Original Article

Genetic analysis and clinical phenotype of two Indian families with X-linked choroideremia

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Purpose: This study aims to describe the phenotype and genotype of two Indian families affected with X-linked choroideremia (CHM). **Materials and Methods:** In these two families, the affected individuals and unaffected family members underwent a comprehensive ophthalmic examination including an optical coherence tomography (OCT) and electroretinogram. Blood samples were collected from the families for genetic analysis. Next generation sequencing (NGS) was done using a panel of 184 genes, which covered previously associated genes with retinal dystrophies. Sequencing data were analyzed for the *CHM*, *RPGR*, and *RP2* genes that have been implicated in CHM and X-linked retinitis pigmentosa (XLRP), respectively. The identified variants were confirmed by Sanger sequencing in available individuals and unrelated controls. **Results:** In two unrelated male patients, NGS analysis revealed a previously reported 3'-splice site change c.820-1G>C in the *CHM* gene in the first family and hemizygous mutation c.653G>C (p.Ser218X) in the second family. The asymptomatic family members were carriers for these mutations. Spectral domain-OCT showed loss of outer retina, preservation of the inner retina, and choroidal thinning in the affected males and retinal pigment epithelial changes in the asymptomatic carriers. The identified mutations were not present in 100 controls of Indian origin. There were no potential mutations found in XLRP-associated (*RPGR* and *RP2*) genes. **Conclusion:** This report describes the genotype and phenotype findings in patients with CHM from India. The identified genetic mutation leads to lack of Rab escort protein-1 (REP-1) or affects the production of a REP-1 protein that is likely to cause retinal abnormalities in patients.

Key words: Choroideremia gene, genetic analysis, next generation sequencing, X-linked chorioretinal dystrophy

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Choroideremia (CHM) (OMIM 303100) is a rare X-chromosome-linked chorioretinal dystrophy characterized by progressive loss of vision due to degeneration of the choroid, retinal pigment epithelium (RPE), and photoreceptor cells in retina.[1,2] Being an X-linked recessive condition, CHM mainly affects males; carrier females are mostly asymptomatic although those with skewed X-inactivation may become symptomatic later in life. Associated with night blindness and reduced peripheral fields, the disease progresses relentlessly till most affected males are legally blind by midlife. Asymptomatic carrier females show irregular pigmentation throughout the fundus.[3] The global prevalence of the disease is estimated at 1 in 50,000–100,000.[4] The highest incidence of CHM is reported in Northern Finland.[5] *CHM* (#300390), encoding the protein Rab escort protein-1 (REP-1), is the gene associated with CHM. The causative gene has been mapped at Xq21.1-q21.3, which consists of 15 exons and encodes the 653 amino acids of intracellular protein known as REP-1. This is involved (Rab GTPases) in the regulation of intracellular vesicle transport and posttranslational modification in lipid prenylation (Rab geranylgeranyltransferase), in addition to vesicle trafficking in various vesicular compartments of the cell.[6,7] The REP-1 protein is actively expressed in ocular and nonocular tissues such as retina, choroid, RPE, and lymphocytes.[8] Mutations in *CHM* gene result in CHM phenotype. The spectrum of *CHM* mutations includes deletions, translocations, and point mutations (including nonsense, frameshift, and splice site) as well as deep intronic mutations.[9-11] In mice, the *CHM* knock out (KO) is embryonically lethal, but conditional KO mice of REP-1 showed age-related changes in RPE.[12,13] Recombinant adeno-associated virus (AAV)-mediated transduction of human REP-1 - restore the enzyme activity; REP-1 *in vitro* in cells from affected patients (CHM), suggesting that gene therapy approach by AAV vectors may be an appealing treatment strategy.[14,15] In the present study, we report for the first time *CHM* gene mutations and associated clinical features in two Indian patients with CHM.

