ORIGINAL ARTICLE

Prediction of Distant Metastasis by Using Reverse Transcriptase–Polymerase Chain Reaction for Epithelial and Variant CD44 mRNA in the Peripheral Blood of Patients With Colorectal Cancer

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Background: Reverse transcriptase–polymerase chain reaction (RT-PCR) has been used to identify small numbers of tumor cells. Molecular detection is thought to provide useful information for the clinical management of postoperative adjuvant therapy regimens.

Objective: To use RT-PCR to identify messenger RNA (mRNA) coding for carcinoembryonic antigen, epithelial and variant CD44, and matrix metalloproteinase 7 in the portal venous and peripheral blood of patients with colorectal carcinoma to predict live or distant metastasis.

Design: Prospective consecutive series.

Setting: University hospital.

Patients and Methods: Portal venous and peripheral blood samples were obtained from 22 patients with colorectal cancer during surgical manipulation. Using complementary DNA primers specific for carcinoembryonic antigen, CD44, and matrix metalloproteinase 7, RT-PCR was performed to detect tumor cells.

Main Outcome Measure: The clinical significance of RT-PCR for epithelial and variant CD44 mRNA in peripheral blood.

Results: During 3 years of follow-up, 2 patients whose peripheral blood had carcinoembryonic antigen and CD44 variant mRNA also had distant metastases (lung or spleen). Expression of epithelial and variant CD44 mRNA in peripheral blood was more highly correlated with the clinical cancer stage than with expression of carcinoembryonic antigen and matrix metalloproteinase 7.

Conclusions: Molecular detection of epithelial and variant CD44 mRNA in the peripheral blood may help determine distant metastases in patients with colorectal carcinoma. Molecular detection in the peripheral blood at surgical treatment suggests that systemic hematogenic tumor cell dissemination is an early event of distant metastasis.

Arch Surg. 2002;137:1069-1073

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OF PATIENTS with colorectal cancer who have curative surgical treatment, 44% develop metastatic disease.[1] The sites of metastases are the lymph nodes, peritoneum, liver, lungs, and bone marrow. Many methods have been established to increase the sensitivity of detection of these early metastases. Cytologic and immunochemical methods have been used to detect colorectal cancer cells in peripheral and mesenteric venous blood–draining tumors.[2,3] However, the prognostic and clinical value of this detection is not clear. Technical advances have also made it possible to detect micrometastases at the molecular level in circulating blood, and recent studies have reported and discussed the clinical significance of such detection.[4,5]

In the present study, we detected the expression of carcinoembryonic antigen (CEA) messenger RNA (mRNA), matrix metalloproteinase 7 (MMP-7) mRNA, and epithelial and variant CD44 mRNA in circulating-blood specimens from patients with colorectal carcinoma. The presence of cancer cells in the blood does not prove that there has been metastasis to distant organs. It seems to be necessary for cancer cells to acquire certain properties for metastasis. Thus, one should consider which tumor marker genes are related to the formation of metastatic foci in distant organs, including the liver, lungs, and spleen. In this study, we selected CD44 variants and the *MMP-7* gene as markers. The CD44 variant exons and MMP-7 are frequently overexpressed in human colorectal carcinoma, and many studies have suggested that CD44 variants and MMP-7 are associated with metastases and invasion,[6-14] with CD44 variants related to the properties of adhesion and MMP-7 re-

lated to tumor invasion. On the basis of our results, we discuss the clinical value of detecting epithelial and variant CD44 and MMP-7 mRNA in the circulating blood.

PATIENTS AND METHODS

PATIENTS

Twenty-two patients with colorectal cancer were enrolled in this study. The locations of the tumor were the ascending colon (n=4), the transverse colon (n=4), the descending colon (n=2), the sigmoid colon (n=6), and the rectum (n=6). All patients received surgical treatment in which the tumors were resected completely. The clinical stages according to TNM staging were stage I (n=4), stage II (n=8), stage III (n=8), and stage IV (n=2). None of the patients had received any previous treatment, including anticancer chemotherapy or radiation therapy. Thirteen patients had well-differentiated adenocarcinoma, 6 patients had moderately differentiated adenocarcinoma, and 3 patients showed other histologic types (2 with mucinous adenocarcinoma and 1 with poorly differentiated adenocarcinoma). Informed consent was obtained from all patients in accordance with the guidelines of the Ethics Committee on Human Research, Wakayama Medical University, Wakayama, Japan.

