Genetics

Mutation Spectrum of the *ABCA4* Gene in 335 Stargardt Disease Patients From a Multicenter German Cohort—Impact of Selected Deep Intronic Variants and Common SNPs

Heidi L. Schulz,[1] Felix Grassmann,[1] Ulrich Kellner,[2,3] Georg Spital,[4] Klaus Rüther,[5] Herbert Jägle,[6] Karsten Hufendiek,[7] Philipp Rating,[8] Cord Huchzermeyer,[9] Maria J. Baier,[1] Bernhard H. F. Weber,[1] and Heidi Stöhr[1]

[1]Institute of Human Genetics, University of Regensburg, Regensburg, Germany
[2]Rare Retinal Disease Center, AugenZentrum Siegburg, MVZ ADTC Siegburg GmbH, Siegburg, Germany
[3]RetinaScience, Bonn, Germany
[4]Department of Ophthalmology, St. Franziskus-Hospital, Münster, Germany
[5]Augenarztpraxis, Berlin, Germany
[6]Department of Ophthalmology, University Hospital Regensburg, Regensburg, Germany
[7]University Eye Hospital Hannover Medical School, Hannover, Germany
[8]Department of Ophthalmology, University Hospital Essen, Essen, Germany
[9]Department of Ophthalmology, University Hospital Erlangen, Erlangen, Germany

Correspondence: Heidi Stöhr, Institute of Human Genetics, University of Regensburg, Franz-Josef-Strauß-Allee 11, D-93053 Regensburg, Germany;
heidi.stoehr@klinik.uni-regensburg.de.

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Purpose. Stargardt disease (STGD1) is an autosomal recessive retinopathy, caused by mutations in the retina-specific ATP-binding cassette transporter (*ABCA4*) gene. To establish the mutational spectrum and to assess effects of selected deep intronic and common genetic variants on disease, we performed a comprehensive sequence analysis in a large cohort of German STGD1 patients.

Methods. DNA samples of 335 STGD1 patients were analyzed for *ABCA4* mutations in its 50 coding exons and adjacent intronic sequences by resequencing array technology or next generation sequencing (NGS). Parts of intron 30 and 36 were screened by Sanger chain-terminating dideoxynucleotide sequencing. An in vitro splicing assay was used to test selected variants for their splicing behavior. By logistic regression analysis we assessed the association of common *ABCA4* alleles while a multivariate logistic regression model calculated a genetic risk score (GRS).

Results. Our analysis identified 148 pathogenic or likely pathogenic mutations, of which 48 constitute so far unpublished *ABCA4*-associated disease alleles. Four rare deep intronic variants were found once in 472 alleles analyzed. In addition, we identified six risk-modulating common variants. Genetic risk score estimates suggest that defined common *ABCA4* variants influence disease risk in carriers of a single pathogenic *ABCA4* allele.

Conclusions. Our study adds to the mutational spectrum of the *ABCA4* gene. Moreover, in our cohort, deep intronic variants in intron 30 and 36 likely play no or only a minor role in disease pathology. Of note, our findings demonstrate a possible modifying effect of common sequence variants on *ABCA4*-associated disease.

Keywords: Stargardt disease, *ABCA4*, mutation screening, genetic risk score

Stargardt disease (STGD1; Mendelian Inheritance in Man [MIM] 248200) is the leading cause of Mendelian macular dystrophy that often manifests in the first two decades, but may also become symptomatic only later in life. It is typically characterized by bilateral progressive loss of central vision, color vision defects, and photophobia. The fundus often presents with yellow-white autofluorescent flecks (fundus flavimaculatus) and chorioretinal atrophy in the macula, typically with an initial foveal sparing and variable extension into the periphery.[1,2]

Autosomal recessive STGD1 is caused by mutations in the *ABCA4* gene.[3] Other retinal phenotypes, such as retinitis pigmentosa and cone–rod dystrophy, have also been linked to recessive mutations in this gene.[4,5] *ABCA4* encodes a member of the ATP-binding cassette (ABC) superfamily of transmembrane proteins specifically localized at the rims and incisures of outer segment discs of rod and cone photoreceptors.[6] In the retina, ABCA4 transports N-retinylidene-phosphatidylethanolamine from the lumen to the cytoplasmic leaflet of photoreceptor disc membranes, a process that together with chemical isomerization facilitates the removal of potentially toxic retinoid compounds.[7] Conversely, impaired ABCA4 function results in an accumulation of bisretinoids such as A2E in the RPE. This debris, also known as lipofuscin, is thought to disturb RPE metabolism and ultimately may cause cell death.[8] Current genotype–phenotype models correlate the degree of residual

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ABCA4 activity with the severity of the retinal disease phenotype.[5,9] In addition, toxic gain-of-function mutations were described in the *ABCA4* gene and were proposed to contribute to disease severity.[10]

To date, more than 900 different mutations including missense, splicing, truncating, and frameshift alterations have been reported in the *ABCA4* gene and shown to be associated with retinal degeneration (HGMD database, build 2015.4) (Qiagen, Hilden, Germany). Of these, 72% were identified in STGD1 patients. Additionally, 1120 rare nonsynonymous variants (frequency $\leq$ 0.005) were found in coding regions or highly conserved exon-flanking intronic sequences of the *ABCA4* gene by the Exome Aggregation Consortium (ExAC) (Cambridge, MA, USA; http://exac.broadinstitute.org; January 2016; in the public domain). These variants encompass 257 known and 863 potential novel disease-associated mutations. *ABCA4* mutations are scattered across all 50 coding exons of the gene, although representative studies from different geographic regions reveal the existence of ethnic founder mutations.[9,11–17]

Despite detection rates of *ABCA4* mutations greatly increasing over recent years by state-of-the-art analytical approaches such as next generation sequencing, homozygous or compound heterozygous mutations are regularly detected in no more than 70% of STGD1 patients, while a significant number of patients carry only a single *ABCA4* mutation (17–30%).[15,17–21] It has been debated whether some of the carriers of monoallelic *ABCA4* mutations could be combined into a subgroup of late-onset STGD1 or may present as age-related macular degeneration (AMD).[22,23] Recently, several deep intronic variants in the *ABCA4* gene were reported to segregate with disease and in some cases to affect correct splicing. It was suggested that these variants represent a novel and as yet rather unexplored type of pathogenic *ABCA4* mutations.[20,21,24–26]

