Circulating Tumor Cell as a Diagnostic Marker in Primary Lung Cancer

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Abstract  Purpose: To investigate the diagnostic performance of circulating tumor cells (CTC) in discrimination between primary lung cancer and nonmalignant diseases as well as in prediction of distant metastasis.

Patients and Methods: We prospectively evaluated CTCs in 7.5-mL samples of peripheral blood sampled from patients with a suspicion or a diagnosis of primary lung cancer. A semiautomated system was used to capture CTCs with an antibody against epithelial cell adhesion molecule.

Results: Of 150 eligible patients, 25 were finally diagnosed as having nonmalignant disease, and 125 were diagnosed as having primary lung cancer with (n = 31) or without (n = 94) distant metastasis. CTCs were detected in 30.6% of lung cancer patients and in 12.0% of nonmalignant patients. CTC count was significantly higher in lung cancer patients than in nonmalignant patients, but a receiver operating characteristic (ROC) curve analysis showed an insufficient capability of the CTC test in discrimination between lung cancer and nonmalignant diseases with an area under ROC curve of 0.598 (95% confidence interval, 0.488-0.708; P = 0.122). Among lung cancer patients, CTC count significantly increased along with tumor progression, especially with development of distant metastasis. The area under ROC curve for CTC count in prediction of distant metastasis was 0.783 (95% confidence interval, 0.679-0.886; P < 0.001). When patients with one or more CTCs were judged as having metastatic disease, sensitivity and specificity of the CTC test were 71.0% and 83.0%, respectively.


Primary lung cancer is the leading cause of cancer death in most industrialized countries, and its high mortality is mainly caused by frequent occurrence of distant metastasis. In fact, ∼40% of lung cancer patients have distant metastasis detectable with current diagnostic modalities such as whole-body computed tomography (CT) and positron emission tomography (PET) scanning (1). More importantly, even in patients without clinically detectable distant metastasis at the time of initial diagnosis, distant metastasis may frequently develop during treatment or long-time follow-up. Thus, in most lung cancer patients, tumor cells may circulate in the blood with or without apparent distant metastasis, and detection of such circulating tumor cells (CTC) may contribute to improvement in diagnosis and therapy of lung cancer patients (2).

However, in spite of many efforts for development of a sensitive detection system of CTCs, clinical significance of CTCs had not been established mainly due to lack of reproducibility and accuracy in detection of CTCs (2, 3). The CellSearch System (Veridex LLC), a semiautomated system for quantitative evaluation of CTCs, has been recently developed, in which CTCs are immunomagnetically captured with an antibody against epithelial cell adhesion molecule (EpCAM; ref. 3). The most important advantage of the CellSearch system is reproducibility across different laboratories, which is validated by a prospective multicenter study in metastatic breast cancer (4). Based on accumulating data supporting the accuracy and precision in evaluating CTCs (5–8), the CTC test has been approved in the United States of America by the Food and Drug Administration for monitoring of blood from metastatic breast and colon cancer patients. In addition, several clinical
studies have revealed that the CTC test is a potentially useful clinical marker in other malignant tumors such as prostate cancer (9–11). In lung cancer, however, little has been reported about the incidence of CTCs (3), and its clinical significance remains unknown. Thus, we now conducted a prospective study to examine the presence and incidence of CTCs in primary lung cancer patients for evaluating its diagnostic performance in discrimination between primary lung cancer and nonmalignant diseases as well as in prediction of distant metastasis.

**Patients and Methods**

**Study design.** Patients who presented with a pathologic diagnosis of primary lung cancer or with a suspicion of primary lung cancer on chest radiograph and/or CT at the Department of Thoracic Surgery, Hyogo College of Medicine hospital and agreed to the purpose of the study were eligible. Patients who have apparent or symptomatic distant metastasis before enrollment were excluded from the study. In addition, patients who had concurrent or prior malignancy treated within in the previous 5 y were excluded. All patients provided written informed consent before enrollment.

A 7.5-ml sample of peripheral blood was collected from each patient, and was served for the CTC test. A complete clinical data including history, physical examination, and laboratory and radiographic studies were also collected. In some primary lung cancer patients who underwent thoracotomy, pulmonary venous blood was also sampled during thoracotomy, and was served for the CTC test for another study (12).

