

MUTATION IN BRIEF

Mutation Analysis of *NR2E3* and *NRL* Genes in Enhanced S Cone Syndrome

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Ten new and seventeen previously reported Enhanced S Cone Syndrome (ESCS) subjects were used to search for genetic heterogeneity. All subjects were diagnosed with ESCS on the basis of clinical, psychophysical and/or electroretinography testing using published criteria. Mutation analysis was performed on the *NR2E3* nuclear receptor gene by single strand conformation analysis and direct sequencing, which revealed either homozygous (N=13) or compound heterozygous (N=11) mutations in 24 subjects (89%), heterozygous mutations in 2 subjects (7%) and no mutations in 1 subject (4%). Fifteen different mutations were identified, including six not previously reported. The subject (Patient A) with no detected *NR2E3* mutation had features not usually associated with ESCS, in particular moderate rod photoreceptor function in peripheral retina and an abnormally thick retinal nerve fibre layer. Mutation analysis of the *NRL*, *CRX*, *NR1D1* and *THRB* genes in this individual revealed a heterozygous one base-pair insertion in exon 2 of the *NRL* gene, which results in a predicted truncation of the *NRL* protein. Loss-of-function *NRL* alleles have not been described previously in humans, but since the same mutation was present in unaffected family members, it raises the possibility that the abnormal ESCS phenotype in Patient A may result from a digenic mechanism, with a heterozygous *NRL* mutation and a mutation in another unknown gene. © 2004 Wiley-Liss, Inc.

KEY WORDS: Enhanced S Cone Syndrome, *NR2E3*, *NRL*, retinal development

INTRODUCTION

The human retina contains a mosaic of photoreceptor cell types consisting of short (S), medium (M) and long

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(L) wavelength cone photoreceptors. Mutations in the nuclear receptor gene, *NR2E3* (MIM# 604485), cause the autosomal recessive disorder Enhanced S Cone Syndrome (ESCS; MIM# 268100), which is a disorder of photoreceptor development with the unique feature of increased function and excess numbers of the minority S (blue) cone photoreceptor type, at the expense of other photoreceptor types (Jacobson et al., 1990, 1991; Roman and Jacobson, 1991; Kellner et al., 1993; Hood et al., 1995; Milam et al., 2002). ESCS also causes night blindness, which results from greatly reduced function of the rod photoreceptors, and some diminution of function of the L and M wavelength (red/green) cone populations. The consequences of such misspecification of developing retinal neurons are uncertain but retinas in ESCS show a constellation of abnormalities including splitting (schisis), yellow flecks, pigment clumping and atrophy and the disorder is a slowly progressive retinal degeneration (Jacobson et al., 1990, 1991; Marmor et al., 1990; Roman and Jacobson, 1991; Kellner et al., 1993; Hood et al., 1995; Milam et al., 2002). The aim of the present investigation was to establish whether ESCS is genetically homogeneous. The results show that while 96% of ESCS subjects have at least one *NR2E3* mutation, there is a small subset without detectable change in the *NR2E3* gene. One subject in this latter group had a heterozygous frameshift mutation in exon 2 of the *NRL* gene and showed an ESCS phenotype with unusual features.

METHODS

Mutation screening

NR2E3 gene

Mutation analysis of the *NR2E3* gene (Acc. no. NM_014249) was performed initially using single strand conformation analysis (SSCA; Vervoort et al., 2000). PCR products were electrophoresed on non-denaturing gels (15ml MDE solution [Flowgen], 1.8µl 20% TBE, 40µl TEMED, 240µl 10% APS, 43 ml dH₂O) overnight in 0.6x TBE at 4°C. The gels were visualised by autoradiography. Samples which showed shifts by SSCA were sequenced directly and compared with control samples using Sequencher 4.0.5 software (GeneCode). If no shift was observed on SSCA of all eight *NR2E3* exons, the entire coding region and exon-intron boundaries were sequenced. Mutations confirmed by sequencing on both strands were further confirmed by DNA digests with restriction enzymes or by PCR with mutation-specific primers. DNA samples from 50 ethnically matched controls (100 chromosomes) were screened for each novel mutation using a specific digest or mutation-specific primers, to exclude polymorphisms.

The 500 bp promoter region of *NR2E3* exon 1 was sequenced using the following primers:-

Forward 5'-GTGGGCAGATCACTTGAGGT-3'

Reverse 5'-GGTCTGGTCTCCATGGGTTA-3'

The *NR2E3* genomic DNA sequences were obtained from the Ensembl Genome Browser (www.ensembl.org) and primers were designed using Primer3 (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi). PCR products were sequenced with both forward and reverse primers to ensure quality.

