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Expression of the 27-kDa heat shock protein (HSP27) in gastric carcinomas and adjacent normal, metaplastic, and dysplastic gastric mucosa, and its prognostic significance

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Abstract *Purpose*: The investigation of heat shock protein 27 (HSP27) expression in gastric cancer and adjacent normal, metaplastic, and dysplastic gastric mucosa and its correlation with clinicopathological parameters and survival of patients. *Methods*: Immunohistochemical methodology was performed on formalin-fixed paraffin-embedded sections by using a monoclonal anti-HSP27 antibody. HSP27 expression was screened and compared in 86 cases of gastric carcinoma and adjacent normal, metaplastic, and dysplastic gastric mucosa. *Results*: In the normal mucosa, HSP27 was detected in 68 out of 86 cases (79%) and was more intense in the surface and upper two-thirds of gastric foveolae. In dysplastic gastric mucosa, HSP27 immunoreactivity was usually higher than that of the adjacent normal epithelium and was parallel to the severity of dysplasia. HSP27 expression was found in 54 out of 86 (62.7%) gastric carcinomas and was significantly related to more than six metastatic lymph nodes ($P = 0.03$). HSP27 expression was also higher in tumors of advanced stage and in those of female patients. HSP27 expression was associated with shorter overall survival in univariate analysis ($P = 0.04$), but this relationship was not retained in multivariate analysis. *Conclusions*: Our findings indicate that: i) HSP27 is commonly expressed in normal gastric epithelium where it seems to exert a protective role; and ii) HSP27 is involved in gastric carcinogenesis and its expression appears to be associated with parameters of unfavorable prognosis and shorter overall survival.

Keywords Heat shock protein · HSP27 · Gastric carcinoma · Immunohistochemistry · Prognosis

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Introduction

Heat shock proteins (HSPs) are highly conserved proteins, which are present in the cells of all studied organisms. Most HSPs are constitutively expressed in eukaryotic cells and participate, mainly through their function as molecular chaperones, in numerous physiological processes, such as three-dimensional folding and intracellular translocation of denatured or newly synthesized proteins, assembly and trafficking of signaling complexes (e.g., steroid hormone receptors), protein secretion and degradation as well as regulation of growth and differentiation (De Maio 1999; Zhang et al. 2001).

The cellular exposure to a wide variety of metabolic and environmental stresses (i.e., elevated temperature, heavy metals, oxidants, ischemia, infections, and drugs) induces expression of HSPs via activation of the heat shock transcription factors (HSFs) (Pirkkala et al. 2001). Activated HSFs bind to the heat shock elements (HSEs) of respective genes potentiating their transcription. HSPs are responsible for the stress response which, unlike apoptosis, protects cells and mediate recovery after injury through repair or degradation of unfolded proteins. HSPs, when present in abnormal levels, have

also been correlated with a number of pathological processes, such as atherosclerosis (Hedges et al. 1999; Bobryshev and Lord 2002), aging (Verbeke et al. 2001), autoimmune disorders (Hayem et al. 1999), and malignant diseases. Their role in neoplasia is evident from their implication in the phenomenon of multidrug resistance (MDR) (Kamishima et al. 1997) and in the regulation of p53 function and apoptosis (Jolly and Morimoto 2000). HSPs comprise a large glycoprotein gene family and are typically named according to their molecular mass, which ranges from 8 kDa to 150 kDa.

