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Immunohistochemical analysis of heat shock proteins in triple negative breast cancer: HSP60 expression is a marker of poor prognosis

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Summary

Triple negative breast cancer (TNBC) is an aggressive and rapidly growing subtype of breast cancer characterized by the lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) expression, rendering it resistant to targeted therapies. In the absence of molecular targets, the pathogenesis of TNBC is not well understood and many studies are focused towards identifying the pathways and molecular signatures associated with the initiation and progression of TNBC. Heat shock proteins emerged as key players in the tumorigenic pathways of several types of cancer including TNBC. In the present study, paraffin-embedded tumor tissues of 66 Jordanian TNBC patients were analyzed, retrospectively, by immunohistochemistry to investigate the expression of HSP90, HSP70, HSP60, and HSP27 in Jordanian TNBC patients. The expression of the aforementioned markers was also examined using disease-free survival (DFS) along with a variety of clinical and pathological characteristics such as age, stage/grade of tumor, and nodal involvement. Positive expression of HSP90, HSP60, HSP27, and HSP70 was shown in 89%, 79%, 76%, and 40% of the cases analyzed, respectively. HSP60 positive expression was found to be significantly correlated with advanced stage (pT3/pT4) of the tumor ($p = 0.05$), nodal involvement ($p = 0.03$), and older age of patients ($p = 0.03$). Among the clinical and pathological variables analyzed in the present study, it was found that advanced stage (pT3/pT4) of the tumor and older age of the patients were significantly associated with worse DFS ($p = 0.001$ and 0.02, respectively). Additionally, positive expression of HSP60 showed to be correlated with worse DFS ($p = 0.05$). This study highlights the importance of HSP60 as a marker of poor prognosis in TNBC patients.

Key words: Breast cancer; Heat shock protein; Immunohistochemistry; Prognosis.

Introduction

Breast cancer is considered the second most commonly diagnosed cancer worldwide accounting for 11.9% of all cancer cases (The International Agency for Research in Cancer (IARC) of the World Health Organization (WHO) in 2013). Estimated new cases in the United States in 2016 are the highest among females, and comprising 29% of cancers in all sites, while estimated deaths in the same year are second in females accounting for 14% of all cancer cases [1]. According to recent data from the Jordanian Cancer Registry, breast cancer accounts for 19.8% of all newly diagnosed cancer cases, and it is considered as the most commonly diagnosed cancer in females representing 36.6% of all cases [2, 3]. It affects Jordanian women at a younger age than in Western countries (median 50 *vs.* 65 years), with reports of common ductal carcinoma among previously lactating women [4]. The delays in presentation, diagnosis, and treatment are the major challenges for breast cancer patients in Jordan [5]. Breast cancer is a heterogeneous disease that comprises multiple distinct types harboring different biological characteristics and clinical behaviors. Molecularly, breast cancer can be classified into different subtypes according to the expression patterns of the steroid receptors of both estrogen (ER) and progesterone (PR), and the human epidermal growth factor receptor-2 (HER2) proteins [6]. Accordingly, breast cancer is classified into five subtypes, namely: normal breast tissue-like subtype, luminal A subtype (ER and PR positive, and HER2 negative), luminal B subtype (ER positive, PR negative, and HER2 positive), HER2 subtype (ER and PR negative, and HER2 positive) and triple negative breast cancer (TNBC) [7]. Triple negative breast cancer (TNBC) is an extremely heterogeneous and clinically aggressive subgroup of breast cancers that is characterized by the lack of expression of ER and PR and the lack of the HER2 expression.

Worldwide, TNBC comprises 15% of all cases of breast

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cancer in women [8], and it poses a severe health problem, despite the fact that it represents a quite small proportion of all breast cancer cases, it is responsible for a disproportionate high number of breast cancer deaths. Additionally, targeted therapy and hormonal therapy that proved to be effective in improving the prognosis of breast cancer has eluded TNBC, given that it is ER and HER2 negative. Due to the extreme heterogeneity of TNBC, there have been fewer advances in finding an appropriate therapeutic regimen that could improve the prognosis of this disease.

Basically, TNBC is characterized by distinct biological features and more aggressive clinical behavior than other molecular subtypes of breast cancer, which pinpoints it as a separate disease entity by itself. Pathologically, TNBC is characterized by having the highest histological grade, high proliferative rate of the tumor cells, large tumor size at diagnosis, and higher prevalence of lymph node metastasis at diagnosis [7, 9, 10]. Clinically, TNBC is more aggressive than other subtypes, which is illustrated by the fact that it is associated with a high recurrence rate occurring between the first and the third years after therapy [10]. Moreover, TNBC is associated with the worst prognosis among the other subtypes, and shorter survival rates where the majority of death cases occur in the first five years after diagnosis. Finally, TNBC has a higher frequency of brain, spinal cord, liver and lung metastases, compared with other types of breast cancer [9, 11-14].