EXTRACTION OF RNA

Blood samples from the portal and peripheral veins (10 mL) and tumor tissue (100 mg) were obtained from each patient during surgical treatment. The mononucleated cell fraction was isolated by centrifugation on a Ficoll-Hypaque gradient at 400*g* for 30 minutes. Total RNA was isolated from mononuclear cells and tumor tissues by guanidinium thiocyanate extraction, using the method described by Chomczynski and Sacchi.[15]

REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION (RT-PCR)

The oligonucleotide primers specific for CEA, CD44, and MMP-7 mRNA were synthesized according to the sequences described by Gerhard et al[16] and Ichikawa et al.[17] To detect CEA expression, the primers used for the first polymerase chain reaction (PCR) were sense, 5′-TCTGGAACTTCTCCTGGTCTCTCAGCTGG-3′, and antisense, 5′-GGGCCACTGCTGGCATCATGATTGG-3′. The heminested sense primer for CEA expression was 5′-TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC 3′, and the antisense primer was the same as that used for the first PCR. The CD44 primers were sense, 5′-TCCCAGACGAAGACAGTC-CCTGGAT-3′, and antisense, 5′-CACTGGGGTGGAATGT-GTCTTGGTC-3′. The MMP-7 primers were sense, 5′-TCTTTGGCCTACCTATAACTGG-3′, and antisense, 5′-CTAGACTGCTACCATCCGTC-3′. Efficient amplification of the RNA samples was confirmed by an internal control by amplification of β-actin mRNA. The primers used for β-actin were sense, 5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′, and antisense, 5′-CGTCATACTCCTGCTTGCTGATCCA-CATCTGC-3′.

Complementary DNA was synthesized from 2 µg of total RNA with a Ready-to-Go T-Primed First-Strand Kit (Amersham Biosciences, Piscataway, NJ) following the manufacturers' instructions. For the first PCR, 50 µL of a solution containing buffer (Takara Bioch Inc, Shiga, Japan), primers (0.4 µmol/L each), 1 µL of complementary DNA, 2.5 U of *Taq* polymerase, and 200µM of deoxynucleoside triphosphate was added to each tube. Twenty amplification cycles were performed at 95°C (1 minute), 55°C for CEA, 67°C for CD44, or 63°C for MMP-7 (1 minute), and 72°C (1 minute), with a final extension step for 10 minutes. Then, 1 µL of the reaction mixture was transferred into a second tube with 50 µL of a solution containing buffer, primers (CD44 and MMP-7 primers were the same as for the first PCR; 0.4µM each), 2.5 U of *Taq* polymerase, and 200µM of deoxynucleoside triphosphate. Thirty amplification cycles were performed at 95°C (1 minute), 67°C for CEA, 55°C for CD44, or 55°C for MMP-7 (1 minute), and 72°C (1 minute), with a final extension step for 10 minutes. We repeated the sensitivity tests and determined the adequate PCR cycle. The most appropriate cycles are 20 for the first PCR and 30 for the second PCR. The PCR products were then electrophoresed on 2% agarose gels containing 1 µg/mL of ethidium bromide and visualized with ultraviolet light. The PCR products were identified by direct sequencing.

STATISTICAL ANALYSIS

The significance of differences was determined by the Fisher exact test. $P<.05$ was considered statistically significant.

RESULTS

To determine the sensitivity of reverse transcriptase–PCR (RT-PCR) for CEA, CD44, and MMP-7, triplicate reconstitution experiments were performed, in which serial 10-fold dilutions of HT29 cells, a human colon cancer cell line, were mixed with 1×10^7 mononucleated cells from a healthy volunteer. In the preliminary study, even 1 tumor cell in 1×10^7 normal cells could be detected by RT-PCR.

