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Genetic Screening in Hereditary Multiple Endocrine Neoplasia Type 1: Absence of a Founder Effect among Japanese Families

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Ten Japanese families with hereditary multiple endocrine neoplasia type 1 (MEN1) were examined. Five DNA polymorphic markers on the long arm of chromosome 11 were analyzed for genetic screening of MEN1 in members of affected families, and disease carriers were identified before clinical manifestations. Unlike MEN1 families in Newfoundland or in Tasmania, no consistent haplotypes were segregated with the disease in the Japanese families when defined by 5 nearby markers. The identification of asymptomatic disease carriers is of substantial clinical importance for early management, genetic counseling and to avoid unnecessary screening for non-disease carriers. However, genetic screening of family members by polymorphic markers could be useful only to each family, and no generally applicable markers were found for Japanese subjects with MEN1.

Key words: Multiple endocrine neoplasia type 1 — Microsatellite polymorphism — Linkage analysis — Polymerase chain reaction — Loss of heterozygosity

Familial multiple endocrine neoplasia type 1 (MEN1) is a hereditary disease characterized by hyperplastic or neoplastic disorder of endocrine organs. This syndrome is inherited in an autosomal dominant manner with a high degree of penetrance.[1)] Most subjects with MEN1 manifest parathyroid hyperfunction in addition to at least one more endocrinopathy, including pancreas tumor, pituitary tumor, and carcinoid tumor. The genetic locus for MEN1 has been assigned to the long arm of chromosome 11 (11q13) by linkage analysis and deletion mapping in tumors,[2,3)] although the gene responsible for this disease is not yet identified.[4,5)] Loss of heterozygosity at this locus in endocrine tumors from subjects with MEN1 has been demonstrated,[6,7)] and it was speculated that this gene may be a tumor suppressor gene.

Analysis of polymorphic markers closely linked to MEN1 such as *PYGM* locus has made it possible to identify carriers of this disease within affected families with considerable accuracy.[8–10)] Completion of the linkage map of the human genome has made linkage analysis more reliable.[11)] In this study, we applied microsatellite polymorphism for genetic screening in families with hereditary MEN1, and identified disease carriers before clinical manifestations. Unlike MEN1 families in Newfoundland[12)] or Tasmania,[13)] an apparent founder effect was not observed among the Japanese families studied.

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MATERIALS AND METHODS

Genetic study Blood samples were obtained from patients with MEN1 and their family members after written informed consent had been obtained. High-molecular-weight DNA was isolated from 0.5 ml of whole blood using a DNA Extractor WB Kit (Wako Pure Chemicals, Osaka) and used for polymerase chain reaction (PCR). Genomic DNA was also prepared from tumor tissues embedded in paraffin by proteinase K digestion and phenol/chloroform extraction. The following oligonucleotide primers were synthesized and used for genotyping five polymorphic markers.

D11S1326,[14)]

forward primer; 5′-TGCCAAGAACAGCAAAA-3′
reverse primer; 5′-GGGGTTCAAAATAAACAAAA-3′

D11S1313,[14)]

forward primer; 5′-CTAAGCATGAAGCCAAGTTA-3′
reverse primer; 5′-AGTTTGACATTAGGGAATTTTG-A-3′

(CA)(GA) repeat in the *PYGM* locus,[15)]

forward primer; 5′-CTAGCAGAGTCCAGGTACTG-3′
reverse primer; 5′-GCTGTCAGGTAGCAACTGAC-3′

D11S913,[14)]

forward primer; 5′-CATTTGGGAAATCCAGAAGA-3′
reverse primer; 5′-TAGGTGTCTTATTTTTTGTTGC-TTC-3′

D11S534,[16]

forward primer; 5′-ATATGGAAACTCTCCGTACT-3′
reverse primer; 5′-GCAACCATGGAGAGTCTGGA-3′

The recombination rate between D11S1326/D11S1313 and D11S913 is 7%.[14] For genotyping, one of the two oligonucleotide primers was end-labeled with [γ-^{32}P]-ATP (ICN, Costa Mesa, CA) and T4 polynucleotide

