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CASE REPORT Open Access

Corneal microstructural changes of precise *CHST6* gene mutation: a case series

Durga Murugan[1*], Senthil Kumar Babu[1], Ezhil Vendhan Kalaimamani[2] and Kamaraj Raju[3]

Abstract

Background Macular corneal dystrophy (MCD) is an inherited, autosomal recessive disorder of defective keratan sulfate (KS) metabolism. It is caused by the mutations in carbohydrate sulfotransferase 6 gene (CHST6) which is essential for the sulfation of KS. Unlike the western world, MCD is the most common corneal stromal dystrophy in India, especially in south Indian population; it could be due to high frequency of consanguineous marriages.

Case presentation This study presents the clinical findings of one North Indian MCD family, including 6 patients and 3 unaffected relatives. We used slit lamp examination and in vivo confocal microscopy for assessment. Mutation screening was performed with Sanger sequencing, and corneal structure was analyzed through histochemistry and immunohistochemistry. Our comparative findings revealed that all the patients identified with the deletion of major portion of *CHST6* that included the Open Reading Frame (ORF). Although all the patients showed significantly reduced central corneal thickness (CCT-250 µm), a drastic decrease in stromal keratocyte count, and depletion of Bowman's layer compared to controls.

Conclusions This study first time revealed that MCD patients from one family with a deletion of major portion of *CHST6* that included ORF leads to severe corneal morphological changes.

Keywords Macular corneal dystrophy (MCD), Carbohydrate sulfotransferase 6 gene (*CHST6*), Open Reading Frame mutation (ORF), In vivo confocal microscope (IVCM), Immunohistochemistry (IHC)

Background

Macular corneal dystrophy (MCD: MIM 217800) is one of the severe form of IC3D category 1 stromal corneal dystrophy [1]. MCD is an inherited autosomal recessive disorder. It is the most common stromal corneal dystrophy in India as opposed to in western countries where it is relatively rare [2–5]. It is immensely prevalent in Iceland, Saudi Arabia and South India due to high degree of consanguinity [3, 6–10].

Carbohydrate sulfotransferase 6 *(CHST6)* is the candidate gene for MCD. *CHST6* encodes an enzyme N-Acetyl-glucosamine-6-sulfotransferase (C-GlcNAc6ST) involved in the sulfation of keratan sulfate (glycosaminoglycan), which plays a role in corneal transparency [11, 12]. Mutations in *CHST6* abolish or reduce the enzyme activity, thus preventing the sulfation of keratan (unsulfated glycosaminoglycan) leading to the accumulation of keratan in stoma (intracellular and extracellular matrix) and keratocytes. Due to lack of treatment options, difficulty in identifying various CHST6 gene mutations and the poor correlation between genotype and phenotype require detailed molecular and histopathological analyses to elucidate the pathogenesis of MCD.

*Correspondence:
Durga Murugan
durga2051991@gmail.com
[1] Central Research Laboratory for Biomedical Research, Vinayaka Mission's Kirupananda Variyar Medical College and Hospitals, Vinayaka Mission's Research Foundation (DU), Salem, Tamil Nadu, India
[2] Department of Ophthalmology, Vinayaka Mission's Kirupananda Variyar Medical College and Hospitals, Vinayaka Mission's Research Foundation (DU), Salem, Tamil Nadu, India
[3] Department of Biotechnology, Rathinam College of Arts and Science, Coimbatore, Tamil Nadu 641021, India

The clinical manifestations of MCD usually starts in the first decade of life leading to progressive visual loss eventually necessitating corneal transplantation by the fifth decade of life [13]. The preferred treatment for MCD has not yet been established. In early stages, Phototherapeutic Keratectomy (PTK) can be done, whereas at advanced stage, it requires full thickness or deep anterior lamellar keratoplasty. Penetrating keratoplasty (PKP) is the final surgical modality followed for MCD patients to rehabilitate the vision. Among stromal corneal dystrophies, MCD is the most common type in India, accounting for approximately 30% of all dystrophies that necessitate PKP [G.K. Vemuganti, personal communication].

