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pSTAT3 expression associated with survival and mammographic density of breast cancer patients

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ABSTRACT

Background: Constitutive activation of STAT3 have been shown in several tumor types including breast cancer. We investigate STAT3 expresion as possible molecular marker for breast cancer early detection, as well as prognostic factor for determination of tumor agressiveness.
Methods: In this study we measure p(Y705)STAT3 expression in tumor and adjacent tissue of breast cancer patients by Western blot. For relapse-free survival (RFS) and overall survival (OS) we used Log-Rank test.
Results: We show that average expression of p (Y705) STAT3 in tumor tissue is higher compared to adjacent tissue. Moreover, we found that patients with HER2 positive receptors had significantly higher pSTAT3 expression compared to HER2 negative patients. We showed that patients with high mammographic density had significantly higher tumor expression of pSTAT3 compared to patients with low mammographic density. Also, we show that pSTAT3 expression correlates with longer RFS in the entire group of patients, as well as in the group of ER positive, in lymph node positive and in older group of breast cancer patients (with age over 50). Furthermore, in the entire group of patients, in ER positive, in lymph node positive and in older group of patient, high expression of pSTAT3 showed a better survival than low expression of pSTAT3.
Conclusion: Considering that the expression of pSTAT3 is associated with longer RFS and survival, it can be used as prognostic tools for determination of group of breast cancer patients with low-risk.

1. Introduction

Recently it has been shown that Signal Transducer and Activators of Transcription (STATs) had an important role in tumor carcinogenesis and that they considered as potential oncogenes. Constitutive activation of STAT3 has been shown in several tumor tipes including breast cancer [1]. It has been shown that STAT3 is protein which activate *c-myc*, cyclin D and Bcl-2, facilitating cells to pass through the critical restriction point and therefore enhances breast cancer cell proliferation [2].

Constitutive activation of STAT3 leads to proliferation of tumor cells and prevents apoptosis, down-regulates the production of numerous proinflamatory cytokines and chemokines and leads to secretion of factors that prevent dendritic cell (DC) maturation that suppresses adaptive antitumor immunity. It is known that invasive tumors need to modulate gene expression in a manner that impairs the activity of innate and adaptive immunity in immune surveillance [3,4]. STAT3 positive tumors achieve this by preventing the production of proinflamatory cytokines, i.e. “danger signals”. Activation of the transcription factor STAT3 in the tumor and adjacent immune cells, as well as, normal epithelial cells, lead to production of cytokines IL-1β, IL-6, IL-10, IL-17, as well as VEGF creating a feedback loop that promotes tumor growth, angiogenesis, evasion of immune surveillance and metastasis [5,6]. It is of importance that activation of STAT3 within tumors is heterogeneous and it has been found that pSTAT3 is highest on the leading edge of tumors and that this is associated with stromal, immune, and endothelial cells. This follows from IL-6 from cancer-associated fibroblasts or myeloid cells that in a feedback loop induces autocrine production of IL-6 and pSTAT3 expression in tumor cells, thus also leading to heterogeneous levels of pSTAT3 [6]. Therefore,

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; RFS, relapse free survival; OS, overall survival

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considering heterogeneous tumor expression of pSTAT3, it is of interest to determine tumor pSTAT3 expression and use it as possible tumor marker for early detection, as well as prognostic marker.

It has been shown that HER2 induce via Src kinases migration of breast cancer cells [7]. Also, activation of STAT3 pathway is shown in ER positive breast cancer lines [8]. Therefore, we investigated association of pathologic parameters and pSTAT3 tumor expression. Recently it has been published in a few studies regarding STAT3 as prognostic factor [9–11], although with controversial results.

It has been shown that amount of mammographic density represent an independent predictive factor of breast cancer risk [12]. The biologic basis for increased risk for breast cancer associated with increased mammographic densities is not understood, and the detailed nature of densities in cancer tissue has not been studied extensively [13–15]. Also, the association between molecular markers in tumor tissue and calcifications on mammograms has not been investigated yet. For this reason, we investigated association of mammographic features and pSTAT3 tumor expression.

Now new aspects of treatment are introduced based on the molecular profile of the tumor, the treatments of various tumors introduce agents that directly or indirectly block the activity of STAT3. Moreover, we investigate STAT3 expresion as possible molecular marker for breast cancer early detection, as well as prognostic factor for determination of tumor agressiveness.

