Usefulness and clinical significance of quantitative real-time RT-PCR to detect isolated tumor cells in the peripheral blood and tumor drainage blood of patients with colorectal cancer

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Abstract. The clinical significance of isolated tumor cells (ITC) circulating in the blood of patients with colorectal cancer is unclear. In this study, we investigated the relationship between the presence of ITC that express carcinoembryonic antigen (CEA) and/or cytokeratin 20 (CK20) transcripts in the blood and the clinicopathological findings and prognosis using the quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) assay. We studied peripheral blood and tumor drainage blood from 167 patients with colorectal cancer. Quantitative real-time RT-PCR assay was able to detect one tumor cell in $3x10^6$ peripheral blood mononuclear cells. Applying a cut-off value, CEA and/or CK20 (CEA/CK20) were detected in 10.2% (17/167) of the patients' preoperative peripheral blood samples and 34.1% (57/167) of the patients' tumor drainage blood samples. In the relationship between the CEA/CK20 of the blood and the clinicopathological factors, a significant correlation was demonstrated between the positivity of marker genes and the depth of invasion, venous invasion, lymph node metastasis, liver metastasis or stage. The disease-free and overall survival of patients with CEA/CK20-positive peripheral or tumor drainage blood was significantly shorter than that of marker gene-negative patients. CEA/CK20 transcripts in tumor drainage blood were independent factors for prognosis in disease-free survival and overall survival. These results suggest that detecting CEA/CK20 mRNA in tumor drainage blood by real-time RT-PCR has prognostic value in patients with colorectal cancer. Large scale and long-term clinical studies are needed to confirm the prognostic value of genetically detecting ITC in the peripheral blood.

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

Key words: colorectal cancer, isolated tumor cells, real-time reverse transcriptase-polymerase chain reaction, CEA, CK20, liver metastasis, tumor drainage blood

Introduction

Colorectal carcinoma is one of the world's most common malignancies. Despite improved therapeutic modalities, approximately 20-45% of patients with colorectal cancer undergoing curative resection subsequently develop local tumor recurrences or metastasis in the lymph nodes, liver, lung and peritoneum (1,2). Liver metastasis is one of the most common and serious problems influencing prognosis (3,4). Detecting liver metastasis early and identifying the patients at risk of recurrence are essential to managing colorectal cancer.

Advances in molecular technology have made it possible to detect even a few tumor cells in the blood, and several authors have described the usefulness of the reverse transcription-polymerase chain reaction (RT-PCR)-based method in increasing the sensitivity to detection (5,6). Using the conventional RT-PCR method, isolated tumor cells (ITC) have been detected in the peripheral blood, mesenteric venous blood drained from tumors, bone marrow and peritoneal washings of patients with colorectal cancer (7-13). Although the conventional nested-primer RT-PCR method is highly sensitive, determining a cut-off level is difficult because of insufficient quantification. However, the recent development of a quantitative real-time RT-PCR system is a breakthrough in the molecular detection of tumor cells (14-19). 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. The quantitative RT-PCR system is used in several clinical fields. For example, it is used to quantify the hepatitis virus

Table I. Sequence of primers and probes.

Target mRNA		Primer/hybridization probe sequence[a] 5'-3'	Amplicon (bp)
CEA			
Primer	Sense	GAC GCA AGA GCC TAT GTA TG	270
	Anti-sense	GGC ATA GGT CCC GTT ATT A	
Probe	Donor	CCC AGA CTC GTC TTA CCT TTC GG-FL	
	Acceptor	LC-AGC GAA CCT CAA CCT CTC CTG C-P	
CK20			
Primer	Sense	GAA GTC GAT GGC CTA CGA AA	331
	Anti-sense	AAC GGG CCT TGG TCT CCT CTA	
Probe	Donor	CTT TGG CCT CTT GAA GGT TCT TCT G-FL	
	Acceptor	LG-GCC ATG ACT TCA TAC TTC TGC CTC A-P	
GAPDH			
Primer	Sense	TGA ACG GGA AGC TCA CTG G	307
	Anti-sense	TCC ACC ACC CTG TTG CTG TA	
Probe	Donor	GAG TGG GTG TCG CTG TTG AAG TCA-FL	
	Acceptor	LC-AGG AGA CCA CCT GGT GCT CAG TGT A-P	

[a]FL, fluorescein; LC, LCRed 640; P, phosphorylated.

and monitor leukemia treatment (20,21). In colorectal cancer, several studies have examined the quantitative detection of ITC in the peripheral blood (17-19). However, the relationship between the presence of ITC in the peripheral blood and prognosis is controversial, and little is known about the clinical significance of ITC in tumor drainage blood.

In this study, we focused on investigating the relationship between ITC in the preoperative peripheral blood and tumor drainage blood and clinicopathological features and prognosis to identify the clinical significance of the ITC in the blood of colorectal cancer patients.