For all patients enrolled, serum levels of carcinoembryonic antigen (CEA) were measured using a commercially available electrochemiluminescence immunoassay system (Roche Diagnostics K.K.) following a manufacturer's instruction; the manufacturer-suggested cutoff point of CEA to discriminate between nonmalignant disease and malignant tumor was 5 ng/mL (13, 14). For patients with a suspicion of primary lung cancer, bronchoscopic and/or trans-thoracic needle biopsy was applied to obtain pathologic diagnosis; if failed, video-assisted thoracoscopic biopsy was done. For lung cancer patients, whole-body CT, brain CT or magnetic resonance imaging (MRI), and PET scanning were routinely conducted to evaluate tumor progression. Clinical stage (c-stage) was determined according to the current tumor-node-metastasis classification as revised in 1997; for patients undergoing surgery for lung cancer, pathologic stage (p-stage) was also determined. This study was approved by the Institutional Review Board of Hyogo College of Medicine.

**Evaluation of CTCs (the CTC test).** CTCs were isolated from peripheral blood using the CellSearch system (Veridex LLC), and the number of CTCs was determined following a manufacturer’s protocol (3). In brief, epithelial cells, which were captured using...
ferroparticles coupled to an anti-EpCAM antibody, were separated in a magnetic field, and the enriched samples were then stained with 4′,6-diamidino-2-phenylindole and anti-cytokeratin-phycoerythrin. Contaminated WBC were excluded by negative selection for CD45. Stained cells were then analyzed on a fluorescent microscope using the Cell track Analyzer II (Veridex LLC). The criteria for each cell to be defined as a CTC are as follows: round to oval morphology, a visible 4′,6-diamidino-2-phenylindole-positive nucleus, positive cytokeratin staining in the cytoplasm, and negative staining for CD45. All evaluations were done by two authors (K.Y. and F.T., both completed the “Cell Interpretation Proficiency Assessment” managed by the Veridex LL for identification of CTCs) independently without knowledge of clinical characteristics of patients.

Statistics. Counts were compared by the χ² test. Continuous data were compared using Student’s t test if the distribution of samples was normal, or using nonparametric tests (Mann-Whitney U test for comparison between two groups and Kruskal-Wallis test for comparison among three or more groups) if the sample distribution was asymmetrical.

Diagnostic performance of CTC count or serum CEA level assessed by constructing a receiver operating characteristic (ROC) curve, and was evaluated by calculating the area under each ROC curve (AUC-ROC; ref. 15). An AUC-ROC of 1 denotes perfect discrimination of a test, whereas an AUC-ROC of 0.5 denotes complete lack of discrimination of a test. P value was calculated for the difference between each AUC-ROC and 0.5 (complete useless test).

For each test, two-sided P values of <0.05 were considered statistically significant. All statistical manipulations were done using the SPSS for Windows software system (SPSS, Inc.).

Results

Patient characteristics. From October 2007 through December 2008, 152 consecutive patients were enrolled in the study.

