Prognostic Value of HSP27 in Head and Neck Squamous Cell Carcinoma: a Retrospective Analysis of 57 Tumours

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Abstract. Background: The aims of the present study were to assess the prevalence of HSP27 expression in oral squamous cell carcinoma (OSCC) and to verify whether HSP27 can be considered to be a marker of prognosis in patients with OSCC. Materials and Methods: The immunohistochemical expression of HSP27 was evaluated in 57 OSCC, who received standard treatment and monitoring. After grouping for HSP27 expression, OSCCs were statistically analysed for the variables age, gender, histological grading, TNM, staging and survival rate. Univariate and multivariate analyses were performed. Results: HSP27 was found to be reduced in 31 OSCC and was normally expressed in 26 OSCC. The pattern and intensity of HSP27 immunolabelling did not show significant differences in relation to any variables retrospectively considered. In terms of prognostic significance, HSP27 reduced expression was found to have an independent association with the poorest overall survival rate (p=0.009; OR= 4.404; CI=1.444:13.427 by Cox regression). Conclusion: HSP27 reduced expression is an early marker of poor prognosis, useful in identifying aggressive biological behaviour in OSCC cases even before relapse.

Heat shock proteins (HSPs) are strongly implicated in the control of cell growth, differentiation and biological behaviour of many human neoplasms (1-5). In fact, HSPs mediate and modulate a wide range of intracellular activities which fulfil protective functions (6, 7), ensure metabolic homeostasis (8, 9) and can participate in several pathogenic processes (10,11). On the basis of their molecular weights, HSPs have been grouped into different sub-families: small (HSP 20-30 KDa), HSP40, HSP70, HSP90 and HSP100; some are cellular proteins normally expressed (12, 13), whereas others are chaperonins of nascent proteins (14).

In oncology, HSPs have been implicated in multidrug resistance (15), in regulation of apoptosis (16) and as modulators of p53 function. In particular, HSP27 is a small HSP abundantly expressed in many tumour cells (17). HSP27 is a cytoplasmic protein that is constitutively present in a broad range of normal tissues and neoplasms. It is normally expressed at low levels in the cytosol of most human cells and has been shown to interact with the actin cytoskeleton, to modulate intracellular reactive oxygen species content and to prevent apoptotic cell death triggered by a variety of stimuli, including tumour necrosis factor alpha (TNF-α) (18) and several commonly used anticancer drugs, such as etoposide (19, 20). Regarding the prognostic value, previous studies by Thor et al. (21), Chamness et al. (22) and Tandon et al. (23) reported that HSP27 overexpression is correlated with reduced overall survival of patients with breast cancer. Later, this conclusion was amended by Conroy et al. (24), who showed that production of anti-HSP27 auto-antibodies also correlated with improved survival in breast cancer patients. But, until now, there is no absolute concordance about the significance of its behaviour and tumoral expression, especially in head and neck squamous cell carcinoma (HNSCC) (25-29). The
majority of findings suggest that HSP27 expression protects cells from apoptosis during inflammation, while the down-regulation in dysplasia could impair the protective mechanism against mutagenesis induced by environmental factors, thus enhancing the transformation of oral epithelial dysplasia into HNSCC.

Since 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, the aims of the present study were to assess the prevalence of HSP27 expression in oral squamous cell carcinoma (OSCC) and to verify whether HSP27 can be considered to be a marker of prognosis in OSCC patients.

Materials and Methods

Fifty-seven patients, affected by histologically-proven OSCC, were consecutively recruited among those surgically treated in a multicentric study between January 1994 and May 2000. The patients included 37 males (64.9%) and 20 females (35.1%). They ranged in age from 18 to 87 years (median age 66 years) at the time of admission; 11 (19.3%) had neck nodes, and none had evidence of distant metastasis. The tumours were classified according to the U.I.C.C. classification (30), including the following Stage grouping: Stage I for 30 OSCC, II for 11, III for 8 and IV for 8. Although recruited in different centres, all OSCCs were treated according to the common and current dedicated guidelines (31). In particular, surgery on T was the treatment of choice and was always performed at the initial course of the protocol with curative intent (i.e., only tumour resections in safe margins were done). When radiotherapy was considered useful, it was usually done 3 weeks after surgery, with external beam and a dose equivalent to 60 or 65 Gy in 6 or 7 weeks. Chemotherapy, when prescribed, consisted of cisplatin (100 mg/m² body-surface area) given as intravenous infusion followed by continuous 24-h intravenous infusion of fluorouracil (1,000 mg/m² per day) for 5 days, globally up to 3 cycles. All patients were followed-up and examined on a monthly basis for the first year after treatment, every 2 months for the second year, and every 3 months thereafter. The follow-up was 32.5 (0.33-72) months. At our baseline, an overall disease-specific survival was calculated to be 72 months for all patients plus cases censored (for death).

