Mutations in the CHST6 Gene in Patients with Macular Corneal Dystrophy: Immunohistochemical Evidence of Heterogeneity

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PURPOSE. Macular corneal dystrophy (MCD) is an autosomal recessive disorder leading to severe visual impairment. The carbohydrate sulfotransferase 6 (CHST6) gene, which encodes the corneal N-acetylgalactosamine 6-O-sulfotransferase on 16q22 has been identified as a causative gene for MCD. The purpose of this study was to identify mutations in CHST6 in Japanese patients with MCD and evaluate them by means of immunohistochemistry.

METHODS. CHST6 was screened in 7 patients and 45 healthy control subjects. Genomic DNA was isolated, and the open reading frame (ORF) of CHST6 was amplified by polymerase chain reaction (PCR). PCR products were analyzed by direct sequencing and restriction enzyme digestion. Immunohistochemistry with a monoclonal anti-keratan sulfate (KS) antibody was performed on corneas from four families of three patients. RESULTS. Three novel mutations (P204Q, R205L, and R177H) and two previously reported mutations (R211W and A217T) were identified in the ORF of CHST6. P204Q, R205L, and R211W were found to be homozygous and R177H and A217T compound heterozygous with R211W on another allele. Immunohistochemistry revealed that R205L homozygous cornea had negative reactivity against the anti-KS antibody, representing type I MCD, and that R211W homozygous and R211W/A217T compound heterozygous corneas had negative or very weak reactivity in the stroma with antibody positive deposits, which were distinct from any previously reported types. CONCLUSIONS. Two mutations (homozygous R211W and compound heterozygous R211W/A217T) should be subclassified immunohistochemically into new phenotypes of MCD. This heterogeneity could provide further insights into the pathogenesis of MCD. (Invest Ophthalmol Vis Sci. 2005;46:3272–3277) DOI:10.1167/iovs.02-0910

Proteoglycans, especially low-sulfated keratan sulfate (KS) proteoglycans are known to be associated with depositions in the cornea in macular corneal dystrophy (MCD). MCD is a rare autosomal recessive characterized by a fine, superficial, and central stromal haze that starts to form at approximately the time of puberty. The haze progressively spreads to the periphery and develops into ill-defined and multiple corneal opacities that result in severe visual disturbance. Immunohistochemistry and immunohistochemical studies have subdivided MCD into two types (type I, type II) and one subtype (type Ia) on the basis of the reactivity against a monoclonal anti-keratan sulfate (KS) antibody (5D4 anti-KS antibody). Type I MCD cornea and serum show negative reactivity against the 5D4 anti-KS antibody, whereas type II MCD cornea and serum show positive reactivity against it. Because the 5D4 anti-KS antibody is highly specific to sulfate residue of KS, patients with MCD type I have been thought to lack normally sulfated KS proteoglycan in the cornea and serum.1–10 The KS proteoglycan synthesized by the cornea with type I MCD has been reported to have little sulfate.11–14 A biochemical assay has confirmed decreased N-acetylgalactosamine 6-O-sulfotransferase (GlcNAc-6-sulfotransferase) activity in MCD cornea.15

Currently, the carbohydrate sulfotransferase 6 gene (CHST6) encoding corneal N-acetylgalactosamine 6-O-sulfotransferase (C-GlcNAc6ST) has been identified as a cause of MCD types I and II.16 C-GlcNAc6ST is an enzyme that transfers sulfate to position 6 of GlcNAc residues and that participates in biosynthesis of KS proteoglycan in the cornea.16,17 KS is a carbohydrate moiety of the major proteoglycans in the corneal stroma and is a highly charged molecule that is responsible for maintaining corneal transparency through the hydrophilic nature of the sulfate moiety. Several missense mutations in CHST6 have been reported in patients with MCD type I,16–19 and deletions and/or rearrangements in the upstream region have been reported in patients with MCD type II.16

In the present study, we screened seven patients from six unrelated Japanese families with MCD to search for mutations in CHST6, and we identified three novel mutations and two previously reported mutations. We also used immunohistochemistry to determine whether these mutations are associated with different expressions of MCD.

MATERIALS AND METHODS

Patients

Seven Japanese patients from six unrelated families who received clinical diagnoses of MCD in Juntendo Hospital were included in this study. The diagnosis in two of them was MCD type I and in one of them was MCD type II. Diagnoses were made by the determination of serum KS with an enzyme-linked immunosorbent assay (ELISA). Informed consents were obtained from all patients and their family members in agreement with the Declaration of Helsinki for research involving human subjects, and approvals for genetic testing were obtained from Juntendo Hospital, Juntendo University, School of Medicine.