Patients and methods

Cell lines. Colorectal cancer HT-29 cells expressing CK20 (American Type Culture Collection, Rockville, MD) and gastric cancer MKN45 cells expressing CEA (IBL, Fujioka, Japan) were cultured in RPMI-medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum (Gibco BRL, Gaithersburg, MD), 100 units/ml penicillin and 100 μg/ml streptomycin. These cells were used for the sensitivity test.

Patients. A total of 167 patients (92 male and 75 female) with colorectal cancer undergoing surgery at three hospitals (Kyushu University, 30 cases; Kumamoto University, 35 cases; and Teikyo University, 102 cases) between 2000 and 2002 were enrolled in this study. The observation period ranged from 22 to 60 months, and the median follow-up period was 30 months. The study protocol conformed to the guidelines of the ethics committees of each university. Written informed consent was obtained from all patients. Their ages ranged from 27-82 years, with a mean age of 67 years. The stages of the tumors were determined according to the Dukes classification system. As a follow-up, all patients were reevaluated at 3-month intervals during the first year, and then at 18 and 24 months and yearly thereafter. Each evaluation consisted of a pertinent medical history, physical examination and repetition of imaging studies, including a CT scan of the abdomen.

Blood sampling and cDNA preparation. As controls, peripheral blood samples collected from 25 healthy volunteers who had no evidence of any disease, and peripheral blood and portal system blood samples collected from 10 patients with benign diseases were prepared. In colorectal cancer patients, blood samples were obtained from the peripheral blood before operation and from the mesenteric vein draining the tumor immediately after laparotomy. These blood samples were collected in PAXgene tubes, stored at -80˚C, and transferred to Teikyo University for real-time RT-PCR assay. Total RNA of the blood samples in PAXgene tubes was extracted using a PAXgene blood RNA kit (Qiagen K.K. GmbH, Germany). Extracted total RNA was reverse transcribed into cDNA using oligo-p$(dT)_{12\text{-}18}$ primers according to the manufacturer's protocol (Invitrogen Corp., CA, USA).

Quantitative real-time RT-PCR. The primer and probe sequences of CEA, CK20 and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), which were utilized as an internal control, are described in Table I. Real-time quantitative RT-PCR of CEA, CK20 and GAPDH transcripts was performed using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany). Five μl of each cDNA was diluted with 15 μl of

