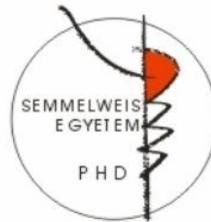


**Genetic causes of hereditary multiple pituitary hormone deficiency. Studies on *PRO1* gene mutations in Hungarian patients**

Abstract of Ph.D. thesis

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## **Introduction**

The most common clinical feature of hereditary multiple pituitary hormone deficiency (MPHD) is a short stature due to undersecretion of growth hormone (GH), which may be accompanied by other signs and symptoms of hypopituitarism. In 1977, GH-preparations have been introduced in Hungary under well-controlled distribution for the treatment of patients and have been since successfully used – together with hormone replacements of other pituitary hormone deficiencies – for the clinical management of patients with MPHD.

During the past 10 years, basic research dealing with pituitary development and differentiation of hormone-producing pituitary cells has contributed to the discovery of novel cellular regulatory mechanisms, whose inborn errors may play a causal role in the development of hereditary MPHD. It has been shown that during different steps of pituitary organogenesis, the differentiation of different pituitary cell lines is controlled by a large number of pituitary transcription factors. Also importantly, germline mutations of genes coding for these transcription factors have been proved to play a causal role in the development of MPHD, and the clinical phenotypes associated with mutations of these genes have been shown to reflect a defective function of the affected genes. In addition,

clinical studies on disease-causing gene mutations have indicated that inborn defects of at least some of the genes coding for pituitary transcription factors are also associated with developmental and functional defects of organs other than the pituitary gland. Most interestingly, a pituitary transcription factor named "paired like homeodomain transcription factor 2" (PITX2) has been implicated in animal models in the regulation of left/right asymmetry of the organism.

In the first part of my thesis, I analyse the outcome of a long-term therapy with a GH-preparation first available for GH replacement of children with GH deficiency in Hungary. Thereafter, I summarize my studies which aimed to investigate the prevalence and spectrum of mutations of two pituitary transcription factor genes in Hungarian patients with MPHD ("prophet of Pit1" transcription factor, *PROPI* gene; "POU domain, class 1, transcription factor 1", *POUIF1* gene). Mutations of these two genes have been shown to be the most common causes of hereditary MPHD in the European population, but considerable differences among different European countries have been also documented. Finally, I present the results of my studies on mutation screening of all coding exons of the *PROPI*, *POUIF1* and *PITX2* genes in a unique patient who presented with MPHD and situs inversus

totalis. To my knowledge, these genes have not been previously investigated in a patient having both MPHD and situs inversus totalis.

### **Aims**

The aims of my research work are summarized as follows:

1. In Hungary, GH-preparations are available under well-controlled distribution for the treatment of children with GH-deficiency since 1977. To analyse the results obtained with a GH-preparation first available in Hungary, I wanted to determine the outcome of long-term treatment with this drug (Grom) in children with idiopathic GH-deficiency.
2. Disease-causing mutations of the *PROPI* gene in patients with MPHD have been first published in 1998, and subsequent studies have indicated marked differences in their prevalence among MPHD patients from different countries. Therefore, the aim of my study was to determine the prevalence and spectrum of mutations of the *PROPI* gene in Hungarian patients with MPHD.

3. With the analysis of clinical, hormonal and genetic findings of patients with hereditary MPHD, I wanted to explore whether patients with disease-causing *PROPI* gene mutations could exhibit differences in clinical and/or hormonal parameters compared to those who do not have mutations of the *PROPI* gene. In addition, my aim was to examine whether any association could exist between the type of disease-causing *PROPI* gene mutations and the clinical and/or hormonal parameters in MPHD patients.
  
4. Previous international studies indicated that mutations of the *POUIF1* gene, which occur less frequently compared to those of the *PROPI* gene in patients with MPHD, are often located in a mutational "hot spot" of exon 6 of the gene. Therefore, I wanted to examine whether mutations in exon 6 of the *POUIF1* gene could be detected in Hungarian patients with MPHD.
  
5. As shown in animal models, the *PITX2* gene plays a role not only in the developmental and functional

regulation of pituitary hormone-producing cells, but it has an impact on the development of right/left asymmetry of the organism. Therefore, I performed mutational analysis of all coding exons of the *PROPI*, *POU1F1* and *PITX2* genes in a patient who had both MPHD and situs inversus totalis in order to examine the possibility of a common genetic background of hereditary MPHD and situs inversus totalis.

