

Retinal Phenotypes in Patients Homozygous for the G1961E Mutation in the *ABCA4* Gene

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PURPOSE. We evaluated the pathogenicity of the G1961E mutation in the *ABCA4* gene, and present the range of retinal phenotypes associated with this mutation in homozygosity in a patient cohort with *ABCA4*-associated phenotypes.

METHODS. Patients were enrolled from the *ABCA4* disease database at Columbia University or by inquiry from collaborating physicians. Only patients homozygous for the G1961E mutation were enrolled. The entire *ABCA4* gene open reading frame, including all exons and flanking intronic sequences,

was sequenced in all patients. Phenotype data were obtained from clinical history and examination, fundus photography, infrared imaging, fundus autofluorescence, fluorescein angiography, and spectral domain-optical coherence tomography. Additional functional data were obtained using the full-field electroretinogram, and static or kinetic perimetry.

RESULTS. We evaluated 12 patients homozygous for the G1961E mutation. All patients had evidence of retinal pathology consistent with the range of phenotypes observed in *ABCA4* disease. The latest age of onset was recorded at 64 years, in a patient diagnosed initially with age-related macular degeneration (AMD). Of 6 patients in whom severe structural (with/without functional) fundus changes were detected, 5 had additional, heterozygous or homozygous, variants detected in the *ABCA4* gene.

CONCLUSIONS. Homozygous G1961E mutation in *ABCA4* results in a range of retinal pathology. The phenotype usually is at the milder end of the disease spectrum, with severe phenotypes linked to the presence of additional *ABCA4* variants. Our report also highlights that milder, late-onset Stargardt disease may be confused with AMD. (*Invest Ophthalmol Vis Sci.* 2012; 53:4458-4467) DOI:10.1167/iovs.11-9166

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Supported in part by grants from the National Eye Institute/NIH EY021163, EY013435, EY019861, and EY019007 (Core Support for Vision Research); Foundation Fighting Blindness (Owings Mills, MD), Foundation Fighting Blindness Canada (Toronto, Ontario, Canada), Canadian Institutes for Health Research (Ottawa, Ontario, Canada), Reseau Vision (Montreal, Quebec, Canada) and unrestricted funds from Research to Prevent Blindness (New York, NY) to the Department of Ophthalmology, Columbia University, The Eye Surgery Fund and the Wynn-Gund Foundation.

Submitted for publication November 27, 2011; revised March 3, 2012; accepted May 21, 2012.

Disclosure: T.R. Burke, None; G.A. Fishman, None; J. Zernant, None; C. Schubert, None; S.H. Tsang, None; R.T. Smith, None; R. Ayyagari, None; R.K. Koenekoop, None; A. Umfress, None; M.L. Ciccarelli, None; A. Baldi, None; A. Iannaccone, None; F.P.M. Cremers, None; C.C.W. Klaver, None; R. Allikmets, None

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Stargardt disease (STGD1) is the most common cause of juvenile macular dystrophy, with an estimated prevalence between 1 in 8,000 and 1 in 10,000.¹ The disease is caused by mutations in the *ABCA4* gene on the short arm of chromosome 1.² The *ABCA4* protein is a member of the ATP-binding cassette superfamily and is the transporter of vitamin A derivatives in the outer segment disc membranes of the photoreceptors. The dysfunctional *ABCA4* protein, due to mutations in the gene, results in formation of vitamin A bisretinoid adducts that are deposited in retinal pigment epithelium (RPE) cells during the process of disc shedding and phagocytosis, eventually leading to cell death and macular degeneration. Given that the carrier frequency for mutations in the *ABCA4* gene has been reported to be as high as 1 in 20,³⁻⁵ it is likely that the prevalence of *ABCA4*-associated phenotypes is much higher than currently estimated.

Apart from STGD1, mutations in the *ABCA4* gene are responsible for additional retinal degeneration phenotypes, including age-related macular degeneration (AMD),⁶ bull's eye maculopathy (BEM),^{7,8} cone-rod dystrophy (CRD), and retinitis pigmentosa (RP).⁹⁻¹³ Understanding the complex interactions between genotype and phenotype in STGD1 long has frustrated ophthalmologists and geneticists, not only because of the large phenotypic variability, but also because of the description of over 700 disease-causing mutations in the *ABCA4* gene.^{8,14-17} Given this heterogeneity, careful pheno-

typing of patients homozygous for specific mutations in *ABCA4* is vital to enhance our understanding of disease expression linked to specific mutations, and the resulting genotype-phenotype correlations.

The missense mutation G1961E occurs in exon 42 of the *ABCA4* gene. While this has been characterized as the most common *ABCA4* mutation, its frequency in the general population varies widely across ethnic groups, from approximately 0.2% in populations of European origin,^{18,19} to approximately 10% in East African (e.g., Somali) populations.²⁰ It is considered a pathologic mutation for three main reasons: (1) it always co-segregates with the disease in families,²¹ (2) other mutations are found rarely in *cis* (i.e., on the same chromosome), and (3) it affects protein function as determined by indirect functional tests (ATP-ase activity and ATP binding).²² The heterozygous G1961E mutation, in combination with a different *ABCA4* allele on the other chromosome, has been associated with a localized disease process that is confined to the posterior pole, normal full-field electroretinogram (ffERG), absence of the “dark choroid” sign on fluorescein angiography (FA), and BEM on fundus autofluorescence (FAF).^{19,23–25} Despite all these data, there still is debate as to whether this mutation causes retinal pathology in homozygosity. We studied patients homozygous for the G1961E allele to demonstrate the pathogenicity of the mutation in homozygosity and to describe further the variation in retinal phenotypes associated with it.

