cess, the timelines for the presentation of amenorrhea, the detection of pituitary tumors and the diagnosis of MEN1 were evaluated in individuals with amenorrhea (Fig. 4a). The interval between age at symptom appearance and that at tumor detection tended to be longer when the tumors were identified above the age of 20. The interval between age at tumor identification and that at MEN1 diagnosis seemed to be also longer after the patients had reached the age of 15 (Fig. 4b). The other symptoms had similar diagnostic intervals.

The correlation between age at the time of detection of tumors and diagnostic intervals was also examined. The interval of peptic ulcers–parathyroid tumors or GEPNETs, and that of urolithiasis–parathyroid tumors were positively correlated with age at tumor detection, meaning that the later age at tumor detection is, the greater the interval of symptom–tumor is. The interval of tumor–MEN1 was negatively correlated with age at tumor detection in the case of fasting hypoglycemia alone, partly because the age at the diagnosis of MEN1 was earlier than that of finding of GEPNETs by detection of other MEN1-related tumors (Table 4).

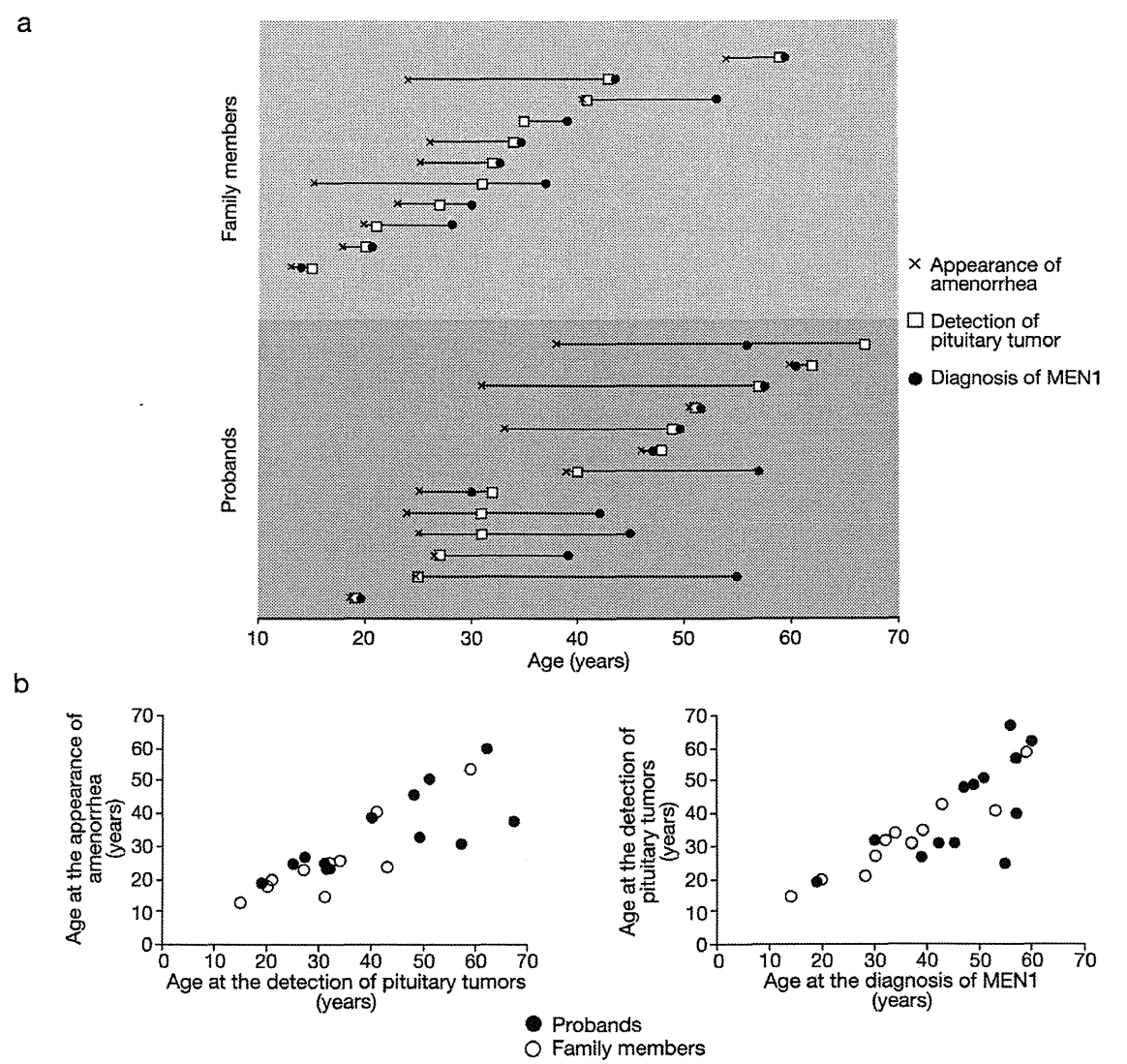

Fig. 4 Diagnostic intervals in individuals with amenorrhea
(a) The timelines for the appearance of amenorrhea, the detection of pituitary tumors and the diagnosis of MEN1 in each patient. (b) The relationship between age at the detection of pituitary tumors and that at the appearance of amenorrhea or between age at the diagnosis of MEN1 and that at the detection of tumors.

Table 4 Correlation between age at tumor detection and diagnostic intervals

Diagnostic intervals	No. of patients	r_s	*p* value
Peptic ulcers–parathyroid tumors	56	0.267	0.047
Parathyroid tumors–MEN1		0.031	0.822
Peptic ulcers–GEPNETs	51	0.465	<0.001
GEPNETs–MEN1		-0.263	0.062
Urolithiasis–parathyroid tumors	79	0.304	<0.001
Parathyroid tumors–MEN1		-0.131	0.251
Fasting hypoglycemia–GEPNETs	37	0.262	0.117
GEPNETs–MEN1		-0.415	0.011
Bone fracture/loss–parathyroid tumors	20	-0.283	>0.050
Parathyroid tumors–MEN1		-0.038	>0.050
Amenorrhea–pituitary tumors	23	0.410	>0.050
Pituitary tumors–MEN1		-0.395	>0.050

r_s : rank correlation coefficient

Discussion

The present study is a nationwide investigation of the diagnosis of MEN1 and MEN1-related endocrine tumors using the clinical data of more than 500 Japanese patients with MEN1. In the present study, initial symptoms appeared in 50% of patients by the fourth decade of life. Parathyroid tumors were the most frequent manifestation and hence the symptoms related to HPT, such as urolithiasis, peptic ulcers, and bone fracture/loss, were frequent in symptomatic cases. As for the diagnosis of three main tumor types and MEN1, the mean age at diagnosis was in the fifth decade of life, supporting previous reports that the mean age at diagnosis was in the fourth to fifth decade of life [4, 10-18].