## **Patients and Methods**

### **Patients**

To analyse the clinical outcome of long-term GH-replacement administered at the time before epiphyseal closure in children with GH-deficiency, the data of 14 patients with hypopituitarism receiving GH-treatment in three pediatric endocrine centres have been reviewed. All patients were treated with a GH-preparation extracted from human pituitary glands (Growth, Serono) for 7 years or longer. At the time of the onset of treatment the mean age of patients was 8 years. The mean duration of Growth treatment was 9.2 years.

Mutational analysis of pituitary transcription factor genes was performed in 35 MPHD patients from 32 families

(13 girls/women; 22 boys/men). Patients were recruited during a three-years period from seven pediatric and adult endocrine centres. Patient enrolment was based on the following inclusion/exclusion criteria: (1) GH deficiency established by endocrinological evaluation, together with at least one other pituitary hormone deficiency; (2) childhood-onset MPHD and (3) absence of history of acquired forms of MPHD (trauma during delivery with perinatal hypoxia; brain trauma; intracranial tumor, tumor of the sellar region, hypothalamic tumor). The mean age of patients at the time of diagnosis of GH-deficiency, which corresponded to the mean age of patients at the onset of GH-replacement therapy was  $7.7\pm 3.6$  years. The mean age of patients at the time of mutational screening was  $21.8\pm 9.3$  years.

Of the 35 MPHD patients enrolled in mutational screening, one patient proved to have situs inversus totalis diagnosed by clinical studies.

### **Anthropometric studies**

Birth weight and height, perinatal history and height of parents were obtained from available medical documents. The height of patients was compared to age- and sex-specific reference data established for Hungarian population and the differences

were expressed as standard deviation scores. Bone age was determined by an X-ray of the non-dominant hand.

### **Hormone measurements and stimulation tests**

Pituitary anterior lobe function was evaluated using measurements of hormone concentration in serum under baseline conditions and during dynamic tests. Evaluation of GH-secreting capacity in children was based on the results of two independent standard dynamic (stimulation) tests.

### **Radiologic imaging studies**

Morphology of the hypothalamic-pituitary region in patients with MPHD was retrospectively analysed using written radiologic evaluation of radiologic exams of the sellar region including X-ray, computed tomography (CT) and magnetic resonance imaging (MRI). Written radiologic evaluation included description of midline structures, radiologic signs of intracranial and intrasellar masses, size and alteration in the homogeneity of the pituitary gland, the presence pituitary hypoplasia or hyperplasia, and the location of the neurohypophysis (normal or ectopic neurohypophysis).

In a patient who had both situs inversus totalis and MPHD functional MRI was also performed.

### **Immunocytochemistry**

In a patient with both situs inversus totalis and MPHD, transnasal brush biopsy was performed and ciliary motility of nasopharyngeal epithel cells was evaluated using a light microscope. Immunofluorescence analysis of the outer dynein arm protein was performed in the laboratory of professor Heymut Omran (Freiburg, Germany) using DNAH5-specific rabbit antibody.

### **Molecular biologic studies**

Mutational analysis of genes encoding pituitary transcription factors (*PROPI*, *POUIF1*, *PITX2*) was performed in the molecular genetic laboratory of the 2nd Department of Medicine, Semmelweis University.

Using commercial kits, genomic DNA was isolated from total peripheral blood obtained in EDTA tubes. DNA samples were stored at  $-20^{\circ}\text{C}$  until use for polymerase chain reaction (PCR). Exons of pituitary transcription factor genes were amplified using PCR. For amplification of exons of the *PROPI* and *POUIF1* genes, sequences of oligonucleotide

primer pairs were obtained from previous publications. For amplification of exons of the *PITX2* gene, oligonucleotide primer pairs were designed using a Primer3 program. Each primer pair was designed to detect not only exonic mutations, but also those occurring in the surrounding intronic regions.

Following PCR, nucleotide sequences of exons were analysed using DNA sequencing instruments (ABI Prism 310 capillary DNA sequencing or LiCOR IR2 DNA sequencing equipments). Forward and reverse sequencing were performed for each exon.

## **Results**

Children with GH-deficiency were treated until epiphyseal closure with the GH-preparation in a total weekly dose between 6 and 18 NE administered in 3 or 4 divided doses per week intramuscularly. Mean growth retardation of children at the time of onset of the treatment was  $-3.85$  SDS, and mean retardation of age-adjusted height as compared to bone age was  $-1.5$  years. The mean duration of Grom treatment was 9.2 years. With the analysis of treatment outcome I found, that after treatment for a longer than 7-yr period, the mean height of children with GH-deficiency reached the normal national

reference range adjusted for age and sex (-1 SDS). This favourable effect of treatment was similar to that published in the international literature. Reviewing patients records I found no side-effects in relation to Grom treatment.

