RESEARCH ARTICLE

Anti-Heat Shock Protein-27 Antibody Levels in Women with Breast Cancer: Association with Disease Complications and Two-Year Disease-Free Survival

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Abstract

Background and Aim: Breast cancer is a major healthcare problem in women. There are many reports about up-regulation of Hsp27 in cancer tissues but less is known about the potential relationship between Hsp27 antibody levels and breast cancer complications. We here investigated concentrations of serum Hsp27 antigen and antibodies in subjects with and without breast cancer and assessed potential associations with two-year disease-free survival, histological grade and number of lymph nodes. **Materials and Methods:** Specifically, serum Hsp27 antigen and antibody levels from 97 patients with breast cancer, and 65 healthy controls were determined by enzyme-linkedimmunosorbent assays (ELISAs). **Results:** Serum Hsp27 and antibody levels were significantly ($p<0.001$) higher in patients with breast cancer compared to the control group, but no relationship were found with two-year disease free survival, histological grade or number of lymph nodes ($p> 0.6$, 0.2 and 0.9 respectively). **Conclusions:** Elevated levels of Hsp27 antibody occur with women with breast cancer but do not appear to be associated with the presence of disease clinical complications.

Keywords: Breast neoplasms - anti-Hsp27 antibody - disease-free survival

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Introduction

Heat shock protein-27 (Hsp27) is a member of a family of proteins whose intracellular expression modulates the ability of cells to respond to several types of injury, heat shock, oxidative stress and other unfavorable conditions in order to offset the deleterious effects of cellular stresses (Ciocca et al., 1993; Ferns et al., 2006; Mehlen et al., 1995). Besides its chaperone function, Hsp27 has also been shown to have an anti-apoptotic role by inhibition of caspase-dependent apoptosis, preventing a wide variety of apoptotic agents from causing cell death (Calderwood et al., 2006; Mehlen et al., 1996; Vidyasagar et al., 2012). This may confers a survival advantage to cells and may contribute to the reduction in apoptosis (Batchelder et al., 2009). With respect to the anti-apoptotic role, Hsp27 is overexpressed in various cancers and display a relationship with patients' survival or response to therapy in specific cancer types and may serve as a biomarker of disease and novel therapeutic target.

Several studies have shown an association between Hsp27 with many types of cancer including gastric, liver, and prostate carcinoma, osteosarcoma, rectal, lung, and breast cancer especially in terms of prognosis and treatment (Ciocca and Calderwood, 2005; Kang et al., 2008; Tweedle et al., 2010; Vidyasagar et al., 2012). It has been reported that the up-regulation of anti-apoptotic pathways induced by Hsp27 enhanced the resistance of cancer cells to apoptosis and may also be implicated in resistance to chemotherapy in breast cancer and is associated with drug-resistant phenotypes (Calderwood et al., 2006). Therefore, it seems to be the most important to control Hsp27 in cancer cells to conquer the cancers.

However, Hsps can also become targets for cancer therapy drugs, as well as targets for the immune system. In vitro studies have shown that Hsps are released from cells exposed to stress, and increased serum concentrations have been implicated in the immune response against HSPs (Burut et al., 2010; Child et al., 1995; Liao et al., 2000). In turn, the immune response against Hsps has been implicated in the pathogenesis of some diseases including atherosclerosis, cardiovascular disease and cancer in the general population (Burt et al., 2009; Mandal et al., 2004; Trieb et al., 2000). After being released into the systemic circulation, Hsp27 can induce an autoimmune response with the production of anti- Hsp27 antibodies (Conroy

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et al., 1998).

The development of breast cancer is a complex molecular process that arise from terminal ductal lobular units (Batchelder et al., 2009). Several lines of evidence exist to indicate that expression of Hsp27 may be of some prognostic value in breast carcinomas (Love and King, 1994; Thor et al., 1991). It is also reported that increased expression of Hsp27 in cancer cells causes the development of chemotherapeutic drug resistance and conversely, down-regulation of Hsp27 in breast cancer cell line MCF-7/MG with antisense- Hsp27 enhanced the sensitivity of the cells to doxorubicin (Oesterreich et al., 1993). Because overexpression of Hsp27 correlates with poor survival in breast cancer patients, investigating whether patients with breast cancer have circulating antibodies to Hsp27, may also be of importance. Conroy et al. have previously reported that high plasma antibody titres against Hsp27 are associated with improved survival in patients with breast cancer (Conroy et al., 1998), but further studies in other groups of subjects may be helpful to investigate a more subtle effect of this Hsp27 and anti- Hsp27 antibody in development of this serious cancer phenotype.

