Immunohistochemical expression of Heat Shock Protein 27, in normal hyperplastic and neoplastic endometrium: Correlation with estrogen and progesterone receptor status, p53, pRb and proliferation associated indices (PCNA, MIB1)

N. Zagorianakou[1], E. Ioachim[1], A. Mitselou[1], E. Kitsou[3], P. Zagorianakou[1], G. Makrydimas[2], M. Salmas[4], N. J. Agnantis[1]

Department of Pathology[1] and Obstetrics and Gynecology[2], Medical School, University of Ioannina, Department of Pathology[3], General Hospital of "G. Hatzikosta", Ioannina, Department of Anatomy[4], University of Athens (Greece)

Summary

Heat shock protein 27 (HSP27) is a relatively small protein produced in response to pathophysiological stress. In the current prospective study the presence and localization of HSP27 was associated with other potential prognostic factors such as: estrogen and progesterone receptors, p53 and proliferative associated indices (MIB1, PCNA). One hundred and twenty-two samples of endometrial tissues (65 endometrial carcinomas, 28 adenomatous hyperplasias, 31 normal endometrium) were studied. Patient records were examined for FIGO stage, grade, and depth of myometrial invasion, histology and lympho-vascular space invasion.

HSPp27 expression was lower in the group of carcinomas when compared with the cases of adenomatous hyperplasias ($p < 0.0001$), normal proliferative ($p < 0.0001$) and secretory endometrium ($p = 0.02$). HSP27 expression was higher in carcinomas from premenopausal women in comparison with women in menopausal status and postmenopausal status. Multivariate tests showed no statistical significance of HSP27 expression according to tumor grade and stage. A positive relationship between the expression of HSP27 expression and estrogen receptors ($p = 0.0018$) as well as with progesterone receptor ($p = 0.0012$) was found with linear regression analysis of variance.

Our data showed that the lower HSP27 expression in endometrial carcinomas in comparison with hyperplastic and normal endometrium may indicate a decreased endogenous protection mechanism against the various stressful stimuli. This expression could be under hormonal control and does not seem to be correlated with other conventional or possible prognostic parameters.

Key words: HSP27; ER; PR; p53; MIB1; PCNA; Endometrial carcinoma.

Introduction

Self-protection and survival characterize the cells of all organisms, which respond with an intrinsic mechanism to Heat shock by activating genes coding for the heat shock proteins (HSPs). Besides heat shock, other environmental stresses like exposure to heavy metals or oxidants and physiologic stresses, ischemia, viral and microbial infections, as well as inflammation, have been mentioned to be responsible for the synthesis of HSPs. The different levels of HSP induction depend on the organism, cell type, cellular conditions, type and intensity of the stimulation [1].

HSP27 is a member of the heat shock protein family, with low molecular weight. Initially it was named 24-kDa protein, and was found in human MCF-7 cells and human breast cancer cell lines [2]. It was characterized as an estrogen regulated protein, because of its expression in several hormone sensitive organs [3, 4]. In adults, HSP27 is found particularly in several cell types, such as breast, uterus, cervix, skin and placenta [1].

This protein, besides its putative role in thermotolerance, is of special clinic interest. Recent reports suggest that elevated expression in cells has been correlated with increased cellular resistance to a variety of cytotoxic agents, including chemotherapeutic drugs, which concern one of the main reasons for treatment failure in cancer chemotherapy [5-7].

HSP27 in epithelial ovarian carcinoma has been identified as an independent prognostic indicator of survival and in the cervix as a marker for cell differentiation [8-10]. The results are conflicting in breast cancer. Several studies demonstrate that it could be correlated with poor prognosis [1, 11, 12]; other studies indicate a good prognosis [13], while others showed no prognostic significance [14]. HSP27 appears to be a favorable prognostic factor in malignant fibrous histiocytoma [15] and in esophageal carcinoma [16], while in gastric carcinoma it has been correlated with poor prognosis [17]. No prognostic value has been attributed in prostate and bladder cancer [18].