<table>
<thead>
<tr>
<th>Primary lung cancer</th>
<th>0</th>
<th>≥1</th>
<th>≥2</th>
<th>≥3</th>
<th>≥5</th>
<th>≥10</th>
<th>≥50</th>
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</thead>
<tbody>
<tr>
<td>All patients (n = 125)</td>
<td>87(69.6%)</td>
<td>38(30.6%)</td>
<td>21(16.8%)</td>
<td>13(10.4%)</td>
<td>9(7.2%)</td>
<td>5(4.0%)</td>
<td>1(0.8%)</td>
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<tr>
<td>Histologic cell type</td>
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<tr>
<td>Squamous cell carcinoma (n = 22)</td>
<td>13(59.1%)</td>
<td>9(40.9%)</td>
<td>4(18.2%)</td>
<td>2(9.1%)</td>
<td>1(4.5%)</td>
<td>1(4.5%)</td>
<td>0</td>
</tr>
<tr>
<td>Adenocarcinoma (n = 85)</td>
<td>63(74.1%)</td>
<td>22(25.9%)</td>
<td>13(15.3%)</td>
<td>8(9.4%)</td>
<td>5(5.9%)</td>
<td>2(2.4%)</td>
<td>0</td>
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<tr>
<td>Other non–small cell carcinoma (n = 9)</td>
<td>8(88.9%)</td>
<td>1(11.1%)</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>Small cell carcinoma (n = 9)</td>
<td>3(33.3%)</td>
<td>6(66.7%)</td>
<td>4(44.4%)</td>
<td>3(33.3%)</td>
<td>3(33.3%)</td>
<td>2(22.2%)</td>
<td>1(11.1%)</td>
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<td>C-stage</td>
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<tr>
<td>I (n = 88)</td>
<td>71(80.7%)</td>
<td>17(19.3%)</td>
<td>8(9.1%)</td>
<td>3(3.4%)</td>
<td>1(1.1%)</td>
<td>1(1.1%)</td>
<td>0</td>
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<tr>
<td>II (n = 3)</td>
<td>3(100%)</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>III (n = 5)</td>
<td>4(80.0%)</td>
<td>1(20.0%)</td>
<td>1(20.0%)</td>
<td>1(20.0%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>IV (n = 29)</td>
<td>9(31.0%)</td>
<td>20(69.0%)</td>
<td>12(41.4%)</td>
<td>9(31.0%)</td>
<td>7(24.1%)</td>
<td>4(13.8%)</td>
<td>1(3.4%)</td>
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<tr>
<td>P-stage</td>
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<tr>
<td>I (n = 67)</td>
<td>55(82.1%)</td>
<td>12(17.9%)</td>
<td>6(9.0%)</td>
<td>1(1.5%)</td>
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<tr>
<td>II (n = 11)</td>
<td>9(81.8%)</td>
<td>2(18.2%)</td>
<td>1(9.1%)</td>
<td>1(9.1%)</td>
<td>1(9.1%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>III (n = 15)</td>
<td>14(93.3%)</td>
<td>1(6.7%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IV (n = 17)</td>
<td>6(35.3%)</td>
<td>11(64.7%)</td>
<td>6(35.3%)</td>
<td>4(23.5%)</td>
<td>1(5.9%)</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Nonmalignant disease</td>
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</tr>
<tr>
<td>All patients (n = 25)</td>
<td>22(88.0%)</td>
<td>3(12.0%)</td>
<td>1(4.0%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 2. A, distribution of CTC count in primary lung cancer patients and in patients with nonmalignant disease. B, ROC curves for CTC count and serum CEA level to discriminate primary lung cancer patients from nonmalignant patients (B).
After enrollment, whole-body CT or PET scanning incidentally revealed a concurrent malignant tumor in two patients (pancreatic cancer and bile-duct cancer in one patient each), and these patients were excluded. Of 150 eligible patients, 17 had been pathologically diagnosed as having primary lung cancer at enrollment. Of 133 patients with a suspicion of lung cancer at enrollment, 108 were finally diagnosed pathologically as having primary lung cancer. Thus, the final diagnosis was primary lung cancer in a total of 125 patients (Fig. 1). The majority of patients had c-stage I disease, and 29 patients were diagnosed as having c-stage IV disease.

Thoracotomy was done for 110 primary lung cancer patients consisting of all (n = 94) c-stage I-IIIA, and one of 2 c-stage IIIb, and 15 of 29 c-stage IV patients. Of 96 patients with c-stage I-III disease, 2 (both c-stage IB patients) were diagnosed as having p-stage IV disease as pulmonary metastases were found during thoracotomy. Finally, distant metastasis was found in a total of 31 primary lung cancer patients (Fig. 1).

The remaining 25 patients were finally diagnosed as having nonmalignant disease by video-assisted thoracoscopic biopsy (Fig. 1); there were 10 patients with organizing organized pneumonia (in 3 patients), 6 with infectious disease, and 2 with lymph-proliferative disease. There was no significant difference in any patient characteristic between lung cancer patients and nonmalignant patients.

**CTCs in primary lung cancer patients and nonmalignant patients.** CTCs were identified in peripheral blood of 3 (12.0%) of 25 nonmalignant patients, and CTC count was "1" (n = 2) or "2" (n = 1). CTCs were identified in peripheral blood of 38 (30.6%) of 125 lung cancer patients, and CTC count was significantly higher compared with that in nonmalignant patients (Table 1; Fig. 2A).

The AUC-ROC for CTC count in discrimination between lung cancer patients and nonmalignant patients was 0.598 (P = 0.122; Fig. 2B). At a cutoff point of 1 for lung cancer (CTC count, ≥1) or nonmalignant patients (CTC count, 0), sensitivity and specificity were 30.4% and 88.0%, respectively (Table 2).

The AUC-ROC for serum CEA level was 0.747 (P < 0.001), suggesting that serum CEA level is a better diagnostic marker for the diagnosis of lung cancer (Fig. 2B). At a cutoff point of 5 ng/mL, sensitivity and specificity were 45.6% and 92.0%, respectively (Table 2).

