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The 2588G→C Mutation in the *ABCR* Gene Is a Mild Frequent Founder Mutation in the Western European Population and Allows the Classification of *ABCR* Mutations in Patients with Stargardt Disease

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Summary

In 40 western European patients with Stargardt disease (STGD), we found 19 novel mutations in the retina-specific ATP-binding cassette transporter (*ABCR*) gene, illustrating STGD's high allelic heterogeneity. One mutation, 2588G→C, identified in 15 (37.5%) patients, shows linkage disequilibrium with a rare polymorphism (2828G→A) in exon 19, suggesting a founder effect. The guanine at position 2588 is part of the 3′ splice site of exon 17. Analysis of the lymphoblastoid cell mRNA of two STGD patients with the 2588G→C mutation shows that the resulting mutant ABCR proteins either lack Gly863 or contain the missense mutation Gly863Ala. We hypothesize that the 2588G→C alteration is a mild mutation that causes STGD only in combination with a severe *ABCR* mutation. This is supported in that the accompanying *ABCR* mutations in at least five of eight STGD patients are null (severe) and that a combination of two mild mutations has not been observed among 68 STGD patients. The 2588G→C mutation is present in 1 of every 35 western Europeans, a rate higher than that of the most frequent severe autosomal recessive mutation, the cystic fibrosis conductance regulator gene mutation ΔPhe508. Given an STGD incidence of 1/10,000, homozygosity for the 2588G→C mutation or compound heterozygosity for this and other mild *ABCR* mutations probably does not result in an STGD phenotype.

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Introduction

Stargardt disease (STGD; MIM 248200) is the most common autosomal recessive macular dystrophy, with an estimated incidence of 1/10,000 (Blacharski 1988). STGD is characterized by onset in the first or second decade of life, decreased central vision, progressive bilateral atrophy of the retinal pigment epithelium, and the appearance of orange-yellow flecks distributed in the posterior pole, sometimes extending beyond the vascular arcade. A milder form of the same disorder, fundus flavimaculatus (FFM), is generally characterized by a later age at onset, slower progression, and more-widespread distribution of the flecks (Stargardt 1909; Franceschetti 1963; Noble and Carr 1979). Linkage studies suggest genetic homogeneity for the recessive forms of these diseases. The STGD/FFM locus was first mapped to 1p22–p21 (Kaplan et al. 1993; Gerber et al. 1995), and subsequently the critical area was refined to a 2-cM interval between D1S406 and D1S236 (Anderson et al. 1995; Hoyng et al. 1996; Weber et al. 1996). In this region, a gene encoding a retina-specific ATP-binding cassette transporter protein (ABCR) was mapped, and it was found to be involved in STGD/FFM (Allikmets et al. 1997*b*). *ABCR* is a large gene consisting of 50 exons (Allikmets et al. 1997*b;* Azarian et al. 1998; Gerber et al. 1998).

Heterozygous mutations in the *ABCR* gene were described in 16% of patients with age-related macular degeneration (AMD; MIM 153800), especially the "dry" form of AMD (Allikmets et al. 1997*a*), indicating that *ABCR* mutations could be a risk factor for this common disease. Dryja et al. (1998) recently discussed the methodology and statistical interpretation of the latter study. Thirteen *ABCR* variants were identified in patients with AMD but were not found, or were found in a lower number, in racially matched controls. This difference, however, was statistically significant only for

Asp2177Asn and Gly1961Glu. Because of the enormous allelic heterogeneity of the *ABCR* gene, statistical significance for most mutations can be obtained only after study of a much larger group of patients and controls than studied by Allikmets et al. (1997*a*). On the basis of the estimated incidence of AMD in the general population (30%) and the identification of *ABCR* mutations in 16% of patients with AMD, heterozygous *ABCR* mutations would be predicted to occur in 4.8% of the control group. If these mutations, when present in both copies of the *ABCR* gene, invariably cause STGD, the incidence of STGD would be six times the observed frequency.

We and others have also implicated the *ABCR* gene in retinitis pigmentosa (RP) and cone-rod dystrophy (CRD), both of which are considered more severe phenotypes than STGD, and have proposed a model that explains how different combinations of mutations can cause distinct chorioretinal disorders (Martinez-Mir et al. 1997, 1998; Rozet et al. 1997; Cremers et al. 1998; van Driel et al. 1998). According to this model, the phenotype is determined by the residual activity of the ABCR protein. The most severe phenotype, atypical RP, is caused by the presence of two *ABCR* null alleles; CRD, by a combination of a null allele and a moderately severe mutation; and STGD, by compound heterozygosity of a null mutation and a mild mutation or two moderately severe mutations. In fact, in a consanguineous family with individuals affected either by RP or by CRD, we found homozygosity for a splice-site mutation (IVS30+1G→T) in patients with RP, whereas patients with CRD had compound heterozygosity for the IVS30+1G→T and IVS40+5G→A mutations (Cremers et al. 1998). The same IVS30+1G→T mutation was identified in STGD patient 7560, who had compound heterozygosity for this mutation and a 2588G→C mutation. On the basis of this finding, we deduced that the less severe CRD and STGD phenotypes in these patients result from the moderate or mild effects on ABCR activity of the accompanying mutation—that is, IVS40+5G→A in CRD and 2588G→C in STGD—compared with the severe effects in RP of IVS30+1G→T, which we consider to be a genuine null allele (Cremers et al. 1998; van Driel et al. 1998). The finding of a homozygous null mutation (1847delA) in a consanguineous family with RP was also reported by Martinez-Mir et al. (1998).

Here, we report on a comprehensive mutation screening of the *ABCR* gene in 40 unrelated western European patients with STGD. Nineteen new mutations were found, including missense, nonsense, and splice-site mutations as well as deletions. The 2588G→C mutation in exon 17 was observed in more than one-third of all patients with STGD. We propose that this mutation is a frequent mild founder mutation in the western European population, which allows us to classify accompanying *ABCR* mutations as severe mutations and to discuss implications for recurrence risk calculations and genotype-phenotype correlations.

