Radiol Oncol 2002; 36(2): 145-51.

Cysteine proteinase inhibitors stefin A and stefin B in operable carcinoma of the head and neck

Primož Strojan[1], Marjan Budihna[1], Lojze Šmid[2], Branka Svetic[3], Ivan Vrhovec[3], Janko Kos[4,5], Janez Škrk[6]

[1]Department of Radiotherapy, [3]Department of Biochemistry, [6]Department of Tumor Biology, Institute of Oncology, Ljubljana, Slovenia, [2]Department of Otorhinolaryngology and Cervicofacial Surgery, University Medical Centre, Ljubljana, Slovenia
[4]Department of Biochemistry and Molecular Biology, Jožef Stefan Institute, Ljubljana Slovenia
[5]Department of Biochemical Research and Drug Design, Research and Development Division, KRKA, d.d., Ljubljana, Slovenia

Purpose. *To evaluate the significance of cysteine proteinase inhibitors stefins (Stefs) A and B for a treatment decision and prognosis in operable squamous cell carcinoma of the head and neck (SCCHN).*
Patients and methods. *Stefs A and B concentrations were determined immunobiochemically using ELISAs in cytosols prepared from the tumor and adjacent normal mucosa from 91 patients with operable SCCHN. The median follow-up period of patients alive at the close-out date was 5.8 years (range, 5-9.3 years).*
Results. *Stef A concentrations were significantly higher in tumor compared to normal mucosa (P=0.05). When a subgroup with clinically palpable node(s) at presentation was taken into consideration (n = 57), a significant difference in Stef A (P = 0.03) and Stef B (P = 0.02) concentrations between those with negative and positive necks, as determined on histopathological examination, was observed. On the univariate survival analysis, higher Stefs' concentrations turned to be prognostically advantageous. Stef A proved its independent prognostic significance also on multivariate setting.*
Conclusions. *With the capability to differentiate between the pN0- and pN+-stages of the disease in the patients originally presented as node-positive, Stefs A and B could be useful markers when deciding on the extent of neck surgery. In addition, both Stefs proved to be reliable prognosticators for survival in patients with operable SCCHN.*

Key words: head and neck neoplasms; carcinoma, squamous cell; cysteine proteinases inhibitors

Received 22 March 2002
Accepted 29 April 2002

Correspondence to: Assist. Prof. Primož Strojan, M.D., Ph.D., Department of Radiation Oncology, Institute of Oncology, Zaloška 2, SI-1000 Ljubljana, Slovenia; Phone: +386 1 232 3063; Fax: +386 1 431 41 80; E-mail: pstrojan@onko-i.si

Acknowledgement: This work was supported by the Ministry of Science and Technology of the Republic of Slovenia Grant J3-3010-0302-01.

Introduction

Alterations in the balance between cysteine proteinases and their endogenous inhibitors have been postulated to contribute to malignant progression. A large body of literature has been accumulating evidence to suggest that they could be used as prognostic markers in a large spectrum of benign and malignant diseases.[1] However, their prognostic significance in the squamous cell carcinoma of the head and neck (SCCHN) was much less investigated. Our research team conducted the most extensive research in this field and arrived at the conclusion that the cytosolic concentrations of the inhibitors stefins (Stefs) A and B were strongly related to the survival probability.[2] The results on their serum concentrations as well as those related to proteinases were negative in this respect.[2,3]

The present study is a re-analysis of the data on Stefs A and B in patients with exclusively operable disease, exploiting the advantage of longer follow-up. In addition to the prognostic value of Stefs A and B, their significance for a treatment decision was analysed.

