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review

Cathepsins and their inhibitors as tumor markers in head and neck cancer

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The invasion and metastasizing of tumor cells is closely connected with the disintegration of basement membranes and extracellular matrix. The carriers of these processes are different proteolytic enzymes, among them cysteine and aspartic cathepsins B, H, L and D as well, a group of ubiquitous lysosomal proteases, and endogenous inhibitors of the former, cystatins. The aim of the present review was to collect the current knowledge on the predictive and prognostic value of cathepsins and their inhibitors in squamous cell carcinoma of the head and neck. In this particular tumor type, the UICC/AJCC TNM-classification system and histopathological characteristics of the tumors were found inadequate to reliably predict either the response to therapy or patients' survival. Moreover, to date, no factor within the wide spectrum of biochemical and histological factors has yet been identified as reliably predicting the natural course of the disease or its response to therapy. To construct a prognostically meaningful tumor profile, new markers are intensively investigated.

Key words: head and neck neoplasms; carcinoma, squamous cell; cathepsins, cystatins; prognosis

Introduction

During the past decades it has been shown that proteases of various classes can act as tumor progression factors in all stages of malignant progression. They can create a local environment that is supportive to the growth, survival, and progression of a tumor through the modulation of growth factor pathways, cell-cell adhesions, and cell-matrix adhesions.[1]

Cathepsins are lysosomal proteolytic enzymes. With regard to their chemical composition, cathepsins are glycoproteins, and the majority of them belong to the group of endopeptidases. In cancer, the most studied cathepsins are those of the cysteine and aspartic classes, cathepsins B, H, L and cathepsin D. Endogenous inhibitors of cysteine cathepsins belong to cystatins, which are subdivided into three families, i.e. stefins, cystatins, and kininogens, and thyropins, whereas the naturally occurring inhibitor of aspartic protease, cathepsin D, has not been found yet in men.[2] Their contribution to the progression of breast, lung, and colorectal cancer has been most extensively investigated at a preclinical level and

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as markers predicting the response to various treatment regimens and prognosis.[3,4]

Head and neck cancer

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most prevalent cancer worldwide, with a global yearly incidence of 500,000.[5] Despite the evolution and refinement of multimodal treatments for the head and neck cancer in the last 20 years, 5-year survival rates have not improved significantly, remaining at the 50% level.[6] Patients grouping with the use of conventional UICC/AJCC TNM staging system and established histopathological characteristics of the primary tumor as well as its metastases on the neck in operated patients allow significant prognostic variation among the individuals within any of these groups.

Compared to the carcinoma of the breast, lung or colorectum, the SCCHN falls into a far less investigated group of cancers. Apart from the studies focused on the activity or level of cathepsins and their inhibitors as determined in matched pairs of tumor tissue and normal mucosa [7], there is only a limited number of reports in literature assessing their predictive or prognostic value in this particular type of cancer. In the present review, clinical data on the predictive and prognostic value of cathepsins B, H, L, and D, and their endogenous inhibitors stefin A, stefin B, and cystatin C in SCCHN have been assembled. The results of our own investigations are also presented and their applicability in routine clinical practice discussed.

Clinical relevance of cathepsins and their inhibitors in squamous cell carcinoma of the head and neck

Markers for diagnosis

The largest pertinent study was reported by Kręcicki and Siewiński[8], who measured serum cathepsin B-like activity in 110 samples from patients with laryngeal carcinoma. Enzyme activity was significantly higher in malignant samples compared to healthy controls, whereas no difference was found between the latter and the group with non-malignant, mainly infectious diseases. In cancer patients, no false-negative serum values of cathepsin B-like activity were obtained. The sensitivity of the assay was calculated to be 100% and the specificity 97.5%.[8] The methodology used in this study was criticized by Bongers *et al*.[9] They claimed that cathepsins B-like activity as determined by Kręcicki and Siewiński is better referred to as "serum-protease-activity". However, when serum-protease-activity was compared between patients with SCCHN and non-cancer controls using the same methodology, and after the adjustment for alcohol and tobacco consumption, no difference was observed between the two groups. We used enzyme-linked immunoassay (ELISA) and also found no alterations in serum cathepsin B between cancer patients and healthy controls, as was the case with cathepsin L.[10] When the same group of patients was tested for cathepsins H and D, a significant increase of both enzymes was found in the sera of patients with cancer.[11,12]

