Cathepsins B, H, and L and Their Inhibitors Stefin A and Cystatin C in Sera of Melanoma Patients

Janko Kos, Borut Štabuc, Ana Schweiger, Marta Krašovec, Nina Cimerman, Nataša Kopitar-Jeralu, and Ivan Vrhovec

Jožef Stefan Institute, Department of Biochemistry and Molecular Biology, 1000 Ljubljana [J. K., A. S., N. K-J.]; Institute of Oncology, 1000 Ljubljana [I. V.]; and KRKA d.d., Research and Development Division, 8000 Nova Nova, Slovenia

ABSTRACT

The levels of cathepsins (Cats) B, H, and L and their inhibitors stefin A and cystatin C were determined in the sera of 43 patients with metastatic melanoma, in 54 patients with treatedcutaneous melanoma, and in 30 healthy blood donors, using quantitative ELISAs. The levels of Cats B and H and cystatin C were significantly higher within the group of metastatic melanoma patients compared with the healthy controls. The median Cat B was 4.8 versus 3.6 ng/ml (P < 0.013), the median Cat H was 13.7 versus 4.9 ng/ml (P < 0.0001), and the median cystatin C was 470 versus 320 ng/ml (P < 0.02). Cat H was also significantly increased within the group of melanoma patients with metastasis, with a median of 9.6 ng/ml. Cat B was found to correlate with Cat L (r = 0.36; P < 0.02) and cystatin C (r = 0.41; P < 0.008). The serum level of Cat H was significantly increased in patients showing no response to the chemoimmunotherapy as compared to the level in responders. Metastatic melanoma patients with high contents of Cat Bi and Cat H experienced significantly shorter overall survival rates than the patients with low levels of each enzyme (Cat B: P < 0.003 and relative risk, 2.5; Cat H: P < 0.006 and relative risk, 2.4, using medians as cutoff values). The other potential factors for prognosis for this group of patients revealed moderate (histological type and age) or no (tumor thickness, sex, and lymph node metastasis) prognostic significance. Similarly, no difference in survival was found for stefin A, cystatin C, and Cat L. These results suggest that the serum levels of Cats B and H could serve as prognostic factors for patients with advanced melanoma.

INTRODUCTION

Tumor invasion and metastasis are associated with proteolytic action of variousproteases. These include serine proteases, urokinase type of plasminogen activator and tissue type of plasminogen activator, matrix metalloproteases, aspartic protease Cat D, and cysteine proteases Cats B, H, and L (1). Increased levels of Cats B, H, and L have been observed in tissues of primary and metastatic tumors in many cancer types (2, 3). These proteases are able to activate other proteolytic enzymes and degrade the proteins of the extracellular matrix or the basement membrane such as laminin, collagen or elastin in vitro (4). Under normal physiological conditions, Cats are localized mostly in lysosomes (5), whereas in tumors, the alterations in expression, processing and/or translocation pathways could provoke increased secretion and uncontrolled extracellular proteolysis. The activities of Cats B, H, and L are regulated by the endogenous CPIs, cystatins, steins, and kininogens, which are present in the cytoplasm (steins A and B) or in extracellular fluids (cystatin C and kininogens; Ref. 6).

In clinical studies of breast (7), head and neck (8), colorectal (9), and lung (10, 11) cancer, overexpression of Cats B and L was shown to correlate with more aggressive tumor behavior, early relapse, and shorter survival. Moreover, the imbalance of the Cat:inhibitor ratio or decreased CPI levels were found to be related to the survival probability in lung (12, 13) and head and neck cancers (8).

