

review

Cysteine cathepsins and their inhibitors in head and neck cancer: an overview of research activities at the Institute of Oncology Ljubljana and ENT Department at the Clinical Center Ljubljana

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To determine the type and extent of the therapy needed for a successful treatment of cancer or to predict clinical outcome, an accurate risk stratification is required. The hypothesis on predictive and prognostic implication of individual cathepsins and their inhibitors originated in their involvement in pericellular proteolysis that participates in virtually all aspects of normal life of a cell and is involved also in the degradation of extracellular matrix barriers during the invasion and metastasizing of tumor cells. The role of cathepsins and their inhibitors in cancer may be categorized as follows: screening markers for diagnosis; predictive markers for lymph node metastasis; predictive markers for response to therapy and for recurrent disease; markers for prognosis. Although the investigations on clinical utility of cathepsins and their endogenous inhibitors in head and neck cancer are limited, the results warranted further evaluation. In the present review, we reported our experience and results gained during a decade of clinically oriented research and made comments on their predictive and prognostic value for routine clinical setting.

Key words: head and neck neoplasms; cysteine endopeptidases; cysteine proteinase inhibitors

Introduction

Head and neck cancer is the sixth most prevalent cancer worldwide. Sites of tumor origin are the organs of the upper aerodigestive tract, *i.e.* oral cavity, pharynx, larynx,

salivary glands, nasal cavity and paranasal sinuses. More than 95% of tumors are of epithelial origin, with alcohol and tobacco abuse being common etiological factors.¹

At presentation, two thirds of patients have locally and/or regionally advanced tumors, and the 5-year survival rates have not improved significantly during the last decades, remaining at 50%.² Conventional UICC/AJCC TNM staging system and established histopathological characteristics allow us only an approximate insight into the inherent biological aggressiveness of individual tumor. At the moment, none of the

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Table 1. Studies on cysteine cathepsins and their inhibitors in head and neck cancer: Ljubljana experience

Study details	Sample type		
	Serum	Cytosol	Tissue section
No. of patients	35, (1998, Ref. 6-8)	Group 1: 45 (1995, Ref. 6, 9, 10) Group 2: 49 (1998, Ref. 11-13) Group 3: 93 (2005) ¹	75 (2005) ¹
Tumor type(s)	OC, OP, HP, L	OC, OP, HP, L	OP
Therapy	SURG+RT	SURG+RT	RT+CT
Analytical methods	Sandwich ELISAs (KRKA dd & Institute Jožef Stefan Ljubljana, Slovenia)		

¹Unpublished data.

OC, Oral cavity; OP, Oropharynx, HP, Hypopharynx; L, Larynx; SURG, Surgery; RT, Radiotherapy; CT, Chemotherapy.

candidate markers within the wide spectrum of biochemical and histological factors adds significantly to the prognostic information obtained from conventional prognosticators.

Cysteine cathepsins B, H and L are lysosomal proteolytic enzymes. They are implicated in virtually all aspects of normal life of a cell as well as in the degradation of extracellular matrix barriers during the invasion and metastasizing of tumor cells. Endogenous inhibitors of cysteine cathepsins constitute a cystatin superfamily, subdivided into several families (stefins, cystatins, kininogens, thyropins). In normal cells, the activation of proteolytic pathways is conducted in cascade manner and controlled by inhibitors. In the tumor tissue, the regulation of this cascade is altered as a result of the modulation of one or more mechanisms regulating the synthesis, transport and release of the involved enzymes and inhibitors.^{3,4}

The predictive and prognostic value of cysteine cathepsins and their inhibitors was widely investigated in breast, lung, and colorectal carcinoma, but not also in head and neck cancer.⁵ The main reasons

are low incidence of the latter and its heterogeneity deriving from the diversity of possible primary sites inside the upper aerodigestive tract, each with its own natural history and treatment outcome.

The aim of the present report was to summarize the results of our research work collected during the last decade in the field of cysteine proteases and their inhibitors in head and neck cancer.

