A novel in-frame deletion in MEN1 (p.Ala416del) causes familial multiple endocrine neoplasia type 1 with an aggressive phenotype and unexpected inheritance pattern

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Received September 16, 2015; Accepted February 15, 2016

DOI: 10.3892/mmr.2016.5462

Abstract. The present study describes a family with multiple endocrine neoplasia type 1 (MEN1) caused by a previously undescribed in-frame deletion c.1246_1248delGCC (Ala416del) in the MEN1 gene. Evidence for the pathogenic character of this mutation, which triggers an aggressive clinical outcome, is demonstrated. Aggregation analysis in the tested family was strongly suggestive of causality of the detected mutation. This was supported by the analysis of LOH (loss of heterozygosity) in tumor-derived DNA and by computational analysis of the functional and structural implications of the mutation. Different phenotypic characteristics were identified among family members, which is typical for MEN1. Additionally, an unexpected disease inheritance pattern was observed in this kindred, in which either all or none of the siblings of one branch inherited the disease.

Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an inherited disorder with high penetrance, which approaches 100% with increasing age (1). The disease occurs with a prevalence of 2-3 per 100,000 in the population (2). It is predominantly characterized by tumors of the parathyroid glands, gastroenteropancreatic tumors, pituitary adenomas, adrenal adenomas, and neuroendocrine tumors of the thymus, lungs or stomach, as well as non-endocrine lesions (2). The expression in terms of tumor localization, age of onset and clinical aggressiveness, may vary even between affected members of the same family. The clinical manifestations of MEN1 are associated with the products of secretion of the tumors rather than the primary sites or metastases, and often appear at a young age (3). Management of MEN1 is based on treatment or prevention of manifestations (4).

The syndrome is caused by inactivating mutations in the tumor suppressor gene MEN1, coding for the 615-amino acid protein menin (5).

MEN1 syndrome is inherited in an autosomal dominant manner, which means that a single inherited mutation in the MEN1 gene predisposes to somatic loss of heterozygosity (LOH) during a patient's lifetime. However, only once LOH has occurred does the disease begin to develop. LOH predominantly occurs in the region at which a mutation was inherited by the patient. This alteration may occur in different tissues; however, a person bearing a single mutation in MEN1 is certain to develop the disease. The location, as well as the order and age of MEN1 manifestations are unpredictable (3). The majority of MEN1 mutations that have been found in affected families result in truncated forms of menin. However, no genotype-phenotype correlations have been proven (2).

In the present study, a kindred with a previously unreported in-frame deletion in the MEN1 gene, with an inheritance that is unexpected for Mendelian diseases was described.

Materials and methods

Subjects and case history. A large Polish kindred was identified, in which 3 generations had MEN1 (Fig. 1). All features presented within the case history occurred prior to the commencement of the study, and all patients were enrolled during the treatment stage. The index patient (II-3) was enrolled into the study aged 50 with suspected MEN1. The patient underwent parathyroidectomy due to primary hyperthyroidism at the age of 20. Somatostatine-receptor scintigraphy showed pathological foci of tracer uptake in the right mesogastrium projecting at the small intestine loop, in the pancreatic tail and in both adrenal glands. An abdominal magnetic resonance imaging scan confirmed the pancreatic tail tumor with a size of 20x15x11 mm; a similar lesion sized 16x13 mm was found in the topography of the inferior duodenal flexure. These
The other brother (II-5) of the index patient was enrolled aged 37 after parathyroidectomy due to primary hyperparathyroidism. She also suffered from nephrolithiasis, euthyroid multinodular goiter and secondary diabetes. She succumbed to hepatic encephalopathy aged 37. Her only son (aged 11) was unavailable for enrollment in the present study.

All tested family members gave their written informed consent for genetic testing. In the case of juvenile members, additional consent was obtained from their legal caretakers. The research has been approved by the local Ethics Committee, (approval no. KBET/70/B/2013).

DNA isolation. Whole peripheral blood samples (2.6 ml) from each patient were collected into EDTA-coated tubes (Sarstedt, Nümbrecht, Germany). DNA was isolated with the QIAamp DNA Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol.

