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.\textsuperscript{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%.\textsuperscript{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
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 a 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

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

<table>
<thead>
<tr>
<th>Study details</th>
<th>Sample type</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>Serum</td>
</tr>
<tr>
<td>Group 1: 45 (1995, Ref. 6, 9, 10)</td>
<td>Group 2: 49 (1998, Ref. 11-13)</td>
</tr>
<tr>
<td>Tumor type(s)</td>
<td>OC, OP, HP, L</td>
</tr>
<tr>
<td>Therapy</td>
<td>SURG+RT</td>
</tr>
<tr>
<td>Analytical methods</td>
<td>Sendwich ELISAs (KRKA dd &amp; Institute Jozef Stefan Ljubljana, Slovenia)</td>
</tr>
</tbody>
</table>

\(^1\) Unpublished data. OC, Oral cavity; OP, Oropharynx; HP, Hypopharynx; L, Larynx; SURG, Surgery; RT, Radiotherapy; CT, Chemotherapy.

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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, ≤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. 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).

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. We observed the same in our series for cathepsin L and both stefins. On the contrary, comparing the pattern of cathepsin B immunostaining between N0-1 and N2-3 subgroups (but not between N0 and N1 subgroups!) in the patients with inoperable oropharyngeal cancer treated with concomitant chemoradiotherapy, the difference

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

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%
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 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.

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).

![Table 2. Cysteine cathepsins as markers for prognosis in head and neck cancer: review of literature](image-url)

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). 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 progno-
sticators in tumor cytosols. In our first two data sets from 1995 and 1998, higher cytosolic concentrations of any of the two ste- 
fins as well as those of cystatin C correlated significantly with longer disease-free inter- 
val on univariate survival analysis.9,11,13 In multivariate model, only stefin A and 
cystatin C retained their independent pro- 
gnostic information. However, when com- 
paring the prognostic strength of the latter 
two, cystatin C lost its significant progno- 
stic power for both survival endpoints un- 
der evaluation, disease-free survival and di- 
ase-specific survival.13

The prognostic strength of stefin A con- 
centration as determined in tumor cytosol 
was reconfirmed recently on an indepen- 
dent dataset of 93 patients with operable 
head and neck cancer (unpublished re- 
sults). After stratifying the patients ac- 
cording to stefin A concentration in 3 sub- 
groups, we recognized an obvious pattern of 
improved survival probability with the in- 
creasing levels of stefin A (Figure 3). The 
maximal difference in survival rates betwe- 
en low and high stefin A subgroups was 
calculated at approximately 400 ng/mgp, 
which classified 29% of tumors as stefin A 
low and the rest as stefin A high. It is inte- 
resting that, in both historical data sets, the 
opimal 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 war- 
ranted further evaluation of cysteine cathe- 
cpsins 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 in- 
stances. In future, larger numbers and mo- 
re homogenous (in regard to primary tumor 
site) populations of patients and standardi- 
zation of analytical methods should be con- 
sidered more rigorously to obtain maxi- 
mally informative results applicable also to 
routine clinical practice.

### Acknowledgement

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histochemical staining.

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