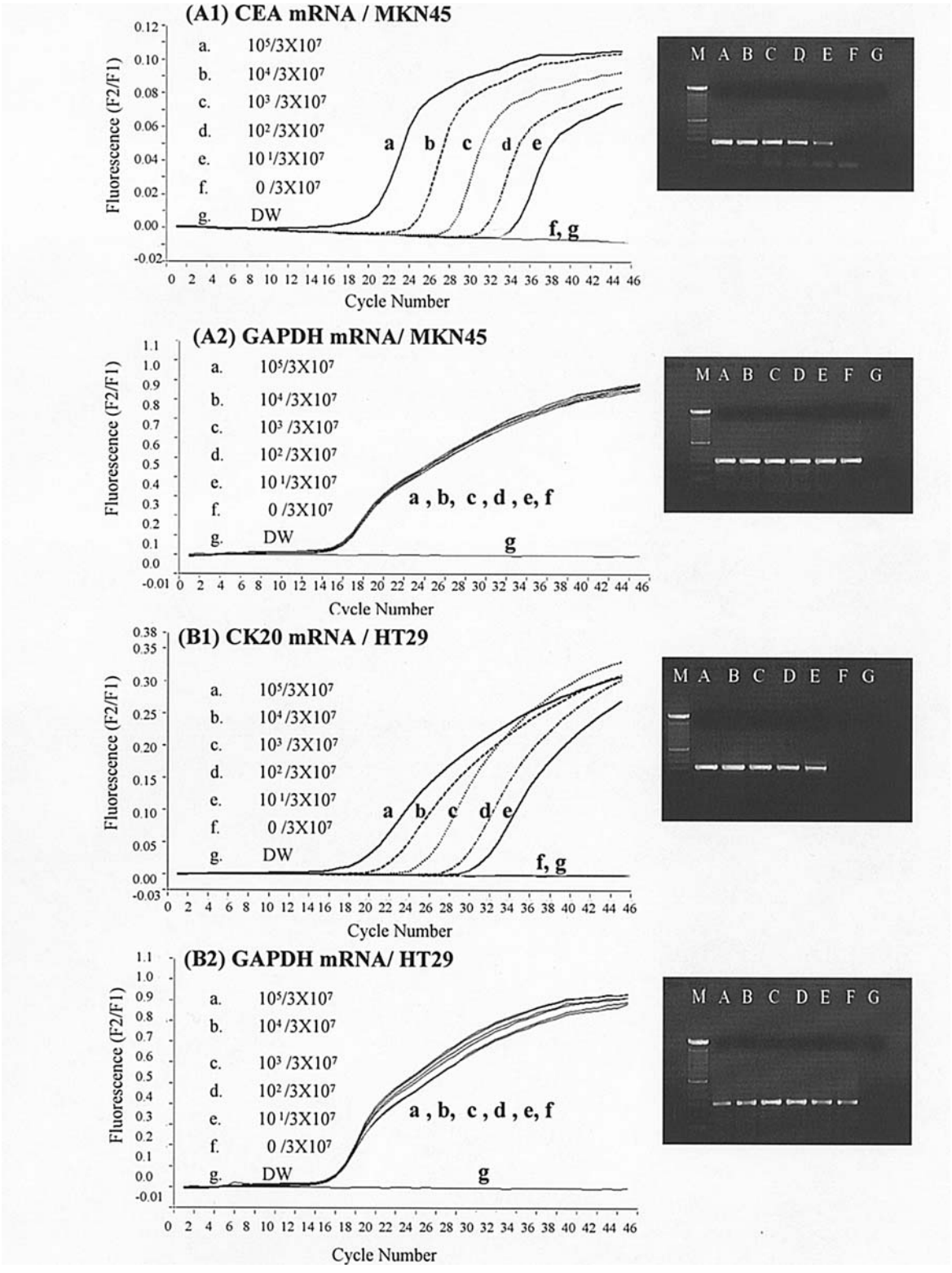

Figure 1. Sensitivity of real-time RT-PCR. To examine the sensitivity of real-time RT-PCR, serial dilutions of CEA-positive MKN45 cells or CK20-positive HT29 ranging from 10^1 to 10^5 were mixed with $3x10^7$ peripheral blood mononuclear cells obtained from healthy volunteers. CEA (A1), CK20 (B1) and GAPDH (A2 and B2) mRNA were measured by real-time RT-PCR. The profile of fluorescence vs. PCR and the results of electrophoresis in a 1.5% agarose gel are shown.

PCR master mix (Lightcycler Faststart DNA Master Hybridization Probes, Roche Diagnostics) containing 0.5 μM of each primer, 0.2 μM fluorescein probe, 0.4 μM LCRed probe and 3 mM $MgCl_2$. For amplification, an initial denaturation at 95˚C for 10 min was followed by 15 sec at 95˚C, 15 sec at 60˚C and 13 sec at 72˚C for GAPDH; 15 sec at 95˚C, 15 sec at 56˚C and 11 sec at 72˚C for CEA; and 15 sec at 95˚C, 15 sec at 56˚C and 14 sec at 72˚C for CK20; for 45 cycles. All samples were measured in duplicate. For external standards for real-time RT-PCR, the PCR product of CEA, CK20 or GAPDH was

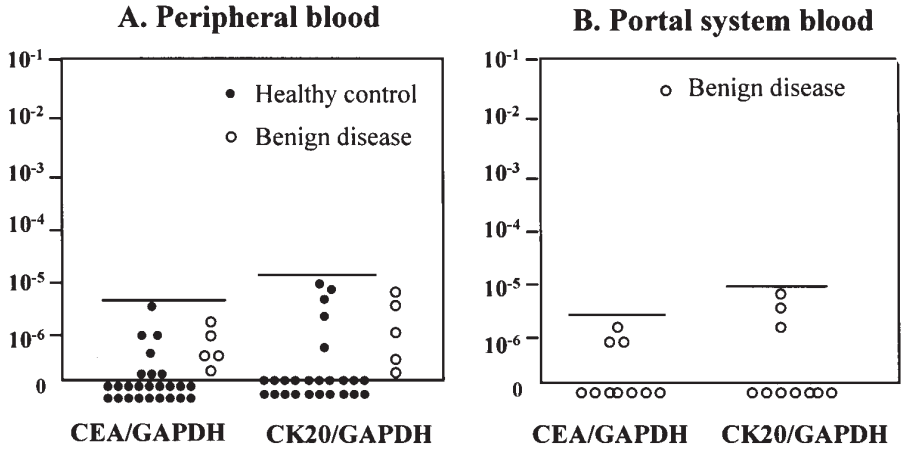

Figure 2. The ratios of CEA:GAPDH and CK20:GAPDH in the peripheral blood and portal system blood of healthy controls and benign disease. To determine the cut-off levels of the peripheral blood, the ratios of CEA:GAPDH and CK20:GAPDH in the peripheral blood of healthy volunteers and patients with benign disease were examined (A). To determine the cut-off levels of tumor drainage blood, the portal system blood of patients with benign disease was examined (B). Each bar shows the cut-off levels for CEA:GAPDH and CK20:GAPDH.

cloned into a separate TOPOTA cloning vector (Invitrogen). The number of duplicate plasmids was estimated by spectrophotometry with the assumption that 1 mol represents $6x10^{23}$ molecules. Dilutions of each plasmid containing 10^1, 10^2, 10^3, 10^4 and 10^5 copies were used as PCR templates to construct a standard curve in each respective PCR run. Quantification of the mRNA in each sample was performed automatically with reference to the standard curve of the plasmid constructed each time, according to the LightCycler software. The expression levels of CEA and CK20 were normalized by GAPDH, and the ratio of CEA or CK20 copies to GAPDH copies was calculated.