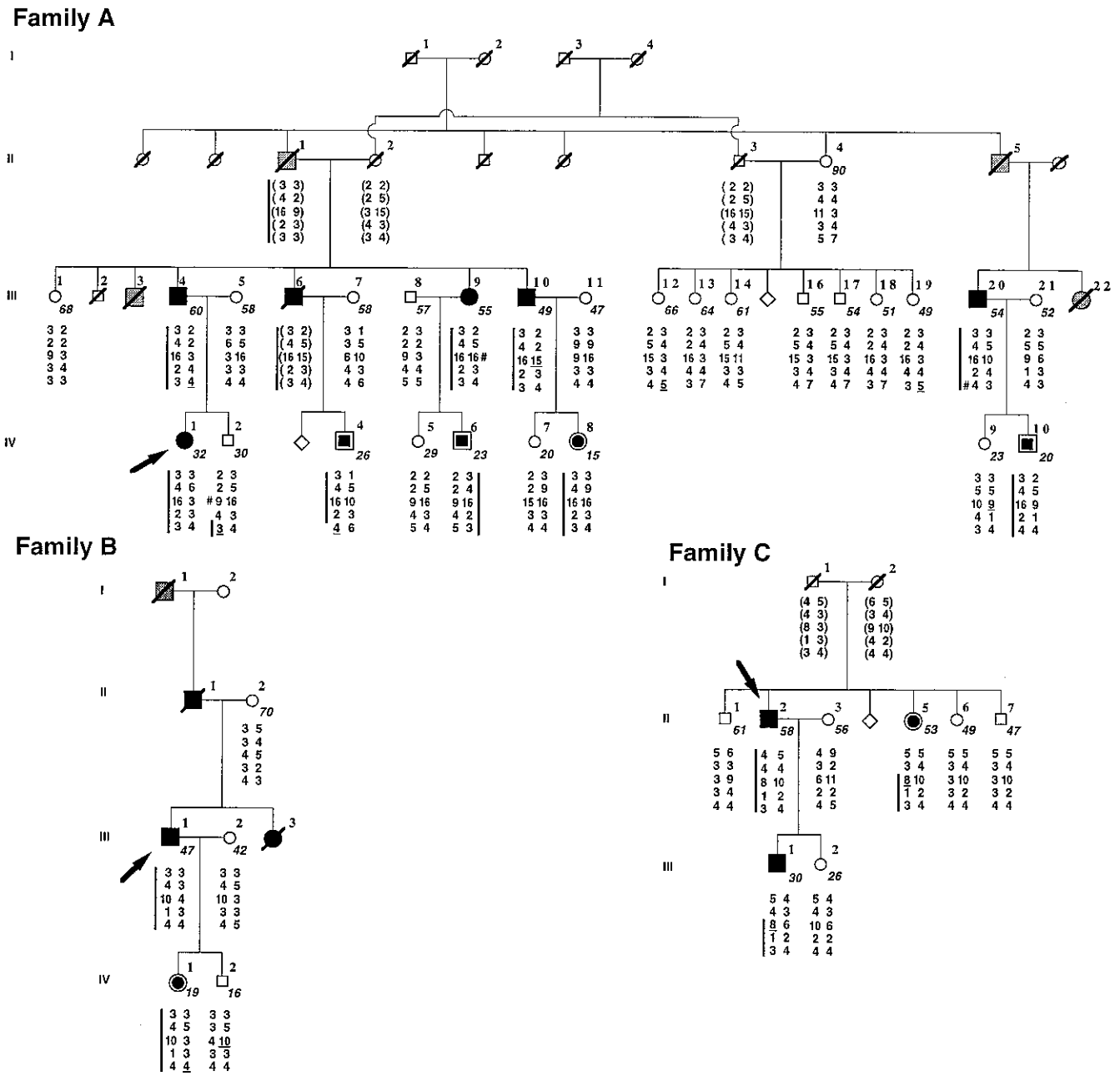
