0013-7227/02/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 87(6):2688–2693

Frequent Occurrence of an Intron 4 Mutation in Multiple Endocrine Neoplasia Type 1

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MEN1 is an autosomal dominant disorder characterized by parathyroid, pituitary, and pancreatic tumors. The MEN1 gene is located on chromosome 11q13 and encodes a 610-amino acid protein. MEN1 mutations are of diverse types and are scattered throughout the coding region, such that almost every MEN1 family will have its individual mutation. To further characterize such mutations we ascertained 34 unrelated MEN1 probands and undertook DNA sequence analysis. This identified 17 different mutations in 24 probands (2 nonsense, 2 missense, 2 in-frame deletions, 5 frameshift deletions, 1 frameshift deletional-insertion, 3 frameshift insertions, 1 donor splice site mutation, and a g→a transition that resulted in a novel acceptor splice site in intron 4). The intron 4 mutation was found in 7 unrelated families, and the tumors in these families varied considerably, indicating a lack of genotype-phenotype correlation. However, this intron 4 mutation is the most frequently occurring germline MEN1 mutation (~10% of all mutations), and together with 5 others at codons 83–84, 118–119, 209–211, 418, and 516, accounts for 36.6% of all mutations, a finding that indicates an approach for identifying the widely diverse MEN1 mutations. (*J Clin Endocrinol Metab* 87: 2688–2693, 2002)

MEN1 IS AN autosomal dominant disorder characterized by the combined occurrence of tumors of the parathyroid glands, the pancreatic islet cells, and the anterior pituitary (1–3). In addition, adrenal cortical tumors, carcinoid tumors, lipomas, facial angiofibromas, and collagenomas have been observed in MEN1 patients (1). The MEN1 gene, which is located on chromosome 11q13 (4, 5), consists of 10 exons (Fig. 1) that encode a 610-amino acid protein referred to as MENIN (6, 7). MENIN is a predominantly nuclear protein that contains 2 nuclear localization signals (NLS) in its C-terminal segment (8). MENIN has been shown to interact directly with the activating protein-1 (AP-1) transcriptional factor JunD to suppress JunD-mediated transcriptional activation (9). In addition, MENIN has been reported to interact with the tumor metastasis suppressor nm23/nucleoside diphosphate (10) and with Smad 3 to inhibit the TGFβ signaling pathway (11). MEN1 mutations are predicted in the main to result in truncated forms of MENIN that lack the NLS sites or to alter binding with JunD (12). Thus, the majority of mutations are likely to be inactivating and consistent with the role of MENIN as a tumor suppressor (12–14). However, all of the more than 440 reported MEN1 germline mutations are scattered throughout the 1830-bp coding region and are of diverse types (12). Thus, approximately 21% are nonsense mutations, approximately 44% are frameshift deletions or insertions, 7% are splice site mutations, approximately 19% are missense mutations, and approximately 9% are in-frame deletions or insertions (12, 15–53). The majority (>80%) of MEN1 families have a unique mutation, although 5 mutations have been observed to occur several times in unrelated families. These 5 mutations, which account for approximately 17% of all germline mutations, consist of deletions, insertions, or missense mutations involving codons 83–84, 118–119, 209–211, 418, and 516 (12). The wide diversity and scattered locations of these mutations have made it difficult to implement mutational analysis in clinical practice. We have undertaken such mutational analysis in 34 unrelated MEN1 probands with the aims of further characterizing the spectrum of abnormalities in this gene and to look for frequently occurring mutations.

Abbreviations: AP-1, Activating protein-1; EBV, Epstein-Barr virus; NLS, nuclear localization signal; nt, nucleotide.

