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Comparative analysis of intraperitoneal minimal free cancer cells between colorectal and gastric cancer patients using quantitative RT-PCR: possible reason for rare peritoneal recurrence in colorectal cancer

Masayasu Hara · Hayao Nakanishi · Qian Jun · Yukihide Kanemitsu · Seiji Ito · Yoshinari Mochizuki · Yoshitaka Yamamura · Yasuhiro Kodera · Masae Tatematsu · Takashi Hirai · Tomoyuki Kato

Abstract Peritoneal recurrence has a much lower incidence in colorectal cancer (CRC) patients than gastric cancer (GC) patients. The aim of this study is to clarify the reason for the rare peritoneal recurrence in CRC as compared with GC. The incidence and the abundance of free tumor cells in the peritoneal lavages from 102 CRC and 126 GC patients who underwent curative surgery were assessed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with carcinoembryonic antigen (CEA) and cytokeratin 20 (CK20) as genetic markers. Prognostic significance of CEA and CK20 mRNA was also compared between CRC and GC after 2 years of follow-up by Kaplan–Meyer method with overall and peritoneal recurrence-free survival as endpoints. Positivity rate and average values of CEA and CK20 mRNA in peritoneal lavages of CRC patients, which are correlated to the depth of tumor invasion (pT category), were essentially the same as those of GC cases. Overall survival was significantly (marginally) worse in CEA mRNA (CK20 mRNA)-positive CRC patients than negatives like GC. However, peritoneal recurrence-free survival was not different between CEA (CK20) mRNA-positive and -negative CRC patients, in quite contrast to GC cases. Multivariate analysis showed that CEA mRNA was an independent prognostic factor for overall survival in GC patients, but not in CRC patients. These results suggest that the rare peritoneal recurrence in CRC patients is not due to the low incidence or the small number of intraperitoneal free cancer cells, but more likely reflects due to the low-peritoneal metastatic potential of CRC cells.

Keywords Quantitative RT-PCR · Peritoneal metastasis · Colorectal cancer · Gastric cancer · Peritoneal lavage cytology

Abbreviations

RT-PCR	Reverse transcription-polymerase chain reaction
qRT-PCR	Quantitative RT-PCR
GC	Gastric cancer
CRC	Colorectal cancer
CEA	Carcinoembryonic antigen
CK20	Cytokeratin 20

M. Hara · H. Nakanishi (✉) · M. Tatematsu
Division of Oncological Pathology, Aichi Cancer Center
Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya, Aichi
464-8681, Japan
e-mail: hnakanis@aichi-cc.jp

M. Hara · Y. Kanemitsu · S. Ito · Y. Mochizuki ·
Y. Yamamura · T. Hirai · T. Kato
Department of Gastroenterological Surgery, Aichi Cancer Center
Central Hospital, Nagoya, Japan

Q. Jun
Department of Colorectal Cancer, Zhejiang Cancer Center,
Hangzhou, China

Y. Kodera
Department of Surgery II, Nogoya University School of
Medicine, Nagoya, Japan

Introduction

Major routes of metastatic spread in colorectal cancers (CRC) are hematogenous metastasis to the liver and lung and regional lymph node metastasis. Peritoneal dissemination is less frequent and therefore prognostically less

important than the other two routes [1]. In contrast, peritoneal metastasis in gastric cancer (GC) is the most frequent pattern of recurrence after curative surgery and it is the most important prognostic factor [2]. The reason for this remarkable difference in peritoneal metastasis between CRC and GC despite the fact that the tumor originates from the same gastrointestinal tract remains largely unknown. Peritoneal metastasis consists of two steps; first, exfoliation of free cancer cells from the serosal surface of the primary tumor into the peritoneal cavity. Second, attachment of intraperitoneal free tumor cells to a preferable site in the peritoneal cavity such as the omentum and mesenterium and subsequent growth and dissemination into the peritoneal cavity [3]. Therefore, the low incidence of peritoneal recurrence in CRC patients may be either due to the low incidence and little exfoliation of free cancer cells from the primary tumors or low-metastatic potential of CRC cells in the peritoneal cavity. For the development of a new preventive modality for peritoneal recurrence in both GC and CRC patients, it is very important to understand the reason for this rare peritoneal recurrence in CRC patients.

Detection of free cancer cells in the peritoneal cavity in gastric, colorectal, pancreatic, and ovarian cancer patients has been performed with peritoneal lavage cytology using Papanicolaou staining [4–6]. The conventional cytology is a reliable and specific method, but has limited usefulness due to its lack of sensitivity. Recent advances in PCR and non-PCR technology such as real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) have allowed sensitive and quantitative detection of free cancer cells in the peritoneal cavity [7–9]. We applied real-time quantitative RT-PCR (qRT-PCR) for the first time to quantify free cancer cells in the peritoneal washes in GC patients and declared the prognostic significance of intra-abdominal carcinoembryonic antigen (CEA) mRNA levels [10, 11]. To date, evidences that CEA mRNA levels are a reliable prognostic factor for assessment of the peritoneal recurrence risk after curative resection in GC patients have been accumulating from a prospective study [12], as well as many retrospective studies [13, 14]. In CRC, however, the prognostic significance of intraperitoneal free cancer cells remains somewhat controversial. Several investigators have reported that overall survival of patients with either cytology or RT-PCR positive for the peritoneal washes were worse than the negatives in CRC patients, but most of them are small-scale study [15–18]. On the other hand, it was reported that intraperitoneal free tumor cells do not influence overall survival of the CRC patients [19, 20]. Furthermore, prognostic significance in terms of peritoneal recurrence-free survival of free cancer cells in the peritoneal washes of CRC patients remains to be elucidated [21].

