Preoperative evidence of circulating tumor cells by means of reverse transcriptase–polymerase chain reaction for carcinoembryonic antigen messenger RNA is an independent predictor of survival in non–small cell lung cancer: A prospective study

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Objective: We conducted a prospective study of 103 consecutive patients with non–small cell lung cancer who underwent a curative lobectomy to test whether circulating tumor cells detected in the peripheral blood by means of reverse transcriptase–polymerase chain reaction of carcinoembryonic antigen messenger RNA is a prognostic indicator independent of tumor stage in patients with non–small cell lung cancer.

Methods: We assayed for carcinoembryonic antigen messenger RNA by means of reverse transcriptase–polymerase chain reaction in peripheral blood taken at the time of diagnosis before an operation and again 2 to 3 weeks after an operation from patients with non–small cell lung cancer who underwent a curative lobectomy between March 1996 and April 1998. We analyzed the prognostic value of carcinoembryonic antigen messenger RNA expression in the patients with non–small cell lung cancer in a univariate and multivariate manner.

Results: Patients with carcinoembryonic antigen messenger RNA in the preoperative blood samples had a poor survival when compared with those without carcinoembryonic antigen messenger RNA. Of these patients, the worst survival was seen in those with carcinoembryonic antigen messenger RNA in the postoperative blood samples. The multivariate stepwise analysis selected the preoperative carcinoembryonic antigen messenger RNA expression ($P = .0004$; relative risk, 0.21) and the pathologic stage of disease ($P = .0002$; relative risk, 1.43) as the independent prognostic factors for survival.

Conclusions: The molecular detection of carcinoembryonic antigen messenger RNA in the preoperative peripheral blood is an independent prognostic factor in patients with non–small cell lung cancer who undergo a curative operation.

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The detachment of tumor cells from the primary site and their consequent release into the circulation is one of the early events in the metastatic cascade. Although the presence of circulating tumor cells does not necessarily correlate with the subsequent appearance of systemic disease, some such tumor cells might have the potential to establish metastases and therefore might have a negative influence on patient prognosis. Because the number of circulating cells might be very small, methods for their detection need to be both sensitive and specific. Morphology, flow cytometry, conventional cytogenetics, and immunocytochemistry have been used to

TABLE 1. Patient characteristics

Characteristic (n = 103)	
Median age, y (range)	68 (35-83)
Sex, n (%)	
Male	76 (74)
Female	27 (26)
Histology, n (%)	
Adenocarcinoma	66 (64)
Squamous cell carcinoma	37 (36)
pT factor,*n (%)	
T1	40 (39)
T2	37 (36)
T3	26 (25)
pN factor, n (%)	
N0	70 (68)
N1	6 (6)
N2	27 (26)
pTNM stage, n (%)	
IA	32 (31)
(−) (−)	18
(+) (−)	10
(−) (+)	1
(+) (+)	3
IB	25 (24)
(−) (−)	16
(+) (−)	6
(−) (+)	0
(+) (+)	3
IIA	4 (4)
(−) (−)	2
(+) (−)	1
(−) (+)	0
(+) (+)	1
IIB	15 (15)
(−) (−)	3
(+) (−)	7
(−) (+)	1
(+) (+)	4
IIIA	27 (26)
(−) (−)	0
(+) (−)	11
(−) (+)	0
(+) (+)	16
Sequence of vessel ligation,*n (%)	
PV first	43 (42)
PA first	35 (34)
Mixed order	25 (24)
RT-PCR for CEA mRNA pattern, n (%)	
(−) (−)	39 (38)
(+) (−)	35 (34)
(−) (+)	2 (2)
(+) (+)	27 (26)

pT, Pathologic T (tumor size); pN, pathologic N (regional lymph node metastasis); pTNM, postoperative pathologic TNM staging determined according to the classification of the International Union Against Cancer.⁹

*The sequence of pulmonary vessel ligation during surgical intervention: *PV first* indicates that the pulmonary vein was ligated before the pulmonary artery, *PA first* indicates that the pulmonary artery was ligated before pulmonary vein ligation, and *mixed order* indicates random ligation of pulmonary vein and pulmonary artery.

