Evaluation of the *ELOVL4*, *PRPH2* and *ABCA4* genes in patients with Stargardt macular degeneration

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Abstract. Mutations in the ATP-binding cassette, subfamily A, member 4 (*ABCA4*), elongation of very long chain fatty acids 4 (*ELOVL4*) and peripherin-2 (*PRPH2*) genes have been identified in patients with Stargardt macular degeneration (STGD). The aim of this study was to investigate which of these genes is responsible for susceptibility in Chinese patients. A total of 41 probands with STGD or suspected STGD were enrolled in the study. The coding regions and adjacent intronic sequences of the *ELOVL4* and *PRPH2* genes and 3 coding exons of the *ABCA4* gene were amplified by polymerase chain reaction (PCR). The nucleotide sequences of the amplicons were determined by Sanger sequencing. Three novel heterozygous missense mutations in the *ABCA4* gene were identified: c:2633C>A (p:Ser878X), c:5646G>A (p:Met1882Ile) and c:6389T>A (p:Met2130Lys). These mutations were not present in 176 normal individuals and were predicted to be pathogenic. Two benign variations were found: a reported variation, c:5682G>C in *ABCA4* and a novel variation, c:699G>A in *ELOVL4*. In addition, 5 single nucleotide polymorphisms (SNPs: rs3812153, rs7764439, rs390659, rs434102 and c:929G>A) were detected in *ELOVL4* and *PRPH2*. The c:929G>A variation has not been previously reported. We conclude that no pathogenic variations in *ELOVL4* and *PRPH2* were detected in the Chinese STGD patients. Our results imply that *ABCA4* is more likely to be significant in Chinese STGD patients.

Introduction

Stargardt macular degeneration [STGD/fundus flavimaculatus (FFM), OMIM #248200] is the most common type of hereditary macular dystrophy, which affects approximately 1 in 10,000 individuals (1-3). Characteristic features include the loss of central vision in the first or second decade of life (4), progressive atrophy of the macula and underlying retinal pigment epithelium and the frequent presence of yellow or white lipofuscin deposits in the posterior pole of the retina (5). To date, mutations in more than 3 genes have been identified as causing Stargardt's disease. STGD1 is the most frequent type of juvenile macular dystrophy, which may be caused by the inheritance of ATP-binding cassette, subfamily A, member 4 (*ABCA4*) in an autosomal recessive manner (6). STGD3 may arise from the autosomal dominant inheritance of elongation of very long chain fatty acids 4 (*ELOVL4*) (7) and has a clinical profile that is very similar to STGD1. Mutations in the peripherin-2 (*PRPH2*) gene are an important cause of multifocal pattern dystrophy with Stargardt-like flecks (8).

The *ABCA4* gene (OMOM: 601691) contains 50 exons and encodes a photoreceptor-specific ATP-binding cassette transporter (ABCR). To date, over 600 different *ABCA4* mutations are known (9,10); however, the most frequent disease-associated *ABCA4* alleles have each been described in only 10% of STGD patients (11). Complex *ABCA4* alleles (2 pathogenic variants in *ABCA4*) are common. Furthermore, approximately 1 in 20 people across all populations carry a potentially disease-associated variant of this gene (12); therefore, the molecular scanning and analyses of *ABCA4* are labor-intensive.

ELOVL4 (OMOM: 605512) contains 6 exons and encodes a putative protein of 314 amino acids which is involved in fatty acid chain elongation. Three disease-associated mutations in exon 6 of *ELOVL4* have been reported (7,13,14). *PRPH2* (OMOM:179605) contains 3 exons. Nine different disease mutations have been identified in *PRPH2* (8,15,16).

