

# The Spectrum of CHM Gene Mutations in Choroideremia and Their Relationship to Clinical Phenotype

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**PURPOSE.** We report the underlying genotype and explore possible genotypic-phenotypic correlations in a large cohort of choroideremia patients.

**METHODS.** We studied prospectively a cohort of 79 patients diagnosed within a tertiary referral service for patients with retinal dystrophies. Phenotypic evaluation consisted of clinical examination, including visual acuity and residual retinal area by fundus autofluorescence (FAF). Genotype was established by sequencing. We also investigated whether particular genotypes were associated with more severe phenotypes by performing analysis of covariance (ANCOVA), with visual acuity and FAF as the dependent variables and age as the covariant.

**RESULTS.** A total of 74 (94%) of patients in our cohort had causative mutations by sequencing, the majority of which were anticipated to be null. Of these, 35 (47%) had insertions and deletions, 13 (18%) had mutations predicted to affect splicing, and 26 (35%) had single point mutations. In the latter case, 13 of 21 (62%) pedigrees with single point mutations were C to T transitions at C-phosphate-G (CpG) dinucleotides. These mutations were spread across 5 of only 24 CpG dinucleotides in the entire *CHM* cDNA. Furthermore, these 5 locations are the only sites at which C to T transitions result in a stop codon. No clear evidence was found for genotype-phenotype correlation except in the instance of a patient with a large deletion involving neighbouring sequences.

**CONCLUSIONS.** In patients with a diagnosis of choroideremia made by a specialty service, there is a high likelihood of establishing a genetic diagnosis. The majority of causative mutations appear to be null and, therefore, may benefit from gene replacement therapy. A disproportionate number of single point mutations observed were C to T transitions, consistent with the evolutionary decay of CpG dinucleotides through methylation and subsequent deamination. Hence, the development of choroideremia in such patients may represent the unwanted consequence of human evolution; de novo mutations are predicted to arise at these sites in future generations. (ClinicalTrials.gov number, NCT01461213.)

Keywords: choroideremia, sequencing, genotyping, phenotyping

Choroideremia (OMIM 303100) is an X-linked recessive retinohoroidal dystrophy, first described by Mauthner in 1871,<sup>1</sup> that affects approximately 1 in 50,000 males. The disease results in stereotypic changes to the ocular fundus that are characterised by centripetal loss of retinal and choroidal tissue. The functional correlates of these structural changes are early loss of scotopic vision and constriction of the visual field, both of which commence in the second decade of life. Central visual function, including visual acuity, typically is well-preserved until the fifth decade of life; however, there is variability in the rate of decline in visual function and the age at which central vision is affected. The causative gene, *CHM* (OMIM #300390), codes for rab-escort protein 1 (REP1),<sup>2</sup> a 653 amino acid polypeptide crucial for intracellular trafficking within the human eye that comparative modeling has shown to be comprised of three principal domains.<sup>3</sup> To date, almost all of

the identified causative mutations appear to be null. Although the clinical phenotype can vary in terms of the age of onset and rate of decline, no evidence has been found for genotype-phenotype correlation.<sup>4,5</sup>

We describe the causative mutations in a large cohort of patients who also were examined clinically and explore potential genotype-phenotype correlations. By so doing, we further aimed to make inferences regarding the importance of particular regions of the *CHM* gene with respect to mutagenesis and to infer the importance of particular regions of the REP1 protein essential for normal function. Finally, we also sought to determine the prevalence of presumed null mutations and the rate of detection of mutations by sequencing. Both of these latter issues are especially important in assessing the potential suitability of gene therapy via a "gene replacement" strategy, a potential treatment that currently is under investigation by our



group (ClinicalTrials.gov number, NCT01461213)<sup>6,7</sup> and by others (ClinicalTrials.gov numbers, NCT02077361, NCT02341807).

## METHODS

The study was approved by the local Institute Review Board before its commencement and conformed to the tenets of the Declaration of Helsinki.

