

Molecular analysis of the *CHST6* gene in Korean patients with macular corneal dystrophy: Identification of three novel mutations

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Purpose: To identify the underlying genetic defect in Korean patients with macular corneal dystrophy (MCD).

Methods: Genomic DNA was isolated from peripheral blood leukocytes of seven patients from six unrelated families with MCD (three men and four women). Polymerase chain reaction was performed for coding regions of the carbohydrate sulfotransferase (*CHST6*), gene followed by bidirectional sequencing. Targeted mutational analysis (exons 4, 11–12, 14) of the transforming growth factor, beta-induced (*TGFBI*) gene was performed for all patients.

Results: All seven patients were found to have compound heterozygous mutations in the *CHST6* gene. In addition to six previously reported mutations, c.95C>A (p.Ser32*), c.521A>G (p.Lys174Arg), c.557C>G (p.Pro186Arg), c.613C>T (p.Arg205Trp), c.820G>A (p.Glu274Lys), and c.1072T>C (p.Tyr358His), three novel mutations were identified in this study, including two missense mutations, c.353C>T (p.Ser118Phe) and c.922C>T (p.His308Tyr), and one frameshift mutation, c.786delC (p.L264Cfs*117). Among the three novel mutations, only the c.353C>T mutation had been reported in the Exon Aggregation Consortium database at an extremely low frequency of 0.00005072. In addition, these three novel mutations were absent from controls in 1,000 genomes, dbSNP, and the TIARA genome database, which is a Korean personal genome database. The most frequent mutation was c.613C>T (p.Arg205Trp), revealed in four unrelated Korean families, which has not previously been reported in other populations. No mutations were detected in the *TGFBI* gene.

Discussion: This is the first report on genetic analysis of Korean MCD patients. Three novel and six previously reported disease-causing *CHST6* mutations were identified, which expands the mutational spectrum of MCD.

Macular corneal dystrophy (MCD, OMIM 217800) is an autosomal recessive corneal disorder characterized by bilateral, progressive, diffuse stromal haze; irregular corneal whitish opacity; and central corneal thinning [1,2]. Over time, the non-transparent areas progressively merge as the entire corneal stroma gradually becomes cloudy, leading to severe visual impairment between 10 and 30 years of age [1,2]. As a far-famed corneal disorder, MCD has been reported in a wide range of prevalence in many different countries [3–12]. In particular, MCD accounts for one-third of every penetrating keratoplasty in Iceland [8]. MCD is also a common type of corneal dystrophy leading to penetrating keratoplasty in Japan, India, and Saudi Arabia [9,10,12].

Histologically, MCD is characterized by the accumulation of glycosaminoglycans (GAGs) between the stromal lamellae, underneath the epithelium, and within keratocytes and endothelial cells [13,14]. Explants from MCD-affected corneas have been reported to synthesize low-sulfated keratan sulfate (KS), suggesting that the sulfate groups in

KS may play critical roles in maintaining corneal transparency [15–17]. N-acetylglucosamine-6-O-sulfotransferase (GlcNAc6ST) activity in the extracts from MCD-affected corneas was much lower than in those in corneas with keratoconus and in normal control corneas [15]. This decrease in GlcNAc6ST activity in corneas with MCD is associated with the occurrence of low- or non-sulfated KS, and this has been proposed as an underlying mechanism for the observed corneal opacity [15,18,19]. Since the carbohydrate sulfotransferase 6 (*CHST6*) gene, located on chromosome 16q22, which encodes the corneal (C)-GlcNAc6ST comprising 395 amino acids, was first identified in 2000 as a candidate gene for MCD in the Japanese population [6], numerous mutations have been reported in MCD patients with different ethnic backgrounds [2–7,9,10]. The *CHST6* gene contains four exons; the coding region is contained only within exon 3 [20]. C-GlcNAc6ST catalyzes the transfer of a sulfate group to N-acetylglucosamine in KS, a common component of corneal proteoglycans [20].

Jee et al. reported that MCD accounts for 12.9% of corneal dystrophy encountered in Koreans, following granular dystrophy (29.2%) and Fuchs' endothelial dystrophy (23.6%) [21]. However, little is known about the molecular characterization of MCD in Koreans. Recently, only one

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homozygous missense mutation (c.613C>T, p.Arg205Trp) has been reported in a 59-year-old Korean woman with MCD [22]. Here, we analyzed the *CHST6* gene in seven Korean MCD patients and identified three novel and six previously reported disease-causing mutations.

