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Elevated Serum Levels of Heat Shock Protein 70 Are Associated with Breast Cancer

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Breast cancer (BC) is the most frequent cause of cancer death in women throughout the world. Thus, it is necessary to establish sensitive screening, diagnosis and treatment methods for BC. Heat shock protein 70 (HSP70) is an important cellular stress response protein that protects cells from apoptosis. Recent studies have shown that serum HSP70 levels may provide clinically important information in various types of cancer. HSP70 is also overexpressed in BC, which is known to be associated with cancer progression, apoptosis and cell proliferation. However, the serum level of HSP70 and its diagnostic and prognostic potential in BC have not been investigated yet. The aim of this study was to determine the usefulness of serum HSP70 level as a *diagnostic test* and its predictive value in patients with BC. This prospective study consisted of 45 female patients diagnosed with BC and 16 healthy women who were matched for age and body mass index (BMI). Enzyme-linked immunosorbent assay (ELISA) technique was used to measure the serum level of HSP70. The serum level of HSP70 was significantly higher in patients with BC than in the healthy control group (5.98 ± 2.05 vs. 1.49 ± 0.47 ng/ml, $p = 0.001$). HSP70 level > 2.41 ng/ml was the best cutoff value to predict BC (97.78% sensitivity and 93.75% specificity). This study shows that HSP70 can be used as an adjunct to other diagnostic tests for BC and may be helpful for identifying patients at increased risk of BC.

Keywords: breast cancer; cut-off value; diagnosis; heat shock protein 70; target therapy
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Introduction

Globally, breast cancer (BC) is the most frequently diagnosed cancer and the leading cause of cancer death in women (Kesson et al. 2012). Increase in molecular and genetic studies relevant to carcinogenesis, which contributes to develop new screening, diagnosis and treatment methods, improves the course of cancer. (Xia et al. 2014; Li et al. 2015).

Heat shock proteins (HSPs) perform essential functions in maintaining cellular homeostasis (Vidyasagar et al. 2012). The eight members of HSPs family are highly homologous, exhibiting between 52 and 99% amino acid identity (Seigneuric et al. 2011). HSPs, as being essential determinants of protein quality control in the cell, are responsible for maintenance of protein homeostasis. They are classified according to the size (Ledford 2011). Under stressful conditions cells may initiate a process of self-destruction known as apoptosis. Cancer cells respond to stress by adaptive changes that increase their ability to tolerate normally lethal conditions. Increased expression of the heat shock protein 70 (HSP70) is an important cellular stress response that protects healthy or cancer cells from apoptosis (Naylor and Hartl 2001).

Recent studies have shown that HSP70 is often highly expressed in various types of cancers (Ledford 2011). A complex network of protein quality-control mechanisms, including chaperoning by HSP70, is not only essential for maintaining the extravagant proteomic lifestyle of cancer cells but also represents an ideal cancer-specific target to be tackled (Bruning and Juckstock 2015). Recent evidence supports that HSP70 is actively involved in tumor cell proliferation, invasion, differentiation, metastases and death (Joly et al. 2010). HSP70 has a pivotal role in several

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checkpoints of apoptosis (Lianos et al. 2015). Therefore, HSP70 has been considered an ideal target for cancer treatment and several clinical trials with HSP inhibitors are currently under way. Serum HSP70 may provide clinically important information in various types of cancer. In recent studies, serum HSP70 has been shown as a biomarker for early detection of pancreatic cancer (Dutta et al. 2012) and as an indicator for high mortality in patients with colorectal cancer without distant metastasis (Kocsis et al. 2011). In addition, its elevated serum levels were associated with increased risk of lung cancer (Suzuki et al. 2006).

Until now, serum levels of HSP70 and their credibility in diagnosing BC have not been investigated. We aimed to investigate the association between serum levels of HSP70 and clinicopathological features in patients with BC, as well as to define whether circulating HSP70 was a useful diagnostic biomarker for BC. Considering that HSP70 is a possible targeted therapy in the future, we also tried to calculate its ideal cut-off value to use whenever necessary.

