A Common Founder Mutation of \textit{CERKL} Underlies Autosomal Recessive Retinal Degeneration with Early Macular Involvement among Yemenite Jews

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\textbf{PURPOSE.} To investigate the genetic basis and clinical manifestations of a characteristic form of retinal degeneration in the Yemenite Jewish population.

\textbf{METHODS.} Haplotype analysis for all known genes and loci underlying autosomal recessive nonsyndromic retinal degeneration was performed in a Yemenite Jewish family segregating autosomal recessive severe retinal degeneration. The causative mutation was detected by direct sequencing of the underlying \textit{CERKL} gene. Generation of additional affected Yemenite Jews was determined. Patients who were homozygous for this mutation underwent ophthalmic evaluation, including funduscopy, electroretinography, electro-oculography, perimetry, and color vision testing.

\textbf{RESULTS.} In the studied Yemenite Jewish family, we found evidence for linkage to the \textit{CERKL} gene. Direct sequencing revealed a novel homozygous splice-site mutation, c.238+1G>A. An in vitro splicing assay demonstrated that this mutation leads to incorrect splicing. c.25G>A was found to cause retinal degeneration in six additional Yemenite Jewish families. The carrier frequency of this mutation in the Yemenite Jewish population is 4.4%. All c.238+1G>A homozygotes manifest widespread progressive impairment of rod and cone function with early macular involvement.

\textbf{CONCLUSIONS.} c.238+1G>A is the second reported mutation of \textit{CERKL} and is a prevalent founder mutation that underlies approximately 33\% of autosomal recessive retinal degeneration cases in the Yemenite Jewish population. It is associated with a characteristic retinal degeneration phenotype with early macular involvement, concomitant progression of rod and cone impairment, and characteristic fundus findings. The identification of this mutation and phenotype will facilitate molecular diagnosis, carrier screening, and genetic counseling in the Yemenite Jewish population. (\textit{Invest Ophthalmol Vis Sci.} 2007;48:5431–5438) DOI:10.1167/iovs.07-0736

\textbf{RESULTS.} Retinitis pigmentosa (RP) is the most common form of hereditary retinal degeneration, with a worldwide prevalence of 1 in 4000.\textsuperscript{1} RP actually encompasses a heterogeneous group of retinal dystrophies characterized by night blindness followed by visual field loss, often resulting in severe visual impairment. In most forms of RP, the disease process initially affects the rod photoreceptors to a more severe degree than the cones.\textsuperscript{1} A related form of retinal dystrophy is cone-rod degeneration (CRD). In CRD, cone involvement initially exceeds that of rods, and thus reduced visual acuity, photophobia, and defective color vision are prominent early symptoms. However, these two heterogeneous clinical entities lie along a spectrum, and in some cases, determining which photoreceptor type is predominately involved and distinguishing between a rod-cone (RP) disease process and a cone-rod (CRD) one may not be entirely possible.\textsuperscript{1,2} Besides being clinically heterogeneous, both RP and CRD are genetically heterogeneous. In most patients, the disease is limited to the eye (nonsyndromic RP/CRD), with no extraocular manifestations, suggesting that the genes involved encode largely eye- and retina-specific products. Nonsyndromic RP and CRD can be inherited as autosomal recessive, autosomal dominant, or X-linked. Mitochondrial and digenic patterns of inheritance have also been described.\textsuperscript{2} Over 50 genes and loci have been implicated in nonsyndromic RP and/or CRD, of which at least 27 are associated with an autosomal recessive mode of inheritance (RetNet-Retinal Information Network; http://www.sph.uth.tmc.edu/RetNet; provided in the public domain by the University of Texas Houston Health Science Center, Houston, TX). One of these loci is RP26, located on 2q31.\textsuperscript{3,4} The gene underlying RP26 is \textit{CERKL}.\textsuperscript{5} To date, only one \textit{CERKL} mutation, p.R257X, has been reported in three unrelated Spanish families segregating nonsyndromic autosomal recessive RP.\textsuperscript{4,5}

