Truncating Mutations in the Carbohydrate Sulfotransferase 6 Gene (*CHST6*) Result in Macular Corneal Dystrophy

Florence Niel,[1] *Pierre Ellies,*[2] *Paul Dighiero,*[3] *Jeannette Soria,*[4] *Celia Sabbagh,*[4] *Chankannira San,*[1] *Gilles Renard,*[2] *Marc Delpech,*[1] *and Sophie Valleix*[1]

Purpose. Identification of mutations in the *CHST6* gene in 15 patients from 11 unrelated families affected with recessive macular corneal dystrophy (MCD).

Methods. Genomic DNA was extracted from peripheral blood leukocytes of the affected patients and their healthy family members, and the mutational status of the *CHST6* gene was determined for each patient by a PCR-sequencing approach. Serum concentrations of antigenic keratan sulfate for each proband were determined by ELISA.

Results. ELISA indicated that all affected patients, except one, were of MCD type I or IA. Fourteen distinct mutations were identified within the *CHST6* coding region: 2 nonsense, 2 frameshift, and 10 missense. Of these, 12 were novel, and a nonsense mutation in the homozygous state is reported for the first time.

Conclusions. These molecular results in French patients with MCD combined with those reported in previous studies indicated *CHST6* mutational heterogeneity. The characterization herein of nonsense mutations is in keeping with the fact that MCD results from loss of function of the *CHST6* protein product. (*Invest Ophthalmol Vis Sci.* 2003;44:2949–2953) DOI:10.1167/iovs.02-0740

Macular corneal dystrophy (MCD; Online Mendelian Inheritance in Man [OMIM] 217800; http://www.ncbi.nlm.nih.gov/Omim/ provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) is an autosomal recessive inherited disorder that is rare in most countries, but very common in Iceland.[1] The onset usually occurs in the first decade of life, and patients with MCD have progressive development in both corneas of punctate gray-white opacities underneath the epithelium, within the corneal stroma, Descemet's membrane, and the corneal endothelium. These abnormal deposits are associated with a central stromal haze that gradually extends to the periphery of the cornea, leading to visual impairment. Previous biochemical studies have indicated that the pathologic corneal deposits most probably result in an accumulation of glycosaminoglycans (GAGs) and that a specific sulfation step of keratan sulfate (KS) is impaired in MCD.[2] KS proteoglycans are the major proteoglycans in the corneal stroma and consist of a linear repeating sequence of disaccharide units of galactose (Gal) and *N*-acetylglucosamine (GlcNAc), which are both sulfated on the C-6 position. Thus, KS proteoglycans are highly negatively charged macromolecules, in part through the addition of these sulfate groups on their sugar residues. MCD has been classified into two major immunophenotypes (types I and II) on the basis of immunohistochemical studies and the detection of the sulfated KS level in blood serum and in corneal tissue. A third subtype, type IA, has been documented in Germany and Saudi Arabia and corresponds to the absence of sulfated KS in the cornea and serum, but its presence in keratocytes.[1,3,4,5] Regardless of the immunotype, the mode of inheritance and the clinical presentation are similar in the two forms. By genetic linkage analysis, the critical region for MCD has been mapped to chromosome 16 (16q22),[6] and the carbohydrate sulfotransferase 6 (*CHST6*; OMIM 605294) gene has been identified as the defective gene.[7] Screening for *CHST6* mutations in patients with MCD has revealed that heterogeneous missense mutations within the coding region accounted for MCD type I, whereas large deletions and/or replacements in the upstream region of *CHST6* result in MCD type II.[7]