MATERIALS AND METHODS

Patients

Only patients who demonstrated phenotypes consistent with *ABCA4* disease (i.e., BEM, STGD1, CRD, or RP) and who were homozygous for the G1961E mutation in the *ABCA4* gene were included. Patients 1–6 were retrieved retrospectively from the database of approximately 600 patients with *ABCA4*-associated diseases at Columbia University. The other six patients were obtained from collaborating retinal physicians by inquiry, and come from a similar pool of retinal dystrophy patients. All patients, except for 7-1 and 7-2 (Jordanian descent), and patient 10 (of Somali descent), were of European ancestry. Age of onset of visual symptoms was recorded for each patient. The duration of symptomatic disease was calculated by subtracting age of onset from the age at most recent examination. As age of onset is a subjective parameter, age at examination provided an objective surrogate parameter for disease duration. This approach has been used successfully before to obtain more precise estimates of disease kinetics in hereditary retinal diseases.²⁶ All patients underwent a complete ophthalmic examination by a single retinal specialist (SHT, RTS, GF, RK, AI, or CCWK), including best-corrected visual acuity (BCVA), slit-lamp biomicroscopy, ocular pressure measurement by applanation tonometry, and fundus examination with direct and indirect ophthalmoscopy following pupil dilation. Patients were classified into one of the four stages of STGD1 as described previously.²⁷ Stage I disease was characterized by central macular atrophy with parafoveal or perifoveal flecks. Where flecks were more numerous and extended anterior to the vascular arcades and/or nasal to the optic disc, then patients were classified as having stage II disease. Although partial resorption of flecks may be present in this stage, more complete resorption of flecks was indicative of stage III disease with choriocapillaris atrophy also within the macula. Widespread RPE and chorioretinal atrophy throughout the fundus defined stage IV disease.²⁷ Based on this classification system, the patients in our study were subdivided into 2 groups, that is those with milder disease (stage I or II) and those with more severe disease (stage III or IV) phenotypes.

The study conformed to the tenets set out in the Declaration of Helsinki, and was approved by the Institutional Review Boards of Columbia University, The University of Illinois at Chicago, McGill

University, The University of Tennessee, and The Erasmus Medical Center. Informed consent was obtained from all participants before their enrollment. Data from four of the patients presented in our study (patients 1, 2, 3, and 7-1) have been reported partially in other studies describing patients with *ABCA4* disease.^{25,28–31} Those studies described some specific features of *ABCA4* disease phenotypes, such as BEM and peripapillary sparing, as well as determining the sequence of retinal and RPE changes in STGD1. In our study, however, we examined the full range of retinal phenotypes in a cohort of patients homozygous for the G1961E mutation to demonstrate the pathogenicity of this mutation in homozygous state.

Fundus Photography and Fluorescein Angiography

Color fundus photographs and FA images were acquired using either a Carl Zeiss Meditech, Inc. (Dublin, CA), Topcon Medical Systems (Paramus, NJ), or Canon USA Inc. (Lake Success, NY) fundus camera with film, or an OIS digital capture system using standard acquisition protocols. Standardized FA was performed using 2.5 mL 25% or 5 mL 10% fluorescein sodium solution injected intravenously in an antecubital vein. The presence of a “dark” or “silent” choroid on FA long has assisted ophthalmologists in making the clinical diagnosis of STGD1. The most commonly quoted frequency for this sign is 85.9%.³² Importantly, its absence does not rule out a diagnosis of *ABCA4* disease. The masking of background choroidal fluorescence occurs due to a build-up of lipofuscin in the RPE causing absorption of short wavelength light. Where extensive disease is present, there can be difficulty identifying the presence or absence of a silent choroid on FA in the presence of numerous window defects and staining of the flecks during the course of angiography.³³ The presence of a ring of hypofluorescence (“dark choroid ring”) in the peripapillary region on FA has been reported in 37% of patients in a cohort with STGD1.³³

Fundus Autofluorescence/Infrared Imaging

FAF and infrared (IR) imaging were performed using a confocal scanning laser ophthalmoscope (Heidelberg Retina Angiograph [HRA] 2 or Spectralis HRA + Optical Coherence Tomography [OCT]; Heidelberg Engineering, Dossenheim, Germany) after pupil dilation with 1% tropicamide eye drops. A 30° field of view at a resolution of 1536 × 1536 pixels was used to obtain these images, which encompassed the entire macula and a portion of the optic disc. For FAF, an optically pumped solid-state laser (488 nm wavelength) was used for excitation and a 495 nm barrier filter was used to block reflected excitation light from the acquired image. Sensitivity adjustment for FAF was performed at 488 nm. Images were averaged computationally to produce a single frame with improved signal-to-noise ratio. The method for IR image acquisition was similar, with an image acquisition wavelength of 830 nm.

In certain cases FAF, IR, FA, and fundus photos were registered for examination of certain regions of interest in the retina using multiple imaging modalities. The registration was performed with MatLab (The MathWorks, Inc., Natick, MA), using previously described custom written software.³⁴

Spectral Domain-OCT (SD-OCT)

SD-OCT images were acquired using the Heidelberg Spectralis HRA + OCT following pupil dilation with tropicamide 1% eye drops. Images were viewed with the Heidelberg Explorer software (Heidelberg Engineering), the point-to-point correlation features of which were used to find corresponding pathologic features on the en face and cross-sectional images. Horizontal line scans were obtained using the “automated retinal tracking” function to achieve an average of 100 images. Resolution and number of images acquired per line scan were varied depending on patient compliance and fixation stability.

TABLE. Summary of Demographic, Clinical, and Functional Data in Patients Homozygous for the G1961E Mutation

Patient #, Sex	Additional <i>ABCA4</i> Mutations	Onset Age (years)	Age at Exam (years)	Duration (years)	VA		Clinical Phenotype	ERG Group	Silent Choroid	Type of Perimetry	Scotoma Location
					OD	OS					
Milder Phenotypes											
1, M		19	34	15	20/150	20/100	I	I	ND	MP-1	Central
2, F		20	21	1	20/25	20/40	I	I	Absent	ND	ND
3, M	T1253M	32	46	14	20/25	20/40	I	I	Absent	GVF	Perifoveal
4, F		43	67	24	20/40	20/150	II	I	ND	MP-1	Central
5, F		48	65	17	20/150	20/200	I	I	ND	MP-1	Central
6, F		64	86	22	20/200	20/200	II	I	Absent	GVF	Central
Severe Phenotypes											
7-1, M	H1838D (Hom)	4	12	8	20/250	20/250	III	III	ND	GVF	Central
7-2, F	H1838D (Hom)	7	13	6	20/200	20/200	IV	III	Peripapillary Ring	GVF	Central
8-1, F	N96K	7	46	39	20/2000	20/2000	III	III	Peripapillary Ring	GVF	Central
8-2, M	N96K	10	49	39	20/400	20/400	IV	ND	ND	GVF	Central
9, F	N96K (Hom)	12	59	47	10/400	10/400	IV	III	ND	ND	ND
10, M		20	51	31	20/25	20/25	III	RP	ND	GVF	Perifoveal

Each number identifies distinct families. Siblings are identified by an additional number after the dash (e.g., 8-1). M, male; F, female; Hom, homozygous; OD, right eye; OS, left eye; ND, not done; MP-1, microperimetry.

