Novel Mutations in the CHST6 Gene Associated With Macular Corneal Dystrophy in Southern India

John F. Warren, MD; Anthony J. Aldave, MD; M. Srinivasan, MD; Eugene J. Thonar, PhD; Abha B. Kumar, MD; Vicky Cevallos, BS; John P. Whitcher, MD, MPH; Todd P. Margolis, MD, PhD

**Objective:** To further characterize the role of the carbohydrate sulfotransferase (CHST6) gene in macular corneal dystrophy (MCD) through identification of causative mutations in a cohort of affected patients from southern India.

**Methods:** Genomic DNA was extracted from buccal epithelium of 75 patients (51 families) with MCD, 33 unaffected relatives, and 48 healthy volunteers. The coding region of the CHST6 gene was evaluated by means of polymerase chain reaction amplification and direct sequencing. Subtyping of MCD into types I and II was performed by measuring serum levels of antigenic keratan sulfate.

**Results:** Seventy patients were classified as having type I MCD, and 5 patients as having type II MCD. Analysis of the CHST6 coding region in patients with type I MCD identified 11 homozygous missense mutations (Leu22Arg, His42Tyr, Arg50Cys, Arg50Leu, Ser53Leu, Arg97Pro, Cys102Tyr, Arg127Cys, Arg205Gln, His249Pro, and Glu274Lys), 2 compound heterozygous missense mutations (Arg93His and Ala206Thr), 5 homozygous deletion mutations (delCG707-708, delC890, delA1237, del1748-1770, and delORF), and 2 homozygous replacement mutations (ACCTAC 1273 GGT, and GCG 1304 AT). One patient with type II MCD was heterozygous for the C890 deletion mutation, whereas 4 possessed no CHST6 coding region mutations.

**Conclusion:** A variety of previously unreported mutations in the coding region of the CHST6 gene are associated with type I MCD in a cohort of patients in southern India.

**Clinical Relevance:** An improved understanding of the genetic basis of MCD allows for earlier, more accurate diagnosis of affected individuals, and may provide the foundation for the development of novel disease treatments.

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**MACULAR CORNEAL DYSTROPHY (MCD) is an autosomal recessive disorder of corneal keratan sulfate (KS) metabolism that is characterized by bilateral progressive corneal clouding.**  
A superficial stromal haze develops in the first to second decade of life, and is followed by an accumulation of irregular, focal, gray-white corneal stromal deposits. These deposits involve the anterior corneal stroma centrally and the deep stroma peripherally. Affected patients typically experience an early-onset, progressive decline in vision, often leading to penetrating keratoplasty. Histopathologic findings in MCD demonstrate abnormal accumulations of glycosaminoglycans in the extracellular stroma, keratocytes, and endothelial cells.

Macular corneal dystrophy is commonly divided into types I, IA, and II, on the basis of analysis of antigenic KS (AgKS) levels in serum and immunohistochemical evaluation of corneal buttons obtained at the time of penetrating keratoplasty. Affected patients with undetectable serum levels of AgKS are classified as having type I MCD, whereas those with low or reference levels are classified as having type II MCD. Type IA MCD is distinguished from type I MCD by the presence or absence, respectively, of highly sulfated AgKS within corneal keratocytes in individuals who have no detectable amounts of this form of AgKS in serum.

Recently, investigators have linked MCD to mutations in a newly recognized carbohydrate sulfotransferase gene (CHST6) on chromosome 16 (16q22). The CHST6 gene product, corneal N-acetylgalactosamine-6-sulfotransferase (C-GlcNAc6ST), is thought to be important in producing sulfated KS, the pre-
Diagnosis of MCD was based on the distinctive clinical features at AEH between January 1, 1990, and January 1, 2000. The database was performed to identify all patients with MCD seen at the University of California–San Francisco (H7177-18489-01), a computerized medical record view board at the AEH and the University of California–San Francisco, where molecular genetic analysis was performed. In addition, a portion of serum from each blood sample was sent to Rush Medical College, Chicago, Ill, for quantification of AgKS levels in serum.

