

ONLINE MUTATION REPORT

Achromatopsia caused by novel mutations in both *CNGA3* and *CNGB3*

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Complete achromatopsia or rod monochromatism is a stationary cone dystrophy, with an incidence of ~1 in 30 000, in which functional cones are absent from the retina.^{1,2} Affected individuals usually present in infancy with nystagmus, poor visual acuity (6/60–6/36), photophobia, and complete colour blindness. Fundal examination is normal, but electroretinography reveals absent photopic (cone) responses and normal scotopic (rod) responses. Individuals with incomplete achromatopsia retain some colour vision.³

Achromatopsia is recessively inherited and genetically heterogeneous. To date, three achromatopsia genes have been characterised, the first two described being *CNGA3*^{4,6} and *CNGB3*,⁷⁻⁹ located at chromosome positions 2q11 and 8q21 respectively. *CNGA3* and *CNGB3* respectively code for the α and β subunits of the cGMP gated cation channel in cone cells. Recently, mutations within a third gene, *GNAT2*, which encodes the α subunit of cone transducin, have also been shown to cause achromatopsia.^{10,11} All these genes encode crucial components of cone specific phototransduction.

Studies of patients with achromatopsia have revealed more than 40 disease causing mutations in *CNGA3*, the majority being missense mutations.^{5,6,12} In the patient groups studied, four *CNGA3* mutations (Arg277Cys, Arg283Trp, Arg436Trp, and Phe547Leu) accounted for 40% of all mutant *CNGA3* alleles. In contrast, only eight different mutations of *CNGB3* have been identified to date, with six of these being nonsense mutations.^{8,9} The most common mutation is a 1 bp frame-shift deletion, 1148delC, which accounts for 84% (199/237) of *CNGB3* mutations.¹⁴

Current estimates suggest that mutations in *CNGB3* account for 40–50% of achromatopsia,¹⁴ with mutations in *CNGA3* contributing a further 20%.³ There is therefore a significant proportion of patients for whom neither *CNGA3* nor *CNGB3* mutations can be found (~30%). *GNAT2* is the third gene to be implicated in achromatopsia, but mutations in this gene are thought to be responsible for <2% of patients affected with this disorder.¹¹ This suggests the presence of further genetic heterogeneity that may extend to a locus on chromosome 14, which was identified as a result of maternal isodisomy.¹⁵

Here we report novel mutations in both *CNGA3* and *CNGB3* in a panel of patients with achromatopsia, which further addresses the question of mutation frequency in the two genes.

PATIENTS AND METHODS

A panel of patients with achromatopsia from 22 different families was ascertained from two British clinical centres. Some of the families were of non-European origin. After informed consent was obtained, blood samples were taken for DNA extraction.

Key points

- Achromatopsia (ACHM) is an autosomal recessive stationary cone dystrophy that occurs in complete and incomplete forms. Individuals with complete ACHM have nystagmus, photophobia, 6/60 vision, and total colour blindness. ACHM is genetically heterogeneous.
- Mutations in the *CNGA3* and *CNGB3* genes, which encode the alpha and beta subunits of the cone cyclic cGMP channel, a crucial component of phototransduction, cause achromatopsia. In addition, mutations in *GNAT2*, which encodes cone specific alpha transducin are a rare cause of achromatopsia.
- We investigated 22 UK achromatopsia families in order to define the *CNGA3* and *CNGB3* mutation spectrum in this heterogeneous population. The diagnosis was confirmed by ophthalmological examination, electroretinography, and psychophysical assessment.
- A high proportion (18/22 kindreds) was found to have a disease associated mutation in either *CNGA3* or *CNGB3*. Five *CNGA3* and two *CNGB3* mutations were novel. The previously described *CNGB3* 1148delC was detected in seven of the nine families with *CNGB3* mutations.
- Overall, we have demonstrated in our cohort of complete achromatopsia patients that mutations in *CNGA3* and *CNGB3* show a similar prevalence, and a high mutation detection rate can be achieved by analysing both genes.