Sensitivity tests of real-time RT-PCR. Sequentially diluted CEA-positive MKN45 cells or CK20-positive HT-29 cells (from 10 to 10^5) were mixed with $3x10^7$ peripheral blood mononuclear cells (PBMC) obtained from healthy volunteers. The elution of RNA, synthesis of cDNA and real-time RT-PCR for CEA, CK20 and GAPDH were performed, as described above. The molecular weights of the PCR products were determined by electrophoresis on 1.5% agarose gels containing ethidium bromide. These experiments were performed in triplicate.

Immunocytochemistry of blood samples. Peripheral blood and tumor drainage blood samples were collected from 102 patients with colorectal cancer in Teikyo University, and the cytology of tumor cells was examined using the immunostaining method. Mononuclear cells from the samples were separated using Ficoll-Paque (Amersham Bioscience Corp.) and leucocytes were removed by negative selection with a magnetic-activated cell separation system (MACS: Miltenyi Biotec, Bergisch Gladbash, Germany) using anti-CD45 mouse monoclonal antibody (Mab)-conjugated microbeads (22). Enriched tumor cells were fixed with a CytoRich Red solution (Medical & Biological Laboratories: MBL, Co., LTD, Nagoya, Japan) and preserved. Then, thin-layer samples were prepared on glass slides using the chamber system (MBL), and stained with the ABC method using an anti-human CEA Mab (Nichirei Corp., Tokyo, Japan) and anti-human cytokeratin Mab (AE1+AE3: Dako Japan Co., Ltd., Tokyo, Japan).

Statistical analysis. The correlation between the presence of CEA and CK20 mRNA in the blood and the various clinical parameters was evaluated using the χ^2 test. Disease-free survival and overall survival were analyzed using the Kaplan-Meier method, and the differences were examined using the log-rank test. Univariate and multivariate analysis were performed using Cox regression analysis. $P<0.05$ was considered statistically significant.

Results

Quantitative range and sensitivity of real-time RT-PCR assay. Real-time RT-PCR monitoring with the LightCycler using hybridization probes allowed the rapid detection of CEA and CK20 mRNA in the patients' samples. With the use of hybridization probes, amplification for 45 cycles and subsequent data analysis were completed within 45 min without the post-amplification procedures, such as gel electrophoresis, which are needed in conventional RT-PCR, and a melting curve analysis using the SYBR-Green I format. Real-time RT-PCR of serially diluted plasmid standards (from 5 to 10^7 copies) provided quantitative data for all markers, and the detection of at least 5-10 copies was possible with reproducibility. The sensitivity of this assay is shown in Fig. 1. This real-time RT-PCR system was able to reliably detect at least 10 and up to 10^5 CK20-positive HT-29 cells or CEA-positive MKN45 cells mixed with $3x10^7$ PBMC from healthy volunteers. On electrophoresis of the PCR products, distinct bands of CEA, CK20 and GAPDH were recognized at this range. These results suggest that the sensitivity of this assay was approximately 1 tumor cell in $3x10^6$ PBMC.

Expression of CEA/CK20 mRNA in the blood of healthy volunteers and benign disease patients. The CEA and CK20 transcript values normalized with GAPDH mRNA levels in the blood of 25 healthy volunteers and 10 with benign diseases were examined to determine the cut-off levels (Fig. 2). Peripheral blood samples from healthy volunteer and patients with benign diseases were used for the control of peripheral blood. Portal system blood samples from patients with benign diseases

Table II. Positive rates of CEA and CK20 mRNA in blood samples.

mRNA	Positive rates (%)	
	Peripheral blood	Tumor drainage blood
CEA and/or CK20	10.2 (17/167)	34.1 (57/167)
$CEA^+/CK20^+$	4.8 (8/167)	15.0 (25/167)
$CEA^+/CK20^-$	3.6 (6/167)	11.4 (19/167)
$CEA^-/CK20^+$	1.8 (3/167)	7.8 (13/167)

Table III. Comparison of CEA/CK20 mRNA positivity in peripheral blood and tumor drainage blood.

Peripheral blood	Tumor drainage blood	Ratio of PCR positive cases (%)[a]
+	+	23.3 (14/60)
-	+	71.7 (43/60)
+	-	5.0 (3/60)

[a](No. of CEA/CK20 positive cases in peripheral blood and/or tumor drainage)/(total no. of CEA/CK20 positive cases) x 100.

were prepared for the control of tumor drainage blood. Based on the range of CEA/GAPDH and CK20/GAPDH, we determined the cut-off value by the 95% confidence intervals (mean plus 1.96 standard deviation) of the control groups. In the peripheral blood samples, cut-off ratios were $4.5x10^{-6}$ in CEA/GAPDH and $1.5x10^{-5}$ in CK20/GAPDH. In portal system blood samples, they were $4.0x10^{-6}$ in CEA/GAPDH and $1.0x10^{-5}$ in CK20/GAPDH.