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. Deparaffinized and rehydrated sections were incubated for 30 min in 3% H₂O₂/methanol to quench endogenous peroxidase activity, and were then rinsed for 20 min with phosphate-buffered saline (PBS) (Bio-Optica M107, Milan Italy). Non-specific protein binding was attenuated by incubation for 30 min with 5% horse serum in PBS. Specimens were incubated overnight with the primary anti-HSP27 monoclonal antibody (Catalogue No. NCL-HSP27; Novocastra Laboratories Ltd., Newcastle, UK) at a dilution of 1:20. The antibody was applied directly to the section, and the slides were incubated overnight (4°C) in a "humified chamber". The sections were washed 3 times with PBS at room temperature. Immune complexes were subsequently treated with the secondary biotinylated antibody and then detected by streptavidin peroxidase, both incubated for 30 min 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 min with 0.1% 3,3’-diaminobenzidine and 0.02% hydrogen peroxide (DAB Substrate Kit, Vector Laboratories). 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 HSP27 expression, 300 cells were examined in at least 5 areas at ×400 magnification, and a mean percentage of positive tumour cells was determined, assigning cases to one of the 3 following categories: a) negative (−), when the frequency of stained cells was <5%; b) reduced, when the stained cells accounted for <80% of positive tumour cells; b) positive, when the stained cells were >80% of the positive tumour cells (32).

Statistical analysis.

a) Univariate analysis. Differences between HSP27 expression and the variables considered were analysed by means of Student-Newmann-Keuls’ test (simple or in multiple comparison) and by ANOVA. The difference was considered significant when the p-value was ≤0.05.

Disease-specific survival curves were calculated according to the product-limit method (Kaplan-Meier algorithm). Time zero was defined as the date of the patient’s initial diagnosis. Patients who were still alive were included in the total number at risk of death only up to the time of their last follow-up. Therefore, the survival rate only changed when death occurred. Patients lost during the follow-up period (i.e., 60 months) were considered as censored. The calculated survival rate was the maximum estimate of the true survival curve. The log rank test was used to compare survival curves, generated by stratifications for a variable of interest.

b) Cox regression analysis. The Cox regression analysis was applied to determine the single contribution of covariates on survival rate. The Cox regression analysis compares survival data while taking into account the statistical value of independent variables, such as age and sex, on whether or not an event (i.e., mortality) is likely to occur. If the associated probability was less then 5% (p<0.05), the difference was considered statistically significant. In the process of doing the regression analysis, odds ratio (OR) and 95% confidence interval (CI) were calculated. Stepwise Cox analysis allowed for the detection of those variables most associated with survival.

Results

Thirty-one OSCC (53.4%) showed reduced expression of HSP27 (5-80% of positive tumour cells) and composed the GROUP I; 26 cases (45.6%) showed a normal HSP27 immunoreactivity (>80% of positive tumour cells) and composed the GROUP II (Figure 1) (Table I). No HSP27-negative OSCC was detected.
Worthy of note, we considered a suprabasal pattern of HSP27, as a parameter of HSP27 expression in the normal human oral mucous epithelium, with positive cells showing cytoplasmic and finely granular staining, while nuclei very rarely showed only a faint positivity. Generally, basal keratinocytes were not positive, even if a slight HSP27 expression was present in some areas. Usually, normal human oral mucous epithelium should include >80% of stained cells (25).

In most cases of the GROUP I, the pattern of HSP27 staining, although always expressed, was not always homogeneous; several cases in G3, in fact, showed a mosaic pattern (alternation of positive and negative areas).

With respect to histological grading, HSP27 immunolabelling was down-regulated in poorly-differentiated areas and up-regulated in highly-differentiated ones, but without any significant difference (p=0.118) when sub-grouping in G1 vs. G2 vs. G3.

The results of the study association after grouping cases by HSP27 expression are detailed in Table I and can be summarised to the extent that no statistical difference exists, with respect to the variables considered at baseline (age, gender, grading, stage grouping).

The second part of the analysis included the study of survival rates with respect to HSP27 expression. The global disease-specific survival rate at 60 months was 48%, irrespectively of the extent of the tumour and the treatment (Figure 2). When the survival rates were calculated distributing the cohort by HSP27, we found that GROUP I had 22% of survival vs 81% in GROUP II (p-value =0.049) (Figure 3).

Finally, among the variables considered in the multilogistic regression analysis, grading, recurrence and HSP27 (adjusted for age and gender) were predictors of outcome (Table II). Specifically, in terms of prognostic significance, HSP27 under-expression was found to have an independent association with the poorest overall survival rate (p=0.009; OR= 4.404; CI= 1.444:13.427 by Cox regression).

**Discussion**

HNSCC is the fifth most common malignancy worldwide (33). Tobacco and alcohol consumption are well-established risk factors for HNSCC. Despite many advances in treatment over the past 30 years, little progress has been made in improving survival rates. Among HNSCC, the most common tumour is the OSCC, which is often discovered in advanced stages with treatment frequently leaving patients disfigured and with debilitating adverse effects of radiations and chemotherapy, compromised speech and swallowing, and significantly diminished quality of life (34). Therefore, the prevention and any innovation that facilitates early detection of this neoplasm, have the potential to improve survival and quality of life.

