Prognostic Significance of Cysteine Proteinases Cathepsins B and L and Their Endogenous Inhibitors Stefins A and B in Patients with Squamous Cell Carcinoma of the Head and Neck[1]

Primož Strojan,[2] Marjan Budihna, Lojze Šmid, Branka Svetic, Ivan Vrhovec, Janko Kos, and Janez Škrk

Departments of Radiotherapy [P. S., M. B.], Biochemistry [B. S., I. V.], and Tumor Biology [J. Š.], Institute of Oncology; University Department of Otorhinolaryngology and Cervicofacial Surgery [L. Š.]; Department of Biochemistry and Molecular Biology, Jožef Stefan Institute [J. K.], and KRKA, d.d., Research and Development Division, Department of Biochemical Research and Drug Design [J. K.], SI-1000 Ljubljana, Slovenia

ABSTRACT

Cysteine proteinases cathepsins (Cats) B and L and their endogenous inhibitors stefins (Stefs) A and B are implicated in the processes of local and metastatic tumor spread. They were identified as potential prognosticators in various malignant diseases, particularly in breast cancer. The aim of the present study was to determine the concentrations of Cats B and L and Stefs A and B in the tumor and adjacent normal tissue samples collected from 49 patients (the present group) with squamous cell carcinoma of the head and neck (SCCHN), using quantitative immunosorbent assays (ELISA; KRKA d.d., Novo mesto, Slovenia). Their clinical significance was compared with that from a previous study (the reference group, 45 patients; Budihna *et al.*, Biol. Chem. Hoppe-Seyler, *377:* 385–390, 1996). The follow-up of patients from the latter report was updated for this purpose.

In the present group, significantly higher concentrations of Cat B ($P < 0.0001$), Cat L ($P < 0.0001$) and Stef A ($P = 0.006$) were found in tumors compared with concentrations in their normal tissue counterparts. Cat concentrations in normal laryngeal tissue were significantly/marginally elevated compared with nonlaryngeal tissue (Cat B, $P = 0.02$; Cat L, $P = 0.06$). The tumor concentration of Cat L was found to correlate with pT classification ($P = 0.005$) and tumor-node-metastasis stage ($P = 0.05$), whereas the concentrations of Stefs A and B correlated with pN classification ($P = 0.007$ and $P = 0.03$, respectively) and tumor-node-metastasis stage of the disease ($P = 0.02$ and $P = 0.03$, respectively). There was no statistically significant difference between low and high Cat B or Cat L groups, regarding either disease-free survival or disease-specific survival, using a minimum *P* approach to determine cutoff concentrations. The risk of disease recurrence and SCCHN-related death was significantly higher in patients with low Stef A ($P = 0.0006$ and $P = 0.0005$, respectively) and Stef B ($P = 0.0009$ and $P = 0.0007$, respectively) tumors, compared with those with high-Stef A and Stef B tumors. These results remained significant even after *P*s were adjusted for a possible bias in the estimated effect on survival. The survival analysis in the reference group also confirmed these findings (Stef A: $P = 0.0009$ and $P = 0.002$, respectively; Stef B: $P = 0.03$ and $P = 0.009$, respectively). To avoid any possible bias arising from the differences between the laboratories that performed the biochemical analysis, the concentrations of both Stefs in the present group and in the reference group were standardized and coupled together to form a uniform group. In univariate survival analysis, standardized values of Stef A and Stef B correlated inversely with the rate of relapse ($P = 0.0000$) and mortality rate ($P = 0.0000$). Multivariate regression analysis showed that the standardized value of Stef A is the strongest independent prognostic factor for both disease-free survival and disease-specific survival. These findings show the specific role of Cats B and L and Stefs A and B in the invasive behavior of SCCHN. Furthermore, Stef A proved to be a reliable prognosticator of the risk of relapse and death in patients with this type of cancer.

INTRODUCTION

Cats[3] B and L are ubiquitous lysosomal proteolytic enzymes present in almost all mammalian cells. They both belong to the cysteine class and participate in the processes of intracellular protein turnover and posttranslation processing of some biologically important protein precursors (1). The activity of Cats B and L is regulated by numerous endogenous inhibitors that, in turn, make up the cystatin superfamily, *i.e.*, Stefs, cystatins, and kininogens. Stefs A and B are the main intracellular inhibitors of cysteine proteinases and belong to family I of the cystatin superfamily of inhibitor proteins (2).

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[1] Supported by the Ministry of Science and Technology of Slovenia Grant J3–7952.
[2] To whom requests for reprints should be addressed, at the Department of Radiotherapy, Institute of Oncology, Zaloška 2, SI-1000, Slovenia. Phone: 386-61-1314225; Fax: 386-61-1314180; E-mail: pstrojan@onko-i.si.

[3] The abbreviations used are: Cat, cathepsin; Stef, stefin; uPA, urokinase-type plasminogen activator; SCCHN, squamous cell carcinoma of the head and neck; DFS, disease-free survival; DSS, disease-specific survival; Ab, antibody; HRP, horseradish peroxidase; RR, relative risk; CI, confidence interval; TNM, tumor-node-metastasis (classification); mgp, mg of total protein; P_{min}, minimum *P*; P_{cor}, corrected *P*; BMDP Biomedical Data Processing.

