Prognosis of Non–Small Cell Lung Cancer Patients by Detecting Circulating Cancer Cells in the Peripheral Blood with Multiple Marker Genes

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ABSTRACT

Purpose: Current lung cancer staging and prognosis methods are based on imaging methods, which may not be sensitive enough for early and accurate detection of metastasis. This study aims to validate the use of a panel of markers for circulating cancer cell detection to improve the accuracy of cancer staging, prognosis, and as a rapid assessment of therapeutic response.

Experimental Design: We analyzed the National Cancer Institute-Cancer Genome Anatomy Project database to identify potential marker genes for the detection of circulating cancer cells in peripheral blood. Nested real-time quantitative PCR and a scoring method using cancer cell load Lc were employed to correlate the amount of circulating cancer cells with clinical outcomes in 54 non–small cell lung cancer (NSCLC) patients. The Kaplan-Meier method was employed for analysis of prognostic variables.

Results: A panel of four marker genes was identified and experimentally validated. With these marker genes, we achieved an overall positive detection rate of 72% for circulating cancer cells in the peripheral blood of NSCLC patients. Patients who had higher Lc values had worse outcomes and shorter survival times. Patients with poor therapeutic response were revealed by positive detection of circulating cancer cells after therapy. The results correlated well with the patients’ survival time.

Conclusion: Circulating cancer cell detection by a panel of markers and the Lc scoring method can supplement the current tumor, node, metastasis staging method for improved prognosis and for rapid assessment of therapeutic response.

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INTRODUCTION

Lung cancer is the leading cause of cancer-related death and non–small cell lung cancer (NSCLC) accounts for ~80% of the cases. Attempts to use serum protein markers for the early diagnosis of lung cancer have not yielded satisfactory results for routine screening, and newly developed early diagnostic methods using serum DNA as a diagnostic marker await further validation (1, 2). Current therapeutic measures remain unable to lower the mortality rate of late-stage lung cancer patients. Surgical resection is still the best cure for the early-stage patients. The tumor, node, metastasis (TNM) classification has been used for cancer staging and prognosis for decades. A large portion of early-stage patients, defined by the current staging system and available imaging modalities, still develop distant metastases although they received surgical removal of the tumor mass. The inability to detect disseminated tumor cells with the current imaging techniques is a major obstacle to accurate cancer staging.

Several research groups have reported the presence of epithelial cancer cells in the bone marrow (3, 4) and in the peripheral blood (5, 6) of patients with carcinoma. In contrast to bone marrow aspirates, peripheral blood samples can be obtained routinely and more readily. Techniques such as immunocytology, flow cytometry (7), and PCR (8–11) have been employed to detect disseminated tumor cells in peripheral blood. The positive detection rate of circulating cancer cells in lung cancer patients was only about 40% when a single marker gene (cytokeratin 19, CK19) was employed (9). To overcome this problem, a multimarker reverse transcription-PCR assay should be beneficial as shown in the diagnosis of melanoma and breast cancer (12, 13).

In this report, we identify a panel of markers for the detection of circulating cancer cells in NSCLC patients by in silico analysis of the National Cancer Institute-Cancer Genome Anatomy Project database (http://cgap.nci.nih.gov/). The load of cancer cells in the circulation was correlated with the clinical outcomes of the patients. An accurate cancer staging method is prerequisite for prescribing optimal therapeutic courses for early- and late-stage lung cancer patients. Detection of the presence of circulating cancer cells in conjunction with the conventional TNM classification may better define the stage and prognosis of cancer patients. This method provides a useful tool to optimize NSCLC treatment and management.

