CLINICAL STUDY

Germline *MEN1* mutations in sixteen Japanese families with multiple endocrine neoplasia type 1 (MEN1)

Noritaka Hai^{1,3}, Norihiko Aoki³, Akira Matsuda^{1,4}, Toru Mori^{1,5} and Shinji Kosugi^{1,2}

Department of ¹Laboratory Medicine and ²Clinical Genetics Unit, Kyoto University School of Medicine, Kyoto 606–8507, Japan, ³Second Division, Department of Internal Medicine, Kinki University School of Medicine, Osaka-Sayama, Osaka 589–0014, Japan, ⁴Department of Internal Medicine, Kin-ikyo Chuo Hospital, Sapporo 007–0870, Japan and ⁵Department of Internal Medicine, Takeda Hospital, Kyoto 606–8558, Japan

(Correspondence should be addressed to S Kosugi, Department of Laboratory Medicine, Kyoto University School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606–8507, Japan; Email: kosugi@kuhp.kyoto-u.ac.jp)

Abstract

Objective: Multiple endocrine neoplasia type 1 (MEN1) is a syndrome of endocrine tumors involving the parathyroids, anterior pituitary and enteropancreatic neuroendocrine tissues, and is inherited in an autosomal dominant manner. Recently, the gene responsible for this syndrome, *MEN1*, was positionally cloned in 11q13. We aimed to assess the significance of *MEN1* gene diagnostics in families with MEN1.

Design: Sixteen probands of familial MEN1 and their 40 family members were subjected to the study. **Methods:** Full-length sequencing of the open reading frame and exon–intron boundaries in the *MEN1* gene was performed with probands of familial MEN1. Family members were examined for the identified mutation in the proband.

Results: We identified heterozygous germline mutations of the *MEN1* gene in all of 16 Japanese MEN1 families examined, achieving the highest detectability of *MEN1* mutations in familial MEN1 among studies that examined more than 10 families. Eleven kinds of the identified *MEN1* germline mutations were novel. More than half were nonsense or frameshift mutations resulting in a premature stop codon (9/15; 60%), and no mutation hot spots or no apparent genotype–phenotype relationships were observed, in support of the results of other studies. We identified 40 mutant *MEN1* gene carriers and 16 non-carriers in the course of the present study in those families.

Conclusions: Analysis of the germline mutations in the *MEN1* gene, providing significantly useful clinical information to probands and family members of MEN1, should be considered as a standard procedure and categorized as belonging to Group 1 cancer predisposition testing by the American Society of Clinical Oncology.

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Introduction

Multiple endocrine neoplasia type 1 (MEN1; online Mendelian inheritance in man (OMIM) no. 131100), first reported by Wermer (1) in 1954, is an autosomal dominant disorder characterized by varying combinations of tumors involving the parathyroids (90–100%), enteropancreatic neuroendocrine tissues (30–75%) and the anterior pituitary (50–65%), with penetrance higher than 90% by 50-year-old patients (2, 3). With the exception of gastrinomas, most of the tumors are non-metastasizing. In addition to mass effects, striking clinical features develop due to hypersecretion of endocrine substances (4).

The *MEN1* locus was mapped by linkage analysis to 11q13 in 1988 (5), and the gene responsible for the disease, *MEN1*, was positionally cloned in 1997 (6). The *MEN1* gene is 9.2 kb in length and contains

10 exons. The 610 amino acid translation product named menin is encoded by exons 2–10, and contains neither a signal peptide nor any transmembrane regions. Northern blotting analysis identified a transcript of 2.8–2.9 kb that was expressed in roughly similar amounts in all adult tissues examined, including leukocytes (6, 7). Menin is localized mainly in the nucleus but has no consensus nuclear localization signal (NLS) (8). Guru *et al.* (8) identified two functionally independent NLS (NLS-1 at residues 479–497 and NLS-2 at residues 588–608) in the C-terminal quarter of menin by site-directed mutagenesis and immunocytochemical analysis. Recently, Agarwal *et al.* (9) reported that menin interacts with the AP1 transcription factor JunD and represses JunD-activated transcription.

Most of the inherited cancer syndromes (with the notable exception of MEN2 and papillary renal cell

carcinoma) are caused by abnormalities in tumor suppressor genes (10). *MEN1* is a putative tumor suppressor gene, and according to Knudson's two-hit model, heterozygous germline mutation of this gene underlies the inherited predisposition to tumorigenesis.

