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ORIGINAL ARTICLE

Clinical and genetic investigation of a large Tunisian family with complete achromatopsia: identification of a new nonsense mutation in *GNAT2* gene

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Complete achromatopsia is a rare autosomal recessive disease associated with *CNGA3*, *CNGB3*, *GNAT2* and *PDE6C* mutations. This retinal disorder is characterized by complete loss of color discrimination due to the absence or alteration of the cones function. The purpose of the present study was the clinical and the genetic characterization of achromatopsia in a large consanguineous Tunisian family. Ophthalmic evaluation included a full clinical examination, color vision testing and electroretinography. Linkage analysis using microsatellite markers flanking *CNGA3*, *CNGB3*, *GNAT2* and *PDE6C* genes was performed. Mutations were screened by direct sequencing. A total of 12 individuals were diagnosed with congenital complete achromatopsia. They are members of six nuclear consanguineous families belonging to the same large consanguineous family. Linkage analysis revealed linkage to *GNAT2*. Mutational screening of *GNAT2* revealed three intronic variations c.119−69G>C, c.161+66A>T and c.875−31G>C that co-segregated with a novel mutation p.R313X. An identical *GNAT2* haplotype segregating with this mutation was identified, indicating a founder mutation. All patients were homozygous for the p.R313X mutation. This is the first report of the clinical and genetic investigation of complete achromatopsia in North Africa and the largest family with recessive achromatopsia involving *GNAT2*; thus, providing a unique opportunity for genotype–phenotype correlation for this extremely rare condition.
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INTRODUCTION

Complete achromatopsia (total color blindness; ACHM2, OMIM 216900; ACHM3, OMIM 262300; ACHM4, OMIM 193340), also referred to as 'rod monochromacy', is a rare congenital hereditary disorder of the retina classified among dyschromatopsia. Its prevalence has been estimated to about 1 in 30 000–50 000.[1–3] Symptoms of the disease usually appear in the fifth month after the birth with congenital nystagmus and severe photophobia under daylight conditions.[3] This total colorblindness is characterized by loss of color discrimination, low visual acuity, photophobia and nystagmus.[3–6]

Rod monochromacy is inherited as an autosomal recessive trait with complete penetrance and is associated with mutations in four different genes. Two genes, *CNGA3* and *CNGB3*, which are more frequently associated to achromatopsia,[7] encode the alpha and beta subunits of cyclic guanosine monophosphate-gated cation channel in cone cells, respectively.[8,9] This channel is involved in cone membrane hyperpolarization during visual transduction. The *GNAT2* gene encoding the alpha subunit of cone transducin G protein has been reported as the third most frequently affected gene, responsible for 2% of achromatopsia cases.[10,11] Recently, the *Pde6c* gene has been involved in achromatopsia in the murine *cpfl1* mutant. Consequently, it has been shown that the human ortholog *PDE6C*, located at 10q24, was also linked to achromatopsia.[12] It encodes the cone cyclic guanosine monophosphate-specific 3′,5′-cyclic phosphodiesterase alpha-subunit and has an important role in signal transduction.

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We report here on a large consanguineous family originating from Southern Tunisia presenting with autosomal recessive complete achromatopsia. On the basis of the informative pedigree comprising at least 12 affected individuals, we have undertaken linkage analysis and identified a novel homozygous nonsense mutation in *GNAT2* that likely impairs visual transduction.

MATERIALS AND METHODS

Patients

Individuals ACH3, ACH4, ACH5 and ACH6 were referred in 2004 to Hedi Rais Institute of Ophthalmology of Tunis by the school doctor for complaints of strong myopia, photophobia and difficulties to read at daylight with better ability to read at night and regression of visual acuity for the elder children. Discussion with clinicians raised awareness of the genetic cause of the disease and seven additional family members presented themselves to the Institute for further clinical investigation.

After informed consent, in accordance with the Declaration of Helsinki, genealogical data and biological material were collected at home of each nuclear family (Figure 1). All patients presented similar clinical features. They were the offsprings of intermarriage between first-degree cousins (Figure 1), had all the same surname and are likely descendant of an Arab tribe that migrated to Southern Tunisia.

