Homozygosity Mapping in Patients with Cone–Rod Dystrophy: Novel Mutations and Clinical Characterizations

Karin W. Littink,[1,2,3] *Robert K. Koenekoop,*[3,4] *L. Ingeborgh van den Born,*[1] *Rob W. J. Collin,*[2,5,6] *Luminita Moruz,*[2,7] *Joris A. Veltman,*[2,8] *Susanne Roosing,*[2] *Marijke N. Zonneveld,*[1,2] *Amer Omar,*[4] *Mahshad Darvish,*[4] *Irma Lopez,*[4] *Hester Y. Kroes,*[9] *Maria M. van Genderen,*[10] *Carel B. Hoyng,*[6] *Klaus Rohrschneider,*[11] *Mary J. van Schooneveld,*[12,13] *Frans P. M. Cremers,*[2,5] *and Anneke I. den Hollander*[2,5,6,8]

Purpose. To determine the genetic defect and to describe the clinical characteristics in a cohort of mainly nonconsanguineous cone–rod dystrophy (CRD) patients.

Methods. One hundred thirty-nine patients with diagnosed CRD were recruited. Ninety of them were screened for known mutations in *ABCA4*, and those carrying one or two mutations were excluded from further research. Genome-wide homozygosity mapping was performed in the remaining 108. Known genes associated with autosomal recessive retinal dystrophies located within a homozygous region were screened for mutations. Patients in whom a mutation was detected underwent further ophthalmic examination.

Results. Homozygous sequence variants were identified in eight CRD families, six of which were nonconsanguineous. The variants were detected in the following six genes: *ABCA4, CABP4*, *CERKL, EYS*, *KCNV2*, and *PROM1*. Patients carrying mutations in *ABCA4*, *CERKL*, and *PROM1* had typical CRD symptoms, but a variety of retinal appearances on funduscopy, optical coherence tomography, and autofluorescence imaging.

Conclusions. Homozygosity mapping led to the identification of new mutations in consanguineous and nonconsanguineous patients with retinal dystrophy. Detailed clinical characterization revealed a variety of retinal appearances, ranging from nearly normal to extensive retinal remodeling, retinal thinning, and debris accumulation. Although CRD was initially diagnosed in all patients, the molecular findings led to a reappraisal of the diagnosis in patients carrying mutations in *EYS*, *CABP4*, and *KCNV2*. (*Invest Ophthalmol Vis Sci.* 2010;51:5943–5951) DOI: 10.1167/iovs.10-5797

From the [1]The Rotterdam Eye Hospital, Rotterdam, The Netherlands; the Departments of [2]Human Genetics and [6]Ophthalmology, the [5]Nijmegen Centre for Molecular Life Sciences, and the [8]Institute for Genetic and Metabolic Disease, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; the [4]McGill Ocular Genetics Laboratory, Montreal Children's Hospital Research Institute, McGill University Health Centre, Montreal, Quebec, Canada; the [7]Center for Biomembrane Research, Department of Biochemistry and Biophysics, Stockholm University, Stockholm, Sweden; the [9]Department of Medical Genetics, University Medical Centre Utrecht, Utrecht, The Netherlands; the [10]Bartiméus Institute for the Visually Impaired, Zeist, The Netherlands; the [11]Department of Ophthalmology, University of Heidelberg, Heidelberg, Germany; the [12]Netherlands Institute of Neuroscience, Amsterdam, The Netherlands; and the [13]Department of Ophthalmology, Academic Medical Centre, Amsterdam, The Netherlands.

[3]These authors contributed equally to the work reported here and therefore should be regarded as equivalent authors.

Supported by the Stichting Wetenschappelijk Onderzoek Oogziekenhuis, the Prof. Dr. H. J. Flieringa Foundation, The Rotterdam Eye Hospital Grant 2005-13 (LIvdB, AIdH, FPMC), Netherlands Organization for Scientific Research Grant 916.56.160 (AIdH), Foundation Fighting Blindness USA Grant BR-GE-0507-0381-RAD (AIdH), the Landelijke Stichting voor Blinden en Slechtzienden (FPMC), Algemene Nederlandse Vereniging ter Voorkoming van Blindheid (FPMC), Canadian Institutes of Health Research (RKK), Foundation Fighting Blindness Canada (RKK), Fonds de la Recherche en Santé Quebec (RKK), National Institutes of Health (RKK), and Réseau de Vision (RKK).

Submitted for publication April 28, 2010; revised June 7, 2010; accepted June 7, 2010.

Disclosure: **K.W. Littink**, None; **R.K. Koenekoop**, None; **L.I. van den Born**, None; **R.W.J. Collin**, None; **L. Moruz**, None; **J.A. Veltman**, None; **S. Roosing**, None; **M.N. Zonneveld**, None; **A. Omar**, None; **M. Darvish**, None; **I. Lopez**, None; **H.Y. Kroes**, None; **M.M. van Genderen**, None; **C.B. Hoyng**, None; **K. Rohrschneider**, None; **M.J. van Schooneveld**, None; **F.P.M. Cremers**, None; **A.I. den Hollander**, None

Corresponding author: Anneke I. den Hollander, Radboud University Nijmegen Medical Centre, Department of Human Genetics, Route 855, PO Box 9101, 6500 HB Nijmegen, The Netherlands, 6500 HD; a.denhollander@antrg.umcn.nl.

Cone–rod dystrophies (CRDs) belong to a heterogeneous group of inherited retinal dystrophies, characterized by the primary dysfunction or loss of cone photoreceptors followed by the dysfunction or loss of rod photoreceptors. The diagnosis is established by documenting decreased visual acuity—usually noticed during childhood—dyschromatopsia, central scotomas on visual field testing, and an electroretinogram (ERG) that shows more severely reduced cone than rod responses.[1] The clinical course involves progressive loss of central vision, followed by peripheral visual field loss and progressive nyctalopia as more rod photoreceptors become involved in the disease process and eventually may lead to complete blindness. CRD often presents as an isolated disease, but can be part of a syndrome as well, as in Bardet-Biedl or Jalili syndrome.[2,3] Isolated CRD can be inherited as an autosomal recessive, autosomal dominant, or X-linked trait and is genetically heterogeneous. Currently, autosomal recessive (ar)CRD is associated with mutations in five genes, of which *ABCA4* is the major contributing gene (40%).[4–7] The other four genes (*ADAM9*, *CERKL*, *PROM1*, and *RPGRIP1*) together account for only a few cases (1%–2% each).[8–11]

Autosomal recessive disorders such as CRD can be caused by homozygous or compound heterozygous mutations. Homozygous mutations are most frequently detected in patients of consanguineous parents or in patients from relatively isolated populations, where the chance that the parents have a

common ancestor is relatively high. However, homozygous mutations are also detected in patients of nonconsanguineous unions, and on the basis of several genetic studies in large cohorts of patients with autosomal recessive diseases from Western European countries, it is estimated that ~35% carry the mutations homozygously.[12–16] A homozygous mutation is likely to reside within a homozygous region that is detectable with a high-resolution single nucleotide polymorphism (SNP) array.[17,18] Mapping of these homozygous regions (homozygosity mapping) may lead to the identification of the genetic defect in consanguineous and in nonconsanguineous patients, as shown as proof of principle in patients with autosomal recessive kidney diseases,[19] and as a successful method for the detection of new disease genes[20–23] and new mutations in known genes implicated in retinal dystrophies.[24]

The goal of this study was to identify and map homozygous regions in a large cohort of CRD patients, mainly born of nonconsanguineous marriages, and to subsequently identify the causal mutations. Using this approach, we identified eight genetic variants, of which seven were novel, in four families and four sporadic patients, respectively. All patients carrying disease-causing mutations were clinically re-evaluated.

