A novel splice site mutation of the \textit{MEN1} gene identified in a patient with primary hyperparathyroidism

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\textbf{Abstract.} Heterozygous germline mutation of the tumor suppressor gene \textit{MEN1} is responsible for multiple endocrine neoplasia type 1 (MEN1), a familial cancer syndrome characterized by pituitary, parathyroid and enteropancreatic tumors. Various mutations have been identified throughout the entire gene region in patients with MEN1 and its incomplete forms often manifested as familial isolated hyperparathyroidism and apparently sporadic parathyroid tumor. Mutation analysis of the \textit{MEN1} gene is a powerful tool for the early diagnosis of MEN1; however, the clinical significance of the identified mutations is not always obvious. In this study, a previously unreported missense \textit{MEN1} mutation, c.824G>T was identified in a patient with primary hyperparathyroidism and evaluated for its pathogenicity. This mutation was predicted to generate a putative missense menin protein, R275M. A stability test of the menin protein demonstrated that the stability of R275M mutant was reduced only slightly as compared with wild type menin, and therefore could not preclude the possibility that it was a rare benign polymorphism. However, further analysis of leukocyte mRNA and minigene experiments indicated that the mutant c.824G>T allele gives rise to abnormally spliced menin mRNA, and thereby confirmed that c.824G>T mutation is causative for MEN1. Thus, leukocyte mRNA analysis has been demonstrated useful to identify a splicing mutation of the \textit{MEN1} gene.

\textbf{Key words}: MEN1, Menin, Splicing, Minigene, Stability

\textbf{MULTIPLE ENDOCRINE NEOPLASIA TYPE 1 (MEN1)} is a relatively rare autosomal dominantly inherited condition characterized by hyperplastic and neoplastic disorder of endocrine organs such as the parathyroid, anterior pituitary and gastroenteropancreatic endocrine tissues [1]. Primary hyperparathyroidism (PHPT) is the most common disorder, and is usually the initial manifestation in MEN1. Its prevalence in MEN1 patients during lifetime is nearly 100\%, and the average age of onset is during the third decade of life, which is much earlier than that of sporadic primary hyperparathyroidism [2, 3]. Anterior pituitary tumors are seen in 40-60\% of MEN1 patients. Among those, prolactinomas are the most common followed by nonfunctioning tumors and growth hormone producing tumors. Gastroenteropancreatic tumors develop in about 60\% of the patients and gastrinoma is the most frequent functioning tumor followed by insulinoma. Other manifestations include adrenal cortex adenomas, which are mostly nonfunctioning, foregut carcinoid tumors and cutaneous tumors.

Germline mutations of the causative gene, \textit{MEN1}, which is localized to human chromosome 11q13 and encodes a 610-amino acid nuclear protein, menin, can be identified in most of the affected subjects [4, 5]. To date, more than 500 different germline \textit{MEN1} mutations have been identified in patients with MEN1. The majority of mutations identified in affected subjects are nonsense and frameshift mutations, which predict premature protein truncations. Splice mutations and large deletions of the \textit{MEN1} gene have also been reported in several families.

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Menin shows no significant homology to other known proteins, and its physiological function is not yet fully understood. Moreover, despite its widespread expression, the molecular basis of its role in tissue-specific tumorigenesis remains elusive [6-10]. Generally, when a missense mutation is identified in an affected subject, examination of the physiological function of the encoded mutant protein is necessary to determine whether the mutation is indeed pathogenic. For menin, however, there are no established parameters that can adequately represent its diverse physiological functions. In the event that no functional assays are available, a linkage study within the affected family may be informative. However, in order to draw a reliable conclusion, it requires a number of affected subjects within the family [11-13], and such analysis is rarely performed in practice. There was a report of a mutation, which was initially considered pathogenic but later turned out to be a rare benign polymorphism [14]. Conversely, a missense mutation initially thought to be a rare polymorphism may be characterized later as a pathogenic mutation with low penetrance.

As is the case for the majority of other hereditary cancer-related genes, MEN1 is categorized as a tumor suppressor gene. Tumor occurrence by mutation of the MEN1 gene can be explained by Knudson’s two-hit theory [3]. In cells containing a heterozygous MEN1 mutation, the function of one allele has already been lost through a germline mutation and cells acquire a tumor phenotype when the function of the remaining wild-type allele is lost somatically during cell division. Indeed, in tumors from MEN1 patients, the wild-type allele is usually deleted and identified as loss of heterozygosity [15-17]. As a result, there should be no functioning menin protein in tumor cells arising in patients with MEN1 mutations.

Genetic analysis of a patient with PHPT revealed a previously unknown single nucleotide substitution in the MEN1 gene, c.824G>T, which can be interpreted as a missense mutation causing an amino acid substitution of arginine by methionine at codon 275. To determine whether the mutation is pathogenic, we examined the characteristics of mRNAs and protein encoded by the mutated MEN1 gene.

