skeletal metastases at the initial staging, and 45 % had SREs.

Zoledronic acid has been used in patients with bone metastases because the drug can reduce the incidence of SREs and delay time to the first SRE [10]. Recently, the non-inferiority of denosumab to zoledronic acid in delaying the time to the first SRE was demonstrated [11]. However, we believe that the efficacy of these drugs cannot be insufficient. The efficacy of chemotherapy against bone lesions in patients with lung cancer has not been reported previously.

Bevacizumab, an anti-vascular endothelial growth factor (VEGF) agent, provides a clinical benefit when combined with platinum-based chemotherapy in first-line therapy against advanced non-squamous (non-Sq) NSCLC [12–14]. In particular, the response rate and progression-free survival (PFS) compared with those of non-bevacizumab-containing chemotherapy are improved by the addition of bevacizumab. Antitumor activity may be induced by the effects of bevacizumab on tumor vasculature, interstitial pressure, and blood vessel permeability, resulting in enhanced delivery of chemotherapy agents to tumor cells [15]. Nagengast et al. [16] demonstrated that bevacizumab distribution to the bone was similar as that to other organs in an ex vivo biodistribution model. Bäuerle et al. [17] reported that bevacizumab significantly inhibited osteolysis, surrounding soft tissue tumor growth, and angiogenesis in an experimental model of breast cancer bone metastasis as visualized on volumetric computed tomography (CT) and magnetic resonance imaging (MRI). Furthermore, the blocking of VEGF–VEGF receptor (VEGFR)-2 signaling inhibited bone metastasis in animal models of lung cancer [18]. Therefore, VEGF was suggested as a therapeutic target for bone metastasis [19]. Thus, we hypothesized that bevacizumab-containing chemotherapy could have some clinical benefit in patients with non-Sq NSCLC and bone metastases. We retrospectively investigated the efficacy of bevacizumab-containing chemotherapy and compared it to that of chemotherapy without bevacizumab in this study.

Patients and methods

Patients

We reviewed electronic medical records of consecutive patients who visited the Shizuoka Cancer Center between January 2007 and December 2011. In addition, electronically stored images were evaluated by a diagnostic radiologist. Eligible patients were pathologically diagnosed with non-Sq NSCLC, received platinum-based first-line chemotherapy, had bone metastases at the time of receiving chemotherapy, had at least 1 evaluable bone lesion according to the Revised Response Evaluation Criteria in Solid Tumors (RECIST) guideline (version 1.1) [20], and received zoledronic acid continuously. We permitted the inclusion of patients who received EGFR tyrosine kinase inhibitors before platinum-based chemotherapy. We selected carboplatin plus paclitaxel and carboplatin plus pemetrexed as the non-bevacizumab-containing chemotherapy regimens because we used only these regimens in combination with bevacizumab in our institution. The patients who received bevacizumab-containing chemotherapy comprised the "BEV group" and those who received chemotherapy without bevacizumab comprised the "non-BEV group".

Evaluation

We evaluated the objective response rate, disease control rate, time to progression of overall disease (TTP), time to progression of bone metastases (B-TTP), overall survival (OS), and proportion of patients with SREs. The response to chemotherapy was accessed according to RECIST criteria (version 1.1). At the initial staging, we performed chest and abdominal CT, brain MRI, and positron emission tomography (PET)–CT/bone scintigraphy. To ascertain disease progression or the relapse of overall disease and bone metastases, patients were evaluated by physical examination, chest radiography, and CT of the chest and abdomen. If bone metastases were detected at the initial staging, the patient was regularly followed up with radiography and CT. If progression of bone metastases was suspected, we additionally performed PET–CT, MRI, or bone scintigraphy, as required. Generally, all patients were evaluated for lesions during and approximately 6–8 weeks after the treatment period.