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Materials and Methods

Patient details and clinical evaluations

This study was approved by the Institutional Ethical Committee and conducted according to the Declaration of Helsinki Principles. All study participants including two patients, two available family members, and 108 normal controls provided a written consent to participate in the study. None of the participants had any systemic illness. There were no hearing or cognitive abnormalities. The proband and family members in two families identified with CHM underwent a complete ophthalmic examination including measurement of visual acuity, intraocular pressure, slit lamp biomicroscopy, detailed fundus examination, fundus photography, fundus autofluorescence (FAF), spectral domain optical coherence tomography (SD-OCT). Four out of the five participants underwent full-field electroretinogram (ERG) according to ISCEV standards.[16] (VERIS systems, USA).

Genetic analysis

A detailed family history was obtained from all participants. DNA was isolated from 5 ml of peripheral blood by the salt precipitation method.[17] Targeted next-generation sequencing (NGS) was carried out using a method described previously that included sample preparation, target enrichment, and exome sequencing data analysis, variant calling.[18] For the variant interpretation, *CHM* (for Choroideremia) and *RPGR* and *RP2* genes (for X-linked retinitis pigmentosa [XLRP]) were included for the annotation, prioritization, and reporting following the ACMG guidelines. To revalidate the potential *CHM* variations in patients and in the available family members, 108 unrelated normal controls were analyzed using polymerase chain reaction (PCR) primers reported elsewhere.[19] The PCR products were sequenced (3500 DX, Lifetech Inc., USA) by Sanger sequencing method. The sequence electropherogram was visualized using the FinchTV software (Geospiza, USA), and the presence of the variation in genes was evaluated by comparing the patient's sequence with the reference sequence (NM_000390.2).

Results

The genetic analysis revealed two previously reported known mutations in the *CHM* gene (p.Ser218X) (c.820-1G>C at intron 6) in two unrelated patients with CHM. There were no potential mutations found in XLRP-associated, *RPGR*, and *RP2* genes.

Family 1: Choroideremia-01

In this family, a 32-year-old male patient (IV: 7) presented with night blindness and defective vision since childhood [Fig. 1]. His vision in both eye was 6/6, N6 on Snellen's charting (RE = −4.00 DS, LE = −3.5 DS). The intraocular pressure and anterior segment examinations in both eyes were normal. The lens was clear. The optic discs were pink with a C/D ratio of 0.1 in both eyes. Bilateral fundus findings included the following: prominent areas of atrophy of both the choroid and RPE in the peripapillary region, patchy pigment clumping, and prominence of large choroidal vessels in the midperiphery, arteriolar attenuation, and scalloped margins of choroidal loss with relative sparing of the central retina. FAF of both maculae showed a widespread loss of the normal autofluorescence. SD-OCT of both eye showed a significant distortion of the outer retinal layers with multiple retinal tubulations [Fig. 2a]. There were no cystoid changes in the macula. Full-field ERGs showed a complete extinction of both the rods and cone responses. His 61-year-old mother was asymptomatic (III: 5), she had undergone cataract surgery a year before this and did not have any significant refractive error before the cataract surgery. Her vision in both eyes was 6/6, N6. Anterior segment examination showed bilateral pseudophakia with a normal intraocular pressure. Fundus examination in both eyes showed widespread RPE alterations with normal retinal and choroidal vessels. FAF both eyes showed patchy hyper- and hypo-autofluorescence throughout the fundus. SD-OCT of both eyes showed significant undulations in the RPE with preservation of all retinal layers [Fig. 2b]. Full-field ERG of both eyes was normal. The 36-year-old sister of the proband was asymptomatic (IV: 5), her vision was 6/6, N6 in both eyes. Intraocular pressures and anterior segments and fundus of both eyes were normal. Both eyes showed a normal autofluorescence and normal layers on the SD-OCT. In the proband (IV: 7), the NGS analysis revealed a reported 3'-splice site (c.820-1G>C) at intron 6 of *CHM* gene on the X-chromosome. Patient's mother