2. Materials and METODS

2.1. Patients

In this study we examine the expression of p (Y705) STAT3 in the tumor and adjacent tissue samples of 80 breast cancer patients (clinical stage I, II and III) by Western blot analysis. The study was carried out after fulfilling all required ethical standards and tumors tissue were investigated according to the ethical standards, with informed consent of patients at the Institute of Oncology and Radiology of Serbia.

2.2. Tissue samples

Malignant and adjacent breast tissues (~100 mg) were quickly weighed and homogenized on ice. Homogenized tissue samples were treated with 200 µl of lysing buffer containing 20 mM Tris-HCl buffer, pH 7.5, 150 mM NaCL, 5 mM EDTA, 1% TRITON X-100, 0.5% for 1 h on 4 °C. After centrifugation at 10,000 rpm for 10 min at 4 °C, the obtained supernatant fluid presents the total cell lysate. The total protein concentrations were measured by Bradford assay that has been adapted to microplates [16]. Tissue lysates equivalent to 50 µg protein were mixed with equal volumes of sample buffer (4% SDS, 20% Glycerol, 0.004% Bromophenol, 0.125 M Tris HCL) and kept at room temperature for 30 min.

2.3. Western blot analysis

Expression of p (Y705) STAT3 was determined according to the method of Horr B et al [17]. Induction of pSTAT3 expression was assessed in cellular lysates by Western blotting using anti-pSTAT3 (BD Biosciences, USA) antibodies. The expression of β actin was used for endogenous control for western blot reaction and immunochistohemistry. The Expression of pSTAT3 in tumor tissue was confirmed by immunochistohemistry.

2.4. Quantification of p (Y705) STAT3 expression

Following Western Blotting, the band of blots was quantified using a scanner equipped with a transparency option interfaced to an IBM PC. Blots were scanned by using image system (Kodak Image 1D 3.6.), in a grey scale mode at 169 mm pixel size and 1250–1650 (X–Y) pixel count, using the autodensity feature on a scale ranging from 0 (clear) to 255 (opaque). The pixel density was used to calculate the integrated density of a selected band. Values of integrated density were reported in volume units of pixel intensity per mm [2]. The integrated density of each band is reported as the mean of three different measurements of the same blot for each sample examined in triplicate.

2.5. Pathological assessment of primary tumors

This study included 80 breast cancer patients ranged from 38 to 82 years (median 59 years) who underwent surgery as primary treatment. According to the TNM classification of the UICC, tumor size (T) was classified by the pathologist after surgery as T1, T2 or T3. The presence of regional lymph node involvement (N) was assessed histologically as No (lymph node negative) or N_+ (lymph node positive). The presence of distant metastases (M) was excluded by clinical, X-ray and ultrasound examination in all cases (Mo). Typing of primary tumors was performed according to the WHO classification, while for grading the Ellis and Elston system was used. Immunostaining was performed on formalin-fixed paraffin-embedded 4 µm tissue sections using the primary mouse monoclonal antibodies for oestrogen receptor (ER), progesterone receptor (PR), HER2 receptor, Citokeratins 5/6 (CK5/6) and epidermal growth factor receptor (EGFR/ HER1) and the primary rabbit monoclonal antibody for Phosho- (Tyr705)Stat3 (Cell Signaling Techology), respectively. Staining was visualized using the Envision method (Dakocytomation, Copenhagen, Denmark) and DAB. For assessment of ER, PR, HER2, CK5/6 and EGFR staining the Allred score was used. Samples were considered hormone receptor negative when staining of both steroid receptors was negative and hormone receptor positive when positive staining for one of the receptors was observed. The DAKO-HercepTest scoring system was used to evaluate the HER2 staining. Samples with a score of 0 or 1 were defined as negative and samples with a score of 2+ or 3+ were defined as positive or strongly positive, respectively [18].

2.6. Image analysis

Each image was read by two radiologists: both radiologists who read an image had to agree on an interpretation before the results were recorded. Mammograms were evaluated according to the American College of Radiology Breast Imaging Reporting and Data system (BIRADS) [19]. These mammograms were classified according to the extent of density in the entire image by four categories (ACR1 [25%], ACR2 [50%], ACR3 [75%], and ACR4 [100%]) [20]. Calcifications were described in morphologic term as pleomorphic, branching, fine linear, amorphous, and punctate. Grouped (clustered, 10 per cm2) pleomorphic, branching, fine linear, and punctate calcification were considered as malignant.

