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ORIGINAL ARTICLE

PTEN, Akt, MAPK, p53 and p95 expression to predict trastuzumab resistance in HER2 positive breast cancer

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Summary

Purpose: Mutations that activate the PIK3CA oncogene and inhibit the tumor suppressor gene PTEN action are commonly found in breast tumors. Akt is a key activator of cell survival. p53 is frequently found mutated in human tumors, and mutant p53 protein actively contributes to tumorigenesis. In selected cases of breast cancer, trastuzumab (TZMB) is incorporated in the primary treatment in the adjuvant and metastatic settings. Many studies have reported that selected patients are resistant to TZMB due to the presence of p95 HER2 fragments. To address this, we analyzed PTEN, Akt, MAPK, p53 and p95 expression in breast cancer patients treated with TZMB.

Methods: Out of 90 patients histologically diagnosed with breast cancer between 2004 and 2011, analyzed were 25 patients with HER2 positive, and estrogen (ER) and progesterone receptors (PR) negative, metastatic or locally advanced disease. All 25 patients were treated with TZMB and resistance to TZMB was assessed. All patients were on anthracycline-and taxane-containing regimens. Tissue samples were obtained from paraffin blocks and evaluated immunohistochemically for PTEN, Akt, MAPK, p53, and p95 expression.

Results: TZMB resistance was detected in 5 (20%) patients. Akt expression was positive in 2 patients (8%) and MAPK, p95, and p53 expression was positive in 1 patient (4%); PTEN expression was negative in 3 patients (12%). No significant differences were found between TZMB resistance and PTEN, Akt, MAPK, p53, and p95 expression. Subgroup analysis was carried out in the neoadjuvant treatment group. Complete pathologic response was detected in 3 patients (21.4%). No statistically significant differences were found between the complete response rate and PTEN, Akt, MAPK, and p95 expression. There was a statistically significant correlation between p53 expression and complete pathologic response (p=0.02).

Conclusion: No statistically significant correlation between TZMB resistance and the expression of these biomarkers was noted. In patients with HER2-positive breast cancer that were treated with 4 dose-dense sequential cycles of doxorubicin and cyclophosphamide, followed by TZMB and paclitaxel combination therapy in the neodjuvant setting, p53 expression could predict complete response to chemotherapy.

Key words: Akt, breast cancer, MAPK, p53, p95, PTEN

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Introduction

TZMB is a humanized monoclonal antibody (mAb) directed against the extracellular domain of the tyrosine kinase receptor HER2. TZMB has shown clinical activity in HER2-overexpressing breast cancers (25–30% of human breast cancers) and is currently approved for patients whose tumors have this abnormality, in both the metastatic and the adjuvant settings [1,2]. Phase II and III trials in patients with metastatic disease showed that TZMB has clinical activity against HER2-positive breast cancer. As a single agent, TZMB produces overall response rates (complete plus partial responses) ranging from 15 to 30% in HER2 positive patients [2,3]. In combination with non-anthracycline conventional agents such as taxanes or vinorelbine, response rates range from 50 to 80% [2,4]. The two most common methods to assess HER2 status are immunohistochemistry and fluorescent *in situ* hybridisation (FISH) [5-7].

The mechanism of TZMB activity is not fully understood. Proposed mechanisms of TZMB activity and resistance to HER2 include inhibition of HER2 shedding and the phosphoinositide-3(OH) kinase (PI3K) -Akt pathway, attenuation of cell signalling, antigen-dependent cellular cytotoxicity, and inhibition of tumor angiogenesis [2]. The most important mechanisms of resistance are increased cell signalling due to phosphatase and tensin homolog (PTEN) loss or increased Akt activity, constitutive activation of the PI3K pathway, and expression of a truncated HER2 receptor called p95HER2 that lacks the extracellular domain needed for TZMB binding [8,9].

In this study we aimed to immunohistochemically analyse the biomarkers that could possibly affect the response to TZMB by determining the expression of PTEN, mitogen-activated protein kinase (MAPK), Akt/MAPK signaling pathway proteins, p95, and p53.

