



Novel mutations of the carbohydrate sulfotransferase-6 (*CHST6*) gene causing macular corneal dystrophy in India

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Purpose: Macular corneal dystrophy (MCD) is an autosomal recessive disorder characterized by progressive central haze, confluent punctate opacities and abnormal deposits in the cornea. It is caused by mutations in the carbohydrate sulfotransferase-6 (*CHST6*) gene, encoding corneal N-acetyl glucosamine-6-O-sulfotransferase (C-GlcNAc-6-ST). We screened the *CHST6* gene for mutations in Indian families with MCD, in order to determine the range of pathogenic mutations.

Methods: Genomic DNA was isolated from peripheral blood leukocytes of patients with MCD and normal controls. The coding regions of the *CHST6* gene were amplified using three pairs of primers and amplified products were directly sequenced.

Results: We identified 22 (5 nonsense, 5 frameshift, 2 insertion, and 10 missense) mutations in 36 patients from 31 families with MCD, supporting the conclusion that loss of function of this gene is responsible for this corneal disease. Seventeen of these mutations are novel.

Conclusions: These data highlight the allelic heterogeneity of macular corneal dystrophy in Indian patients.

The corneal dystrophies are a heterogeneous group of disorders that may lead to severe visual impairment [1]. Macular corneal dystrophy (MCD, MCDC1 [OMIM 217800]) is an autosomal recessive disorder clinically characterized by bilateral corneal opacification. Initially the patients have diffuse, fine superficial clouding in the central stroma. The opacities extend through the entire thickness of the cornea and involve the central and peripheral corneal stroma. The corneal stroma is often thinner than normal [2-4]. The corneal endothelium is involved and guttae form on the Descemet's membrane [5]. The prevalence of MCD varies immensely in different parts of the world but in most populations the condition is rare. In some countries MCD accounts for 10-75% of the corneal dystrophies requiring corneal grafting [6,7].

Keratan sulfate (KS) is the major corneal glycosaminoglycan [8] and is a component of three corneal proteoglycans (lumican, keratocan, and mimecan). Sulfate ions contribute significantly to the negative charge of proteoglycans and in the cornea this highly anionic charge on KS is believed to contribute to its ability to imbibe water and influence corneal hydration, which must be critically controlled for the maintenance of corneal transparency [9]. During corneal development, sulfation of poly lactosamine (non-sulfated precursor of KS) occurs at later stages and is required for attainment of full corneal transparency [10]. Macular corneal dystrophy arises

due to a failure to synthesize normal keratan sulfate proteoglycan within the cornea [11-13] and corneas with this disorder accumulate a glycoaminoglycan within the keratocytes, corneal endothelium, Bowman's layer, Descemet's membrane, and extracellularly in the stroma.

MCD is divided into three immunophenotypes (MCD types I, IA, and II) based on the reactivity of the patient's serum and corneal tissue to an antibody that recognizes sulfated KS, although these subtypes are clinically indistinguishable from each other [14-16]. In MCD type I neither the serum nor the corneal tissue contain antigenic KS (AgKS). In MCD type IA, sulfated KS is absent in the cornea and the serum but can be detected in the keratocytes [16]. MCD type II is characterized by the presence of AgKS in the corneal tissue and normal levels of AgKS in the serum. After identifying the locus for MCD on chromosome 16 [17] and fine mapping the gene [18,19], mutations in the carbohydrate sulfotransferase gene (*CHST6*) encoding corneal N-acetyl glucosamine-6-O-sulfotransferase (C-GlcNAc-6-ST) were identified as the cause of MCD types I and II [20]. While mutations of the coding region of *CHST6* were found in MCD types I, IA, and II, deletions and rearrangements of the upstream region of *CHST6* as well as missense mutations have been reported in MCD type II [20-22].