### Clinical Examination Functional Assessment

Clinical examination consisted of full medical and ophthalmic history, anterior and posterior segment examination, and assessment of IOP. Visual acuity was assessed using a Bailey-Lovie style letter chart.<sup>8</sup> For illustrative purposes, the visual acuities in the 7 patients who were count fingers (CF) or less were converted to logMAR values, as outlined by Holladay.<sup>9</sup> A diagnosis of choroideremia, based upon clinical findings alone, was made by a senior clinician with expertise in inherited retinal disease (REM or SMD).

### Structural Assessment

Fundus autofluorescence (FAF) imaging was performed with a Heidelberg Spectralis optical coherence tomography (OCT)-confocal scanning laser ophthalmoscopy (cSLO) machine with the BluePeak blue laser autofluorescence module (Heidelberg Engineering GmbH, Heidelberg, Germany) according to the manufacturer's protocols. Variations in corneal curvature were accounted for by entering the patient's keratometry values ("C-curve"). For assessing areas of autofluorescence, FAF imaging by the Spectralis with the 30° and 55° fields was performed. Fundus autofluorescence images were imported into Adobe Photoshop (Version 12; Adobe Systems, Inc., San Jose, CA, USA) and adjusted to similar levels of contrast. The area of autofluorescent zones was estimated using an automated pixel count using the Histogram function at 400% zoom, which then was converted into area measurements in mm<sup>2</sup> using the imported scale bar.

### Genotyping

DNA extraction using the Chemagic Magnetic Separation Module 1 (PerkinElmer Chemagen Technologie GmbH, Baesweiler Germany) was performed on whole blood specimens. Bidirectional sequencing was used to explore the 15 *CHM* gene exons and the intron/exon junctions. Amplification by polymerase chain reaction (PCR) was performed using standard conditions, with annealing of primers at 60°C. Then, 15 µL of PCR product was purified by removing Effluvia (dNTPs and primers) using Agencourt AMPure XP (Beckman Coulter Genomics, Danvers, MA, USA). The BigDye Terminator 3.1 Cycle Sequencing Kit (ABI; Life Technologies, Waltham, MA, USA) was used for sequencing, using the same primers as PCR, with the exception of exons 3 and 13, where alternative sequencing primers were used to account for repetitive sequences in the PCR products. Final purification was performed with Agencourt CleanSEQ (Beckman Coulter Genomics) and sequence analysis was performed using the 3730xL DNA Analyzer (ABI; Life Technologies). *CHM* exonic deletions and duplications were sought using multiplex ligation dependent probe amplification (MLPA) using a commercially available SALSA MLPA kit (P366 *CHM*-RP2-RPGR; MRC Holland, Amsterdam, The Netherlands). Reactions were conducted according to the manufacturer's instructions and

analyzed with the 3130xL DNA Analyzer (ABI; Life Technologies).

Known nonpathogenic polymorphisms were excluded from the analysis (defined as variants described on nondisease-associated variant databases with quoted frequency data).<sup>10,11</sup> Frameshift, nonsense, and splice site (affecting positions ±1, ±2 at the intron/exon junction) changes were considered pathogenic, as were deletions or duplications involving one or more exons. In cases of single exon deletions (determined by MLPA), genomic DNA was sequenced to exclude the occurrence of a rare single nucleotide polymorphism (SNP) at the relevant primer binding site. In the absence of further information, remaining changes were considered to be of uncertain significance. Full details of the sequencing methods and interpretation of genetic data have been reported previously.<sup>10,11</sup>

### Phenotype–Genotype Correlations

The majority of causative mutations, regardless of mechanism, were predicted to be null. We categorized mutations by mechanism into small deletions, small insertions, and insertion/deletions, large deletions (≥1 exon), splice site mutations, missense mutations, premature termination codons (i.e., point mutations resulting in a stop codon), and also grouped together patients in whom no mutation could be identified. We also identified those patients with mutations within 55 base pairs (bp) of the final exon junction as such mutations generally are not thought to result in nonsense mediated decay (NMD).<sup>12</sup> This would, in turn, enable us to speculate on the importance of the C-terminus in *REPI* functioning. Additionally, we compared mutations by mechanism. Results then were analyzed for correlation to visual function (best corrected visual acuity [BCVA]) and structure (FAF area, where available) by analysis of covariance (ANCOVA) with age as the controlled variable and using the data from right eyes in analysis.

