Journal of Cancer Research and Treatment, 2018, Vol. 6, No. 2, 47-53
Available online at http://pubs.sciepub.com/jcrt/6/2/4

DOI:10.12691/jcrt-6-2-4

Using a Panel of Heat Shock Proteins for Diagnosis and Prognosis of Breast Cancer

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Abstract **Purpose:** The current work aimed to evaluate the diagnostic and prognostic role of a panel of heat shock proteins; HSP27, HSP60, HSP70 and HSP90, in sera of breast cancer patients, in comparison with CA 15.3 as the standard marker in breast cancer management. **Methodology:** The study included 248 females diagnosed with primary breast cancer and 232 normal healthy females. Patients were managed by modified radical mastectomy or breast conservative surgery then received adjuvant therapy and clinically followed up for 5 years. In sera of all patients and controls; HSP27, HSP60, HSP70 and HSP90 were measured by ELISA while CA 15.3 was measured by IRMA. **Findings:** Pre-treatment serum levels of HSP27, HSP60, HSP70 and HSP90 were significantly elevated in breast cancer patients than in controls, furthermore, they were significant as diagnostic markers, especially when using a panel of HSP70≥9.2 ng/ml and HSP60≥7.5 ng/ml with AUC, sensitivity and specificity of 0.995, 98% and 95%; respectively. As prognostic markers, patients with elevated serum HSP27, HSP60 or HSP70 had significantly lower DFS than patients having lower serum levels. In addition, in patients with at least two HSPs of the three above their cutoffs, the HR increased to 4.65, and it jumped to 17.88 for patients having all three HSPs elevated above their respective cutoff values. CA 15.3 was not significant as diagnostic or prognostic marker. **Conclusion:** A panel of pre-treatment serum HSP70≥9.2 ng/ml and HSP60≥7.5 ng/ml may be useful for screening of populations at high risk of developing breast cancer. For prediction of treatment response, patients who have elevated pre-treatment serum HSP27, HSP60 or HSP70 had significantly lower DFS. In addition, the risk of relapse in patients having at least two HSPs and patients having all three HSPs elevated above their respective cutoffs were 4.65-times and 17.88-times that of patients with HSPs below their respective cutoffs.

Keywords: *breast cancer, heat shock proteins, diagnosis, prognosis*

Cite This Article: Ebtsam R. Zaher, Mahmoud A. Hemida, Mohammad M. El-Hashash, and Heba G. El-Sheridy, "Using a Panel of Heat Shock Proteins for Diagnosis and Prognosis of Breast Cancer." *Journal of Cancer Research and Treatment*, vol. 6, no. 2 (2018): 47-53. doi: 10.12691/jcrt-6-2-4.

1. Introduction

Breast cancer is the most frequent cancer among women in 140 of 184 countries worldwide including Egypt, and the second leading cause of cancer-related mortality in females. Notably, the incidence rates vary dramatically across the globe, being always highest in more developed regions. [1] At present, breast cancer detection relies mostly on mammography; however, mammography screening has generated controversy due to the risks of false-positive results. Mammography also has limited sensitivity for detection of tumors in dense breast tissue. [2] There is thus an urgent need for early biomarkers that could predict disease outcome, providing prognostic information to the clinician for treatment stratification. In addition, blood testing is more acceptable by patients and would also circumvent the problems associated with imaging high-density breast tissue. There are currently no blood-borne biomarkers recommended for breast cancer diagnosis or screening. CA15-3 is perhaps the best known, non-invasive marker of breast cancer, although its recommended clinical use is restricted to monitoring of patients with metastatic disease during active therapy.

Heat shock proteins (HSPs) are intracellular, evolutionary conserved proteins with a very important role in maintaining homeostasis of the cells by holding and folding other proteins as well as by protecting the genetic information. HSPs are usually divided into families according to their molecular weight; 10 kD (HSP10), 27 kD (HSP27), 40 kD (HSP40), 60 kD (HSP60), 70 kD (HSP70), 90 kD (HSP90), and 110 kD (HJSP110). These HSPs primarily play essential, but diverse roles in tumorigenesis and metastasis formation, by promoting autonomous cell proliferation and inhibiting apoptosis. Due to the loss of p53 function and the greater expression of the proto-oncogenes HER2 and c-Myc, the transcription of HSPs is increased in certain tumor cells. [3]

Although HSPs are intracellular, they can be released from the cells and become detectable in the blood as soluble HSPs. Since the serum levels of soluble HSPs can markedly change in different diseases, measurement of

HSP concentration may provide clinically important information. [4]

The current work aimed to evaluate the diagnostic and prognostic role of a panel of heat shock proteins; HSP27, HSP60, HSP70 and HSP90, in sera of breast cancer patients.

