Identification of Novel Mutations in the Carbohydrate Sulfotransferase Gene (CHST6) Causing Macular Corneal Dystrophy

Mobamed F. El-Asbry,1,2,5 Mai M. Abd El-Aziz,1 Simon Wilkins,4 Michael E. Cheetham,4 Susan E. Wilkie,1 Alison J. Hardcastle,1 Stephanie Halford,1 Ahmed Y. Bayoumi,2 Linda A. Ficker,7 Stephen Tuft,3 Shomi S. Bhattacharya,1 and Neil D. Ebenezer1

PURPOSE. Macular corneal dystrophy (MCD) is a rare corneal dystrophy that is characterized by abnormal deposits in the corneal stroma, keratocytes, Descemet’s membrane, and endothelium, accompanied by progressive clouding. It has been classified into three immunophenotypes—MCD types I, IA, and II—according to the serum level of sulfated keratan sulfate (KS) and immunoreactivity of the corneal tissue. Recently, mutations in a new carbohydrate sulfotransferase gene (CHST6) encoding corneal glucosamine N-acetyl-6-sulfotransferase (C-GlcNac-6-ST) have been identified as the cause of MCD. Mutation screening of the CHST6 gene has been undertaken to identify the underlying mutations in five unrelated British families with MCD.

METHODS. DNA was extracted from venous blood obtained from all participants, and the coding region of CHST6 was amplified by polymerase chain reaction (PCR). The PCR products were analyzed by direct sequencing and restriction enzyme digestion. Enzyme-linked immunosorbent assay (ELISA) was performed to assess the presence of KS in serum from the probands of MCD-affected families participating in the study.

RESULTS. Six novel missense mutations—four homozygous and two compound heterozygous—were identified in the CHST6 gene. The ELISA showed that the disease in all patients participating in the study was of MCD type I, including the subtype IA.

CONCLUSIONS. These novel mutations are thought to result in loss of corneal sulfotransferase function, which would account for the MCD phenotype. (Invest Ophthalmol Vis Sci. 2002;43: 377–382)

Macular corneal dystrophy (MCD, On-line Mendelian Inheritance in Man (OMIM) 217800; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD, and available at http://www3.ncbi.nlm.nih.gov/omim) is an autosomal recessive disorder characterized by bilateral progressive stromal clouding and central corneal thinning.1,2 MCD accounts for 10% to 75% of the corneal dystrophies that require penetrating keratoplasty, depending on the population.3,4 The disease becomes evident in the first decade of life, defined by fine, diffuse, superficial clouding in the central stroma that extends to the periphery and usually involves the entire thickness of the cornea by the second decade of life. These opacities are more superficial and prominent in the central cornea and are deeper and more discrete in the periphery.

MCD has been classified into three immunophenotypes—I, IA, and II—based on measurement of the serum level of sulfated keratan sulfate (KS) by enzyme-linked immunosorbent assay (ELISA)5 and an immunohistochemical evaluation of the corneal tissue.6 In MCD type I, neither the cornea nor the serum contains appreciable levels of sulfated KS, whereas in MCD type II, there is detectable KS in the cornea and serum.7 The third subtype, IA, in which sulfated KS is absent in the cornea and the serum but can be detected in the keratocytes, has been identified in subjects from Saudi Arabia.8 All three immunohistochemical subtypes have the same clinical phenotype.

Histologically, MCD is characterized by accumulation of glycosaminoglycans between the stromal lamellae, underneath the epithelium, and within the keratocytes and endothelial cells.9,10 An abnormality in the metabolism of KS has been implicated in the pathogenesis of MCD11 and has been attributed to an error in a specific sulfotransferase involved in the sulfation of KS.12–14 MCD types I and II had previously been linked to chromosome 16 (16q22).15–17 Recently, mutations in a new carbohydrate sulfotransferase gene (CHST6) encoding corneal glucosamine N-acetyl-6-sulfotransferase (C-GlcNac-6-ST) have been identified as the cause of MCD.18 A number of missense mutations were described in patients with MCD type I18–20 or type IA.20 MCD type II, deletions and/or rearrangements in the upstream region of CHST618 and recently a missense mutation20 were reported.