2.7. Statistical analysis

We measured expression of p (705) STAT3 in tumor and adjacent tissue and data obtained were analyzed by the parametric Student T test. The expression of pSTAT3 and clinicopathological features was analyzed using parametric Student T test. 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 $p < 0.05$. Survival as evaluated with Kaplan-Meier product-limit method. Median with corresponding 95% CI and log-rank test were used for relapse-free survival (RFS) and overall survival (OS). Reported P values were not corrected for multiple testing. Analyses were performed with Statistical Package for Social Sciences, Version 11.5 (SPSS, Inc., Chicago, IL, USA).

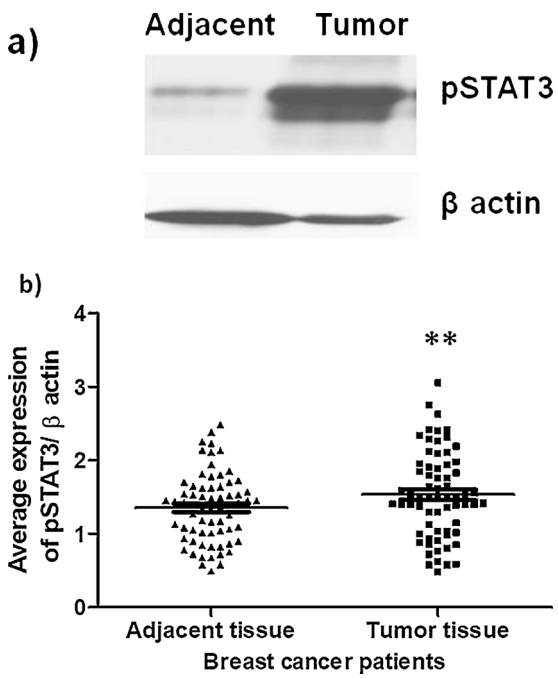

Fig. 1. (a) Representative blots showing p(Y705)STAT3 expression in tumor and adjacent tissue and expression of β actin as control. (b) The mean values with standard deviation (mean ± SD) of p(Y705)STAT3 expression show significant increase of p(Y705)STAT3 in tumor tissue compared to adjacent tissue. Values of integrated density were reported in volume units of pixel intensity per mm2.

3. Results

3.1. pSTAT3 expression and clinico-pathological characteristics

We show that expression of phospho(705)STAT3 detected by Western blotting was found in 67 of 80 investigated patients with breast carcinomas. Furthermore, we show the expression of pSTAT3 in tumor and adjacent tissue of breast cancer patients and expression of β actin as control used in this study (Fig. 1a). Each measurement of pSTAT3 expression was conferred with expression of β actin. We confirmed that expression of phospho(705)STAT3 detected by immunochistochemistry was found in 63 of 80 investigated breast cancer patients (Fig. 2a). Average expression of p(Y705)STAT3 in breast cancer patients show that pSTAT3 had more intense band in tumor tissue when compared to adjacent normal tissue ($p = 0.03$, Student T test) (Fig. 1A, B).

We found no significant difference in pSTAT3 expression between patients who are over 50 years old than patients with breast cancers who have less than 50 years (0.94 ± 0.26 vs. 1.21 ± 0.36, $p = 0.09$, Student T test) (Table 1.) Also there was no significant difference in pSTAT3 expression between patients with carcinoma ductale and carcinoma lobulare (1.24 ± 0.37 vs. 1.13 ± 0.4), $p = 0.42$, Student T test). More detailed analyze show that there was no association between p(Y705)STAT3 tumor expression and tumor size (1.35 ± 0.23 vs. 1.21 ± 0.41 vs. 1.13 ± 0.39, $p = 0.51$, Student T test), as well as between lymph node positive (LN+) and lymph node negative breast cancer patients (LN_+ vs. LN_0 1.19 ± 0.40 vs. 1.21 ± 0.38, $p = 0.60$, Student T test) (Table 1.).

3.2. pSTAT3 expression in tumor tissue with respect to receptor status

We analyzed pSTAT3 expression with respect to expression of ER, PR and HER2 expression in tumor tissue samples. There is no difference in pSTAT3 tumor expression in breast cancer patients with ER + compared to patients with ER- (1.39 ± 0.15 vs. 1.16 ± 0.17, $p = 0.235$, Student T test) (Fig. 2D). However, we found significant difference in pSTAT3 expression between HER2+ (+2, +3) and HER2- (0, +1) patients (2.0 ± 0.12 vs. 1.49 ± 0.07, $p = 0.034$, Student T test) (Table 1.). Moreover, in this study we show that patients with

