Clinical and genetic features of Hungarian achromatopsia patients

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Purpose: To describe the clinical features and molecular genetic findings in a collection of Hungarian achromatopsia patients.

Methods: Twelve patients with congenital achromatopsia from nine Hungarian families were analyzed in this study. The patients underwent standard ophthalmological examination including detailed full-field electroretinography and color vision testing. In two patients, dark adaptation and spectral luminosity tests were also performed. PCR/RFLP analysis and DNA sequencing was applied for mutation screening of CNGA3 and CNGB3. Heterologous minigene expression was used to evaluate transcript splicing of a new intronic mutation in CNGB3.

Results: Mutations in CNGA3 were present in four families and mutations in CNGB3 in the remaining five families, including mutations known from Western European patient samples and two new CNGB3 mutations: c.112C>T/Gln38X and c.1663-5T>G. Heterologous expression in COS7 cells shows that the latter induces a splicing defect through the activation of a cryptic splice site 4 bases upstream of the genuine splice site. The patients presented with a clinical picture typical for congenital achromatopsia and there was no significant difference in the phenotype of subjects with either CNGA3 or CNGB3 mutations based on standard ophthalmological examination. However, we assume residual cone function in a subject homozygous for the Phe547Leu mutation in CNGA3 based on prior detailed psychophysical testing (i.e., dark adaptation and spectral luminosity).

Conclusions: Mutations in CNGA3 and CNGB3 account for achromatopsia in Hungarian patients including known mutations and a few new CNGB3 mutations. While standard ophthalmological examination revealed a phenotype of complete achromatopsia, we show that thorough psychophysical testing can help to identify subjects with some minute cone function.

Typical complete achromatopsia (i.e., complete achromatopsia with reduced visual acuity, mediated only by rods), also referred to as rod monochromacy (ACHM2, OMIM 216900; ACHM3, OMIM 262300; ACHM 4, OMIM 139340), is a rare, autosomal recessive inherited congenital retinal disorder. Its prevalence has been estimated at about 1 in 30,000 to 50,000 [1,2]. The disease is characterized by photophobia, nystagmus, severely reduced visual acuity, and complete inability to discriminate colors. ERG recordings and psychophysical tests typically show a complete absence of cone function supporting the original theory of Galezowski in 1868 [3], who proposed that visual function in complete achromats is mediated wholly by rods, the "pure rod theory".

Some of the few histological investigations, however, have revealed that some cones, even morphologically intact ones, may be present in the retina of achromats [4,5]. Similarly, some preserved cones have been noticed in animal models for achromatopsia. These cones are described as morphologically malformed even in very young animals and eventually degenerate in older ones [6,7].

Despite the progressive degeneration observed in animal models, human achromatopsia is regarded as a stationary disorder, since the symptoms do not progress after childhood [5].

Because of the rarity of achromatopsia and the nonspecific early symptoms (such as inability to fixation, nystagmus, photophobia) in childhood, it is often misdiagnosed as some other, relatively more common disorder, such as Leber congenital amaurosis, Stargardt macular dystrophy, optic nerve atrophy, early onset nystagmus of uncertain origin. In contrast to achromatopsia, these disorders are characterized by a progressive loss of vision, and therefore early diagnosis would be very helpful in clinical practice and patient counselling.

In recent years there has been considerable progress in elucidating the genetic basis of achromatopsia. So far, three genes have been associated with achromatopsia: CNGA3 and CNGB3 encoding the α- and β-subunit of the cone photoreceptor cGMP-gated channel, and GNAT2 encoding the α-subunit of the cone G-protein transducin [8-12]. Mutations in CNGA3 and CNGB3 account for about 25% and 45% to 50% of achromatopsia cases, respectively [13-15]. In contrast, mutations in GNAT2 have only been found in a few families. However, these figures stem from the analysis of mostly Northern European and US patients and may be biased due to some highly frequent founder mutations [13,16].

Here we report the molecular genetic analysis and clinical investigation of Hungarian achromatopsia patients. While Hungarian people are of mixed origin with a considerable genetic contribution from eastern and southeastern European populations, most common western CNGA3 and CNGB3 mutations were also found in Hungarian achromats in addition to a few new exclusive CNGB3 mutations.