Table 1 Cysteine proteinases and their endogenous inhibitors in squamous cell carcinoma of the head and neck: a review of the literature

Author (Reference)	Enzyme/ Inhibitor	Samples		Assay	Correlation with	
		Origin	*n*		Histopathological prognostic factors	Survival[a]
Siewinski *et al.* (30)	CPI[b]	Serum	86	EA	TNM stage	n.r.
Kręcicki and Siewiński (31)[c]	Cat B	Serum	110	EA	TNM stage	n.r.
Bongers *et al.* (32)	Cat B	Serum	20	EA	No	n.r.
Russo *et al.* (33)[c]	Cats B, L	Tissue	71	EA	Cat B: DNA ploidy Cat L: DNA ploidy, Grade, TNM stage	Cat L ↑
Budihna *et al.* (34)	Cats B, H, L Stefs A, B	Tissue	45	ELISA	Cat B: Site of origin Stef A: T stage	Cat L ↑ Stefs A, B ↓
Šmid *et al.* (35)[c]	Cat B Stefs A, B	Tissue	25	ELISA	Stefs A, B: T stage	Stefs A, B ↓
Strojan *et al.* (36)	Cat H	Serum	35	ELISA	No	No

[a] Correlation with survival is indicated as follows: ↑, correlation of high levels with poor prognosis; ↓, correlation of low levels with poor prognosis.
[b] CPI, cysteine proteinase inhibitors; EA, enzyme activity; n.r., not reported.
[c] Included only patients with laryngeal carcinoma.

Under certain conditions the Cats are secreted from cells, mainly as inactive precursors that require activation by another protease (3). It has been proposed that extracellular Cats B and L, together with the members of other classes of proteinases, *i.e.*, Cat D, palsminogen activators, and matrix metalloproteinases, are all subject to a cascade-like manner of activation. Because of altered or compromised regulation of the cascade response in tumor tissue, enzyme activation results in uncontrolled proteolysis of the extracellular matrix components, *i.e.*, laminin, fibronectin, and type IV collagen, which is believed to be a preliminary condition for local and metastatic tumor spread (3).

Active serine protease uPA plays a pivotal role in the cascade. It converts proenzyme plasminogen into the fibrinolytic peptide plasmin, which is capable of degrading most extracellular proteins either directly or indirectly through activation of other proteinases (4). Activation of the precursor form of uPA into the enzymatically active uPA results from the proteolytic activity of Cats B and L (5, 6), and, *vice versa*, pro-Cat B can be activated by uPA (7). As it was shown *in vitro*, the activation of both Cats is presumably caused by proteolytic action of aspartic protease Cat D (8), although it has been reported that the processing of pro-Cats B and L to active enzymes can also be an autocatalytic process (9, 10). With its ability to be autoactivated in an acid microenvironment, Cat D adopts the role of initiator of the proteolytic cascade (11). All of the three Cats—B, L, and D—can also directly degrade extracellular matrix proteins (12–14).

A large body of literature has accumulated to suggest that cysteine proteinases and Stefs are involved in tumor invasion and metastasis. Alterations in the expression at mRNA and protein levels, as well as in the activity and trafficking of enzyme and inhibitor molecules, have been found to correlate with malignancy of various murine and human tumors (3). In clinical studies of malignant melanoma (15) and breast (16–21), colorectal (22, 23), lung (24–27), gastric (28), and brain (29) cancers, overexpression of Cats B and L, imbalance of the Cat:inhibitor ratio, or decreased total inhibitor activity have all been shown to be related to survival probability.

Cysteine proteinases and their endogenous inhibitors in SCCHN have seldom been studied. The prognostic value of these enzymes and inhibitors have been evaluated in four of seven published papers on this topic (Table 1, Refs. 30–36). Russo *et al.* (33) reported better DFS in patients who had tumor:normal tissue ratio of Cat B activity below or equal to one. The results published by our group in 1996 (34) found that a better prognosis correlated with lower concentrations of Cat L in tumor tissue for both DFS and DSS analysis. In the same study, high concentrations of Stef A and Stef B proved to be strong indicators of the rate of relapse and the duration of survival after treatment for SCCHN (34). These results were reconfirmed by a subsequent analysis in a subpopulation of patients with laryngeal cancer (35). Recently, we found that low serum and tissue levels of the least studied of cysteine proteinases, Cat H, correlated with both better DFS and better DSS (36).

In the present study, the prognostic significance of tumor concentrations of Cats B and L, and Stefs A and B were analyzed in a new, independent group of patients with SCCHN (hereafter, the "present group"). We decided to repeat the analysis as it was designed in our previous study (34) for the following reasons: (*a*) the findings of the latter study had not yet been confirmed or opposed by any other study (see Table 1); (*b*) the reported results warranted further evaluation; (*c*) the test kits used for the biochemical analysis of Cats B and L had been upgraded and had been used in several clinical studies on malignant melanoma (15) and breast (18, 19), colorectal (23), and lung (26) cancers. Modified assays assure more precise measurement of enzyme concentrations and allow comparisons with values measured in other tumor types. For a more relevant estimate of the prognostic significance of the studied Cats and Stefs, the follow-up of the patients from our previous study (hereafter, the "reference group"; Ref. 34) was updated, and their survival results were included in this report.

PATIENTS AND METHODS

Patients. Forty-nine patients (4 females, 45 males) with primary SCCHN, undergoing curative surgical treatment between November 1995 and December 1996 at the University Department of Otorhinolaryngology and Cervicofacial Surgery in Ljubljana,

Slovenia, entered the study. Clinical data on patient age, tumor site, stage of disease, histopathological grade, and extracapsular tumor spread were available for all of the cases (Table 2). The International Union Against Cancer TNM (37) classification was used for tumor staging, and the histopathological grade was defined according to WHO criteria (38).

Surgical procedure comprised the resection of the primary tumor together with a surrounding margin of normal tissue and removal of the related regional lymph nodes on the neck. Forty-two of the patients received postoperative radiotherapy at the Institute of Oncology, Ljubljana, Slovenia, because of an advanced stage of the disease, residual growth after surgery, extranodal tumor spread, or the presence of neoplastic emboli in the lymphatic vessels. Radiotherapy was performed on a Cobalt-60 unit or a 5-MV linear accelerator, with a daily dose of 1.8–2 Gy, 5 days per week. A total radiation dose of 50–66 Gy (median, 56 Gy) was delivered through two opposing lateral fields to the primary tumor and regional lymph nodes, with the spinal cord shielded after a dose of 40–44 Gy. One anterior field was added to cover the lower neck. If there was no residual tumor after surgery (32 patients), 50–56 Gy were given. However, in cases of extracapsular rupture (16 patients) or microscopic residuum (1 patient), the dose was increased to 63 or 66 Gy, respectively.

