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Characterization of Mutations in Patients with Multiple Endocrine Neoplasia Type 1

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Summary

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disorder characterized by tumors of the parathyroids, pancreatic islets, and anterior pituitary. The MEN1 gene, on chromosome 11q13, has recently been cloned, and mutations have been identified. We have characterized such MEN1 mutations, assessed the reliability of SSCP analysis for the detection of these mutations, and estimated the age-related penetrance for MEN1. Sixty-three unrelated MEN1 kindreds (195 affected and 396 unaffected members) were investigated for mutations in the 2,790-bp coding region and splice sites, by SSCP and DNA sequence analysis. We identified 47 mutations (12 nonsense mutations, 21 deletions, 7 insertions, 1 donor splice-site mutation, and 6 missense mutations), that were scattered throughout the coding region, together with six polymorphisms that had heterozygosity frequencies of 2%–44%. More than 10% of the mutations arose de novo, and four mutation hot spots accounted for >25% of the mutations. SSCP was found to be a sensitive and specific mutational screening method that detected >85% of the mutations. Two hundred and one MEN1 mutant-gene carriers (155 affected and 46 unaffected) were identified, and these helped to define the age-related penetrance of MEN1 as 7%, 52%, 87%, 98%, 99%, and 100% at 10, 20, 30, 40, 50, and 60 years of age, respectively. These results provide the basis for a molecular-genetic screening approach that will supplement the clinical evaluation and genetic counseling of members of MEN1 families.

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Introduction

Multiple endocrine neoplasia type 1 (MEN1; MIM 131100 [http://www3.ncbi.nlm.nih.gov:80/htbin-post/Omim/dispmim?131100]) is an autosomal dominant disorder characterized by the combined occurrence of tumors of the parathyroid glands, the pancreatic islet cells, and the anterior pituitary (Wermer 1954; Thakker 1995; Trump et al. 1996). In addition, adrenal cortical tumors, carcinoid tumors, and lipomas have also been observed in association with MEN1. The autosomal dominant inheritance of this disease indicates that 50% of the children of a patient with MEN1 will have the mutant gene and be at a high risk of developing endocrine tumors. The identification of such individuals, together with an earlier detection of the development of tumors, may help to reduce the morbidity and mortality of these patients (Trump et al. 1996). The recent isolation of the MEN1 gene in conjunction with the characterization of mutations in MEN1 patients (Agarwal et al. 1997; Chandrasekharappa et al. 1997; European Consortium on MEN1 1997*b*), may facilitate this assessment.

The MEN1 gene, which represents a putative tumor-suppressor gene (Varmus 1984; Thakker 1993; Brown and Solomon 1997), had been localized, by genetic-mapping studies, to chromosome 11q13 (Larsson et al. 1988; Thakker et al. 1989), within a <300-kb region flanked centromerically by PYGM and telomerically by D11S1783 (European Consortium on MEN1 1997*a*). Characterization of genes from this region led to the identification of the MEN1 gene (Chandrasekharappa et al. 1997; European Consortium on MEN1 1997*b*), which consists of 10 exons (fig. 1) that encode a novel 610-amino-acid protein, referred to as "MENIN" (Chandrasekharappa et al. 1997). The functional role of MENIN, which does not have homologies to other proteins that are involved in cell proliferation and control of the cell cycle (Pang and Thakker 1994; Brown and Solomon 1997; Chandrasekharappa et al. 1997; European Consortium on MEN1 1997*b*), remains to be elu-

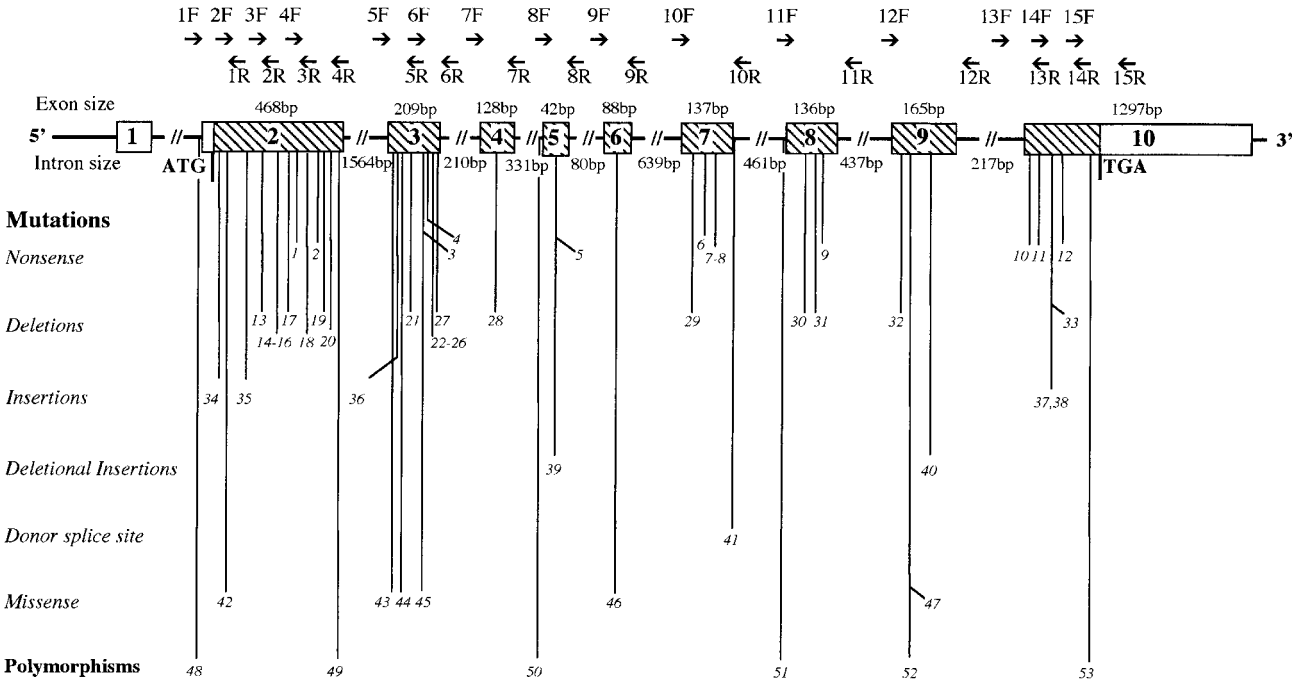

Figure 1 Schematic representation of the genomic organization of the MEN1 gene. The human MEN1 gene consists of 10 exons that span >9 kb of genomic DNA and encodes a 610-amino-acid protein (Chandrasekharappa et al. 1997; European Consortium on MEN1 1997*b*). The 1.83 kb coding region is organized into nine exons (exons 2–10 [*hatched boxes*]) and eight introns (*thicker horizontal line* [not drawn to scale]). The sizes of the exons (range 42–1,297 bp) and introns (range 80–1,564 bp) are shown, and the start (ATG) and stop (TGA) sites in exons 2 and 10, respectively, are indicated. Exon 1, the 5′ part of exon 2 and the 3′ part of exon 10 are untranslated (*nonhatched boxes*). The locations of 15 pairs of the primers (*arrows* [F = forward; and R = reverse]) that were used to amplify the exons and exon/intron boundaries are shown. The sites of the 47 mutations (12 nonsense mutations, 21 deletions, 5 insertions, 2 deletional insertions, 1 donor splice-site mutation, and 6 missense mutations) and of six different polymorphisms are shown below; the details of each of these are provided in table 1.

cidated. However, inactivating mutations of the MEN1 gene (Agarwal et al. 1997; Chandrasekharappa et al. 1997; European Consortium on MEN1 1997*b*), which is ubiquitously expressed as a 2.9-kb transcript, with an additional 4.2-kb transcript being present in pancreas and thymus (European Consortium on MEN1 1997*b*), are associated with the development of endocrine tumors in MEN1 families. We have therefore undertaken studies to characterize the spectrum of MEN1 mutations, with the aims of evaluating the reliability of SSCP for their detection and of defining the age-related penetrance of this disorder, so as to supplement the clinical evaluation of MEN1 patients and their families.