Case Presentation

A 33-year-old woman at the 24th week of gestation was referred to our department due to severe hypercalcemia (Ca 17.5 mg/dL). Based on a markedly elevated level of plasma intact PTH (1425 pg/mL, normal range; 10-65 pg/mL), a diagnosis of PHPT was made. Cervical ultrasonography and MRI revealed a large parathyroid nodule with cystic change. An enlarged parathyroid gland and right lobe of thyroid gland were surgically removed. The removed parathyroid tumor was 5.5 × 2.5 × 2.5 cm in size, and microscopically, chief cells were massively proliferated. After surgery, her serum calcium level normalized and intact PTH decreased to 19.2 pg/mL. Imaging studies for pituitary and enteropancreas performed after parathyroidectomy revealed no abnormal findings. Results of biochemical studies are summarized in Table 1. Based on the young age of onset of PHPT, genetic testing for the MEN1 mutation was proposed [18, 19]. Written informed consent was obtained from the patient before genetic testing. The full sequence of the coding region of the MEN1 gene showed a heterozygous single nucleotide substitution, c.824G>T (Fig. 1A). This nucleotide substitution occurred at the last nucleotide of exon 5, and if it does not affect splicing, this mutation was predicted to substitute amino acid codon 275 of menin from arginine (AGG) to methionine (ATG). Screening of family members revealed that her father, 68 years old, had hypercalcemia (10.9 mg/dL) and an increase in intact PTH level (125.3 pg/mL). Imaging studies revealed an enlarged parathyroid nodule, but he declined any treatment beyond regular screening. He refused genetic testing.

Materials and Methods

Stability analysis of variant menin

The intracellular stability of missense menin variants was evaluated using a quantitative fluorescent immuno-histochemical method as described previously [20, 21]. Briefly, WI38VA13 cells were transfected with a bicis-

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**Table 1 Results of biochemical studies**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH (ng/mL)</td>
<td>0.7</td>
</tr>
<tr>
<td>IGF-1 (ng/mL)</td>
<td>264</td>
</tr>
<tr>
<td>PRL (ng/mL)</td>
<td>10.8</td>
</tr>
<tr>
<td>Insulin (fasting)</td>
<td>5.0</td>
</tr>
<tr>
<td>Glucose (fasting)</td>
<td>88</td>
</tr>
<tr>
<td>Gastrin (pg/mL)</td>
<td>35</td>
</tr>
<tr>
<td>Glucagon (pg/mL)</td>
<td>68</td>
</tr>
</tbody>
</table>

GH, Growth Hormone; IGF-1, Insulin-like Growth Factor-1; PRL, Prolactin
Novel MEN1 splice mutation

The control plasmid expressing both FLAG- and Myc-tagged wild-type menin. As a known unstable control, L22R variant expression plasmids were used. The mean of analyzed nuclei number was 24 per transfection and the minimum was 9 per transfection. Mutant menin was located mainly in the nucleus although the cytoplasm was also faintly stained in some cells. Only nuclear staining was analyzed.

Analysis of menin mRNA in blood cells

RNA was isolated from whole blood with the LeukoLOCK™ total RNA isolation system (Ambion,
Austin, TX, USA), and treated with RNase-Free DNase set (QIAGEN, Hilden, Germany). cDNA was synthesized with oligo dT primer using SuperScript III (Invitrogen, Carlsbad, CA, USA). The cDNA was amplified by PCR with primers 3-3 (5’-acctggcagggcagcaagc-3’) and 7-3 (5’-gtagccagaggatcaggtgg-3’), which were designed on the basis of the sequences of exon 3 and exon 7 of the MEN1 gene, respectively. The PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The DNA fragments were excised from the gel and purified with UltraClean 15 DNA purification kit (MO BIO Laboratories, Carlsbad, CA, USA), then sequenced directly, or cloned into pCR2.1-TOPO TA vector (Invitrogen, Carlsbad, CA, USA) and sequenced with a BigDye terminators v1.1 cycle sequencing kit (Applied biosystems, Foster City, CA, USA).

**Sequencing analysis of tumor DNA**

Tumor DNA was extracted with DEXPAT™ (TAKARA BIO, Shiga, Japan) and amplified by PCR with primers 56-1 (5’-aaggacccgttctcctccctgt-3’) and 56-2 (5’-ggcccctgcctcagccactgttag-3’), which were designed on the basis of intron sequences upstream of exon 5 and downstream of exon 6, respectively. The PCR product was sequenced directly as described above.

