Clinical and molecular genetic examinations of patients with congenital achromatopsia

Doctoral thesis

Dr. Varsányi Balázs

Semmelweis University
Clinical Medicine Doctoral School

Supervisor: Dr. Farkas Ágnes
Opponents: Dr. Pámer Zsuzsanna
Dr. Holub Marianna

Chief of examination committee: Prof. Dr. Németh János
Members of examination committee: Dr. Holub Marianna
Dr. Milibák Tibor

Budapest
2006.
Introduction

Congenital achromatopsia, also referred to as rod-monochromacy, or total colour-blindness (ACHM2: OMIM 216900; ACHM3: OMIM 262300; ACHM4: OMIM 139340), is a stationery, recessively inherited disorder of the retina. The disease is characterized by inability to discriminate colours, low visual acuity (0.1-0.2), photophobia and nystagmus.

Since the basis of the disorder is the lack of functioning cones, the patients’ vision is mediated only by rods. This explains the clinical signs, as cones are responsible for sharp and colour vision.

The estimated prevalence of achromatopsia is 1:30000-1:50000 worldwide. Accordingly, the estimated number of the patients in the Hungarian population is about 200-300. However, only a small portion of this number is diagnosed.

In recent years there has been considerable progress in elucidating the genetic basis of achromatopsia. Mutations in the genes coding cone-specific proteins involved in the phototransduction cascade lead to the lack or dysfunction of these proteins. So far, three genes have been associated with achromatopsia: CNGA3 and CNGB3 encoding the $\alpha$- and $\beta$-subunit of the cone photoreceptor cGMP-gated channel, and GNAT2 encoding the $\alpha$-subunit of the cone G-protein transducin. Mutations in CNGA3 and CNGB3 account for about 25% and 45% to 50% of achromatopsia cases, respectively. In about 20-25% of the patients with the clinical diagnosis of achromatopsia the genetic background is still unknown.

There is only a few data on the histological changes in the retina of patients with achromatopsia, mainly from post mortem, or animal model examinations.

There is an incomplete form of achromatopsia, characterized by some residual cone function, thus residual colour discrimination function. The definition of this form is not absolutely clear. The residual cone function is detectable mainly by psychophysical (colour-vision) test. The genetic background could be identical to that of the complete form (autosomal recessive inheritance, involvement of
CNGA3 gene). Usually there is no detectable cone function using full field ERGs, but some reports report some residual photopic response.

**Purposes**

1. The spectral sensitivity properties of the functioning photoreceptors are known neither in the complete, nor in the incomplete form of achromatopsia. These properties are important in distinguishing the two forms, but also in understanding the pathomechanisms behind the disease. Thus our purpose was to find a method
   a. to assess the spectral sensitivity of individual functioning photoreceptors
   b. to measure the spectral luminosity maximum of patients with achromatopsia
   c. to distinguish the complete and incomplete forms of the disease.

2. Molecular genetic examinations are useful tools for prove the clinical diagnosis. Financial and temporal effectiveness could be improved by well-designed screening protocol. So our aim was
   a. to analyse the prevalence of the mutations in the achromatopsia-related genes in great number of patients, and
   b. to set up a screening protocol for the routine molecular genetic examination of achromatopsia regarding the most frequent mutations.

3. In 20-25% of the patients with achromatopsia the molecular genetic background is still unknown, so our aim was to find new candidate genes for achromatopsia. We performed linkage analysis on chromosome 14, as previous publications suggested relation between this chromosome and achromatopsia.

4. No reports were published on the molecular genetic examinations of Hungarian patients with achromatopsia. By analyzing a relatively large number
of patients we described the molecular genetic background of Hungarian patients with achromatopsia, and also compared it to that of European data.

5. The mutational spectra of the CNGA3 and the CNGB3 genes differ significantly. We aimed to compare the clinical features of patients with different genetic background (genotype-phenotype comparison)

6. There are only a few reports on the morphological changes related to achromatopsia, and only from post mortem and animal model experiments. So our aim was to analyse the retinal structure of patients with achromatopsia in vivo.

7. Achromatopsia is a stationery disorder. To examine the possible age-related, degenerative changes we tried to model the time-course of the disease by comparing the functional and morphological results of “younger” and “older” patients.
Patients and methods

We analysed the results of electrophysiological, psychophysical morphological and molecular genetic examinations carried out on twelve patients with achromatopsia in nine Hungarian families.

