[CANCER RESEARCH 57, 4736–4738, November 1, 1997]

Advances in Brief

PTEN/MMAC1 Mutations in Endometrial Cancers

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Abstract

Endometrial carcinomas represent the most common gynecological cancer in the United States, yet the molecular genetic events that underlie the development of these tumors remain obscure. Chromosome 10 is implicated in the pathogenesis of endometrial carcinoma based on loss of heterozygosity (LOH), comparative genomic hybridization, and cytogenetics. Recently, a potential tumor suppressor gene, *PTEN/MMAC1*, with homology to dual-specificity phosphatases and to the cytoskeletal proteins tensin and auxillin was identified on chromosome 10. This gene is mutated in several types of advanced tumors that display frequent LOH on chromosome 10, most notably glioblastomas. Additionally, germ-line mutations of *PTEN/MMAC1* are responsible for several familial neoplastic disorders, including Cowden disease and Bannayan-Zonana syndrome. Because this locus is included in the region of LOH in many endometrial carcinomas, we examined 70 endometrial carcinomas for alterations in *PTEN/MMAC1*. Somatic mutations were detected in 24 cases (34%) including 21 cases that resulted in premature truncation of the protein, 2 tumors with missense alterations in the conserved phosphatase domain, and 1 tumor with a large insertion. These data indicate that *PTEN/MMAC1* is more commonly mutated than any other known gene in endometrial cancers.

Introduction

The molecular pathogenesis of endometrial carcinoma is largely unknown (1). The most commonly observed defect is a profound instability of microsatellite sequences found in about 20% of tumors (2, 3). This type of microsatellite instability is seen in sporadic as well as tumors from hereditary nonpolyposis colorectal cancer, of which endometrial carcinoma is the second most common noncolonic tumor (4, 5). Microsatellite instability is likely a result of defective DNA mismatch repair. Additionally, a smaller percentage of tumors have activating alterations of the *K-ras* oncogene or alterations in the *p53* tumor suppressor gene (1). Recently, several studies have examined LOH[2] in endometrial carcinoma to elucidate regions of the genome that could harbor tumor suppressor genes. Chromosome 10 has been shown to contain significant LOH in endometrial carcinomas (6, 7). These studies have implicated two commonly deleted areas, one in the 10q22–24 region and a more distal region at 10q25–26 (7, 8). Additionally, conventional cytogenetic banding techniques and comparative genomic hybridization methods have implicated chromosome 10 in endometrial carcinomas (9, 10).

A candidate tumor suppressor gene, *PTEN/MMAC1*, was recently isolated from the 10q23–24 region and found to be mutated in several cancer types that display LOH in this region (11, 12). Of these, mutations in *PTEN/MMAC1* are most frequently found in advanced glioblastomas. The fact that mutations were seen in advanced tumors suggested that the gene was involved in disease progression and thus led to its nomenclature (mutated in multiple advanced cancer 1). *PTEN/MMAC1* is also mutated in several other tumor types including those of the prostate and the breast. Germ-line mutations of *PTEN/MMAC1* are also responsible for Cowden disease and Bannayan-Zonona syndrome (12, 13). These syndromes are characterized by several neoplastic conditions, some of which are benign in nature. These data strongly suggest that the *PTEN/MMAC1* gene is involved in the pathogenesis of several varied neoplasms. Based on these findings and the clear involvement of this chromosomal region in endometrial cancers, we examined the *PTEN/MMAC1* gene for mutational inactivation in a large panel of endometrial carcinomas.

Materials and Methods

Clinical Specimens. Snap-frozen tissue samples were obtained from 70 women who underwent hysterectomy for endometrial adenocarcinoma at Duke University Medical Center between 1990 and 1996. There were 16 well-differentiated cancers, 29 moderately differentiated cancers, and 25 poorly differentiated cancers. Staging was performed in accordance with the Fédération Internationale des Gynaecologistes et Obstetristes staging system. Histological evidence of metastatic disease was found outside the uterus in 17 of 70 cases (24%). Complete clinicopathological information was abstracted from the hospital records. Genomic DNA and total RNA were isolated according to established protocols. Cases with identical mutations were genotyped with the D1S80 marker using the AmpliFlp kit (Applied Biosystems, Foster City, CA).