In the present study, we quantitatively measured intraperitoneal free cancer cells using dual marker qRT-PCR

and compared peritoneal recurrence between CRC and GC patients with curative resection. We found that exfoliation of free cancer cells into the peritoneal cavity occurs in CRCs essentially to the same extent as in GCs, but the rate of peritoneal recurrence was remarkably low in the former. Possible reason for the low-peritoneal recurrence in the CRC patients as compared with GCs will be discussed.

Materials and methods

Patients

A total of 128 CRC patients and 131 GC patients were enrolled in this study. All patients underwent operation at the Department of Gastroenterological Surgery, Aichi Cancer Center Central Hospital. In CRCs, total 128 patients include 126 primary cancer (64 colon cancer and 62 rectal cancer patients) and two patients with peritoneal recurrence disease. These patients underwent curative and non-curative operation from June 2001 to August 2003. Median follow-up period was 672 days, ranging from 119 to 1,147 days. The population included 19 patients with synchronous liver metastases, six patients with peritoneal metastases at laparotomy, four patients with positive cytology (three primary and one recurrence), and three patients with distant metastases (two lungs and one bone). Lymph node metastases were observed in 63 patients. There were 12 patients with T1 (mucosal to submucosal invasion), 93 patients with T2 (muscularis propria to subserosal invasion), 18 patients with T3 (serosal invasion), and three patients with T4 (invasion to adjacent tissues). The definitions of pT category (depth of cancer invasion) of the UICC classification for GC and for CRC are different. Therefore, the pT category used in this study was graded according to the UICC classification for GC based on the histological examination of resected specimens to compare CRC and GC using the same criteria. Among these 128 CRC patients, curative operations with R0 resection were performed in 102 patients, some of whom (42 patients) had lymph node metastasis. There were 12 patients with T1, 83 patients with T2, five patients with T3, and two patients with T4. Patient characteristics are shown in Table 1.

In GCs, the population included 126 patients with curative resection and five patients with peritoneal metastasis. These patients underwent operation from July 2001 to August 2003. Median follow-up period was 669 days, ranging from 141 to 1,306 days. Population characteristics of 126 GC patients with curative resection are also summarized in Table 1. Preoperative and intraoperative chemotherapy and radiation therapy were not performed in this series. The sites of the recurrence were judged based on

Table 1 Characteristics of colorectal cancer and gastric cancer patients enrolled in this study

	Colorectal cancer patients		Gastric cancer patients	
	All patients[a] $n = 126$ (%)	Curative operation $n = 102$ (%)	All patients[a] $n = 132$ (%)	Curative operation $n = 126$ (%)
Age (mean)	63.3	61.6	62.9	62.5
Gender				
Male	71 (56.3)	48 (47.1)	89 (67.4)	84 (66.7)
Female	55 (43.7)	54 (52.9)	43 (32.6)	42 (33.3)
Depth of tumor invasion[b]				
T1	12 (9.5)	12 (11.8)	50 (37.9)	50 (39.7)
T2	93 (73.8)	83 (81.4)	39 (29.5)	38 (30.2)
T3	18 (14.3)	5 (4.9)	35 (26.5)	33 (26.2)
T4	3 (2.4)	2 (2.0)	8 (6.1)	5 (4.0)
Lymph node metastasis				
Positive	66 (52.4)	42 (41.2)	65 (49.2)	59 (46.8)
Negative	60 (47.6)	60 (58.8)	67 (50.8)	67 (53.2)
Histologic type				
Well	10 (7.9)	10 (9.8)	19 (14.4)	18 (14.3)
Moderately	103 (81.7)	85 (83.3)	35 (26.5)	35 (27.8)
Poor	13 (10.3)	7 (6.9)	78 (59.1)	73 (57.9)
Hepatic metastasis				
Positive	19 (15.1)	0 (0)	1 (0.8)	0 (0)
Negative	107 (84.9)	102 (100)	131 (99.2)	126 (100)
Distant metastasis				
Positive	3 (2.4)	0 (0)	1 (0.8)	0 (0)
Negative	123 (97.6)	102 (100)	131 (99.2)	126 (100)
Visible peritoneal metastasis				
Positive	6 (3.2)[c]	0 (0)	5 (3.8)	0 (0)
Negative	122 (96.8)	102 (100)	127 (96.2)	126 (100)

[a] All patients; patients with curative and non-curative operation

[b] T1; mucosal to submucosal invasion, T2; muscularis propria to subserosal invasion, T3; serosal invasion, T4; invasion to adjacent tissue (pT category based on the UICC classification for gastric cancer)

[c] Four primary with synchronous peritoneal metastasis and two recurrence with metachronous metastasis

radiological or cytopathological evidence. Local recurrences of rectal cancer were distinguished from the peritoneal metastases, because of possibly inadequate local excision or unresected lymphatic permeation. Patient's written informed consent was obtained from all patients examined in this study.

