A Longitudinal Study of Stargardt Disease: Quantitative Assessment of Fundus Autofluorescence, Progression, and Genotype Correlations

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Purpose. We characterized subtypes of fundus autofluorescence (AF) and the progression of retinal atrophy, and correlated these findings with genotype in Stargardt disease.

Methods. Full clinical examination and AF imaging was undertaken in 68 patients with Stargardt disease. The baseline data were compared to those at follow-up. Patients were classified into three AF subtypes: type 1 had a localized low signal at the fovea surrounded by a homogeneous background, type 2 had a localized low signal at the macula surrounded by a heterogeneous background with numerous foci of abnormal signal, and type 3 had multiple low signal areas at the posterior pole with a heterogeneous background. At baseline, there were 19 patients with type 1, 41 with type 2, and 8 with type 3 disease. The areas of reduced AF signal were measured and rate of atrophy enlargement (RAE) was calculated as the difference of the atrophy size over time (mm^2) divided by the follow-up interval (years). Molecular screening of *ABCA4* was undertaken.

Results. The mean follow-up interval was 9.1 years. A total of 42% cases with type 1 disease progressed to type 2, and 12% with type 2 progressed to type 3. The RAE (mm^2/y) based upon baseline AF subtypes was significantly different; 0.06 in type 1, 0.67 in type 2, and 4.37 in type 3. *ABCA4* variants were identified in 57 patients. There was a significant association between AF subtype and genotype.

Conclusions. The AF pattern at baseline influences the enlargement of atrophy over time and has genetic correlates. These data are likely to assist in the provision of counseling on prognosis in Stargardt disease and be valuable for future clinical trials.

Keywords: Stargardt, ABCA4, autofluorescence

Stargardt disease is the most common inherited macular dystrophy, and is associated with a variable phenotype and disease severity.[1–12] Stargardt disease typically presents with central macular atrophy and yellow-white flecks at the posterior pole, primarily at the level of the RPE. Progressive retinal degeneration over time, including development/resorption of flecks, atrophy enlargement, and deterioration of retinal function, has been reported in Stargardt disease.[7–9] Mutations in the gene *ABCA4* underlie Stargardt disease, and also have been implicated in cone dystrophy, cone-rod dystrophy, and "retinitis pigmentosa."[2,9,10,13–17] The *ABCA4* gene encodes a transmembrane rim protein in the outer segment discs of photoreceptors that is involved in active transport of retinoids from photoreceptor to RPE.[18–24] Failure of this transport results in accelerated deposition of a major lipofuscin fluorophore, N-retinylidene-N-retinylethanolamine (A2E), in the RPE.[20–22] A2E and other lipofuscin fluorophores are elevated dramatically in the RPE of postmortem samples from patients with Stargardt disease and in *ABCA4* knockout mice (*abca4*$^{-/-}$).[19,25] Over time, A2E-associated cytotoxicity is believed to cause RPE dysfunction and cell death, with subsequent photoreceptor cell loss.[26,27]

Autofluorescence (AF) imaging can provide useful information about the distribution of lipofuscin in the RPE, and give indirect information on the level of metabolic activity of the RPE; lipofuscin levels are determined largely by the rate of turnover of photoreceptor outer segments.[28,29] The abnormal accumulation of lipofuscin, the presence of active and resorbed flecks, and RPE atrophy leads to a characteristic appearance on AF imaging in Stargardt disease; very low AF signals in photoreceptor and RPE atrophy, and foci with low or high AF signals due to flecks.[5,6,10,30]

Table 1. Definition of Fundus AF Subtypes in Stargardt Disease

Type 1	Localized low AF signal at the fovea surrounded by a homogeneous background with/without perifoveal foci of high or low signal
Type 2	Localized low AF signal at the macula surrounded by a heterogeneous background and widespread foci of high or low AF signal extending anterior to the vascular arcades
Type 3	Multiple areas of low AF signal at posterior pole with a heterogeneous background with/without foci of high or low signal

It has been challenging in Stargardt disease to establish comprehensive genotype–phenotype correlations due to the variable phenotype and the heterogeneity of *ABCA4*; more than 700 sequence variants have been reported.[1,2,9–15,31–44] A previous cross-sectional study of 43 patients with Stargardt disease demonstrated that AF patterns appeared to relate to functional abnormalities.[5] A recent small AF study ($n = 12$) demonstrated variable rates of enlargement of RPE atrophy in Stargardt disease, with a strong association between atrophy enlargement and electrophysiological grouping.[6] Chen et al.[30] also have reported the progressive change in the area of atrophy in 52 patients with Stargardt disease over a mean follow-up of 2.92 years; with variable atrophy progression demonstrated, and an association with electrophysiological findings. However, comprehensive investigations over a long-term follow-up of a large cohort of patients with Stargardt disease, including AF imaging, clinical assessment, and molecular analysis still are lacking.

The purpose of this study was to characterize the subtypes of AF and investigate the enlargement of RPE atrophy in patients with Stargardt disease in a longitudinal survey with a mean follow-up of 9 years. This study also provided an opportunity to investigate the association of these AF subtypes and atrophy progression with the detailed clinical and molecular genetic findings.

Methods

Patients

A cohort of 68 patients with a clinical diagnosis of Stargardt disease and a minimum of 6 years of follow-up were ascertained at Moorfields Eye Hospital.

For the purpose of this study, patients with a clinical history compatible with Stargardt disease and clinical signs of bilateral macular atrophy, with or without surrounding flecks, were included. The clinical features of 42 patients in this cohort have been described partially in an earlier report, which did not include AF findings.[9] The panel included five sibling pairs. After informed consent was obtained, blood samples were taken for DNA extraction and mutation screening of *ABCA4*. The protocol of the study adhered to the provisions of the Declaration of Helsinki and was approved by the local Ethics Committee of Moorfields Eye Hospital.