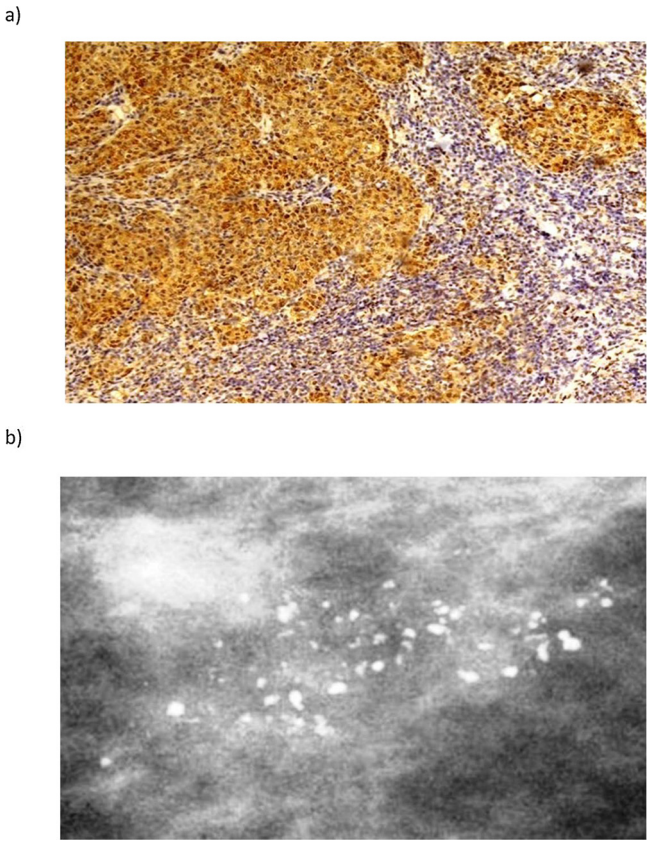

Fig. 2. (a) A mammogram of a 48-year-old woman with high grade, ductal carcinoma exhibits cluster of punctate microcalcifications, characterize as BIRADS 5. (b) Photomicrograph of tumor sample of breast cancer patient displaying a ductal carcinoma (grade 2) with marked nuclear and cytoplasmic staining of p(TYR705)STAT3 (figure is at 100 magnification).

Table 1
Patients characteristics.

Characteristics	Patients n(%)	p(Y705)STAT3	p
Age			
< 50	23(28.75%)	0.94 ± 0.26	
> 50	57(71.25%)	1.21 ± 0.36	0.09
Tumor type			
Ca ductale invasivum	46 (57.5%)	1.24 ± 0.37	
Ca lobulare invasivum	34 (42.5%)	1.13 ± 0.40	0.42
Primary tumor size			
T1	28 (35%)	1.13 ± 0.39	
T2	38 (47.5%)	1.21 ± 0.41	
T3	15 (18.5%)	1.35 ± 0.23	0.51
Lymph node status			
Negative	45 (56.25%)	1.21 ± 0.38	
Positive	35 (43.75%)	1.19 ± 0.4	0.6
HER2 status			
Negative	69 (86.25%)	1.49 ± 0.07	
Positive	11 (13.75%)	2.0 ± 0.12	0.034
ER status			
Negative	13 (16.25%)	1.16 ± 0.17	
Positive	67 (83.75%)	1.39 ± 0.15	0.235
Molecular classification			
Basal-like	9 (11.25%)	1.15 ± 0.39	
HER2+ subtype	4 (5%)	1.76 ± 0.33	
Luminal A	60 (75%)	1.56 ± 0.17	
Luminal B	7 (8.75%)	1.31 ± 0.35	0.036

Table 2
Mammographic features stratified according to pSTAT3 expression.

Characteristics	Patients n(%)	p(Y705)STAT3	*p*
Mammographic densities			
ACR1/ACR2(25-50%)	60 (78.67%)	1.05 ± 0.09	
ACR3/ACR4(25-50%)	20 (21.33%)	1.27 ± 0.06	0.042
Calcifications			
No	62 (77.5%)	1.18 ± 0.06	
Yes	18 (22.5%)	1.27 ± 0.10	0.405

basal-like cancers had significantly lower pSTAT3 expression (1.56 ± 0.33 vs. 1.17 ± 0.37, p = 0.036, Student T test) compared to patients with luminal A tumors (Table 1.).

