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Somatic Mutations of the *PTEN/MMAC1* Gene in Fifteen Japanese Endometrial Cancers: Evidence for Inactivation of Both Alleles

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Loss of heterozygosity (LOH) of chromosome 10q is observed in approximately 40% of endometrial cancers. Mutations in *PTEN/MMAC1*, a gene recently isolated from the 10q23 region, are responsible for two dominantly inherited neoplastic syndromes, Cowden disease and Bannayan-Zonana syndrome. Somatic mutations of this gene have also been detected in sporadic cancers of the brain, prostate and breast. To investigate the potential role of this putative tumor suppressor gene in endometrial carcinogenesis as well, we examined 46 primary endometrial cancers for LOH at the 10q23 region, and for mutations in the entire coding region and exon-intron boundaries of the *PTEN/MMAC1* gene. LOH was identified in half of the 38 informative cases, and subtle somatic mutations were detected in 15 tumors (33%). Our results suggest that of the genes studied so far in endometrial carcinomas, *PTEN/MMAC1* is the most commonly mutated one, and that inactivation of both copies by allelic loss and/or mutation, a pattern that defines genes as "tumor suppressors," contributes to tumorigenesis in endometrial cancers.

Key words: *PTEN/MMAC1* — Endometrial cancer — Tumor suppressor gene — Chromosome 10q

Endometrial cancer is the most common gynecological malignancy in the United States and the fifth most common cause of cancer in women worldwide.[1] Like many other solid tumors, carcinomas of the endometrium are now believed to develop through a multi-step process involving activation of oncogenes and inactivation of tumor suppressor genes.[2–6] Yet the molecular events underlying tumor development and/or progression of this type of tumor have not been characterized well. Until now, the most commonly observed alteration in endometrial tumors has been microsatellite instability, a phenomenon first detected in tumors among patients suffering from HNPCC, a hereditary nonpolyposis colorectal cancer syndrome. In HNPCC families, where defective alleles of DNA-repair genes are associated with the instability of microsatellite loci, endometrial cancer is the second most common type of tumor. In fact, 17–23% of all endometrial cancers examined, sporadic or inherited, have been characterized by this form of genetic alteration.[7–9] Although alterations of the *K-ras* oncogene or the *p53* tumor suppressor gene also occur in endometrial cancers, the frequency of those mutations appears to be low.[10]

Many tumor suppressor genes are inactivated by intragenic mutations in one allele, accompanied by loss of a chromosomal region containing the other allele that can be detected by loss of heterozygosity (LOH). Endometrial cancers have shown a high frequency of allelic loss on chromosome 10.[11–13] Two regions are commonly deleted, one at 10q22–24 and the other at 10q25–26.[12,13] Analyses of homozygous deletions affecting chromosome 10q23 led to the recent identification of a novel tumor suppressor gene, designated *PTEN/MMAC1*.[14,15] Germline mutations of *PTEN/MMAC1* are responsible for two dominantly inherited neoplastic syndromes, Cowden disease and Bannayan-Zonana syndrome.[16,17] *PTEN/MMAC1* encodes a 403-amino-acid, dual-specificity phosphatase that contains a region homologous to tensin and auxillin, cytoskeletal proteins that interact with adhesion molecules.[14,15,18] Somatic mutations of this gene have been detected in tumors of the brain, prostate and breast.[14,15]

To determine the role of *PTEN/MMAC1* alterations in the development and/or progression of endometrial tumors, we examined this gene for mutation and allelic loss in 46 primary endometrial cancers, and looked for correlations between *PTEN/MMAC1* mutations and certain clinicopathological parameters. Here we report evidence that both alleles of the *PTEN/MMAC1* gene are inactivated in a large proportion of endometrial cancers.

