Circulating Tumor Cells in Perioperative Esophageal Cancer Patients: Quantitative Assay System and Potential Clinical Utility

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Abstract

Purpose: To establish a quantitative system for evaluating the role of circulating tumor cells (CTC) in peripheral blood samples in patients who undergo surgery for treatment of esophageal cancer.

Experimental Design: One hundred fifty-five peripheral blood samples from 53 esophageal cancer patients were collected before surgery (B-1), immediately after surgery (B0), and on the 3rd day postoperatively (B+3). Eighty-nine samples from 22 benign patients who underwent thoracotomy and 30 healthy volunteers were obtained as controls. A real-time reverse transcription-PCR quantitative analysis system based on carcinoembryonic antigen (CEA) mRNA gene expression was designed for detection of CTC.

Results: This developed system can detect CEA mRNA-positive cells down to 3 cells per milliliter of peripheral blood. The cells in negative control groups were lower than the detection limit. The medians of 188 [95% confidence interval (95% CI), 155-498], 1513 (95% CI, 660-7,974) and 707 (95% CI, 737-3,005) CEA mRNA-positive cells per mL with the *CEA*-positive rates of 28.3%, 60.4%, and 42.9% in B-1, B0, and B+3 peripheral blood samples were obtained, respectively. There was statistically significant difference between B-1 and B0 ($P = 0.0001$) and between B-1 and B+3 ($P = 0.0209$). Fifty percent of patients with $R > 0.4$ showed metastasis in 1 year after surgery, whereas the probability was only 14.3% for patients with $R < 0.4$ (where R is CTC ratio of B+3 to B0, $P = 0.043$).

Conclusions: Esophageal cancer operation results in tumor cells dissemination and significant increase of CTC in peripheral blood, which is related to the developed metastasis. CTC are helpful for evaluating micrometastasis and have the potential for predicting recurrence in esophageal cancer.

Esophageal cancer is one of the lethal cancers with a poor prognosis. Its survival rate is only 15% at 5 years (1) and remains less than 20% to 30% at 2 years even after surgical resection (2). Long-term observation suggests that the micrometastasis in esophageal cancer patients might exist before surgery, which is also enhanced upon surgical manipulation due to the shedding of tumor cells into bloodstream in animal studies (3–5). Although surgery is the current gold standard for treatment of this aggressive cancer, and the postoperative mortality has also been declined by improving the complete resection and using current treatment strategies such as adjuvant chemotherapy and radiotherapy after complete surgery, the presence of disseminated tumor cells in lymph nodes (6) or blood circulation (7, 8) is related to metastatic relapse, which remains the most frequent cause of cancer-related deaths (9). Thus, an accurate specific system for quantification of circulating tumor cells (CTC) in esophageal cancer patients is desired for monitoring the dissemination of tumor cells in peripheral blood, which is helpful to assess the extent of tumor cells dissemination in peripheral blood and the development of distant metastasis.

The micrometastasis of esophageal cancer patients has been studied using classic reverse transcription-PCR (RT-PCR) coupled with gel-electrophoresis measurement to detect the tumor cells in bone marrow (10), lymph node (11–14), and peripheral blood (15–17). However, this technique can only obtain the positive or negative result and is unable to reflect quantitatively the dissemination status of tumor cells. This work uses a real-time quantitative RT-PCR method for analysis of carcinoembryonic antigen (CEA) mRNA expression to achieve the quantification of CTC in esophageal cancer patients. As well known, real-time quantitative RT-PCR is a sensitive, quantitative, and contamination-free technique with numerous

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advantages over classic RT-PCR and relative quantitative PCR based on $2^{-\Delta\Delta Ct}$ method (18). It has been extensively applied to detect micrometastasis in breast (19–21), colorectal (22), prostatic (23), and lung cancers (24). Owing to the high efficiency of reverse transcription and PCR amplification, real-time quantitative RT-PCR could be extremely easily affected by various factors during manipulation, leading to possible conflicting results of CTC detection even if the used procedures are similar or the same. Thus, it is crucial to choose appropriate controls and standardize all the uncertain factors in each step for minimizing the systematic errors in quantification.