Clinical Assessment

We assessed 68 patients on at least two occasions, with the first and most recent visits taken as the baseline and "follow-up" examinations, respectively, for the purposes of data analysis. A full medical history was obtained and a comprehensive ophthalmologic examination performed. The age of onset was defined as the age at which visual loss was first noted by the patient. The duration of the disease was calculated as the difference between age at onset and age at the baseline examination when AF imaging was obtained. The interval of observation was determined by the difference between the age at baseline and the age at the most recent "follow-up" examination when AF imaging was done.

Best-corrected Snellen visual acuity was converted to equivalent logMAR visual acuity,[45] and visual acuity reduction was calculated as the difference between logMAR visual acuity at baseline and follow-up.

Fundus AF Imaging

The AF imaging was performed using a confocal scanning laser ophthalmoscope (cSLO). Baseline images were obtained before 2003 using a Zeiss prototype cSLO (SM 30-4024, excitation light 488 nm, barrier filter 521 nm, field of view 30° × 30°; Carl Zeiss Meditec, Oberkochen, Germany).[5,29,46–48] From 2003 to 2009, images were obtained using an HRA2 (excitation light 488 nm, barrier filter 500 nm, field of view 30° × 30°; Heidelberg Engineering GmbH, Heidelberg, Germany).[47] After 2009, images were obtained using the Spectralis with viewing module version 5.1.2.0 (excitation light 488 nm, barrier filter 500 nm, fields of view 30° × 30° and 55° × 55°; Heidelberg Engineering GmbH).[49]

Patients were classified into 3 AF subtypes based on a recent report of AF findings in Stargardt disease:[10] type 1 – localized low AF signal at the fovea surrounded by a homogeneous background, with/without perifoveal foci of high or low AF signal; type 2 – localized low AF signal at the macula surrounded by a heterogeneous background, and widespread foci of high or low AF signal extending anterior to the vascular arcades; and type 3 – multiple areas of low AF signal at the posterior pole with a heterogeneous background, with/without foci of high or low AF signal (Table 1, Fig. 1). In previously published reports, the progression of atrophy has been influenced by two patterns of background AF ("homogeneous" and "heterogeneous"),[6] and multiple atrophic lesions at the posterior pole have been associated with a more rapid functional deterioration.[9] The data of AF subtypes obtained at follow-up were compared to those at baseline. A patient (patient 61) who had an asymmetric AF subtype was excluded from the AF subtype analysis.

Areas of low AF signal were measured using custom software (Retinal analysis tool; Halfyard AS, Fitzke FW, University College London [UCL] Institute of Ophthalmology, London, UK). With reference to a given distance between the center of the optic nerve head and the foveola, which is defined as 15°, this software enables measurement of the dimensions of the area tracked manually and computation of the size expressed in square degrees automatically (Fig. 2). The significant low gray scale point on the images was decided upon by agreement between the two investigators (KF, RM) and the dimension of the area within the tracked line of low gray scale was calculated.

All the values in square degrees were converted to square millimeters using the previously reported conversion factor (1° = 0.3 mm; Fitzke FW. *IOVS* 1981;20(suppl):ARVO Abstract 144). Only low AF signal lesions of >0.18 mm^2 in size were considered. The total area of atrophy was calculated by summation of all the measured low signal lesions. All of these measurements were conducted by two investigators (KF, RM), and the averaged values were used for final analyses. The rate of atrophy enlargement (RAE, mm^2/y) was calculated as follows according to previous reports[6,30]: size of the area of atrophy at last follow-up minus size of the area of atrophy at baseline (mm^2) divided by the follow-up time (years) (Fig. 2).