Patients and methods

Patients

Ninety-one previously untreated patients with primary operable squamous cell carcinoma of the head and neck entered the study. The routine diagnostic work-up comprised a clinical examination and endoscopy of the aerodigestive tract, chest X-ray, and standard haematological and biochemical tests. In all patients, therapeutic surgery of the primary tumour related to the lesion extension, and neck node dissection were performed. Postoperative radiotherapy was applied in all but nine patients with low-risk disease. The radiotherapy doses were adapted to the disease extent and ranged between 50-66 Gy (median, 56 Gy). Patients were irradiated on a Cobalt-60 unit or a 5-MV linear accelerator, with a daily dose of 1.8-2 Gy, 5 days per week. Tumors were staged after the histopathological examination of surgical specimens (pathological stage) according to UICC TNM classification[4], clinical N-stage before surgery was also recorded. The histopathological grade was defined according to WHO criteria.[5] Clinical features of the patients and their tumors are shown in Table 1.

As of October 31, 2001 (close-out date), 53 patients died: 22 due to the disease recurrence and/or dissemination and 31 due to causes other than the treated malignant disease. Thirty-three patients were alive with no signs of the disease and five patients were lost from follow-up; they were considered to be ineligible for the analysis of survival. The median follow-up period of all eligible patients as calculated from the date of surgery was 4.3 years

Table 1. Patient and tumor characteristics ($n = 91$)

Patients

Age: Median, 58 years (range, 37-72 years)

Sex: Female, 6; Male, 85

Tumors

Site: Oral cavity, 16
Oropharynx, 21
Hypopharyngis, 11
Larynx, 43

pTNM-stage:

	pN0	pN1	pN2	pN3	total
pT1	3	2	2	0	7
pT2	13	4	13	1	31
pT3	15	2	11	0	28
pT4	10	2	13	0	25
total	41	10	39	1	91

Degree of differentiation: $Grade_1$, 1
$Grade_2$, 71
$Grade_3$, 12
$Grade_x$, 7

Extracapsular extension: 34[a]

Tumor emboli in lymph node vessels: 7[a]

[a] pN+ patient only, n = 50 .

(range, 0.1-9.3 years), and those alive at the last follow up examination was 5.8 years (range, 5-9.3 years).

Biochemical analysis of stefins

For biochemical analysis of Stef A and Stef B, tissue specimens from the primary tumor and adjacent normal mucosa (matched pairs) were collected during surgery. The tissue cytosols were prepared as described in details elsewhere.[6] For quantitative analysis of Stefs A and B in tissue cytosols, commercially available ELISAs (KRKA d.d., Novo mesto, Slovenia) were used, as developed at Jožef Stefan Institute, Ljubljana, Slovenia.[6] The concentrations of Stefs in tissue cytosols were expressed as ng/mg of total protein (ng/mgp).

Statistical analysis

The differences between the median concentrations of each of the Stefs in match pairs and various prognostic groups were determined using nonparametric Wilcoxon signed rank test and Mann-Whitney U-test. In the analysis of the disease-free survival (DFS, local and/or regional recurrence and/or systemic dissemination considered as event) and the disease-specific survival (DSS, deaths from disease-unrelated causes censored), Kaplan-Meier product-limit method[7] was used, and the differences between the groups were tested by the log-rank test.[8] The patients were grouped according to the cut-off concentrations of Stef A and Stef B, at which maximal differences in the survival rates were determined. All tests were two-sided and the results were considered statistically significant at the probability level of 0.05.

Results

The distribution of Stefs A and B concentrations in matched pairs is represented in Figure 1. Stef A concentrations were significantly higher in tumors compared to normal mucosa (467 *vs.* 346 ng/mgp, $P = 0.05$); however, the difference in Stef B concentrations was not significant (285 *vs.* 269 ng/mgp, $P > 0.05$). At presentation, 34 patients were staged as node-negative (clinical stage, cN_0) and 57 as node positive (cN_+). When a subgroup with clinically positive neck nodes was taken into consideration, a significant difference in Stef A (536 *vs.* 380 ng/mgp, $P = 0.03$) and Stef B (382 *vs.* 240 ng/mgp, $P = 0.02$) concentrations was observed between those with negative and those with positive necks, as determined by histopathological examination after surgery (Figure 2). In node-negative subgroup, however, this difference didn't reach the level of statistical significance. No statistically important relationship was observed between

