SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

New genotype-phenotye correlations in inherited ophthalmological disorders

by

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	Ákos Skribek, MD, PhD

The Examination takes place at the 2.405 room of the Department of Human Genetics, Faculty of Medicine, University of Debrecen, at 11:00, 3rd of May, 2018

Head of the **Defense Committee**:	Bálint Nagy, PhD, DSc
Reviewers:	Andás Penyige, MD, PhD
	Nicolette Sohár, MD, PhD
Members of Defense Committee:	Gábor Méhes, MD, PhD, DSc
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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 13:00, 3rd May, 2018

INTRODUCTION

Many out of the 6000 known inherited disorders affect the eye due to its complex structure and signaling mechanism. Mutations responsible for inherited eye disorders are often found in genes which play a crucial role in the development of the eye or in maintaining its function. The abnormalities can occur isolated or as a part of a multiorgan syndrome. The severity of the clinical manifestation, the progression and the tissues which are affected by the disease show a high diversity. Many genetic eye disorders cannot be diagnosed solely based upon clinical examination, the definite diagnosis can rather be achieved with molecular genetic testing. The diagnosis of cases with so-far undetermined genetic origin has always been a challenge due to the time consuming and expensive tools available. However, it has become easier with the recent developments of high through-put genetic testing technology, such as whole exome sequencing and clinical exome sequencing. These new thechniques provide an affordable tool to study genetic diseases of undetermined origin. Here we report three novel genotype-phenotype correlations revealed in inherited ophthalmological disorders associating with myopia. The background of each disease is reviewed critically under separate subheadings in the following section.

Does nonsyndromatous inherited high myopia exist or is it rather cone dystropy with color vison defect? Is Bornholm eye disease stacionery or progressive?

The long- and middle-wavelength sensitive cone opsin genes (L and M-opsin genes; *OPN1LW, OPN1MW*, respectively) reside in a head-to-tail tandem array on Xq28. Rare exon 3 interchange haplotypes are a recently described group of mutations in the opsin genes on chromosome X, which involve amino acid residues 153, 171, 174, 178, and 180, and their name is the acronym of the one letter code of specific amino acids present at these locations. Normal opsin genes specify the haplotypes LVAIS (Leucin, Valin, Alanin, Isoleucin, Serin) and MVAIA (Metionin, Valin, Alanin, Isoleucin, Alanin) in the L and M genes, respectively. Disease causing, also known as toxic exon 3 interchange haplotypes have been commonly reported in Bornholm eye disease (BED), originally described in a family from the Danish island of Bornholm. Although the second family with BED was diagnosed in Minnesota, the family had a Danish origin. Affected males had infantile onset myopia with astigmatism, decreased visual acuity from childhood, subnormal photopic, and normal scotopic electroretinogram (ERG) parameters with normal macular appearance. The disease showed no

progression with age. The first family had a deutan while the second had a protan color vision defect.

The disease was accounted for by the LVAVA interchange haplotype in one of the first two genes in the opsin gene array in both families. Interestingely, exon 3 interchange haploytpes equally associate with either protan or deutan color vision defects. The explanation for this observation was revealed by gene expression experiments. Rare interchange haplotypes have been shown to cause skipping of exon 3, a frame shift and premature termination of translation. In gene expression experiments, no mRNA expression was detected as a consequence of the LIAVA haplotype, however, there was a residual low level of correctly spliced opsin mRNA containing the LVAVA or MVAVA haplotypes indicating some amount of functionally normal opsin proteins. Accordingly, color vision defect was caused in BED patients with color vision defect by the lack of either L- or M-opsin genes in the first two positions of the opsin gene array rather than the direct opsin inactivating effect of the LVAVA or MVAVA haplotypes. These orservations suggest that LVAVA and MVAVA haplotypes may have a dominant effect on the development of myopia, a key element of BED, however, have little or no effect on the color vision.

Interestingly, the LVAVA haplotype differs from other interchange mutations in terms of disease progression as well. According to its original description, BED is a nonprogressive disease. However, a spectral-domain optical coherence tomography (SD-OCT) and adaptive optics scanning laser ophthalmoscopy analysis of two patients with the LVAVA-only genotype showed characteristic signs of progressive macular dystrophy including overall retinal thinning, mottling of photoreceptor inner segments, and structural disruption of the inner retina and the outer nuclear layer. This finding indicated that the LVAVA haplotype may cause progressive degenerative changes that results in damage to neighboring cells in addition to those expressing the mutated opsin. Moreover, these patients gradually developed clinical signs of BED during their elementary school years, representing a somewhat older age at disease onset than observed in other BED patients.

