Prediction of Peritoneal Micrometastasis by Peritoneal Lavaged Cytology and Reverse Transcriptase-Polymerase Chain Reaction for Matrix Metalloproteinase-7 mRNA

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ABSTRACT

Purpose: Peritoneal dissemination is the most common cause of death associated with gastric cancer. In this study, we report the significance of molecular diagnosis of peritoneal dissemination by means of matrix metalloproteinase-7 (MMP-7) reverse transcriptase-PCR (RT-PCR) assay using preoperative peritoneal lavaged fluid.

Experimental Design: Preoperative peritoneal lavage by paracentesis was performed on 152 patients with gastric cancer. The peritoneal lavaged fluid was subjected to RT-PCR analysis with primers specific for MMP-7 and conventional cytological Papanicolaou examination.

Results: The MMP-7 RT-PCR assay was able to detect cancer cells at densities even lower than 10 cells/sample. There was no signal of MMP-7 mRNA from mesothelial cells, fibroblasts, peripheral blood, and lavaged fluid from patients with benign disease. Cytological examination and MMP-7 RT-PCR assay results were positive for 27 (18%) and 28 (18%) samples, respectively. The sensitivity for the prediction of peritoneal dissemination by cytology and MMP-7 RT-PCR assay were 46% and 33%, but the combination analysis using both parameters improved the sensitivity rate with 62%. Logistic regression analysis revealed that the cytological examination and MMP-7 RT-PCR assay are independent predictors of peritoneal dissemination.

Conclusion: The combination of cytological examination and RT-PCR assay of preoperative peritoneal lavaged fluid is a highly efficient and reliable method for the selection of patients for adjuvant i.p. chemotherapy.

INTRODUCTION

The prognosis of patients with gastric cancer is strongly affected by the degree of lymph node metastasis and serosal invasion. Peritoneal dissemination is the most common mode of metastasis in advanced gastric cancer. Even after extended lymphadenectomy, peritoneal recurrence develops in approximately half of the patients (1).

Peritoneal recurrence develops from micrometastasis, which is already seeded before operation, and micrometastasis originates from peritoneal free cancer cells (2). However, the morphological identification of micrometastasis in the peritoneum can be particularly difficult, and peritoneal wash cytology has a low sensitivity rate for the detection of peritoneal dissemination (3, 4).

Recently, new ways of detecting micrometastasis with the aid of immunohistochemical (5) and molecular biological (6) methods have been developed, and the rate of false-negative diagnosis has been reduced. Benevolo et al. (5) reported that with immunohistochemical detection of peritoneal micrometastasis in gastric cancer it was possible to identify free cancer cells in 35% of the patients, with a 14% improvement over routine cytopathology results (6).

Furthermore, Kodera et al. (7) found that the use of RT-PCR for CEA mRNA resulted in a detection rate of free cancer cells of 28% (41 of 148), with a 14% higher detection rate than for peritoneal wash cytology. However, the sensitivity of the CEA RT-PCR assay for the detection of peritoneal micrometastasis still remains low. For this reason, development of new molecules for RT-PCR assay with high accuracy is eagerly awaited.

Of the more than 17 MMPs, Yonemura et al. (8) reported that MMP-7 is selectively produced from gastric cancer cells and expressed in peritoneal dissemination from gastric cancer. This suggested to us that detection of MMP-7 mRNA in the peritoneal lavaged fluid might indicate the existence of peritoneal free cancer cells and micrometastasis.

In this study, we report the clinical significance of a new molecular diagnosis method to detect i.p. micrometastasis with a combination of peritoneal wash cytology and RT-PCR assay for MMP-7 mRNA using preoperative peritoneal lavaged fluid.

