Heat Shock Proteins hsp27 and hsp70: Lack of Correlation with Response to Tamoxifen and Clinical Course of Disease in Estrogen Receptor-positive Metastatic Breast Cancer (A Southwest Oncology Group Study)1

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ABSTRACT

In this study, we tested the hypothesis that heat shock proteins (hsps) 27 and 70 are associated with clinical resistance to tamoxifen. hsp27 is, like progesterone receptor, an estrogen-regulated protein. hsp70 is also of interest because of its interaction with estrogen receptors and because hsp70 is a component of the molecular chaperone machinery functioning in the assembly and trafficking of steroid receptors. In addition, hsps in general help protect cells against noxious stimuli and stress, and their expression has been linked to drug resistance. The study involved 205 tumors from estrogen receptor-positive tamoxifen-treated breast cancer patients with metastatic disease. All patients received daily tamoxifen as initial therapy for metastatic disease. The study began in 1982, and follow-up is now 9 years. hsp27 and hsp70 were detected by immunohistochemistry and scored according to the nuclear and/or cytoplasmic content. Expression of hsp27 or hsp70 was unrelated to estrogen receptor content, progesterone receptor content, menopausal status, age, and presence of visceral disease. Cytoplasmic and nuclear hsp27 positivities were weakly and inversely related to each other (P = 0.05). There was a significant association between cytoplasmic hsp27 and cytoplasmic hsp70 content (P < 0.001), as well as between nuclear hsp70 and nuclear hsp27 content (P = 0.001). Cytoplasmic and nuclear hsp70 were also associated (P = 0.02). However, increased hsp27 and hsp70 expression (nuclear or cytoplasmic) was not significantly associated with response to tamoxifen, time to treatment failure, or survival. Thus, this study clarifies the lack of clinical utility of hsp27 and hsp70 in predicting the response to tamoxifen in an estrogen receptor-positive breast cancer population.

INTRODUCTION

Tamoxifen is used to treat patients with early-stage and advanced breast cancer. The selection of patients likely to respond to tamoxifen therapy includes the measurement of ERs3 and PRs in tumor tissue (1). However, only about 60% of patients with ER-positive tumors and 70% of those with ER- and PR-positive tumors respond to endocrine therapy. Thus, even in receptor-positive patients, tamoxifen resistance is a major problem. There are a number of reasons that might explain this failure, including methodological errors in receptor determination, receptor loss during tamoxifen treatment, receptor mutations, low tumor tamoxifen levels, and emergence of tamoxifen metabolites with less potent antiestrogenic and more estrogenic properties (2–5). Alterations in molecular pathways important in apoptosis, growth factor interactions, and stress responses might also result in tamoxifen resistance.

We are conducting a series of studies evaluating different molecular markers as possible predictors for the novo tamoxifen resistance in ER-positive tumors (6, 7), from a prospective trial of the SWOG (8). In the present report, we have analyzed two hsps, hsp27 (M, 27,000) and hsp70 (M, 70,000), in these tumors. hsp27 is, like PR, an estrogen-regulated protein, its presence in breast tumors correlated with that of ER (9, 10), and in an earlier study hsp27 was associated with a higher response rate to hormonal therapy in a group of ER-positive patients (11). hsp70 is also of interest because of its correlation/interaction with ER

1 The abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; hsp, heat shock protein; SWOG, Southwest Oncology Group.

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and because the protein is a component of the molecular chaperone machinery functioning in the assembly and trafficking of steroid receptors (12–14). hsp70 expression could influence ER function and changes in hormonal sensitivity. Finally, hsps in general help to protect cells against noxious stimuli and stress, and their expression has been linked to resistance to cytotoxic drugs (15, 16). Thus, the presence of hsps could reduce the effectiveness of treatment.

PATIENTS AND METHODS

Patient Selection. The study was performed on 205 formalin-fixed paraffin blocks retrieved from SWOG protocol 8228 (8). SWOG 8228, a prospective trial designed to investigate the prognostic significance of PR levels in ER-positive breast cancer patients treated with tamoxifen, was opened in 1982 and closed in 1987. Further details on patients and tumor specimens have been recently published (6).

Treatment and Response Criteria. In the initial phase of the SWOG 8228 study, 87 patients were treated with tamoxifen, 10 mg p.o. twice a day; the dose was changed to 10 mg/m² p.o. twice a day for the remaining 255 patients. For those evaluated in SWOG 9314, 56 received tamoxifen 10 mg twice daily and 149 received 10 mg/m² twice daily.

