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Short Report

Allelic heterogeneity of the carbohydrate sulfotransferase-6 gene in patients with macular corneal dystrophy

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Macular corneal dystrophy (MCD) is an autosomal recessive disorder characterized by grayish white opacities in the cornea. It is caused by mutations in the carbohydrate sulfotransferase-6 (*CHST6*) gene, which codes for the enzyme corneal *N*-acetylglucosamine-6-sulfotransferase. This enzyme catalyzes the sulfation of keratan sulfate, an important component of corneal proteoglycans. We screened 31 patients from 26 families with MCD for mutations in the coding region of the *CHST6* gene. Twenty-six different mutations were identified, of which 14 mutations are novel. The novel mutations are one nonsense mutation found in one patient (Trp2Ter), one frameshift (insertion plus deletion) mutation in two patients (His335fs), and 12 missense mutations (Leu3Met, Ser54Phe, Val56Arg, Ala73Thr, Ser98Leu, Cys165Trp, Ser167Phe, Phe178Cys, Leu193Pro, Pro204Arg, Arg272Ser, and Arg334Cys) in 11 patients. These data demonstrate a high degree of allelic heterogeneity of the *CHST6* gene in patient populations with MCD from Southern India, where this disease may have a relatively higher prevalence than in outbred communities.

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Key words: carbohydrate sulfotransferase – CHST6 – macular corneal dystrophy – mutations

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Macular corneal dystrophy (MCD; MCDC1, MIM217800) is an autosomal recessive disorder characterized clinically by the formation of opacities in the corneal stroma that usually become evident in childhood or adolescence. A diffuse fine symmetric clouding in the central corneal stroma and discrete white opacities develop that extend to the periphery and eventually involve the entire thickness of the cornea, leading to visual impairment (Fig. 1). Corneas with MCD are distinguished histochemically by the presence of intra- and extracellular deposits that stain positively for glycosaminoglycans in the Bowman layer, stroma, Descemet membrane, and endothelium (1). Biochemical studies on surgically excised corneal tissues revealed a defect in the sulfation of keratan sulfate (KS) moieties in the KS-containing proteoglycans (2–4). Although MCD is clinically homogenous, it can be divided into three immunophenotypes, MCD types I, IA, and II, based on the immunohistochemical reactivity to anti-KS antibody in the cornea and serum (5–7). The gene responsible for MCD was mapped to chromosome 16q22.1 (8), and several years later, after fine mapping of the gene (9), mutations in the carbohydrate sulfotransferase-6 (*CHST6*) gene were found in patients with MCD (9, 10). The *CHST6* gene codes for corneal *N*-acetyl glucosamine sulfotransferase, an enzyme that specifically transfers the sulfate group on to the unsulfated KS proteoglycan. In the absence of this sulfation reaction, unsulfated proteoglycan precursors presumably accumulate in the cornea and give rise to the characteristic deposits seen in MCD.

We screened the *CHST6* gene for mutations in 31 patients from 26 Indian families with MCD, and herein, we report 14 novel mutations in this gene.

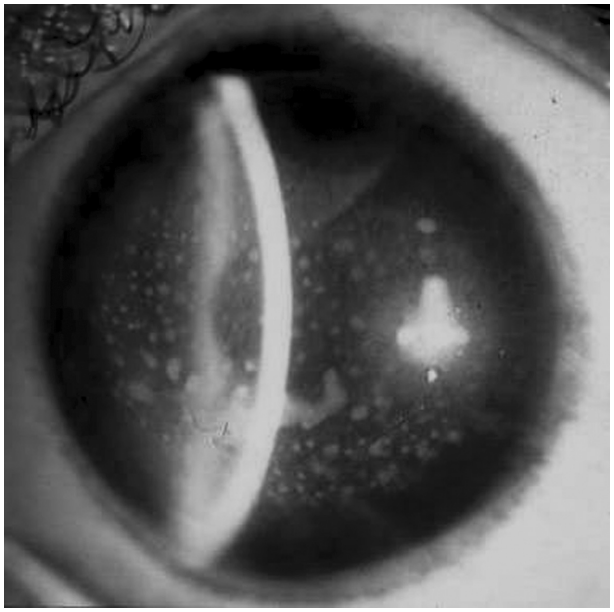

Fig. 1. Clinical photo of patient with macular corneal dystrophy (MCD). Slit lamp view of cornea of patient with MCD showing rounded stromal opacities.