MATERIALS AND METHODS

Patients and Specimens. Peripheral blood samples were obtained with informed consent from 54 patients with histologically documented NSCLC in the National Taiwan University Hospital. Staging procedures included chest radiography, bronchoscopy, brain and thoracic computed tomography, sonography, and bone scintigraphy. The follow-up period of the lung cancer
patients was up to 85 months for stage I patients who were still alive in May 2003. The normal control population consisted of 24 healthy volunteers with no history or present diagnosis of malignancy. Among the 54 patients, 32 were men and 22 were women with median age of 65 years (SD = 11, range = 28-81). In the healthy control group, 16 were men and 8 were women with median age of 57.5 years (SD = 15.1, range = 27-78). Adenocarcinoma lung cancer cell lines (A549, CL3, H928, CL1-0, CL1-5, CRL-5865, CRL-5806, and CRL-5807) and squamous carcinoma lung cancer cell lines (NCI-H520, H2981, CRL-5802, and HTB-54) were used to validate the candidate marker genes and for analyzing the correlation cell number.

**Identification of Candidate Marker Genes.** To take advantage of the vast information of the expressed sequence tags databases generated with cancer cell lines, we used the cDNA Digital Gene Expression Displayer developed by the Cancer Genome Anatomy Project (14) to identify genes that were differentially expressed between lung cancer cells and leukocytes. The Digital Gene Expression Displayer program identified differentially expressed genes among 47,036 sequences in five lung cancer cDNA libraries and 21,460 sequences in six leukocyte cDNA libraries with a P filter set at 0.01. The differentially expressed genes were ranked by sequence odds ratio. The genes with the highest sequence odds ratios were selected as candidate marker genes for quantitative PCR (qPCR) assay.

**Sample Collection and RNA Preparation.** The blood sample collection and RNA preparation methods were the same as described by Peck et al. (9). Briefly, two samples were collected from each subject with Vacutainers (Becton Dickinson, Rutherford, NJ). The first tube with 1 to 2 mL of peripheral blood was discarded and only the second tube with 3 to 4 mL of blood was assayed to avoid epithelial cell contamination by the needle puncture. The blood was collected from each subject with Vacutainers (Becton Dickinson, Rutherford, NJ). The first tube with 1 to 2 mL of peripheral blood was discarded and only the second tube with 3 to 4 mL of blood was assayed to avoid epithelial cell contamination by the needle puncture. The blood was collected from each subject with Vacutainers (Becton Dickinson, Rutherford, NJ). The first tube with 1 to 2 mL of peripheral blood was discarded and only the second tube with 3 to 4 mL of blood was assayed to avoid epithelial cell contamination by the needle puncture. The blood was collected from each subject with Vacutainers (Becton Dickinson, Rutherford, NJ). The first tube with 1 to 2 mL of peripheral blood was discarded and only the second tube with 3 to 4 mL of blood was assayed to avoid epithelial cell contamination by the needle puncture.

**Nested Reverse Transcription-PCR Assay.** To detect a few cancer cells in the circulation, a highly sensitive nested reverse transcription-PCR is necessary. cDNA was derived from 1 to 2 mL of total RNA by random primed reverse transcription, and nested PCR was used to amplify the candidate marker gene transcripts for detection. The primer sets are listed in Table 1. The first round (outer) of the nested PCR was done using 1 μL of 20-fold diluted cDNA with a PCR mixture containing 0.1 μmol/L outer primer pair, 0.2 mmol/L deoxyribonucleotide triphosphate, 50 mmol/L Tris-HCl (pH 8.3), 10 mmol/L KCl, 5 mmol/L (NH4)2SO4, 2 mmol/L MgCl2, and 0.75 units of FastStart Taq DNA polymerase (Roche, Mannheim, Germany) in a total volume of 12.5 μL. The PCR conditions were one cycle at 94°C for 7 minutes followed by 25 cycles at 94°C for 50 seconds, 60°C for 50 seconds, 72°C for 35 seconds, and a final extension at 72°C for 10 minutes.

For the second round (inner) of the nested PCR amplification, the reaction mixture contained 2 μL of the first round PCR product, 0.25 μmol/L inner primers, and SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) in a total volume of 20 μL. The real-time qPCR assays were done with an ABI prism 7000 SDS (Applied Biosystems) instrument. The real-time qPCR condition was 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds, 60°C for 25 seconds, and 72°C for 35 seconds.

