Patient backgrounds

Among the 560 registered patients, 238 (42·5%) were men and 310 (55·4%) were women. No information was provided for 12 (2·1%) patients. Female predominance has also been observed in a European MEN1 database.[14] Four hundred and two patients (71·8%) had familial and 94 (16·8%) had sporadic MEN1. No information was provided for 64 (11·4%) patients.

Age at diagnosis of MEN1 was 47·5 ± 13·5 years (range, 11–75 years; median, 49) for probands (including apparent sporadic cases), and 38·5 ± 15·4 years (range, 6–78 years; median, 36) for family members. Average age at diagnosis for family members was about 10 years younger than that for probands ($P < 0·001$).

Initial symptoms

Clinical symptoms that patients experienced before the diagnosis of MEN1 are presented in Fig. 1. Approximately 18% (47/265) of probands were asymptomatic and found to have either hypercalcaemia or pancreatic tumours during health screening or hospital visits for other purposes. Hypercalcaemia-related symptoms such as peptic ulcer (23·0%), urolithiasis (19·2%) and fracture/decreased bone mineral density (10·2%) were commonly seen. In more than a third of patients whose disease had been identified based on being family members, MEN1 was diagnosed before manifestation of clinical symptoms. In other words, nearly two-thirds of family members had already developed clinical symptoms when the proband was diagnosed as having MEN1.

Prevalence of tumours

Primary hyperparathyroidism, gastroenteropancreatic neuroendocrine tumours (GEPNET), and pituitary tumours were seen in 90·4%, 56·1% and 47·5% of patients, respectively (Fig. 2). Excluding 24 presymptomatic cases diagnosed by genetic testing, none of whom has yet developed a neoplastic disease, the prevalences of these tumours were 94·4%, 58·6% and 49·6%, respectively. These numbers were similar to those previously reported for patients in

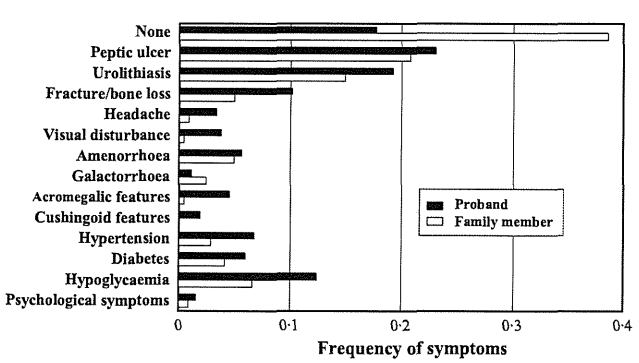

Fig. 1 Symptoms appeared before diagnosis of MEN1. Frequency of symptoms patients experienced before diagnosis of MEN1. Closed columns; proband and apparently sporadic cases (n = 265), open columns; family members of probands (n = 220). As some patients had more than one symptom before diagnosis of MEN1 was made, sum of frequency of each symptom exceeds 100%.

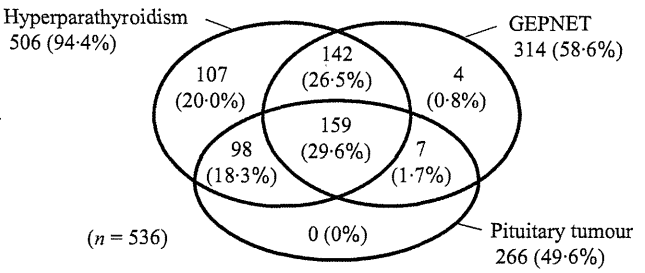

Fig. 2 Triad prevalence. The prevalence of the triad of primary hyperparathyroidism, gastroenteropancreatic neuroendocrine tumour (GEPNET) and pituitary tumour is shown schematically.

western countries.[5] Adrenal cortex tumours were seen in 20·1% and thymic/bronchial tumours in 8·4% (excluding genetically diagnosed presymptomatic cases). Although thymic carcinoid tumour is considered to occur predominantly in males and smokers,[16,17] it is noteworthy that 37% of patients (men, 17; women, 10) with thymic carcinoid tumours were women. Among those 10 female patients, three had already died and one patient is lost to follow-up. The cause of death of all three deceased patients was malignant progression of thymic carcinoid tumour. Only one patient was an apparent smoker, and six patients were nonsmokers. No information on smoking habits was provided for the other three patients.

Primary hyperparathyroidism Age at diagnosis of primary hyperparathyroidism was 46·8 ± 13·1 years (range, 11–78; median, 48) for probands (including apparent sporadic cases), and 39·8 ± 14·6 years (range, 16–78; median, 37) for family members. Diagnosis of primary hyperparathyroidism for family members was made at significantly younger age than probands ($P < 0·001$). We previously reported a positive correlation between age and the plasma concentration of parathyroid hormones in patients with MEN1,[18] but no such correlation was observed in the present study (data not shown).

Surgery for primary hyperparathyroidism was performed in 77·7% (393/506) of patients. Among them, 44 underwent parathyroidectomy prior to the diagnosis of MEN1, and single gland parathyroidectomy was performed for more than half of these patients.

GEPNET Age at diagnosis of GEPNET was 46·0 ± 14·5 years (range, 9–74; median, 47) for probands (including apparent sporadic cases), and 42·7 ± 16·9 years (range, 8–78; median, 42·5) for family members. Although the difference in average age at diagnosis for family members was only 3·3 years younger than probands, there was a statistically significant difference between two groups (P = 0·002). The majority of patients had multiple tumours. A single tumour was detected in 26·1% (82/314) of patients, 28·7% (90/314) had 2–4 tumours, 6·7% (21/314) had 5–10 tumours, and 4·1% (13/314) had 11 or more tumours. No information on the number of tumours was provided for 34·4% (108/314) of patients, possibly reflecting inability to detect duodenal gastrinoma by conventional imaging modalities.

Information on hormone secretion, obtained in 280 cases, is summarized in Table 1. Among functioning tumours, gastrinoma was the most common followed by insulinoma. The prevalence of

Table 1. Functional details and management of GEPNETs

Secreted hormone	*n** (%)†	Management		
		Surgical (%)‡	Nonsurgical (%)‡	Unknown (%)‡
Nonfunctioning§	91 (29·0)	31 (34·1)	44 (48·4)	16 (17·5)
Gastrin	91 (29·0)	47 (51·6)	37 (40·7)	7 (7·7)
Insulin	69 (22·0)	63 (91·3)	2 (2·9)	4 (5·8)
Glucagon	19 (6·1)	13 (68·4)	4 (21·1)	2 (10·5)
VIP	3 (1·0)	2 (66·7)	0 (0·0)	1 (33·3)
Somatostatin	3 (1·0)	3 (100·0)	0 (0·0)	0 (0·0)
ACTH	2 (0·6)	2 (100·0)	0 (0·0)	0 (0·0)
GRH	2 (0·6)	2 (100·0)	0 (0·0)	0 (0·0)
Unknown	41	24	6	11
Total	314	180 (57·3)¶	95 (30·3)¶	39 (12·4)¶

*As some patients had multiple functioning tumours, sums of the numbers of patients with each tumour exceed the total number of patients with GEPNETs (314).
†Percentage of the total number of patients with GEPNETs.
‡Percentage of each functioning tumour.
§'Nonfunctioning' indicates patients with nonfunctioning tumours only. Patients with both functioning- and nonfunctioning tumours are not included.
¶Percentage of patients receiving designated management among the total number of patients with GEPNETs.

insulinoma was apparently higher in Japanese (22·0%) than in the western patients (about 10%).[9,19] Surgery was performed for 57·3% (180/314) of GEPNET patients. More than 80% of functioning tumours, other than gastrinomas, were surgically treated. Approximately 50% of gastrinomas were managed without surgery.

