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LABORATORY STUDY

Novel mutations in of the *ABCR* gene in italian patients with Stargardt disease

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Abstract

***Purpose*: Stargardt disease (STGD) is the most prevalent juvenile macular dystrophy, and it has been associated with mutations in the *ABCR* gene, encoding a photoreceptor-specific transport protein. In this study, we determined the mutation spectrum in the *ABCR* gene in a group of Italian STGD patients.**

***Methods*: The DNA samples of 71 Italian patients (from 62 independent pedigrees), affected with autosomal recessive STGD, were analysed for mutations in all 50 exons of the *ABCR* gene by the DHPLC approach (with optimization of the DHPLC conditions for mutation analysis) and direct sequencing techniques.**

***Results*: In our group of STGD patients, 71 mutations were identified in 68 patients with a detection rate of 95.7%. Forty-three mutations had been already reported in the literature, whereas 28 mutations had not been previously described and were not detected in 150 unaffected control individuals of Italian origin. Missense mutations represented the most frequent finding (59.2%); G1961E was the most common mutation and it was associated with phenotypes in various degrees of severity.**

***Conclusions*: Some novel mutations in the *ABCR* gene were reported in a group of Italian STGD patients confirming the extensive allelic heterogeneity of this gene—probably related to the vast number of exons that favours rearrangements in the DNA sequence.**

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Keywords: ABCR gene; mutations; macular dystrophies; Stargardt disease

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Introduction

Stargardt disease (STGD), the most prevalent inherited juvenile macular dystrophy, is a progressive juvenile-to-young-adult onset macular degeneration characterized by severe reduction of central visual acuity and normal peripheral vision.[1,2] The onset of the disease occurs usually around 10 years of age, and the first symptom is a reduction of central visual acuity. Rare late clinical pictures, appearing even after 50 years of age, have also been described.[3]

STGD is distinguished by a diffuse accumulation of toxic metabolites (lipofuscins) within the retinal pigment epithelium (RPE) with consequent disomogeneity and focal hypertrophy of the same RPE, and subsequent photoreceptors atrophy.[4]

Fundoscopy usually manifests a macular atrophy, often associated with typical fishtail white-yellowish spots spread at the posterior pole, and sometimes at retinal midperiphery (flecks). In the past, the presence of flecks was considered a well-marked disease ('fundus flavimaculatus'), which could be associated or not with atrophic maculopathy. Currently, macular atrophy and fundus flavimaculatus (and their possible association) are considered variants of the basically same disease.[3]

Fluorangiography often reveals a homogeneous dark aspect of the choroid ('dark or silent choroid'), determined by the masking effect of lipofuscin storage in RPE on the underlying choroidal fluorescence. ERG abnormalities can be recorded in advanced stages of the disease.

Stargardt disease is predominantly inherited as an autosomal recessive trait; however, autosomal dominant forms have been described. It has been associated with alterations in the gene encoding the photoreceptor-specific ATP-binding cassette (ABC) transporter (*ABCR*). This gene (OMIM no. 601691) is localized in chromosome 1p22, and it codes for a 2.273 amino-acid protein expressed in the rims of rod and cone outer

segment disks.[5] As with other ABC transporters, ABCR consists of a consensus ATP-binding region of approximately 90–110 amino acids, which includes two Walker motifs (A and B regions), a linker or dodecapeptide region, which lies between these Walker motifs (also known as the C region) and some additional regions of homology upstream and downstream from the Walker A and B motifs. Although A, B, and C regions are well conserved across all known organisms, they are usually associated with less conserved transmembrane (TM) domains consisting of six TM helices, which confer substrate specificity.[6,7]

ABCR is a transport protein located in the rim of photoreceptor discs and involved in the transport of all-trans-retinal through the disc membrane, and then from the disc lumen to the photoreceptor cytoplasm. ABCR dysfunction reveals an accumulation of all-trans-retinal in the photoreceptors discs and in the RPE. All-trans-retinal is then converted into a substance called A2-E, a major component of lipofuscin, which determines a toxic effect leading to the disruption of the RPE and the overlying photoreceptors.[8]

Several series of mutation analyses have confirmed that homozygous and compound heterozygous mutations in *ABCR* are responsible for recessive STGD.[9–13]

ABCR has also been singled out as a possible cause of other diseases with similar clinical macular abnormalities. Gene mutations have been observed in families manifesting cone-rod dystrophy,[14] retinitis pigmentosa,[15] and age-related macular degeneration (AMD).[16]

All studies investigating *ABCR* in eye diseases report a broad mutation spectrum and high allelic heterogeneity. This phenomenon might be due to the long gene sequence (50 exons) and to ethnic variability.