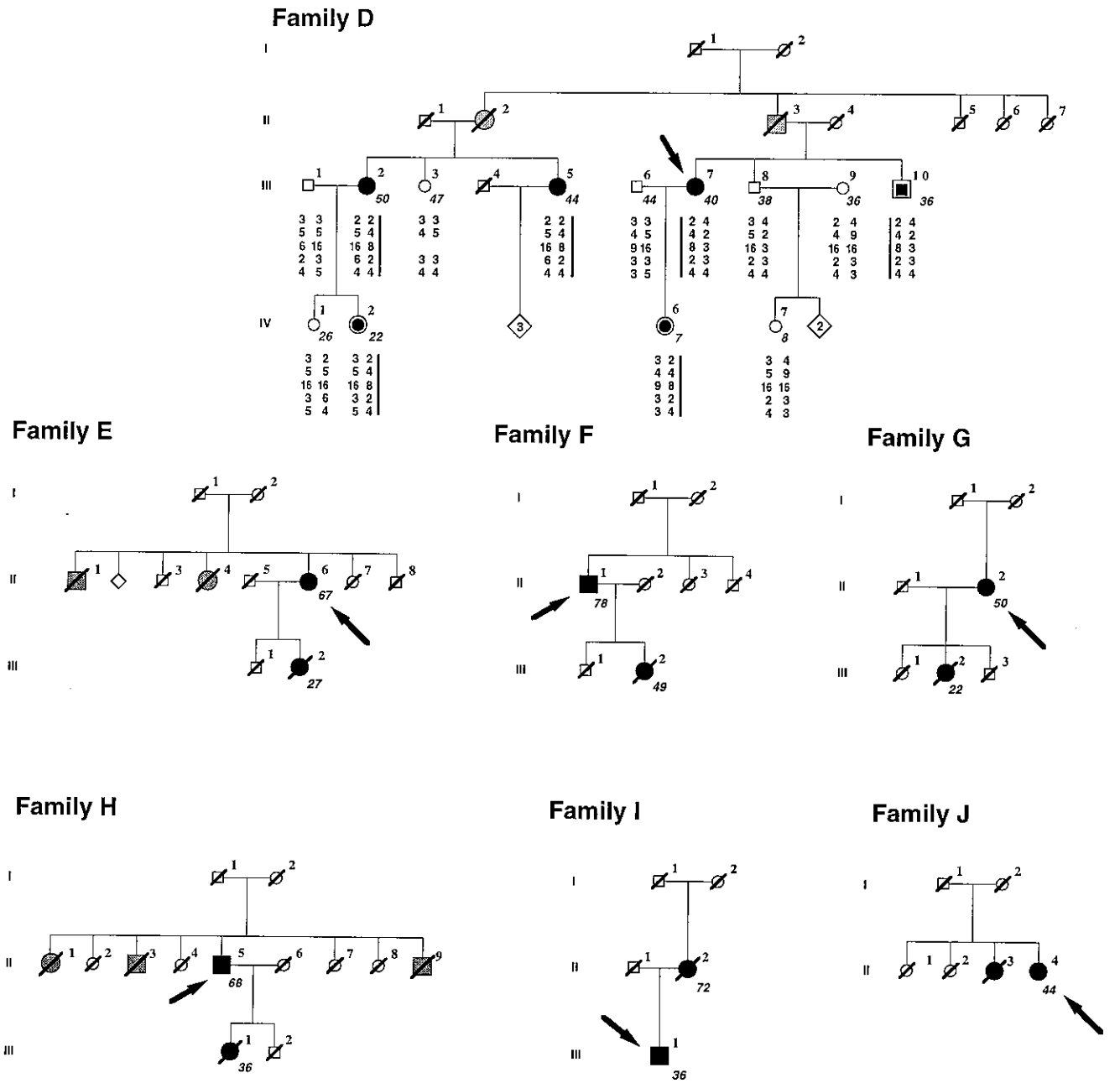