Pituitary tumour Age at pituitary tumour diagnosis was 46·1 ± 14·4 years (range, 11–75; median, 49) for probands (including apparent sporadic cases), and 38·9 ± 13·4 years (range, 14–69; median, 36) for family members ($P < 0·001$, probands *vs* family members).

Table 2. Functional details and management of pituitary tumours

Secreted hormone	*n* (%)*	Management		
		Surgical (%)†	Nonsurgical (%)†	Unknown (%)†
Nonfunctioning‡	75 (28·2)	19 (25·3)	49 (65·3)	7 (9·3)
PRL	97 (36·5)	22 (22·7)	63 (64·9)	12 (12·4)
GH	34 (12·9)	23 (67·6)	5 (14·7)	6 (17·7)
PRL + GH	9 (3·4)	5 (55·6)	4 (44·4)	0 (0·0)
ACTH	10 (3·8)	7 (70·0)	3 (30·0)	0 (0·0)
TSH	1 (0·4)	1 (100·0)	0 (0·0)	0 (0·0)
Unknown	39	5	10	25
Total	266	82 (30·8)	134 (50·4)	50 (18·8)

*Percentage of the total number of patients with pituitary tumours.
†Percentage of each functioning tumour. ‡Nonfunctioning indicates patients with apparently detectable pituitary tumor without any evidence of abnormal secretion of hormones.

Information on hormone secretion, obtained in 227 cases, is summarized in Table 2. Nonfunctioning tumours and prolactinomas accounted for 28·2 and 36·5% of pituitary tumours, respectively. Tumour sizes at diagnosis ranged from 2 mm to 100 mm, and 58·3% were microadenomas.

Surgery was performed for 30·8% (82/266) of patients with a pituitary tumour. Nonfunctioning tumours and prolactinomas were generally either followed up periodically or treated with a dopamine agonist.

Genetic testing

Among registered patients, 419 (74·9%) underwent genetic testing. Germline mutations of the *MEN1* gene were found in 347 (82·8%) patients belonging to 180 families. The mutation positive ratio was 91·7% in familial cases (negative 5·8%, unknown 2·5%) and 49·3% in apparently sporadic cases (negative 49·3%, unknown 1·4%) (Fig. 3 and Table 3). A small deletion was the most common type of mutation and 74·4% (134/180) of families had truncating mutations. As previously reported,[20] mutations were detected throughout the coding region and exon–intron boundaries, and no genotype–phenotype correlations were observed. During this study, we identified several novel mutations which will be reported elsewhere (manuscript in preparation).

Mortality

Among registered patients, 36 had already died and causes of death were related to MEN1 in 21 of these. Distant metastases of pancreatic tumours and thymic carcinoids were the cause of death in 62% (13/21). Age at diagnosis of deceased patients was 44·2 ± 12·7 years (range, 17–66; median, 42), and age at the time of death 57·1 ± 13·0 years (range, 24–80; median, 56). Age at diagnosis did not differ significantly between deceased and surviving patients ($P = 0·232$).

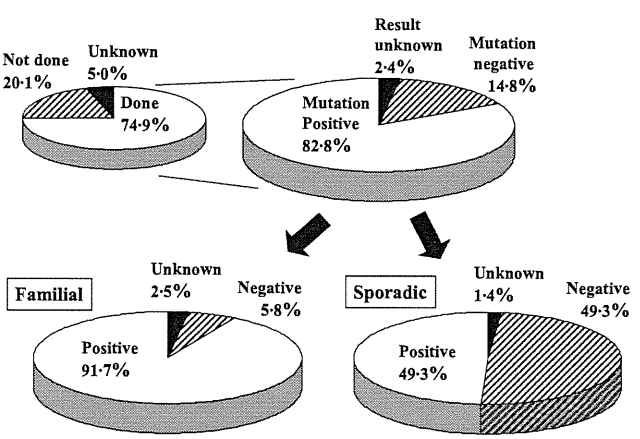

Fig. 3 Genetic testing of *MEN1* gene. Among registered patients, 74·9% had undergone germline *MEN1* gene analysis, and mutations were found in 82·8% of these patients. While germline mutations were detected in over 90% patients with a family history, mutations were found in <50% of sporadic cases.

Table 3. Types and distribution of germline *MEN1* mutations

Exon	2	3	4	5	6	7	8	9	10	Others	Total number of families
Missense mutation	7	6	7	1		3	4	5	5		38
Nonsense mutation	3	3	1			1	1	6	8		23
Small deletion	19	17	2	2	3	2	2	12	10		69
Small insertion	10		1			1	1	1	16		30
Small indel								2			2
Splice mutation							1			9	10
Large deletion							1			2	3
Unknown	2									3	5
Total number of families	41	26	11	3	3	7	10	26	39	14	180

Discussion

The aims of the MEN Consortium of Japan are to clarify the clinical features of Japanese patients with MEN1 and provide information useful to clinicians facing difficulties in managing these patients. To collect enough information to be representative of the entire Japanese MEN1 patient population, we have established a database for collection of detailed information on Japanese patients with MEN1.

The estimated prevalence of MEN1 is 2–3/100 000 live births in Caucasians.[4] Reliable data on the prevalence of MEN1 in Asian countries including Japan are lacking. We previously reported that MEN1 prevalence may be similar in Japanese and Caucasians.[8] If this is the case, there would be about 4000 patients in Japan. Our present study is not an epidemiological surveillance and therefore cannot determine the prevalence of MEN1. Furthermore, our database includes deceased patients and some lost to follow-up. In one epidemiological study performed in a community hospital in Okinawa, Japan, the incidence of primary hyperparathyroidism among hospitalized patients was 1·5/10 000.[21] In another study, Kihara *et al.*[22] identified 16 patients with *MEN1* gene mutation from 466 Japanese patients with primary hyperparathyroidism (mutation positive rate 3·4%). The incidence of primary hyperparathyroidism varies even among population-based studies in western countries,[23,24] and some have suggested a lower incidence of primary hyperparathyroidism among Asian populations.[25,26] The incidence of MEN1 in the Japanese population might be lower than that of western populations, and further investigation is warranted.

Among registered cases, 81·4% (456/560) of reports were from members of the Consortium. This may indicate that patients are already concentrated in several specialized hospitals in Japan. Alternatively, work-ups for patients suspected of having MEN1 might be insufficient in other hospitals. In the present study, the response rate of nonmember physicians to the call for registration was only 17·5%. Furthermore, 70% of respondents did not have any experience of patients with MEN1. One of the reasons for the low rate of response and experience is that we sent request letters to the board of representatives of the Japan Endocrine Society, which includes many basic scientists, obstetricians and paediatric endocrinologists, who rarely see patients with MEN1. In our earlier study, which surveyed experience of familial endocrine tumour syndromes among Japanese physicians, only 15% of respondents had experience with clinical care of patients with MEN1 despite 75% of respondents being either endocrine surgeons, urologists or internists.[27] Many clinicians may not be fully aware of the possibility of MEN1 when they see patients with endocrine tumours, and patients with MEN1 may be left undiagnosed because of insufficient work-ups for other endocrine lesions, family history, or genetic information.

Although the penetrance of most of MEN1-related lesions was not apparently different from those of previous reports,[5,19] the high incidence of thymic carcinoid tumours in female patients was an exception. In previous reports, thymic carcinoid tumours were predominantly seen in male patients and they are likely to be a smoker.[16,17] Female Japanese patients who developed thymic carcinoid tumours manifested typical features of MEN1, and all but one patient had either a germline *MEN1* mutation or family history of MEN1. Seven patients had had surgery, and the tumour was confirmed pathologically. Thus, there is little doubt that the incidence of thymic carcinoid tumours in Japanese female patients with MEN1 is higher than in western populations. The reason of this ethnic difference is unclear and requires further investigation. This result also provides an important suggestion on clinical surveillance of female patients as the risk of thymic carcinoid tumours could be underestimated when currently available epidemiological data are applied to Japanese patients.