Electroretinogram

Ganzfeld fERGs were recorded from both eyes of each patient with DTL, conjunctival HK-loop, or corneal contact lens (ERG Jet or Henkes) electrodes according to published methods³⁵⁻³⁷ in compliance with the International Society for Clinical Electrophysiology of Vision (ISCEV) standards in scotopic and photopic states to assess retinal function.³⁸ The minimum protocol that incorporates the rod-specific and standard dark-adapted bright flash combined rod and cone ERG responses were recorded after a minimum of 20 minutes of dark adaptation. Following light adaptation, the transient photopic ERG and the photopic 30 Hz flicker ERG were recorded. The amplitude and timing of the responses recorded from both eyes of each patient were compared to normative data ranges for each lab. Amplitudes reduced below 2 SDs from the mean were considered abnormal. Patients were divided into 3 groups based on the fERG results. Patients with ERG group I disease had normal amplitude of the photopic and scotopic ERGs; ERG group II disease patients had relative loss of cone-driven function, while ERG group III patients had abnormally reduced scotopic and photopic ERGs.³⁹ With advanced STGD1, photopic and scotopic responses may become diminished below the recording threshold, whereby it becomes difficult to distinguish ERG group III disease from a rod-cone pattern of dysfunction electrophysiologically. In this situation a presenting history of nyctalopia, rather than light aversion and/or central or color vision problems, would suggest a diagnosis of RP rather than ERG group III disease.²⁴

Perimetry

The visual field was evaluated in some patients using the MP-1 microperimeter (Nidek Technologies Inc., Padova, Italy). Patients were asked to maintain fixation on a red cross (2° in diameter) for the duration of the test. The non-tested eye was occluded throughout the procedure. Microperimetry was performed subsequently and the sensitivity of the central visual field was determined using a 10-2 program. "White" test lights (stimulus size Goldmann III, duration 200 ms) were presented on a dim "white" background (1.27 cd/m²) using a 4-2 threshold. For the 10-2 program, 68 samplings covering an area of 20° in diameter were tested.²⁵ Where microperimetry was not available, kinetic perimetry was performed using the Goldmann visual

field test (GVF, model 940; Haag-Streit AG, Koniz, Switzerland) employing standard test targets and strategies.

Genetic Analysis

In our protocol, screening for mutations in the *ABCA4* gene commences with the ABCR600 microarray (Asper Ophthalmics, Tartu, Estonia; in the public domain <http://www.asperbio.com/genetic-tests/panel-of-genetic-tests/stargardt-disease-cone-rod-dystrophy-abca4>), which currently detects 615 known mutations in the *ABCA4* gene.^{3,25,40} To confirm the presence of variants detected by the array, as well as to exclude the presence of additional disease causing mutations in the gene, the entire *ABCA4* gene subsequently was sequenced in each patient. Sequencing of the *ABCA4* was performed by either the Sanger method or by next generation sequencing on the Roche 454 platform.^{17,28}

RESULTS

Clinical, genetic and demographic information for all patients is summarized in the Table. Depending on the availability of the testing methods described below, each patient had a selection of these performed. We included 12 patients (5 men, 7 women) in this series. Mean ages at onset and last examination were 23.8 (range 4-64) and 45.8 (range 12-86) years, respectively. The mean symptomatic duration of disease was 21.9 years (range 1-47). Unilateral BCVA ranged from 20/25-20/2000. All patients were homozygous for the G1961E mutation in the *ABCA4* gene. Patient 9 was double homozygous for the N96K mutation together with G1961E. For patients 8-1 and 8-2, N96K was detected on only one allele in each patient. All 3 of these patients were of Italian origin. Two other siblings (patients 7-1 and 7-2), who belonged to a consanguineous Jordanian family, also had the H1838D mutation detected in homozygosity, in addition to being homozygous for the G1961E mutation. These two patients exhibited the more complex phenotype of atrophic STGD1 macular changes associated with peripheral, classic bone spicule-like deposits as seen in RP. Patient 3 had an additional heterozygous T1253M variant. Interestingly, patient 10 was of Somali descent and had no additional possibly disease-causing