Subjects and Methods

Patients and families

Thirty-four unrelated probands with MEN1 were studied, and 21 of these had a family history of MEN1. A total of 189 family members (88 males and 101 females) were included in the study; 71 were affected, and 118 were unaffected. MEN1 was diagnosed in a family if 2 or more

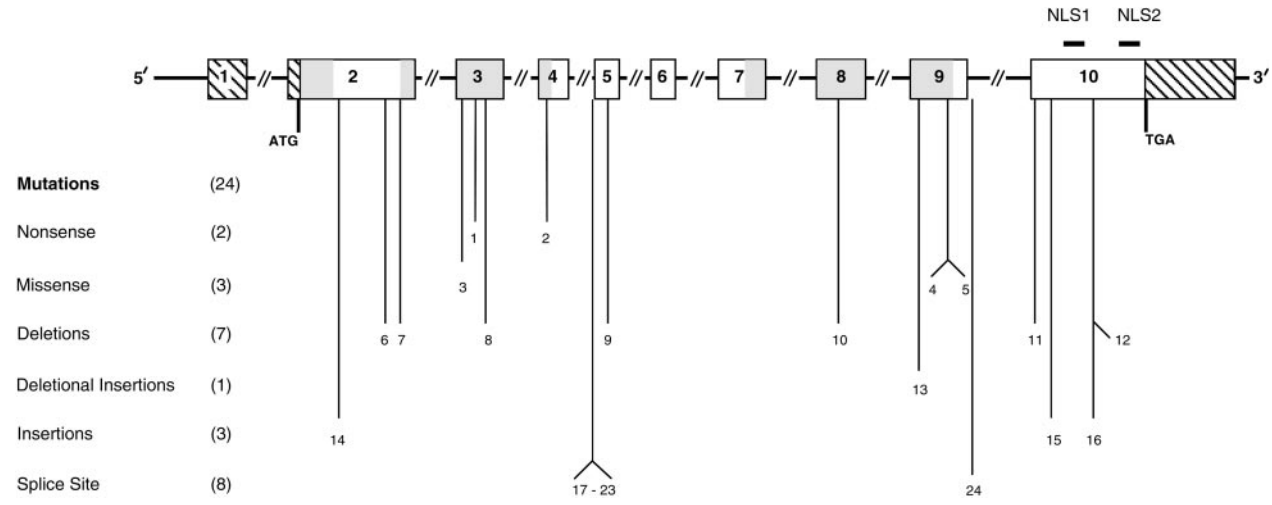

FIG. 1. Schematic representation of the genomic organization of the MEN1 gene, illustrating the locations of the identified germline mutations (see Table 1). The human MEN1 gene consists of 10 exons (indicated by *boxes*) that span more than 9 kb of genomic DNA and encodes a 610-amino acid protein. The 1.83-kb coding region is organized into 9 exons (exons 2–10) and 8 introns (indicated by a *line*, but not to scale). The sizes of the exons range from 41–1297 bp, and those of the introns range from 80–1564 bp. 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 (indicated by the *hatched boxes*). The locations of the two NLS that are at codons 479–497 and 588–608 are represented by *thick horizontal lines*. The locations of the 3 JunD-interacting domains that are formed by codons 1–40 (exon 2), 139–242 (exons 2–4), and 323–428 (exons 7–9) are indicated by the *shaded boxes*. The sites of the 24 germline mutations (2 nonsense mutations, 3 missense mutations, 7 deletions, 1 deletional insertion, 3 insertions, and 8 splice site mutations) identified by the present study are shown below, and details of each of these are provided in Table 1.