**Semiquantification of the Nested PCR Results.** By using a real-time qPCR instrument, the threshold cycle (C_T), the fractional cycle number at which the SYBR Green I fluorescence exceeded a set level above baseline, was determined. We used GAPDH mRNA as an internal control. The relative amount of mRNA, normalized against the GAPDH mRNA, was expressed as ΔC_T = C_T(GAPDH) - C_T(marker gene). If the fluorescence signal was undetected after 40 cycles, the C_T value was given the maximum cycle number of 40 for analysis convenience. The differential expression ratio of a candidate marker gene, Q, for patients versus normal controls was calculated by Q = 2^[-ΔΔC_T], mean of ΔC_T in normal. To estimate the number of circulating cancer cells, we normalized the differential expression ratio of each marker gene to take into account the different amount of the marker gene transcripts. We then summed up the expression ratios of the marker genes to estimate the cancer cell load in the circulation in a semiquantitative way. The formula for normalizing the expression ratio, E_i, of a marker gene is E_i = Q_i, where i is the patient index, j is the marker gene index, Q_i,j is the differential expression ratio of marker gene j in patient i, Q_i,j is the mean and σ_j is the SD of the expression ratios of the 54 patients for marker gene j. The load of cancer cells in the circulation of a patient is defined as L_C = \sum_{j=1}^{n} E_j, where n is the number of marker genes. In this study, the value of L_C ranged between ~2.2 and 8.4.

**Statistical Analysis.** Fisher’s exact test and Student’s t test were used to compare the clinicopathologic characteristics of patients with low and high L_C values. All statistical tests were two sided. Survival time of the patients was calculated from the day of specimen collection. Survival curves were obtained by the Kaplan-Meier method. The difference of survival times between two groups was analyzed with the log-rank test. P < 0.05 was considered statistically significant.

**RESULTS**

**Marker Genes for Detecting Circulating NSCLC cells.** The in silico Digital Gene Expression Displayer program search...
of the National Cancer Institute-Cancer Genome Anatomy Project database yielded 85 overexpressed genes with a sequence odds ratio > 16 between the lung cancer cDNA libraries and the leukocyte cDNA libraries. These candidate genes were further verified by nested real-time qPCR. All of the cancer cell lines listed in the MATERIALS AND METHODS and pooled peripheral blood mononuclear cells from 12 healthy controls were used as samples in the first round verification. Fifty-nine candidate marker genes showed >2-fold differential expression ratios, but only 19 genes had differential expression ratios > 100,000. Marker genes with large differential expression ratios are required to detect rare circulating cancer cells in blood samples containing millions of peripheral blood mononuclear cells. By using real-time qPCR to detect the presence of 19 candidate genes in the clinical specimens of 54 NSCLC patients and 24 normal controls, four marker genes including CK19 were identified to show positive detection in at least two NSCLC patients. The four marker genes are listed in Table 2. The first three marker genes had negligible expression in the blood samples of normal controls by the real-time nested qPCR assay and are labeled as genes A to C. The fourth gene, labeled as gene D, had high expression in the cancer patient group versus the normal control group, but had residual expression in normal peripheral blood mononuclear cells. Therefore, we set the positive detection threshold at 99% confidence interval in the normal control group to avoid false positive detection.

Enhancement of Positive Detection Rate with Multiple Genes. As shown in Fig. 1A, the positive detection rate of circulating cancer cells in NSCLC patients was 41%, 11%, 39%, and 11% for genes A to D, respectively. The fraction of positives among all the patients are indicated in the columns.

Increasing the number of marker genes raised the positive detection rate for NSCLC patients to 72% (39 of 54) compared with 41% (22 of 54) positive detection rate using the CK19 marker gene alone (Fig. 1B). These results prove that using multiple markers indeed improves the positive detection rate. The B marker gene was not detected in squamous cell lung cancer patients, but the other three genes were detected in patients with adenocarcinoma or squamous carcinoma. Further analysis of these marker genes did not show statistically significant results for lung cancer subtyping.