Although the association of tumor levels of Cat and inhibitors with malignant progression is becoming well established, the relationship of their extracellular levels to clinical factors remains less clear. Changes in cysteine endopeptidase-like activity or in the CP:CPI ratio have been studied in serum of patients with breast, lung, colorectal, and larynx cancers (14), in the ascitic fluid of patients with ovarian carcinoma (15), and in the urine of patients with colorectal cancer (16). Significantly increased levels of concentrations of the Cat B, H, and L proteins, determined by ELISA, were found in the sera of patients with breast carcinoma (17), hepatocellular carcinoma (18), and ductal pancreatic carcinoma (19) and in the bronchoalveolar lavage fluid of lung cancer patients (20). To our knowledge, the correlation of the levels of extracellular Cats in cancer patients with the outcome of the disease has not been demonstrated thus far.

MM represents 1% of all cancer patients, with rapidly
increasing incidence in many countries. Despite extensive attempts to improve treatment, the outlook of MM patients remains poor, with constant increase in mortality (21). In addition to pathohistological and clinical characteristics as prognostic factors of survival, several molecular markers have been studied to predict the course of disease and enhance treatment planning (22, 23). Cats B, H, and L have been suggested as markers for malignancy of melanolytic tumors (24). In immunohistochemical analysis, stronger staining intensities were observed in metastatic and advanced primary melanomas than in early primary melanomas and pigmented naevis. Overexpression of these proteins in melanolytic cells may result in increased secretion, indicating presumably malignant progression and predicting survival probability.

Our aim was to examine serum levels of Cats B, H, and L and their protein inhibitors stefin A and cystatin C in patients with metastatic melanoma using quantitative ELISAs. The serum values have been compared with those of patients with treated primary tumors with no metastases and with a control group of healthy donors. For melanoma patients with metastases, we correlated protein serum levels to clinical features, considering especially the correlation of individual protein values with survival rate.

PATIENTS AND METHODS

Patients

This study was performed on groups of 30 healthy blood donors (group A), 54 patients with previously treated cutaneous melanoma with no evidence of metastatic disease (group B), and 43 patients with metastatic melanoma (group C).

Group A included 17 men and 13 women with a mean age of 37 years (range, 21–49). From their histories of disease, it was evident that none suffered from any infectious disease at least 1 month prior to collection of blood samples. None had ever undergone any treatment for cancer or other chronic diseases, such as diabetes, hypertension, or rheumatoid arthritis. Hematological and biochemical blood tests and hepatitis A and B markers were within normal limits.

Group B included 17 men and 37 women with a mean age of 48 years (range, 21–77), all of stage I according to the WHO. For all patients, a cutaneous melanoma was surgically removed between 14 and 130 months before they entered the study (mean postoperative period, 26 months). Twenty-four patients received a 6-month postoperative adjuvant immunotherapy with IFN-α. In all 24 patients, immunotherapy was completed at least 8 months before they entered the study. No evidence of metastatic spread was detected in any of those patients either at the time of blood sample collection or 6 months later. Hematological and biochemical blood tests were within normal limits. No patient underwent any chronic disease treatment for at least 6 months prior to blood sample collection.

Group C included 19 men and 24 women with a mean age of 45 years (range, 21–70), all of stage III according to WHO. All patients had biopsy proven metastatic melanoma, with Eastern Cooperative Oncology Group performance status ≤2 and normal organ function. None had previously undergone any chemotherapy or immunotherapy or any other chronic disease treatment. Of 43 patients of group C, the first relapse was detected in 28, and the second and third were detected in 16. They all received only surgical treatment, which was in all cases completed at least 3 months before entering the study. Metastases were detected in soft tissue (25 patients), in lung (6 patients), and in bones (2 patients). Multiple metastases spread was discovered in 10 patients. The patients with metastatic spread in liver or brain were not included in the study.

After blood sample collection for examination of Cats and inhibitors, all of the patients of group C were treated with chemoimmunotherapy: day 1, a 10-h infusion of 2 mg/m² vinblastine, and 40 mg/m² lomustine p.o.; days 2–5, a 2-h infusion of 20 mg/m² cisplatin; and days 3–6, 6 MUI IFN-α 2b s.c. The treatment cycle was repeated every fourth week (25).

The survival of these patients was defined as the interval between date of blood sample collection and date of last observation or death.

