AMERICAN ACADEMY™
OF OPHTHALMOLOGY

Photoreceptor Progenitor mRNA Analysis Reveals Exon Skipping Resulting from the *ABCA4* c.5461-10T→C Mutation in Stargardt Disease

Riccardo Sangermano, MSc,[1,2] *Nathalie M. Bax, MD,*[3] *Miriam Bauwens, MSc,*[4] *L. Ingeborgh van den Born, MD, PhD,*[5] *Elfride De Baere, MD, PhD,*[4] *Alejandro Garanto, PhD,*[1,2] *Rob W.J. Collin, PhD,*[1,2] *Angelique S.A. Goercharn-Ramlal, PhD,*[1] *Anke H.A. den Engelsman-van Dijk, BSc,*[1] *Klaus Rohrschneider, MD, PhD,*[6] *Carel B. Hoyng, MD, PhD,*[3] *Frans P.M. Cremers, PhD,*[1,2,*] *Silvia Albert, PhD*[1,2,*]

Purpose: To elucidate the functional effect of the *ABCA4* variant c.5461-10T→C, one of the most frequent variants associated with Stargardt disease (STGD1).

Design: Case series.

Participants: Seventeen persons with STGD1 carrying *ABCA4* variants and 1 control participant.

Methods: Haplotype analysis of 4 homozygotes and 11 heterozygotes for c.5461-10T→C and sequence analysis of the *ABCA4* gene for a homozygous proband. Fibroblasts were reprogrammed from 3 persons with STGD1 into induced pluripotent stem cells, which were differentiated into photoreceptor progenitor cells (PPCs). The effect of the c.5461-10T→C variant on RNA splicing by reverse-transcription polymerase chain reaction was analyzed using PPC mRNA. In vitro assays were performed with minigene constructs containing *ABCA4* exon 39. We analyzed the natural history and ophthalmologic characteristics of 4 persons homozygous for c.5461-10T→C.

Main Outcome Measures: Haplotype and rare variant data for *ABCA4*, RNA splice defects, age at diagnosis, visual acuity, fundus appearance, visual field, electroretinography (ERG) results, fluorescein angiography results, and fundus autofluorescence findings.

Results: The frequent *ABCA4* variant c.5461-10T→C has a subtle effect on splicing based on prediction programs. A founder haplotype containing c.5461-10T→C was found to span approximately 96 kb of *ABCA4* and did not contain other rare sequence variants. Patient-derived PPCs showed skipping of exon 39 or exons 39 and 40 in the mRNA. HEK293T cell transduction with minigenes carrying exon 39 showed that the splice defects were the result of the c.5461-10T→C variant. All 4 subjects carrying the c.5461-10T→C variant in a homozygous state showed a young age of STGD1 onset, with low visual acuity at presentation and abnormal cone ERG results. All 4 demonstrated severe cone–rod dystrophy before 20 years of age and were legally blind by 25 years of age.

Conclusions: The *ABCA4* variant c.5461-10T→C is located on a founder haplotype lacking other disease-causing rare sequence variants. In vitro studies revealed that it leads to mRNA exon skipping and ABCA4 protein truncation. Given the severe phenotype in persons homozygous for this variant, we conclude that this variant results in the absence of *ABCA4* activity. *Ophthalmology 2016;■:1–11* © *2016 by the American Academy of Ophthalmology.*

Supplemental material is available at www.aaojournal.org.

Biallelic variants in the gene encoding the ATP-binding cassette transporter type A4 (*ABCA4*) have been identified in approximately 75% of cases with autosomal recessive Stargardt disease (STGD1)[1–5] and in approximately 30% of patients with autosomal recessive cone–rod dystrophy (CRD).[6] In severe CRD cases, the phenotype can resemble retinitis pigmentosa. More recently, late-onset STGD1[7] and STGD1 cases with a fine granular pattern with peripheral spots on autofluorescence examination[8] have been associated with the presence of 1 or 2 *ABCA4* variants. The clinical variability observed in *ABCA4*-associated cases can be explained by a genotype–phenotype correlation model in which the residual activity of the mutant ABCA4 protein determines the clinical phenotype.[2,9] Persons with severe CRD carry 2 *ABCA4* null alleles, whereas persons with STGD1 carry 2 moderately severe variants or a combination of a mild and a severe variant.[2,9] The ABCA4 protein has been proposed to act as a flippase for 11-*cis* and *all-trans* isomers of *N*-retinylidene-phosphatidylethanolamine across disc membranes, thereby

http://dx.doi.org/10.1016/j.ophtha.2016.01.053
ISSN 0161-6420/16

facilitating the removal of retinal products from the disc membranes and preventing the accumulation of potentially toxic bisretinoid compounds (e.g., A2E) in photoreceptor and retinal pigment epithelial cells.[10,11]

To provide an accurate prognosis for persons with *ABCA4* variants, it is important to assess the functional consequences of the approximately 1000 *ABCA4* variants that have been reported to date. This is straightforward for protein-truncating variants (i.e., stop mutations, frameshift mutations, canonical splice-site mutations), but much more difficult for rare missense mutations (i.e., amino acid substitutions) and noncanonical splice variants (i.e., RNA splice variants outside the conserved intronic dinucleotides at the ends of introns) with an unclear functional effect. The pathogenicity of selected amino acid substitutions has been studied by assessing mutant ABCA4 protein stability, ATP-binding properties, ATPase activity, and mislocalization.[12–15]

An unusually high proportion of STGD1 cases from northern Europe and the United States (approximately 30%) shows only 1 *ABCA4* variant, despite comprehensive Sanger sequencing and deletion analysis of the 50 protein coding exons.[1–4,16,17] Very recently, deep-intronic variants (i.e., variants not located near the coding exons) have been identified that can explain a proportion of these so-called missing mutations.[16–19] The identification of both *ABCA4* alleles and experimental proof that they have an effect on ABCA4 function also are crucial for the selection of persons with STGD1 for upcoming gene-specific trials, such as gene augmentation (i.e., https://clinicaltrials.gov/ct2/show/NCT01367444?term=ABCA4&rank=8 and https://clinicaltrials.gov/ct2/show/NCT01736592?term=ABCA4&rank=12) or other types of treatment.