In endometrial carcinoma, which is the most common malignancy of the female genital tract and the third most common cancer in women, a limited number of studies have been performed aiming to explore the biological significance of HSP27 [19-25]. Their results have shown that increased levels of HSP27 are correlated with increased tumor cell differentiation [20-22]. In addition, HSP27 expression was found to be higher in endometrioid lesions,

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non- or minimally invasive lesions, and early-stage cancers [23]. Lower levels of HSP27 were found in patients who had tumor recurrences and in patients who died from the disease [23, 24]. Furthermore, its expression was characterized as an independent prognostic indicator in patients with endometrial carcinoma [23].

The aim of this study was to examine HSP27 expression in a series of endometrial carcinomas, hyperplasia and normal endometrium, in an attempt to clarify its role in the development and progression in endometrial tumors. We also searched for a possible correlation between HSP27 expression and estrogen expression (ER) and progesterone receptor (PR) status, as well as, with p53 and proliferation-associated indices, PCNA and MIB1.

Materials and Methods

A total of 65 surgical specimens, from patients with a diagnosis of primary endometrial cancer of endometrioid type were selected. All patients underwent a total abdominal hysterectomy/bilateral salpingo-oophorectomy and none received preoperative pelvic radiation. All the specimens were surgically resected at the Department of Gynecology, University Hospital of Ioannina. In addition, curetted samples from 28 cases of endometrial hyperplasia, 16 cases of proliferative phase and 15 cases of secretory phase of normal endometrium were also studied.

Neoplastic tissue including adjacent hyperplastic or non-neoplastic tissue were fixed routinely in 10% buffered formalin and embedded in paraffin. Each specimen was examined histologically on H&E-stained slides. All the cases were analyzed for age, FIGO stage, grade, histological type, depth of myometrial invasion, and lympho-vascular space invasion. Histological grading and surgical staging were based on the revised FIGO criteria [26].

Immunohistochemistry:

Out of one or two selected paraffin blocks from each case, 4 μm tissue sections were placed on poly-L-lysine-coated glass slides. Consequently the sections were deparafinised in xylene and dehydrated.

All sections were treated for 30 min with 0.3% hydrogen peroxide (in methanol) to quench endogenous peroxidase activity and then were incubated with primary antibodies. We used the method of the avidin-biotin-peroxidase complex and developed the chromogen with immersion of the slides in a diaminobenzidine-H2O2 substrate for five min. The slides were counterstained in Harris' haematoxylin, dehydrated and mounted. To assess the specificity of the reaction, positive and negative control slides were used in each run. Tissue sections subjected to the procedure expected for incubation with the primary antibody were used as a "negative" substitute. The sources of the antibodies and dilutions are shown in Table 1.

Table 1. — *Antibodies used.*

Antibodies	Supplier	Dilution	Incubation time
HSP27 (2B4)	Novocastra	1:40	1 hour
ER (M7047)	Dako	1:50	1 hour
PR (M3569)	Dako	1:75	1 hour
p53 (DO7, IgG2b)	Ylem	1:200	1 hour*
MIB1 (Ki-67 paraffin)	Dako	1:50	1 hour
PCNA (PC-10)	Dako	1:50	1 hour

* with microwave oven antigen retrieval.

Immunohistochemical evaluation:

HSP27 immunostaining was determined based on the percentage of carcinoma cells stained in each section (0, < 10; 1, 10-25%; 2, 26-50; 3, > 50%), the intensity (1, weak; 2, moderate; 3, strong), and the heterogeneity (-1, marked; 0, moderate; +1 mild). Only cytoplasmic staining was noted. Heterogeneity was defined as no uniform immunostaining in a tumor section. Multiplying the percentage of cells stained by the intensity and then adding the heterogeneity score calculated the final score. The highest score possible was 10 and the lowest 0. The scoring system was the same as previously published by Geisler *et al.* [10].

Estrogen receptor (ER) and progesterone receptor (PR) immunostaining: ER and PR expression were evaluated by examination of the positive epithelial cell nuclei (0, no positive cells; 1, < 10%; 2, 11-50%; 3, 51-80%; 4, > 80% positive cells). An immunoreactivity score of 2 and higher was considered to be positive [27].

p53 immunoreactivity: Based on other studies, only cases showing staining at least 10% of endometrial cell nuclei were considered to be p53 positive [28].