The *MEN1* gene was isolated in 1997 and has enabled clinicians to offer a molecular diagnosis to patients suspected to have MEN1 as well as presymptomatic genetic testing for at-risk family members.[28] Early identification of mutation carriers is expected to permit early diagnosis of each lesion and early intervention, both of which are anticipated to improve patient outcomes.[29,30] However, our data indicate that presymptomatic genetic testing is not proactively applied in Japan. Indeed, the age distribution pattern of affected family members at the time of the diagnosis of MEN1 did not change after identification of the *MEN1* gene. Average age at diagnosis of family members was 38·0 ± 14·3 years (range, 11–70; median, 39) before 1996, and 38·9 ± 16·2 years (range, 6–78 years; median, 34 years) after 2001 (P = 0·94). Insufficient application of genetic testing may reflect the limited availability of genetic counselling in Japan.[31] Despite national and international guidelines recommending genetic counselling before and after presymptomatic genetic testing, only a small minority of Japanese physicians

are experienced in clinical genetics. Furthermore, there are only about 100 certified genetic counsellors in Japan. In this era of genetic medicine, more human resources in clinical genetics are urgently needed in Japan.

Our database has limitations. The registered clinical information contains considerable qualitative and quantitative variations. Significant portions of the questionnaires were unanswered or the response given was 'unknown'. Although criteria for registration have been applied as described in Patients and Methods section of this manuscript, procedures for diagnosis and surveillance of MEN1 may vary among different hospitals. Unfortunately, our questionnaire did not ask about methods of diagnosis and surveillance of neoplastic lesions identified in patients. So we are cautious to draw epidemiological conclusions by comparison of our data with those of others. This kind of information should be included in our database after future revision.

As patients with MEN1 develop various endocrine and nonendocrine diseases in different organs, medical personnel from different specialties participate in the management of a single patient. Thus, sharing clinical information with participating staff members may be challenging unless well-organized management teams are responsible for comprehensive care. The long history of each patient may also make it difficult to collect accurate and complete medical information, as patients may change physicians and/or hospitals during the course of their disease. Ideal management of patients with MEN1 requires integrated medical care by a multidisciplinary team involving endocrinologists, endocrine surgeons, neurosurgeons, abdominal surgeons, urologists, as well as experienced nurses and genetic counsellors. Institutions with such teams tend to be limited, underscoring the need for MEN1 patients to be managed in regional core hospitals.

Collection and analysis of clinical information makes it possible to understand the clinical features of rare diseases and to standardize their medical management. In this regard, our database is a potentially powerful tool for improving the future management of Japanese patients with MEN1. Although we are currently collecting only descriptive information, adding other data such as details of imaging studies, biochemical assays and histology will further enhance the value of the database.

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Competing interests/financial disclosure

The authors have nothing to disclose.

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ORIGINAL

A novel splice site mutation of the *MEN1* gene identified in a patient with primary hyperparathyroidism

Yuko Nagamura[1)], Masanori Yamazaki[2)], Satoko Shimazu[1)], Kenji Sano[3)], Toshihiko Tsukada[1)] and Akihiro Sakurai[2), 4)]

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

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

Key words: MEN1, Menin, Splicing, Minigene, Stability

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

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

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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

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

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

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

Case Presentation

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

Materials and Methods

Stability analysis of variant menin

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

Table 1 Results of biochemical studies

			Reference range
GH	(ng/mL)	0.7	<1.0
IGF-1	(ng/mL)	264	121-436
PRL	(ng/mL)	10.8	1.4-10.8
IRI (fasting)	(μU/mL)	5.0	<10
Glucose (fasting)	(mg/dL)	88	<110
Gastrin	(pg/mL)	35	37-172
Glucagon	(pg/mL)	68	23-197

GH, Growth Hormone; IGF-1, ********; PRL, Prolactin; IRI, **********

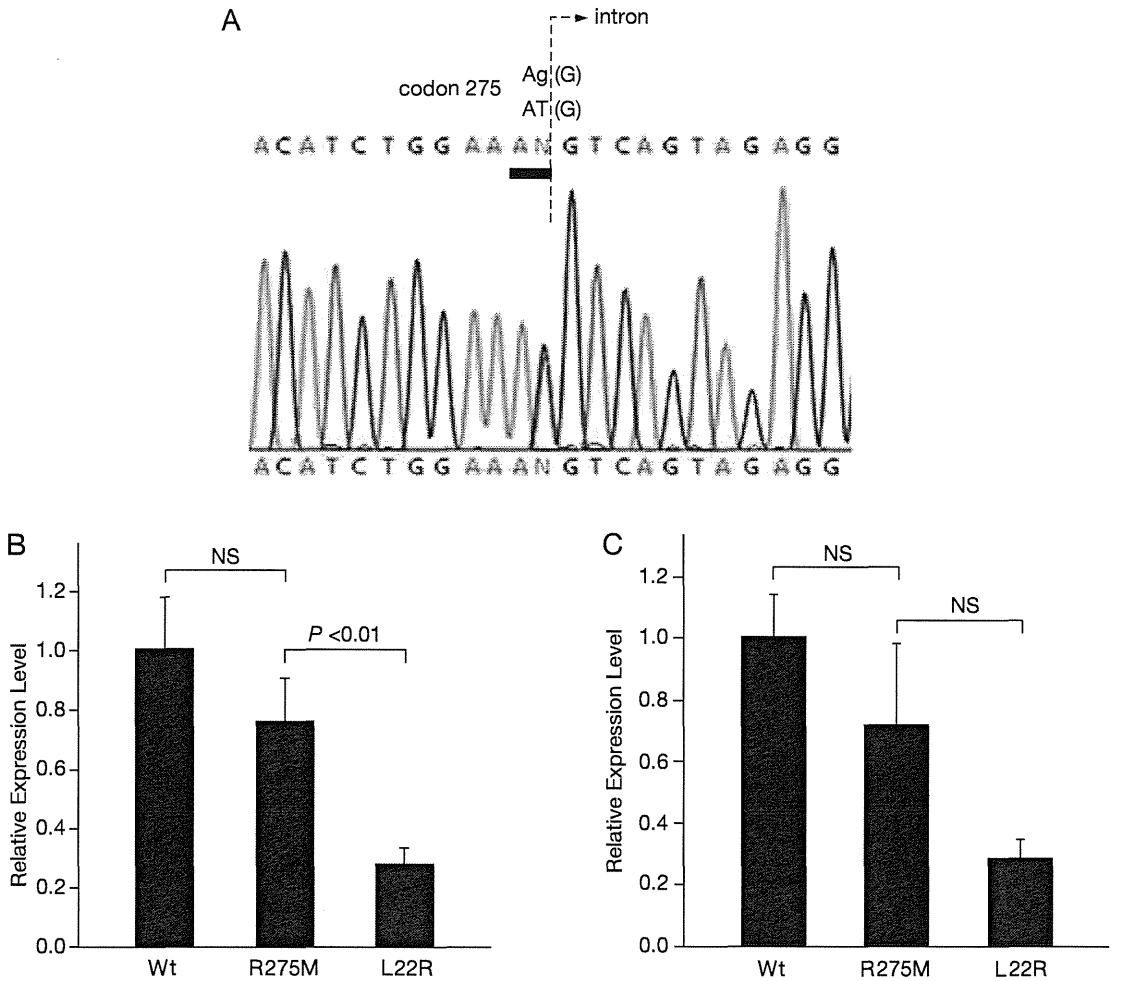

Fig. 1 Stability of menin missense mutant
Genomic DNA was isolated from whole blood of the patient for genetic testing. Sequencing analysis of *MEN1* gene identified c.824G>T mutation (A). This nucleotide substitution is predicted to generate putative missense menin protein, R275M. Mutant menin protein 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 (B) or FLAG-tagged mutant and Myc-tagged wild type menin (C). 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). The thin bars represent standard error of the mean of three independent transfection experiments. NS, not statistically significant ($P>0.05$)

tronic 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. 48 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. The mean of analyzed nuclei number was 24 per transfection and the minimum was 9 per transfection. Mutant menin was located mainly in the nucleus although the cytoplasm was also faintly stained in some cells. Only nuclear staining was analyzed.

