Identification of Genetic Defects in 33 Probands with Stargardt Disease by WES-Based Bioinformatics Gene Panel Analysis

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Abstract

Stargardt disease (STGD) is the most common hereditary macular degeneration in juveniles, with loss of central vision occurring in the first or second decade of life. The aim of this study is to identify the genetic defects in 33 probands with Stargardt disease. Clinical data and genomic DNA were collected from 33 probands from unrelated families with STGD. Variants in coding genes were initially screened by whole exome sequencing. Candidate variants were selected from all known genes associated with hereditary retinal dystrophy and then confirmed by Sanger sequencing. Putative pathogenic variants were further validated in available family members and controls. Potential pathogenic mutations were identified in 19 of the 33 probands (57.6%). These mutations were all present in \textit{ABCA4}, but not in the other four STGD-associated genes or in genes responsible for other retinal dystrophies. Of the 19 probands, \textit{ABCA4} mutations were homozygous in one proband and compound heterozygous in 18 probands, involving 28 variants (13 novel and 15 known). Analysis of normal controls and available family members in 12 of the 19 families further support the pathogenicity of these variants. Clinical manifestation of all probands met the diagnostic criteria of STGD. This study provides an overview of a genetic basis for STGD in Chinese patients. Mutations in \textit{ABCA4} are the most common cause of STGD in this cohort. Genetic defects in approximately 42.4% of STGD patients await identification in future studies.

Introduction

Stargardt Disease (STGD), with a world-wide prevalence of at least 1:10,000 [1], is the most common hereditary macular degeneration in juveniles. It is characterized by macular dystrophy associated with loss of central vision in the first or second decade of life, a “beaten-metal” appearance in the fovea or parafoveal region, yellowish flecks around the macula or in posterior area of the retina, progressive atrophy of the bilateral foveal retinal pigment epithelium, and the “dark choroid” sign on Fundus Fluorescein Angiography (FFA) in 80% of patients [2,3].
STGD is frequently transmitted as an autosomal recessive trait but rarely as an autosomal dominant trait [4,5].

To date, mutations in five genes have been reported to be responsible for classic STGD or “Stargardt-like” disease (S1 Table): fatty acid elongase 4 (ELOVL4, MIM: 605512) [6–8], prominin 1 (PROM1, MIM: 604365) [5,9], peripherin 2 (PRPH2, MIM: 179605) [10,11], Bestrophin 1 (BEST1, MIM: 607854) [12] and ATP-binding cassette, sub-family A, member 4 (ABCA4, MIM: 601691) [13]. Mutations in these genes are responsible for over half of STGD cases, based on analysis of individual gene or a subset of genes [14,15]. Recently, targeted exome sequencing on 144 or 213 genes detected mutations in 100% (6/6) or 68% (59/88) of families with STGD [15,16], where 13% (11/88) of the families with atypical STGD had mutations in genes other than STGD-associated genes [15]. Additional comprehensive studies are expected to enrich the understanding of the genetic defects responsible for STGD.

In the present study, whole exome sequencing was performed on 33 probands with STGD, and potential pathogenic mutations were detected in 19 of these 33 patients.

Materials and Methods

Patients

All 33 probands from unrelated families and available family members were collected from Zhongshan Ophthalmic Center, Sun Yat-sen University. Written informed consent was obtained from participants or their guardians before the collection of their peripheral venous blood and clinical data. This study was approved by the Institutional Review Board of the Zhongshan Ophthalmic Center and was adherent to Declaration of Helsinki. Genomic DNA was extracted from the leukocytes of peripheral venous blood of each participating individual, as well as 96 unrelated healthy individuals [17] and 228 unrelated controls.

