthalmologists tend to diagnose Norrie disease, and retinal specialists, seeing older patients who lack deafness and mental retardation, tend to diagnose FEVR. An important finding from this cohort is that only two of the 13 (15%) index cases had the additional extraocular features of Norrie disease. Thus, paediatric ophthalmologists should be less pessimistic about the extraocular manifestations in full-term infants with retinal detachment. As all the participants enrolled in this study were born prior to 2000, it is now evident that most do not have extraocular features of Norrie disease.

The *NDP* gene has been described as playing a causal role in the genetic eye diseases Norrie disease, X-linked FEVR, Coat's disease and ROP. Our study detected two novel *NDP* gene missense mutations in patients diagnosed with Norrie disease and FEVR, respectively, and a deletion within the untranslated exon 1 CT repeat in a three ROP patients and one unaffected premature control.

A novel His43Arg mutation in exon 2 was detected in a pair of twin boys born at 38 weeks displaying the clinical features of severe Norrie disease, including bilateral total retinal detachment and hearing loss. This His43Arg mutation was not detected in ROP or FEVR patients, or unaffected controls. A His43Gln transition has been reported in a French kindred exhibiting the ocular features of Norrie disease associated with learning difficulty but not deafness.[20] Other mutations previously described in this region include a His42Arg transition in an X-linked FEVR kindred,[21] a Tyr44Cys in a Norrie disease patient,[22] and a Tyr44stop mutation identified in a Japanese kindred affected with Norrie disease.[23] These amino acids are in close proximity to one of the critical cysteine residues involved in the cysteine knot motif (Cys39) and mutations within this region appear to produce a range of severe phenotypic features.[24]

A second novel missense mutation was identified in a single FEVR patient. It was not found in our ROP patients nor in any of the controls. To our knowledge the Leu103Val mutation in exon 3 has not been previously described. Mutations in this region include Lys104Gln and Ala105Thr in less severe Norrie disease patients.[25,26] Other *NDP* gene mutations identified in X-linked FEVR patients were also located in exon 3, at amino acid positions 121 and 124.[1,27]

Amplification of sequencing products from all samples indicated that microdeletions encompassing the *NDP* gene observed in some Norrie disease patients[28] were not present in any of the remaining male Norrie disease/FEVR patients in our study. There remain 11 Norrie/FEVR patients in this dataset where no mutations were detected in the *NDP* gene. This phenomenon has also been observed in another Norrie disease dataset.[20] Given that the remainder of the Norrie/FEVR patients did not strictly fit the criteria for clinical diagnosis of Norrie disease, it is possible that the disease in the remaining patients arises from other genetic causes. However, we cannot rule out the possibility that mutations affecting *NDP* gene expression or function may exist outside the exonic regions examined in this study. Subsequent screening of our Norrie/FEVR patient samples have revealed mutations in the *FZD4* gene in two individuals and mutations in the *LRP5* gene in one further patient.[7] The *FZD4* and *LRP5* genes are both located within the *EVR1* locus mapped to chromosome 11[29] and have been associated with FEVR disease.[7]

Although it is evident that the *NDP* gene plays a role in X-linked FEVR, its role in ROP is less clear. Mutations in exon 3 (Arg121Trp and Leu108Pro) have been found in a small number of patients with severe ROP.[6] However, screening for these mutations (and a two further mutations associated with FEVR (Ala105Thr and Val60Glu) in Kuwaiti premature babies revealed that these mutations, although present in the population, were not associated with ROP disease.[11] In the same population, Haider *et al*. have identified a c597c>a polymorphism, which was present in 83% of advanced stage ROP cases compared with 0% of mild regressing ROP and 10% of controls.[30] This c597c>a transition does not result in an amino acid change at Ala63 and the authors suggest that this polymorphism may cause a splicing error or influence expression of the *NDP* gene. Kim *et al*. have examined the *NDP* gene for all known *NDP* gene mutations in 18 ROP patients from the Korean population and did not identify any mutations in this group.[13] It has been postulated that ethnic variation may account for the observed differences in the evidence presented in these studies.[11]

In our study, screening a group of patients with severe and mild ROP for any mutations of the *NDP* gene did not result in detection of any of the above missense mutations. We did, however, identify deletions within the CT repeat region in one male patient with stage 2 regressed ROP with mild hypermetropia and in twin boys diagnosed with stage 3

regressed ROP. The twin boys were examined at 5 years of age and showed no ocular abnormalities. It is possible that the presence of this mutation together with prematurity and oxygen therapy may contribute to the development of regressed ROP observed in these patients; however, it appears that these factors are not sufficient to cause severe ROP. Talks *et al*. noted dizygotic twins where one had a CT deletion and severe ROP, and the other had no CT deletion and mild ROP.[15] The functional importance of the CT repeat region was examined by Kenyon and Craig who showed that deletion of the entire CT repeat region reduces efficient expression of the gene in *in vitro* transfection studies.[31] Other studies have suggested that deletions and insertions within this region are associated with a small number of cases of severe ROP[10,15] as well as Norrie disease.[14] It is possible that other factors may be contributing to the development of severe disease in these cases.

Genotype-phenotype comparisons of the *NDP* gene show no clear correlation. Norrie disease, FEVR or Coat's disease have been reported to arise from the same *NDP* mutations.[5,17] Disruption of the CT repeat region is reported to be associated with cases of both Norrie disease and severe ROP.[14,15] Difficulties may also arise in the clinical distinction of these diseases, especially for affected babies born prematurely and of low birthweight. This may be pertinent for the few cases where *NDP* gene mutations have been found to be associated with severe ROP.

Retinal disease may be attributable to *NDP* gene mutations in a minority of patients diagnosed with Norrie disease and FEVR, and possibly, a small number of ROP patients in our dataset. Functional roles for the *NDP*, *LRP5* and *FZD4* genes have been described in the Wnt signalling pathway and it appears that these genes interact in this pathway to regulate retinal and inner ear vasculature development and maintenance.[2] This new evidence provides some clues as to the mechanism of retinal dysfunction and it appears likely that this group of clinically similar diseases arise as a result of mutations within one or more genes that interact within the same signalling pathway.

The results of this study suggest that mutations within the *NDP* gene do not contribute to disease in the majority of ROP, Norrie disease and FEVR patients investigated, nor the individual with Coat's disease. Although two novel mutations within the *NDP* gene were identified, there were a number of Norrie disease/FEVR patients in whom no mutation was found. Furthermore, disruption of the CT repeat region of the *NDP* gene was not sufficient to cause severe ROP disease even in the presence of associated factors of prematurity, low birthweight and exposure to oxygen in our population.

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