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Somatic Mutations in MEN Type 1 Tumors, Consistent with the Knudson "Two-Hit" Hypothesis

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MEN type 1 is an autosomal dominant disorder characterized by the combined occurrence of tumors of the parathyroids, anterior pituitary, and pancreatic islet cells. The MEN1 gene, which is located on chromosome 11q13, consists of 10 exons and encodes a 610-amino acid protein named MENIN. The observation of LOH involving 11q13 in MEN type 1 tumors and the inactivating germline mutations found in patients suggest that the MEN1 gene acts as a tumor suppressor, in keeping with the "two-hit" model of hereditary cancer. The second hit in MEN type 1 tumors typically involves large chromosomal deletions that include 11q13. However, this only represents one mechanism by which the second hit may occur, and the other mechanisms, such as intragenic deletions or point mutations that inactivate the gene, have not been reported in MEN type 1 tumors. We have therefore undertaken studies to search for such mutations in six MEN type 1 tumors (four parathyroid tumors, one insulinoma, and one lipoma) that did not have LOH at 11q13 as assessed using the flanking markers D11S480, D11S1883 and PYGM centromerically and D11S449 and D11S913 telomerically. This revealed four somatic mutations, which consisted of two missense mutations and two frameshift mutations in two parathyroid tumors, one insulinoma, and one lipoma. Thus, our results, which represent the first small intragenic somatic mutations reported in MEN type 1 tumors, provide further evidence that the role of the MEN1 gene is consistent with that of a tumor suppressor gene, as postulated by Knudson's "two-hit" hypothesis. (*J Clin Endocrinol Metab* 86: 4371–4374, 2001)

MEN type 1 (MEN1) is an autosomal dominant disorder characterized by the combined occurrence of tumors of the parathyroids, anterior pituitary, and pancreatic islet cells. Other associated features include adrenal cortical tumors, angiofibromas, collagenomas, carcinoid tumors, and lipomas (1, 2). The MEN1 gene is located on chromosome 11q13 and encodes a 610-amino acid nuclear protein, referred to as MENIN (3–5), which interacts with the activator protein 1 (AP1) transcription factor JunD to inhibit JunD-activated transcription (6). Heterozygous germline MEN1 mutations have been identified in patients with familial and isolated forms of MEN1 and in patients with familial isolated primary hyperparathyroidism (4, 5, 7–10). These mutations are scattered throughout the coding region, and the majority of the mutations are nonsense or frameshift deletions, duplications, or insertions that are likely to result in a functional loss of the MENIN protein (10). The observation of LOH involving chromosome 11q13 in MEN1 tumors and the inactivating germline mutations in patients suggest that the MEN1 gene acts as a tumor suppressor, in keeping with the two-hit model of hereditary cancer as originally postulated by Knudson for retinoblastoma (11). This is further supported by the observations of somatic MEN1 mutations and chromosome 11q13 LOH in non-MEN1 sporadic (*i.e.* nonfamilial) forms of parathyroid adenomas (12), gastrinomas (13), insulinomas (13), glucagonomas (14), and bronchial carcinoids (15). In the MEN1 and non-MEN1 tumors, the second hit typically involves LOH of a large chromosomal region that includes chromosome 11q13 (16–18). However, this LOH represents only one mechanism by which the second hit may occur, and the other mechanisms, involving smaller deletions and point mutations that may also inactivate the gene, have not been reported in MEN1 tumors. We looked for such abnormalities in MEN1 tumors that did not show LOH at 11q13, with the aim of further elucidating the molecular genetic mechanisms that lead to tumor formation in MEN1.

Abbreviations: MEN1, MEN type 1.

Subjects and Methods

Patients and tissue specimens

MEN1 was diagnosed if two or more MEN1-related tumors had been demonstrated in a patient (1, 2). A family history of MEN1 was established in three of the probands, two males and one female (Table 1), but not in the other three male probands. The six MEN1 tumors (four parathyroid, one insulinoma, and one lipoma) were obtained at surgery and stored at −70 C. Venous blood samples were obtained from the probands and relatives. Leukocyte and tumor DNA were extracted as previously described (17).

Microsatellite polymorphisms analysis and LOH studies

The microsatellite polymorphisms from the chromosome 11q13 loci D11S480, D11S1883, and PYGM, which were located centromeric to MEN1, and the loci D11S449 and D11S913, which were located telomeric to MEN1 were detected by PCR and ^{32}P-radiolabeled oligonucleotide primers, as previously described (19, 20). The polymorphisms obtained from the leukocyte and tumor DNA of each patient were compared and assessed for tumor LOH, as previously described (19). The results of tumor LOH at the D11S480 and PYGM loci have been previously reported (19) in the four parathyroid tumors and the insulinoma.