The gene encoding CEA is one of the most widely expressed genes in tumor cells. The CEA mRNA expression occurs in 92% to 95% of carcinomas of gastrointestinal tract (25). The *CEA*-specific RT-PCR method has been widely used to detect CTC in blood from cancer patients since established in 1994 (26). This study established a standardized quantitative assay system, with which the numbers of tumor cells in peripheral blood during surgical manipulation could be detected accurately and directly. The results indicated the dissemination of tumor cells in circulation during and after surgery could be monitored, which could be further developed for evaluating micrometastasis and predicting recurrence in esophageal cancer.

Materials and Methods

Patients. According to the rules of the local ethical committee, 155 peripheral blood samples were collected from 53 patients without distant metastasis of esophageal squamous carcinoma (mean age of 58.1) during September to December 2005 in the Tumor Hospital of Jiangsu Province. These samples were collected at three time points, before surgery (B-1), immediately after surgery (B0), and the 3rd day postoperatively (B+3), by central venipuncture after discarding the first 5 mL of blood to avoid epithelial contamination from venipuncture.

Fifty-nine peripheral blood samples were collected from 22 patients without any evidence of malignancy, who underwent thoracotomy over the same period (seven peripheral blood samples missed at B+3 time point) to compose an ideal negative control group. Thirty peripheral blood samples from 30 healthy volunteers formed another negative control group.

Eight tissue samples were obtained from these patients during surgery and shock-frozen in liquid nitrogen for later RNA extraction as positive quality controls for real-time quantitative PCR.

Cell line and cell culture. The human esophageal squamous carcinoma cell line TE-10 expressing *CEA* gene was obtained from Shanghai Institute for Biological Sciences and cultured in RPM1 1640 containing 2 mmol/L glutamine and supplemented with 15% fetal serum at 37°C in air containing 5% CO_2.

To assess the sensitivity and get a calibration curve of the system for CEA mRNA detection, TE-10 cells grown in monolayer were collected by digesting the monolayer with PBS (pH 7.4) containing 0.25% trypsin and 0.53 mmol/L EDTA for 5 min at 37°C. After the digested monolayer was washed with PBS, the obtained cells were counted, and their viability was assessed by trypan blue dye exclusion. Finally, the TE-10 cells were serially diluted with PBS and mixed with 2 mL blood obtained from healthy volunteers to form a concentration gradient of tumor cells ranging from 1 to 10^5 cells per milliliter of blood.

Sample processing and cDNA synthesis. Five milliliters of each peripheral blood sample were divided as follows: 1.0 mL for counting monocytes, 2.0 mL for RNA extraction, and 2.0 mL for repetition. The samples for RNA extraction were processed immediately after collection and distribution to avoid RNA degradation. Mononuclear blood cells were firstly harvested by density centrifugation (1,800 rpm for 20 min) with lymphocytes separation medium and washed with PBS (800 rpm for 10 min). They were then counted. The total RNA in these cells was extracted using TRI REAGENT (Molecular Research Center, Inc.), and its purity and quality were measured by UV-visible spectrophotometer (Bio-Tek); 1% agarose gel electrophoresis was used to assess the integrity of the obtained RNA. cDNA with a total volume of 20 μL was synthesized using commercially available RevertAid First Strand cDNA Synthesis kit #k1622 containing reverse transcriptase (Fermentas) according to the recommended protocol by the manufacturer.

Real-time quantitative PCR. Real-time quantitative PCR of the target *CEA* gene and *GAPDH* as internal control was carried out with icycler iQ Multicolor Real-time PCR Detection System (Bio-Rad Laboratories, Inc.). According to their sequences, amplification primers and Taqman probes of the two genes were synthesized (DaAn Gene Co. Ltd.) as follows: *CEA*, forward primer 5′-CCGCATACAGTGGTCGAGAGA-3′, reverse primer 5′-GTGTAGGGTGTAGAATCCTGTGTCA-3′, probe 5′-CCCAATGCATCCCTGCTGATCCAG-3′; *GAPDH*, forward primer 5′-CCACCCATGGCAAATTCC-3′, reverse primer 5′-TGGGATTTCCATTGATGACAAG-3′, probe 5′-TGGCACCGTCAAGGCTGAGAACG-3′.

The probes were labeled at the 5′-end with reporter dye molecule FAM and the 3′-end with quencher dye TAMRA. The primers were designed to extend across at least one intron so that eventual DNA contamination would not pose a significant problem. Corresponding length of PCR products of CEA and GAPDH mRNA was 92 and 69 bp, respectively. PCR was done with 400 ng of total RNA.