DNA PREPARATION

After receiving informed consent from each study participant, a blood sample and buccal epithelial swab were obtained. Approximately 3 mL of peripheral blood was drawn from each subject by means of standard phlebotomy. In addition, samples of buccal mucosal epithelium were obtained by twirling a cytology brush (CytoSoft brush CP-5B; Medical Packaging Corporation, Camarillo, Calif) vigorously against the buccal epithelium. Genomic DNA was prepared from the buccal epithelial cells and/or blood leukocytes using a spin protocol (QIAamp DNA Mini Kit; Qiagen Inc, Valencia, Calif).

POLYMERASE CHAIN REACTION AMPLIFICATION

The CHST6 coding region was amplified by means of polymerase chain reaction (PCR) using primers designed to create 3 overlapping amplicons. The oligonucleotide primers used were identical to those reported by Akama et al, with the exception of the middle coding region reverse primer (5’-TCCGTGGGTTGATGTTATGGAT-3’). Each reaction was performed in a 30-µL mixture containing 25 µL of PCR premix (100mM Tris hydrochloride [pH, 8.3], 100mM potassium chloride, 400µM of each deoxynucleotide phosphate, and proprietary concentrations of magnesium chloride and PCR enhancer) (FailSafe PCR 2×PreMix D; Epicentre, Madison, Wis), 0.2µM of each primer, 1.5 U of DNA polymerase (AmpliTaq; Applied Biosystems, Foster City, Calif), and approximately 100 ng of genomic DNA. Thermal cycling was performed with the following program: initial denaturation for 3 minutes at 96°C, 35 cycles of 96°C for 30 seconds, 57°C for 30 seconds, and 72°C for 45 seconds, and final extension for 5 minutes at 72°C (GeneAmp PCR System 9700; Applied Biosystems).

DNA SEQUENCING

Amplified DNA was column purified (QIAquick PCR purification kit; Qiagen Inc) and sequenced directly according to the protocols accompanying the cycle sequencing kit (BigDye Terminator kit; Applied Biosystems). A genetic analyzer (ABI Prism 310; Applied Biosystems) was used to collect and analyze the sequence data. Nucleotide sequences were compared with the published CHST6 complementary DNA sequence.

AgKS QUANTIFICATION

An epitope present on long KS chains was quantified using a well-characterized enzyme-linked immunosorbent assay that uses the 1/20/3-D-4 monoclonal antibody. Levels of AgKS in serum are reported here as equivalents of a standard of AgKS highly purified from human costal cartilage.

RESULTS

A total of 75 affected patients, representing 51 different families, were enrolled in the study. On the basis of undetectable levels of serum AgKS, 70 of the affected patients (47 families) were classified as having type I MCD. The other 5 affected patients (4 families) had serum levels of AgKS that were low or within the reference range (103-210 ng/mL) and were classified as having type II MCD. Levels of AgKS within the reference range were maintained.
were found to possess only 1 observed. Two patients (same family) with type I MCD
whereas normal amplification of other genomic sites was
region primer pairs produced a detectable amplicon,
plification. In these patients, none of the
open reading frame on the basis of failure of PCR am-
lies were presumed to have a deletion of the entire
1 affected individual. Three patients from 2 unrelated fami-
dehletion (del1748-1770) was identified in a family with
erozygotes (Arg93His and Ala206Thr). A 23-nucleotide
detected in 7 patients from 3 families. Both affected in-
tients from 19 families, and 2 homozygous replacement
708, delC890, and delA1237) were identified in 26 pa-
three homozygous frameshift mutations (delCG707-
Arg127Cys, Arg127Gln, His249Pro, and Glu274Lys).
acid substitutions revealed that 11 (85%) of 13 would re-
sites. Examination of the R groups of the predicted amino
11 (85%) of 13 were found to occur at highly conserved
of the number of gene defects associated with type I MCD.
CHST6, carbohydrate sulfotransferase gene; MCD, macular corneal dystrophy.