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METHODS

Patients: Twelve achromates from nine Hungarian families were involved in this study. They were informed about the objectives of the study and consented to participate. The patients were examined and followed-up at the 2nd Department of Ophthalmology, Semmelweis University, Budapest, between 1998 and 2002. Venous blood samples were taken for genetic analysis from the patients and some available relatives.

Each family is assigned a consecutive letter of the alphabet in the order in which they were first admitted to the clinic. In Family E, the mother and both of her children were affected which initially precluded the establishment of the mode of inheritance (Figure 1). In Family H, both children were affected, while in the other families there were only single affected subjects. The patients originate from different parts of Hungary, but now they all live in or around Budapest.

Clinical ophthalmologic examinations: The clinical diagnosis was based on photophobia, nystagmus, low visual acuity, and the results of color vision tests (Ishihara plates, Farnsworth Dichotomous D15 test) and full-field electroretinography (ERG). The ERGs were obtained with "Retiport" system (Roland Consult, Brandenburg, Germany) under sc-

otopic and photopic conditions (with white light stimuli), according to the Standards of the International Society for Clinical Electrophysiology of Vision (ISCEV) [17]. In two of the children, visual acuity measurements and color vision tests could not be performed, the fundus examination and the ERG were done under mild general anesthesia, using Tomey PE-400 ERG setup.

Molecular genetic analysis: The genetic analysis was carried out at the Molecular Genetic Laboratory of the University Eye Hospital, Tübingen, Germany. Genomic DNA was extracted from venous blood samples according to standard procedures. Index patients were selected for mutation screening of the CNGA3 and the CNGB3 gene. Initially the most prevalent mutations were screened for by RCP/RFLP analysis. This includes the c.847C>T mutation in CNGA3 associated with a gain of a *Dde*I site. In CNGB3 we assayed for the c.819_826del8 mutation associated with a loss of a *Hin*fI site, the c.886_896del11insT mutation associated with a loss of an *Apo*I site, the c.991-3T>G mutation associated with a gain of a *Hin*fI upon amplification with a mismatch containing reverse primer (5'-GATTAAATTCAAAAAATGAAGTGACT-3'), and the c.1006G>T mutation associated with a loss of an *Apo*I site. Digested PCR products were separated on 2.5% agarose gels and visualized by ethidium bromide staining in comparison to a normal control subject. Eventually the coding exons of CNGA3 and/or CNGB3 were sequenced after PCR amplification with primers located in flanking intron sequences. PCR products were purified by Exo-SAP treatment (ExoSAP-IT, Amersham Biosciences, Freiburg, Germany) and sequenced employing BigDye Terminator chemistry (Applied Biosystems, Darmstadt, Germany). Sequences were separated on an ABI Prism 3100 capillary sequencer (Applied Biosystems) and inspected manually and additionally by computer-aided alignments (SeqMan; Lasergene, Madison, WI). Segregation analysis within families was done by PCR/RFLP or sequencing of the appropriate exons.

TABLE 1. MUTATIONS IN HUNGARIAN ACHROMATOPSIA PATIENTS

Gene	Exon/intron	Alteration in nucleotide sequence	Alteration in polypeptide/mRNA
CNGA3	Exon 5	c.488C>T	Pro163Leu
CNGA3	Exon 7	c.847C>T	Arg283Trp
CNGA3	Exon 7	c.1641C>T	Phe547Leu
CNGB3	Exon 1	c.112C>T	Gln38X
CNGB3	Exon 6	c.819_826del8bp	Pro273fs
CNGB3	Exon 10	c.1148delC	Thr383fs
CNGB3	Intron 14	c.1663-5T>G	Splicing mutation

