



Mutation screening of patients with Leber congenital amaurosis or the enhanced S-cone syndrome reveals a lack of sequence variations in the *NRL* gene

Ceren Acar,^{1,2} Alan J. Mears,¹ Beverly M. Yashar,^{1,3} Anjali S. Maheshwary,¹ Sten Andreasson,⁴ Alfonso Baldi,⁵ Paul A. Sieving,⁶ Alessandro Iannaccone,⁷ Maria A. Musarella,⁸ Samuel G. Jacobson,⁹ Anand Swaroop^{1,3}

Departments of ¹Ophthalmology and Visual Sciences and ³Human Genetics, University of Michigan, Ann Arbor, MI; ²Department of Biology, Hacettepe University, Ankara, Turkey; ⁴Department of Ophthalmology, University of Lund, Lund, Sweden; ⁵Department of Biochemistry, Section of Molecular Pathology, II Università di Napoli, Naples, Italy; ⁶National Eye Institute, Bethesda, MD; ⁷Department of Ophthalmology, University of Tennessee Health Science Center, Memphis, TN; ⁸Department of Ophthalmology, SUNY Downstate Medical Center, Brooklyn, NY; ⁹Scheie Eye Institute, University of Pennsylvania, Philadelphia, PA

Purpose: To determine if mutations in the retinal transcription factor gene *NRL* are associated with retinopathies other than autosomal dominant retinitis pigmentosa (adRP).

Methods: Genomic DNA was isolated from blood samples obtained from 50 patients with Leber Congenital Amaurosis (LCA), 17 patients with the Enhanced S-Cone Syndrome (ESCS), and a patient with an atypical retinal degeneration that causes photoreceptor rosettes with blue cone opsin. The 5' upstream region (putative promoter), untranslated exon 1, coding exons 2 and 3, and exon-intron boundaries of the *NRL* gene were analyzed by direct sequencing of the PCR-amplified products.

Results: Complete sequencing of the *NRL* gene in DNA samples from this cohort of patients revealed only one nucleotide change. The C->G transversion at nucleotide 711 of *NRL* exon 3 was detected in one LCA patient; however, this change did not alter the amino acid (L237L).

Conclusions: No potential disease causing mutation was identified in the *NRL* gene in patients with LCA, ESCS, or the atypical retinal degeneration. Together with previous studies, our results demonstrate that mutations in the *NRL* gene are not a major cause of retinopathy. To date, only missense changes have been reported in adRP patients, and sequence variations are rare. It is possible that the loss of *NRL* function in humans is associated with a more complex clinical phenotype due to its expression in pineal gland in addition to rod photoreceptors.

The neural retina leucine zipper (*NRL*) gene encodes a member of the Maf-subfamily of bZIP (basic region leucine zipper) transcription factors [1]. Multiple isoforms of *NRL* are generated by differential phosphorylation and their expression appears to be restricted to the rod photoreceptors [2] or pineal gland (A.J. Mears, unpublished data). *NRL* is shown to regulate the expression of several phototransduction genes [3-5]. Stringent regulation of rhodopsin is achieved by *NRL* in synergy with the cone-rod homeobox transcription factor *CRX* [6,7], whereas the regulation of the gene for rod-specific cGMP phosphodiesterase β -subunit involves *NRL* and *SP4* [8]. Since mutations in phototransduction genes lead to retinal diseases (RetNet), it was anticipated that sequence changes in photoreceptor-specific transcription factors would also cause retinopathies. For example, mutations in *CRX* have been identified in a broad spectrum of retinal diseases, including retinitis pigmentosa, cone-rod dystrophy, and Leber congenital amaurosis (LCA) [9-13]. *NRL* mutation screenings have so far revealed five missense mutations in the *NRL* gene in autosomal dominant retinitis pigmentosa (adRP) and one potential mu-

tation in a patient with simplex RP [14-18]. Based on in vitro rhodopsin promoter activity assays, it was hypothesized that the first reported *NRL* mutation (S50T) results in a gain of function leading to higher activity of the mutant protein [15].