Patients and Methods

Patients

The families of 63 unrelated MEN1 probands were ascertained, and a total of 947 members (479 males and 468 females; age range 2–89 years [mean ± SD 36 ± 18 years]) were assessed (Trump et al. 1996). MEN1 was diagnosed in a family if two or more MEN1-related tumors had been demonstrated in at least one individual; other related members were considered affected only if they had clinical, radiological, and/or surgical evidence of MEN1-related tumors or repeated biochemical evidence of one of the MEN1-associated tumors, as reported elsewhere (Trump et al. 1996). A family history of MEN1 could be established in 57 of the probands (family-size range 3–80 members), and there were 269 affected members (134 males and 135 females) and 672 unaffected members (342 males and 330 females). A familial basis for MEN1 could not be established in six of the MEN1 probands (three males and three females; age range 32–52 years), all of whom had at least three MEN1-related tumors. Venous blood samples were obtained from 189 affected members (92 males and 97 females; age range 8–85 years [mean ± SD 40 ± 16 years]) and 396 unaffected members (184 males and 212 females; age range 2–89 years [mean ± SD 34 ± 19 years]) of the 57 MEN1 families and from the 6 nonfamilial MEN1 patients. A total of 342 tumors occurred in the 195 affected members (189 familial and 6 nonfamilial), and these consisted of 179 parathyroid tumors,

81 pancreatic islet–cell tumors (52 gastrinomas, 17 insulinomas, 6 glucagonomas, and 6 nonfunctioning tumors), 66 anterior-pituitary tumors (55 prolactinomas, 4 somatotrophinomas, 3 corticotrophinomas, and 4 nonfunctioning tumors), 7 carcinoid tumors, 6 adrenal cortical tumors, and 3 lipomas.

DNA Hybridization and Microsatellite Polymorphism Analysis

DNA from leukocytes was prepared by standard methods (Thakker et al. 1989), and RFLPs and microsatellite polymorphisms were detected by methods described elsewhere (Thakker et al. 1989; Pang et al. 1996; European Consortium on MEN1 1997*c*). Ten polymorphic microsatellite loci (CNTF, D11S480, D11S1883, D11S457, PYGM, D11S449, D11S913, D11S97, INT2, and D11S533) from chromosome 11q13 were used as described elsewhere (Pang et al. 1996; European Consortium on MEN1 1997*c*) to determine the haplotypes around the MEN1 locus, and the five loci (D11S1883, PYGM, D11S449, D11S97, and D11S533) each with eight or more alleles and a heterozygosity >75% (Pang et al. 1996; European Consortium on MEN1 1997*c*) were used to exclude nonpaternity, as described elsewhere (Pearce et al. 1995; Pang et al. 1996).

DNA Sequence Analysis of the MEN1 Gene

DNA sequence abnormalities were initially sought in each of the 63 MEN1 probands, by SSCP analysis (Grompe 1993; Pearce et al. 1995; Lloyd et al. 1997). Fifteen pairs of primers (European Consortium on MEN1 1997*b*) were used for the PCR amplification of the nine coding exons of the MEN1 gene (fig. 1) and of their corresponding 16 exon/intron boundaries, with conditions as described elsewhere (European Consortium on MEN1 1997*b*). The PCR products were analyzed for SSCPs, by the Phast electrophoresis system (Pharmacia Biotech), as reported elsewhere (Pearce et al. 1995; Lloyd et al. 1997), by the method of silver staining, with 0.025 M aqueous silver nitrate for 10 min. Genomic DNA samples from 10 unrelated normal individuals were also used, as controls, in the SSCP analysis. The DNA sequence of abnormal SSCPs was determined by the use of *Taq* polymerase cycle sequencing and a semiautomated detection system (ABI 373A sequencer; Applied Biosystems) (Pearce et al. 1995; Lloyd et al. 1996, 1997). In addition, these DNA sequence abnormalities were confirmed either by restriction-enzyme analysis of genomic PCR products obtained by the use of the appropriate primers, or by allele-specific oligonucleotide (ASO) hybridization analysis (Thakker et al. 1993), or by agarose-gel electrophoresis (GE) (Pearce et al. 1995; Lloyd et al. 1996, 1997). These DNA sequence abnormalities were also demonstrated to cosegregate with the disorder and to be absent as common polymorphisms in DNA obtained from 55 unrelated normal individuals (27 males and 28 females). In order to assess the sensitivity and specificity of SSCP analysis in the detection of the mutations, the DNA sequence of the entire 2.79 kb of the coding sequence, together with the exon/intron boundaries of the MEN1 gene, was determined and compared with the SSCP results obtained in 10 MEN1 probands; the mutations in 3 of these probands, from families 18, G/87, and H/94, have been reported elsewhere (European Consortium on MEN1 1997*b*). Each of the 10 probands had developed parathyroid, pancreatic islet–cell, and anterior-pituitary tumors. Southern and northern blot hybridization analyses were used to assess, respectively, genomic deletions of the MEN1 gene and the associated abnormal sizes of the mRNA transcripts, as described elsewhere (Thakker et al. 1993; Pearce et al. 1995; Lloyd et al. 1996, 1997; European Consortium on MEN1 1997*b*).

Statistical Analysis

The means, SDs, standard error of the means, differences between means, and χ^2 values were calculated by the Microsoft Excel program. In addition, nonparametric tests were performed by use of the Mann-Whitney U-test with the STATA program.

Results

Mutational Detection by SSCP

The analysis of 945 SSCPs obtained from the 63 unrelated MEN1 patients revealed a total of 90 abnormal bands (fig. 2). Twenty-seven of the MEN1 patients had one abnormal SSCP, 30 patients had two abnormal SSCPs, 1 patient had three abnormal SSCPs, and 5 patients had no SSCP abnormalities. The occurrence of two or more SSCP abnormalities in a patient suggested the occurrence of polymorphisms, and DNA sequence analysis of all the 90 SSCP abnormalities revealed that 46 (51%) of the SSCP abnormalities were mutations (table 1 and fig. 1), that 38 (42%) were the result of six different polymorphisms, and that 6 (7%) were associated with a normal DNA sequence and thus were false positives. In addition, DNA sequence analysis of the entire 2.79-kb coding region and exon/intron boundaries in 10 MEN1 probands revealed the presence of eight mutations—Trp183stop, Glu388stop, 83–84del4bp (which was observed twice), 210–211del4bp, 327del1bp, 373del1bp, and Trp183Ser (table 1). Seven of these eight mutations (i.e., >85%) could be detected by SSCP analysis, and only the Glu388stop mutation (table 1) could not be detected by SSCP analysis. Mutations were not detected in the remaining two MEN1 unrelated probands, from families 3/92 and 5/94 (Trump et al. 1996),