MIB1 and PCNA immunostaining: Only nuclear epithelial staining was considered positive for MIB1 and PCNA expression and for statistical analysis the cases were divided into groups using a semi quantitative approach; into two groups for MIB1 (< 10%, > 10% of positive cells) and into three groups for PCNA (< 10%, 10-50%, > 50% of positive cells). The subcategories were chosen after it was observed that the cases were divided into nearly equal groups. The staining was evaluated in the areas with well-preserved tissue and away from artifacts. All slides were reviewed and scored in a blind test by two pathologists. Differences in the interpretation were reconciled by re-review of slides separately or jointly with a double-headed microscope.

Statistical analysis:

All data were entered into a microcomputer and statistical analysis was performed using the SPSS for Windows (version 6) statistical package. The association of continuous variables was confirmed using the Student's *t*-test, one-way analysis of variance (one-way ANOVA), logistic regression, and χ^2 test.

Results

HSP27 expression in carcinomas

Localization of HSP27 was detectable only in the cytoplasm of the benign and malignant epithelial cells of all cases in variant amounts (Figure 1-4).

The mean value of the HSP27-positive-staining group was 29.74%. A statistically significant lower HSP27 expression in endometrial carcinomas was compared with the cases of adenomatous hyperplasias ($p < 0.0001$) as well as with the normal proliferative ($p < 0.0001$) and secretory phase of endometrium ($p = 0.02$) (Figure 5).

HSP27 expression was higher in carcinomas from premenopausal women in comparison with women in menopausal status and postmenopausal status.

Multivariate tests showed no statistical significance of HSP27 expression according to tumor grade and stage. A positive relationship between the expression of HSP27 expression and estrogen receptors ($p = 0.0018$) as well as with progesterone receptors ($p = 0.0012$) was found (Figures 6, 7) with linear regression analysis of variance.