Whole exome sequencing

Genomic DNA from the 33 probands was initially analyzed by whole exome sequencing, as described previously [18]. Briefly, genomic DNA was fragmented and the fragments containing coding regions was captured by an Agilent SureSelect Human All Exon Enrichment Kit V4 array. The exome-enriched DNA fragments were sequenced with an Illumina HiSeq2000 at an average sequencing depth of 125-fold. All sequencing reads were aligned to the consensus sequence (UCSC hg19) with Burrows-Wheeler Aligner (BWA) (http://bio-bwa.sourceforge.net/) and variants were detected with SAMtools (http://samtools.sourceforge.net/). In the current study, variants for all genes related to retinal dystrophy based on RetNet (https://sph.uth.edu/RetNet/home.htm, accessed on August 14, 2014) were selected and then analyzed as follows: 1) exclusion of variants in non-coding regions or synonymous variants without affecting splicing site predicted by BDGP (http://www.fruitfly.org/seq_tools/splice.html); 2) exclusion of variants with minor allele frequency >0.01 determined with 1000 Genomes (http://browser.1000genomes.org/index.html), the Exome Variant Server (http://evs.gs.washington.edu/EVS/), ExAC (http://exac.broadinstitute.org/) and in-house controls; 3) exclusion of single heterozygous variants in genes responsible for recessive diseases; 4) exclusion of variants predicted to be benign by all three online tools (SIFT: http://sift.jcvi.org/; PolyPhen-2: http://genetics.bwh.harvard.edu/pph2/; and Proven: http://provean.jcvi.org/); and in-house controls. After the above four steps, the remaining variants were then confirmed by Sanger sequencing and were further evaluated in available family members as well as in controls.
Sanger Sequencing

For each candidate variant, primers were designed with the online tool Primer3 (http://primer3.ut.ee/) to amplify the genomic fragment harboring the variant (S2 Table). The amplicons were analyzed on an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA) with a BigDye Terminator cycle sequencing kit version 3.1. The sequencing results were aligned with consensus sequences from the Genome Bioinformatics database (http://genome.ucsc.edu/) to identify variants by SeqmanII program of DNASTAR Lasergene package (Lasergene version 7.1; DNASTAR, Madison, WI, USA).

Results

In total, 800 variants were initially detected in 163 genes by whole exome sequencing initially. After multi-bioinformatics filtering and confirmation, 28 potential pathogenic mutations were identified in 19 of the 33 probands (57.6%) (Table 1, S1 Fig). These mutations were all present in ABCA4, involving 13 novel and 15 known mutations. ABCA4 mutations were identified in 19 probands, including one proband with a homozygous mutation and another 18 probands with compound heterozygous mutations. Analysis of controls and available family members in 12 of the 19 families further support the pathogenicity of these variants (Fig 1). No potential mutations were detected in the other four STGD-associated genes (ELOVL4, PROM1, PRPH2, and BEST1) or in the 209 genes responsible for other forms of retinal dystrophy. Potential ABCA4 variants were identified in 8 probands (S3 Table), including 4 probands with two or more heterozygous variants but only one of them was predicted to be pathogenic, and 4 probands with only one heterozygous variant predicted to be pathogenic.

Clinical data of the 19 probands with ABCA4 mutations were listed in Table 2. Most of the probands had complaint of poor vision in their first decades. Ophthalmoscope examination of the fundus revealed one or more of typical clinic features, including a “beaten-metal” appearance in the foveal or parafoveal macula, yellowish flecks around the macula or in posterior retina, retinal pigment epithelium (RPE) atrophy and pigment disorder to different degrees, and a “dark” or “silent” choroid on Fundus Fluorescein Angiography (FFA) (Fig 2).

Discussion

In this study, we identified 28 mutations of ABCA4 in 19 of 33 (57.6%) unrelated probands with STGD. No potential pathogenic variations were detected in other four STGD-related genes and 209 genes responsible for other forms of retinal dystrophies listed in RetNet (S3 Table).