Mutational analysis of the *PROPI* gene was performed in 35 patients with MPHD, of which 15 patients (43%) had disease-causing mutations. Of the 15 patients, 10 had homozygous *PROPI* gene mutations (301-302delGA in exon 2 in 4 patients, 150delA in exon 2 in 4 patients, 217C>T [Arg73Cys] in exon 2 in one patient, and 349T>A [Phe117Ile] in exon 3 in one patient. In addition, two different heterozygous *PROPI* gene mutations were found in 5 patients. In the latter cases mutational analysis of the *PROPI* gene was also performed in DNA samples obtained from the parents of patients to examine whether the two heterozygous mutations could be inherited in separate alleles. The results of these studies showed that each of the 5 patients had compound heterozygous mutations of the *PROPI* gene (150delA and 301-302delGA in 3 patients, 150delA and 349T>A [Phe117Ile] in one patient, and 296C>T [Arg99Stop] and 301-302delGA in one patient. When the relative frequencies of the mutant alleles were analysed, the results showed that about 80% of the mutant

alleles were attributed to those containing the 150delA and 301-302delGA mutations of the *PROPI* gene.

In addition to *PROPI* gene mutations, the 27T>C polymorphism in exon 1 not resulting in an amino acid change (Ala9Ala) was detected in a heterozygous form in 6 patients and in a homozygous form in 4 patients. No polymorphism was found in exon 2. The 424G>A (Ala142Thr) polymorphism in exon 3 was present in a homozygous form in one patient and in a heterozygous form in 8 patients.

After the completion of the *PROPI* gene mutation screening, the patients with MPHD were divided into two groups. One group consisted of patients who had disease-causing homozygous or compound heterozygous mutations (15 patients), whereas the other group included patients who did not have mutations (20 patients). When clinical data of the two groups of patients were separately analysed, the results showed that GH-deficiency was diagnosed at earlier age of life in patients with *PROPI* gene mutations compared to those without gene mutations ( $6.3 \pm 1.6$  and  $8.9 \pm 4.4$  years,  $p < 0.05$ ), but the severity of growth retardation at the time of diagnosis of GH-deficiency or the age of patients at the time of manifestation of other pituitary hormone deficiencies (TSH, LH, FSH and ACTH) were similar in the two groups of

patients. ACTH-deficiency was detected in 3 patients with *PROPI* gene mutations and in 6 patients who had no *PROPI* gene mutations. Insufficient TSH secretion appeared early in both groups; TSH-deficiency was documented in all patients with *PROPI* gene mutations as well as in most patients without *PROPI* gene mutations at the time of diagnosis of GH-deficiency. LH- and FSH-deficiency was diagnosed at the time of expected puberty in both groups. At the time of my studies, 22 patients (11 in each group) belonged to the postpubertal age, of which none of the patients had spontaneous puberty.

In one patient who had *PROPI* gene mutation a transient enlargement of the pituitary gland was observed by MRI, whereas the other patients with or without *PROPI* gene mutations had a normal or hypoplastic pituitary by radiologic imaging. The location of the posterior lobe of the pituitary gland was normal in all patients with *PROPI* gene mutations, but an ectopic posterior lobe was found in 7 patients who had no *PROPI* gene mutations.

In 15 patients who had no disease-causing mutations within the three coding exons of the *PROPI* gene, the exon 6 of the *POUIF1* gene containing a mutational "hot spot" was also examined but no mutations were found.

In a patient who had both MPHD and situs inversus totalis, symptoms of hypopituitarism due to an insufficient replacement therapy was observed. Dysmorphic features were absent. The diagnosis of situs inversus totalis was confirmed by physical examination, electrocardiogram, echocardiography, chest x-ray, abdominal ultrasound, enterography, and gastroscopy. The patient was right-handed. Brain MRI revealed a hypoplastic anterior lobe and an ectopic posterior lobe of the pituitary gland, but other structural abnormalities were absent. Functional MRI showed left-hemisphere activation during language tests. Light microscopic examination of nasopharyngeal epithelial cells obtained by brush biopsy indicated normal ciliary motility, and immunofluorescence analysis using a DNAH5-specific antibody showed normal localization of the outer dynein arm protein. Mutational analysis of the three coding exons of the *PROPI* gene revealed a heterozygous 27T>C polymorphism not resulting in an amino acid change (Ala9Ala), but disease-causing mutations were absent. Nucleotide sequence analysis of exons 1-6 of the *POUIF1* gene and exons 4-7 of the *PITX2* gene was also performed, but no disease-causing mutations were found.