**CTCs in primary lung cancer patients.** Among primary lung cancer patients, small-cell lung cancer patients showed a significantly higher CTC count than non–small cell cancer patients (Fig. 3A).

CTC count significantly increased along with increase in c-stage (Fig. 3B). The difference in CTC count between c-stage I disease and c-stage IV disease was statistically significant, but the difference among c-stage I to III diseases did not reach a statistically significance.

CTC count significantly increased along with increase in p-stage, and was significantly higher in p-stage IV disease than any other p-stage disease (Fig. 3C). Of noted, two patients, who migrated from c-stage IB to p-stage IV disease due to pulmonary metastases found during thoracotomy, had one or more CTCs (CTC count, 1 and 3 in one patient each) in peripheral blood.

**CTC test for prediction of distant metastasis.** Metastatic lung cancer patients showed a significantly higher CTC count...
than primary lung cancer patients without distant metastasis (Fig. 4A). The AUC-ROC for CTC count for prediction of the absence or presence of distant metastasis among primary lung cancer patients was 0.783 ($P < 0.001$; Fig. 4B), suggesting that CTC count was a useful surrogate marker of distant metastasis. At a cutoff point of 1 for metastatic lung cancer (CTC count, $\geq 1$) or nonmetastatic lung cancer patients (CTC count, 0), sensitivity and specificity were 71.0% and 83.0%, respectively (Table 2).

In contrast, the AUC-ROC for serum CEA for prediction of distant metastasis failed to reach a statistical significance ($P = 0.136$; Fig. 4B).

When the diagnostic performance of the CTC test in prediction of distant metastasis was evaluated among all patients enrolled in the present study (all patients with a suspicion or a diagnosis of lung cancer), similar results were obtained (Table 2; Fig. 4C and D).

**Discussion**

The present study is the first detailed study evaluating CTCs in patients with a suspicious or a diagnosis of primary lung cancer using the CellSearch system. We first showed that CTC count was significantly higher in lung cancer patients than in nonmalignant patients, but the discriminatory capability might be insufficient with an AUC-ROC of 0.598 ($P = 0.122$). Second, we showed that CTC count significantly increased along with tumor progression, especially with development of distant metastasis, and the CTC test showed a moderate diagnostic performance in discrimination between nonmetastatic patients and metastatic patients with an AUC-ROC of 0.783 showing a significantly better capability compared with a completely useless test with an AUC-ROC of 0.5 ($P < 0.001$). Furthermore, these ROC-curves suggested that the optimal cutoff point in diagnosis of lung cancer was 1 (patients with one or more CTCs are judged to be “positive”).

Although intense efforts have been continued for development of useful biomarkers, many have not been established for diagnosis or decision-making in therapy of lung cancer (16, 17). Because only a small tumor specimen can be obtained by usual diagnostic modalities such as fine-needle aspiration and transbronchial biopsy, development of biomarkers that are evaluated in peripheral blood samples would be valuable. In addition, blood-based biomarkers have a great advantage of being evaluated easily and repeatedly. Among blood-based biomarkers, serum CEA is most extensively examined, and several studies showed that serum CEA was a useful marker not only to discriminate lung cancer, especially adenocarcinoma of the lung, from benign diseases but also to determine tumor progression and prognosis (13, 14). Despite these promising results, routine use of serum CEA in clinical practice is not recommended mainly due to its insufficient sensitivity and/or specificity as well as lack of reproducibility of the results (16, 17). Thus, we assessed the diagnostic performance of CTC count in comparison with that of serum CEA in the present study.

