A Whole MEN1 Gene Deletion Flanked by Alu Repeats in a Family with Multiple Endocrine Neoplasia Type 1

Atsushi Fukuuchi1, Yuko Nagamura2, Hiroko Yaguchi2, Naganari Ohkura2, Takao Obara3 and Toshihiko Tsukada2

1Department of Breast and Endocrine Surgery, Mitsui Memorial Hospital, Tokyo, 2Tumor Endocrinology Project, National Cancer Center Research Institute, Tokyo and 3Department of Endocrine Surgery, Tokyo Women's Medical University, Tokyo, Japan

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Multiple endocrine neoplasia type 1 is an autosomal dominant cancer syndrome characterized by pituitary, parathyroid and enteropancreatic endocrine tumors, which is caused by germline mutations of the tumor suppressor gene MEN1. In the case reported here, the patient had family with this disease whose germline MEN1 mutation was undetectable by conventional sequencing analysis. Further investigations involving polymorphism analyses, gene dose assay and nucleotide sequencing identified a large germline deletion of approximately 29 kilobase pairs spanning the whole MEN1 gene. The deletion was flanked by Alu repetitive sequences, suggesting unequal homologous recombination as the deletion mechanism. The polymorphism linkage data suggested that an asymptomatic son of the proband did not carry the family mutation. More direct evidence was obtained by gene dose assay and deletion-specific polymerase chain reaction, which demonstrated the normal MEN1 gene dosage and the absence of the deletion breakpoints in this asymptomatic subject and thus definitely excluded the possibility of disease predisposition.

Key words: multiple endocrine neoplasia type 1 – MEN1 – deletion – Alu repeat

INTRODUCTION

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant familial cancer syndrome characterized by combined occurrence of parathyroid, pituitary, enteropancreatic and other endocrine and mesenchymal tumors, and has been estimated to occur at a prevalence of 2–20 per 100,000 in the general population (1,2). The correct diagnosis of this syndrome is mandatory for the management of this disease, because therapeutic options for tumors associated with this syndrome are often different from those for the similar but non-hereditary endocrine tumors and because MEN1 manifestations should be carefully and periodically explored in asymptomatic individuals at risk in the affected pedigree as well as in the patients with early signs of MEN1 (1,2).

The tumor suppressor gene MEN1 has been identified as the causative gene of this syndrome (3). The MEN1 gene comprises 10 exons distributed over 7 kilobase pairs (kb) in the chromosome region 11q13, and encodes a 610-amino acid protein, menin, whose biochemical function has not been fully understood (4). Various heterozygous germline mutations of this gene have been detectable in approximately 90 and 70% of familial and sporadic MEN1 patients, respectively (5).

Germline mutation analysis of the MEN1 gene is a powerful tool for the definitive diagnosis of suspicious cases as well as recognition or exclusion of the predisposition to MEN1 in asymptomatic members at risk in the affected family. However, the conventional DNA test for MEN1 mutation, which analyses only exons and their flanking sequences amplified by polymerase chain reaction (PCR), often fails to detect causative mutations even in typical MEN1 patients (1,2,5). These mutation-negative patients may carry mutations in the untested part of the gene such as
the promoter region or introns. Large genomic deletions, which vary widely in size ranging from a single exon to a very large chromosomal region, can also cause MEN1, but would escape detection by conventional mutation analysis, because PCR products from the normal allele mask the deletion in the disease chromosome (6). Characterization of the cryptic mutations in these apparently mutation-negative patients is important in view of their potential diagnostic value.

We previously reported the first case of MEN1 caused by a large germline deletion of the MEN1 gene (6), and demonstrated that gene dose mapping successfully delimited the deletion boundaries and led to the determination of exact deletion breakpoints (7). Here we describe another family with typical MEN1 caused by a germline deletion of the whole MEN1 gene. The identification of the gene deletion contributed to the clinical decision making in this family. The deletion breakpoints in the present case were found to be within the Alu repetitive elements scattered around the MEN1 gene. This is the first report of the MEN1 gene deletion possibly mediated by recombination between Alu repetitive sequences.

