CLINICAL INVESTIGATION

STEFIN A AND STEFIN B: MARKERS FOR PROGNOSIS IN OPERABLE SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

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Purpose: The aim of this study was to test the hypothesis about the protective role of high stefin A and stefin B concentrations in operable carcinoma of the head and neck.

Methods and Materials: Stefins A and B concentrations were measured in tissue cytosols of nontumorous mucosa and primary tumors from 92 patients. For quantitative analysis of stefins in tumor cytosols, commercially available enzyme-linked immunosorbent assays were used.

Results: Stefin A was upregulated in 53 patients (higher concentrations were measured in tumor samples than in nontumorous mucosa) and was downregulated in 39 patients. The corresponding numbers for stefin B were 49 and 43, respectively. A significantly higher proportion of downregulated cases were found among patients with disease re-appearance. In the Cox model, high stefin A concentrations appeared as independent predictors for favorable disease-free survival. Assuming a “broken stick” model, a significant increase in the recurrence rate after the threshold of 1063 ng/mgp (the 64th percentile in the group) was found, the hazard ratio reaching 3% of the reference value with doubling of the level of stefin A. These results were reconfirmed after pooling the data with two historical data sets into a uniform series involving 182 patients.

Conclusions: A group of patients at high risk for disease progression was identified, characterized by the downregulated stefin A protein in the tumor compared with the nontumorous mucosa. Stefin A was recognized as a promising candidate marker for prognosis in patients with operable carcinoma of the head and neck.

STEFIN A, STEFIN B, SQUAMOUS CELL CARCINOMA, HEAD-AND-NECK CANCER, PROGNOSIS.

INTRODUCTION

To distinguish biologically more aggressive and less aggressive head-and-neck carcinomas within each traditional risk category, numerous new prognostic factors have been evaluated on genetic, mRNA, or protein levels. The recent implementation of microarray technology for biologic profiling of tumors has confirmed the multifactorial origin of carcinogenesis (1, 2). Among the factors that promote tumor growth and invasion, several protease systems, involved in proteolytic degradation of extracellular matrix components, have been studied, including papain-like lysosomal cysteine proteases such as cathepsins B and L, as well as their physiologic inhibitors cystatins, i.e., cystatin C, stefin A, and stefin B (3, 4).

Alterations in the expression of cystatins at mRNA and protein levels, as well as in trafficking and activity, have been found to correlate with the malignant phenotype of the tumor both in vitro and in vivo. Transfection of various carcinoma cells with cDNA encoding human cystatin C and the subsequent protein overproduction resulted in a suppression of their invasiveness and metastasis formation (5–7). Furthermore, silencing of cystatin M in metastatic oropharyngeal cancer cell line MDA-686Ln augments the enzymatic activities of cathepsins B and L, significantly enhances proliferation, and increases the in vitro transmigration and Matrigel invasion of these cells (8). Using the same model, overexpression of cystatin C and stefin A was found to block TNF-α and TRAIL-mediated apoptosis of tumor cells, which was hypothesized to augment their metastatic potential, most likely by inhibiting cathepsin B (9, 10).

In our previous studies on operable head-and-neck carci-

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noma, high levels of cysteine protease inhibitors in tumor tissue homogenates appeared as prognostically advantageous. Specifically, in two independent but smaller prospective cohorts of patients, this relationship was confirmed for stefin A (11), stefin B (12), and cystatin C (13). The main purpose of the present study was to test prospectively the hypothesis about the protective role of high stefin A and stefin B levels in patients with comparable clinical characteristics. Furthermore, a pooled analysis with the use of all three data sets, the present one and the two historical sets (11, 12), was performed.

METHODS AND MATERIALS

Patients and therapy

Between January 1997 and December 1998, 92 patients with operable squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, and larynx were prospectively enrolled in the study. Of the cohort, 90% were men, and the median age was 58.8 years (range, 36.5–79.9 years). All patients gave their informed consent to participate in the study.

All patients underwent curative surgery and 84 patients had postoperative radiotherapy. Details on surgical procedure, indications for postoperative irradiation and on radiotherapy technique and doses were described previously (11–13). The tumors were staged using the criteria of the International Union Against Cancer (UICC) TNM staging system (14). Postoperatively, 59 (64%) tumors were staged as locally advanced pT3 or pT4. Nodal infiltration with tumor cells was determined in 54 (59%) cases, with extracapsular tumor spread in 27 (Table 1).