In 1991, a factual investigation of 106 Japanese patients with MEN1 was conducted [19]. The first clinical manifestations were symptoms associated with HPT such as nephrolithiasis (32%), pituitary tumors (26%), peptic ulcers (28%) and hypoglycemia (13%), which appeared at an average age of 34.4 years in familial MEN1 and 33.3 years in sporadic cases. However, the mean age at MEN1 diagnosis was 46.2 years in familial cases and 41.3 years in sporadic ones, indicating that many years elapsed before diagnosis of MEN1 depending on the symptoms. There is little difference between these results and our data, indicating that MEN1 diagnosis is still delayed, i.e. little or no progress has been made in early diagnosis.

Delayed diagnosis of MEN1 has also been observed in other countries. A study in the U.S. showed that the mean age at onset of urolithiasis to be 26.9 years, which is 8.1 years earlier than that in the present study, whereas the mean time elapsing from the appearance of this symptom to the diagnosis of MEN1 was 17.2 years [12]. Another study in the same country also found that mean onset of the first clinical signs or symptoms occurred 7.6 years before diagnosis of MEN1 [11]. In an analysis of three apparently unrelated Brazilian MEN1 clusters, the age at diagnosis of HPT was 40.68 years on average although the first episode of renal calculi occurred at an average age of 23.14, which is approximately 12 years earlier than that in the present study [13]. Moreover, in a study in the Netherlands, the median interval between the occurrence of the first manifestations and MEN1 diagnosis was 9.5 years [14].

The present analysis raises two considerable and debatable issues regarding the presentation of initial symptoms and the timing of diagnosis between probands and family members for the early diagnosis of MEN1. One is that early recognition of symptoms does not necessarily contribute to early diagnosis of tumors and subsequent MEN1. In other words, typical symptoms linked to MEN1 diagnosis are overlooked. The time-to-diagnosis of MEN1 depending on amenorrhea due to pituitary tumors was strikingly prolonged despite the recognition of symptom at younger age. Also, in the case of fasting hypoglycemia, early diagnosis of GEPNETs and subsequent MEN1 was not necessarily made although the diagnostic interval of GEPNETs–MEN1 was negatively correlated with age at tumor identification. In patients with peptic ulcers from GEPNETs, tumor diagnosis was obviously delayed though MEN1 diagnosis was made before or shortly after tumor identification.

For patients with parathyroid tumors, urolithiasis, bone fracture or loss, and peptic ulcers were the symptoms leading to MEN1 diagnosis soon after the tumors were found. From these facts, it is supposed that the interval between the onset of symptoms and tumor detection may be prolonged in part because some patients did not wish to consult a doctor by themselves in spite of having symptoms. Moreover, it is also assumed that family members may not have sufficient knowledge of the disease from which the probands are suffering.

The second issue is that both the mean age of appearance of typical symptoms and the mean age at diagnosis of parathyroid and pituitary tumors or MEN1 were significantly lower in family members than in probands, whereas the mean age at diagnosis of GEPNETs was not. This finding may partly reflect the earlier recognition of symptoms in family members. It is natural that family members can more rapidly identify symptoms based on probands' experiences and genetic analysis. However, in our data, like the two other tumor types, there was no significant difference in diagnostic intervals of GEPNETs between probands and their family members. It has been previously demonstrated that GEPNETs were found at a younger age and with shorter delay between symptom presentation and tumor diagnosis in familial cases than in sporadic ones [20]. Hence, our data suggest that prospective screening and surveillance strategies for GEPNETs might be inadequate for family members.

Considering the circumstances mentioned above, we infer that the important factors causing delayed diagnosis and inadequate tumor screening are on the side of health care professionals. Indeed, Christopoulos *et al.* [12] have cited a lack of further examination beyond the scope of standard urological investigation in patients with urolithiasis by urologists and emphasized the responsibility of urologists suspecting HPT to appropriately refer patients for formal endocrinology consultations. It should be recognized that medical practitioners, such as physicians and co-medical staff, may insufficiently understand the characteristics of MEN1 and related disorders. In addition, the genetic testing of MEN1 for family members before the manifestation of tumors is not general in our country and a special screening strategy for family members has not been clearly shown in the existing guideline for management of MEN1. Given these facts, for appropriate screening of related endocrine tumors, we should consider improved sharing of medical information on patients and their family members among medical practitioners who examine the same patients and provide not only probands but also the family members with sufficient information of MEN1.

That said, we recognize that there are limitations of the collection and analysis of our data. The clinical data were collected with both qualitative and quantitative questionnaires, some portions of which were less informative. In the case of patients having a long history of MEN1, there is the possibility of fragmentary data collection since they may change physicians and/or hospitals. In fact, not all data were necessarily complete and thus we had to extract only the analyzable data.

In conclusion, there is a long interval not only between the appearance of initial symptoms and the diagnosis of MEN1-related endocrine tumors but also between tumor detection and MEN1 diagnosis, resulting in delayed diagnosis of MEN1 and related endocrine tumors. The symptoms of MEN1 related diseases can first be diagnosed and treated by urologists, gastroenterologists, and gynecologists, among other specialist. However, there may exist a lack of acknowledgement of MEN1 and related endocrinopathy and insufficient sharing of medical information on the same patients and their family members among involved medical practitioners. Providing family members with information of MEN1 is also insufficient. Further steps should be taken not only to promote understanding of MEN1 and prepare an environment for optimal medical information sharing among medical professionals but also to accelerate the education of family members by health care professionals.

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Disclosure

The authors have nothing to disclose.

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Clinical features of insulinoma in patients with multiple endocrine neoplasia type 1: analysis of the database of the MEN Consortium of Japan

Akihiro Sakurai[1), 2)], Masanori Yamazaki[2)], Shinichi Suzuki[3)], Toshihiko Fukushima[3)], Tsuneo Imai[4)], Toyone Kikumori[4)], Takahiro Okamoto[5)], Kiyomi Horiuchi[5)], Shinya Uchino[6)], Shinji Kosugi[7)], Masanobu Yamada[8)], Izumi Komoto[9)], Kazuhiro Hanazaki[10)], Masatsune Itoh[11)], Tatsuya Kondo[12)], Masatomo Mihara[13)] and Masayuki Imamura[9)]

