Original article

Detection of cancer cells and gene expression of cytokines in the peritoneal cavity in patients with gastric cancer

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Abstract

Background. The gene expression of the cytokines interleukin-2 (*IL-2*) and *IL-10* in peritoneal washings was examined in relation to the presence of cancer cells in the peritoneal cavity in patients with gastric cancer.

Methods. Total RNA was extracted from 50-ml peritoneal wash samples from 124 patients (gastric cancer, $n = 110$; controls, $n = 14$). Carcinoembryonic antigen (CEA) messenger RNA (mRNA) was used to identify the number of cancer cells in peritoneal wash samples by a real-time reverse transcription-polymerase chain reaction (RT-PCR) method, which method was also used to assay the *IL-2* and *IL-10* gene expression levels.

Results. In the 14 control samples, CEA mRNA was not detected, while CEA mRNA was detected in 2 of the 51 stage I gastric cancer patients. Thus, the specificity of this method for the detection of cancer cells in peritoneal wash samples was 97% (63/65). The CEA-based real-time RT-PCR method demonstrated greater prognostic impact than the traditional cytological method. *IL-2* gene expression in peritoneal wash samples that were CEA mRNA-positive was suppressed compared with that in peritoneal wash samples that were CEA mRNA-negative, while *IL-10* gene expression did not differ according to the CEA mRNA findings.

Conclusion. The detection of small numbers of cancer cells in peritoneal wash samples from patients with advanced gastric cancer is a good marker for peritoneal metastatic recurrence. In the peritoneal cavity, cancer cells may escape from immune surveillance by controlling the expression of cytokines.

Key words Cytokine · Gastric cancer · Intraperitoneal free cancer cells · Real-time reverse transcriptase polymerase chain reaction

Introduction

In advanced gastric cancer, peritoneal metastasis or intraperitoneal free cancer cells are important factors for a poor prognosis [1–3]. Peritoneal washing cytology has been introduced for the detection of cancer cells in the peritoneal cavity in patients with gastric cancer [4]. However, with this method, it is difficult to identify small numbers of cancer cells in peritoneal washings. Recent advances in the real-time reverse transcription-polymerase chain reaction (RT-PCR) method have enabled us to identify small numbers of cancer cells in the peritoneal cavity [5]. In the present study, we investigated the clinical importance of the detection of small numbers of cancer cells in peritoneal wash samples from gastric cancer patients.

The peritoneal cavity is thought to be a compartment in which immunologic host-tumor interaction can occur. Recent studies have demonstrated that the cytokine network plays important roles in the immunosuppressive and immunostimulatory properties of cancer-related ascites fluid [6,7]. To clarify the relationships between the gene expression levels of cytokines and the detection of cancer cells in the peritoneal cavity, we examined the gene expression levels of interleukin-2 (*IL-2*) and *IL-10* in peritoneal wash samples obtained from gastric cancer patients.

Methods

Sample collection

A total of 110 gastric cancer patients (72 men and 38 women; mean age, 66 years; range, 26–88 years) and 14 patients with cholecystolithiasis (controls, 6 men and 8 women; mean age, 50 years; range, 14–83 years) operated between January 2002 and March 2005 were enrolled. In the 110 patients with primary gastric cancer

(stage I, $n = 51$; II, $n = 14$; III, $n = 20$; and IV, $n = 25$), gastrectomy was performed in 96 patients; gastrectomy was not performed in the remaining 14 patients because of peritoneal dissemination (12 cases) or extended lymph node metastasis (2 cases). Aliquots of 150 ml of saline were introduced into the Douglas cavity at the beginning of the operation and aspirated soon after gentle stirring. Then 50-ml the peritoneal washings was submitted to cytological diagnosis and the remaining 50 ml was prepared for RNA extraction. The present study conformed to the ethical standards of the World Medical Association Declaration of Helsinki. Tumor stage and pathological classification were described according to the *Japanese Classification of Gastric Carcinoma* [8].

Cytological diagnosis of intraperitoneal free cancer cells

The collected saline peritoneal wash sample was centrifuged for 5 min at 2000g. The cell pellet was smeared onto four slides, two for Papanicolaou staining and two for Giemsa staining. These specimens were examined at the pathology laboratory at our hospital by physicians licensed by the Japanese Society of Clinical Cytology.

Human gastric cancer cell lines

Human gastric cancer cell lines (MKN-28, MKN-45, MKN-74, and KATO-III) were maintained in RPMI-1640 medium (Cosmo Bio, Tokyo, Japan) supplemented with 10% fetal calf serum (Cansera International, Ontario, Canada) in a humidified atmosphere containing 5% CO₂ at 37°C.

