Novel mutations of CHST6 in Iranian patients with macular corneal dystrophy

Shiva Akbari Birgani,1 Zivar Salehi,1 Masoud Houshmand,2 Mohamad Javad Mohamadi,1 Leila Azzadeh Promehr,1 Zahra Mozafarzadeh1

1Department of Biology, Guilan University of Medical Science, Rasht, Iran; 2National Research Center for Genetic Engineering and Biotechnology, Tehran Iran

Purpose: To characterize mutations within the carbohydrate sulfotransferase 6 (CHST6) gene in Iranian subjects from 12 families with macular corneal dystrophy (MCD).

Methods: Genomic DNA was extracted from peripheral blood of 20 affected patients and 60 healthy volunteers followed by polymerase chain reaction (PCR) and direct sequencing of the CHST6 coding region. The observed nucleotide sequences were then compared with those found by investigators in other populations with MCD and in the controls.

Results: Analysis of CHST6 revealed 11 different mutations. These mutations were comprised of six novel missense mutations (p.F55L, p.P132L, p.S136G, p.C149Y, p.D203Y, and p.H249R), one novel nonsense mutation (p.S48X), one novel frame shift (after P297), and three previously reported missense mutations (p.P31L, p.C165Y, and p.R127C). The majority of the detected MCD mutations are located in the binding sites or the binding pocket, except the p.P31L and p.H249R mutations.

Conclusions: Nucleotide changes within the coding region of CHST6 are predicted to significantly alter the encoded sulfotransferase within the evolutionary conserved sequences. Our findings show that CHST6 mutations are responsible for the pathogenesis of MCD in Iranian patients. Moreover, the observation that some cases of MCD cannot be explained by mutations in the coding region of CHST6 suggests that MCD may result from possible upstream rearrangements in the CHST6 genomic region.

Corneal dystrophies are a heterogeneous group of disorders that may lead to severe visual impairment of which macular corneal dystrophy (MCD; OMIM 217800) is transmitted as an autosomal recessive trait [1]. The prevalence of MCD varies immensely in different parts of the world, but in most populations, the condition is rare. As a treatment, penetrating keratoplasty is performed when useful vision has been lost. Reportedly, in Iceland and Japan, MCD accounts for 75% and 10%, respectively, of the corneal dystrophies requiring corneal grafting [2,3]. Clinical symptoms usually manifest within the first decade of life, and MCD patients show spotted opacity in both corneas. These abnormal deposits are associated with a central stromal haze that gradually extends to the periphery of the cornea, leading to visual impairment, and patients require keratoplasty [4]. MCD has been subdivided into three immunophenotypes (MCD types I, IA, and II) based on the reactivity of the patient's serum and corneal tissue to monoclonal anti-keratan sulfate antibody (5D4 anti-KS antibody) [5].

Biochemical studies have indicated that a specific sulfation step of keratan sulfate is impaired in MCD, resulting in an accumulation of glycosaminoglycans (GAGs). Keratan sulfate proteoglycan, the most abundant carbohydrate in the cornea, plays an important role in maintenance of corneal transparency [6,7]. Keratan sulfate consists of a linear poly-N-acetyllactosamine chain that carries sulfate residues on C-6 of N-acetylgalcosamine (GlcNAc) and galactose (Gal). Because the sulfation of carbohydrates affects their biochemical characteristics such as water solubility and electrical charge, this modification appears to be important for the function of keratan sulfate proteoglycans in the cornea. Considering the importance of the sulfated carbohydrates of keratan sulfate in the normal function of the cornea, it has been suggested that lack of this sulfation step is a major cause of MCD [6,8,9].

It has also recently been shown that there is a failure in the activity of corneal GlcNAc 6-O-sulfotransferase (C-GlcNAc6ST), which transfers sulfate to position 6 of GlcNAc residues and this enzyme participates in biosynthesis of corneal keratan sulfate proteoglycan. In the cornea of patients with MCD, a decrease in C-GlcNAc6ST activity results in the formation of poorly sulfated or non-sulfated keratan sulfate and causes corneal opacity [10,11].

