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ORIGINAL ARTICLE

PTEN protein expression in postmenopausal steroid receptor positive early breast cancer patients treated with adjuvant tamoxifen

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Summary

Purpose: *Since one of possible causes of resistance to antiestrogen therapy in steroid receptor positive (SR^+) breast cancer (BC) patients is an alteration of PTEN (phosphatase and tensin homolog deleted on chromosome 10) signaling pathways, the aim of this study was to determine the PTEN protein expression in postmenopausal patients with steroid SR^+ BC treated with adjuvant tamoxifen, to investigate the association of PTEN protein expression with tumor histology, size and grade, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) statuses and disease outcome.*

Methods: *This was a retrospective analysis of 78 postmenopausal stage I/II SR^+ BC patients treated with adjuvant tamoxifen. PTEN protein expression and ER, PR and HER2 status were determined using immunohistochemistry.*

Results: *The distribution of PTEN protein expression according to tumor histology was as follows: $PTEN^+$ status in 27/43 (62.8%) patients with ductal and in 26/35 (74.3%) patients with lobular carcinomas; and $PTEN^-$ status in 16/43 (37.2%) patients with ductal and in 9/35 (25.7%) patients with lobular carcinomas. Disease relapse was observed in 38/78 patients: 14/53 (26.4%) of $PTEN^+$ BC subgroup and 24/25 (96%) of $PTEN^-$ subgroup (x^2, $p=0.018$). There were no significant associations between PTEN protein expression and tumor histology, size and grade, and ER, PR and HER2 expression. Patients with $PTEN^-$ had significantly shorter disease-free interval (DFI) and overall survival (OS) (for both, log rank test, $p<0.01$) compared to $PTEN^+$ BC patients.*

Conclusion: *Our results suggest that PTEN protein expression might be of prognostic significance in postmenopausal SR^+ BC patients treated with adjuvant tamoxifen.*

Key words: breast carcinoma, PTEN, steroid receptors, tamoxifen

Introduction

Tamoxifen is a nonsteroid selective estrogen receptor modulator which is been used in the treatment of BC for more than 30 years [1]. It has a remarkable antiestrogen activity in the breast and, until recently, it has been a standard first-line therapy in postmenopausal patients with ER^+ BC, both in adjuvant and metastatic settings [2-4]. However, one third of the patients with SR^+ are resistant to tamoxifen, whereas one third who initially respond, develop resistance [2]. The molecular mechanism which is responsible for the antiestrogen-resistant phenotype, is still unknown. A possible cause of resistance is alteration of the PTEN/PI3K/Akt (Phosphatase and tensin homolog deleted on chromosome 10/phosphatidyl-inositol 3-kinase/serine-threonine protein kinase) signaling pathway [5].

A number of studies show that a reduced PTEN expression is related to the stage of disease, histological tumor grade, nodal status and SR, whereas fewer authors do not confirm this correlation [5-8].

Studies of BC cell lines show that PI3K/Akt pathway could modulate ERα activity and in that way cause resistance to tamoxifen [5,9]. Campbell et al. showed that increased expression of PI3K and Akt activates ERα in BC cells and in that way protects malignant cells from apoptosis caused by tamoxifen [5]. Pfeiler et al. reported that the apoptotic effect of tamoxifen could depend on PTEN expression, in a way that inadequate PTEN expression leads to modified cascade of apoptot-

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ic signals, which makes malignant cells "escape" from antitumor therapy [10]. Shoman et al. demonstrated a significant correlation between decreased PTEN expression in ERα$^+$ BC and resistance to tamoxifen [2].

Unlike the above mentioned authors, Frogne et al. did not notice decreased PTEN protein expression in BC cell lines overexpressing Akt and which also had acquired resistance to antiestrogen therapy [11].

PTEN is a tumor supressor gene which has a significant role in the negative regulation of PI3K/Akt pathway; the gene encodes a multifunctional phosphatase which regulates cell cycle, apoptosis and cell adhesion [7,12-14]. Its main substrate is phosphatidylinositol 3,4,5 triphosphate (PIP3) which is a product of PI3K; an increase of PIP3 causes shift of Akt to cell membranes where Akt is activated by kinases, the activity of which also depends on PIP3 [15]. PTEN protein catalyses the removal of phosphate from PIP3, transforming it into biphosphate (PIP2) and in that way blocks cell proliferation and cell survival [7,16]. Loss of PTEN function results in accumulation of PIP3 and in activation of the PI3K/Akt pathway which stimulates cell proliferation and invasion and inhibits apoptosis [17,18].