MCD is one of the most common types of corneal dystrophy in India and constitutes about 30% of all dystrophies requiring penetrating keratoplasty (G.K. Vemuganti, personal communication). We screened 36 patients with MCD belonging to 31 families for mutations in the *CHST6* gene. Our study revealed 22 mutations 17 of which are novel, consisting of deletions, insertions, missense, and nonsense mutations.

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METHODS

Clinical evaluation and patient selection: The study had the approval of Institutional Review Boards of the L. V. Prasad Eye Institute and Duke University Medical Center and conformed to the tenets of the declaration of Helsinki. Informed consent was obtained from all participants for clinical and molecular genetic studies. Patients were evaluated clinically by a corneal specialist (M.S.S.). Patients were diagnosed as having MCD based on typical clinical features. All patients had a characteristic haze and whitish nodular lesions involving the central cornea. According to the patients, clinical symptoms first became apparent at 13 to 49 years of age (Table 1). Decreased visual acuity was the common presenting complaint (found in 85% of patients) followed by irritation and tearing (45% of patients) and the presence of a white opacity (20% of patients). Among patients who did not undergo penetrating keratoplasty, the visual acuity ranged from counting fingers to 20/30. Climatic droplet keratopathy (spheroidal degeneration) was associated in 2 patients (5%), who not only had the typical features of MCD, but also golden-colored spherical deposits in the superficial cornea. None of the patients had raised intraocular pressure or posterior segment abnormalities. Out of 17 patients who underwent penetrating keratoplasty (PK), the grafts remained clear in all except 5 cases for

the period of follow up, which ranged from 0 to 14 years. The grafts failed in five cases and in all of these instances the grafted tissue became edematous, without clinical evidence of recurrent MCD. In 17 patients who had undergone keratoplasty (8 unilateral grafts, 9 bilateral grafts) diagnosis was confirmed by histopathologic examination. Of the 36 patients studied, 14 were sporadic cases with no other affected relatives while the rest had familial disease. Consanguinity was known to be present in 20 out of 31 families.

PCR amplification and sequence analysis: Genomic DNA was extracted from 8-10 ml of blood samples obtained from all the patients involved in the study and also from the normal controls without any corneal diseases. The *CHST6* gene is 16.9 kb in length and consists of 4 exons, of which only exon 4 contains the coding sequence of 1,189 bp. For amplification of the *CHST6* coding region, 3 primer pairs were used for 5', middle, and 3'-segments as described by Akama et al. [18]. All PCR reactions were done (thermal cycler PTC 200 MJ Research, Watertown, MA) using 75 ng genomic DNA in 25 µl reaction containing 1X PCR buffer (200 µM of dNTPs, 0.5 µM of each primer, 4% dimethyl sulfoxide [DMSO] and 1 U *Taq* polymerase). PCR products were purified on Amicon columns (Microcon PCR; Millipore, Bedford, MA) and directly sequenced using fluorescent dideoxynucleotides (Big Dye