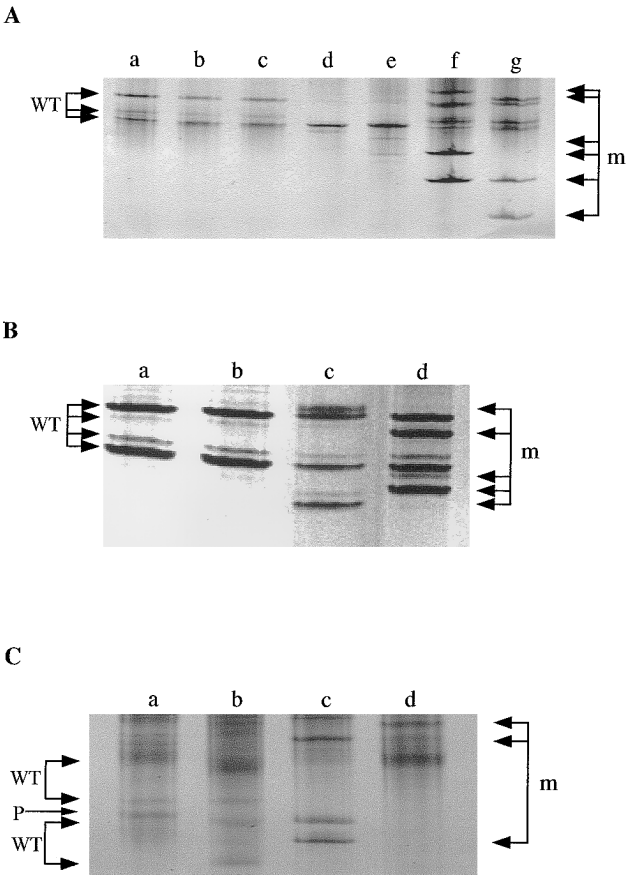

Figure 2 Detection of eight mutations, in exon 2 (*A*), exon 5 (*B*), and exon 9 (*C*), by SSCP. *A,* Results of SSCP analysis of the Gly42Asp (lane d) mutation (table 1), the 10-bp insertion in codons 63–66 (lane e), the 15-bp insertion in codons 34–38 (lane f), and the 11-bp deletion of codons 69–73 (lane g), together with samples from three unrelated normal controls (lanes a–c). The mutant bands (m), lanes d–g, differed from the three wild type (WT) bands. *B,* Results of SSCP analysis of the deletional insertion at codon 262 (lane c) and the Lys262stop mutation (lane d) (table 1), together with bands for samples from two unrelated normal controls (lanes a and b). The mutant bands (m) differed from the four wild-type (WT) bands (lanes a and b). *C,* Results of SSCP analysis of the deletional insertion at codons 440 and 441 (lane c), the Asp418Asn mutation (lane d), and the Asp418Asp polymorphism, C/T (lane a) and C/C (lane b), from two unrelated normal controls. The mutant bands (m) and the polymorphic band (P) differed from the wild-type (WT) bands. SSCP analysis was successful in detecting 85% of the MEN1 mutations.

in whom previous haplotype analysis, using polymorphic loci from chromosome 11q13, had demonstrated cosegregation with MEN1 (European Consortium on MEN1 1996; Pang et al. 1996). These results indicate that ~80% of MEN1 patients are likely to have mutations within the MEN1 coding region and its exon/intron boundaries. Southern and northern blot hybridization analysis in the 63 MEN1 probands did not detect additional abnormalities, indicating, respectively, the absence of genomic deletions and abnormally associated mRNA transcripts. Thus, a total of 47 mutations, 43 of which occurred in familial MEN1 and 4 of which occurred in patients with nonfamilial MEN1 (table 1), were identified in this analysis of 63 unrelated MEN1 patients.

Mutations in MEN1

The 47 mutations occurred throughout the coding exons of the MEN1 gene and also involved one donor splice site (table 1 and fig. 1). Thus, 12 nonsense mutations, 21 deletions, 7 insertions, 1 donor splice-site mutation, and 6 missense mutations were detected. Approximately 80% of these mutations are likely to result in a truncated MEN1 protein and thus to be inactivating, which is consistent with the tumor-suppressor role proposed for the MEN1 gene (Larsson et al. 1988; Thakker et al. 1989). Each of these mutations in the 47 MEN1 probands was confirmed and demonstrated to cosegregate with the disease in the 43 families, either by restriction-enzyme analysis (fig. 3), ASO hybridization analysis (fig. 4), or GE (table 1). In addition, the absence of the DNA sequence abnormalities in 110 alleles from 55 unrelated normal individuals established that these abnormalities were mutations and unlikely to be polymorphisms that would be expected to occur in >1% of the population. A more detailed examination of the mutations, together with the associated clinical abnormalities, revealed several interesting findings. First, four different mutations (table 1) were found to occur in two or more unrelated families, as demonstrated by microsatellite polymorphism haplotype analysis at the chromosome 11q13 loci D11S1883, D11S457, PYGM, and D11S449 (data not shown) (European Consortium on MEN1 1996, 1997*c;* Pang et al. 1996). The 4-bp deletion (CAGT) of codons 210 and 211 in exon 3 was observed in five unrelated families; the 4-bp deletion (GTCT) of codons 83 and 84 in exon 2 was observed in three unrelated families; the Gln349stop in exon 7 was observed in two unrelated families; and the poly$(C)_7$ tract of codons 514–516 in exon 10 was observed to have insertional or deletional mutations in three unrelated families. These four DNA sequences were involved in >25% of the 47 mutations, suggesting that these codons may be particularly prone to mutations; for example, the 4-bp deletion (CAGT) in exon 3 (fig. 1) accounted for >10% of all the mutations and thus may represent a mutation hot spot in the MEN1 gene. Second, correlations between the mutations and the clinical manifestations of MEN1 could not be established either between the families (table 1) or even within the families (figs. 3 and 4); for example, the five unrelated families with the same 4-bp deletion (CAGT) in codons 210 and 211 were found to have a wide range of MEN1-asso-