Analysis of menin mRNA in blood cells

RNA was isolated from whole blood with the LeukoLOCK^{TM} total RNA isolation system (Ambion,

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

Sequencing analysis of tumor DNA

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

Minigene analysis of c.824G>T mutant

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

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

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

Results

Stability of variant menin R275M

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

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

Given that the c.824G>T mutation occurred at an exon-intron junction, this mutation could act as a splicing mutation rather than a simple missense mutation. The menin mRNA of the patient was therefore examined for evidence of abnormal mRNA splicing. PCR amplification with primers on exons 3 and 7 of the patient's blood cell cDNA generated several fragments in addition to the predicted wild type 400-bp cDNA (Fig. 2A). Direct sequencing of the normal-sized fragment with a primer on exon 5 showed only normal sequence and the mutation identified in the germline was not detected (Fig. 2B). The three additional fragments of 360 bp,

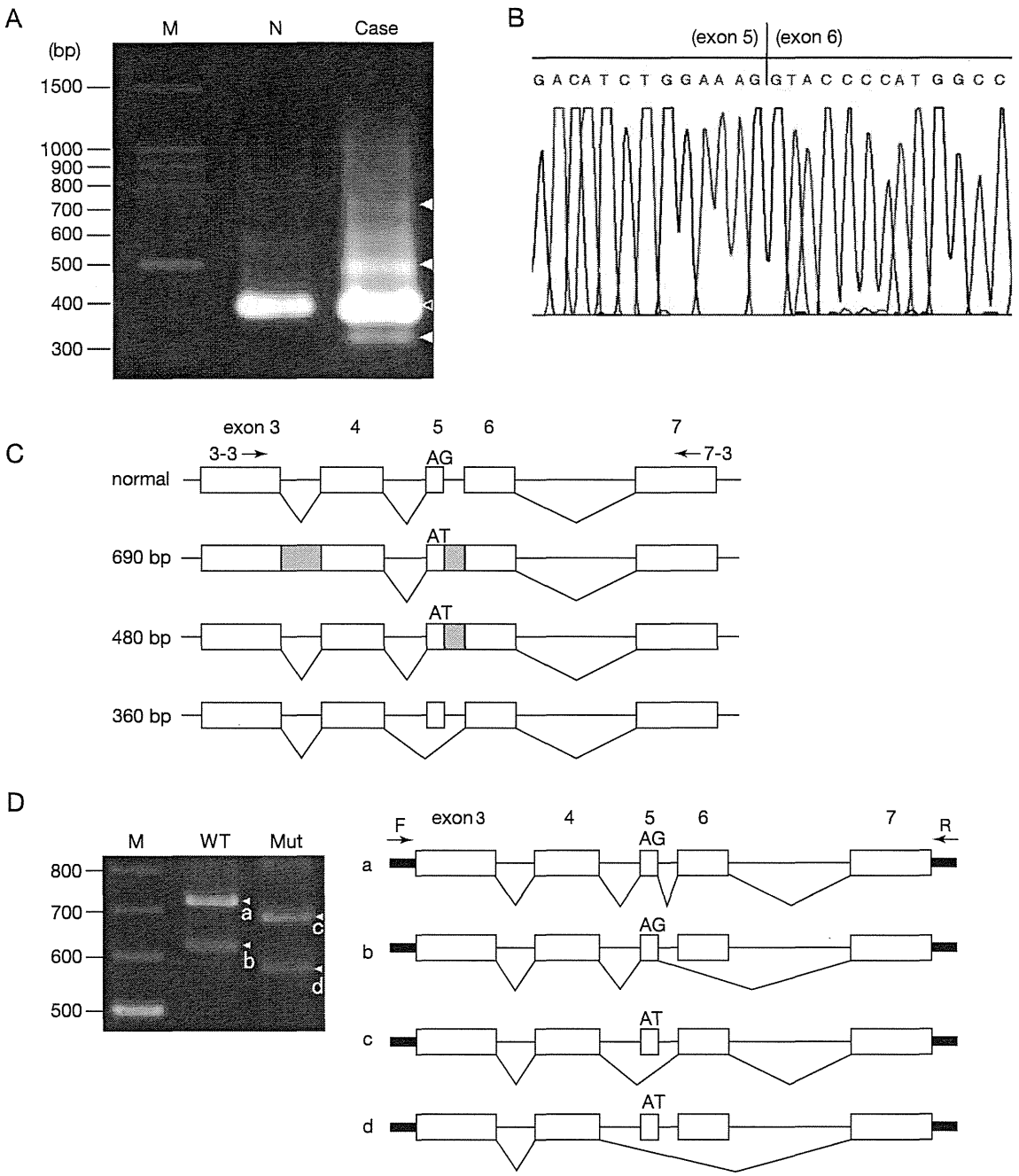

Fig. 2 *MEN1* mRNA in the patients with c. 824G>T mutation

A. The patient's blood cell cDNA was amplified with primers on *MEN1* exons 3 and 7, and separated on agarose gel (Case) along with that from a normal subject (N). The PCR product of the normal size (open triangle) and abnormal PCR products (solid triangles) were excised and subjected to either direct sequencing (normal fragment) or sequencing after cloning (690-bp, 480-bp and 360-bp abnormal fragments). M: size marker. B. Direct sequencing of the normal-sized cDNA fragment analyzed with a sequencing primer on exon 5. The mutated sequence at the 3' end of exon 5 was not detectable. C. Structures of normal-sized, 690-, 480- and 360-bp cDNAs. Open boxes and closed boxes indicate exons and unspliced introns, respectively. V-shaped lines below each diagram indicate the splicing events that give rise to each mRNA. Normal-sized cDNA contained only the wild type sequence (AG) at the exon-intron junction while the 690- and 480-bp cDNAs contained only the mutant sequence (AT). The positions of the PCR primers used are shown above as arrows. D. mRNA from the wild type (WT) and c.824G>T mutant (Mut) minigenes. The structures of PCR products a, b, c and d identified on agarose gel (left) were analyzed by sequencing and shown in the right. The wild type and mutant sequences at the 3' end of exon 5 is shown as AG and AT, respectively. Thick lines represent minigene-specific regions of the transcripts where PCR primers anneal (F and R, shown by arrows).

480 bp and 690 bp were cloned and sequenced (Fig. 2A, C). The 360-bp fragment lacked exon 5; the 480-bp fragment contained an unspliced 80-bp intron sequence following the mutated exon 5; and the 690-bp fragment contained a 210-bp intron sequence following exon 3 as well as the previously observed 80-bp intron sequence following the mutated exon 5. Similar intron retention between exons 3 and 4 in menin mRNA induced by a distant splicing mutation has been reported previously [22]. These findings suggest that the c.824G>T mutation causes aberrant mRNA splicing, and that all detectable menin mRNA splicing variations potentially cause protein truncation by frame-shift or a cryptic stop codon within unspliced intron sequence.