The present study was aimed to investigate changes in serum HSP antigen and anti- Hsp27 antibody concentrations associated with breast cancer complications and 2-year disease-free survival and also to correlate them with clinical features and prognosis of the disease.

Materials and Methods

Subjects

Sera from 97 patients with breast cancer (mean age: 47.2 years, range 27.0–74.0) diagnosed at Omid and Ghaem hospitals (Mashhad, Iran) between 2009 and 2011 along with sera from 65 genetically unrelated-age matched healthy female controls with no evidence of cancer or a family history of the disease were analyzed in this study. All serum samples were collected before the patients with breast cancer underwent chemotherapy. Samples were stored at -70.0°C until analysis. Informed consent was obtained from all participants using protocols approved by the Ethics Committee of the Mashhad University of Medical Science (MUMS). All the necessary information related to clinical characteristics including taking any medications affecting on the pro-oxidant - antioxidant balance such as vitamins, retinoic acid, beta-carotene, and the absence of systemic disease as well as histological status including the stage of disease and tumor grade were extracted from patient's archival records. The numbers of patients having early stage tumor were 74.4%. Subjects who were in metastatic stage and patients with systemic diseases such as cardiovascular disease, and those taking vitamin D supplements were excluded from the study. Patient's 2-year disease free survival data were obtained and this was then related to serum anti-Hsp27 antibodies levels.

Methods

Antibody titres were tested by a solid-phase-bound antigen indirect enzyme-linked immunosorbent assay (ELISA).Shortly, ELISA plates were coated with 100ng human Hsp27 in Carbonate-Bicarbonate buffer (pH 9.6) in PBS (100 ml well^{-1}) overnight at 4°C then blocked with Goat serum 2%(v/v) in PBS at 37°C for 30 min. After washing, serum samples (100 ul well^{-1}, diluted 1:100, 30 min, and 25°C) were tested for their antibody titres against Hsp27 by peroxidase-conjugated IgG (100 ul well^{-1}, diluted 1:500, 30 min, 25°C). The plates were developed using 100 ul substrate and were read on an ELISA reader at 450 nm after subtraction of non-specific binding.

Statistical analysis

Categorical data were analyzed by the X2 test or Fisher's exact test. All tests were 2-sided. The association between anti-Hsp27titres and clinical parameters was assessed using the Mann–Whitney U-test. The significance level was set at a value of $P < 0.05$. All normally distributed values are given as mean ± S.D. and all tests were performed at least in duplicate. Two year disease free Survival was estimated by the Kaplan-Meier estimator from lifetime data. Data were analyzed using Cox's proportional hazards model and other related techniques including log-rank to determine the association of serum anti-Hsp27 with disease survival.

Results

Serum anti-Hsp27 levels in patients and healthy controls.Serum anti-Hsp27antibody levels in patients with breast cancer in comparison with healthy control group showed that the frequency of anti-Hsp27 were significantly higher in the women with breast cancer than in the matched controls ($p<0.001$). To investigate the relationship between 2-year disease free survival and serum anti-Hsp27 antibody titres, 97.0 subjects were followed up for 24 months. Of these, 69 patients (71.1%) showed no relapse during the study and 28 patients (28.9%) undergone disease recurrence.