To further explore the genetic architecture of ABCA4 pathology in STGD1, we analyzed the *ABCA4* gene in a large German cohort of 335 STGD1 patients referred to our molecular diagnostics unit at the Institute of Human Genetics, Regensburg, between 2008 and 2015. The molecular analysis included sequencing of the 50 coding exons/conserved splice sites of the *ABCA4* gene as well as two regions in intron 30 and 36 reported earlier to harbor deep intronic disease-associated variants. Moreover, functional studies were performed to assess pathogenicity of selected *ABCA4* variants. Finally, a multivariate logistic regression model was used to investigate the role of common *ABCA4* variants with allele frequencies $>$ 0.01.

Materials and Methods

Patients

Sequence analysis included a total of 335 patients (191 female, 144 male) with definite or suspected clinical diagnosis of STGD1 who underwent routine genetic testing at the Institute of Human Genetics Regensburg. Referrals between 2008 and 2015 were included in the study. A positive family history of STGD1 was reported for 35 cases; 131 individuals had a negative family history, and no further information on the familial status of retinal degeneration was available for 169 patients. Information on ethnicity was mainly provided by the patients themselves or was estimated from their first or last name. A total of 283 patients were Caucasian (80%), 30 patients were from the Middle East, 1 patient was from Asia, and for 21 patients ethnicity could not unanimously be determined. A control group of 779 probands free of retinal disease was recruited from the Lower Franconian region in Germany[27] and used to assess the role of common variants in STGD1 pathology. In accordance with the German Genetic Diagnostics Act, written informed consent was obtained from all subjects. This study adhered to the tenets of the Declaration of Helsinki.

Molecular Analysis

For DNA extractions, EDTA peripheral blood samples were obtained from all patients. Mutational analyses of the coding exons and the flanking intronic sequences [–12 bp/+6 bp covering the key donor ((C|A) A G G T (A|G) A G T) and acceptor (Y_{10} N (C|T) A G (G|A)) splice signals at the exon–intron boundaries[28]] of the *ABCA4* gene (NM_00350.2) were mainly performed (186 patients) by a custom-designed GeneChip CustomSeq Resequencing Array (RetChip) (Affymetrix, Santa Clara, CA, USA). Multiplex-PCR products were amplified by exon flanking oligonucleotide primer pairs (oligonucleotide sequences will be provided upon request). The data were analyzed with the Affymetrix GeneChip Analysis Software (GSEQ) and the SeqC module (JSI medical systems, Kippenheim, Germany) as previously described.[29] For 149 patients, mutation testing was performed by next generation sequencing (NGS) on an ION Torrent semiconductor personal sequencing machine (Life Technologies, Darmstadt, Germany) upon multiplex-PCR amplification of the fragments with the STARGARDT MASTR kit (Multiplicom, Niel, Belgium). Regions with a depth of sequence coverage below 30× were reanalyzed by Sanger chain-terminating dideoxynucleotide sequencing on an ABI 3130xl instrument (Applied Biosystems, Carlsbad, CA, USA). Next generation sequencing data were analyzed within the CLC Genomics Workbench (CLC bio, Aarhus, Denmark). Potentially disease-relevant variants identified by RetChip or NGS were routinely confirmed by Sanger sequencing. To test for deep intronic sequence variants in 236 patients, primer pairs ABCA4-Exon30.1 fwd (5′-TCT GAA GTT CCC ACA TAG TAC TTC C-3′)/rev (5′-GAT TGT GTT TGA GTG GCT AGG G-3′) and ABCA4-Exon36.1 fwd (5′-ACT ACC ACC TTC CTG ACA ACC AA-3′)/rev (5′-CTA CAT CTC TCT CCA TAG GCT CAG A-3′) were used to amplify and sequence a 607- and 535-bp fragment within *ABCA4* intervening sequences 30 and 36, respectively.

Disease-related variants were submitted to the ClinVar database (Acc. Nos. SCV000281879–SCV000281965, http://www.ncbi.nlm.nih.gov/clinvar/; in the public domain). All variants are described according to current nomenclature guidelines (http://www.hgvs.org/mutnomen; in the public domain).

Bioinformatic Analyses and Variant Classification

Variants were classified as recommended by the American College of Medical Genetics and Genomics (ACMG) standards and guidelines and the Association for Molecular Pathology (AMP) Clinical Practice Guidelines and Reports without discrimination between benign or likely benign and were based on population data, computational data, previous publications, and functional data. Variant frequency in non-Finnish Europeans was taken from the browser of the Exome Aggregation Consortium (ExAC). A variant was classified as benign if the minor allele frequency (MAF) was $\geq$0.005 except for variant c.2588G>C. Variants with MAF below 0.005 or with an unknown frequency were further tested for disease relevance by prediction algorithms of the programs MutationTaster (Mutation Taster, http://www.mutationtaster.org/; in the public domain) and PolyPhen-2 (PolyPhen2, http://genetics.bwh.harvard.edu/pph/; in the public domain). A sequence variant was classified as a variant of uncertain

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significance (vus) when none or only one of the two programs predicted a possible pathogenic effect. Exceptions to this rule included missense mutations well documented in the literature to be disease associated in STGD1 patients. Such variants were nonetheless classified as "likely pathogenic." When both programs, MutationTaster and PolyPhen-2, predicted a pathogenic effect, the variants were classified as "likely pathogenic." Stop and frameshift mutations as well as variants with experimental evidence of ABCA4 dysfunction were classified as pathogenic. In addition, rare variants were further analyzed for a potential effect on correct splicing by using the interface provided by the software package Alamut (Interactive Biosoftware, Rouen, France; Alamut Visual 2.7.1), which combines five algorithms (SpliceSiteFinder, MaxEntScan, NNSPLICE, GeneSplicer, and Human Splicing Finder). All tools provide prediction scores for the normal and the mutant allele. Variants were selected for further testing by in vitro minigene splicing assays when the score differences calculated by four prediction programs were >10%.