\textit{CERKL} encodes a novel ceramide kinase-like protein. Ceramide is the metabolic product of sphingophospholipids, which are part of the cell membrane, especially in neuronal cells. Generation of ceramide by hydrolysis of sphingophospholipids (e.g., sphingomyelin) is associated with the induction of apoptotic cell death and other stress-related responses. Termination of the second-messenger actions of ceramide is mediated by ceramidase, which hydrolyzes it to form sphingosine. However, ceramide can also be phosphorylated to ceramide-1-phosphate (C1P) by ceramide kinase (CERK).\textsuperscript{6} Both CERK and its homologue, CERKL, harbor a similar kinase domain related to the diacylglycerol kinases (DAGKs). However, unlike CERK, CERKL does not phosphorylate ceramides, and its kinase activity remains to be shown. Moreover, the \textit{CERK} gene is highly and ubiquitously expressed, whereas \textit{CERKL} expression is lower and restricted to a smaller variety of tissues.\textsuperscript{7,8} In the mouse retina \textit{Cerkl} is predominantly expressed.
in the ganglion cell layer and to a lesser extent in the inner nuclear and photoreceptor cell layers.4

In the present study, we sought to identify a common founder mutation of CERKL, which underlies approximately 33% of autosomal recessive retinal degeneration cases in the Yemenite Jewish population. Of interest, this mutation is associated with a severe form of retinal degeneration with early macular involvement, in which roughly parallel cone and rod involvement and dysfunction seem to occur.

**MATERIALS AND METHODS**

**Patients and Control Subjects**

Affected individuals and their healthy family members were ascertained through Bnai Zion and Hadassah-Hebrew University Medical Centers. Anonymous DNA control samples of various Jewish populations were obtained from the National Laboratory for the Genetics of the Israeli Population at Tel Aviv University. The study was performed in accordance with the Declaration of Helsinki, and written informed consent was obtained from all participants. The research was approved by the local institutional review boards of all participating medical centers and by the National Helsinki Committee for Genetic Research in Humans.

**Clinical Evaluation**

A full ophthalmic examination, including assessment of visual acuity, ocular motility, pupillary reaction and a biomicroscopic slit-lamp and dilated fundus examination, was performed in all patients. Kinetic perimetry (Goldmann visual fields, targets V4e and III4e), color vision testing (Ishihara 38-plate and the Farnsworth-Munsell D-15 tests), full-field electroretinography (ERG), and electro-oculography (EOG) were performed according to the patient’s ability and compliance, as previously described.9 Briefly, full-field ERGs were recorded with corneal electrodes and a computerized system (UTAS 3000; LKC, Gaithersburg, MD). In the dark-adapted state, a rod response to a dim blue flash and a mixed cone–rod response to a white flash were acquired. Cone responses to 30-Hz flashes of white light were acquired under a background light of 21 cd/m². All ERG responses were filtered at 0.3 to 500 Hz and signal averaging was used. EOG was performed according to the ISCEV (International Society for Clinical Electrophysiology of Vision) standard, with bilateral skin electrodes on both canthi, and the Arden ratio (light peak to dark trough) was derived.10

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**FIGURE 1.** Pedigrees and haplotype analysis at the RP26 locus. (A) Seven unrelated Yemenite Jewish families segregating the c.238+1G>A mutation. Haplotype analysis performed on family TB32 demonstrates cosegregation of a haplotype of three polymorphic marker alleles, linked to the RP26 locus on 2q31.3, with autosomal recessive retinal degeneration in this family. Box: mutation-bearing haplotype. (B) Haplotypes of c.238+1G>A-bearing chromosomes from seven Yemenite Jewish families. The haplotypes, represented by vertical bars, are composed of several polymorphic markers flanking the c.238+1G>A mutation (m) of the CERKL gene (hatched box), and extending approximately 2.6 Mb on 2q31.3. D2S2261, D2S2310, D2S364, and D2S2273 are microsatellite repeats. rs155100, rs155102, and D81A are SNPs. Box: the minimal conserved haplotype shared by all c.238+1G>A-bearing chromosomes.
Haplotypic Analysis

Genomic DNA was extracted from venous blood samples according to a standard protocol. DNA samples were PCR amplified with fluorescent dye-labeled primers flanking microsatellite repeat markers linked to all known autosomal recessive RP and CRD loci and genes (RetNet). PCR products were separated by electrophoresis (310 Genetic Analyzer; Applied Biosystems, Inc. [ABI], Foster City, CA), and genotypes were determined (Genescan and Genotyper software, using a Gene analyzer; Applied Biosystems, Inc., Foster City, CA). Analysis of single-nucleotide polymorphisms (SNPs) was performed by direct sequencing.