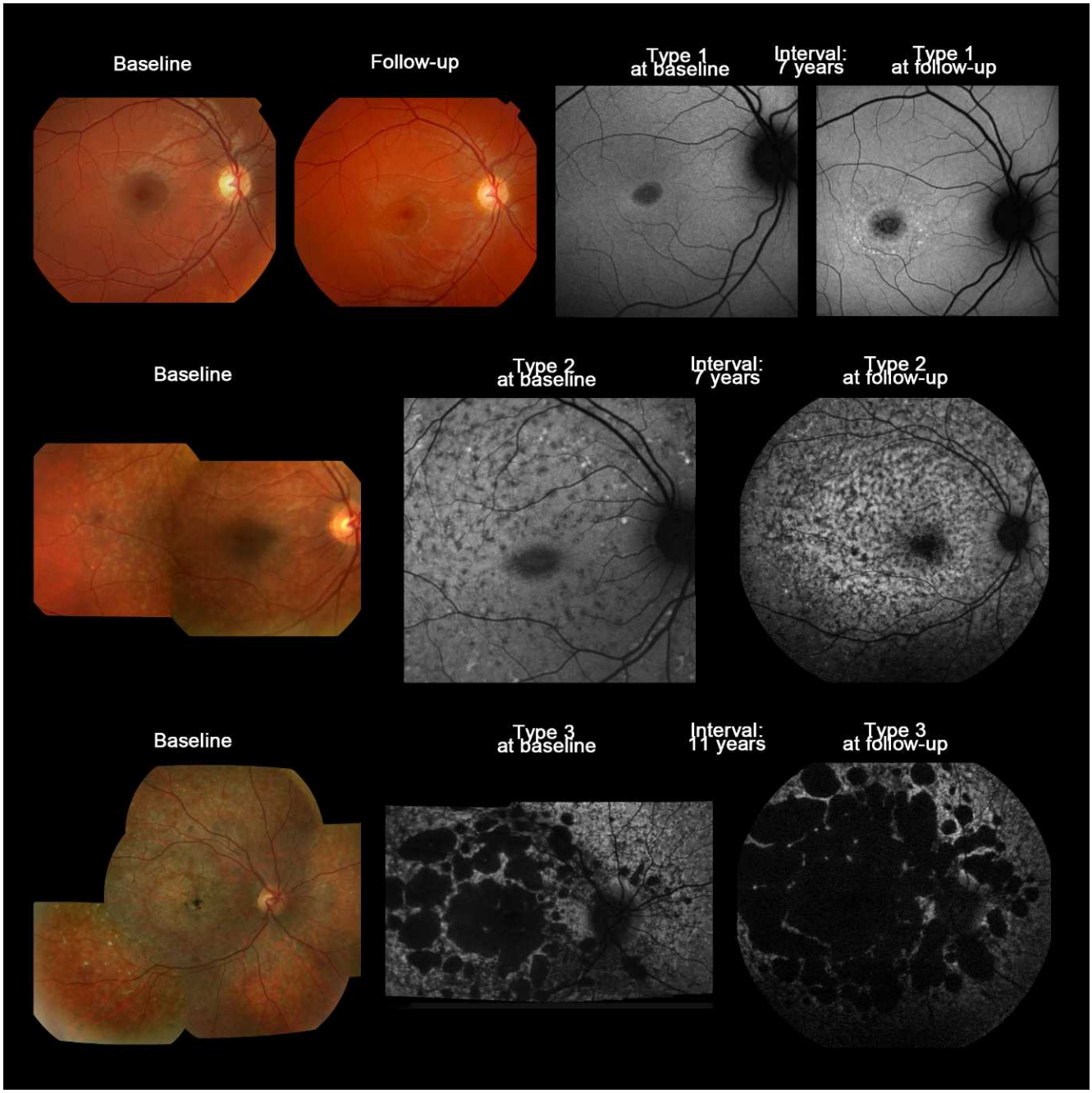

Figure 1. Color fundus photographs and AF images of three representative cases with Stargardt disease, illustrating the three AF subtypes in subjects where there was no subtype transition over time (patients 7, 50, and 54). *Top row*: Color fundus photographs of patient 7 showing subtle RPE changes at the fovea at baseline with a mild increase in the degree of atrophy at follow-up. AF imaging demonstrates a localized low signal lesion with a relatively high signal edge and a homogeneous background at baseline, and a low signal foveal lesion surrounded by patchy small foci with high signal and a homogeneous background at follow-up; consistent with AF type 1 at baseline and follow-up. *Middle row*: Patient 50 had macular atrophy surrounded by yellowish-white flecks extending anterior to the vascular arcades at baseline, and a low signal area at the macula surrounded by high and low foci throughout the posterior pole (AF type 2) at baseline and follow-up, with a heterogeneous background at baseline and follow-up. *Bottom row*: Patient 65 had extensive areas of atrophy throughout the posterior pole, extending beyond the vascular arcades, with yellowish-white and atrophic flecks at baseline, and multiple areas of low signal with heterogeneous background (AF type 3) at baseline and follow-up.

Where possible, AF images with a 30° × 30° field were used for RAE analysis ($n = 68$ at baseline and $n = 67$ at follow-up). For patients with AF images available in both eyes at baseline and follow-up ($n = 61$), the eye used for analysis was selected according to the Random Integer Generator (available in the public domain at http://www.random.org/), and for individuals with AF imaging available in only one eye ($n = 7$), that eye was selected for analysis. Patients who had central atrophy that extended beyond the limits of the AF images obtained with field of view 30° × 30° were excluded from the size of atrophy and RAE analyses ($n = 6$).

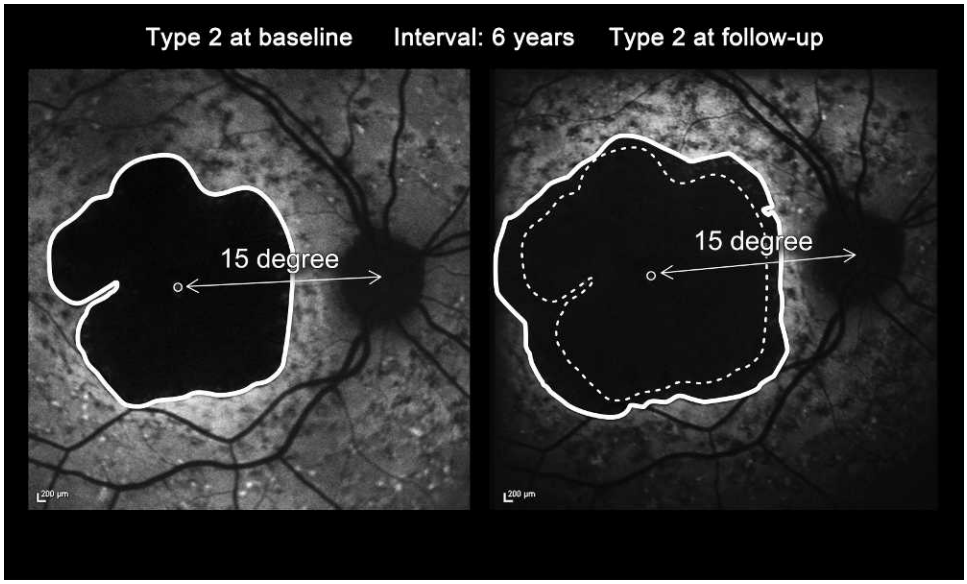

FIGURE 2. Measurement of the low signal area of AF and calculation of the rate of atrophy enlargement in a representative case with Stargardt disease. The area of low AF signal (patient 51) was measured using custom software, which enables measurement of the dimensions of the area outlined manually (*white line* in the *left* and *right* images) and the automatic computation of the area expressed in square degrees to facilitate the appreciation of the relation to the patient's visual function; with a given distance between the center of the optic nerve head and the foveola defined as 15° (marked in the images). Atrophy enlargement over time was calculated as the difference between the size of atrophy at baseline and follow-up (*broken white line* and *continuous white line* of the *right* image). The rate of atrophy enlargement (mm^2/y) was obtained as the atrophy enlargement (mm^2) divided by the follow-up time (years).

Mutation Screening

Mutation screening was performed using the single-stranded conformation polymorphism (SSCP) strategy in 35 subjects,[50] and the arrayed primer extension (APEX) microarray (ABCR400 chip; Asper Ophthalmics, Tartu, Estonia) in 33 patients.[51] All the variants detected were confirmed with direct Sanger sequencing. Direct Sanger sequencing also was performed in siblings of probands and parents when available to confirm segregation of alleles.

