Clinical and molecular genetic study of 12 Italian families with autosomal recessive Stargardt disease

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ABSTRACT. Stargardt disease was diagnosed in 12 patients from 12 families using complete ophthalmologic examination, fundus photography, fundus autofluorescence, and spectral-domain optical coherence tomography. DNA was extracted for polymerase chain reaction (PCR) and direct DNA sequencing (*ABCA4* gene). Genetic counseling and eye examination were offered to 16 additional family members. Various patterns of presentation were observed in patients with clinical diagnoses of Stargardt disease. The genetic study identified 2 mutations in 75% of families (9/12); a second mutation could not be found in the remaining 25% of families (3/12). The most frequent mutation was G1961E, found in 17% of families (2/12). This finding is similar to that of a previous analysis report of an Italian patient series. Four new mutations were also identified: Tyr1858Asp, Leu1195fsX1196, p.Tyr850Cys, and p.Thr959Ala. Our results suggest that PCR and direct DNA sequencing are the most appropriate techniques for the

analysis of the *ABCA4* gene. However, this method requires supplementation with specific PCR analysis to diagnose large deletions. The study of the families identified healthy carriers and affected subjects in presymptomatic stages and was also useful for evaluating the risk of transmission to progeny. Combined ophthalmologic and genetic evaluation enabled better clinical management of these families.

Key words: *ABCA4*; Mutation; Stargardt disease

INTRODUCTION

Autosomal recessive Stargardt disease (STGD) is caused by mutations in the *ABCA4* gene (Allikmets, 1997) and manifests with progressive loss of central visual function owing to macular degeneration. The mean age of onset is the second decade, although cases with onset in the first or after the fourth decade have also been reported (Fishman, 1976).

The disease accounts for 7% of all retinal dystrophies and has a frequency of approximately 1 per 10,000. It is the most common form of macular degeneration in young people (Weleber, 1994; Molday et al., 2009). The *ABCA4* gene (NCBI Ref-Sec: NM_000350.2) is composed of 50 coding exons that produce a transcript with a 7309-bp coding region (Allikmets et al., 1997; Nasonkin et al., 1998) comprising coding 131 polymorphisms and more than 500 mutations associated with STGD. This gene is considered one of the most polymorphic genes in the human genome (Kaplan et al., 1993; Gerber et al., 1995). The *ABCA4* gene codes for a membrane protein of the ATP-binding cassette family expressed in the external segments of retinal photoreceptors (Allikmets et al., 1997). This protein is an ATP-dependent membrane transporter that translocates N-retinylidene-phosphatidylethanolamine, a lipid substrate involved in the chemical cycle of vision (Beharry et al., 2004), and it is composed of 12 transmembrane regions that form a transport channel, 2 large extracellular domains that recognize a lipid substrate and 2 triphosphate nucleotide-binding domains that are hydrolyzed to produce the energy necessary for translocation (Illing et al., 1997; Bungert et al., 2001). Lack of this protein increases levels of retinal all-trans, protonated *N*-retinylidene-phosphatidylethanolamine, and phosphatidylethanolamine in the retina that lead to the formation of toxic compounds in the cells of the retinal pigmented epithelium, in turn inducing apoptosis and retinal degeneration (Weng et al., 1999; Sparrow et al., 2000; Molday et al., 2009).

The usual strategies for finding mutations in the *ABCA4* gene are DNA analysis through denaturing high-performance liquid chromatography (DHPLC) combined with direct sequencing of fragments with heteroduplex peaks, direct DNA sequencing of the entire coding region of the gene, and microarray analysis, such as the ABCR400 chip (Asper Ophthalmics, Tartu, Estonia), which identifies the 400 most typical mutations (Jaakson et al., 2003). Multiplex ligation-dependent probe amplification is now increasingly used to supplement previous strategies and identify large deletions.

Therapies currently being tested for autosomal recessive STGD include recombinant proteins (mainly anti-apoptotic growth factors such as nerve growth factor) (Lambiase et al., 2010; Colafrancesco et al., 2011) injected into the posterior chamber of the eye and drugs that decrease the concentrations of retinal 11-cis in the photoreceptors through sight cycle inhibitors such as *N*-(4-hydroxyphenyl) retinamide or isotretinoin (Golczak et al., 2005; Travis et

al., 2007; Molday et al., 2009). Innovative approaches such as gene therapy and stem cells are also being explored, but clinical application is not imminent (Cideciyan et al., 2009; Molday et al., 2009; Dahlmann-Noor et al., 2010).

