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# NOVEL MUTATIONS IN SERBIAN MEN1 PATIENTS: GENOTYPE-PHENOTYPE CORRELATION

## NOVOOTKRIVENE MUTACIJE KOD PACIJENATA SA MEN1 SINDROMOM U SRBIJI: KORELACIJA GENOTIPA I FENOTIPA

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## Summary

**Background:** Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant cancer syndrome characterized by the occurrence of primary hyperparathyroidism (PHPT), pituitary adenoma (PA) and pancreatic neuroendocrine tumor (pNET). Whether the underlying mutations in *MEN1* gene predict clinical presentation of affected heterozygotes or not, is still a matter of a debate.
**Methods:** Clinical and genetic analysis of 90 consecutive MEN1 patients was performed in a retrospective, single – center study.
**Results:** *MEN1* mutation was found in 67 (74.4%) patients belonging to 31 different families. Twenty nine different heteozygous mutations were found, including 6 novel point mutations (W220G, 941delG, 1088del7, 1184insA, 1473del10, 1602del17) and one large deletion of exon 8. Truncating mutations predicted development of pNETs (OR=5.8, 95% CI 1.7 – 19.7%) and PHPT (OR=4.3, 95% CI 1.5 – 12.4%).
**Conclusions:** Large number of novel mutations among MEN1 patients confirmed previously reported data. PNETs and PHPT were more frequent in patients with truncating mutations.

**Keywords:** MEN1, genotype, phenotype, novel mutations

## Kratak sadržaj

**Uvod:** Multipla endokrina neoplazija tip 1 (MEN1) predstavlja autozomalno dominantni kancerski sindrom koji se karakteriše pojavom primarnog hiperparatireoidizma (PHPT), tumora hipofize i pankreasnih neuroendokrinih tumora (pNET). U kojoj meri postojanje heterozigotne mutacije u *MEN1* genu određuje kliničku sliku nosilaca i dalje predstavlja predmet diskusije.
**Metode:** U okviru retrospektivne studije jednog centra, sprovedeno je kliničko i gensko ispitivanje 90 uzastopnih pacijenata sa MEN1 sindromom.
**Rezultati:** Mutacija u *MEN1* genu nađena je kod 67 (74,4%) pacijenata koji su pripadali 31 različitoj porodici. Identifikovano je dvadeset devet različitih heterozigotnih mutacija, uključujući i 6 novootkrivenih (W220G, 941delG, 1088del7, 1184insA, 1473del10, 1602del17) i jednu veliku deleciju 8. egzona. Mutacije koje dovode do skraćenja proteina predvidele su pojavu pNET (OR=5,8, 95% CI 1,7 – 19,7%) i PHPT (OR=4,3, 95% CI 1,5 – 12,4%).
**Zaključak:** Veliki broj novootkrivenih mutacija među MEN1 pacijentima je u skladu sa prethodno objavljenim podacima. Pankreasni NET i PHPT su bili značajno češći kod pacijenata sa mutacijama koje dovode do skraćenja proteina.

**Ključne reči:** MEN1, genotip, fenotip, novootkrivene mutacije

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*List of abbreviations:* MEN1, multiple endocrine neoplasia type 1; PHPT, primary hyperparathyroidism; PA, pituitary adenoma; pNET, pancreatic neuroendocrine tumor, Smad3, SMAD family member 3; NFkB, nuclear factor kappa B; LOH, loss of heterozigosity; CDKN1B, cycline dependant kinase inhibitor N1B; MLPA, multiplex ligation-dependent probe amplification; MEN4, multiple endocrine neoplasia type 4; AIP, aryl hydrocarbon receptor-interacting protein; GPR101, G protein-coupled receptor 101; CHES1, checkpoint suppressor 1; FIHP, familial isolated hyperparathyroidism; IC, index case; FM, family member; AA, adrenal adenoma; tNET, thymic neuroendocrine tumor.

## Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant cancer predisposition syndrome characterized by the occurrence of primary hyperparathyroidism (PHPT), pituitary adenoma (PA) and pancreatic neuroendocrine tumor (pNET). Additionally, other endocrine and non-endocrine tumors can be present, such as adrenal tumors, duodenal, thymic and lung NETs, lipomas, facial angiofibromas, and colagenomas, in more than 20 different tumor combinations described (1). It has a high penetrance, and more than 95% of carriers develop disease by the age of 40 years (2). The prevalence is estimated to be 1/30 000 to 1/50 000 in general population (3).

