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Genetic Analysis of *CHST6* Gene in Indian Families with Macular Corneal Dystrophy

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Abstract

Background: Unlike the western world, Macular Corneal Dystrophy (MCD) is the most common corneal stromal dystrophy in India. It is caused by mutations in the carbohydrate sulfotransferase 6 (*CHST6*) gene. So far, there are limited numbers of reports on *CHST6* screening. Therefore, our screening of *CHST6* gene in Indian families with MCD will be useful for genetic diagnosis, carrier detection and genetic counseling to families included in this study and other families with similar disease condition.

Purpose: The main purpose of this study was to perform the genetic analysis of 55 Indian MCD families.

Methods: We have recruited 55 affected, 11 unaffected members from 55 MCD families along with 100 controls. All the study subjects underwent ocular examination before collecting the blood for the screening of *CHST6* gene. Polymerase chain reaction was performed followed by bi-directional sequencing. The novel mutations were predicted by Polyphene-2, SIFT, Mutation taster and SOPMA tool.

Results: We identified 14 different mutations, 3 known SNP's in 44 MCD patients amongst 6 were novel. Also 2 hotspot mutations were identified amongst the 14 mutations. We could not identify any mutations in the coding region of *CHST6* gene in 11 MCD patients (20%).

Conclusion: Our study identified six novel mutations which will add up to the list of already known mutations identified in different ethnic populations. Our study also increases the mutational landscape of *CHST6* gene. We concluded due to genetic heterogeneity there might be some other gene involved in Indian MCD patients who are negative for *CHST6* mutations.

Keywords: *CHST6* gene; Cornea; Heterogeneity; Mutations;

Abbreviations

MCD: Macular Corneal Dystrophy; *CHST6*: Carbohydrate Sulfotransferase 6; SIFT: Sorting Intolerant From Tolerant; SOPMA: Self-Optimized Prediction Method with Alignment;

Introduction

Macular corneal dystrophy is an autosomal recessive disorder, caused by *CHST6* gene [1]. It was first described by Groenouw in 1890. Due to high degree of consanguinity in some ethnic population, this disorder is highly prevalent in Saudi Arabia and South Indian population [2,3]. The Clinical manifestations of MCD usually occurs by irregular, focal haze formation that leads to grey-white opacities eventually leading to decreased visual acuity by the fifth decade of life [4]. Slit lamp view of cornea of patient with MCD showing diffusely distributed, rounded stromal opacities (Figure 1). In early stages, Photo Therapeutic Keratectomy (PTK) can be done, whereas in advanced disease stage, it requires full thickness or deep anterior lamellar keratoplasty [5,6]. The recurrence rate of corneal opacities is more than 40% in macular corneal grafts even after 10 years of Penetrating Keratoplasty (PKP) [7].

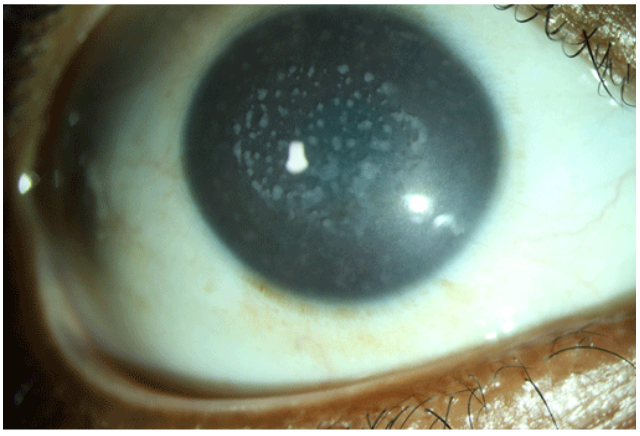

Figure 1: Slit lamp view of cornea with macular dystrophy demonstrating multiple irregular grey-white opacities with intervening central stromal haze.

CHST6 gene is the only candidate gene so far known in MCD and has been further screened in different ethnic populations across the world [2,3,8-17]. It is located on chromosome 16q22 [18,19]. It encodes a golgi resident enzyme N-acetyl glucosamine-6-O-sulfotransferase (C-GlcNac-6-ST) that catalyses the sulfation of keratan sulfate (KS) essential for corneal transparency [20-22]. Defect in *CHST6* gene results in unsulfated keratan sulfate deposition eventually leading to MCD phenotype [23].