None of the healthy volunteers (n=5) had CEA, epithelial or variant CD44, or MMP-7 mRNA expressed in their peripheral blood; 3 patients with colorectal adenoma showed expression of CEA, epithelial and variant CD44, and MMP-7 mRNA in adenoma tissues but had no expression in portal venous and peripheral blood samples. All 22 patients with colorectal cancer showed expression of CEA, epithelial and variant CD44, and MMP-7 mRNA in all tumor tissues (**Table 1**). Tumor cells were detected in portal venous and peripheral blood samples of patients with stage I, II, III, and IV disease by using the RT-PCR assay for CEA, whereas the expression of epithelial and variant CD44 was detected in portal venous and peripheral blood samples of patients with stage II, III, and IV disease. The rates of expression of epithelial and variant CD44 were correlated with TNM stage in portal venous and peripheral blood. In all tumor tissues, including liver metastatic lesions, MMP-7 mRNA was expressed. However, there was no expression of MMP-7 mRNA in the portal venous or peripheral blood of any patients, including patients with liver metastases. The expression of these tumor marker genes in 2 representative cases is shown in **Figure 1**. The pattern of expression of CEA mRNA in patients with stage I and II cancers was similar to that in patients with stage III and IV cancers (**Table 2**). The CD44 variant mRNA were detected in 1 (8%) of the portal venous and peripheral blood samples from patients with localized disease (stage I and II) and were detected in 9 portal venous blood samples (90%) and 6 peripheral blood samples (60%) of patients with stage III and IV disease, including patients with regional lymph node involvement or metastatic lesions (portal venous blood, $P<.001$; peripheral blood,

Table 1. Clinicopathological Characteristics of Patients With Colon Cancer and Detection of CEA, CD44 Variants, and MMP-7 mRNA by Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)*

Patient No.	Tumor		Tumor Invasion				TNM Stage	Gene Expression by RT-PCR								
								CEA			CD44 Variants			MMP-7		
	Location	Histological Characteristics	Depth	LY	V	N		T	D	P	T	D	P	T	D	P
1	Transverse	Well differentiated	M	N	N	N	0	Y	N	N	Y	N	N	Y	N	N
2	Sigmoid	Well differentiated	PM	Y	Y	N	I	Y	N	N	Y	N	N	Y	N	N
3	Descending	Well differentiated	PM	Y	N	N	I	Y	N	N	Y	N	N	Y	N	N
4	Sigmoid	Well differentiated	SM	Y	Y	N	I	Y	Y	N	Y	N	N	Y	N	N
5	Rectum	Well differentiated	PM	Y	Y	N	II	Y	N	N	Y	N	N	Y	N	N
6	Sigmoid	Well differentiated	SS	N	N	N	II	Y	N	N	Y	N	N	Y	N	N
7	Ascending	Mucinous	SS	Y	Y	N	II	Y	Y	N	Y	N	N	Y	N	N
8	Ascending	Well differentiated	SS	Y	Y	N	II	Y	Y	N	Y	N	N	Y	N	N
9	Transverse	Well differentiated	SS	Y	Y	N	II	Y	Y	Y	Y	N	N	Y	N	N
10	Rectum	Well differentiated	SS	Y	Y	N	II	Y	Y	Y	Y	N	N	Y	N	N
11	Ascending	Mucinous	SI	Y	Y	N	II	Y	N	N	Y	N	N	Y	N	N
12	Ascending	Poorly differentiated	SI	Y	Y	N	II	Y	Y	Y	Y	Y	Y	Y	N	N
13	Descending	Well differentiated	SS	Y	Y	Y	III	Y	N	N	Y	N	N	Y	N	N
14	Rectum	Moderately differentiated	SS	Y	Y	Y	III	Y	Y	N	Y	Y	N	Y	N	N
15	Sigmoid	Well differentiated	SS	Y	Y	Y	III	Y	Y	N	Y	Y	N	Y	N	N
16	Transverse	Well differentiated	SE	Y	Y	Y	III	Y	Y	N	Y	Y	N	Y	N	N
17	Rectum	Moderately differentiated	SS	Y	Y	Y	III	Y	Y	Y	Y	Y	Y	Y	N	N
18	Rectum	Moderately differentiated	SS	Y	Y	Y	III	Y	Y	Y	Y	Y	Y	Y	N	N
19	Sigmoid	Moderately differentiated	SS	Y	Y	Y	III	Y	Y	Y	Y	Y	Y	Y	N	N
20	Rectum	Well differentiated	A2	Y	Y	Y	III	Y	Y	Y	Y	Y	Y	Y	N	N
21	Transverse	Moderately differentiated	SE	Y	Y	Y	IV	Y	Y	Y	Y	Y	Y	Y	N	N
22	Sigmoid	Moderately differentiated	SS	Y	Y	Y	IV	Y	Y	Y	Y	Y	Y	Y	N	N