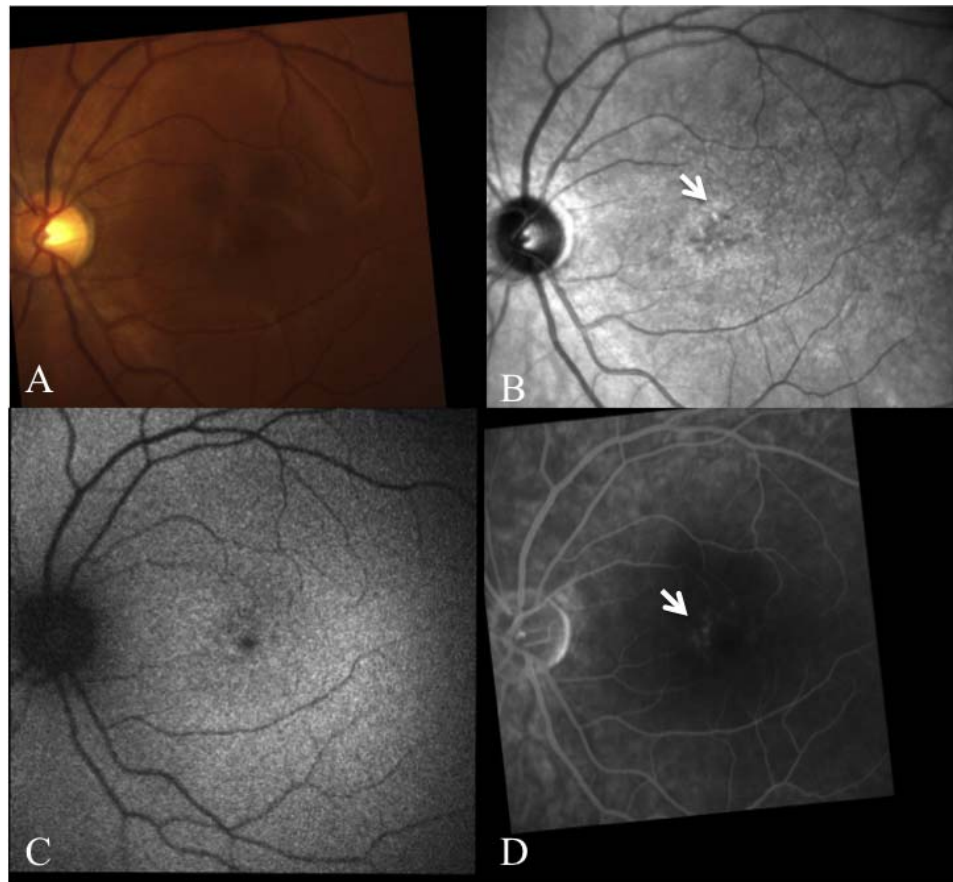


FIGURE 1. Fundus photographs (A, B) with corresponding FA images (C, D) of 2 patients (7-2 and 8-1, respectively) with severe STGD1 phenotypes. Note the severe central macular atrophy in both patients, most obvious on FA. While both patients had evidence of peripheral intraretinal pigmentation on fundus photography (*black arrows*), these were more obvious in patient 7-2. Given the extensive atrophy of the RPE in both patients, detection of the dark choroid sign was not possible. However, partial dark choroid rings were observed in the peripapillary regions (C, D; *white arrows*).

mutations in the *ABCA4* gene. Family members (parents and siblings) were available in all cases except for patients 6 and 10. The phase and true homozygosity of the G1961E mutation were determined by segregation analyses. Hemizygosity, that is the homozygous appearance due to the deletion on the other chromosome, was ruled out in all cases.

Patients with Milder Phenotype (Stages I and II)

Of the 6 patients with milder phenotypes based on clinical examination, four had stage I and 2 had stage II disease (Figs. 1A–D, 2A, 2B, 3A, 3B). Five of these patients had BCVA of 20/150 or better in at least one eye (median uniocular BCVA for this group 20/120). Ages of onset ranged from 19–64 years. Patients 2 and 3 had unilateral visual acuities equal to the best in the study, that is 20/25. While the former had the shortest disease duration of any patient in the study, the latter had evidence of relative foveal sparing despite a symptomatic disease duration of 14 years. The single patient (patient 6) in this milder disease subgroup, with visual acuity of 20/200 bilaterally, had a symptomatic disease duration of 22 years and had co-existing primary open-angle glaucoma. Interestingly, she had an age at onset of 64 years, the latest of all patients in this study, and had the oldest age (86 years) at last examination. In fact, this patient had been misdiagnosed initially with AMD, although no drusen (or flecks) were noted on fundus examination at any time during follow-up. Only one patient in this group (patient 3) had a heterozygous T1253M variant

detected in the *ABCA4* gene, in addition to the homozygous G1961E mutation.

FAF images for this group of patients with milder phenotypes revealed subtle focal FAF changes as seen in patient 2 (Fig. 1C), central macular patchy hypo/hyperautofluorescence surrounded by a hyperautofluorescent halo as seen in patient 5 (Fig. 2A), as well as geographic atrophy (GA) in patients 1 and 4.

FA images were available for 3 patients and there was no evidence of the dark choroid sign or choroidal neovascularization (Figs. 1D, 3A). Window defects, consistent with atrophy of the RPE on FA, were present in both eyes of patients 2, 3, and 6. There also was FA evidence of relative bilateral foveal sparing in patient 3.

Perimetric data were available on 5 of the 6 patients in this subgroup. Four of these patients had evidence of central scotomata, while one (patient 4) had preserved central visual function with perifoveal scotomata in keeping with the evidence of relative structural foveal sparing observed on FA. All patients had normal ffERG and, therefore, were classified as having ERG group I STGD1.

Patients with Severe Phenotype (Stages III and IV)

Of the 6 patients with more severe disease phenotypes, three each had stages III and IV disease (Figs. 4A–D, 5A–C). Five of the 6 patients in this subgroup had an onset of visual symptoms at ≤ 12 years of age and all of these had BCVA of

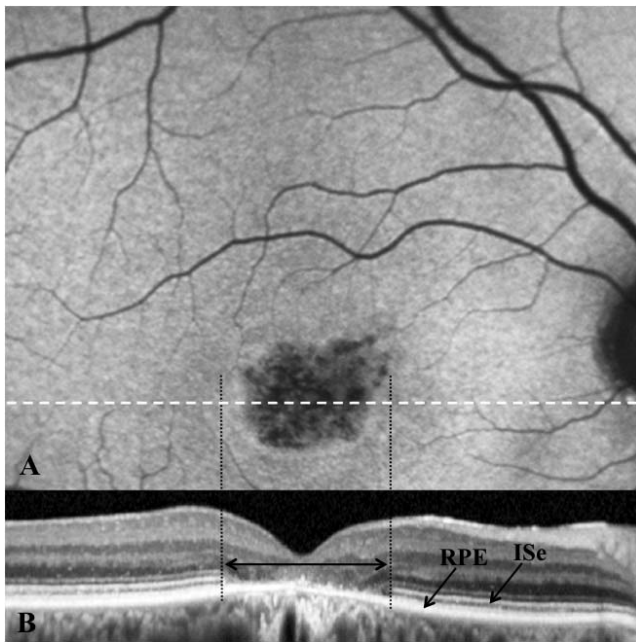


FIGURE 2. FAF and SD-OCT of the right eye of patient 5. Simultaneous FAF (A) and SD-OCT (B) images were acquired. Note the central macular FAF abnormalities with alternating hypo/hyperautofluorescence surrounded by a perimeter of relative hyperautofluorescence. This corresponded with abnormalities on SD-OCT with absence of the ISe and thinning and irregularity of the RPE (*double headed black arrow*). Note also the absence of focal hyperautofluorescence, suggestive of flecks, on FAF.