CHST6 Analysis

Genomic DNA was isolated from peripheral blood leukocytes (white blood cells) by standard techniques. Three pairs of primers were used...
Table 1. Mutations Identified within the CHST6 Gene in Patient with MCD

<table>
<thead>
<tr>
<th>Family</th>
<th>ID</th>
<th>Serum KS* (ng/µL)</th>
<th>Mutation in DNA</th>
<th>Transition in Protein</th>
<th>Type of DNA Change</th>
<th>Mutation in Control Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>ND</td>
<td>1303G→A</td>
<td>P204Q</td>
<td>Homozygous</td>
<td>0/90</td>
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<tr>
<td>B</td>
<td>2</td>
<td>ND</td>
<td>1306G→T</td>
<td>R205L</td>
<td>Homozygous</td>
<td>0/90</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>&lt;3</td>
<td>1325C→T†</td>
<td>R211W</td>
<td>Homozygous</td>
<td>0/90</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>ND</td>
<td>1325C→T†</td>
<td>Heterozygous</td>
<td>0/90</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>5</td>
<td>&lt;3</td>
<td>1322G→A</td>
<td>R177H</td>
<td>0/90</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>6</td>
<td>125</td>
<td>1341G→A‡</td>
<td>A217T</td>
<td>0/90</td>
<td></td>
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<tr>
<td></td>
<td>7</td>
<td>ND</td>
<td>1325C→T†</td>
<td>R211W</td>
<td>0/90</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td>152 ± 48</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* Serum KS was determined by immunoassay as described in the Methods section.
† The mutation was previously reported by Akama et al.16
‡ The mutation was previously reported by Akama et al.17

to amplify the region of the open reading frame (ORF) of CHST6. For the 5' coding region, CK71h-intron (5'-GCCCTTAACCGCTGGCCTCT-3') designed by Akama et al.16 and 1022B (5'-ATAGGACTCACAACCTTGCG-CCA-3') were used; for the middle coding region, 743F (5'-GCAAGCCTCTCTCTTCCTCTCT-3') and 1578B (5'-TGAGACTCTGAGCCCGATGAAAG-3') were used, and for the 3' coding region, 1324F (5'-GGGAGCAAGACGCAGAAGCAAGG-3') and 1907B (5'-TAGCGCCTGTCTACAATCGT-3') were used. Each PCR was performed on a 50-µL reaction mixture consisting of genomic DNA (100 ng), dNTPs, 10× PCR buffer, 5× buffer (Q Buffer: Qiagen, Crawley, UK), and Taq polymerase (Qiagen). 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 45 seconds, annealing for 45 seconds at 56°C (for the 5' coding region) or at 58°C (for the middle coding region and the 3' coding region), extension at 72°C for 1 minute, and a further extension step at 72°C for 7 minutes. The amplification products were analyzed by agarose gel electrophoresis to assess for the presence of aberrant transcripts, purified with a kit (High Pure PCR Product; Roche Diagnostics, Indianapolis, IN), and sequenced on both strands by an automatic fluorometric DNA sequencer (model 373A; Applied Biosystems, Foster City, CA) according to the manufacturer’s recommendations.

To exclude the possibility that the mutations were polymorphisms, the 835-bp PCR products of the middle coding region from control subjects were analyzed by restriction digestion with enzymes SacII, StyI, and Apa LI. The SacII produces two fragments of length 562 and 273 bp from wild-type allele. This was used for detecting P204Q and R205L substitution, because either mutation disrupts a SacII site in the PCR fragment. The StyI produces three fragments of length 310, 283, and 242 bp from wild-type allele, which was used for detecting the mutation of A217T because the mutation of A217T disrupts a single StyI site. For detecting the mutation R177H, ApaLI, which produces three fragments of 329, 294, and 212 bp, was used, because the R177H mutation creates an ApaLI site in the PCR products. For SacII digestion, the reaction buffer contained 6 U of enzyme, 2 µg of PCR product, 10× T buffer (Takara, Tokyo, Japan), 0.01% BSA, and water, to a final volume 15 µL (Takara, Tokyo, Japan), and performed at 57°C for 2 hours. For StyI digestion, reaction buffer contained 10 U of enzyme, 2 µg of PCR product, 10× H buffer (Takara), and water, to a final volume of 12 µL (Takara), and was incubated overnight at 37°C. For ApaLI digestion, reaction buffer contained 4 U of enzyme, 2 µg of PCR product, 10× L buffer (Takara), and water, to a final volume of 12 µL (Takara), and was incubated overnight at 37°C. Digestion products were electrophoresed on 2% (for SacII) or 3% (for StyI and ApaLI) agarose gels and visualized by ethidium bromide staining and fluorescence under UV light.

