Motility Related Protein 1 (MRP1/CD9) Expression in Colon Cancer

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ABSTRACT

It is important to detect genes that may be good prognostic markers for colon cancer patients. With this in mind, we identified the motility related protein-1 (MRP1/CD9) gene in human colon tissues. The aim of this study was to clarify the significance of MRP1/CD9 gene expression in human colon cancers. We performed the differential mRNA display technique between tumor/normal paired samples of the colon and identified MRP1/CD9. Eighty-two surgical specimens of primary colorectal cancer were analyzed by means of reverse transcription-PCR for the MRP1/CD9 gene. Its expression status and clinicopathological variables were analyzed univariately and multivariately. The MRP1/CD9 mRNA expression was positive in 56 cases and negative in 26 cases. The MRP1/CD9 negative cases showed a significantly higher frequency of venous-vein invasion and liver metastasis, or a worse prognosis than the MRP1/CD9 positive cases (P < 0.05). Multivariate analysis with the Cox regression model disclosed that MRP1/CD9 expression was an independent prognostic factor distinct from the lymph node status. The findings imply that the study of MRP1/CD9 expression may be useful for predicting prognosis of patients with colorectal cancer.

INTRODUCTION

We have used the techniques of subtractive cDNA cloning or differential display between tumor/normal paired samples of gastrointestinal cancers to determine potential new prognostic markers (1–3). We have recently identified one gene, which is identical to MRP1, by differential display of colon cancer normal samples.

MRP1 is a transmembrane glycoprotein that is identical to the CD9 antigen (4). MRP1/CD9 belongs to a structurally distinct family of cell membrane glycoproteins called the transmembrane-4 superfamily (5, 6). The alteration in expression of these cell membrane glycoproteins may be associated with tumor cell progression or metastasis. For example, one of these cell membrane glycoproteins, ME491/CD63, is reported to be inversely associated with metastasis of melanomas (7). Similarly, another cell membrane glycoprotein, KAI-1/CD82, may function as a metastasis suppressor factor (8, 9).

Concerning MRP1/CD9, its expression has been reported to be inversely correlated with tumor cell growth or metastasis by both in vitro studies (10) and clinical studies of lung or breast cancers (11, 12). To our knowledge, however, there is no information on the expression of MRP1/CD9 in human colorectal cancers. We herein report the isolation of MRP1/CD9 mRNA from differential displays, the results of RT-PCR of this gene expression in clinical colon cancer tissues, and a correlation between the gene expression status and clinicopathological factors. Interestingly, this study disclosed that the MRP1/CD9 expression may be a new prognostic marker for patients with colorectal cancer.

MATERIALS AND METHODS

Identification of MRP1/CD9 from Differential Displays.

The mRNA obtained from human colon cancer tissue and that from corresponding normal tissue were analyzed by the modified protocol of fluorescent differential display (13). One band that was remarkably reduced in cancer tissue compared with normal tissue was cut, cloned, and sequenced.

Northern Blot.

To confirm the expression of the gene isolated from differential displays, Northern blots were performed as described elsewhere (1, 2). The paired samples of tumor/normal tissues obtained from six cases of colon cancer were used. These six cases were randomly selected from the 82 patients described below.

Clinical Samples and RNA Extraction.

Eighty-two fresh surgical specimens of primary colon or rectal cancer were used. The samples were kept at −90°C until use and total RNA was prepared (1, 2). To avoid the contamination by genomic DNA, 50 μg of total RNA was treated with one unit of DNase I (Message clean kit, Gen Hunter Corp.) at 37°C for 1 h in the presence of one unit of RNase inhibitor, followed by phenol/ chloroform purification and ethanol precipitation. The treated RNA was stored at −90°C until use.