Material and Methods

Patients with STGD

Forty patients with STGD—29 from The Netherlands, 11 from Germany, and 5 from Sweden—were included in the mutation analysis. The presence of the founder mutation 2588G→C and the polymorphic variant 2828G→A was investigated in a larger group of 68 patients with STGD, mainly from The Netherlands (n = 47) and from Germany (n = 16). Control individuals were from The Netherlands.

The family history of, and ophthalmologic information on, the patients with STGD were reviewed by four experienced ophthalmologists in three different centers, the Department of Ophthalmology, Nijmegen (C.B.H.), The Netherlands Ophthalmic Research Institute (N.T.), and the Augenklinik, Heidelberg (K.R. and A.B.). For the purpose of the study, the essential features of STGD were the following: (1) a retinal disorder in families with more than one affected individual, compatible with autosomal recessive inheritance; (2) onset of symptoms before the age of 30 years; (3) bilateral central vision loss with "beaten-metal" foveal changes and/or yellow pigment epithelium deposits ("flecks") in the posterior pole of the eye; (4) normal caliber of the retinal vessels and no pigmented bone-spicules in the retinal periphery; (5) normal scotopic electroretinogram; and (6) typical "dark choroid" in fluorescein angiography. Four STGD patients (numbers 7550, 8189, 8270, and 8272) showed a relatively large number of flecks and therefore might be considered to have the FFM type of STGD. Some peripheral involvement was seen in three STGD patients (numbers 8228, 8257, and 8434). We have not listed the age at onset of the patients with STGD, which varies from 10 to 30 years, because we observed a large intrafamilial variability.

All human studies were reviewed by the Nijmegen Medical Ethics Commission. All individuals gave their informed consent prior to inclusion in the study. Some of the patient data included in this study have been analyzed previously, either as part of an STGD linkage study (Hoyng et al. 1996) or in a study describing the molecular genetic analysis of a family with RP/CRD (Cremers et al. 1998). Patient 8414 belongs to family ST1, patient 7552 to family ST6, and patient 7569 to family ST5 (Hoyng et al. 1996). The mutation analyses of STGD patients 7560 and 8439 are described elsewhere (Cremers et al. 1998).

SSCP and Sequence Analysis of the ABCR *Gene*

The oligonucleotides employed for SSCP and direct sequencing of exons 4, 7, 9, 10, 15–21, and 23–50 are described elsewhere (Gerber et al. 1998). The sequences of the primers used to amplify the remaining exons are given in table 1. DNA amplifications were performed in 96-well trays in a thermocycler (MJ Research). SSCP was performed by use of a 38:2 acrylamide:bisacrylamide gel, with or without 10% (w/v) glycerol, at 4°C. Nucleotide sequence analysis was performed with a dye terminator sequencing kit on an automated sequencer (Applied Biosystems 373), as described elsewhere (de Kok et al. 1995).

Southern Blot Analysis

Southern blot analysis was performed as described elsewhere (Huber et al. 1994). To show intragenic deletions, we hybridized pools of genomic DNA fragments spanning exons 1–4, 15–25, 27, 33–36, 43–46, and 48 and analyzed *Eco*RI-digested genomic DNA of patients with STGD.

Allele-Specific Oligonucleotide Hybridization Analysis

Allele-specific oligonucleotide (ASO) hybridization and washing was performed as described by Shuber et al. (1993).

Reverse Transcription (RT) and Nested PCR Analysis

To study the functional consequences of the splice-site mutation in exon 17, we reverse transcribed the *ABCR* mRNA by use of random hexamers and amplified the cDNA by use of primers 2359f (5′-TGTCCATGCAGATGATGCTC-3′) and 2360r (5′-GGAGTCGTGTATTCCTTCTG-3′). We followed this with a nested PCR that used primers 2070f (5′-TGCGTGCTATGGCTTACTCGC-3′) and 2071r (5′-TGGATCCTCCGTTTC CTCTG-3′). The primary cDNA product encompasses nt 2513–2748; the nested cDNA segment contains nt 2541–2721 (nucleotide numbering according to Allikmets et al. 1997*b*).

Statistical Analysis

One- and two-sided Fisher's exact tests were used to compare the frequencies of both the 2588G→C and 2828G→A alleles among patients with STGD (21/68 = 0.309; 95% confidence interval [CI] 0.202–0.433) and ethnically matched controls (2/154 = 0.013; 95% CI 0.002–0.046). This difference is highly significant ($P <$ .001) when either the one- or two-sided Fisher's exact test is applied. The 2828G→A variant was present without the 2588G→C mutation in 0/68 patients with STGD (95% CI 0.000–0.050) compared with 9/154 controls (0.058; 95% CI 0.027–0.108). This difference is significant ($P = .041$) when the one-sided Fisher's exact test is applied but not when the two-sided test is used ($P = .061$). The probability of not finding one 2828G→A allele without the 2588G→C mutation in 68 patients with STGD was calculated as $(145/154)^{68} =$ 1.7% (95% CI 0.4%–15.5%). For this analysis, we did not use data from the group of 311 controls, because only half of them thus far have been investigated for the presence of the 2828G→A variant. The 2588G→C mutation was identified in 15/50 (0.3) mutant *ABCR* alleles. If we assume a random occurrence of the mild 2588G→C mutation in patients with STGD, the a priori chance of not finding any patient homozygous for this mutation is $[1 - (15/50)^2]^{68} =$ 0.2% (95% CI 0.0%–10.9%).