[1)] *Department of Medical Genetics, Shinshu University School of Medicine, Matsumoto 390-8621, Japan*
[2)] *Division of Diabetes, Endocrinology and Metabolism, Department of Internal Medicine, Shinshu University School of Medicine, Matsumoto 390-8621, Japan*
[3)] *Department of Breast, Endocrine and Thyroid Surgery, Fukushima Medical University, Fukushima 960-1295, Japan*
[4)] *Department of Breast and Endocrine Surgery, Nagoya University Hospital, Nagoya 466-8550, Japan*
[5)] *Department of Endocrine Surgery, Tokyo Women's Medical University, Tokyo 162-8666, Japan*
[6)] *Noguchi Thyroid Clinic and Hospital Foundation, Beppu 874-0932, Japan*
[7)] *Department of Medical Ethics/Medical Genetics, Kyoto University School of Public Health, Kyoto 606-8501, Japan*
[8)] *Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, Maebashi 371-8511, Japan*
[9)] *Department of Surgery, Kansai Electric Power Company Hospital, Osaka 553-0003, Japan*
[10)] *Department of Surgery, Kochi Medical School, Kochi University, Nankoku 783-8505, Japan*
[11)] *Department of Pediatrics, Kanazawa Medical University, Uchinada 920-0293, Japan*
[12)] *Department of Metabolic Medicine, Kumamoto University Faculty of Life Sciences, Kumamoto 860-8556, Japan*
[13)] *Department of Molecular Endocrinology and Metabolism, Tokyo Medical and Dental University, Tokyo 113-8519, Japan*

Abstract. More than 50% of patients with multiple endocrine neoplasia type 1 (MEN1) develop gastroenteropancreatic neuroendocrine tumors (GEPNETs), and insulinoma is the second most common functioning GEPNET. Compared to other functioning and nonfunctioning GEPNETs in MEN1, insulinoma is considered to develop at a younger age. To clarify the clinical features of insulinoma developed in Japanese patients with MEN1, a recently constructed database of Japanese MEN1 patients was analyzed. Among 560 registered cases, insulinoma was seen in 69 patients and information on age at diagnosis was available for 54 patients. Tumors predominantly occurred in the body and tail of the pancreas. The mean age at diagnosis of insulinoma (34.8 ± 16.7 yrs) was significantly younger than that of gastrinoma (50.6 ± 14.3 yrs) and nonfunctioning tumor (44.7 ± 13.3 yrs) in patients with MEN1. Patients diagnosed as having insulinoma during middle-age (30 - 49 yrs) tended to have a long period from appearance of hypoglycemic symptoms to diagnosis of the tumor. Of note, 13 patients (24%) were diagnosed as having insulinoma before 20 yrs of age. Such young onset was not seen in other GEPNETs. Since the development of GEPNETs during adolescence is quite rare, insulinoma diagnosed before 20 yrs strongly suggests the presence of MEN1 and warrants further investigation, including *MEN1* genetic testing. Also, clinicians should be aware that insulinoma can often be missed in middle-aged patients.

Key words: Multiple endocrine neoplasia type 1 (MEN1), Database, Adolescence, Hypoglycemia

MULTIPLE ENDOCRINE NEOPLASIA TYPE 1 (MEN1) is an autosomal dominantly inherited endocrine tumor syndrome characterized by tumor development in various endocrine- and non-endocrine organs such as parathyroid hyperplasia, gastroenteropancreatic neuroendocrine tumor (GEPNET), anterior pituitary adenoma and adrenal cortex adenoma [1, 2]. Less frequent tumors include cutaneous tumor, lipoma, and thymic- and bronchial neuroendocrine tumors. MEN1 is clinically diagnosed by confirming neoplastic diseases in at least two of the commonly affected organs, namely, parathyroid, endocrine pancreas and anterior pituitary [3]. For family members of the patient, the

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Correspondence to: Akihiro Sakurai, M.D., Ph.D., Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan. E-mail: aksakura@shinshu-u.ac.jp

presence of one lesion is sufficient to establish a diagnosis. Most subjects with MEN1 carry a heterozygous germline mutation in the *MEN1* gene, which localizes to chromosome 11q13 [4]. The *MEN1* gene consists of 10 exons and encodes the 610 amino-acid nuclear protein menin [5]. Based on data on the incidence of primary hyperparathyroidism (PHPT) and other epidemiological studies, the prevalence of MEN1 in western countries has been estimated to be about 2-3 cases/100,000 individuals [6].

PHPT is the most common, and usually the initial manifestation of MEN1 [7]. Its prevalence during lifetime for gene carriers is nearly 100% and the average age of onset is during the third decade of life, about 30 years earlier than sporadic primary hyperparathyroidism [8].

GEPNET is the second most common endocrine manifestation in MEN1 after PHPT, and its prevalence is about 50 - 60% [3, 9, 10]. Since many patients with PHPT remain asymptomatic, GEPNET can be the initial manifestation that leads to the diagnosis of MEN1 in up to 50% of patients [11, 12]. The most common form of functioning GEPNET in MEN1 is gastrinoma, which exists predominantly in the duodenal mucosa. Other forms of functioning tumors seen in MEN1 include insulinoma, glucagonoma, somatostatinoma and VIPoma. Nonfunctioning tumors are also common and usually multiple in MEN1.

Among GEPNETs in MEN1, insulinoma is considered to develop during early age [1, 9]. Compared to other MEN1-related GEPNETs, insulinoma occurs more often in patients younger than 40 years old, and therefore could be the initial manifestation. Accordingly, early recognition of insulinoma could lead to early diagnosis of MEN1 in patients.

To ascertain the clinical features and current management of MEN1, and to provide information useful to clinicians facing difficulties in managing MEN1 patients, we established a MEN study group in 2008 designated the "MEN Consortium of Japan", and constructed a database of Japanese patients with MEN [10]. Here we report clinical features of insulinoma in patients with MEN1 who were registered in the database of the MEN Consortium of Japan.

Patients and Methods

We analyzed the clinical data of patients with MEN1 collected by the MEN Consortium of Japan. Procedure of data collection and general characteristics of patients with MEN1 have been recently reported [10]. Physicians and surgeons in Japan reported clinical information of their MEN1 patients according to questionnaires provided by the MEN Consortium. The study was reviewed and approved by the Institutional Review Board of Shinshu University School of Medicine.

After verification of registered data, 560 cases were considered eligible for the analysis. Data on patients with GEPNETs were extracted from the database and the clinical features were analyzed. Because of the retrospective design, the diagnostic criteria of MEN1-related tumors could have varied between hospitals. Thus we judged appropriateness of diagnosis of insulinoma by confirmation of inappropriate hypersecretion of insulin, amelioration of clinical symptoms after surgery, and results of immunostaining of the resected tumor. When diagnosis of insulinoma was uncertain or registered information was insufficient, data was verified by directly communicating with attending physician of each patient. Results are expressed as mean ± SD. Since most of our data was not normally distributed, significance of differences was determined by Mann-Whitney-Wilcoxon test or Fisher's exact test wherever appropriate. $p < 0.05$ was considered significant.