Table 1

Mutations, Polymorphisms, and Clinical Details of MEN1 Patients and Families

Number[a]	Exon or Intron	Codon[b]	Base Change[c]	Confirmed by	No. of Tumors			
					Parathyroid	Pancreatic[d]	Pituitary[e]	Other[f]
Nonsense:								
1	Exon 2	Arg98stop	CGA→TGA	*Bgl*II	2	1 (G)	1 (P)	0
2[g]	Exon 2	Trp126stop	TGG→TAG	*Nde*I	2	0	1 (P)	0
3[h,i]	Exon 3	Trp183stop	TGG→TGA	*Bst*NI	5	3 (G, I, N)	2 (P, Co)	2 (A, C)
4	Exon 3	Glu191stop	GAG→TAG	ASO	3	1 (G)	0	0
5	Exon 5	Lys262stop	AAG→TAG	ASO	1	1 (I)	1 (P)	0
6	Exon 7	Trp341stop	TGG→TAG	*Bst*NI	3	1 (G)	2 (P)	0
7	Exon 7	Gln349stop	CAG →TAG	*Bst*NI	2	1 (N)	1 (P)	1 (L)
8	Exon 7	Gln349stop	CAG→TAG	*Bst*NI	3	1 (G)	0	2 (A, L)
9[h,j]	Exon 8	Glu388stop	GAG→TAG	*Mnl*I	6	2 (G, Gg)	1 (P)	1 (C)
10	Exon 10	Gln453stop	CAG→TAG	*Mwo*I	1	1 (I)	1 (P)	0
11[k]	Exon 10	Arg460stop	CGA→TGA	*Dde*I	1	1 (I)	0	0
12	Exon 10	Arg527stop	CGA→TGA	*Dde*I	6	1 (N)	1 (P)	0
Deletions:								
13[k]	Exon 2	69–73: fs42aaX	11 bp: CCCGACCCGCC	GE	1	1 (I)	0	0
14[h]	Exon 2	83/84: fs32aaX	4 bp: GTCT	GE	2	3 (G, G, Gg)	3 (P, P, N)	1 (A)
15	Exon 2	83/84: fs32aaX	4 bp: GTCT	GE	2	1 (G)	0	0
16[h]	Exon 2	83/84: fs32aaX	4 bp: GTCT	GE	17	3 (G, G, I)	1 (P)	0
17	Exon 2	90: fs25aaX	2 bp: AT	GE	3	2 (G, Gg)	1 (P)	0
18[l]	Exon 2	118/119: del1aa	3 bp: GAA	*Mbo*II	3	1 (G)	0	0
19	Exon 2	131: fs53aaX	1 bp: C	*Hae*II	2	2 (G, I)	0	0
20	Exon 2	134: fs20aaX	1 bp: C	*Afl*II	1	1 (G)	3 (P)	0
21[g]	Exon 3	174: fs9aaX	1 bp: C	*Mnl*I	8	3 (G, G, I)	4 (P, P, P, S)	0
22	Exon 3	210/211: fs11aaX	4 bp: CAGT	GE	3	1 (G)	0	1 (C)
23	Exon 3	210/211: fs11aaX	4 bp: CAGT	GE	2	1 (I)	1 (P)	0
24	Exon 3	210/211: fs11aaX	4 bp: CAGT	GE	2	1 (G)	2 (P, N)	0
25	Exon 3	210/211: fs11aaX	4 bp: CAGT	GE	2	1 (G)	2 (P)	0
26[hi]	Exon 3	210/211: fs11aaX	4 bp: CAGT	GE	3	3 (G, Gg, N)	1 (P)	0
27	Exon 3	214: fs9aaX	1 bp: G	*Msp*I	4	1 (G)	1 (P)	0
28[l]	Exon 4	236–239: del3aa	9 bp: TGGCGTTCA	GE	2	2 (G, I)	1 (P)	0
29[h]	Exon 7	327: fs53aaX	1 bp: T	*Nla*IV	3	1 (G)	2 (P)	1 (A)
30[l]	Exon 8	358/359: del1aa	3 bp: GAG	GE	2	1 (G)	0	0
31[h]	Exon 8	373: fs2aaX	1 bp: C	ASO	9	2 (G)	3 (P)	0
32[k]	Exon 9	406–408: fs42aaX	5 bp: CCCTG	GE	1	1 (I)	0	0
33	Exon 10	516: fs42aaX	1 bp: C	ASO	1	1 (G)	1 (P)	0

Insertions:								
34[m]	Exon 2	34–38: ins5aa	15 bp: TGGTGCTCCTTTCCT	GE	1	1 (G)	1 (P)	0
35[g]	Exon 2	63–66: fs51aaX	10 bp: CCAGCCCAGC	GE	2	1 (G)	0	0
36[k]	Exon 3	162/163: fs22aaX	5 bp: GTTGG	GE	1	2 (G, Gg)	0	1 (A)
37	Exon 10	516: fs14aaX	1 bp: C	ASO	3	2 (G, N)	1 (P)	0
38	Exon 10	516: fs14aaX	1 bp: C	ASO	1	1 (G)	1 (P)	0
Deletional insertions:								
39	Exon 5	262: ss,fs18aaX	ctagA→tcagCC	*Pvu*II	3	2 (G)	3 (P)	0
40	Exon 9	440/441: fs5aaX	TT→GAAA	*Taq*I	3	1 (G)	1 (N)	0
Donor splice sites:								
41[g]	Exon 7	nt 6024: fs1aaX	gtgag→atgag	ASO	2	1 (G)	1 (P)	0
Missense mutations:								
42	Exon 2	Gly42Asp	GGC→GAC	ASO	6	1 (G)	1 (S)	0
43	Exon 3	Ala160Pro	GCT→CCT	ASO	6	2 (G)	1 (P)	0
44	Exon 3	Ala164Asp	GCC→GAC	*Hae*III	1	2 (G, N)	0	0
45[h,i]	Exon 3	Trp183Ser	TGG→TCG	*Bst*NI	7	3 (G, I, I)	3 (Co, Co, S)	1 (A)
46[g]	Exon 6	Ala284Glu	GCA→GAA	*Mbo*II	2	1 (G)	1 (P)	0
47	Exon 9	Asp418Asn	GAC→AAC	ASO	2	0	0	1 (C)
Polymorphisms:								
48	Intron 1 (−15)	nt2049	C →G (6%)	ASO				
49	Exon 2	Ser145Ser	AGC→AGT (2%)	*Alu*I				
50	Intron 4 (−9)	nt5168	G→A (3%)	*Alu*I				
51	Intron 7 (−3)	nt6620	C→G (2%)	*Dde*I				
52	Exon 9	Asp418Asp	GAC→GAT (44%)	ASO				
53	Exon 10	Glu536Glu	GAG→GAA (2%)	*Nla*IV				

[a] Refers to location of mutation, as illustrated in figure 1.

[b] For deletions, insertions, and donor splice-site mutations, "fs" denotes a frameshift, which is followed by the number of amino acids ("aa") and the stop codon, which is denoted by "X," and "ss" denotes splice site alterations.

[c] Uppercase letters denote an exon sequence, and lowercase letters denote an intron sequence. The percentages denote frequency of heterozygosity.

[d] G = gastrinoma; I = insulinoma; Gg = glucagonoma; and N = nonfunctional tumor.

[e] P = prolactinoma; S = somatotrophinoma; Co = corticotrophinoma; and N = nonfunctional tumor.

[f] A = adrenal cortical tumor; C = carcinoid tumor; and L = lipoma.

[g] De novo mutation.

[h] The entire coding region and exon/intron boundaries were examined by DNA sequence analysis.

[i] Reported previously, by European Consortium on MEN1 (1997*b*).

[j] Not detected by SSCP.

[k] Nonfamilial MEN1.

[l] Deletion does not cause frameshift.

[m] Insertion does not cause frameshift.

