High HSP90 Expression Is Associated with Decreased Survival in Breast Cancer

Elah Pick,[1] Yuval Kluger,[3] Jennifer M. Giltnane,[2] Christopher Moeder,[2] Robert L. Camp,[2] David L. Rimm,[2] and Harriet M. Kluger[1]

Departments of [1]Medicine and [2]Pathology, Yale University School of Medicine, New Haven, Connecticut and [3]Department of Cell Biology, New York University, New York, New York

Abstract

The heat shock protein HSP90 chaperones proteins implicated in breast cancer progression, including Her2/neu. HSP90-targeting agents are in clinical trials for breast cancer. HSP90 expression is high in breast cancer cell lines, yet no large studies have been conducted on expression in human tumors and the association with clinical/pathologic variables. Tissue microarrays containing 10 cell lines and primary specimens from 655 patients with 10-year follow-up were assessed using our automated quantitative analysis (AQUA) method; we used cytokeratin to define pixels as breast cancer (tumor mask) within the array spot and measured HSP90 expression within the mask using Cy5-conjugated antibodies. We similarly assessed estrogen receptor, progesterone receptor, and Her2/neu expression. HSP90 expression was more variable in human tumors than in cell lines ($P < 0.0001$). High HSP90 expression was associated with decreased survival ($P = 0.0024$). On multivariable analysis, high HSP90 expression remained an independent prognostic marker. High HSP90 expression was associated with high Her2/neu and estrogen receptor, large tumors, high nuclear grade, and lymph node involvement. Although HSP90 levels were high in all our cell lines, expression in tumors was more variable. High HSP90 expression in primary breast cancer defines a population of patients with decreased survival. Evaluation of HSP90 expression in early-stage breast cancer may identify a subset of patients requiring more aggressive or pathway-targeted treatment. Prospective studies are needed to confirm the prognostic role of HSP90, as well as the predictive role of HSP90 expression in patients treated with HSP90 inhibitors. [Cancer Res 2007;67(7):2932–7]

Introduction

Heat shock chaperone proteins are necessary for cell survival during stress. HSP90, a 90 kDa heat shock protein, is an abundant chaperone essential for cellular quality control. It maintains the function of major cellular proteins, including hormone receptors, protein kinases, and proteins controlling cell cycle and apoptosis. It exhibits dual functionality by not only conserving the folding of newly translated proteins (thus preserving the conformational maturation of polypeptides) but also by facilitating degradation of unfolded proteins (1, 2). HSP90 functions with co-chaperones such as HSP70 (1), and chaperones over 100 known "client" proteins (1, 3). It is required for stability of proto-oncogenes important for breast cancer growth and survival, including estrogen receptor (ER), progesterone receptor (PR), and Her2/neu, as well as proteins downstream of Her2/neu, such as AKT, c-SRC, and RAF (3).

HSP90 stabilizes and protects proteins bearing individual amino acid mutations from proteasomal degradation. For example, HSP90 protects constitutively active B-RAF bearing the V599E mutation, which is present in ∼60% of melanomas. Although mutated B-RAF is chaperoned and protected by HSP90, wild-type B-RAF is not (2).

The NH_2-terminal domain of HSP90 binds ATP and other purine analogues. The COOH-terminal domain is responsible for HSP90 dimerization, which is required for biological activity. Two different binding sites at the NH_2 and COOH termini bind the chaperoned proteins. In cancer cells, HSP90 has higher affinity for the NH_2-terminal ligands than HSP90 in normal cells (4). When HSP90 interacts with a co-chaperone protein called CHIP (carboxyl-terminal HSP70-interacting protein), it promotes ubiquitination and degradation of some client proteins (5, 6).

HSP90 expression in malignant cells is reported to be constitutive, and is 2- to 10-fold higher than in normal cells, suggesting a crucial role in survival and growth of cancer cells, and can thus serve as an effective drug target (7). Inhibition of HSP90 in tumor cells results in dissociation from client proteins, induces apoptosis, and reduces chemotherapy resistance in aggressive cancers (5).