Loss of PTEN function can take place due to mutations, deletion or methylation of the gene promoter [19]. Deletions and mutations of the PTEN gene often take place in numerous primary sporadic carcinomas such as BC, endometrial and ovarial carcinomas, carcinoma of urinary bladder, prostate carcinoma, small cell carcinoma of the lung, thyroid carcinoma as well as malignant melanoma [20-23]. Germ line mutations are a cause of Cowden syndrome and also a cause of 4 rare autosomal dominant diseases with similar clinical pictures which display predisposition for occurrence of different types of carcinoma [23,24].

A frequent cause of loss of PTEN function is loss of heterozygosity (LOH) which is found in 30-40% of sporadic BC [14,24,25-27]. PTEN mutations are rare, and are found in about 5-12% of sporadic BC [27]. A reduced or absent PTEN expression is in the range from 8-50% in sporadic BC [5,27].

The aim of this study was to determine the PTEN protein expression in postmenopausal patients with SR$^+$ BC treated with adjuvant tamoxifen, to evaluate the association of PTEN protein expression with tumor histology, size and grade, ER, PR and HER2 statuses and disease outcome.

Methods

This retrospective analysis included 78 postmenopausal ER$^+$ and/or PR$^+$ BC patients with node negative, grade 3 tumors, or 1-3 positive axillary lymph nodes of any tumor grade that were diagnosed at the Institute for Oncology and Radiology of Serbia between 1988 and 1995. All of them were treated with radical mastectomy followed by postoperative radiotherapy (to the regional axillary, supraclavicular and infraclavicular lymph nodes, or internal mammary region with total dose [TD] of 48 Gy in 22 fractions) in the majority of patients (n=70). According to the Protocol for Diagnosis and Treatment of Cancers of the Institute for Oncology and Radiology of Serbia at that time [28], all of them received adjuvant tamoxifen 20 mg/day for 5 years as the only adjuvant therapy.

Formalin-fixed, paraffin-embedded tissue samples were sectioned at 5 μm thick sections and stained with hematoxylin-eosin (HE). The histological type, grade, ER, PR and HER2 status were determined. For histological grading the Scarff-Bloom-Richardson scoring system was used: high (G 1), medium (G 2) and low (G 3) [29].

A manual immunohistochemical technique was used with primary monoclonal mouse anti-human PTEN clone (1:100, Clone 6H2.1, Dako) with EnVision$^+$ system (HRP Labelled Polymer, K4000, Dako) and chromogen Dako Dab liquid (K3468). Labelled streptavidin-biotin (LSAB) method together with immunoperoxidase were used according to the recommended procedure for commercial primary monoclonal mouse antibody: Anti-Human ERα clone (1:50; Clone 1D5; Dako) and Anti-Human PR clone (1:50; Clone PgR 636; Dako); as for polyclonal rabbit antibody Anti-Human c-erbB2/HER2 Oncoprotein (1:300; Dako) with Dako LSAB^{TM+}/HRP kit (K0679). Slices were contrasted with Mayer hematoxylin.

The immunoreactivity of PTEN was assessed using the semiquantitative method based on the score of percentage of stained cells-cytoplasm/nuclei (0: no immunoreactivity; 1: 1-10%; 2: 11-50%; 3: 51-100%) and intensity of staining (0: no immunoreactivity; 1: reduced staining intensity relative to the corresponding normal cells; 2: same as normal cells staining; 3: mildly increased staining; 4: moderately increased staining; 5: intensely increased staining) [30]. For the internal positive control, immunoreactivity of normal surrounding breast tissue (duct epithelium, myoepithelial cells, endothelium, fibrocytes and nerves) was used [30]. PTEN status was defined as follows: positive if score ≥ 4, negative if score <4.

The evaluation of SR (ER, PR) was based on the scoring system which included the percentage of stained malignant nuclei (0-5) and their intensity of staining (0-3) [31]. ER/PR status was defined as follows: positive if score ≥ 4, negative if score <4 [31].

HER2 status was determined using the DAKO scoring system and $HER2^+$ status was defined if immunohistochemistry (IHC) score 2^+ and 3^+ [32].

Retesting of all histological slices (HE, immunoreactivity of PTEN, ER, PR and HER2) was performed by two independent pathologists.

Statistical analyses

For testing the association of PTEN protein expression and tumor histology, size and grade, ER, PR and HER2 statuses Pearson x^2 and Fisher's exact tests were used. The endpoints of disease outcome were DFI and overall OS. DFI was defined as the time from BC surgery to locoregional recurrence, second primary cancer in the contralateral breast or distant metastases, while OS was defined as the time from BC surgery to death for any reason. Kaplan-Meier function estimates were plotted to compare the survival distributions (time until progression and time until death) by PTEN status.