A formalin-fixed paraffin-embedded (FFPE) post-operative parathyroid gland was obtained from the index patient. Seven sections, with a thickness of 10 µm each, were cut from a region that contained ~70% cancerous tissue, as assessed by light microscopy (Olympus BX51 with 40x UPlanFLN eyepiece; Olympus Corporation, Tokyo, Japan), which involved the fixation of the material in formalin, which was then processed by the routine method and embedded in paraffin. Sections (4 µm) were cut from paraffin blocks and stained with standard hematoxylin and eosin (H&E; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for histological examination. Corresponding paraffin cube containing tumor tissue was selected on the basis of a comparison with H&E slides. DNA from these sections was isolated using the NucleoSpin FFPE DNA kit (Machery-Nagel, Düren Germany). As a negative somatic control, a mixture of two randomly selected healthy post-operative FFPE parathyroid glands (which were removed together with the thyroid during surgery for non-parathyroid associated reasons from patients unrelated to the tested family and negative for MEN1 syndrome) were used.
Sequencing

Amplification of products for sequencing. The 9 coding exons of MEN1 (according to transcript variant 1, RefSeq NM_000244.3; ncbi.nlm.nih.gov/nuccore) were sequenced for the index patient, her siblings and children, and only the one exon in which the mutation was found, for the remaining participants. For PCR, 25 µl reaction mixtures with HotStarTaq polymerase (Qiagen) were set up for each exon according to the standard recommendations of the manufacturer. The mixtures contained 0.2 µM each of the appropriate forward and reverse primer (Institute of Biochemistry and Biophysics Polish Academy of Sciences, Warsaw, Poland), and 100 ng DNA. Primers are listed in Table I. Reaction conditions: Initial denaturation at 95˚C for 15 min; 35 cycles including denaturation at 95˚C for 30 sec, annealing at 59˚C for 30 sec, and elongation at 72˚C for 30 sec; final elongation at 72˚C for 10 min. Samples were amplified in a Mastercycler realplex2 (Eppendorf, Hamburg, Germany).

Product purification and visualization. The quality of the products was assessed by 2% agarose electrophoresis in TAE buffer (Tris base, Thermo Fisher Scientific, Inc.; acetic acid, Chempur, Piekary Slaskie, Poland; EDTA, Avantor Performance Materials Poland S.A., Gliwice, Poland) and visualized with ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA). The remaining PCR products were purified with the QIAquick PCR Purification kit (Qiagen), according to the manufacturer's protocol.

Sequencing PCR. The sequencing PCR reaction mixture included 1.25 µl BigDye Terminator v3.1 (Thermo Fisher Scientific, Inc.), and 200 µM each of the appropriate dNTP. The sequencing reaction was carried out according to the manufacturer's protocol.

Table I. Primers used in polymerase chain reaction.

<table>
<thead>
<tr>
<th>Amplified region</th>
<th>Primer designation</th>
<th>Primer sequence</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 2</td>
<td>2_F</td>
<td>5'-AACCTTAGGGACCCCTGG-3'</td>
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</tr>
<tr>
<td></td>
<td>2_R</td>
<td>5'-ATAACACCTGCCGAACCTCA-3'</td>
<td></td>
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<tr>
<td>Exon 3</td>
<td>3_F</td>
<td>5'-CCCTTTCCCATGTAAAGC-3'</td>
<td>322</td>
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<tr>
<td></td>
<td>3_R</td>
<td>5'-GGTGCCCTGGCTACTACAG-3'</td>
<td></td>
</tr>
<tr>
<td>Exon 4</td>
<td>4_F</td>
<td>5'-CCCTTTCCCTGGCTGATTTTC-3'</td>
<td>264</td>
</tr>
<tr>
<td></td>
<td>4_R</td>
<td>5'-CCCAACAGCAAGTCAAGTCTG-3'</td>
<td></td>
</tr>
<tr>
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<td>5'-CTAAGGACCGTTCTCCTCC-3'</td>
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<td></td>
<td>5-6_R</td>
<td>5'-CCTGCCCTCAGCCACTGTAG-3'</td>
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<tr>
<td>Exon 7</td>
<td>7_F</td>
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<td></td>
<td>7_R</td>
<td>5'-GGAAACTGATGGAGGGGAAG-3'</td>
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<tr>
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<td>271</td>
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<td></td>
<td>8_R</td>
<td>5'-ATGCGCTGTAAGAAGGGAG-3'</td>
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<tr>
<td>Exon 9</td>
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<td>293</td>
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<tr>
<td></td>
<td>9_R</td>
<td>5'-AAAAAGTCTGACAAGGCGGT-3'</td>
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<tr>
<td>Exon 10</td>
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<td>5'-TCCTGGAGTTCAGCCAC-3'</td>
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<td>5'-GAACATGAGGCTAGAGTG-3'</td>
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<td>--_f</td>
<td>5'-ACCCAGAGCCAAGGGTTC-3'</td>
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<td>('ext')</td>
<td>2_r</td>
<td>5'-ATTGACATGAGCCGTCTAG-3'</td>
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<tr>
<td>Inner region</td>
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</table>