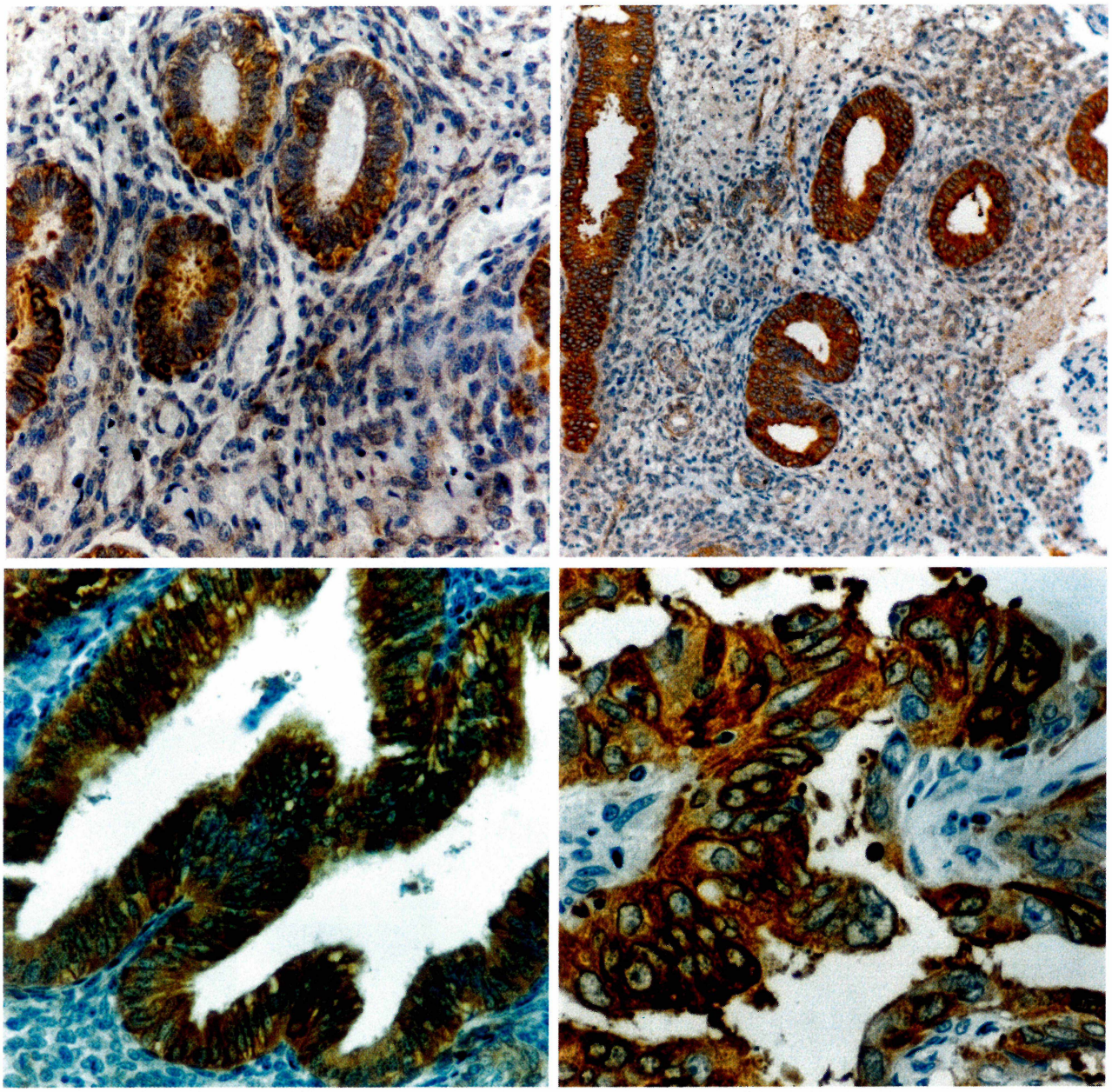

Figure 1. — HSP27 expression in the end of the proliferative phase of normal endometrium (ABC method; original magnification x 200).
Figure 2. — Normal secretory endometrium showing high epithelial reactivity for HSP27 protein expression (ABC method; original magnification x 100).
Figure 3. — High HSP27 expression in a epithelial cells in a hyperplastic lesion (ABC method; original magnification x 400)
Figure 4. — Tumor cell HSP27 expression in a case of endometrial carcinoma (ABC method; original magnification x 400).

No statistical correlation between HSP27 expression and p53 and the associated proliferative indices was detected (Table 2).

HSP27 expression in adenomatous hyperplasias

The mean value of HSP27 expression in hyperplastic cases was 73.11%. This expression did not show any important statistical correlation with ER or PR status. A positive, statistically significant correlation between HSP27 expression and p53 ($p = 0.042$) was detected. No statistically significant correlation between HSP27 expression and MIB1 or PCNA was found.

HSP27 expression in normal endometrium

In the proliferative phase of normal endometrium the mean value of HSP27 expression was 73.13%. No correlation between its expression with ER, PR status or with the other examined biological markers was found.

In the secretory phase of normal endometrium the mean value of HSP27 expression was 53.33%. A positive,

Table 2. — *HSP27 expression with clinicopathological parameters and other potential biological prognostic markers.*

	hsp27 expression		
	negative	positive	p value
Menopausal status			
< 45	4	1	
45-55	4	13	p = 0.003
> 55 meta	10	28	p = 0.0065
Grade			
1	8	13	
2	6	20	NS
3	4	11	
FIGO			
1	12	21	
2	3	7	NS
3	2	7	
4	1	1	
ER			
< 10	17	33	p = 0.0018
> 10	1	10	
PR			
< 10	16	34	p = 0.0012
> 10	3	10	
p53			
< 10	14	34	NS
> 10	5	10	
MIB1			
< 10	16	38	NS
> 10	3	7	
PCNA			
< 10	6	17	
10-50	3	11	NS
> 50	10	17	

* with microwave oven antigen retrieval.