TNBC was discovered and described in medical literature about eight years ago [15] and extensive research was conducted since then to find an optimal therapeutic approach for such a heterogeneous disease, albeit the disease is still anonymous with no specific molecular targets, which leaves the clinicians with no choice, except to rely on non-specific cytotoxic therapeutic agents [16, 17]. Many studies are underway to understand the exact pathways involved in the initiation and progression of TNBC, and to identify molecular signatures specific for the disease which could be the basis for developing targeted therapies and help improve the outcome of the disease. Heat Shock Proteins (HSPs) have emerged as key player in the tumorigenic pathways of many cancers, including breast cancer.

HSPs are a group of highly conserved family of proteins that act as molecular chaperones and their expression is induced as a result of different kinds of stress conditions, such as: heat shock, oxidative stress, irradiation, and chemical stress [18]. HSPs have been classified according to their molecular size into six families: HSP100, HSP90, HSP70, HSP60, HSP40, and the small HSPs like HSP27 [19]. HSPs are involved in a variety of functions including protein folding, assembly and disassembly of protein complexes, transport of proteins across cellular membranes, degradation of denatured proteins, regulation of transcription factors, and cell survival as they were found to be involved in many cytoprotective mechanisms [20-23]. Recent studies have proved that HSPs have a key role in many signaling pathways responsible for tumor cell proliferation, differentiation, invasion, metastases, and growth [23], in which it was described that their role in tumor initiation and progression is manifested by the growing list of client proteins they transiently interact with to regulate tumor growth, apoptotic inhibition, angiogenesis, and metastasis. In addition, HSPs were found to be overexpressed in a plethora of cancers, such as colorectal cancer, prostate cancer, breast cancer, endometrial cancer, pancreatic cancer, bladder cancer, cervical cancer, gastric cancer, and leukemia [24-31]. Taken together, HSPs are known to be involved in the tumorogenic pathways of a wide variety of cancers and their level of expression is an indicator of poor prognosis, metastasis, and resistance to radiation and chemotherapy. Previous studies have shown that specific members of the HSP family including HSP90, HSP70, HSP60, and HSP27 are shown to be highly expressed in breast cancer tissues and were associated with poor prognosis [24, 32-36]; however, few studies have investigated the expression levels of the aforementioned proteins specifically in TNBC patients. Furthermore, although breast cancer is one of the leading causes of cancer deaths in women in Jordan and in the Arab world, there are no data investigating the prognostic role of heat shock proteins in breast cancer.

In this study, we aimed to identify signature markers of TNBC with prognostic value. A retrospective investigation of archived tissues of triple negative breast cancer patients was conducted. Clinicopathological characteristics were determined and analyzed including: age, TNM stage, grade, nodal status, and recurrence date. Furthermore, formalin-fixed, paraffin-embedded tissues were analyzed for the expression levels of HSP90, HSP70, HSP60, and HSP27 by immunohistochemistry. Additionally, we have analyzed the prognostic value of HSPs expression in regards with the clinical and pathological variables investigated in this work for TNBC patients in Jordan.

Materials and Methods

This study was performed on archival paraffin embedded blocks of 66 cases of triple negative breast cancer collected from the department of pathology in King Abdullah University Hospital and Al-Basheer Hospital between the years 2004 and 2015. Cases were diagnosed as TNBC by certified pathologists at the Department of Pathology in both hospitals, that carried out morphological examination of the tumor tissues based on their histological criteria. Negative expression of the nuclear receptors ER and PR, and a score of 0 or 1 for the expression of HER2 were criteria required to diagnose the case as a triple negative. Complete pathological records were obtained for all patients from both hospitals. Tumors were categorized according to the WHO classification system, into invasive ductal carcinoma (IDC) in 55 cases (83%), medullary carcinoma in eight cases (12%), metaplastic carcinoma in two cases (3%) and only one case of invasive lobular carcinoma (ILC). Tumors were staged according to the American Joint Committee on Cancer (AJCC/UICC, TNM staging, 7th edition) and graded according to the Nottingham grading system (Modified Bloom's Richardson score). Complete clinical records

were available for all cases for further analysis. Approval for this work was obtained from the Faculty of Medicine Research Ethics Committee at the Jordan University of Science and Technology.