Expression of CEA/CK20 mRNA in the blood of colorectal cancer patients. The positive rates of either CEA or CK20 or both (CEA and/or CK20) in the peripheral blood and tumor drainage blood samples were 10.2% (17/167) and 34.1% (57/167), respectively (Table II). The positive rate of marker genes in the tumor drainage blood was 3.3 times higher than that of the peripheral blood. In these samples, the positive rates of CEA^+ $CK20^+$ were only 4.8% (8/167) in the peripheral blood and 15.0% (25/167) in the tumor drainage blood. The positive rate of CEA was higher than that of CK20. To increase the detection rates of the marker genes, we designated CEA- and/or CK20 (CEA/CK20)-positive cases as marker gene-positive cases in the following analysis. Next, we examined the individual correlations between the results obtained in the peripheral blood and in tumor drainage blood (Table III). The ratio of patients that were PCR-positive in tumor drainage blood but not in peripheral blood was 71.7% (43/60), and 23.3% (14/60) showed PCR positivity in both peripheral and tumor drainage blood samples. In contrast, the ratio of patients that showed PCR positivity in peripheral blood samples was 5.0% (3/60). These results suggest a high detection ratio of marker genes in tumor drainage blood.

Table IV. Relationship between peripheral blood CEA/CK20 mRNA positive rates and clinicopathological factors.

Variables	No. of patients (n=167)	PCR positive (n=17)	Positive rate (%)	P-value
Tumor size (cm)				
<5	114	12	9.4	0.827
≥ 5	53	5	10.5	
Histological type				
Well	114	9	7.9	0.197
Moderate	36	4	11.1	
Poor, Muc	17	4	23.5	
Depth of invasion				
≤ mp	43	1	2.3	0.025
≥ ss(a1)	124	16	12.9	
Lymphatic invasion				
ly (-)	104	9	8.7	0.401
ly (+)	63	8	12.7	
Venous invasion				
v (-)	75	2	2.7	0.002
v (+)	92	15	16.3	
Lymph node metastasis				
n (-)	92	5	5.4	0.024
n (+)	75	12	16.0	
Liver metastasis				
H (-)	138	8	5.8	0.001
H (+)	29	9	31.0	
Peritoneum dissemination				
P (-)	155	15	9.7	0.473
P (+)	12	2	16.7	
Dukes stage				
A	36	0	0.0	0.003
B	48	3	6.3	
C	44	5	11.4	
D	39	9	23.1	

Relationship between CEA/CK20 mRNA and clinicopathological factors. The relationship between the expression of CEA/CK20 in peripheral blood and tumor drainage blood

Table V. Relationship between tumor drainage blood CEA/CK20 mRNA positive rates and clinicopathological factors.

Variables	No. of patients (n=167)	PCR positive (n=57)	Positive rate (%)	P-value
Tumor size (cm)				
<5	114	35	30.7	0.216
≥ 5	53	22	41.5	
Histological type				
Well	114	33	28.9	0.120
Moderate	36	16	44.4	
Poor, Muc	17	8	47.1	
Depth of invasion				
≤ mp	43	3	7.0	<0.001
≥ ss(a1)	124	54	43.6	
Lymphatic invasion				
ly (-)	104	30	28.9	0.066
ly (+)	63	27	42.9	
Venous invasion				
v (-)	75	18	24.0	0.012
v (+)	92	39	42.4	
Lymph node metastasis				
n (-)	92	18	19.6	<0.001
n (+)	75	39	52.0	
Liver metastasis				
H (-)	138	36	26.1	<0.001
H (+)	29	21	72.4	
Peritoneum dissemination				
P (-)	155	51	32.9	0.240
P (+)	12	6	50.0	
Dukes stage				
A	36	0	0.0	<0.001
B	48	12	25.0	
C	44	20	45.5	
D	39	25	64.1	

and the clinicopathological factors were examined. In preoperative peripheral blood, a significant relationship was demonstrated between positivity for CEA/CK20 and the depth of tumor invasion, venous invasion, lymph node metastasis, liver metastasis or stage (Table IV). In tumor drainage blood, a significant correlation was observed between CEA/CK20 expression and the depth of tumor invasion, venous invasion, lymph node metastasis, liver metastasis or stage (Table V). These results suggest that the presence of ITC in the peripheral blood and tumor drainage blood correlates with parameters associated with tumor progression.

Table VI. Incidence of metachronous liver metastasis in patients with CEA/CK20 mRNA (%).[a]

Peripheral blood	Tumor drainage blood
37.5 (3/8)	25.0 (8/32)

[a](No. of patients with metachronous liver metastasis)/(no. of CEA/CK20 positive patients who underwent curative surgery) x 100.

Table VII. Comparison of immunocytochemistry and real-time RT-PCR on paired samples.

PCR status of samples	Positive rates of immunocytochemistry (%)[a]	
	Peripheral blood	Tumor drainage blood
PCR positive	58.3 (7/12)	63.2 (24/38)
PCR negative	0.0 (0/90)	0.0 (0/64)

[a](No. of immunocytochemistry positive samples/no. of PCR positive or PCR negative samples) x 100.