Materials and Methods

This clinical trial was conducted at the department of medical oncology of our hospital. A total number of 57 patients with BC were admitted into the study. Twelve patients were excluded from the study. BC patients who had undergone chemotherapy or radiation therapy and patients with non-regulated chronic diseases (kidney diseases, cardiovascular disorders, rheumatological diseases, etc.) were excluded. Forty-five patients and sixteen healthy women who were matched for age and body mass index (BMI) were enrolled in the study. The patients' characteristics with respect to age, BMI, menopausal status, histopathological type, tumor invasion depth, lymph node metastasis status, grade, and estrogen receptor (ER), progesterone receptor (PR) and HER2 (human epidermal growth factor receptor 2), lymphovascular invasion (LVI), perineural invasion (PNI) and stage were recorded for data analyses. Our hospital's ethical committee approved the study and written informed consent was provided by the patients prior to the assessment.

Tumor differentiation was defined according to the World Health Organization (WHO) classification of tumors (World Health Organization 2003). The pathological tumor stage was defined according to the 7th edition of the tumor-node-metastasis (TNM) classification of the American Joint Committee on Cancer (AJCC) (Edge et al. 2010). ER, PR and HER2 data were obtained from patients' pathology records. Biologic subclassification using ER, PR, and HER2 was performed. Luminal A tumors were defined as ER+, PR+, and HER2−; Luminal B tumors were defined as ER+, PR−, and HER2− or ER+, PR+, and HER2+; HER2 like tumor was defined as ER−, PR+, and HER2+; and triple negative tumors were negative for ER, PR, and HER2 (Huober et al. 2010).

Blood sample collection

Blood samples of patients were drawn before initiating chemo(radio)therapy and/or surgical resection of tumor. After an overnight fasting period, venous blood samples were drawn from the antecubital vein of BC patients and healthy controls. Blood samples were collected in blood collection tubes and centrifuged at 4,000 g (10 min) to remove the serum. Serum samples were kept at −80°C until the analysis of HSP70 levels.

Measurement of HSP70

The serum level of HSP70 was assessed by the enzyme-linked immunosorbent assay (ELISA) technique. Serum HSP70 was analysed using Human Heat Shock Protein 70 (HSP70), (Cat no: 20,959) purchased from Bio Medical Assay (BMASSAY, Beijing, China) following the manufacturer's instructions. Their levels were expressed as ng/ml. The detection and quantification limits were set at < 0.05 ng/ml for HSP70. The ELISA kit shows no cross reactivity with any of the cytokines. The intra-assay coefficient variations (CV) of HSP70 was 8.2%.

Other variables

Complete blood count was determined in a Coulter LH 750 autoanalyser (Beckman Coulter, CA, USA). Serum CA 15-3 (Cancer Antigen 15-3) were determined by Beckman Coulter AU 5800 chemistry auto-analyser and DXI 800 systems by using commercial kits (Beckman Coulter, CA, USA). Reference range: Ca 15-3: 0-31.3 U/ml.

Statistical analysis

Statistical analysis was done by NCSS (Number Cruncher Statistical System) 2007&PASS (Power Analysis and Sample Size), 2008 Statistical Software (Utah, USA). During the evaluation of study variables, descriptive statistical methods (mean, standard error, median, rate and ratio) were used. For a comparison of variables of normal distribution, the t-test for independent samples was used and the Kruskal Wallis test and the Mann-Whitney U test were used for the comparison of variables with non-normal distribution. Receiver operating characteristic (ROC) analysis was used for the determination of the possible use of the markers for clinical differentiation between the patient and the control groups. When the area under the curve was found to be significant, the cut-off values were determined and sensitivity and specificity for that particular cut-off point were calculated as well. Results were evaluated in confidence interval. Statistical significance was accepted as $p < 0.05$ in all tests.

Results

Forty-five females with BC and sixteen healthy females (control group) were included in the study. The descriptive features of the patients are summarized in Tables 1 and 2. The mean HSP70 levels of patients and controls were 5.98 ± 2.05 ng/mL and 1.49 ± 0.47 ng/mL, respectively. They were higher in BC patients and also they were statistically significant ($p = 0.001$) (Table 3).