detect circulating tumor cells.¹ However, these techniques are relatively insensitive and dependent on the interaction of antibodies with tumor-associated cell-surface antigens. These methods might yield false-positive results if the antibodies cross-react with normal antigens or if the tumor antigens are presented on host immune cells.^{1,2}

Recent developments in molecular technology, including the advent of the polymerase chain reaction (PCR), have permitted the sensitive detection of circulating tumor cells in the peripheral blood.^{1,3} Several researchers have used the gene encoding carcinoembryonic antigen (CEA) as the target gene because CEA messenger RNA (mRNA) can be detected in almost all epithelial cells, including cancer cells, but not in nonepithelial cells.^{4,5} The reverse transcriptase-polymerase chain reaction (RT-PCR) amplification method for CEA mRNA is an efficient means of detecting circulating cancer cells in the peripheral blood.⁶ If CEA mRNA is detected in blood samples, this implies the presence of ectopic epithelial cells and hence, presumably, cancer cells.

However, CEA is not a specific marker of lung cancer cells, and the technique does not allow for specific identification of tumor cells. Therefore, a positive finding of CEA mRNA might merely indicate the presence of epithelial cells. In addition, the relationship between circulating tumor cells and the development of metastatic disease is not fully understood, and there is ample evidence in the literature that many patients with malignant disease have evidence of circulating tumor cells, yet most of these patients do not have systemic disease.⁷

Given this background, we conducted a prospective study of 103 consecutive patients with non-small cell lung cancer (NSCLC) who underwent a curative lobectomy to test whether circulating tumor cells detected in the peripheral blood by means of RT-PCR of CEA mRNA are a prognostic indicator in patients with NSCLC. The results of the RT-PCR amplification for CEA mRNA in the preoperative and postoperative blood samples from these patients and its relationship to clinicopathologic factors have been described in detail elsewhere.⁸ In brief, the occurrence of positive CEA mRNA expression in the preoperative blood samples was significantly higher in the pathologic T3 (pT3) tumors (85%) compared with the pT1 and pT2 tumors (48% and 57%, respectively; $P = .0048$), in the pathologic N1 (pN1) and pN2 tumors (67% and 100%, respectively) compared with the pN0 tumors (44%, $P < 0.0001$), and in pathologic TNM (pTNM) stage IIA, IIB, and IIIA disease (50%, 73%, and 100%, respectively) compared with stage IA and IB disease (41% and 36%, respectively; $P < .0001$). Similarly, the occurrence of positive CEA mRNA expression in the postoperative blood samples was significantly higher in the pT3 tumors (50%) compared with the pT1 and pT2 tumors (18% and 24%, respectively; $P = .0090$), in the pN1 and pN2 tumors (17% and 59%, respectively) com-

Figure 1. Overall survival in 103 patients with NSCLC who underwent a curative lobectomy in terms of pTNM stage of disease.

pared with the pN0 tumors (17%, $P = .0007$), and in pTNM stage IIA, IIB, and IIIA disease (25%, 33%, and 59%, respectively) compared with stage IA and IB disease (13% and 12%, respectively; $P = .0002$). Now sufficient time has elapsed to analyze the prognosis of patients with NSCLC in a prospective study.

Patients and Methods

Patients

The 103 patients with NSCLC enrolled in this study underwent a curative lobectomy with mediastinal lymph node dissection (R2) at the Department of Surgery II, Kumamoto University Hospital, and at the Department of Thoracic Surgery, Kumamoto Chuo Hospital, between March 1996 and April 1998. Informed consent was obtained from each patient, and the study was approved by the ethics committees of our institutes. The clinicopathologic characteristics of the 103 patients are summarized in Table 1.⁹ None of these patients with NSCLC in this study received preoperative or postoperative chemotherapy or radiotherapy.

Immunohistochemical staining for CEA revealed that positive immunostaining was observed in the tumor cells of all the NSCLC specimens, including squamous cell tumors and nonsquamous cell tumors, although the staining intensity and the extent of cancer cells varied.