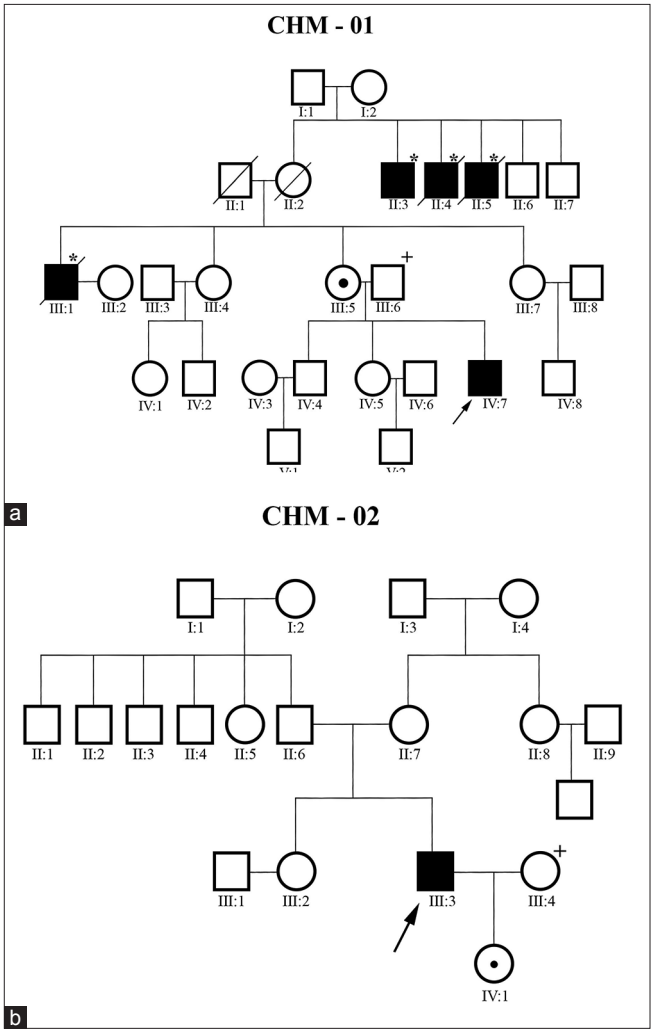

Figure 1: (a and b) Pedigrees of two unrelated families (choroideremia-01, choroideremia-02) with choroideremia. The asterisk denotes the individuals had an unknown cause of blindness. Arrow indicates the proband in each family. The '+' symbol denotes the individuals did not consent for the genetic analysis

