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# **Molecular Genetic Study of Egyptian Patients with Macular Corneal Dystrophy**

**Running title: Molecular analysis of Egyptian patients with MCD**

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## **Abstract**

**Aim:** To identify the underlying genetic defect in Egyptian patients with macular corneal dystrophy (MCD).

**Methods:** A clinical and molecular genetic study was performed on eleven patients from six families with MCD. Clinical diagnosis was confirmed by slit lamp biomicroscopy and histopathological examination of corneal buttons following keratoplasty. The coding region of the carbohydrate sulfotransferase (*CHST6*) gene was amplified by polymerase chain reaction (PCR) in all affected subjects. This was followed by direct sequencing and restriction digest analyses. Enzyme-linked immunosorbent assay (ELISA) of antigenic keratan sulfate (KS) in patients' serum was also performed.

**Results:** Six homozygous mutations of which three are novel were identified within the coding region of *CHST6* in six unrelated MCD families. The barely detectable level of antigenic KS in the serum of the affected individuals indicated that they all have MCD type I, including the subtype IA.

**Conclusions:** This is the first report of molecular genetic analysis of MCD in the Egyptian population. Our data indicates the extensive allelic heterogeneity within *CHST6* and further support its essential role in maintaining corneal transparency.

## Introduction

Macular corneal dystrophy (MCD, OMIM 217800), is an autosomal recessive disorder characterised by superficial clouding of the central corneal stroma which progresses to involve the whole corneal thickness.<sup>1,2</sup> The prevalence of MCD varies across the world with the highest incidence in India<sup>3-5</sup> and Saudi Arabia.<sup>6</sup>

Three MCD immunophenotypes, I, IA and II, have been described based on the serum level of sulphated keratan sulphate (KS) and an immunohistochemical evaluation of the corneal tissue.<sup>6-8</sup> Type I and type II are characterised by respective absence and presence of sulphated KS in the serum.<sup>7,8</sup> Subtype IA was described in patients from Saudi Arabia, in which sulphated KS is absent in the serum and cornea but can be detected in the keratocytes.<sup>6</sup>

Mutations within the carbohydrate sulfotransferase (*CHST6*, OMIM 605294) gene on chromosome 16q22 have been identified as the cause of MCD.<sup>9</sup> So far, more than 120 missense, nonsense and frameshift mutations have been reported in MCD type I and type IA.<sup>10-15</sup> In MCD type II patients, large deletions and rearrangements in the upstream region of *CHST6* were initially described.<sup>9</sup> Nonetheless, nucleotide changes within the coding sequence of *CHST6* have been subsequently discovered.<sup>10, 11, 16</sup> Moreover, *CHST6* mutations could not be identified in some MCD cases suggesting genetic heterogeneity as the aetiology in these cases.<sup>10, 13</sup>

The aim of this study was to identify the underlying genetic defect in six MCD families of Egyptian origin by undertaking mutation screening of the *CHST6* gene together with antigenic detection of sulphated KS.

## **Methods**

### **Patients and Clinical Evaluation**

Twenty four members from six consanguineous families (**figure 1**) with MCD and with no systemic manifestations in addition to 100 control individuals of Egyptian origin were included in the study. An informed consent was obtained from all participants for clinical and molecular genetic studies. The research followed the tenets of the Declaration of Helsinki.

Full ophthalmological examination was performed by MFE and OS. Clinical diagnosis was confirmed by slit lamp biomicroscopy and histopathologic examination of corneal buttons from all patients after keratoplasty.

### **Molecular diagnosis**

#### ***DNA extraction and PCR amplification***

Genomic DNA was extracted from peripheral blood lymphocytes according to the standard procedures. The coding region of *CHST6* was amplified by polymerase chain reaction (PCR) using the primers we have previously published.<sup>17</sup> Each PCR was performed in 25- $\mu$ l reaction mixture containing genomic DNA (100 ng), primers (0.4  $\mu$ M each), MgCl<sub>2</sub> (1.5-2.5 mM), deoxynucleoside triphosphate (dNTPs; 0.2 mM), 1xPCR buffer (Bioline, UK) and Taq polymerase (0.5 U; Bioline, UK). Amplification reactions were performed under the following conditions: 3 minutes of denaturation at 94 °C followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 57 to 65 °C for 30 seconds, extension at 72 °C for 30 seconds, and a further extension step at 72 °C for 5 minutes. For GC rich regions, buffer 3 (containing 22.5 mM MgCl<sub>2</sub>, supplied with Boehringer Mannheim GmbH-Germany) together with dimethyl sulphoxide DMSO 5%

(Sigma Aldrich, UK) or Ready mix (AB-0795; ABgene, UK) were used. Also, KOD Hot Start DNA Polymerase (Novagen, UK) was used whenever necessary.