Taken together, our understanding on the genotype–phenotype correlation associating to the LVAVA haplotype has largely developed since the first description of BED as a stationary disease with color vision deficiency. Recent papers suggest that LVAVA expressing cones remain functional in early life and degenerate later. However, there is no direct clinical evidence available so far that BED or exon 3 interchange haplotypes cause progressive retinal dystrophies.

Permanent neonatal diabetes associated with retinal dystrophy- the role of the *NEUROD1* gene

Neurod1 is a tissue-specific basic helix loop helix (bHLH) transcription factor that plays an important role in the development and maintenance of neuronal elements and the endocrine pancreas. It also plays a key role in maintaining normal glucose homeostasis. Most of our knowledge on the function of the *NEUROD1* gene comes from animal experiments. The effect of *NEUROD1* null mutation in mice can not be investigated by usual knockout models due to its lethality. *NEUROD1* null mutant mice kept alive by either a transgene encoding the mouse *NEUROD1* gene under the insulin promoter or by crossing the null mutation into a different genetic background showed concordant neuronal phenotypes, including ataxic gait, impaired balance, circling, impaired cerebellar function and epilepsy. Abnormal hearing and vision are caused by sensory defects of the inner ear and the neural retina.

To define the role of *NEUROD1* in the retina, Ochocinska et al. used *NEUROD1* conditional knockout mice (cKO). They observed that two-month-old *NEUROD1* cKO retinas underwent dramatic changes, including reductions in rod- and cone-driven electroretinograms and disorganized outer segments. Furthermore, photoreceptors totally disappeared in later ages. These results suggested that *NEUROD1* is involved in retinal development and also plays a crucial role in the maintenance of receptor homeostasis. Heterozygous loss-of-function mutations in the human *NEUROD1* gene have been described in the background of maturity-onset diabetes of the young (MODY) and late-onset diabetes – diseases without ophthalmological or other neural abnormalities. In contrast, homozygous *NEUROD1* mutations in humans were not known until Cabezas et al. (2010) described two unrelated patients with two different homozygous loss-of-function *NEUROD1* mutations. Both single base pair duplication (c.364dupG) and two base pair CT deletion (c.427_428del) result in a frameshift and a premature truncation of the C-terminus of the expressed protein (p.Asp122Glyfs*12 and p.Leu143Alafs*55, respectively), leading to mutated proteins completely lacking the transactivation domain. These two probands were diagnosed with permanent neonatal diabetes (PNDM) and showed similar neurologic abnormalities, including cerebellar hypoplasia, developmental delay and visual and hearing impairment. Interestingly, the rescued *NEUROD1*-null mice and the two *NEUROD1*-deficient patients showed similar disease manifestations except epilepsy, which was only observed in the mice. However, the ophthalmic phenotype was not investigated in detail, leaving many questions unanswered

about the structural and functional consequences of a homozygous null *NEUROD1* mutation on the human eye.

Oculodentodigital dysplasia

Oculodentodigital dysplasia (ODDD) is a rare congenital autosomal dominant disease. There are only 300 ODDD cases have been published so far. ODDD is caused by mutations in the *GJAI* gene encoding the connexin 43 protein that is responsible for the normal development of the eye. The disorder is characterized by developmental anomalies of the face, eyes, teeth and limbs. Typical dysmorphic signs include long and narrow nose with prominent nasal bridge and hypoplastic alae nasi, dry and sparse hair and eyebrows. Its main ophthalmic features include microcornea, microphthalmos, glaucoma, iris atrophy, cataract and optic nerve atrophy. To date, more than 60 connexin43 mutations have been reported in the *GJA1* gene. However, for most of these mutations, a detailed genotype-phenotype correlation has not yet been documented.

AIMS

The aim of our study was to investigate patients with rare inherited ophthalmological disorders either of uncertain origin or with unknown genotype-phenotype correlation. The included diseases and related scientific questions are listed below:

1. Our goal was to determine the pathogenic mutation in a large multi-generational family with X-linked high myopia and to provide detailed clinical characterization of the affected male family members. At the time of patient enrolment, no specific genes or mutations were known to associate with X-linked non-syndromic high myopia. However, linkage studies have earlier suggested specific chromosomal regions that cosegregated with the disease in families with X-linked high myopia.

2. Since the causative gene of the X-linked non-syndromic high myopia was unknown, we could not use targeted Sanger sequencing to reveal the disease causing mutation. Therefore, we intended to set up two next generation genetic methods: X-chromosome arrayCGH and clinical exome sequencing to identify the pathogenic mutation. We also aimed to create an effective algorithm for the identification of the specific alterartions capable to deal with the huge amount of data of the next generation sequencing.

