Synonymous Variant in the *CHM* Gene Causes Aberrant Splicing in Choroideremia

Mariana Matioli da Palma,[1] Fabiana Louise Motta,[1] Caio Perez Gomes,[2] Mariana Vallim Salles,[1] João Bosco Pesquero,[2] and Juliana Maria Ferraz Sallum[1,3]

[1]Department of Ophthalmology, Federal University of São Paulo, São Paulo, Brazil
[2]Department of Biophysics, Federal University of São Paulo, São Paulo, Brazil
[3]Instituto de Genética Ocular, São Paulo, Brazil

Correspondence: Juliana Maria Ferraz Sallum, Rua Botucatu, 822 Vila Mariana, São Paulo, Brazil; juliana@pobox.com.

MMP and FLM contributed equally to this work.

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Purpose. Choroideremia is an inherited retinal degeneration caused by 280 different pathogenic variants in the *CHM* gene. Only one silent/synonymous variant (c.1359C>T; p.(Ser453=)) has been reported and was classified as inconclusive based on in silico analysis. This study elucidates the pathogenicity of this variant also found in a Brazilian patient.

Methods. Ophthalmological examinations such as color fundus photography, spectral-domain optical coherence tomography, fundus autofluorescence, and macular integrity assessment microperimetry were performed. The subjects' total RNA was extracted from peripheral blood cells. cDNA was synthesized and the amplification between exon 10 and 14 of the CHM mRNA was performed. The amplification products were sequenced by Sanger sequencing and the results were aligned to the reference sequence.

Results. The synonymous variant c.1359C>T p.(Ser453=) in the *CHM* gene is associated with an error in mRNA processing, leading preferentially to production of an aberrant transcript without exon 11 (p.(Gln451Phefs*3)). This anomalous mRNA production is related to typical choroideremia phenotype.

Conclusions. These molecular findings reinforce the need for more detailed investigation of silent variants in patients with well-defined phenotype of retinal dystrophies. Molecular and clinical findings provided evidence that c.1359C>T (p.(Gln451Phefs*3)) in *CHM* should be considered a disease-causing variant.

Keywords: *CHM* gene, aberrant splicing, synonymous variant, choroideremia

Choroideremia (MIM 303100) is an X-linked recessive retinal degeneration that affects approximately 1:50,000 males.[1,2] The symptoms include early nyctalopia with progressive constriction of peripheral visual fields.[3] The central vision is preserved until approximately 40 years of age, when the central vision loss usually starts.[4,5] Pathogenic variants in the *CHM* gene are related to choroideremia.[1–5]

According to the Human Gene Mutation Database, more than 280 variants in the *CHM* gene have already been associated with choroideremia,[6] but only 1 silent/synonymous variant (c.1359C>T; p.(Ser453=)) has been reported.[7] Until now, there has been no substantial molecular evidence that this variant actually affects the correct splicing process. This synonymous variant also was found in one Brazilian patient who has advanced choroideremia and was classified as inconclusive based on in silico analysis. For this reason, a detailed ophthalmologic investigation and RNA analysis were performed on this patient and his daughter. This study describes the pathogenicity of the c.1359C>T variant.

Methods

Subjects

The Ethics Committee in Research of Federal University of São Paulo approved this study (CEP:1191.0071.10/2018). Both subjects provided written informed consent for the use of personal medical data for scientific purposes and publication. This study was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and its subsequent amendments.

Ophthalmic Investigations

The best corrected visual acuity (BCVA) was measured in each eye using the Early Treatment Diabetic Retinopathy Study charts at 4 m. The following examinations were performed: color fundus photography (VISUCAM 500, Zeiss, Oberkochen, Germany), spectral domain optical coherence tomography (OCT) (Spectralis, Heidelberg Engineering, Heidelberg, Germany), and fundus autofluorescence

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(FAF) (HRA2, Heidelberg Engineering) in the patient and his daughter. Macular integrity assessment (MAIA) microperimetry (Cetervue, Padova, Italy) using the 10-2 pattern was conducted on the proband.