3.3. Mammographic features

Mammograms of 80 patients with patohistologically confirmed cancer according to American College of Radiology (ACR) showed 60 patients with ACR1 or ACR2 density and 20 patients with ACR3 and ACR4 mammographic density. Mammograms of patients showed 18 patients with grouped calcifications with or without masses and specifically 9 patients had pleomorphic calcifications, 3 patients had punctate calcification (Table 2.), 3 patients had branching calcifications and 3 patients had fine linear type of calcification. In Fig. 2b we show a mammogram of a 48-year-old with high grade ductal carcinoma exhibits cluster of punctate microcalcifications characterize as BIRADS 5. In Fig. 2a we show a photomicrograph of tumor sample of same patients with hormone receptor positive ductal carcinoma (grade 2) with marked nuclear and cytoplasmatic expression of p(TYR705)STAT3.

In the entire group of patients more detailed analyze showed that patients with high mammographic density had significantly higher expression of pSTAT3 in their tumor tissue compared to tumor expression in patients with low mammographic density (1.27 ± 0.06 vs. 1.05 ± 0.09, p = 0.042, Student T test) (Table 2.). There was no association between pSTAT3 tumor expression and presence of calcification on mammograms (1.27 ± 0.10 vs. 1.18 ± 0.06, p = 0.405, Student T test) (Table 2.).

3.4. Survival analysis

When we analyzed all patients, patients with higher expression of pSTAT3 (detected by Western blot) in tumor tissue had a significantly longer RFS than those with low expression of pSTAT3 (p = 0.002, Log-Rank test) (Fig. 3A). Also, patients with higher expression of pSTAT3 (detected by immunochistochemistry) had a significantly longer RFS than those with low expression of pSTAT3 (p = 0.003, Log-Rank test) (data not shown). Moreover, in the group of ER positive patients, patients with the expression of pSTAT3 in tumor tissue had a significantly longer RFS than those with low expression of pSTAT3 (p = 0.014, Log-Rank test) (Fig. 3B). ER positive patients with expression of pSTAT3 by immunochistochemistry had significantly longer RFS (p = 0.004, Log-Rank test) (data not shown). Also, when patients with lymph node involvement (LN+) were analyzed, patients with the expression of pSTAT3 in tumor tissue had a significantly longer RFS than those with low expression of pSTAT3 (p = 0.006, Log-Rank test) (Fig. 4C). Lymph node positive patients with expression of pSTAT3 by immunochistochemistry had significantly longer RFS (p = 0.03, Log-Rank test) (data not shown). However, in group of patients with negative lymph node, there was no significant difference in RFS between the high pSTAT3 expressing group and the low pSTAT3 expressing group (p = 0.09, Log-Rank test) (data not shown). Patients with age over 50 had a significantly longer RFS when they have the expression of pSTAT3 in tumor tissue than those with low expression of pSTAT3 (p = 0.008, Log-Rank test) (Fig. 3D). Older patients with expression of pSTAT3 by immunochistochemistry had significantly longer RFS (p = 0.01, Log-Rank test) (data not shown).

When we analyzed all patients, patients with high pSTAT3 expression detected bz Western blot in tumor tissue had significantly longer OS compared to those with low expression of pSTAT3 (p = 0.006, Log-Rank test) (Fig. 4A). In group of investigated patients survival rate at 5 years was 82.8% (70.1%–97.8%). Furthermore, patients with ER positive receptors and high expression of pSTAT3 in tumor had significantly longer OS compared to ER positive patients with low expression of pSTAT3 (p = 0.028, Log-Rank test) (Fig. 4B). In the group of hormone positive patients survival rate at 5 years was 85.1% (72.6%–99.7%). In the group of patients with lymph node involvement, there was significant difference in prognosis between the high pSTAT3 expressing group and the low pSTAT3 expressing group (p = 0.018, Log-Rank test) (Fig. 4C). In the group of lymph node positive patients survival rate at 5 years was 86.7% (71.06%–100%). In the group of patients with age over 50, those with the high expression of pSTAT3 in tumor tissue showed a significantly longer OS than those with low expression of pSTAT3 (p = 0.024, Log-Rank test) (Fig. 4D). In the group of older patients survival rate at 5 years was 81.2% (66.2%–99.7%)

4. Discussion

Identification of novel biomarkers for breast cancer patients that would enable a safer risk assessment and prognosis of these patients, as well as the identification of target molecules for therapeutics represent important targets in the treatment of breast cancer. It has been shown that STAT3 signaling pathway is included in apoptosis inhibition, cancer cell proliferation, angiogenesis induction and avoiding of immune response (1, 7, and 21). In our research we show that malignant tissue had expression of pSTAT3 compared to adjacent peritumor tissue. These results are in concordance with previous published studies based on immunohistochemistry as detection method that show a higher level of pSTAT3 in malignant tissue compared to adjacent tissue [7,21,22], while in our study we used Western blot, a more sensitive method. In this sense, our results indicate that pSTAT3 expression could be used as a diagnostic marker for breast cancer patients.

