Genetic analysis of CHST6 and TGFBI in Turkish patients with corneal dystrophies: Five novel variations in CHST6

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Purpose: To identify pathogenic variations in carbohydrate sulfotransferase 6 (CHST6) and transforming growth factor, beta-induced (TGFBI) genes in Turkish patients with corneal dystrophy (CD).

Methods: In this study, patients with macular corneal dystrophy (MCD; n = 18), granular corneal dystrophy type 1 (GCD1; n = 12), and lattice corneal dystrophy type 1 (LCD1; n = 4), as well as 50 healthy controls, were subjected to clinical and genetic examinations. The level of antigenic keratan sulfate (AgKS) in the serum samples of patients with MCD was determined with enzyme-linked immunosorbent assay (ELISA) to immunophenotypically subtype the patients as MCD type I and MCD type II. DNA was isolated from venous blood samples from the patients and controls. Variations were analyzed with DNA sequencing in the coding region of CHST6 in patients with MCD and exons 4 and 12 in TGFBI in patients with LCD1 and GCD1. Clinical characteristics and the detected variations were evaluated to determine any existing genotype–phenotype correlations.

Results: The previously reported R555W mutation in TGFBI was detected in 12 patients with GCD1, and the R124C mutation in TGFBI was detected in four patients with LCD1. Serum AgKS levels indicated that 12 patients with MCD were in subgroup I, and five patients with MCD were in subgroup II. No genetic variation was detected in the coding region of CHST6 for three patients with MCD type II. In other patients with MCD, three previously reported missense variations (c.1A>T, c.738C>G, and c.631 C>T), three novel missense variations (c.164 T>C, c.526 G>A, c. 610 C>T), and two novel frameshift variations (c.894_895 insG and c. 462_463 delGC) were detected. These variations did not exist in the control chromosomes, 1000 Genomes, and dbSNP.

Conclusions: This is the first molecular analysis of TGFBI and CHST6 in Turkish patients with different types of CD. We detected previously reported, well-known hot spot mutations in TGFBI in the patients with GCD1 and LCD1. Eight likely pathogenic variations in CHST6, five of them novel, were reported in patients with MCD, which enlarges the mutational spectrum of MCD.

Corneal dystrophies (CDs) are a group of hereditary disorders that affect one or several layers of the cornea and are usually bilateral, symmetric, and progressive. Affected patients generally suffer from recurrent erosions and/or progressive visual deterioration due to the increasing corneal opacity. Keratoplasty is still the most common treatment method when CD leads to important visual impairment [1]. Although CDs are relatively common in Turkey due to the high rate of consanguinity [2] and are among the most common indications of keratoplasty in Turkey [3], until now no comprehensive study has investigated genotype–phenotype properties of CDs.

During the past decade, important advances have been made in determining the genetic basis of CDs. According to the updated classification based on this increasing knowledge, CDs are divided into four groups: epithelial and subepithelial dystrophies, epithelial-stromal transforming growth factor, beta-induced (TGFBI); Gene ID: 7045, OMIM 601692) dystrophies, stromal dystrophies, and endothelial dystrophies [4]. The causative genes have been mapped, and the specific mutations are known for several types of CDs [5,6].

The carbohydrate sulfotransferase-6 (CHST6; Gene ID: 4166, OMIM 605294) gene, on chromosome 16q22, encodes enzyme N-acetylgalcosamine-6-sulfotransferase (GlcNAc6ST) [5,7]. Mutations in CHST6 cause the deposition of low or unsulfated keratan sulfate (KS) in the corneal stroma [8] and result in autosomal recessively inherited macular corneal dystrophy (MCD, OMIM 217800). MCD causes bilateral, progressive corneal clouding and irregular corneal opacities [5]. MCD is the most common stromal CD in Iceland where the gene pool is small [9]. MCD is also common in countries such as Saudi Arabia and Turkey due to the high rate of consanguinity [2,10]. There are three
After informed consent was obtained, patient selection and clinical evaluation: 

Determination of serum AgKS: The serum AgKS levels were determined in 18 patients with MCD and 50 controls with enzyme-linked immunosorbent assay (ELISA) using the Human Keratan Sulfate (KS) ELISA Kit (East Bio Pharm, Hangzhou, China) as previously described [19]. Serum AgKS levels that were 10 ng/ml or below were interpreted as MCD types I/IA and levels of 100 ng/ml or above as normal or MCD type II [20].