## RESULTS

### Spectrum of Genetic Mutations

Of 79 patients who had undergone clinical examination, 74 had mutations identified (94%), making this the largest cohort of patients from a single center with identified mutations described. The mutations in our cohort are summarized in Figure 1 and Tables 1 to 5. The majority of mutations were predicted to be null. Two patients were identified in whom expression of a truncated protein was anticipated because of premature stop codons occurring downstream of 55 bp from the final exon junction. One patient was identified with a missense mutation (c.104T>C, p.Leu35Pro) known to be associated with low levels of *REPI* expression.<sup>13</sup> Mutations of uncertain significance included one patient with noncontiguous duplications of exons 1 to 2 and 9 to 12 who was subsequently demonstrated in our laboratory to have severely reduced expression of *REPI* in peripheral blood by Western blotting (Edwards TL, Patricio MI, Williams J, Simunovic MP, MacLaren RE, submitted for publication, 2016). One patient was identified in whom a 6 bp (in frame) insertion at c.343 results in p.Ala115delinsValPheThr. One patient was identified with a small (in frame) deletion of 9 bp (c.872\_880delAAAAGC-GAA). Finally, four new variants presumed to cause aberrant splicing were identified (Table 3); one patient with a previously described c.940+3delA mutation of similarly uncertain significance was identified.<sup>10</sup> Most small intragenic mutations were located within the region 700 to 900 (Fig. 1); one striking

TABLE 1. Summary of Major Deletions

Instances	No. Pedigrees	Major Deletion	Previously Reported?
2	2	All 15 exons	Yes <sup>10</sup>
1	1	Whole gene with contiguous regions containing <i>POU3F4</i> and <i>ZNF711</i> (6.05Mb deletion Xq21.1-q21.31)	Yes <sup>16</sup>
2	2	Exons 9–15 deleted	Yes <sup>10</sup>
1	1	Exons 3 and 4 deleted	Yes <sup>18</sup>
1	1	Exons 10 and 11 deleted	Yes <sup>10</sup>
1	1	Exons 10–15 deleted	Yes <sup>10</sup>
2	1	Exon/intron 9 deleted	Yes <sup>10</sup>

Instances refers to number of subjects affected, pedigrees refers to the number of families affected (i.e., affected patients could come from the same, or a different, pedigree).

feature of this region is the presence of 5 CpG sites in which C to T transitions result in a premature stop codon. Mutations to 4 of these sites were reported previously by McTaggart et al.<sup>14</sup> to be recurrent, that is, observed in multiple unrelated pedigrees. Furthermore, these are the only CpG sites (of a total of 24) in which C to T transition results in a stop codon.

### Correlation Between Mutation and Visual Function

The majority of *CHM* mutations are predicted to be null, either through major deletions, or more commonly, through nonsense mutations. One mutation resulted from noncontiguous exon duplications and two occurred within 55 bp of the final intron/exon junction and would not be predicted to result in NMD. In view of the small number of subjects with such mutations, it is difficult to establish their effects on phenotype (Fig. 1). Analysis of covariance (with age as the covariant) does not suggest a significant difference between mutations involving the C-terminus and those occurring upstream of this location ( $P > 0.05$ ) in terms of logMAR acuity (Fig. 2). Similarly, no correlation between genotype by category and logMAR acuity was observed ( $P > 0.05$ ; Fig. 2), nor was a correlation between genotype and FAF area observed ( $P > 0.05$ ; Fig. 3).