RT-PCR for CEA mRNA

Blood samples were taken from an antecubital vein in 103 patients with NSCLC at the time of diagnosis before the operation and again 2 to 3 weeks after the operation. Total RNA extraction and the RT-PCR for CEA mRNA were performed as described previously.¹⁰ An aliquot of the RT reaction mixture in all samples was subjected to RT-PCR for β -actin to monitor cDNA synthesis. This RT-PCR assay could detect CEA mRNA at a cell frequency as low as 10 malignant cells per 10^7 normal cells in an in vitro model.⁸

Figure 2. Overall survival in 103 patients with NSCLC who underwent a curative lobectomy in terms of preoperative and postoperative CEA mRNA status.

Figure 3. Overall survival in 103 patients with NSCLC who underwent a curative lobectomy in terms of CEA mRNA expression pattern.

Figure 4. Overall survival in 103 patients with NSCLC who underwent a curative lobectomy in terms of CEA mRNA expression pattern according to the pTNM stage.

At risk:	PA-first	35	27	21	19	18	5	1	0
	Mixed	25	19	15	11	9	6	2	0
	PV-first	43	36	26	24	19	10	2	0

Figure 5. Overall survival in 103 patients with NSCLC who underwent a curative lobectomy in terms of the sequence of pulmonary vessel ligation.

The technique is also highly specific because no control blood samples from healthy volunteers were positive for CEA mRNA.⁸ In addition, another in vitro experiment showed that none of the lysed cancer cells mixed with normal blood were positive for CEA mRNA, suggesting that the detection of CEA mRNA by means of the RT-PCR might represent viable, rather than dead, cancer cells in the circulation.⁸

Survival Analysis

The major statistical end point of this study was survival, and this was calculated from the day of operation to the day of death or the

last known date alive. Event time distribution was estimated with the method of Kaplan and Meier.¹¹ Differences between death distributions were tested for statistical significance with the log-rank test.¹² For simultaneous control for the effects of many variables on differences in death rates, a multivariate proportional hazards regression model was used.¹³

Results

Univariate Analysis

The 103 patients in the present analysis constitute 100% of our previous study population.⁸ There is no statistical difference in survival between patients with adenocarcinoma and those with squamous cell carcinoma ($P = .4240$). Figure 1 shows overall survival curves generated on the basis of pT factor, pN factor, and pTNM stage of disease. As expected, each of these factors was found to have a significant effect on survival when evaluated in a univariate analysis.

When the patient prognosis was analyzed in terms of the results of the RT-PCR amplification for CEA mRNA in the preoperative and postoperative blood samples, patients with positive CEA mRNA blood samples had a significantly shorter survival when compared with those with negative CEA mRNA samples (Figure 2). Patients with CEA mRNA in the preoperative blood samples had a poor survival; of these patients, the worst survival was seen in those with CEA mRNA in the postoperative blood samples (Figure 3). In contrast, patients with persistently negative CEA mRNA blood samples had better survivals than did patients with any other expression patterns for CEA mRNA (Figure 3). Next, the survival analysis regarding CEA mRNA expression patterns was repeated separately according to the

pTNM stage. Patients with persistently positive CEA mRNA blood samples are likely to have a poorer prognosis when compared with patients with any other expression patterns for CEA mRNA, although a statistically significant difference in survival was obtained only in stage IB disease ($P = .0163$, Figure 4).

We also examined whether the sequence of pulmonary vein (PV) and pulmonary artery (PA) ligation in curative lobectomy for NSCLC affects the prognosis of patients. As shown in Figure 5, there was no statistical difference in survival among 3 patterns of pulmonary vessel ligation sequence. The median survival by patient characteristics is shown in Table 2.