MATERIALS AND METHODS

Samples and DNA preparation Tumors and corresponding noncancerous tissues were obtained from 46 patients with sporadic endometrial cancers who underwent surgery at Nippon Medical School; none had undergone previous radiotherapy or chemotherapy. Genomic DNA was

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extracted from 9 frozen tissues in a manner described previously[19] and from 37 paraffin-embedded tissues that were dissected under microscopic observation to separate cancer cells from normal cells, using the "DEXPAT" (TaKaRa, Tokyo) system according to the manufacturer's directions. Histopathological diagnoses and clinical stages were classified according to the criteria of WHO and the International Federation of Gynecology and Obstetrics, respectively.[20, 21]

LOH analysis Matched samples of normal and tumor genomic DNA from all 46 cases were analyzed for LOH with three microsatellite markers mapped in the 10q23 region surrounding *PTEN/MMAC1* (D10S1744, D10S1765 and AFM280WE1). Polymerase chain reaction (PCR) experiments, electrophoresis and autoradiography were carried out as described elsewhere.[22]

Mutation analysis All samples were screened for mutations by PCR-single strand conformational polymorphism (SSCP) analysis of the entire *PTEN/MMAC1* coding region and exon-intron boundaries, using PCR primers designed to amplify 27 overlapping segments (Table I). The length of the PCR products subjected to SSCP analysis ranged from 102 bp to 128 bp. Each segment was amplified in a volume of 10 μl containing 20 ng of genomic DNA, 1× PCR reaction buffer (Boehringer Mannheim, Mannheim, Germany), 200 m*M* of each dNTP, 2 pmol of each primer, 2 μCi of [α-^{32}P]dCTP (3000 Ci/mmol, 10 mCi/ml) and 0.5 units of *Taq* polymerase (Boehringer Mannheim). Cycling conditions were 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min (Gene Amp PCR 9600 System, Perkin-Elmer Cetus, Norwalk, CT). Each PCR product was mixed with 10 μl of loading buffer (95% formamide, 20 m*M* EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol), heated at 94°C for 5 min, rapidly cooled on ice, and applied to a 5% polyacrylamide gel containing 5% glycerol in 0.5× TBE buffer.[23] Electrophoresis was performed under two different conditions to improve resolution for detecting different types of SSCP variants: 120 V for 16 h at room temperature, and 200 V for 16 h at 4°C. Gels were dried and autoradiographed with intensifying screens. For sequence analysis, 2 μl of each genomic DNA that showed variant bands in PCR-SSCP analysis was used as a template for PCR amplifica-

Table I. Sequence of *PTEN/MMAC1* Primers Used for PCR-SSCP Analysis

Exon	Sense primer (5′-3′)	Antisense primer (5′-3′)
1-a	GCCATCTCTCTCCTCCTTTT	AGGTCAAGTCTAAGTCGAATC
1-b	AAAGAGATCGTTAGCAGAAACA	CTAAGAGAGTGACAGAAAGGTA
2-a	GATTGCTGCATATTTCAGATATT	TCAATATTGTTCCTGTATACGC
2-b	ATCCAAACATTATTGCTATGGG	ATGAATATAAACATCAATATTTGAAA
3	TTTTTGTTAATGGTGGCTTTTTG	TTAGAAGATATTTGCAAGCATAC
4	GCAAAAGATAACTTTATATCACTT	TCGGGTTTAAGTTATACAACATA
5-a	AGTTTTTTTTTCTTATTCTGAGGT	GGTCAAGATCTTCACAAAAGG
5-b	CTTTTGAAGACCATAACCCAC	CCAGCTTTACAGTGAATTGCT
5-c	GATCTTGACCAATGGCTAAGT	TTGCCCCGATGTAATAAATATG
5-d	TGTAAAGCTGGAAAGGGACG	TCTGGTCCTTACTTCCCCAT
5-e	TCGGGGCAAATTTTTAAAGGC	TCCAGGAAGAGGAAAGGAAA
6-a	CATAGCAATTTAGTGAAATAACT	CAGGTAGCTATAATAATACACAT
6-b	TTCTGTCCACCAGGGAGTAA	GTTTCAAACATCATCTTGTGAAA
6-c	CCTGTTAAAGAATCATCTGGAT	GTTCCAATACATGGAAGGATG
7-a	TGACAGTTTGACAGTTAAAGG	GTCGTGTGGGTCCTGAATTG
7-b	TTGTGGTCTGCCAGCTAAAG	ACTCTACTTTGATATCACCACA
7-c	GACAAGTTCATGTACTTTGAGT	ACGAAAGTAAAGTACAAACCTTT
7-d	GATATCAAAGTAGAGTTCTTCC	GGATATTTCTCCCAATGAAAG
8-a	TAATTAAATATGTCATTTCATTTCTT	TTCTGAGGTTTCCTCTGGTC
8-b	GACAAAATGTTTCACTTTTGGG	ACTGCAAATGCTATCGATTTCT
8-c	GGAAACCTCAGAAAAAGTAGAA	TGTTAAAGTAAGTACTAGATATTC
8-d	ATCGATAGCATTTGCAGTATAG	TATCGGTTGGCTTTGTCTTTAT
8-e	GAATATCTAGTACTTACTTTAACA	CACCAACCCCCACAAAATGT
9-a	TGAGTCATATTTGTGGGTTTTC	TACAGAAGTTGAACTGCTAGC
9-b	CTGTACTTCACAAAAACAGTAG	GTCAGTGGTGTCAGAATATCT
9-c	CTGTAACACCAGATGTTAGTG	TGTAATTTGTGTATGCTGATCTT
9-d	CTGACACCACTGACTCTGAT	TTTTCATGGTGTTTTATCCCTC