HSP90 has several protein- and nucleotide-binding sites, and many products specifically bind HSP90, providing candidate therapeutic compounds. The antibiotic geldanamycin binds HSP90 and promotes proteolytic degradation of client oncoproteins, such as V-SRC and Her2/neu (8). Additional HSP90 inhibitors include 17-allylamino-17-demethoxygeldanamycin (17-AAG), 17-dimethyla-minoethylamino-17-demethoxy-geldanamycin (17-DMAG), radicicol, novobiocin, purines, cisplatin, and histone deacetylase inhibitors, as reviewed (9). Geldanamycin, for example, binds HSP90 and inhibits ATP binding, ATP-dependent HSP90 chaperone activity, and promotes binding of CHIP, thus promoting the protein degradation function of HSP90 (3, 5).

17AAG, the clinical derivative of geldanamycin, was the first HSP90 inhibitor to enter clinical trials. Successful modulation of HSP90 by 17AAG has been shown, and 17AAG selectively binds active forms of HSP90 found in tumor cells (4, 10). Her2/neu is one of the client proteins that are most sensitive to the effects of geldanamycin and 17AAG. In the presence of geldanamycin, CHIP facilitates ubiquitination and degradation of Her2/neu (11). A clinical trial is ongoing to assess the efficacy of 17-AAG plus trastuzumab in Her2/neu–overexpressing breast cancer (12). In phase I studies at clinically tolerated doses, molecular changes consistent with intratumor cellular changes (such as down-regulation of C-RAF and CDK4 proteins) were observed (9). 17-AAG is being assessed for other diseases and with other antineoplastic agents.

Most published works show HSP90 expression in breast cancer cell lines (5, 8). Two small studies have assessed HSP90 expression in malignant breast tumors, showing increased expression compared with normal breast tissue, but intensity of expression and association with clinical variables were not assessed (13, 14). To the best of our knowledge, no large-scale quantitative studies have been conducted on HSP90 expression in clinical breast cancer specimens assessing the association with outcome.

Given the importance of HSP90 in malignant progression and the current development of HSP90 targeting agents in breast cancer, we assessed its expression in a panel of cell lines, and confirmed high expression in all breast cancer cell lines, as described by others (4, 8, 13). We studied specimens from a large historical patient cohort and assessed the association with survival and other clinical variables. To obtain more accurate, objective expression measures, we used our newly developed method of automated, quantitative analysis (AQUA) of tissue microarrays. This method has been validated and has proven to be more accurate than pathologist-based scoring of brown stain (15, 16). As is the case with some other targeted therapies, it is possible that response to HSP90-targeting drugs might be associated with expression levels of the target and

its client proteins in tumors, and quantitative assays need to be developed to predict response. Other markers that have both prognostic and predictive value, such as Her2/neu and hormone receptors, have significantly affected our ability to appropriately select therapeutic regimens for breast cancer.

Materials and Methods

Cell lines and Western blots. Chinese hamster ovary (CHO), SKOV3 (human ovarian cancer), MDA-MB-436, MDA-MB-468, MCF-7, T47D, and SKBR3 (human breast cancer) cell lines were purchased from American Type Culture Collection (Manassas, VA). Western blotting of protein extracts was done using standard methods. HSP90 was detected by 1-h incubation with mouse anti-HSP90 IgG (BD Transduction, San Jose, CA) at 1:500. Protein loading was assessed using mouse anti-γ-tubulin (Sigma-Aldrich, St. Louis, MO) at 1:5,000.

