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Detection of Carcinoembryonic Antigen-expressing Free Tumor Cells in Peritoneal Washes from Patients with Gastric Carcinoma by Polymerase Chain Reaction

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Cytological examination of peritoneal washes is a useful predictor of peritoneal recurrence in gastric carcinoma patients. In the present study, even more sensitive detection of free cancer cells could be achieved through amplification of carcinoembryonic antigen (CEA) mRNA by means of the reverse transcriptase-polymerase chain reaction (RT-PCR). CEA was first confirmed to be present in all the gastric cancer cell lines examined, irrespective of the differentiation degree, and absent in blood and mesothelium, indicating the specificity of this approach for detection of carcinoma cells in peritoneal lavage fluid. In sensitivity tests, CEA RT-PCR proved to be capable of detecting 10 carcinoma cells per sample. Peritoneal washes of 15 of 48 gastric carcinoma patients, including all 10 patients with positive cytology results, proved positive for CEA mRNA. None of the 5 patients with benign disease was positive. Moreover, a close association with the depth of cancer invasion was established. The results indicate that the assay is more sensitive for detection of free carcinoma cells in the peritoneal cavity than conventional cytology. This is the first study to suggest the feasibility of the RT-PCR method for prediction of peritoneal recurrence in gastric cancer patients.

Key words: RT-PCR — Gastric carcinoma — Peritoneal dissemination — Peritoneal wash cytology — Carcinoembryonic antigen

The prognosis of gastric carcinoma has improved in Japan due to the early diagnosis attributable to the nationwide practice of mass screening.[1] However, gastric carcinomas invading as far as the gastric serosa still carry a poor prognosis, with a 5-year survival of less than 35%.[2] Peritoneal dissemination is reported to be the most frequent type of recurrence after curative resection in such cases.[3] Free cancer cells derived from serosal invasion might be an indicator of early peritoneal seeding with subsequent formation of metastatic colonies. Their detection, therefore, is likely to be a useful tool for prediction of outcome in patients with advanced gastric carcinomas. Indeed, cytologic examination of abdominal washes obtained at laparotomy has been a gold standard for this purpose, with a clear association between a positive result and a low survival rate.[4] Conventional cytology, however, lacks sensitivity and some patients with negative cytology results have nevertheless been found to have recurrence in the form of peritoneal dissemination.[5]

The extreme sensitivity of reverse transcriptase-polymerase chain reaction (RT-PCR) techniques has been found to allow diagnosis of micrometastases based on tissue-specific mRNA expression of tumor cells in peripheral blood, bone marrow, lymph nodes and cerebrospinal fluid.[6–10] In the present study, we therefore applied the RT-PCR technique to detect free gastric carcinoma cells in peritoneal washes in the hope that minute quantities of free cancer cells that might be missed by conventional cytology, would thereby be identified, allowing a more accurate prediction of outcome for patients with gastric carcinoma.

MATERIALS AND METHODS

Cell lines and tissues Six human gastric cancer cell lines and 1 colon carcinoma cell line were used in this study. HSC-39 and HSC-43, poorly differentiated gastric carcinoma cell lines derived from ascites in patients with peritonitis carcinomatosis,[11] were kindly provided by Dr. K. Yanagihara (Hiroshima University, Hiroshima). The poorly differentiated and moderately differentiated carcinoma cell lines, MKN45, MKN28 and MKN74 were obtained from the Japanese Cancer Research Resources Bank (Tokyo). GLM-3, a well differentiated carcinoma cell line, and COLM-1, a moderately differentiated, carcinoembryonic antigen (CEA)-producing colon carcinoma cell line were established in our own laboratory. They were all cultured in RPMI 1640 or DMEM media (Nissui Pharmaceutical, Tokyo) supplemented with 10% fetal calf serum (GIBCO BRL, Gaithersburg, MD), 100 unit/ml penicillin and 100 μg/ml streptomycin. Tissue

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samples from colon, stomach, lymph nodes and spleen were obtained at autopsy from non-cancerous patients and peripheral venous blood samples were taken with informed consent from healthy volunteers. Primary human cultured mesothelial cells were also obtained from peritoneal washes of a patient with a gastric cancer localized in the mucosa of the stomach using the method described previously.[12, 13]