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2 The abbreviations used are: RT-PCR, reverse transcriptase PCR; CEA, carcinoembryonic antigen; MMP, matrix metalloproteinase.
PATIENTS AND METHODS

Patients and Cell Lines. Preoperative peritoneal lavage was performed in 152 patients with gastric cancer who intended to undergo gastrectomy at Kanazawa University, Ulsan University, and Tsuruga National Hospital between January 1996 and December 1999. The patients included 90 men and 62 women and ranged in age from 29 to 82 years (median, 62 years). These patients underwent gastrectomy with lymph node dissection. The follow-up period ranged from 8 to 47 months, and the median follow-up period was 2.4 years.

All of the resected specimens were histologically examined by H&E staining according to the general rules of the Japanese Classification of Gastric Carcinoma (9). As a positive control of RT-PCR for MMP-7 mRNA, the gastric cancer cell line, MKN-45-P, was cultured in RPMI 1640 medium with 10% FCS (Life Technologies, Inc.). MKN-45-P has been established as a highly metastatic cell line on the nude mouse peritoneum and is known to overexpress MMP-7 mRNA and protein (8,10). The fibroblast cell line KMST-6 and human mesothelial cells were also cultured and used as a negative control (10). Samples from normal gastric mucosa were obtained at endoscopic biopsy from a noncancerous patient, and peripheral venous blood samples were taken with informed consent from healthy volunteers.

Preoperative Peritoneal Wash Examination. The study consisted of 152 patients with gastric cancer and 26 with benign disease including cholelithiasis, ileus, appendicitis, and gastric adenoma. A preoperative peritoneal wash was done by a puncture at the point on the Monro-Richter line under local anesthesia with 20 ml of 1% xylocain, 1000 ml of saline was injected into the abdominal cavity. One hour later, a part of injected saline was withdrawn, and the recovered fluid was centrifuged at 2000 rpm for 10 min. The pellets of lavaged fluid were dissolved in ISOGEN RNA extraction buffer (Nippon Gene, Tokyo, Japan) and stored at −80°C until use. A part of each peritoneal wash fluid was examined cytologically after conventional Papanicolaou staining. Peritoneal dissemination from 10 gastric cancer patients was surgically removed and stored at −80°C until use.

RT-PCR. RT-PCR analysis was done with the modifications of Conboy et al. (12). Total RNAs were extracted from the pellets of peritoneal lavaged fluid, peritoneal dissemination, normal gastric mucosa, MKN-45-P cells, a fibroblast cell line of KMST-6, human mesothelial cells, and peripheral blood using a guanidinium-isothiocyanate-phenol-chloroform-based method (13). The prepared RNA was mixed with oligodeoxythymidy-late primer and was reverse-transcribed with AMV reverse transcriptase (Life Sciences, St. Petersburg, FL), followed by PCR amplification (Perkin-Elmer Corp., Norwalk, CT) with specific primers. PCR amplification was done for 1 min at 94°C, 1.5 min at 58°C, and 2 min at 72°C for 27 cycles, then by three cycles of 1 min at 94°C, 2 min at 58°C, and 5 min at 72°C. The PCR products were electrophoresed on 2% agarose gels and transferred to a nylon membrane filter. The transferred products were hybridized overnight to a 32P-end-labeled probe specific for the target cDNA fragment. The autoradiogram was exposed for 4–5 h with two intensifying screens at −80°C. Sequences of sense and antisense primers for the MMP-7 gene were 5′-ATGTTAAACTCCCGCTCATA-3′ (sense primer) and 5′-CAGCATACAGGAAGTTAATCC-3′ (antisense primer), respectively (PCR product, 481 bp). The probe oligonucleotide for MMP-7 was 5′-CCTACTGATGTCAGCAGTT-3′. Primer sequences for β-actin were 5′-TTGAAAGGTAGTTCTCTG-3′ (sense) and 5′-GAAAATCTCTGCCACCAACCTT-3′ (antisense; PCR product, 591 bp), and the probe oligonucleotide was 5′-ACTGACTACCTCATGAAGAT-3′.