<table>
<thead>
<tr>
<th>Variables</th>
<th>GROUP I</th>
<th>GROUP II</th>
<th>Statistical data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases</td>
<td>57</td>
<td>26(45.6)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 65 years</td>
<td>29</td>
<td>14</td>
<td>1.34 1.04 p=0.282*</td>
</tr>
<tr>
<td>&gt; 65 years</td>
<td>28</td>
<td>17</td>
<td>1.64 1.02</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>38</td>
<td>20</td>
<td>1.44 0.97 p=0.228*</td>
</tr>
<tr>
<td>Female</td>
<td>19</td>
<td>11</td>
<td>1.57 1.16</td>
</tr>
<tr>
<td>Staging</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>18</td>
<td>13</td>
<td>1.83 1.04 p=0.118*</td>
</tr>
<tr>
<td>G2</td>
<td>25</td>
<td>12</td>
<td>1.48 0.96</td>
</tr>
<tr>
<td>G3</td>
<td>14</td>
<td>6</td>
<td>1.07 1.07</td>
</tr>
<tr>
<td>IV</td>
<td>8</td>
<td>4</td>
<td>1.25 0.88</td>
</tr>
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</table>

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

Despite the identification and extensive study of several potential tumour markers, none has been found to have clinical utility as a diagnostic marker or screening tool for HNSCC. A number of novel molecular approaches to detect clonal genetic alterations in the blood and saliva of HNSCC patients have been developed for the early detection of this tumour. These include the detection of specific patterns of microsatellite markers (35), promoter hypermethylation (36), and mitochondrial DNA mutations in patient saliva and serum (37). Among the various HSP proteins, HSP27 and HSP70 have been shown to have a strong association with cancer, since alterations of their expression levels during carcinogenesis have been reported (38). Several mechanisms have been proposed to account for the HSP27-mediated negative regulation of programmed cell death. This small HSP specifically interacts with cytochrome c when released from the mitochondria to the cytosol, thus preventing the formation of the apoptosome. A premitochondrial effect at a higher HSP27 expression level has also been described (39). In vitro, this protein behaves as an ATP-independent chaperone that helps in the refolding of denatured proteins (40), a property that could be of key importance for understanding its in vivo functions. Parcellier et al. identified another role for HSP27, that of facilitating the activation of the ubiquitin-proteasome pathway (41, 42). One of the consequences of this effect is an increase in NF-κB activity through enhanced degradation of its main inhibitor, I-κBα.
Since the suppression of apoptosis is an important function of NF-κB, increased NF-κB activation may contribute to the anti-apoptotic effect of HSP27. After induction, the protein becomes phosphorylated while, simultaneously, translocated from the cytoplasm to within or around the nucleus (43). Phosphorylation is a key regulator of HSP27 function occurring at serine residues 78 and 82 (44) through interaction with a specific kinase, but it may be activated by several different signal transduction mechanisms. The presence of the protein contributes to cell survival after diverse stress insults (45). It has been proposed that HSP27 modulates reactive oxygen species via a glutathione-dependent pathway (46), thereby protecting intracellular proteins and explaining, in part, the protective effect of HSP27 against chemotherapeutic agents (47).

Although HSP expression has been recognised as a possible prognostic factor in some tumours, so far the data are limited and often contradictory. Some studies reported the reduced expression of HSP27 as a negative prognostic factor in oesophageal carcinoma (32), while others related its overexpression to poor diagnosis in astrocytoma (48).

Regarding OSCC, no correlation was reported between HSP27 staining and survival period, stage, lymph node metastasis or histological grade by Ito et al. (27). In addition, findings by Mese et al. (26) did not reveal any association with clinical stage and lymph node metastasis, but did show an inverse association with the survival period.

Regarding the histological grading, no statistical association was found in the present study with respect to HSP27 expression degree, but it was observed that HSP27 immunolabelling was down-regulated in poorly-differentiated areas, with a mosaic pattern in G3, and up-regulated in highly-differentiated ones. This observation has also been reported for the epithelium of other sites, such as the cervical epithelium, in normal epidermis and in skin neoplasms, where HSP27 expression was correlated to the degree of cellular differentiation (49). Mese et al., in their paper concerning OSCC, also reported a higher percentage of reduced HPS 27 expression in poorly-differentiated (58%) and moderately-differentiated (66%) OSCC than in well-differentiated OSCC (29%) (26).
The present study principally focused on the HSP27 as a possible prognostic factor for OSCC by means of Cox regression analysis. The main finding is that HSP27 expression in OSCC is related to a significantly better survival at 60 months, consistent with findings reported by Fortin et al. in oro-pharyngeal carcinomas treated with radiotherapy (28). In spite of the limited sample size, our finding suggests an important role for this protein among early prognostic tumour markers.

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