Cell lines

In this study, ten GC cell lines including GCIY, MKN-28, MKN-45, MKN-74, HSC-43, GLM-1, GLM-2, GLM-4, NUGC-4, and KATO-III, and ten CRC cell lines including LS174T, COCM-1, COLM-1, COLM-2, COLM-3, COLM-4, COLM-5, COLM-6, CaCo-2, and HT-29 were used to compare CEA mRNA expression between CRC and GC cells. Primary mesothelial cells were chosen as negative controls. Human CRC cell lines, LS174T and COCM-1,

and GC cell lines, NUGC-4, GCIY, MKN-28, MKN-45, and MKN-74, were obtained from RIKEN cell bank (Tsukuba, Japan). GLM-1, GLM-2, GLM-4, COLM-1, COLM-2, COLM-3, COLM-4, COLM-5, and COLM-6 cell lines were established in our laboratory from liver metastasis [22, 23]. HSC-43 was kindly provided by Dr. Yanagihara (National Cancer Center Research Institute, Tokyo, Japan). These cell lines were cultured in the same method as described previously [22].

Peritoneal washes

Peritoneal washes were obtained during laparotomy. At the beginning of each operation, 100 ml saline was introduced into the Douglas cavity and paracolic cavity near the tumors. After gentle stirring, these fluids were aspirated into the sterile tube. One half of each wash was sent to the

Division of Cytology at the Central Clinical Laboratory, Aichi Cancer Center Hospital for routine cytopathology with conventional Papanicolaou staining. The other half of the wash was sent to the Division of Oncological Pathology, Aichi Cancer Center Research Institute to measure CEA, and cytokeratin 20 (CK20) mRNA levels. Intact cells collected from the lavages by centrifugation at 1,800 rpm for 10 min were washed with phosphate buffer saline (PBS), dissolved in ISOGEN-LS, RNA extraction buffer (Nippon Gene, Tokyo, Japan) and stored at –80°C until analysis.

cDNA synthesis

Frozen peritoneal wash samples and cell lines in ISOGEN-LS were thawed and total RNA was extracted using guanidinium–isothiocyanate–phenol–chloroform method. Since cells are usually few in wash fluids, we added 2 µl of glycogen solution (20 mg/ml) (Boehringer, Mannheim, Germany) per tube as a carrier to improve RNA recovery before isopropanol precipitation. Extracted total RNA (up to 5 µg) was incubated with 50 ng of random hexanucleotide primer (Invitrogen, Carlsbad, CA, USA) in a volume of 9 µl for 10 min at 70°C. After chilling on ice, 4 µl of five fold synthesis buffer, 2 µl of 100 mM dithiothreitol, 4 µl of 2.5 mM each dNTP, and 1 µl of SuperScript II RNase H⁻ reverse transcriptase (200 U/µl, Invitrogen) were added. The reaction mixture was incubated at 42°C for 40 min and terminated by heating at 70°C for 15 min. The resultant first-strand cDNA was stored at –80°C until analysis.

Real-time quantitative RT-PCR

Single-step real-time qRT-PCR was performed using CEA- and CK20-specific oligonucleotide primers and two fluorescent hybridization probes (donor and acceptor) on the LightCycler instrument (Roche Diagnostics, Mannheim, Germany). To quantify and prove the integrity of the isolated RNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also analyzed by real-time RT-PCR using the hybridization probe method. The sequences of the primers and probes for CEA, CK20, and GAPDH used in this study are the same as described previously [12, 24]. All primers and probes were synthesized and purified by reverse-phase HPLC at Nihon Gene Research Laboratories (Sendai, Japan).

Amplification by PCR using a LightCycler proceeded in a 10 µl volume consisting of master mix containing Taq DNA polymerase, dNTP mixture and buffer (Light-Cycler DNA Master hybridization probes, Roche Diagnostics), 4.0 mM MgCl$_2$, 0.5 µM sense and anti-sense primer, 0.4 µM of each probe, and 1 µl of template cDNA in the LightCycler capillaries. Before amplification, primer elongation was blocked by adding 0.1 µl of anti-Taq DNA polymerase antibody (TaqStart antibody, Clontech Lab., CA, Palo Alto, USA) to the reaction mixture at room temperature for 5 min. Antibody was inactivated at 95°C for 90 s and then CEA and CK20 was amplified by 50 cycles at 95°C (0 s) for denaturation, 50°C (55°C for CK20) (10 s) for annealing, and 72°C (10 s) for extension. The same temperature profile was used to amplify GAPDH except for the extension step, which was 72°C for 20 s. Six external CEA and CK20 mRNA standards were prepared by tenfold serial dilution (1–10^5 cells) of cDNA equivalent to 1×10^6 COLM-2 cells (a colon cancer cell line that highly expresses CEA and CK20) spiked into 1×10^7 peripheral blood leukocytes. Each run consisted of external standards, a negative control without a template and patient samples with unknown mRNA concentrations. The higher CEA and CK20 mRNA value of two washes (Douglas cavity and Paracolic cavity) from each patient was selected. If at least one CEA (CK20) mRNA value from the two washes was above the cut-off value, the patient was considered positive for CEA mRNA.

