

## Cathepsin H in squamous cell carcinoma of the head and neck

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**Purpose.** To estimate a prognostic value of cathepsin H (CH) in squamous cell carcinoma of the head and neck (SCCHN).

**Materials and methods.** CH concentration was measured using a quantitative immunosorbent assay (ELISA; KRKA d.d., Novo mesto, Slovenia) in serum samples from 35 patients, obtained at surgery (Serum no.1) and 7-407 days (median, 55 days) after therapy (Serum no.2). As control, CH concentration as measured in sera from 30 healthy volunteers was used from the study of Kos et al. (Clin Cancer Res, 1997). The prognostic significance of serum concentration of CH was compared with that of its tissue concentration from the study of Budihna et al. (Biol Chem Hoppe-Seyler, 1996), and for this purpose the follow-up of patients from the latter report was updated.

**Results.** A significantly elevated concentration of CH was measured in Serum no.1 as compared to Serum no.2. (8.9 vs. 8.0 ng/mls,  $P=0.04$ ) or the sera from healthy volunteers (8.9 vs. 4.9 ng/mls,  $P<0.0001$ ). The CH concentration in Serum no.1 appeared to be grade dependant ( $G_{T+2}$  vs.  $G_3$ , 9.1 vs. 5.0 ng/mls,  $P=0.06$ ); no correlation was observed with other established prognostic factors or the presence of subsequent recurrence/dissemination of the disease. The time of Serum no.2 collection did not influence the CH concentration in these samples. There was a trend towards a better prognosis with increasing levels of CH in Serum no.1 in both the analysis of disease-free survival (DFS) and disease-specific survival (DSS). The maximal differences in survival rates between patients with low and high CH levels were calculated at cut-off concentration 10.7 ng/mls (DFS: 60 vs. 89%,  $P=0.13$ ; DSS: 65 vs. 86%,  $P=0.25$ ). The results of tissue concentration of CH were equivocal, with the maximal difference between low and high CH groups at cut-off concentration 720 ng/mgp (DFS: 14 vs. 48%,  $P=0.04$ ; DSS: 23 vs. 60%,  $P=0.27$ ).

**Conclusions.** Our results provide indirect evidence for the specific role of CH in the processes of invasion and metastasis in SCCHN. Besides, its serum and particularly tissue concentration might also be of prognostic value in this particular type of cancer.

**Key words:** cathepsins, cathepsin H; head and neck neoplasm, carcinoma, squamous cell; prognosis

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## Introduction

Cathepsin H (CH) is an ubiquitous lysosomal enzyme and belongs to the class of cysteine proteinases, such as cathepsins B (CB), L (CL) and S. In the cell, it is involved in the processes of intracellular protein turnover and post-translation processing of some biologically important protein precursors.<sup>1</sup> The proteolytic activity of CH is regulated by the endogenous protein inhibitors of cystatin superfamily, i.e. stefins, cystatins and kininogens.<sup>2</sup>

Biochemically, CH is a glycoprotein. Mature enzyme molecule of human kidney consists of 220 amino acid residues,<sup>3</sup> and is found as a single chain form of 28 kDa or two chain form of 24 kDa (heavy chain) and 4 kDa (light chain).<sup>4</sup> Besides endopeptidase activity, it possesses also aminopeptidase activity, which is a unique feature when compared with other lysosomal cathepsins.<sup>5</sup> For optimal activity, CH requires acid pH, and is unstable at neutral or alkaline pH values.<sup>1</sup>

In normal cells, CH molecules are stored in lysosomes, but under certain conditions they can be secreted from the cell, mainly in the inactive precursor form. Their activation could result in uncontrolled extracellular proteolysis, which is implicated in pathogenesis of various diseases, also of cancer.<sup>6</sup> It has been demonstrated for CH to participate actively in the degradation of extracellular matrix components,<sup>7</sup> which is believed to be a crucial step in local invasion and metastatic spread of tumour cells.<sup>6</sup> Increased protein and/or activity levels of the enzyme have been determined in tumour tissue and sera of patients with breast cancer,<sup>8</sup> glioma<sup>9</sup> and malignant melanoma,<sup>10,11</sup> compared to adjacent normal tissue or sera of healthy controls. The opposite relation has been established in squamous cell carcinoma of the head and neck (SCCHN).<sup>12,13</sup> In a clinical study on melanoma, the serum concentration of CH was shown to be related with the survival probability,<sup>11</sup> whereas in the head and neck cancer, no statistically significant differ-

ence but only a trend was observed between groups with low and high concentration of the enzyme in tumour tissue.<sup>13</sup>

In present study, we examined the levels of CH in sera from patients with SCCHN (Group 1) using a quantitative immunosorbent assay (ELISA). In an attempt to ensure more reliable evaluation of the results, the follow-up of the patients from our previous study (Group 2),<sup>13</sup> in which the survival significance of enzyme concentration in tumour tissue had been investigated, was updated, and the results presented here.

