Clinical Significance of Circulating Tumor Cells and Free DNA in Non-small Cell Lung Cancer

KAZUTOSHI ISOBE1, YOSHINOBU HATA2, KUNIHIKO KOBAYASHI3, NAO HIROTA1, KEITA SATO1, GO SANO1, KEISHI Sugino1, SUSUMU SAKAMOTO1, YUJIRO TAKAI1, KAZUTOSHI SHIBUYA1, KEIGO TAKAGI2 and SAKAE HOMMA1

Departments of 1Respiratory Medicine, 2Chest Surgery, and 3Surgical Pathology, Toho University Omori Medical Center, Ota-ku, Tokyo, Japan; 4Department of Respiratory Medicine, Saitama International Medical Center, Saitama Medical University, Hidaka city, Saitama, Japan

Abstract. Aim: This pilot study assessed correlations between circulating tumor cells (CTCs) and circulating free DNA (cfDNA) of metastatic non-small cell lung cancer (NSCLC) after acquisition of resistance to epidermal growth factor receptor tyrosine kinase inhibitors. Patients and Methods: CTCs were counted using the CellSearch system (Veridex). cfDNA was analyzed for EGFR mutation status by the Cycleave real-time PCR assay. Results: Twenty-four patients participated in this study. CTCs were detected in 8 of 24 cases (33.3%), at a mean of 2.6 CTCs per 7.5 ml blood (range: 1-24). EGFR mutations in cfDNA were detected in 6 out of 24 cases (25%). The EGFR mutation detection rates in cfDNA were significantly higher in patients with ≥2 CTCs per 7.5 ml (100%) than in those with <2 CTCs per 7.5 ml (10%) (p=0.0001). Conclusion: The presence of CTCs was correlated with the positivity of EGFR mutation in cfDNA.

An activating mutation of the epidermal growth factor receptor (EGFR) gene is a promising target for the therapy of non-small cell lung cancer (NSCLC). The recommended strategy for treating NSCLC with an active EGFR mutation is to use EGFR tyrosine kinase inhibitors (EGFR-TKI), such as gefitinib or erlotinib (1-3). NSCLC patients with an active EGFR mutation treated with EGFR-TKI as first-line therapy had longer progression-free survival (PFS) than those who underwent platinum-based chemotherapy (4, 5). Therefore, detection of EGFR mutations in patients with metastatic NSCLC is an important factor in selecting therapy for the individual patient.

However, most patients have disease relapse within one year after the initial EGFR-TKI treatment (6). A secondary resistant T790M mutation has been identified in around 50% of patients with such acquired resistance to EGFR-TKI. Other mechanisms responsible for acquired resistance have been reported to be mesenchymal epithelial transition (MET) amplification and hepatocyte growth factor (HGF) production (7). In order to overcome the acquired resistance, new EGFR-TKIs active despite the T790M mutation, MET-inhibitors for the MET amplification, and transient phosphatidylinositol 3-kinase (PI3K) inhibition for HGF-mediated MET activation are all under investigation (8-10). The optimal management of patients with acquired EGFR-TKI resistance should be considered according to the mechanism of acquired resistance. Improved non-invasive methods for serial monitoring of the mutation status of EGFR in patients with metastatic NSCLC during treatment are therefore desirable.

Correspondence between EGFR mutation status in circulating free DNA (cfDNA) and in tumor tissue has been reported (11, 12), although the molecular mechanisms causing cfDNA release into the bloodstream have not yet been unequivocally determined. In addition, the prognostic significance of EGFR mutation status in circulating tumor cells (CTCs) from NSCLC patients has also been reported (13). CTCs are extremely rare cells that originate from the tumor mass and circulate freely in the peripheral blood. They are reported to represent a novel and promising prognostic factor in breast, prostate, and colorectal cancer (14-16). Although the clinical significance of CTCs in lung cancer patients remains unclear, several studies on this subject were reported in recent years (13, 17-19). It is a matter of great concern whether these newly developed techniques are suitable for clinical use.
The aim of the present study was to assess the correlation between blood content of CTCs or cfDNA of patients with metastatic NSCLC with acquired resistance to EGFR-TKI.

**Patients and Methods**

**Study design.** This was a single-institution prospective study conducted at Toho University Omori Medical Center (Tokyo, Japan), and was approved by its Ethical Committee. Blood samples (20 ml) were collected after development of EGFR-TKI resistance from 24 patients with primary lung cancer who had EGFR mutations at the primary site. Prior to their participation in the study, patients were examined to ensure that they met the following criteria: (a) histological or cytological diagnosis of NSCLC; (b) clinical stage IV or recurrence with distant metastases after surgical resection, (c) EGFR mutation at the primary site, (d) less than one month since acquiring EGFR-TKI resistance, and (e) informed consent of the patient, which was obtained before enrollment in the study according to institutional guidelines.