Formalin fixed, paraffin embedded tissues diagnosed as TNBC were used in this study. The tissue samples were cut into 4-μm thick sections on coated slides using the rotary microtome and prepared for immunostaining. Sample sections were deparaffinized then rehydrated in a series of graded alcohol from a concentration of 100% into 70% for one minute each, then finally in a distilled water tank for two minutes. Antigen retrieval was performed in high pH citrate buffer with a pH=9 at 98ºC for 28 minutes using the PT Link platform. After retrieval, sample sections were washed with phosphate buffer saline (PBS) and the endogenous peroxidase activity was blocked with 3% hydrogen peroxide for ten minutes. Immunostaining for the target proteins HSP90, HSP70, HSP60, and HSP27 was performed manually on the sample sections using commercially available rabbit anti- human HSP90, rabbit anti- human HSP70, mouse anti- human HSP27, and rabbit anti-human HSP60 primary antibodies. About 200 μl of each antibody at dilution of 1:200 for anti-HSP90, HSP70, and HSP60 and 1:100 for anti- HSP27 were added to each sample section and incubated at room temperature for 45 minutes, and subsequently washed with PBS. After that, the sample sections were incubated at room temperature with HRP conjugated universal secondary antibody for 30 minutes and then washed thoroughly with PBS. Immunoreactivity was visualized with 3,3diaminobenzidine (DAB) chromagen and then counterstained with Mayer's hematoxylin. Finally, the sample sections were dehydrated then mounted with Diamount solution. Tissues known to express the target proteins were used as positive control (colon and appendix) and negative controls were performed by omitting the primary antibody step. Immunostained sections were examined under the light microscope by two independent certified pathologists (H.Z. and I.M.) blinded from any knowledge of patients' clinicopathological data. In all specimens, ten fields were examined at ×400 magnification and at least 100 cells were evaluated in each area in order to assess the tumor as a whole. Positively stained cells were counted and the intensity of the stain was also evaluated. Considering the percentage of positively stained cells and the stain intensity, a scoring system was designed with a cut off value of less than 20% positively stained cells. Positively stained cells of less than 50% and moderate stain intensity or positively stained cells of more than 50%, and a weak staining intensity were considered as weakly stained (+) positive samples. Percentage of more than 50% positively stained cells and strong or moderate stain intensity was considered as strongly stained (++) positive samples.

Pearson's X^2 test of independence and Fisher's exact test were used to investigate the association between the clinicopathological variables and the expression of HSP90, HSP70, HSP60, and HSP27. Spearman's rank correlation coefficient (r_s) test was used to evaluate the correlation between the expressions of the aforementioned heat shock proteins. The r_s test is defined as a measure of statistical dependence between two variables, to evaluate the linear relationship between them. Correlation was considered significant at the 0.05 level (one tailed). Disease-free survival (DFS) curves were constructed using the Kaplan-Meier method. Accordingly, DFS was defined as the period from the time of diagnosis to death from any cause or recurrence of the disease or last contact. The survival curves were compared using the log rank test and Breslow test. A value of $p \leq 0.05$ was considered statistically significant. Statistical analysis for all the data was carried out using the Statistical Package for the Social Sciences program (SPSS 21.0).

Table 1. — *Histopathological and clinical characteristics of the 66 triple negative breast cancer patients.*

Clinicopathological characteristics	Number	Percentage
Age (years)		
≤ 50	36	55%
> 50	30	45%
Grade		
G1	3	5%
G2	14	21%
G3	49	74%
Stage		
pT1	5	7%
pT2	29	44%
pT3	17	26%
pT4	5	8%
N/A	10	15%
Lymph node involvement		
N0	12	17%
N1	7	11%
N2	8	12%
N3	10	15%
Nx	29	45%
Pathological type		
Invasive ductal carcinoma (IDC)	55	83%
Medullary carcinoma	8	12%
Metaplastic carcinoma	2	3%
Invasive lobular carcinoma (ILC)	1	2%
LVi		
Present	28	42%
Absent	38	58%
Tumor size		
< 5	32	48%
≥ 5	22	34%
N/A	12	18%
Recurrence		
Yes	25	38%
No	41	62%
Follow up (months)		
Median	10	
Range	(1-81)	

