

HSP90 as a marker of progression in melanoma

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Background: HSP90 chaperones molecules critical for cell survival and malignant progression, including mutated B-raf. HSP90-targeting agents are in clinical trials. No large studies have been conducted on expression of HSP90 in melanomas.

Materials and methods: Tissue microarrays containing 414 nevi, 198 primary and 270 metastatic melanomas were assessed using our automated quantitative analysis (AQUA) method of *in situ* protein measurement; we use S-100 to define pixels as melanocytes (tumor mask) within the array spot, and measure HSP90 expression within the mask using Cy5-conjugated antibodies.

Results: HSP90 expression was higher in melanomas than nevi ($P < 0.0001$) and higher in metastatic than primary specimens ($P < 0.0001$). No association was seen between high HSP90 expression and survival in the primary or metastatic patient subsets. In primary melanomas, high HSP90 expression was associated with higher Clark level ($P = 0.0167$) and increased Breslow depth ($P < 0.0001$).

Conclusions: HSP90 expression was significantly higher in tumors than nevi and was associated with disease progression, indicating that it might be a valuable drug target in melanoma, as well as a useful diagnostic marker. Prospective studies are needed to confirm the diagnostic role of HSP90, as well as the predictive role of HSP90 expression in patients treated with HSP90 inhibitors.

Key words: chaperones, diagnostic markers, HSP90, progression markers, therapeutic targets

introduction

Cellular heat shock proteins (HSPs) are found in all cells in many life forms and are present under normal cellular conditions, but are up-regulated under conditions of increased stress, including cancer [1]. HSPs are cytoplasmic molecules that facilitate protein folding, ensuring both proper folding upon synthesis and refolding under conditions of denaturing stress [2]. HSPs also act as chaperones, shuttling proteins to different intracellular locations; they transport proteins across plasma membranes within a cell and transport proteins to the proteasome for degradation [1].

HSPs have significant overlap in their functions and are classified by their molecular weight. HSP90 is a 90-kDa protein, which protects its 'client' proteins from deterioration caused by environmental stress, including chemotherapy, thus promoting chemotherapy resistance and cell survival. More than 100 client proteins have been identified for HSP90, many of which are important in the progression of cancer [3, 4], including MEK, CDK4, CDK6, AKT, Apaf-1, h-Tert, VEGFR and c-MET [5]. Thus, inhibition of HSP90 can have

devastating effects on cell viability, as it plays a role in the regulation of signal transduction, cell cycle control and apoptosis [6, 7].

Over the last decade, the focus of anticancer drug development has shifted towards targeted therapies. Examples include imatinib and gefitinib and the therapeutic antibodies trastuzumab, cetuximab and bevacizumab [8]. There are many signaling pathways involved in malignancy; therefore, targeting a single molecule in solid tumors is not always effective. Given that HSP90 chaperones numerous proteins implicated in carcinogenesis, inhibiting HSP90 could result in inhibition of several pathways at once, providing more effective antitumor therapy.

Studies have shown that the Ras/Raf/mitogen-activated protein kinase signaling pathway is mutationally activated in 60%–70% of melanomas [9]. These B-raf mutations are usually in the kinase domain, and in melanomas these are usually V600E missense mutations. Targeting mutated B-raf in melanoma is the subject of ongoing clinical trials. Da Rocha Dias et al. and Grbovic et al. [10, 11] showed that HSP90 chaperones mutated B-raf, but not wild-type B-raf. Therefore, use of HSP90 inhibitors in B-raf-driven melanoma could result in B-raf degradation, resulting in inhibition of cell proliferation.

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The incidence of cutaneous melanoma in the United States is rising faster than that of any other malignancy, with 59 990 expected new cases in 2007, and 8110 expected deaths from metastatic disease [12]. Given that there is no effective therapy for unresectable melanoma, there is an urgent need for improved systemic therapies, as melanoma is usually resistant to standard chemotherapy. Novel therapeutic approaches, such as targeting HSP90, are being assessed. Preclinical studies have shown activity of HSP90-targeting therapies in melanoma [13].