Sample Collection

Blood samples of patients with metastatic disease (group C) were collected 1–3 days prior to the first cycle of chemoimmunotherapy. For patients with no evidence of metastatic disease (group B) the blood samples were collected on regular follow-up. The quantity of blood was 5 ml per patient. The blood was centrifuged at 3000 rpm, and serum was stored at −20°C until it was analyzed.

Antigens

Human Cats B, H, and L were isolated and characterized in our laboratory as described (6, 26). They were used for immunization of animals and as standards for assay calibration curves. For stefin A and cystatin C assays, recombinant proteins, expressed in E. coli (27, 28), were used for immunization and for preparation of the calibration curves.

ELISAs

Human Cats B, H, and L, stefin A, and cystatin C were analyzed using ELISAs (sandwich ELISAs, KRKat d.d.) developed at Jožef Stefan Institute. The components were purified and characterized, and the tests were optimized as described (29, 30). The linearity of ELISAs was tested by serial dilution of serum samples to the levels encompassing the range of the assays. The measured values of diluted samples were subsequently compared with the calibration values. A microplate reader (SLT 400AT, SLT, Salzburg, Austria) was used to measure absorbency in ELISAs. The Cat and inhibitor protein was expressed in ng/ml of serum.

Cat B Protein

For determination of Cat B antigen, sheep and rabbit polyclonal Abs (IgG), purified from antisera by immunoaffinity chromatography on immobilized human Cat B, were used as capture and detection Abs, respectively. Sera in a 1:2 dilution were added to the wells of a microtiter plate that had previously been precoated with rabbit anti-Cat B IgG. After 2 h of incubation at 37°C, the wells were washed and filled with sheep anti-Cat B IgG conjugated with HRP. After a further 2 h 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 Cat B, was
Concentration profile of Cats B, H, and L, stefin A, and cystatin C in the sera of melanoma patients and healthy controls. The mean levels, obtained for control sera, have been normalized to a relative concentration of 1.0.

The differences in Cats and inhibitors content were tested by Mann-Whitney and Kruskal-Wallis tests. Correlations among the Cats and inhibitors levels were defined by Spearman linear regression analysis. Overall survival analysis was calculated according to the Kaplan-Meier method (31), using the log rank test for statistical significance between subgroups of patients, and to the Cox proportional hazards model (32). In all tests, two-sided $P$ values below 0.05 were considered significant.

RESULTS

Distribution of Cats and Inhibitors. The relative concentrations of enzymes and inhibitors measured in normal sera (group A), in the sera of patients with nonmetastatic disease (group B), and in the sera of patients with metastatic melanoma (group C) are shown in Fig. 1. The values for groups B and C were calculated by dividing the means obtained for melanoma patients by the means of control sera. The mean levels of Cats and inhibitors in control sera were $3.6 \pm 0.21$ ng/ml (mean $\pm$ SE) for Cat B, $4.7 \pm 0.79$ ng/ml for Cat H, $25.3 \pm 2.08$ ng/ml for Cat L, $4.7 \pm 0.39$ ng/ml for stefin A, and $13.4 \pm 0.91$ ng/ml for cystatin C.
Cathpsins and Their Inhibitors in Melanoma Sera