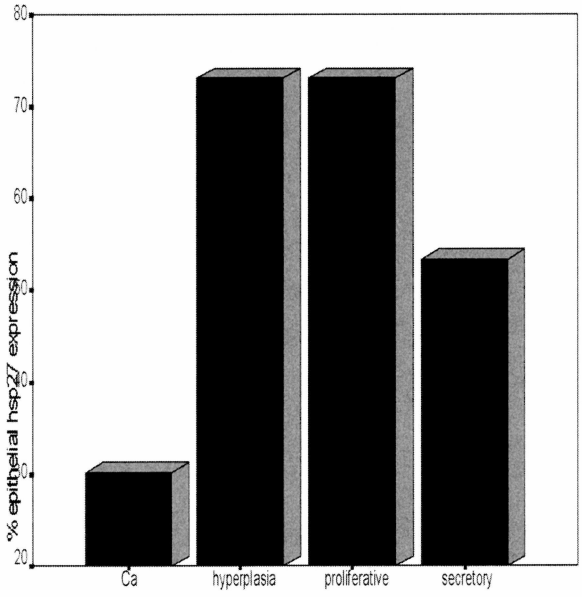

Figure 5. — Epithelial HSP27 expression in normal, hyperplastic and malignant endometrium (mean value).

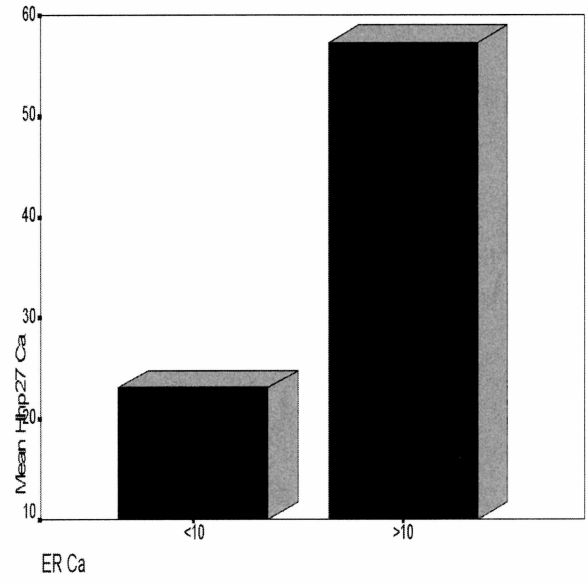

Figure 6.

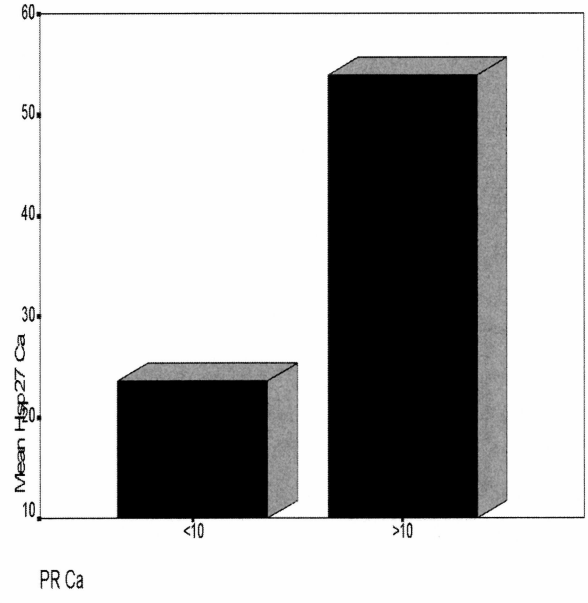

Figure 7.

statistically significant correlation between its expression and ER status (p = 0.034) was detected.

Discussion

The proposed role of heat shock protein 27 is to increase the thermal tolerance of a cell after exposure to heat shock or another stressor. In addition, HSP27 may act as a chaperone protein, binding complexes and affecting proteolysis. Furthermore, this protein could create a resistance to chemotherapy after no lethal heat shock or other non-lethal stress [1, 2, 5-7, 29]. In human tumors the role of HSP27 is unknown and there are controversial

results about the prognostic contribution. The results of the present study showed that lower HSP27 expression in carcinomas is comparable with hyperplastic and normal endometrium according to other investigators [25]. Thus, we can hypothesize that in endometrial carcinomas the absence or lower expression of HSP27 may creat a defective mechanism against stressful conditions. In addition, this event could take place in the late stage of endometrial carcinogenesis.