The diagnostic value of the inhibitors of cysteine proteases was first studied by Siewiński *et al*.[13] Using their method they were able to discriminate between total inhibitory activity (free molecules and enzyme-inhibitor complexes) and that of active (enzyme-free) and latent (enzyme-inhibitor complexes) forms of specific papain-like cysteine inhibitors; however, the method had no potential to assess the contribution of individual inhibitors. A significantly higher total inhibitory activity (and of latent fraction) and lower activity of active fraction of the inhibitors were found in cancer patients compared to healthy controls or patients with inflammatory diseases. On the contrary, our ELISA measurements allowed quantification

of specific inhibitors but were not able to recognize different forms of the inhibitor molecules. Significantly higher stefin A [10] and cystatin C [14] levels were measured in the patients' sera than in controls, whereas levels of stefin B were significantly lower.[10]

Due to accompanying alterations of cathepsins and their inhibitors in non-malignant conditions[1], it seems that their screening perspectives are quite limited. Furthermore, considerable overlap of their concentration ranges between patients and healthy controls or those with benign diseases further reduces their diagnostic strength.

Predictive markers for lymph node metastasis

The presence of lymph node metastases is the single most adverse independent prognostic factor in SCCHN that, in comparison to node negative patients, reduces a 5-year overall survival rate by about 50%.[15] The use of tumor-related histopathological factors in predicting lymph node metastases or advanced imaging techniques is not a reliable method. Furthermore, one should be aware that up to two-thirds of clinically N0 necks are classified as node-free on histopathological examination after surgery (pN0) and that some morbidity resulted even after most strictly limited dissections on the neck, and *vice versa*, a substantial proportion of clinically positive necks are actually pN0.[16]

The predictive value for lymph node metastasis most often correlated with increased Cathepsin D immunohistochemical staining. Grandour-Edwards *et al*.[17] reported on 34 patients with oral cavity, oropharyngeal and hypopharyngeal SCCs. In node negative group, 13/15 (87%) tumors were found to be cathepsin D negative, whereas 11/19 (58%) pN+ tumors stained positive for cathepsin D. When adjusted for tumor stage and grade, cathepsin D positivity was nearly twice as likely to be associated with node metastasis.[17] In two other studies, including exclusively oral cavity tumors, cathepsin D immunoreactivity correlated significantly with the pN-stage of the disease [18,19], whereas in the study of Resnick *et al*, limited to laryngeal tumors, no such relationship was found.[20] Of the other cathepsins, a statistically insignificant trend of higher levels of intensity of immunoreactivity in pN+ group compared to pN0 group of oral cavity tumors was also reported for cathepsin B [18] and cathepsin L.[21]

The cathepsin inhibitors were studied by our team only. In the subgroup of patients with operable tumors (various subsites) and clinically positive neck nodes at presentation, stefin A and stefin B concentrations emerged as significant predictors of lymph-node involvement with tumor cells, i.e. pN-stage (Figure 1).[22] Differentiating between the patients with nodes enlarged due to inflammation and those with metastatic nodes, a portion of patients could be spared more aggressive therapy and treatment-related side effects. On the other hand, in the patients with clinically undetectable nodes at diagnosis, stefins had no potential in predicting pN-stage of the disease. However, clinical relevance of this finding is limited if surgery is technically correctly performed because no adjuvant therapy is indicated in pN0 subgroup, whereas pN+ patients are highly curable with a moderate-dose of postoperative radiotherapy, i.e. ≥95% cure rate at a dose of 50 Gy.[23]

Predictive markers for response to therapy and for recurrent disease

When assessing the efficiency of particular therapy by monitoring the presence of tumor cells in the body, surgery-based therapies and non-surgical therapies should be evaluated separately regarding the differences in mechanisms of tumor cell eradication.

Unfortunately, no study was found to have analyzed the predictive value of cathepsins and their inhibitors for response and for disease recurrence to non-surgical therapies, i.e.