Patients were divided into subgroups, according to presence or absence of metastasis, histological grade of cancer, histopathological subtypes, positive and/or negative results for ER, PR, HER2, TN (+), LVI and/or PNI, existence of obesity, menopausal status, existence of comorbidity, level of serum CA 15-3 and presence or absence of leukocytosis, thrombocytosis, anemia. Multiple comparisons were performed to determine whether there was any difference in serum HSP70 levels among subgroups. Comparison of patients with and without leukocytosis showed that those with leukocytosis had significantly lower serum HSP70 levels than those without leukocytosis (4.36 ± 1.63 vs. 6.16 ± 2.08, respectively; $p = 0.037$) (Table 2). And the rest of the subgroup comparisons showed no statis-

Table 1. Comparison of clinicopathologic features and serum HSP70 level in patients with breast cancer.

$n = 45$		n	%	HSP 70 Mean ± S.D. (Median)	p value
Estrogen receptor	(+)	32	71.1	5.90 ± 2.04 (5.73)	0.661
	(−)	13	28.9	6.17 ± 2.16 (7.20)	
Progesteron receptor	(+)	27	60.0	5.61 ± 2.10 (5.45)	0.126
	(−)	18	40.0	6.54 ± 1.91 (7.01)	
Triple negative	Yes	10	22.2	6.61 ± 1.98 (7.27)	0.253
	No	35	77.8	5.80 ± 2.07 (5.72)	
HER2	(+)	8	17.8	5.72 ± 2.25 (5.22)	0.657
	(−)	37	82.2	6.04 ± 2.04 (5.73)	
Lymphovascular invasion	(+)	20	44.4	5.68 ± 2.19 (5.17)	0.386
	(−)	25	55.6	6.22 ± 1.95 (5.97)	
Perineural invasion	(+)	15	34.9	6.30 ± 1.90 (6.58)	0.583
	(−)	30	65.1	5.98 ± 2.13 (5.48)	
Metastasis	Yes	5	11.1	6.96 ± 2.36 (7.20)	0.309
	No	40	88.9	5.86 ± 0.01 (5.61)	
Grade	1	2	4.9	7.08 ± 1.91 (7.08)	
	2	29	70.7	5.82 ± 2.05 (5.51)	0.382
	3	10	24.4	6.67 ± 2.12 (6.89)	
Histopathological subtype	IDC	33	73.3	6.21 ± 1.99 (6.23)	
	ILC	2	4.4	5.48 ± 0.04 (5.48)	0.463
	Others	10	22.2	5.33 ± 2. 41(4.67)	
Tumor size	T1	10	22.2	5.90 ± 1.59 (5.48)	
	T2	24	53.3	5.65 ± 2.11 (5.59)	0.522
	T3	6	13.3	6.84 ± 2.49 (7.54)	
	T4	5	11.1	6.71 ± 2.21 (6.90)	
Lymph node metastasis	N0	25	55.6	5.77 ± 1.75 (5.72)	
	N1	6	13.3	6.59 ± 2.43 (7.20)	0.773
	N2	9	20.0	6.32 ± 2.67 (6.90)	
	N3	5	11.1	5.72 ± 2.27 (4.62)	
Stage	I	6	13.3	5.71 ± 1.01 (5.48)	
	II	21	46.7	6 ± 2.09 (5.73)	0.759
	III	13	28.9	5.70 ± 2.32 (4.62)	
	IV	5	11.1	6.96 ± 2.36 (7.20)	

S.D., standard deviation; HSP70, heat shock protein 70; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; HER2, human epidermal growth factor receptor.

tical differences ($p \geq 0.05$) (Table 1).

As previous studies indicated that increased BMI was associated with the increased risk of BC in Okinawa (Tamaki et al. 2014), we wanted to determine whether there was an association between HSP70 and obesity. Therefore we divided patients into two subgroups, according to BMI above (obese) or below (non-obese) 30 kg/m^2 (World Health Organization 2011). No relation has been found between HSP70 and obesity ($p \geq 0.05$).

As a result, we found a significant difference in serum HSP70 levels between patients and healthy controls. We also tried to find an optimal cut-off value for HSP70. ROC analysis were used to determine a cut-off value. The area under a ROC curve for HSP70 to detect BC was determined at > 2.41 ng/ml. Using the cut-off value of HSP70 > 2.41 ng/ml, there was sensitivity at 97.78%, specificity at 93.75%, positive predictive value at 97.78%, negative predictive value at 93.75% and accuracy at 96.72% (AUC: 0.996 [0.986-1.000]; $p = 0.001$) (Fig. 1).

