Prospective study of the quantitative carcinoembryonic antigen and cytokeratin 20 mRNA detection in peritoneal washes to predict peritoneal recurrence in gastric carcinoma patients

NAOYUKI TAMURA, HISAE IINUMA and TADAHIRO TAKADA

Department of Surgery, Teikyo University School of Medicine, Tokyo, Japan

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Abstract. The prediction of peritoneal recurrence in gastric cancer patients is extremely important in preventing an unfavorable prognosis. In this prospective study, we examined the usefulness of the carcinoembryonic antigen (CEA) and cytokeratin 20 (CK20) mRNA detection in peritoneal washes as a prophylactic tool for peritoneal recurrence. Peritoneal washes were obtained from 164 patients with gastric carcinoma during a laparotomy. CEA, CK20 and glyceraldehyde-3-phosphate-dehydrogenase mRNA levels in the peritoneal washes were detected by real-time reverse transcription-polymerase chain reaction (RT-PCR) using LightCycler. Molecular detection of the CEA and CK20 mRNA in the peritoneal washes by real-time RT-PCR showed a significant correlation with tumor size, histological type, depth of invasion, lymphatic invasion, venous invasion, lymph node metastasis, peritoneal dissemination, stage and cytology. The peritoneal recurrence-free survival and overall survival rates of CEA and CK20 mRNA-positive patients were significantly worse than those of marker gene-negative patients. The CEA and CK20 mRNA levels in the peritoneal washes were a significant independent prognostic factor. In conclusion, our prospective study demonstrates that the detection of CEA and CK20 mRNA by quantitative real-time RT-PCR is a useful tool for identifying patients at high risk of peritoneal recurrence who may need adjuvant chemotherapy.

Correspondence to: Dr Hisae Iinuma, Department of Surgery, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-0003, Japan
E-mail: iinuma@med.teikyo-u.ac.jp

Abbreviations: ICT, isolated tumor cells; RT-PCR, reverse transcription-polymerase chain reaction; CEA, carcinoembryonic antigen; CK20, cytokeratin 20; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase

Key words: gastric cancer, peritoneal recurrence, isolated tumor cells, real-time reverse transcriptase-polymerase chain reaction, peritoneal wash, carcinoembryonic antigen, cytokeratin 20

Introduction

Although the prognosis of gastric cancer has improved with the development of early diagnosis and new therapy strategies, it still remains one of the main causes of cancer death in Japan (1). Peritoneal dissemination is reported to be the most frequent type of gastric cancer recurrence (2). About 50% of patients with serosal-invasive gastric carcinoma develop peritoneal recurrence and die within 2 years, even if curative resection is performed (3,4). Isolated tumor cells (ITC) derived from serosal invasion could be indicators of early peritoneal seeding with the subsequent formation of peritoneal dissemination (5). The detection of ITC in peritoneal washes by cytology has been applied clinically to assess early peritoneal recurrence (6,7). However, conventional cytology, by the Papanicolaou staining of peritoneal washes, lacks sensitivity and peritoneal recurrence can occur in cytology-negative patients (8). The development of new techniques that can overcome the low sensitivity of conventional cytology is essential.

In order to increase the sensitivity of conventional peritoneal lavage cytology, a highly sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) system to detect intraperitoneal ITC has been developed (9,10). Furthermore, another approach using real-time RT-PCR, has improved RT-PCR precision by setting accurate cut-off values (11-13). Using real-time RT-PCR, many investigators have analyzed the prognostic value of the carcinoembryonic antigen (CEA) mRNA in peritoneal washes. However, a multi-marker for gastric cancer cells is required in order to overcome the heterogeneity of gastric cancer (14,15). Although there are several prospective studies on the ITC of breast cancer patients, there are only a few for gastric cancer patients (16,17). In this prospective study, using a multiple real-time RT-PCR system, we examined whether the levels of CEA and cytokeratin 20 (CK20) mRNA in peritoneal washes can predict peritoneal recurrence in gastric cancer patients.