ABCR mutations in STGD have been investigated in several different ethnic groups. Italian STGD patients had been analysed for *ABCR* mutations in three earlier studies. The first study considered 11 families coming from southern Italy,[10] and the second studied 36 families originating from various regions of northern, central, and southern Italy.[11] In addition, another study on an Italian sample considered only patients with biallelic disease mutations for a genotype–phenotype correlation.[17]

In this study, the mutation spectrum of the *ABCR* gene was determined in another group of Italian patients affected with autosomal recessive STGD. Our series consisted of 62 families, originating for the most part from the central Italy. Consequently, it can be said that our sequence represents a large and homogeneous sampling.

Materials and methods

Clinical evaluation

Sixty-two Italian families, some members of which were affected by autosomal recessive STGD, were recruited through the Hereditary Retinal Degenerations Referral Center of the Eye Clinic, University of Florence.

Criteria for the STGD phenotype included the following: (1) juvenile-to-adult symptom onset; (2) bilateral central vision loss; (3) macular dystrophy and/or atrophy (beaten bronze appearance or large patch of atrophy); (4) normal calibre of retinal vessels; (5) absence of pigmented bone spicules; and (6) normal electroretinogram.

The fluorangiographic phenomenon of 'dark choroid' and the presence of yellow-white flecks were not considered among the obligatory inclusion criteria, even if they could be appreciated in most of the patients in our series.

Seventy-one patients with a clinical diagnosis of STGD were included in the study (40 males and 31 females). The mean age was 36.6 years (± 15.8 years); (range: 10–75 years); visual acuity ranged from 1/30 to 8/10, with an average value of 2.31/10 (± 1.9/10). In 48 patients, fundoscopy confirmed the presence of flecks at the posterior pole, whereas in 40 patients, the phenomenon of dark choroid could be observed during the fluorangiographic examination.

DNA extraction and PCR amplification

After obtaining the written informed consent and a complete family history, 10 ml of peripheral blood taken from the antecubital vein using EDTA-containing vials were collected. DNA was extracted from 200 l of peripheral blood with Biorobot EZ1 (Qiagen GmbH, Hilden, Germany).

Coding regions, intron/exon boundaries, and 5′ and 3′ regions of *ABCR* were amplified in 50 reactions. The PCR amplification was performed using the Core System-Robotic Station (Beckman Coulter, CA, USA). Cycling parameters for each reaction were optimized for all the exons.

The PCR amplification of 50 exons and flanking intronic regions of the *ABCR* gene was performed using 50–100 ng of genomic DNA. The primers used were those recommended in Rivera *et al*.,[9] and are exhibited in Table 1.

Amplification was performed in 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.3, 5 mmol/l MgCl2, 200 μmol/l dNTPs, and 0.5 μmol/l for each primer set. AmpliTAq DNA polymerase (1 U; Ampli TAq Gold, Applied Biosystems, Foster City, CA, USA) was added for each 25 μl reaction. PCR was performed by employing a