Splicing Evaluation of *CHM* Gene

The subjects' total RNA was extracted from the peripheral blood cells using the Tempus system (Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized by SuperScript III Reverse Transcriptase (Thermo Fisher Scientific), and amplification between exons 10 and 14 of the *CHM* mRNA was performed using GoTaq Green Master Mix 2x (Promega Corporation, Madison, WI, USA) and primers 5'TCTTCGCCATTCAGTACAGTGC3' and 5'AGAAGATGTGCAAGTCAAATGAACC3'. The PCR products were separated by electrophoresis in 2% agarose gel, bands were extracted from the gel, DNA was recovered by centrifugation, and Sanger sequencing was performed. The 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) was used for bidirectional sequencing and Geneious 10.2.5 software (Biomatters, New Zealand) for sequence alignment. The molecular analyses were based on references: NG_009874, NM_000390.3 and NP_000381.1.

Results

Phenotype Description

The proband was a 58-year-old man who presented with the complaint of nyctalopia since childhood and progressively decreasing vision during the previous 6 years. His BCVA was 20/50 in the right eye and 20/80 in the left eye. The patient had no family history of vision impairment.

Slit-lamp examination showed an unremarkable anterior segment. Funduscopy showed diffuse retinal atrophy and depletion of choriocapillaris with scleral exposure and visible choroidal vessels; the retinal vessels were unaffected (Figs. 1A, B).

FAF imaging showed preserved islands of autofluorescence including the fovea in the right eye (Fig. 1C) and immediately bellow the fovea in the left eye (Fig. 1D). These areas were surrounded by an extensive hypoautofluorescence that corresponded to RPE loss.

The MAIA microperimetry showed good fixation stability bilaterally and low retinal sensitivity with an average threshold of 0.0 dB in the right eye and 1.4 dB in the left eye. A large number of scotomatous points were seen bilaterally. Colored points seen were correlated with areas of preserved autofluorescence, an anatomic/functional correlation (Figs. 1E, F).

The OCT confirmed extensive outer retinal degeneration with outer retinal atrophy and diffuse thinning of all retinal layers including the central macula and retinal laminar disorganization. The choroidal vessels were thin in the right eye (Fig. 1G) and even thinner in the left eye (Figs. 1H, I).

The proband had only one daughter, who was without ophthalmological complaint. Her BCVA was 20/20 in each eye. The funduscopy showed a mottled pigment with hyperpigmentation in the peripheral retina. FAF revealed a mosaic pattern in posterior pole with areas of reticular hyperautofluorescent and hypoautofluorescence of the RPE in a fishnet pattern (Figs. 2A, B). The OCT showed preservation of retinal and choroidal layers (Figs. 2C, D).