Clinical and electrophysiological examinations

ACH1-G multiplex nuclear family and all other available family members underwent the ophthalmological examination, including best corrected visual acuity, slit lamp examination of the anterior segments, funduscopy for the differential diagnosis with cone dystrophy, fluorescein angiography if macular changes were suspected and electroretinographic recordings in accordance with the standard protocol of the International Society Of Clinical Electrophysiology Of Vision.[13] All individuals who exhibited nystagmus or low-visual acuity had color vision tests using the Farnsworth–Munsell 100 Hue and a full-field electroretinogram (ERG) using the vision monitor Métrovision (Pérenchies, France). Complete achromatopsia was diagnosed in younger patients on the basis of a simplified method using electroluminescent diode stimulation at red light.[14] Optical coherence tomography examinations were performed with dilated pupils by analyzing the B-scans in a carrier and her affected son ACH22 (OTI, Ophthalmic Technologies, Toronto, Canada).

Molecular investigation

Blood samples were taken for DNA extraction from peripheral blood leukocytes by salting out.[15] Genotypes for all available family members were determined using microsatellite markers D2S2311 (AFMb355zg1) and D2S2175 (AFMa153zg5), D8S167 and D8S273 (AFM179yf6), D1S2778 (AFMb338wd9), D1S418 (AFM197yg1), and D10S185 (AFM019th6) overlapping the *CNGA3*, *CNGB3*, *GNAT2* and *PDE6C* genes, respectively.[16]

In addition, two primers, GNAT2M1 and GNAT2M2, were designed to flank the short tracks of nucleotide repeats of *GNAT2* at the ACHM4 locus (Supplementary data 1). The genotyping protocol was performed as reported previously.[17]

Mutational screening of *CNGA3* and *GNAT2* exons has been performed by direct sequencing using the Big Dye terminator Kit (Applied Biosystems, Foster City, CA, USA) in an ABI Prism 3130 sequencer (Applied Biosystems).

RESULTS

Clinical data

Ophthamological and general data of 35 individuals belonging to five nuclear families including patients and their relatives when available are summarized in Table 1. For the majority of the patients, parents reported pendular nystagmus in early infancy.

The rod monochromacy includes nystagmus, diminished visual acuity, normal fundus, abolished photopic response in ERG, loss of