TABLE 1. MEN1 mutations

Number[a]	Exon/intron	Codon/nt	Base change[b]	Confirmed/detected by	Predicted effect[c]
Nonsense mutations					
1	Exon 3	191/nt 4422	*G*AG → *T*AG	ASO	Glu → Stop
2	Exon 4	227/nt 4742	TA*C* → TA*G*	*Nsp* I	Tyr → Stop
Missense mutations					
3	Exon 3	154/nt 4312	A*G*C → A*T*C[d]	*Alu* I	Ser → Ile
4	Exon 9	418/nt 7262	*G*AC → *A*AC	SSCP	Asp → Asn
5	Exon 9	418/nt 7262	*G*AC → *A*AC	SSCP	Asp → Asn
Deletions					
6	Exon 2	118-119/nt 2641	3 bp: GAA	*Mbo* II	del 1aa (Lys)
7	Exon 2	134/nt 2689	1 bp: C	*Afl* II	fs 19 aa X
8	Exon 3	209-211/nt 4480	4 bp: CAGT	GE	fs 11 aa X
9	Exon 5	264/nt 5184	61 bp[d]	GE	del 12 aa, ss, fs 11 aa X
10	Exon 8	363/nt 6660	3 bp: GAG	SSCP	del 1 aa (Glu)
11	Exon 10	453/nt 7584	1 bp: C[d]	*Fnu* 4HI	fs 4 aa X
12	Exon 10	516/nt 7773	1 bp: C	SSCP	fs 42 aa X
Deletional insertions					
13	Exon 9	400-403/nt 7210	AGGTTCCGC → GGGT[d]	GE	fs 4 aa X
Insertions					
14	Exon 2	63/nt 2475	5 bp: CCAGC	GE	fs 57 aa X
15	Exon 10	463/nt 7616	8 bp: GAGGCCGA[d]	GE	fs 96 aa X
16	Exon 10	516/nt 7773	1 bp: C	SSCP	fs 14 aa X
Splice site mutations					
17	Intron 4	nt 5168	g → a	*Nla* IV	ss, fs 9 aa X
18	Intron 4	nt 5168	g → a	*Nla* IV	ss, fs 9 aa X
19	Intron 4	nt 5168	g → a	*Nla* IV	ss, fs 9 aa X
20	Intron 4	nt 5168	g → a	*Nla* IV	ss, fs 9 aa X
21	Intron 4	nt 5168	g → a	*Nla* IV	ss, fs 9 aa X
22	Intron 4	nt 5168	g → a	*Nla* IV	ss, fs 9 aa X
23	Intron 4	nt 5168	g → a	*Nla* IV	ss, fs 9 aa X
24	Intron 9	nt 7361–7371	del 11 bp: gtgagggacag	GE	ss, fs 75 aa X

Frame shift, in frame and splice site mutations are numbered with reference to the *MEN1* genomic sequence U93237 (GenBank). ASO, Allele specific oligonucleotide hybridisation; GE, agarose gel electrophoresis; SSCP, single stranded conformational polymorphism analysis; nt, nucleotide.

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

[b] *Upper case letters* denote exonic sequence, and *lower case letters* denote intronic sequence.

[c] For deletions, insertions, and splice-site mutations, fs; denotes a frame shift, which is followed by the number of amino acids (aa) before the stop codon, which is denoted by X. ss, Splice site alterations; del, deletion.

[d] Novel MEN1 mutations.

MEN1-related tumors had been demonstrated in at least 1 individual; other relatives were considered affected only if they had clinical, radiological, and/or surgical evidence of associated tumors as previously reported (3). Twelve MEN1 probands and their families in whom MEN1 mutations had not been previously detected (14) by the use of single-strand conformational polymorphism analysis were included in this study. Informed oral consent was obtained from all of the individuals, using guidelines approved by the local ethics committee at Hammersmith Hospital (London, UK).

DNA sequence analysis of the MEN1 gene

Leukocyte DNA was extracted and used with the previously reported 15 pairs of primers for PCR amplification of the 9 coding exons of the MEN1 gene and their corresponding 17 intron/exon boundaries (7, 14). The DNA sequence of gel-purified PCR products was determined by the use of *Taq* polymerase cycle sequencing (14) and a semiautomated detection system (ABI 373XL sequencer, PE Applied Biosystems, Foster City, CA). DNA sequence abnormalities were confirmed either by restriction endonuclease analysis of genomic PCR products obtained by the use of appropriate primers, allele-specific oligonucleotide hybridization analysis, or agarose gel electrophoresis. 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). The 47 mutations identified by our previous study (14) were pooled with those identified in this study to determine the frequency of each mutation.

RT-PCR

RT-PCR was used to investigate mRNA splicing abnormalities, using total RNA extracted from Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines from three unrelated probands and a normal individual as previously described (54). The DNA sequences of these RT-PCR products were then determined using methods previously reported (14).