TABLE 2. Summary of Minor Deletions and Insertions

Instances	No. Pedigrees	Location	cDNA effect	Predicted Effect	Previously Reported?
1	1	Exon 1	c.179delA	Frameshift	No
1	1	Exon 5	c.343_344ins6 p.(Ala115delinsValPheThr)	Uncertain	No
3	2	Exon 6	c.492_493delGA	Frameshift	Yes <sup>10</sup>
2	2	Exon 6	c.525_526delAG	Frameshift	Yes <sup>20</sup>
1	1	Exon 6	c.529delG	Frameshift	Yes <sup>14</sup>
1	1	Exon 6	c.535_538delGAAA	Frameshift	Yes <sup>10</sup>
2	2	Exon 6	c.649_652delTACT	Frameshift	Yes <sup>19</sup>
2	2	Exon 6	c.652_655delTTCAC	Frameshift	Yes <sup>13</sup>
1	1	Exon 7	c.737_741delTAATC	Frameshift	Yes <sup>10</sup>
1	1	Exon 8	c.872_880delAAAAGCGAA	Uncertain	Yes <sup>10</sup>
1	1	Exon 8	c.886delA	Frameshift	No
1	1	Exon 9	c.1079delA	Frameshift	Yes <sup>10</sup>
1	1	Exon 9	c.1099_1100insTACC	Frameshift	No
1	1	Exon 11	c.1300_1303delGTGG	Frameshift	Yes <sup>10</sup>
1	1	Exon 12	c.1335dupA	Frameshift	No
1	1	Exon 14	c.1584_1587delTGTT	Frameshift	No
1	1	Exon 15	c.1762_1765del	Frameshift	Yes <sup>10</sup>
1	1	Exon 16	c.1780delC	Frameshift	Yes <sup>10</sup>

Instances refers to number of subjects affected, pedigrees refers to the number of families affected (i.e., affected patients could come from the same, or a different, pedigree).

### Correlation Between Mutation and Systemic Disease

One patient was identified with a “choroideremia plus” syndrome.<sup>15</sup> This individual has a history of deafness and developmental delay, and was demonstrated to have a large deletion involving the neighbouring *POU3F4* and *ZNF711* genes. The former is a transcription factor implicated in X-linked congenital deafness,<sup>16</sup> while the latter is a zinc finger protein previously described as a cause of X-linked “mental retardation.”<sup>17</sup>

### DISCUSSION

We identified a total of 48 unique mutations in 74 patients from a cohort of 79 affected males examined clinically who had been diagnosed with choroideremia. Thus, the rate of detection (94%) was higher than that reported previously using the same molecular diagnostic techniques in patients referred from a variety of centers<sup>10</sup> (rather than from a single center with particular interest in choroideremia and inherited eye disease).

In keeping with previous reports, the majority of identified mutations were anticipated to be null.<sup>18–23</sup> This, combined with the low rate of patients in whom sequencing failed to identify a genetic cause, reaffirms the logic of a gene-replacement strategy as a possible means of treatment, such as that currently undertaken at our center.<sup>6,7</sup>

In our cohort, there was a notable absence of disease-causing missense mutations that also would cause protein misfolding. The paucity of missense mutations in choroideremia is in contrast to the majority of human genetic diseases (which are caused predominantly by such mutations). This may be due to the role of REP1 as a chaperone protein, because the folding may be determined by the Rab27a ligand, rather than posttranslational modification of the REP1 protein itself. Since Rab27a binds equally well to REP2, which shares less than 80% sequence homology to REP1,<sup>24</sup> it is logical to assume that a significant number of single amino acid substitutions in the REP1 sequence would be well tolerated. A preponderance of nonsense mutations has been observed similarly in mutations to other GTPase regulators, such as

TABLE 3. Summary of Splice Site Mutations

Instances	No. Pedigrees	Location	cDNA Effect	Predicted Effect	Previously Reported?
3	3	Intron 1	c.49+2dupT	Splice site	No
2	2	Intron 2	c.116+1G>A	Splice site	Yes <sup>21</sup>
1	1	Intron 3	c.189+1G>C	Splice site	Yes <sup>10</sup>
1	1	Exon 7	c.703-1_727del/insTTAGA	Splice site	No
1	1	Intron 7	c.819+1G>A	Splice site	Yes <sup>10</sup>
1	1	Intron 7	c.819+1G>T	Splice site	No
2	2	Intron 8	c.940+2T>C	Splice site	Yes <sup>10</sup>
2	2	Intron 8	c.940+3delA	Splice site	No

Instances refers to number of subjects affected, pedigrees refers to the number of families affected (i.e., affected patients could come from the same, or a different, pedigree).