Tissue extraction

For biochemical analysis of stefins, two tissue samples of 50 to 200 mg, representing matched pairs, were obtained from the tumor and the adjacent normal tissue during surgery. Immediately after removal, they were immersed in liquid nitrogen, and fat and necrotic parts of the tissue were carefully removed. Pulverization was performed on the frozen tissue with a microdismembranator (Mikro-Dismembranator, B. Braun Biotech International, Melsungen, Germany) for 60 s at maximum power. The resulting tissue powder was suspended in the extraction buffer (pH 8.5), containing 0.02 mol/L Tris-HCl, 0.125 mol/L NaCl, 2% Triton X-100, and was shaken for 3 h at 4°C. The obtained suspension was centrifuged for 30 min at 100,000 g at 4°C to obtain the supernatant fraction, i.e., cytosol, which was divided into aliquots and stored at −70°C until use. Total protein concentration in tissue cytosol was determined according to the Pierce assay.

Determination of stefin A and stefin B

Stefin A and stefin B protein concentrations in tissue cytosols were determined by enzyme-linked immunosorbent assays (Sandwich ELISAs, KRKA d.d., Novo Mesto, Slovenia), as suggested by the manufacturer. The characteristics of the assays, linearity, and precision controls were reported previously (15). The concentration of stefins was expressed in nanograms per milligram of total protein (ng/mg).

Tumor and normal tissue samples in 1:100 and 1:80 dilutions, respectively, controls, and calibrators were added to the wells of a microtiter plate that had been precoated with the corresponding capture anti-stefin antibody. After a 2-h incubation at 37°C, the wells were washed and filled with the detection anti-stefin antibody conjugated with horseradish peroxidase. The enzyme-linked complex was detected by incubation with TMB substrate solution (3,3,5,5-tetramethyl benzidine and hydrogen peroxide). The intensity of the developed color was read at 450 nm using an ELISA microtiter plate reader and was proportional to the concentration of a particular stefin in the specimen within the working range of the test. The stefin concentration was calculated from the corresponding calibration curve obtained by plotting the inhibitor concentration of the calibrators vs. the absorbance at 450 nm.

Statistical analysis

The results were analyzed using SPSS (release 11.0, SPSS Inc., Chicago, IL) and R (Release 2.2.1, R Foundation for Statistical Computing, Vienna, Austria) statistical packages. All of the tests were two-sided, and the results were considered significant at a probability level ≤5%.

Nonparametric tests (Wilcoxon signed-rank test, Mann-Whitney U test, Spearman rank correlation) were used for comparative analysis of biochemical variables and clinical and histopathologic parameters. The frequency distribution of categorical variables was tested using a Chi-square test and Fisher exact test. Univariate analysis of the patients’ survival was carried out using the Kaplan-Meier product-limit method (16) and log-rank comparison to evaluate the difference between the survival curves (17). The primary
92 samples was analyzed, no statistically significant differences between the extremes (20). Because both stefin A and stefin B seem to be associated with the hazard ratio for DFS to the stefin A and stefin B concentrations used only as a graphical evaluation of the functional form relating the hazard ratio for DFS to the stefin A and stefin B concentrations (18). Because both stefin A and stefin B seem to be associated with DFS only when exceeding a certain value, a flexible methodology for analyzing their effect was used, with the advantage of avoiding arbitrary categorization and its subsequent loss of information (19). Thus, a “broken stick” model of the form

$$\beta (V - V_0),$$

where $V$ is the measured value, $V_0$ is the cut-off value and the plus denotes that only the part where $V$ is greater than $V_0$ is used, was proposed. Both $\beta$ and $V_0$ were estimated simultaneously by maximizing the Cox partial likelihood in a model using no additional covariates (19).

In pooled analysis we reanalyzed individual patient data from the present and the two historical data sets (11, 12). Because the method of determination of stefins was improved over time and to avoid any possible bias arising from the differences between the laboratories that performed biochemical analysis, a common scale for inhibitory levels was introduced. For each data set, we ranked the stefin A and stefin B measurements and divided the ranks by the number of patients. Thus inhibitor levels were converted to fractional ranks (between 0 and 1). In this way, equal fractional ranks are comparable across data sets with different numbers of patients included, allowing an assumption that the use of different methodology for stefin determination did not influence the rank of stefin A and stefin B levels profoundly. The hazard ratio (HR) for the ranked variables represents the differences between the extremes (20).