Discussion

HSPs have been found to play key roles in tumor immunity mediated by antigen presenting cells, T cells and natural killer (NK) cells. Most immunotherapeutical approaches exploit the carrier function of HSPs for tumor specific antigenic peptides. This “chaperokine” function of members of the HSP70 family results in a non-specific stimulation of the innate immune system. Under stressful

Table 2. Assessment of the relation between HSP70 and obesity, smoking, comorbidity, menopausal status, or blood biochemistry results.

n = 45		*n*	%	HSP 70 Mean ± S.D. (Median)	*p* value
BMI	≥ 30 kg/m^2	18	40	6.48 ± 2.12 (6.26)	0.267
	< 30 kg/m^2	27	60	5.65 ± 1.98 (5.51)	
Smoker	Yes	10	22.2	5.84 ± 2.03 (6.02)	0.722
	No	35	77.8	6.02 ± 2.09 (5.72)	
Comorbidity	Yes	23	51.1	5.62 ± 2.09(5.45)	0.177
	No	22	48.9	6.36 ± 2.04 (7.13)	
Menopausal Status	Pre	31	68.9	6.31 ± 2.16 (6.28)	0.138
	Post	14	31.1	5.26 ± 1.64 (4.62)	
Leucocytosis	≥ 11 (× 10^3/mm^3)	5	11.6	4.36 ± 1.63 (3.98)	0.037
	< 11 (× 10^3/mm^3)	40	88.4	6.16 ± 2.08 (5.85)	
Anemia	< 12 g/dL	15	33.3	6.29 ± 2.13 (5.72)	0.501
	≥ 12 g/dL	30	66.7	5.83 ± 2.03 (5.86)	
Trombocytosis	≥ 400 (× 10^3/mm^3)	5	11.1	6.04 ± 2.55 (5.72)	0.964
	< 400 (× 10^3/mm^3)	40	88.9	5.97 ± 2.02 (5.85)	
Ca 15-3	≥ 31 U/L	6	14.3	6.47 ± 2.47 (6.70)	0.641
	< 31 U/L	39	85.7	5.94 ± 2.06 (5.73)	

S.D., standard deviation; BMI, body mass index; HSP70, heat shock protein 70; Ca 15-3, carcinoic antigen 15-3.

Table 3. Comparison of age and HSP70 level in patients with breast cancer and in healthy controls.

	Patients (*n* = 45) Mean ± S.D.	Control (*n* = 16) Mean ± S.D.	*p* value
Age (year)	57.6 ± 10.6	52.56 ± 6.99	0.083
HSP70 (ng/ml)	5.98 ± 2.05	1.49 ± 0.47	**0.001**

S.D., standard deviation; HSP70, heat shock protein 70.

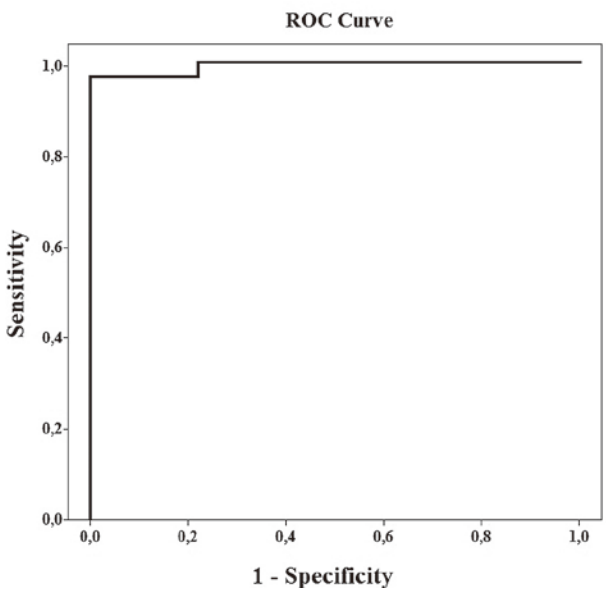

Fig. 1. Receiver operating characteristics (ROC) curve for serum HSP70 for the diagnosis of breast cancer.
Area under the curve (AUC) for HSP70 is 0.996 with p = 0.001.

cellular conditions, elevated HSP70 levels allow the cells to cope with increased levels of unfolded and denatured proteins (Suto and Srivastava 1995; Asea et al. 2000; Radons and Multhoff 2005).