Based on the histochemical features, MCD is classified into three immunophenotypes I, II and IA [24-26]. Type I is characterized by absence or low level of sulfated KS (AgKS) in cornea as well as serum. Type II is characterized by normal or marginally reduced level of AgKS in cornea as well as serum. Type IA is characterized by low level of antigenic AgKS in serum and detectable level in keratocytes [26].

The main purpose of this study was to screen the coding region of *CHST6* gene in 55 Indian families with MCD.

Materials and Methods

Patients

The subjects for this study (cases and controls) were recruited from the outpatient services of a tertiary eye care hospital in South India (Aravind Eye Care System, Madurai, India). The study adhered to the tenets of the Declaration of Helsinki, and ethics committee approval was obtained from the Institutional Ethics Committee of the Aravind Eye Care System. All subjects read and signed informed consent except for illiterate subjects, who had the information leaflet read out and provided a thumb impression for participation consent.

Genetic Analysis

DNA extraction and PCR amplification

With informed consent from the study subjects, 5 ml of blood was collected from all the study subjects and genomic DNA was extracted by salting out method [27]. There are four coding regions in *CHST6* gene [18,19]. Out of these, exon 3 is unique as already reported in several independent studies. Based on the uniqueness of exon 3, we have also targeted the exon 3 by using predesigned primers [16]. 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 minutes at 96°C, followed by 37 cycles of 96°C for 30 seconds, 60°C for 30 seconds of annealing, 72°C for 45 seconds with a final extension at 72°C for 5 minutes

Sanger Sequencing

The amplified DNA products were purified by QIA quick PCR purification kit method (Bio Basic Inc.,) followed by cyclic PCR. Bi-directional sequencing was performed (3130 Genetic Analyser; Applied Biosystems) and the results were compared with the reference sequences of *CHST6* gene using BLAST and Chromas lite (2.1) software.

Bioinformatics Analysis

The Self-Optimized Prediction Method with Alignment (SOPMA) tool (https://npsa prabi.ibcp.fr/NPSA/npsa_sopma.html) was used for the prediction of secondary structure of *CHST6* protein. In addition, Polyphene 2, SIFT and Mutation taster were used for predicting the pathogenicity of novel mutations identified in *CHST6* gene and also the conservation of the novel mutations identified was being analysed by CLUSTALW (1.2.2) multiple sequence alignment software (http://www.clustal.org/omega).

Results

We have recruited 55 families with MCD at the cornea unit of Aravind eye care system. Out of the 55 families, 30 families were carrying history of consanguineous marriages. All the study subjects were screened for *CHST6* gene mutations. Interestingly, we identified 6 novel mutations (Ser53X, Ser81X, Val172Met, Arg202His, Ser248Asp, Glu274Gln) in 7 different MCD families and 8 known mutations (H42Y, S53L, R93H, R127C, Q58X, V66VfsX3, Q182RfsX198, N194_196delinsRC) in 29 different MCD families. Also we have identified 3 known single nucleotide polymorphisms (R50C, R205Q, R205W) in 8 families described in Table 1. Rest of the unaffected 11 family members was negative for these mutations. In addition, 100 age matched controls were also negative for these mutations. We considered 6 novel sequence variants as mutations based on the following criteria:

1. The six novel changes were segregating in the family in an autosomal recessive fashion.

2. All the six novel mutations were absent in the following databases (Ensemble, HGVS, 1000 genome project)

3. Three of the novel missense mutations were highly conserved (Val172Met, Ser248Asn, Glu274Gln) and one novel missense mutation was not conserved (Arg202His) across different orthologous species (Figure 2)

4. Mutation taster predicted all the novel mutations to be disease causing and the polyphen2, SIFT programme predicted all are damaging.

A novel homozygous missense mutation Val172Met was identified in a patient from family 5 with consanguinity, Arg202His was identified in a patient from family 42 with consanguinity, Ser248Asn was present in a patient from family 25 with consanguinity, Glu274Gln was identified in a patient from family 9 with consanguinity (Table 1). Additionally, a novel homozygous nonsense mutation Ser53X was identified in 2 patients (patient & patient's younger brother) from family 8 without consanguinity but with MCD history (Figure 3a). One more novel homozygous nonsense mutation Ser81X was identified in a patient from family 36 without consanguinity and MCD history.