Mutational analysis: After informed consent was obtained, peripheral blood samples (5 ml) were obtained from each subject for DNA isolation and molecular analysis. DNA was extracted using the spin column–based nucleic acid purification method (MN Macherey-Nagel, Düren, Germany). The exon–intron boundaries in the neighbourhood of the coding region and the coding region of CHST6, as well as exons 4 and 12 of TGFBI, were amplified with PCR using the newly designed primers listed in Table 1. Each PCR was performed in a 50 μl reaction mixture containing genomic DNA (100 ng), primers (20 pmol/μl each), MgCl2 (25 mM), deoxynucleoside triphosphate (dNTPs; 2.5 mM), PCR buffer (Fermentas, Burlington, Canada), and Taq polymerase (0.5 IU/μl; Fermentas). The PCR protocol was as follows: initial denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s; and final extension at 72 °C for 3 min. PCR products were purified using Wizard® SV Gel and PCR Clean-Up System kits (Promega, Madison, WI). Bidirectional sequencing was performed by using BigDye Terminator Mix, version 3.1 (Applied Biosystems Inc., Foster City, CA) and analyzed on an ABI-3100 genetic analyzer (Applied Biosystems). The chromatograms were analyzed with the ChromasPro software version 1.7.7 (Technelysium, South Brisbane, Australia). The pathogenicity of novel missense variations was evaluated with the SIFT and PolyPhen-2 software programs. Additionally, conservation of the involved amino acids among several sulfotransferases of human and other species was evaluated with the Chou–Fasman protein secondary structure prediction method (Chou, P.Y. and Fasman, G.D. Accurate Prediction of Protein Structure and the Secondary Structure of Proteins, Nature 272, 665–671, 1978) using the WebLogo program (Crooks, G.E., Hon, G., Chandonia, J.-M., and Brenner, S.E. WebLogo: A sequence logo generator, Bioinformatics 24, 746–748, 2008).
mouse origins was evaluated using Clustal Omega. Human Genome Variation Society (HGVS) nomenclature was used for the description of all detected variations.

RESULTS
Clinical findings: Thirty-four Turkish patients with CD were included in the study. Patients were unrelated as we questioned their family history in three generations. Eighteen of the patients were clinically diagnosed with MCD (Figure 1A), 12 with GCD1 (Figure 1B), and four with LCD1 (Figure 1C). Demographic characteristics, family history, known consanguinity, clinical findings, and previous therapeutic procedures, as well as the genetic analysis results for TGFBI and CHST6, are summarized in Table 2 and Table 3, respectively. In all patients, the eyes were affected bilaterally. The CCT of seven patients with MCD without previous surgical procedures was measured with ultrasonic pachymetry, and the mean values were 456.4±23.6 µm (435–497 µm) in the right eye and 458.4±31.2 µm (430–520 µm) in the left eye.

Sulfated KS level in patients’ serum: The AgKS level was detected in 17 patients with MCD and 50 control patients. The mean concentration of AgKS was 259.3±66.4 ng/ml (120–384 ng/ml) in the control patients. Twelve patients were diagnosed with MCD type I because the concentration of sulfated KS in their serum was less than 10 ng/ml (Table 3). Five patients were diagnosed with MCD type 2 because the mean AgKS level in their serum was 204±72.7 ng/ml (125–305 ng/ml).

Molecular findings: Exons 4 and 12 in TGFBI were analyzed in 12 patients with GCD1 and four patients with LCD1. In all patients with GCD1, the c.1663 C>T (p. R555W) variation was detected in TGFBI exon 12 (Figure 2A). This variation was heterozygous in 11 patients and homozygous in one patient (Figure 2B). In two patients with GCD1, the c.1620 C>T (p. F540F) variation was detected heterozygously. This synonymous variation was included in the dbSNP database as a polymorphism. In all patients with LCD1, the c.370 T>C (p. R124C) variation was detected heterozygously in exon 4 in TGFBI (Figure 2C). These variations were not detected in 100 control chromosomes.