In this investigation we show that there is no association of pSTAT3 tumor expression and clinical stage of the disease. In accordance with this result, we show that pSTAT3 tumor expression is not associated with tumor size or lymph node involvement. Other studies had controversial results regarding association of p(TYR705)STAT3 tumor expression and tumor size [10] or lymph node involvement [9,21], although these studies were based on nuclear pSTAT3 expression. It is possible that activation of pSTAT3 pathway is present during cancerogenesis, as well as in all clinical stage of the disease.

In our study we show that there is no significant difference in p (tyrosine 705)STAT3 tumor expression in patients with ductal carcinoma compared to expression in patients with lobular carcinoma. Furthermore, we show that there is no difference of pSTAT3 expression and age of breast cancer patients. Other studies showed similar results [22,23], although in these studies pSTAT3 nuclear localization was detected, while in our study we detected cell expression of pSTAT3. These results sugest that pSTAT3 activation is an important event in cancerogenesis, independent from tumor type or age of patients.

The published study showed that breast cancer lines with ER and PR positive receptors are associated with STAT3 activation [24]. We show that there is no difference in expression of pSTAT3 in tumor tissue beetwen patients with hormone receptor positive and hormone receptor negative patients. It has been shown that HER2 can activate STAT3 signaling pathway [25]. Also, HER2 induce via Src kinases migration of breast cancer cells [7]. Other study has shown that Src kinases through pSTAT3 activation transform breast cancer cells [26]. In our study we show that patients with HER2 overexpression had highier tumor expression of p(tyrosine 705)STAT3 compared to patients with HER2 negative receptors. In this regard, it is of importance to follow pSTAT3 expression in HER2 positive breast cancer patients as it may affect the