METHODS

Subjects

A total of 126 probands and 13 affected siblings with known or suspected arCRD or isolated CRD were included in this study by ophthalmologists from The Netherlands (LIvdB, MMvG, CBH, MJvS), Germany (KR), and Canada (RKK). The study was approved by the ethics review board of the participating centers, and all patients signed an informed consent that adhered to the tenets of the Declaration of Helsinki.

After the genetic defect was detected, clinical data were retrospectively reviewed, and patients were invited for ophthalmic examination by their ophthalmologists. Clinical evaluation included best corrected projected Snellen visual acuity, objective refractive error after cycloplegia, biomicroscopy, and funduscopy. Visual fields were assessed with Goldmann kinetic perimetry (targets V-4e and I-4e). Color vision was tested with the American Optical Hardy-Rand-Rittler Test (AO-HRR), Farnsworth D-15 panel (saturated and desaturated), or Ishihara color plates. Spectral-domain optical coherence tomography (OCT) and fundus autofluorescence (FAF) imaging (Spectralis; Heidelberg Engineering, Heidelberg, Germany) were performed in four patients. Fundus photographs were made in all patients.

Homozygosity Mapping

Blood samples for molecular genetic testing were obtained from all probands and affected family members. Total genomic DNA was extracted from leukocytes by a standard salting-out procedure.[25] DNA samples of 90 probands, mainly from The Netherlands and Germany, were screened for known mutations in *ABCA4* by the *ABCA4* arrayed-primer extension (APEX) microarray (Asper Ophthalmics, Tartu, Estonia).[26] The 46 probands and 13 siblings who did not carry known mutations in *ABCA4* were genotyped (GeneChip Mapping 250K *Nsp*I array; Affymetrix, Santa Clara, CA), containing 262,000 SNPs. Forty-nine probands, mainly from eastern Canada, were included in the study in a later stage and were not prescreened for known mutations in *ABCA4*. These samples were genotyped on an SNP array (GeneChip Genome-Wide Human SNP Array 6.0; Affymetrix), containing 906,600 polymorphic SNPs. Array experiments were performed according to protocols provided by the manufacturer. For both array platforms, genotypes were called by allied software (Genotype Console; Affymetrix). For the 250K-analyzed samples, the default confidence threshold was adjusted to 0.3 for samples, with a quality control value of <93%. All samples analyzed on the 6.0 array had a quality control value of >93%, and for those samples, default settings were used. Regions of homozygosity were determined by PLINK software,[27] with a sliding window of 50 SNPs and allowing 2 heterozygous SNPs (miscalls) and 10 missing SNPs (no calls) per window. Because the 250K and 6.0 arrays contain different SNP densities, we defined regions to be homozygous when they contained 200 or more consecutive homozygous SNPs on the 250K array and 600 or more consecutive homozygous SNPs on the 6.0 array. The minimum length of the regions appeared to be ~3 Mb, on average.

Analysis of Homozygous Regions

Homozygous regions were ranked based on the number of SNPs. SNP positions were derived from the UCSC human genome browser build hg18, March 2006 (http://genome.ucsc.edu provided in the public domain by UCSC Genome Bioinformatics, University of California at Santa Cruz, Santa Cruz, CA). All arCRD and autosomal dominant (ad) CRD genes residing in a homozygous region were selected for mutation analysis. We also analyzed all autosomal recessive retinal dystrophy genes (derived from RetNet: http://www.sph.uth.tmc.edu/RetNet; provided in the public domain by the University of Texas Houston Health Science Center, Houston, TX) residing in the six largest homozygous regions of each patient or, in case of multiple affected siblings, in the six largest homozygous regions shared by the siblings. Finally, we determined overlap of the detected homozygous regions with known, published retinal dystrophy loci (RetNet).

Sequence Analysis

We selected a total of 15 retinal dystrophy genes for mutation analysis. Six genes known to be associated with arCRD and adCRD were analyzed (arCRD genes: *ABCA4*, two patients; *ADAM9*, three patients; and *CERKL*, three patients; *PROM1*, three patients. adCRD genes: *SEMA4A*, two patients; and *UNC119*, two patients). In addition, nine genes associated with other retinal dystrophies were screened (*CABP4*, *KCNV2*, and *RLBP1* each in two patients; *C2orf71, CNGA1*, *IDH3B*, *RDH5*, *RDS*, and *RP1* each in one patient). All coding exons of the selected genes were PCR amplified and analyzed in sense or antisense direction on an automated sequencer (BigDye Terminator, ver. 3, on a 3730 DNA analyzer; Applied Biosystems, Inc., Foster City, CA). Primers were designed using Primer3 software.[28] Ethnically matched control individuals were screened for newly identified mutations by using the amplification-refractory mutation system (ARMS) or restriction fragment length polymorphism.[29] Primer sequences and PCR conditions are available on request.

RESULTS

Patient Cohort and ABCA4 Prescreening

A total of 126 probands and 13 affected siblings who received a clinical diagnosis of arCRD or isolated CRD were included in this study. This cohort included 10 families with two or three affected siblings. Six sporadic CRD patients and one CRD family were reported to be consanguineous; the remaining patients and families were reported to be nonconsanguineous. Prescreening of 90 probands, using the *ABCA4* APEX microarray (Asper Ophthalmics),[26] revealed mutations in *ABCA4* in 31 patients (1 homozygous, 15 compound heterozygous, and 15 heterozygous), of which 19 have been published previously.[4,7] All patients carrying one or two *ABCA4* mutations were excluded from further research.

Homozygosity Mapping

A total of 59 CRD samples were analyzed for 262,000 SNPs (250K array; Affymetrix) and 49 samples were analyzed for 906,600 SNPs (6.0 array; Affymetrix), and homozygous regions were determined.