HSPs are highly expressed in several malignancies. For example, HSP70 overexpression is a marker of non-small cell lung cancer (NSCLC), cholangiocarcinoma, colorectal cancer, early hepatocellular and prostate cancer (Chuma et al. 2003; Abe et al. 2004; Kocsis et al. 2011; Sato et al. 2012; Zimmermann et al. 2012). In addition, overexpression of HSP70 was shown as a marker for advanced disease in BC immunohistochemically (Murphy 2013).

HSP70 is indeed one of the most cytoprotective proteins ever described, blocking all main cell death types. The search for inhibitors of HSP70 has dramatically increased over the last two years. One interesting observation is that HSP70 inhibition or depletion provokes tumor regression in syngeneic animals by inducing an immune anti-tumor response (Schmitt et al. 2006). This immune response might be explained by the inhibiting effect of HSP70 in activating myeloid suppressive cells (Chalmin et

al. 2010).

Overexpression of HSP70 has been shown as a marker for advanced disease and lymph node metastasis in colorectal carcinoma (Lazaris et al. 1997; Hwang et al. 2003), BC and undifferentiated ovarian cancer (Athanassiadou et al. 1998). In cervical cancer, it is correlated with tumor size and increased proliferation (Ralhan and Kaur 1995). In esophageal squamous cell carcinoma, the expression of HSP70 correlated inversely with depth of invasion ($p < 0.05$), stage ($p < 0.05$), and relationship and it correlated positively with lymphocyte infiltration ($p < 0.05$) (Nakajima et al. 2002). Levels of HSP70 can be correlated with the stage in malignant melanoma (Lazaris et al. 1995) and oral carcinoma (Kaur et al. 1998), as well as high grade and poor overall survival in bladder cancer (Syrigos et al. 2003). Finally, intense staining of HSP70 is associated with shorter survival and poor prognosis in BC (Murphy 2013).

Cancer cells can actively (exosomal) and/or passively (by dying tumour cells) release HSP70 into the extracellular space (Vega et al. 2008; Gehrmann et al. 2014). Also in animal model studies a correlation between serum Hsp70 levels and tumor volume has been indicated (Bayer et al. 2014). Thus serum HSP70 levels had been investigated in some cancer studies. In pancreatic cancer patients, serum HSP70 level was found significantly increased and it could be useful as an additional biomarker for the detection of pancreatic cancer (Dutta et al. 2012). Also serum HSP70 has been shown as an indicator for high mortality in patients with colorectal cancer and its serum levels has been shown to predict increased risk of lung cancer (Suzuki et al. 2006; Kocsis et al. 2011). Based on these studies we intended to determine the serum levels of HSP70 in BC patients. In our study, the HSP70 level was significantly higher in patients with BC than the healthy controls, whereas no significant difference was observed in the levels of HSP70 among the BC subtypes. The serum HSP70 level was significantly lower in patients with BC with leucocytosis. This could be due to the anti-apoptotic effects of HSP70. But as we did not show the cellular expression of HSP70 in pathology specimens of BC patients, we could not determine the definitive source of elevated serum HSP70 in patients.

The cut-off value of serum HSP70 has been investigated in various types of cancer. In cholangiocarcinomas, the cut-off value for HSP70 is 5.67 ng/mL (Sato et al. 2012). The cut-off point for plasma HSP70 levels in patients with localized untreated prostate cancer is 1.15 ng/mL (Abe et al. 2004). We tried to determine an optimal cut-off value for HSP70. In our study, the cut-off value of HSP70 levels in patients with BC were > 2.41 ng/mL which is close to the value of cholangiocarcinomas.

As a result, in our study, BC patients had higher HSP70 values than the healthy controls and we found an inverse relationship between the leukocyte count and HSP70 levels. We determined a cut-off value for HSP70 for detecting BC and to the best of our knowledge, this is the first study that provides this information for BC patients. This information suggests the potential use of this marker in the clinic for BC screening and diagnosis. Further studies are needed to define the important effects of HSP70 in BC patients, especially to examine therapies targeting this protein for cancer therapy.

We have not made a sample size method in order to reach the necessary number for our groups of patients. As the number of patients and the duration of follow-up were not large enough for accurate statistical calculation, the relation between HSP70 and prognosis could not be determined. Also we did not show the cellular expression of HSP70 in pathology specimens.

Conflict of Interest

The authors have no conflict of interest.

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