Calculation of results and experimental controls. PCR product of CEA mRNA in positive control was purified and cloned into pMD-18T. The recombination plasmids were further purified and quantified with UV-visible spectrometry. The obtained solution was diluted at different dilutions to prepare the standard solutions (N_1, copies per microliter). A curve showing the linear correlation between the cycle threshold (C_t) value and logarithm of plasmids or CEA mRNA copies of the quantified vectors was then obtained. This calibration curve was modified for each run to eliminate the errors caused by the difference in PCR efficiency. The calibration curve showing the correlativity between the total CEA mRNA copies (N_2) and the number of CEA mRNA-positive cells per milliliter was obtained with the serial diluted suspensions of TE-10 cells as standard samples.

The number of CEA mRNA-positive cells was calculated with the obtained C_t value and the two calibration curves obtained above. N_1 was first obtained from the C_t value. N_2 was then calculated according the equation: $N_2 = \frac{N_1 \times 20 V_1}{V_2}$, where 20 was the total volume of cDNA synthesized in microliters, V_1 was the total volume of total RNA extracted from 2.0 mL peripheral blood sample, and V_2 was the volume corresponding to 2.0 μg total RNA for reverse transcription, which was ascertained by UV-visible spectrometry at A_{260}.

The reliability of the results was assessed by comparing the expression of GAPDH or CEA mRNA with those from preliminary experiments. Total RNA was extracted from 10 healthy volunteers for GAPDH mRNA and from positive quality control for CEA mRNA. Real-time quantitative PCR was done for 10 times with 400 ng of total RNA each time, from which the average C_t values ($\bar{C}_t$) and deviations ($\bar{d}$) for GAPDH and CEA mRNA were calculated, respectively. If the obtained C_t value of GAPDH mRNA was beyond the range of $\bar{C}_t \pm 4\bar{d}$, or that of CEA mRNA in each run was not in the range of $\bar{C}_t \pm$ SD, we ran the sample again.

Statistics. Data analysis was carried out with Stata statistical packages. Mann-Whitney test was used to compare the CEA mRNA levels in peripheral blood between (*a*) B-1 and B0 and (*b*) B0 and B+3. When all results of CEA mRNA expression were negative at B-1, B0, and B+3, the data would be abandoned in statistics. When one or two of three results were positive, the other expression would be treated as 1 for statistical convenience. The relationship between the amount of CEA mRNA-positive cells in peripheral blood sample and classic prognostic factors, such as the tumor location, size, tumor-node-metastasis stage, and pathologic stage, was investigated using Man-Whitney test (for comparison between two groups) or Kruskal-Wallis test (for comparison among three groups), respectively. The

relationship between expression of CEA mRNA and classic prognostic factors was analyzed using the χ^2 test. $P < 0.05$ was considered significant.

Results

Evaluation of the system. In preliminary experiments, CEA and GAPDH mRNA extracted from TE-10 cells were amplified. No amplification product was detectable in absence of RT enzyme, indicating the primers were appropriately designed to avoid the contamination from DNA derived from the processed pseudogene. All eight tissue samples showed positive results after real-time quantitative RT-PCR of CEA mRNA. The $\bar{C}_t$ and SD values of target CEA mRNA in the positive control were 29.7 and 0.91 (n = 10, inter-assess), whereas these values of GAPDH mRNA from healthy volunteers were 14.8 and 0.33 (n = 10, inter-assess), respectively.

The calibration curves for detection of mRNA-positive cells were shown in Fig. 1. The minimum amount of 3 CEA mRNA-positive cells per milliliter of peripheral blood could be detectable, which was used as the detection limit of the proposed method. To assess the effect of leukocyte concentration in peripheral blood on the detection, 2×10^3 TE-10 cells were added to 2.0 mL peripheral blood containing different concentrations of leukocytes (5×10^6 to 1×10^7 per milliliter). The results of detectable TE-10 cells gave a coefficient of variation of 9.5% (n = 10, inter-assess).