Mutation Analysis

Specific primers were used to PCR amplify and sequence the 14 coding exons of CERKL, including intron-exon boundaries. Primer sequences are available in Supplementary Table S1, online at http://www.iovs.org/cgi/content/full/48/12/5431/DC1. PCR products were purified with a PCR clean-up system (Wizard SV; Promega Corp., Madison, WI). Mutation screening was performed by direct sequencing (Big Dye terminator cycle sequencing kit on a 3130xl Genetic Analyzer; ABI). To detect c.238+1G>A and p.D81A by restriction endonuclease-based assays, CERKL exons one and two, respectively, were PCR amplified in a 25-μL reaction volume. Twenty microliters of the products were digested overnight in a 30-μL volume with BstUI (10 U, 50°C) or HpaI (5 U, 37°C), respectively, and 1× the recommended buffer (New England BioLabs, Beverly, MA). The entire reaction volume (30 μL) was visualized by electrophoresis on a 2% agarose gel. Expected band sizes for exon 1 are 708 and 79 bp for the wild-type (wt) allele and 511, 197, and 79 bp for the c.238+1G>A mutant allele. Expected band sizes for exon 2 are 520, 213, and 167 bp for the p.81D allele and 687 and 213 bp for the p.81A allele.

RESULTS

Novel CERKL Mutation in a Yemenite Jewish Family

We ascertained a large Israeli Jewish family of Yemenite origin (family TB32, Fig. 1A). The family is not known to be consanguineous, but both parents originated from the same small village in Yemen. Both parents had normal vision and there was no known history of retinal degeneration in previous generations. Yet, five of their 14 offspring had severe retinal degeneration with early macular involvement diagnosed in their late teens to early 20s. We concluded that the most probable mode of inheritance in this family is autosomal recessive.

In Vitro Splicing Assay

To create wt and mutant minigene constructs, DNA fragments harboring CERKL exons 1 or 2, each flanked by 50 to 220 bp of intronic sequences, were PCR-amplified from genomic DNA derived from a patient or from a normal control subject. The two fragments were inserted in tandem into a mammalian expression vector (pCMV-Script; Stratagene, La Jolla, CA; Fig. 3B). Constructs were transfected into NIH3T3 cells, using a transfection reagent (JetPEI; Polyplus-Transfection, Illkirch, France). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (Biological Industries, Beit Haemek, Israel) and maintained at 37°C and 5% CO2. Twenty-four hours after transfection, total RNA was extracted from cells (TRI reagent; Sigma-Aldrich, St. Louis, MO). Reverse transcription was performed with 1 μg of total RNA in a 20-μL reaction volume using 200 U of M-MLV reverse transcriptase and 100 ng of random primers (Stratagene). Two microliters of cDNA were subjected to PCR amplification with the following primers, located in CERKL exons 1 and 2, respectively: 5′-GAGCGAGCCTGCGGTGGC-3′ and 5′-GCAGATGAAGAGTGTGATAC-3′. Cycling conditions were 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 45 seconds, and a final step of 72°C for 10 minutes.

GenBank accession number

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FIGURE 2. CERKL gene and splice isoforms. The location of the p.R257X mutation described by Tuson et al.4 and the c.238+1G>A mutation described herein are shown on a schematic representation of the CERKL gene (drawn to scale). Also shown are the nine reported CERKL splice isoforms (variants a–i). Filled boxes: coding exons; open boxes: noncoding exons.
**CERKL**, the mutated gene in the original RP26 family, was initially reported to comprise 13 exons.\(^9\) Nine alternatively spliced isoforms of **CERKL** have been submitted to GenBank.\(^8\) Comparison of each of the nine **CERKL** splice variants to the human genome sequence at the UCSC Genome Browser revealed that the gene actually comprises 15 exons, 14 of which are coding (Fig. 2). In most splice variants (8/9) the first exon is exon 1, which includes a translation initiation codon. However, one of the reported splice isoforms (variant i) starts with an alternative, noncoding first exon (0). In this isoform, the open reading frame starts within exon 4. An additional exon (4a) is included in only one of the splice variants (b). The different splice variants encode nine protein products, varying in length from 173 to 558 amino acids (Fig. 2).