Agents that target HSP90 are currently in clinical development. As with many other targeted therapies, efficacy of these agents might be dependant on target expression within the disease population. A phase I clinical trial assessing the first HSP90 inhibitor to enter the clinic (17-AAG) included 11 metastatic melanoma patients, two of whom had prolonged stable disease (defined as less than a 50% reduction and less than a 25% increase in the sum of products of two perpendicular diameters of all measured lesions and the appearance of no new lesions) 15 and 41 months, indicating that this agent has clinical activity in melanoma [14]. Therefore, we sought to determine the expression patterns of HSP90 in a large cohort of melanomas and to determine the differences in expression between benign and malignant tissue. Two small studies have reported the presence of HSP90 in 10 and 23 melanomas, respectively [13, 15]. A review of the literature did not reveal large-scale studies assessing HSP90 expression patterns in melanocytic lesions. Other HSPs such as HSP70 have also been identified in melanoma cell lines and small cohorts of patients [16, 17]. We utilized our newly developed method of automated quantitative analysis (AQUA) of tissue microarrays (TMAs) to assess expression of HSP90. This method has been validated and proven to be more accurate than pathologist-based scoring of 3,3'-diaminobenzidine stain [18]. Here, we found higher HSP90 expression in tumors than nevi and higher expression in metastatic than primary specimens.

material and methods

cell lines and western blots

CHO (Chinese hamster ovary) and MCF-7 (human breast cancer) cell lines were purchased from The American Type Culture Collection (Manassas, VA). YUMAC, YUSAC, YUSOC, YUGEN8, YUFIC and YULAC cell lines were established from tumors of patients treated at Yale University as previously described [19]. Mel501 and Mel928 cells were obtained from Steven Rosenberg at the Surgery Branch, National Cancer Institute, Bethesda, MD. Western blotting of protein extracts was carried out 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 rabbit anti- β -actin (Sigma-Aldrich, St Louis, MO) at 1 : 5000.

TMA construction

TMAs were constructed as previously described [19]. A cohort of 230 primary melanomas, each measuring 0.6 mm in diameter, were spaced 0.8 mm apart on glass slides. For comparison of expression, specimens from a series of 293 metastatic patients were placed on the slides. Specimens and clinical information were collected with approval of a Yale Institutional Review Board. The specimens were resected from 1959

to 2000. The cohort has been described and validated in numerous publications [19]. Pellets of 15 melanoma cell lines were embedded as described [20]. The benign nevus array contained 540 nevi and 40 melanomas and cell lines that were also present on the tumor array and used as controls.

immunohistochemistry

One set of two slides (each containing a core from different areas of tumor for the same patient) was stained for HSP90. Staining was carried out for AQUA as described [19]. Briefly, slides were incubated with the primary antibody; mouse monoclonal anti-HSP90 at 1 : 500, diluted in Tris-buffered saline containing phosphate-buffered saline. Goat anti-mouse horseradish peroxidase-decorated polymer backbone (Envision, Dako, Carpinteria, CA) was used as a secondary reagent. To create a tumor mask, slides were simultaneously incubated with rabbit anti-S100 at 1 : 200 and conjugated to Alexa 546 (Molecular Probes, Inc., Eugene, OR). HSP90 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 [18] and demonstrated in Figure 1. Briefly, monochromatic, high-resolution (1280 \times 1024 pixel) images were obtained of each histospot. Tumor was distinguished from stromal elements by S-100 signal. Coalescence of S-100 at the cell surface was used to localize cell membranes, and DAPI was used to identify nuclei. The target (HSP90) signal 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 0–255 (the AQUA score). No HSP90 staining was seen in the nuclei, and

