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ORIGINAL ARTICLE

The influence of PTEN protein expression on disease outcome in premenopausal hormone receptor-positive early breast cancer patients treated with adjuvant ovarian ablation: a long-term follow-up

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Summary

Purpose: *All breast cancer (BC) patients with detectable hormone receptors (HR) expression should be offered endocrine therapy (ET). In premenopausal patients, tamoxifen and/or ovarian suppression (OvS)/ablation (OA) may improve disease outcome. Alteration of phosphatase and tensin homolog (PTEN) signaling pathways could be one of the possible mechanisms of resistance to antiestrogen therapy. The purpose of this study was to investigate the association of PTEN protein expression with prognostic factors such as tumor histology and grade, estrogen receptor (ER) and progesterone receptor (PgR) status, human epidermal growth factor receptor 2 (HER2) and disease outcome in premenopausal patients with HR-positive early BCs treated with adjuvant OA.*

Methods: *We analyzed a group of premenopausal early stages I/II HR-positive BC patients who had undergone radical mastectomy followed only with adjuvant OA by irradiation. ER and PgR contents were determined by classical biochemical dextran-coated charcoal (DCC) method, HER2 status by chromogen in situ hybridization (CISH) analysis and PTEN status by immunohistochemistry (IHC).*

Results: *Sixty-six premenopausal patients included into this analysis were followed for a median of 17 years (range 1-29). Compared to PTEN-positive BCs, PTEN-negative BCs were significantly more frequently associated with lobular tumor histology ($p<0.05$), higher ER content ($p<0.05$), and had significantly decreased disease-free survival (DFS) and overall survival (OS) ($p<0.01$ for both) compared to patients with PTEN-positive BCs.*

Conclusions: *It seems that PTEN status determined by protein expression may discriminate between subgroups with poor and good prognosis in premenopausal HR-positive BC patients receiving adjuvant OA.*

Key words: *early breast cancer, hormone receptors, PTEN*

Introduction

Approximately 25% of BC patients are premenopausal at the time of diagnosis [1] and of these, 60% have HR-positive tumors [2]. According to recommendations, all patients with detectable ER expression, defined as ≥1% of invasive cancer cells, should be offered ET with or without chemotherapy (CT) and HER2-directed therapy, if indicated [3]. Tamoxifen for 5 years was historically a standard adjuvant ET for premenopausal patients and is still recommended for low-risk luminal/HER2-negative BCs [3]. For those with intermediate/high risk (intermediate/low ER/PgR expression, higher tumor grade, Ki 67 >15-20%, node-positive status, tumor size ≥5 cm) OvS added to tamoxifen or aromatase

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inhibitors (AIs) may improve the disease outcome [4-7] and extended tamoxifen up to 10 years from surgery may be an option in patients remaining premenopausal [3,8].

OA by surgical oophorectomy or ovarian irradiation was the first endocrine therapy introduced for premenopausal patients with BC [9-11]. Potentially reversible OvS using luteinizing hormone releasing hormone (LH-RH) agonists was introduced in early nineties and today is a preferred adjuvant estrogen deprivation therapy [12].

Although adjuvant ET reduces the relative risk of disease relapse in HR-positive BC patients by approximately 40% [13], recently published data [14] showed that the risk of developing distant metastases 5-20 years from diagnosis after stopping ET at 5 years is dependent predominantly on TN status at diagnosis. This analysis revealed that up to 40% of patients with T1-T2 BCs experienced disease relapse within 20 years from surgery. This means that resistance to antiestrogen therapy develops in some patients and the underlying molecular mechanisms to endocrine resistance are still being investigated.

Alteration of PTEN as part of phosphatidyl inositol 3 kinase (PI3K)/Akt signaling pathway could be one of the possible mechanisms causing resistance to antiestrogen therapy [15-17]. *PTEN* is one of the most frequently mutated human tumor suppressor genes. Its product is PTEN protein, with predominantly lipid phosphatase activity. PTEN protein is a negative regulator of PI3K/Akt signaling pathway by converting inositol triphosphate into inositol bisphosphate, thus inhibiting survival and proliferative pathways. [16,17]. Also, PTEN blocks cells in G1 phase of the cell cycle and enable apoptosis through AKT-dependent mechanism [15].