MEN1 syndrome is caused by mutations in *MEN1* tumor suppressor gene, mapped to 11q13 chromosome (4). The gene encodes 610 amino acid protein menin. Menin is a nuclear, ubiquitously expressed, scaffold protein that interacts with numbers of protein partners (JunD, Smad3, NFkB) involved in diverse cellular processes. Loss of heterozigosity (LOH) in tumor tissues demonstrates tumor suppressor role of menin in these tumors (5). So far, more than 450 different mutations scattered all over the gene were identified, with no hot spots or genotype-phenotype correlation (6). Neither the type of the mutation nor a position within the gene appears to have any effect on the phenotype. However, there are some exceptions, showing that mutations leading to a truncated menin are related to higher prevalence of thymic and malignant pancreatic NETs (7, 8). A large GTE study (*Groupe des Tumeurs Endocrines*) also confirmed the lack of direct genotype-phenotype correlation, but had shown that patients with mutations affecting the JunD interacting domain had a higher risk of death secondary to a MEN1 tumor (9).

About 10% of MEN1 patients may not harbor mutations in *MEN1* gene (MEN1 phenocopy) (1). These patients may have whole gene deletions or mutations in the promoter and untranslated regions which cannot be detected routinely. Furthermore, other genes may be responsible for development of MEN1-like syndrome, such as *CDKN1B* (10). Nevertheless, sporadic occurrence of the tumors cannot be excluded (11). Here we present the results of genetic analysis of *MEN1* gene in Serbian MEN1 patients in correlation to patients' clinical presentation.

## Materials and Methods

*Patients*

This retrospective study was performed at the Clinic for Endocrinology, Diabetes and Metabolic Diseases in Belgrade, Clinical Center of Serbia. Genetic analysis of *MEN1* gene was performed at the same institution. In the period from January 2004 until December 2016 MEN1 syndrome was diagnosed in 90 consecutive patients according to following criteria: 1) clinical – two or more major endocrine tumors: parathyroid, pituitary or pNET, 2) familial – one major tumor and a first degree relative with clinical diagnosis of MEN1, 3) genetic – mutation in MEN1 gene, including those with no clinical signs of MEN1 (12). All the patients underwent routine, site-specific, diagnostic procedures according to current diagnostic guidelines, to confirm the presence of tumors (CT/MRI, Octreoscan/$Ga^{68}$ PET CT, biochemical and hormonal measurements, histopathological analysis after the surgery or biopsy) (13–19). Genetic analysis *MEN1* gene performed in all patients. Data from patients' medical records were retrospectively studied and analyzed. MEN1 patients were classified as familial cases if two or more members of the pedigree were diagnosed with MEN1 tumors. Patients with no MEN1 tumors or mutation in the family where classified as sporadic, irrespective of patient's mutational status. Age at onset was defined as the age at which the first tumor occurred. Informed consent was obtained from all patients included in the study. All procedures were carried out in conformance with the Declaration of Helsinki ethical guidelines. The study was approved by the institutional Ethical committee.

*Genetic analysis*

Mutational analysis was performed on genomic DNA, extracted from peripheral blood leukocytes using Pure Link Genomic DNA Mini Kit (Termo Fisher Scientific, USA), according to the manufacturer's instructions. The entire coding region plus flanking splice sites of *MEN1* (exons 2–10) were analyzed by PCR sequencing using specific primers. Direct DNA sequencing using the Big Dye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA) was performed on automated ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, USA) and analyzed with ABI DNA Sequencing Analysis Software v5.2.

All patients negative for point mutations were screened for larger deletions in *MEN1* gene, using the SALSA MLPA P017-C1 MEN1 kit (MRC-Holland, Netherlands). Coffalyser.NET Software (MRC-Holland, Netherlands) was used for fragment analysis and comparative analysis of MLPA samples. DNA samples obtained from healthy control individuals and negative control (no-DNA control) were included in MLPA analysis. Probe ratios below 0.7 or above 1.3 were considered as cut-off values for heterozygous deletion or amplification, respectively.

