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# Reduced PTEN expression predicts relapse in patients with breast carcinoma treated by tamoxifen

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**Tamoxifen treatment substantially improves the 10-year survival of women with estrogen-receptor (ER)-α-positive tumors. However, approximately one-third of all breast cancer patients with ER-α-positive tumors progress on antiestrogen therapy. The molecular mechanism(s) involved in antiestrogen-resistant phenotype of breast carcinoma is not completely understood. The *PTEN* (phosphatase and tensin homolog deleted on chromosome Ten) gene is a novel candidate tumor suppressor that plays an important role in cell cycle regulation and apoptosis by regulating Protein kinase-B/Akt activity. Previous studies have shown that PTEN downregulation in breast cancer is associated with high-grade tumor, distant metastases and poorer disease-free survival. Decreased PTEN and/or increased protein kinase B/Akt activity in breast cancer cells has recently been associated with resistance to tamoxifen-induced apoptosis. In this study, we have evaluated PTEN expression by immunohistochemistry in 100 tamoxifen-treated ER-α-positive breast cancer patients. Reduced PTEN protein expression was associated with shorter relapse-free survival. When stage I patients were analyzed separately, reduced PTEN expression was a strong predictor of both, shorter relapse-free survival and shorter disease-specific survival. An association of reduced PTEN expression with shorter relapse-free survival and disease-specific survival in stage I patients was still observed after stratification by stage, axillary lymph node status, tumor size, grade, and expression of ER-α, progesterone receptor, and Her-2/*neu*. In summary, our results showed a strong association between downregulation of PTEN expression in ER-α-positive tumors and failure to tamoxifen treatment.**
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Tamoxifen, a selective estrogen receptor modulator, is standard endocrine treatment for hormone receptor-positive breast cancer, both in the initial adjuvant therapy and as treatment of patients with metastatic disease. However, approximately one-third of patients with estrogen receptor (ER)-α-positive tumors are refractory to tamoxifen therapy and approximately one-third of patients who initially respond to tamoxifen develop resistance.[1–3] Most breast cancers with acquired endocrine resistance retain ER-α expression, which suggests that the loss of ER-α is not a common cause of resistance to endocrine therapy.[4] The mechanisms associated with resistance to tamoxifen therapy are not completely understood.[5–7]

The phosphatase and tensin homolog deleted on chromosome ten (*PTEN*) is a lipid phosphatase that antagonizes signal transduction downstream of PI-3 (phosphatidylinositol-3) kinase by dephosphorylating phosphatidylinositol-triphosphate (PtdInsP) and suppresses cell growth through the negative regulation of cell cycle and cell survival.[8–12] Downregulation of PTEN is associated with increased PI-3 kinase activity with subsequently higher levels of 3′-phosphorylated phosphoinositides, which bind to and activate PK-B/Akt. Activated PK-B/Akt promotes cell survival by phosphorylating and modulating the activity of various transcription factors.[13,14] Recently, it has been demonstrated *in vitro* that PI-3/Akt pathway modulates ER-α activity and confers tamoxifen resistance to MCF-7 breast cancer cells.[15–17]

PTEN mutations in metastatic prostate cancer are associated with resistance to antiandrogen therapy.[18,19]

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The prognostic significance of PTEN protein loss in breast cancer is not well established. A few studies have demonstrated reduced PTEN expression in breast cancer and its association with stage, grade, lymph node metastases and steroid receptor status.[20–24] To date, none of the studies have examined prognostic significance of reduced PTEN protein expression in ER-α-positive breast cancer patients and its potential association with unresponsiveness to tamoxifen.

The main objective of this study was to determine whether PTEN protein expression as determined by immunohistochemistry is related to the outcome of the patients who received adjuvant hormonal therapy, tamoxifen. Association of PTEN expression with stage, grade, tumor size, and tumor expression of estrogen receptor-alpha (ER-α) progesterone receptor (PR), and Her-2/*neu* expression was also evaluated.

## Materials and methods

### Patients

In all, 100 patients with ER-positive breast cancer, who were treated with antiestrogen, tamoxifen, were randomly selected from database obtained from Saskatoon Cancer Center. 'Relapse' was defined as occurrence of local recurrence, regional lymph node metastases or distant metastases. All the charts were reviewed to obtain relevant clinical information, recurrence, treatment and follow-up. Two pathologists reviewed slides of the primary breast tumor and representative blocks of formalin-fixed/paraffin-embedded tissue were obtained from the pathology archives. The tumors were graded according to modified Bloom and Richardson grading criteria.

### Immunohistochemistry

The PTEN, monoclonal mouse anti-human PTEN clone (1:200, Santa Cruz Biotech, Santa Cruz, CA, USA); ER-α clone 6F11 (1:15, Novocastra, Vector labs Burlington, ON, Canada); PR clone 16 (1:100, Novocastra, Vector labs Burlington, ON, Canada); and c-erbB2 (Her-*2/neu*) polyclonal (1:300, Dako, Massisauga, ON, Canada) were obtained. Immunohistochemistry was performed on the Ventana NEXES automated immunohistochemistry instrument (Ventana Medical systems, Inc, Tucson, AZ, USA) with an avidin–biotin system on 6 $\mu$M formalin-fixed paraffin-embedded sections from a representative block, containing both normal and tumor tissue. Slides were cut on a superfrost plus slides and baked overnight at 37°C. When required antigen retrieval was performed with 6 mM citrate pH 6.0 in a microwave oven.

