Clinical significance of intraperitoneal CD44 mRNA levels of magnetically separated CD45-negative EpCAM-positive cells for peritoneal recurrence and prognosis in stage II and III gastric cancer patients

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Abstract. Peritoneal recurrence of gastric cancer patients is a serious problem. Recently, the CD44 molecule was reported to be a marker of cancer stem cells in gastric cancer. In this study, we examined the prognostic significance of CD44 mRNA levels in magnetically separated CD45-negative EpCAM-positive ($CD45^-EpCAM^+$) cells, in the peritoneal washes collected from gastric cancer patients with TNM stage II and III. A total of 147 gastric cancer patients with stage II (n=75) and III (n=72) were included. All patients were negative by peritoneal cytology. Peritoneal washes of the Douglas pouch were collected and used for pathological cytology and molecular diagnosis. Prior to molecular diagnosis, $CD45^-EpCAM^+$ cells were separated from peritoneal washings by an auto-magnetic-activated cell separation system. CD44 and CEA mRNA levels of the $CD45^-EpCAM^+$ fraction were detected by real-time RT-PCR. The CD44 mRNA and CEA mRNA levels in the peritoneal washes showed a significant correlation with tumor size and stage. In patients with stage III, peritoneal recurrence-free survival rates (PRFS) and overall survival rates (OS) of CD44 $mRNA^+$ or CEA $mRNA^+$ patients were significantly worse than those of marker gene-negative patients. In stage II patients, PRFS and OS of CD44 $mRNA^+$ patients were significantly worse than those of marker gene-negative patients. In the Cox regression hazard model analysis, the presence of CD44 mRNA in peritoneal washes was found to be an independent prognostic factor for PRFS and OS in stage II and III. In contrast, CEA mRNA levels of these samples showed a prognostic value only in stage III. Our results suggest that CD44 mRNA of magnetically separated $CD45^-EpCAM^+$ cell fraction of peritoneal washes is a useful genetic marker for predicting high-risk individuals among gastric cancer patients with stage II and III.

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Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction; CEA, carcinoembryonic antigen; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase

Key words: gastric cancer, peritoneal recurrence, cancer stem cell, free cancer cells, real-time reverse transcriptase-polymerase chain reaction, peritoneal washing, CD44, carcinoembryonic antigen

Introduction

Gastric cancer still remains the most common cancer in Japan (1). Peritoneal recurrence is the most frequent event in gastric cancer patients, and it is known that the prognosis of these patients is poor (2,3). Peritoneal recurrence presumably arises from the intraperitoneal seeding of cancer cells, and the presence of free cancer cells in the peritoneal fluid is an early event in peritoneal seeding (4-6). In gastric cancer, positive intraperitoneal washes cytology (cy^+) is regarded as distant metastasis (M1), and cy^+ patients are classified as stage IV in the International Union Against Cancer (UICC)-TNM and the Japanese Classification of Gastric Carcinoma (JCGC) (7,8). However, peritoneal recurrence sometimes occurs in TNM stage II and III patients because of the low sensitivity of conventional pathological cytology techniques (9). Therefore, more highly sensitive methods based on the reverse transcriptase-polymerase chain reaction (RT-PCR) are of interest. The clinical significance of detecting free cancer cells using genetic markers such as tumor-associated antigens [carcinoembryonic antigen (CEA)] or epithelial cell antigens [cytokeratin 20 (CK20)] (10-13) has been reported. However, only few reports have been published concerning the significance of these genetic markers in each staging of gastric cancer patients.