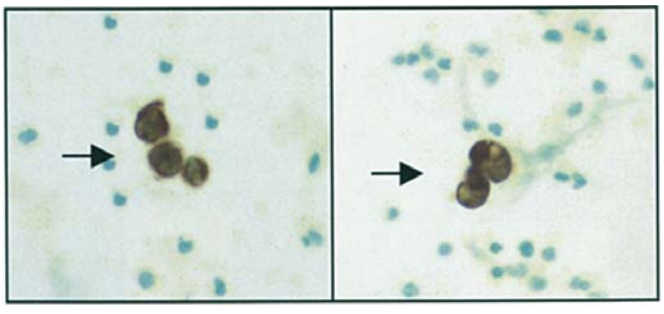

Figure 3. Immunocytochemistry of tumor cells in blood samples. Tumor drainage blood samples were collected from CEA/CK20 real-time RT-PCR positive cases. Tumor cells of blood samples were enriched by MACS and stained with a mixture of anti-human CEA and anti-human cytokeratin monoclonal antibodies. The arrow shows CEA- and cytokeratin-positive tumor cells (x400).

Next, we assessed the relationship between the detection of the CEA/CK20 transcript and metachronous liver metastasis. After curative surgery, metachronous liver metastasis developed in 37.5% (3/8) of patients with marker genes in their peripheral blood and 25.0% (8,11) of patients with marker genes in their tumor drainage blood (Table VI).

Comparison of immunocytochemistry and real-time RT-PCR. We compared immunocytochemistry and real-time RT-PCR using paired blood samples for the validation of tumor cells (Table VII). CEA/CK20 positive tumor cells were detected in 58.3% (7/12) of the peripheral blood samples and 63.2%

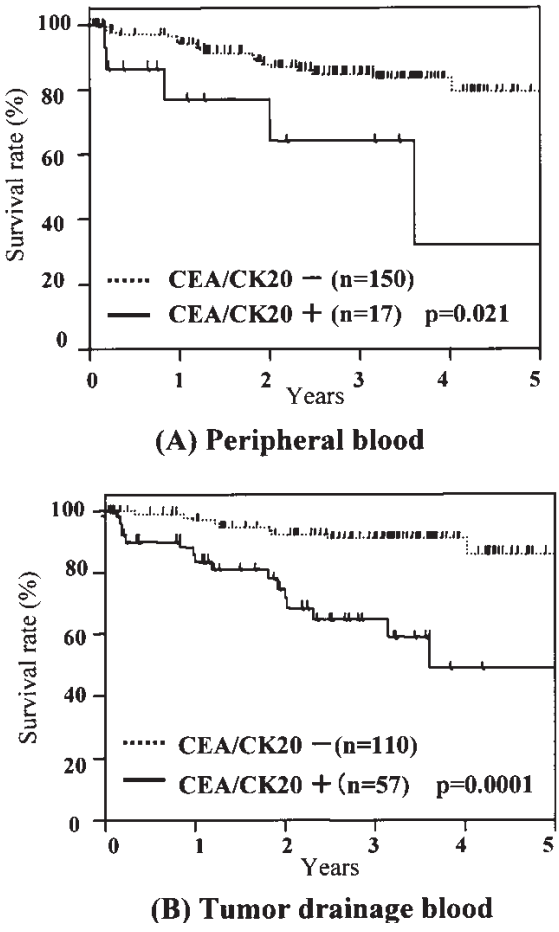

Figure 4. Overall survival according to CEA/CK20 real-time RT-PCR results. Overall survival for all patients (n=167) was analyzed by Kaplan-Meier method. Peripheral blood (A) and tumor drainage blood samples (B) were used in this study. In peripheral and tumor drainage blood, significant differences were shown between the CEA/CK20-positive and CEA/CK20-negative groups.

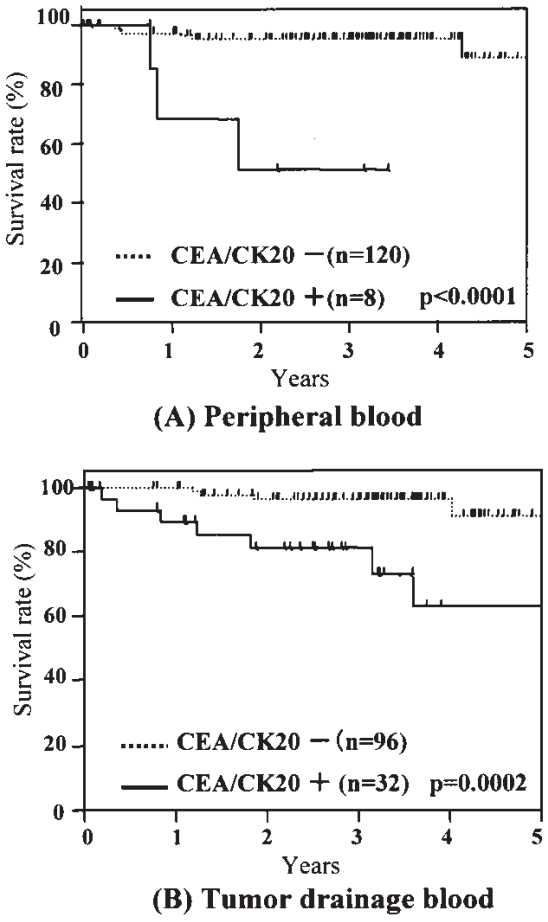

Figure 5. Disease-free survival according to CEA/CK20 real-time RT-PCR results. Disease-free survival for 128 cases who underwent curative surgery was analyzed by Kaplan-Meier method. Peripheral blood (A) and tumor drainage blood samples (B) were used in this study. In peripheral and tumor drainage blood, significant differences were shown between the CEA/CK20-positive and CEA/CK20-negative groups.