To detect mutations in the **CERKL** gene, we determined the sequence of each of the 14 coding exons (exons 1–13), including exon-intron boundaries, in one of the affected individuals from family TB32. We identified two homozygous single-base changes, both of which were found to cosegregate with retinal degeneration in this family.

The first identified homozygous change is an A to C transversion located in exon 2, which leads to the substitution of alanine for aspartic acid at position 81 of the **CERKL** protein (variants a–h; p.D81A). Aspartic acid at position 81 is conserved in mouse, dog, and chimpanzee **CERKL** proteins; however, in rat Cerkl, there is a glycine at this position. Moreover, we screened a panel of 112 Yemenite Jewish control DNA samples and found a carrier frequency of 9% for the p.81A allele. We concluded that p.D81A is most probably a common polymorphism in the Yemenite Jewish population.

The second identified homozygous change is a G-to-A transversion located at the conserved donor site of exon 1 (c.238+1G>A; Fig. 3A). This change is expected to lead to altered splicing of exon 1. Exon 1 is included in eight of nine **CERKL** splice variants and harbors the translation initiation codon (Fig. 2). Due to the limited expression pattern of **CERKL**, we could not evaluate the effect of c.238+1G>A on splicing in patient-derived RNA. Alternatively, we used an in vitro splicing assay approach. For this purpose, we created a minigene construct. This construct harbors **CERKL** exons 1 and 2, flanked by 50 to 220 bp of intronic sequences, downstream of a CMV promoter (Fig. 3B). Constructs harboring either the wt or the c.238+1G>A allele were transfected into NIH3T3 cells, followed by RNA extraction and RT-PCR analysis with primers located in each of the exons. RNA derived from the wt construct was correctly spliced, whereas RNA derived from the mutant construct yielded two types of aberrantly spliced products. In one of these products, the entire intron was retained, whereas in the second product the intron was only partially retained, due to the use of a cryptic donor site, located 202 bp upstream of exon 2 (Figs. 3B, 3C). In silico analysis of this cryptic donor site (using Splice Site Prediction by Neural Network) yielded a relatively low score of 0.75 (in comparison to a score of 0.98 in the original donor site). Retention of intron 1 is expected to lead to premature translation termination, after the insertion of 115 incorrect amino acids, starting at position 80 of the **CERKL** protein. The in vitro splicing assay we performed demonstrates that an exon 1 donor splice site harboring the c.238+1G>A change is not efficiently recognized by the mammalian splicing machinery. Although the exact effect of this splicing mutation on **CERKL** transcripts in human retina is not known, the expected outcome is incorrect splicing, leading to an abnormal protein product.

**FIGURE 3.** Analysis of the c.238+1G>A mutation. (A) Nucleotide sequence traces of **CERKL** exon 1 in a noncarrier individual (wt), an individual heterozygous for the c.238+1G>A substitution (het), and an affected individual homozygous for the mutant allele (mut). The exon-intron boundary is marked. (B) Minigene constructs and products obtained in the in vitro splicing assay. Shown are the constructs, which include **CERKL** exons 1 and 2 (boxes), flanked by 50 to 220 bp of intronic sequences (straight lines). Constructs harbor either the wt or the c.238+1G>A allele were transfected into NIH3T3 cells, followed by RNA extraction and RT-PCR analysis with primers used for RT-PCR analysis (arrows) and the obtained splicing products. (C) Wt and mutant constructs were transfected into NIH3T3 cells, followed by RNA extraction and RT-PCR analysis with primers located in each of the exons. The wt construct (W) yielded the expected product of 183 bp, indicating correct splicing. The mutant construct (M) yielded two types of products, of 367 and 562 bp, derived from aberrantly spliced mRNA molecules. RT-PCR analysis of RNA extracted from HEK-295 cells served as a positive control for the expected **CERKL** splicing product. β-Actin (Actb; 437 bp product) served as an internal control for RNA quality and quantity.
Founder Mutation in the Yemenite Jewish Population