Methods

Patient selection

Patients with pathologically diagnosed breast cancer between 2004 and 2011 were studied. Ninety patients were assessed and, based on the inclusion and exclusion criteria (Table 1), 25 of them were finally selected who were HER2-positive, hormone receptor-negative, with metastatic or locally advanced disease. All of the patients were treated with TZMB along with anthracycline-and taxane-containing regimens. Gemcitabine, cisplatin, capecitabine and navelbine-containing regimens were given as second- and third-line therapies. Tissue samples were obtained from paraffin blocks and evaluated for PTEN, MAPK, Akt, p53 and p95 expression using immunohistochemical methods.

Table 1. Inclusion and exclusion criteria

Inclusion criteria	*Exclusion criteria*
HER2 3+ by IHC	HER 2 (-, 1+,2+) by IHC
ER and PR negative by IHC	ER/PR positive by IHC
Locally advanced (T3-4*,N2-3)	Early stage (T0-1-2/N0-1)
Metastatic stage	
Invasive ductal carcinoma	Other histologic subtypes (medullary, adenoid cystic etc.)
	Bilateral breast carcinoma.
	Pregnancy
	Male breast cancer
	Discontinuation of therapy (cardiotoxicity etc.)
	Not enough pathologic specimen for IHC

IHC:immunohistochemistry, ER: estrogen receptor, PR: progesterone receptor
*Except T4d (inflammatory carcinoma)

Hormone receptors and HER2 determination

ER, PR and HER2 expression was determined by immunohistochemistry. Specifically, for HER2 expression the HERCEP test™ was used and a score of 3+ was accepted as positive

PTEN, MAPK, Akt, p53, and p95 assessment

Tumor tissue was fixed in 10% formaldahyde and 5 μm sections were stained with hematoxylin and eosin. Staining was assessed with light microscopy. Histological cross sections were taken to special slides with polylysin for immunohistochemical staining. Immunohistochemistry was carried out using the avidin-biotin peroxidase complex method (DAKO, North America Inc., Biotinylated Link Universal), and streptavidin horseradish peroxidase (HRP) (rabbit, mouse, goat; K 0690). The antibodies used were, PTEN (mouse pri-

mary monoclonal antibody, Biogenex 28H6, USA), MAPK mouse monoclonal primary antibody (NCL – MKK4, Leica Biosystems, United Kingdom), Akt (phosphorylated) mouse monoclonal antibody (NCL -L -Akt Phos, Leica Biosystems, United Kingdom), p53 mouse primary monoclonal antibody (NCL-L-p53-D07, Leica Biosystems, United Kingdom), and Anti-p95 NBS1 antibody [Y112] – ChIP Grade (ab 32074) (ABCAM, USA).

Paraffin-embedded sections 5 μm in diameter were incubated at 60°C for 30-45 min and washed for 10 min in xylol. Paraffin was removed after a 5-min incubation in 95% alcohol, and 3% H_2O_2 was used for blocking endogenous peroxidase activity. Slides were placed in a microwave oven (Beko, 1550 model, Turkey), incubated for 7 min, and washed for 3-5 min in TRIS-buffered saline (TBS) at pH 7.2-7.4. Primary antibody was applied as follows: PTEN (1/20), MKK4 (1/30), p53 (1/80), alkaline phosphatase (1/50), and p95 NBS1 (1/80). These were incubated for 2 h and washed in TBS for 3-5 min. Secondary antibody was applied and incubated for 20 min at room temperature. HRP-streptavidin was applied on slides and incubated for 30 min. Slides were then washed in TBS for 3-5 min. Aminoethylcarbazol (AEC) chromogen was applied and slides were stained with Mayer hematoxylin for 1-3 min, washed for 3-5 min, and were coverslipped in a water-based closure material (DAKO, North America Inc., USA).

Immunohistochemical evaluation

Preparations were evaluated by two independent pathologists in the Department of Pathology of Cukurova University. The investigators were not informed about the clinical and demographic characteristics of the patients. All of the samples were evaluated by the same pathologists. The slides stained with PTEN, MAPK, Akt, p53 and p95 antibodies were examined using a light microscope at a magnification of ×400. No staining or very low staining was considered negative and moderate or intense staining was considered positive.

