Clinical significance of circulating tumor cells in blood by molecular detection and tumor markers in esophageal cancer

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Background. *The clinical significance of circulating tumor cells in the blood during surgery has not been elucidated in esophageal squamous cell carcinoma (ESCC). We evaluated the relationship between circulating tumor cells and clinicopathologic findings, compared with that of serum squamous cell carcinoma (SCC) antigen and carcinoembryonic antigen (CEA), in ESCC.*

Methods. *Blood samples from 54 consecutive patients were obtained from the peripheral artery and the superior vena cava at three points in time: immediately before surgery, and before and after tumor resection. CEA-specific reverse transcriptase-polymerase chain reaction (RT-PCR), which can quantify circulating tumor cells in blood, was performed. The preoperative values of serum SCC antigen and CEA were also obtained for all patients.*

Results. *CEA messenger RNA (CEA mRNA) was detected in the blood of 31 out of 54 patients (57.4%). CEA mRNA positivity was detected most frequently after tumor resection and correlated with nodal status and stage grouping. The incidence of total recurrence and blood-borne recurrence was significantly greater in patients with CEA mRNA positivity than in those with CEA mRNA negativity (P = .036 and .0026, respectively). Preoperative serum levels of SCC antigen and CEA did not correlate with clinicopathologic findings and tumor recurrence.*

Conclusions. *CEA mRNA detected by RT-PCR was more predictive of tumor recurrence than serum tumor markers. Effective adjuvant therapy is recommended for patients with CEA mRNA positive expression. (Surgery 2003;133:162-9.)*

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PATIENTS WITH ADVANCED esophageal squamous cell carcinoma (ESCC) have frequent tumor recurrence even after R0 resection.[1] Blood-borne recurrence is one of the most common modes in gastrointestinal carcinoma. Shedding of cancer cells into the bloodstream during surgical manipulation of a primary tumor has been observed in animal models.[2,3] Furthermore, shedding of cancer cells into the bloodstream increased the rate of distant metastasis.[4,5] Recently, using reverse transcriptase-polymerase chain reaction (RT-PCR), isolated tumor cells in the lymph nodes, bone marrow, and blood have been detected in patients with ESCC.[6,7] Various genes are currently used to detect circulating tumor cells in the blood. Carcinoembryonic antigen (CEA) is one of the most widely expressed genes in cancer cells, occurring in 92% to 95% of carcinomas of the gastrointestinal tract.[8] Since the CEA-specific RT-PCR method was established by Gerhard et al[9] in 1994, circulating tumor cells have been detectable in blood obtained from cancer patients.[10]

Serum CEA, first identified as a tumor-specific antigen,[11] is used in the preoperative evaluation of patients with colon and gastric cancer. The preoperative value of serum CEA is related to tumor growth and clinical outcome.[12-15] Squamous cell carcinoma-related antigen (SCC antigen), a subfraction of tumor antigen-4 (TA-4), was first extracted and purified from uterine SCC,[16] and the measurement of SCC antigen has predictive usefulness in SCC of the cervix and esophagus.[17-19]

In the current study, we performed CEA-specific RT-PCR on blood samples from the peripheral artery and the superior vena cava to investigate the presence or absence of circulating tumor cells in their relation to the time course of surgery. We compared CEA mRNA expression and conventional serum tumor markers in relation to clinicopatho-

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logic factors, including tumor recurrence, and especially blood-borne recurrence.

MATERIAL AND METHODS

Study group. The subjects of this study were 54 consecutive patients with esophageal cancer who underwent R0 resection in the First Department of Surgery in Kagoshima University between January 1997 and April 1999. There were 52 men and 2 women ranging in age from 38 to 83 years (mean, 65.3 years). Based on the TNM classification,[20] 16 patients had pT1 tumors, 6 patients had pT2 tumors, 24 patients had pT3 tumors, and 8 patients had T4 tumors. Eight tumors were located in the upper one-third of the esophagus, 22 tumors in the middle one-third, and 24 tumors in the lower one-third. Pathologically, all tumors were SCCs (12 well-differentiated, 25 moderately differentiated, and 17 poorly differentiated). Radiochemotherapy before surgery was performed in 14 patients. Neoadjuvant therapy consisted of radiotherapy of 40 Gy and chemotherapy using 2 anticancer agents, cisplatin (5 mg/m^2) and 5-fluorouracil (350 mg/m^2) for 4 weeks.

