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Norrie disease gene sequence variants in an ethnically diverse population with retinopathy of prematurity

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Purpose: Retinopathy of prematurity (ROP) is a leading cause of visual loss in the pediatric population. Mutations in the Norrie disease gene (NDP) are associated with heritable retinal vascular disorders, and have been found in a small subset of patients with severe retinopathy of prematurity. Varying rates of progression to threshold disease in different races may have a genetic basis, as recent studies suggest that the incidence of NDP mutations may vary in different groups. African Americans, for example, are less likely to develop severe degrees of ROP. We screened a large cohort of ethnically diverse patients for mutations in the entire NDP.

Methods: A total of 143 subjects of different ethnic backgrounds were enrolled in the study. Fifty-four patients had severe ROP (Stage 3 or worse). Of these, 38 were threshold in at least one eye (with a mean gestational age of 26.1 weeks and mean birth weight of 788.4 g). There were 36 patients with mild or no ROP, 31 parents with no history of retinal disease or prematurity, and 22 wild type (normal) controls. There were 70 African American subjects, 55 Caucasians, and 18 of other races. Severe ROP was noted in 29 African American subjects, 17 Caucasians, and 8 of other races. Seven polymerase chain reaction primer pairs spanning the NDP were optimized for denaturing high performance liquid chromatography and direct sequencing. Three primer pairs covered the coding region, and the remaining four spanned the 3' and 5' untranslated regions (UTR).

Results: Six of 54 (11%) infants with severe ROP had polymorphisms in the NDP. Five of the infants were African American, and one was Caucasian. Two parents were heterozygous for the same polymorphism as their child. One parent-child pair had a single base pair (bp) insertion in the 3' UTR region. Another parent-child pair had two mutations: a 14 bp deletion in the 5' UTR region of exon 1 and a single nucleotide polymorphism in the 5' UTR region of exon 2. No coding region sequence changes were found. No polymorphisms were observed in infants with mild or no ROP, or in the wild type controls.

Conclusions: Of the six sequence alterations found, five were novel nucleotide changes: One in the 5' UTR region of exon 2, and four in the 3' UTR region of exon 3. The extent of NDP polymorphisms in this large, racially diverse group of infants is moderate. NDP polymorphisms may play a role in the pathogenesis of ROP, but do not appear to be a major causative factor.

Retinopathy of prematurity (ROP) is a leading cause of visual loss in the pediatric population [1]. ROP is a retinal vascular disorder affecting infants with low birth weight and preterm gestational age [2]. With preterm birth, anterior radial retinal vessel growth emanating from the optic nerve is disrupted, and abnormal blood vessel growth at the vanguard between vascularized and avascular retina can occur. In severe cases of ROP, the abnormal blood vessels damage the retina due to scarring with traction, often with detachment, causing blindness. Infants with birth weights less than 1250 g have a 40-66% chance of developing ROP, and a nearly 20% chance of developing severe ROP [3]. As the survival rate of very low birth weight infants rises, the number of infants with decreased vision secondary to ROP has risen [3]. Ablative treatments available for severe ROP such as cryotherapy or laser photocoagulation are only 40-50% effective at preventing retinal detachment or macular scarring [4,5]. It is estimated that even with treatment, more than 2000 infants were affected by the cicatricial changes of ROP in the United States in 1991 [4].

Norrie disease (ND) is an inherited X-linked recessive disorder characterized by bilateral retinal fibrosis and detachments, accompanied by progressive hearing loss and central nervous system dysfunction in a third of patients [6]. Literature reviews note approximately 300 reported cases, initially only from Scandinavia, but subsequently from various other countries and in all races [7-9]. The Norrie disease gene (NDP) maps to chromosome Xp11.2-11.3 and is expressed in the retina, choroid, and brain [10]. It is a three exon gene with a 1.85 kb transcript [11,12]. NDP encodes for the protein Norrin, which is 133 amino acids in length, and is involved in blood vessel formation and neural differentiation pathways [13,14]. Its exact function is unknown, but it has structural similarity to the "cysteine knot" growth factors such as transforming

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growth factor, and sequence homology to other genes involved in regulation of cellular proliferation [15].

Sequence variants in the NDP have been identified in a small number of patients with ROP and in patients with X-linked familial exudative vitreoretinopathy (FEVR), an inherited proliferative retinal disease that phenotypically resembles ROP with retinal vascular arrest [16,17]. Many of the retinal complications associated with FEVR, such as retinal folds, macular ectopia, and combined tractional and exudative retinal detachments, are identical to those observed in severe ROP [18].