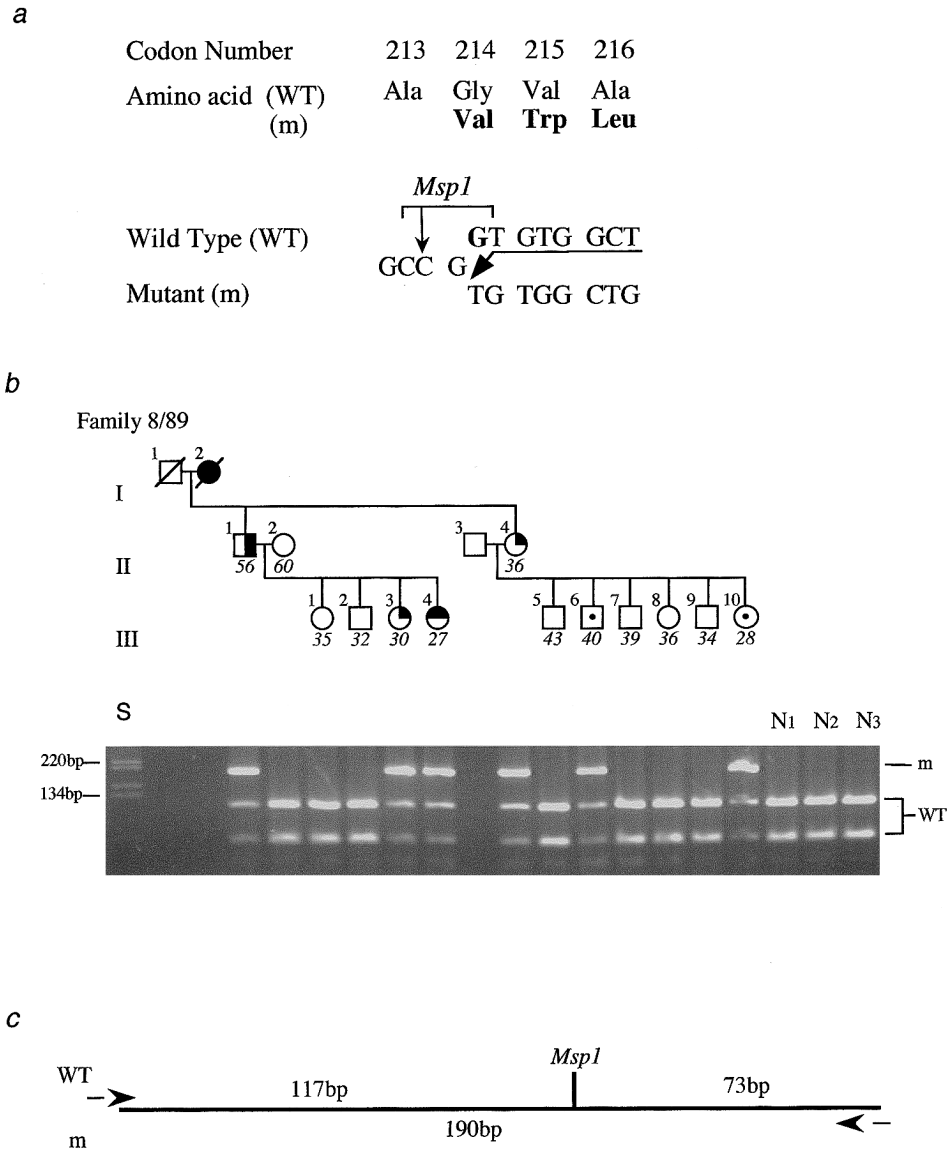

Figure 3 Detection of mutation in exon 3 in family 8/89, by restriction-enzyme analysis. SSCP and DNA sequence analysis of individual II-1 revealed a 1-bp deletion at the second position (GGT) of codon 214 (*a*). The deletion has caused a frameshift that continues to codon 223 before a stop codon (TGA) is encountered in the new frame (table 1). The 1-bp deletion results in the loss of an *Msp*I restriction-enzyme site (C/CGG) from the normal (i.e., wild-type [WT]) sequence (*a*), and this has facilitated the detection of this mutation in the other affected members (II-4, III-3, and III-4) of this family (*b*). The mutant (m) PCR product is 190-bp, whereas the wild type (WT) products are 117 and 73 bp (*c*). The affected individuals were heterozygous, and the unaffected members were homozygous, for the wild-type sequence. Individuals III-6 and III-10, who are 40 and 28 years of age, respectively, are mutant-gene carriers who are clinically and biochemically normal; this is due to the age-related penetrance of this disorder. Individuals are represented as male (*squares*), female (*circles*), unaffected (*unblackened/unmarked symbols*), affected with parathyroid tumors (*symbols with blackened upper-right quadrant*), affected with gastrinoma (*symbols with blackened lower-right quadrant*), affected with prolactinoma (*symbols with blackened upper-left quadrant*), and unaffected mutant-gene carriers (*symbols containing a black dot*). Individual I-2, who is now deceased but was known to be affected (tumor details are not known), is represented by a blackened symbol. Below each symbol, the individual's age either at diagnosis or at the time of the last biochemical screening is given. The standard-size marker (S), in the form of a 1-kb ladder, is shown. Cosegregation of this mutation with MEN1 in family 8/89, in conjunction with its absence in 110 alleles from 55 unrelated normal individuals (N_1–N_3 are shown) indicates that it is not a common DNA sequence polymorphism.