Results

DNA sequence analysis of the entire 2.79-kb coding region and exon-intron boundaries in the 34 MEN1 probands revealed the presence of 24 mutations. These 24 mutations (Table 1 and Fig. 1) consisted of 2 nonsense mutations ($Glu^{191}Stop$ and $Tyr^{227}Stop$); 3 missense mutations ($Ser^{154}Ile$, and $Asp^{418}Asn$, which was found twice); 2 in-frame deletions at codons 118–119 and 363; 5 frameshift deletions at codons 134, 209–211, 264, 453, and 516; 1 frameshift deletional insertion at codons 400–403; 3 frameshift insertions at codons 63, 463, and 516; 1 donor splice site mutation [nucleotide (nt) 7361 del 11 bp] in intron 9; and 7 acceptor splice site mutations (g→a transition at nucleotide 5168 in intron 4; Fig. 2). The 7 MEN1 families with the intron 4 acceptor splice site mutation were established to be unrelated on the basis of the absence of a common shared haplotype, as assessed previously (55) by use of 5 closely linked chromosome 11q13 polymorphic markers whose locus order is 11cen-D11S1883-D11S457-PYGM-MEN1-D11S1783-D11S449–11qter (56). Each of the DNA sequence abnormalities was confirmed and demonstrated to cosegregate with the disease by restriction enzyme analysis (Fig. 2), allele-specific oligonucleotide hybridization analysis, or agarose gel electrophoresis (Table 1). In addition, the absence of these DNA sequence abnormalities in 110 alleles from 55 unrelated normal individuals indicated that they were unlikely to be polymorphisms that would be expected to occur in more than 1% of the population. Nineteen of these mutations (2 nonsense, the 9 frameshift deletions or insertions, and 8 splice site mutations) are predicted to result in truncated forms of MENIN (Table 1) that would lack the NLS sites (Fig. 1), whereas another 4 mutations ($Ser^{154}Ile$, the in-frame deletion of codon 363, and $Asp^{418}Asn$, which occurred twice) involve the JunD-binding domains (Fig. 1) and are predicted to prevent MENIN's repressive action on JunD-mediated transcription (9). The effects of the in-frame deletion of Lys^{119} are more difficult to predict, as this site does not directly alter any of the JunD-binding domains or the NLS sites. However, it is important to note that the Lys^{119} deletion has been reported in 8 other unrelated MEN1 families, and it may be that Lys^{119} is involved in interactions with other proteins such as Smad3 and nm23/nucleoside diphosphate (10, 11) or that the Lys^{119} deletion results in a small conformational change that may affect protein function.

The g→a transition at nucleotide 5168 in intron 4, which had been previously considered to be a polymorphism (14) on the basis of its intronic location and lack of disruption to the naturally occurring acceptor splice site, has been reported

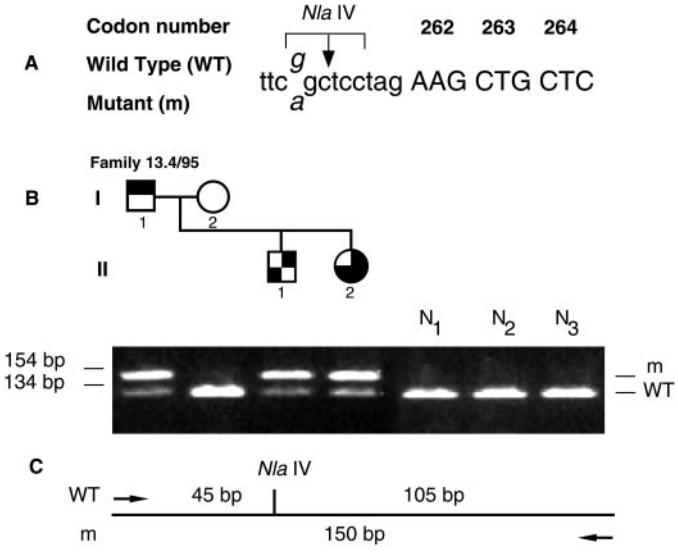

FIG. 2. Detection of mutation in intron 4 in family 13.4/95 by restriction enzyme analysis. DNA sequence analysis of individual I-1 revealed a g→a transition at nt 5168 (−9 nt from codon 262; A). The transition results in a novel consensus acceptor splice site tcag, in which ag is the invariant dinucleotide (59). Use of this novel acceptor splice site would lead to a frameshift (see Fig. 3), which predicts the incorporation of nine missense amino acids (Table 1) after which a Stop codon is encountered. The g→a transition results in the loss of an *Nla*IV restriction enzyme site (ggc/tcc) from the wild-type (WT) sequence (A), and this has facilitated the detection of this mutation in the other affected members (II.1 and II.2) of this family (B). The mutant (m) PCR product is 150 bp (C), whereas the wild-type (WT) products are 105 and 45 bp (not shown). The affected individuals are heterozygous (WT/m), and the unaffected spouse (I.2) and control unrelated normals (N_1 to N_3) are homozygous for the wild-type allele (B). Individuals are represented as males (*squares*), females (*circles*), unaffected (*open symbol*), affected with parathyroid tumors (*filled in upper right quadrant*), affected with prolactinoma (*filled in lower right quadrant*), affected with gastrinoma (*filled in lower left quadrant*), and affected with bronchial carcinoid (*filled in upper left quadrant*). The positions of the size markers (1-kb ladder) at 154 and 134 bp are shown. Cosegregation of this mutation with MEN1 in family 13.4/95 and its absence from 110 alleles of 55 unrelated normal individuals (N_1–N_3 shown), thereby indicating that it is not a common DNA sequence polymorphism, were demonstrated.