PTT. Five μg of total RNA were reverse-transcribed into cDNA using Superscript II reverse transcriptase and random hexamers according to the manufacturer's recommendations (Life Technologies Inc., Gaithersburg, MD). The entire *PTEN/MMAC1* gene was amplified using the Expand high-fidelity PCR system (Boehringer Mannheim, Indianapolis, IN) with a PCR cycling profile of 20 s at 95°C, 20 s at 55°C, and 2 min at 70°C, repeated 35 times. Primers for PCR were GGATCCTAATACGACTCACTATAGGGAGACCACCATGACAGCCATCATCAAAGAG and GGTCCATTTTCAGTTTATTCAA. PCR product (3 μl) was used in a coupled transcription and translation reaction (Promega, Madison, WI) that incorporated [^{35}S]methionine to label the resultant proteins. Labeled proteins were analyzed on 10 and 4–15% SDS polyacrylamide gels.

SSCA. This assay was performed as described previously (14). The intron sequences of *PTEN/MMAC1* were determined by sequencing the product of FA/RP primers described (11). The 9 exons of *PTEN/MMAC1* were amplified using 11 pairs of primers based on the FA/RP sequences. Exons 5 and 8 divided were amplified with two overlapping primer pairs. Normal and tumor DNAs were amplified using a step-down cycling profile of 7 cycles of 20 s at 95°C, 20 s at 55°C, and 30 s at 72°C, followed by an additional 30 cycles with an annealing temperature of 48°C, or alternatively, 37 cycles with an annealing temperature of 48°C. PCR was performed using Amplitaq or Amplitaq Gold (for exon 1) with appropriate buffers from the manufacturer (Applied Biosystems). The products were labeled with [^{33}P]dATP, which was incorporated during the PCR. Primers for the exons were: exon 1, CAGAAGAAGCCCCGCCACCAG and AGAGGAGCAGCCGCAGAAATG; exon 2, TTTCAGATATTTCTTTCCTTA and AACAAGAATATAAAACATCAA; exon 3, TAATTTCAAATGTTAGCTCAT and AAGATATTTGCAAGCATACAA; exon 4, GTTTGTTAGTATTAGTACTTT and ACAACATAGTACAGTACATTC; exon 5, TATTCTGAGGTTATCTTTTA and CTTTTCCAGCTTTACAGTGAA (first pair) and GCTAAGTGAAGATGACAATCA and AGGAAAAACATCAAAAAATAA (second pair); exon 6, TTGGCTTCTC-

Received 8/26/97; accepted 9/22/97.

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[2] The abbreviations used are: LOH, loss of heterozygosity; SSCA, single-strand conformational analysis; PTT, protein truncation test.

Table 1 *PTEN/MMAC1 mutations in primary endometrial carcinomas*

Mutations are named according to the suggested nomenclature (17).

Case	Nucleotide	Codon	Base change	Amino acid change	Stage	Grade[a]	Histology[b]
1	40	14	Del A	Stop 23	IA	1	E
2	80	26	Del AT	Stop 42	IC	3	E
3	117	40	G>T	Stop 40	IIIA	2	E
4	117	40	Del ATT	Del Ile 40	IB	1	E
	969	323	Ins A	Stop 324			
5	187	63	Del A	Stop 98	IB	1	E
	969	323	Ins A	Stop 324			
6	202	68	Ins TAT	Stop 74	IA	1	E
7	313	104	Ins T	Stop	IIC	3	E
8	387	128	Del A	Stop 133	IB	2	E
9	388	129	C>G	Gly to Arg	IB	1	E
10	389	129	G>A	Gly to Glu	IB	2	E
11	640	214	C>T	Stop 214	IC	2	AS
12	640	214	C>T	Stop 214	IA	3	E
13	727	242	Del 13bp	Stop 251	IB	2	E
14	799	267	Del A	Stop 275	IIIC	3	E
15	799	267	Del A	Stop 275	IC	1	E
	956	323	Ins A	Stop 324			
16	802	268	Ins 171bp	Ins 57 AA	IVB	3	AS
17	864	288	Del 5bp	Stop 295	IVB	2	E
18	938	313	Del A	Stop 315	IB	3	AS
19	968	322	Del A	Stop 343	IB	3	E
20	969	323	Ins A	Stop 324	IC	3	E
	1048	350	Ins A	Stop 360			
21	984	328	Del 4bp	Stop 343	IA	1	E
22	987	329	Del 4bp	Stop 343	IB	1	E
23	1003	335	C>T	Stop 335	IA	1	E
24	1012	338	Del T	Stop 343	IA	1	E

[a] Grade: 1, well differentiated; 2, moderately differentiated; 3, poorly differentiated.
[b] Histology: E, endometrioid; AS, adenosquamous.