Response to treatment was defined as a patient’s having either complete response, partial response, or prolonged stable disease with a time to treatment failure of more than 6 months (6). Prolonged stable disease was included as a response to treatment, because patients with prolonged disease stabilization in response to tamoxifen clearly benefited clinically, and because objective benefit is difficult to assess in patients with osseous disease. Time to treatment failure was defined as the time from registration to first occurrence of progression, discontinuation of treatment, or death.

Immunohistochemical Analysis and Scoring. A 5-μm section of submitted paraffin blocks was first stained with H&E to verify that adequate numbers of invasive tumor cells were present and that fixation quality was sufficient for immunohistochemistry. Immunohistochemical staining was performed as described elsewhere (6). For hsp27 immunostaining, specimens were incubated with mouse monoclonal antibody G3.1 (Neo-markers, Fremont, CA) at a 1:8000 dilution for 1 h at room temperature. For hsp70 immunostaining, slides were incubated with mouse monoclonal antibody BRM22 (Sigma Chemical Company, St. Louis, MO) at a 1:8000 dilution for 1 h at room temperature. The specificity of these two monoclonal antibodies has been tested by our group using Western blot analysis (17, 18).

Tumors were scored according to the estimated proportion of tumor cells that were positively stained (6, 7, 17). Scoring criteria based on the estimated fraction of positively staining cells were as follows: 0, none; 1, less than 1/100; 2, 1/100–1/10; 3, 1/10–1/3; 4, 1/3–2/3; and 5, greater than 2/3. This scoring system was applied separately for the cytoplasmic and for the nuclear localization of the hsps. The cut points were: cytoplasmic hsp27 staining, ≥3; nuclear hsp27 staining, ≥1; cytoplasmic hsp70 staining, ≥4; and nuclear hsp70 staining, >4. These cut points were prospectively selected based on previous studies (17) and on unpublished results obtained from our tumor bank.

### Table 1: Correlation of hsp27 score with patient and tumor characteristics

<table>
<thead>
<tr>
<th>Score</th>
<th>Cytoplasmic scores</th>
<th>Nuclear scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>(n = 69)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>(n = 136)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>(n = 175)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>(n = 26)</td>
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</tr>
</tbody>
</table>

Table 1 Correlation of hsp27 score with patient and tumor characteristics

Statistical Analysis. χ² tests were used to compare hsp27 and hsp70 immunohistochemistry with dichotomized patient and tumor characteristics. Estimation of time to treatment failure and overall survival was performed using the Kaplan-Meier method. Log-rank statistics were used to compare time to treatment failure and survival. Multivariate analyses were performed using Cox’s partially nonparametric model for censored survival data. The association of response with hsp27 and hsp70 and other characteristics was analyzed using logistic regression. All reported Ps were two sided.

RESULTS

hsp Expression in Cytoplasmic and Nuclear Compartments. hsp27 was predominantly expressed in the cytoplasm of tumor cells; however, in some instances, hsp70 immunostaining was also detected in the nuclei, although at low intensity. In contrast, hsp70 was clearly seen in the cytoplasm and/or in the nuclei of tumor cells. Because nuclear expression/translocation of hsps has been noted in stressed cells, evaluation of these two hsps was independently performed in the cytoplasmic and nuclear cell compartments. Also, in preliminary studies, the localization/quantification of hsp27/70 appeared as important to discriminate the response of breast tumor cells to chemotherapy (19).

Correlation of hsps with Patient/Tumor Characteristics. Expression of hsp27 in the cytoplasmic cell compartment was first compared with other tumor and patient characteristics. Positivity was unrelated to the main baseline parameters examined (Table 1). Table 1 also shows that cytoplasmic and nuclear...
hsp27 positivities were inversely related to each other ($\chi^2$ P = 0.05). Stronger associations were noted between cytoplasmic hsp27 and cytoplasmic hsp70 content ($\chi^2$ P < 0.001).

Nuclear hsp27 expression was not correlated with any patient and tumor characteristics under study (Table 1); positivity was associated with nuclear hsp70 expression (P = 0.001).

