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Predictive biological markers determining the Neoadjuvant Chemotherapy Response in Locally Advanced Breast Cancer

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1. Abstract

1.1. Aim

In the present study, we aimed to determine whether expression of HSP-27 along with bcl-2 and p53 may be associated with chemotherapy response and outcome in locally advanced breast cancer patients treated with anthracycline-based neoadjuvant chemotherapy.

1.2. Method

Biopsy materials of 34 patients, were examined by immunohistochemistry using specific monoclonal antibodies for HSP-27, bcl-2 and p53 along with c-erbB2, estrogen and progesteron receptor.

1.3. Results

Of those (n=34) studied in the present study, 20 (59%) were diagnosed with inflammatory breast cancer, and 14 patients (41%) had noninflammatory breast cancer. Patients with a poor response to anthracycline-based NAC were more likely to have HSP-27 expressions in both pre-NAC (NAC-unresponsive; 5/5, 100% vs NAC-responsive; 4/9, 44%; p=0.06) and post-NAC tumor samples (NAC-unresponsive; 5/5, 100% vs NAC-responsive; 4/9, 44%; p=0.06) that did not reach the statistical significance. Patients with inflammatory LABC were more likely to have c-erbB2 (9/16, 56% vs 1/9, 11%; p=0.034) and HSP-27 expressions (9/16, 56% vs 1/8, 12.5%; p=0.05) in preNAC tumor samples. The 3-year overall survival (OS) rate was 50%. Factors associated with poor OS rate were presentation with inflammatory breast cancer, poor chemotherapy response, high TLI and non-luminal tumor biology. Patients with $HSP\text{-}27_{post\text{-}NAC}$ expression were found to have significantly decreased disease free survival time ($HSP\text{-}27_{post\text{-}NAC}$-positive, 8 months± 4 months [95% CI: 4-15] vs $HSP\text{-}27_{post\text{-}NAC}$-negative, 16 months± 3 months [95% CI: 10-22]; p=0.05). Patients with $p53_{TOTAL}$ expressions were found to have a shorter locoregional recurrence free time compared to those with $p53_{TOTAL}$-negative expression (15 months± 2 months [95% CI: 12-18] vs 28 months± 8 months [95% CI: 13-42]; p=0.04). Patients with a poor chemotherapy response, high nuclear grade, c-erbB-2_{TOTAL} positivity, $HSP\text{-}27_{postNAC}$ expression, ER or PR negativity following chemotherapy were more likely to have distant metastases. Finally, patients with $p53_{TOTAL}$ expression were more likely to develop breast cancer recurrence either locoregional or distant recurrences.

1.4. Conclusion

In this study, it has been shown that HSP-27 may play a role in anthracycline resistance and in the development of distant metastases while p53 may act as a poor prognostic factor in LABC. Further larger studies are required regarding the interactions HSP-27 positivity and anthracycline responsiveness and the prognostic significance of p53 expressions.

2. Introduction

Locally advanced breast cancer (LABC) is more often seen in most low-middle resource countries due to the lack of early diagnosis strategies [1]. Neoadjuvant therapy (NAC) has been studied widely for the treatment of LABC followed by locoregional therapy. It allows to evaluate the NAC response that is administered preoperatively and to deescalate breast and axillary surgery [2]. Response to NAC has been found to be associated with better outcome in LABC. Therefore, biological markers predicting chemotherapy response should be identified to avoid ineffective treatments and improving chemotherapy benefit and outcome. Heat shock proteins (HSPs) are molecular chaperones including HSP-27, HSP40, HSP60, HSP70, and HSP90 defined according to their molecular weight. HSPs take part in diverse physiological processes to maintain cellular homeostasis. HSPs also play an essential role in cancer cell proliferation and metastasis, and anti-cancer drug resistance [3,4]. HSP-27 overexpression in cancer cells was shown to be involved in doxorubicin resistance [5-9]. However, its prognostic role has been controversial in breast cancer requiring more studies [10-13]. Similar to heat shock protein expressions, controversial findings were reported regarding an association between chemotherapy response and immunohistochemical expression of p53 on tumor in patients with locally advanced breast cancer receiving NAC [14-19]. However, a poorer survival has been demonstrated in those with p53 expression [20]. Contrarly, bcl-2 expression was found to be associated with improved survival, while lack of bcl-2 expression was associated with a response to anthracycline based chemotherapy in patients with LABC [21, 22].In the present study, we aimed to determine whether expressions of HSP-27 along with bcl-2 and p53 may be associated with chemotherapy response and outcome in locally advanced breast cancer patients treated with anthracycline-based neoadjuvant chemotherapy.