Various studies have sought correlations between mutations, severity, and evolution of STGD for genetic counseling purposes. Although preliminary and fragmented, these studies have established that mutations in the active site of the nucleotide-binding domain 2 of the protein (for example, the mutation C2150Y) are the most severe, followed by large deletions leading to truncated proteins. Mutations responsible for single amino acid substitutions are linked to milder phenotypes with late onset and slow progression (Rozet et al., 1999; Briggs et al., 2001; Cideciyan et al., 2009).

Pilot studies have endeavored to relate mutations of the *ABCA4* gene to age of onset of STGD. The predictive model proposed by Cideciyan et al. (2009) uses 2 mutations that give rise to a truncated protein as a reference. The mutations are associated with an intermediate age of onset of 10.7 years. Comparison of a series of patients in which various mutations were associated with the reference mutation has quantified the contribution of the mutations to phenotype and age of onset, and an algorithm to estimate age of onset (limited to the panel of mutations studied) has been proposed. Estimates showed a consistency better than a decade (Cideciyan et al., 2009).

In Italian statistical samples, more than 100 probands have been described from all regions of the country. The most interesting aspect is that mutation G1961E is the most common in Italy and is associated with a milder phenotype (Simonelli et al., 2005; Passerini et al., 2010; Sodi et al., 2010).

In this study, we evaluated subjects with clinical diagnoses of STGD through ophthalmic as well as genetic examinations.

MATERIAL AND METHODS

Genetic study

Blood samples from patients and 16 additional family members were sent to MAGI Laboratory (MAGI Non-Profit Human Medical Genetics Institute, Rovereto, Italy) and used to extract DNA with a kit based on salting out (Blood DNA Kit E.Z.N.A.; Omega Bio-Tek Inc., Norcross, GA, USA). All 50 exons of the *ABCA4* gene in the intron/exon junction region were amplified using polymerase chain reaction (PCR) and sequenced with a Beckmann Coulter CEQ 8000 sequencer (Beckmann Coulter, Milano, Italy). Primer sequence, PCR conditions, and sequencing information are available on request.

Clinical study

Twelve patients with suspected STGD (from 12 families) were examined at the eye clinic, in the Department of Clinical Science "Luigi Sacco", Sacco Hospital, University of Milan, Italy. A team of ophthalmologists and medical geneticists (the latter from MAGI Non-Profit Human Medical Genetics Institute) performed a series of clinical and other tests to reach a diagnosis. The diagnosis was confirmed with genetic testing whenever possible.

All research procedures were performed according to institutional guidelines and the Declaration of Helsinki. Patients enrolled in the study signed 2 informed consents - one for genetic testing and another to make the clinical and genetic data available for research and publication. The nature of the procedure was fully explained.

Patients with clinically suspected STGD underwent second-level examinations with fundus photography, fundus autofluorescence (FAF), and spectral-domain optical coherence tomography (SD-OCT). The criteria for clinical diagnosis of STGD were bilateral progressive central visual loss, macular dystrophy or atrophy, and the presence of yellowish-white flecks.

Demographics, age of onset, and duration of the disease were recorded for subjects with definite diagnosis of STGD. The age of onset was defined as the age at which visual loss was first noted or Stargardt disease was suspected on a clinical basis.

FAF images and SD-OCT scans were obtained using a confocal scanning laser ophthalmoscope (Spectralis Heidelberg Retinal Angiography, Heidelberg Engineering, Heidelberg, Germany). FAF was obtained at 30 and 55 degrees to allow evaluation of the mid-periphery. We evaluated macular autofluorescence, the presence of flecks, and involvement of the mid-periphery. SD-OCT was performed as a single 30-degree horizontal scan through the fovea using 1536 A-scan/B-scan and 100 frames for image averaging to eliminate speckle noise. We evaluated alterations of inner segment/outer segment photoreceptors and retinal pigmented epithelium.

Bioinformatics study

The electropherograms of all amplified fragments were compared with the reference sequences using Basic Local Alignment Search Tool (BLAST) 2 sequences (http://blast.ncbi.nlm.nih.gov). The fragments containing sequence variations were then analyzed using nucleotide BLAST to determine whether the sequence variations were present in the data bank and coded as polymorphisms. For the identification of mutations, the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk) and the Retina International database (http://www.retina-international.org) were also consulted. This analysis enabled us to classify the sequence variations (if present in the databases) as mutations or polymorphisms.