METHODS

This study was performed according to the principles of the Declaration of Helsinki, after approval by the Institutional Review Board of Seoul St. Mary's Hospital, College of Medicine, Catholic University of Korea, Seoul, Korea (KC14RISI0419). Informed consent was obtained from all patients.

Patient selection and clinical evaluation: The medical records of seven patients from six unrelated families with MCD in Seoul St. Mary's Hospital were investigated. Seven patients were diagnosed as having MCD based on typical clinical features after evaluation by a corneal specialist (M.S.K.) and referred for molecular confirmation. Thorough ocular examinations were performed, including best-corrected visual acuity (BCVA), intraocular pressure (IOP), refractive measurement, central corneal thickness, and slit lamp biomicroscopy of the anterior segment and fundus. The age, gender, visual acuity, family history, and previous ocular records of the patients were reviewed. The obtained pedigrees were consistent with an autosomal recessive inheritance pattern, and there was no known consanguinity in all six families.

Mutational analysis: After receiving informed consent, blood samples were collected from all patients for DNA extraction and *CHST6* gene analysis. In addition, targeted mutational analysis (exons, 4, 11–12, 14) of the transforming growth factor, beta-induced (*TGFBI*) gene was performed for all patients. Genomic DNA was extracted from the peripheral blood leukocytes using the QIAmp DNA Mini Kit (Qiagen, Hamburg, Germany). Polymerase chain reaction (PCR) was performed using previously published primer sets for *CHST6* [6]. All the coding exons and the flanking intron/exon boundaries of *CHST6* were amplified. The PCR amplicons were bidirectionally sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The chromatograms were analyzed with the Sequencher software version 5.0 (Gene Codes, Ann Arbor, MI). Mutations were confirmed by sequencing two or more independent PCR reactions. When a novel missense mutation was identified, *in silico* analysis was performed using [Polyphen-2](#) and [SIFT](#). In cases of missense mutations, conservation of the involved amino acids among several sulfotransferases of human and mouse origin was investigated using

[Clustal Omega](#). DNA from available family members was sequenced to determine whether the mutation cosegregated with the phenotype within the pedigree. All mutations were described according to the Human Genome Variation Society nomenclature, and GenBank accession numbers [NM_021615.4](#) and [NM_000358.2](#) were used for *CHST6* and *TGFBI* alignment, respectively.

RESULTS

Molecular analysis of the CHST6 gene: The patients' molecular findings in the present study are summarized in Table 1. Nine different mutations within the coding region of the *CHST6* gene were identified in seven patients from the six unrelated families (Table 1). In addition to six previously reported mutations (c.95C>A, c.521A>G, c.557C>G, c.613C>T, c.820G>A, and c.1072T>C), three novel mutations, c.353C>T, c.786delC, and c.922C>T, were identified. There were seven missense mutations, one nonsense mutation, and one frameshift mutation. The c.613C>T (Arg205Trp) mutation was the most frequently detected mutation in our study. No mutations were detected in the *TGFBI* gene.

Figure 1 shows the nucleotide sequences in the three novel mutations, c.353C>T, c.786delC, and c.922C>T. The c.922C>T mutation detected in a 41-year-old female (patient 2) was a missense mutation resulting in a histidine-to-tyrosine substitution at codon 308. Another missense c.353C>T mutation, detected in one family (patients 5–1 and 5–2), led to a serine-to-phenylalanine substitution at codon 118. Patient 6 was a 15-year-old female and had a novel mutation (c.786delC, p.L264Cfs*117), which resulted in premature termination due to a frameshift. The clinically unaffected father and brother carried either one of the two mutations, which implied MCD as a recessive inherited disorder. Among the three novel mutations, only the c.353C>T mutation had been reported in the Exon Aggregation Consortium (ExAC) database at an extremely low frequency of 0.00005072. In addition, these three novel mutations were absent from controls in 1,000 genomes, dbSNP, and the [TIARA](#) genome database, a Korean personal genome database.