(24/38) of the tumor drainage blood samples collected from PCR-positive patients. In contrast, tumor cells were not detected in the peripheral and tumor drainage blood samples, which were PCR-negative. These results suggest that real-time RT-PCR is more sensitive than immunocytochemistry. Fig. 3 shows a representative pattern of tumor cell staining with the mixture of CEA and cytokeratin antibodies.

Prognostic value of CEA/CK20 mRNA in the blood. The Kaplan-Meier survival curves show the overall survival and disease-free survival rates according to the CEA/CK20 gene expression status. The overall survival of patients with positive marker genes in their peripheral blood and tumor drainage blood showed a significantly poorer prognosis than that of marker gene-negative patients (Fig. 4). Disease-free survival was analyzed for 128 patients with colorectal cancer who underwent curative surgery (Fig. 5). In this analysis, the patients whose peripheral blood and tumor drainage blood was CEA/CK20 transcript positive showed a significantly poorer prognosis than patients who were marker gene negative. Next, we examined the univariate Cox proportional hazard regression analysis in overall survival (Table VIII) and disease-free survival (Table IX), and significant relationships were shown concerning the depth of invasion, lymphatic invasion, venous invasion, stage and CEA/CK20 in tumor drainage blood. Then, multivariate Cox regression analysis was performed for factors that showed significance in univariate analysis, and it was demonstrated that CEA/CK20 in tumor drainage blood showed significance for overall and disease-free survival. These results suggest that CEA/CK20 mRNA in tumor drainage blood possesses prognostic value in terms of survival.

Discussion

The molecular detection of ITC has attracted attention as a more sensitive and objective method than immunocytochemistry. Our results also demonstrate the superior sensitivity of molecular detection of ITC in the blood as compared with that of immunocytochemistry. Although the conventional nested-primer RT-PCR method showed high sensitivity, determining an accurate cut-off level has been difficult because of insufficient quantification. In contrast, the quantitative real-time RT-PCR system has several advantages over conventional RT-PCR. One advantage is the quantification of the initial template copy number based on the fact that the cycle number

Table VIII. Univariate and multivariate analysis for the prediction of overall survival.

	Univariate analysis			Mutlivariate analysis		
Variables	Regression coefficient	Hazard ratio	P-value	Regression coefficient	Hazard ratio	P-value
Tumor size	-0.227	0.797	0.266		-	
Depth of invasion	-7.294	0.001	<0.001	-6.450	0.002	0.098
Lymphatic invasion	-1.032	0.356	<0.001	-0.700	0.496	0.001
Venous invasion	-0.822	0.440	0.001	-0.351	0.704	0.134
Dukes stage	-1.502	0.223	<0.001	-0.918	0.367	0.001
CEA/CK20 in PB	0.506	1.658	0.078		-	
CEA/CK20 in DVB	0.878	2.406	<0.001	0.417	1.517	0.042

PB, peripheral blood; DVB, tumor drainage blood.

Table IX. Univariate and multivariate analysis for the prediction of disease-free survival.

	Univariate analysis			Mutlivariate analysis		
Variables	Regression coefficient	Hazard ratio	P-value	Regression coefficient	Hazard ratio	P-value
Tumor size	-0.167	0.846	0.632		-	
Depth of invasion	-7.349	0.001	0.002	-0.438	0.645	0.357
Lymphatic invasion	-0.955	0.385	0.002	-0.185	0.830	0.470
Venous invasion	-0.921	0.398	0.006	-0.492	0.611	0.074
Dukes stage	-9.905	0.001	<0.001	-0.918	0.399	0.001
CEA/CK20 in PB	0.842	2.321	0.075		-	
CEA/CK20 in DVB	0.999	2.716	0.001	0.557	1.744	0.031

PB, peripheral blood; DVB, tumor drainage blood.

at which the sample fluorescence exceeds the background level is inversely correlated with the starting copy number. The quantification of a low-level of transcription allowed the establishment of accurate cut-off values for the expression of target genes and thus improved the precision of RT-PCR. Another advantage is the rapid amplification with the capillary tube. With this method, the 45-cycle PCR was finished within 45 min. In addition, with the hybridization probe designed inside the primers, the specificity of this assay increased as compared with the SYBR-Green I format, which includes non-specific products, such as a primer dimmer. Therefore, real-time RT-PCR seems to be more suitable for routine testing than conventional RT-PCR to detect small amounts of ITC (14-21). In our study, the sensitivity of tumor cells was one tumor cell per $3x10^6$ PBMC, which is an improvement over the results of previous reports (16,17).