Fig. 1. Pedigrees of ten families with MEN1 are shown. For families A, B, C and D, alleles for the analyzed loci are indicated below each individual. Alleles shown in this figure are, from the top (centromeric) to the bottom (telomeric), D11S1326, D11S1313, (CA) (GA) repeat in the *PYGM* locus (*PYGM*-CAGA), D11S913, and D11S534. Paternal and maternal haplotypes are aligned at left and right, respectively. D11S1326 and D11S1313 are localized very close to each other, but the relative positions of the two loci are not known. The MEN1 locus exists between *PYGM*-CAGA and D11S913. Haplotypes linked to ↗

kinase (Takara Biomedicals, Kyoto), and approximately 50,000 cpm of ^{32}P-labeled primer was included in the PCR mixture. Each PCR cycle consisted of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C. Amplified fragments were separated by gel electrophoresis in 5% polyacrylamide gels containing 8 *M* urea. Gels were dried in vacuum and exposed to Fuji New RX X-ray film for 24 h.



↘ MEN1 in each family are marked with bars. Haplotypes inferred from the analyses of other family members are shown in parenthesis. When meiotic crossing-over was observed, the nearest telomeric markers are underlined. Probands in each family are indicated by arrows. "#" denotes *de novo* mutation. Closed symbol, affected with MEN1; shaded symbol, suspected MEN1 but insufficient clinical information for diagnosis; closed symbol within open symbol, possible MEN1 carrier identified by genetic analysis.

Table I. Clinical Features of Affected Subjects with Hereditary MEN1

Family	Subject	Affected organs					
		Parathyroid	Pancreas	Adrenal	Pituitary	Carcinoid	Others
A	III-4	+	Ins, Gast	NF	NF	Thymus	
	III-6	+	Ins, Gast		GH		
	III-9	+	NF		PRL		Thyroid adenoma
	III-10	+	NF	NF			
	III-20	+	Gast	NF			
	IV-1	+	Ins		GH		
B	II-1	+				Thymus	
	III-1	+	Som			Duodenum	
	III-3	+	Ins				
C	II-2	+	Gluc				
	III-1		Ins				
D	III-2	+	Gast				
	III-5	+	Gast				
	III-7	+	Ins, Gluc	Cortisol			
E	II-6	+	Gast	Cortisol	NF		
	III-2	+					
F	II-1	+	Gast	NF	NF	Duodenum	GRH[a)]
	III-2	+			NF	Duodenum	
G	II-2	+			NF		
	III-2		Gast				
H	II-5	+	Gast		PRL	Duodenum	
	III-1	+	NF		PRL		
I	II-2	+	Ins				
	III-1	+	NF			Thymus	
J	II-3	+	VIP				
	II-4	+	Gluc		GH, PRL		

Affected lesions were indicated by oversecreted hormones. All parathyroid lesions were adenoma or hyperplasia with hypersecretion of parathyroid hormone. For carcinoids, the localization is shown.
Ins, insulin; Gast, gastrin; Gluc, glucagon; Som, somatostatin; VIP, vasoactive intestinal polypeptide; GH, growth hormone; PRL, prolactin; GRH, growth hormone-releasing hormone; NF; non-functioning tumor.
a) Blood GRH level was elevated, but its origin could not be identified.

Subjects The pedigrees of affected families analyzed in this study are illustrated in Fig. 1, and clinical features of the affected subjects are summarized in Table I. Diagnosis of MEN1 was made on the basis of evidence of hypersecretion or neoplastic lesions in at least two endocrine organs. Carcinoid tumor was counted as one endocrine lesion. For children of affected subjects, diagnosis was made on the basis of evidence of one lesion. All these diagnoses had been made before genetic analysis. All families except family D have lived in Nagano prefecture of Japan (northwest of Tokyo, 13,584 km^2, population 2.15 million) for more than three generations.

In family A, II-1 and II-4, II-2 and II-3 were descendants of I-1 and I-2, and I-3 and I-4, respectively, but there was no consanguinity. II-1 died at the age of 52 due to massive hematemesis. Endocrinological examinations had, however, not been performed. II-5 had gastrinoma and died at the age of 52. III-22 died at the age of 40 with massive bleeding from long-term (over 20 years) gastric ulcer. She had not undergone endocrine function study. III-3 died of massive hematemesis at the age of 29. He also had an acromegalic appearance.

In family B, I-1 had long-term gastric ulcer, but the primary cause of death is unknown. II-1, who had hyperparathyroidism, died of distant metastasis of malignant thymic carcinoid. II-2 is 70 years old and has no neoplastic disease or endocrinopathy.

In family C, I-1 and I-2 died of cerebral infarction and acute myocardial infarction, respectively. Endocrine function tests had not been performed before death.

In family D, II-2 died of perforation of the stomach. No detailed record of her clinical history was available. II-3 died of heart failure at the age of 54, but the primary cause of death is unknown.

In family E, II-1 and II-4 had histories of recurrent peptic ulcer and urolithiasis. Both are dead and there is no information on endocrinopathy.

In family H, I-1 died of cerebral hemorrhage at the age of 59. He had been receiving medication for hypertension. I-2 also had hypertension, and died at the age of 74.

There are no records of their endocrine function. II-1 died of hepatic carcinoid at the age of 50. II-3, who is 71 years old, had a history of urolithiasis more than twenty years ago. He has not received any medical care since then. II-9 had hyperparathyroidism, urolithiasis and peptic ulcer.