Concerning the diagnostic performance in discrimination between nonmalignant patients and lung cancer patients, serum CEA showed a moderate performance (AUC-ROC, 0.747). However, the CTC test showed an insufficient performance (AUC-ROC, 0.598), which may be mainly due to its low sensitivity. In fact, when patients with one or more CTCs were judged as having lung cancer (cutoff point, 1), sensitivity was only 30.4%, whereas specificity was as high as 88.0%. And, if the cutoff point was elevated to 3, only 10.4% of lung cancer patients were correctly judged to be positive, whereas...
no nonmalignant patients were wrongly judged to be positive (both specificity and positive predictive value, 100%). These results indicate that the CTC test is characterized by low sensitivity and negative predictive value as well as high specificity and positive predictive value. Here, at a cutoff point of 1, attention should be paid to the fact that specificity or positive predictive value did not reach 100% (88.0% and 92.7%, respectively). In other words, some patients (7.3%, 3 of 41 patients) were finally diagnosed as having nonmalignant diseases, whereas one or two “tumor cells” were identified in peripheral blood. These results are similar as those in a previous study showing that up to three CTCs were detected in a small subset of healthy volunteers or nonmalignant patients. Such “false-positive” results may be classified into “true” false-positive results or “false” false-positive results. A true false-positive result, indicating that patients without any malignant tumor are judged to have a malignant tumor according to the CTC test, can be brought by several factors as follows: (a) contamination of epithelial cells in blood samples due to a variety of technical issues such as inappropriate blood sampling, (b) false-positive staining of contaminated nonepithelial cells for cytokeratin/4′,6-diamidino-2-phenylindole during sample processing, (c) inappropriate judgment in identification of CTCs by researchers. A false false-positive result can occur, when detected CTCs are originated from a clinically undetectable malignant tumor. An individual reason for three false-positive cases in the present study remains unclear, and we will continue to watch for development of malignant tumors.

The CTC test showed a significant diagnostic performance to predict the absence or presence of distant metastasis (AUC-ROC, 0.783; \( P < 0.001 \)), whereas serum CEA showed an insufficient performance. Thus, when CTCs are identified in peripheral blood of patients with a suspicion or a diagnosis of lung cancer, whole-body PET-scanning and brain magnetic resonance imaging (or CT) for evaluation of possible distant metastasis should be routinely performed even in clinical T1N0 cases evaluated with thoracic CT scanning. However, at a cutoff point of 1, specificity or negative predictive value
of the CTC test for prediction of distant metastasis was not 100% (83.0% and 89.7%, respectively), indicating that whole-body PET scanning and brain magnetic resonance imaging (or CT) are still necessary even when the CTC test was negative. The most important advantage of the CTC test may be its capability of detecting micrometastasis undetectable with routine diagnostic modalities. In the present study, CTCs were detected in 17 (19.3%) of 88 c-stage I patients, and the presence of distant metastasis was actually confirmed in resected specimens in 2 of 17 CTC-positive c-stage I patients. The most interesting and important question is whether distant metastasis may develop after thoracotomy in CTC-positive patients without detectable metastasis. CTCs detected in clinically “nonmetastatic” patients may represent true CTCs or nonmalignant cells that are contaminated or incorrectly identified as CTCs, which may be answered after a longer follow-up of patients. In addition, basic research studies should be done to reveal whether circulating epithelial cells defined as “CTCs” are true tumor cells and whether CTCs can actually grow at distant organs to form metastatic foci. More experimental and clinical studies are needed before the CTC test can be used in clinical practice for decision making of therapy of lung cancer patients.

The CellSearch system, used in the present study, is the only commercially available system for detection and identification of CTCs. However, the most critical issue in the use of the CellSearch system may be its low sensitivity for detecting CTCs, which suggest a need for a more sensitive detection system. Recently, a novel microfluidic platform for detecting CTCs (“CTC chip”) has been developed (18, 19). This CTC chip consists of an array of 78,000 microposts coated with anti-EpCAM antibodies, and CTCs are captured by interaction of these cells with the EpCAM-coated microposts under laminar flow conditions. The CTC chip may provide a higher sensitivity in identification of CTCs, as a pilot study showed that CTCs were detected in most blood samples taken from patients with a variety of malignant tumor including lung, prostate, pancreatic, breast, and colon cancer (18). Of noted, CTCs were detected in all blood samples (n = 55) taken from non–small cell lung cancer patients, and the mean CTC count was very high (155 cells/mL) compared with that in the present study. When the CTC chip can be available in future, a comparative study between the CellSearch system and the CTC chip system may be conducted. In parallel with a longer follow-up of patients enrolled in the present study, we are now conducting following studies to reveal biological and clinical significance of CTCs: (a) in vitro and in vivo studies to assess viability and capability to form metastatic foci of CTCs captured from lung cancer patients, (b) molecular studies to reveal biological characteristics such as mutations in the epidermal growth factor receptor gene of CTCs, (c) translational studies to evaluate clinical value of the CTC test as a surrogate of therapeutic effect, and (c) clinical studies to assess the nature of CTCs in pulmonary venous as mentioned in the “Patients and Methods” section of the article (12).

In conclusion, CTC is a surrogate of distant metastasis in primary lung cancer patients, which can be useful in clinical practice. Further studies to confirm clinical value of the CTC test are warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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