Indicates the additional mismatch that was introduced into the Wm2 primer in order to enhance mutant allele specificity.
SKALNIAK et al. MEN1 p.ALA416del-OBSERVED PHENOTYPES AND INHERITANCE

Scientific, Inc.), 0.16 µM of the appropriate forward or reverse primer, and 20 ng of the appropriate purified product. PCR was conducted under conditions recommended by the manufacturer. Specifically, PCR was conducted in Mastercycler RealPlex2 (Eppendorf, Hamburg, Germany) under the following conditions: Initial denaturation at 96°C for 1 min; 25 cycles including denaturation at 96°C for 10 sec, annealing at 55°C for 5 sec and elongation at 60°C for 4 min.

Ethanol precipitation. To purify products after the sequencing PCR, 2 µl of 1.5 M sodium acetate/250 mM EDTA buffer, pH >8.0 were added to 10 µl of the reaction mixture. After pipetting, 80 µl of 95% ethanol were added, the samples were centrifuged for 15 min at 10,000 x g, and the supernatant discarded. The pellets were washed with 75% ethanol and centrifuged for 2 min in 10,000 x g. After dissolving the supernatants, DNA pellets were left to air-dry, and dissolved in 20 µl nuclease-free water (Ambion; Thermo Fisher Scientific, Inc.). The whole procedure was conducted at room temperature. The purified products were separated on the ABI3500 sequencer (Thermo Fisher Scientific, Inc.).

Sequence analysis. The obtained sequences were aligned to the reference NC_000011.10 with SeqScape software (version 2.7; Thermo Fisher Scientific, Inc.). After identification of an exon-shortening event, the overlapping sequence resulting from the heterozygous deletion was analyzed manually using FinchTV (version 4.0; Geospiza, Inc, Seattle, WA, USA).

Multiplex ligation-dependent probe amplification (MLPA). MLPA was performed with use of SALSA MLPA probemix P017-C1; lot C1-0711 (MRC-Holland, Amsterdam, the Netherlands). The reaction was performed according to the manufacturer’s protocol, using 100 ng DNA. Results were analyzed with Coffalyser.net (version 131123; MRC-Holland; Amsterdam, Netherlands).

Testing for LOH. After an initial PCR with external primers, three quantitative PCRs per sample were performed with external primers (‘ext’), and with internal primers specific for the wild-type allele (‘wt’) or for the mutant allele (‘mut’). Each sample was run in triplicate. Primer sequences are presented in Table I.

At the time of primer design it was assured that the tested patients and controls did not bear the rs2071313 polymorphism in germline material, in order to avoid lack of primer binding due to this change.

The 10 µl real-time PCR mix contained 5 µl RT 2X PCR Master mix SYBR-C (A&A Biotechnology, Gdynia, Poland), one of 4 successive ten-fold dilutions of the external-PCR product, and standardized amounts of primers: 6 µM each for the ‘wt’ and the ‘mut’ reactions, and 3 µM forward and 6 µM reverse primer for ‘ext’. The reaction was set on ice; the prepared samples were put into the heated thermal cycler. Reaction conditions were as follows: 40 cycles consisting of 95°C for 5 sec, 59°C for 10 sec, 72°C for 8 sec and 80°C for 15 sec; followed by a single step of 95°C for 15 sec. Fluorescence was measured after each 80°C step. A melting curve analysis step was added after the reaction. Each sample was run in triplicate. All reactions were run in the Mastercycler realplex2 (Eppendorf, Hamburg, Germany).

The comparative Cq method was used to evaluate the copy number in tested samples, with DNA from the index patient as a reference (‘IP blood’), which contains 1 wt and 1 mut copy. Amplification efficiencies (E) were included into the normalized copy number ratio equation EΔΔCQ (6).

Average values and standard errors were calculated from three independent experiments.

Results

DNA analysis. A heterozygous in-frame deletion, c.1246_1248delGCC, was identified by sequencing the MEN1 gene for the index patient, II-3. At the protein level, this leads to the deletion of alanine at position 416 (p.Ala416del). According
to available variation databases, accessed on August 24, 2015 through the Genome Browser at UCSC Genome Bioinformatics (7) and The Universal Mutation Database (8), the identified mutation has not been reported previously.

The index patient’s two adult children, III-3 and III-4 (at that time aged 24 and 22), did not exhibit any clinical manifestation to suggest MEN1, and the absence of any variant in MEN1 was confirmed by sequencing of the whole coding region.