tion under conditions described elsewhere.[22] Each PCR product was purified using "SUPREC-02" (TaKaRa), according to the manufacturer's instructions. Direct sequencing of purified PCR products was performed with a ^{32}P-end-labeled primer using the "Thermo Sequenase cycle sequencing kit" (Amersham, OH), according to the manufacturer's instructions. After electrophoresis at 1800 V for 2–3 h, gels were dried and exposed to X-ray film at room temperature for 16–24 h.

RESULTS

LOH analysis Three microsatellite markers in the 10q23 region containing the *PTEN/MMAC1* gene, D10S1744, D10S1765 and AFM280WE1, were used for LOH analysis. Among the 46 endometrial cancers examined, five were excluded from LOH analysis since they showed replication errors (RER). Thirty-eight patients in our study were informative, and LOH at one or more loci was

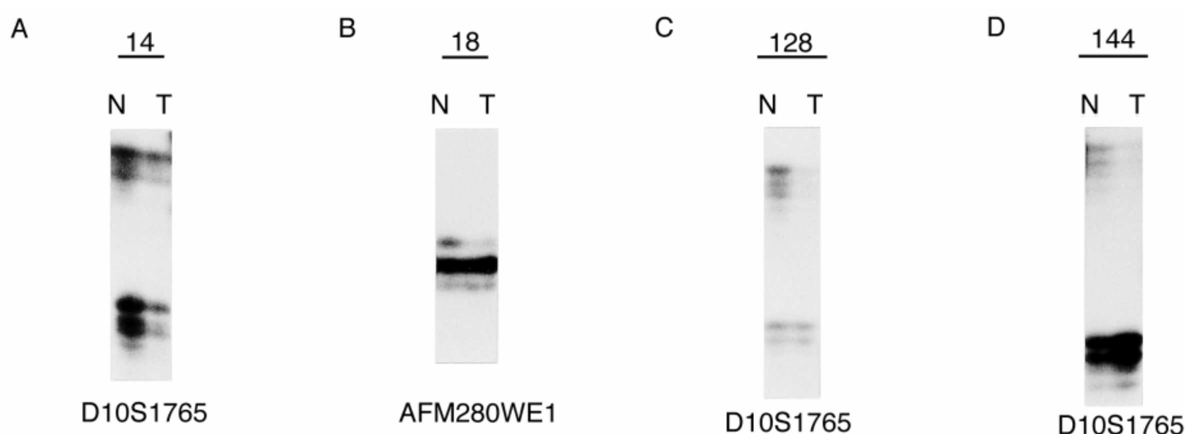

Fig. 1. Representative autoradiograms of the LOH analyses. Case numbers are shown at the top of each panel; T and N, matched DNA samples isolated from tumor and normal tissues, respectively. Tumor samples show loss of one allele when compared to the normal heterozygote.