Materials and methods

Prospective study design. This study was a prospective cohort study aimed at validating the ability of quantitative CEA and CK20 mRNA detection in peritoneal washes in order to predict peritoneal relapse. A total of 164 patients (106 men and 58 women) with gastric cancer were studied

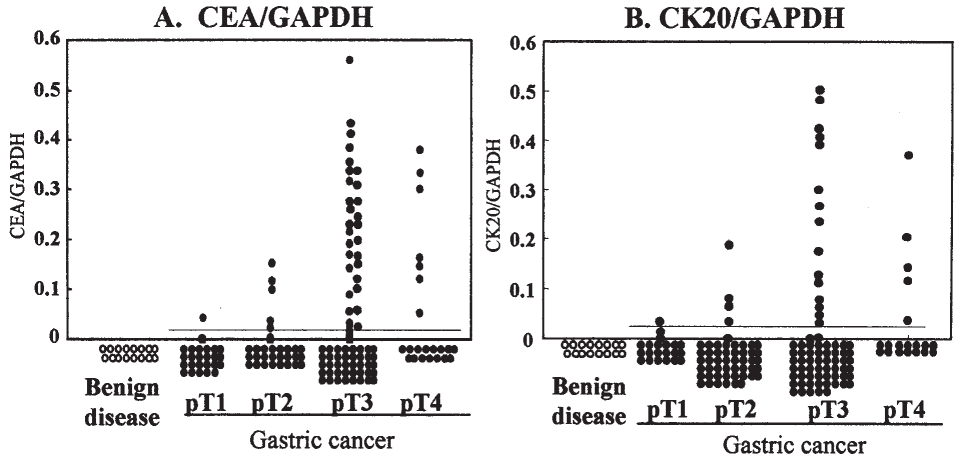

Figure 1. CEA/GAPDH and CK20/GAPDH levels in benign disease and gastric cancer patients. The CEA/GAPDH (A) and CK20/GAPDH levels (B) in the peritoneal washes from benign disease and gastric cancer patients are plotted. The CEA/GAPDH and CK20/GAPDH values correlate with the depth of invasion. The bars indicate the cut-off values of CEA/GAPDH (0.004) and CK20/GAPDH (0.02).

between 2000 and 2005. The median follow-up period was 26 months (range, 18-65 months). The depth of cancer invasion (pT categories) and stage was evaluated histologically according to the TNM classification (18). This study was approved by the institutional review board of our hospital, and all patients provided written informed consent.

Peritoneal washes. Peritoneal washes, collected from 164 patients with gastric cancer and 16 patients with benign disease, were prepared. At the beginning of each surgery, 100 ml saline was introduced into the Douglas cavity and left in subphrenic space and aspirated after gentle stirring. One half of each wash was used for cytopathology and the other half for real-time RT-PCR assay. Cells collected from the peritoneal washes by centrifugation were rinsed with phosphate-buffered saline (PBS), dissolved in TRIzol RNA extraction buffer (Invitrogen, Carlsbad, CA, USA) and stored at -80°C.

Quantitative real-time RT-PCR. Total RNA was extracted using a guanidinium-isothiocyanate-phenol-chloroform-based method using TRIzol. cDNA was synthesized from total RNA by SuperScropt II RNase H reverse transctiptase (Invitrogen) according to the manufacturer's instructions. Two-step real-time quantitative RT-PCR of CEA, CK20 and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA was performed using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) as described previously (11). In brief, each cDNA was diluted with PCR master mix (Lightcycler Faststart DNA Master Hybridization Probes, Roche Diagnostics) containing primers, a fluorescein probe, an LCRed probe and $MgCl_2$ and was amplified by a LightCycler instrument. All samples were measured in duplicate. As an external standard, the PCR product of CEA, CK20 or GAPDH was cloned into a TOPOTA cloning plasmid vector (Invitrogen). The mRNA in each sample was quantified automatically with reference to the standard curve of the plasmid, according to the LightCycler software. The levels of CEA and CK20 mRNA were normalized by GAPDH, and the ratio of CEA or CK20 copies to the GAPDH copies (CEA/GAPDH, CK20/GAPDH) was calculated. The cut-off values of the CEA/GAPDH and CK20/GAPDH were determined by reference to the receiver operating characteristics (ROC) curve, and we selected 0.004 in CEA/GAPDH and 0.02 in CK20/GAPDH as the cut-off values (12,13).

Postoperative surveillance. The follow-up program, consisting of an interim history, physical examination, hematology and blood chemistry, was performed every 3 months for the first operative year and every 6 months thereafter. A computed tomography or abdominal ultrasonography were performed every 6 months. Evidence of peritoneal recurrence was diagnosed comprehensively with parameters including paracentesis and autopsy.

Statistical analysis. Survival was analyzed using the Kaplan-Meier method, with death and a clinical diagnosis of peritoneal recurrence as the end points. Univariate and multivariate analyses were performed using Cox regression analysis. A P-value of $P<0.05$ was considered statistically significant.