Genetic Risk Score Calculation

Genotyping of controls was performed on a HumanExome Bead Chip (Illumina, San Diego, CA, USA) by the International AMD Genomics consortium.[30] Association testing and genetic risk score (GRS) calculations were performed as described by Grassmann et al.[31] In brief, logistic regression analysis was used to assess the association of common alleles (MAF > 0.01) present in the coding regions of the *ABCA4* gene. Since variants rs1800717 and rs6666652 are highly correlated (pairwise $r^2 = 1$, based on the EUR panel in the 1000 Genomes Project, Build 20130502, http://www.internationalgenome.org/; in the public domain), only rs6666652 was used in the analysis. Variants with a MAF ≤ 0.01 and rare deep intronic variants were not included in further calculations. Initially, we determined the odds ratios (OR), 95% confidence intervals (95% CI), and *P* values for each common variant. We adjusted the resulting *P* values according to the false discovery rate (FDR)[32] and considered variants with an FDR < 0.05 to be statistically significant. A GRS was calculated from significantly associated variants for all controls and patients from a multivariate logistic regression model. This was done by multiplying the number of risk-modulating alleles (which can be either 0, 1, or 2) with the respective logarithmic odds ratio (log odds, computed from the multivariate model); then the sum of all weighted genotypes was calculated. Finally, a constant (−1.0503) was subtracted from the score to center the risk score distribution of the controls on zero.

Minigene Assay

Functional consequences of putative splicing variants were analyzed with the pSPL3-based exon trapping system.[33] Exons harboring spliceogenic variants and control exons were PCR amplified from genomic DNA using a 7:1 ratio of Taq:Pfu polymerase and oligonucleotide primer pairs flanked by restriction sites for *Eco*RI and *Bam*HI (Supplementary Table S1). The amplified products were cloned into the pSPL3b vector. HEK293 cells were transfected in duplicate with 3 µg construct DNA using *Trans*IT-LT1 Transfection Reagent (Mirus Bio, Madison, WI, USA) and harvested after 48 hours; total RNA was extracted using RNeasy Micro Kit (Qiagen). Reverse transcription–PCR was performed with the RevertAidH First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) and a pSPL3-specific SD6 (5′-TCT GAG TCA CCT GGA CAA CC-3′) and SA4 (5′-CAC CTG AGG AGT GAA TTG GTC G-3′) primer pair in combination with oligonucleotide primers for the housekeeping genes *ACTB* (forward 5′-GAC ATC CGC AAA GAC CTG TA-3′; reverse (5′-CAG GAG AGC AAT GAT CTT GA-3′) and *GUSB* (forward 5′-GAT CCA CCT CTG ATG TTC AC-3′; reverse (5′-TAT TCC CCA GCA CTC TCG TC-3′). Polymerase chain reaction products were separated by electrophoresis on a 2% agarose gel, excised, cloned, and sequenced. Fragment analysis was done by PCR amplification with fluorescein amidite (FAM)–labeled SD6 using Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific). Samples and a LIZ Size Standard (Applied Biosystems, Darmstadt, Germany) were run on an ABI 3130xl sequencer and analyzed with the Peak Scanner software (Applied Biosystems).

Results

Identification of *ABCA4* Mutations

Mutational analysis of the *ABCA4* gene was performed in 335 STGD1 patients and identified 189 unique sequence variations, of which 171 were listed in the non-Finnish European ExAC cohort as a rare variant with MAF ≤ 0.005. Of the 171 rare *ABCA4* variants, 148 were classified as pathogenic or likely pathogenic and 23 as vus (Supplementary Table S2). Twenty-eight percent (48/171) of the rare alleles constitute novel as yet unpublished variants (Table 1). Missense mutations represent 59.6% (102/171) followed by nonsense and frameshift mutations (15.8%; 27/171), splice site mutations (13.5%; 23/171) and synonymous variants (8.8%; 15/171). Of note, variant c.2588G>C is present with a MAF > 0.005 (0.0063; ExAC) but was classified as pathogenic based on its known deleterious effect on ABCA4 protein function.[9,34,35]

The most common pathogenic or likely pathogenic mutations in our cohort were c.5882G>A (51 of 396 mutant alleles), c.[3113C>T(;)1622T>C] (36/396), c.2588G>C (28/396), and c.5461-10T>C (23/396), which is consistent with our earlier report of frequent mutations in 144 German STGD1 patients.[12] Together, these sequence changes account for 34.8% (138/396) of disease-associated variants. Ninety-nine mutations were identified in only one patient (Supplementary Table S2).

Impact of Selected *ABCA4* Sequence Variants on RNA Splicing Efficiency

Twenty-eight sequence variants were considered to have a potential impact on splicing of the mature *ABCA4* transcript based on results from the bioinformatic analysis (Supplementary Table S2). Experimental evidence for splicing defects was previously published for five of these (c.768G>T, c.2588G>C, c.4253+5G>A, c.5461-10T>C, c.5714+5G>A) (Supplementary Table S2). Here, functional pSPL3-based minigene assays were performed for another 22 variants (Table 2; Supplementary Fig. S1). The analysis of cDNAs derived from transfected HEK293 cells revealed aberrant pre-mRNA splicing for all of the 13 variants affecting canonical GT-AG splice sequences and for c.4773+3A>G located in the conserved donor splice site of *ABCA4* exon 33. This includes three mutations changing the canonical GT to the weaker GC 5′ splice site, which is present in ~1% of authentic introns.[36] The absence of functional donor or acceptor splice sites led to exon skipping, intron retention, and use of cryptic splice sites (Table 2; Supplementary Fig. S1), likely leading to truncated protein products or nonsense-mediated mRNA decay (NMD).