TABLE 1. MUTATIONS OF THE *CHST6* GENE AMONG PATIENTS WITH MACULAR CORNEAL DYSTROPHY

Family	Mutation	Mutation type	Consequence in protein	Restriction site change	Age (yrs) of onset	Consanguinity	Corneal graft
1	c.708-732del	Deletion (25 bp)	FS at R5	-	14	N	N
2	c.744C>T	Nonsense	Q18X	Xmi1	26	N	Bilateral
3	c.786-792del	Deletion (7 bp)	FS at P31	-	26	Y	N
4	c.847G>A	Missense	G52D	Hinf1	13	Y	N
5	c.847G>A	Missense	G52D	Hinf1	40	Y	Bilateral
6	c.850C>T*	Missense	S53L	Eco241	23	Y	Unilateral
7	c.850C>T*	Missense	S53L	Eco241	20	N	N
8	c.872delC	Deletion (1 bp)	FS at F60	Mse1	15	Y	N
9	c.890delC*	Deletion (1 bp)	FS at V66	-	43	N	N
10	[c.985C>G, c.986C>G] + c.1012T>C	Missense	S98W+F107S	-	14	N	Bilateral
11	c.1012T>C*	Missense	F107S	-	10	Y	Unilateral
12	c.1012T>C*	Missense	F107S	-	15	Y	N
13	c.1055C>G	Missense	F121L	BseN1	23	Y	N
14	c.1061G>A	Nonsense	W123X	Cfr131	16	N	Bilateral
15	c.1151C>A	Nonsense	C153X	-	34	N	Unilateral
16	c.1279insACG	Insertion	R195-196ins	-	10	Y	Bilateral
17	c.1296C>A	Missense	R202S	Hph1	21	Y	Bilateral
18	c.1303C>A*	Missense	P204Q	Cfr421	49	Y	N
19	c.1304-1306 del3insAT*	Deletion + insertion	FS at P204	Cfr421	25	N	N
20	[c.1321C>T + c.1355C>G]	Missense	S210F+D221E	-	29	Y	N
21	c.1353G>T	Missense	D221Y	Nmuc1	19	Y	Bilateral
22	c.1348insCTG	Insertion (in frame)	W219-220ins	-	17	N	Unilateral
23	c.1348insCTG	Insertion (in frame)	W219-220ins	-	15	Y	Unilateral
24	c.1348insCTG	Insertion (in frame)	W219-220ins	-	12	Y	N
25	c.1348insCTG	Insertion (in frame)	W219-220ins	-	14	Y	N
26	c.1355C>G	Missense	D221E	Nmuc1	13	Y	Unilateral
27	c.1355C>G	Missense	D221E	Nmuc1	childhood	N	Bilateral
28	c.1355C>G	Missense	D221E	Nmuc1	35	Y	Unilateral
29	c.1617G>T	Nonsense	G309X	Tspr1	22	Y	N
30	c.1731G>T	Nonsense	E347X	-	34	N	Unilateral
31	c.1731G>T	Nonsense	E347X	-	15	Y	Bilateral

Shown are details of probands of the families studied and mutations identified. The asterisks (“*”) designate mutations reported previously. FS indicates a frameshift. The last column indicates whether corneal grafts were performed and, if yes, whether they were bilateral or unilateral.

Terminator Kit; Applied Biosystems Incorporated, Foster City, CA) on an automated DNA sequencer (ABI Prism 377). The sequences were compared with the published cDNA sequence of *CHST6* (AF219990) and mutations identified were excluded from 75 control DNA samples using restriction enzymes where appropriate. For mutations without changes in restriction enzyme sites, single strand conformation polymorphism (SSCP) using fragments <300 bp in length amplified with overlapping sets of primers or direct sequencing was performed.

RESULTS

In the population analyzed, mutations in the coding region of *CHST6* were identified in all cases. We found 22 different mutations and none of them were detected in a control population of the same ethnic origin as determined by restriction enzyme digests or SSCP analysis on at least 150 chromosomes, suggesting that they are likely to be pathogenic mutations.

Out of the 22 identified mutations, there were 4 deletions, 2 insertions, 1 complex mutation consisting of deletion and insertion, 5 nonsense and 10 missense mutations (Table 1). A homozygous 25 bp deletion was identified (family 1), resulting in a frameshift at arginine-5, followed by 55 amino acids of altered reading frame before a stop codon. A second homozygous deletion of 7 bp was found in family 3, producing a frameshift at proline-31, with termination after 36 amino acids. In family 8, homozygous deletion of a single base C-872 was found, leading to a frameshift at phenylalanine-60, with termination after eight amino acids. A heterozygous deletion of C-890 predicting a frameshift at valine-66 and immediate termination of protein at amino acid 70 was found in family 9. In this family, the second mutation has not been identified upon sequencing of the coding region. A complex homozygous mutation, consisting of a deletion of GCG at nucleotide 1304 and an insertion of AT was seen in family 19, which resulted in a frameshift at proline-204. A 174 amino acid polypeptide of altered reading frame is predicted after the frameshift.