Statistics

The SPSS 18.0 package program was used for the statistical analysis of data. Categorical measurements were summarised as numbers and percentages, whereas numeric measurements were given as average and standard deviation (SD). The x^2 test was performed to compare categorical measurements between different groups. Log-rank test under Kaplan-Meier analysis was performed to evaluate the correlation between survival and expression of PTEN, MAPK, Akt, p53 and p95. The level of statistical significance was put at 0.05 for all tests.

Results

The mean age of the 25 patients was 54±12 years. On entering the therapeutic protocol 14 patients (56%) had locally advanced and 11 (44%) had metastatic disease (Table 2). Modified radical mastectomy and adjuvant radiation therapy were applied to patients with locally advanced stage. Twelve patients (48%) were premenapausal and 13 (52%) postmenauposal. All of the patients were treated with TZMB-containing combination regimens: dose-dense doxorubicin (60 mg/m^2) plus cyclophosphamide (600 mg/m^2) every 2 weeks for 4 cycles and paclitaxel (175 mg/m^2) every 2 weeks for 4 cycles, given as neoadjuvant therapy in the locally advanced group. TZMB was given in combination with paclitaxel.TZMB first loading dose was 4mg/kg, followed by 2 mg/kg every week for 9 weeks and then 6mg/kg every 3 weeks for 52 weeks in the locally advanced group and until progression in the metastatic group.

Tumor paraffin-embedded specimens showed that all of the patients were HER2 3(+) immunohistochemically.

TZMB resistance was detected in 5 (20%) patients.

Table 2. Patient and disease characteristics (N=25)

Characteristics	
Mean age, years±SD	54±12
Stage	Locally advanced, N=14 (56%) (noninflammatory)
	Metastatic, N=11 (44%)
Grade	II (N=15; 60%)
	III (N=10; 40%)
Trastuzumab resistance*	5 patients (20%)
	2 patients (8%) in the locally advanced group
	3 patients (12%) in the metastatic group

* Resistance to trastuzumab was defined as development of resistant disease within one year of treatment initiation

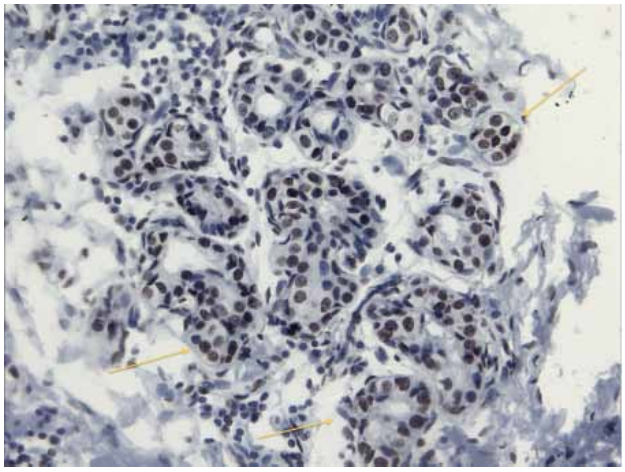

Figure 1. PTEN expression (light microscope, ×200).

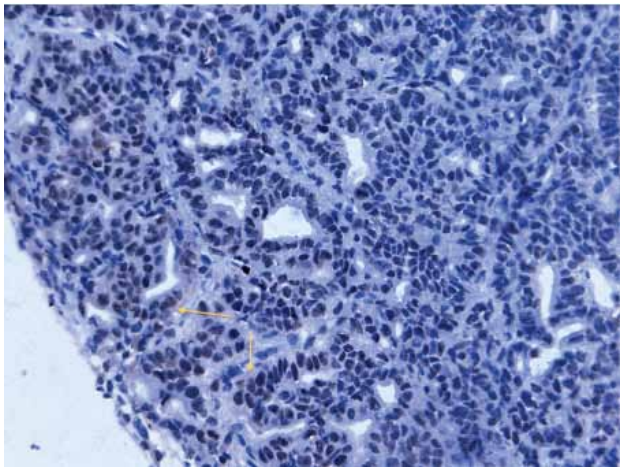

Figure 2. Akt expression (light microscope, ×200).