DNA sequence analysis of the MEN1 gene

Fifteen pairs of primers were used for PCR amplification of the nine coding exons and adjoining splice junctions of the MEN1 gene, as previously described (5, 8). The DNA sequence was determined (8) using the Thermo Sequenase II DNA Polymerase Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Arlington Heights, IL) and a semiautomated detection system (ABI 373 sequencer; PE Applied Biosystems, Foster City, CA). DNA sequence abnormalities were con-

TABLE 1. Clinical and genetic findings in six MEN1 patients and tumors

Tumor No.	Tumor Type	Family history	Germline mutation	Somatic mutation	Other tumors in patient	D11S480	D11S1883	PYGM	Exon 9	D11S449	D11S913
1	Parathyroid	No	agCT-atCT int 7	N/D	PTH, PRL, GAS	+	+	+	○	+	+
2	Parathyroid	Yes	N/D	Val121Asp	PTH, GH, GAS	+	+	+	○	+	+
3	Parathyroid	No	4-bp del 210–211	1-bp del 39	PTH, PRL	○	+	+	+	+	+
4	Parathyroid	Yes	3-bp del 359[a]	N/D	PTH	+	+	+	+	+	○
5	Insulinoma	No	N/D	Trp183Gly	PTH, PRL, GAS, CAR, LIP	+	+	+	○	+	○
6	Lipoma	Yes	4-bp del 210–211[a]	1-bp del 51	PTH, PRL	N/T	+	N/T	○	+	+

PTH, Multiglandular parathyroid disease; GAS, gastrinoma; GH, somatotrophinoma; PRL, prolactinoma; LIP, lipoma; CAR, carcinoid; +, retention of heterozygosity; ○, uninformative marker; N/T, marker not tested; N/D, not detected.

[a] Mutation previously reported (9).

firmed by either restriction enzyme analysis or gel electrophoresis analysis of genomic PCR products as described previously (8).

Results

The six tumors were analyzed for LOH using the five polymorphic loci from 11q13 whose order around MEN1 has been established as 11cen-D11S480-D11S1883-PYGM-MEN1-D11S449-D11S913–11qter (5, 20). All of the tumors were heterozygous for the flanking markers D11S1883 and D11S449, thus indicating an absence of large chromosomal deletions involving the MEN1 locus in these tumors (Table 1). To further elucidate the molecular mechanisms underlying tumorigenesis in MEN1, we performed DNA sequence analysis of the entire 1.83-kb coding region and exon/intron boundaries of the MEN1 gene in the six tumor samples. Direct sequencing of PCR-amplified tumor DNA revealed a total of eight heterozygous mutations (Table 1). These consisted of two missense mutations, two 1-bp deletions, two 4-bp deletions, one 3-bp deletion, and one acceptor splice site mutation. In each case the mutations were confirmed independently by restriction enzyme analysis or gel electrophoresis analysis of genomic PCR products in the six tumors and matching leukocyte DNA (Fig. 1). Four of the eight mutations were detected in both normal and tumor DNA (Table 1), consistent with a germline origin. The remaining four mutations (no. 2, 3, 5, and 6, Table 1) were found only in tumor DNA, consistent with a somatic origin, and included two 1-bp deletions and two missense mutations. The two 1-bp deletions were observed in a parathyroid adenoma (1bp del 39) and in a lipoma (1bp del 51; Fig. 1) from two unrelated patients (no. 3 and 6, Table 1) with a germline 4-bp deletion at codons 210–211. The two missense mutations consisted of a T→A transversion at codon 121 (Val121Asp) in a parathyroid adenoma and a T→G transversion at codon 183 (Trp183Gly) in an insulinoma (Table 1) and were identified in the two patients (no. 2 and 5) in whom germline mutations of the coding region were not detected. In addition, sequence analysis of exon 9 revealed the polymorphic heterozygous sequence GAC/GAT coding for aspartic acid at codon 418 in the two tumors (no. 3 and 4, Table 1), thereby confirming the absence of large intragenic deletions in these tumors.