The *CHST6* gene has been grouped into the GST-family (galactose/*N*-acetylgalactosamine/*N*-acetylglucosamine 6-*O*-sulfotransferases) including a group of Golgi enzymes that transfer sulfate from 3′phosphoadenosine5′phospho-sulfate to the 6-hydroxyl group of galactose, *N*-acetylgalactosamine (GalNac), glucose, or *N*-acetylglucosamine (GlcNac) in nascent glycoproteins. The *CHST6* gene, also called *GST-4B*, encodes a corneal *N*-acetylglucosamine-6-*O*-sulfotransferase (C-GlcNAc6ST) that initiates sulfation of KS chains on proteoglycans (PG). The gene consists of four exons, but its open reading frame (ORF) is contained only within exon 4.[7,8] This gene is located 50 kb downstream of a highly similar GST gene, *GST-4A* or *CHST5*, which encodes an intestinal isoenzyme of *N*-acetylglucosamine-6-*O*-sulfotransferase.

We report the mutational spectrum in the *CHST6* gene of 15 patients with MCD from nine French and two Maghreb (North African) kindreds. Fourteen distinct mutations in *CHST6* coding sequences were identified: 2 nonsense mutations, 2 frameshift mutations, and 10 missense mutations. Of these, 12 were novel, and nonsense mutations in *CHST6* were reported for the first time.

Methods

Patients

Eleven unrelated families with MCD, nine of French and two of Maghreb origins were studied, including in total, 15 patients. The pedigrees of each family were established, and all were consistent with a recessive autosomal transmission of the corneal disease. No patients had

From the [1]Laboratory of Biochemistry and Molecular Genetics, Hospital, Cochin, Paris, France; the [2]Department of Ophthalmology, Hospital Hôtel-Dieu, Paris, France; the [3]Department of Ophthalmology, Hospital of Poitiers, France; and the [4]Laboratory of Biochemistry A, Hospital, Hôtel-Dieu, Paris, France.

Submitted for publication July 22, 2002; revised October 17, and November 19, 2002; accepted November 23, 2002.

Disclosure: **F. Niel**, None; **P. Ellies**, None; **P. Dighiero**, None; **J. Soria**, None; **C. Sabbagh**, None; **C. San**, None; **G. Renard**, None; **M. Delpech**, None; **S. Valleix**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Paul Dighiero, Centre Hospitalo-Universitaire de Poitiers, Department of Ophthalmology, 2, rue de la Milétrie, BP 577-86021 Poitiers, France; p.dighiero@chu-poitiers.fr.

Copyright © Association for Research in Vision and Ophthalmology

evidence of other ocular or systemic clinical abnormalities. All patients gave their informed consent before inclusion in the study, which was approved by Hôtel-Dieu Hospital Ethics Committee and conformed with the provisions of the Declaration of Helsinki.

Determination of Sulfated KS in Serum

Serum concentrations of antigenic KS in each patient and control subject were determined by a solid-phase competitive immunoassay, as described previously.[9] The antigen used for coating was bovine nasal cartilage proteoglycan KS (BNC-PG; ICI, Aurora, OH), and the antibody was a monoclonal Ig antibody against KS (clone 5-D-4) that specifically recognizes an antigenic determinant in the polysaccharide structure of KS (ICI). The binding of antibody against KS to KS-coated plates is competitively inhibited by the KS in the solution to be measured. The amount of antibody bound to the polystyrene plate is revealed using a peroxidase-labeled anti-mouse IgG (obtained from Miles, Elkhart, IN). The standard curve was performed using serial dilutions of a pool of normal plasma. The results of antigenic KS (aKS) levels obtained from patients with MCD were expressed as a percentage of KS present in normal plasma (normal levels were found, ~190–250 ng/mL). Patients with aKS levels less than 10% were classified as MCD types I and IA. Probands from families 1, 2, and 7 were found to have aKS levels less than 1%, those from families 8 and 9 had aKS levels less than 5%, and probands from families 3, 5, 6, and 10 showed aKS levels less than 10%. In contrast serum concentrations of aKS for probands from family number 11 were found to be high (90%), and these patients were classified as having MCD type II. Samples from family 4 were not available.

DNA Extraction

Genomic DNA was extracted by standard methods from peripheral blood leukocytes samples collected from the 15 patients and their healthy family members (35).

