Correlation between PTEN Expression and PI3K/Akt Signal Pathway in Endometrial Carcinoma*

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Summary: In order to investigate the role of the PTEN expression in carcinogenesis and development of endometrial carcinoma and clarify whether and how PTEN and PI3K/Akt pathway relate to endometrial carcinoma, the expression of PTEN and phospho-Akt was detected by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) methods and Western-blot from 24 cases of endometrial carcinoma, 10 cases of endometrial atypical hyperplasia, 10 cases of normal endometrium. SP immunohistochemical methods were used to measure levels of PTEN protein expression in following 5 study groups: 31 cases of endometrium in proliferative phase, 30 cases of endometrium in secretory phase, 71 cases of endometrial hyperplasia, 25 cases of atypical hyperplasia and 73 cases of endometrial carcinoma. Immunostaining score of PTEN was 3.39±0.15 in proliferative phase, 1.90±0.21 in secretory phase, 3.34±0.29 in endometrial hyperplasia, 0.62±0.11 in atypical hyperplasia and 0.74±0.19 in endometrial carcinoma, respectively. PTEN mRNA relative value in normal endometrium, endometrial hyperplasia, endometrial atypical hyperplasia, and endometrial carcinoma was 2.45±0.51, 2.32±0.32, 0.46±0.11, and 0.35±0.13 respectively. The expression levels of PTEN mRNA and protein in patients with endometrial carcinoma and atypical hyperplasia were significantly lower than in those of proliferative phase and with endometrial hyperplasia. The level of PTEN expression in patients with endometrial carcinoma was significantly related to tissue type (P<0.05), differentiation (P<0.05) and clinical stage (P<0.05), but not to depth of myometrium invasion (P>0.05). Western blot analysis revealed that Phospho-Akt level in PTEN negative cases was significantly higher, and there was a negative correlation between PTEN and phospho-Akt (r=−0.8973, P<0.0001). It was suggested that loss of PTEN expression was an early event in endometrial tumorigenesis. The phosphorylation of Akt induced by the loss of PTEN took part in the tumorigenesis and development of endometrial carcinoma.

Key words: endometrial neoplasm; PTEN; Akt; immunohistochemistry

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 induces genetic alterations, resulting in the emergence of malignant transformation of human endometrium.

PTEN is a tumor suppressor gene located on 10q23, and alterations of this gene have been identified in a large fraction of cancers. PTEN mutates in 30%–50% of endometrial cancer cases, a rate that is among the highest of any type tumor analysed to date, and PTEN is also suggested to play a “gatekeeper” role for the PTEN gene in the endometrium. PTEN is a lipid phosphatase dephosphorylating the 3-position of phospatidylinositol 3,4,5-triphosphate, a second messenger of phospatidylinositol 3-kinase (PI3K). PTEN antagonizes PI3K activity and negatively regulates its downstream target, the serine/threonine kinase Akt. Phosphorylated and activated Akt modulates the activity of a variety of downstream proteins that relate to cell survival and proliferation. In present study, we detect the PTEN mRNA and protein expression in a spectrum of precisely classified endometrial tissues to investigate the relationship between changes in PTEN structure and function and the carcinogenesis and development of endometrial carcinoma. Additionally, to know whether and how PTEN and the PI3K/Akt pathway relate to endometrial cancer, we examined the expression of PTEN and phospho-Akt protein in clinical specimens of endometrial cancers.

1 MATERIALS AND METHODS

1.1 Tissue Samples

Snap-frozen endometrial samples were obtained from 54 women undergoing surgery at Tongji hospital from Apr. 2005 to Oct. 2007, including 24 cases of endometrial carcinoma, 10 cases of endometrial atypical hyperplasia, 10 cases of endometrial hyperplasia, and 10

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cases of normal endometrium. Tissue samples were immediately frozen in liquid nitrogen. Paraffin-embedded specimens were collected from 230 patients who were pathologically verified with endometrial carcinoma (73 cases), endometrial atypical hyperplasia (25 cases), endometrial hyperplasia (71 cases), endometrium in proliferative phase (31 cases) and secretory phase (30 cases). Among 73 cases of endometrial carcinoma, there were 57 cases of endometrioid adenocarcinoma, 5 cases of papillary serous adenocarcinoma, 6 cases of clear cell carcinoma, 3 cases of adeno-squamous carcinoma, 1 case of adenoacanthoma, and 1 case of squamous carcinoma. All patients were not subjected to radiotherapy or chemotherapy. The clinical stage was based on FIGO (1988).