Blood samples. Blood samples were obtained from 54 patients with esophageal carcinoma, 20 patients with benign disease (15 with cholecystolithiasis, 3 with inflammatory bowel disease, and 2 with benign pancreatic disease), and 20 healthy volunteers. We obtained consent for blood collection from all patients and healthy volunteers. All subjects, excluding the 20 healthy volunteers, underwent esophagectomy. During general anesthesia, blood samples from the peripheral artery were obtained from a catheter used to monitor arterial blood pressure. During general anesthesia, blood samples from the superior vena cava were obtained from a catheter used to monitor central vein blood pressure.

Blood samples were obtained three times, once before and twice during surgery. For the purposes of this study, the phrase "before surgery" is defined as just after anesthesia, "before resection" is defined as just after thoracotomy, and "after resection" is defined as just after tumor resection. To prevent any contamination of epithelial cells, 5-mL samples of heparinized blood were obtained from each of three catheters inserted into the peripheral artery and the superior vena cava. Peripheral venous blood was used as the blood sample from healthy volunteers. The initial 20 mL of blood was discarded to protect the mixture from epithelial cells. In this report, we refer to the blood samples obtained once before and twice during surgery as "in-operation" blood samples.

Cell lines. To prepare for CEA-specific RT-PCR, 3 carcinoma cell lines, TE-1 (esophageal cancer cell line), MCF-7 (breast cancer cell line), and MKN-45 (gastric cancer cell line) were used. Lymphocytes were collected from healthy volunteers with no signs of epithelial malignancy. After lymphocytes were isolated from peripheral blood by gradient centrifugation, the mononuclear cell layer was collected. Cell lines were serially 10-fold diluted in 2-5 x 10^7 lymphocytes, to give ratios ranging from 1:10 to 1:10^7 carcinoma cells:lymphocytes.

RNA extraction. The fraction of nucleated cells was obtained using Mono-poly resolving medium (Dainippon Pharmaceutical, Osaka, Japan). Blood samples were diluted by the addition of 2 mL of 0.05 M phosphate-buffered saline (PBS). Diluted samples were layered on 4 mL of Mono-poly resolving medium. Each sample was centrifuged at 400 X *g* at room temperature for 20 minutes. The cell layer was then collected into 40 mL of PBS. The fraction of nucleated cells was collected after being centrifuged at 250 X *g* at 4°C for 15 minutes. The resulting fraction of nucleated cells was suspended in 1 mL of ISOGEN (Nippon Gene, Toyama, Japan) and stored at -80°C until use.

Total RNA was extracted using ISOGEN. The RNA extraction method used conformed to the manufacturer's protocol. Total RNA was dissolved in 10 μl of diethyl pyrocarbonate (DEPC)-treated water. The volume and quality of obtained total RNA was assessed by an absorption measurement at 260 nm and 280 nm using UV-Visible Spectrophotometer (BioSpec-1600; Shimadzu, Kyoto, Japan). Prior to the synthesis of complementary DNA (cDNA), Deoxyribonuclease I (DNase I) (Stratagene, La Jolla, Calif) was added to each sample of total RNA. A half-unit of DNase I and 1 μl of 10x Buffer (Takara Shuzo, Otsu, Japan) were added to 5 μg of total RNA in a total volume of 9.5 μl. The reaction mixture was incubated for 60 minutes at 37°C. Next, 1.5 μl of 20-mM EDTA was added. An 11-μl aliquot of reaction mixture was incubated for 20 minutes at 70°C and quickly chilled on ice.

After 50 ng of random hexamer was added, 5 μg of total RNA in a volume of 12 μl was incubated for 10 minutes at 72°C and quickly chilled on ice. One μl of 10x Buffer, 2 μl of 25-mM $MgCl_2$, 1 μl of 0.1 M dithiothreitol (DTT), 1 μl of 10-mM deoxynucleotide triphosphate (dNTP) mix, and 2 μl of DEPC water were added to the reaction mixture. A 19-μl aliquot of reaction mixture was incubated for 10 minutes at 25°C. Next, 100 units of SuperScript II Reverse Transcription (RT) were added to the reaction mixture, which was then incubated for 15

minutes at 25ºC, 90 minutes at 42ºC, 15 minutes at 70ºC, and then quickly chilled on ice. One unit of *E. coli* ribonuclease H (RNase H; All Life Technologies, Rockville, Md) was added to the reaction mixture. A 20-µl aliquot of reaction mixture was incubated for 40 minutes at 37ºC. Next, 5 µl of Tris-EDTA was added to cDNA, which was then stored at -20ºC until use.