3. Our goal was to determine the effect of a known homozygous *NEUROD1* null mutation in humans thus to provide insight into the role of the *NEUROD1* gene in retinal homeostatis. To achieve that we aimed to characterize its functional and anatomical consequences in the human retina. We intended to use long term follow-up to provide information on the patomechanism, progression and prognosis of the disease.

4. Our aim was to identify the causative mutation in a patient with characteristic dysmorphic features of Oculodentodigital dysplasia and to document the ophthalomological phenotype in detail. To date, more than 60 mutations are described in the *GJA1* gene. However, for most of these mutations no detailed genotype-phenotype correlation has yet been documented. We intended to compare our results with the previously described ODDD cases and other diseases associated with microphthalmos.

PATIENTS AND METHODS

Patients

Three different diseases were identified in three unrelated families. The ophthalmological examinations were performed at the Department of Ophthalmology, University of Deberecen. The genetic investigations were performed at the Division of Clinical Genetics, Department of Laboratory Medicine, University of Debrecen. We obtained informed consent from all participants who underwent physical and genetic examinations. The research was performed according to the Declaration of Helsinki and was approved by the local institutional review board.

Case I.

We performed clinical and genetic examinations in a six-generation family with X-linked high myopia. Living family members unanimously confirmed that male family members of the second generation suffered from serious visual disability, however its cause remained undisclosed. All affected members of the family were males who developed substantial myopia before school age. The youngest patient had no complaints, while others suffered from photophobia, gradually increasing difficulty identifying mixed colors and distinguishing between small color hue differences. Patients complained neither of night blindness nor a decline in their visual acuity. Even the oldest living affected family member considered his visual acuity satisfactory to maintain his regular work and everyday life. Genetic and clinical investigations were performed in case of these family members: VI:6 (11- years old), V:1 (46 years old, proband), V:3 (42-years old), IV:5 (62-years old) and IV:7 (51-years old).

Case II.

We annually examined a female patient suffering from neonatal diabetes caused by a homozygous loss-of-function mutation in the NEUROD1 gene between 2009 (age 14) and 2014 (age 19). The patient was diagnosed with intrauterin retardation, developmental delay, severe hearing impairment, cerebellar hypoplasia, and had normal exocrin function of the pancreas. The patient had normal blood glucose control using insulin supplementation. The patient complained about slowly progressive blurry vision, constriction of the visual field, and difficulties seeing at night or in dim light, beginning in early childhood. Her best corrected visual acuity showed a slow decrease during the investigation period, ranging from 20/25 to

15/25. Her refractive error did not show any changes during the investigation period and remained -7.5D spherical with +3.0D cylindrical correction for both eyes. Automated full-size visual field perimetry showed concentric constriction of the visual field, sparing the central 30 degrees in both eyes, in 2013. The previously performed genetic investigation identified a homozygous loss-of-function mutation in NEUROD1 gene (c.427_428delCT (p.Asp122Glyfs*12). However, detalied ophthalmological description of the effect of the homozygous *NEUROD1* null mutation has not yet been documented.

Case III.

The 28-year-old male patient was investigated with dysmorphic features suggestive of ODDD, such as long and narrow nose, abnormally shaped small teeth with enamel hypoplasia and high arched palate. He was born with bilateral syndactyly of fingers IV-V which was surgically treated. His toes were normal. The detailed ophthalmological examination was completed by anterior segment OCT, Pentacam and the determination of axial length. The patient was the only child of his parents who were free from any symptoms of ODDD. The available family members were also examined (mother, and the father of the mother). To confirm the clinical diagnosis, genetic analysis was performed.

Ophthalmological investigations

Color fundus photographs were taken with a Zeiss FF450+IR fundus camera (Carl Zeiss AG, Jena, Germany) mounted with a ZK-5 color sensor (Allied Vision Technologies GmbH, Stadtroda, Germany) and operated with Zeiss Visupack 4.4 software. Optical coherence tomography and confocal-scanning laser fundus autofluorescence imaging were performed with SpectralisOCT (Heidelberg Engineering, Heidelberg, Germany). Electroretinography was executed with Ganzfeld Q400 equipment (Roland Consult GmbH, Brandenburg, Germany) using standard ISCEV parameters. Measures of photoreceptor dysfunction were defined as: mild (70%–99% of normal amplitude), moderate (30%–69% of normal), severe (1%–29% of normal), or undetectable. The visual field was investigated with an Octopus 900 automated static perimeter, using its standard white/white full-size visual field program (Haag Streit AG, Koenitz, Switzerland). Autorefractometry was performed using a Topcon KR 8100 equipment (Topcon Corp., Tokyo, Japan). Color vision was tested under standardized conditions, with pseudoisochromatic plates (Tafeln und Prüfung des Farbensinnes, 29. Auflage, 2002), the Farnsworth Munsell 100-hue test and the anomaloscope Nagel type II test.