Nonnull variants were analyzed using two software prediction programs: Sorting Intolerant from Tolerance (SIFT, available in the public domain at http://sift.jcvi.org/),[52] and PolyPhen2 (available in the public domain at http://genetics.bwh.harvard.edu/pph/index.html).[53] All variants were compared to variants in the Exome Variant Server; National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project, Seattle, Washington (available in the public domain at http://snp.gs.washington.edu/EVS/).

Patients harboring two or more mutations were classified into two mutually exclusive genotype groups on the basis of the molecular analysis: patients with at least one null variant, (group A) and subjects with two or more missense variants (group B). Only patients harboring two or more likely disease-causing variants were included to investigate genotype–phenotype correlations. Null variants were those that would be expected to affect splicing, or to introduce a premature truncating codon in the protein if translated. One disease-associated intronic change with uncertain effect was treated as a null allele due to the associated severe clinical phenotype previously reported.[9,33]

Statistical Analysis

Statistical methods are provided in Supplementary Material S1.

RESULTS

Clinical Findings

We included in the study 68 patients with a clinical diagnosis of Stargardt disease. The clinical findings are summarized in Supplementary Table S1. There were 36 female (53%) and 32 male patients (47%). All complained of central visual loss, with a median age of onset of 19.0 years (range, 5–48 years) and a median duration of disease of 9.0 years (range, 0–47 years). One patient had relative foveal sparing in the left eye on AF imaging at presentation (patient 24; age of onset, 48 years). The median ages at baseline and at follow-up were 30.5 and 39.0 years (range, 8–58 and 18–67), respectively. The mean follow-up interval was 9.1 years (range, 6–13). Seven patients (10%) presented before 16 years of age and 61 patients (90%) presented after 16 years. The median logMAR visual acuities at baseline and at follow-up were 1.00 (range, 0.0–1.98) and 1.00 (0.0–2.28), respectively, with a median logMAR visual acuity reduction during the follow-up interval of 0.15 (range, −0.78–1.28).

Color fundus photographs and AF images of representative cases are shown in Figures 1 and 3; with three representative cases without AF type transition during follow-up in Figure 1, and two cases with AF type transition in Figure 3.

Fundus AF Findings

The AF imaging at baseline was obtained with the Zeiss system in 52 patients and with the HRA2 in 16 subjects. The AF imaging at follow-up was obtained with the HRA2 or Spectralis (field of view 30° × 30°) in all 68 individuals, with additional wide-field images (55° × 55°) undertaken in 19 patients. Complete AF data sets were available at baseline and follow-up with few exceptions; in patient 28, AF images were unavailable of the right eye at baseline and follow-up, and six patients

