Histol Histopathol (2004) 19: 119-128

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

HSP 27 as possible prognostic factor in patients with oral squamous cell carcinoma

L. Lo Muzio[1], R. Leonardi[2], M.A. Mariggiò[3], M.D. Mignogna[4], C. Rubini[5], A. Vinella[3], G. Pannone[6], L. Giannetti[7], R. Serpico[8], N.F. Testa[8], G. De Rosa[6] and S. Staibano[6]

[1]Institute of Dental Sciences, University of Ancona, Ancona, Italy, [2]Department of Medical and Surgical Sciences, II Dental Unity, University of Catania, Catania, Italy, [3]Department of Biomedical Sciences and Human Oncology - Section of General Pathology and Experimental Oncology, University of Bari, Bari, Italy [4] Department of Dental Sciences, University of Naples Federico II, Naples, Italy, [5]Institute of Pathological Anatomy, University of Ancona, Ancona, Italy, [6]Department of Biomorphological and Functional Sciences, Pathology Unit, University of Naples Federico II, Naples, Italy, [7]Institute of Dentistry and Surgery, University of Modena, Modena, Italy and [8]Department of Dental Sciences and Surgery, University of Bari, Bari, Italy

Summary. HSP27 belongs to the Heat shock protein (HSP) family, which plays essential functions in cells under physiological conditions and prevents stress-induced cellular damage. The aim of this study was to investigate the biological role of HSP27 in oral tumorigenesis. Materials and methods: Seventy-nine cases of oral squamous cell carcinoma and 10 cases of normal mucosa were analysed for HSP27 expression by immunohistochemistry. Moreover, the western blot analysis was performed on two cases of normal mucosa and five cases of OSCC. Results: Normal oral mucosa showed a suprabasal expression of HSP27. Twenty-four cases of SCC (30.7%) showed a diffuse staining for HSP27, and 48 cases (60.3%) showed instead a decrease in staining, which was diffuse, homogeneous, or with alternation of positive and negative areas in a single tumor ("mosaic" pattern). Only 7 cases of OSCC (7.5%) were completely negative for HSP27. Frequency of lymph node metastases was higher in HSP27-negative tumours (3/7, 42.8%) than in HSP-reduced (16/48, 33.3%) or positive ones (5/26, 19.2%). Regard staging, stages I and II had a higher score than stages III and IV (stage I > stage II > stage III > stage IV). There was also a statistically significant correlation between HSP27 expression and grade: HSP27 expression was reduced in poorly differentiated tumours ($P < 0.05$). When analysed for prognostic significance, patients with negative/reduced HSP27 expression had poorer survival rates than the group with positive HSP27 expression ($P < 0.05$). The statistical analysis of these findings showed no significant correlation between HSP27 expression, sex, and tumour size. Conclusion: Cases with reduced expression were more aggressive and poorly differentiated. These data suggest that HSP27 expression may be useful in order to identify cases of oral squamous cell carcinoma with more aggressive and invasive phenotype providing novel diagnostic and prognostic information on individual patient survival with oral cancers.

Key words: HSP27, Squamous cell carcinoma, Oral mucosa, Mouth, apoptosis

Offprint requests to: Prof Lorenzo Lo Muzio, Via Carelli 28, 71100 Foggia, Italy. Fax: (39) 881-685809. e-mail: lomuziol@tin.it

Introduction

Heat shock protein (HSP) family is a large family which plays essential homeostatic functions in cells under physiological conditions and in protecting cells against damage in stressful conditions. On the basis of their molecular weights HSPs have been grouped into different sub-families: small (HSP 20-30-KDa), HSP40, HSP60, HSP70, HSP90 and HSP100. Intracellulary, HSPs, the most conserved proteins throughout evolution (Lindquist and Craig, 1988), are involved in several processes such as protein denaturation-renaturation, folding-unfolding, transport-translocation, activation-inactivation, and secretion (Frydman et al., 1994).

Experimental evidence suggests that HSPs may promote tumorigenesis by suppressing apoptosis (Jaattela, 1999). On the contrary, it seems that some members of HSPs play important roles in the immune response against cancer (Multhoff et al., 1997).

HSP27 is a cytoplasmic protein that is constitutively expressed in a broad range of normal tissues and neoplasms. HSP27 is constitutively expressed at low levels in most human tissues (Lindquist and Craig, 1988). After induction, the protein becomes phosphorylated and simultaneously translocated from the cytoplasm to within or around the nucleus (Lavoie et

al., 1993). The phosphorylation process is a key regulator of HSP27 functions: it occurs at serine residues 78 and 82 by a specific kinase and may be activated by several different signal transduction mechanisms. Squamous tissues in several sites have been found to strongly express the 27-Kda stress-response peptide in suprabasal layers.

A differential pattern of HSP27 "in vitro" expression levels has been correlated to malignant transformation in experimental models (La Thangue and Latchman, 1988). Fibrosarcoma cells and breast cancer lines, transfected with HSP27, exhibited delayed tumor progression and a lower proliferation rate in comparison to controls (Blackburn et al., 1997; Kindas-Mugge et al., 1998). On the other hand HSP27 overexpression in malignant cells obtained from a colon adenocarcinoma cell clone increased their tumorigenicity in syngeneic animals (Garrido et al., 1998). Breast cancer cell lines overexpressing HSP27 displayed altered cell morphology and decreased cell motility, as well as enhanced proliferation (Lemieux et al., 1997).

Clinico-pathological and immunohistochemical studies attempting to correlate the expression level of HSP27 protein in tumor cells with tumor progression and clinical outcome have also provided contradictory results (Langdon et al., 1995; Storm et al., 1996; Hsu and Hsu, 1998; Geisler et al., 1999; Vegh et al., 1999; King et al., 2000). Therefore, it may be assumed that there are wide variations in the degree of expression between different cell types and tissues (Ciocca et al., 1993b; Dunn et al., 1993).

In squamous cell carcinoma of the tongue no correlation has been reported between HSP27 staining and survival period, stage, lymph node metastasis or histological grade (Ito et al., 1998). HSP27 expression was markedly lower in Barrett's metaplasia and oesophageal adenocarcinomas than in normal mucosa, but it was higher in oesophageal squamous cell carcinomas (Soldes et al., 1999). Similarly Lambot et al. (2000) observed an increased HSP27 protein expression in malignant areas, from dysplastic lesions to invasive carcinoma, being highest in the less differentiated areas. On the other hand, Kawanishi et al. (1999) also reported a reduced expression of HSP27 in patients with esophageal squamous cell carcinoma (Kawanishi et al., 1999) and therefore, they believed that HSP27 should be considered an independent prognostic factor of this disease (Kawanishi et al., 1999).

The frequency of squamous cell carcinoma of the oral mucosa is rapidly increasing and, in particular, it constitutes the most frequent malignant tumour of the oral cavity. The incidence of metastasis depends on the degree of cellular differentiation, degree of invasion and site of the primary tumour. However, the clinical behaviour of this tumour is difficult to predict based on classical histopathological parameters alone. Biological markers that can identify the lesions with an aggressive phenotype and worse prognosis need to be identified.

