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Heat shock protein 27 is expressed in normal and malignant human melanocytes *in vivo*

Background: Heat shock proteins (HSPs) are a family of highly conserved proteins found ubiquitously in mammalian cells, believed to be regulators of normal cell physiology and the cellular stress response. In addition, the small 27-kDa heat shock protein (HSP27) has previously been found to be a differentiation marker for keratinocytes and a prognostic marker associated with increased survival in certain cancerous tumors.
Methods: Using immunohistochemistry on routinely processed paraffin sections, we examined skin biopsies from 15 invasive melanomas, 13 intradermal nevi, and two compound nevi immunostained with a mouse monoclonal antibody to HSP27. In addition, cultured melanocytes were heat stressed at 45°C for 1 h and then fixed and immunostained in order to localize HSP27 expression intracellularly.
Results: We found cytoplasmic and strong perinuclear staining of HSP27 in melanocytes in normal skin, in melanomas, and in nevi. Nuclear reactivity was absent. In addition, in cultured non-malignant melanocytes, HSP27 expression relocated from the cytoplasm to the nucleus with heat stress.
Conclusions: To our knowledge, this investigation is the first to demonstrate that HSP27 is expressed in melanocytes in normal skin, in nevi, and in non-malignant cultured melanocytes.

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Heat shock proteins (HSPs) are a family of highly conserved proteins found in all organisms,[1] and their expression is increased after heat stress as well as a host of environmental insults such as UV, metabolic poisons, alcohol, heavy metals, and mechanical stretching.[2,3] HSPs are also expressed in non-stress conditions where they are believed to regulate protein synthesis, folding, assembly and degradation, signal transduction, and cell proliferation.[1] Small molecular weight HSPs also act as molecular chaperones protecting peptides from misfolding and irreversible denaturation, and some are reported to have anti-apoptotic[4,5] and actin-stabilizing properties.[6]

A member of the small HSP family, 27-kDa heat shock protein (HSP27), is expressed in a variety of normal human tissues such as breast, uterus, oviduct, vagina, and skin.[7] Its normal intracellular location is in the cytoplasm, near the Golgi complex.[8] When cells are heat stressed or exposed to other oxidative stresses, HSP27 relocates from the cytoplasm to the nucleus.[9,10] Hence, the localization pattern of HSP27, whether nuclear or cytoplasmic, may be a marker in

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determining which cells are responding to stressful conditions, and thus may be a useful diagnostic tool.

Recent studies have also suggested that HSP27 expression by malignant cells may serve as a prognostic factor. In certain tumors, such as astrocytic brain tumors, squamous cell carcinoma of the esophagus, and malignant fibrous histiocytoma[11–13], increased-HSP27 expression is correlated with better patient survival. On the other hand, in gastric and hepatocellular carcinoma increased-HSP27 expression by tumor cells has been correlated with worse survival.[14,15] To confuse matters further, HSP27 is not a prognostic marker for either better or worse survival in uveal melanomas.[16]

In keratinocytes, HSP27 expression serves as a marker of differentiation, both *in vitro*[17]and *in vivo*.[18] We previously demonstrated a gradient of HSP27 expression within normal human epidermis, with little expression within the basal layer, and increased expression in the suprabasilar, differentiated layers.[18] Kindas-Mugge et al.[17] demonstrated that keratinocytes cultivated under high calcium conditions that induce differentiation revealed a two-fold increase in HSP27 expression. In addition, another study correlated high HSP27 expression with increasing epidermal differentiation in developing fetal epidermis.[19] In contrast, HSP27 expression in melanocytes has not been studied extensively. Although previous studies have demonstrated HSP27 expression in cultured malignant melanocytes,[20,21] there are no published reports concerning HSP27 expression in normal non-malignant melanocytes *in vitro*. In addition, the only published study[22] commenting on melanocyte HSP27 expression *in vivo* concluded that HSP27 is not expressed in melanocytes. Hence, while HSP27 is established as a differentiation marker in keratinocytes, its ability to serve as a marker for the differentiated phenotype in other skin cell types, such as melanocytes, is not clear.

The present study was performed to investigate whether HSP27 is expressed in melanocytes present in normal human skin and whether its expression is changed in either nevi or melanomas. This investigation is the first to demonstrate that HSP27 is expressed in melanocytes in normal skin, in nevi, and in non-malignant cultured melanocytes. Further investigations are needed to elucidate the relationship between HSP27 and malignancy in cutaneous pathology.

