

Estrogen Receptor Gene Expression and Its Relation to the Estrogen-Inducible HSP27 Heat Shock Protein in Hormone Refractory Prostate Cancer

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BACKGROUND. The recent discovery of the classical estrogen receptor α (ER α) in androgen-insensitive prostate cancer has shed new light on the role of estrogens in endocrine therapy failure. To get more information on downstream events of estrogen signaling in these tumors, we investigated the relation between ER α gene expression, and the estrogen-inducible heat shock protein HSP27 in recurrent prostatic adenocarcinomas.

METHODS. Palliative transurethral resection specimens from 50 patients with androgen-insensitive disease were submitted for study. Messenger RNA in situ hybridization for the ER α and immunohistochemistry of the HSP27 protein were performed on adjacent sections of an equal number of prostate cancer tissue with and without ER α protein expression.

RESULTS. Cancerous lesions lacking the nuclear ER α at the protein level revealed ER α mRNA expression in 15 of 25 cases (60%). A coordinate expression of ER α mRNA and HSP27 was observed in 33 of 40 cases (83%), although a significant correlation between ER α protein and HSP27 expression was not obtained. Conversely, 90% of neoplastic lesions without detectable levels of ER α mRNA and protein also lacked HSP27 immunoreactivity.

CONCLUSIONS. ER α gene expression at the mRNA level significantly correlated with the immunoprofile of the estrogen-inducible HSP27 protein in androgen-insensitive prostatic adenocarcinomas. This may indicate that these tumors harbor functional active estrogen receptors promoting transcriptional activity of the HSP27 gene. Determination of the receptor status by immunohistochemistry is unable to identify neoplastic lesions with established ER α mRNA expression in a substantial number of cases. HSP27 may be an additional surrogate biomarker for estrogen-regulated growth in androgen-insensitive prostate cancer. *Prostate* 45:36–41, 2000. © 2000 Wiley-Liss, Inc.

KEY WORDS: heat shock protein HSP27; estrogen receptor; prostate cancer; endocrine therapy failure

INTRODUCTION

Androgen deprivation or peripheral blockade of androgen action remains the critical therapeutic option for the treatment of advanced and metastatic prostate cancer [1,2]. Despite the impressive symptomatic response to endocrine therapy, most (if not all) advanced prostate cancers relapse to androgen-insensitive disease after androgen deprivation therapy [1,2]. The average survival of patients with recurrent disease is only 4–5 months [1,2]. Unfortunately, there are no effective alternative therapies for these patients. Understanding the molecular mechanisms of tumor

progression and endocrine therapy failure is therefore of paramount importance. The current concept of androgen-independent prostatic growth involves a growing number of factors, including androgen receptor (AR) gene mutations, AR amplification, and alter-

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native (androgen-independent) growth factor pathways [3]. We have recently shown that a substantial number of recurrent prostatic adenocarcinomas after androgen deprivation therapy express the classical estrogen receptor α (ER α) at protein and mRNA levels [4]. It has been suggested that prostate cancer cells may survive in an androgen-deprived milieu by using estrogens for their continuous maintenance and growth [4]. Nevertheless, it is currently unknown whether the ER α present in tumor samples is functionally active and promotes transcriptional activity of estrogen-regulated genes.

In estrogen target cells and estrogen-dependent neoplasias (e.g., endometrial and breast cancer), a number of regulatory peptides have been identified that can be induced through an ER-mediated process, including cathepsin D, pS2, and the heat shock protein HSP27 [5–8]. The latter (formerly known as ER-D5 or estrogen-regulated antigen) has been widely used as surrogate marker for estrogen signaling to predict the response to endocrine therapy in breast and endometrial carcinomas [5–7].

Heat shock proteins function as molecular chaperons enabling cells to survive and recover from stressful conditions, including heat shock, UV radiation, cytotoxic drugs, and hormonal stress [6]. In normal human tissue, HSP27 is expressed in a variety of epithelial and stromal cell types with the highest amount in estrogen target organs of the female reproductive tract [6]. Few studies have been conducted in prostate cancer, leaving conflicting results [9–12]. For example, Storm and coworkers failed to demonstrate detectable levels of HSP27 in 36 radical prostatectomy specimens from patients with prostate cancer [10]. The most extensive study was performed in tissue microarray specimens and revealed HSP27 expression in 31% of hormone-refractory tumors, in 5% of primary tumors, and in 0% of benign prostate tissue [12].