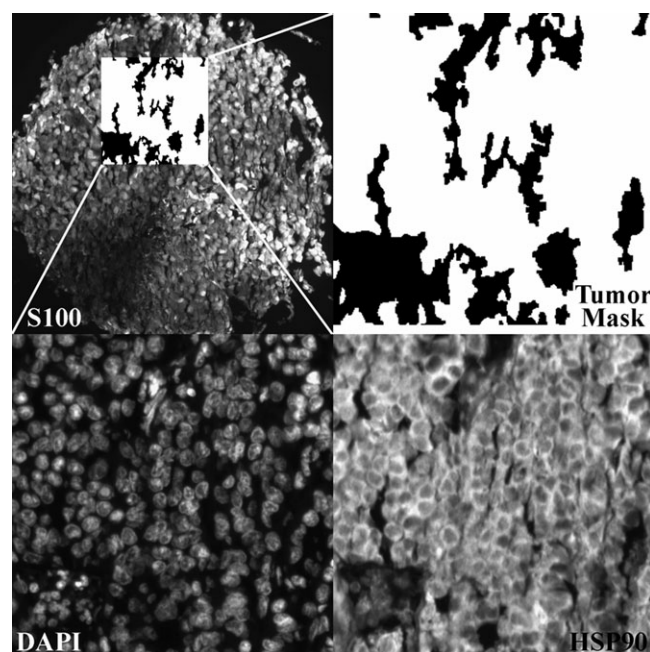


Figure 1. Automated quantitative analysis (AQUA) of *in situ* protein levels: AQUA uses S100 to define the tumor or nevus mask (top two panels), 4',6-diamidino-2-phenylindole (DAPI) to define the nuclear compartment (left lower panel) and subtracts the nuclear compartment from the S100 mask to define the cytoplasmic compartment. HSP90 (lower right panel) expression is measured within the nuclear and cytoplasmic compartments, within the tumor mask, and each clinical case is assigned a score based on pixel intensity per unit area.

only cytoplasmic expression was analyzed. We excluded histospots with few tumor cells, selecting >3% as a threshold for inclusion.

data analysis

JMP version 5.0 software was used (SAS Institute, Cary, NC). The prognostic significance of parameters was assessed using the Cox proportional hazards model with survival as an end point. The association between continuous HSP90 AQUA scores and other clinical/pathological parameters was assessed by analysis of variance.

results

Western blots showed high HSP90 expression in all melanoma cell lines compared with CHO cells, with the exception of mel928 (Figure 2A).

To account for intratumor heterogeneity of HSP90 expression, two separate melanoma TMAs, each containing a core from a different area of tumor for each patient, were stained. Positive HSP90 immunoreactivity was observed predominantly in the cytoplasmic compartment; hence, the nuclear compartment was not analyzed. We did not see differences in staining patterns within the tumor mask within a histospot. The matching cell line controls on the arrays had very high correlations for HSP90 expression ($R = 0.86$), and the tumors were somewhat more heterogenous ($R = 0.5$), as shown in Figure 2B.

AQUA scores from both slides were combined to give a single dataset. Of the 523 melanoma histospots on each slide,

234 had cores that were interpretable on both slides and 241 were interpretable for one slide. Spots were deemed uninterpretable if they had insufficient tumor, loss of tissue or an abundance of necrotic tissue. For patients who had two interpretable histospots, a composite score was formed by averaging the two scores. For patients with only one interpretable core, the single score was used. The combined dataset had scores for 468 melanoma patients: 198 primary specimens and 270 metastatic specimens. For the histospots on the nevus array, we obtained 414 scores.

AQUA scores for melanoma specimens ranged from 3.45 to 136.68. Figure 2C shows a frequency distribution of HSP90 expression for melanomas, cell lines and nevi. The nevi predominantly had low HSP90 scores. HSP90 scores in melanomas were relatively normally distributed and higher than nevi. The cell lines had variable HSP90 expression, higher than that of nevi and melanomas.

Using analysis of variance, we found that HSP90 expression was significantly higher in malignant than benign specimens ($P < 0.0001$) and higher in metastatic than in primary lesions ($P < 0.0001$), as shown in Figure 2D. The mean AQUA scores for HSP90 were 7.76 in nevi, 31.53 in primary lesions, 42.03 in metastatic lesions and 37.62 in all malignant lesions. Seventeen percent of the melanomas had HSP90 scores lower than the 95th percentile score for nevi.