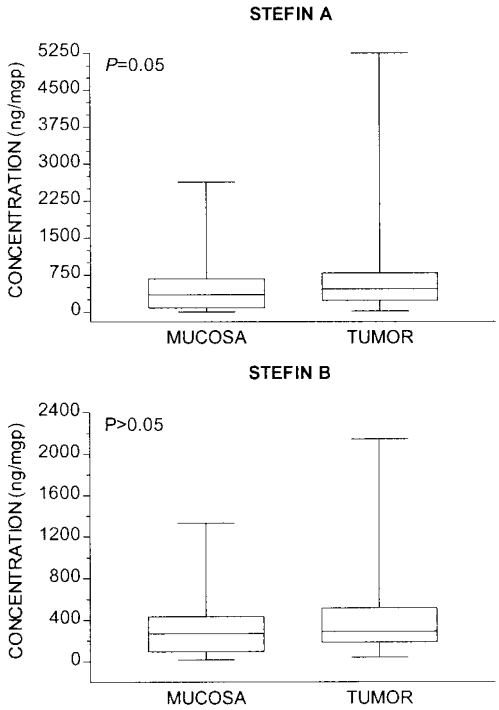

Figure 1. Concentrations of stefins in tumor and normal mucosa (n = 91). The top and the bottom of the box represent the 25th and 75th percentiles, respectively, and the ends of the bars represent the rang. The line in the box is the median value.

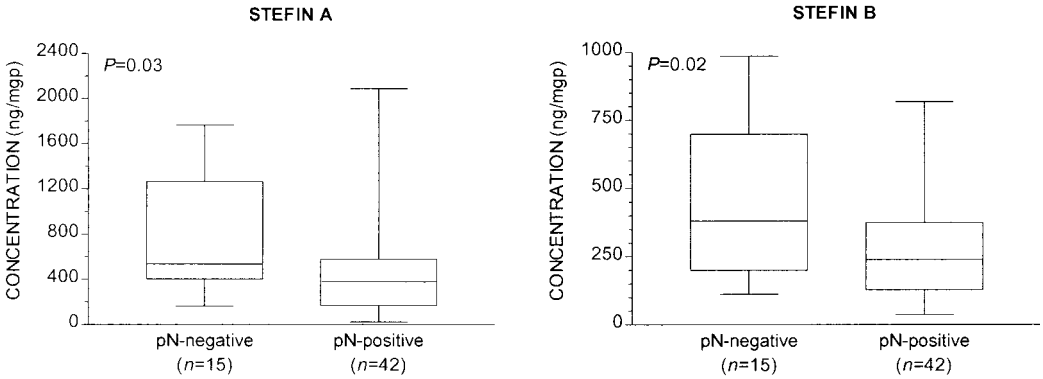

Figure 2. Distribution of tumor concentrations of stefins between patients with histopathologically determined negative and positive necks, as measured in a group with clinically palpable nodes at presentation. The top and the bottom of the box represent the 25th and 75th percentiles, respectively, and the ends of the bars represent the rang. The line in the box is the median value. n, number of samples.