Offspring of an affected individual are at a 50% risk of inheriting the mutant *MEN1* gene and developing various endocrine tumors. Earlier detection of these tumors by screening may help to reduce the morbidity and mortality in this high-risk population. It is important to study the role of *MEN1* gene diagnostics in young individuals at risk to assess the value of identifying or excluding the presence of a mutation before the onset of symptoms. Accurate determination of gene carriers at the preclinical stage will help to answer the crucial question of whether earlier diagnosis and treatment can reduce morbidity and mortality.

To date, of ~300 *MEN1* families analyzed, >80% were shown to be positive for *MEN1* gene mutation (11–16). *MEN1* mutations have been characterized as follows: (a) more than half of the mutations cause truncation of menin, probably resulting in loss-of-function; (b) there are no differences in the nature or distribution of *MEN1* mutations between familial and sporadic cases; (c) there are no apparent hot spots for *MEN1* mutation; (d) at least 10% of *MEN1* mutations are *de novo*; and (e) there is no apparent genotype–phenotype relationship.

In the present study, we analyzed and identified *MEN1* germline mutations in all of 16 *MEN1* families examined, supporting the practical importance of *MEN1* gene diagnostics in following up family members of patients with *MEN1*.

Subjects and methods

Clinical diagnosis

The diagnosis of *MEN1* was based on the presence of tumors in two or more of the three principal systems; i.e. parathyroid, anterior pituitary and enteropancreatic neuroendocrine tissues (6). Diagnosis of familial *MEN1*

required at least one first-degree relative with a tumor in one or more of these systems. Detailed family histories were obtained from the probands and available family members. Individual tumors were classified according to the clinical features (6).

Genomic DNA extraction, PCR and direct sequencing

Procedures followed were in accordance with the ethical methods of the institutional committee. Prior to participation in this study, informed consent was obtained from all subjects. Genomic DNA was extracted using a blood kit (Wako, Osaka, Japan) from the patients and their family members (17). Each exon was amplified by PCR with a pair of primers derived from the flanking introns (Table 1). Exons 5 and 6 were co-amplified with the intervening intron. The 3'-end of intronic primers were 38–96 nucleotides distant from the 5'- or 3'-end of the exons. The following PCR conditions were applied unless otherwise noted in Table 1. The PCR reactions were performed in 50 µl buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris–HCl, pH 8.3) containing 0.2 mM each dNTP, 0.5 µM each primer, 100 ng genomic DNA and 1.0 U TaKaRa Taq DNA polymerase (TaKaRa, Tokyo, Japan). Samples were denatured for 5 min at 94 °C and then subjected to 35 cycles consisting of 1 min at 94 °C, 1 min at 58 °C (exon 2, 7 and 10 were 60 °C), and 1 min at 72 °C using a thermal sequencer (TSR-300; Iwaki, Kyoto, Japan). The last extension was carried out at 72 °C for 10 min. The PCR products were purified after agarose gel electrophoresis using Ultra-free-MC filter units (Millipore, Bedford, MA, USA). Nucleotide sequences of exonic regions from nucleotide 88 to 1988 covering the full-length coding region, and those of intronic regions at exon–intron boundaries containing at least 38 nucleotides, where critical sequences for splicing are expected to exist (18), were determined in both orientations in all the probands by direct sequencing with a DNA sequencer 373A (Perkin-Elmer, Foster City, CA, USA) and Dye Terminator Cycle Sequencing kits (Perkin-Elmer) according to

Table 1 The primers and conditions for PCR amplification of *MEN1* gene coding regions.

Exon	Forward primer ^a	Reverse primer ^a	Size ^b (bp)	Temperature ^c (°C)	PCR buffer
2	AAGCAGGGGAGCTGTGCGTGT	CACCTGCCGAACCTCACAAAGG ^d	685	60	N5 ^e
3	TCACTACCTGGCCCTTTCCC ^d	TGGAGTCCCTTGGGTGGCTTG ^d	346	58	S ^f
4	CCCTGAAGCAGGCACAGGGTG ^d	CTGCCAGGGTCCCACAGCAA ^d	256	58	S
5+6	CCACCTCTGCCCCGATAGGCTA ^d	CTCATCTGCCAGATGAGGGC ^d	362	58	S
7	CATTGTGCCAGCAGGGCAGC ^d	TGGACGAGGGTGGTTGGAAAC ^d	274	60	S
8	CTGGGGCTACCCCCGATGGTG ^d	CCATGGCCCTGTGGAAGGGAG ^d	268	58	S
9	CTGGCCTGTGCCCTCTGCTAA ^d	AAAGTCTGACAAGCCCGTGGC ^d	302	58	S
10	TTCGGTGCCGATGGGACTGAG	GGGCTCAGAGTTGGGGGACTA ^d	666	60	S10 ^g