Based on the data related to the mean Hsp27 antibody levels in patients with relapse and non-recurrent, stage of disease and also the presence of estrogen receptor (ER) or lymph node involvement, there were no significant relationships between antiHsp27 antibody levels in patients with and without relapse ($p = 0.6$), stage of the disease (Stages I and II in comparison with stage III) ($p =$

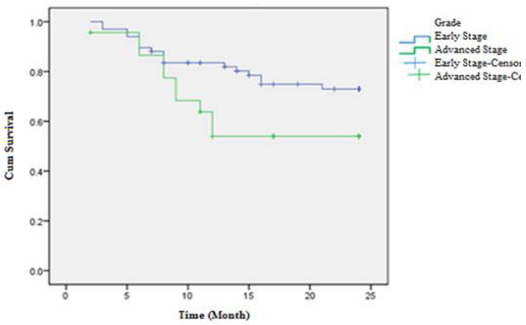

Figure 1a. Diagram Comparing the Survival of Patients with Early-Stage and Patients with Advanced Stage Disease

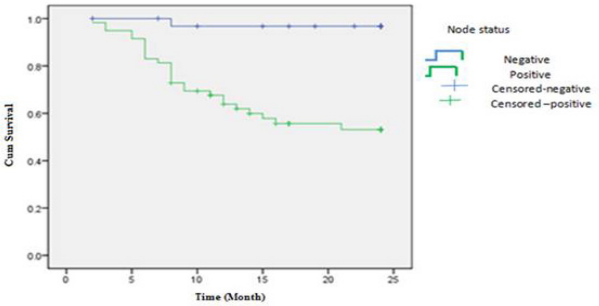

Figure 1b. Diagram Comparing the Survival of Patients With and Without Node Involvement

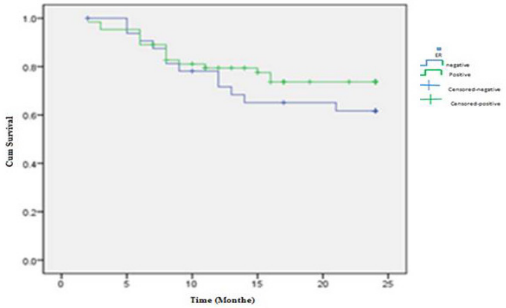

Figure 1c. Diagram Comparing the Survival of Patients Regard to the ER Status.

0.16), patients with and without ER (p = 0.89) and node-negative and node-positive women (p = 0.91).

Associations between cancer stage, node positivity, ER status and anti-Hsp27 levels at 2-year disease-free survival in breast cancer patients

The mean time to recurrence in patients with early stage disease was 0.8 ± 20.2 years and this was 1.7 ± 16.8 for patients with advanced stage of the disease (Figure 1a). Log-rank test analysis showed no significant difference between diagram of a 2-year disease-free survival in patients in the first stage of their disease, with patients in the advanced stage of the disease (p = 0.06). Node-positive patients showed a significantly reduced 2-year disease-free survival than node-negative women (p < 0.001) (Figure 1b). Patients who were ER positive did not differ significantly for 2-year disease free survival (Figure 1c). Cox regression analysis was used to investigate the relationship between Hsp27 antibody levels and 2-year disease-free survival. The hazard ratio in a stratified Cox regression model was 1.0 (p = 0.005). However the serum anti-Hsp27 antibody concentration had no significant relationship with 2-year survival (p = 0.92).

Multivariate analysis in which serum anti- Hsp27 antibody concentrations, lymph node, and estrogen receptor status, stage of disease and age were put into, the Cox regression model showed that only lymph node status was a significant predictor of 2 year disease free survival. The risk of recurrence in node-positive patients was 13.8 times greater than that in patients without node involvement (p = 0.01) (Table 1).

Discussion

In this follow-up study, we have investigated serum anti- Hsp27 antibody levels in women with breast cancer in Iran. We have also evaluated the relationship between some features of disease including, node status, estrogen receptor status, stage of disease and age with 2-year disease-free survival. Our data show evidence of a significantly elevated level of anti- Hsp27 antibodies in subjects with breast cancer than healthy individuals. Consistence with previous studies, our results were in line with overexpression of Hsp27 protein and development of the immune response to this protein in patients with breast cancer (Conroy et al., 1998; Love and King, 1994; Thor et al., 1991). Given the fact that Hsp27 is an intracellular protein, the mechanism involved giving rise to the immune response to Hsp27 is not fully understood, although it is possible that Hsp27 is released from apoptotic cancer cells and the results to date firmly establish the extracellular presence of Hsp27, indicating its secretion from tumor cells (Batulan et al., 2016). Some key findings indicate that Hsp27 like other secretory proteins lacking the signal peptide for the classical pathway, may exit eukaryotic cells via the lysosomes and/or exosomes and raising the possibility of direct protein translocation (Batulan et al., 2016). It is important to find whether Hsp27 and its antibody concentrations could be considered as markers