The median age of these patients was 60 years, with a range of 37–72 years, and all of them were eligible for follow-up. As of August 31, 1999, disease recurrence and/or distant dissemination was diagnosed in 12 patients, and 10 of the 12 died of disease-related causes. Ten other patients died from causes other than the treated malignant disease. The median follow-up time for patients alive at the last follow-up examination was 30 months, with a range of 24–40 months.

Tissue Extraction. For biochemical analysis of Cats and Stefs, two tissue samples of 200–500 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; fat and necrotic parts of the tissue were carefully removed. Pulverization was performed on the frozen tissue with a Mikro-Dismembranator (Braun, Melsungen, Germany) for 60 s at a maximum power, and the resulting tissue powder was suspended in extraction buffer consisting of 5 mM Na_2HPO_4, 1 mM monothioglycerol, and 10% v/v glycerol (pH 7.4). The suspension was centrifuged for 45 min at 100,000 × *g* to obtain the supernatant, *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 method of Bradford (39).

Assays for Cats B and L and Stefs A and B. For quantitative analysis of Cats B and L and Stefs A and B in tissue cytosols, commercially available modified specific ELISAs (sandwich ELISAs, KRKA d.d., Novo mesto, Slovenia) were used, as developed at Jožef Stefan Institute, Ljubljana, Slovenia (40). Human Cats B and L (2), and recombinant Stefs A and B expressed in *Escherichia coli* (41), were used for immunization of animals and as standards for assay calibration curves. The linearity of ELISAs was tested by serial dilutions of tissue cytosol samples to the levels encompassing the range of assays. The measured values of diluted samples were subsequently compared with the calibration values. For the determination and characteristics of recovery and within-run and between-run coefficients of variance, see Kos *et al.* (40)

The diluted tissue cytosol was added to wells of a microtiter plate that had been precoated with the corresponding capture anti-Cat/Stef Ab. After a 2 h-incubation at 37°C, the wells were washed and filled with detection anti-Cat/Stef Ab conjugated with HRP. After an additional 2-h incubation at 37°C, the peroxidase substrate 3,3,5,5-tetramethyl benzidine (Sigma Chemical Co., St. Louis, MO) was added in the presence of hydrogen peroxide. The amount of degraded substrate, as a measure of bound immunocomplexed Cat/Stef, was visualized by absorbency at 450 nm, using a microplate reader (Model 450, Bio-Rad, USA). The particular Cat/Stef concentration was calculated from the corresponding calibration curve and expressed in ng/mgp.

For Cat B ELISA, immobilized rabbit and HRP-conjugated sheep polyclonal anti-Cat B IgG were used as capture and detection Abs, respectively. Tumor samples in 1:100 dilution and normal samples in 1:10 dilution were used in the assay. The detection limit of the assay was 0.9 ng/ml sample.

For Cat L ELISA, immunoselective polyclonal sheep anti-Cat L IgG was used for immobilization and as an HRP-conjugated Ab for detection. Tumor samples were diluted to 1:4, and normal tissue samples were diluted to 1:2. The detection limit of the assay was 1.7 ng/ml.

For Stef A ELISA, monoclonal murine anti-Stef A Abs were used in the assay as capture Abs and as HRP-conjugated detection Abs. Tumor and normal tissue samples in 1:100 dilution were used. The detection limit of the assay was 1 ng/ml.

For Stef B ELISA, monoclonal murine anti-Stef B Abs were used for capture and as HRP-conjugated Abs for detection. Tumor and normal tissue samples in 1:100 dilution were used. The detection limit of the assay was 1 ng/ml.

The polyclonal Abs used for Cats B and L ELISA recognize precursor molecules, enzyme-inhibitor complexes, and mature forms of the enzymes. According to the manufacturer, ELISAs for Cat B and Cat L show no cross-reactivity. The monoclonal Abs used for Stefs A and B ELISA bind enzyme-inhibitor complexes and free antigens and exhibit no cross-reactivity between the Stefs.

Reference Group. Forty-one patients (1 female, 40 males), aged 40–69 years (median, 56 years) with primary SCCHN, diagnosed and treated between June 1992 and August 1993, were eligible for survival analysis. Treatment regimen, sampling and cytosol preparation were essentially the same as described above. By March 31, 1999, 26 patients were dead; 14 from the disease and 12 from causes unrelated to the disease. Disease recurrence/dissemination was diagnosed in 16 patients. Median follow-up of those alive was 69 months, with a range of 58–75 months. Concentrations of Cat B and both Stefs were determined in tissue samples from all of the 41 patients, and that of Cat L from 23 of the 41 patients, using test kits from the same manufacturer as those used in the present study. However, in the period between both studies, the manufacturer modified the test kits for Cat B and Cat L to upgrade their specificity potential by improving the stability and by using different lots of standards and Abs.

For details on the therapy, tumor characteristics, and the results of biochemical and statistical analysis of the reference group see Budihna *et al.* (34).

Statistical Analysis. The results were analyzed using a PC computer and BMDP software package (BMDP Statistical Software, Los Angeles, CA). All of the tests were two-sided and the results were considered statistically significant at a P of 0.05. The differences between the median concentrations of Cats and Stefs in matched pairs of tumor and normal tissue samples were determined by the Wilcoxon signed-rank test. The Mann-Whitney U test was used to test the relationship between the median values of the tumor tissue enzyme and inhibitor concentrations in different groups of patients. The strength of the association between individual Cat and Stef concentrations was tested using the Spearman rank correlation (r_s).

