Loss of PTEN expression is an independent predictor of favourable survival in endometrial carcinomas

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Background: We and others previously reported the prognostic significance of PTEN mutational status on favourable survival in endometrial carcinomas. Here, we demonstrate that loss of PTEN expression in immunohistochemistry is an independent prognostic marker for favourable survival in endometrial carcinomas.

Methods: We conducted immunohistochemical analyses of PTEN, PIK3CA, phosphorylated Akt (p-Akt), and p27 in primary endometrial carcinomas from 221 patients. Mutation of PTEN was analysed further.

Results: Expression of PTEN was lost in 56 patients (25%), and PIK3CA was overexpressed in 159 patients (72%). Overexpression of PIK3CA was associated with p-Akt overexpression (P < 0.001), which was in turn associated with loss of nuclear p27 expression (P = 0.028). Loss of PTEN expression was found to be associated with endometrioid histology (P = 0.03), and was inversely associated with the presence of lymphovascular space invasion (P = 0.03). Univariate and multivariate survival analyses revealed that factors of PTEN loss, age < 70, histological grade 1, early International Federation of Gynecology and Obstetrics (FIGO) stage, and absence of lymphovascular invasion were independent prognostic indicators for better overall survival (P = 0.03, 0.04, 0.01, < 0.001, and 0.03, respectively). The subset analysis showed a stronger tendency of PTEN loss towards favourable survival in advanced-stage (III and IV) disease than in early-stage (I and II) disease (P = 0.05 vs 0.14). Moreover, our mutational analysis demonstrated that PTEN expression loss was associated with PTEN-truncating mutations (P = 0.03).

Conclusion: The current observations further support the prognostic significance of PTEN aberration on favourable outcome in endometrial carcinomas, providing useful implications for the individualised management of the disease.
we demonstrate that loss of PTEN expression is a significant and independent prognostic factor for favourable survival in the disease. Our observation presents additional evidence for the prognostic significance of PTEN aberration on favourable outcome in endometrial carcinoma, further providing significant implications for the management of the disease including molecular targeted therapies.

MATERIALS AND METHODS

Patients and specimens. The Ethical Committee of the University of Tsukuba Hospital approved the study protocol. All patients diagnosed with endometrial carcinoma, who were treated in the Department of Obstetrics and Gynecology at the University of Tsukuba Hospital between 1999 and 2009, were identified through our database. A total of 221 patients with endometrial carcinomas were included in the present study, and their medical records were reviewed. A median follow-up duration was 59 months (range, 3–119 months). All patients provided written informed consent. Staging was performed based on the criteria of International Federation of Gynecology and Obstetrics (FIGO). Endometrioid adenocarcinomas were subclassified into three grades (G1, G2, and G3) according to the FIGO criteria. Table 1 summarises the patient characteristics.

Treatment. The operative procedure included hysterectomy, bilateral salpingo-oophorectomy, and systematic aortic and pelvic lymph-node dissection. Radical hysterectomy or semiradical hysterectomy (with removal of vaginal cuff and partial resection of vesico-uterine ligament) was performed on patients with positive findings on cervical stromal invasion by MRI, and simple hysterectomy (with removal of vaginal cuff and partial resection of vesico-uterine ligament) was performed on patients with positive peritoneal cytology, adnexal/peritoneal involvement, or pelvic-/aortic-node metastases were treated with combination chemotherapy of paclitaxel and carboplatin. Small-pelvis irradiation (with the lower superior border of field) was indicated for patients with adnexal/peritoneal involvement or deep pelvic irradiation (with the lower superior border of field) was indicated for patients with adnexal/peritoneal involvement or deep muscular invasion (more than two-thirds depth in endometriod G1, and more than one-half in G2 or other histotypes). Whole-pelvis or peri-aortic irradiations were administered to pelvic or aortic node-positive patients, respectively.