To get more information on estrogen signaling in prostate cancer tissue we screened 50 androgen-insensitive prostatic adenocarcinomas for the presence of the estrogen-inducible HSP27 protein and investigated its relation to ER α gene expression.

MATERIALS AND METHODS

Tissue Selection

Formalin-fixed, paraffin-embedded tissue sections were obtained from palliative transurethral resection (TUR) specimens from 50 patients with recurrent prostate cancer after orchiectomy. The patients' age ranged from 49 to 82 years (mean age 68 years). The material had been selected from a larger series of recurrent prostatic adenocarcinoma screened for the presence or

absence of the nuclear ER α at the immunohistochemical level. An equal number of ER α positive ($n = 25$) and ER α negative ($n = 25$) cases were submitted for study. In situ hybridization (ISH) and immunohistochemical (IHC) analyses were performed on adjacent sections. From each patient, one or two tissue blocks were examined. All cases were poorly differentiated prostatic adenocarcinomas with some degree of regressive changes after androgen deprivation therapy. No particular grading system was applied to these recurrent lesions.

Immunohistochemical Analysis

Tissue sections were deparaffinized, rehydrated through graded alcohol, and subsequently incubated in H₂O₂ (0,3%) to block endogenous peroxidase. For microwave-based antigen retrieval, sections were microwaved (750W for 5 min and 450W for 5 min) in 10 mM citrate-buffer, pH 6.0. After pretreatment, the sections were incubated for 30 min in a normal rabbit serum (Dako, Hamburg, Germany). The mouse monoclonal antibody NCL-ER-6F11, directed against the full-length ER molecule (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) was used in a dilution of 1:200. Detection was achieved by the avidin-biotin-peroxidase complex method and a signal amplification technique using biotinylated tyramine, as described recently in greater detail [4]. To localize the HSP27 protein, we used the mouse monoclonal antibody NCL-HSP27-2B4 (Novocastra Laboratories) in a dilution of 1:50. Before application of the primary antibody, the slides were microwaved (750W for 2.5 min and 360W for 10 min) as described above. Detection was achieved by the avidin-biotin-peroxidase complex method (Dako, Hamburg, Germany) according to the manufacturer's instructions. The peroxidase reaction was developed by 3,3'-diaminobenzidine (Sigma, Deisenhofen, Germany) leaving a brown-colored end product. Negative controls were performed on consecutive sections by replacing primary antibodies with a nonimmune mouse serum.

In Situ Hybridization Analysis

ISH for detection of ER α mRNA in tissue sections was performed as described recently [4]. Twenty-four base cDNA oligonucleotide antisense and sense probes (antisense: 5'-CTC CAG CTC GTT CCC TTG GAT CTG-3'; sense 5'-CAG ATC CAA GGG AAC GAG CTG GAG-3') complementary to human ER α mRNA coding for amino acids 17–24 (GenBank accession number M12674) were synthesized by MWG-Biotech (Ebersberg, Germany). The oligonucleotides (antisense probe and sense probe for negative con-