Mutation analyses have been performed as a cost-effective method of diagnosis (9,17). However, to date, mutation analyses of Chinese patients with STGD are rare (18). In order to evaluate the role that the *ELOVL4*, *PRPH2* and *ABCA4* genes play in STGD in Chinese patients, we analyzed the coding exons and the adjacent regions of the *ELOVL4* and *PRPH2* genes and 3 coding exons of the *ABCA4* gene in 41 patients from unrelated Chinese families with STGD or suspected STGD.

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Table I. Primers used for the amplification and sequencing of *ELOVL4*, *PRPH2* and *ABCA4* genes.

Gene	Exon and direction	Primer sequence (5'-3')	Size of amplified fragment (bp)	Annealing temperature (˚C)	GC buffer
ELOVL4	1 F	ccttgaggagcaggagaaga	446	60[a]	Yes
	1 R	gaggggaggccttaacattc			
	2 F	tgggactcaaaggacagtga	577	64[a]	Yes
	2 R	aactttcaatgccagaacagc			
	3 F	agcaatcggaatgcatgaaa	423	57	Yes
	3 R	ggggacagagcaagaaactg			
	4 F	cccatggagagatgcttagg	480	57	No
	4 R	aaaaagaaatgaacatggaaatg			
	5 F	tctagcttaatctgaagggaaaac	498	58[a]	No
	5 R	caaagatttgctgggaccaa			
	6 F	catgggagccagaaaacaat	597	57	No
	6 R	tcataaataaaacatctgggtatgg			
PRPH2	1-1 F	gtgggactcgacatgggtag	590	64[a]	Yes
	1-1 R	ggtgtctgtgtcccggtagt			
	1-2 F	cgaaagaggagcgatgtgat	587	57	Yes
	1-2 R	ccctcacatacgcagcaata			
	2 F	cacagcaaatatataccaagtgtgc	500	57	Yes
	2 R	cagctccactgaaggctgtt			
	3 F	accaacccacactccacagt	486	57	Yes
	3 R	attccaccgtcagggagagt			
ABCA4	17 F	agatcttatagaactgcggtaagg	233	57	Yes
	17 R	atagagggccacctctgtga			
	40 F	tttggctcttgctcagttcc	337	57	Yes
	40 R	gggctcctgaggaaagaaat			
	47 F	catcccacaggcaagagatt	267	57	Yes
	47 R	gcagcaggactcttccaagt			

ELOVL4, elongation of very long chain fatty acids 4; *PRPH2*, peripherin-2; *ABCA4*, ATP-binding cassette, subfamily A, member 4; F, forward sequence; R, reverse sequence; Yes, GC buffer was used in the amplifications. [a]PCR began at a temperature 4˚C above the annealing temperature.

Materials and methods

Subjects. Probands with STGD or suspected STGD from 41 unrelated families were enrolled in this study. Written informed consent was obtained from the participating individuals or their guardians prior to the collection of clinical data and genomic samples. This study was approved by the Internal Review Board of the Zhongshan Ophthalmic Center, Guangzhou, China. Thorough clinical ophthalmic examinations were performed, including ophthalmic and family history, funduscopic examination and best corrected visual acuity. Genomic DNA was prepared from venous blood from each participating individual (19).

Mutation detection. Genomic DNA was prepared from peripheral leukocytes as described previously (20). Table I lists the primers used to amplify the coding exons and adjacent introns of the *ELOVL4* gene (NCBI human genome build 37.3, NG_009108.1 for genomic DNA, NM_022726.3 for mRNA and NP_073563.1 for protein), *PRPH2* gene (NG_009176.1 for genomic DNA, NM_000322.4 for mRNA and NP_000313.2 for protein) and *ABCA4* gene (NG_009073.1 for genomic DNA, NM_000350.2 for mRNA and NP_000341.2 for protein). Only 3 exons (exon 17, 40 and 47) were screened. Touchdown polymerase chain reaction (PCR) was performed with a temperature decrease of 0.5˚C per cycle from 64˚C for the first 15 cycles, and continued at 57˚C (the annealing temperature) for the remaining 21 cycles. The PCR marked by 'a' began at a temperature 4˚C above the annealing temperature, then decreased by 2˚C per 5 cycles and was continued at the optimal annealing temperature (listed in Table I) for the remaining 25 cycles. The DNA sequences of the amplicons were identified using the ABI BigDye Terminator cycle sequencing kit version 3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI 3100 Genetic Analyzer (Applied Biosystems). Sequencing results and consensus sequences from the NCBI human genome database were compared using the SeqManII program of the Lasergene software package (DNASTAR Inc., Madison, WI, USA) and then aligned to identify variations. Each variation was confirmed by bidirectional sequencing. Mutation description followed the recommendations of the Human Genomic Variation Society (HGVS). Variations detected in patients were further evaluated by sequencing 176 normal individuals (controls).