*CEA indicates carcinoembryonic antigen; MMP-7, matrix metalloproteinase 7; mRNA, messenger RNA; LY, lymphatic vessel; V, vascular; N, lymph node; T, tumor tissue; D, drainage (portal) venous blood; P, peripheral blood; M, mucosa; PM, muscularis propria; SM, submucosa; SS, subserosa; SI, serosa, infiltrating; SE, serosa, exposed; and A2, obvious invasion to the adventitia.

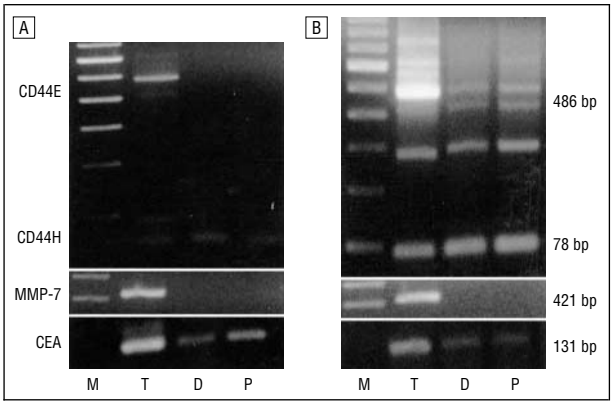

Figure 1. Reverse transcriptase–polymerase chain reaction assays for carcinoembryonic antigen (CEA), CD44, and matrix metalloproteinase (MMP-7) messenger RNA (mRNA) in tumor tissue, portal venous blood, and peripheral blood of 2 representative patients with colorectal cancer. Patient 10 with stage II cancer (A) showed expression of CEA mRNA in all samples and of epithelial and variant CD44 and MMP-7 mRNA in tumor tissues, but not in portal venous or peripheral blood. Patient 20 with stage III cancer (B) showed expression of CEA mRNA in all samples and also expression of epithelial and variant CD44 mRNA in all samples. However, MMP-7 mRNA was expressed in tumor tissues but not in portal venous or peripheral blood. CD44E indicates epithelial CD44; CD44H, hematopoietic CD44; M, molecular markers; T, tumor tissue; D, drainage (portal) venous blood; P, peripheral blood; and bp, base pair.

P=.02). Two patients with liver metastases had positive expression of epithelial and variant CD44 in all samples, including tumor tissues, portal venous blood, peripheral blood, and liver metastatic foci. Furthermore, patients with expression of epithelial and variant CD44 mRNA had cancer recurrences by 3 years after surgical treatment. Patient 20 had lung metastasis after 1 year, and patient 21 had spleen metastasis after 6 months (**Figure 2**). Of 6 patients with stage III or IV disease who had CEA and CD44 variant mRNA expressed in their peripheral blood, 3 had metastases to distant organs. Of 4 patients with stage III or IV disease but without DEA and CD44 variant mRNA, none had metastases to distant organs (χ^2=2.86; P=.09).

Table 2. TNM Stage and Reverse Transcriptase–Polymerase Chain Reaction Detection of CEA and CD44 Variant mRNA*

TNM Stage	mRNA Expression			
	CEA		CD44 Variant	
	D	P	D	P
I and II (n = 12)	6 (50)	3 (25)	1 (8)	1 (8)
III and IV (n = 10)	9 (90)	6 (60)	9 (90)†	6 (60)‡

*Data are given as the number (percentage) of subjects. CEA indicates carcinoembryonic antigen; mRNA, messenger RNA; D, drainage (portal) venous blood; and P, peripheral blood.
†P<.001 compared with stage I and II.
‡P = .02 compared with stage I and II.

