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 tumors. However, the clinical significance of MEN1 gene variants, especially missense and in-frame mutations as well as some splicing mutations, is not always obvious. We have previously shown that mutant menin proteins associated with MEN1 are rapidly degraded by the ubiquitin-proteasome pathway. We also demonstrated by a fluorescent immunocytochemical stability test that the stability of missense and in-frame deletion mutants varies widely but that unstable mutants were found only in MEN1 and related disorders and not in normal polymorphisms. In the present study, we evaluated by this stability test the pathogenicity of a novel MEN1 missense mutation, c.1118C>T, encoding a P373L mutant menin, identified in a suspected MEN1 patient. The results demonstrated that the mutant menin is highly unstable, indicating that this mutation is causative for MEN1. These findings encouraged us to proceed with presymptomatic genetic screening for this mutation among the family members, which resulted in the identification of asymptomatic mutation carriers. Thus, the information from the menin stability test was useful for genetic diagnosis and counseling of MEN1 in the case with a previously unreported MEN1 missense mutation.

Key words: MEN1, Menin, Stability, Missense mutation
other known proteins and its physiological function is not fully understood, there are no established parameters that can adequately represent impaired function of mutant menin [10-14].

We previously reported that missense mutant menin proteins associated with MEN1 are unstable and rapidly degraded through ubiquitin-proteasome pathway [15]. More detailed analysis by a newly developed fluorescence immuno-cytochemical method revealed that the stability of missense and in-frame deletion mutants varies widely but that unstable mutants were found only in MEN1 and related disorders and not in normal polymorphisms [16]. We recently encountered a suspected MEN1 patient with a previously unreported missense mutation in the MEN1 gene. To assess the pathogenicity of this mutation, we examined the stability of the menin protein encoded by this mutant MEN1 gene.

**Case Presentation**

A 56-year-old woman was referred to Shinshu University Hospital. She had been diagnosed with primary hyperparathyroidism (PHPT) and undergone parathyroidectomy when she was 45 years old. Three enlarged glands were removed but the fourth gland was not found. She had been followed-up before being referred to us. An abdominal CT scan identified multiple contrast-enhanced nodular lesions (3-12 mm in diameter) in her pancreas, based upon which she was suspected as having MEN1. She was eucalceemic but her plasma PTH level was elevated (II-2, Table 1). Other biochemical studies including fasting plasma levels of gastrin, insulin and glucose, and glucagon revealed no abnormalities. Pancreas tumors were thus considered nonfunctioning. MRI imaging for pituitary gland revealed no abnormal findings and plasma levels of prolactin and IGF-1 (insulin-like growth factor I) were within normal range. Genetic testing of the patient, performed after obtaining written informed consent, revealed a heterozygous single nucleotide substitution (c.1118C>T) in the MEN1 gene, which was predicted to substitute amino acid codon 373 of menin from proline (CCC) to leucine (CTC). This mutation has neither been reported [3] nor registered to mutation database (The Human Gene Mutation Database, http://www.hgmd.cf.ac.uk/ac/index.php).

Her 82-year-old mother had a history of PHPT and had undergone a single gland parathyroidectomy at the age of 69. She is currently eucalceemic (Table 1) and is receiving no medication except oral antidiabetic drugs for her type 2 diabetes. Periodic surveillance including imaging studies for pituitary and abdomen and biochemical and endocrine function tests are performed at another hospital and no MEN1-related diseases have been identified. Genetic analysis revealed that she also had the same mutation.