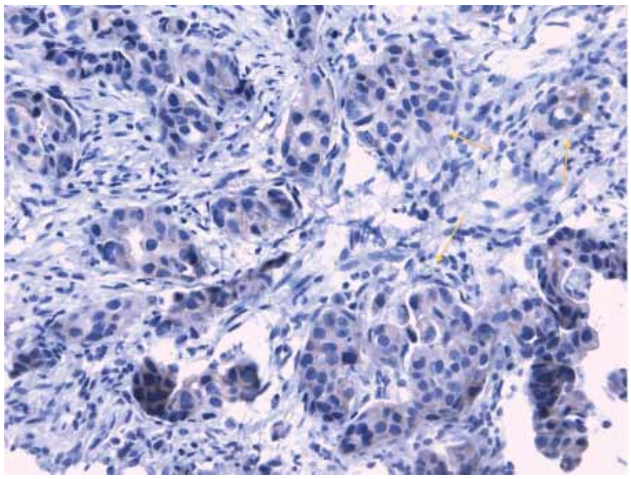

Figure 3. MAPK expression (light microscope, ×400).

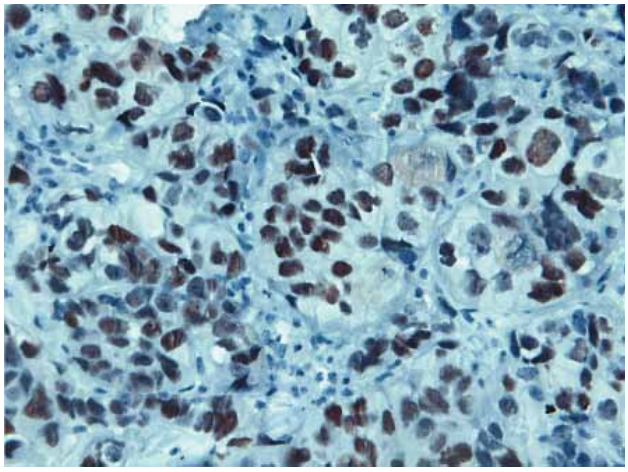

Figure 4. p53 expression (light microscope, ×400).

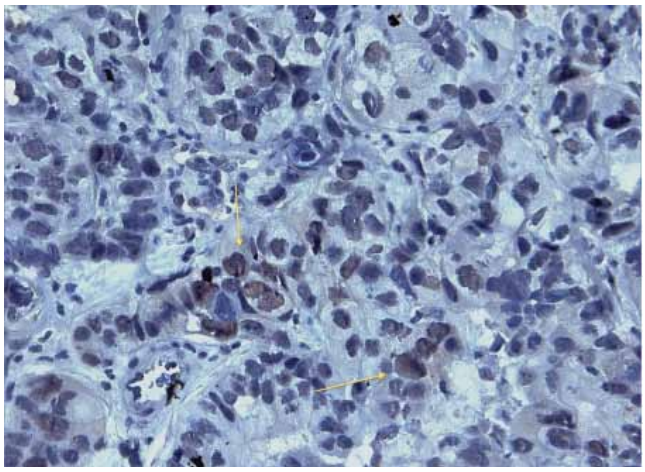

Figure 5. p95 expression (light microscope,×400).

Akt expression was positive in 2 (8%) patients, while MAPK, p95, and p53 expression were positive in 1 patient (4%) each. In contrast, PTEN expression was negative in 3 (12%) patients . PTEN, Akt, MAPK, p53 and p95 expression varied in different tumor tissue samples (Figures 1-5). All biomarker expressions (negative/positive) were compared to each other and no statistically significant correlation was found between expression rates of PTEN, Akt, MAPK, p53, p95 on tumor tissue samples (p=0.378, 0.694, 0.564, 0.301 and 0.475, respectively). Also, no significant differences were found between TZMB resistance and PTEN, Akt, MAPK, p53, and p95 expression (p=0.11, 0.79, 0.95, 0.88, and 0.74, respectively; Table 3).

