

**Short Report: Novel non-contiguous exon duplication in choroideremia**

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Word count: 2460

**Acknowledgements**

19 This work was funded in part by a grant to TE from the Nuffield Trust. This work  
20 was also funded by the Oxford University Hospitals Foundation Trust NIHR  
21 Biomedical Research Centre and Fight for Sight.

22 **Conflict of Interest**

23 The authors declare no relevant conflict of interest

## Abstract

The importance of establishing a genetic diagnosis in patients with a choroideremia phenotype has been underscored by the advent of gene replacement therapy for this condition. Here we describe a complex imbalance at the *CHM* locus in a male patient with classical disease. At the DNA level, this imbalance consists of two non-contiguous duplications (exons 1-2, and 9-12). Further characterization suggests the generation of two independent *CHM* transcriptional units, one of which may produce a deleted form of the REP1 protein. Expression of such a type of aberrant protein in photoreceptors may have important implications when considering gene therapy for this disorder.

Key Words- Retinal Dystrophy, Ophthalmology, Choroideremia, Genetic Testing, Structural Variation, Copy Number Variation

## Introduction

Choroideremia (OMIM phenotype reference: 303100) is an X-linked retinal degeneration that results in progressive concentric field loss during early adulthood, before involving central vision typically by the fourth decade<sup>1</sup>. It is due to mutations in *CHM*, on chromosome Xq21 (OMIM gene reference: 300390), that lead to deficiency of the Rab escort protein 1 (REP1), causing under-prenylation of Rab GTPases, essential for membrane trafficking and vesicle transport in photoreceptors<sup>2</sup>.

The majority of disease-associated mutations in *CHM* result in a loss of detectable REP1 and include whole and partial deletions gene deletions, and

single nucleotide variants leading to premature termination of protein translation through a variety of mechanisms<sup>3-6</sup>. Mutations maintaining the *CHM* reading frame are rare; however, missense variants predicted to result in decreased protein stability, as well as variants leading to in-frame exon skipping have also been reported<sup>3,5</sup>.

Here we present a novel *CHM* variant identified in a male patient with typical choroideremia consisting of two non-contiguous duplications that has been further characterized at the RNA level by Reverse Transcription PCR (RT-PCR).

## **Patients and Methods**

The patient is a 44 year-old male with a history of 'retinitis pigmentosa' (RP) who first noticed a decline in night vision during late childhood. He had no associated systemic features and there was no known family history of eye disorders. The best-corrected visual acuity was 6/24 and 6/15 in the right and left eye respectively. Fundoscopy (Spectralis, Heidelberg Engineering, Germany) showed extensive symmetrical chorioretinal atrophy with sparing of the posterior poles over an area corresponding to a well-demarcated island of residual fundus autofluorescence (Figure 1). Spectral domain optical coherence tomography (Spectralis, Heidelberg Engineering, Germany) revealed widespread chorioretinal thinning in those regions outside the island of autofluorescence. A topographical map of macular threshold sensitivity was performed by microperimetry testing (Maia, CenterVue, Italy), which showed relatively sustained retinal function over the island of autofluorescence, which was extinguished precipitously outside its border.

The coding regions of 55 genes associated with RP were sequenced in DNA isolated from venous blood, using a custom design Haloplex enrichment kit (Agilent Technologies) on the Illumina MiSeq platform. Data analysis was performed using an in-house pipeline.

Dosage analysis of *CHM* was performed using MLPA (Probe mix P366, MRC-Holland, Amsterdam, Netherlands) with data analysis performed in Coffalyser (MRC-Holland). REP1 protein expression was assayed using immunoblot on lysed primary fibroblasts obtained from patients' skin biopsies (UK REC reference 15/WA/0087) probed with an anti-REP1 monoclonal antibody (EMD Millipore, Hertfordshire, UK) with  $\beta$ -actin used as a loading control.

For RT-PCR analysis, total RNA from whole blood was extracted using the QIAamp RNA Blood Mini Kit (Qiagen, Manchester, UK). cDNA synthesis was performed using random primed synthesis with SuperScript III (Life Technologies, Paisley, UK). cDNA libraries were amplified using KAPA 2G Fast polymerase (Kapa Biosystems, Cape Town, South Africa) prior to Sanger sequencing on an ABI 3730 using BigDye V3.1 chemistry (Life Technologies, Netherlands).

Variants are described based on the RefSeq *CHM* transcript NM\_000390.3.

Work carried out in this study has received ethics approval from the London – West London & GTAC research ethics committee (reference 15/LO/1379) as part of the REGENERATE study (ClinicalTrials.gov ref NCT02407678).