Though no loss of function mutation has been identified in the human *NRL* gene, the targeted deletion of *Nrl* in mouse (*Nrl*^{-/-}) provided considerable insights into its in vivo function [19]. The retinas of *Nrl*^{-/-} mice showed a dramatic phenotype with a complete loss of rod function (as measured by electroretinography) and rod-specific phototransduction proteins (as revealed by RNA and protein expression studies). Instead, there was enhanced S-cone (short wavelength / UV-sensitive) function, associated with increased expression of S-opsin and many cone-specific proteins. This phenotype showed similarity to the *rd7* mouse retina [20,21] and the human retinal disease, Enhanced S-cone Syndrome (ESCS) [22,23]; both of these are associated with mutations in the transcription factor *NR2E3* [20,24]. ESCS is associated with night blindness and heightened sensitivity to short wavelength light, originally hypothesized [22] and recently proven to be mediated by an increase in S-cones [25]. Sensitivity to long (red) and medium (green) wavelength light is altered to varying degrees and the extent of visual loss due to degeneration varies considerably within the ESCS phenotype [22,23,25]. It has, therefore, been

Correspondence to: Anand Swaroop, Ph.D., Department of Ophthalmology and Visual Sciences, W. K. Kellogg Eye Center, University of Michigan, 1000 Wall Street, Ann Arbor, MI, 48105; Phone: (734) 763-3731; FAX: (734) 647-0228; email: swaroop@umich.edu

proposed that NR2E3 plays a crucial role in photoreceptor differentiation. In the *Nrl*^{-/-} mouse retina, the *Nr2e3* transcript was undetectable and the temporal expression data suggested that *Nrl* is upstream of *Nr2e3* in the transcriptional hierarchy associated with photoreceptor cell fate determination [19].

Based on *NRL*'s synergistic interaction with *CRX*, the phenotype of the *Nrl*^{-/-} mouse retina, and its involvement in regulating phototransduction proteins, we hypothesized that loss of function mutations in the human *NRL* gene might result in severe retinopathy and/or congenital blindness, such as in the disease category of LCA. In addition, since *NRL* is upstream of NR2E3 in transcriptional hierarchy in the retina, we postulated that sequence variations in *NRL* may modify clinical manifestation of ESCS patients carrying *NR2E3* mutations. We also tested the hypothesis that *NRL* may be abnormal in an unusual retinal degeneration (proven not to be ESCS) that shows extensive rod loss but photoreceptor rosettes with blue cone opsin immunoreactivity, as determined by postmortem retinal histopathology in one of two affected siblings [26]. The finding in *rd7* and *Nrl*^{-/-} mice of photoreceptor rosettes and increased S cones warranted inclusion of this atypical retinopathy in the cohort screening.

Here, we report the results of direct sequencing of the 5' upstream region, untranslated exon 1, the coding exons 2 and 3, and exon-intron boundary regions of *NRL* in a cohort of 68 patients with the above-mentioned retinopathies.

METHODS

Mutation screening, described in this report, included unrelated patients with the following retinopathies: LCA (50 patients), ESCS (17 patients), and an atypical retinal degeneration (1 patient). These patients were of different races and ancestry. A majority of LCA patients had been screened for mutations in *RPE65*, *GUCY2D*, and *CRX*, and no disease-causing mutations were identified ([27] and authors' unpublished data). Of the 17 ESCS patients, 13 had been screened for mutations in the *NR2E3* gene; all ESCS patients, screened so far, have at least one allelic and potentially disease-causing variation in the *NR2E3* gene [24,25]. However, phenotypic variations in affected patients prompted us to evaluate a possible modifier effect of *NRL*. The atypical retinal degeneration patient was the affected sibling of an eye donor (Case 2 in reference [26]) and had no previous molecular investigations.