3. Material and Methods

Patients with locally advanced breast cancer (LABC) who were treated with anthracycline-based preoperative neoadjuvant chemotherapy (NAC) were included in the present retrospective study. Tumor paraffin block sections were obtained from archival

tissue material of biopsy samples or surgical specimens for immunohistochemical staining to study different markers. Ethical approval was obtained from the Istanbul University, Istanbul Faculty of Medicine.

4.1. Patients and Tissue Material

Thirty-four patients were diagnosed with locally advanced breast cancer at Istanbul University Istanbul Faculty of Medicine, Department of General Surgery. Based on the results of the previous pathology reports, patients who underwent surgery following NAC due to locally advanced breast cancer (LABC) were included in the study. The AJCC Staging 8th edition has been used in the clinical and pathological TNM classification [23]. Patients with inflammatory (n=20) and non-inflammatory breast cancer (n=14) were analyzed according to the clinical and/or pathological characteristics. Diagnosis of inflammatory breast cancer was based on the distribution of inflammatory signs including skin edema and redness and pathological findings of malignant subdermal lymphatic involvement. Clinical chemotherapy response was evaluated according to WHO criteria as complete response, partial response, stable disease and disease progression as determined by physical exam and imaging [24]. Nineteen patients (56%) received 3 cycles either FAC (5-Florouracil, 500 mg/m2, adriamycine, 50 mg/m2, cyclophosphamide, 500 mg/m2) or FEC (5-Florouracil, 500 mg/m2, epirubicine, 80 mg/m2, cyclophosphamide, 500 mg/ m2) regimen every 3 week before surgery. Patients with a poor response to 2 cycles anthracyclines (n=6) received 3 cycles taxanes (100 mg/m2), whereas 4 patients were treated with preoperative radiotherapy.The remaining responsive patients completed 4 cycles of adjuvant anthracycline based-chemotherapy followed by adjuvant radiotherapy and hormonotherapy if their tumor expressed ER and/or PR. Of 34 patients, only 3 cases with cT2N1 disease underwent breast conserving surgery with axillary lymph node dissection. Modified radical mastectomy was performed in the remaining 31 patients. Radiotherapy (RT) was given 45 to 50 Gy in 25 fractions to the whole breast in patients treated with breast conserving surgery or chest wall for mastectomy, and axillary (level I-II-III) and supraclavicular lymph node regions with/without the mammary internal lymph node region. Boost RT for tumor bed was also delivered to patients treated with breast conserving surgery. Boost doses are 10 to 16 Gy in 5 to 8 fractions. Patients with a poor response to anthracyclines further received taxanes before surgery or radiotherapy.

4.2. Immunohistochemical Staining

Biological markers were studied in tumor paraffin block sections of 34 patients obtained before starting with chemotherapy (n = 25) or in surgical specimen after NAC (n=15) by immunohistochemistry (IHC). Estrogen (ER) and progesterone receptors (PR)and human epidermal growth factor 2 (HER2), formerly described as c-erbB2, Ki-67, p-53, bcl-2, and heat shock protein-27 (HSP-27) were examined by IHC. ER, PR, Ki-67, c-erbB2, p-53, HSP-27 and bcl-2 expressions were assessed on 5-µm formalin-fixed paraffin-embedded slides. Sections were incubated with primary antibodies for ER (mouse mAb; NCL-ER-6F11, Novocastra) at 1:40 dilution, for PR (mouse mAb; NCL-PGR, Novocastra) at 1:40 dilution, for Ki-67 (mouse mAb, Novocastra, NCL-Ki67-MM1) at 1:50 dilution, for c-erbB-2 (rabbit policlonal Ab; DAKO, A 0485) at 1:400 dilution, HSP-27 (Mouse mAb; NCL-HSP-27, Novocastra) at 1:30 dilution, bcl-2 (mouse mAb; Clone-124, DAKO) at 1:40 dilution, and p-53 (mouse mAb; M 7001, DO-7 clone, DAKO) at 1:100 dilution. The avidin-biotin complex method was used to assess the immunohistochemical expression of biomarkers by using biotin-streptavidine-peroxidase system (LAB VISON; TR-125-HL) for c-erbB-2 staining and (DAKO, K4005) for the other markers studied. Sections were then counterstained with hematoxylin and eosin.