Results

Sixty-six patients diagnosed with TNBC were selected in a retrospective analysis of their pathological records carried out between the years 2004 and 2015 in the Department of Pathology at the King Abdullah University Hospital and Al-Basheer Hospital. All patients were females who underwent radical mastectomy for the tumor and most of them underwent axillary lymph node resection afterwards. The mean age of the cohort was 49 ± 11.6 years with a median of 48 and a range of 30-87 years. Complete pathological reports were available for all selected patients. Table 1 shows the major histopathological and clinical features of this cohort. It is shown that most of the cases were IDC (83%) and eight (12%) cases were medullary carcinoma. Regarding the grade of the tumor, most of the cases were poorly differentiated in which three (5%) cases were

Table 2. — *Expression levels of the examined markers. (-): negative, (+): weak expression, and (++): strong expression.*

Heat shock proteins	-	+	++
HSP90			
Number	7	14	45
Percentage	11%	21%	68%
HSP70			
Number	39	26	1
Percentage	59%	39%	2%
HSP27			
Number	16	11	39
Percentage	24%	17%	59%
HSP60			
Number	14	0	52
Percentage	21%	0%	79%

G1, 14 (21%) cases were G2, and 49 (74%) cases were G3. TNM staging of the tumors showed that five (7%) cases were Stage PT1, 29 (44%) cases were Stage PT2, 17 (26%) cases were Stage PT3, and five (8%) cases were Stage PT4. Furthermore, 37 (55%) patients underwent axillary lymph node resection, and it was found that seven (19%) of them were Stage N1, eight (22%) were Stage N2, ten (27%) were Stage N3, and 12 (32%) showed no lymph node involvement (N0). Complete clinical reports were available for all patients for further analysis. Clinical follow-up for this cohort was available for up to 81 months with a median of 10 months, and 25 (38%) cases of the cohort experienced recurrence (Table 1).

Protein expression was investigated using the immunohistochemistry procedure for the following heat shock proteins: HSP90, HSP70, HSP60, and HSP27. The four markers showed differential expression in the TNBC cases. A scoring system of negative, weak (+) and strong expression (++) was introduced in this study depending on the percentage of positively stained cells and the intensity of the staining. Table 2 represents the scoring data for each marker in the investigated cases. Accordingly, HSP60, HSP90, and HSP27 showed a strong expression in the tumor cases. Regarding HSP90, it was found that 45 (68%) cases showed a strong expression (++) of HSP90 (Figure 1A), 14 (21%) cases showed a weak expression (+) of HSP90 (Figure 1A), and only seven (11%) cases showed a negative expression (Figure 1A). Interestingly, HSP70 was found to have the lowest expression in TNBC, in which most of the cases (39, 59%) showed a negative expression (Figure 1B), while 26 (39%) cases exhibited a weak expression of HSP70 (Figure 1B). HSP60 was positively expressed in 52 (79%) cases and it showed a strong expression in all of them (Figure 1C), while 14 (21%) cases showed a negative expression of HSP60 (Figure 1C). HSP27 was found to be strongly expressed (++) in 39 (59%) cases (Figure 1D) and weakly expressed in 11 (17%) cases (Figure 1D), while it was found to be negatively expressed in 16 (24%) cases (Figure 1D). Expression of

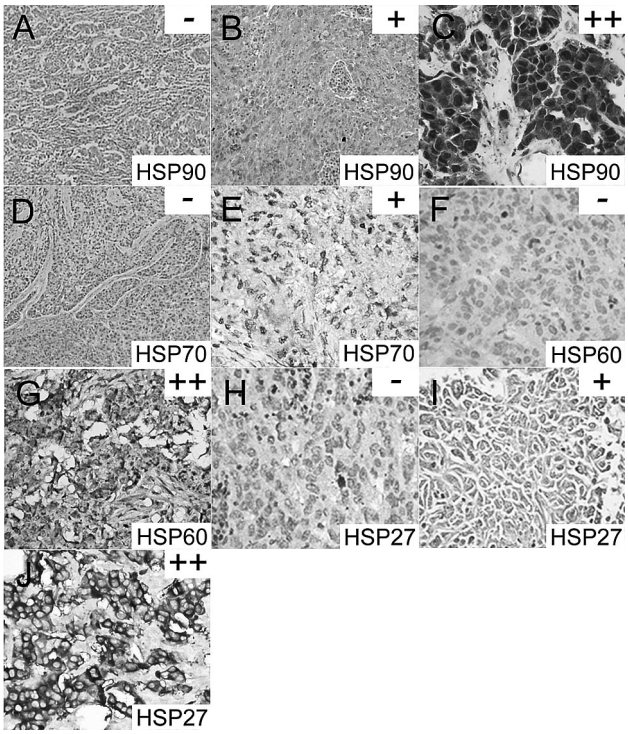

Figure 1. — Representative cases of immunohistochemical staining of the examined markers expression in tumor cells of TNBC. (A) Negative, weak, and strong cytoplasmic and nuclear staining of HSP90. (B) Negative and weak cytoplasmic staining of HSP70. (C) Negative and strong granular cytoplasmic staining of HSP60. (D) Negative, weak cytoplasmic and strong cytoplsmic and membranous staining of HSP27. Pictures were taken on magnifications of ×100 and ×400.

