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Detection of Tumor Cell Contamination in Peripheral Blood by RT-PCR in Gastrointestinal Cancer Patients

We analyzed the peripheral blood of patients with gastrointestinal tract cancer at different stages to assess the presence of carcinoembryonic antigen (CEA) mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR), which we used as an indicator for micrometastatic malignant cells. A total of 35 gastric, 24 colorectal, 4 esophageal and 4 biliary tract cancer patients and nine normal healthy subjects were studied. No CEA mRNA was detected in the nine normal healthy volunteers. CEA mRNA was detected in 100% (10/10) of metastatic, 33.3% (3/9) of early gastric cancer (EGC), and 18.8% (3/16) resectable gastric cancer patients, respectively. In colorectal cancer, 55.6% (5/9) of metastatic cancers were positive for CEA mRNA, and 26.7% (4/15) Duke stage B/C showed positive. One patient with stage III gastric cancer who was negative CEA mRNA initially and turned positive during follow-up, developed multiple bone metastasis one month later. Another stage III patient, who was positive for CEA mRNA, preoperatively revealed early relapse in two months. These results suggest that the identification of circulating tumor cells using RT-PCR for the detection of CEA mRNA is feasible and this analysis may be a promising tool for early detection of micrometastatic circulating malignant cells in patients with gastrointestinal tract cancer.

Key Words: *Gastrointestinal neoplasms; Neoplasm metastasis*

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INTRODUCTION

Recurrence and distant metastasis are still major problems in the treatment of solid tumors, and the prognosis of patients with malignant tumors deteriorates with metastatic spreading of the underlying disease. Even with the recent development of image studies and other diagnostic measures, it is not always possible to detect metastasis in the very early stage of disease. It has also been reported that up to 20% of the cases diagnosed as lymph node negative in routine sections do contain micrometastasis on reexamination by serial sectioning (1). Moreover, at the time of primary surgery, approximately 90% of all patients with breast cancer are free of metastases, but almost 50% of them will eventually develop relapse in 5 years suggesting micrometastasis exists and may play a key role in relapse (2). Recent advances in molecular technology make it possible to detect small numbers of circulating tumor cells in the peripheral blood or bone marrow (3).

Since carcinoembryonic antigen (CEA) mRNA is expressed in almost all epithelial cells, but not in non-epithelial cells, it would be a useful indicator for micrometastatic tumor cells (4-6). In this study, we analyzed the peripheral blood of patients with gastrointestinal tract cancer at different stages to assess the presence of CEA mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR), which we used as an indicator for micrometastatic malignant cells.

MATERIALS AND METHODS

Patient populations

Thirty-five gastric cancer, 24 colorectal cancer, 4 esophageal cancer, and 4 biliary tract cancer patients were studied. The tumor, node, metastases system (TNM) classification was used for esophageal, gastric, and biliary tract cancer and Dukes' classification was used for colorectal carcinoma. All patients who had undergone curative or noncurative surgery followed postoperative pathological stage. Other metastatic or relapsed patients were staged clinically using abdominal computed tomography, gastro-

fiberscopy, and bone scan. Nine normal healthy subjects were studied as a negative control.

Cell lines and peripheral blood samples

A colon cancer cell line, Colo 201, was used to test the potential sensitivity of RT-PCR in the detection of tumor cells in the blood. Mononuclear cells from normal healthy donors and patients were separated from 10 to 14 mL of peripheral blood by Ficoll-Hypaque gradient method.

RNA preparation

RNA from peripheral mononuclear cells was obtained by the thiocyanate, phenol-chloroform method described by Chomczinski and Sacchi (7). Total RNA was spectrophotometrically quantified at 260/280 nm, and its quality was tested in 1% agarose gel to find intact 28S and 18S RNAs.