Recently, the new concept of cancer stem cells has focused on trying to devise novel diagnostic and therapeutic procedures (14). Cancer stem cells have been defined as a unique subpopulation in tumors that possess the ability to initiate tumor growth and sustain tumor self-renewal (15-17). Accumulating evidence shows that cancer stem cells are associated with metastasis, resistance to chemotherapy and radiotherapy, and recurrence. Regarding the marker expression of cancer stem cells, some cancer specific expression patterns exist. Although there are some discrepancies, most

cancer stem cells express $CD44^+CD24^-$/lowESA^+ in breast cancer, $CD133^+$ and $EpCAM^{high}CD44^+$ ($CD166^+$) in colon cancer, $CD133^+$ in brain cancer, and $CD44^+CD24^+ESA^+$ or $CD133^+$ in pancreatic cancer (18-22). Recently, Takaishi *et al* defined the existence of gastric cancer initiating cells in the $CD44^+$ population of gastric cancer cell lines (23). Other publications have suggested that CD44 may be more specific markers for stem cells (24). However, it is known that CD44 is expressed not only in cancer stem cells but also in a variety of cells include the blood cells. Therefore, for the detection of CD44 originated in cancer stem cells in peritoneal washes, deletion of $CD44^+$ blood cells from peritoneal washes is essential.

In this study, we evaluated the usefulness of CD44 mRNA in peritoneal washes for predicting peritoneal recurrence and prognosis in gastric cancer with TNM stage II and III. Especially, we separated the CD45-negative and EpCAM-positive cell fractions ($CD45^-EpCAM^+$) from peritoneal washes by the Auto-MACS in order to detect the CD44 mRNA originated in cancer cells.

Patients and methods

Subjects and study design. A total of 147 gastric cancer patients with stage II (n=75) or III (n=72) were studied. The patients were patological cytology negative. This study was a retrospective analysis of clinical outcome coupled with analysis of stored cells separated from peritoneal washings. The samples of peritoneal washes were collected from 2000 to 2006. Conventional pathological cytology of peritoneal washes was negative in all patients. Tumors were staged according to the TNM classification and the categories were determined from pathological findings based on surgically resected specimens (7,8). The median follow-up period was 37 months (range, 7-68 months). This study was approved by the institutional review board of Teikyo University Hospital, and all patients provided written informed consent.

Peritoneal washes and magnetic cell separation. Peritoneal washes collected from gastric cancer patients and 20 patients with benign disease (gallstone patients) were prepared. At the beginning of each operation, 100 ml saline was introduced into the Douglas pouch and aspirated after gentle stirring. One half of each wash was used for cytopathology and the other half was used for the genetic examination. For the genetic examination, cancer-enriched cells were separated from peritoneal washes by two-steps Auto-MACS system. First, blood cells were removed by negative selection using the anti-CD45 mouse mAb-conjugated microbeads (CD45 microbeads), and then cancer cells were enriched by positive selection using the anti-EpCAM mouse MAb-conjugated microbeads (EpCAM microbeads) described previously (25,26). Finally, ($CD45^-EpCAM^+$) were collected and stored at -80˚C for RNA extraction procedure.

Quantitative real-time RT-PCR. Total RNA of samples was extracted using a guanidinium-isothiocyanate-phenol-chloroform-based method using TRIzol (Invitrogen, Carlsbad, CA). One-step real-time quantitative RT-PCR for CD44, CEA and GAPDH mRNA was performed using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany). The sequences of the primers and probes for CEA and GAPDH, and the PCR conditions have been described previously (12). The primers and probe for CD44 mRNA were as follows: sense, 5'-TCCAGGCAACTCCTAGTAGTA-3'; antisense, 5'-CTGTCCCTGTTGTCGAAT-3'; and probes, 5'-AAACAG CTACCCGAGAGGAACAGTGGTTTGG-3'-fluorescein (donor) and 5'-LCRed640-AACAGATGGCATGAGGGATA TCGCCAAACAC-3'-phosphorylated (acceptor). For amplification of CD44, initial denaturation at 95˚C for 10 min was followed by 20 sec at 95˚C, 20 sec at 60˚C and 20 sec at 72˚C. All samples were measured in duplicate. As an external standard, the PCR product of each target gene was cloned into a TOPO TA cloning plasmid vector (Invitrogen). The mRNA in each sample was quantified automatically with reference to the standard curve of the plasmid, using LightCycler software. The levels of CEA and CD44 mRNA were normalized to GAPDH, and the ratios of the CEA and CD44 copy numbers to the GAPDH copy numbers (CEA/GAPDH and CD44/GAPDH) were calculated. The cut-off values for CEA/GAPDH and CD44/GAPDH were determined as the 95% confidence intervals (mean plus 1.96 standard deviation) of the peritoneal washes from 20 patients with benign disease.