*Statistical analysis*

Results for continuous variables are presented as mean values ± standard deviation (SD). For dichotomous variables, results are expressed as percentages. Dichotomous variables were analyzed by $\chi^2$ test. Binary logistic regression was applied to calculate the odds ratio to assess association between mutation and expected phenotype. The results of this analysis were expressed as odds ratios (OR) and their 95% condense intervals (95% CI). P-value less than 0.05 was considered statistically significant.

## Results

A total of 90 consecutive MEN1 patients, of which 63 (70%) were index cases, were studied during the 12 years period, mean follow up period 10.9 ± 8.47 years. Mean age at diagnosis was 38.8 ± 18.3 years, and there was an overall female predominance 61 (67.8%) among patients. *MEN1* gene alterations were found in 67 (74.4%) patients. MEN1-phenocopy was found in 23 (25.6%) among all patients, or in 36.5% among index cases. All these patients had negative family history. Twenty seven patients (27/67, 40.3%) were family members, belonging to 31 different families. Fifty eight (64.4%) cases were classified as familial, and 32 (35.6 %) as sporadic.

The most frequent tumor observed was parathyroid hyperplasia/adenoma (65, 72.2%), with the average age at onset of 44.7 ± 14.5 years. Pituitary adenoma was present in 52 (57.8%), age at onset was 39.2 ± 15.7 years. Prolactinoma (36.5%) and nonfunctioning adenomas (34.6%) were the most frequent. PNETs were found in 26 patients (28.9%), average age at onset was 43.1 ± 14.4 years, with insulinoma and gastrinoma as the most frequent ones (26.9% and 23.1%, respectively). Additionally, adrenal tumor was present in 13 (14.4%) patients (age at onset 49.4 ± 11.9 years) and lung NETs in 8 (8.9%) patients (age at onset 45.8 ± 12.3 years). Duodenal, thymic and small intestinal NETs were diagnosed in 1 (1.1%) patient, each. Fourteen patients (14/67, 20.9%) were asymptomatic mutation carriers, mean age 16.5 ± 13.5 years, mean follow up period 5.4 ± 4.8 years.

Mutational analysis revealed 28 different germline point mutations scattered all over the coding region (exons 2, 3, 4, 5, 7, 8 and 10), one point mutation in intron 4 and one large deletion of exon 8 (*Figure 1*). The majority of mutations resulted in truncation of the menin protein (40, 44.4%). We found 11 (37.9%) different frameshift, 6 (20.7%) nonsense, 9 (31%) missense, 2 (6.9%) different in-frame deletions and 1 (3.5%) splice-site mutation. The most frequent mutation found was missense P188L mutation, found in 5 (7.9%) index cases. All these index cases presented with pituitary adenoma only, whilst their siblings were asymptomatic carriers. P188L mutation did not predicted the presence of PA (p=0.07).

One large deletion of exon 8, MEN1ex8del, and 6 point mutations were novel: W220G in exon 4, 941delG and 1088_1095del7 in exon 7, 1184_1185insA in exon 8 and 1473_1483del10 and 1602_1618del17 in exon 10. Detailed characteristics, expected effects and their phenotypes are shown in *Table I*.

There was no correlation between the position within the gene or the type of mutation with the phenotype, or the age at onset ($p > 0.05$). Truncating mutations were significantly more frequent in patients with pNETs than in patients without (20 (83.3%) vs. 4