Due to lack of treatment options, complexity in identifying multiple CHST6 gene mutations and poor genotype–phenotype correlation needed an extensive molecular and histopathological approaches for understanding the pathogenesis of MCD. So far, there are limited genetic studies of *CHST6* mutations and their effects on cornea for understanding the genotype and phenotype correlations. A spectrum of *CHST6* mutations was identified throughout the world to expand the mutational landscape. But their clinical significance related to disease-causing mutations remains unclear. The clinical importance of the specific mutations is helpful in disease management by providing patients and clinicians with useful information regarding the disease prognosis. Understanding the genetic aspects and structural changes of MCD is essential for the earlier accurate diagnosis, better treatments and preventive therapy. In this scenario, the present study was focused to elucidate the relationship between *CHST6* mutations and their effect on MCD cornea.

Case presentation

Pedigree analysis

In the present study, one Indian family were recruited with the previous family history of MCD. The present study was approved by the Institutional Ethics Committee (IEC- Tracking number: VMKVMC&H/IEC/22/38) of Vinayaka Mission's Kirupananda Variyar Medical College and Hospitals, Salem, India. Informed consent was taken from all the study participants.

Initially, 2 females (III:3, III:6) were identified with MCD by the comprehensive ophthalmic examinations including visual acuity and slit-lamp biomicroscope (SLM-3X-Slit-Lamp, China). Based on their family history, all other available family members and relatives were included in the study. After completing the ophthalmic examinations totally 6 MCD patients (III:1, III:3, III:6, IV: 1, IV:2, IV:5) and 3 unaffected relatives (III:8, III:10, IV: 9) were included (Fig. 1). Ocular details of MCD proband and available family members listed in Table 1.

With the severe visual problems MCD patient III:3 and III:6 were suggested for PKP surgery. Before surgery we have done IVCM analysis for microstructural changes in cornea. After surgery we have collected 2 corneal buttons for the histochemistry and immunohistochemistry analysis.

In 2016, the proband III:3 (47/F) was done with the PKP surgery in left eye (LE); in 2019, PKP was done in the right eye (RE) due to severe gray-white opacities