SUBJECTS AND METHODS

SUBJECTS

The proband (Fig. 1, I.2) was a 58-year-old man with pituitary adenoma, gastrinomas, parathyroid hyperplasia and large lipomas on the back. He was operated on for a pituitary adenoma and received postoperative irradiation at the age of 41, and was re-operated on the following year. The diagnosis of Zollinger–Ellison syndrome was made when he was 42 years old, and the gastrinomas were resected. He showed hyperparathyroidism at the age of 57, for which subtotal parathyroidectomy was conducted. The pathological diagnosis was parathyroid hyperplasia. His 21-year-old son (II.1) had prolactinoma diagnosed and resected at the age of 20. He also showed hypercalcemia. A younger sister of the proband (I.4) had hyperparathyroidism and the parathyroid tumor was resected. An elder sister (I.1) and another son (II.2, 20 years old) exhibited no manifestation of MEN1. The parents of the proband had been deceased for decades. No further information was available about the parents.

Informed consent was obtained for MEN1 gene analysis from four family members: I.2, I.4, II.1 and II.2. This study was approved by the ethics committee for gene analysis research in the Mitsui Memorial Hospital and National Cancer Center.

CONVENTIONAL SEQUENCING ANALYSIS OF THE MEN1 GENE

All protein-coding regions of the MEN1 gene, which are encoded by exons 2–10 (Fig. 2), were amplified by PCR with a thermal cycler (Robocycler Gradient, Stratagene, La Jolla, USA) in a 50 or 100 μl reaction mixture containing 100–250 ng genomic DNA, Taq DNA polymerase (TaKaRa Taq, Takara Bio, Tokyo, Japan) and one of the following primer pairs: Men 2-1 and Men 2-2 for exon 2; Men 3-1 and Men 56-2 for exons 3–6; Men 7-1 and Men 8-2 for exons 7 and 8; Men 9-1 and Men 10-2 for exons 9 and 10. The primer sequences were described previously (8). The PCR conditions were as follows: 1 min at 95°C for 1 cycle; 1 min at 95°C, 1 min at 64°C for exon 2, 64°C for exons 3–6, 70°C for exons 7–8, or 68°C for exons 9–10, and 2 min at 72°C for 30–31 cycles; and 5 min at 72°C for 1 cycle. PCR products were purified with the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and subjected to nucleotide sequencing by the dideoxy chain termination method with the BigDye Terminator Cycle Sequencing kit (AppliedBiosystems, Foster City, USA) and analyzed by an automated genetic analyzer, ABI PRISM310 (AppliedBiosystems). Additional primers described previously (8) were also used for the sequencing analysis.

MICROSATELLITE LENGTH POLYMORPHISM ANALYSIS

Microsatellite length polymorphism around the MEN1 gene was analysed at the DNA marker loci D11S4940, D11S4946 and PYGM (Fig. 2) as described previously (6). Genomic DNA was amplified by PCR with a pair of primers, one of which was labeled with fluorescein. The PCR products were run on the automated genetic analyser, and their sizes were estimated by use of the GeneScan analysis software (AppliedBiosystems).

GENE DOSE MAPPING

The copy numbers of specific nucleotide sequences around the MEN1 gene were estimated by the previously reported gene dose assay utilizing double competitive PCR (6,7). The target sites U2, D1, D2, and D3 were the same as described by Kikuchi et al. (7) and the target site K119 was the same as described by Kishi et al. (6). The hMLH1 gene was used as the reference sequence as described previously (9). Each sample was amplified in duplicate, and the mean values were used for calculation of sequence copy numbers. The PCR
primers and internal standards used for this assay were described previously (6,7,9).

**PCR AND SEQUENCING OF THE DELETION BOUNDARIES**

PCR primers were designed within the region where the deletion breakpoints were mapped by the gene dose mapping (Fig. 2). The long-range PCR was conducted in a 50 μl reaction mixture containing 2.5 units of TaKaRa LA-Taq (Takara Bio), 200 ng genomic DNA, 0.5 μM each primer and components recommended by the enzyme supplier. Genomic DNA was amplified by PCR in a thermal cycler (Robocycler Gradient) under the following conditions: 30 s at 98°C for 1 cycle; 30 s at 98°C, 10 min or 5 min at 72°C for 30 cycles; 10 min at 72°C for 1 cycle. The primers were 24 or 25 base pairs (bp) in size. The position number of the 5′-end nucleotide assigned in the BAC clone 137c7 (GenBank accession number: AC000134) was used for the name of each primer.

Short DNA fragments (<4 kb) were amplified by PCR under the same conditions as MEN1 gene exons as described above except for annealing temperatures, which were adjusted for each target sequence. PCR products were separated by electrophoresis on 0.6–1.0% agarose gel and visualized by ethidium bromide staining. Patient-specific PCR products were purified from the gel by use of a DNA purification kit (UltraClean 15, Mo Bio Laboratories, Carlsbad, USA) and analysed by direct nucleotide sequencing.