Peripheral blood samples were obtained from the subjects after informed consent, and the research procedures were in accordance with institutional guidelines and the Declaration of Helsinki. Genomic DNA was isolated from the peripheral blood using standard methods. The 5' upstream region (potential promoter region) and the three exons of *NRL* were PCR-amplified using *LA* Taq polymerase (Panvera-Takara, Madison, WI) and sequenced using the following primers, designed from the human genomic sequence (GenBank Accession number AL136295): 5' upstream region (-603 to -1; forward primer: 5'-TAT TTG GGT GGC CTC AGA AG-3', reverse primer: 5'-AGA GGG GGT TCT AGG TGA GC-3', forward nested sequencing primer: 5'-CTC CCA AGC TGG ATT AGC AA-3'), exon 1 (forward and sequencing primer: 5'-CTC

AGA GAG CTG GCC CTT TA-3', reverse primer: 5'-AGA GGG GGT TCT AGG TGA GC-3'), exon 2 (forward and sequencing primer: 5'-CCA TGT GCT CCA GAC CTC TC-3', reverse primer: 5'-CTC TCT TGG GCA GTCC TCC T-3'), exon 3 (forward primer: 5'-GGG GAT CCC AGA GAC GAG-3', reverse and sequencing primer: 5'-AAG GCG CTC TGG TAA CGA T-3'). PCR products were purified either with Qiaquick columns (Qiagen, Valencia, CA) or with the MultiScreen PCR system (Millipore, Bedford, MA). Products were sequenced by the University of Michigan DNA sequencing core using reagents (BigDye version 1) and automated sequencers (models 373, 377 and 3700) from Applied Biosystems (Foster City, CA), according to the manufacturer's protocols. Sequence variation was confirmed by manual sequencing with the ³³P-ThermoSequenase Cycle Sequencing Kit (Amersham Pharmacia Biotech, Piscataway, NJ).

RESULTS

DNA samples from 68 index patients were used for PCR with four primer sets that amplified 603 base pairs upstream of the transcription start site (putative promoter region), the three exons (including the complete coding region of 237 amino acids) and the exon-intron boundary regions of *NRL*. This comprehensive sequence analysis did not reveal any sequence change in 67 of the 68 samples. In one LCA patient, a C->G transversion was detected at nucleotide position 711 of the *NRL* coding sequence. This change was, however, in the wobble base and did not alter the amino acid residue (L237L).

DISCUSSION

In vitro studies and the identification of potential "hypermorphic" missense mutations associated with adRP in humans have implicated *NRL* as a major regulator of rod photoreceptor genes. Targeted disruption in mouse suggests an essential role for *Nrl* in the terminal differentiation of rod photoreceptors. In its absence, at least some of the rod precursors appear to develop into S-cones. With such a critical role in both photoreceptor development and function, the human *NRL* gene is an excellent candidate for retinopathies, such as LCA, ESCS, and the atypical retinal degeneration with S-cone rosettes. The lack of sequence changes in our cohort of patients suggests that mutations in *NRL* do not account for a significant proportion of these cases. It should, however, be noted that we can not rule out the possibility of intronic sequence variations, which result in splicing errors. Together with previously published studies, the mutation screenings of the *NRL* gene in 677 index patients with retinopathies (18 adRP, 23 arRP, and 12 LCA [14]; 200 adRP [15,16]; 130 adRP and 37 simplex RP [17]; 189 adRP [18]; this study) have identified only six potential disease-causing mutations in three codons (S50T: four apparently-related families in the UK [16]; S50L: two patients; S50P, P51T, P51L, and G122E). None of these changes are observed in control individuals. All of these mutations appear to originate independently and alter the putative transactivation domain of the *NRL* protein.