HSP27 was originally identified as a 24-kDa protein in the human MCF-7 cell line exposed to the action of estrogens, as well as in human breast cancer cells (Adams and McGuire 1985). While HSP27 is constitutively expressed at low levels in the cytoplasm of many human cells, its abundance increases following exposure to a wide range of stimuli. In addition to stress signals, proinflammatory cytokines, growth factors, and autonomic neurotransmitters induce phosphorylation of HSP27 and its subsequent translocation into or around the nucleus. Unphosphorylated HSP27 is normally present in the cytosol in the form of high-molecular-weight multimers/large oligomers which function as molecular chaperones. In vitro experiments have shown that HSP27 large oligomers can rescue several heat-denatured proteins, by absorbing them on their surface, preventing their heat-induced aggregation and maintaining them in a folding-competent state. Phosphorylation results from activation of p38 mitogen-activated protein kinase (MAPK), which phosphorylates downstream kinases that, in turn, phosphorylate HSP27 at specific serine residues leading to the dissociation of HSP27 into small oligomers, dimers, and monomers (Kyriakis and Avruch 2001). Although small oligomers do not possess chaperone activity in vitro, in vivo growing of cells shows that cell-cell contacts induce formation of large oligomers, regardless of the status of HSP27 phosphoacceptor serines (Bruey et al. 2000b). Unphosphorylated HSP27 is a F-actin modulating protein inhibiting actin polymerization (Smoyer and Ransom 2002), while phosphorylated HSP27 stabilizes actin fibers, restores heat-shock disrupted actin stress fibers, and increases cortical polymerized F-actin content, membrane ruffling, pinocytosis, and cell motility (Landry and Huot 1999; Piotrowicz et al. 1998). HSP27 and HSP70 bear antiapoptotic activities and have thus been implicated in carcinogenesis. HSP27, in the form of large oligomers, interferes with the mitochondrial pathway of caspase-dependent cell death by binding to cytochrome-c released from the mitochondria and preventing the cytochrome-c-mediated interaction of Apaf-1 with procaspase-9 (Bruey et al. 2000a).

Immunohistochemical study of the distribution of HSP27 in various tissues revealed that this protein is highly expressed in the epithelium of many estrogen-target organs such as uterus (Padwick et al. 1994), vagina (Ciocca et al. 1992), and the skin (Gandour-Edwards et al. 1994). However, subsequent studies documented that HSP27 expression can also be detected in neoplasms of the esophagus (Lambot et al. 2000), brain (Assimakopoulou and Varakis 2001), and soft tissue and skin (Gandour-Edwards et al. 1994). The present study aimed at investigating the expression of HSP27 in gastric cancer and adjacent normal, metaplastic and dysplastic gastric mucosa and correlating its tumor expression profile with the clinicopathological data and overall survival of the patients.

Materials and methods

Specimens, clinicopathological and follow-up data

Eighty-six cases of gastric carcinoma surgically removed by total or subtotal curative gastrectomy (R0 = no residual tumor) from 44 men and 42 women were included in this study. The age of the patients ranged from 37 years to 85 years (mean age: 64.52, SD = 10.04 years). The number of lymph nodes that were resected in each case ranged from 15 to 106 (average number: 28 lymph nodes). Sections from tumors and gastric mucosa adjacent to the tumor, as well as ≈5-cm away from it, were fixed in 4% neutral buffered formaldehyde and embedded in paraffin. Gastric dysplasia was graded into low and high grades according to Padova International Classification (Rugge et al. 2000), while intestinal metaplasia was subtyped as immature in all cases after staining with Alcian Blue (pH 1.0). Gastric dysplasia in this case is defined as a preneoplastic change, and normal, metaplastic, and dysplastic gastric mucosa approximately 5-cm away from the tumor was evaluated. Gastric carcinomas were classified according to Lauren histological types (Lauren 1965) and staged according to Union International Contre le Cancer (UICC) criteria [Sobin and Wittekind (1997) TNM classification of malignant tumors, 5th edn.]. Among the 86 patients with gastric carcinoma, 71 were followed-up for a period of 9–163 months (median: 22 months; mean: 33.8 months). The remaining 15 patients were lost from follow-up.

Immunohistochemistry

HSP27 was detected by immunohistochemistry on paraffin-embedded sections using the standard avidin-biotin-peroxidase (ABC) technique. Microwave irradiation in citric buffer (pH 6.0) was employed as antigen retrieval method. The primary mouse monoclonal antibody against HSP27 (Biogenex, San Ramon, Calif., USA) was diluted 1:80 with phosphate-buffered saline (PBS) and applied on tissue sections for 1 h at room temperature. Antigen binding was detected using the Vectastain ABC Elite peroxidase kit (Vector Laboratories, Burlingame, USA). Sections from breast carcinomas reactive to HSP27 immunostaining were used as positive controls, whereas sections from gastric carcinomas, in which the primary monoclonal antibody was omitted, were used as negative controls.