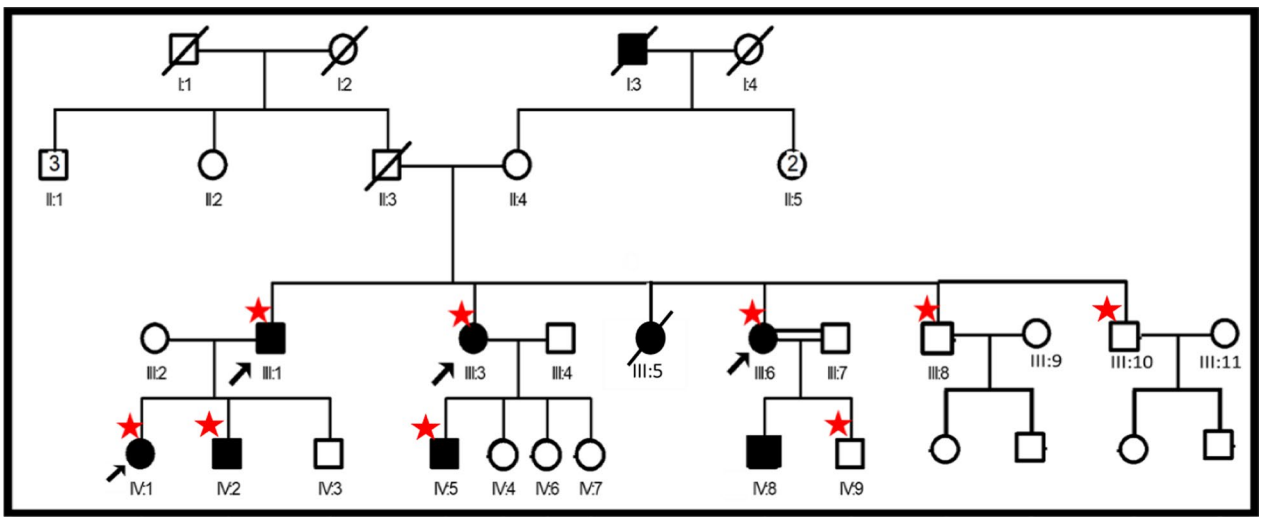

Fig. 1 Pedigree analysis of MCD patient family. I, II, III, IV indicate generations; 1, 2, 3, 4 indicate individulas; squares indicate males; circles indicate females; solid square and arrow indicate affected individual; open squares and circles indicate unaffected individuals; parallels indicate consanguinity; asterisk* indicate available samples for analysis

Table 1 Histochemistry, immunohistochemistry, IVCM and immunophenotype findings of MCD patients harboring deletion of ORF mutation in *CHST6*

Family I.d	Age/sex	RE/LE	Age at PKP	Mean VA		Corneal features				OE	FH/C	Mutation	IVCM	Histochemistry (Alcian blue staining)	IHC (5D4)	IP Classification	CCT (μm)
				RE	LE	OS	OD	AC	Lens								
III:1	49/M	**Graft**	RE-42 LE-40						Not clear	Same	+	ORF deletion	–	–	–	–	–
III:3	47/F	RE	44 Y	6/18P	1/60	C+MP	A+M+D	Q	Clear	S/P PTK	+	ORF deletion	Epithelial, BL scar with highly light reflective deposits; light reflective clump of stromal deposits; loss of K, disorganized CF; polymegathism	Subepithelial clumps of GAGs deposits; abnormal clump of GAGs in BM; loss of BL; intra/extracellular GAGs; abnormal K, CF	Faint KS expression only in K, Reduced E thickness, BL destruction	**IA**	191
III:6	37/F	RE	34Y	HM	HM	C+MP+P	A+M+D	Q	PCIOL	Same	+	ORF deletion	Epithelial, BL scar with highly light reflective deposits; light reflective clump of stromal deposits; loss of K, disorganized CF; polymegathism	Subepithelial clumps of GAGs deposits; abnormal clump of GAGs in BM; loss of BL; intra/extracellular GAGs; abnormal K, CF	Faint KS expression only in K, Reduced E thickness, BL destruction	**IA**	274
III:8	45/M	Normal							Clear	Same	+	NO	–	–	–	–	–
III:10	35/M	Normal							Clear	Same	+	NO	–	–	–	–	–
IV:1	17/F	**Graft**	RE-15 LE-12	1/6	6/9	C+MP+P	A+M+D	Q	Clear	Same	+	ORF deletion	–	–	–	–	–
IV:2	15/M	**Graft**	RE-9 LE-1 0	6/6	6/9	C+MP+P	A+M+D	Q	Not clear	Same	+	ORF deletion	–	–	–	–	–
IV:5	19/M	**Graft**	LE-7 RE-14	1/60	6/9P	C+MP+P	A+M+D	Q	PCIOL	Same	+	ORF deletion	–	–	–	–	–
IV:9	19/M	Normal							Clear	Same	+	NO	–	–	–	–	–