Minigene analysis of c.824G>T mutation

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

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

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

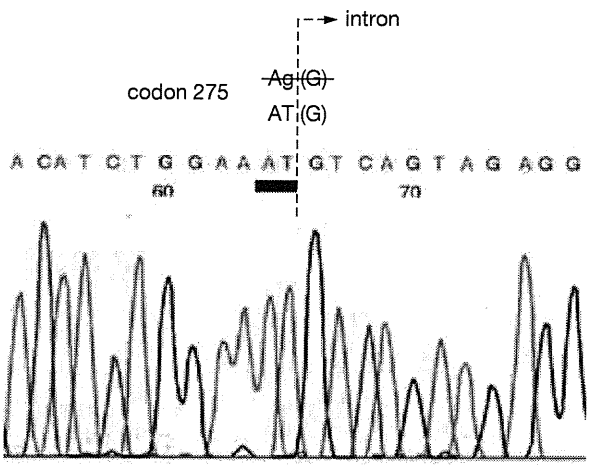

Fig. 3 Loss of the wild type allele of the *MEN1* gene in the parathyroid gland obtained from a patient with c.824G>T mutation
DNA isolated from parathyroid tissue was amplified and sequenced. Note that only mutant allele was seen in the tumor. Compare the sequence to that shown in Fig. 1A.

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

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Disclosure Summary

All authors have nothing to disclose.

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ORIGINAL

Delay in the diagnosis of multiple endocrine neoplasia type 1: typical symptoms are frequently overlooked

Masanori Yamazaki[1)], Shin-ichi Suzuki[2)], Shinji Kosugi[3)], Takahiro Okamoto[4)], Shinya Uchino[5)], Akihiro Miya[6)], Tsuneo Imai[7)], Hiroshi Kaji[8)], Izumi Komoto[9)], Daishu Miura[10)], Masanobu Yamada[11)], Takashi Uruno[12)], Kiyomi Horiuchi[4)], Ai Sato[1)], Akira Miyauchi[6)], Masayuki Imamura[9)], Akihiro Sakurai[1), 13)] and MEN Consortium of Japan

[1)] *Division of Diabetes, Endocrinology and Metabolism, Department of Internal Medicine, Shinshu University School of Medicine, Matsumoto 390-8621, Japan*
[2)] *Department of Breast, Endocrine, and Thyroid Surgery, Fukushima Medical University, Fukushima 960-1295, Japan*
[3)] *Department of Medical Ethics/Medical Genetics, Kyoto University School of Public Health, Kyoto 606-8501, Japan*
[4)] *Department of Endocrine Surgery, Tokyo Women's Medical University, Tokyo 162-8666, Japan*
[5)] *Noguchi Thyroid Clinic and Hospital Foundation, Beppu 874-0932, Japan*
[6)] *Department of Surgery, Kuma Hospital, Kobe 650-0011, Japan*
[7)] *Department of Breast and Endocrine Surgery, Nagoya University, Nagoya 466-8550, Japan*
[8)] *Department of Physiology and Regenerative Medicine, Kinki University School of Medicine, Osaka 589-8511, Japan*
[9)] *Department of Surgery, Kansai Electric Power Company Hospital, Osaka 553-0003, Japan*
[10)] *Department of Breast and Endocrine Surgery, Toranomon Hospital, Tokyo 105-8470, Japan*
[11)] *Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, Maebashi 371-8511, Japan*
[12)] *Department of Surgery, Ito Hospital, Tokyo 150-8308, Japan*
[13)] *Department of Medical Genetics, Shinshu University School of Medicine, Matsumoto 390-8621, Japan*

Abstract. The morbidity and mortality of individuals with multiple endocrine neoplasia type 1 (MEN1) can be reduced by early diagnosis of MEN1 and related endocrine tumors. To find factors contributing to early diagnosis, we collected clinical information on MEN1 patients through a MEN study group, "MEN Consortium of Japan" and analyzed the time of initial symptom-dependent detection of parathyroid tumors, gastro-entero-pancreatic neuroendocrine tumors (GEPNETs) and pituitary tumors, and that of tumor detection-dependent MEN1 diagnosis in 560 patients. Main tumors were identified up to 7.0 years after symptoms appeared and there was no difference in age at the diagnosis of GEPNETs alone between probands and family members. In patients with typical symptoms (peptic ulcers, urolithiasis, fasting hypoglycemia, bone fracture/loss and amenorrhea), the mean interval between symptom manifestation and tumor detection was extended up to 9.6 years. In particular, 21.7% (5/23) of patients with amenorrhea were diagnosed with pituitary tumors in under one year. In patients with peptic ulcers (from parathyroid tumors or GEPNETs) and urolithiasis (from parathyroid tumors), the interval was positively correlated with age at tumor detection. The interval between tumor detection and MEN1 diagnosis was also prolonged to approximately four years in patients with fasting hypoglycemia (from GEPNETs) and amenorrhea. A substantial delay in the diagnosis of symptom-related tumors and subsequent MEN1 and inadequate screening of GEPNETs in family members were indicated. A greater understanding of MEN1 may assist medical practitioners to make earlier diagnoses, to share patients' medical information and to give family members sufficient disease information.

Key words: Multiple endocrine neoplasia type 1 (MEN1), Endocrine tumor, Diagnosis

MULTIPLE ENDOCRINE NEOPLASIA TYPE 1 (MEN1) is a rare autosomal dominantly transmitted hereditary disorder with an estimated prevalence of about one in 30000 individuals [1]. MEN1 is generally diagnosed by detection of at least two of the three principal endocrine tumors, i.e. parathyroid tumors, gastro-entero-pancreatic neuroendocrine tumors (GEPNETs) and pituitary tumors [2]. Some patients can also develop thymic and broncho-pulmonary neuroendocrine tumors (NETs), adrenocortical tumors, facial angiofibromas, collagenomas and lipomas. Parathyroid

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

tumors, pathologically identified as hyperplasia or adenoma, are the most common and frequent manifestation, occurring in more than 85-95% of patients with MEN1 [3-5]. GEPNETs, which mainly consist of gastrinomas (manifested as Zollinger-Ellison syndrome), insulinomas (also manifested as fasting hypoglycemia) and nonfunctioning tumors, occur in approximately 40-60% of affected individuals [3-5]. Anterior pituitary tumors are evident in about 30-50% of patients [3-5]. Among functioning tumors, prolactinomas, which induce hyperprolactinemia, are the most common. Amenorrhea, galactorrhea and infertility are typical symptoms in women, whereas hypogonadism and sexual dysfunction occur in men [1]. Nonfunctioning tumors also frequently develop. Germline mutations of the *MEN1* gene, which encodes the tumor suppressor protein menin, are identified in most patients [3].

While prognosis of MEN1 has improved regularly, GEPNETs and thymic NETs can increase the risk of death because they have high potential for malignant transformation [4]. Indeed, some reports have shown that patients with MEN1 may have a shorter life expectancy due to these malignancies [6, 7]. The early diagnosis of MEN1 and disease-related endocrinopathy is therefore necessary to reduce morbidity and mortality. Patients diagnosed with MEN1 are fewer in number than predicted from the estimated prevalence and it therefore seems likely that many patients remain undiagnosed or are diagnosed only after a significant time-lag [8, 9].

In the present study we used a recently established clinical database of Japanese patients with MEN1 to evaluate factors contributing to the early diagnosis of MEN1 through an analysis of the interval between the appearance of initial symptoms and the diagnosis of MEN1 or MEN1-related main endocrine tumors.

