Patterns and Intensities of Near-Infrared and Short-Wavelength Fundus Autofluorescence in Choroideremia Probands and Carriers

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PURPOSE. To ascertain cellular constituents within islands of preserved retina in choroideremia (CHM) by multimodal imaging.

METHODS. CHM probands (16) and female carriers (9) of CHM were studied. Near-infrared autofluorescence (NIR-AF; 787-nm excitation; emission, >830 nm), short-wavelength autofluorescence (SW-AF; 488-nm excitation, 500- to 680-nm emission), and spectral-domain optical coherence tomography (SD-OCT) images were acquired with a confocal scanning laser ophthalmoscope. SW-AF intensities were measured by quantitative fundus autofluorescence (qAF), and NIR-AF intensity profiles were analyzed. Retinal thicknesses and visual acuity were measured.

RESULTS. In 19 of 31 eyes of affected males, islands of preserved NIR-AF signal were also visible as fluorescence signal in SW-AF images. Notable in 12 eyes were areas of speckled SW-AF that was hypoafluorescent in the NIR-AF image. Islands of preserved NIR-AF and SW-AF signal were often associated with the presence of visible but thinned outer nuclear layer and discontinuous interdigitation zone, ellipsoid zone, and external limiting membrane. NIR-AF profiles revealed that even in areas of preserved retina, the NIR-AF signal from retinal pigment epithelium (RPE) melanin is greatly reduced. qAF was reduced overall. The fundus of carriers was characterized by a mosaicism in which patches of reduced NIR-AF colocalized with reduced SW-AF.

CONCLUSIONS. In CHM-affected males, the presence of RPE was indicated by an NIR-AF signal and the absence of hypertransmission of OCT signal into the choroid. RPE preservation was associated with better visual acuity. In carriers, patches of reduced SW-AF colocalized with decreased NIR-AF and qAF was severely reduced.

Keywords: X-linked choroideremia, quantitative fundus autofluorescence, near-infrared autofluorescence

Choroideremia (CHM) is an X-linked recessive disorder that affects ~1 in 50,000 individuals1 and is characterized by the progressive degeneration of photoreceptor cells, retinal pigment epithelium (RPE), and the underlying choroid in affected males. CHM typically has onset in juveniles and is attributable to pathogenic variants in the CHM gene (OMIM 300390)2,3 that encodes the ubiquitously expressed Rab escort protein 1 (REP-1)4 expressed by rods and RPE.5 REP1 participates in the lipid modification (prenylation) of Rab proteins. The latter are vital regulators of intracellular vesicular transport and organelle movement. The deficiency in REP-1 can be compensated for by REP2 in most cell types but, as evidenced by CHM, adequate compensation does not occur in retina6;7; the result is widespread chorioretinal atrophy.8,9 One of the Rabs affected in CHM is Rab27a, which is required for melanosome movement into the apical processes of RPE cells.10 Disease-associated variants in CHM consist predominantly of null or loss-of-function alleles;2 however, phenotypes can vary across individuals. Disease onset in affected males is generally marked by slowed dark adaptation, followed by progressive constriction of the visual field and degeneration of RPE, choroid, and photoreceptor cells. Spectral-domain optical coherence tomography (SD-OCT) imaging of affected males has revealed zones of macular sparing bordered by the loss of interdigitation zone (IZ) and ellipsoid zone (EZ), with outer nuclear layer (ONL) thinning, and increased signal transmission posterior to RPE/Bruch’s membrane.11-13 Foveal structure and function can be retained for several decades.12-15
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Heterozygous female carriers of CHM mutations are typically asymptomatic. However, some carriers report nyctopia in middle and late life, and full-field electroretinography (ERG) recordings can be abnormal. In short-wavelength autofluorescence (SW-AF) images, patches of hyperautofluorescence alternate with hypoautofluorescence, a mosaicism that is considered to reflect random X-inactivation. By multifocal ERG, abnormal responses corresponding to the mosaic pattern can be recorded. In SD-OCT scans, female carriers occasionally present with hyperreflective foci that interrupt photoreceptor-attributable bands and correspond to hyperautofluorescent foci in the macula.