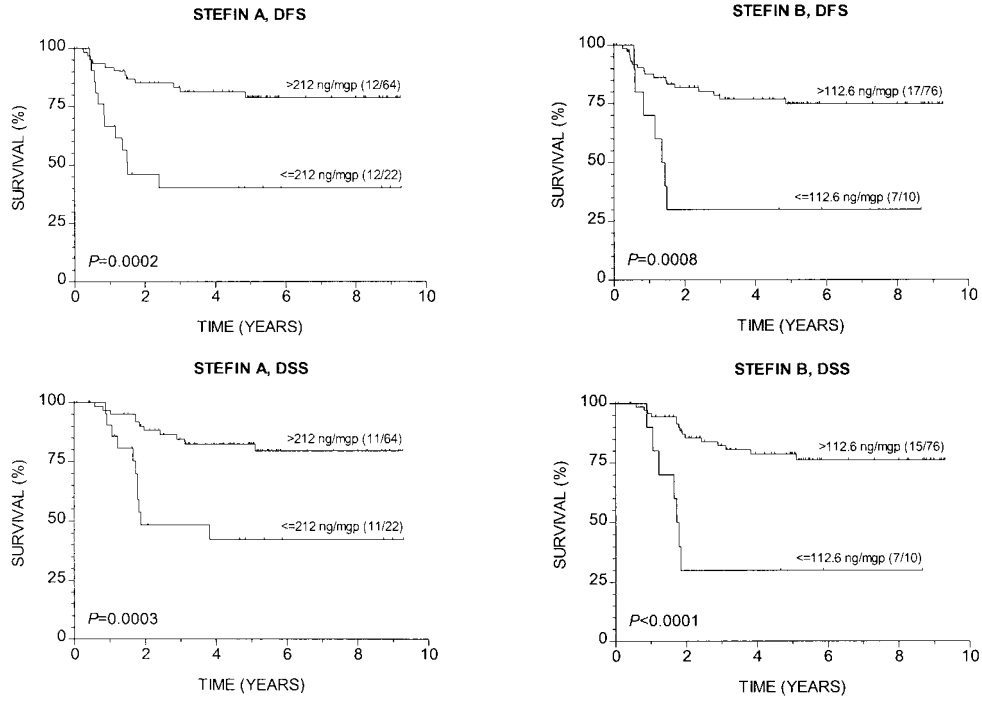

Figure 3. Disease-free survival (DFS) and disease-specific survival (DSS) of patients with respect to the optimal cut-off concentrations of stefin A and stefin B. The numbers in parentheses indicate the number of events/total in each group.

Stef concentrations and other established prognostic factors.

On univariate analysis, longer DFS and DSS correlated with higher concentrations of Stef A (P_{DFS} = 0.0002, P_{DSS} = 0.0003) and Stef B (P_{DFS} = 0.0008, P_{DSS} < 0.0001) (Figure 3). In addition, the pN_0- and lower overall stage of the disease, the absence of extracapsular extension and tumor emboli in lymph node vessels were also harbingers of favourable outcome

Table 2. Univariate and multivariate analysis of survival ($n = 86$)

Variable		Disease-free survival				Disease-specific survival			
		Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	n	%, 5-yr	*P*-value	*P*-value	RR	%, 5-yr	*P*-value	*P*-value	RR
Stefin A									
≤ 212 ng/mgp	22	40	0.0002	0.02	0.31	42	0.0003	0.04	0.35
> 212 ng/mgp	64	79				83			
Stefin B									
≤ 112.6 ng/mgp	10	30	0.0008	NS		30	<0.0001	0.06	0.33
> 112.6 ng/mgp	76	75				79			
Age									
≤58 yrs	43	73	NS	–		75	NS	–	
> 58 yrs	43	65				68			
pT-stage									
T_{1-3}	62	73	NS	–		79	NS	–	
T_4	24	60				66			
pN-stage									
N_0	37	80	0.08	NS		88	0.01	NS	
N_{1-3}	49	61				60			
pTNM									
$Stage_{I-III}$	36	85	0.01	NS		91	0.001	0.07	3.84
$Stage_{IV}$	50	57				59			
Tumor site									
Larynx	38	77	NS	–		80	NS	–	
Non-larynx[a]	48	64				66			
Tumor differentiation									
$Grade_{1-2}$	70	70	NS	–		74	NS	–	
$Grade_3$	12	73				73			
Extracapsular spread									
Negative	52	81	0.003	0.05	4.71	87	0.0003	0.03	6.13
Positive	34	50				47			
Tumor emboli									
Negative	76	71	0.03	NS		75	0.03	NS	
Positive	7	50				33			

[a]Oral cavity, oropharynx and hypopharynx.

n, Number of patients; RR, Risk ratio; NS, Not significant.

(Table 2). Radiation dose and other classical prognostic factors didn't come out as prognostically important. In Cox multivariate regression analysis for DFS and DSS, only Stef A tumor concentrations ($P_{DFS} = 0.02$, $P_{DSS} = 0.04$) and extracapsular extension were retained in the final model (Table 2).