Immunohistochemistry. Immunohistochemistry was performed as described previously (Abe et al, 2013). Antibodies used were P13 Kinase p110z (rabbit monoclonal, 1:200; Cell Signaling, Danvers, MA, USA), Anti-Human PTEN (6H2.1) (mouse monoclonal, 1:100; Cascade, Winchester, MA, USA), Phospho-Akt (Ser473) (rabbit monoclonal, 1:50; Cell Signaling), and Anti-p27 (mouse monoclonal, 1:100; BD Pharmingen, Franklin Lakes, NJ, USA). The corresponding normal endometria or normal endometria and endometrial carcinomas. For normal control, normal endometria from 15 women were examined, and >90% of the specimens were scored as 6 for PTEN, p-Akt, and PIK3CA, and >85% were positive for p27, respectively.

DNA extraction and PTEN mutational analysis. Genomic DNA was extracted from tumour areas of formalin-fixed, paraffin-embedded archival tissues with a Dnasey Blood & Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Mutational analysis for PTEN was performed as previously described. Briefly, aberrant bands revealed by SSCP analysis were excised from the gel, amplified by PCR, purified, and submitted to the Operon Biotechnologies (Tokyo, Japan) for direct sequencing.

Statistical analysis. Differences in proportions were evaluated by the Fisher’s exact test. Kaplan–Meier survival curves were calculated and compared statistically using the log-rank test.

Table 1. Patient characteristics

| Characteristic | Number n = 221 |
|---------------|---------------|
| Median age (range) | 57.0 (26–84) |
| FIGO stage |
| I | 128 |
| Ia | 22 |
| Ib | 76 |
| Ic | 30 |
| II | 26 |
| IIa | 10 |
| IIb | 16 |
| III | 43 |
| IIIa | 20 |
| IIlc | 23 |
| IV | 24 |
| IVa | 2 |
| Nb | 22 |
| Histotype |
| Endometrioid | 196 |
| G1 | 115 |
| G2 | 56 |
| G3 | 25 |
| Serous | 12 |
| Adenosquamous | 4 |
| Clear cell | 4 |
| Poorly differentiated | 1 |
| Undifferentiated | 1 |
| Mixed epithelial | 3 |
| Primary treatment |
| Surgery | 221 |
| Lymphadenectomy | 171 |
| Lymph-node sampling | 21 |
| Chemotherapy | 60 |
| TC | 55 |
| CAP | 4 |
| Irradiation | 58 |

Abbreviations: CAP = cyclophosphamide, doxorubicin, and cisplatin combination; FIGO = International Federation of Gynecology and Obstetrics; TC = paclitaxel and carboplatin combination.
The Cox proportional hazard model was used for the univariate and multivariate analyses.

RESULTS

Our IHC analyses in 221 endometrial carcinomas showed that PTEN expression was lost in 56 cases (25%), PIK3CA was overexpressed in 159 (72%), p-Akt was overexpressed in 189 (86%), and nuclear p27 expression was lost in 143 (65%) (Table 2). Moreover, overexpressed PIK3CA was significantly associated with p-Akt overexpression ($P < 0.001$), which in turn significantly correlated with negative nuclear p27 expression ($P = 0.03$) (Table 2). These observations are consistent with the signal transduction mechanism where upregulation of PI3 kinase leads to phosphorylation of Akt, which in turn results in translocation of p27 from nucleus to cytoplasm. This consistency strengthens the validity of our IHC analyses.

We subsequently investigated the relationships between IHC results and clinicopathological parameters (Table 3). Loss of PTEN expression was found to be associated with endometrioid histology ($P = 0.03$), and was inversely associated with the presence of lymphovascular invasion ($P = 0.03$). Negative nuclear p27 expression was associated both with endometrioid histology and with $G_1$ ($P = 0.008$ and 0.016, respectively). Negative PTEN showed trends towards younger age ($P = 0.10$), obesity ($P = 0.17$), and less lymph-node metastases (data not shown).

| Expression                  | Number, $n = 221$ | (+), $n = 189$ | (−), $n = 32$ | $P$-value |
|-----------------------------|-------------------|----------------|---------------|-----------|
| Negative PTEN (IHS = 0)     | 56 (25%)          | 51 (27%)       | 5 (16%)       | 0.19      |
| Overexpressed PIK3CA (IHS > 6) | 159 (72%) | 148 (78%)       | 11 (34%)       | 1.6E −06  |
| Overexpressed p-Akt (IHS > 6) | 189 (86%) | —              | —             | —         |
| Negative nuclear p27 (0%) | 143 (65%)         | 128 (68%)       | 15 (47%)       | 0.03      |

Abbreviations: IHC = immunohistochemical; IHS = IHC score; p-Akt = phosphorylated Akt.