In family J, I-1 died of cerebral hemorrhage at the age of 47. I-2 is 83 years old and has no evidence of endocrinopathy. II-3 died of WDHA syndrome at the age of 29.

Clinical examination Clinical examination for affected subjects and family members included determination of the levels of at least serum calcium, phosphate, parathyroid hormone, glucose, insulin, gastrin and prolactin. In selected cases, pancreatic function was assessed with stimulation tests. Roentgenogram of the sella, computed tomography scan and echogram of the parathyroid, pancreas and adrenal cortex were performed for most cases.

RESULTS

The *PYGM* locus has been assigned as centromeric with respect to the MEN1 locus with a recombination rate of less than 1%.[3] (CA)(GA) repeat polymorphism in the *PYGM* locus (*PYGM*-CAGA) is highly polymorphic, and different allele frequencies have been observed among three racial groups, Pima Indians, American blacks, and Caucasians.[15] Therefore we first examined the allele distribution of *PYGM*-CAGA in Japanese. As shown in Table II, thirteen alleles ranging in size from 160 to 190 bp were observed for the *PYGM*-CAGA. This result shows that *PYGM*-CAGA is also highly polymorphic in Japanese, and could be a suitable marker for genetic screening of MEN1. To obtain more reliable information on segregation of the MEN1 gene, we also typed several polymorphic markers which were assigned as centromeric (D11S1326, D11S1313) and telomeric (D11S913, D11S534) of *PYGM*. Allele frequencies of these polymorphic markers in Japanese are also shown in Table II. All markers were highly polymorphic for Japanese, as was the case for CEPH families or Caucasians,[11, 15, 16] and thus could be used for the following study.

Results of genotyping for affected families are shown in Fig. 1. In family A, affected subjects, III-4, III-9, III-10 and IV-1 carried the same haplotype, *cent*-3-4-16-2-3-*qter*, and so this haplotype was thought to be linked to abnormal MEN1 gene. To confirm this, possible loss of heterozygosity was examined in the resected parathyroid adenoma of IV-1. As shown in Fig. 2, the putative normal allele (allele 3) of *PYGM*-CAGA was deleted in the tumor, in accordance with the two-hit theory of tumor suppressor genes originally postulated by Knudson.[17] III-1, the only living sibling without MEN1 at the age of 68, did not carry this haplotype. If we suppose that no meiotic recombination had occurred, genotyping of III-1 and her siblings would enable us to infer the haplotypes of II-1 and II-2 (predicted haplotypes in parenthesis). IV-6 and IV-8 inherited the risk haplotype. IV-2 and IV-4 inherited only part of this

Table II. Distribution of Polymorphic Dinucleotide Repeat Alleles in Japanese

Marker	D11S1326		D11S1313		*PYGM*-CAGA		D11S913		D11S534	
	size (bp)	freq.	size (bp)	freq.	size (bp)	freq.	size (bp)	freq.	size (bp)	freq.
Allele 1	255	0.03	204	0.03	190	0.07	227	0.09	244	0
2	253	0.21	202	0.17	188	0.04	225	0.24	242	0.03
3	251	0.58	200	0.11	186	0.06	223	0.37	240	0.24
4	249	0.17	198	0.13	184	0.03	221	0.30	238	0.24
5	247	0.01	196	0.43	182	0			236	0.26
6			194	0.07	180	0.07			234	0.09
7			192	0	178	0.07			232	0.11
8			190	0	176	0.20			230	0.03
9			188	0.06	174	0.14				
10					172	0.07				
11					170	0.07				
12					168	0				
13					166	0				
14					164	0.04				
15					162	0.01				
16					160	0.11				

Genotypes of 70 chromosomes from 35 unrelated subjects were examined. These subjects are patients in our out-patient clinic and have no endocrinological complications. They all live in Nagano Prefecture of Japan. freq, frequency. Allele 1 of D11S534 was observed in European Caucasians.[16]

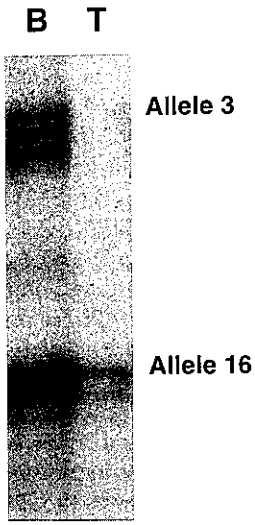


Fig. 2. Allelic loss in a parathyroid tumor of a subject with MEN1. *PYGM*-CAGA of IV-1 of family A is shown. B, DNA from leukocytes; T, DNA from parathyroid adenoma. Note the loss of the normal allele (allele 3) in tumor DNA (see Fig. 1).