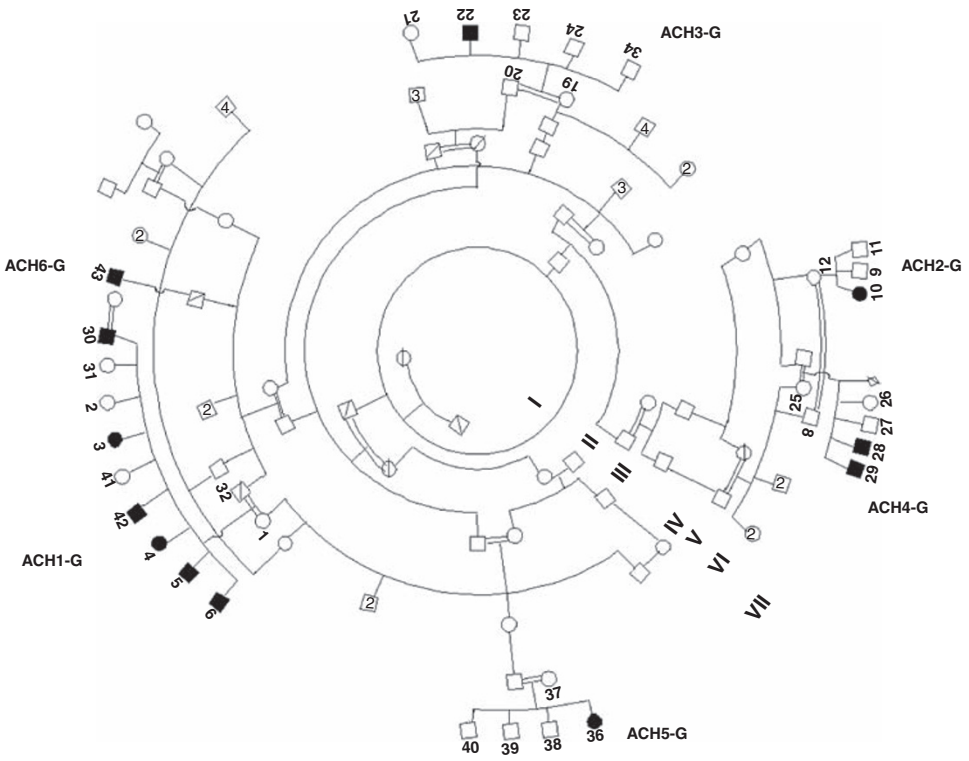

Figure 1 The Tunisian complete achromatopsia genealogy ACH-G. Each generation is designated by Roman numerals (I–VII). Squares and circles indicate male and female members, respectively. Slashes indicate that an individual is deceased. Shaded symbols indicate affected individuals. Double lines indicate consanguineous mating.

Table 1 Ophthalmological and general informations of patients with complete achromatopsia and their relatives

				Visual acuity						
Nuclear family	*Individual*	*Age at last examination/sex*	*Status*	*RE*	*LE*	*Nystagmus*	*Photophobia*	*Color vision*	*Cone ERG*	*Fundus*
	ACH 1	53/F	Unaffected	20/20	20/20	No	No	Normal	Normal	Normal
	ACH 2	27/F	Unaffected	—	—	No	No	—	—	Normal
	ACH 3	25/F	Affected	20/400	20/400	++	Yes	None	No response	Normal
	ACH 4	17/F	Affected	20/400	20/400	++	Yes	None	No response	Normal
ACH1-G	ACH 5	13/M	Affected	20/200	20/200	++	Yes	None	No response	Normal
	ACH 6	11/M	Affected	20/100	20/100	++	Yes	None	No response	Normal
	ACH 41	22/F	Unaffected	—	—	No	No	—	—	—
	ACH 42	19/M	Affected	—	—	++	Yes	—	—	—
	ACH 30[a]	37/M	Affected	20/100	20/100	+	Yes	None	No response	Normal
	ACH 32	58/M	Unaffected	—	—	No	No	Normal	Normal	Diabetic retinopathy
	ACH 31	29/F	Unaffected	—	—	No	No	—	—	Normal
	ACH 8	50/M	Unaffected	—	—	No	No	Normal	Normal	Normal
	ACH 9	23/M	Unaffected	20/20	20/70	No	No	—	Normal	Normal
ACH2-G	ACH 10	11/F	Affected	20/400	20/400	++	Yes	None	No response	Normal
	ACH 11	21/M	Unaffected	—	—	No	No	—	—	Normal
	ACH 12	44/F	Unaffected	—	—	No	No	Normal	Normal	Normal
	ACH 19	50/F	Unaffected	20/20	20/20	No	No	—	Normal	Normal
	ACH 20[b]	58/M	Unaffected	20/25	20/70	No	No	—	Normal	Normal
	ACH 21	22/F	Unaffected	20/20	20/20	No	No	—	Normal	—
ACH3-G	ACH 22	15/M	Affected	20/400	20/400	++	Yes	None	No response	Normal
	ACH 23	19/M	Unaffected	20/20	20/20	No	No	—	Normal	—
	ACH 24	24/M	Unaffected	20/20	20/20	No	No	—	Normal	—
	ACH 34	27/M	Unaffected	20/20	20/20	No	No	—	Normal	—
	ACH 25	F	Unaffected	—	—	No	No	—	—	Normal
	ACH 26	F	Unaffected	—	—	No	No	—	—	—
ACH4-G	ACH 27	M	Unaffected	—	—	No	No	—	—	—
	ACH 28	7/M	Affected	20/400	20/400	++	Yes	—	No response	Normal
	ACH 29	M	Affected	<20/400	<20/400	++	Yes	None	—	Normal
	ACH 35	8/F	Unaffected	20/20	20/25	No	—	—	Normal	—
	ACH 36	8/F	Affected	20/400	20/400	+	Yes	—	No response	Normal
	ACH 37	35/F	Unaffected	20/20	20/20	No	No	—	Normal	Normal
ACH5-G	ACH 38	17/M	Unaffected	20/20	20/20	No	No	—	Normal	—
	ACH 39	14/M	Unaffected	20/20	20/20	No	No	—	Normal	—
	ACH 40	12/M	Unaffected	20/20	20/20	No	No	—	Normal	—
ACH6-G	ACH 43	10/M	Affected	—	—	++	Yes	—	—	—