Time to progression was measured from the start of first-line chemotherapy to the date of an event of documented disease progression/recurrence or the last follow-up visit. B-TTP was measured from the start of first-line chemotherapy to the date of an event of documented progression of bone metastases and/or SRE or the last follow-up visit. Cases of TTP or B-TTP were censored under the following conditions: no progression or recurrence of overall disease or bone metastases and death. The incidence of SREs accounted for all events that occurred from the start of platinum-based chemotherapy to the date of first progression of overall disease or the last follow-up visit. SREs included a pathologic fracture, spinal cord compression, and the need for bone radiation or surgical therapy. OS was measured from the start of first-line chemotherapy to the date of death or the last follow-up visit.

Statistical analysis

All categorical variables, objective response rates, and incidences of SREs were analyzed and compared between the BEV and non-BEV groups using the χ^2 test or Fisher's exact test, as appropriate. The distributions of TTP, B-TTP, and OS were estimated using the Kaplan–Meier method, and the BEV and non-BEV groups were compared using the log-rank test. All p values were two-sided, and values less than 0.05 were considered statistically significant. All analyses were performed using JMP 9 software (SAS Institute, Cary, NC). This study was approved by the Institutional Review Board of Shizuoka Cancer Center.

Results

A total of 25 patients, 13 patients in the BEV group and 12 patients in the non-BEV group, were eligible for this retrospective study. Patient characteristics are shown in Table 1. In the BEV and non-BEV groups, the median ages of patients were 63 and 67 years, respectively. In total, 11 of 13 (85 %) patients in the BEV group and 9 of 12 (75 %) patients in the non-BEV group were men. The BEV group included 11 (85 %) current or ever smokers, and the non-BEV group included 7 (58 %) current or ever smokers. The numbers of patients with an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–1 were 12 in the BEV group and 11 in the non-BEV group, and 1 patient in each group had an ECOG PS of 2. The EGFR status was not examined in 5 patients in the non-BEV group, but no statistically significant difference in EGFR status was found between the 2 groups ($p = 0.41$).

The administered chemotherapy regimens are shown in Table 1. In the BEV group, 6 patients were treated with carboplatin, paclitaxel, and bevacizumab, whereas 7 patients were treated with carboplatin, pemetrexed, and bevacizumab. In the non-BEV group, 11 patients received carboplatin plus paclitaxel, and 1 patient received carboplatin plus pemetrexed.

The response rates for overall disease were 54 % in the BEV group and 8 % in the non-BEV group ($p = 0.01$; Table 2). The disease control rates for overall disease were 100 % in the BEV group and 50 % in the non-BEV group ($p = 0.01$; Table 2). The response rates for bone metastases were 23 % in the BEV group and 0 % in the non-BEV group ($p = 0.038$; Table 3). The disease control rates for bone metastases were 100 % in the BEV group and 67 % in the non-BEV group ($p = 0.01$; Table 3).

The Kaplan–Meier curve for B-TTP is shown in Fig. 1. The median B-TTPs were 13.7 months in the BEV group and 4.3 months in the non-BEV group ($p = 0.06$). The Kaplan–Meier curve for TTP is shown in Fig. 2. The median TTPs were 5.7 months in the BEV group and 2.6 months in the non-BEV group ($p = 0.17$). Overall disease progression was observed in 12 of 13 patients in the BEV group and in all patients in the non-BEV group. The median OS was 6.6 months (range, 4.0–34.7 months) in the non-BEV group and this was not reached (range, 6.6 months-) in the BEV group ($p = 0.13$). In the present study, the median follow-up duration was 15.1 months.

Skeletal-related events occurred in 3 patients (23 %) in the BEV group and in 6 patients (50 %) in the non-BEV group (Table 4). The types of SREs were as follows: 3 instances of the need for bone radiation, 1 instance of spinal cord compression in the BEV group, and 5 instances of the need for bone radiation, 1 bone surgery, and 1 pathologic fracture in the non-BEV group.