in four unrelated probands to result in the occurrence of a novel acceptor splice site (22–24, 34, 35). Furthermore, use of this novel acceptor splice site has been demonstrated by an analysis of RNA obtained from one MEN1-associated insulinoma (34) and one MEN1-associated adrenal cortical adenoma (35). These analyses in tumors revealed that use of this novel acceptor splice site leads to incorporation of the 7-bp 5′ to the naturally occurring acceptor splice site, with a resultant frameshift and premature termination. Our finding of this mutation in seven unrelated MEN1 families indicates that this germline mutation is likely to have a more frequent occurrence than previously envisaged. In addition, our studies showing the expression of the mutant and wild-type alleles in EBV-transformed lymphoblastoids confirm that the mutation causes a shift in the reading frame that results in a premature termination (Fig. 3). However, it has been postulated that the mutant MEN1 allele may still produce a small amount of normal mRNA generated by correct splicing at the naturally occurring acceptor splice site, and that this may be associated with a milder form of MEN1 (50). Indeed, the

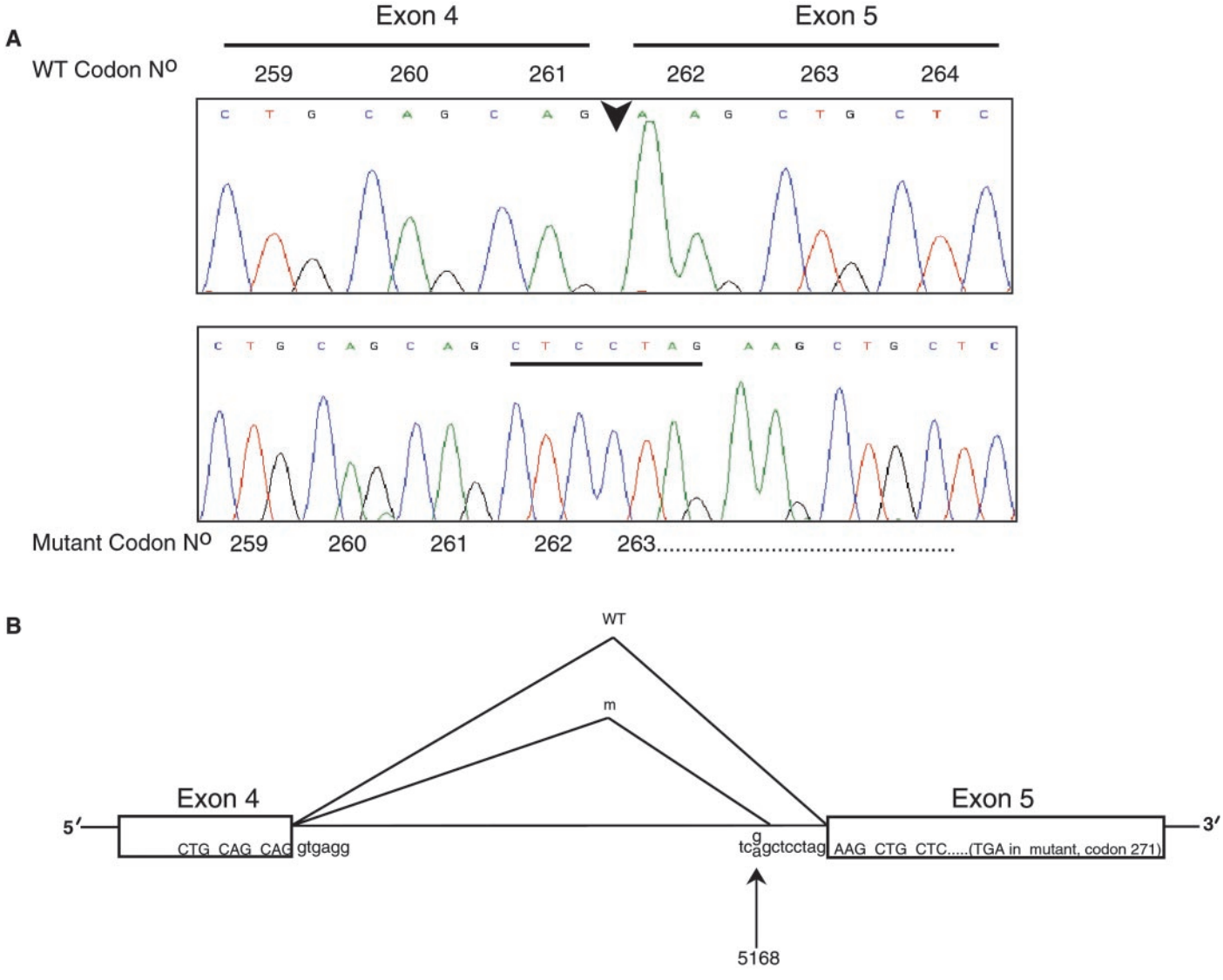

FIG. 3. Abnormal mRNA splicing due to intron 4 mutation. The g→a transition at nucleotide 5168 in intron 4 (Fig. 2) leads to the occurrence of a novel consensus acceptor splice site (tcag) at position 5166–5169 (B). The possible mRNA splicing abnormalities were investigated by the use of RT-PCR using total RNA obtained from EBV-transformed lymphoblastoids of patient I.1 from family 13.4/95 (Fig. 2) and a control normal individual. Only one RT-PCR product was obtained