Supplementary Table S1, http://www.iovs.org/cgi/content/full/51/11/5943/DC1, gives an overview of the number and

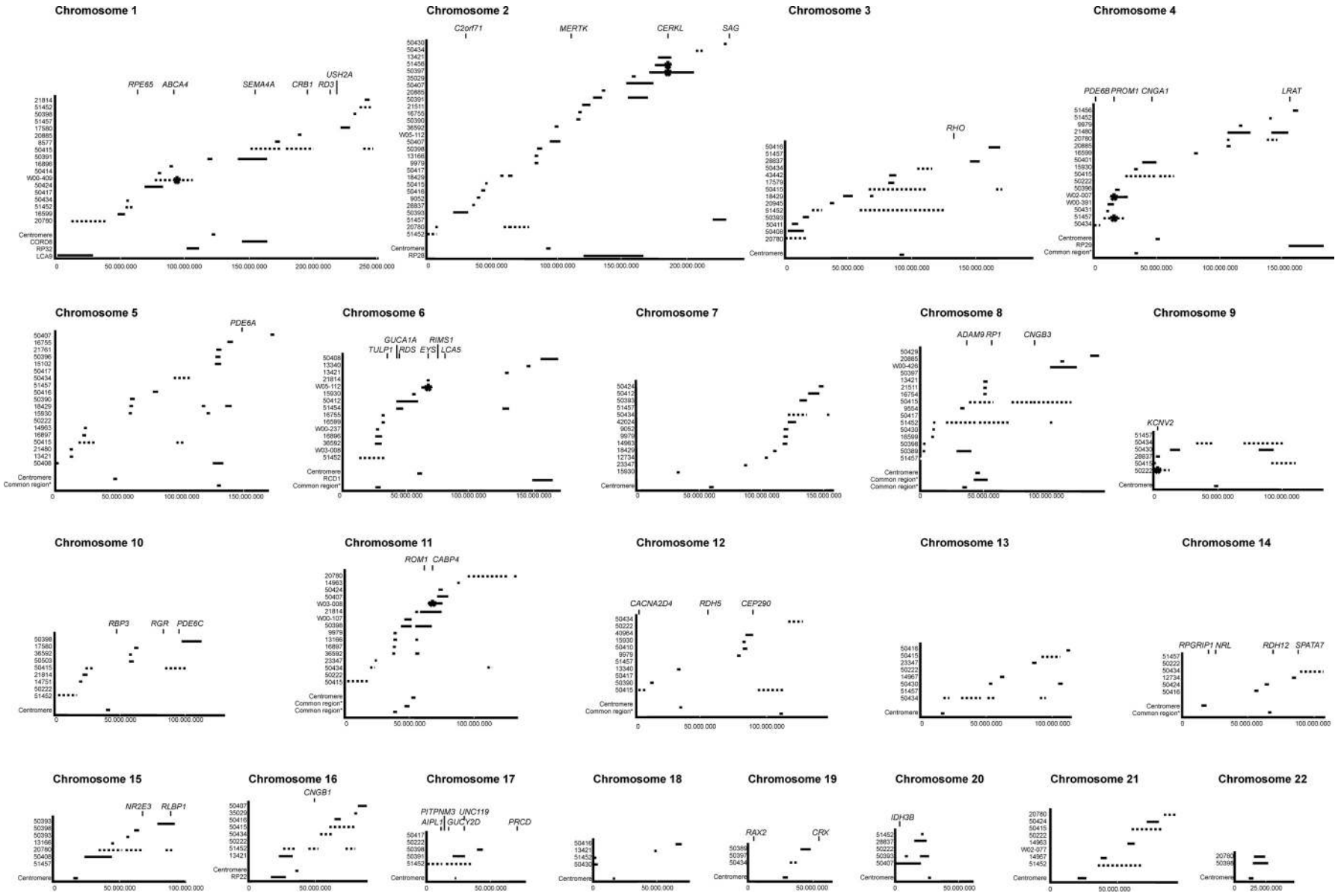

FIGURE 1. Chromosome plots showing homozygous regions identified in 108 patients with cone–rod dystrophy. The chromosomal positions are plotted on the *x*-axis, and the patient numbers are displayed on the *y*-axis. Centromeric positions, common homozygous regions,[30] and retinal dystrophy loci are indicated in the lower part of each plot, and the chromosomal position of retinal dystrophy genes are indicated at the top of each plot. W*xx-xxx* numbers in the *y*-axis designate homozygous regions that are shared by all affected siblings of a family. *Black lines*: homozygous regions in nonconsanguineous patients; *dotted lines*: homozygous regions in consanguineous patients; *gray lines*: a mutation identified in another homozygous region of that patient; *asterisk*: a mutation identified at that position.

Table 1. Overview of the Genes in Which Mutations Were Detected, and the Size Ranking of the Homozygous Regions in Which These Genes Resided

Retinal Dystrophy Gene	Patient or Family ID (Siblings, *n*)	Consanguinity	Country (Origin)	Ranking of Region (*n*)	Size (Mb)	% of Genome That Is Homozygous (Regions > ~3 Mb)	Homozygous Mutations	Reference for Mutation
ABCA4	W00–409 (3)	Yes	Germany (Iraq)	1st (1)	27.8	5.7*	c.160T>G; p.C54G	Ozgül et al.[31]
ABCA4	50417	No	Canada (Pakistan)	5th (10)	4.2	1.5	c.6729+5_19del15; splice defect	This study
CABP4	W03–008 (2)	No	Netherlands	1st (2)	8.9	0.9*	c.646C>T; p.Arg216X	Littink et al.[32]
CERKL	50397	No	Canada	1st (4)	28.4	1.6	c.375C>G; p.C125W	This study
CERKL	51456	No	Canada (French Canadian)	1st (2)	10.6	0.5	c.847C>T; p.R283X	This study
EYS	W05–112 (2)	No	Netherlands	2nd (2)	5.0	0.9*	c.9468T>A; p.Y3156X	Collin et al.[22]
KCNV2	50222	Yes	Netherlands (Morocco)	3rd (16)	10.9	7.0	c.162C>A; p.Y54X	This study
PROM1	W02–077 (2)	No	Netherlands (Greece)	2nd (6)	10.4	1.2*	c.1142–1G>A; splice defect	This study

* In the case of multiple affected siblings, the mean percentage of the genome that is homozygous was calculated.

Table 2. Overview of Characteristics of the Missense Variants Detected in CRD Patients

Gene	Sequence Change	Protein Change	BLOSUM 62 Score*	Polyphen	SIFT Prediction (Score)†	Grantham Score‡	Segregation Analysis	Presence in Control Individuals	Conclusion
ADAM9	c.2400G>T	p.Q800H	3	Possibly damaging	Not tolerated (0.03)	24	Homozygously present in unaffected brother	Excluded in 210 Dutch alleles§	Not pathogenic
CERKL	c.375C>G	p.C125W	−2	Probably damaging	Tolerated (0.08)	215	Homozygously present in affected brother	Excluded in 200 alleles	Pathogenic
PROM1	c.1946C>T	p.S649L	−3	Possibly damaging	Tolerated (0.14)	145	Both parents heterozygous, wild-type in nonaffected brother	Present homozygously in 1/90 control individuals	Not pathogenic

* The more negative the BLOSUM62 score, the more likely that a variant is pathogenic.
† SIFT score of <0.05 is predicted to be pathogenic.
‡ Grantham score >60, possible damage.
§ No ethnically matched controls available.