Evaluation of HSP27 expression

The immunostained sections were evaluated and scored by two independent pathologists without prior knowledge of the clinicopathological characteristics. In agreement with previous studies (Villaseca et al. 1997), cases displaying immunostaining in more than 5% of tumor cells were regarded as positive. Cases with interobserver differences were re-assessed by simultaneous examination of the specimens by the two pathologists in a double-headed light microscope. In normal, metaplastic, and dysplastic gastric mucosa the staining was classified as low and high according to the intensity. In addition, in normal mucosa, the staining differences in gastric foveolae and glands were also recorded.

Statistical analyses

The correlation of the HSP27-positive and HSP27-negative tumors with the clinical and pathological features of the cases was performed by using the Fisher exact test. Results were considered statistically significant only if $P < 0.05$ was present. Survival rates were estimated by the Kaplan-Meier method and compared using the log-rank test. Multivariate analysis was performed by the Cox proportional hazards model. The statistical software package SPSS 6.0 was employed for data management and analysis.

Results

Normal, metaplastic, and dysplastic gastric mucosa

HSP27 immunoreactivity was observed in 68 out of 86 cases (79%) of tumor-adjacent normal gastric mucosa and it was localized in the subnuclear cytoplasmic region of gastric epithelial cells. The staining was more intense in the epithelial cells of the surface and the upper two-thirds of gastric foveolae and weaker in the lower third, whereas deep gastric glands were mostly negative (Fig. 1A). A band-like enhancement of staining was locally observed on the cell surface of the upper two-thirds of the gastric pits. No significant difference in staining intensity between fundic and pyloric glands was detected. In mucosal folds, the intensity of immunostaining was higher than that of the adjacent epithelial cells in one-third of the cases examined. Intestinal metaplasia of the immature type found in 41 cases showed negative ($n = 22$) or low ($n = 15$) immunostaining, while high staining was obtained only in four cases. In low- and high-grade gastric dysplasia high levels of HSP27 immunoreactivity were observed in 50% and 76.5% of the cases, respectively (Fig. 1B). Detailed scoring of HSP27 immunostaining in normal gastric mucosa, intestinal metaplasia, and dysplasia is presented in Table 1.

Gastric carcinomas

In gastric carcinomas, HSP27 immunostaining was diffuse cytoplasmic with perinuclear intensification in some neoplastic cells. In addition to cytoplasmic, local nuclear staining was observed in nine out of 50 intestinal, seven out of 19 diffuse, and eight out of 17 mixed carcinomas. A strong, band-like staining of luminal cell surface was detected in about half of the well-differentiated intestinal carcinomas and in the glandular element of a few moderately differentiated intestinal carcinomas (Fig. 1C). Noteworthy was the enhancement of HSP27 nuclear and