Despite histopathologic studies, in vivo imaging, and the availability of mouse models, it remains uncertain as to whether degeneration is initiated in photoreceptor cells or in RPE cells, with SW-AF emission originating from retinal bisretinoid lipofuscin and NIR-AF from RPE melanin. We have also implemented quantitative approaches to analyze SW-AF by quantitative fundus autofluorescence (qAF) and to measure NIR-AF intensities.

METHODS

Patients, Clinical Evaluation, and Genetic Testing

A retrospective observational study of images acquired from patients presenting to the Department of Ophthalmology Columbia University was performed. Institutional review board/ethics committee approval was obtained under protocol 5002553135 (ClinicalTrials.gov numbers, NCT0192553135, NCT02407678, and NCT02341807), and recent results from a phase 1/2 clinical trial indicate that persistent, clinically significant visual acuity gains can be achieved in eyes in which rapid visual acuity loss would otherwise be expected. The aim of this study was to correlate SW-AF and near-infrared fundus autofluorescence (NIR-AF) signal with SD-OCT images in affected males and female carriers of CHM to better identify the cellular constituents in preserved central islands of the retina. In the healthy eye, SW-AF and NIR-AF are primarily generated in RPE cells, with SW-AF emission originating from retinal bisretinoid lipofuscin and NIR-AF from RPE melanin.

RESULTS

Fundus Imaging in Affected Patients

The study cohort consisted of 16 affected patients (31 eyes); one eye of one patient was excluded because the advanced stage of CHM precluded the acquisition of NIR-AF and qAF images. All affected patients were male with a mean age of 44.9 years (range, 10.2–77.2) at the time of examination. BCVA ranged from 20/20 to 20/150 (Table). Demographic, clinical, and genetic data along with familial relationships are summarized in the Table. All images were evaluated independently by two investigators (MP, JRS). Disease-causing mutations in the CHM gene were detected in all affected patients and carriers who underwent genetic screening. P9 and C4 were not screened but were clinically diagnosed and reported a positive family history of CHM, respectively. In total, 19 unique variants
were detected in the cohort (Supplementary Table S1), the majority of which were frameshifts (26.3%), large deletions (21.1%), and non-coding or intronic (26.3%). All intronic mutations occurred within canonical splice sites (±1 and ±2) and are strongly predicted to result in skipping of the subsequent or adjacent exon. All missense variants are predicted to be pathogenic or previously associated with CHM (single nucleotide polymorphism).

Patients exhibited varying degrees of chorioretinal atrophy. In color fundus photographs, regions of advanced degeneration and widespread depigmentation were pale due to reflection of light from bare sclera (Fig. 1A: proband/patient 7 [P7], white asterisk). In areas of exposed choroid, pigment and choroidal vessels were visible (Fig. 1 A: P7, P5). The choroidal vessel patterns visible in the color fundus photographs were often associated with the presence of pigmented bands (Fig. 1D: P7, white asterisk and bracket). Areas of RPE depigmentation were typically associated with thinning of outer retina.

In 8 patients (13 eyes), distinct islands of preserved RPE in SW-AF images presented as NIR-AF signals were often associated with the presence of a visible but thinned ONL, nondetectable IZ and EZ, and external limiting membrane (ELM) that were discernible to the central island of residual AF included finger-like extensions below, was typically associated with thinning of outer retina.
variable extents or absent (Fig. 1D: P7, P5, P15, green bracket).
In the SD-OCT scan presented for P7, outer retinal tubulations
(ORTs) were visible in an island that exhibited NIR-AF signal
but was devoid of SW-AF (Fig. 1D: P7, red asterisk). Here,
hyperreflectivity associated with the dense pigment reduced
transmission of the OCT signal into the choroid (Fig. 1D: P7,
red asterisk). Foci of the SW-AF signal in the absence of NIR-AF
appeared to be associated with thinned ONL and discontinu-
onous or loss of IZ, EZ, and ELM bands along, with hyper-
transmission into the choroid (Fig. 1C: P5, AF blue arrowhead;
D: P5, blue asterisk).

Preservation of the fovea and parafovea as exhibited by P15
was identified by the increased central NIR-AF signal
and decreased foveal SW-AF, both of which are characteristic of the
macula in healthy eyes (Fig. 1B, 1C: P15). In addition, the SD-
OCT image revealed a relatively intact outer retina (Fig. 1D:
P15, green bracket). Of additional interest is the speckled
autofluorescence that characterized the extrafoveal region in
the SW-AF image. This brightly speckled macular area in the
SW-AF image was hypoautofluorescent in the NIR-AF image
(Figs. 1B, 1C: P15).