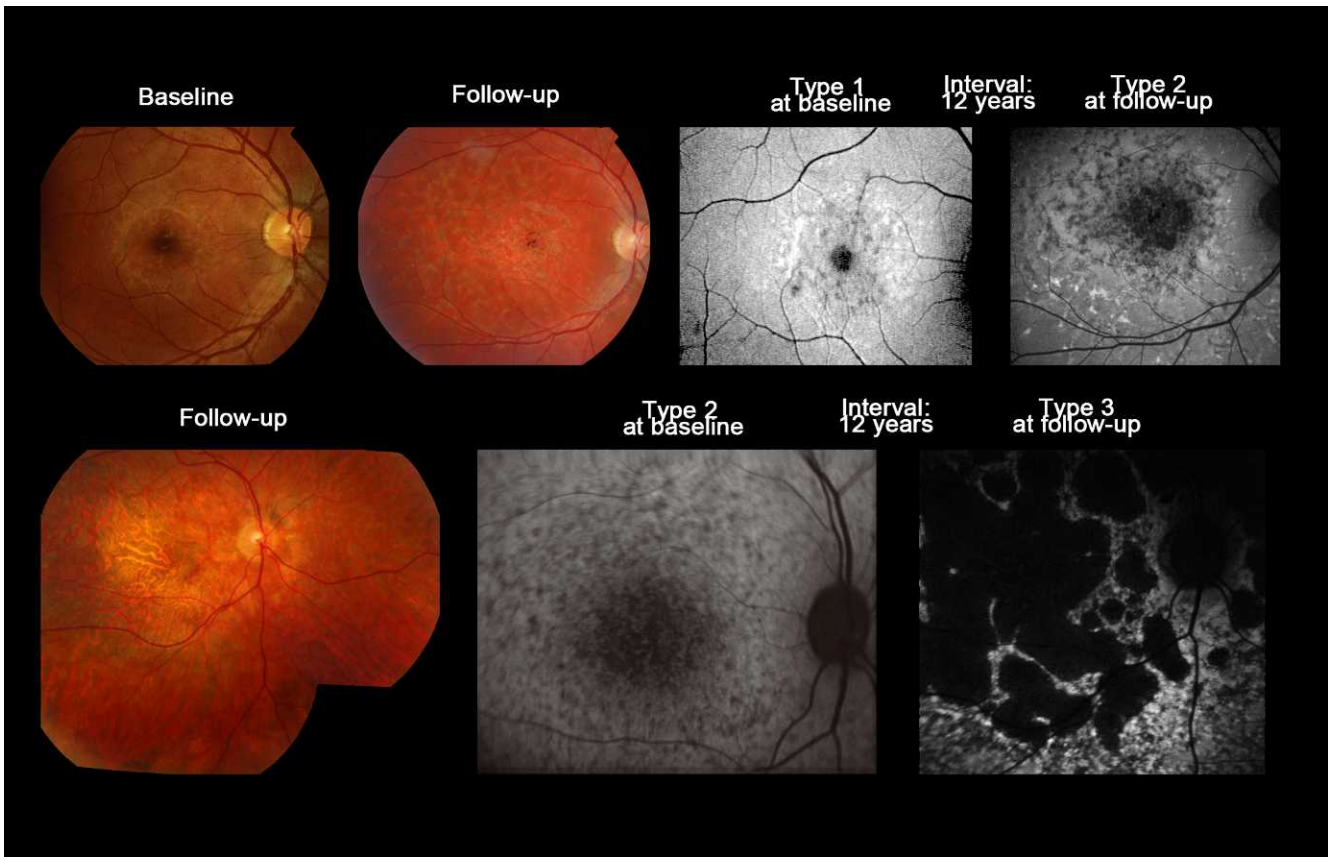

Figure 3. Color fundus photographs and AF images of two representative cases with Stargardt disease, showing AF subtype transition over time (patients 12 and 59). *Top row*: Color fundus photographs of patient 12 showing mild foveal atrophy surrounded by parafoveal yellowish-white flecks at baseline and more marked macular atrophy surrounded by numerous atrophic flecks extending anterior to the vascular arcades at follow-up. AF imaging demonstrates localized low signal at the fovea surrounded by a homogeneous background with high and low signal parafoveal foci at baseline, and a macular low signal lesion surrounded by numerous high and low signal foci throughout the posterior pole with a heterogeneous background at follow-up, consistent with transition from AF type 1 to type 2. *Bottom row*: Patient 59 had extensive areas of atrophy extending beyond the vascular arcades with atrophic flecks at follow-up. AF imaging showed subtype transition; a mottled macular low signal lesion surrounded by numerous low signal foci with a heterogeneous background (type 2) at baseline, and multiple areas of low signal throughout the posterior pole with a heterogeneous background (type 3) at follow-up. The central atrophy at follow-up extended beyond the limits of the AF image ("beyond the scope").

(patients 11, 31, 36, 39, 44, 56) had unavailable AF images at baseline of the left eye.

At baseline, 67 patients were classified based on the AF findings into three subtypes: 19 patients (28%) in type 1, 41 (61%) in type 2, and 7 (10%) in type 3. At follow-up there were 11 (16%) in type 1, 44 (66%) in type 2, and 12 (18%) in type 3 (Table 2). All patients had a symmetrical AF subtype between eyes, except for patient 61 with type 3 in the right and type 2 in the left at baseline, with type 3 chosen as the overall classification for this patient.

A total of 13 patients (28%) showed AF subtype transition during follow-up; 8/19 (42%) subjects from AF type 1 to AF type 2, and 5/41 (12%) individuals from AF type 2 to AF type 3 (Table 2). Three of five sibships were concordant for AF subtype at baseline (patients 31 and 37; 35 and 36; and 63 and 64) and two of five were discordant (patients 13 and 23; and 18 and 23); all the sibships were concordant at follow-up (Supplementary Table S1).

A total of 13 patients (19%) at baseline and 2 (2%) at follow-up had a single small area of low signal (<0.18 mm^2) in the selected eye and were recorded as having zero mm^2 of atrophy (Supplementary Table S1). There were six patients who had atrophy in the selected eye extending beyond the limits of the AF image at baseline and, thereby, they were excluded from the quantitative analyses (Supplementary Table S1). The median total size of atrophy at baseline and follow-up was 1.12 mm^2 (range, 0.00–27.23) and 5.32 mm^2 (range, 0.00–62.58), respectively. The median RAE over time was 0.45 mm^2/y (range, 0.00–5.89). The concordance correlation coefficient revealed significant agreement between the two observers' measurements (concordance correlation coefficient was 0.99). The clinical features of each baseline AF subtype are summarized in Table 3 and Figure 4. The median size of

Table 2. Distribution and Transition of AF Subtypes of 67 Patients With Stargardt Disease

	AF Type at Follow-up		
	Type 1	**Type 2**	**Type 3**
AF type at baseline			
Type 1, $n = 19$	11	8	0
Type 2, $n = 41$		36	5
Type 3, $n = 7$			7
Total, $n = 67$	11	44	12

One patient was excluded for this analysis due to an asymmetric AF subtype at baseline (patient 61).

Table 3. Clinical Features and Quantitative AF Data Associated With AF Subtype at Baseline in 67 Patients With Stargardt Disease

	Median Age of Onset, y	Median Duration, y	Median Age, y		Mean Interval, y	LogMAR Visual Acuity			Median Size of Atrophy, mm²		Rate of Atrophy Enlargement, mm²/y
			BL	FU		BL	FU	Reduction	BL	FU	
Type 1, $n = 19$	24.0	3.0	29.0	36.0	9.2	0.78	1.00	0.22	0.00	1.00	0.06
Type 2, $n = 41$	18.0	9.0	31.0	38.0	9.0	1.00	1.00	0.22	1.91	8.52	0.67
Type 3, $n = 7$	8.0	27.0	37.0	43.0	9.1	1.98	1.78	0.00	20.54	57.17	4.37
Total, $n = 67$	19.0	9.0	31.0	39.0	9.1	1.00	1.00	0.12	1.12	5.32	0.45

One patient was excluded from baseline AF subtype analysis due to an asymmetric AF subtype at baseline (patient 61). The age of onset was defined as the age at which visual loss was first noted by the patient. The duration of disease was calculated as the difference between age at onset and age at the baseline examination when AF imaging was obtained. The interval of observation was determined by the difference between the age at baseline and the age at the most recent "follow-up" examination at which AF imaging was obtained. The rate of atrophy enlargement (mm²/y) was calculated as follows: size of the area of atrophy at last follow-up minus size of the area of atrophy at baseline (mm²) divided by the follow-up time (years). BL, baseline; FU, follow-up.

atrophy and median RAE associated with each baseline AF subtype are shown in Table 3 and Figure 4. There was a statistically significant difference between AF type 1 and 3, and type 2 and 3 in terms of age of onset ($P = 0.003$ and 0.016, respectively). In respect to duration of disease, there were statistically significant differences between AF types 1 and 2, types 1 and 3, and types 2 and 3 ($P = 0.018$, 0.002, and 0.001, respectively). There also was a statistically significant difference in logMAR visual acuity at baseline between AF types 1 and 3, and types 2 and 3 ($P = 0.003$ and 0.003, respectively), and in logMAR visual acuity reduction between types 1 and 3, and types 2 and 3 ($P = 0.042$ and 0.008, respectively). With respect to size of atrophy at baseline and RAE, statistically significant differences were seen between types 1 and 2, types 1 and 3, and types 2 and 3 ($P = 0.000$, $P = 0.049$, $P = 0.000$ for size of atrophy at baseline, and $P = 0.000$, $P = 0.019$, 0.014 for RAE, respectively). However, there were no statistically significant differences between AF subtypes in terms of other parameters, including age at baseline and follow-up interval.