All seven missense mutations identified in Korean MCD patients, including two novel mutations, were predicted to have a pathogenic effect by Polyphen-2 and SIFT software, which produced results designated as “probably damaging” and “not tolerated,” respectively (Table 1). In addition, multiple protein sequence alignment analyses between several sulfotransferases of human and mouse origin demonstrated that the majority of the amino acids substituted in the missense mutations identified in Korean MCD patients were highly conserved residues, while the amino acids substituted

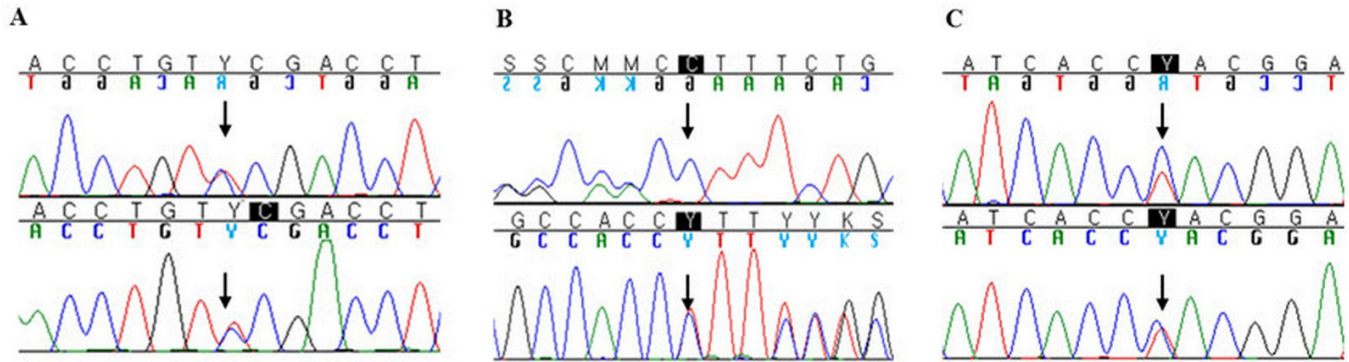


Figure 1. Sequencing chromatograms of the three novel *CHST6* mutations identified in this study. **A:** c.353C>T (p.Ser118Phe); **B:** c.786delC (p.L264Cfs*117); **C:** c.922C>T (p.His308Tyr).

in the newly identified p.Ser118Phe and p.His308Tyr mutations showed a lesser degree of conservation (Table 2).

Ocular phenotypes associated with mutations: Table 3 shows the ocular phenotypes of seven patients from six unrelated families tested (three males and four females). All seven patients were found to have compound heterozygous mutations in the *CHST6* gene. Slit lamp examination of all affected patients revealed bilateral stromal corneal cloudiness. Multiple irregular-shaped, gray-white, poorly delineated opacities were found involving the center of the cornea (Figure 2A–C) and extending to the peripheral limbus in some patients (Figure 2D). The two chief complaints were

progressive visual disturbance and photophobia. Participants from a wide range of age at detection revealed various visual acuity. Central corneal thickness was decreased in all 12 eyes that were examined at initial visit. The mean values of central corneal thickness measured by ultrasound pachymetry were 450.3±32.8 μm and 446.0±31.2 μm in the right and left eyes, respectively.

Five eyes from three patients received therapeutic penetrating keratoplasty. In patient 1, carrying the c.[521A>G];[613C>T] mutations, penetrating keratoplasty was performed in both eyes at the age of 38 in another hospital. This patient is currently waiting for a repeat penetrating

TABLE 1. MUTATION ALLELES OF THE *CHST6* GENE IDENTIFIED IN 7 KOREAN PATIENTS WITH MCD.

Gene	Nucleotide change	Amino acid change	Mutation effect	Number of families	Number of individuals	Polyphen	SIFT	Reference
	c.95C>A	p.Ser32*	Nonsense mutation	1	1	-	-	Chinese ⁷
	c.353C>T	p.Ser118Phe	Missense mutation	1	2	Probably damaging	Not tolerated	Novel
	c.521A>G	p.Lys174Arg	Missense mutation	1	1	Probably damaging	Not tolerated	Japanese ⁶
	c.557C>G	p.Pro186Arg	Missense mutation	1	1	Probably damaging	Not tolerated	African American ⁵
CHST6	c.613C>T	p.Arg205Trp	Missense mutation	4	4	Probably damaging	Not tolerated	Korean ²⁴
	c.786delC	p.L264Cfs*117	Frameshift mutation	1	1	-	-	Novel
	c.820G>A	p.Glu274Lys	Missense mutation	1	1	Probably damaging	Not tolerated	Japanese ⁶ , Indian ⁹
	c.922C>T	p.His308Tyr	Missense mutation	1	1	Probably damaging	Not tolerated	Novel
	c.1072T>C	p.Tyr358His	Missense mutation	1	2	Probably damaging	Not tolerated	British ⁴ , Chinese ³