Table 1  Concentrations (ng/ml) of Cat B, Cat H, Cat L, stefin A, and cystatin C in sera of healthy controls (group A), patients with no metastases (group B), and patients with metastases (group C)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median</th>
<th>Range</th>
<th>Median</th>
<th>Range</th>
<th>Median</th>
<th>Range</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat B</td>
<td>3.6</td>
<td>(2.6–5.6)</td>
<td>3.7</td>
<td>(2.1–6.3)</td>
<td>4.8</td>
<td>(3.0–7.0)</td>
<td>A:C &lt; 0.013</td>
</tr>
<tr>
<td>Cat H</td>
<td>4.9</td>
<td>(2.0–8.0)</td>
<td>9.6</td>
<td>(2.0–8.5)</td>
<td>13.7</td>
<td>(3.0–25.0)</td>
<td>A:C &lt; 0.0001</td>
</tr>
<tr>
<td>Cat L</td>
<td>24.0</td>
<td>(18.0–29.0)</td>
<td>20.0</td>
<td>(11.0–33)</td>
<td>21.0</td>
<td>(13.0–35.0)</td>
<td>B:C &lt; 0.001</td>
</tr>
<tr>
<td>Stefin A</td>
<td>3.2</td>
<td>(1.2–6.4)</td>
<td>2.5</td>
<td>(1.1–8.5)</td>
<td>2.4</td>
<td>(1.1–4.8)</td>
<td>NS**</td>
</tr>
<tr>
<td>Cystatin C</td>
<td>320</td>
<td>(200–400)</td>
<td>360</td>
<td>(290–480)</td>
<td>470</td>
<td>(210–750)</td>
<td>A:C &lt; 0.02</td>
</tr>
</tbody>
</table>

** NS, not significant.

for Cat L, 3.4 ± 0.21 ng/ml for stefin A, and 310 ± 21.2 ng/ml for cystatin C. For all antigens, the control sera levels were defined as a relative concentration of 1.0. Median serum levels of Cats and inhibitors are shown in Table 1. The Cat B median was significantly increased within the group of patients with metastatic melanoma compared with patients with no metastasis or with controls (P < 0.013; see Table 1). Cat H levels were significantly increased within the groups of metastatic and non-metastatic patients compared with the control group (group A versus group C; P < 0.0001); they were the highest for metastatic melanoma patients. Cystatin C levels were significantly increased within the group of metastatic melanoma patients when compared with the control group (P < 0.02). The level within the nonmetastatic group was not significantly different from the control level. The levels of Cat L and stefin A were not statistically different among the groups of controls, nonmetastatic, and metastatic patients.

Patient Treatment. Objective response to treatment was observed in 36% of patients (95% CI, 26–52%). Of 16 responding patients, 5 had complete remission. The median survival of patients with objective response was 16.0 months (95% CI, 8.9–23.1), and that of patients with no response or stagnation was 9.0 months (95% CI, 4.0–14.0); however, the difference was not statistically significant (P = 0.16). In 27 nonresponders, metastatic progress was noted in soft tissues (4 patients), lung (2 patients), liver (4 patients), bone (1 patient), brain (5 patients), and in multiple organs, particularly in soft tissues of lung and liver (12 patients).

Relationship to Clinical Characteristics. The levels of Cats B and L and inhibitors, measured in sera before starting the treatment, exhibited no significant difference between responders and nonresponders, whereas the level of Cat H was significantly higher within nonresponders (16.0 ± 0.9 ng/ml (mean ± SE)) compared with responders (12.7 ± 1.3 ng/ml; P < 0.03). No correlation has been found with the number or localization of metastasis, the number of relapses, patient age, sex, or histological type. Among the Cats and inhibitors, significant correlation has been found between Cat B and Cat L (r = 0.36; P < 0.02) and between Cat B and cystatin C (r = 0.41; P < 0.008).

Prognostic Significance. Cats and inhibitors were analyzed as dichotomized variables using median values as the cutoff points to divide the patients into low- and high-level groups. To test the survival probability, Kaplan-Meier analysis was performed using log-rank tests for evaluation of the differences among the groups. The difference in survival probability rate was the most significant between patients with low versus high Cat B levels (P < 0.0027). Patients with high Cat B serum levels had a poor prognosis (Fig. 2). Using the Cox univariate model, the RR for patients with a high Cat B level compared with those with a low level was 2.6 (95% CI, 1.3–5.0). Significant differences were also observed between the groups of patients with low versus high Cat H serum levels (P < 0.0073; Fig. 3), with a RR of 2.4 (95% CI, 1.2–4.8). For age and histological type of primary tumors (nodular histological type versus other histological types), the difference was only marginally significant (P < 0.04 and P < 0.05, respectively). For Cat L, cystatin C, and stefin A, no significant difference was observed between high- and low-level groups, using medians as cutoff values. Likewise, no difference in survival probability rate was observed for sex and lymph node localization when the patients were divided into positive versus negative. The results of univariate analysis are summarized in Table 2.

