Radiol Oncol 2008; 42(2): 69-81. *doi:10.2478/v10019-008-0006-4*

Cysteine cathepsins and stefins in head and neck cancer: an update of clinical studies

Primož Strojan

Department of Radiation Oncology, Institute of Oncology, Ljubljana, Slovenia

Background. *Cancer of the head and neck represents a diverse group of malignant diseases; so far, no factor in a wide spectrum of biochemical and histological candidate-markers has yet been identified to predict reliably the natural course of the disease or its response to the therapy to be used in routine clinical practice. Among the factors that promote tumor growth and invasion, several protease systems, implemented in proteolytic degradation of extracellular matrix components, were studied, including papain-like lysosomal cysteine proteases (e.g. cathepsins B and L) and their physiological inhibitors cystatins (e.g. stefins A and B, cystatin C). The aim of the present report is to review the published studies on clinical applicability of cysteine cathepsins and their endogenous inhibitors stefins in squamous cell carcinoma of the head and neck and to present recent research results from this area conducted jointly by the Institute of Oncology Ljubljana and ENT Department of the University Medical Center Ljubljana, Slovenia.*

Conclusions. *According to our experience, immunohistochemical staining of cysteine cathepsins and stefins seems to be of limited value for predicting either treatment response or patients' survival. However, the results of studies on stefin A in tumor tissue cytosols should be considered hypothesis-generating and deserves further evaluation in the frame of prospective controlled multicentric clinical study.*

Key words: head and neck cancer; cathepsins; stefins; prognosis

Introduction

Cancer of the head and neck represents a diverse group of malignant diseases arising from mucosa of the upper aerodigestive tract, major salivary glands and nodes from the neck. The majority of tumors is of squamous cell origin and alcohol and tobacco abuse are the two most important etiological factors. Surgery and radiotherapy are standard treatment options with systemic therapy being added to irradiation of the patients with increased risk for disease recurrence.[1]

To distinguish biologically more aggressive and less aggressive head and neck carcinomas within each traditional risk-category, numerous new prognostic factors were evaluated on genetic, mRNA or protein levels. Among the factors that promote tumor growth and invasion, several protease

Received 16 May 2008
Accepted 23 May 2008

Correspondence to: Assoc. Prof. Primož Strojan, M.D., Ph.D., Department of Radiation Oncology, Institute of Oncology, Zaloška 2, SI-1000 Ljubljana, Slovenia; Phone: +386 1 5879 110; Fax: + 386 1 5879 400; E-mail: pstrojan@onko-i.si

The research was supported by the Slovenian Research Agency Grant P3-0307. The article was presented at the 5th Conference on Experimental and Translational Oncology, Kranjska gora, Slovenia, March 26-30, 2008.

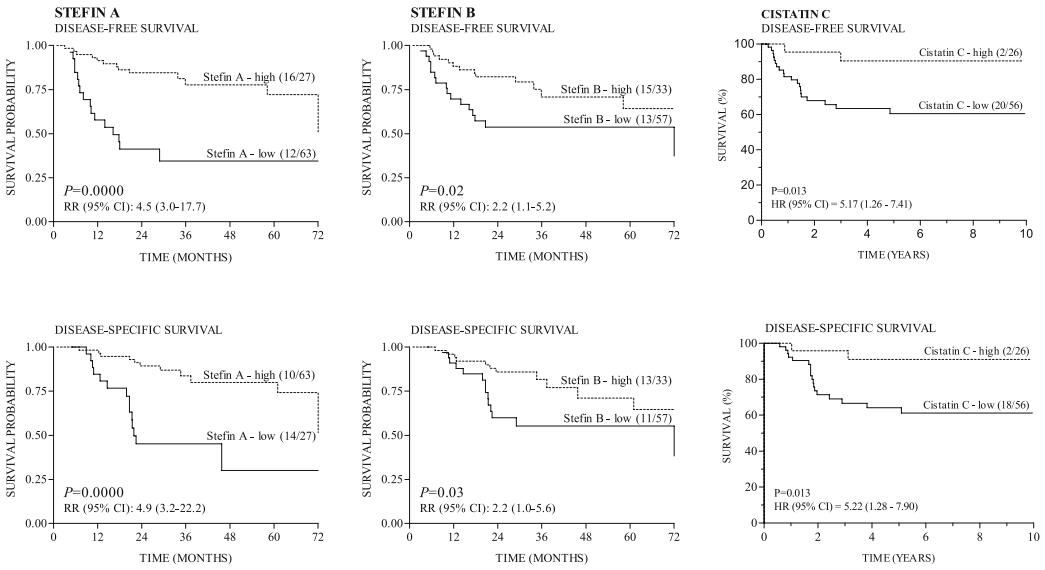

Figure 1. Actuarial disease-free survival and disease-specific survival as a function of stefin A, stefin B, and cystatin C status. The numbers in parentheses indicate the number of recurrences or deaths/total in each group.

systems, implemented in proteolytic degradation of extracellular matrix components, were studied, including papain-like lysosomal cysteine proteases, such as cathepsins B (CB) and L (CL), and their physiological inhibitors cystatins (e.g. stefins A [SA] and B [SB], cystatin C [CC]).[2] Recently, the involvement of cysteine cathepsins and stefins in apoptotic death of tumor cells, triggered also by irradiation and chemotherapeutics, was confirmed in several systems.[3]

The aim of the present report is to review the published studies on clinical applicability of cysteine cathepsins and their endogenous inhibitors stefins in squamous cell carcinoma of the head and neck and to present recent research results from this area collected jointly at the Institute of Oncology Ljubljana and ENT Department of the University Medical Center Ljubljana, Slovenia. In all our studies, the same kits of reagents were used for the determination of studied cathepsins and stefins, *i.e.* the commercially available ELISAs developed at the Jožef Stefan Institute.[4]

What do we know?