Tissue microarray construction. Tissue microarrays were constructed as previously described (17). Three hundred thirty-one node-negative and 324 node-positive breast cancer cores, each measuring 0.6 mm in diameter, were spaced 0.8 mm apart. Specimens and clinical information were collected with approval of the Yale University Institutional Review Board. The cohort has been described and validated in numerous publications (17). Pellets of cell lines (BT474, MDA-MB-231, MDA-MB-435, MDA-MB-436,
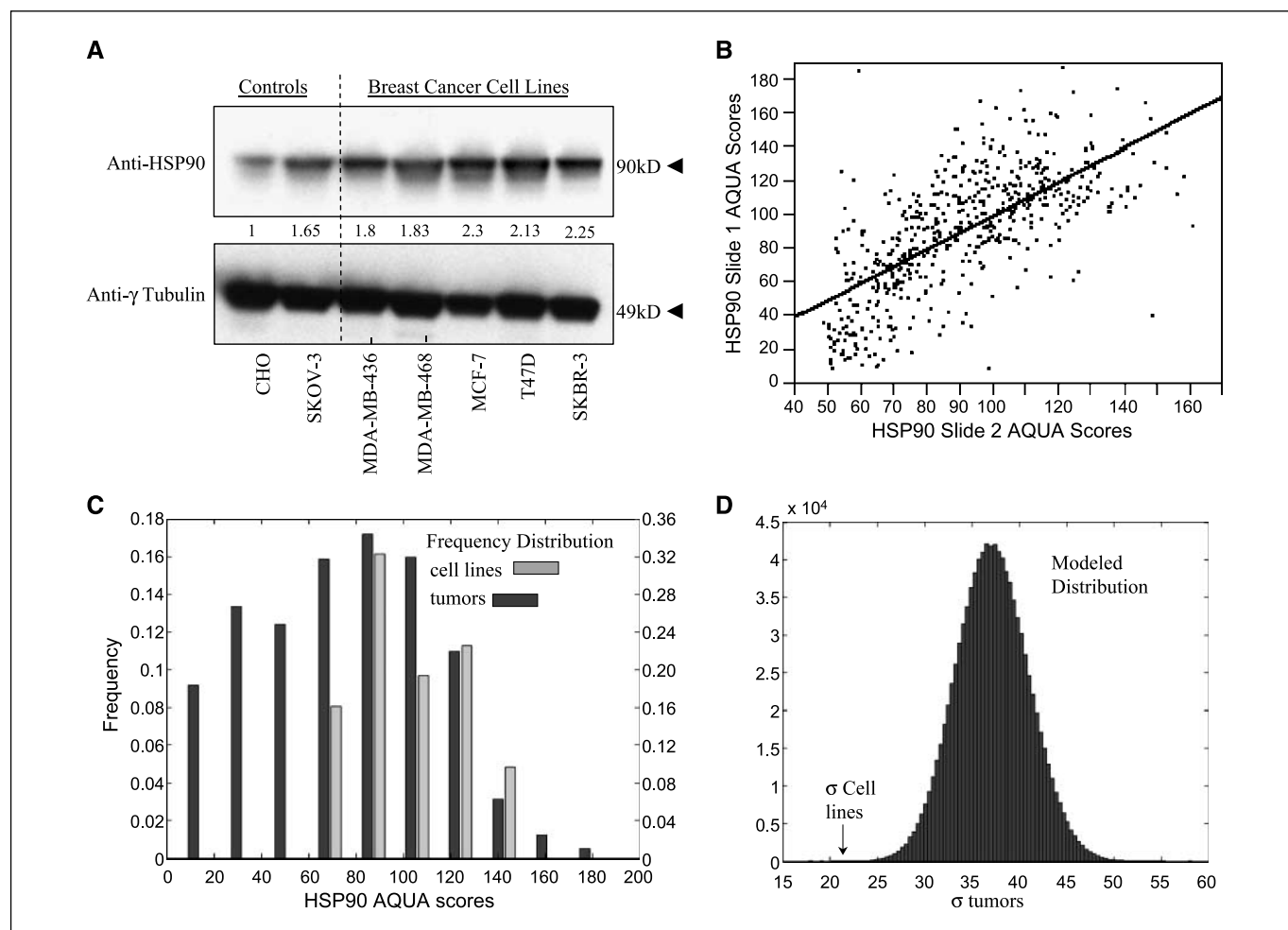