The identification of mRNA abnormalities is hampered by the photoreceptor-specific expression of *ABCA4*.[5] This is particularly important for noncanonical splice variants with unknown effects on splicing. The third most frequent *ABCA4* variant, c.5461-10T→C (previously denoted as IVS38-10T→C; Cornelis S, Cremers FPM, unpublished data, 2015), on the basis of prediction programs has a very small effect on splicing efficiency. In our classical STGD1 patients, it is found very often together with the mild c.2588G→C (p.Gly863Ala)/(p.Gly863del) variant, suggesting that c.5461-10T→C represents a severe *ABCA4* variant.

In this study, we investigated the effect of the c.5461-10T→C variant on splicing by analyzing mRNA from photoreceptor progenitor cells (PPCs) derived from persons with STGD1 who carry this variant in a homozygous or heterozygous state. In addition, we performed in vitro minigene splicing studies to show that this variant results in truncation of the predicted ABCA4 protein.

Methods

Subjects and Clinical Evaluation

We ascertained 15 persons with STGD1 carrying the c.5461-10T→C variant in a homozygous state (patients 1–4) or compound heterozygous state (patients 5–15) from the Netherlands and Germany. In addition, we studied 2 individuals with STGD1 with a single *ABCA4* allele (patients 16 and 17) and a control. Genotype data for patients 1 through 17 can be found in Supplemental Table 1 (available at www.aaojournal.org). This study was approved by the institutional review board and adhered to the tenets of the Declaration of Helsinki. We studied all recent and available retrospective data of patients 1 through 4. This included history on initial symptoms, age at diagnosis, regression of Snellen best-corrected visual acuity (converted in Fig 1 to equivalent logarithm of the minimum angle of resolution visual acuity), and description of the fundus abnormalities. Thereafter, we also examined available color vision testing results, visual fields (Goldmann), fundus photography, fluorescein angiography, fundus autofluorescence imaging (confocal scanning laser ophthalmoscope [Spectralis; Heidelberg Engineering, Heidelberg, Germany]), spectral-domain optical coherence tomography (Spectralis), and full-field electroretinography (ffERG).

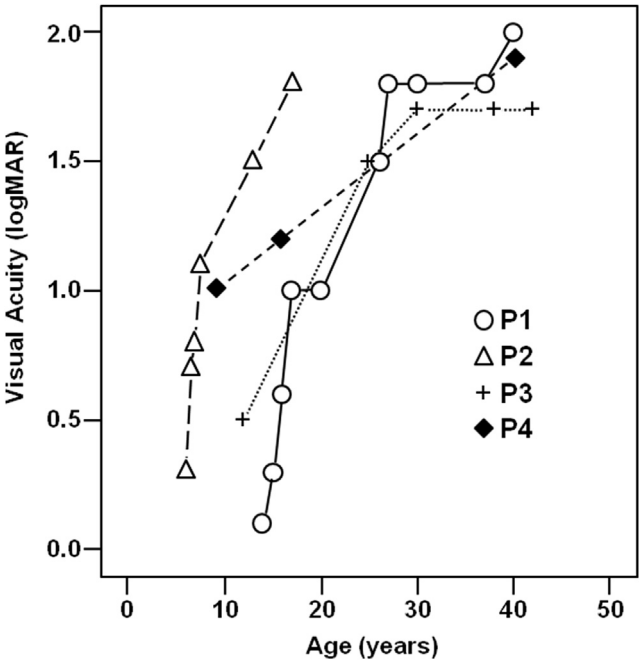

Figure 1. Graph showing visual acuity in 4 persons with Stargardt disease with the homozygous c.5461-10T→C *ABCA4* mutation. Snellen best-corrected visual acuity (BCVA) was converted to equivalent logarithm of the minimum angle of resolution (logMAR) visual acuity: logMAR 0.5 = 20/60 Snellen; logMAR 1.0 = 20/200 Snellen; logMAR 1.4 = 20/500 Snellen = blindness (World Health Organization criteria). Age at diagnosis for these persons was between 6 and 14 years; legal blindness was observed in all 4 patients before the age of 25 years. P = patient.

Molecular Genetic Analyses

In Silico Analysis of the *ABCA4* c.5641-10T→C Variant on Splicing. The possible effect of the noncanonical splice variant c.5461-10T→C in *ABCA4* on splicing was assessed by the use of 5 algorithms (SpliceSiteFinder, MaxEntScan, NNSPLICE, GeneSplicer, and Human Splicing Finder) via Alamut Visual software version 2.7 (Interactive Biosoftware, Rouen, France; www.interactive-biosoftware.com).

Haplotype Analysis. Eighteen single nucleotide polymorphisms (SNPs) were selected from within the *ABCA4* gene and