Patients and methods

Patients and sample collection

Thirty-one patients from 26 families were evaluated clinically by a corneal specialist (MSS). The diagnosis of MCD was based upon the distinctive clinical features, and in 20 families, this was confirmed by a histopathologic examination of the excised corneal buttons. Patients underwent complete eye examination. This included visual acuity assessment, slit-lamp examination, intraocular pressure, and fundus examination. In patients where fundus details were not visible, ultrasound B-scan examination was performed. The various slit lamp findings seen in patients with MCD were full thickness corneal haze from limbus to limbus, anterior stromal nodular deposits, corneal thinning, and endothelial deposits.

Age of onset according to the patients ranged from 7 to 33 years. Of the 31 patients studied, nine were sporadic cases with no affected relatives, while 22 patients from 17 families had a positive family history. Consanguinity was known to be present in 20 of 26 families. Peripheral blood samples were collected from 31 patients after obtaining informed consent, and genomic DNA was extracted from the leucocytes. The study protocol adhered to the tenets of the declaration of Helsinki, and the research was done after the prior approval by the institutional review board of L.V. Prasad Eye Institute.

Polymerase chain reaction amplification and sequence analysis

The *CHST6* gene is 16.9 kb in length and consists of four exons, of which only third exon contains the coding region. The coding region was amplified using polymerase chain reaction (PCR) with the primers described previously (9). Conditions for PCR amplification and sequencing were as reported previously (11). The nucleotide sequences were compared with the published cDNA sequence of the *CHST6* gene (GenBank NM_021615).

DNA from 75 unrelated healthy Indian individuals, without a history of MCD, was screened for the sequence changes identified, using restriction enzymes whenever appropriate to confirm pathogenicity. For mutations that did not change any restriction site, direct sequencing or single-strand conformation polymorphism analysis was carried out using primers designed to amplify overlapping fragments of <300 base pairs in length for amplification as described previously (12).

For one patient (no. 39, Table 1) having the two consecutive heterozygous base changes [c.293C > T; c.294C > G], we designed primers specific for mutant and wild-type sequences. We performed allele-specific PCR to know whether the two sequence changes are in *cis* or *trans*. PCR was done with the patient and normal control DNAs at an annealing temperature of 57 °C. As an additional control, we used DNA from an MCD patient having the change [c.293C > G; c294C > G] identified in a previous study (11). This sample differs from the one in the present study by one base in the region tested. Sequences of oligonucleotides were

CHST-ASOF1 [wild type (forward)]: GCGAC CTGGTGCGCT**CC**

CHST-ASOF2 [Mutant (forward)]: GCGACC TGGTGCGCT**TG**

CHST-ASOR [Common (reverse)]: GGGTCG CTGAGCAGCGG

The residues in bold at the 3′ end of the forward primers are the bases that are mutated.

To evaluate the upstream region of the *CHST6* gene in patients with heterozygous mutations in the coding region, we performed PCR on genomic DNA according to the method of Akama et al. (9) using the primers and conditions described on the Nature Genetics website (http://www.nature.com/ng/supplementary_info/ng1000_237/ng1000_237_S1.doc), with the following exceptions: primer F2M was replaced by 5′-CCACAGCCAATTCCATCTTGGATTTTC TC-3′ and primer R2 was replaced by 5′-CATTA