Cut-off value for CEA and CK20 mRNA

A cut-off value for CEA mRNA (0.1) was previously determined based on the Receiver Operating Characteristic (ROC) curve analysis performed as a retrospective study of GC patients using qRT-PCR. CEA mRNA value more or less than 0.1 was judged as positive or negative for qRT-PCR, respectively. A cut-off value for CK20 mRNA (0) was determined as reported previously [25].

Statistical analysis

The CEA and CK20 mRNA values and the mRNA positivity rates among each pT category were compared using the Kruskal–Wallis test and Fisher's exact test, respectively. Survival was analyzed by Kaplan–Meier curves with death and a clinical diagnosis of peritoneal recurrence as endpoints. Cancer deaths resulting from other types of metastasis in the absence of clinical signs of peritoneal recurrence were treated as censored. Multivariate analysis using the Cox regression hazards model identified independent prognostic factors. Tumor grade, lymph node metastasis, and depth of tumor invasion were selected as covariates, along with CEA mRNA status.

Results

CEA and CK20 mRNA level in the peritoneal washes of colorectal and gastric cancer patient

Real-time qRT-PCR method allowed sensitive and quantitative detection of CEA (CK20) mRNA ranging from 1 (10) to 1×10^5 COLM-2 colon carcinoma cells expressing CEA and CK20. In peritoneal washes, CEA mRNA was detected in 29 (23.0%) of 126 CRC patients, but not in the benign counterparts. The average values of CEA mRNA (T1: 1.1, T2: 24.7, T3: 340.3 and T4: 106.8) (Fig. 1a) and CK20 mRNA (T1: 0, T2: 19.8, T3: 20.7 and T4: 728.0) in CRC patients were correlated with the depth of tumor invasion according to the pT category of UICC classification for GC. Similar correlation was observed in GC cases for average CEA mRNA values (T1: 0.07, T2: 5.6, T3: 48.8 and T4: 8800.0) (Fig. 1b) and CK20 mRNA values (T1:0, T2: 0.97, T3: 10.7 and T4: 282.5). Positivity rate for CEA mRNA and CK20 mRNA with the depth of tumor invasion (Fig. 2) were not significantly different between CRC and GC patients at any stages (CEA, T1: $P = 0.83$, T2: $P = 0.13$, T3: $P = 0.09$, T4: $P = 0.5$; CK20, T1: $P > 0.99$, T2: $P = 0.73$, T3: $P = 0.45$, T4: $P > 0.99$), indicating that in CRC patients, tumor cells also exfoliated into the peritoneal cavity from primary tumor at a level comparable to that of GC patients in terms of tumor cell numbers and incidence. Based on eight patients with benign disease and six CRC patients with macroscopic peritoneal deposits, sensitivity of qRT-PCR (CEA and CK20) and cytology was

calculated to be 100% (6/6) and 50% (3/6), respectively, and specificity was 100% (8/8) and 100% (8/8), respectively.
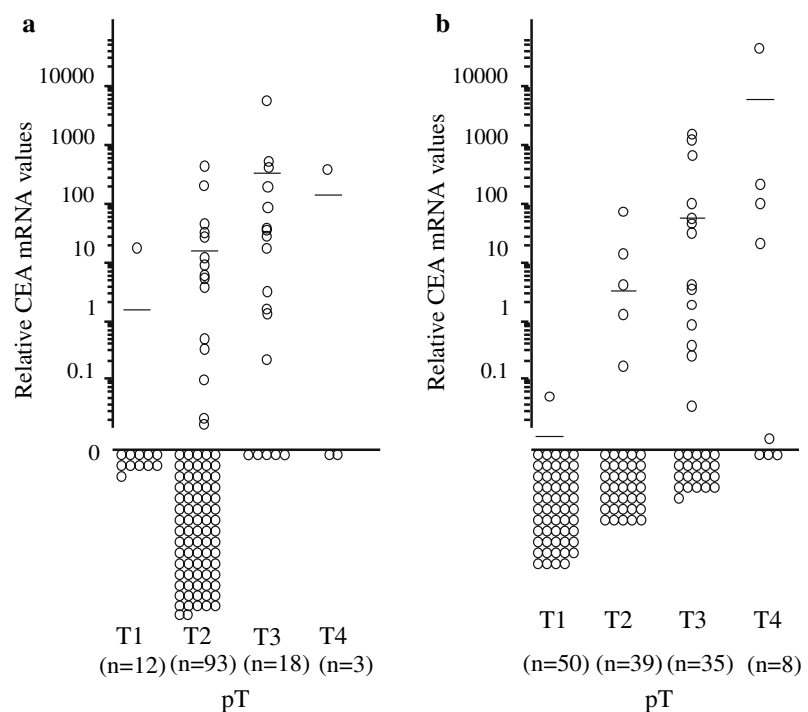
CEA and CK20 mRNA expression in colorectal cancer and gastric cancer cell lines