Data Presentation and Statistical Analysis. All of the statistical calculations were done with SPSS statistical software. The figures show the mean plus or minus the SD. The statistical calculations were done with SPSS statistical software. The chi square test was used to analyze data. The outcomes of the different groups of patients were compared by the generalized Wilcoxon test. To clarify the status of i.p. MMP-7 mRNA and cytology as the predictor of peritoneal dissemination, logistic regression analysis was performed. A Cox proportional hazard model was done to identify the independent prognostic factors.

RESULTS

MMP-7 Expression from Peritoneal Dissemination. A specific DNA fragment of MMP-7 with a corresponding size of 418 bp was amplified by RT-PCR for the detection of MMP-7 mRNA extracted from MKN-45-P (Fig. 1). Fig. 1 shows a result of a serial dilution experiment of MMP-7 producing MKN-45-P cells with PBS buffer. The MMP-7 RT-PCR assay was capable of detecting tumor cells of smaller than 10.

The MMP-7-specific transcripts were detected in all of 10 samples of peritoneal dissemination (Fig. 2) but were not detected in normal gastric mucosa, mesothelial cells, peripheral blood, and a fibroblast cell line (KMST-6; Fig. 3).

Results of Preoperative Peritoneal Wash Assay. MMP-7 RT-PCR assay and cytology gave 28 (18%) and 27 (18%) positives of 152 peritoneal wash samples from patients with gastric cancer, respectively (Table 1), whereas none of 26 patients with benign disease showed positive results for MMP-7 RT-PCR assay and peritoneal lavaged cytology. The result of MMP-7 RT-PCR assay significantly correlated with that of
cytology (P = 0.003; Table 1). However, 17 (14%) of 125 cytologically negative patients were positive for MMP-7 RT-PCR assay, and 16 (13%) of 124 patients with a negative study of MMP-7 RT-PCR assay had positive cytology. By the combination analyses of MMP-7 RT-PCR and cytology, a positive rate of either MMP-7 RT-PCR or cytology was 29% (44 of 152), with an 11% increase as compared with a positive rate of cytology alone (18%; 27 of 152).

The correlations between the clinicopathological factors and RT-PCR assays and cytology are summarized in Table 2. Positive RT-PCR assay significantly correlated with lymph node metastasis, serosal invasion, infiltrating type, vessel invasion, and advanced cancer but not with histological type. Cytologically positive tumors had significantly higher incidences of lymph node metastasis, serosal invasion, vessel invasion, advanced tumor, and poorly differentiated type. Cytology, and MMP-7 RT-PCR are the significant prognostic factors for poor prognosis.

As shown in Fig. 4, cytologically positive patients had significantly poorer prognoses than cytologically negative patients (P < 0.001). In addition, there was a significant survival difference between patients with a negative study and with a positive examination for MMP-7 RT-PCR assay (Fig. 5). There was no 2-year survivor in patients with a positive examination for either MMP-7 RT-PCR assay or cytology.

When the patients were subdivided according to the results of cytology and MMP-7 RT-PCR assay, patients with a negative assay in cytological and MMP-7 RT-PCR examination survived significantly longer than those with positive results in cytology or MMP-7 RT-PCR assay (Fig. 6). The prognosis of patients with positive MMP-7 RT-PCR assay and negative cytology was also very poor, as was the survival of cytologically positive patients.

Multivariate ANOVA was performed by the Cox stepwise regression analysis to identify individual variables that were significant in terms of survival (Table 3). Among eight factors that were found to affect the outcome of patients by univariate analysis, lymph node metastasis, cytological examination, and a combination analysis of cytological and MMP-7 RT-PCR assay were judged as independent prognostic factors (Table 3). However, serosal involvement, vessel invasion, histological type, and MMP-7 RT-PCR assay alone were not independent prognostic factors. However, a combination assay of cytology and

Table 1: Correlation of cytology and MMP-7 RT-PCR assay in the preoperative peritoneal lavaged fluid

<table>
<thead>
<tr>
<th>Preoperative peritoneal lavaged cytology</th>
<th>MMP-7 RT-PCR assay</th>
<th>P</th>
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<tr>
<td>Negative</td>
<td>108 (86%)</td>
<td>17 (14%)</td>
</tr>
<tr>
<td>Positive</td>
<td>16 (59%)</td>
<td>11 (41%)</td>
</tr>
</tbody>
</table>