## Results

Next Generation Sequencing (NGS) of 55 genes associated with RP did not detect a pathological sequence variant in our patient; however, read depth analysis was suggestive of a putative complex rearrangement in *CHM* which was confirmed using Multiplex Ligation-dependent Probe Amplification (MLPA, Figure 2A). The relative copy number of exons 1-2 and exons 9-12 were doubled, indicating the presence of two non-contiguous *CHM* exon duplications.

An identical MLPA result was observed when analyzing DNA extracted from the patient's mother, consistent with X-linked inheritance and her carrier status.

However, because MLPA is unable to provide positional information with regards to the insertion point of duplicated sequence, there remained the possibility of an intact *CHM* gene with the duplicated material inserted elsewhere in the genome in both the patient and his mother.

To explore this, an assay of REP1 using immunoblot, was performed (Figure 2B). An absence of REP1 in cultured skin fibroblasts was confirmed, consistent with pathogenicity of the non-contiguous duplication, and a clinical diagnosis of choroideremia. Efficacy of the assay was demonstrated by detection of REP1 in two positive control (non-choroideremia) cell lines.

As immunoblot analysis did not detect any REP1 signal in our patient, we next sought to fully characterize the molecular nature of the non-contiguous duplication. We chose an approach based on analysis of *CHM* RNA using RT-PCR. cDNA libraries were initially amplified using seven different primer sets designed across splice sites throughout the reference *CHM* transcript (NM\_000390.3, results not shown). In this initial analysis, a primer pair amplifying between exons 8 and 13 failed to generate a product from the

patient's library, indicating that the non-contiguous duplication was affecting normal transcript production between these sites.

A second set of PCRs was carried out using three different primer sets, one amplifying between exons 1 and 12, a second between exons 1 and 15, and a final set between exons 1 and 13. As shown in Figure 3A, the exon 1-12 primer set produced a predominant product in the control sample consistent with the normal pattern of *CHM* splicing. When using the patient's cDNA library as template, a band matching that in the control and a second product of approximately 475 bp was generated. Using both the exon 1-15 and exon 1-13 primer sets a single product corresponding to the normal *CHM* transcript was amplified from the control library, while two main products of significantly smaller size were amplified from the patient library confirming loss of normal transcript expression.

To better characterize the smaller products from the patient sample, purified products from the exon 1-15 primer set (identified in Figure 3A by asterisks) were sequenced. Figure 3B shows sequence analysis of the products from the patient's library, revealing the predominant product (\*2 in Figure 3A) to be composed of exons 1-2 spliced directly onto exons 9-15; analysis of the product from the control library (\*1 in Figure 3A) confirmed normal splicing of exons 2-3 (see Figure 3C). The smaller, less intense, product from the patient sample (\*3 in Figure 3A) appeared to be generated through an alternate splicing event between exons 9 and 12.

## **Discussion**

140 In this case, we have characterized a novel, pathogenic, non-contiguous  
141 duplication in *CHM* comprising exons 1-2 and exons 9-12. Based on the results  
142 of cDNA analysis in this patient, we propose the non-contiguous duplication  
143 consists of two *CHM* transcriptional units (see Figure 3D). The first composed of  
144 exons 1 to 12 (Unit A), and the second containing the duplicated exons (1, 2, 9,  
145 10, 11, and 12) along with single copy exons 13-15 (Unit B). Using HGVS  
146 nomenclature, this variant is best described at the RNA level as two transcripts  
147 originating from a single chromosome: r.[1511\_\*3450del;117\_1166del].

148 Given that Unit A is predicted to lack the transcription termination and  
149 polyadenylation signals of wild type *CHM* it is unclear whether it will produce  
150 functional mRNA transcripts. Conversely, the presence of both the proximal and  
151 terminal segments of the *CHM* gene in Unit B is consistent with the production  
152 of functional mRNA capable of protein translation; the predominant transcript  
153 from this unit would be predicted to result in a REP1 protein with an in-frame  
154 deletion of 350 amino acids from Arg40 to Arg389 (HGVS:  
155 p.(Arg40\_Arg389del)). Such putative protein was also predicted to be translated  
156 in a choroideremia patient with a simple deletion of *CHM* exons 3-8<sup>7</sup>, and may  
157 evade detection by our anti-REP1 monoclonal antibody, which was raised  
158 against the C-terminus of recombinant human REP1( i.e. exons 6-15 inclusive).

159 One explanation for how this rearrangement may have occurred is displayed in  
160 Figure 3E. Misalignment and subsequent recombination between a normal X  
161 chromosome and one containing a *CHM* gene with a deletion of exons 3-8, as  
162 also reported in a family by McTaggart *et al*, may have generated this complex  
163 locus<sup>7</sup>.