**Evaluation of CTCs.** Ten milliliters of blood was evacuated into CellSave Preservative Tubes, maintained at room temperature and processed within 72 hours of collection. The CTCs were captured and quantitatively evaluated in peripheral blood using the semiautomated CellSearch system (Veridex, LLC, Raritan, N.J., USA) (20), following the manufacturer’s protocol. In brief, epithelial cells were captured using ferroparticles coupled to a monoclonal antibody against epithelial cell adhesion molecule and separated in a magnetic field, and the enriched samples were then stained with 4’, 6-diamidino-2-phenylindole (DAPI) and an anticytokeratin antibody conjugated with phycoerythrin. Contaminating white blood cells were excluded by negative selection for CD45. Stained cells were then analyzed under a fluorescence microscope using the Cell Track Analyzer II (Veridex LLC). The CTC criteria were as follows: round to oval morphology, a visible DAPI-positive nucleus, cytokeratin staining in the cytoplasm, and no staining for CD45. All evaluations were performed without knowledge of the clinical characteristics of the patients.

**Detection of EGFR mutations in cfDNA.** Separated serum was stored at −80°C until use. cfDNA was extracted and purified using a QIAamp Circulating Nucleic Acid Kits (Qiagen, Hilden, Germany) following the manufacturer’s protocol and stored at −20°C until use. EGFR mutation status in cfDNA was analyzed using the cycleave real-time PCR assay (exon 18, 20, and 21) and fragment analysis (exon 19) (21). The cycleave PCR technique is based on a chimeric DNA-RNA-DNA probe labeled with a fluorescent dye and quencher at each end. The RNA sequence of the probes corresponds to that of the wild-type and point mutations labeled with 5-carboxyfluorescein (FAM) and 6-carboxy-X-rhodamine (ROX), respectively. When mutant molecules are present in the sample and PCR-amplified DNA generates a complete hybrid with the RNA portion of the mutant probe, RNase-H digests the probe at the RNA-DNA heteroduplex into two pieces, leading to a significant increase in fluorescence intensity by separation of the fluorescent dye from the quencher. The intensity of the wild-type probe served as an internal control for the assay. To detect deletions and insertions of the gene, common fragment analysis is used (21). Sample DNA is amplified with a FAM-labeled primer set. PCR products are electrophoresed on a sequencer. When a deletion mutation is present, PCR amplifies the shorter segment of DNA, which creates a new peak in an electropherogram.

**Statistical analysis.** Analyses were conducted using SPSS® software for Windows®, version 12.0 (SPSS Inc., Tokyo, Japan). Differences in survival between patients with CTCs (≥1 CTC in 7.5 ml of blood) and those without, and between patients in whom EGFR mutation had or had not been detected in cfDNA at the time of collecting blood samples were compared. The detection rate of EGFR mutations in cfDNA in serum from patients with or without CTCs had or had not been detected in cfDNA at the time of collecting blood samples were compared. The detection rate of EGFR mutations in cfDNA in serum from patients with or without CTCs was compared using the Chi-square test. Survival curves were drawn by the Kaplan-Meier method and statistical analysis was performed by a log-rank test.

**Results**

**Patients entered.** Between June and November 2010, 24 patients participated in this pilot study. Their characteristics are shown in Table I. There were 6 males and 18 females with a mean age of 64.1 (25 to 82) years. The major histological subtype was adenocarcinoma (23/24) and clinical stage was stage IV (19/24). The locations of the EGFR mutations in the primary site were exon 18 G719C in exon 19 19del G719C+L858R 19del L858R G719C+L858R G719C

