Cystatin-like Metastasis-associated Protein mRNA Expression in Human Colorectal Cancer Is Associated with Both Liver Metastasis and Patient Survival

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ABSTRACT

Purpose and Experimental Design: We previously reported that an increased expression of cystatin-like metastasis-associated protein (CMAP) mRNA is involved in liverspecific metastasis in a mouse model. We also identified its human homologue and showed that the expression of CMAP in various human cancer cell lines correlated with the description of malignancy in these cell lines. However, there is still no information available on the clinical significance of CMAP expression in human cancer specimens. Thus, we studied the CMAP expression levels using a real-time quantitative reverse transcription-PCR for 79 patients with colorectal cancer, including 17 cases with liver metastasis.

Results: The mean expression level of CMAP in tumor tissue specimens was significantly higher than in the corresponding normal tissue specimens (P < 0.05). A higher expression of CMAP was significantly correlated with liver metastasis (P < 0.01) as well as a less differentiated histological type (P < 0.05) of colorectal cancer. An increased expression of CMAP was also identified as the strongest independent factor for liver metastasis based on a multivariate analysis (P < 0.001). Furthermore, the prognosis of the patients with a higher expression of CMAP was significantly worse than those with a lower expression (5-year survival rate; 49.7% and 75.0%, respectively, P = 0.038).

Conclusions: These findings imply that the expression level of CMAP in human cancer may be a new biomarker for both liver metastasis and the patient’s outcome.

INTRODUCTION

We previously identified a novel metastasis-associated gene with a differential display system in murine carcinoma cells showing a high rate of metastasis to the liver (1). The protein coded by this gene was named CMAP and showed a 22.1–28.1% homology to human family II cystatins. Two other groups have also independently reported the same cDNA sequence of CMAP in so-called leukocystatin (2) or cystatin F (3) without identifying the first methionine in the cDNA or noting the relationship of the gene to cancer metastasis.

Cysteine proteinases, such as cathepsin B, H, and L, are regulated by endogenous cysteine proteinase inhibitors named cystatins. The cystatin superfamily is composed of at least four families of closely related proteins, including stefins (family I), cystatins (family II), kininogens (family III), and various structurally related but noninhibitory proteins of family IV (4, 5). In the processes of carcinoma invasion and metastasis, both cysteine proteinases and cystatins have been shown to participate in the dissolution and remodeling of connective tissue and basement membranes (6, 7).

Our previous study (1) revealed that CMAP mRNA was selectively overexpressed in all murine liver metastatic tumors but not in any pulmonary metastatic tumors examined. The transfection of CMAP antisense DNA into highly metastatic liver cells greatly decreased their CMAP expression and metastatic potential, thus indicating that CMAP is involved in the liver metastatic ability after the intravasation of malignant cells. The human homologue of CMAP was also found in some malignant human cancer, including gastrointestinal cancers by a PCR-based strategy, although the studies were performed with tumor cell lines. For more exact information on the relationship between CMAP expression and malignant progression, including metastasis in human cancer, an analysis of surgical or biopsy specimens is required.

This study focused on identifying whether the expression of CMAP mRNA is also involved in liver metastasis of human cancer. Interestingly, the CMAP expression status in tumor tissue determined by a real-time quantitative RT-PCR showed a strong association with both colorectal liver metastasis and the patient prognosis.

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2 The first two authors contributed equally to this study.

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4 The abbreviations used are: CMAP, cystatin-like metastasis-associated protein; RT-PCR, reverse transcription-PCR.
MATERIALS AND METHODS

Seventy-nine patients with colorectal cancer who underwent surgery at our institutes were entered in this study. The resected tumor and paired nontumor tissue specimens were immediately frozen in liquid nitrogen and kept at \(-70^\circ\text{C}\) until the extraction of RNA. Written informed consent was obtained from all patients. Among these 79 patients, 17 patients had synchronous hepatic metastases. All patients were closely followed after surgery at regular 1-month intervals. The follow-up ranged from 3 to 63 months with a median of 41 months.