Previously, different sequencing technologies have been used to detect genetic defects of STGD patients in different populations [14–16,19–26]. Mutations in ABCA4 are the most common cause of STGD in previous studies [15,19,21,25]. Similarly, mutations ABCA4 have been detected in 57.6% STGD probands in our Chinese cohort. Several specific alleles have been reported in different populations [19–21], but no specific alleles were found in Chinese population in the present study due to the limited number of patients. The 15 known ABCA4 mutations were also found in the present study. Different patients with known mutations have different phenotypes, previously reports revealed that c.4773+1G>T and c.5196+1G>A associated with AMD (Age-related Macular Degeneration) and c.164A>G (p.H55R) associated with for cone-rod dystrophy [27–29], while homozygous c.4773+1G>T and compound heterozygous c.5196+1G>A and c.164A>G were associate with STGD in our study. Such varied phenotypes may be determined by the second mutation [30]. Three novel ABCA4 mutations were identified in three small families (QT085, QT302, and QT358) where family members was not available for analysis. These mutations occur in different protein domains, including
## Table 1. The ABCA4 causative variants in 19 Chinese probands with Stargardt disease.

| Patient ID | Nucleotide | Amino Acid | State of Change | Computational Prediction | Allele Frequency in Reported Populations | Reported |
|------------|------------|------------|-----------------|--------------------------|------------------------------------------|-----------|
| QT058      | c.6173T>G  | p.L2058R   | Het             | PrD                      | 0/192 0/456                              | Novel     |
|            | c.4773+1G>T| Splicing   | Het             | SSA                      | - 0/456                                  | Pang et al. 2002; Riveiro-Alvarez et al. 2013 |
| QT085      | c.6173T>G  | p.L2058R   | Het             | PrD                      | 0/192 0/456                              | Novel     |
|            | c.5932delA | p.K1978Qfs13| Het             | NA                       | 0/192 0/456                              | Novel     |
| QT292      | c.6389T>A  | p.M2130K   | Het             | PoD                      | - 0/456                                  | Yi et al. 2012 |
|            | c.6118C>T  | p.R2040*   | Het             | NA                       | 2/121394 0/192                           | Baum et al. 2003 |
| QT302      | c.6816+1G>A | Splicing   | Het             | SSA                      | - 0/456                                  | Robert et al. 2014 |
|            | c.555delA  | p.T1519Rfs*7| Het             | NA                       | 0/192 0/456                              | Novel     |
| QT398      | c.4352+1G>A | Splicing   | Het             | SSA                      | 1/121268 - 0/456                         | Ernest et al. 2009 |
| QT431      | c.5646G>A  | p.M1882I   | Het             | PoD                      | - 0/456                                  | Lewis et al. 1999; Wiszniewski et al. 2005; Heathfield et al. 2013 |
|            | c.1804C>T  | p.R602W    | Het             | PoD                      | 6/119038 - 2/456                         | Zernant et al. 2011 |
| QT458      | c.4555delA | p.T1519Rfs*7| Het             | NA                       | 0/192 0/456                              | Novel     |
|            | c.164A>G   | p.H55R     | Het             | PoD                      | - 0/456                                  | Thiadens et al. 2012 |
| QT727      | c.161-2A>G | Splicing   | Het             | SSA                      | 0/192 0/456                              | Novel     |
|            | c.101_106del| p.S34_L35del| Het             | NA                       | 0/192 0/456                              | Novel     |
| QT833      | c.2424C>G  | p.Y808*    | Het             | NA                       | - 0/456                                  | Zhou et al. 2014 |
|            | c.1560delG | p.V521Sfs*47| Het             | NA                       | 0/192 0/456                              | Novel     |
| QT1137     | c.6284A>T  | p.D2095V   | Het             | PoD                      | 0/192 0/456                              | Novel     |
|            | c.22C>T    | p.QG*      | Het             | NA                       | 0.0001 0/192 0/456                       | Novel     |
| QT1160     | c.240_241del| p.C81Ffs*17| Het             | NA                       | 0/192 0/456                              | Novel     |
|            | c.101_106del| p.S34_L35del| Het             | NA                       | 0/192 0/456                              | Novel     |
| QT1175     | c.4195G>T  | p.E1399*   | Het             | NA                       | 2/120596 - 0/456                         | Novel     |
|            | c.2894A>G  | p.N965S    | Het             | PoD                      | 0.0001 21/121302 - 0/456                  | Allikmets et al. 1997; Shanks et al. 2013; Bertelsen et al. 2014 |
| QT1182     | c.4773+1G>T | Splicing   | Hom             | SSA                      | - 0/456                                  | Pang et al. 2002; Riveiro-Alvarez et al. 2013 |
| QT1198     | c.5446G>A  | p.M1882I   | Het             | B                        | 3/121340 - 0/192                         | Zernant et al. 2011 |
|            | c.2894A>G  | p.N965S    | Het             | PrD                      | 21/121302 - 0/456                         | Allikmets et al. 1997; Shanks et al. 2013; Bertelsen et al. 2014 |
| QT1200     | c.6563T>C  | p.F2188S   | Het             | B                        | 0.0005 2/121380 - 0/456 1/456            | Fukui et al. 2002 |
|            | c.858+2T>A | Splicing   | Het             | SSA                      | - 0/456                                  | Zhang et al. 2014 |
| QT1230     | c.6317G>C  | p.R2106P   | Het             | PrD                      | 0/192 0/456                              | Novel     |
|            | c.101_106del| p.S34_L35del| Het             | NA                       | 0/192 0/456                              | Novel     |
| QT1277     | c.6479+2T>C | Splicing   | Het             | SSA                      | 0/192 0/456                              | Novel     |