Discussion

To the best of our knowledge, the present study is the first report to evaluate the bone-specific efficacy of

Table 1 Patients characteristics and chemotherapy regimens

		BEV	Non-BEV	*P* value
Number		13	12	–
Age	Median (range)	63 (35–75)	67 (40–76)	0.3255
Sex	M/f	11/2	9/3	0.5476
Smoking	Yes/no	11/2	7/5	0.1394
PS	0/1/2	3/9/1	0/11/1	0.9530
EGFR	Mt/wt/unknown	4/9/0	2/5/5	0.4054
Regimen of chemotherapy	CBDCA + PTX	–	11	–
	CBDCA + PEM	–	1	
	CBDCA + PTX + BEV	6	–	
	CBDCA + PEM + BEV	7	–	

Mt mutation, *Wt* wild type, *CBDCA* carboplatin, *PTX* paclitaxel, *PEM* pemetrexed, *BEV* bevacizumab

Table 2 Response and control rates for overall disease

Best response	BEV ($n = 13$)	Non-BEV ($n = 12$)	P value
PR	7	1	
SD	6	5	
PD	0	6	
Response rate	54 %	8 %	0.01
Disease control rate	100 %	50 %	0.01

PR partial response, *SD* stable disease, *PD* progressive disease

Table 3 Response and control rates for bone metastases

Best response	BEV ($n = 13$)	Non-BEV ($n = 12$)	P value
PR	3	0	
SD	10	8	
PD	0	4	
Response rate	23 %	0 %	0.04
Disease control rate	100 %	67 %	0.01

PR partial response, *SD* stable disease, *PD* progressive disease

chemotherapy in patients with bone metastases from NSCLC. In addition, it was important to evaluate the bevacizumab-mediated potentiation of chemotherapeutic efficacy against bone metastases. In the present study, in the BEV group, the response and disease control rates for bone metastases were 23 and 100 %, respectively, and the median B-TTP was 13.7 months.

Rosen et al. [10, 21] reported a Phase 3 trial of zoledronic acid. Among 254 patients who received zoledronic acid 4 mg, 124 patients (49 %) had NSCLC and 207 patients (82 %) received chemotherapy. The best bone response rate as per the original criteria was 8 %, and the disease control rate for bone metastases was 29 %. In this study, by using the RECIST guideline (version 1.1), the response rate for bone metastases was 0 % and the disease control rate for bone metastases was 67 % in the non-BEV group. In contrast, the response rate for bone metastases was 23 % and the disease control rate for bone metastases was 100 % in the BEV group. Although different bone lesion response criteria were used for the Phase 3 trial of zoledronic acid and this study, administration of bevacizumab-containing chemotherapy showed some potential for eliciting an effect on bone metastases. In the same Phase 3 trial, the median B-TTP of patients who received zoledronic acid 4 mg was 145 days, and the proportion of patients with at least 1 SRE over a period of 9 months was 38 %. In this study, the median B-TTPs were 130 days in the non-BEV group and 412 days in the BEV group. In terms of the proportion of patients with SREs, 50 % of patients in the non-BEV group and 23 % of patients in the BEV group had SREs until the first progression of overall disease or the last follow-up visit. These results suggest that bevacizumab-containing chemotherapy specifically controlled bone lesions as well as systemic lesions.

The antitumor activity of bevacizumab-containing chemotherapy is believed to be the result of enhanced chemotherapy delivery to tumor cells [15]. Bevacizumab distribution to bone was similar as that to other organs in ex vivo biodistribution analysis [16]. Inhibiting VEGF–VEGFR-2 signaling inhibited bone metastasis in animal models of lung cancer with bone metastasis [18]. Solares et al. [22] reported a patient with lung adenocarcinoma and bone metastases in whom a complete response was achieved with carboplatin, paclitaxel, and bevacizumab. Paule and Brion [23] reported that 2 patients with renal cell carcinoma (RCC) and bone metastases who were treated with the anti-VEGFR inhibitor sunitinib experienced long-term survival and stabilization of bone metastases. They concluded that VEGF-targeted agents such as sunitinib

