ORIGINAL ARTICLE

The Molecular Genetic Cause and Clinical Findings in two Probands with Stargardt Disease

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SUMMARY

Purpose: The aim of our study was to describe the phenotype and to perform molecular genetic investigation in two probands of Czech origin diagnosed with Stargardt disease (STGD).
Methods: Both males underwent ocular examination including assessment by high-resolution spectral domain optical coherence tomography (SD-OCT). DNA was isolated from venous blood. Mutation detection was performed using the ABCA4 genotyping microarray (Asper Ophthalmics, Estonia).
Results: The best corrected visual acuity in proband 1 (aged 39 years) was 0.1 bilaterally, and 0.05 in proband 2 (aged 26 years). Fundus examination showed typical multiple yellow-white lesions and macular atrophy. Alterations of retinal pigment epithelium, retinal thinning and disruption of the photoreceptor inner segment ellipsoid band were detected with an SD-OCT. Two known diseasecausing mutations in ABCA4 were identified in proband 1; c.4234C>T, p.(Gln1412*) in exon 28; and c.5882G>A, p.(Gly1961Glu) in exon 42. Only one pathogenic change was detected in proband 2; c.1988G>A, p.(Trp663*) in exon 14. A second change, anticipated because of the recessive status of the disease, was not identified.
Conclusion: The frequency and full spectrum of ABCA4 mutations in Czech patients with inherited retinal disorders is yet to be established. The inability to detect a second pathogenic change in ABCA4 coding sequences in proband 2 warrants further investigation.

Key words: Stargardt disease, ABCA4, mutation, SD-OCT

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INTRODUCTION

Stargardt disease and fundus flavimaculatus are variants of a hereditary disease which afflicts the retinal pigment epithelium (RPE) and the photoreceptors. A characteristic clinical finding is the presence of yellowish stains on the macula on the level of the RPE, together with atrophy of the macula. Stargardt disease is typically manifested as an irreversible reduction of central visual acuity, beginning in childhood or early adulthood, although symptoms of the disease may appear also later. During the initial manifestations, the macula may have a normal appearance, later small hyperpigmentations may appear, subsequently it takes on an appearance of wrought bronze and atrophy progressively occurs, sometimes with yellowish stains around the edges or diffused throughout the entire fundus. Their appearance and size varies to an extremely large degree. Pigment shifts may also appear later in the periphery of the retina. In the final result, the disease may lead to total blindness. The prevalence of the disease is relatively high, and it is estimated that 1 person per 8-10 000 of the population is afflicted (10).
Today autofluorescence of the fundus (7, 22, 47) is considered to be the most reliable method for identifying Stargardt disease and especially the scope of pathological changes. Optical coherence tomography with spectral domain (SD-OCT), fluorescence angiography, examination of the visual field and electroretinography are also used in diagnostics (23, 25, 28, 40, 41). SD-OCT enables display of the changes from the early stages of the disease. A typical finding is irregularity of the RPE, disorganisation to loss of the line of ellipsoids of the internal segments of the photoreceptors, in the literature also indicated as a line of the junction of the internal and external segments of the photoreceptors, loss of the external nuclear layer and attenuation of the neuroretina (32).
Stargardt disease is most frequently inherited as an autosomal recessive disease (STGD1). The disease is conditioned by mutations in the ABCA4 gene (ATP-binding cassette, subfamily A, member 4), localised on the chromosome 1p22, which codes the retinally specific ATP-binding transport protein located on the edges of the photoreceptor discs in the outer segments of the rods and cones (6). ABCA4 helps remove potentially toxic retinal compounds from the photoreceptors following excitation. The impairment of this transport after phagocytosis of the outer segments of the photoreceptors leads to their accumulation in the RPE and the formation of lipofuscin deposits. The accumulating toxic compounds then lead to apoptosis of the RPE, and because the survival of the photoreceptors is dependent on the RPE, they subsequently

degenerate (29).
In rare cases, diseases similar to Stargardt disease may generate mutations in the genes ELOVL4 (ELOVL fatty acid elongase 4) (STGD3) and PROM1 (prominin 1) (STGD4) (14, 45, 49, 52).