Figure 1. *A,* Western blots of a panel of cell lines show high expression in all breast cancer cell lines and lower expression in CHO cells. The numbers show the ratios of band intensity in the cell lines relative to that in CHO cells, normalized to the γ-tubulin loading. *B,* regression plot for scores from the two breast cancer arrays stained for HSP90 (*R* = 0.69). *C,* frequency distributions of tumors (*dark gray columns, scale on left Y axis*) and cell lines (*light gray columns, scale on right side Y axis*). *D,* histogram of standard deviations (σ) of 100,000 computer-generated random selections of 31 tumor AQUA scores from the 1,144 tumor scores obtained from the two arrays. The SD from the 31 cell line AQUA scores obtained from the arrays was 22.55 (*solid arrow*), significantly lower than those of the tumors (*P* < 0.0001).
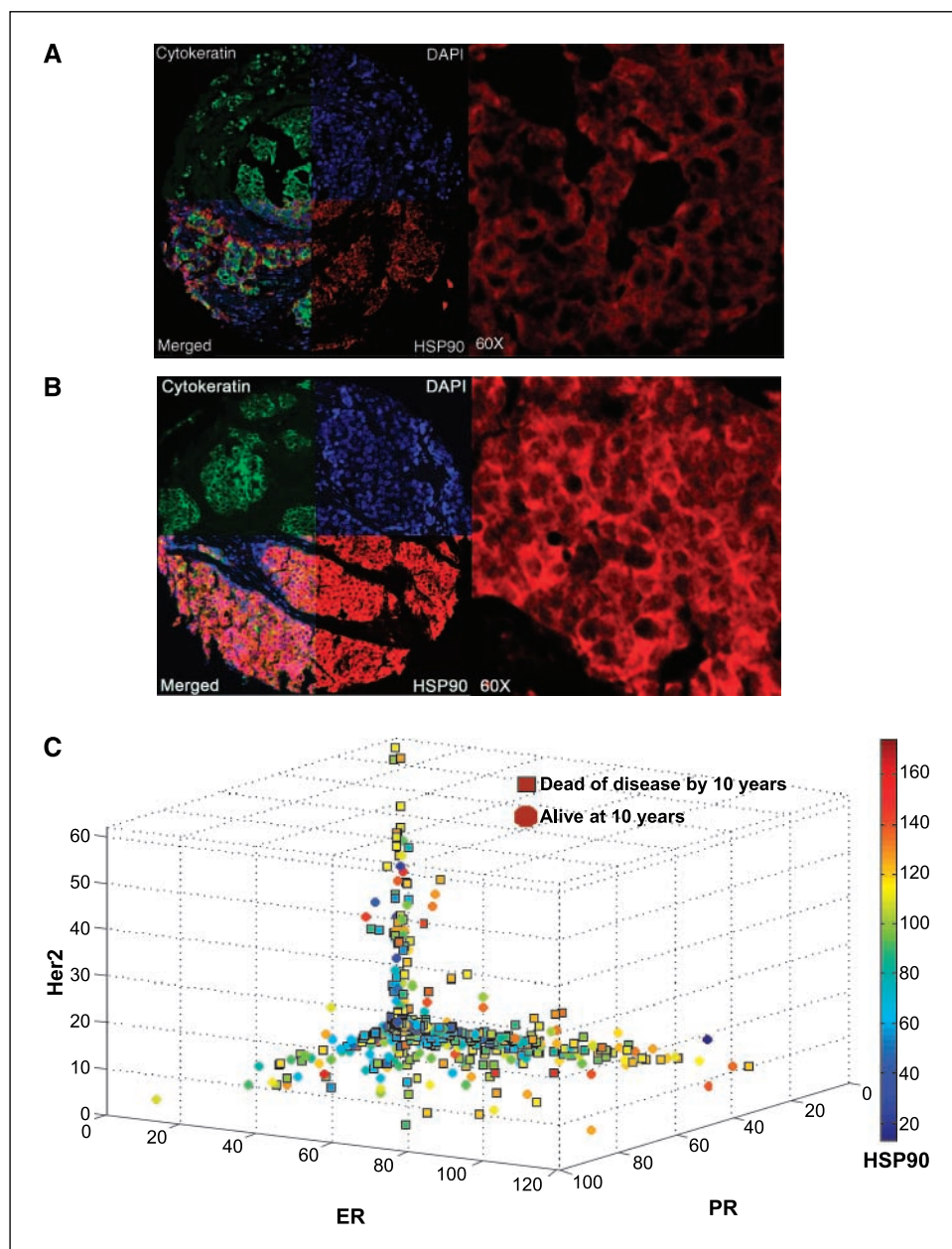
Figure 2. Low (*A*) and high (*B*) membranous HSP90 expression in a breast cancer histospot using cytokeratin to the define tumor mask, DAPI to define the nuclear compartment, and Cy5 for target (HSP90) identification at ×10 and ×60 magnifications. *C,* three-dimensional scatter plot for ER, PR, and Her2/neu expression, with HSP90 expression color-coded as in the bar on the right. *Squares,* patients who were dead of breast cancer at 10 y; *circles,* patients who were alive at 10 y.