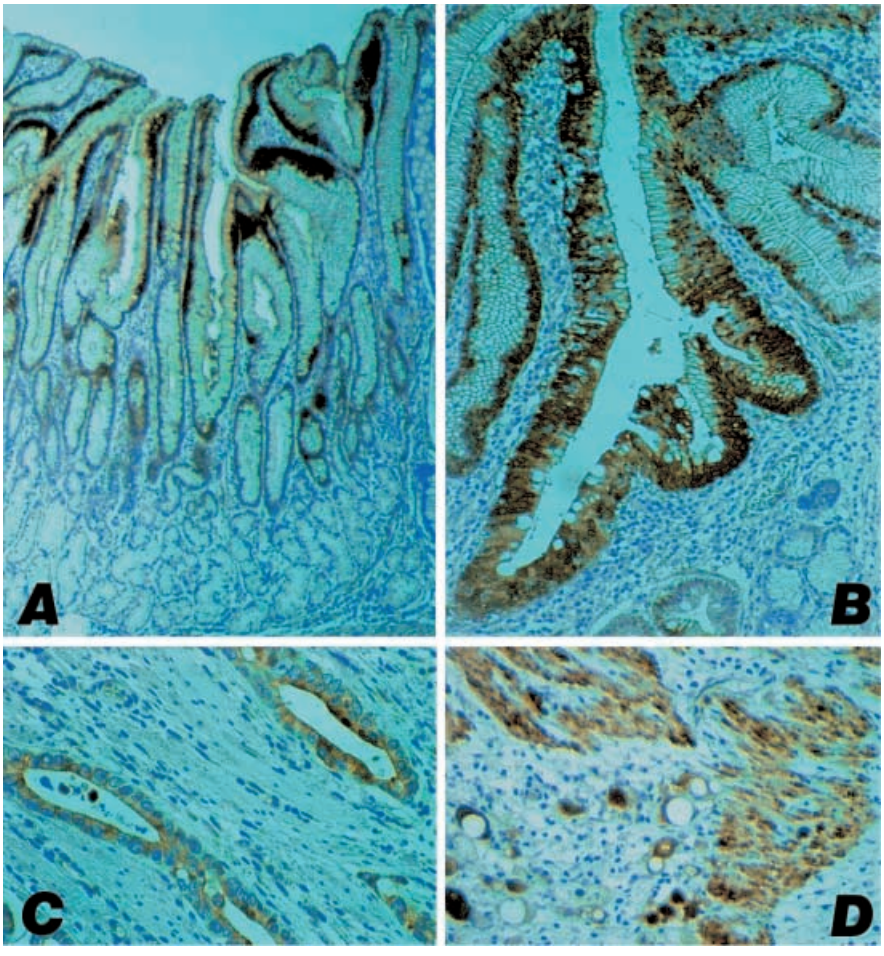

Fig. 1. **A** HSP27 immunostaining in normal gastric mucosa. Subnuclear cytoplasmic staining is observed in the epithelial cells of the surface and the upper parts of gastric pits, while deep glands are negative. Intestinal metaplasia (*right side*) exhibits very weak staining (×16). **B** Dysplastic gastric epithelium displaying diffuse cytoplasmic HSP27 immunostaining, which is more intense than that of the adjacent normal epithelium (×40). **C** HSP27 immunostaining in a well-differentiated gastric carcinoma of the intestinal type. Cytoplasmic immunostaining with a band-like intensification on the luminal surface of the carcinoma cells is shown (×80). **D** HSP27 immunostaining in a diffuse gastric carcinoma infiltrating the smooth muscle. Strong nuclear and cytoplasmic staining in both carcinoma cells and smooth muscle cells is shown (×80)

Table 1. Heat shock protein 27 (HSP27) expression in normal, metaplastic and dysplastic gastric mucosa

	Total Number	HSP27[a]		
		–	Low	High
Gastric foveolae	86	18 (21%)	26 (30.2%)	42 (48.8%)
Gastric glands	86	78 (90.6%)	8 (9.4%)	0
Intestinal metaplasia[b]	41	22 (53.6%)	15 (36.6%)	4 (9.8%)
Gastric dysplasia				
Low grade	20	2 (10%)	8 (40%)	10 (50%)
High grade	17	1 (6%)	3 (17.6%)	13 (76.5%)

[a]Staining intensity
[b]Immature in all cases

cytoplasmic immunostaining in smooth muscle cells of the lamina muscularis propria in the regions of active tumor infiltration, compared to the regions with no tumor infiltration that were usually stained weakly (Fig. 1D).

Overall, out of 86 cases of gastric carcinomas, 54 (62.7%) were positive and 32 (37.3%) were negative for HSP27 expression. Table 2 presents the correlation of HSP27 expression with clinicopathological characteristics of the cases. Significantly higher HSP27 expression was found in tumors with metastases to lymph nodes, especially in those with more than six ($P = 0.03$). HSP27 expression was also higher in tumors of advanced (III and IV) UICC stage ($P = 0.10$) and in tumors of female versus male patients ($P = 0.17$). Comparison of nuclear and cytoplasmic HSP27 immunostaining revealed that the former was significantly associated with gastric carcinomas of diffuse/mixed type ($P = 0.03$), advanced UICC stage ($P = 0.04$), and higher tumor grade ($P = 0.05$) (Table 3).

Survival analysis revealed that patients with HSP27-positive tumors had a significant survival disadvantage in comparison to patients showing HSP27-negative tumors. Patients with HSP27-positive tumors had a median survival of 17 months (95% confidence interval: 8–28 months), whereas patients with HSP27-negative tumors had a median survival of 33.3 months (95% confidence interval: 10–62 months) ($P = 0.04$, Fig. 2).