## Patients and methods

Patients from both groups were treated with curative intent at the University Department of Otorhinolaryngology and Cervicofacial Surgery, and at the Institute of Oncology, Ljubljana, Slovenia. The study protocol was approved by the Medical Ethics Committee at the Ministry of Health of the Republic of Slovenia, and all included patients gave their consent to voluntary participation in the study. The concentration of CH was measured at the Departments of Biochemistry of the Institute of Oncology (serum samples, Group 1) and of the Jožef Stefan Institute (tissue samples, Group 2), Ljubljana, Slovenia.

### Patients

#### Group 1.

Between November 1995 and December 1996, 35 patients (two females, 33 males) with primary SCCHN entered the study. Median age of the patients was 58 years, range 37-71 years. They were primarily operated on and 30 of them were postoperatively irradiated because of an advanced stage of the disease, residual growth after surgery, extranodal spread of the tumour or the presence of neoplastic emboli in the lymphatic vessels.

Tumours were staged according to UICC TNM classification,<sup>14</sup> and histopathological grade was defined according to WHO criteria.<sup>15</sup> The clinical and histopathological tumour characteristics are listed in Table 1.

All the 35 patients were eligible for the analysis of disease-free survival (DFS) and disease-specific survival (DSS), which were defined as the time intervals between the date of surgery and the date of disease recurrence/dissemination (DFS), or disease-related death or the last follow up (DSS). In 11 patients, the recurrent disease and/or distant dissemination were diagnosed during follow-up period, and 9/11 died of disease-related causes. Four patients died due to causes unrelated to the treated malignant disease and were censored at the last follow-up in the analysis of DSS. Median follow-up of those alive at the last follow-up examination was 28 months, range 23-40 months.

#### Group 2.

Cathepsin H concentration was determined in 21/45 male patients, aged 38 to 66 years (median, 53 years), with primary SCCNH, who were included in the study between June 1992 and August 1993.<sup>13</sup> Their tumour parameters are shown in Table 1. Seventeen patients were primarily operated on and 16 of them were postoperatively irradiated for the same indications as those in Group 1. Four patients received irradiation treatment alone. For more detailed information on the patients and therapy see Budihna *et al.*<sup>13</sup>

Eighteen patients were included in the analysis of DFS and DSS; three patients were lost to follow-up. In 11 patients, the disease recurrence/dissemination was diagnosed. The disease was the cause of death in nine patients and the disease-unrelated causes in four patients. Median follow-up of patients alive was 69 months, range 64-75 months.

#### *Sampling and biochemical assay of cathepsin H*

##### Group 1.

Five-ml samples of venous blood were collected on the day of surgery (Serum no.1) and 7-407 days (median, 55 days) after therapy (Serum no.2). Blood sampling was co-ordinated with the routine blood collection for preoperative and control laboratory tests. Thirty min. after withdrawal, blood was centrifuged at 1000xg/10 min. The serum was stored at -70°C until analysed.

Human CH concentration was analysed using a specific enzyme-linked immunoassay (sandwich ELISA; KRKA d.d., Novo mesto, Slovenia), developed at the Jožef Stefan Institute, Ljubljana, Slovenia.<sup>16</sup> Human liver CH was isolated and characterised as described,<sup>4</sup> and was used for immunisation of animals and as a standard for assay calibration curve. Utilising sheep polyclonal antibody for capture, and murine 2E3 monoclonal horseradish peroxidase-labeled antibody for detection, both raised to human antigen, the assay was able to detect a mature protein, a precursor molecule and enzyme-inhibitor complexes. The assay characteristics regarding linearity, recovery, within-run and between-run precision, and detection limit enable satisfactory application of the assay on serum and tissue samples.<sup>16</sup> The CH concentration was expressed in ng/ml of serum (ng/mls).