<table>
<thead>
<tr>
<th>DNA Change</th>
<th>Amino Acid Substitution</th>
<th>R-Group Change</th>
<th>No. of Patients</th>
<th>No. of Families</th>
</tr>
</thead>
<tbody>
<tr>
<td>T757G</td>
<td>Leu22Arg</td>
<td>Nonpolar to basic</td>
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<td>1</td>
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<tr>
<td>C816T</td>
<td>His42Tyr</td>
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<td>1</td>
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<td>Arg50Cys</td>
<td>Basic to polar</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>G641T</td>
<td>Arg50Leu</td>
<td>Basic to nonpolar</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>C850T</td>
<td>Ser53Leu</td>
<td>Polar to nonpolar</td>
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<td>4</td>
</tr>
<tr>
<td>C850T heterozygous</td>
<td>Ser53Leu</td>
<td>Polar to nonpolar</td>
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<td>1</td>
</tr>
<tr>
<td>G892C</td>
<td>Arg97Pro</td>
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<td>1</td>
</tr>
<tr>
<td>G979A</td>
<td>Cys102Tyr</td>
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<td>2</td>
</tr>
<tr>
<td>C1071T</td>
<td>Arg127Cys</td>
<td>Basic to polar</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>G1306A</td>
<td>Arg206Gln</td>
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<td>2</td>
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<tr>
<td>A1438C</td>
<td>His249Pro</td>
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<tr>
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<td>Glu274Lys</td>
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<td>1</td>
</tr>
<tr>
<td>G970A; G1308A</td>
<td>Arg93His; Ala206Thr</td>
<td>Basic to basic; nonpolar to polar</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

Abbreviations: CHST6, carbohydrate sulfotransferase gene; MCD, macular corneal dystrophy.

<table>
<thead>
<tr>
<th>DNA Change</th>
<th>Protein Change</th>
<th>No. of Patients</th>
<th>No. of Families</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replacement</td>
<td>194 Asn Leu Arg 196; ACCTAC 1273 GGT</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Replacement</td>
<td>Major</td>
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<td>1</td>
</tr>
<tr>
<td>GCG 1304 AT</td>
<td>Major</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Deletion CG707-708</td>
<td>Major</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Deletion C890</td>
<td>Major</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Deletion A1237</td>
<td>Major</td>
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<td>12</td>
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<tr>
<td>Deletion 1748-1770</td>
<td>Major</td>
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<td>1</td>
</tr>
<tr>
<td>Deletion of open reading frame</td>
<td>Absent</td>
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</tr>
</tbody>
</table>

Abbreviations: CHST6, carbohydrate sulfotransferase gene; MCD, macular corneal dystrophy.

In this study of 51 families with MCD, 20 different type I disease-associated CHST6 coding region mutations were identified. These included 13 point mutations, 5 deletions, and 2 replacements (deletion-insertion). This represents the largest cohort of patients with MCD to undergo mutational analysis, and results in a doubling of the number of gene defects associated with type I MCD. Furthermore, 17 of the mutations identified in our cohort were novel (C1071T, G1512A, and deletion of the open reading frame have been previously reported). The open reading frame deletions discovered in 2 of our families may also be novel gene defects; however, more ac-
curate mapping of these mutations will be required to make this determination.

Despite significant heterogeneity in the location and type of coding region mutations identified in our patient cohort, each was predicted to cause significant structural or site-specific changes in the encoded protein, C-GlcNAc6ST. Most of the deletion and insertion mutations were found to cause a reading frame shift, resulting in major changes in the primary protein structure. The amino acid substitutions (point mutations) were shown to occur at highly conserved sites (11/13) and/or result in a significant change in polarity or charge (11/13). An additional 1 of 13 would result in substitution of tyrosine for cysteine, with the resulting loss of a potential disulfide bonding site and insertion of a large cyclical R group, likely to have profound consequences on protein structure.22

Consistent with a pattern of autosomal recessive inheritance, all 68 of the individuals in our cohort with 2 CHST6 coding region mutations (66 homozygous and 2 compound heterozygous) were affected by corneal opacities characteristic of MCD. These same 68 subjects were found to have uniformly low (undetectable) levels of serum AgKS. The results of the measurement of AgKS levels in serum, if viewed as a surrogate for C-GlcNAc6ST activity, indicate total (or near total) absence of enzymatic activity in cases of type I MCD. The 24 relatives who were heterozygous for coding region mutations were clinically and serologically indistinguishable from those with 2 normal copies of the gene. These findings support our conclusion that the CHST6 gene defects identified in this patient cohort are responsible for causing corneal opacification by disruption of C-GlcNAc6ST function.