The coding region and the flanking exon–intron boundaries in exon 4 of CHST6 were analyzed in 18 MCD probands. Eight variations were detected in 18 patients: three previously reported missense variations (c. 1A>T, p. M1L; c.738C>G, p.C246W; and c.631 C>T, p. R211W), three novel missense

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**Table 1. Primer list of CHST6 and TGFBI genes.**

| Gene | Primers (5’-3’) | Tm(°C) | PCR product |
|------|----------------|--------|-------------|
| **CHST6** | | | |
| Primer 1 | F: C T C G G G T C T G G T G G T A G A A T C T T | 60.9 | 658 bp |
| | R: TTGAAAGACGCACTCTCCTT | 61.1 |  |
| Primer 2 | F: C T C T T C A G T G G C C G T G A G | 62.5 | 626 bp |
| | R: TTAGCCGATTCTGGAGCAA | 62.6 |  |
| Primer 3 | F: G C A G A A A T C C G T G C T C T A C | 61.9 | 505 bp |
| | R: AGAGAAAGAAACGTCAGTCCTT | 60.4 |  |
| **TGFBI** | | | |
| Exon 4 | F: T C G T C C T C T C T C A C C T G T A G A | 59.0 | 548 bp |
| | R: AACATGTTTCCAGCCCTCGT | 59.3 |  |
| Exon 12 | F: A A C C A A G G T G T G T G C A T T C C | 59.0 | 415 bp |
| | R: TTTAGTCCGGCCACCTCCTT | 59.0 |  |

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Figure 1. AS photographs of patients. A: Macular corneal dystrophy patient (patient 9). B: Patient with granular corneal dystrophy type 1 (patient 5). C: Patient with lattice corneal dystrophy type 1 (patient 2).
| Patient no | Age/Sex | Diagnosis | Family history | Consanguinity | Age at diagnosis | BCVA (OD/OS) | Treatment | Variation |
|------------|---------|-----------|----------------|---------------|-----------------|-------------|-----------|-----------|
| 1          | 28/F    | LCD1      | +              | -             | 18              | 0.8/0.7     | -         | R124C/-   |
| 2          | 23/F    | LCD1      | -              | -             | 18              | 0.2/0.4     | -         | R124C/-   |
| 3          | 45/M    | LCD1      | +              | -             | 24              | 0.05/0.4    | OD ALK    | R124C/-   |
| 4          | 40/M    | LCD1      | -              | -             | 20              | 0.4/0.5     | OD-OS PTK | R124C/-   |
| 5          | 36/F    | GCD1      | +              | +             | 15              | 0.05/0.05   | OD PPK    | R555W/-   |
| 6          | 35/M    | GCD1      | +              | +             | 20              | 0.05/0.3    | -         | R555W/-   |
| 7          | 18/F    | GCD1      | +              | -             | 10              | 0.9/0.8     | -         | R555W/-   |
| 8          | 53/M    | GCD1      | +              | -             | 30              | 0.4/0.4     | -         | R555W/-   |
| 9          | 27/M    | GCD1      | +              | -             | 20              | 0.05/0.05   | OD-OS ALK | R555W/R555W |
| 10         | 42/F    | GCD1      | +              | +             | 18              | 0.3/0.4     | -         | R555W/-   |
| 11         | 26/M    | GCD1      | -              | -             | 18              | 0.5/0.3     | OS PTK    | R555W/-   |
| 12         | 54/F    | GCD1      | +              | -             | 30              | 0.6/0.5     | -         | R555W/-   |
| 13         | 40/M    | GCD1      | +              | -             | 20              | 0.1/0.2     | OD ALK    | R555W/-   |
| 14         | 43/M    | GCD1      | +              | +             | 15              | 0.05/0.05   | OD-OS PPK | R555W/-   |
| 15         | 35/F    | GCD1      | -              | -             | 20              | 0.4/0.3     | -         | R555W/-   |
| 16         | 33/F    | GCD1      | +              | -             | 18              | 0.8/0.7     | -         | R555W/-   |

OD: Right eye; OS: Left eye; ALK: Anterior lamellar keratoplasty; PPK: Partial penetrating keratoplasty; PTK: Phototherapeutic keratectomy, NA: Nonavailable
### Table 3. Clinical findings of Turkish MCD patients carrying CHST6 gene variations.