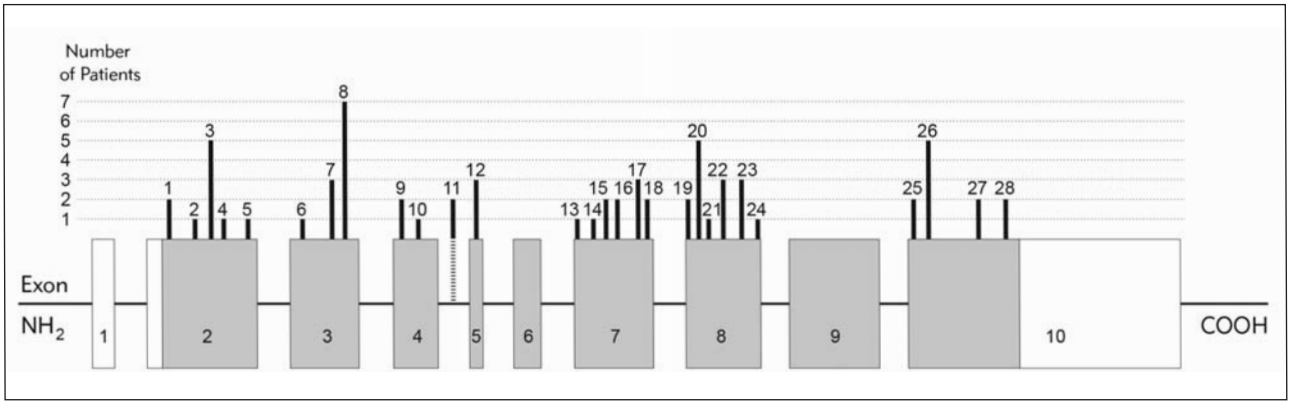


**Figure 1** Schematic presentation of *MEN1* gene and mutations found

Exons are marked with numbers 1 – 10. Coding region of *MEN1* gene is indicated by shaded region, untranslated regions are indicated by open boxes. Germline point mutations are presented with vertical lines. The scale on the left represents the number of affected patients. Numbers above vertical lines represent reported mutations as follows: 1- M1V; 2- Y77X; 3- 359_362del4; 4- 247delC; 5- H139R; 6- C165Y; 7- A176S; 8- P188L; 9- W220G; 10- 865del4; 11- IVS4as -1G A; 12- 794_802del9; 13- 941delG; 14- H317Y; 15- 960delG; 16- 1088_1095del7; 17- 979delT; 18- W341X; 19- E392X; 20- E358X; 21- R355W; 22- Q395X; 23- Y351H; 24- 1184_1185insA; 25- 1473_1483del10; 26- 1546_1547insC; 27- R527X; 28- 1602_1618del17. Large deletion of exon 8 (MEN1ex8del) is not shown on the graphic.

**Table I** Characteristics of six novel point mutations and a large deletion.

| Mutation | Exon | Nucleotide change | Mutation type | Clinical presentation |
|---|---|---|---|---|
| W220G | 4 | TGG GGG | Missense | IC: PHPT (52), AA (52)<br>FM: pNET (30) |
| 941delG | 7 | TATC(G)GGAT | Frameshift | IC: PA (28), pNET (29) |
| 1088_1095del7 | 7 | CA(CTGTCGC)AAC | Frameshift | IC: pNET (31), PHPT (52), tNET (54)FM: Asymp. (19) |
| 1184_1185insA | 8 | AGCCA(/A)G | Frameshift | IC: PA (61), PHPT (63) |
| 1473_1483del10 | 10 | AAG(GTGCGCATAG)TGAGC | Frameshift | IC: pNET (51), PHPT (51), AA (51), PA (52)FM: pNET (30) |
| 1602_1618del17 | 10 | AGCAC(GGCTCAGGTGCCAGCAC)CC | Frameshift | IC: PHPT (25)FM: PHPT (50), lungNET (52), AA (52) |
| MEN1ex8del* | 8 | precise position and the number of deleted bp not known | In-frame deletion | IC: PA (19), PHPT (22) |

IC – index case, FM – family member, lungNET – lung neuroendocrine tumor, PA – pituitary adenoma, Asymp – asymptomatic, PHPT – primary hyperparathyroidism, AA – adrenal adenoma, pNET – pancreatic neuroendocrine tumor, tNET – thymic neuroendocrine tumor

(16.7%) respectively, p= 0.003; OR=5.8, 95% CI 1.7 – 19.7%). The majority of these mutations (76.2%) were in exon 7, 8 and 10 (codons 314 – 527). The type of pNET did not correlate to the effect of mutations ($p > 0.05$). Truncating mutations were also more frequent in patients with PHPT than in those without (31 (72.1%) vs. 12 (27.9%) respectively, p=0.006; OR= 4.3, 95% CI 1.5 – 12.4%), scattered all over the coding region. There were no difference in detection of truncating mutations in patients with or without PA, lung NET or adrenal adenoma ($p > 0.05$).