4.3. Immunohistochemical Evaluation

The intensity, staining percentage, and pattern of staining (nuclear, membranous, cytoplasmic) were assessed for the markers studied in the present study. The intensity was scored as low, moderate, and strong compared with background staining. The percentages of positive cells were estimated by calculating the ratio of the positively stained cells as counting at least 1000 invasive tumor cells. The expression patterns were nuclear for ER, PR, Ki67, p-53, whereas the staining was cytoplasmic for HSP-27 and bcl-2 and membranous for c-erbB-2 (Figures 1a-d). ER and PR were considered positive if the nuclear staining was >10%. HER2/neu was considered positive in the statistical analyses if >10% of the tumor cells showed a complete and strong membranous staining (3+). Any staining ≥20% was considered positive for Ki-67, HSP-27 and p-53 expressions, whereas any staining ≥15% was considered positive for bcl-2 expression (Table 1).

4.4. Assessment of Thymidine Labeling Index

Thymidine labeling index was determined in fresh tumor samples obtained from surgical biopsies of patients with breast cancer as

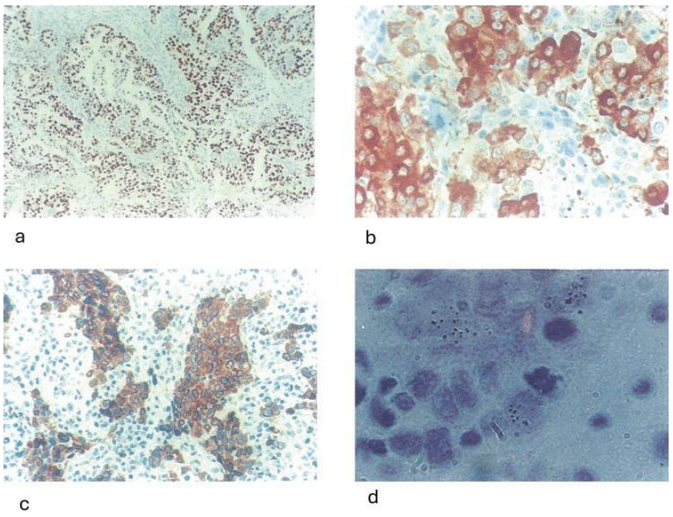

Figure 1: Expression of biomarkers.
A) High p53 expression (strong nuclear staining, *X125* magnification)
B) High HSP-27 expression (strong cytoplasmic staining, *X500* magnification) in a patient with a poor chemotherapy response to anthracycline containing regimen
C) High bcl-2 expression (strong cytoplasmic staining, *X500* magnification)
D) Autoradiography of a patient with a high thymidine labeling index (TLI)

described before [25]. Briefly, the minced tumor fragments were placed in 2 mL of 199 medium (Biological Industries, Kibbutz Beit Haemek, Israel) containing 20% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel), streptomycin 100microg/ml, penicillin 100 U/mL, and 6 micro Ci/mL H_3-thymidine with specific activity 5 Ci/mol (Radiochemical Center, Amersham Life Science, UK). After 1 hour incubation at 37°C in shaker water bath, the tumor fragments were washed 3 times in phosphate-buffered solution, and fixed in buffered 10% formalin solution dehydrated in alcohol, and embedded in paraffin. The 5-µm formalin-fixed paraffin-embedded sections were coated with emulsion film (Ilford K2, Mobberley Cheshire, UK) in a dark room and exposed at 4°C for 3–5 days. Autoradiographies were then developed at 18°C, and fixed in a standard fixer. The slides were stained with hematoxylin and eosin at 4°C. A total of 1000–3000 cells were counted to determine the ratio of labeled cells. A tumor cell was considered labeled with thymidine when it contained at least 20 grains overlying the nucleus. Thymidine labeling index was estimated as the percentage of epithelial cells labeled with thymidine. Values less than 3% were considered as low TLI, whereas values equal to or more than 3% were considered high TLI based on previous studies [26].

