

*Clinical and genetic screening of multiple endocrine  
neoplasia type 1*

PhD thesis

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## Introduction

Multiple endocrine neoplasia type 1 (MEN 1 syndrome; Wermer syndrome) is characterized by the combined occurrence of tumours of the anterior pituitary gland, parathyroid glands and endocrine pancreas. In addition to these major lesions, several other tumours may also develop, such as carcinoid, tumours of the adrenal glands and several non-endocrine tumours. The clinical symptoms of MEN 1 depend on the involved glands and the presence or absence of hormonal hypersecretion.

According to the international literature, the prevalence of MEN 1 is estimated as 1/30000 in the general population. The prevalence of the disorder in Hungary is unknown, but it is likely similar to that found in international studies. In my work I summarize our clinical and genetic findings in MEN 1 syndrome, focusing on the opportunity for the application of current international guidelines in Hungarian patients, and taking advantage our experience, with a genetic screening program.

The *MEN1* gene, responsible for the disease, is a tumoursuppressor gene. It consists of one untranslated exon and nine exons. Previous studies have identified more than 400

germline and somatic mutations, spreading across all the coding sequence of the *MEN1* gene. Most of the mutations predict premature protein truncation. There is no mutational “hot spot” or genotype-phenotype correlation either. The gene encodes a 610 amino acid protein, named menin. Menin does not show homologies to any other known proteins. Via its interactions menin takes part in the regulation of cell cycle, cell growth and genome stability.

According to current guidelines, *MEN1* gene mutation screening is recommended in index patients with familial or sporadic MEN 1 and in those familial or sporadic cases who are suspicious or atypical of the disorder. When mutation is detected in the index patient, genetic testing of first-degree relatives can be used to identify mutation carriers and non-carriers. It has been noted, however, that when current guidelines for *MEN1* gene mutation screening in index patients are applied in clinical practice, the efficacy of mutation detection largely depends on clinical manifestations and the family history of the index patient. Therefore, in the present study we also investigated the prevalence and spectrum of *MEN1* gene mutations in Hungarian patients with familial and sporadic MEN 1 and in those with suspected MEN 1. When

mutation was detected, first-degree relatives of the index patient were also tested.

Genetic screening of the *MEN1* gene is time-consuming but it plays a key role when international guidelines in the patients' management are applied. The most frequently used methods for screening the *MEN1* gene are single strand conformational polymorphism (SSCP), denaturant gradient gel electrophoresis (DGGE), temporal temperature gradient gel electrophoresis (TGGE) and conformational sensitive gel electrophoresis (CSGE). Thereafter, the localized genetic alterations can be identified by direct sequencing, both in patients and asymptomatic family members. One main advantage of the genetic screening is that exclusion of the carrier-status in family members of a MEN 1 patient makes unnecessary the later clinical follow-up, while detection of the carrier-status can help to diagnose the potentially developing tumors in an early stage. Besides family screening, the genetic methods are also helpful in the management of patients with a suspicion of MEN 1 syndrome or a MEN 1-like state (e.g. familial isolated hyperparathyroidism or recurrent hyperparathyroidism; 2 or more parathyroid adenomas under 30 yrs; gastrinoma or multiple islet cell tumors at any age).

## Aims

First in Hungary we have started to perform genetic screening of MEN 1 patients at the 2<sup>nd</sup> Department of Medicine, in collaboration with the Department of Physiology, Semmelweis University, Budapest in September 2001. The aims of my work, including the genetic screening of 32 patients with MEN 1 syndrome or MEN 1 like state, were the following:

1. After a systematic analysis of the advantages and disadvantages of the “pre-screening” methods described in the literature, I wanted to establish a reliable, easily reproducible, cost-effective and fast screening method for detecting genetic alterations in the coding region of the *MEN1* gene.

2. To achieve these goals I made efforts to work out the optimal parameters of the temperature gradient gel electrophoresis systems and to define the specificity and sensitivity of this “pre-screening” method.

3. After the “pre-screening” of the patients with MEN 1 or MEN 1-related state, I wanted to identify the *MEN1* mutations by direct DNA sequencing in order to analyze the mutational spectrum of the Hungarian patients and its relation to those published in the international literature through the.

4. I wanted to study, whether in these patients a correlation could exist between the number and type of the MEN 1-associated manifestations or other phenotypic features and the efficacy of the mutation screening.

5. I wanted to investigate the value of genetic screening among the first-degree relatives of MEN1 gene mutation-carrier patients.

