Genetic Heterogeneity in Microcornea-Cataract: Five Novel Mutations in CRYAA, CRYGD, and GJA8

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PURPOSE. To unravel the molecular genetic background in families with congenital cataract in association with microcornea (CCMC, OMIM 116150).

METHODS. CCMC families were recruited from a national database on hereditary eye diseases; DNA was procured from a national gene bank on hereditary eye diseases and by blood sampling from one large family. Genomewide linkage analysis, fine mapping, and direct genomic DNA sequencing of nine cataract candidate genes were applied. Restriction enzyme digests confirmed identified mutations.

RESULTS. Analyses of 10 Danish families with hereditary congenital cataract and microcornea revealed five novel mutations. Three of these affected the crystallin, α-A gene (CRYAA), including two mutations (R12C and R21W) in the crystallin domain and one mutation (R116H) in the small heat shock domain. One mutation (P189L) affected the gap junction protein α 8 (GJA8), and one mutation (Y134X) was detected in crystallin γ-D (CRYGD).

CONCLUSIONS. The identification of a CRYGD mutation adds another gene to those that may be mutated in CCMC and underscores the genetic heterogeneity of this condition. Three CRYAA mutations at the R116 position, in association with CCMC, suggest that R116 represents a CCMC-mutational hotspot. The CCMC phenotype demonstrates variable expression including additional malformation of the anterior segment of the eye, confirm that dedicated cataract genes may be involved in the largely unknown developmental molecular mechanisms involved in lens-anterior segment interactions. (Invest Ophthalmol Vis Sci. 2007;48: 3937–3944) DOI:10.1167/iovs.07-0013

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The association of developmental cataract and microcornea (congenital cataract-microcornea syndrome, microcornea-cataract [CCMC], OMIM 116150) makes up a distinct phenotype within the group of autosomal dominant congenital cataracts. In 1952, Friedman and Wright described a five-generation family in whom CCMC segregated, and subsequently reports of seven families with CCMC have been published. Polomeno et al. first identified an autosomal dominant locus, CMC3, and up to now six mutations in four genes and one locus have been reported in autosomal dominant CCMC.

CCMC has been considered a rare phenotype. The condition may, however, be more common than hitherto estimated. Among 60 Indian probands, Devi and Vijayalakshmi ascertained 11 subjects with CCMC (18%), and Auffarth and Völker were able to recruit 79 patients. The frequency of CCMC may be underestimated because the focus often is on the sight-threatening cataract, and corneal diameters may pass unnoticed. Another reason for underestimation is a misclassification of microcornea as microphthalmos. Congenital cataract is frequently associated with either condition, and attention is sometimes not directed toward the size of the bulb or the presence or absence of a corneoscleral groove, which is a differentiating sign.

From a clinical point of view, the distinction between cataracts with normal corneal dimensions and CCMC is important because it has repeatedly been shown that CCMC is a significant risk factor for the subsequent development of glaucoma. Within families the association between microcornea and cataract is consistent, though variations occur in the corneal diameter. Yet CCMC has also been reported in association with other developmental defects of the anterior chamber of the eye, primarily with iris coloboma or Peters anomaly, implicating a close pathogenetic relation between congenital cataract and various kinds of anterior chamber dysgenesis. It is, therefore, important to outline these phenotypes and unravel their genetic backgrounds to gain insight into the roles of different genes and, in turn, their differential roles in lens development and its relation to the architecture of the anterior segment of the eye.

As part of a larger study on hereditary cataract, we analyzed 10 families with supposed autosomal dominant CCMC; likely disease-causing mutations were identified in five of the families.