HSP90, HSP70, and HSP27, mainly presented a cytoplasmic and occasionally nuclear and membranous pattern of expression, while HSP60 presented a granular cytoplasmic pattern of expression in all the investigated cases.

Spearman's rank correlation coefficient was evaluated in this study to determine the linear relationships between the expression of HSP90, HSP70, HSP60, and HSP27 (Table 3). Accordingly, a significant negative yet weak correlation was found between the HSP60 expression and the HSP27 expression (r_S = -0.294, p = 0.008). No significant correlation was found between the expression levels of the other markers.

To understand the effect of the differential expression of HSPs in the tumor cases, HSPs expression was assessed for correlation with the clinicopathological variables of the cohort. Table 4 illustrates the correlation between HSP60 expression and the clinicopathological variables. Collectively, HSP60 positive expression was found to be significantly associated with patients' age (age ≥ 50, p = 0.03), advanced stage of the tumor (p = 0.05), and positive lymph node involvement (p = 0.03); while a marginal trend was found

Table 3. — *Correlation between the expression levels of HSP90, HSP70, HSP27, and HSP60 in all triple negative tumor samples. r_s: Spearman's rank correlation coefficient. p: p-value.*

	HSP90	HSP70	HSP27	HSP60
HSP90	-	r_s = -0.014	r_s = -0.195	r_s = 0.182
		p = 0.4	p = 0.06	p = 0.07
HSP70		-	r_s = 0.039	r_s = -0.021
			p = 0.3	p = 0.4
HSP27			-	r_s = -0.294
				p = 0.008*
HSP60				-

* *Statistically significant.*

Table 4. — *Frequencies of HSP60 expression according to the clinicopathological characteristics.*

Clinicopathological characteristics	HSP60 expression		p-value
	Negative	Positive	
Age (years)			0.03*
< 50	11 (17%)	25 (38%)	
≥ 50	3 (4%)	27 (41%)	
Grade			0.09
Low grade	6 (9%)	11 (17%)	
High grade	8 (12%)	41 (62%)	
Stage			0.05*
Early stage	10 (18%)	23 (41%)	
Advanced stage	2 (3%)	21 (38%)	
pT classification			0.1
T1	3 (5%)	2 (4%)	
T2	5 (9%)	24 (43%)	
T3	4 (7%)	13 (23%)	
T4	0 (0%)	5 (9%)	
Lymph node involvement			0.03*
Negative	12 (18%)	29 (44%)	
Positive	2 (3%)	23 (35%)	
pN classification			0.1
N0	12 (18%)	29 (44%)	
N1	0 (0%)	7 (11%)	
N2	0 (0%)	8 (12%)	
N3	2 (3%)	8 (12%)	
All cases	14 (21%)	52 (79%)	