| Patient no | Age/Sex | Family history | Consanguinity | Age at diagnosis | VA(OD/OS) | CCT(OD/OS) | Treatment | KS level (ng/ml) | Immunotype | Variation |
|------------|---------|----------------|---------------|-----------------|-----------|------------|-----------|------------------|------------|-----------|
| 1          | 32/M    | +              | +             | 18              | 0.05/0.7  | NA         | OD-OS PPK | 0               | I          | C246W     |
| 2          | 42/M    | +              | +             | 15              | 0.05/0.05 | NA         | OD-OS PPK | 125             | II         | M1L/-     |
| 3          | 27/M    | +              | -             | 15              | 0.4/0.3   | NA         | OD-OS PPK | 0               | I          | P204S     |
| 4          | 33/M    | -              | +             | 10              | 0.05/0.05 | NA         | OD-OS PPK | 0               | I          | Q298fs    |
| 5          | 21/F    | -              | -             | 13              | 0.6/0.5   | 482/475    | -         | 146             | II         | -         |
| 6          | 24/M    | -              | -             | 10              | 0.1/0.2   | 452/444    | OD-OS PPK | 0               | I          | R155fs/P204S|
| 7          | 55/F    | -              | +             | 20              | 0.05/0.05 | NA         | OD-OS PPK | 0               | I          | Q298fs    |
| 8          | 63/M    | +              | +             | 20              | 0.05/0.05 | NA         | OD-OS PPK | 0               | I          | V176M     |
| 9          | 29/F    | -              | -             | 15              | 0.1/0.1   | NA         | OD-OS PPK | 241             | II         | V176M/F55S|
| 10         | 27/M    | +              | +             | 15              | 0.2/0.4   | 445/430    | -         | 0               | I          | R211W     |
| 11         | 55/M    | +              | +             | 20              | 0.16/0.1  | NA         | OD-OS PTK | 0               | I          | Q298fs    |
| 12         | 25/M    | +              | +             | 15              | 0.16/0.2  | 440/462    | -         | 0               | I          | R211W     |
| 13         | 27/F    | -              | -             | 18              | 0.2/0.3   | NA         | OD ALK   | 203             | II         | -         |
| 14         | 48/M    | -              | +             | 18              | 0.1/0.2   | NA         | OD PPK   | NA              | NA         | Q298fs    |
| 15         | 37/F    | +              | +             | 15              | 0.05/0.05 | 435/438    | OS PPK   | 0               | I          | V176M     |
| 16         | 26/F    | +              | +             | 15              | 0.05/0.2  | NA         | -         | 305             | II         | -         |
| 17         | 18/F    | +              | +             | 14              | 0.05/0.05 | 497/520    | OS PTK   | 0               | I          | R211W     |
| 18         | 32/M    | -              | +             | 18              | 0.4/0.6   | 444/440    | OD-OS PTK | 0               | I          | Q298fs    |

OD: Right eye; OS: Left eye; ALK: Anterior lamellar keratoplasty; PPK: Partial penetrating keratoplasty, PTK: Phototherapeutic keratectomy, NA: Nonavailable
variations (c.164 T>C, p. F55S; c.526 G>A, p. V176M; and c. 610 C>T, p. P204S), and two novel frameshift variations (c.894_895 insG, p. Q298fs and c. 462_463 delGC, p. R155Afs). The variation c.894_895 insG (p. Q298fs) was the most common variation in CHST6 in the study. No variation was detected in three of the five patients with MCD type II. A homozygous or compound heterozygous variation was detected in the coding region in all patients with MCD type I. Patient 14 who could not be immunophenotyped had a homozygous p. Q298fs variation. All patients with known consanguinity had homozygous variations with the exception of patient 2, who was heterozygous for c.1A>T (p. M1L).