### RESULTS

#### Concentrations of stefins in tumor and mucosa

The concentrations of stefin A and stefin B in the tissue cytosols are presented in Table 2. When the whole group of 92 samples was analyzed, no statistically significant differences in the distribution of stefin A or stefin B concentrations was observed between tumor and nontumorous tissue samples. However, after grouping the samples on the basis of the stefin A difference as calculated in matched pairs of tumor tissue and nontumorous mucosa, a 5.35-fold median increase (i.e., upregulation) in inhibitor concentration was measured in tumor samples compared with their control counterparts in a group of 53 cases (58%). In the other 39 cases (42%), a median decrease (i.e., downregulation) in stefin A concentration by a factor of 2.66 was measured. The corresponding values for stefin B were 4.39 (49 cases, 53%) and 2.79 (43 cases, 47%), respectively. The mucosal concentrations of either of the stefins were significantly higher in the patients with downregulated inhibitor concentration than in those with upregulated inhibitor (Table 2).

Between stefin A and stefin B, a highly significant correlation was found when either mucosal ($R_S = 0.887$, 95% CI, 0.834–0.926, $p < 0.0001$) or tumor ($R_S = 0.594$, 95% CI, 0.439–0.716, $p < 0.0001$) concentrations were compared. The difference between tumor and mucosal stefin A and stefin B concentrations was congruent (i.e., both either positive or negative in the same patient) in 87% of patients.

#### Relation to clinical and histopathologic parameters

In nontumorous mucosa samples, the stefin A and stefin B concentrations were significantly influenced by the site of sampling, being higher in hypopharyngeal tissue samples than in samples from other primary tumor subsites: stefin A, 1808 vs. 590 ng/mgp, $p = 0.017$; stefin B, 637 vs. 167 ng/mgp, $p = 0.009$. Compared with other tumor subsites, a significantly higher proportion of cases with a negative (downregulated) tumor-to-mucosa concentration difference of stefin A (67% vs. 38%, $p = 0.038$) and stefin B (80% vs. 40%, $p = 0.005$) was found in hypopharyngeal lesions.

The relationship between tumor stefin concentrations and the established patient-related and disease-related prognostic factors were also analyzed. For any of the studied inhibitors, no correlation was found with the patients’ age and

### Table 2. Stefin A and stefin B concentrations in tissue homogenates of tumor and adjacent nontumorous mucosa

<table>
<thead>
<tr>
<th>Patients</th>
<th>Stefin A (ng/mgp)</th>
<th>Stefin B (ng/mgp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n$</td>
<td>Median</td>
</tr>
<tr>
<td>All</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucosa</td>
<td>92</td>
<td>759.5</td>
</tr>
<tr>
<td>Tumor</td>
<td>92</td>
<td>795</td>
</tr>
<tr>
<td>Upregulated cases*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucosa†</td>
<td>53</td>
<td>244</td>
</tr>
<tr>
<td>Tumor</td>
<td>53</td>
<td>1059</td>
</tr>
<tr>
<td>Downregulated cases*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucosa†</td>
<td>39</td>
<td>1690</td>
</tr>
<tr>
<td>Tumor</td>
<td>39</td>
<td>468</td>
</tr>
</tbody>
</table>

*Abbreviation: $n$ = number of samples.

† Mucosa, upregulated cases vs. downregulated cases: stefin A, $p<0.0001$; stefin B, $p<0.0001$. 

end point of survival analysis was event-free survival (i.e., disease-free survival [DFS]) in which local recurrence, regional recurrence, and systemic dissemination were each considered as an event. The survival times were calculated from the date of surgery. As the sample size was small, a Cox model allowing splines was used only as a graphical evaluation of the functional form relating the hazard ratio for DFS to the stefin A and stefin B concentrations (18). Because both stefin A and stefin B seem to be associated with DFS only when exceeding a certain value, a flexible methodology for analyzing their effect was used, with the advantage of avoiding arbitrary categorization and its subsequent loss of information (19). Thus, a “broken stick” model of the form

$$\beta (V - V_0),$$

where $V$ is the measured value, $V_0$ is the cut-off value and the plus denotes that only the part where $V$ is greater than $V_0$ is used, was proposed. Both $\beta$ and $V_0$ were estimated simultaneously by maximizing the Cox partial likelihood in a model using no additional covariates (19).

In pooled analysis we reanalyzed individual patient data from the present and the two historical data sets (11, 12). Because the method of determination of stefins was improved over time and to avoid any possible bias arising from the differences between the laboratories that performed biochemical analysis, a common scale was introduced. For each data set, we ranked the stefin A and stefin B measurements and divided the ranks by the number of patients. Thus inhibitor levels were converted to fractional ranks (between 0 and 1). In this way, equal fractional ranks are comparable across data sets with different numbers of patients included, allowing an assumption that the use of different methodology for stefin determination did not influence the rank of stefin A and stefin B levels profoundly. The hazard ratio (HR) for the ranked variables represents the differences between the extremes (20).
sex, histopathologic tumor grade, or pT, pN, and overall UICC pTNM stage of disease, and the presence of extracapsular tumor extension. The same was observed when the frequency distribution of upregulated and downregulated cases was compared among different prognostic subgroups. The only exception was the primary tumor site, as described above.