Table 1: Clinical Details of the MCD Patients with Mutations

Family/ Patient number	Mutation[a]	Mutation type	Amino acid R-group change	Age(yrs)/ Sex		Novel/ Previously reported[b]
1	No mutation			32/M	No	
2	c.614G>A (p.R205Q) dbSNP:rs377706989	Homozygous missense	Basic to polar	35/M	No	India
3	c.172C>T (p.Q58X)	Homozygous nonsense	Basic to Polar	44/M	Yes	France
4	No mutation			26/M	No	
5	c.514G>A (p.V172M)	Homozygous missense	Non-polar to non-polar	44/M	Yes	Novel
6	c.198delC (p.V66VfsX3)	Heterozygous deletion	Non-polar to non-polar	18/M	No	India
7	No mutation			10/M	Yes	
8	c.158C>A (p.S53X)	Homozygous nonsense	Polar to stop codon	30/M	No	Novel
9	c.820G>C (p.E274Q)	Homozygous missense	Acidic to polar	60/M	Yes	Novel
10	No mutation			66/M	No	
11	c.614G>A (p.R205Q) dbSNP:rs377706989	Heterozygous missense	Basic to polar	23/M	No	India
12	c.158C>T (p.S53L)	Homozygous missense	Polar to basic	32/M	Yes	Hotspot mutation India, America
13	No mutation			50/F	Yes	
14	c.545delA (p.Q182RfsX198)	Heterozygous deletion	Polar to basic	47/F	Yes	Hotspot mutation India
15	c.158C>T (p.S53L)	Homozygous missense	Polar to non-polar	16/F	Yes	Hotspot mutation India, America
16	c.158C>T (p.S53L)	Homozygous missense	Polar to non-polar	32/M	Yes	Hotspot mutation India, America
17	c.379C>T (p.R127C)	Homozygous missense	Basic to polar	33/M	No	Saudi Arabia, India
18	No mutation			20/M	Yes	
19	No mutation			38/F	Yes	
20	c.545delA (p.Q182RfsX198)	Homozygous deletion	Polar to basic	28/F	Yes	Hotspot mutation India
21	No mutation			26/F	No	
22	c.545delA (p.Q182RfsX198)	Homozygous deletion	Polar to basic	27/F	No	Hotspot mutation India
23	No mutation			28/F	Yes	
24	c.545delA (p.Q182RfsX198)	Homozygous deletion	Polar to basic	19/F	Yes	Hotspot mutation India
25	c.743G>A (p.S248N)	Homozygous missense	Polar to Polar	37/M	Yes	Novel
26	c.614G>A (p.R205Q)	Homozygous missense	Basic to polar	12/M	No	India

27	c.124C>T (p.H42Y)	Homozygous missense	Basic to polar	30/F	No	India
28	c.820G>C (p.E274Q)	Heterozygous missense	Acidic to polar	47/F	Yes	India
29	c.148C>T (p.R50C) dbSNP:rs28937877	Homozygous missense	Basic to polar	40/M	No	India
30	c.379C>T (p.R127C)	Homozygous missense	Basic to polar	22/M	No	Saudi Arabia, India
31	c.614G>A (p.R205Q) dbSNP:rs377706989	Homozygous missense	Basic to polar	52/F	No	India
32	No mutation			52/F	Yes	
33	c.158C>T (p.S53L)	Homozygous missense	Polar to non-polar	20/M	No	Hotspot mutation India, America
34	c.158C>T (p.S53L)	Homozygous missense	Polar to non-polar	35/F	Yes	Hotspot mutation India, America
35	c.545delA (p.Q182RfsX198)	Heterozygous deletion	Polar to Basic	50/M	No	Hotspot mutation India
36	c.242C>A (p.S81X)	Homozygous nonsense	Polar to stop codon	33/M	No	Novel
37	c.613C>T (p.R205W) dbSNP:rs750219546	Homozygous missense	Basic to non-polar	46/M	No	Korea
38	c.545delA (p.Q182RfsX198)	Homozygous deletion	Polar to basic	30/F	Yes	Hotspot mutation India
39	c.545delA (p.Q182RfsX198)	Homozygous deletion	Polar to basic	22/M	Yes	Hotspot mutation India
40	c.158C>T (p.S53L)	Homozygous missense	Polar to non-polar	30/M	Yes	Hotspot mutation India, America
41	c.148C>T (p.R50C) dbSNP:rs28937877	Homozygous missense	Basic to polar	25/F	No	India
42	c.290G>A (p.R202H)	Homozygous missense	Basic to basic	21/M	Yes	Novel
43	c.545delA (p.Q182RfsX198)	Homozygous deletion	Polar to basic	36/M	No	Hotspot mutation India
44	c.545delA (p.Q182RfsX198)	Heterozygous deletion	Polar to basic	61/M	No	Hotspot mutation India
45	c.158C>T (p.S53L)	Homozygous missense	Polar to non-polar	25/M	Yes	Hotspot mutation India, America
46	c.545delA (p.Q182RfsX198)	Heterozygous deletion	Polar to basic	60/M	No	Hotspot mutation India
47	c.545delA (p.Q182RfsX198)	Homozygous deletion	Polar to basic	27/F	Yes	Hotspot mutation India
48	c.158C>T (p.S53L)	Homozygous missense	Polar to non-polar	38/F	Yes	Hotspot mutation India, America
49	c.158C>T (p.S53L)	Homozygous missense	Polar to non-polar	31/M	Yes	Hotspot mutation India, America