Results

The median age of the analyzed 78 postmenopausal SR^+ patients was 60 years (range 43-81). The median follow-up period was 114 months (range 11-228). Ductal carcinoma was diagnosed in 43/78 (55.1%) and lobular carcinoma in 35/78 (44.9%) patients. Axillary lymph node metastases were found in 73 (93.6%) patients. ER^+ status was found in all 78 (100%) patients; PR^+ was found in 65/78 (83.3%), whereas PR^- status was detected in 13/78 (16.7%); $HER2^-$ status was found in 66/78 (84.6%), whereas $HER2^+$ status was present in 12/78 (15.4%). Patient characteristics are shown in Table 1.

In the analyzed material, normal gland epithelium showed PTEN immunoreactivity both in the cytoplasm and nuclei. In case of absence of normal breast tissue, immunoreactivity of fibrocytes, nerves and endothelium was used as internal control (Figure 1). There was considerable difference in PTEN expression between malignant cells within the same tumor (heterogeneity of PTEN immunoreactivity); immunoreactivity was present in the cytoplasm and nuclei of malignant cells (Figures 2, 3).

Table 1. Patient characteristics

Characteristics	*n (%)*
Age, years, median (range)	60 (43-81)
Tumor histology	
Ductal invasive	43/78 (55.1)
Lobular invasive	35/78 (44.9)
Tumor size	
T1	40 (51.3)
T2	35 (44.9)
T3	3 (3.8)
Tumor grade	
1	7 (9)
2	62 (79.5)
3	9 (11.5)
Nodal status	
Negative	5 (6.4)
Positive	73 (93.6)
SR status	
ER^+/PR^+	65 (83.3)
ER^+/PR^-	13 (16.7)
HER2 status	
Positive	12/78 (15.4)
Negative	66 (84.6)
PTEN status	
Positive	53 (67.9)
Negative	25 (32.1)

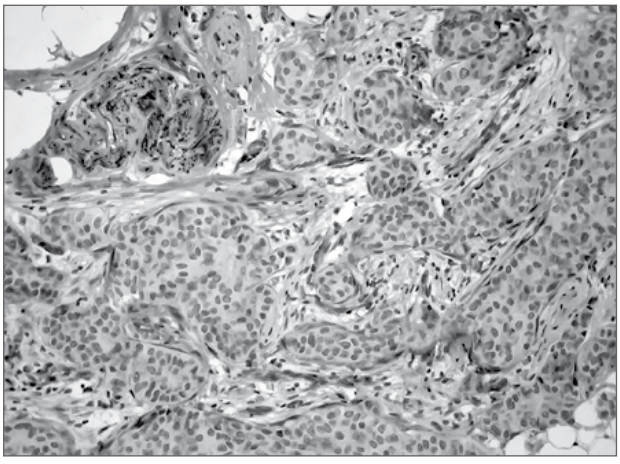

Figure 1. PTEN positive immunohistochemical staining of nerves and fibrocytes but negative for tumor cells (×100).

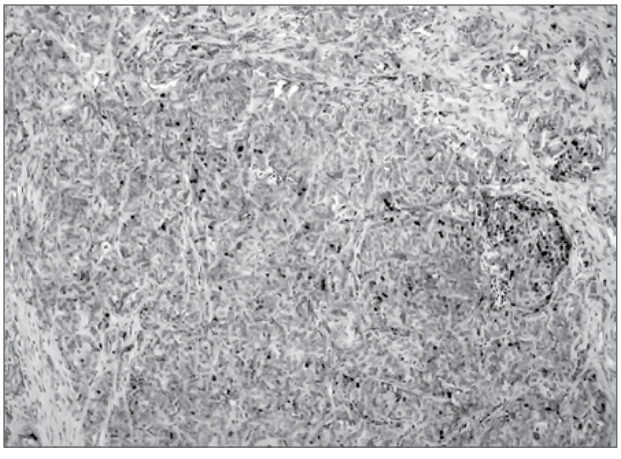

Figure 2. Heterogeneity of nuclear PTEN immunoreactivity in tumor cells (×40).