**Table 1** Serum and plasma concentrations of biochemical parameters of P373L mutation carriers

<table>
<thead>
<tr>
<th>Age (year)</th>
<th>Sex</th>
<th>Calcium (mg/dL)</th>
<th>Phosphate (mg/dL)</th>
<th>Intact PTH (pg/mL)</th>
<th>Prolactin (ng/mL)</th>
<th>IGF-1 (ng/mL)</th>
<th>Glucose (mg/dL)</th>
<th>Insulin (μU/mL)</th>
<th>Gastrin (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-2**</td>
<td>F</td>
<td>10.0</td>
<td>2.7</td>
<td>57</td>
<td>9.1</td>
<td>ND</td>
<td>188</td>
<td>24.0</td>
<td>77</td>
</tr>
<tr>
<td>II-2**</td>
<td>F</td>
<td>9.9</td>
<td>2.7</td>
<td>119</td>
<td>12.2</td>
<td>193</td>
<td>99</td>
<td>7.0</td>
<td>39</td>
</tr>
<tr>
<td>III-1</td>
<td>M</td>
<td>10.0</td>
<td>3.9</td>
<td>85</td>
<td>6.5</td>
<td>215</td>
<td>82</td>
<td>9.7</td>
<td>52</td>
</tr>
<tr>
<td>III-2</td>
<td>M</td>
<td>9.6</td>
<td>3.4</td>
<td>32</td>
<td>14.1</td>
<td>ND</td>
<td>86</td>
<td>8.5</td>
<td>102</td>
</tr>
<tr>
<td>III-3</td>
<td>M</td>
<td>10.6</td>
<td>3.1</td>
<td>64</td>
<td>12.2</td>
<td>ND</td>
<td>105</td>
<td>6.1</td>
<td>84</td>
</tr>
</tbody>
</table>

* I-2 has diabetes and is receiving medication. Insulin and glucose of I-2 were measured 2 hours after meal. ** Proband. ND, not determined. IGF-1, insulin-like growth factor I.
Unstable missense menin protein

the proband and her mother were undertaken at other hospitals more than 10 years ago.

Materials and Methods

The intracellular stability of missense menin variants was evaluated using a quantitative fluorescent immunohistochemical method as described previously [15, 16]. Briefly, WI38VA13 cells were transfected with a bicistronic plasmid expressing N-terminal FLAG-tagged and Myc-tagged proteins: one protein was wild type menin, which served as an internal control for transfection efficiency, and the other was the variant menin to be tested. Forty eight hours after transfection, expressed proteins were stained with FITC-labeled anti-FLAG antibody and Cy3-labeled anti-c-myc antibody, and quantified by fluorescence microscopic digital photography and an image analysis software. The ratios of the mean numerical value of fluorescence intensity for mutant menin to that for wild type menin in each nucleus was calculated, and normalized by the ratio obtained from the control plasmid expressing both FLAG- and Myc-tagged wild-type menin. As a known unstable control, L22R variant expression plasmids were used. Mutant menin was located mainly in the nucleus although the cytoplasm was also faintly stained in some cells. Only nuclear staining was analyzed.

To measure the degradation rate of menin proteins, 293T cells were transfected with plasmids expressing FLAG-tagged menin, and 28 hr after transfection, 20 μg/mL of cycloheximide (CHX) was added into the culture medium to prevent further protein synthesis. Whole-cell lysates were prepared from samples taken at 0 hr (control) and 6 hr after adding CHX, and analyzed by Western blotting with an alkaline phosphatase-conjugated anti-FLAG monoclonal antibody coupled with CDP-Star reagent. The membranes were exposed to X-ray films, and density of the target bands were scanned with a densitometer. 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 P373L

The intracellular stability of the putative products of the c.1118C>T mutation, P373L, was examined by comparing the relative expression levels of mutant vs. wild-type menin proteins 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 for each mutant, 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 test showed that stability of the P373L mutant was comparable to that of the L22R mutant (Fig. 1A and 1B).

To confirm that the lowered protein level of the mutant was due to rapid protein degradation, the effects of CHX on the amounts of menin proteins were analyzed. The results demonstrated the rapid reduction of P373L mutant after 6-hr treatment with CHX, while the amount of the wild type menin was almost unaffected (Fig. 1C). These findings suggest that the c.1118C>T mutation is likely a pathogenic mutation causing MEN1.