Molecular genetic methods

Isolation of genomic DNA

Genomic DNA was isolated from EDTA or citrate anticoagulated blood using the QIAamp Blood Mini kit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany).

Array CGH

The copy number variations (CNVs) of X chromosome were analyzed by comparative genomic hybridization array (array CGH: Roche, NimbleGen, Madison, WI, USA) on genomic DNA from the proband sent to the NimbleGen custom microarray services facility (NimbleGen Systems of Iceland, LLC, Reykjavik, Iceland). The genomic DNA labelled with Cy3 or Cy5 using NimbleGen Dual Color DNA labelling kit and co-hibridizyed to the arrays (Nimble Gen CGH Services: Guide to your CGH data v5p1). For data analysis SingleMap (version 1.9) was used. The design of the array was based on the human reference genome NCBI36/hg18.

Sanger sequencing of RPGR ORF15

The genomic DNA of the index patient was screened for disease-associated sequence alterations in exon ORF15 of *RPGR* gene by sequencing of PCR products. The amplification consisted of 35 cycles. We applied previously published and self-designed primers. Cycling conditions of PCR reactions were in case of 15.1, 15.2, 15.3, and 15.5 fragments: initial denaturation 95°C for 10 minutes, denaturation 94°C for 30 seconds, annealing 55°C for 30 seconds, and elongation 72°C for 1 minute. The amplification consisted of 40 cycles. The conditions of the 982-bp length 15.4 fragment was: initial denaturation 95°C for 10 minutes, denaturation 94°C for 45 seconds, annealing 60°C for 45 seconds, and elongation 72°C for 1.5 minutes. Polymerase chain reaction amplicons were identified with ABI BigDye Terminator cycle sequencing kit version 3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI 310 sequencer (Applied Biosystems). The sequenced profiles were compared with the retinitis pigmentosa GTPase regulator (RPGR) reference genome (NM_001034853) in National Center for Biotechnology database.

Clinical exome sequencing

Clinical exome sequencing was performed on the sample of the proband (V:1) and his mother (IV:3). Exome sequencing was carried out by TruSight One Sequencing Panel (Illumina, San Diego, CA, USA) that covers 4813 clinically relevant genes. Library preparation was done according to the manufacturer's instruction and sequencing was performed using Illumina Miseq system (Illumina). At least 20-fold coverage was observed in 92% of the target regions. The reads were mapped against the human reference genome NCBI37/hg19. For bioinformatic analysis NextGene Software version 2.3.4 was used (SoftGenetics, State College, PA, USA). The analysis pipeline was based on X-linked recessive inheritance. The coding regions of X-linked genes and the splicing regions (±5 bp) were analyzed. The silent and noncoding variants were excluded. Ensembl (http://ensembl.org), HGMD (http://hgmd.cf.ac.uk), and ExAC (http://exac.broadinstitute.org) and dbSNP (http://.ncbi.nlm.nih.gov/projects/SNP/) databases were used for analysis of the variants.

Validation and segregation testing of OPN1LW, OPN1MW és CACNA1F variants

Putative variants detected by clinical exome sequencing in *OPN1LW*, *OPN1MW* (c.532A>G/p.Ile178Val; c.538T>G/p.Ser180Ala) and CACNA1F (c.1843G>T/p.Ala615Ser) genes were confirmed by Sanger sequencing. Cycling conditions of PCR reactions were: initial denaturation 95°C for 10 minutes, denaturation 94°C for 30 seconds, annealing 55°C for 30 seconds, and elongation 72°C for 1 minute. Polymerase chain reaction amplicons were sequenced on an ABI310 sequencer (Applied Biosystems). These analyses were performed in all available family members (IV:3, IV:5, IV:7, V:1, V:3, VI:6).

Sanger sequencing of GJA1 gene

To confirm the clinical diagnosis of ODDD, *GJA1* gene was sequenced by Sanger sequencing. Exon 1 is an untranslated region, and the coding sequence starts from exon 2. We amplified the *GJA1* exon 2 using polymerase chain reaction (PCR) and designed primer pairs with mismatches to avoid the amplification of the *GJA1P1* pseudogene. The cycling conditions of PCR reaction were: initial denaturation 95°C for 10 minutes, denaturation 94°C for 30 seconds, annealing 55°C for 30 seconds, and elongation 72°C for 1 minute. The amplicons were identified with ABI BigDye Terminator cycle sequencing kit version 3.1 (Applied Biosystems, Foster City, CA) on an ABI310 sequencer (Applied Biosystems). The

sequenced profiles were compared with the *GJA1* reference genome (NM_001034853) in National Center for Biotechnology database (NM_000165).