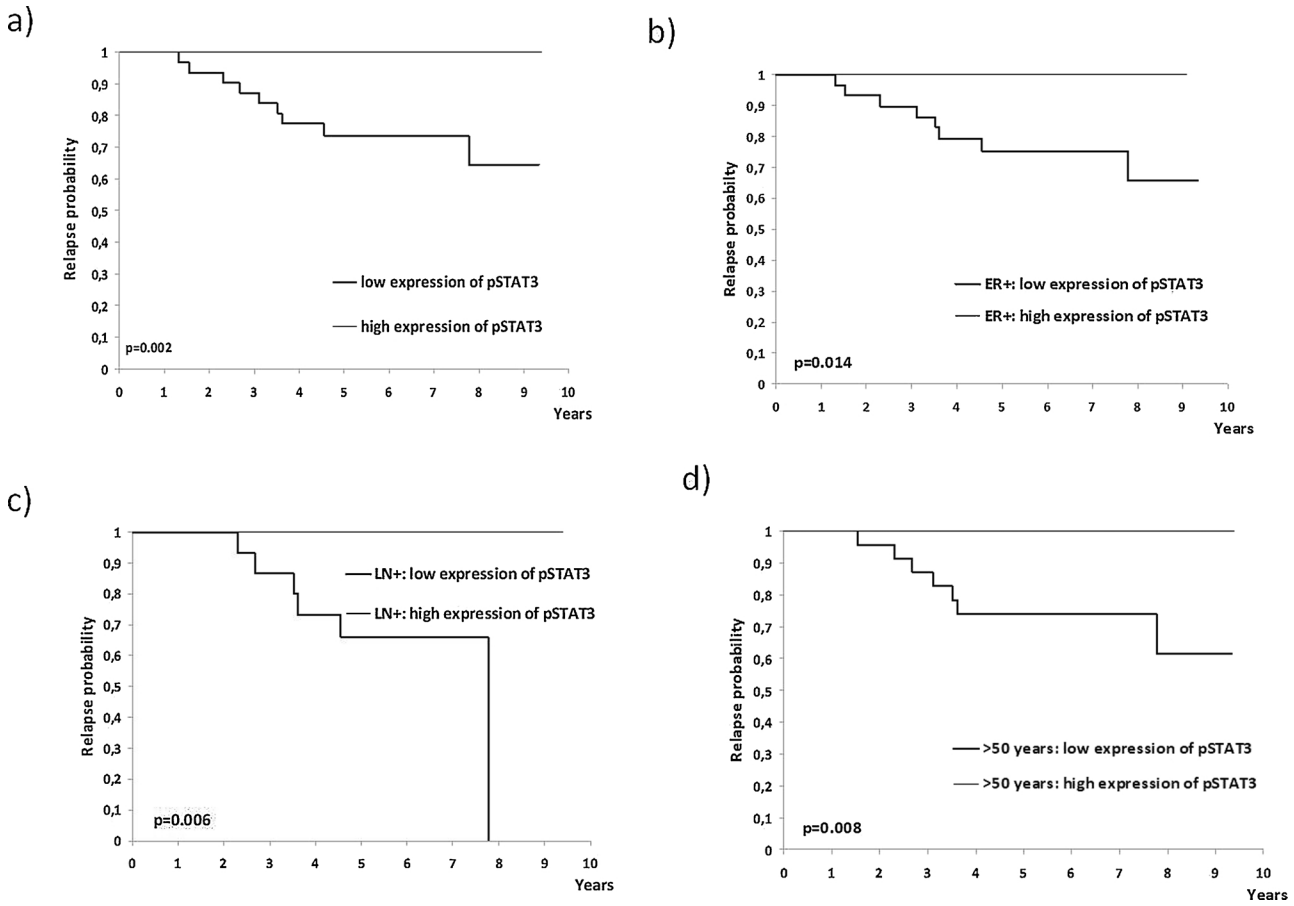

Fig. 3. (a) Patients with the expression of p(Y705)STAT3 in tumor tissue had a significantly longer RFS than those with low expression of p(Y705)STAT3 in the entire group of patients (p = 0.002, Log-Rank test), (b) in the group of ER positive patients (p = 0.014, Log-Rank test), (c) in the group of lymph node positive patients (p = 0.006, Log-Rank test) and (d) in group of patients with age over 50 (p = 0.008, Log-Rank test).

choice of therapy.

Calcifications represent one of the most important features of breast carcinoma. It is well documented that calcifications represent one of the earliest mammographically detectable changes associated with in situ and invasive breast carcinomas in asymptomatic women [27,28]. Seo at al. found that HER2 positive cancers were associated with suspicious calcifications at mammography [29]. Considering that in our group of investigated patients there was a small number of patients who had HER2 positive breast cancers and presence of calcifications on mammograms (only 5 patients), we did not investigate association of STAT3 expression and HER2 positive patients. We found that there is no association between pSTAT3 tumor expression and presence of calcifications on mammograms; however patients with calcifications had higher expression of pSTAT3. Recently it has been shown that high mammographic density on mammograms can be a predictor of poor prognosis of breast cancer patients [30]. For this reason we investigated and we show that patients with high mammographic density (ACR3 or ACR4) had significantly higher tumor p(tyrosine705)STAT3 expression compared to patients with low mammographic density. These results provide insight into the biological processes in breast cancer defined by association of pSTAT3 and mammographic density and could be of use in defining novel therapeutic and management strategies.

As novel predictive marker, pSTAT3 was investigated in a few studies showing controversial results [9,21,31]. In the present study we show that pSTAT3 expression correlates with longer RFS in the entire group of patients, as well as in the group of ER positive, in lymph node positive and in the older group of breast cancer patients (with age over 50). Furthermore, in the entire group of patients, in ER positive, in lymph node positive and in group of breast cancer patient with age over 50 high expression of pSTAT3 showed a better survival than the low expression of pSTAT3. Doled Filhart et all showed that STAT3 expression is associated with RFS in patients that are lymph node negative, although only nuclear expression showed significantly longer survival [23]. On the other hand, Berclaz et al did not detect prognostic value of pSTAT3 [21]. Different group of investigated patients and number of patients could be the reason for the difference in the obtained results [9,21,32]. In this regard, in our study we used Western blot as very sensitive method for pSTAT3 detection, as well as immunohistochemistry, although both methods are semiquantitative. In present study, ER positive patients with high cytoplasmatic expression of pSTAT3 had longer survival. On the contrary to this, Aleksandarany et al showed that ER patients had longer survival with high nuclear pSTAT3 expression [21]. This result suggest that STAT3 signaling present one of most sustained events in tumorigenesis of invasive breast cancer patients with less aggressive tumor types.

Considering that the expression of pSTAT3 is associated with longer RFS and survival, it can be used as prognostic tools for determination of group of breast cancer patients with low-risk. Also, the shown association of pSTAT3 expression with mammographic density indicates further investigations regarding molecular basis of density.