MDA-MB-453, MDA-MB-468, MCF-7, SKBR3, T47D, and ZR571) with 2- to 4-fold redundancy were embedded as described (17).

Immunohistochemistry. One set of two slides (each containing a core from different areas of the tumor for the same patient) was stained for each target marker (HSP90, ER, PR, and Her2/neu). Staining was done for AQUA as described (17). Briefly, slides were incubated with primary antibodies, mouse monoclonal anti-HSP90 at 1:500, mouse monoclonal anti-ER (DAKO, Carpinteria, CA) at 1:50, mouse monoclonal anti-PR at 1:50 (DAKO), and rabbit anti-Her2/neu at 1:8,000 (DAKO) diluted in TBS containing PBS. Goat anti-mouse (or anti-rabbit for Her/neu) horseradish peroxidase–decorated polymer backbone (Envision, DAKO) was used as a secondary reagent. To create a tumor mask, slides were simultaneously incubated with anticytokeratin of a different species (rabbit for HSP90, ER, and PR; mouse for Her2/neu) at 1:200, conjugated to Alexa 488 (Molecular Probes, Inc., Eugene, OR). The target antibody was visualized with Cy5-tyramide (NED Life Science Products, Boston, MA). Coverslips were mounted with ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA).

Automated image acquisition and analysis. Images were analyzed using algorithms that have been extensively described (16). Briefly, monochromatic, high-resolution (1,280 × 1,024 pixel) images were obtained of each histospot. Tumor was distinguished from stromal elements by cytokeratin signal. Coalescence of cytokeratin at the cell surface was used to localize cell membranes, and DAPI was used to identify nuclei. The target signal (HSP90, ER, PR, or Her2/neu) from the pixels within the membrane area or nucleus of tumor cells was normalized to the area of tumor mask and scored on a scale of 0 to 255 (the AQUA score). We excluded histospots with few tumor cells, arbitrarily selecting >3% as a threshold for inclusion.

Data analysis. JMP version 5.0 software was used (SAS Institute, Cary, NC). The prognostic significance of variables was assessed using the Cox proportional hazards model with survival as an end point. Continuous AQUA scores of target expression were dichotomized by the median, and associations with clinical and pathologic variables were done using the χ^2 test. Survival curves were generated using the Kaplan-Meier method, with significance evaluated using the Mantel-Cox log-rank test.

Results

Western blots showed high HSP90 expression for all breast cancer cell lines, and lower HSP90 for CHO cells, as shown in Fig. 1*A*, yet expression of HSP90 in breast cancer specimens was variable as detailed below.