Real-time RT-PCR assay

The peritoneal washings were centrifuged for 10 min at 400g. Total RNA from the pellets of the peritoneal washings was isolated using RNeasy Mini Kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized with 1 µg of total RNA with Ready-to-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and the final volume of cDNA solution was adjusted to 33 µl with diethylpyrocarbonate (DEPC)-treated water. Primers and TaqMan (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) probes for CEA were as follows: forward primer, 5'-GGCCACTGTCGGCATCAT-3'; reverse primer, 5'-GGAAGAAGCAAAACAAGTGTGAGTTCATTTCAGGA-3'. Primers and TaqMan probes for TATA box-binding protein (TBP; as the endogenous RNA control), IL-2, and IL-10 were obtained from Perkin-

Elmer Applied Biosystems. The PCR solution (50 µl) was composed of 1 µl of cDNA solution, 5 pmol of the forward and reverse primers, 10 pmol of internal probe, and TaqMan Universal PCR Master Mix (Perkin-Elmer Applied Biosystems). PCR was carried out after incubation at 50°C for 2 min and denaturing at 95°C for 10 min, 45 cycles at 95°C for 15 s, and 61°C for 1 min. Quantification of gene expression was performed by a real-time quantitative RT-PCR (Gene Amp 5700 Sequence Detection System [Perkin-Elmer Applied Biosystems]) [9].

Relative numbers of cancer cells in the peritoneal wash samples

Different concentrations of KATO-III cells (10 to 10^6) were added to 1.5-ml peripheral blood (PB) samples from one healthy volunteer (6×10^6 peripheral blood granulocytes). CEA mRNA expression levels from different concentrations of KATO-III were identified by the real-time RT-PCR. The experiments were repeated three times. When the plots represented the log of KATO-III cells in 6×10^6 granulocytes on the x axis and cycle numbers on the y axis, an equation was derived from the line of the CEA calibration curve [10]. The formula for CEA was $y = 40 - 3x$ ($r^2 = 0.994$). For each of the experimental samples, the amounts of CEA mRNA were determined from this standard curve. TBP transcripts were monitored as a control to quantify the transcripts.

Quantification of cytokine gene expression

The method for quantification of cytokine gene expression was described previously [11]. Briefly, for each reaction tube, the fluorescence signal of the reporter dye was divided by the fluorescence signal of the passive reference dye to obtain the normalized reporter signal (R_n). The threshold line was set at $R_n = 0.1$. The point at which the amplification plot crossed this threshold was defined as C_t , which represented the cycle number at this point. Each sample was normalized by the gene expression of *TBP*. *TBP* was chosen because there are no known retropseudogenes [12], and the gene expression level of *TBP* is lower than those of *IL-2* and *IL-10*. The cDNAs were prepared from 1.5-ml PB samples from eight healthy volunteers [9]. The ΔC_t values for *IL-2* and *IL-10* in the PB samples were determined by subtracting the *IL-2* and *IL-10* C_t values from the *TBP* C_t value. These mean ΔC_t values for *IL-2* and *IL-10* in the PB samples were used as calibrators (2.0 and 1.0, respectively). In each sample, the expression levels of the target genes were obtained by the following formulas, $\Delta C_t \text{ IL-2 (sample)}/\Delta C_t \text{ IL-2 (calibrator)}$ and $\Delta C_t \text{ IL-10 (sample)}/\Delta C_t \text{ IL-10 (calibrator)}$. Since $\Delta C_t \text{ IL-2}$

(calibrator) was 2.0 and $\Delta\text{Ct } IL-10$ (calibrator) was 1.0, the relative expression levels of the *IL-2* gene and *IL-10* gene in this sample were $\Delta\text{Ct } IL-2$ (sample)/2.0 and $\Delta\text{Ct } IL-10$ (sample)/1.0.

Statistical analysis

The χ^2 test and Fisher's exact probability test were used to compare the distribution of individual variables among the patient groups. Differences in numerical data between two groups were evaluated using the Mann-Whitney *U*-test, and the Kruskal-Wallis test was used for evaluating differences among three or more groups. Survival rates were calculated using the Kaplan-Meier method. The log rank test was used for comparisons of two survival curves. A *P* value of less than 0.05 was considered statistically significant.