DISCUSSION

MM is a very unpredictable disease, affecting different populations with different rates and prognosis. Several clinical and pathohistological factors have been evaluated for disease-free and overall survival to predict the course and the outcome of the disease. Among them, tumor thickness (33), depth of invasion (34), histological type, tumor site, ulceration, sex, age, mitotic activity, microsatellites, vascular invasion, and regression were reported to be the most relevant in stage I (35). However, in stages II and III, the importance of these prognosticators is limited and the outcome of the disease is influenced more by the number of positive lymph nodes, time delay to node dissection, and number and localization of metastatic sites (36). For this group of patients, new, independent biological factors for the estimation of prognosis are needed to individualize the therapy. Because chemotherapy for patients with disseminated melanoma was shown to be of limited value, new markers could also allow for the selection of patients who might benefit from this treatment from the nonresponders who should not be exposed to the toxic effects of the chemotherapy.

Serine proteases, aspartic proteases, metalloproteases, and cysteine proteases have been shown to associate with malignant progression (3). It is believed that the concerted action of proteases of all classes is required for degradation of extracel-
Fig. 2 Prognostic significance of Cat B in the sera of patients with metastatic melanoma disease (n = 43). Survival probability rate was calculated according to the Kaplan-Meier method. A median level of 4.8 ng/ml was used as a cutoff value to divide patients into low- and high-Cat B groups. The log rank test was used to analyze the difference between the groups.

Fig. 3 Prognostic significance of Cat H in the sera of patients with metastatic melanoma disease (n = 42). A median level of 13.7 ng/ml was used as a cutoff value to divide patients into low- and high-Cat H groups.

The role of Cats in the serum of cancer patients still remains unclear. Their catalytic capability is rather limited because the majority of Cats B, H, and L is released from normal and tumor cells in the latent, precursor form, and their serum levels are much lower than those determined in tumors. Moreover, the serum concentrations of cystatin C and kininogens, potent inhibitors of cysteine proteases, are in large excess over protease levels and ensure effective in vivo inhibition. In addition to CPIs, serum contains high concentrations of α2-macroglobulin, the major scavenger of endopeptidases, including Cats B and L, but not of Cat H, which is an exopeptidase (aminopeptidase; Ref. 40). After initial proteolytic cleavage and entrapping of active enzyme by α2-macroglobulin, the complex is bound to the macrophage receptors and cleared from the circulation. The rate of cleavage is presumably related to the endopeptidase
Table 2  Univariate analysis of Cat B, Cat H, Cat L, stefin A, cystatin C, and other potential factors for prognosis for metastatic melanoma patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cutoff value (median)</th>
<th>n</th>
<th>Median survival (months) (95% CI)</th>
<th>P (log rank)</th>
<th>RR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat B</td>
<td>&lt;4.8 ng/ml</td>
<td>29</td>
<td>24.0 (1.3-5.0)</td>
<td>0.003</td>
<td>2.5 (1.3-5.0)</td>
</tr>
<tr>
<td></td>
<td>≥4.8 ng/ml</td>
<td>14</td>
<td>7.0 (5.4-8.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cat H</td>
<td>&lt;13.7 ng/ml</td>
<td>21</td>
<td>34.1 (16.1-51.9)</td>
<td>0.006</td>
<td>2.4 (1.2-4.8)</td>
</tr>
<tr>
<td></td>
<td>≥13.7 ng/ml</td>
<td>21</td>
<td>8.0 (6.3-9.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histological type</td>
<td>Nonnodular</td>
<td>16</td>
<td>20.0 (0.4-39.6)</td>
<td>0.05</td>
<td>1.4 (0.6-3.8)</td>
</tr>
<tr>
<td></td>
<td>Nodular</td>
<td>27</td>
<td>13.0 (6.9-19.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>&lt;43 years</td>
<td>13</td>
<td>30.0 (4.2-55.8)</td>
<td>0.04</td>
<td>1.1 (1.0-1.2)</td>
</tr>
<tr>
<td></td>
<td>≥43 years</td>
<td>30</td>
<td>11.0 (5.6-18.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
<td>24</td>
<td>30.0 (4.3-55.7)</td>
<td>NS&quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>19</td>
<td>12.0 (0.5-24.5)</td>
<td></td>
<td></td>
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<tr>
<td>Lymph node metastases</td>
<td>Positive</td>
<td>16</td>
<td>22.0 (0.4-59.2)</td>
<td>NS&quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>25</td>
<td>13.0 (4.8-21.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cat L</td>
<td>&lt;21.0 ng/ml</td>
<td>22</td>
<td>22.0 (3.6-40.4)</td>
<td>NS&quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥21.0 ng/ml</td>
<td>20</td>
<td>8.0 (2.7-13.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystatin C</td>
<td>&lt;470 ng/ml</td>
<td>20</td>
<td>15.0 (9.3-20.7)</td>
<td>NS&quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥470 ng/ml</td>
<td>23</td>
<td>13.0 (3.0-22.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stefin A</td>
<td>&lt;2.4 ng/ml</td>
<td>22</td>
<td>15.0 (0.5-31.1)</td>
<td>NS&quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥2.4 ng/ml</td>
<td>21</td>
<td>14.0 (9.5-18.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

