Truncating Mutations in the Carbohydrate Sulfotransferase 6 Gene (CHST6) Result in Macular Corneal Dystrophy

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PURPOSE: Identification of mutations in the CHST6 gene in 15 patients from 11 unrelated families affected with recessive macular corneal dystrophy (MCD).

METHODS: Genomic DNA was extracted from peripheral blood leukocytes of the affected patients and their healthy family members, and the mutational status of the CHST6 gene was determined for each patient by a PCR-sequencing approach. Serum concentrations of antigenic keratan sulfate for each proband were determined by ELISA. Results. ELISA indicated that all affected patients, except one, were of MCD type I or IA. Fourteen distinct mutations were identified within the CHST6 coding region: 2 nonsense, 2 frameshift, and 10 missense. Of these, 12 were novel, and a nonsense mutation in the homozygous state is reported for the first time.

CONCLUSIONS. These molecular results in French patients with MCD combined with those reported in previous studies indicated CHST6 mutational heterogeneity. The characterization herein of nonsense mutations is in keeping with the fact that MCD results from loss of function of the CHST6 protein product. (Invest Ophthalmol Vis Sci. 2003;44:2949–2953) DOI:10.1167/iovs.02-0740

Macular corneal dystrophy (MCD; Online Mendelian Inheritance in Man [OMIM] 217800; http://www.ncbi.nlm.nih.gov/Omim/ provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) is an autosomal recessive inherited disorder that is rare in most countries, but very common in Iceland.1 The onset usually occurs in the first decade of life, and patients with MCD have progressive development in both corneas of punctate gray-white opacities underneath the epithelium, within the corneal stroma, Descemet’s membrane, and the corneal endothelium. These abnormal deposits are associated with a central stromal haze that gradually extends to the periphery of the cornea, leading to visual impairment. Previous biochemical studies have indicated that the pathologic corneal deposits most probably result in an accumulation of glycosaminoglycans (GAGs) and that a specific sulfation step of keratan sulfate (KS) is impaired in MCD.2 KS proteoglycans are the major proteoglycans in the corneal stroma and consist of a linear repeating sequence of disaccharide units of galactose (Gal) and N-acetylgalactosamine (GlcNAc), which are both sulfated on the C-6 position. Thus, KS proteoglycans are highly negatively charged macromolecules, in part through the addition of these sulfate groups on their sugar residues. MCD has been classified into two major immunophenotypes (types I and II) on the basis of immunohistochemical studies and the detection of the sulfated KS level in blood serum and in corneal tissue. A third subtype, type IA, has been documented in Germany and Saudi Arabia and corresponds to the absence of sulfated KS in the cornea and serum, but its presence in keratocytes.1,5,6,7 Regardless of the immunotype, the mode of inheritance and the clinical presentation are similar in the two forms. By genetic linkage analysis, the critical region for MCD has been mapped to chromosome 16 (16q22),6 and the carbohydrate sulfotransferase 6 (CHST6; OMIM 605294) gene has been identified as the defective gene.7 Screening for CHST6 mutations in patients with MCD has revealed that heterogeneous missense mutations within the coding region accounted for MCD type I, whereas large deletions and/or replacements in the upstream region of CHST6 result in MCD type II.7

The CHST6 gene has been grouped into the GST-family (galactose/N-acetylgalactosamine/N-acetylgalactosamine 6-O-sulfotransferase) including a group of Golgi enzymes that transfer sulfate from 3’phosphoadenosine5’phospho-sulfate to the 6-hydroxyl group of galactose, N-acetylgalactosamine (GalNac), glucose, or N-acetylgalactosamine (GlcNac) in nascent glycoproteins. The CHST6 gene, also called GST-4B, encodes a corneal N-acetylgalactosamine-6-O-sulfotransferase (C-GlcNAc6ST) that initiates sulfation of KS chains on proteoglycans (PG). The gene consists of four exons, but its open reading frame (ORF) is contained only within exon 4.7,8 This gene is located 50 kb downstream of a highly similar GST gene, GST-4A or CHST5, which encodes an intestinal isoenzyme of N-acetylgalactosamine-6-O-sulfotransferase.

We report the mutational spectrum in the CHST6 gene of 15 patients with MCD from nine French and two Maghreb (North African) kindreds. Fourteen distinct mutations in CHST6 coding sequences were identified: 2 nonsense mutations, 2 frameshift mutations, and 10 missense mutations. Of these, 12 were novel, and nonsense mutations in CHST6 were reported for the first time.