### Staining Interpretation

A semiquantitative scale was used to score the staining intensity for PTEN, ER-α, PR, and Her-2/*neu*. Expression of ER-α and PR was considered low if less than 50% cells were positive and negative if less than 10% of cells were positive.[25] Expression of Her-2/*neu* was not considered overexpressed if scored less than 3+ as described previously.[26] Since cutoff levels for reduced PTEN expression by using immunohistochemical methods are not defined so far, we have scored both, staining intensity and the percent of positive cells by using *H*-score (histo-score). *H*-score was calculated as follows: $H\text{-score} = (\%1 + \text{cells} \times 1) + (\%2 + \text{cells} \times 2) + (\%3 + \text{cells} \times 3)$. The mean PTEN score was used as a cutoff point to designate reduced expression. *H*-score PTEN value was categorized for the purpose of summarizing the data for Table 3.

### Axillary Lymph Node Status

Axillary lymph node status was defined as follows: a score of 0 for absence of axillary lymph node metastases, a score of 1 for 1–3 involved lymph nodes and a score of 2 for 4 or more involved lymph nodes.

### Statistical Analysis

Statistical analyses were conducted with SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA), using $\chi^2$, Spearman correlation, Fisher's exact (two-tailed *P*-values) and Linear-by-Linear Association $\chi^2$-tests to determine the associations between different variables. Cox regression analysis was conducted to estimate relative risks (with 95% confidence intervals).[27] Relapse-free survival and disease-specific survival were calculated by the Kaplan–Meier survival estimates and the log-rank test from the date of diagnosis until last contact or relapse (for relapse-free survival) or death from disease (for disease-specific survival).[28] The level of significance was set at 0.05.

## Results

Patients' characteristics are summarized in Table 1. The mean patient follow-up was 6 years (range 4–10). In total, 97 patients had breast carcinomas with histology of invasive ductal carcinoma, while the rest were invasive lobular carcinomas. The presence of axillary lymph node metastases was strongly associated with shorter relapse-free survival ($P<0.0001$) and shorter disease-specific survival ($P<0.0001$). Stage was equally significant predictor of relapse-free survival ($P<0.0001$) and disease-specific survival ($P<0.0001$). However, about 50% of patients with stage I relapsed in approximately 90 months and 30% of the stage I patients died in the same period.

## PTEN Expression in Invasive Breast Cancer

The results of immunohistochemical analyses are summarized in Table 2. In 80% of the cases, normal breast parenchyma was also present in the biopsy. The epithelial cells of normal terminal ducts and lobules showed strong nuclear staining with PTEN antibodies (Figure 1). The intensity and degree of PTEN expression in adjacent neoplastic cells varied considerably. Strong PTEN staining was detected

**Table 1** Patients' characteristics

| | *Total* |
|---|---|
| Age in years (mean) | 28–93 (62) |
| Follow-up in months (mean) | 12–120 (72) |
| *Stage* | |
| I | 59 |
| II | 36 |
| III | 2 |
| IV | 3 |
| *Tumor size (cm)* | |
| <2 | 61 |
| 2–4 | 33 |
| >4 | 6 |
| *Lymph node metastasis* | |
| Yes | 33 |
| No | 67 |
| Total | 100 |

**Table 2** Tumor immunophenotype[a]

| | *No recurrence (%)* | *Recurrence (%)* | *Total* |
|---|---|---|---|
| *PTEN (P < 0.0001, FET)* | | | |
| High expression | 35 (81.4) | 25 (43.9) | 60 (60.0) |
| Low expression | 8 (18.6) | 32 (56.1) | 40 (40.0) |
| *ER-α (P = 0.227, FET)* | | | |
| High expression | 23 (53.5) | 23 (40.4) | 46 (46.0) |
| Low expression | 20 (46.5) | 34 (59.6) | 54 (54.0) |
| *PR (P = 0.410, FET)* | | | |
| High expression | 19 (44.2) | 20 (35.1) | 39 (39.0) |
| Low expression | 24 (55.8) | 37 (64.9) | 61 (61.0) |
| *Her-2 (P = 0.682, FET)* | | | |
| High expression | 24 (55.8) | 35 (61.4) | 59 (59.0) |
| Low expression | 19 (44.2) | 22 (38.6) | 41 (41.0) |

[a]High expression = 3+ and 4+, low expression = 0–2+.
FET = Fisher's exact test, two-tailed.