Figure 1. IHC staining patterns of PTEN, PIK3CA, p-Akt, and p27 in normal endometria and endometrial carcinomas. PTEN, PIK3CA, and p-Akt, ×100; p27, ×400.
Next, we compared survival curves according to protein expressions (Figure 2). Patients with loss of PTEN expression showed significantly improved overall survival compared with those without PTEN expression loss (Figure 2A, \( P = 0.016 \)). In contrast, PIK3CA overexpression, p-Akt overexpression, and negative p27 did not show significant differences in overall survival (Figures 2B–D). When compared in subsets of stage I/II and III/IV, negative PTEN showed more favourable survival in advanced disease than in early disease (Figures 2E and F). Negative PTEN showed trends towards favourable survival, when compared in subsets of stage I/II, negative PTEN showed more favourable survival in advanced disease than in early disease (Figures 2E and F). Negative PTEN showed trends towards favourable survival, when compared in subsets of stage I/II and III/IV, negative PTEN showed more favourable survival in advanced disease than in early disease (Figures 2E and F). Negative PTEN showed trends towards favourable survival, when compared in subsets of stage I/II and III/IV, negative PTEN showed more favourable survival in advanced disease than in early disease (Figures 2E and F).

We further conducted univariate and multivariate analyses of prognostic factors for overall survival. Among various prognostic factors, loss of PTEN expression, age \( \geq 70 \), G1, FIGO stage III/IV, muscular invasion >1/2, and presence of LVI were found to be significant in the univariate analysis (\( P = 0.03, <0.001, <0.001, <0.001, <0.001, <0.001, <0.001, <0.001, <0.001 \), and \( <0.001 \); respectively; Table 4). Among those significant factors, the following multivariate analysis demonstrated that loss of PTEN expression, age \( \geq 70 \), G1, FIGO stage III/IV, and presence of LVI remained to be significant and independent factors (\( P = 0.03, 0.04, 0.01, <0.001, <0.001, <0.001, <0.001 \), and 0.03; respectively; Table 4).

Finally, we performed PTEN mutational analysis using DNAs from 33 archival tumour specimens (Table 5). A total of 27 mutations were detected in 19 samples. Mutations in exon 5 (9) and frameshift and missense mutations (12 each) were most frequent. Loss of PTEN expression (IHS = 0) in immunohistochemistry showed no correlation with the presence of PTEN mutation (67% vs 52%, \( P = 0.49 \); Table 6). Interestingly, however, loss of PTEN expression was found to be significantly associated with the presence of frameshift or non-sense mutations, which result in PTEN protein truncation (67% vs 24%, \( P = 0.03 \); Table 6).

**DISCUSSION**

Inactivation of the PTEN gene is the most frequent genetic defect in endometrial carcinoma. The most commonly observed PTEN defect is inactivation of both alleles by large deletion and mutation in each allele. Our IHC analyses showed that loss of PTEN expression (IHS = 0) was associated with endometrioid histology and absent lymphovascular invasion (Table 3). This observation suggests that tumours with loss of PTEN expression may have more indolent biological behaviour compared with tumours without PTEN loss. Furthermore, our survival analyses demonstrated that loss of PTEN expression was a significant and independent prognostic predictor for favourable survival in endometrial carcinoma (Table 4), keeping in line with the previously published findings on mostly limited sample size where PTEN mutation is associated with favourable prognosis (Risinger et al, 1998; Minaguchi et al, 2001; Sun et al, 2001; Salvesen et al, 2004). Collectively, our above findings utilising larger sample size suggest that loss of PTEN expression may have prognostic impact on survival through more indolent biological tumour behaviour, further confirming the prognostic significance of PTEN aberration.