**Table I. Patient characteristics (n=24).**

<table>
<thead>
<tr>
<th>Age (years) range</th>
<th>25-82</th>
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<tbody>
<tr>
<td>Mean</td>
<td>64.1</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6</td>
</tr>
<tr>
<td>Female</td>
<td>18</td>
</tr>
<tr>
<td>ECOG Performance status</td>
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</tr>
<tr>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>23</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>19</td>
</tr>
<tr>
<td>Recurrence †</td>
<td>5</td>
</tr>
<tr>
<td>EGFR mutation in primary site</td>
<td></td>
</tr>
<tr>
<td>G719C</td>
<td>1</td>
</tr>
<tr>
<td>G725A+19del</td>
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</tr>
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<td>19del</td>
<td>9</td>
</tr>
<tr>
<td>G719C+L858R</td>
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</tr>
<tr>
<td>L858R</td>
<td>11</td>
</tr>
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<td>Previous EGFR-TKI</td>
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<tr>
<td>Gefinitib</td>
<td>10</td>
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<td>Gefinitib and Erlotinib</td>
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ECOG: Eastern Cooperative Oncology Group; †after surgical resection; EGFR: epidermal growth factor receptor; L858R: exon 21 L858R; 19del: exon 19 deletion; G725A: exon 19 deletion; G719C: exon 18 G719C; EGFR-TKI: EGFR-tyrosine kinase inhibitor.
CTCs and cfDNA in serum. CTCs were detected in 8 out of the 24 cases (33.3%). The mean number of CTCs per 7.5 ml blood was 2.6 (range: 1-24). The characteristics of the 8 patients with CTCs and the other 16 were compared. No significant differences were found between the two groups with regard to age, sex, sites of distant metastases, times since diagnosis, smoking history, or tumor size.

EGFR mutations in cfDNA were detected in 6 out of the 24 cases (25%). The locations of these EGFR mutations were identical with those at the primary site in each patient. Additional mutations including T790M were not detected. L858R in four patients and 19 del in one patient with CTCs, and L858R in one patient without CTCs were found (Table II).

The rates of EGFR mutations in cfDNA were compared between patients with and without CTCs. The EGFR mutation rate in cfDNA was significantly higher in patients with CTCs (62.5%; 5 out of 8 cases) than in patients without (6.3%; 1 out of 16 cases, p=0.002). Moreover, EGFR mutation detection rates in cfDNA were significantly higher in patients with ≥2 CTCs in 7.5 ml (4 out of 4 cases) than in those with <2 CTCs in 7.5 ml (10%: 2 out of 20) (p=0.0001) (Table III).

Survival, CTCs and cfDNA. Survival of patients with or without CTCs at the time of collecting blood samples was compared. Patients with CTCs (≥1 in 7.5 ml) had a significantly shorter survival time than those without CTCs [median of 3.0 months vs. not reached, p=0.012, hazard ratio (HR)=2.9, 95% confidence interval (CI)=1.6-54.1 months] (Figure 1). There was no significant difference in survival between patients in whom EGFR mutation had or had not been detected in cfDNA [median not reached (n=18) vs. 3.9 months (n=6), respectively p=0.71, HR=1.1, 95% CI=0.4-3.1 months].

Discussion

CTCs are derived from clones in the primary tumor (22), suggesting that they may be useful for early diagnosis and prognosis. In addition, CTCs may play a major role in characterizing genetic and immunophenotypic changes occurring with tumor progression, thereby helping to guide targeted therapy (13). The CellSearch CTC test has been approved by the Food and Drug Administration of the United States for monitoring the number of CTCs in patients with metastatic breast, colorectal or prostate cancer. In two studies using the CellSearch system for patients with metastatic NSCLC, CTCs were detected in 30.6% (range of 0-62 per 7.5 ml blood) (23) and 32% (range of 0-146 per 7.5 ml blood) of cases (17). In those studies, patients were not subclassified according to EGFR mutation status. In our present study of metastatic NSCLC patients with activating EGFR mutations and acquired resistance, CTCs were detected at a similar rate of 33.3% (range of 0-24 per 7.5 ml blood).

Regarding the number detected, Tanaka et al. (23) reported that the CTC count increased significantly with
had metastatic disease. Allard et al. (83.0% and respectively, for patients with one or more CTCs who showed a significantly shorter survival than those without CTCs (median survival time=3.0 months vs. not reached, p=0.012, hazard ratio=2.9, 95% confidence interval=1.6-54.1 months).

Survival of patients from time of study entry. Patients with circulating tumor cells (CTCs) (≥1 in 7.5 ml) showed a significantly shorter survival than those without CTCs (median survival time=3.0 months vs. not reached, p=0.012, hazard ratio=2.9, 95% confidence interval=1.6-54.1 months).

![Figure 1. Survival of patients from time of study entry. Patients with circulating tumor cells (CTCs) (≥1 in 7.5 ml) showed a significantly shorter survival than those without CTCs (median survival time=3.0 months vs. not reached, p=0.012, hazard ratio=2.9, 95% confidence interval=1.6-54.1 months).](image)