COMMENT

For patients with colorectal carcinoma, distant metastasis is one of the most common recurrence patterns after

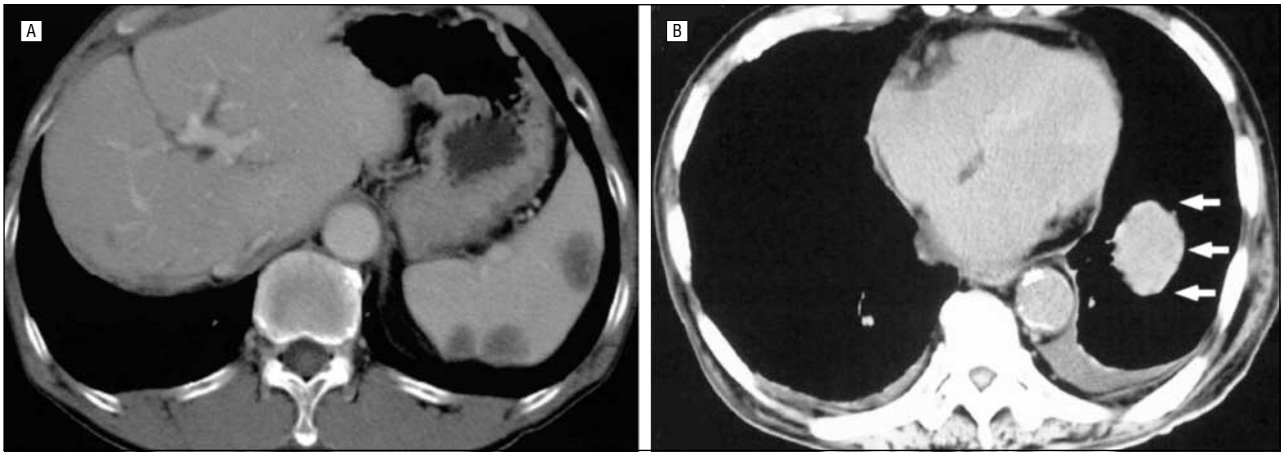

Figure 2. A, Plain computed-tomographic (CT) scan of the upper abdomen demonstrates spleen metastasis 6 months after surgical treatment in patient 21. B, Plain CT scan of the chest demonstrates lung metastasis 1 year after surgical treatment in patient 20 (arrows indicate the lung metastasis). Epithelial and variant CD44 messenger RNA were expressed in the peripheral blood of 2 patients during the surgical treatment.

surgical treatment.[18-21] In particular, colorectal cancer patients with stage III disease often have distant metastasis and poor survival.[8,18] The presence of tumor cells in peripheral blood is by itself not sufficient to prove metastasis to distant organs. Rather, it seems to be necessary for cancer cells to acquire some additional properties for metastasis. For improving survival of patients with stage III disease, early detection of the cancer cells with the properties necessary for metastasis in portal venous or peripheral blood is required.

Using RT-PCR, the dissemination of tumor cells in the blood has been shown in patients with breast cancer,[18,22] gastrointestinal cancer,[18] malignant melanoma,[23-25] and lung cancer.[26] Colorectal cancer cells are detected by using RT-PCR for CEA,[18-20,27] cytokeratin 19,[28] cytokeratin 20,[29] K-*ras*,[30] and *p53*[28] mutations, and CEA mRNA is the most commonly used marker for detection of cancer cells in the bloodstream of patients with colorectal cancer. Positive expression of CEA mRNA in the bloodstream indicates the existence of viable cancer cells, which have been released or detached from the primary tumor beds.[18-20] We examined CEA mRNA for detection of circulating cancer cells; however, there was no significant difference between patients with stage I and II vs stage III and IV cancers. Indeed, the portal vein is the main drainage vein for colorectal cancer, and CEA mRNA was expressed in portal venous blood, but the presence of cancer cells in the bloodstream was not sufficient to cause metastasis. Therefore, in addition to examining CEA mRNA in the portal venous and peripheral blood samples of patients with colorectal cancer, we also analyzed the expression of CD44 and MMP-7 mRNA, which are likely candidates for conferring properties to promote metastasis.