Given the tumour suppressor function of PTEN (Minaguchi et al, 1999), one would expect that PTEN inactivation would imply poor prognosis. Endometrial cancers develop through accumulation of multiple genetic and epigenetic aberrations. Some tumours acquire malignant characteristics through PTEN inactivation, while others do so through aberrations of other genes; those aberrations may lead to more aggressive phenotype due to more detrimental molecular events than PTEN inactivation does. Meanwhile, another possible explanation for the impact of PTEN inactivation on good prognosis may be tumour suppressive roles of Akt, the pivotal effector downstream of PTEN (Wyszomierski and Yu, 2005). Akt reportedly blocks cancer cell mortality and invasion through the transcription factor NFAT (Yoeli-Lerner et al, 2005) or downregulation of RHO activity (Liu et al, 2006). The inhibitory effect of Akt activation on cancer cell cycle has also been reported (Kodama et al, 2002). Another study has indicated that Akt activation can promote tumorigenesis, but suppresses tumour invasion (Hutchinson et al, 2004). In our study, however, neither p-Akt nor p27 did show any prognostic significance for survival, although both proteins showed the expression patterns that are consistent with the signalling pathway. Accordingly, the observed PTEN impact on survival is not likely to be attributed to functions of downstream effectors but rather PTEN genetic aberration itself.

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**Table 3. Relationship between IHC results and clinicopathological features**

| Clinicopathological variables | PTEN expression | PIK3CA overexpression | p-Akt overexpression | Nuclear p27 expression |
|-----------------------------|-----------------|-----------------------|----------------------|------------------------|
| **Negative** | **Positive** | **P** | **Negative** | **Positive** | **P** | **Negative** | **Positive** | **P** |
| Age \( \geq 70 \) | 5 (9%) | 31 (19%) | 0.10 | 26 (16%) | 10 (16%) | 1 | 31 (16%) | 5 (16%) | 1 |
| Pre-menopause | 19 (34%) | 44 (27%) | 0.31 | 43 (27%) | 20 (32%) | 0.51 | 49 (26%) | 14 (44%) | 0.055 |
| Null parity | 11 (20%) | 26 (16%) | 0.54 | 25 (16%) | 12 (19%) | 0.55 | 33 (17%) | 4 (13%) | 0.61 |
| BMI > 30 | 11 (20%) | 19 (12%) | 0.17 | 20 (13%) | 10 (16%) | 0.26 | 24 (13%) | 6 (19%) | 0.40 |
| DM | 8 (14%) | 31 (18%) | 0.55 | 25 (16%) | 14 (23%) | 0.24 | 32 (17%) | 7 (22%) | 0.46 |
| Endometrioid (vs Non-endometrioid) | 53 (98%) | 143 (88%) | 0.03 | 139 (87%) | 57 (92%) | 0.48 | 171 (90%) | 25 (78%) | 0.064 |
| G1 | 30 (54%) | 86 (52%) | 0.88 | 84 (53%) | 32 (52%) | 0.88 | 102 (54%) | 14 (44%) | 0.34 |
| Mi > 1/2 | 17 (30%) | 64 (12%) | 0.34 | 55 (35%) | 26 (42%) | 0.35 | 68 (36%) | 13 (41%) | 0.69 |
| LVI | 14 (25%) | 70 (42%) | 0.025 | 58 (36%) | 26 (42%) | 0.54 | 71 (38%) | 13 (41%) | 0.84 |
| FIGO stage III/IV | 12 (29%) | 51 (31%) | 0.87 | 46 (29%) | 21 (34%) | 0.52 | 57 (30%) | 10 (31%) | 1 |

Abbreviations: BMI = body mass index; DM = diabetes mellitus; FIGO = International Federation of Gynecology and Obstetrics; IHC = immunohistochemical; LVI = lymphovascular invasion; Mi = muscular invasion; p-Akt = phosphorylated Akt.
Figure 2. Kaplan–Meier curves for overall survival according to protein expression levels in endometrial carcinomas. (A) Patients with negative PTEN (n = 56) vs positive PTEN (n = 165); (B) patients with overexpressed PIK3CA (n = 159) vs without overexpressed PIK3CA (n = 62); (C) patients with overexpressed p-Akt (n = 189) vs without overexpressed p-Akt (n = 32); (D) patients with negative nuclear p27 (n = 143) vs positive nuclear p27 (n = 78); (E) patients with negative PTEN (n = 40) vs positive PTEN (n = 114) in early-stage disease (stages I and II); (F) patients with negative PTEN (n = 14) vs positive PTEN (n = 51) in advanced disease (stages III and IV); (G) patients with negative PTEN (n = 53) vs positive PTEN (n = 143) in pure endometrioid disease; (H) patients with negative PTEN (n = 3) vs positive PTEN (n = 22) in disease other than pure endometrioid histology.
Table 4. Univariate and multivariate analyses of prognostic factors for overall survival