Table 1. Mutations in *CHST6* gene among patients with macular corneal dystrophy

Family/Patient number	Mutation[a]	Mutation type	Consequence in protein	Restriction site change (if any)	Novel/previously reported[b]
32	[c.6G > A; c.7C > A] + [c.6G > A; c.7C > A]	Nonsense + missense	Trp2X + Leu3Met		Novel
33	c.16_40del	Deletion (25 bp)	Val6fs		11
34	c.161C > T	Missense	Ser54Phe	*Mbo*II	Novel
35	[c.166G > A; c.167T > G][c.500C > T] + ;[=]	Missense	Val56Arg + Ser167Phe	*Mbi*I + *Eco*57I	Novel
36	c.180delC	Deletion (1 bp)	Phe60fs		11
37	c.180delC	Deletion (1 bp)	Phe60fs		11
38	c.217G > A	Missense	Ala73Thr	*Bsh*1236I	Novel
39	[c.293C > T; c.294C > G] + [=]	Missense	Ser98Leu		Novel
40	c.369G > A	Nonsense	Trp123X	*Cfr*13I	11
41	c.391T > C	Missense	Ser131Pro		18
42	c.459C > A	Nonsense	Cys153X		11
43	c.494G > C; c.495C > T	Missense	Cys165Ser		16
44	c.495C > G	Missense	Cys165Trp		Novel
45	c.533T > G	Missense	Phe178Cys		Novel
46	[c.545delA] + [c.827 T > C]	Deletion Missense	Gln182fs + Leu276Pro	*Pst*I	15, 21
47	c.578T > C	Missense	Leu193Pro		Novel
48	c.604C > A	Missense	Arg202Ser		11
49	c.611C > G	Missense	Pro204Arg		Novel
50	c.656_657insCTG	Insertion (in frame)	Ala219_Arg220insTrp		11
51	c.656_657insCTG	Insertion (in frame)	Ala219_Arg220insTrp		11
52	c.663C > G	Missense	Asp221Glu		11
53	c.661G > T	Missense	Asp221Tyr		11
54	c.814C > A	Missense	Arg272Ser	*Alu*I	Novel
55	c.1000C > T	Missense	Arg334Cys	*Hae*II	Novel
56	c.1002_1012delinsTTG	Frameshift	His335fs	*Nla*III	Novel
57	c.1002_1012delinsTTG	Frameshift	His335fs	*Nla*III	Novel

This table summarizes the *CHST6* gene mutations identified in probands from each of the 26 families. The family/patient numbers are a continuation of cases after those previously reported (11).

[a]Mutation nomenclature is according to the current recommendations in http://www.hgvs.org/mutnomen/recs.html#DNA.

[b]Whether mutations are novel or reported and the reference of the first report, is shown.

GACACCTCACCTGCTTTGGC-3′. In numerous analyses the original primers (9) did not yield consistent results, perhaps because of mispairing due to the high degree of similarity of CHST5 and *CHST6*. We have hence modified the method using new primers that cover areas where there is less homology between CHST5 and *CHST6*. The annealing temperatures were adjusted to 58 and 62 °C for the primer pairs F2/R2 and F2M/R2, respectively. All amplicons indicating upstream DNA rearrangements were checked using sequencing methods. A new primer, R2M (annealing temperature 61 °C), was introduced to pair with F2M and produce an amplicon on the 3′ side of region 'B' [as defined by Akama et al. (9)]. The 3′ end of this amplicon was sequenced using a forward primer, 5′-GCAGAGGTTGCACACACCTGTC-3′. In our experience, these changes produced a more robust method that yielded more consistent results. Additionally, because two of the resulting amplicons were extended into less homologous areas, their sequences could be more readily verified. All PCR amplicons were electrophoresed on 2% agarose gels, and the gels were documented using the BIOCHEMI IMAGE ACQUISITION AND ANALYSIS Software (UVP BioImaging Systems, Upland, CA).