Further mutation screening in ESCS Patient A

In an ESCS patient in whom no *NR2E3* mutation was identified (Table 1, #20, or Patient A), other molecular studies were performed. Since *NR2E3* is proposed to be downstream of *NRL* in the transcriptional regulatory hierarchy (Mears et al., 2001), we examined the *NRL* gene in the patient and 4 family members and evaluated the segregation of *NRL* alleles. The three exons and the upstream region of the *NRL* gene were directly sequenced as described (Bessant et al., 1999; Acar et al., 2003).

Analysis of *NRL*, *NR1D1*, *CRX* and *THRB* genes

A 600 bp 5' upstream region (potential promoter region) as well as the three exons of *NRL* were amplified via PCR with LA Taq polymerase (Panvera-Takara, Madison, WI) and the following oligonucleotide primers: 5' upstream region and exon 1: (forward and sequencing primer) 5'-CTC CCA AGC TGG ATT AGC AA-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 GTC CTC CT-3' exon 3: (forward primer) 5'-ACT GGA GCA ACG GTC AGC-3' (reverse and sequencing primer) 5'-CAG CCC CCA CTA CAC CAC-3'. PCR products were purified with Qiaquick columns (Qiagen, Valencia, CA). The purified PCR products were then 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). All sequence data were read

using Chromas version 1.45 software (Griffith University, Southport, Australia). The complete coding region and exon-intron boundaries of *CRX* were sequenced in Patient A using the primers and conditions described by Swain et al. (1997) and, for *NR1D1* (Acc. no. NM_021724), using the following primers:-.

Exon 1 F 5'-CTAAACACGCCACCCTGACT-3'
 Exon 1 R 5'-GACTCCTCAAGTGTGCTCC-3'
 Exons 2,3,4 F 5'-GCAGTCGATATTCCTCAAGGA-3'
 Exons 2,3,4 R 5'-GCAACAGGATGAGAACAGCA-3'
 Exon 5 F 5'-AGCAATCCCTCCTAGGGAAA-3'
 Exon 5 R 5'-GGAAAAGATTGAAGGAGGGG-3'
 Exon 6 F 5'-GCTGAGTGTGGGGATAGCAT-3'
 Exon 6 R 5'-CAAGGTCAAAGCAGGAAGC-3'
 Exon 7 F 5'-TCTCCTACCTCAGCCTTCCA-3'
 Exon 7 R 5'-GACTCCCTTGCTTTTTGCTG-3'
 Exon 8 F 5'-AGGTGACACTTGAGTTGGGG-3'
 Exon 8 R 5'-AATAGGGGAGGAGGGGAAGT-3'

The *NR1D1* genomic DNA sequences were obtained from the Ensembl Genome Browser (www.ensembl.org) and primers were designed using Primer3 (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi). All PCR products were sequenced with both forward and reverse primers to ensure quality.

Exons 1, 9 and 10 of the *THRB* gene were sequenced in Patient A (H.A. Chapman Institute of Medical Genetics, Tulsa, OK; University of Pennsylvania DNA Sequencing Facility, Philadelphia, PA; Sharon et al., 2003).

Comparative sequence analysis was carried out using the CLUSTAL W (1.82) multiple sequence alignment program (<http://www.ebi.ac.uk/>). Input sequences included human NR2E3 (NP_055064), human tailless (QY466), mouse tailless (Q64104), *Xenopus laevis* tailless (P70052), *Drosophila melanogaster* tailless (A35602), human retinoic receptor- β (NP_068811), rat retinoic acid receptor α (Q05343), chicken transcription factor 2 (Q90733).

The mutation nomenclature uses the first nucleotide of the ATG codon in the cDNA (NM_014249.2) as +1, and the first codon of the protein as +1 (NM_014249.2).

Clinical studies

All ESCS patients included in the study underwent clinical examinations and were diagnosed as having enhanced S cone function by psychophysical testing and/or electroretinography (ERG). In a proband without *NR2E3* mutation (Patient A), the following retinal function studies were specifically performed: static threshold perimetry in the dark-adapted (500- and 650-nm stimuli) and light-adapted (600-nm stimulus on 2.7 log td white background; 440-nm stimulus on a 4.0 log td yellow background) states using a modified automated perimeter, to determine the retinal sensitivities to light of different wavelengths (Humphrey Field Analyzer, San Leandro, CA); full-field electroretinography (ERG) to determine the electrical responses of the retina to light; and cross-sectional retinal imaging with optical coherence tomography (OCT3, Zeiss Humphrey Instruments, Dublin, CA). The methods used to characterize phenotype were those used in our previous studies of ESCS-*NR2E3* (Jacobson et al., 1990, 1991; Roman and Jacobson, 1991; Kellner et al., 1993; Hood et al., 1995; Haider et al., 2000; Jurklies et al., 2001; Milam et al., 2002; Cideciyan et al., 2003). Informed consent for all procedures was obtained from subjects after the nature of the studies had been explained. Research procedures were in accordance with institutional guidelines and the Declaration of Helsinki.

