Loss of PTEN expression followed by Akt phosphorylation is a poor prognostic factor for patients with endometrial cancer

N Terakawa, Y Kanamori and S Yoshida
Department of Obstetrics and Gynecology, Tottori University School of Medicine, Yonago, Japan
(Requests for offprints should be addressed to Y Kanamori, Department of Obstetrics and Gynecology, Tottori University School of Medicine, 36-1 Nishimachi, Yonago 683-8504, Japan; Email: kanamori@grape.med.tottori-u.ac.jp)

Abstract
To clarify whether and how PTEN and the phosphatidylinositol 3-kinase/Akt pathway relates to endometrial cancer we examined the expression of these pathway-related proteins in patients with endometrial cancer. Of 103 endometrial cancers, 37 (36%) showed negative immunohistochemical staining for PTEN. Western blotting revealed that the level of phosphorylated Akt expression in PTEN-negative cases was significantly higher compared with that in positive cases. We found a significant inverse correlation between PTEN and phosphorylated Akt. The present study indicates the phosphorylation of Akt accompanied by the loss of PTEN in clinical specimens of endometrial cancers.

In order to investigate the relationship between PTEN expression and prognosis in endometrial cancer, 98 patients with advanced endometrial cancer were newly enrolled. The survival rate for PTEN-positive patients was significantly higher than that for PTEN-negative or -heterogeneous staining patients. Of the 98 patients, 25 underwent radiation therapy, 62 received chemotherapy after surgery, and the remaining 11 did not have any postoperative treatment. When patients underwent chemotherapy, the survival rate for PTEN-positive cases was clearly higher than that for PTEN-negative or -heterogeneous cases (62.4 vs 11.8%). Subsequent multivariate analysis revealed that PTEN staining was an independent prognostic factor for patients undergoing chemotherapy. The current study demonstrates that PTEN-positive staining is a significant prognostic indicator of favorable survival for patients with advanced endometrial cancer who undergo postoperative chemotherapy.

Introduction
Endometrial cancer is one of the most common malignancies of the female genital tract. Despite its prevalence, the molecular mechanisms of endometrial carcinogenesis have been poorly understood. Unopposed estrogen promotes cell proliferation and accumulation of genetic alterations, resulting in the emergence of malignant transformation of human endometrium. MLH-1 promoter hypermethylation and mutations in the K-ras, beta-catenin and insulin-like growth factor-II receptor genes were reported in endometrial cancer, but the extent of these alterations was limited (Berkhuck & Boyd 1995, Matias-Guiu et al. 2001).

PTEN is a tumor suppressor gene located on 10q23, and alterations of this gene have been identified in a large fraction of cancers (Di Cristofano & Pandolfi 2000). PTEN mutates in 30–50% of endometrial cancer cases, a rate that is among the highest of any type tumor analyzed to date (Risinger et al. 1997, Tashiro et al. 1997). The mutations are also seen in about 20% of cases of endometrial hyperplasia, a precursor of endometrial cancer (Levine et al. 1998, Maxwell et al. 1998). Recently, it has been shown that progesterone treatment of cultured endometrial stromal cells up-regulates PTEN level, and estradiol induces PTEN phosphorylation in the cells. These findings suggest that PTEN is strongly involved in the development and/or progression of endometrial cancer.
PTEN is a lipid phosphatase dephosphorylating the 3-position of phosphatidylinositol 3,4,5-triphosphate, a second messenger of phosphatidylinositol 3-kinase (PI3K) (Maehama & Dixon 1998, Di Cristofano & Pandolfi 2000). PTEN antagonizes PI3K activity and negatively regulates its downstream target, the serine/threonine kinase Akt (Stambolic et al. 1998). Phosphorylated and activated Akt modulates the activity of a variety of downstream proteins that relate to cell survival and proliferation (Di Cristofano & Pandolfi 2000).

To know whether and how PTEN and the PI3K/Akt pathway relate to endometrial cancer, we examined the expression of those pathway-related proteins such as PTEN, Akt, Bad and p27 in clinical specimens of endometrial cancers. Additionally, to clarify the prognostic significance of PTEN, we investigated the relationship between its expression and prognosis in another series of advanced endometrial cancers.

Materials and methods

Specimen collection

Paraffin-embedded tumor tissues were collected from 103 patients with endometrial cancer. The histological type was endometrioid in all subjects. The tumor samples were also obtained at the time of surgery, snap-frozen, and stored at \(-80^\circ\text{C}\) for Western blotting. For survival analysis, 98 paraffin-embedded specimens were collected from patients with advanced endometrial cancer (stage IIIc or IV) in another setting. All patients provided informed consent for research use of their specimens.