Abbreviations: ERG, electroretinogram; F, female; LE, left eye; M, male; RE, right eye.
Visual acuity corresponds to the best corrected visual acuity. The signs '+' and '++' indicate the transient and severe nystagmus.
[a]Oligospermia.
[b]Cataract and pseudophakia.

color vision and photophobia. Among 12 individuals known to be affected in the family, 7 were fully examined, 3 others partially and ACH42, ACH43 were non-cooperative (Table 1).

Three affected schoolchildren ACH3, ACH4, ACH5 and their first cousin ACH10, followed since 2004, mentioned that they saw better at night, suggesting a day blindness. Their oldest brother, ACH30, was seen at the Institute for an evaluation of complaints of nystagmus and visual impairment at his workplaces. All examined patients had poor visual acuity ranging from 20/200 to 20/400 and nystagmus (Table 1).

On the last examination, in March 2007, the proband ACH3 and her young siblings ACH4, ACH5 and ACH6 exhibited decreased vision. Although electroretinographic recordings of the scotopic b2 waves were normal in dark-adapted conditions, the photopic components were not recordable (Figure 2) reflecting a visual function mediated entirely by rods. Color vision was tested monocularly and revealed a lack of color axis. None of the affected subjects could name any color correctly (Table 1).

No abnormalities were observed in fundus among patients except for ACH30 and ACH4 who had 4 and 5 mm diameters of peripheral congenital hypertrophy, respectively. The retina was normal in the patient ACH22 and the healthy subject ACH19 in optical coherence by tomography examinations (Figure 3). All affected individuals had similar clinical characteristics for rod monochromacy and a different course for the pendular nystagmus. A better improvement