$\leq 20/200$ bilaterally (median unocular BCVA for the group 20/320). Only patient 10 from Somalia, with an onset of disease at 20 years of age, had visual acuities of 20/25 bilaterally. These 6 patients had the most severe fundus abnormalities of our study sample, with marked atrophy of the retina and RPE, pigment clumping, optic disc pallor, and attenuation of the retinal vessels detected to varying degrees (Figs. 4A, 4B, 5A, 5B). Among the 2 patients with FA images available (Figs. 4C, 4D), there was no evidence of a dark choroid, in the presence of extensive angiographic evidence of advanced disease. This is not surprising given the funduscopic evidence of extensive atrophy of the RPE. However, a partial, faint peripapillary dark choroidal ring was detected in both patients on FA, corresponding to areas of better preserved retina and RPE.²⁵

SD-OCT imaging of the macula of patient 10 (Fig. 5C) showed an island with relative sparing of the inner segment ellipsoid band of the photoreceptors (ISe), as well as of the outer nuclear layer (ONL) in the central macula consistent with the measured visual acuity. This region was surrounded by macular regions where there was absence of the ISe, and obvious atrophy of the inner and outer retina (Fig. 5C). These changes were in keeping with phenotypes seen in RP.

Five of the 6 patients in this subgroup had perimetric data available. Central scotomata with reduced visual sensitivity more peripherally were detected in 4 patients. In keeping with the relatively preserved foveal structure described above, patient 10 had evidence of relative sparing of central visual function on GVF testing. While there was reduced pericentral visual sensitivity bilaterally in this patient, the peripheral portions of the visual fields, however, were well preserved (Figs. 5D, 5E).

ffERG results were available for 5 of the 6 patients, and showed a cone and rod pattern of dysfunction (ERG group III disease) in 3. Importantly, while the photopic and scotopic ERGs appeared equally severely affected for patients 9 and 10,

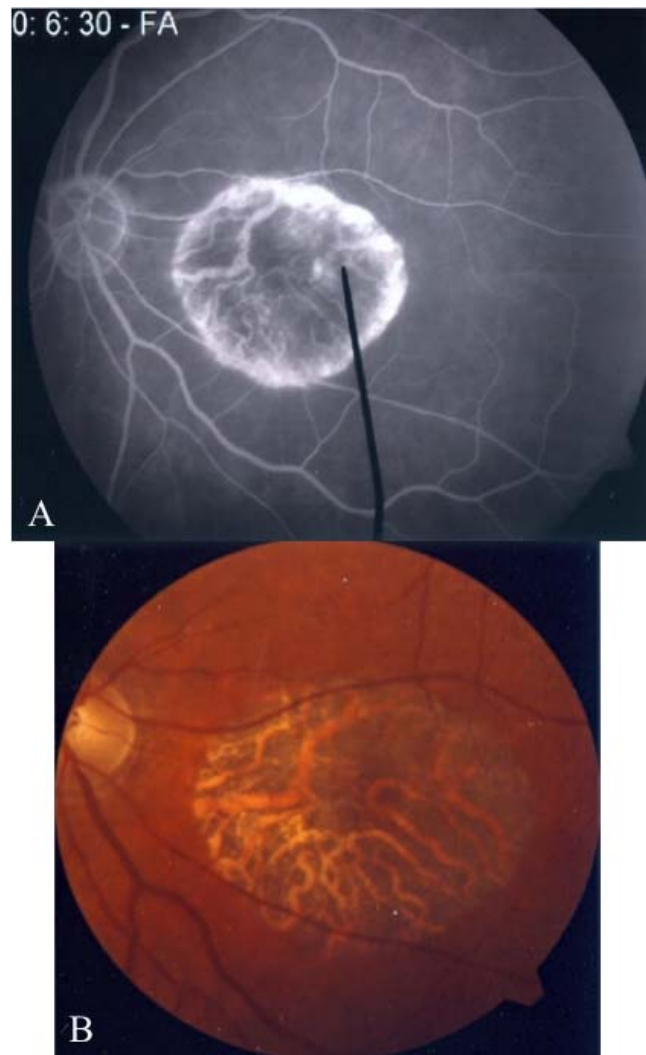


FIGURE 3. FA (with fixation target) and fundus photograph of the left eye of patient 6. The FA (A) showed absence of the dark choroid, as well as evidence of choroidal atrophy (relative hypofluorescence) in the central macula within the region of GA. There was persistence of choroidal perfusion immediately inside the boundary of the region of GA, as evidenced by the irregular ovoid ring of hyperfluorescence in the macula. There was no evidence of flecks or drusen on FA or fundus photography (B).

the history of early central vision loss for the former suggested a cone-rod rather than rod-cone etiology of disease in keeping with a diagnosis of ERG group III disease. In contrast, nyctalopia was the earliest visual symptom for patient 10, which is more consistent with a rod-cone pattern of disease, and a diagnosis of RP. Interestingly, 5 of 6 patients in this group, with the exception of patient 10, harbored heterozygous or homozygous *ABCA4* variants in addition to G1961E.