Clinical assessment

A full medical and ophthalmic history was obtained and an ophthalmological examination performed. Patients with clinical findings consistent with a diagnosis of achromatopsia underwent electrodiagnostic assessment that included a flash electroretinogram (ERG) and pattern ERG, according to the protocols recommended by the International Society for Clinical Electrophysiology of Vision.¹⁶⁻¹⁸ Psychophysical testing included the use of the American Optical Company, Hardy, Rand, and Rittle plates, Sloan achromatopsia plates, Farnsworth D-15, the Mollon-Reffin (MR) minimal test,¹⁹ a computerised colour vision test,^{20,21} and an anomaloscope. The Sloan achromatopsia plates, Farnsworth D-15, and the

Abbreviations: ERG, electroretinogram; MR, Mollon-Reffin minimal test; SNPs, single nucleotide polymorphisms

MR test were all performed under CIE Standard Illuminant C from a Sol-Source easel lamp (Gretag Macbeth).

Individuals were diagnosed as affected based on the presence of characteristic clinical and psychophysical findings, and electrophysiological evidence of absent or severely reduced photopic ERG, with normal scotopic responses.

Molecular genetic analysis

Total genomic DNA was extracted from blood samples using a Nucleon[®] Biosciences kit. The genomic structure of both *CNGA3* and *CNGB3* was established using published information^{5,8,9} and by screening the NCBI high throughput genomic sequences database with coding sequence. The coding sequences of *CNGA3* and *CNGB3* were amplified by PCR in each individual using primer sequences and conditions as published previously.^{6,8} PCR reactions (50 µl) were performed as follows: 1×NH₄ buffer, 1 mmol/l MgCl₂, 200 µmol/l each dNTP, 10 pmol/l each of sense and antisense primers, 200 ng–1 µg DNA, 1 U BioTaq. After resolution on a 1% LMT agarose gel, products were excised and eluted. Direct sequencing of PCR products was carried out on an ABI 3100

Genetic Analyser using the original PCR primers in the sequencing reactions. The sequence was examined for alterations utilising Sequencing Analysis (ABI Prism[™]) and GeneWorks[™] software.

GenBank sequences were used to construct an alignment of channel protein sequences and to analyse the evolutionary conservation of the corresponding amino acid positions. The sequences used were the cGMP gated (CNG) channel cone α subunits of human (accession number AF065314), mouse (AJ243933), and chicken (X89598) photoreceptors, the rod α subunits of human (S42457) and mouse (XM132106), the cone β subunits of human (AF272900), mouse (NM_013927), and dog (AF490511), and the rod β subunits of human (AF042498) and rat (NM_031809). Sequences were aligned using Clustal W.²²

RESULTS

Clinical assessment

At least one affected individual was assessed from each family, and the clinical data obtained are summarised in table 1. All examined patients had complete achromatopsia