## Conclusions

With the analysis of the clinical and hormonal parameters and with mutational screening of three pituitary transcription factor genes (*PROPI*, *POUIF1* and *PITX2*) in patients with inherited form of MPHD, the conclusions of my research work are as follows:

1. The results of my studies showed that after treatment for a longer than 7-yr period with a GH-preparation (Grom) available under well-controlled distribution in Hungary, the mean height of children with GH-deficiency reached the normal national reference range adjusted for age and sex. The GH-preparation used in this study has been since replaced by recombinant human GH-preparations, but the first experience with GH-replacement in children has been well utilized when novel recombinant GH-preparations have become available.
2. I established clinical criteria for selection of patients with hereditary MPHD for screening disease-causing mutations of all coding sequences of the *PROPI* gene, and these criteria were used for recruitment of 35 MPHD patients from 32 families for genetic

studies. The patients were recruited from 7 pediatric and adult endocrine centres during three years. With these studies I showed that when applying clinical criteria for patient selection, screening of *PROPI* gene mutations offers a highly efficient means to detect hereditary form of MPHD in Hungarian patients. Of the 35 patients, 15 patients (43%) had homozygous (10 patients) or compound heterozygous (5 patients) mutations of the *PROPI* gene. All mutations have been already described in the literature. It was also found that more than 80% of mutant alleles were accounted for by those containing the 150delA and 301-302delGA mutations of the *PROPI* gene. These findings indicated a high relevance of mutational "hot spots" of the *PROPI* gene in Hungarian patients with MPHD and they also offered an opportunity for the development of rational and cost-effective screening strategy. Based on these observation it can be proposed that gene regions containing the 150delA and 301-302delGA mutation sites should be screened first when *PROPI* gene mutations are analysed in Hungarian patients with MPHD.

3. When clinical and hormonal findings of MPHD patients with and without *PROPI* gene mutations were compared, the results showed that GH-deficiency was diagnosed at earlier age of life in patients with *PROPI* gene mutations, but the severity of growth retardation at the time of diagnosis of GH-deficiency or the age of patients at the time of manifestation of other pituitary hormone deficiencies (TSH, LH, FSH and ACTH) were similar in the two groups of patients. It is therefore possible, that clinical manifestations of growth retardation in patients with *PROPI* gene mutations may become apparent at earlier age compared to those without *PROPI* gene mutations. Therefore, *PROPI* gene mutation screening may be more relevant in children, in whom growth retardation is diagnosed at younger ages.
  
4. In 15 MPHD patients without *PROPI* gene mutations, the exon 6 of the *POU1F1* gene containing a mutational "hot spot" was also examined but no mutations were found. Thus, these

results do not support a significant role of the mutational "hot spot" of the *POUIF1* gene in Hungarian MPHD patients, although a potential role of mutations located in exons other than exon 6 cannot be excluded.

5. I introduced a method for the detection of mutations of coding exons and intronic sequences close to exons of the *PITX2* gene, a pituitary transcription factor gene that plays a role not only in pituitary development and differentiation but also in the lateralization of organs. With the use of this method, I performed mutational analysis of all coding exons of this gene in an exceptionally unique patient who had both situs inversus totalis and MPHD, but no mutation was found. Also, other studies failed to confirm that situs inversus totalis in this patient was due to Kartagener syndrome, and mutational analysis of the *PROPI* and *POUIF1* genes revealed normal nucleotide sequences. The *PROPI*, *POUIF1* and *PITX2* gene mutations have not been previously investigated in a patients with both MPHD and situs inversus totalis. Thus, the findings failed to indicate

that mutations of these genes are involved in the pathomechanism of situs inversus totalis associated with MPHD.

### **List of publications**

#### **Original articles directly related to the doctoral thesis:**

**Halász Z**, Gács G, Sólyom J, Péter F, Muzsnai Á, Kozári A, Soltész Gy. (1994) Influence of long-term Grom treatment on target height in hypopituitary dwarfism. *Gyermekegyógyászat*, 5: 432-434. (in Hungarian)

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**Halász Z**, Sólyom J. Pharmacotherapy of endocrine diseases. In: Fekete F és Gyurkovits K (szerk), *Pediatric pharmacotherapy*. B+V Lap- és Könyvkiadó Kft, Gyoma, 2006: 386-418. (in Hungarian)

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**Halász Z**, Majnik J, Tőke J, Bertalan R, Tömböl Zs, Balogh K, Patócs A, Tóth M, Rác K. (2006) Mutation analysis of pituitary transcription factors coding genes in Hungarian patients with childhood-onset multiple pituitary hormone deficiency. *Magy Belorv Arch* S1:39. (MEAT, 2006. Debrecen) (in Hungarian)

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