**Fundus Imaging in CHM Carriers**

Nine heterozygous carriers were also included in the study (18
eyes). The mean age in the carrier group was 54 (29.3–75.5)
and visual acuity ranged from 20/20 to 20/200 (Table).
In SW-AF and NIR-AF images, 9 carriers (18 eyes) exhibited
AF mottling with mosaic-like patterns of alternating hypo- and
iso-AF extending throughout the posterior pole (18/18 eyes)
(Figs. 2B, 2C). The areas of reduced NIR-AF in the mosaicism
colocalized with foci of reduced SW-AF, although the contrast
between hypo- and hyper-AF signal in the NIR-AF images was
more pronounced than the contrast in corresponding areas in
the SW-AF images (Figs. 2B1, 2C1).

Carrier 3 presented with more advanced peripapillary and
macular atrophy in the color fundus image (Fig. 2D, carrier 3),
with nummular areas of reduced NIR-AF and SW-AF signal (Fig.
2B, carrier 3). In SD-OCT scans acquired from carrier 3,
thinning of the outer retina was readily observable as was an
ORT and hypertransmission into the choroid (Fig. 2A, carrier
3). In all 9 carriers, hyperreflective disturbances of the EZ and
IZ were observed in the SD-OCT scans, and hypertransmission
into the choroid could be observed in association with these
irregularities (Fig. 2A, carrier 1). These aberrations were also
visible as bright hyperautofluorescent flecks in both SW-AF and
NIR-AF images (Fig. 2B, carriers 1 and 2).

**qAF in CHM-Affected Proband**

To assess SW-AF intensities, qAF levels were measured in those
patients for whom qAF imaging was available (6 affected
patients, 7 eyes; P1, P5, P7, P9, P10, and P15; age, 31–67 years)
and who exhibited foveal sparing. To better visualize the
distribution of SW-AF intensities in relation to the central
retinal islands, we constructed qAF maps (scaled from 0–1200
qAF units) and compared CHM patients to age-matched healthy
eyes. The distribution of qAF signal in the CHM-affected
patients (Fig. 3A: P15, P6, P10) was nonuniform and distinctly
different than in the healthy age-similar eyes (Fig. 3C).
Specifically, throughout the macula, qAF was profoundly reduced in the probands not just in the areas of choreoretinal atrophy but also in the central areas of spared retina. Measurements were acquired within a foveal segment (1°). In both fundus zones, the qAF values of the probands were either within or below the range of the lower 95% CI in healthy eyes (Fig. 3D).

qAF in CHM Carriers

Short wavelength fundus autofluorescence was measured by qAF in 6 carriers (carriers 1, 4, 5, 7, 8, and 9; 12 eyes; age, 29–65 years) for whom qAF images were available. Color-coded qAF images revealed both an overall decrease in qAF and local increases and decreases associated with the mosaicism of the fundus (Figs. 4A, 4B: carriers 4, 1, and 5). SW-AF levels within the macula (qAFs) in the eyes of heterozygous carriers fell below or within the range of the lower 95% CI in healthy eyes (Fig. 3D).

Quantitation of NIR-AF in Patients and Carriers

A semiquantitative analysis of NIR-AF intensities was performed using images acquired from 9 probands (P6, P7, P9, P10, P11, P12, P14, P15, and P16; 18 eyes; age, 10.2–77.2 years) and 5 carriers (C7, C8, and C9; 6 eyes; age range, 29.3–64.7 years).

Intensities extracted from horizontal NIR-AF profiles through the fovea (Figs. 5A, 5B), revealed that for CHM probands, the mean NIR-AF intensity was below the 95% CI for healthy eyes (Fig. 5C, green trace versus red and yellow). Each NIR-AF profile spanned atrophic regions and islands of preserved tissue, thereby representing the signal from both exposed choroid and relatively preserved RPE. Notably, even in the central portions of the profiles corresponding to residual retina, the NIR-AF signal was below the normal range (Fig. 5C).