Table 1

Oligonucleotides Flanking *ABCR* Exons

	Forward Primer		Reverse Primer		T_a
Exon	Code	Sequence	Code	Sequence	(°C)[a]
1	1607	GGCTCTTAACGGCGTTTATG	1632	CCCCACAGTTCCAACCTG	55
2	1646	GACAAAAGGCCCAGACCAAAG	1647	GGATCTGAGGAGGGCTGTG	58
3	1635	GTCTCCATGACTAATCCCG	1636	ACTTCCTCCCCCGCATGG	55
5	1649	CCCATTTCCCCTTCAACAC	1681	TTGCCTTTCTCAGGCTGGG	56
6	1650	CTTTCCTACCACAGGGCAG	1750[b]	AGGAATCACCTTGCAATTGG	58
8	1752	ATTGGCCTCACAGCAGATTA	1684	TTAACCAACATGAGAGGCC	56
11	1740	CAAGACCAAAGATCCTATGG	1686	GCTATNTTCAAGGGGCCCA	58
12	1744	GGTCCTCCTCACACTCTCTC	1688	AATTTCCCACTGACTTTGGAG	59
13	1745	GAGGTATGAGTGAGCTATCC	1746	CCCCCATTAGCGTGTCATG	58
14	1689	GTACAGAGCACTCTACTATTC	1747	GGGGAAAGGAACCAAAGTATTC	58
22	1965	CACCCTCCACAGCCCCTTAAC	1577[c]	TCATTGTGGTTCCAGTACTCAG	58

Note.—Sequences 5′– 3′ of forward and reverse primers.

[a] T_a = annealing temperature.

[b] Reverse primer reported elsewhere by Gerber et al. (1998).

[c] Reverse primer reported elsewhere by Allikmets et al. (1997*b*).

For this calculation we used the heterozygote frequency of mild *ABCR* mutations in the panel of 40 patients with STGD, because this group was investigated comprehensively for mutations.

Results

Mutation Spectrum

To investigate the presence of mutations in the *ABCR* gene of patients with STGD, we analyzed all 50 exons of the gene by use of the PCR-SSCP technique, followed by direct sequencing of exons showing aberrant migration. In addition, we performed Southern blot analysis on ~50% of the *ABCR* gene in patients with STGD by using pools of *ABCR* exons as probes. We found 29 different mutations, 19 of which had not been reported previously (table 2). In total, 50 mutations were identified in 80 putative *ABCR* alleles (62.5%). Missense mutations constitute the largest group, accounting for 19 (38%) of 50 mutations. Five (10%) of the 50 mutations are nonsense mutations, 7 (14%) are splice-site mutations, and 3 (6%) are small deletions generating frameshifts. In STGD patient 7679, a pool of genomic probes consisting of exons 20–24 hybridized to an 8-kb band instead of the normal 12-kb band (fig. 1). Hybridization with each single exon as a probe showed the lower band when exon 17, 19, or 23 was used but not when exon 20, 21, or 22 was used (data not shown), indicating the presence of a 4-kb deletion encompassing exons 20–22. Finally, a G→C change at nt 2588—which will be discussed later—accounts for 15 (30%) of the 50 mutations. Frameshift mutations all result in the truncation of the second ABC-domain and are considered pathological. To investigate the possibility that missense and splice-site mutations represent polymorphic variants, we analyzed 100 control individuals from The Netherlands. Except for the 2588G→C mutation, none of these mutations was observed in the controls. Of the 40 patients with STGD, mutation analysis showed both *ABCR* gene mutations in 14 patients (fig. 2), one *ABCR* mutation in 22 patients, and no mutation in 4 patients.

Polymorphisms in the ABCR *Gene*

Sequencing of *ABCR* exons showed 14 nucleotide alterations that can be regarded as polymorphisms (table 3). Six DNA variants are silent mutations; four are intronic changes that, according to Shapiro and Senapathy (1987), are not predicted to affect splicing efficiency when they are located in a splice site. Finally, four missense mutations (Arg943Gln, Asn1868Ile, Pro1948Leu, and Ser2255Ile) have been found at a relatively high frequency in the control population, by us or by others (Allikmets et al. 1997*a*). Except for Arg943Gln, the occurrence of which is discussed later, carrier frequencies in the control population are similar to heterozygote frequencies in patients with STGD, which strongly suggests that these amino acid changes do not contribute to pathological lesions. The observed carrier frequencies of these missense mutations in the general population (6.9%–10.3%) are predicted to yield homozygotes for these mutations at an incidence of ~1/830–1/380. On the basis of the known incidence of STGD, these mutations, therefore, are unlikely to result in pathological lesions.

The 2588G→C Mutation Is a Frequent Founder Mutation in the Western European Population

Because of its high frequency in the group of patients with STGD, we determined the presence of the 2588G→C mutation in 311 control individuals from The Netherlands. By use of ASO hybridization analysis, we ascertained that 9 (2.9%) of 311 control individuals carried this mutation. We noticed a co-occurrence of the 2588G→C mutation with one of the polymorphisms, 2828G→A (previously denoted asArg943Gln; Allikmets et al. 1997*a*), described. This suggested the possibility that a 2588G→C mutation might have occurred on an *ABCR* allele that carried the rare 2828G→A polymorphism. We therefore investigated a total of 68 patients with STGD and 154 control individuals for the presence of both variants. Most of the patients with STGD were from The Netherlands ($n = 47$) or from Germany ($n = 16$). In figure 3, we show the ASO hybridization result for 17 mers specific for the 2588G→C (fig. 3*A, C*) and 2828G→A (fig. 3*B, D*) DNA variants in 40 of 68 tested patients with STGD and 4 patients with other chorioretinal diseases (fig. 3*A, B*) and in 40 of 154 analyzed control individuals (fig. 3*C, D*). Both *ABCR* gene variations were found to coexist in 21 of 68 patients with STGD (data partially shown, fig. 3*A, B*) and in 2 of 154 controls (data partially shown, fig. 3*C, D*). This difference is highly significant ($P < .001$) when the two-sided Fisher's exact test is applied. In contrast, the 2828G→A polymorphism alone, without co-occurrence of the 2588G→C mutation, was found in 9 of 154 control individuals (data partially shown, fig. 3*C, D*) but not in any of the 68 patients with STGD. This difference is significant ($P = .041$) when the one-sided Fisher's exact test is applied but not significant ($P = .061$) when the two-tailed Fisher's exact test is used. Clearly, nt 2588C and nt 2828A are in linkage disequilibrium, and the high frequency of the 2588G→C mutation in the western European population results from a founder effect. The founder mutation was virtually equally frequent in Dutch ($15/47 = 31.9\%$) and German ($5/16 = 31.3\%$) patients with STGD.