RESULTS

Ophthalmological findings in the family with X-linked high myopia and late-onset cone dystrophy

We have investigated a six-generation family with X-linked high myopia, including three of the precedent generations. Living family members unanimously confirmed that male family members of the second generation suffered from serious visual disability, however its cause remained undisclosed. One family member (subject IV:9) refused to participate in the study. We have followed the proband (patient V:1) for 8 years. Previous clinical data were available on patient IV:5 that were retrospectively analyzed. All affected members of the family were males who developed substantial myopia before school age. The refraction of patient IV:5 remained stable thereafter, while others experienced a gradual increase of myopic refraction throughout their life. Spherical equivalent of patients was between −5.0 and −21.0 diopters upon examination (patient IV:5 underwent corneal refractive surgery at the age of 40, therefore we used preoperative refractive data). The youngest patient had no complaints, while others suffered from photophobia, gradually increasing difficulty identifying mixed colors and distinguishing between small color hue differences. Patients complained neither of night blindness nor a declined in their visual acuity. Even the oldest living affected family member (IV:5, 62-years old) considered his visual acuity satisfactory to maintain his regular work and everyday life. A significant decline in visual acuity could only be detected in the two oldest patients (IV:5, IV:7), both older than 50 years. A deutan/protan color blindness developed gradually and simultaneously along with the visual acuity decline in these patients, which could not be detected in younger individuals. However, patient VI:6 was found to have a mild deutan color vision defect with pseudoisochromatic charts and the Nagel anomaloscope at the age of 11 years. On the other hand, he performed well on the FM 100 hue test excluding a severe color vision defect. Ophthalmoscopic examination revealed myopic fundus changes with a central pigmented epithelial layer atrophy in the proband and patients V:3, IV:5 and IV:7, all older than 40 years. The 11-year-old patient (VI:6) had normal fundus appearance. Autofluorescent fundus images showed central patchy drop-outs in older patients (V:1, V:3, IV:5 and IV:7) indicating severe central pigmented epithelial layer atrophy. Central hyperreflective ring, a characteristic sign of cone dystrophies, was detected in patients IV:7 and VI:6. Spectral-domain OCT examination showed disruption of the pigmented epithelial layer, a thinning of the outer nuclear layer and photoreceptor outer segments in patients older than 40 years. The 11-year-old patient (VI:6) showed normal

scotopic rod response and mildly decreased response upon maximal scotopic light stimulation (ERG 3.0). Moderately reduced amplitudes were registered under single photopic stimulation and a mild reduction was detected in the response for photopic 30-Hz flicker stimulation of cone photoreceptors. Patients V:1 (proband) and IV:7 showed severely reduced rod response, moderately reduced amplitudes to maximal scotopic light stimuli and undetectable electrical activity of cone photoreceptors under photopic conditions in response to single or flicker stimuli. Patient IV:5 (62-years old) showed moderately reduced rod response, mildly reduced amplitudes to maximal scotopic light stimuli, and undetectable electrical activity of cone photoreceptors under photopic conditions in response to single or flicker stimuli.

Molecular genetic findings in the family with X-linked high myopia and late onset cone dystrophy

No chromosomal rearrangements or copy number variations of the X chromosome could be detected with array CGH. Mutations in the *RPGR* gene on Xp21 are the most common causes of X-linked cone dystrophy. ORF15 of *RPGR* gene has been identified as a mutation hotspot. In the proband we found an already known in-frame 12-nt deletion polymorphism in nucleotides 3074_3085 resulting in elimination of four amino acids (rs201134185, c.3074_3085delTGGAAGGGGAGG, p.Val1025_Glu1028del). The deletion has no clinical significance.

Clinical exom sequencing detected 8174 variants int he porband and 8041 variants in the mother. To decrease the number of the potential variants the following filter parameters were used: In the first round, we focused on the X- linked inheritance, and the missense mutations. We detected 47 hemizygous variants in the proband and 50 heterozygous variants in the proband's mother on the X chromosome. Seventeen of these variants were found in both samples. Ensembl, dbSNP, and Exac databases checked the minor allele frequencies of these variants. Filtering for minor allele frequencies (MAF) and clinical significance yielded three variants putatively associating to diseases in our pedigree.

