Identification of Two Novel Mutations in *ABCA4* Gene in a Patient With Stargardt Disease

Masoumeh Mohebbi[1], Matin Heidari[2], Mansour Heidari[3]

[1] *Farabi Eye Research Center, Farabi Eye Hospital, Tehran University of Medical Sciences, Tehran, Iran*

[2] *Department of Molecular Biology, Islamic Azad University of Qom, Qom, Iran*

[3] *Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*

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Abstract- Herein we investigated mutations in the *ABCA4* gene in an Iranian patient with Stargardt disease using whole exome sequencing (WES). We evaluated genetic alterations in a 13-year-old Iranian girl with Stargardt disease and her family using WES. The target sequences for the proband and her parents were then amplified through polymerase chain reaction (PCR) and the obtained products were screened for mutations in *ABCA4* gene by Sanger chain terminating dideoxy nucleotide sequencing. Two novel potentially pathogenic mutations in compound heterozygous state (c.2713del p.E905Rfs*27 and c.5172G>A p.W1724X) were identified in *ABCA4* gene which may contribute to the proband's Stargardt disease phenotype. In general, the WES successfully identified novel causal mutations in *ABCA4* gene which may be used for genetic counseling, prenatal diagnosis (PND), and preimplantation genetic diagnosis (PGD) of Stargardt disease.

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Introduction

Stargardt disease (OMIM 248200) was accounts 7% of all congenital retinal disorders (1,2). The prevalence of Stargardt disease is 0.1-0.125% worldwide with onset in childhood or early adulthood (2,3). Patients with Stargardt disease primarily suffer from progressive loss of central vision, and present retinitis pigmentosa, yellow-white auto-fluorescent flecks (fundus flavimaculatus), beaten-bronze display, and chorioretinal atrophy in macula associated with accumulation of lipofuscin inmaculae or central and peripheral retina (4,5). Pathologic changes primarily involve pervasive lipofuscin deposition in retinal pigment epithelium, accumulation of toxic metabolite, and excessive photoreceptor cell death (6). Various mutations in the ATP binding cassette subfamily A member 4 gene (*ABCA4*) with autosomal recessive inheritance patterns are believed to cause Stargardt disease. Moreover, several Stargardt disease-like phenotypes with autosomal dominant inheritance have been reported which are associated with mutations in the prominin 1 (PROM1), ELOVL fatty acid elongase 4 (ELOVL4), crumbs 1 cell polarity complex component (CRB1), bestrophin 1 (BEST1), and peripherin 2 (PRPH2) (7,8).

The *ABCA4* gene encoded photoreceptor cell-specific *ABCA4* membrane protein contains two exocytoplasmic domains, two transmembrane domains, and two cytoplasmic domains. The encoded protein transports all-trans retinal (produced by retinoid cycle) from intra-disc space to photoreceptor cytoplasm and prevents its accumulation in retinal pigment epithelium cells and photoreceptors outer segments (9,10). The mutant forms of *ABCA4* gene mainly encode dysfunctional *ABCA4* proteins, also known as Rim proteins, and lead to disruption and/or degeneration of photoreceptor (11). Mutations in the *ABCA4* gene have been described as responsible for several abnormalities such as Stargardt disease, retinitis pigmentosa-19 (RP19), cone-rod dystrophy 3 (CORD3), fundus flavimaculatus (FFM),

Corresponding Author: M. Heidari
Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
Tel: +98 2188953005, E-mail address: mheidari@sina.tums.ac.ir

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age-related macular degeneration 2, and early-onset severe retinal dystrophy (12,13). So far, more than 800 different mutations such as splicing site, nonsense, frameshift, missense, and small insertion/deletion have been reported in the *ABCA4* gene and been associated with retinal degeneration (14). Previous studies reported that homozygous or compound heterozygous mutations in *ABCA4* are detected in 70% of patients with Stargardt disease, whereas a significant number of patients have a single mutation in *ABCA4* gene (15,16).

Analyzing samples from an Iranian family affected by Stargardt disease through next generation sequencing (NGS) and Sanger sequencing methods, the present study identified two novel variants in a compound heterozygous state (c.2713del p.E905Rfs*27 and c.5172G>A p.W1724X) in *ABCA4* gene which can potentially be pathogenic.

Methods and Materials

Case presentation

A 13-year-old Iranian girl with Stargardt disease were considered for genetic screening at the Aria Gene Medical Genetics Laboratory, Qom, Iran. She was born after a full-term pregnancy without serious complications and was offspring of unrelated marriage (Figure 1A). Stargardt disease was diagnosed clinically according to retinal yellowish flecks, progressive bilateral central vision loss, onset in the first or second decades, macular atrophy or dystrophy with a beaten-bronze appearance, and lack of pigmented bone spicules. The study was approved by the Institutional Review Board (IRB) of Qom University of Medical Sciences and conducted according to the ethical standards of the Declaration of Helsinki. Informed consents was obtained from the patient, her parents, and all healthy controls.