Table 1 Summary of clinical findings and patient genotypes

Family	Patient	Status	Age (years)	Visual acuity OD-OS	Photopic (cone) ERG	Sequence variants	
						<i>CNGA3</i>	<i>CNGB3</i>
1	RM1	A	10	6/60–6/60	Absent		1148delC/595delG
	RM2	A	8	3/60–3/60	Absent		1148delC/595delG
	RM3	N	28	–	–		1148delC/+
	RM4	N	26	6/6–6/6	–		595delG/+
2	RM5	A	38	6/60–6/60	Absent		1148delC/1148delC
	RM6	A	39	6/60–3/60	Absent	Arg223Trp/?	
3	RM7	A	28	6/60–6/60	Absent	Arg223Trp/?	
	RM8	A	16	6/60–6/60	Absent	Thr245Met/?*	
4	RM9	N	43	–	–	Thr245Met/+	
	RM10	A	44	3/60–3/60	Absent		1148delC/?
5	RM11	N	70	–	–		1148delC/+
	RM12	A	15	6/36–6/36	Absent	Gly548Arg/Gly548Arg	
6	RM13	A	9	3/60–3/60	Absent	Arg23Stop/Arg23Stop	
	RM14	N	37	6/6–6/6	–	Arg23Stop/+	
7	RM15	N	52	6/6–6/6	–	Arg23Stop/+	
	RM16	A	19	6/60–6/60	Absent	Gln196Stop/Gln196Stop	
8	RM17	N	40	–	–	Gln196Stop/+	
	RM18	A	26	6/36–6/36	Absent	1443insC/Arg569His	
9	RM19	A	19	6/36–6/36	Absent	Arg221Stop/Arg221Stop	
	RM20	A	14	6/60–6/60	Absent		1148delC/1148delC
10	RM21	A	20	6/60–6/60	Absent		1148delC/1148delC
	RM22	N	50	–	–		1148delC/+
11	RM23	N	55	–	–		1148delC/+
	RM24	A	12	3/60–3/60	Absent	Arg436Trp/?	
12	RM25	N	43	6/6–6/6	–	Arg436Trp/+	
	RM26	N	40	–	–	+/?	
13	RM27	A	34	6/36–6/36	Absent		Phe525Asn/Phe525Asn
	RM28	A	36	6/36–6/36	Absent		Phe525Asn/Phe525Asn
14	RM29	N	61	–	–		Phe525Asn/+
	RM30	N	59	–	–		Phe525Asn/+
15	RM28	A	5	6/60–6/60	Absent		1148delC/1148delC
	RM29	A	6	6/60–6/60	Absent	Phe547Leu/Phe547Leu	
16	RM30	A	2	6/60–6/60	Absent		1148delC/1148delC
	RM31	A	24	6/60–6/60	Absent		1148delC/1148delC
17	RM32	A	5	6/60–6/60	Absent	Phe547Leu/Phe547Leu	
	RM33	A	12	6/60–6/60	Absent		Ile307Val/?*
18	RM34	N	30	–	–		Ile307Val/+
	RM35	A	12	6/60–3/60	Absent	No mutations detected	
19	RM36	A	32	6/60–6/60	Absent	No mutations detected	
	RM37	A	41	6/60–6/60	Absent	No <i>CNGA3</i> or <i>CNGB3</i> mutations detected.	
20	RM38	A	20	6/60–6/60	Absent	GNAT2 mutation identified. ¹⁰	
	RM39	N	33	6/6–6/6	–		

*Substitutions at non-conserved residues that are unlikely to be disease associated (see text).

Family 12 has additional 5'UTR and Asn27Ser mutations in *CNGB3* as discussed in the Results section.

N, normal; A, affected individuals.

with photophobia, variable degrees of nystagmus, decreased visual acuity (6/60–6/36), and absent photopic ERG with normal rod responses. Affected individuals in whom psychophysical testing was performed displayed no residual colour vision. The unaffected family members who were assessed were found to have normal ophthalmological examination.

Molecular genetics

The eight coding exons of *CNGA3* and the 18 coding exons of *CNGB3* were screened for mutations in the panel of 22 small families/probands with achromatopsia. All mutations and genetic variants were assigned a nucleotide/amino acid number starting at the first translated base/codon of the *CNGA3* and the *CNGB3* genes according to GenBank entries NM_001298 and NM_019098, respectively.

CNGA3 mutation analysis

For consistency with previous studies, the numbering of the *CNGA3* sequence excludes the 55 amino acids (165 nucleotides) encoded by exon 2b, reported recently.⁵ Nine different mutations of *CNGA3* were found that would appear to be disease associated. Of these, five are new mutations that have not previously been reported: a homozygous Arg23Stop mutation in family 7, a homozygous Gln196Stop mutation in family 8, a homozygous Arg221Stop mutation in family 10, a 1443insC (Ile482fs) frameshift mutation as a compound heterozygote with the previously reported⁵ Arg569His substitution in family 9, and a homozygous Gly548Arg substitution in family 6 (table 2). For the novel missense mutation (Gly548Arg), 100 control chromosomes were screened for mutations but none was found. Affected individuals in six of the families are homozygous and one family is a compound heterozygote. In families 3 and 12, only one mutation in each was identified. However, these two mutations, Arg223Trp and Arg436Trp, have both been reported previously as disease causing,^{5,6} so it is probable that the second mutation was missed in these families by our current mutation screening strategy.