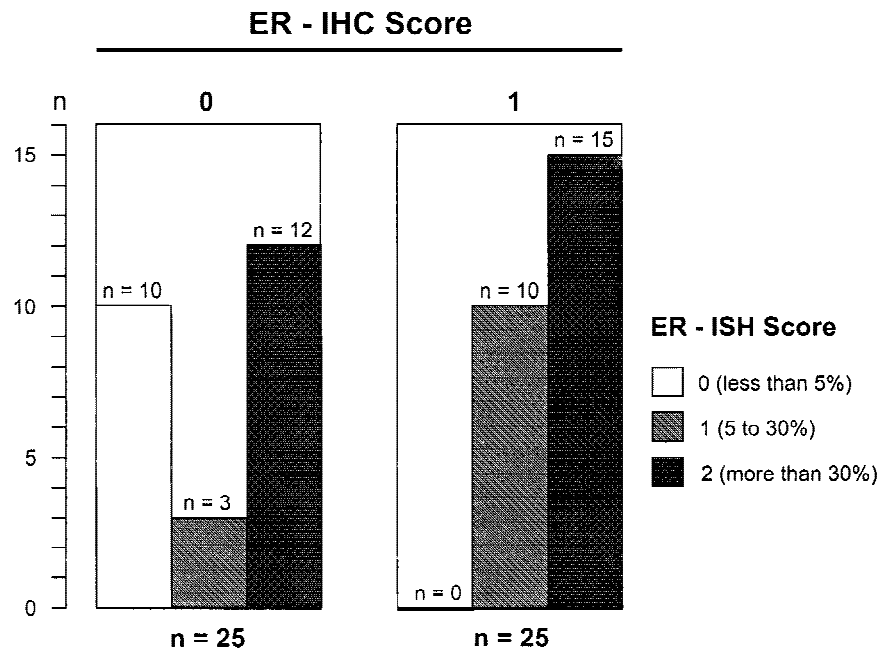


Table I. ER α mRNA Expression in 50 Recurrent Prostatic Adenocarcinomas With and Without Detectable Levels of the ER α Protein by Immunohistochemistry. According to the study design, an equal number of neoplastic lesions with and without detectable levels of ER α protein were screened for the presence of ER α mRNA. The immunohistochemical (IHC) and in situ hybridization (ISH) score was evaluated by a grading system ranging from 0 (less than 5% positive tumor cells), 1 (5 to 30% positive tumor cells), to 2 (more than 30% positive tumor cells). Twenty-five ER α negative neoplastic lesions revealed ER α mRNA expression in 15 cases (60%). The remaining (ER α positive) lesions expressed ER α mRNA at variable degree. The ER α was undetectable by both immunohistochemistry and in situ hybridization analysis in 10 of 50 cases. Association between ER α protein and mRNA expression reached the statistical level of significance ($P = 0,01$). N = number of patients.

trols) were biotin-16-2'-desoxyuridine-5-triphosphate (bio-16-dUTP; Boehringer, Mannheim, Germany) 3'-tailed using the DNA tailing kit (Boehringer, Mannheim). The labeling procedure was performed according to the manufacturer's instructions (Boehringer, Mannheim). The efficiency of labeling reaction was checked on dot blot dilutions series. Detection of ER α mRNA in tissue sections was achieved by a nonradioactive ISH procedure described recently [4]. To prove the specificity of the hybridization process, the following negative controls were performed in each case. The slides were hybridized with the corresponding sense probe. Second, the hybridization procedure was performed by omitting the sense and antisense probe. In addition, slides pretreated with RNase (Boehringer, Mannheim) were hybridized as described above.

Quantitative Analysis of Staining

IHC and ISH staining results were classified into three categories: (0) less than 5% positive tumor cells; (1) 5–30% positive tumor cells; (2) more than 30% positive tumor cells. The percentage of positive staining recorded in the entire cancerous lesion present in one

or two tissue blocks was estimated at low power magnification ($\times 100$).

Statistical Analysis

Contingence table and χ -square analyses were used to study the relation between HSP27, ER α protein, and ER α mRNA expression (e.g., Pearson's χ -square, the likelihood-quotient- χ -square, and Spearman's coefficient of correlation for the statistical computation of significance). The statistical analyses were performed with the SPSS-software (SPSS ASC GmbH, Erkrath, Germany). $P < 0,05$ was regarded as statistically significant.

RESULTS

HSP27, ER α protein, and ER α mRNA expression was detected in both epithelial and stromal compartments of the neoplastic lesions. The data outlined below refer to the localization of these markers in tumor cells but not in the host tissue. According to the study design, an equal number of ER α positive ($n = 25$) and ER α negative ($n = 25$) neoplastic lesions were submitted for study. Tumors expressing the ER α protein also