According to the results of previously reported studies there is an association between decreased PTEN expression in ER-positive BC and resistance to tamoxifen [16,18]. PTEN downregulation in BC was associated with high tumor grade, distant metastases and poorer DFS [16-18]. It was also shown that decreased PTEN and/or increased Akt activity in breast cancer cells were associated with resistance to tamoxifen-induced apoptosis. [16].

In our earlier analysis we found that PTEN loss had been associated with significantly decreased in both DFS and OS in postmenopausal patients with HR-positive BCs treated with adjuvant tamoxifen alone [18]. The purpose of this research was to examine if PTEN protein expression had an influence on disease outcome in HR-positive premenopausal BC patients treated with adjuvant OA only.

Methods

Patient selection

Our study included a group of premenopausal patients with HR-positive early breast cancers with node-negative grade 3 tumors, or 1-3 positive axillary lymph nodes of any tumor grade. Women were considered premenopausal if they had regular menstruation or had their normal last menstrual period within the last 12 months before surgery. All patients were diagnosed at the Institute for Oncology and Radiology of Serbia from 1988 to 1994 and, according to the current Protocol for the diagnosis and the treatment of breast cancer patients [19], all of them had undergone radical breast surgery followed with adjuvant OA only without postoperative RT. Ovarian ablation was done by ovarian irradiation with a total dose (TD) of 12 Gy in 4 fractions.

Study objective

The purpose of this study was to estimate the influence of PTEN protein expression in this group of patients on disease outcomes. Primary endpoints were: a) DFS, defined as the time from radical breast surgery to loco-regional recurrence and/or distant metastasis and/or contralateral breast cancer and/or primary tumor of other organ and/or death without disease relapse; b) breast cancer specific survival (BCSS), defined as the time from breast surgery to death from breast cancer; and c) OS, defined as the time from breast surgery to death from any cause.

Histology

All specimens were reviewed at the Department of Pathology, Institute for Oncology and Radiology of Serbia. Formalin-fixed, paraffin-embedded tissue samples were sectioned at 5μm thick sections and stained with hematoxylin-eosin. The histological type and grade were determined. For histological grading the Scarff-Bloom-Richardson scoring system was used: high (G1), medium (G2) and low (G3).

Immunohistochemistry for PTEN analysis

A manual immunohistochemical technique was used with primary monoclonal mouse anti-human PTEN clone (1:100, Clone6H2.1, Dako, Denmark) with EnVision+ system (HRP Labelled Polymer, K4000, Dako, Denmark) 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™+/HRPkit (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 immumoreactivity; 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). For the internal positive control, immunoreactivity of normal surrounding breast tissue (duct epithelium, myoepithelial cells, endothelium, fibrocytes and nerves) was used. PTEN status was defined as follows: positive if score ≥4, negative if score <4. Retesting of all histological slices (immunoreactivity of PTEN) was performed by two independent pathologists.

Steroid receptor analysis

ER and PgR contents of breast tumor cytosols were measured using the classical biochemical dextran-coated charcoal (DCC) method [20]. ER≥10 fmol/mg protein, and PgR≥20 fmol/mg protein were defined as positive status.