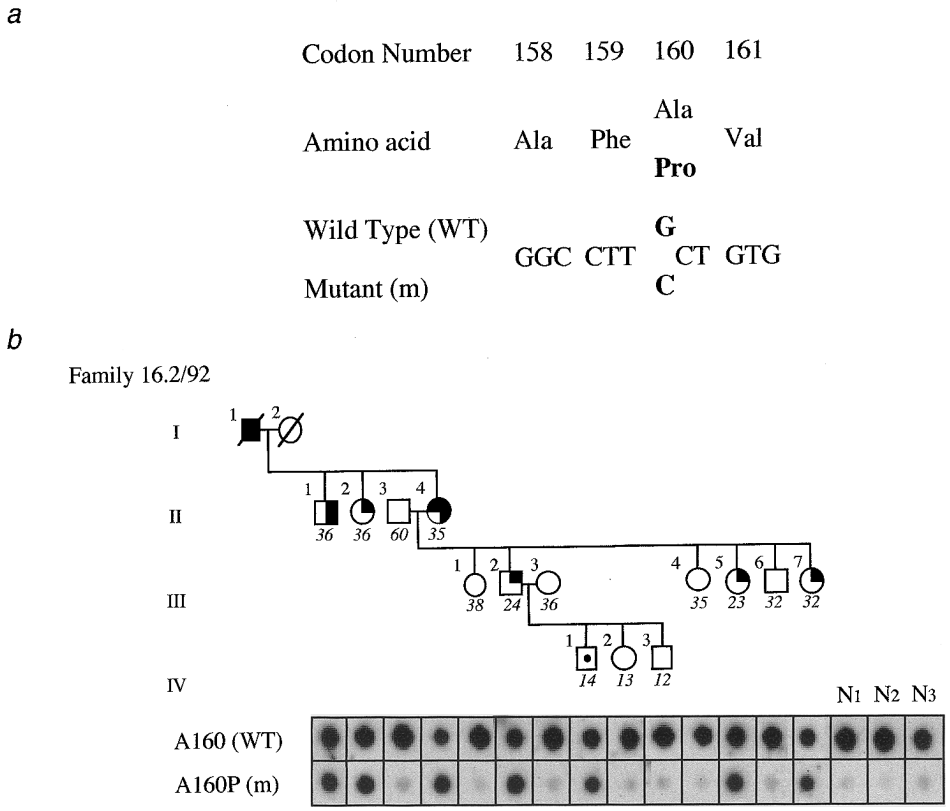

Figure 4 Missense mutation in exon 3 of the MEN1 gene in family 16.2/92. SSCP and DNA sequence analysis of individual II-4 revealed a G→C transversion at codon 160 (*a*), thus altering the wild-type (WT) sequence GCT, encoding an alanine (A), to the mutant (m) sequence, CCT, encoding a proline (P). The cosegregation of this mutation (A160P; table 1) in family 16.2/92, as well as its absence from 55 unrelated normal individuals (N_1–N_3 are shown), were demonstrated by ASO hybridization analysis (*b*), since it was not associated with an alteration of a restriction-enzyme site. Thus, the three unrelated normal individuals (N_1–N_3), the unaffected spouses II-3 and III-3, and the unaffected individuals III-1, III-4, III-6, IV-2, and IV-3 were found to have only the wild-type (WT) sequence A160 and none of the mutant (m) sequence (A160P) and were therefore homozygous for the wild-type sequence. However, all of the affected members (II-1, II-2, II-4, III-2, III-5, and III-7) have both the wild-type and mutant sequences. In addition, individual IV-1, who is 14 years of age and remains clinically and biochemically unaffected, is also heterozygous (WT/m), and this individual is thus a mutant-gene carrier who illustrates the age-related penetrance of this autosomal dominant disorder. The symbols representing the individuals and their ages are as described in figure 3.

ciated tumors (table 1); all the affected members had parathyroid tumors, but mutations 24–26 (table 1) were associated with gastrinomas and prolactinomas, whereas mutation 23 was associated with an insulinoma and a prolactinoma, and mutation 22 was notably absent of anterior-pituitary tumors and was associated with a gastrinoma and carcinoid tumors. Third, a comparison between the 47 MEN1 patients (and their families) in whom mutations were detected and the 16 MEN1 patients (and their families) in whom mutations were not detected revealed no significant phenotypic differences. Thus, the distribution of tumors in the patients and their families with (n = 277 tumors) and without (n = 65 tumors; distribution percentage shown in parentheses) mutations, respectively, was parathyroid tumors 53% (48%), gastrinomas 16% (14%), insulinomas 5% (6%), glucagonomas 2% (1%), nonfunctioning pancreatic islet–cell tumors 2% (0%), prolactinomas 15% (20%), somatotrophinomas 1% (2%), corticotrophinomas 1% (0%), nonfunctioning anterior-pituitary tumors 1% (1%), adrenal cortical tumors 2% (2%), carcinoid tumors 1% (4%), and lipomas 1% (2%). Fourth, five of the mutations (Trp126stop, 174del1bp, 63–66dup10bp, the donor splice-site mutation [nt6024], and Ala284Glu) were demonstrated to arise de novo (fig. 5), by the use of combined mutational and haplotype analysis (Pang et al. 1996; European Consortium on MEN1 1997*c*) that utilized polymorphic loci from chromosome 11q13. Haplotype studies have previously been used to predict MEN1 mutant-gene carrier status (Larsson et al. 1992, 1995), but our demonstration of the occurrence of de novo mutations of the MEN1 gene in