TABLE 1. Summary of NR2E3 and NRL Mutation Analyses in ESCS Patients

| Patient | NR2E3 nucleotide change [#] | Traditional nomenclature | NR2E3 amino acid change [#] | NRL change | Geographic and/or ethnic origin(s) | Reference |
|-------------------|--------------------------------------|--------------------------|--------------------------------------|---------------------|------------------------------------|------------|
| 1 | c.119-2A>C c.119-2A>C | IVS1-2A>C IVS1-2A>C | | ND | Syrian/Lebanese | b, c |
| 2 | c.1220T>A c.1220T>A | | p.M407K p.M407K | Normal | Spanish/Italian | b |
| 3 | c.925C>G c.932G>A | | p.R309G p.R311Q | ND | Italian | b, d |
| 4 | c.119-2A>C c.310C>T | IVS1-2A>C | p.R104W | ND | British/American | b |
| 5 | c.701G>C | | p.W234S | Normal | British/German | b |
| 6 | c.119-2A>C c.1095C>G | IVS1-2A>C | p.P365P | ND | German | This study |
| 7 | c.290G>A c.290G>A | | p.R97H p.R97H | ND | British | b, c |
| 8 | c.119-2A>C c.788T>C | IVS1-2A>C | p.L263P | ND | British/Italian | This study |
| 9 | c.119-2A>C c.932G>A | IVS1-2A>C | p.R311Q | Normal | Western European | b, d |
| 10 | c.119-2A>C c.932G>A | IVS1-2A>C | p.R311Q | Normal | Ashkenazi Jewish | b, d |
| 11 | c.932G>A c.932G>A | | p.R311Q p.R311Q | Normal | Ashkenazi Jewish | b, c |
| 12 | c.932G>A c.932G>A | | p.R311Q p.R311Q | Normal | Ashkenazi Jewish | b, d |
| 13 | c.932G>A c.932G>A | | p.R311Q p.R311Q | Normal | Ashkenazi Jewish | b, d |
| 14 | c.119-2A>C c.119-2A>C | IVS1-2A>C IVS1-2A>C | | ND | Ashkenazi Jewish/ Italian | b |
| 15 | c.932G>A | | p.R311Q | Normal | Ashkenazi Jewish | b, d |
| 16 | c.119-2A>C c.119-2A>C | IVS1-2A>C IVS1-2A>C | | ND | Cuban | b |
| 17 | c.932G>A c.932G>A | | p.R311Q p.R311Q | Normal | Ashkenazi Jewish | b, d |
| 18 | c.932G>A c.932G>A | | p.R311Q p.R311Q | Normal | Ashkenazi Jewish | b, d |
| 19 | c.119-2A>C c.194-202del9 | IVS1-2A>C | p.N65_C67del ^a | ND | Ashkenazi Jewish | b |
| 20 (Patient A) | | | | c.353insC (p.L75fs) | Italian | This study |
| 21 | c.119-2A>C c.767C>A | IVS1-2A>C | p.A256E | ND | British/American Indian | This study |
| 22 (Patient B) | c.119-2A>C c.1007T>C | IVS1-2A>C | p.L336P | ND | British | This study |
| 23 | c.119-2A>C c.481delA | IVS1-2A>C | | ND | German | This study |
| 24 | c.119-2A>C c.119-2A>C | IVS1-2A>C IVS1-2A>C | | ND | German | This study |
| 25 | c.119-2A>C c.119-2A>C | IVS1-2A>C IVS1-2A>C | | ND | German | This study |
| 26 | c.263G>T c.263G>T | | p.G88V p.G88V | ND | German | This study |
| 27 | c.481delA c.1057C>G | | p.L353V | ND | German | This study |

Novel mutations are shown in bold. NRL mutation analyses determined to be normal were reported previously in Acar et al. (2003). ^aN65_C67del was previously reported as del67_69 by Haider et al. (2000). Reported previously in: ^bHaider et al., 2000; ^cMiano et al., 2000; ^dMilam et al., 2002. ND, not determined. [#]The mutation nomenclature uses the first nucleotide of the ATG codon in the cDNA (NM_014249.2) as +1, and the first codon of the protein as +1 (NM_014249.2).

RESULTS

Mutation analysis of NR2E3

Mutation analysis of the *NR2E3* gene was carried out in 40 subjects, 27 of whom were unrelated and diagnosed clinically with ESCS. The subjects included 10 new ESCS patients and 17 reported previously (Haider et al., 2000; Miano et al., 2000; Milam et al., 2002). The results are shown in Table 1. At least one *NR2E3* mutation was found in 26 of the 27 subjects (96%) and no mutations were found in 1 subject (4%). Thirteen ESCS subjects were homozygous (48%) and 11 subjects compound heterozygous (41%) for known or proposed disease-causing mutations in *NR2E3*. Two ESCS subjects (#5, 15) were heterozygous for established disease-causing mutations, suggesting that rare intronic or other non-coding mutations may have been missed.