Five nonsense mutations were found in 6 families, involving the formation of stop codons at glutamine-18 (Q18X) in family 2, tryptophan-123 (W123X) in family 14, cysteine-153 (C153X) in family 15, glycine-309 (G309X) in family 29 and glutamic acid-347 (E347X) in families 30 and 31.

Two insertions resulting in an in-frame insertion of a single amino acid were found. A recurrent homozygous insertion of CTG at nucleotide 1348 in families 22-25 resulted in the insertion of an extra amino acid tyrosine, after alanine-219. Homozygous insertion of ACG in family 16 resulted in the insertion of an arginine residue after leucine-195. Since there is an "ACG" sequence from nucleotides 1277-1279 of the *CHST6* cDNA, the insertion of an ACG either at position 1276 or at 1279 would produce the same result. Hence the insertion could have occurred at either of these positions with the resulting amino acid sequence being identical in both cases.

Apart from these, we identified 10 missense mutations in 16 families (Table 1). Homozygous mutations identified were glycine-52 to aspartic acid (G52D) in 2 families, serine-53 to leucine (S53L) in 2 families, phenylalanine-107 to serine

(F107S) in 2 families, phenylalanine-121 to leucine (F121L) in one family, arginine-202 to serine (R202S) in one family, proline-204 to glutamine (P204Q) in one family, aspartic acid-221 to glutamic acid (D221E) in 3 families, and aspartic acid-221 to tyrosine (D221Y) in one family. Two novel compound heterozygous missense mutations were identified in families 10 and 20. In family 10, changes of serine-98 to tryptophan (S98W) and phenylalanine-107 to serine (F107S) were found in two affected members of the family. In family 20, the proband was a compound heterozygote for serine-210 to phenylalanine (S210F) and aspartic acid-221 to glutamic acid (D221E) missense mutations.

We failed to detect any apparent relationship between *CHST6* mutations and the clinical manifestations in the patients with MCD. Families with the same mutations had variations in the clinical features. For example, age of onset as well as presence of deposits up to the corneal limbus varied among members of families 26-28 which had the D221E change (Table 1). Truncating and non-truncating mutations did not cause apparent differences in the age of onset of the cases.

DISCUSSION

Corneal N-acetyl glucosamine-6-O-sulfotransferase catalyzes the transfer of a sulfate group to C6 of N-acetyl glucosamine in keratan sulfate, a common component of corneal proteoglycans. Structurally, this enzyme shares conserved sequences with many sulfotransferases (NCBI Conserved Domain Database), consisting of a single α/β domain [23]. It is presumed to play a key role in the production of corneal keratan sulfate [24,25].

We studied a cohort of 36 patients (31 unrelated) with MCD, which is a common corneal dystrophy in India. Twenty-two mutations including 17 novel mutations were identified. A notable feature of the mutations in this series is that 10 are predicted to result in a premature termination of the encoded protein and would be expected to produce functionally null alleles. As shown in Table 1 the majority of the unrelated patients (28) had homozygous pathogenic mutations within the coding region of *CHST6*. Two patients (from families 10 and 20), were compound heterozygotes for two missense mutations. However, in 1 family (family 9), only a single heterozygous change was detected within the coding region of *CHST6*. Because the second mutation was not identified in the coding region, it is possible that it is located in the promoter or in a non-coding region of the gene.

Overall, the data indicate a high degree of heterogeneity among the patients studied. Similar heterogeneity has been a feature in other studies carried out on individuals with MCD in other populations including those from Britain [22], France [26], Iceland [21], India [27], Japan [28], Saudi Arabia [29], United States of America [30], and Vietnam [31]. Warren et al. [27] in their study on MCD in a Southern Indian population also noted a high degree of mutational heterogeneity. We found only three of the mutations that were reported by them in our patient population (see below). The spectrum of mutations described in their study comprised missense, deletions,

and complex mutations. In contrast to our study they did not observe any nonsense mutations [27]. Since our patients represent diverse regions, our data do not permit conclusions as to whether any of the mutations are specific to an ethnic subgroup. An analysis of a larger cohort of patients would be required to determine if nonsense mutations are a significant type of mutation in MCD patients.