4.5. Statistical Analysis

The SPSS software package (SPSS, Inc., Chicago, IL) was used for statistical analysis. Categorical variables were evaluated by two-tailed Fisher's exact test. A p value equal or less than 0.05 was considered statistically significant. Correlations between expression of biomarkers were determined by Spearman's test. Kaplan Meier survival analyses were used to estimate the survival, whereas the outcome between groups with different biomarker expressions was compared by log rank test. Disease free survival (DFS) was analyzed by considering both the locoregional recurrences including breast, chest wall and regional lymph nodes and distant recurrences.

5. Results

Of those (n=34) studied in the present study, 20 (59%) were diagnosed with inflammatory breast cancer, and 14 patients (41%) had noninflammatory breast cancer. The mean age of the cohort was 44.2±12.5. The demographic and clinical characteristics of patients were shown in Table 2. According to the TNM staging, the majority of patients had stage IIIB disease (85.3%), whereas only 5 patients had stage IIIA disease (14.7%). Most patients presented with clinically T4 (64.7%) and node-positive disease (70.6%). Of those with inflammatory breast cancer, 18 had clinical signs such as skin edema and/or erythema, and 14 (70%) had malignant subdermal lymphatic invasion.

5.1. Expression of Biomarkers

Based on the Spearman correlation test, the expression of hormone receptors including ER and PR have shown significant positive correlations with bcl-2 and HSP-27 expressions, and negative correlations with c-erbB-2 and TLI expressions studied in biopsy and surgical specimen before and after NAC (Table 3). Patients not responding well to anthracycline-based NAC were more likely to have HSP-27 expressions in both pre-NAC (NAC-unresponsive; 5/5, 100% vs NAC-responsive; 4/9, 44%; p=0.06) and post-NAC tumor samples (NAC-unresponsive; 5/5, 100% vs NAC-responsive; 4/9, 44%; p=0.06) that did not reach the statistical significance.

Patients with inflammatory LABC were more likely to have c-erbB2 (9/16, 56% vs 1/9, 11%; p=0.034) and HSP-27 expressions (9/16, 56% vs 1/8, 12.5%; p=0.05) in pre-NAC tumor samples. In both pre- and post-NAC samples, patients with inflammatory breast cancer were more likely to have high TLI expressions (5/8, 63% vs 3/11, 27%; p=0.14) and low bcl-2 (2/16, 12.5% vs 6/14, 43%; p=0.07) and low PR expressions (3/18, 17% vs 7/14, 50%; 0.05) compared to those with non-inflammatory LABC that did not reach the statistical significance (Table 4).

5.2. Outcome

Overall, the mean overall survival time and disease free survival time were 47 months± 5.5 months (95% CI: 37-54) and 17 months± 2 months (95% CI: 13-21) in the present cohort. The 3-year overall survival was found to be 50%. Factors associated with poor OS were presentation with inflammatory breast cancer, poor chemotherapy response, high TLI and non-luminal tumor biology (Table 5). The overall mean systemic recurrence free time (SRFT) was 20 months± 5 months (95% CI: 11-30), and the mean local recurrence free survival time was 19 months± 3 months (95% CI: 13-25). "Patients with HSP-27 TOTAL expression were found to have decreased SRFT (HSP-27-positive, 8 months± 4 months [95% CI: 4-15] vs HSP-27-negative, 20 months± 5 months [95% CI: 11-30]; p=0.09). Similarly, patients with $HSP\text{-}27_{post\text{-}NAC}$ expression were found to have significantly decreased disease free survival time ($HSP\text{-}27_{post\text{-}NAC}$-positive, 8 months± 4 months [95% CI: 4-15] vs $HSP\text{-}27_{post\text{-}NAC}$-negative, 16 months± 3 months [95% CI: 10-22]; p=0.05). Patients with $p53_{TOTAL}$ expressions were found to have a shorter locoregional recurrence free time compared to those with $p53_{TOTAL}$-negative expression (15 months± 2 months [95% CI: 12-18] vs 28 months± 8 months [95% CI: 13-42]; p=0.04). Furthermore, patients with a younger age (<44), and high TLI_{TOTAL} were more likely to develop locoregional recurrences. Patients with a poor chemotherapy response, high nuclear grade, c-erbB-2_{TOTAL} positivity (p=0.04), $HSP\text{-}27_{postNAC}$ expression, ER or PR negativity following chemotherapy were more likely to have distant metastases. Increased positivities of high expression of p53/HSP-27 (p=0.07) were also observed in patients with recurrences (local and/or distant) either before or following chemotherapy. Finally, patients with $p53_{TOTAL}$ expression were more likely to develop breast cancer recurrence as either locoregional or distant recurrences (Table 6).