Mutation Screening

To identify genomic rearrangements in the 5′upstream region of *CHST6* gene by polymerase chain reaction (PCR), we used the four primer sets previously reported.[7] The coding *CHST6* region was screened by a PCR-sequencing approach with three primer sets, as published by Akama et al.,[7] but we replaced the last primer set with these primers: 5′-CAGCCAAGGCTCTGGCGC-3′ and 5′-CACCATG-CACTCTCCTCCCG-3′. The PCR products were purified (QIAQuick PCR Purification Kit; Qiagen, Chatsworth, CA) and sequenced on both strands with a kit (Big Dye Terminator; Applied Biosystems, Foster City, CA). The samples were resolved on an automatic fluorometric DNA sequencer (Prism 377; Applied Biosystems). Each electropherogram was compared with the nucleotide sequence of the *CHST6* human complementary DNA (GenBank accession number: AF219990; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).

Results

An enzyme-linked immunosorbent assay (ELISA) was performed to measure KS concentrations in serum obtained from all the probands of MCD-affected families participating in this study (see the Methods section). The results indicated that all affected patients, except for those in family 11, were of MCD type I, including the subtype IA. For family 11, the KS levels in the serum of the two probands were normal, and therefore they were classified as having type II MCD.

No genomic rearrangements in the 5′noncoding region of *CHST6* were detected in any patients, whereas 14 distinct sequence changes, illustrated in Figure 1, were identified within the *CHST6* coding region. Of which 12 mutations (L15P, Q82X, L152P, C102G, P204G, N61T, N70L, Q58X, Y68H, S131P, 1055-1056insC, 962-965delGCT→insA) have not been identified. Missense mutations identified were classified as potentially pathogenic according to their absence in 60 normal individuals (120 chromosomes in total), a correct segregation of each mutation within MCD-affected families and the predicted effect on the amino acid sequence of the gene product. All mutations are detailed in Table 1. Except for R166P and L200R, all the mutations identified appeared to be private variants.

Probands from four families (2, 4, 5, and 6) were homozygous, whereas most of the probands were compound heterozygous with at least one identified allele. In three families (9, 10, and 11) only one mutated allele was detected after all the entire coding of *CHST6* was sequenced. Analysis of the 5′upstream region of *CHST6* in these patients did not indicate rearrangements similar to those observed in patients with MCD type II. However, a large deletion or any other molecular defect in this region not detected with the primer sets used could not be excluded. Among the 19 mutations identified in patients with MCD, there were 13 missense, 3 nonsense, and 2 frameshift deletions/insertions. In addition, null mutation on both *CHST6* alleles were identified in probands of family 2.

Four different truncating mutations were detected in families 1, 2, 7, and 9 that are expected to cause loss of *CHST6* gene expression. These consisted of two nonsense mutations producing a codon stop and two insertions/deletions of a number of nucleotides that are not an exact multiple of 3, causing therefore a shift in the translational reading frame and introducing a premature termination codon not far downstream of the mutation site. In family 1, the proband was compound heterozygous for C→T and T→C transitions at nucleotide positions 936 and 736, respectively, corresponding to Q82X and L25P mutations at the protein level. The Q82X mutation produced a premature termination codon simply by converting a glutamine into a stop codon. The nucleotide change found on the other allele is expected to lead to the replacement of a leucine by a proline residue at amino acid position 25. Although leucine and proline are both nonpolar aliphatic residues, an aberrant proline residue is expected to introduce a bend in the protein chain and therefore impairs its flexibility.

In family 2 in which the parents were first cousins, the three affected children were homozygous for a C→T transition at nucleotide position 864 predicting the replacement of a glutamine by a stop codon at position 58 of the protein (Q58X).

In families 7 and 8, the mutation 1055-1056insC and the complex mutation 962-965delGCT→insA each introduced a frameshift in translation, which results in a premature termination codon at amino acid positions 107 and 221.