Recent reports have provided additional evidence to indicate that C-GlcNAc6ST plays an important role in maintaining corneal transparency through the production of properly sulfated KS. In vitro assays have confirmed that C-GlcNAc6ST catalyzes the transfer of sulfate to position C-6 of GlcNAc.23-25 The C-GlcNAc6ST activity is undetectable in extracts from corneas affected by MCD,26 and is similarly abolished in cells transfected with C-GlcNAc6ST containing MCD-associated amino acid substitution.24 There is heavy labeling of abnormal unsulfated KS in the deposits found in the stroma, keratocytes, and endothelium of corneas with type I MCD compared with healthy controls.26 Finally, analysis of KS chain fine structure in corneas with MCD revealed significantly reduced chain size and chain sulfation compared with healthy tissue.27 It is intriguing that such a large number and wide variety of novel mutations were discovered distributed throughout the CHST6 coding sequence. This suggests 2 additional conclusions. First, in contrast to the BIGH3 gene, with its hot spots for corneal dystrophy–associated amino acid substitution,28 the CHST6 gene does not demonstrate a highly conserved number of disease-causing mutations. Indeed, the CHST6-encoded sulfotransferase does not seem particularly robust, with inactivation resulting from changes at a number of different locations. Second, it appears that the southern Indian population is genetically distinct from other populations previously studied.

Our cohort included only 5 patients classified as having type II MCD. These patients had clinical findings consistent with the diagnosis of MCD and levels of serum AgKS that were low or within the reference range. Akama et al8 discovered that their patients with type II MCD possessed upstream rearrangements or deletions that presumably resulted in altered promoter function and reduced cornea cell-specific transcription of the CHST6 gene. They very accurately mapped a deletion that appeared to have resulted from recombination between the highly similar sequences surrounding the CHST5 and CHST6 genes. Using the same PCR primers, we failed to find the same changes in our patients with type II MCD. One of our patients with type II MCD was found to be heterozygous for a type I disease-causing mutation (delC890). This is not surprising because the type II phenotype was shown to be dominant in compound heterozygotes (individuals possessing types 1 and II alleles).9 Our discovery of a family with type I MCD that included 2 affected individuals, both of whom carry only 1 type I allele (Ser53Leu heterozygotes), was surprising. We are continuing to investigate these families in an effort to better define their disease-causing genetic defects.

The use of buccal epithelial cells as a source of genomic DNA is a feature of this study worthy of special mention. Although blood samples were obtained from all subjects for measuring serum levels of AgKS, all of the genetic testing was performed on DNA extracted from swabs of the buccal mucosa. There are a number of advantages to this approach. First, buccal swabbing is quicker and easier to perform, and does not induce the anxiety associated with needles and blood. Second, mucosal epithelium is a much more efficient source of genomic DNA than blood, as, in the latter, only the white blood cell fraction is nucleated. Third, the risks of exposing investigators to blood-borne pathogens can be decreased. Finally, DNA is more stable on a buccal swab than in a tube of blood. Blood must be refrigerated until the time of DNA extraction, or frozen for long-term storage. In contrast, DNA is readily isolated from buccal epithelial cells even after storage for weeks to months at room temperature.29,30

The great advances made recently in the understanding of the genetic basis of the corneal dystrophies provide clinicians with the ability to confirm or refute diagnoses that previously were based primarily on phenotypic criteria. By identifying the distinct mutations responsible for each form of corneal dystrophy, including macular dystrophy, we will be better able to predict the prognosis for affected patients and their offspring, and properly counsel them. The identification of causative mutations in genes such as CHST6 that result in dystrophic corneal opacification is the first step toward understanding the role that such genes play in maintaining corneal clarity.

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