6. Discussion

In the present study, biomarkers that could predict resistance to neoadjuvan chemotherapy based on a anthracycline containing regimen have been studied in patients with locally advanced breast cancer. Of note, the majority as 59% of the present cohort consisted of patients with inflammatory breast cancer that distinguished our study from others. Our results suggest that HSP-27 could play a role

Table 1: Evaluation of the immunohistochemical staining of HSP-27, p53, and bcl-2 expressions.

	Percentage (%)	Score	Staining Intensity	Score	Staining Percentage	Expression score	High expression Score
HSP-27	10-20%	1	low	0	20%	2	≥4
	20-60%	2	intermediate	2			
	60-100%	3	strong	3			
p-53	1-10%	1	low	0	20%	2	≥4
	10-20%	2	intermediate	2			
	20-60%	3	strong	3			
	60-100%	4					
Bcl-2	10-30%	1	low	0	15%	1	
	30-60%	2	intermediate	2			
	60-100%	3	strong	3			

Table 2: Clinical and pathological characteristics of patients.
na: not applicable. Missing data were excluded since nuclear and histologic grade could not be determined due to the neoadjuvant chemotherapy.

	Overall (N=34, %)	Non-inflammatory Locally Advanced Breast Cancer (n=14)	Inflammatory Breast Cancer (n=20)
Menopausal status			
Premenopausal	22 (64.7%)	10(71.4%)	12 (60%)
Postmenopausal	12 (35.3%)	4 (28.6%)	8 (40%)
Clinical TNM-T category			
T2	3(8.8%)	3 (17.6%)	
T3	9 (26.5%)	9 (52.9%)	3 (15%)
T4	22 (64.7%)	22 (64.7%)	17 (85%)
Clinical TNM-N category			
N0	10 (29.4%)	2 (14.3%)	8 (40%)
N1	7 (20.6%)	1 (7.2%)	6 (30%)
N2	17 (50%)	11 (78.5%)	6 (30%)
Stage			
IIIA	5 (14.7%)	5 (35.7%)	na
IIIB	29 (85.3%)	9 (64.3%)	20 (100%)
Tumor pathology			
Invasive ductal	31 (91.2%)	13 (93%)	18 (90%)
Invasive lobular	1 (2.9%)	0(0%)	1 (5%)
Mixed	2 (5.9%)	1 (7%)	1 (5%)
Nuclear grade			
intermediate	13 (54.2%)	5 (50%)	8 (57.1%)
high	11 (45.8%)	5 (50%)	6 (42.9%)
Histologic grade			
intermediate	11 (45.8%)	4 (40%)	7 (50%)
high	13 (54.2%)	6 (60%)	7 (50%)

Table 3:Correlation of different biomarkers studied by immunohistochemistry.
ER=estrogen receptor, PR=progesteron receptor, TLI= Thymidine labeling index.

Biopsy material before neoadjuvan chemotherapy		
Biomarker expression scores	**Spearman Correlation coefficient**	**P value**
ER_{preNAC} vs PR_{preNAC}	0.82	0.0001
ER vs bcl-2	0.44	0.034
PR vs bcl-2	0.54	0.004
ER vs HSP-27	0.33	0.03
ER vs c-erbB2	-0.66	0.01
PR vs c-erbB2	-0.31	0.085
bcl-2 vs c-erbB2	-0.36	0.05
ER vs TLI	-0.81	0.028
PR vs TLI	-0.81	0.03
Bcl-2 vs TLI	-0.76	0.049
HSP-27 vs TLI	-0.59	0.044
Surgical specimen after neoadjuvan chemotherapy		
$ER_{postNAC}$ vs $PR_{postNAC}$	0.63	0.012
ER vs bcl-2	0.48	0.008
PR vs bcl-2	0.56	0.04
ER vs c-erbB2	-0.45	0.01
PR vs c-erbB2	-0.66	0.008
bcl-2 vs c-erbB2	-0.58	0.03

Table 4: Expressions of biomarkers in patients with locally advanced breast cancer (LABC).
PR=progesteron receptor, TLI= Thymidine labeling index.