TABLE 2. MULTIPLE SEQUENCE ALIGNMENT OF SULFOTRANSFERASES SHOWING CONSERVATION OF AMINO ACID RESIDUES MUTATED IN KOREAN PATIENTS WITH MCD.

Sulfotransferase	S118F	K174R	P186R	R205W	E274K	H308Y	Y358H
Human carboxy- drate sulfotrans- ferase 6 precursor (GI:11055976)	L S D L	L K E V	L Y P L	D P R A	R F E D	T H G S	L G Y R
Human carbohydrate sulfotransferase 5 (GI:21362052)	L S A F	L K E V	L Y P L	D P R A	R F E D	T H G S	L G Y R
Human N-acetyl- glucosamine 6-O-sulfotrans- ferase (GI:4927114)	Q S S L	L K E V	L Y P L	D P R A	R Y E D	T R G K	L G Y R
Human L-selectin ligand sulfotransferase (GI:13897504)	Q S S L	L K E V	L Y P L	D P R A	R Y E D	T R G K	L G Y R
Human chon- droitin 6-sulfo- transferase-2 (GI:930944)	T A A L	I K D V	L V P L	D P R A	R Y E D	T R G A	L A Y P
Human keratan sulfate Gal- 6-sulfotransferase (GI:2887403)	T D R I	I K T V	L R A L	D P R G	R Y E D	T R G D	L G Y K
Mouse L-selectin ligand sulfo- transferase (GI:5596406)	Q S S L	L K E V	L Y P L	D P R A	R Y E D	T R G K	L G Y L
Mouse carboxy- drate sulfotrans- ferase 5 precursor (GI:9910284)	I S D L	L K E V	L Y P L	D P R A	R Y E D	T H G S	L G Y R
Mouse chondroitin 6-sulfotransferase (GI:3253091)	T Q F L	L K A V	L Q P L	D P R A	R Y E D	T Q A T	F G Y K

TABLE 3. CLINICAL FINDINGS OF KOREAN MCD PATIENTS CARRYING CHST16 MUTATIONS.

Case No.	Age/Gender	Mutation	Age at detection (years)	Family History	VA (Rt / Lt)	Initial Pachy (Rt / Lt)	ECD (Rt / Lt)	Therapeutic procedures	Final VA (Rt / Lt)
1	50/Male	c.521A>G / c.613C>T	15	+	NA	NA	NA	Keratoplasty, Both eyes	0.4 / 0.25
2	41/Female	c.820G>A / c.922C>T	41	-	1.0 / 1.0	441 / 432	1377 / 2237	-	NA
3	44/Male	c.557 C>G / c.613 C>T	42	-	0.4 / 0.32	492 / 498	-	-	NA
4	35/Male	c.95C>A / c.613 C>T	15	-	0.16 / 0.125	482 / 460	-	Keratoplasty, Right eye	0.5 / 0.8
5-1	15/Female	c.353 C>T / c.1072 T>C	11	+	0.8 / 0.8	407 / 405	-	-	0.4 / 0.4
5-2	19/Female	c.353 C>T / c.1072 T>C	15	+	0.63 / 0.32	425 / 445	-	-	0.32 / 0.2
6	15/Female	c.613C>T / c.786delC	10	-	0.4 / 0.4	455 / 436	-	Keratoplasty, Left eye	0.1 / 0.5

keratoplasty in the left eye due to the recurrence of corneal opacity. Patient 5-1, carrying the c.[95C>A];[613C>T] mutations, received penetrating keratoplasty in both eyes. Patient 6, carrying the c.[613C>T];[786delC] mutations, received penetrating keratoplasty only in the left eye. Basophilic deposits between the stromal lamellae and within keratocytes and endothelial cells were positive to Alcian blue and periodic acid-Schiff stains, but negative to Congo red and Masson's trichrome stain, which are consistent with the accumulation of GAGs (Figure 3). The BCVA improved after surgery in these three eyes without any postoperative complications. The clarity of grafted corneas was maintained throughout the follow-up period, which ranged from 14 to 48 months.