METHODS

We examined two unrelated patients with clinical symptoms of Stargardt disease. Best corrected visual acuity was determined using ETDRS optotypes. Close-up vision was tested using Jäger tables with a scope from the smallest to the largest text of 1-12. We conducted a biomicroscopic examination of the fundus in mydriasis, colour photography of the eyeground, photography in red-free light and examination of the autofluorescence of the fundus (Visucam 200, Carl Zeiss Meditec AG, Germany), which utilises the fluorescence capability of lipofuscin to highlight its distribution and accumulation and to differentiate the damaged receptors of the retina and the damaged RPE. In addition, an examination of the visual field was conducted by static perimetry (M-700, Medmont International Pty Ltd., Vermont, Australia), as well as examination of contrast sensitivity on a Pelli-Robson table (HS Clement Clarke International, Essex, Great Britain), examination by SD-OCT with axial resolution of 5 µm and transversal resolution of 15 µm (RTVue, Optovue, Inc, Fremont, USA) and an instrument which combines SD-OCT with axial resolution of 6 µm and a scanning laser ophthlmoscope (Spectral OCT/SLO, OTI Ophthalmic Technologies Inc., Canada).
We took a 3 ml sample of venous blood into EDTA test tubes and isolated the genome DNA using the system

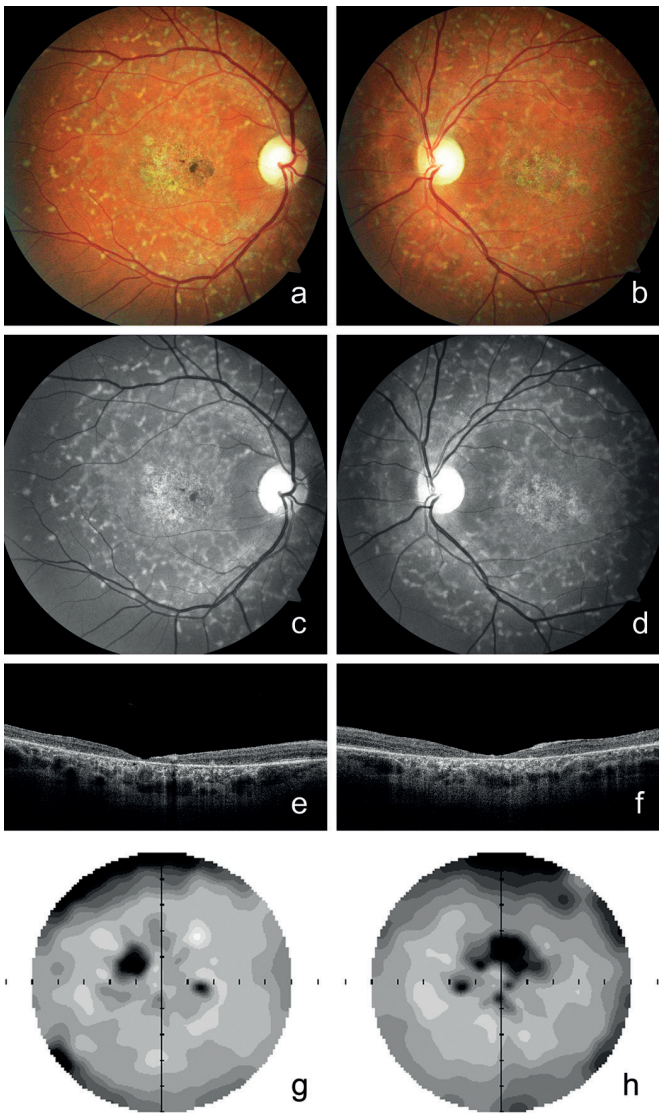

Fig. 1 Clinical finding in proband 1 examined at the age of 39 years. Photographs of the fundus, documenting yellowish deposits and atrophy of the retinal pigment epithelium with an appearance of wrought bronze in the macula of the right a) and left eye b). Yellowish deposits of various shapes on the posterior pole better visible in red-free light on images of right c) and left eye d). Horizontal SD-OCT scan of macula of right e) and left eye f) with attenuation of the neuroretina in the macula, absence of a line of ellipsoids of the internal segments of the photoreceptors and atrophy of the retinal pigment epithelium. Visual field of right g) and left h) eye with paracentral blind spots, validity reduced by loss of fixation.