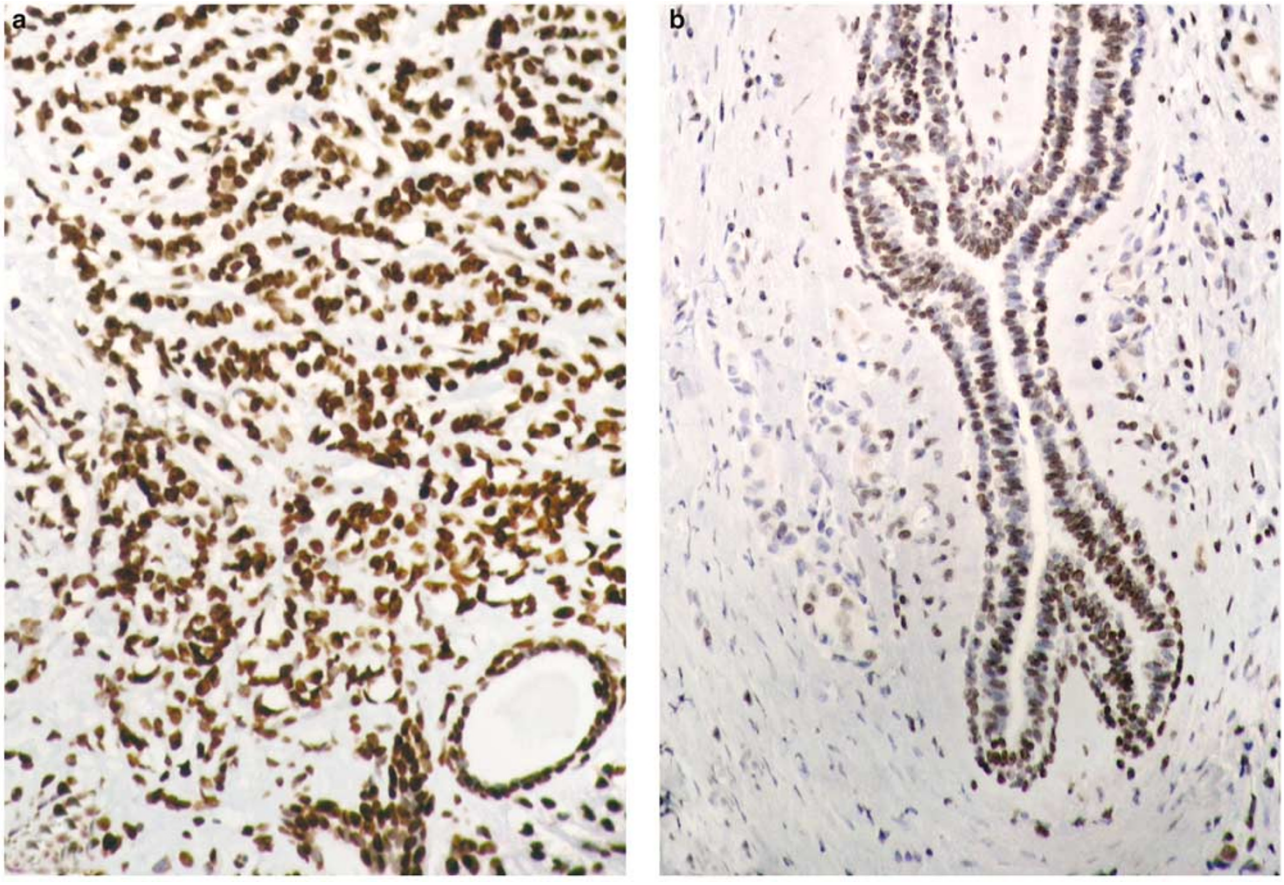


**Figure 1** (**a**) PTEN is strongly expressed in the nuclei of the breast cancer cells as well as in the benign duct epithelial cells (right lower corner). (**b**) PTEN staining is barely detectable in the neoplastic cells, while strongly expressed in the benign duct epithelial cells.

in 81% of nonrecurrence group, while only 43% in the recurrence group showed similar staining ($P<0.0001$, Fisher's exact test).

PTEN expression was analyzed in relation to clinicopathological parameters including age of the patients, stage, lymph node status, tumor grade, size, and tumor expression of ER-α, PR, and Her-2/*neu*. No correlation was found between tumor grade and expression of PTEN ($P=0.078$, Spearman correlation, Figure 2a) and no correlation was found for PTEN and PR expression ($P=0.17$). However, there was a positive correlation between expression of PTEN and ER-α ($P=0.022$) (Table 3 and Figure 2b). Strong negative association between PTEN expression and the presence of lymph node metastasis was found ($P=0.006$). In addition, there was no association between PTEN expression and the age of the patients, tumor grade, size, or expression of Her-2/*neu*. As expected, there was a strong positive association between ER-α and PR expression ($P<0.0001$). Negative correlation between ER-α expression and the size of the tumor ($r=-0.24$, $P=0.016$) was noted. PR expression also had negative association with Her-2/*neu* ($P=0.008$). Her-2/*neu* expression was positively associated with the presence of axillary lymph node metastases ($P=0.004$). The presence of axillary lymph node metastases was associated with higher grade ($P=0.017$) and larger size of the tumor ($P=0.017$). Stage and grade were also positively associated ($P=0.002$) as well as stage and the size of the tumor ($r=0.47$, $P<0.0001$).

### PTEN Expression, Relapse-Free Survival and Disease-Specific Survival

*Univariate analysis*

Reduced expression of PTEN was significantly associated with shorter relapse-free survival ($P=0.0015$) and there was a trend for shorter disease-specific survival ($P=0.0636$) (Figure 3). Most significant observation of this study was that the reduced expression of PTEN was associated with both, shorter relapse-free survival ($P=0.0003$) and shorter disease-specific survival ($P=0.0136$) in stage I patients (Figure 4). When patients were stratified by high *vs* low levels ($\geq 50\%$ cells positive *vs* $<50\%$ cells positive) of ER-α expression, reduced PTEN expression was again associated with less favorable outcome in both groups of patients (Figure 5). In stage I patients with low expression of ER-α