In family 8, the two probands were compound heterozygous for C102G and P204G mutations. These involve cysteine and proline residues respectively, which may play key roles in the protein conformation. Indeed, cysteine is often involved in disulfide bounding. As no other amino acid has a side chain with a sulfhydryl group, there is a strong pressure to conserve cysteine residues which are among the least mutable of the amino acids. In the case of the P204G mutation, proline and glycine are both non–polar aliphatic residues, but each of their lateral chains are significantly different. Proline is unusual in that the side chain connects the nitrogen atom of the NH2 group to the central carbon atom expecting to generate a rigid conformation of the protein, whereas glycine, which has the smallest side chain among the amino acids, enhances the flexibility of the protein. Similarly, P204G is a missense mutation located in the 3′ phosphate-binding (3′PB) domain of the enzyme which interacts with 3′phosphoadenosine 5′phosphosulfate (PAPS), the sulfate donor for C-GlcNAc6ST.

In two families (5 and 6), the probands were homozygous for a G→C transversion at nucleotide 1189, leading to the

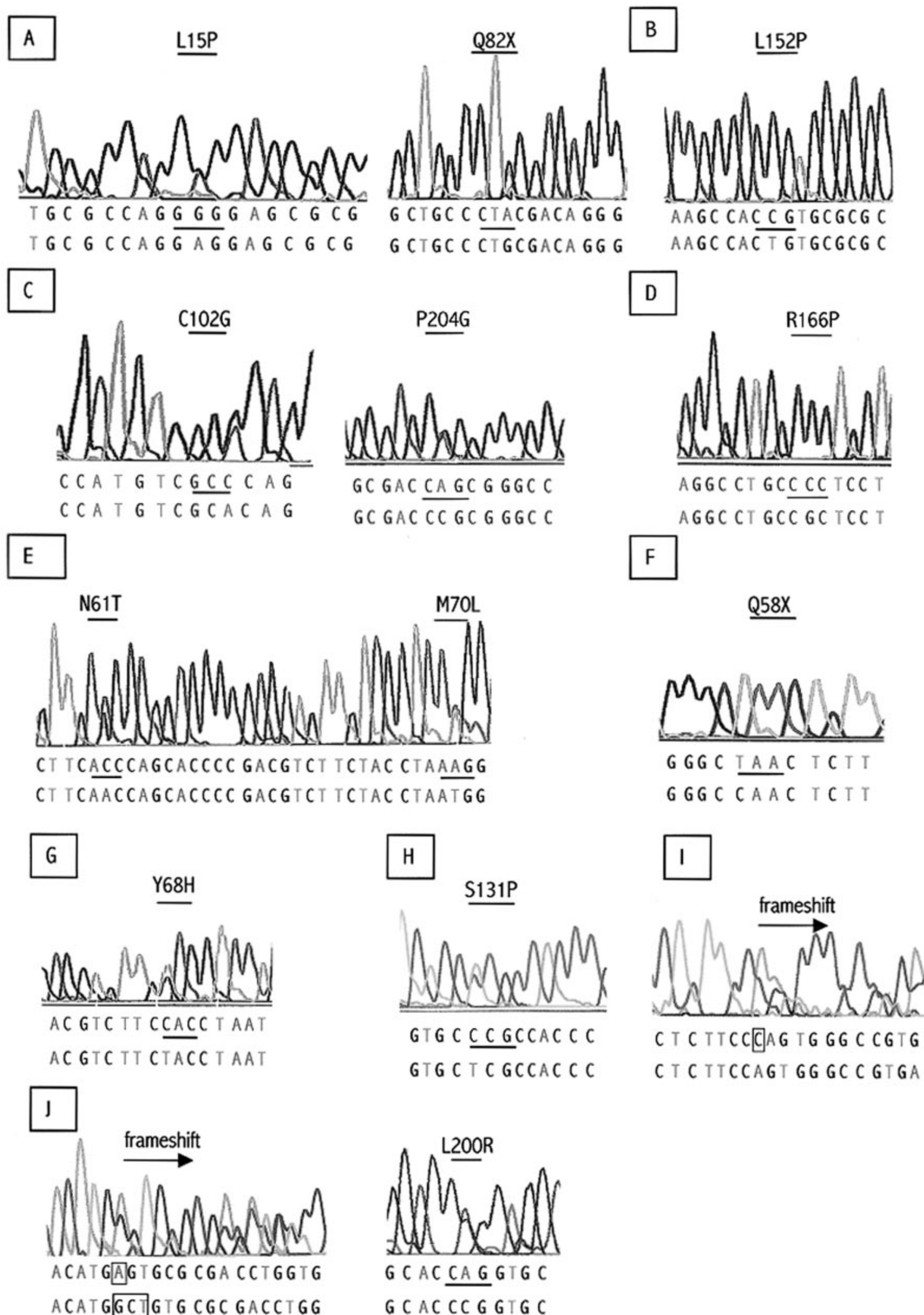

FIGURE 1. Identification of *CHST6* mutations. In each panel (**A**–**I**), are shown the mutated DNA-sequence electrophoregrams of one proband from each MCD-affected family. All the mutations were confirmed by sequencing in both directions, but only one sense (forward or reverse) is displayed for each case. The positions of the mutations are underlined in the corresponding DNA sequence. Sequences shown are the antisense sequences for the L15P, Q82X, and C102G mutations.

Table 1. Mutations Detected in the *CHST6* Coding Regions

	Allele 1		Allele 2	
Family	**Nucleotide Changes**	**Mutations**	**Nucleotide Changes**	**Mutations**
1	736T→C	L15P	936C→T	Q82X
2	864C→T	Q58X	864C→T	Q58X
3	874A→C	N61T	891T→A	M70L
4	1147T→C	L152P	1147T→C	L152P
5	1189G→C	R166P	1189G→C	R166P
6	1189G→C	R166P	1189G→C	R166P
7	962-965 delGCT→insA	Frameshift (ter221)	1291T→G	L200R
8	996T→G	C102G	1303C>A	P204G
9	1055-1056insC	Frameshift (ter107)	?	
10	894T→C	Y68H	?	
11	1083T→C	S131P	?	

replacement of an arginine by a proline residue at position 166 of the enzyme (R166P). This mutation has been reported in Icelandic patients with MCD.[10]