Postoperative surveillance. The follow-up program, which consisted of interim history, physical examination, hematology and blood chemistry, was performed every 3 months for the first operative year and every 6 months thereafter. Computed tomography or abdominal ultrasonography was examined every 6 months. Evidence of peritoneal recurrence was diagnosed comprehensively using various methods, including paracentesis and autopsy.

Statistical analysis. The sample size of this study was determined by use of SAS v9.2 software (SAS Institute, Inc., Tokyo). Peritoneal recurrence-fee survival (PRFS) was analyzed using the Kaplan-Meier method, with death and a clinical diagnosis of peritoneal recurrence as end-points. Overall survival (OS) was analyzed with death as end-points. P-values are estimated by log-rank tests. Univariate and multivariate analysis were performed using Cox regression hazard model analysis. $P<0.05$ was considered significant. Data were analyzed using JMP 7.0 software (SAS Institute, Inc.).

Results

Cut-off levels for CEA and CD44 mRNA. To determine the cut-off values for CEA/GAPDH and CD44/GAPDH, we examined the peritoneal washes of benign disease patients. As cut-off values, we determined 0.14 for CEA/GAPDH and 0.76 for CD44/GAPDH to be the 95% confidence interval (mean plus 1.96 standard deviation) (data not shown).

Positive rates and the sensitivity and specificity of genetic markers. The PCR positive rates of CEA mRNA, CD44 mRNA were examined in all patients (n=147), and the results are shown in Table I. The positive rates of CEA (33.33%) were higher than that of CD44 (27.89%). The peritoneal recurrence rates of patients with stage II or III were 12% (9/75) and 27.78% (20/72), respectively. Next, we examined the sensi-

Table I. Comparison of positive rates and the sensitivities and specificities of CD44 and CEA mRNA for peritoneal recurrence.

Genetic markers	Positive rate (%)	Sensitivity (%)	Specificity (%)
CD44$^+$	27.89 (41/147)	51.72 (15/29)	77.97 (92/118)
CEA$^+$	33.33 (49/147)	55.17 (16/29)	72.03 (85/118)

The positive rates of each genetic marker, and the sensitivities and specificities for peritoneal recurrence were examined in CEA$^+$ and CD44$^+$ groups.

Table II. Relationships between clinicopathological factors and CD44 mRNA positivity rates.

Variables	No. of patients (n=147)	CD44 positive cases (n=41)	Positivity rates (%)	P-value
Age (years)	66±11			
Gender				
Male	97	28	28.87	0.714
Female	50	13	26.00	
Tumor size (cm)				
<5	35	5	14.29	0.039[a]
≥5	112	36	32.14	
Histological type				
G1	17	5	29.41	1.000
>G2	130	36	27.69	
Depth of invasion				
pT1	7	0	0.00	0.191
>pT2	140	41	29.29	
Lymphatic invasion				
L0	30	6	20.00	0.361
L1	117	35	29.91	
Venous invasion				
V0	34	11	32.35	0.519
V1	113	30	26.55	
Lymph node metastasis				
pN0	7	0	0.00	0.191
>pN1	140	41	29.29	
TNM stage				
II	75	10	13.33	<0.001[a]
III	72	31	43.06	

Table III. Relationships between clinicopathological factors and CEA mRNA positivity rates.