RT-PCR

cDNA was synthesized from 5 μg of total RNA in a 25 μL reaction mixture containing 5 μL of 5× reverse transcriptase reaction buffer, 200 μM dNTP, 100 μM of random hexamer, 50 units of RNasin, 2 μL of 0.1 M dithiothreitol, and 200 units of Moloney leukemia virus reverse transcriptase. The mixture was incubated at 37℃ for 60 min, heated to 95℃ for 10 min, and then chilled on ice. CEA-specific oligonucleotide primers used for nested PCR were as follows: A: 5'-TCTGGAACTTCTCCTGGTCTCTCAGCTGG-3'; B: 5'-TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC-3'; C: 5'-GGGCCACTGTCGGCATCATGATTGG-3'. The first PCR product exhibited a 160 bp fragment and the second PCR product, a 131 bp fragment. The nested PCR was performed according to the method described by Gerhard et al. (5). Briefly, for the first PCR, 80 μL containing chelating buffer, 2.5 mM $MgCl_2$, 200 μM dNTP, and 25 pmoles of primer A and B were added to the eppendorf tube. Thirty-five cycles of amplification were performed in a thermocycler (Robocycler 40: Stratagene, La Jolla, CA) at 95℃ for 1 min and 72℃ for 2 min with a final extension step performed for 10 min at 72℃. Five microliters of the reaction were transferred into a second eppendorf tube containing 200 μM dNTP, 1.5 mM $MgCl_2$, 2.5 U *Taq* polymerase and 20 pmoles of primer B and C. Thirty-five cycles were performed at 95℃ for 1 min, 69℃ for 1 min, and 72℃ for 1 min, with the final extension step performed for 10 min at 72℃.

To ensure the RNA was of sufficient purity for RT-PCR, a PCR assay with primers specific for the gene β-actin cDNA was carried out in each case. The primer sequences for β-actin primers were as follows: 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3' and 5'-CTAGAAGCATTGCGGTGGACGATGGAGGG-3'. Each series of RT-PCR reactions included RNA-negative samples and normal healthy blood samples as a negative control and Colo 201 cell samples as a positive control, respectively.

RESULTS

Marker CEA mRNA detection sensitivity

Total RNA was isolated from colon cancer cell lines and quantitated to determine the sensitivity of the assay. Serially diluted Colo 201 cells from 10^4 to 10^1 were mixed in 10^6 mononuclear cells from normal healthy subjects, and RT-PCR was performed. CEA mRNA could be detected at the level of 10^1 colon cancer cells in 10^6 mononuclear cells (1:100,000) (Fig. 1).

RT-PCR results and clinical data

Among the 35 gastric cancer patients, 16 patients (45.7%) were positive for CEA mRNA in the peripheral blood cells. According to each stage, CEA mRNA was detected in 10 out of 10 (100%) metastatic or relapsed, 3 out of 9 (33.3%) EGC, and 3 out of 16 (18.8%) resectable gastric cancers (Table 1). Representative results are shown in Fig. 2. There was a significant difference between resectable gastric cancer and metastatic gastric cancer in CEA mRNA positivity [6/25 (24%) vs 10/10 (100%), p=0.01]. The median numbers of lymph node metastasis were four (range: 2-8) in CEA mRNA positive resectable gastric cancer and seven (range: 0-22) in CEA

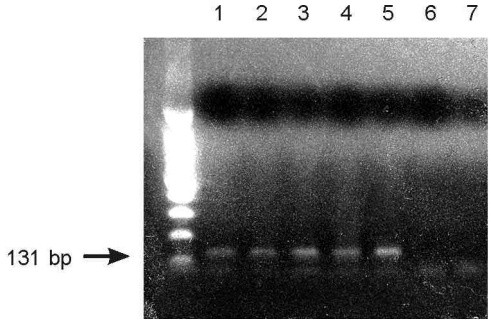

Fig. 1. Sensitivity test for CEA mRNA RT-PCR. Colo 201 cells were serially diluted and mixed with 10^6 mononuclear cells obtained from healthy normal subjects and RT-PCR was performed. Size marker: 100 bp. Lane 1-4: 10^1, 10^2, 10^3, 10^4 Colo 201 cells in 10^6 mononuclear cells, respectively. Lane 5: 10^6 Colo 201 cells, Lane 6: 10^6 mononuclear cells, Lane 7: no RNA.