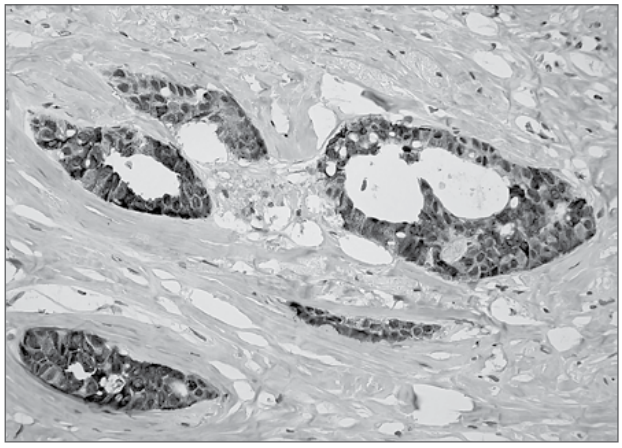

Figure 3. Immunohistochemical staining demonstrates cytoplasmic PTEN positivity in tumor cells (×40).

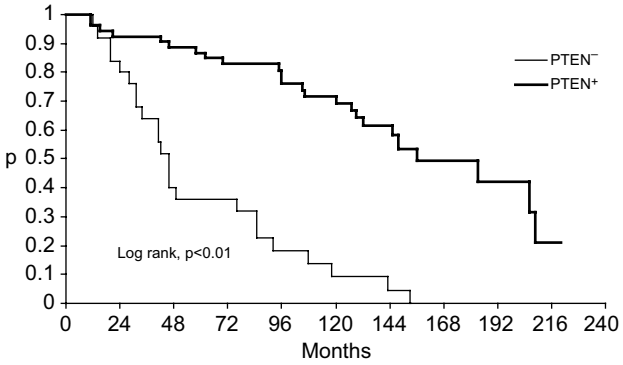

Figure 4. Disease-free interval in PTEN$^-$ breast cancer patients was significantly shorter in comparison to PTEN$^+$ breast cancer patients.

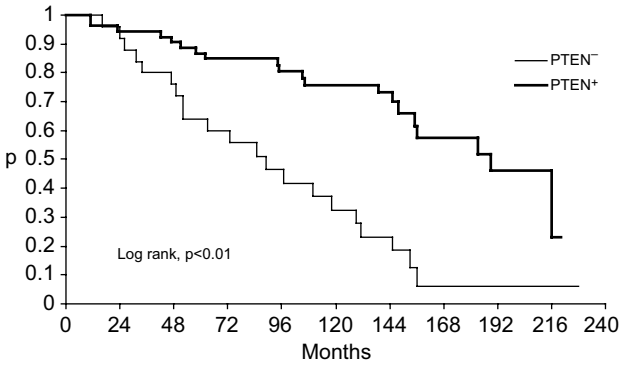

Figure 5. Overall survival in PTEN$^-$ breast cancer patients was significantly reduced in comparison to PTEN$^+$ breast cancer patients.

No significant association was detected between PTEN status and histological type: among patients with ductal carcinoma PTEN$^+$ and PTEN$^-$ status was found in 27/43 (62.8%) and 16/43 (37.2%) patients, respectively; among patients with lobular carcinoma PTEN$^+$ and PTEN$^-$ status was found in 26/35 (74.3%) and 9/35 (25.7%) patients, respectively. There was no statistically significant association between PTEN protein expression and tumor size (Fisher exact test; p=0.09), histological type (Pearson x^2 test; p=0.28) and tumor grade (Fisher exact test; p=0.20). Furthermore, there was no difference between PTEN protein expression and ER$^+$/PR$^+$ and ER$^+$/PR$^-$ phenotypes of BC (Fisher exact test; p=0.75) and HER2 status (Fisher exact test; p = 0.32).

Concerning disease outcome, positive PTEN protein expression was confirmed in 39/40 (97.5%) patients without disease relapse, and in 14/38 (36.84%) patients with disease relapse (Pearson x^2 test; p <0.001). Absence of PTEN protein and relapse of disease were found in 24/25 (96%) patients, whereas only one patient with PTEN$^-$ BC (4%) did not develop disease relapse (Pearson x^2 test; p=0.018) (Table 2). Furthermore, patients with PTEN$^-$ BC had significantly shorter both DFI (log rank test; p=0.0) and OS (log rank test; p=0.0)

Table 2. PTEN protein expression in patients with and without disease relapse

PTEN protein expression	*With relapse, (n=38) n (%)*	*Without relapse, (n=40) n (%)*	*Total*	*p-value*
Positive	14 (17.94)	39 (50.00)	53 (67.94)	<0.09
Negative	24 (30.76)	1 (1.28)	25 (32.06)	=0.018