The cells expressing CEA mRNA were lower than the detection limit in either the samples from 22 patients without

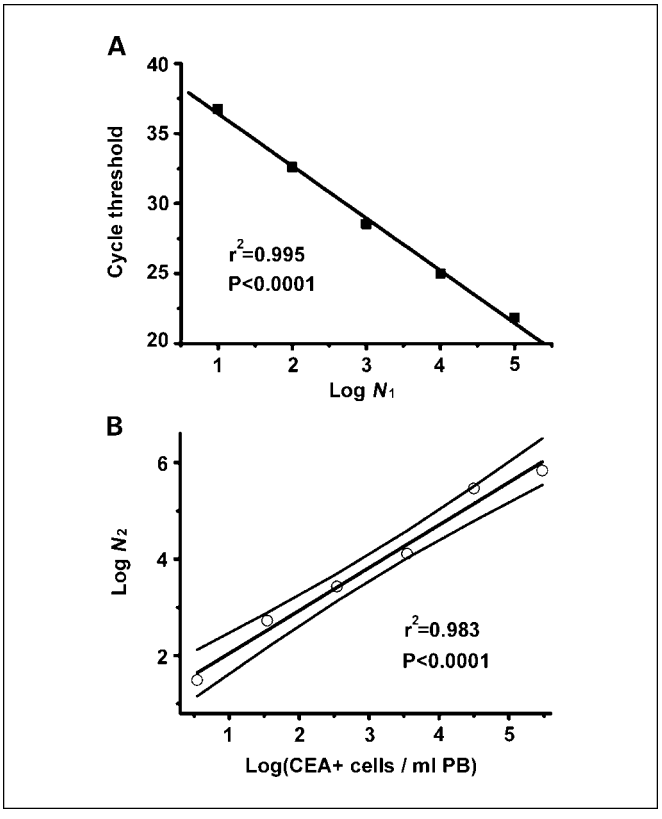

Fig. 1. Calibration curves for quantification. *A,* CEA mRNA copies. *B,* CEA mRNA-positive cells. 95% confidence interval. N_1, CEA mRNA copies per microliter; N_2, standardized CEA mRNA copies.

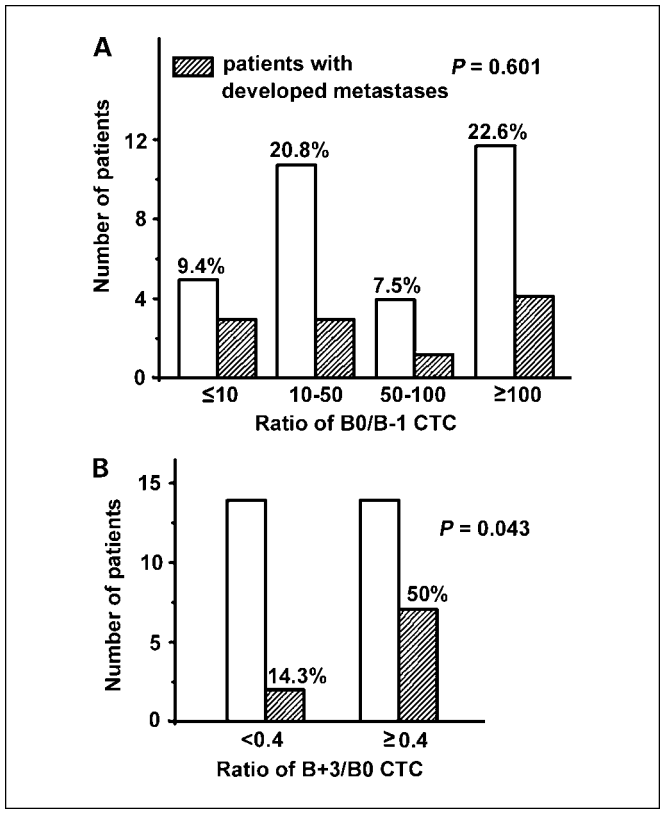

Fig. 2. Distributions of CEA mRNA-positive cells numbers and developed metastasis. *A,* B0 to B-1; *B,* B+3 to B0. If cells number was lower than the detection limit, its value was treated as 2 for calculating convenience. The percentages were the proportion of the patient number with marked ratios in (*A*) 53 patients and (*B*) 14 patients. *P* values were calculated using χ^2 test.

any evidence of malignancy who underwent thoracotomy at three time points or the samples from 30 healthy volunteers. Although the scale of the control group was not large due to the limited amount of such patients in our hospital during the period, data from these two groups could be regarded as competent controls.