Subgroup analysis was performed in the metastatic and neoadjuvant treatment groups that were treated

Table 3. TZMB resistance and PTEN, Akt, MAPK, p53, p95 expression

		TZMB resistance		*p-value*
		Yes	*No*	
		N (%)	*N (%)*	
PTEN	Positive	2 (8)	13 (52)	0.11
	Negative	3 (12)	7 (28)	
Akt	Positive	3 (12)	11 (44)	0.79
	Negative	2 (8)	9 (36)	
MAPK	Positive	2 (8)	4 (16)	0.95
	Negative	16 (64)	3 (12)	
p53	Positive	2 (8)	6 (24)	0.88
	Negative	3 (12)	14 (56)	
p95	Positive	1 (4)	4 (16)	0.74
	Negative	6 (24)	14 (56)	

TZMB: Trastuzumab

with TZMB-containing regimens. Pathological complete responses were detected in 3 (21.4%) patients in the neoadjuvant group of patients. No significant differences were found between complete response rates and PTEN, Akt, MAPK, and p95 expression (p=0.275, 0.154, 0.452, and 0.542, respectively). There was a significant positive correlation between p53 expression and complete pathologic response (p=0.02). p53 expression was high in the group of patients treated with a TZMB combination in the neoadjuvant setting.

Discussion

TZMB has significantly improved disease-free and overall survival when combined with chemotherapy in patients with breast cancers treated in both adjuvant and metastatic settings [2,12-16]. Additive cytotoxic interactions between TZMB and other agents, including paclitaxel, have been shown in clinical practice [17]. However, a subset of patients who initially received TZMB as adjuvant therapy eventually developed recurrent, HER2 resistant tumors within 1 year of treatment [18]. The emergence of refractory tumors has become a significant clinical problem as evidenced by the numerous preclinical studies investigating possible etiologies for this resistance, such as HER2 downstream signaling, crosstalk pathways, and HER2 gene mutations [19].

Resistance to TZMB therapy occurs in both de novo and acquired forms. The de novo resistance results from genetic changes in tyrosine kinases receptors and their downstream cellular pathways. The main genetic changes in this case include deficient PTEN [12] or mutated PIK3CA genes [13] that cause constitutive activation of the PI3K pathway and expression of a truncated HER2 receptor (called p95HER2) that lacks the extracellular domain needed for TZMB binding [8].

HER2 expression in tumor tissue is a guide for TZMB treatment. We aimed to investigate which biomarkers in tumor tissue can predict resistance to TZMB therapy. Like HER2 overexpression, PTEN loss and PI3K signaling pathways are important factors for resistance, while p95 and p53 expression could also predict resistance.

Preclinical studies indicate synergistic antitumor activity when TZMB is combined with a number of anticancer drugs, but also additive cytotoxic interactions between TZMB and other agents, including paclitaxel, have been shown [17]. Mutated or downregulated PTEN leads to its loss of function, a phenomenon described in nearly 50% of breast cancer cases [20]. Because PTEN has inhibitory effects on PI3K, loss of PTEN function constitutively maintains the activity of the PI3K/Akt pathway [12] that inhibits cell cycle arrest and apoptosis mediated by TZMB. Patients with breast cancer lacking PTEN expression but overexpressing HER2 respond more poorly to TZMB therapy than patients with normal PTEN expression [20]. In our study, we detected PTEN in tumor tissue that overexpressed HER2, but there was no correlation between TZMB resistance and PTEN loss. This result indicates that PTEN is not predictive of the TZMB response like HER2. Therefore, PTEN loss could provide another indication for TZMB resistance. In fact, in patients who had poor response to TZMB therapy but high-level PTEN expression and inhibition of PI3K/Akt pathway, it is possible that another therapeutic opportunity exists. This theory is supported by the positive response to TZMB and lapatinib in patients with PTEN loss and PI3K activation [21].