## **Patients and methods**

### **Patients**

The diagnosis of MEN 1 was established in patients who had at least two of the three major lesions. Familial MEN 1 was diagnosed when the MEN 1 patient had at least one first-degree relative in whom at least one of the three main target organ was affected. Patients with MEN 1-related state had one of the three main lesions plus at least one other lesion, or multiple parathyroid tumors with onset before the age of 30 y, recurrent primary hyperparathyroidism or familial isolated primary hyperparathyroidism. Of the 32 index patients studied (12-81 yrs; average: 41.9 yrs), 19 patients met the diagnostic criteria of MEN 1 (6 patients with familial and 13 patients with sporadic MEN 1; 12-81 yrs; average: 40.31 yrs) and 13 patients

had a MEN 1-related state (2 familial and 11 sporadic; 17-66 yrs; average: 44.23 yrs).

## **Methods**

1. Genomic DNA was prepared from peripheral blood samples using DNA isolation kits. DNA samples were stored at -20°C
2. All exons of the MEN1 gene were amplified in polymerase chain reaction (PCR). For TGGE screening one primer from each primer pair contained a 40-base GC-rich sequence (GC-clamp) to prevent complete denaturation of the PCR product during the electrophoresis. Primers were designed based on the study of Morelli et al. For sequencing, the same primers were used without GC-clamps, but with an M13-tail. After the traditional PCR protocol, the PCR-amplified products were allowed to form heteroduplexes by a denaturation at 95°C for 5 min followed by an incubation at room temperature for 1 hour.
3. There are several softwares to optimize the electrophoretic parameters for a TGGE analysis. In my work I compared two softwares: WinMelt and Poland, which both calculate and graph the theoretical melting domains of the amplified fragments upon DNA sequence. The denaturing conditions of a DNA are depending not only on its sequence but the concentration of DNA or buffers, which makes it difficult to

model or reproduce the electrophoretic environment by computers. The description of TGGE parameters were proved to be much more reliable by using an experimental perpendicular electrophoresis of each amplified exon, because the controlled temperature gradient and a stable environment (constant salt- and DNA-concentration) allowed us to estimate the start- and end-points to be estimated by a correlation of the temperature scale to the denaturation profile.

4. TGGE was performed by using two mutation detection system: DCode Universal Mutation Detection System (BioRad) and TGGE standard system (Whatman Biometra).

5. The genetic alterations localized by TGGE were identified by automatic sequencing (AbiPrism, Genetic analyser, Applied Biosystems).

## **Results**

Using TGGE, heterozygous alterations were detected in exon 2 (4 patients), exon 3 (9 patients), exons 5-6 (one patient), exon 8 (one patient) and exon 9 (18 patients), whereas homozygous alterations were found only in exon 9 (3 patients).

DNA sequencing revealed 10 different mutations in the *MEN1* gene (two deletions: nt359del4bp, nt738del4bp; 2

insertions: nt317ins5bp, nt1657insC; 3 nonsense: E26X, Q209X, C354X and 3 missense mutations: A91V, G28A, L301R). Five of the 10 mutations were novel mutations (E26X, C354X, A91V, G28A, L301R). In addition to *MEN1* gene mutations, 4 different polymorphisms (S145S, R171Q, D418D, L432L) have also been revealed. However, in one patient who had the nt1657insC mutation in exon 10 by direct DNA sequencing, the TGGE analysis of the same exon was false negative. In another patient, who proved to have the E26X mutation in exon 2 by DNA sequencing, PCR amplification of exon 2 for TGGE analysis was repetitively unsuccessful and, therefore, TGGE analysis of this exon could not be performed.

Family screening was performed in 6 symptomatic and 15 asymptomatic first-degree relatives of the index patients with disease-causing *MEN1* gene mutations. In one family, the genetic screening revealed the presence of *MEN1* gene mutation in an asymptomatic young boy, but in all other cases the results of the genetic testing was identical with the clinical findings. In two families both parents of the index patients proved to be genetically negative suggesting that the mutations occurred de novo. In two other families the inherited or de novo origin of the mutations in index patients could not be

examined because of the lack of appropriate DNA samples from family members.

