Title

In vivo imaging of cone mosaic in patient with *GNAT2* variant associated achromatopsia

Running title; Cone mosaic of patient with *GNAT2* variant ACHM

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Abstract

Purpose: The two most common causative genes for achromatopsia (ACHM) are the *CNGA3* and *CNGB3* genes, and the other gene variants including *GNAT2* account for only a small portion of the patients with ACHM. The cone mosaics in eyes with mutations of *CNGA3* and *CNGB3* are severely disrupted, however the cone mosaics in patients with *GNAT2*-associated ACHM have been reported to have a contiguous pattern in the adaptive optics (AO) retinal images. The purpose of this study was to analyze the cone mosaics of another case of *GNAT2*-associated ACHM.

Patient and methods: The patient was a 17-year-old Japanese boy. Comprehensive ocular examinations including fundus photography, electroretinography (ERGs), optical coherence tomography (OCT), and whole exome analysis were performed. The cone mosaics were recorded with a flood-illuminated AO fundus camera, and the cone densities of the patient were compared to those of 10 normal control eyes.

Results: The patient had the typical phenotype of ACHM, and a novel homozygous variant of c.730_743del in *GNAT2* was identified. The fundus did not show any specific abnormalities, and the ellipsoid zone in the OCT images was present. The AO fundus image showed clearly defined cone mosaics around the fovea. The cone densities of the patient at 500 μm from the fovea were reduced by 15-30% compared to those of normal subjects.

Conclusions: This is the first report of Japanese patient with ACHM with a novel *GNAT2* variant. ACHM eyes with this *GNAT2* variant had preserved cone structure with loss of function.

Introduction

Congenital achromatopsia (ACHM) is an autosomal recessive retinal abnormality that affects approximately 1 in 30,000 people [1]. The symptoms and signs of achromatopsia are present at birth or early infancy, and individuals with ACHM are characterized by pendular nystagmus, poor visual acuity, severe photophobia, and an absence or markedly altered color vision. The fundus usually appears normal, and the electroretinographic (ERG) findings indicate an absence of cone function with normal rod function [2].

To date, six genes have been reported to cause ACHM. The two most common causative genes are *CNGA3* [3] which encodes the alpha-subunit and *CNGB3*, [4] which encodes the beta-subunit of the cGMP-gated cation channel. Sequence variants of *CNGA3* and *CNGB3* have been reported to account for 70% to 80% of the ACHM in the world [5-8].

Another causative gene for ACHM is *GNAT2* which encodes the alpha-subunit of transducin, *PDE6C, PDE6H*, and *ATF6,* but accounts for only a small proportion of eyes with ACHM [9,10].

The advancement of retinal imaging technology has allowed investigators to examine

the outer retinal architecture in eyes with ACHM. Several optical coherence tomographic (OCT) studies have shown different degrees of disruption of the hyperreflective photoreceptor band known as the ellipsoid zone (EZ), an optically empty cavity in the outer retina of the fovea [11-13].

Adaptive optics (AO) retinal imaging has enabled clinicians to view *en face* images of the cone photoreceptor mosaics of human eyes *in situ* [14-16]. Carroll et al. reported a severely disrupted cone mosaic pattern in the fovea and parafovea in eyes with ACHM [17]. Nine molecularly-confirmed individuals with ACHM caused by variants of *CNGA3* and *CNGB3* were reported to have residual cone structure but most were abnormal with reduced reflectance. More recently, Scoles et al. examined four patients with ACHM associated with *CNGA3* and *CNGB3* variants with a “split detector” AO scanning light ophthalmoscope (AOSLO). They reported that the cone inner segment structure was substantially retained although the cone outer segments were disrupted in these patients [18]. These results indicated that the disrupted cone outer segments were related to the pathology of ACHM.

However, the results of two other studies of ACHM eyes with the *GNAT2* variant showed a greater degree of preservation of the outer retinal architecture in the SD-OCT and AOSLO images. Sundaram et al. reported that 3 of 4 subjects with the *GNAT2* variant had an intact EZ in the SD-OCT images [13]. Dubis et al. reported that

the number of cone cells in two brothers with *GNAT2*-associated ACHM was preserved with nearly contiguous cone mosaics [19]. However, the cone mosaics obtained by AOSLO were blurred in other subjects with ACHM caused by other genes variants [19]. These results indicated that the disease mechanism of *GNAT2*-associated ACHM might be unique and may be due to cone dysfunction rather than a cone structural disorder.

