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

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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.

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Key Words: Circulating tumor cells (CTCs), circulating free DNA (cfDNA), lung cancer, epidermal growth factor receptor (*EGFR*), CellSearch system, Cycleave real-time PCR assay.

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.

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

	n
Age (years) range	25-82
Mean	64.1
Gender	
Male	6
Female	18
ECOG Performance status	
0	8
1	12
2	4
Histology	
Adenocarcinoma	23
Squamous cell carcinoma	1
Clinical stage	
IV	19
Recurrence†	5
EGFR mutation in primary site	
G719C	1
G725A+19del	2
19del	9
G719C+L858R	1
L858R	11
Previous EGFR-TKI	
Gefitinib	10
Erlotinib	6
Gefinitib and Erlotinib	8

ECOG: Eastern Cooperative Oncology Group; †after surgical resection; *EGFR*: epidermal growth factor receptor; L858R: exon 21 L858R; 19del: exon 19 deletion; G725A: exon 18 G725A; G719C: exon 18 G719C; EGFR-TKI: EGFR-tyrosine kinase inhibitor.

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 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

Table II. *Detection of circulating tumor cells (CTCs) and epidermal growth factor receptor (EGFR) mutation in circulating free DNA (cfDNA) (n=24).*

Case	*EGFR* mutation in primary site	CTCs (n/7.5 ml)	*EGFR* mutation in cfDNA
1	L858R	24	L858R
2	19del	14	19del
3	G719C+L858R	8	L858R
4	L858R	3	L858R
5	L858R	1	L858R
6	19del	1	Wild-type
7	L858R	1	Wild-type
8	L858R	1	Wild-type
9	L858R	0	L858R
10	G725A+19del	0	Wild-type
11	L858R	0	Wild-type
12	L858R	0	Wild-type
13	19del	0	Wild-type
14	L858R	0	Wild-type
15	19del	0	Wild-type
16	19del	0	Wild-type
17	19del	0	Wild-type
18	L858R	0	Wild-type
19	19del	0	Wild-type
20	L858R	0	Wild-type
21	19del	0	Wild-type
22	G725A+19del	0	Wild-type
23	19del	0	Wild-type
24	19del	0	Wild-type

L858R: exon 21 L858R; 19del: exon 19 deletion; G725A: exon 18 G725A; G719C: exon 18 G719C; G725A: exon 18 G725A.

1 case, exon 19 deletions in 11 (of whom two had a double mutation in exon 18 G725A), and exon 21 L858R in 12 (of whom one had a double mutation in exon 18 G791C).

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).

Table III. *Relationship between presence of circurating tumor cells (CTCs) and detection of epidermal growth factor receptor (EGFR) mutation in circulating free DNA (cfDNA).*

	EGFR mutation in cfDNA, no. patients		
	Positive	Negative	Detection rate of *EGFR* mutation
≥2 CTCs per 7.5 ml	4	0	100%*
<2 CTCs per 7.5 ml	2	18	10%*

*p=0.0001.

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 patients. 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

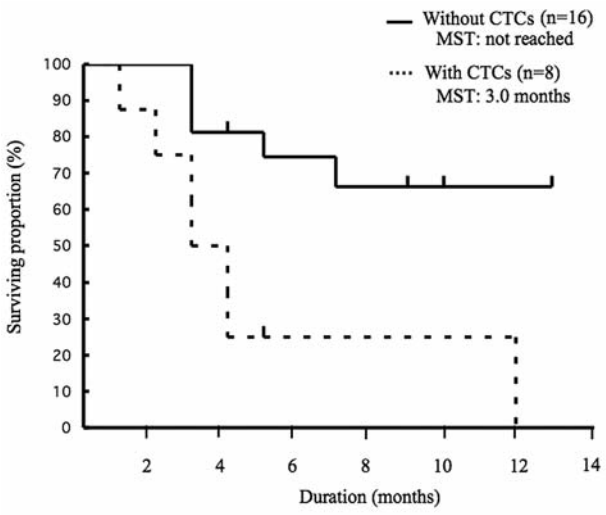

Figure 1. *Survival of patients from time of study entry. Patients with circulating tumor cells (CTCs) (≥1 in 7.5 ml) showed a significantly shorter survival than those without CTCs (median survival time=3.0 months vs. not reached, p=0.012, hazard ratio=2.9, 95% confidence interval=1.6-54.1 months).*

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≥IIIA), 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 could 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.