Family ID (number in pedigree), *IP* Immunophenotype Classifications, *VA* visual acuity, *FH* family history, + means presence of FH. *RE* right eye, *LE* left eye, *OE* Other Eye, *C* central, *MP* middle periphery, *P* periphery, *A* anterior stroma, *M* middle stroma, *D* deep stroma, *PCIOL* Posterior chamber intraocular lenses, *S/P* status post phototherapeutic keratectomy, *PKP* penetrating keratoplasty, *AC* anterior chamber, *Q* quiet, *Ho.M* Homozygous missense, *Ho.D* Homozygous deletion, *He.D* Heterozygous deletion, *BM* basement membrane, *BL* Bowman's layer, *DM* Descemet's membrane, *S* stroma, *E* endothelium, *K* keratocytes, *KS* keratan sulfate, *CF* collagen fibrils, *NF* nerve fibers, *GAG's* glycosaminoglycan's, *O* opacity, *μm* micro meter, *ng/ml* nanogram per milli-liter, *IVCM* in vivo confocal microscope, *HC* histochemistry, *IHC* immunohistochemistry, *5D4-MoAb* 5D4 monoclonal antibody, *CCT* central corneal thickness

(Blood, Cornea available). Elder son of III:3 was the another MCD proband IV: 5 (19/M) who had PKP in the early stage of life; (LE-7 years, RE-14 years) due to sever visual problems (Blood sample only available). Remaining 3 daughters (IV:4, IV:6, IV:7) are normal without any corneal dystrophies (Samples not available) (Fig. 1).

In 2016, with consanguineous marriage another proband III:6 (37/F) had PKP in the LE; PKP in the RE in 2019 (Blood, Cornea available). Elder son IV: 8 (21/M) of the proband III:6 already had PKP in the early stage (samples not available) and younger son IV: 9 (19/M) was normal while came to the hospital (Blood sample only available).

MCD proband III: 1 (49/M) had PKP in 2012 in both eyes (Blood sample only available). Elder daughter IV: 1 (17/F) who had PKP in both eyes (Blood sample only available). Younger son IV: 2 (15/M) had PKP in both eyes (Blood sample only available) but RE showed failed corneal graft. Younger son IV: 3 (12/M) was normal (samples not available) (Fig. 1).

After completion of ophthalmic examinations family members III:8 (35/M) (Blood sample only available), III:10 (33/M) (Blood sample only available) were included and they did not show any phenotype of corneal dystrophy (normal) and rest of the family members (details not available) are also normal (Fig. 1).

Genetic analysis

5 ml of peripheral blood samples were collected from 6 MCD patients and 3 unaffected relatives and 10 healthy volunteers without any corneal dystrophy (controls). Genomic DNA was extracted by salting out method. There are four coding regions in *CHST6* gene. Out of these, exon 3 is unique as already reported in several independent studies [5]. Based on the uniqueness of exon 3, we have also targeted the exon 3 by using predesigned primers [6]. Each PCR was carried out in a 50 µl reaction mixture containing 100 ng of genomic DNA, 1X buffer (PCR buffer (10 mM TRIS hydrochloride, pH 8.3; 50 mM potassium chloride; 1.5 mM magnesium chloride and 0.001% gelatin)), 0.5 pmol of each primer 200 µM of deoxynucleotide triphosphate and 1 U of Taq DNA polymerase (Sigma-Aldrich). Amplification was performed in a DNA Thermal cycler (Applied Biosystems-Invitrogen). The thermal cycling program started with an initial denaturation of 10 min at 96 °C, followed by 37 cycles of 96 °C for 30 s, 60 °C for 30 s of annealing, 72 °C for 45 s with a final extension at 72 °C for 5 min [7]. Bidirectional Sanger sequencing (ABI 3130 genetic analyzer, Applied Biosystems, Foster City, CA) was performed for mutation screening as per earlier published methods and conditions [6, 7]. Sequencing results were analyzed using Chromas Lite (2.1) software and compared with the *CHST6* reference sequence using the Basic Local Alignment Search Tool (BLAST) tool. This analysis presumed all the MCD patients showed deletion of major portion of *CHST6* that included ORF. In these patient's none of the *CHST6* coding region primer pairs produced a detectable amplicon on PCR, whereas normal amplifications of the other genomic sites were observed.