## Discussion

This study represents a single-center mutational analysis in 90 consecutive patients who fulfilled current diagnostic criteria for MEN1 syndrome. MEN1 gene alterations were found in 74.4%. Conversely, MEN1 phenocopy was diagnosed in 25.6% of all patients, or in 36.5% of index cases, which corresponds with the data in the literature. The percentage of mutation-negative patients, or so-called MEN1 phenocopy patients, varies widely in the literature, from 10 to 60% among index cases (11, 20–24). At some extent, this might be attributed to limitations of available diagnostic molecular techniques in the past. Only one study prior to this implemented MLPA analysis, that probably increased sensitivity of *MEN1* gene analysis (11). Nonetheless, only one patient in our study was found to have large chromosomal deletion. Mutations in other genes may cause MEN1-like syndromes, such as *CDKN1B* that causes MEN4 syndrome, *AIP*, *GPR101*, or rarely, other cell-cycle inhibitors genes (10, 25, 26). However, alterations in these genes explain only a small subset of *MEN1* mutation-negative cases (26, 27). Recently, a study had shown that none of these patients had positive family history and more than two occurring tumors that developed later in life, suggesting possible sporadic occurrence of two neuroendocrine tumors (11). Finally, phenocopy may occur in other cancer syndromes, suggesting the role of modifier genes (28, 29).

Our study revealed 28 different point mutations scattered throughout the coding region of *MEN1* gene and intron 4, and one large deletion of exon 8. As in other studies, frameshift and missense mutations were the most frequent (1, 30). The most frequently found mutation P188L (exon 3, codon 188), was a missense mutation of uncertain significance, causing nucleotide substitution CCC CTC, that was previously reported in sporadic pNETs and primary hyperparathyroidism (31, 32). In our study, P188L was found in 5 index cases which presented with pituitary adenoma solely. However, the mutation was not predictive for development of PA. In addition to mutations that we reported previously, we found 6 novel mutations in exon 4, 7, 8 and 10, and this result supports previously published data on high prevalence of novel *MEN1* gene mutations among MEN1 patients (33-35). Mutation W220G in exon 4 which resulted in substitution of trhyphtophan to glycine (TGG GGG) at codon 220, was classified as missense. It is likely that the mutation cause the disease, but this is not possible to determine since missense mutations do not predict obvious inactivation of menin. Its pathologic nature should be verified by tracking with the

disease trough multiple generations (36). All other novel point mutations changed the reading frame that lead to a premature stop codon at indicated amino acids. All affected patients had at least two major MEN1 tumors and segregation with the disease in family members was observed.

Our study has shown that truncating mutations correspond with higher OR for developing of both PHPT and pNET in comparison to nontruncating, but we have not found the influence upon development of the specific type of pNET. Although majority of studies had shown that there was no direct relationship between genotype and phenotype, there are some exceptions. In a study of Vierimaa et al, truncating frameshift and nonsense mutations (1657insC, R527X) have significantly higher OR for developing nonfuntioning pNET, compared with in-frame/missense mutations 1466del12, D418N and G156R (37). The same study has shown that nontruncating in-frame/missense mutations have higher risk for developing gastrinoma (37). Truncating mutations are more prevalent in $MEN1_{Burin}$ phenotypic variant, presenting with high prevalence of prolactinoma and low prevalence of gastrinoma (38, 39). It has been shown that truncating mutations involving checkpoint kinase 1 CHES1 domain (codons 428–610) of the *MEN1* gene have higher prevalence of malignant and aggressive pNETs (40). There was also a high prevalence of truncating mutations in MEN1-related thymic carcinoids, although when compared with the prevalence of truncating mutations in all reported MEN1 mutations, this was not statistically significant (41). MEN1 mutations have been reported in 42 families with isolated hyperparathyroidism (FIHP), and 38% of these are missense mutations that are less likely to result in a truncated, inactivated protein. This proportion of missense mutations among FIHP families is significantly higher than in all MEN1 patients (20%) (30). However, despite all these aforementioned studies, the largest genotype-phenotype study on 806 MEN1 patients failed to demonstrate direct genotype–phenotype correlation. Nevertheless, the same study showed that patients with mutations affecting the JunD interacting domain had a higher risk of death secondary to a MEN1 tumor (9).

In conclusion, we report 6 novel *MEN1* mutations that are likely to cause the disease. It is in accordance with previously published data on high prevalence of novel mutations among MEN1 patients. Our study confirmed the higher frequency of truncating mutations among patients with pNETs, and showed high prevalence in patients with PHPT. Larger studies would possibly reveal closer relationships between specific mutations and their clinical appearance.

## Conflict of interest statement

The author stated that she has no conflicts of interest regarding the publication of this article.

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