**Minigene analysis of c.824G>T mutant**

DNA fragment containing the sequence between the 5’ end of exon 3 and 3’ end of exon 7 of the MEN1 gene was amplified by PCR with primers containing EcoRI or SalI recognition sites (5’-gaattcgcaccaaattggacagctccggtgtgg-3’ and 5’-gtgacactctgtctgcagcaagtcggtgtctcc-3’), using human genomic DNA (Clontech, Mountain View, CA, USA) as a template. The PCR products were cloned into pCR-Blunt II-TOPO vector (Invitrogen) prior to confirmation by nucleotide sequencing that the insert sequence was identical to the published MEN1 gene sequence (GenBank accession No. U93237), then excised and transferred to the mammalian expression vector, pCMV-Tag2 (Stratagene, La Jolla, CA, USA). A c.824G>T mutant minigene was constructed by introducing the mutation into the wild-type minigene using the QuikChange Site-Directed Mutagenesis kit (Stratagene).

Minigene was introduced into WI38VA13 cells with FuGENE6 (Roche Diagnostics, Indianapolis, IN, USA). Total RNA was extracted 24 hr after transfection with QIAshredder and RNeasy Mini kit (QIAGEN), treated with DNase, and subjected to cDNA synthesis as described above. The cDNAs were amplified by PCR with primers (5’-gattacaagcatgacgacgataag-3’ and 5’-ggcgaattgggtacacttacctgg-3’) designed to anneal to the 5’ and 3’ minigene-specific regions of the transcripts. The PCR products were separated on a 3% agarose gel, visualized by ethidium bromide staining, and excised and directly sequenced as described above.

These studies were approved by the Institutional Review Board of both the National Cancer Center Research Institute and Shinshu University School of Medicine.

**Results**

**Stability of variant menin R275M**

The intracellular stability of the putative products of the c.824G>T mutation, R275M was examined by comparing the relative expression levels of mutant vs. wild-type menin protein expressed from a bicistronic plasmid. The L22R mutant, a disease-causing mutation associated with typical MEN1, was used as a positive control for unstable menin. Two plasmids were constructed, one expressing FLAG-tagged wild type menin and Myc-tagged mutant menin, the other expressing FLAG-tagged mutant menin and Myc-tagged wild type menin. Using either construct, the stability test showed that the stability of the R275M mutant was not significantly different from that of wild type menin (Fig. 1 B, C). The stability of the R275M mutant suggests that the c.824G>T mutation may not cause MEN1 if its primary effect was the amino acid substitution [21].

**Menin mRNA in blood cells of the patient with c.824G>T mutation**

Given that the c.824G>T mutation occurred at an exon-intron junction, this mutation could act as a splicing mutation rather than a simple missense mutation. The menin mRNA of the patient was therefore examined for evidence of abnormal mRNA splicing. The PCR products were separated on a 3% agarose gel, visualized by ethidium bromide staining, and excised and directly sequenced as described above.
Novel MEN1 splice mutation

Fig. 2  MEN1 mRNA in the patients with c. 824G>T mutation
A. The patient’s blood cell cDNA was amplified with primers on MEN1 exons 3 and 7, and separated on agarose gel (Case) along with that from a normal subject (N). The PCR product of the normal size (open triangle) and abnormal PCR products (solid triangles) were excised and subjected to either direct sequencing (normal fragment) or sequencing after cloning (690-bp, 480-bp and 360-bp abnormal fragments). M: size marker. B. Direct sequencing of the normal-sized cDNA fragment analyzed with a sequencing primer on exon 5. The mutated sequence at the 3’ end of exon 5 was not detectable. C. Structures of normal-sized, 690-, 480- and 360-bp cDNAs. Open boxes and closed boxes indicate exons and unspliced introns, respectively. V-shaped lines below each diagram indicate the splicing events that give rise to each mRNA. Normal-sized cDNA contained only the wild type sequence (AG) at the exon-intron junction while the 690- and 480-bp cDNAs contained only the mutant sequence (AT). The positions of the PCR primers used are shown above as arrows. D. mRNA from the wild type (WT) and c.824G>T mutant (Mut) minigenes. The structures of PCR products a, b, c and d identified on agarose gel (left) were analyzed by sequencing and shown in the right. The wild type and mutant sequences at the 3’ end of exon 5 is shown as AG and AT, respectively. Thick lines represent minigene-specific regions of the transcripts where PCR primers anneal (F and R, shown by arrows).
480 bp and 690 bp were cloned and sequenced (Fig. 2A, C). The 360-bp fragment lacked exon 5; the 480-bp fragment contained an unspliced 80-bp intron sequence following the mutated exon 5; and the 690-bp fragment contained a 210-bp intron sequence following exon 3 as well as the previously observed 80-bp intron sequence following the mutated exon 5. Similar intron retention between exons 3 and 4 in menin mRNA induced by a distant splicing mutation has been reported previously [22]. These findings suggest that the c.824G>T mutation causes aberrant mRNA splicing, and that all detectable menin mRNA splicing variations potentially cause protein truncation by frame-shift or a cryptic stop codon within unspliced intron sequence.