DISCUSSION

In this report, we describe *CHST6* mutations in Korean MCD patients. Three novel mutations were identified in this study, including two missense mutations, c.353C>T (p.Ser118Phe) and c.922C>T (p.His308Tyr), and one frameshift mutation c.786delC (p.L264Cfs*117). The most frequent mutation was the c.613C>T (p.Arg205Trp) mutation revealed in four unrelated Korean families, which has not previously been reported in other populations.

CHSTs are a family of related enzymes that catalyze the sulfation of specific carbohydrates [20]. The N-acetylglucosamine-6-O-sulfotransferase (GlcNAc6ST) enzyme encoded by the *CHST6* gene transfers sulfate to N-acetylglucosamine

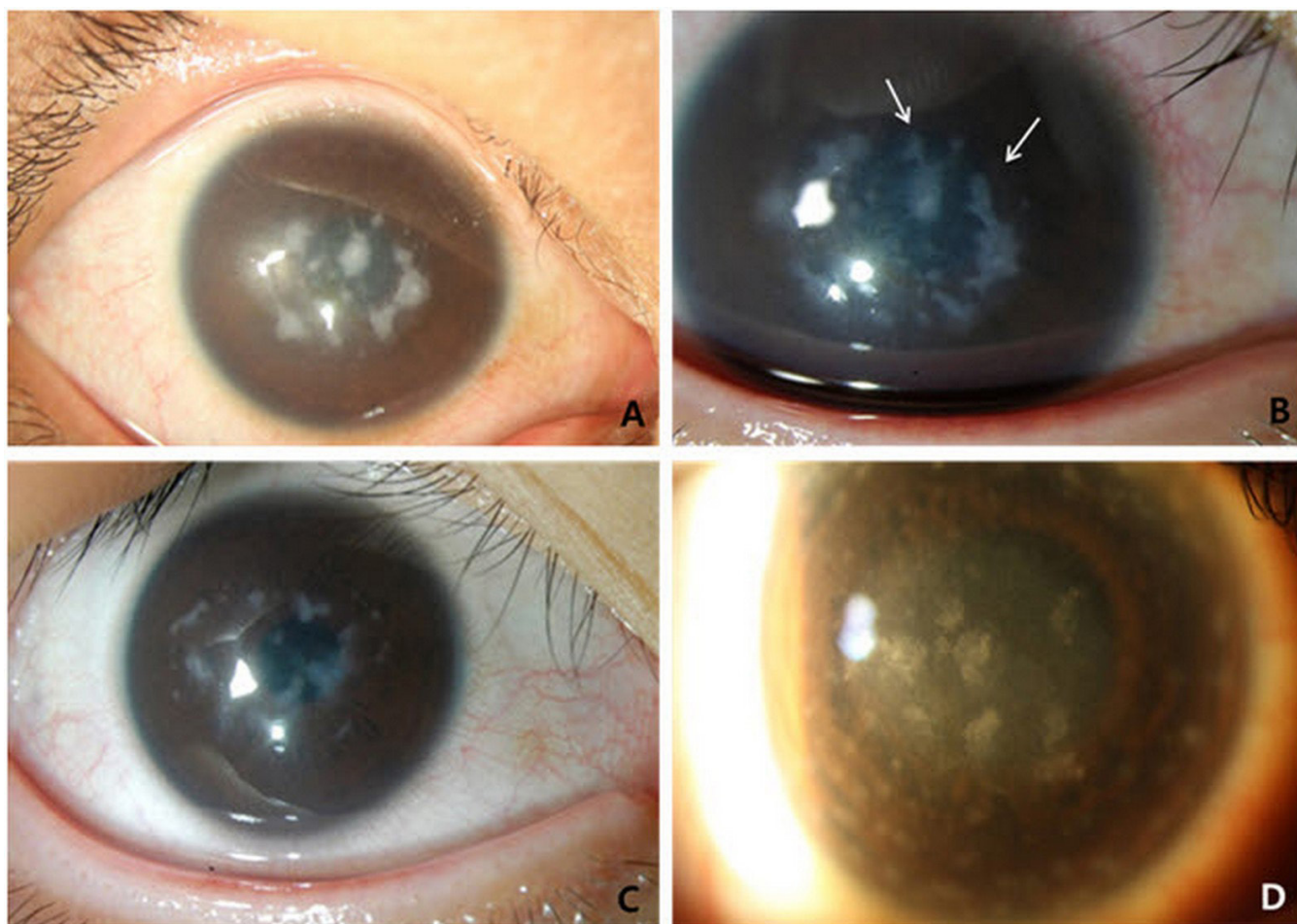


Figure 2. Slit lamp photography of patients with MCD. **A, B:** A 15-year-old female carrying p.Arg205Trp/p.L264Cfs*117 in the *CHST6* gene (patient 6) revealed diffuse ground-glass-like stromal haze and multiple gray-white opacities with irregular borders. Arrows (**B**) indicate newly developed opacities three years after initial diagnosis (**A**). The patient complained of severe photophobia, which required penetrating keratoplasty in her left eye. **C:** A 19-year-old female carrying p.Ser118Phe/p.Tyr358His in the *CHST6* gene (patient 5-2) shows coalescence of individual stromal opacities. The absence of clear areas between corneal opacities is a characteristic finding, unlike granular dystrophy. **D:** A 44-year-old male carrying p.Pro186Arg/p.Arg205Trp in the *CHST6* gene (patient 3) has multiple, irregular, gray-white opacities extending to the limbus in both eyes.

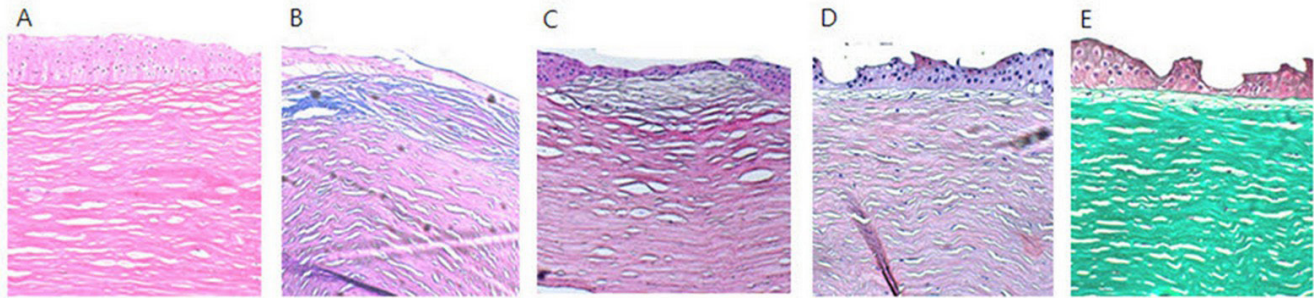


Figure 3. Histopathological findings in a 15-year-old female patient (patient 6). **A:** Hematoxylin and eosin stain. **B:** Alcian blue stain. **C:** Periodic acid-Schiff stain. **D:** Congo red stain. **E:** Masson's trichrome stain. Basophilic deposits between the stromal lamellae and within keratocytes and endothelial cells were positive for Alcian blue and periodic acid-Schiff stain, but negative for Congo red and Masson's trichrome stain, which are consistent with the accumulation of glycosaminoglycans.