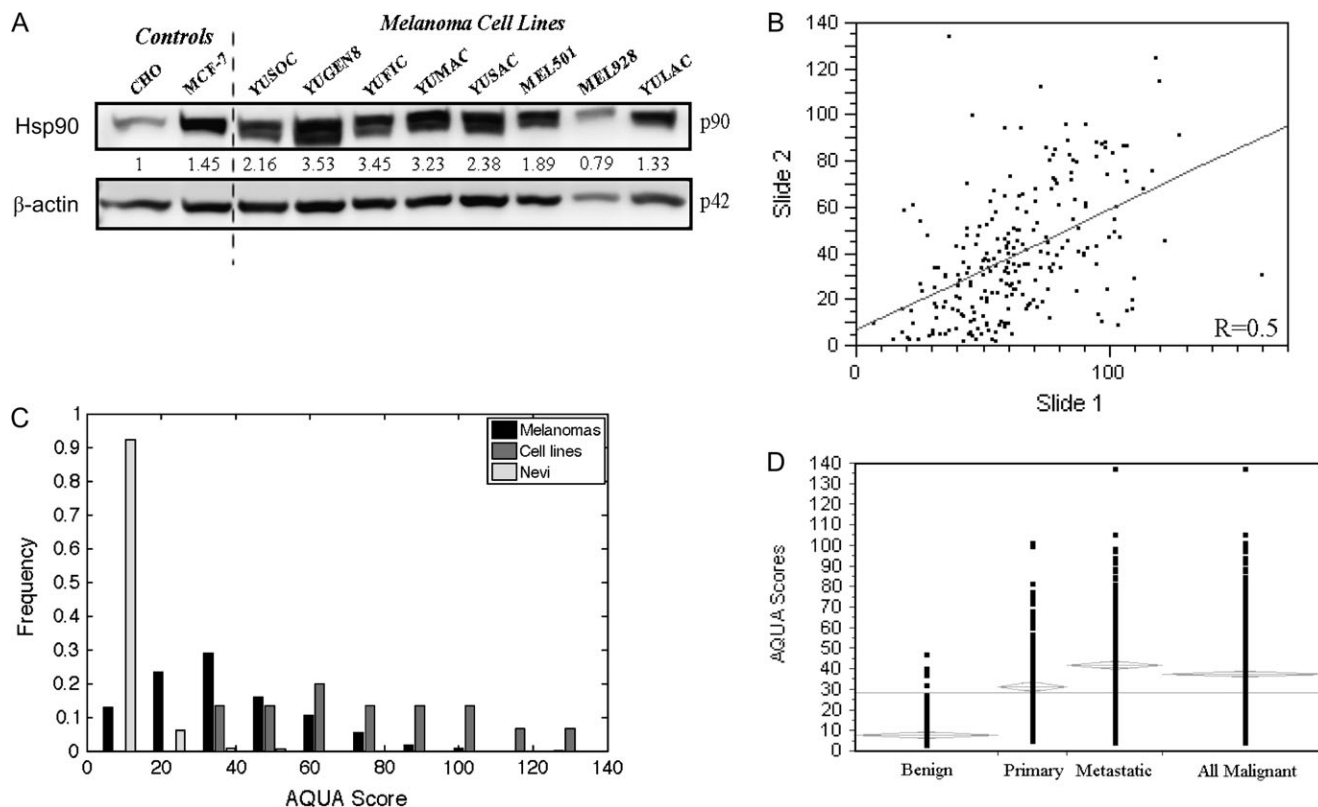


Figure 2. Western blots (A) of a panel of cell lines shows high expression in all melanoma cell lines than in (CHO) Chinese hamster ovary cells, with the exception of Mel928. The numbers show the ratios of band intensity in the cell lines relative to that in CHO cells, normalized to the β-actin loading. Cell lines from left to right: CHO, MCF-7, YUSOC, YUGEN8, YUFIC, YUMAC, YUSAC, Mel501, Mel928 and YULAC. Regression plot (B) for scores from the two melanoma tissue microarrays stained for HSP90 ($R = 0.5$). Frequency distributions (C) of melanomas (black bars), cell lines (gray bars) and nevi (white bars). Analysis of variance (D) comparing the means of benign, primary, metastatic and all malignant (primary and metastatic combined) specimens.

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By Cox univariate and multivariate survival analyses of raw AQUA scores, we found no association between high HSP90 and melanoma-specific survival in the primary specimen cohort ($P = 0.39$). The only variables associated with survival on multivariate analysis were Breslow depth and Clark level.