" NS, not significant.

activity, and one may speculate that Cat L, which is considered the most powerful cysteine protease (6), is cleared faster than Cat B, whereas Cat H is not entrapped by α2-macroglobulin.

Although the activation of serum cysteine proteases, particularly in certain microenvironments, cannot be completely ruled out, Cats are present in serum mainly as inactive forms, and their levels probably only reflect increased expression and secretion from tumor cells and/or from tumor-associated cells, such as macrophages, fibroblasts, or infiltrated lymphocytes.

Increased expression and release of Cats B, H, and L has been detected in various murine (41) and human (42, 43, 44) melanoma cells. CPIs have been demonstrated to depress the motility and adherence of human A2058 melanoma cells (42). In B16a murine cells, the expression and the activity of Cat B was found to be much higher than that of Cat L (3). Using immunohistochemical analysis, the overexpression of Cats B, H, and L has been demonstrated also in primary and metastatic human melanoma tumors (24). The highest levels of all of the Cats have been observed in metastatic tumors, suggesting a correlation of Cats B, H, and L with the metastatic potential of human melanolytic tumors.

In this study, we observed a significant increase of Cat B (1.3-fold) and Cat H (1.5-fold) in the sera of patients with metastatic melanoma compared with patients with primary lesions, whereas for Cat L, no such difference was observed. This is consistent with the immunohistochemical study of Kageshita et al. (24), which revealed among the Cats the weakest expression and staining intensity for Cat L in primary and metastatic melanomas. These findings support the idea that the role of Cat L in melanoma progression is less important than that proposed for some other tumors (7, 45).

Compared to the control serum levels, Cat H was elevated in primary melanoma patients, whereas increased Cat B levels were found only in metastatic melanoma patients. Therefore, Cat B overexpression seems to be associated with the metastatic potential of melanomas as shown previously by cytochemical techniques for B16a and B16-F1 cells (3). On the other hand, increased Cat H serum levels in patients with primary melanoma suggest the correlation of this enzyme with earlier steps in malignant progression and indicate a possible diagnostic application of Cat H for melanolytic tumors.

Cat H was found to be significantly increased within the group of patients, with no response to the combined chemoimmunotherapy compared with the group of the responders. The potential of this enzyme to predict the effectiveness of the therapy could be of significant clinical importance for the selection of a patient’s individual treatment.