Table 2

Mutations in the *ABCR* Gene in 40 Patients with STGD

Exon	DNA Change	Effect	Cases (*n*)	Reference
Missense:				
1	52C→T	Arg18Trp	1[a]	Gerber et al. (1998)
12	1622T→C	Leu541Pro	1	Rozet et al. (1998)
13	1822T→A	Phe608Ile	1	This study
16	2453G→A	Gly818Glu	1	Allikmets et al. (1997*b*)
19	2870A→G	Gln957Arg	1	This study
20	2932G→T	Gly978Cys	1	This study
21	3113C→T	Ala1038Val	2	Allikmets et al. (1997*b*)[b]
23	3335C→A	Thr1112Asn	1	This study
30	4469G→A	Cys1490Tyr	1	This study
35	4892T→C	Leu1631Pro	1	This study
35	4918C→T	Arg1640Trp	1	Rozet et al. (1998)[b]
38	5381C→A	Ala1794Asp	1	This study
39	5537T→C	Ile1846Thr	2	This study
42	5882G→A	Gly1961Glu	2	Allikmets et al. (1997*a*)
44	6079C→T	Leu2027Phe	1	Allikmets et al. (1997*b*)[b]
Nonsense:				
1	45G→A	Trp15stop	1	This study
14	2041C→T	Arg681stop	1	This study
28	4200C→A	Tyr1400stop	1	This study
28	4234C→T	Gln1412stop	2	This study
Deletion:				
2	108delT	Frameshift	1	This study
20–22	del exons 20–22	Deletion/frameshift	1	This study
36	5161delAC	Frameshift	1	This study
40	5668delTTC	DelPhe1890	1	This study
Splice site:				
6	768G→T	Splice mutation	1	This study
16	IVS16+1G→A	Splice mutation	1	This study
30	IVS30+1G→T	Splice mutation	2	Cremers et al. (1998)
39	IVS39+6T→C	Splice mutation	1	This study
40	IVS40+5G→A	Splice mutation	2	Cremers et al. (1998)
Splice site/missense:				
17	2588G→C	DelGly863/Gly863Ala	15	Allikmets et al. (1997*b*)

[a] Homozygous.
[b] Data were erroneously published with a different nomenclature.

Consequences of the 2588G→C Mutation for the ABCR *mRNA and Predicted Protein*

The 2588G→C mutation is located in the first nucleotide position of exon 17 (fig. 4*A*, *upper panel*) and therefore is part of its splice acceptor site. The nucleotide change could result in a missense mutation, causing an exchange of an alanine for glycine at amino acid residue 863, but could also affect the splicing at the 3′ splice site of exon 17. We identified a cryptic 3′ splice site 3 bp downstream (nt 2591) in exon 17 (fig. 4*A*, *lower panel*). According to Shapiro and Senapathy (1987), the splice potential score of the normal splice site is 86.3, whereas the score of the cryptic splice site is 81.2. The G→C change at nt 2588 reduces the splice potential score of the normal splice site to 80.3 and simultaneously increases the score of the cryptic splice site to 92.9. We hypothesized that the cryptic splice site might be used

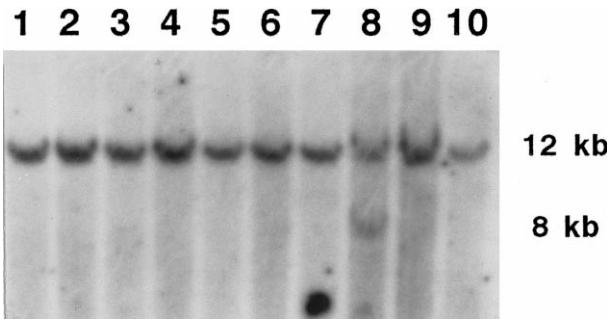

Figure 1 Southern blot analysis of 10 STGD patients' genomic DNA with a pool of genomic probes spanning exons 20–24. Patient 7679 (*lane 8*) shows a 4-kb deletion encompassing exons 20–22.

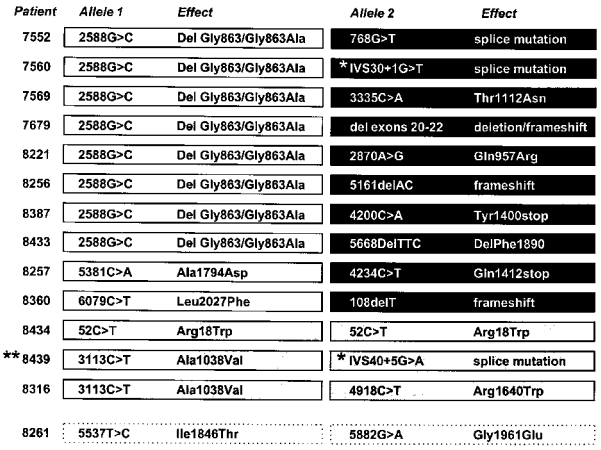

Figure 2 Patients with STGD in whom both *ABCR* mutations were identified, with classification of each mutation on the basis of the residual activity of the ABCR protein. In black boxes are indicated mutations that cause a complete loss of ABCR activity; in gray boxes, moderate mutations; and in white boxes, mild mutations. The DelGly863/Gly863Ala mutation is considered a mild mutation, and therefore all accompanying mutations are severe or null mutations. The Tyr1400stop and 108delT mutations are also considered null mutations, and therefore the accompanying mutations, Ala1794Asp and Leu2027Phe, respectively, are considered mild mutations. Arg18Trp has been found homozygously in patient 8434 and, according to the model, is considered a moderate mutation. The classification of the mutations in STGD patients 8316 and 8439 is described in detail in the Discussion section. The mutations marked by one asterisk (*) were previously found in patients with RP (homozygosity for IVS30+1G→T) or patients with CRD (compound heterozygosity for IVS30+1G→T and IVS40+5G→A [Cremers et al. 1998]). Patient 8439 (**) was found to carry a Leu541Pro as a third mutation. The Ile1846Thr and Gly1961Glu mutations found in patient 8261 cannot be classified.

in the mutant allele, thereby resulting in a deletion of Gly863.