on KS GAG chains [17,20]. Abnormalities in the synthesis of this enzyme have been identified as a cause of MCD [6,15,18,19]. Unsulfated poly-lactosamine chains are likely to be less water soluble than the fully sulfated KS because of the decrease in GAG chain polarity in the absence of sulfate esters [20]. The degradation of unsulfated KS proteoglycans may also be inhibited by its insolubility [20]. The intracellular and extracellular accumulation of poorly or non-sulfated KS in the corneal stroma, corneal endothelium, and Descemet membrane resulted in corneal opacity in patients with MCD [1,2,13,14].

The present study revealed the mutational heterogeneity of the *CHST6* gene in Korean MCD patients (Table 1 and Table 3). It is noteworthy that the most frequent abnormality found in this study was the c.613C>T mutation, leading to an arginine-to-tryptophan substitution at codon 205. Recently, this mutation was reported in a 59-year-old Korean woman with MCD, but was not previously reported in other populations [22]. The amino acid substitutions at codon 205 have been previously identified in Japanese (p.Arg205Leu from c.614G>T) and Indian (p.Arg205Gln from c.614G>A) MCD patients, but they are different from the mutation detected in our study [9,23]. The identified c.613C>T (p.Arg205Trp) mutation was located in the RX7S sequence in the 3'-phosphate binding domain, which was one of the two binding sites for the high-energy sulfate donor in the C-GlcNAc6S protein [23-25]. The sequence between the two binding sites is thought to contribute to a binding pocket that interacts with an acceptor to bring it into apposition with the sulfate donor [24,25]. It is known that mutations of these binding motif sequences abolish HKN-1 sulfotransferase activity [23].