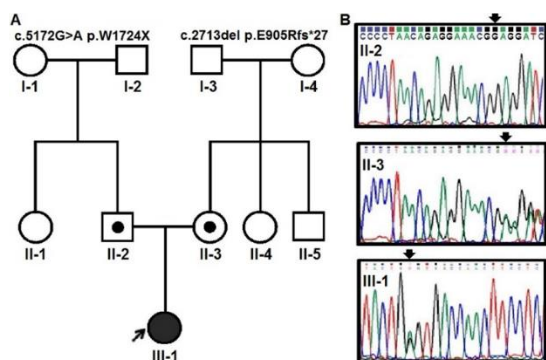

Figure 1. Pedigree analysis and molecular evaluation of a patient with *ABCA4* gene mutation. (A) The pedigree of the affected patient (arrow indicates the proband); (B) The affected patient is compound heterozygous and both parents are single heterozygous

Genomic DNA extraction

Genomic DNA was extracted from peripheral blood lymphocytes (5 ml) of the proband and her parents using a DNA purification kit (Roche, Switzerland) according to the manufacturer's instructions. The quantity and purity of the extracted genomic DNA was evaluated using a NanoDrop instrument (Thermos Fisher Scientific, USA). The samples OD 260/280 ratio between 1.7-1.9 was selected for molecular analysis. Moreover, electrophoresis on 1% agarose gel was performed to evaluate the quality of extracted genomic DNA. The DNA samples with appropriate quality and quantity were selected and stored at -20° C until molecular analysis (17).

Whole-exome sequencing (WES)

WES was employed to enrich all coding regions of proband and her parents. Exome sequence capture was performed using the SureSelect Human All Exon V5 Kit (Agilent Technologies, USA) accordance to the manufacturer’s instructions. The capture library was sequenced via 2×150 paired end sequencing on a Hiseq2000 Sequencer (Illumina, USA) (18).

Analysis of sequencing data

The sequence reads were aligned to human reference genome and processed using SAMtools. The single nucleotide polymorphisms (SNPs) and small deletions/insertions (indels) were analyzed using Genome Analysis Toolkit (GATK) and VarScan software. Variants were annotated using the ANNOVAR software. Variants with low-quality scores (depth <10 or genotype quality <20) or in homozygous state were filtered out. The nonsense mutations and low-frequency frameshift mutations were considered as pathogenic. The missense mutations were analyzed using MutationTaster, Polyphen-2, SIFT, REVEL, PROVEAN, MetaLR, MetaSVM, M-CAP, and FATHMM for assessment of pathogenic potential. The variants shared by the studied patient, but not in the healthy control, were remained. The variants co-segregating with autosomal dominant disease and genes annotated to be associated with disease were filtered then prioritized. The variants that passed these filtering was considered as pathogenic mutations (19).

Sanger sequencing

The Sanger sequencing was used to validate candidate Stargardt disease-causing mutations in proband and her family. The target exons containing mutations of *ABCA4* gene were amplified using designed primers by Primer3 software. The fragment of *ABCA4* gene with target

mutation was amplified using 10 pmol of each forward and reverse primers, template DNA (60 ng), dNTPs (200 μM), DNA polymerase (0.2 U Taq), $MgCl_2$ (0.5 mM), and PCR buffer in 25 μl total volume. The PCR conditions include initial denaturation (1 cycle for 3 min at 95° C), denaturation (35 cycles for 30 sec at 95° C), annealing (35 cycles for 30 sec at 60° C), extension (35 cycles for 30 sec at 60° C), and final extension (1 cycle for 10 min at 72° C). The amplified fragments were sequenced using ABI 3130 automated sequencer (Applied Biosystems, Forster City, CA, USA), and the sequences were assembled and analyzed by Mutation Surveyor software (20).

Results

Clinical findings

In present study, an Iranian family with a child suffering from Stargardt disease was examined (Figure 1A). The proband, a 13-year-old Iranian girl, presented poor visual acuity at 6 years of age. She was sensitive to light when she was 6-11 years old and permanently lost her sight later. The clinical examination of the proband showed photoreceptor and retinal pigment epithelium cells degeneration and bilateral macular atrophy. The fundus imaging showed yellowish flecks and beaten-bronze in macular area (Figure 2A). Moreover, the fundus fluorescein angiography showed hyper-fluorescent flecks in mid-peripheral retina, and fluorescence blocking formation in macular area due to pigment mottling (Figure 2B). The optical coherence tomography showed retinal outer layer attenuation, photoreceptor outer segments, choroidal reflectivity increment, and hyper-reflective deposition between retinal pigment epithelium layer (Figure 2C). Therefore, the proband was diagnosed with Stargardt disease. Her 32-year-old mother and 40-year-old father had no ocular abnormalities.