Table 2. Correlation of HSP27 expression with clinicopathological characteristics of gastric carcinoma cases. (*pts* patients, *Pos* positive, *Neg* negative, *Met* metastases)

	Total number of pts	HSP27		P
		Pos Number of pts	Neg Number of pts	
Gender				
Male	44 (51.2%)	25 (56.8%)	19 (43.2%)	0.17
Female	42 (48.8%)	29 (69%)	13 (31%)	
Age				
≤ 65	43 (50%)	25 (58.1%)	18 (42.9%)	0.25
> 65	43 (50%)	29 (67.4%)	14 (32.6%)	
Tumor size				
≤ 5	49 (57%)	32 (65.3%)	17 (34.7%)	0.36
> 5	37 (43%)	22 (59.5%)	15 (40.5%)	
UICC stage				
I	6 (7%)	3 (50%)	3 (50%)	0.10*
II	18 (20.9%)	9 (50%)	9 (50%)	
III	51 (59.3%)	32 (62.8%)	19 (37.2%)	
IV	11 (12.8%)	10 (90.9%)	1 (9.1%)	
Histological type				
Intestinal	50 (58.1%)	29 (58%)	21 (42%)	0.37*
Diffuse	19 (22.1%)	12 (63.2%)	7 (36.8%)	
Mixed	17 (19.8%)	13 (76.5%)	4 (23.5%)	
Grade				
I	16 (18.6%)	9 (56.3%)	7 (43.7%)	0.67*
II	23 (26.7%)	16 (69.6%)	7 (30.4%)	
III	47 (54.7%)	29 (61.7%)	18 (38.3%)	
Met. lymph nodes				
0	15 (17.4%)	6 (40%)	9 (60%)	0.03*
1–6	39 (45.4%)	23 (59%)	16 (41%)	
> 6	32 (37.2%)	25 (78.1%)	7 (21.9%)	

*P (Test for trend)

Table 3. Correlation of nuclear and cytoplasmic HSP27 immunoreactivity with UICC stage, histopathological type, and tumor grade

	Tot. positive cases	HSP27		*P**
		Nuclear	Cytoplasmic	
UICC stage				
I, II	12 (21.8%)	2 (16.67%)	10 (83.33%)	0.04
III, IV	43 (78.2%)	22 (51.16%)	21 (48.84%)	
Histological type				
Intestinal	30 (54.5%)	9 (30%)	21 (70%)	0.03
Diffuse/mixed	25 (45.5%)	15 (60%)	10 (40%)	
Grade				
I, II	25 (45.5%)	7 (28%)	18 (72%)	0.05
III	30 (54.5%)	17 (56.67%)	13 (43.33%)	

*Fisher exact test (two–tailed)

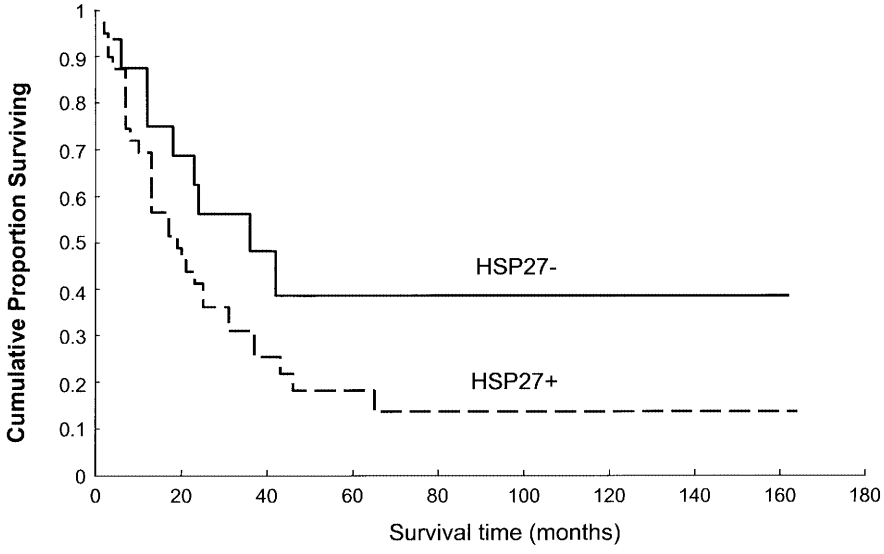

Fig. 2. Kaplan-Meier survival plot according to HSP27 expression (log-rank test, $P = 0.04$)