Table 1 Primers used for the PCR amplification of the *ABCR* gene

Exon	*Sense primer (5′–3′)*	*Antisense primer (5′–3′)*	*Annealing temperature (°C)*
1	AATCTGGTCTTCGTGTGGTC	GTTTATTTGCTCCACACCTC	60
2	AATCTCTTAGCACCACTGAAC	AGGCCCAGACCAAAGTCTC	60
3	CCTGCTTGGTCTCCATGAC	ACGTGAAGGGGTGTGCAAC	60
4	CCTTATTAATGAGGCTTTGTC	ATAGGTGAGGGAAATGATGC	60
5	CCATTTCCCCTTCAACACCC	GTGCTTCCCTCCCCTCCAG	62
6	CTACCACAGGGCAGTTTCTA	CAGGAATCACCTTGCAATTG	60
7	GATCAGACTGTGCCTATGTG	ATAAGTGGGGTAAATGGTGG	60
8	GAGCATTGGCCTCACAGCAG	CCCCAGGTTTGGTTTCACC	60
9	AGGTTACAAGCAATGGGGAG	TCTGGGAGGTCCAGGGTA	62
10	ATCTTTGTCTGGTTTTAGGC	CCCCCCTTACTCTGATCAT	60
11	GAATTTCTAAGCAGAGCAGTG	AGCTCTGGCCCCACTCATG	60
12	AGTTGAGTCTTTGCAGTTGG	CTGACTTTGGAGAAATGCAG	58
13	TCGGGAGGTGTGAGTGAGC	TTAGCGTGTCATGGAGGAGG	62
14	ATTCTGCCTCTACCAGGTAC	AATCCAGGCACATGAACAGG	60
15	AGGCTGGTGGGAGAGAGC	GGACTGCTACGGACCATTC	60
16	CTGTTGCATTGGATAAAAGGC	GATGAATGGAGAGGGCTGG	60
17	CTGCGGTAAGGTAGGATAGGG	CACACCGTTTACATAGAGGGC	62
18	CTCTCCCCTCCTTTCCTG	GCCTTTTCCTCGCCTCTG	60
19	TGGGGCCATGTAATTAGGC	TGGGAAAGAGTAGACAGCCG	58
20	GCCCTCCTAAGGCATGTTG	TATCTCTGCCTGTGCCCAG	62
21	GTAAGATCAGCTGCTGGAAG	GAAGCTCTCCTGCTCCAAGC	62
22	AGGTACCCCCACAATGCC	AGCCCAGCCCAGGAGACT	60
23	TTTTTGCAACTATATAGCCAGG	AGCCTGTGTGAGTAGCCATG	60
24	GCATCAGGGAGAGGCTGTC	CCAGACGGAACCCAAGTATG	60
25	GGTAACCTCACAGTCTTCC	GGGAACGATGGCTTTTTGC	58
26	TCCCATTATGAAGCAATACC	CCTTAGACTTTCGAGATGG	58
27	GAGATCCAGACCTTATAGGC	GTTATAACCCATGCCTGAAG	60
28	ACGTGTGACATCTCCATGCC	CCCTTCTAAGCAGCATGTGA	60
29	AGGCTCTGAGTTGCATGATG	CTGCCATCTTGAACCCACC	60
30	ACTTTGAGGCTGATTATGGAA	CCCCGTTGTTTGGAGGTC	58
31	TATAAGTCCTCAAGTTCCAAG	AATATCTTCTACAGGGAGCC	58
32	TAACGGCACTGCTGTACTTG	TCATGGCTGTGAGGTGTGC	60
33	TTCATGTTTCCCTACAAAACCC	AAAATCCTACTCAAATCTCCAG	60
34	GCTTAACTACCATGAATGAG	TCAGCAGGAGGAGGGATG	56
35	TAACTAGCTGTTAATGCAGCG	AAGAGTGGAGAAGGTGACAA	58
36	GTATCTTCTCCTCCTTCTGC	CACACAAGCTCCACCTTGG	60
37	CAGGTCTGAGAGGTTAAGTG	CCACCAGGCTTCTCTTCAG	60
38	GGAATGGAATGTGGAACTCC	ACACATACTCTACTATCCTAC	60
39	GGTTTGCCCCGTTTCCAAC	TCCCAGCTTTGGACCCAG	60
40	AGGTCTGTGGGGTGAGCTG	TCTGGATGCCCTGAGCTGC	60
41	GAAAGGACAGTGCCAAGGAC	TCTAACCAGCACCTCCAAAC	60
42	CCGTCTCAGTTCTCAGTCC	AGAGCTGATGTTCGGAAGCC	60
43	CTTACCCTGGGGCCTGAC	TCAGAGCCACCCTACTATAG	60
44	GAAGCTTCTCCAGCCCTAGC	TGCACTCTCATGAAACAGGC	62
45	CTTGTCTTCTCCAAATGGCA	TTTAAGCCCTTGGTGCGGC	60
46	GAAGCAGTAATCAGAAGGGC	CCTCACATTCTTCCATGCTG	60
47	CACATCCCACAGGCAAGAG	ATCCACAGAAGGCAACAAGG	60
48	AGGCCCAACCACTAACAGAG	ACACTGGGTGTTCTGGACC	60
49	GTGTAGGGTGCTGTTTTCC	CAAGCTGTGGACTGCATAAG	60
50	AAACCAAGATGACGCGAGTC	GGAACGAGCGGTGTGAAAG	60