Ljubljana experience

Our experience originated in 1995. During a decade of systematic research, we tested the prognostic and predictive role of cysteine cathepsins and their inhibitors in several independent groups of patients and in different types of biological samples, *i.e.* serum, tissue cytosols, and recently also tissue sections (Table 1).⁶⁻¹³

We gained the most extensive experience from the studies on cytosols prepared from the tumor tissue of operable tumors, treated with surgery and postoperative radiotherapy. However, the main drawback of these studies was a relative heterogeneity of

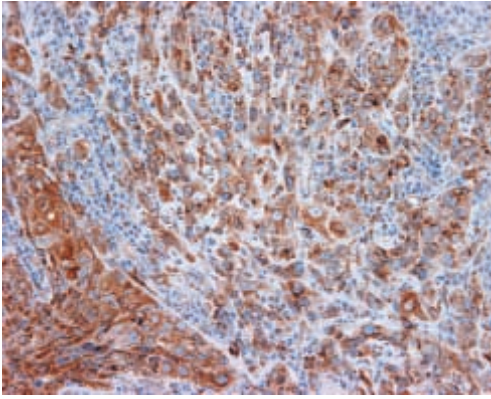


Figure 1. Cathepsin B immunohistochemistry is strongly positive in the cells of moderately differentiated invasive squamous cell carcinoma.

the included primary tumors; one half of them were laryngeal tumors, whereas the others originated from the oral cavity, oropharynx or hypopharynx. From this viewpoint, much more homogenous was a group of patients from our recent study on tissue sections in which only those with inoperable carcinoma of the oropharynx were included; they were treated uniformly with irradiation and concomitant chemotherapy with Mitomycin C and Bleomycin. Highly positive and extensive immunohistochemical reaction in tumor cells (more than 50% of the cells with positive cytoplasmic reaction) was observed in the case of cathepsin B (Figure 1) and stefin A, whereas cathepsin L and stefin B immunohistochemistry was less pronounced (minimal, $\leq 10\%$ positive cells) or modest (10-50% positive cells). Analyzing non-malignant stromal cells in the tumors, reactivity to the cathepsins and stefins were recognized also in lymphocytes and ductal cells. The immunohistochemical reaction in the former case was scored as modest and in the latter case as minimal (unpublished data).

Furthermore, in all groups of patients, the same kits for biochemical determination of studied cathepsins and stefins were used, *i.e.* the commercially available ELISEs

developed at the Jožef Stefan Institute. Because the tests kits have been modified during the years as has also been the methodology for tissue cytosol preparation, the results of measurements in individual groups are not directly comparable.

Prediction of lymph node metastasis

The possibility to predict cervical lymph node infiltration with tumor cells from a primary tumor biopsy specimen would be of critical importance for treatment optimization. The presence of lymph node metastases is the single most adverse prognostic factor in head and neck cancer, reducing 5-year overall survival rate up to 50% compared to node negative patients.¹⁴ Primary tumor-related histopathologic factors (site, T-stage, grade, growth pattern, thickness, perineural infiltration, and others) are not reliable enough in predicting lymph node metastases. Consequently, up to one third of clinically node negative necks at presentation are bearing lymph nodes infiltrated with tumor cells and, vice versa, a significant proportion of patients with palpable neck nodes or radiologically determined neck disease were actually disease free on the neck. In the latter case, nodal enlargement is caused by inflammatory processes in the affected node(s).¹⁵

The results of immunohistochemical studies published so far, analyzing the potential of cysteine cathepsins and their inhibitors for predicting tumor cell infiltration of regional lymphatics, were not conclusive.¹⁶⁻¹⁸ We observed the same in our series for cathepsin L and both stefins. On the contrary, comparing the pattern of cathepsin B immunostaining between N_{0-1} and N_{2-3} subgroups (but not between N_0 and N_+ subgroups!) in the patients with inoperable oropharyngeal cancer treated with concomitant chemoradiotherapy, the difference

