**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-
dominant glycosaminoglycan expressed in the adult cornea. Lack of activity of this enzyme is thought to result in the production of unsulfated KS, leading to a loss of transparency in the corneas of affected patients. Mutations within the coding region of CHST6 have been found to be associated with type I MCD, whereas deletions and rearrangements in the upstream regulatory region have been identified in patients with type II MCD. Despite the variety of mutations reported, all of the gene defects are associated with a common disease phenotype.8-11

In this study, we sought to further characterize the role of the CHST6 gene in MCD through the identification of causative mutations in a large cohort of affected patients in southern India. The relatively high prevalence of MCD in southern India is probably a result of the high frequency of consanguineous marriage within this population. Because many patients with MCD have been carefully followed up for years at the Aravind Eye Hospital (AEH), Madurai, India, we were able to enroll a large series of patients to undergo molecular genetic analysis.

METHODS

After study approval was obtained from the institutional review board at the AEH and the University of California–San Francisco (H7177-18489-01), a computerized medical record search was performed to identify all patients with MCD seen at AEH between January 1, 1990, and January 1, 2000. The diagnosis of MCD was based on the distinctive clinical features (Figure), and in most cases was confirmed by results of histopathologic examination of the excised corneal buttons. All cases were consistent with an autosomal recessive inheritance pattern. Families with affected patients older than 20 years were contacted by telephone or letter and asked to participate in the study. Unrelated, unaffected, healthy volunteers were recruited to serve as control subjects. All study subjects returned to AEH for slitlamp examination and collection of blood samples and buccal mucosal swabs. Family members were considered unaffected if they were older than 20 years and without clinical evidence of MCD. All samples were transported to 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,8 with the exception of the middle coding region reverse primer (5’-TCCGTGGGTGATGTTATGGAT-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) (FallSafe 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 1/20/5-D-4 monoclonal antibody.12-14 Levels of AgKS in serum are reported here as equivalents of a standard of AgKS, 103-210 ng/mL and were classified as having type II MCD. Levels of AgKS within the reference range were

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
detected in the serum of all 33 unaffected relatives and 48 controls (range, 134-515 ng/mL).

Analysis of the CHST6 coding region identified 20 distinct genetic defects (Table 1 and Table 2). Eleven homozygous missense mutations were identified in 29 patients from 20 different families (Leu22Arg, His42Tyr, Arg50Cys, Arg50Leu, Ser53Leu, Arg97Pro, Cys102Tyr, Arg127Cys, Arg205Gln, His249Pro, and Glu274Lys). Three homozygous frameshift mutations (delCG707-708, delC890, and delA1237) were identified in 26 patients from 19 families, and 2 homozygous replacement mutations (ACCTAC1273GGT and GCG1304AT) were detected in 7 patients from 3 families. Both affected individuals in 1 family were found to be compound heterozygotes (Arg93His and Ala206Thr). A 23-nucleotide deletion (del1748-1770) was identified in a family with 1 affected individual. Three patients from 2 unrelated families were presumed to have a deletion of the entire CHST6 open reading frame on the basis of failure of PCR amplification. In these patients, none of the CHST6 coding region primer pairs produced a detectable amplicon, whereas normal amplification of other genomic sites was observed. Two patients (same family) with type I MCD were found to possess only 1 CHST6 coding region mutation (heterozygous for Ser53Leu). One patient with type II MCD was heterozygous for the frameshift mutation delC890. No coding region mutations were identified in the other 4 patients with type II MCD.

Most (24/31 [77%]) of the unaffected relatives were identified as heterozygous for a mutation detected in their affected relatives. Parents or children were heterozygous in 11 (92%) of 12 cases. The 1 exception was the father of a patient with a homozygous point mutation (C1071T). Paternity testing was not performed. Siblings were heterozygous in 12 (80%) of 15 cases and second-degree relatives in 1 (25%) of 4 cases. As anticipated, the son of a patient with a presumed deletion of the open reading frame had a wild-type genotype, and therefore could not be positively classified. Of the 48 controls, one was discovered to be heterozygous for a presumed disease-causing mutation (C1071T).

We performed multiple alignment analysis (Clustal W, version 1.8) to compare the amino acid sequence of the CHST6 gene product, C-GlcNAc6ST, with that of 5 closely related members of the sulfotransferase enzyme family. Of the missense mutations identified in this study, 11 (85%) of 13 were found to occur at highly conserved sites. Examination of the R groups of the predicted amino acid substitutions revealed that 11 (85%) of 13 would result in a change in polarity or charge (Table 1).

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-

<table>
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<th>Protein Change</th>
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<th>No. of Families</th>
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<td>194 Asn Leu Arg 196; 194 Arg Cys 195</td>
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<tr>
<td>Deletion of open reading frame</td>
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Abbreviations: CHST6, carbohydrate sulfotransferase gene; MCD, macular corneal dystrophy.
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.23-25

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 codin 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 disrupting 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 corneae affected by MCD,24 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,20 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 corneal 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 I 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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