Fifteen different mutations were identified in the ESCS subjects, including six that have not been reported previously. The most common mutations were the intron 1 splice acceptor mutation c.119-2A>C (IVS1-2A>C), which was found in 19 out of 50 proposed disease causing alleles (38%), and a p.R311Q (c.932G>A) missense substitution, present in 14 alleles (28%) – both reported by Haider et al. (2000). Only one other allele, a novel one basepair deletion (c.481delA) was found in more than one individual (#23, 27).

The six newly identified *NR2E3* mutations which are proposed to be disease-causing include, firstly, deletion of a single nucleotide at position 481 (c.481delA) in exon 4. This causes a frameshift in the amino acid sequence, leading to 17 abnormal amino acids followed by a premature stop codon. The c.481delA mutation is predicted to delete the entire ligand binding domain. It was found in two unrelated German subjects (#23, 27), as a compound heterozygous change together with either c.119-2A>C (IVS1-2A>C) or p.L353V mutations. The second novel mutation (p.L263P; c.788T>C) occurs in exon 6 and is predicted to cause a non-conservative missense substitution at a site within the ligand binding domain that is conserved in 8 members of the nuclear receptor family (human NR2E3 (hsNR2E3), human tailless (hsTll), mouse tailless (mmTll), *Xenopus laevis* tailless (xlTll), *Drosophila melanogaster* tailless (dmTll), human retinoic receptor- β (hsRar β), rat retinoic acid receptor α (rnRar α), chicken transcription factor 2 (ggTf2)). The third proposed novel mutation (c.1095C>G) is a synonymous (p.P365P) substitution which is predicted to create a strong cryptic splice donor site 8 nucleotides from the end of exon 7, in the context of what appears to be a weak normal splice donor site, with a GA in the +1 and +2 positions instead of the canonical intronic GT. The c.1095C>G mutation is adjacent to a GT dinucleotide in exon 7 and increases the predicted strength of this cryptic site from 70.4% to 83.9% of the highest scoring splice donor sequence, compared with 20.6% for the normal sequence, based on the predictions of Senapathy et al. (1990) (<http://home/snafu.de/probins/Splice/SpliceApplet.html>). If the new cryptic splice site is preferentially used, it would result in 10 novel amino acids followed by a premature stop codon, deleting the terminal 45 amino acids, and severely truncating the ligand binding domain. Expression of *NR2E3* is thought to be confined to the retina, and it was not possible to test the effect of the proposed c.1095C>G mutation on splicing by searching for abnormal splicing products using reverse transcriptase polymerase chain reaction (RT-PCR) of mRNA from blood cells. The ESEfinder program (<http://exon.cshl.edu/ESE/>) (Cartegni et al., 2003) failed to identify an exonic splicing enhancer (ESE) effect of the proposed c.1095C>G mutation.

The fourth novel mutation (p.L336P; c.1007T>C) is a non-conservative missense substitution of proline for leucine in exon 7, at a site that is conserved in 5/8 members of the nuclear receptor family within the ligand binding domain (hsNR2E3, dmTll, hsRar β , rnRar α , ggTf2). The fifth novel mutation is a c.263G>T nucleotide substitution in exon 3, which would give rise to a glycine to valine substitution at residue 88 (p.G88V) within the DNA binding domain. This residue is conserved in 5/8 members of the nuclear receptor family (hsNR2E3, hsTll, mmTll, xlTll, dmTll) but is a basic residue in the retinoic acid receptors and chicken transcription factor 2. The sixth novel mutation is a c.1057C>G nucleotide substitution, which would give rise to a predicted leucine to valine substitution at codon 353 (p.L353V) within the ligand binding domain. Although this is a conservative amino acid substitution, it occurs within the ligand binding domain at a site that is absolutely conserved in all eight members of the nuclear receptor family. None of these proposed mutations were present in 100 ethnically matched control chromosomes.