As it is well known, carcinogenesis is a multistage process involving the activation of oncogenes and the inactivation of tumour suppressor genes. In this context, most human tumours are characterized by an imbalance of regulatory mechanisms controlling cell-cycle progression and/or the cell death/viability balance, or apoptosis.

Apoptosis has become a basic tool in developing cancer research and establishing new cancer strategies. Apoptosis, or programmed cell death, is a genetically controlled process, which maintains developmental morphogenesis (Vaux et al., 1994) and homeostasis of differentiated organisms by removing senescent, unneeded or dangerous cells (Sternberg, 1997). Aberrations of this process leading to aberrantly reduced cell death are thought to participate in cancer by promoting increased resistance to therapy and favouring the insurgence of transforming mutations (Jameson, 1998).

Considerable interest has recently been focused on the identification of regulators of apoptosis, which may potentially influence the cell death/cell viability balance in cancer. In addition to pro- and anti-apoptotic bcl-2 molecules (Reed, 1998), and a second gene family of Inhibitor of Apoptosis (IAP) (Crook et al., 1993), several studies have recently focused on the role of HPSs in human cancers.

To our kwnolegde only three studies on a few cases examined HSP27 expression in oral squamous cell carcinoma (Fortin et al., 2001; Leonardi et al., 2002; Mese et al., 2002). In order to understand the biological role of HSP27 in oral tumorigenesis, we studied HSP27 expression in oral cancers by immunohistochemistry and western blot in samples of OSCCs and in oral cell lines, correlating its expression with clinico-pathological features

Materials and methods

Selection of cases

The study population consisted of 57 men and 22 women with a mean age of 63,88 years (age range 20-81). 35 cases were stage I, 20 stage II, 6 stage III, and 18 stage IV. All patients were analysed for survival rates (the follow-up time was in all cases > 3 years). Survival was calculated from the date of surgery to the date of the latest clinical follow-up or death by disease. Patients who died of postoperative complications were excluded from the study.

None of the patients had been treated previously. They received surgical treatment with curative intention. No patients had multicentric lesions.

Seventy-nine samples from paraffin-embedded and ten from frozen specimens of primary oral squamous cell carcinomas, and 7 from paraffin-embedded specimens of lymph node and tissutal metastases of oral squamous cell carcinomas (OSCCs) were used for the study. Specimens were fixed in 10% neutral-buffered formalin.

The histopathological grading was assessed on

paraffin H&E - stained sections. Tumor extent was classified according to the TNM system by UICC (Sobin and Witterkind, 1997) and tumors were classified as grade 1, 2 or 3, according the WHO classification of histological differentiation.

Ten paraffin-embedded and five frozen specimens of healthy oral mucosa were obtained from patients who had undergone routine oral surgical procedures, with the informed consent of the donors.

Immunohistochemistry

Four-μm serial sections from formalin-fixed, paraffin-embedded blocks of representative areas of tumour were cut for each case. Only sections containing sufficient epithelium to assess the antibody reactivity with 1000 cells were considered for this study.

Immunohistochemistry was then performed on the remaining sections mounted on poly-L-lysine-coated glass slides. Deparaffined and rehydrated sections were incubated for 30 minutes in 3% H_2O_2/methanol to quench endogenous peroxidase activity, and then rinsed for 20 minutes with phosphate-buffered saline (PBS) (Bio-Optica M107, Milan Italy). Non-specific protein binding was attenuated by incubation for 30 minutes with 5% horse serum in PBS. Specimens were incubated overnight with the primary anti-HSP27 monoclonal antibody (Catologue No. NCL-HSP27; Novocastra Laboratories Ltd, Newcastle, U.K.) at a dilution of 1:20. The antibody was applied directly to the section and the slides were incubated overnight (4 °C) in a "humidified chamber". The sections were washed three times with PBS at room temperature. Immune complexes were subsequently treated with the secondary byotinilated antibody and then detected by streptavidin peroxidase, both incubated for 30 minutes at room temperature (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). After rinsing with 3 changes of PBS the immunoreactivity was visualized by development for 2 minutes with 0,1% 3,3'-diaminobenzidine and 0,02% hydrogen peroxide (DAB substrate kit, Vector Laboratories Burlingame Calif.). Sections were counterstained with Mayer's haematoxylin, mounted with permanent mounting medium and examined by light microscopy.

Positive controls consisted of tissue specimen sections of breast carcinoma with known antigenic reactivity. A negative control was performed in all cases by substituting the primary antibody for normal rabbit serum. Negative controls in all instances resulted in a negative immunoreactivity for HSP27. To evaluate the HSP27 expression, 300 cells were examined in at least five areas at x400 magnification, and a mean percentage of positive tumour cells was determined assigning cases to one of the three following categories: a) positive (+), when the stained cells accounted >80% of the total; b) reduced (±), when the stained cells were comprised from 5 to 80% of the total cell population; and c) negative (-), when the frequency of stained cells was <5% (Kawanishi et al., 1999).

Western blot analysis

Two specimens of normal mucosa and five cancerous specimens were used for western blot analysis.

Proteins were extracted with T-Per Tissue (Pierce, Rockford, IL) containing protease inhibitors (Sigma Chemical Co., St. Louis, MO). The protein concentration was determined by Bradford protein Assay (Sigma) using BSA (Sigma) as a standard. Thirty μg of proteins were solubilized in Laemmli's sample buffer by boiling for 8 min and subjected to 10% SDS-PAGE, followed by electroblotting onto a nitrocellulose membrane (Bio-Rad, Richmond, CA). The membrane was blocked for 1 hour at room temperature with PBS buffer containing 5% non-fat dry milk powder and 0.1% Tween 20. Western blot analysis was performed using a monoclonal antibody anti-HSP27 (Catalogue No. NCL-HSP27; Novocastra Laboratories Ltd, Newcastle, U.K.) at a dilution of 1:250 in PBS buffer containing 5% non-fat dry milk powder and 0.1% Tween 20 for 60 min at room temperature. Incubation with a secondary HRP-conjugated goat antimouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at dilution of 1:10000 was performed under the same conditions. For detection of the immunocomplex, the SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL) was used. Anti-beta-actin mouse monoclonal antibody (clone AC-15; Sigma) was used for normalization of western blot analysis.

Image analysis

Autographs obtained by western blot analysis were subjected to densitometric analysis (Gel Doc 2000, Bio-Rad, Richmond, CA) and the signal intensity of HSP27 and beta-actin was quantified. The mean values for individual samples were normalised to the values for the matching actin signal to compensate for any variation in protein loading and concentration.