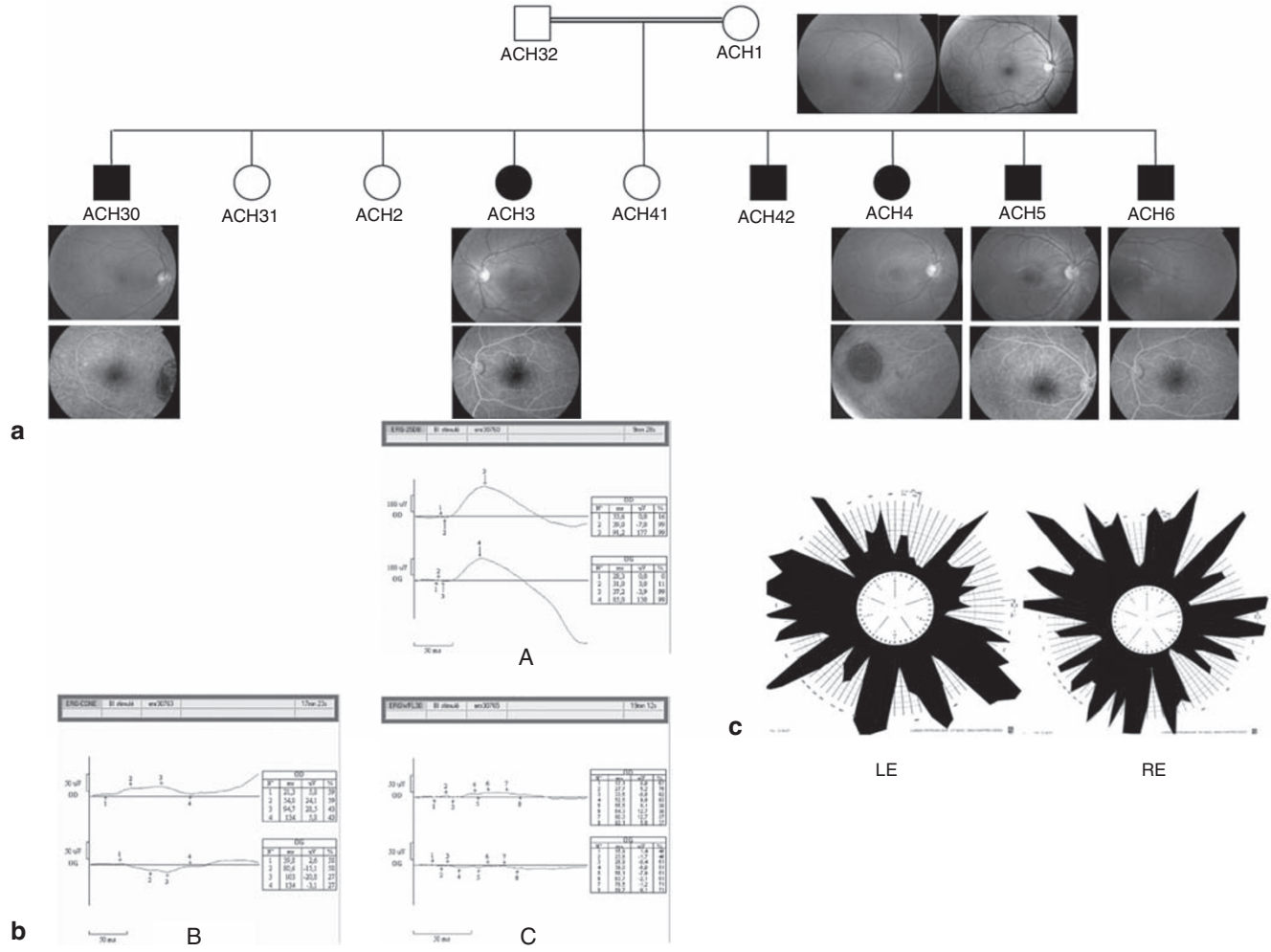

Figure 2 Ophthalmological findings in ACH1-G family. (**a**) Funduscopy and angiography. The angiography and the fundus examination have a normal appearance in affected patients. Hypertrophy of retinal pigmentary epithelium was found on peripheral fundi in ACH30 and ACH4. (**b**) Electroretinogram recordings in ACH3. (A) Scotopic phase: response 25 Db on blue light stimulus is normal; (B and C) photopic phase: no response on cone (B) and cone-flicker stimulus (C). (**c**) The Farnsworth–Munsell 100 hue color vision results of the affected ACH5. The illumination was provided by neon light. This pattern is same for all investigated achromatopsic patients. LE, left eye; OD, optical density; RE, right eye.

of nystagmus was noticed for the 11-year-old girl ACH10. This was not the case for their cousins ACH6 (10-year-old) and ACH22 (15-year-old, at the time of examination).

In conclusion, diagnosis of complete achromatopsia was established for ACH-G family. The genealogic tree of this family is suggestive of an autosomal recessive mode of inheritance of the disease.

Genetic analysis to the *CNGA3*, *CNGB3* and *PDE6C* genes

As a sporadic Tunisian achromatopsia case has been reported with p.Pro372Ser mutation within *CNGA3* gene,[18] patient ACH4 from family ACH1-G, was screened for this mutation. Direct sequencing of exon 7 excluded the p.Pro372Ser mutation in that patient. Because this finding could not exclude occurrence of another mutation in *CNGA3*, linkage was first investigated in the nuclear and multiplex ACH1-G family.

Genotype analysis showed that for both D2S2311 and D2S2175 markers, affected individuals did not share the same genotype. This was extended with haplotype analysis that showed that affected individuals ACH30 and ACH3, and his healthy sibling ACH2 had the same haplotype, thus suggesting exclusion of *CNGA3* in this family. Using a similar strategy, haplotype analysis excluded linkage to *CNGB3* and *PDE6C* genes in this family.