We identified only one nucleotide substitution in the *NRL* gene in our study and this does not result in an amino acid

change (L237L). Only two nucleotide changes (in addition to the disease-causing mutations) in the coding region of *NRL* have been reported previously but were erroneously described as L155L and V245V [17]. These two silent polymorphisms were actually L208L and L237L, the latter being the same as identified in this study. As illustrated, even these are not commonly observed in the population. A relative lack of polymorphic amino acid changes and/or single nucleotide variations in *NRL* is rather remarkable, particularly in light of the frequency of haplotype variations observed in human genes [28]. A comprehensive analysis of single nucleotide variations from 313 human genes has identified on average one single nucleotide polymorphism (SNP) every 185 bases (SNPs per kbp: 3.4 in the coding regions, 5.3 in the 5' untranslated regions, 5.9 in 5' upstream region, 6.5 in the exon-intron boundaries, and 7.0 in the 3' untranslated regions) [28]. Thus, one would predict identification of 8-9 SNPs in the *NRL* sequence analyzed in this study (a total of 1702 bp). The discovery of a single SNP in our study is significantly different from the expected 8-9 SNPs ($P < 0.01$).

Although the photoreceptor phenotype of *Nrl*^{-/-} mice suggested there may be human retinal diseases other than adRP that may be caused by *NRL* gene mutations, not a single mutation in our cohort of patients was observed. It is possible that the function(s) of *NRL* in mouse and human retina is different. Though both species have rod-dominant retinas the photoreceptor topology is distinct and humans have three different cone types [29]. In humans, additional transcription factors may compensate for the loss of *NRL* and the adRP clinical phenotype can be accounted by a dominant gain of function mutation [15]. Conversely, it is plausible that, in humans, *NRL* may have alternative functions and the loss of *NRL* would result in a more complex phenotype. For example, in mice, we detect the expression of *Nrl* in the pineal gland and the loss of *Nrl* radically affects pineal gene expression and circadian behavior (A.J. Mears, unpublished data). Continued analysis of *NRL* mutations in both animal models and human patients will therefore be necessary to gain further insight into the specific roles of this important retinal transcription factor.

ACKNOWLEDGEMENTS

We thank Debra Breuer, Elaine deCastro, Suja Hirianna, Leigh Gardner, Mirna Mustapha-Chaib, Adam Reddick, Jindan Yu, and Sepideh Zareparsis for their advice and assistance. This research was supported by grants from National Institutes of Health (EY11115, EY13385, EY13729), Foundation Fighting Blindness (Owings Mills, MD), Research to Prevent Blindness (New York, NY), Daniel Matzkin Research Fund (Philadelphia, PA), Elmer and Sylvia Sramek Charitable Foundation (Chicago, IL), Scientific and Technical Research Council of the Turkish Republic (TUBITAK), and the State Planning Organization of Turkey (DPT; Grant number 00K120660).

REFERENCES

1. Swaroop A, Xu JZ, Pawar H, Jackson A, Skolnick C, Agarwal N. A conserved retina-specific gene encodes a basic motif/leucine zipper domain. *Proc Natl Acad Sci U S A* 1992; 89:266-70.