Nucleotide sequence position based on GenBank accession numbers AF065314 (CNGA3) and AF272900 (CNGB3) with the adenosine of the annotated start codon denoting nucleotide position 1.
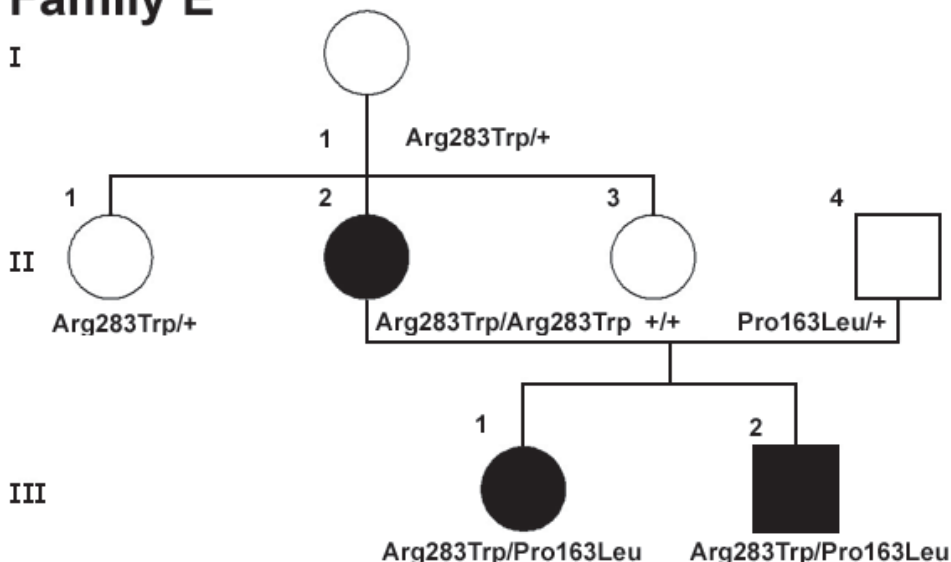

Figure 1. Pedigree of family E displaying pseudo-dominant inheritance of achromatopsia. The genotypes are provided for all subjects available for molecular genetic analysis. Closed circles represent affected and open circles represent unaffected family members.

Heterologous splicing assay: A 7910 bp genomic segment covering exon 14 to exon 15 and flanking sequences of introns 13 and 15 of CNGB3 were amplified from genomic DNA applying Pfu Turbo polymerase (Stratagene, Heidelberg, Germany) and blunt-end cloned into the pPCR-Script Amp SK(+)vector using the PCR-Script Amp Cloning Kit (Stratagene). The insert was excised by digestion with *Bam*HI and *Not*I and cloned into *Bam*HI/*Not*I-digested pSPL3_2096 (a derivate of pSPL3 [Life Technologies, Gaithersburg, MD] with a stuffer fragment cloned into the original *Not*I site). The construct was partially sequenced including vector/insert boundaries and exon/intron borders. COS7 cells were cultured in DMEM supplemented with 5% (v/v) nonessential amino acids and 10% fetal calf serum. Cells were transfected with CNGB3 constructs using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) 36 h after transfection total RNA was prepared using TRIZOL reagent (Invitrogen) and reverse tran-

scribed with either oligo-dT or SD6 primer and AMV reverse transcriptase (RNA PCR Kit; Takara Gennevilliers, France). The resulting cDNA was PCR-amplified with pSPL3 exon primers, and the RT-PCR products were either directly sequenced or sequenced after an intermitting cloning step.

RESULTS

Molecular genetic findings: The genetic basis of the disorder could be resolved in all twelve patients. In four of the nine families causative mutations were found in the *CNGA3* gene, while the five others had mutations in the *CNGB3* gene (Table 1).