Briefly, sera in 1:4 dilution were added to the wells of microtiter plate that had previously been precoated with capture antibodies. After a 2h-incubation at 37°C, the wells were washed and filled with detection antibodies. After further 2 hrs. of incubation at 37°C, peroxidase substrate 3,3',5,5'-tetramethyl benzidine (Sigma Chemical Co., St. Louis, MO) in the presence of hydrogen peroxide was added. The amount of degraded substrate, as a measure of bound immunocomplexed CH, was visualised by absorbance

at 450 nm, and the CH concentration was calculated from the calibration curve.

As controls, the results of CH concentration measurements in the sera of 30 healthy volunteers (13 females and 17 males, aged 21-49 years, mean 37 years) were used from the study of Kos *et al.* (with the permission of authors).<sup>11</sup> The serum collection, test kit and the time of biochemical analysis were exactly the same as in our study.

## Group 2.

In 17 surgically treated patients, two tissue samples weighing 200-500 mg, representing matched pairs, were obtained from each tumour and the adjacent normal tissue. In four non-operated patients, only a tumour specimen was obtained during diagnostic endoscopy. The tissue cytosol was prepared as described.<sup>13</sup>

The cytosolic concentration of CH was determined by enzyme-linked immunosorbent assay (sandwich ELISA) using sheep immunoselective IgG as capture antibody, and rabbit peroxidase-labeled anti-CH IgG for detection, as described by Kos *et al.*<sup>12</sup> The CH concentration was expressed in ng/mg of total protein (ng/mg).

## Statistics

The results were analysed using a PC computer and BMDP software package (BMDP Statistical Software, Los Angeles, CA). All the tests were two-sided and the results were considered statistically significant at the probability level of 0.05.

The difference between the median concentrations of CH in match pairs of Serum no.1 and no.2, and of tumour and normal tissue samples were determined by the Wilcoxon signed rank test. The Mann-Whitney *U*-test was used to test the relation of Serum no.1 and tumour tissue concentration of the enzyme to clinical and histopatho-

logical prognostic factors, and to subsequent recurrence/dissemination of the disease, and to calculate the difference in serum levels of CH between controls and cancer patients. The relationship between CH concentration in Serum no.2 and the time of its collection was established by Spearman's rank correlation. In the survival analysis, Kaplan-Meier product limit method was used,<sup>17</sup> and the difference between groups was tested by the log-rank test.<sup>18</sup> Patients were grouped according to the cut-off concentration of CH, at which maximal difference in the survival rates was determined.

## Results

### Group 1.

A significantly elevated concentration of CH was measured in Serum no.1 as compared to Serum no.2. (8.9 vs. 8.0 ng/mls,  $P=0.04$ ; Figure 1) or sera from healthy volunteers (8.9 vs. 4.9 ng/mls,  $P<0.0001$ ). Among the clinical and histopathological prognostic factors under investigation, only the histopathological grade appeared to be related to the CH concentration as measured in Serum no.1

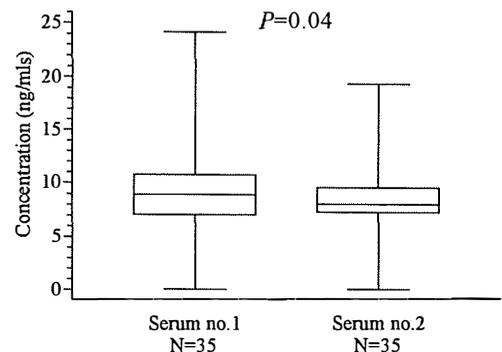


Figure 1. Concentration of cathepsin H in Serum no.1 and Serum no.2 (Group 1). The top and the bottom of the box represent the 25 and 75<sup>th</sup> percentiles, respectively, and the ends of the bars represent the range. The line in the box is the median value. N, number of samples.

**Table 1.** Clinical and histopathological characteristics of tumours and corresponding concentrations of cathepsin H in Serum no.1 (Group 1) and tumour tissue (Group 2).