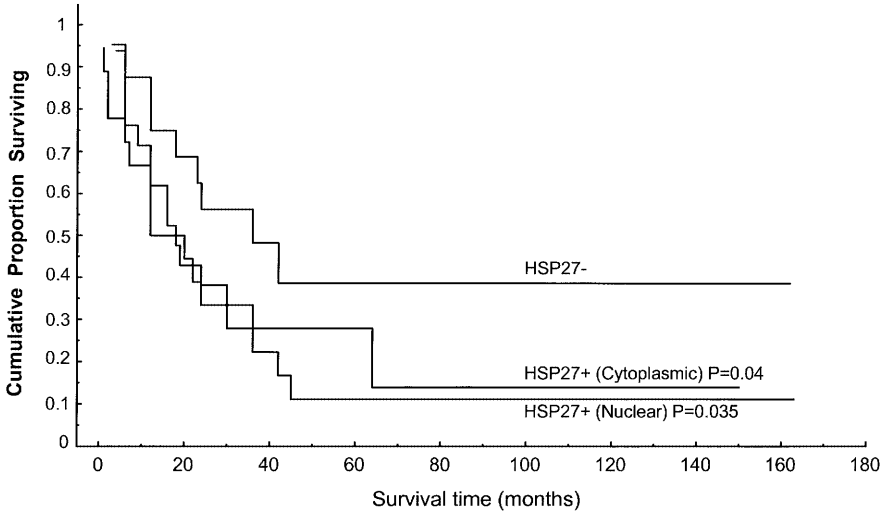

Fig. 3. Kaplan-Meier survival plot according to nuclear HSP27 expression (log-rank test, $P = 0.035$) and cytoplasmic only HSP27 expression (log-rank test, $P = 0.04$)

Patients with tumors displaying a nuclear (in addition to cytoplasmic) HSP27 staining pattern had a median survival of 16 months (95% confidence interval: 7–27 months) ($P = 0.035$, Fig. 3). Patients with tumors displaying only a cytoplasmic HSP27 staining pattern had a median survival of 18 months (95% confidence interval: 8–30 months) ($P = 0.04$, Fig. 3). However, using the Cox proportional hazards model, HSP27 failed to retain its prognostic significance when analyzed together with UICC stage. Advanced UICC stage was the

only significant independent factor of unfavorable outcome ($P = 0.0034$).

Discussion

HSP27 was found to be commonly expressed in the upper parts of normal gastric mucosa. This expression was greatly reduced along the gastric pits, shifting to negative in the epithelium of gastric glands. Subnuclear cytoplasmic staining was observed in the epithelial cells of the surface and the upper parts of the gastric pits. This observation may reflect a functional role of the above protein that may be elicited in response to a variety of aggressive factors (e.g., hydrochloric acid, non-steroid anti-inflammatory drugs, ethanol, *Helicobacter pylori* infection) acting mainly at the surface of gastric mucosa. Exposure of rat gastric mucosa cells to restraint and water immersion stress resulted in rapid activation of HSF-1 and the extent of this activation was inversely related to the severity of mucosal damage (Rokutan 2000). Activation of the heat shock protein or stress response in gastric mucosa pretreated with mild irritants, increases resistance against subsequent insults, a phenomenon known as adaptive cytoprotection. The role of HSP27 in mucosal defense is further supported by our finding that HSP27 immunostaining is enhanced in the epithelium of mucosal folds, which represent sites of prolonged action of irritant chemical or bacterial factors.

Mounting evidence suggests that HSP27 is an estrogen-inducible protein (Porter et al. 2001). Both tumorous and non-tumorous tissues of gastric cancer patients express estrogen receptor alpha and beta (ERα and ERß) mRNA (Takano et al. 2002). Recurrent androgen-insensitive prostate adenocarcinomas express classical ERα, promoting transcriptional activation of HSP27 (Bonkhoff et al. 2000). It is possible that in many gastric carcinomas HSP27 expression may be under hormonal control. This hypothesis is in concert with our finding of augmented HSP27 expression in tumors of female patients.