tumor progression, especially with the development of distant metastasis in patients with lung cancer. The sensitivity and specificity of the CTC test was 71.0% and 83.0%, respectively, for patients with one or more CTCs who had metastatic disease. Allard et al. (20) recommended the cut-off as ≥2 CTCs per 7.5 ml blood because eight out of 145 samples from healthy women (5.5%) had one CTC, and no samples were found to contain ≥2 CTCs. Detection of ≥2 CTCs occurred in 20% (34 out of 168) of lung cancer cases. Whereas the recommended cut-off for screening of lung cancer might be ≥2 CTCs, cut-off levels appropriate for the purpose of the examination should be set for each study. Krebs et al. (17) showed a prognostic significance of the number and change in the number of CTCs for lung cancer outcome and response to therapy using a cut-off of 5 CTCs. Among untreated patients with lung cancer (n=101, stage IIIB), those with <5 CTCs had significantly longer progression-free survival (PFS) and overall survival (OS) compared to patients with ≥5 CTCs before chemotherapy (PFS=6.8 vs. 2.4 months; OS=8.1 vs. 4.3 months). These 101 patients were also not subclassified by EGFR mutation status. Further studies are needed to define prognostically relevant CTC cut-off levels.

cfDNA is thought to originate from apoptotic and necrotic cells of the primary or metastatic tumor, which discharge DNA early during tumorigenesis. EGFR mutations in cfDNA have been reported in 22.5-35.0% of patients with metastatic NSCLC patients (13, 24), and 23.7% of patients with stage IIIB to IV adenocarcinoma (25). In our study, EGFR mutations in cfDNA were detected in 6 out of 24 cases (25%). Although cfDNA is a valuable source for monitoring EGFR mutation status in patients with NCLC with activating EGFR mutations, the detection rate was very low. Maheswaran et al. (13) reported that direct analysis of CTCs for EGFR mutation status, using the CTC-chip and Scorpion-Amplification Refractory Mutation System (Scorpion-ARMS), was more sensitive than genotyping of cfDNA. Although their CTC-chip was highly sensitive and is expected to be promising, no large-scale study to confirm the usefulness of the CTC-chip exists thus far, suggesting that this method might not yet be sufficiently mature for clinical use. Goto et al. (25) reported that the detection rates of EGFR mutations in stage IIIB to IV adenocarcinoma in the IRESSA Pan-Asia Study (IPASS) were 23.7% in cfDNA and 61.5% in tumor tissue-derived DNA. PFS was significantly longer with gefitinib than carboplatin/paclitaxel in the cfDNA EGFR mutation-positive subgroup (HR=0.29; 95% CI=0.14-0.60; p<0.001), although cfDNA had a high rate of false negatives (56.9%). cfDNA is expected to play an important role in guiding targeted therapy as will CTCs, but sensitivity remains an unsolved problem. The mechanism of cfDNA release from primary and metastatic tumors during the metastatic process has not been fully determined (26, 27). Allelic imbalances, such as loss of heterozygosity and microsatellite instability, which we thought to be a biomarker of prostate cancer, was found in cfDNA from 58 patients with metastasis (28). In our study, the detection rate of EGFR mutations in cfDNA was significantly higher in patients with CTCs than in those without (p=0.002). Thus, the detection of EGFR mutations in cfDNA is affected by the presence of CTCs in the peripheral blood of patients with NSCLC with EGFR mutations, indicating that the tumor-derived component of total cfDNA might be mainly from CTCs, rather than from the primary or metastatic tumor itself.

The secondary resistance mutation, T790M, is found in 52% of patients with acquired EGFR-TKI resistance (7). In our study, the locations of EGFR mutations in cfDNA were identical with those at the primary sites. No additional mutations, including T790M, were detected. There may be at least three possible reasons for this. Firstly, the possibility of false-negatives should be considered because of the limited sensitivity of Cycleave analysis; this was reported to be capable of detecting EGFR mutations in samples containing 5% mutant alleles (29), but new methods with higher sensitivity have been reported since (30, 31). Kuang et al. (30) reported that they were able to detect T790M in cfDNA of 35% of patients. Moreover, Taniguchi et al. (31) proposed a highly sensitive method for cfDNA, which showed that 72.7% of patients with acquired EGFR-TKI resistance had an activating EGFR mutation and 43.5% had also acquired the T790M mutation. Secondly, the suggested indolent characteristics of patients with T790M...
might play a role in this phenomenon (32). Oxnard et al. (32) reported that patients with T790M were more likely to experience disease progression at an existing site (90%) rather than in a new organ system (10%). Of ten patients with wild-type EGFR in cfDNA, re-biopsy for resistant tumors revealed the T790M mutation in five (50%). Therefore, T790M might tend not to be released into circulating blood at the beginning of acquired resistance. Thirdly, the small sample size of our study is a limitation. Further studies are needed to clarify relationships between the presence of T790M and CTCs, and patient prognosis after acquiring EGFR-TKI resistance.

In conclusion, we found that the presence of CTCs was correlated with the positivity of EGFR mutation in cfDNA.

Conflicts of Interest Statement

None declared.

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