Discussion

Loss of tumor heterozygosity involving chromosome 11q13 has been demonstrated in over 90% of MEN1 tumors, and this has generally been taken as evidence that the MEN1 gene acts as a tumor suppressor gene, consistent with Knudson's "two-hit" hypothesis (11, 16–18). However, the observation of LOH at 11q13 does not exclude the involvement of other tumor suppressor genes within this region, and this suggestion has gained support from the results of deletion mapping studies in MEN1 tumors that have identified a locus distal to the MEN1 gene, in the interval between D11S4907/D11S4908 and D11S987 (21). Our demonstration of somatic mutations in the MEN1 gene in the absence of chromosomal deletions at 11q13 suggests that sequential inactivation of the MEN1 gene is an essential step in the development of MEN1 tumors and that it probably precedes the unmasking of any other putative tumor suppressor genes in

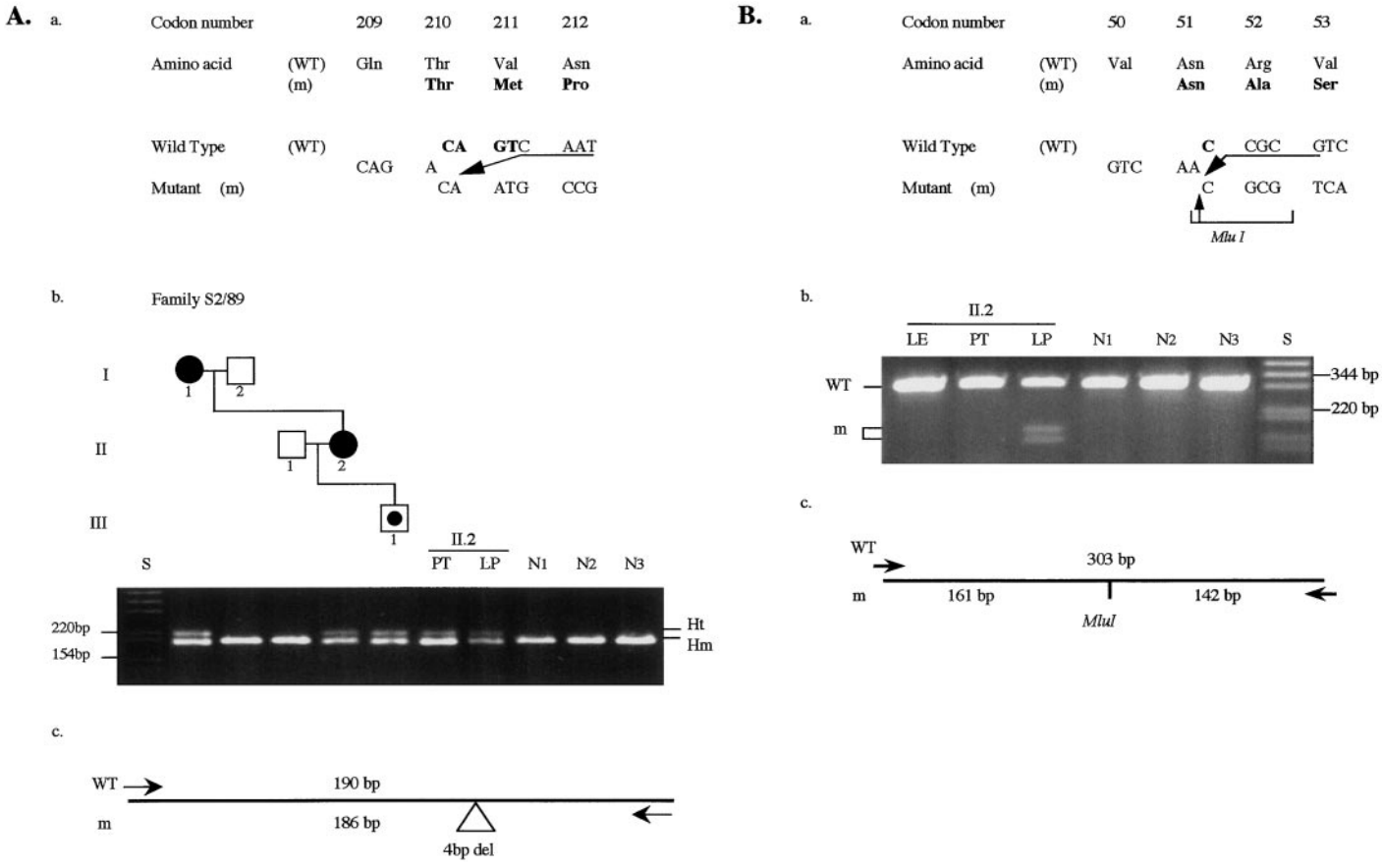

FIG. 1. DNA sequence analysis of the MEN1 gene. The detection of germline and somatic mutations in a lipoma (tumor 6, Table 1) from an MEN1 patient is illustrated. DNA sequence analysis of the lipoma (LP) from individual II.2 revealed two mutations, one germline (A) and one somatic (B). A, The germline mutation consisted of a 4-bp deletion (CAGT) involving codons 210 and 211. The mutation is predicted to result in a frameshift with the introduction of 11 missense amino acids followed by a premature stop at codon 222 (a). After PCR amplification of the mutation, the PCR products result in the formation of homoduplexes (Hm) and heteroduplexes (Ht), which facilitate the detection of the mutation in the family members and in a parathyroid tumor (PT) from individual II.2 by agarose gel electrophoresis (b and c). The absence of this 4-bp deletion in 110 alleles from 55 unrelated normal individuals (N1–N3 are shown) established that this was not a common sequence polymorphism. B, The somatic mutation in the lipoma (LP) from individual II.2 consisted of a 1-bp deletion (C) at codon 51. The mutation resulted in the gain of a *Mlu*I restriction enzyme site, which facilitated the detection of the mutation (b and c) and the demonstration of its absence in the leukocytes (LE) and parathyroid tumor (PT) from individual II.2 and in 55 normal individuals (N1–N3 are shown). After PCR amplification and *Mlu*I digestion, a 303-bp product was obtained for the wild-type (WT) allele, whereas two products of 161 and 142 bp were obtained for the mutant (m) alleles, which were resolvable by agarose gel electrophoresis (b and c). Individual I.1 had suffered from parathyroid tumors and gastrinoma; II.2 suffered from parathyroid tumors, prolactinoma, and lipoma; and individual III.1, who is 18 yr old, is asymptomatic, biochemically normal, and represents a carrier (*dot in the middle of symbol*) for the MEN1 mutation. There is an age-related penetrance for MEN1 (8). *Squares*, Males; *circles*, females; *open symbols*, unaffected; *filled symbols*, affected.