The Molecular Genetic Laboratory of the University Eye Clinic, Tübingen has the world’s largest DNA-sample collection of patients with achromatopsia, owning more than 1000 samples from more than 300 families worldwide. The collection enlarges with about 40-50 new families a year. We analysed these samples for describing the mutational spectra of the achromatopsia related genes, and for finding new candidate genes.

Molecular genetic examinations

The DNA samples from patients and relatives were amplified by polymerase chain reaction (PCR) technique. The nucleotide sequence of CNGA3 and CNGB3 genes were analysed by restriction fragment length polymorphism (RFLP) and direct sequencing.

For finding new candidate genes we performed linkage analysis on chromosome 14 using microsatellite-markers.

Clinical examinations on the retinal function

The retinal function of the patients with achromatopsia was analysed by different psychophysical (subjective) and electrophysiological (objective) methods. Some of the formers were conventional ophthalmologic test, as the dark adaptation, colour-vision tests (Ishihara, Farnsworth 15D Hue). Besides we examined the patients’ colour vision by some special tests. Relative brightness matching test was developed by the Department of Mechatronics, Optics and Instrumentation Technology, Budapest University of Technology and Economics (BMGE MOM). This test is based on the comparison of the brightness of different basic colours, and thus the functioning photoreceptors could be assessed. Spectral luminosity testing is to assess the spectral luminosity maximum derived from the spectral
sensitivity of the functioning photoreceptors. For this test a new device was set up in cooperation with the BMGE-MOM, by which the spectral luminosity of patients with achromatopsia could be examined more precisely.

Full field electroretinography, as an objective functional test is crucial in the clinical diagnosis of achromatopsia. To distinguish the complete and incomplete form of the disease, and to examine the residual cone function in more details, we performed full field ERG with colour stimuli.

*Morphological examinations*

For the in vivo morphological examination of the retinal structure of patients with achromatopsia we used optical coherence tomography (OCT). This non-invasive, non-contact examination was carried out using a “third generation” Stratus OCT device, with an axial resolution of 10μm. We measured the total macular volume (TMV) and the thickness of different parts of the retina. The results were compared to those of healthy control subjects.

*Statistical methods*

The normality of the data was checked by Shapiro-Wilks W test. In most of the cases the data had normal distribution, and we used two-sample t-probe to compare the results of the different groups. If the data did not have normal distribution Mann-Whitney-U test was used for the comparison. Statistical significance was stated at p<0,01.

Statistical calculations were performed using Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA) software.
Results

Molecular genetic examinations

The clinical diagnosis of achromatopsia was confirmed by the molecular genetic analysis in 248 of 341 families. In most of the cases (163/248, 65.7%) a causative mutation was found in the CNGB3 gene. In one third if the cases (80/248, 32.2%) mutations in the CNGA3 gene were found in the background of the disease. Seven patients of five families had mutations in the GNAT2 gene.

Analyzing the prevalence of the different mutations, an RFLP-based screening protocol was developed for the 5 most frequent CNGB3, and the most frequent CNGA3 mutation. With this protocol the results of the molecular genetic examinations could be done in one day in about 80% of the cases.

The linkage analysis on chromosome 14 showed a marked positive LOD-score in three regions, around markers D14S275, D14S258 and D14S985 (1.8; 2.4; 1.9, respectively). These scores are not high enough to state a linkage between these regions and achromatopsia, but encouraging for further examinations.

The molecular genetic examinations confirmed the clinical diagnosis of achromatopsia in all the twelve Hungarian patients attended at our Clinic. Mutations in CNGA3 were present in six patients in four families and mutations in CNGB3 in the remaining six patients of five families. These mutations were mainly previously known, frequent mutations, but there were also two new mutations found. The c.112C>T/Gln38X leads to premature translation termination, thus to a largely truncated polypeptide. The c.1663-5T>G is an intronic splice site mutation leading to 4bp insertion and thus frame-shift during the translation. The pathogenicity of this mutation was proved by heterologous splicing assay.
Distinction of between the complete and incomplete forms of achromatopsia

Psychophysical test were suitable to distinguish the complete and incomplete forms of the disease during clinical examinations. Using spectral luminosity testing and relative brightness matching we were able to detect some residual colour-vision (thus cone function) characteristics of the incomplete form in two of the patients.