To determine the carrier frequency of the c.238+1G>A allele, we screened a panel of Yemenite Jewish control DNA samples. c.238+1G>A was found heterozygously in five of 112 Yemenite Jewish individuals, thus indicating a carrier frequency of 1:22 (4.4%; 95% CI 0.2%–8.7%) in this population. c.238+1G>A was not detected among 294 non-Yemenite Jewish control DNA samples, derived from Ashkenazi, Iraqi, Moroccan, and Sephardic Jews. The carrier frequency of c.238+1G>A in Yemenite Jews suggests a retinal degeneration incidence rate of approximately 1 per 2000 (95% CI 1/1,000,000–1/526), based on random mating and complete penetrance. Since additional retinal degeneration–causing mutations may be present in this population, the actual risk may be somewhat higher.

To identify additional families in which retinal degeneration is caused by the c.238+1G>A mutation, we screened a panel of 20 unrelated Yemenite Jewish patients diagnosed with RP or CRD. Six of them were found to be homozygous for c.238+1G>A (Fig. 1A). Analysis of additional family members demonstrated cosegregation of the mutation with retinal degeneration in each of these families. In total, homozygosity for c.238+1G>A was found in 7 (33%) of 21 unrelated Yemenite Jewish families with nonsyndromic autosomal recessive retinal degeneration.

All chromosomes harboring c.238+1G>A shared the same haplotype of three polymorphic markers, which span 171 kb.

Figure 4. Fundus findings and perimetry in CERKL c.238+1G>A homozygotes. Individual II-3, family HD50: fundus photographs (A, C) and an autofluorescence image (B) at the age of 18 years, showing early signs of maculopathy. Individual II-1, family HD50: fundus photographs at the ages of 21 (D) and 27 (E, F) years, as well as Goldmann kinetic visual fields in the same eye at the ages of 21 (M) and 27 (N) years, showing progression of disease (black, isopter-target V4e; blue, target III4e). Individual II-3, family HD312: fundus photographs at the age of 28 (G, H), showing more advanced maculopathy, few pigment clumps, and mid-peripheral changes. Individual II-7, family TB32: fundus photographs at the age of 50 years (I, J) showing severe macular and peripheral changes. Individual II-1, family HD9: fundus photographs at the age of 53 (K, L) showing severe macular and peripheral changes. Individual II-1, family HD9: fundus photographs at the age of 53 (K, L), and Goldmann kinetic visual fields in the same eye at the age of 27 years (O; black, isopter target V4e).
Table 1. Clinical Data of Individuals Homozygous for the CERKL c.238+1G>A Mutation

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<th>Color Vision</th>
<th>EOG† Arden Ratio (%)</th>
<th>fERG Rod (µV)‡</th>
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EOG, electro-oculogram; fERG, full-field electroretinogram; Ish, Ishihara Pseudolochromatic Color Test; D-15, Farnsworth Dichotomous Test Panel D-15; R, Right eye; L, left eye; BE, both eyes; ND, not done; WNL, within normal limits; HM, hand movement.

* Visual acuity (best corrected).
† Normal Arden ratio > 185%.
‡ Rod response b-wave amplitude: normal, >200 µV; mixed cone-rod response amplitudes: normal a-wave, >90 µV; normal b-wave, >400 µV; 30-Hz cone flicker response: normal amplitude (Amp), >60 µV, normal implicit time (IT), ≤ 35 mS.
Most c.238+1G>A-bearing chromosomes had an identical haplotype of marker alleles farther downstream and upstream from this region; however, we did find evidence of historical meiotic recombinations of closely linked markers (Fig. 1B). The conserved haplotype of the region surrounding the c.238+1G>A mutation suggests a common origin for this mutation in the Yemenite Jewish population.