(Continued)
Table 1. (Continued)

| Patient ID | Nucleotide Change | Amino Acid Change | State | Splicing Site Prediction | 1000G Allele Frequency | ExAC Allele Frequency | NC Allele Frequency | RC Allele Frequency | Reported References |
|------------|-------------------|-------------------|-------|--------------------------|------------------------|-----------------------|-------------------|-------------------|---------------------|
| QT1317     | c.5196 +1G>A      | Splicing defect   | Het   | SSA                       | NA                     | NA                    | 3/49858           | -                 | 0/456               | Allikmets et al. 1997; Wiszniewski et al. 2005 |
| QT1317     | c.5646G>A         | p.M1882I          | Het   | PoD                       | D                      | D                     | 3/121340          | -                 | 0/456               | Zernant et al. 2011 |
| QT1317     | c.4622T>C         | p.L1541P          | Het   | PrD                       | D                      | D                     | NA                | NA                | 0/192               | Novel               |
| MD19       | c.4793C>G         | p.A1598G          | het   | PoD                       | D                      | N                     | NA                | 0.0001            | 0/456               | Mauger et al. 2000; Cideciyan et al. 2009; Burke et al. 2010 |
| MD19       | c.634C>T          | p.R212C           | het   | D                         | D                      | D                     | NA                | 0.0002            | 14/120056           | Gerber et al. 1998; Thiadens et al. 2012 |

The following abbreviations are used: P/SS, PolyPhen-2/Splice Site Prediction; 1000G, 1000 Genomes; EVS, Exome Variant Server; ExAC, Exome Aggregation Consortium; Het, heterozygous; Hom, homozygous; NC, normal control; RC, relative control; PrD, probably damaging; PoD, possibly damaging; B, benign; SSA, splicing site abolished; N, neutral; D, damaging; and NA, not applicable; -, not done.

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Fig 1. The ABCA4 mutation and the pedigree. Under each individual, + indicates a wild type allele, and M indicates a mutant allele.