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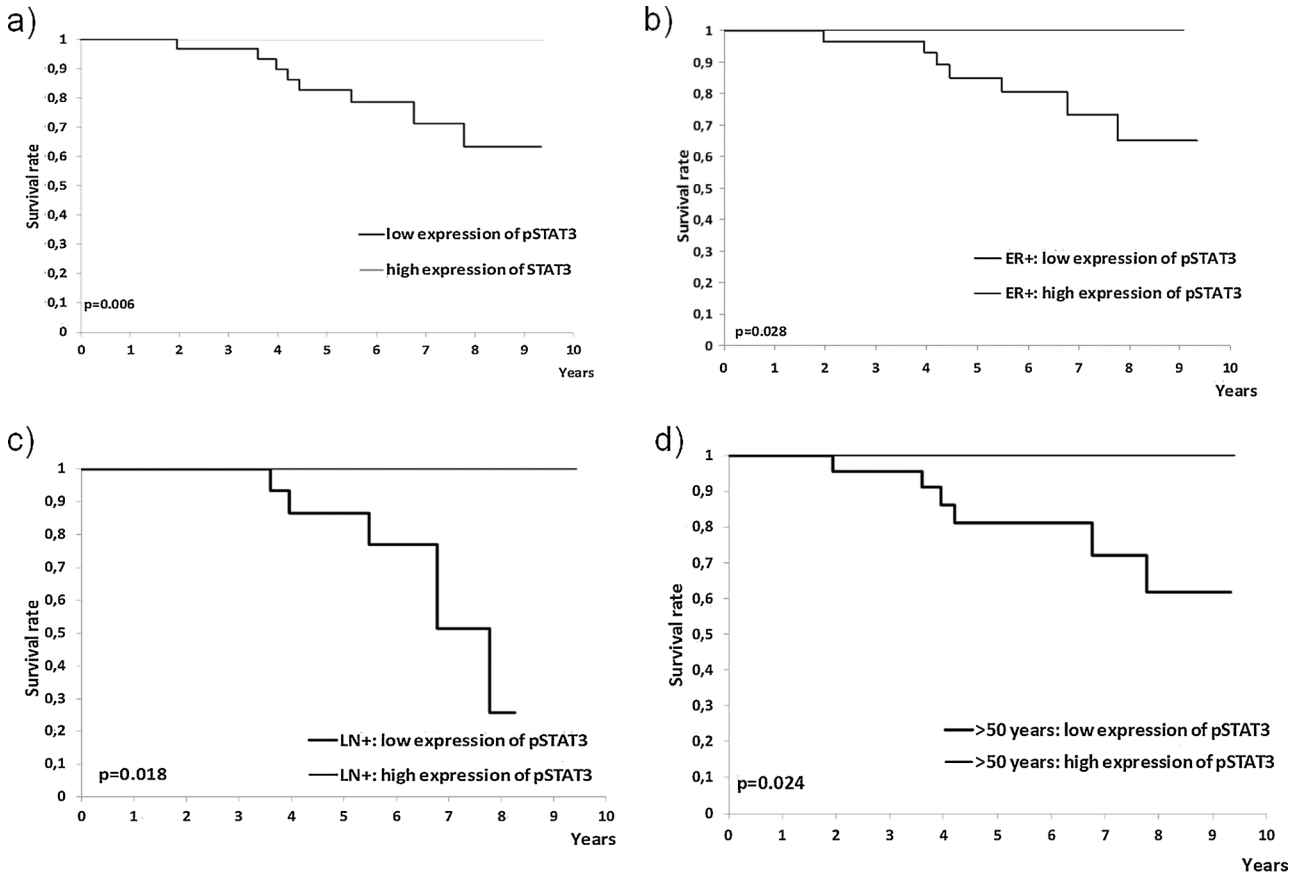

Fig. 4. (a) Patients with the expression of p(Y705)STAT3 in tumor tissue had a significantly longer OS than those with low expression of p(Y705)STAT3 in the entire group of patients ($p = 0.006$, Log-Rank test), (b) in the group of ER positive patients ($p = 0.028$, Log-Rank test), (c) in the group of lymph node positive patients ($p = 0.018$, Log-Rank test) and (d) in group of patients with age over 50 ($p = 0.024$, Log-Rank test).

Competing interests

None declared.

Patient consent

Obtained.

Ethics approval

Ethical committee of the Institute of Oncology and Radiology of Serbia.

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