### ***Mutation detection***

Initially, PCR products were purified by adding Exosap [1 U shrimp alkaline phosphatase (SAP, QAmersham LifeScience, Buckinghamshire, UK; ExoSAP-IT®, USB Corporation) and 1 U Exonuclease I (United States Biochemicals, Ohio, USA) to 1 µl of the PCR product and incubated at 37 °C for 15 minutes then at 80 °C for another 15 minutes to deactivate the enzyme. Sequencing reaction was then performed as previously described.<sup>18</sup> Direct sequence analysis of all probands was performed on the automated fluorescence DNA sequencer (ABI 3730, Applied Biosystems), according to manufacture's instructions. Subsequently, the data were analysed using SeqMan<sup>TM</sup> 4.03 software (DNASTAR Inc).

All changes were assigned a nucleotide number starting at the first translation base of *CHST6* according to the GenBank entry NM\_021615.

### ***Confirmation of mutations***

A restriction enzyme digest analysis was performed as an independent method to study the segregation of one of the novel mutations and to confirm its absence in the matching control population. The first pair of mutation detection primers were used to PCR-amplify a 500 bp product, which was subsequently digested for an hour at 37°C with 1 unit *SacI* enzyme (New England BioLabs, UK) and analysed on 2% agarose gel. The restriction enzyme reaction was carried out as previously described.<sup>18</sup>

For mutations that did not create or abolish a restriction enzyme site, direct sequence analysis was used to test their segregation within the family and to prove their absence in control population.

### **Multiple Sequence alignment**

To assess the significance of the identified changes, an alignment of the amino acid sequence of *CHST6* with other carbohydrate sulfotransferases using CLUSTAL W (1.82) program was carried out.

### **Assay of sulfated KS in the serum**

The concentration of sulfated KS in the serum of MCD patients was determined by an indirect enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody 5D4 (ICN Biochemicals Ltd., Basingstoke, UK), as previously described.<sup>19</sup> The plates were read at 410 nm with a reference wavelength of 490 nm on a flow plate reader (ICN Biochemicals Ltd.). The ELISA was sensitive to KS concentrations of 10 to 10,000 ng/ml.

## Results

### *Clinical examination*

Slit lamp examination of all affected individuals revealed bilateral superficial stromal cloudiness studded by small, irregular, rounded gray-white opacities affecting both the centre and periphery of the cornea typical of MCD. Each family had a pedigree structure consistent with the autosomal recessive pattern of inheritance (**figure 1**).

### *Mutation screening*

Sequence analysis of the *CHST6* coding region led to the identification of six different mutations in six patients with MCD (Table 1). All were homozygous which is consistent with the recessive pattern of inheritance in the families studied.

Four of the identified mutations are missense, **p.L22P**, **p.R50S**, **p.D221E** and **p.E274K**, and were detected in families A, B, C and D; respectively. In family A, a novel **c.65T>C** transition at the second nucleotide position of codon 22 was identified, resulting in a leucine to proline substitution (**figure 2A&B**). A previously unreported **c.148C>A** transversion at the first nucleotide position of codon 50 was found, with an arginine to serine substitution in family B (**figure 2C&D**). In a third family (C) a previously reported **c.663C>G** transversion, changed the amino acid from aspartic to glutamic acid at the third nucleotide position of codon 221<sup>4,5</sup> (**figure 2E&F**). The fourth missense change, was previously identified in MCD patients from India and Japan<sup>3,9</sup> as a **c.820G>A** transition at the first nucleotide position of codon 274 and was detected in the proband of family D leading to a glutamic acid to lysine substitution (**figure 2G&H**). The remaining two mutations were deletions: 1) the first was a novel 2-bp deletion at nucleotide position 606\_607 (**figure 2I&J**) in family E leading to a frameshift and premature termination



codon; 2) the second was a deletion of the *CHST6* gene open reading frame (ORF) in the sixth family (F) which was previously reported in patients from Japan, India and United States.<sup>3, 11, 12</sup> The ORF deletion was identified by the respective absence and presence of PCR products for the *CHST6* coding region in the affected and unaffected members of family F (**figure 3 A**).

#### ***Pathogenicity of mutations***

None of the novel changes was detected in 100 ethnically matching control individuals, as determined by restriction enzyme digest and/or direct sequence analysis, confirming that they are likely to be pathogenic mutations. For example, p.R50S mutation in family B was shown to segregate with the disease phenotype by restriction enzyme analysis (**figure 3B**).

#### ***Conservation of mutations***

Multiple sequence alignment revealed that all the identified missense mutations were either highly conserved across other carbohydrate sulfotransferases or located within the hydrophobic site or the 5' and 3' –phosphate binding (PB) domains of *CHST6* (**figure 4**).