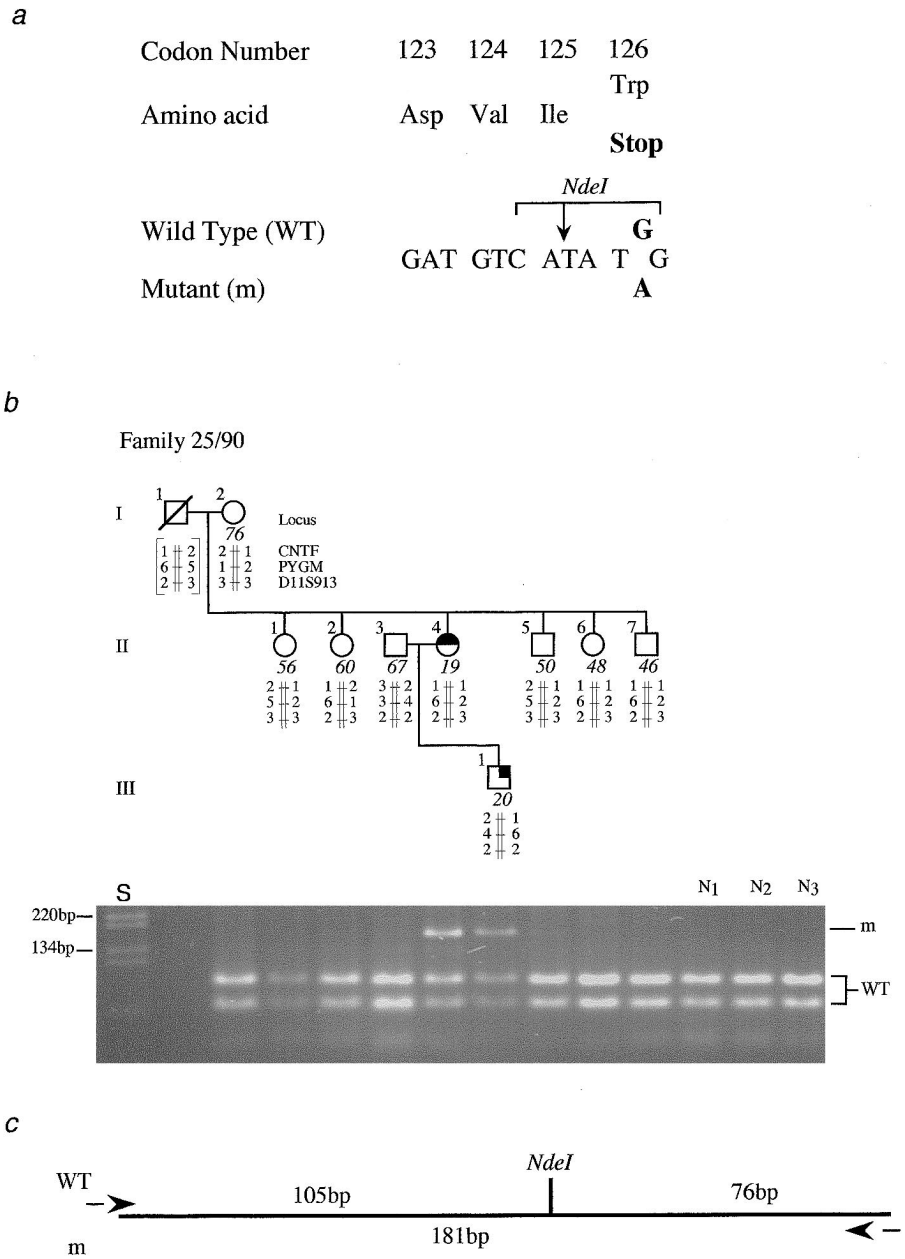

Figure 5 De novo heterozygous nonsense mutation of the MEN1 gene in family 25/90. SSCP and DNA sequence analysis of affected individual II-4 revealed a G→A transition at codon 126. The wild-type (WT) DNA sequence at codon 126 is TGG coding for a tryptophan (W) residue, whereas the mutant (m) sequence is TAG encoding a stop (X) signal (*a*). This nonsense mutation, W126X, resulted in the loss of an *Nde*I site, which facilitated its detection in family 25/90 (*b*). The restriction-enzyme map (*c*) of the PCR product shows that the wild-type (WT) DNA sequence, which has one *Nde*I site, is associated with cleaved PCR products of 105 and 76 bp, whereas the mutant (m), which has lost the *Nde*I site, is associated with a PCR product of 181 bp. Analysis of family 25/90 (*b*) revealed that the affected members II-4 and III-1 were heterozygous for the W126X mutation and that both shared the (1,6,2) haplotype, whose elements were obtained with the polymorphic loci CNTF, PYGM, and D11S913, respectively. The MEN1 gene is located in the interval between PYGM and D11S913 (European Consortium on MEN1 1996, 1997*a*). An examination of the haplotypes (paternal and maternal haplotypes are shown on the left and right, respectively) in the other family members, who are all unaffected, revealed that individuals II-2, II-6, and II-7 had also inherited the (1,6,2) haplotype from the deceased unaffected father, I-1 (whose haplotype is shown in square brackets). Thus, individuals II-2, II-6, and II-7 have the haplotype that is associated with the disease in individuals II-4 and III-1, but they themselves do not have MEN1 or the W126X mutation. These findings indicate that the W126X mutation is likely to have arisen de novo in individual II-4 and that her affected son III-1 has inherited it. The symbols representing the individuals and their ages are as described in figure 3. A total of five such de novo mutations (table 1) were identified.

Table 2

Details on 201 MEN1 Mutant-Gene Carriers

	Affected		Unaffected:
	Group A	Group B	Group C
No. of individuals	100	55	46
Male:female ratio	1:1	0.6:1	1.39:1
Mean ± SD age (years)[a]	35 ± 12	25 ± 10	17 ± 10
Age range (years)	11–70	8–46	2–50

[a] The Mann-Whitney U-test resulted in $P < 10^{-35}$ for group A compared with group B, $P < 10^{-29}$ for group A compared with group C, and $P < 10^{-28}$ for group B compared with group C; and the difference-of-means test resulted in $P < .001$ for all three comparisons.

>10% of the families (table 1) indicates that such haplotype analysis is not reliable (fig. 5) and requires cautious interpretation.

Mutant-Gene Carriers

The identification of mutations in the 43 MEN1 families (table 1), together with their confirmation by either restriction-enzyme analysis, ASO hybridization analysis, or GE, facilitated the detection of mutant-gene carriers. The normal or mutant-gene carrier status (figs. 3 and 4) was established in 320 members and 73 spouses from the 43 MEN1 families. Two hundred and one mutant-gene carriers were identified, and ~50% of these had presented with clinical symptoms (group A; table 2 and fig. 6), 27% were asymptomatic and had been detected by biochemical screening (group B), and 23% had remained unaffected and were biochemically normal (group C). The remaining 119 members, together with the 73 spouses, were all unaffected and normal-gene carriers. The mean age of group B was significantly ($P < .001$) lower than that of group A (table 2), confirming that biochemical screening is useful in the earlier detection of the disease. In addition, the mean age of group C was significantly lower than those of group A ($P < .001$) and group B ($P < .001$), indicating the nonpenetrance of the mutant gene in this younger group. These results were also confirmed by nonparametric analysis using the Mann-Whitney U-test. A further analysis of groups B and C revealed that only 17 of the 55 members of group B, compared with 31 of the 46 members of group C ($\chi^2 = 13.39$; $P < .0005$), were <20 years of age, which corresponds to the reported (Trump et al. 1996) age at conversion from an unaffected to an affected phenotype. The age-related penetrance (i.e., number of MEN1 affected by a given age ÷ [affected + unaffected mutant-gene carriers by the given age] × 100%) of MEN1 was calculated from the ages of the 201 mutant-gene carriers (fig. 6). This revealed that, although the mutant MEN1 gene is nonpenetrant in all three mutant-gene carriers <5 years of age, thereafter the

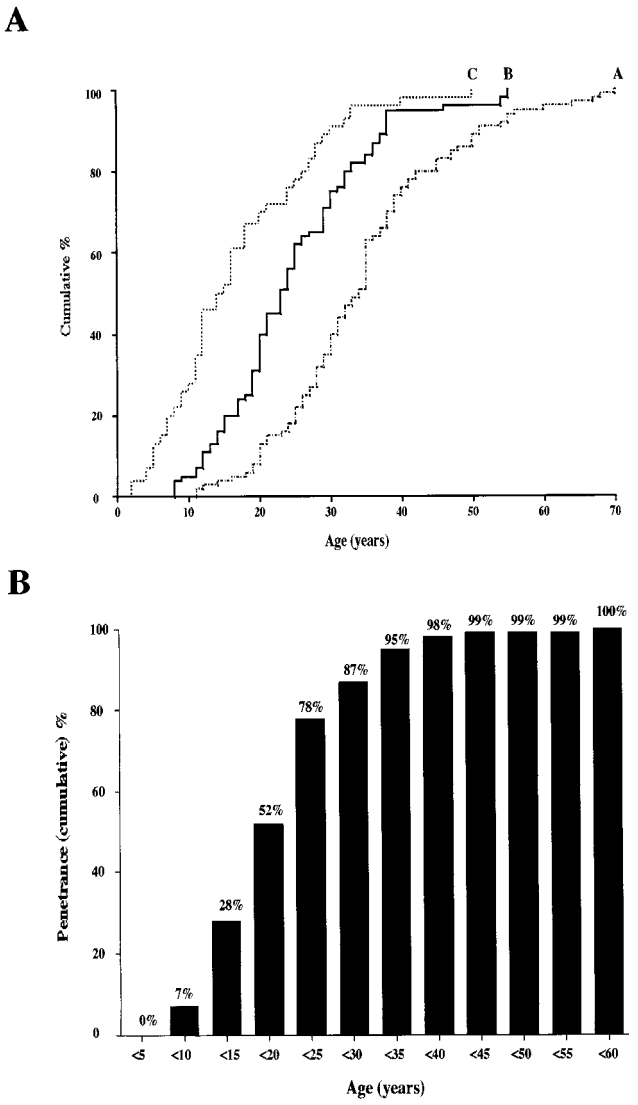

Figure 6 Age distributions (*A*) and age-related penetrances (*B*) of MEN1, determined on the basis of analysis of 201 mutant-gene carriers. The age distributions were determined for three groups of MEN1 mutant-gene carriers (table 2) from the 43 families in whom the mutations were detected (table 1). The 100 members of group A presented with symptoms, whereas the 55 members of group B were asymptomatic and were diagnosed by biochemical screening (Thakker 1995; Trump et al. 1996). The 46 members of group C represent those individuals who are MEN1 mutant-gene carriers (figs. 3 and 4) and who remain asymptomatic and biochemically normal. The ages included for members of groups A, B, and C are those at the onset of symptoms, at the finding of the biochemical abnormality, and at the last clinical and biochemical evaluation, respectively. Groups B and C contained members who were significantly younger ($P < 0.001$) than those in group A (table 2). The younger age of the group C mutant-gene carriers is consistent with an age-related penetrance for MEN1, and this was calculated (*B*) for the first 6 decades. The age-related penetrances (i.e., the proportion of mutant-gene carriers with manifestations of the disease by a given age) rose steadily, from 7% in the <10-years-old group, to 52%, 87%, 98%, 99%, and 100%, by the ages of 20, 30, 40, 50, and 60 years, respectively.

mutant gene has a high penetrance, being 52% penetrant by 20 years of age and fully (100%) penetrant by 60 years of age. Thus, the residual risk (i.e., 100 − age-related penetrance %) that an unaffected 20-year-old mutant-gene carrier will develop MEN1 tumors is 48%, and these risk assessments will help in the clinical evaluation of familial MEN1 patients.