The c.1843G>T (p.Ala615Ser) variant in the *CACNA1F* gene had a MAF less than 0.01 and the gene is known to be associated with X-linked incomplete congenital stationary night blindness and Åland island eye disease. The mutation could not be detected in any other family members by Sanger sequencing. We found two variants (c.532A>G, p.Ile178Val and c.538T>G, p.Ser180Ala) in the *OPN1LW* gene and one variant (c.532A>G, p.Ile178Val) in the *OPN1MW* gene, which are known to form the LVAVA and MVAVA rare interchange haplotypes in the L- and M-opsin genes, respectively. No other nucleotides were detected in

corresponding positions in any of the reads indicating only mutated opsin genes in the array. Sanger sequencing unequivocally confirmed these haplotypes with only one nucleotide detectable at each position in every affected family member. However, there were nucleotides coding a leucine and a methionine simultaneously detected at amino acid position 153, originating from the L- and M-opsin genes, as expected. Based on these results we can conclude that the LVAVA and MVAVA haplotypes cosegregated with the disease in the entire family. We could not detect any additional nucleotide variations including SNPs or any other mutations in the L- and M-opsin genes of the proband and his mother. *OPN1MW2*, an additional M-opsin gene was only detected in the mother. We checked an exonic (*OPN1MW*, c.849A and *OPN1MW2*, c.849C) and an intronic (*OPN1MW*, c.984+59 and OPN1MW2, c.984+59C) site that distingusihes between the two M opsin genes. The mother of the proband displayed only the nucleotides specific for the *OPN1MW2* gene in the corresponding positions, while we failed to detect any of these nucleotides in the proband. No other nucleotides were detected and the coverage was zero in these positions in the proband. Most likely, one allele of the mother contains normal L and M opsin followed by an *OPN1MW2* gene, while the other allele, transmitted to the proband, has mutated opsin genes and has no *OPN1MW2* gene. Based on NGS sequencing data therefore we can assume, that the proband has no additional M genes. NGS data are also in line with the clinical findings of young patients having normal color vision, suggesting one red and one green opsin gene in the first two places following the promoter, which are the only opsin genes translated in the array.

Results of the ophthalmological examinations in the female patient with homozygous *NEUROD1* null mutation associated with progressive rod-cone dystrophy

The anterior segment did not show any morphological changes in the 21-year-old patient with *NEUROD1* null mutation. Scheimpflug imaging of the anterior segment showed regular oblique corneal astigmatism. Corneal thickness was near normal, with a central corneal thickness of 600 μm. No signs of Keratoconus could be detected with Scheimpflug imaging using the Belin-Ambrosio analysis. Anterior chamber depth and anterior chamber angle were within the normal range. Dilated funduscopy revealed optically clear media throughout the cornea, lens, and vitreous. The retinal pigmented epithelial layer showed mottling at the posterior pole and diffuse atrophy in the periphery. However, neither bone spicule formation nor pigment clumping could be observed. Unlike the pale and waxy optic disc associated with retinitis pigmentosa (RP), the optic disc in her case was slightly pale but had a near normal appearance. The diameter of retinal vessels was apparently normal, and

vascular attenuation could not be observed. The central foveal spot was enlarged. Diabetic retinopathy was not observed during the investigation period. Fundus autofluorescence imaging presented a dark fovea surrounded by a hyperreflective ring, as seen in other hereditary retinal dystrophies. Increased autofluorescence of the choroid was detected, most likely due to the atrophy and mottling of the RPE in the posterior pole. No typical spot or patchy reflectance of lipofuscin could be seen. Optical coherence tomography showed reduced retinal thickness. Outside the fovea, the neurosensory retina was composed of only six layers, lacking the external limiting membrane, photoreceptor outer segment, and photoreceptor inner segment. An optically dense discoid remnant of photoreceptors was detected in the central fovea. The extent of the disc representing photoreceptors in the fovea showed constriction during the investigation period, indicating the progressive loss of cone photoreceptors.

Electric signals of retinal origin could not be differentiated from background noise with any of the standard ERG settings, including the dark-adapted 0.01 ERG (rod response), dark-adapted 3.0 ERG (maximal combined rod-cone response), dark-adapted 3.0 oscillatory potentials, light-adapted 3.0 ERG (single-flash photopic ERG), and light-adapted 3.0 flicker ERG (30 Hz flicker) settings. Peripapillary nerve–fiber layer thickness showed normal values in both eyes.