Subjects and Methods

Patients and data collection

We analyzed the clinical data of patients with MEN1 collected by the MEN Consortium of Japan, a voluntary research group which has established a national database for patients with MEN1. In brief, physicians and surgeons in Japan reported clinical information of their MEN1 patients according to questionnaires provided by the MEN Consortium. The required information included gender, birth date, family history, initial symptoms, the dates of lesion detection and diagnosis of MEN1, medical and surgical managements of all lesions and their outcomes, and both pathological and genetic information. As of April 2011, surveillance had been completed for 582 cases. After verification, 560 cases were considered to be eligible for the present analysis. The general characteristics of patients with MEN1 have been recently reported [5]. In brief, primary hyperparathyroidism, GEPNETs, and pituitary tumors were seen in 90.4%, 56.1%, and 47.5% of MEN1 patients respectively. Approximately 18% of probands were asymptomatic, while nearly two-thirds of family members had already developed clinical symptoms when the probands were diagnosed with MEN1. Frequent initial symptoms seen in both probands and family members were peptic ulcers, urolithiasis, hypoglycemia, bone fracture/loss and amenorrhea. Age at diagnosis was calculated by the date of diagnosis and birth date. Intervals were also calculated by age of appearance of initial symptoms and/or age at diagnosis. This study was approved by the Institutional Review Board of Shinshu University School of Medicine and other Universities/Facilities enrolled in this study.

Statistical analysis

We used the mean (S.D.) for analysis of the descriptive data. To find a significant difference in each item between probands (including apparently sporadic cases) and their family members, the Mann-Whitney's U test was conducted. The correlation between age at tumor detection and diagnostic intervals was evaluated by Spearman's rank correlation. A p value of less than 0.05 was considered statistically significant.

Results

Appearance of initial symptoms

The mean age of appearance of initial symptoms in patients with MEN1 is shown in Table 1. Age-specific appearance of initial symptoms was observed to be 36.9% at 30 years, 59.4% at 40 years, 76.9% at 50 years and 92.3% at 60 years for all symptomatic patients (Fig. 1). Initial symptoms were recognized more by family members than by probands ($p<0.001$).

Diagnostic timing of MEN1 and related main endocrine tumors

The mean age at diagnosis of MEN1 or related classical endocrine tumors is shown in Table 1. The cumulative percentage of patients with tumors or MEN1 are also illustrated in Fig. 2. There were no great differ-

Table 1 Age of appearance of initial symptoms and at diagnosis of endocrine tumors or MEN1

	All	Probands	Family members	*p* value[a]
Appearance of initial symptoms				
No. of patients	325	185	140	
Mean age ± S.D., years	37.7 ± 14.8	40.4 ± 14.4	34.1 ± 14.5	<0.001
Diagnosis of endocrine tumors				
Parathyroid tumors				
No. of patients	394	209	185	
Mean age ± S.D., years	43.5 ± 14.2	46.8 ± 13.1	39.8 ± 14.6	<0.001
GEPNETs				
No. of patients	248	148	100	
Mean age ± S.D., years	44.7 ± 15.6	46.0 ± 14.5	42.7 ± 16.9	0.069
Pituitary tumors				
No. of patients	197	118	79	
Mean age ± S.D., years	43.2 ± 14.4	46.1 ± 14.4	38.9 ± 13.4	<0.001
MEN1				
No. of patients	418	207	211	
Mean age ± S.D., years	42.9 ± 15.1	47.5 ± 13.5	38.5 ± 15.4	<0.001

[a] Probands *vs* family members

ences in mean age at diagnosis among the three main endocrine tumor types. The cumulative percentage of patients who had developed parathyroid tumors at the ages of 20, 30, 40 and 50 years were 4.3%, 22.6%, 43.9% and 65.2% respectively. Age-related penetrance of GEPNETs or pituitary tumors was almost equal to that of parathyroid tumors. However, parathyroid and pituitary tumors were diagnosed earlier in family members than probands ($p<0.001$).

The mean age at diagnosis of MEN1 was similar to those of the main endocrine tumors. The cumulative percentage of patients with MEN1 at the ages of 20, 30, 40 and 50 years were 6.7%, 24.4%, 44.7% and 64.4% respectively. Family members were also diagnosed as having MEN1 earlier than probands.

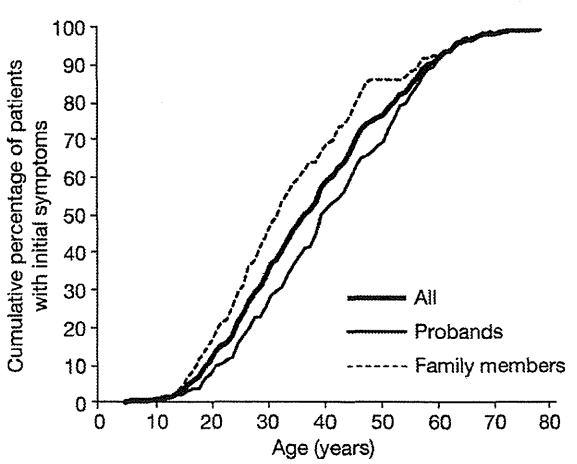

Fig. 1 Age of first appearance of symptoms
Cumulative percentage of patients according to age of appearance of initial symptoms are indicated in probands and apparently sporadic cases, family members of the probands, and all symptomatic patients.

Interval between appearance of initial symptoms and diagnosis of MEN1

As mentioned above, representative initial symptoms were peptic ulcers, urolithiasis, hypoglycemia, bone fracture/loss and amenorrhea. The temporal relationship between the occurrence of the symptoms and the diagnosis of corresponding endocrine tumors (symptom–tumor) or between tumor detection and MEN1 diagnosis (tumor–MEN1) was analyzed. The age of appearance of typical symptoms and mean diagnostic intervals are shown in Tables 2 and 3, respectively.

Peptic ulcers as an initial symptom of parathyroid tumors or GEPNETs

Patients with parathyroid tumors and GEPNETs (gastrinomas) can develop peptic ulcers. The mean age of appearance of initial symptoms was in the fifth decade of life. There was no significant difference in the mean age of appearance of peptic ulcers between probands and family members.

Twenty (35.7%) of 56 patients were diagnosed with parathyroid tumors less than one year after the symptoms appeared, whereas more than 10 years elapsed before diagnosis in 17 patients (30.4%). However, 51 (81.0%) of 63 patients were diagnosed as having MEN1 in less than one year (Fig. 3a). The mean inter-