Shastry et al. [16] identified mutations in the NDP in four of 16 infants of both sexes with advanced ROP, and in none of 50 healthy controls [16]. All four patients had missense mutations in the third exon of NDP. This observation suggested the first possible genetic link to ROP susceptibility. Subsequent studies of NDP in ROP patients have been reported [19-23]. The type of sequence variant has varied from report to report. Many of the studies were performed in populations of single ethnic backgrounds, and some with partial NDP gene sequencing.

Different incidences of progression to threshold ROP in different races have been well documented. The CRYO-ROP study found that African American infants have a lower risk

Table 1. Demographics of enrolled subjects

Subject type	Subjects (% total)	Male	Gestational age	Birth weight
Affected	54 (38%)	34	25.5 weeks	773 g
Control	36 (25%)	19	27.7 weeks	968 g
Parents	31 (22%)	11		
Wild type	22 (16%)	10		
Total	143	71 (50%)		

Subject type	African American	Caucasian	Other
Affected	29	17	8
Control	25	7	4
Parents	10	20	1
Wild type	6	11	5
Total	70 (49%)	55 (39%)	18 (13%)

The table shows a demographic summary of the 143 subjects of this study. Affected subjects had stage 3 ROP or greater. Controls were premature with no or minimal ROP. Parent subjects are parents of affected babies. Wild type subjects have no history of ocular diseases.

Table 2. Norrie disease gene primers

Exon	Primer	Position on contig NT_079573	Primer sequence 5'-3'	Size (bp)
1	NDG-01F	6682461	TGGCATTCCCATTTGCTAGT	369
	NDG-01R	6682829	AGGATGAAATGCTCGGTTTG	
2	NDG-02F	6697148	TGGGTTCCATTAGTGGTTCTG	476
	NDG-02R	6697646	GGCTTCTTGCCTGTTTCTGA	
3	NDG-03F	6705972	TGCTGTTTTACCTGGCTAAGG	350
	NDG-03R	6706321	GCTGGTCGAACTGCCTCTAC	
3	NDG-04F	6706222	CCGGTACATCCTCTCCTGTC	364
	NDG-04R	6706584	TGTATGAGGGCCCACTTTTT	
3	NDG-05F	6706518	TTGGCTCTCAATGCTGTTTG	349
	NDG-05R	6706866	GCTGTCAAGAGTTCCAGCATC	
3	NDG-06F	6706797	CAGCCAGCGAACTGACATTA	348
	NDG-06R	6707144	TTAGAGAATGATGCCCGTGA	
3	NDG-07F	6707064	GCATGCAAATTAGACAACCAA	312
	NDG-07R	6707375	AGGAGATGCTCAAGCACTAGC	

Seven primers were designed for mutation screening. The forward primers are named with a terminal “F”; the reverse primers are named with a terminal “R”.

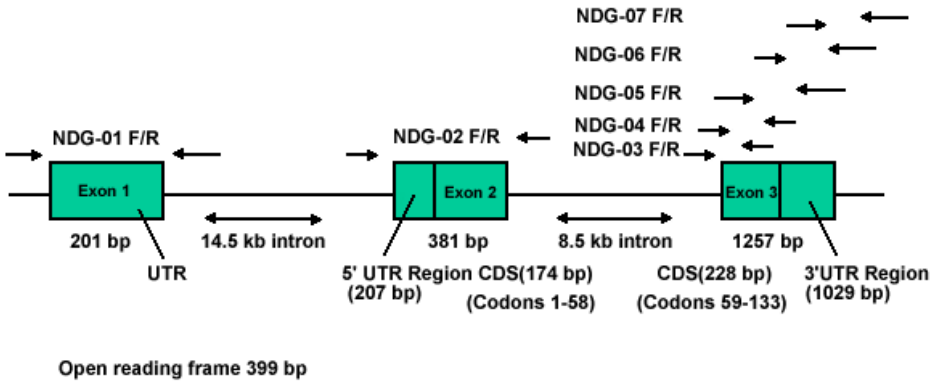

Figure 1. Norrie disease gene structure. The intron-exon structure of the Norrie disease gene (contig NT_079573). The coding sequence (CDS) is contained within exons 2 and 3. Each of the seven overlapping primer pairs is delineated with arrows as NDG01 through NDG07. The forward primers are named with a terminal “F”; the reverse primers are named with a terminal “R”.