Further molecular and phenotypic analyses of a patient without NR2E3 mutations

The ESCS patient without any *NR2E3* mutations was analysed further. Patient A (#20) was screened for mutations in the 500 nucleotide sequence immediately upstream of the *NR2E3* translation start site in exon 1, including the putative basal promoter region, but no sequence variation was found. Since *NR2E3* is proposed to be

downstream of *NRL* in the transcriptional regulatory hierarchy (Mears et al., 2001), the *NRL* gene was examined in this patient and 4 family members to evaluate the segregation of *NRL* alleles. A heterozygous insertion of a single cytosine residue after nucleotide 353 in exon 2 of the *NRL* gene (c.353insC) was identified. This insertion is predicted to result in a frameshift (L75fs), altering the leucine residue at codon 75 to a proline, and causing premature truncation of the *NRL* protein with 18 novel residues at the carboxyl terminus (Fig. 1). In addition to the patient, this sequence variation was in the father and two siblings, who were clinically unaffected. The *NR1D1* and *CRX* genes were also screened in Patient A for sequence variation but none was found. Sequence variation in *THRB* was not identified in the coding regions of exons 1, 9 and 10. At position 1536 in exon 10, a T to C transition resulted in a synonymous substitution (p.F417F) (which was also present in the unaffected father of the patient).

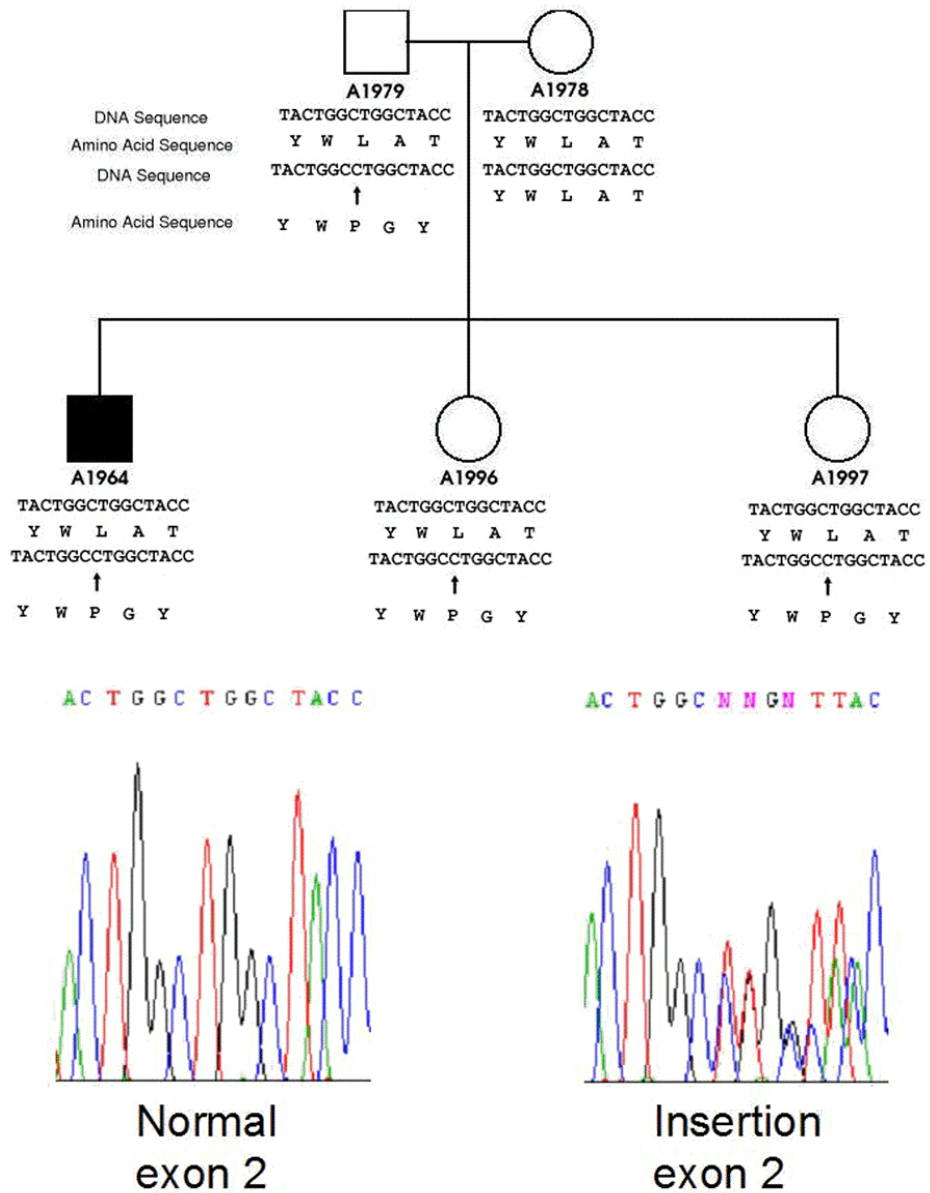


Figure 1. Segregation of putative *NRL* null allele (L75fs) in exon 2 of Patient A (shaded), his parents and two sisters. The normal allele and sequence is shown above and the arrowed mutant sequence below. The corresponding normal and mutant electropherograms are also shown.