At the moment, only cytosolic concentrations of cystatins from the tissue of operable head and neck carcinomas were found to correlate with the patients' survival. In our initial set of studies, high levels of SA, SB and CC in tissue homogenates from two independent, but smaller prospective cohorts of patients appeared prognostically advantageous (Table 1, Figure 1).[5-7] The issue of the protective role of high levels of cysteine protease inhibitors in tissue homogenates was raised also following the survival analysis of the patients with breast[8] and lung[9,10] carcinoma.

The results of the studies on the serine protease system inhibitor (plasminogen activator inhibitor type 1, PAI-1) in tumor tissue extracts of breast carcinoma,[11] SA immunohistochemistry in breast cancer sections,[12] and on various cystatins from the serum of patients with colorectal carcinoma,[13] lung carcinoma and non-Hodgkin's lymphomas[14] are contrary to the above hy-

Table 1. Clinical studies on cysteine cathepsins and their endogenous inhibitors in tissue cytosols conducted at the Institute of Oncology Ljubljana and ENT Department of the University Medical Center Ljubljana, Slovenia, 1995 – 2007

Study details	Study no., Year		
	I, 1995	II, 1998	III, 2006
No. of patients	45	49	92
Sex (female/male)	2/43	4/45	9/83
Age (in years)[1]	55 (40 – 69)	60 (37 – 72)	59 (37 – 80)
Primary tumor site			
Larynx	25	20	43
Nonlarynx[2]	20	29	49
T-stage			
pT_{1+2}	14	23	33
pT_{3+4}	31	26	59
N-Stage			
pN_0	18	24	38
pN_{1-3}	27	25	54
Overall TNM stage			
S_{I+II}	7	10	18
S_{III+IV}	38	39	74
Extranodal tumor spread[3]			
Negative	6	9	27
Positive	19	16	27
Unknown	2	0	0
Mode of therapy			
Surgery	2	7	8
Surgery + radiotherapy	39	42	84
Radiotherapy	4	0	0
Analytical method	ELISA	ELISA	ELISA
Reference(s) No.	5	6, 7	27

[1]Median (range).

[2]Oral cavity, oropharynx, hypopharynx.

[3]pN_{1-3} patients only.

[4]Sandwich ELISAs, KRKA dd & Institute Jožef Stefan Ljubljana, Slovenia.

pothesis. However, the observed variations in the relationship between the cystatin levels and survival probability could be attributed to the differences between the serine and cysteine proteases in regulatory mechanisms operating during tumor progression,[15] to the inherent variations between the biological samples of different types, and to the systemic response to malignant disease, which influence also the extracel-

lular (*i.e.* serum) levels of cystatins.[13] The importance of variations in methodology used for the preparation of biological samples of different types and of their inherent characteristics influencing quantitative (and most probably also qualitative) relations between individual enzymes and inhibitors were clearly exposed in a comparative study on pairs of different biological samples obtained from the same patients with breast carcinoma. For example, the authors identified CB cytosolic levels, but not also CB immunostaining in tumor cells, as prognostically important.[16]

Much less data exist on the clinical applicability of cysteine cathepsins and stefins determined in other types of biological samples. In the serum, alterations in activity and concentration levels of studied enzymes and inhibitors between patients and healthy controls were found to be highly variable and influenced by other non-malignant disease conditions, mainly inflammational.[17-22] Thus, any interpretation of the results from pertinent studies would be only speculative. More convincing is the observation by a small but homogenous study group in regard to cancer type and treatment mode, reported by Kręcicki and Siewiński.[17] In 25 post-laryngectomy patients, serum CB-like activity was constantly declining, reaching normal values within four months post-surgery. In other 14 patients failing treatment, the mean serum values of CB activity dropped in the first month after surgery, but rapidly increased in the subsequent tests. The elevation had occurred at least two months before clinical evidence of metastases or recurrent tumor became apparent.[17] No persuasive evidence on the prognostic value of serum measurements of cysteine cathepsins and their inhibitors was provided so far.

The data on the immunohistochemically determined expression profile of cysteine cathepsins are available from a limited number of rather small series and only for oral cavity tumors, but not also for pharyngeal or laryngeal carcinomas; the same finding was also referred to their possible prognostic significance.[23-26] So far, to the best of our knowledge, stefins have not been subjected to immunohistochemical evaluation in any of the studies conducted on head and neck carcinomas. The results on spatial distribution of CB and CL immunoreactivity, with perinuclear positivity mainly manifested intracellularly and on the membrane surface outside the tumor cells, reflect their physiological role and are consistent throughout the studies.[23-26]

Clinical studies, 2006 – 2008

Tissue homogenate (cytosol)

With the aim to test prospectively the hypothesis about the protective role of high SA and SB levels in the patients with operable tumors, their concentrations were measured in tissue cytosols of non-tumorous mucosa and primary tumor from 92 patients.[27] All patients underwent curative surgery and 84 patients had postoperative radiotherapy. Fifty-nine (64%) tumors were staged as locally advanced pT3-T4, and nodal infiltration with tumor cells was determined in 54 (59%) cases, with extracapsular tumor spread in 27 of them.