50	c.158C>T (p.S53L)	Homozygous missense	Polar to non-polar	17/F	No	Hotspot mutation India, America
51	c.581_586 delACCTACinsGGT (p.N194_R196delinsRC)	Deletioninsertion mutation	Non-polar to polar	39/M	Yes	India
52	No mutation			36/F	Yes	India
53	c.278G>A (p.R93H)	Homozygous missense	Basic to Basic	40/M	Yes	India
54	c.148C>T (p.R50C) dbSNP:rs28937877	Homozygous missense	Basic to polar	37/F	Yes	India
55	c.581_586 delACCTACinsGGT (p.N194_R196delinsRC)	Deletioninsertion mutation	Non-polar to polar	44/M	No	India

Table 1. Details of CHST6 gene mutations identified in 55 MCD patients. Mutationa represents the nomenclature of mutations according to the current recommendations in http://www.hgvs.org/mutnomen/recs.html#DNA. Reportedb represents the novel or previously reported mutations in the specific population. dbSNP represents single nucleotide polymorphism.

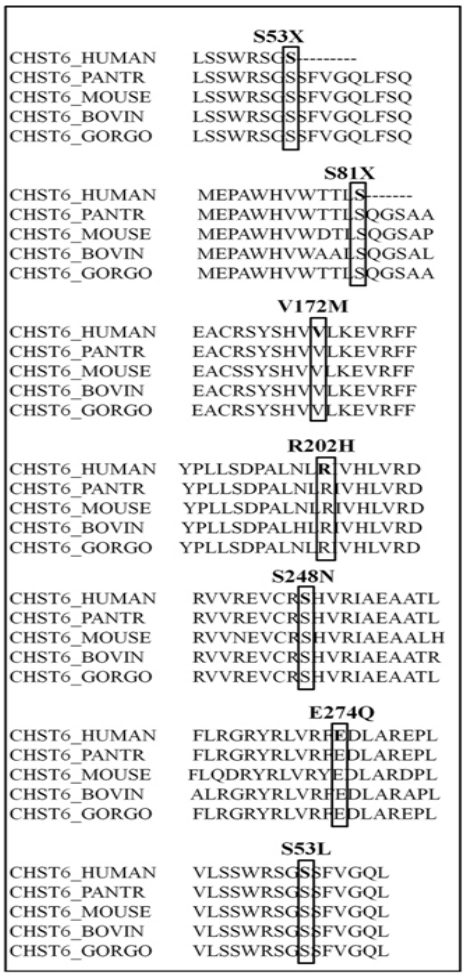

Figure 2: Protein sequence alignment showing the conservation of amino acids in CHST6 among related mammalian species. Boxes indicate the position of mutated residue in orthologs.

In addition, we have identified two hotspot mutations in the coding region of *CHST6* gene i.e. a missense mutation serine-53 to leucine (Ser53Leu) was identified in 10 patients from 10 different families (Figure 3b). We also observed a homozygous single base pair deletion (Glu182ArgfsX198) in 7 patients from 7 different families and heterozygous deletion in 4 patients from 4 different families (Figure 3c). This deletion mutation resulted in frame shift at glutamine-182 with termination after 198 amino acids leading to altered reading frame.

A heterozygous deletion (Q182RfsX198-deletion) mutation was identified in a patient from family 44. Further, pedigree analysis revealed that the propand's father, propand's elder sister and propand's elder brother had the same eye problem (No genomic DNA for analysis). But propand's two sons are phenotypically and genotypically normal. We believe, either it could be uniparentaldisomy or the second mutation may be present in the deep intronic region or regulatory region which was not covered by Sanger sequencing.