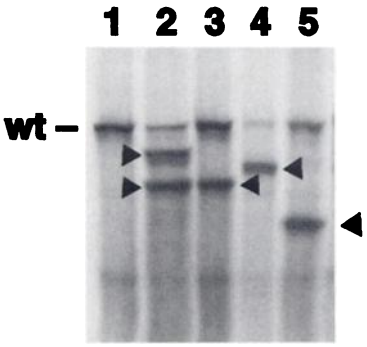

Fig. 1. PTT of *PTEN/MMAC1* in primary endometrial cancers. *Lane 1* represents the protein generated from normal uterus and its position is denoted by *wt* (wild-type). *Lanes 2–5* contain shorter peptides indicative of mutant *PTEN/MMAC1* and are marked with an *arrowhead*, residual wild-type product is present in all cases, most likely due to the presence of nonneoplastic cells in the tissue biopsies.

TTTTTTTTCTG and ACATGGAAGGATGAGAATTTC; exon 7, CCTGTGAAATAATACTGGTATG and CTCCCAATGAAAGTAAAGTACA; exon 8, ACACATCACATACATACAAGTC and GTGCAGATAATGACAAGGAATA (first pair) and TTAAATATGTCATTTCATTTCTTTTTC and CTTTGTCTTTATTTGCTTTGT (second pair); and exon 9, TTCATTTTAAATTTTCTTTCT and TGGTGTTTTATCCCTCTTGAT.

Sequencing. PCR products from mutant PTTs or SSCAs were directly purified on a Wizard PCR prep column (Promega), and a portion was directly sequenced with the Thermosequenase kit (Amersham, Arlington Heights, IN). Samples with alterations were repeated in both tumor and normal DNA from the patient.

Results and Discussion

The entire coding region of *PTEN/MMAC1* was examined using both an RNA-based (PTT) and a DNA-based (SSCA) mutation screening test. Both tests were used to maximize mutation detection. Mutations were identified in 24 of 70 (34.3%) unselected endometrial cancers (Table 1). Most mutations resulted in premature termination of the *PTEN/MMAC1* message, either by small deletions or insertions and in several cases by missense alteration. Examples of cases detected by the protein truncation test are depicted in Fig. 1, *Lane 2* (case 20), *Lane 3* (case 23), *Lane 4* (case 22), and *Lane 5* (case 17), which clearly show peptides smaller than the wild-type peptide (*Lane 1*). Representative mutations detected by SSCA are shown in Fig. 2 for two cases with different alterations in exon 3. PCR products amplified from the patient's tumor DNA contain aberrantly migrating bands, whereas those amplified from normal lymphocyte DNA migrated with the wild-type pattern (Fig. 2). The exact nucleotide changes and subsequent effect on possible translated proteins is summarized for these and all mutations in Table 1. Three representative examples of the direct sequence analysis are depicted in Fig. 3. Several other missense alterations were detected in tumors. These changes were present in the corresponding normal DNA and were not additionally analyzed.

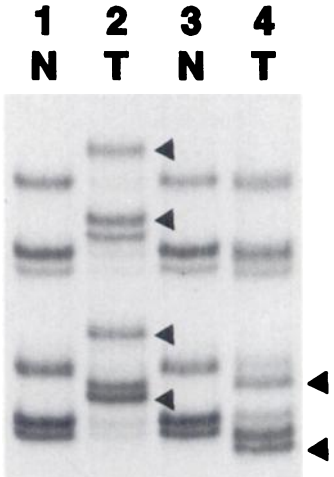

Fig. 2. SSCA analysis of *PTEN/MMAC1* exon 3. Altered mobility bands present in PCR products amplified from the patients' tumor DNA are seen in *Lane 2* (case 6) and *Lane 4* (case 5). PCR products amplified from the patients' normal DNA do not contain alterations indicating the somatic nature of these changes.