Intestinal metaplasia showed decreased staining for HSP27, compared to adjacent normal epithelium, whereas low- and high-grade dysplastic epithelium exhibited high immunopositivity in 50% and 76.5% of the studied material, respectively. Similarly, expression of HSP27 was found in 62.7% of gastric carcinomas. These findings indicate that in a significant number of cases, HSP27 synthesis may be up-regulated in the course of neoplastic transformation of gastric epithelium. Similar results have been reported concerning normal esophagus, dysplastic lesions, and esophageal carcinoma (Lambot et al. 2000). HSP27 is present only in the upper epithelial layer of normal esophagus and its expression increases from dysplasia to invasive carcinoma, achieving highest levels in the more undifferentiated areas. HSPs include anti-apoptotic and pro-apoptotic proteins that function at key points of the apoptotic pathway. Due to its apoptosis-inhibitory effect, HSP27 seems to play an important role in the process of carcinogenesis (Jolly and Morimoto 2000).

In gastric carcinomas, increased HSP27 expression may also be attributed to the biological stress exerted on cancer cells by various metabolic perturbations, such as anoxia and pH changes within the tumor. This elevated stress is also evident from the different pattern of HSP27 expression in the tumors, as compared to the non-neoplastic mucosa. Specifically, HSP27 expression was detected not only in the cytoplasm but also in the nuclei of some carcinomas, particularly of the diffuse and mixed type, and was associated with factors of unfavorable prognosis such as advanced UICC stage and higher tumor grade, and therefore lower survival. This change in intracellular localization of HSP27 has also been found in experimental studies, in which HSPs accumulated in the nuclei during heat shock action and returned to the cytoplasm after shock removal (Arrigo et al. 1988). HSP70 rescues unfolded nuclear proteins during stress by driving them to nucleolus, thus preventing their aggregation in the nucleus and reducing damage to other nuclear components (Nollen et al. 2001).

Significantly higher HSP27 expression was observed in tumors with metastases to lymph nodes and those of advanced UICC stage. Several studies have demonstrated that HSP27 expression is associated with aggressive tumor behavior contributing to the metastatic process. HSP27 appears to confer protection to metastatic cells, to increase cell invasiveness (Lemieux et al. 1997), and cell migration (Hedges et al. 1999). Noteworthy is the implication of HSP27 in the angiogenetic process. Vascular endothelial growth factor (VEGF), which plays a pivotal role in angiogenesis, activates the p38 MAPK cascade leading to phosphorylation of HSP27 and subsequent actin reorganization and endothelial cell migration (Rousseau et al. 1997).

Our observation of the intense nuclear and cytoplasmic HSP27 immunoreactivity in the smooth muscle cells of the lamina muscularis propria, at the sites of active tumor infiltration, is another indication for the activation of heat shock protein response as a result of the biologic stress exerted to the normal tissue during tumor expansion.

Univariate analysis revealed that tumor HSP27 expression was related to lower overall survival of the patients, albeit the median follow-up period was only 22 months. This is supported by its association with parameters of unfavorable prognosis, such as more than six metastatic lymph nodes, and, to a lesser degree, the advanced stage. Similar findings were reported concerning patients with resectable stage IV gastric cancer (Takeno et al. 2001). The same study revealed that mutant p53 protein overexpression bears prognostic significance in stage IV gastric cancer patients. While HSP70 is expressed in gastric carcinomas, its expression is not associated with gastric cancer prognosis (Maehara et al. 2000). Other analyses indicate that HSP27 carries a negative prognostic value in osteosarcoma (Uozaki et al. 2000) and prostate cancer (Cornford et al. 2000).

In conclusion, the data presented in this study imply that HSP27 displays a protective role as a molecular chaperone in normal gastric mucosa and is vigorously involved in the process of gastric carcinogenesis. In gastric cancer, the HSP27 expression profile seems to be related to parameters of poor outcome and decreased survival of the patients. The development of therapeutic strategies targeting to modulation of HSP27 expression in gastric cancer cells may allow control over tumor expansion and improvement of patient survival.

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