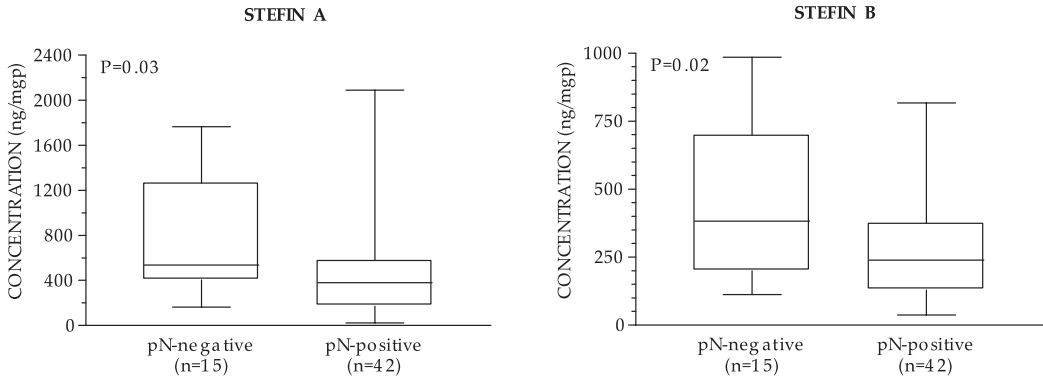


Figure 2. Distribution of tumor concentrations of stefins A and B between patients with histopathologically determined negative (pN0-stage) and positive (pN+stage) necks, as measured in a group with clinically palpable nodes at presentation (cN+stage). The top and the bottom of the box represent 25th and 75th percentiles, respectively and the end of the bars represents the rang. The line in the box is the median value. N, Number of patients. Reproduced by kind permission of Radiology & Oncology from Strojan P et al, *Radiol Oncol* 2002; 36: 145-6.

reached the level of statistical significance (Fisher exact test, $P=0.03$; unpublished data). However, as the key question is how to differentiate node-negative from node-positive necks, we have concluded that cathepsin B immunohistochemistry of primary tumor biopsy sample has no clinical implications in predicting the presence of metastases in the cervical lymphatics.

More encouraging were the results from the tissue cytosols. In the operated patients with clinically positive neck nodes at presentation (*i.e.* before surgery), a statistically significant difference in stefin A and stefin B cytosolic concentrations was calculated between the subgroup of patients who were actually disease-free in the neck and those with metastases confirmed on histopathological examination of the resected specimen (Figure 2).¹² This observation pointed out the ability of stefins to differentiate between the nodes enlarged due to inflammation and those with deposits of tumor cell, and raised a possibility of sparing a portion of cN₊ patients from more aggressive therapy and treatment related side effects. On the other hand, in the patients with clinically undetectable nodes at dia-

gnosis, stefins had no potential to predict pN-stage of the disease.

Prediction of response to therapy

Tumor regression during external beam radiotherapy course is an independent predictive factor of local control in head and neck carcinomas. However, the regression is sequential: the maximum clearance rates for the primary were recorded during the treatment, whereas they were delayed for the nodes, with the maximal complete regression rate at about two months after irradiation.^{19,20} One of the important mechanisms underlying tumor regression after ionizing radiation or chemotherapy is cell disintegration via apoptotic pathways in which cysteine cathepsins and their inhibitors have also been suggested to participate actively.²¹

According to our experience, only cathepsin B immunostaining showed some potential for predicting treatment failure (Fisher exact test, $P=0.034$; unpublished data). The latter was defined as no tumor response or only partial response (less than 100%

Table 2. Cysteine cathepsins as markers for prognosis in head and neck cancer: review of literature

Studies	Cathepsin B	Cathepsin H	Cathepsin L
Positive ¹	Russo et al, 1995 (Ref. 23) Oblak et al, 2005 ³	Strojan et al, 1999 (Ref. 6)	Budihna et al, 1996 (Ref. 9)
Negative ²	Budihna et al, 1996 (Ref. 9) Strojan et al, 2000 (Ref. 11) Kawasaki et al, 2002 (Ref. 17) Strojan et al, 2005 ³	Kawasaki et al, 2002 (Ref. 17)	Russo et al, 1995 (Ref. 23) Strojan et al, 2000 (Ref. 11) Kawasaki et al, 2002 (Ref. 17) Oblak et al, 2005 ³ Strojan et al, 2005 ³
No. of patients	404	96	387

¹Prognostic value of cysteine cathepsins was confirmed.