Haplotype analysis to the ACHM4 locus

ACH patients and family members were subtyped with five microsatellite markers (D1S2778, GNAT2M1, GNAT2M2, D1S2726 and D1S418) overlapping the *GNAT2* gene region. For each family, the most likely haplotype was constructed by visual inspection. Affected offsprings in each family showed homozygosity by descent at the ACHM4 locus, whereas none of the unaffected individuals were homozygous for the two closest markers flanking *GNAT2* gene. All affected members in each family presented the same homozygous haplotype for the three closest markers to *GNAT2*, D1S2778, GNAT2M1 and GNAT2M2. D1S2726 marker was non-informative marker in this family (Supplementary data 2). A maximal two point lod score of 4.33 at $\theta=0$ was obtained for the marker GNAT2M2 (Supplementary data 3). Affected offsprings of nuclear families ACH2G, ACH5G, ACH6G and ACH7G shared the same disease haplotype (2-5-3-284-2) for the markers encompassing *GNAT2*

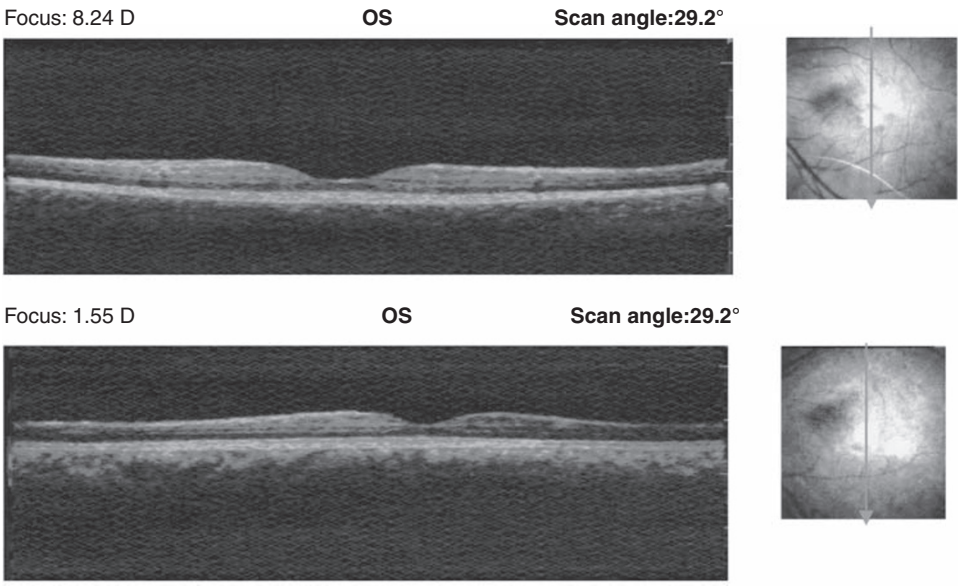

Figure 3 The optical coherence tomography image of the central retina of a healthy carrier subject ACH19 (top) and the ACH22 patient with achromatopsia (bottom).

region. This suggests that these families likely share a common mutation inherited from a common ancestor.

Mutation analysis in *GNAT2* gene

Mutational screening was performed for one patient (ACH30) who carries the morbid haplotype (2-5-3-284-2). It revealed a novel homozygous nonsense mutation c.937C>T in exon 8 that co-segregated with three intronic variants c.119−69G>C, c.161+66A>T and c.875−31G>C in all affected individuals (Figure 4).

This mutation results in the substitution of an arginine (CGA) for a stop codon (TGA) at position 313 (p.R313X) (Figure 4).

The p.R313X mutation truncates 41 amino acids at the C terminus of the alpha transducin protein. It likely interrupts interaction with photoactivated rhodopsin, resulting in a failure of visual transduction.

DISCUSSION

In this paper, we report a clinical and genetic study of GNAT2 achromatopsia patients in a seven-generation consanguineous Tunisian family, the third and largest ever reported so far. Until now two sporadic GNAT2 achromatopsia cases and four patients belonging to two families were been reported worldwide.[11]

A total of 12 individuals were diagnosed as having total color blindness and others were suspected to be affected based on familial history. The relatively high number of affected individuals is likely the result of nonrandom mating in a highly endogamous family. This condition is similar to CNGB3 achromatopsia among the Pingelapese islanders.[19]

Among the patients, one individual (ACH30) was infertile. Oligospermia was diagnosed and no chromosomal abnormalities in 3 Mb R-banded karyotype were identified. The infertility may reflect a hormonal disturbance, as previously reported in a case of total color blindness.[20,21] Other phenotypes were observed in the family, we report most of them being developmental diseases, other ocular diseases (nystagmus and strabismus) or neurological defects (mental and motor impairment).