Profiles of probands with central islands not visibly detectable in NIR-AF images (Figs. 5B, yellow trace; 5C, NIR-AF/C0) fell below the 95% CI of probands with a preserved island emitting detectable NIR-AF signal (Figs. 5A, red profile; 5C, NIR-AF+).

Profiles of the carriers (Fig. 5C: blue trace) showed variability, with the central foveal values being below the healthy 95% CI (Fig. 5C, below, green trace).

Retinal Thickness and Visual Acuity in Relation to NIR-AF Signal

FT and SFT were measured in all 16 probands (31 eyes). In the group with detectable NIR-AF signal emitted from the retinal island, the average (±SD) FT and SFT were higher (FT, 191.6 μm ± 52.06; SFT, 148.4 ± 81.8) than in those with no NIR-AF signal originating from the central island (FT, 155.08 ± 102.5;
SFT, 124.6 ± 74.3); however, that difference was not statistically significant ($P = 0.21$ and $P = 0.58$, Wilcoxon-Mann-Whitney Test). The Bland-Altman analysis (Supplementary Fig. S1A) of the FT measurements obtained from the two observers revealed the mean of the differences to be 6.23 ($±15.21$, SD of the difference between the observers). The 95% limits of agreement between observers (estimated as $±1.96 \times SD$) were −23.6 to 36.0, indicating that measurements by observer 1 could be 23.6 units below or 36 units above observer 2. In the case of choroidal thickness (Supplementary

**Figure 3.** qAF color-coded images of P15, P6, and P10 (A). Corresponding SW-AF images (B). Healthy age-similar qAF color-coded image (C). qAF values acquired from foveal area (1° eccentricity; circle in C) and plotted as a function of age for healthy subjects (blue circles), CHM probands (P1, P5, P7, P9, P10, and P15; red circles), and CHM carriers (carriers 1, 3, 4, 7, 8, and 9; yellow circles) (D).

**Figure 4.** qAF in CHM carriers. qAF color-coded images of CHM carrier 4, carrier 1, and carrier 5 (A). Corresponding SW-AF images (B). Healthy (age 55 years) qAF color-coded image (C). qAF$_8$ values (yellow circles) acquired from 8 concentric segments (7°–9° eccentricity; outlined in C) and plotted as a function of age for carriers 1, 4, 5, 7, 8, and 9 (D). Mean (solid black line) ± 95% CIs (dashed lines).
FIGURE 5. NIR-AF signal intensity profiles for CHM probands and carriers. NIR-AF images with profile overlay and SW-AF images for P15 (A) and P6 (B). Intensity profiles are presented as mean (solid line) and 95% CIs (dashed line). Horizontal intensity profiles through the fovea are shown for 6 patients exhibiting a central NIR-AF signal (NIR-AF+: red profile) and for 3 patients having reduced or absent central NIR-AF signal (NIR-AF−: yellow profile). Comparison is made to intensity profile constructed from 19 healthy eyes (green profile) (C, above). Horizontal profiles through the fovea are shown for 3 carriers (blue profile) and compared to healthy eyes (green profile) (C, below).
to the group with absent NIR-AF signal (logMAR equivalent AF form in photoreceptor cells before phagocytic transfer to images (Fig. 1D). Even in healthy eyes, the fluorophores of SW-discontinuous IZ, EZ, and ELM reflectivity layers in SD-OCT ating photoreceptor cells that are represented in P6 by the elevated SW-AF in the absence of NIR-AF signal is also observed atrophy. In support of this explanation, we have noted that alternative source of this aberrant SW-AF signal 39 is degener-

hypertransmission into the choroid was observed (Fig. 1D). A zone associated with this area, EZ and ELM were discontinuous, and visible (Fig. 1C). A comparable but smaller area of SW-AF in NIR-AF signal and hypertransmission of OCT signal into the choroid. Taken together, these features indicated that RPE and photoreceptor cells were at least partially intact.

In association with preserved retinal islands at other locations, we observed that SW-AF signal could coexist with absent or appreciably reduced NIR-AF signal. For instance, in P6, the loss of RPE in residual fovea was indicated by the deficiency in NIR-AF signal and hypertransmission of OCT signal into the choroid due to reduced reflectance by RPE melanin (Figs. 1B, 1D). Nonetheless, an area of speckled SW-AF signal was also visible (Fig. 1C). A comparable but smaller area of SW-AF emission was detected in the fundus of P5, even as the NIR-AF signal was diminished (Figs. 1B, 1C). In the SD-OCT scan associated with this area, EZ and ELM were discontinuous, and hypertransmission into the choroid was observed (Fig. 1D).