Results of the ophthalmological and genetic investigations in the patient with Oculodentodigital dysplasia

The 28-year-old male patient was investigated with dysmorphic features suggestive of ODDD, such as long and narrow nose, abnormally shaped small teeth with enamel hypoplasia and high arched palate. The patient was the only child of the parents. He was born with bilateral syndactyly of fingers IV-V which was surgically treated. His toes were normal. The patient's corneal diameter was 9,51 and 9,56 mm OD and OS, respectively, representing microcornea on both sides. His best corrected visual acuity was 1.0 on both eyes, however his refractive error was -6.0Dsph+3.0Dcyl in his right eye and -5.0Dsph+2.5Dcyl in his left eye. Axial length (22.63 mm OD and 22.49 mm OS), corneal thickness (542 μm OD and 544 μm OS) and intraocular pressure (14 Hgmm in both eyes) were normal. Optic nerve head appeared healthy in both eyes. The anterior chamber depth was 2.34 mm (OD) and 2.26 mm (OS), the anterior chamber angle was 23,8°-26,7° (OD) and 22.9°-25.4° (OS) representing a shallow anterior chamber with a narrow anterior chamber angle. Gonioscopy also revealed a

narrow angle, however it reached as much as Schaffer grade III. The ocular fundus was normal in both eyes. The parents of the patient were free from any symptoms of ODDD. The father of the patient had no refractive errors, however the mother had a moderate myopia (SE -6.0D OD/-4.5D OS). Moreover, the father of the mother had a high myopia (SE -18.0D OD and -20.0D OS). Sequence analysis detected a pathogenic heterozygous missense mutation, c.413G>A, p.Gly138Asp in the cytoplasmic loop of connexin 43 protein. This mutation was previously reported in a patient with ODDD.

DISCUSSION

New genotype-phenotype correlations

LVAVA/MVAVA opsin interchange haplotypes associated with high mopia and late-onset cone dystropy

We have investigated a three-generation family with X-linked high myopia. Initially, the proband was suspected to suffer from nonsyndromatous X-linked high myopia. However, diminished rod and extinguished cone responses upon ERG examination indicated a simultaneous X-linked cone dystrophy confirmed by the macular dystrophy and the combined red and green color blindness (blue monochromatism) of older family members. We failed to detect any mutations in the ORF15 of the *RPGR* gene, a known locus for X-linked cone dystrophy. X chromosome-specific high-resolution array CGH detected neither rearrangements nor copy number variations in the proband. Clinical exome sequencing analysis identified three putative variants including c.1843G>T (p.Ala615Ser) in the *CACNA1F* gene and the LVAVA and MVAVA rare interchange haplotypes in exon 3 of the L- and M-opsin genes, respectively. The pathogenic role of the *CACNAF1* mutation was already questioned by the noncorresponding clinical phenotype. Moreover, the mutation could not be detected with Sanger sequencing in any other family members, ultimately excluding it as a cause of the disease. On the other hand, Sanger sequencing in all affected family members, making them the only possible cause of the disease, could confirm the LVAVA and MVAVA haplotypes. Normal haplotypes were detected with neither of the sequencing methods indicating a mutated-only genotype in the opsin gene array in all patients. The LVAVA haplotype was found to cause the disease in the first two families reported with BED. However, findings in our family showed striking differences when compared with these original reports: our patients retained normal visual acuity and color vision even in their middle ages and only thereafter did the disease lead to progressive blue monochromatism and macular dystrophy, a phenotype previously not described in association with rare interchange haplotypes. We reported a novel phenotype that has not been previously reported to associate to any of the rare exon 3 interchange haplotypes. This novel genotype-phenotype correlation highlighted two new findings:

1. Interchange haplotypes do not only cause stationer BED but can also lead to progressive retinal dystrophy.

2. The LVAVA haplotype does not associate with non-syndromic high myopia as suggested in two Chinese family previously. In contrast to that, here we show that LVAVA haplotype associates with late-onset syndromic high myopia.

Homozygous NEUROD1 null mutation associated with a new syndromic progressive rod-cone dystrophy

NEUROD1 takes part in the development of the endocrine pancreas and neuronal elements, including the retina. Most of our knowledge about the function of the protein comes from animal experiments. Most of our knowledge on the function of the *NEUROD1* gene and protein comes from animal models. *NEUROD1* seems to be necessary for the maintenance of normal photoreceptor structure and function. The absence of *NEUROD1* leads to severe retinal dystrophy. Our study demonstrates the first detailed description of an ophthalmological phenotype caused by a homozygous *NEUROD1* null mutation in humans and indicates that it causes severe rod–cone dystrophy. Although it resembles retinitis pigmentosa in many aspects, it can be clearly distinguished from it. The relatively spared pigmented epithelial layer, the normal appearance of the optic disc and retinal vessels, and the disc-shaped remnant of cone photoreceptors in the fovea are characteristic hallmarks of the disease, distinguishing it from any other hereditary retinal dystrophies. The total lack of rod photoreceptors, sharply demarcated from relatively spared foveal photoreceptors is also an unusual phenotype. The findings of our report confirm the observations of knockout animal models. In the present study we provided the first detailed description of the ophthalmological consequences of a homozygous *NEUROD1* mutation in humans. Our results are the first to show that the loss of *NEUROD1* has similar effects on the human retina as have been previously shown in animal experiments. Moreover, we proved that the *NEUROD1* gene has a crucial role in retinal homeostasis in humans.