In our study, mutation screening of CHST6 in five unrelated British families with MCD and with no clinically apparent systemic manifestations, revealed four homozygous and two compound heterozygous mutations in CHST6, all of the missense type. All the mutations involve the substitution of amino acids highly conserved across related carbohydrate sulfotransferases and/or represent the nonconservative substitution of nonpolar for polar residues and are thought to result in loss of CHST6 gene function.

MATERIALS AND METHODS

Clinical Diagnosis

The study had the approval of Moorfields Eye Hospital Ethics Committee and conformed to the tenets of the Declaration of Helsinki. Informed consent was obtained from all participants for clinical and
molecular genetic study. Autosomal recessive MCD in our patients of white origin was diagnosed on the basis of pedigree structure and the following clinical features: bilateral symmetrical superficial stromal cloudiness studied by small, irregular, rounded, gray-white anterior stromal patches. The diagnosis was confirmed by histopathologic examination of corneal buttons from all patients after keratoplasty.

### Mutation Detection

Genomic DNA was extracted from 10-mL blood samples obtained, according to standard protocols, from all subjects involved in the study. The coding region of CHST6 was amplified by polymerase chain reaction (PCR). Each PCR was performed in a 50-μL reaction mixture containing genomic DNA (200 ng), primers (0.4 μM each), MgCl₂ (1.5–2 mM), deoxynucleoside triphosphates (dNTPs; 0.2 mM), 1× PCR buffer (containing 10 mM Tris-HCl [pH 8.3], 50 mM KCl, and 0.1% gelatin) and Taq polymerase (0.5 U; Bioline, UK). Amplification reactions were performed under the following conditions: 5 minutes of denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 60 to 68°C for 1 minute, and a further extension step at 72°C for 1 minute. For amplification of the CHST6 coding region the following primer pairs were used: for the 5’-coding region, CK71M-F1 (5’-GAGCCGCTGGCAGAAATC-3’) and CK71M-R2 (5’-ACCGCTGCGCTCTC-3’), and for the 3’-coding region the following primer pairs were designed: CK71h-F1 (5’-GAGATGGAGGTGTTATGTCG-3’) and CK71h-R1 (5’-GACGATGTCCTGGTGC-3’); CK71M-F2 (5’-GCTGAACCTAGAATGCTG-3’) and CK71M-R2 (5’-ATCGTTGGATGTTATGGA-3’); and for the 3’-coding region the following primer pair was designed: CK71L-F (5’-GAGCCGCTGGCAGAACATG-3’) and CK71L-R (5’-TGCAACGTAGATGGCCTC-3’). A mismatch primer pair was also designed, sulfofhin-F (5’-CTGTTGACATGGGATGG-3’) and sulfofhin-R (5’-CAACCGTTGGTGCAGAGG-3’) to confirm one of the homozygous mutations (in family A, F107S) which does not alter any known restriction enzyme site. The mismatch primer created an HinfI site in conjunction with the mutation.

For direct sequencing,PCR products were purified (Qiagen PCR purification kit; Qiagen, Crawley, UK) and sequenced using an automatic fluorescence DNA sequencer (ABI Prism 373A; Perkin Elmer, Foster City, CA), according to the manufacturers’ instructions. Nucleotide sequences were compared with the published cDNA sequence of CHST6 (GenBank accession number AF219990). GenBank is provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD, and is available at http://www.ncbi.nlm.nih.gov/genbank), and mutations identified were excluded from 100 control chromosomes of white origin by restriction enzyme digestion.

#### Restriction Enzymes

Five enzymes were used to confirm the mutations identified, HinfI, with recognition site 5’...GANTC...3’, was used to study the homozygous T1012C and G906T in families A and E, respectively. Apatl, with recognition site 5’...GGGCC...3’, and BstNI, with recognition site 5’...CC...3’, were also used to confirm the heterozygous changes C785T and T1291G in families B and C, respectively. Similarly, DrdI, with recognition site 5’...GACNNNNNGNCT...3’, was used to confirm the homozygous C1309T mutation in family D. And finally, BanII, with recognition site 5’...G(A/G)GC(T/C)C...3’, was used to confirm the homozygous G905T change in family E.