Both stefins were found to be associated significantly with the disease-free survival probability only when exceeding a certain value. Thus, a flexible methodology for analyzing their effect – a "broken stick" model – was employed, with the advantage of avoiding arbitrary categorization and its subsequent loss of information:[28]

$$\beta(V - V_0)_+$$

(where V is the measured value, V_0 is the cut-off value and the *plus* denotes that only

Table 2. Concentrations of stefin A and stefin B in tissue cytosols of match-pairs of tumor and adjacent non-tumorous mucosa

Patients	Stefin A (ng/mgp)				Stefin B (ng/mgp)			
	N	Median	Range	P-value	*n*	Median	Range	P-value
All								
Mucosa	92	759.5	7 – 4878	0.36	92	187.5	6 – 1736	0.98
Tumor	92	795	80 – 5320		92	203.5	28 – 1974	
Upregulated[1]								
Mucosa[2]	53	244	7 – 4878	<0.0001	49	54	6 – 703	<0.0001
Tumor[3]	53	1059	115 – 5320		49	294	57 – 1974	
Downregulated cases[1]								
Mucosa[2]	39	1690	196 – 4877	<0.0001	43	388	58 – 1736	<0.0001
Tumor[3]	39	468	80 – 2074		43	167	28 – 495	

[1]Patients with increased (upregulated cases) and decreased (downregulated cases) concentration of inhibitor

In tumor compared to mucosa.

[2]Mucosa, upregulated cases *vs.* downregulated cases: stefin A, P<0.0001; stefin B, P<0.0001.

[3]Tumor, upregulated cases *vs.* downregulated cases: stefin A, P<0.0001; stefin B, P<0.004.

N, Number of samples.

the part where V is greater than V_0 is used). Both beta and V_0 were estimated simultaneously by maximizing the Cox partial likelihood in a model using no additional covariates.[28] The model assumed no effect of the log of stefin A up to the cut-point value, which was calculated to be the 64th percentile in the group, and a linear effect afterwards. In the multivariate analysis, a significant decrease in the risk of disease re-appearance to only 3% (*i.e.* by 97%) of the reference value was observed after doubling the stefin a concentration above the calculated cut-off. In the case of SB, all patients with an inhibitor value exceeding the cut-off point (the 78th percentile in the group) were censored and no further calculations were performed.

These results were reconfirmed after pooling the data with two historical data sets[5,6] into a uniform series of 182 patients. For each data set, we ranked the results of individual SA measurements; thus, the inhibitor levels were converted to fractional ranks (between 0 and 1) and the equal fractional ranks became comparable across the data sets.[11] Again, the optimal cut-off point for SA was found at the 63th percentile in the group, after which the risk of disease reappearance was reduced, reaching 53% of the reference value as the fractional rank of SA increased by 0.1 (Table 2).

The observed prognostic strength of SA forced us to study further the quantitative relationship between SA and SB and two cysteine cathepsins, which was simultaneously determined in the tissue homogenates from the same group of 92 patients, but had no impact on the patients' prognosis at all (Table 3). Analyzing the whole group of 92 samples, there was no differences observed in SA and SB concentrations between tu-

Table 3. Multivariate analysis on prognostic value of stefin A as determined in cytosols of tumor tissue: pooled analysis (N = 182)

	Disease-free survival		
Variable	**HR**	**95% CI**	**P-value**
Stefin A rank[1, 2]	0.53	0.35 – 0.82	0.004
Extracapsular extension			
Negative[3] *vs.* positive	2.44	1.31 – 4.52	0.005
pT-stage			
pT_{1+2+3} *vs.* pT_4	2.05	1.12 – 3.74	0.020
Primary tumor site			
Larynx *vs.* nonlarynx[4]	2.05	1.04 – 4.03	0.037

[1]After the threshold.
[2]The hazard ratio is given for a difference in 0.1 fractional rank.
[3]Patients without extension of tumor tissue beyond nodal capsule and those with pN_0-stage of disease were included.
[4]Oral cavity, oropharynx, hypopharynx.
HR, Hazard ratio; CI, Confidence interval.

mor and mucosa. However, after stratifying the patients according to SA (and SB as well) differences as calculated in matched pairs of tumor tissue and non-tumorous mucosa, SA was found upregulated in 53 patients (higher concentrations were measured in tumor samples than in non-tumorous mucosa) and was downregulated in 39 patients; the corresponding numbers for SB were 49 and 43, 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 concentration and the opposite was calculated for their tumor concentrations. Between SA and SB, a highly significant correlation was found when either mucosal (R_S=0.887, P<0.0001) or tumor (R_S=0.594, P<0.0001) concentrations were compared. The difference between tumor and mucosal SA and SB concentrations was congruent (*i.e.* both either positive or negative in the same patient) in 87% of patients. A significantly higher proportion of downregulated cases were found among the patients with disease re-appearance (70% *vs.* 35%, p=0.005) who had significantly lower tumor concentrations of SA and SB compared to those experiencing successful treatment. [27]

The crucial observation from this study would be that, in the patients with inherently low SA concentrations in non-tumorous mucosa (upregulated ceases), the CB and CL mucosal concentrations were significantly lower compared to those patients with high mucosal concentrations of SA (downregulated cases) (Figure 2). It seems that, in normal tissue, the ability of inhibitory component (*i.e.* stefins) of cysteine proteolytic system is well adapted to the proteolytic capacity of proteases (i.e. CB and CL), suggesting an active buffer role of stefins.