We previously outlined the importance of PTEN loss in the constitutive activation of the PI3K/Akt pathway that leads to TZMB resistance. This pathway can also be activated via PI3K mutations. PIK3CA encodes the catalytic subunit of PI3K which is a gene frequently mutated in breast cancer and promotes TZMB insensitivity

in breast cancer cells *in vitro* [21,22]. *In vivo* studies also emphasise the anti-TZMB role played by mutated versions of PIK3CA when it is applied in situations where PTEN expression is reduced [12]. In parallel, HER2-amplified breast cancer cell lines that contain PIK3CA hotspot mutations are more resistant to TZMB than mutant-free cells [23]. Therefore, the PI3K/Akt pathway provides another landmark for TZMB efficacy [24]. From this signaling pathway we analysed MAPK, Akt, PTEN, p95 and p53 expression using immunohistochemistry

Gallardo et al. reported their immunohistochemistry results for HER2, ER/PR, epidermal growth factor 1-receptor (EGFR), α-insulin-like growth factor 1-receptor (IGF1R), PTEN, p110α, phosphorylated Akt (pAkt), pBad, pmTOR, pMAPK, MUC1, Ki67, p53 and p27 in 155 breast cancers and mutation analysis of PIK3CA and PTEN, and PTEN promoter hypermethylation. Their results support the hypothesis that mechanisms of TZMB resistance are related to deregulation of the PTEN/PI3K/Akt/mTOR pathway, and/or EGFR and IGF1R overexpression in a subset of HER2-positive breast carcinomas [25].

The loss of PTEN expression and pAkt is thought to be involved in the mechanism leading toTZMB resistance in patients with HER2-positive breast cancer. The present study included 14 patients with breast cancer who received TZMB-containing neoadjuvant chemotherapy Three patients achieved complete pathological response. The intensity of staining was evaluated for each biomarker, and the correlation between the immunohistochemical profile and the clinical outcome were analysed. The changes in the immunohistochemical profiles between specimens obtained before and after TZMB-containing neoadjuvant chemotherapy were evaluated for the patients with residual tumors. PTEN was positive in 14% of the patients (n=6/44) and pAkt in 80% (n=35/44). The expression of both PTEN and pAkt were not correlated with complete pathological response. Persistent HER2 overexpression after neoadjuvant chemotherapy was significantly associated with recurrence. Among the 27 patients with residual cancer, the percentage of patients with HER2-positive or pAkt-positive tumors was low, but in those with PTEN expression was high. The present study suggested that neither the immunohistochemical expression of PTEN nor the expression of pAkt was associated with the clinical outcome of TZMB-containing neoadjuvant chemotherapy. Except among patients with complete pathological remission, the persistent overexpression of HER2 may be a poor prognostic factor [26].

The most significant finding of our study was the correlation between p53 expression and complete pathological response in patients receiving a TZMB-containing therapy, as described in methods. Kandioler-Eckersberger et al. reported that the clinical response to fluorouracil, epirubicin, and cyclophosphamide is dependent on the wild type p53 and the cytotoxicity of paclitaxel is related to mutated p53. The efficiency of paclitaxel during mitosis might be supported by the lack of G1 arrest due to p53 defective function. Based on the results of that study, patients with p53-deficient tumors may benefit from paclitaxel treatment (p=0.011) [27]. Similar results were found in our study in the group of patients whose tumors were HER2 3(+) by immunohistochemistry. Arribas et al. reported that the expression of p95HER2 is predictive for poor prognosis and correlates with TZMB resistance [28], but in our study we did not find such a correlation.

In conclusion, although HER2 expression in breast cancer is an important predictive factor for treating patients with TZMB, some patients do not respond to this treatment. This study focused on determining the reason for resistance. Immunohistochemistry was used because it is an easy and accessible test available to clinicians. We found that although HER2 was predictive, PTEN, pAkt, MAPK, p53 and p95 could not be used for prediction of TZMB resistance. In contrast, p53 expression can predict complete response to TZMB-containing neoadjuvant chemotherapy in HER2-positive breast cancer patients.

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