The PCR products digested by HinfI, BstNI, and BanII restriction enzymes were analyzed by 6% nondenaturing polyacrylamide gel electrophoresis (Protogel; National Diagnostics, Atlanta, GA) and were visualized by staining with ethidium bromide. Products digested by Apal and DrdI were analyzed on 3% agarose gels (Bio-Rad, Herts, UK).

### Assay of Sulfated KS in Serum

Inhibition ELISA for KS detection using the monoclonal antibody 5-D-4 (ICN Biochemicals Ltd., Basingstoke, UK) was performed to assess the presence of KS in serum obtained from the probands of MCD-affected families participating in the study. The method was performed as described previously, except that an antibody (Aggreкан; Sigma Chemical Co., Poole, UK) was used to coat the plates and 2’azino-di-[3-ethylbenzthiazoline sulfonate 6] (ABTS; Roche Molecular Biochemicals, Lews, UK) was used as the substrate. The plates were read at 410 nm with a reference wavelength of 490 nm on a flow plate reader (ICN Biochemicals Ltd.). The ELISA was sensitive to KS concentrations of 10 to 10,000 ng/mL.

#### Table 1. Mutations Identified within the CHST6 Gene in MCD-Affected Families

<table>
<thead>
<tr>
<th>Family</th>
<th>Nucleotide Change</th>
<th>Type of Nucleotide Change</th>
<th>Amino Acid Change</th>
<th>Type of Amino Acid Substitution</th>
<th>Type of Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1012T→C</td>
<td>Homozygous</td>
<td>F107S</td>
<td>Polar→nonpolar</td>
<td>Nonconservative</td>
</tr>
<tr>
<td>B</td>
<td>[783C→T+1291T→G]</td>
<td>Heterozygous</td>
<td>P31S</td>
<td>Nonpolar→polar</td>
<td>Non-conservative</td>
</tr>
<tr>
<td>C</td>
<td>[783C→T+1291T→G]</td>
<td>Heterozygous</td>
<td>L200R</td>
<td>Non-polar→acidic</td>
<td>Non-conservative</td>
</tr>
<tr>
<td>D</td>
<td>1309C→T</td>
<td>Homozygous</td>
<td>A206V</td>
<td>Non-polar→non-polar</td>
<td>Conservative</td>
</tr>
<tr>
<td>E</td>
<td>906C→T</td>
<td>Homozygous</td>
<td>P72S</td>
<td>Non-polar→polar</td>
<td>Non-conservative</td>
</tr>
</tbody>
</table>

#### Figure 1. DrdI restriction digest on 3% agarose gels. Lane 1: undigested PCR product (400 bp); lane 2: digested mutant allele (360 bp) from the proband of family D; lane 3: digested mutant and undigested wild-type alleles from the proband’s mother (homozygous); lanes 4 and 5: unaffected proband’s sisters (homozygous) with undigested wild-type alleles; lane M: dX174 RF DNA HindIII marker.
RESULTS

Six novel missense mutations were identified within the coding region of the CHST6 gene in five unrelated families with MCD type I (Table 1). None of these changes was detected in the control population of the same ethnic origin, as determined by restriction enzyme digest on 100 chromosomes, confirming that they are likely to be pathogenic mutations. In addition, they were shown to segregate with the disease by sequencing and restriction enzyme analyses (Fig. 1).

Four of these mutations are homozygous and were detected in families A, D, and E. In family A, a T1012C transition at the second nucleotide position of codon 107 was identified, resulting in a phenylalanine-to-serine substitution (Figs. 2A, 2B). In family D, a C1309T transition at the second nucleotide position of codon 206 was found, with an alanine-to-valine substitution (Figs. 2G, 2H). In family E, two consecutive homozygous changes were identified. The first is a G905T transversion, located at the third nucleotide position of codon 71, which changes the amino acid from glutamic acid to aspartic acid. The second change, a C906T transition located at the first nucleotide position of codon 72 leads to a proline-to-serine substitution in the same patient (Figs. 2I, 2J). Although the first mutation predicts a change in the amino acid within the same group, the second alteration changes the amino acid residue to a different anionic group.

