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.

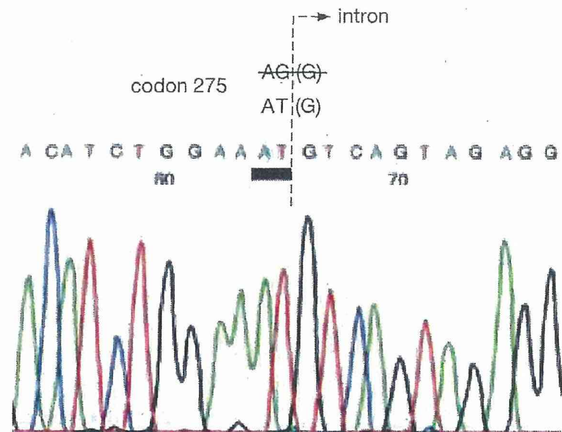

Fig. 3 Loss of the wild type allele of the *MEN1* gene in the parathyroid gland obtained from a patient with c.824G>T mutation
DNA isolated from parathyroid tissue was amplified and sequenced. Note that only mutant allele was seen in the tumor. Compare the sequence to that shown in Fig. 1A.

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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ORIGINAL

Application of an intracellular stability test of a novel missense menin mutant to the diagnosis of multiple endocrine neoplasia type 1

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Abstract. Germline *MEN1* mutation analysis is a powerful tool for an early diagnosis of multiple endocrine neoplasia type 1 (MEN1), an autosomal dominant familial cancer syndrome characterized by the parathyroid, 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

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]. Germline mutation of the causative gene, *MEN1*, which encodes 610 amino-acid residue nuclear protein menin, can be identified in the most of the affected subjects [2, 3]. *MEN1* is a tumor suppressor gene and tumorigenesis in MEN1 by *MEN1* gene mutations can be explained by Knudson's two-hit theory [4], i.e., function of one allele is lost by a germline mutation and the inactivation of the remaining wild type allele by somatic mutation leads to tumor development.

The majority of mutations identified in affected subjects are nonsense and frameshift mutations. Splice mutations and large deletion of the *MEN1* gene have also been reported in several families [3]. It is obvious that these mutations cause loss of function of the gene and are pathogenic. On the other hand, when a novel missense mutation or an in-frame deletion or addition is identified, molecular diagnosis of MEN1 is not so simple since the pathogenicity of these mutations is not clear *per se*. Although 26% and 48% of germline *MEN1* mutations associated with MEN1 and familial isolated hyperparathyroidism, respectively, are missense mutations or in-frame deletions [3], evidence for the pathogenicity of these mutations was lacking in many cases [5-9]. As menin shows no significant homology to

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

		I-2*	II-2**	III-1	III-2	III-3	Normal range
Age (year)		82	57	31	28	24	
Sex		F	F	M	M	M	
Calcium	(mg/dL)	10.0	9.9	10.0	9.6	10.6	8.8-10.1
Phosphate	(mg/dL)	2.7	2.7	3.9	3.4	3.1	2.7-4.0
Intact PTH	(pg/mL)	57	119	85	32	64	10-65
Prolactin	(ng/mL)	9.1	12.2	6.5	14.1	12.2	< 12.7
IGF-1	(ng/mL)	ND	193	215	ND	ND	37-266 (II-2) 85-369 (III-1)
Glucose	(mg/dL)	188	99	82	86	105	70-109
Insulin	(μU/mL)	24.0	7.0	9.7	8.5	6.1	1.84-12.2
Gastrin	(pg/mL)	77	39	52	102	84	< 200

* 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

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 immunocytochemical 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 eucalcemic 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 eucalcemic (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.

Tumor specimen was not available as surgeries for

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.

