Phenotypic features of *CRB1* associated early-onset severe retinal dystrophy and the different molecular approaches to identify the disease-causing variants

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ABSTRACT

Purpose: To determine the molecular genetic basis of an early-onset severe retinal dystrophy in three unrelated consecutive patients of Czech origin and to describe their ocular phenotype.

Methods: DNA samples from two probands were analyzed using a genotyping microarray (Asper) followed by either target analysis of 43 genes implicated in retinal disorders by next generation sequencing or whole-exome sequencing, respectively. The third proband underwent conventional Sanger sequencing of *CRB1* based on her ocular findings.

Results: All three probands harboured a known disease-causing mutation c.2843G>A; p.(Cys948Tyr) in the *CRB1* gene. One individual was homozygous for this mutation, while in the other two probands c.2308G>A; p.(Gly770Ser) and c.3121A>G; p.(Met1041Val) were also identified in the heterozygous state, respectively. Both variants were novel and evaluated by in silico analysis as pathogenic. A false negative result was observed in one of the two samples examined by the genotyping microarray. Disease onset in all patients was before the age of 7 years. Hypermetropic refractive error, bilateral nummular retinal pigmentation, retinal thickening and cystoid spaces in the macula were observed in two probands, aged 6 and 7 years. The third proband aged 28 years, had bone spicule-like pigmentary changes associated with increased retinal nerve fiber layer.

Conclusions: The first study reporting on the molecular genetic cause of non-syndromic early-onset severe retinal dystrophy in Czech patients identified one homozygous and two compound heterozygote probands with *CRB1* mutations. Retina nerve fibre layer measurements should be considered an integral part of the clinical evaluation of retinal dystrophies. Detailed clinical examination and imaging can both direct molecular screening and help to confirm or refute disease-causation of identified variants.

Keywords: *CRB1*; early-onset retinal dystrophy; whole exome sequencing; optical coherence tomography

INTRODUCTION

Retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA) are clinically heterogeneous monogenic disorders characterized by death of rods followed by cones and abnormalities in the retinal pigment epithelium (RPE) [1-3]. RP leads to nyctalopia and loss of peripheral visual field in the initial stages, progressing to loss of central vision in advanced disease [3]. LCA presents earlier, by definition there is a bilateral severe visual impairment, often associated with nystagmus, before the age of one year [2]. However, there is significant clinical and genetic overlap with early-onset severe retinal dystrophy, also referred to as severe early-childhood onset retinal dystrophy, which presents before the age of 5 – with such children often also labeled as having LCA [4].

LCA and RP are also genetically extremely heterogeneous, to date more than 100 disease-causing genes have been identified, with many more genes remaining to be discovered (RetNet, <http://www.sph.uth.tmc.edu/RetNet/>) [5]. Early-onset severe retinal dystrophies are caused by mutations in at least 22 genes; some of which are also implicated in RP [6]. Mutations in the crumbs homolog 1 gene (*CRB1*, OMIM 604210) have been associated with a wide range of phenotypes including LCA, RP, cone-rod dystrophy, and RP with Coat's-like vasculopathy [1].

Next generation sequencing (NGS) is becoming increasingly used in both research and molecular diagnostics of genetically heterogeneous conditions, including retinal dystrophies such as RP and LCA. The two common approaches are custom-designed target enrichment permitting analysis of disease-associated gene panels and whole-exome sequencing (WES) [5].

In this study we report three different paths leading to the identification of disease-causing mutations in three consecutive Czech probands presenting with visual loss due to early-onset severe retinal dystrophy and describe their ocular phenotype in detail.

METHODS

Ophthalmological examination

Three consecutive probands with no family history of inherited retinal disorders underwent detailed ocular examination in the Department of Ophthalmology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, followed by molecular genetic investigation. Probands 1 and 3 requested ophthalmological assessment to confirm and better characterize the clinical diagnosis of an early-onset retinal dystrophy. Proband 2 was referred after she had sought preconception clinical genetic counseling. Probands and/or their legal guardians signed informed consent prior to inclusion in our study, which adhered to the Helsinki Declaration and was approved by the local institutional review board.