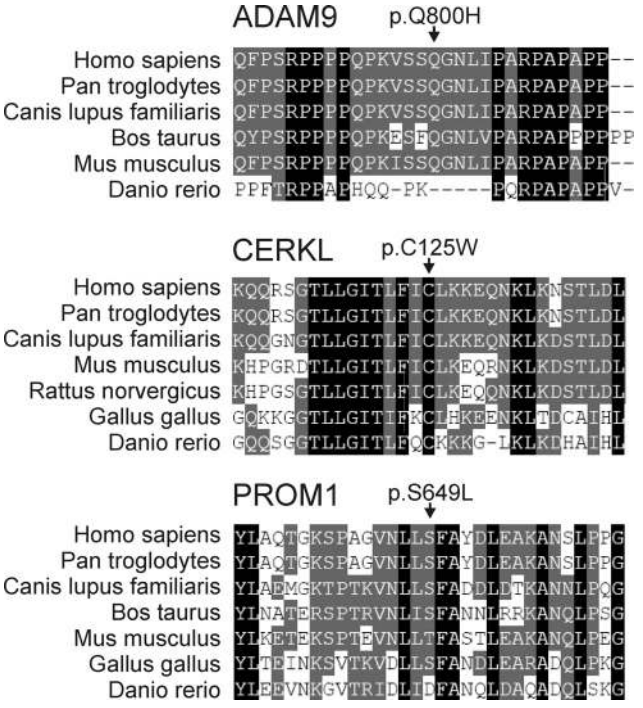

FIGURE 2. Evolutionary conservation of the three missense variants detected in *ADAM9*, *CERKL*, and *PROM1*. The p.C125 residue in CERKL shows full conservation up to *Danio rerio* (zebrafish). The p.Q800 in ADAM9 is fully conserved in at least five of six residues, and the p.S649 residue in PROM1 shows a similar amino acid in six of seven residues, but both appear to be nonpathogenic. *White* lettered residues on a *black* background are fully conserved. *White* letters on a *gray* background are relatively well-conserved or form a block of similar amino acids. Accession numbers of the protein sequences used for sequence comparison are as follows. ADAM9: *Homo sapiens*, NP_003807.1; Pan troglodytes, XP_519719; *Canis lupus familiaris*, XP_532798.2; *Mus musculus*, NP_031430.1; *Rattus norvergicus*, NP_001014772.1; *Gallus gallus*, NP_001026567.1; *Danio rerio*, NP_001107911.1. CERKL: *Homo sapiens*, NP_963842.1; Pan troglodytes, XP_515955.2; *Canis lupus familiaris*, XP_545552.2; *Mus musculus*, NP_001041641.1; *Rattus norvergicus*, XP_578135.2; *Gallus gallus*, XP_421973.2; *Danio rerio*, NP_001082943.1. PROM1: *Homo sapiens*, NP_006008.1; Pan troglodytes, XP_517115.2; *Canis lupus familiaris*, XP_545934.2; *Bos taurus*, XP_875477.2; *Mus musculus*, NP_032961.1; *Gallus gallus*, XP_001232165.1; *Danio rerio*, NP_001108615.1.

sizes of the homozygous regions for each patient and the percentage of the total genome that was homozygous. Significant homozygous regions were identified in 76 (77%) of the nonconsanguineous patients with CRD. The average number of homozygous regions in these patients was three (range, 1–10), and the average total length of homozygous regions was 17.5 Mb (range, 1.7–83.3 Mb), which corresponds to 0.5% (range, 0.1%–2.9%) of the genome. Twenty-three (23%) nonconsanguineous patients carried no significant homozygous regions. All consanguineous patients carried multiple large homozygous regions; on average, each patient carried 18 (range, 8–27) homozygous regions, with a total length of 204.3 Mb (range, 107.3–332.9 Mb), which corresponds to 7.2% of the genome (range, 3.8%–11.7%).

In affected siblings, we compared the individual homozygous regions and included only those regions that were shared between siblings and displayed the same haplotype. In two nonconsanguineous families, no shared homozygous regions were present. Five families (of which one was consanguineous) had one shared homozygous region, and three families had two.

TABLE 3. Clinical Features of Patients in Which a Genetic Defect Was Identified

Patient ID (Family Number)	Gene Defect	Age (y)	Age of Diagnosis	Visual Acuity (Best Eye)	Color Vision	Goldmann Perimetry	ERG, Rod*	ERG, Cone†
CRD Patients								
16875 (W00–409)	*ABCA4*, p.C54G	17	15	20/200	Diffuse errors	Small central scotoma	Subnormal	NR
16876 (W00–409)	*ABCA4*, p.C54G	15	14	20/400	Diffuse errors, mostly protan axis	Large central scotoma	↓	↓↓
16877 (W00–409)	*ABCA4*, p.C54G	13	8	20/300	Diffuse errors	NP	↓↓	NR
50417	*ABCA4*, c.6729+5_19del15	48	30	20/400	Abnormal	Central scotoma and decreased sensitivity	NR	NR
50397 (W09–0340)	*CERKL*, p.C125W	48	30	20/50	Abnormal	Central scotoma	↓↓	↓↓
54684 (W09–0340)	*CERKL*, p.C125W	51	28	3/400	Abnormal	Central scotoma	NR	NR
51456	*CERKL*, p.R283X	53	29	LP	NP	Not measurable	NR	NR
20695 (W02–077)	*PROM1*, c.1142–1G>A	18	13	20/200	Severely disturbed	Substantial central scotoma	↓↓	NR
20696 (W02–077)	*PROM1*, c.1142–1G>A	16	9	20/125	Severely disturbed	Central scotoma with relative ring scotoma	NR	NR
Patient Affected by Cone Dystrophy with Supernormal Rod ERG Responses								
50222	*KCNV2*, p.Y54X	11	10	20/32	Moderately disturbed	Normal	↑, delayed	↓

* Isolated rod response.

† Single flash cone response; ↓, reduced; ↓↓, severely reduced; ↑, supernormal; LP, light perception; NR, no responses; NP, not performed.

Figure 1 presents an overview of all detected homozygous regions sorted by chromosome. In these plots, known retinal dystrophy loci, centromeric regions (which are less likely to recombine), and common homozygous regions are indicated. A common homozygous region was defined as a region of ≥100 homozygous SNPs present in ≥25% of individuals in a cohort of 144 healthy controls.[30] A small number of homozygous regions seemed to be shared among four or six individuals. However, when patients who carried mutations in another homozygous region, common homozygous regions, and regions close to the centromere were excluded, only regions shared among a maximum of three patients remain. This finding indicates that no major novel locus for arCRD was detected in this cohort by our homozygosity mapping approach.

Mutation Analysis

Mutation analysis of four arCRD genes (*ABCA4*, *ADAM9*, *CERKL*, and *PROM1*) in a total of 11 probands (eight sporadic CRD patients and three families) revealed sequence variants in seven of them (five sporadic patients and two families; Table 1). Screening of 9 other retinal dystrophy genes in a total of 12 probands led to the identification of novel homozygous mutations in one sporadic patient (*KCNV2*) and in one family (*CABP4*; Table 1).