Individuals with *GNAT2*-associated ACHM account for only 1-2% of all cases of ACHM [9], and the two brothers with *GNAT2*-associated ACHM were the only ones examined by AO imaging. Thus, the purpose of this study was to examine the cone mosaics in a case with *GNAT2*-associated ACHM.

Patient and methods

This was an observational case study. The procedures used were approved by the Institutional Review Board of Nagoya University Graduate School of Medicine (1067-3). A signed informed consent was obtained from the patient, and all of the procedures conformed to the tenets of the Declaration of Helsinki.

The patient had a comprehensive ophthalmological examination including visual acuity measurements, dilated ophthalmoscopy, Goldmann kinetic perimetry, Farnsworth dichromatic test (PD-15), full-field electroretinography (ERG; UTAS, LKC

Technologies, USA), spectral domain optical coherence tomography (SD-OCT; Spectralis, Heidelberg Engineering, Germany), and fundus autofluorescence imaging (FAF; 200Tx, optos, UK) at Nagoya University. Full-field ERGs were recorded from the patient under the guidelines of the International Society for Clinical Electrophysiology of Vision (ISCEV) Standards [20] using a Burian–Allen bipolar contact lens electrode (Hansen Ophthalmic Laboratories, Iowa City, IA).

High-resolution *en face* images of the cone photoreceptor mosaics were obtained with a flood-illuminated AO fundus camera (rtx1; Imagine Eyes, Orsay, France) as described. [21,22]. The AO fundus images were exported and overlaid on the SLO images using Adobe Photoshop CS4 (Adobe Systems, Inc, San Jose, CA).

The cell densities of both eyes of the patient and 10 eyes of 10 axial length-matched normal controls (23.4 + 0.7 mm, Mean + SD) were measured at 500 μm and 1000 μm from the fovea on the nasal and temporal side. The area of analysis was 200 × 200 pixels that corresponded to about 160 μm square. The automatically calculated cell density was subsequently corrected manually. We did not assess the cones within 1° from the foveal center because the cone density is too high and the cone diameter is too small within the area in normal eyes and exceeded the resolution limits of the camera (250 line pairs/mm).

Blood samples were taken from the patient for genetic analysis. Whole exome

sequencing was done according to the published protocol of the National Institute of Sensory Organs (NISO), a customized analysis protocol for the Japanese population [23].

Results

The patient was a 17-year-old Japanese boy. He was referred to Nagoya University with severe photophobia, poor visual acuity, and color blindness that were detected shortly after birth. There was no family history of any ophthalmic diseases and no consanguinity. A comprehensive ocular examination showed that his decimal best-corrected visual acuity (BCVA) was 0.09 with -1.5 diopters sphere (DS) combined with −1.5D cylinder (DC) ax 70º OD, and 0.08 with -2.75 DS combined with −2.0 DC ax 180º OS. The PD-15 test yielded several crossing lines between deutan and tritan axis in both eyes, and the results indicated that the patient was totally color blind (Fig. 1a and b). A central scotoma was detected by Goldmann perimetry, but slit-lamp and ophthalmoscopic examinations found no abnormalities (Fig. 1c). Fundus autofluoresence showed no specific abnormal patterns (Fig. 1d).

Horizontal and vertical cross sectional images of the fovea recorded by SD-OCT are shown in Figures 1e and 1f. In both images, the EZ was clearly intact but the cone interdigitation zone (CIZ) appeared to be unified with the EZ and was not identified

distinctly. A bulging of the EZ at the central fovea, termed the foveal bulge, was found in the vertical image of SD-OCT but not in the horizontal scan images because the image was not centered.

The amplitudes of the full-field scotopic ERGs (dark-adapted 0.01, 3.0 and 10.0) were within the range of normal eyes but the photopic ERGs, light-adapted 3.0 and 30 Hz flicker, were non-recordable (Fig. 2). These ERG results indicated normal rod functions but an absence of cone functions. The ERG results were compatible with ACHM.

A homozygous variant of c.730_743del (p.H244fs) was detected by whole exome sequencing analysis (mRNA reference sequence: NM_005272). Other candidate variants reported in earlier studies as causative genes for ACHM, *CNGA3*, *CNGB3, PDE6C, PDE6H*, and *ATF6*, were not present. Five other heterozygous missense variants were detected in the genes reported in Retnet™ (https://sph.uth.edu/retnet/home.htm) including *TTLL5, RBP4, PNPLA6, PCDH15* and *MFN2*. In silico bioinformatic analyses were performed to predict the pathogenicity of all of these identified variants. Variants in *RBP4, PNPLA6* were predicted as pathogenic in the software prediction programs of Sorting Intolerant from Tolerant (SIFT) and PolyPhen2. But we concluded that the ACHM in this patient was caused by a *GNAT2* variant from the inheritance pattern.