Materials and methods

Sample collection

Cutaneous tissue specimens that had been excised for pathologic examination were obtained from the surgical pathology files at the University of California-Davis Medical Center from 30 different individuals (10 male, 20 female, median age 44, range 14–91 years). The specimens included 15 invasive melanomas, 13 intradermal nevi, and two compound nevi were biopsied from various sites (back, abdomen, groin, extremities, neck, face, and scalp). The tumor thickness of the 15 invasive melanoma cases ranged from 0.25 to 3.5 mm.

Immunohistochemistry

All tissues were submitted in 10% buffered formalin, routinely processed and embedded in paraffin for tissue sectioning. Paraffin sections were examined by immunohistochemical technique utilizing an immunoglobulin G (IgG) monoclonal antibody to HSP27 (StressGen Biotechnologies Corp, Victoria, BC, Canada) at a dilution of 1 : 1000. Briefly, 5-μm sections were deparaffinized, rehydrated, and endogenous peroxidase-blocked with 3% H_2O_2 in absolute methanol. After blocking with 10% normal equine serum, a 1-h incubation at room temperature with the primary monoclonal antibody was performed, followed by a phosphate-buffered saline (PBS) rinse. A biotin-conjugated equine antimouse secondary antibody (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1 : 1000 followed. After PBS rinsing, an avidin-biotin complex (ABC Elite, Vector Laboratories) was applied. After PBS rinsing, diaminobenzidene-peroxidase (ScyTek Laboratories, Logan, UT, USA) was applied to achieve a permanent color change in the reactive cells.

Cases were scored on a 0 (no reactivity), 1 (<50% reactivity), and 2 scale (>50% reactivity) semiquantitatively using an Olympus BX40 microscope by a dermatopathologist (MAF). Abundance of the antigen was not quantified; any reactivity to HSP27 staining in the cutaneous sections was scored as a positive sample. As a negative control, one section from each sample was prepared for immunohistochemistry as outlined above, except the HSP27 primary antibody was omitted.

Melanocyte cell culture

Normal human epidermal melanocytes were isolated from primary keratinocyte cultures derived from neonatal foreskins, obtained with an approved protocol from the University Institutional Review Board.[23] The initial isolation of the keratinocytes is the same procedure as we have reported previously.[24] Briefly, foreskins were trimmed of excess subcutaneous tissue, cut into small pieces and trypsinized overnight at 4°C. Epidermis was scraped from the dermis, dispersed into a single-cell suspension and plated onto a mitomycin C-treated 3T3 feeder layer, as previously described.[25] After the cells in culture formed 6–12 cells per colony, the medium was changed to keratinocyte growth

medium (KGM, Cascade Biologics Inc., Portland, OR, USA). Once the cell culture reached 20% confluency, the melanocytes were isolated by selective trypsinization[26] and plated onto new dishes in melanocyte growth medium (MGM, Cascade Biologics Inc., Portland, OR, USA), a M154-based medium supplemented with penicillin-G, streptomycin, amphotericin B, bovine pituitary extract, fetal bovine serum, bovine insulin, bovine transferrin, basic fibroblast growth factor, hydrocortisone, heparin, and phorbol 12-myristate 13-acetate. After cultures reached about 70% confluency, cells were treated with 100 μg/ml of geneticin (G418 Sulfate, Gibco BRL, Grand Island, NY, USA) for 3–5 days to selectively eliminate any fibroblast contamination.[27] To test the purity of melanocyte cultures, cells were recovered, passed onto collagen I-coated glass coverslips and probed with the NK1/beteb antibody (Caltag Laboratories, Burlingame, CA, USA) specific to melanocytes.[28] Cultures were only used if they were free of contaminating keratinocytes and fibroblasts. Cells were maintained at 37°C/5% CO_2.