Results

Expression of CEA/CK20 mRNA in benign disease and gastric cancer patients. Fig. 1 shows the CEA/GAPDH and CK20/GAPDH values in peritoneal washes from benign disease and gastric cancer patients. As cut-off values, we determined 0.004 in CEA/GAPDH and 0.02 in CK20/GAPDH in order to allow maximal sensitivity (85%) and maximal specificity (83%) by reference to the ROC curve (data not shown). There was no detection of CEA and CK20 values in the samples from benign disease patients. In the gastric cancer samples, the positive rates of CEA/ GAPDH were 4% (1/25), 10% (5/50), 43% (30/70) and 42% (7/19) in pT1, pT2, pT3 and pT4 patients, respectively. The positive rates of CK20/GAPDH were 4% (1/25), 8% (4/50), 21%

Table I. The relationship between the clinicopathological factors and CEA/CK20 mRNA positive rates in peritoneal washes.

Variables	Number of patients (n=164)	PCR positive (n=45)	Positive rate (%)	P-value
Tumor size (cm)				
<5	53	9	16.9	0.0328
≥5	111	36	32.4	
Histological type				
Well, Moderate	82	12	14.6	0.0002
Poor, Undifferentiated	82	33	40.2	
Depth of invasion				
pT1	25	1	4	<0.0001
pT2	50	6	12	
pT3	70	30	42.8	
pT4	19	8	42.1	
Lymphatic invasion				
ly (-)	56	3	5.4	<0.0001
ly (+)	108	42	38.9	
Venous invasion				
v (-)	50	8	16	0.0245
v (+)	114	37	32.5	
Lymph node metastasis				
n (-)	43	2	4.7	<0.0001
n (+)	121	43	35.5	
Liver metastasis				
H (-)	156	42	26.9	0.5258
H (+)	8	3	37.5	
Peritoneum dissemination				
P (-)	149	33	22.2	<0.0001
P (+)	15	12	80	
Stage				
I, II	68	6	8.8	<0.0001
III, IV	96	39	40.6	
Cytology				
CY (-)	140	28	20	<0.0001
CY (+)	24	17	70.8	

(15/70) and 26% (5/19) in pT1, pT2, pT3 and pT4 patients, respectively.

Relationship between CEA/CK20 mRNA and clinicopathological factors. Although the results of RT-PCR were separated into 4 groups depending on the status of the CEA and CK20 mRNA expression (CEA and/or CK20, $CEA^+/CK20^+$, $CEA^+/CK20^-$, $CEA^-/CK20^+$), we selected CEA and/or CK20 (CEA/CK20) as RT-PCR-positive cases, due to the highest sensitivity (data not shown). We examined the relationship between CEA/CK20 mRNA in the peritoneal washes and the clinicopathological factors (Table I). A significant relationship was demonstrated between the positivity for CEA/CK20 and tumor size, histological type, depth of tumor invasion, lymphatic invasion, venous invasion, lymph node metastasis, peritoneal dissemination, stage and cytology.

Correlation between survival and CEA/CK20 mRNA. Kaplan-Meier survival curves show the overall and peritoneal recurrence-free survival of gastric carcinoma patients stratified according to the results of the CEA/CK20 mRNA expression status. The overall survival of patients with a positive CEA/CK20 expression in the peritoneal washes showed a significantly poorer prognosis than that of CEA/CK20-negative patients (Fig. 2). Peritoneal recurrence-free survival was analyzed for the gastric cancer patients following curative resection. Patients who were CEA/CK20 mRNA-positive in the peritoneal washes showed a significantly poorer prognosis than patients who were marker gene-negative (Fig. 3). Fig. 4 shows the overall survival rates in cytology-negative patients. In this analysis, patients who were CEA/CK20 mRNA-positive showed a significantly poorer prognosis than patients who were marker gene-negative.

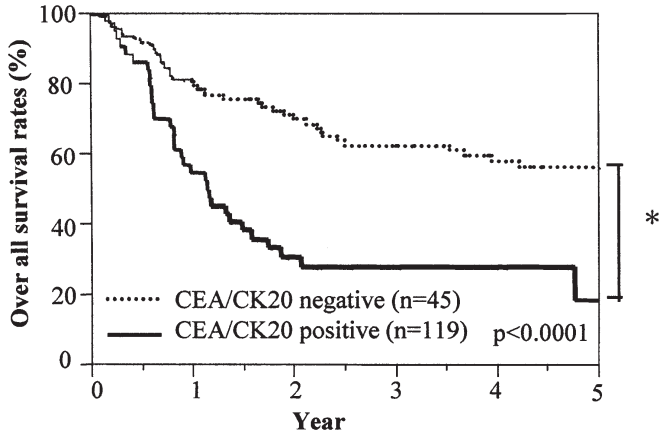

Figure 2. Overall survival curves stratified according to the results of the CEA/CK20 real-time RT-PCR. The overall survival for all the 164 patients was analyzed by the Kaplan-Meier method, and a significant difference was shown between the CEA/CK20-positive and CEA/CK20-negative groups.