2. Subjects and Methods

The study included 480 females in two groups; group 1 included 248 females diagnosed with primary breast cancer free of metastasis and Group 2 included 232 normal healthy females matching group 1 in age as a control group. The study was approved by the local ethics committee and each subject provided an informed written consent before sampling. The study was conducted during the period from October 2012 till October2017.

Clinicopathological data of patients were recorded including age, menopausal status, tumor size, nuclear grade, histological type, status and number of positive axillary lymph nodes, presence of lymphovascular invasion, status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (Her-2) expression. Breast cancer staging was defined according to the eighth edition of the Cancer Staging Manual of the AJCC. [5]

Patients were treated primarily with Modified Radical Mastectomy or conservative breast surgery with or without reconstruction according to stage. [6] Adjuvant chemotherapy regimens included 3-6 anthracycline-based regimens with or without the addition of taxanes. [7] Postoperative radiotherapy was administered according to our institutional guidelines. Adjuvant hormonal treatment was administered according to hormonal receptor status. Patients were clinically followed up for a mean time of 5 years (49-66 months) after completing chemotherapy.

A single blood sample was drawn from each patient before surgery and from each control subject. After sampling, sera from all samples were stored at -70°C till time of assay.

HSP27 was assayed by Human HSPB1 (HSP27) ELISA Kit (Thermo Fisher Scientific Inc., USA), HSP70 was assayed by Human HSPA4 (HSP70) ELISA Kit (Thermo Fisher Scientific Inc., USA) and HSP90 was assayed by Human HSP90 alpha Platinum ELISA kit (Thermo Fisher Scientific Inc., USA). HSP60 was assayed by HSP60 (human), ELIZA kit (Enzo Life Sciences Inc., N.Y., U.S.A.)

According to the manufacturer's instructions, 100µl of each standard, sample or control are dispensed into appropriate wells and after incubation for 2.5/1 hr, wells were aspirated and washed. 100µl of biotinylated antibody were incubated for 1hr at room temperature (RT) then wells were aspirated and washed. 100µl of streptavidine-HPR solution were incubated for 45/30 min. at RT then wells were aspirated and washed. 100µl of TMB substrate were incubated for 30 min. then absorbance was measured at 450 nm. A standard curve was constructed for each of the assayed parameters by plotting the average absorbance for each standard concentration on the Y-axis versus the corresponding parameter concentration on the X-axis. Sample and controls concentrations were assessed by interpolation from the standard curves.

CA 15.3 was measured in all serum samples collected by Immunoradiometric Assay (IRMA) using a CA 15-3-IRMA kit (DIAsource ImmunoAssays S.A., Nivelles, Belgium). According to maneufacturer's instructions, 20µl of each sample were diluted with 500µl diluent and 50 µl of diluted sample or calibrator were dispensed into antibody-coated tubes. The tubes were aspirated and washed twice, then 50µl of ^{125}I-labelled anti-CA 15.3 were added to each tube, after incubation for 90 min/RT on shaker, tubes were aspirated, washed twice and counted on a Gamma Counter. A standard curve was constructed by plotting the % binding for each standard concentration on the Y-axis versus concentration on the X-axis. Sample concentrations were determined by interpolation from the standard curve.

3. Statistical Analysis

Statistical analysis was performed using the SPSS (Statistical Package for the Social Sciences) software version 22.0 (SPSS Inc., Chicago, IL, USA).

For variables' description, the mean (M) and standard deviation (SD) were used for quantitative, normally distributed variables and the median and range were used for the description of quantitative, randomly distributed variables.

The diagnostic accuracy of biochemical parameters was assessed using Receiver Operator Characteristic (ROC) curve. For the Area Under the Curve (AUC) of a parameter to be significant, its 95% lower confidence interval should be above 0.50. Sensitivity and specificity were calculated using the cutoff with the highest diagnostic accuracy obtained from the curve. Mann Whitney-U test was used to study the statistical significance in the association of quantitative variables with clinicopathological features of breast cancer patients. The difference was considered significant at $p \leq 0.05$.