Three different missense mutations were found in *CNGA3*, all of them already reported in previous studies [13]. Pseudodominant inheritance of achromatopsia could be established in family E. While the affected mother was homozygous for the Arg283Trp mutation, her affected children were
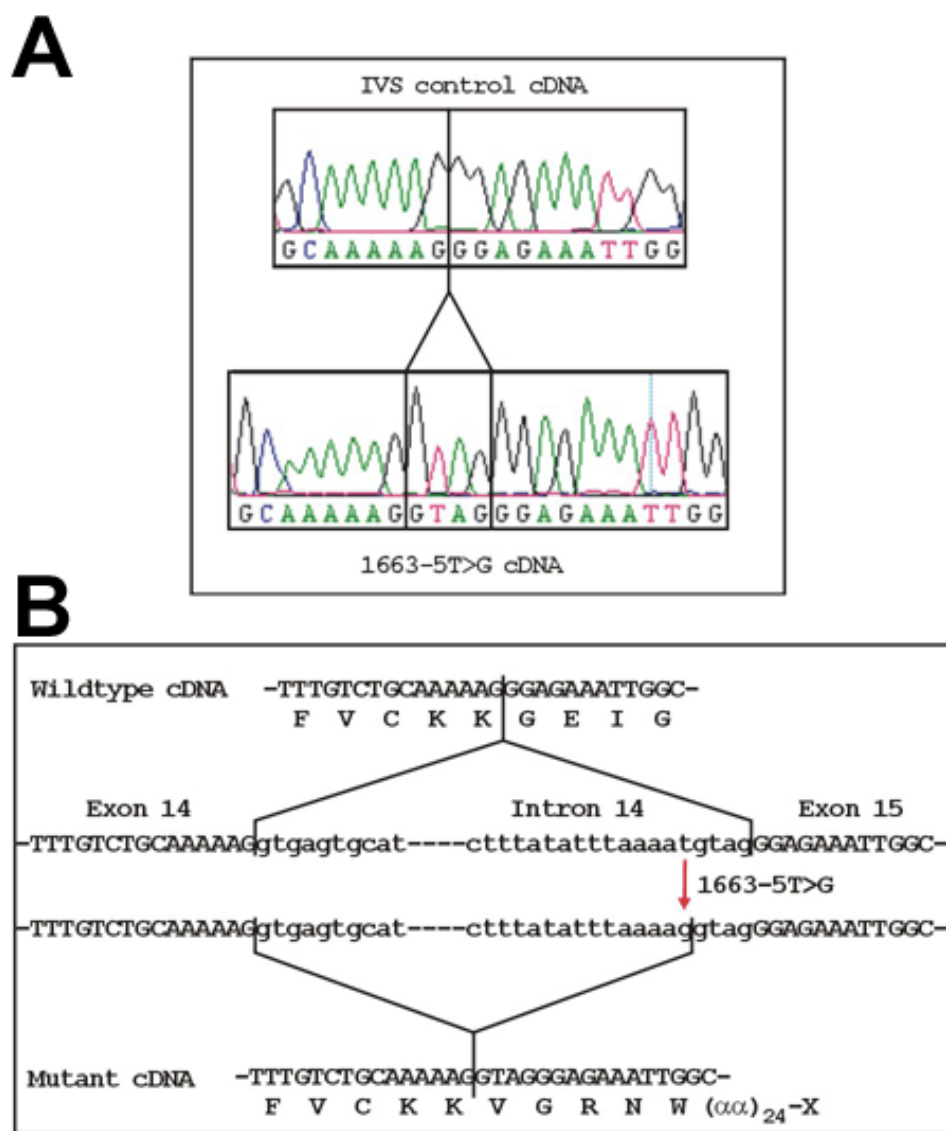

Figure 2. Characterization of the c.1663-5T>G splicing mutation. Analysis of RT-PCR products obtained from heterologous expression of minigene constructs in COS7 cells. **A**: Sequence traces of RT-PCR products obtained from COS7 cells transfected with wildtype (top) and mutant plasmid constructs (bottom). **B**: Scheme of the aberrant splicing caused by the c.1663-5T>G mutation.

compound heterozygous for the Arg283Trp and the Pro163Leu mutations, the latter inherited from the unaffected father (Figure 1).

Four different mutations were found in the *CNGB3* gene. While the Pro273fs and the Thr383fs mutations represent common mutations, the Gln38X and the c.1663-5T>G mutation are new mutations that have not been found in other achromatopsia patients so far [14]. The c.112C>T/Gln38X mutation introduces a stop codon that results in early translation termination and thus most likely represents a null allele. The effect and pathogenicity of the c.1663-5T>G mutation is less clear since it affects parts of the splice acceptor site of intron 14 that are less stringently conserved. In order to evaluate the effect of the c.1663-5T>G mutation, we tested this mutation in a heterologous splicing assay. A 7910 bp genomic segment covering exon 14 to 15 plus parts of the flanking introns of *CNGB3* were amplified from the patient and a control subject and cloned into the pSPL3 exon trapping vector. Constructs were transiently expressed in COS7 cells and RNA extracted for RT-PCR analysis. Sequence analysis showed that RT-PCR products obtained from cells transfected with the mutant construct contained a 4 bp insertion between exons 14 and 15 (Figure 2). This insertion is identical to the 4 bases just upstream from the regular exon 15 boundary and is most likely the result of the use of a cryptic splice acceptor site that is activated by the c.1663-5T>G mutation (Figure 2).