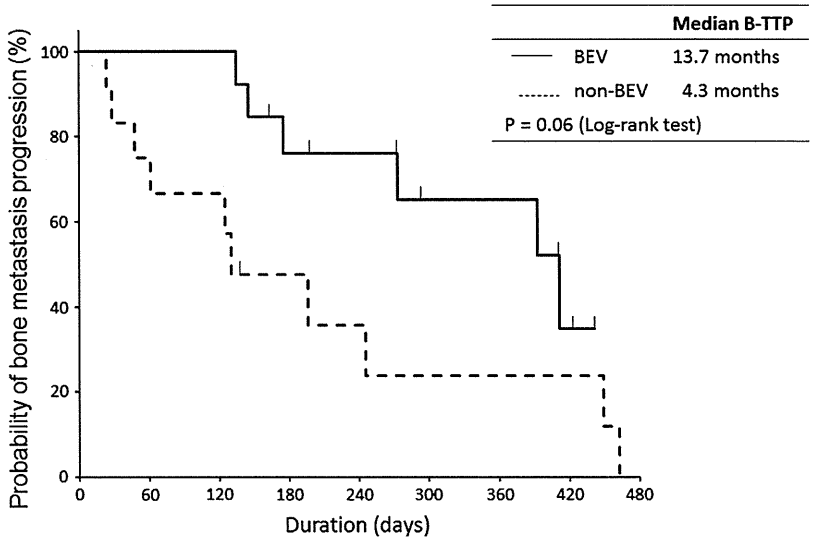

Fig. 1 Kaplan–Meier plot of time to progression of bone metastases (B-TTP) of patients who received chemotherapy containing bevacizumab (BEV group) or lacking bevacizumab (non-BEV group). The median B-TTPs were 13.7 months in the BEV group and 4.3 months in the non-BEV group ($p = 0.06$)

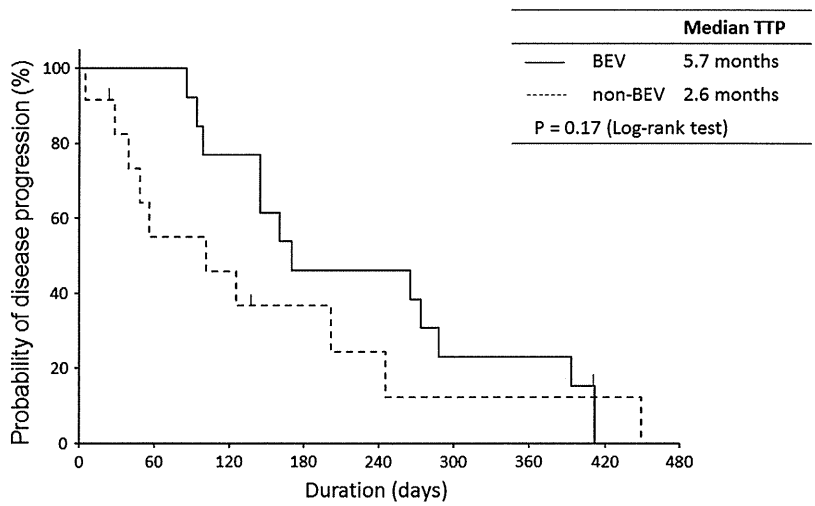

Fig. 2 Kaplan–Meier plot of time to progression of overall disease (TTP) of patients who received chemotherapy containing bevacizumab (BEV group) or lacking bevacizumab (non-BEV group). The median TTPs were 5.7 months in the BEV group and 2.6 months in the non-BEV group ($p = 0.17$)

Table 4 Proportion of patients with SREs until the first documented event of disease progression

	BEV $n = 13$	Non-BEV $n = 12$
SREs*	3 (23 %)	6 (50 %)
Radiation to bone	3	5
Surgery to bone	0	1
Spinal cord compression	1	0
Pathologic fracture	0	1

SRE skeletal-related events

* $P = 0.16$

may be effective treatments for bone metastases. Furthermore, a retrospective analysis reported that sunitinib plus bisphosphonates such as zoledronic acid and pamidronate improved the response rate, PFS, and OS in cases of RCC with bone metastases [24]. In our study, the response rates for bone metastases were 23 % in the BEV group and 0 % in the non-BEV group. These results might validate the clinical efficacy of bevacizumab-containing chemotherapy against bone metastases.

This study has several limitations. The sample size was small. This was a retrospective study with an inherent potential for bias. The collection of clinical characteristics and treatment response data was retrospective, and the follow-up interval for physical examinations was indefinite. Therefore, future studies are warranted to investigate larger sample sizes.