Table 1. Patients characteristics in gastric cancer

Patient	Age	Sex	Status	Stage	CEA mRNA
1	67	F	Relapsed	IV	+
2	64	F	Relapsed	IV	+
3	45	M	Metastatic	IV	+
4	67	M	AGC	IIIb	−
5	58	F	Metastatic	IV	+
6	51	M	AGC	IIIb	−
7	43	M	Metastatic	IV	+
8	52	M	AGC	IIIb	−
9	59	F	AGC	IIIb	−
10	63	F	AGC	IIIa	−
11	62	M	AGC	IIIb	−
12	65	M	AGC	IIIa	−
13	63	M	AGC	IIIb	−
14	60	F	AGC	IIIa	−
15	44	F	Metastatic	IV	+
16	50	M	AGC	IIIb	+
17	34	F	AGC	IIIb	−
18	62	M	EGC	Ib	−
19	50	M	Relapsed	IV	+
20	63	F	EGC	Ia	+
21	65	M	AGC	IIIb	−
22	55	M	AGC	IIIa	+
23	36	M	EGC	Ib	+
24	63	M	Relapsed	IV	+
25	37	M	AGC	IIIa	−
26	66	M	AGC	IIIb	+
27	26	F	EGC	Ia	+
28	71	M	AGC	IIIb	−
29	48	F	EGC	Ia	−
30	59	M	EGC	Ib	−
31	60	M	EGC	Ib	−
32	39	M	EGC	Ib	−
33	61	M	EGC	Ib	−
34	42	M	Metastatic	IV	+
35	58	F	Metastatic	IV	+

AGC, advanced gastric cancer; EGC, early gastric cancer

negative group, respectively. But no difference was noted in lymph node metastasis between CEA mRNA positive and negative group. Two (No. 16, 26) of three patients, who were positive for CEA mRNA preoperatively, developed early relapse in 1 month and 2 months, respectively. Meanwhile, only two (No. 4, 13) of 13 CEA mRNA negative patients developed relapse after 16 months and 36 months postoperatively. One stage III gastric cancer patient (No. 13) was negative initially, but during the follow-up CEA mRNA turned positive and one month later he was diagnosed to have multiple bone metastasis.

In 24 colorectal cancer patients, CEA mRNA was detected in nine patients (37.5%). Whereas 5 out of 9 (55.6%) metastatic or relapsed cancers were positive for CEA mRNA, 4 out of 15 (26.7%) Duke stage B/C showed positive (Table 2, Fig. 3). No difference was noted

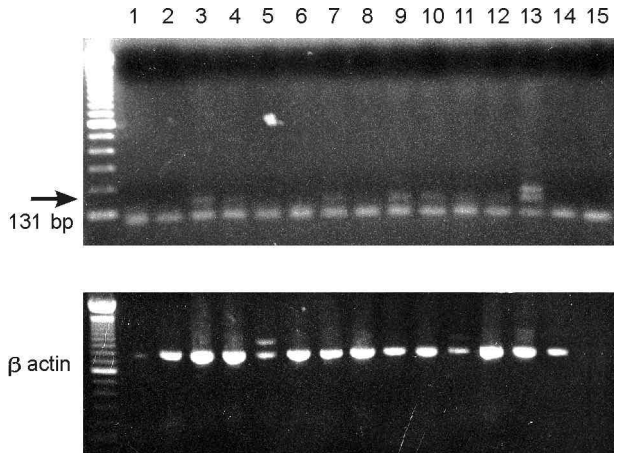

Fig. 2. Representative RT-PCR data for CEA mRNA in gastric cancer patients. Lane 1-4: early gastric cancer, Lane 5-8: advanced gastric cancer, Lane 9-12: Metastatic or relapsed, Lane 13: Colo201 cells as a positive control, Lane 14: normal healthy blood as a negative control. Lane 15: dH_20 without RNA as a negative cotnrol. β actin serves as an internal control.

Table 2. Patients characteristics in colorectal cancer

Patient	Age	Sex	Status	Stage	CEA mRNA
1	59	M		B2	−
2	53	F	Relapsed	D	−
3	61	M	Metastatic	D	+
4	40	F	Metastatic	D	+
5	64	F		B2	−
6	60	M		B2	−
7	38	M	Metastatic	D	−
8	61	F		C2	−
9	40	M	Relapsed	D	−
10	50	F		C2	−
11	57	M		C2	−
12	40	M	Relapsed	D	−
13	41	M		C2	−
14	52	M	Metastatic	D	+
15	57	M		B2	+
16	38	M		B2	−
17	45	F		C2	−
18	60	F		C2	+
19	34	M		C2	−
20	67	M	Metastatic	D	+
21	34	M		B2	+
22	62	M		B1	+
23	45	F		C2	−
24	50	F	Metastatic	D	+

Stage, modified Dukes classification

between Duke B/C and Duke D in CEA mRNA positive rate (p=0.212). Two out of 4 esophageal cancers and one out of 4 biliary tract cancer patients were also positive for CEA mRNA (Table 3, Fig. 4).