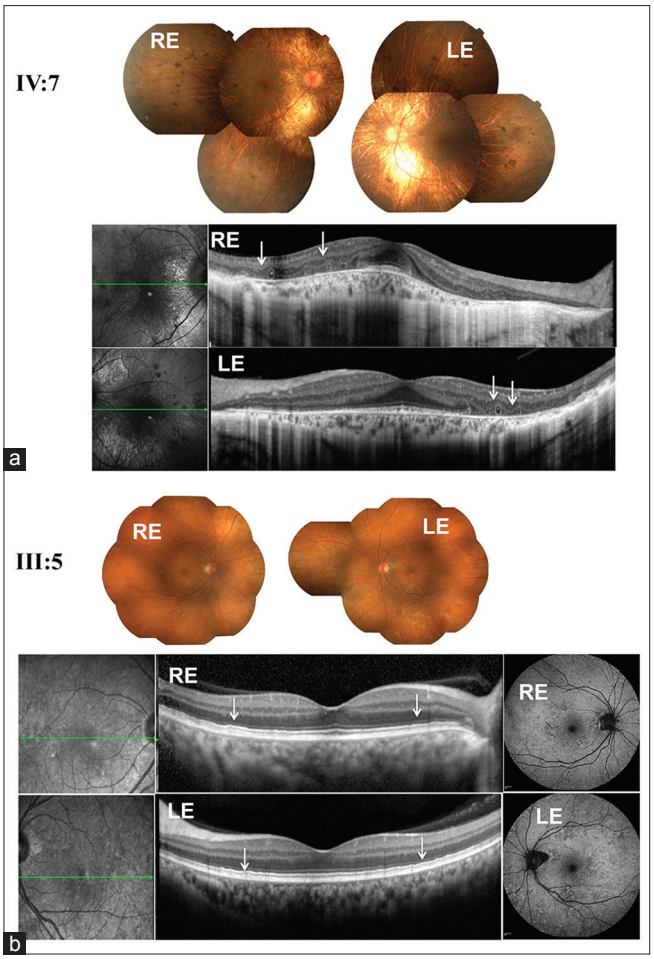

Figure 2: Clinical features from choroideremia-01 family. (a) The fundus pictures of both eyes in patient IV: 7 from family choroideremia-01 (top), lower pictures show the infrared images and the spectral-domain optical coherence tomography of both the eyes. Note the preservation of inner retinal layers, disruption of outer retinal layers, retinal tubulation (white arrows), and choroidal thinning. (b) Fundus pictures, fundus autofluorescence, and the spectral domain optical coherence tomography of the asymptomatic mother (III: 5). Top-fundus pictures of the right and left eyes. Bottom right-fundus autofluorescence of the right and left eyes showed the patchy autofluorescence. Lower left-Infrared and spectral-domain optical coherence tomography images of the eyes showing the retinal pigment epithelial irregularities (white arrows)

(III: 5) was a heterozygous carrier for the mutation which was absent in the patient's sister (IV: 5). This variation leads to altered splice site that results in skipping of exon 7. It codes for amino acids 274 (Val-274) to 314 (Gly-314); however, the codon for Gly314 was shared between exon 7 and exon 8, and thus will lead to a frameshift in the open reading frame. Ophthalmic examination of the proband's father (III: 6) was normal. Historically, the mother's brother and three of her uncles were legally blind (not examined), likely due to advanced CHM.

Family-2: Choroideremia-02

A 39-year-old male patient (III: 3) presented with night blindness since the age of 5 years, other family members had no visual complaints, and there was no history of consanguinity among the parents [Fig. 1]. Ophthalmic examination showed a visual acuity of 6/9, N6 in both eyes (RE = −4.00 DS/−1.75 DC 80°, LE = −4.5 DS/−1.0 DC 110°). Intraocular pressures and anterior segment were normal in both eyes. The lens was clear in both eyes. Fundus evaluation showed normal discs with a C/D ratio of 0.5. Bilateral fundus findings included the following: Areas of prominent loss of choroid and RPE nasal to the disc and arteriolar attenuation with prominent choroidal vessels in the midperiphery. Macula showed patchy pigment clumping, scalloped areas of choroidal loss, and a very small island of normal retina. FAF showed very small areas of hyperautofluorescence at the macula, corresponding to the remaining visual field. SD-OCT showed advanced loss of photoreceptors with choroidal thinning with retinal tubules in the outer nuclear layer at the junction of the normal and atrophic retina. There were a few cystoid changes at the macula in the LE; there were no cystoid changes in the RE. Visual fields showed a small island of remaining visual field on the Humphrey's 10-2 [Fig. 3a]. Full field ERG showed an extinguished rod response with minimal cone response. Genetic analysis of the patient (III: 3) revealed a C to G (c.653C>G) *de novo* hemizygous nucleotide change found in the *CHM* gene. It was a known nonsense mutation resulting in a premature stop codon (p.Ser218X) found at exon 5 in the canonical transcript (NM_0003 90.2). The truncated protein would lack a major portion of 436 amino acids at the C-terminal region of the REP-1. The patient's 13-year-old daughter (IV: 1) was asymptomatic, had a heterozygous change (c.653C>G, p.Ser218X) in the *CHM*. Her uncorrected vision of 6/6, N6 both eyes, intraocular pressure, and anterior segment examination was normal in both eyes. Fundus of both eyes showed normal discs, normal retinal, and choroidal blood vessels with areas of retinal pigment alterations throughout the retina. FAF both eyes showed patchy hyperautofluorescence throughout the fundus. SD-OCT of both eyes showed RPE undulations corresponding to the areas of pigmentation [Fig. 3b]. Full-field ERG in both eyes was normal. Neither the mother nor the sister was available for ophthalmic or genetic evaluation.