Fig. 2 Expression of MMP-7 mRNA in peritoneal dissemination from 10 patients. Total RNAs were extracted from peritoneal dissemination, using a guanidinium-isothiocyanate-phenol-chloroform-based method. The prepared RNA was mixed with oligodeoxythymidylate primer and was reverse-transcribed with AMV reverse transcriptase, followed by PCR amplification with specific primers of MMP-7. MMP-7 mRNA was detected in all of the peritoneal dissemination.

Fig. 3 Expression of MMP-7 mRNA from normal gastric mucosa, a fibroblast cell line of KMST-6, MKN-45-P, and the samples of peritoneal wash fluid.

Fig. T-10: Tissues of peritoneal dissemination from ten patients

RT-PCR for MMP-7 mRNA in peritoneal wash fluid

<table>
<thead>
<tr>
<th>case number</th>
<th>MPM-7</th>
<th>β-actin</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
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</tr>
<tr>
<td>3</td>
<td></td>
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<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
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</table>
MMP-7 RT-PCR examination revealed an independent prognostic factor. The relative risk for death in cytologically positive patients was 3.8-fold higher than it was in cytologically negative patients. Patients with positive MMP-7 RT-PCR assay and negative cytology have a 2.5-fold higher relative risk for death than those with negative examination for both cytology and MMP-7 RT-PCR assay.

**Peritoneal Wash Assay as a Risk Factor for Peritoneal Recurrence.** Table 4 shows the relation between peritoneal dissemination and peritoneal wash assay. The sensitivity rates of lymph node metastasis, serosal invasion, histological type, and vessel invasion for the prediction of peritoneal dissemination ranged from 82 to 94%, but the specificity rates of these factors were low, with a range of 44 to 74%. In contrast, the sensitivity rates of cytology and MMP-7 RT-PCR assay were 46% and 33%, respectively, but their specificity rates were 95% and 88%, respectively.

Table 5 shows the cause of death after operation in terms of the status of cytology and MMP-7 RT-PCR assay. Peritoneal dissemination in cytologically positive patients was more frequently found (23 of 27; 85%) than in patients with a negative cytology (25 of 125; 20%; \( P < 0.001 \); Table 5). Patients with...
positive MMP-7 RT-PCR assay had a significantly higher incidence of peritoneal dissemination (16 of 28; 57%) than those with a negative examination (32 of 124; 26%; \( P < 0.01 \)). In addition, peritoneal dissemination was developed significantly higher in 68% (30 of 44) of patients with positive results in MMP-7 RT-PCR assay or cytology than it was in 17% (18 of 108) of patients with negative results for both examinations (\( P < 0.01 \)).

In a multivariate model using logistic stepwise regression analysis, serosal invasion, histological type, peritoneal cytological examination, MMP-7 RT-PCR assay, and a combination assay of cytology and MMP-7 RT-PCR assay were significantly and independently related to peritoneal dissemination (Table 4). Lymph node metastasis, infiltrating pattern, vessel invasion, and lymphatic invasion lost significance for peritoneal recurrence. Peritoneal cytological examination was the most significant factor for the prediction of peritoneal dissemination with a relative risk of 5.3. Patients with a positive MMP-7 RT-PCR assay had a 2-fold relative risk for peritoneal dissemination as compared with those with a negative assay.

If the assay was judged as positive when either cytological examination or MMP-7 RT-PCR assay showed positive results, the sensitivity rate for peritoneal dissemination was 62%, with 16% and 29% improvement over that of cytology and MMP-7 RT-PCR assay alone.

Furthermore, the relative risk for peritoneal dissemination in patients with positive examination in either cytology or MMP-7 RT-PCR assay showed the highest value of 5.5 among nine factors (Table 4).