Results

Sensitivity and reliability of CEA mRNA probes

CEA mRNA expression was detected in all four cell lines (MKN-28, MKN-45, MKN-74, and KATO-III). The CEA mRNA probe can detect 10 cancer cells in 6×10^6 granulocytes. We found no CEA mRNA expression in the peritoneal wash samples taken from the 14 noncancerous patients. However, we found CEA mRNA expression in the peritoneal wash samples from 2 of the 51 stage I gastric cancer patients. One was a 60-year-old man and the disease was classified as stage IA. He had 42 CEA-positive cells/ 6×10^6 granulocytes in the peritoneal wash sample and was followed for 33 months without recurrence. The other patient was a 76-years-

old man whose disease was classified as stage IB. He had 19 CEA-positive cells/ 6×10^6 granulocytes in the peritoneal wash sample and was followed for 34 months without recurrence. So, we decided that the results for these two stage I patients were false-positives. From our data, the specificity of CEA for the detection of small numbers of cancer cells in peritoneal wash samples was 97% (63/65) and CEA mRNA was thought to be a sensitive and reliable probe to identify small numbers of cancer cells.

Peritoneal metastasis and cytological findings in peritoneal washings

Macroscopic peritoneal metastasis (P) was detected in 16 patients and intraperitoneal free cancer cells recognized by cytology (CY) were detected in 16 patients. We divided the 110 gastric cancer patients into four subgroups according to P and CY status. Eighty-eight patients were CY(-)/P(-), 6 were CY(-)/P(+), 6 were CY(+)/P(-), and 10 were CY(+)/P(+).

CEA mRNA expression in peritoneal washing from gastric cancer patients

CEA mRNA was detected in the peritoneal wash samples from 33 gastric cancer patients (range, $4 - 1.5 \times 10^6/6 \times 10^6$ granulocytes). When we used the cutoff value (10 cancer cells/ 6×10^6 granulocytes) used in a previous report [5], CEA mRNA was detected in the peritoneal wash samples of patients 27 (24.5%). Figure 1 shows the relative numbers of cancer cells in the peritoneal wash samples of the 110 gastric cancer patients. Positivity for CEA mRNA in the peritoneal wash samples was as follows: 7/88 (8%) with CY(-)/P(-), 4/6 (67%) with

Fig. 1. Relative numbers of cancer cells in the peritoneal wash samples of 110 gastric cancer patients CY, peritoneal free cancer cells detected by cytology; P, macroscopic peritoneal metastasis

Table 1. Detection of CEA mRNA expression in peritoneal wash samples from patients with gastric cancer

	<i>n</i>	CY (+)	CEA mRNA (+)
Stage			
I	51	0	2 (4%)
II	14	0	0
III	20	0	4 (20%)
IV	25	16	21 (84%)

CY, peritoneal free cancer cells recognized by cytology

CY(-)/P(+), 6/6 (100%) with CY(+)/P(-), and 10/10 (100%) with CY(+)/P(+). CEA mRNA was detected in all 16 patients with CY(+). However, in 2 of the 6 patients with CY(-)/P(+), we failed to find cancer cells in the peritoneal cavity by real-time RT-PCR. The correlation between the detection of CEA mRNA and tumor stage is indicated in Table 1.

Correlation between detection of cancer cells in peritoneal washings and cytokine gene expression

IL-2 gene expression was detected in 11/14 (79%) of the control patients and in 85/110 (77%) of the gastric cancer patients, and *IL-10* gene expression was detected in all of the control and in 108/110 (98%) of the gastric cancer patients. In the 110 gastric cancer patients, the mean *IL-2* gene expression level in the 27 patients who were CEA-positive (1.1; range, 0–3.6) was lower than that in the 83 CEA-negative patients (1.8; range, 0–5.8; $P = 0.044$). On the other hand, the mean *IL-10* gene expression level in the 27 CEA-positive patients (8.2; range, 3–15) was not different from that in the 83 CEA-negative patients (8.7; range, 0–17.8; $P = 0.466$).

Correlation between detection of cancer cells in peritoneal washings and patients' prognoses

The overall 3-year survival rate of the 88 patients with CY(-)/P(-) was 78%; all 6 of the patients with CY(-)/P(+) died within 19 months after surgery (mean, 11.8 months); all 6 of the patients with CY(+)/P(-) died within 9 months (mean, 6.8 months); and all 10 of the patients with CY(+)/P(+) died within 14 months (mean, 6.7 months). No difference was observed in mean survival periods after surgery among the three groups, of CY(-)/P(+), CY(+)/P(-), and CY(+)/P(+). CEA mRNA was detected in 4 of the 20 patients with stage III disease, and all of these 20 patients underwent curative gastrectomy. The disease-free survival curves of the 16 CEA mRNA-negative patients and the 4 CEA-positive patients are shown in Fig. 2. All 4 patients who were CEA mRNA-positive developed peritoneal metastasis and 3 of the 4 died within 18 months after opera-

Fig. 2. Disease-free survival curves of 16 stage III gastric cancer patients who were carcinoembryonic antigen (CEA) mRNA-negative and 4 stage III patients who were CEA-mRNA positive are shown. The difference was significant ($P = 0.002$).

tion. The remaining patient was alive with peritoneal metastasis in June 2005 (22 months after surgery). Five of the remaining 16 stage III patients developed recurrence foci, in the liver ($n = 2$), lymph node ($n = 2$), and peritoneum ($n = 1$).