Among seven missense mutations of the *CHST6* gene, the c.521A>G (p.Lys174Arg), c.557C>G (p.Pro186Arg), c.820G>A (p.Glu274Lys), and c.1072T>C (p.Tyr358His)

mutations have been previously identified to be responsible for MCD in Japanese [6], African-American [5], Indian [9], British [4], and Chinese [3] patients (Table 1). The c.353C>T (p.Ser118Phe) and c.922C>T (p.His308Tyr) mutations were detected for the first time in the present study. These two novel missense mutations were predicted to have a pathogenic effect by PolyPhen-2 and SIFT software, which produced results designated as "probably damaging" and "not tolerated," respectively. We also searched the genome databases, including ExAC, 1,000 genomes, dbSNP, and an ethnic-specific Korean personal genome database, TIARA. Only the p.Ser118Phe mutation had been reported in ExAC, though at an extremely low frequency of 0.00005072. A mutation of this low frequency, especially in a recessive disorder, could be considered to have moderate evidence of pathogenicity, according to the American College of Medical Genetics and Genomics (ACMG) standards and guidelines for the interpretation of sequence variants. Additionally, the multiple protein sequence alignment analysis between several sulfotransferases of human and mouse origin demonstrated that the novel mutations substitute relatively well-conserved amino acid residues, which can also be regarded as a supporting evidence of pathogenicity of these novel missense mutations (Table 2).

Generally, nonsense or frameshift mutations in the *CHST6* gene occur less frequently in MCD [1,2,26]. The nonsense mutation of the *CHST6* gene was first identified in three patients in one French family, who experienced rapid visual deterioration at an early age and all required keratoplasty in the second decade of life [27]. A genetic analysis of German patients with MCD demonstrated that frameshift mutations in the *CHST6* gene have a tendency to be associated with more severe clinical phenotypes with much deeper corneal deposits [28]. Gruenauer-Kloevekorn et al. suggested that the application of therapeutical options such as primary

phototherapeutic keratectomy or primary penetrating keratoplasty could be based on the molecular genetic findings in each patient [28]. However, Sultana et al. reported that there were no consistent differences in phenotype between patients with the various amino acid substitutions and truncating mutations [29]. In our study, two different kinds of nonsense or frameshift mutations were detected in two families (patients 4 and 6).

All of the study participants carried compound heterozygous mutations in the *CHST6* gene, which may be related to the non-consanguinity of the study participants. They had bilateral corneal cloudiness and corneal thinning, which are characteristic features of MCD [1,2]. The reduced corneal thickness is expected to result from the dysregulation of KS proteoglycan synthesis or catabolism [30]. Although different mutations of the *CHST6* gene were confirmed in each patient, we did not notice any phenotypic differences according to the identified genotype (Table 3). Further studies with larger numbers of patients are required to delineate the genotype-phenotype correlation of MCD.

This study had some limitations. Relatively small numbers of patients were analyzed, and the parents and/or siblings of patients were not thoroughly examined. The serum levels of KS and the immunohistochemical reaction to the corneal tissue were not sufficiently evaluated to characterize the immunophenotype of MCD. However, recently there has been increased awareness of and interest in genetic diseases in Korea. Genetic analysis of Koreans with MCD could provide valuable information for correct diagnosis, carrier detection, and genetic counseling in patients and their families.

In conclusion, this is the first report of a genetic analysis of Korean MCD patients. Three novel and six previously reported disease-causing *CHST6* mutations were identified, which expands the mutational spectrum of MCD.

ACKNOWLEDGMENTS

The authors thank all the patients for participating in this study. This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2013R1A1A2006801). The authors declare no conflict of interest. Man Soo Kim (mskim@catholic.ac.kr) and Myungshin Kim (microkim@catholic.ac.kr) contributed equally.

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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 26 October 2015. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.