Results and discussion

Screening of *CHST6* gene identified 26 mutations in 26 families. Two probands had single heterozygous mutations, one proband had a compound heterozygous mutation and all the remaining 23 probands had homozygous mutations. Mutations identified (details in Table 1) consisted of 18 missense mutations (17 patients; three heterozygous), three nonsense mutations (three patients), three deletions (one heterozygous, two homozygous in four patients), one complex mutation (deletion + insertion; two patients), and one insertion (two patients). These sequence changes were absent in 75 unrelated normal individuals.

Novel mutations identified in 13 unrelated patients consisted of one nonsense mutation, one frameshift, and 12 missense mutations. A novel homozygous nonsense mutation predicting a truncation at tryptophan-2 was found in one patient (Table 1). Interestingly, this individual was found to have two homozygous changes, i.e. Trp2Ter and Leu3Met (Fig. 1B). The second mutation is predicted to create a methionine codon just downstream of the termination codon, thus raising the possibility of a re-initiation of translation at this position, causing a protein product that lacks the first three amino acids. Two factors make the synthesis of such a protein unlikely. The initiation codon created as a result of this mutation does not conform to the Kozak sequence because it lacks the highly conserved purine residues located three and six bases upstream of the initiation site (13) and would therefore be expected to function weakly or not at all. In addition, the existence of the nonsense codon may target the mRNA for degradation.

Apart from the Leu3Met mutation discussed above, there were 11 novel missense mutations, three heterozygous and eight homozygous, in 10 patients (Table 1). These were Ser54Phe, Val56Arg, Ala73Thr, Ser98Leu, Ser167Phe, Cys165Trp, Phe178Cys, Leu193Pro, Pro204Arg, Arg272Ser, and Arg334Cys. One complex heterozygous allele with two changes, i.e. Val56Arg and Ser167Phe, was found in one patient (Family 35), and one heterozygous allele with a change of Ser98Leu was found in another patient (Family 39). The mutations were in Family 35, the Val56Arg and Ser167Phe changes, was confirmed to be in *cis* using RFLP analysis as the two mutations resulted in changes in restriction sites for *Mbi*I and *Eco*57I (data not shown). The Ser98Leu mutation involved two consecutive heterozygous base changes [c.293C > T; c.294C > G]. These were tested for being in *cis* or *trans* using allele-specific PCR (Fig. 2). Normal control DNA gave the expected amplified product with the ASOF1 allele-specific primer complementary to the normal sequence but did not show amplification with the mutant allele-specific primer ASOF2 (Fig. 2, lanes 1 and 4). The DNA from Family 39 (Fig. 2, lanes 3 and 5) amplified with allele-specific primers ASOF1 and ASOF2 for both

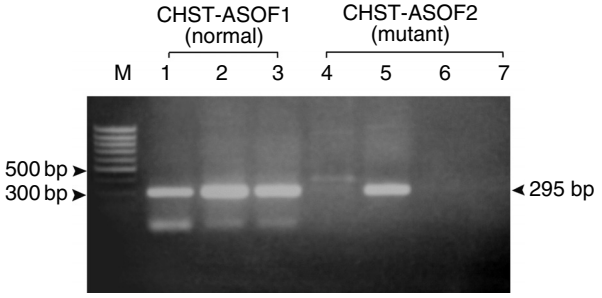

Fig. 2. Results of allele-specific polymerase chain reaction. Details of the assay are in the text. Lanes 1 and 4: DNA from normal control. Lanes 2 and 6: DNA from macular corneal dystrophy family 10 [11]. Lanes 3 and 5: DNA from proband of Family 39. Lane 7: negative control. CHST-ASOF1 forward allele-specific primer for normal sequence. CHST-ASOF2 forward allele-specific primer for mutant sequence. Reverse primer used was common to both sets of reactions. M-DNA size standards.

normal and mutant sequences, respectively (see *Methods*). DNA from MCD Family 10 having the [c.293C > G; c294C > G] mutation identified in a previous study (11) amplified with the ASOF1 primer but not with ASOF2 (Fig. 2, lanes 2 and 6). These results indicate that the two base changes at c.293 and c.294 are in *cis* in Family 39.