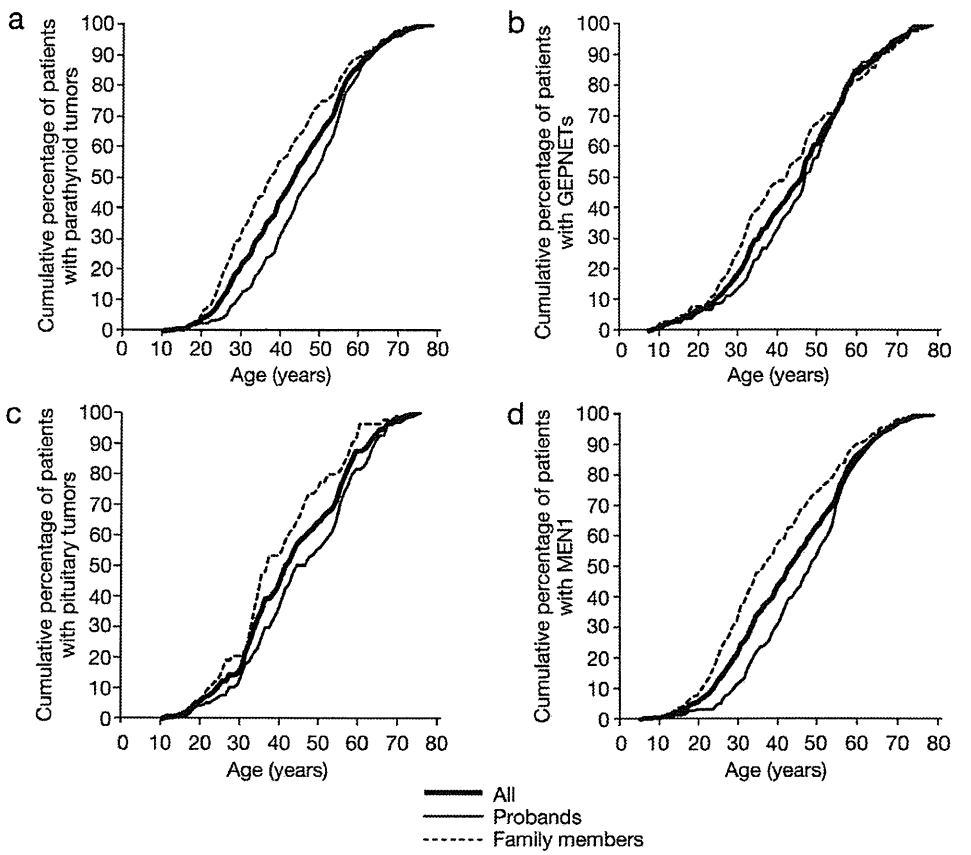

Fig. 2 Age at diagnosis of MEN1 and related classical endocrine tumors
Data are shown as cumulative percentage of patients according to age at diagnosis in probands and apparently sporadic cases, family members of probands, and all patients. (a) parathyroid tumors (b) GEPNETs (c) pituitary tumors (d) MEN1.

Table 2 Age of first appearance of typical symptoms

Initial symptoms (causative tumors)	All	Probands	Family members	*p* value[a]
Peptic ulcers (parathyroid tumors)				
No. of patients	56	34	22	
Mean age ± S.D., years	44.9 ± 12.8	45.5 ± 11.6	43.9 ± 14.8	0.387
Peptic ulcers (GEPNETs)				
No. of patients	53	33	20	
Mean age ± S.D., years	42.8 ± 13.7	42.5 ± 13.1	43.3 ± 15.0	0.854
Urolithiasis (parathyroid tumors)				
No. of patients	82	45	37	
Mean age ± S.D., years	34.5 ± 11.9	38.4 ± 11.3	29.8 ± 11.1	0.003
Fasting hypoglycemia (GEPNETs)				
No. of patients	39	25	14	
Mean age ± S.D., years	29.8 ± 17.0	30.5 ± 17.9	28.6 ± 15.7	0.930
Bone fracture/loss (parathyroid tumors)				
No. of patients	24	17	7	
Mean age ± S.D., years	46.2 ± 17.0	48.3 ± 16.1	41.0 ± 19.2	0.308
Amenorrhea (pituitary tumors)				
No. of patients	23	13	10	
Mean age ± S.D., years	30.5 ± 12.8	34.0 ± 12.2	25.9 ± 12.5	0.062

[a] Probands *vs* family members

Table 3 Diagnostic intervals

	All	Probands	Family members	*p* value [a]
Peptic ulcers–parathyroid tumors–MEN1				
Peptic ulcers–parathyroid tumors				
No. of patients	56	34	22	
Mean interval ± S.D., years	7.3 ± 9.3	6.0 ± 8.3	9.3 ± 10.5	0.205
Parathyroid tumors–MEN1				
No. of patients	63	37	26	
Mean interval ± S.D., years	0.4 ± 2.4	0.4 ± 2.3	0.4 ± 2.5	0.478
Peptic ulcers–GEPNETs–MEN1				
Peptic ulcers–GEPNETs				
No. of patients	53	33	20	
Mean interval ± S.D., years	9.6 ± 10.9	8.0 ± 10.8	12.3 ± 10.8	0.067
GEPNETs–MEN1				
No. of patients	58	35	23	
Mean interval ± S.D., years	-0.8 ± 6.2	0.5 ± 6.3	-2.9 ± 5.5	0.022
Urolithiasis–parathyroid tumors–MEN1				
Urolithiasis–parathyroid tumors				
No. of patients	82	44	38	
Mean interval ± S.D., years	8.0 ± 10.6	7.7 ± 10.6	8.4 ± 10.7	0.575
Parathyroid tumors–MEN1				
No. of patients	93	48	45	
Mean interval ± S.D., years	1.0 ± 3.5	0.9 ± 2.4	1.2 ± 4.5	0.963
Fasting hypoglycemia–GEPNETs–MEN1				
Fasting hypoglycemia–GEPNETs				
No. of patients	39	25	14	
Mean interval ± S.D., years	3.3 ± 9.7	4.3 ± 8.5	1.5 ± 11.7	0.767
GETNETs–MEN1				
No. of patients	37	23	14	
Mean interval ± S.D., years	4.2 ± 11.0	3.5 ± 10.6	5.3 ± 11.8	0.732
Bone fracture/loss–parathyroid tumors–MEN1				
Bone fracture/loss–parathyroid tumors				
No. of patients	23	16	7	
Mean interval ± S.D., years	2.0 ± 4.7	1.6 ± 3.5	2.9 ± 7.1	0.967
Parathyroid tumors–MEN1				
No. of patients	26	16	10	
Mean interval ± S.D., years	0.5 ± 1.7	0.4 ± 1.5	0.5 ± 2.0	0.476
Amenorrhea–pituitary tumors–MEN1				
Amenorrhea–pituitary tumors				
No. of patients	23	13	10	
Mean interval ± S.D., years	7.0 ± 8.5	7.5 ± 10.0	6.4 ± 6.4	0.662
Pituitary tumors–MEN1				
No. of patients	24	13	11	
Mean interval ± S.D., years	4.1 ± 8.4	5.2 ± 10.9	2.8 ± 4.1	0.835

[a] Probands *vs* family members

val of peptic ulcers–parathyroid tumors was 7.3 years, but that of parathyroid tumors–MEN1 was only 0.4 years. Each interval was not significantly different between probands and family members.

GEPNETs were diagnosed almost simultaneously with the finding of ulcers in 16 (30.2%) of 53 patients and 21 (39.6%) of 53 patients were diagnosed with GEPNETs after more than 10 years. Those who were diagnosed with MEN1 in under one year accounted for 50 (86.2%) of 58 patients with GEPNETs (Fig. 3b). The mean time of peptic ulcers–GEPNETs was 9.6 years. However, the mean intervals of GEPNETs–MEN1 showed negative values in all patients' and family members' groups because MEN1 diagnosis was made from 1 to 15 years before GEPNETs were found through the detection of primary hyperparathyroidism in 20 patients (8 probands and 12 family members). There was a significant difference in the time of GEPNETs–MEN1 between probands and family members (p=0.022).

Urolithiasis as an initial symptom of parathyroid tumors

The mean age of the first appearance of urolithiasis was in the fourth decade of life. The symptoms were recognized in family members 8.6 years earlier than by probands (p=0.003).

Thirty (36.6%) of 82 patients were diagnosed as having parathyroid tumors less than one year after initial colic attacks, but in 26 (31.7%) of all patients 10 years and over elapsed before tumor diagnosis. MEN1 was diagnosed less than one year after detection of parathyroid tumors in 68 (71.6%) of 93 patients with tumors and more than 80% of probands or family members were diagnosed with MEN1 within five years (Fig. 3c). The mean number of years for diagnostic intervals of urolithiasis–parathyroid tumors and parathyroid tumors–MEN1 were 8.0 and 1.0 respectively. There were no significant differences in both diagnostic intervals between probands and family members.