from the normal individual, but two products were obtained from the patient (data not shown). DNA sequence analysis of these RT-PCR products revealed that the normal (wild-type) product consisted of exon 4 spliced to exon 5, but that the mutant product contained an extra 7 nucleotides (CTCCTAG, *underlined*) spliced between exons 4 and 5 (site indicated by *arrowhead*, A). These findings indicate that the novel acceptor splice site, which results from the g→a transition at position 5168, is being used (A). The mutant mRNA, if translated, would predict a frameshift that results in nine missense amino acids followed by a premature stop signal (TGA) at codon 271 in exon 5. Thus, utilization of this novel acceptor splice site would result in a truncated protein that would lack one of the three JunD-binding domains, in exons 7, 8, and 9, and the two NLS sites, in exon 10 (Fig. 1). EBV-transformed lymphoblastoid total RNA was available from two of the six other unrelated probands with this intron 4 mutation, and use of this demonstrated the same mRNA splicing abnormality to be present.

TABLE 2. MEN1 associated tumors in seven unrelated families with the $g \rightarrow a$ novel acceptor splice site mutation in intron 4

	11.3/90	15/91	23.2/96	2/96	13.4/95	20/96	13.2/97
Parathyroid adenoma	+	+	+	+	+	+	+
Pancreatic tumor	−	+[a]	+[a,b]	−	+[a]	+[c]	+[a]
Pituitary tumor	+[d]	+[d]	+[d]	+[d]	+[d]	+[d]	+[e]
Carcinoid	−	−	−	−	+	−	−
Adrenal adenoma	−	−	−	−	−	+	+
Lipoma	−	−	−	−	−	+	−

+, Presence of at least one tumor of that type in the family; −, absence of tumor; [a] gastrinoma; [b] pancreatic polypeptideoma; [c] nonfunctioning neuroendocrine tumor; [d] prolactinoma; [e] somatotrophinoma.