METHODS

Patients

Eleven unrelated families with MCD, nine of French and two of Maghreb origins were studied, including in total, 15 patients. The pedigrees of each family were established, and all were consistent with a recessive autosomal transmission of the corneal disease. No patients had
evidence of other ocular or systemic clinical abnormalities. All patients
gave their informed consent before inclusion in the study, which was
approved by Hôpital-Dieu Hospital Ethics Committee and conformed with
the provisions of the Declaration of Helsinki.

**Determination of Sulfated KS in Serum**

Serum concentrations of antigenic KS in each patient and control
subject were determined by a solid-phase competitive immunoassay, as
described previously.

The antigen used for coating was horine nasal cartilage proteoglycan KS (BNC-PG; IC, Aurora, OH), and the antibody
was a monoclonal Ig antibody against KS (clone 5D-4) that specifically
recognizes an antigenic determinant in the polysaccharide structure of
KS (IC). The binding of antibody against KS to KS-coated plates is
competitively inhibited by the KS in the solution to be measured. The
amount of antibody bound to the polystyrene plate is revealed using a
peroxidase-labeled anti-mouse IgG (obtained from Miles, Elkhart, IN).

The standard curve was performed using serial dilutions of a pool of
normal plasma. The results of antigenic KS (aKS) levels obtained from
normal plasma. The results of antigenic KS (aKS) levels obtained from
patients with MCD were expressed as a percentage of KS present in
normal plasma. Normal levels were found, ~190–250 ng/mL. Patients
with aKS levels less than 10% were classified as MCD types I and IA.
Probands from families 1, 2, and 7 were found to have aKS levels less
than 1%, those from families 8 and 9 had aKS levels less than 5%, and
probands from families 3, 5, 6, and 10 showed aKS levels less than 10%.

In contrast serum concentrations of aKS for probands from family
number 11 were found to be high (90%), and these patients were
classified as having MCD type II. Samples from family 4 were not
available.

**DNA Extraction**

Genomic DNA was extracted by standard methods from peripheral
blood leukocytes samples collected from the 15 patients and their
healthy family members (35).

**Mutation Screening**

To identify genomic rearrangements in the 5’upstream region of
CHST6 gene by polymerase chain reaction (PCR), we used the four
primer sets previously reported.

The coding region of the CHST6 gene was compared with the nucleotide sequence of the
human complementary DNA (GenBank accession number: AF219990;
http://www.ncbi.nlm.nih.gov/Genbank; provided in the public do-
main by the National Center for Biotechnology Information, Bethesda,
MD).

**RESULTS**

An enzyme-linked immunosorbent assay (ELISA) was per-
formed to measure KS concentrations in serum obtained from all
the probands of MCD-affected families participating in this
study (see the Methods section). The results indicated that all
affected patients, except for those in family 11, were of MCD
type I. Including the subtype IA. For family 11, the KS levels in
the serum of the two probands were normal, and therefore
they were classified as having type II MCD.

No genomic rearrangements in the 5’noncoding region of
CHST6 were detected in any patients, whereas 14 distinct sequence changes, illustrated in Figure 1, were identified
within the CHST6 coding region. Of which 12 mutations
(L15P, Q82X, L152P, C102G, P204G, N61T, N70L, Q58X,
Y68H, S131P, 1055-1056insC, 962-965delGCT→insA) have not
been identified. Missense mutations identified were classified as potentially pathogenic according to their absence in 60
normal individuals (120 chromosomes in total), a correct seg-
regation of each mutation within MCD-affected families and
the predicted effect on the amino acid sequence of the gene
product. All mutations are detailed in Table 1. Except for
R166P and L200R, all the mutations identified appeared to be
private variants.

Probands from four families (2, 4, 5, and 6) were homozy-
gous, whereas most of the probands were compound heterozy-
gous with at least one identified allele. In three families (9, 10, and
11) only one mutated allele was detected after all the entire
coding of CHST6 was sequenced. Analysis of the 5’upstream
region of CHST6 in these patients did not indicate rearrange-
ments similar to those observed in patients with MCD type II.
However, a large deletion or any other molecular defect in this
region not detected with the primer sets used could not be
included. Among the 19 mutations identified in patients with
MCD, there were 13 missense, 3 nonsense, and 2 frameshift
deletions/insertions. In addition, null mutation on both CHST6
alleles were identified in probands of family 2.