Slit lamp examination

Slit lamp analysis was done in 6 MCD patients, 3 unaffected relatives and 10 healthy individuals. Slit lamp view of control cornea showed normal corneal cells from epithelium to endothelium (Fig. 2A-a). Slit lamp view of cornea with MCD (III: 3) demonstrated multiple, irregular, gray-white opacities with intervening, central stromal haze (Fig. 2B-a). The slit lamp image of the MCD proband (III: 6) revealed diffuse, fine, symmetric clouding in the central corneal stroma, with discrete white opacities emerging that extend towards the periphery and progressively involve the entire thickness of the cornea (Fig. 2C-a).

IVCM examination

To analyze the corneal cellular structure of MCD patient, in vivo laser confocal microscope (Olympus Europa, Hamburg, Germany) was performed on 6 MCD patients, 3 unaffected relatives and 10 healthy individuals (controls). After capturing each location across corneal layers, six distinct IVCM images were selected and represented the microstructural findings of MCD patients compared to the control. Control eye (A-1,2,3,4,5,6) showed normal cellular morphology of different corneal layers from epithelium to endothelium.

Sequential IVCM images of 47-year-old MCD proband III: 3 (visual acuity 1/60) and 37-year-old MCD proband III: 6 (visual acuity 1/60) showed fine, light reflective scattered deposits in supra basal epithelium (Fig. 2B-1, C-1) without clear borders and without distinct nuclei. The Bowman's layer was not clear due to highly reflective, clumps of deposits that extended from the basal membrane to the underlying stroma (Fig. 2B-2, C-2). A very thin, unbranched nerve fibers were seen in immediately after the Bowman's layer and immediately before the anterior stroma. The anterior stroma appeared totally bright and fibrotic. Abnormal light reflected keratocyte cell nucleus were surrounded by the diffuse deposits (Fig. 2B-3, C-3). Highly light reflective diffuse deposits were observed throughout the anterior and middle stroma, accompanied by a loss of keratocyte cells. In the middle stroma, abnormally extended keratocyte cell and nucleus were surrounded by the diffuse deposits. Short length of collagen lamellae was seen among the highly reflected deposits surrounded the keratocyte