Biomarker expression	**Inflammatory group**	**Non-inflammatory group (%)**	***P* value**
c-erbB-2_{preNAC}	9/16 (56%)	1/9 (11%)	0.034
HSP-2_{7preNA}C	9/16 (56%)	1/8 (12.5%)	0.05
Bcl-2_{TOTAL}	2/16 (12.5%)	6/14 (43%)	0.07
PR_{TOTAL}	3/18 (17%)	7/14 (50%)	0.05
High TLI_{TOTAL}	5/8 (63%)	3/11 (27%)	0.14

Table 5: Factors associated with overall survival (OS).
I-LABC= inflammatory locally advanced breast cancer;NI-LABC= non-inflammatory locally advanced breast cancer; neoadjuvant chemotherapy= NAC, ER=estrogen receptor, PR=progesteron receptor, TLI= Thymidine labeling index.

	Mean OS time (month)	2-year OS (%)	P value
Overall (n=34)	47±5.3 (37-54)	62	
Clinical Presentation Type:			0.07
I-LABC	32±5.2 (22-42)	33	
NI-LABC	56±6.3 (44-68)	76	
NAC response			0.06
NAC response (+)	55±6 (42-67)	82	
NAC response (-)	33±5 (22-43)	39	
TLI			0.013
TLI(+)	31±6 (20-42)	45	
TLI(-)	61±6 (49-73)	80	
			0.006
Nonluminal (ER- and PR- total)	30±4 (22-38)	42	
Luminal (ER+ or PR+, total)	65±6 (54-76)	88	

Table 6: Factors associated with breast cancer recurrence in patients receiving anthracycline-based chemotherapy.
Missing data were excluded from the analysis.
LRR=locoregional recurrence, SR=systemic recurrence, I-LABC= inflammatory locally advanced breast cancer,
NI-LABC= non-inflammatory locally advanced breast cancer, neoadjuvant chemotherapy= NAC, ER= estrogen receptor,
PR= progesteron receptor, TLI= Thymidine labeling index.

Factors affecting outcome	LRR (+)	p	Systemic recurrences (+)	p	LRR or SR(+)	P
Overall	7/34 (21%)		16/34 (47%)		18/34 (53%)	
Clinical presentation		0.38		0.7		0.24
I-LABC	5/20 (25.0%)		10/20 (50%)		12/20 (60%)	
NI-LABC	2/14 (14.29%)		6/14 (43%)		6/14 (43%)	
Age		0.06		0.30		0.40
<44	6/18 (33.33%)		10/18 (56%)		11/18 (61%)	
≥44	1/16 (6.25%)		6/16 (38%)		7/15 (47%)	
Chemotherapy response		0.4		0.01		0.02
(+)	2/20 (10%)		5/20 (25.0%)		7/20 (35%)	
(-)	5/14 (35.71%)		11/14 (78.6%)		11/14 (78.57%)	
Nuclear grade		0.40		0.04		0.25
2	3/14 (25%)		5/14 (36%)		7/13 (54%)	
3	4/12 (33%)		9/12 (75%)		9/12 (75%)	
ER (postNAC) (n=16)		0.6		0.07		0.09
negative	2/9 (22.2%)		7/9 (77.8%)		8/10 (80.0%)	0.09
positive	1/7 (14.3%)		2/7 (28.6%)		2/6 (33.3%)	
PR (postNAC) (n=16)		0.7		0.02		0.08
negative	2/10 (20%)		8/10 (80%)		8/10 (80%)	
positive	1/6 (16.7%)		1/6 (16.7%)		2/6 (33.3%)	
High-TLI (TOTAL)		0.06		0.2		0.2
negative	1/11 (9.1%)		5/11 (45.5%)		5/11 (45.5%)	
positive	3/6 (50%)		6/8 (75.0%)		6/8 (75.0%)	
High c-erbB2 (TOTAL)		0.6		0.04		0.08
negative	4/19		6/19 (31.6%)		8/19 (42.1%)	
positive	3/13		9/13		9/12 (75%)	
Bcl-2 (TOTAL)		0.7		0.17		0.21
negative	6/22 (27.3%)		13/22 (59.1%)		11/22 (50%)	
positive	0/8 (0%)		2/7 (28.6%)		2/8 (25%)	
p53-high (TOTAL)		0.23		0.32		0.07
negative	2/16 (12.5%)		6/16		6/15 (40%)	
positive	3/9 (33.3%)		5/9		7/9 (77.8%)	
HSP27 (postNAC)		0.24		0.06		0.2
negative	2/5		1/5		2/5	
positive	1/9		7/9		7/9	