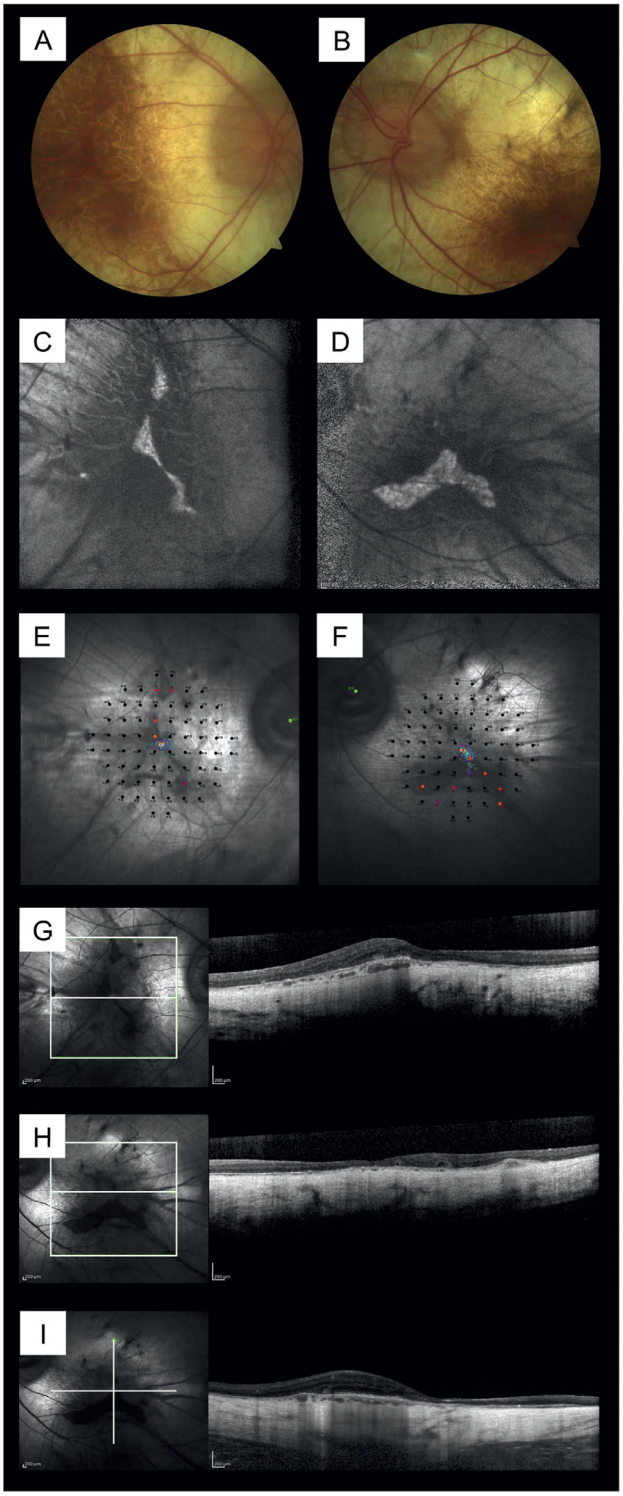

Figure 1. Retinal imaging findings. (**A,B**) Retinographies of both eyes show a pale fundi appearance. Autofluorescence images of the central retina show the preserved autofluorescence in a stellate-shape (**C**) more linearly and centrally in the right eye and (**D**) more inferiorly in the left eye. MAIA microperimetry images show a large number of scotomatous points bilaterally (black color). (**E,F**) The specific color points seen are correlated with areas of preserved autofluorescence. The horizontal line scan from the OCT images of the macula in the (**G**) right eye and (**H**) left eye; (**I**) the vertical line scan in the left eye shows diffuse thinning of all retinal layers with retinal laminar disorganization and decrease in choroidal thickness.