Discussion

In the present study we showed that Stef A and Stef B concentrations could be useful markers when deciding on treatment intensity, and reliable prognosticators in patients with SCCHN. With the analysis restricted exclusively to the patients with operable disease and

the maturity of follow-up data, places a higher emphasis on the reliability of present results.

As in our previous study on more heterogeneous group of patients,[2] only Stef A, but not Stef B, concentrations differed between tumor and normal mucosa. However, in numerous other reports on their levels and/or activity in malignant tissue compared with normal tissue the results are inconclusive, too.[1] It seems that a significant elevation of inhibitor production is not the only option when proteolytic activity in tumor tissue is increased: it is more likely that the ratio between active/non-active or functioning/malfunctioning inhibitor molecules determines the net proteolytic potential in the cells.

The new and the most important finding in the present study is that, in patients with clinically positive neck nodes at presentation (cN_+), Stef A and Stef B concentrations emerged as reliable predictors of lymph-node involvement with tumor cells. From clinical point of view, this differentiation is of utmost importance because, in a considerable proportion of patients with SCCHN (in the present series, 26 %), the nodes are enlarged due to inflammation rather than tumor infiltration. Those patients could be spared of more aggressive therapy, i.e. extensive neck surgery and/or high-dose radiotherapy, and treatment related side-effects. In this context, even though there is an overlap of individual values of inhibitor concentrations between those with pathologically positive and negative necks, Stefs alone or in combination with other biological or clinical markers that would increase their diagnostic accuracy warrant further evaluation.

In patients with clinically undetectable nodes at diagnosis (cN_0), Stefs had no potential to predict pN-stage of the disease, which is not of critical importance from clinical perspective. In this group, 76% of patients were without tumor cells at histopathological examination after neck dissection. If surgery is technically correctly performed, postoperative radiotherapy is not indicated and its side effects could be avoided. Only a minority of patients (24 %) were found to have a microscopic tumor deposits in the neck nodes which are highly curable with a moderate-dose radiotherapy, i.e. ≥ 95 % cure rate with 50 Gy.[9] As the pattern of spread of neoplastic cells from the primary tumor to regional lymph nodes is predictable,[10] the risk of geographic miss could be neglected. In addition, those neck regions with the highest risk for bearing micrometastases are usually in immediate vicinity of the primary. The most welcome consequence is that when there is an indication for primary to be irradiated postoperatively, the majority if not all nodal basins at risk are also included in the high-dose irradiation volume. Following these propositions, neck surgery is not always necessary prior to irradiation and these patients can be spared of its morbidity.

The results of univariate analysis of the survival showed that the patients with Stef A or Stef B concentrations above the calculated cut-off concentrations do significantly better than those with lower concentrations of either inhibitor. In addition, Stef A tumor concentration turned to be independent prognosticator for the risk of relapse and death in our group of patients. These results are consistent with our earlier observation[2] and further support the concept of protective role of Stefs A and B, previously raised in the studies on carcinoma of the breast[11] and lung.[12] The studies that contradict this assumption are those by Kuopio et al.[13] on breast cancer and by Kos et al.[14] on colorectal cancer. However, in the first, the Stefs' content was determined immunohistochemically, while in the other, their extracellular, i.e. serum concentrations were measured, which may reflect the involvement of Stefs in mechanisms other than the control of extracellular matrix degradation and invasion of tumor cells. Because the origin of inhibitor molecules in different sample types and/or modes of quantification of their content diffe-

red substantially from the type of analysis used in our and related reports,[11,12] a simple comparison would be inadmissible. According to our experience, the prognostic potential of immunohistochemically determined Stef expression and their serum concentrations in SCCHN is yet to be defined.[3,15]

On the basis of the presented results our conclusions would be as follows: (1) With the capability to differentiate between pathologically positive and negative necks in patients originally presented as node-positive, Stef A and Stef B could be useful markers when deciding on the extent of neck surgery; (2) Stefs A and B proved to be reliable prognosticators for the survival of patients with operable SCCHN.