PATIENTS, MATERIALS, AND METHODS

Patients

Patient data were retrieved from a registry on hereditary eye diseases at the Kennedy Institute-National Eye Clinic (www.kisoe.eu), formerly the National Eye Clinic for the Visually Impaired (NEC). Ten families affected by CCMC were included in the study, and results were obtained in one large family (CCMC0106; Fig. 1) and in four small families (CCMC0101, CCMC0103, CCMC0108, CCMC0109; Fig. 2). During home visits, blood samples were collected from 15 persons (seven unaffected and eight affected members) in family CCMC0106, and
stored DNA samples from the ophthalmic gene bank at the Kennedy Institute-National Eye Clinic were obtained from one affected person each from the nine small families. Blood samples from additional affected family members were procured if possible. At the time of this study, most patients were either aphakic or had artificial lenses. Therefore, cataract phenotypes were retrospectively obtained from the patient files at NEC. Microcornea was defined as a horizontal corneal diameter smaller than 11 mm in persons 7 years of age and older. During home visits, the corneal diameter was measured with a simple rule. The study adhered to the tenets of the Declaration of Helsinki and was approved by the Copenhagen Scientific Ethical Committee. All subjects gave informed written consent to participate in the study. Genomic DNA was extracted from whole blood using standard procedures. Screening of mutations and polymorphisms in the healthy population was performed on 170 independent persons selected from the Copenhagen Family Bank.21

Genome Linkage Analysis

Genomewide linkage analysis using a linkage map (ABI 10cM; Applied Biosystems, Foster City, CA) was performed for family CCMC0106. Fine mapping of the candidate region was performed with radioactive-labeled STS markers D21S1890 and D21S1885 (Table 1). Two-point LOD scores for initial exclusion and mapping were calculated using the program LIPED.22

DNA Sequencing

Direct DNA sequencing of the genes CRYAA, CRYBA1, CRYBB1, CRYBB2, CRYGC, CRYGD, GJA3, GJA8, and HSF4 were carried out using sequencing technology and a sequencing apparatus (BigDye version 1.1 and ABI 3130; Applied Biosystems). PCR and sequencing primers (primers sequences and amplification conditions available on request) were designed using a primer23 from TAG Copenhagen A/S (Primer 3; Copenhagen, Denmark). All exons and exon/intron border regions were bidirectionally sequenced and aligned to the GenBank sequences (accession no. NM_000394, CRYAA; NM_006891, CRYGD; NM_005267, GJA8), and genomic intron sequences were aligned to human reference assembly hg17 (NCBI Build 35 from UCSC http://genome.ucsc.edu/). Polymerases were purchased from New England Biolabs (Taq DNA polymerases; Ipswich, MA), Qiagen (HotStartTaq DNA Polymerase; Hilden, Germany), and Invitrogen (Platinum Taq DNA polymerase; Carlsbad, CA). Reactions were carried out in 15-μL volumes according to the manufacturers’ protocols. PCR reactions
were analyzed by 2% agarose gel electrophoresis by staining with ethidium bromide, 1×TBE, before sequencing. Sequence analyses were carried out with the appropriate software (Chromas or ChromasPro; Technelysium Pty. Ltd., Tewantin, Australia). One person from each of the nine CCMC families, with the exception of CCMC0106, was chosen for DNA sequencing of the nine cataract candidate genes.

**Mutation Analysis**

Identified mutations were analyzed by restriction enzyme digestes. All restriction enzymes were purchased from New England Biolabs, and digests were conducted in accordance with the manufacturer’s protocols in a reaction volume of 20 μL using 2 to 4 μL PCR product and 5 to 10 U enzyme. Digested PCR products were analyzed by 2% agarose, 1×TBE, or 20% acrylamide gel electrophoresis, 1×TBE, respectively, and DNA was visualized by staining with ethidium bromide.

**RESULTS**

**Molecular Findings**

Overall, the analyses revealed mutations in five (50%) of the examined families. All identified mutations were novel; three resided in CRYAA, one in GJA8, and one in CRYGD (Table 2). Linkage data in family CCMC0106 are summarized in Table 1, and the results of sequencing are summarized in Figures 1 and 2.