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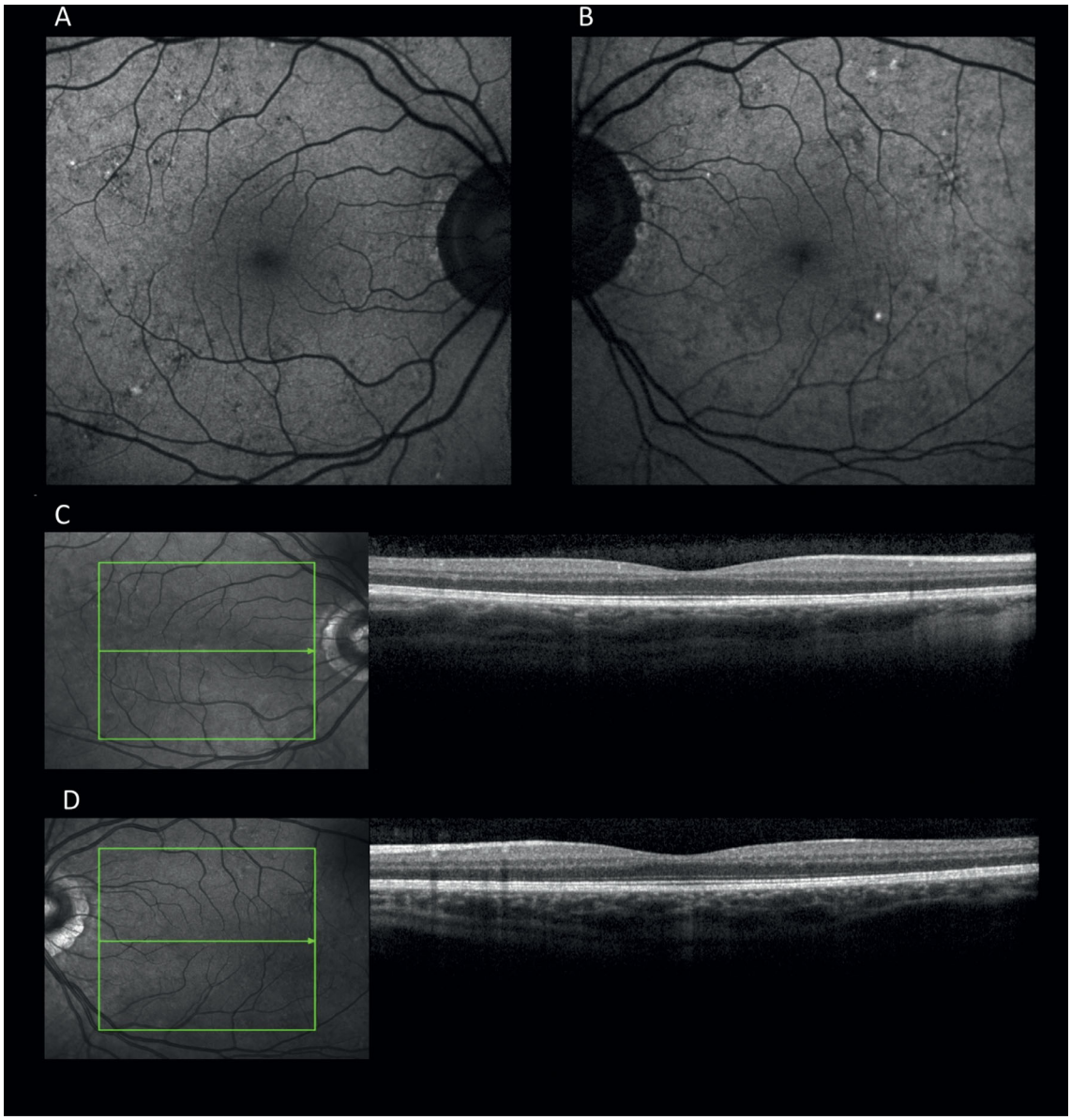

Figure 2. Imaging findings of the unaffected carrier daughter. (**A,B**) Autofluorescence of both eyes showing areas of hyperautofluorescence and hypoautofluorescence in a fishnet pattern. The horizontal line scan from the OCT images of the macula in the (**C**) right eye and (**D**) left eye show preservation of retinal and choroidal layers.

Based on the ophthalmologic investigation, the proband had a clinical diagnosis of choroideremia with a typical chorioretinal phenotype and had a carrier daughter. His molecular test, performed in a commercial laboratory, was classified as inconclusive. Sanger sequencing analyses of all *CHM* exons and exon/intron boundaries suggested a synonymous variant in the CHM gene (c.1359C>T, p.(Ser453=)) as likely disease-causing, which was the only one found in the sequenced *CHM* regions. Although this variant has already been reported,[7] its pathogenic effect has not been evaluated in vitro.

Molecular Investigation

To elucidate the pathogenicity of the c.1359C>T p.(Ser453=) variant in the *CHM* gene, reverse-transcriptase (RT) PCR followed by Sanger sequencing was performed on the proband and his carrier daughter. Analysis of the fragments in agarose gel showed that the heterozygotic daughter had a main 343 base pair (bp) fragment corresponding to the wild type (WT) transcript and a smaller secondary 279-bp band, whereas the hemizygote proband had a 279-bp band with significant intensity, which was denominated alternative transcript 1 (Alt1), and a less intense 174-bp fragment, denominated alternative transcript 2 (Alt2) (Fig. 3A).