Immunohistochemistry

Four micron meter section was cut from the paraffin blocks of a primary uterine tumor. Each section was mounted on a silane-coated glass slide, deparaffinized, and soaked for 15 min at room temperature in 0.3% \(\text{H}_2\text{O}_2/\text{methanol}\) to block endogenous peroxidase. A mouse monoclonal anti-PTEN antibody, PTEN A2B1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), was applied for 2 h at 37°C. The primary antibody was visualized using the Histofine Simple Stain PO(M) kit (Nichirei, Tokyo, Japan) according to the instruction manual. The slide was counterstained with hematoxylin. For routine histological studies, sections were also stained with hematoxylin and eosin. The status of PTEN staining was evaluated according to the following criteria: a positive case was defined as one in which all of the tumor cells were stained, a heterogeneous case was defined as one with both staining and non-staining tumor cells and a negative case was defined as one with no staining of any tumor cells.

Figure 1 Comparison of the expression levels of PTEN (A), phospho-Akt (B) and phospho-Bad (C) between PTEN-positive and PTEN-negative staining cases. Means and S.D. are shown. Vertical axes show intensity of the bonds.
Western blotting

The frozen specimen was homogenized in a lysis buffer containing 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.1% SDS, 1 mM dithiothreitol, 10 mM NaF, 2 mM Na3VO4, and 1 × Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Ingelheim, Germany). The lysate was centrifuged and supernatant was prepared. Protein concentration of the supernatant was measured by Bradford’s assay (Bradford 1976). Seventy micrograms of each protein sample were separated by 14% SDS-PAGE, blocked in 2.5% skim milk/TPBS (1 × PBS, 0.1% Tween-20) for PTEN and p27, or in 5% BSA/TPBS for phospho-Akt and phospho-Bad. Those samples were probed with each primary antibody overnight at 4°C. The source of anti-PTEN is described above; anti-p27 was obtained from Santa Cruz Biotechnology, and anti-phospho-Akt (Ser473) and anti-phospho-Bad (Ser136) from New England Biolabs (Beverly, MA, USA). Following incubation with horseradish peroxidase-conjugated secondary antibody, protein signals were detected using enhanced chemiluminescence (Amersham). The immunoblots were quantitated using a public domain NIH image program (written by Wayne Rasband, NIH).

Statistical analysis

We used an unpaired t-test to analyze the differences in expression level of proteins. A Pearson’s correlation test was performed to examine the relationship. Patient survival distribution was calculated using the Kaplan–Meier method. The significance of the survival rate in each group was examined by the log-rank test. Multivariate analysis was performed using the Cox’s proportional-hazards regression model with the Stat View Version 5.0-J program (Hulinks, Tokyo, Japan). The χ2 test and the unpaired t-test were used to examine the relationship between prognostic factors and PTEN staining. P < 0.05 was considered statistically significant.

Results

There were 37 PTEN-negative cases (36%), 16 heterogeneous cases (15%) and 50 positive cases (49%) in the immunohistochemical analysis of 103 endometrial cancers. According to the analysis, we selected PTEN-positive or PTEN-negative endometrial cancers. Twenty of the protein samples of tumors (ten positive and ten negative) were examined for the expression of PTEN, phospho-Akt, phospho-Bad and p27 by Western blotting. The level of PTEN expression in negative cases was significantly lower than in the positive cases (Fig. 1A). In contrast, the phospho-Akt level in negative cases was significantly higher (Fig. 1B). A significant inverse correlation between PTEN and phospho-Akt expression was observed (Fig. 2). The phospho-Bad level in negative cases was significantly higher (Fig. 1C). The expression level of p27 did not differ between positive and negative cases.

In 98 patients for survival analysis, the result of immunohistochemical staining of PTEN was negative in 44 patients (45%), heterogeneous in 20 (20%) and positive in 34 (35%). PTEN-negative or -heterogeneous staining was observed in 64 patients (65%) with advanced endometrial cancer. The
survival rate for patients with PTEN-positive staining was significantly higher than that for patients with PTEN-negative or -heterogeneous staining (Fig. 3A). PTEN staining status was not associated with patient age, International Federation of Gynecology and Obstetrics (FIGO) stage, myometrial invasion or histological grade (Table 1). Of the 98 patients, 25 underwent radiation therapy, 62 received chemotherapy after surgery, and the remaining 11 did not have any postoperative treatment. Survival did not differ between the radiation therapy and chemotherapy groups (Fig. 3B). Among patients receiving radiation therapy, PTEN expression did not relate to survival (Fig. 3C). In contrast, the survival rate for PTEN-positive cases was significantly higher than that for negative or heterogeneous cases when patients underwent chemotherapy (62.4 vs 11.8%, Fig. 3D). Subsequent multivariate analysis revealed that FIGO stage, myometrial invasion and PTEN staining were independent prognostic factors (Table 2).

Discussion

We examined the expression of PTEN and the PI3K/Akt pathway-related proteins according to the status of PTEN staining in clinical specimens of endometrial cancer. Akt was significantly phosphorylated in tumor tissue with loss of PTEN expression, and that phosphorylated Akt expression was negatively correlated with PTEN expression. This finding supports the basic evidence that Akt activation accompanied by PTEN inactivation is a key step for the development and/or progression of cancers (Stambolic et al. 1998, Di Cristofano & Pandolfi 2000). Interestingly, Bad, a pro-apoptotic factor, was more phosphorylated in PTEN-negative tumors, but not extremely, suggesting that Bad may be a target for Akt phosphorylation in endometrial cancer. The present study suggests that the phosphorylation of Akt caused by loss of PTEN may be involved in the mechanism of carcinogenesis by preventing apoptosis relating to Bad in patients with endometrial cancer.