The three novel nonsense mutations that introduce stop codons at Arg23, Gln196, and Arg221 would all generate (if translated and not subjected to nonsense mediated mRNA decay) significantly truncated proteins that would be unlikely to retain any normal function. The frameshift mutation (Ile482fs) would truncate the protein after adding five aberrant amino acids. The novel Gly548Arg is in the cGMP binding domain and is highly conserved across avian cone and mammalian rod and cone CNG channel α subunit proteins (table 3). The locations of these mutations in the α channel protein are shown in fig 1.

A homozygous Thr245Met substitution was found in the affected individual in family 4. Unlike the above mutations however, Thr245 is not conserved across other CNG

Table 2 *CNGA3* disease causing mutations in families with achromatopsia

Mutation			
Family	Type	Nucleic acid change	Amino acid substitution
7	Hm	67C→T	Arg23Stop
8	Hm	586C→T	Gln196Stop
10	Hm	661C→T	Arg221Stop
3	Ht	667C→T	Arg223Trp
12	Ht	1306C→T	Arg436Trp
9	Ht	1443insC , 1706G→A	Ile482fs , Arg569His
15	Hm	1641C→A	Phe547Leu
18	Hm	1641C→A	Phe547Leu
6	Hm	1642G→A	Gly548Arg

Ht, heterozygous; Hm, homozygous.
Mutations in **bold** represent novel changes.

Table 3 Amino acid conservation in cone and rod α subunits of CNG channel proteins at sites of missense mutations in *CNGA3*

Residue	Human cone α	Mouse cone α	Human rod α	Mouse rod α	Chicken cone α
245	Thr	Lys	Ser	Ala	Gln
548	Gly	Gly	Gly	Gly	Gly

sequences. Its role in disease pathogenesis is therefore less certain and so it has not been included in the list of novel disease associated mutations.

In addition to the above, three single nucleotide polymorphisms (SNPs) were found, a silent T→C transversion at nucleotide 72 that has been reported previously,⁵ a silent C→T change at nucleotide 1278, and a G→A change at nucleotide 171 that causes a Met57Ile substitution. This latter change was found as a polymorphism in eight of the families.

CNGB3 mutation analysis

The Thr383 frameshift (1148delC) mutation has been previously identified as the most common disease causing mutation.^{8,12} In the present study, this 1 bp deletion was found to segregate with disease status in seven of the nine families with *CNGB3* mutations.

Two novel sequence variants in the coding region of *CNGB3* were also identified (table 4). A 595delG frameshift mutation (Glu198fs) that results in a truncated polypeptide of 200 amino acids was found as a compound heterozygote in family 1 and a Phe525Asn substitution as a homozygous change in family 13. Phe525 is highly conserved across other CNG receptor subunits (table 5) and the mutation was not found in a screen of 100 control chromosomes. The locations of the novel Glu198fs and Phe525Asn mutations in the β channel protein are shown in fig 1.

An Ile307Val substitution was detected in affected and unaffected individuals in family 19. However, this is unlikely to represent a disease causing mutation, as it is a very conservative substitution at a site that is not conserved across species (table 5). In fact, Val307 is encoded by the normal canine gene.

In addition, five SNPs were identified. The common 892A→C (Thr298Pro) and 2264A→G (Glu755Gly) polymorphisms have been previously reported.⁸ New silent polymorphisms are 487T→C, 1752G→A, and 2214A→G.