Peritoneal washes The study population consisted of 48 patients with gastric carcinomas and 5 with benign disease including cholelithiasis and leiomyoma of the uterus, undergoing surgery at Aichi Cancer Center Hospital (Nagoya). Ascites fluid was collected from the Douglas cavity at laparotomy. In the absence of ascites, 50 ml of saline was introduced into the Douglas cavity at the beginning of each operation and aspirated after gentle stirring. These washes were centrifuged at 2000 rpm for 10 min to collect intact cells, rinsed with phosphate-buffered saline (PBS), dissolved in ISOGEN RNA extraction buffer (Nippon Gene, Tokyo) and stored at −80°C until use. A part of each peritoneal wash was examined cytopathologically after conventional Papanicolaou staining. The depth of cancer invasion was evaluated by histological procedures according to the Japanese Classification of Gastric Cancer.[2, 14] Serum CEA levels were measured with a radiometric immunoassay kit (Dainabot, Tokyo).

RT-PCR Frozen samples in ISOGEN were thawed and total RNA was extracted using a guanidinium-isothiocyanate-phenol-chloroform based method as reported previously.[15] Since the number of cells in the wash fluids is usually small, we added glycogen for molecular biology (Boehringer Mannheim, Germany) as a carrier to improve recovery of RNA before ethanol precipitation. First-strand cDNA was synthesized using SuperScript II reverse transcriptase (GIBCO BRL) and random hexanucleotide primers (Pharmacia Biotech, Sweden). Five microgram aliquots of total RNA were preincubated with 50 ng of random hexamer in 9 μl of solution for 10 min at 70°C. After chilling on ice, 4 μl of 5-fold synthesis buffer [250 m*M* Tris-HCl, pH 8.3, 375 m*M* KCl, 15 m*M* $MgCl_2$], 2 μl of 100 m*M* dithiothreitol, 4 μl of 2.5 m*M* (each) dNTP and 1 μl of SuperScript II reverse transcriptase (200 units/μl) were added. The reaction mixture was then incubated for 1 h at 37°C and the reaction was terminated by heating at 90°C for 10 min and stored at −80°C until analysis. The integrity of isolated RNA was proven by RT-PCR analysis of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described previously.[16] Only samples with positive bands for GAPDH underwent the steps described below.

CEA-specific oligonucleotide primers synthesized for the nested RT-PCR analysis were those reported by Gerhard *et al.*[17] The sequences of the three primers used were: A (outer sense), 5′-TCTGGAACTTCTCCTGGTCTCTCAGCTGG-3′, B (antisense), 5′-TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC-3′, C (inner sense), 5′-GGGCCACTGTCGGCATCATGATTGG-3′. The first-round PCR was carried out in a reaction mixture (10 μl) containing 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 200 μ*M* dNTP, 1.5 m*M* $MgCl_2$, 0.25 unit of Taq DNA polymerase (Perkin Elmer Cetus, Branchburg, NJ), 0.2 μ*M* primers A and B, and 1 μl of template cDNA. Twenty rounds of amplification were performed in a thermocycler (MJ Research Inc., Watertown, MA) at 94°C (1 min) and 72°C (2 min and 30 s), with a final extension step at 72°C for 10 min. One microliter aliquots of reaction product were then transferred into second tubes containing the identical reaction mixture except for the primers, C and B. Twenty more cycles were run at 94°C (1 min) and 72°C (2 min and 30 s) with a final extension step for 10 min. Five microliter quantities of the second-round PCR products were then run on 2% agarose gels, followed by ethidium bromide staining. Samples with visible 131-base-pair bands were designated as positive. All necessary precautions against contamination of PCRs were rigorously applied.[18] The nucleotide sequences of the PCR products were confirmed by the dideoxy chain termination sequencing method.[19]

RESULTS

CEA RT-PCR successfully amplified a specific PCR fragment with the expected size (131 bp). This band was confirmed to be identical with the CEA cDNA sequence by direct sequencing (data not shown). Fig. 1A shows the results of a cell spiking study performed using serial dilutions of the colorectal carcinoma cell line COLM-1 in PBS buffer. The CEA RT-PCR assay was capable of detecting 10 tumor cells per wash sample in the presence of glycogen as a carrier. In the absence of the carrier, the sensitivity was decreased 10–100 fold. The CEA-specific band was detected in all the gastric carcinoma cell lines tested, even in two poorly differentiated carcinoma lines from ascites, with somewhat lower detection levels than the CEA-producing colon carcinoma cell line (data not shown). In normal tissues, specific transcripts were detected in the stomach and colon, but not in the lymph nodes, spleen, peripheral blood and primary cultured human mesothelial cells, the latter two being major constituents of peritoneal washes (Fig. 1B).