Table 3. Disease free interval (DFI) and overall survival (OS) (months) according to tumor histology and PTEN status

	Ductal carcinoma				*Lobular carcinoma*			
	DFI median, (95% CI)	*p-value*	*OS median, (95% CI)*	*p-value*	*DFI median, (95% CI)*	*p-value*	*OS median, (95% CI)*	*p-value*
PTEN$^+$	206 (>132)	<0.01	183 (>155)	<0.01	Not reached (148)	<0.01	189 (>148)	<0.01
PTEN$^-$	43.5 (31-118)		97 (>52)		46 (>148)		63 (>49)	

compared to $PTEN^+$ BC patients (Figures 4, 5). These differences between $PTEN^+$ and $PTEN^-$ BC patients remained statistically significant in ductal and lobular subgroups for both DFI (both log rank tests; $p < 0.01$) and OS (both log rank tests; $p < 0.01$) (Table 3).

The risk for disease relapse and death were 6-fold higher (HR 6.04, 95% CI 3.23-11.3, likelihood ratio test, $p=3\times10^{-8}$) and more than 3.5-fold higher (HR 3.68, 95% CI 1.96-6.89, likelihood ratio test, $p=7\times10^{-5}$), respectively in $SR^+/PTEN^-$ BC patients in comparison with $SR^+/PTEN^+$ BC patients treated with adjuvant tamoxifen only.

Discussion

Loss of PTEN expression was detected in 32.05% of SR^+ postmenopausal BC patients. Several studies reported loss of PTEN in 28-40% of BC patients [2,6,8,27,33]. The different literature results can be attributed to the small number of evaluated patients as well as to inadequate classification of PTEN expression as a dichotomous variable e.g. positive/negative. Another possible cause of difference in frequency of reduced PTEN expression could be the use of different antigens in different studies and lack of standardized interpretation of PTEN expression.

Our results concerning the absence of correlation between PTEN expression, tumor size, histological type and grade of carcinoma, are also consistent with the literature [6,27,33].

There are controversial results between mutual correlation of altered PTEN expression and SR status. While some authors confirm a correlation between reduced PTEN expression and negative ER/PR status, others state that a reduced PTEN expression is associated with high ER expression [5,6,14,34]. Unlike other authors, we analyzed subgroups of patients with ER^+/PR^+ status and with ER^+/PR^- status in relation to PTEN expression and found no association between the two. Neither did we find an association between HER2 status and PTEN expression, in concordance with the results of Shoman et al. [2], Buse et al. [8], and Panigrahi et al. [35]. Although we did not confirm HER2 status in IHC 2^+ BC by CISH, it seems that the level of HER2 expression determined by IHC did influence the prognosis in BC patients: the higher the HER2 expression the worse the prognosis [36].

Our results showed that reduced/absent PTEN protein expression was associated with a shorter DFI and OS in postmenopausal SR^+ breast cancer patients treated with adjuvant tamoxifen only. Shoman et al. stated the same results related to DFI [2]. This was the only study which looked for a predictive role of PTEN expression in relation to response to antiestrogen therapy. Unlike these authors, we found that postmenopausal $PTEN^+/SR^+$ BC patients treated with adjuvant tamoxifen had significantly longer OS compared to $PTEN^-/SR^+$ BC patients. Such results could imply that a loss of PTEN expression directly or indirectly influences the effects of tamoxifen.

It is well known that estradiol has stimulating effects on breast epithelium. Tamoxifen as a partial ER antagonist, binds to ERα and causes inhibition of the estrogen-dependent activation of AF-2 region. The results of Campbell et al. on tumor cell lines showed that activated PI3K/Akt pathway leads to a hormone-independent activation of ERα and, as a result, inhibits tamoxifen-induced apoptosis [5]. In this way, these authors postulate a new mechanism of resistance to tamoxifen and at the same time a role of PTEN as a negative regulator of PI3K/Akt signaling pathway. If this hypothesis is confirmed in the future, standardization of immunohistochemical method of PTEN detection and standardization of interpretation of the obtained results will be necessary. The caveats of our study refer to the evaluated group which was completely SR^+ and mostly HER2 negative. So we were not able to test an association between $SR^-/HER2^+$ status and PTEN expression.

In conclusion, we found reduced/absent PTEN protein expression in one third of postmenopausal SR^+ BC patients. Also, our results might imply that reduced PTEN protein expression is a predictive factor for resistance to antiestrogen therapy. It is necessary to do further research in order to confirm the importance of PTEN/PI3K/Akt signaling pathway as a possible target in SR^+ breast carcinoma.

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