Further, we hypothesized that, after malignant transformation of previously normal mucosal cells with inherently low cathepsin

and stefin levels (upregulated group), a significant and synchronous increase on both enzymatic and inhibitory side of proteolytic tandem occurred, gaining a more favorable prognosis of these patients. On the other hand, in the patients with originally high levels of cysteine cathepsins and stefins in normal mucosa (downregulated group), the malignant transformation resulted in an additional raise of the enzymes not being followed by an adequate adjustment of the inhibitors. The concentrations of the latter were found to be even depressed significantly compared to those of mucosa. Such pattern of quantitative relationships in cysteine proteolytic system contributes to a switch in cellular mechanisms at different levels toward more invasive cell phenotypes, resulting in an increased risk for disease recurrence or systemic failures. Furthermore, because in tumor tissue, no difference in concentrations of either CB or CL was observed between the down- and upregulated cases, it appears that the proteolytic balance after the malignant transformation is mainly determined by the changes on the stefin side (Figure 3).

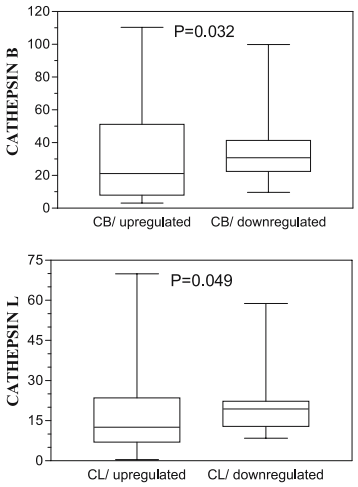

Figure 2. Cathepsin B and cathepsin L mucosal concentrations in patients grouped according to the stefin A difference as calculated in matched pairs of tumor tissue and non-tumorous mucosa.

Immunohistochemistry

Recently, we determined immunohistochemically the labeling pattern and expression profile of CB and CL and SA and SB in the tissue sections of 75 unresectable squamous cell carcinomas of the oropharynx treated with concomitant chemoradio-

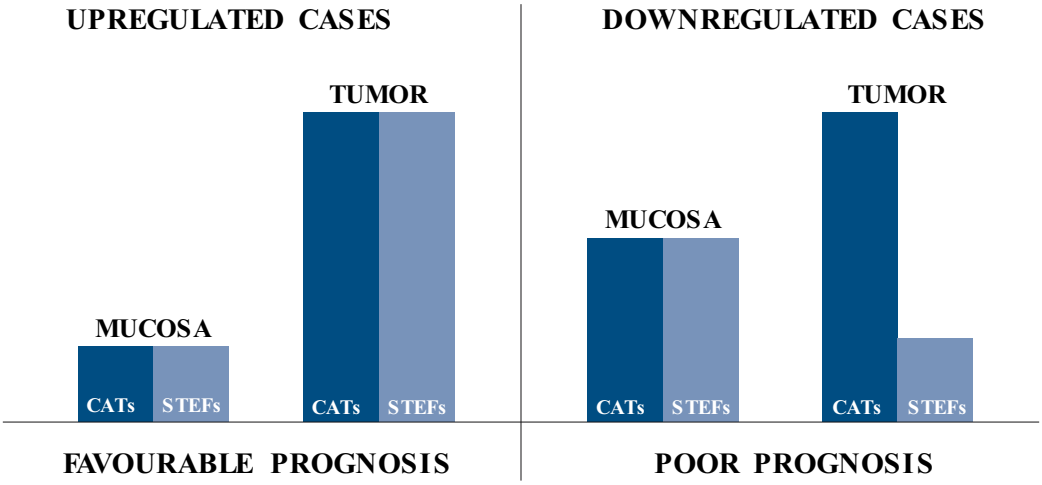

Figure 3. Relationship (schematic) between tumor and mucosal levels of cysteine cathepsins and stefins in down- and upregulated group of patients (in regard to the stefin A concentrations).