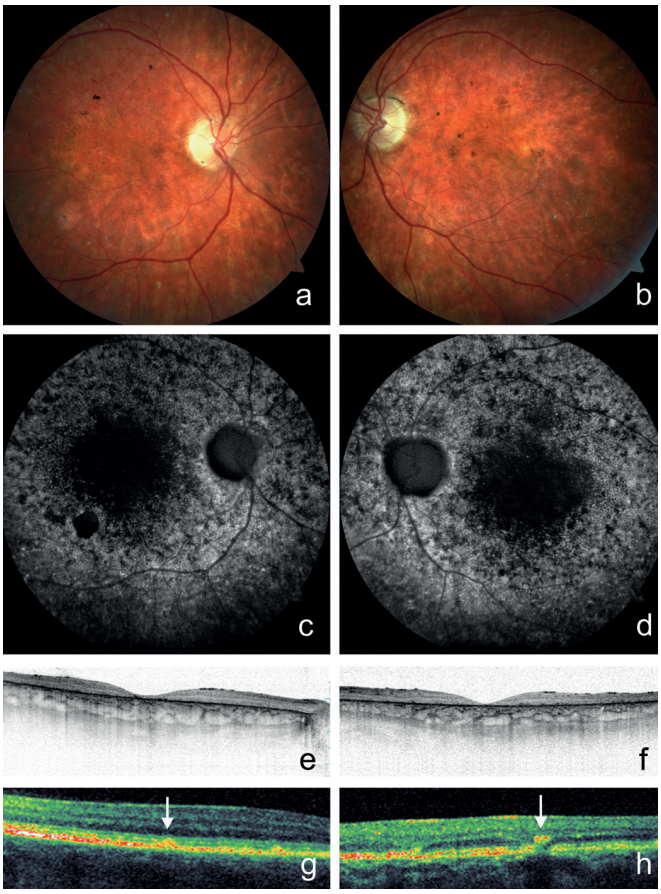

Fig. 2 Clinical finding in proband 2 examined at the age of 26 years. Colour photograph of fundus, documenting atrophy of the neuroretina and retinal pigment epithelium in the macula of the right a) and left eye b), with small yellowish-white deposits in the periphery. Lack of autofluorescence of the macula of the right c) and left eye d) with deposit increase of autofluorescence of the fundus in areas of accumulation of lipofuscin. Horizontal SD-OCT scan of macula of right e) and left eye f), demonstrating attenuation of the neuroretina, absence of line of ellipsoids of internal segments of photoreceptors and atrophy of the retinal pigment epithelium. Hyperreflexive deposits divided into type 1 g) and type 2 h) according to depth of embedding in retina and corresponding to yellowish-white stains (indicated by arrows) on SD-OCT of left eye.

FLEX STAR (AutoGen, USA) according to the instructions of the manufacturer. For identification of the mutations we used a genotyping microchip for the gene ABCA4, version 11.0 (Asper Ophthalmics, Estonia), detecting 558 known mutations and single-nucleotide polymorphisms.
The nomenclature of the description of the mutations was based on the recommendation of the Human Genome Variation Society (http://www.hgvs.org/rec.html), in which the first adenine of the initialisation codon ATG in the coding DNA is always indicated with the number 1. NM_000350.2 was used as a referential sequence. The research was conducted in accordance with the Helsinki declaration. Both patients signed written declarations of informed consent.