The statistical assessment of patient survival was carried out using the Kaplan-Meier product-limit method (42) and log-rank comparison to evaluate the differences between the survival curves (43). The primary end points of survival analysis were DFS and DSS; the latter was calculated by censoring disease-unrelated deaths. In both types of cases, the survival times were calculated from the date of surgery. Concentrations of each individual Cat/Stef were dichotomized according to their optimal cutoff concentrations, which were determined among the measured values using the criterion of maximal difference in the survival rates between low and high Cat/Stef groups (*i.e.*, minimum P approach, P_{min}). Because this approach has proved to be associated with a considerable inflation in type I error rate, thus falsely claiming a factor to be prognostically relevant, all of the P_{min} values < 0.05 were corrected according to the recommendation of Altman *et al.* (44), using the following formula:

$$P_{cor} \approx -3.13\, P_{min}\,(1 + 1.65 \log P_{min})$$

In cases in which $P_{min} < 0.05$, the RR and 95% CI were also calculated. Because the test kits for Cats B and L analysis had been modified in the period between both studies, the results of the measurements for both groups were considered incomparable. The results, therefore, were statistically evaluated for each group separately, which precluded the use of multivariate analysis because of the small number of patients in these groups. Although the same test kits were used for Stefs A and B determination in both the present and the reference group, these parameters were analyzed as a uniform group, only after standardizing their concentrations, to avoid any possible bias arising from the differences between the laboratories that performed biochemical analysis (for the present group, Institute of Oncology; for the reference group, Jožef Stefan Institute). Thus, the measured concentrations (X_i) were standardized using group-specific mean (X_{mean}) and SD values according to the following formula:

$$\frac{(X_i - X_{mean})}{SD}$$

The standardized concentrations, thus, have a mean of 0 and an SD of 1 within each group. These were then used to perform a multivariate analysis according to Cox's proportional hazard model (45).

The study protocol was approved by the Medical Ethics Committee at the Ministry of Health of the Republic of Slovenia, and all of the included patients gave their informed consent to voluntary participation in the study.

RESULTS

The relationships between the concentrations of Cats and Stefs as measured in tumor and normal tissue samples are shown in Fig. 1. The concentrations of both studied enzymes and Stef A were significantly higher in tumors than in their normal counterparts (Cat B: 422 *versus* 85 ng/mgp, $P < 0.0001$; Cat L: 12.7 *versus* 5.1 ng/mgp, $P < 0.0001$; Stef A: 451 *versus* 294 ng/mgp, $P = 0.006$); however, the concentration of Stef B was not significantly higher (288 *versus* 268 ng/mgp, $P > 0.05$).

A significantly higher concentration of Cat B was found in the samples of normal laryngeal tissue, compared with those of nonlaryngeal tissue, *i.e.*, of the oral cavity, oro- and hypopharynx (124 *versus* 70 ng/mgp, $P = 0.02$). The site of normal tissue sampling also seemed to be related to Cat L concentration (7.8 *versus* 4.2 ng/mgp, $P = 0.06$); however, for the Stefs, no such trend was observed. The distribution of tumor concentrations of the studied Cats and Stefs according to various clinical and histopathological prognostic factors and to the outcome of disease is given in Table 2. For Cat L, a significant relationship was found with local tumor extent ($P = 0.005$) and TNM stage of the disease ($P = 0.05$). Tumor concentration of Stef A was significantly related to nodal involvement ($P = 0.007$) and TNM stage ($P = 0.03$), as was the case in Stef B ($P = 0.02$ and $P = 0.03$, respectively). Lower Stef concentrations were measured in tumor tissue from patients with recurrent disease, compared with those without evidence of recurrent disease during follow-up (Stef A, $P = 0.01$; Stef B, $P = 0.003$). Among Cats and inhibitors, a significant correlation has been found between Cat B and Cat L ($r_s = 0.67$, $P < 0.0001$), between Stef A and Stef B ($r_s = 0.69$, $P < 0.0001$) and between Cat L and Stef A ($r_s = 150$ 0.32, $P = 0.03$).

Univariate Survival Analysis. To allow visualization of the association of Cat B and Cat L with DFS and DSS, enzyme concentrations were dichotomized according to the P_{min} approach. Although no statistically significant difference was observed between low and high Cat groups at any chosen cutoff value, a trend toward higher survival probability was apparent for lower Cat L levels. No such trend was determined for Cat B.

In the case of Stefs A and B, patients with lower tumor content of either inhibitor showed a poorer prognosis. There was a trend toward longer survival with increasing levels of Stef A and Stef B in the analysis for both DFS and DSS (Table 3). The maximal differences in the survival rates between low and high Stef groups were calculated to be 303 ng/mgp for Stef A and 240 ng/mgp for Stef B. Using these values as cutoff concentrations, 29% of the tumors were classified as Stef A-low and 35% as Stef B-low. The differences in DFS and DSS rates between low and high Stef groups were highly significant for both inhibitors, as shown in Table 4. After the correction of P_{min} as calculated by means of log-rank test for a possible bias in the estimated effect on survival, the corresponding values of P_{cor} for Stef A were 0.02 and 0.02 (DFS and DSS, respectively), and for Stef B 0.03 and 0.02. The corresponding figures for the reference group are also presented in Table 4.