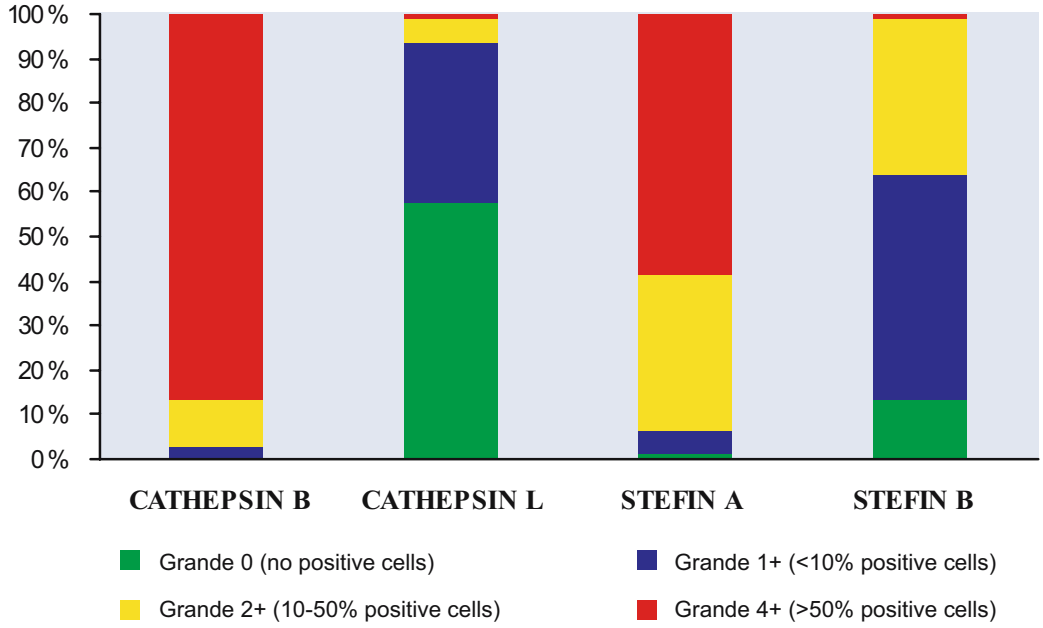

Figure 4. Immunohistochemical staining for cathepsins and stefins in tumor cells

therapy with mitomycin C and bleomycin. The secondary objective was to estimate the possible predictive and prognostic significance of the observed immunohistochemical reactions in this particular cancer type. The study population was intentionally homogenized by limiting the entry criteria to unresectable tumors of one subsite inside the upper aerodigestive tract, treated uniformly in order to minimize the impact of some well-established prognostic indicators on treatment results. According to the UICC TNM staging criteria, 67% of patients had stage IV disease. Because the intensity of immunohistochemical staining followed the variations in proportion of positively stained cells, as it was previously observed in breast (16) and rectal (29) carcinomas, a semiquantitative four grade (0–3+) scoring system was used for estimating the percentage of positively stained cells in tissue sections.

Tumor cells and stromal lymphocytes stained for all four studied parameters: in tumor cells, the most extensive staining was observed for CB and SA, whereas CL and SB yielded much lower immunoreactivity scores (Figure 4). The comparable CB and CL immunohistochemical profiles were described in the study on oral cavity tumors by Vigneswaran *et al.*,[23] whereas conflicting results from some other studies could have resulted from the differences in analytical procedures used (antigens, reagents), low sample numbers in some series,[24,25] and from the inherent biological characteristics of the site of tissue sampling (oral cavity *vs.* oropharynx *vs.* other tumor types).[6,7,27] The observed perinuclear cathepsin positivity mainly manifested intracellularly and on the membrane surface outside the tumor cells, was more consistent throughout the studies (Figure 5a).[23-26] Exclusively intracellular immunostaining for stefins reflected the lack of secretory signal sequences on corresponding genes (Figure 5b).[2] Contrary to our observation, in the sections of breast carcinoma tissue and malignant brain tu-

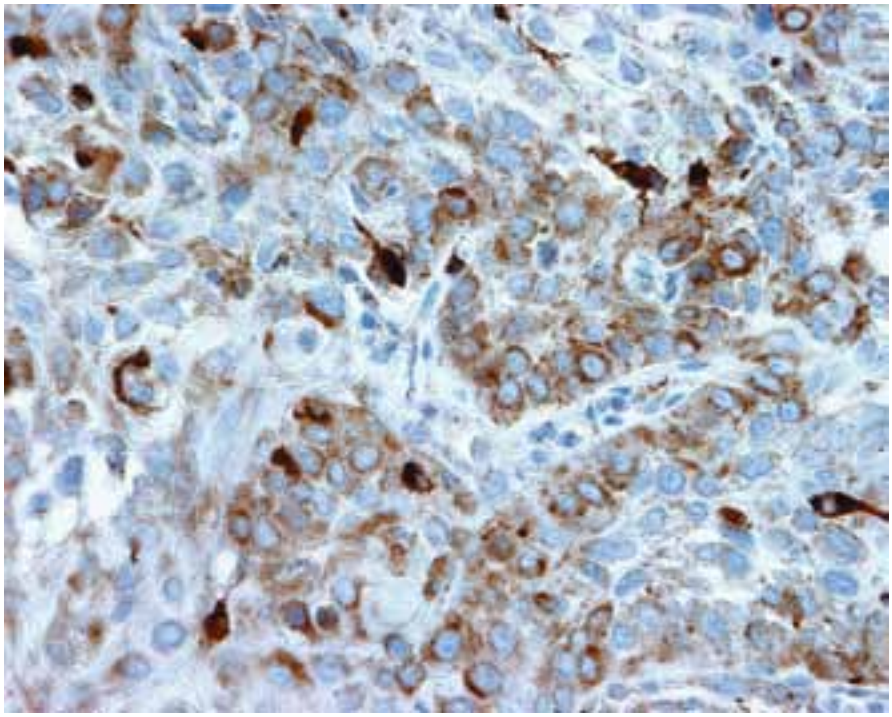

Figure 5a. Immunohistochemical staining for cathepsin B: predominant perinuclear pattern.

mors, SA and/or SB immunoreactivity was described in a minority of cases or was only sporadic.[6,12,30]

CB and SA scores were found to be predictive for the tumor origin within the oropharynx, and the balance in the tandem CB-SB inclined toward enzymatic component correlated with more advanced tumors (P=0.049) and residual disease two months after therapy (P=0.024). While the value of correlation observed between the CB and SA immunohistochemical scores and the origin of primary tumor is debatable, the domination of enzymatic over inhibitory component in the pair CB-SB linked to a more aggressive disease phenotype suggests a pivotal role of enzyme-to-inhibitor score ratio over the expression levels of individual parameters. The predictive significance of the cathepsin-stefin ratio for the incidence of pelvic metastases has also been reported for the prostate carcinoma.[31]