this region, which may be involved in the molecular etiology of MEN1 tumors. In keeping with the Knudson hypothesis (11) these somatic mutations would be expected to involve the allele that does not already harbor the germline mutation (2, 16, 17). Indeed, it has already been established that somatic LOH in MEN1-associated insulinomas (16) and parathyroid tumors (17) occurs on the allele that does not have the germline mutation. However, a similar demonstration of the locations of the somatic and germline mutations on different alleles in tumors 3 and 6, which would require the subcloning of PCR products spanning codons 39–211 in plasmids together with their sequencing, is not possible. This is because both the somatic and germline mutations are separated by more than 2 kbp of genomic DNA, thereby making it difficult to obtain reliable PCR products, and the use of RT-PCR products is not feasible, as RNA from the tumors is not available. However, the occurrence of the four somatic mutations solely in the tumors and their absence in other tissues, *e.g.* leukocyte DNA, together with previous reports (16, 17) showing that the somatic and germline abnormalities do occur on separate alleles are consistent with a tumor suppressor gene role for the MEN1 gene. Indeed, our findings of somatic mutations in the MEN1 tumors are similar to those reported for retinoblastoma (22), which is the paradigm model for Knudson's "two-hit" hypothesis (11).

Our identification of these somatic mutations in two parathyroid tumors, a lipoma and an insulinoma, from four MEN1 patients is also consistent with the idea that the same genetic deregulation may initiate neoplastic processes in different cell types. However, it is important to note that two of the MEN1 tumors studied did not harbor somatic mutations of the coding region or LOH, and several explanations may

be considered, including the failure of PCR to detect LOH in these tumors because of admixture of normal DNA or the involvement of mechanisms of gene inactivation, such as hypermethylation (23), or mutations of the promoter or noncoding regions. Interestingly, the MEN1 gene is known to contain a CpG-rich area in its 5′-region, and hypermethylation of such regions in the promoters of the VHL gene in von Hippel-Lindau and the hMLH1 gene in colorectal carcinoma has been reported to be a frequent cause of the somatic second hit in the tumorigenesis of these disorders (24–26). In summary, we report that somatic point mutations of the MEN1 gene, in addition to LOH, may act as the second hit in the etiology of MEN1 tumors, in keeping with the proposed role as a tumor suppressor for the MEN1 gene.