Identification of new mutations

Sequence variations not found in the literature or databases were further investigated using BLAST-x and BLAST 2 sequences to characterize amino acid substitutions or premature stop codons. Sequence variations at intron/exon junctions were analyzed using GeneNet2 (http://genenet2.uthsc.edu), which predicts the efficiency of the splicing process. Single amino acid substitutions were also studied with UniProt (http://www.uniprot.org/) and tBLASTx to forecast the effect of the substitution. If the amino acid in the substitution position is highly conserved in evolution, substitution with an amino acid belonging to the same class (e.g., polar) is presumed to cause a protein functional defect.

The possible mutations were then sought with PCR combined with DNA sequencing of 100 healthy subjects from the same geographic area. If the variation was found in other DNA, it was considered a polymorphism; if not, the hypothesis of mutation was confirmed.

RESULTS

Genetic evaluation

In 9 of 12 subjects, 2 mutations were identified and in 3 of 12 only 1 mutation was found (Table 1). During our analyses we encountered 4 new mutations that we characterized bioinformatically.

Table 1. List of mutations and optical informations in our study population.

No. of patients	Subject	Allele 1	Allele 2	Age of diagnosis (years)	Visual acuity	
					Right eye	Left eye
1	F1					
	ID81	Tyr1858Asp	Met1Val; Arg2030Gln	22	20/50	20/32
2	F2					
	ID220	Ile156Val	Gly607Arg; Gly1961Glu	30	20/800	20/400
3	F3					
	ID362	Met1Val	Gly1961Glu; Arg2030Gln	60	20/40	20/32
4	F4					
	ID197	Asp1532Asn	Arg2030term	40	20/32	20/32
5	F6					
	ID363	Tyr362Term	Gly863Ala	16	20/200	20/250
6	F7					
	ID365	Arg1098Cys	Cys1488Arg	50	20/32	20/800
7	F8					
	ID394	Arg18Trp	Val767Asp	10	20/800	20/800
8	F9					
	ID396	IVS40+5G>A	IVS13+1G>A	19	20/40	20/50
9	F10					
	ID366	p.Gln1513Profs*42	-	20	20/200	20/200
10	F12					
	ID377	Leu1195Argfs*2	-	50	20/32	20/20
11	F13					
	ID4	Cys2150Tyr	-	70	20/400	20/400
12	F17					
	ID457	p.Tyr850Cys	p.Thr959Ala	50	20/20	20/40

F1 = family 1; ID = reference code to a specific patient.

In family F1 we found the new mutation Tyr1858Asp, in which an amino acid with an aromatic ring with high stearic hindrance was substituted for another having an alcohol group but only small variations in polarity (from acid polarity to neutral). Amino acid 1858 is situated in the extracytoplasmic region of the bridge protein between 2 alpha helixes. During evolution, the amino acid tyrosine has been conserved in that position of the protein in all species of animals since amphibians. A reasonable assumption is that substitution of the amino acid would severely impair protein function. In this family, a segregation study located the new mutation in the transposition (on the second allele) with respect to the other 2 known mutations on the first allele.

In family F12 we found a new mutation, p.Leu1195fsX1196, featuring the insertion of 2 bases into exon 24. This insertion leads to a reading frame shift in the coding region downstream, which results in a change in amino acid 1195 from leucine to arginine and the appearance of a stop codon in the next position, generating a truncated protein.

In family F17 we found two 2 new mutations, p.Tyr850Cys and p.Thr959Ala. The

first involved the substitution of an apolar amino acid with a polar one. This amino acid lies in the transmembrane domain of the protein. During evolution, tyrosine is conserved in that position in all vertebrates. The second mutation featured the substitution of a polar amino acid with an apolar one. This amino acid lies in the cytoplasmic domain of the protein. During evolution, threonine is conserved in that position in all vertebrates.

Clinical study

Twenty-four eyes of 12 consecutive patients with suspected STGD referred to Sacco Hospital from March 2009 to October 2010 were studied. Five patients were male and 7 were female. Mean age was 54 years (range, 19 to 78 years).

The age of onset of the disease of the subjects ranged from 10 to 70 years (mean age of onset was 35 years). According to previous studies, early onset is considered to occur before 20 years and late onset is that occurring after 20 years of age.