1.2 RT-PCR

Total RNA was extracted from 50-100 µg endometrial samples using the TRIZOL reagent kit (GIBCO, China) according to the instructions. cDNA was synthesized from 4 µg of total RNA in a final volume of 20 µL Tris-HCL (pH 8.4), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L dNTP, 1 µL cDNA, 2.5 U of Taq DNA polymerase, and 400 mmol/L primers for 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 60 s in a MJ Research thermal cycler. Oligonucleotide primers for human PTEN gene were synthesized by Bo Ya Company (China) based on the published cDNA sequences (Genebank). Primers sense: 5′-CAGAAAGACTTGAAGGCCTAT-3′; antisense: 5′-AGCCAGAAGTTGAAAAGCTCAA-3′ (586 bp). β-actin sense: 5′-AGCAGAGAATGGAAAGTCAAA-3′; antisense: 5′-ATGCTGCTTACATGTCTCAG-3′ (266 bp). The PCR products were electrophoresed on 1.5% agarose gels. β-actin was also amplified to ensure the quality of the RNA and reagents used to in the reactions and used as the standard for semi-quantitative comparisons.

1.3 Immunohistochemistry

Endometrial tissues were fixed by formalin and embedded in paraaffin following the standard of histological practices. The samples were cut into 4-µm sequential sections, which were stained with hematoxylin-eosin staining for pathological diagnosis. Immunohistochemical staining for PTEN was carried out by using the streptavidin-peroxidase technique with SP kit (Zymed, USA). The primary antibodies were goat polyclonal PTEN antibody (1:200 dilution; Santa Cruz, USA). The status of PTEN staining was evaluated morphometrically with a computer-assisted image-analyzer (HPIAS-1000 high precise color-image measure system, Tongji Qianping Image Engineer Company, China) by randomly selecting 10 fields of each section. Negative controls were prepared by PBS substitution for the primary antibodies staining, and normal endometrium sections used as positive controls were included in each staining run.

1.4 Western Blot Analysis

The frozen specimen was homogenized in a lysis buffer containing 20 mmol/L Tris-HCL at pH 7.5, 137 mmol/L NaCl, 1% NP-40, 10% glycerol, 1 mmol/L phenyimethylsulfonyl fluoride and 5 µg/mL of aprotinin. The lysate was centrifuged at 12 000 g for 15 min at 4°C and then transferred to a new tube. This preparation was stored at –80°C. Soluble proteins (50 µg) were subjected to 12% SDS-PAGE and transferred to PVDF membrane by electrophoresis in a semidry chamber (Biometra, Germany). The membrane was blocked for 2 h with a blocking buffer containing 5% fat-free dry milk in TBST at room temperature. Proteins were identified by immunoblot analysis using anti-PTEN goat polyclonal antibody (diluted 1:1000, Santa Cruz, USA), anti-phospho-Akt rabbit polyclonal antibody (diluted 1:1000, New England Biolabs, USA) and anti-Akt rabbit polyclonal antibody (diluted 1:1000, New England Biolabs, USA) containing 5% fat-free dry milk overnight at 4°C. After washing in TBST, the immunoreactive proteins were visualized using HRP labeled horse anti-goat IgG (diluted 1:2000) and anti-rabbit IgG (diluted 1:2000) and the enhanced chemiluminescence (ECL) detection system (Pierce Co., USA).

1.5 Statistical Analysis

All statistical analyses were performed using SAS 8.1 software. Statistical analysis was done by analysis of variance (ANOVA) followed by multiple comparison. A Pearson’s correlation test was performed to examine the relationship and values of P<0.05 were considered statistically significant.