Conflict of Interest Statement

None declared.

Acknowledgements

We thank Atsushi Kakimoto, Yoshio Inoue, Yuji Shimoda, Satoshi Natsume of SRL Inc. (Tokyo Japan), and Akiyoshi Yutani, Tomoki Shirai, Motoki Yamagata of Astra Zeneca Inc. (Tokyo Japan) for their help in designing the study.

References

1 Azzoli CG, Baker S Jr, Temin S, Pao W, Aliff T, Brahmer J, Johnson DH, Laskin JL, Masters G, Milton D, Nordquist L, Pfister DG, Piantadosi S, Schiller JH, Smith R, Smith TJ, Strawn JR, Trent D and Giaccone G: American Society of Clinical Oncology Clinical Practice Guideline update on chemotherapy for stage IV non-small-cell lung cancer. J Clin Oncol *27*: 6251-6256, 2009.

2 Ettinger DS, Akerley W, Bepler G, Blum MG, Chang A, Cheney RT Chirieac LR,D'Amico TA, Demmy TL, Ganti AK, Govindan R, Grannis FW Jr, Jahan T, Jahanzeb M, Johnson DH, Kessinger A, Komaki R, Kong FM, Kris MG, Krug LM, Le QT, Lennes IT, Martins R, O'Malley J, Osarogiagbon RU, Otterson GA, Patel JD, Pisters KM,Reckamp K, Riely GJ, Rohren E, Simon GR, Swanson SJ, Wood DE and Yang SC: Non-small cell lung cancer. J Natl Compr Cancer Netw *8*: 740-801, 2010.

3 Fukuoka M, Wu YL, Thongprasert S, Sunpaweravong P, Leong SS, Sriuranpong V Chao TY, Nakagawa K, Chu DT, Saijo N, Duffield EL, Rukazenkov Y, Speake G, Jiang H, Armour AA, To KF, Yang JC and Mok TS: Biomarker analyses and final overall survival results from a phase III, randomized, open-label, first-line study of gefitinib *versus* carboplatin/paclitaxel in clinically selected patients with advanced non-small-cell lung cancer in Asia (IPASS). J Clin Oncol *29*: 2866-2874, 2011.

4 Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, Gemma A, Harada M, Yoshizawa H, Kinoshita I, Fujita Y, Okinaga S, Hirano H, Yoshimori K, Harada T, Ogura T, Ando M, Miyazawa H, Tanaka T, Saijo Y, Hagiwara K, Morita S and Nukiwa T: Gefitinib or chemotherapy for non-small cell lung cancer with mutated *EGFR*. N Engl J Med *362*: 2380-2308, 2010.

5 Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, Seto T, Satouchi M, Tada H, Hirashima T, Asami K, Katakami N, Takada M, Yoshioka H, Shibata K, Kudoh S, Shimizu E, Saito H, Toyooka S, Nakagawa K and Fukuoka M: Gefitinib *versus* cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. Lancet Oncol *11*: 121-128, 2010.

6 Sequist LV, Bell DW, Lynch TJ and Haber DA: Molecular predictors of response to epidermal growth factor receptor antagonists in non-small cell lung cancer. J Clin Oncol *25*: 587-595, 2007.