Tumour characteristics	Group 1		P-value	Group 2		P-value
	No. of patients	Concentration (ng/ml) <sup>a</sup>		No. of patients	Concentration (ng/ml) <sup>a</sup>	
<i>Localisation</i>						
Larynx	11	9.6 (6.1 - 24.1)	NS	12	1.0 (0.6 - 2.3)	NS
Non-larynx <sup>b</sup>	24	8.5 (0.0 - 15.7)	NS	9	0.7 (0.5 - 7.1)	
<i>T-stage</i>						
T <sub>1+2</sub>	18	7.6 (0.0 - 15.7)	NS	5	0.9 (0.5 - 7.1)	NS
T <sub>3+4</sub>	17	9.3 (4.7 - 24.1)		16	0.9 (0.5 - 2.3)	
<i>N-stage</i>						
N <sub>0</sub>	19	8.9 (5.0 - 24.1)	NS	6	0.9 (0.7 - 2.3)	NS
N <sub>1-3</sub>	16	8.9 (0.0 - 15.7)		15	1.0 (0.5 - 7.1)	
<i>TNM-stage</i>						
Stage <sub>I+II</sub>	8	7.5 (5.0 - 10.2)	NS	2	(0.7 - 1.0)	-
Stage <sub>III+IV</sub>	27	9.3 (0.0 - 24.1)		19	1.0 (0.5 - 7.1)	
<i>Histopathologic grade</i>						
G <sub>1+2</sub>	28	9.1 (4.7 - 24.1)	0.06	17	0.9 (0.5 - 7.1)	-
G <sub>3</sub>	3	5.0 (0.0 - 8.8)		2	(0.5 - 1.4)	
Unknown	4	-		2	-	
<i>Extranodal tumour spread</i>						
Negative	4	7.9 (4.7 - 14.7)	NS	4	1.3 (0.5 - 2.0)	NS
Positive	12	9.2 (0.0 - 15.7)		9	0.9 (0.5 - 7.1)	
Unknown	0	-		2	-	

<sup>a</sup> Median (range).

<sup>b</sup> Oral cavity, oropharynx, hypopharynx.

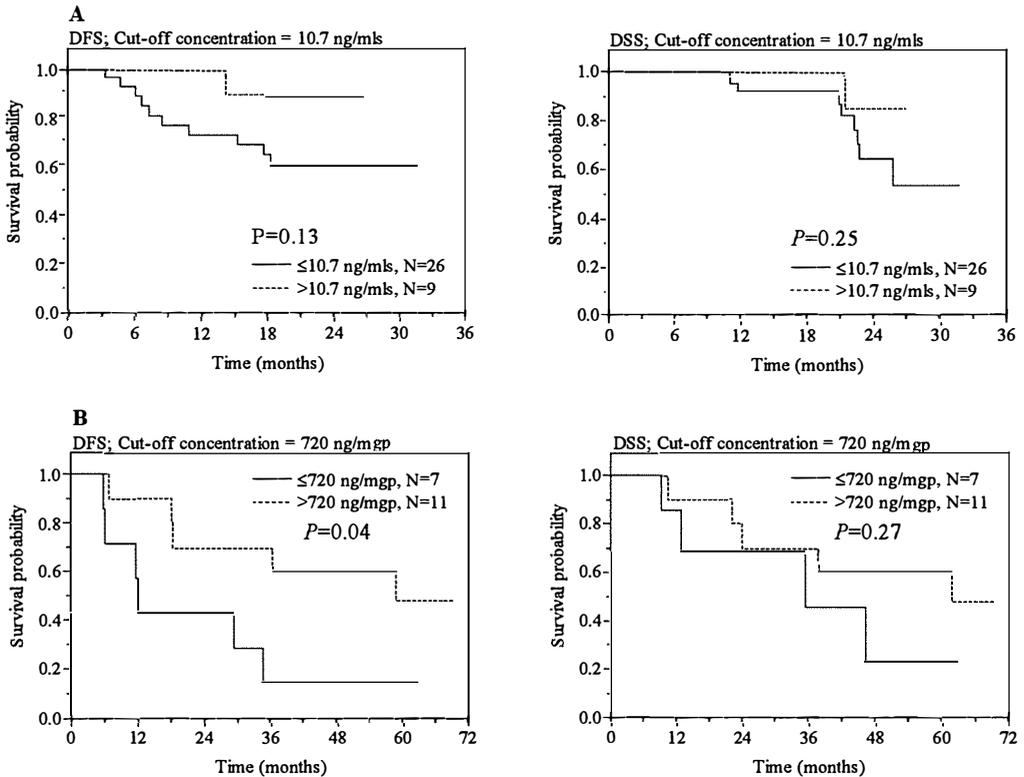
NS, not significant.