Figure 2 (next page). The genetic structure of the Norrie disease gene showing its DNA and amino acid sequence. Known Norrie disease causing gene mutations are highlighted in orange and the resulting protein alteration is given. The positive control samples (sample identification numbers 367, 481, 484, and 758) used in this study with known mutations are highlighted in red and underlined. Positive control 367 had a deletion of “A” at bp position 741. Positive control 481 had “AA” instead “GC” at bp position 792. Positive control 484 had a “C” instead “G” at bp position 698. Positive control 758 had an insertion of “CTC” at bp position 676.


CCTCTCTCTCTCTCCCTCTCTCTCTCCCTCTCTCTCTCTCCCTGTGTCGCTTAAACAACAGTCCTAACTTTTGTGTGTTGCA
AATATAAAAGGCAAGCCATGTGACAGAGGGACAGAAGAACAAAAGCATTTGGAAGTAACAGGACCTCTTTCTAGCTCTCAGA
AAAGTCTGAGAAGAAAGGAGCCCTGCGTTCCCCTAAGCTGTGCAGCAGATACTGTGATGATGGATTGCAAGTGCAAAGAGTA
AGACAAAACTCCAGCACATAAAGGACAATGACAACCAGAAAGCTTCAGCCCGATCCTGCCCTTTCCTTGAACGGGACTGGAT
CCTAGGAGGTGAAGCCATTTCCAATTTTTTGTCCTCTGCCTCCCTCTGCTGTTCTTCTAGAGAAGTTTTTCCTTACAACA
ATGAGAAAACAT
METArgLysHis
GTACTAGCTGCATCCTTTTCTATGCTCTCCCTGCTGGTGATAATGGGAGATACAGACAGT
ValLeuAlaAlaSerPheSerMETLeuSerLeuLeuValIleMETGlyAspThrAspSer
533
530|
AAGCGC
AAAACGGACAGCTCATTCATAATGGACTCGGACCCTCGACGCTGCATGAGGCACCACTAT
LysThrAspSerSerPheIleMETAspSerAspProArgArgCysMETArgHisHisTyr
LysArg
R41K
H42R
582
AAT
GTGGATTCTATCAGTCACCCATTGTACAAGTGTAGCTCAAAGATGGTGCTCCTGGCCAGG
ValAspSerIleSerHisProLeuTyrLysCysSerSerLysMETValLeuLeuAlaArg
Asn
K58N
634(#2619)
TAG
TGCGAGGGGCACTGCAGCCAGGCGTCACGCTCCGAGCCTTTGGTGTCGTTCAGCACTGTC
CysGluGlyHisCysSerGlnAlaSerArgSerGluProLeuValSerPheSerThrVal
Ter
E76Ter
676(#758)
696 698(#484)
TTCCTCCGT
TGGCCG
CTCAAGCAACCCTTCCGTTCCTCCTGTCACTGCTGCCGGCCCCAGACTTCCAAGCTGAAG
LeuLysGlnProPheArgSerSerCysHisCysCysArgProGlnThrSerLysLeuLys
PheLeuArg
90InsCTC
TrpPro
C96W
R97P
741(#367)
769
721
731
736
767
ACA
CCG
GGCTC-
TGCTGG
GCACTGCGGCTGCGATGCTCAGGGGGCATGCGACTCACTGCCACCTACCGGTACATCCTC
AlaLeuArgLeuArgCysSerGlyGlyMETArgLeuThrAlaThrTyrArgTyrIleLeu
Thr
Pro
Gly
CysTrp
A105T
L108P
Y120C
C110G
R121W
111DelA
791
792(#481)
TAA
TCCTGTCACTGCGAGGAATGCAATTCCTGAGGCCCGCTGCTGTGTGTGGCTTCTGGATGG
SerCysHisCysGluGluCysAsnSerSTP
Ter
C128Ter
GACAACTGTAGAGGCAGTTCGACCAGCCAGGGAAAGACTGGCAAGAAAAGAGTTAAGGCAAAAAAGGATGCAACAATTCTCC
CGGGACTCTGCATATTCTAGTAATAAAGACTCTACATGCTTGTTGACAGAGAGAGATACTCTGGGAACTTCTTTGCAGTTCC
CATCTCCTTTCTCTGGTACAATTTCTTTTGGTTCATTTTCAGATTCAGGCATTTTCCCCCTTGGCTCTCAATGCTGTTTGGG
TTTCCAACAATTCAGCATTAGTGGGAAAAAGTGGGCCCTCATACACAAGCGTGTCAGGCTGTCAGTGTTTGGTGCACGCTGG
GGAAGAATTTACTTTGGAAAGTAGAAAAGCCCAGCTTTTCCTGGGACATCTTCTGTTATTGTTGATGTTTTTTTTTACCTTG
TCATTTTGGTCTAAGGTTGCCATTGCTGCTAAAGGTTACCGATTTCAAAGTCCAGATACCAAGCATGTGGATATGTTTAGCT
ACGTTTACTCACAGCCAGCGAACTGACATTAAAATAACTAACAAACAGATTCTTTTATGTGATGCTGGAACTCTTGACAGCT
ATAATTATTATTCAGAAATGACTTTTTGAAAGTAAAAGCAGCATAAAGAATTTGTCACAGGAAGGCTGTCTCAGATAAATTA
TGGTAAAATTTTGTAAGGGAGCAGACTTTTAAAGACTTGCACAAATACGGATCCTGCACTGACTCTGGAAAAGGCATATATG
TACTAGTGGCATGGAGAATGCACCATACTCATGCATGCAAATTAGACAACCAAGTATGAATCTATTTGTGGGTGTGCTATAG
CTTTAGCGTGTCACGGGCATCATTCTCTAATATCCACTTGTCCATGTGAAACATGTTGCCAAAATGGTGGCCTGGCTTGTCT
TCTGAACGTTTGGTTCAAATGTGTTTTGGTCCTGGAGGCTCAAATTTTGAGTTATTCCCACGTTTTGAAATAAAAAGAGTAT
ATTCAAAAAAAAAAAAAAAAAA