RESULTS

The scope of the clinical examination of the probands depended on the availability of the above-stated methods at the individual workplaces.
Proband 1 had seen equally well in both eyes in childhood, but from the age of 15 years had observed a gradual, progressive deterioration of central visual acuity and photophobia in harsh lighting. Symptoms of Stargardt disease were first described upon an examination of the fundus at the age of 25 years. Visual acuity was 0.16 in both eyes without correction. Close-up vision was reduced bilaterally to reading text with Jäger number 6. At the age of 30 years, visual acuity in both eyes for distance vision was retained with correction of -1.5D, but close-up vision was reduced to reading text with Jäger number 8. At the last examination at the age of 39 years, visual acuity was reduced to 0.1 bilaterally, in the right eye with correction of -2.75D = -1.0 Dcyl ax 20° and in the left eye with correction of -3.0D. Close up the patient could read text with Jäger number 10. In the maculas of both eyes there was a horizontal oval deposit of an irregular disorder of the RPE, with an appearance of wrought bronze, biomicroscopic size of 1.7 of the diameter of the disc of the optic nerve, there was a lack of a foveolar reflex, and at the nasal edge of the macula of the right eye there was a small, web-like pigmented rounded deposit with a size of 0.5 of the diameter of the disc of the optic nerve. A yellowish deposit was present on the level of the RPE around the maculas by the capillary arcades and nasally from the papillas, the retinal capillaries appeared normal (fig. 1a-D). The finding on SD-OCT revealed bilateral attenuation of the neuroretina in the macular region, with atrophy of the outer nuclear layer, absence of a line of ellipsoids of the internal segments of the photoreceptors and atrophy of the RPE with more pronounced display of the structures of the choroid (fig. 1e, f). The smallest thickness of the neuroretina in the foveas of both eyes was 20 µm. An examination of the visual field demonstrated bilateral paracentral scotomas in the upper region, the larger in the right eye, the validity of the examination was reduced by the loss of fixation (fig. 1g, h).
In proband 2 the disease of the retina was manifested in a deterioration of close-up vision at the age of 6 years. The patient did not suffer from nyctalopia or photophobia. Upon our examination at the age of 26 years, visual acuity in both eyes was 0.05, close up the patient could not read even the largest Jäger table. Clinically we found advanced manifestations of the disease with atrophy of the neuroretina and the retinal pigment epithelium in the macula. Yellowish-white deposits were dispersed around the macula and outer capillary arcades (fig. 2a, b). Autofluorescence of the fundus showed a disorder of distribution. In the areas of atrophy of the RPE it was entirely lacking, whereas in its surrounding area we observed regions with irregularly grainy autofluorescence. By contrast, in areas with an accumulation of lipofuscin deposits, autofluorescence was increased (fig. 2c, d). Upon an SD-OCT examination, extensive atrophy of the outer nuclear layers was recorded bilaterally, accompanied by a lack of a line of ellipsoids of the internal segments of the photoreceptors, atrophy of the RPE and attenuation of the neuroretina (fig. 2e, f). Hyperreflexive deposits were also documented in various depths of the retina, corresponding to yellowish-white stains (fig. 2g, h). The minimum height of the neuroretina in the fovea of the right eye was 35 µm, and in the left eye 20 µm. An examination of the visual field by static perimetry was not reliable, due to the inability to fix.

Molecular genetic analysis

In proband 1, two causal mutations were detected in gene ABCA4: a substitution of cytosine by thymine (c.4234C>T) in exon 28, leading to the formation of a termination codon in position 1412 p.(Gln1412*). The second pathogenic mutation c.5882G>A is found in exon 42, and its presence means a substitution of the amino acid glycine by glutamine in position 1961 p.(Gly1691Glu).
In proband 2 only one known mutation c.1988G>A was demonstrated in exon 14, causing a classification of a termination codon in position 663 p.(Trp663*).

DISCUSSION

Our study is the first to describe the molecular genetic cause of Stargardt disease in patients of Czech origin. At the same time the ocular finding was also documented in detail using modern display methods such as SD-OCT and examination of autofluorescence of the fundus.
Stargardt disease was first described by Karl Stargardt in 1909, and ranks amongst the most common hereditary diseases of the macula. This disease involves a bilateral deterioration of visual acuity, usually beginning in childhood or early adulthood, as we observed also in our patients. Usually patients suffer from progressive loss of visual acuity up to the value of around 0.1, although the degree of loss of visual acuity depends on the age at which the first manifestations of the diseases appeared. In proband 2 the first symptoms of the disease appeared earlier than in proband 1, and in accordance therewith the patient also had a greater loss of visual acuity, which was also below the average of visual acuity usual in the final stage.
The finding on the eyeground was also more advanced in proband 2 than in proband 1, despite the fact that the patient was younger at the time of our examination. Deposits of an irregular atrophy of the RPE were found in the maculas in proband 1, as well as partial choriocapillaris with an appearance of wrought bronze, in proband 2 atrophy of the retina including the RPE was already present. In both probands we observed typical yellowish to yellowish-white stains of various shapes in the area of the posterior pole and also in the periphery. Generally in accordance with the published data, the stains are frequently of low contrast in the biomicroscopic examination and on the colour photograph of the fundus, and are better visible in red-free light, though are best and most clearly perceptible upon examination of autofluorescence of the fundus, in which they demonstra-