To monitor the synthesis of cDNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RT-PCR was performed using published GAPDH primer.[22] The GAPDH up-primer was 5'-TCCCATCACCATCTTCCCA-3' and the GAPDH down-primer was 5'-CATCACGCCACAGTTTCC-3'. Next, 0.5 µl of cDNA, 5 µl of 10x PCR Buffer, 200 µM of dNTP mixture, 0.2 µM of up-primer, 0.2 µM of down-primer, and 1.25 units of Taq DNA polymerase (all Takara Shuzo, Otsu, Japan) was added to a 40-µl sample of PCR mixture. Denaturation for 4 minutes at 95ºC was carried out.

Amplification was performed with denaturation for 4 minutes at 95ºC, annealing for 1 minute at 57ºC, extension for 50 seconds at 72ºC for 40 cycles, followed by a final extension for 9 minutes at 72ºC. The GAPDH RT-PCR product was separated using the 2% agarose gel electrophoresis in tris-acetate EDTA (TAE) buffer and visualized by ethidium bromide staining. The RT-PCR product was detected as a 390-base pair (bp) fragment.

RT-PCR. To more accurately detect CEA mRNA, we performed nested RT-PCR. We used 3 different CEA primers designed by Gerhard et al.[9] The CEA-A primer was 5'-tctggaacttctcctggtctctcagctgg-3', the CEA-B primer was 5'-TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC-3', and the CEA-C primer was 5'-GGGCCACTGTCGGCATCATGATTGG-3'. In the first RT-PCR, 0.5 µl of cDNA, 2 µl of 10x PCR Buffer, 200 µM of dNTP mixture, 0.2 µM of CEA primer A and primer B, and 0.5 units of Taq DNA polymerase (all Takara Shuzo, Otsu, Japan) were added to a 20-µl aliquot of reaction mixture. Denaturation for 4 minutes at 95ºC was carried out. Amplification was performed with denaturation for 30 seconds at 95ºC, annealing for 1 minute at 69ºC, extension for 30 seconds at 72ºC for 30 cycles, followed by a final extension for 6 minutes at 72ºC. In nested RT-PCR, 1 µl of first PCR product, 4 µl of 10x PCR Buffer, 200 µM of dNTP mixture, 0.2 µM of CEA primer B and primer C, and 1 unit of Taq DNA polymerase (all Takara Shuzo, Otsu, Japan) were added to a 40-µl aliquot of reaction mixture. Denaturation for 4 minutes at 95ºC was carried out. Amplification was performed with denaturation for 30 seconds at 95ºC, annealing for 1 minute at 64ºC, and extension for 30 seconds at 72ºC for 32 cycles, followed by a final extension for 6 minutes at 72ºC. The CEA RT-PCR product was separated using 2% agarose gel electrophoresis in TAE buffer and visualized by ethidium bromide staining. The RT-PCR product was detected as a 131-base pair (bp) fragment. CEA-specific nested RT-PCR was performed twice per same sample. Samples were judged as positive for CEA mRNA only when 131-base pair fragment was detected in both assays. Three kinds of samples (water, the stock sample obtained from healthy volunteers, and the stock sample obtained from the MCF-7 cell line) were always used for quality control in each assay.

Determination of SCC and CEA in serum samples. Serum samples were obtained from all patients within the 2 weeks before surgery. Serum levels of SCC and CEA were determined using a commercial enzyme immunoassay kit (SCC antigen and CEA; Dainabot, Tokyo, Japan). Patients whose serum levels were greater than 1.5 ng/mL SCC, or 5 ng/mL CEA, were usually considered to be SCC positive, or CEA positive, respectively.

Clinical follow-up. All patients were followed up after discharge by physical examination, routine blood tests, serum tumor marker tests and x-ray examination every 1 to 3 months, by computed tomography every 3 to 6 months, and by ultrasonography every 6 months. Follow-up data after surgery were obtained for all patients, with a median follow-up period of 30 months (range, 2-60 months).