Results

General features of insulinoma in MEN1

As previously reported [10], GEPNETs were diagnosed in 314/536 patients (58.6%), excluding 24 patients diagnosed by presymptomatic genetic testing. Insulinoma was diagnosed in 69 patients, among whom 41 patients had clinically proven hypoglycemia or clinical histories symptomatic of hypoglycemia prior to diagnosis. Asymptomatic hypoglycemia was identified during initial work-up for MEN1 or during surveillance in majority of other patients.

Information on localization of the tumor was available for 41 patients. Only three (7%) patients had insulinoma in the pancreas head and 30 (73%) patients developed insulinoma in the distal portion of pancreas. Eight (20%) patients had multiple insulinomas existing in both the head and body-tail region of the pancreas. Information on the tumor size was obtained from 29 patients and ranged from 1.5 mm to 94 mm (25.4 ± 19.5 mm).

Surgical resection of insulinoma was performed for 91% (63/69) of patients, a proportion significantly higher than that for gastrinoma (52%) or for nonfunctioning tumor (34%). Operative procedures are summarized in

Table 1. Given that the majority of insulinoma occurred in the body or tail of the pancreas, distal pancreatectomy is the most commonly selected procedure, followed by enucleation of the tumor. Localization of the tumor was not provided for 28 patients, which probably reflects incomplete clinical record such as loss of old chart or change of hospital during clinical course. Indeed, 9 patients underwent distal pancreatectomy, indicating that they had insulinoma in the pancreas tail. It is possible that exact localization of the tumor was unable to determine in some patients because the tumor was too small to detect by imaging study. Reoperation was performed for 8 patients due to metachronous tumor development.

Information on pathological diagnosis was given for 38 patients. Diagnosis of well-differentiated endocrine tumor and well-differentiated endocrine carcinoma was made for 18 and 1 patients, respectively. In other 19 patients, tumor was pathologically diagnosed as adenoma. No tumors were pathologically diagnosed as either endocrine cancer or poorly differentiated endocrine carcinoma. One patient with "malignant insulinoma" was registered, but pathological information was not given.

Age at diagnosis of insulinoma

Ages at diagnosis of insulinoma, gastrinoma and nonfunctioning tumor are summarized in Table 2 and Fig. 1. Since nonfunctioning tumors frequently accompany other functioning tumors, patients with nonfunctioning tumors only are included in the "nonfunctioning tumor" group. Patients included in the "insulinoma" and "gastrinoma" groups may have other functioning- or nonfunctioning tumors. For example, patients having both insulinoma and gastrinoma are included in both groups.

The average age at diagnosis of GEPNETs in Japanese patients with MEN1 was 44.7 ± 15.6 years, about 10 years younger than sporadic patients [13, 14]. When compared based on function of the tumor, insulinoma in MEN1 was diagnosed significantly earlier than gastrinoma and nonfunctioning tumor in MEN1 (Table 2). In insulinoma, there was no discernible peak age of diagnosis and it varied widely from the second to sixth decade of life. 33/54 (61%) patients were diagnosed before age of 40 (Fig. 1), consistent with a previous report [9]. In the database of MEN Consortium of Japan, 16 patients developed GEPNETs before age 20 yrs, 13 of which had insulinoma. The other two patients had nonfunctioning tumors, and information on the function of the tumor was not available in one patient.

Mean ages at first appearance of hypoglycemic symptoms and at diagnosis of insulinoma are shown in Fig. 2. Insulinoma was diagnosed within 1 year from the onset of hypoglycemic symptoms in about 50% of patients. In a significant number of patients however, a long period - up to 30 years in one case - elapsed between the appearance of symptoms and the diagnosis of insulinoma. The delay in diagnosis was particularly evident in patients diagnosed with insulinoma between age 30 and early 50s and was observed in both probands and family members. The delay in diagnosis of insulinoma is well known and the mean duration of symptoms at diagnosis has been reported as 3 - 4 years [15, 16], while the age dependence of the delay in diagnosis has not previously been demonstrated.

Table 1 Surgical procedure undertaken for insulinoma in patients with MEN1

procedure	number of patients	(%)
distal pancreatectomy	20	32
tumor enucleation	14	22
distal pancreatectomy plus tumor enucleation	5	8
total pancreatectomy	2	3
others	2	3
unknown	20	32

Table 2 Age at diagnosis of GEPNETs registered with the MEN Consortium of Japan database

	insulinoma	gastrinoma	NF tumor	insulinoma *vs.* others	
				vs. gastrinoma	*vs.* NF tumor
all (yr [a]) (range)	34.8 ± 16.7 (8-68)	50.6 ± 14.3 (20-78)	44.7 ± 13.3 (19-74)	$p < 0.001$	$p = 0.001$
proband (yr [a]) (range)	37.6 ± 19.0 (9-68)	47.9 ± 13.4 (20-74)	47.9 ± 12.0 (19-74)	$p = 0.017$	$p = 0.068$
family member (yr [a]) (range)	29.3 ± 13.0 (8-47)	56.2 ± 14.4 (33-78)	40.8 ± 14.1 (22-71)	$p < 0.001$	$p = 0.022$
proband *vs.* family member	$p = 0.136$	$p = 0.030$	$p = 0.038$		