* *Statistically significant.*

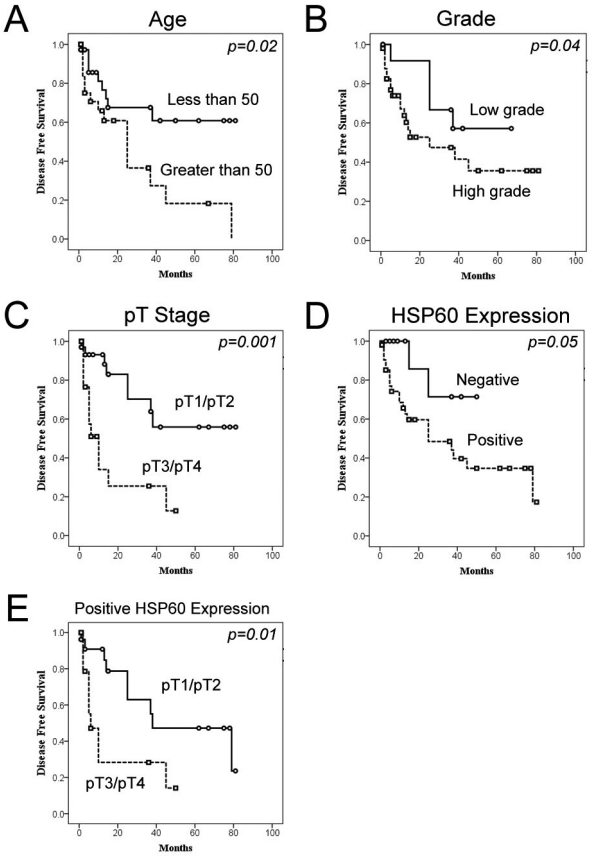

Figure 2. — Kaplan-Meier curves of the analyzed variables. (A) Disease-free survival curve according to the age of the patients. (B) Disease-free survival curve according to the grade of the tumor. (C) Disease-free survival curve according to the pT stage of the tumor. (D) Disease-free survival curve according to Hsp60 expression. (E) Disease-free survival curve according to tumor stage stratified to Hsp60 positive expression.

with high grade of the tumor (p = 0.09). On the other hand, HSP90, HSP70, and HSP27 positive expression showed no significant association with the investigated clinicopathological variables.

The DFS of all 66 cases included in this study was evaluated to show the prognostic value of HSPs expression in TNBC. Characteristics analyzed were patients' age, tumor stage and grade, lymph node involvement, and expression levels of the examined markers (HSP90, HSP70, HSP60, and HSP27). Regarding the clinicopathological variables of the patients, it was found that mean DFS times for patients with an age of less than 50 years and more than 50 years were 54 and 29 months, respectively. Patients with an age of more than 50 years had a significantly worse DFS compared to patients with an age of less than 50 (log rank X^2= 5.207, p = 0.02, Figure 2A). Mean DFS times for patients with low grade and high grade tumors were 48 and 38 months, respectively. Patients with high grade tumors had a marginal significant worse DFS compared to patients with low grade tumors (BreslowX^2= 3.038, p = 0.08, Figure 2B). The mean DFS times for patients with early stage tumors (pT1 and pT2) and late stage tumors (pT3 and pT4) were 55 and 16 months, respectively. Patients with late stage tumors had a significantly worse DFS compared to patients with early stage tumors (log rank X^2= 1.620, p = 0.001, Figure 2C). Mean DFS times for patients with negative and positive lymph nodes involvement were 49 and 32 months, respectively. The authors did not see any differences in DFS between the two groups (log rank X^2=1.477, p = 0.2).

Regarding the positive expression of the analyzed markers, we found that the mean DFS times for patients with negative and positive expression of HSP60 were 41 and 38 months, respectively. Patients with a positive expression of

HSP60 had a significantly worse DFS compared to patients with a negative expression of HSP60 (Breslow X^2= 3.829, p = 0.05, Figure 2D). Stratifying for positive HSP60 expression, patients with advanced stage tumors (pT3 and pT4) had a significantly worse DFS than patients with early stage tumors (pT1 and pT2), as the mean DFS times for patients with early and advanced stage of tumors were 49 and 17 months, respectively (log rank X^2= 5.742, p = 0.01, Figure 2E). In contrast, we could not find any significant effect of the positive expression for HSP90, HSP70, and HSP27 on the DFS times of the patients.

Discussion

TNBC is a heterogeneous and clinically aggressive subgroup of breast cancers that poses a severe health problem worldwide due to the lack of a specifically designed targeted therapy against it [8]. Many studies are underway to identify the exact molecular markers involved in the signaling pathways of TNBC that causes its tumor progression in order to develop targeted therapeutic regimens against it. Heat shock proteins are considered as one of the major emerging possible markers of cancer with active research ongoing to determine their role in cancer pathogenesis. Many studies have proved that HSPs have a major role in cancer pathogenesis as most of its client proteins are involved in maintaining tumor progression; also it was found to be highly expressed in a plethora of cancers [23, 25, 26, 28]. Regarding breast cancer, it was found that HSP90, HSP70, HSP60, and HSP27 are highly expressed in breast tumor tissues and associated with poor prognosis and aggressive behavior [24, 32-36], but few studies have investigated the expression levels of the aforementioned proteins and their prognostic role in TNBC. Accordingly, the present study focused on evaluating the expression levels of HSP90, HSP70, HSP60, and HSP27 in TNBC tissues in order to further investigate their possible role in TNBC pathogenesis and prognosis; thus, they might have a possible therapeutic role using the newly emerging inhibitors of HSPs.