Of the eight exonic sequence changes included in the minigene assay, three stop mutations and four missense mutations were predicted to create cryptic canonical splice sites, whereas the synonymous mutation c.3813G>A could

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Table 1. Novel Rare Sequence Variants in the *ABCA4* Gene

Position on Chr. 1, hg19	Exon	Variant, NM_000350.2	Protein	Classification
94578624	2	c.67-2A>G	p.(?)	p
94578623	2	c.67-1G>C	p.(I23Afs*24)	p
94578603	2	c.86T>G	p.(L29R)	lp
94577116	3	c.180G>C	p.(A60A)	vus
94577090	3	c.206G>A	p.(W69*)	p
94574258	4	c.317A>T	p.(Y106F)	vus
94546177	8	c.956T>G	p.(L319R)	vus
94546124	8	c.1009T>C	p.(F337L)	vus
94543445	11	c.1357-2A>G	p.(D453Gfs*14)	p
94528844	12	c.1584C>A	p.(Y528*)	p
94528814	12	c.1614C>T	p.(A538A)	vus
94528736	12	c.1692A>G	p.(P564P)	vus
94528709	12	c.1719G>A	p.(M573I)	lp
94528277	13	c.1793T>G	p.(V598G)	vus
94528179	13	c.1891G>A	p.(G631R)	lp
94528152	13	c.1918C>G	p.(P640A)	lp
94517233	17	c.2609C>T	p.(P870L)	lp
94510310	20	c.2919-10T>C	p.(?)	vus
94510279	20	c.2940G>C	p.(L980F)	vus
94508989	21	c.3093delA	p.(G1032Efs*52)	p
94502701	25	c.3813G>A	p.[(?, p.G1203Dfs*10)]	p
94502343	26	c.3815T>C	p.(I1272T)	lp
94497591	27	c.3871C>T	p.(Q1291*)	p
94497562	27	c.3900A>G	p.(R1300R)	vus
94496083	29	c.4254-1G>C	p.(?)	p
94495989	29	c.4347G>T	p.(W1449C)	lp
94495186	30	c.4354G>T	p.(E1452*)	p
94488974	32	c.4635C>T	p.(S1545S)	vus
94488969	32	c.4640delA	p.(K1547Rfs*34)	p
94486835	35	c.4979C>T	p.(P1660L)	lp
94485197	36	c.5137C>A	p.(Q1713K)	lp
94485181	36	c.5153T>G	p.(V1718G)	lp
94485145	36	c.5189G>A	p.(W1730*)	p
94480196	38	c.5363C>T	p.(P1788L)	lp
94476924	39	c.5478C>T	p.(N1826N)	vus
94476889	39	c.5513A>G	p.(H1838R)	lp
94476844	39	c.5558C>A	p.(A1853D)	lp
94476464	40	c.5606C>T	p.(P1869L)	lp
94476414	40	c.5656G>A	p.(G1886R)	lp
94476352	40	c.5714+4C>T	p.(?)	vus
94474314	41	c.5828T>C	p.(L1943P)	lp
94473286	43	c.5909T>C	p.(L1970P)	lp
94473253	43	c.5942C>G	p.(T1981R)	lp
94473222	43	c.5973G>C	p.(V1991V)	vus
94471067	44	c.6077T>C	p.(L2026P)	lp
94466664_94466663	46	c.6283-3_6283-2delinsAG	p.(D2095Gfs*2)	p
94466618	46	c.6326T>C	p.(L2109P)	lp
94463433	48	c.6713A>G	p.(Q2238R)	lp

lp, likely pathogenic; p, pathogenic.

weaken the donor splice site of exon 25 (Table 2). Four exonic variants revealed both correct and aberrant splice products in various proportions. Synonymous mutation c.3813G>A and missense mutations c.4469G>A, c.4919G>A, and c.6647C>T led to incorrectly spliced transcripts that match the predictions from the bioinformatic analysis (Table 2; Supplementary Fig. S1). Hence, these four variants initially classified as likely pathogenic or vus were reclassified as being pathogenic (Supplementary Table S2). Sequence variants c.122G>A/p.(W41*), c.179C>T/p.(A60V), c.3808G>T/p.(E1270*), and c.5189G>A/p.(W1730*) revealed no effect on mRNA splicing in the in vitro assay.

Disease-Associated ABCA4 Alleles in 335 STGD Patients

Two or more likely pathogenic or pathogenic changes in the *ABCA4* gene were found in 151 of the 335 STGD1 patients. In another seven patients a deleterious mutation was detected in addition to a vus, eventually raising the overall detection rate of two expected mutations to 47.2% (Supplementary Table S3). This includes 14 cases with null mutations: c.[2692G>T(;)5461-10T>C(;)5606C>T], c.[5018+2T>C(;)6386+2C>G], c.[296dupA];[5018+2T>C], c.[3813G>A(;)3813G>A], c.[4234C>T(;)5714+5G>A],

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Table 2. Functional Analysis of Putative Splice Mutations in the *ABCA4* Gene