Features of the disease expression in Patient A at age 28 years are illustrated (Fig. 2). The patient recalled night vision disturbances and reduced ability to see clearly from childhood. Between ages 5 and 12 years, visual acuities were reduced to 20/50 in each eye (with a +3.00 spherical equivalent refractive error) and there were abnormal retinal fundus features such as clumped pigment and yellow lesions in the region of the vascular arcades and more peripheral retina. The clinical diagnosis until age 28 was chorioretinitis (inflammation of the choroid and retina) of uncertain etiology. In his early 20's, visual acuity further declined to 20/80; this change in vision coupled with the observation of apparent optic nerve elevation in both eyes led to extensive work-ups for cerebral abnormalities or systemic inflammation. Results of all investigations were normal. Kinetic visual fields examined over the course of a decade showed mid-peripheral relative scotomas (blind spots) but otherwise a wide peripheral extent of function.

At age 28 years, an electroretinogram (ERG) was performed (Fig. 2A, upper). To a dim blue flash of light in the dark-adapted state, normal subjects show a rod ERG b-wave but Patient A had no detectable response, indicating severe rod dysfunction. For comparison, Patient B (#22), a 13 year-old compound heterozygote for *NR2E3* mutation (c.119-2A>C, p.L336P) also has no detectable response (Fig. 2A). A bright white flash in the dark-adapted state elicits a response resulting from both rod and cone components in the normal subject; the same flash on a light-adapting background produces a smaller, faster response from cones. To this light stimulus in Patients A and B, there are similar-appearing negative waveforms, regardless of whether the individual was dark- or light-adapted, and the waveforms are unlike those of the normal subject. Compared to the same results in Patient B, the light-adapted waveform in Patient A is lower in amplitude than the dark-adapted waveform. Cone flicker stimuli elicit responses in both patients that are abnormally reduced in amplitude and delayed in timing.

Psychophysical testing with chromatic perimetry (Fig. 2A, lower) in normal subjects (average data displayed; n=18, ages 15-57 years) shows at least 3 log units of rod sensitivity across the visual field. Unusually for ESCS, Patient A revealed peripheral rod-mediated vision (with between 2 and 3 log units of rod sensitivity) but otherwise little or no detectable rod function. Patient C (sibling of Patient B) had essentially no measurable rod-mediated vision across the visual field. Normal L/M and S cone function (average data; n=11; ages 19-57 years) decline with increasing eccentricity from the central field. Patients A and C have very reduced L/M cone sensitivities, mainly showing function in the central field. S cone function in Patient A was subnormal near fixation and in the mid-periphery, normal in the pericentral field, and supernormal at loci in the far periphery. Patient C has normal S cone function centrally, scattered loci in the mid and far periphery with subnormal results, and peripheral loci with supernormal function. There is relatively greater S cone than L/M cone sensitivity outside the central visual field in both patients, whereas the relationship is the opposite throughout the visual field of normal subjects.