Statistical analysis

Data were analysed using GraphPad Prism software version 3.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Significant differences ($P<0.05$) between groups were determined using one-way analysis of variance and the Student-Newman-Keuls-test. Survival analysis was computed by comparing positive (+), when the stained cells accounted >80% of the total, reduced (±), when the stained cells were comprised from 5 to 80% of the total cell population, and negative (-), when the frequency of stained cells was <5%. Survival curves were analyzed according to the method of Kaplan-Meier and for differences between curves the p value was calculated by

the log-rank test. A p value of less than 0.05 was accepted as statistically significant.

Results

Immunohistochemistry

HSP27 expression in normal oral mucosa

Normal human oral mucous epithelium had a suprabasal pattern of HSP27 expression (Fig. 1). The labeling was cytoplasmic and finely granular with nuclei very rarely showing only a faint staining and becoming stronger from the parabasal layer to the surface layer. Generally basal keratinocytes were not immunolabelled by anti-HSP27 antibody, although a slight expression of HSP27 was recorded in some areas. Thus, normal epithelium included >80% of stained cells.

HSP27 expression in oral squamous cell carcinoma

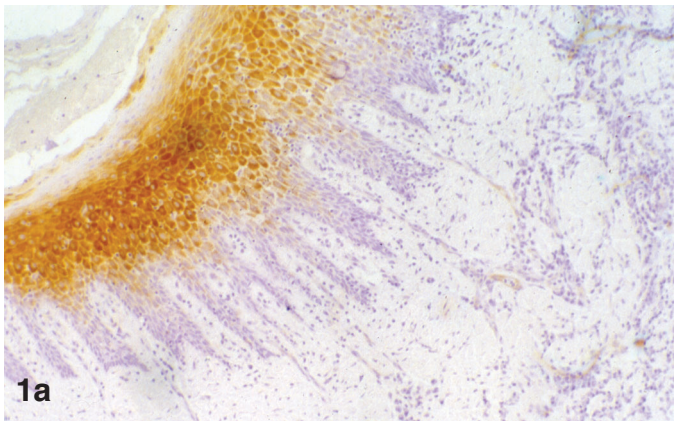
Various staining patterns were observed for HSP27 expression in OSCCs (Fig. 1). Seven cases (7.5%) showed <5% of positive tumor cells and were classified as negative (-) (Table 1). In 48 cases (60.3%), the positive tumor cells were comprised between 5 and 80%

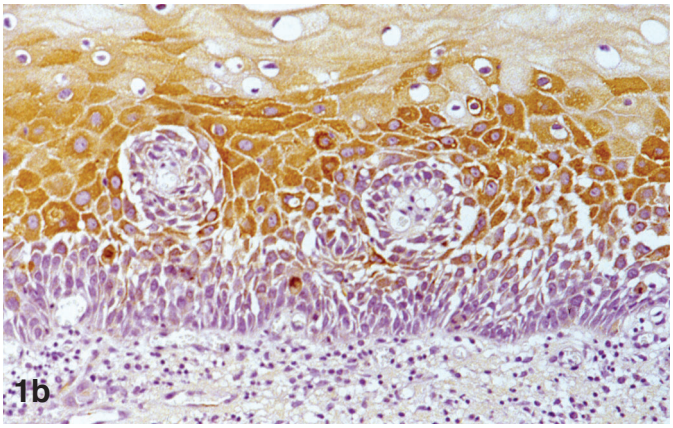
Table 1. Statistical analysis of HSP27 expression and associated clinico-pathological findings in oral SCCs.

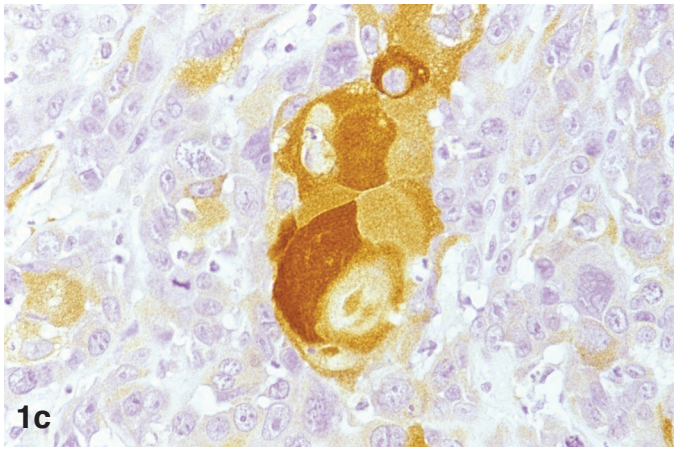
VARIABLES	No.	SCORE - n. (%)	SCORE ± n. (%)	SCORE + n. (%)	MEAN	STANDARD DEVIATION	STANDARD ERROR	p <0.05	STATISTICAL DATA			
Cases	79	7 (8.8)	48 (60.7)	24 (30.3)								
Age												
< 65 years	39	5	26	8	1.077	0.579	0.092	Yes°	P = 0.0396			
> 65 years	40	2	22	16	1.350	0.579	0.091					
Sex												
Male	57	6	34	17	1.193	0.610	0.080	No°	P = 0.5948			
Female	22	1	14	7	1.273	0.550	0.117					
Grading										Mean Diff.	q	P value
G1	18	1	7	10	1.500	0.618	0.145		grade 3 vs grade1	-0,6176	4,723	< 0.01
G2	41	2	27	12	1.244	0.537	0.083	Yes*	grade 3 vs grade 2	-0,3615	3,242	< 0.05
G3	20	4	14	2	0.900	0.552	0.123		grade 2 vs grade 1	-0,2561	2,343	> 0.05
Size												
< 1.5 cm	27	4	32	9	1.185	0.681	0.131	No°	P = 0.7478			
> 1.5 cm	52	3	16	5	1.231	0.546	0.075					
Lymph node metastasis												
Negative	55	4	32	19	1.273	0.591	0.079	No°	P = 0.1929			
Positive (N+)	24	3	16	5	1.083	0.583	0.119					
Staging										Mean Difference	P value	
I	35	4	18	13	1.257	0.657	0.111		stage 4 vs stage 1	-0.1460	>0.05	
II	20	0	15	5	1.250	0.444	0.099	No*	stage 4 vs stage 2	-0.1389	>0.05	
III	6	1	3	2	1.167	0.752	0.307		stage 4 vs stage 3	-0.05556	>0.05	
IV	18	2	12	4	1.111	0.583	0.137		stage 3 vs stage 1	-0.09048	>0.05	
									stage 3 vs stage 2	-0.08333	>0.05	
									stage 2 vs stage 1	-0.007143	>0.05	
Recidive												
Yes	17	2	13	2	1.000	0.500	0.121	No°	P = 0.1424			
No	62	5	35	22	1.242	0.619	0.078					

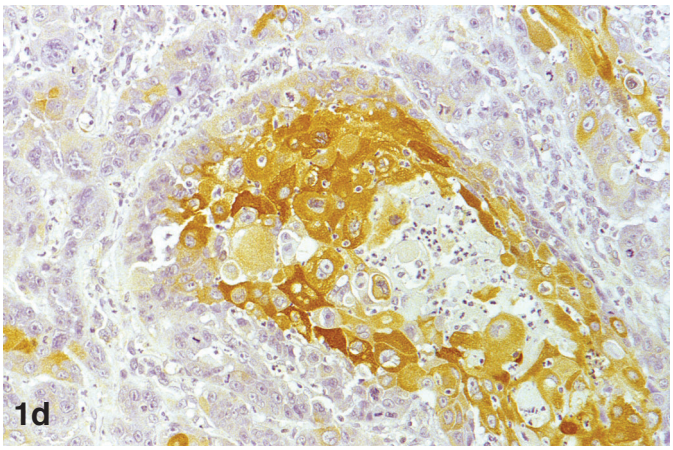
°:Student-Newmann-Keuls' test. *:One-way Analysis of Variance (ANOVA) and Student-Newman-Keuls Multiple Comparisons Test.