All three RT-PCR products were sequenced. As expected, WT fragment corresponded to the normal transcript of the *CHM* gene (NM_000390.3) with preserved exon-exon junctions. However, the Alt1 fragment presented with exon 11 skipping, whereas in the Alt 2, exons 10 and 11 were absent (Fig. 3B). In both alternative transcripts, there was a premature stop codon formation in the subsequent exon to the exon skipped, meaning in exon 12 of WT transcript (Figs. 3B,C). The respective likely protein

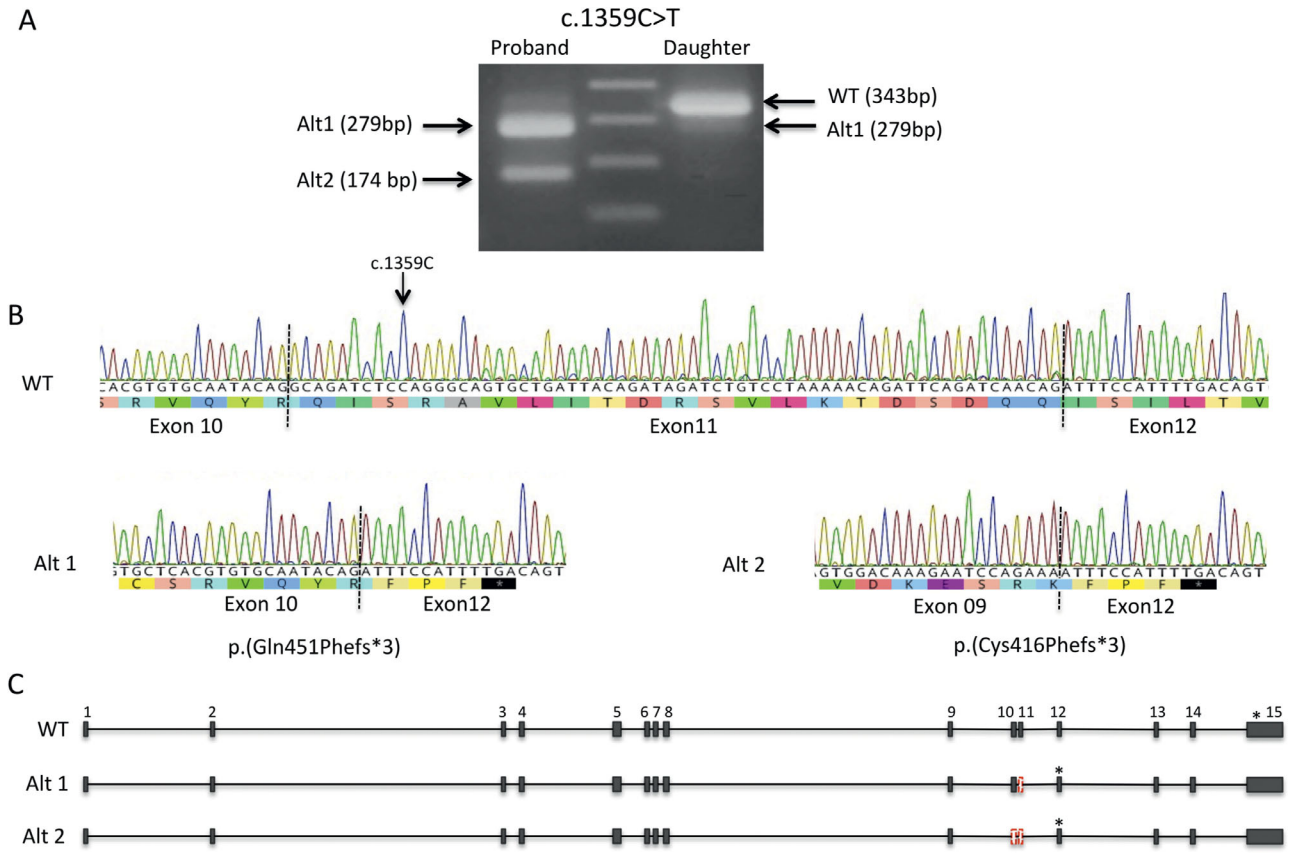

Figure 3. The effect of the c.1359C>T silent variant on RNA processing. (**A**) Agarose gel electrophoresis separation of the *CHM* transcripts amplified (*arrows*) from the proband hemizygote and his heterozygote daughter. (**B**) Electropherograms of RT-PCR products from three different transcripts: WT, Alt1, and Alt2, show the exonic origin of the sequences and protein sequences; the *arrow* indicates the position of c.1359 in the WT transcript. (**C**) Schematic representation of the three different transcripts identified in this family: *black boxes* indicate exons present in the transcript, *red dashed boxes* indicate exons skipped in the alternative transcripts, and *asterisks* indicate the location of the stop codon in each transcript.

consequences of Alt1 and Alt2 are p.(Gln451Phefs*3) and p.(Cys416Phefs*3).