Survival analysis
As of October 31, 2005 (close-out date), the median follow-up period for all 92 patients was 4.8 years (range, 0.4–8.8 years) and 8.0 years (range, 2.0–8.8 years) for those alive at the last follow-up examination (all patients but 1 were followed ≥5.8 years). Disease recurrence was diagnosed in 20 patients: isolated local or regional recurrence in 2 patients each, simultaneous local and regional disease reappearance in 4 patients, systemic dissemination only in 11 patients, and in conjunction with neck recurrence in 1 patient. The causes of death were as follows: disease recurrence or distant dissemination in 20 patients, and causes other than the treated malignant disease in 38 patients.

The DFS at 5 years was 77%. On univariate analysis, DFS was significantly influenced by primary tumor site, by pT-, pN-, and overall UICC pTNM stage of the disease, and by the presence of extracapsular tumor spread (Table 3). The tumor concentrations of stefins were also predictive for DFS. In tumor samples from the patients who experienced treatment failure, lower concentrations of stefin A and stefin B were measured compared with those experiencing successful treatment of malignant disease (stefin A: 426 vs. 943.5 ng/mgp, p = 0.001; stefin B: 165.5 vs. 230 ng/mgp, p = 0.060), and a significantly higher proportion of downregulated cases were found among the patients with disease reappearance (stefin A: 70 vs. 35%, p = 0.005; stefin B: 70 vs. 40%, 0.018). There was a gradual trend toward improved DFS rates at 5 years with increasing concentrations of stefin A but not also of stefin B (Fig. 1).
1063 ng/mgp (the 64th percentile in the group), and in the stefin B it was 333 ng/mgp (the 78th percentile in the group).

To evaluate the connection between stefin A and DFS survival, we applied the Cox model using the estimated optimal cut-off point for the logarithm of stefin A and pT-stage as possible confounder. The model assumed no effect of the log of stefin A up to the cut-point value of 1064 ng/mgp and a linear effect afterward. As shown in Table 4, the results of multivariate analysis were significant, confirming that the increase of stefin A concentration above the threshold value of 1064 ng/mgp reduces the risk of disease reappearance significantly. The HR of 0.03 for the logarithm of stefin A implies that after doubling of stefin A concentration above the calculated cut-off value the hazard decreases to only 3% (or, in other words, by 97%) of the reference value. However, as the confidence interval is rather broad, the HR reduction may not be that substantial. In the case of stefin B, all patients with an inhibitor value exceeding 333 were censored. Consequently no further calculations were performed.

### Pooled analysis

The survival data for 90 patients with operable squamous cell carcinoma of the head and neck from our previous two studies (historical groups in Refs. 11, 12) were updated. By the close-out date, the median follow-up of those alive was 9.5 years (range, 2.4–12.8 years). A total of 28 patients experienced disease reappearance, and 60 patients died: 24 from the disease and 36 from causes unrelated to the disease.

After pooling the data from all three data sets, a uniform group of 182 patients with 48 events was formed, eligible for the maximum likelihood estimate and survival analysis. The optimal cut-off point for stefin A was calculated to be the 63rd percentile in the group. Considering the number of events, four variables were introduced into the Cox multivariate model. In the final model, pT-stage, primary tumor site, extracapsular extension, and logarithm of stefin A rank were retained (Table 4). A significant decrease in the recurrence rate after the cut-off value was observed, reaching 53% as the fractional rank of stefin A increases by 0.1.
The presented results confirmed the hypothesis about the protective role of high levels of stefins A and B in tissue homogenates. Furthermore, although no difference in stefin A and stefin B concentrations was found in the total population of tumor homogenates compared with normal mucosa, two groups of patients with downregulated and upregulated stefin concentrations were distinguished. Assessing the biologic meaning of this finding in relation to the treatment outcome, we observed a significantly higher proportion of downregulated cases among the patients with recurrent disease. Moreover, in this group, the survival probability at 5 years was significantly lower compared with the patients with upregulated inhibitors (data not shown).