Many target genes, such as CEA, cytokeratin (CK7, CK8, CK18, CK19, CK20) and mucin 1, are reportedly able to detect ITC (23-28). Because there is no specific marker for colorectal cancer, the detection of disseminated colorectal tumor cells is based on epithelial markers. Several studies have reported that CEA and CK20 are reliable target genes for the detection of disseminated colorectal cancer cells (26-28). However, some papers reported that these marker genes have relatively high background levels in normal control samples, which cause the problem of false positives (29,30). In contrast, in our study, primers and probes for CEA and CK20 were designed to amplify short amplicons spanning exon-intron boundaries to prevent the detection of genomic DNA. Therefore, the expression of CEA and CK20 mRNA of healthy control blood was at very low levels and we were able to establish accurate cut-off levels based on the 95% confidence intervals of the control group. Regarding the positive identification of CEA and CK20, colorectal carcinoma cells were reportedly detected in 11-41% of peripheral blood samples and in 30-42% of tumor drainage vein blood samples (18,19). Our data have shown that the PCR-positive rate in tumor drainage blood was 34.1%, which was 3.3 times higher than the 10.2% in peripheral blood, which is consistent with the results of the previous report.

The relationship between CEA and CK20 mRNA expression in peripheral blood samples and clinicopathological parameters remains unclear (19-24). Recently, Ito *et al* and Schuster *et al* reported that they found no significant relationship between the results of CEA mRNA expression in preoperative peripheral blood and clinicopathological factors using

quantitative real-time RT-PCR (17,18). In contrast, Miura *et al* reported that the CEA mRNA level was significantly higher in Dukes D patients than in the other clinical stages (19). In the present study, we have shown a significant relationship between the CEA/CK20 transcripts in preoperative peripheral blood and the depth of tumor invasion, venous invasion, lymph node metastasis, liver metastasis and tumor stage. Interestingly, the sensitivity of the detection of ITC in tumor drainage blood is higher than that in peripheral blood in our study. The relationship between the positive rates of gene markers in tumor drainage blood and clinicopathlogical factors is also unclear. Using the conventional RT-PCR method, Taniguchi *et al* reported a significant relationship between the CEA mRNA expression in tumor drainage blood and lymph node metastasis, lymphatic invasion, vascular invasion and histological type (10). Yamaguchi *et al* also found a significant relationship between CEA and CK20 mRNA expression and the depth of invasion, lymph node metastasis and stage using conventional RT-PCR (11). Using real-time RT-PCR, we have demonstrated that a significant correlation exists between the positive rates of CEA/CK20 mRNA in tumor drainage blood and the depth of invasion, vascular invasion, lymph node metastasis, liver metastasis and stage.

Identifying colorectal cancer patients with a high-risk of recurrence in the liver is important for improved prognosis. Little is known, however, about the relationship between the presence of ITC in the blood and recurrence in the liver. In this study, we found that the incidence of metachronous liver metastasis in PCR positive patients who underwent curative surgery was 37.5% in the peripheral blood and 25.0% in the tumor drainage blood. The relatively low positive rates of these gene transcripts may be attributed to the blood sampling. As Glavers *et al* suggested, cancer cells may be discontinuously shedding into the bloodstream (31). Another reason may be the complexity of the metastasis formation, such as the scattering of ITC from the original sites, spreading into the blood circulation, arresting in the small vessels, the adherence of tumor cells to the vascular endothelium, migration into the extracellular space, establishment of the micro-environment, escape from the host defense mechanism and growth as micrometastases.

Next, we evaluated the prognostic value of ITC in the peripheral blood and tumor drainage blood of patients with colorectal cancer. Using conventional RT-PCR and peripheral blood, Wyld *et al* reported no correlation between the presence of CK20 mRNA in the blood and survival (8). In contrast, recent quantitative RT-PCR-based studies have indicated that the disease-free survival of patients with CEA mRNA-positive peripheral blood was significantly worse than that of patients who were CEA mRNA negative (18). In this study, we have shown that Kaplan-Meier survival curves of overall survival and disease-free survival were significantly shorter in patients with CEA/CK20 mRNA-positive peripheral blood or tumor drainage blood than in cases negative for these marker genes. Furthermore, it was shown that the CEA/CK20 mRNA in the tumor drainage blood was an independent prognostic factor for survival by Cox multivariate analysis. To our knowledge, our study is the first to demonstrate the prognostic value of ITC in tumor drainage blood detected by real-time RT-PCR in colorectal cancer patients. One of the reasons why we could not clarify the prognostic relevance of CEA/CK20 in peripheral blood may have been the small number of patients. To confirm the prognostic value of genetically detecting ITC in the peripheral blood, we are presently undertaking a large-scale clinical study.

In conclusion, this analysis demonstrates that the detection of ITC in tumor drainage blood is associated with prognosis, and this strategy may be useful for the selection of patients who need postoperative adjuvant therapy.

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