Discussion

This is the first report to describe the genotype and phenotype findings in patients with CHM from India. Variety of *CHM* mutations known to cause X-linked inherited or sporadic CHM have been summarized in databases (https://grenada.lumc.nl/LOVD2/Usher_montpellier/home.php?select_db=CHM, http://www.retina-international.org/sci-news/databases/mutation-database/chm-mutation/). *CHM* (REP-1 is ubiquitously expressed, has a vital role in prenylation of Rab proteins, which are essential for vesicle trafficking in endocytic and exocytic pathways. In CHM patients, loss of function of REP-1 due to *CHM* mutations may cause progressive degeneration of choroid, photoreceptors, and RPE.[20,21] The clinical features associated with CHM include night blindness, visual field constriction, reduced visual activity, and progressive chorioretinal degeneration. Some of these clinical features may overlap with XLRP, one of the more severe forms of RP.[22] Symptoms of night blindness and constriction of peripheral visual fields are common in both diseases. High myopia and waxy pallor of discs are more commonly seen in RP. Fundus in CHM is characterized by chorioretinal scalloped atrophy initiated from the midperiphery without affecting the macula till the late stages. In contrast, due to a greater degree of RPE migration seen in RP, bony spicules are commonly

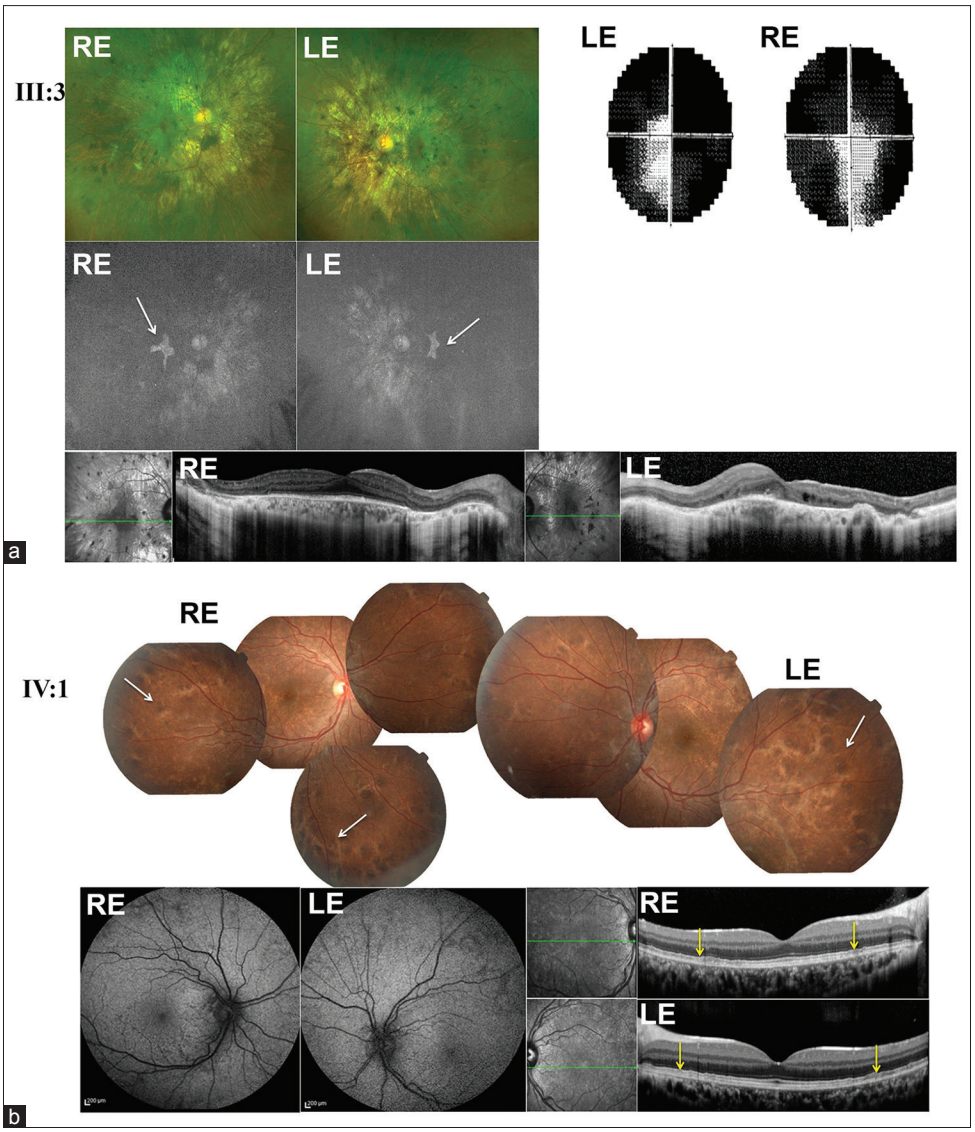

Figure 3: Clinical features from choroideremia-02 family. (a) Clinical details of patient (III: 3). Top left-FP (Optos). Middle-fundus autofluorescence showed a small area of normal AF (white arrows). Lower-spectral domain optical coherence tomography showed the loss of outer retinal layers, choroidal thinning, and preservation of the inner retinal architecture. The LE shows a few cystoid spaces in the macula. Top right: Humphrey visual fields (10-2) showing the remaining extremely small visual fields. (b) Clinical details of the daughter (IV: 1). Top-FP, showing the extensive retinal pigment epithelial pigmentation throughout the fundus (white arrows). Lower left –fundus autofluorescence showing the patchy AF. Lower right-infrared and the spectral-domain optical coherence tomography pictures showing the retinal pigment epithelial irregularity (yellow arrows)