The p.C54G change in *ABCA4* (family W04-009) is a known mutation that was not yet present on the 2001 version of the *ABCA4* APEX array at the time that the patient's DNA was screened.[31] The other mutation in *ABCA4* (c.6729+5_19del15) is new and was not detected in 180 control alleles. This mutation may result in defective splicing, since the splice site consensus score decreases from 81.8 to 61.7, as calculated by the method of Shapiro and Senapathy.[33] A homozygous nonsense mutation (c.847C>T; p.R283X) and a homozygous missense mutation (c.375C>G; p.C125W), both novel, were identified in *CERKL*. The missense mutation replaces a cysteine with a tryptophan, which does not occur commonly during evolution (BLOSUM62 score, −2; Table 2).[34] The mutation affects a fully conserved residue (Fig. 2). It was also identified homozygously in the patient's affected sibling (patient 54684) and was not present in 200 ethnically matched control alleles and is therefore likely to be pathogenic. The novel splice site mutation in *PROM1* (c.1142-1G>A) affects the invariable AG-dinucleotide of the splice acceptor site of intron 10 and is predicted to impair splicing. The missense changes in *PROM1* (c.1946C>T; p.S649L) and *ADAM9* (c.2400G>T; p.Q800H) both affect highly conserved residues (Fig. 2). However, the variant in *PROM1* was identified homozygously in 1 of 90 ethnically matched control individuals and is therefore likely to be nonpathogenic. The *ADAM9* variant was detected homozygously in an unaffected brother and is therefore likely to be nonpathogenic as well (Table 2). The amino acid sequences are aligned in commercial software, using default settings (Vector NTI software; Invitrogen, Breda, The Netherlands). Protein sequences of CERKL, PROM1 and ADAM9 orthologs were derived from the NCBI database (http://www.ncbi.nlm.nih.gov/homologene/National Center for Biotechnology Information [NCBI], Bethesda, MD).

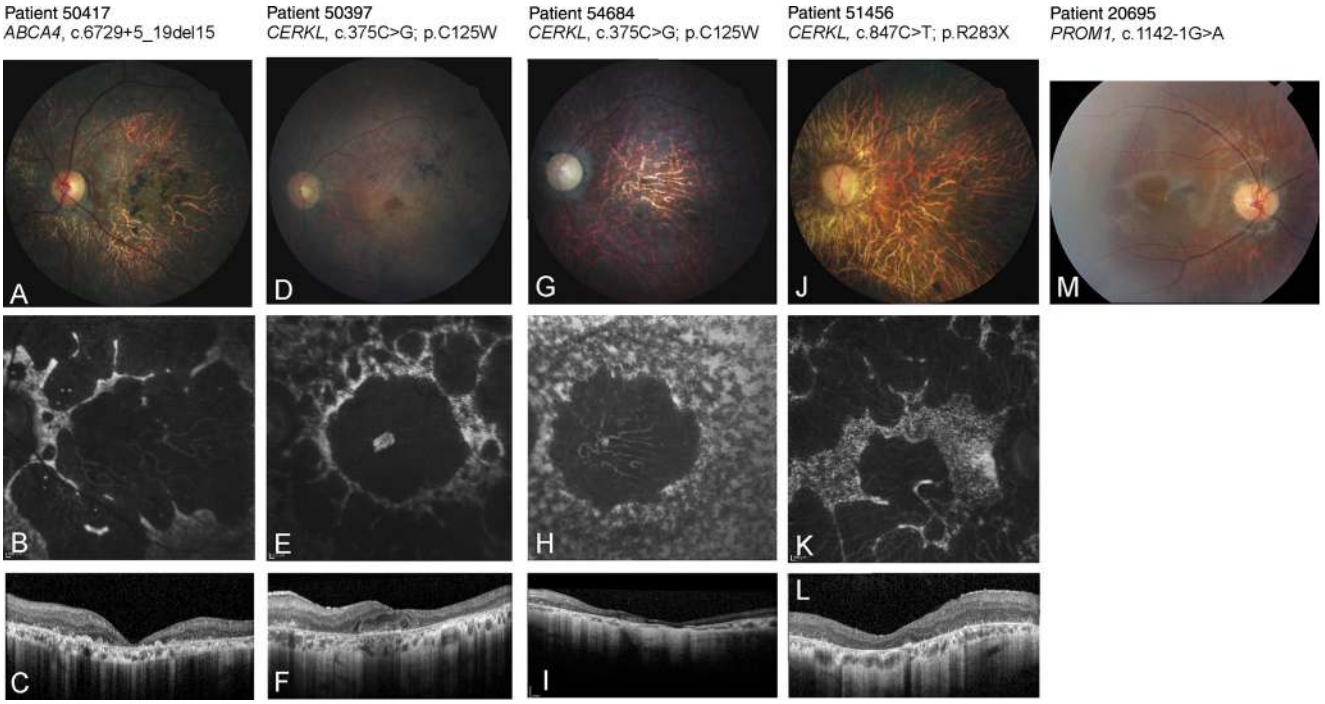

Figure 3. Fundus photographs, autofluorescence, and spectral-domain OCT images of CRD patients with conclusive genetic defects. The retina of patient 50417 (*ABCA4*, c.6729+5_19del15; age 44) showed (**A**) extensive RPE cell loss, choroidal sclerosis, and pigmentary macular changes on funduscopy; (**B**) marked loss of central lipofuscin accumulation on FAF; and (**C**) a disorganized retina in which the six layers were not detectable, the fovea was extremely thin, with loss of the inner–outer segment junction. Extensive debris accumulation was noted. Patient 50397 (*CERKL*, p.C125W; age 48) had (**D**) a pigmentary maculopathy with choroidal sclerosis, (**E**) absence of central FAF, except in a tiny island, indicating nearly complete loss of lipofuscin deposition, and (**F**) very extensive retinal remodeling and an unusual subretinal cystic structure in the fovea. His older brother (54684, age 51), although carrying the same mutation in *CERKL*, had (**G**) extensive retinal, RPE, and optic disc atrophy and sclerosis, (**H**) loss of central FAF, with a peculiar salt-and-pepper pattern of FAF outside the central retina, and (**I**) thinning and disorganization of the retina. A *CERKL* nonsense mutation (p.R238X) in patient 51456 (age 53) resulted in (**J**) extensive choroidal sclerosis, (**K**) loss of FAF, and (**L**) retinal remodeling with loss of inner–outer segment junction. The retina of patient 20695 (*PROM1*, c.1142-1G>A; age 18) showed (**M**) central atrophic lesions in the fovea and severely attenuated retinal vessels. No autofluorescence and spectral-domain OCT images were available for this patient.