Of 98 advanced cases, 64 (65%) showed negative or heterogeneous PTEN staining; their survival rate was significantly lower than that of PTEN-positive cases. Risinger et al. (1998) reported that PTEN mutation was associated with favorable survival in endometrial cancer. Their data conflict with our results. As advanced-stage cancers were intentionally overrepresented in their study (40% included), selection bias may be involved. Furthermore, the frequency of PTEN mutation was higher in early-stage and non-metastatic disease. It is not consistent that the frequency of PTEN mutation decreases along with disease progression in the same cancer. Minaguchi et al. (2001) reported that PTEN mutation only outside exons 5–7 was associated with better survival, although mutation in any exon of PTEN was not. Because exons 5–7 contain a phosphatase domain and a C2 domain, which are important domains for tumor-suppressor function.
Table 1 Prognostic factors and PTEN staining

<table>
<thead>
<tr>
<th>PTEN staining</th>
<th>Negative/Heterogeneous (n = 64)</th>
<th>Positive (n = 34)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>58.1</td>
<td>59.4</td>
<td>0.50</td>
</tr>
<tr>
<td>Range</td>
<td>30–75</td>
<td>36–78</td>
<td></td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIIc</td>
<td>55</td>
<td>32</td>
<td>0.32</td>
</tr>
<tr>
<td>IV</td>
<td>9</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Myometrial invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1/2</td>
<td>14</td>
<td>9</td>
<td>0.51</td>
</tr>
<tr>
<td>≥1/2</td>
<td>50</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>21</td>
<td>16</td>
<td>0.38</td>
</tr>
<tr>
<td>G2</td>
<td>25</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>18</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Multivariate analysis of patients undergoing postoperative chemotherapy

<table>
<thead>
<tr>
<th>Prognostic variables</th>
<th>Coefficient</th>
<th>S.E.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIGO stage</td>
<td>−1.047</td>
<td>0.487</td>
<td>0.031</td>
</tr>
<tr>
<td>Myometrial invasion</td>
<td>−2.225</td>
<td>1.027</td>
<td>0.030</td>
</tr>
<tr>
<td>PTEN staining</td>
<td>1.085</td>
<td>0.507</td>
<td>0.032</td>
</tr>
</tbody>
</table>

(Lee et al. 1999, Minaguchi et al. 2001), their data can be interpreted as demonstrating that patients with PTEN mutations of functionally important exons have poor prognosis. In another report, methylation of the PTEN promoter was associated with advanced stage in endometrial cancer (Salvesen et al. 2001), supporting our hypothesis that loss of PTEN function might relate to poor prognosis.

The survival rate for PTEN-positive cases was significantly higher than that for negative or heterogeneous cases, but PTEN-staining status was not associated with the known prognostic factors. We therefore tried to elucidate why PTEN-positive patients showed more favorable survival. In our series, 87 patients (89%) received postoperative treatment due to advanced stage and lymph node metastasis. Among patients undergoing chemotherapy, PTEN-positive cases showed clearly better survival compared with negative or heterogeneous cases (62.4 vs 11.8%). Multivariate analysis revealed that PTEN-positive staining was a significant prognostic indicator of favorable survival for patients who underwent chemotherapy. The favorable survival for PTEN-positive patients undergoing chemotherapy would certainly contribute to overall better survival of PTEN-positive patients in advanced endometrial cancer.

It has been shown that PTEN dephosphorylates focal adhesion kinase and inhibits cell migration, spreading and focal adhesion formation (Tamura et al. 1998). Indeed, PTEN deficiency led to increased cell motility in PTEN−/− fibroblasts and reintroducing the PTEN gene reduced the enhanced motility of the cells (Liliental et al. 2000). PTEN inactivation may lead to a more aggressive and metastatic phenotype and may be directly involved in progression of endometrial cancer.

Overexpression of the PTEN gene increases the chemosensitivity of bladder cancer cells and enhances the sensitivity to irradiation in malignant glioma cells (Wick et al. 1999, Tanaka et al. 2000). Our present results suggest that PTEN-positive tumors might be more sensitive to chemotherapy than negative or mixed tumors. The relationship between PTEN and chemosensitivity should be studied in endometrial carcinoma. In contrast, we failed to show that PTEN expression was associated with survival for patients receiving radiation therapy. Thus, the effectiveness of radiation therapy may be limited in the advanced stage of endometrial carcinoma.

The present study indicates that positive staining of PTEN is a significant prognostic indicator of favorable survival for patients with advanced endometrial cancer who undergo postoperative chemotherapy.

References

Levine RL, Cargile CB, Blazes MS, van Rees B, Kurman RJ & Ellenson LH 1998 PTEN mutations and microsatellite