The CD44 variants containing the products of variant exons 8 through 10 (CD44 v8-10) play an important role in the adhesion of tumor cells to the capillaries of distant organs in the metastatic process.[31] Moreover, the level of expression of CD44 v8-10 is significantly higher in cancers associated with liver metastasis than in those without liver metastasis. In addition, expression of CD44 v8-10 in liver metastases is stronger than that in primary colorectal cancers.[6] Therefore, expression of CD44 v8-10 has emerged as an independent prognostic indicator.[7] It has been reported that CD44 variant exon 6 (CD44 v6) expression in tumors is associated with tumor-related death in patients with colorectal cancer. Expression of CD44 v6 has prognostic value independent of the Dukes classification and reflects the propensity for metastasis after apparently curative surgical treatment.[8] Epithelial and variant CD44 were detected in 8% of portal venous and peripheral blood samples of patients with stage I and II disease, whereas they were detected in 90% of portal venous and 60% of peripheral blood samples of patients with stage III and IV disease. Two patients with liver metastases had epithelial and variant CD44 expressed in all samples, including tumor tissue, portal venous blood, peripheral blood, and liver metastatic lesions. The presence of CEA and CD44 variant mRNA may indicate hematogenous metastasis. Based on 3 years of follow-up data, 2 patients whose peripheral blood had expression of CEA and CD44 variant mRNA had distant metastases (lung or spleen). In the present study, the pattern of expression of CEA mRNA in stage I and II cancers was similar to that in stage III and IV cancers. The CD44 variant mRNA were detected in 8% of peripheral blood samples of patients with stage I and II disease and in 60% of peripheral blood samples of patients with stage III and IV disease. Therefore, RT-PCR for epithelial and variant CD44 mRNA may be a more sensitive tool than RT-PCR for CEA for delineating a high-risk group for hematogenous metastasis. In our results, expression of epithelial and variant CD44 mRNA in portal venous blood could not predict liver metastasis. On the other hand, expression of epithelial and variant CD44 mRNA in peripheral blood could predict distant metastasis (lung or spleen). Therefore, the detection of epithelial and variant CD44 expression in peripheral blood might provide the tool for diagnosis of distant metastasis and therapy for prevention of recurrence of stage III colorectal cancer.

Matrilysin, or MMP-7, is a matrix metalloproteinase that may be involved in the metastasis of colorectal cancer[14] because of its ability to degrade the extracellular matrix, especially type IV collagen, a major compo-

nent of the basement membrane.[32] We chose MMP-7 mRNA because it is known to be epithelium-specific, unlike the other MMPs. Most MMPs are secreted not only from epithelial cells, but also from mesenchymal cells. In colonic tissue, MMP-7 is detected consistently in tumors but not in normal surrounding mucosa or mononucleated cells.[11] Therefore, the detection of MMP-7 mRNA in lymph nodes and blood is definite proof of the presence of colorectal cancer cells. Our study demonstrates that MMP-7 mRNA is expressed in all tumor tissues, but not in all portal venous and peripheral blood samples, even in patients with stage III and IV disease, which means that adherence and interaction with mesenchymal cells seems to be necessary for expression of MMP-7 mRNA in cancer cells.[32] These results suggest that MMP-7 gene expression may be associated with cell invasion. Therefore, expression of MMP-7 mRNA in portal venous and peripheral blood is not a useful tool for predicting liver and distant metastases.

In conclusion, expression of epithelial and variant CD44 mRNA of circulating cancer cells in peripheral blood may provide the ability to predict the hematogenous dissemination of cancer cells in patients with colorectal cancer. The present study suggests that the detection of cancer cells by RT-PCR assays for epithelial and variant CD44 mRNA may be more useful than CEA mRNA for predicting metastases in patients with stage III disease. These differences seem most evident in patients with stage III disease. Currently, chemotherapy is the standard of care for these patients.[33] Further data on genetic changes in response to chemotherapy would be enlightening. Further investigations are required to determine the usefulness of RT-PCR assay for epithelial and variant CD44 mRNA.

This study was supported by the Mitsui Life Social Welfare Foundation, Tokyo, Japan.

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