To assess the significance of the identified missense mutations, an alignment of protein sequences of the conserved domains from several sulfotransferases of human and mouse origin was examined. As can be seen from Table 2, all residues mutated are highly conserved, suggesting that these amino acids are essential for the structure and function of the protein. Five mutations involve the sulfate donor 3'-phospho adenosine 5'-phosphosulfate (PAPS) [32]. Mutations G52D and S53L are located within the 5'-PSB (5'-phosphosulfate-binding) domain which interacts with the 5'-phosphate group of PAPS and is highly conserved among all sulfotransferases known to date [23]. The R202S, P204Q, and S210F involve the 3'-PB (3'-phosphate-binding) domain, the highly conserved domain interacting with the 3'-phosphate of PAPS. Five of the mutations identified in the present study were previously reported; c.850C>T (S53L), c.890delC, and del1304-1306insAT were reported in Indian patients [27], the F107S mutation has been reported previously in a British family [22] and the P204Q

mutation has been recently reported in Japanese patients [28].

The only substitution that involved replacement with a chemically similar amino acid is D221E (Table 1). The occurrence of this change in probands from 3 families (Table 1, families 26-28) as a homozygous change and in one family (family 20) as a heterozygous change, together with its absence in 75 unrelated control individuals supports the conclusion that it is pathogenic. Structural modeling of normal and mutant proteins as well as in vitro studies on the properties of the wild type and mutant proteins may be required to determine the exact consequences of this change. It is possible that a mutation of aspartic acid-221 causes changes in local structure or alters protein stability or processing, thus leading to deficient enzyme activity.

Analysis of clinical parameters indicated that most patients in our study had similar features. There were no consistent differences in phenotype between patients with the various amino acid substitutions and truncating mutations. Biochemical studies on the enzymatic activity of the mutant proteins identified in our study may help elucidate the role of specific residues on the function of the *CHST6* gene product.

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TABLE 2. SEQUENCE ALIGNMENT OF SULFOTRANSFERASES SHOWING CONSERVATION OF AMINO ACIDS MUTATED IN MCD PATIENTS

	G52D, S53L	S98W	F107S	F121L	R202S, P204Q	D221E/Y	S210F
Human corneal GlcNAc 6-O-sulfotransferase (AF219990)	S G S S	R S V F	D V F D	L F Q W	V R D P	A R D N	L R S R
Human intestinal GlcNAc 6-O-sulfotransferase (AF246718)	S G S S	R S I F	D V F D	F F N W	V R D P	A R D N	L R S R
N-acetylglucosamine- 6-O-sulfotransferase (human) (AF131235)	S G S S	R A V F	S V F D	L F Q W	V R D P	M I D S	F R S R
Human chondroitin 6-sulfotransferase-2 (AB037187)	T G S S	R S L F	S V L R	L F R W	F R D P	L R E S	H N S R
Mouse L-selectin ligand sulfotransferase (AF131235)	S G S S	R S V F	S V F D	L F Q W	V R D P	V V D S	F R S R
N-acetylglucosamine- 6-O-sulfotransferase (Mouse) (AF176841)	S G S S	R S V F	D V F D	L F Q W	V R D P	A R D N	L R S R
Mouse chondroitin 6-sulfotransferase (AB008937)	T G S S	K Q L L	Y V L E	L F R R	V R D P	Y E N W	L A S R

The protein sequences of the sulfotransferases shown were aligned using the software Omega version 2.0 (Oxford Molecular Ltd., Cambridge, England). Residues mutated along with flanking amino acids are shown in this table. Mutations are indicated at the top of each column. GenBank accession numbers of the sequences are given in parentheses.

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