| Prognostic factor | HR (95% CI) | P-value | HR (95% CI) | P-value |
|-------------------|-------------|---------|-------------|---------|
| PTEN negative (vs positive) | 0.20 (0.05–0.86) | 0.03 | 0.21 | 0.05–0.88 | 0.03 |
| PIK3CA overexpression (vs remainder) | 0.68 | 0.32–1.43 | 0.31 | — | — |
| p-Akt overexpression (vs remainder) | 0.92 | 0.35–2.42 | 0.87 | — | — |
| Nuclear p27 negative (vs positive) | 0.76 | 0.37–1.60 | 0.47 | — | — |
| Age ≥ 70 (vs < 70) | 3.67 | 1.69–7.98 | <0.001 | 2.44 | 1.06–5.63 | 0.04 |
| G1 (vs G2–3/non-endometrioid) | 0.16 | 0.06–0.42 | <0.001 | 0.28 | 0.11–0.76 | 0.01 |
| FIGO stage III/IV (vs I/II) | 10.9 | 4.44–26.9 | <0.001 | 5.70 | 2.17–15.0 | <0.001 |
| MI > 1/2 (vs ≤ 1/2) | 9.50 | 3.62–24.9 | <0.001 | 2.24 | 0.76–6.60 | 0.14 |
| LVI present (vs absent) | 7.42 | 3.02–18.2 | <0.001 | 3.01 | 1.14–7.95 | 0.03 |

Abbreviations: CI = confidence interval; FIGO = International Federation of Gynecology and Obstetrics; HR = hazard ratio; LVI = lymphovascular invasion; MI = muscular invasion; p-Akt = phosphorylated Akt.

Table 5. PTEN mutational status and IHC score in endometrial carcinoma cases

| Case | Histotype | Mutated exons | NCL | AA | NCL | AA | NCL | AA | Mut outside exons 5–7 | PTEN truncation | IHS |
|------|-----------|---------------|-----|----|-----|----|-----|----|----------------------|-----------------|-----|
| 1    | G2        | 8             | 963_964insA | T321fs*3 | 1 | 1 | 1 | 0 |
| 2    | G1        | 8             | 1008C>A | Y336* | 1 | 1 | 1 | 0 |
| 3    | G2        | 0             | 0 | 0 | 0 | 4.5 |
| 4    | Mixed     | 0 | 0 | 0 |
| 5    | G1        | 0 | 0 | 0 | 6 |
| 6    | Mixed     | 0 | 0 | 0 | 0 |
| 7    | G2        | 0 | 0 | 0 | 6 |
| 8    | G1        | 5             | 389G>C | R130P | 1 | 0 | 0 | 6 |
| 9    | G1        | 5             | 388C>G | R130G | 1 | 0 | 0 | 6 |
| 10   | G1        | 8             | 907delI | L333fs*4 | 1 | 1 | 1 | 0 |
| 11   | G1        | 7             | 800delI | K267fs*9 | 1 | 0 | 1 | 6 |
| 12   | Clear cell | 0 | 0 | 0 | 6 |
| 13   | G1        | 0 | 0 | 0 |
| 14   | G2        | 5, 8          | 388C>G | R130G | 1 | 0 | 0 | 6 |
| 15   | G1        | 0 | 0 | 0 |
| 16   | G1        | 3, 5, 6, 6    | 208C>A | L70I | 431A>C | K144Y | S17C>T | R173C | 601G>T | E201* | 1 | 0 | 1 | 0 |
| 17   | G2        | 7             | 640_655>ACT | Q214fs*3 | 1 | 0 | 1 | 0 |
| 18   | G2        | 5             | 389G>A | R130Q | 1 | 0 | 0 | 4 |
| 19   | G2        | 5, 9          | 405_406delAT | I133fs*4 | 1028TT>G | V343G | 1 | 0 | 1 | 0 |
| 20   | G1        | 1             | 64_70del7bp | D225fs*2 | 1 | 1 | 1 | 0 |
| 21   | G1        | 7             | 710delI | K237fs*19 | 1 | 0 | 1 | 0 |
| 22   | G1        | 2             | 80G>A | Y27C | 1 | 1 | 0 | 3 |
| 23   | Mixed     | 0 | 0 | 0 | 3 |
| 24   | G2        | 0 | 0 | 0 | 3 |
| 25   | Clear cell | 1             | 38_39insC | K13fs*30 | 1 | 1 | 1 | 6 |
| 26   | G1        | 5, 6          | 440_441insA | K147fs*32 | S625_576>0 | Y188fs*8 | 1 | 0 | 1 | 3 |
| 27   | G1        | 2             | 103A>G | M35V | 1 | 1 | 0 | 3 |
| 28   | G1        | 0 | 0 | 0 | 9 |
| 29   | G1        | 5, 5          | 263A>G | Y88C | 276C>G | D92E | 1 | 0 | 0 | 9 |
| 30   | G2        | 0 | 0 | 0 | 0 |
| 31   | G2        | 0 | 0 | 0 | 9 |
| 32   | G2        | 0 | 0 | 0 | 3 |
| 33   | G1        | 6, 7          | 611delC | P204fs*17 | 796A>T | K26A* | 1 | 0 | 1 | 3 |