Further, a known heterozygous single base pair deletion was also identified in one of the patient from family 6 causing a frame shift at valine-66 (Val66ValfsX3) resulting in a premature termination codon at amino acid residue 2. The second mutation in the patient could be either present in deep intronic or regulatory region of *CHST6* gene which was not covered by Sanger sequencing. Furthermore, in family 3, we have identified a homozygous nonsense mutation (Glu58X) in a patient and his affected sibling that leads to the formation of truncated protein (Table 1). Interestingly, we have also identified known 6 bp deletion (homozygous state) and 3 bp Insertion (Asn194_Arg196delinsArgCys) in two patients belonging to two unrelated families (family 51 and 55) that results in frame shifting at asparagine-194 position (Table 1).

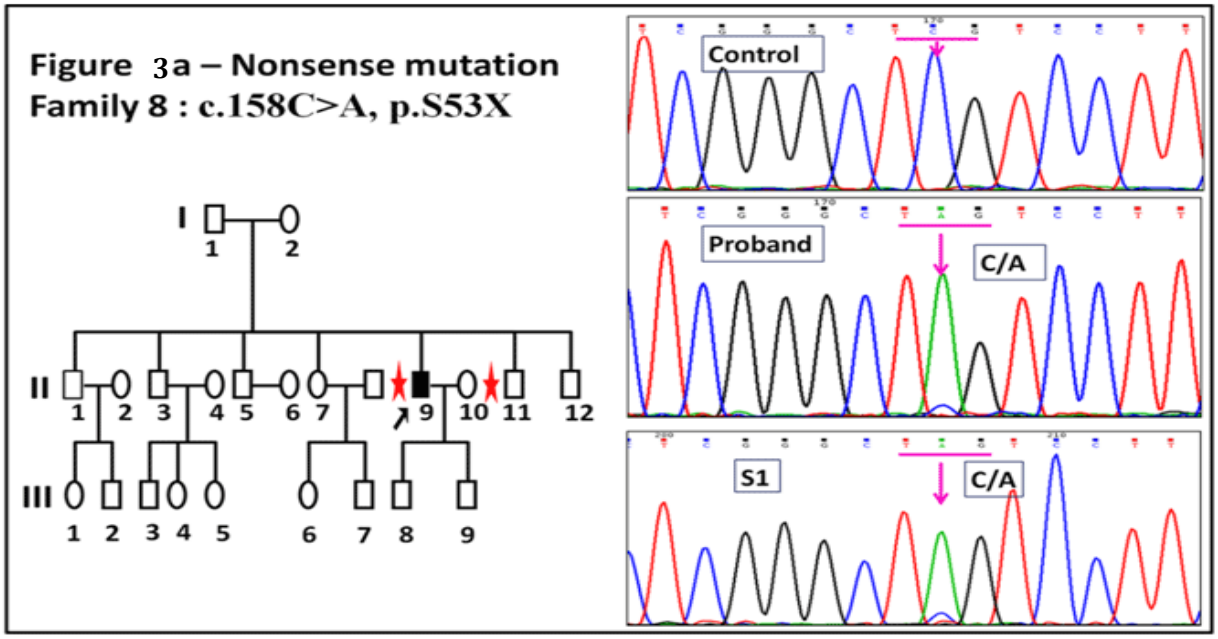

Figure 3a: Mutation analysis of *CHST6* using bi-directional sequencing. I,II,III indicate generation 1,2,3; squares indicate males; circles indicate females; solid square and arrow indicate affected individual; open squares and circles indicate unaffected individuals; parallels indicate consanguinity; asterisk* indicate available sample for analysis; in the chromatogram arrow indicate codon subjected to change, and S1 indicate sibling 1.