TABLE 4. Overview of Funduscopy, Autofluorescence Imaging and SD-OCT of CRD Patients

Patient ID (Family Number)	Gene Defect	Funduscopy	Autofluorescence	Spectralis OCT
16875 (W00–409)	*ABCA4*, c.160T>G, p.C54G	Central glittering maculopathy and some peripheral fishtail pigment clusters	NP	NP
16876 (W00–409)	*ABCA4*, c.160T>G, p.C54G	Central glittering maculopathy and some peripheral fishtail pigment clusters	NP	NP
16877 (W00–409)	*ABCA4*, c.160T>G, p.C54G	Granular macular changes with irregular RPE	NP	NP
50417	*ABCA4*, c.6729+5_19del15, splice defect	Advanced pigmentary maculopathy	Striking macular absence of FAF	Loss of the IS-OS junction, massive debris accumulation between the retina and RPE, retinal thickening and foveal thinning
50397 (W09–0340)	*CERKL*, c.375C>G, p.C125W	Perifoveal atrophy with a central RPE island	Striking macular absence of FAF with remaining central island	Single retinal cyst formation
54684 (W09–0340)	*CERKL*, c.375C>G, p.C125W	Advanced foveal and macular atrophy, choroidal sclerosis, and sclerosis in the macula	Striking macular absence of FAF	Extensive retinal remodeling, severe diffuse retinal thinning of one to two layers
51456	*CERKL*, c.874C>T, p.R283X	Choroidal sclerosis and sclerosis in the macula	Striking macular absence of FAF	RPE thinning
20695 (W02–007)	*PROM1*, c.1142–1G>A, splice defect	Central atrophic lesions in the macula, severely attenuated retinal vessels	NP	NP
20696 (W02–007)	*PROM1*, c.1142–1G>A, splice defect	Discrete central atrophy in the macula, severely attenuated retinal vessels, granular pigmentation in periphery	NP	NP

NP, not performed.

Overlap with Retinal Disease Loci

One homozygous region in a nonconsanguineous Dutch CRD family from this cohort overlapped with the RP25 locus, which led to the discovery of *EYS* and the identification of a p.Y3156X mutation in the affected siblings. We published this finding elsewhere.[22] In addition, several other homozygous regions were detected that overlapped with previously published retinal dystrophy loci: LCA9, RP22, RP28, RP29, RCD1, and most interesting, the 5.8 Mb CORD8 locus.[35,36] Two CRD patients of this study show an overlap with the entire CORD8 locus, a region that harbors more than 100 genes, of which *SEMA4A* was excluded as being causative. No other obvious candidate genes were identified in this region. Since most of the published retinal dystrophy loci are very large, the overlap with one of our homozygous regions may be coincidental.

Clinical Features

All 14 patients from eight families in which mutations were identified underwent detailed ophthalmic examinations. An overview of the clinical data is presented in Table 3. Ten patients showed clear signs of CRD. Remarkably, four patients from three families (two patients with mutations in *CABP4*, one with *EYS* mutations, and one with *KCNV2* mutations), originally thought to have cone–rod dysfunction and therefore entered in this study, appeared not to be affected with CRD. We have described the phenotype of the patients carrying mutations in *CABP4* and in *EYS* in other reports.[22,32] The fourth patient, carrying a homozygous *KCNV2* mutation, initially received a diagnosis of CRD. However, additional ERG testing at the age of 11 showed supernormal and delayed rod responses and decreased cone responses, characteristic of cone dystrophy with supernormal rod responses (RCD3; OMIM entry 610024; Online Mendelian Inheritance in Man; http://www.ncbi.nlm.nih.gov/Omim/ provided in the public domain by NCBI). The detection of the novel p.Y54X mutation in *KCNV2* confirmed the corrected diagnosis, since this phenotype is specifically associated with mutations in *KCNV2.*[37]

The remaining patients showed the typical signs of CRD, with a wide range of phenotypic features and severity. Visual acuity ranged from 20/50 at age 48 in patient 50397 (*CERKL*, p.C125W) to light perception (LP) at age 53 in patient 51456 (*CERKL*, p.R283X). All patients experienced color vision abnormalities, central defects of the visual field on Goldmann perimetry, and, when measurable, more decreased cone than rod responses. Fundus photographs, spectral-domain OCT, and FAF images of selected patients are presented in Figure 3 and described in Table 4.

In summary, macular appearances ranged from subtle RPE changes (*PROM1*) to pronounced atrophy of the RPE and choriocapillaris (*CERKL*). FAF showed an absence of autofluorescence in the macula in most of the patients. In vivo retinal architecture performed by spectral-domain OCT, showed a variety of patterns, ranging from severe retinal thinning (*CERKL*) to thickening (*CERKL*), loss of architecture (*ABCA4*), cysts (*CERKL*), and accumulation of subfoveal hyperreflective debris (*ABCA4*).

DISCUSSION

Homozygosity mapping in 95 probands and 13 affected siblings revealed significant homozygous regions in 77% of nonconsanguineous patients and in all consanguineous patients and led to the identification of the causative genetic defect in six nonconsanguineous probands and in two consanguineous probands. The percentage of homozygous regions in nonconsanguineous

patients seems to be high compared with those in other studies, in which homozygous regions were detected in 5% to 52% of patients from outbred populations.[24,38,39] This disparity could be explained by the less stringent software settings that we used to avoid false-negative regions. In addition, the cutoff for minimum length of homozygous regions varies among the different studies. Results of genotyping studies in large cohorts show that individuals from outbred populations commonly carry homozygous regions, although the regions are usually shorter than those in individuals from isolated populations.[17,40]

In all patients in whom a mutation was detected, the retinal phenotypes were reassessed. The mutations residing in *ABCA4*, *PROM1*, and *CERKL*, all previously associated with CRD,[4,9,10] indeed caused a CRD phenotype, although with variable characteristics. Three siblings carrying mutations in *ABCA4* and two siblings with *PROM1* mutations showed a decrease in visual acuity during childhood (8–15 years of age), which is consistent with the previously described *ABCA4*- and *PROM1*-related phenotypes.[9,41] One patient (50417) carrying a defective splice site in *ABCA4* had a later age at onset (30 years), which suggests that this mutation could have a moderately damaging effect on the protein. The three patients carrying mutations in *CERKL* had an age at onset of approximately 30 as well, but their visual acuities at the age of 50 differed considerably, even in the two brothers carrying the same mutation (20/50 at age 48 and 3/400 at age 51, respectively, and light perception at age 53 in the third patient). A wide variety of visual acuities in CRD patients with *CERKL* mutations were described by Aleman et al.,[10] as well.

A variability in retinal and macular appearances, retinal OCT findings and FAF patterns was noted, even for the same gene in different families (Figs. 3D–I versus 3J–L) and the same mutation in the same family (Figs. 3D–F versus 3G–I). A variety of maculopathies were documented, with geographic atrophy, choroidal sclerosis, and hyperpigmentation. Detailed OCT studies revealed retinal remodeling, loss of retinal layers, debris accumulation between the retina and RPE, single cysts, a subretinal cystic structure, and foveal thinning. Finally, FAF showed a complete absence of autofluorescence in the macular region in most patients, suggesting integral RPE and photoreceptor loss. Documenting structural (OCT) and functional (FAF) disease patterns in genotyped patients are crucial for future therapeutic trials and deciding the appropriate therapeutic modalities for the genotyped patients (gene replacement and drug trials in relatively intact retinas versus cell replacements in relatively destroyed maculas) but also for refining the clinical diagnosis and the visual prognosis.