Playing an important role in apoptosis, in one of the basic mechanisms of tumor cell killing with irradiation and chemotherapeutics,[32] the high expression level of cathepsins and stefins was hypothesized to predict a favourable response to chemoradiation. However, the observed association between strong immunostaining for CB (or CB-SB tandem) and locoregional treatment failure two months after therapy contradicts the proapoptotic role of cysteine cathepsins suggested in preclinical studies.[3] The opposing roles of cysteine cathepsins in oral squamous cell carcinoma apoptosis have been suggested recently by Johansson *et al.*.[33] Intracellularly, they were recognized as promoters of apoptosis, whereas in extracellular compartments, cysteine cathepsins seem to be involved in shedding Fas

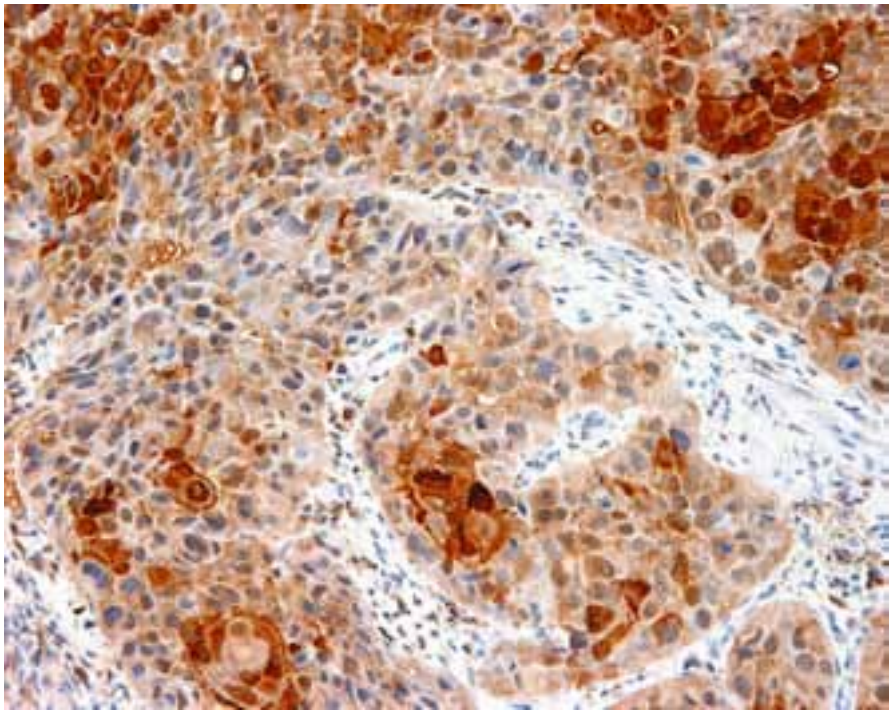

Figure 5b. Positive cytoplasmic immunohistochemical staining for stefin A.

death receptors on the cell surface and thus act to prevent apoptosis. The chemoresistance of laryngeal carcinoma cells with increased level of CB[34] and of glioblastoma cells with increased level of CL,[35] as well as unchanged TNF-α mediated apoptotic activity in HeLa cells after the transfection of CB and CL,[36] also support the hypothesis that high levels of cathepsin expression may not result in the enhanced response of tumor cells to proapoptotic stimuli.

Another reason for this discrepancy might be hypoxia mediated inhibition of TRIAL-induced apoptosis of tumor cells. The prevention of Bax activation and protection of mitochondrial stability with the inhibition of cathepsin translocation by hypoxia might be a mechanism by which tumor cells survive against tumor therapies.[37] On the other hand, hypoxia was demonstrated to increase CB expression and activity and to down-regulate its inhibitors, SB and CC, resulting in an increased residual activity of CB and, consequently, enhanced invasive and /or metastatic potential of hypoxic tumor cells.[38] Thus, the relationship between tumor hypoxia, a frequent and prognostically unfavourable feature of advanced disease, as was the case in our patients, cathepsin and stefin expression levels or activity, and apoptosis is to be determined.[39]

Only CB immunostaining showed some prognostic potential on univariate survival analysis, with low scores being prognostically advantageous over more extensive immunoreactivity (Figure 6). However, after testing CB in multivariate model, it did not appear as an independent prognostic factor. In regard to other tumor types, immunohistochemical labeling for CB was found to be of prognostic value in malignant brain tumors and colorectal carcinoma,[40,41] but not

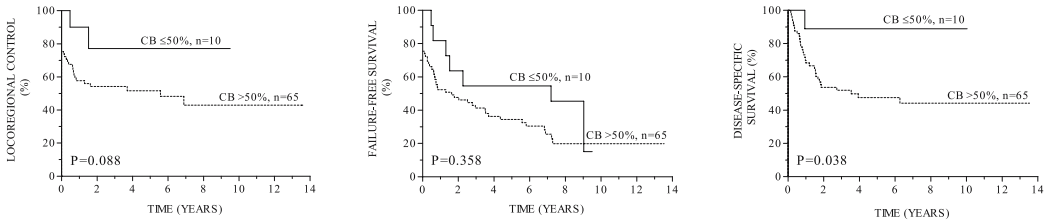

Figure 6. Actuarial survival of patients according to immunohistochemical staining for cathepsin B. **a)** locoregional control; **b)** failure-free survival; **c)** disease-specific survival.