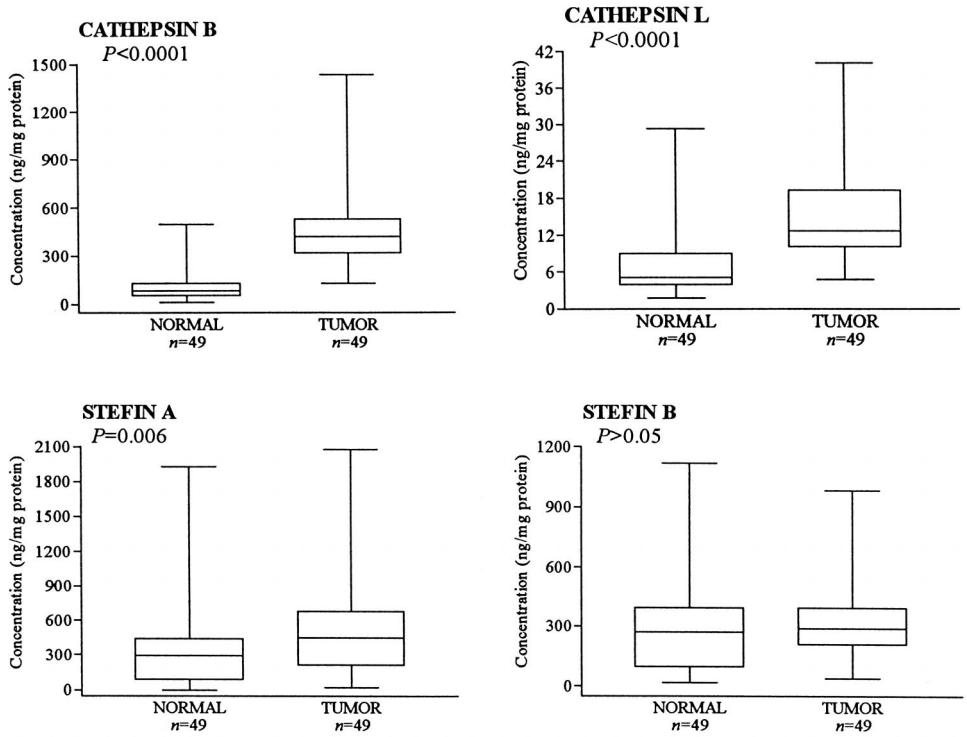

Fig. 1 The relationships between normal and tumor tissue concentrations of Cats B and L and Stefs A and B. The *top* and the *bottom of the box*, the 25th and 75th percentile, respectively; the *ends of the bars*, the range; the *line in the box*, the median value; *n*, number of samples included in the study.

Group with Standardized Concentration. The concentrations of Stefs A and B were standardized, this being expressed as the number of SDs from the mean for each group, *i.e.*, the present and the reference one. The values obtained were coupled together, forming a uniform group of 90 patients eligible for survival analysis. For Stef A, the optimal cutoff value for both DFS and DSS was the 30th percentile in the group, giving in both cases a P_{min} of 0.0000 and a P_{cor} of 0.004 (the lowest *P* as calculated by BMDP statistics is 0.0000; in these cases the value of 0.0001 was used for the P_{cor} calculation). As compared with tumors with low standardized values of Stef A, tumors with high values showed an increased rate of relapse and disease-related deaths, with RR of 4.5 and 4.9, respectively (Table 5; Fig. 2). The optimal cutoff value for Stef B was the 37th percentile in the group. Using this cutoff point to discriminate between low and high Stef B tumors, we calculated P_{min} of 0.02 and 0.03 for DFS and DSS, respectively, with corresponding RRs of 2.2 and 2.2 (Table 5; Fig. 2). None of the listed P_{min} values for Stef B turned out to be statistically significant after applying the correction formula (DFS, $P_{cor} = 0.37$; DSS, $P_{cor} = 0.46$).

Multivariate Analysis. In Cox multivariate regression analyses for DFS and DSS, the relative prognostic strengths of standardized Stef A and Stef B tumor concentrations were compared with those of the established prognostic factors that were found to be significant indicators of patient's survival in univariate analysis. Among the parameters tested, only tumor site, nodal status, TNM stage of disease, and extracapsular tumor spread met the inclusion criteria. Stef levels were dichotomized according to the cutoff values corresponding to the 30th and 37th percentiles in the group. Variables were eliminated from the model singly in a backward fashion and reincluded only if *P* was < 0.05. As shown in Table 5, only Stef A and extracapsular tumor spread were retained in the final model.

DISCUSSION

The role of cysteine proteinases and their endogenous inhibitors in the invasive behavior of SCCHN has not yet been extensively studied yet. Therefore, the present study, including the published report on the reference group (34), could be considered as the first attempt to extensively evaluate the prognostic significance of Cats B and L and Stefs A and B in this particular type of cancer. A critical review of the relevant literature has identified Cats B and L and Stefs A and B as the most promising among biological prognostic factors in the proteinase group to be studied to date for different cancers (3), in addition to the aspartic proteinase Cat D (46), and serine proteinase uPA and its inhibitors (47).

An important finding in the present study is that tumor levels of Stef A can reliably predict the clinical outcome of SCCHN, whereas the prognostic relevance of Stef B seems less clear than that of Stef A. In contrast to breast (16–20), colorectal (22, 23), lung (24–26), gastric (28), and brain (29) cancer, Cat B and Cat L failed to demonstrate any predictive value in SCCHN.

Cats B and L. In our study, significantly higher concentrations of both Cat B and Cat L were found in the cytosols of tumors than in normal tissue, which indicated that the invasive behavior of SCCHN is associated with the action of cysteine proteinases. This same relationship between the Cat B and Cat

Table 2 Concentration profile of Cats B and L and Stefs A and B by clinical and histopathological prognostic factors and by outcome of disease