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Table 2. Clinical features of Stargardt disease probands with the ABCA4 mutations identified in this study.

| Patient ID | Variations | Gender | Age at First BCVA | Fundus examination | Dark Choroid |
|------------|------------|--------|-------------------|-------------------|--------------|
| QT058      | c.[6173T>G];[4773+1G>T] | F      | 18                | PV                | Present      |
| QT085      | c.[6173T>G];[5932delA] | M      | 9                 | PV                | NA           |
| QT292      | c.[6389T>A];[618C>T] | M      | 25                | PV                | NA           |
| QT302      | c.[6816+1G>A];[4555delA] | M      | 17                | PV                | NA           |
| QT398      | c.[4352+1G>A];[1804C>T] | M      | 8                 | PV                | NA           |
| QT431      | c.[5646G>A];[1804C>T] | F      | 12                | PV                | NA           |
| QT458      | c.[4555delA>G];[164A>G] | F      | 20                | PV                | NA           |
| QT727      | c.[161-2A>G];[101_106del] | F      | 15                | PV                | NA           |
| QT833      | c.[2424C>G];[1560delG] | F      | 9                 | PV                | NA           |
| QT1137     | c.[6284A>T];[22C>T] | M      | 7.5               | PV                | Present      |
| QT1160     | c.[240_241del];[101_106del] | F      | 11                | PV                | Present      |
| QT1175     | c.[4195G>T];[2894A>G] | F      | 11                | PV                | NA           |
| QT1182     | c.[4773+1G>T];[4773+1G>T] | M      | 7                 | NA                | NA/NA        |
| QT1198     | c.[5646G>A];[2894A>G] | M      | 8                 | PV                | BMS; PD      |
| QT1200     | c.[6563T>C];[858+2T>A] | M      | 26                | PV                | Not Present  |
| QT1230     | c.[6317G>C];[101_106del] | F      | 8.5               | PV                | BMS; PD      |
| QT1277     | c.[6479+2T>C];[5196+1G>A] | F      | 7                 | PV                | NA           |
| QT1317     | c.[5646G>A];[4622T>C] | F      | 10                | PV                | NA           |
| MD19       | c.[4793C>G];[634C>T] | M      | 12                | NA                | NA           |

The following abbreviations are used: M, male; F, female; EC, early childhood; NA, not available; OD, right eye; OS, left eye; PV, poor vision; BCVA, Best corrected visual acuity; PD, pigment disorder; BMS, beaten-metal sign; FFA, Fundus Fluorescein Angiography; YF, yellowish fleck.

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Fig 2. Fundus photographs and Fundus Fluorescein Angiography from QT058 and QT1137 respectively. The ABCA4 mutations were listed above each photograph identified in this study. OD and OS represent right and left eyes, respectively. Fundus photographs showed yellowish flecks in all posterior of the retina and macular atrophy. FFA showed “dark choroid” sign. More clinical information about these patients is listed in Table 2.

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K1978Qfs*13 and L2058R in cytosolic nucleotide binding domain 1 and T1519Rfs*7 in exocytoplasmic domains 2.

Except for ABCA4 mutations in 19 of 33 probands, potential mutations have not been detected in the other 14 probands after analysis of other 213 genes in which mutations were responsible for different forms of retinal dystrophy based on comprehensive whole exome analysis. No mutations identified in other genes except for ABCA4 may partly because of the limited number of probands analyzed. A previous study [15] also reported no pathogenic mutations in about 30% of patients with STGD. This suggests that as yet unidentified genes may also contribute to STGD. Therefore, further bioinformatics analysis on data resulted from whole exome sequencing may lead to find new genes responsible for STGD in the remaining probands without mutations. In addition, mutations in the noncoding regions may be missed by whole exome sequencing. Additional analysis using different strategies are expected to find such mutations, especially for those probands with single pathogenic variants in recessive genes.

Supporting Information

S1 Fig. Sequence chromatography. 38 sequence changes that were detected in 19 probands with Stargardt disease are shown (left column) and compared with corresponding normal sequences (right column).

S1 Table. Genomic information of the five known genes responsible for STGD.

S2 Table. Primers used in Sanger sequencing.

S3 Table. Potential pathogenic ABCA4 variants in other 8 probands with Stargardt disease.

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Author Contributions

Conceived and designed the experiments: QZ. Performed the experiments: WX XX SL XJ. Analyzed the data: WX QZ. Contributed reagents/materials/analysis tools: XX SL XJ XG QZ. Wrote the paper: WX XX SL XJ XG QZ.

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