**DISCUSSION**

Recently, RT-PCR-based screening methods for the detection of micrometastasis from clinical specimens have become standard procedures. To establish assays with high accuracy, however, the marker molecules should be selectively expressed from cancer cells, not from normal tissues. For the detection of micrometastasis in lymph nodes, cytokeratin 19 and CEA have been used as target genes (14, 15). The cytokeratin 19 RT-PCR assay is a reliable method for the detection of occult bone marrow micrometastasis and is a good prognostic indicator for breast cancer (16). Furthermore, this assay was found to be more sensitive than immunocytochemistry (16). Our experiences showed, however, that cytokeratin 19 mRNA was also detected in the peritoneal lavaged fluid of patients with benign disease. Accordingly, cytokeratin RT-PCR assay cannot be used as a diagnostic tool for the detection of peritoneal micrometastasis. For this purpose, CEA is generally accepted as the only specific marker. Nakanishi et al. (6) reported a high sensitivity of the CEA RT-PCR assay for the detection of peritoneal micrometastasis and a 20% improvement in the positive rate, as compared with that for cytology alone. However, there are some cancer cells that do not produce CEA, so they cannot be detected with the CEA RT-PCR method. Moreover, some false-positive results have been reported for the CEA RT-PCR assay (6). A main source of such false-positive results is thought to be amplification of low-level transcribed CEA from the peritoneal inflammatory cells. Thus, the development of more sensitive methods has been anticipated. In the study presented here, we used a highly sensitive and specific RT-PCR method for the detection of carcinoma cells in the peritoneal cavity. Our results show that MMP-7 mRNA was not expressed by fibroblasts, peripheral blood, mesothelial cells, normal gastric mucosa, or the perito-
Molecular Diagnosis of Peritoneal Dissemination

RT-PCR assay is considered to be superior to that of CEA for early gastric cancers, so that the specificity of MMP-7 RT-PCR assay showed no positive results for T1 tumors, in which the peritoneum recurrence is almost nil (6,17). In contrast, our MMP-7 RT-PCR assay showed no positive results of peritoneal dissemination. As reported by Bando et al., MMP-7 RT-PCR assay for MMP-7 to be an independent predictor for peritoneal dissemination. Our previous studies (8) of MMP-7 in gastric cancer showed that MMP-7 is selectively expressed by cancer cells, not by normal gastric mucosa. MMP-7 mRNA was detected in only 28 (53%) of 58 primary tumors, but the peritoneal dissemination exclusively expressed the MMP-7 mRNA and protein. Furthermore, MMP-7 expression in the primary tumor correlated well with peritoneal dissemination. It is generally accepted that peritoneal dissemination originates from peritoneal free cancer cells exfoliated from serosa of primary tumors. We speculated that the reason for the high frequency of MMP-7 expression in peritoneal dissemination was that peritoneal free cancer cells expressing MMP-7 may selectively metastasize to the peritoneum, because MMP-7 is an essential molecule for the invasion into the subperitoneal tissue (8). We also hypothesized that MMP-7 must be a key enzyme for the formation of peritoneal dissemination and that MMP-7 may be a good marker for peritoneal dissemination from gastric cancer. For these reasons, we selected MMP-7 as a candidate molecule for RT-PCR assay.

Our RT-PCR-based assay for the detection of MMP-7 mRNA from peritoneal lavaged fluid was confirmed as a very sensitive method for the detection of even small numbers of peritoneal free cancer cells. Sensitivity tests proved the method to be capable of detecting cancer cells of at a density of even less than 10 cells/sample. CEA RT-PCR assay yielded positive results for 6.8–9% of T1 tumors, in which the peritoneum recurrence is almost nil (6,17). In contrast, our MMP-7 RT-PCR assay showed no positive results for early gastric cancers, so that the specificity of MMP-7 RT-PCR assay is considered to be superior to that of CEA RT-PCR assay.