Sequence analysis revealed the presence of the c.1246_1248delGCC mutation in the other affected family members, II-4, II-5, II-6 and III-2. This mutation was not found in any of the four asymptomatic children of patient II-4 (III-5, III-6, III-7 and III-8). MLPA was performed for all affected family members, revealing no copy number changes among any exon or exon part of the MEN1 gene. (data not shown).

In order to confirm that the detected c.1246_1248delGCC mutation was causative of the disease in this family, the post-operative FFPE parathyroid tissue from the index patient (II-3) was analyzed for an additional, somatic MEN1 gene-function disrupting event (LOH), which typically occurs as a large deletion in any region of the gene, but predominately on that region of the wild-type allele in which the germline mutation occurs on the other allele.

MLPA, although suggestive for a large deletion encompassing the region with the mutation, gave ambiguous results, most probably because of the poor quality of the DNA, reflected by a poor Coffalyser Analysis Score for those samples.

The numbers of wild-type and mutated alleles were determined by relative quantification. Average values of 3 independent experiments are shown in Fig. 2. As expected, in the blood sample obtained from a healthy family member (the index patient’s daughter, ‘CTRL blood’) and in healthy parathyroid tissue (‘CTRL tissue’) the number of wild-type alleles was twice that of the index patient, and the mutated allele was absent. Results from the transformed parathyroid tissue obtained from the index patient (‘IP tissue’) revealed that the relative quantities of the wild-type and the mutated allele were 0.33 and 1.81, respectively. If the LOH is due to a copy loss without mutant allele duplication, these values represent ~double of the factual amounts as, in fact, there is only one remaining copy (the mutated) left in the sample. The finding of 0.33 wt copy numbers in the sample (where 0 wt copy numbers would typically be expected) may arise from the surrounding tissue, which may have a different number of wt copies, as FFPE slices containing ~70% tumor tissue were used for analysis.

In silico analysis of the mutation. In the wild-type protein, alanine-416 is located in an α-helix near the disordered structure of the menin protein. According to SWISS-MODEL prediction (9), the deletion causes a disruption of the N-terminal end of this helix (Fig. 3). The tool PROVEAN marked this mutation as ‘deleterious’ with a score of -10.97 (10). According to SIFT Indel, the mutation is ‘damaging’ to the protein, with a confidence score of 0.894, which indicates that it affects a Pfam domain and that the deletion is not located in a disordered region (11). Indeed, MEN1 is conserved in bilateria (pfam05053), and Ala416 is located in a highly conserved region of the protein (12).

Discussion

It has previously been demonstrated that genetically diagnosed patients with MEN1 present with biochemical changes 10 years prior to the signs and symptoms of the disease (13), and an earlier diagnosis would allow for more effective management of the disease. In addition, patients who do not harbor a MEN1 mutation may be prevented from undergoing unnecessary examination and lifelong surveillance (14). However, predictive testing can be offered to family members only after the disease-causing nature of a variant has been unequivocally established (4). It is therefore of importance for the patients and their family to confirm the pathogenic character of their mutation in MEN1.

Large deletions and mutations at conserved donor and acceptor splice sites, or mutations which introduce a premature stop codon in the protein-coding region of MEN1 (nonsense mutations and frameshift insertions or deletions) are explicitly predicted to be disease causing (4). In the case of any other variant, it has yet to be elucidated whether this is a pathogenic or a neutral change. The present study provides evidence that in the in-frame deletion c.1246_1248delGCC in the MEN1 gene, which, at the protein level, leads to the deletion of alanine at position 416 in menin, is a disease causing mutation. In order to confirm this finding, the post-operative FFPE parathyroid tissue from the index patient (II-3) was analysed for LOH in the region of the MEN1 gene. The analysis of this large kindred resulted in a further notable observation. In this family, either all or none of the siblings inherited the disease. Statistically, this is not impossible, but taking into account Mendelian inheritance patterns, this observation may be noteworthy.

The present study is of importance as it characterizes a newly discovered pathogenic mutation which may be useful for any researcher or physician that encounters the same mutation in another family or patient, but is unable to assess its pathogenic status. In order to obtain more information about the inheritance of this mutation, further investigations involving the partners of the affected patients are required. The assessment of environmental factor influence on the development of the disorder would also be beneficial, however, for a small group of patients, this kind of investigation may be difficult and have limited statistical power.

Acknowledgements

This study was supported by the Polish Ministry of Science and Higher Education (grant no. K/ZDS/003796). The authors would like to thank Dr Łukasz Skalniak for his assistance in experimental design. The authors would also like to thank the described family for their collaboration.

References