In families B and C two heterozygous changes were identified, with a different mutation on each allele (i.e., compound heterozygotic mutations), which could account for such autosomal recessive inheritance. The first heterozygous change, a C783T transition, occurred at the first nucleotide position of codon 31, predicting a change of amino acid proline to serine (Figs. 2G, 2H). In family E, two consecutive homozygous changes were identified. The first is a G905T transversion, located at the third nucleotide position of codon 71, which changes the amino acid from glutamic acid to aspartic acid. The second change, a C906T transition located at the first nucleotide position of codon 72 leads to a proline-to-serine substitution in the same patient (Figs. 2I, 2J). Although the first mutation predicts a change in the amino acid within the same group, the second alteration changes the amino acid residue to a different anionic group.
The other change, a T1291G transversion, was identified at the second nucleotide position of codon 200, changing the amino acid from leucine to arginine (Figs. 2E, 2F).

To assess the significance of these mutations, an alignment of the primary sequence of CHST6 with other published carbohydrate sulfotransferases, 23–27 a computer program for simultaneous alignment of many nucleotide or amino acid sequences to detect or demonstrate homology between new sequences and existing families of sequences (Clustal W, ver. 1.7; SGI, Mountain View, CA) was used28 (Fig. 3). With the exception of a P31S substitution (families B and C) that represents a nonconservative change but occurs at a position where residue type is not conserved across the carbohydrate sulfotransferases, all the mutations occur at positions where the residue type is highly conserved across all the carbohydrate sulfotransferases. Furthermore, mutations F107S (family A), L200R (families B and C), and P72S (family E) are all nonconservative changes involving substitution of a nonpolar residue for a polar residue that may affect CHST6 enzyme activity. Two of the substitutions are conservative changes, E71D (acidic→acidic, family E) and A206V (nonpolar→nonpolar, family D). Of these, E71D occurs in tandem with the P72S substitution (which is more likely to be disease causing) and thus may not itself have a major deleterious effect. It is not

**FIGURE 3.** Alignment of human CHST6 and other carbohydrate sulfotransferases peptide sequences. A computer program29 was used for multiple alignment of amino acid sequences. The positions of mutated amino acids in patients with MCD reported in this study are shaded in gray. The 5' and 3' PB site motifs29,30 are boxed. Note that two of the mutations occur at highly conserved positions within the 3' PB. The peptide sequences compared are I-GlcNAc6ST (intestinal N-acetylglucosamine-6-sulfotransferase),23 HecGlcNAc6ST (human high-endothelial-cell GlcNAc-6-sulfotransferase),24 GlcNAc6ST (human GlcNAc-6-sulfotransferase),25 Chon6ST (human chondroitin-6-sulfotransferase),26 KSG6ST (human keratan sulfate Gal-6-sulfotransferase).27

(Figs. 2C, 2D). The other change, a T1291G transversion, was identified at the second nucleotide position of codon 200, changing the amino acid from leucine to arginine (Figs. 2E, 2F).

To assess the significance of these mutations, an alignment of the primary sequence of CHST6 with other published carbohydrate sulfotransferases,25–27 a computer program for simultaneous alignment of many nucleotide or amino acid sequences to detect or demonstrate homology between new sequences and existing families of sequences (Clustal W, ver. 1.7; SGI, Mountain View, CA) was used28 (Fig. 3). With the exception of a P31S substitution (families B and C) that represents a nonconservative change but occurs at a position where
immediately apparent, however, why the A206V substitution should be disease causing, although conservative substitutions elsewhere in CHST6 have been reported to underlie MCD type I in Icelandic families.19

In the alignment, 5'- and 3'-phosphate-binding (PB) domains that interact with adenosine 3'-phosphate 5'-phosphosulfate (PAPS), a sulfate donor for sulfotransferases, are boxed (Fig. 3).29,30 These domains form part of the vital active site region of the enzyme. Two of the new mutations identified in this study, L200R and the conservative substitution A206V, occur within the 3'-PB domain.