in anthracycline resistance (5-9), while other markers including p53, and TLI were found as prognostic factors affecting survival [20, 25, 26]. In concordance with other studies [5-9], we similarly found increased HSP-27 expressions in patients with a poor response to anthracycline-based NAC in both pre-NAC and post-NAC tumor samples compared to those with good responders to NAC. Previous in vitro studies indicated elevated HSP-70 and HSP-27 levels associated with doxorubicin resistance in human breast cancer cells [5, 6]. HSP-27 transfected MDA-MB-231 breast cancer cells were shown to display a 3-fold elevated resistance to anthracyclines [7]. Anchorage-dependent proliferation and anchorage-independent growth were also increased 2-4-fold in these transfectants. When these cells are transfected with an antisense HSP-27 construct, they are rendered sensitive to anthracyclines with decreased anchorage-dependent as well as anchorage-independent cell growth. Vargas-Roig et al similarly reported increased HSP-27 expressions on tumor samples following anthracycline-containing NAC [8]. They also observed that patients with high cytoplasmic expression (>66%) of HSP-27 had shorter DFS in concordance with our findings. Of note, patients with inflammatory LABC in the current study were more likely to have c-erbB2 and HSP-27 expressions in pre-NAC tumor samples indicating that these biomarkers are associated with poor prognosis. However, Nadin et al could not find any correlations between HSPs expression and the clinical and pathological response to neoadjuvant therapy in 60 patients presented with LABC [9]. Oesterreich et al studied the prognostic power of HSP-27 expression measuring HSP-27 levels in 425 patients with an immunohistochemistry (IHC) study analyzing 788 patients [10]. Similar to our findings in the present study, HSP-27 levels correlated positively with ER status (P = 0.0001 in Western blot and IHC study), progesterone receptor status (P = 0.0001 in Western blot and IHC study). However, there was no significant associations between HSP-27 expression and disease-free survival or overall survival. Nevertheless, high HSP-27 levels correlated modestly with shorter disease-free survival as we demonstrated in the current study. Zhuang et al recently reported that an elevated HSP-27 tested by immunohistochemistry indicated a poor prognosis in patients with LABC (n=103) having a lower DFS and OS compared to others [11]. Love et al further analyzed HSP-27 expression in 361 patients with primary breast cancer [12]. In both node-negative and node-positive women, ER+ HSP-27- patients were found to have a longer DFS than those with ER- HSP-27+ expression. However, there was no relationship between HSP-27 and overall survival. Finally, Thanner et al evaluated HSP-27 expression by immunohistochemistry in 191 patients with node-negative disease [13]. At a median follow-up of 177 months, no association was found between DFS and HSP-27 expression, whereas OS (p=0. 02) and survival after first recurrence (p=0.01) were significantly decreased.