[a], values are mean age ± SD. NF tumor, nonfunctioning tumor

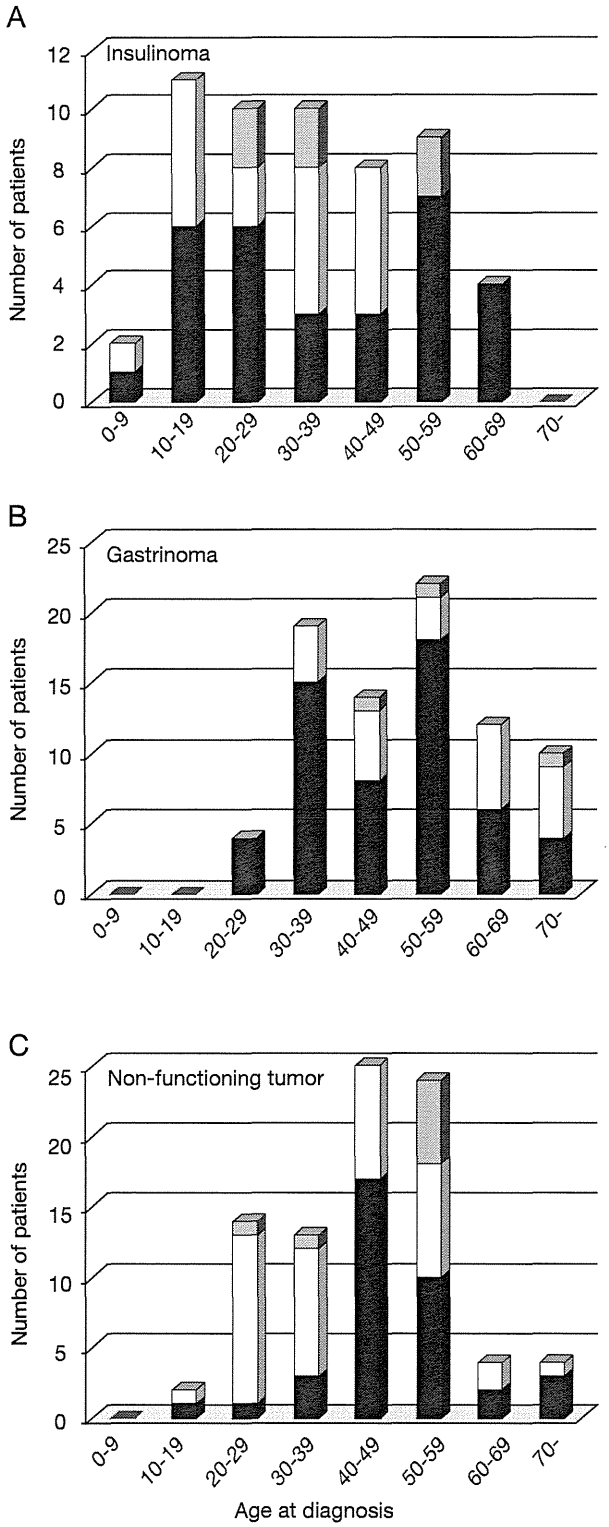

Fig. 1 Age at diagnosis of GEPNETs in patients with MEN1 Number of patients who developed insulinoma (A), gastrinoma (B) and non-functioning tumor (C) and their age at diagnosis is shown. Closed bar, open bar and gray bar represent proband, family member and patient without information of family history, respectively.

Age dependence of clinical features

Patients with insulinoma could be categorized into three groups according to their age at diagnosis of insulinoma: (1) a younger group diagnosed before age 30 with a short symptomatic period before diagnosis; (2) a middle-aged group with a lengthy symptomatic period before diagnosis, and (3) an aged group who had a brief or non-existent symptomatic period before diagnosis. A comparison of the three groups is summarized in Table 3. Only patients whose age at diagnosis of insulinoma was available are shown. In the younger group, 13 patients (57%) were probands, that is, no family members had been previously diagnosed with MEN1 when insulinoma was detected in these individuals. In the middle-aged group, 6/18 (33%, $p = 0.19$ *vs.* younger group) individuals were probands. In the aged group, 11 patients (85%, $p = 0.03$ *vs.* younger group) were probands. Information on family history was not available for the other two patients. 51 patients (94%) had undergone surgery followed by pathological confirmation of insulinoma. Due to the development of multiple tumors, the majority of patients in the middle-aged- and aged groups had undergone partial pancreatectomy, while enucleation of the tumor was performed in 13 of 23 patients (57%) in the younger group. The proportion of patients who had undergone enucleation was significantly higher in the younger group compared to that of the middle-aged group (2/18, 11%, $p < 0.01$ *vs.* younger group) but was not statistically different from that in the aged group (4/13, 31%, $p = 0.18$ *vs.* younger group). The age at diagnosis of PHPT increased along with increasing age at diagnosis of insulinoma, reflecting the fact that PHPT and insulinoma were simultaneously identified in many of middle-aged- and aged group patients. The status of the *MEN1* gene had been analyzed in most patients and pathologic germline mutations were identified in all but 3 patients (mutation positive rate 92.5%).

Discussion

In the present study, we used a recently established database of Japanese MEN1 patients [10] to analyze the clinical features of insulinoma in these patients. After gastrinoma, insulinoma is the second most frequent functioning GEPNET in MEN1, and is often the first symptom which patients with MEN1 experience. The average age of onset of insulinoma is younger than other MEN1-related GEPNETs and it

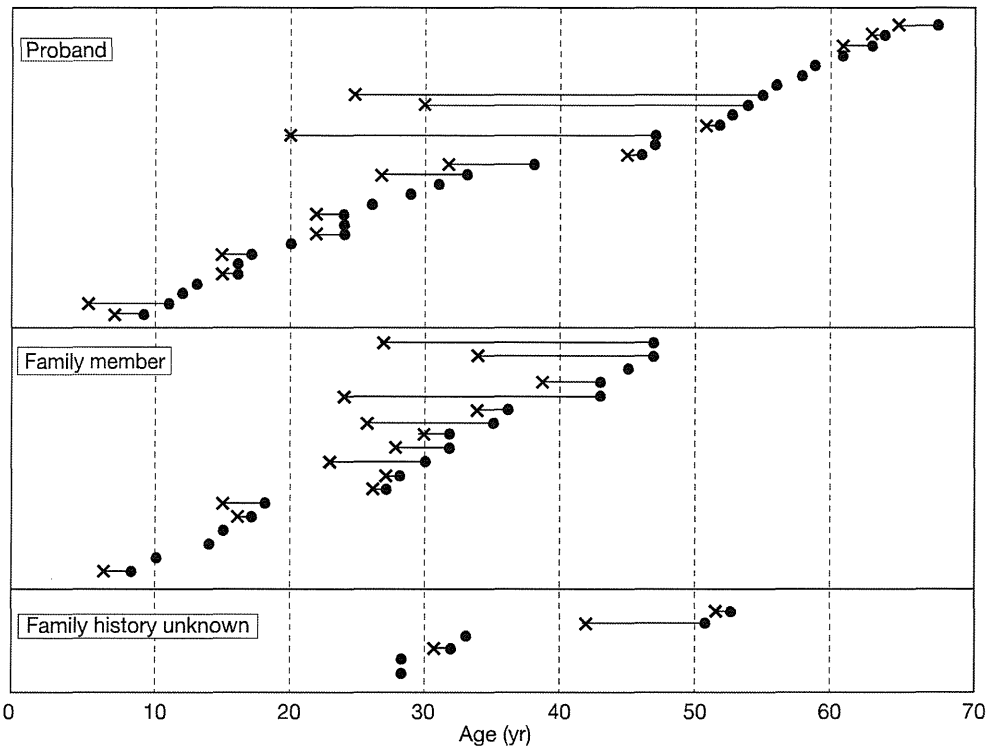

Fig. 2 Age at appearance of hypoglycemic symptom and diagnosis of insulinoma in patients with MEN1
Age at appearance of hypoglycemic symptoms and diagnosis of insulinoma in each patient was shown. Each closed circle represents a single patient and aligned according to the age at diagnosis of insulinoma. X represents age at appearance of hypoglycemic symptoms or confirmation of hypoglycemia, and is shown only if insulinoma was diagnosed more than one year after the appearance of hypoglycemic symptoms.