The Spearman rank correlation test was applied for assessment of the relationships between parameters, including age of onset and size of atrophy at baseline, age at baseline and size of atrophy at baseline, duration of disease and size of atrophy at baseline, age of onset and RAE, age at baseline and RAE, duration of disease and RAE, and size of atrophy at baseline and RAE. There was a statistically significant correlation between age at baseline and size of atrophy at baseline ($\rho = 0.402$, $P < 0.0015$), duration of disease and size of atrophy at baseline ($\rho = 0.626$, $P < 0.0001$), age at baseline and RAE ($\rho = 0.369$, $P < 0.0037$), duration of disease and RAE ($\rho = 0.607$, $P < 0.0001$), and size of atrophy at baseline and RAE ($\rho = 0.767$, $P < 0.0001$). A tendency of negative correlation also was suggested between age of onset and RAE ($\rho = -0.191$, $P = 0.133$).

Molecular Genetics

Likely disease-causing variants in *ABCA4* were detected in 57 of 68 patients; with two or more variants identified in 27 patients and one variant in 30 subjects (Supplementary Table S1). Detailed results including in silico analysis are shown in Supplementary Table S2. A total of 45 variants was found in 57 patients; 13 null mutations, including one disease-associated intronic change and one predicted to affect splicing; and 32 missense variants. A total of 22 patients harbored at least one null variant, with a single subject having two null mutations. Of these 45 variants 43 have been reported previously and 2 are putative novel mutations: c.93G>A, p.Tyr31* and c.617_618delCG, p.Ser206Argfs*320 (Supplementary Tables S1, S2). The 26 patients harboring two or more disease-causing variants were classified into two genotype groups (Table 4); there were 14 patients with at least one null variant and 12 with two or more missense variants.

Genotype–AF Phenotype Correlations

The association between AF subtype and genotype group is shown in Table 4 and Figure 5. In 26 patients with two or more likely disease-causing variants, there was a statistically significant association between AF subtype classification and genotype group classification at baseline and follow-up ($\gamma = -0.567$ and $\gamma = -0.646$, respectively). There was a suggestion of a difference between the two genotype groups, in terms of proportion of AF subtypes 1 and 3. The proportion of AF subtype 1 was 21% at baseline and 7% at follow-up for the null variant genotype group, compared to 36% at baseline and 25% at follow-up for the missense variant genotype group. The proportion of AF subtype 3 was 21% and 36% at baseline and follow-up for the null variant genotype group, compared to 0% and 8% at baseline and follow-up, respectively, for the missense variant genotype group.

Four of nine patients from eight families harboring the variant c.5461-10 T>C were classified into type 3 AF at baseline and three (including one sibling pair) of these subjects had atrophy that extended beyond the limits of the AF image obtained (Supplementary Table S1). The median size of atrophy at baseline and follow-up in the remaining six patients was 0.92 and 6.29 mm², respectively. The median RAE of these patients harboring the variant c.5461-10 T>C was 0.45 mm²/y.

Four of eight patients from eight families harboring the missense variant p.Leu2027Phe had small central atrophy (<0.18 mm²) at baseline (Supplementary Table S1). The median size of atrophy at baseline and median RAE of these eight patients was 0.27 mm² and 0.39 mm²/y. respectively, which are less than the median values for the entire cohort of 68 patients (1.12 mm² and 0.45 mm²/y). The median size of atrophy at baseline and the median RAE of the five patients from five families harboring the missense variant p.Gly1961Glu also was relatively small; 0.47 mm² and 0.20 mm²/y respectively (Supplementary Table S1).

Discussion

This study assessed longitudinal changes in RPE atrophy by undertaking AF imaging in a large well-characterized cohort of patients with Stargardt disease; 84% of subjects harbored at least one likely disease-causing *ABCA4* allele. The findings herein assist in providing improved advice on prognosis and