Table II. Sequence variation of *ABCA4*, *PRPH2* and *ELOVL4* genes in STGD patients.

Gene	Exon	Variation	Effect	Homo/hetero	PolyPhen-2 prediction	Alignment	Note
Pathogenic							
ABCA4	17	c:2633C>A	p:Ser878X	Hetero			Novel
	40	c:5646G>A	p:Met1882Ile	Hetero	Possilby damaging	Highly conserved	Novel
	47	c:6389T>A	p:Met2130Lys	Hetero	Possilby damaging	Highly conserved	Novel
Benign							
ABCA4	40	c:5682G>C	p:Leu1894Leu		Benign		Reported
ELOVL4	6	c:699G>A	p:Thr233Thr	Hetero	Benign		Novel
	6	c:895A>G	p:Met299Val	Homo and hetero			rs3812153
PRPH2	1	c:318T>C	p:Val106Val	Homo and hetero			rs7764439
	3	c:910C>G	p:Gln304Glu	Homo and hetero			rs390659
	3	c:929G>A	p:Arg310Lys	Homo			Novel
	3	c:1013A>G	p:Asp338Gly	Homo and hetero			rs434102

STGD, Stargardt macular dystrophy; *ABCA4*, ATP-binding cassette, subfamily A, member 4; *ELOVL4*, elongation of very long chain fatty acids 4; *PRPH2*, peripherin-2; homo, homogeneous; hetero, heterogeneous.

Table III. Clinical information on individuals with *ABCA4* variations.

ID Number	Mutations		Gender	Age at onset	First symptom	Best corrected visual acuity		Macula	Posterior retina
	ccds	pro				OD	OS		
QT223	2633C>A	Ser878X	M	19	Blurred vision	0.1	0.1	Foveal reflex was blunted	Yellow and white exudation
QT292	6389T>A	Met2130Lys	M	>10	Blurred vision	0.1	0.2	Pigmental proliferation. Gold metal reflex	Normal
QT431	5646G>A	Met1882Ile	F	10	Blurred vision	0.2	0.2	Pigmental disorder. Foveal reflex was blunted	Normal

ABCA4, ATP-binding cassette, subfamily A, member 4; M, male; F, female; OD, *oculus dexter* (right eye); OS, *oculus sinister* (left eye).

The possible functional effect of amino acid substitution due to mutation was predicted using the Sorting Intolerant From Tolerant (SIFT) program and the Polymorphism Phenotyping v2 (PolyPhen-2) online tool (http://genetics.bwh.harvard.edu/pph2/index.shtml).

Results

After sequencing 3 exons of the *ABCA4* gene from 41 STGD or suspected STGD patients, 3 novel heterozygous missense mutations in the *ABCA4* gene were identified (Table II and Fig. 1A): c:2633C>A (p:Ser878X) mutation in exon 17, c:5646G>A (p:Met1882Ile) mutation in exon 40, and c:6389T>A (p:Met2130Lys) mutation in exon 47. All were absent from the 176 normal individuals and were predicted to be pathogenic. The C>A change at residue 2633 in exon 17 of the *ABCA4* gene, where serine was replaced by a stop codon at codon 878, resulted in a premature termination. The 2 missense mutations (p:Met1882Ile and p:Met2130Lys) were predicted by PolyPhen2 to be damaging and were highly conserved for *ABCA4* (Fig. 1B). In addition, a previously reported synonymous variation, c:5682G>C (p:Leu1894Leu), was observed in exon 40 of *ABCA4* (17).