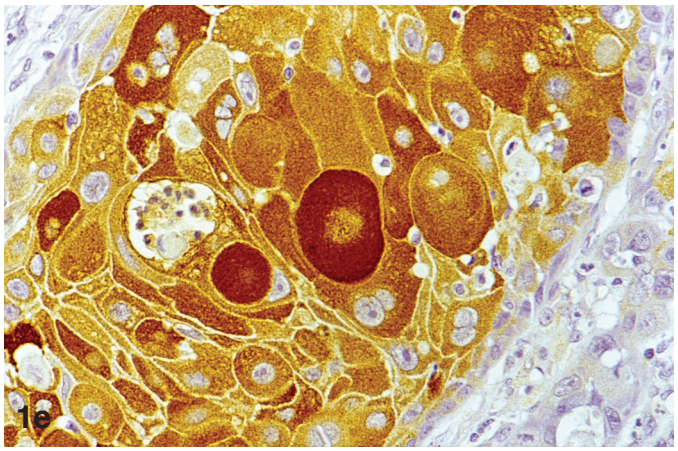
Fig. 1.a. Normal pattern of staining for HSP27 in oral epithelium, with a cytoplasmatic membrane localization in suprabasal layers (LSAB-AP, x 160). **b.** Normal pattern of staining for HSP27 in oral epithelium at higher magnification in intermediate-superficial layers (LSAB-AP, x 250). **c.** OSCC 1 showing intense cytoplasmatic signal for HSP27 (LSAB-AP, x 400). **d.** A mosaic pattern with positive areas and negative areas HSP27 immunolabelling being down-regulated in poorly-differentiated areas and up-regulated in highly-differentiated ones. (LSAB-AP, x 250). **e.** Higher magnification: a mosaic pattern with positive areas and negative areas HSP27 immunolabelling being down-regulated in poorly-differentiated areas and up-regulated in highly-differentiated ones (LSAB-AP, x 400). **f.** Complete loss of immunostaining for HSP27 in a case of G2 OSCC, with sparse horn pearls exhibiting cytoplasmatic localization of the signal (LSAB-AP, x 160). **g.** Complete loss of immunostaining for HSP27 in a case of G2 OSCC, with sparse horn pearls exhibiting cytoplasmatic localization of the signal (LSAB-AP, x 160). **h.** Higher magnification: complete loss of immunostaining for HSP27 in a case of G2 OSCC, with sparse horn pearls exhibiting cytoplasmatic localization of the signal and intense lymphoid infiltration (LSAB-HRP, x 250)

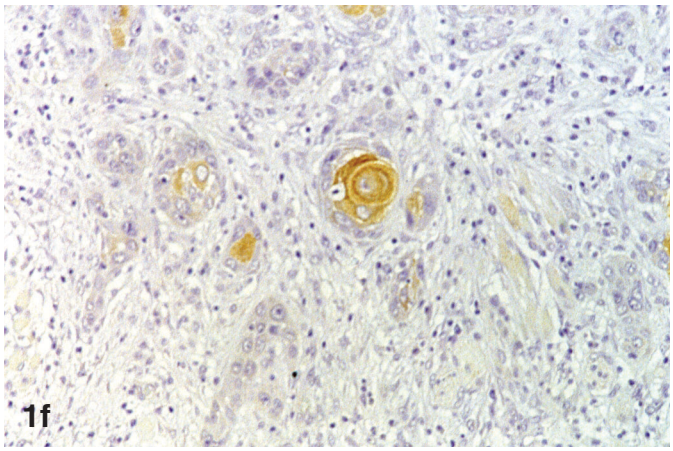

1a

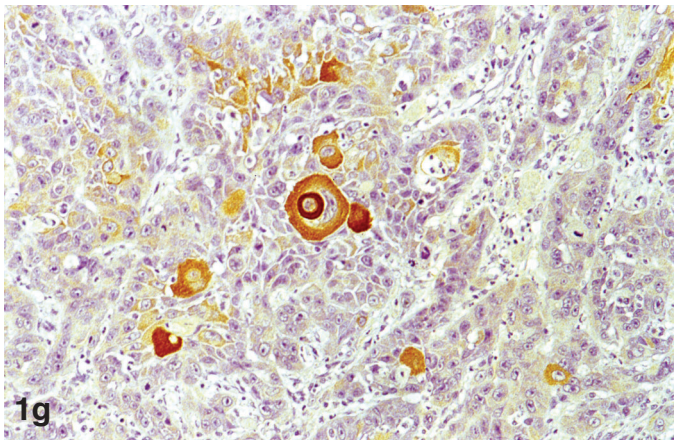

1b

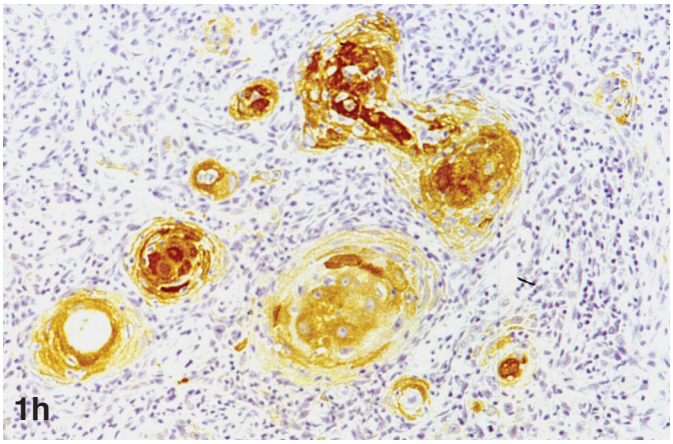

1c


1d


1e


1f


1g


1h

and were evaluated as reduced (±) and in 24 cases (30.7%), the positive tumor cells were >80% and were evaluated as positive (+). In ± cases the pattern of staining was not always homogeneous (diffuse decrease in HSP27 expression); some cases, in fact, showed a mosaic pattern (alternation of positive and negative areas). So, in OSCC both low and high HSP27 levels of expression could be appreciated HSP27 immunolabelling being down-regulated in poorly differentiated areas and up-regulated in highly differentiated ones.