The clinical investigation of ACH-G family was strongly consistent with rod monochromacy. The reduced visual acuity, nystagmus and photophobia were the main presenting complaints. Fundus was normal and abnormalities were observed in photopic responses, thus excluding cone dystrophy and cone-rod dystrophy. Only two patients presented a small peripheral hypertrophy of retinal pigmentary epithelium (ACH4, ACH30) that could be an uncommon fortuitous clinical association. ERG recordings allowed a differential diagnosis of Leber congenital amaurosis by showing normal rod responses in the 10 examined patients. Extinguished photopic recordings and recessive inheritance of the disease in ACH-G family were inconsistent with the incomplete achromatopsia, and argue for the complete form. Color discrimination test also exhibited a lack of the three-color axis in Ishihara plates in youngest patient ACH10, and Farnsworth–Munsell 100 Hue test in all examined patients. Thus far, complete ophthalmological examination indicated that electroretinographic analysis remains the most reliable method for complete achromatopsia diagnosis.[14]

The identification of the novel mutation p.Arg313X in *GNAT2* gene together with the report of a previous Tunisian sporadic case carrying a homozygous P372S mutation in *CNGA3* gene,[18] highlights the genetic heterogeneity of the ACH in Tunisian population. These findings further delineate the genetic heterogeneity of rare autosomal recessive diseases in Tunisia, as reported previously for different conditions.[22–26] Compared with the other two genes known to cause achromatopsia *CNGA3* and *CNGB3*, *GNAT2* is only a minor achromatopsia locus, which account for 2% of the cases.[7]

As this is the largest sibship affected with GNAT2 achromatopsia, this family gave a unique opportunity for phenotype-genotype analysis and comparison to other complete achromatopsia subtypes. Although some *CNGB3* affected subjects and carriers presented a macular atrophy and peripheral granularity, respectively,[27] no macular atrophy was identified in ACH-G patients' and carriers' fundi. Only, the carrier ACH32 fundus exhibited a diabetic retinopathy (Table 1). Likewise, retinal layers exploration by optical coherence tomography revealed no change in the thickness of the central retina in carrier ACH19 and her affected son ACH22, whereas a thinning of the retina was reported in CNGB3 achromatopsic patients.[28] In ERG, ACH-G patients have revealed a normal rod-mediated function, whereas rod-b wave amplitude could be reduced in CNGA3 and CNGB3 patients.[27] Moreover, although maximal flash a-wave was reduced and b-wave