References

1. Kos J, Lah TT. Cysteine proteinases and their endogenous inhibitors: target proteins for prognosis, diagnosis and therapy in cancer. [Review]. *Oncol Rep* 1998; **5:** 1349-61.

2. Strojan P, Budihna M, Šmid L, Svetic B, Vrhovec I, Kos J, et al. 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. *Clin Cancer Res* 2000; **6:** 1052-62.

3. Strojan P, Budihna M, Šmid L, Svetic B, Vrhovec I, Škrk J. Cathepsin B and L and stefin A and B levels as serum tumour markers in squamous cell carcinoma of the head and neck. *Neoplasma* 2001; **48:** 66-71.

4. Sobin LH, Wittekind Ch. TNM classification of malignant tumours. *International Union Against Cancer (UICC)*. 5th ed. New York: Wiley-Liss; 1997. p. 20-37.

5. Azzopardi JG, Chepizk OF, Hartman WH. *International histological classification of tumours no.2: histological typing of breast tumours.* 2nd ed. Geneva: World Health Organisation; 1981.

6. Kos J, Šmid A, Krašovec M, Svetic B, Lenarčič B, Vrhovec I, et al. Lysosomal proteases cathepsins D, B, H, L and their inhibitors stefins A and B in head and neck cancer. *Biol Chem Hoppe-Seyler* 1995; **376:** 401-5.

7. Kaplan EL, Meier P. Nonparametric estimation from incomplete observation. *J Am Stat Assoc* 1958; **53:** 457-81.

8. Peto R, Pike MC, Armitage P, Breslow NE, Cox DR, Howard SV, et al. Design and analysis of randomised clinical trials requiring prolonged observation of each patients. II. Analysis and examples. *Br J Cancer* 1977; **35:** 1-39.

9. Withers HR, Peters LJ, Taylor JMGP. Dose-response relationship for radiation therapy of subclinical disease. *Int J Radiat Oncol Biol Phys* 1995; **31:** 353-9.

10. Grégoir V, Coche E, Cosnard G, Hamoir M, Reychler. Selection and delineation of lymph node target volumes in head and neck conformal radiotherapy. Proposal for standardizing terminology and procedure based on the surgical experience. *Radiother Oncol* 2000; **56:** 135-50.

11. Lah TT, Kos J, Blejec A, Frkovič-Georgijo S, Golouh R, Vrhovec I, et al. The expression of lysosomal proteinases and their inhibitors in breast cancer: possible relationship to prognosis of the disease. *Pathol Oncol Res* 1997; **3:** 89-99.

12. Ebert E, Werle B, Jülke B, Kopitar-Jerala N, Kos J, Lah T, et al. Expression of cysteine proteinase inhibitors stefin A, stefin B and cystatin C in human lung tumors. *Adv Exp Med Biol* 1997; **421:** 259-65.

13. Kuopio T, Kankaanranta A, Jalava P, Kronqvist P, Kotkansalo T, Weber E, et al. Cysteine proteinase inhibitor cystatin A in breast cancer. *Cancer Res* 1998; **58:** 432-6.

14. Kos J, Krašovec M, Cimerman N, Nielsen HJ, Christensen IJ, Brünner N. Cysteine proteinase inhibitors stefin A, stefin B, and cystatin C in sera from patients with colorectal cancer: relation to prognosis. *Clin Cancer Res* 2001; **6:** 505-11.

15. Strojan P, Šmid L, Budihna M, Gale N, Svetic B, Vrhovec I, et al. The expression of stefins A and B in supraglottic carcinoma: immunobiochemical and immunohistochemical study. In: Bosatra A, Gale N, Michaels L, Pavelić K, Vizjak A, Zidar N, et al, editors. *Epithelial tumours of the head and neck. Proceeding of the XXXIst memorial meeting for professor Janez Plečnik, Ljubljana 2000.* Ljubljana: Institute of Pathology, Faculty of Medicine University of Ljubljana; 2000. p. 38-42.