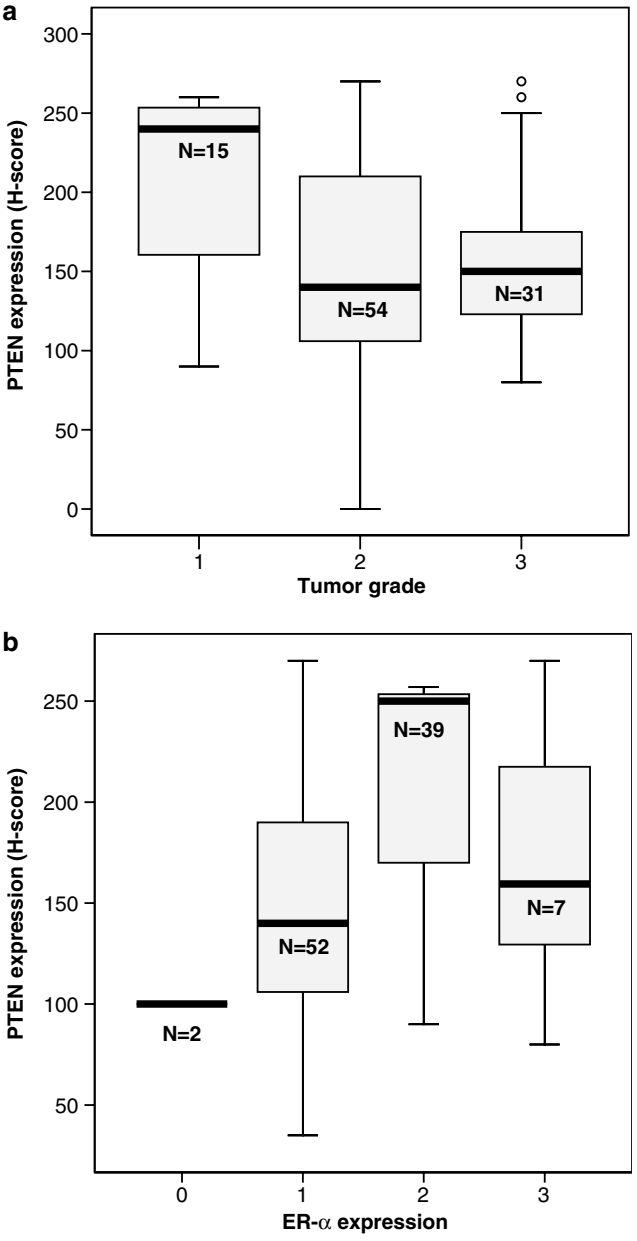


**Figure 2** Distribution of PTEN scores according to tumor grade (**a**) and estrogen receptor-α expression (**b**).

**Table 3** PTEN expression has positive correlation with ER-α expression ($P=0.022$, Spearman correlation)

| *PTEN* | *ER-α (%)* | | | | *Total (%)* |
|---|---|---|---|---|---|
| | *0* | *1+* | *2+* | *3+* | |
| 1+ | 0 (0.0) | 7 (13.5) | 0 (0.0) | 0 (0.0) | 7 (7.0) |
| 2+ | 2 (100.0) | 18 (34.6) | 2 (28.6) | 11 (28.2) | 33 (33.0) |
| 3+ | 0 (0.0) | 16 (30.8) | 2 (28.6) | 18 (46.2) | 36 (36.0) |
| 4+ | 0 (0.0) | 11 (21.2) | 3 (42.9) | 10 (25.6) | 24 (24.0) |
| Total (%) | 2 (100.0) | 52 (100.0) | 7 (100.0) | 39 (100.0) | 100 (100.0) |