Table II. *PTEN/MMAC1* Mutations in Primary Endometrial Cancers

Sample	10q LOH[a]	Exon/intron	Codon	Mutation	Predicted effect	Stage	Grade[b]	Histology[c]
174	−	exon 1	13–14	A ins	stop at 43	IIa	1	E
160	+	exon 2	33	3 bp del	Ilu deletion	Ib	1	
144	+	exon 5	92	GAC to GGC	Asp to Gly	Ic	1	E
116	RER	exon 5	93	CAT to TAT	His to Tyr	IIb	2	
102	N	exon 5	124	TGT to AGT	Cys to Ser	IIa	1	E
14	+	exon 5	133	GTA to ATA	Val to Ile	Ic	2	E
112	+	exon 5	133	GTA to ATA	Val to Ile	Ib	2	E
120	N	exon 6	170	AGT to AAT	Ser to Asn	IIIc	3	E
148	−	exon 7	245–247	8 bp del + A ins	stop at 253	Ib	1	
146	RER	exon 7	265–267	A del	stop at 275	Ib	1	
18	+	intron 7	first base	GT to TT	splice variant	Ia	3	E
128	+	exon 8	298	CAA to TAA	Gln to stop	IIIc	1	
20	RER	exon 8	319–320	4 bp del	stop at 319	Ib	1	E
154	+	exon 8	319–320	4 bp del	stop at 319	Ic		E
136	−	exon 8	321–323	A del	stop at 343	Ib	1	

a) N: not informative.
b) Grade: 1, well differentiated; 2, moderately differentiated; 3, poorly differentiated.
c) Histology: E, endometrioid.

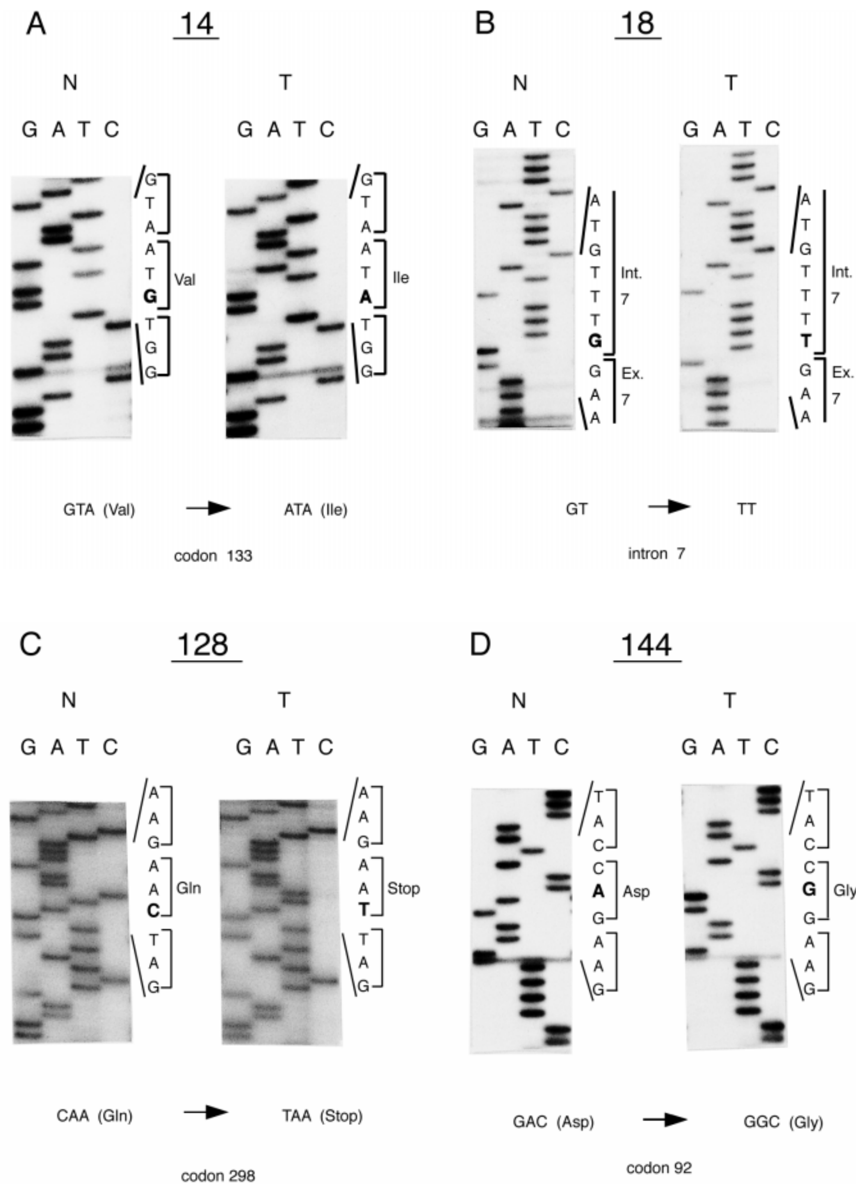

Fig. 2. Sequence analysis of PCR products of tumor DNA and corresponding normal DNA from the four patients whose tumor DNA revealed aberrant bands by PCR-SSCP analysis. Case numbers are shown at the top of each panel; T and N, matched DNA samples isolated from tumor and normal tissues, respectively.

detected in 19 (50%) of the tumors examined. Representative autoradiograms for four of these cases are shown in Fig. 1.