^aFrom 5' to 3'; ^bsize of PCR product; ^cannealing temperature; ^dthe primers also used for sequencing; ^ebuffer N5: 15 mM ammonium sulfate, 2.0 mM MgCl₂, 60 mM Tris–HCl, pH 10.0, 5% (v/v) DMSO; ^fstandard buffer as described in Subjects and Methods; ^gbuffer S10: standard buffer with 10% (v/v) DMSO.

the manufacturer's protocol. Primers used for sequencing are also listed in Table 1 and 5'-GAACCTTAGCG GACCCTGGGA-3' and 5'-TTCCCGAGCTCACCTTCCA GC-3' for forward direction of exon 2, 5'-ATAGACAGG TCGGCCACGGGA-3' for reverse direction of exon 2, 5'-GCCCCTGCCTCAGCCACTGTT-3' for reverse direction of exons 5 and 6, 5'-GGTCCTGGAGTTCCAGCCACT-3' and 5'-AAGCCTCCTGGGACTGTCGCT-3' for forward direction of exon 10 and 5'-ATGCTGCGGGTGCTGGCACCT-3' for reverse direction of exon 10. When PCR products of two different sizes were separated on agarose or 6% non-denaturing polyacrylamide gels, each DNA band was purified and sequenced separately. When a mutation was identified, family members were examined for the mutation by restriction analysis, polyacrylamide gel electrophoresis or direct sequencing (Table 2). Mutations are named according to standard nomenclature (19).

All mutations were confirmed to be heterozygous and none of the patients was found to have more than one *MEN1* gene mutation. All mutations (Table 2) were confirmed by at least two independent experiments.

Results

We identified heterozygous germline *MEN1* mutations in all the probands of 16 unrelated Japanese *MEN1* families (Table 2). We identified 24 mutant gene carriers and 16 non-carriers among the family members; the non-carriers had no *MEN1*-related lesions (Table 2). The occurrence of tumors in the three major systems in all *MEN1* mutant carriers examined in this study was 32/40 (80%) for the parathyroids, 8/40 (20%) for the anterior pituitary and 17/40 (43%) for enteropancreatic neuroendocrine tissues.

In total, 15 kinds of heterozygous *MEN1* germline mutations were identified in 16 families. Eleven kinds were novel for germline *MEN1* mutations (Table 2). The 15 kinds of mutations consisted of four missense mutations, five deletions, three insertions, one nonsense mutation and two abnormal splicings. More than half were nonsense or frameshift mutations resulting in a premature stop codon (9/15; 60%, the incidence similar to that reported by other groups (70/107; 65%) (11, 12)). Further, no mutation hot spots or apparent genotype-phenotype relationships were observed among the 40 carriers of *MEN1* mutation.

It is likely that the three missense base substitutions identified in this study (W183R, G225R, C241Y and S253P) were missense mutations and not common benign polymorphisms because they were not detected in 94 independent alleles. Further, the existence of the missense mutations and appearance of *MEN1*-related lesion(s) in the family members were never contradictory.

Intronic nucleotide alteration was found in two probands. Unfortunately, however, no fresh blood samples for RNA preparation were obtained in the two

cases; we failed to demonstrate direct evidences for transcript abnormality. A mutation involving a conserved splice donor site, 1460+1del14insAT, was found in case 11. The first 14 nucleotides of intron 9 were deleted but replaced with an AT dinucleotide. In case 7, we identified a base substitution, 894-9G→A, creating a new AG at the junction of intron 4 and exon 5. Four mutant carriers among the family members in this study had *MEN1* lesions and five members without the mutation had no *MEN1*-related lesions, suggesting that this substitution was strongly related to the manifestation of *MEN1*. No 894-9G→A base substitution was detected in 94 independent alleles.