Analysis of c-erbB-2 gene amplification

The amplification of *c-erbB-2* gene was retrospectively determined from formalin-fixed and paraffin-embedded tissue blocks by CISH method. Zymed Laboratories Inc., USA, has produced a SpoT-Light HER-2 test, which consists of a dual-chain DNA labeled digoxigen. It specifically binds to the *c-erbB-2* gene locus on the 17q12-21 chromosome. The aim of the method is that it allows the detection of gene amplification by using a conventional peroxidase reaction under a light microscope of classically prepared tissue [21]. Interpretation of the results (according to the manufacturer's recommendations): amplification of *c-erbB-2* gene if there are >10 signals or large clusters in the nucleus in more than 50% of the cells; poor amplification if there are 6-9

Table 1. Patient, disease and therapy characteristics in the whole group and according PTEN categories

Characteristics	*n (%)*	*PTEN-positive*	*PTEN-negative*	*Fisher Exact test*
Age, years				ns*
Mean (SD)	44.56 (100)	44.68 (4.38)	44.26 (5.24)	
Median (range)	45 (35-54)	45 (35-52)	43 (36-54)	
Age, years				ns
≤45	37 (56.06)	27 (57.45)	10 (52.63)	
>45	29 (43.94)	20 (42.55)	9 (47.37)	
Clinical TNM stage				ns
II A	58 (87.88)	44 (93.62)	14 (73.68)	
II B	8 (12.12)	2 (6.38)	3 (26.32)	
Tumor histology				p<0.05
Ductal	36 (54.55)	21 (44.68)	8 (42.11)	
Lobular	29 (43.94)	16 (34.04)	11 (57.89)	
Other	1 (1.52)	10 (21.28)	0 (0)	
Tumor size (cm)				ns
0-2	3 (4.55)	26 (55.32)	10 (52.63)	
2-5	53 (80.30)	21 (44.68)	8 (42.11)	
> 5	10 (15.15)	0 (0)	1 (5.26)	
Tumor grade				ns
1	3 (4.55)	3 (6.38)	0 (0)	
2	53 (80.30)	37 (78.72)	16 (84.21)	
3	10 (15.15)	7 (14.89)	3 (15.79)	
Nodal status				ns
Negative	6 (9.09)	5 (10.64)	1 (5.26)	
Positive	60 (90.91)	42 (89.36)	18 (94.74)	
ER values (fmol/mg protein)				p<0.05*
Mean (SD)	32.44 (39)	25.89 (30.68)	48.63 (51.99)	
Median (range)	22 (0-199)	18 (0-183)	29 (8-199)	
PgR values (fmol/mg protein)				ns*
Mean (SD)	102.6 (104.52)	108.9 (11.95)	87 (84.05)	
Median (range)	65 (14-511)	70 (14-511)	59 (16-330)	
HER-2 status (CISH)				ns
Negative	56 (84.85)	39 (82.98)	17 (89.47)	
Positive	10 (15.15)	8 (17.02)	2 (10.53)	
Total	66 (100)	47 (100)	19 (100)	-

*Wilcoxon rank sum test, ns : not significant,CISH : chromogenic *in situ* hybridization, SD : standard deviation

signals or points of *c-erbB-2* gene present in more than 50% of the cells; without amplification if 1-5 signals or points of the *c-erbB-2* gene in the nucleus in >50% of the cells.

Statistics

For normal distribution data testing, the Kolmogorov-Smirnov and Shapiro-Wilk tests were used. Descriptive methods (frequencies, percent, mean, median, standard deviation and range) were used to summarize the data. The statistical significance level was set at $p<0.05$. For comparison of disease and treatment characteristics among different risk subgroups the Wilcoxon rank sum and Fisher exact tests were used. Survival was evaluated with Kaplan-Meier product-limit method. Median with corresponding 95% CI and log-rank test were used for DFS, BCSS and OS. The statistical analysis was done with the program R version 3.3.2 [22].

Results

Patient characteristics and PTEN expression

Characteristics of patients, disease and therapy are shown in Table 1. Sixty-six premenopausal HR-positive early BC patients with median age 45 years (range 35-54) were included into this analysis. Most of them were diagnosed with stage IIA (88%); also, more than 80% of patients had pT2 and grade 2 BCs and 90% had N1-3 status. All of them had ER-positive and/or PgR-positive tumors, while HER2-positive status was found in 15% of the patients.