In conclusion, this study indicates that bevacizumab might potentiate the antitumor activity of chemotherapy against both systemic disease and bone metastases, thereby prolonging bone-specific TTP and reducing the incidence of SREs.

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Conflict of interest The authors declare that they have no conflict of interest.

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Interstitial Lung Disease Associated with Gefitinib in Japanese Patients with *EGFR*-mutated Non-small-cell Lung Cancer: Combined Analysis of Two Phase III Trials (NEJ 002 and WJTOG 3405)

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Objective: Interstitial lung disease associated with gefitinib is a critical adverse reaction. When geftinib was administered to *EGFR*-unknown patients, the interstitial lung disease incidence rate was approximately 3–4% in Japan, and usually occurs during the first 4 weeks of treatment. However, it has not been fully investigated in *EGFR*-mutated patients.
Methods: We collected clinical records of participants of two Phase III trials (WJTOG 3405 and NEJ 002), which compared gefitinib with platinum doublet chemotherapy. All patients were *EGFR* mutated, chemo-naïve and had good performance status.
Results: A total of 402 patients were enrolled in this study. In the gefitinib arm, 10 (5.0%) of 201 patients developed interstitial lung disease, of whom five (2.5%) were Grade 3 or greater, with two deaths (1.0%). In contrast, only one patient developed interstitial lung disease (Grade 1) in the chemotherapy arm. With regard to gefitinib, smoking history was significantly associated with developing interstitial lung disease (odds ratio 0.18; 95% confidence interval: 0.05–0.74; $P = 0.01$). The cumulative incidence rate of interstitial lung disease was similar in the 0–4, 5–8 and 9–12 week time periods. However, between smokers and never-smokers, cumulative incidence rates in the first 4 weeks were significantly different (4.7% versus 0%, $P = 0.03$). Three of 10 patients developed interstitial lung disease after 8 weeks of gefitinib administration (days 135, 171 and 190, respectively).
Conclusions: Among *EGFR*-mutated patients, the incidence of interstitial lung disease associated with gefitinib was not different from that in previous reports. Smoking history was associated with developing interstitial lung disease, and smokers had a higher incidence rate of interstitial lung disease in the first 4 weeks.

Key words: epidermal growth factor receptor mutation – gefitinib – epidermal growth factor receptor-tyrosine kinase inhibitor – interstitial lung disease – Japanese

INTRODUCTION

The recent introduction of targeted agents has dramatically changed the treatment of non-small-cell lung cancer (NSCLC). Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) is a prototype of such therapy which targets NSCLC harboring the *EGFR* mutation (1,2). EGFR-TKIs have demonstrated a higher response rate and longer progression-free survival than platinum doublet chemotherapy (3–6). Common adverse events associated with EGFR-TKIs include skin rash, diarrhea and hepatotoxicity. Interstitial lung disease (ILD) is a rare but potentially fatal adverse event (7). The incidence of ILD has been reported to be higher in Japanese than in Caucasians. Two large, multi-institutional studies in Japan (8–10) reported that its incidence is 3.5–4.0%, compared with just 0.3% in the USA (11). They also suggested that male gender, history of smoking, poor performance status, pre-existing lung disorder and prior history of chemotherapy were predictive risk factors (8–10).

Today, clinical guidelines recommend that administration of EGFR-TKIs should be limited to *EGFR*-mutated patients, reflecting the high efficacy of this drug in this patient population (12). Since it is known that *EGFR* mutation is relatively rare in males or smokers, which are known risk factors of ILD, ILD incidence might be lower in patients with *EGFR* mutation. However, a detailed investigation of ILD associated with EGFR-TKIs among *EGFR*-mutated patients has not been done. Therefore, we conducted a combined analysis of two Phase III trials that compared gefitinib with platinum doublet chemotherapy in Japanese NSCLC patients with *EGFR* mutation.