1650insC was identified in two of our 16 unrelated Japanese *MEN1* families and by other groups (11-15). The two families were confirmed to be independent because bases at a polymorphic site (cDNA 1731) on the mutant allele were different (guanine in case 12 and adenine in case 13). Seven consecutive cytosines are present in this region, and such repeat sequences are prone to mutation due to polymerase slippage error (20). 299insCCAGC and 357del4 were identified in unrelated families both in the present study (cases 1 and 2 respectively) and by other investigators (11-15, 21). These mutations are present in short repeats and are probably due to replication errors.

1823delCT identified in case 16 causes a frameshift and is located at the most 3'-site in the *MEN1* gene among all the mutations identified to date. This mutation produces a mutant menin protein consisting of 571 normal and 23 alternative amino acids caused by the frameshift. 1823delCT is expected to lack one NLS. No clinical differences were identified in the present study between patients with nonsense or frameshift mutations retaining one NLS (cases 12-16 and their family members) and patients with mutations lacking both NLS.

We identified three major polymorphisms; C2249G (33/108; 31%), D418D (GAC→GAT, 38/93; 41%) and A541T (GCA→ACA, 24/94; 26%). Frequencies of C2249G and A541T in Japanese subjects were higher than those in Caucasian populations (11-13) as reported previously (21, 22).

Discussion

Full-length sequencing of the open reading frame of the *MEN1* gene is relatively simple because the coding region of the *MEN1* cDNA is as short as 2 kb. We achieved the highest detectability (16/16; 100%) of *MEN1* mutations in familial *MEN1* among studies which examined more than 10 families. *MEN1* germline mutations were identified in more than 85% of *MEN1* families investigated when full-length sequencing of the open reading frame was performed (11, 12, 15), and in about 60% when some screening technique such as single strand conformational variant analysis was applied (13, 14, 16). Therefore, analysis of the *MEN1*

Table 2 Summary of clinical features of the probands of familial MEN1 in the present study.

Case	Age ^a	Sex	Parathyroid lesions	Anterior pituitary lesion	Enteropancreatic neuroendocrine lesions	Other lesions	Germline <i>MEN1</i> mutation ^b
1	54	F	Single	—	Insulinoma	—	299insCCAGC ^e
2	34	M	Single	PRL	Multiple carcinoids	—	357del4
3	30	M	Multiple	PRL	Multiple NFTs	—	W183R (TGG→CGG) ^f
4	62	M	Single	—	Gastrinoma	—	G225R (GGA→AGA) ^f
5	38	F	Single	—	NFT	Adrenal NFT	C241Y (TGT→TAT) ^f
6	46	F	Multiple	—	Multiple insulinomas	—	S253P (TCG→CCG) ^f
7	51	F	Single	—	insulinoma	—	894-9G→A ^f
8	20	F	Single	—	Inappropriate secretion of insulin	—	1001delC ^f
9	50	F	Single	—	NFT	—	1142delG ^f
10	26	F	Multiple	PRL	—	Lipoma	Q450X (CAG →TAG)
11	32	F	Multiple	—	NFT	—	1460+1del14insAT ^f
12	24	F	Multiple	—	WDHA syndrome	—	1650insC
13	64	F	Single	PRL	Gastrin, calcitonin-secreting tumor	—	1650insC
14	44	F	Single	PRL	Zollinger–Ellison syndrome	—	1667insT ^f
15	75	M	Multiple	—	VIPoma, duodenal carcinoid	—	1717delA ^f
16	39	M	Single	PRL	Hypergastrinemia	Adrenal NFT	1823delCT ^f

^aAge at genetic analysis; ^bstandard nomenclature (19); ^c number of retained wild-type amino acids; ^dincluding proband; ^einsertion of CCAGC at 299 (after 298 and before 299); ^fnovel mutation.

PRL, prolactinoma; NFT, non-functioning tumor; AS, abnormal splicing; WDHA, watery diarrhea hypokalemia achlorhydria; VIP, vasoactive intestinal polypeptide.