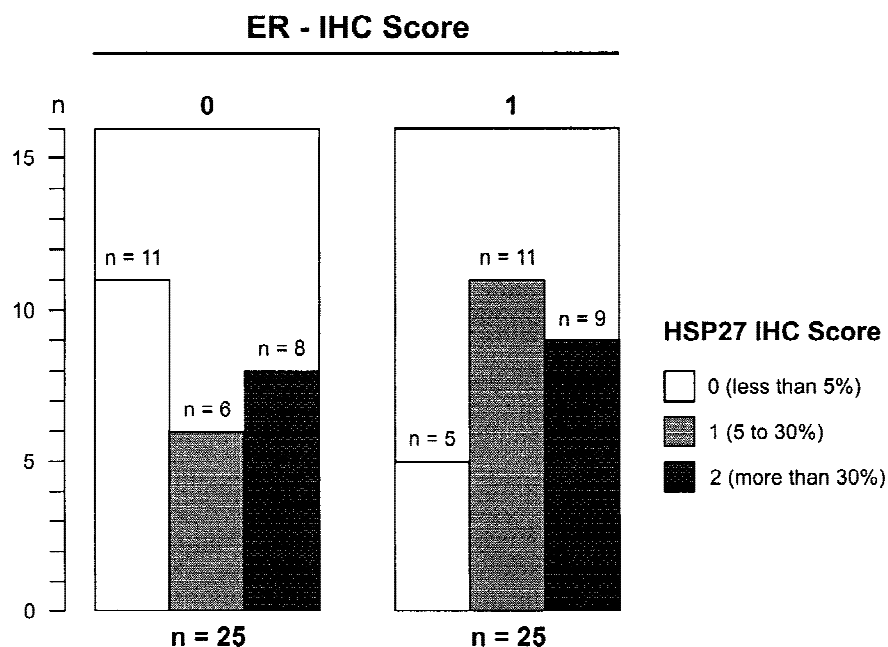
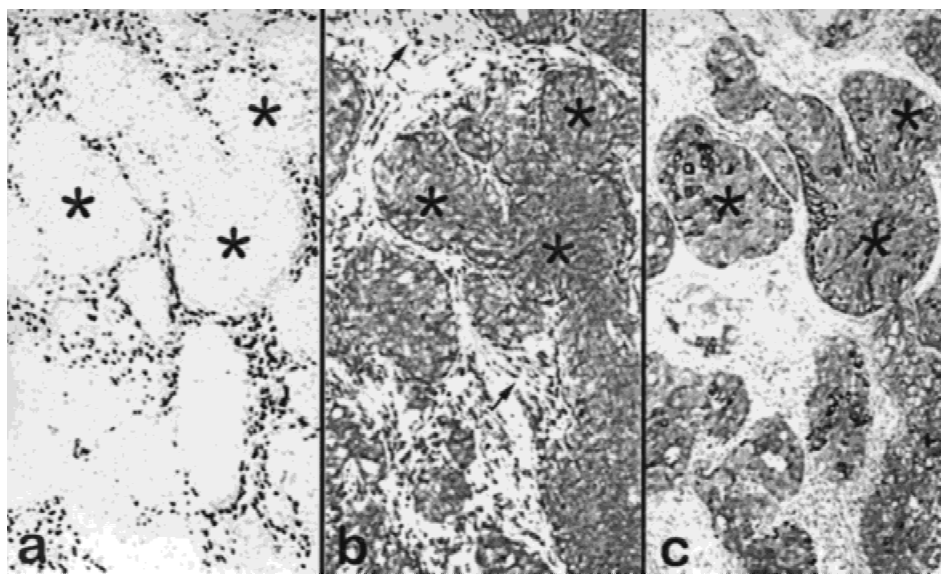


Table II. Immunoprofile of the HSP27 Protein in 50 Recurrent Prostatic Adenocarcinomas With and Without Detectable Levels of the ER α Protein by Immunohistochemistry. The immunohistochemical (IHC) score for ER α and HSP27 was evaluated by a grading system ranging from 0 (less than 5% positive tumor cells), 1 (5 to 30% positive tumor cells), to 2 (more than 30% positive tumor cells). ER α positive lesions (n = 25) revealed HSP27 expression in 20 cases (80%). In ER α negative tumors, the HSP27 protein was also undetectable in 11 cases (44%). The correlation between both markers, however, did not reach the statistical level of significance ($P = 0,151$). A substantial number of ER α negative lesions revealed HSP27 immunoreactivity (14 of 25 cases). N = number of patients.

Fig. 1. Recurrent prostatic adenocarcinoma. Immunohistochemistry reveals strong ER α positivities in stromal cells, whereas tumor cells lack the nuclear ER α (a): On adjacent sections, high steady-state levels of ER α mRNA (b) and strong HSP27 immunoreactivity (c) are detected in the cancerous lesion. Note that both markers are partially co-expressed. In the host tissue some stromal cells reveal ER α mRNA expression (arrow), whereas HSP27 is not detectable in the stromal compartment of this tumor. Asterisks indicate corresponding tumor areas in adjacent sections. Original magnification: a, b, c, $\times 50$.



revealed high steady-state levels of ER α mRNA. The 25 neoplastic lesions lacking the nuclear ER α expressed ER α mRNA in 15 of 25 cases (60%). The differential expression of ER α protein and mRNA levels is summarized in Table I.