of developing severe disease than their Caucasian counterparts of similar birth weight and gestational age [24]. The reasons for this difference are unknown. Thus, a genetic study of African American infants with severe ROP is of special interest, as these patients may have a different genetic susceptibility makeup.

The current study addressed whether NDP sequence variants are associated with ROP staging in a large heterogeneous group of at risk infants of varying ethnic backgrounds. Unlike previous studies on NDP sequence screening in association with ROP [16,22], all three exons were screened, including splice sites, and the 5' and 3' untranslated regions (UTR).

METHODS

Subject enrollment and data collection: The study protocol was approved by the Children's Hospital of Philadelphia, the University of Maryland, and the Children's National Medical Center Institutional Review Boards on Human Subjects Research, and adhered to the tenets of the Declaration of Helsinki. Written parental consent was obtained from all enrolled families. Information recorded for premature infants included race, birth weight, gestational age, major medical problems, stage and zone of most advanced ROP, and whether treatment was required. A complete ophthalmic examination by a study investigator, including a dilated fundus examination by indirect ophthalmoscopy was performed. Subjects were recruited from both inpatient and outpatient settings, and were identified during routine ROP screening examinations and follow-up visits from three institutions with level-3 neonatal intensive care units. Eligibility criteria for participation included: Birth weight less than 1500 g; gestational age less than 34 weeks; and a medical condition stable enough to allow for the collection of blood samples. Any major co-morbid eye disease was grounds for exclusion. Patients who were categorized as control subjects were defined as premature with no or minimal ROP (equal to or less than Stage 2). Patients who were categorized as affected subjects had prethreshold ROP (Stage 3 with no Plus disease), threshold ROP (Stage 3 with Plus disease), or greater staging in at least one eye. The control group was included in order to compare children with a similar demographic baseline and set of environmental stressors to the affected children. A subset of parents of children with ROP was also enrolled for study. A final group of wild type control subjects with no history of ocular disease was included for comparison. Table 1 displays the demographics of enrolled subjects.