Clinical features: The results of the ophthalmologic examinations are summarized in Table 2. All patients had varying degrees of nystagmus and complaints of photophobia. The degrees of these subjective symptoms were categorized as slight, moderate, and severe. The degree of the nystagmus was less in older patients, as described by other authors [18].

Best corrected visual acuity was 0.2 or worse in all 12 patients. No significant color vision was observed in any of the examined patients. Color test revealed a complete confusion in test plate arrangements, and no definite axis was found using the Farnsworth 15D Hue test. Color tests were not performed in two young children (D II:1 and F II:1).

The fundus appearance varied among subjects. The children (3 to 9 years) displayed no pathological changes. Among the adults, two (A II:1, B I:1) showed marked maculopathy, while in the others pigment mottling on the posterior pole was found.

Scotopic ERG responses were normal in all patients. Under light-adapted conditions, no response over threshold noise was observed in any patient, neither for single flash nor for cone-specific 30 Hz flicker stimulation.

DISCUSSION

In recent years considerable progress has been made in the understanding of the genetic basis of achromatopsia. Prior studies including patients mainly from Western and Northern European countries and the US have shown that mutations in *CNGA3* and *CNGB3* account for the majority of achromatopsia cases [13,14]. Mutation spectra for *CNGA3* and *CNGB3* have been established, showing that some few common mutations significantly contribute to the relative prevalence of *CNGA3* and *CNGB3* mutant alleles in achromatopsia. For instance, the Thr383fs mutation in *CNGB3* represents 70% of CNGB3 mutant disease alleles and is involved in at least 40% of all achromatopsia cases [14]. In order to extend the population basis of prior investigations we have now analyzed twelve achromatopsia patients from nine Hungarian families. Some of the most common *CNGA3* and *CNGB3* mutations, namely

TABLE 2. CLINICAL FINDINGS IN HUNGARIAN ACHROMATOPSIA PATIENTS

Family	Patient	Age	Gender	Photophobia	Visual acuity (right/ left eye)	Nystagmus	Gene	Allele 1	Allele 2
B	I:1	63	Female	+ +	0.2 /0.2	+	CNGA3	Phe547Leu	Phe547Leu
D	II:1	4	Male	+ + +	0.15/0.15	+	CNGA3	Arg283Trp	Phe547Leu
E	III:1	11	Female	+ + +	0.1 /0.1	+ + +	CNGA3	Pro163Leu	Arg283Trp
E	III:2	8	Male	+ + +	0.03/0.1	+ + +	CNGA3	Pro163Leu	Arg283Trp
E	II:2	38	Female	+ +	0.1 /0.15	+ + +	CNGA3	Arg283Trp	Arg283Trp
F	II:1	3	Male	+ + +		+ +	CNGA3	Phe547Leu	Phe547Leu
A	II:1	36	Male	+ + +	0.2 /0.1	+ +	CNGB3	Thr383fs	Thr383fs
C	II:1	10	Male	+ +	0.1 /0.1	+ + +	CNGB3	Thr383fs	Thr383fs
G	I:1	65	Male	+ +	0.2 /0.2	+	CNGB3	Thr383fs	Splice mutation
H	II:1	12	Female	+ + +	0.1 /0.1	+ + +	CNGB3	Gln38X	Pro273fs
H	II:2	9	Male	+ + +	0.1 /0.1	+ + +	CNGB3	Gln38X	Pro273fs
I	II:1	22	Male	+ + +	0.2 /0.06	+ + +	CNGB3	Pro273fs	Thr383fs

Clinical diagnoses of patients in this study were based on photophobia, nystagmus, low visual acuity, and color vision tests and electroretinography. The patients presented with a clinical picture typical for congenital achromatopsia. There were no significant differences in the phenotypes of subjects with CNGA3 or CNGB3 mutations. Photophobia and nystagmus were graded as slight (+), moderate (++), and severe (+++). All patients listed had preserved responses for the scotopic ERG, but their responses to the photopic and 30 Hz Flicker ERGs were extinguished. Patients D/II:1 and F/II:1 were too young to assess their color vision; all other patients listed lacked color discrimination. Visual acuity could not be assessed in patient F/II:1.