Cytoplasmic hsp70 expression showed a weak association with menopausal status (Table 2), whereas high nuclear hsp70 expression revealed a weak association with longer disease-free interval (P = 0.03). The association between cytoplasmic and nuclear hsp70 expression was modest (P = 0.02).

**hsps and Response to Tamoxifen.** We next examined whether the expression of the hsps in each of the cell compartments might be correlated with response to tamoxifen. For this purpose, a response was defined as a complete response, a partial response, or stable disease for >6 months. As can be seen in Table 3, only elevated hsp70 nuclear expression appeared weakly associated with the response to tamoxifen (65% response in hsp70-positive tumors versus 51% in hsp70-negative tumors; $P = 0.06$). Logistic regression analysis incorporating three variables (menopausal status, PR $\geq$10 fmol/mg cytosol protein, and ER $\geq$50 fmol/mg cytosol protein), which were found important to predict response to tamoxifen in the SWOG 8228 study (8), did not change the statistical results obtained.

**hsps and Time to Treatment Failure and Survival.** Cytoplasmic/nuclear hsp27 and cytoplasmic hsp70 content were not significantly associated with time to treatment failure (log-rank $P$s, 0.78, 0.27, and 0.07), whereas nuclear hsp70 was associated (log-rank $P$, 0.02). After adjustment for variables previously identified as important (menopausal status, PR $>$100 fmol/mg cytosol protein, and disease-free interval $>$3 years), cytoplasmic hsp70 became statistically significant ($P = 0.05$), whereas nuclear hsp70 lost its significance ($P = 0.09$).

None of the hsps examined were significantly associated with survival, either alone or after adjustment for the variables found important in the SWOG 8228 study (adjusted Ps were 0.30 for cytoplasmic hsp27, 0.11 for nuclear hsp27, 0.67 for cytoplasmic hsp70, and 0.57 for nuclear hsp70; survival log ranks, 0.57, 0.23, 0.57, and 0.1).

**DISCUSSION**

Unfortunately, in the present study, we found that determination of hsp27 and hsp70 were of no clinical value in predicting the response to tamoxifen. We have tested a relatively large and homogeneous group of patients with a long follow-up period, and in these patients, elevated hsp70 nuclear content showed only a weak association with response to tamoxifen ($P = 0.06$). Moreover, relatively high cytoplasmic and nuclear hsp70 content were only weakly correlated with a longer time to treatment failure (much better predictors were PR content, menopausal status, and disease-free interval); therefore, hsp70 determination will not be clinically useful for this purpose. We asked 12 questions, i.e., the association of each of 4 hsps (cytoplasmic/nuclear hsp27/70) with each of 3 end points, and tested each of these 12 in two ways (adjusted and unadjusted). A small number of marginally “significant” results was not unexpected even if there were no associations. Taking into consideration the lack of a convincing association between any of the hsps with the clinical end points tested, other cut points were not explored.

Biologically, testing of hsp27 and hsp70 was attractive because hsp27 is under estrogen regulation like PR, and because both hsps interact with ER. In addition, these hsps interact with many other proteins (10, 20–23). Cells with elevated expression of hsps may show increased survival against heat shock and other stressful conditions (10, 15, 16, 23–25), and they seem also to be involved in cell growth and differentiation (18, 26). However, the complexity of biological functions attributed to these hsps and the fact that the gene expression is controlled by several transcription factors/stimuli (10, 27–29) may obscure a clear association with response to a therapy such as tamoxifen. Also, the tumors were examined before tamoxifen administration, and subsequent changes of the hsps in the tumor cells are unknown.

In previous studies, the expression of hsp27 and hsp70 was related to the presence of ER, with these two hsps more frequently found in ER-positive tumors/cells (9, 13). We have now found that elevated expression of hsp27 and hsp70 were not correlated with the amount of ER, although all of the tumors examined in this study were ER positive. Although an initial study involving a small number of patients reported that hsp27-positive tumors had a better response to endocrine therapy (11), other studies testing a relatively low number of breast cancer patients have not confirmed this finding (30–32). Consistent with this, in the present report, we have found that hsp27 (and
also hsp70 was not related with response or survival in advanced breast cancer patients treated with tamoxifen.

REFERENCES
Heat shock proteins hsp27 and hsp70: lack of correlation with response to tamoxifen and clinical course of disease in estrogen receptor-positive metastatic breast cancer (a Southwest Oncology Group Study).

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