The novel mutations identified in our study may affect structurally and functionally important domains of the translated protein. Two novel mutations (E26X in exon 2 and C354X in exon 8) were nonsense mutations. These two mutations cause early termination in gene translation and truncation of protein product, resulting in a loss of nuclear localization sequences and the proline-rich region of the protein. The other three novel mutations were missense mutations. The first missense mutation, A91V was detected in a MEN 1 family and its presence in family members coincided with the clinical findings, suggesting its pathogenic role in the development of the disease. Similar alanine to valine substitutions in other regions of the MEN1 gene, such as A242V and A535V, have also been reported as disease-causing mutations in MEN 1 patients. Although the functional consequence of A91V mutation is unknown, it may affect the function of the Smad3 and nm23 interacting domains of menin. The second novel missense mutation, L301R was found in a large MEN 1 family and segregation with the disease in family members was observed, suggesting that the L301R is likely to be a disease-causing mutation. Disease-causing MEN1 gene

mutations leading to the substitution of apolar leucine with the positively charged arginine (e.g. L22R or L89R) have also been described. The mechanism by which the L301R affect the function of the translated protein is unknown but it may alter the GTPase consensus sequence and may influence interactions with PEM and nm23. The clinical significance of the third novel missense mutation, G28A needs further assessment, because this mutation was found in a young patient with recurrent isolated primary hyperparathyroidism, and the first-degree relatives including the asymptomatic parents had negative genetic testing. Mutations resulting in a change of alanine to glycine have already been published in other genes as the molecular cause of reduced protein affinity or functionally changed protein structure. It is also possible, that the G28A mutation of the MEN1 gene results in disturbances in protein interactions with JunD and nm23 or it alters the structure of the first leucine zipper-like motif of the translated protein. In addition, missense MEN1 gene mutations may influence the protein product by other mechanisms, including a rapid degradation of the protein by the ubiquitin-proteasoma pathway<sup>49</sup>. Such a mechanism has already been considered to explain how different missense mutations spreading the MEN1 coding region and affecting different menin structural domains

can result the same clinical symptoms without genotype-phenotype correlations.

The difference in the effectiveness of mutation detection between familial and sporadic cases has been already noted in previous studies, as the mutation detection rate in clinically diagnosed familial MEN 1 was in a range of 80% to 90%, in contrast to the presence of mutations in 30% to 52% of sporadic MEN 1 patients. In patients who met the diagnostic criteria of sporadic MEN 1, a failure to find *MEN1* gene mutations could be related to the presence of disease phenocopy, large deletions that are missed by DNA sequencing, intronic mutations not detected by currently used primers, or mutations in regulatory or nonintronic untranslated region of the *MEN1* gene that are not tested in our and earlier studies. Our finding of only one mutation-positive patient among the 13 patients who had a MEN 1-related state but did not meet the diagnostic criteria for familial or sporadic disease is also consistent with earlier observations showing a low detection rate of *MEN1* gene mutations in these patients. In a recent study including the largest cohort of patients who underwent *MEN1* gene mutation testing, the mutation detection rate depended on the number of MEN 1-related tumors and the family history of index patients. When the detection rate of

*MEN1* gene mutations in our patients was correlated with the number of main MEN 1 tumors and family history, the results showed the presence of mutations in 100% and 0% of familial cases with two and one main MEN 1 tumors, respectively, and in 50%, 18%, and 9% of sporadic cases with three, two and one main MEN 1 tumors, respectively. Thus, our data support the proposal, that the best predictors of a positive genetic test may be the number of main MEN 1 tumors and the family history. In our patients with a MEN 1-related state, the high prevalence of adrenal adenomas and its association with either pituitary tumor or hyperparathyroidism but not with *MEN1* gene mutation, suggest that the presence of adrenal tumors has a low prediction of *MEN1* mutations.

## **Conclusions**

In my work I performed the genetic analysis of the entire coding region of the *MEN1* gene. Because of the large size of the gene, the great number of possible germline mutations and the absence of mutational “hot spot” I used TGGE for the localization of the altered gene-fragments, and I identified the mutations by direct DNA sequencing.

1. I showed that the available softwares for primer design improve the sensitivity of TGGE. In my hand, the experimental perpendicular electrophoresis was the most effective for the optimization of the electrophoretic parameters.

2. I found that TGGE gave false negative result during screening of exon 10. Therefore, TGGE is not recommended to screen this exon.

3. I have managed to separate two different frequent polymorphisms (D418D and L432L) in exon 9 by TGGE, and I recommend the use of D418D and L432L heterozygous samples as internal controls during the screening of this exon.

4. Direct sequencing the MEN1 gene in our patients proved that the most frequent polymorphisms of the MEN1 gene are as frequent in Hungarian patients, as described in the international literature.

5. I showed, that in 10 of the 32 patients with MEN 1 syndrome or MEN 1-like state had disease-causing mutations (31.25%), of the 10 mutations 5 were not described in the literature (A91V, G28A, E26X, L301R, C354X).

6. I showed that in patients with MEN 1 or MEN 1-like state the prevalence of disease-causing mutations is higher in familiar cases than in sporadic cases. The efficacy of mutation

detection is better in cases with 2 or 3 MEN 1-associated lesions than in those with only 1 MEN 1-associated tumor.

## **Publications**

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