Real-Time Quantitative RT-PCR. Total RNA was extracted from the surgical samples or HL-60 human leukemia cells by the acid-phenol guanidinium method (8, 9). cDNA was synthesized with random hexamer primer and M-MLV reverse transcriptase (Life Technologies, Inc.). The quantification of the mRNA levels was performed on an ABI Prism 7700 Sequence Detection System (PE Biosystems). Primers and TaqMan probe for human CMAP were designed using Primer Express software. To avoid the amplification of contaminating genomic DNA, a TaqMan probe was placed at the junction of exons 3 and 4. To standardize the amount of total RNA added to each reaction, \(\beta\)-actin mRNA levels were measured as an endogenous control by using TaqMan PDAR Control Reagents. To quantitate the amount of specific mRNA in the samples, a standard curve was generated for each run using five points (20–0.002 ng) of the HL-60 cDNA. In each reaction for tissue samples, 16 ng of cDNA were added in 50 \(\mu\)l of total reaction mixture, and both the CMAP and \(\beta\)-actin PCRs were carried out in the same tube in triplicate. The relative expression levels of CMAP were obtained by normalizing the amount of CMAP mRNA divided by that of \(\beta\)-actin mRNA in each sample (10, 11). The sequences for the TaqMan probes and primers were as follows: sense primer 5'–GTCTGGATGACTGTGACTTCCAAA–3'; antisense primer 5'–AGTGGACAACGGAGAAGCGCA–3'; and probe 5'–CAACCAACCTTTGAGCAGCCAGT–3'.

Statistical Methods. For continuous variables, the data were expressed as the means \(\pm SD\). The relationship between the CMAP mRNA expression and the clinicopathological factors was analyzed using the \(\chi^2\) test and Student’s \(t\) test. The surviving curves were plotted according to the Kaplan–Meier method, and the generalized Wilcoxon test was applied to compare the survival curve. A multivariate adjustment was also made using a stepwise regression analysis. All tests were analyzed using the StatView software package (Abacus Concepts, Inc.), and the findings were considered significant when \(P < 0.05\).

RESULTS

Expression Value of CMAP mRNA. We determined the levels of CMAP mRNA expression by comparisons with human leukemia cell line HL-60 as the quantifying standard, which expresses human CMAP sufficiently. The mean expression level of CMAP mRNA in tumor tissue, 0.062 \(\pm 0.074\), was significantly higher than 0.043 \(\pm 0.037\) in the corresponding normal tissue (\(P = 0.042\)). The cases with values of less than the mean expression level (0.062) in tumor tissue were considered to be a low expression group (\(n = 55\)), whereas those with values \(\geq 0.062\) were considered to be a high expression group (\(n = 24\)). The clinical implications of CMAP expression in patients with colorectal cancer were evaluated by comparisons between these two groups.

Expression of CMAP mRNA and Clinicopathological Characteristics. The clinicopathological factors analyzed are shown in Table 1 in relation to the CMAP mRNA expression status. A high expression group showed a significantly higher frequency of moderately or poorly differentiated tumors (\(P = 0.011\)). Furthermore, the incidence of liver metastasis in the high expression group (11 of 24, 46%) was significantly higher (\(P = 0.0008\)) than that in the low expression group (6 of 55, 11%). In contrast, other pathological variables such as the serosal invasion, lymph node metastasis, lymphatic invasion, venous invasion, and peritoneal dissemination were not associated with the CMAP expression status. Because only 1 patient with lung metastasis was included in this study, the relationship between the CMAP expression status and lung metastasis was not considered for a statistical analysis. Table 1 shows the results of a multivariate adjustment with a stepwise regression analysis. In addition to histological type, liver metastasis was selected as the strongest independent factor (\(F = 14.22\)) for patients in the high CMAP expression group (\(P < 0.001\)).

We also compared the cumulative survival rate of the low expression (\(n = 55\)) and high expression (\(n = 24\)) group as

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinicopathological data and CMAP mRNA expression in the tumor tissue specimens of 79 patients with colorectal carcinoma</th>
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<tbody>
<tr>
<td>Variables</td>
<td>Low expression(^a)</td>
</tr>
<tr>
<td>Sex (male:female)</td>
<td>23:32</td>
</tr>
<tr>
<td>Age</td>
<td>67.9 (\pm 9.2)</td>
</tr>
<tr>
<td>Site</td>
<td>[Details not shown]</td>
</tr>
<tr>
<td>Histological grade(^b)</td>
<td>[Details not shown]</td>
</tr>
<tr>
<td>Liver metastasis</td>
<td>[Details not shown]</td>
</tr>
<tr>
<td>Peritoneal dissemination</td>
<td>[Details not shown]</td>
</tr>
<tr>
<td>Duke’s classification</td>
<td>[Details not shown]</td>
</tr>
</tbody>
</table>

\(^a\) Low and high groups were determined by a mean value of CMAP mRNA (0.062).

\(^b\) G1, well-differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma.
shown in Fig. 1. The high expression group showed a significantly poorer prognosis ($P = 0.038$) than the low expression group (5-year survival rate; 49.7 and 75.0%, respectively).

### DISCUSSION

We previously reported the identification and cloning of a novel cDNA for a $\text{CMAP}$ and its close correlation with the liver metastatic potential of cells in the murine system (1). In this study, we tested this correlation in human colorectal cancer, which is the most common human cancer known to develop liver metastasis (12).