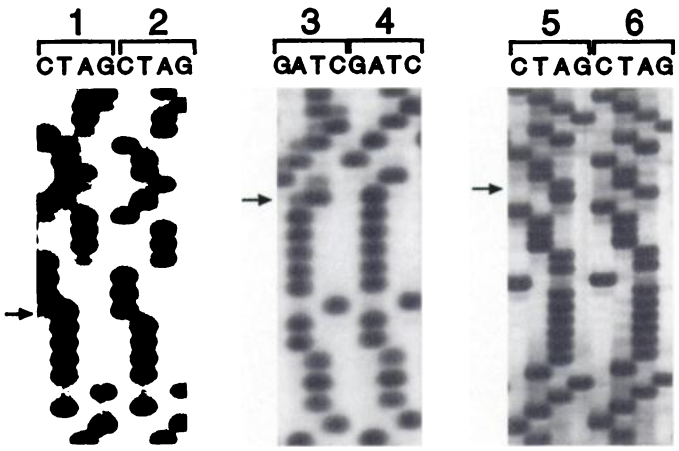

Fig. 3. Representative examples of *PTEN/MMAC1* sequence alterations. *Sequence 1* (case 7) shows an additional A (nucleotide 313) in a short homopolymeric run; the alteration is absent in the patient's germ-line DNA, *sequence 2*. *Sequence 3* (case 18) shows deletion of an A nucleotide in the $(A)_6$ homopolymeric run present at nucleotides 963–969; the change is not present in the patient's normal tissue (*sequence 4*). Single nucleotide insertion of an A residue (case 15) at position 956, *sequence 5*; lack of alteration in the PCR product generated from normal DNA, *sequence 6*.

Of the 24 cases with mutations, several are particularly interesting. Tumors from two patients (cases 9 and 10, Table 1) have missense alterations in codon 129 of the putative phosphatase domain, suggesting that this residue may be functionally important. Additionally, several cancers displayed frameshifts in short repeat sequences. Of these repeats, four tumors had somatic gain or loss of 1 bp within the $(A)_6$ repeat beginning at nucleotide 963, and two tumors had a somatic loss in the $(A)_6$ repeat beginning at position 795. All of these tumors have previously been shown to contain high levels of microsatellite instability (2, 14). Repeat sequences are particularly prone to polymerase slippage events in tumors with microsatellite instability, and mononucleotide repeats within other cancer genes have been similarly disrupted. The best-studied example involves the $(T)_{10}$ repeat of *TGFBRII*, which is mutated in almost all colon cancers with microsatellite instability (15). Interestingly, the *TGFBRII* repeat is only rarely altered in endometrial cancers with microsatellite instability (16). The *TGFBRII* repeat is wild-type in all 70 endometrial cancers examined in this study,[3] indicating that the *PTEN/MMAC1* mutations described here are necessary for tumorigenesis. The possibility that *PTEN/MMAC1* is a specific mutational target gene in endometrial and other tumor types with microsatellite instability warrants further study.

Cancers in which *PTEN/MMAC1* was mutated did not have a striking clinical phenotype. Mutations were seen in 17 of 53 (32%) cases in which the cancer was confined to the uterus and in 4 of 17 (24%) cases with metastatic disease. Although the highest frequency of mutations was seen in Stage IA well-differentiated cases with no myometrial invasion (86%), only seven such cases were examined. These very early lesions were underrepresented in our data set because there often is too little tumor available for research samples. No *PTEN/MMAC1* mutations were seen in papillary serous or clear cell cancers, but again, the number of cases studied was small (six cases). The involvement of *PTEN/MMAC1* in recurrent or metastatic tumors was not assessed; it is possible that these lesions may harbor a higher frequency of alteration.

The frequency of *PTEN/MMAC1* mutations described in this report is severalfold higher than that described for any other gene mutated in endometrial cancers, including *K-ras* and *p53* (1). Based on these data, *PTEN/MMAC1* mutations seem to represent the most common defined genetic alteration identified to date in endometrial cancers.

[3] J. I. Risinger, A. Berchuck, and J. C. Barrett, unpublished results.

Acknowledgments

We thank Dr. Roger Wiseman, Dr. Cynthia Afshari, and N. Keith Collins for critical review of the manuscript.

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