Using genetic linkage analysis, the critical region for MCD has been mapped to chromosome 16 (16q22). The carbohydrate sulfotransferase 6 gene (CHST6) was identified within the MCD critical region encoded C-GlcNAc6ST, and mutations in CHST6 have been shown to be the fundamental defect in MCD [12,13].
In the present study, which is the first comprehensive work studying Iranian subjects with MCD, we screened 20 patients with MCD from 12 families to search for mutations in \( \text{CHST6} \). We identified seven novel mutations and four previously reported disease-causing mutations.

### METHODS

**Patients:** This study was performed in accordance with the Declaration of Helsinki and with the approval of the ethics board of the Amiralmomenin Hospital and Guilan University (Rasht, Iran). Twenty patients from 12 unrelated Iranian families who received clinical diagnosis of MCD were included in this study. Diagnosis for MCD in affected individuals was made by cornea specialists at the Amiralmomenin Hospital (associated with Guilan University of Medical Sciences). All patients were older than 25 years, and the obtained pedigrees were consistent with an autosomal recessive inheritance pattern. There was not any known consanguinity in the patients' families. Sixty unrelated, unaffected, and healthy volunteers were recruited to serve as controls.

Blood samples were obtained from a peripheral blood vessel from affected patients and healthy volunteers. Genomic DNA was extracted by standard procedure using the PAX gene blood DNA kit (Qiagen, Valencia, CA).

**DNA analysis:** The coding region of \( \text{CHST6} \) was amplified using polymerase chain reaction (PCR) and the primers designed to create three overlapping amplicons. The oligonucleotide primers used were identical to those reported by Akama and associates [12].

Each reaction was performed in a 50-µl volume comprising of genomic DNA (100 ng), 10X PCR buffer, 0.2 µM dNTP, 1.5–2 µM of MgCl\(_2\), 0.2 µM of each primer, 1 U of Taq DNA polymerase (Qiagen). The thermal cycling was performed using the following protocol 2 min at 96 °C and 35 cycles of 30 s at 96 °C, 30 s at 57 °C, 45 s at 72 °C, and final extension 7 min at 72 °C followed by a 4 °C hold cycle.

The PCR products were then purified using the QIAquick PCR purification kit (Qiagen) and sequenced on both strands by the ABI BigDye terminator chemistry and an ABI Prism 3700 instrument (Applied Biosystems, Foster City, CA). Sequences were analyzed using the Sequencher software (Gene Codes Corporation, Ann Arbor, MI) and compared with the nucleotide sequence of the \( \text{CHST6} \) human cDNA (GenBank accession number AF219990).

### RESULTS

A total of 20 affected patients representing 12 different families were enrolled in the study. The most abnormalities found in this study were single base changes in the coding region of \( \text{CHST6} \) that altered a coded amino acid (Table 1), seven of which were homozygous. The nucleotide changes included c.92C>T, c.143C>A, c.165C>A, c.379C>T, c.406A>G, and c.746A>G, resulting in a proline to a leucine substitution (p.P31L), a serine to stop codon substitution (p.S48X), a phenylalanine to a leucine substitution (p.F55L), an arginine to a cysteine substitution (p.R127C), a serine to a glycine (p.S136G), and a histidine to an arginine substitution (p.H249R).

In family C, an insertion of a single base pair between nucleotides 891 and 892 was identified, resulting in a frame shift after codon, P297 (p.P297fs).

In addition, a compound heterozygosity was detected in family E. In this family, a heterozygous changes, c.395C>T and c.494G>A, was identified, predicting amino acid changes of proline to leucine (p.P132L) and cysteine to tyrosine (p.C165Y).

In two families, only one heterozygous pathogenic change was observed in the coding \( \text{CHST6} \) sequence. In family F, we found c.446G>A, which replaces a cysteine to a tyrosine (p.C149Y). In family H, c.607G>T was found, replacing an aspartic acid to a tyrosine (p.D203Y).