To assess the association between HSP90 expression and commonly used clinical and pathological parameters in the subset of primary specimens, we used analysis of variance. HSP90 expression was associated with high Clark level ($P = 0.0167$) and Breslow depth ($P = 0.0015$) (Table 1).

discussion

Our purpose was to quantitatively assess HSP90 expression on a large number of melanomas and nevi in an automated, objective fashion. Furthermore, we evaluated the association between expression of HSP90 and clinical and pathological variables. HSP90 expression was significantly higher in malignant than in benign specimens and in metastatic versus primary specimens. HSP90 expression was more variable in tumors than in nevi, and there was a small subset of 17% of the malignant specimens that had expression levels lower than the 95th percentile for nevi. Among primary specimens, HSP90 expression was higher in Clark level IV–V specimens and in specimens deeper than 2 mm.

We also assessed HSP90 expression in a panel of cell lines. In previous studies in breast cancer [20], we found that all cell lines had high expression, and the range of expression in cell lines was less variable than in breast tumors. In our melanoma studies, we again demonstrated high expression in most cell lines, but found it to be more variable than in breast cancer. This might reflect the fact that here we used patient-derived early passage cell lines in addition to established, higher passage cell lines.

Higher HSP90 expression in malignant specimens may have important implications in the diagnosis of melanoma. Immunohistochemical biomarkers can be useful when the diagnosis of malignancy is questionable.

High HSP90 expression in malignant specimens also has important therapeutic implications. HSP90 client proteins are involved in many pathways implicated in malignant progression [5]. Inhibiting HSP90 results in degradation of

these proteins and subsequent cell death. There are currently >20 ongoing clinical trials with HSP90 inhibitors [21]. HSP90 inhibitors selectively target malignant cells over normal cells [22]. The HSP90 inhibitor 17-AAG has 100-fold higher affinity for the tumor-based conformation of HSP90 than HSP90 in normal cells [23]. *In vivo* inhibition is seen at concentrations that do not have significant toxic side-effects in patients [21]. Examples of HSP90 inhibitors include Geldanamycin and its clinical derivatives (17-AAG and 17-DMAG), radicicol, novobiocin, purines, pyrazoles, cisplatin and histone deacetylase inhibitors, as extensively reviewed [24, 25].

In breast cancer, HSP90 inhibitors are currently being studied in clinical trials in combination with trastuzumab, which inhibits Her2/neu. Similarly, in melanoma combinations of HSP90 inhibitors and therapy targeted at critical client proteins (such as inhibitors of mutated B-raf) might be synergistic. Further work is needed to characterize the client protein profile of HSP90 in melanoma in order to begin testing combinations of targeted therapies.

Although most melanomas had high HSP90 expression, there remained a subset that demonstrated relatively low levels. Further work is needed to determine whether there is a threshold HSP90 level, below which HSP90 inhibitors are ineffective. The association between HSP90 expression levels in tumors and response to therapy is unknown. As with some other targeted therapies (such as trastuzumab and antihormonal therapy in breast cancer), higher levels of target expression might be required for a tumor to respond to therapy. It is, however, also possible that the reverse might in fact be true for HSP90 and lower expressers might respond to therapy or there might be no association between HSP90 expression and response to HSP90-targeted therapies. Given that all of our melanoma cell lines except for mel928 had relatively high levels of HSP90 (possibly as a response to tissue culture conditions), the association between HSP90 expression levels and response to therapy will most likely need to be assessed *in vivo*. Our data indicate that at the very least, expression of HSP90 and its critical clients should be assessed in specimens from melanoma patients treated with HSP90 inhibitors.

In summary, we found that HSP90 expression is significantly higher in melanomas than nevi, making HSP90 an attractive drug target for this disease and a diagnostic marker that is worthy of further evaluation. Additional work is needed to elucidate the biological significance of high HSP90 expression and assess the association with response to HSP90 inhibitors.

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Table 1. Association between HSP90 expression in primary melanomas and other clinical and pathological variables

Clinical variable	P value
Clark level (I–III versus IV–V)	0.0167 ^a
Age (≤50 versus >50 years)	0.1432
Breslow depth (≤2 versus >2 mm)	<0.0001 ^a
Ulceration (present versus absent)	0.1713
Sex (female versus male)	0.7270
Survival (Cox)	0.39

The association between HSP90 expression and clinical variables was assessed by analysis of variance and the association with survival by the Cox proportional hazards model.

^aSignificance was determined using a cut-point of <0.05.

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