Disease free survival (DFS) methods were applied using metastasis or recurrence as end point. The survival curve was calculated by the Kaplan-Meier method, based on the log-rank test and by Cox-regression analysis. Hazard ratios (HRs) with 95% confidence intervals (CIs) were used to convey the effect of the marker on DFS.

4. Results

The study included 248 breast cancer patients and 232 females as controls. The clinicopathological characterization of the patients' group is presented in Table 1.

The age of the cohort ranged from 37 to 74 years, with a median of 48 years. The mean tumor size was 4.6 cm with an SD of 3.4 cm. Half the cases were of histological grade II with only 8.9% of grade I, but regarding the clinical stage, the majority of cases (48.4%) were of grade III. Most of the cases were positive for estrogen (71.0%) and progesterone (66.5%) receptors. More than half the cohort showed positive lymph node involvement (56.5%) and vascular invasion (57.3%).

Table 2 represents serum levels of HSP27, HSP60, HSP70, HSP90 and CA 15.3 in breast cancer patients and controls. At diagnosis, levels of all HSPs were

significantly elevated in sera of breast cancer patients than in control. Mean HSP27 in breast cancer patients was 13.81 ng/ml while in controls the mean was 3.25 ng/ml, the two means were significantly different (p <0.001). The same was true for HSP60 levels (12.28 vs 4.62 ng/ml; respectively, p =0.005), HSP70 levels (15.51 vs 6.57 ng/ml; respectively, p <0.001) and HSP90 levels (54.67 vs 27.54 ng/ml; respectively, p =0.037). CA 15.3 mean level in breast cancer patients was higher than control level (28.73 vs 11.61 ng/ml; respectively), however, the difference was not statistically significant (p =0.082).

The diagnostic potential of all studied parameters were assessed by ROC curves, Figure 1. All HSPs shows statistical asymptomatic significance (p <0.001, <0.001, 0.017 and 0.037 for HSP70, HSP60, HSP27 and HSP90; respectively), but not CA 15.3 (p=0.354). Cutoff values were chosen for all parameters so as to keep the specificity above 80%, as shown in Table 2. HSP70 showed the highest AUC (0.918) and at a cutoff value of 9.2 ng/ml, it had a sensitivity of 88.5% and specificity of 85.7%. HSP60 showed a high AUC of 0.890, and at a cutoff 7.6 ng/ml, its sensitivity and specificity were 80.9% and 83.2%; respectively. HSP27 showed an AUC of 0.801 and its optimum cutoff was 6.1 ng/ml with a sensitivity of 70.1% and specificity of 81.3%. HSP90 curve had an AUC of 0.773, and at a cutoff value of 35.0 ng/ml, its sensitivity and specificity were 70.1% and 84.5%; respectively. CA 15.3 showed the least AUC (0.606), its optimum cutoff was 20.7 ng/ml with a sensitivity of 47.3% and specificity of 82.1%. The addition of HSP70 and HSP60 resulted in an increased AUC to 0.995 which was significantly higher than either HSP70 or HSP60 alone (p =0.005 and <0.001; respectively) with 98% sensitivity and 95%specificity. None of the other combinations resulted in a significant increase in AUC than HSP27 alone.

Table 1. Description of the clinicopathological features of breast cancer patients

Characteristic		Breast cancer (248) Number (%)
Age (years)	median	48
	Range	37 - 74
Tumor Size (Mean± SD; cm)		4.6 ± 3.4
Histological Grade	I	22 (8.9)
	II	125 (50.4)
	III	101 (40.7)
Clinical Stage	I	22 (8.9)
	II	106 (42.7)
	III	120 (48.4)
Estrogen receptor	-ve	72 (29.0)
	+ve	176 (71.0)
Progesterone receptor	-ve	83 (33.5)
	+ve	165 (66.5)
Lymph node invasion	absent	108 (43.5)
	Present	140 (56.5)
Vascular invasion	absent	106 (42.7)
	Present	142 (57.3)

Table 2. Levels of all parameters investigated presented as range and M±SD in breast cancer and control groups.