te hyperautofluorescence on a background of an accumulation of lipofuscin in the RPE (46, 47). In proband 1 the stains were larger, forming web-like patterns, and were of a yellowish colour, in proband 2 they were smaller in size, more isolated and rounded, of yellowish-white colour.

Upon SD-OCT examination we recorded an attenuation of the neuroretina, lack of a line of ellipsoids of the internal segments of the photoreceptors and hyperreflexive deposits located in the outer layers of the neuroretina in both patients. According to localisation this is divided into two groups. Type 1 deposits are of a cupola shape and are located in the level or closely above the level of the inner side of the line of the RPE. The reflexivity of both objects may be similar, and they are difficult to differentiate upon SD-OCT examination. Type 2 deposits appear as small, linear hyperreflexive lesions encroaching into the outer nuclear layer of the retina, and may be separated from the line of the RPE (46). We observed lesions of both types in both of our probands.

Reduction of visual acuity corresponds to a diminution of receptors, which also correlates with the extent of changes visible upon examination of the autofluorescence of the fundus. By contrast, reduction of the height of the retina need not necessarily be accompanied by a loss of photoreceptors, and despite progressive atrophy of the intraretinal layers, visual acuity may be better than could be expected (15).

In proband 1 the examination of the visual field by static perimetry was less valid due to loss of fixation, in proband 2 it was not possible to conduct this examination whatsoever for the same reason. A typical finding upon perimetric examination in the case of Stargardt disease is central scotoma.

A positive family anamnesis for Stargardt disease was not present in either patient, which is in accordance with autosomal recessive heredity and thus with STGD1, conditioned by mutations in the gene ABCA4. Mutations in this gene cause a range of autosomal recessive retinal degenerations with affliction of the macula, in addition to Stargardt disease also autosomally recessive cone-rod dystrophies and retinitis pigmentosa (9, 12, 17, 26). Certain variants of this gene are linked also to an increased risk of age related macular degeneration (3, 5), nevertheless all the studies confirm this association (4, 42).

The gene ABCA4 has 50 exons, codes protein with a size of 2273 amino acids, and is therefore relatively large. The performance of screening by conventional mutation analysis using Sanger sequencing would be demanding both in terms of time and financially, and as a result a cheaper method of detection of mutations using a genotyping microchip was selected, which uses APEX technology ("arrayed primer extension") (24). At the time of the examination of both probands, this microchip contained 558 various pathogenic sequential variants and benign polymorphisms (51), and has already been used for systematic screening of retinal diseases associated with mutations in the gene ABCA4 in a range of previous studies due to its favourable price to performance ratio (20, 34, 44). Both mutations are found by this method in an average of 40% of patients with diagnosed classic STGD1, in a further 40% of patients one mutation is determined and in 20% no mutation is detected (19). False positives or negatives upon use of a genotyping microchip is less than 2%, nevertheless if conditions so allow it is suitable to demonstrate the presence of the determined mutations also by another method, most commonly by Sanger sequencing (24, 34). The disadvantage of a genotyping microchip is that it is possible to examine the presence of only known sequential variants, and so new mutations are not detected. At present there is a shift towards new generation sequencing technology in the determination of mutations, which demonstrates a favourable price to performance ratio and also enables the detection of hitherto undocumented sequential variants (19, 51).