Statistical evaluation. Data were analyzed statistically using the chi-square test and Student *t* test. A *P* value of less than .05 was considered to be significant.

RESULTS

Sensitivity of CEA mRNA by RT-PCR. CEA mRNA was detected in TE-1, MCF-7, and MKN cell lines. The lower limit of detection was CEA-positive cancer cells diluted among 1 X 10^6 normal lymphocytes. No signals of CEA mRNA were detected in either blood from patients with noncancerous disease or in lymphocytes from healthy volunteers.

Relationship between CEA mRNA expression and tumor markers, and clinicopathological findings. Of 54 patients with esophageal carcinoma, 31 patients (57.4%) were positive for CEA mRNA (Fig 1). CEA mRNA positivity occurred in 23 (42.6%) in the peripheral artery and in 17 (31.5%) in the superior vena cava. CEA mRNA positivity did not differ significantly between the two blood sample sites (*P = .32)*. When detection of CEA mRNA before surgery, before resection,

Table I. Expression of CEA mRNA during surgery

Before surgery	*Before resection*	*After resection*	*Number of patients*
-	-	-	23
-	+	-	1
-	-	+	21
-	+	+	4
+	-	-	2
+	-	+	3
+	+	-	0
+	+	+	0

Table II. Clinicopathologic findings according to RT-PCR results

	RT-PCR(+) (n = 31)	*RT-PCR(-) (n = 23)*	*p value*
Age (mean ± SD)	62.3 ± 8.6	69.3 ± 6.7	0.002
pT			0.21
pT1	7	9	
pT2	2	4	
pT3	16	8	
pT4	6	2	
pN			0.011
pN0	6	12	
pN1	25	11	
pM			0.98
pM0	23	18	
pM1	8	5	
Stage			0.048
I	2	7	
IIA	1	3	
IIB	4	4	
III	16	4	
VIA	3	1	
VIB	5	4	
Location			0.72
Upper	4	4	
Middle	14	8	
Lower	13	11	
Histology			0.98
Well	7	5	
Mod	14	11	
Poor	10	7	
Lymphatic invasion			0.16
Positive	23	12	
Negative	8	11	
Venous invasion			0.16
Positive	13	8	
Negative	8	11	
Neoadjuvant therapy			0.77
Yes	9	5	
No	22	18	

Well, Well-differentiated squamous cell carcinoma; mod, moderately-differentiated squamous cell carcinoma; poor, poorly-differentiated squamous cell carcinoma.

and after resection was compared, it was found that 5 (9.3%) of 54 patients showed positive CEA mRNA signals before surgery, 5 (9.3%) before resection, and 28 (51.9%) after resection. Of the latter 28 patients, 21 had positive CEA mRNA expression only after resection. CEA mRNA expression before resection only was detected in 1 patient, and before surgery only in 2 patients (Table I).

Although the positivity rates for CEA mRNA tended to be higher as the tumor invaded deeper layers, significant differences were not found by

Table III. Clinicopathologic findings according to serum SCC or CEA level

	SCC(+) (n = 24)	*SCC(-) (n = 30)*	*p value*	*CEA(+) (n = 14)*	*CEA(-) (n = 40)*	*p value*
Age (mean ± SD)	66.7 ± 8.4	64.2 ± 8.6	0.30	64.9 ± 5.1	65.4 ± 9.5	0.85
pT			0.29			0.54
pT1	6	10		6	10	
pT2	2	4		1	5	
pT3	10	14		6	18	
pT4	6	2		1	7	
pN			0.77			0.91
pN0	7	11		4	14	
pN1	17	19		10	26	
pM			0.26			0.52
pM0	16	25		12	29	
pM1	8	5		2	11	
Stage			0.30			0.40
I	4	5		3	6	
IIA	1	3		0	4	
IIB	1	7		4	4	
III	10	10		5	15	
VIA	2	2		1	3	
VIB	6	3		1	8	
Location			0.44			0.95
Upper	3	4		2	5	
Middle	12	10		6	16	
Lower	9	16		6	19	
Histology			0.05			0.59
Well	9	3		2	10	
Mod	8	17		8	17	
Poor	7	10		4	13	
Lymphatic invasion			0.97			0.71
Positive	15	20		8	27	
Negative	9	10		6	13	
Venous invasion			0.64			0.55
Positive	8	13		4	17	
Negative	16	17		10	23	
Neoadjuvant therapy			1.00			0.04
Yes	6	8		7	7	
No	18	22		7	33	