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References

1. **Marx SJ** 1998 Multiple endocrine neoplasia type 1. In: Vogelstein B, Kinzler KW, eds. The genetic basis of human cancer. New York: McGraw-Hill; 489–506
2. **Thakker RV** 1995 Multiple endocrine neoplasia type 1. In: DeGroot LJ, ed. Endocrinology, 3rd Ed. Philadelphia: W.B. Saunders; 2815–2831
3. **Guru SC, Goldsmith PK, Burns AL, et al.** 1998 Menin, the product of the MEN1 gene, is a nuclear protein. Proc Natl Acad Sci USA 95:1630–1634
4. **Chandrasekharappa SC, Guru SC, Manickam P, et al.** 1997 Positional cloning of the gene for multiple endocrine neoplasia-type 1. Science 276:404–407
5. **European Consortium on MEN1** 1997 Identification of the multiple endocrine neoplasia type 1 (MEN1) gene. Hum Mol Genet 6:1177–1183
6. **Agarwal SK, Guru SC, Heppner C, et al.** 1999 Menin interacts with the AP1 transcription factor JunD and represses JunD-activated transcription. Cell 84: 730–735
7. **Agarwal SK, Kester MB, Debelenko LV, et al.** 1997 Germline mutations of the MEN1 gene in familial multiple endocrine neoplasia type 1 and related states. Hum Mol Genet 6:1169–1175
8. **Bassett JH, Forbes SA, Pannett AA, et al.** 1998 Characterization of mutations in patients with multiple endocrine neoplasia type 1. Am J Hum Genet 62: 232–244
9. **Teh BT, Esapa CT, Houlston R, et al.** 1998 A family with isolated hyperparathyroidism segregating a missense MEN1 mutation and showing loss of the wild-type alleles in the parathyroid tumors. Am J Hum Genet 63:1544–1549
10. **Pannett AA, Thakker RV** 1999 Multiple endocrine neoplasia type 1. Endocr Relat Cancer 6:449–473
11. **Knudson AG, Jr** 1971 Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci USA 68:820–823
12. **Heppner C, Kester MB, Agarwal SK, et al.** 1997 Somatic mutation of the MEN1 gene in parathyroid tumours. Nat Genet 16:375–378
13. **Zhuang Z, Vortmeyer AO, Pack S, et al.** 1997 Somatic mutations of the MEN1 tumor suppressor gene in sporadic gastrinomas and insulinomas. Cancer Research 57:4682–4686
14. **Hessman O, Lindberg D, Skogseid B, et al.** 1998 Mutation of the multiple endocrine neoplasia type 1 gene in nonfamilial, malignant tumors of the endocrine pancreas. Cancer Res 58:377–379
15. **Debelenko LV, Brambilla E, Agarwal SK, et al.** 1997 Identification of MEN1 gene mutations in sporadic carcinoid tumors of the lung. Hum Mol Genet 6:2285–2290
16. **Larsson C, Skogseid B, Oberg K, Nakamura Y, Nordenskold M** 1988 Multiple endocrine neoplasia type 1 gene maps to chromosome 11 and is lost in insulinoma. Nature 332:85–87
17. **Thakker RV, Bouloux P, Wooding C, et al.** 1989 Association of parathyroid tumors in multiple endocrine neoplasia type 1 with loss of alleles on chromosome 11. N Engl J Med 321:218–224
18. **Friedman E, Sakaguchi K, Bale AE, et al.** 1989 Clonality of parathyroid tumours in familial multiple endocrine neoplasia type 1. N Engl J Med 321: 213–218
19. **Williamson C, Pannett AA, Pang JT, et al.** 1997 Localisation of a gene causing endocrine neoplasia to a 4 cM region on chromosome 1p35–p36. J Med Genet 34:617–619
20. **European Consortium on MEN1** 1996 Definition of the minimal MEN1 candidate area based on a 5-Mb integrated map of proximal 11q13. Genomics 37:354–365
21. **Chakrabarti R, Srivatsan ES, Wood TF, et al.** 1998 Deletion mapping of endocrine tumors localizes a second tumor suppressor gene on chromosome band 11q13. Genes Chromosomes Cancer 22:130–137
22. **Hogg A, Bia B, Onadim Z, Cowell J K** 1993 Molecular mechanisms of oncogenic muations in tumours from patients with bilateral and unilateral retinoblastoma. Proc Natl Acad Sci USA 90:7351–7355
23. **Tycko B** 2000 Epigenetic gene silencing in cancer. J Clin Invest 105:401–407
24. **Herman JG, Latif F, Weng Y, et al.** 1994 Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. Proc Natl Acad Sci USA 91:9700–9704
25. **Prowse AH, Webster AR, Richards FM** 1997 Somatic inactivation of the VHL gene in Von Hippel-Lindau disease tumors. Am J Hum Genet 60:765–771
26. **Herman JG, Umar A, Polyak K, et al.** 1998 Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci USA 95:6870–6875