seen throughout the fundus. In early stages of XLRP, the choroid is relatively normal in comparison to CHM where there is extensive chorioretinal degeneration. Carriers of both diseases exhibit characteristic pigmentary alterations in the fundus. Some carriers of XLRP may show golden tapetal-like fundus reflex.

We, therefore, carried out a genetic analysis using the combination of targeted NGS (panel of gene test) and validation with Sanger sequencing on *CHM*, *RPGR*, and *RP2* genes, the latter two associated with XLRP. High-resolution imaging of the choroid is useful to assess the severity and determine the progression of the disease. In both our patients, we noted that photoreceptor degeneration was less severe over the areas of relatively preserved RPE.[23] The latter observation was further confirmed by OCT findings that retinal lamination and thickness remained remarkably normal in areas in which the RPE was preserved in contrast to generalized RPE and choriocapillaris atrophy.[23] It has been shown that RPE changes precede retinal degeneration.[23,24] FAF of both patients (IV: 7, III: 3) showed a very small island of autofluorescence in the macula, corresponding to the area of functioning RPE. FAF of both the carriers showed speckled autofluorescence throughout the fundus. This corresponds to the mosaic pattern of retinal dysfunction known to occur in carriers of CHM,[25] which occurs due to lyonization, the process of random inactivation of X-chromosomes in retinal cells, as in every somatic diploid cell.[26] In patients with CHM, an abnormal thickening has been noted around the parafoveal region on the OCT early in the second decade of life. Observing a significant alteration in the laminar organization of the retina in patients with CHM; Jacobson *et al*. hypothesized that these