To account for intratumor heterogeneity, two separate slides, each containing a core from a different area of the tumor for each patient, were used to evaluate expression of each marker. HSP90 and Her2/neu did not have nuclear staining, and only membranous/cytoplasmic compartments were analyzed, and vice versa for ER and PR. By performing log regression analysis for each marker, we found that scores for matching histospots were highly correlated [R = 0.69 for HSP90 (Fig. 1*B*), R = 0.74 for ER, R = 0.79 for PR, and R = 0.92 for Her2/neu]. AQUA scores ranged from 9.0 to 174 for HSP90, 2.29 to 105.39 for ER, 2.34 to 111.92 for PR, and 1.08 to 69.03 for Her2/neu.

We assessed HSP90 AQUA scores in cell lines embedded on the arrays (31 total cell line measurements), and compared them with the 1,144 tumor AQUA scores from the two arrays. Figure 1*C* shows frequency distributions of tumors and cell lines and Fig. 1*D* shows a histogram of standard deviation values (σ) of 100,000 computer-generated random selections of 31 tumor AQUA scores from the 1,144 tumor scores (median 37.35). The SD from the 31 cell line AQUA scores was 22.55 (*solid arrow*), which is significantly lower than those of the tumors ($P < 0.0001$).

Examples of weak and strong HSP90 tumor staining are shown in Fig. 2*A* and *B*.

Scores from the two slides were combined for a single data set. Of the 683 tumor histospots on each slide stained for HSP90, 470 were interpretable for both cores and 213 for one core. Spots were deemed uninterpretable if they had insufficient tumor, loss of tissue, or abundant necrosis. For patients who had two interpretable histospots, a composite score was formed by taking the average of the two scores. For patients with one interpretable core, the single score was used. The combined data set for HSP90 had 617 patients. For ER, PR, and Her2/neu (stained on earlier cuts in the array block), we obtained scores for 646, 642, and 654 patients, respectively.

Table 1 shows associations between continuous AQUA scores for HSP90, ER, PR, and Her2/neu and survival for the entire cohort, and for node-negative and node-positive subsets, by Cox univariate analysis. High HSP90 expression was associated with decreased survival for all patients ($P = 0.0024$) and for the node-negative subset ($P = 0.0145$), but not for the node-positive subset ($P = 0.4278$).

A three-dimensional scatter plot of ER, PR, and Her2/neu scores with HSP90 scores color-coded is shown in Fig. 2*C*. Low HSP90
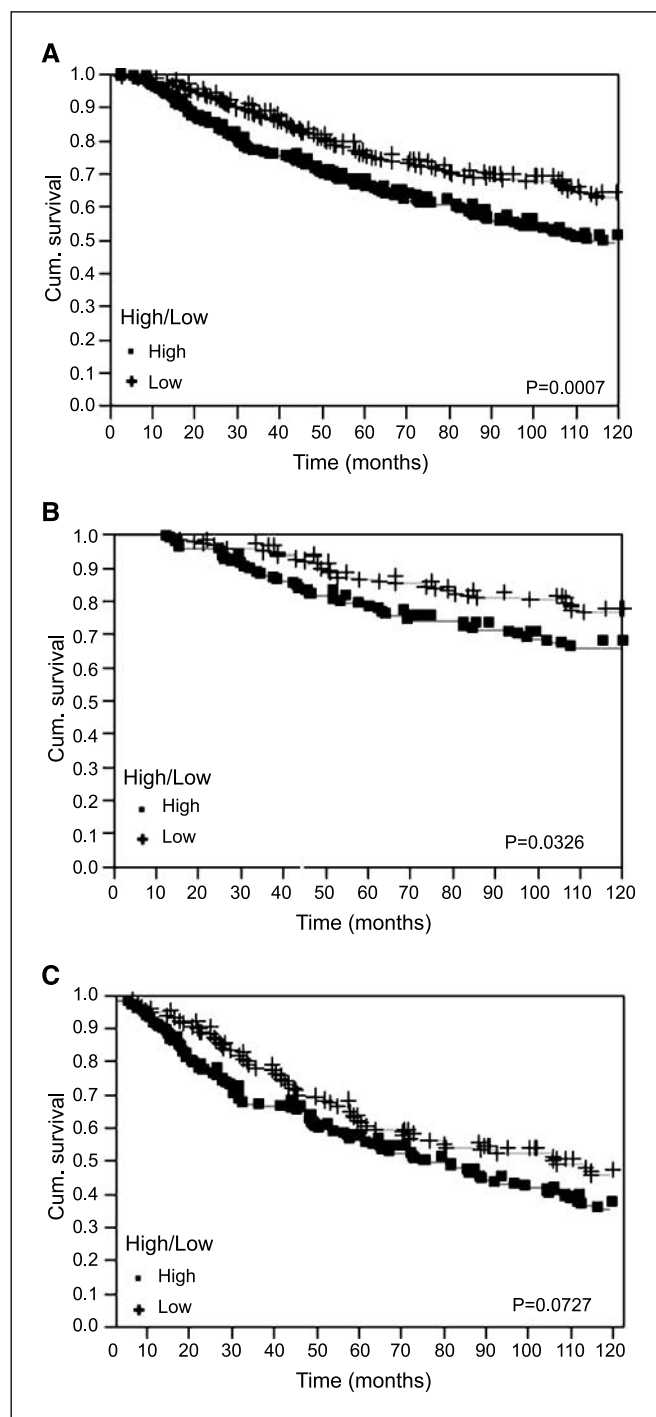
Figure 3. Kaplan-Meier survival curves for HSP90 AQUA scores divided by the median score for the entire cohort of patients (*A*), node-negative patients (*B*), and node-positive patients (*C*).