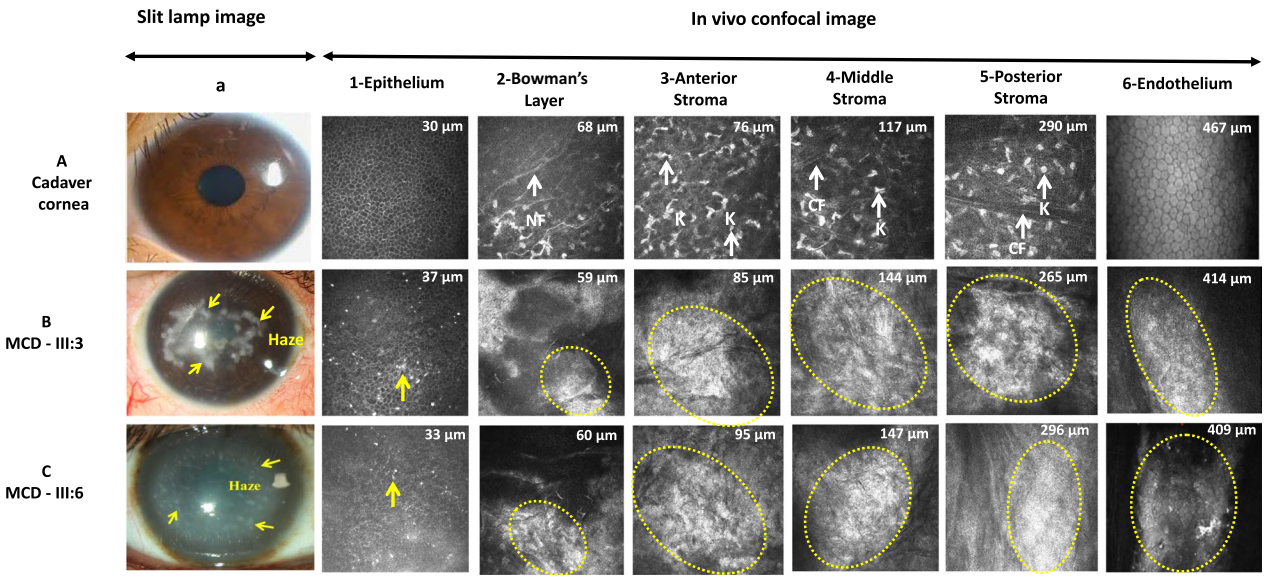

Fig. 2 Slit lamp and IVCM images of MCD cornea. The cornea was scanned from epithelium to endothelium (1–6). Slit lamp (a) and IVCM images (1–6) of proband III: 3, proband III: 6 with deletion of major portion of *CHST6* that included ORF. Slit lamp (image a) and sequential in vivo confocal images (images 1–6) of a healthy subject (**A**-control) and selected MCD patients (**B**, **C**) representing different corneal layers. Control eye (**A-1,2,3,4,5,6**) showed normal cellular morphology of different corneal layers. Proband III:3, III:6 showing scattered deposits in epithelium. Bowman's layer (**B-2**, **C-2**) existing with cluster of deposits and loss of NF (nerve fibers). Anterior stroma, middle stroma and posterior stroma (**B-3,4,5** and **C-3,4,5**) showing highly reflective clump of deposits and reduced keratocyte cells (K) count and disrupted collagen fibers (CF). Endothelium (**B-6**, **C-6**) with scattered deposits along with polymegathism

nucleus. Crisscross collagen fibers were not seen in the middle and posterior stroma (Fig. 2B-4,5, C-4,5). A homogeneous reflective material with dark striae like images was observed throughout the stroma, along with highly reflective deposits. Uniformly, scattered bright diffuse deposits were seen in the corneal endothelium. Abnormal polymegathism was observed in endothelial cells (Fig. 2B-6, C-6).

Histochemistry (HC) analysis

Corneal buttons were processed and stained with Alcian blue (AB) staining according to the published protocol [14]. The processed corneal sections were observed by phase contrast Microscope (Nikon Eclipse Ti2, Japan), inverted phase contrast microscope (Nikon Eclipse TS100, Japan) and analyzed for GAG deposits.

These corneas showed a markedly very thin, irregular, attenuated epithelium and focal BL breaks and destruction (★) (Fig. 3B-1, C-1). The surrounding stroma showed evidence of edema. While compared to control, thinning of the stroma (two–threefold decrease) and severely disorganized collagen lamellae was seen in these patients. There was diffuse, subepithelial and stromal inter-lamellar irregular GAG deposits along with the abnormal keratocytes (K). The Descemet's membrane is intact but much thickened. The corneal endothelial cells are absent or markedly reduced and highly abnormal deposits. Severe corneal morphological changes were observed in these patients with a deletion of major portion of *CHST6* that included ORF leads to severe corneal morphological changes (Fig. 3B-1, C-1).

Immunohistochemistry (IHC) analysis

Immunostaining was performed on the deparaffinized and rehydrated sections according to the protocols [4]. Immunostained corneal sections were visualized using Confocal Laser Scanning Microscope (Leica SP8 confocal microscope, Germany). Processed sections without primary antibody were taken as negative control.

Severe corneal morphological changes and reduction in CCT (CCT-231 µm) were seen in MCD patients detected with deletion of major portion of *CHST6* that included ORF. These patients classified under immunophenotype IA due to faint KS expression (IHC-5D4 anti-KS Ab) perceived only in the stromal keratocytes (K), while the other corneal layers showed no KS expression (no immunoreactivity). BL destruction (☆) (Fig. 3B-2, C-2) was observed in the central cornea, whereas peripheral cornea was normal. Abnormal keratocytes with reduced count was observed in stroma.