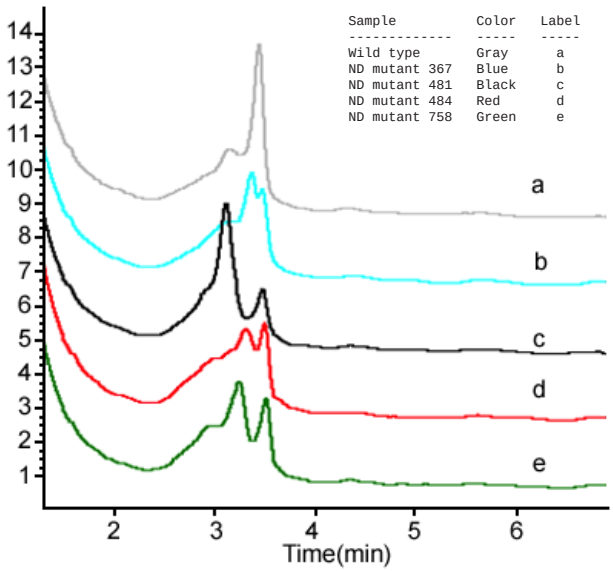

Figure 3. Denaturing high performance liquid chromatography waveforms of known Norrie disease gene mutations for amplicon NDG-03 (the 5' end of exon 3). Subject samples are from a wild type (control) individual and four subjects with known Norrie disease mutations serving as positive controls. Sample identification numbers (367, 481, 484, and 758) correspond to the known mutations listed in Figure 2. Note that each of the known Norrie disease samples (positive controls) have a distinct waveform from each other and from the normal control.

Collection of samples and DNA extraction: DNA was extracted from peripheral blood or buccal swab samples. Leukocytes were extracted from 250 µl of venous blood and stored at -4 °C until DNA extraction was performed. White blood cell pellets were then incubated with lysis buffer and proteinase K at 37 °C. Three stage organic solvent extraction was performed using phenol, phenol-chloroform, and chloroform. In the final phase of extraction, ice cold isopropanol was added to precipitate the DNA. Strands of DNA were centrifuged into a pellet at 1600 rpm. Pellets were allowed to dry, and then dissolved in 30 µl of low Tris. DNA samples were stored at 4 °C. Buccal swabs were processed with the Puregene nucleic acid extraction kit (Gentra Systems Inc., Minneapolis, MN) according to the manufacturer's instructions.

Polymerase chain reaction amplification: Figure 1 shows the genetic structure of NDP. It is a 28 kb gene composed of three exons, and encodes a 133 amino acid protein (Norrin) from a portion of the second and third exons. Exon 1 is untranslated. The total transcript length is 1.85 kb, and the open reading frame is 399 bp (contig NT_079573). We designed and optimized seven novel sets of forward and reverse (NDG F/R) polymerase chain reaction (PCR) primer pairs (Table 2). These primers encompass intron-exon boundaries, exon 1, the 5' untranslated (UTR) region of exon 2, the coding sequences (CDS) of exon 2 and 3, and the 3' UTR of exon 3.

All PCR volumes were 50 µl and contained 38.5 µl water, 0.5 µl of 5 U/µl AccuType DNA polymerase (Stratagene, La Jolla, CA), 3 µl of 25 mM dNTP (deoxynucleotide substrate), 5 µl of 10X AccuType reaction buffer (Stratagene, La Jolla, CA), 1 µl of 100 ng/µl DNA and 1 µl of 20 mM of each forward and reverse primer. The amplification conditions involved an initial warming at 95 °C for 10 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 15 s. The final extension at 72 °C was for 4 min. Amplified products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide.

Denaturing high performance liquid chromatography (DHPLC): Four genomic DNA samples with known NDP mutations were used as positive controls in optimizing the temperature dependent denaturing conditions of the DHPLC protocol. The mutations are highlighted in Figure 2, which is the base pair and amino acid sequence of the NDP, and our compilation of all reported Norrie disease causing mutations of the NDP gleaned from the literature. The numbers given with each mutation (367, 481, 484, and 758) are laboratory identifying numbers.

Since all positive control DNA had known mutations only in exon 3, no alteration or shift in waveform profile is seen or expected for other exons relative to the control. Figure 3 shows optimized waveforms for PCR amplicon 3, defined by primer pair NDG-03 F/R that encodes for the 5' end of exon 3, and encompasses the coding region of that exon. The optimized melting temperature is 56 °C for this amplicon. The control waveform is at the top, followed by distinctively different waveform profiles for the four mutant DNA positive controls tested (Figure 3). All mutant DNA samples have known base pair mutations on exon 3, and were therefore expected to show differences in amplicon denaturing patterns.

Since ND is an X-linked disorder, samples expected to be heterozygous (females) were run without mixing, but those expected to be hemizygous (males) were mixed with a female wild type control, heated, and cooled prior to running. A mixture of 15 µl of each amplicon from subject DNA and from a sequence verified control was heated for 5 min at 95 °C, and then cooled to room temperature. An aliquot (5 µl) of the DNA mixtures was directly injected into a separation column. Each fragment was analyzed using three partially denaturing temperatures, which were based on fragment melting profiles. Fragments containing heteroduplexes (with a shorter retention time compared to normal controls) were sequenced to confirm putative sequence variations. Transgenomic WAVE DNA Fragment Analysis System (Transgenomic, Inc., Omaha, NE) and associated WAVEMAKER software were used.