Mutation Associated with Severe Retinal Degeneration with Early Macular Involvement

A characteristic ocular phenotype is associated with the c.238+1G>A mutation. In all individuals homozygous for the mutation, early macular involvement accompanied by rod dysfunction is a key feature (Fig. 4, Table 1). These findings often led to confusion in making the initial clinical diagnosis, with some patients diagnosed as manifesting RP, whereas in others the phenotype was described as CRD. The full-field ERG findings did not help in clarifying the clinical dilemma, as often a similar degree of rod and cone dysfunction was present rather than preferential involvement of one photoreceptor type or the other (Table 1). Visual dysfunction, including mild night and central vision impairment, usually manifests during the teen years. On fundus examination, macular changes were documented as early as 15 years of age. In two sisters at the ages of 18 and 21, signs of maculopathy were evident (Figs. 4A, 4B, 4D). Whereas the retinal periphery appears preserved (Fig. 4C). Visual field testing shows a corresponding central scotoma in the 21-year-old patient, with relatively preserved peripheral vision (Fig. 4M). Over time, macular changes grow more severe and peripheral involvement becomes apparent, with few pigment spots in the mid periphery accompanied by salt-and-pepper–like changes (Figs. 4E–4H). These findings correlate with progressive loss of visual fields, including enlargement of central scotomas and increasing peripheral constriction (Figs. 4N, 4O). In late stages of disease, widespread macular and patchy peripheral retinal atrophy ensues, with pigmented changes in the form of clumps of varying sizes and bone spicule–like deposits (Figs. 4I–4L). The severe early atrophic macular changes with relatively large pigment clumps seem to be characteristic features of the retinal degeneration associated with the CERKL c.238+1G>A mutation.

DISCUSSION

The molecular causes of hereditary retinal degenerations are strikingly heterogeneous. More than 25 genes and loci are associated with autosomal recessive RP and/or CRD. The contribution of each one of these genes to the overall prevalence of retinal degeneration is relatively small, and for many of them, pathogenic mutations have been reported in only a few families worldwide. Because of this situation, genetic analysis in patients with RP or CRD and their families is very complicated. To date, only one disease allele can become prevalent in a population either because the heterozygous state is associated with a selective advantage and/or because there was a change in frequency of the mutant allele caused by random genetic drift. The Yemenite Jewish population is ancient. The first Jews arrived in Yemen from the land of Israel approximately 2500 years ago. Over the past 130 years, most Yemenite Jews have immigrated to Israel or the United States. Currently, only a few hundred Jews remain in Yemen, and the majority of Yemenite Jews (~174,600 people) reside in Israel. Of interest, several genetic disorders are found with a relatively high frequency among Yemenite Jews. At least some of them, including phenylketonuria and metachromatic leukodystrophy, are caused by prevalent founder mutations segregating in the Yemenite Jewish population.

A characteristic and consistent retinal phenotype, including both rod and cone degeneration with severe early macular involvement and clumped pigmentation, is associated with the CERKL c.238+1G>A mutation. Of note, early macular involvement was also reported in the original RP26 Spanish family, which segregated a different CERKL mutation, p.R257X, suggesting that this may be a common outcome of CERKL mutations. Another unique feature, found both in our patients and in the Spanish family, is the concomitant involvement of both rods and cones as reflected in the full-field ERG findings (Table 1). There are two possible alternative explanations for these findings: Either intact CERKL activity is required within both types of photoreceptors, or it points to a disease mechanism that primarily affects another cell type that interacts with and affects both rods and cones.

The activity of the CERKL protein is unknown. Previous studies identified homology to CERK; however, no kinase activity of CERKL could be demonstrated in vitro. In the mouse retina, CERKL was shown to be expressed in both the ganglion and photoreceptor cell layers. Additional studies of CERKL spatial and temporal expression patterns and characterization of CERKL biochemical properties will be required to ascertain the role of this protein in retinal function and disease.

Acknowledgments

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References

APPENDIX

The URL for the Web sites, all in the public domain, for data presented herein are as follows:

- UCSC Genome Bioinformatics, http://genome.ucsc.edu/