7 Yano S, Yamada T, Takeuchi S, Minami Y, Yatabe Y, Mitsudomi T, Tanaka H, Kimura T, Kudoh S, Nokihara H, Ohe Y, Yokota J, Uramoto H, Yasumoto K, Kiura K, Higashiyama M, Oda M, Saito H, Yoshida J, Kondoh K and Noguchi M: Hepatocyte growth factor expression in *EGFR* mutant lung cancer with intrinsic and acquired resistance to tyrosine kinase inhibitors in a Japanese cohort. J Thorac Oncol *6*: 2011-2017, 2011.

8 Kobayashi S, Boggon TJ, Dayaram T, Jänne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG and Halmos B: *EGFR* mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med *352*: 786-792, 2005.

9 Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N,Gale CM, Zhao X, Christensen J, Kosaka T, Holmes AJ, Rogers AM, Cappuzzo F, Mok T, Lee C, Johnson BE, Cantley LC and Jänne PA: *MET* amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science *316*: 1039-1043, 2007.

10 Donev IS, Wang W, Yamada T, Li Q, Takeuchi S, Matsumoto K, Yamori T, Nishioka Y, Sone S and Yano S: Transient PI3K inhibition induces apoptosis and overcomes HGF-mediated resistance to EGFR-TKIs in *EGFR* mutant lung cancer. Clin Cancer Res *17*: 2260-2269, 2011.

11 Kimura H, Kasahara K, Kawaishi M, Kunitoh H, Tamura T, Holloway B and Nishio K: Detection of epidermal growth factor receptor mutations in serum as a predictor of the response to gefitinib in patients with non-small cell lung cancer. Clin Cancer Res *12*: 3915-3921, 2006.

12 Kimura H, Suminoe M, Kasahara K, Kunitoh H, Sone T, Araya T, Tamori S, Koizumi F, Nishio K, Miyamoto K, Fujimura M and Nakao S: Evaluation of epidermal growth factor receptor mutation status in serum DNA as a predictor of response to gefitinib (IRESSA). Br J Cancer *97*: 778-784, 2007.

13 Maheswaran S, Sequist LV, Nagrath S, Ulkus L, Brannigan B, Collura CV, Inserra E, Diederichs S, Iafrate AJ, Bell DW, Digumarthy S, Muzikansky A, Irimia D,Settleman J, Tompkins RG, Lynch TJ, Toner M and Haber DA: Detection of mutations in *EGFR* in circulating lung-cancer cells. N Engl J Med *359*: 366-377, 2008.

14 Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW and Hayes DF: Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med *351*: 781-791, 2004.

15 Cohen SJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, Picus J, Morse M, Mitchell E, Miller MC, Doyle GV, Tissing H, Terstappen LW and Meropol NJ: Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. J Clin Oncol *26*: 3213-3221, 2008.

16 Scher HI, Jia X, de Bono JS, Fleisher M, Pienta KJ, Raghavan D and Heller G: Circulating tumour cells as prognostic markers in progressive, castration-resistant prostate cancer: a re-analysis of IMMC38 trial data. Lancet Oncol *10*: 233-239, 2009.

17 Krebs MG, Sloane R, Priest L, Lancashire L, Hou JM, Greystoke A, Ward TH, Ferraldeschi R, Hughes A, Clack G, Ranson M, Dive C and Blackhall FH: Evaluation and prognostic significance of circulating tumor cells in patients with non-small cell lung cancer. J Clin Oncol *29*: 1556-1563, 2011.

18 Wu C, Hao H, Li L, Zhou X, Guo Z, Zhang L, Zhang X, Zhong W, Guo H, Bremner RM and Lin P: Preliminary investigation of the clinical significance of detecting circulating tumor cells enriched from lung cancer patients. J Thorac Oncol *4*: 30-36, 2009.

19 Okumura Y, Tanaka F, Yoneda K, Hashimoto M, Takuwa T, Kondo N and Hasegawa S: Circulating tumor cells in pulmonary venous blood of primary lung cancer patients. Ann Thorac Surg *87*: 1669-1675, 2009.