**FIGURE 2.** Genetic analysis of families CCMC0101 (A), CCMC0103 (B), CCMC0108 (C), and CCMC0109 (D). Filled symbols: affected persons. Open symbols: unaffected persons. a) Family members who underwent DNA sequencing. (A) CCMC0101. Restriction enzyme HhaI digest of the CRYAA PCR product shows three fragments (84 bp, 96 bp, 248 bp) for the wild-type alleles in the control person (N) and the mutant allele fragments (84 bp, 344 bp) plus the wild-type allele fragments in affected persons II:2 and III:3. Size marker (M) is a 50-bp ladder. (U) Uncut PCR product, 2% agarose gel electrophoresis. The DNA sequence chromatogram shows the c.34C>T transition in exon 1 as a C/T double peak. (B) CCMC0103. Restriction enzyme BspI digest of the GJA8 PCR product shows two wild-type allele fragments (115 bp and 127 bp) for the control (N) and an additional fragment (154 bp) representing the mutant allele in the affected persons II:7 and III:2. Size marker (M) is a 100-bp ladder. (U) Uncut PCR product, 2% agarose gel electrophoresis. The DNA sequence chromatogram shows the c.565C>T mutation in exon 2 as a C/T double peak. (C) CCMC0108, the restriction enzyme MspI digest of the CRYAA PCR product, shows three wild-type allele fragments (107 bp and 117 bp as one band, and 205 bp) for the control (N) and an additional fragment (322 bp) representing the mutant allele in affected person III:4. Size marker (M) is a 100-bp ladder. (U) Uncut PCR product, 2% agarose gel-electrophoresis. The DNA chromatogram shows the c.61C>T mutation in exon 1 as a C/T double peak. (D) CCMC0109, the restriction enzyme DdeI digest of the CRYGD PCR product shows the wild-type allele fragments (152 bp and 225 bp) for the control person (N) and two additional two fragments (52 bp and 100 bp) representing the mutant allele in affected person II:4. Size marker (M) is a 50-bp ladder, 2% agarose gel electrophoresis. The DNA sequence chromatogram shows the c.418C>A mutation in exon 3 as a C/A double peak.
2. DNA from 170 unrelated unaffected persons from the same ethnic background (340 chromosomes) served as controls (data not shown).

Phenotypes

We identified 10 families with apparent autosomal dominant developmental cataract and microcornea from approximately 80 families (12.5%) with autosomal dominant developmental cataracts.

Clinical findings are summarized in Table 2. Information on cataract morphology was present in most of the patient’s files. Corneal diameters varied between 8 and 10 mm, whereas most eyes had normal keratometer readings. Nystagmus was present in some families and absent in others, depending primarily on the degree of visual impairment during the first months of life. Cataract phenotypes varied but often involved the nuclei with cortical laminar elements and anterior and posterior polar opacities to a variable extent. Most cataracts had a clear peripheral zone. In some patients, cataract progression during the first years of life was noted.

DISCUSSION

This study adds five novel mutations to those previously reported in association with cataract and microcornea (Table 3). Three of the mutations reported here are located in the α-crystallin gene CRYAA. Among the total number of cataract mutations in this gene, four missense mutations are reported in association with CCMC (Table 3), and three are reported in association with congenital cataract without anterior segment anomalies (CC)25 (Figs. 3A, 3B).

The R116C mutation has been reported twice in CCMC families12,13 and once in a CC family with additional microphthalmia and coloboma.26 The identification in a Danish family of a new and distinct CRYAA mutation involving the same amino acid position strongly underscores the importance of the arginine 116 residue. The crystallin A type of small heat shock proteins is found in many different phyla, and all share a common structure of an N-terminal less-conserved (disordered) region, a conserved α-crystallin domain (ACD), and a short C-terminal (Figs. 3A, 3B). The ACD region may be involved in aggregation and disaggregation of larger protein complexes, whereas the N-terminal and the C-terminal regions are suggested to play a role in oligomerization.27 Crystal-structure investigations have shown R116 to be located in the oligomer interface of the β7-strand in the ACD region (Fig. 3C), which may be part of a conserved exterior salt bridge in the α-crystallin oligomer.15,27–29 Alignment of the primary sequence for small heat shock proteins from bacteria, plants, and mammals, including human CRYAB and CRYAA, demonstrate that the arginine 116 residue is among the highest conserved positions of the ACD region (Fig. 3A). In addition, a mutation of the identical arginine residue in CRYAB has been reported in a desmin-related myopathy,30 which underscores the importance of arginine 116. Studies in a two-hybrid system assay31 showed that the R116C mutation decreased αA-crystallin interactions with βB- and γC-crystallins, whereas those with αB-crystallin and heat-shock protein Hsp27 increased, suggesting that the mutation destabilizes αA-crystallin so that it sets loose its own chaperone properties and is bound by other chaperones.