*Spectral luminosity testing (SL)* showed a spectral sensitivity maximum at 558.7 nm (SD=6.4) in healthy control subjects, which value corresponds to the photopic (cone derived) sensitivity maximum known from the literature. In five of the six patients with achromatopsia examined with this method 509.6 nm (SD=7.2) was measured, which is the spectral sensitivity maximum of the rods. One patient was most sensitive to the 539.8 nm light; in this case incomplete form of achromatopsia is presumptive.

*Relative brightness matching* test was carried out in seven patients with achromatopsia. In five cases ratios typical of rod function only were calculated. In the two other cases ratios revealing protanopia /green monochromacy were measured, implying functioning photoreceptors different from rods (cones), i.e. the incomplete form of achromatopsia. In one of them the SL also showed incomplete form, while in the other case only the RBM implied the incomplete form. The different spectral sensitivity of the functioning photoreceptors in the two patients could account for the different results.

As an objective test, full field ERG with colour stimuli was also used. This method was not proved to be useful in distinguishing the complete and incomplete form of achromatopsia. No detectable photopic (cone) response was observed with any colour stimuli in any of the patients, even in the ones with presumably incomplete form of the disease.
**Morphological examination**

OCT showed a significant thinning of the retina of patients with achromatopsia and also remarkable structural changes within the foveola, compared to the central retina of healthy control subjects. We found remarkable difference between the results obtained from different methods; with automated methods the retinal thickness was found lower of 15-30 μm than with manual measurements. The morphological changes, most prominent in the foveola, could be the due to the qualitative and/or quantitative alteration of the non-functioning cones and also the cone-related structures.

Only a few reports were reported on the morphological changes of the retina in achromatopsia (from post mortem and animal model examinations). Contrary, OCT offers a non-invasive, in vivo method to examine the morphological structure of the patients’ retina. It also seems a suitable method helping to establish indication for gene therapy, and monitoring the time course of the disease.

**Modelling the time course of the disease**

Achromatopsia is a stationery disease. The quality of life of the patients could even improve, if they learned how to use their remaining vision. We tried to model the age-related changes in the disease by comparing the results of the “younger” and “elder” patients. The functional and morphological follow-up of the patients could help in designing a gene-therapeutic window. With ERG the scotopic rod- and maximal responses were analysed, and in the “elder” patients a slight increase in the latencies was found, without any change in the response amplitudes. With OCT significant thinning was observed in the “elder” patients compared to the “younger” ones. This could be due to some degeneration of the cone-related structures in the retina. These findings emphasise the importance of the proper early diagnosis, and the need for early gene therapeutic intervention. Thus, the proper examination of the early, not so specific signs is crucial.
Conclusions

1. a. We lay down that *spectral luminosity testing* and the *relative brightness measurements* are appropriate methods to assess the individual spectral sensitivity of the functioning photoreceptors.

b. We demonstrated that *spectral luminosity testing* and *relative brightness measurements* are appropriate methods to distinguish the complete and incomplete forms of congenital achromatopsia.

c. We developed a new setup for measuring the spectral luminosity maximum, which is more precise and more tolerable by patients with congenital achromatopsia, than the former methods.

d. Our measurements proved that the spectral luminosity maximum is at lower wavelength in patients with achromatopsia than in healthy control subjects.

e. We confirmed the known electroretinographic features of achromatopsia (i.e. normal scotopic and extinguished photopic responses) using modern setups, however found ERGs with colour stimuli not suitable to distinguish the complete and incomplete form of the disease.

2. a. We described the mutational spectra of CNGA3 and CNGB3 genes by examining a great number of patients with achromatopsia.

b. Regarding the most frequent mutations we set up a fast and effective screening protocol for the molecular genetic examinations of patients with achromatopsia.

3. a. We performed molecular genetic analysis in Hungarian patients with achromatopsia for the first time. Analyses confirmed the clinical diagnosis of achromatopsia in all cases. We described that the mutational spectrum of the Hungarian patients does not differ substantially from that of Western-European samples.
b. We described the c.1663-5T>G and the c.112C>T mutation first in the international literature by examining the Hungarian patients.

c. Molecular biological experiments proved that the c.1663-5T>G mutation is a causative mutation in achromatopsia, and that is involved in the incomplete form of achromatopsia.

4.

No clinical (phenotypic) difference was found between patients carrying mutation in CNGA3 and CNGB3 genes, which have significantly different mutational spectra.