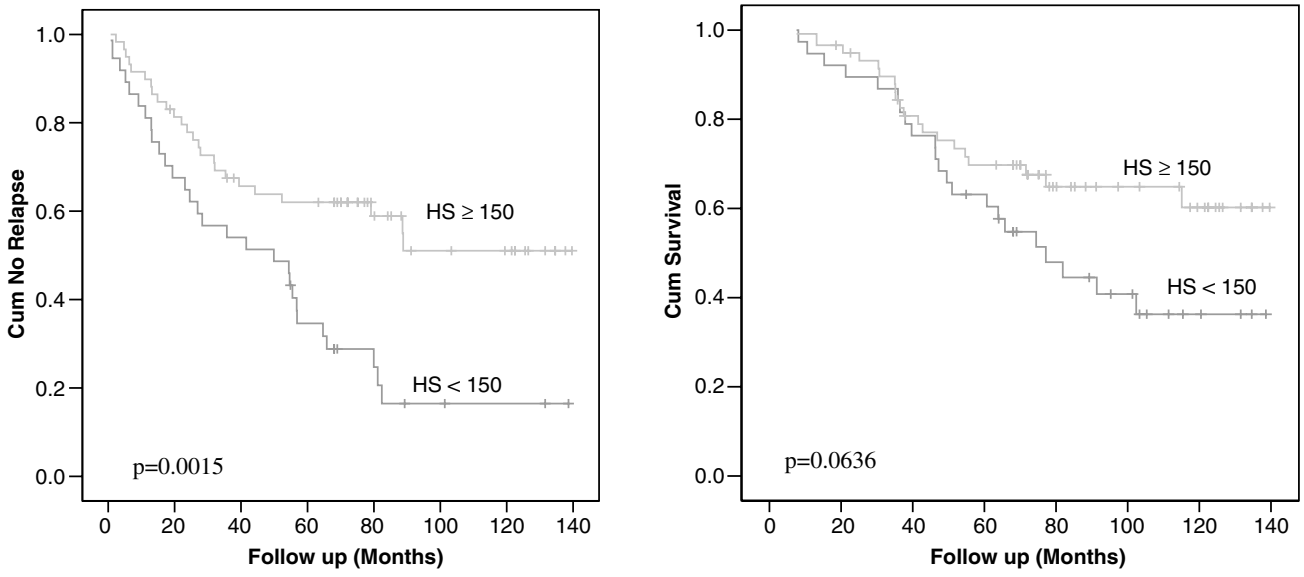


**Figure 3** Patients with tumors that do not show downregulation of PTEN expression have significantly longer relapse-free survival as well as statistical trend for longer disease-specific survival.

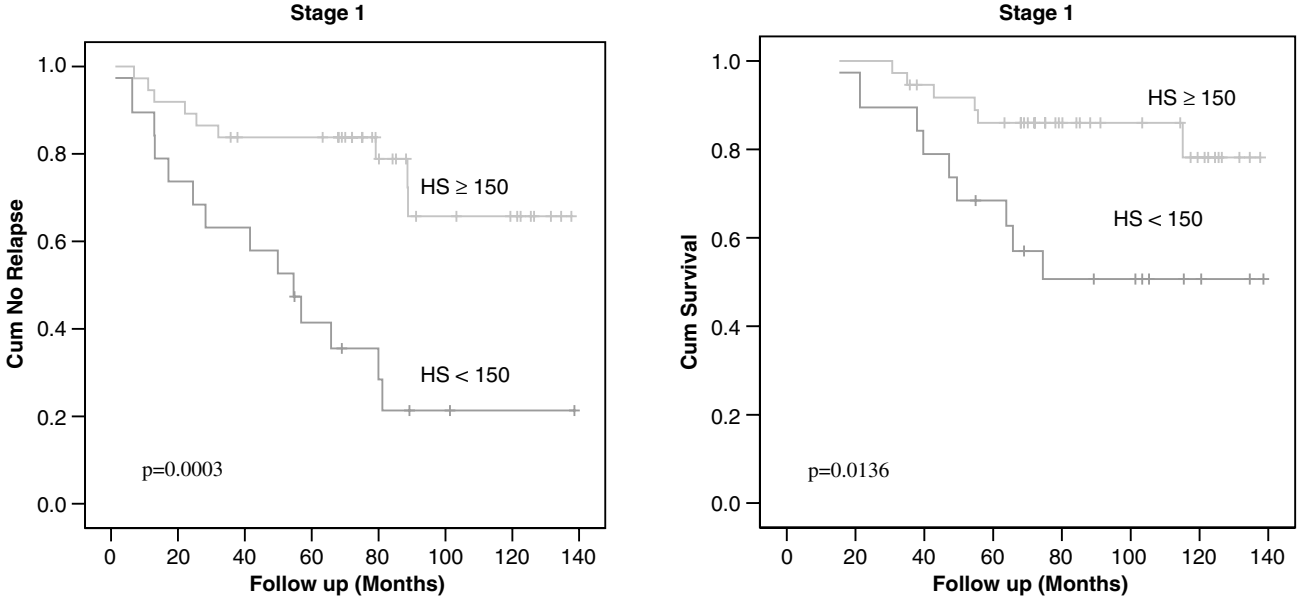


**Figure 4** Patients with stage 1 tumors that do not show downregulation of PTEN expression have markedly longer relapse-free survival as well as significantly longer disease-specific survival.

(<50% cells positive), the difference in relapse and survival was very large based on PTEN expression. Only 30% patients relapsed and 25% died with normal expression of PTEN, while as many as 90% relapsed and 65% died if they had tumors with reduced expression of PTEN (Figure 6).

Strong expression of Her-2/*neu* ($P=0.031$) was also associated with shorter relapse-free survival. No such association between relapse-free survival was found for the size of the tumor or expression of ER-$\alpha$ or PR. Expression of PR was associated with longer disease-specific survival ($P=0.016$), while overexpression of Her-2/*neu* was associated with shorter disease-specific survival ($P=0.020$). There was no association of disease-specific survival with age of the patients, tumor size, grade, or ER-$\alpha$ expression.

*Cox regression analysis*

Cox regression analysis showed that association between reduced expression of PTEN and shorter relapse-free survival was still observed after stratification by stage, tumor size or grade, lymph node