Immunostaining of HSP27 in cultured melanocytes

Melanocytes were plated on glass, collagen I-coated coverslips (Vitrogen, Cohesion Technologies, Palo Alto, CA, USA). Collagen coating was done by immersing coverslips in 60 μg/ml of collagen diluted in medium 154 at 37°C for a minimum of 1 h. Coverslips were then washed three times with medium 154 before melanocytes were plated. Cells were cultured on coverslips 48 h before heat treatment and immunostaining. For heat treatment, cultured cells were subjected to 45°C in an incubator for 60 min. Following heat treatment, the cultured cells were returned to a 37°C incubator for 30 min to recover prior to fixation. Coverslips were fixed 3.7% formaldehyde for 10 min at room temperature, permeabilized with −20°C acetone for 3 min and washed with PBS. Non-specific binding was blocked by incubation in 10% normal goat serum diluted in PBS for 1 h at room temperature. Coverslips were then incubated in mouse anti-HSP27 (1 : 500) (Stressgen Bioreagents, Victoria, BC, Canada) at 37°C for 1 h. Coverslips were then washed three times in PBS, incubated for 1 h at 37°C in goat anti-mouse fluorescein isothiocyanate (FITC) (1 : 500, Sigma-Aldrich, St. Louis, MO, USA), washed three times in PBS, incubated for 10 min in equilibrium buffer and mounted in SlowFade Light (Molecular Probes, Eugene, OR, USA). Cells were viewed with epifluorescence optics on a Nikon Diaphot microscope.

Results

In unstressed cultured melanocytes (Fig. 1A), HSP27 was expressed within the cytoplasm with limited nuclear expression. After stressing, cultured melanocytes by exposure to 45°C for 1 h, strong nuclear and cytoplasmic HSP27 was observed (Fig. 1B).

Sections of normal skin, adjacent to benign intradermal nevi (Fig. 2) demonstrated cytoplasmic immunostaining with the HSP27 antibody in suprabasilar epidermal keratinocytes. There was immunostaining for HSP27 in the basal cell layer of epidermis keratinocytes. However, staining intensity gradually increased from the basal cells to the keratinocytes of the granular layer in line with previous reports.[7,18,29] Immunostaining was homogeneously distributed throughout the keratinocyte cytoplasm, and nuclear expression was absent. Junctional melanocytes (Fig. 2 – identified by arrows) demonstrated cytoplasmic staining of HSP27 in 26/30 cases with occasional strong perinuclear staining and an absence of nuclear reactivity (Table 1).

In 15 melanocytic nevi (13 intradermal nevi – Fig. 3 and two compound nevi samples – Fig. 4), there was cytoplasmic staining in both junctional and dermal nests with focal perinuclear staining (Fig. 3). In the 15 melanoma samples examined, most but not all of the lesional melanocytes expressed HSP27 (Fig. 5). This is in contrast to nevi where 100% of dermal and junctional nests were immunopositive (Table 1). In 10 of the 15 melanoma samples, HSP27 staining was seen

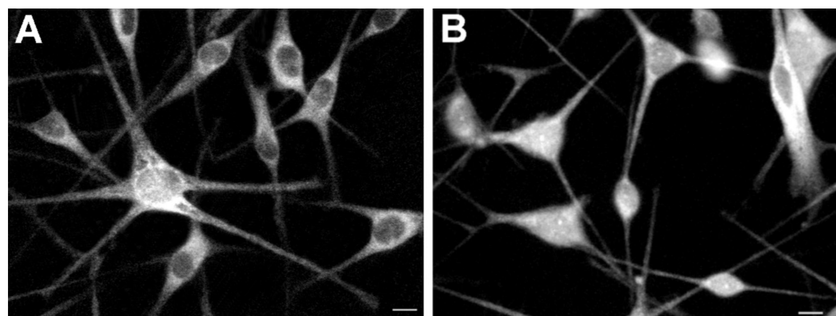

Fig. 1. Human melanocytes express heat shock protein 27 (HSP27) in culture. A) Unstressed cultured melanocytes demonstrate strong cytoplasmic HSP27 staining and limited nuclear expression. Bar: 10 μm. B) Melanocytes stressed with 45°C for 1 h reveal strong cytoplasmic HSP27 and nuclear HSP27 staining.

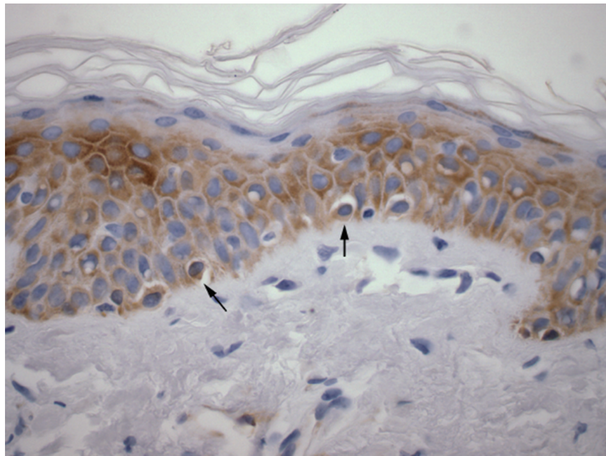

Fig. 2. Heat shock protein 27 (HSP27) is expressed in normal human epidermal melanocytes. Normal adult skin demonstrates cytoplasmic HSP27 staining of melanocytes with the basal cell layer (arrows). Note the strong cytoplasmic staining of keratinocytes with gradual increased intensity from the basal to the granular layer.

in the cytoplasmic areas of melanocytes located in junctional nests (Fig. 5C). In addition, strong perinuclear cytoplasmic staining was observed in some melanocytes that stained for HSP27 in melanoma samples.