The cataract phenotypes in the R116 mutation CCMC families (Tables 2, 3) show similarities consisting of nuclear opacities with fans,13 ramifications in different directions (this study), or zonular elements13 and nuclear cataract in one CC family.26

The two novel CRYAA mutations, R12C and R21W, are located in the N-terminal, and the two arginine residues are conserved in several of the small heat shock proteins (Fig. 3A). The phenotypes for these two mutations are similar and consist of a central, zonular cataract with varying involvement of the anterior and posterior poles.

Several mutations are known in GJA8 (Fig. 4A), three of which are associated with the CCMC phenotype (Table 3). Two of the CCMC associated mutations, P189L in the Danish family CCMC0103 and R198Q in an Indian family,8 resided in

<table>
<thead>
<tr>
<th>Marker</th>
<th>Mbp*</th>
<th>0.00</th>
<th>0.01</th>
<th>0.05</th>
<th>0.10</th>
<th>0.20</th>
<th>0.30</th>
<th>0.40</th>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td>2.25</td>
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<td>1.21</td>
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<td>0.40</td>
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<td>0.47</td>
<td>0.38</td>
<td>0.24</td>
<td>0.10</td>
</tr>
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</table>

* Distances in mega base pairs according to UCSC May 2004, NCBI Build 35.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Family</th>
<th>No. Affected Examined</th>
<th>Corneal Diameter (mm)</th>
<th>Corneal Curvature Radii (mm)</th>
<th>Cataract Phenotype</th>
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<td>R12C</td>
<td>CCMC00101</td>
<td>1</td>
<td>9.5</td>
<td>6.7–7.2</td>
<td>Posterior polar progressing to dense nuclear and laminar, with involvement of anterior and posterior poles</td>
</tr>
<tr>
<td>CRYAA</td>
<td>R21W</td>
<td>CCMC00108</td>
<td>4</td>
<td>8–10</td>
<td>6.4–7.2</td>
<td>Central and laminar with varying opacification of anterior and posterior poles</td>
</tr>
<tr>
<td>CRYAA</td>
<td>R116H</td>
<td>CCMC00106</td>
<td>8</td>
<td>8–10.5</td>
<td>6.8–7.7</td>
<td>Nuclear with polar and/or equatorial opacification of anterior and posterior poles</td>
</tr>
<tr>
<td>GJA8</td>
<td>P189L</td>
<td>CCMC00103</td>
<td>4</td>
<td>10</td>
<td>7.1–8.7</td>
<td>Star-shaped nuclear opacity with a whitish central core</td>
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<td>CRYGD</td>
<td>Y134X</td>
<td>CCMC00109</td>
<td>2</td>
<td>10</td>
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</table>
the second extracellular domain. Both mutant amino acid positions are highly conserved in different gap junction proteins and between species (Fig. 4B). P189 is part of a conserved proline-cysteine-proline motif, which includes one of the three conserved cysteines in EC2. This Pro-Cys-Pro motif is conserved in all human gap junction proteins, /H9251/H9253/-type (Fig. 4B), and the pathogeneity of this proline residue is further stressed by an analogous mutation, P187L (Fig. 4B), in the GJA3 protein associated with the CC phenotype.32 A third CCMC-associated mutation in an Indian family 8 is located in the first transmembrane domain. The CCMC phenotype in both Indian families, but not in the Danish family, was associated with myopia (Table 2).