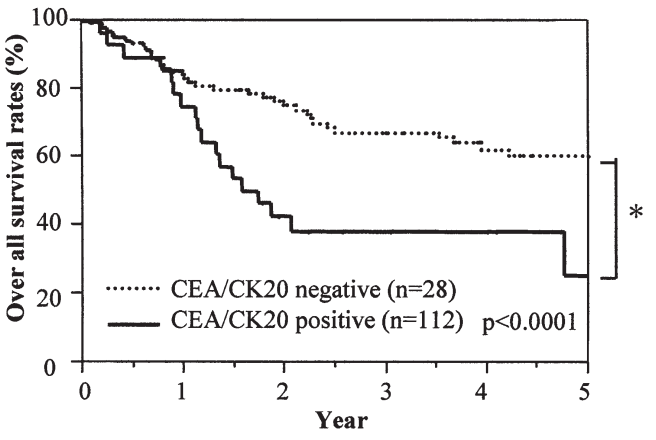

Figure 4. Overall survival curves of the cytology-negative patients stratified according to the results of the CEA/CK20 real-time RT-PCR. The overall survival of the 140 cytology-negative patients was analyzed by the Kaplan-Meier method, and a significant difference was shown between the CEA/CK20-positive and CEA/CK20-negative groups.

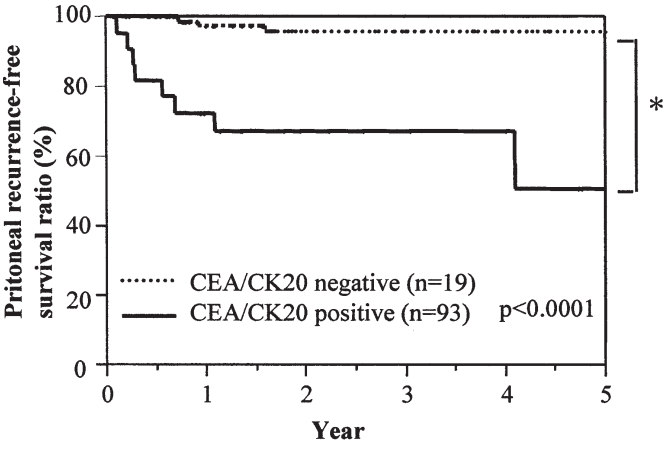

Figure 3. Peritoneal recurrence-free survival curves stratified according to the results of the CEA/CK20 real-time RT-PCR. The peritoneal recurrence-free survival for the 112 curative patients was analyzed by the Kaplan-Meier method, and a significant difference was shown between the CEA/CK20-positive and CEA/CK20-negative groups.

Univariate and multivariate analysis of prognostic factors. Tables II and III show the univariate and multivariate Cox proportional hazard regression analyses for the overall and peritoneal recurrence-free survival. In the univariate analyses, significant relationships were shown concerning the depth of invasion, lymph node metastasis, histological type and CEA/CK20 mRNA. Then multivariate analyses were performed for the factors that showed significance in the univariate analyses. The CEA/CK20 mRNA, depth of invasion and lymph node metastasis showed significance for overall survival, and the CEA/CK20 mRNA showed significance for peritoneal recurrence-free survival. These results suggest that CEA/CK20 mRNA in peritoneal washes possess an independent prognostic value for peritoneal recurrence and survival.

Discussion

This prospective study using the multiplex real-time RT-PCR demonstrated that the detection of CEA/CK20 mRNA in the peritoneal washes of gastric cancer patients is an independent predictive factor of peritoneal recurrence and prognosis.

Locally advanced gastric cancer has a high probability of developing into peritoneal carcinomatosis (11). The molecular detection of ITC in the peritoneal washes of patients with gastric cancer is a more sensitive predictive marker for peritoneal recurrence than conventional cytology. Using CEA mRNA as a marker gene, several studies have reported the usefulness of the molecular detection of ITC by real-time RT-PCR as a prognostic factor (12,13). Recently,

Table II. Univariate and multivariate analysis of the prognostic factors in overall survival.