In family 12 with a heterozygous *CNGA3* mutation (Arg436Trp), a single T→G nucleotide change was also found

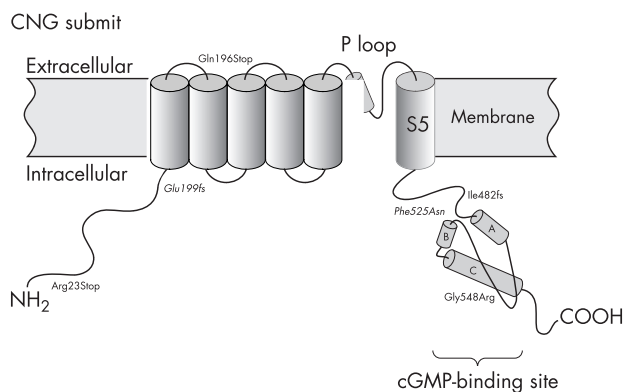


Figure 1 Model of CNG subunit showing the approximate position of the novel *CNGA3* and *CNGB3* (*italic*) mutations.

Table 4 *CNGB3* disease causing mutations in families with achromatopsia

Family	Mutation		
	Type	Nucleic acid change	Amino acid substitution
1	Ht	595delG , 1148delC	Glu199fs , Thr383fs
19	Ht	919A→G	Ile307Val*
2	Hm	1148delC	Thr383fs
5	Ht	1148delC	Thr383fs
11	Hm	1148delC	Thr383fs
14	Hm	1148delC	Thr383fs
16	Hm	1148delC	Thr383fs
17	Hm	1148delC	Thr383fs
13	Hm	1573/4TT→AA	Phe525Asn

Ht, heterozygous; Hm, homozygous, fs, frameshift.

Mutations in **bold** represent novel alterations.

*Substitution at non-conserved residue that is unlikely to be disease associated (see text).

Table 5 Amino acid conservation in cone and rod β subunits of CNG channel proteins at sites of missense mutations in *CNGB3*

Residue	Human cone β	Mouse cone β	Dog cone β	Human rod β	Rat rod β
307	Ile	Leu	Val	Leu	Leu
525	Phe	Phe	Phe	Phe	Phe

in the 5'UTR of the *CNGB3* gene, 36 bp upstream of the ATG start codon. This is present in the heterozygous state in the affected male subject RM24, and was inherited from his mother RM26 (table 1). He also has a second β subunit variant that encodes an Asn27Ser substitution inherited from his father, RM25. As a heterozygous *CNGA3* mutation had been already detected in this family, it is probable that both the 5'UTR and the Asn27Ser substitution are polymorphisms. However, in the absence of a second *CNGA3* mutation in this family, the possibility remains that the achromatopsia may originate from an interaction between the *CNGA3* and *CNGB3* mutations.

Following the exclusion of a mutation in both *CNGA3* and *CNGB3* in family 22, a novel homozygous frameshift mutation in *GNAT2* (c842_843insTCAG; M280fsX291) was identified.¹⁰

DISCUSSION

Mutations in the genes *CNGA3* and *CNGB3* encoding the α and β subunits of cone photoreceptor CNG channels have been described in subjects with achromatopsia.^{5 8 9 13} These proteins are functionally important, therefore, in all three classes of human cones. Moreover, analysis of the homologous *CNGA3* knockout mouse model shows complete absence of physiologically measurable cone function, a decrease in the number of cones in the retina, and morphological abnormalities of the remaining cones.²³

Unlike previous studies, which indicated that approximately 25% of patients with achromatopsia have alterations in *CNGA3*⁵ and 40–50% in *CNGB3*,^{8 14} we have found a more even distribution of mutations across the two genes; 41% of families with mutations in *CNGA3* and 36% with mutations in *CNGB3*. We failed to find CNG mutations in five families, although two of these had heterozygous substitutions (Thr245Met in *CNGA3* and Ile307Val in *CNGB3*) that are most probably not disease associated, as neither residue is

conserved in other CNG receptors. However, final confirmation of the lack of disease association will require a functional assay of the altered protein.