HSP27 has been shown to be directly related to estrogen receptor status in endometrial carcinomas according to other studies [23, 24]. It has been shown that HSP27 is estrogen-inducible in estrogen-dependent cell lines [30, 31]. In addition, endometriod tumors showed significantly higher HSP27 immunostaining than non-endometriod [23]. Paradoxically, HSP27 expression did not correlate with ER status in hyperplastic and normal proliferative endometrium, while a positive relationship of its expression with ER status was found in normal secretory endometrium and in the group of carcinomas. In hyperplastic and normal proliferative endometrium, where the estrogen receptor status is in high levels, HSP27 expression is unrelated to these receptors. In normal secretory endometrium and in endometrial carcinomas the level of estrogen receptors decreased in addition to lower HSP27 expression, probably because of the stressful conditions. The results of the present study also showed a positive relationship of HSP27 expression with PR status in endometrial carcinomas in contrast to the findings of other investigators [24], suggesting that HSP27 maybe regulated by the synergistic action of estrogen and progesterone.

A statistically important correlation was noticed between HSP27 expression and premenopausal status. The expression decreased with age, which can be explained by estrogen status, as it is more activated in premenopausal women and becomes less activated in menopausal women.

In this study we failed to demonstrate a correlation of HSP27 expression with tumor grade or stage. However, other investigators have shown increased HSP27 expression to be correlated with increased tumor cell differentiation [20-22].

Furthermore, it has been shown that HSP27 is also involved in cell proliferation and several studies correlated its expression with proliferative associated-indices. Khalid *et al.* [32] showed a positive relationship of HSP27 expression with Ki-67 labeling index in human astrocytomas, in contrast to the findings of other studies [33,34]. Moreover, no correlation of HSP27 expression with p53 was found [33, 34] which is in accordance with our results. Vargas-Roig *et al.* [35] found an inverse relationship of HSP27 expression with PCNA proliferating index in breast carcinomas, and this finding added evidence to the concept that HSP27 maybe involved in cell growth arrest. In our study we did not find such a correlation, suggesting probable different pathways of cell proliferation.

Therefore, we may hypothesize that in hyperplastic and normal proliferative endometrium a high level of HSP27 occurs as a result of the activated estrogen status, which stimulates cells to grow and to induce HSP27. In contrast, in normal secretory endometrium and in carcinomas the expression of HSP27 decreases, due to the loss of function of ER, but in these cases probably a synergetic action with hormonal status takes place, which may protect cells from various stressful conditions. In addition, the results of the current study showed no specific prognostic evaluation of HSP27 expression in endometrial carcinomas.

References

[1] Ciocca D.R., Oesterreich S., Chamnss G.C., McGuire W.L., Fuqua S.A.: "Biological and clinical implications of heat shock protein 27000 (HSP27): a review". *J. Nat. Cancer Institute,* 1993, *85,* 1558.

[2] Mahvi D.M., Carper S.W., Storm F.K.: "Over-expression of 27-kDa-heat shock protein in MCF-breast cancer cells: Effects on lymphocyte killing by natural killer and gamma delta T cells". *Cancer Immunol. Immunother.,* 1993, *37,* 181.

[3] Ciocca D.R., Adams D.J., Edwards D.P., Bjercke R.J., McGuire W.L.: "Distribution of an estrogen-induced protein with a molecular weight of 24,000 in normal and malignant tissues and cells". *Cancer Res.,* 1983, *31,* 1204.

[4] Adams D.J., McGuire W.L.: "Quantitative enzyme-linked immunoassorbent assay for the estrogen related Mr 24,000 protein in human breast tumors: Correlation with estrogen and progesterone receptors". *Cancer Res.,* 1985, *45,* 2445.