2. Swain PK, Hicks D, Mears AJ, Apel IJ, Smith JE, John SK, Hendrickson A, Milam AH, Swaroop A. Multiple phosphorylated isoforms of *NRL* are expressed in rod photoreceptors. *J Biol Chem* 2001; 276:36824-30.
3. Rehemtulla A, Warwar R, Kumar R, Ji X, Zack DJ, Swaroop A. The basic motif-leucine zipper transcription factor *Nrl* can positively regulate rhodopsin gene expression. *Proc Natl Acad Sci U S A* 1996; 93:191-95.
4. Kumar R, Chen S, Scheurer D, Wang QL, Duh E, Sung CH, Rehemtulla A, Swaroop A, Adler R, Zack DJ. The bZIP transcription factor *Nrl* stimulates rhodopsin promoter activity in primary retinal cell cultures. *J Biol Chem* 1996; 271:29612-8.
5. Lerner LE, Gribova YE, Ji M, Knox BE, Farber DB. *Nrl* and *Sp* nuclear proteins mediate transcription of rod-specific cGMP-phosphodiesterase beta-subunit gene: involvement of multiple response elements. *J Biol Chem* 2001; 276:34999-5007.
6. Mitton KP, Swain PK, Chen S, Xu S, Zack DJ, Swaroop A. The leucine zipper of *NRL* interacts with the *CRX* homeodomain. A possible mechanism of transcriptional synergy in rhodopsin regulation. *J Biol Chem* 2000; 275:29794-9.
7. Chen S, Wang QL, Nie Z, Sun H, Lennon G, Copeland NG, Gilbert DJ, Jenkins NA, Zack DJ. *Crx*, a novel *Otx*-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. *Neuron* 1997; 19:1017-30.
8. Lerner LE, Gribova YE, Whitaker L, Knox BE, Farber DB. The rod cGMP-phosphodiesterase beta-subunit promoter is a specific target for *Sp4* and is not activated by other *Sp* proteins or *CRX*. *J Biol Chem* 2002; 277:25877-83.
9. Rivolta C, Berson EL, Dryja TP. Dominant Leber congenital amaurosis, cone-rod degeneration, and retinitis pigmentosa caused by mutant versions of the transcription factor *CRX*. *Hum Mutat* 2001; 18:488-98.
10. Sohocki MM, Sullivan LS, Mintz-Hittner HA, Birch D, Heckenlively JR, Freund CL, McInnes RR, Daiger SP. A range of clinical phenotypes associated with mutations in *CRX*, a photoreceptor transcription-factor gene. *Am J Hum Genet* 1998; 63:1307-15.
11. Swain PK, Chen S, Wang QL, Affatigato LM, Coats CL, Brady KD, Fishman GA, Jacobson SG, Swaroop A, Stone E, Sieving PA, Zack DJ. Mutations in the cone-rod homeobox gene are associated with the cone-rod dystrophy photoreceptor degeneration. *Neuron* 1997; 19:1329-36.
12. Freund CL, Gregory-Evans CY, Furukawa T, Papaioannou M, Looser J, Ploder L, Bellingham J, Ng D, Herbrick JA, Duncan A, Scherer SW, Tsui LC, Loutradis-Anagnostou A, Jacobson SG, Cepko CL, Bhattacharya SS, McInnes RR. Cone-rod dystrophy due to mutations in a novel photoreceptor-specific homeobox gene (*CRX*) essential for maintenance of the photoreceptor. *Cell* 1997; 91:543-53.
13. Swaroop A, Wang QL, Wu W, Cook J, Coats C, Xu S, Chen S, Zack DJ, Sieving PA. Leber congenital amaurosis caused by a homozygous mutation (R90W) in the homeodomain of the retinal transcription factor *CRX*: direct evidence for the involvement of *CRX* in the development of photoreceptor function. *Hum Mol Genet* 1999; 8:299-305.
14. Farjo Q, Jackson A, Pieke-Dahl S, Scott K, Kimberling WJ, Sieving PA, Richards JE, Swaroop A. Human bZIP transcription factor gene *NRL*: structure, genomic sequence, and fine linkage mapping at 14q11.2 and negative mutation analysis in patients with retinal degeneration. *Genomics* 1997; 45:395-401.
15. Bessant DA, Payne AM, Mitton KP, Wang QL, Swain PK, Plant C, Bird AC, Zack DJ, Swaroop A, Bhattacharya SS. A mutation in *NRL* is associated with autosomal dominant retinitis