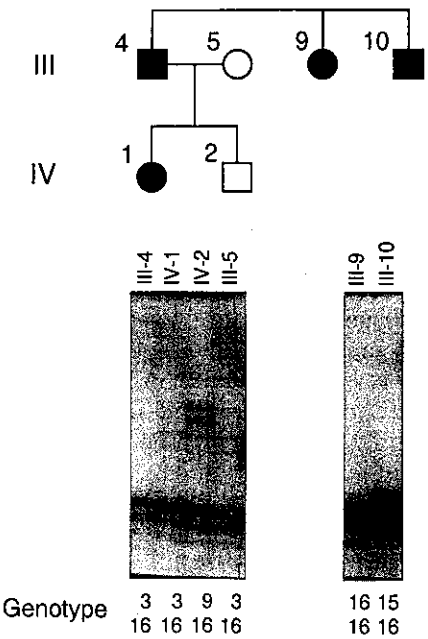


Fig. 3. *De novo* mutations in *PYGM*-CAGA. Part of an autoradiogram showing *PYGM*-CAGA polymorphism in family A. IV-2 carries allele 9, which is not seen in either III-4 or III-5. III-9 and III-10 carry different alleles, which should be identical (see Fig. 1).

haplotype, with meiotic crossing-over between D11S913 and D11S534. IV-4, IV-6 and IV-8 were thus diagnosed as MEN1 carriers, and IV-2 as a non-carrier.

Since II-4 shared no common haplotypes with II-1, II-1 and II-4 were regarded as having inherited different haplotypes from their parents, I-1 and I-2. As a matter of fact, there are no MEN1 patients among II-4 and her descendants. Genotyping of II-4 and her children enabled us to predict the haplotype of II-3.

We have no available clinical records which suggest that II-5, a sibling of II-1, was affected with other endocrinopathies than gastrinoma. However, a family study strongly indicates that he was a carrier of MEN1 gene since his son (III-20) is affected with MEN1 and the clinical history of his daughter (III-22) suggests she might have been affected with Zollinger-Ellison syndrome. In fact, III-20 carries a haplotype which is highly homologous to that identified in other affected subjects in this family. The allele of D11S534 in a paternal allele of III-20 (allele 4) does not match that of other affected members who carry allele-3, and allele-4 is not seen in either II-1 or II-4. This allele had probably been generated *de novo* in III-20 or II-5. Alternatively, allele-3 in the affected haplotype of II-1 might have been generated *de novo*. The putative risk haplotype of III-20 was transmitted to IV-10, who was thus diagnosed as a possible MEN1 carrier. Similar conversions were also observed in the maternal allele of *PYGM*-CAGA of III-9 (allele 15→16), and in the paternal allele of *PYGM*-CAGA of IV-2 (allele 3→9) (Fig. 3). For the latter cases, the possibility of non-paternity should be considered since a new mutation involving a change of multiple repeats (loss of six repeats in this case) is very rare. However, we did not examine this possibility further because the patients' informed consent covered only examination of the MEN1 locus, so assessment of parentage was regarded as improper.

We next typed other affected families. In family B, it is suspected that I-1 and II-1 were affected with MEN1 from their clinical histories. Although the genotype of II-1 was not available, the affected haplotype could be identified by typing II-2 and III-1. The risk haplotype in III-1 was transmitted to IV-1.

In family C, different alleles of *PYGM*-CAGA and other telomeric markers were transmitted from the proband (II-2) to affected (III-1) and healthy (III-2) offspring, identifying a risk haplotype in this family. Part of this haplotype, including the MEN1 locus, was also transmitted to II-5, who has no clinical evidence of MEN1 at the age of 53.