Variables	No. of patients (n=147)	CEA positive cases (n=49)	Positivity rates (%)	P-value
Age (years)	66±11			
Gender				
Male	97	36	37.11	0.178
Female	50	13	26.00	
Tumor size (cm)				
<5	35	6	17.14	0.024[a]
≥5	112	43	38.39	
Histological type				
G1	17	7	41.18	0.585
>G2	130	42	32.31	
Depth of invasion				
pT1	7	1	14.29	0.425
>pT2	140	48	34.29	
Lymphatic invasion				
L0	30	7	23.33	0.278
L1	117	42	35.90	
Venous invasion				
V0	34	12	35.29	0.837
V1	113	37	32.74	
Lymph node metastasis				
pN0	7	1	14.29	0.425
>pN1	140	48	34.29	
TNM stage				
II	75	19	25.33	0.036[a]
III	72	30	41.67	

tivity and specificity for peritoneal recurrence of CD44 and CEA (Table I). The CD44 group showed a higher specificity (77.97%) and lower sensitivity (51.72%) than those of CEA group (specificity 72.03%, sensitivity 55.17%).

Relationships between clinicopathological factors and genetic markers. Tables II and III show the relationships between the CD44 mRNA or CEA mRNA expression (in CD45$^-$EpCAM$^+$ cells fractions) in peritoneal washes and clinicopathological factors. Significant relationships were demonstrated between positivity for CD44 expression and tumor size and tumor stage (Table II). The positivity for CD44 mRNA did not show any significant relationships with histological type, depth of invasion, lymphatic invasion, venous invasion and lymph

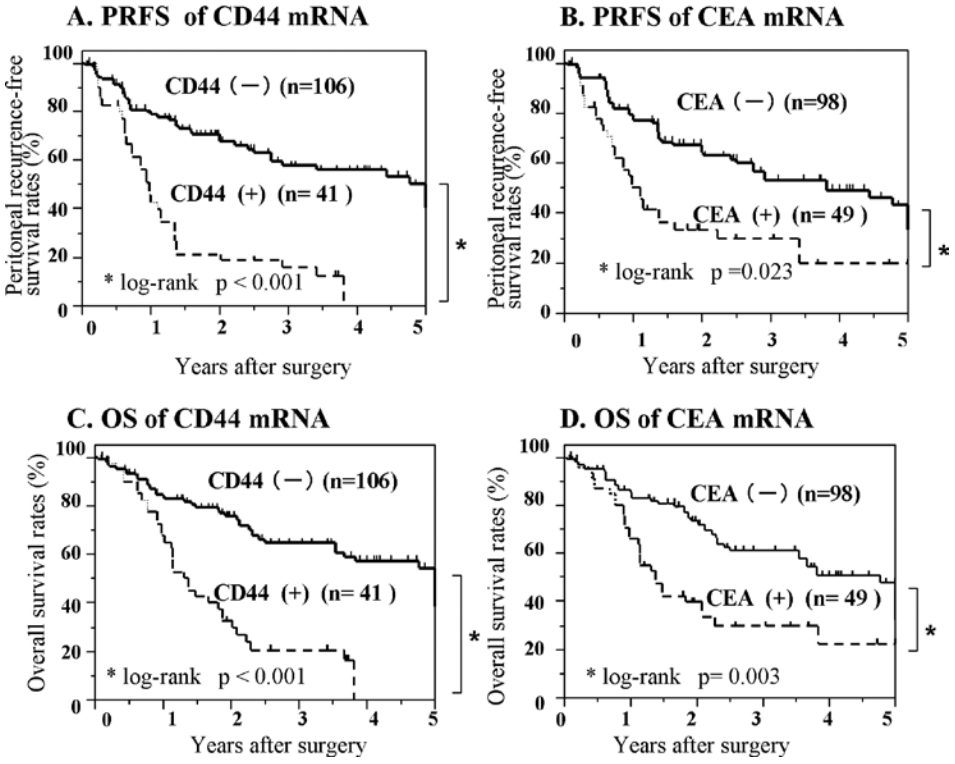

Figure 1. PRFS and OS stratified according to the results of CD44 or CEA in all patients. PRFS and OS for the patients with stage II and III (n=147) were classified based on CD44 mRNA (A, C) and CEA mRNA (B, D) and analyzed by the Kaplan-Meier method. Significant differences exist between the CD44-positive and negative groups, and CEA-positive and negative groups.