For each exon, the forward primer, the reverse primer, and the annealing temperature are indicated.

multiblock MWG PCR System. Cycling parameters for the reactions were optimized for each exon.

Mutational analysis

Sequence variations were detected by denaturing high-performance liquid chromatography (DHPLC) using the WAVE™ DNA Fragment Analysis System equipped with a DNAsep column (Transgenomics, San Jose, CA, USA).[18,19] The conditions for DHPLC were developed on the basis of exon-specific melting profiles predicted by NAVIGATOR™ Software. PCR products were examined for heteroduplexes by subjecting 5 μl of each PCR product to a denaturation step process (5 min at 95°C),

Table 2 Summary of the mutations identified in the *ABCR* gene in our series of STGD Italian patients

Patient	*Allele 1 mutation*	*Allele 2 mutation*
S 1	R212C	T1019M
S 8	V1433I	V1433I
S 21	A1598D	A1598D
S 33	**N96K**	G978D
S 56	A1598D	G1961E
S 70	R212C	T1019M
S 71	W700X	WT
S 74	6750delA	V767D
S 77	G1961E	WT
S 82	**Q21X**	G1961E
S 106	C1177X	G1961E
S 107	C1177X	G1961E
S 114	**T970P-F1015E**	-
S 115	**T970P-F1015E**	-
S 120	**N415K**	G1961E
S 162	324-327insT	324-327insT
S 181	W1408X	G1961E
S 190	C1177X	A1598D
S 201	G1961E	WT
S 202	Q21X	**T970P-F1015E**
S 213	**M840R**	G1961E
S 231	WT	WT
S 236	C1177X	G1961E
S 237	WT	WT
S 241	V256 splice	WT
S 246	IVS6-1g>t	R1108C
S 260	**L2221P**	**5109delG**-I156V
S 321	**IVS9+1G>C**	**S1099X**
S 328	**IVS42+4delG**	IVS35+2t>c
S 346	E2096K	WT
S 347	IVS28+5g>a	WT
S 353	**P1484S**-G1961E	P68L
S 354	**P1484S**-G1961E	P68L
S 355	**P1484S**-G1961E	P68L
S 360	G1961E	5961delGGAC
S 364	IVS35+2t>c	G1961E
S 365	L541P/A1038V	G1961E
S 377	**IVS42+4delG**	IVS35+2t>c
S 380	R653C	WT
S 413	R212C	T1019M
S 414	A1598D	G1961E
S 417	**G1078E**	G1961E
S 438	R1055W	WT
S 440	**4021ins24bp**	T1526M-G1961E
S 449	W1479X	**L2140Q**
S 450	W1479X	**L2140Q**
S 474	W1461X	G 1977S
S 486	WT	WT
S 492	R1098C/L1970F	**6548insTGAA**
S 528	**T977P**	IVS40+5g>a
S 531	**G690V**	**Q1332X**
S 532	**R572X**	**L1473M**-4733delGTTT
S 535	IVS40+5g>a	5917delG
S 550	IVS40+5g>a	**6750delA**
S 555	250insCAAA	WT
S 556	250insCAAA	WT
S 575	N96H	G1961E
S 590	W821R	IVS40+5g>a
S 592	V931M	R1108C
S 593	V767D	R2030X
S 594	G172S	G1961E
S 602	P1380L	G1961E
S 607	**E616K**	**L1580S-K2172R**
S 640	250insCAAA	S1696N
S 694	IVS35+2t>c	G1961E
S 725	IVS13+1g>a	**Q1376 splice**
S 731	L541P-A1038V	G1961E
S 755	N965S	IVS40+5g>a
S 789	E1087K	G1977S
S 968	T1019M	G1961E
S 992	R212C	G1961E