An AO montage image of the fovea of the patient is shown in Figure 3. Well-defined cone photoreceptor mosaics were present in the patient. Magnified AO images centered at 500 μm, and 1000 μm nasal and temporal from the fovea also showed clearly defined cone mosaics (Figs. 3d and 3e). The integrity of the cone photoreceptors in the AO images of 10 normal control eyes was compared to that of this patient. The cone mosaics of the patient (Figs. 3d and 3e) were comparable to that of normal subject (Figs. 3f and 3g). The cone density at the foveal center was too high and the cone diameter too small and exceeded the resolution limits of the camera in the eyes of both the patient and controls (Fig. 3b* and controls; data not shown).

The cell densities of the eyes of the patient and 10 eyes of axial length-matched normal controls were measured at 500 μm and 1000 μm from the fovea on both the nasal and temporal sides. The average axial length of both eyes of the patient was 23.9 mm and those for normal control eyes were 23.4 + 0.7 mm (Mean + SD). The results of the cone density measurements are shown in Figure 4. The cell densities of the right and left eyes were similar. The cone densities of the patient at 1000 μm from the fovea in both nasal and temporal sides were similar to the mean of the normal subjects. However, those at 500 μm from the fovea were 15% to 30% lower than that of normal controls, even though the cone mosaic of the patient appeared to be

contiguous. The value at 500 μm from the fovea was below the mean -2 standard deviations of that of the normal subjects. The cone density of our control data was comparable to those of previous histological data [24] and AO image data [25] as shown in Figure 4.

Discussion

The *GNAT2* gene encodes the cone photoreceptor-specific alpha-subunit of transducin, a G-protein of the phototransduction cascade, which was first shown to be the causative gene for ACHM by Kohl et al. in 2002 [9]. However, only a small number of case reports have been published since then because of the rarity of *GNAT2*-associated ACHM [13,26,27]. *GNAT2*-associated ACHM has not been reported in the Japanese population as far as we searched “Pubmed” and “Ichushi”.

We have presented the ocular phenotype of a Japanese individual with *GNAT2*-associated ACHM who had a novel homozygous variant, c.730_743del (p.H244fs). ACHM is classified as complete ACHM and incomplete ACHM in which some cone function is preserved. We diagnosed this patient as a complete ACHM from the severe reduction of the visual acuity, abnormal color vision tests, and absence of the photopic ERGs. The cone mosaics detected by a flood-illuminated AO fundus camera are supposed to be the cone outer segments [18], and our results

indicated that our patients had morphologically normal cone outer segments. Interestingly, the results of the other tests indicated that the cones were not functioning normally.

Previous OCT studies of individuals with ACHM showed that 3 of the 4 subjects with the *GNAT2* variants, homozygous variant of c.843-844insAGTC, had an intact EZ despite a low retinal sensitivity determined by microperimetry [13]. The other case had a defective EZ in the central fovea with intact EZ in other areas [13]. Our case had an intact EZ with a foveal bulge which suggests an increase of the foveal cone outer segment density [28]. The AO image of the central fovea showed blurred cone mosaics because their density exceeded the resolution limits of the AO camera. This would then indicate that the cone density was greater than 30 000/mm^2 [29]. Thus, the AO images confirmed the high concentration of cone outer segments in the central fovea.

One question still remains on why the CIZ was not identified in our case. This was also reported in the OCT images of the reported cases [13]. The CIZ represents the outer segments of the cone photoreceptors which can be detected by the AO flood camera. Contiguous cone mosaics in the AO fundus image and invisible CIZ in the OCT image appear to be contradictory. One plausible explanation for this is that the

averaging of 100 images of the SD-OCT image blurred the fine structures of the cones because of nystagmus.

In an earlier study, Dubis et al. demonstrated clear differences in the cone mosaics between ACHM caused by *CNGA3/B3* and by *GNAT2* variants [19]. They reported that two brothers who had the homozygous variants, c.843-844insAGTC of *GNAT2,* had photoreceptor densities only slightly lower than that of normal eyes, and an almost normal contiguous mosaic pattern at the fovea. Thus, the question arises on whether their findings could extend to other subjects with *GNAT2*-associated ACHM or were restricted to the specific variants of the brothers. The results of our patient showed that the cone mosaic were contiguous and comparable to that of normal subjects. Our data also indicated that the cone density was reduced compared to that of normal subjects only at the central fovea but it was within the normal range at 1000 μm from the fovea. These results suggest that ACHM with the *GNAT2* variant had preserved cone structure with an altered function, which is different from the patients with the other ACHM genotypes, all of whom had markedly reduced cone structure at the parafoveal location [19].