This method gave 15 positives out of 48 patients with gastric cancer (31%), while none of the 5 patients with benign disease was positive (Fig. 2). The association between PCR results, depth of invasion and results of conventional cytology is summarized in Table I. The positive rate with the RT-PCR assay correlated significantly with the depth of cancer invasion in terms of

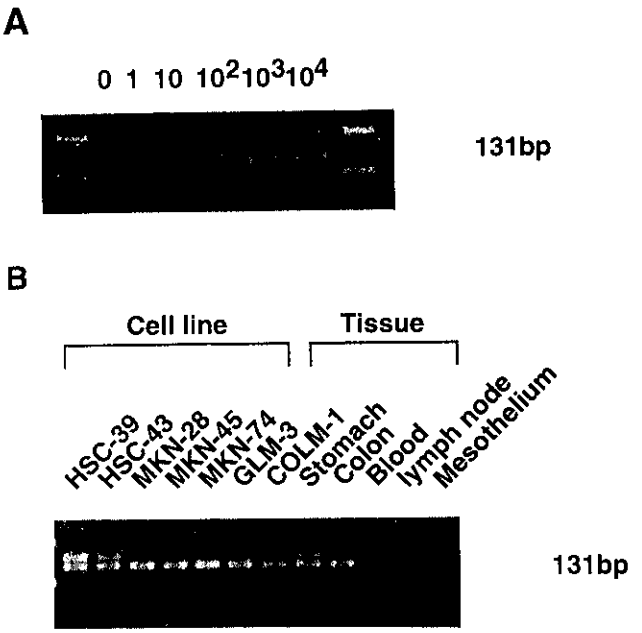

Fig. 1. Sensitivity (A) and specificity (B) of the CEA RT-PCR assay. A, Total RNA was extracted from serially diluted COLM-1 colon carcinoma cells in PBS with the aid of glycogen as a carrier and analyzed by nested-primer RT-PCR. B, CEA mRNA expression in various gastric and colonic cancer cell lines, and normal tissues was analyzed by RT-PCR. The results of agarose gel electrophoresis, stained with ethidium bromide, are shown.

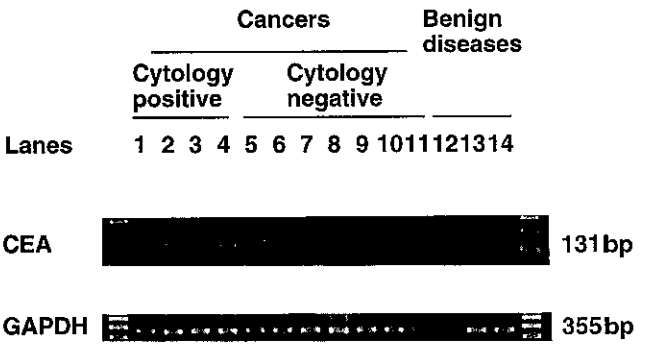

Fig. 2. Detection of carcinoma cells in peritoneal washes from gastric cancer patients and benign controls by CEA RT-PCR. Representative RT-PCR results of 4 washes from cytology-positive patients (lanes 1–4), 7 from cytology-negative patients (lanes 5–11) and 3 from patients with benign disease (lanes 12–14) are shown. Lanes 5, 6, 7, 8, 9 correspond to the patient numbers 11, 12, 13, 14, 15 in Table II.