20 Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, Tibbe AG, Uhr JW and Terstappen LW: Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res *10*: 6897-6904, 2004.

21 Yatabe Y, Hida T, Horio Y, Kosaka T, Takahashi T and Mitsudomi T: A rapid, sensitive assay to detect *EGFR* mutation in small biopsy specimens from lung cancer. J Mol Diagn *8*: 335-341, 2006.

22 Fehm T, Sagalowsky A, Clifford E, Beitsch P, Saboorian H, Euhus D, Meng S, Morrison L, Tucker T, Lane N, Ghadimi BM, Heselmeyer-Haddad K, Ried T, Rao C and Uhr J: Cytogenetic evidence that circulating epithelial cells in patients with carcinoma are malignant. Clin Cancer Res *8*: 2073-2084, 2002.

23 Tanaka F, Yoneda K, Kondo N, Hashimoto M, Takuwa T, Matsumoto S, Okumura Y, Rahman S, Tsubota N, Tsujimura T, Kuribayashi K, Fukuoka K, Nakano T and Hasegawa S: Circulating tumor cell as a diagnostic marker in primary lung cancer. Clin Cancer Res *15*: 6980-6986, 2009.

24 Bai H, Mao L, Wang HS, Zhao J, Yang L, An TT, Wang X, Duan CJ, Wu NM, Guo ZQ, Liu YX, Liu HN, Wang YY and Wang J: Epidermal growth factor receptor mutations in plasma DNA samples predict tumor response in Chinese patients with stages IIIB to IV non-small cell lung cancer. J Clin Oncol *27*: 2653-2659, 2009.

25 Goto K, Ichinose Y, Ohe Y, Yamamoto N, Negoro S, Nishio K, Itoh Y, Jiang H, Duffield E, McCormack R, Saijo N, Mok T and Fukuoka M: Epidermal growth factor receptor mutation status in circulating free DNA in serum: From IPASS, a phase III study of gefitinib or carboplatin/paclitaxel in non-small cell lung cancer. J Thorac Oncol *7*: 115-121, 2012.

26 Pantel K and Brakenhoff RH: Dissecting the metastatic cascade. Nat Rev Cancer *4*: 448-456, 2004.

27 Gupta GP and Massagué J: Cancer metastasis: building a framework. Cell *127*: 679-695, 2006.

28 Schwarzenbach H, Alix-Panabières C, Müller I, Letang N, Vendrell JP, Rebillard X and Pantel K: Circulating-free tumor DNA in blood plasma as a marker for circulating tumor cells in prostate cancer. Clin Cancer Res *15*: 1032-1038, 2009.

29 Pao W and Ladanyi M: Epidermal growth factor receptor mutation testing in lung cancer: searching for the ideal method. Clin Cancer Res *1*: 4954-4955, 2007.

30 Kuang Y, Rogers A, Yeap BY, Wang L, Makrigiorgos M, Vetrand K, Thiede S, Distel RJ, and Jänne PA: Noninvasive detection of *EGFR* T790M in gefitinib- or erlotinib-resistant non-small cell lung cancer. Clin Cancer Res *15*: 2630-2636, 2009.

31 Taniguchi K, Uchida J, Nishino K, Kumagai T, Okuyama T, Okami J, Higashiyama M, Kodama K, Imamura F and Kato K: Quantitative detection of *EGFR* mutations in circulating tumor DNA derived from lung adenocarcinomas. Clin Cancer Res *17*: 7808-7815, 2011.

32 Oxnard GR, Arcila ME, Sima CS, Riely GJ, Chmielecki J, Kris MG, Pao W, Ladanyi M and Miller VA: Acquired resistance to EGFR tyrosine kinase inhibitors in *EGFR*-mutant lung cancer: distinct natural history of patients with tumors harboring the T790M mutation. Clin Cancer Res *17*: 1616-1622, 2011.

Received March 18, 2012
Revised April 19, 2012
Accepted April 23, 2012