***PTEN/MMAC1* mutation** We screened 46 primary endometrial cancers for mutations in the *PTEN/MMAC1* gene by means of PCR-SSCP and sequencing analyses after having determined the exon-intron boundary sequences for all nine exons. Somatic mutations identified in 15 (33%) of the tumors included six missense mutations, four frameshifts, three nonsense mutations, one 3-bp in-frame deletion, and one splicing consensus mutation (Table II).

Of the six tumors with missense mutations, cases 14 and 112 exhibited the same G-to-A transition at the first nucleotide of codon 133 (exon 5), which would result in a substitution of Ile for Val at this site in the putative phosphatase domain (V133I) (Fig. 2A). In another example involving this domain, tumor 144 showed an A-to-G transition at the second nucleotide of codon 92, which would

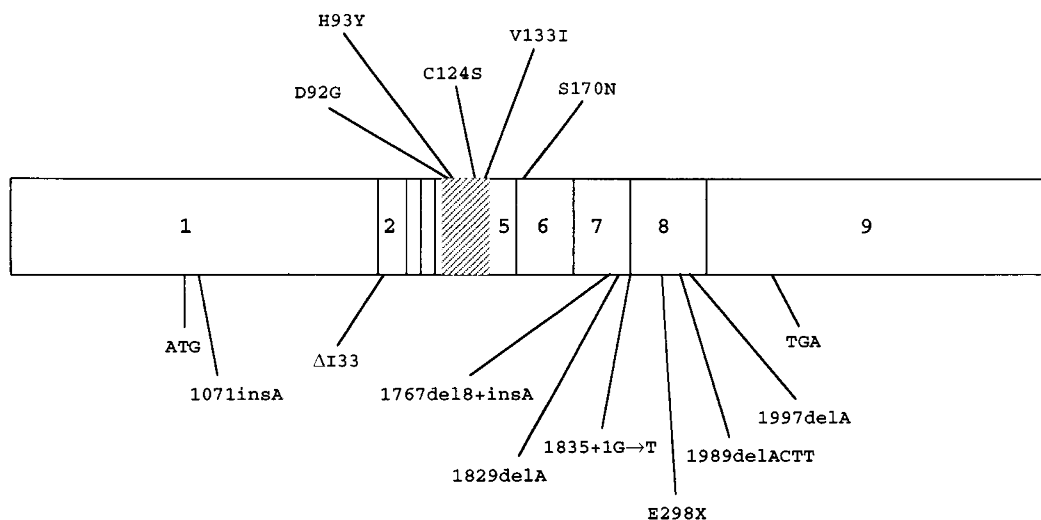

Fig. 3. Summary of mutations identified in primary endometrial cancers. The locations of the six missense mutations (V133I was encountered twice) are shown above a diagram of the *PTEN/MMAC1* gene. Four frameshift mutations, one in-frame deletion of a single amino acid, one nonsense mutation, one 8-bp deletion, a 1-bp insertion, and a G-to-T substitution in the first base of intron 7 are shown below the gene diagram. The 1989delACTT mutation was also encountered twice. Mutation abbreviations follow standard nomenclature.[36] Locations of the start (ATG) and stop (TGA) codons are indicated. ▒ putative phosphatase domain.

result in a substitution of Gly for Asp (D92G) (Fig. 2D). Other missense mutations in the putative phosphatase domain were present in tumors 102 and 116. Tumor 128, an example of nonsense mutation, showed a C-to-T transition at the first nucleotide of codon 298, which caused a nonsense mutation in place of Gln (E298X) (Fig. 2C). Another type of mutation is shown in Fig. 2B, where in tumor 18, the G-to-T transition at the first nucleotide in intron 7 would alter the consensus sequence at a splice-donor site (1835+1G→T). Mutations were detected in six of the nine exons, the majority of them in exons 5 (33%) and 8 (27%). Five of the six missense mutations identified were located in the putative phosphatase domain in exon 5, and this domain is critical for the function of the PTEN/MMAC1 protein (Fig. 3).