Exon	Variant, NM_000350.2	Predicted Effect*	Protein	Major Missplicing†	Predicted Protein of Major Misspliced Transcript
Variants in canonical and conserved splice sites					
2	c.67-1G>C	Loss of AS	NA	Skipping of exon 2	p.(I23Afs*24)
2	c.160+2T>C	Loss of DS	NA	Retention of intron 2	p.(?)
9	c.1239+1G>C	Loss of DS	NA	Skipping of exon 9	p.(T367Sfs*6)
11	c.1357-2A>G	Loss of AS, Cryptic AS	NA	Cryptic AS in intron 10	p.(D453Gfs*14)
13	c.1937+1G>A	Loss of DS	NA	Cryptic DS in exon 13 and intron 13	p.[(Y603_S646del, F647*)]
14	c.2160+1G>T	Loss of DS	NA	Retention of intron 14	p.(?)
29	c.4352+1G>A	Loss of DS	NA	Skipping of exon 29	p.(S1418_P1451delinsR)
33	c.4773+3A>G	Weakens DS	NA	Skipping of exon 33 and 34	p.(Y1557Cfs*45)
35	c.5018+2T>C	Loss of DS	NA	Retention of intron 35, skipping of exon 35	p.[(?, V1617Afs*113)]
36	c.5196+1G>A	Loss of DS	NA	Retention of intron 36	p.(?)
36	c.5196+2T>C	Loss of DS	NA	Retention of intron 36	p.(?)
37	c.5312+1G>A	Loss of DS	NA	Skipping of exon 37	p.(N1734Gfs*14)
46	c.6283-3_6283-2delinsAG	Loss of AS, Cryptic AS	NA	Cryptic AS in intron 45	p.(D2095Gfs*2)
46	c.6386+2C>G	Loss of DS	NA	Cryptic DS in exon 46	p.(V2114Hfs*5)
Exonic variants					
2	c.122G>A	Cryptic AS	p.(W41*)	None	–
3	c.179C>T	Cryptic DS	p.(A60V)	None	–
25	c.3808G>T	Cryptic DS	p.(E1270*)	None	–
25	c.3813G>A	Weakens DS	p.(E1271E)	Retention of intron 25	p.(?)
30	c.4469G>A	Cryptic DS	p.(C1490Y)	Cryptic DS in exon 30	p.(C1490Efs*12)
35	c.4919G>A	Cryptic AS	p.(R1640Q)	Cryptic AS in exon 35	p.(V1617_R1640del)
36	c.5189G>A	Cryptic AS	p.(W1730*)	None	–
48	c.6647C>T	Cryptic DS	p.(A2216V)	Cryptic DS in exon 48	p.(A2216_Q2243del)

AS, conserved acceptor site; DS, conserved donor site.
* Alamut Software: SpliceSiteFinder-like, MaxEntScan, NNSPLICE, GeneSplicer, Human Splicing Finder.
† Misspliced transcripts found in control and mutant constructs are not listed.

c.[4773+3A>G(;)5461-10T>C], c.[5461-10T>C];[5461-10T>C], c.[67-1G>C];[67-1G>C], c.[1A>G; 6089G>A];[5714+5G>A], c.[5714+5G>A(;)5714+5G>A], c.[5917delG];[5917delG], and c.[5714+5G>A];[5917delG]. The latter two combinations were found in two patients (ID 86 and ID 255), and an additional 58 cases were carrying a null and a missense mutation (Supplementary Table S3). Compound heterozygosity or homozygosity could be confirmed by segregation analysis in 27 patients. One pathogenic or likely pathogenic mutation was identified in 82 individuals, one vus in 13 cases resulting in a detection rate of 28.4% for carriers of a single *ABCA4* mutation (Supplementary Table S3). Complex alleles verified by testing the parent(s) of the respective patients were c.[1A>G; 6089G>A] in patient 219, c.[1609C>T; 5881G>A] in patient 314, c.[1622T>C; 3113C>T] in patients 1, 13, 106, 124, 273, 282, and 311, c.[2588G>C; 3289A>T] in patient 75, and c.[2588G>C; 5153T>G] and c.[5318C>T; 6148G>C] together in patient 69. In a total of 89 patients (26.6%) no disease-associated variant was found in the *ABCA4* gene.

Frequency of Deep Intronic Variants in Putative Alternative Exons of the *ABCA4* Gene

To investigate the frequency of deep intronic variants in the *ABCA4* locus in our patient cohort, we sequenced genomic regions encompassing putative alternative exons in intron 30 and 36, namely, exon 30.1, exon 36.01, and exon 36.1. These regions were previously identified by RNA sequencing of retinal tissue (Supplementary Fig. S2).[24,37,38] Ten different sequence variants were found in 236 patients, of which four (c.4539+2028C>T, c.4540-2036C>A, c.5196+1013A>G, c.5196+1056A>G) were defined as rare variants with a MAF $\leq$ 0.005 in the 1000 Genomes catalog, phase 3 (Table 3; Supplementary Fig. S2). These changes were each identified once in the 472 alleles analyzed. Among these, c.4539+2028C>T and c.5196+1056A>G have been described previously by Braun et al. (2013)[24] as V5 and V3, respectively, and were later also found in another cohort of STGD1 patients.[20] Deep intronic variants c.4540-2036C>A and c.5196+1013A>G are reported for the first time. The c.5196+1056A>G (V3) variant is predicted to strengthen a cryptic donor splice site. Reverse transcriptase–PCR analysis using keratinocyte mRNA of patients carrying the V3 variant revealed the inclusion of a 177-bp segment into the *ABCA4* transcript (termed "alternative exon 36.01"), thus providing experimental evidence for a loss-of-function effect of this variation.[24] Patient 230 in our cohort carried the V3 variant and a second variant (c.2588G>C) annotated as pathogenic. This suggests that the V3 mutation is indeed the second disease-associated allele in our patient. A novel sequence change, c.5196+1013A>G, was found in patient 79 in combination with c.1654G>A, a likely pathogenic missense mutation [p.(V552I)]. Similar to the V3 variant, c.5196+1013A>G is predicted to strengthen a cryptic donor splice site and thus may also result in deleterious effects upon splicing. Based on bioinformatics, variants c.4539+2028C>T (V5) and c.4540-2036C>A are not predicted to affect splicing. The c.4539+2028C>T (V5) sequence change was identified in patient 42 with no other probable mutant *ABCA4* allele; variant c.4540-2036C>A was detected in patient 159 in addition to pathogenic mutation c.[1622T>C(;)3113C>T] and likely pathogenic mutation c.3261A>C. A disease associ-

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Table 3. Deep Intronic Variants in the *ABCA4* Gene