(G<sub>1+2</sub> vs. G<sub>3</sub>, 9.1 vs. 5.0 ng/mls,  $P=0.06$ ) (Table 1). The duration of time interval between the completion of therapy and Serum no.2 collection did not influence the enzyme concentration ( $R_s=-0.12$ ,  $P=0.50$ ). In patients with subsequently diagnosed locoregional recurrence or distant dissemination of the disease, CH concentration in Serum no.1 was insignificantly lower than in those with no evidence of active disease at the last follow-up examination (8.4 vs. 9.1 ng/mls,  $P=0.45$ ). At any concentration level of CH in Serum no.1 used as a cut-off concentration, a better survival of patients was associated with high enzyme concentration, but the differences in DFS- and DSS-rates between low and high enzyme groups did not reach the level of statistical significance. The difference in DFS rates was maximal at a cut-off concentration 10.7

ng/mls (74<sup>th</sup> percentile; 60 vs. 89% at 2 years,  $P=0.13$ ), as it was for DSS (65 vs. 86% at 2 years,  $P=0.25$ ) (Figure 2A).

#### Group 2.

The CH concentration was significantly higher in normal tissue samples than in their tumour counterparts (2.2 vs. 0.9 ng/mgp,  $P=0.001$ ) with tumour to normal tissue ratio of median concentrations 0.42. No correlation was observed between tumour CH concentration and the established prognostic factors (Table 1) or the presence of subsequent recurrence/dissemination of the disease (for details see Budihna *et al.*<sup>13</sup>). In the analysis of DFS and DSS, the maximal differences in survival rates were calculated using cut-off concentration 720 ng/mgp (39<sup>th</sup> percentile). In



**Figure 2.** Disease-free survival (DFS) and disease-specific survival (DSS) of patients with respect to the cut-off concentrations of cathepsin H at which maximal differences in survival rates were calculated. A, Group 1; B, Group 2. N, number of patients.

both cases better survival was associated with a high concentration of CH, and the difference in DFS between the groups was statistically significant (DFS: 14 vs. 48% at 5 years,  $P=0.04$ ; DSS: 23 vs. 60% at 5 years,  $P=0.27$ ) (Figure 2B).

## Discussion

Possible clinical significance of the cysteine proteinases as prognosticators of disease recurrence and patient survival based on their involvement in proteolytic processes leading to the invasion and metastasising of tumour cells.<sup>6</sup> The most investigated enzymes of cysteine class are CB and CL, whereas the

role of CH in the invasive behaviour of cancerous cells and particularly its prognostic value is much less investigated.<sup>19</sup> In present study, the concentration of CH was measured in sera from patients with SCCHN (Group 1). The prognostic significance of the enzyme concentration was estimated and compared with updated results from the study of Budihna *et al.*,<sup>13</sup> who determined CH levels in tissue cytosols (Group 2).

In Group 1 patients, a significantly higher serum concentration of CH was measured compared to control sera of healthy volunteers. The same relationship was reported by Gabrijelčič *et al.*<sup>8</sup> for breast cancer and Kos *et al.*<sup>11</sup> for malignant melanoma, indicating that a markedly increased proportion of the

enzyme is secreted from the malignant cells rather than being routed to lysosomes as it is usual in normal cells. Besides, the concentration of CH in Serum no.2 was significantly reduced as compared to that in Serum no.1, although Spearman's statistics showed no correlation between Serum no.2 enzyme values and the time of its collection. It appears that the decrease in proteolytic activity in the treated area as a result of the resection/destruction of the gross tumour burden results in a decrease in serum CH levels, which is most probably gradual, slowly approaching normal value, as it has already been established for aspartic proteinase cathepsin D (CD).<sup>20</sup>

The only correlation observed was between serum concentration of CH and histopathological grade, and even in that case it was of marginal statistical significance. A higher enzyme concentration was associated with a lower histopathological grade of tumours, i.e. with biologically less aggressive form of the disease. Bearing in mind the tumour-to-normal tissue relation of cytosolic concentration of CH, the established difference logically reflects successive steps in malignant transformation of the cell, from normal - with the highest enzyme concentration - to well, moderately and - finally - to poorly differentiated cell with the lowest enzyme concentration. To our knowledge, besides in head and neck cancer a decreased expression of CH in tumour tissue has been determined also in lung cancer (Schweiger *et al.*, unpublished data). On the other hand, Gabrijelčič *et al.*<sup>8</sup> demonstrated that CH concentration was higher in malignant than in non-malignant samples from breast cancer patients, and that it increased with the histopathological grading, which is just the opposite of what we have found in head and neck cancer. The results in glioma<sup>9</sup> and malignant melanoma<sup>10</sup> are consistent with those in breast cancer.<sup>8</sup> We can speculate that characteristics of the tissue of origin may also play a

role in the expression of enzyme, resulting in the differences observed between various tumour types. Among murine tissues, the highest concentration of CH was found in the kidney, followed by vagina, liver, lung and spleen,<sup>21</sup> while much less is known about its distribution in human.