164

165 Production of a deleted form of REP1 could have implications on gene therapy.  
166 To-date, clinical trials have been performed on a null background with respect  
167 to REP1 expression <sup>8,9</sup>. Indeed, complete loss of protein in the majority of  
168 patients has been highlighted as an advantage in determining dosage levels in  
169 clinical trials as compared to disorders such as *RPE65*-associated Leber  
170 Congenital Amaurosis (LCA) <sup>9,10</sup>.

171 Detailed assessment of choroideremia patients with missense variants versus  
172 those with absent REP1 is not suggestive of phenotypic differences in these  
173 cohorts <sup>4,6</sup>. However, the impact of residual mutant REP1 expression on clinical  
174 outcome in the context of gene therapy remains to be assessed, with  
175 suggestions that interference could impact success rates <sup>6</sup>. Furthermore, it is  
176 interesting to note that misfolding of mutant RPE65 has been shown to lead to  
177 cytotoxic aggregates in the photoreceptors of LCA patients: an alternate  
178 pathogenic mechanism that cannot be treated through gene replacement  
179 therapy that should also be explored in rare cases of choroideremia maintaining  
180 mutant REP1 expression <sup>11</sup>.

181 To the best of our knowledge, this represents the first complex rearrangement  
182 to be described in *CHM*, and highlights the utility of supplementing NGS with  
183 further genetic testing when there is a high index of clinical suspicion.

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224 **Titles and Legends to Figures**

225 **Figure 1. Clinical phenotype**

226 (A) Colour fundus photographs; (B) autofluorescence images (Spectralis,  
227 Heidelberg Engineering, Germany); (C) microperimetry (Maia, CenterVue, Italy)  
228 and; (D) optical coherence tomography scans (Spectralis, Heidelberg  
229 Engineering, Germany).

230 **Figure 2. Multiplex Ligation-dependent Probe Amplification and REP1**  
231 **expression.**

232 (A) The relative copy number of exons 1-2 and exons 9-12 were doubled,  
233 indicating the presence of two non-contiguous exon duplications. (B)  
234 Immunoblot demonstrating absence of REP1 expression in the patient's skin  
235 fibroblasts. A band of the correct size (83 kDa) was detected in the fibroblasts of  
236 2 other male patients with no mutation in CHM (Control #1 and #2).  $\beta$ -actin was  
237 used as a loading control. Key: CHM=patient sample; Control #1 and #2 =  
238 control samples from unaffected males #1 and #2.

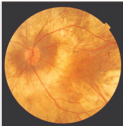
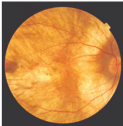
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240 **Figure 3. Proposed organization of the *CHM* transcription units and a**  
241 **model for their generation based on PCR and Sanger sequencing analysis**

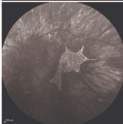
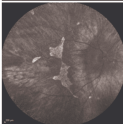
242 (A) PCR analysis using primers amplifying between exons 1-12, exons 1-15 and  
243 exons 1-13 for the wild-type (control) and mutant (patient) *CHM* cDNA library as  
244 a template. Products marked with asterisks were further purified prior to Sanger  
245 sequencing. (B) The top chromatogram, generated from the larger of the two  
246 patient fragments (\*2 in Panel A), demonstrates splicing between exons 2 and 9

247 generating a transcript consisting of exons 1-2 spliced onto exons 9-15. The  
248 lower chromatogram highlights splicing between exons 9 and 12 observed in  
249 the smaller of the two products resulting in an alternative transcript lacking  
250 exons 10 and 11 (\*3 in Panel A). (C) A portion of sequencing data from the  
251 product generated by PCR using primers specific to exons 1 and 15 and using  
252 the control cDNA library as a template demonstrating the expected pattern of  
253 splicing between exons 2 and 3 (\*1 in Panel A). (D) We propose that the *CHM*  
254 locus in our patient consists of two individual transcription units (A and B). (E)  
255 We propose a model where misalignment and subsequent recombination  
256 between a normal X chromosome and one containing a *CHM* gene with a  
257 deletion of exons 3-8 results in the generation of this complex locus.

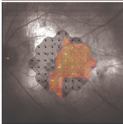
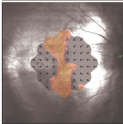
A



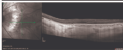
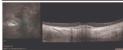
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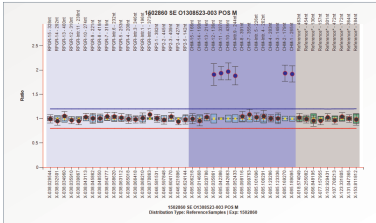
C



D



A



B