To investigate the effect of the 2588G→C mutation, we isolated RNA from Epstein-Barr virus–transformed lymphoblastoid cells of a control individual and of two STGD patients carrying the mutation, performed RT-PCR, and amplified nt 2541–2721 of the *ABCR* cDNA containing part of exon 16, exon 17, and part of exon 18. Nested PCR products showed a clear band at the expected size (181 bp), an aspecific band of ~200 bp, and no evidence of smaller-sized products indicative of exon 17 skipping (fig. 4*B*). Direct sequencing of the main PCR product derived from a control individual showed that only the normal splice site at position 2588 was used (fig. 4*D, panel I*). In contrast, the cDNA from STGD patients 7552 and 7560, both heterozygous carriers of the 2588G→C mutation, showed two overlapping sequences, one being the result of cryptic splicing at position 2591, causing the predicted 3-bp deletion, and the other being a missense mutation resulting from the splicing at the mutant cytosine at 2588 (fig. 4*A, D, panel II*). PAGE analysis of [^{32}P]-labeled cDNA PCR products under denaturating conditions showed a similar intensity of the signal from both transcripts, indicating that the splice sites at positions 2588 and 2591 are approximately equally used (fig. 4*C*). In both patients, the cDNA product of the second allele, carrying a different mutation not affecting exon 17, was not detected. In the sequence displayed in figure 4*D, panel II,* there is no evidence of a cytosine at 2588 in the reverse sequence of the cDNA. Apparently, in both patients the mutation on the other allele resulted in a very low quantity or absence of the mRNA. Genomic sequencing showed a splice-site mutation (768G→T) in exon 6 in patient 7552; in patient 7560, a splice-site mutation (IVS30+1G→T) was identified in exon 30.

Discussion

Identification of a Frequent Western European ABCR *Founder Mutation*

Mutation analysis of the *ABCR* gene in 40 western European patients with STGD yielded 29 different *ABCR* mutations among a total of 50. One mutation (2588G→C) is of particular interest because of its high heterozygote frequency both among European patients with STGD (21/68 = 30.9%) and among control individuals from the Dutch population (2.9%). There was no significant difference between the heterozygote frequency in Dutch (15/47 = 31.9%) and German (5/16 = 31.3%) patients with STGD. Very likely, the population incidence of 2588G→C carriers in the German population is similar to that in the Dutch. In

Table 3

Polymorphisms in the *ABCR* Gene

Exon	DNA Change	aa Change/SPS[a]	Controls[b] (*n* [%])
19	2828G→A	Arg943Gln	11/154 (7.1)
28	4203C→A	Pro1401Pro	ND
39	IVS38-10T→C	SPS: 92.0→90.5	ND
40	5603A→T	Asn1868Ile	11/108 (10.2)
40	5682G→C	Leu1894Leu	ND
42	IVS41-11G→A	SPS: 94.4→94.4	ND
42	5843/4CA→TG	Pro1948Leu	9/130 (6.9)
42	5844A→G	Pro1948Pro	ND
45	6249C→T	Ile2083Ile	ND
46	6285T→C	Asp2095Asp	ND
48	6693C→T	Ile2231Ile	ND
48	IVS48+21C→T	…	ND
49	6764G→T	Ser2255Ile	6/58 (10.3)[c]
49	IVS48-3T→C	SPS: 87.3→95.6	ND

[a] SPS = splice potential score according to Shapiro and Senapathy (1987).
[b] ND = not determined.
[c] Result reported by Allikmets et al. (1997*a*).

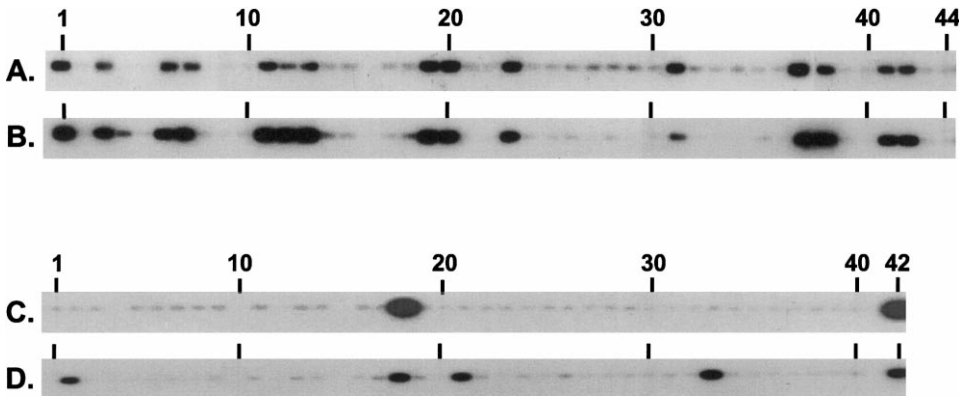

Figure 3 ASO hybridization analysis of the 2588G→C mutation and the 2828G→A polymorphism in patients with STGD and control individuals—analysis of the mutant cytosine at position 2588 (*A*) and the polymorphic adenine at position 2828 (*B*) in 40 patients with STGD and 4 patients (*lanes 5, 16, 30, and 34*) with other chorioretinal diseases and analysis of the mutant cytosine at position 2588 (*C*) and the mutant adenine at position 2828 (*D*) in 40 control individuals and STGD patient 7550 (*lane 42*). An approximately equal amount of DNA was loaded onto the agarose gel and blotted. In *B, lane 4,* patient 7553 shows a weak signal. By sequence analysis, we have shown that this results from overflow of material from lane 3.

the United States, this mutation was found at a much lower heterozygote frequency both in patients with STGD (13/150 = 8.7%) and in the control population (2/220 = 0.9%) (Allikmets et al. 1997*a*). A carrier frequency of 2.9% is similar to that of another mild autosomal recessive mutation, the IVS8-5T splice-site mutation in the *CFTR* gene; approximately twice as high as the carrier frequency of the most frequent severe autosomal recessive mutation in The Netherlands, the *CFTR* ΔPhe508 mutation (Tuerlings et al. 1998); and similar to the carrier frequency of the 30delG mutation in the *connexin 26* gene in Mediterranean countries (Denoyelle et al. 1997; Estivill et al. 1998). Surprisingly, the 2588G→C mutation was not mentioned at all by Rozet et al. (1998), who analyzed 55 European and North African patients with STGD/FFM for *ABCR* mutations. Possibly, they did not consider this to be a pathological mutation because of its high carrier frequency in the control population.