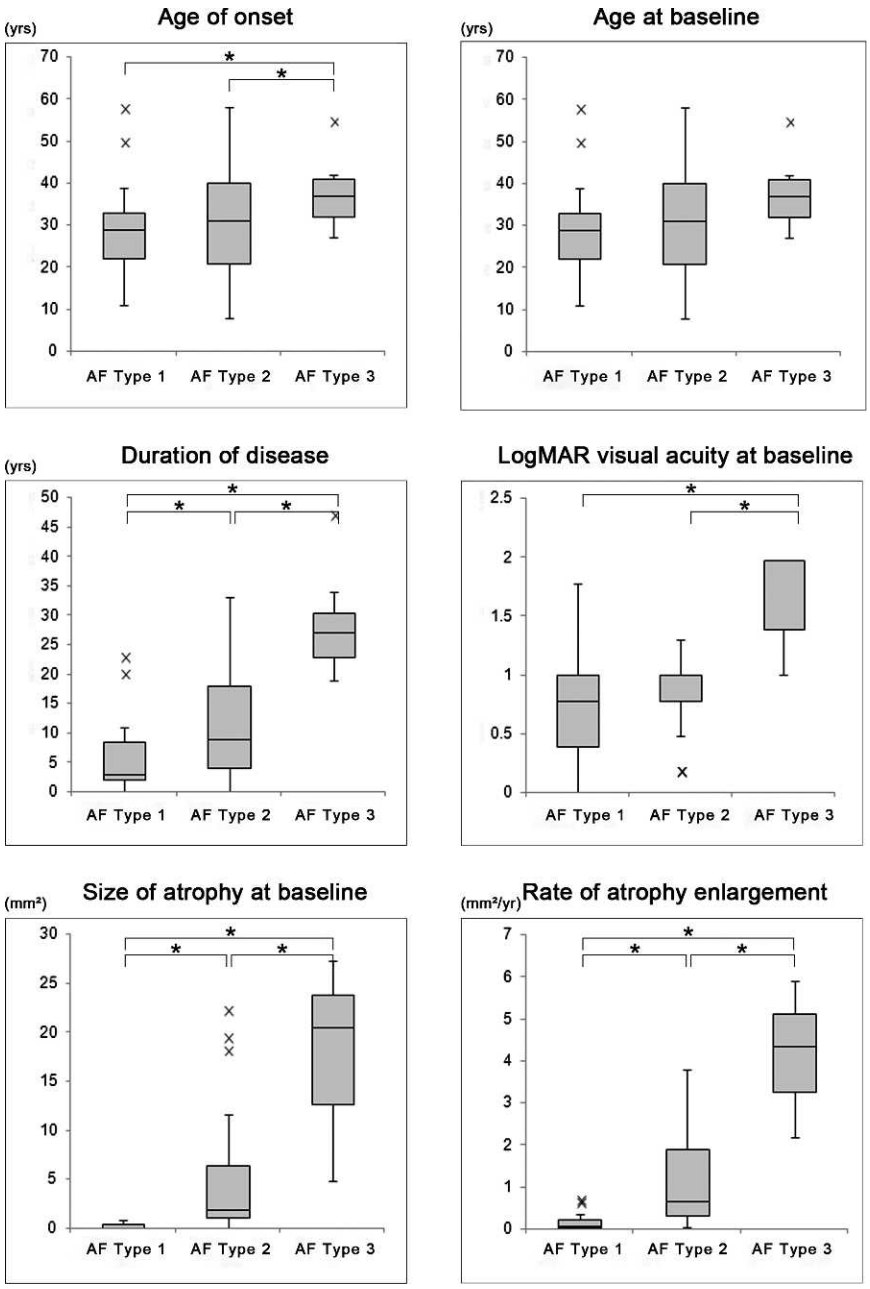

FIGURE 4. A comparison of selected clinical features and quantitative AF imaging data associated with each AF subtype at baseline in 67 patients with Stargardt disease, showing significant differences in age of onset, duration of disease, visual acuity at baseline, size of atrophy at baseline, and rate of atrophy enlargement. The *boxes* show the median, and 25% and 75% confidence interval (lower and upper quartiles). The *whiskers* extend to what could be considered the 95% confidence interval. *Crosses* represent values outside the 95% confidence interval. *Statistically significant differences.

may inform patient selection for future therapeutic interventions for *ABCA4*-related retinopathy.

We classified patients into three AF subtypes at baseline. Of patients with type 1 AF at baseline, 58% remained in type 1 AF at follow-up, whereas 12% of patients with type 2 AF showed transition to type 3 AF. Type 3 appears to be a comparatively distinct phenotype, given the fact that only a relatively small number of subjects had progression from AF type 2 to type 3, and none of the type 1 AF patients showed transition to type 3 AF. Statistically significant differences between baseline AF types in RAE also were demonstrated, suggesting a less severe and more slowly progressive phenotype in type 1 AF, and more severe and more rapid enlargement of atrophy in type 3 AF. The correlation between the total size of atrophy and RAE also supports this proposal.

There was one patient (patient 61) with an asymmetric AF subtype at baseline, type 3 in the right eye and type 2 in the left, with type 3 in both eyes at follow-up. Many possible factors may have a role in interocular asymmetry, including anisometropia, skewed X-inactivation of a modifier (in females), differences in mitochondrial sequences, somatic mutation, epigenetic differences, and "stochastic" factors (e.g., small initial differences in gene expression leading to significant differences later).

The clinical characteristics of each AF subtype showed significant differences in terms of age of onset, duration of

TABLE 4. Association Between AF Subtype and Genotype in 26 Patients With Two or More Disease-Causing *ABCA4* Variants

	Patients Harboring at Least One Null Variant, $n = 14$		Patients Harboring Two or More Missense Variants, $n = 12$	
	BL	FU	BL	FU
AF type 1	3 (2)	1	5 (2)	3
AF type 2	8 (2)	8	7 (1)	8
AF type 3	3	5	0	1
Total, $n = 26$	14	14	12	12

One patient harboring null variants was excluded from this analysis due to an asymmetric AF subtype at baseline (patient 61). The number of patients who showed AF subtype transition is shown in parentheses.

disease, baseline logMAR visual acuity, and logMAR visual acuity reduction. Patients with type 1 AF at baseline had a later onset of central visual loss and better visual acuity, compared to patients with type 3 AF at baseline. Correlation between duration of disease and size of atrophy, and duration of disease and RAE also were established; thereby supporting the recent suggestion that a longer disease duration is associated with more extensive and more rapidly progressive central retinal atrophy.[30]

Overall, the findings of this study suggested that patients with Stargardt disease showing localized foveal atrophy have milder progression of central atrophy compared to subjects with multiple atrophic lesions who have more rapid loss of central retinal structure over time. In contrast, patients in the "intermediate" group, with macular atrophy and a heterogeneous background, have a more variable area of atrophy and atrophy enlargement. An association between the pattern of functional loss detected on electrophysiology and the RAE was suggested in previous reports[6,30]; therefore, electrophysiological assessment may assist in the characterization of patients with an "intermediate" phenotype. Furthermore a comprehensive study of the relationship over a long-term follow-up period between AF and electrophysiology in a larger cohort of patients with Stargardt disease would be valuable.