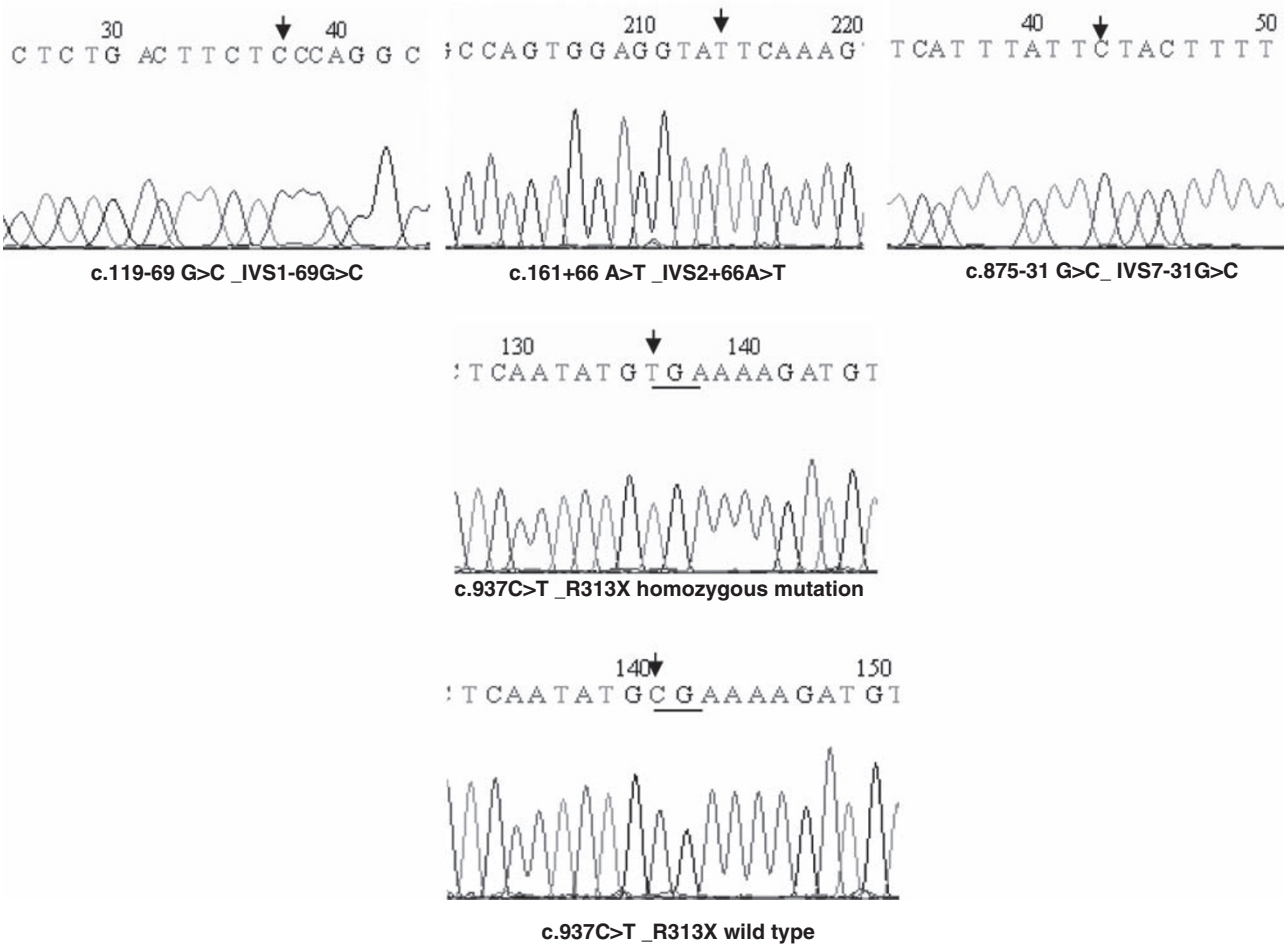

Figure 4 The *GNAT2* mutations in nuclear families segregating with achromatopsia. Electropherograms of mutant sequence (top) and corresponding wild-type reverse-strand sequence (bottom). The arrow indicates the nucleotide site substitution.

was normal in CNGA3 and CNGB3 affected individuals, maximal flash a and b-waves were both reduced for ACH-G patients. Under photopic conditions, a clearly abolished photopic b-wave and flicker responses with no residual cone function was observed in ACH-G patients, whereas a progressive loss of residual cone function was reported in CNGA3 and CNGB3 patients.[29] Therefore, GNAT2 achromatopsia seems to be more severe than CNGA3 and CNGB3 phenotypes (Supplementary data 4).

As with all the *GNAT2* mutations observed,[10,11,30] the p.Arg313X results in a premature translation termination. It is located in a receptor contact site α4-β6 loop of Gα transducin[31] leading to a truncated alpha G-transducin short of 41 amino acids in the carboxy terminal region. This region contains a conserved motif IKENLKDCGLF common to the Gα group of G proteins. It is essential for the interaction with intermediates states of metarhodopsin, meta-Ib as well as meta-II.[32] Therefore, we could hypothesize that p.Arg313X leads to a disruption of heterotrimeric G-protein signaling, which couples light-activated cone visual pigments to the visual transduction cascade. That might explain extinguished photopic b-wave and flicker responses found in GNAT2 achromatopsia Tunisian patients.

To our knowledge, this is the first report of a detailed clinical GNAT2 achromatopsia phenotype in a large pedigree. Recently, both cone-mediated ERG response and visual acuity in a GNAT2 mouse model *cpfl3* were rescued using targeted adeno-associated virus gene therapy.[33] Unraveling the genetic basis of the disease in the reported family is an essential step for future access to gene therapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Mutation nomenclature

We have chosen to number the A of the start codon (ATG) of the GenBank *GNAT2* cDNA sequence (NM_005272.3) as nucleotide +1.

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