In family 4, the proband was found to be homozygous for a T→C transition at nucleotide position 1147 expected to result in the replacement of a leucine by a proline residue at position 152 of the protein sequence (L152P). The proband of family 3 was compound heterozygous for the N61T and M70L mutations. Although these amino acid changes are conservative, methionine at position 70 is highly conserved across species and between members of the sulfotransferase gene family. The M70L mutation therefore probably affects the protein structure or its function.

In families 10 and 11, only one mutated allele was detected in all the patients corresponding to Y68H and S131P mutations at the protein level, respectively. These amino acid changes are nonconservative, and tyrosine at position 68 and serine at position 131 of the protein are both highly conserved.

Discussion

These results from French families affected by MCD clearly indicate that the majority of *CHST6* mutations are private, demonstrating therefore that the *CHST6* gene is subject to strong allelic heterogeneity. Apart from the R166P mutation reported previously, *CHST6* molecular heterogeneity has also been observed in Japanese, British, Indian, and Saudi Arabian MCD-affected families. Therefore, several different mutations have been identified in the *CHST6* gene (Bao W, Smith CF, Al-Rajhi A, ARVO Abstract 2609, 2001; Warren JF, Aldave AJ, Thonar EJ, Margolis TP, Whitcher JP, Srinivasan M, ARVO Abstract 2870, 2001).[7,10,11] No nonsense mutation has been identified in patients with MCD so far, and the identification of such mutations is in keeping with the fact that MCD results from loss of function of the C-GlcNAc6ST protein. It has been reported that most chain termination mutations may result in the generation of an unstable mRNA, which undergoes rapid degradation. These mutations are thus expected to be associated with a clinically more severe phenotype. We noted that the three homozygous patients of family 2 experienced rapid visual deterioration at an early age, and all required keratoplasty in the second decade of life. The other novel *CHST6* mutations identified among our patients are missense mutations that involved conserved residues of the enzyme after alignment of various sequences of sulfotransferases. Although it is difficult to predict whether these mutations are disease-causing in the absence of functional studies, it indicates that mutations affecting conserved residues occur in critically important regions, destabilizing an essential structure or impeding gene function in a way not yet known.