The cytosine at 2588 is situated at the first position of exon 17 and shows linkage disequilibrium with a polymorphism (2828G→A) situated in exon 19. In all 21 patients with STGD and the two healthy individuals known to carry the 2588G→C mutation, the 2828G→A variant was also present. In the control population, the 2828G→A polymorphism was observed without the 2588G→C mutation in 9 (5.8%) of 154 individuals. We did not, however, find the 2828G→A variant without the 2588G→C mutation in a total of 68 patients with STGD. Given an occurrence of the single 2828G→A variant in 9 of 154 control individuals, the chance of not finding this allele without the 2588G→C mutation in 68 patients with STGD is highly unlikely ($P = .017$; 95% CI 0.004–0.155). From this we conclude that the 2588G→C allele is the more recent and probably the pathological mutation. Without a test that can assay ABCR activity, it is difficult to assess the functional consequences of both DNA variants. The 2828G→A alteration results in a nonconservative Arg943Gln mutation. Our RT-PCR studies suggest that the 2588G→C mutation does not result in exon skipping but leads to a mixture of mutant ABCR proteins either lacking Gly863 or carrying a nonconservative Gly863Ala mutation. Whether the 2588G→C mutation acts on its own or synergistically with the 2828G→A variant might be resolved when an STGD patient carrying the 2588G→C—but not the 2828G→A—alteration is identified. Currently, we have no explanation for the high carrier frequency of the 2588G→C *ABCR* mutation. By extending the haplotype containing the 2588G→C *ABCR* mutation, we will investigate the extent of the observed linkage disequilibrium. Possibly a beneficial mutation in a closely linked gene has resulted in the increased frequency of this mutation. Alternatively, the high incidence might be attributable to genetic drift, a phenomenon that is not uncommon in the Dutch population (Peelen et al. 1997; Petrij-Bosch et al. 1997).

Classification of ABCR *Mutations*

In the Introduction, we referred to a model that correlates phenotypes in several chorioretinal disorders with the residual activity of the ABCR protein (Cremers et al. 1998; Martinez-Mir et al. 1998; van Driel et al. 1998). In this model, *ABCR* mutations are classified as mild, moderate, or severe, indicated in the scheme in white, gray, or black boxes, respectively (fig. 5). On the basis of the combinations of *ABCR* mutations identified

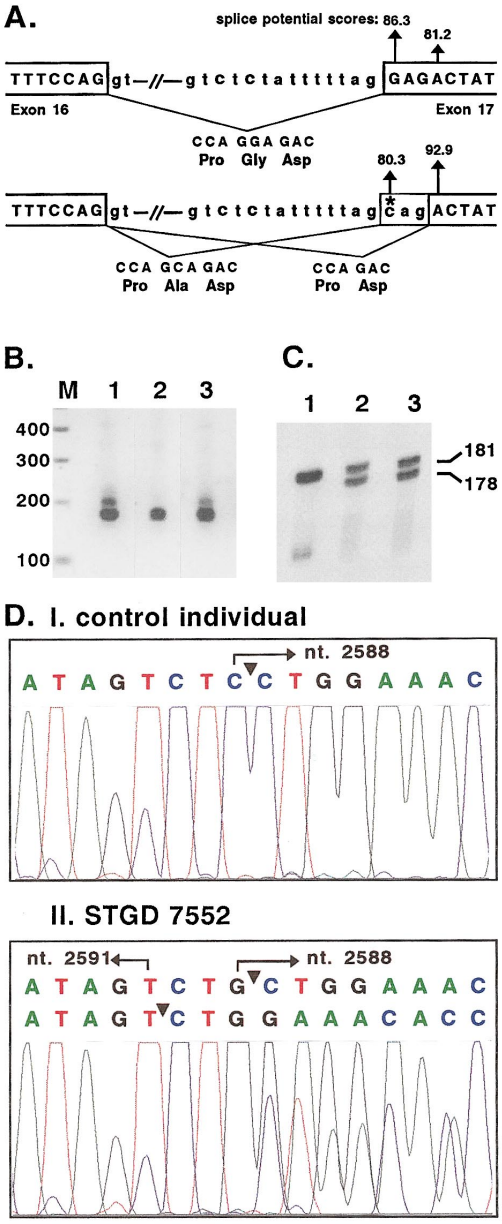

Figure 4 RT-PCR analysis of the 2588G→C mutation in two STGD patients and a control. *A*, Potential splice variants for intron 16. *Upper scheme*, normally spliced sequence; *lower panel*, splice variants using the mutant cytosine (nt 2588) and the adenine at nt 2591. The former results in Gly863Ala; the latter, in DelGly863. Splice potential scores were determined by the method of Shapiro and Senapathy (1987), where the frequencies of the −14 to +1 nucleotides in the 3′ splice sites are weighed. The asterisk (*) denotes the mutant cytosine. *B*, RT-PCR results for a control (*lane 1*) and for patients 7552 (*lane 2*) and 7560 (*lane 3*). *C*, PAGE analysis of [^{32}P]-labeled RT-PCR products shows approximately equal intensities for the 181-bp RT-PCR product resulting from splicing at the mutant cytosine at 2588 and the 178-bp product resulting from cryptic splicing at the adenine at 2591. *Lane 1*, control; *lane 2*, patient 7552; and *lane 3*, patient 7560. *D*, Sequence analysis of the major PCR products shown in *B*. *Panel I*, reverse cDNA sequence of RT-PCR product of control; *panel II*, reverse cDNA sequences of the two RT-PCR products of patient 7552. Because of the 3-bp deletion, the sequences following the mutant guanine at 2588 in the *upper sequence* show overlapping peaks. For clarity, both mutant cDNA sequences are indicated. Triangles denote positions of the exon-exon junctions.

in patients with RP, CRD, and STGD, we proposed that a combination of two severe mutations leads to an RP phenotype and compound heterozygosity for a severe and a moderate mutation leads to CRD, whereas a combination of either a severe and a mild mutation or two moderate mutations results in STGD (Cremers et al. 1998; van Driel et al. 1998). We also proposed, on the basis of this model, that the 2588G→C mutation is a

mild mutation, and that any mutation accompanying the 2588G→C mutation in a patient with STGD should be a severe missense or a null mutation.