Serum levels (ng/ml)	Controls	Breast cancer	p
HSP27	0.00 – 8.13 3.25 ± 1.72	1.12 – 47.45 13.81 ± 11.37	<0.001
HSP60	2.61 – 8.97 4.62 ± 2.17	3.87 – 35.11 12.28 ± 7.15	0.005
HSP70	2.33 – 19.21 6.57 ± 2.07	3.17 – 45.61 15.51 ± 7.22	<0.001
HSP90	10.10 – 89.28 27.54 ± 27.48	10.00 – 290.70 54.67 ± 46.16	0.037
CA 15.3	0 – 29.18 11.61 ± 8.37	0 – 83.11 28.73 ± 28.51	0.082

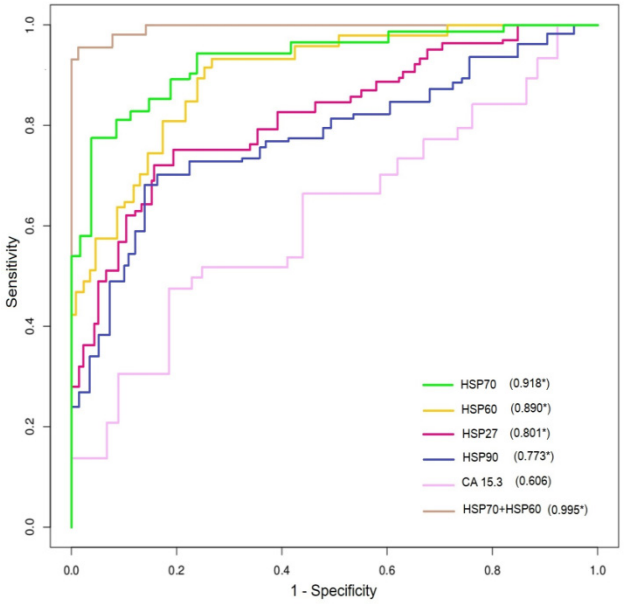

Figure 1. ROC curve for all parameters under investigation

Table 3. Diagnostic potential of HSPs and CA 15.3 for breast cancer presented as AUC, optimum cutoff, sensitivity and specificity as depicted from ROC curves

Parameter	AUC	p	Cutoff (ng/ml)	Sensitivity	Specificity
HSP70	0.918	<0.001	9.2	88.5	85.5
HSP60	0.890	<0.001	7.6	80.9	83.2
HSP27	0.801	0.008	6.1	75.1	81.3
HSP90	0.773	0.013	35.0	70.1	84.5
CA 15.3	0.606	0.354	20.7	47.3	82.1

Table 4. Association between serum HSPs and CA 15.3 levels and clinicopathological features of breast cancer patients

Clinicopathologic parameter	HSP27	HSP60	HSP70	HSP90	CA 15.3
Tumor size	0.077	0.286	0.152	0.063	**0.037**
Clinical Stage	0.339	0.472	0.419	**0.041**	0.197
Histological Grade	0.267	0.389	**0.047**	0.117	0.405
Estrogen Receptor	**0.020**	0.118	0.762	0.825	0.383
Lymph Node +ve	**0.043**	**0.027**	0.591	0.078	0.174
Vascular Invasion	0.719	**0.002**	0.083	0.216	0.538

Mann-Whitney *U* test, the test is considered significant when p ≤ 0.05.

The association between elevated serum levels of each parameter above its respective cutoff value and the clinicopathological features of breast cancer patients have been investigated, Table 4. Only few associations have been found; including; elevated HSP27 with positive estrogen receptor and lymph node metastasis, elevated HSP60 with lymph node metastasis and vascular invasion, elevated HSP70 with higher histological grade, elevated HSP90 with advanced clinical stage and elevated CA 15.3 with larger tumor size.

After follow up of breast cancer patients for 5 years, 170 patients (68.5%) remained free while 78 patients (31.5%) relapsed. Patients were analyzed for DSF according to each of the studied parameters (Figure 2). Breast cancer patients with HSP27 ≥6.1 ng/ml had a significantly lower DFS than patients with HSP27 <6.1 ng/ml (27.0 vs 48.8 months; p<0.001). Also patients with HSP60 ≥7.6 ng/ml had a significantly lower DFS than patients with HSP60 <7.6 ng/ml (25.6 vs 49.6 months; p<0.001), and patients with HSP70 ≥9.2 ng/ml had a significantly shorter DFS than patients with HSP70 <9.2 ng/ml (24.9 vs 48.1 months; p<0.001). Meanwhile neither HSP90 nor CA 15.3 elevation above their respective cutoffs reflect any significant difference on DFS of breast cancer patients (p=0.186 and 0.325; respectively).