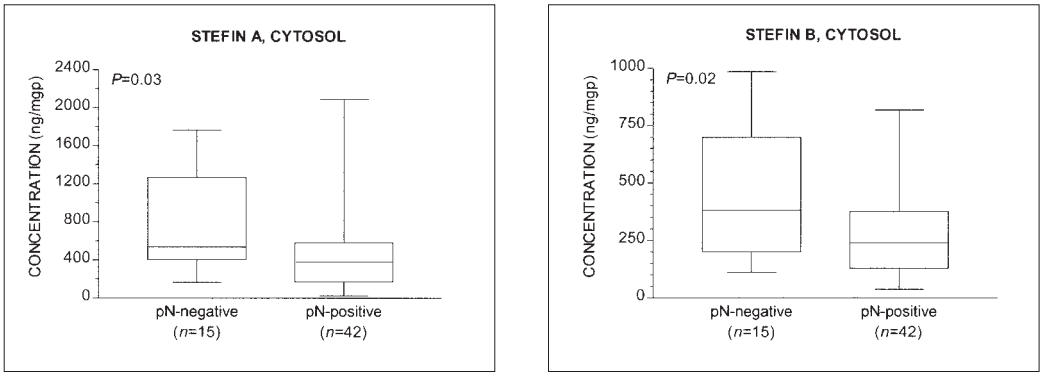

Figure 1. Distribution of tumor concentrations of stefins between patients with histopathologicaly determined negative and positive necks, as measured in a group with clinically palpable nodes at presentation. The top and the bottom of the box represent the 25th and 75th percentiles, respectively, and the ends of the bars represent the rang. The line in the box is the median value. *n*, number of samples. (Reproduced by kind permission of Radiology & Oncology from Strojan P *et al*., *Radiol Oncol* 2002; **36:** 145-6.)

radiotherapy and/or chemotherapy. For the patients successfully treated with surgery for larynx carcinoma, Kręcicki and Siewiński reported a constant decline in serum cathepsin B-like activity, which normalized within four months of the operation.[8] In the subgroup of patients in whom the treatment failed, the mean serum values of cathepsin B-activity dropped in the first month after surgery, but rapidly increased in subsequent assays. The elevation occurred in all cases at least two months before clinical evidence of metastases or a recurrent tumor became apparent.[8]

According to our experience with more heterogeneous group of tumors treated with surgery and postoperative radiotherapy, cytosolic level of stefin A [24,25] and pretreatment serum level of cathepsin L [10] were found predictive for disease outcome, which, in turn, reflected the tumor response to applied therapy. In the same population the additional sample of serum was collected during regular follow-up visits 7 to 407 days (median, 59 days) after the completion of all therapies. No correlation was found between post-treatment concentrations of any of the studied cathepsins or inhibitors and the time of serum sampling. However, when only those patients with a time interval of more than 45 days (n = 14) from the completion of therapy to the serum sampling were considered, a trend of gradual decline in stefin B (unpublished data) and cystatin C concentrations was observed with the increasing time delay (Figure 2).[14] It appears that the decrease in enzyme and inhibitor activity in the treated area after the resection of the gross tumor burden was followed by a transient elevation of their serum levels, likely due to the inflammatory response of the tissues confined in the irradiation field during postoperative radiotherapy. The cutoff time of 45 days concurs well with the duration of radiomucositis as seen in clinics, gradually subsiding in 4–6 weeks following the end of a radiotherapy course.[26]

Markers for prognosis

The prognostic relevance of cathepsin D was studied most frequently (Table 1). It showed a universal trend of higher survival probability in the patients with low cytosolic or serum levels of the enzyme [24,27-29], and low intensity level of immunoreactivity.[19] The prognostic reliability of cathepsin D was proved in three [27,30,31] out of four [32] studies that utilized multivariate analysis. The same relation between enzyme expression and survival was ob-

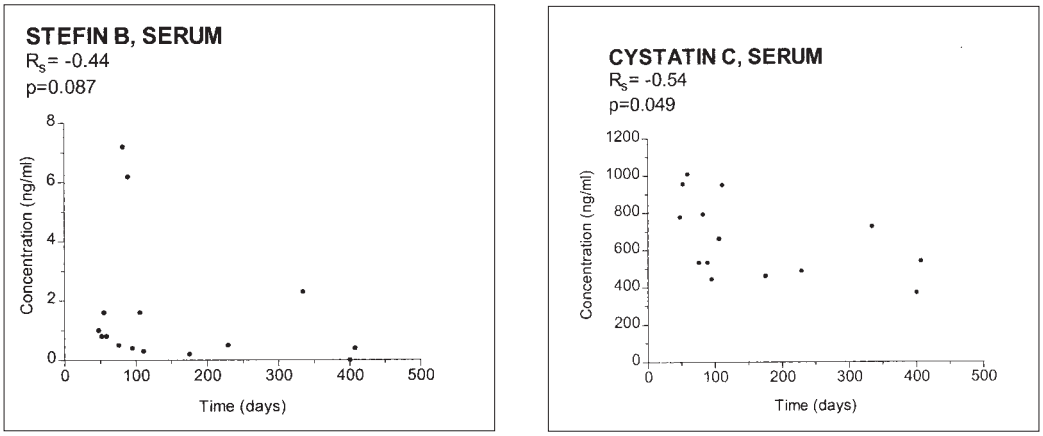

Figure 2. Relationship between post-therapy concentration of stefin B and cystatin C, and the time interval from the completion of therapy to serum sampling in non-relapsed patients with a time delay of more than 45 days (n = 14).

served for cathepsin B [10,25,33] and cytosolic levels of cathepsin L [24,25], whereas higher levels of cathepsin L in the serum was identified as prognostically superior [10], as was the case in cathepsin H.[11,24] However, due to univariate setting of survival analysis, the prognostic information collected from these studies leaves much to be desired.