There were 2 variations in *ELOVL4* and 4 variations in the *PRPH2* gene (Table II). For the novel variation c:699G>A in *ELOVL4* observed in 2 patients, the substitution did not change the amino acid. Five single nucleotide polymorphisms in the *ELOVL4* and *PRPH2* genes (rs3812153, rs7764439, rs390659, rs434102 and c:929G>A) were detected. The homozygous variation (c:929G>A) has not been previously reported.

Patients with pathogenic mutations in *ABCA4* had moderately reduced visual acuity in the second decade of life (Table III). All patients showed a blunted foveal reflex. The patient with a substitution of Ser878X had yellow or white

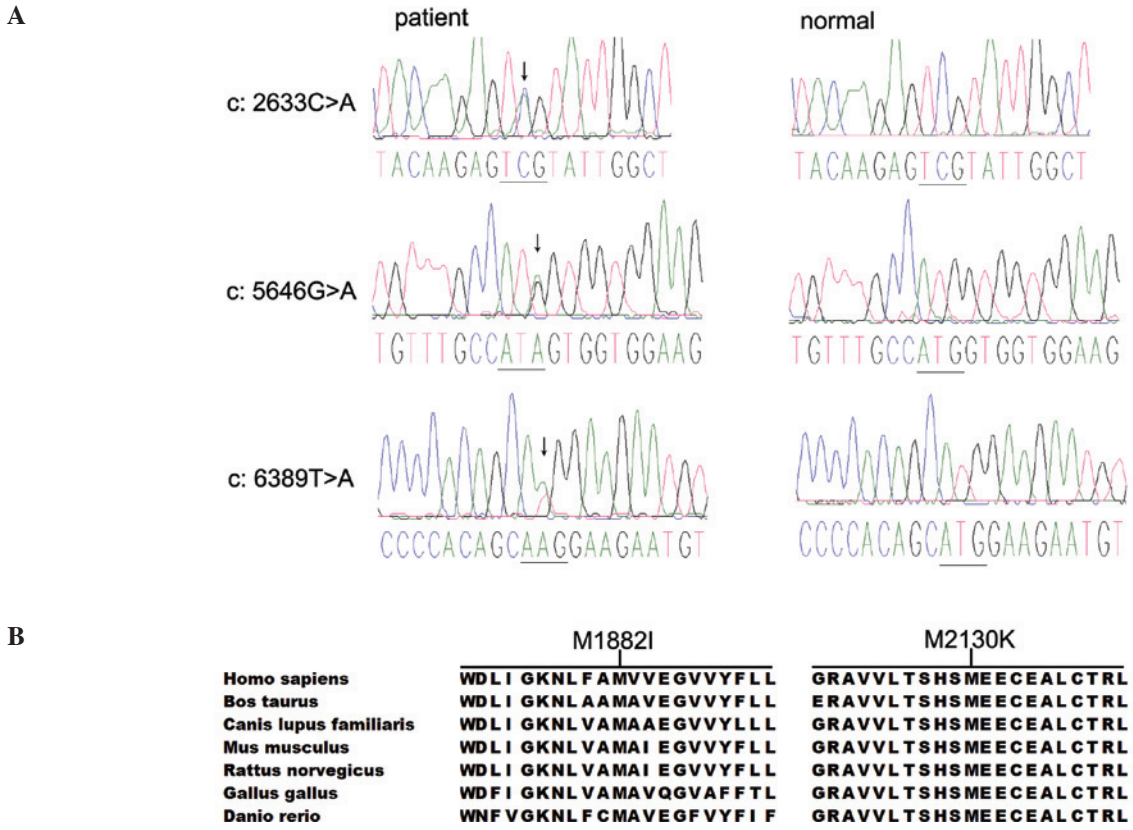

Figure 1. Sequence chromatography and protein sequence alignment of ATP-binding cassette, subfamily A, member 4 (*ABCA4)* orthologs. (A) Novel sequence changes (3) detected in the probands with *ABCA4* are shown (left column) compared with the corresponding normal sequences (right column). (B) Protein sequences are shown. Regions with the novel p:M1882I and p:M2130K mutations are highly conserved.

lipofuscin deposits in the posterior pole of the retina, while the patient with Met2130Lys presented with a macula having a bronze metal appearance.

Discussion

STGD presents in a wide range of phenotypes from mild FFM to multifocal pattern dystrophy. There is not an absolute STGD clinical phenotype; it has a wide degree of variability. All clinical features are also characteristic of age-related macular degeneration (21). The correct diagnosis and precise clinical data may be helpful for mutation-disease association studies. STGD1 may be distinguished from other macular degenerations by its autosomal recessive pattern of inheritance, and its hallmark feature is the presentation of a dark choroid during fluorescein angiography examination. There is not an absolute STGD3 clinical phenotype, except for its expression as an apparently dominant form. FFM is a late-onset, moderate form of STGD which is caused by mutations in *PRPH2*.