Table 5 represents the HR of each of the significant parameters (HSP27, HSP60 and HSP70) on DFS of breast cancer patients. The highest impact on survival was for HSP60≥6.1 ng/ml, with a HR of 2.34 (p<0.001). HSP70 and HSP27 also had a significant impact on DFS (p<0.001 and <0.001; respectively) with HRs 2.10 for HSP70 and 1.72 for HSP27. When analyzing the impact of having at least two HSPs of the three above their cutoffs, the HR increased to 4.65, and it jumped to 17.88 for the group of patients having all three HSPs elevated above their respective cutoff values.

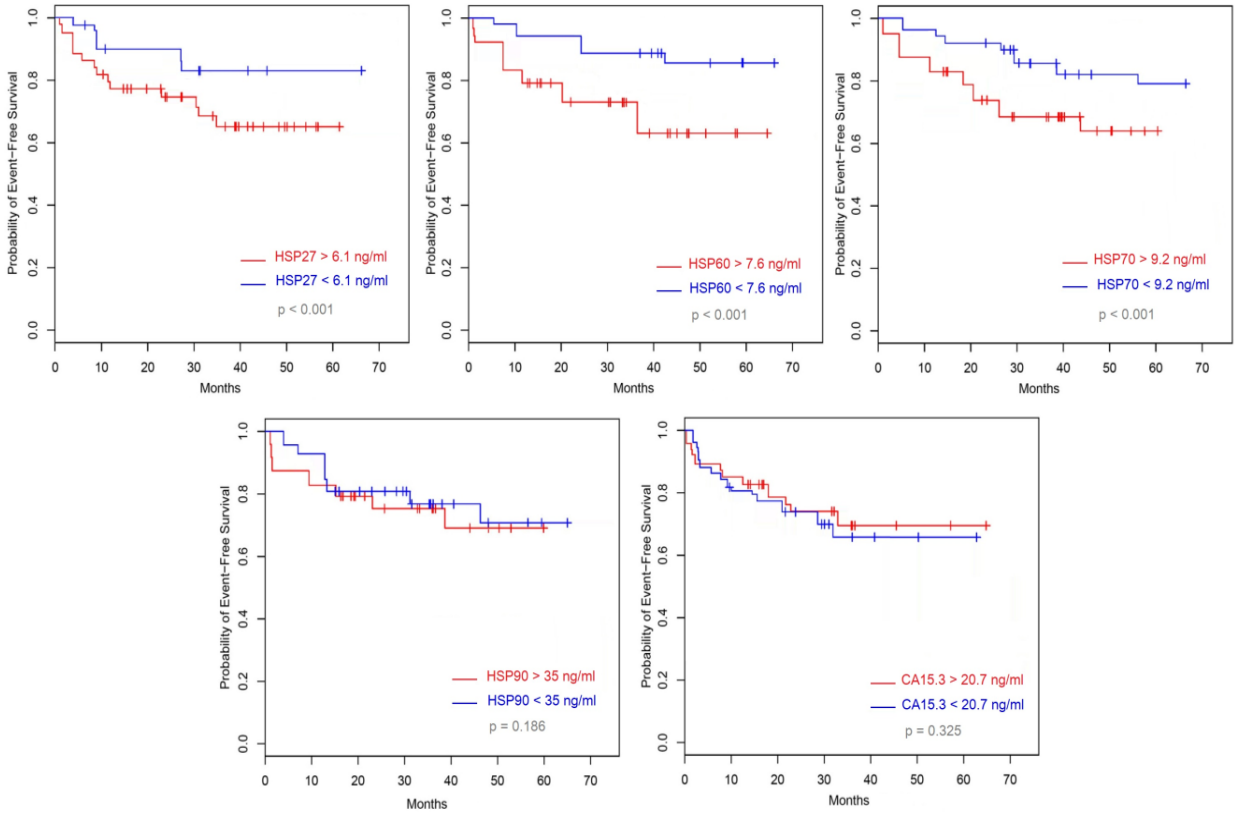

Figure 2. Kaplan-Meier disease-free survival curves of HSP27, HSP60, HSP70, HSP90 and CA 15.3

Table 5. Impact of elevated serum HSPs levels on disease-free survival of breast cancer patients