In all of the samples (n = 30), endothelial cells, sebaceous glands, and sweat ducts were reactive to HSP27 immunostaining, while variable staining was observed in a minority of smooth muscle arrector pili. In contrast, an earlier study demonstrated negative HSP27 expression in endothelial cells.[22] Dermal components such as fibroblasts, adipose tissue, and peripheral nerves did not appear to demonstrate HSP27 immunoreactivity in agreement with an earlier report.[22]

Discussion

This investigation demonstrates that HSP27 is expressed in human melanocytes *in vivo* in normal skin, in nevi, and in melanomas. As both nevi and invasive melanoma have similar patterns of HSP27 expression, HSP27 expression cannot be used to distinguish between melanocytes in nevi vs. melanoma. At this point, the cellular function of HSP27 in melanocytes in human skin remains enigmatic.

As HSP27 is a prognostic factor associated with increased survival in certain malignancies,[30] we initially sought to determine whether its presence could likewise be used as a marker of melanocyte malignant transformation. We first examined the expression of HSP27 in cultured human melanocytes and found robust intracellular expression. After application of heat stress, HSP27 translocated from the cytoplasm into nuclear aggregates, consistent with published reports in keratinocytes when stressed by various stimuli.[9,10] Although HSP27 expression has been previously observed in cultured human melanoma cells,[21,31,32] this is the first demonstration of the protein within normal human non-malignant melanocytes in culture. To assure that these findings were not limited to cultured cells or an artifact of *in vitro* cultivation, we examined HSP27 expression in melanocytes present within human skin as well. We found that HSP27 was expressed in single melanocytes within the normal epidermis.

We initially hypothesized that HSP27 might be a prognostic factor for tumor aggressiveness. Supporting this hypothesis is the work of Aldrian and colleagues,[20] who overexpressed HSP27 in a melanoma cell line and demonstrated a marked change in the cells that expressed HSP27, correlating with a less invasive cell phenotype. These changes included a more epithelioid colony morphology in culture, less invasive ability in extracellular matrix-coated filter assay, loss of $\alpha v\beta 3$ integrin, and a decrease in metalloproteinase expression, thought to mediate cell invasiveness. These findings are consistent with the notion that HSP27 expression is somehow protective against the malignant phenotype and may underlie the good clinical prognosis seen in patients with a subset of HSP27-expressing tumors. HSP27 is reported to be a prognostic factor in certain tumors such as astrocytic brain tumors, squamous cell carcinoma of the esophagus, and malignant fibrous histiocytoma.[11–13] In uveal melanomas, however, HSP27 is not a prognostic factor.[33] In this study, we demonstrate that HSP27 cannot be used to distinguish between melanocytes in nevi vs. melanomas because HSP27 is not differentially expressed in melanomas as compared to nevi. Further studies are needed to determine whether HSP27 expression can used as a prognostic marker in skin malignancies.

In addition, HSP27 expression and cellular localization *in vivo* does not appear to change in response to exogenous stress in the same way that it does *in vitro*. A recent *in vivo* study has concluded that there is no difference in intracellular HSP27 localization in melanocytes in uveal melanomas after treatment with

Table 1. Expression of HSP27 in melanocytes*

	Perilesional single melanocytes along dermal/epidermal junction	Melanocytes in junctional nests	Melanocytes in the dermis
Melanoma (n = 15)	13/15 (87%)	10/15 (67%)	13/15 (87%)
Nevi (n = 15)	13/15 (87%)	2/2 (100%) (compound)	15/15 (100%)

HSP27, heat shock protein 27.
*Number of samples that stain positively for HSP27.