In 8/15 STGD patients with the 2588G→C mutation, we found a mutation on the other *ABCR* allele (fig. 2). In three of these patients—patients 7679, 8256, and 8387—the second mutation truncates the ABCR protein and can be considered a null allele. RT-PCR results indicate that in patient 7560, only the allele carrying the 2588G→C mutation generates a detectable transcript, strongly suggesting that IVS30+1G→T is a severe mutation (fig. 4*B–D*). RT-PCR analysis of RNA from STGD patient 7552, who has compound heterozygosity for a 768G→T splice-site mutation and the 2588G→C founder mutation, led to the same result, indicating that the 768G→T mutation also is a severe mutation (fig. 4*B–D*). Moreover, we found the 768G→T mutation homozygously in a patient with RP (current authors, unpublished data). Two patients carried missense mutations. The Gln957Arg mutation in STGD patient 8221 represents a nonconservative amino acid change located nine amino acids aminoterminal to the first Walker A motif. Moreover, the Gln957 residue is found also in other members of the ABC1 subfamily (mouse Abcr, mouse Abc1, mouse Abc2, and human ABCC [Luciani et al. 1994; Klugbauer and Hofmann 1996; Azarian and Travis 1997]). The Thr1112Asn mutation in STGD patient 7569 represents a conservative change, but all members of the ABC1 subfamily contain a Thr at this position, which is located 19 residues carboxyterminal to a Walker B motif. Finally, patient 8433 shows a deletion of a Phe residue at position 1890, an amino acid that is conserved in four of five members of the ABC1 subfamily and is located in the transmembrane domain XII. Hence, in accordance with our model, most, if not all, of the mutations accompanying the 2588G→C mutation can be considered severe mutations.

We have also classified, on the basis of our model, other mutations in different grades of severity (fig. 2). The 4234C→T mutation in STGD patient 8257 and the 108delT mutation in patient 8360 are null mutations, and therefore the respective accompanying mutations, Ala1794Asp and Leu2027Phe, most likely are mild mutations. In accordance with this classification, the Leu2027Phe change is a conservative change, and the Ala1794Asp exchange affects an amino acid residue that is not conserved between the five members of the ABC1 subfamily. On the basis of the co-occurrence of the IVS40+5G→A mutation with the IVS30+1G→T mutation in a patient with CRD (Cremers et al. 1998), the former mutation is considered a moderate mutation. Consequently, the accompanying mutation in a patient with STGD should also be a moderate mutation. Thus, we classified the Ala1038Val mutation, which previously was erroneously indicated as Ala1028Val (Lewis et al., in press), as a moderate mutation. In patient 8439, we identified a third mutation, Leu541Pro, which is not indicated in figure 2. Leu541Pro was also found to accompany the Ala1038Val mutation in a patient with FFM, as described by Rozet et al. (1998), but not in our patient 8316. Whether only one mutation or both mutations, possibly acting in concert, contribute to pathological abnormalities cannot be predicted. Arg18Trp is present homozygously in patient 8434 and therefore is considered a moderate mutation. This is a nonconser-

Table 4

Estimated Incidences of Combinations of Different Types of *ABCR* Mutations and Predicted Associated Phenotypes

Combination of *ABCR* Alleles	Incidence	Phenotype
Mild/+	1/25	Normal[a]
Moderate/+	1/100	Normal[a]
Severe/+	1/125	Normal[a]
Mild/mild	1/2,500	Normal[a]
Mild/moderate	1/10,000	Normal[a]
Mild/severe	1/12,500	STGD
Moderate/moderate	1/40,000	STGD
Moderate/severe	1/50,000	CRD
Severe/severe	1/62,500	RP

[a] In these individuals, combinations of *ABCR* mutations might be a risk factor for AMD.

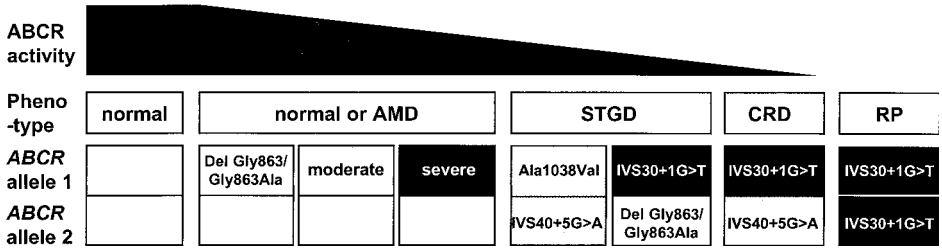

Figure 5 Phenotype-genotype correlation for *ABCR* mutations depicting the inverse correlation between ABCR activity and the severity of the associated chorioretinal disease. Combinations of null mutations (given in black boxes) invariably result in (atypical) RP; combinations of null and moderately severe (gray boxes) mutations result in CRD; and combinations of two moderately severe mutations or a null mutation and a mild mutation (white boxes) result in STGD; a single *ABCR* mutation might be a risk factor for developing AMD.

vative change that affects an amino acid residue found in three of five members of the ABC1 subfamily. Similarly, Arg1640Trp, which was found together with Ala1038Val in patient 8316, was present homozygously in a patient with STGD described by Rozet et al. (1998) and therefore is a moderate mutation. Our data do not allow us to classify the Gly1961Glu and accompanying Ile1846Thr mutations found in patient 8261. We recognize that the proposed classification is not based on functional studies of the ABCR protein and therefore should be considered tentative. Clearly, to test this model, future studies should focus on the elucidation of the function of the ABCR protein.