Fasting hypoglycemia as an initial symptom of GEPNETs

Fasting hypoglycemia, which is a specific symptom of GEPNETs (insulinomas), appeared at a mean age of 29.8 years. There was no significant difference in age at symptom presentation between probands and family members. Twenty-one (53.8%) of 39 patients with hypoglycemia were diagnosed as having GEPNETs less than one year after the first attack and more than 10 years elapsed before tumor detection in five patients (12.8%). Fourteen (56.0%) of 25 probands and seven (50.0%) of 14 family members also had tumors detected in under one year. In contrast, the rate of diagnosis of MEN1 in less than one year was 59.5% of all patients with GEPNETs (Fig. 3d). The mean intervals of fasting hypoglycemia–GEPNETs and GEPNETs–MEN1 were 3.3 and 4.2 years respectively. Neither diagnostic interval was significantly different between probands and family members.

Bone fracture/loss as an initial symptom of parathyroid tumors

Bone fracture or loss was detected in the fifth decade of life. The period required for recognition of this symptom was greater than that of urolithiasis. There was no difference in the age of appearance of the symptom between probands and family members.

Parathyroid tumors were identified in less than one year in 16 (69.6%) of 23 patients with bone fracture or loss and after more than 10 years in only 2 patients (8.7%). Twenty-three (88.5%) of 26 patients were diagnosed with MEN1 less than a year after tumor detection and no patients were diagnosed with the disease after more than 10 years (Fig. 3e). The mean intervals of bone fracture/loss–parathyroid tumors and parathyroid tumors–MEN1 were 2.0 and 0.5 years respectively. There was no significant difference in either diagnostic intervals between probands and family members.

Amenorrhea as an initial symptom of pituitary tumors

Amenorrhea is often induced by hypersecretion of prolactin due to physiologic stimuli, systemic disorders or drugs, while it is also one of the symptoms related to pituitary tumors. Amenorrhea was recognized at a mean age of 30.5 years. While symptoms were present in probands 8.1 years later than in family members, the mean age in probands was not significantly different from that in family members. Surprisingly, 5 (21.7%) of 23 patients with amenorrhea were diagnosed with pituitary tumors less than one year after this symptom appeared, whereas in 5 patients (21.7%) more than 10 years elapsed prior to tumor diagnosis. One (10.0%) of 10 family members with amenorrhea had tumors detected less than one year, compared to four (30.8%) of 13 probands. Fourteen (58.3%) of 24 patients with pituitary tumors had MEN1 diagnosed in less than one year (Fig. 3f). The mean diagnostic intervals of amenorrhea–pituitary tumors and pituitary tumors–MEN1 were 7.0 and 4.1 years respectively, indicating that it was likely that the diagnosis of pituitary tumors and subsequent MEN1 was not readily made. There were no significant differences in both intervals between probands and family members.

The correlation between age at tumor diagnosis and diagnostic intervals in individuals with an initial symptom

To clarify the cause of the delayed diagnostic pro-

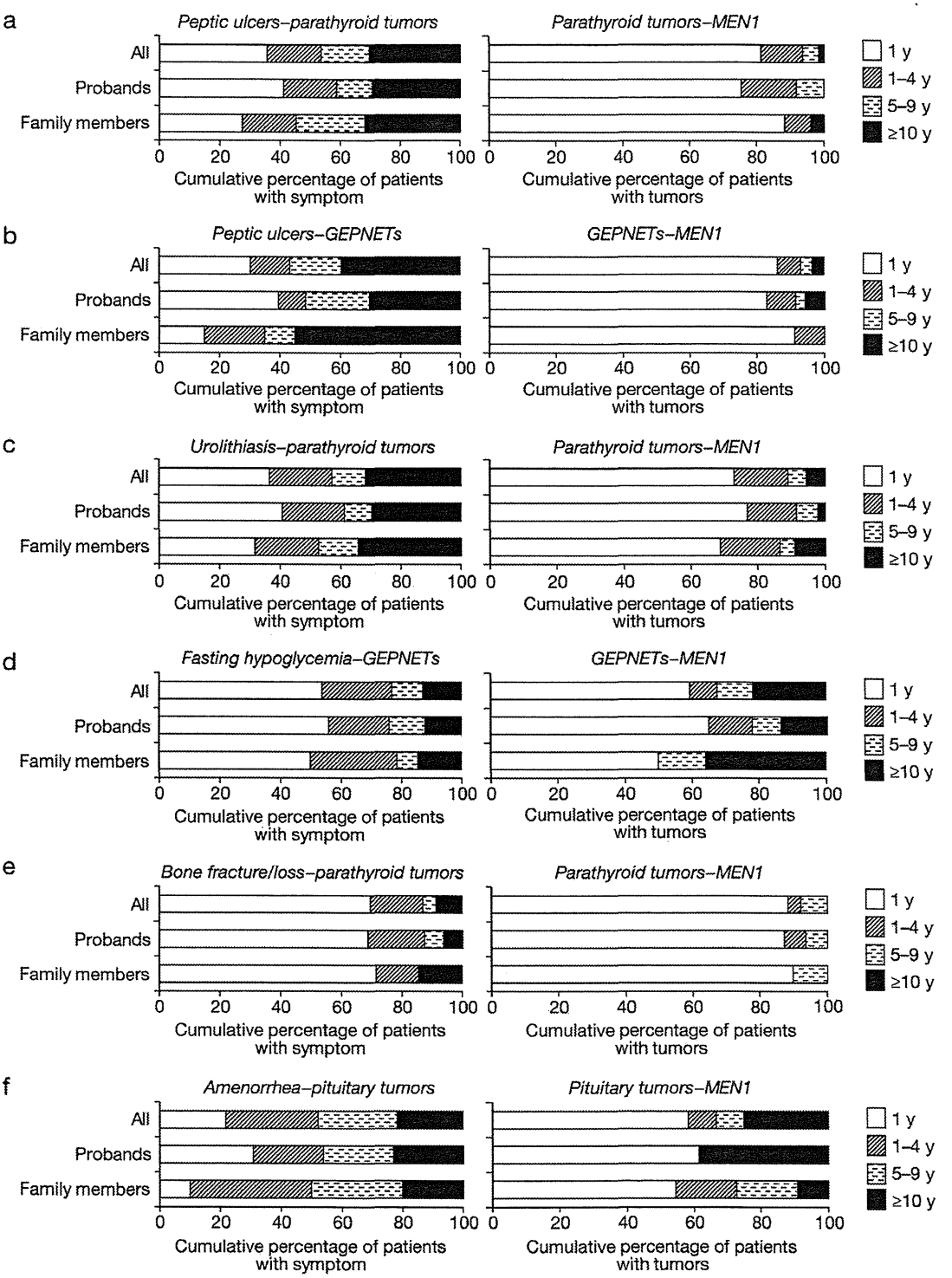

Fig. 3 Diagnostic intervals in patients with typical initial symptoms
Intervals were divided into two periods; between the appearance of each specific initial symptom and the detection of tumors, and between the detection of tumors and the diagnosis of MEN1. Data are shown for probands and apparently sporadic cases, family members of the probands, and all patients with each symptom. (a) urolithiasis–parathyroid tumors–MEN1 (b) peptic ulcers–parathyroid tumors–MEN1 (c) peptic ulcers–GEPNETs–MEN1 (d) fasting hypoglycemia–GEPNETs–MEN1 (e) bone fracture/loss–parathyroid tumors–MEN1 (f) amenorrhea–pituitary tumors–MEN1.