Circulating Cancer Cell Load and Patient Outcome. To investigate the correlation between the number of circulating cancer cells and patient outcome, we used cancer cell load $L_c$ (see MATERIALS AND METHODS) as a measure of the number of cancer cells in circulation. By definition, $L_c$ increases with the number of circulating cells and/or the number of detected marker genes in the circulating cancer cells. We spiked different numbers of lung cancer cells (CL1-0) into peripheral blood mononuclear cells to determine the correlation between the number of circulating cancer cells and $L_c$ values (Fig. 2A). The results show a good correlation ($r = 0.99$) between cancer cell number and $L_c$ value.

To determine whether $L_c$ can be used as an indicator of patient outcome, we analyzed the $L_c$ value versus the survival time of stage I to IIIa patients who received surgical resection. We found that patients who survived for more than 5 years had low $L_c$ values (<1; data not shown). Therefore, we set $L_c = 1$ as the threshold for the subsequent prognosis studies in the 54 lung cancer patients. We divided the patients at different stages into two groups ($L_c \geq 1$ or $L_c < 1$) and examined their survival time. As expected, late-stage patients had shorter survival times than did early-stage patients (Fig. 2B, inset). The cancer load ($L_c$) study further distinguished that for patients of the same stage, those with low $L_c$ had longer survival time than those with high $L_c$ value (Fig. 2B). For the operable early-stage patients, the survival time is highly correlated ($P = 0.002$) with $L_c$ value. We then used the $L_c$ value to analyze the prognosis of late-stage patients (stages IIIb and IV) with Kaplan-Meier survival plots. The survival time difference was more significant by using $L_c$ as a classification parameter (Fig. 3B, $P = 0.006$) than by using the traditional TNM classification (Fig. 3A, $P = 0.03$). The number of patients in each group is shown in parentheses in the figures. We further used the $L_c$ parameter to divide the patients of the same stage into low ($L_c < 1$) and high ($L_c \geq 1$) value groups and examined the survival plots. The survival time of stage IIIb patients with low and high $L_c$ value was significantly different.

### Table 2

List of a panel of four marker genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Title</th>
<th>Genbank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Homo sapiens keratin 19 (KRT19)</td>
<td>NM_002276</td>
</tr>
<tr>
<td>B</td>
<td>Ubiquitin thiolesterase</td>
<td>NM_004181</td>
</tr>
<tr>
<td>C</td>
<td>Highly similar to HSFIB1 for fibronectin</td>
<td>NM_054034</td>
</tr>
<tr>
<td>D</td>
<td>Tripartite motif-containing 28 (TRIM28)</td>
<td>NM_005762</td>
</tr>
</tbody>
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![Fig. 1](clincancerres.aacrjournals.org) Analysis of positive detection rates with the multimarker gene panel. A, positive detection rate for the individual four marker genes. B, positive detection rate increases with the number of marker genes used in the assay.
The same analysis on stage IV patients did not achieve statistical significance (Fig. 3D, \( P = 0.09 \)). No statistically significant association was found between \( L_c \) values and clinicopathologic variables such as age, smoking, histologic type, and stage, with the exception of gender (Table 3). A search of the available literature reports on the four marker genes did not offer a plausible explanation for this gender bias. An investigation on this finding will be conducted in a separate study.

**Assessment of Therapy Efficacy.** We further examined whether cancer cell load could be employed to monitor the efficacy of therapy. Fig. 4 shows the assessment of six patients before and after chemotherapy. The stage I, II, and IIIa patients received surgical resection, whereas the stage IIib and IV patients received chemotherapy. “d” indicates that the samples were collected before therapy, “dn” indicates that the samples were collected on the \( n \)th day after therapy, and “/” indicates the course number of chemotherapy. The stage I patient had negative \( L_c \) values before and after treatment and was still alive on the last follow-up date (72.3 months). The \( L_c \) values decreased for the stage II and IIib patients after therapy. The stage IIIa and IV patients had higher \( L_c \) values after the last course of treatment and had short survival times. These results suggest that measurement of the cancer cell load can be used to reveal whether therapy is efficacious.