In the last two families (K and L), no presumable pathogenic change was detected. In addition, a single novel nucleotide

| Family | Number of patients | Zygosity | Nucleotide change | Protein change | Disease-causing or not |
|--------|--------------------|----------|------------------|----------------|-----------------------|
| A      | 3                  | Homozygous | c.746A>G         | p.H249R        | +                     |
| B      | 3                  | Homozygous | c.92C>T          | p.P31L         | +                     |
| C      | 2                  | Homozygous | c.891–892insC    |                |                       |
| D      | 1                  | Homozygous | c.143C>A         | p.S48X         | +                     |
| E      | 4                  | Heterozygous | c.494G>A       | p.C165Y        | +                     |
| F      | 1                  | Heterozygous | c.395C>T       | p.P132L        | +                     |
| G      | 1                  | Homozygous | c.379C>T         | p.R127C        | +                     |
| H      | 1                  | Heterozygous | c.607G>T       | p.D203Y        | +                     |
| I      | 1                  | Homozygous | c.406A>G        | p.S136G        | +                     |
| J      | 1                  | Heterozygous | c.165C>A      | p.F55L         | +                     |
| K      | 1                  | No mutations |               |                | -                     |
| L      | 1                  | No mutations |               |                | -                     |

In the table “+” denotes that the observed changes are Disease-causing, and “-” denotes that the observed changes are not Disease-causing.

In the present study, which is the first comprehensive work studying Iranian subjects with MCD, we screened 20 patients with MCD from 12 families to search for mutations in \( \text{CHST6} \). We identified seven novel mutations and four previously reported disease-causing mutations.
polymorphism, c.405C>T (a heterozygous change), was found in family K. It was considered a non-pathogenic sequence variant because it had no effect on the amino acid.

The amplified PCR products from 60 control individuals were analyzed for each alteration using direct sequencing, none of which indicated the mutations seen for the patients.

DISCUSSION
In this study, we examined the CHST6 coding region for mutations in 20 individuals with MCD from 12 Iranian families. Sequencing analysis revealed six novel missense mutations (p.F55L, p.P132L, p.S136G, p.C149Y, p.D203Y, and p.H249R), one nonsense mutation (p.S48X), one frame shift (p.P297fs), and three previously reported missense mutations (p.P31L [13], p.C165Y [14], and p.R127C [15]) in CHST6 in the Iranian patients with MCD.

CHST6 encodes the C-GlcNAc6ST, comprising 395 amino acids. This enzyme is localized in the Golgi apparatus and is a member of the Gal/GalNAc/GlcNAc 6-O sulfotransferase (GST) family. The C-GlcNAc6ST contains a short cytosolic tail at the NH2-terminal, a single transmembrane span, and a COOH-terminal domain that contains two putative binding sites for the high energy sulfate donor (adenosine3′-phosphate-5′phosphosulfonate [PAPS]). One of the binding sites is the 5′-phosphosulfate binding site (5′-PSB), corresponding to amino acid 49–56, and the other is the RX-S 3′-phosphate binding site (3′-PB), corresponding to amino acid 202–210 (Figure 1). The sequence between the two binding sites is thought to contribute to a binding pocket that interacts with an acceptor (GlcNAc) to bring it into apposition with the sulfate donor [16].

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 [17].

The identified mutation of p.F55L is located in the 5′-PSB domain. The mutations of p.R127C, p.P132L, p.S136G, p.C149Y, p.C165Y are located in the sequence between the 5′-PSB domain, and the 3′-PB domain. The mutation of p.D203Y is located in the RX-S sequence for the 3′-PB domain. Whereas the mutations of p.P31L, p.H249R are neither located in the binding motifs nor in the binding pocket.

Amino acid changes at positions 203 and 249 have been previously reported by Akama et al. [12] and Warren et al. [18], respectively, but they are different from the mutations identified in the present study. We have observed c.607G>T causing a replacement of an aspartic acid to a tyrosine (p.D203Y) while in Japanese patients with MCD, a change at nucleotide position 609 (c.609C>A) causes the replacement of an aspartic acid to a glutamic acid at the same protein position (p.D203E) [12]. In Iranian subjects, a change at nucleotide position 746 (c.746A>T) leads to the replacement of a histidine to an arginine at position 249 of the protein (p.H249R) while in Indian patients with MCD, Warren et al.
inactivation of the sequence changes identified are responsible for producing [22]. Additional evidence to support our findings that the reading frame was identified as the cause in this individual origin with MCD was studied. Deletion of the whole open proteins that they encode does not occur [21].

translation so production of the potentially toxic truncated terminate translation more than 50–55 nucleotides upstream NMD in mammalian cells generally degrades mRNAs that mutations may lead to the elimination of the protein due to nonsense-mediated decay of the mRNA (NMD). NMD in mammalian cells generally degrades mRNAs that terminate translation more than 50–55 nucleotides upstream of a splicing-generated exon-exon junction [19,20]. NMD downregulates spliced mRNAs that prematurely terminate translation so production of the potentially toxic truncated proteins that they encode does not occur [21].