Detection of CEA mRNA-positive cells in peripheral blood samples from esophageal cancer patients. One hundred fifty-five peripheral blood samples from 53 esophageal cancer patients (four samples of B+3 were absent because of sampling failure) were analyzed. The medians of CEA mRNA-positive cells at B-1, B0, and B+3 were 188 (95% confidence interval, 155-498), 1,513 (95% confidence interval, 660-7,974), and 707 (95% confidence interval, 737-3,005) cells per milliliter of peripheral blood, respectively. The change in the number of CEA mRNA-positive cells upon surgery was shown in Fig. 2A.

Using the minimum amount of detectable cells as a cutoff, 28.3% (15 of 53), 60.4% (32 of 53), and 42.9% (21 of 49) of B-1, B0, and B+3 peripheral blood samples were considered CEA mRNA positive, respectively. The CEA mRNA expression status in different samples was shown in Table 1. The distribution of CEA mRNA-positive cells at different time points was illustrated in Fig. 3.

Clinical correlation. With different classic prognostic factors of gender, age, tumor location, size, depth, pathologic stage, and lymph node status, the mean numbers of detectable CEA mRNA-positive cells in peripheral blood collected at the same time points did not show statistical difference except the mean

Table 1. Results of the real-time quantitative PCR for *CEA* expression

PB (B-1)	PB (B0)	PB (B+3)	No. patients (%)
+	+	+	12/49 (24.5)
+	+	-	2/49 (4.1)
-	+	+	9/49 (18.4)
-	+	-	6/49 (12.2)
-	-	-	20/49 (40.8)

NOTE: -, cell number is lower than the detection limit.

numbers at B0 for the ages <60 and >60. However, the positivity of CEA mRNA-expressed cells was statistically related to some factors, such as lymph node status and pathologic stage (Table 2). Patients with positive lymph nodes, who were known to have a worse survival, showed higher positivity of CEA mRNA-expressed cells than those with negative lymph nodes; and patients with stage III disease, who had a worse outcome than those with stage II, seemed to have a high positivity of CEA mRNA-expressed cells.

When 155 peripheral blood samples from 53 esophageal cancer patients were considered as three groups according to the time points, the median of CEA mRNA-positive cells showed statistically significant difference between B-1 and B0 ($P = 0.0001$) and between B-1 and B+3 ($P = 0.0209$), but no statistically significant difference occurred between B0 and B+3 ($P = 0.4396$). When the patients were divided into different subgroups according to each classic prognostic factor, statistically significant difference from B-1 to B+3 could be observed except two when the tumors were at the upper location and in the pathologic stage II (Table 3).

Follow-up. The follow-up of 45 patients who were successfully sampled at B+3 was available in 1 year. Thirteen patents showed the developed metastasis, and three of them died of the metastasis. In these 13 patients, four came from those showing undetectable CTC at all three sampling points, and nine came from 28 patients who showed detectable CTC at B0. The developed metastasis was statistically irrelative to the ratio of B0/B-1 CTC (Fig. 2A). To assess the correlation between the CTC level and metastasis possibility, another variable R was defined as the ratio of CTC values at B+3 to B0. Fifty percent (7 of 14) of patients who had the $R > 0.4$ produced the developed metastasis, which was significantly higher than 14.3% (2 of 14) of patients who showed the $R < 0.4$ ($P = 0.043$), as shown in Fig. 2B.

Discussion

Since its establishment, real-time quantitative RT-PCR has been considered as a sensitive method to detect CTC and applied in various carcinomas (27–29). It provides a formidable tool for cancer therapy personalization (30–32). However, experimental variation of this technique is easily brought due to the complicated sample processing and highly susceptive amplification (33, 34). The obtained results indicated acceptable specificity of the proposed procedure. No evidence indicated that the stimulating factors in thoracotomy manipulation could change the CEA mRNA expression, although cytokines, steroids, or environmental factors could modulate the expression of some tissue-specific genes such as *CK19* (35); 59.2% (29 of 49) of patients at B0 and 42.9% (21 of 49) of patients at B+3 showed CEA mRNA expression, and 20 patients (20 of 53, 40.8%) had no evidence of measurable CTC at B-1, B0, and B+3. These results were similar to those of 51.9% immediately after resection (17) and 36.8% in follow-up period (36). Such positive detection rate of CTC was due to the CTC level lower than the detection limit or absent *CEA* expression in esophageal cancer tumor cells, which could be overcome using multiple-marker detection (37).