For MCD families 1 to 8 in which the two mutated alleles were characterized, the molecular data were in good agreement with the immunochemical classification. In families 9, 10, and 11, we failed to identify the second mutated allele. However, KS levels in serum showed that probands from families 9 and 10 were of type I and IA, and those from family 11 were of type II. Because a mutation in the coding regions of *CHST6* was identified in patients in family 11, suggesting MCD type I, we concluded that these patients in fact had a combination of type I and type II, because the MCD type II phenotype was dominant over that of type I.[7]

The data were also analyzed for genotype–phenotype relationships, but no clear-cut correlations between each mutation and disease phenotype were obvious, except for three families (families 1, 2, and 5) in which all the affected patients had undergone bilateral keratoplasty in the second decade of life. The presence of multiple private mutations imply that screening of the *CHST6* gene will not be straightforward and will require sequence analysis of all the coding region in addition to the 5′ upstream *CHST6* region. However, the characterization of the spectrum of *CHST6* gene mutations now allows us to offer genetic testing to unaffected younger siblings in our French MCD families.

In normal corneas, KSs comprise 4% unsulfated, 42% monosulfated, and 54% disulfated disaccharides with number of average chain lengths of 14 disaccharides.[12] The sulfation of corneal KS is catalyzed by at least two different sulfotransferases in the Golgi apparatus; one enzyme, the KS Gal-6-sulfotransferase (*KSGAL6ST* or *CHST1*) catalyzes the sulfation at position 6 of the Gal residue, whereas *N*-acetylglucosamine-6-sulfotransferase (C-GlcNAc6ST/*CHST6*) catalyzes sulfation at position 6 of the nonreducing end of GlcNAc residues.[7,13,14] Biochemical studies confirmed that C-GlcNAc6ST transfers sulfate only onto the C-6 of GlcNAc residues and demonstrated that missense mutations in *CHST6* abolish the sulfotransferase activity of the corneal enzyme, resulting in the lack of highly sulfated KS in the corneal stroma of patients with MCD type I.[15] It has also been noted that Gal residues are not sulfated at position 6 in MCD corneas suggesting therefore that sulfation of GlcNAc residues must be required for sulfation of Gal by HKSG6ST.[14] Furthermore, recent studies have indicated that the sulfation of GlcNAc residues is tightly coupled with the elongation of sugar chains because, in addition to the absence of KS chain sulfation, the KS chain size is reduced to three to four disaccharides in MCD type I corneas as well as in cartilages. These data support the assumption that defect in

C-GlcNAc6ST alters the matrix organization of both corneas and cartilages although MCD phenotype is apparently restricted to the cornea.[12] Therefore, it is not inconceivable that other molecular defects in the *CHST6* gene may underlie related inherited corneal and/or skeletal dystrophies, as it was observed for the recently identified diastrophic dysplasia sulfate transporter gene (*DTDST*). Loss-of-function mutations in the *DTDST* gene lead to defective sulfate uptake and proteoglycan sulfation and cause three related autosomal recessive skeletal dysplasias of increasing severity, depending on the residual activity of the enzyme.[16] These examples of inherited diseases illustrate the critical role of carbohydrate sulfation in the organization of the extracellular matrix of the cornea, the cartilage, and bones.