It is notable that previously only one patient of Iranian origin with MCD was studied. Deletion of the whole open reading frame was identified as the cause in this individual [22]. Additional evidence to support our findings that the sequence changes identified are responsible for producing inactivation of the CHST6 gene product is provided by the fact that no mutations were detected in the CHST6 coding region from control individuals.

In one family (family L), there were no coding region mutations, and in another family (family K), the nucleotide change had no effect on protein phenotype. These cases of MCD can not be explained by mutations in the coding sequence of CHST6, suggesting that MCD may result from possible upstream rearrangements such as the promoter, which is also part of CHST6 and has not been analyzed in the present study. It is intriguing that such a large number and wide variety of novel mutations were discovered throughout the CHST6 coding sequence. This suggests that in contrast to TGFB1 (transforming growth factor, beta induced) with its hot spots for corneal dystrophy-associated amino acid substitution, CHST6 does not demonstrate a highly conserved number of disease-causing mutations [23].

In summary, we identified seven novel and three previously reported CHST6 mutations in our panel consisting of 20 Iranian MCD patients from 12 families. Further analysis of these mutations may help to achieve a clearer understanding of the molecular events leading to the characteristic lesions of MCD.

ACKNOWLEDGMENTS

We would like to thank the Iranian families for consenting to participate in this study. We would also like to thank Miss Zahra Shaygan for valuable assistance and Dr. Ghader Bashiri for a critical reading of the manuscript.

REFERENCES

1. Klintworth GK. Research into the pathogenesis of macular corneal dystrophy. Trans Ophthalmol Soc U K 1980; 100:186-94. [PMID: 6973846]

2. Jonasson F, Johannsson JH, Garner A, Rice NS. Macular corneal dystrophy in Iceland. Eye 1989; 3:446-54. [PMID: 2606219]

3. Santo RM, Yamaguchi T, Kanai A, Okisaka S, Nakajima A. Clinical and histopathologic features of corneal dystrophies in Japan. Ophthalmology 1995; 102:557-67. [PMID: 7724173]

4. Vance JM, Jonasson F, Lennon F, Sarrica J, Damji KF, Stauffer J, Pericak-Vance MA, Klintworth GK. Linkage of a gene for macular corneal dystrophy to chromosome 16. Am J Hum Genet 1996; 58:757-62. [PMID: 8644739]

5. Yang C, SundarRaj N, Thonar EJ, Klintworth GK. Immunohistochemical evidence of heterogeneity in macular corneal dystrophy. Am J Ophthalmol 1988; 106:65-71. [PMID: 3293458]

6. Hassell JR, Newsome DA, Krachner JH, Rodrigues MM. Macular corneal dystrophy: failure to synthesize a mature keratan sulfate proteoglycan. Proc Natl Acad Sci USA 1980; 77:3705-9. [PMID: 6447876]

7. Funderburgh JL, Funderburgh ML, Mann MM, Conrad GW. Physical and biological properties of keratan sulphate proteoglycan. Biochem Soc Trans 1991; 19:871-6. [PMID: 1794576]

8. Nakazawa K, Hassell JR, Hascall VC, Lohmander LS, Newsome DA, Krachner J. Defective processing of keratan sulfate in macular corneal dystrophy. J Biol Chem 1984; 259:13751-7. [PMID: 6238957]

9. Klintworth GK, Meyer R, Dennis R, Hewitt AT, Lenz ME, Hassell JR, Stark WJ Jr, Kuettner KE, Thonar EJ. Macular corneal dystrophy. Lack of keratan sulfate in serum and cornea. Ophthalmic Paediatr Genet 1986; 7:139-43. [PMID: 2951638]

10. Hasegawa N, Takayoshi T, Kato T, Miyajima H, Furuhata A, Nakayasu K, Kanai A, Habuchi O. Decreased GlcNac 6-O-sulfotransferase activity in the cornea with macular corneal dystrophy. Invest Ophthalmol Vis Sci 2000; 41:3670-7. [PMID: 11053262]