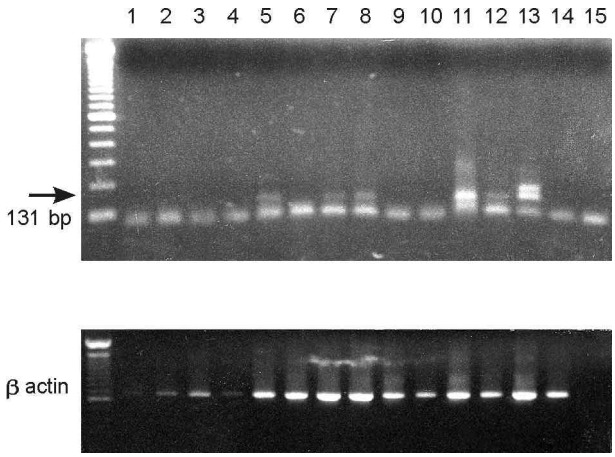

Fig. 3. Representative RT-PCR data for CEA mRNA in colorectal cancer patients. Lane 1-8: Duke stage B and C, Lane 9-12: Duke stage D, Lane 13: Colo 201 cells as a positive control, Lane 14-15: normal healthy blood and dH_20 without RNA as a negative control, respectively. β actin serves as an internal control.

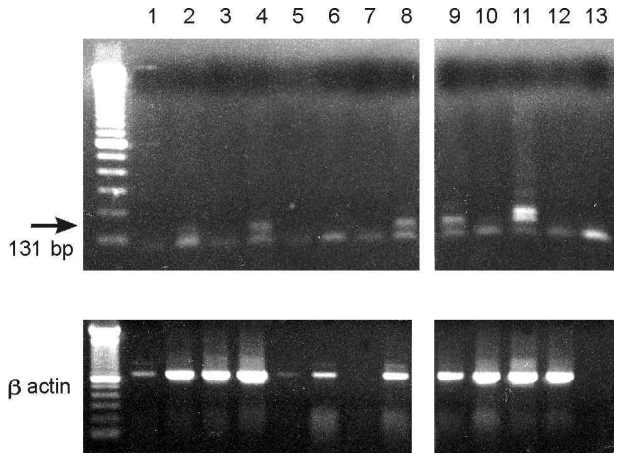

Fig. 4. Representative RT-PCR data for CEA mRNA in esophageal and biliary tract cancer patients. Lane 1-4: esophageal cancer, Lane 5-8: biliary tract cancer, Lane 9-10: advanced gastric cancer patients who were negative initially and converted to positive during the follow-up, Lane 11: Colo 201 cells as a positive control, Lane 12-13: normal healthy blood and dH_20 without RNA as a negative control, respectively. β actin serves as an internal control.

Table 3. Patients characteristics in esophageal cancer and biliary tract cancer

Patient	Age	Sex	Stage	CEA mRNA
1	65	M	IV	-
2	60	M	IV	+
3	65	M	IV	-
4	76	F	III	+
5	50	M	IV	-
6	38	M	IV	-
7	66	M	IV	+
8	57	M	III	-

No. 1-4, esophageal cancer; No. 5-8, biliary tract cancer

DISCUSSION

Our study demonstrated that 28 out of 67 (41.8%) patients with various kinds of gastrointestinal tract cancer showed CEA mRNA positive, suggesting that significant numbers of patients are contaminated with circulating malignant cells in peripheral blood (Table 4). The current study revealed that 6 (24%) out of 25 gastric cancer patients who underwent curative resection showed CEA mRNA positive, whereas CEA mRNA was detected in the peripheral blood samples of all 10 (100%) metastatic or relapsed patients. Also in colorectal cancer, 4 (26.6%) of 15 resectable stage and 5 (55.6%) of 9 metastatic cancer also showed CEA mRNA positive. These findings support the hypothesis that solid cancers are systemic diseases and can disseminate into the microcirculation (8, 9). It is also very interesting to note that many patients with early gastric cancer (3/9, 33.3%) or Duke stage B patients (3/7, 42.8%) are positive for CEA mRNA, indicating that cancer cells can disseminate even in the early stage of disease. Postoperative adjuvant chemotherapy is highly recommended for those patients who are positive for CEA mRNA.