Table 3 Comparison of patients with insulinoma diagnosed at different age

age at diagnosis of insulinoma	before 30 yrs (younger group)	30-49 yrs (middle-aged group)	50 yrs or older (aged group)
number of patients	23	18	13
sex male/female	12/11	4/14	6/7
family history proband/family member/unknown	13/8/2	6/10/2	11/0/2
age at diagnosis of insulinoma (yr[a]) (range)	19.0 ± 6.9 (8-29)	38.7 ± 6.7 (30-47)	57.4 ± 5.3 (51-68)
duration between appearance of symptom and diagnosis (yr[a]) (range)	1.1 ± 1.6 (0-6)	6.8 ± 8.0 (0-27)	5.5 ± 9.9 (0-30)
surgery for insulinoma[b] total/partial/enucleation/unknown	1/7/13/4	0/11/2/4	1/7/4/2
recurrence of insulinoma Yes/No (%)	4/19 (17%)	1/17 (6%)	0/13 (0%)
PHPT Yes/No (%) age at diagnosis (yr[a]) (range)	23/0 (100%) 28.9 ± 11.8 (11-54)	17/1 (100%) 40.5 ± 9.9 (24-62)	12/1 (92%) 57.2 ± 4.7 (50-62)
pituitary tumor Yes/No (%) age at diagnosis (yr[a]) (range)	8/15 (35%) 24.8 ± 7.5 (11-35)	12/6 (67%) 41.7 ± 11.1 (27-62)	5/8 (38%) 53.0 ± 16.1 (25-64)
MEN1 mutation Yes/No /Not done	18/1/4	10/1/7	8/1/4

Data do not include patients whose age at diagnosis is not known.
[a], values are mean age ± SD. [b], When patients underwent partial pancreatectomy and enucleation of the tumor simultaneously, both procedures were counted independently.

often occurs before adolescence [1, 9, 17, 18]. In the MEN Consortium database, 13% of probands and 7% of affected family members had symptoms related to hypoglycemia before the diagnosis of MEN1.

Insulinoma was detected at a variety of ages before 70 years old and there was no apparent peak age of diagnosis (Fig. 1). It is particularly notable that a significant proportion of patients was diagnosed before age 20 yrs, which is in clear contrast to gastrinoma and nonfunctioning GEPNET; which were rarely if ever diagnosed before age 20 yrs. Interestingly, when the timing of appearance of hypoglycemia or suspected symptoms was closely investigated, many patients who were diagnosed with insulinoma during middle-age (30 - 49 years) had manifested hypoglycemic symptoms much earlier (Fig. 2), suggesting earlier development of insulinoma. It is rather surprising that 7/18 patients diagnosed between age of 30 to 49 had either a hypoglycemic episode or had clinical histories symptomatic of hypoglycemia before age 30 yrs. The reason for this delay of diagnosis is not the subject of the present study, but it is possible that nonspecific symptoms and/or a relatively indolent clinical course may have delayed the point at which those patients visited hospital. Social factors such as work or family affairs may also have detracted from their willingness to visit hospital. The disproportionate female predominance among patients in the middle-aged group may reflect such social factors (Table 3). This issue should be further clarified and may provide a clue to earlier diagnosis of insulinoma. Moreover, symptoms caused by hypoglycemia may have been misdiagnosed as other disorders, such as psycho-neurological diseases [19-21].

In contrast to the other age groups, patients whose insulinoma was detected after age 50 had distinct clinical features. These patients were predominantly probands, and insulinoma was simultaneously diagnosed with PHPT. 5/13 patients (38%) had not experienced hypoglycemic symptoms before diagnosis. The exact age of onset of insulinoma in these aged patients is unknown, but it is reasonable to assume that their insulinomas had developed earlier in their life but were asymptomatic for a long period. Indeed, small- and clinically silent insulinomas are often not diagnosed until incidentally found at autopsy [22].

An interesting question is whether insulinoma develops earlier than other GEPNETs in MEN1 or is simply diagnosed earlier than other tumors because the clinical symptoms of insulinoma tend to appear earlier. Diagnosis of insulinoma may be relatively straightforward once hypoglycemia with elevated level of plasma insulin is confirmed. However, because of the nonspecific nature of hypoglycemic symptom such as fatigue, confusion, weakness or behavioral change, the duration of the symptomatic period before diagnosis can be lengthy [23]. In patients with nonfunctioning tumors, age at diagnosis among probands showed a peak during the 40s, while those tumors were frequently found at a younger age in family members (Fig. 1C), suggesting that a long period had elapsed before the nonfunctioning tumors in probands were identified. In contrast, the age at diagnosis of gastrinomas in family members was unexpectedly older than that of the probands (Table 2). This may be attributable to the fact that gastrinoma in MEN1 is usually diagnosed by clinical symptoms and/or elevated plasma levels of gastrin, and not by imaging studies [11].

Recently, Ito *et al.* reported results of a nationwide epidemiological surveillance of GEPNETs in Japanese [14]. They estimated the total number of patients treated for pancreas NETs in the year 2005 was 2,845 (95% CI 2,455-3,507). 10% of patients with pancreas NET and 14% of patients with insulinoma had MEN1, a proportion higher than other studies which reported the proportion of MEN1 among patients with insulinoma as 4-8% [24-26]; this may be due in part to a relatively high prevalence of insulinoma in Japanese patients with MEN1 [10]. The MEN Consortium database confirmed 314 patients with GEPNETs, most of whom are currently alive and under follow-up. It is likely that many patients with MEN1 with GEPNETs have been registered on both GEPNET surveillance and the MEN1 database.

According to Ito *et al.*, only 1% of patients with GEPNETs were diagnosed before age of 20 years [14]. Although they did not show age-related prevalence of each functioning tumor, our present results suggest a high probability of the presence of MEN1 in patients with insulinoma diagnosed at young age, especially before 20 years old. Clinical guidelines for MEN1 released in 2001 recommended *MEN1* genetic analysis for all patients with gastrinoma, based on the fact that 25% of gastrinoma patients have MEN1 [3]. We propose that *MEN1* gene analysis should be offered to patients who have developed insulinoma before age of 20 years, even if they have no other MEN1-related diseases or family history of MEN1. Indeed, since most subjects with *MEN1* mutation do not man-

ifest hyperparathyroidism or pituitary tumors before the age of 20 years, biochemical- and imaging studies for the purpose of surveillance of MEN1 will be of limited clinical utility. Among 13 patients who developed insulinoma before age of 20 years, only 4 patients manifested other MEN1-related diseases before the age of 20 years.

In conclusion, we analyzed the clinical features of insulinoma in patients with MEN1. While insulinoma occurs in a wide range of age groups, development of the tumor in younger patients was characteristic. Insulinoma before age of 20 years may be a strong indication of presence of MEN1, and detailed surveillance of MEN1 is warranted in these cases. Clinicians should also be aware that insulinoma can often be missed in middle-aged patients.