Variable	*n*	Concentration (ng/mg protein)[a]			
		Cat B	Cat L	Stef A	Stef B
Age (yr)					
≤ 60	24	428 (132–1434)	11.6 (7.8–40.1)	436 (22–2077)	328 (37–985)
>60	25	417 (163–963)	4.7 (4.7–26.9)	451 (56–1241)	283 (73–818)
P		NS[b]	NS	NS	NS
Tumor site					
Larynx	20	425 (241–1434)	16.0 (4.7–26.9)	510 (56–1764)	302 (73–985)
Nonlarynx[c]	29	422 (132–957)	11.4 (6.7–40.1)	420 (22–2077)	284 (37–869)
P		NS	NS	NS	NS
pT classification					
T_{1+2}	23	422 (132–957)	10.6 (6.7–40.1)	498 (56–2077)	371 (73–869)
T_{3+4}	26	425 (185–1434)	18.1 (4.7–26.9)	416 (22–1764)	259 (37–985)
P		NS	0.005	NS	NS
pN classification					
N_0	24	409 (163–1434)	13.5 (4.7–26.9)	575 (79–1764)	375 (96–985)
$N_{1–3}$	25	423 (132–957)	12.3 (6.7–40.1)	356 (22–2077)	241 (37–818)
P		NS	NS	0.007	0.02
TNM stage					
S_{I+II}	10	401 (163–862)	9.9 (7.9–26.9)	652 (186–1289)	455 (248–869)
S_{III+IV}	39	422 (132–1434)	13.3 (4.7–40.1)	407 (22–2077)	265 (37–985)
P		NS	0.05	0.03	0.03
Histopathological grade					
G1+2	40	415 (132–1434)	13.9 (4.7–40.1)	455 (22–1764)	302 (37–985)
G3	5	462 (163–519)	11.1 (8.5–21.0)	364 (56–554)	265 (73–371)
P		NS	NS	NS	NS
Extracapsular spread					
Negative	9	467 (286–957)	13.9 (7.8–26.9)	308 (56–605)	241 (37–625)
Positive	16	399 (132–713)	11.2 (6.7–40.1)	385 (22–2077)	246 (91–818)
P		NS	NS	NS	NS
Outcome of disease					
Without RD	37	413 (132–1434)	11.9 (4.7–26.9)	488 (22–2077)	341 (73–985)
With RD	12	466 (164–713)	15.0 (7.8–40.1)	209 (77–536)	199 (37–443)
P		NS	NS	0.01	0.003

[a] Median (range).
[b] NS, not significant; RD, recurrent disease.
[c] Oral cavity, oropharynx, hypopharynx.

Table 3 DFS and DSS as a function of Stef A and Stef B status

Variable	*n*	2-yr DFS % ± SE	2-yr DSS % ± SE
Stef A, ng/mgp[a]			
≤205	12	48 ± 15	52 ± 16
>205–451	13	74 ± 13	70 ± 14
>451–690	12	73 ± 13	82 ± 12
>690	12	100	100
Stef B, ng/mgp[a]			
≤197	12	48 ± 15	46 ± 15
>197–288	13	66 ± 14	79 ± 13
>288–443	12	83 ± 11	83 ± 11
>443	12	100	100

[a] Tumors were divided into four groups with an approximately equal number of patients based on the quartiles of the concentrations of Stef A and Stef B.

L activity levels observed in the sera of SCCHN patients compared with that in healthy controls were reported by Kręcicki and Siewiński (31) and by Bongers *et al.* (32). A comparison between the present and the reference group found that enzyme levels were three times lower in the present group than in the reference group. However, in both groups, the ratio of the median concentrations between tumor and normal tissue was of the same magnitude [5.0 *versus* 5.0 for Cat B and 2.5 *versus* 1.7 for Cat L (Ref. 34)], which confirms the reliability of the observed differences between tumor and normal tissue. A strong correlation has been found between individual Cat B and Cat L tumor values, which suggests that both enzymes are up-regulated in tumors in the same manner. On the other hand, only Cat L and Stef A levels correlated significantly, which indicates a difference in tumor-associated inhibition between both enzymes. In both groups of patients, the present and the reference one, substantially higher tumor and normal tissue concentrations of Cat B were measured compared with Cat L. The same findings were reported in breast (17–19), lung (26), and gastric (28) cancers. These data support the idea that the lower expression of Cat L may be compensated for by a high rate of effectiveness, particularly considering that both the activation (18) and the proteolytic activity of the enzyme (1, 2) resulted in at least a 10-times-faster degradation of protein substrates than the other proteinases, including Cats B and H (1).

In addition, we found that cytosols prepared from normal laryngeal tissue samples contained higher concentrations of Cat B and Cat L than cytosols from the oral cavity and pharynx. The same

Table 4 Univariate analysis for DFS and DSS of patients with SCCHN in the present and reference groups

		DFS[a]					DSS[b]				
Variable	*n*	%	P_{min}	RR	95% CI	P_{cor}	%	P_{min}	RR	95% CI	P_{cor}
Present group[c]											
Cat B											
≤437	28	81	NS[d]				80	NS			
>437	21	63					74				
Cat L											
≤11.2	19	88	NS				88	NS			
>11.2	30	65					71				
Stef A											
≤303	14	41	0.0006	6.2	2.7–37.6	0.02	43	0.0005	7.5	3.1–58.4	0.02
>303	35	88					90				
Stef B											
≤240	17	46	0.0009	6.7	2.3–27.4	0.03	47	0.0007	9.0	2.7–40.0	0.02
>240	32	90					93				
Reference group[e]											
Cat B											
≤1086	20	73	NS				72	NS			
>1086	21	44					58				
Cat L											
≤46.3	18	89	0.01	0.1	0.01–0.5	NS	89	0.02	0.2	0.01–0.7	NS
>46.3	5	40					40				
Stef A											
≤231	12	24	0.0009	4.4	2.4–31.3	0.03	28	0.002	4.4	2.1–32.4	0.06
>231	29	70					78				
Stef B											
≤114	6	33	0.02	3.4	1.3–42.4	NS	33	0.009	4.1	1.9–76.7	NS
>114	35	63					71				

[a] Concentrations of Cats and Stefs were expressed in ng/mgp.
[b] RR, 95% CI and P_{cor} were calculated only in the cases of $P_{min} < 0.05$.
[c] Calculated at 2 years.
[d] NS, not significant.
[e] Calculated at 5 years.