One patient with *EYS* mutations received a diagnosis of CRD, but her brother was found to have RP. Mutations in *EYS* have been shown to be causative of ~5% to 12% of arRP cases, whereas no mutations have been found in additional CRD patients.[42–45] *EYS* is therefore not a frequent cause of CRD. The clinical diagnoses of the patients carrying mutations in *CABP4* and *KCNV2* were revised to congenital cone–rod synaptic disorder and cone dystrophy with supernormal rod responses, respectively.[32,37,46] These revisions illustrate the complexity of diagnosing retinal dystrophies, the significant clinical overlap of genetically distinct disorders, and the power and utility of molecular genetic testing.

Overall, the percentage of CRD cases that can be attributed to each gene in this cohort is ~1% for *PROM1* (1/123), ~2% for *CERKL* (2/123), and between ~27% (33/123) to ~34% (31/90) for *ABCA4*, which is consistent with reported findings in previous publications.[4–7,9,10] We identified *EYS* mutations in 1% (1/123) of the CRD patients, but no other mutations in CRD patients were identified in other studies.[42,43] The percentages include patients in which homozygous and heterozygous *ABCA4* mutations were detected by APEX screening (Asper Ophthalmics). For these calculations, we excluded the four patients in whom other clinical diagnoses had been established.

Which patients are most suitable for mutation detection by homozygosity mapping? Gibson et al.[40] showed that long homozygous segments are common in unrelated individuals from the HapMap database. However, long and numerous homozygous segments usually indicate parental relatedness.[17,18] Also, the average genome-wide homozygosity in nonconsanguineous patients of the entire cohort was 0.5% (0%–2.9%), whereas the average was 1.06% (0.5%–1.6%) in patients in whom homozygous mutations were identified. Although Carothers et al.[47] showed that the percentage of homozygosity is unreliable in predicting relatedness, our data suggest that the chance of finding a homozygous mutation is higher in patients with a high percentage of homozygosity. Knowing the family origin provides a better chance of finding a homozygous defect. In at least four of our patients, the paternal and maternal grandparents originated from the same geographic region; one family from a province in Greece, one from the south-west of The Netherlands, one from the east of The Netherlands, and one family from the French-Canadian founder population. Therefore, detailed information about the origin of grandparents of a patient is desired when considering whether homozygosity mapping will be efficient in identifying a genetic defect in a particular patient. Furthermore, for four of eight probands in which the genetic defect was identified, one or more affected siblings were available for homozygosity mapping. In agreement with the findings of Woods et al.,[48] families with two or more affected siblings provided the most power for pinpointing the homozygous region containing the mutation.

The homozygosity data generated in this study are likely to be a valuable resource for the detection of novel disease genes. The discovery of the *EYS* gene in a family of this cohort, for example, shows how relatively small homozygous regions in nonconsanguineous families can fine map the causative locus and thereby facilitate the identification of a new causative gene. Moreover, such data may be valuable now that next-generation sequencing is emerging, which enables the screening of all genes within a homozygous region in one experiment.[49]

In conclusion, this study shows that homozygosity mapping can lead to the identification of novel genetic defects in consanguineous as well as nonconsanguineous families. Our results show that many more CRD genes may exist. Finding them and identifying the associated detailed phenotypes will provide more insight into patients suitable for gene-specific therapies.

Acknowledgments

The authors thank Christel Beumer, Saskia van der Velde-Visser, and Jinny Conte for technical assistance; Suzanne Keijzers-Vloet and Irene Janssen for performing the microarray experiments; and Kentar ArimadyoSulakso and Almudena Avila-Fernández for assistance in mutation analysis.

References

1. Szlyk JP, Fishman GA, Alexander KR, Peachey NS, Derlacki DJ. Clinical subtypes of cone-rod dystrophy. *Arch Ophthalmol.* 1993;111:781–788.
2. Hamel CP. Cone rod dystrophies. *Orphanet J Rare Dis.* 2007;2:7–14.
3. Jalili IK, Smith NJ. A progressive cone-rod dystrophy and amelogenesis imperfecta: a new syndrome. *J Med Genet.* 1988;25:738–740.
4. Maugeri A, Klevering BJ, Rohrschneider K, et al. Mutations in the ABCA4 (ABCR) gene are the major cause of autosomal recessive cone-rod dystrophy. *Am J Hum Genet.* 2000;67:960–966.