Inhibition ELISA performed on serum from the patients with MCD showed little or no inhibition of the monoclonal antibody 5-D-4. Control experiments using increasing KS concentrations effectively inhibited 5-D-4 binding. These data from our patients are consistent with MCD type I or type IA.

DISCUSSION

In this study we collected five unrelated British families with clinically and histopathologically diagnosed MCD and screened the CHST6 gene, which is reported to be involved in the pathogenesis of this disorder by affecting the synthesis of KS.

KS proteoglycans (lumican, keratan, and mimecan) are present in the cornea as the major class of proteoglycans and are thought to play an important role in corneal transparency.31–35 The sulfate group of KS appears to be crucial for its biological function, because the degree of sulfation of KS increases during corneal development34,35 and the synthesis of unsulfated KS is observed in the corneas of patients with MCD.36,37 In view of the autosomal recessive inheritance of the condition, MCD probably results from a deficiency in sulfotransferase specific for proper sulfation of KS.10

We have identified six novel missense mutations in the probands from all families participating in this study. The mutations were detected by direct sequencing of CHST6 PCR products. Using restriction enzyme analysis, we were able to confirm the mutations in the probands and exclude them from unaffected family members and 50 normal individuals. To measure levels of sulfated KS in the serum of our patients with MCD, ELISA was performed and revealed barely detectable levels in all patients, confirming that disease in our patients was of MCD type I or IA.

All mutations detected occur at positions in the protein where the residue type is highly conserved across carbohydrate sulfotransferases and/or involve nonconservative substitutions of nonpolar residues for polar residue. One possible exception is E71D (family E) but this occurs as part of a double, homoyzygous substitution with P72S. It seems unlikely that E71D (family E) but this occurs as part of a double, homozygous substitution with P72S. It seems unlikely that E71D is chemically similar. Two mutations occur in the 3'-PB domain, an essential part of the active site responsible for PAPS binding. Of these, L200R involves a nonconservative substitution of a polar for a nonpolar residue at a site that is completely conserved across carbohydrate sulfotransferases. The A206V substitution, although representing a conservative change at a position that is not so highly conserved across carbohydrate sulfotransferases, is nonetheless predicted to result in a structural change at a highly sensitive region of the protein and, when modeled on the crystal structure of mouse estrogen sulfotransferase, result in nonfunctionality due to structural constraints (data not shown).

It has recently been shown that there is a decrease in the activity of N-acetylglucosamine-6-sulfotransferase (GlcNAc6ST) in the cornea of patients with MCD and that this results in the formation of poorly or nonsulfated KS and causes corneal opacity.38 More recently, it has been reported that mouse intestinal GlcNac6-ST (mGlcN6ST) has the same activity as human corneal GlcNac6-ST (C-GlcNA6ST) suggesting that mGlcN6ST is the orthologue of human C-GlcNA6ST and functions as a sulfotransferase to produce KS in the cornea. Moreover, the amino acid substitutions in human C-GlcNA6ST resulting from missense mutations in CHST6 found in patients with MCD abolished the sulfotransferase activity by functional inactivation rather than protein degradation or mislocalization.39

The homozygous mutations detected in our patients infers an essential role of C-GlcNA6ST, the CHST6 protein product, in the production of normally functioning KS, whereas its inactivation is responsible for the MCD phenotype. Furthermore, the heterozygous changes identified affected both alleles in two families, suggesting that these compound heterozygous mutations could also result in the MCD phenotype.

We conclude that mutated carbohydrate sulfotransferase could result in loss of function of the sulfotransferase enzyme required for proper sulfation of KS, an essential element for corneal transparency, leading to the MCD phenotype.

Acknowledgments

The authors thank John Dart, David Gartry, and Frank Larkin for help with the clinical data; the genetic nurses, Catherin Willis and Catherine Plant, for samples collection; Nigel Fullwood for advice and guidance; and all patients who participated in this study.

References