Table 1. Cox Regression Analysis Assessing the Hazard Ratio for the Studied Factors

Variable		Hazard Ratio	β	P-value	Confidence interval %95	
					Lower bound	Upper bound
Level of Serum oxidant- anti oxidant		1.0	0.003	0.595	0.99	1.01
antibody Hsp27 serum		0.4	-0.936	0.487	0.03	5.38
Age		1.0	0.006	0.774	0.96	1.05
ER hormone receptor	negative	1.0	-	-	-	-
	positive	0.6	-0.506	0.219	0.27	1.35
Stage of disease	early	1.0	-	-	-	-
	advance	1.6	0.460	0.403	0.54	4.65
Involved node	negative	1.0	-	-	-	-
	positive	13.8	2.63	0.011	1.82	105.20

ER, estrogen receptor; Hsp27, Heat shock protein-27; Bold values represent significant P value.

of disease or whether they are etiological factors in cancer development perhaps via the formation of immune complexes or by other related mechanisms.

However there were no significant differences in serum levels of anti- Hsp27 antibodies between patients who relapsed during a period of 2 years follow-up and those who did not have recurrent disease. Nor were there significant differences in serum anti- Hsp27 antibody titres in patients with breast cancer at an early stage (stage I, II) versus those with advanced stage (stage III) disease; nor women with tumors that were estrogen-receptor positive or negative (ER+ and ER-) or those with and without lymph node involvement. Conroy et al showed that an increased level of anti- Hsp27 antibody is associated with improved long term survival, and that this difference becomes more evident after 5.0 years (Conroy et al., 1998). We did not find any relationship between serum anti- Hsp27 antibody levels and 2 year disease free survival which is similar to the results of Love et al, and Thanneri et al (Love and King, 1994; Thanner et al., 2005).

Didelot and colleges (2007) have reported, in contrast to intracellular Hsps that present a cytoprotective role, some extracellular Hsps such as Hsp27, have immunogenic properties and may induce inflammatory response or Vega et al (2008) have shown that membrane-bound Hsps in vesicles were able to activate macrophages. Not surprisingly we expected to detect an increasing trend in serum levels of anti-Hsp27 antibody with regards to disease progression, but our finding do not support it. One possibility to account for this may attributed to the tolerance of the immune system to the tumor cells resulted from tumor derived exosomes harboring Hsps in the membrane, which need to be further explored in future studies to determine whether this is indeed the case. Another possibility can be explained by the fact that Hsp27 might not be a direct tumor antigen but involved only in antigens presentation to the surface of tumor cells. Hence, with this in mind, it can be concluded that anti-tumor immune responses appears to be directed against some tumor antigens associated with hsps. It should be noted that this description must be interpreted with caution.

Despite being a preliminary study and providing some evidence of the relationship between anti-Hsp27 antibodies and breast cancer disease complications, there are certain limitations to this study such as a cross-sectional study design with a relatively small sample size, restricting the ability to identify causal biological mechanisms underlying possible association and also the reduced power of the analyses due to lower number of control subjects compared with patients. However, a key strength here is ability to control many confounding factors due to selecting high matched control subjects. Another limitation is related to the serum samples, even were stored in appropriate condition, the possibility of protein degradation would not improbable. Finally, the lack of similar studies in other populations to confirm our findings.

In conclusion, an increase in tissue levels of Hsp27 in breast cancer patients may lead to the induction of an immune. Serum anti- Hsp27 antibody level was not associated with 2-year disease free survival in women with breast cancer and cannot be accounted as predictive value for the progression of cancer stages with high risk of mortality. The findings could be significant for understanding the possible role of antibodies in the pathogenesis of breast cancer and for prognosis of disease advancement. However, more extensive studies are needed to determine the cut-off point level for such evaluations.

Conflict of interest

The authors declare that they have no conflict of interest.

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