We screened the *ELOVL4* and *PRPH2* genes in 41 STGD patients or suspected STGD patients. Six variations were detected, but there were no pathogenic variations in *ELOVL4* and *PRPH2*. *ELOVL4* encodes an enlongase enzyme involved in the elongation of very long-chain fatty acids. All 3 known disease mutations are located in exon 6, resulting in a truncated protein missing the C-terminal segment (7,13,14). It is notable that all mutations were detected in large autosomal dominant families. In our study, most of the probands were sporadic, and there was no large autosomal dominant family in our STGD subjects. Although mutations in *PRPH2* are a significant cause of multifocal pattern dystrophy with Stargardt-like flecks (7,8), there was no pathogenic variation in *PRPH2* in our subjects. Similarly, Zernant *et al* did not find disease-associated mutations in the peripherin/*RDS* or *ELOVL4* genes of 30 to 40 STGD patients with no mutations in *ABCA4* (22) and Lai *et al* did not find disease-associated mutations in the *RDS* or *ELOVL4* genes in an autosomal dominant STGD3-like macular dystrophy pedigree (18). Extensive family data and typical clinical features may be helpful for pinpointing the possible genetic causes (23).

Mutations in *ABCA4* are responsible for almost all cases of classic Stargardt's disease (24). Frequent ethnic group-specific *ABCA4* alleles have been identified (25). We screened the 3 exons (17, 40 and 47) of *ABCA4* as disease-associated mutations in these exons have been reported more often in the Asian population (27-29). We found 3 novel mutations in the 3 exons: c:2633C>A (p:Ser878X), c:5646G>A (p:Met1882Ile) and c:6389T>A (p:Met2130Lys). The 3 missense variations were pathogenic, since the individuals with those mutations were STGD patients confirmed by precise clinical data. We found 3 novel variations in only 3 exons, which expanded the mutation spectrum of *ABCA4*. There are more than 600 variations and some complex alleles in *ABCA4*. Genetic analyses of *ABCA4* are complicated. High-throughput and cost-effective screening tools are helpful, including the *ABCA4* genotyping microarray (26) and next-generation sequencing strategy (22).

We will screen all 50 exons of *ABCA4* with high-flow analysis techniques in future studies.

In conclusion, our results not only reveal that no pathogenic variations in *ELOVL4* and *PRPH2* were detected in our Chinese STGD patients but also imply that *ABCA4* is likely to be significant in Chinese STGD patients.

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