This method could detect CEA mRNA-positive cells down to 3 cells per milliliter of peripheral blood, indicating high sensitivity due to the high RNA extraction efficiency and the standardized detection procedure. The leukocytes showed little effect on CEA mRNA detection. The coefficient of variation of 9.5% for CEA mRNA-positive cell detection at different concentrations of leukocytes indicated that the effect of leukocyte change during surgery was negligible. It means that the proposed calibration curve method was superior to relative quantitative PCR, and its sensitivity and specificity were enough to determine the CTC for elucidating the effect of esophageal cancer surgery on CTC in the peripheral blood.

The results showed that the peripheral blood samples from nine esophageal cancer (9 of 49, 18.4%) patients (5/4 for N_0/N_1 of lymph node status, 3/0/6 for I/II/III of pathologic stage, and 3/4/2 for $pT_1/pT_2/pT_3$ of tumor depth) without preoperative CTC were found to contain tumor cells at both B0 and B+3. Five of them showed the developed metastasis. Twelve esophageal cancer patients (12 of 49, 24.5%) showed peripheral blood–positive results of CEA mRNA expression at all three time points; moreover, the number of CEA mRNA-positive cells in peripheral blood samples was significantly elevated ($P = 0.0003$) from the median value of 209 to that of 4,062 following the tumor removal manipulation. The median value of 1,178 at B+3 was still higher than that before surgery, which did not significantly decrease when compared with the number of CEA mRNA-positive cells at B0. Although six patients (4/2 for N_0/N_1 of lymph node status, 3/1/2 for I/II/III of pathologic stage, and $pT_1/pT_2/pT_3$ of tumor depth) showed negative results of CEA mRNA expression in peripheral blood samples collected at both B-1 and B+3, all of them showed positive results at B0. The tumor resection led to significant

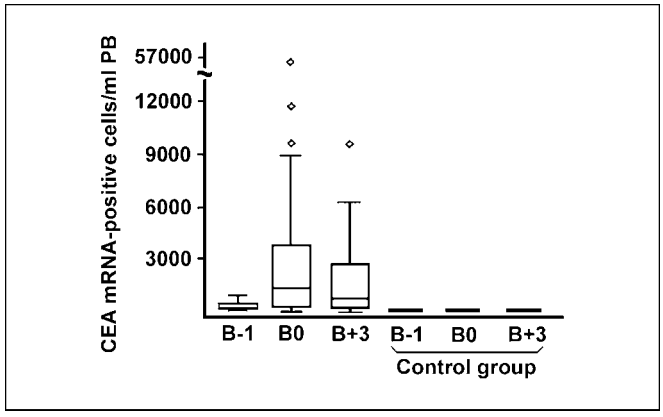

Fig. 3. Box-whisker comparative plots of the numbers of CEA mRNA-positive cells per milliliter of peripheral blood of esophageal cancer patients before surgery (B-1; $n = 53$), immediately after surgery (B0; $n = 53$), and on the 3rd day after surgery (B+3; $n = 49$).

increase of CEA mRNA-positive cells in peripheral blood samples. The facts supported the hypothesis that surgical manipulation would cause tumor cells to shed into peripheral blood. The disappearance of CEA mRNA-positive cells in peripheral blood samples collected from these six patients at B+3 might be attributed to the functions of body immune system because significant increases of T-cell counts were also observed in these patients (data not shown).

Pantel et al. (38, 39) suggested that the detection and characterization of disseminated tumor cells in patients with cancer could provide important information about the cascade of metastatic events, and it was important to know how many disseminated epithelial cells were actually of neoplastic origin. The extent of CEA mRNA-positive cells increased upon surgery revealed the proportion of CEA mRNA-positive cells entering into peripheral blood. Five patients showed a consecutive increase from B-1 to B+3 in the number of CEA mRNA-positive cells, and three of them had lymph node–positive disease, suggesting that tumor cells originated from other minimal focus might also enter into peripheral blood by the stimulation of surgical operation in these patients. The positivity of patients with lymph node–negative disease was 26.3%, 52.6%, and 34.2% at B-1, B0, and B+3, respectively, whereas it was 33.3%, 80%, and 53.3% for patients with lymph node–positive disease, respectively. Furthermore, the median value of CEA mRNA-positive cells at B+3 in the patients with the lymph node metastasis beside tumor tissue was 2,689 (n = 9), and the value in the patients with the lymph node metastasis at other location was 163 (n = 6). These results implied that the regional lymph nodes, particularly the lymph node metastasis beside tumor tissue, might lead to higher positivity of CEA mRNA cells or more tumor cells dissemination in peripheral blood. This information would be complementary with those reported with pathologically lymph node–negative patients (11, 40).