The nucleotide sequences of the three previously reported variations in CHST6 in the patients are shown in Figure 3. Figure 4A shows the c.894_895insG variation in CHST6 that was detected in five unrelated patients. This variation results in a frameshift after codon 298 and early stop codon formation in codon 304. Figure 4B shows the c.462_463 delGC variant, which results in a frameshift after codon 155 (p.R155Afs). This variant was found only in patient 6, who is a compound heterozygote with the other novel variant c. 610 C>T (p. P204S). The c.610 C>T (p. P204S) variant was detected in two patients and leads to a proline to serine substitution (Figure 5A). Another novel missense variation, c. 526 G>A, was homozygously and compound heterozygously detected (Figure 5B,C). This mutation leads to a valine to methionine substitution at codon 176. The last novel missense variation, c.164T>C, was compound heterozygously detected in patient 9. This mutation leads to a phenylalanine to serine substitution at codon 55 (Figure 5D). When the pathogenic effect of the three novel missense variations was evaluated with SIFT and PolyPhen-2 in silico analysis software, the results were “probably damaging” and “not tolerated,” respectively (Table 4). Additionally, all newly detected variations in the CHST6 gene were not included in 100 control chromosomes, 1,000 Genomes, the dbSNP database, Exome Aggregation Consortium (ExAC), and the Human Gene Mutation Database (HGMD). Additionally, amino acid sequence analyses between several sulfotransferases of human and mouse origin revealed that the amino acids substituted in the missense variations detected in the patients were highly conserved residues (Table 5).

DISCUSSION

In this study, we describe variations in CHST6 and TGFBI in 34 unrelated Turkish patients with CD. We detected five novel and three previously reported variations in CHST6 in patients with MCD and two previously reported variations in TGFBI in patients with GCDI and LCDI. TGFBI: Since Munier et al. reported mutations in TGFBI in 61 indexed patients with CD (30 LCD, 12 GCD, seven ACD, eight RBCD, and four TBCD) in 2002, this gene has been investigated in autosomal dominant epithelial-stromal CDs in numerous populations [6]. TGFBI encodes keratoepithelin (transforming growth factor beta-induced protein, TGFBIp) that was described as an extracellular matrix protein [13]. Two mutation hot spots correspond to keratoepithelin arginine residues at positions 124 and 555, where 50% of mutations were detected [21]. Five genotype–phenotype correlations were detected in these studies: R555Q in TBCD,
R555W in GCD1, R124C in LCDI, R124H in ACD, and R124L in RBCD [6]. In addition to these defined correlations, molecular studies in a growing number of patients revealed mutational and phenotypic heterogeneity. To date, more than 60 mutations have been reported in TGFBI [6,16,22-24]. We detected the R555W mutation in patients with GCD1 in agreement with previous reports from Turkey [17,18] and from other populations [6,25-31]. The R555W mutation was heterozygous in 11 patients with GCD1 but homozygous in patient 9 who had known consanguinity in the family. His BCVA was severely affected although he was 27 years old and had undergone early keratoplasty surgery in both eyes. The phenotype of this patient was more severe than that of patients who carry the R555W mutation heterozygously. This

Figure 3. Sequencing chromatograms of previously reported variations in CHST6 identified in this study. A: The c.738 C>G (p.C246W) variation (homozygote) in macular corneal dystrophy (MCD) patient 1. B: The c.1 A>T (p.M1?) variation (heterozygote) in MCD patient 2. C: The c.631 C>T (p.R211W) variation (homozygote) in MCD patient 10.

Figure 4. Sequencing chromatograms of novel frameshift variations in CHST6 detected in this study. A: The c.894_895 insG (p.Q298fs) variation (homozygote) in macular corneal dystrophy (MCD) patient 4. B: The c.462_463delGC (p.R155Afs) variation (heterozygote) in MCD patient 6.
condition was reported in the literature for patients with GCD and ACD and explained by semidominance: Patients who are homozygous for ACD (R124H) or GCD (R555W) mutations have been reported, and these patients were more severely affected than heterozygotes [32-34]. Furthermore, the clinical severity of the disease was different in patients with GCD although they carried the same variation in one allele and had similar ages. Patient 12 was a 54-year-old woman. Her BCVA was above 0.4 in both eyes, and she had no previous surgery. However, patient 14 was a 43-year-old man. His BCVA was below 0.1 in both eyes, and he had undergone keratoplasty surgery in both eyes. Phenotypical differences in patients with the same mutation have been reported in the literature even between members of the same family [25]. This condition could be explained by two mechanisms: There could be other genetic variations in TGFBI that we did not analyze.