2 RESULTS

2.1 Expression of PTEN Protein in Endometrium Tissues

Immunohistochemical analysis of 230 cases of endometrium tissues revealed that PTEN was located in cytoplasm and nuclei, and its immunohistochemical score in proliferative phase, secretory phase, endometrial hyperplasia, endometrial atypical hyperplasia, and endometrial carcinoma groups was 3.39±0.15, 1.90±0.21, 3.34±0.29, 0.62±0.11, and 0.74±0.19, respectively. ANOVA analysis showed that the expression levels of PTEN in endometrial carcinoma and endometrial atypical hyperplasia groups were statistically significantly lower than in proliferative phase and secretory phase groups (P<0.01), those in proliferative phase group higher than in secretory phase group (P<0.05), and there was no significant difference between proliferative phase group and endometrial hyperplasia group (P>0.05). There was significant difference in the PTEN expression between simple endometrial hyperplasia, cystic endometrial hyperplasia or adenosomatous endometrial hyperplasia groups and endometrial atypical hyperplasia group (P<0.01), but there was no significant difference among simple endometrial hyperplasia, cystic endometrial hyperplasia and adenosomatous endometrial hyperplasia groups (P>0.05).

Among 73 cases of endometrial carcinoma, the PTEN immunostaining intensity in endometrioid adenocarcinoma was stronger than other histological types of endometrial carcinomas (P<0.005). The level of PTEN expression in patients with endometrial carcinoma was significantly related to differentiation (The expression of PTEN in high differentiated endometrial carcinoma was significantly higher than that in middle-low differentiated one) (P<0.05) and clinical stage (P<0.05), but not to depth of myometrium invasion (P>0.05) (table 1, fig. 1–3).
**Table 1 PTEN protein expression in endometrial tissues**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cases</th>
<th>Immunohistochemical score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferative phase</td>
<td>31</td>
<td>3.39±0.15</td>
</tr>
<tr>
<td>Secretary phase</td>
<td>30</td>
<td>1.90±0.21</td>
</tr>
<tr>
<td>Endometrial hyperplasia</td>
<td>71</td>
<td>3.34±0.29</td>
</tr>
<tr>
<td>Simple endometrial hyperplasia</td>
<td>24</td>
<td>3.34±0.35</td>
</tr>
<tr>
<td>Cystic endometrial hyperplasia</td>
<td>32</td>
<td>3.07±0.49</td>
</tr>
<tr>
<td>Adenomatous endometrial hyperplasia</td>
<td>15</td>
<td>3.60±0.36</td>
</tr>
<tr>
<td>Endometrial atypical hyperplasia</td>
<td>25</td>
<td>0.62±0.11</td>
</tr>
<tr>
<td>Endometrial adenocarcinoma</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>Histological classification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>57</td>
<td>0.74±0.19</td>
</tr>
<tr>
<td>Others</td>
<td>16</td>
<td>2.59±0.18</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>32</td>
<td>1.33±0.43</td>
</tr>
<tr>
<td>Middle-low</td>
<td>25</td>
<td>0.38±0.16</td>
</tr>
<tr>
<td>Invasion of myometrium</td>
<td></td>
<td></td>
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<tr>
<td>None</td>
<td>12</td>
<td>1.00±0.25</td>
</tr>
<tr>
<td>≤1/2</td>
<td>29</td>
<td>0.65±0.17</td>
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<tr>
<td>&gt;1/2</td>
<td>16</td>
<td>1.06±0.19</td>
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<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
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<tr>
<td>I - II</td>
<td>51</td>
<td>0.92±0.16</td>
</tr>
<tr>
<td>III-IV</td>
<td>6</td>
<td>0.11±0.06</td>
</tr>
</tbody>
</table>

2.2 Expression of PTEN mRNA among Histological Types of Endometrium

By semi-quantitative RT-PCR, we measured expression levels of PTEN mRNA in 4 groups. mRNA relative value in endometrioid adenocarcinoma, endometrial atypical hyperplasia, endometrial hyperplasia, and normal endometrium groups was 0.35±0.13, 0.46±0.11, 2.32±0.32, and 2.45±0.51, respectively. Our results suggested that levels of PTEN mRNA in endometrioid adenocarcinoma group were significantly lower than in normal endometrium and endometrial hyperplasia (P<0.01). Additionally, products of PTEN mRNA in endometrial atypical hyperplasia group were significantly decreased as compared with normal endometrium and endometrial hyperplasia groups (P<0.01).