These signs of RPE atrophy (reduced or absent NIR-AF with hypertransmission into the choroid) concomitant with the presence of a SW-AF signal are inconsistent with the assumption that SW-AF originates only from RPE cells. An alternative source of this aberrant SW-AF signal 32 is degener-

ating photoreceptor cells that are represented in P6 by the discontinuous IZ, EZ, and ELM reflectivity layers in SD-OCT images (Fig. 1D). Even in healthy eyes, the fluorophores of SW-AF form in photoreceptor cells before phagocytic transfer to RPE. 36 Accordingly, in the absence of NIR-AF, the SW-AF emission may originate from the photoreceptor outer and inner segments that are degenerating secondary to RPE atrophy. In support of this explanation, we have noted that elevated SW-AF in the absence of NIR-AF signal is also observed at positions of SD-OCT-detectable photoreceptor cell degeneration in other retinal disorders. 59, 60

In NIR-AF images of CHM-affected patients, we also observed melanin signal in zones where there was definitely no SW-AF signal. Without the availability of an NIR-AF image, a residual island in P7 (Fig. 1B) might have been overlooked. In this example presented for P7 (Figs. 1A–D), hyperpigmentation in color fundus photographs colocalized with a veil of AF in the NIR-AF images, an absence of SW-AF, thinned ONL, a loss of EZ and ELM reflectivity layers, and the presence of an ORT, yet no hypertransmission into the choroid in the SD-OCT scan. A zone having NIR-AF emission in P6 is also devoid of SW-AF (Figs. 1B–D). One might suggest that the AF findings indicated the presence of RPE that had retained melanin (the NIR-AF signal) but were devoid of lipofuscin (absence of SW-AF signal). However, because SW-AF is attenuated by RPE melanin, it is also possible that the reduced SW-AF signal is a product of increased melanin absorbance of the SW-AF exciting light. Interestingly, we found that qAF intensities mapped to the fundus by scaled color-coding were profoundly reduced not only in CHM-affected patients but also in female CHM carriers. As demonstrated in CHM carrier 1 (Fig. 4A), this qAF reduction was not attributable to outer retinal degeneration in SD-OCT scans (Fig. 2A). Moreover, the signal intensity was nonuniform in both NIR-AF and SW-AF images. Specifically, in CHM carriers, patches of reduced AF in SW-AF images colocalized with reduced AF in NIR-AF images. It has been suggested that nonuniform melanin distribution associated with CHM/REP-1 dysfunction represents an X-linked manifestation of altered RPE melanosome movement. 10 However, we have also studied melanin pigment mosaicism in NIR-AF images of X-linked albinism carriers (GPR143/OA1), and unlike in CHM carriers, patches of reduced signal in NIR-AF images of GPR143/OA1 carriers colocalize with increased signal (not reduced signal) in the SW-AF modality. 57 Moreover, because the fundus changes in CHM carriers develop with age 34 and exhibit progression, 41 random X-inactivation of REP-1 leading to melanosome dysfunctioning 10 is not sufficient to explain the mosaicism. This is an interesting issue that we are currently exploring. Whether RPE cells or photoreceptor cells are the first to express the disease has been an issue of uncertainty. We observed that choroidal vasculature was preserved even in areas exhibiting outer retinal atrophy, as indicated by the loss of EZ and ELM and increased OCT signal transmission into the choroid (Fig. 1, P5), which is indicative of RPE loss.

Other observations indicate that a disease process takes place in the RPE layer before the photoreceptors are affected in CHM. For instance we observed ORTs in the outer retina of both CHM-affected patients and carriers. The formation of ORTs is considered to reflect a survival response by photoreceptors, as it is caused by mutations in the gene C1QTNF5, which encodes a protein expressed by RPE. 44 This too suggests that RPE may be involved in the survival response by photoreceptors, is considered to reflect a survival response by photoreceptors, is considered to reflect a survival response by photoreceptors.
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References


11. Huang AS, Kim LA, Fawzi AA. Clinical characteristics of a large