**Minigene analysis of c.824G>T mutation**

The effect of the c.824G>T mutation on mRNA splicing was examined by minigene experiments (Fig. 2D). The wild type minigene construct generated a normally spliced transcript containing all of exons 3-7 and a splicing variant which lacked exon 6. The mutant construct generated a transcript lacking exon 5 and its variant which lacked both exons 5 and 6, and failed to generate a normally spliced transcript. The deletion of exon 6 in the transcripts of both constructs may be a consequence of artificial gene structure and experimental conditions. These findings strongly suggest that normally spliced mRNA is not generated from the c.824G>T mutant allele of the patient.

**Loss of wild type allele of the MEN1 gene in parathyroid tissue obtained from a patient with c.824G>T mutation**

We next examined whether the wild type allele is lost by a second hit in the tumor cells of a patient with c.824G>T mutation. DNA was isolated from tumor cells as described in the Materials and Methods and sequenced. As shown in Fig. 3, only the mutant allele was detectable in tumor cells, confirming the loss of the wild type allele.

**Discussion**

Identification of the MEN1 gene in 1997 enabled early diagnosis of MEN1 even when patients had developed only a single tumor [4]. Moreover, early or presymptomatic diagnosis of at risk relatives became possible. In the case of frameshift mutation, nonsense mutation or large deletion, it is relatively straightforward to consider those lesions as pathogenic because MEN1 gene is a tumor suppressor gene. However, when identified mutations are missense mutations or in-frame deletions, molecular diagnosis of MEN1 is not so simple, since the pathogenicity of these mutations is not clear *per se*. Furthermore, when the mutation exists near exon-intron junction, possible deleterious effects of the mutation on splicing have to be considered. Indeed, pathogenic aberrant splicing caused by point mutations are often overlooked as routine genetic testing examines only genomic DNA [23].

In the present report, we examined the pathogenicity of nucleotide substitution of the MEN1 gene which exists at the last nucleotide of the exon 5. Using analysis of leukocyte mRNA and minigene experiments, our present study clearly demonstrated that the c.824G>T mutation is a splice site mutation causing protein truncation, rather than a missense mutation. Because of nonsense-mediated mRNA decay, it is often difficult to detect aberrantly spliced mRNAs transcribed from a mutant tumor suppressor gene in leukocytes. Nevertheless, the leukocyte mRNA analysis in our case proved useful in demonstrating a splicing mutation of the MEN1 gene. Analysis of the MEN1 mutation database revealed that 9% and 14% of MEN1 germline mutations identified in patients with MEN1 and familial isolated hyperparathyroidism, respectively, were splice mutations [5]. Also in our recent report on
Japanese patients with MEN1, 5.6% (10/180) of germline MEN1 mutations were splice mutations [24]. However, evidence of aberrant splicing has not always been demonstrated.

In our case, the patient had PHPT but no other MEN1-related tumors. Screening of family members revealed that her father also had PHPT. Since her father declined any further examination, it is unknown whether he had other MEN1-related diseases. Results of our mRNA analysis gave us a rationale to survey the patient with the same protocol as that for patients with typical MEN1.

Menin is considered to function as a scaffold protein for other cellular proteins, and its physiological function appears to be diverse including regulation of cell cycle, transcription, DNA repair, chromatin remodeling, and apoptosis [6-10]. Tissue-specific regulation of endocrine function and cellular proliferation by menin has also been reported [25-29]. There have been studies that examined molecular and physiological function of menin, but these studies examined only specific functions among diverse roles of menin and none of the methods used in these reports are capable of evaluating the function of menin as a whole. In this regard, lack of wild type protein in tumor cells may be the most reliable information which suggests pathogenicity of the mutation. In our present study, we could clearly demonstrate that tumor cells have only mutant allele (Fig. 3), and that mutant allele does not produce normally spliced mRNA, indicating no functional menin protein in tumor cells (Fig. 2).

In conclusion, we examined the pathogenicity of novel nucleotide substitution in the MEN1 gene identified in a patient with PHPT using a menin stability test and analysis of menin mRNA. Our results clearly demonstrated that the mutation, c.824G>T, is indeed pathogenic.

Acknowledgments

This work was supported by the Grant-in-Aid from the Ministry of Health, Labor and Welfare for the 3rd-term Comprehensive 10-Year Strategy for Cancer Control and National Cancer Center Research and Development Fund (21-8-6 and 23-A-11), and the Grant from the Ministry of Health, Labour and Welfare for Research on intractable diseases (H22-Nanchi-Ippan-105). S. S. was a recipient of the research resident fellowship from the Foundation for Promotion of Cancer Research, Japan.

Disclosure Summary

All authors have nothing to disclose.

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