Table 5 Univariate and multivariate analysis for DFS and DSS

	DFS				DSS			
Variable	Univariate *P*	Multivariate *P*	RR	95% CI	Univariate *P*	Multivariate *P*	RR	95% CI
Stef A, standardized								
High *vs.* low	0.0000	0.0000	0.19	0.09–0.42	0.0000	0.0000	0.15	0.06–0.36
Extracapsular spread								
Negative[a] *vs.* positive	0.001	0.0002	4.7	2.1–10.6	0.0000	0.0001	6.8	2.7–17.4
TNM stage								
$S_{I–III}$ *vs.* S_{IV}	0.004				0.0007			
pN classification								
N_0 *vs.* $N_{1–3}$	0.02				0.004			
Stef B, standardized								
High *vs.* low	0.02				0.03			
Tumor site								
Larynx *vs.* nonlarynx[b]	0.03				0.05			

[a] In the negative extracapsular spread group, patients without extension of tumor tissue beyond nodal capsule and those without regional metastases (i.e., N_0) were included.
[b] Oral cavity, oropharynx, hypopharynx.

trend was observed in the reference group (34). However, in the cases of Cat D (48) and uPA (49) this difference was also found to be statistically significant. The absence of the difference in corresponding tumor concentrations of both Cats between the groups could probably be explained by the fact that: (*a*) the patients with laryngeal tumors experienced significantly longer DFS and DSS (univariate analysis, $P = 0.03$ and $P = 0.05$, respectively; Table 5); and (*b*) low Cat B and/or Cat L tumor/serum levels or activity correlated with better survival probability of patients with various tumor types (15, 17–20, 22–26, 28, 29). Consequently, the elevation of the enzyme concentrations in the patients with laryngeal tumors may be less than in the nonlaryngeal group, and the difference in the elevations results in the equalization of their concentrations between the groups.

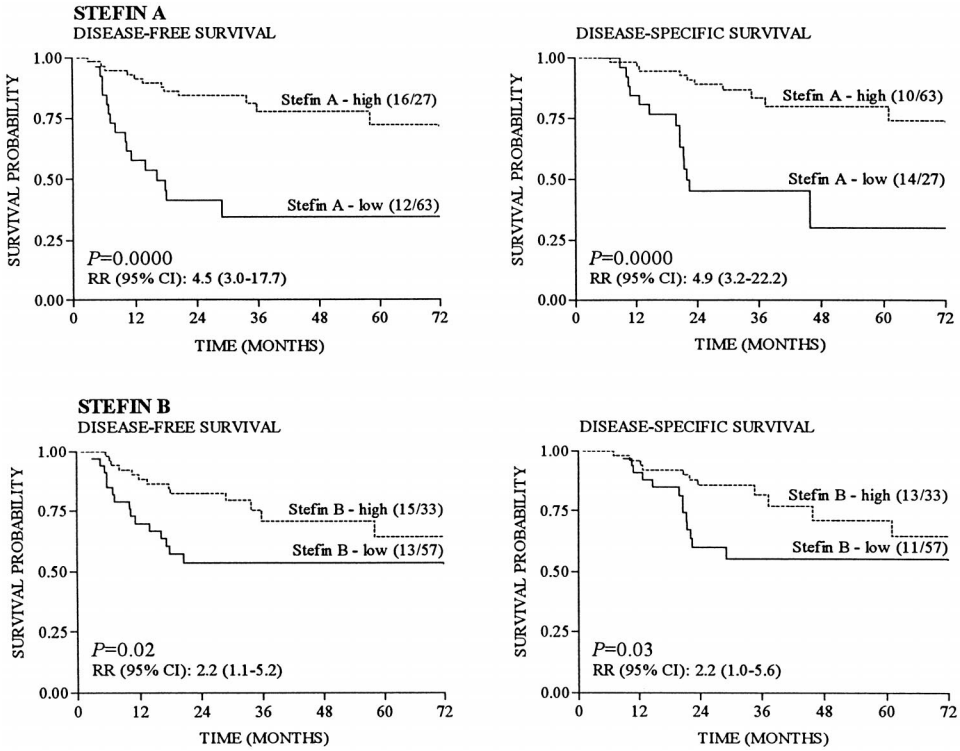

Fig. 2 Actuarial DFS and DSS as a function of Stef A and Stef B status in group with standardized concentrations. Concentrations (X_i) of both Stefs as measured in the present and the reference groups were standardized using group-specific mean (X_{mean}) and SD values according to the formula $(X_i - X_{mean}) \div SD$. The standardized concentrations from both groups were coupled together and analyzed as a uniform group. Cutoff values were calculated according to P_{min} approach. *Numbers in parentheses*, the number of recurrences or deaths/total in each group.

Furthermore, we observed that higher Cat L concentration was associated with advanced (T_{3+4} and S_{III+IV}) disease, as opposed to early (T_{1+2} and S_{I+II}) disease. It could be speculated that the increased production and/or secretion of Cat molecules is a consequence of progressive dedifferentiation and/or destruction of cells and of changes in the malignant potential in tumors of a higher T stage and overall TNM stage. This is in agreement with the results obtained by Russo *et al.* (33), who reported higher Cat B and L tumor activity to be associated with DNA aneuploid multiclonal (*i.e.*, more malignant) tumors compared with DNA aneuploid monoclonal or diploid (*i.e.*, less malignant) tumors. The trend in the differences in Cat tumor levels observed between the patients with and without disease recurrence in the present group and in the reference group (34) supports these findings.

Survival analysis in the reference group showed that patients with low tumor Cat L levels survived significantly longer than those with high enzyme levels. However, after correcting the P_{min} as proposed by Altman *et al.* (44), these differences lost their statistical significance. The results in the present group also deny the prognostic significance of Cat L, although the trend toward longer survival was also limited to lower levels of the enzyme. For Cat B, neither a significant difference nor a trend were observed in the studied groups. On the contrary, Russo *et al.* (33) reported the high tumor:mucosa ratio of Cat B activity to be significantly related to the risk of relapse in laryngeal cancer. In addition, longer survival probability was found to be related to a low tumor or serum concentration and/or activity levels of either Cats in patients with malignant melanoma (15) and breast (17–20), colorectal (22, 23), lung (24–26), gastric (28), and brain (29) cancer. The only exception is the study of Budihna *et al.* (16), in which an inverse relationship between Cat B and prognosis in breast carcinoma was observed.