Discussion

This report describes the second case of choroideremia in a patient with a synonymous variant (c.1359C>T p.(Ser453=)) in the *CHM* gene. Based on these findings, the report found that this variant disrupts an exonic splicing enhancer that is a binding site for serine- and arginine-rich splicing factor 1, resulting in exon skipping and possibly the translation of truncated proteins, as previously predicted by an in silico evaluation.[7] The Alt1 (i.e., the main aberrant transcript produced) has p.(Gln451Phefs*3) as its likely protein consequence. The previously reported case[7] and the findings of this study reinforce the pathogenicity of variant c.1359C>T in the *CHM* gene.

The current patient had advanced choroideremia and he is older than the first and unique reported case that has the same mutation, a 34-year-old man of Cuban descent with 20/20 in both eyes and a milder form of the disease.[7] The ophthalmic characteristics of choroideremia manifest early in life and usually begin with night blindness in the first or second decade and progress to visual field constriction. Chorioretinal atrophy begins at the periphery and extends centrally, with macular sparing until the fifth decade. At this time, the visual acuity deteriorates.[1–5] Choroideremia is a progressive disease, which explains the latest stage of the disorder of the current 58-year-old Brazilian patient compared with the 34-year-old Cuban descendent.

His daughter, who is an asymptomatic carrier, had good visual acuity 20/20 bilaterally with no complaints. Her dilated fundus examination demonstrated a mottled pigment with hyperpigmentation in the peripheral retina. The autofluorescence revealed a mosaic pattern in posterior pole with areas of reticular hyperautofluorescent and hypoautofluorescence of the RPE in a fishnet pattern. (Figs. 2A, B). This phenotype is consistent with a heterozygous carrier woman with an X-linked inherited retinal disease.[8–10] The classic phenotypes of the Brazilian patient who has choroideremia and his carrier daughter reinforce the pathogenicity of this variant.

The ophthalmologic examination of the current patient showed extensive atrophy in the fundus photographs and OCT images, which showed a delaminated and thinned retina, the latest stage of the disease.[11,12] The preserved islands of retinal tissue observed in FAF allow him to have moderate visual acuity. Generally, there is a degree of symmetry between the two eyes in these areas on FAF images[13,14]; nevertheless, asymmetry may be found in the later disease stages, as in the proband.[4] Until now, according

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to the literature, there has not been any genotype-phenotype correlation in choroideremia.[1,15–19]

Sanger sequencing of all *CHM* exons and exon/intron boundaries found only one synonymous variant (c.1359C>T, p.(Ser453=)). This variant was considered to have a high likelihood of being disease-causing, via the observed skipping of *CHM* exon 11 reported here. Therefore, a consideration of this study is that it was not demonstrated that this variant alters splicing directly (e.g., via an in vitro splicing assay after introducing the c.1359 C>T change in the WT *CHM* sequence). Furthermore, the observed skipping of *CHM* exon 11 should be due to the presence of a deep-intronic variant associated to the c.1359 C>T. Because of this, a sequence analysis of intron 10 in the proband and his daughter was performed and did not identify any additional variants, supporting the likely pathogenicity of the c.1359C>T missense variant.

Choroideremia is an inherited chorioretinal dystrophy for which clinical trials of gene therapy are ongoing.[20–24] The molecular and clinical findings in the current case provide strong evidence that c.1359C>T (p.(Gln451Phefs*3)) in the *CHM* gene should be considered a disease-causing variant. Moreover, patients with this variant should be considered as candidates for future *CHM*-related therapies.

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