To compare CEA and CK20 mRNA expression between CRC and GC, we measured the mRNA of ten CRC and ten GC cell lines by qRT-PCR. Mean (±SD) CEA/GAPDH ratios in CRC and GC cell lines were 170.4 ± 189 (range 0.31–490.4) and 225.8 ± 326 (range 0–798.7), respectively. CEA mRNA was not detected in primary mesothelial cells as negative control. Although CEA mRNA expression of some poorly differentiated GC cell lines was lower than that of CRC cell lines, average CEA and CK20 mRNA expression per cell base was not significantly different between gastric and CRC cell lines ($P = 0.3$ and 0.16, respectively) (Fig. 3a, b, c and d).

Clinicopathological features of CEA and CK20 mRNA positive colorectal cancer patients

Table 2 shows the clinicopathological features of CEA mRNA-positive patients among 126 primary CRC patients. The univariate analysis showed that CEA mRNA positivity in the peritoneal washes correlated with the depth of tumor invasion (pT) ($P < 0.0001$), peritoneal metastasis ($P < 0.0001$), histology ($P <0.0001$), hepatic metastasis ($P < 0.0001$), and lymphatic metastasis ($P = 0.004$).

Fig. 1 Relative CEA mRNA values of peritoneal washes from colorectal cancer patients (**a**) and gastric cancer patients (**b**) measured by qRT-PCR according to the depth of tumor invasion (pT category)
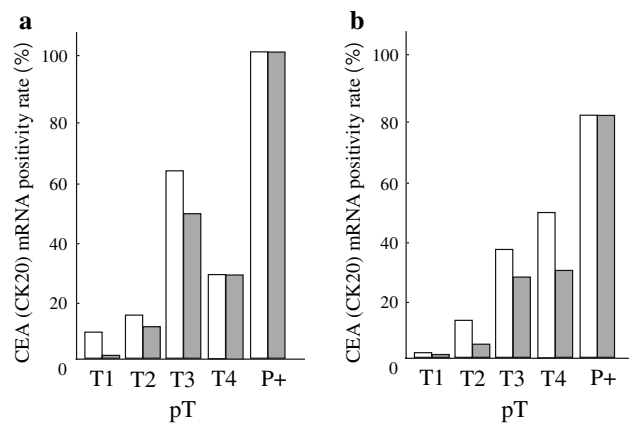
Fig. 2 CEA and CK20 mRNA positivity rate of peritoneal washes from colorectal cancer patients (**a**) and gastric cancer patients (**b**). CEA mRNA (*white bar*), CK20 mRNA (*gray bar*). P+ indicates six patients with synchronous and metachronous peritoneal metastasis. Positivity rate for CEA mRNA and CK20 mRNA with pT category was not significantly different between colorectal and gastric cancer patients at any stages ($P > 0.09$)

Among these, however, only histology ($P = 0.01$) and pT ($P = 0.002$) remained a significant covariate correlating CEA mRNA by multivariate analysis (Logistic analysis). In CK20 mRNA-positive CRC patients, only pT ($P = 0.004$) and lymphatic metastasis ($P = 0.008$) remained significant by multivariate analysis (data not shown).

Prognostic significance of CEA mRNA in peritoneal washes of colorectal and gastric cancer patients

The recurrence patterns of CRC and GC patients who underwent curative resection is shown in Table 3. The recurrence rate was almost the same between colorectal and GC patients (13.7% = 14/102 vs. 15.1% = 19/126), but the site of recurrence differed remarkably. In GC patients, peritoneal recurrence accounted for more than half of the recurrences (10/19), whereas virtually no peritoneal recurrence was observed in CRC patients. Overall survival was significantly worse ($P = 0.008$) in CEA mRNA-positive patients and marginally worse ($P = 0.08$) in CK20 mRNA-positive patients than the mRNA-negative patients in CRC patients (Figs. 4a, 5a), although the extent of significance is less than that of GC cases (Fig. 4c). Peritoneal recurrence-free survival of CEA mRNA-positive GC patients was also significantly worse than the negatives (Fig. 4d), but, this was not the case at all with CRCs in which no peritoneal recurrence was observed in CEA and CK20 mRNA-positive patients (Figs. 4b, 5b). Even three patients who were classified as positive by conventional cytology have not developed peritoneal metastasis in the CRC cases.

Multivariate analysis of prognostic factors

A Cox regression analysis with overall survival as an endpoint was performed to determine independent prognostic factors among covariates including tumor grade, pT, lymph node metastasis, cytology, and CEA mRNA status of peritoneal washes of patients who underwent curative resection. Although CEA mRNA of peritoneal washes ($P = 0.04$) was an independent prognostic factor in GC patients, lymph node metastasis ($P = 0.008$), but not CEA mRNA of peritoneal washes ($P = 0.23$), proved to be an independent prognostic factor in CRC patients (Table 4).