If the family that was subsequently shown to have a homozygous mutation in *GNAT2*¹⁰ is included, 23% (5/22) of families did not appear to have a disease causing mutation in either the *CNGA3* or *CNGB3* gene. This is somewhat lower therefore than the ~30% of patients who did not appear to have either a *CNGA3* or *CNGB3* mutation in previous investigations,¹¹ and may reflect sampling bias arising from the smaller number of families examined in this study. Alternatively, as some of our families are not of European origin, the difference may reflect a different prevalence of *CNGA3* and *CNGB3* disease mutations in different populations.

This study has identified five previously unreported *CNGA3* mutations and two previously unreported *CNGB3* mutations. Of these, one of the *CNGA3* mutations and one of the *CNGB3* mutations are missense mutations resulting in the replacement of a highly conserved amino acid. The remaining novel mutations result in premature termination of translation and therefore severely truncated polypeptides lacking important functional elements such as the pore or cGMP binding domain.

Homozygous mutations were present in 12 families (which may reflect consanguinity within the families, although data relevant to this were not collected), while compound heterozygous mutations were found in two families. Only single heterozygous mutations could be found in *CNGA3* in three families and in *CNGB3* in one family; the most likely explanation for this is that the second mutation is in either an intron or promoter region that is outside the regions sequenced, or an as yet unidentified exon. It is also possible that a larger deletion may be present that would not have been detected by our screening strategy.

The majority of *CNGA3* mutations identified to date are missense mutations, indicating that there is little tolerance for substitutions with respect to functional and structural maintenance of the channel polypeptide. This notion is supported by the high degree of evolutionary conservation among CNG channel α subunits. By contrast, only one of the previously identified mutations of *CNGB3* was a missense mutation, with the others being either stop or frameshift mutations. In the present study, however, one of the two novel mutations is a missense mutation, indicating that functional constraints are as important for the β as for the α subunit, and this is reinforced by the evolutionary conservation of residues at these sites in the β subunit genes of rods and cones. The novel Gly548Arg missense mutation in *CNGA3* adds to the large cluster of 15 different mutations in the cGMP binding domain, indicating that an altered binding affinity for cGMP may be responsible for lack of function of the channel.

The Arg436Trp mutation in *CNGB3* that is present in the heterozygous state in patient RM24 has been previously found in one case of complete and three cases of incomplete achromatopsia.⁵ In all instances, the affected individuals were compound heterozygotes, although as for RM24, the second mutation for one of the incomplete achromatopsia was not identified. From this, it is evident that the severity of the disorder may vary depending on the particular combination of mutant alleles.

There is to date a far greater allelic heterogeneity of *CNGA3* mutations (over 50 described) compared with *CNGB3* mutations (only 10 described). As it is known that *CNGA3* subunits can form functional homomeric channels when expressed alone whereas *CNGB3* subunits cannot,²⁴ it is possible that mutant β subunits are only deleterious and therefore detected if they interact in an inhibitory fashion

with wild type α subunits. However, this interpretation is not supported by recent work²⁵ in which the channel proteins were expressed in *Xenopus* oocytes. In this system, mutant Thr383fs β subunits do not prevent homomeric α channel formation and function. Additionally, it is also unlikely that homomeric α channels are sufficient for normal channel function in intact cone photoreceptors.

An autosomal recessive canine form of cone degeneration of that occurs naturally in the Alaskan Malamute and German Shorthaired Pointer breeds is phenotypically similar to human achromatopsia. Canine *CNGB3* mutations have recently been identified in both of these breeds, thereby establishing these affected dogs as the only naturally occurring large animal model of human achromatopsia.²⁶ Such models provide a valuable system for exploring disease mechanisms and evaluating the potential for gene therapy.

The three achromatopsia disease genes described to date, *CNGA3*, *CNGB3*, and *GNAT2*, all encode proteins that are restricted to the cone phototransduction cascade. This confirms that all three classes of cone photoreceptor utilise a common cone specific cGMP gated channel and a common α transducin subunit. As other components of phototransduction such as the β and γ transducin subunits and the phosphodiesterase subunits are cone specific, it follows that these are also candidates for achromatopsia disease genes.

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