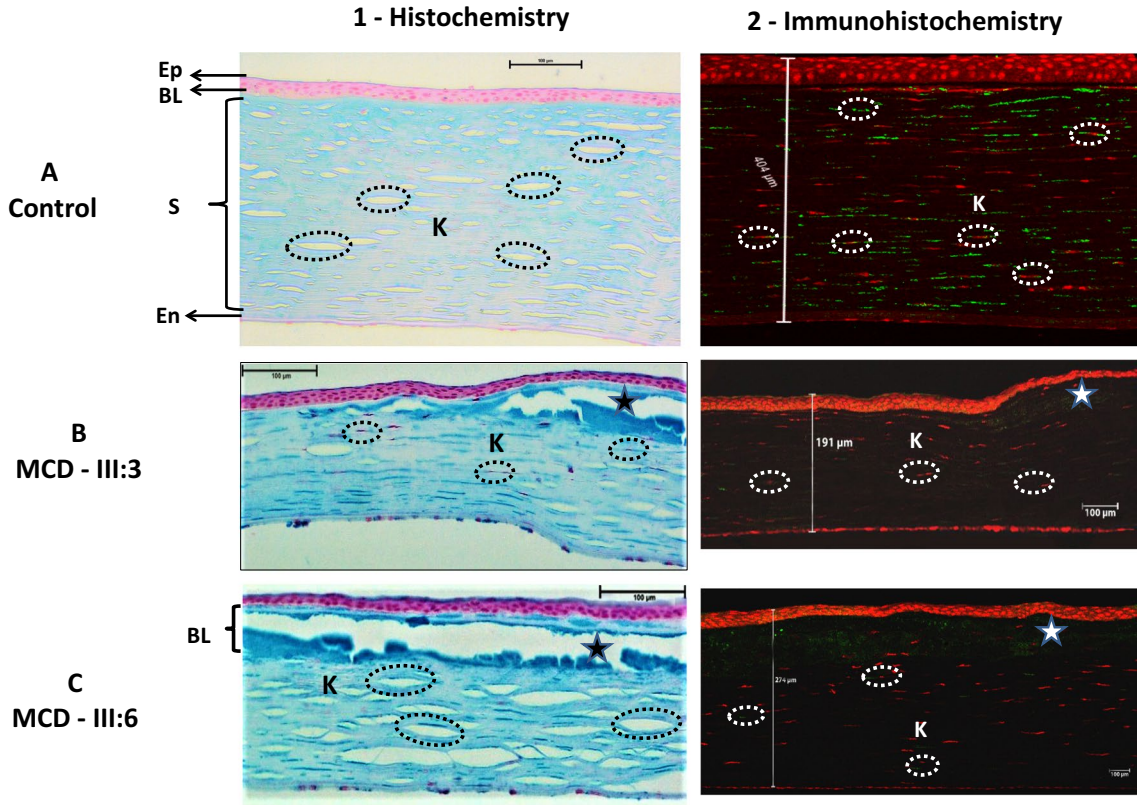

Fig. 3 Histochemistry and Immunohistochemistry images of MCD cornea. Images left side represents AB-stained corneal sections and right side represents immunostained corneal sections. Ep, Epithelium; BL, Bowman's layer; BM, Basement membrane; S, Stroma; DM, Descemet's membrane; EN, Endothelium; K, Keratocytes. **A** Corneal layers marked in the control (cadaver) showing normal morphology. Probands (III:3, III:6) showing severe morphological alterations in the Bowman's layer and anterior stroma due to abnormal, amorphous, finely granulated GAGs deposits (★, ☆) with altered collagen lamellae and altered keratocyte (K) cell shape (**B-1**, **C-1**). IHC also showed the presence of KS only in stroma with less number and reduced CCT (**B-2**, **C-2**)