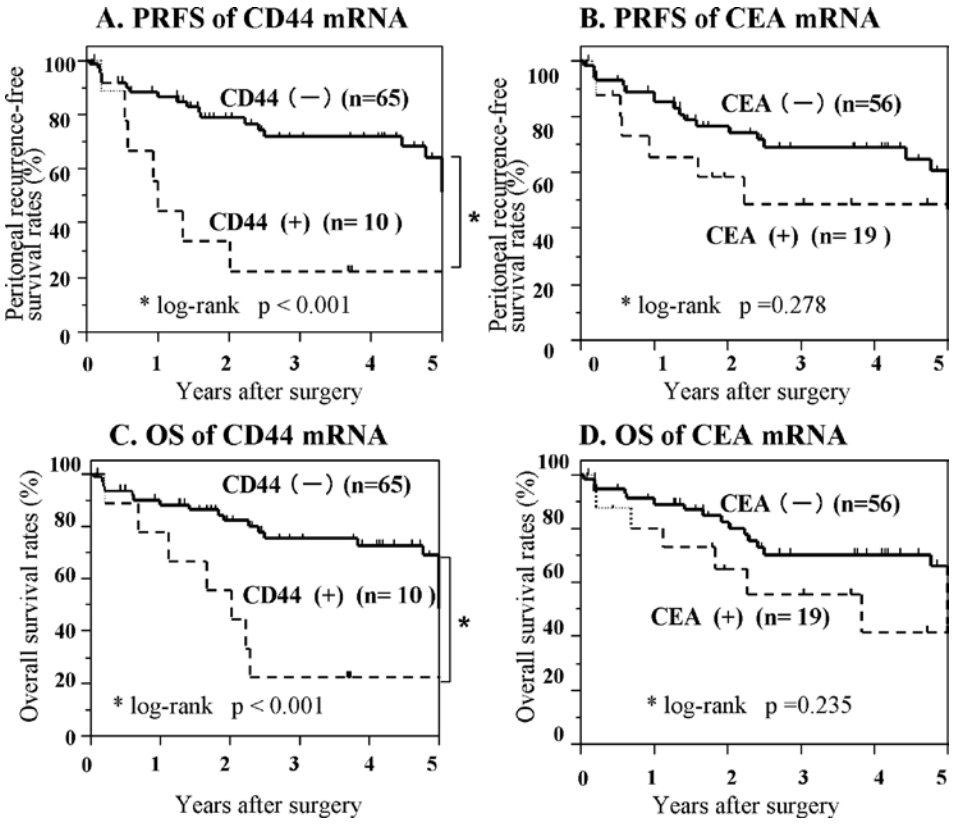

Figure 2. PRFS and OS stratified according to the results of CD44 or CEA in stage II patients. PRFS and OS of the stage II patients (n=75) were classified based on CD44 mRNA (A, C) and CEA mRNA (B, D) and analyzed by the Kaplan-Meier method. Significant differences exist between the CD44 mRNA-positive and negative groups.