Because the RT-PCR-based assay is a new method for the detection of micrometastasis, there are no reports on the correlation between the results of RT-PCR and long-term survival or recurrence. Kodera et al. (18) reported 2-year follow-up results for the CEA RT-PCR assay, demonstrating that it is a promising method for the prediction of a poor outcome and peritoneal recurrence. Our median follow-up period of 2.4 years proved that MMP-7 RT-PCR assay results constitute an independent prognostic factor and a predictor of peritoneal dissemination. Furthermore, patients with positive MMP-7 RT-PCR assay results had a poorer prognosis and a significantly higher mortality of peritoneal dissemination as compared with those with negative cytology and RT-PCR assay results. This indicates that MMP-7 RT-PCR assay results also present a reliable prognostic factor.

In addition, logistic regression analyses showed the RT-PCR assay for MMP-7 to be an independent predictor for peritoneal dissemination. As reported by Bando et al. (3), the presence of i.p. free cancer cells proved by peritoneal lavaged cytology indicates the existence of i.p. micrometastasis, and all of the patients with positive cytology died of peritoneal recurrence even after curative resection. This means that positive results of the MMP-7 RT-PCR assay strongly suggest the existence of i.p. free cancer cells and micrometastasis.

By combining cytology and MMP-7 RT-PCR, the sensitivity rate for the prediction of peritoneal dissemination was improved to 11% (17 of 152) over the prediction by routine cytology. Thus, the combination of conventional cytology and RT-PCR-based assays for the analysis of preoperative peritoneal lavaged fluid appears to result in improved accuracy for the prediction of peritoneal micrometastasis, so that patients at high risk of recurrence in the peritoneum can be identified preoperatively. Therefore, the preoperative assay of peritoneal lavaged can be considered a reliable method for the selection of patients for whom adjuvant i.p. chemotherapy can be expected to be effective (19). We are now using a combination of MMP-7

### Table 4

<table>
<thead>
<tr>
<th>Peritoneal recurrence</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>94%</td>
<td>52%</td>
</tr>
<tr>
<td>Serosal invasion</td>
<td>92%</td>
<td>60%</td>
</tr>
<tr>
<td>Infiltrating pattern</td>
<td>78%</td>
<td>74%</td>
</tr>
<tr>
<td>Lymphatic invasion</td>
<td>84%</td>
<td>44%</td>
</tr>
<tr>
<td>Vessel invasion</td>
<td>78%</td>
<td>58%</td>
</tr>
<tr>
<td>Histological type</td>
<td>82%</td>
<td>63%</td>
</tr>
<tr>
<td>Cytology</td>
<td>46%</td>
<td>95%</td>
</tr>
<tr>
<td>MMP-7 RT-PCR</td>
<td>33%</td>
<td>88%</td>
</tr>
<tr>
<td>Cytology and MMP-7</td>
<td>62%</td>
<td>88%</td>
</tr>
</tbody>
</table>

* Cytoplogy (+) or MMP-7 RT-PCR (+) vs. cytology (−) and MMP-7 RT-PCR (−).

### Table 5

<table>
<thead>
<tr>
<th>n</th>
<th>Peritoneal dissemination</th>
<th>Lymph node</th>
<th>Liver</th>
<th>Others</th>
<th>No recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy(−) and MMP7(−)</td>
<td>108</td>
<td>18 (16.6%)</td>
<td>7 (6.6%)</td>
<td>9 (8.6%)</td>
<td>1 (0.9%)</td>
</tr>
<tr>
<td>Cy(−) and MMP7(+)</td>
<td>17</td>
<td>7 (41.2%)</td>
<td>1 (5.9%)</td>
<td>5 (29.4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Cy(+) and MMP7(−)</td>
<td>16</td>
<td>14 (88.5%)</td>
<td>1 (6.3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Cy(+) and MMP7(+)</td>
<td>11</td>
<td>9 (81.8%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>2 (18.2%)</td>
</tr>
</tbody>
</table>
RT-PCR, CEA RT-PCR, and conventional cytology in a search for even greater improvement in the accuracy of peritoneal dissemination prediction.

REFERENCES
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