Relative anterior microphthalmos associated with oculodentodigital dysplasia

ODDD is an extremely rare congenital disorder caused by mutations in the *GJA1* gene coding the connexin 43 protein. The 28-year-old male patient showed characteristic facial features of ODDD. Detailed ophthalmologic investigation detected a shallow anterior chamber with a narrow anterior chamber angle, microcornea, a normal axial length and a refractive myopia in both eyes referring to relative anterior microphthalmos.

Genetic analysis detected a heterozygous missense mutation in the *GJA1* gene

(c.413G>A, p.Gly138Asp). However, the ophthalmological phenotype of the present case differs from those observed in ODDD patients so far, in that our patient had a normal axial length. To the best of our knowledge, this is the first report on ODDD associating to relative anterior microphthalmos and myopia. Our results indicate that microphthalmos is not an obligate feature of ODDD and microcornea can associate with a normal axial length in ODDD patients. This observation expands the known phenotypic spectrum of the ODDD and may add to the understanding of genetic factors influencing axial length of the eyeball.

In the present work we provided genuine novel information on the genotype-phenotype association of three rare inherited ophthalmologocal disorders associating with myopia. Our results add substantially to the current knowledge on these disorders and provide useful information for the practicing geneticists and ophthalmologists.

New results of the dissertation

1. Taking the advantage of the interdisciplinary cooperation of the Department of Ophthalmology and the Department of Laboratory Medicine, Divison of Clinical Genetics, we determined new genotype-phenotype correlations of patients with inherited ophthalmologycal disorders. We combined succesfully traditional genetic investigations and new generation methodologies, classical ophthalmological examinations with modern imaging and electrophysiological investigations to detect the genetic causes and the exact phenotype of the disases.

2. The combination of the LVAVA/MVAVA haplotype and the related ophthalmological phenotype was described for the first time in the literature.

3. The association of late-onset cone-rod dystrophy and X-linked high myopia with toxic haplotypes was previously unknown. Thus, we have contributed to a more accurate understanding of the genotype-phenotype correlations associated with rare exon 3 *interchange* haplotypes.

4. We noticed that the specific symptoms of progressive cone dystrophy (visual acuity decrease, color vision deficiency) appear only in later ages, which call attention to the importance of detailed family studies.

5. Our results confirmed that the color vision defect is not the direct consequence of the toxic haplotype, it is due to the late-onset cone dystrophy.

6. Based on our results the diagnosis of a previously reported non-syndromatous X-linked high myopia should be reconsidered, thus re-opening the question whether there is no non-syndromatous X-linked high myopia.

7. We performed the first detailed ophthalmological description of a progressive retinal dystrophy caused by a homozygous *NEUROD1* null mutation (c.427_428delCT, p.Leu143Alafs*55) in a patient with permanent neonatal diabetes, neurological defects and severe visual impairments. We determined in detail the anatomical and functional consequences of the homozygous *NEUROD1* mutation in the human retina. We were

the first to report that the *NEUROD1* null mutation causes severe retinal dystrophy in humans similar to that seen in animal experiments.

8. We detected a previously described missense mutation in the *GJA1* gene (c.413G>A, p.Gly138Asp) in a patient with oculodentodigital dysplasia. A new phenotype was revealed by detailed ophthalmological examination, namely the simultaneous presence of anterior microphthalmos and myopia. Our results indicate that microphthalmos is not an obligate feature of ODDD and microcornea can associate with a normal axial length in ODDD patients.

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List of publications related to the dissertation

1. Orosz, O., Fodor, M., Balogh, I., Losonczy, G.: Relative anterior microphthalmos in oculodentodigital dysplasia.
   Indian J. Ophthalmol. 66 (2), 334-336, 2018.
   IF: 0.835 (2016)
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   Invest. Ophthalmol. Vis. Sci. 58 (3), 1834-1842, 2017.
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   Mol. Vis. 5 (21), 124-130, 2015.
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