[5] Ciocca D.R., Suzann A.W. Fuqua, Sarah Lock-Lim, David O. Toft, William J. Welch, William L. McGuire: "Response of human breast cancer cells to heat shock and chemotherapeutic drugs". *Cancer Res.,* 1992, *52,* 3648.

[6] Oesterreich S., Weng C.N., Qiu M., Hilsenbeck S., Osborne C.K., Suzann A. W., Fuqua A.W.: "The small heat shock protein HSP27 is correlated with growth and drug resistance in human breast cancer cell lines". *Cancer Res.,* 1993, *53,* 4443.

[7] Kamishima T., Fukuda T., Yoshiya N., Suzuki T.: "Expression and intracellular localization of heat shock proteins in multidrug resistance of a cisplatin resistant human ovarian cancer cell line". *Cancer Lett.,* 1997, *116,* 205.

[8] Geisler J.P., Hans E., Geisler H.E., Tammela J., Wiemann M.C., Zhou Z. *et al.*: "Heat shock protein 27: An independent prognostic indicator of survival in patients with epithelial ovarian carcinoma". *Gynecol. Oncol.,* 1998, *69,* 14.

[9] Park C.S., Joo I.S., Song S.Y., Kim D.S., Bae D.S., Lee J.H.: "An Immunohistochemical analysis of heat shock protein 70, p53 and estrogen receptor status in carcinoma of the uterine cervix". *Gynecol. Oncol.,* 1999, *74,* 53.

[10] Geisler J.P., Geisler H.E., Tammela J., Wiemann M.C., Zhou Z., Miller G.A., Crabtree W.: "Heat shock protein 27: An independent prognostic indicator of survival in patients with epithelial ovarian carcinoma". *Gynecol. Oncol.,* 1998, *69,* 14.

[11] Nakapoulou K., Lazaris A.C., Baltas D., Giannopoulou I., Kavanzaw N., Tsonou A.: "Prognostic evaluation of estrogen regulated protein immunoreactivity in ductal invasive (NOS) breast cancer". *Virchows Arch.,* 1995, *427,* 33.

[12] Storm F.K., Mahvi D.M., Gilchrist K.W.: "Lack of association between tumor necrosis and HSP27 expression in primary breast cancer". *J. Surg. Oncol.,* 1996, *61,* 14.

[13] Seymour L., Bezwoda W.R., Meyer K.: "Tumor factors predicting for prognosis in metastatic breast cancer". *Cancer,* 1990, *66,* 2390.

[14] Tetu B., Brisson J., Landry J., Huot J.: "Prognostic significance of heat shock protein-27 in node positive breast carcinoma: An immunohistochemical study". *Breast Cancer Res. Treat.,* 1995, *36,* 93.

[15] Tetu B., Lacasse B., Boucard H.L., Lagace R., Huot J., Landry J.: "Prognostic influence of HSP-27 in malignant fibrous histiocytoma: a clinicopathological and immunohistochemical study". *Cancer Res.,* 1992, *52,* 2325.

[16] Kawanishi K., Shiozaki H., Doki Y., Sakita I., Inoue M., Yano M. *et al.*: "Prognostic significance of heat shock protein 27 and 70 in patients with squamous cell carcinoma of esophagus". *Cancer,* 1999, *85,* 1649.