HSP-27 overexpression was shown to increase cancer cell survival against the chemotherapeutic agents including doxorubicin, herceptin/trastuzumab, gemcitabine, 5-FU, temozolomide, and paclitaxel [14]. Conversely, HSP-27 inhibition increased the efficacy of those chemotherapy drugs, both in vitro and in vivo. HSP-27 most commonly contributed to the upregulation of Akt/mTOR signaling cascade and inactivation of p53, thus inhibiting the chemotherapy-mediated induction of apoptosis. Geisler et al investigated any association between p53 status and expression of c-erbB-2, bcl-2, and histological grading and to the response to doxorubicin monotherapy in 90 patients with locally advanced breast cancer [15]. Similarly, factors including expression of c-erbB-2 (p= 0.041), a high histological grade (p= 0.023), and lack of expression of bcl-2 (p= 0.018) predicted chemoresistance. No statistically significant association between p53 immunostaining and response to NAC could be estimated. These findings support the hypothesis that other defects may act in concert with loss of p53 function, causing resistance to doxorubicin in breast cancers.However, Fernández-Sánchez et al demonstrated in 60 patients with LABC receiving FAC regimen that positive immunoreactivity to p53 had a lower response to neoadjuvant chemotherapy with anthracycline [16]. Furthermore, in a Phase I/pilot clinical trial, p53 expression was found to be increased in 4 of 10 tumor samples in 24 h or 48 h after induction doxorubicine therapy [17]. Similarly, Prisack et al evaluated the expression of biomarkers in pre-treatment biopsies from 517 patients with LABC [18]. Patients with high grade tumors, lower ER, PR, bcl-2 or a higher proliferation had a significantly greater benefit from chemotherapy, while p53 staining did not have any predictive value. However, a higher pathologic complete response rate was obtained in those with high p53 immunostaining studied in 91 patients with LABC receiving anthracyline containing regimen as reported by Mukerjee et al [19].

In the current study, we report here a shorter locoregional recurrence time and increased breast cancer recurrence in those patients with p53 expressions. Similarly, Bonnefoi et al [20] demonstrated a poorer survival in patients with p53 expression presenting with LABC (n=448). In the multivariate analysis, p53 positivity was associated with a shorter progression-free survival [hazard ratio (HR) = 1.96; 95% CI 1.33-2.91; P = 0.0008) and a shorter overall survival (HR = 1.98; 95% CI 1.28-3.06; P = 0.002). However, Vargas-Roig et al could not find any correlation between p53 expression and survival [27].

Expression of anti-apoptotic proteins such as bcl-2 may confer chemotherapy resistance [21]. Absence of bcl-2 expression in pre-chemotherapy specimens was associated with more frequent complete pathological response (58% vs.20%; p = 0.04). Keam et al also studied biological markers including ER, PR, p53, c-erbB2, bcl-2, and Ki-67 by immunohistochemistry in a total of 145 stage II and III breast cancer patients received neoadjuvant docetaxel/doxorubicin chemotherapy [22]. Low histologic grade, positive hormone receptors, positive bcl 2 and low level of Ki-67 were associated with prolonged recurrence free survival. In addition, positive ER and positive bcl-2 dere associated with prolonged OS.

Proliferative activity of the tumor cells utilizing TLI has been a reliable and reproducible method in the past. As a dynamic measurement of de novo DNA synthesis, TLI reflects the percentage of cells in the S-phase fraction of the cell cycle, and has been utilized as a prognostic factor in the past [25, 26]. We previously have shown TLI-index as a poor prognostic factor in patients with LABC (n=71) treated with anthracycline based NAC regimen [25, 26]. In univariate analysis, patients with inflammatory breast cancer, high TLI-index (> or = 2.62%), lymph node (LN) positivity or > 3 positive lymph nodes following neoadjuvant chemotherapy and without any response to neoadjuvant chemotherapy were found to have worse DFS and OS-rates and high local and systemic recurrence rates. In multivariate analysis, TLI was estimated as the most powerful independent factor affecting the OS in LABC patients among the other established clinical and biological parameters (p= 0.02). Similar to our previous findings, we have shown here that factors associated with poor OS rate were presentation with inflammatory breast cancer, poor chemotherapy response, high TLI and non-luminal tumor biology.

In conclusion, the present study has shown that HSP-27 may play a role in anthracycline resistance and in the development of distant metastases while p53 may act as a poor prognostic factor in LABC. Further larger studies are required regarding the interactions HSP-27 positivity and anthracycline responsiveness and the prognostic significance of p53 expressions. Future studies are warranted to utilize the HSP-27 or bcl-2 proteins as potential therapeutic targets for anti-cancer treatment to increase the efficacy of systemic therapies [28, 29].

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Author Contributions

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Methodology and immunohistochemistry and analysis of the data: NC, ST, EY, OD

Writing of the manuscript: NC

Review-editing of the manuscript: ST, AI, OD, MM, VO, AB, AA, MP, MK, TD, EY

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