RT-PCR Analysis.

cDNA was synthesized from 2.5 μg of total RNA (14, 15). The oligonucleotide primer pairs for MRP1/CD9 were synthesized (sense primer, 5′-TGCATCTCG-
TATCCAGCGCCA-3'; antisense primer, 5'-CTCAGGGATG-TAAGCTGACT-3'). The oligonucleotide primer was end-labeled with radiisotupe. The PCR was performed according to Higashiyama et al. (11). RT-PCR was performed with and without addition of reverse transcriptase to eliminate genomic DNA contamination. GAPDH amplification was used as an internal control (14, 15). Aliquots of the amplified DNA were electrophoresed on 2% agarose gels, dried and exposed to an imaging plate, and analyzed by a Bio-Image analyzer (Fuji, Kanagawa, Japan).

The expression of MRP1/CD9 was corrected for that of GAPDH in each case. The cases with values of equal or more than 0.5 were considered as positive and those with values of less than 0.5 were considered as negative.

**Clinicopathological Data.** The clinical data variables as shown in Table 1 were all available for evaluation. Follow-up data were also available in all patients. The data were compared between MRP1/CD9 positive and negative cases.

**Statistical Analysis.** The BMDP Statistical Package program (BMDP, Los Angeles, CA) for the main frame computer (4381; IBM, Armonk, NY) was used for all analyses. Associations between the variables were tested by Fisher’s exact test. The BMDP PIL program was used for survival analysis (Kaplan-Meier method) and testing equality of survival curves (Mantel-Cox method). The BMDP P2L program was used for multivariate adjustments for all covariates simultaneously, with a backward stepwise logistic regression analysis.

**RESULTS**

**Isolation of MRP1/CD9.** One result of differential displays of subsets of cDNA between samples of two tumor/normal pairs of colorectal tissues is shown in Fig. 1A. The PCR product present in the normal samples and absent in the tumor samples and that gave rise to MRP1/CD9 is shown by an arrow (Fig. 1A).

**Northern Blot Analysis.** Individual bands of interest from the differential display gels were cut out, amplified by PCR, purified, and used as probes in Northern analysis to determine the relative expression between a larger number of tumor and normal pairs of RNAs, each pair from the same patient. Those with a significantly different expression in at least several pairs of cancer/normal samples were further examined. One such PCR product is the subject of this report. As shown in Fig. 1B, all six normal samples showed a MRP1/CD9 mRNA signal, whereas four of the six cancer samples showed a very weak signal. The results prompted us to further examine the significance of the difference between the positive and the negative MRP1/CD9 cases.

**RT-PCR and Clinicopathological Data.** The patients consisted of 50 men and 32 women. The ages ranged from 40–82 years and the mean was 66.1 years. The clinicopathological factors analyzed are shown in Table 1 in relation to the MRP1/CD9 expression status.

RT-PCR results demonstrated that 56 cases (68.3%) were positive and 26 cases (31.7%) were negative. Representative results of 15 tumors are shown in Fig. 2. As shown in Table 1, there were no significant differences between the MRP1/CD9 expression status and the age, sex, histological differentiation, depth of tumor invasion, lymph-vessel invasion, lymph node metastasis, peritoneal dissemination, or Dukes’ stage. In contrast, the MRP1/CD9 expression was significantly associated with venous-vessel invasion ($P < 0.05$) and liver metastasis ($P = 0.05$).

The survival curve of 82 patients in relation to the MRP1/CD9 status is shown in Fig. 3. The patients with positive expression showed a significantly better survival than those with negative expression ($P < 0.05$).

Each of the 10 variables of age, sex, histological differentiation, depth of tumor invasion, lymph-vessel invasion, venous-vessel invasion, lymph node metastasis, liver metastasis, peritoneal dissemination, and MRP1/CD9 status was used in Cox regression analysis. Consequently the lymph node metastasis and the MRP1/CD9 status were disclosed to be significant prognostic factors as shown in Table 2.

