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

Keisuke Kurose, Koichi Bando, Koichi Fukino, Yuichi Sugisaki, Tsutomu Araki and Mitsuru Emi

Department of Molecular Biology, Institute of Gerontology, Nippon Medical School, 1-396 Kosugi-cho, Nakahara-ku, Kawasaki 211-8533, Department of Obstetrics and Gynecology and Division of Surgical Pathology, Nippon Medical School Hospital, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603

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. 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. 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. 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.

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. Two regions are commonly deleted, one at 10q22–24 and the other at 10q25–26. Analyses of homozygous deletions affecting chromosome 10q23 led to the recent identification of a novel tumor suppressor gene, designated PTEN/MMAC1. Germline mutations of PTEN/MMAC1 are responsible for two dominantly inherited neoplastic syndromes, Cowden disease and Bannayan-Zonana syndrome. PTEN/MMAC1 encodes a 403-amino-acid, dual-specificity phosphatase that contains a region homologous to tension and auxillin, cytoskeletal proteins that interact with adhesion molecules. Somatic mutations of this gene have been detected in tumors of the brain, prostate and breast.

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

To whom correspondence should be addressed.
extracted from 9 frozen tissues in a manner described previously\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 mM of each dNTP, 2 µCi of [α-\textsuperscript{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 mM 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.\textsuperscript{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  | GCCATCTCTCTCTCTTTTTT | AGGTCAAGCTTAAGTGAATC  |
| 1-b  | AAAAGAATGCCTGACAAACAAAACA | CTAAGAGAGTCAGACAAAAAGGT |
| 2-a  | GATTTGTCATATTTCTTGATATT | TCAATTTGCTCTGCTATACGC |
| 2-b  | ATCCAAACATTATGCTATGGG | ATGAATATACATCAATATTTGAAA |
| 3    | TTTTTGTTAATTTGCTTTTTTT | TTGAGAATATTTGCTTGACAT |
| 4    | GCAAAAGATAAATTTTATATCTT | TGGGGTTTTAAGTATACACAT |
| 5-a  | AGTTTTTTTTTCTTTATCTTGAGGT | GTGCAAGATCTCTCACAAGGAG |
| 5-b  | CTTTGAAGCATATACCCACCA | CCAGCTCTAAGTGAATGCT |
| 5-c  | GATCTTGACCAATGCTATGGTGTTAGT | TTGCCCCGATGTAATAAAAATG |
| 5-d  | TGGTTAAAAGGGGGAAGGGAC | TCTGGTCTTACCTCCCAT |
| 5-e  | TCGGGCAAATT TTAAAAAGGC | TCCAGGAAAGGAGGAAGGAA |
| 6-a  | CATAGCAATTTGTAATAAATC | CAGTATGCTTAATAAATACAT |
| 6-b  | TGTCTTCTCACCAGGGAGTAA | GTTTCAACATCATCTTGTGAAA |
| 6-c  | CCTGTTAAAGAATCATCTGAGAT | GTTCATTACATGGAAGGATG |
| 7-a  | TGGACGTGTGGCTGGTTAAGG | GTCTGGTGGTCTGGATTG |
| 7-b  | TGTGGTGGTCCCCAGCTAAAG | ACTCTACTTTGATATACCACA |
| 7-c  | GAACATGTCATGTACATTGAGT | AGCAAAAGTAAGTACAAACCTT |
| 7-d  | GATATCAACTGATAAGTCTTCC | GGATATTGTCTCTAAAAGGAG |
| 8-a  | TAAATTTATTGCTCTATTCTGCATT | TCTGAACCTTTCTCTGGTC |
| 8-b  | GACAAATGTTGCTCTATCTTTGG | ACTGCAAAATGTCTATCGATT |
| 8-c  | GCAAATCTACAGGAAAAAGTAAAG | TTGTAAAGTAAGTACAGATATT |
| 8-d  | ATGGATGGATTTCAGGCTATAG | TATGCGTGGGTGCTTCTCTT |
| 8-e  | GATAATCTTAGACTTACTTTAAAACA | CACCAACCTCCACAAAATG |
| 9-a  | TGAATCATTATTTGGGTGTTC | TACAGAAATGTTGAACATCTAG |
| 9-b  | CTGCTTCTTAAACCAAGCTAG | GTCACTGGTGTCAGAATAATCT |
| 9-c  | CTGTAACACCAGATGTTAGTG | TGAATTTGTTATATCTTAT |
| 9-d  | CTGACACACTGACTGAGAT | TTTTACAGTGGTTTATCCCTC |
tion under conditions described elsewhere. 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 3P-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

Table II. PTEN/MMAC1 Mutations in Primary Endometrial Cancers

| Sample | 10q LOH | Exon/intron | Codon | Mutation | Predicted effect | Stage | Grade | Histology |
|--------|---------|-------------|-------|----------|-----------------|-------|-------|-----------|
| 174    | −       | exon 1      | 13–14 | A ins    | stop at 43      | IIa   | 1     | E         |
| 160    | +       | exon 2      | 33    | 3 bp del | Ile deletion    | Ib    | 1     |           |
| 144    | +       | exon 5      | 92    | GAC to GGC| Asp to Gly    | lc    | 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   | Ia    | 1     | E         |
| 14     | +       | exon 5      | 133   | GTA to ATA| Val to Ile  | lc    | 2     | E         |
| 112    | +       | exon 5      | 133   | GTA to ATA| Val to Ile  | lb    | 2     | E         |
| 120    | N       | exon 6      | 170   | AGT to AAT| Ser to Asn  | IIIe  | 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 | la    | 3     | E         |
| 128    | +       | exon 8      | 298   | CAA to TAA| Gla to stop | IIIe  | 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    | lc    | 1     | E         |
| 136    | −       | exon 8      | 321–323| A del   | stop at 343    | lb    | 1     |           |

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

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.
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. 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.

“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. 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, 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; 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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