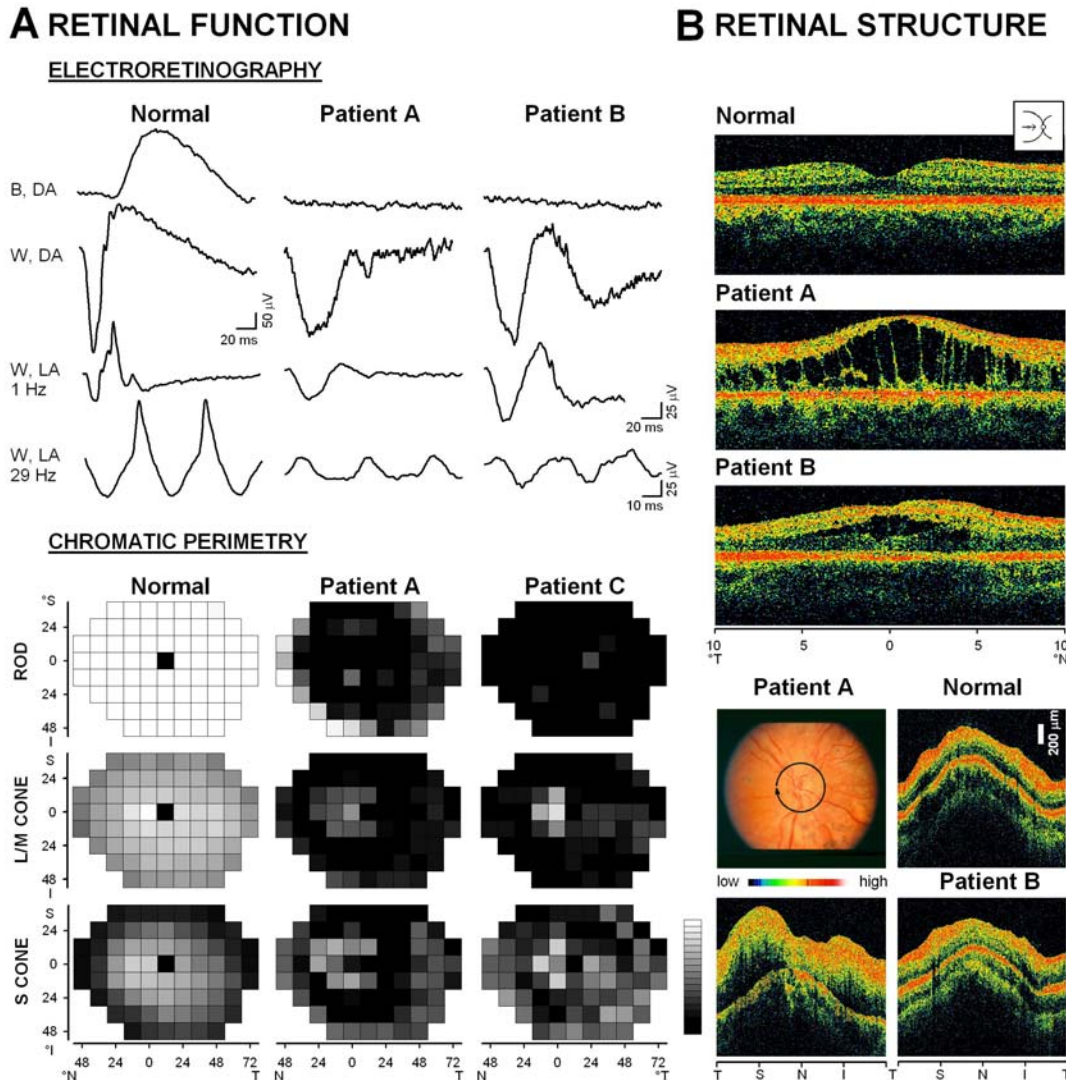


Figure 2. Phenotype of a patient without *NR2E3* mutation but with heterozygous *NRL* mutation. (A) Retinal function by electroretinography (upper panels) and static threshold color (chromatic) perimetry (lower panels) in a normal subject (left column) compared with an ESCS patient without *NR2E3* mutation (Patient A, middle column) and ESCS patients with *NR2E3* mutations (sibling Patients B,C, right column). Blue light flashes in the dark-adapted (B, DA) state; a white light flash, dark-adapted (W, DA), and the same stimulus in the light-adapted state (W, LA 1Hz); and white flickering light (W, LA 29 Hz) produce very different waveforms in the patients compared with the normal. Calibrations apply to responses above and to the left. Chromatic perimetry results are displayed as grey scale maps of rod (upper row), L/M cone (middle row), and S cone (lower row) function. Rod sensitivity is to a 500nm stimulus, DA (when sensitivity differences between 500 and 650nm stimuli indicate rod function); L/M cone sensitivity is to a 650nm stimulus, DA, in the patients and during the cone plateau of dark adaptation in normals. S cone sensitivity is to 440nm on a yellow background. Grey scale represents 3 (maximum, white) log units of sensitivity for rod and L/M cone maps, and 2.5 log units for S cones. In addition to the dramatic differences between patient and normal data, notable features in the maps include: retained peripheral regions of rod function in Patient A versus Patient C; and the regions of greater S cone function compared with L/M cone function in both patients, in contrast to the opposite relationship across the normal visual field. (B) Cross-sectional images of the right eye by optical coherence tomography (OCT) of the central retina (upper 3 panels; inset shows scan location and direction) and of an annulus (diameter, 3.4mm) around the optic nerve (lower 3 panels; location and direction of circular scan are shown on retinal photograph from Patient A). T, temporal; S, superior; N, nasal; I, inferior. Eccentricities from 0 (fovea) in perimetry and OCT are in degrees on visual field and retina, respectively.

Examination of the microstructure of the normal central retina using optical coherence tomography (OCT) shows a foveal depression, parafoveal thickening and distinct retinal layers or laminae previously demonstrated to correspond to the nuclear and synaptic layers of the retina (Huang et al., 1998, 2000; Jacobson et al., 2003). Patient

A has loss of normal foveal architecture due to schisis (splitting of retinal layers); there is separation of laminae with interconnecting pillars of tissue, indicating disruption of retinal structure. Patient B also has loss of normal structure but less profound elevation and schisis than in Patient A. Scans encircling the optic nerve in normal subjects show nerve fibre layer (NFL) thickness is greatest at the superior and inferior poles of the optic nerve with less NFL thickness temporally and nasally. Patient B has NFL thickness that falls within normal limits, but Patient A has greatly increased thickness (Jacobson et al., 2003).