PCR product purification and sequencing: PCR products were purified using QIAquick purification columns (Qiagen, Valencia, CA) and sequenced using BigDye™ Terminator version 3.1 on an ABI 3700® DNA Analyzer (Applied Biosystems, Foster City, CA).

Statistical Methods: To determine if there is a significant association between the studied genetic alterations in NDP and the occurrence of stage 3 ROP, basic likelihood statistics were calculated. Using a null hypothesis that the observed mutation frequency in NDP gene among ROP patients does not differ from expected frequency in control samples, a Yates corrected χ^2 analysis was performed on the data.

RESULTS

A total of 143 subjects were screened (Table 1). These subjects included 54 infants with severe ROP, 36 control infants, 31 parents of ROP subjects, and 22 wild type normal controls (Table 1). There were 29 African American subjects, 17 Caucasian, and eight other races in the affected group; 34 of these subjects were male. There were 25 African American subjects, seven Caucasian, and four other races in the control group; 19

Table 3. Norrie Disease sequence alterations identified in study subjects

Patient number (Original sample number)	Genetic alteration	Region	Sex/ Race	Study group and ROP findings	Birth weight (g)	Gestational age (weeks)
1 (ROP19)	mRNA position 1572 G>A heterozygous	3' UTR	F/AA	Affected; Stage 3 (threshold)	427	26
2 (ROP31)*	mRNA position 1253; 1 bp insertion	3' UTR	M/AA	Affected; Stage 3 (threshold)	772	25
3 (ROP12)	mRNA position 824 G>A	3' UTR	F/AA	Affected; Stage 3 (prethreshold)	720	25
4 (CR0037)	mRNA position 824 G>A	3' UTR	M/AA	Affected; Stage 3 (threshold)	1218	28
5 (CR0024)*	mRNA position 1103 A>G	3' UTR	M/AA	Affected; Stage 3 (threshold)	539	28
6 (CR001)*	mRNA position 28; 14 bp deletion	5' UTR exon 1	F/C	Affected; Stage 3 (threshold)	633	27
7 (CR001-2nd polymorphism)*	mRNA position 298 T>C heterozygous	5' UTR exon 2	F/C	Affected; Stage 3 (threshold)	633	27

These Norrie disease gene polymorphisms were found in our study subjects. These subjects were all African-American (AA) or Caucasian (C). Asterisks (*) indicate the same polymorphism was found in a parent of the affected child.

were male. The parental group contained 10 African American subjects, 20 Caucasian, and one of another race. The wild type group contained six African American subjects, eleven Caucasian, and five others. The mean gestational age of the affected infants was 25.5 weeks (range, 23.0 to 32.0 weeks) and the mean birth weight was 773 g (range, 367-1440 g). In the control group, the mean gestational age was 27.7 weeks (range, 24.0 to 34.0 weeks), and the mean birth weight was 968.0 g (range, 650 to 1360 g).

The ROP findings in the severely affected children were: Less or equal to prethreshold in either eye (n=16), and threshold or worse (more advanced staging) in at least one eye (n=38). The group with threshold or worse included five children who developed partial or total retinal detachment (stage 4 or 5) in at least one eye. The prethreshold group consisted of eight African American subjects, five Caucasians, and three of other races. The threshold group consisted of 21 African American subjects, twelve Caucasians, and five of other races.

Six infants with severe ROP and 3 of their parents had NDP sequence alterations identified (Table 3). Six of the 54 (11%) severe ROP patients carried NDP sequence alterations. None of the 36 mild ROP control group infants or any of the 22 wild types had NDP sequence alterations. A total of 6/54 of severe ROP patients and 3/89 unaffected persons had sequence alterations (p=0.063). There was no statistically significant difference between the incidence of NDP polymorphisms in ROP affecteds or in unaffected subjects.

Five subjects had various single base pair alterations in the 3' UTR. Two of the subjects were a mother and her son (ROP 31), who each had a single nucleotide insertion in the 3' UTR. Two unrelated affected subjects (ROP 12 and CR0037) had the same polymorphism (824 bp G>A). Two of the subjects, a father and his daughter (CR001) had both a 14 bp deletion in the 5' UTR of exon 1 and a single base pair alteration in the 5' UTR of exon 2 (Figure 4). A 14 bp deletion in the 5' UTR of exon 1 has been previously reported in ROP patients of another study [20].