Ophthalmological examination included Snellen visual acuity extrapolated to decimal values, slit-lamp biomicroscopy and dilated retinal assessment. All patients also had standard or wide-field colour fundus photography and autofluorescence imaging [Visucam 200 (Carl Zeiss Meditec AG, Jena, Germany), FF 450 plus IR with filters for fundus autofluorescence (Carl Zeiss) and P200C (Optos Plc, Dunfermline, UK)]. Macular architecture was studied and peripapillary retinal nerve fiber layer (RNFL) thickness measurements were established by circular scans 3.5-3.6 mm in diameter around the optic nerve using spectral domain optical coherence tomography [SD-OCT, Spectral OCT/SLO (OTI Ophthalmic Technologies Inc., Toronto, Canada) and Spectralis (Heidelberg Engineering GmbH, Heidelberg, Germany)].

Molecular genetic analysis

DNA was extracted from white blood cells using the Nucleon Genomic DNA Extraction Kit BACC3 (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions.

Several approaches were undertaken to detect the disease-causing variants: (i) a genotyping microarray of 780 known pathogenic and rare sequence variants in 15 genes associated with early-onset retinal dystrophies (LCA version 9.0, Asper, Tartu, Estonia) was used in proband 1, and (ii) a genotyping microarray covering 594 pathogenic and rare sequence variants in 19 genes implicated in autosomal recessive RP (AR RP, version 5.3, Asper) in proband 2.

Subsequently, because of the negative results of genotyping microarray testing in proband 1, standard PCR was used to amplify 43 genes known to be implicated in retinal dystrophies at the time of the analysis and the products were sequenced on the Ion PGM sequencer (Life Technologies, Darmstadt, Germany) with coverage of at least 25x in more than 97.4% of the analyzed regions. Detected variants were validated by conventional Sanger sequencing. This analysis was performed as a fee for service in a certified diagnostic laboratory (Zentrum für Humangenetik Regensburg, Germany).

Although results from genotyping microarray testing suggested the likely disease-causing gene by identification of one known mutation in *CRB1* in proband 2, she was subjected to WES, because she also requested carrier screening for mutations in the *ABCA4* gene. WES was performed as a fee for service (Axeq Technologies, Rockville, MD, USA) using a TruSeq exome enrichment kit (Illumina, Inc., San Diego, CA, USA) in accordance with the manufacturer's recommendations, with subsequent analysis on an Illumina HiSeq 2000. Reads were aligned to the hg19 human reference sequence using BWA (<http://bio-bwa.sourceforge.net/>). Variants were called by SAMTOOLS (<http://samtools.sourceforge.net/>). 91.2% of target regions were covered more than 10x. The presence of possibly disease-causing variants identified by WES was verified by Sanger sequencing.

In proband 3 all *CRB1* exons were Sanger sequenced from the outset based on the clinical and imaging findings suggesting possible involvement of *CRB1*. Primers used for amplification of *CRB1* exons and Sanger sequencing reactions are shown in Supplementary Table 1.

Only changes with a minor allele frequency <0.001 in variation datasets: the Exome Aggregation Consortium (ExAC) release version 0.3 (<http://exac.broadinstitute.org>) showing exome sequencing data from more than 60,000 unrelated individuals of different origins and the Exome Variant Server (EVS), NHLBI GO Exome Sequencing Project, Seattle, WA (<http://evs.gs.washington.edu/EVS/>) release version: v.0.0.25; which includes data for more than 6,000 exomes, were considered as possibly pathogenic. In addition, we have used in house NGS data from 1057 Czech unrelated individuals (available through projects of The National Centre for Medical Genomics) to check for population specific variant frequencies. The effect of detected novel missense variants was further evaluated using PolyPhen-2 [7], MutPred [8], SNPs&GO [9], PROVEAN [10], SIFT [11] and MutationTaster [12]. Description of the nucleotide and predicted protein changes was as recommended by the Human Genome Variation Society (www.hgvs.org) [13, 14]. Conservation analysis of affected amino acid residues was performed using T-coffee [15].

