Prognostic Values of Cathepsin B and Carcinoembryonic Antigen in Sera of Patients with Colorectal Cancer

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ABSTRACT

The level of cathepsin B (Cat B) was determined in sera obtained preoperatively from 325 patients with colorectal cancer using an ELISA. Control sera from 90 healthy blood donors were analyzed. The levels of Cat B detected included all forms that were present in the sera, i.e., mature enzyme, precursor molecule, and enzyme-inhibitor complexes. The level of Cat B was significantly increased in sera of patients with colorectal cancer. The median level was 10.7 ng/ml versus 2.1 ng/ml in controls (P < 0.0001). A correlation between Cat B serum level and advanced Dukes' stage (P < 0.003) was found, whereas no associations have been found with age, sex, or level of carcinoembryonic antigen (CEA). In survival analysis, the patients with high serum Cat B experienced significantly lower survival probability. At the optimal cutoff value of 9.4 ng/ml, the relative hazard ratio was 1.8 (95% confidence interval, 1.1-2.8; P = 0.016) in the univariate Cox proportional hazards model. The median observation time was 4.4 years (range, 3.2-5.5 years). In multivariate analysis, Dukes' stage was the strongest prognostic variable, followed by age, whereas serum Cat B and CEA were not significant prognostic factors in this model, in accordance with their association with Dukes' stage. When the data for Cat B and CEA were combined, CEA-positive patients were further separated by Cat B into high- and low-risk groups. Patients with high serum levels of both molecules had significantly shorter survival (relative hazard ratio of 2.2; 95% confidence interval, 1.5-3.2; P < 0.0001), as compared with patients with low levels of both molecules.

INTRODUCTION

Tumor invasion and metastasis are associated with proteolytic activity of various proteases. These include the serine proteases, the matrix metalloproteases, the aspartic protease Cat D, and the cysteine proteases Cat B, Cat H, and Cat L (1), all proposed to mediate the degradation of extracellular matrix proteins in a cascade-like manner. Furthermore, Cat D has been shown to activate pro-Cat B at an acidic pH (2), and Cat B, in turn, is able to activate pro-urokinase-type plasminogen activator (3), enhancing subsequent plasmin generation. In vitro Cat B also degrades the proteins of the extracellular matrix and the basement membrane, including laminin, elastin, fibronectin, proteoglycans, and collagen (4-7). The proteolytic activity of Cat B is regulated by the endogenous protein inhibitors cystatins, stefins, and kininogens that are present in cytoplasm (stefins A and B) or in extracellular fluids (cystatin C and kininogens; Ref. 8). Under normal physiological conditions, Cat B is localized mostly in lysosomes, whereas in tumors, alterations in expression, processing, and/or translocation pathways may provoke increased secretion and uncontrolled extracellular proteolysis (9).

Regulatory mechanisms for Cat B in malignant and normal cells appear to be complex and remain to be elucidated. Increased levels of Cat B have been observed in tissues of primary and metastatic tumors in many cancer types (10, 11). Overexpression of Cat B in murine tumor cells was shown to correlate with their metastatic capability (11). In clinical studies of breast (12), head and neck (13), colorectal (14), and lung cancers (15, 16), increased tumor tissue Cat B activity and protein concentration correlated with more aggressive tumor behavior, early relapse, and shorter survival.

Significantly increased levels of Cat B have also been found in sera of patients with breast (17), liver (18), pancreatic (19), and melanoma (20) cancers. For metastatic melanoma patients, we demonstrated the correlation of high Cat B serum levels with shorter overall survival time (20).

In tumors of colon and rectum, high Cat B-like activity and mRNA content have been found (21-24). In these studies, tumor-specific increase was found to be greater in earlier stage tumors (Dukes' A and B) than in more advanced tumors (Dukes' C and D; Refs. 22 and 23), whereas immunohistochemical studies (14) revealed stronger staining intensities in advanced colorectal tumors. Different colonic cell lines were also

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3 The abbreviations used are: Cat, cathepsin; CEA, carcinoembryonic antigen; RHR, relative hazard ratio; CI, confidence interval.
shown to be a source of Cat B-like protease, actively secreted from the cells in a latent precursor form (21, 25).

The aim of this study was to examine the levels of Cat B in sera of patients with colorectal cancer using a quantitative immunosorbent assay (ELISA). The serum levels have been compared with those of a control group of blood donors and analyzed with respect to serum levels of CEA, an established tumor marker in colorectal carcinoma (26–28). The results have also been tested for their relationship to clinical features, considering especially the correlation of individual protein values with the survival rate.