The residues involved in missense mutations are fairly well-conserved among various sulfotransferases of human and mouse origin as shown in the partial sequence alignment in Table 2, suggesting that mutations at these sites may be deleterious to the function of the enzyme. Ser54Phe and Val56Arg are located in the conserved 5′ phosphosulfate-binding loop, while Arg202Ser and Pro204Arg are present in the 3′-phosphate-binding domain (14). These domains are required for binding of the sulfate donor 3′-phosphoadenosine-5′-phosphosulfate. Scores from the Blosum 62 substitution matrix for the various amino acid missense mutations in Table 2 have negative values suggesting that these substitutions are unlikely to occur and are therefore possibly unfavorable.

A complex mutation consisting of a deletion plus an insertion resulting in a frameshift at histidine-335 was identified in two patients (Table 1).

The remaining 12 mutations identified in 13 unrelated patients in the present study have been reported previously (Table 1), mostly in Indian patients (11, 15). Consistent with our earlier observations (11), data from the present study demonstrate further allelic heterogeneity in MCD in an Indian patient population. Similar allelic heterogeneity has been observed among patients from several populations including Saudi Arabian (16), British (17), French (18), Vietnamese (19) Southern Indian (15), Icelandic (10), and American (20, 21).

We identified both mutant alleles in all except two patients who had single heterozygous changes (Table 1). Analysis of the upstream regions of CHST5 and *CHST6* in these patients for deletions/rearrangements (9) revealed no alterations (data not shown).

A major proportion of the data available on mutations in MCD so far is from the Indian population. We have analyzed 57 unrelated Indian probands with MCD for mutations in *CHST6* (this study; 11). Of the 47 mutations that we identified in *CHST6* in India in this study and an earlier one (11), 30 were not previously reported. Recurrent mutations in the population studied include c.656_657insCTG, giving rise to an in-frame insertion of tryptophan at position 219 (Ala219_Arg220insTrp), and a missense mutation leading to change of aspartic acid-221 to glutamic acid (Asp221Glu). Both these mutations were each found in 6/57 patients. A deletion of C at position 872 of the cDNA, producing a frameshift at phenylalanine-60, was also recurrent in our patient population, and it was found in 3/57 patients [Table 1; (11)]. MCD represents one of the major inherited corneal disorders among patients presenting at our tertiary care institution in Southern India, accounting for at least one-third of all corneal grafts performed on patients with corneal dystrophies. In general, autosomal recessive corneal disorders appear to predominate over the autosomal dominant types in this patient population, possibly reflecting the high prevalence of consanguinity found in communities from Southern India.

Mutations are distributed throughout the length of the protein, which shares blocks of conserved sequences with other sulfotransferases and is predicted to form a tertiary structural domain that is conserved among sulfotransferase enzymes (NCBI Conserved Domain Database, URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = cdd) (22). A comparison of the mutational spectrum that we obtained with that reported in other studies is shown in Table 3. Missense mutations appear to be the most frequent cause of MCD, with 136/210 (64%) patients studied from different populations having missense mutations (Table 3). Also, of 99 different mutations in *CHST6* identified so far in patients with MCD, 68 are missense. In Indian patients, null mutations (nonsense and frameshift) account for the remaining one-third to one-half of patients (Table 3). The smaller number of patients studied in other ethnic groups precludes any analysis of the relative frequencies of the other categories of mutation. The vast number of missense mutations in *CHST6* all resulting in a fairly uniform clinical picture seem to suggest that none of the resultant mutant proteins found in MCD patients would have any residual activity or that the activity is impaired below a critical level sufficient for disease. Our further studies are aimed at understanding the impact of these pathogenic alterations on the structure of corneal *N*-acetyl glucoamine 6-*O*-sulfotransferase enzyme using structural modeling.