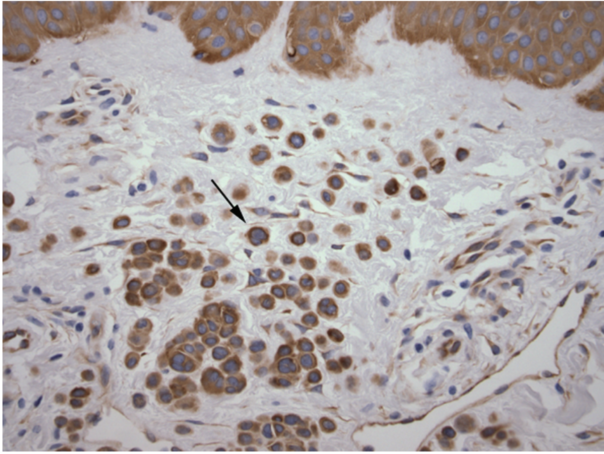

Fig. 3. Heat shock protein 27 (HSP27) is expressed in melanocytes in intradermal nevi. An intradermal nevus of an adult demonstrates cytoplasmic HSP27 staining of melanocytes with occasional strong perinuclear staining (arrow).

either radiation or thermotherapy as compared to non-treated melanomas.[16] However, in the study, there is a minimum of a week between the treatment of a uveal melanoma and enucleation. Therefore, the authors attribute the lack of HSP27 expression *in vivo* to the long-time interval after the chemical or thermal stress and suggest that the stress may have induced localization changes at an earlier time point then they examined. Indeed, an earlier study[34] in a fibrosarcoma and monoblastoid cell line demonstrates that after heat stress, HSP27 levels return to normal in 20–24 h. These findings suggest that the transient nature of HSP27 expression may explain the lack of stress-induced HSP27 expression *in vivo*.

HSP27 is a marker of keratinocyte differentiation, with more differentiated, suprabasilar keratinocytes of human epidermis expressing higher levels of HSP27 than the basilar keratinocytes.[19] In our study, we were unable to ascribe a role for HSP27 expression to melanocyte differentiation. While the antigens S100 and HMB-45 have value as a melanocyte lineage marker,[35] to our knowledge, there is, as yet, no definitive melanoma-specific differentiation marker. Finding such a marker would be a great advantage, as distinguishing between borderline benign nevi and melanoma can be difficult, making misdiagnosed melanomas an all too frequent occurrence.[36] This diagnostic challenge is exacerbated by the fact that histologic differentiation between borderline benign and malignant melanocytic lesions has poor reproducibility and large interobserver variation.[37,38] Thus, we embarked on this study hoping that HSP27 expression would be decreased in melanocytes with presumed altered differentiation, such as those in melanomas. Although there is some variability in the expression of HSP27 in dermal melanocytes within melanoma as compared to the intradermal components of nevi (Table 1), the differences are not striking. Unfortunately, there is little difference in HSP27 expression in melanocytes in melanomas as compared to those in normal skin and nevi.

While HSP27 expression cannot be used to distinguish between melanocytes in nevi and melanomas, its expression may have biological relevance in terms of immune modulation of tumor rejection. Recent studies have shown that vaccination with large HSP-antigen peptide complexes renders mice resistant to the malignancies from which the HSPs were isolated.[39,40] The HSP-antigen complex causes the antigen to be absorbed into an antigen-presenting cell and be presented to the immune system.[41] It has been proposed that HSPs be used to stimulate the immune system against melanomas by injecting HSP-antigen peptide complexes isolated via chromatography from the primary tumor into patients.[42] Hence, investigating the expression of HSP27 in melanocytic lesions may lead to potential therapeutic interventions in the future.

Complicating the assessment of the biologic relevance of HSP27 expression in melanocytes is the fact that phosphorylation of this stress protein when exposed to a diverse array of stimuli alters its function.[10] Phosphorylated HSP27 is correlated with smooth muscle and mesangial cell contraction[43,44] as well as actin stabilization[6,45] which plays an important role in the regulation of structural organization in a cell. This initial study did not evaluate the phosphorylation status of the expressed HSP27. Further studies are underway to elucidate the role of phosphorylated HSP27 in intracellular localization of melanocytes *in vivo*.

Our study is the first to report the expression of HSP27 expression in melanocytes in normal epidermis, compound nevi, intradermal nevi, melanomas, and normal non-malignant cultured melanocytes.