According to the immunohistochemical analysis of the TNBC archived tissues included in this study, we found that HSP60, HSP90, and HSP27 were highly expressed in most of the TNBC tissues, while the expression of HSP70 was mainly reduced in the samples analyzed. HSP90 was also found to be highly expressed in TNBC reported by several studies. Accordingly, Lu *et al.* reported a higher expression of HSP90 in TNBC compared to ER+/PR+/Her2+ breast cancer [37]. Another study was carried out on 23 gene expression datasets of 4,010 breast cancer patients of different subtypes, including TNBC. The study analyzed the expression of different genes and it found that HSP90 is up-regulated in HER2-/ER+ breast cancer and in TNBC [38]. It is worth noticing that the study reported an up-regulation of HSP90 at the RNA level. Therefore, this suggests that HSP90 is up-regulated in breast cancer in both RNA and protein levels, given that consistent results have been found at the protein level. Moreover, several studies reported a significant increase in HSP90 expression in invasive breast cancer cases of ER+ and/or HER2+ subtypes [35, 36, 39, 40]. In contrast, other studies reported a significant decrease in HSP90 expression in breast cancer tissues of ER+ and/or HER2+ subtypes [39, 41, 42]. These studies were performed on breast cancers of lobular neoplasia and infiltrative lobular carcinoma types, and it was reported that these types tend to have a decreased expression of HSP90, while an elevated HSP90 expression is a marker of breast ductal carcinomas [41]. Therefore, the difference in HSP90 expression between TNBC and some other cases of different subtypes of breast cancer could be explained by the notion that TNBC is characterized by having a pathological type of IDC in most cases [43].

Concerning HSP70, we found a major decrease in its expression level, as 59% of the cases analyzed in the present study had a negative expression of HSP70 and the remaining cases showed a weak expression of HSP70 (Table 2). These findings are in contradiction with the literature. Both Barnes *et al.* and Torronteguy *et al.* reported an elevated expression of HSP70 in breast cancer of ER+ and/or HER2+ subtypes [44, 45]. Similarly, Sun *et al.* reported an up-regulated expression of HSP70 in metastatic TNBC [46]. Moreover, HSP70 was also reported to be expressed at higher levels in TNBC tissues compared to ER+/PR+/Her2+ breast cancer [37]. This major discrepancy in the present results with the literature regarding HSP70 expression could be explained by the notion that besides the role of HSP70 in tumorigenesis, it was proven that HSP70 has an anti-tumor immune response activity in which it assists antigen presentation of tumor peptides on the tumor cells in order to develop an immune response against it; thus, aggressive tumor cells tend to down-regulate HSP70 expression levels to evade immuno-surveillance and enhance growth of tumor cells [22]. Moreover, this discrepancy might be attributed to several other differences between the present study and other previous studies; including choice of antibody used, differences in antigen retrieval protocols, choice of immunohistochemistry protocol, using methodologies other than immunohistochemistry, selection of patients concerning their ethnicity, stage, and grade of the tumor, sample size, and finally this discrepancy reflects the heterogeneous nature of TNBC [8]. Regarding the study of Chang *et al.*, unlike the present study, they used a mass spectrometer (LC/MS) analysis to evaluate the differential expression of several proteins in TNBC tissues [37], while Zhang *et al.* used immunohistochemistry protocol to evaluate the expression of HSP70, but with a different choice of antibody used and different antigen retrieval protocol [46].

Few studies have investigated the expression of HSP60 in breast cancers and there is no specific study performed

on TNBC; the present results were consistent with studies showing that HSP60 expression is up-regulated in breast tumor tissues [47-50]. This is the first study analyzing the expression of HSP60 on TNBC archived tissues immunohistochemically.

Regarding HSP27 expression, several studies were consistent with the present results, and reported an elevated expression of HSP27 in breast cancer tissues [51-54]. Similarly, an increased abundance of HSP27 in TNBC tissues compared to normal breast tissues was reported in a proteomics profiling study using tandem mass spectrometry [55], which suggests a consistent up-regulation of HSP27 expression using other methods than immunohistochemistry.

To understand the effect of the differential expression of HSPs in the tumor cases, HSPs expression was assessed for correlation with the clinicopathological variables of analyzed patients in this study. HSP60 expression was found to be significantly associated with several clinicopathological parameters of the corresponding patients, in which positive HSP60 expression was found to be significantly associated with advanced stage (pT3/pT4) of the tumor ($p = 0.05$, Table 4), positive nodal involvement ($p = 0.03$, Table 4), and with an age of more than 50 years ($p = 0.03$, Table 4). To the best of our knowledge, there are no other studies investigating the role of HSP60 expression on TNBC, but other studies done on ER+ and/or HER2+ breast cancers have also found a correlation between HSP60 positive expression and advanced stage of the tumor, and positive nodal status [48-50, 56]. These findings suggest that HSP60 expression is correlated with a more aggressive phenotype of tumors. On the other hand, we did not find any significant correlation between the positive expression of HSP90, HSP27, and HSP70 and the histopathological parameters of the patients included in this study.