All cases of metastasis showed HSP27 positivity. The frequency of lymph node metastases was higher in HSP27-negative tumours (3/7, 42.8%) than in HSP-reduced (16/48, 33.3%) or positive (5/26, 19.2%).

Western blot and image analysis

HSP27 protein was expressed either in normal mucosa or in cancerous samples to various extents. Normal mucosa show lowed HSP27 expression, while well differentiated oral carcinomas showed an HSP27 over-expression. Moderately- or poorly-differentiated carcinomas had a reduced HSP27 expression (Fig. 2)

Densitometric analysis revealed relatively arbitrary levels of expression which were, respectively: 5 and 23 in normal mucosa, 80 and 53 in well-differentiated carcinomas, 28 and 17 in moderately-differentiated carcinomas, and 3 in poorly-differentiated carcinoma. HSP27 expression levels were relative to actin expression.

Statistical analysis

There was no statistically significant correlation between HSP27 expression, sex and tumour size, staging or the presence of lymph node metastases (Table 1), even if tumour without lymph node metastases had score higher for HSP27 than tumour with metastases. Regarding staging, low stages had a higher score than high stages (stage I > stage II > stage III > stage IV). Patients > 65 years had a higher score for HSP27. HSP27 expression was reduced in poorly-differentiated tumours, and these differences were statistically significant ($p < 0.05$). When analysed for prognostic significance, patients with negative/reduced HSP27 expression had poorer survival rates than the group with positive HSP27 expression (Fig. 3). This difference in survival rates was statistically significant ($p < 0.05$).

Discussion

Among the several HSP proteins, HSP 27 and HSP 70 have been shown to have a strong association with cancer, showing, during carcinogenesis, alteration of their expression levels, either increasing or decreasing (Ferrarini et al., 1992).

Although HSP expression has been recognized as a possible prognostic factor in some tumors, the data are limited and often contradictory. Some studies reported the reduced expression of HSP27 as a negative prognostic factor in esophageal carcinoma (Kawanishi et al., 1999), while others related its over-expression to poor prognosis in astrocytoma (Hitotsumatsu et al., 1996) and gastric carcinoma (Ciocca et al., 1993b) or to increased tumor drug resistance in breast carcinoma (Fuqua et al., 1994). Other studies reported no significance of HSP27 expression in carcinomas of prostate and bladder (Storm et al., 1993), and breast (Tetu et al., 1995).

HSP27 expression has been found absent in skin cancers, particularly in poorly-differentiated areas, while it has been shown in the supra-basal layers of normal skin, and increased in all epithelial layers in inflammatory skin lesions (Gandour-Edwards et al., 1994; Trautinger et al., 1995b). HSP27 seems to play an important role in keratinocytic tumorogenesis, by delaying tumor development, as shown by experimental studies (Kindas-Mugge and Trautinger., 1994). The data concerning esophageal squamous cell carcinoma are contradictory; one study correlated the over-expression

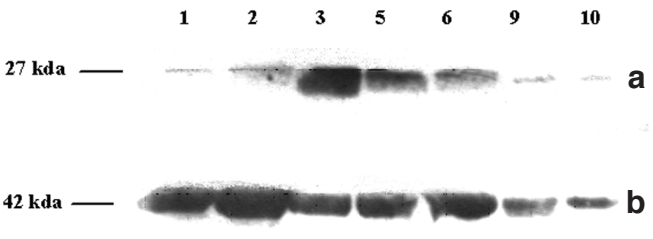


Fig. 2. a. Expression levels of HSP27 in normal mucosa, lanes 1-2, and in oral carcinomas, lanes 3-10. Lanes 3, 5: well-differentiated carcinomas; lanes 6, 9: moderately-differentiated carcinomas; lane 10: poorly-differentiated carcinoma. **b.** Expression of actin analysed as control for sample loading, protein integrity and to normalize densitometric analysis of HSP27 expression.

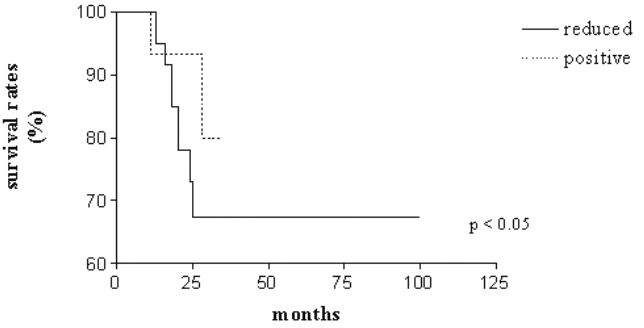


Fig. 3. Survival analysis is computed by comparing negative (-)/reduced (±) and positive (+) carcinomas for HSP27 staining. Patients with negative/reduced score had poorer survival rates than patients with positive staining. This difference in survival rates is statistically significant ($p<0.05$).

of HSP27 to poor histological differentiation (Lambot et al., 2000), while other recent data suggested that the expression of HSP27 is reduced in this carcinoma and that this reduction is associated with poor postoperative survival (Kawanishi et al., 1999). Probably the discrepancy between these findings was due to a different study population in the two studies: the first study examined carcinoma occurring in smokers and/or alcohol abusers, so these high levels of HSP27 can be explained by the stress response of esophageal mucosa to these irritant agents (Lambot et al., 2000).

A study on cancer of the tongue showed a positivity for HSP27 in several cases (18/24) with no correlation between HSP27 staining and survival, stage, lymph node metastasis, histological grade or p53 immunostaining (Ito et al., 1998). Similar data were obtained by another study on head-neck carcinoma with HSP27 positivity in 86% of cases (43/50) without prognostic significance for 5-year survival (Gandour-Edwards et al., 1998).

Other studies examined HSP27 expression in oral carcinomas (Fortin et al., 2001; Leonardi et al., 2002; Mese et al., 2002). Fortin examined 81 patients with oral cavity and oropharyngeal carcinomas. 23% of HSP-27-positive patients had neck failure compared with 51% of HSP27-negative ones (p= 0.02). In a Cox model, HSP27 (p=0.05) was associated with neck failure and HSP27 expression may play a role in predicting nodal failure(Fortin et al., 2001). Recently Mese et al. (2002) examined 40 specimens of oral squamous cell carcinoma for HSP27 expression by immunohisto-chemistry and correlated with clinical stage, lymph node metastasis, histological grade and survival period. HSP27 staining was positive (+) in 20 (50%), weak or negative (-) in 20 (50%). There was no correlation between HSP27 expression and clinical stage, lymph node metastasis and histological grade. However, when compared with clinicopathological features, the expression of HSP27 correlated inversely with survival period. This study suggests that the expression of HSP27 is frequently promoted in patients with oral squamous cell carcinoma and should be considered an independent prognostic factor of OSCC. In our early study we showed no (5 cases, 29%) or low (8 cases, 46.4%) HSP27 staining in oral premalignant lesions (Leonardi et al., 2002), while in OSCC both low and high HSP27 levels of expression were observed. HSP27 immunolabelling was down-regulated in poorly differentiated areas and up-regulated in highly differentiated ones. These findings indicated that HSP27 expression seems to protect cells from apoptosis during inflammation, while the down-regulation in dysplasia could impair the protective mechanism against mutagenesis induced by environmental factors and thus enhancing the transformation of oral epithelial dysplasia into OSCCs (Leonardi et al., 2002). A study on OSCC lines showed that recombinant clones overexpressing Hsp27 were more resistant to IFN-gamma-induced cell death than parent cells. Conversely, cells expressing a dominant-negative mutant of Hsp27, in which three serine residues (15, 78 and 82) were replaced by glycine, were hypersensitive to the effects of IFN-gamma and exhibited a typical apoptotic phenotype (Yonekura et al., 2003). These data suggest that IFN-gamma suppresses Hsp27 expression in oral SCC cells and blocks the inhibitory effects of this molecular chaperone on apoptotic cell death (Yonekura et al., 2003). Hsp27 plays a crucial role in the inhibition of apoptosis of oral SCC cells and Hsp27 plays a significant role in the IFN-gamma-induced sensitization of oral SCC cells to anticancer drugs (Yonekura et al., 2003).