Discussion

As for genetic markers for detection of micrometastasis in CRCs, a number of candidates genes have been reported. Among these, CEA and CK20 is reportedly the most useful genetic marker for RT-PCR to detect free tumor cells in the peritoneal lavage in CRC patients [16, 26]. In fact, we found that CRC cell lines expressed CEA and CK20 mRNA at a similar level. CEA mRNA values in the peritoneal washes of CRC patients were also comparable to CK20 mRNA. Furthermore, neither CEA nor CK20 mRNA was detected in the peritoneal washes of eight patients with benign disease and of the peripheral blood leukocytes from ten healthy volunteers. These findings indicate that CEA and CK20 mRNA can be equally used as reliable parameters for the number of intraperitoneal tumor cells in CRC patients.

To date, few survival analyses were performed with an adequate sample size specific to peritoneal washes in CRC patients [16–20]. In the present study, to resolve some controversy on the prognostic significance of intraperitoneal free tumor cells in CRC patients, we conducted abovementioned, dual marker qRT-PCR analysis on the relationship between free tumor cells and peritoneal recurrence with a sufficient sample size. We here demonstrated that the incidence of peritoneal recurrence was not different between CEA (CK20) qRT-PCR-positive and -negative CRC patients. Previously, Yamamoto et al. and Kanellos et al. reported that the peritoneal recurrence rate was significantly increased in patients with positive cytology than the negatives and that cytology was an independent prognostic predictor of cancer-specific survival [21, 27]. In contrast, Vogel et al. reported in their immunohistochemical study that microscopic intraperitoneal free tumor cells do not influence survival time after R0 resection in CRC patients [19]. Our present results are consistent with the Vogel's work. This is probably because the detection sensitivity of Vogel's work (62–73%) and ours (60%) were much higher than with conventional cytology (6–30%) in

Fig. 3 Comparison of CEA and CK20 mRNA expression between colorectal (**a, c**) and gastric cancer cell lines (**b, d**). Relative CEA mRNA expression was calculated as CEA mRNA value relative to GAPDH mRNA value (CEA/GAPDH ratio). Average CEA and CK-20 mRNA were not significantly different between colorectal and gastric cancer cell lines ($P = 0.30$ and 0.16, respectively)
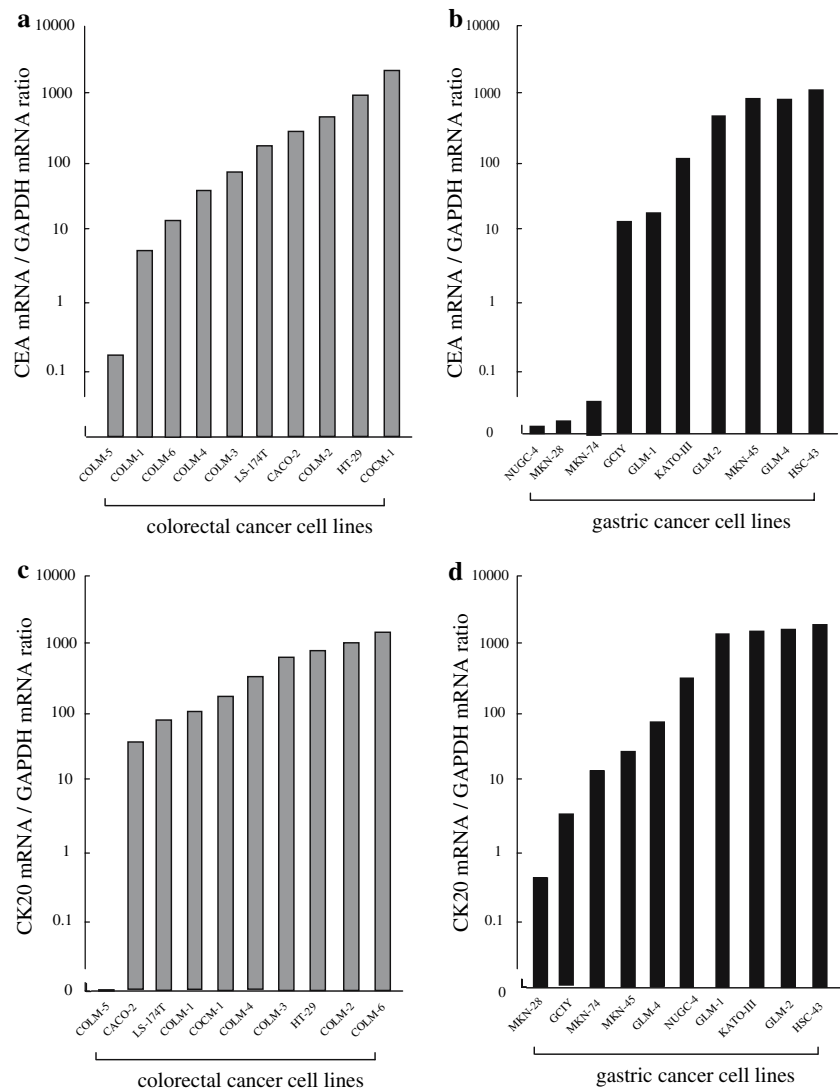
patients with serosal invasion. Abundant free tumor cells detectable by low-sensitive cytology may lead to at least in part peritoneal recurrence, but a small number of intra-peritoneal free tumor cells detected only by high-sensitive immunohistochemistry (Vogel's work) or qRT-PCR (our study) do not result in peritoneal recurrence in CRC patients, suggesting that prognostic significance depends on the number of disseminated intraperitoneal free tumor cells.