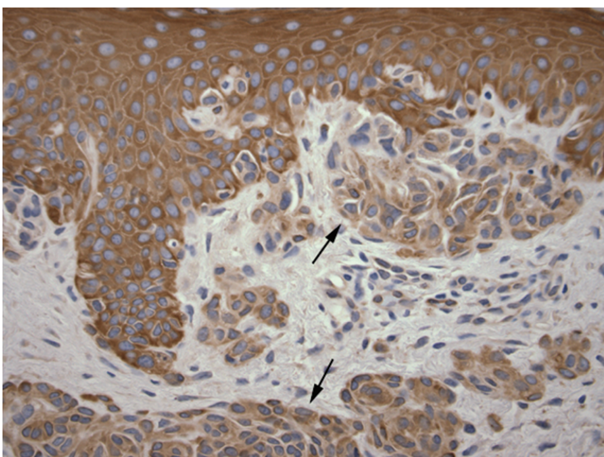

Fig. 4. Heat shock protein 27 (HSP27) is expressed in melanocytes throughout compound nevi. A compound nevus in an adult shows cytoplasmic HSP27 staining of melanocytes in junctional and dermal nests (arrows).

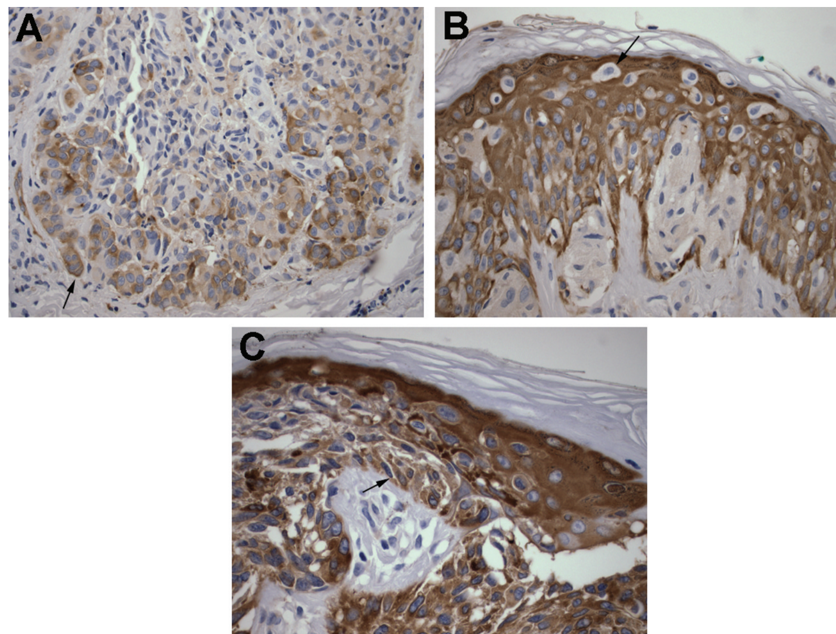

Fig. 5. Heat shock protein 27 (HSP27) is variably expressed in melanocytes in melanomas. A) An invasive melanoma in an adult with a tumor depth of 2.35 mm reveals cytoplasmic HSP27 staining of melanocytes in dermal nests (arrow). B) The same melanoma specimen reveals negative HSP27 staining in a majority of melanocytes in the epidermis (arrow). C) A different malignant melanoma in an adult with a tumor depth of 0.25 mm reveals cytoplasmic HSP27 staining of melanocytes in the epidermis (arrow).

This is in contrast to a prior report, which evaluated HSP27 expression in normal skin and within epidermal neoplasms.[22] The authors comment that they did not observe HSP27 expression in melanocytes within normal skin. They report that HSP27 immunostaining of keratinocytes in the basal layer, where melanocytes reside, to be weak or absent but well visualized in the rest of the epidermis. In contrast, in our study, keratinocytes demonstrated strong HSP27 immunostaining throughout the epidermis including the basal layers as compared to melanocytes. Hence, the parameters for the Trautinger et al. immunolocalization study may have been optimized for visualization of HSP27 of keratinocytes, thereby obscuring expression in the melanocytes.

In conclusion, our data clearly show cytoplasmic HSP27 immunoreactivity in melanocytes in normal skin, in nevi, and in invasive melanoma. Cytoplasmic HSP27 expression was demonstrated in normal, single melanocytes within the epidermis, or grouped melanocytes within the dermis and dermal/epidermal junction in intradermal, and in the malignant-transformed cells of melanomas. We also demonstrated that HSP27 is present in normal non-malignant cultured melanocytes and relocates from the cytoplasm to the nucleus when subjected to heat stress. In addition, we demonstrated that HSP27 expression cannot be used to distinguish between melanocytes in nevi vs. melanomas. Further studies are needed to elucidate the precise role of HSP27 as a diagnostic, prognostic, and differentiation marker in cutaneous pathology.

Acknowledgements

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