MATERIALS AND METHODS

Patients
Three hundred twenty-five patients with histologically verified colorectal cancer were included in the study. Clinical data, such as age, sex, Dukes’ stage, and survival after the operation, were registered for each patient. As a control group, sera from 90 healthy blood donors were included. The patients’ characteristics are shown in Table 1.

Sample Collection
Five-mI blood samples were collected preoperatively from patients scheduled to undergo elective colorectal cancer surgery. The blood was clotted at 4–8°C and subsequently centrifuged at 3000 rpm. The sera were stored at −80°C until they were analyzed.

Antigen
Human Cat B antigen was isolated and characterized in our laboratory as described (8). It was used for immunization of animals and as a standard for preparing the calibration curve.

Determination of Cat B and CEA

Cat B ELISA. Human Cat B was analyzed using an ELISA (sandwich ELISA; KRKA d.d., Novo mesto, Slovenia), developed at Jožef Stefan Institute (Ljubljana, Slovenia). The components were purified and characterized, and the test was optimized as described (20, 29). The antibodies used for Cat B ELISA recognize precursor molecule and enzyme-inhibitor complexes, as well as the mature form of the enzyme (29).

The linearity of ELISA was tested by serial dilution of serum samples to the levels encompassing the range of the assay (20). The measured values of diluted samples were subsequently compared with the standard values. The recovery was tested by the addition of different amounts of antigen to the serum samples with known antigen concentration and varied from 85 to 96%, comparing expected versus observed concentrations. A microplate reader (SLT Rainbow; SLT, Salzburg, Austria) was used to measure absorbance in ELISA. Cat B protein was expressed in ng/ml of serum. The detection limit of the assay was 0.9 ng/ml. Sera, in 1:2 dilution, were added to wells of a microtiter plate, and the assay was performed further as described (20).

CEA. CEA serum level was determined by Immulite CEA assay (EURO/PPC Ltd.), according to the instructions of the manufacturer. CEA protein was expressed in ng/ml of serum. When serum CEA levels exceeded the range of the standard curve, the sample was further diluted and reanalyzed.

Statistical Methods

For descriptive statistics, SPSS PC software was used (Release 6.0; SPSS Inc., Chicago, IL). The differences in Cat B content between two or more groups were tested using rank sum tests.

For analysis of survival, SAS software (Release 6.12; SAS Institute Inc., Cary, NC) was used. Survival curves were estimated using the product limit method of Kaplan-Meier (30), and homogeneity between strata was tested using the log-rank test. The proportional hazards model of Cox (31) was used for multivariate analysis. The covariates Cat B and CEA were scored by dichotomization by their medians. The search for an optimal cutoff point for the dichotomization of Cat B was done by randomizing the data into two groups of approximately the same size. For one of the groups (optimization group or group A), the cutoff point (between the 25th and 75th percentiles) maximizing the partial likelihood of the Cox proportional hazards model was calculated, and this cutoff point was tested on the other group (validation group or group B). In all tests, two-sided Ps below 5% were considered significant.

RESULTS

Distribution of Cat B and CEA. The level of Cat B was significantly increased in sera of patients with colorectal cancer when compared to healthy controls. The medians were 10.7 ng/ml (range, 1.0–140 ng/ml) and 2.1 ng/ml (range, 1.5–40.0 ng/ml), respectively ($P < 0.0001$). The distribution of Cat B serum values from patients was approximately log-normal and is shown in Fig. 1. A correlation between high Cat B level and advanced Dukes’ stage was found ($P < 0.003$), whereas there was no association between serum Cat B and age or sex.

The median serum CEA level was 3.6 ng/ml, and when it was treated as continuous variable, a significant association between CEA and Dukes’ stage was found ($P < 0.001$, $x^2$ test). The levels of Cat B and CEA among Dukes’ stages are shown in Table 2.