| Nucleotide change | Amino acid change | Variation type | Immunotype | Patient no | Polyphen | SIFT | HGMD No |
|-------------------|-------------------|----------------|------------|------------|----------|------|---------|
| c.738 C>G         | p.C246W            | missense       | I          | 1          | 1 (probably damaging) | 0 (not tolerated) | CM065078 |
| c.1 A>T           | p. MIL             | First codon loss | II         | 1(2:heterozygote) | MD       | MD   | CM0650699 |
| c.894_895 insG    | p. Q298fs          | frameshift     | I          | 5(4,7,11,14,18: heterozygote) | MD       | MD   | Novel |
| c.610 C>T         | p. P204S           | missense       | I          | 3(3: homozygote; 6: compound heterozygote) | 0,01 (not tolerated) | Novel |
| c.462_463 del GC  | p. R155Afs         | frameshift     | I          | 1(6:compound heterozygote) | MD       | MD   | Novel |
| c.526 G>A         | p. V176M           | missense       | I,II       | 2(8,15:homozygote; 9:compound heterozygote) | 0 (not tolerated) | Novel |
| c.631 C>T         | p. R211W           | missense       | I          | 3(10,12,17: homozygote) | 0 (not tolerated) | CM002586 |
| c.164 T>C         | p. F55S            | missense       | I          | 1(9:compound heterozygote) | 0,968 (probably damaging) | 0 (not tolerated) | Novel |
or there could be other genetic and/or environmental factors that affect protein–protein interactions [35,36] or protein degradation mechanisms [37,38], which should be evaluated to reveal the exact mechanism of corneal deposit formation in TGFBI-associated CDs.

In patients with LCD1, R124C was the most common mutation in TGFBI, but various mutations were detected in exons 4, 11, and 12 in previous studies [6,24-31,39-41]. In all of the present patients with LCD1, a heterozygous R124C mutation was detected in exon 4 in TGFBI. There was no known consanguinity in the LCD families, and the patients had similar phenotypes, except patient 3 in whom the disease showed asymmetry between two eyes. The asymmetric progression of LCD was reported in the literature [25,34], but further studies are needed to explain this asymmetry by identifying other pathogenic factors that underlie amyloid formation in the cornea. This study was the first report to examine genotype–phenotype relations in Turkish patients with LCD1.

**CHST6**: In this study, the coding region of CHST6 was analyzed in 18 unrelated patients with MCD. MCD was the most frequent CD type in the study population. MCD is also one of the most common CD observed in patients undergoing cornea transplantation in Saudi Arabia and India due to the high rate of consanguinity as in Turkey [10,42-44].

CHST6 encodes the enzyme N-acetyl-glucosamine-6-O-sulfotransferase (C-GlcNAc6ST), a member of the carbohydrate sulfotransferase family. C-GlcNAc6ST contains a short cytosolic tail at the N-terminal, a single transmembrane span, and a C-terminal domain that contains two sulfate donor [adenosine 30-phosphate-50-phosphosulphonate (PAPS)] binding sites, the catalytic domain, and an area that determines carbohydrate specificity in vivo [7,45,46]. The mutations of these three important sequences were reported to deteriorate the enzyme function and cause the deposition of low or unsulfated KS in the corneal stroma, leading to MCD [47-49].

There are three clinically indistinguishable immunophenotypes of MCD (I, IA, and II) based on the absence or presence of AgKS in the serum and corneal tissue [11]. In the present study population, we detected AgKS levels only in the serum samples of patients with MCD as histopathological cornea material was not available. We detected a variation in the coding region of CHST6 in all patients with type I, but not in three of the five patients with MCD type II. Akama et al. detected deletions or rearrangements of the upstream region of CHST6 in patients with MCD type II and suggested that MCD type II is characterized by the absence of mutations in the coding region in one or two alleles [5]. In the present study, we analyzed only the coding sequence. Thus, it is possible that the variation was located in the promoter or in a non-coding upstream or downstream region of this gene. Additionally, we could not correlate MCD diagnosis with histopathology in these three patients. Thus, these cases without coding region mutations might represent phenocopies. Furthermore, genetic heterogeneity could explain the patients without coding region mutations reported in the present study and in previous studies [50-55]. In one of the patients with type II MCD, we detected only a single heterozygous M1L variation that was previously reported in patients with MCD type I homozygously [56,57]. Nowinska et al. detected this start codon alteration heterozygously in a Polish patient with MCD with an unknown immunophenotype [50]. There could be a second undetected variation outside the coding region of CHST6 in the present patient, or genetic heterogeneity could be another possible explanation in this case. In the fifth patient with MCD type II, we detected two novel heterozygote missense variations. One missense variation, V176M, was also homozygously detected in two patients.
with MCD type I. Although Akama et al. proposed that MCD types I and II were discernible by the type of CHST6 variation [5], recent reports and the present study suggest that there is no correlation between immunophenotypes and variations in CHST6 [58-60]. The same variation could be associated with all three immunophenotypes as reported from a single family in India [20].