HSP27 immunoreactivity was detected in 34 of 50 specimens evaluated. In ER α positive lesions, a coordinate expression of the ER α protein and HSP27 was

observed in 20 cases (80%). Nevertheless, HSP27 immunoreactivity was also present in 14 of 25 ER α negative lesions (Table II). Consequently, statistical analysis failed to demonstrate a significant correlation between ER α and HSP27 immunoreactivity ($P = 0,151$). However, when ISH results were included in the analysis, the relation between the ER α status and HSP27 expression became more evident. In fact, the

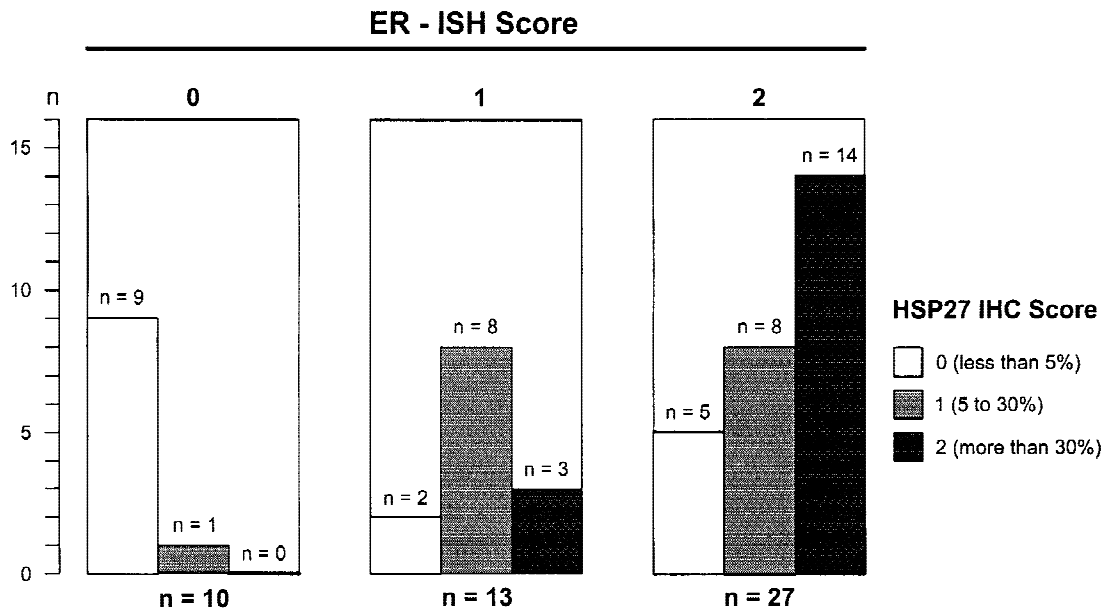


Table III. Comparative Evaluation of ER α mRNA and HSP27 Protein Expression in 50 recurrent Prostatic Adenocarcinomas With and Without Detectable Levels of the ER α Protein by Immunohistochemistry. The immunohistochemical (IHC) and the in situ hybridization (ISH) score was evaluated by a grading system ranging from 0 (less than 5% positive tumor cells), 1 (5 to 30% positive tumor cells), to 2 (more than 30% positive tumor cells). 82.5% of recurrent adenocarcinoma with established ER α mRNA expression also revealed HSP27 immunoreactivity (33 of 40 cases). Among the 10 neoplastic lesions lacking ER α mRNA, only one lesion showed weak HSP27 positivities. A statistically highly significant association was found between the ER-ISH and the HSP27-IHC score (coefficient of correlation 0,519 at $P < 0,001$). N = number of patients.

HSP27 protein was undetectable in 90% of tumors lacking both the ER α protein and mRNA expression (9 of 10 cases). Only one of these ER negative lesions revealed focal HSP27 immunoreactivity. A coordinate expression of ER α mRNA and HSP27 was found in 33 of 40 cases (83%) (Fig. 1). Statistical analysis revealed a highly significant association between ER α mRNA and the amount of HSP27 detectable in the neoplastic lesions ($P < 0,001$; coefficient of correlation: 0,519). Table III summarizes the differential expression of ER α mRNA steady-state levels and HSP27 immunoreactivity.