Bold values indicate novel mutations.

followed by a renaturing step (30 min at 37°C). The PCR products were then separated through a 5% linear acetonitrile gradient. Commercially available WAVE Optimized™ buffers (A, B, and D) and Syringe Solution (Transgenomics) were used to provide highly reproducible retention times. Samples showing heteroduplex by DHPLC were finally sequenced on an automated sequencing system (ABI Prism 3100 genetic Analyser) using Big Dye Terminator chemistry (Applied Biosystems). PCR products were purified according to QIAquick PCR Purification Kit (Quiagen GmbH). Finally, data obtained from the Sequence Analysis Software (Applied Biosystems) were aligned with the wild-type *ABCR* gene sequence (GenBank database; http://www.ebi.ac.uk/). According to the EMQN Best Practice Guidelines, a sequence mismatch was considered as a disease-causing mutation only if it is absent in 150 healthy controls,[18–20] associated with amino-acidic change, confirmed by a new independent PCR, and, whenever possible, by restriction enzyme digestion. As a final point, the degree of evolutionary conservation of the affected residue, directly related to its importance in the functional protein structure, was assessed by interspecies amino-acid alignment analysis.

Results

Seventy-one Italian patients with a diagnosis of STGD (from 62 independent pedigrees) were clinically examined. DNA samples of the affected individuals were analysed for mutations in all 50 exons of the *ABCR* gene by DHPLC approach and by direct sequencing techniques. Seventy-one mutations were identified in 68 patients with a detection rate of 95.7%. Forty-three mutations had already been reported in the literature,[5,10–14,17–24] whereas 28 mutations had not been and, further, were not detected in 150 unaffected control individuals (300 chromosomes) of Italian origin.[18–20] The mutations identified in our series are reported in Table 2.

Forty-two mutations (59.2%) were missense mutations, 10 (14.1%) were nonsense mutations, 9 (12.6%) were splice mutations, and 10 (14.1%) were frameshift mutations.

The majority of patients (42/71, 59.1%) were compound heterozygotes for two missense mutations or one nonsense and one missense mutations, and 12 patients (16.9%) were simple heterozygotes.
Only three patients were found to be homozygous for *ABCR* variants (324-327insT, V1433I, and A1598D). Potential complex alleles were identified in 11 patients (15.5%). Unfortunately, because of a lack of parental DNA, the exact genotype was not determined in nine cases.

G1961E was the most common mutated allele among our STGD patients of Italian origin. It was found, always in heterozygosis, in 40.8% of our series (29/71; and then in 20.4% of our STGD alleles). The next most common mutated allele was the splicing mutation IVS40 + 5G → A, reported, always in heterozygosis, in 8.4% (6/71) of our series (and then in 4.2% of our STGD alleles). In our series, the detected mutations occurred with equal frequency in predicted conserved regions and in non-conserved regions.

Thirty different alterations were present in >1% of the control alleles and were classified as polymorphisms (Table 3).

Table 3 Summary of the polymorphic variants identified in the *ABCR* gene in our series of STGD Italian patients

Location	*Polymorphic variants*	*Number of alleles*
Exon 3	IVS3 + 26a > g	14
Exon 5	D159	1
Exon 6	R212H	6
Exon 7	IVS7-32t > c	9
Exon 10	H423R	12
Exon 13	D644	1
Exon 14	IVS14 + 50t > c	1
Exon 15	IVS15-13t > c	2
Exon 16	IVS16-13c > t	1
Exon 19	R943Q	3
Exon 20	L1988	1
Exon 23	Q1169	4
Exon 23	IVS23 + 25g > a	2
Exon 24	T1176	6
Exon 24	K1182	3
Exon 28	P1401	1
Exon 33	IVS33-39t > c	2
Exon 34	IVS34 + 16insgtt	4
Exon 38	D1817Q	7
Exon 40	N1868I	3
Exon 40	L1894	16
Exon 41	L1938	15
Exon 42	P1948	23
Exon 44	I2023	5
Exon 44	IVS44-16g > a	5
Exon 44	IVS44 + 77g > a	1
Exon 45	I2083	5
Exon 46	D2095	19
Exon 48	IVS48 + 21c > t	3
Exon 49	S2255I	5

Discussion

We have optimized DHPLC conditions for mutation analysis of all 50 exons of the *ABCR* gene in a study group of Italian STGD patients.