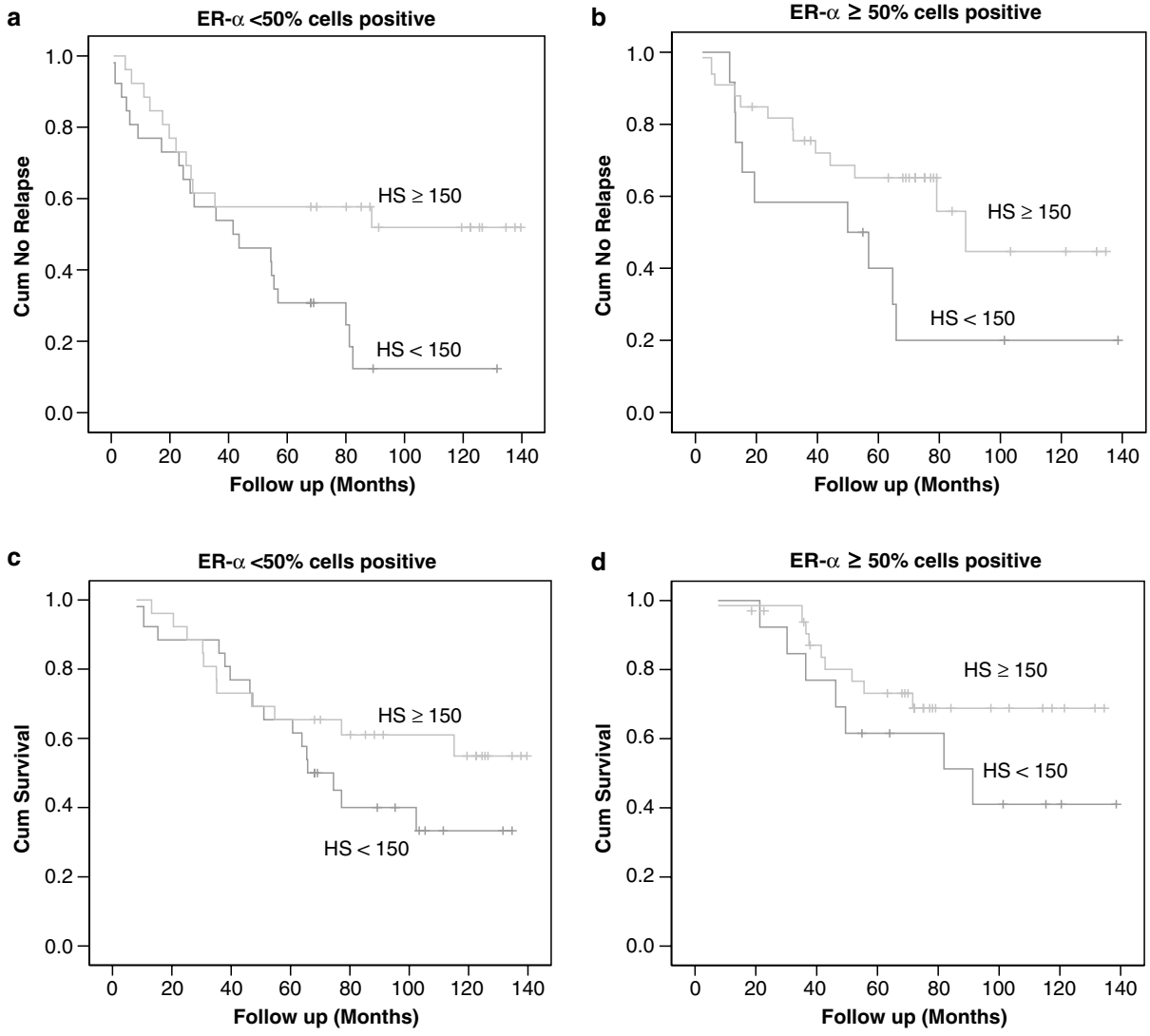


**Figure 5** Reduced PTEN expression is associated with shorter relapse-free survival ($P=0.0030$) (**a**) and (**b**) and statistical trend for shorter disease-specific survival (**c**) and (**d**) particularly in patients with tumors weakly positive for ER-α.

status, and tumor expression of ER-α, PR or Her-2/*neu* (Table 4).

An association of reduced PTEN expression with shorter relapse-free survival and disease-specific survival in stage I patients was still observed after stratification by tumor size and grade, and tumor expression of ER-α, PR, or Her-2/*neu* (Table 5).

## Discussion

The dual-specificity phosphatase PTEN/MMAC1 has recently been identified as the frequently mutated tumor suppressor gene in various human tumors including endometrial carcinoma, malignant melanoma, high-grade glioma and metastatic prostate cancer.[29–33] There is an increased predilection for breast cancer caused by germline mutations of PTEN (lifetime risk >50% in patients with Cowden's disease) however, mutations are rare in sporadic breast cancer.[34–36]

Previous studies have demonstrated association between loss of PTEN expression or reduced PTEN expression in breast cancer with stage, lymph node status, disease-related death, and loss of ER.[20–24,37] Two of these studies included small number of unselected cases.[20,24] The largest of these studies, also the only follow-up study, included 151 breast cancer cases and showed loss of PTEN expression in 48% of tumors, significantly higher than in our study.[22] The difference in the sensitivity of the immunohistochemical analysis may be responsible for this discrepancy. Otherwise, our results in relation to outcome of the patients and association of reduced PTEN expression with adverse clinicopathological variables were similar. All of the studies reported an association between reduced (or loss of) PTEN expression and advanced breast