DISCUSSION

Estrogens have been widely used in the medical treatment of advanced prostate cancer. It is well established that the therapeutic efficiency of estrogens refers to their suppressive effect on the pituitary release of gonadotrophins, blocking the testicular output of androgens [13]. The recent discovery of the classical ER α in a significant number of androgen-insensitive prostatic adenocarcinomas, however, suggests that estrogens can also affect androgen-insensitive tumor growth through a receptor-mediated process [4]. This concept may have clinical implications. For example, tumor cells expressing the ER α are potentially estro-

gen-responsive and may survive in an androgen-deprived milieu by using estrogens. Results of the current study may provide further information on estrogen signaling in hormone refractory prostate cancer.

Evaluating the ER α status of these tumors, it is important to note that a significant number of neoplastic lesions lacking the receptor protein express high steady-state levels of ER α mRNA. In our series, this situation was encountered in 60% of cases investigated. There are several ways to explain this observation. Immunolocalization of the nuclear ER α depends on a number of factors, including tissue processing, antibody specificity, sensitivity, and antigen retrieval methods. Until recently, the nuclear ER α was undetectable in prostate cancer cells [4], which obviously reflects the difficulty to assess the ER status by current available IHC methods. Alternatively, the low immunohistochemical detection rate could also be related to the stability of the receptor protein. It is conceivable that receptor saturation by ligand binding and subsequent degradation of the ER-ligand complex accounts for the difference between ER α mRNA and protein expression. Irrespective of possible explanations, current IHC methods appear insufficient to determine the ER status in recurrent prostatic adenocarcinomas.

Another important issue refers to the functional sig-

nificance of the ER α detected in prostate cancer tissue. In breast and endometrial carcinomas, the estrogen-inducible HSP27 protein has been widely used as a marker for a functional ER apparatus [5,6], although more recent studies have failed to demonstrate a significant correlation between the HSP27 status and response to tamoxifen treatment or survival [14,15]. Results of the current study revealed HSP27 expression in 68% (34 of 50 cases) of androgen-insensitive prostatic adenocarcinomas. Bubendorf and coworkers reported HSP27 positivity in 31% of hormone-refractory tumors analyzed in tissue microarrays [12]. This apparent discrepancy may reflect a sampling error due to the small amount of tissue present in the microarray specimens. Surprisingly, the amount of HSP27 detected in the current study significantly exceeded the levels of ER α evaluated by IHC. In our series of 25 ER α negative lesions, HSP27 was identified in 14 cases (56%). This discrepancy obviously reflects the low detection rate of the ER α by current IHC methods. Using *in situ* hybridization, we were able to demonstrate a significant association between ER α mRNA and HSP27 expression in recurrent prostatic adenocarcinoma ($P < 0,001$). In more than 80% of cases, both markers were closely related. Divergent findings (i.e., ER α mRNA positive and HSP27 negative, or vice versa) were encountered in 18% (respectively 10%) of cases. The close relation between HSP27 and ER α mRNA expression described here suggests that the HSP27 gene is a target of estrogen signaling mediated by the ER α . Simultaneous detection of HSP27 and ER α mRNA or protein in prostate cancer tissue may therefore provide additional information on the functional ER status and estrogen-regulated growth.

The role of heat shock proteins in prostate cancer progression is currently unknown. Nevertheless, HSP27 has been recognized as an important component of the molecular chaperone machinery protecting cells in various stressful conditions including hormonal stress caused by androgen withdrawal [6]. Further investigations are required to establish the prognostic significance of HSP27 in androgen-insensitive prostate cancer growth.

CONCLUSIONS

Detection of the estrogen-inducible HSP27 in a substantial number of androgen-insensitive prostatic adenocarcinoma and its close relation to ER α mRNA expression may indicate that these tumors harbor a functional active estrogen receptor. Although downstream events of estrogen signaling remain poorly understood, HSP27, by its function as a molecular chap-

erone, may play a protective role in hormonal stress situation caused by androgen deprivation. Based on the current information, HSP27 is a promising, additional biomarker for estrogen signaling in neoplastic tissue obtained from patients with recurrent disease. It may be useful in clinical trials aimed to investigate the efficiency of antiestrogens in the treatment of androgen-insensitive prostate cancer.

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