According to PTEN status the patient group was divided into two subgroups: PTEN-positive [47/66 (71%)] and PTEN-negative [19/66 (29%)]. Compared to PTEN-positive BCs, PTEN-negative BCs were significantly more frequently associated with lobular tumor histology ($p<0.05$) and a higher ER content ($p<0.05$).

Disease outcome and survival analysis

After a median follow-up period of 17 years (range 1-29) disease relapse was diagnosed in 37/66 (56.1%) of the patients while 34/66 (51.5%) patients died and 2 of them died without BC relapse or primary tumors in other organs (Table 2). Bone lesions were the most frequent sites of distant metastases (localizations of BC relapse sites are shown in Figure 1).

Most of PTEN-negative patients (17/19) experienced disease relapse and all of them died of cancer. On the contrary, less than 50% of patients with PTEN-positive BCs experienced disease relapse and about one third died (Table 2). Disease relapse and mortality rates were significantly higher in the PTEN-negative subgroup in comparison to the PTEN-positive subgroup ($p<0.01$ for both; Table 2). Since almost all patients who died suffered from BC relapse there was only a slight difference between OS and BCSS (Table 3). Patients with PTEN-negative BCs had significantly decreased both DFS ($p<0.001$;Figure 2) and OS ($p<0.01$;Figure 3) compared to patients with PTEN-positive BCs.

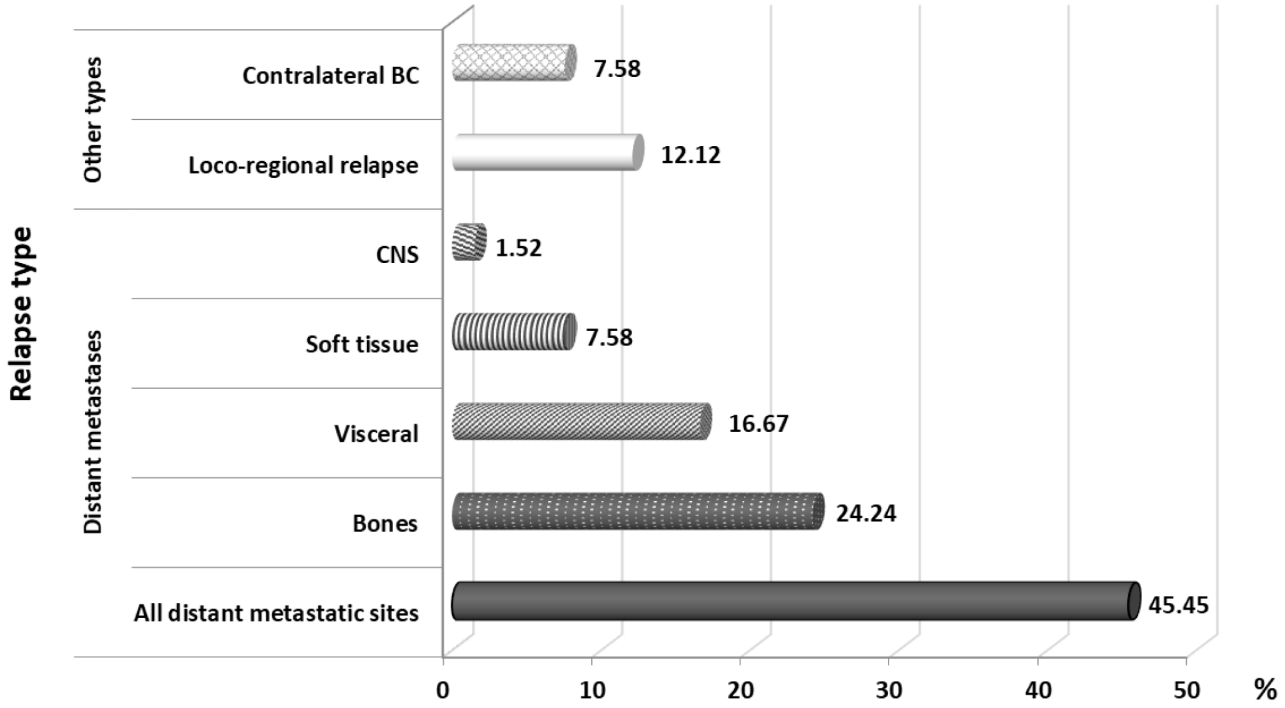

Figure 1. Disease relapse sites.