In this study the pattern of HSP27 protein expression in oral mucosa, using immunohistochemistry, revealed intense expression restricted to the suprabasal layer. HSP27 was not present in the mitotic basal layer. Similar data have been described in tongue epithelium (Ito et al., 1998) and in the esophageal stratified squamous epithelium (Soldes et al., 1999). This pattern of staining suggests that the level of HSP27 may increase both with the differentiation of oral epithelium or following exposure to damaging agents. In epithelium of other sites, such as cervical epithelium, in normal epidermis and in skin neoplasms, HSP27 expression correlates to the degree of cellular differentiation (Gandour-Edwards et al., 1994; Trautinger et al., 1995c). Recent evidence suggested that HSP27 is involved in the regulation of epidermal differentiation (Jantschitsch et al., 1998) and its absence in the upper epidermal layers may be a marker for epidermal malignancy (Trautinger et al., 1995b).

Multiple studies have demonstrated the protective effect of HSP27 protein against a wide variety of cytotoxic stresses (Mehlen et al., 1995; Richards et al., 1996a). Therefore, as has been suggested for esophageal stratified squamous epithelium (Soldes et al., 1999), the abundance of HSP27 in the oral mucosa may represent one of the cellular mechanisms by which the oral mucosa is protected against certain damaging agents.

The highest levels of HSP27 expression, with an increase of immunoreaction products especially in the basal layer, have been reported in some inflammatory skin lesions (Gandour-Edwards et al., 1994; Trautinger et al., 1995a), in esophagitis (Lambot et al., 2000) and during wound healing (Laplante et al., 1998). Thus it is reasonable to assume that HSP27 over-expression may serve a protective function against the inflammatory-stressful environment. In fact HSP27 protein has been demonstrated to protect against a variety of environmental stresses including heat shock, oxidative injury, heavy metals, and tumor necrosis factor-alfa (TNF-alfa) (Mehlen et al., 1995; Richards et al., 1996b; Wu and Welsh, 1996).

Moreover, HSP27 expression has been correlated to malignant transformation of preneoplastic or potentially malignant states in many body sites (Trautinger et al., 1995c; Soldes et al., 1999; Lambot et al., 2000), even though there are differences in the degree of HSP27 expression between cell types and tissues (Ciocca et al., 1993a; Dunn et al., 1993).

OSCCs showed positive levels of HSP27 staining, especially in the highly differentiated area. This result is consistent with in "vitro" findings that HSP27 gene transfection within epidermal squamous cell lines resulted in a lower proliferation rate (Kindas-Mugge et al., 1996). The less differentiated area showed a reduced expression of HSP27 or an absence of immunopositivity.

Our findings indicated that HSP27 expression is altered in OSCC, where both low and high HSP27 levels of expression could be appreciated, HSP27 immunolabelling being down-regulated in poorly-differentiated areas and up-regulated in highly-differentiated ones. Various staining patterns were observed for HSP 27 expression in OSCCs. 55 cases (70%) showed a reduction in HSP27 expression, while 24 cases (30%) showed no reduction. In reduced cases the pattern was not always homogeneous with a diffuse decrease in HSP27 expression, but some cases showed a mosaic pattern with positive areas and negative areas. There was a statistically significant positive correlation between HSP27 expression and patient survival. When analysed for prognostic significance, patients with negative/reduced HSP27 expression had poorer survival rates than the group with positive HSP27 expression and this difference was statistically significant ($p<0.05$).

Western blot analysis, though performed in few samples, confirmed the immunohistochemical results.

Patients with > 65 years showed higher score for HSP27, because in these patients well differentiated OSCC prevailed, while in < 65 years old patients low differentiated were more numerous.

The function of this protein in tumor cells is still unclear and remains controversial. Many studies have reported a probable association between HSPs and cell proliferation and the prevention of apoptosis helping cells to survive, but this event appears strange in OSCCs where the loss of HSP expression is associated with poor prognosis. Differentiation is an important cellular process that regulates the clonal expansion of the cell population. Mehelen et al. (1997) reported that over-expression of HSP27 is associated with reduced growth of stem cells, not only with differentiation (Mehlen et al., 1997). So high levels of HSP27 can be correlated with well-differentiated tumours and with slow growth, even if in some tumors high levels of HSP27 expression were correlated with growth of poorly-differentiated types and poor prognosis (Assimakopoulou et al., 1997). Probably, the correlation between reduced expression and poor survival can be explained by another HSP function. The recent discovery of HSP families on the surface of tumor cells, with an immunological consequence, has suggested their possible role as tumor antigens or tumor antigen carriers (Srivastava and Heike; Srivastava, 1994; Srivastava et al., 1994). A study of HSP expression in esophageal carcinomas showed leukocyte infiltrations in tumoral areas expressing HSP, possibly due to stimulation by some antitumor immunity system (Kawanishi et al., 1999). Thus, patients with HSP-positive tumors can be expected to have a better prognosis.

Regarding other clinopathological data there was no statistically significant correlation between HSP27 expression and sex, tumour size, staging or the presence of lymph node metastases, even if tumour without lymph node metastases had scored higher for HSP27 than tumour with metastases. Frequency of lymph node metastases was higher in HSP27-negative tumours (3/7, 42.8%) than in HSP-reduced (16/48, 33.3%) or positive ones (5/26, 19.2%). With regard to staging, low stages had higher scores than high stages (stage I > stage II > stage III > stage IV). Conversely, the correlation between HSP27 expression and grade was statistically significant ($p<0.05$), showing reduced expression in poorly-differentiated tumours and normal expression in well-differentiated tumours.

So it seems that there is a relationship between HSP27 expression and some clinical-histological findings of oral cancer (grade and survival). Clearly these results support the need for a larger study to verify the possible role of HSP27 as a prognostic factor able to identify patients at risk of more aggressive and disseminated disease. This may be potentially relevant for implementation of closer follow-up protocols and/or alternative therapeutic regimens.