**DISCUSSION**

In this study, we employed *in silico* analysis to identify a panel of marker genes for NSCLC prognosis and assessment of therapy efficacy. Based on our experimental results, the National Cancer Institute–Cancer Genome Anatomy Project database and the Digital Gene Expression Displayer program are useful tools for identifying differentially expressed genes between two pools of samples provided that a sufficient number of expressed sequence tags libraries for the tissue of interest are archived in the database. The differentially expressed genes can be further developed into marker genes for diagnostic or prognostic purposes by experimental verification procedures such as real-time qPCR.

NSCLC is heterogeneous with respect to histology and biological characteristics (15). Individual NSCLC cells within a tumor and in different patients’ tumors express different amounts of marker gene transcripts. The heterogeneity of marker gene expression levels in NSCLC limits the reliability of an assay method with a single-marker detection scheme. Several literature reports have described PCR methods for the detection of tumor cells dispersed in the circulation (8–11). However, not one tumor marker is consistently and specifically expressed in all of the primary tumors of a particular malignancy (16, 17). Literature reports have also shown that a panel of marker genes provides a more reliable and informative approach than a single-marker assay for the detection of melanoma and breast cancer cells in blood (12, 13). Such assays for lung cancer have been limited by the availability of molecular markers (18). To overcome this obstacle, we developed an effective method to identify marker genes for NSCLC and showed the use of a multimarker reverse transcription-qPCR assay for detecting metastatic lung cancer cells in blood with a good detection rate.
The positive detection rate was significantly improved by using a panel of four marker genes as compared with using a single CK19 marker gene, which is a characteristic marker to epithelial cells. Other than CK19, the roles of the other three marker genes in cell differentiation, carcinogenesis, tumor invasion, and metastasis are not yet certain. Literature reports characterizing the marker genes and their association with cancers are available for ubiquitin thiolesterase and fibronectin but not TRIM28.

Ubiquitin thiolesterase, also called protein gene product 9.5 (PGP9.5), is a neurospecific peptide that functions to remove ubiquitin from ubiquitinated proteins and prevents them from targeted degradation in proteasomes. PGP9.5 can contribute to the uncontrolled growth of somatic cells (19). Recent studies have identified PGP9.5 as a marker for invasive colorectal cancer (20) and as a prognostic factor in pancreatic cancer (21) and NSCLC (22). In our study, the PGP9.5 gene was preferentially expressed in stage IIIb and IV NSCLC (17%) compared with early-stage (I, II, and IIIa) patients. This result is similar to previous findings (22).

Fibronectin, an extracellular matrix protein, is produced by a wide variety of cells such as fibroblasts (23), endothelial cells (24), epithelial cells (25), macrophages (26), and some carcinoma cells (27, 28). Fibronectin plays several important roles in cell adhesion, morphology, migration, oncogenic transformation, neovascularization, tumor invasion, and metastasis (29, 30). The intracellular expression of fibronectin has been reported to be stronger in the invading parts of most papillary thyroid carcinomas tumors compared with the central tumor regions (31). Up-regulation of fibronectin has also been reported in several other human cancers and its overexpression may be tumor promoting in hepatoblastoma (32) and ovarian cancer (33).

Using the four marker genes, the positive detection rates of patients with circulating cancer cells were 67% (4 of 6) for stage I, 100% (4 of 4) for stage II, 67% (6 of 9) for stage IIIa, 67% (14 of 21) for stage IIIb, and 79% (11 of 14) for stage IV. The detection rate indicates the sensitivity of using the four markers for detecting the presence of circulating NSCLC cells. The detection rate, however, does not provide information on the number of circulating cancer cells and the prognosis, which are better measured and assessed by the cancer cell load $L_c$. Our data showed that 16.7% of stage I, 50% of stage II, 22% of stage IIIa, 24% of stage IIIb, and 28.6% of stage IV patients had $L_c > 1$.