#### ***Sulphated KS level in patient's serum***

All patients who participated in the study had MCD type I since the concentration of sulfated KS in their serum was less than 10 ng/ml (**table 1**) in comparison to the control individuals whose sulfated KS level was within the normal range (112-617 ng/ml).<sup>20</sup>

## Discussion

Herein, we report on the mutation screening of *CHST6* gene in six Egyptian families with macular corneal dystrophy type I. Six independent mutations, four missense and two deletions, were identified. Of these three were novel whereas the others were previously reported in MCD patients from India, Japan and United States.<sup>3-5, 9, 10</sup>

All missense mutations detected in this study occurred at positions in the protein where the residue type is highly conserved across carbohydrate sulfotransferases and/or within important domains that are essential for maintaining the structure and function of the protein (**figure 4**). For example, the non-conservative p.R50S substitution is located within the 5'-PB domain, an essential part of the active site responsible for adenosine 3'-phosphate 5'-phosphosulfate (PAPS) binding. This is essential for forming the vital active site of the enzyme by donating the sulfate that is required for the sulfotransferases activity.<sup>21</sup> Similarly, the p.E274K involving non-conservative substitution of acidic for basic residues is highly conserved across other carbohydrate sulfotransferases.

On the other hand, the previously unreported p.L22P involved substitution of chemically similar amino acid nevertheless its position within the hydrophobic site of CHST6 could be sufficient to alter the protein stability. Likewise, p.D221E which was previously reported in MCD patients from India<sup>4, 5</sup> represents an example of chemically similar amino acid substitution causing MCD phenotype. Moreover, we have previously shown that the conservative p.A206V mutation is when modeled on the crystal structure of mouse estrogen sulfotransferase, result in a structural change at a highly sensitive region of the protein.<sup>17</sup> Hence, this is denoting that chemically related amino acid changes within *CHST6* can be disease causing.

The two deletions identified here (**table 1**) are both predicted to lead to premature stop codons. Therefore, the disease mechanism in these families could be due to complete absence of a functional protein since mRNA with premature stop codons are known to be unstable and eventually undergo nonsense mediated decay.<sup>22</sup> These mutations may therefore be expected to be associated with an early onset and/or severe form of MCD. However, we did not observe any correlation between types of *CHST6* mutations and the clinical phenotype of MCD patients.

Overall, the number of missense mutations predicted to lead to a conserved amino acid change or affect important domains within *CHST6* in our study is two times that for nucleotide deletions. Even though the number of patients we have studied may not be statistically significant for this conclusion but this is consistent with previous results.<sup>10</sup> Moreover, the number of mutations we and others have previously identified in MCD patients of multiracial origin<sup>10, 11-15</sup> in addition to those reported here denotes strong allelic heterogeneity within *CHST6*.

In conclusion, this is the first report of a genetic study of MCD families from Egypt. Six independent mutations have been identified in six families with MCD type I. Three mutations were novel implying that they could be private for the Egyptian population. Recruiting additional families from with MCD phenotype is underway in order to study the mutational spectrum of *CHST6* in MCD patients of Egyptian origin.

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## Figure legends

**Figure 1:** MCD families who participated in the study. Open and closed symbols indicate unaffected and affected individuals, respectively. Deceased family members are denoted by diagonal slashes, asterisks highlight individuals examined both clinically and genetically and arrows mark the probands in each family. Consanguineous marriages are denoted by double lines.

**Figure 2:** Electropherograms of *CHST6* gene mutations in the probands of MCD families and the corresponding normal sequence in controls. (A) Normal sequence at nucleotide position 65 (B) homozygous c.65T>C in the proband of family A (C) Normal sequence at nucleotide position 148 (D) homozygous c.148C>A in the proband of family B (E) Normal sequence at nucleotide position 663 (F) homozygous c.663C>G in the proband of family C (G) Normal sequence at nucleotide position 820 (H) homozygous c.820G>A in the proband of family D (I) Normal sequence at nucleotide positions 606 and 607 (J) homozygous delGC between nucleotide positions 606 and 607 in the proband of family E.

**Figure 3:** (A) Cosegregation of *CHST6* open reading frame deletion within family F using 4 pairs of primers spanning the whole coding region of the gene (a-d). Lanes 1-3 with absent PCR products represent affected members whereas unaffected siblings (lanes 4-8) showed PCR products corresponding to the expected size of the amplified fragments. (B) *SacI* restriction digest analysis in family B showing segregation of the p.R50S mutation. The recognition sequence for the enzyme is 5`...GAGCTC...3`, which identify the mutant-type allele only. Hence, all affected individuals (lanes 1, 2, 7 and 8) are



completely digested (300 and 200 bp) representing the mutant homozygous allele (A/A). Lanes 3-5 (Heterozygous parents and the proband's brother) on the other hand show partially digested products (500, 300 and 200 bp) representing both mutant (A/A) and wild (C/C) type alleles. Lane 6 (the proband's normal sister) show a completely undigested product (500 bp) depicting the wild type allele (C/C). M is a one Kb smart ladder.