Four different truncating mutations were detected in fami-
lies 1, 2, 7, and 9 that are expected to cause loss of CHST6 gene
expression. These consisted of two nonsense mutations pro-
ducing a codon stop and two insertions/deletions of a number
of nucleotides that are not an exact multiple of 3, causing
therefore a shift in the translational reading frame and intro-
ducing a premature termination codon not far downstream of
the mutation site. In family 1, the proband was compound
heterozygous for C→T and T→C transitions at nucleotide
positions 936 and 736, respectively, corresponding to Q82X
and L25P mutations at the protein level. The Q82X mutation
produced a premature termination codon simply by converting
a glutamine into a stop codon. The nucleotide change found on
the other allele is expected to lead to the replacement of a
leucine by a proline residue at amino acid position 25. Al-
though leucine and proline are both nonpolar aliphatic resi-
dues, an aberrant proline residue is expected to introduce a
bend in the protein chain and therefore impairs its flexibility.

In family 2 in which the parents were first cousins, the three
affected children were homozygous for a C→T transition at nucleotide
position 864 predicting the replacement of a glu-
tamine by a stop codon at position 58 of the protein (Q58X).

In families 7 and 8, the mutation 1055-1056insC and the
complex mutation 962-965delGCT→insA each introduced a
frameshift in translation, which results in a premature termi-
nation codon at amino acid positions 107 and 221.

In family 8, the two probands were compound heterozy-
gous for C102G and P204G mutations. These involve cysteine
and proline residues respectively, which may play key roles in the
protein conformation. Indeed, cysteine is often involved in
disulfide bonding. As no other amino acid has a side chain
with a sulphydryl group, there is a strong pressure to conserve
cysteine residues which are among the least mutable of the
amino acids. In the case of the P204G mutation, proline and
proline are both nonpolar aliphatic residues, but each of their
lateral chains are signi-
cantly different. Proline is unusual in
that the side chain connects the nitrogen atom of the NH2
group to the central carbon atom expecting to generate a rigid
conformation of the protein, whereas glycine, which has the
smallest side chain among the amino acids, enhances the flex-
ibility of the protein. Similarly, P204G is a missense mutation
located in the 3’ phosphate-binding (3’PB) domain of the
enzyme which interacts with 3’ phosphoadenosine 5’phospho-
sulfate (PAPS), the sulfate donor for C4GlCNac6ST.

In two families (5 and 6), the probands were homozygous
for a G→C transversion at nucleotide 1189, leading to the
Figure 1. Identification of CHST6 mutations. In each panel (A–I), are shown the mutated DNA-sequence electrophoregrams of one proband from each MCD-affected family. All the mutations were confirmed by sequencing in both directions, but only one sense (forward or reverse) is displayed for each case. The positions of the mutations are underlined in the corresponding DNA sequence. Sequences shown are the antisense sequences for the L15P, Q82X, and C102G mutations.
replacement of an arginine by a proline residue at position 166 of the enzyme (R166P). This mutation has been reported in Icelandic patients with MCD.\textsuperscript{10}

In family 4, the proband was found to be homozygous for a T→C transition at nucleotide position 1147 expected to result in the replacement of a leucine by a proline residue at position 152 of the protein sequence (L152P). The proband of family 3 was compound heterozygous for the N61T and M70L mutations. Although these amino acid changes are conservative, methionine at position 70 is highly conserved across species and between members of the sulfotransferase gene family. The M70L mutation therefore probably affects the protein structure or its function.

In families 10 and 11, only one mutated allele was detected in all the patients corresponding to Y68H and S131P mutations at the protein level, respectively. These amino acid changes are nonconservative, and tyrosine at position 68 and serine at position 131 of the protein are both highly conserved.

**DISCUSSION**

These results from French families affected by MCD clearly indicate that the majority of CHST6 mutations are private, demonstrating therefore that the CHST6 gene is subject to strong allelic heterogeneity. Apart from the R166P mutation reported previously, CHST6 molecular heterogeneity has also been observed in Japanese, British, Indian, and Saudi Arabian MCD-affected families. Therefore, several different mutations have been identified in the CHST6 gene (Bao W, Smith CF, Al-Rajhi A, ARVO Abstract 2609, 2001; Warren JF, Aldave AJ, Thonar EJ, Margolis TP, Whitcher JP, Srinivasan M, ARVO Abstract 2870, 2001).\textsuperscript{7,10,11} No nonsense mutation has been identified in patients with MCD so far, and the identification of such mutations is in keeping with the fact that MCD results from loss of function of the C-GlcNAc6ST protein. It has been reported that most chain termination mutations may result in the generation of an unstable mRNA, which undergoes rapid degradation. These mutations are thus expected to be associated with a clinically more severe phenotype. We noted that the three homozygous patients of family 2 experienced rapid visual deterioration at an early age, and all required keratoplasty in the second decade of life. The other novel CHST6 mutations identified among our patients are missense mutations that involved conserved residues of the enzyme after alignment of various sequences of sulfotransferases. Although it is difficult to predict whether these mutations are disease-causing in the absence of functional studies, it indicates that mutations affecting conserved residues occur in critically important regions, destabilizing an essential structure or impeding gene function in a way not yet known.