The nonsense mutation Y134X is the first crystallin γ-D mutation identified in association with CCMC (Fig. 5A). The mutation carries the systematic names c.418C>A at the nucleotide level and Y134X at the protein level, respectively, according to the standard rules of nomenclature33 (see Fig. 5A and legend for explanation). The Y134X nonsense mutation is located in exon 3, the last exon in the CRYGD gene (Fig. 5B), which also codes for the legal CRYGD protein stop codon. Transcripts of both the mutant Y134X and the second known nonsense mutant W157X (Fig. 5B) probably escape the nonsense-mediated decay of RNA (NMD) pathway,34 and the aberrant mRNAs are instead translated to truncated protein products. These truncated protein products may act by a dominant
negative mechanism, giving rise to the cataract phenotypes in both cases. The mechanisms through which protein abnormalities cause loss of lens transparency are still speculative. Functional studies on specific crystallin gene mutations have predicted increased light scatter because of reduced protein solubility, protein precipitation secondary to aberrant protein–

A. GJA8

B. Human

Dog

Mouse

Sheep

Golden hamster

Chick

Danio

GJA8

GJA10

GJA7

GJA12

GJA4

GJA1

GJA5

GJA8

GJA10

GJA7

GJA12

GJA4

GJA1

GJA5

A. CRYGD

B. (A) Six CRYGD mutations in association with the OC phenotype have been reported in 10 family studies. Until now the systematic name for all CRYGD mutations (except the E107A mutation) has used the N-terminal–processed CRYGD protein, which starts with glycine at position 2 in the translated mRNA. According to the nomenclature for the description of sequence variations, the first methionine in the coding sequence should be assigned position 1, and the adenine in the corresponding start ATG codon should be assigned position +1. The translated and the processed CRYGD protein sequences are aligned, and the mutation nomenclatures are shown for both sequences with the position of the Greek key motifs inserted between the two sequences. (B) The structure of the CRYGD protein is represented by four Greek key motifs organized in two separate domains, which are encoded by exons 2 and 3, respectively. The CRYGD protein spans 174 amino acids, and the six known plus the novel CCMC-associated Y134X mutation (shaded in yellow) are shown. Both the Y134X and the W157X mutations are located in the fourth Greek key motif, resulting in a new illegal stop codon in the last exon.
protein interaction, misfolding, and altered aggregation properties leading to intranuclear amyloid-like inclusions and enlarged interfiber spaces as likely mechanisms involved in cataractogenesis.\textsuperscript{35,36} Lens opacification with \textit{GJA3} and \textit{GJA8} mutations has been shown to result in absent or defective gap junctions and hemichannels,\textsuperscript{37–39} which theoretically may alter intercellular signaling and intracellular ionic concentrations, giving rise to secondary changes in the molecular properties of crystallins. Among nonstructural functions of crystallin genes, their properties as chaperones with structural similarities to small heat shock proteins genes also seem to play a role in cataract formation. Recently it was shown that the major lenticular protein chaperones, \(\alpha\)- and \(\beta\)-crystallin, increased the solubility and reduced the size of \(\alpha\)/\(\beta\)-crystallin aggregates.\textsuperscript{40} The small heat shock transcription factor \(Hsf4\), in which four different mutations in cataract families have been reported, has been shown to exert transcriptional activity on other heat shock protein genes.\textsuperscript{41}

Cataract phenotypes in CCMC show considerable clinical heterogeneity with regard to morphology, age of onset, and progression, and consistent genotype–phenotype relations for the common cataract types have not been recognized. A wide and flourishing spectrum of descriptive terms for basically similar conditions might have contributed to this failure. We were able to identify five novel mutations by screening 10 CCMC families in nine already known cataract genes. These results stress the close relations between simple cataract and cataracts involving microcornea and raise the question whether the two conditions should be considered separate ontological entities. The latter view seems to be justified by the fact that most published families present either the CC or the CCMC phenotype. In addition, microcornea was never observed as an isolated trait in our study. The numbers of known mutations in CC and CCMC are, however, still limited, which hampers evaluation of whether the appearance of CC or CCMC phenotypes depends on specific mutations or closely linked modifying elements.

Substantial experimental evidence has documented that proper differentiation of the neural crest-derived structures of the anterior chamber depends on molecular signals from the lens epithelium.\textsuperscript{32,33} Determining whether the final growth of the cornea late in fetal life is influenced by similar mechanisms must await the identification of these pathways.

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**References**