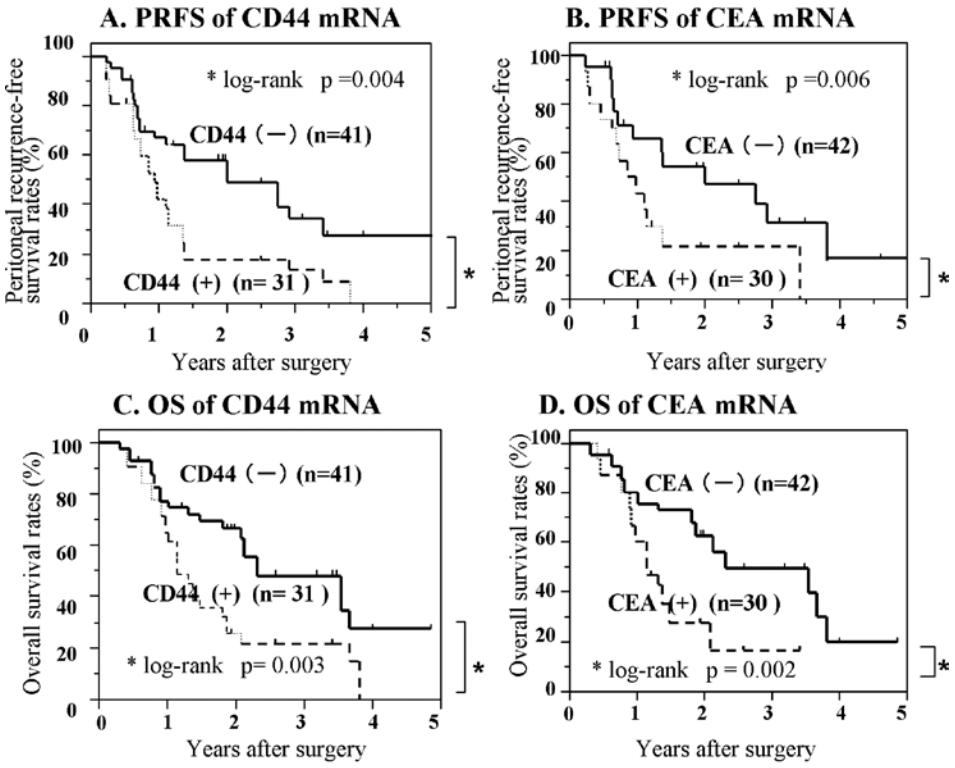

Figure 3. PRFS and OS stratified according to the results of CD44 or CEA in stage III patients. PRFS and OS for the patients with stage III (n=72) were classified based on CD44 mRNA (A, C) and CEA mRNA (B, D) and analyzed by the Kaplan-Meier method. Significant differences exist between the CD44 mRNA-positive and negative groups, and CEA mRNA-positive and negative groups.

Table IV. Multivariate analysis of prognostic factors for PRFS and OS in all patients.

	Peritoneal recurrence-free survival			Overall survival		
	RC[a]	HR[b]	P-value	RC	HR	P-value
CD44 mRNA	-0.91	0.40 (0.24-0.66)	0.001[c]	-9.45	0.39 (0.23-0.65)	0.001[c]
Tumor size	0.28	1.33 (0.73-2.53)	0.359	0.27	1.31 (0.72-2.52)	0.386
Lymph node metastasis	1.65	5.19 (1.08-93.11)	0.037[c]	1.61	5.02 (1.05-90.08)	0.042[c]
TNM stage	0.69	2.00 (1.17-3.52)	0.011[c]	0.67	1.95 (1.14-3.44)	0.015[c]
CEA mRNA	-0.57	0.57 (0.35-0.92)	0.022[c]	-0.65	0.52 (0.32-0.86)	0.011[c]
Tumor size	0.17	1.19 (0.66-1.56)	0.465	0.15	1.17 (0.66-1.51)	0.495
Lymph node metastasis	1.65	5.22 (1.09-93.67)	0.037[c]	1.60	4.97 (1.04-89.18)	0.044[c]
TNM stage	0.88	2.42 (1.44-4.20)	0.001[c]	0.86	2.37 (1.40-4.13)	0.001[c]

[a]RC, regression coefficient. [b]HR, Hazard ratio (95% confidence interval).

node metastasis. Significant relationships were demonstrated between positivity for CEA mRNA expression and tumor size and tumor stage (Table III). The positivity for CEA mRNA did not show any significant relationships with histological type, depth of invasion, lymphatic invasion, venous invasion and lymph node metastasis.

Correlation between genetic markers and survival. Kaplan-Meier peritoneal recurrence-free survival (PRFS) curves and overall survival (OS) curves were compared according to the expression status of CD44 mRNA or CEA mRNA in all patients (Fig. 1). In this analysis, the $CD44^+$ group showed significantly worse PRFS and OS than those of the $CD44^-$ group ($P<0.001$ in PRFS and OS). The CEA^+ group also showed significantly worse PRFS and OS than those of the CEA^- group ($P=0.023$ in PRFS, $P=0.003$ in OS). Next, we examined the PRFS and OS at each stage according to the PCR status (Figs. 2 and 3). In stage II, the PRFS and OS of the $CD44^+$ groups were significantly worse than those of $CD44^-$ groups ($P<0.001$ in PRFS and OS) (Fig. 2). In contrast,

Table V. Multivariate analysis of prognostic factors for PRFS and OS in stage II patients.