Table 2. Disease outcome and PTEN protein expression

Outcome and PTEN expression	*n (%)*	*PTEN-positive n (%)*	*PTEN-negative n (%)*	*Fisher exact test*
Relapse				p<0.01
No	29 (43.94)	27 (57.45)	2 (10.53)	
Yes	37 (56.06)	20 (42.55)	17 (89.47)	
Outcome				p<0.01
Alive	32 (48.48)	30 (63.83)	2 (10.53)	
Dead	34 (51.52)	17 (36.17)	17 (89.47)	

Table 3. Survival analysis for the patient whole group

	Number of events	Median (Years)	*95%CI*	*Log-rank, p*
DFS	29/66	15.6	10.5	< 0.01
BCSS	32/66	23	14.2	< 0.01
OS	34/66	23	14.2	< 0.01

DFS : disease-free survival, BCSS : breast cancer-specific survival, OS : overall survival, CI : confidence interval

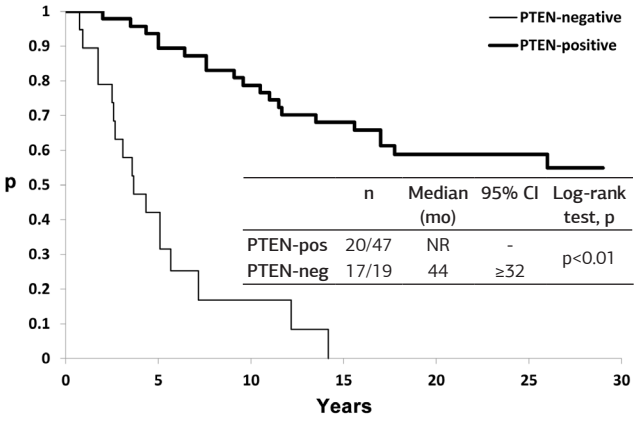

Figure 2. Disease-free survival of patients with PTEN-positive and PTEN-negative breast cancers.

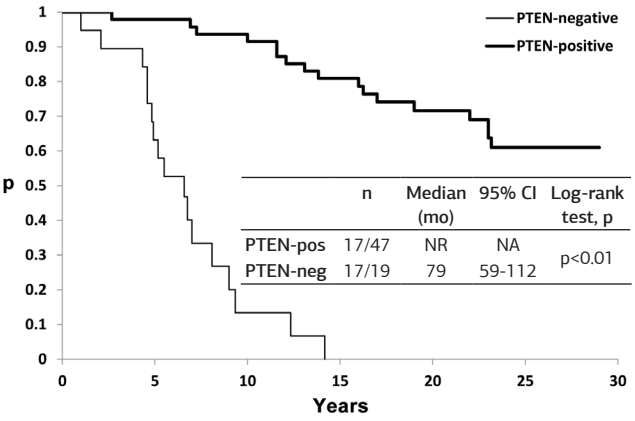

Figure 3. Overall suvrival of patients with PTEN-positive and PTEN-negative breast cancers.

Discussion

The first results on the efficacy of OA on disease outcome in BC patients were reported by the Early Breast Cancer Trialists' Collaborative Group meta-analysis [9]. It was a comprehensive investigation encompassing 13 trials of OA (ablation by surgery or irradiation) and 4 trials of OvS investigating the influence of estrogen deprivation therapy vs. no such treatment on disease outcome in 2102 women under 50 years of age. The results revealed that 15-year OS rate was highly improved among those allocated to OA (52.4 vs. 46.1%, log rank test, p=0.001), as was the recurrence-free survival (45.0 vs. 39.0%, log rank test, p=0.0007) [9].