DISCUSSION

We illustrated the phenotypic expression of retinal disease associated with homozygous G1961E mutation in the *ABCA4* gene. It was suggested previously that G1961E was not pathogenic, at least in homozygosity, for two reasons. First, the mutation is detected at approximately 10% frequency in the general population from Somalia, predicting that 1/100 Somalis are homozygous for G1961E.²⁰ Since, as stated in that

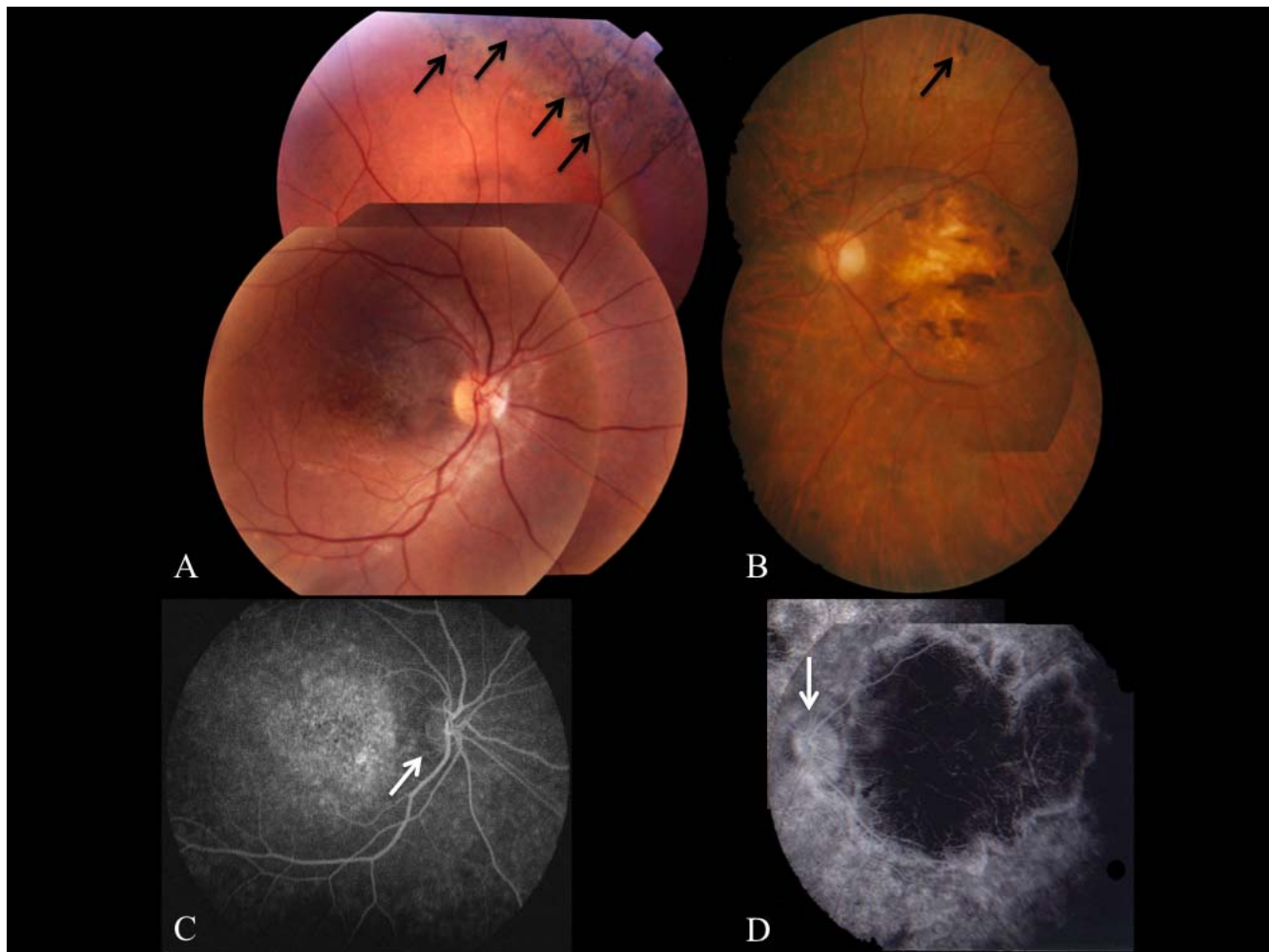


FIGURE 4. Fundus photographs (A, B) with corresponding FA images (C, D) of 2 patients (7-2 and 8-1, respectively) with severe STGD1 phenotypes. Note the severe central macular atrophy in both patients, most obvious on FA. While both patients had evidence of peripheral intraretinal pigmentation on fundus photography (*black arrows*), these were more obvious in patient 7-2. Given the extensive atrophy of the RPE in both patients, detection of the dark choroid sign was not possible. However, partial dark choroid rings were observed in the peripapillary regions (C, D; *white arrows*).

study, “Stargardt disease is not known to be 100 times more prevalent in Somalia than in the United States, it suggests that G1961E does not frequently cause disease in the homozygous state.” Secondly, The same group presented an asymptomatic 25-year-old Somali man homozygous for G1961E (Shankar SP. *IOVS* 2006;47:ARVO E-Abstract 1699).

These interesting observations can be interpreted as follows. It is true that the G1961E mutation is frequent in East Africa. We have genotyped sizable populations from Kenya, Ethiopia, Egypt, the Middle East, Europe, and so forth, and confirmed that the frequency is the highest (8–10%) in the general population from the Horn of Africa region (Somalia/Kenya/Ethiopia) and, gradually and rapidly, becomes lower in the neighboring regions. This mutation is very rare in West Africa (and, consequently, in African Americans) and in European populations. One possibility is that the G1961E mutation is pathogenic in Europeans and not in East Africa, that is the change happened twice or multiple times. This is not, however, the case as the haplotype on which this mutant allele resides (Fig. 6) is the same across all populations (data not shown). In addition, in our study 50% (3 of 6) of patients with homozygous G1961E and no additional *ABCA4* variants had a late disease onset, over 25 years of age. Therefore, it is not entirely surprising that a

patient could have a normal eye examination at 25 years of age and experience symptomatic disease expression much later in life. However, patient 10, who also did not carry any other *ABCA4* mutations, was Somali with an age of onset at 20 years and a disease pattern consistent with RP, highlighting the phenotypic variability associated with *ABCA4* mutations, even in relatively genetically homogeneous ethnic groups.