Acknowledgements

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Table 2. Sequence alignment of sulfotransferases showing conservation of amino acids mutated in macular corneal dystrophy patients

Sequence	Ser54Phe Val56Arg	Ala73Thr	Ser 98 Leu	Cys165Trp Ser167Phe	Phe178Cys	Leu193Pro	Pro204Arg	Arg272Ser	Arg334Cys
Human corneal GlcNAc 6-*O*-sulfotransferase (NM_021615)	**S** F **V**	G – E P **A**	W – R **S**	V F – A **C** R **S**	– V R **F**	F – P A **L**	N – R D **P**	R – V **R**	F E – A W **R** H
Human intestinal GlcNAc 6-*O*-sulfotransferase (AF246718)	**S** F **V**	G – E P **A**	**W** – R **A**	V F – A **C** R **S**	– V R **F**	F – P A **L**	N – R D **P**	R – V **R**	F E – A W **R** H
N-acetylglucosamine 6-*O*-sulfotransferase (human)(AF131235)	**S** F **V**	G – E P **A**	W – R **S**	V F – A **C** R **S**	– V R **F**	F – P S **L**	N – R D **P**	R – V **R**	Y E – A W **R** W
Human chondroitin 6-sulfotransferase-2 (AB037187)	**S** F **L**	G – E P **M**	W – R **S**	L F – E **C** R **K**	– V R **L**	L – P G **L**	N – R D **P**	R – L **R**	Y E – A W **R** E
Mouse$_L$-selectin ligand sulfotransferase (AF109155)	**S** F **L**	G – E P **A**	W – R **S**	I F – A **C** R **S**	– V R **F**	L – P S **L**	N – R D **P**	R – L **R**	Y E – A W **R** W
N-acetylglucosamine 6-*O*-sulfotransferase (Mouse) (AF176841)	**S** F **V**	G – E P **A**	W – R **S**	V F – A **C** S **S**	– V R **F**	F – P A **L**	N – R D **P**	R – V **R**	Y E – A W **R** H
Mouse chondroitin 6-sulfotransferase (AB008937)	**S** F **V**	G – E P **L**	W – K **Q**	L L – A **C** R **R**	– V R **I**	R – L R **L**	D – R D **P**	R – V **R**	Y E – K W **R** F
Blosum62 substitution score[a]	−2 −3	0	−2	−2 −2	−2	−3	−2	−1	−3

The protein sequences of the sulfotransferases (GenBank accession numbers of the sequences in parentheses) were aligned using the software OMIGA ver 2.0 (Oxford Molecular Ltd, Cambridge, England). Shown above are sequence alignments surrounding the site of novel missense mutations identified in this study. Only partial sequences consisting of four amino acids surrounding the mutant residues are shown for clarity. Residues mutated are highlighted in bold, and mutations are indicated at the top of each column.
[a]Substitution scores on the Blosum-62 matrix for each of the mutations. The values were obtained from http://www.expasy.org/cgi-bin/blosum.pl.

Table 3. CHST6 gene mutations in macular corneal dystrophy patients from different populations

Country of origin of patients	Number of unrelated patients/ families	Missense mutations	Nonsense mutations	Deletion/ Deletion + insertion	Insertion	Compound heterozygous mutations[a]	Upstream deletion/ re-arrangement	No mutation[b]	Reference
India	108	52	9	35	7	1	0	3	[11, 15] present study
America	24	17	1	0	0	1	0	1	[21]
Iceland	6	5	0	0	0	1	NT	0	[10]
Britain	5	5	0	0	0	0	NT	0	[17]
Japan	20	13	0	1	1	1	4	0	[9, 23]
Saudi Arabia	12	12	0	0	0	0	NT	0	[16]
Italy	3	2	0	0	0	0	1	0	[24]
France	11	7	1	0	1	2	0	0	[18]
Vietnam	21	14	1	0	3	3	0	0	[19]

NT, not tested. The table shows the total number of patients screened and the numbers of patients having each of the different types of mutations.
[a]Patients who were heterozygous for two different types of mutation.
[b]No mutations were detected in the regions screened.

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