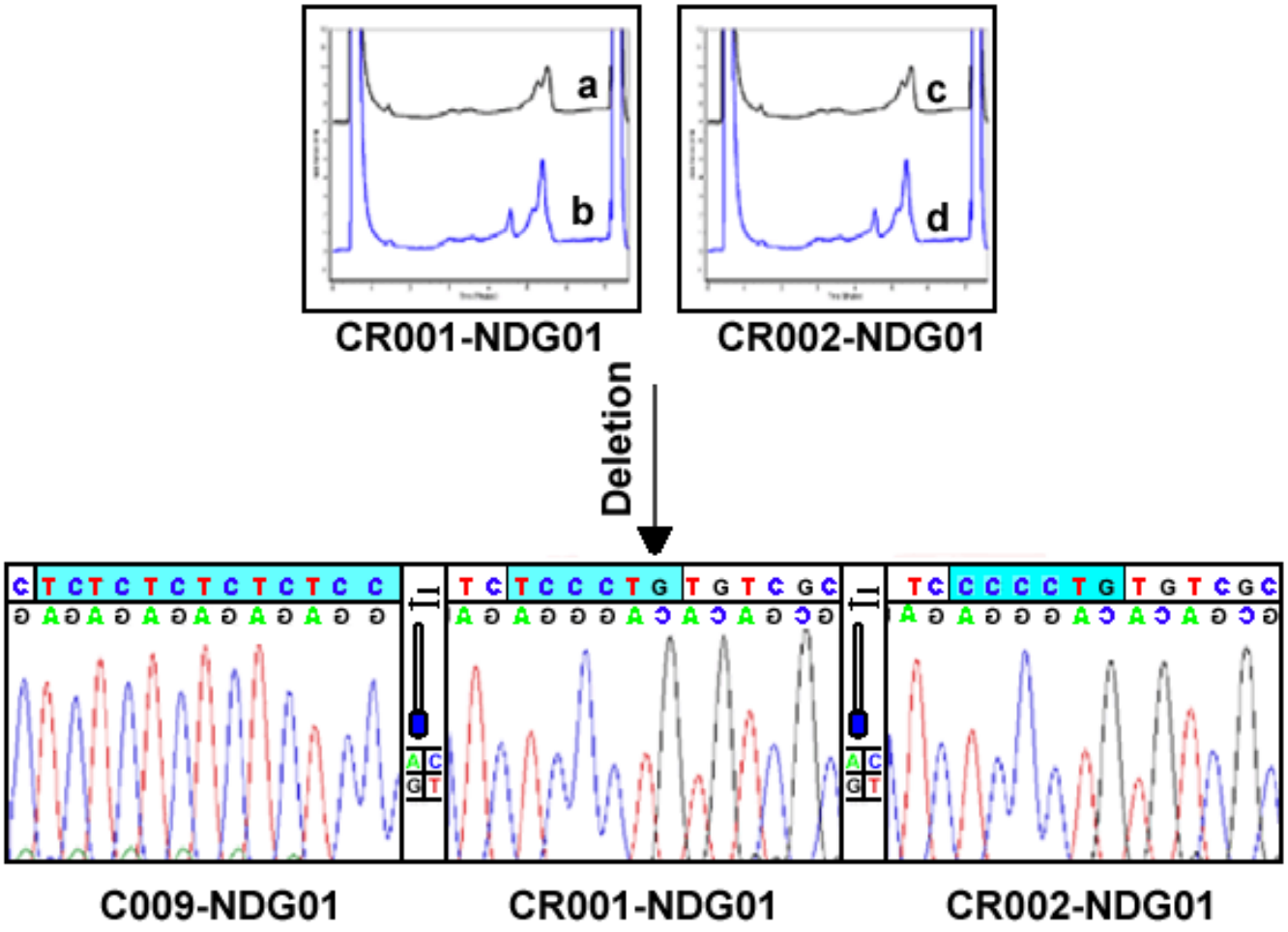

Figure 4. Denaturing high performance liquid chromatographs with corresponding base pair sequence changes. Example of denaturing high performance liquid chromatography waveform chromatographs with their corresponding sequences changes identified by direct sequencing. Samples C009 (wild type control), CR001 (daughter) and CR002 (father) are shown. The DHPLC waveforms show homoduplex controls in black and heteroduplex polymorphisms in color. The X-axis represents time in min; the y-axis is a measure of absorbency in μV. Sample sequence alteration is highlighted in blue.