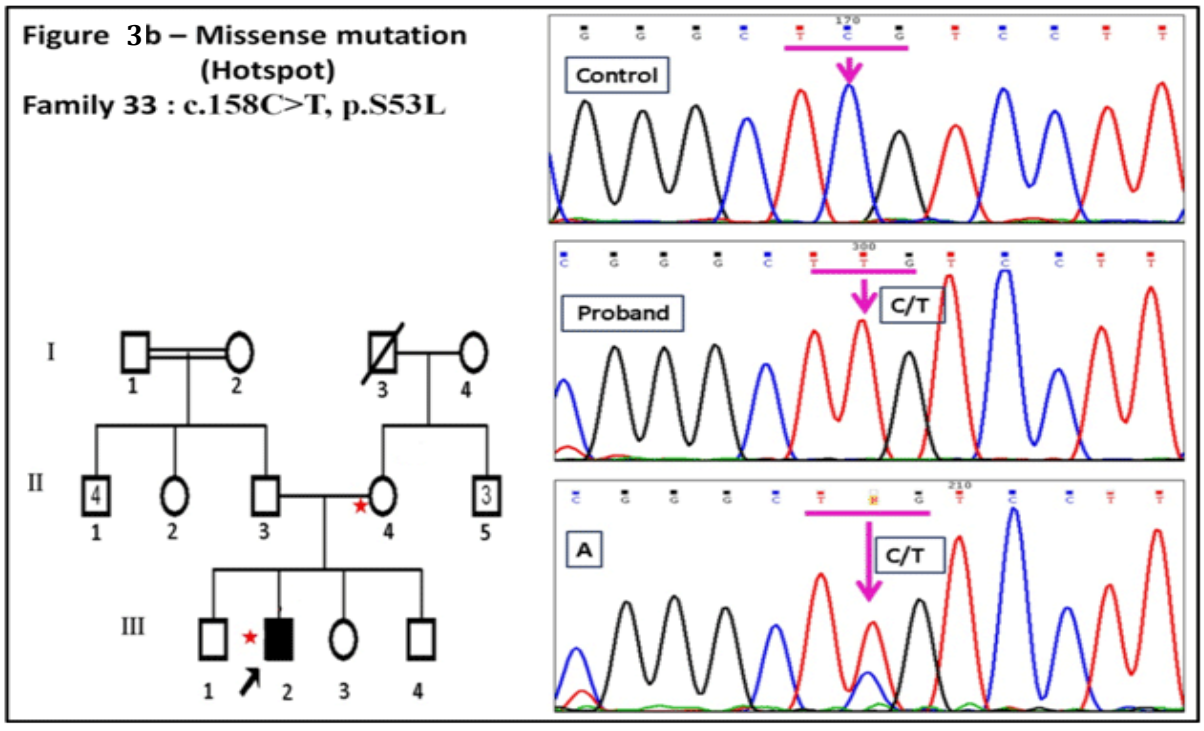

Figure 3b: I,II,III indicate generation 1,2,3; squares indicate males; circles indicate females; solid square and arrow indicate affected individual; open squares and circles indicate unaffected individuals; parallels indicate consanguinity; a slash through a square or circle indicate deceased individuals; asterisk* indicate available sample for analysis; in the chromatogram arrow indicate codon subjected to change, and A indicate propand's mother.