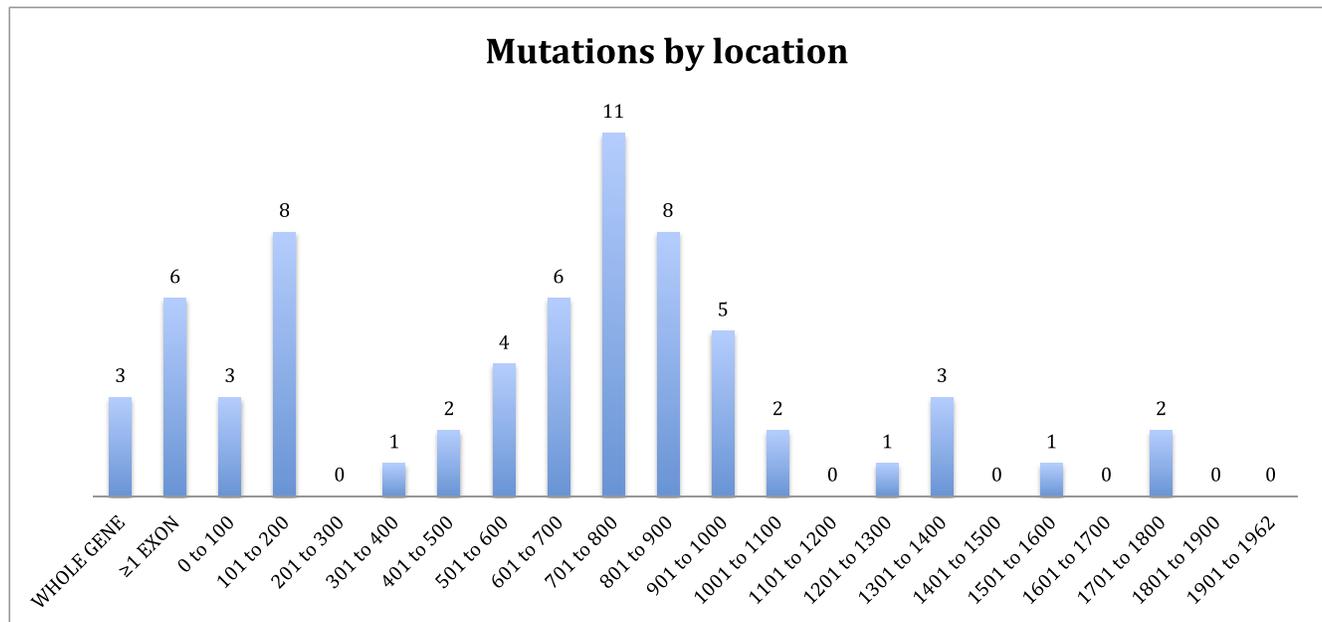


FIGURE 1. Number of pedigrees by cDNA location of mutations. The two mutations between 1701 and 1800 occur within 55 bp of the final exonic junction and are anticipated to result in expression of a truncated REP1 protein.

TABLE 4. Point Mutations

Instances	No. Pedigrees	Location	cDNA Effect	Predicted Effect	Previously Reported?
1	1	Exon 2	c.104T>C	p.Leu35Pro	Yes <sup>10</sup>
1	1	Exon 3	c.126C>G	p.Tyr42X	Yes <sup>10</sup>
1	1	Exon 3	c.130G>T	p.Arg44X	Yes <sup>15</sup>
1	1	Exon 3	c.173G>A	p.Trp58X	Yes <sup>10</sup>
1	1	Exon 6	c.655C>T	p.Gln209X	No
1	1	Exon 7	c.698C>A	p.Ser233X	No
5	3	Exon 7*	c.715C>T	p.Arg239X	Yes <sup>23</sup>
2	2	Exon 7*	c.757C>T	p.Arg253X	Yes <sup>22</sup>
3	2	Exon 7*	c.799C>T	p.Arg267X	Yes <sup>19</sup>
2	2	Exon 7*	c.808C>T	p.Arg270X	Yes <sup>21</sup>
5	4	Exon 8*	c.877C>T	p.Arg293X	Yes <sup>20</sup>
1	1	Exon 11	c.1264C>T	p.Gln422X	Yes <sup>10</sup>
1	1	Exon 11	c.1347C>G	p.Tyr449X	Yes <sup>10</sup>

\* CpG sites. Instances refers to number of subjects affected, pedigrees refers to the number of families affected (i.e., affected patients could come from the same, or a different, pedigree).