RESULTS

Proband 1 was noted by her parents to have visual impairment and nyctalopia at the age of 3 years. At 6 years of age her best-corrected visual acuity (BCVA) was 0.1 in the right eye and 0.16 in the left eye. When first examined proband 2 was 28 years old with hand movement vision in both eyes. She was diagnosed with retinal dystrophy when she was 7 years old but admitted to suffer from nyctalopia and reduced visual acuity prior to this age. In the third

proband reduced BCVA was noticed at the age of 6. Examination at the age of 7 years revealed BCVA 0.12 in the right eye and 0.16 in the left eye.

Proband 1 and 3 had nummular retinal pigmentation located predominantly in the mid-periphery (Fig. 1a, c, d, f), and on SD-OCT had increased retinal thickness with loss of normal retinal lamination (Table 1) and intra-retinal cystoid spaces in the macula (Fig. 1j, l).

At presentation at age 28 years proband 2 had advanced disease, with pigmentary deposits resembling bone spicules (Fig. 1e), no cystoid spaces in the macular area were observed using SD-OCT, blood vessels were attenuated, and the optic disc was pale (Table 1). Repeat examination at the age of 30 years showed disrupted macular architecture (Fig. 1k) and increased RNFL thickness in all quadrants above 2 SD compared to the SD-OCT normative database (Fig. 1n-q). Autofluorescence imaging revealed in all three probands a generalised reduction in autofluorescence, with an irregular pattern in proband 1 and 2 and scattered focal areas of increased signal. Patient 3 had a relatively increased signal at the macula and the arcades, other areas were hypofluorescent (Fig. 1i).

All three probands had a known disease-causing variant, c.2843G>A; p.(Cys948Tyr) in the *CRBI* gene. Proband 3 was homozygous for this mutation, while proband 1 and 2 were compound heterozygotes and carried one of two novel *CRBI* mutations c.3121A>G; p.(Met1041Val) and c.2308G>A; p.(Gly770Ser), respectively (Fig. 2).

Results of six prediction tools supported the pathogenic nature of these novel mutations (Supplementary Table 2). In addition, all three mutations had very low frequencies in public variant databases (Table 2) consistent with causing a recessive trait and were absent in 1057 ethnically matched control samples. High conservation across species further supported their pathogenicity (Supplementary Figure 2).

The genotyping microarray detected the known variant c.2843G>A in proband 2, but not in proband 1. In proband 2, although we expected to find a second mutation in *CRBI*, we did not

opt for Sanger sequencing of *CRB1* coding regions, but decided to perform WES. Under the primary assumption of a recessive trait being implicated in proband 2, search in the 22 genes associated with early-onset retinal dystrophies did not reveal two rare variants other than in *CRB1*. The rationale to perform WES in proband 2 was that the partner had Stargardt disease (MIM #248200, caused by recessive mutations in *ABCA4*) [18] and the reason for initiating molecular genetic investigation was that the couple sought preconception counseling. As it has been reported that the frequency of *ABCA4* mutation carriers in a general population may be relatively high [19], arguably warranting preconception screening in partners of patients with *ABCA4* related disease [20], we decided that WES was more cost-effective than setting up conventional Sanger sequencing for the detection of *CRB1* and *ABCA4* coding region variants.

CRB1 variants in proband 3 were identified by targeted Sanger sequencing after clinical examination revealed phenotype similarities with proband 1. The various approaches used in the detection of *CRB1* mutations are schematically depicted in Supplementary Figure 1.

DISCUSSION

Herein we report for the first time on the molecular genetic causes of non-syndromic early-onset severe retinal dystrophy in patients from the Czech Republic. We have used three different approaches to detect the disease-causing variants including WES, genotyping microarray and candidate gene screening based on detailed phenotyping.

Although the three probands were consecutive, they all carried *CRB1* mutations, of which two were novel. Their pathogenic nature was supported by in silico analysis. Segregation testing confirmed that the observed sequence variants were inherited from each parent in families 1 and 2; in family 3 a paternal sample was not available therefore a de novo origin of

c.2843G>A or a larger deletion on the second allele could not be excluded, however, de novo mutations are very rare and larger deletions have not been reported in *CRB1* [1].