Multivariate Analysis

To verify the independent nature of the prognostic value of CEA mRNA expression, we used multivariate analysis. Cox regression analysis of overall survival, allowing for age, sex, histologic type, pT factor, pN factor, pTNM stage, sequence of pulmonary vessel ligation, preoperative CEA mRNA status, and postoperative CEA mRNA status, showed that preoperative CEA mRNA expression is the single independent prognostic factor regarding survival ($P = .0001$; relative risk, 0.17; Table 3). To eliminate the effect of the inclusion of less important variables into the model, we also performed stepwise regression analysis with a 5% significance level. Through a stepwise method, the model selected the pathologic stage of disease ($P = .0002$; relative risk, 1.43) and the preoperative CEA mRNA expression ($P = .0004$; relative risk, 0.21; Table 4). Age, sex, histologic type, pT factor, pN factor, sequence of vessel ligation, and postoperative CEA mRNA expression were not independent prognostic factors.

Discussion

An unresolved and most important question to clinicians is whether circulating tumor cells detected in the peripheral blood by means of RT-PCR of CEA mRNA have a prognostic value. Despite the detection of a small amount of tumor cells in the peripheral blood, lymph nodes, and bone marrow by means of molecular analysis, such as PCR-based methods, the exact relationship between these cells and the development of recurrent disease in malignancies is not fully understood. However, recent reports provided data indicating that the presence of occult cancer cells in lymph nodes or bone marrow increases the risk of relapse in patients with colorectal¹⁴ and breast¹⁵ cancer. In the present study we prospectively tested the prognostic value of the presence of CEA mRNA in the peripheral blood of patients with resectable NSCLC before and after an operation by means of an RT-PCR assay. We have demonstrated that patients with CEA mRNA in the preoperative blood samples had poor survival; of these patients, persistent evidence

TABLE 2. Median survival times by patient characteristics

Characteristic	Median survival (mo)	P value (log rank)
Histology		
Adenocarcinoma	40.0	
Squamous cell carcinoma	24.0	.4240
pT factor		
T1	>34.0*	
T2	30.0	
T3	11.5	.0001
pN factor		
N0	>53.0*	
N1	27.0	
N2	12.0	.0001
pTNM stage		
IA	>53.0*	
IB	>51.0*	
IIA	>14.0*	
IIB	14.0	
IIIA	12.0	.0001
Sequence of vessel ligation†		
PV first	35.0	
PA first	>28.0*	
Mixed order	24.0	.7270
Preoperative CEA mRNA expression		
(-)	>26.0*	
(+)	14.0	.0001
Postoperative CEA mRNA expression		
(-)	>51.0*	
(+)	14.0	.0001
RT-PCR for CEA mRNA pattern		
(-)(-)	>24.0*	
(+)(-)	14.0	
(-)(+)	>26.0*	
(+)(+)	13.0	.0001

*The survival times were represented as the longest follow-up period of the patients in a group because the median survival times were not able to be calculated because of the small number of dead patients in a group.

†The sequence of pulmonary vessel ligation during surgical intervention: *PV first* indicates that the pulmonary vein was ligated before the pulmonary artery, *PA first* indicates that the pulmonary artery was ligated before pulmonary vein ligation, and *mixed order* indicates random ligation of pulmonary vein and pulmonary artery.

of circulating tumor cells, as determined by using RT-PCR for CEA mRNA, showed worse prognosis than those patients in whom evidence for circulating tumor cells no longer exists. We also revealed that the preoperative CEA mRNA expression is a prognostic indicator independent of tumor stage.

The results of the current study should be interpreted with appropriate caution, taking into account the small sample size and the limited observation time. However, the major clinical implication of our findings might be seen in the potential opportunity for therapeutic setting and monitoring. Patients with CEA mRNA in the preoperative, postoperative, or both blood samples might represent evidence of residual disease, and thus even after curative operations

TABLE 3. Cox proportional hazards regression analysis of variables

Variable	z Score	SE	P value	Relative risk
Age (<60 y vs ≥60 y)	−0.61	0.22	.201	1.68
Sex (male vs female)	0.86	0.30	.423	0.54
Histology (adenocarcinoma vs squamous cell carcinoma)	0.40	0.29	.157	1.50
pT factor (T1 vs T2 vs T3)	0.04	0.33	.898	1.04
pN factor (N0 vs N1 vs N2)	−0.47	0.31	.133	0.62
pTNM stage (IA vs IB vs IIA vs IIB vs IIIA)	0.50	0.27	.062	1.65
Vessel ligation (PV first vs PA first vs mixed order)*	−0.62	0.33	.062	0.54
Preoperative e CEA mRNA expression (+ vs −)	−1.79	0.45	.0001	0.17
Postoperative CEA mRNA expression (+ vs −)	−0.47	0.38	.211	0.62

*The sequence of pulmonary vessel ligation during surgical intervention: *PV first* indicates that the pulmonary vein was ligated before the pulmonary artery, *PA first* indicates that the pulmonary artery was ligated before pulmonary vein ligation, and *mixed order* indicates random ligation of pulmonary vein and pulmonary artery.