corresponded with remodeling of the retina in CHM.[21] The earliest abnormality noted was retinal thickening, explained by photoreceptor damage and stress activating Muller cells, the main glial cells in the retina, noted as retinal thickening on the OCT. In advanced stages, there is photoreceptor dysfunction and death and loss of normal retinal lamination.[21] SD-OCT in both male patients (IV: 7 and III: 3) showed the presence of multiple outer retinal tubulations (ORT), significant choroidal thinning, and relative preservation of the inner retinal layers. There was a central preservation of the photoreceptor layer and external limiting membrane, possibly corresponding to the remaining visual fields. The choroidal thickness underlying areas of preserved photoreceptors appeared normal, with a sharp reduction in the thickness at the transition zone between normal and atrophic photoreceptors. The tubules were seen prominently at the junction where inner segment/outer segment (IS/OS) loss was noted. There was cystoid spaces at the macula distinguishable from the choroidal tubules only in the left eye of the patient (CHM-02_III: 3) in the second family. Zweifel *et al*.[27] first described the "ORT" on the SD-OCT. They hypothesized that these were formed by the rearrangement of the photoreceptor layer in response to retinal injury in many conditions including CHM.[27] These have been known to develop from the gradual invagination of outer retinal structures at the junction of intact and atrophic retina.[28] The unaffected individuals (III: 5, IV: 1) were asymptomatic carriers showed irregularities in RPE and more prominent outside the macula. The IS/OS junction, although undulated, was intact throughout the macula. The RPE showed focal disruption corresponding to the undulations. FAF of the second carrier showed speckled autofluorescence, corresponding to the irregular RPE. In the first family, the patient (IV: 7) had a 3′-splice site (c.820-1G>C) variation at intron 6 of *CHM* gene associated with ORT. Interestingly, this variation was found in heterozygous state in a female with the family history of CHM, showed a detectable level of subretinal hemorrhage in the fundus examinations.[29] Previously, skipping of exon 7 at mRNA level (3′-splice site variation) in *CHM* gene was found in Italian patients with CHM.[30] This variation (c.820-1G>C) found in 3′-splice acceptor site of intron 6 of *CHM* can produce altered or shorted REP-1 protein due to alternate splice event that is the likely cause of retinal abnormalities in the patient. Furthermore, a novel variation (c.819+2T>A), at the donor splicing site of intron 6 of the *CHM,* was found in families with CHM associated with various clinical manifestations indicating the intrafamilial variable expressivity.[31] In the first family (CHM-01), the proband's mother (III: 5) was asymptomatic and showed significant RPE alterations in the fundus examination which is consistent with previous findings.[31]

In the second family (CHM-02), proband (III: 3) showed clinical features of advanced CHM such as hyperautofluorescence at the macula, loss of photoreceptors with choroidal thinning with retinal tubules associated with nonsense mutation (p.Ser218X). This mutation leads to premature stop codon at C-terminal region that forms a part of the GDI domain (Rab GDP dissociation inhibitors)[32] that has functional and regulatory role on posttranslational modification (prenylation) and vesicular membrane trafficking.[33] There was a known mutation (p.Tyr2l7fs*230) at the adjacent position of Ser 2I7 that has been implicated in CHM.[11] Previously, mutation in C-terminal region of REP-1 protein (p.Ser218Lysfs*13) was reported in CHM patients from Chinese and Italian origins.[10,30,34]

Since the clinical features of CHM overlap with XLRP, we suggest that the panel of targeted gene sequencing by NGS may be a convenient tool for genetic testing.[18] Advances in gene therapy and cell-based therapy for CHM holds promise with progress in science.[35,36]

Conclusion

This is the first report to describe the genotype and phenotype findings in two patients with CHM from India. Furthermore, these mutations might play a role in pathogenesis consistent with previously reported genotype/phenotype correlations in patients with CHM. In addition, NGS panel of gene sequencing is an efficient method that is useful in accurate molecular diagnosis of asymptomatic carriers and helps in genetic counseling.

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Conflicts of interest

There are no conflicts of interest.

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