in carcinomas of the breast and oral cavity.[16,26] In head and neck carcinomas, more convincing results were reported from biochemical studies (see above, Refs.[6,7,27]). The importance of complexity of interactions between individual enzymes and inhibitors in biological samples of different types for prediction of survival was clearly exposed in the study by Lah *et al*.[16] In the samples obtained from the same patients with breast carcinoma, the authors identified CB cytosolic levels, but not also CB immunostaining in tumor cells, as prognostically important, thereby suggesting the existence of inherent variations between biological samples of different types. Furthermore, the prognostic importance of individual parameters in a particular cancer type might vary across different patient subgroups, stratified according to well-established prognostic factors. For example, the prognostic reliability of SA immunostaining in breast cancer was reported to be N stage dependent,[42] whereas in prostate carcinoma, the CB-SA ratio reliably differentiated less aggressive from more aggressive subpopulations of tumors within an individual Gleason score.[31]

Conclusions

The knowledge on predictive and prognostic value of cysteine proteases and their endogenous inhibitors in squamous cell carcinoma of the head and neck is scanty. According to our experience, immunohistochemical staining of cysteine cathepsins and stefins seems to be of limited value in this respect. However, the determination of SA in tumor tissue cytosols certainly deserves further evaluation: (*i*) SA confirmed its prognostic value in three independent data sets, with high levels being prognostically advantageous; and (*ii*) considering the differences in inhibitor concentrations in matched pairs of tumor and mucosa samples, two populations of tumors were clearly identified. This observation has strong prognostic implications because downregulated cases are at an increased risk for disease recurrence. These results should be considered hypothesis-generating and should encourage a prospective controlled and multicentric evaluation of cytosolic SA as a promissing prognostic indicator in head and neck cancer on sufficiently large number of patients and with standardized analytical method for SA determination.

Acknowledgement

The author thanks to Professor Nina Gale for providing photographs on cathepsin B immunohistochemical staining and to all colleagues who actively participated in the presented studies: Professors Marjan Budihna, Janez Škrk, Lojze Šmid, Janko Kos, and colleagues Ivan Vrhovec, Branka Svetic, Irena Oblak and Aleksander Aničin.

References

1. Vokes EE, Weichselbyum RR, Lippman SM, Hong WK. Head and neck cancer. *N Engl J Med* 1993; **328:**184-94.

2. Strojan P. Cathepsins and their endogenous inhibitors in clinical oncology. *Radiol Oncol* 1996; **30:** 120-33.

3. Stoka V, Turk B, Turk V. Lysosomal cysteine cathepsins: signaling pathways in apoptosis. *Biol Chem* 2007; **388:** 555-60.

4. Kos J, Šmid L, Krašovec M, Svetic B, Lenarcic 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.

5. Budihna M, Strojan P, Šmid L, Skrk J, Vrhovec I, Zupevc A, et al. Prognostic value of cathepsins B, H, L, D and their endogenous inhibitors stefins A and B in head and neck carcinoma. *Biol Chem Hoppe Seyler* 1996; **377:** 385-90.

6. Strojan P, Budihna M, Šmid L, Vrhovec I, Skrk J. Prognostic significance of cysteine proteinases 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.

7. Strojan P, Oblak I, Svetic N, Šmid L, Kos J. Cysteine proteinase inhibitor cystatin C in squamous cell carcinoma of the head and neck: relation to prognosis. *Br J Cancer* 2004; **90:** 1961-8.

8. Lah TT, Kos J, Blejec A, Frkovic-Georgio 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.

9. Knoch H, Werle B, Ebert W, Spiess E. Imbalance between cathepsin B and cysteine proteinase inhibitors is of prognostic significance in human lung cancer. *Int J Oncol* 1994; **5:** 77-85.

10. 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 tumor tissue. *Adv Exp Med Biol* 1997; **421:** 259-65.

11. Look MP, van Putten WL, Duffy MJ, Harbeck N, Christensen IJ, Thomssen C, et al. Pooled analysis of prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in 8377 breast cancer patients. *J Natl Cancer Inst* 2002; **94:** 116-28.

12. 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.

13. Kos J, Krasovec M, Cimerman N, Nielsen HJ, Christensen IJ, Brunner N. Cysteine proteinase inhibitors stefin A, stefin B, and cystatin C in sera from patients with colorectal cancer: relation to prognosis. *Clin Cancer Res* 2000; **6:** 505-11.

14. Kos J, Lah T. Role of cystatins and stefins in cancer. In: Zerovnik E, Kopitar-Jerala N, Uversky V, editors. *Human stefins and cystatins.* New York: Nova Science Publishers, Inc; 2006. p. 233-6.

15. Levičar N, Kos J, Blejec A, Golouh R, Vrhovec I, Frkovic-Grazio S, et al. Comparison of potential biological markers cathepsin B, cathepsin L, stefin A and stefin B with urokinase and plasminogen activator inhibitor-1 and clinicopathological data of breast carcinoma patients. *Cancer Detect Prev* 2002; **26:** 42-9.

16. Lah TT, Kalman E, Najjar D, Gorodetsky E, Brennan P, Somers R, et al. Cells producing cathepsins D, B and L in human breast carcinoma and their association with prognosis. *Hum Pathol* 2000; **3:** 149-60.

17. Kręcicki T, Siewiński M. Serum cathepsin B-like activity as a potential marker of laryngeal carcinoma. *Eur Arch Otorhinolaryngol* 1992; **249:** 293-5.

18. Bongers V, Konings CH, Grijpma AM, Steen I, Braakhuis BJM, Snow GB. Serum proteinase activities in head and neck squamous cell carcinoma patients. *Anticancer Res* 1995; **15:** 2763-6.