Table I. Summary of CEA RT-PCR Results for Lavaged Peritoneal Fluid in Patients with Gastric Cancer

Depth of invasion[a)]	No. of patients	No. of positive cases	
		CEA RT-PCR	Cytology
Benign disease	5	0 (0%)	0 (0%)
Gastric carcinoma			
t1	22	2 (9%)	0 (0%)
t2	13	3 (23%)	1 (8%)
t3	11	8 (73%)	7 (64%)
t4	2	2 (100%)	2 (100%)
Total	48	15 (31%)	10 (21%)

a) t classification: t1, mucosa to submucosa; t2, muscularis propria to subserosa; t3, serosa-exposed; t4, serosa-infiltrating.

t-number, ranging from 2 of 22 (9%) t1 (mucosal to submucosal invasion), to 3 of 13 (23%) t2 (muscularis propria to subserosal invasion), 8 of 11 (73%) t3 (serosal invasion) and 2 of 2 (100%) t4 lesions (invasion to adjacent tissue) ($P<0.0001$, χ^2 test). Patients with a positive cytology included 9 with serosal (t3–t4) and 1 with subserosal invasion (t2), all of these being also positive in the PCR analysis. In addition, 5 gastric cancer patients with negative cytology exhibited amplification of the CEA sequence, but the expression levels as evaluated from the density of the specific band on agarose gel seemed to be lower than those of positive cytology cases (Fig. 2). These latter included 2 patients with class III cytology and 3 with class I or II. The class III patients had positive serum CEA and nodal metastasis, but the others exhibited no such clinicopathological features (Table II).

Of the 2 patients with class III cytology and positive PCR results, one was subsequently found to have re-elevated serum levels of the tumor marker CA19-9, and is currently under evaluation as to the type of recurrence. The other patient died from recurrent disease with lymph-node and bone metastasis. The presence or absence of peritoneal metastasis could not be determined because of the lack of an autopsy examination. The 3 patients with class I-II cytology and positive PCR results have been followed postoperatively without signs of recurrence for 372 to 665 days. The 33 patients with negative PCR results are disease-free to date (294 to 659 days) with one exception, who died from bone marrow metastasis complicated by disseminated intravascular coagulation.

DISCUSSION

The prognosis of gastric cancer patients with peritoneal dissemination is invariably poor, and even intraperitoneal administration of anticancer drugs has so far proved unsuccessful for treatment.[20)] However, there is a report claiming that the survival of individuals with

Table II. Clinical Features of Gastric Cancer Patients with Positive CEA RT-PCR Findings

Patients No.	Age/ Sex	Histology[a]	Depth of invasion[b]	Metastasis		CEA in serum (μg/ml)	Cytology
				Lymphnode	Peritoneum		
1	62/F	por	si	NT	+	− (4.5)	class V
2	51/M	por	si	NT	+	− (2.3)	class V
3	45/F	por	se	+	+	++ (70.9)	class V
4	69/M	mod	se	+	+	+ (5.8)	class V
5	62/M	por	se	+	+	− (1.9)	class V
6	47/F	por	se	+	+	− (0.3)	class V
7	76/M	mod	se	+	+	− (3.3)	class V
8	64/M	por	se	+	−	+ (7.9)	class V
9	39/F	por	se	+	−	− (0.9)	class V
10	70/F	sig	ss	+	−	− (1.0)	class V
11	55/M	muc	se	+	−	++ (133.0)	class III
12	79/F	mod	ss	+	−	+ (5.2)	class III
13	68/F	por	mp	−	−	− (0.9)	class I
14	43/M	sig	sm	−	−	− (0.4)	class II
15	45/M	mod	m	−	−	− (2.2)	class I

a) mod, moderately-differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma; sig, signet-ring cell carcinoma; muc, mucinous adenocarcinoma.
b) m, mucosa; sm, submucosa; mp, muscularis propria; ss, subserosa; se, serosa-exposed; si, serosa-infiltrating.

serosal invasion, but without macroscopic evidence of peritoneal dissemination can be significantly improved by intraoperative chemohyperthermia, a prophylactic treatment for peritoneal recurrence.[21] This implies that the detection of possible minimal residual disease in the abdominal cavity, which is occasionally unsuccessful by conventional cytology, could in future have potential predictive value, in addition to the pre-existing prognostic factors.[22] It has been shown that quantification of CEA protein levels in peritoneal washes can be a sensitive and useful predictor for peritoneal recurrence in gastric cancer patients.[23–25]