Data from other European populations indicate that *CRB1* disease-causing changes are found in approximately 10% of probands presenting with an early-onset severe retinal dystrophy and c.2843G>A has been shown to be the most common mutation observed in patients with *CRB1*-related disease [1] with prevalence ~2 heterozygous carriers per 10,000 inhabitants (ExAC dataset).

A previous study independently validating the Asper genotyping microarray in 153 patients with an early-onset severe retinal dystrophy has established that the rate of false negativity (bidirectional call failure) is 0.47% [21]. In our study the known pathogenic *CRB1* mutation c.2843G>A was not detected in one of the two samples examined by this technique.

Although no unequivocal genotype-phenotype correlation has been established in early-onset severe retinal dystrophies, certain clinical features may be indicative of *CRB1*-related disease. The most prominent are hypermetropic refractive error, nummular pigmentation, intraretinal cystoid spaces, retinal thickening with loss/reduced retinal lamination, and occasionally there may also be sparing of para-arteriolar RPE [22, 23]; all of these features were observed in proband 3, and lead us to screen *CRB1* by Sanger sequencing as an initial approach. In proband 2, with advanced disease, the aforementioned clinical examination features were not readily recognizable and she was initially referred to a geneticist with a diagnosis of autosomal recessive RP, which resulted in genetic screening using an AR RP microarray. However, in a follow-up examination with SD-OCT we were able to document altered retinal lamination with increased RNFL thickness; as this feature has been to the best of our knowledge only observed in patients with early-onset severe retinal dystrophy due to *CRB1* mutations [24] we suggest that RNFL measurements should pose an integral part of clinical evaluation in retinal dystrophies as they may point to the disease-causing gene directly.

Our study describes a range of approaches to establish the underlying molecular genetic basis of childhood-onset inherited retinal disease, highlights the value of a personalized approach, and the importance of detailed clinical examination and imaging to both direct molecular screening and help to confirm or refute disease-causation of identified variants.

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Conflict of Interest: All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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Fig. 1 Retinal findings in three probands with *CRB1* related disease.

Right fundus photographs showing nummular pigmentation in proband 1 aged 6 years (a), bone spicules and nummular pigmentation in proband 2 aged 28 years (b), and retinal pigment epithelium (RPE) changes with peripheral nummular pigmentation and mild bone spicules in proband 3 aged 7 years (c); pigmentary deposits shown in detail in proband 1 (d), 2 (e) and 3 (f). Generally decreased autofluorescence (obtained using different types of fundus cameras) in all three individuals, with isolated hyperfluorescent dots in the mid-periphery in proband 1 (g), irregular pattern in proband 2 due to RPE mottling (h), and relative preservation of the RPE along the vascular arcades and in the macula in proband 3 (i). Horizontal spectral domain optical coherence tomography of the macular area documenting intraretinal cystoid spaces and parafoveal thickening and loss of normal retinal lamination in proband 1 (j), disruption of the inner segment ellipsoid layer, absence of the outer limiting membrane, some debris in the outer nuclear layer, loss of distinct retinal lamination, atrophy of the RPE and thinner choroid in proband 2 (k), and intraretinal cystoid spaces in proband 3 (l). Ultra-widefield fundus photography highlighting mid-peripheral pigment clumping and blood vessel attenuation in the right eye of proband 2 (m). Thickened retinal nerve fibre layer (delineated by red and blue line) and comparison to normative database (curve above 2 SD) in the right (n, o) and left eye (p, q) of proband 2.

Fig. 2 Detected *CRB1* mutations and their segregation with the families.

Pedigree of proband 1 (a), capillary sequencing chromatograms of novel c.3121A>G; p.(Met1041Val) in a heterozygous state (b), pedigree of proband 2 (c), capillary sequencing image of novel c.2308G>A; p.(Gly770Ser) in a heterozygous state (d), pedigree of proband 3 (e), capillary sequencing image of c.2843G>A; p.(Cys948Tyr) in a homozygous state (f).