cases (*dark blue*) are not observed at the higher end of ER scores, and very few are seen with high Her2/neu or PR. Patients with low ER, PR, and Her2/neu levels had variable HSP90 levels.

Continuous HSP90 AQUA scores were dichotomized arbitrarily by the median score, reflecting the use of routine statistical divisions in the absence of underlying justification for division of expression levels. Kaplan-Meier survival curves are shown in Fig. 3. Log-rank analysis revealed a significant association with survival in

Table 1. Association between HSP90, ER, PR, and Her2/neu and breast cancer–specific survival by Cox univariate analysis with 10-y follow-up

	P			
	HSP90	ER	PR	Her2/neu
Node-positive patients	0.4278	0.0254	0.0012	0.0050
Node-negative patients	0.0145	0.7413	0.5107	0.1845
All patients	0.0024	0.0491	0.0032	0.0002

the entire cohort (Fig. 3*A*, log-rank *P* = 0.0007), and in the node-negative subset of patients (Fig. 3*B*, log-rank *P* = 0.0326), but not in the node-positive subset (Fig. 3*C*, log-rank *P* = 0.0727).

Using the Cox proportional hazards model, we did multivariable analyses. HSP90 expression retained its independent prognostic value, as did tumor size, nodal status, Her2/neu, and PR (Table 2).

To assess associations between HSP90 and other clinical and pathologic variables, we binarized the continuous AQUA scores by the median for each marker. We found a strong association between high HSP90 and high nuclear grade. Weaker associations were found between HSP90 and high Her2/neu, high ER, large tumors, and nodal metastases (Table 3).

Discussion

We quantitatively assessed expression of HSP90 and its clients Her2/neu, ER, and PR on a large primary breast cancer cohort and evaluated the association between expression and breast cancer-specific survival. Our AQUA method gives objective, continuous measures of expression, rather than arbitrary pathologist-based divisions of staining into ordinal scores, or "high/low." HSP90 expression was significantly more variable in tumors than in our cell line controls. There was a significant association between high HSP90 expression and survival in the entire cohort and among node-negative patients. On multivariable analysis, HSP90 expression retained its prognostic significance. When scores were split into high and low categories, an association was seen between high HSP90 scores and high nuclear grade. Weaker associations were seen with ER and Her2/neu expression.