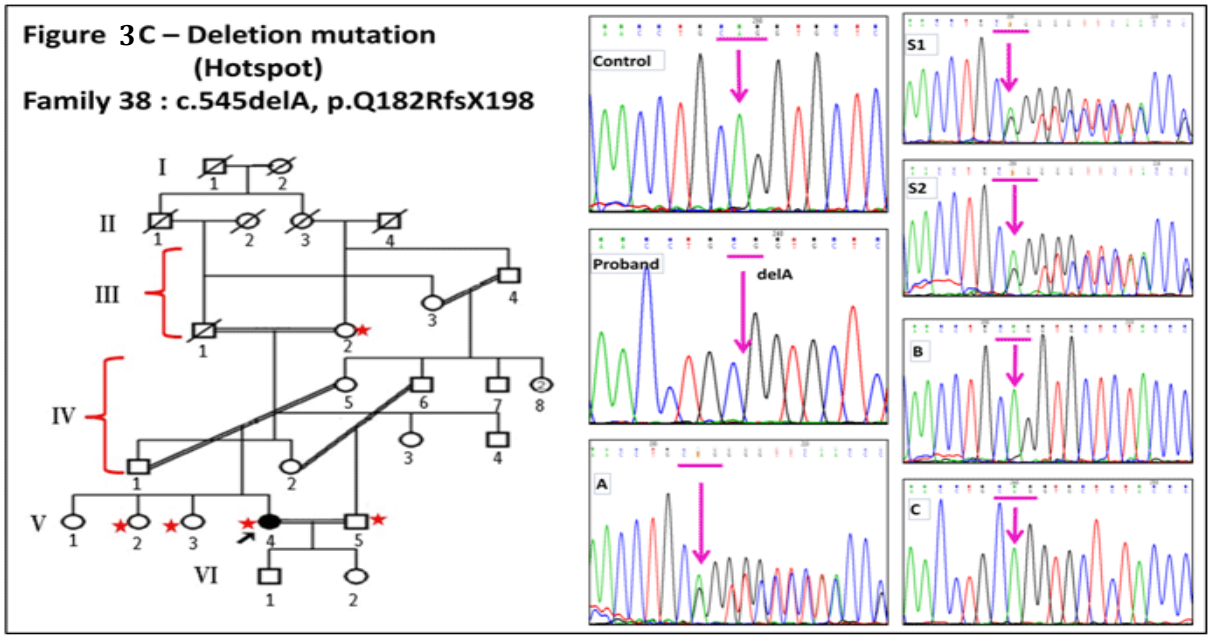

Figure 3c: I,II,III,IV,V,VI indicate generation 1,2,3,4,5,6; squares indicate males; circles indicate females; solid circle and arrow indicate affected individual; asterisk* indicate available sample for analysis; open squares and circles indicate unaffected individuals; parallels indicate consanguinity; a slash through a square or circle indicate deceased individuals; in the chromatogram arrow indicate codon subjected to change, S1,S2 indicate sibling 1,2; A indicate propand's mother; B indicate propand's husband; C indicate propand's grandmother.

Moreover, we have identified 3 known single nucleotide polymorphisms (SNP's) (rs28937877, rs377706989 and rs750219546) in eight different patients (Table 1). Of these, rs750219546 has already been identified in the ethnic cohort of Korean population providing additional support for their study.

Overall, we have sequenced 55 families, out of these families; we failed to identify any mutation in 11 MCD patients. Amongst these, 2 patients from two unrelated families had consanguinity (7,13) in their family, but had no mutations in the entire coding region of *CHST6* gene.

Discussion

In our current study, we have analysed 55 MCD patients including their 11 unaffected family members and 100 controls from the same ethnic background for the screening of *CHST6* gene. Interestingly, we have identified 6 novel mutations in six MCD patients and 11 different known mutations in 38 patients. Previous studies have shown in Saudi Arabian and South Indian population that the prevalence of MCD is high; most probably due to the higher frequency of consanguineous marriage in these ethnic population [2,3]. In our current study, out of 55 families studied, we have also analysed 30 south Indian families with consanguineous marriage.

Table 2: Secondary Structure Analysis using SOPMA

State of amino acid	Control		Nonsense p.S53X		Nonsense p.S81X		Missense p.V172M		Missense p.R202H		Missense p.S248N		Missense p.E274Q		Hotspot-missense p.S53L	
		%		%		%		%		%		%		%		%
Alpha helix	190	48.1	15	28.85	28	35	194	49.11	190	48.1	184	46.58	190	46.1	191	48.35
Extended strand	58	14.68	14	26.92	20	25	54	13.67	58	14.68	50	12.66	58	14.68	58	14.68
β turn	31	7.85	3	5.77	8	10	31	7.85	31	7.85	0	0	31	7	32	8.1
Random coil	116	29.37	20	38.46	24	30	116	29.37	116	29.37	161	40.76	116	30.37	116	28.86
Instability index (II)	US	46.24	US	58.93	US	50.87	US	46.24	US	46.24	US	45.13	US	45.13	US	45.1

Details of the secondary structure of *CHST6* gene proteins. % represents the percentage of each secondary elements present in the protein. II represents the Instability index of unstable protein (US).

CHST6 gene encodes an enzyme C-GlcNac-6-ST which utilizes a sulfate group donor 3'-phospho-5'-adenylyl sulfate (PAPS) to catalyse the transfer of sulfate group to position 6 of N-acetylglucosamine (GlcNac) residues of keratan sulfate [28]. Keratan sulfate plays a central role in the developing cornea for the acquisition of transparency and in the adult cornea for the maintenance of transparency [11,29]. The keratan sulfate is impaired in MCD cornea resulting corneal cloudiness leads to impaired vision.

The identified known mutations have been observed among patients from several populations included India Saudi Arabia, Korea, Egypt, Japan, America and France [2,3,1517,18,23]. This additionally supports our findings showed a high degree of mutational heterogeneity among the patients studied.