In family D, although there was no clinical evidence, transmission of a common haplotype to three affected subjects (III-2, III-5 and III-7) strongly suggests that II-

Table III. Genotyping of MEN1 Subjects for Polymorphic Markers on Chromosome 11

Family	F-A	F-B	F-C	F-D	F-E	F-F	F-G	F-H	F-I	F-J
D11S1326	3	3	4	2	2, 4	3, 3	2, 4	2, 2	3, 3	2, 3
D11S1313	4	4	4	4	2, 2	3, 4	2, 2	4, 4	3, 5	2, 5
PYGM-CAGA	16	10	8	10	9, 16	16, 16	9, 16	11, 16	9, 10	10, 10
D11S913	2	1	1	2	2, 3	4, 4	2, 3	3, 4	2, 2	3, 4
D11S534	3	4	3	4	4, 4	4, 4	4, 4	4, 4	4, 6	4, 4

F-A to F-I are genotypes of polymorphic markers in probands of family A to family I, respectively. For families A to D, only risk haplotypes are shown. For other families, genotypes of the probands are shown.

Table IV. Affected Subjects Identified after Genetic Screening

Family	Subject	Lesion	Elevated hormone		Normal
A	IV-6	Pituitary	PRL	32 ng/ml	1.5–9.7
		Parathyroid	PTH-intact	69 pg/ml	15–50
			Ca	10.8 mg/dl	8.5–10.2
			IP	3.7 mg/dl	2.5–4.5
		Pancreas	NF		
A	IV-8	Parathyroid	PTH-intact	75 pg/ml	14–66
			Ca	10.4 mg/dl	8.6–10.1
			IP	3.9 mg/dl	2.2–4.1
B	IV-1	Parathyroid	PTH-intact	52 pg/ml	15–50
			Ca	10.9 mg/dl	8.5–10.2
			IP	3.2 mg/dl	2.5–4.5
D	III-10	Parathyroid	PTH	2900 pg/ml	160–520
			Ca	11.0 mg/dl	8.5–10.0
			IP	3.9 mg/dl	2.4–5.0
		Pancreas[a)]	Gastrin	480 pg/ml	<200
			Glucagon	190 pg/ml	40–180

NF, non-functioning tumor.
a) Tumor was not identified.

2 and II-3 were also affected with MEN1, or were at least asymptomatic carriers of the abnormal gene. The putative risk haplotype was transmitted to IV-2 and IV-6. III-10 also carried this haplotype.

Risk haplotypes in families A to D were thus different, which indicates that genetic abnormalities in each family had occurred independently, or if they have separated from common affected ancestors, this should have occurred in early generations. For six other affected families, families E to J, we could obtain blood samples for genetic analysis only from probands. Due to the lack of genetic information from other family members in these families, the phased haplotype segregated with the disease was not determined. The results are summarized in Table III. No apparent similarity of genotypes to those of families A to D were observed. For instance, allele 16 of *PYGM*-CACA, which was seen in the affected haplotype of family A, was observed in families E, F, G, and H. However, none of these families shared the same haplotype as was seen in family A, *cent*-3-4-16-2-3-*qter*. On the other hand, the genotypes of families E, F, G and H were highly similar to each other, and the probands of families E and G had identical genotypes. Some of these families thus could have a common founder. Although genotyping of other family members is necessary to examine this possibility, we were unable to do so because either we could not contact them, or we could not obtain their informed consent.

Genotyping of family members in families A to D identified putative genetic carriers in their youngest generations (Fig. 1). To confirm the reliability of our genetic screening and to detect asymptomatic manifestation of MEN1, most of the carriers and some of the non-carriers underwent clinical examinations for endocrinopathies (in family D, only III-10 took the tests). As shown in Table IV, four genetically identified carriers were diagnosed as MEN1. None of the non-carriers showed abnormal findings.