Stefin A, an intracellular protein inhibitor of cysteine proteases, exhibited low values within both groups of patients and controls. In both groups of patients, its level was not statistically significantly lower than the controls. The decrease in expression or inhibitory capability of stefin A, found in various tumors (38, 39, 46), has been proposed as the cause of increased tumor associated proteolytic activity.

In contrast to stefin A, cystatin C is an extracellular inhibitor of cysteine proteinases present in high content in cerebrospinal fluid, seminal plasma, serum, and other body fluids (47). It is one of the most potent inhibitors of cysteine proteinases and
is able to inhibit very effectively the proteolytic activity of Cat B and H in the enzyme-inhibitor ratios demonstrated in serum samples. Among patients and controls, its concentration profile is similar to that of Cat H, exhibiting the highest level for metastatic patients (Fig. 1). Association of increased cystatin C with advanced melanoma is consistent with recent findings that reveal a correlation of high plasma levels of inhibitors of metalloproteases and serine proteases with tumor progression or poor prognosis (48, 49).

Cystatin C may be elevated in malignant sera to balance the increased values of Cats B and H. Its values correlated significantly with Cat B but not with Cat H. On the other hand, cystatin C was found, like creatinine or $\beta_2$ microglobulin, to be a good indicator of glomerular filtration rate (50). Thus, it is also possible that the difference in cystatin C level reflects alterations in renal function and is not directly related to malignant progression. We have demonstrated a significant correlation between cystatin C and creatinine values ($r = 0.3; P < 0.003$).

Cat B, as well as cystatin C, correlates with Cat L. This relationship has been found in tissue cytosols of various tumors (7, 14, 29, 51), suggesting similar regulation of these two enzymes in malignant processes, whereas the regulatory mechanism of Cat H is probably distinct. However, the absence of increase of Cat L in malignant sera may indicate also differences in secretion or clearance between Cat B and Cat L.

The analysis of the relationship of the variables with the survival probability using Kaplan-Meier or Cox univariate models revealed the most significant prognostic value for Cat B. The patients with high Cat B serum levels were associated with significantly lower survival probability rates than the patients with low Cat B levels. A strong prognostic impact of Cat B is consistent with previous findings, confirming the association of its overexpression with the metastatic potential of melanoma cells (3). In this study, Cat H was also demonstrated as a significant prognosticator for the survival of metastatic melanoma patients, with a RR close to that of Cat B. Similar to Cat B, the patients with high level of Cat H experienced shorter survival.

Some of the factors being suggested as prognosticators in stage I (33, 34, 35) exhibited moderate (histological type and age) or no (sex and tumor thickness) prognostic impact for the stage III patients of group C included in our study. Similarly, we were not able to demonstrate any prognostic value for lymph node metastasis and the levels of stefin A and cystatin C. For Cat L, the median survival of patients with low levels is apparently longer than that of the patients with high levels (22.0 versus 8.0 months; see Table 2), but the difference is not statistically significant ($P = 0.12$). The lack of prognostic impact of Cat L in serum is contrary to results obtained in tissue cytosols of breast tumors (7), and this remains to be clarified.

In conclusion, our preliminary study reveals the association of higher serum levels of Cats B and H with the group of patients with metastatic melanoma disease. Cat H was also increased in stage I patients and has been shown to correlate with response to the therapy. Moreover, the prognostic significance of Cats B and H has been defined, revealing strong correlation of higher levels of these two enzymes, with a shorter survival rate of metastatic melanoma patients. To our knowledge, this is the first time that the serum levels of cysteine proteases have been demonstrated to be of prognostic significance. This finding could be of considerable clinical interest for other cancers, also, because the assessment of prognostic factors in serum is more convenient than in tumor cytosols. However, a study of larger populations is needed to evaluate the prognostic importance and clinical relevance of these new factors in metastatic melanoma.

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