The most important finding in the present study results from comparative analysis of the intraperitoneal free tumor cells between CRC and GC patients. We clearly demonstrated that although CRC cells exfoliated into the peritoneal cavity at a level similar to GC in terms of incidence and cell number, the patient outcome was completely different between the two cancers. Peritoneal recurrence occurred in ~50% of the CEA mRNA-positive GC patients, but never at all in CRC patients with curative resection. In

the present study, indeed, overall survival was worse in CEA (CK20) mRNA-positive CRC patients than negatives similar to the previous report by Vogel et al. [19], but multivariate survival analysis showed that only lymph node metastasis was an independent prognostic factor, suggesting that shorter overall survival with CEA (CK20) mRNA-positive CRC patients is associated with lymph node metastasis, not peritoneal recurrence. These results strongly suggest that the rare peritoneal recurrence in CRC patients is not due to low incidence or a small number of intraperitoneal free cancer cells, but to the low-metastatic potential of intraperitoneal CRC cells, in quite contrast to GC cells.

A number of pathological factors and genes such as adhesion molecules have been reported to be associated with metastasis in CRCs [28]. Among these factors, specific histological type such as poorly differentiated adenocarcinoma and mucinous carcinoma have a known

Table 2 Univariate and multivariate analysis of 126 colorectal cancer patients

	Univariate			Multivariate		
	CEAmRNA-	CEAmRNA+	P	Hazard ratio	95% Cl	P
Histologic type						
Well + moderately	93	20	<0.0001	1	1.562–34.12	0.01
Poor + mucinous	4	9		7.0		
Depth of tumor invasion[a]						
T1–T2	90	15	<0.0001	1	2.269–35.87	0.002
T3–T4	7	14		8.7		
Hepatic metastasis						
Negative	89	18	<0.0001	1	0.443–8.177	0.3
Positive	8	11		2.0		
Lymphatic metastasis						
Negative	57	7	0.004	1	0.706–7.052	0.2
Positive	40	22		2.2		
Distant metastasis						
Negative	96	27	0.07	1	0.024–105.3	0.9
Positive	1	2		1.4		
Cytology						
Negative	96	27	0.07	1	0.147–83.22	0.5
Positive	1	2		2.7		

[a] T1; mucosal to submucosal invasion, T2; muscularis propria to subserosal invasion, T3; serosal invasion, T4; invasion to adjacent tissue (based on the UICC classification for gastric cancer)

Table 3 Pattern of recurrence of colorectal cancer and gastric cancer patients who underwent curative resection

	Colorectal cancer patients				Gastric cancer patients			
	Total	(%)	PCR+	PCR–	Total	(%)	PCR+	PCR–
Recurrence[a]	14	(13.7)	5	9	19	(15.1)	9	10
Liver	10	(9.8)	3	7	3	(2.4)	0	3
Lung	4	(3.9)	0	4	1	(0.8)	1	0
Peritoneum	0	(0)	0	0	10	(7.9)	8	2
Local	2	(2.0)	1	1	1	(0.8)	0	1
Bone	2	(2.0)	1	1	2	(1.6)	1	1
Lymph node	0	(0)	0	0	7	(55.6)	3	4
No recurrence	88	(86.3)	11	77	107	(84.9)	10	97
Total	102	(100)	16	86	126	(100)	19	107

[a] Primary recurrence site

tendency to disseminate into the peritoneal cavity as compared with differentiated adenocarcinoma [29–32]. In fact, CEA mRNA positivity rate of peritoneal washes in CRC patients was found to be significantly higher in the (poorly differentiated + mucinous) type (69% = 9/13) than differentiated type (18% = 20/113) in the present study. To elucidate whether the peritoneal metastatic potential differs depending on the histological type of CRC, we tested peritoneal metastatic capability of two moderately differentiated colonic cancer cell lines (COLM-2 and COLM-3), one poorly differentiated colonic cancer cell line (COLM-

5), one mucinous colonic cancer cell line (COLM-6), and two moderately differentiated and poorly differentiated GC cell lines (MKN-28 and GCIY) in nude mouse xenograft models. Our preliminary results showed that COLM-2 and COLM-3 cell lines produced a small peritoneal metastasis at omentum 2 months after intraperitoneal injection (average tumor weight 0.42 and 0.28 g, respectively), whereas COLM-5 and COLM-6 cell lines generated relatively large intraperitoneal metastatic tumors (average tumor weight 2.05 and 1.25 g, respectively). GC cell lines (MKN-28 and GCIY) formed large metastatic tumors in the