- pigmentosa. *Nat Genet* 1999; 21:355-6.
16. Bessant DA, Payne AM, Plant C, Bird AC, Swaroop A, Bhattacharya SS. NRL S50T mutation and the importance of 'founder effects' in inherited retinal dystrophies. *Eur J Hum Genet* 2000; 8:783-7.
 17. Martinez-Gimeno M, Maseras M, Baiget M, Beneito M, Antinolo G, Ayuso C, Carballo M. Mutations P51U and G122E in retinal transcription factor NRL associated with autosomal dominant and sporadic retinitis pigmentosa. *Hum Mutat* 2001; 17:520.
 18. DeAngelis MM, Grimsby JL, Sandberg MA, Berson EL, Dryja TP. Novel mutations in the NRL gene and associated clinical findings in patients with dominant retinitis pigmentosa. *Arch Ophthalmol* 2002; 120:369-75.
 19. Mears AJ, Kondo M, Swain PK, Takada Y, Bush RA, Saunders TL, Sieving PA, Swaroop A. Nrl is required for rod photoreceptor development. *Nat Genet* 2001; 29:447-52.
 20. Akhmedov NB, Piriev NI, Chang B, Rapoport AL, Hawes NL, Nishina PM, Nusinowitz S, Heckenlively JR, Roderick TH, Kozak CA, Danciger M, Davisson MT, Farber DB. A deletion in a photoreceptor-specific nuclear receptor mRNA causes retinal degeneration in the rd7 mouse. *Proc Natl Acad Sci U S A* 2000; 97:5551-6.
 21. Haider NB, Naggert JK, Nishina PM. Excess cone cell proliferation due to lack of a functional NR2E3 causes retinal dysplasia and degeneration in rd7/rd7 mice. *Hum Mol Genet* 2001; 10:1619-26.
 22. Jacobson SG, Marmor MF, Kemp CM, Knighton RW. SWS (blue) cone hypersensitivity in a newly identified retinal degeneration. *Invest Ophthalmol Vis Sci* 1990; 31:827-38.
 23. Jacobson SG, Roman AJ, Roman MI, Gass JD, Parker JA. Relatively enhanced S cone function in the Goldmann-Favre syndrome. *Am J Ophthalmol* 1991; 111:446-53.
 24. Haider NB, Jacobson SG, Cideciyan AV, Swiderski R, Streb LM, Searby C, Beck G, Hockey R, Hanna DB, Gorman S, Duhl D, Carmi R, Bennett J, Weleber RG, Fishman GA, Wright AF, Stone EM, Sheffield VC. Mutation of a nuclear receptor gene, NR2E3, causes enhanced S cone syndrome, a disorder of retinal cell fate. *Nat Genet* 2000; 24:127-31.
 25. Milam AH, Rose L, Cideciyan AV, Barakat MR, Tang WX, Gupta N, Aleman TS, Wright AF, Stone EM, Sheffield VC, Jacobson SG. The nuclear receptor NR2E3 plays a role in human retinal photoreceptor differentiation and degeneration. *Proc Natl Acad Sci U S A* 2002; 99:473-8.
 26. Milam AH, Jacobson SG. Photoreceptor rosettes with blue cone opsin immunoreactivity in retinitis pigmentosa. *Ophthalmology* 1990; 97:1620-31.
 27. Lotery AJ, Namperumalsamy P, Jacobson SG, Weleber RG, Fishman GA, Musarella MA, Hoyt CS, Heon E, Levin A, Jan J, Lam B, Carr RE, Franklin A, Radha S, Andorf JL, Sheffield VC, Stone EM. Mutation analysis of 3 genes in patients with Leber congenital amaurosis. *Arch Ophthalmol* 2000; 118:538-43.
 28. Stephens JC, Schneider JA, Tanguay DA, Choi J, Acharya T, Stanley SE, Jiang R, Messer CJ, Chew A, Han JH, Duan J, Carr JL, Lee MS, Koshy B, Kumar AM, Zhang G, Newell WR, Windemuth A, Xu C, Kalbfleisch TS, Shaner SL, Arnold K, Schulz V, Drysdale CM, Nandabalan K, Judson RS, Ruano G, Vovis GF. Haplotype variation and linkage disequilibrium in 313 human genes. *Science* 2001; 293:489-93.
 29. Curcio CA, Sloan KR, Kalina RE, Hendrickson AE. Human photoreceptor topography. *J Comp Neurol* 1990; 292:497-523.