In the previous report (1), we analyzed the expression levels of $\text{CMAP}$ in several organ tissues of mice and found its expression to be detected only in lymphoid organs such as thymus, spleen, and lymph node. Thus, this unique distribution of $\text{CMAP}$ in lymphoid organs suggested that $\text{CMAP}$ possesses a physiological association with the immune system. However, there was no information on the $\text{CMAP}$ mRNA expression in human tissues, including human cancer. We thus determined its expression levels in surgically resected colorectal cancer tissues. Although no $\text{CMAP}$ expression was observed in the mouse large intestine, $\text{CMAP}$ expression was detectable in both normal and tumor tissue specimens of human colorectal cancer. One possible explanation for this discrepancy might be attributable to differences in the species studied. Another explanation is the differences in the detection systems for the $\text{CMAP}$ and its close correlation with the liver metastasis was statistically confirmed by both a univariate and multivariate analysis. Proteinase inhibitors such as cystatins are generally considered to possess antimetastatic activities because proteinases are usually up-regulated and/or abnormally activated in metastatic tumor cells (13–15). We used the differential display method to isolate the cancer-related genes not only in murine cell lines (1) but also in clinical carcinoma specimens (16–18). One such successfully obtained gene was cystatin B (16), the expression of which was drastically suppressed in the tumor tissue of esophageal carcinoma when compared with its corresponding normal tissue. The cystatin B expression demonstrated an inverse relation to lymph node metastasis of the esophageal carcinoma and consequently to the stage of disease.

Although the results of this study are consistent with our previous report in the murine system (1), these results appear to be contradictory to the general view of cystatins (13–15), including our previous findings in patients with esophageal cancer (16). However, the production levels of cysteine proteinase inhibitors may possibly increase to compensate for the excessively induced cysteine proteinase in metastatic tumor cells, and as a result, an imbalance may occur between cysteine proteinase and its inhibitor. Such an imbalance seems to play an important role in cancer invasion and metastasis (5, 19).

On the other hand, several studies have also reported a relationship between the activities of proteinase inhibitors and the prognosis of cancer patients, however, the results remain controversial (20–29). In breast (20), lung (21), and head and neck (22) tumors, higher levels of stefin A or stefin B were shown to correlate with a favorable prognosis. On the other hand, the risk of dying has been shown to be significantly higher in patients with increased levels of cysteine proteinase inhibitors in recent studies of breast (23) and colorectal (24) tumors. Furthermore, our results are also consistent with recent reports revealing a correlation between both the increased levels of metalloproteinase inhibitors (25–26) and serine proteinase inhibitors (27–29) and the poor prognosis of cancer patients. For tissue inhibitor of metalloproteinase-1 and plasminogen activator inhibitor-1, it has been proposed that besides proteinase inactivation, additional tumorigenic functions may contribute to a worse prognosis, although no such tumorigenic functions were found for $\text{CMAP}$. Therefore, the precise function of $\text{CMAP}$ in the patient prognosis remains unclear. However, $\text{CMAP}$ may protect producer cells themselves from either excessive proteolysis or from an attack of exogenous proteinases. Alternatively, high levels of $\text{CMAP}$ may also protect the provisional pericellular matrix of nascent capillaries forming behind the proteinase promoted extensions (27, 29).

In this study, we confirmed the close correlation between the tumor expression levels of $\text{CMAP}$ and liver metastasis even for human colorectal cancer. Therefore, it is possible that a determination of the $\text{CMAP}$ mRNA expression of human cancer may help in the identification of patients at high risk for liver

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**Table 2** Significant variables determined by a stepwise regression model for $\text{CMAP}$ expression in the tumor tissue specimens of 79 patients with colorectal carcinoma

<table>
<thead>
<tr>
<th>Variables</th>
<th>Coefficient</th>
<th>$F$</th>
</tr>
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<tbody>
<tr>
<td>Histological grade</td>
<td>0.295</td>
<td>8.67</td>
</tr>
<tr>
<td>Liver metastasis</td>
<td>0.378</td>
<td>14.22</td>
</tr>
</tbody>
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**Fig. 1** Comparison of the Kaplan-Meier survival curves in the low expression group ($n = 55$) versus the high expression group ($n = 24$). The survival rate for patients in the high expression group was significantly lower than that for patients in the low expression group ($P = 0.038$).
metastasis, and these patients could thereby benefit from careful examinations and extensive treatments for liver metastasis. Recently, the genomic localization of human CMAP was determined by fluorescence in situ hybridization at our laboratory (30). Additional elucidation of the mechanisms by which the increased CMAP expression affects the liver-specific metastatic potential of colorectal cancer is expected to provide a new molecular target for the treatment of liver metastasis.

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