Fig. 4 Comparison of overall (**a, c**) and peritoneal recurrence-free survival (**b, d**) between 90 curatively resected advanced colorectal (**a, b**) and 76 advanced gastric cancer patients (**c, d**) with positive and negative for CEA mRNA
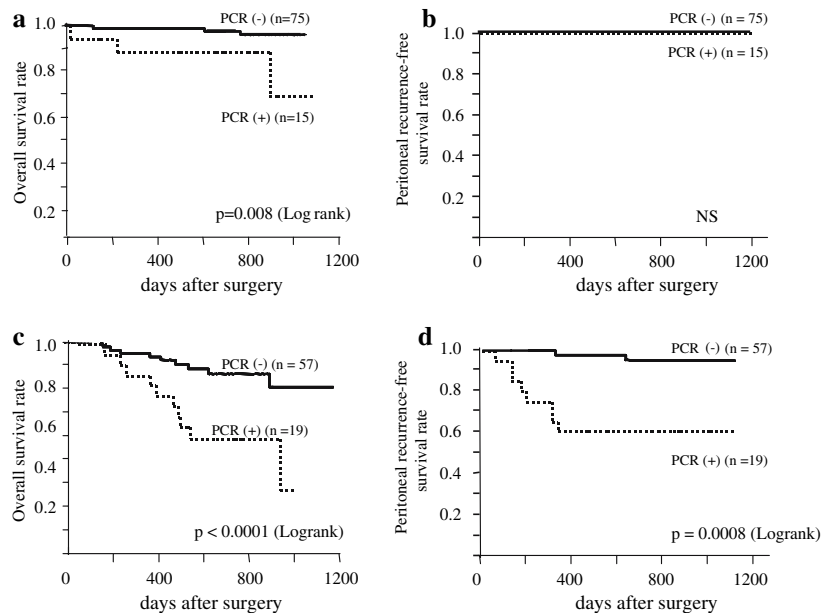

Fig. 5 Overall survival (**a**) and peritoneal recurrence-free survival (**b**) of 83 curatively resected advanced colorectal cancer patients with positive and negative for CK20 mRNA
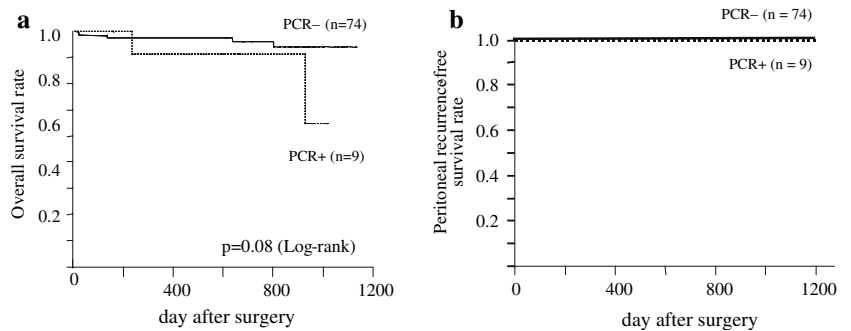

Table 4 Multivariate analysis of prognostic factors in 102 colorectal cancer and 126 gastric cancer patients who underwent curative resection with overall survival as an end point

Variable	Colorectal cancer ($n = 102$)			Gastric cancer ($n = 126$)		
	Risk ratio	95% Cl	P	Risk ratio	95% Cl	P
Histology						
Differentiated	1		0.20	1		0.09
Undifferentiated	2.00	0.662–5.193		3.63	0.804–16.388	
Depth of tumor invasion[a]						
T1–T2	1		0.30	1		0.001
T3–T4	1.72	0.651–4.195		12.2	2.646–56.510	
Lymph node metastasis						
Negative	1		0.008	1		0.86
Positive	2.67	6.25e200		1.12	0.327–3.780	
CEA mRNA						
Negative	1		0.23	1		0.04
Positive	1.72	0.651–7.680		2.81	1.034–7.626	

[a] T1; mucosal to submucosal invasion, T2; muscularis propria to subserosal invasion, T3; serosal invasion, T4; invasion to adjacent tissue (based on the UICC classification for gastric cancer)

peritoneum (average tumor weight 2.92 and 3.48 g, respectively). These results indicate significantly slower metastatic growth potentials of moderately differentiated colonic cancer cell lines than poorly differentiated/mucinous colonic cancer cell lines and GC cell lines (unpublished results), suggesting the low-metastatic ability of differentiated type CRC cells experimentally, even if it is still preliminary. Taken together, these results strongly suggest that low-peritoneal metastatic potential of CRCs are largely due to the low-metastatic potential of well to moderately differentiated cancers, a major subtype of CRCs.

In conclusion, we quantitatively demonstrated for the first time that the rare peritoneal recurrence in CRC patients is not due to the low incidence of exfoliation into the peritoneal cavity and small number of intraperitoneal free tumor cells. The present clinical findings and experimental evidence with CRC and GC cell lines further suggest that intraperitoneal free CRC cells have a low risk for generating peritoneal recurrence if the number of free tumor cells is limited within a range detectable only by sensitive qRT-PCR and histology of tumor cells is restricted to the well to moderately differentiated type. Understanding the reason for the low-metastatic potential of the differentiated type CRC s may provide fresh insight into the development of a new therapeutic modality against gastric as well as CRC peritoneal metastasis.

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