Disclosure of Interest

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

Yuko Nagamura[1], Masanori Yamazaki[2], Satoko Shimazu[1], Toshihiko Tsukada[1] and Akihiro Sakurai[2, 3]

[1] *Division of Familial Cancer Research, National Cancer Center Research Institute, Tokyo 104-0045, Japan*
[2] *Division of Diabetes, Endocrinology and Metabolism, Department of Internal Medicine, Shinshu University School of Medicine, Matsumoto 390-8621, Japan*
[3] *Department of Medical Genetics, Shinshu University School of Medicine, Matsumoto 390-8621, Japan*

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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Correspondence to: Akihiro Sakurai, M.D., Ph.D., Department of Medical Genetics, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621 Japan.
E-mail: aksakura@shinshu-u.ac.jp

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.

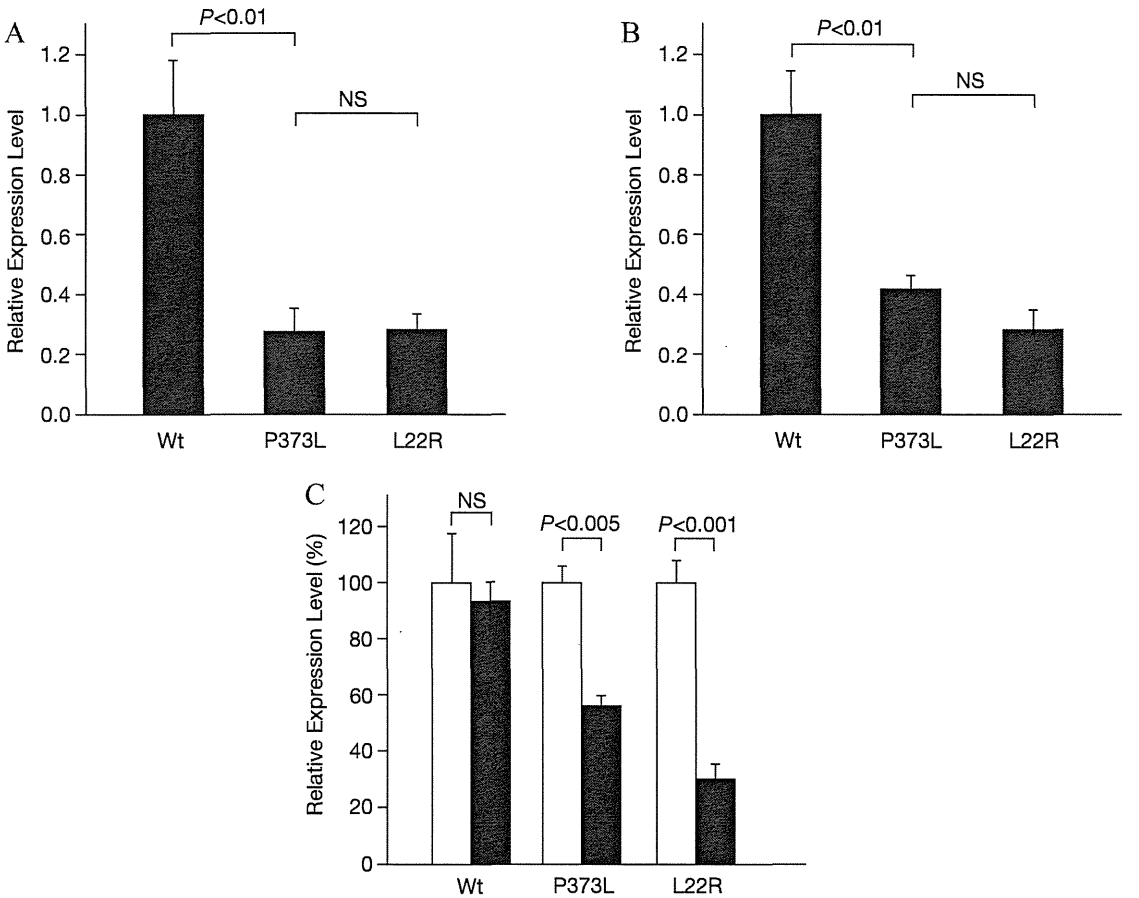

Fig. 1 Stability of missense mutant menin
Mutant menin protein was coexpressed with wild type menin in culture cells by transfection of a bicistronic plasmid vector expressing either FLAG-tagged wild type and Myc-tagged mutant menin (A) or FLAG-tagged mutant and Myc-tagged wild type menin (B). The relative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

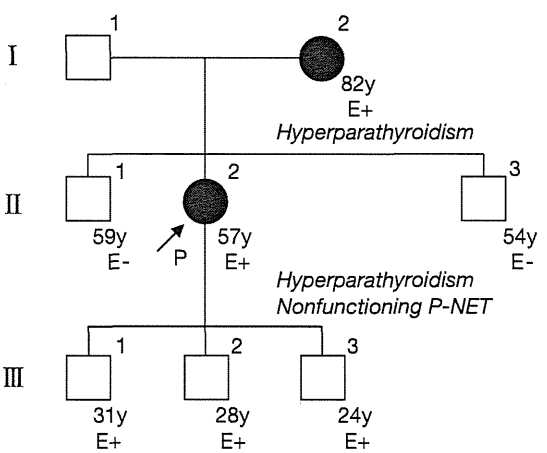

Fig. 2 Pedigree of the patient with P373L mutation
Open square, male; Solid circle, female; E+, have had genetic analysis and P373L mutation was identified; E-, have had genetic analysis and P373L mutation was not identified; P, proband; P-NET, pancreas neuroendocrine tumor

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.

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Clinical Practice Guidelines for Multiple Endocrine Neoplasia Type 1 (MEN1)

Rajesh V. Thakker, Paul J. Newey, Gerard V. Walls, John Bilezikian, Henning Dralle, Peter R. Ebeling, Shlomo Melmed, Akihiro Sakurai, Francesco Tonelli, and Maria Luisa Brandi

Academic Endocrine Unit (R.V.T., P.J.N., G.V.W.), University of Oxford, Oxford OX3 7LJ, United Kingdom; Division of Endocrinology (J.B.), Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York 10032; Department of General Surgery (H.D.), Martin Luther University, 06097 Halle-Wittenberg, Germany; Faculty of Medicine, Dentistry and Health Sciences (P.R.E.), University of Melbourne, Melbourne, Victoria 3010, Australia; Division of Endocrinology and Metabolism (S.M.), Cedars-Sinai Medical Center, Los Angeles, California 90048; Department of Medical Genetics (A.S.), Shinshu University School of Medicine, 390-8621 Matsumoto, Japan; and Departments of Clinical Physiopathology (F.T.) and Internal Medicine (M.L.B.), University of Florence, 50134 Florence, Italy

Objective: The aim was to provide guidelines for evaluation, treatment, and genetic testing for multiple endocrine neoplasia type 1 (MEN1).