²Prognostic value of cysteine cathepsins was not confirmed.

³Unpublished data.

regression) to applied chemoradiotherapy evaluated locally and regionally two months after finishing all therapies. We found low cathepsin B immunostaining being uniformly predictive (10/10 cases) for favorable clinical response; however, a substantial proportion of patients with highly positive CB staining were also complete responders (41/65 patients). It seems that cathepsin B immunohistochemistry *per se* is not specific enough and should not be used as a predictive marker of the tumor response to applied therapy independently from other markers. Evaluation in combination with other candidate markers is warranted.

The observation on low cathepsin B immunostaining being predictive for the favorable response of the tumor to radiochemotherapy directly contradicts the recognition of cathepsins as promoters of apoptosis which, in turn, leads into the reduction of cell number and, finally, the volume of the tumor. It seems that other, cathepsin B independent molecular mechanisms are involved in the irradiation induced apoptotic pathways. On the other hand, because complete tumor regression was recorded in a substantial proportion of patients with strongly positive cathepsin B tumors as well,

we hypothesized that the ratio between the cathepsins and their inhibitors may also play a decisive role. For example, an evaluated inhibitor expression, blocking the intrinsic cathepsin B activity, was shown to rescue the tumor cells from TNF-induced apoptosis in experimental setting of the cell lines derived from primary and metastatic lesions of oropharyngeal squamous cell carcinoma.²²

Markers for prognosis

In head and neck cancer, the prognostic value of cysteine cathepsins was studied much less extensively than in breast, lung, or colorectal carcinoma (Table 2).^{6,9,11,17,23} With the exception of cathepsin H, the trend of higher survival probability correlates with lower levels of cathepsin B and cathepsin L. In the studies on tissue cytosols, however, no strong relationship with prognosis was established. As an immunohistochemical marker, only cathepsin B showed some association with the outcome of the disease; the latter was not confirmed on multivariate analysis (unpublished results).

We recognized stefins A and B and cystatin C as the most influential prognos-

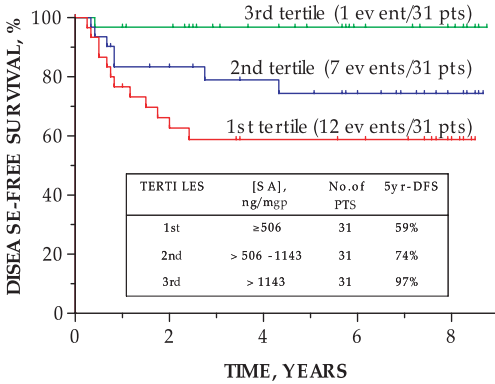


Figure 3. Disease-free survival as a function of stefin A status.

sticators in tumor cytosols. In our first two data sets from 1995 and 1998, higher cytosolic concentrations of any of the two stefins as well as those of cystatin C correlated significantly with longer disease-free interval on univariate survival analysis.^{9,11,13} In multivariate model, only stefin A and cystatin C retained their independent prognostic information. However, when comparing the prognostic strength of the latter two, cystatin C lost its significant prognostic power for both survival endpoints under evaluation, disease-free survival and disease-specific survival.¹³

The prognostic strength of stefin A concentration as determined in tumor cytosol was reconfirmed recently on an independent dataset of 93 patients with operable head and neck cancer (unpublished results). After stratifying the patients according to stefin A concentration in 3 subgroups, we recognized an obvious pattern of improved survival probability with the increasing levels of stefin A (Figure 3). The maximal difference in survival rates between low and high stefin A subgroups was calculated at approximately 400 ng/mgpp, which classified 29% of tumors as stefin A low and the rest as stefin A high. It is interesting that, in both historical data sets, the optimal cut-off concentration fell into the

same range of measured values as it was the case in our recent group, *i.e.* around 30th percentile. On multivariate analysis, stefin A appeared as the strongest independent predictor of a disease-free survival in the model, irrespective of whether it was tested as continuously or categorically variable.