Because of the absence of other histologies among the above listed tumors [*i.e.*, sarcoma, lymphoma, hepatocellular, transitional cell, and skin (excluding melanoma) carcinoma, hypernephroma, and so forth; Refs. 16–25, 28], one could speculate that the prognostic relevance of Cats B and L might be determined by histological tumor type. However, a rather small incidence of some tumor types and/or small number of patients in certain studies, including ours, might be the reason for the false-negative results. In addition, different methods used for the detection of Cats, *i.e.*, recognizing different forms of the enzymes, may also be a source of discrepancies when evaluating Cats' prognostic role in various types of cancers. For example, Werle *et al.* (50) found only a fraction of Cat B active at a physiological pH of 7.5 to be of prognostic significance in squamous cell carcinoma of the human lung.

Stefs A and B. We found that only Stef A, but not Stef B, concentration differed significantly between tumor and normal tissue. However, it was not changed to the extent as in Cat L and particularly in Cat B (1.5 *versus* 2.5 *versus* 5.2). Reports on cysteine proteinase inhibitors are contradictory regarding their levels and/or activity in malignant tissue compared with normal tissue (3). These discrepancies could be attributed to different causes of changed inhibitory capacity of Stefs associated with the malignant transformation of cells, such as: (*a*) reduced or insufficiently elevated Stef concentrations to effi-

ciently compensate for increased Cats (18); and (*b*) changes in the molecular structure of Stefs without a change in their total concentration, thereby decreasing their affinity for the targeted Cats (51). The resulting imbalance between Cat and Stef components of the proteolytic cascade is of critical importance for tumor cell invasion after proteolytic degradation of extracellular matrix components (18, 25, 52).

Moreover, a strong correlation has been found between Stef A and Stef B tumor concentrations, indicating that in individual tumors the expression of these two inhibitors is, to some extent, related. Comparing the concentrations of both Stefs, we have found that, in tumor and in normal tissue, Stef A content was approximately 1.6- and 1.1-fold, respectively, more than the content of Stef B. The corresponding numbers for the reference group were 1.7 and 1.5. The relationship between the levels of Stefs A and B found in malignant and normal tissue, which were both of squamous cell origin, is in accordance with the reported differences in the distribution of inhibitors among various tissues: the presence of Stef B in different tissues is relatively uniform, whereas Stef A is abundant primarily in various types of epithelial cells and in some cell types of the lymphoid tissue (2, 3).

In the present group, higher concentrations of both Stefs correlated significantly with less aggressive forms of disease, *i.e.*, lower node (N_0) and overall TNM stages (S_{I+II}). In the reference group, on the other hand, a higher content of both Stefs was measured in advanced stages of disease (34). Nevertheless, the protective role of the studied inhibitors found in the present study was also confirmed by the increased Stef A and Stef B concentrations in tumors of patients without recurrence of the disease. In addition, the results of univariate survival analysis in the present group and in the reference group showed that patients with Stef A or B concentration above the calculated cutoff concentrations do significantly better than those with a lower concentration of either inhibitor. The concept of the protective role of Stef A and Stef B is further supported by the survival results of Lah *et al.* (18) in breast carcinoma and by Knoch *et al.* (25) and Ebert *et al.* (27) in lung carcinoma. The only study that contradicts this assumption is that of Kuopio *et al.* (21), in which the risk of breast-cancer-related deaths was found to be associated with Stef A-positive immunohistochemical staining of neoplastic cell subpopulations within the tumors. In contrast to the latter report, the type of analysis used in the above listed studies (18, 25, 27), including ours (*i.e.*, measurements from homogenized tumor tissue specimens), does not allow for differentiation between neoplastic and nonneoplastic cells to be the source of Stef A. Because of this difference, the opposing results of these studies should be interpreted with caution and require further evaluation.

The present study and that of Budihna *et al.* (*i.e.*, the reference group; Ref. 34) are the first studies evaluating the prognostic value of these two inhibitors in SCCHN. The most important finding is that high levels of both Stefs in tumor tissue, and especially Stef A, predict longer survival of patients with this type of cancer. This statement is based on several facts: (*a*) in two independent groups, *i.e.*, the present and the reference one, the probability of survival depended significantly on tumor Stefs concentrations; (*b*) even after applying the correction formula for P_{min}, the differences in survival probability remained statistically significant for Stef A in both groups, and for Stef B in the present group; (*c*) in the case of Stef A, the optimal cutoff point was exactly the same in the present and the reference groups, *i.e.*, the 29th percentile in both groups; (*d*) using the standardized values of inhibitor concentrations, both Stefs retained their prognostic significance at the calculated cutoff points, which for Stef A, was very close to that for the concentrations measured, *i.e.*, the 30th percentile. In the case of Stef A, P_{cor} also reached the level of statistical significance; and (*e*) in multivariate analysis, the standardized values of Stef A turned out to be the most significant predictor of both the DFS and the DSS.

In conclusion, our data provide evidence that cysteine proteinases Cats B and L and their endogenous inhibitors Stefs A and B are implicated in the invasive behavior of SCCHN. In addition, higher tumor levels of both Stefs correlated significantly with longer survival probability in univariate survival analysis, and Stef A proved to be a reliable prognosticator in multivariate survival analysis. On the basis of the presented results, additional studies are required: (*a*) to evaluate the prognostic relevance of both Stefs—and that of Stef A in particular—in a more homogeneous and larger group of patients with SCCHN; and (b) to determine the value of their clinical applicability with respect to the selection of treatment in individual patients with this type of cancer.

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Prognostic Significance of Cysteine Proteinases Cathepsins B and L and Their Endogenous Inhibitors Stefins A and B in Patients with Squamous Cell Carcinoma of the Head and Neck

Primoz Strojan, Marjan Budihna, Lojze Smid, et al.

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