Combinations of Severe/Severe and Mild/Mild ABCR *Mutations Do Not Cause STGD*

On the basis of our results, one third to one half of all *ABCR* mutations observed in patients with STGD can be considered null mutations. Additional evidence for the model depicted in figure 5 is the absence of null mutations in both *ABCR* alleles of patients with STGD in our study, as well as in ~150 STGD patients from the United States (Lewis et al., in press). The only suspicious mutation in this respect is a homozygous deletion of five amino acids (ValValAlaIleCys) after amino acid 1681 of the ABCR protein, which affects the last predicted transmembrane domain of the ABCR protein (Allikmets et al. 1997*b*). In our study, the 2588G→C mutation was found in 15/50 mutant *ABCR* alleles. If the founder mutation is present randomly in patients with STGD, 1/11 patients with STGD, on average, would be predicted to be homozygous for the 2588G→C mutation. Thus far, we have not identified any homozygotes or compound heterozygotes for this mild mutation in a total of 68 patients with STGD (this study; current authors, unpublished data [$P = .002$; 95% CI 0.000–0.109]). On the basis of the heterozygote frequency of the 2588G→C mutation alone, homozygosity for this mutation would be responsible for STGD in ~1/4,800 individuals. On the basis of a conservative estimate of a total carrier frequency of all mild mutations of 1/25, homozygosity or compound heterozygosity for two mild *ABCR* mutations would occur in 1/2,500 individuals. To the best of our knowledge, only one study has been performed that estimates the incidence of STGD in the American population at 1/10,000 (Blacharski 1988). Although we cannot exclude the very real possibility that STGD is underdiagnosed, it is clear that combinations of mild mutations are unlikely to result in STGD. Recently, Dryja et al. (1998) noted a discrepancy between the observed frequency of *ABCR* variants in patients with AMD, which yields a carrier frequency of *ABCR* mutations in the general population of 4.8%, and the "observed" frequency of 0.45%. In this calculation, Dryja et al. (1998) included only the Asp2177Asn mutation, excluding the 2588G→C mutation (given as Gly863Ala by Allikmets et al. 1997*a,* 1997*b*) and the nonconservative missense mutations Val643Gly and Gly818Glu. Together, these mutations were found in 5/220 controls (2.25%), much closer to the carrier frequency expected on the basis of the incidence of AMD. As indicated above, we here propose the existence of a relatively large pool of mild *ABCR* variants in the general population, which, when present in both *ABCR* alleles, do not lead to STGD, RP, or CRD but may confer an increased risk of developing AMD.

Estimates of Incidences of Mutant ABCR *Alleles and Their Involvement in CRD and RP*

In table 4, we estimate the carrier frequencies of moderate and severe *ABCR* alleles, as well as the incidences of different retinal disorders associated with *ABCR* mutations, on the basis of the following findings and assumptions: (1) ~25% of patients with STGD carry two moderate mutations (see fig. 2); (2) the incidence of STGD is ~1/10,000 (Blacharski 1988); and (3) in view of the carrier frequency of the founder mutation alone, the total carrier frequency for mild mutations in the European population can conservatively be estimated as ~0.04. Finally, to perform the following calculations, we assume that a combination of mild/moderate mutations does not result in STGD. If we assume an STGD incidence of 1/10,000, the incidences of combinations of mild/severe and moderate/moderate *ABCR* mutations are ~1/12,500 and ~1/40,000, respectively. From this, it follows that the carrier frequencies of severe and moderate *ABCR* mutations will be ~0.008 and ~0.01, respectively. From this calculation, we deduce that *ABCR* mutations might be a relatively frequent cause of autosomal recessive CRD and autosomal recessive RP. This calculation also shows that healthy individuals have a much greater chance to carry a mild than a severe *ABCR* mutation, which has implications for STGD recurrence risk calculations. On the basis of our calculations, a child of a healthy individual and a patient with STGD has a 1 in 50 chance of inheriting a mild *ABCR* mutation, a 1 in 200 chance of inheriting a moderate mutation, and a 1 in 250 chance of inheriting a severe mutation from the healthy parent. In the majority of cases, the parent with STGD will carry a mild and a severe *ABCR* mutation, and the child will have a risk of ~1/83 (1/100 + 1/500) of developing STGD, 1/400 of developing CRD, and 1/500 of developing RP.

The questions remain whether combinations of mild/mild and moderate/mild *ABCR* mutations increase the risk of developing AMD and whether the classification of *ABCR* mutations as proposed in this study also relates to their penetrance in causing AMD. Given the high carrier frequency of the 2588G→C mutation in western Europe, some of these questions can now be addressed.

Implications for STGD Linkage Analysis

The high carrier frequency of mild *ABCR* mutations in the general population complicates linkage analysis in the ~1/50 families with STGD in which one of the parents is predicted to carry two mild *ABCR* mutations. Because this parent will not show retinal pathological changes, the results of haplotype analysis will be misleading. In a previous study, we indeed observed a family, ST6, in which haplotype analysis demonstrated that one affected female subject carries only one disease allele (Hoyng et al. 1996). The affected subject (individual 5) and her affected brothers carry a severe paternal 768G→T splice-site mutation, and her brothers both carry the mild, maternally inherited founder mutation, 2588G→C, as well. A logical explanation would be that her healthy mother carries two mild *ABCR* mutations. We ruled out homozygosity of the mother for the 2588G→C mutation (current authors, unpublished data), but she could very well have compound heterozygosity for this mutation and another mild *ABCR* mutation. Similarly, Anderson et al. (1995) described a family with STGD (AR088) in which two affected siblings apparently received different disease haplotypes from one of their parents. Weber et al. (1996) found evidence for nonallelic heterogeneity in 3/21 families with STGD, which, in some cases, might be explained by the presence of mild mutations in both *ABCR* alleles of one of the parents in the respective families.

In conclusion, we have identified a remarkably frequent mild *ABCR* mutation in the western European population that enables a differentiation of *ABCR* mutations into different classes of severity and that has important consequence for recurrence risk calculation in families with STGD.

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Electronic-Database Information

The accession number and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim (for STGD [MIM 248200] and AMD [MIM 153800])

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