We identified 6 novel mutations across the *CHST6* gene in 6 MCD patients as described in the result section. Of these, we have identified a novel missense mutation in patient from the family 9 that leads to the replacement of glutamic acid to glutamine (E274Q) while in an independent studies from different ethnic background (Egypt, Japanese, American) with MCD; they have identified the same missense mutation (E274K) though the glutamic acid was replaced by lysine and the same mutation was identified in a patient from family 28, but in a heterozygous state suggesting that the second mutation may be present in the deep intronic or regulatory region of *CHST6* gene which was not covered by Sanger sequencing [15,18]. This mutation was highly conserved across different orthologous species (Figure 2). Polyphene 2, SIFT and Mutation taster also predicted the mutation was pathogenic in nature. In addition, we have used SOPMA tool that also predicted the un-stability (instability index 45.13) in the protein structure. Moreover, additional evidences also supports our findings that the secondary structure of protein was altered by the missense mutation. Which causes protein un-stability thus leads to deficient enzyme activity [12].

Interestingly, we have also identified a novel homozygous nonsense mutation (S53X) in a patient from family 36 was highly conserved across different orthologous species. Polyphene 2, SIFT and Mutation taster also predicted the mutation was pathogenic. SOPMA also predicted that this cause changes in protein stability (instability index 58.93) that leads to the formation of unstable protein. We have identified one more novel homozygous nonsense mutation (S81X) was highly conserved with the un-stability (instability index 50.87) in the protein structure that leads to the formation of unstable protein. These nonsense mutations may lead to the absence of proteins due to nonsense-mediated decay (NMD) of the mRNA suggesting that these mutations might be expected to be associated with an early onset and/or severe form of MCD affecting both the eyes [14].

In addition, we have identified one novel homozygous missense mutation (R202H). It was not conserved across the species but Polyphene 2, SIFT and Mutation taster predicted the mutation as pathogenic in nature. Though, this particular change was not conserved. However, its mild secondary structural modifications with instability index 46.24 may be responsible for the loss of enzyme activity.

Apart from the novel mutations described above; we have identified 12 known mutations including a hotspot deletion mutation (Q182RfsX198) in the coding region of the *CHST6* gene that causes frameshift changes in the upstream region of *CHST6* gene due to nucleotide sequence similarity of CHST5 and *CHST6* genes and the adjacent regions or it could be due to chromosomal crossover in CHST5 and *CHST6* genes [18]. Previous studies have revealed that frameshift mutations of *CHST6* gene may lead to severe MCD phenotypes with much deeper grey white deposits [30].

Additionally, we have also observed a hotspot missense mutation (S53L) in 10 patients from different MCD families that leads to unstable protein (instability index 45.10) this mutation was already reported in 7 patients from seven South Indian families and the same mutation was identified in an American population suggesting a hotspot mutation [2,12,19]. Previous studies suggested that this mutation might be present in the 3'-phosphate-binding domain of C-GlcNac-6-ST enzyme. This region of the *CHST6* gene contains an active site which might be a mutational hotspot [31].

We failed to identify mutations in 11 patients from 11 different families out of 55 families screened. Warren, et al. also did not identify *CHST6* gene mutations in 4 of 51 families screened suggesting that the mutations in these families may be present in a yet to be identified gene or may be present in a deep intronic or promoter region which can be explored by means of extensive linkage analysis [32]. And also this may be asociated with MCD type II phenotype caused by genetic abnormalities in the upstream of *CHST6* gene [8].

Taken together, our results indicate the high degree of mutational heterogeneity in Indian population. The coding region of *CHST6* gene significantly affected by mutations leads to unstable protein products with altered secondary structure. All the altered amino acid residues are evolutionary conserved among other mammalian species indicating severe functional loss of *CHST6* gene. Functional characterization of these novel mutations identified may help to improve the understanding of the disease pathogenesis.

Conclusion

In conclusion, we identified 6 novels, 8 reported and 2 hotspot mutations in 44 MCD patients from Indian population. Therefore, our screening of *CHST6* gene in Indian families with MCD will be useful for genetic diagnosis, carrier detection and genetic counseling to families included in this study and other families with similar disease condition. In addition, our data showed high degree of allelic and locus heterogeneity exists for MCD.

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Declarations

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B. Conflict of Interest All authors of this manuscript declare that they have no conflict of interest.

C. Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Ethical Committee of the Aravind Eye Care System, Madurai, Tamilnadu, India and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent Informed consent was obtained from all individual participants included in the study.

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