Position on Chr. 1, hg19	Variant, NM_000350.2	Predicted Effect*	MAF, 236 STGD1 Patients	MAF, Controls†	Reference
Exon 30.1					
94493231	c.4539+1770C>A	–	0.055	0.061	–
94493000	c.4539+2001G>A	–	0	–	20, 24–26
94492973	c.4539+2028C>T	–	0.002	–	20, 24
94492937	c.[4539+2064C>T; 5461-1389C>A]	–	0	–	20
94492773	c.4540-2169A>G	–	0.432	0.431	–
94492681	c.4540-2077C>T	–	0.002	0.008	–
94492640	c.4540-2036C>A	–	0.002	–	–
Exon 36.01/36.1					
94484239	c.5196+899C>T	–	0.032	0.023	–
94484125	c.5196+1013A>G	Strengthens cryptic DS	0.002	–	–
94484123	c.5196+1015A>G	–	0.053	0.058	–
94484082	c.5196+1056A>G	Strengthens cryptic DS	0.002	–	20, 24
94484060	c.5196+1078delA	–	0.021	0.023	–
94484002	c.5196+1136C>A	Novel cryptic AS	0	0	25
94484001	c.5196+1137G>A	Strengthens cryptic AS	0	–	20, 21, 24–26
94483979	c.5196+1159G>A	Novel cryptic AS	0	0.002	25
94483922	c.5196+1216C>A	Strengthens cryptic DS	0	–	24

AS, conserved acceptor site; DS, conserved donor site.
* Alamut Visual 2.7.1.
† 1000 Genomes Project.

ation of these intronic variants in our patients is thus unclear. Six previously reported intronic sequence changes (c.4539+2001G>A, c.4539+2064C>T, c.5196+1136C>A, c.5196+1137G>A, c.5196+1159G>A, c.5196+1216C>A) were not found in our patient cohort (Table 3).

The Contribution of Common *ABCA4* Variants to STGD1

We noticed a striking difference in the frequencies of common *ABCA4* variants (MAF $\geq$ 0.01) in the patient versus the control group (Supplementary Table S2; Fig. 1). This led us to investigate whether such variants may exert an effect on the risk for developing STGD1. Genotype information on 16 common *ABCA4* variants was available for the 335 patients and 781 German controls from Lower Franconia. For each variant, risk association was computed applying a logistic regression model. We found statistically significant association for six variations (c.1268A>G, c.4203C>A, c.5603A>T, c.5682G>C, c.5843C>T, c.6249C>T) (FDR $<$ 0.05, Supplementary Table S4). To estimate the independence of the associated variants, we fit a multivariate logistic regression model incorporating these variations. In the multivariate model, association of variant c.4203C>A (rs1801666) with disease risk was no longer statistically significant, probably due to linkage disequilibrium with the strongly associated variant c.5603A>T (rs1801466, $r^2 = 0.38$). The remaining five risk-modulating variants were found to be independently associated with disease risk (Table 4). Repeating the analysis excluding the data from 52 samples of non-European or unknown ancestry, similar results were obtained, including the significant and independent association of the five risk-modulating variants (c.1268A>G, c.5603A>T, c.5682G>C, c.5843C>T, c.6249C>T) with similar effect sizes.

Assuming that common pathogenic mutations emerged on common haplotypes tagged by associated variants, our analysis may measure their impact on disease risk rather than that of the common associated variant. We therefore evaluated the correlation between common mutations in our cohort (c.1622T>C, c.2588G>C, c.3113C>T, c.4234C>T, c.5461-10T>C, c.5714+5G>A, c.5882G>A, c.5917delG) and the five risk-modulating variants (c.1268A>G, c.5603A>T, c.5682G>C, c.5843C>T, c.6249C>T). Three frequent mutations (c.2588G>C, c.5461-10T>C, c.5882G>A) were found to be potentially correlated ($R^2 > 0.05$) with common STGD1-associated variants. Subsequently, the association analysis was repeated by excluding individuals carrying any of three correlated pathogenic mutations c.5882G>A, c.2588G>C, and c.5461-10T>C from the analysis. Still, STGD1 association of common variants c.1268A>G and c.5603A>T remained highly significant (*P* values of 3.45E-05 and 4.82E-05, respec-

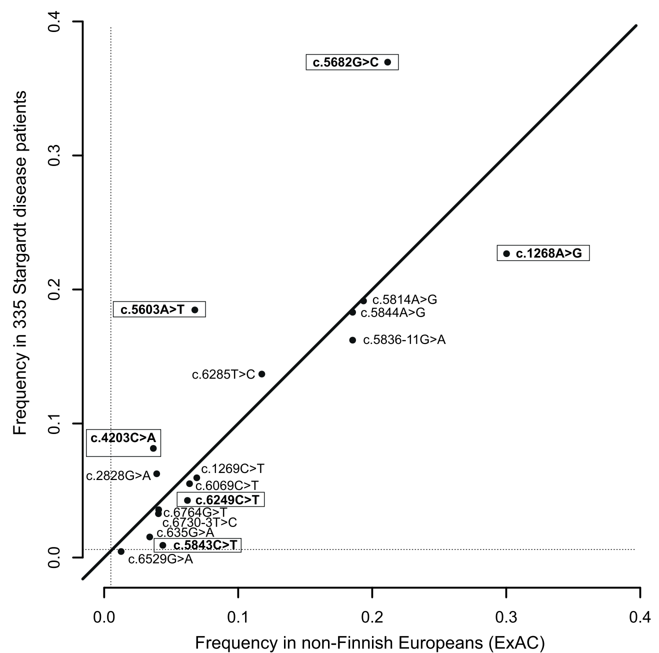

Figure 1. Frequency of common *ABCA4* variants in patients with Stargardt disease and European non-Finnish controls. Six variants that were found to be significantly enriched in either patients or controls are *framed*.

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Table 4. Association of Common *ABCA4* Variants With Stargardt Disease

Variant	Effect of Variant	SNP ID, dbSNP	MAF		Reference Allele	Effect Allele	Univariate LR				Multiple LR	
			Controls, $n = 779$	Cases, $n = 335$			Odds Ratio, Univariate	95% CI	*P* Value	*Q* Value	Odds Ratio, Multivariate	95% CI
Adverse												
c.5603A>T	p.(N1868I)	rs1801466	0.074	0.185	A	T	2.69	2.05–3.54	1.28E-12	2.04E-11	1.97	1.34–2.91
c.5682G>C	p.(L1894L)	rs1801574	0.261	0.370	G	C	1.64	1.35–1.99	5.59E-07	4.47E-06	1.40	1.08–1.80
Protective												
c.1268A>G	p.(H423R)	rs3112831	0.300	0.227	A	G	0.67	0.54–0.83	3.06E-04	1.05E-03	0.67	0.53–0.84
c.5843C>T	p.(P1948L)	rs56142141	0.040	0.009	C	T	0.21	0.08–0.46	3.29E-04	1.05E-03	0.17	0.06–0.38
c.6249C>T	p.(I2083I)	rs1801359	0.067	0.049	C	T	0.59	0.38–0.90	1.75E-02	4.67E-02	0.65	0.41–1.00

LR, logistic regression; dbSNP, The Single Nucleotide Polymorphism Database (https://www.ncbi.nlm.nih.gov/projects/SNP, in the public domain).