Presymptomatic genetic testing for offspring of the proband

We confirmed an association between the mutation and phenotype in the elder generation of the family (generations I and II, Fig. 2) before offering presymptomatic genetic testing for her offspring. II-1 and II-3 did not have c.1118C>T mutation, and no abnormal findings were found by biochemical and imaging studies. Genetic testing of three sons (III-1,2,3, Fig. 2) was then performed and revealed that they all had c.1118C>T mutation. Although they were asymptomatic, biochemical screening indicated that they had early stage endocrine abnormalities, consistent with results of genetic testing. III-1 was eucalcemic but intact PTH level was above the normal range. Prolactin level of III-2 was slightly elevated, and III-3 had hypercalcemia with unsuppressed PTH (Table 1). Although observed biochemical changes were subtle and imaging studies failed to detect any abnormalities in either individual, it is likely that they had already developed the disease. Indeed, in contrast to sporadic PHPT, a significant proportion of PHPT developed in MEN1 patients show marginal biochemical abnormalities [17]. Future surveillance for three sons was thus warranted.
Fig. 1 Stability of missense mutant menin
Mutant menin protein was coexpressed with wild type menin in culture cells by transfection of a bicistronic plasmid vector expressing either FLAG-tagged wild type and Myc-tagged mutant menin (A) or FLAG-tagged mutant and Myc-tagged wild type menin (B). The relative expression levels of mutant to wild type menin were compared with those of control plasmid expressing FLAG-tagged and Myc-tagged wild type menin proteins (Wt). Degradation rate of menin proteins was evaluated by CHX experiments (C). The open and closed bars indicate the control and CHX-treated samples, respectively. The data are expressed as relative values, with the control levels of each menin protein being a hundred per cent. The thin bars represent standard error of the mean of three independent transfection experiments. P373L and L22R represent the missense menin mutant identified in this study and that previously reported to cause typical MEN1, respectively. NS, not statistically significant ($P > 0.05$).

Fig. 2 Pedigree of the patient with P373L mutation
E+, have had genetic analysis and P373L mutation was identified; E-, have had genetic analysis and P373L mutation was not identified; P, proband; P-NET, pancreas neuroendocrine tumor
Unstable missense menin protein and stability of missense menin tested in various non-endocrine as well as in endocrine cells [15, 16]. We are also aware that the stability of menin missense mutants is highly variable and that some mutants associated with typical MEN1 are comparatively stable [16, 19]. Therefore, the pathogenicity of a missense mutation giving rise to a stable mutant menin should be interpreted cautiously.

In conclusion, we examined the pathogenicity of novel nucleotide substitution in the \textit{MEN1} gene using a menin stability test. Our results strongly suggest that c.1118C>T mutation is pathogenic. The future collection of data on the stability of missense menin protein will be of value in understanding the molecular pathogenicity of menin variants.

Discussion

In the present study, we examined stability of the mutant menin protein identified in a family with MEN1. Our case had PHPT with involvement of multiple glands and pancreas endocrine tumors. Her mother also had a history of PHPT, but she was diagnosed with PHPT at the age of 69 and only one gland was affected. Her mother has remained eucalcemic for 13 years since single gland parathyroidectomy and no other endocrine diseases had developed to date, which is an atypical clinical course of MEN1. Therefore, we were cautious to conclude that the c.1118C>T missense mutation was pathogenic based only on the segregation pattern. Although an association of a different mutation P373S at the same codon with typical MEN1 had previously been reported [18], there are a number of examples that different amino acid substitution at the same codon exerts different clinical consequences.

Our present study demonstrated that the P373L missense menin protein is highly likely pathogenic as this protein is apparently unstable compared to wild-type menin. This finding encouraged us to offer presymptomatic genetic testing for her sons, which resulted in early diagnosis of the disease. Since the menin stability test we established focuses on the stability of protein rather than its specific function, it enables a more comprehensive verification of pathogenicity of mutant menin. It might be argued that our \textit{in vitro} method, which quantitates proteins in fibroblast-derived culture cells, may not reflect menin stability in endocrine cells. However, we have previously demonstrated an apparent correlation between the clinical phenotype and stability of missense menin tested in various non-endocrine as well as in endocrine cells [15, 16]. We are also aware that the stability of menin missense mutants is highly variable and that some mutants associated with typical MEN1 are comparatively stable [16, 19]. Therefore, the pathogenicity of a missense mutation giving rise to a stable mutant menin should be interpreted cautiously.

In conclusion, we examined the pathogenicity of novel nucleotide substitution in the \textit{MEN1} gene using a menin stability test. Our results strongly suggest that c.1118C>T mutation is pathogenic. The future collection of data on the stability of missense menin protein will be of value in understanding the molecular pathogenicity of menin variants.

Disclosure Summary

All authors have nothing to disclose.

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