Acknowledgments

The authors thank the patients and their family members for their devoted participation in this study.

References

1. Jonasson F, Oshima E, Thonar EJMA, et al. Macular corneal dystrophy in Iceland: a clinical, genealogic, and immunohistochemical study of 28 patients. *Ophthalmology.* 1996;103:1111–1117.
2. 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;77:3705–3709.
3. Yang CJ, SundarRaj N, Thonar EJ, Klintworth GK. Immunohistochemical evidence of heterogeneity in macular corneal dystrophy. *Am J Ophthalmol.* 1988;106:65–71.
4. Klintworth GK, Oshima E, Al-Rajhi A, et al. Macular corneal dystrophy in Saudi Arabia: a study of 56 cases and recognition of a new immunophenotype. *Am J Ophthalmol.* 1997;124:9–18.
5. Cursiefen C, Hofman-Rummelt C, Schlotzer-Schrehardt U, et al. Immunohistochemical classification of primary and recurrent macular corneal dystrophy in Germany: subclassification of immunophenotype IA using a novel keratan sulfate antibody. *Exp Eye Res.* 2001;73:593–600.
6. Vance JM, Jonasson F, Lennon F, et al. Linkage of a gene for macular corneal dystrophy to chromosome 16. *Am J Hum Genet.* 1996;58:757–762.
7. Akama TO, Nishida K, Nakayama J, et al. Macular corneal dystrophy type I and type II are caused by distinct mutations in a new sulphotransferase gene. *Nat Genet.* 2000;26:237–241.
8. Hemmerich S, Lee JK, Bhakta S, et al. Chromosomal localization and genomic organization for the galactose/N-acetylgalactosamine/N-acetylglucosamine 6-O-sulfotransferase gene family. *Glycobiology.* 2001;11:75–87.
9. Thonar EJ, Lenz ME, Klintworth GK, et al. Quantification of keratan sulfate in blood as a marker of cartilage catabolism. *Arthritis Rheum.* 1985;28:1367–1376.
10. Liu NP, Dew-Knight S, Rayner M, et al. Mutations in corneal carbohydrate 6 gene (CHST6) cause macular corneal dystrophy in Iceland. *Mol Vis.* 2000;6:261–264.
11. El-Ashry MF, Abd El-Aziz MM, et al. Identification of novel mutations in the carbohydrate sulfotransferase gene (*CHST6*) causing macular corneal dystrophy. *Invest Ophthalmol Vis Sci.* 2001;41:3670–3677.
12. Plaas AH, West LA, Thonar EJA, et al. Altered fine structure of corneal and skeletal keratan sulfate and chondroitin/dermatan sulfate in macular corneal dystrophy. *J Biol Chem.* 2001;276:39788–39796.
13. Fukuta M, Inazawa J, Torii T, Tsuzuki K, Shimada E, Habuchi O. Molecular cloning and characterization of human keratan sulfate Gal-6-sulfotransferase. *J Biol Chem.* 1997;272:32321–32328.
14. Hasegawa N, Torii T, Kato T, et al. Decreased GlcNac 6-*O*-sulfotransferase activity in the cornea with macular corneal dystrophy. *Invest Ophthalmol Vis Sci.* 2000;41:3670–3677.
15. Akama, TO, Nakayama J, Nishida K, et al. Human corneal GlcNac 6-O-sulfotransferase and mouse intestinal GlcNac 6-O-sulfotransferase both produce keratan sulfate. *J Biol Chem.* 2001;276:16271–16278.
16. Hastbacka J, Superti-Furga A, Wilcox WR, Rimoin DL, Cohn DH, Lauder ES. Atelosteogenesis type II is caused by mutations in the diastrophic dysplasia sulfate-transporter gene (DTDST):evidence for a phenotypic series involving three chondrodysplasias. *Am J Hum Genet.* 1996;58:255–262.