Table 1. Prognostic relevance of cathepsins and their endogenous inhibitors in squamous cell carcinoma of the head and neck

Author (Ref.)	No.of patients	Tumor site	Method	Prognostic significance
Cathepsin D				
Maurizi *et a.*[27]	63	Larynx	IRA	↑, MVA
Lazaris *et al.*[30]	64	Larynx	IHC	↑, MVA
Seiwerth *et al.*[31]	61	Larynx	IHC	↑, MVA
Kawasaki *et al.*[19]	78	Oral cavity	IHC	↑, UVA
Cathepsin B				
Russo *et al.*[33]	68	Larynx	EA	↑, UVA
Cathepsin H				
Strojan *et al.*[11]	18	All sites	ELISA	↓, UVA
Cathepsin L				
Budihna *et al.*[24]	23	All sites	ELISA	↑, UVA
Strojan *et al.*[10]	35	All sites	ELISA	↓, UVA
Stefin A				
Strojan *et al.*[25]	90	All sites	ELISA	↓, MVA
Stefin B				
Strojan *et al.*[25]	90	All sites	ELISA	↓, MVA
Cystatin C	82			
Strojan *et al.*[34]		All sites	ELISA	↓, MVA

IRA, immunoradiometric assay; IHC, immunohistochemical analysis; EA, enzyme activity; ELISA, enzyme-linked immunosorbent assay; MVA, multivariate analysis; UVA, univariate analysis; ↑, correlation of high levels with poor prognosis; ↓, correlation of low levels with poor prognosis.

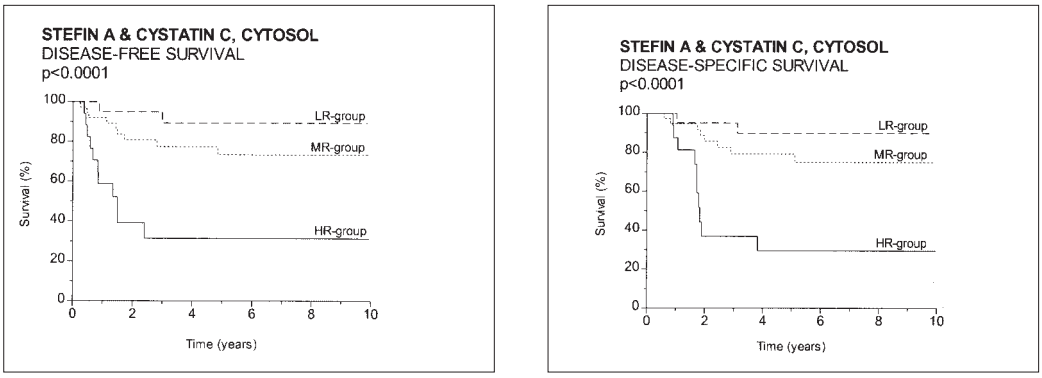

Figure 3. Prognostic significance of the combination of cystatin C and stefin A concentrations: disease-free survival and disease-specific survival. LR, Low-risk group (high stefin A and high cystatin C, *n*=23); MR, Medium-risk group (high stefin A and low cystatin C *or* low stefin A and high cystatin C, *n*=41); HR, High-risk group (low stefin A and low cystatin C, *n*=18).

The results on the prognostic value of cathepsin inhibitors were provided by our team only.[22,24,25,28,34] In operable SCCHN, higher cytosolic concentrations of stefin A, stefin B and cystatin C strongly correlated with longer survival probability in univariate survival analysis, which concurs with the concept of protective role of high levels of cysteine protease inhibitors in tissue homogenates. In multivariate analysis, only stefin A and cystatin C retained their independent prognostic information. However, when comparing the prognostic strength of stefin A with that of cystatin C, the latter lost its significant prognostic power. In addition, the combination of the two inhibitors, stefin A and cystatin C could further stratify the risk of adverse event (Figure 3).[34] No prognostic information was provided from the serum levels of any of the studied inhibitors.[10,14]

Conclusions

Although the clinical utility of cathepsins and their endogenous inhibitors in the management of SCCHN remains open to investigation, it is evident that they exhibit potential in the clinical setting, particularly as markers for lymph node metastasis, for monitoring the presence of tumor cells in the body, and for prognosis. In future studies, larger numbers of patients with clinically more homogenous characteristics should be included and a comparison between various analytical procedures should be focused on. It seems, however, that clinical value of tumor marker profiling in SCCHN patients would be further improved by combining the predictive information from several markers with unrelated or mutually opposing biological roles.

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