HSP	No. of patients (%) N = 248	5-year survival rate	HR (95% CI)	*p*
HSP27				
≥6.1 ng/ml	186 (75.0 %)	65.1 %	1.72 (1.16–3.27)	<0.001
<6.1 ng/ml	62 (25.0 %)	79.0 %		
HSP60				
≥7.6 ng/ml	198 (79.8 %)	64.1 %	2.34 (1.28-4.89)	<0.001
<7.6 ng/ml	50 (20.2 %)	86.0 %		
HSP70				
≥9.2 ng/ml	219 (88.3 %)	66.7 %	2.10 (1.03-4.49)	<0.001
<9.2 ng/ml	29 (11.7 %)	82.8 %		
2 HSPs				
2/3 HSPs ≥ cutoff	116 (46.8 %)	39.7 %	4.65 (2.42-8.30)	<0.001
Less than 2 HSPs ≥cutoff	132 (53.2 %)	93.9 %		
3 HSPs				
3 HSPs ≥ cutoff	82 (33.1 %)	7.3 %	17.88 (8.91-28.64)	<0.001
Less than 3 HSPs ≥cutoff	166 (66.9 %)	98.7 %		

5. Discussion

Virtually all cells respond to stress through the activation of primitive, evolutionarily conserved genetic programs that maintain homeostasis and assure survival. *Stress adaptation* is a common paradigm found throughout nature in which a primary exposure of a cell to a stressful stimulus results in an adaptive response, which is not unique to the original stimulus. [8] The heat shock response is an ancient, highly conserved, endogenous stress adaptation defense mechanism characterized by the rapid upregulation of a specific class of proteins known as heat shock proteins (HSPs). [9] The structure, mode of regulation, and function of HSPs are phylogenetically conserved among different species, and HSPs have been isolated from virtually every class of living organism to date, including both prokaryotes and eukaryotes. These proteins range in molecular weight from 7 kDa to 110 kDa and have been found in virtually every part of the cell. By convention, the HSPs are grouped and classified into families based upon their molecular weight. [10]

HSPs often act in concert, in large multiprotein complexes known as molecular chaperones. HSPs are involved in the repair of cellular damage induced by the stress, which is necessary for insult resolution. Moreover, HSP confer protection from subsequent insults, which has been coined stress tolerance. [11] The HSPs are generally thought to maintain cellular homeostasis by acting as molecular chaperones, facilitating the proper folding and assembly of nascent polypeptides and assisting in the refolding and stabilization of damaged peptides. [8,13] HSPs have traditionally been considered to be exclusively intracellular proteins, however, they can also be found in the extracellular environment. How HSPs are found in the extracellular medium is still a debated issue since HSPs lack the consensus signal for secretion via the classical Golgi pathway. The first hypothesis was attributed to spontaneous cell death leading to HSP extravasation. However, it was found that undamaged, live cells released HSPs by an active non-classical secretory pathway. [14] Since that time, there has been a virtual explosion of literature on the biology of extracellular HSPs. Perhaps even more intriguing is the relatively recent recognition that extracellular HSPs possess the ability to stimulate many cells of the innate and adaptive immune systems. [15]

HSPs functions are altered in oncogenesis allowing malignant transformation with upregulation of stress-related genes and increased synthesis of intracellular and extracellular HSPs. This results in HSPs being tumor-protective through mechanisms such as anti-oxidative processes, the prevention of protein denaturation, anti-apoptotic activity, and even the direct suppression of the immune system. It has also been shown that stress proteins participate in the folding of numerous proto-oncogenes and oncogenes. [16] It is unclear how HSPs become overexpressed in cancer. One hypothesis is that the physiopathological features of tumor microenvironment tend toward HSP induction. Another hypothesis is that HSPs may well be related to the genetic changes associated with tumor progression. [17] Also Oncoproteins may appear during carcinogenesis and these mutated and conformationally altered proteins may elicit a dramatic change in the HSP context and behavior. However, the exact mechanisms are yet to be determined although they likely involve molecular changes common to a wide range of cancer cells. [18]

HSPs expression has been extensively studied and they were found overexpressed in a wide range of malignant cells and tissues. Also the diagnostic and prognostic roles of HSPs overexpression have been widely evaluted. However, the serum profile of HSPs has been studied to a much lesser extent as their presence in the serum of cancer patients is still a new research area and to date they have never been evaluated as a panel in cancer.

The current work aimed to evaluate the diagnostic and prognostic role of a panel of heat shock proteins; HSP27, HSP60, HSP70 and HSP90, in sera of breast cancer patients, in comparison with CA 15.3 as the standard marker in breast cancer management.