**RESULTS**

**POLYMORPHISM ANALYSIS SUGGESTED MEN1 GENE DELETION**

The proband (Fig. 1, I.2) was a patient having tumors in all three principal MEN1-related endocrine tissues. In spite of the typical MEN1 phenotype, conventional sequencing analysis of the PCR-amplified protein-coding exons of the MEN1 gene revealed no causative germline mutation. As expected, his affected son (II.1) and asymptomatic son (II.2) also exhibited only normal MEN1 gene sequences. However, the comparison of their sequences revealed that the proband and the affected son did not share the same single nucleotide polymorphisms (SNPs) at codon 418 in exon 9 and codon 541 in exon 10, both of which were previously described as normal polymorphisms (3). The proband showed GAT at codon 418 and GCA at codon 541, both in the apparently homozygous state. In contrast, the affected son had single sequences GAC at codon 418 and ACA at codon 541 (Fig. 1). These findings suggested that the proband and his
affected son had a deletion mutation of the \textit{MEN1} gene resulting in the loss of at least exon 9 and exon 10 of the gene.

Microsatellite length polymorphism analysis revealed that \textit{D11S4940} and \textit{PYGM} markers showed heterozygosity in all four subjects examined (Fig. 3). In contrast, \textit{D11S4946} showed apparently homozygous patterns in all these subjects. However, the proband (I.2) and his affected son (II.1) did not share the identical peaks for \textit{D11S4946}, excluding the possibility of homozygosity at this site. Therefore, the \textit{D11S4946} marker site, which is located approximately 500 bp upstream from the transcription start point of the \textit{MEN1} gene (Fig. 2), was likely to be deleted. Together with the SNPs data, these findings suggested that the causative germline mutation in this family was a large deletion spanning at least from the promoter region to exon 10 of the \textit{MEN1} gene. Heterozygosity of \textit{D11S4940} and \textit{PYGM} indicated that these sequences were retained in the disease chromosome and therefore the deletion size was estimated to be at most 300 kb.

\textbf{Gene Dose Mapping}

In order to confirm the gene deletion and localize it more precisely, the sequence copy number in the proband was measured by the gene dose assay (6,7,9) at approximately 20-kb-upstream (U2) and 22-kb- (D2), 29-kb- (D3) and 39-kb-downstream (D1) sites from the gene as well as within exon 2 (K119) of the \textit{MEN1} gene (Fig. 2). The proband’s estimated sequence copy number of exon 2 was approximately 0.9 (normal value = 2.0) while all other sites measured (U2, D2, D3 and D1) showed almost normal values (2.2, 1.6, 1.8 and 1.7, respectively). Thus, gene dose mapping indicated the deletion of exon 2 and the retention of all other sites where the copy numbers were measured. The affected son and the proband’s sister also showed a half reduction of the exon 2 sequence (data not shown). These findings, together with the polymorphism data, indicated that the upstream breakpoint of the deletion was located between the 20-kb-upstream (U2) and the 500-bp-upstream (\textit{D11S4946}) sites of the \textit{MEN1} gene, and the downstream breakpoint was located between exon 10 (codon 541) and the 22-kb-downstream site (D2).

\textbf{Nucleotide Sequences of the Deletion Boundaries}

As a first step to obtain DNA fragments containing the deletion breakpoints, long-range PCR was conducted with primers designed to hybridize to the sequences at approximately 6-kb intervals within the region where the deletion breakpoints were mapped by the gene dose assay (Fig. 2). When the primer pair of 182044 and 143586 was used, a patient-specific PCR product of approximately 8.5 kb was generated (Fig. 4A). The primer pair of 176015 and 149248 did not generate patient-specific PCR products (data not shown), suggesting that the upstream deletion end was located between 182044 and 176015 (Fig. 2). Likewise, the primer pair of 182044 and 149248 did not generate patient-specific PCR products (data not shown), suggesting that the downstream deletion end was located between 149248 and 143586 (Fig. 2).

Next, short-range PCR was conducted with several primer pairs designed to hybridize to the regions where the upstream and downstream deletion ends were mapped by long-range PCR. A patient-specific PCR product of 800 bp was generated in all three patients by PCR with an upstream primer 177020 and a downstream primer 146736 (Fig. 4B).