Large studies in elderly subjects of East African descent could reveal the correlation between the homozygous G1961E mutation and eye disease in those populations. In other populations, for example those of European descent, a normal visual function and a normal clinical examination do not exclude *ABCA4* disease, especially when caused by the G1961E mutation in homozygosity. A likely scenario has the G1961E mutation arising once, in East Africa, and then lost (eliminated by selective pressure) in other regions due to its pathogenicity and earlier onset in heterozygosity with other, more severe, *ABCA4* alleles. It is possible that these alleles are rare in East Africa and the mild G1961E mutation itself does not result in the disease during reproductive age, and, therefore, is not under selective pressure.

Another proof of pathogenicity for the homozygous G1961E comes from allele frequency calculations. The mutation is

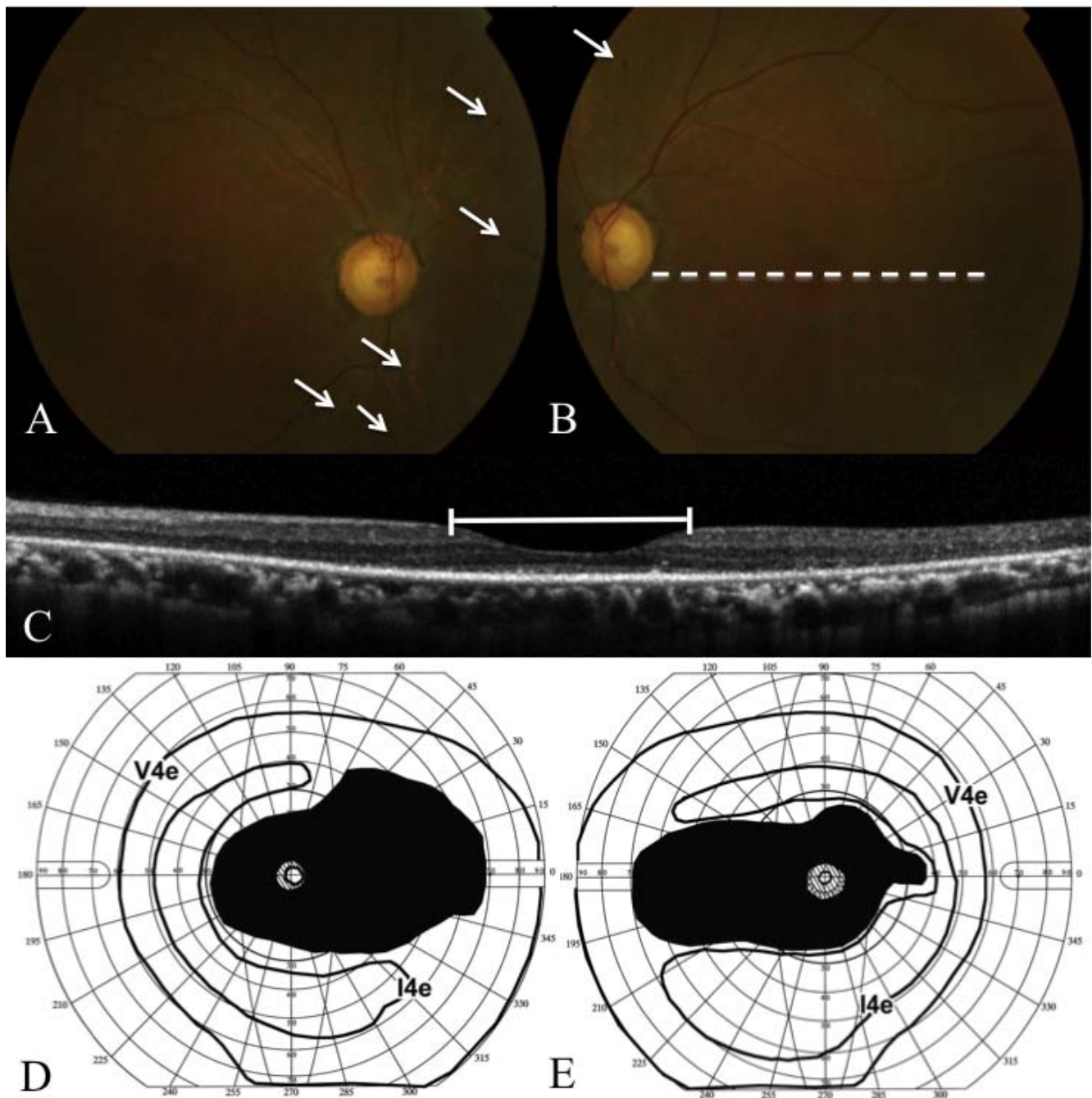


FIGURE 5. Fundus photographs, SD-OCT, and GVF results for patient 10 with a diagnosis of RP. Fundus photographs of the right (**A**) and left (**B**) eye are presented. Note the optic disc pallor bilaterally with large round excavated cups. There also was arteriolar narrowing bilaterally with retinal pigment epithelial atrophy along the vascular arcades. A grayish sheen was noted in the mid-periphery bilaterally with areas of intraretinal pigmentation (*white arrowheads*). (**C**) SD-OCT image of the left eye corresponding with the dashed line in image (**B**). The white line indicates the horizontal extent of the central macular region with relative preservation of the inner segment ellipsoid band of the photoreceptors and the outer nuclear layer. Outside this region the photoreceptors, as well as the inner retina, are markedly atrophic. (**D**, **E**) GVF of the right and left eye, respectively. Visual function in the central 5° was intact. Absolute ring scotomata were present in the mid-periphery at eccentricities from 5° up to 70° bilaterally. Visual function in the far periphery remained intact.

found in 10% of STGD1 patients in heterozygous state, so homozygotes, if causal, should account for 1% of all STGD1 patients. We have detected 6 G1961E homozygotes after screening approximately 600 random STGD1 patients, which is the exact predicted frequency of homozygosity.