Both mutations in the gene ABCA4 demonstrated in proband 1 had previously been documented in connection with STGD1 in cases from various populations. Whilst it is relatively rare and has been found only in a number of families in the USA and in the Danish population (28, 35), in a number of populations it is the most commonly detected pathogenic sequential variant (1, 11, 20, 24). The mutation present in proband 2 was determined in one patient of German origin and in one family from the USA (10, 33). The failure to find a second pathogenic variant may be caused by the occurrence of a new, hitherto undocumented mutation. Nevertheless, the sequence of the gene ABCA4 is markedly variable, and even the use of the newest technologies does not lead to the detection of one or more mutations in a range of patients. Hitherto undetected changes in ABCA4 may be located in regulatory sequences or deep in introns, with a pathogenesis which is difficult to demonstrate (8).

The effect of mutations on the function of ABCA4 is diverse, leading for example to a reduction of expression, non-binding of substrate, retention in the endoplasmatic reticule, reduction of ATPase activity, which all leads in its final result to a reduction of the transport activity of this protein. These various functional results probably share in a wide spectrum of phenotype expression of retinal disease on a background of mutation in ABCA4 (29). The first pathogenic variant demonstrated in proband 1 leads to a formation of a termination codon in the second half of the gene, the result of which is premature termination of translation and therefore a truncation of protein with serious impairment of its function. The second mutation p.(Gly1961Glu) determined in proband 1 is missense in its nature, i.e. it leads to a substitution of one amino acid with another. It is assumed that this causes an alteration of the function of the protein by reducing the bond of ATP and ATPase activity (43). This mutation is located outside of the functional domain of ABCA4 and is linked to a milder clinical expression of the disease (18, 38), which would correspond also in the finding in proband 1 in comparison with proband 2. As a consequence of the sequential pathogenic variant found in proband 2, a termination codon is formed in the first quarter of the gene, and the coded protein is thus probably entirely non-functional, alternatively a degradation of mRNA may occur on a background of the defensive cell mechanism nonsense mediated decay (21).

The frequency of incidence of changes in ABCA4 has not been determined in the ordinary Czech population. It is known that this gene contains an extremely large quantity of polymorphisms, frequently with an unclear influence on the function of the protein (37, 48). It is estimated that approximately one in twenty individuals could be a carrier of some type of potentially pathological mutation in ABCA4, and it is therefore possible that the prevalence of retinal diseases associated with this gene is higher than is currently estimated (10, 24, 28, 50). Within the framework of family planning of patients with retinal

dystrophy conditioned by mutations in ABCA4, a request for screening of ABCA4 in a healthy partner is therefore not entirely unjustified. Precisely due to the high frequency of pathogenic changes, recessive diseases occurring on a background of mutations in this gene may appear also in several generations of a single family ("pseudodominance"), which hampers differential diagnostics and genetic consultancy (10, 34, 36).

Demonstration of causal mutations enables us to offer carriers of pathogenic mutations (e.g. parents of an already afflicted child) prevention of transmission of the disease to the next generation through the help of pre-implantation diagnosis (13). In 2010 a study was published describing this procedure in a man with Stargardt disease and his partner, who was demonstrated to be a carrier of a pathogenic allele in ABCA4. Their progeny would therefore have a 50% risk of contracting Stargardt disease upon spontaneous conception (39).

No effective treatment exists for Stargardt disease to date. On the basis of a study of animal models demonstrating increased formation of toxic compounds in the internal segments of the photoreceptors upon exposure to light, patients are recommended to protect their eyes with sunglasses and not to supplement their diet with vitamin A (16, 27, 31, 34). A pilot study, within the framework of which patients with diagnosis of retinal degeneration associated with ABCA4 were supplemented with lutein for a period of 6 months, led to an increase in the optical density of macular pigment in two thirds of eyes, but there was no improvement in central visual acuity (2). Knowledge of the precise molecular diagnosis may be one of the requirements for classification for clinical tests of gene therapy or stem cell therapy, which have been commenced recently. Since 2011 the first clinical study on humans examining the safety of a lentivirus vector developed for gene therapy of Stargardt disease has been under way. In future medical interventions may also focus on the removal of toxic derivates of vitamin A (30), and the effect of neuroprotectives is being tested (www.clinicaltrials.gov). Until the time when the results of these clinical trials are known, however, patients with retinal disease conditioned by mutations in the gene ABCA4 are merely referred for various corrective aids for the visually impaired.

LITERATURE

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