**DISCUSSION**

Identifying new genes that are associated with tumor growth, metastasis, and prognosis is very important to advance the understanding of cancer biology (16). Recent progress in molecular biology techniques allows us to identify such genes in several kinds of cancers. We have been identifying genes that are differentially expressed between tumor and normal samples of the gastrointestinal tract by means of cDNA subtraction or differential display methods (1–3). Accordingly, one such gene, MRP1/CD9, was identified and is the subject of this report. The MRP1/CD9 gene encodes a 24–27 kDa glycoprotein that contains four hydrophobic domains and an extracellular N-glycosylated domain; the latter may function as a cell surface-anchored receptor (6, 17, 18). The precise physiological functions of MRP1/CD9 remain unknown;

<table>
<thead>
<tr>
<th>Variable</th>
<th>MRPI/CD9 expression</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>37</td>
<td>13</td>
</tr>
<tr>
<td>Female</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>Histologic differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>Moderate</td>
<td>29</td>
<td>14</td>
</tr>
<tr>
<td>Poor</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Depth of invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within the wall</td>
<td>37</td>
<td>13</td>
</tr>
<tr>
<td>Beyond the wall</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>Lymph vessel invasion</td>
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<td></td>
</tr>
<tr>
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<td>24</td>
<td>12</td>
</tr>
<tr>
<td>Present</td>
<td>32</td>
<td>14</td>
</tr>
<tr>
<td>Venous-vessel invasion</td>
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<td></td>
</tr>
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<td>Absent</td>
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<td>9</td>
</tr>
<tr>
<td>Present</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>32</td>
<td>13</td>
</tr>
<tr>
<td>Present</td>
<td>24</td>
<td>13</td>
</tr>
<tr>
<td>Liver metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>52</td>
<td>19</td>
</tr>
<tr>
<td>Present</td>
<td>4</td>
<td>7</td>
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<tr>
<td>Peritoneal dissemination</td>
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<td></td>
</tr>
<tr>
<td>Absent</td>
<td>53</td>
<td>24</td>
</tr>
<tr>
<td>Present</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Dukes’ stage</td>
<td>A/B</td>
<td></td>
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<tr>
<td>A</td>
<td>32</td>
<td>13</td>
</tr>
<tr>
<td>C/D</td>
<td>24</td>
<td>13</td>
</tr>
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</table>

" NS, not significant.
Fig. 1 A, differential display of RNA subsets between colon cancer (T) and adjacent non-neoplastic colon (N). The PCR band present in the normal sample but absent from the paired colon cancer sample, which represents MRP1/CD9, is shown by the arrow. B, northern hybridization with tumor (T) and nontumor (N) paired RNA samples from six patients. The MRP1/CD9 expression is very weak in tumor tissue in four cases. The filter was stripped and rehybridized to a GAPDH probe to verify that the mRNA is intact and that equal amounts of RNA are loaded onto the gels.

Fig. 2 RT-PCR of MRP1/CD9 in 15 representative cases. RT-PCR of GAPDH was used as an internal control. The expression of MRP1/CD9 corrected for that of GAPDH was calculated and a ratio ≥0.1 was considered positive, whereas a ratio <0.1 was considered negative. Lanes 2, 3, 4, 6, 7, 8, 9, 10, 11, and 14 are positive, and Lanes 1, 5, 12, 13, and 15 are negative.

Fig. 3 The survival curve of colorectal cancer patients according to the expression status of MRP1/CD9. The patients with positive MRP1/CD9 expression show a better survival rate than those with negative MRP1/CD9 expression (P < 0.05).

Table 2 Results of a multivariate analysis

<table>
<thead>
<tr>
<th>Variables</th>
<th>RC</th>
<th>SE</th>
<th>RC/SE</th>
<th>Odds ratio</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Lymph node metastasis</td>
<td>2.418</td>
<td>0.863</td>
<td>2.80</td>
<td>1.2</td>
<td>0.001</td>
</tr>
<tr>
<td>MRP1/CD9 expression</td>
<td>-1.785</td>
<td>0.725</td>
<td>-2.46</td>
<td>0.168</td>
<td>0.010</td>
</tr>
</tbody>
</table>

RC, regression coefficient.