Polymorphisms in the MEN1 gene

Six polymorphisms, which were all detected by SSCP analysis and confirmed by DNA sequence analysis together with restriction-enzyme and ASO hybridization analysis, were observed (table 1) in the MEN1 gene. Three of these polymorphisms occurred in introns, and the three polymorphisms that occurred in exons 2, 9, and 10 all involved the third base of the codon and did not lead to an alteration of the encoded amino acid. The range of heterozygosity frequencies of these polymorphisms was 2%–44% (table 1), and >75% of all the polymorphisms were due to the C→T transition of codon Asp418Asp in exon 9 (fig. 2). The polymorphisms in codons 145 and 418 have been observed by others (Agarwal et al. 1997; Chandrasekharappa et al. 1997), at similar frequencies—0.7% and 54%, respectively. The importance of these polymorphisms lies in their recognition and distinction from the DNA sequence abnormalities that represent mutations. This is of particular note in the detection of mutations in exon 9, which contains the Asp418Asp polymorphism that has a 44%–54% heterozygosity frequency in the population, and the inclusion of appropriate controls in the SSCP analysis of exon 9 (fig. 2) will help to distinguish between the polymorphism and mutations.

Discussion

Our results, which have identified 39 different mutations of the MEN1 gene in 47 unrelated patients and their families, are consistent with our previous prediction, which was based on an absence of common ancestral haplotypes and a lack of linkage disequilibrium in these families (European Consortium on MEN1 1997*c*). These 47 mutations (table 1) , which are comparable to those of a recent report (Agarwal et al. 1997), consisted of nonsense mutations (26% vs. 22% [all percentages are those of the present study vs. those reported by Agarwal et al. 1997), deletions (45% vs. 51%), insertions (14% vs. 4%), donor splice-site (2% vs. 0%) mutations, and missense (13% vs. 23%) mutations. The mutations are scattered throughout the coding region of the MEN1 gene (fig. 1) and do not correlate with the phenotypes observed in the MEN1 patients and their families. The majority (>85%) of the 47 mutations are nonsense mutations , frameshift deletions, or insertions that are likely to result in a functional loss of the MENIN protein and are thus in keeping with the proposed role of the MEN1 gene as a tumor-suppressor gene (Larsson et al. 1988; Thakker et al. 1989; Thakker 1993). In addition, the high proportion (60%) of deletions and insertions that were observed among the 47 MEN1 mutations is similar to that observed (50%) in the tumor-suppressor retinoblastoma gene (Blanquet et al. 1995; Lohmann et al. 1996). Furthermore, the independent and multiple occurrences of these types of MEN1 mutations in codons 83/84, 210/211, and 516, which, respectively, account for 6%, 11%, and 6% of all the 47 mutations, indicate that these may represent hot spots for small deletions and insertions (table 1). Such deletional and insertional hot spots may be associated with DNA sequence repeats that may consist of long tracts of either single nucleotides or shorter elements, ranging from dinucleotides to octanucleotides (Ripley et al. 1986; Krawczak and Cooper 1991). An examination of the DNA sequence in the vicinity of codons 83/84 in exon 2, and codons 210/211 in exon 3 revealed the presence of CT and CA dinucleotide repeats, respectively, flanking the 4-bp deletions; these would be consistent with a replication-slippage model (Ripley et al. 1986; Cooper and Krawczak 1991; Krawczak and Cooper 1991) in which there is misalignment of the dinucleotide repeat during replication, followed by excision of the resulting 4-bp single-stranded loop. The deletions and insertions of codon 516 involve a poly$(C)_7$ tract, and a slipped-strand mispairing model is also the most likely mechanism to be associated with this mutation hot spot (Cooper and Krawczak 1991). Thus, the MEN1 gene appears to contain DNA sequences that may render it susceptible to such deletional and insertional mutations. The mutational diversity within the 2,790 bp of the MEN1 gene coding region and exon/intron boundaries makes mutational screening for MEN1 by a direct DNA sequencing approach in patients considered to suffer from MEN1 time consuming and unfeasible. We have therefore explored the use of the SSCP technique, for the more rapid screening of MEN1 mutations. Our results demonstrate that SSCP is a useful, sensitive, and specific method that is successful in the detection of >85% of the MEN1 mutations. However, the presence of polymorphisms may hinder the detection of mutations by SSCP, and the use of appropriate controls (fig. 2) is required in order to facilitate the interpretation of these results, particularly in the case of exon 9, which contains a common polymorphism. Our analysis did not detect mutations in 16 of the 63 unrelated MEN1 probands, and this may partly be due to the limited sensitivity of SSCP, but it is important to note that direct DNA sequencing of the 2,790 bp of the MEN1-coding region and exon/intron boundaries also did not detect mutations in 2 of the 10 MEN1 probands. This failure to

detect mutations is unlikely to be due to genetic heterogeneity, which has been reported in one kindred (Stock et al. 1997), since cosegregation between MEN1 and closely flanking polymorphic markers from chromosome 11q13 has been established by previous studies (European Consortium on MEN1 1996, 1997*c;* Pang et al. 1996) in these families. A more likely explanation is that these MEN1 patients may harbor mutations in the promoter regions or UTRs, which remain to be investigated.

Our finding of five de novo mutations (table 1), which represent >10% of the mutations, together with their inheritance in subsequent generations, has important clinical implications. Thus, the development of MEN1 tumors in a patient without a family history of the disorder does not necessarily imply that the siblings are at risk, even if they have inherited the chromosome 11q13 haplotype that segregates with the disease (fig. 5). In addition, the subsequent inheritance of such a de novo mutation from the parent places the children at risk and in need of clinical and biochemical evaluation. Our analysis of the MEN1 mutant-gene carrier status in 320 (165 unaffected and 155 affected) members of 43 MEN1 families has helped to determine the age-related penetrance of this disorder, and this also has important clinical implications. The MEN1 mutant gene has a high penetrance (fig. 6) after the age of 5 years and is 52% and 100% penetrant by the ages of 20 years and 60 years, respectively. In addition, biochemical screening can detect the onset of the disease ~10 years earlier (table 2 and fig. 6) and before individuals are symptomatic, thereby providing an opportunity for earlier treatment. Our data suggest the following possible combined molecular genetic and clinical approach for MEN1 patients and their families. Identification of the mutation in a proband may be undertaken primarily by SSCP analysis, initially directed at the mutation hot spots in exons 2, 3 and 10 (table 1), and the DNA sequences of any abnormalities may be determined. Confirmation of the mutation and its subsequent detection, to identify mutant-gene carriers, may be undertaken either by restriction-enzyme, ASO, GE, or SSCP analysis. Clinical and biochemical evaluation, as described elsewhere (Thakker 1995; Trump et al. 1996), can then be commenced in mutant-gene carriers after the age of 5 years and can be repeated at 6–12-mo intervals (Trump et al. 1996). An application of this proposed approach will help to identify those individuals who are at a higher risk and will enable the earlier detection of MEN1 tumors, thereby facilitating appropriate genetic counseling and management of patients with this inherited endocrine disorder.

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