TABLE 3. Frequently involved MEN1 germline mutation sites

Number[a]	Mutation site[b]	Frequency (n = 71)[c,d]
	83–84	4.2%
6	118–119	2.8%
8	209–211	8.5%
4 and 5	418	4.2%
12 and 16	516	7.0%
17 to 23	nt 5168	9.9%
Total		36.6%

[a] Refers to location of mutation, as illustrated in Fig. 1 and Table 1.
[b] Refers to the codon number, except in the case of mutation numbers 17–23, which occurred in intron 4 and the nucleotide (nt) is given.
[c] Mutations from present and previous (14) study combined.
[d] These frequencies when calculated using the reported 440 germline mutations (12,15–53) are as follows: codon 83– 84, 5.2%; codon 118–119, 1.8%; codon 209–211, 2.5%; codon 418, 2.3%; codon 516, 5.4%; nt 5168 (intron 4), 1.0%; total, 18.2%.

absence of reported pituitary tumors in four MEN1 families (23, 24, 34) with this germline mutation has been suggested to support this hypothesis (50). Our findings of this mutation in seven unrelated families enabled us to examine this hypothesis and to assess for a genotype-phenotype correlation. The seven unrelated families with the same g→a mutation were found to have a wide range of MEN1-associated tumors (Table 2). Thus, all of the affected families had parathyroid and pituitary tumors, but families 11.3/90 and 2/96 did not have pancreatic tumors, whereas families 15/91, 23.2/96, 13.4/95, 20/96, and 13.2/97 had gastrinomas or other neuroendocrine pancreatic tumors. Furthermore, family 13.4/95 (Fig. 2) had carcinoid tumors, family 20/96 had adrenal cortical tumors and lipomas, and family 13.2/97 had adrenal cortical tumors. Thus, there is a lack of genotype-phenotype correlation in these seven families with the same g→a intron 4 mutation, and this is similar to our previous observations in five unrelated MEN1 families who had the same 4-bp deletion of codons 209–211 (14). These findings also indicate that the g→a intron 4 mutation is not associated with a milder form of MEN1 as previously suggested (50), and that patients with this mutation may also develop anterior pituitary and carcinoid tumors.

Discussion

Our results, which have identified 17 different mutations in 24 of 34 MEN1 probands investigated, reveal that the majority (16 of 17) are likely to result in a functional loss of MENIN either by loss of 1 of the NLS sites or by an abnormality in 1 of the JunD-binding domains. Thus, most of the MEN1 mutations are predicted to be inactivating and thus consistent with the findings of previous studies (6, 13, 14) and with the role of MENIN as a tumor suppressor gene (12–14). The mutations are scattered throughout the coding region of the MEN1 gene and do not correlate with the phenotypes in the patients and families. Five of these mutations have not been previously observed. Furthermore, our studies indicate that the g→a transition that results in a novel acceptor splice site in intron 4 is a frequently occurring mutation. This mutation, which has previously been reported in 4 unrelated probands, was found to occur in 7 unrelated families, indicating that its frequency in our total series of 71 mutations [47 previously reported (14) and 24 mutations from the present study] is approximately 10%. Thus, this mutation in intron 4 is the most frequently occurring MEN1 mutation (Table 3). Indeed, mutations at 6 sites in the MEN1 gene account for 36.6% of the 71 mutations from our previous (14) and present studies. Furthermore, mutations at each of these 6 sites may be readily detected by the use of an appropriate restriction enzyme, single-strand conformational polymorphism, or gel electrophoresis. These data indicate that an initial examination using the appropriate method to detect mutations at these 6 frequently involved sites may be worthwhile, as it will identify over one third of the MEN1 mutations. This approach may help to reduce some of the costs and time involved in detecting the widely diverse and scattered mutations in the MEN1 gene. Finally, the absence of mutations within the coding region of the MEN1 gene in 10 of the 34 probands suggests that further searches of the untranslated regions or intronic sequences for alterations at lariat branch sites (57) or for cryptic splice site formation (58) may be warranted.

Acknowledgments

We are grateful to M. Aitken, D. Brenton, O. M. Edwards, R. A. Eeles, J. R. Farndon, S. Hodgson, K. Jones, V. Murday, S. O'Rahilly, S. Price, S. Shallet, P. J. Trainer, and J. Walker for access to the patients and families.

Received November 19, 2001. Accepted March 4, 2002.

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This work was supported by the Medical Research Council, United Kingdom (to J. J.O.T., S.A.F., A.A.J.P., J.H.D.B., B.H., P.T.C., and R.V.T.) and the Rhodes Foundation (P.D.L.).

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