Abbreviations: AA = amino acid; IHC = immunohistochemical; IHS = IHC score; Mut = mutation; NCL = nucleotide.
which may represent the biological and clinical characteristics of tumour.

The antibody used for our PTEN immunohistochemistry, that is, 6H2.1, recognises the C-terminal 100 amino acids of PTEN protein. Theoretically, not all mutations of PTEN may be detected as altered protein expression. Frameshift and nonsense mutations that result in truncating the C-terminal 100 amino acids of PTEN should be observed as null staining, while missense mutations may not be recognised by altered staining. In fact, our mutational analysis demonstrated that loss of PTEN expression was not associated with PTEN mutational status, but rather with the presence of frameshift or nonsense mutations that produce truncated PTEN proteins (Table 6). It can be speculated that PTEN-truncating mutations may spare functionally important regions of PTEN protein, leading to better outcome, compared with tumours with other PTEN mutations and mutations of other genes. Indeed, we previously reported that PTEN mutation only outside exons 5–7 was an independent prognostic factor for favourable survival in endometrial carcinoma, possibly due to incomplete disruption of protein function by sparing functionally important elements located inside exons 5–7 (Minaguchi et al., 2001). However, the current study failed to find statistically significant correlation between loss of PTEN expression and mutations only outside exons 5–7 (Table 6). This discordance may be due to small sample size for mutational analysis in the current study. In any case, together with the published findings, our results suggest that PTEN truncation may be due to small sample size for mutational analysis in the current study. Furthermore, PTEN truncation mutations may also be due to more indolent biological behaviour of tumour.

Table 6. Relationship between PTEN mutational status and IHC results

| PTEN IHC | Mutation in any exon | P-value | Mutation only outside exons 5–7 | P-value | PTEN truncation | P-value |
|---------|---------------------|---------|--------------------------------|---------|----------------|---------|
| IHS = 0 | 8/12 (67%)          | 0.49    | 4/12 (33%)                      | 0.38    | 8/12 (67%)     | 0.03    |
| HIS > 0 | 11/21 (52%)         |         | 3/21 (14%)                      |         | 5/21 (24%)     |         |
| IHS < 6 | 13/21 (62%)         | 0.72    | 5/21 (24%)                      | 1.0     | 11/21 (52%)    | 0.07    |
| HIS > 6 | 6/12 (50%)          |         | 2/12 (17%)                      |         | 2/12 (17%)     |         |

Abbreviations: IHC = immunohistochemical; IHS = IHC score.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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