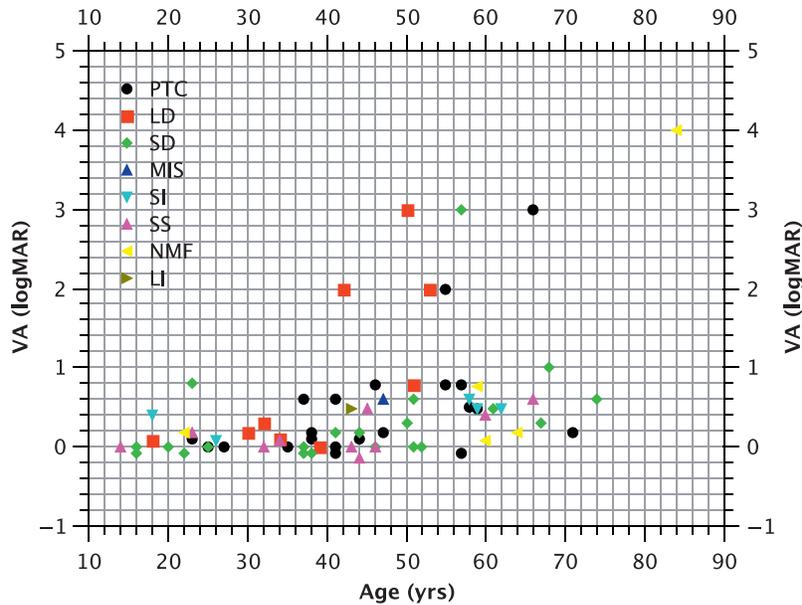


FIGURE 2. Visual acuity (logMAR) versus age (years). PTC, premature termination codon; LD, large deletion ( $\geq 1$  exon); SD, small deletion; MIS, missense mutation; SI, small insertion or insertion/deletion; SS, splice site mutation; NMF, no mutation found; LI, large insertion.

*RPGR* and *KRIT1*. Possible explanations (some more plausible than others) have been advanced previously for this phenomenon.<sup>4</sup> One possibility is that some missense mutations may result in milder or different phenotypes that previously have evaded detection/recognition. With the advent of rapid sequencing, the likelihood of such occurrences is becoming increasingly remote. A further suggestion is that some missense mutations may exert a dominant negative effect and thereby disrupt the activity of *REP2*. Therefore, such mutations might be anticipated to be lethal in utero in males, while female carriers might remain largely asymptomatic by virtue of lyonization. The fact that *REP1* is a monomer makes this possibility less likely. However, a further (and related) possibility to be considered is that mutations resulting in the expression of a protein product also might be less amenable to

a gene replacement strategy because expressed mutant proteins could interfere with wild-type function. Even if this were to be the case, such mutations form the minority of cases; furthermore, overexpression may negate such hypothesized effects.

Two nonsense mutations were observed to occur within 55 bp of the final intron-exon junction and, therefore, would not be anticipated to result in NMD.<sup>12</sup> Previous studies of the *REP1* molecule suggest that the C-terminus acts as mobile shield that covers a conserved hydrophobic patch on the surface of *REP1* and its presence is proposed to be essential for the maintenance of solubility.<sup>25</sup> Furthermore, the C-terminus is essential for membrane attachment.<sup>26</sup> The fact that C-terminus involving mutations are pathogenic further suggests that it is essential for the normal functioning of *REP1* in vivo. It should