DISCUSSION

Although most of the neoplastic lesions in subjects with MEN1 are benign, malignant tumors are also seen. The mean age of death in subjects with MEN1 is significantly younger than that of the normal population, primarily as a result of peptic ulcer and complications related to malignant neoplasms.[18, 19] Identification of asymptomatic carriers in MEN1 families is therefore important for the early detection and early management of complications. Periodical biochemical screening for members of MEN1 families has been successful for this purpose.[20] However, this screening program is costly and life-time screening would be required for non-disease carriers in an affected family. On the other hand, genetic diagnosis could be made at (or even before) birth based on a single blood sample to extract genomic DNA. This would also be beneficial to avoid unnecessary follow-up for non-disease carriers. Although the gene for MEN1 has not yet been identified, a recent genetic map has provided many polymorphic markers closely linked to the MEN1 locus, and has made linkage analysis more accurate.[11] We performed genetic screening of MEN1 in Japanese families using multiple polymorphic markers and identified asymptomatic disease carriers. It is generally accepted that the *PYGM* locus is the most useful polymorphic marker for the genetic screening of MEN1 because the recombination rate is low, and *PYGM* is highly polymorphic.[3, 8, 15] However, we found *de novo* mutations in the dinucleotide repeat polymorphic region (*PYGM*-CAGA) of this locus in several cases. *De novo* meiotic mutations were not observed for other polymorphic markers, D11S1326, D11S1313 and D11S913, and a possible mutation was seen for D11S534 in one subject (III-20 of family A, described above). This implies that this region could be relatively unstable and thus linkage analysis using only this locus could be misleading. Multiple polymorphic markers located both centromeric and telomeric of the MEN1 locus should be used for linkage analysis. *PYGM* contains another highly polymorphic dinucleotide repeat segment, the (AT)n repeat element, and this element should also be useful.[15]

Ideally, the logarithm of likelihood ratio for linkage (LOD) score should be calculated for the families we studied to confirm linkage between the disease and the MEN1 locus. However, this was not possible because the number of subjects eligible for analysis was not enough for reliable estimation. This is important, because in some hereditary diseases, genetic heterogeneity has been reported.[21, 22] The possibility of a genetic variation among different racial groups could also be considered. However, all reported MEN1 families demonstrate tight linkage at the long arm of chromosome 11 (11q13) regardless of their ethnic origin[2, 3, 8–10, 23, 24] and no other genes or genetic loci linked to the MEN1 phenotype have been reported. Loss of heterozygosity at chromosome 11 in tumors from MEN1 patients was observed in Japanese patients as well as in patients from other ethnic groups,[25, 26] and we also observed loss of the normal allele in a parathyroid tumor of one affected subject (Fig. 2). These observations clearly imply that all familial MEN1 is linked to chromosome 11q13 and there are no racial variations. If the inferred haplotypes of II-1 in family A (Fig. 1 in parenthesis) could have been confirmed, the LOD score for linkage between MEN1 phenotype and *PYGM*-CAGA in this family (allele-16) would have been 2.966 at $\theta = 0.01$. The calculated chance of double recombination both centromeric (between *PYGM*-CAGA and MEN1 locus) and telomeric (between MEN1 locus and D11S913) to the MEN1 locus is less than approximately 0.0002.

It is of interest that II-5 of family C, who carries a common allele with the proband (allele 8 of *PYGM*-CAGA), showed no clinical evidence of MEN1 at the age of 53. It is possible that II-5 carries the abnormal MEN1 gene, but is not clinically affected due to incomplete penetrance. About 15% of subjects with abnormal MEN1 gene would be negative in biochemical screening at fifty years old.[27] However, using sophisticated screening programs including stimulation tests, nearly 100% of asymptomatic carriers could be identified at this age.[27] Despite our proposal, she has not been willing to take those tests. Alternatively, mutation in the MEN1 gene might have occurred sporadically in the proband. Parents of the proband (I-1 and I-2) have already died and it is uncertain if one of them were affected with MEN1. The reported causes of their death were cerebral infarction and acute myocardial infarction.

$MEN1_{Burin}$ is a hereditary syndrome of prolactinoma, hyperparathyroidism and carcinoids. The gene for this disease is mapped to chromosome 11q, the same region as typical MEN1.[12] Linkage analysis of four Newfoundland families with $MEN1_{Burin}$ revealed a founder effect. In Tasmania, a careful survey of family trees revealed that over 150 MEN1 patients could be traced back to a single founder.[13] This type of study has not been performed in Japanese subjects. Our present results demonstrate that, in contrast to MEN1 families in Newfoundland or Tasmania, no apparent founder effect exists among MEN1 families in Japan. Risk haplotypes of affected families were not homologous, and in family C, there was a possibility of a *de novo* mutation in the MEN1 gene of the proband. It is thus concluded that mutations of MEN1 gene in these families have arisen independently or they have separated from common affected ancestors in early generations. One aim of genetic diagnosis of hereditary diseases is to decrease morbidity and mortality due to those diseases by prevention, early detection, and early

management through intensive follow-up of individuals at risk. However, during the present study, various family members were unwilling to take genetic tests. If this strategy is to be utilized, the ethical problems, legal issues and psychological impact on individuals have to be carefully considered and resolved.[28, 29]

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