Arg283Trp and Phe547Leu in *CNGA3*, and Pro273fs and Thr383fs in *CNGB3*, were also present in Hungarian patients, in accordance with the reported prevalence of the mutations. Four of the six patients with a mutation in CNGB3 carried the Thr383fs mutation (6 of the 12 alleles), while two were heterozygous for the Pro273fs mutation (2 of the 12 alleles).

Hungarian people are of mixed origin, historically with a Caucasian ancestry. Due to the intensive immigration during the past 1000 years, there is a considerable genetic similarity to the neighboring European populations [19]. In the present study of Hungarian patients with achromatopsia, the most common Western European and North American mutations have been found, similar to other diseases. Gyurus et al. [20] examined triplet repeat length polymorphism at five different disease loci in Hungarians, Varga et al. [21] checked HLA-alleles in patients with rheumatoid arthritis.

In addition, we could identify two new CNGB3 mutations, including a nonsense mutation Gln38fs and an intronic mutation that causes aberrant mRNA splicing at the intron 14/exon 15 junction. These findings in Hungarian patients further support the previous observations that missense mutations predominate in *CNGA3*, while most *CNGB3* mutations result in a truncated polypeptide [13,14].

Besides reporting on the achromatopsia patients in Hungary, the study aimed to compare the clinical aspects of the patients with mutations in *CNGA3* and *CNGB3* genes. We found no significant difference between the visual acuity measured by Snellen chart of patients in the *CNGA3* and *CNGB3* groups. Photophobia and nystagmus measured by subjective scale (slight, moderate, and severe) showed no difference between the two groups, either. No detectable color vision was observed in any of the patients. The results of the color tests were inconsistent in all of the examined patients. Using the Farnsworth 15D Hue test, no definite axis was found in any patient.

The only known objective method, the ERG, showed essentially preserved rod-function in all patients. Since the refractory period of rods is relatively long (approximately 50 ms), responses to 30 Hz flicker stimulus represent only the cone function, while single flash stimuli could stimulate some rods as well, even in light-adapted patients [17,22]. Both single flash and 30 Hz Flicker stimuli were examined and no response was recorded from any of the patients to either method. This indicates no ERG-measurable cone function in our patients with achromatopsia.

Prior to the molecular genetic analysis, Farkas et al. [23] reported two achromatopsia cases (both of them are involved in this study as well), in which dark adaptation and spectral luminosity examinations were also performed. The results of these two psychophysical examinations showed some remarkable difference between the two patients [23]. Genetic analysis revealed that the two patients carry mutations in different achromatopsia genes. One subject, with a monophasic dark adaptation curve and maximal spectral luminosity at 507 nm, is homozygous for the 1148delC/Thr383fs mutation in the *CNGB3* gene (A II:1). The monophasic adaptation curve im-

plies that this patient has only one type of photoreceptor, and since this luminosity maximum is characteristic for rod photoreceptors, these are most likely rods. The other patient, showing an abnormal biphasic dark adaptation curve (and with a maximal spectral luminosity at 527 nm), is homozygous for the c.1641C>T/Phe547Leu mutation in the *CNGA3* gene (B I:1). The biphasic-like dark adaptation curve and the higher spectral luminosity maximum suggest that, in addition to the rods, there may be other functioning photoreceptors present in the retina of this patient.

Regarding the prior psychophysical data, some measurable differences are present between two genetically confirmed achromatopsia patients. With only a limited number of cases it is not clear, however, whether these differences are due to the different genetic alterations or some other individual factors. To confirm these differences, further examinations (not only dark adaptation and spectral luminosity in each patient, but also more detailed analysis of the rod function by electroretinography) are needed.

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