In the current study, mean serum levels of all HSPs under investigation; namely HSP27, HSP60, HSP70 and HSP90; in breast cancer patients were significantly higher than in controls. Similar findings have been reported for each HSP individually, where HSP27 [19], HSP60 [20], HSP70 [21] and HSP90 [22]. However, the exact serum levels in cancer and control subjects were largely variable, depending on the method used for quantification and the antibody used for detection.

For diagnosis, we found that the best markers were serum HSP70 and HSP60 and to a lesser extent HSP27 and HSP90. A panel of HSP70 and HSP60 resulted in a much higher prognostic significance (AUC=0.995), with 98% sensitivity and 95% specificity. Meanwhile, CA 15.3 did not show any diagnostic significance, or added any value to other HSPs under investigation. Controversial reports regarding the diagnostic significance of HSPs were found, however, their clinical use might be largely hindered by the fact that they are widely upregulated in several cancer types and not specific for breast cancer. [14,16,18,23,24] However, a panel of more than one serum HSP could be a good candidate for a universal cancer detection marker(s). In that regard, the excellent sensitivity and specificity of serum HSP70/HSP60 as a panel in breast cancer may be of significance for screening or follow up of populations at high risk of developing breast cancer.

Serum HSPs levels also did not present enough significant association with clinicopathological characters of breast cancer patients, as the few significant associations found in the current study would not be sufficient to abrogate the classical histological evaluations that are currently in use.

After follow up of breast cancer patients for 5 years, 170 patients (68.5%) remained free while 78 patients (31.5%) relapsed. For prognostic evaluation, monitoring of serum HSPs levels after treatment were not found beneficial, due to huge discrepancy in data after treatment with chemo- and radiotherapy (data not shown) which necessitates further data analysis for that aspect. Instead, initial serum HSPs levels at presentation were evaluated for prediction of treatment outcome presented as DFS.

When patients were analyzed for DSF according to each of the studied parameters, only HSP27, HSP60 and HSP70 showed a significant impact on DFS of breast cancer patients. The highest impact on survival was for

HSP60≥6.1 ng/ml, with an HR of 2.34 (p<0.001). HSP70 and HSP27 also had a significant impact on DFS (p<0.001 and <0.001; respectively) with HRs 2.10 for HSP70 and 1.72 for HSP27. Meanwhile neither HSP90 nor CA 15.3 above their respective cutoffs reflect any significant difference on DFS of breast cancer patients (p=0.186 and 0.325; respectively).

Expression patterns of HSPs have been extensively studied as prognostic markers on various tumor tissues, and many HSPs were proven to be worthy prognostic markers in breast cancer tissues especially HSP70 and HSP90. [18] That was the main rational behind targeting HSPs as a new way of therapy, since they seem to be crucially required for cancer progression and metastasis. [25,26,27] However, prognostic value of serum HSPs has been rarely discussed. We came into only one study reporting HSP27 as a useful prognostic marker, after only 12 months of follow up. [28]

Several molecular mechanisms are involving HSPs in resistance to cancer therapies including; (1) as molecular chaperones they can confer cytoprotection by repairing more efficiently the damaged proteins resulting from cytotoxic drug administration, (2) protecting cancer cells from apoptosis, [29] (3) protecting the microvasculature inside tumors, [30] and (4) enhancing DNA repair [31].

When analyzing the impact of having at least two HSPs of the three above their cutoffs, the HR increased to 4.65, and it jumped to 17.88 for the group of patients having all three HSPs elevated above their respective cutoff values. Only 7.3% of patients having all 3 HSPs elevated above their cutoff levels remained free of disease after 5 years, which conveys the significance of using this panel for prediction of treatment outcome.

In conclusion, pre-treatment serum levels of HSP27, HSP60, HSP70 and HSP90 were significantly elevated in breast cancer patients than in controls, furthermore, they were significant as diagnostic markers, specially using a panel of HSP70≥9.2 ng/ml and HSP60≥7.5 ng/ml with AUC, sensitivity and specificity of 0.995, 98% and 95%; respectively. As predictive markers for response to treatment, patients with elevated serum HSP27, HSP60 or HSP70 had significantly lower DFS than patients having lower serum levels. In addition, in patients with at least two HSPs of the three above their cutoffs, the HR increased to 4.65, and it jumped to 17.88 for patients having all three HSPs elevated above their respective cutoff values.

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