The plot of individual Cat B values versus the individual

<table>
<thead>
<tr>
<th>Table 1 Patient characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic</td>
</tr>
<tr>
<td>No. of colorectal cancer patients</td>
</tr>
<tr>
<td>Median age (yr)</td>
</tr>
<tr>
<td>Sex (no. of patients)</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Median observation time (yr)</td>
</tr>
<tr>
<td>Tumor stage (no. of patients)</td>
</tr>
<tr>
<td>Dukes’ A</td>
</tr>
<tr>
<td>Dukes’ B</td>
</tr>
<tr>
<td>Dukes’ C</td>
</tr>
<tr>
<td>Dukes’ D</td>
</tr>
</tbody>
</table>

* Numbers in parentheses represent ranges.
* Numbers in parentheses represent percentages.
CEA values is shown in Fig. 2. The Spearman rank correlation is 0.15 ($P = 0.01$), indicating a weak association.

**Survival Analysis.** The median values were used to dichotomize the Cat B and CEA levels to study their prognostic significance by univariate analysis. For each of these two molecules, overall survival (deaths of all causes) was compared for patients with levels below and above the median values. As seen in Fig. 3A, with this cutoff value, CEA significantly predicted prognosis (RHR = 1.8; 95% CI, 1.4–2.5; $P < 0.0001$). A similar but not statistically significant trend was observed for Cat B (RHR = 1.3; 95% CI, 1.0–1.8; $P = 0.07$; Fig. 3B).

To search for more optimal Cat B cutoff point than the arbitrarily selected median value, the patients were randomly divided into two groups, one denoted group A (optimization group) and the other denoted group B (validation group). There was no statistical difference between the two groups with respect to clinical parameters (sex, age, and Dukes’ stage) or Cat B values. The optimal cutoff point was estimated to be 9.4 ng/ml. This value was the 39th percentile in group A. Using this cutoff point to divide the group A patients, the RHR between the low versus the high group was 1.7. To validate this cutoff point, we applied it to the group B patients. The 36th percentile of group B was 9.4 ng/ml, and using this cutoff point, a significant difference between the low and high Cat B groups was seen (RHR = 1.8; 95% CI, 1.1–2.8; $P = 0.016$). Patients with high Cat B experienced worse prognosis (Fig. 3C).

A multivariate analysis was performed to compare the prognostic value of serum Cat B levels with that of other parameters. The medians of Cat B and of CEA for the total patient population were used as cutoff values. Variables were eliminated from the model singly in a backward fashion and reincluded only if $P$ was <0.05. As seen in Table 3, only age and Dukes’ stage were retained in the final model.

It was then studied whether combination of Cat B and CEA

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**Table 2** Distribution of serum Cat B and CEA among Dukes’ stages

<table>
<thead>
<tr>
<th>Dukes’ stage</th>
<th>Median (ng/ml)</th>
<th>High/low*</th>
<th>Median (ng/ml)</th>
<th>High/low*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8.7</td>
<td>15/13</td>
<td>1.6</td>
<td>6/22</td>
</tr>
<tr>
<td>B</td>
<td>10.7</td>
<td>72/43</td>
<td>2.7</td>
<td>43/72</td>
</tr>
<tr>
<td>C</td>
<td>10.0</td>
<td>54/40</td>
<td>3.4</td>
<td>43/51</td>
</tr>
<tr>
<td>D</td>
<td>13.2</td>
<td>63/25</td>
<td>20.6</td>
<td>68/20</td>
</tr>
</tbody>
</table>

* Median of total population was used as cutoff value.

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*Fig. 1* Distribution of Cat B in the preoperative sera of 325 patients with colorectal cancer. Arrow, position of the optimized cutoff value.

*Fig. 2* Relationship between Cat B and CEA levels in patients’ sera ($n = 325$; $r = 0.15$; $P = 0.01$, Spearman’s rank correlation). Concentrations of both factors are expressed in ng/ml of serum.
Table 3 Univariate and multivariate analysis of survival in 325 patients with colorectal cancer

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>RHR (95% CI)</td>
</tr>
<tr>
<td>Cat B</td>
<td>0.07</td>
<td>1.3 (1.0-1.7)</td>
</tr>
<tr>
<td>CEA</td>
<td>0.0001</td>
<td>1.8 (1.4-2.5)</td>
</tr>
<tr>
<td>Dukes’ B</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Dukes’ C</td>
<td>&lt;0.0001</td>
<td>2.3 (1.4-3.7)</td>
</tr>
<tr>
<td>Dukes’ D</td>
<td>&lt;0.0001</td>
<td>10.0 (6.4-15.6)</td>
</tr>
<tr>
<td>Sex</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.0009</td>
<td>1.0 (1.0-1.05)</td>
</tr>
</tbody>
</table>

* Dukes’ A is a baseline.