Recently reported results from the joint analysis of SOFT and TEXT trials [6] showed that dual estrogen blockade by OvS plus aromatase inhibitor (exemestane) significantly reduced the BC recurrence rate compared to the combination of OvS and selective ER modulator (tamoxifen) (hazard ratio for recurrence 0.66, 95%CI 0.55-0.80, p<0.001) in premenopausal HR-positive early BC patients (44% of them had received also an adjuvant CT). SOFT study [7] showed that adding OvS for 5 years to tamoxifen did not significantly improve recurrence rate compared to tamoxifen alone. This study confirmed that younger premenopausal patients had high 5-year DFS even in the absence of adjuvant CT (5-year freedom from BC was 95.8% for tamoxifen alone, 95.1% for OvS+tamoxifen and 97.1 for OvS+exemestane). Of note is that most of these women were over 40 years of age (90%), node-negative (90%), pT1 (85%) and tumor grade 1/2 (90%). However, there was no difference in

5-year mortality rate between the therapy arms. TEXT [6] and SOFT [7] trials' results confirmed the data from ABCSCG-12 study where premenopausal early HR-positive BC patients were treated with OvS and tamoxifen or anastrozole with or without zoledronic acid [23]. The 5-year DFS rate for tamoxifen and anastrozole subgroups without zoledronic acid were 11.7 and 12.6%, respectively (not significant), even though they were largely untreated with adjuvant CT (>85%) and received adjuvant ET for 3 years. Differently from SOFT and TEXT study population, ABCSG-12 trial recruited 30% node-positive BC patients.

According to Early Breast Cancer Trialists Collaborative Group (EBCTCG) meta-analysis [24], 5 years of adjuvant tamoxifen reduced the risk of BC relapse and death from BC by 40%, independently from menopausal status, age or adjuvant chemotherapy. ATLAS study showed that 10-year of adjuvant tamoxifen therapy additionally decreased the absolute mortality from BC by 3% in patients with HR-positive BCs [8]. The aTTom study confirmed that 10 years of adjuvant tamoxifen in comparison with 5 years of adjuvant tamoxifen reduced the risk from BC relapse during years 7-9 [rate ratio (RR) 0.84 (95%CI 0.73-0.95)] and after year 10 [RR 0.75 (95%CI 0.66-0.86)], and risk of dying after year 10 (RR 0.86 (95%CI 0.75-0.97)] [25].

It is well known that more than half of recurrences in ER-positive early BC patients occur after more than 5 years from diagnosis and radical surgery. A recently reported EBCTCG analysis on 20-year risk of BC distant recurrence after stopping ET at 5 years showed that the cumulative incidence of BC recurrence is dependent on initial TN status [14]. Thus, during 15-year period from the end of adjuvant ET patients with T1N0 and T2N4-9 had cumulative incidence of BC distant recurrence of 13% and 41%, respectively. Tumor grade and Ki67 index had only moderate prognostic value for distant recurrence, while PgR and HER2 status were not predictive at all. In our analysis we noticed disease relapse in 56% of the patients, which is in accordance with EBCTCG analysis given that 85% of our patients had initially T1 and T2 BCs.

Although nowadays OA/OvS as only adjuvant ET is thought to be an insufficient adjuvant treatment in early BC, patients treated as ours are a valuable group suitable for the research on the influence of estrogen deprivation on disease outcome in various subtypes of BC. Our previous research on the influence of HER2 status on disease outcome in the same group of patients did not show that HER2-positive status was associated with higher recurrence rate [26]. However, among a subgroup of patients with disease relapse, patients with HER2-positive BCs, meaning triple-positive BCs, had a trend toward longer disease free interval [median 11 years (95%CI 1.8-17.8)] compared to patients without HER2-amplified BCs [median 4.5 years (95%CI 0.5-15.6)], Mann-Whitney Exact Test, p=0.049)].