We identified 45 likely disease-causing *ABCA4* variants, with two putative novel mutations detected. A total of 26 patients harbored two or more likely disease-causing variants; there was a statistically significant association between AF subtype classification and genotype group classification at baseline and follow-up. A difference was suggested between genotype groups in terms of proportion of AF subtypes 1 and 3, in keeping with more deleterious genetic variants being associated with a more severe and progressive AF phenotype. Sodi et al.,[54] in an AF study of 20 patients, also concluded that the presence of two severe mutations was associated with a larger area of macular atrophy.

Consistent with previous reports, four of nine patients with the c.5461-10 T>C variant had a severe phenotype, with multiple large areas of atrophy.[9,33,55,56] In contrast, there were three patients with the c.5461-10 T>C variant with a lower atrophy enlargement, all of whom also harbored missense variants (p.Gly1961Glu in one patient, and p.Leu2027Phe in the remaining two subjects); considered to be associated with a milder phenotype.[2,10,54,56,57] The substitutions p.Leu2027Phe, and to a greater extent p.Gly1961Glu, also were associated with a milder AF phenotype in our study. Larger numbers of patients are needed to investigate the phenotypic characteristics of other rare alleles.

There are several potential limitations of this study, many of which are inherent to retrospective studies, including study population selection, the variable number and interval of examinations during follow-up, the definition of atrophic lesions on AF images, AF subtype classification, and the strategy of mutation screening and genotype grouping. Of the recruited patients, 62% at baseline and 54% at follow-up were in AF subtype 2, with the number of patients with AF subtype 3 being relatively small; a larger prospective cohort will be helpful to gain an improved understanding of the pathophysiological features associated with AF subtype 3 and also address many of the aforementioned limitations of retrospective studies.

This study was designed to observe the progression between the two time points (baseline and follow-up) and the assumption of linear progression was made for atrophy enlargement in keeping with previous reports.[6,30] This study has not examined the linearity of change between baseline and follow-up testing; a prospective study with additional more frequent time point sampling will help address this pertinent question. It is possible that progression will be linear in some individuals and nonlinear in others, in keeping with the commonplace phenotypic heterogeneity of inherited retinal disorders.

The definition of the significant low gray scale on AF images can be challenging—nevertheless it is very reassuring that the two investigators in this study showed statistically significant

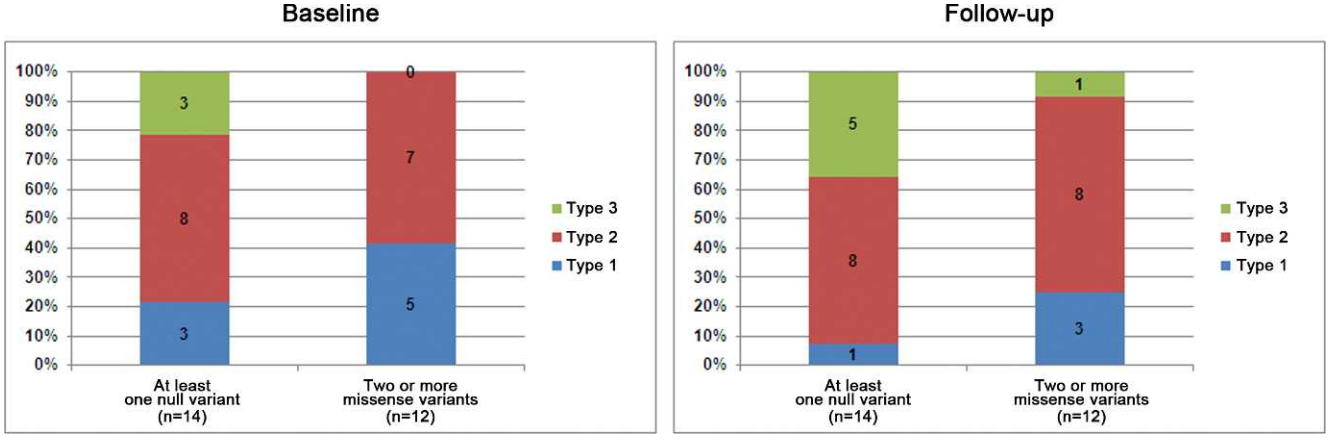

FIGURE 5. The association between genotype group and AF type at baseline and follow-up. The proportion of each AF subtype for each genotype group is shown in the *bar graphs*. In 26 patients with two or more likely disease-causing variants, there was a statistically significant association between AF subtype classification and genotype group classification at baseline and follow-up.

agreement in atrophy measurement. Software, which defines and calculates the atrophic lesions automatically, may arguably be more reliable in performing quantitative analysis, although this also is not without its limitations. Many of the older AF images at baseline in our study could not be analyzed with such recent software. Quantitative analysis of optical coherence tomography data also will have an important role in future studies of atrophy progression. In addition, wide-field AF imaging systems also may aid a more comprehensive assessment in terms of AF type classification (less atrophic lesions "beyond the scope" of the acquired image) and quantitative atrophy measurement.

Two different gene screening methods (SSCP and microarray) were applied in our cohort due to the technological advances made during the period of this study. In keeping with previous studies, we were able to undertake segregation analysis in a limited number of cases due to the unavailability of other family samples. Undoubtedly, more advanced recent mutation analysis, such as PCR enrichment–based next-generation sequencing (NGS), will result in a higher mutation detection rate, including identifying the often "missing" second *ABCA4* allele, which will allow more informed genotype–phenotype correlations to be investigated.[33,58] The genotype–phenotype correlations have been investigated by comparing the *ABCA4* gene mutations with the clinical features in this study; however, it is likely that other factors must be considered, including environmental, genetic, and epigenetic modifiers.

This study has investigated AF subtypes/patterns in a longitudinal survey, and determined changes in AF pattern and progression of atrophy. It has highlighted that patients with localized foveal atrophy tend to experience milder progression, including milder visual acuity loss, whereas those subjects with multiple large areas of retinal atrophy had severe progression and severe visual acuity reduction. These data assist counselling with regard to visual prognosis, and may help in study design and patient selection of clinical trials for *ABCA4*-related retinopathy.

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