	Peritoneal recurrence-free survival			Overall survival		
	RC[a]	HR[b]	P-value	RC	HR	P-value
CD44mRNA	-1.37	0.25 (0.11-0.67)	0.008[c]	-1.36	0.26 (0.10-0.69)	0.009[c]
Lymph node metastasis	1.37	3.93 (0.80-71.01)	0.102	1.39	4.01 (0.82-72.16)	0.095
CEA mRNA	-0.33	0.72 (0.31-1.84)	0.464	-0.37	0.69 (0.30-1.77)	0.416
Lymph node metastasis	1.46	4.29 (0.88-77.53)	0.077	1.47	4.35 (0.89-78.25	0.073

[a]RC, regression coefficient. [b]HR, Hazard ratio (95% confidence interval).

Table VI. Multivariate analysis of prognostic factors for PRFS and OS in stage III patients.

	Peritoneal recurrence-free survival			Overall survival		
	RC[a]	HR[b]	P-value	RC	HR	P-value
CD44 mRNA	-0.74	0.48 (0.27-0.85)	0.011[c]	-0.77	0.46 (0.26-0.82)	0.009[c]
Histological type	-1.02	0.36 (0.14-1.25)	0.099	-1.12	0.33 (0.12-1.13)	0.074
CEA mRNA	-0.73	0.48 (0.27-0.87)	0.017[c]	-0.90	0.41 (0.21-0.77)	0.006[c]
Histological type	-1.02	0.38 (0.12-1.15)	0.107	-1.08	0.34 (0.13-1.18)	0.084

[a]RC, regression coefficient. [b]HR, Hazard ratio (95% confidence interval).

CEA^+ groups did not show the significant differences in PRFS and OS between the CEA^- groups (P=0.278 in PRFS, P=0.235 in OS). In stage III, the PRFS and PS of $CD44^+$ group were significantly worse than those of marker-negative group (P=0.004 in PRFS, P=0.003 in OS). Furthermore, PRFS and OS of CEA^+ groups were significantly worse than those of CEA^- groups (P=0.006 in PRFS, P=0.002 in OS) (Fig. 3). These results suggest that the CD44 mRNA in peritoneal washes significantly associated with PRFS and OS in stage II and III patients, and CEA mRNA are associated with PRFS and OS in stage III patients.

Cox univariate and multivariate analysis of prognostic factors. First, we analyzed the prognostic value of CD44 and CEA mRNA for all patients. Table IV shows the multivariate Cox proportional hazard regression analysis for PRFS and OS in all patients. Multivariate analyses were evaluated in factors which showed significance in univariate analyses. CD44 and CEA were analyzed in separate group. In these analyses, CD44, lymph node metastasis and tumor stage showed significance for PRFS and OS. Next, we evaluated the prognostic value of these factors in each tumor stage. In analysis of patients with stage II, CD44 mRNA showed significance for PRFS and OS (P=0.008 in PRFS, P=0.009 in OS) (Table V). In contrast, CEA mRNA did not show the significance for PRFS and OS. In analysis of patients with stage III, CD44 mRNA showed significance for PRFS and OS (P=0.011 in PRFS, P=0.009 in OS) (Table VI). CEA mRNA also showed significance for PRFS and OS (P=0.017 in PRFS, P=0.006 in OS). These results suggest that the CD44 mRNA in peritoneal washes possesses independent prognostic value for PRFS and OS in stage II and III, and CEA mRNA possesses prognostic value for PRFS and OS in stage III patients.

Discussion

This study demonstrated that the CD44 mRNA levels of $CD45^-EpCAM^+$ cell fractions in peritoneal washes of gastric cancer patients with stage II and III are an independent prognostic factor for PRFS and OS.