Conclusions

The results presented in this overview warranted further evaluation of cysteine cathepsins and their inhibitors as predictive and prognostic markers in head and neck cancer. In particular, this is the case when analyzing the stefin A concentrations from tissue cytosols. The latter confirmed its prognostic value in three independent data sets, given identical results in all three instances. In future, larger numbers and more homogenous (in regard to primary tumor site) populations of patients and standardization of analytical methods should be considered more rigorously to obtain maximally informative results applicable also to routine clinical practice.

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Odpornost na komplement ovira onkološko zdravljenje

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Izhodišča. Različne *in vitro* raziskave, ki so bile narejene v zadnjih dveh desetletjih, jasno kažejo, da je odpornost človeških tumorskih celic na avtologi komplement pogojena z na membrano vezanimi regulatornimi proteini komplementa (mCRP). Takšna proteina sta CD55 in CD46, najpomembnejšo vlogo pa ima CD59. Ta imunska dogajanja zelo vplivajo na potek bolezni, kar potrjujejo novejša klinična raziskava. Odpraviti odpornost na komplement obeta izboljšanje zdravljenja bolnikov z različnim rakom, s tem pa tudi izboljšanje napovedi izhoda bolezni. V pričujočem kratkem preglednem članku podrobneje predstavljamo: (1) nevtralizacijo proteinov mCRP z monoklonskimi ali rekombinantnimi protitelesi in (2) strategijo »utišanja« genov za proteine mCRP z delovanjem na nivoju RNA ob uporabi siRNA.

Zaključki. Ker so proteini mCRP prisotni v vseh normalnih tkivih endotelnih celic parenhimskih organov (jetra, ledvica, itd...) in v krvnih celicah, je zelo pomembno, da je blokiranje delovanja proteinov mCRP selektivno in da tako ne prizadene zdravega tkiva. Čeprav so prvi rezultati ohrabrujoči, je vplivanje na delovanje proteinov MCRP, da bi izboljšali imunoterapijo, še vedno velik izziv v klinični praksi.

Katepsini cisteinske skupine in njihovi inhibitorji pri raku glave in vratu: pregled raziskovalnega dela na Onkološkem inštitutu Ljubljana in Kliniki za otorinolaringologijo Kliničnega centra Ljubljana

Strojan P

Za odločitev o vrsti in intenzivnosti terapije, potrebne za uspešno ozdravitev raka, kot tudi za napoved izida bolezni je potrebna natančna ocena agresivnosti bolezni. Hipoteza, ki predpostavlja napovedni in prognostični pomen posameznih katepsinov in njihovih inhibitorjev, temelji na vpletenosti enih in drugih v obcelične proteolitične procese. Ti so sestavni del večine aktivnosti, povezanih z življenjem normalne celice, kot tudi procesov, povezanih z razgradnjo zunajceličnega matriksa med procesom invazije in zasevanja tumorskih celic. Vlogo katepsinov in njihovih inhibitorjev pri raku lahko razčlenimo na naslednje skupine: markerji za presejanje; markerji za napoved prisotnosti zasevkov v področnih bezgavkah; markerji za napoved odgovora na zdravljenje in ponovitev bolezni; prognostični markerji. Čeprav je raziskav s področja katepsinov in njihovih endogenih inhibitorjev pri raku glave in vratu malo, rezultati opravičujejo nadaljna preučevanja. V pričujočem pregledu smo predstavili naše izkušnje in rezultate iz desetletnega obdobja klinično usmerjenega raziskovalnega dela in podali mnenje o njihovi napovedni in prognostični vlogi za potrebe vsakodnevne klinične prakse.