Control Subjects
Nucleotide substitutions observed in this screening were researched in a control group obtained from 45 individuals who had no visual impairment. Our control group was not age or sex matched with the experimental group, and no ophthalmic examination was performed in these individuals.

Immunohistochemistry and Immunoassay
Cornal buttons available from patients with MCD and peripheral corneas from donors at autopsy were stained by an avidin-biotin immunofluorescence complex technique, with a monoclonal anti-KS antibody (5D4; Seikagaku, Tokyo, Japan), as has been described.15 Minor changes were made for the cornea from patient 2 and the half of the cornea from patient 7, which were embedded in paraffin instead of being frozen in optimal cutting temperature compound (OCT; Tissue Tek II, Baxter Scientific, Columbia, MD), after having been fixed in phosphate-buffered saline containing 4% paraformaldehyde. For negative control specimens, the antibody was preincubated for 30 minutes with 1 mg/mL of shark KS (Seikagaku), and then used in place of the primary antibody. Cryostat sections (7 µm) and paraffin-embedded sections (5 µm) were also stained with hematoxylin-eosin and Hale’s colloidal iron. The serum KS level was determined by an inhibition ELISA, as described previously.15,20

RESULTS
DNA Analysis
Direct sequencing of the CHST6 gene from patients with MCD was performed, and three novel and two previously reported missense mutations were identified (Table 1). Forty-five control chromosomes were analyzed for each alteration by direct sequencing and/or restriction digestion of PCR products, and none of the mutations was found among them. Patient 1 was homozygous for the transition G1303→A, which encodes a missense proline-to-glutamine substitution at codon 204 (P204Q). Patient 2 was homozygous for the transversion G1306→T, which encodes a missense arginine-to-leucine substitution at codon 205 (R205L). Patients 3 and 4 were homozygous for the transition C1323→T, which encodes a missense arginine-to-tryptophan substitution at codon 211 (R211W). Three patients from two families were found to be compound heterozygous for the R211W and other missense mutations. The other mutations were the transition G1222→A, encoding an arginine-to-histidine substitution (R177H) in patient 5 and

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FIGURE 1. Paraffin sections of cornea of patient 2 stained with hematoxylin-eosin (A), Hale’s colloidal iron (B), and 5D4 anti-KS antibody (C, E). (D) Phase-contrast micrograph of (C). For a negative control, antibody was preincubated with 1 mg/ml shark cartilage KS before staining (E). Neither the corneal stroma nor the accumulations were stained (compare with the normal control processed in the same manner in Fig. 3A).

FIGURE 2. Histologic appearance of corneas of patient 3 in frozen sections. Hematoxylin-eosin (A) and Hale’s colloidal iron (B) preparations illustrate abnormal accumulations in the interlamellar stroma. Immunofluorescence with 5D4 anti-KS antibody (C) and its phase contrast (D) demonstrate that the accumulations were positively stained with the antibody, in comparison with the negative control (E). Note that positive staining was present in the posterior stroma, but was diffuse and weak in contrast to the normal control (Fig. 3B).
the transition G1341→A encoding an alanine-to-threonine substitution (A217T) in patients 6 and 7.

An immunoassay for serum KS was performed in three patients, showing that patients 3 and 5 did not have any detectable serum KS and patient 6 had a normal level.