**Figure 4:** Alignment of human CHST6 and other carbohydrate sulfotransferases peptide sequences using CLUSTAL 2.0.10 multiple sequence alignment software. The positions of missense mutations in MCD patients reported in this study are boxed. The hydrophobic site and the 5' and 3' PB site motifs are also shown.

**Table 1:** Summary of mutations identified in the *CHST6* gene in Egyptian families with MCD type I

Family ID/ patient no.	Serum KS level (ng/ml)	DNA change	Protein change	Type of protein change	Type of mutation	Status of the change
A/II-1	<3	c.65T>C	p.L22P	Non polar→Non Polar	Missense	Homozygous
B/II-2	0	c.148C>A	p.R50S	Basic→Uncharged	Missense	Homozygous
C/II-1	<9	c.663C>G	p.D221E	Acidic→Acidic	Missense	Homozygous
D/II-1	<3	c.820G>A	p.E274K	Acidic→Basic	Missense	Homozygous
E/II-2	<2	c.606_607delGC	p.R202RfsX18	N/A	Frameshift	Homozygous
F/II-3	<6	delORF	Major	N/A	Deletion ORF	Homozygous

ID: identification; no: number; KS: keratan sulphate; ORF: open reading frame; N/A: not applicable

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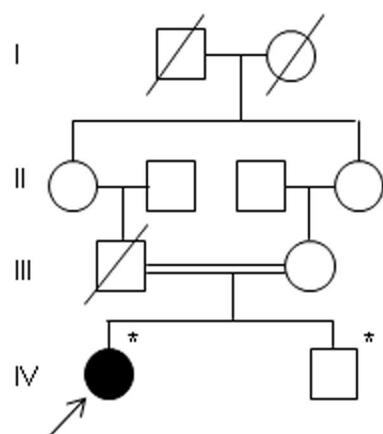
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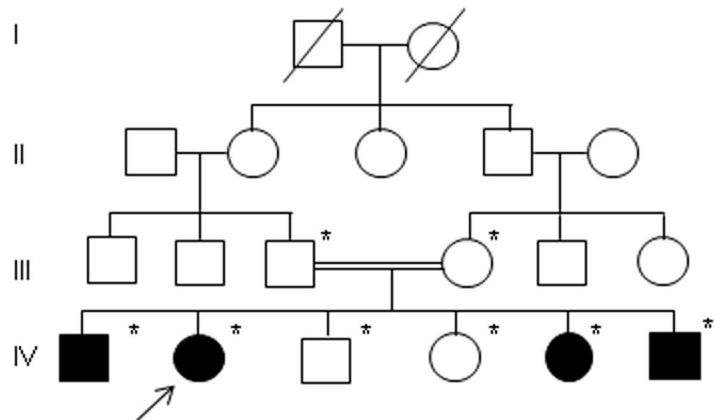
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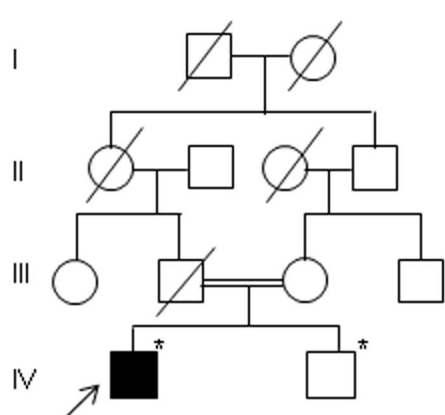
Family A



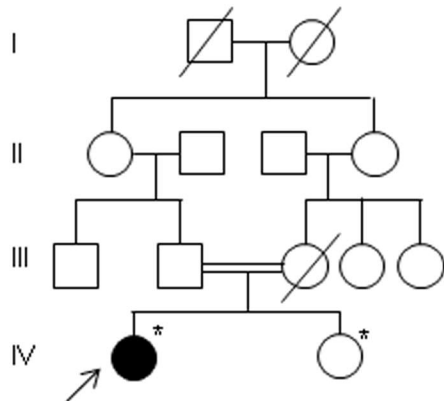
Family B



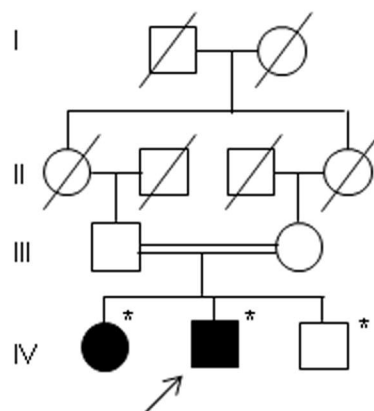
Family C



Family D



Family E



Family F