However, the previous of OCT images of the EZ of eyes with *GNAT2*-associated ACHM varied from a lack of the EZ in the central fovea to the existence of a foveal bulge [13]. These results might indicate that the cone numbers of these patients also

varied, and a larger number of cases need to be studied to determine the exact relationship between the genotype and cone mosaics.

The reason why the eyes with *GNAT2*-associated ACHM had preserved cone outer segment is still unknown. *GNAT2,* G Protein Subunit Alpha Transducin 2, encodes the alpha-subunit of transducin which is necessary for the hyperpolarization of the cones. Transducin activates the phosphodiesterase (PDE) and hydrolyzes the diffusible messenger cGMP. The resulting decrease in the cytoplasmic free cGMP concentration leads to the closure of the cGMP-gated channels on the plasma membrane. *CNGA3/B3* encodes the subunit of the cGMP-gated cation channel which is an important structural protein in the cones besides its role as the Na^{+}/Ca^{2+} influx pathway. Thus, the loss of CNGA3/ B3 would not only impair the phototransduction cascade but could also lead to structural alterations of the cone outer segments [30, 31]. On the other hand, a lack of GNAT2 which is not a structural protein might impair only the phototransduction of the cone cells. In support of our idea, *Gnat* ^{cpfl3} mice, which is an animal model of *GNAT2*-associated ACHM, were reported to have normal cone outer segment structure and a normal number of cone outer segments, even with reduced levels of cone alpha-transducin, and extinguished photopic ERGs [32].

There are several limitations in our study. We used flood-illuminated AO fundus camera which has a pixel resolution of 1.6 μm but this is not high enough to evaluate

259 the condition of the rod and cone mosaics of the central fovea. Customized high
260 resolution AO SLO or “split detector” AOSLO should be useful for analyzing the exact
261 condition of the foveal area of ACHM patients.
262 In conclusion, we reported the first case of a Japanese subject with ACHM
263 associated with a *GNAT2* variant, and the cone mosaics were nearly contiguous. Our
264 results indicate that an assessment of the cone photoreceptor mosaics can provide
265 important information on the pathological mechanism ACHM.
266

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Figure legends

Fig. 1 Farnsworth dichromatic test (PD-15), fundus photograph, fundus autofluorescent (FAF) images, and spectral domain optical coherence tomographic (SD-OCT) images of a subject with *GNAT2*-associated achromatopsia (ACHM). The results of PD-15 are typical of complete achromatopsia for the right eye (a) and left eye (b). Fundus photograph (c) and fundus autofluorescent (FAF) image (d) show no abnormalities (images of left eye are shown). Spectral domain optical coherence tomographic (SD-OCT) images of the left eye of this subject (c, horizontal section; d, vertical section) showed intact ellipsoid zone (EZ)

Fig. 2 Full-field ERGs recorded under conditions conforming to the guidelines of the International Society for Clinical Electrophysiology of Vision (ISCEV) Standards of a patient with ACHM (left) and normal subject (right). The scotopic ERGs are within the normal limits but the photopic ERGs are absent in this subject

Fig. 3 Adaptive optics (AO) fundus images of an ACHM patient.
(a) AO fundus images of the left eye of the patient are superimposed on the fundus image recorded by near-infrared reflectance. A magnified image of the white square in the AO image (a) is shown in (b). Magnified images of the white square white

391 squares (c, d, and e) in (b) are shown in (c), (d) and (e) respectively. Asterisk
392 indicates the center of the fovea. (f) and (g) are cone mosaics of representative
393 control subjects. The magnified scale of (d), (e), (f), and (g) is same. Scale bar is 50
394 μm. (f) and (d) are images 1000 μm from the fovea on the nasal side. (e) and (g) are
395 images 500 μm from the fovea on the nasal side
396
397 **Fig. 4** Cone cell density at 500 μm and 1000 μm from the fovea on the nasal and
398 temporal sides of the fovea.
399 The cell densities of the both eyes of the patient (○:left and ●: right) and mean ± 2
400 SD of the 10 eyes of the age- and axial length-matched normal controls(◆) are
401 shown. N1000 and N500 indicate 1000 μm and 500 μm retinal eccentricities nasal to
402 the fovea. T500 and N1000 indicate 500 μm and 1000 μm retinal eccentricities
403 temporal to the fovea. The cone densities of the previous histological data of normal
404 subject of Crucio(◇) [24] and AO image data of Lombardi (■) [25] are shown
405 together