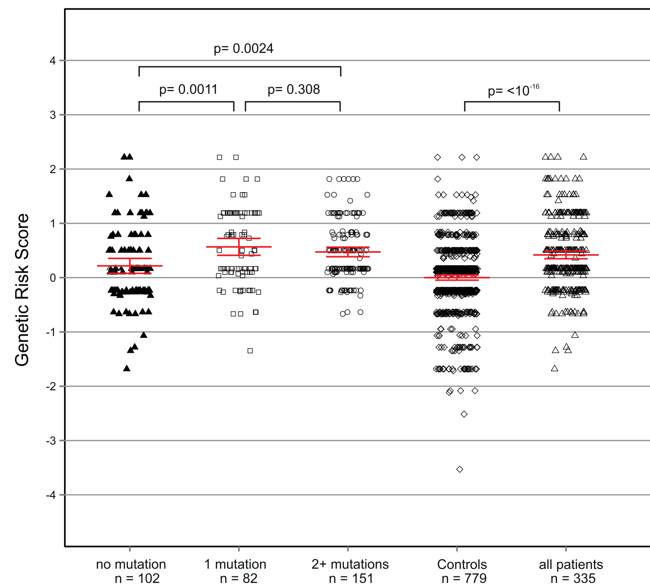

Figure 2. Genetic risk score (GRS) for patients and controls stratified according to the number of *ABCA4* mutations or disease status. An alpha estimate of –1.0503 was used to center the GRS on zero for the controls. *Red bars* represent the mean GRS and confidence intervals.

tively). Similarly, removing patients carrying one of the eight most common STGD1 mutations (c.5882G>A, c.3113C>T, c.1622T>C, c.2588G>C, c.5461-10T>C, c.5714+5G>A, c.4234C>T, or c.5917delG) from the analysis resulted in a statistically significant association for the common variants c.5603A>T and c.1268A>G (*P* values of 0.000273 and 0.0109, respectively) (Supplementary Table S5). For variant c.6249C>T, the association was no longer significant after removal of patients carrying common mutations, although the variant still revealed a similar effect size. The latter finding suggests that loss of significance is likely due to lack of statistical power caused by the reduced number of individuals in the subsequent analyses (from $n = 335$ to $n = 247$ to $n = 197$ patients, Supplementary Table S5). In contrast, variant c.5682G>C showed a reduced effect size and also a lack of statistically significant association in both subsequent analyses.

To further evaluate the role of these common variants in retinal pathology, a GRS was calculated for the STGD1 patients in our cohort and the 781 control individuals. This was computed as the sum of risk-increasing and protective alleles weighted by the relative effect size of each of the risk-modulating variants from the multivariate logistic regression model. An alpha estimate of –1.0503 was used to center the GRS on zero for the controls. Patients diagnosed with STGD1 have a significantly higher mean GRS (0.418; *P* value $< 10^{-16}$, 95% CI: 0.3479–0.4887) compared to controls (0.0, 95% CI: –0.0466 to 0.0466) (Fig. 2). Importantly, patients with one, two, or more disease-associated *ABCA4* mutations have a significantly higher GRS than those with none ($P = 0.0011$ and 0.0024; respectively) (Fig. 2), implying a risk-increasing effect of common *ABCA4* alleles when combined with a single defective copy of the *ABCA4* gene.

Discussion

Almost 20 years after the identification of *ABCA4* as the causative gene for autosomal recessive STGD1,[3] the genetics of this common form of hereditary macular degeneration still have not fully been clarified. Our study describes the findings

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of mutational profiling of the *ABCA4* gene in the largest cohort of STGD1 patients published so far. A total of 171 unique rare sequence variants, 48 of which constitute novel, so far unreported, mutations, were identified in the coding region, and an additional four rare deep intronic variants were detected in intron 30 and 36 of the *ABCA4* gene. In 47% of patients, two causative *ABCA4* mutations were found. This is at the lower end of the mutation detection rate in STGD1 patients, with other studies identifying biallelic mutations between 55% and 80%,[17,19,21,39,40] but may be explained by a combination of factors. First, clinical heterogeneity and variable diagnostic criteria inherent to multiclinic retrospective cohort studies may have led to uncertainties in clinical diagnosis in some patients of our cohort. In addition, small deletions/insertions may have been missed due to limitations in the detection accuracy of this type of mutation by the resequencing array and ION Torrent semiconductor technology. Finally, large rearrangements within the *ABCA4* locus that have been shown to occur in 0.3% to 2.2% of the analyzed chromosomes in previous reports[9,26,41] would not have been detected by the mutational screening in this study.