DISCUSSION

Fifty *NR2E3* mutations were found in the 27 ESCS subjects, which included 10 subjects previously unreported. This is a large proportion (93%) of the possible disease-causing alleles, and includes 15 different mutations, of which 6 are novel. The proposed novel mutations each occur at conserved residues either within the ligand binding domain (p.L263P, p.L336P, p.L353V), DNA binding domain (p.G88V) or are predicted to truncate the ligand binding domain (c.481delA, c.1095C>G). Two novel missense mutations (p.L263P, p.L336P) involve the introduction of a proline, which is known to have helix-breaking properties, into the ligand binding domain, consistent with impaired function, and the others (p.G88V, p.L353V) occur at highly conserved residues. Four of the six novel mutations occur in compound heterozygotes with established disease-causing mutations (c.1095C>G, c.481delA, p.L263P, p.L336P). Two previously reported mutations account for two-thirds of all identified mutations – the splice acceptor mutation in intron 1 (c.119-2A>C, formerly IVS1-2A>C) and the missense substitution in the ligand binding domain (R311Q). The results are summarised in Table 1, which shows that 13 ESCS subjects were mutation homozygotes (48%), 11 were compound heterozygotes (41%), 2 were heterozygotes (7%) and a single subject (4%) showed no *NR2E3* mutations.

The high yield of disease-causing mutations suggests, firstly, that there is considerable specificity to the ESCS diagnosis and, secondly, that most *NR2E3* mutations are detectable by analysis of the coding and splice sites. The possibility remains that heterozygous deletions were present as the second disease-causing allele in some ESCS subjects carrying single mutations, since this was not excluded by Southern analysis or quantitative PCR. The identified mutations are highly clustered within the conserved and functionally important ligand binding and DNA binding domains. These results suggested that further analyses of the subject (Patient A; #20) with an unequivocal diagnosis of ESCS who did not show *NR2E3* mutations might be fruitful. This led to the identification of a heterozygous single nucleotide insertion in exon 2 of the *NRL* gene, which is predicted to result in a frameshift (L75fs) and truncation of the *NRL* protein. However, since the same mutation was found in the father and two siblings of Patient A, it is not disease-causing *per se*. Nevertheless, this is the first report of a putative null allele of *NRL* identified in mutation screening studies (Farjo et al., 1997; Bessant et al., 1999; Milla et al., 2002; DeAngelis et al., 2002; Acar et al., 2003). The possibility remains that *NR2E3* mutations were missed in Patient A and that the *NRL* null mutation is an unrelated rare variant. However, the fact that two ESCS-associated mutations were found in 89% of patients prompts the hypothesis that *NRL* may be acting digenically or as a heterozygous (epistatic) modifier of another disease-causing locus/allele (Badano et al., 2003), leading to the variant ESCS phenotype in Patient A. Since *NRL* is part of a transcription regulatory complex with *CRX* and *NR1D1* *in vivo* and synergistically activates the expression of rhodopsin (Mitton et al., 2000; Hong et al., personal communication), both of these genes were analysed. However, no sequence change was identified in *CRX* or *NR1D1*. In addition, the retina-specific exon 1 of the thyroid hormone receptor beta, *THRB*, and exons 9 and 10, which include the majority of reported mutations in this gene, were also excluded as sites of mutation in Patient A. A digenic mechanism has been proposed in Bardet-Biedl syndrome, an autosomal recessive syndromal form of retinal dystrophy, in which three disease susceptibility alleles at two loci appear to influence disease expression (trialelic inheritance) (Katsanis et al., 2001; Badano et al., 2003).

Two unusual manifestations in ESCS Patient A are the detectable, albeit reduced, rod function by dark-adapted perimetry in the peripheral retina and a greatly increased nerve fibre layer thickness, as measured near the optic nerve. It is of interest that among mice with genetically-engineered or naturally-occurring diseases that lead to an increased S cone population, some do exhibit rod function (Akhmedov et al., 2000; Ng et al., 2001). Abnormally increased nerve fibre layer thickness has been documented in another early-onset retinal disease and thought to be due to interrupted naturally-occurring apoptosis during normal human retinal development (Jacobson et al., 2003). The relationship of these findings to the heterozygous *NRL* mutation is uncertain. The possibility that a loss-of-function *NRL* mutation might produce an ESCS-like phenotype is emphasised by the finding that *Nrl*^{-/-} knockout mice showed enhanced S cone function and increased expression of S opsin and other cone-specific proteins, similar to ESCS (Mears et al., 2001). This is likely to result from a severe reduction or absence of *Nr2e3* transcripts in *Nrl*^{-/-} retinas (Mears et al., 2001). The caveat is that *Nrl*^{+/-} mice were indistinguishable from wildtype,

leading to our speculation that another unknown disease-causing allele may be contributing to the phenotype in Patient A.

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