Participants: The group, which comprised 10 experts, including physicians, surgeons, and geneticists from international centers, received no corporate funding or remuneration.

Process: Guidelines were developed by reviews of peer-reviewed publications; a draft was prepared, reviewed, and rigorously revised at several stages; and agreed-upon revisions were incorporated.

Conclusions: MEN1 is an autosomal dominant disorder that is due to mutations in the tumor suppressor gene *MEN1*, which encodes a 610-amino acid protein, menin. Thus, the finding of MEN1 in a patient has important implications for family members because first-degree relatives have a 50% risk of developing the disease and can often be identified by *MEN1* mutational analysis. MEN1 is characterized by the occurrence of parathyroid, pancreatic islet, and anterior pituitary tumors. Some patients may also develop carcinoid tumors, adrenocortical tumors, meningiomas, facial angiofibromas, collagenomas, and lipomas. Patients with MEN1 have a decreased life expectancy, and the outcomes of current treatments, which are generally similar to those for the respective tumors occurring in non-MEN1 patients, are not as successful because of multiple tumors, which may be larger, more aggressive, and resistant to treatment, and the concurrence of metastases. The prognosis for MEN1 patients might be improved by presymptomatic tumor detection and undertaking treatment specific for MEN1 tumors. Thus, it is recommended that MEN1 patients and their families should be cared for by multidisciplinary teams comprising relevant specialists with experience in the diagnosis and treatment of patients with endocrine tumors. (***J Clin Endocrinol Metab* 97: 2990–3011, 2012**)

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Abbreviations: AIP, Aryl hydrocarbon receptor-interacting protein; CT, computed tomography; ECL, enterochromaffin-like; FBHH, familial benign hypocalciuric hypercalcemia; FIHP, familial isolated hyperparathyroidism; FIPA, familial isolated pituitary adenoma; HPT-JT, hyperparathyroid-jaw tumor; LOH, loss of heterozygosity; MDT, multidisciplinary team; MEN, multiple endocrine neoplasia; MEN1, MEN type 1; MEN2, MEN type 2; MLL1, mixed lineage leukemia protein 1; MRI, magnetic resonance imaging; MTC, medullary thyroid carcinoma; mTOR, mammalian target of rapamycin; NET, neuroendocrine tumor; TKR, tyrosine kinase receptor; VIP, vasoactive intestinal polypeptide; VIPoma, VIP-secreting tumor; ZES, Zollinger-Ellison syndrome.

Summary of Recommendations

General recommendations

Patients with multiple endocrine neoplasia (MEN) type 1 (MEN1) and their families should be managed by a multidisciplinary team (MDT) consisting of relevant specialists with experience in the management of endocrine tumors (2|⊕⊕○○).

MDT representation should include specialist physicians (*e.g.* endocrinologist, gastroenterologist, and oncologist) in the management of neuroendocrine tumors (NET), endocrine surgeons, histopathologists (with expertise in NET), radiologists (including those with expertise in nuclear medicine), and clinical geneticists (2|⊕⊕○○).

Genetic testing

MEN1 germline mutation testing should be offered to index patients with MEN1 and their first-degree relatives. This includes relatives who are either asymptomatic or who have clinical manifestations of MEN1 (1|⊕⊕⊕○).

MEN1 germline mutation testing of asymptomatic relatives should be offered at the earliest opportunity because MEN1 manifestations may occur by the age of 5 yr (2|⊕⊕○○).

MEN1 germline mutation testing may be recommended in individuals with an atypical MEN1 phenotype (*e.g.* multigland hyperparathyroidism) (2|⊕⊕○○).

All individuals offered *MEN1* mutation testing should be provided with genetic counseling before testing (1|⊕⊕⊕⊕).

MEN1 germline mutation testing should be undertaken by a clinical genetics laboratory accredited in mutation analysis of the *MEN1* gene (1|⊕⊕⊕⊕). If a coding region *MEN1* mutation is not identified, then testing for partial or whole-gene deletion, or haplotype analysis of the *MEN1* locus, or analysis of other genes should be considered (1|⊕⊕⊕○).

Relatives of a patient with a known *MEN1* mutation should be offered *MEN1* germline mutation analysis before biochemical and radiological screening tests for the detection of MEN1 tumors, so as to avoid the burden of undergoing multiple tests involving different modalities and to reduce financial costs (1|⊕⊕⊕○).

Individuals who are found to have a *MEN1* germline mutation should be screened regularly (*e.g.* on an annual basis) for development of MEN1-associated tumors (1|⊕⊕⊕○).

Screening for tumors

Individuals identified as having a high risk of developing MEN1-associated tumors [*e.g.* index cases (*i.e.* MEN1 patients) and their relatives who have been identified as having a *MEN1* mutation] should be offered a program of combined clinical, biochemical, and radiological screening as detailed below. The nature and timing of screening will depend on local resources, clinical judgment, and patient preferences (2|⊕⊕○○).

Parathyroid tumors

Diagnosis

Screening for primary hyperparathyroidism should include annual assessment of plasma calcium and PTH concentrations (1|⊕⊕⊕⊕).

Treatment

Surgery performed by an experienced endocrine surgeon is the treatment of choice, although the optimum timing has not been defined. Conventional open bilateral exploration with subtotal parathyroidectomy (at least 3.5 glands) or total parathyroidectomy is recommended (1|⊕⊕⊕○). Concurrent transcervical thymectomy is also suggested at the time of surgery (2|⊕⊕○○). Total parathyroidectomy with autotransplantation may be considered (2|⊕⊕⊕○). Minimally invasive parathyroidectomy is usually not recommended because multiple glands are typically affected (1|⊕⊕⊕○).

Pancreatic NET

Diagnosis

Screening for gastropancreatic NET should include, as a minimum, an annual plasma biochemical evaluation of a fasting gastrointestinal tract hormone profile that includes measurement of gastrin, glucagon, vasointestinal polypeptide, pancreatic polypeptide, chromogranin A, and insulin with an associated fasting glucose level (2|⊕⊕○○).

A consensus for optimum radiological screening has not been established and will depend on local resources, clinical judgment, and patient preferences. A suggested minimum imaging protocol includes annual pancreatic and duodenal visualization with magnetic resonance imaging (MRI), computed tomography (CT), or endoscopic ultrasound (2|⊕⊕○○).

Treatment

The main aim is to maintain patients disease- and symptom-free for as long as possible and to maintain a good quality of life (1|⊕⊕⊕⊕).

The aim of treatment for individuals with symptomatic functioning pancreatic NET including insulinoma is to achieve cure, if possible, by surgery (1|⊕⊕⊕⊕).

The extent of disease should be evaluated fully before planning specific therapy (1|⊕⊕⊕○).