gene in *MEN1* is clinically significant, although a small proportion of cases of familial *MEN1* might be due to germline mutations in unknown genes. This is in marked contrast to hereditary non-polyposis colorectal cancer which can be caused by a mutation in different genes and genetic analysis of which is categorized as belonging to Group 2 cancer predisposition testing by the American Society of Clinical Oncology (23, 24). Genetic analysis is essential because half or a quarter of *MEN1* cases will be missed by examination of only symptomatic abnormalities or by biochemical screening respectively (12). Thus, among 201 *MEN1* mutant carriers, 100 (50%) had clinical symptoms, 55 (27%) had biochemical abnormalities without clinical symptoms and 46 (23%) were unaffected both clinically and biochemically (12). As it is highly likely that germline *MEN1* mutations will be found in a proband of familial *MEN1*, it is also likely that we shall be able to determine whether a patient's family member is a mutant gene carrier or not. If a family member of *MEN1* who had histories of urolithiasis or gastric ulcer is shown to be without *MEN1* mutation, it relieves anxiety concerning possible development of *MEN1*-related lesions. Definite and simple determination of carrier status is an apparent superior point of genetic diagnosis in comparison with biochemical screening which can be meaningful with positive results, but never exclude carrier status. It was proposed that mutant *MEN1* gene carriers should be followed up for early diagnosis of *MEN1*-related lesions by annual biochemical testing and radiological examinations every 3–5 years (3). Medical care is dependent on the results of gene diagnostics; analysis of the *MEN1*

gene should thus be considered a standard procedure. We propose that analysis of the *MEN1* gene should be categorized as belonging to Group 1 cancer predisposition testing by the American Society of Clinical Oncology (23, 24).

Thakker *et al.* (25) suggested three potential hot spots for *MEN1* mutations; i.e. codons 83 and 84, 209–211 and 514–516. Mutations involving these regions are caused by mechanisms such as DNA-polymerase slippage errors due to short repeats (12), resulting in deletions and insertions but not missense mutations. It is necessary to clarify whether the missense mutations or in-frame deletions actually affect unknown function(s) of menin (11). It is of note that 45 reported missense mutations and in-frame deletions and four novel missense mutations in the present study were at residues conserved between human and mouse homologues (26).

Exon skipping was expected in case 11 with 1460+1del14insAT (27, 28), but genomic sequences such as AGGTCTGGG (seven out of nine identical) or ACAGGTG (seven out of seven identical), highly homologous to and at a short distance from the original splice site ACAG/gtgaggg, may be used as cryptic sites (28). It has been reported that there are no AG dinucleotides in the acceptor site from –15 to –5 (28, 29). Therefore, 894–9G→A creating a new AG at the junction of intron 4 and exon 5 can be a new splice-acceptor site leaving seven bases at the 3'-end of intron 4. Mutch *et al.* (16) directly confirmed this splice abnormality in the transcript. Furthermore, the same mutation was identified constitutionally in a sporadic case with adrenocortical tumor which also showed a somatic stop codon mutation (30). On the basis of these

Table 2 continued

Location	Mutant truncated protein ^c	Identified polymorphic site			Family examination ^d	Identified carrier	Identified non-carrier	Reference
		Genome 2249	cDNA 1364	cDNA 1731				
Exon 2	69	C/C	C/C	G/A	Sequence	1	2	—
Exon 2	84	C/C	C/T	G/A	Electrophoresis	2	0	—
Exon 3	Missense	C/G	C/C	G/A	<i>Sma</i> I	3	0	31
Exon 4	Missense	C/G	C/T	G/A	<i>A/w</i> I	4	1	—
Exon 4	Missense	C/C	C/T	G/A	—	1	0	—
Exon 4	Missense	C/G	C/C	G/A	Sequence	2	0	32
Intron 4	AS	C/G	C/T	G/G	<i>Alu</i> I	5	5	33
Exon 6	297	C/C	C/C	A/A	—	1	0	—
Exon 7	344	C/G	C/C	G/A	Sequence	3	0	—
Exon 9	449	C/C	T/T	G/G	—	1	0	—
Intron 9	Possible AS	C/C	C/T	G/A	—	1	0	34
Exon 10	515	G/G	C/C	G/G	—	1	0	35
Exon 10	515	C/C	C/T	G/A	—	1	0	—
Exon 10	519	C/G	C/T	G/G	—	1	0	36
Exon 10	535	C/G	C/T	G/G	<i>Dde</i> I	10	4	37
Exon 10	571	G/G	C/C	G/G	<i>Xho</i> I	3	4	38,39

three reports, we conclude that 894–9G→A is an apparently disease-causing mutation, although Bassett *et al.* (12) reported that this alteration was a benign polymorphism with an incidence of 3%. A possible explanation for their results is that the alteration gives rise to the MEN1 phenotype in most populations but not in a specific population, which would suggest the influence of different modifying backgrounds or different environmental influences. Otherwise, it might have been simply due to a typographical error.

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