Nucleotide sequencing analysis of the patient-specific fragments and comparison with the registered human genomic sequences (a BAC clone 137c7) revealed the deletion of the region between nucleotide positions 147205 and 176686 (Fig. 5). The deletion was approximately 29 kb in size, spanning from a 13-kb upstream site to a 9-kb downstream site of the \textit{MEN1} gene. The normal sequences spanning the two deletion breakpoints were highly homologous to each other. The homologous regions were approximately 300 bp and were identified as Alu repetitive elements (10) oriented in the same direction. As a result of the highly repetitive nature of the boundary sequences, the deletion breakpoints could not be determined precisely, but probably fell within an exactly identical 10-bp region within the Alu repeats (Fig. 5).

\textbf{Genetic Status of the Asymptomatic Son}

The 20-year-old asymptomatic son of the proband (II.2) was tested for the gene mutation. His SNPs at codon 418 and
codon 541 (Fig. 1) and microsatellite polymorphisms at D11S4940 and D11S4946 (Fig. 3) were indistinguishable from those of his affected father and, therefore, were not informative for the prediction of his genetic status. However, he did not share the PYGM peaks common to all three symptomatic subjects (Fig. 3). This finding suggested that he was a non-carrier of the mutation. The gene dose assay revealed that he showed the normal copy number of exon 2 (data not shown) while all other symptomatic individuals showed half-reduction of the sequences, confirming that he was not a deletion carrier. Finally, he was tested for deletion breakpoints. The deletion-specific PCR product was not observed with his genomic DNA (Fig. 4B). This result was consistent with that of the gene dose assay, and thus definitely excluded the possibility of MEN1 predisposition in the asymptomatic son.

**DISCUSSION**

This family presented a typical collection of MEN1-related tumors and was readily diagnosed as having the MEN1 trait. However, the causative germline mutation was not identified by the conventional mutation screening. The first evidence suggesting the MEN1 gene deletion in this family was the incompatible SNPs found in the proband and his affected son, which prompted us to analyse microsatellite polymorphisms around the MEN1 gene. These polymorphism analyses suggested the whole MEN1 gene deletion in the affected family members and its absence in the asymptomatic son. However, the evidence was not so definite at this point that we could rely on it in the clinical decision making, because, in linkage analyses, naturally occurring recombination between the gene and polymorphic markers may lead to wrong answers. Therefore, we proceeded to characterize the mutation more precisely. The demonstration of reduced gene dosage and the identification of deletion breakpoints in the affected members confirmed the family mutation as a large deletion. In addition, the normal gene copy number and the absence of the deletion breakpoints in the asymptomatic son definitely excluded the genetic predisposition to MEN1. Consequently, he could avoid the unnecessary and costly periodic tests for endocrine tumors, which would have been conducted for life if his genetic status remained unknown (1,2).

Large germline deletions of a tumor suppressor gene are thought to account for a significant portion of causative
mutations of familial cancer syndromes. Several different techniques for detecting deletions have been used, including Southern blot analysis (11), fluorescence in situ hybridization (12) and long-range PCR (13), but no single method is sufficient to identify a variety of large deletions. About 10 and 30% of the familial and sporadic MEN1 patients, respectively, are apparently mutation-negative cases, a portion of which is thought to be caused by large germline deletions of the MEN1 gene. However, such MEN1 gene deletions have rarely been reported (6,14,15). The lack of simple and sensitive detection techniques appears to have the apparently mutation-negative cases left unexplored for large deletions. We previously developed a gene dose assay of the MEN1 gene region and used it to identify deletion breakpoints in a family with MEN1 caused by a whole MEN1 gene deletion (7). We also developed a similar assay for the APC gene and verified its usefulness in the detection of the whole APC gene deletion (9). In the present study, the gene dose assay was used to identify the deletion breakpoints in another MEN1 family and successfully delimited the deletion boundaries, leading to the identification of the breakpoints.

The genomic deletion determined in this study was approximately 29 kb in length and spanned the whole MEN1 gene. Both boundaries of the deletion contained Alu repetitive sequences of approximately 300 bp (10). This is different from the previously described MEN1 gene deletion, the breakpoints of which showed only a 3-bp repeat sequence and were not flanked by interspersed repetitive elements. The repetitive sequences such as Alu elements have often been implicated in gene rearrangements (10). The large genomic deletion in the family presented here may have been generated by non-allelic homologous recombination between the Alu sequences. This is the first report demonstrating the involvement of Alu elements in the MEN1 gene deletion.

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References