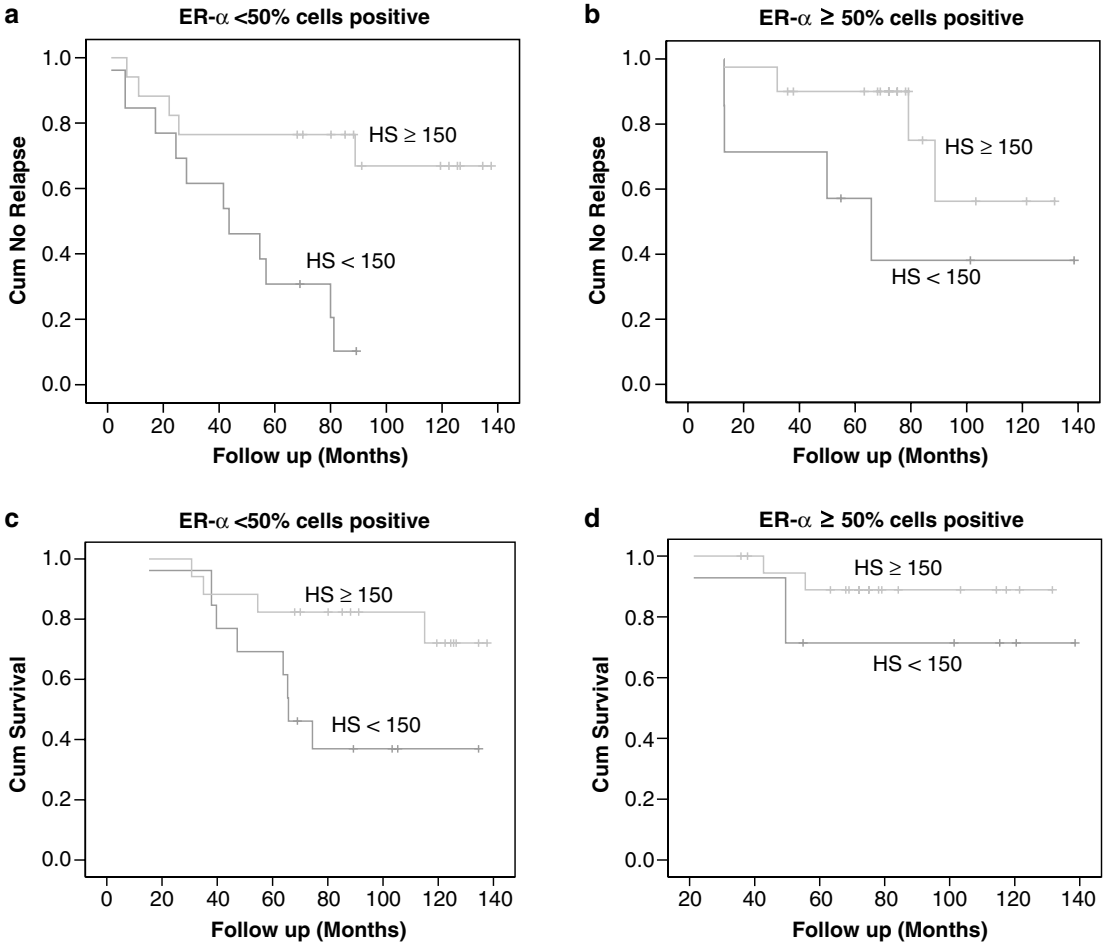


**Figure 6** In stage I patients, reduced PTEN expression has even stronger association with shorter relapse-free survival (**a**, **b**) and disease-specific survival (**c**, **d**) particularly in patients with tumors weakly positive for ER-α (10–50% positive cells) (**a**, **c**).

**Table 4** Cox regression analysis estimated prognostic value by hazard ratios (HR) and 95% CI* for relapse-free survival and disease-specific survival

| *Variable* | *Relapse-free survival* | | | *Disease-specific survival* | | |
|---|---|---|---|---|---|---|
| | P** | *HR* | *95% CI* | P** | *HR* | *95% CI* |
| PTEN (per score point) | 0.001 | 0.54 | 0.373–0.785 | 0.218 | 0.775 | 0.517–1.162 |
| Age (per year) | 0.204 | 1.02 | 0.992–1.039 | 0.642 | 1.006 | 0.981–1.032 |
| Stage | 0.004 | 2.05 | 1.263–3.336 | 0.032 | 1.830 | 1.055–3.175 |
| Axillary LN metastases (none *vs* 1–3 *vs* ≥4) | 0.032 | 1.61 | 1.043–2.492 | 0.237 | 1.374 | 0.811–2.328 |
| Tumor size (per cm) | 0.96 | 1.01 | 0.739–1.376 | 0.909 | 1.022 | 0.701–1.490 |
| Tumor grade (per point) | 0.80 | 0.94 | 0.593–1.498 | 0.886 | 0.962 | 0.566–1.635 |
| ER-α (per score point) | 0.46 | 1.11 | 0.841–1.469 | 0.483 | 1.114 | 0.824–1.507 |
| PR (per score point) | 0.74 | 1.05 | 0.810–1.348 | 0.196 | 0.826 | 0.618–1.104 |
| Her-2 (per score point) | 0.04 | 1.23 | 1.008–1.502 | 0.309 | 1.127 | 0.895–1.420 |

*CI denotes confidence interval.
**P*-values were computed from Wald statistics.

cancer stage, suggesting that loss of PTEN expression might have prognostic relevance. Similar to other studies, there was a significant positive association between PTEN expression and ER-α.[20,22] However, our study is unique in that we have evaluated the potential significance of reduced PTEN expression as a prognostic marker of response to tamoxifen treatment.