We found no significant association between the presence or absence of mutations in the *PTEN/MMAC1* gene and either clinical stage or histopathological type.

Two-hit inactivation of the *PTEN/MMAC1* gene We examined the allelic status of the *PTEN/MMAC1* gene in our panel of endometrial cancers by combining the results of LOH analysis and direct sequencing. In each of the 15 tumors in which a subtle mutation was identified, we were able to determine that the mutated sequence was retained in the tumor DNA whereas the wild-type sequence had been lost. These findings were supported by the results of LOH analyses, as seven of the ten mutation-containing tumors that were informative for microsatellite markers exhibited loss of an allele.

DISCUSSION

The results reported here represent the first extensive screening for *PTEN/MMAC1* gene mutations in endometrial cancers in Japan. Among 46 primary tumors examined we identified 15 mutations, all of them somatic alterations. The frequency of *PTEN/MMAC1* mutations in the Japanese patients was 33%. In the United States, Tashiro *et al.*[24] and Risinger *et al.*[25] found *PTEN/MMAC1* mutations in 34–50% of endometrial cancers from Caucasian women. Our findings indicate that *PTEN/MMAC1* mutations are common in endometrial cancers regardless of ethnicity.[24, 25] In the present study of Japanese endometrial cancers, mutations were detected in six of the nine exons, the majority of them in exons 5 (33%) and 8 (27%). Most missense mutations identified were located in exon 5. In previous reports that analyzed brain, prostate, breast and endometrial tumors, as well as inherited neoplastic syndromes, the majority of *PTEN/MMAC1* mutations were also detected in exons 5, 7 and 8.[14, 24, 26–28] Of those, most of the missense mutations were identified in exon 5. The mutation spectrum noted in these studies indicates that exon 5, encoding the putative phosphatase domain, and exons 7 and 8, encoding a potential tyrosine kinase phosphorylation site, are essential for the function of the PTEN/MMAC1 protein.

Our studies revealed LOH in 19 (50%) of the 38 informative tumors and RER in 5 (11%) of the 46 tumor specimens. Mutations were detected in 3 of 5 (60%) RER-

positive and 12 of 41 (29%) RER-negative endometrial tumors. Tashiro *et al*. also reported that *PTEN/MMAC1* was mutated more often in RER-positive endometrial cancers than in RER-negative tumors.[24] In the majority of the tumors in which we identified point mutations, we demonstrated that both alleles of *PTEN/MMAC1* were inactivated. Our results suggest that inactivation of both copies of this gene is required for development and/or progression of endometrial cancer; this conclusion would fulfill the criterion of the "two-hit" concept defining tumor suppressor genes, as proposed by Knudson.[29]

"Classical" tumor suppressor genes responsible for inherited neoplastic syndromes, such as *Rb*, *VHL* and *WT1*, have been shown to undergo "two-hit" inactivation in sporadic tumors as well as in familial tumors of the same tissue type.[30–32] It is true that germline mutations of *PTEN/MMAC1* gene are responsible for two dominantly inherited neoplastic syndromes, Cowden disease and Bannayan-Zonana syndrome, where the risk of breast and thyroid cancers is elevated in carriers of mutant alleles.[16, 17] However, contrary to expectations based on the behavior of classical tumor suppressor genes, somatic mutation of the *PTEN/MMAC1* gene is seldom found in sporadic breast or thyroid cancers[14, 15, 33–35]; moreover, Cowden disease is not associated with an elevated risk of endometrial cancer. The results of the present study and other studies have indicated that somatic "two-hit" *PTEN/MMAC1* mutations are frequent in sporadic endometrial cancers. We suggest that *PTEN/MMAC1* may belong to a novel and distinct class of tumor suppressor genes, in that it fulfills Knudson's "two-hit" inactivation hypothesis in one type of sporadic cancer (endometrial), but not in the familial cancers (thyroid and breast) for which germline mutations of the *PTEN/MMAC1* gene are responsible.

In the study reported here, we identified frequent allelic losses and somatic mutations of the *PTEN/MMAC1* gene in primary endometrial cancers from Japanese women, and showed inactivation of both alleles in most of the informative cases. We suggest that *PTEN/MMAC1* is the most commonly altered gene in endometrial cancers and that this tumor suppressor gene undergoes "two-hit" inactivation during endometrial carcinogenesis.

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