Six patients had other *ABCA4* variants on the same chromosome with G1961E. One patient harbored a heterozy-

gous T1253M variant, which previously has been reported sometimes to form a complex allele with G1961E.⁴¹ It is predicted to give rise to an amino acid change that lies outside the functional domain of the *ABCA4* protein, it never occurs without G1961E, and, therefore, its pathogenicity has not been confirmed. The N96K missense change, which previously has been reported as a disease causing mutation in the *ABCA4*

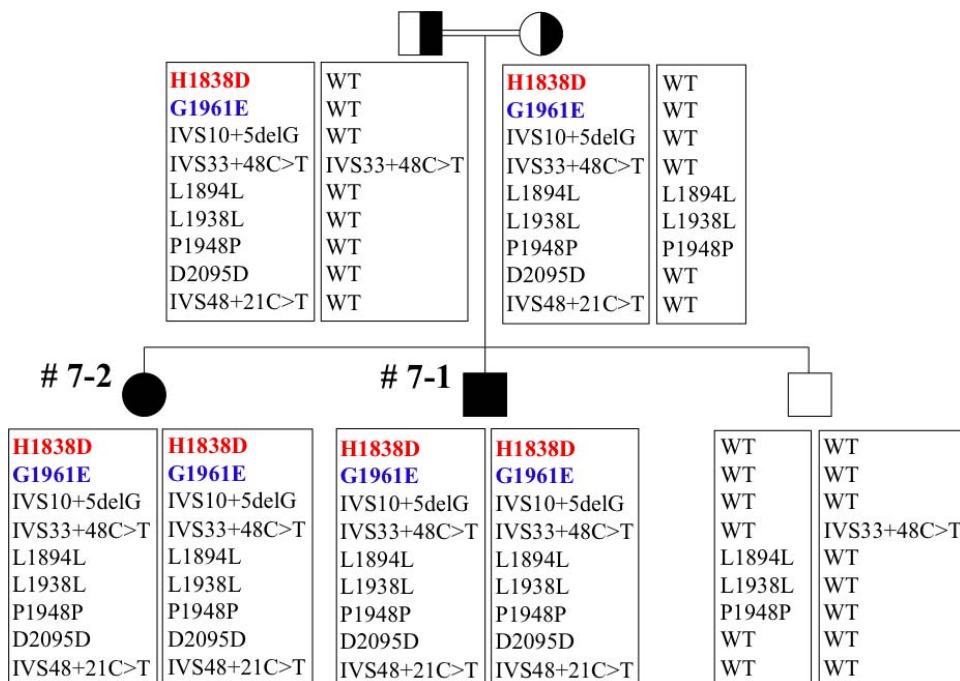


FIGURE 6. Pedigree for family 7. Patients 7-1 and 7-2 were offspring from a consanguineous marriage. The haplotype for the G1961E mutation, together with the H1838D mutation in this case, is demonstrated clearly. All other variants on the disease haplotype, including the IVS10+5delG, have no effect on splicing or are deemed pathogenic by any other criteria, that is they are frequent (>5%) in the general population and they do not segregate with the disease without the G1961E mutation.

gene,^{42,43} was detected 4 times in this cohort, that is twice in 2 siblings heterozygous for this mutation (patients 8-1 and 8-2), and once in a double homozygous patient (patient 9). In all 3 cases, this additional mutation yielded a severe disease phenotype with extensive retinal atrophy, primarily affecting the macular region. These genetic findings in our patients of Italian origin are in keeping with recent reports of this mutation in Italian populations with STGD1.⁴³ The H1838D mutation has been reported previously in patient 7-1.³¹ This variant clearly has a profoundly deleterious effect on *ABCA4* protein function, at least when present in conjunction with the G1961E in homozygosity, giving rise to a severe, early-onset and complex phenotype in both siblings from a consanguineous family of Jordanian descent.

In general, patients homozygous for the G1961E mutation demonstrated a later onset of visual symptoms than typically would be seen in STGD1.^{27,44,45} However, despite the better prognosis for overall retinal function in these G1961E homozygous patients (as determined by normal fERG) the final visual acuity can still reach 20/200. Previous reports have focused mainly on the phenotypes of patients with G1961E in simple or compound heterozygosity. The majority of those patients revealed a milder retinal disease phenotype confined to the central macula, with absence of the dark choroid sign on FA, and with normal fERG.^{24,25,46} This milder phenotype correlates with the observation that although there is a reduction in ATPase activity with the G1961E mutation, there is comparable yield to the levels seen in the wild-type.⁴⁶ However, its basal ATPase activity appears inhibited rather than stimulated by retinal.²²

While all other patients had a clinical diagnosis of STGD1 before genetic confirmation, patient 6 initially was diagnosed with dry AMD with GA, due to the late onset of disease and the absence of flecks on clinical examination. During investigation of the genetic causes of AMD the patient was screened for mutations in the *ABCA4* gene and the homozygous G1961E

mutation was detected, highlighting an important issue in crossover between AMD and *ABCA4*-associated disease phenotypes. This crossover may explain partly the underestimation of the prevalence of STGD1, that is “milder” mutations have later onset and result in atypical STGD1 phenotypes similar to, and likely misdiagnosed as, AMD. This is important especially when we consider that vitamin A/beta-carotene supplementation is not recommended for patients with *ABCA4*-linked retinopathies,⁴⁷ whereas it is part of the traditional supplementation regimen that has been investigated in AMD.⁴⁸ With the opportunity for gene therapy of *ABCA4*-linked diseases also approaching (see ClinicalTrials.gov Identifier: NCT01367444), this differential diagnostic distinction may have additional therapeutic ramifications.

In summary, our findings demonstrate the pathogenicity of the G1961E mutation by the phenotypic manifestation of STGD1 patients where only the G1961E mutation was detected in homozygosity. As observed previously in patients compound heterozygous for the G1961E mutation, phenotypic expression was somewhat atypical for classical STGD1, with the traditional findings of retinal flecks and a dark choroid effect on FA largely absent in association with the G1961E mutation in homozygosity. Furthermore, clinicians must consider late-onset STGD1 also in the differential diagnosis of AMD-like, atrophic phenotypes. Lastly, a normal visual acuity and a normal clinical examination should not exclude a diagnosis of *ABCA4* disease, especially when the G1961E mutation is the putative genetic cause.

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