An important question relates to the high proportion of carriers of single *ABCA4* mutations consistently observed in numerous studies regardless of the ethnic background of the patients.[14,15,18,19,21,39,40,42] A single mutant *ABCA4* allele was found in almost 30% of the 335 individuals in our patient group, a number representing the upper end of the reported ranges. Mutations in noncoding regions of the *ABCA4* gene locus have been proposed as a common source for a second causative mutation. More specifically, several studies have described the occurrence of deep intronic variants in intron 30 and 36 in a significant proportion of patients (7.9–17.9%) with single *ABCA4* mutations.[20,21,24–26] Indeed, there is some experimental evidence that a few of these variants activate/strengthen cryptic splice sites leading to the inclusion of alternative exons in the mature transcript.[24] In our cohort, of 95 STGD patients with a single pathogenic/likely pathogenic *ABCA4* mutation, only 2 were found to carry an additional rare intronic variant in intron 30 or 36 (2.1%). Two other novel rare intronic variants were detected in a patient with two causative *ABCA4* mutations and a patient with no *ABCA4* mutations, respectively. From these data, we conclude that causative *ABCA4* mutations in intron 30 and 36 may contribute to STGD1 pathogenesis only in a minority of cases. Similarly, in a small German cohort of 46 patients with single heterozygous *ABCA4* variants, no mutations were found in these intronic regions.[25]

To further add to the discussion on the impact of deep intronic variants to STGD1 disease, the full sequence analysis of the entire *ABCA4* locus including exonic as well as intronic sequences will be needed independent of the patient's mutation status. It was estimated that approximately 95% of multiexon transcripts undergo alternative splicing.[43] While the key components and mechanisms involved in mRNA processing are reasonably well understood, high-confidence predictions on splicing effects of defined sequence changes remain inaccurate. In this context, it is of note that the extent and variability of alternative splicing in the human retina were found to be remarkable,[37] although the functional consequences of the alternative transcripts remain obscure. Approximately 13% of novel mRNA junctions were expressed in the retina at levels similar to or higher than the reference transcript. Deep RNA sequencing of three healthy human retinas revealed at least 11 alternatively spliced exons and 71 alternative exon/intron junctions,[37] implying the possibility of additional deep intronic regions as candidates to potentially harbor mutations affecting the correct splicing of the *ABCA4* transcript. Considering that 7.6% of STGD1 patients of a Belgian cohort carried a founder deep intronic c.4539+2001G>A mutation,[25] it is conceivable that a significant fraction of the German STGD1 patients carry as yet unknown mutations in the noncoding regions of the *ABCA4* gene.

To address the strikingly high frequency of carriers with only a single identified *ABCA4* disease allele, we assessed the contribution of common *ABCA4* variants on disease development. To this end, our findings reveal that variants c.1268A>G (OR 0.6682; CI 0.531–0.836), c.5843C>T (OR 0.1678; 0.063–0.377), and c.6249C>T (OR 0.6483; CI 0.407–1.001) exert a protective effect, while variants c.5603A>T (OR 1.9652; CI 1.335–2.909) and c.5682G>C (OR 1.3973; CI 1.082–1.800) confer an increased risk for developing STGD1. These results are in agreement with and extend previous studies that identified c.5603A>T as a risk-increasing allele[44] and c.1268A>G as a protective variant.[40] Our data now facilitate a GRS estimate that coalesces the total effect of protective and risk-increasing alleles similarly to the model developed to assess the genetic risk for AMD.[31] Interestingly, the GRS in the group of patients with only a single disease-associated *ABCA4* mutation is significantly higher than in patients with no *ABCA4* mutation, suggesting that common variants are involved in conferring risk for STGD1.

Simultaneous loss of significant association with STGD1 and effect size was observed for common variant c.5682G>C when the three most frequent pathogenic mutations were excluded from the analysis. This could be due to linkage disequilibrium between certain pathogenic mutations and c.5682G>C, implying that the common *ABCA4* allele either has no effect on the pathogenicity of the rare variant or adds to the damaging effect of the mutation. Furthermore, c.5682G>C may act as a risk-increasing allele only in *trans* when combined with specific pathogenic mutations. The assumption that the presence of a common genetic variant with an adverse effect on ABCA4 function actually triggers the deleterious consequences of the pathogenic mutation could explain why some of the common mutations have a frequency of >1% in certain populations but do probably not cause STGD1 in the respective carriers. For example, the allele frequency of pathogenic mutation c.5882G>A/p.(G1961E) is 1.4% in the South Asian population (ExAC) and even higher in Somalia,[45] although a higher prevalence of STGD1 in these countries has not been reported. Alternatively, a protective *ABCA4* allele may ameliorate a pathogenic mutation in *trans*, although only in combination with a less severe *ABCA4* allele. In this context, it should be noted that protective variant c.6249C>T is five times more frequent in the African than in the European or Asian populations.

The GRS model may also explain another discrepant aspect in our patient cohort. Fourteen STGD1 patients in our study each harbor two null mutations in the *ABCA4* gene. Compound heterozygosity/homozygosity could be confirmed in seven of these patients. In accordance with the hypothesis by Maugeri et al.,[9] the presence of the severe mutations in our patients would strongly suggest a cone–rod dystrophy or an atypical retinitis pigmentosa phenotype, thus reflecting the greatly reduced or even absent functional activity of ABCA4. A review of the clinical data, however, verified that all 14 patients presented with typical symptoms of STGD1 disease with age of onset ranging between 4 and 60 years. More precisely, eight patients presented with initial symptoms at the age of 11 or younger; two patients were under 20; and the remaining four patients were 30 to 60 years old when they first noticed vision problems. In another report, one-third of the nontruncating *ABCA4* alleles were found to cause more severe disease than premature truncations.[10] Thus, specific common *ABCA4* variants may alleviate deleterious mutations leading to asymptomatic, mild, or less severe disease and vice versa. Taken

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together, future studies aiming at investigation of the influence of common variations and rare mutations on disease risk are necessary, ideally in a prospective cohort setting.

In summary, despite certain shortcomings in terms of possible uncertainties in clinical diagnosis and the paucity of detecting large copy number variations (CNVs) or mutations in noncoding regions, the present study further broadens our knowledge about the genetic basis of STGD1 disease. First, we expanded the mutational spectrum of STGD1 by identifying an additional 48 novel pathogenic/likely pathogenic gene variants in *ABCA4*. Our findings also add to the clarification of the role of deep intronic variants in putative alternative exons within intron 30 and 36, which seem to represent only a minor proportion of disease-associated variants. Finally, an in-depth analysis of common variants at the *ABCA4* gene locus revealed risk-altering properties for several common variants, particularly in those patients who carry only a single disease-associated *ABCA4* allele. Furthermore, GRS data can be used to ascertain the risk of a patient with a single *ABCA4* mutation and may further refine our understanding of *ABCA4*-associated retinal pathology.

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