To date, 165 variations in CHST6 in patients with MCD from different populations have been reported in the HGMD. This means strong allelic heterogeneity in CHST6. The identification of eight variations in 15 patients with MCD in the present study also supports allelic heterogeneity in this gene.

The most common variation detected in the patients with MCD was c.894_895insG (p. Q298fs). This variation was homozygously detected in five unrelated patients with MCD type I. This insertion variation was not previously reported and resulted in the occurrence of a frameshift after the 298th glutamine residue and premature stop codon formation. This variation was found in ten of 36 alleles in the present study population. This could be explained in two ways. First, this could be a hot spot mutation, but this variation was not detected in other patients from different populations studied until now. Second, this variation could be a founder mutation, but to evaluate a founder effect, a haplotype analysis of the family members of these patients, whom we could not reach, should be performed. The other novel frameshift variation detected in the present study was c.462_463delGC (p.R155Afs). This deletion, detected in patient 6 compound heterozygously, resulted in the occurrence of a frameshift after the 155th arginine residue and premature stop codon formation. According to the recommended standards for the interpretation of sequence of the American College of Medical Genetics and Genomics (ACMG), these two variations belong to the PVS1 null variant class that can often be assumed to disrupt gene function by leading to a complete absence of the gene product by a lack of transcription or nonsense-mediated decay of an altered transcript [61]. Null variants were reported less frequently than missense variants in MCD [4,62]. In French and German families, frameshift mutations correlated with more severe phenotypes and earlier requirement of keratoplasty [51,56]. However, El-Ashry et al. and Sultana et al. observed that there is no consistent phenotypic difference between truncating and missense mutations in their study populations [43,63]. In the present study, we detected two frameshift variations that resulted in premature stop codon formation in six patients of whom four underwent keratoplasty (66%). We detected missense variations in nine patients of whom six underwent keratoplasty (66%). Regarding previous treatments, other phenotypic properties listed in Table 3 were not different between patients carrying missense and frameshift variations in accordance with reports from Egypt and India [43,63].

The second common variation in the present study was c. 631 C>T (p. R211W), which was previously reported in populations from Iceland [5] and Japan [49]. The other previously reported variation homozygously detected in patient 1 was c.738 C>G (p. C246W). This missense variation was reported in populations from North America in 2006 [62].

In the present study, we also detected three novel missense variations in patients with MCD: c.610 C>T (p. P204S) was located in the RX7S sequence for the 3'-PB domain, c.526 G>A (p. V176M) was located in the sequence between the 5'-PSB domain and the 3'-PB domain called the binding pocket, and c.164 T>C (p. F55S) was located in the 5'-PSB domain. All the novel variations were located in the binding sites or binding pocket that was important for enzyme function [45,46]. In addition, none of these missense variations was detected in the 100 control chromosomes or the ExAC, 1000 Genomes, and dbSNP databases. These three novel missense variations were predicted to be pathogenic by SIFT and PolyPhen-2 software (Table 4). Moreover, the amino acid sequence analysis between several sulfotransferases of human and mouse origin demonstrated that the novel variations substitute well-conserved amino acid residues, which supports the pathogenic effect of these novel missense variations (Table 5). However, functional studies are warranted to determine the exact consequences of these changes for protein functions.

Analysis of clinical parameters between different variations and between different immunophenotypes indicated that most patients in the present study had similar features as reported in various studies in different populations [5,12,22]. As a result, it was not possible to detect consistent genotype and phenotype correlations in patients with MCD.

This study presents some limitations. The patient population was small, and the families of the patients were not methodically evaluated.

In conclusion, this is the first molecular analysis of TGFBI and CHST6 in Turkish patients with CD. Well-known mutations in TGFBI were detected in patients with GCD1 and LCD1, supporting the existence of hot spot mutations in this gene. Moreover, five novel and three previously reported likely pathogenic variations in CHST6 were identified in patients with MCD, which highlights the allelic heterogeneity of this gene.
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