The percentage of patients with high $L_c$ value is greater for the late-stage patients than for the early-stage patients except for the stage II patients. We looked into this issue and found that the markers have different detection rates for different histologic types of NSCLC. The markers are more sensitive for detecting squamous carcinoma (85.7%; 12 of 14) compared with adenocarcinomas (68.6%; 24 of 35) and others (60%, 3 of 5). An investigation on the histologic types of the NSCLC patients revealed that the available stage II patients were composed of three (75%) squamous carcinoma and one (25%) adenocarcinoma patients, whereas the other stage patients were composed of 11% to 33% squamous carcinoma, 50% to 71% adenocarcinoma, and 5% to 22% poorly differentiated cell type patients. The higher detection rate of the stage II patients can therefore be attributed to the limited number of clinical samples and their histologic composition.
The data shown in Figs. 2B and 3 indicate that patients of the same stage who had higher \( L_c \) had worse outcomes. The results indicate that \( L_c \) measurement is a supplementary tool to the traditional TNM staging method to better predict the outcome of cancer patients. In traditional metastasis models, metastatic cells are rare and arise during late stages of tumor progression (34). These models have been challenged by recent studies, which reported that most cancer cells in a primary tumor have a “metastatic phenotype” (35, 36). TNM classification is based on pathologic observations to determine the cancer progression stage by a set of guidelines using tumors size, metastases in the surgically removed regional lymph nodes, and the presence of distant metastases as parameters. Although the presence of metastases in the axillary lymph node predicts the development of distant metastases in breast carcinoma patients, 20% to 30% of the patients who were free of axillary lymph node metastases also developed disease at distant sites (37). This observation indicates that breast tumor cells can bypass the lymph nodes and disseminate directly through the blood to distant organs. Our observation of the presence of circulating cancer cells in the stage I NSCLC cancer patients also concur with the above findings in breast cancer patients.

Our data show that detection of circulating cancer cells is a valid supplement to the TNM method for better cancer staging. The two methods combined together provide better information for designing lung cancer treatment strategies. In this study, the 5-year survival rate of early-stage (I–IIIa) NSCLC patients was 30% to 50% after surgical resection. A more precise staging method that includes detection of circulating cancer cells will aid in deciding whether adjuvant therapeutic regimen in addition to tumor resection are beneficial to the patients. Early-stage patients who have circulating cancer cells in their peripheral blood may actually have later-stage disease. An additional C factor for detection of circulating cancer cells or disseminated tumor cells should make the TNM staging method more precise for prognosis and therapeutic purposes. On the other hand, although it is statistically more significant to use \( L_c \) for classifying late-stage patients compared with TNM staging \((P = 0.006 \text{ versus } P = 0.03 \text{ in Fig. 3B and } A)\), the available data do not warrant using \( L_c \) alone for cancer staging. The detection of circulating cancer cells is more appropriate as a supplementary factor to the TNM staging.

At present, a reliable serologic biomarker assay for assessing the treatment response of NSCLC patients is not available. Two courses of chemotherapy are traditionally given before imaging is done to evaluate the treatment response of NSCLC patients. We showed in a limited number of patients that the \( L_c \) could be used for monitoring therapeutic response. Because different cancer cell types have different levels of marker gene expression, patients with higher \( L_c \) values do not necessarily have more circulating cancer cells in their peripheral blood than do patients with lower \( L_c \) values. Nevertheless, this semiquantitative approach is useful for measuring the relative cancer cell load in a patient’s peripheral blood to monitor the effectiveness of treatment. This method highlights an alternative approach to rapidly assess the treatment response of NSCLC patients. This method may therefore help to design more comprehensive and reasonable therapeutic regimens at earlier dates for NSCLC patients.

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