Cancer stem cells have been characterized as a subpopulation in cancers that possess the ability to initiate tumor growth and sustain tumor self-renewal (14-17). Accumulating evidence suggests the existence of cancer stem cells in various solid tumors, and the detection of cancer stem cells is therefore important in the accurate diagnosis and effective treatment of cancer patients (18-22). Previously, we reported that the molecular detection of free cancer cells in peritoneal washes of curative colorectal cancer patients, using multimarkers including CD133 which is cancer stem-like cell marker, are useful in prognosis (23). However, the clinical significance of cancer stem-like cells in peritoneal washes of gastric cancer has not been published. Takaishi *et al* reported that $CD44^+$ gastric cancer cells showed the properties of self-renewal and the ability to form differentiated progeny, consistent with the cancer stem cell phenotype (23). CD44 is

a class I transmembrane glycoprotein, and its transcripts are subject to alternative splicing, which creates more than 10 different isoforms (24). It can act as a ligand-binding receptor for extracellular matrices and as a specialized platform for growth factors and matrix metalloproteinases, and it has been shown to be a down-regulation target of the Wnt/β catenin pathway (27). Several recent studies have suggested that CD44 may play an important role in the tumorigenicity of cancer stem cells. It was also reported that CD44 may be more specific marker for cancer stem cells as compared to other cancer stem cell markers (24). However, it is also known that CD44 is expressed not only in cancer stem cells but also in a variety of other cells, such as blood cells. Therefore, we first separated $CD45^{-}EpCAM^{+}$ cells from peritoneal washes using the Auto-MACS system, and then measured the CD44 mRNA and CEA mRNA levels. Cancer cell enrichment using the EpCAM microbeads has been reported in various cancers and applied in the CellSearch system which has been approved by FDA (28,29).

In previous studies, general markers such as CEA and CK20 mRNA were used as target genes for peritoneal free cancer cells in gastric cancer. In this study, we subjected stage II and III patients with peritoneal cytology negative. In our analysis, CD44 mRNA levels showed significant relationships between the tumor size and stage, and these results are same in those of CEA. Previous studies which include the stage I-IV gastric cancer patients, had demonstrated that the significant correlation with CEA mRNA levels and tumor size, depth of invasion, lymph node metastasis, lymphatic invasion, venous invasion, peritoneal dissemination and stage (13). Concerning the prognostic value of detecting free cancer cells in peritoneal washes using the CEA mRNA, several papers have reported on the usefulness of CEA mRNA of peritoneal washes as a sensitive predictive tool for intraperitoneal recurrence in gastric cancer patients (10,11,30,31). In these published studies, predictive values for peritoneal recurrence of CEA mRNA in each stage were not examined. However, the selection of high-risk patients with stage II and III is desired in order to select those who are in need of adjuvant chemotherapy. In this study, our data showed that CEA mRNA levels of peritoneal washes are predictive marker for peritoneal recurrence and prognosis in stage III patients but not stage II. In contrast, the CD44 mRNA levels of peritoneal washes are of predictive value for peritoneal recurrence and prognosis in both stage II and III patients. These results were apparent in both the Kaplan-Meier survival analysis and the Cox proportional hazard mode analysis. To the best of our knowledge, this is the first study to have demonstrated the predictive values for peritoneal recurrence and poor prognosis of CD44 mRNA in peritoneal washes in gastric cancer patients with stage II. As to the reason for the different properties of free cancer cells in stage II, we speculate that $CD44^{+}$ cells may be more aggressive than CEA^{+} cells and a small number of CD44 cells may be associated with the peritoneal recurrence in patients with stage II. However, further study is necessary in order to investigate this in detail.

In conclusion, our results demonstrated that CD44 mRNA, which is known as a cancer stem-like cell marker, may be a useful biomarker for the selection of high-risk patients in stage II gastric cancer patients who are in need of adjuvant chemotherapy. A further large-scale study is needed in order to validate our findings.

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