In our study, reduced PTEN expression was significantly associated with shorter relapse-free survival in breast cancer patients on tamoxifen treatment and also with both, shorter relapse-free

**Table 5** Stage I patients only: the Cox regression analysis estimated prognostic value by hazard ratios (HR) and 95% CI* for relapse-free survival and disease-specific survival

| *Variable*** | *Relapse-free survival* | | | *Disease-specific survival* | | |
|---|---|---|---|---|---|---|
| | P*** | *HR* | *95% CI* | P*** | *HR* | *95% CI* |
| PTEN (per score point) | <0.0001 | 0.384 | 0.237–0.622 | 0.032 | 0.577 | 0.349–0.953 |
| Age (per year) | 0.055 | 1.038 | 0.999–1.077 | 0.193 | 1.031 | 0.985–1.079 |
| Tumor size (per cm) | 0.741 | 1.170 | 0.460–2.975 | 0.702 | 1.255 | 0.392–4.015 |
| Tumor grade (per point) | 0.098 | 0.529 | 0.249–1.124 | 0.211 | 0.560 | 0.226–1.390 |
| ER-α (per score point) | 0.705 | 1.088 | 0.702–1.687 | 0.664 | 1.129 | 0.653–1.954 |
| PR (per score point) | 0.509 | 0.874 | 0.586–1.304 | 0.073 | 0.608 | 0.353–1.048 |
| Her-2 (per score point) | 0.005 | 1.585 | 1.153–2.179 | 0.025 | 1.567 | 1.058–2.319 |

*CI denotes confidence interval.
**Constant or linearly dependent covariates stage = 1 and axillary LN metastases = 0 excluded from analysis.
****P*-values were computed from Wald statistics.

survival and shorter disease-specific survival for stage I patients. These findings were independent of stage or lymph node status and expression of ER-α, PR, or Her-2/*neu* in tumors. These findings suggest that loss of PTEN may indirectly or directly interfere with cellular effects of tamoxifen.

The PTEN gene encodes a dual specificity phosphatase that opposes the effects of PI-3 kinase by dephosphorylating its lipid products.[38] These products are required for activation of protein kinase B (PK-B), also known as Akt, a serine/threonine kinase involved in cell growth and survival.[39] PTEN may thus inhibit carcinogenesis through its inhibitory effect on PKB/Akt-3 signal transduction pathway. PK-B/Akt-3 inhibits apoptosis, induced by wide variety of agents, and promotes cell cycle progression.[40] Therefore, cells with high Akt-3 levels or reduced PTEN are more resistant to chemotherapeutic agents.[15–17,40–43] There is a significant negative correlation between low cellular levels of ER-α, and PTEN on one side and elevated Akt-3/PK-B on the other.[37,44–46] Functional association between PTEN and estradiol is supported by the observation that in knockout mice with a mammary-specific deletion of the *PTEN* gene, exposure to estradiol enhanced development of mammary tumor early in life.[47] It appears that estradiol downregulates PTEN activity by increasing its phosphorylation.[48,49] In general, estradiol promotes proliferation of mammary epithelial cells and inhibits apoptosis,[50] which provides the basis for treatment with antiestrogen tamoxifen.[51] Tamoxifen, a partial ER antagonist, binds to ER-α and mediates its effects via inhibiting the estrogen-dependent activation of AF-2 region. A recent *in vitro* study showed that in breast cancer cells, increased PI-3 kinase and Akt activate ER-α and protect breast cancer cells from tamoxifen-induced apoptosis.[17] Campbell *et al*[17] clearly demonstrated a molecular link between activation of the PI-3 kinase/Akt pathway, hormone-independent activation of ER-α and inhibition of tamoxifen-induced apoptosis, and proposed a new cellular mechanism for tamoxifen resistance. The capacity to overcome mechanisms that promote apoptosis is essential for the development and progression of cancer, and the P1-3 kinase/Akt pathway, which is negatively regulated by PTEN, provides such a mechanism by transmitting a strong survival signal.[52,53]

In our study, ER-α-positive breast tumors with reduced PTEN expression had shorter relapse-free survival, which suggests poor response to tamoxifen therapy. The full significance of this finding is not clear as our study is limited by the lack of ER negative and no tamoxifen treatment control. We also did not correlate the level of PTEN protein expression with response to other chemotherapeutic agents as reduced PTEN expression and/or activation of PI 3-K/Akt pathway has been shown to confer resistance to chemotherapy.[54–57] However, in the light of information from cell culture studies, it may be proposed that reduced PTEN expression can result in increased activity of PK-B/Akt antiapoptotic pathway, which interferes with cellular actions of tamoxifen resulting in tumor recurrence. PTEN overexpression has also been shown to inhibit cell migration, whereas antisense PTEN enhanced cell migration.[58,59] An association of reduced PTEN expression with the presence of axillary lymph node metastases as well as distant metastases and death from disease suggests that loss of PTEN may also be involved in promotion of the invasive properties of the tumor.

The most significant observation in our study was a very strong association between reduced PTEN protein expression with relapse- and disease-related death in stage I patients. If these findings can be confirmed in future studies, we would like to propose that an attempt to standardize methods for immunohistochemical detection of PTEN should be made, similar to that for Her-2/*neu*, so that interpretation of the immunostaining may be relevant for clinical practice.

In conclusion, we have found significant association between reduced PTEN protein expression and adverse prognostic factors in breast cancer as well as

shorter relapse-free survival and disease-specific survival. These results generally concur with previous studies implicating PTEN as a candidate tumor suppressor in breast cancer. Further study of this gene, especially in relevance to tamoxifen response and association with other breast cancer markers, appears warranted.

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