Two parent-affected offspring subjects' pairs (ROP 31 and CR001) shared sequence alterations. No parent had a history of eye disease, ROP, or premature birth. Of interest, one parent had two children with premature birth, both of whom developed advanced ROP requiring treatment. This mother's polymorphism was identified in her son (ROP 31), but not in her daughter.

Five of the affected patients with NDP mutations were African American and one was Caucasian. This represents 5 out of 29 (17%) of severely affected African American patients and one out of 17 (6%) of severely affected Caucasian patients.

DISCUSSION

In this study of a large, ethnically diverse group of premature infants, we identified NDP sequence alterations in 11% of affected patients with severe ROP. These patients were recruited from a cross section of patients screened for ROP in three major metropolitan areas in the United States. The gene sequence screening encompassed all three exons of the NDP, the intron-exon boundaries, and the 5' and 3' UTRs. There was no statistically significant increase in the rate of NDP polymorphisms found in the ROP severely affected patients, nor was there a single consistent sequence alteration found in the patients who did have polymorphisms identified. Our results suggest that while NDP polymorphisms do exist in patients with ROP, they do not play a major role in disease pathogenesis. However, this report adds to the body of literature, generally supporting other studies that have found low incidences of NDP polymorphisms in ROP severely affected patients but not in controls [16,19-23]. The statistical power of the study would be increased by recruitment of a greater number of patients.

Shastry and collegues previously reported coding sequence mutations (R121W, L108P) in four of twelve patients with advanced ROP [16]. These patients had Stage 4 or 5 ROP, and all were Caucasian. The same investigators later reported results for a group of ROP patients of differing ethnic backgrounds where 2% harbored NDP sequence alterations [20]. No coding sequence mutations were noted, but one patient had a 14 bp deletion in the 5' UTR of exon 1. In our current study, we also found a 14 bp deletion in the 5' UTR of exon 1 in one patient and her father (Figure 4) [20].

Talks et al. [21] reported two out of 31 (6%) infants with advanced ROP and NDP sequence alterations. They found two different deletion sequence variants in the UTR. Both infants had Stage 4 or 5 ROP in both eyes [21]. They did not find sequence variants in 85 normal controls or in 26 premature infants with mild ROP.

Haider and colleagues reported a C-<A597 polymorphism in the UTR region of 83% of 24 patients with Stage 4 or 5 ROP, and in none of 115 control babies [22]. The significance of this sequence variant is unclear, as it does not result in an amino acid alteration. The authors speculate that the polymorphism could cause splice site errors or interact with other, unidentified upstream mutations to alter protein function. Only exon 3 was screened. Like Shastry et al. [16] these authors screened more patients with stage 4 and 5 ROP than in most other studies [16,22].

The sequence alterations identified in the current study, similar to those found by Haider et al. [22] and Talks et al. [21] were not in the coding sequence. However, upstream and downstream regulatory site mutations are increasingly being recognized as possible gene modifiers. Alterations in the 3' UTR have been shown to affect RNA stability and sequestration within the cell, and could therefore have an effect on protein quantity and localization [25]. Localization signals occur in the 3' UTR of several different species, including human. These are large redundant elements spread throughout the 3' UTR that help to determine the routing of messenger RNA in the cell [26]. Specific proteins, such as ribonucleoproteins, bind to the localization sequences and form granules. The resulting complexes coordinate transport and translation of the mRNA. Kislauskis et al. [25] reported that varying isoforms of the 3' UTR sequence directed mRNAs to different cytoplasmic compartments. The mRNA localization within the cell may also be regulated by extracellular signaling and by growth factors. Since Norrin is bound to the extracellular matrix and is involved in signaling and cell-cell interactions, alterations in Norrin function may have manifold downstream effects on protein synthesis in the cell. As Norrin is also involved in early retinal development, we postulate that NDP sequence alterations may contribute to increased susceptibility to environmental insults that also occur during early retinal development.

We were particularly interested in screening African American patients with severe ROP, since in general this demographic group has a lower statistical probability of progression to threshold disease. We theorized that those infants from this demographic who do develop severe ROP may harbor a genetic predisposition. However, the incidence of NDP polymorphisms among severely affected African American infants (17%) was similar to that of severely affected Caucasian infants (5%), thus NDP mutations do not explain the differential risk of ROP progression of disease.

This study is the first to report NDP sequence alterations in parents of offspring who develop severe ROP. Our study of a large, ethnically diverse population of ROP patients, suggests that NDP sequence variants are not associated with disease status in the majority of patients. In this and other studies, NDP sequence alterations have been found in a small number of patients with ROP.

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