In 95.7% of STGD patients (68/71), we reported some mutations of the *ABCR* gene. This detection rate is in conformity with Fumagalli *et al*[11] (94.4%), and it is somewhat higher than Simonelli *et al*[10] (73%).

This might be due to mild technological differences in mutation analysis, to the strict clinical inclusion criteria, and/or to the lack of a functional analysis to select disease-causing mutations.

In our series, we detected 71 mutations. Missense mutations made up the majority (59.2%), followed by nonsense (14.1%) and frameshift (14.1%) mutations. Splicing mutations represented 12.6%. These data are in agreement with the results of earlier investigations in the Italian population and with other studies in a sizeable series of patients.[9,12,25]

We identified 28 novel *ABCR* mutations, confirming and further highlighting the high heterogeneity of the *ABCR* gene in STGD. The frequent report of novel mutations is probably related to the enormous number of exons in the *ABCR* gene, which favours rearrangement in the DNA sequence.

The majority of patients (59.1%) were compound heterozygotes. Only three patients were found to be homozygous for variants. A single disease allele was found in 16.9% of our series. No disease allele was reported in 4.3% of our patients. Potential complex alleles were identified in 11 patients (15.5%). Possible unidentified mutations might be located in parts of the gene that had not been screened; alternatively, our investigation technique could have failed to detect certain variations. The possible influence of modifier genes in the clinical expression of the *ABCR* gene variants must be considered.

Concurring with earlier studies,[9,11,13,26] *ABCR* mutant alleles are distributed throughout the entire coding sequence, and no mutational hotspots seem to exist.

In our series, mainly consisting of patients coming from central Italy, G1961E was the most common mutant allele, in congruence with other studies performed in distinct dissimilar European populations.[9,20] Nevertheless, the frequency of G1961E mutation (20.4% of our STGD alleles) was higher than in the other Italian

studies where this mutation was detected in 11.1[10] and 9.7% [11] of the screened alleles.

In earlier reports, G1961E was considered a mutation with a low pathogenetic influence,[10,13] but in our series, it may be associated with STGD phenotypes of a varying severity. Therefore, it can be speculated that its clinical expression depends on the mutation severity on the fellow allele, as suggested by earlier genotype/phenotype studies.[17,21]

Still, although the A1038V mutation is commonly reported in the literature,[9,13,27] in our series, it was detected in only two patients (1.4% of the patients, 0.7% of the alleles) within the L541P-A1038V complex allele. Moreover, in the Italian studies, this mutation was reported in 4.1[10] and 5.5%[11] of the alleles. These data suggest a possible regional distribution of specific mutations.

In our series, the reported mutations occurred with equal frequency in predicted conserved regions and in non-conserved regions. These data underscore the importance of screening the entire coding sequence of the *ABCR* to search for mutant alleles and not merely to seek out that portion predicted to encode conserved domains.

Affected members of the same family occasionally showed variable phenotypes (various age of onset, different visual loss severity, presence of flecks, or dark choroid), in agreement with some earlier reports of reduced penetrance and variable clinical expressivity of the *ABCR* mutations in STGD.[27–31] The variable expressivity of *ABCR* mutations might be due to the influence of environmental factors or unknown modificator genes. This suggests that molecular genetic results must be interpreted with caution especially in the context of genetic counselling for family planning.

In our series, the homozygous independent genomes (three unrelated patients) continuously showed a rapidly progressive clinical course, even if with a variable age of onset of the disease (8, 31 and 38 years). The presence of flecks and the phenomenon of dark choroid could be appreciated in all three patients.

In conclusion, 28 novel *ABCR* mutations were identified in our series of 71 Italian patients with diagnoses of STGD. A concentrated knowledge of the *ABCR* mutation spectrum, and further information about genotype/phenotype correlations are essential to understand the physiopathology of STGD and to evaluate the chances for patients to be referred to future possible therapeutic options.

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