2.3 Correlation between PTEN and phospho-Akt Expression

In 24 cases of endometrioid carcinomas, the level of phospho-Akt expression in PTEN negative cases was significantly higher than in PTEN positives cases (fig. 6). However, the total Akt was not accompanied with alteration of PTEN expression in different specimens. A significant inverse correlation between PTEN and phospho-Akt expression was observed (r=-0.8973, P<0.01, fig. 7).
The mechanism of the involvement of PTEN in the development and progression of endometrial carcinoma was not elucidated. Those previous studies suggested that the loss of PTEN function with subsequent activation of PI3K/Akt signalling pathway contributes to carcinogenesis. However, those findings have not been confirmed in patients with endometrial carcinoma. Our data showed that Akt was significantly phosphorylated in endometrioid endometrial adenocarcinoma and may be a new target for prevention and therapy of cancer.

As we all know, the evolvement of the estrogen-related endometrioid adenocarcinomas is as follows: normal endometrium → cystic endometrial hyperplasia → adenomatous endometrial hyperplasia → endometrial atypical hyperplasia → carcinoma in situ → invasive carcinoma. Frequently the advanced carcinomas are temporally and spatially associated with endometrial hyperplasia. To test our hypothesis that changes in PTEN structure and function are among the earliest events in the pathway to endometrial cancer and precancer, we measured the expression of PTEN in endometrial hyperplasia and found that the differences were very significant between endometrial atypical hyperplasia and the three subtypes of endometrial hyperplasia, but there was no significant difference among the three subgroups of endometrial hyperplasia. It is suggested that PTEN mutation under unopposed estrogen conditions would not functionally regulate the proliferation of endometrium, which results in a high risk of developing carcinoma. PTEN is a major gene involved in the pathogenesis of endometrioid endometrial adenocarcinoma and may be a new target for prevention and therapy of cancer.

We subsequently explored the relationship between the PTEN expression and clinicopathological characteristics. Endometrioid adenocarcinoma versus other histological types of endometrial carcinomas by loss of PTEN expression showed that endometrioid adenocarcinoma had a statistically significant decrease in the PTEN expression compared with other histological types of endometrial carcinoma. Our data suggest that two major pathogenetic variants of endometrial carcinomas, endometrioid and serous, seem to evolve via divergent pathways. It has been reported that endometrioid adenocarcinoma and its precursors have sevenfold higher PTEN mutation rates than papillary serous type. Our observation inferred that these two histological types of endometrial cancers seemingly originated from different endometrial stem cells and further supported the dualistic model of endometrial carcinogenesis incorporating a “classic” estrogen-driven pathway and an “alternative” pathway seemingly unrelated to hormones. We also observed that the level of PTEN expression in patients with endometrial carcinoma was significantly related to pathological grades and clinical stages, but not to depth of myometrium invasion, which suggested that the lower differentiation of tumors has the more frequency of loss PTEN expression, in turn the worse prognosis.

3.3 Correlation between PTEN and PI3K/Akt Signal Pathway

The PTEN expression was examined by RT-PCR methods and SP immunohistochemical methods in endometrial carcinoma, endometrial atypical hyperplasia, endometrial hyperplasia and normal endometrium. The PTEN expression mRNA and protein levels in endometrial carcinoma and endometrial atypical hyperplasia groups were significantly lower than in proliferative phase and secretory phase groups (P<0.01), suggesting that the expression of PTEN is repressed at the transcriptional and translational levels, which is essential for inactivated PTEN.
endometrial carcinoma with a loss of PTEN expression, and that phospho-Akt expression was negatively correlated with the PTEN expression. This finding supports the basic evidence that Akt activation accompanied by PTEN inactivation is a key step in the development and progression of endometrial cancer.

Our observations have demonstrated that complete inactivation of PTEN occurs in the great majority of endometrial carcinomas, especially those of the endometrioid subtypes, and even in majority of precancers. Decreased PTEN expression or function is a marker of the earliest endometrial precancers, and activation of Akt caused by the loss of PTEN may be involved in the mechanism of carcinogenesis in patients with endometrial carcinoma.

REFERENCES

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