[17] Harisson J.D., Jones J.A., Ellis I.O., Morris D.L.: "Oestrogen receptor D5 antibody is an independent negative prognostic factor in gastric cancer". *Br. J. Surg.,* 1991, *78,* 334.
[18] Storm F.K., Mahvi D.M., Gilchrist K.W.: "HSP-27 has no diagnostic or prognostic significance in prostate or bladder cancer". *Urology,* 1993, *42,* 379.
[19] Devaja O., King R.J., Papadopoulos A., Raju K.S.: "Heat-shock protein 27 and its role in female reproductive organs". *Eur. J. Gynaecol. Oncol.,* 1997, *18,* 16.
[20] Ciocca D.R., Puy L.A., Fasoli L.C.: "Studies of estrogen receptor, progesterone receptor, and estrogen-regulated Mr 24,000 protein in patients with carcinomas of the endometrium and cervix". *Cancer Res.,* 1989, *4,* 4298.
[21] Ciocca D.R., Stati A.O., Amprino de Castro M.M.: "Colocalization of estrogen and progesterone receptors with an estrogen regulated heat shock protein in paraffin sections of human breast and endometrial cancer tissue". *Breast Cancer Res. Treat.,* 1990, *16,* 243.
[22] McGuire W.L., Dressler L.G., Sledge G.W., Ramzy I., Ciocca D.R.: "An estrogen-regulated protein in normal and malignant endometrium". *J. Steroid. Biochem.,* 1986, *24,* 155.
[23] Geisler J.P., Geisler H.F., Tammela J., Miller G.A., Wiemann M.C., Zhou Z.: "A study of heat shock protein 27 in endometrial carcinoma". *Gynecol. Oncol.,* 1999, *72,* 347.
[24] Raju K.S., King R.J., Kaern J., Sumner D., Abeler V.M., Mandalaya S., Trope C.: "Influence of HSP27 and steroid receptor status on provera sensitivity, DNA-ploidy and survival of females with endometrial cancer". *Int. J. Gynecol. Cancer,* 1995, *5,* 94.
[25] Wataba K., Saito T., Fukunaka K., Ashihara K., Nishimura M., Kudo R.: "Over-expression of heat shock proteins in carcinogenic endometrium". *Int. J. Cancer,* 2001, *91,* 448.
[26] FIGO stages-1988 revision. *Gynecol. Oncol.,* 1989, *35,* 125.
[27] Segreti E.M., Novotny D.B., Soper J. T., Mutch D.G., Creasman W.T., McCarty K.S.: "Endometrial cancer: histologic correlates of immunohistochemical localization of progesterone and estrogen receptor". *Obstet. Gynecol.,* 1989, *73,* 780.
[28] Sung C.J., Zheng Y., Quddus M.R., Kang X., Zhang Z.F., Lauchlan S.C., Zheng W.: "p53 as a significant prognostic marker in endometrial carcinoma". *Int. J. Gynecol. Cancer,* 2000, *10,* 119.
[29] Lindquist S., Craig E.A.: "The heat shock proteins". *Ann. Rev. Genet.,* 1988, *22,* 631.
[30] Edwards D.P., Adams D.J., Savage N., McGuire W.L.: "Estrogen induced synthesis of specific proteins in human breast cancer cells". *Biochem. Biophys. Res. Commun.,* 1980, *93,* 804.
[31] Masiakowski P., Breathnach R., Bloch J., Gannon F., Krust A., Chambon P.: "Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell line". *Nucleic Acids Res.,* 1982, *10,* 7895.
[32] Khalid H., Tsutsumi K., Yamashita H., Kishikawa M., Yasunaga A., Shibata S.: "Expression of the small heat shock protein (HSP) 27 in human astrocytomas correlates with histologic grades and tumour growth fractions". *Cell. Mol. Neurobiol.,* 1995, *15,* 257.
[33] Fortin A., Raybaud-Diogene H., Tetu B., Deschenes R., Huot J., Landry J.: "Overexpression of the 27 KDa heat shock protein is associated with thermoresistance and chemoresistance but not with radioresistance". *Int. J. Radiat. Oncol. Biol. Phys.,* 2000, *46,* 1259.
[34] Malusecka E., Zborek A., Krzyzowska-Gruca S., Krawczyk Z.: "Expression of heat shock proteins HSP70 and HSP27 in primary non-small cell lung carcinomas. An immunohistochemical study". *Anticancer Res.,* 2001, *21,* 1015.
[35] Vargas-Roig L.M., Fanelli M.A., Lopez L.A., Gago E.E., Tello O., Aznar J.C., Ciocca D.R.: "Heat shock proteins and cell proliferation in human breast cancer biopsy samples". *Cancer Detect. Prev.,* 1997, *21,* 441.

Address reprint requests to:
N. J. AGNANTIS, M.D.
Department of Pathology
Medical School
University of Ioannina
45110 Ioannina (Greece)