5. Ducroq D, Rozet JM, Gerber S, et al. The ABCA4 gene in autosomal recessive cone-rod dystrophies. *Am J Hum Genet.* 2002;71:1480–1482.
6. Fishman GA, Stone EM, Eliason DA, Taylor CM, Lindeman M, Derlacki DJ. ABCA4 gene sequence variations in patients with autosomal recessive cone-rod dystrophy. *Arch Ophthalmol.* 2003; 121:851–855.
7. Klevering BJ, Yzer S, Rohrschneider K, et al. Microarray-based mutation analysis of the ABCA4 (ABCR) gene in autosomal recessive cone-rod dystrophy and retinitis pigmentosa. *Eur J Hum Genet.* 2004;12:1024–1032.
8. Hameed A, Abid A, Aziz A, Ismail M, Mehdi SQ, Khaliq S. Evidence of RPGRIP1 gene mutations associated with recessive cone-rod dystrophy. *J Med Genet.* 2003;40:616–619.
9. Pras E, Abu A, Rotenstreich Y, et al. Cone-rod dystrophy and a frameshift mutation in the PROM1 gene. *Mol Vis.* 2009;15:1709–1716.
10. Aleman TS, Soumittra N, Cideciyan AV, et al. CERKL mutations cause an autosomal recessive cone-rod dystrophy with inner retinopathy. *Invest Ophthalmol Vis Sci.* 2009;50:5944–5954.
11. Parry DA, Toomes C, Bida L, et al. Loss of the metalloprotease ADAM9 leads to cone-rod dystrophy in humans and retinal degeneration in mice. *Am J Hum Genet.* 2009;84:683–691.
12. Yzer S, Leroy BP, De Baere E, et al. Microarray-based mutation detection and phenotypic characterization of patients with Leber congenital amaurosis. *Invest Ophthalmol Vis Sci.* 2006;47:1167–1176.
13. Wissinger B, Gamer D, Jagle H, et al. CNGA3 mutations in hereditary cone photoreceptor disorders. *Am J Hum Genet.* 2001;69: 722–737.
14. Krone N, Braun A, Roscher AA, Knorr D, Schwarz HP. Predicting phenotype in steroid 21-hydroxylase deficiency?—comprehensive genotyping in 155 unrelated, well defined patients from southern Germany. *J Clin Endocrinol Metab.* 2000;85:1059–1065.
15. Sandoval N, Platzer M, Rosenthal A, et al. Characterization of ATM gene mutations in 66 ataxia telangiectasia families. *Hum Mol Genet.* 1999;8:69–79.
16. Roux AF, Faugere V, Le Guedard S, et al. Survey of the frequency of USH1 gene mutations in a cohort of Usher patients shows the importance of cadherin 23 and protocadherin 15 genes and establishes a detection rate of above 90%. *J Med Genet.* 2006;43:763–768.
17. McQuillan R, Leutenegger AL, Abdel-Rahman R, et al. Runs of homozygosity in European populations. *Am J Hum Genet.* 2008; 83:359–372.
18. Broman KW, Weber JL. Long homozygous chromosomal segments in reference families from the centre d'Etude du polymorphisme humain. *Am J Hum Genet.* 1999;65:1493–1500.
19. Hildebrandt F, Heeringa SF, Ruschendorf F, et al. A systematic approach to mapping recessive disease genes in individuals from outbred populations. *PLoS Genet.* 2009;5:e1000353.
20. den Hollander AI, Koenekoop RK, Yzer S, et al. Mutations in the CEP290 (NPHP6) gene are a frequent cause of Leber congenital amaurosis. *Am J Hum Genet.* 2006;79:556–561.
21. den Hollander AI, Koenekoop RK, Mohamed MD, et al. Mutations in LCA5, encoding the ciliary protein lebercilin, cause Leber congenital amaurosis. *Nat Genet.* 2007;39:889–895.
22. Collin RWJ, Littink KW, Klevering BJ, et al. Identification of a 2 Mb human ortholog of Drosophila eyes shut/spacemaker that is mutated in patients with retinitis pigmentosa. *Am J Hum Genet.* 2008;83:594–603.
23. Thiadens AA, den Hollander AI, Roosing S, et al. Homozygosity mapping reveals PDE6C mutations in patients with early-onset cone photoreceptor disorders. *Am J Hum Genet.* 2009;85:240–247.
24. den Hollander AI, Lopez I, Yzer S, et al. Identification of novel mutations in patients with Leber congenital amaurosis and juvenile RP by genome-wide homozygosity mapping with SNP microarrays. *Invest Ophthalmol Vis Sci.* 2007;48:5690–5698.
25. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 1988;16:1215.
26. Jaakson K, Zernant J, Kulm M, et al. Genotyping microarray (gene chip) for the ABCR (ABCA4) gene. *Hum Mutat.* 2003;22:395–403.
27. Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet.* 2007;81:559–575.
28. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol.* 2000;132:365–386.
29. Little S. Amplification-refractory mutation system (ARMS) analysis of point mutations. *Curr Protoc Hum Genet.* 2001;chapter 9:unit 9.8.
30. Lencz T, Lambert C, DeRosse P, et al. Runs of homozygosity reveal highly penetrant recessive loci in schizophrenia. *Proc Natl Acad Sci U S A.* 2007;104:19942–19947.
31. Ozgul RK, Durukan H, Turan A, Oner C, Ogus A, Farber DB. Molecular analysis of the ABCA4 gene in Turkish patients with Stargardt disease and retinitis pigmentosa. *Hum Mutat.* 2004;23:523.
32. Littink KW, van Genderen MM, Collin RWJ, et al. A novel homozygous nonsense mutation in CABP4 causes congenital cone-rod synaptic disorder. *Invest Ophthalmol Vis Sci.* 2009;50:2344–2350.
33. Shapiro MB, Senapathy P. RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. *Nucleic Acids Res.* 1987;15:7155–7174.
34. Henikoff S, Henikoff JG. Amino acid substitution matrices from protein blocks. *Proc Natl Acad Sci U S A.* 1992;89:10915–10919.
35. Khaliq S, Hameed A, Ismail M, et al. Novel locus for autosomal recessive cone-rod dystrophy CORD8 mapping to chromosome 1q12–q24. *Invest Ophthalmol Vis Sci.* 2000;41:3709–3712.
36. Ismail M, Abid A, Anwar K, Mehdi SQ, Khaliq S. Refinement of the locus for autosomal recessive cone-rod dystrophy (CORD8) linked to chromosome 1q23–q24 in a Pakistani family and exclusion of candidate genes. *J Hum Genet.* 2006;51:827–831.
37. Wu H, Cowing JA, Michaelides M, et al. Mutations in the gene KCNV2 encoding a voltage-gated potassium channel subunit cause "cone dystrophy with supernormal rod electroretinogram" in humans. *Am J Hum Genet.* 2006;79:574–579.
38. Harville HM, Held S, Diaz-Font A, et al. Identification of 11 novel mutations in 8 BBS genes by high-resolution homozygosity mapping. *J Med Genet.* 2010;47:262–267.
39. Li LH, Ho SF, Chen CH, et al. Long contiguous stretches of homozygosity in the human genome. *Hum Mutat.* 2006;27:1115–1121.
40. Gibson J, Morton NE, Collins A. Extended tracts of homozygosity in outbred human populations. *Hum Mol Genet.* 2006;15:789–795.
41. Klevering BJ, Blankenagel A, Maugeri A, Cremers FPM, Hoyng CB, Rohrschneider K. Phenotypic spectrum of autosomal recessive cone–rod dystrophies caused by mutations in the *ABCA4* (*ABCR*) gene. *Invest Ophthalmol Vis Sci.* 2002;43:1980–1985.
42. Littink KW, van den Born LI, Koenekoop RK, et al. Mutations in the EYS gene account for ~5% of autosomal recessive retinitis pigmentosa and cause a fairly homogeneous phenotype. *Ophthalmology*. Published online May 27, 2010.
43. Bandah-Rozenfeld D, Littink KW, Ben-Yosef T, et al. Novel null mutations in the EYS gene are a frequent cause of autosomal recessive retinitis pigmentosa in the Israeli population. *Invest Ophthalmol Vis Sci.* In press.
44. Abd El-Aziz MM, O'Driscoll CA, Kaye RS, et al. Identification of novel mutations in the ortholog of *Drosophila* eyes shut gene (EYS) causing autosomal recessive retinitis pigmentosa. *Invest Ophthalmol Vis Sci.* 2010;51:4266–4272.
45. Audo I, Sahel JA, Mohand-Said S, et al. EYS is a major gene for rod-cone dystrophies in France. *Hum Mutat.* 2010;31:E1406–E1435.
46. Zeitz C, Kloeckener-Gruissem B, Forster U, et al. Mutations in CABP4, the gene encoding the Ca2+-binding protein 4, cause autosomal recessive night blindness. *Am J Hum Genet.* 2006;79: 657–667.
47. Carothers AD, Rudan I, Kolcic I, et al. Estimating human inbreeding coefficients: comparison of genealogical and marker heterozygosity approaches. *Ann Hum Genet.* 2006;70:666–676.
48. Woods CG, Cox J, Springell K, et al. Quantification of homozygosity in consanguineous individuals with autosomal recessive disease. *Am J Hum Genet.* 2006;78:889–896.
49. Metzker ML. Sequencing technologies: the next generation. *Nat Rev Genet.* 2010;11:31–46.