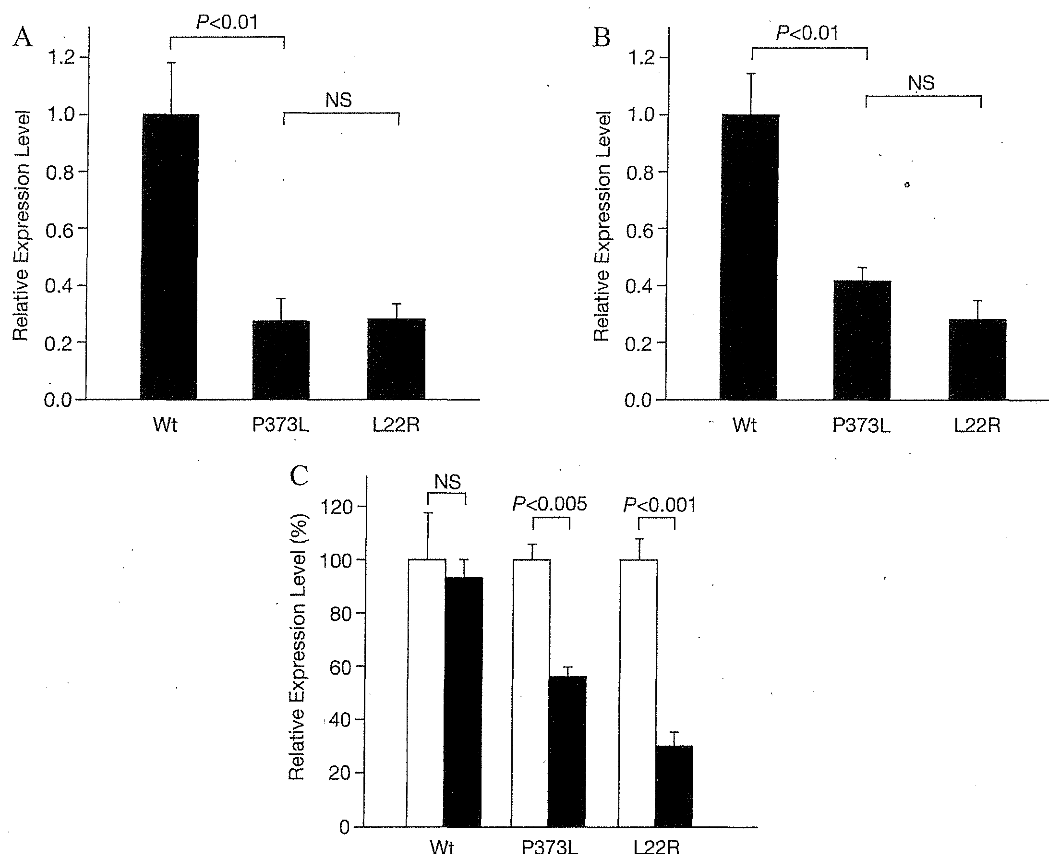

Fig. 1 Stability of missense mutant menin
Mutant menin protein was coexpressed with wild type menin in culture cells by transfection of a bicistronic plasmid vector expressing either FLAG-tagged wild type and Myc-tagged mutant menin (A) or FLAG-tagged mutant and Myc-tagged wild type menin (B). The relative 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 ($P>0.05$).

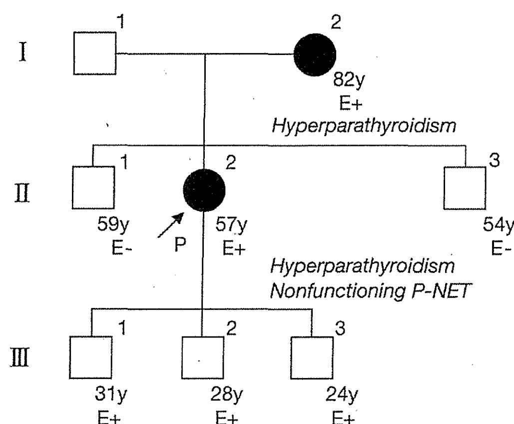

Fig. 2 Pedigree of the patient with P373L mutation
E+, have had genetic analysis and P373L mutation was identified; E-, have had genetic analysis and P373L mutation was not identified; P, proband; P-NET, pancreas neuroendocrine tumor

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 *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 *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.

Acknowledgments

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ORIGINAL ARTICLE

An analysis of genotype–phenotype correlations and survival outcomes in patients with primary hyperparathyroidism caused by multiple endocrine neoplasia type 1: the experience at a single institution

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Abstract
Purpose To examine the clinical characteristics and survival outcomes of patients with primary hyperparathyroidism (PHPT) in multiple endocrine neoplasia type 1 (MEN1) in relation to the *MEN1* gene mutation.
Methods The study population included the patients, positive for the *MEN1* gene mutation, who underwent parathyroidectomy between 1983 and 2009 at a single tertiary referral center. Manifestations of the syndrome, other tumors and causes of death were retrospectively correlated with the specific types and locations of *MEN1* gene mutations.
Results Thirty-two patients from 19 families were diagnosed as having MEN1 on genetic examinations. Mutations were most common in exons 2, 7 and 10. A phenotypic analysis of the main MEN1 tumor types among the 32 patients revealed that PHPT was the most common (100 %), followed in order by pancreatic neuroendocrine tumors (PNETs) (53 %) and pituitary tumors (38 %). Death due to MEN1-related disease occurred in five patients (16 %), including malignant PNET in three cases (exons 2, 3), pituitary crisis in one case (exon 2) and thymic cancer in one case (large deletion).
Conclusions Premature deaths related to MEN1 are due to the development of malignant PNET, pituitary crisis or thymic tumors associated with mutations in exons 2, 3 and a large deletion.

Keywords Multiple endocrine neoplasia type 1 · Primary hyperparathyroidism · Genotype–phenotype analysis

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Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal-dominant disease characterized by tumors of the parathyroid, pancreas and pituitary. Its prevalence has been estimated to be 1–10 per 100,000 individuals [1]. The clinical manifestations of MEN1 include hyperparathyroidism (over 90 % of cases), pancreatic islet cell tumors (30–80 % of cases) and pituitary tumors (15–50 % of cases) [1, 2]. Other complications such as facial angiofibromas, lipomas, carcinoids and adrenal tumors have also been identified [1, 2].

The locus of the gene responsible for MEN1 was assigned to the long arm of chromosome 11 in 198 [3, 8] and the gene was isolated by positional cloning in 1997 [4]. *MEN1* is a tumor-suppressor gene spanning 9 kb and containing 10 exons. It encodes the 610-amino-acid protein known as menin [5].

Investigators have attempted to correlate various mutations of *MEN1* with clinical manifestations or patient survival; however, no significant correlations have emerged. We herein present an analysis of the genotype–phenotype expression and genotype–prognosis correlations based on long-term observation of MEN1 patients who underwent