11. Akama TO, Nakayama J, Nishida K, Hiraoka N, Suzuki M, McAuliffe J, Hindsgaul O, Fukuda M, Fukudal MN. Human corneal GlcNac 6-O-sulfotransferase and mouse intestinal GlcNac6-O-sulfotransferase both produce keratan sulfate. J Biol Chem 2001; 276:16271-8. [PMID: 11278593]

12. Akama TO, Nishida K, Nakayama J, Watanabe H, Ozaki K, Nakamura T, Dota A, Kawasaki S, Inoue Y, Maeda N, Yamamoto S, Fujiwara T, Thonar EJ, Shimomura Y, Kinoshita S, Taniyama A, Fukuda MN. Macular corneal dystrophy type I and type II are caused by distinct mutations in a new sulfotransferase gene. Nat Genet 2000; 26:237-41. [PMID: 11017086]

13. Klintworth GK, Smith CF, Bowling BL. CHST6 mutations in North American subjects with macular corneal dystrophy: a comprehensive molecular genetic review. Mol Vis 2006; 12:159-76. [PMID: 16568029]

14. Liskova P, Veraitch B, Jirsova K, Filipc M, Neuworth A, Ebenezer ND, Hysi PG, Hardcastle AJ, Tuft SJ, Bhattacharya SS. Sequencing of the CHST6 gene in Czech macular corneal dystrophy patients supports the evidence of a founder mutation. Br J Ophthalmol 2008; 92:265-7. [PMID: 17962390]
15. Bao W, Smith CF, al-Rajhi A, Chandler JW, Karcioglu ZA, Akama TO, Fukuda MN, Klintworth GK. Novel mutations in the CHST6 gene in Saudi Arabic patients with macular corneal dystrophy. ARVO Annual Meeting; 2001 April 29-May 4; Fort Lauderdale, FL.

16. Kakuta Y, Pedersen LG, Pedersen LC, Negishi M. Conserved structural motifs in the sulfotransferase family. Trends Biochem Sci 1998; 23:129-30. [PMID: 9584614]

17. Ong E, Yeh JC, Ding Y, Ding Y, Hindsaul O, Pedersen LC, Negishi M, Fukuda M. Structure and function of HNK-1 sulfotransferase: identification of donor and acceptor binding sites by site directed mutagenesis. J Biol Chem 1999; 274:25608-12. [PMID: 10464296]

18. Warren JF, Aldave AJ, Srinivasan M, Thonar EJ, Kumar AB, Cevallos V, Whitcher JP, Margolis TP. Novel mutations in the CHST6 gene associated with macular corneal dystrophy in southern India. Arch Ophthalmol 2003; 121:1608-12. [PMID: 14609920]

19. Maquat LE. Nonsense-mediated mRNA decay: splicing, translation and mRNA dynamics. Nat Rev Mol Cell Biol 2004; 5:89-99. [PMID: 15040442]

20. Nagy E, Maquat LE. A rule for termination-codon position within intron-containing genes: when nonsense affects RNA abundance. Trends Biochem Sci 1998; 23:198-9. [PMID: 9644970]

21. Maquat LE. Nonsense-mediated mRNA decay in mammals. J Cell Sci 2005; 118:1773-6. [PMID: 15860725]

22. Young RD, Akama TO, Liskova P, Ebenezer ND, Allan B, Kerr B, Caterson B, Fukuda MN, Quantock AJ. Differential immunogold localisation of sulphated and unsulphated keratan sulphate proteoglycans in normal and macular dystrophy cornea using sulphation motif-specific antibodies. Histochem Cell Biol 2007; 127:115-20. [PMID: 16944190]

23. Munier FL, Fruch BE, Othenin-Girard P, Uffer S, Cousin P, Wang MX, Heon E, Black GC, Blasi MA, Balestrazzi E, Lorenz B, Escoto R, Barraquer R, Hoeltzenbein M, Gloor B, Fossarello M, Singh AD, Arsenijevic Y, Zografos L, Schorderet DF. BIGH3 mutation spectrum in corneal dystrophies. Invest Ophthalmol Vis Sci 2002; 43:949-95. [PMID: 11923233]