For the detection of tumor cells in lymph node or bone marrow, immunocytochemical methods are most commonly used (10, 11). But those methods have insufficient sensitivity (12, 13), and the interpretations are subjective and time-consuming because a large number of blood cell smears have to be inspected. The recent development of molecular technology makes it possible to detect small

Table 4. Summary of CEA mRNA results in gastrointestinal tract cancer patients

GI tract cancer	No. of positive / No. of patients (%)			
	Early	Advanced	Metastatic or relapsed	Total
Gastric	3/9 (33%)	3/16 (19%)	10/10 (100%)	16/35 (45.7%)
Colorectal	3/7 (43%)	1/8 (13%)	5/9 (56%)	9/24 (37.5%)
Esophageal		1/1 (100%)	1/3 (33%)	2/4 (50%)
Biliary tract		0/1 (0%)	1/3 (33%)	1/4 (25%)

numbers of circulating cancer cells in the peripheral blood or bone marrow (3). The highly sensitive RT-PCR is widely used to detect a tumor cell among 10^6 mononuclear cells (5, 6, 14-18). In our study, we can detect CEA mRNA by RT-PCR in 10 tumor cells out of 10^6 mononuclear cells with the same sensitivity as previously described (5, 6).

The most commonly used markers for the detection of tumor cell contamination are cytokeratin (CK8, CK18, CK19, or CK20) based on specific traits of the tissue from which most of epithelial cell tumors originate (14-16). Also certain antigens expressed by tumor cells such as CEA, estrogen receptors, or tyrosine hydroxylase are used (5, 6, 18). But the markers for detecting tumor cells in blood samples should not be expressed by peripheral blood mononuclear cells. In that sense, cytokeratin markers (CK8, CK18, CK19) or estrogen receptors are not considered adequate for detecting tumor cells because they are expressed to a greater or lesser extent by peripheral blood mononuclear cells (17, 18). We found that CEA mRNA was not detected in peripheral blood mononuclear cells from the normal healthy subjects, suggesting CEA mRNA may be a reliable and reproducible marker for the detection of tumor cell contamination.

Many studies have reported that tumor cell contamination in bone marrow is associated with poor clinical prognosis such as tumor stage, nodal status, and tumor grading and also associated with early relapse in breast cancer patients (2). It has been reported that the detection of tumor cells in the peripheral blood correlated with clinical stage and was an independent prognostic factor for recurrence of disease in malignant melanoma (19, 20). Recently, Mori et al. reported that among 62 gastric and breast cancer patients who underwent curative surgery, CEA mRNA was detected in 12 cases and four of these 12 cases developed metastatic disease after surgery whereas none of 50 cases negative by RT-PCR developed metastasis (21). Our study also showed that two of three resectable advanced gastric patients developed recurrence in two months postoperatively, whereas only two out of 19 CEA mRNA negative patients developed relapse, and the relapse free interval was 16 months and 36 months, respectively. These findings suggest that tumor cell contamination may be associated with early relapse in gastric cancer. We also found that many patients with early gastric cancer or early stage colorectal cancer were positive for CEA mRNA, but no recurrence was noted in those patients. The clinical significance of CEA mRNA positive in these patients group should be elucidated in the near future.

In conclusion, our study indicates that the identification of circulating tumor cells using RT-PCR to detect CEA mRNA in gastrointestinal cancer is feasible, and this analysis may be a simple and promising tool for early detection of micrometastatic circulating tumor cells in patients with gastrointestinal tract cancer. Further studies to determine whether the presence of circulating malignant cells is associated with clinical prognosis will need the enrollment of large number of patients and serial follow-up.

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