The subpopulations of the patients with down- and upregulated cystatins have already been described in the literature. In head-and-neck carcinoma patients, a comparable pattern in inhibitor alterations between nonmalignant mucosa and tumors was observed in the case of cystatin C protein, even though these changes had no implication for prognosis (13). In the study by Lah et al. (21), lower cysteine protease inhibitor activity in the breast carcinoma tissue (i.e., in the low-activity group) was associated with significantly higher increases of cathepsin B and cathepsin L activity, and more patients developed poorly differentiated and hormone receptor–negative tumors than in the high-activity group. However, recurrences were reported with the same percentage in both groups (21). To the contrary, in the subsequent study, Lah et al. reported a $\geq3$-fold decrease in tumor stefin A compared with the adjacent normal breast parenchyma as the most significant prognosticator for survival (22). No significant relationship between alterations in cysteine protease inhibitor activity and survival in non–small-cell lung cancer patients was observed (23).

The second observation that we wish to point out is the correlation between the high levels of tumor stefins and favorable prognosis. The issue of the protective role of high levels of cysteine protease inhibitors in tissue homogenates was raised after the survival analysis of patients with carcinoma of the breast (22), lung (23, 24), and head and neck (11–13). On the other hand, this concept contradicts what was observed for the serine protease system inhibitor, plasminogen activator inhibitor type 1, in tumor tissue extracts of breast carcinoma (20), for stefin A immunohistochemistry in breast cancer sections (25), or various cystatins from the serum of patients with colorectal carcinoma (26), lung carcinoma, and non-Hodgkin’s lymphomas (27). However, the observed variations in the relationship between the measured levels of cystatins and survival probability are to be expected because of differences in regulation between serine and cysteine proteases during tumor progression (28), inherent variations between the biologic samples of different types, and because the extracellular (i.e., serum) levels of cystatins may reflect not only the changes in their local expression in tumors but also a systemic response to malignant disease (26).

With regard to head-and-neck tumors, we identified stefin A and stefin B as significant predictors of disease recurrence in the univariate analysis of two smaller sets of patients with operable tumors (11, 12). In the pooled analysis of the two, stefin A emerged as the most significant prognosticator of all tested variables from the multivariate model (12). Comparing these findings with the results of the present study we may conclude that high stefin A levels confirmed their favorable prognostic potential. In multivariate analysis, we found a significant decrease in the hazard ratio for disease recurrence, reaching 3% of the initial value with doubling of the level of stefin A, after the threshold of 1064 ng/mg (64th percentile in the group). Furthermore, after pooling the data of all three data sets with a total of 182 patients, the optimal cut-off point was the 63th percentile in the group and again high stefin A appeared as a significant predictor for favorable DFS in the Cox multivariate model.

After the resection of the gross tumor burden, the aim of postoperatively administered radiotherapy is to eradicate the microscopic tumor cell aggregates that escape surgical excision. The main mechanism of killing these cells in the hypoxic environment of the post-resection bed is apoptosis (29). The potential of cystatins to regulate tumor cell apoptosis was demonstrated in several studies (9, 10, 30); however, the results are contradictory. Two opposing roles of cysteine cathepsins in oral squamous cell apoptosis have been suggested recently by Johansson et al. (31). Intracellularly, they were recognized as promoters of apoptosis, whereas in extracellular compartments, cysteine cathepsins seemed to be involved in shedding Fas death receptors on the cell surface and act to prevent apoptosis. Whether the impairment of extracellular cathepsins by stefins potentially secreted from tumor or adjacent cells contributes to the promotion of the death signal and consequently the apoptosis of tumor cells remains to be elucidated. However, this process together with the role the stefins have in the reduction of the degradation of extracellular matrix and tumor cell invasion, may explain the survival results in the present and above-listed studies (11, 12, 22–24) and, specifically, the prognostic advantage of the upregulated ratio in stefin levels between nonmalignant mucosa and tumor tissue samples. On the other hand, in the downregulated cases, low inhibitor levels in the extracellular compartment are supposed to be insufficient for effective enzyme inhibition and resulted in a switch to a tumor-promoting mode.

**CONCLUSIONS**

Our results point to stefin A as a promising candidate marker in patients with operable carcinoma of the head and neck. We identified a group of patients at particularly high risk for disease progression characterized by downregulated stefin A protein in the tumor compared with the nonmalignant mucosa and by stefin A concentration below the cut-off point as determined from the maximum likelihood estimate.
of the threshold model. Thus a significant decrease in treatment failure should be expected, reaching 3% of the reference value with doubling the level of stefin A, above the threshold of 1063 ng/mgp. These results were reconfirmed after pooling the data of all three data sets into a uniform series involving 82 patients.

REFERENCES