Variables	Univariate analysis			Multivariate analysis		
	Regression coefficient	Hazard ratio	P-value	Regression coefficient	Hazard ratio	P-value
Depth of invasion	1.143	3.137	<0.001	0.794	2.212	0.006
Lymph node metastasis	0.650	1.961	<0.001	0.574	1.775	0.001
Histological type	-0.315	0.730	0.007	-0.136	0.872	0.255
CEA/CK20 mRNA	0.497	1.643	<0.001	0.319	1.376	0.009

Table III. Univariate and multivariate analysis of the prognostic factors in peritoneal recurrence-free survival.

Variables	Univariate analysis			Multivariate analysis		
	Regression coefficient	Hazard ratio	P-value	Regression coefficient	Hazard ratio	P-value
Depth of invasion	0.953	2.593	0.020	0.379	1.461	0.268
Lymph node metastasis	0.276	1.318	0.001	0.605	1.831	0.179
Histological type	-0.089	0.410	0.008	-0.573	0.563	0.111
CEA/CK20 mRNA	1.322	3.753	<0.001	1.032	2.806	0.001

another report suggested the necessity of multiple genetic markers for the accurate measurement of ITC (14,15). In this study, we added CK20 to CEA, which has been reported to show a correlation with lymph node micrometastasis in gastric cancer (19).

The development of the quantitative real-time RT-PCR system is a breakthrough in the molecular detection of tumor cells (20-27). Quantifying the low-level expression of marker genes allows accurate cut-off values to be established, thus improving the precision of molecular detection and allowing high reproducibility. Although this quantitative real-time RT-PCR system has been applied in the detection of ITC in the peritoneal washes of gastric cancer patients, the cut-off value remains controversial. In this preliminary study, we tried to determine the cut-off value based on the mean + 2 standard deviation (SD) of the control samples collected from benign disease patients. In these control samples, however, the CEA and CK20 mRNA levels were undetectable and this resulted in a high positive rate in the early-stage patients. We determined the cut-off value of the CEA/GAPDH and CK20/GAPDH by reference to the ROC curve described previously (12,13). It should be noted that a few pT1 patients showed low levels of CEA or CK20 mRNA. Some studies have reported that the low specificity of CEA mRNA to the cancer cells is the reason for these false-positives. However, as we have previously demonstrated, the designs of primers and probes of CEA and CK20 mRNA in our study were highly specific to the cancer cells, and these genes were hardly detected in the blood samples from healthy volunteers (11). This could be attributed to the illegitimate expression of target genes in non-carcinoma cells, such as the mesothelium cells. Marutsuka *et al* reported that lymph node dissection opened the lymphatic channels and spread viable cancer cells into the peritoneal cavity in non-serosa-invasive gastric carcinoma patients (20). As our samples were collected at the time of laparotomy before lymph node dissection, the true reason for PCR-positive cases in early-stage patients requires further research.

Concerning the relationship between the CEA/CK20 mRNA in peritoneal washes and clinicopathological factors, we have demonstrated a significant relationship between the CEA/CK20 mRNA and tumor size, histological type, depth of tumor invasion, lymphatic invasion, venous invasion, lymph node metastasis, peritoneum dissemination, stage and cytology. Oyama *et al* reported that the positive rate of CEA mRNA in peritoneal washes showed a significant correlation with lymph node metastasis, peritoneal metastasis and serosal invasion (21). Another study also reported that the positivity of CEA and CK20 mRNA correlated with the histological type and lymph node metastasis, thereby supporting our results (20).

Next, we evaluated the prognostic value of CEA/CK20 mRNA in peritoneal washes. We have shown that the Kaplan-Meier survival curves of overall and peritoneal recurrence-free survival were significantly worse in the CEA/CK20 mRNA-positive patients than in the cases negative for these marker genes. Furthermore, Cox multivariate analysis has shown that the CEA/CK20 mRNA in the peritoneal washes was an independent prognostic factor for survival. Another study demonstrated the same prognostic values of the CEA mRNA expression in the peritoneal washes in gastric cancer patients following curative resection (13). To our knowledge, this is the first prospective study evaluating the prognostic value by multi-marker (CEA and CK20) detection of real-time RT-PCR in peritoneal washes as a reliable prognostic indicator of peritoneal recurrence.

In conclusion, we have demonstrated that CEA and CK20 mRNA detection by quantitative real-time RT-PCR is a useful tool for identifying patients at high risk of peritoneal recurrence. Curative gastric cancer patients with CEA and CK20 mRNA-positive results in peritoneal washes may need adjuvant chemotherapy based on the results of the molecular detection of ITC.

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