Visual acuity ranged from 20/800 to 20/20 with a mean of 20/64. FAF showed several patterns:

- abnormal hypo- and hyperautofluorescence in focal deposits distributed at the posterior pole were found in 7 eyes (27%), 3 of which showed focal areas of atrophy in the macular region;
- multiple confluent foci of low autofluorescence in the macular region (macular dystrophy) surrounded by a ring of hyperautofluorescence were found in 6 eyes (23%);
- confluent areas without autofluorescence (atrophy) were found in 4 eyes (15%) in the macular region, and
- a central area without autofluorescence (macular atrophy) was found in 9 eyes (35%).

All patients showed flecks in various stages at the posterior pole, 4 had flecks in the middle periphery, and 2 displayed peripapillary flecks.

SD-OCT revealed disorganization or loss of inner segment/outer segment junction or retinal pigmented epithelium in all eyes except one.

No particular patterns were found in patients with new mutations.

In patients with the G1961E mutation, the most frequent *ABCA4* alleles in Italian STGD patients, we observed various clinical features: one patient had late onset of the disease (30 years) and showed multiple foci of hypoautofluorescence surrounded by a hyperautofluorescent area in the macular region. One patient had very late onset of the disease (60 years) and confluent areas of atrophy.

DISCUSSION

In this study, we found various patterns of presentation in subjects with STGD. This finding is consistent with previous observations (Cremers et al., 1998). However, in other studies, the authors have been able to correlate specific mutations with phenotypes of STGD (Passerini et al., 2010).

On the contrary, our results revealed major heterogeneity in patients with specific genetic mutations, making the correlation with specific mutations impossible. For example,

patients with the G1961E mutation had extremely different ages of onset and fundus findings. We cannot exclude the possibility that this result is due to our small sample size, which in this sense is a limitation of the present study.

In previous studies, age of onset has been considered a criterion defining disease severity: cases with earlier onset generally develop more severe fundus alterations. However, in our study, some patients with late onset developed severe fundus alterations. In particular, 4 patients reported early onset, and the severity of clinical features correlated well with this feature; 8 patients reported later onset, but 4 of them had clinical features strongly suggesting earlier onset. In our opinion, age of onset is not always a helpful indicator of severity. A possible explanation is that the diagnosis or suspected diagnosis of STGD is usually made when visual symptoms occur and the patient is referred for ophthalmological evaluation. Nevertheless, atrophic changes may also involve the macula but spare the fovea. In these instances, patients may not experience a significant loss in visual acuity or seek clinical evaluation, delaying the age of diagnosis. In these cases the age of onset may be inaccurately determined as late.

In this study, we used PCR and direct sequencing of the entire coding region and intron/exon regions of the *ABCA4* gene to identify mutations. We succeeded in identifying 2 mutations in 75% of families (9/12), but were unable to identify a second mutation in 25% (3/12). The most frequent mutation was G1961E, found in 17% of families. This percentage is similar to that reported for other patient populations in Italy (Sodi et al., 2010).

In families in which only 1 mutation was found, large deletions were also likely; indeed, the literature indicates large deletions in at least 11% of such families (Sodi et al., 2010). The results of the present research prompt us to add a method suitable for identifying large deletions to our diagnostic strategy.

Automation enables the performance of the genetic test we propose (DNA extraction, PCR, and direct sequencing of DNA) in 4 working weeks at a cost of US$2500. Our method is more sensitive - recognizing up to 10% more mutations - than that using DHPLC. With DHPLC, sequences with heteroduplex peaks require direct sequencing of DNA in any case. Because the *ABCA4* gene has many polymorphisms, various laboratories have discarded this approach, as it requires the sequencing of approximately half the fragments amplified.

A microarray approach (such as that using the ABCR400 chip by Asper Ophthalmics) would not have identified 3 of the mutations found in 25% of our population (3/12 families) or the second mutation found in 8% (1/12), presumably carriers of large deletions in 1 allele. In the families in our study, the use of DHPLC would have required direct sequencing of 42% of the DNA (5/12 families). Our approach, therefore, remains the most reliable, rapid, and economical method for genetic testing.

Once the sequence variations of the proband were identified, we checked for them in other family members. This approach enabled us to find healthy carriers and other affected family members, even those in presymptomatic stages. Thus, we were able to offer more complete genetic and clinical counseling. In our experience, patients want to know how symptoms are likely to progress and the risk of transmitting STGD to their children. They are also willing to take part in clinical trials, and to do so, they need to know what mutations are responsible for the disease.

We were able to identify 4 new mutations in the *ABCA4* gene responsible for STGD. Our data suggests that the best approach for genetic evaluation is PCR combined with direct sequencing of the entire coding region and intron/exon regions.

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