5.

a. We examined and described the retinal morphology of patients with achromatopsia using in vivo methods for the first time.

b. We described that the foveolar structure of the patients differs significantly from that of control subjects, and the thickness of the neuroretina is significantly lower than that of control subjects.

6.

a. No significant difference was found with electroretinography in the retinal function of “younger” and “older” patients.

b. Using optical coherence tomography a significant thinning was observed in the “older” patients compared to the “younger” ones.

c. In our opinion, optical coherence tomography could be a suitable method in designing the gene therapy of patients with achromatopsia, and also to follow-up the morphological changes induced by the intervention.
Publication list

Publications on the subject:

   *Molecular Vision*; 11:996-1001 (*IF: 2.9*)

   *CNGB3* mutations account for almost 50% of all cases with autosomal recessive achromatopsia
   *European Journal of Human Genetics*, Volume 13, 3 (*IF: 2.741*)

Citeable abstracts on the subject:

   Optical coherence tomography in patients with achromatopsia
   *Ophthalmic Research*, Volume 36, Suppl. 1 (*IF: 1.0*)

   New methods in examination of achromatopsia
   *Ophthalmic Research*, Volume 37, Suppl. 1 (*IF: 1.0*)

   Comparison of measurements with OCT2 and OCT3 in patients with achromatopsia. *Ophthalmic Research*, Volume 37, Suppl. 1. (*IF:1.0*)
Lectures held on the subject:

1. TDK Conference, 2003, Budapest
   **Varsányi Balázs**, Farkas Ágnes:
   Klinikai és molekuláris genetikai vizsgálatok eredményeinek összevetése veleszületett teljes színvakság esetében (Clinical and genetic features of Hungarian achromatopsia patients)

2. PhD Conference 2004, Budapest
   **Varsányi Balázs**, Farkas Ágnes:
   A spektrális luminozitás mérése congenitalis achromatopsiában (Spectral luminosity testing in patients with achromatopsia)

   **Varsányi Balázs**, Somfai Gábor Márk, Farkas Ágnes:
   Optical coherence tomography in patients with achromatopsia

   **Varsányi B.**, Samu K., Wenzel K.:
   Pszichofizikai vizsgálatok achromatopsiás betegeken (Psychophysical testing in patients with achromatopsia)

5. Colouristic Symposium, May 30- 1 June, 2005, Eger
   **Varsányi B.**, Samu K., Wenzel K.:
   Achromatopsiás betegek vizsgálata pszichofizikai módszerekkel (Psychophysical methods in colour vision testing of patients with achromatopsia)

   **Varsányi B.**, Samu K., Wenzel K., Farkas Á.:
   Új módszerek az achromatopsiás betegek vizsgálatában (New methods in examination of achromatopsia)

7. EVER 2005 Congress, October 5-8, 2005, Vilamoura, Portugal
   **Varsányi B.**, Samu K., Wenzel K., Farkas Á.:
   New methods in examination of achromatopsia

8. CME Course for Ophthalmologists, January 16, 2006, Budapest
   **Varsányi Balázs**:
   A színlátás objektív vizsgálata (Objective methods in colour vision testing)
Posters on the subject:

1. EURETINA Congress, 2005. May 19-21, Barcelona, Spain

2. EVER 2005 Congress, 2005. October 5-8, Vilamoura, Portugal
   Varsányi B., Somfai G.M., Lesch B., Farkas Á.: Comparison of measurements with OCT2 and OCT3 in patients with achromatopsia

Other lectures, posters:

   Vámos R., Farkas Á., Lesch B., Varsányi B.: A Leber-féle congenitális amaurosisról – 2 eset kapcsán

2. Congress of the Hungarian Ophthalmologic Society , June 9-11, 2005, Szeged
   Farkas Á., Vámos R., Lesch B., Varsányi B., Salacz Gy.: A szemészeti diagnózis jelentősége a retina dystrophiaval társult tünetegyüttesek korai felismerésében

3. SOE Congress, September 25-29, 2005, Berlin
   Lesch B., Szabó V., Vámos R., Somfai G., Hargitai J., Varsányi B., Farkas Á.: Optical coherence tomography and electroretinography findings in 4 Hungarian families with x-linked juvenile retinoschisis

4. Pro Retina Research-Colloquium, April 7-8, 2006, Potsdam
   Vámos R., Hargitai J., Varsányi B., Lesch B., Szabó V., Farkas Á.
   Research on inherited retinal diseases at the University Eye Clinic, Budapest