Well, Well-differentiated squamous cell carcinoma; mod, moderately differentiated squamous cell carcinoma; poor, poorly differentiated squamous cell carcinoma.

tumor depth. The incidence of lymph node metastasis was higher in patients with CEA mRNA expression than in those without CEA mRNA expression ($P = .011$), and the positivity rate increased with tumor stage grouping ($P = .048$). With the exception of age, no other factors (eg, location, histology, lymphatic invasion, venous invasion, neoadjuvant therapy) were significantly associated with the expression of CEA mRNA (Table II). No significant associations were found between serum SCC antigen or CEA, except for neoadjuvant therapy and clinicopathologic factors (Table III).

Comparison between CEA mRNA expression and serum tumor markers. High values of serum SCC antigen and CEA were found in 24 (44.4%) and 14 (25.9%) patients, respectively. Of the 31 patients with RT-PCR-positive results, 15 (48.4%) and 6 (19.4%) patients were positive for SCC antigen and CEA, respectively. The relationships between CEA mRNA expression and serum SCC antigen or CEA were not significant (Table IV).

Prediction of tumor recurrence by CEA mRNA expression and tumor markers. Tumors recurred in 30 patients following surgery. The numbers of patients with blood-borne, both blood-borne and lymph node, lymph node, and local recurrence were 19, 3, 4, and 4, respectively. Disease recurred in 21 (67.7%) of the 31 patients with positive CEA mRNA expression and in 9 (39.1%) of the 23 patients with negative CEA mRNA expression. The incidence of recurrence was significantly greater in patients with CEA mRNA positivity than in those with negativity ($P = .036$). When only blood-borne recurrence was analyzed, the difference was highly significant, with 18 (58.1%) of 31 patients with CEA mRNA positivity experiencing disease recur-

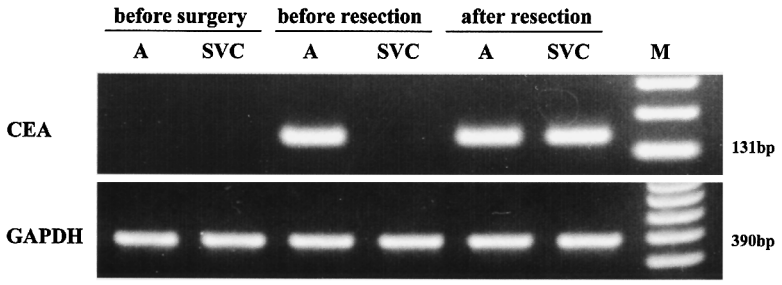

Fig 1. An example of expression of carcinioembryonic antigen (CEA) mRNA. CEA mRNA was positive in the artery before and after resection, and in the SVC after resection. *A*, peripheral artery; *SVC*, superior vena cava; *M*, marker.

Table IV. Relationship between RT-PCR results and tumor markers

	RT-PCR positive (n = 31)	*RT-PCR negative* (n = 23)	*p* value
SCC antigen			0.69
Positive	15	9	
Negative	16	14	
CEA			0.33
Positive	6	8	
Negative	25	15	

Table V. Mode of recurrence according to CEA mRNA expression

	CEA- mRNA expression		
Mode of recurrence	*Positive (n = 31)*	*Negative (n = 23)*	*p value*
Blood-borne	15	4	
Blood-borne and lymph node	3	0	p = 0.0026
Lymph node	1	3	
Local	2	2	
Total	21	9	p = 0.036