Si and Hersey (20) reported similar prognostic significance of MRP1/CD9 gene expression in melanoma patients. In our present study of colon cancer, the reduced expression of MRP1/CD9 in the tumor tissue was significantly associated with venous-vessel invasion and liver metastasis. The patients with reduced MRP1/CD9 expression in colon cancer tissue showed a significantly worse prognosis than those with MRP1/CD9 expression, and this is similar to the results of lung or breast cancers or melanoma. Interestingly, the multivariate analysis clarified that the expression status of MRP1/CD9 was an independent prognostic factor in addition to the status of lymph node metastasis.

In addition to MRP1/CD9, the expression of two other members of the transmembrane 4 superfamily have been correlated with metastasis. KAI1/CD82 was identified as a metastasis-suppressor gene for prostate cancer, and its protein expression was downregulated during the progression of human prostatic cancer (8, 21, 22). A similar finding was recognized in pancreatic cancer (23) and non-small cell lung cancer (9). In the latter, KAI1/CD82 was disclosed to be an independent prognostic factor. Another member, ME491/CD63, was identified in human melanoma cells. The ME491/CD63-transfected melanoma cells showed much lower growth rates in athymic nude mice and showed a reduced ability for metastasis in the peritoneal cavity and s.c. sites (7). Similar results

however, the highly preserved DNA sequence throughout evolution suggests an important role for this gene. Several possible functions have been reported, including participation in signal transduction, antigen presentation, cell proliferation, cell adhesion, or cell motility (4-6, 17, 18).

Recent studies of clinical samples of human cancers demonstrated the relation between the reduced expression of MRP1/CD9 and aggressive behavior of the tumor. Higashiyama et al. (11) reported that cases of lung adenocarcinoma with reduced MRP1/CD9 expression had a significant tendency to show lymph nodes metastasis, an advanced stage of disease, and a worse prognosis. Miyake et al. (12, 19) demonstrated that reduced MRP1/CD9 expression in breast cancer was significantly associated with lymph node metastasis and a worse prognosis.
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were demonstrated in ME491/CD63-transfected NIH-3T3 cells (24). Thus, ME491/CD63 and KAI1/CD82, as well as MRPI/CD9, may have a role that acts to suppress tumor invasion, progression, or metastasis. A recent study (5) disclosed the existence of a transmembrane 4 superfamily network on the cell surface, further supporting the intimate correlation between MRPI/CD9, ME491/CD63, and KAI1/CD82.

The reasons why the reduced expression of the MRPI/CD9 gene is associated with poor prognosis remain uncertain. There are several possible explanations postulated. One possibility relates to cell motility and metastasis. Cell motility was suppressed in various types of cultured cells transfected with MRPI/CD9 cDNA (25). In addition, the metastatic potential of mouse melanoma BL6 transformants that expressed the MRPI/CD9 gene was lower than that of the parent BL6 cells (10). These reports suggest a negative role for MRPI/CD9 in tumor motility and metastasis and support the results of the clinical samples mentioned above. Our present study showing the association between the reduced expression of MRPI/CD9 and venous-vessel invasion may also suggest this possibility.

Cajot et al. (26) demonstrated interesting findings from differential display analysis between matched primary and metastases-derived human colon carcinoma cell lines. They identified the MRPI/CD9 gene and described that MRPI/CD9 was highly expressed in the primary tumor-derived cell line that displayed a higher migration potential, whereas it was expressed little in the metastasis-derived cell line that displayed a lower migration potential. Our results support their statement that cancer cell motility may favor local dissemination from the primary cancer site, whereas metastasis formation at a distant site (which includes capillary arrest and adhesion to the endothelium, followed by extravasation in the distant organ) may require down-regulation of MRPI/CD9 expression.

In conclusion, the status of MRPI/CD9 may be a new prognostic marker for patients with colon cancer. More work is required to clarify the precise mechanism of the MRPI/CD9 gene expression in cancer progression or metastasis.

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Clinical Cancer Research

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