In conclusion, these data suggest that HSP27 expression may be useful for the identification of cases of oral squamous cell carcinoma with more aggressive and invasive phenotype. Cases with reduced expression were more aggressive and poorly differentiated, so HSP27 expression seems to provide novel diagnostic and prognostic information on individual patient survival with oral cancers.

Acknowledgements. This investigation was supported by the Italian Programma di Ricerca Scientifica di Rilevante Interesse Nazionale – anno 2002 from the MIUR and the Progetto di Ateneo - anno 2003 from the University of Ancona, Italy.

References

Assimakopoulou M., Sotiropoulou-Bonikou G., Maraziotis T. and Varakis I. (1997). Prognostic significance of Hsp-27 in astrocytic brain tumors: an immunohistochemical study. Anticancer Res. 17, 2677-82.

Blackburn R.V., Galoforo S.S., Berns C.M., Armour E.P., McEachern D., Corry P.M. and Lee Y.J. (1997). Comparison of tumor growth between hsp25- and hsp27-transfected murine L929 cells in nude mice. Int. J. Cancer 72, 871-877.

Ciocca D.R., Oesterreich S., Chamness G.C., McGuire W.L. and Fuqua S.A. (1993a). Biological and clinical implications of heat shock protein 27,000 (Hsp27): a review. J. Natl. Cancer Inst. 85, 1558-1570.

Ciocca D.R., Oesterreich S., Chamness G.C., McGuire W.L. and Fuqua S.A. (1993b). Biological and clinical implications of heat shock protein 27,000 (Hsp27): a review. J. Natl. Cancer Inst. 85, 1558-1570.

Crook N.E., Clem R.J. and Miller L.K. (1993). An apoptosis-inhibiting

baculovirus gene with a zinc finger-like motif. J. Virol. 67, 2168-2174.

Dunn D.K., Whelan R.D., Hill B. and King R.J. (1993). Relationship of HSP27 and oestrogen receptor in hormone sensitive and insensitive cell lines. J. Steroid Biochem. Mol. Biol. 46, 469-479.

Ferrarini M., Heltai S., Zocchi M.R. and Rugarli C. (1992). Unusual expression and localization of heat-shock proteins in human tumor cells. Int. J. Cancer 51, 613-619.

Fortin A., Raybaud-Diogene H., Tetu B., Huot J., Blondeau L. and Landry J. (2001). Markers of neck failure in oral cavity and oropharyngeal carcinomas treated with radiotherapy. Head Neck 23, 87-93.

Frydman J., Nimmesgern E., Ohtsuka K. and Hartl F.U. (1994). Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. Nature 370, 111-117.

Fuqua S.A., Oesterreich S., Hilsenbeck S.G., Von Hoff D.D., Eckardt J. and Osborne C.K. (1994). Heat shock proteins and drug resistance. Breast Cancer Res. Treat. 32, 67-71.

Gandour-Edwards R., McClaren M. and Isseroff R.R. (1994). Immunolocalization of low-molecular-weight stress protein HSP 27 in normal skin and common cutaneous lesions. Am. J. Dermatopathol. 16, 504-509.

Gandour-Edwards R., Trock B.J., Gumerlock P. and Donald P.J. (1998). Heat shock protein and p53 expression in head and neck squamous cell carcinoma. Otolaryngol. Head Neck Surg. 118, 610-615.

Garrido C., Fromentin A., Bonnotte B., Favre N., Moutet M., Arrigo A.P., Mehlen P. and Solary E. (1998). Heat shock protein 27 enhances the tumorigenicity of immunogenic rat colon carcinoma cell clones. Cancer Res. 58, 5495-5499.

Geisler J.P., Geisler H.E., Tammela J., Miller G.A., Wiemann M.C. and Zhou Z. (1999). A study of heat shock protein 27 in endometrial carcinoma. Gynecol. Oncol. 72, 347-350.

Hitotsumatsu T., Iwaki T., Fukui M. and Tateishi J. (1996). Distinctive immunohistochemical profiles of small heat shock proteins (heat shock protein 27 and alpha B-crystallin) in human brain tumors. Cancer 77, 352-361.

Hsu P.L. and Hsu S.M. (1998). Abundance of heat shock proteins (hsp89, hsp60, and hsp27) in malignant cells of Hodgkin's disease. Cancer Res. 58, 5507-5513.

Ito T., Kawabe R., Kurasono Y., Hara M., Kitamura H., Fujita K. and Kanisawa M. (1998). Expression of heat shock proteins in squamous cell carcinoma of the tongue: an immunohistochemical study. J. Oral Pathol. Med. 27, 18-22.

Jaattela M. (1999). Escaping cell death: survival proteins in cancer. Exp Cell Res. 248, 30-43.

Jameson J.L. (1998). Principles of molecular medicine. Humana Press. Totowa, New Jersey.

Jantschitsch C., Kindas-Mugge I., Metze D., Amann G., Micksche M. and Trautinger F. (1998). Expression of the small heat shock protein HSP 27 in developing human skin. Br. J. Dermatol. 139, 247-253.

Kawanishi K., Shiozaki H., Doki Y., Sakita I., Inoue M., Yano M., Tsujinaka T., Shamma A. and Monden M. (1999). Prognostic significance of heat shock proteins 27 and 70 in patients with squamous cell carcinoma of the esophagus. Cancer 85, 1649-1657.

Kindas-Mugge I., Micksche M. and Trautinger F. (1998). Modification of growth in small heat shock (hsp27) gene transfected breast carcinoma. Anticancer Res. 18, 413-417.

Kindas-Mugge I., Herbacek I., Jantschitsch C., Micksche M. and Trautinger F. (1996). Modification of growth and tumorigenicity in epidermal cell lines by DNA-mediated gene transfer of M(r) 27,000 heat shock protein (hsp27). Cell. Growth Differ. 7, 1167-1174.

Kindas-Mugge I. and Trautinger F. (1994). Increased expression of the M(r) 27,000 heat shock protein (hsp27) in in vitro differentiated normal human keratinocytes. Cell Growth Differ. 5, 777-781.

King K.L., Li A.F., Chau G.Y., Chi C.W., Wu C.W., Huang C.L. and Lui W.Y. (2000). Prognostic significance of heat shock protein-27 expression in hepatocellular carcinoma and its relation to histologic grading and survival. Cancer 88, 2464-2470.

La Thangue N.B. and Latchman D.S. (1988). A cellular protein related to heat-shock protein 90 accumulates during herpes simplex virus infection and is overexpressed in transformed cells. Exp. Cell Res. 178, 169-1679.

Lambot M.A., Peny M.O., Fayt I., Haot J. and Noel J.C. (2000). Overexpression of 27-kDa heat shock protein relates to poor histological differentiation in human oesophageal squamous cell carcinoma. Histopathology 36, 326-330.