The expression of the examined HSPs in the present study was further analyzed using the Spearman's rank correlation test to investigate a possible association between their expression levels. It was found that the expression of HSP27 and HSP60 were negatively correlated ($r_S = -0.294$, $p = 0.008$, Table 3), indicating that when one of them is highly expressed the other one will be down-regulated. As far as we know, this is the first study reporting a negative correlation between HSP27 and HSP60 expression in TNBC. This phenomenon might be explained by the dual roles of HSP60 in apoptosis, in which it can act as either an anti-apoptotic factor that promotes cancer cell survival, or as a pro-apoptotic factor that actually promotes cancer cell death [57]. Given that HSP27 is an anti-apoptotic protein [58], we postulate that an increased expression of HSP27 might have a role in down- regulating the expression of HSP60 when it acts as a pro-apoptotic protein, as a self protecting mechanism of cancer cells.

DFS analysis was conducted to examine the effect of HSPs and the clinicopathological variables on the recurrence rates of the cohort. Regarding the clinicopathological variables of the studied patients, we found that patients with an age of more than 50 years had a significantly worse DFS compared to patients with an age of less than 50 years according to log rank test ($p = 0.02$, Figure 2A). Additionally, patients with late pT stage (pT3/ pT4) tumors had a significantly worse DFS than those with an early pT stage (pT1/ pT2) tumors according to the log rank test ($p = 0.001$, Figure 2C). Previous studies have reported similar results associating late stage tumors with worse DFS in TNBC patients [7, 9, 10].

DFS analysis was also performed to examine the effect of HSP60 positive expression on the recurrence rates of the patients analyzed in this study, and it was found that patients with positive expression of HSP60 had a significantly worse DFS compared to patients with a negative expression of HSP60 according to the Breslow test ($p = 0.05$, Figure 2D). Also, HSP60 positive expression was significantly associated with worse DFS in advanced stage (pT3/ pT4) subgroup according to log rank test ($p = 0.01$, Figure 2E). Such results strongly suggest a potential prognostic value of HSP60 in TNBC, given that HSP60 positive expression is not only significantly associated with an aggressive phenotype of the tumor, but also it significantly increases the recurrence rates of TNBC patients. Several studies support the present findings in breast cancer of other subtypes like ER+ and/or HER2+ subtypes [48-50, 56], but as far as the present authors know, the prognostic value of HSP60 in TNBC was not previously examined.

Collectively, the present study demonstrated an increased expression of HSP60 in TNBC tissues. HSP60 was found to be strongly expressed in most of the analyzed cases (79%) and their strong expression was significantly associated with advanced stage of the tumor, older age, and positive nodal status, which suggest that HSP60 expression is associated with aggressive phenotype of the tumor. In addition, HSP60 expression influenced the recurrence rates of the analyzed TNBC patients. Such findings are considered to be a strong evidence of a potential prognostic role of HSP60 expression in TNBC. The importance of HSP60 expression in TNBC and its prognostic role is that it can be used in the treatment management and personalized medicine of patients with more aggressive TNBC, in which inhibitors targeted against the down-regulation of HSP60 could be developed and used as treatment plan for TNBC patients with advanced stage of tumor and positive nodal status. Nonetheless, few inhibitory drugs were developed to target HSP60, such as epolactaene and mizoribine which are still in the pre-clinical stages and have not yet been tested on human cancers [59].

Indeed, TNBC is an extremely aggressive and heterogeneous disease that demonstrates poor prognosis and survival outcome in patients, and given that it is triple negative regarding ER, PR, and HER2, targeted therapies like tamoxifen and Herceptin are of no value for the treatment of

TNBC, with no other option for therapy except chemotherapy. Accordingly, this highlights the urgent importance of developing targeted therapies against certain markers involved in the initiation and progression of this disease. Future research should be highly directed toward getting a deeper look behind what actually occurs on the regulatory level of HSP60 and examine the client proteins involved in its activity. Finally, according to the present results, HSP60 represents a strong candidate for the development of efficient inhibitors against its activity in the fight against TNBC.

Conclusion

HSP60 positive expression was found to be significantly correlated with advanced stage (pT3/pT4) of the tumor, nodal involvement, and older age of patients. Advanced stage (pT3/pT4) of the tumor and older age of the patients were significantly associated with worse DFS and positive expression of HSP60 was shown to be correlated with worse DFS.

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