Recently, highly sensitive methods using RT-PCR for tissue-specific mRNA have been used to detect small numbers of cancer cells in various clinical samples. For gastric cancers, cytokeratin 19 and CEA have been used as target genes for micrometastases in lymph nodes.[26, 27] In the present study, we applied a highly sensitive and specific RT-PCR method to detect carcinoma cells in peritoneal washes of gastric cancer patients for the first time. Since cytokeratin 19, but not CEA transcripts were found to be expressed by mesothelial cells (data not shown), the latter was selected as the target gene for RT-PCR. Leucocytes, another major constituent of peritoneal washes, express a number of CEA-related genes,[28] therefore, we carried out PCR using stringent conditions with an annealing temperature of 72°C to avoid false-positive results due to the presence of inflammatory cells. All of the peripheral blood samples obtained from 20 healthy volunteers as well as peritoneal washes of 5 patients with benign disease were thereby found to be negative for CEA transcripts, confirming the specificity of the method. CEA RT-PCR was found to be capable of detecting the poorly differentiated gastric carcinoma cells that are common in patients with peritoneal dissemination, although the detection level was decreased 5–10 fold as compared with differentiated colon carcinoma cells that show high CEA production. The sensitivity of 10–100 carcinoma cells per sample is comparable to that previously reported for CEA RT-PCR detection of micrometastases in bone marrow and lymph nodes.[17, 26]

The RT-PCR assay demonstrated CEA mRNA in the wash fluid from all 10 patients with positive cytology. In addition, 5 patients with negative cytology were found to be positive by CEA RT-PCR. Two of these 5 patients demonstrated subserosal and serosal invasion (t2–t3 group) and class III cytology. These results indicate that CEA RT-PCR is more sensitive than conventional cytology and sometimes provides the correct diagnosis when conventional cytology yields only suspicious results. Recurrence data for patients positive for PCR and negative for cytology are still preliminary, but one of the class III patients was found on the 302nd postoperative day to have a re-elevated serum CA19-9 level without evident hematogenous metastasis. Our PCR analysis might thus be concluded to have been more informative as far as this particular patient was concerned. Moreover, it was encouraging to note that there is no evidence of peritoneal recurrence to date for any of the 33 patients with negative PCR results.

Of the 35 patients with invasion confined to the stomach wall (t1–t2 group), 5 (16%) were positive for CEA mRNA by RT-PCR. Since this approach is considered to detect intact exfoliated tumor cells in the peritoneal cavity rather than exudates from primary tumors, the positive results for 2 early gastric cancer patients suggest the presence of a route of penetration of the stomach wall by tumor cells without direct serosal invasion. Lymphatic channels or micrometastasis in lymphnodes are possible routes. This idea is supported at least partly by the fact that peritoneal recurrence after curative surgery does occur, even if the incidence is very low at less than 1% of early gastric cancers, as reported previously.[29] However, we cannot exclude the possibility that these cases may represent false positives, either due to contamination of the wash fluid by epithelial cells from the skin during incision of the abdominal wall or the presence of abundant inflammatory cells or mesothelial cells with nonspecific, constitutive, low-level (illegitimate) CEA expression. Combination studies with other molecular diagnostic markers specific for malignant neoplasms are now under way in our laboratory to eliminate this possibility.

The present RT-PCR judged 3 of 13 patients (23%) with serosal (t3) or adjacent tissue invasion (t4) as negative, suggesting that in these cases, most of the tumor cells could not attach to the peritoneum and died soon after penetrating the serosal surface. One possible explanation for this is the lack of expression of cell adhesion molecules such as CD44 and β_1 integrin, which could lead to the failure of adhesion of cancer cells to mesothelium and the subsequent development of peritoneal dissemination, as demonstrated for gastric and ovarian cancers.[13, 30]

The observed close association between RT-PCR positivity and depth of invasion, and the recurrence data suggest the feasibility of using CEA RT-PCR to predict peritoneal recurrence of gastric cancer, especially in cases of advanced lesions with or without serosal invasion. A large-scale long-term follow-up study is currently under way in our hospital to ascertain the actual peritoneal recurrence in cytology-negative and PCR-positive patients, and to confirm that the negative patients remain disease-free.

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