Loss of PTEN protein expression in our study was recorded in 29% of premenopausal HR-positive BC patients. Several studies reported loss of PTEN in 28-40% of BC patients [17]. The difference in PTEN-negative status rate may possibly be caused by the difference in tissue preparation and methodology of PTEN determination, lack of standardization in PTEN status interpretation and small number of patients [18]. In our previous analysis done on postmenopausal patients with the same prognostic factors at diagnosis who received adjuvant tamoxifen for 5 years, with median follow up period of 9.5 years (range 1-19), the DFS and OS were significantly reduced in PTEN-negative vs. PTEN-positive BC patients (PTEN-negative BC patients had 6.5 fold increased risk for disease relapse and 3.5 fold increased risk for dying compared to PTEN-positive BC patients) [18].

Like in our current analysis of premenopausal patients, other authors found no associations between PTEN protein expression and tumor histology, size and grade in postmenopausal patients [18]. There was also no association between PTEN protein expression and HER2 status. Previously reported studies emphasized how important may be the determination of tumor PTEN expression since loss of PTEN expression may be a potential cause of resistance to trastuzumab [27-29]. Binding to HER2 receptor, trastuzumab stabilizes and activates the PTEN tumor suppressor and consequently down-regulates the PI3K/Akt signaling pathway. When the expression of PTEN is reduced or abrogated, this sequence of events is interrupted and the antitumor effects of trastuzumab are impaired [30,31]. Patients with HR-positive/HER2-positive (triple-positive) BCs in our analysis did not receive CT and trastuzumab, but only estrogen deprivation therapy. However, only 2/10 HER2-positive BC patients had PTEN-negative status and both of them died. On the contrary, 8/10 HER2-positive BCs were also PTEN-positive, 5 of them had disease relapse, while 4 patients died (data not shown). The efficacy of trastuzumab in triple-positive BC patients had been challenged in the Loi et al. report on the predictive factors for response to trastuzumab in these patients [32]. A secondary analysis of the HERA trial showed that significant effect of trastuzumab therapy was noticed in all patients except for ER-positive/HER2 low FISH ratio (≥2 to <5) subgroup. Of note, among 1720

triple-positive BC patients in the HERA study, 90% of them received adjuvant ET (17% premenopausal patients were treated with OA/OvS) [32]. The results of our analysis confirm that some triple-positive BC patients can achieve prolonged survival in the absence of adjuvant CT and HER2-directed therapy.

Activating alterations in the PI3K/Akt-mTOR signaling pathway (including PTEN as its critical negative regulator) are one of the most frequent genetic events in cancer and a major target for drug development (PI3K or mTOR inhibitors). Combination of PI3K inhibition with endocrine therapy has a synergistic effect *in vitro* and *in vivo* and might help overcome resistance to endocrine agents [33].

The caveats of our study are the small number of patients and its retrospective nature, although all patients had a prospective follow-up. However, the advantage of this analysis is that it included a highly selective, homogeneous group of patients according to prognostic factors (all HR-positive, node 0-3 positive patients, treated with adjuvant estrogen deprivation therapy without the influence of CT) treated according to current Protocols for the diagnosis and treatment of BCs [19]. Finally, the long term follow up indicates that half of initially premenopausal patients treated with adjuvant ET only are alive, and most of them are free from BC.

In conclusion, our results might imply that *PTEN* status determined with its protein expression may discriminate subgroups with poor and good prognosis in premenopausal HR-positive BC patients receiving adjuvant OA. Although its predictive value for response to CT is not confirmed, PTEN status might identify patients at higher risk for development of endocrine resistance thus being more suitable for adding adjuvant CT. Also, further research on potential therapeutic targets relating to PTEN/PI3K/Akt signaling pathway in luminal BCs is warranted.

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Conflict of interests

The authors declare no conflict of interests.

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