Discussion

The combinatory findings of mutation analysis, corneal morphological alterations and immunophenotypes are presented in the current study which revealed severe corneal structural changes in patients with deletion of major portion of *CHST6* that included ORF. MCD is a disease of keratan sulfate (KS) metabolism, where KS is a major corneal glycosaminoglycan (GAG) involved in the organization of collagen fibrils [15]. An ordered lattice structure of regular interspaced, tightly packed collagen fibrils in stroma is the major cardinal requirement for the optical clarity and hence transparency. Basically, sulfation of KS mediated by corneal sulfotransferases is vital and failure to make bridges due to abnormally sulfated or unsulfated KS between collagen fibrils results in disturbed lattice arrangement and reduced interfibrillar spacing, thus reduced corneal thickness in MCD corneas compared to the normal cornea [4]. Mutations in highly conserved sites of corneal glucosamine N-acetyl-6-sulfotransferase (C-GlcNAc6ST) might affect the sulfation reaction could impact the irregular collagen fibril organization and undulated arrangement of collagen fibers leading to dark striae like appearances throughout the stroma [15, 16]. Till now, about 193 *CHST6* mutations have been reported which include missense, deletions (del), nonsense, deletion–insertion (delins), and complete deletion of ORF. These MCD linked diverse mutations identified among different ethnicities suggesting mutational heterogeneity [17].

Besides, slit lamp examination and IVCM findings reflected severe corneal structural changes of MCD patients detected with deletion of major portion of *CHST6* that included ORF. Further, it has been suggested in a study by Niel and coworkers that *CHST6* frameshift mutations may lead to severe MCD phenotypes with much deeper deposits [17].

Analysis of corneal morphology by AB staining targeting the presence of glycosaminoglycan (or GAG deposits) and immunohistochemical investigation of the KS immunoreactivity to anti-KS 5D4 MoAb in the corneal

sections revealed thin epithelium and thick BL with focal rupture or depletion along with the abnormal keratocytes in patients with deletion of major portion of *CHST6* that included ORF. Earlier studies reported that BL with some discontinuities together with clusters of abnormal, anomalous granular material, which could correspond to the irregular hyperreflective areas as observed with IVCM [8, 18–21]. Previous literatures-based IVCM analysis suggested that more wound healing might have happened in these patients due to stromal edema with highly light reflective clumps of deposits [22–25]. Due to enriched keratocyte cells and collagen lamellae, the anterior stroma is crucially affected by keratan deposits along with the loss of Bowman's layer and nerve fibers compared with the posterior stroma. Deep stromal involvement, Bowman's layer depletion, and changes in collagen fibril organization might result in early loss of vision which may lead to PKP in the early stage of life.

Though, this cumulative study shed some insights into the association between corneal microstructural changes of specific *CHST6* mutations of MCD patients. However, additional studies with larger sample size will be useful to increase knowledge toward mutational analysis for understanding the MCD pathogenesis. We did not perceive any other significant genotype and phenotype correlation of MCD based on corneal morphology by in vivo and in vitro analysis.

Conclusion

This study first time revealed that severe corneal microstructural alterations in Macular corneal dystrophy patients identified with deletion of major portion of *CHST6* that included ORF. Further, advanced studies will be crucial to improve the knowledge about MCD pathogenesis toward earlier diagnosis, better treatments and preventive therapy.

Abbreviations

MCD	Macular corneal dystrophy
CHST6	Carbohydrate sulfotransferase 6
C-GlcNAc6ST	N-acetyl-glucosamine-6-sulfotransferase
ORF	Open Reading Frame mutation
PKP	Penetrating keratoplasty
KS	Keratan sulfate
IVCM	In vivo confocal microscope
HC	Histochemistry
IHC	Immunohistochemistry

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Author contributions

DM had designed the work, collected data and wrote the case series. SKB, KR had critically reviewed the case series. All the authors read and approved the final case series.

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Availability of data and materials

All data are available upon request.

Declarations

Ethics approval and consent to participate

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or compare ethical strand.

Consent for publication

Written informed consent was obtained from the family for this publication.

Competing interests

The authors declare that they have no competing interests.

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