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

20. Strojan P, Budihna M, Šmid L, Svetic B, Vrhovec I, Kos J, et al. Cathepsin H in squamous cell carcinoma of the head and neck. *Radiol Oncol* 1999; **33:** 143-51.

21. Siewiński M, Kręcicki T, Jarmułowicz J, Berdowska I. Cysteine proteinase inhibitors in serum of patients with head and neck tumors. *Diagn Oncol* 1992; **2:** 323-6.

22. Strojan P, Svetic B, Šmid L, Kos J. Serum cystatin C in patients with head and neck carcinoma. *Clin Chim Acta* 2004; **344:** 155-61.

23. Vigneswaran N, Zhao W, Dassanayake A, Muller S, Miller DM, Zacharias W. Variable expression of cathepsin B and D correlates with highly invasive and metastatic phenotype of oral cancer. *Hum Pathol* 2000; **31:** 931-7.

24. Macabeo-Ong M, Shiboski CH, Silverman S, Ginzinger DG, Dekker N, Wong DT, et al. Quantitative analysis of cathepsin L mRNA and protein expression during oral cancer progression. *Oral Oncol* 2003; **39:** 638-47.

25. Nikitakis NG, Rivera H, Lopes MA, Siavash H, Reynolds MA, Ord RA, et al. Immunohistochemical expression of angiogenesis-related markers in oral squamous cell carcinoma with multiple metastatic lymph nodes. *Am J Clin Pathol* 2003; **119:** 574-86.

26. Kawasaki G, Kato Y, Mizuno A. Cathepsin expression in oral squamous cell carcinoma: Relationship with clinicopathologic factors. *Orla Surg Oral Med Oral Patol Oral Radiol Endod* 2002; **93:** 446-54.

27. Strojan P, Aničin A, Svetic B, Pohar M, Šmid L, Kos J. Stefin A and stefin B: markers for prognosis in inoperable squamous cell carcinoma of the head and neck. *Int J Radiat Oncol Biol Phys* 2007; **68:** 1335-41.

28. Bossard N, Descotes F, Bremond AG, Bobin Y, De Saint Hilaire P, Golfier F, et al. Keeping data continuous when analyzing the prognostic impact of the tumor marker: an example with cathepsin D in breast cancer. *Breast Cancer Res Treat* 2003;82:47-59.

29. Castiglioni T, Merino MJ, Elsner B, Lah TT, Sloane BF, Emmert-Buck MR. Immunohistochemical analysis of cathepsin D, B and L in human breast cancer. *Hum Pathol* 1994; **25:** 857-62.

30. Strojnik T, Židanik B, Kos J, Lah TT. Cathepsin B and L are markers for clinically invasive types of meningeomas. *Neurosurgery* 2001; **48:** 598-605.

31. Sinha AA, Quast BJ, Wilson MJ, Fernandes ET, Reddy PK, Ewing SL, et al. Prediction of pelvic lymph node metastasis by the ratio of cathepsin B to stefin A in patients with prostate carcinoma. *Cancer* 2002; **94:** 3141-9.

32. Kim R, Emi M, Tanabe K, Uchida Y, Arihiro K. The role of apoptotic or nonapoptotic cell death in determining cellular response to anticancer treatment. *Eur J Surg Oncol* 2006; **32:** 269-77.

33. Johansson AC, Norberg-Spaak L, Roberg K. Role of lysosomal cathepsins in napthazarin- and Fas-induced apoptosis in oral squamous cell carcinoma cells. *Acta Oto-Laryngol* 2006; **126:** 70-81.

34. Osmak M, Svetic B, Gabrijelčič-Geiger, Skrk J. Drug-resistant human laryngeal carcinoma cells have increased levels of cathepsin B. *Anticancer Res* 2001; **21:** 481-4.

35. Zajc I, Hreljac I, Lah T. Cathepsin L affects apoptosis of glioblastoma cells: a potential implication in the design of cancer therapeutics. *Anticancer Res* 2006; **26:** 3357-64.

36. Gewies A, Grimm S. Cathepsin-B and cathepsin-L expression levels do not correlate with sensitivity of tumor cells to TNF-alpha-mediated apoptosis. *Br J Cancer* 2003; **89:** 1574-80.

37. Nagaraj NS, Vigneswaran N, Zacharias W. Hypoxia inhibits TRAIL-induced tumor cell apoptosis: involvement of lysosomal cathepsins. *Apoptosis* 2007; **12:** 125-39.

38. Wickramasinghe NS, Banerjee K, Nagaraj NS, Vigneswaran N, Zacharias W. Hypoxia alters cathepsin B/inhibitor profiles in oral carcinoma cell lines. *Anticancer Res* 2005; **25:** 2841-9.

39. Horsman MR. Measurement of tumor oxygenation. *Int J Radiat Oncol Biol Phys* 1998; **42:** 701-4.

40. Strojnik T, Kos J, Židanik B, Golouh R, Lah T. Cathepsin B immunohistochemical staining in tumor and endothelial cells is a new prognostic factor for survival in patients with brain tumors. *Clin Cancer Res* 1999; **5:** 559-67.

41. Campo E, Munoz J, Miquel R, Palacín A, Cardesa A, Sloane BF. Cathepsin B expression in colorectal carcinomas correlates with tumor progression and shortened patient survival. *Am J Pathol* 1994; **145:** 301-9.

42. Elzagheid A, Kuopio T, Pyrhönen S, Collan Y. Lymph node status as a guide to selection of available prognostic markers in breast cancer: the clinical practice of the future? *Diagn Pathol* 2006; **1:** 41.