Immunohistochemical Analysis

Histochemical analysis was performed in patients 2, 3, 6, and 7. Corneal sections from all patients displayed the presence of extracellular blue accumulations in the stroma, detected by Hale’s colloidal iron staining. Immunohistochemistry for the 5D4 anti-KS antibody showed variability in the staining patterns among the corneas from the patients. Corneal sections from patient 2 showed no immunoreactivity in the stroma, keratocytes, and deposits (Fig. 1). Such a characteristic is consistent with type I MCD, although an immunoassay for serum was not possible in this case. Corneal sections from patient 3, in whom type I MCD had been diagnosed by immunoassay, unexpectedly showed positive immunoreactivity in the accumulations (Fig. 2). In addition, significantly decreased and uneven positive staining appeared in the stroma in contrast to the normal control (Fig. 3). The intensity of the staining in the stroma was weaker anteriorly than it was posteriorly, which may reflect an artifact of tissue processing. A different staining pattern was present in the cornea of patients 6 and 7 (data not shown for patient 6), in whom type II MCD had been diagnosed by immunoassay (Fig. 4). These two patients showed positive immunoreactivity in the accumulations, but negative reactivity in the stroma.

DISCUSSION

The present report identified three novel missense mutations (P204Q, R205L, and R177H) and two previously reported missense mutations (R211W and A217T) in the \( \text{CHST6} \) gene, by direct sequencing and restriction enzyme digestion in patients with MCD in Japanese families. Four patients from different families were homozygous for P204Q, R205L, and R211W and three patients from two families were compound heterozygous for R177H and A217T, as with the R211W substitution on the other allele.

\( \text{CHST6} \) encodes an enzyme that is a member of the Gal/GalNAc/GlcNAc 6-\( \text{O} \)sulfotransferase (GST) family and catalyzes the sulfation of position 6 of \( \text{O}-\text{GlcNAc} \) in \( \text{N}-\text{acetylglactosamine} \). Within the GST family and other glycosaminoglycan sulfotransferases, two putative binding sites for the high-energy sulfate donor, 3’-phosphoadenosine 5’-phosphosulfate (PAPS) have been pointed out. One is the 5’-PSB binding site (5’-PSB) and another is the RX-S 3’-phosphate binding site (3’-PB). The sequence between the two motifs is

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**Figure 3.** Immunostaining of human cornea with 5D4 anti-KS antibody for normal control subjects. Before immunohistochemistry, the corneal section was embedded in paraffin (A) or frozen in OCT (B).

**Figure 4.** Corneal sections from patient 7 stained with hematoxylin-eosin (A) and Hale’s colloidal iron (B), with 5D4 anti-KS antibody (C, D, E), and (F) Phase-contrast micrograph of (D). Sections in (A), (B), (D), (E), and (F) were processed from paraffin-embedded tissue and (C) frozen tissue. Note that the stroma showed no reactivity with the antibody, which is the same reactivity as the negative control (F), and the extracellular deposits in the stroma and in the subepithelial lesion showed positive reactivity.
thought to contribute to a binding pocket that interacts with an acceptor (GlcNAc) to bring it into apposition with the sulfate donor.\textsuperscript{21} It is reported that mutations of these binding motif sequences abolish the sulfotransferase activity in the case of other glycosaminoglycan sulfotransferases, indicating important catalytic roles of these amino acid residues\textsuperscript{22}. Most of the mutations for MCD type I were reported to be located in the binding sites or the binding pocket, with exceptions of the E274K,\textsuperscript{16} A217T,\textsuperscript{16} and P31S\textsuperscript{19} mutations (Fig. 5). The identified mutations of P204Q and R205L are located in the RX-S sequence for the 3'-PB domain, corresponding to amino acid 202-210; R211W is located in the region that is adjacent to the RX-S sequence in 3'-PB domain, and R177H is located in the sequence between the 5'-PSB domain, corresponding to amino acid 49-56, and the 3'-PB domain, whereas A217T is neither located in the binding motifs nor in the binding pocket. However, it has been reported that the A217T mutation indeed leads to a failure of synthesis of sulfated KS.\textsuperscript{17}

Cases of MCD are classified into three subtypes—I, II, and la—according to the immunohistochemistry of the cornea and immunoreactivity of the serum with the 5D4 anti-KS antibody that recognizes sulfate moieties of the KS chain. Precisely, mutations in the coding region of \textit{CHST6} in type I MCD must cause inactivation of GlcNAc6ST and synthesis of undersulfated KS proteoglycans, leading to an absence of immunoreactivity against the 5D4 anti-KS antibody in the serum and stromal matrix. Mutations in the upstream region of \textit{CHST6} in type II MCD promote loss of corneal epithelium-specific \textit{CHST6} expression, resulting in normal immunoreactivity in the serum and stromal matrix.\textsuperscript{24}