Table VI. Mode of recurrence according to serum SCC or CEA

	SCC antigen		*CEA*	
Mode of recurrence	*Positive* (n = 24)	*Negative* (n = 30)	*Positive* (n = 14)	*Negative* (n = 40)
Blood borne	9	10	4	15
Blood borne and lymph node	2	1	0	3
Lymph node	3	1	0	4
Local	1	3	1	3
Total recurrence	15	15	5	25

rence, compared with 4 (17.4%) of 23 patients with negativity ($P = .0026$) (Table V). Five patients with positive CEA mRNA expression before surgery had recurrent disease (2 lung metastasis, 2 liver metastasis, and 1 local recurrence). The mean period between surgery and recurrence in patients with positive CEA mRNA expression before surgery and in those with positive CEA mRNA expression before or after resection was 10.9 months and 19.1 months, respectively. Although the duration between surgery and recurrence was shorter in the former than in the latter, significant difference was not found ($P = .27$).Of 24 patients with high levels of SCC antigen, 15 (62.5%) experienced disease recurrence, compared with 15 (50%) of 30 patients with normal values of SCC antigen, a difference which was not significant. Five (35.7%) of 14 patients with CEA positivity and 25 (62.5%) of 40

patients with CEA negativity had recurrent disease, and again the difference in recurrence rates was not significant (Table VI).

DISCUSSION

One of the most crucial aspects of the diagnosis and treatment of patients with ESCC is the prediction of blood-borne metastasis. In the present study, we investigated circulating tumor cells in blood from 2 different vessels in relation to the time course of surgery. The detection rate of circulating tumor cells was not significantly different between the peripheral artery and the superior vena cava as collection sites, but the positivity rate increased significantly after tumor resection, compared with before surgery and before resection. In our previous report on gastric cancer, the incidence of positive CEA mRNA did not differ at the various blood sampling sites (the portal vein, peripheral artery, and superior vena cava), and the positivity rate of CEA mRNA was again higher after tumor resection than before resection.[21] Thus, the same pattern in detection rates of CEA mRNA by sample site and time course was found in both esophageal and gastric cancer. It is therefore suggested that tumor cells may detach from primary tumors and flow into the blood vessels as a result of surgical maneuvers.

In the current study, CEA mRNA expression was significantly related to lymph node metastasis and stage grouping. It has been reported that CEA mRNA expression is related to lymph node metastasis and lymphatic and vascular invasion in carcinoma of the stomach and the colorectum.[21-24] The presence of circulating tumor cells in blood may reflect tumor extension and the potential for tumor metastasis. Positive rates of serum CEA in ESCC ranged from 17% to 39%, and our result (25.9%) falls within this range.[25-28]

In the present study, the preoperative CEA value did not correlate with any clinicopathologic findings. Clark et al[28] also reported that there was no relationship between preoperative CEA elevation and the stage of the tumor or the patient's survival. However, postoperative CEA elevation was highly predictive of recurrent disease.[28] In our series, elevation of SCC antigen was found before surgery in 24 (44.4%) of 54 patients, but no clinicopathologic factors correlated with the value of SCC antigen. The positive rate of SCC antigen reportedly ranges from 17% to 43%.[19,25,26] Some authors[19] have reported that SCC antigen reflects tumor volume and clinical stage, but others[25,26,29] do not, including our data. Recently, serum CYFRA 21-1 was found to be of clinical significance in ESCC.[26,29] In the present study, we did not examine this marker in all patients, and so we could not compare CYFRA 21-1 with SCC antigen or CEA. CEA mRNA expression was not related to the elevation of SCC antigen or CEA, and our results indicated that CEA mRNA expression predicted tumor progression more accurately than did serum tumor markers.

Recurrent disease, especially blood-borne metastasis, was more frequently seen in patients with CEA mRNA positivity than in those with negativity. In our previous reports on gastric cancer, circulating tumor cells detected in the blood by RT-PCR during surgery were related to hepatic metastasis after surgery.[21] Taniguchi et al[23] reported that the presence of CEA mRNA in the blood was a useful indicator of circulating tumor cells and a major prognostic factor for patients with colorectal carcinoma. In our results, neither tumor markers, SCC antigen, nor CEA were related to tumor recurrence. These results indicate that CEA mRNA may prove to be the most important of those reviewed.

In the present study of esophageal carcinoma, we have demonstrated the significance of circulating tumor cells detected in the blood by RT-PCR for the prediction of recurrence, compared with serum SCC antigen and CEA. CEA mRNA expression was most accurate in predicting tumor recurrence after surgery for esophageal carcinoma. Patients with positive CEA mRNA have a high risk of blood-borne metastasis, and therefore effective chemotherapy is desirable to prevent tumor recurrence in such patients.

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