* NS, not significant.

would increase the prognostic stratification of the patients. Using Cat B and CEA median values for the total patient population as a cutoff value, further stratification of risk within the group of CEA-positive patients was observed, whereas no difference in survival was found for CEA-negative patients. Kaplan-Meier curves of the four CEA-Cat B combinations are shown in Fig. 4. Patients with both factors above the cutoff point (median) had significantly lower survival probability, as compared with those with CEA+/Cat B− (RHR = 1.5; 95% CI, 1.1–1.8; P = 0.05) or with CEA−/Cat B− (RHR = 2.2; 95% CI, 1.5–3.2; P < 0.0001).

DISCUSSION

Colorectal carcinoma is the second highest cause of cancer mortality (32). For prediction of prognosis in this type of malignancy, the most important factor is the extent of invasion of the primary tumors (Dukes’ staging). Among several tumor markers that are also suggested to correlate with prognosis in colorectal carcinoma, CEA represents the most widely accepted one (33). Increased preoperative serum levels of CEA in colorectal carcinoma patients were shown to correlate with shorter disease-free and overall survival periods (26). However, the rather low sensitivity of serum CEA, the association with Dukes’ stage, different secretion rates of individual tumors, and nonspecific elevations of serum CEA reduce its prognostic impact and indicate the need for additional biological factors for the estimation of prognosis.

Tumor tissue level of Cat B has been shown to correlate with survival in various cancer types (12, 15, 16), including colorectal cancer (14). It has been shown that patients with higher content or increased proteolytic activity of Cat B in tissue cytosols of primary tumors had significantly higher risk of recurrence or death than did patients with a low content of the enzyme. The occurrence of Cat B in sera of cancer patients and its relationship to clinical parameters is less clear. In serum, the catalytic capability of Cat B is rather limited because the majority of the protein is released from the normal and tumor cells in its latent precursor form. Moreover, the serum concentrations of cysteine protease inhibitors cystatin C and kininogens are in large excess over Cat B level and ensure effective in vivo inhibition. Additionally, serum contains also high concentration of α2-macroglobulin, a major scavenger of all endopeptidases. However, α2-macroglobulin probably inhibits powerful endopeptidase Cat L better than it inhibits Cat B because the rate
of initial proteolytic cleavage, necessary for the entrapment of active enzymes by α2-macroglobulin, is related to the endopeptidase activity (20, 34). Although the activation of Cat B in serum cannot be completely ruled out, particularly not in certain microenvironments, it is present in serum predominantly as an inactive enzyme, reflecting increased expression and secretion from tumor and/or tumor-associated cells.

This study shows that higher serum levels of Cat B are associated with shorter overall survival of patients with colorectal cancer. This result is strongly supported by the fact that the survival analysis revealed very similar results (RHR values), when performed on two independent patient populations, i.e., on groups A and B. Furthermore, the association of higher levels of Cat B with poor prognosis is in accordance with the significant correlation between serum Cat B and more advanced Dukes’ stage, an established prognostic factor in colorectal cancer. Because detection of enzymatic activity of Cat B is dependent on various inhibitors and activators, the quantitative immunoassay, detecting all forms of Cat B in serum, should provide more useful clinical information.

CEA levels also correlated strongly with the tumor stage. However, a very weak correlation has been found between individual CEA and Cat B serum values, suggesting independent regulation of these two tumor-associated factors. Univariate analysis provided similar prognostic values for CEA and Cat B (RHR = 1.8). In multivariate analysis, including Dukes’ stage, both variables were left out from the model because, in this patient’s population, they were not independent of Dukes’ stage.

When the data of Cat B and CEA were combined with regard to survival probability, significant improvement of prognostic impact has been obtained when compared with the individual values of either CEA or Cat B. Interestingly, the combination of both factors further stratified the risk of death for CEA-positive patients only, whereas no such difference has been obtained for CEA-negative patients. The survival probability rate was significantly decreased for the group of patients with high levels of both variables, as compared with patients with one or both factors being negative.

In conclusion, our data provide evidence for possible clinical application of Cat B serum levels for prediction of survival of patients with colorectal cancer. The prognostic value of this new factor was considerably increased when combined with CEA. Further studies should evaluate the clinical relevance of the combination of these two independent prognostic factors and their impact on the selection of treatment for the individual patient.

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