Table 4. Final stepwise regression analysis

Variable	z Score	SE	P value	Relative risk
pTNM stage	0.35	0.09	.0002	1.43
Preoperative CEA mRNA expression	−1.58	0.44	.0004	0.21

for early stage NSCLC, consideration might be given for adding combined multimodal therapy. The current study might also indicate the possibility that evaluating a patient's peripheral blood status at appropriate intervals after the operation and before recurrence could help to determine the remaining potential for recurrence. Although several studies have demonstrated the general benefit of adjuvant chemotherapy, individual risk evaluation is still warranted to determine a patient's optimal dosage, drug selection, and duration of therapy. In addition, thus far there are no clinically relevant means available to determine the efficacy of adjuvant therapy in an individual patient until the disease recurs. Monitoring the extent of minimal residual disease in patients with NSCLC after primary treatment might help to individually tailor a patient's therapy, potentially improving an individual's chance for long-term remission.

Surgeons always worry that the surgical manipulation of a tumor during an operation might enhance the shedding of cancer cells into the bloodstream, resulting in an increase in the occurrence of distant metastases. There is some evidence indicating that intraoperative manipulation of a tumor can enhance metastasis formation.^{16,17} We recently reported that video-assisted thoracic surgery for NSCLC might increase intraoperative hematogenous dissemination of tumor cells compared with an operation by means of conventional open thoracotomy, as judged by detection of circulating tumor cells expressing CEA mRNA by RT-PCR.¹⁸ One surgical technique that might prevent such shedding is to ligate the efferent vessels first. In lung carcinoma operations, ligating the PV before ligating the PA has been postulated as a way to prevent tumor cell dissemination

into the bloodstream. Although there has been considerable debate over this recommendation, it has not been rigorously tested.

Using the RT-PCR amplification of CEA mRNA, we previously examined whether vessel ligation sequence affects the presence of circulating cancer cells and have demonstrated that ligation of the PV before ligation of the PA might partly prevent the release of tumor cells into the bloodstream during a pulmonary lobectomy for NSCLC.¹⁰ These results seem to support the opinion that the PV should be ligated first before the PA during surgical intervention and that this order of vessel ligation might improve the prognosis of patients with NSCLC. However, the present study demonstrated that there was no difference in survival among the 3 forms (PV first, PA first, and mixed) of pulmonary vessel ligation. It is conceivable that most of the cancer cells shed into the bloodstream during the operation ultimately will be destroyed by natural defense mechanisms, and very few of the tumor cells succeed in establishing secondary tumors. However, it is also possible that some tumor cells shed into the bloodstream by the surgical manipulation might survive in a selected subset of patients. The long-term follow-up of our patient cohort will answer the important question of whether the sequence of vessel ligation will actually affect the prognosis of patients with NSCLC who undergo a curative lobectomy and will also provide data on the prognostic relevance of circulating tumor cells in relation to a minimally invasive operation, such as video-assisted lobectomy.

In conclusion, this is the first report showing that the molecular detection of CEA mRNA in the peripheral blood is an independent prognostic factor in patients with NSCLC. Although large studies are still needed to explore the possibility of implementing a surrogate marker in the adjuvant setting, we believe that there is increasing evidence that detecting occult metastatic cells in the follow-up of patients with cancer is feasible.¹⁹ This new approach might, in the future, help clinicians to estimate an individual's pending

risk for recurrence and to individualize the duration of treatment according to the presence or absence of occult metastatic cells in the circulation of patients with NSCLC.

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