For MCD families 1 to 8 in which the two mutated alleles were characterized, the molecular data were in good agreement with the immunochemical classification. In families 9, 10, and 11, we failed to identify the second mutated allele. However, KS levels in serum showed that probands from families 9 and 10 were of type I and IA, and those from family 11 were of type II. Because a mutation in the coding regions of CHST6 was identified in patients in family 11, suggesting MCD type I, we concluded that these patients in fact had a combination of type I and type II, because the MCD type II phenotype was dominant over that of type I.\textsuperscript{7}

The data were also analyzed for genotype–phenotype relationships, but no clear-cut correlations between each mutation and disease phenotype were obvious, except for three families (families 1, 2, and 5) in which all the affected patients had undergone bilateral keratoplasty in the second decade of life. The presence of multiple private mutations imply that screening of the CHST6 gene will not be straightforward and will require sequence analysis of all the coding region in addition to the 5′ upstream CHST6 region. However, the characterization of the spectrum of CHST6 gene mutations now allows us to offer genetic testing to unaffected younger siblings in our French MCD families.

In normal corneas, KSs comprise 4% unsulfated, 42% monosulfated, and 54% disulfated disaccharides with number of average chain lengths of 14 disaccharides.\textsuperscript{12} The sulfation of corneal KS is catalyzed by at least two different sulfotransferases in the Golgi apparatus; one enzyme, the KS Gal-6-sulfotransferase (KSGAL6ST or CHST1) catalyzes the sulfation at position 6 of the Gal residue, whereas N-acetylglucosamine-6-sulfotransferase (C-GlcNAc6ST/CHST6) catalyzes sulfation at position 6 of the nonreducing end of GlcNac residues.\textsuperscript{7,13,14} Biochemical studies confirmed that C-GlcNAc6ST transfers sulfate only onto the C-6 of GlcNac residues and demonstrated that missense mutations in CHST6 abolish the sulfotransferase activity of the corneal enzyme, resulting in the lack of highly sulfated KS in the corneal stroma of patients with MCD type I.\textsuperscript{1,5} It has also been noted that Gal residues are not sulfated at position 6 in MCD corneas suggesting therefore that sulfation of GlcNAc residues must be required for sulfation of Gal by HKSG6ST.\textsuperscript{1,4} Furthermore, recent studies have indicated that the sulfation of GlcNAc residues is tightly coupled with the elongation of sugar chains because, in addition to the absence of KS chain sulfation, the KS chain size is reduced to three to four disaccharides in MCD type I corneas as well as in cartilages. These data support the assumption that defect in

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<th>Family</th>
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<td>936C→T</td>
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<tr>
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<td>891T→A</td>
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<td>1147T→C</td>
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<tr>
<td>5</td>
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<td>R166P</td>
<td>1189G→C</td>
<td>R166P</td>
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<tr>
<td>6</td>
<td>1189G→C</td>
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<td>1189G→C</td>
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<td>7</td>
<td>902-906</td>
<td>Frameshift</td>
<td>1291T→G</td>
<td>L200R</td>
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<tr>
<td>8</td>
<td>delGCT→insA</td>
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<tr>
<td>9</td>
<td>996T→G</td>
<td>C102G</td>
<td>1305C→A</td>
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<tr>
<td>10</td>
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<td>12</td>
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C-GlcNAc6ST alters the matrix organization of both corneas and cartilages although MCD phenotype is apparently restricted to the cornea. Therefore, it is not inconceivable that other molecular defects in the CHST6 gene may underlie related inherited corneal and/or skeletal dystrophies, as it was observed for the recently identified diastrophic dysplasia sulfate transporter gene (DTDST). Loss-of-function mutations in the DTDST gene lead to defective sulfate uptake and proteoglycan sulfation and cause three related autosomal recessive skeletal dysplasias of increasing severity, depending on the residual activity of the enzyme. These examples of inherited diseases illustrate the critical role of carbohydrate sulfation in the organization of the extracellular matrix of the cornea, the cartilage, and bones.

Acknowledgments
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References