In the present study, immunohistochemistry using the 5D4 anti-KS antibody demonstrated rather complex results. The cornea of patient 2 with the R205L homozgyous mutation showed no reactivity with the antibody. The negative reactivity in the cornea with this mutation must be attributable to synthesis of a completely inactive form of the sulfotransferase. The cornea of patient 3 with the homozgyous R211W mutation, who had type I diagnosed by immunoassay, demonstrated immunopositive interlamellar accumulations. From such a finding, it was obvious that this patient did not have typical type I or type la disease and that her disease should be subclassified into a phenotype of MCD type I. In contrast, histochemistry of the corneas of patients 6 and 7 showed an absence of immunoreactivity in the stromal matrix and positive reactivity in the corneas of patients 6 and 7 showed an absence of immunoreactivity in the serum and stromal matrix.

In type I MCD, the chain length of KS is reduced to three to four disaccharides in the cornea, whereas the average chain length of KS from normal cornea is approximately 14 disaccharides.\textsuperscript{21} The low sulfation and short chain length of KS observed in type I MCD may allow it to precipitate and to be replaced easily, thus producing various results in immunoreactivity, depending on the epitope concentrations, as is shown in our immunohistochemistry. At the same time, it should be noted that immunoreactive deposits were seen in a cornea with a homozgyous R211W mutation that failed to synthesize highly sulfated KS when cotransfected with KSgal6ST into HeLa cells.\textsuperscript{16,17} Also, it should be mentioned that highly sulfated KS is detected in the serum with the compound R211W/A217T mutation. These findings may support the idea that GlcNAc6ST activity derived from C-GlcNAc6ST may be retained at a low but significant level in the cornea and also in the cartilage, from which part of the circulating KS may derive. Alternatively, some other unknown enzymes may participate in sulfation of KS in vivo.

Five enzymes of GlcNAc 6-O-sulfotransferase that constitute members of the GST family have been cloned and characterized in humans so far. Among them, the three GST genes \textit{CHST4} (HEC-GlcNAc6ST, GST5),\textsuperscript{25} \textit{CHST5} (I-GlcNAc6ST, GST4),\textsuperscript{26} and \textit{CHST6} (C-GlcNAc6ST, GST4b)\textsuperscript{18,19} have been reported to form a conspicuous cluster on human chromosome 16 in long-arm region 23.1-23.2.\textsuperscript{27} \textit{CHST5} and \textit{CHST6} are two highly homologous GlcNAc 6-O-sulfotransferase isozymes that are encoded by a tandem gene, presenting different substrate specificities—for example, \textit{CHST6} catalyzes the sulfation of

![Figure 5. Amino acid sequence of \textit{CHST6} showing the location of mutations in patients with MCD. Bold letters with arrowheads: missense mutations reported in this study. These and previously reported mutations are marked by amino acid position. The R50, K174, D203, E274, and R211 mutations were reported in Akama et al.,\textsuperscript{16} the A217 mutation in Akama et al.,\textsuperscript{17} and the A128 and R166 mutations in Liu et al.\textsuperscript{18} The P31, P72, P107, L200, and A206 mutations were reported in El-Ashry and El-Aziz.\textsuperscript{19} Shaded area: 5' phosphate binding site (5'PSB) and 3' phosphate binding site (3'PB).]
of keratan sulfate, whereas CHST5 does not. Moreover, they are expressed in a limited array of tissues: HEK-GlcNAc6ST is expressed in the high endothelial venule, CHST5 in the small intestine and colon, and CHST6 in the brain and cornea. Although CHST6 is identified as a cause of MCD and is strongly expressed in the brain, no neurologic disturbances have been clinically reported in patients with MCD. It is very likely that other keratan GlcNAc 6-O-sulfotransferases substitute for the CHST6-induced loss of function in the brain. In addition, a substantial increase in sulfation and tissue concentration of other glycosaminoglycans of chondroitin-dermatan sulfate is reported in MCD cornea.24 These facts bring us to assume that in patients with MCD other GlcNAc6STs may compensate for the deficiency of CHST6 in the brain and probably in some cases in the cornea and the cartilage. This could be an explanation for the immunohistochemical heterogeneity that we found in our sample.

In summary, we identified three novel mutations and two previously reported mutations on CHST6 in patients in Japanese families with MCD. We also demonstrated immunohistochemical heterogeneity in the cornea with a R211W homozygous mutation and a R211W/A217T compound heterozygous mutation. Further analysis of these mutations regarding their heterogeneity should help achieve a clearer understanding molecular mechanisms of MCD.

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References