Discussion

Recently, it has been shown that small numbers of cancer cells in peritoneal washings can be detected by using real-time RT-PCR [13]. For detecting intraperitoneal free cancer cells, a CEA mRNA probe is widely used, and real-time RT-PCR using a CEA mRNA probe is a more reliable method for identifying small numbers of cancer cells than cytology [4], but we need to pay careful attention when analyzing the results. The reliability of this marker for the detection of small numbers of cancer cells has been discussed. Kodera et al. [13] reported that the specificity of CEA in the detection of intraperitoneal free gastric cancer cells was 94%. In the present study, we found that 2 of 65 cases were false-positives (0/14 in the controls and 2/51 in stage I gastric cancer patients). Thus from our data, the specificity of CEA in the detection of small numbers of cancer cells from peritoneal wash samples was 97% (63/65). Of our 20 stage III patients, 4 were CEA mRNA-positive. All 4 of these patients developed peritoneal metastasis within 18 months after operation. This result suggests that the use of real-time RT-PCR analysis of peritoneal wash samples is applicable for patients with advanced gastric cancer, because, CEA mRNA-based real-time RT-PCR is a strong prognostic marker for these patients.

Six of our 16 patients with histologically confirmed peritoneal metastasis showed no free cancer cells by cytology, and we failed to find cancer cells in the peritoneal wash samples in 2 of these 6 patients even by the real-time RT-PCR method. These 2 patients underwent gastrectomy but died within 8 months due to peritoneal metastasis after operation. Peritoneal metastasis is widely recognized as a result of cancer-cell seeding from the primary tumor. However, another possibility is that, in some patients with peritoneal metastasis, lymphatic metastases to the peritoneal cavity may occur from metastatic lymph nodes or from the primary tumors. In such cases, peritoneal metastatic tumors may not face the peritoneal cavity directly and there may be no free cancer cells in the peritoneal cavity. Real-time RT-PCR is a useful method to detect small numbers of free cancer cells in the peritoneal cavity; however, not all patients with peritoneal metastasis may have free cancer cells in the peritoneal cavity.

Tumor-bearing hosts develop antitumor immune responses. In advanced gastric cancer, free cancer cells are frequently detected in the peritoneal cavity. Thus, the peritoneal cavity is a compartment in which immunologic host-tumor interaction can occur. IL-2 and IL-12 are produced by T cells and can affect many different cell types, such as lymphocytes, neutrophils, and macrophages. In particular, IL-2 activates natural killer (NK) cells, and CD4+ and CD8+ T cells (cytotoxic T lymphocytes), resulting in tumor destruction. Thus, IL-2 and IL-12 have been used for intraperitoneal immunotherapy in patients with various cancers, including gastric cancer [14–16]. In the present study, we found that the gene expression of *IL-2* was suppressed in the peritoneal cavity of patients who had free cancer cells in the peritoneal cavity. Peritoneal washings may contain not only immunocompetent cells but also tumor cells and normal mesothelial cells. So, this result suggests that cancer cells in the peritoneal cavity may suppress *IL-2* gene expression in immunocompetent cells or normal mesothelial cells, and may change the environment of the peritoneal cavity so that it is suitable for tumor growth. Another possibility, that the low expression level of *IL-2* in CEA-positive samples may correlate with a high cancer/T-cell ratio in the samples, can also be considered. On the other hand, Majima et al. [7] reported that the IL-10 protein level in peritoneal washings from patients with gastric cancer increased according to tumor progression. IL-10, a type-2 cytokine, inhibits type-1 immune responses, and recent studies have reported that patients with carcinoma of the digestive tract more frequently showed type-2 cytokines in the peripheral blood (PB) in comparison with normal subjects [17]. However, our results demonstrated that the gene expression level of *IL-10* in peritoneal washings from patients who were CEA

mRNA-positive did not differ from that in the peritoneal washings from patients who were CEA mRNA-negative. So, the role of a type-2 cytokine such as IL-10 in tumor progression in the peritoneal cavity in patients with advanced gastric cancer remains unclear.

Recently, a contrary finding on IL-2 function was reported, in which endogenous IL-2 promoted tumor cells growth and protected these cells from apoptosis [18]. Thus, the role of cytokines produced by cells in the peritoneal cavity in tumor growth in gastric cancer patients is still unclear. In future, the relationships between the gene and protein expressions of cytokines and tumor growth should be clarified.

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