Apart from CH (present study), elevated serum values of CB<sup>22</sup> and aspartic proteinase CD<sup>20</sup> have also been reported in patients with SCCHN. Furthermore, in serial sampling of serum, CB has also proved to be a useful marker in following response to therapy in laryngeal carcinoma.<sup>22</sup> As observed by Krecicki and Siewinski,<sup>22</sup> in all 14 patients who failed after surgery a significant increase in CB activity was measured at least two weeks before clinical evidence of metastases or signs of recurrent disease. In our study, serum (i.e. Serum no.2) was collected also after therapy. However, due to a wide time span in its sampling, it is not possible to draw any conclusion on the potential role of CH as a marker of treatment response or early indicator of treatment failure.

The survival analysis revealed an indisputable trend towards a better prognosis with increasing serum and tissue levels of CH in both the analysis for DFS and DSS. There is only the study of Budihna *et al.*<sup>23</sup> on breast cancer that has recognised high tissue concentration of CB as prognostically superior. All other reports,<sup>19</sup> including that on the prognostic significance of serum CH in melanoma patients,<sup>11</sup> have correlated elevated serum and/or tissue levels of cysteine proteinases with worse survival. This difference could reflect not only the specific characteristics of individual cancer types but also the selection criteria for cut-off concentration. For example, in melanoma study<sup>11</sup> the same test kit was used for biochemical analysis of serum CH as in ours and the results of both studies are comparable. However, while we used the optimal cut-off concentration at which the maximal difference was calculated

between low and high CH groups, in the other study<sup>11</sup> a median value was chosen for this purpose. The difference in DFS-rates as calculated with respect to tissue cut-off concentration of CH was of statistical significance, which was not the case with its serum concentration. Nevertheless, the follow-up in Group 1 is probably too short to yield a more reliable estimation rather than a trend only.

For CH measurement enzyme-linked immunoassay was used. With the first version of the test kit, using polyclonal antibodies for capture and for detection,<sup>12</sup> the enzyme concentration in tissue cytosol was determined in Group 2 patients.<sup>13</sup> The results were in the range of the amounts obtained for cathepsins by protein purification and enzyme activity measurements.<sup>24</sup> When the same test kit was used for quantitative determination of CH in sera from patients with breast cancer,<sup>8</sup> enzyme levels were 50-100 times higher than those of CB and CL, suggesting that they were overestimated, most probably due to a non-specific reaction of polyclonal antibodies with other serum components. For that reason, a more sensitive and specific ELISA utilising mono- and polyclonal antibodies and capable of detecting distinct CH forms was developed.<sup>16</sup> It was used in the present study for the determination of CH concentration in the serum from patients with SCCHN, and previously by Kos *et al.*<sup>11</sup> in patients with malignant melanoma. The concentration ranges agree well between the studies and are consistent with the amounts reported for CB and CL.<sup>11</sup> The assay has already been in use for the analysis of CH concentration in tissue cytosols of the human heart, muscle and kidney.<sup>16</sup> The results were consistent with the levels determined in rats using an immunoassay based on polystyrene beads coated with anti-rat CH IgG,<sup>21</sup> and were approximately 50-times higher than those in patient sera. The ratio between cytosolic concentration of CH as measured in cancerous patients<sup>13</sup> and different human tis-

sues<sup>16</sup> was about 10:1. However, when we attempted to use a modified poly-mono version of the assay for enzyme determination in tissue cytosols of patients in Group 1, inconsistency between CH concentrations as measured upon different dilutions of individual sample within the working range of the assay was observed and the analysis was not carried out.

To conclude, in this report we have shown that CH concentration in serum from patients with SCCHN was elevated as compared to normal serum from healthy volunteers. Further, considering the tissue concentration of CH as well, less aggressive forms of the disease were found to be associated with higher levels of the enzyme. In DFS and DSS analysis, a trend towards better prognosis was observed with high concentrations of CH as measured in both the serum and the tissue samples. Although our results should still be confirmed by a more extensive study with appropriate follow-up, it appears that also in SCCHN CH concentration might be of prognostic importance.

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