Staging of primary breast cancer is important for determining prognosis and selecting patients for adjuvant chemotherapy, which reduces the risk of relapse and death. Molecular markers in primary specimens highly associated with survival, such as HSP90, could improve staging for node-negative patients, and enable us to identify high-risk patients more likely to benefit from adjuvant chemotherapy, thus avoiding chemotherapy toxicity for the vast majority of patients who are cured by local therapy alone (18, 19). Similarly, such markers could be used to avoid aggressive therapy in the smaller subset of node-positive patients who are cured without additional systemic chemotherapy (20).

In addition to a potential role as a prognostic marker, our findings have very important implications for therapeutic applications of HSP90 inhibitors in breast cancer. As with some other targeted therapies, there might be an association between target expression and response to therapy. Preclinical studies with HSP90 inhibitors have focused on expression of HSP90 clients and have not

included assessment of HSP90 itself (2, 6, 8). HSP90 levels increase in stressed cells, and it is unclear whether cell stress is necessary for efficacy of HSP90 inhibitors. We were unable to assess the association between HSP90 expression levels and response to therapy in breast cancer cell lines *in vitro*, as all of our breast cell lines in the panel expressed high HSP90 levels, as has been the case in studies by other researchers (13). However, in our clinical specimens, there is definite variability in HSP90 expression, and HSP90 expression defines two distinct populations of patients with clear differences in prognosis. The association between HSP90 expression as well as expression of client proteins and response to HSP90 targeting therapies is subject to further investigation.

In addition to metastatic breast cancer patients for whom novel therapies are clearly needed, there remains a percentage of node-negative and node-positive patients whose disease recurs despite aggressive therapy and who could benefit from novel therapies such as HSP90 inhibitors. The addition of HSP90 inhibitors to current therapy or as an alternative to standard chemotherapy could improve the therapeutic ratio and outcome of patients. However, our data suggest that patient selection, appropriate characterization of individual tumor biology, and perhaps intervention with additional rationally targeted agents might be necessary to achieve optimal results.

In summary, our study shows a strong association between high HSP90 expression and decreased survival in primary breast cancer, both among the entire patient cohort and among the node-negative subset. Greater variability in HSP90 expression was seen in tumors than in cell lines. Prospective studies are needed to confirm the prognostic role of HSP90, as well as the predictive value of HSP90 expression in patients treated with HSP90 inhibitors, preferably using quantitative methods of protein measurement. Further work is needed to elucidate the biological significance of high HSP90 expression and response to HSP90 inhibitors. Future clinical trials incorporating HSP90 inhibitors for breast cancer should stratify patients based on HSP90 expression, with the goal of developing an assay for improved patient selection for HSP90 inhibitors.

Acknowledgments

Table 3. Association between high HSP90 expression and commonly used clinical and pathologic variables

Variable	χ^2	*P*
Tumor size, >2 cm	8.343	0.0039
Age at diagnosis, <50 y	0.752	0.3858
Nuclear grade, 2–3/3	21.849	<0.0001
ER*	5.132	0.02
PR*	0.553	0.4573
Her2/neu*	9.621	<0.0019
Nodal positivity	5.844	0.0156

*HSP90, ER, PR, and Her2/neu AQUA scores were binarized by the median score.

Table 2. Cox multivariate analysis of the association between survival and other clinical and pathologic variables

Variable	95% CI	*P*
Tumor size, >2 cm	1.070–1.192	<0.0001
Age at diagnosis, <50 y	0.875–1.690	0.2449
Nuclear grade, high (2–3/3)	0.680–1.525	0.9296
Nodal status, node negative	0.321–0.601	<0.0001
ER, lower ER expression	0.983–1.012	0.7060
PR, lower PR expression	0.971–0.996	0.0086
Her2/neu, higher Her2/neu expression	1.009–1.039	0.0019
HSP90, higher HSP90 expression	1.001–1.011	0.0193

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