The survival status of CTC in peripheral blood was still unknown. Mehes et al. (41) reported that apoptotic CTC in peripheral blood had a significant percentage in breast cancer, whereas Meng et al. (42) observed the CTC could remain for as long as 22 years. Here, CEA mRNA-positive cells increased significantly at B0 and maintained at a higher expression level at B+3 than B-1. It suggested that the tumor cells not only shed into the bloodstream by esophageal cancer operation manipulation but also existed to some extent at B+3. This would increase the possibility of neoplasia. The data from follow-up in 1 year showed the increasing possibility. When the ratio of CTC values at B+3 to B0 was >0.4, 50% of patients produced the developed metastasis, whereas it occurred in only 14.3% of patients who showed the ratio < 0.4. At the same time, 69% of patients (9 of 13) with developed metastasis had detectable CTC at both B0 and B+3. Only 4 of 13 patients with no detectable CTC showed the developed metastasis due to other factors, which needed some new techniques for further study.

Another interesting observation was that the extent of tumor cells dissemination into peripheral blood by surgical manipulation was larger in esophageal cancer than those in breast cancer (11). The surgical manipulation of breast cancer showed the relative increase of median of *CK19*-positive cells in peripheral blood from 109 to only 179. These phenomena might be helpful to reveal the reason of high recurrence and poor survival in esophageal cancer patients. To testify this, further investigations with these patients would be carried out.

Table 2. Correlation between CEA mRNA-positive cells detectable at B-1, B0, or B+3 and classic prognostic factors in 53 esophageal cancer patients undergoing resection

Prognostic factors	B-1	B0	B+3
Gender			
P	0.609	0.226	0.201
Male	10/38 (26%)	21/38 (55%)	13/35 (37%)
Female	5/15 (33%)	11/15 (73%)	8/14 (57%)
Age (y)			
P	0.226	0.124	0.243
≤60	11/32 (34%)	22/32 (69%)	14/28 (50%)
>60	4/21 (19%)	10/21 (48%)	7/21 (33%)
Tumor location			
P	0.555	0.786	0.689
Upper	2/6 (33%)	4/6 (67%)	3/6 (50%)
Middle	12/39 (31%)	24/39 (62%)	16/36 (44%)
Lower	1/8 (13%)	4/8 (50%)	2/7 (29%)
Pathologic tumor size (cm)			
P	0.338	0.899	0.249
≤3.0	9/26 (35%)	15/26 (58%)	12/26 (46%)
3.0-5.0	5/17 (29%)	11/17 (65%)	8/16 (50%)
>5.0	1/10 (10%)	6/10 (60%)	1/7 (14%)
Pathologic stage			
P	0.814	0.032	0.044
0-I	6/22 (27%)	12/22 (55%)	8/21 (38%)
II	3/13 (23%)	5/13 (38%)	2/11 (18%)
III	6/18 (33%)	15/18 (83%)	11/17 (65%)
Tumor depth			
P	0.911	0.071	0.389
pT_{is}-pT_1	8/26 (31%)	14/26 (54%)	9/25 (36%)
pT_2	5/20 (25%)	11/20 (55%)	8/18 (44%)
pT_3	2/7 (29%)	7/7 (100%)	4/6 (67%)
Lymph node status			
P	0.609	0.014	0.201
N_1	5/15 (33%)	12/15 (80%)	8/14 (57%)
N_0	10/38 (26%)	20/38 (53%)	13/35 (37%)

NOTE: *P* values were calculated using χ^2 test.

Table 3. Difference of the amounts of peripheral blood CEA mRNA-positive cells of esophageal cancer patients from B-1 to B+3 classified with classic prognostic factors

Classic prognostic factors	*P*
Tumor location (*n*)	
Upper (6)	0.3836
Middle (8)	0.0001
Lower (39)	0.0384
Pathologic stage (*n*)	
0-I (22)	0.0030
II (13)	0.0749
III (18)	0.0001
Tumor depth (*n*)	
pT_{is}-pT_1 (26)	0.0020
pT_2 (20)	0.0008
pT_3 (7)	0.0074
Pathologic tumor size cm (*n*)	
≤3.0 (26)	0.0013
3.0-5.0 (17)	0.0016
>5.0 (10)	0.0028

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