Langdon S.P., Rabiasz G.J., Hirst G.L., King R.J., Hawkins R.A., Smyth J.F. and Miller W.R. (1995). Expression of the heat shock protein HSP27 in human ovarian cancer. Clin. Cancer Res. 1, 1603-1609.

Laplante A.F., Moulin V., Auger F.A., Landry J., Li H., Morrow G., Tanguay R.M. and Germain L. (1998). Expression of heat shock proteins in mouse skin during wound healing. J. Histochem. Cytochem. 46, 1291-1301.

Lavoie J.N., Gingras-Breton G., Tanguay R.M. and Landry J. (1993). Induction of Chinese hamster HSP27 gene expression in mouse cells confers resistance to heat shock. HSP27 stabilization of the microfilament organization. J. Biol. Chem. 268, 3420-3429.

Lemieux P., Oesterreich S., Lawrence J.A., Steeg P.S., Hilsenbeck S.G., Harvey J.M. and Fuqua S.A. (1997). The small heat shock protein hsp27 increases invasiveness but decreases motility of breast cancer cells. Invasion Metastasis 17, 113-123.

Leonardi R., Pannone G., Magro G., Kudo Y., Takata T. and Lo Muzio L. (2002). Differential expression of heat shock protein 27 in normal oral mucosa, oral epithelial dysplasia and squamous cell carcinoma. Oncol. Rep. 9, 261-266.

Lindquist S. and Craig E.A. (1988). The heat-shock proteins. Annu. Rev. Genet. 22, 631-767.

Mehlen P., Preville X., Chareyron P., Briolay J., Klemenz R. and Arrigo A.P. (1995). Constitutive expression of human hsp27, Drosophila hsp27, or human alpha B-crystallin confers resistance to TNF-α and oxidative stress-induced cytotoxicity in stably transfected murine L929 fibroblasts. J. Immunol. 154, 363-374.

Mehlen P., Mehlen A., Godet J. and Arrigo A.P. (1997). hsp27 as a switch between differentiation and apoptosis in murine embryonic stem cells. J. Biol. Chem. 272, 31657-31665.

Mese H., Sasaki A., Nakayama S., Yoshioka N., Yoshihama Y., Kishimoto K. and Matsumura T. (2002). Prognostic significance of heat shock protein 27 (HSP27) in patients with oral squamous cell carcinoma. Oncol Rep. 9, 341-344.

Multhoff G., Botzler C., Jennen L., Schmidt J., Ellwart J. and Issels R. (1997). Heat shock protein 72 on tumor cells: a recognition structure for natural killer cells. J. Immunol. 158, 4341-4350.

Reed J.C. (1998). Bcl-2 family proteins. Oncogene 17, 3225-3236.

Richards E.H., Hickey E., Weber L. and Master J.R. (1996a). Effect of overexpression of the small heat shock protein HSP27 on the heat and drug sensitivities of human testis tumor cells. Cancer Res. 56, 2446-2451.

Richards E.H., Hickey E., Weber L. and Master J.R. (1996b). Effect of

overexpression of the small heat shock protein HSP27 on the heat and drug sensitivities of human testis tumor cells. Cancer Res. 56, 2446-2451.

Sobin L.H. and Wittekind C.H. (1997). UICC: TNM classification of malignant tumours. Wiley-Liss Inc. New York. ed. 5.

Soldes O.S., Kuick R.D., Thompson I.A., 2nd, Hughes S.J., Orringer M.B., Iannettoni M.D., Hanash S.M. and Beer D.G. (1999). Differential expression of Hsp27 in normal oesophagus, Barrett's metaplasia and oesophageal adenocarcinomas. Br. J. Cancer 79, 595-603.

Srivastava P.K. (1994). Heat shock proteins in immune response to cancer: the Fourth Paradigm. Experientia 50, 1054-1060.

Srivastava P.K. and Heike M. (1991). Tumor-specific immunogenicity of stress-induced proteins: convergence of two evolutionary pathways of antigen presentation? Semin. Immunol. 3, 57-64.

Srivastava P.K., Udono H., Blachere N.E. and Li Z. (1994). Heat shock proteins transfer peptides during antigen processing and CTL priming. Immunogenetic 39, 93-98.

Sternberg S.S. (1997). Histology for pathologist. Lippincott-Raven Publishers. Philadelphia.

Storm F.K., Mahvi D.M. and Gilchrist K.W. (1993). Hsp-27 has no diagnostic or prognostic significance in prostate or bladder cancers. Urology 42, 379-382.

Storm F.K., Mahvi D.M. and Gilchrist K.W. (1996). Heat shock protein 27 overexpression in breast cancer lymph node metastasis. Ann. Surg. Oncol. 3, 570-573.

Tetu B., Brisson J., Landry J. and Huot J. (1995). Prognostic significance of heat-shock protein-27 in node-positive breast carcinoma: an immunohistochemical study. Breast Cancer Res. Treat. 36, 93-97.

Trautinger F., Kindas-Mugge I., Barlan B., Neuner P. and Knobler R.M. (1995a). 72-kD heat shock protein is a mediator of resistance to ultraviolet B light. J. Invest. Dermatol. 105, 160-162.

Trautinger F., Kindas-Mugge I., Dekrout B., Knobler R.M. and Metze D. (1995b). Expression of the 27-kDa heat shock protein in human epidermis and in epidermal neoplasms: an immunohistological study. Br. J. Dermatol. 133, 194-202.

Trautinger F., Kindas-Mugge I., Dekrout B., Knobler R.M. and Metze D. (1995c). Expression of the 27-kDa heat shock protein in human epidermis and in epidermal neoplasms: an immunohistological study. Br. J. Dermatol. 133, 194-202.

Vaux D.L., Haecker G. and Strasser A. (1994). An evolutionary perspective on apoptosis. Cell 76, 777-779.

Vegh G.L., Fulop V., Liu Y., Ng S.W., Tuncer Z.S., Genest D.R., Paldi-Haris P., Foldi J., Mok S.C. and Berkowitz R.S. (1999). Differential gene expression pattern between normal human trophoblast and choriocarcinoma cell lines: downregulation of heat shock protein-27 in choriocarcinoma in vitro and in vivo. Gynecol. Oncol. 75, 391-396.

Wu W. and Welsh M.J. (1996). Expression of the 25-kDa heat-shock protein (HSP27) correlates with resistance to the toxicity of cadmium chloride, mercuric chloride, cis-platinum(II)-diammine dichloride, or sodium arsenite in mouse embryonic stem cells transfected with sense or antisense HSP27 cDNA. Toxicol. Appl. Pharmacol. 141, 330-339.

Yonekura N., Yokota S., Yonekura K., Dehari H., Arata S., Kohama G. and Fujii N. (2003). Interferon-gamma downregulates Hsp27 expression and suppresses the negative regulation of cell death in oral squamous cell carcinoma lines. Cell. Death Differ. 10, 3133-3122.

Accepted October 10, 2003