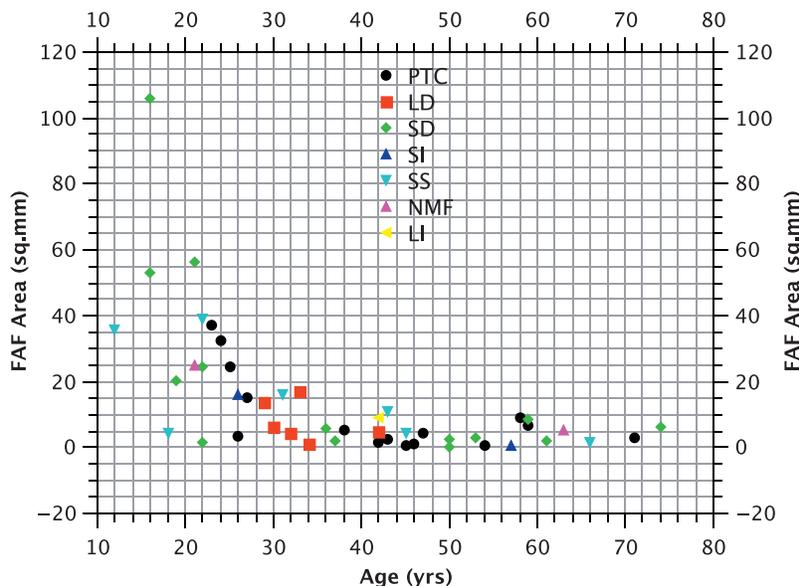


FIGURE 3. Fundus autofluorescence area (mm<sup>2</sup>) versus age (years).

TABLE 5. Insertions

Instances	No. Pedigrees	cDNA Effect	Predicted Effect	Previously Reported?
1	1	Noncontiguous duplication of exons 1-2 and 9-12	Unknown	No

Instances refers to number of subjects affected, pedigrees refers to the number of families affected (i.e., affected patients could come from the same, or a different, pedigree).

be noted that some caution must be exercised in the interpretation of these findings: in particular, it has been argued that X-chromosome coded genes may be less likely to be subject to NMD than autosomes,<sup>27</sup> and so the differential effects of the location of mutations with respect to the final intron-exon junction may be blunted.

We found no evidence for genotype-phenotype correlation by genetic mechanism with one exception: a single patient with a large mutation involving the *CHM* gene as well as the neighboring *POU3F4* and *ZNF711* genes. Mutations to *POU3F4* have been identified as a cause of sensorineural hearing loss and mutations of *ZNF711* have been described previously as a cause of intellectual impairment. As would be anticipated, this patient's medical history was notable for hearing impairment and developmental disability. Choroideremia with deafness and development delay (syn. Xq21 deletion syndrome) was first described by Ayazi in a single pedigree.<sup>28</sup> Although subsequent studies localized this syndrome to Xq21.1-21.3, the first complete molecular analysis of the syndrome was performed only recently by Iossa et al.,<sup>17</sup> who similarly identified loss of the *CHM*, *POU3F4*, and *ZNF711* genes.

The most frequently encountered mutation, in terms of number of pedigrees affected, occurred at a CpG site (877 C>T, Arg293X). Furthermore, the majority of point mutations occurred at the only 5 CpG sites that would code for a stop codon as the result of a C>T transition. Such transitions are believed to occur with high frequency in evolution through inherent instability via methylation and subsequent deamination. Furthermore, these mutations were found to be recurrent, that is, they each occurred in more than one pedigree. Given the known instability of CpG sites and our observations, we hypothesized that these 5 mutations would be anticipated to be encountered with increased frequency in future generations. We also may assume that similar C>T transitions are occurring in the other CpG sites but we do not detect them, either because they are silent (i.e., if C>T is at codon position 3) or because the single amino acid change does not significantly impair REP1 function, for the reasons explained above.

In summary, therefore, we found no evidence for a correlation between different *CHM* mutations and phenotype in our cohort. Therefore, what factors may account for phenotypic variation among choroideremia patients? First, it may be that such correlations elude us because of the small numbers of patients with certain mutations, as outlined above. Leaving this possibility aside, it is further possible that polymorphisms in other genes whose products participate in the prenylation reactions facilitated by *REP1* enable *REP2* to partially compensate for the loss of the former, thereby blunting the effects of null mutations. We found clinical evidence to support the assertion that the C-terminus of REP-1 is crucial for its correct functioning, as two of our patients had mutations in this region, which are anticipated to result in an expressed, though aberrant, protein product. Furthermore, we found that 62% of the unique point mutations responsible for choroideremia in our cohort occurred at 5 CpG sites in which C>T transition results in a stop codon. Such mutations are recurrent and may represent the unwanted consequence of

CpG instability that is a feature in the evolution of humans from the great apes.<sup>29,30</sup> Our data affirmed that patients screened in specialist centers are highly likely to have an identified causative mutation revealed by sequencing (94% of patients in our sample) and confirmed that a gene replacement approach is a logical treatment strategy to be explored.

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