Detection of *ABCA4* mutation using WES

The WES was used to identify disease-causing mutations in the *ABCA4* gene of the proband which revealed a novel heterozygous deletion mutation c.2713del p.E905Rfs*27 in this gene. Moreover, we found another novel heterozygous missense mutation c.5172G>A p.W1724X in the *ABCA4* gene of this patient. These suggested co-segregation of the compound heterozygous variants (c.2713del and c.5172G>A) with Stargardt disease in this family.

Confirmation of detected *ABCA4* mutation using sanger sequencing

Sanger sequencing using designed primers was conducted to confirm the novel mutations (c.2713del and c.5172G>A) in the *ABCA4* gene identified by WES. We found the two detected genetic variants of *ABCA4* gene were in the compound heterozygous state in the proband (Figure 1B). Her mother was in heterozygous state for c.2713del mutation, while her father was in heterozygous state for c.5172G>A mutation (Figure 1B).

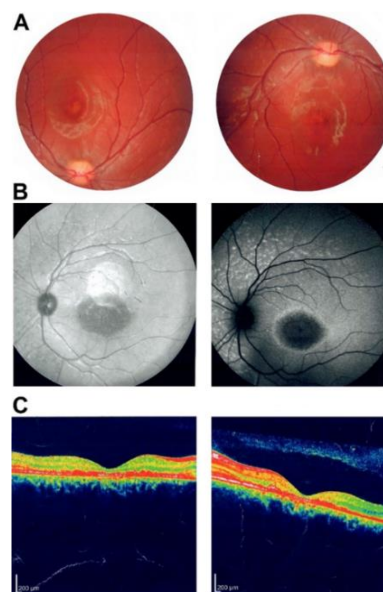

Figure 2. Clinical characteristics of the studied patient. (A) Fundus photographs: yellowish flecks and beaten-bronze appearance in maculae, and some pigment mottling. (B) Fundus fluorescein angiography: fluorescence blocking in maculae, and hyper-fluorescent flecks into mid-peripheral retina. (C) Optical coherence tomography: photoreceptors outer segments, choroidal reflectivity increment, retinal outer layers attenuation, and hyper-reflective deposition between retinal pigment epithelium

Discussion

Stargardt disease, an autosomal recessive disease, is a juvenile macular degeneration due to accumulation of lipofuscin in retinal pigment epithelial (21). The disease symptoms typically develop early in life, with patients often suffering from visual acuity loss as a result of yellowish lipofuscin flecks deposition and macular atrophy in retinal pigment epithelial (1). High expression of several related genes with degenerative macular dystrophies in photoreceptor cells play important role in small molecule transport, photo-transduction, photoreceptor structure, visual cycle (5).

In our study, Stargardt disease was clinically diagnosed in an Iranian patient. The proband's age of onset was 6 years when she presented with central visual acuity loss. We observed high lipofuscin flecks

accumulation in retinal pigment epithelial, and a significant decrease in retinal thickness in central foveola. No ocular abnormalities observed in other family members.

WES combined with Sanger sequencing were performed to identify potential disease-causing genetic variants in this patient. Two compound heterozygous variants (c.2713del p.E905Rfs*27 and c.5172G>A p.W1724X) in *ABCA4* gene were identified in proband. The parents of studied patient with heterozygous mutation in *ABCA4* (c.2713del or c.5172G>A) were healthy, which can be due to partly normal function of *ABCA4* protein. This suggested the potential role of identified compound heterozygous mutations in phenotype of the proband.

Evidence demonstrated that most patients with Stargardt disease have one missense mutation and one null mutation or two missense mutations in compound heterozygous status (22,23). The *ABCA4* protein on the photoreceptor cells contains two nucleotide binding domains (NBD1 and NBD2) (24,25). Various mutations that alter NBD1 or NBD2 cause *ABCA4* proteins. Thus, compound heterozygotes or homozygotes mutations in the *ABCA4* gene can decrease function of *ABCA4* protein, and leads to progressive degeneration of photoreceptors, and other clinical features of the Stargardt disease.

The amino acid deletion of the hydrophilic glutamic acid at position 905 (p.E905Rfs*27) may impact the function or structure of protein. This alteration located in the NBD1 probably damages the *ABCA4* protein binding sites (12). Moreover, the change of tryptophan residue to unknown amino acid residue at position 1724 (p.W1724X) may also impact NBD1 (26). Moreover, previous studies reported various mutations in compound heterozygous status in patients with Stargardt disease (12). The compound heterozygous or homozygous mutations are identified in more than 70% of patients with Stargardt disease (26).

In general, we detected two novel pathogenic variants in compound heterozygous status (c.2713del p.E905Rfs*27 and c.5172G>A p.W1724X) in *ABCA4* gene responsible for Stargardt disease phenotype in an Iranian patient. These results can refine genetic counseling and treatments of patients with *ABCA4*-caused Stargardt disease.

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