PATIENTS AND METHODS

Patient Selection and Treatment Methods

We collected the clinical records of participants of two Phase III trials (WJTOG 3405 (3) and NEJ 002 (4)). These trials compared gefitinib with platinum doublet chemotherapy in Japanese NSCLC patients with *EGFR* mutation. *EGFR* mutation was screened by PCR-based methods as previously described (13,14). All of the participants were required to be chemo-naïve, with Eastern Cooperative Oncology Group performance status (ECOG PS) of 0–1 and aged between 20 and 75 years, with adequate organ function. Patients with active infectious disease or severe heart disease were excluded. All patients were confirmed not to have pulmonary fibrosis by chest computed tomography (CT) within 1 month prior to registration. Both studies were approved by the institutional review board at each participating site.

Eligible patients were randomly assigned to receive either gefitinib (250 mg daily) or standard chemotherapy. The latter consisted of paclitaxel 200 mg/m^2 plus carboplatin (area under the curve of six) in NEJ 002 or docetaxel 60 mg/m^2 plus cisplatin 80 mg/m^2 in WJOG 3405, every 3 weeks. All participants who had received at least one dose of a study drug were included in the safety analysis.

Baseline data were collected for each patient, including information on sex, age, history of smoking, ECOG PS, tumor histology, clinical stage and type of *EGFR* mutation.

Evaluation of ILD and Statistical Analysis

All patients were assessed by chest CT for their response to treatment every 2 months. The diagnosis of ILD was based on clinical manifestations (worsening dry cough or dyspnea within days to weeks), accompanied by interstitial pulmonary infiltrates on a chest X-ray and a chest CT (15). Close investigation, such as blood and bacterial examination, was required in the protocols to exclude other ILDs. Bronchoalveolar lavage was also recommended, if possible. ILD was assessed according to the National Cancer Institute

Table 1. Baseline characteristics of the patients in the gefitinib arm

	Total ($n = 201$)	Non-ILD ($n = 191$)	ILD ($n = 10$)	P value
Age (years)				
Mean	64	64	63	0.67
Range	34–75	34–75	56–75	
Sex (no.)				
Male	71	65	6	0.17
Female	130	126	4	
Smoking status (no.)				
Never	137	134	3	0.01
Previous/current	64	57	7	
ECOG performance status (no.)				0.35
0	111	107	4	(PS 0 versus 1)
1	89	83	6	
2	1	1	0	
Histology (no.)				
Ad	187	180	7	1.0
Other	14	14	0	
Clinical stage (no.)				
IIIB	25	25	0	0.52
IV	129	122	7	
Post-operative relapse	47	44	3	
Type of *EGFR* mutation				
Exon 19 del	108	104	4	0.42
L858R	85	80	5	
Other	8	7	1	

ILD, interstitial lung disease; ECOG, Eastern Cooperative Oncology Group; *EGFR*, epidermal growth factor receptor.

Common Terminology Criteria (NCI-CTC, version 3.0). All events were assessed by investigators at first; then severe cases were confirmed by independent committees based on medical, pathological and radiological findings.

Differences between covariates in patients with or without ILD were analyzed using Fisher's exact tests or Pearson's tests. The Kaplan–Meier method was used to estimate the cumulative incidence rate of ILD, and differences according to the smoking status were analyzed by the log-rank test. All the analyses were performed using JMP version 7 (SAS Institute Inc., USA).

RESULTS

In WJOG 3405, 177 patients were randomized and 175 were included in the safety analysis. In NEJ 002, 230 patients were randomized and 227 were included in the safety analysis. In our study a total of 402 patients were enrolled, half of them in the gefitinib arm.

Baseline characteristics of the patients were well balanced between the treatment groups. As previously reported (3,4), about two-thirds of patients were female, the median age was 64 years, 65% were never-smokers, 55% had an ECOG PS of 0 and 95% had adenocarcinoma.

At the time of data cut-off, the median duration of gefitinib treatment was 165 days (WJTOG 3405) and 308 days (NEJ 002); the median number of chemotherapy cycles was four. In the gefitinib arm, 10 (5.0%) of 201 patients developed ILD, of whom five (2.5%) were Grade 3 or greater, with two deaths (1.0%). In contrast, only one patient developed ILD (Grade 1) in the chemotherapy arm.

The background and clinical course of the patients in the gefitinib arm are summarized in Tables 1 and 2. The clinical background of patients who developed ILD and those who did not showed no difference other than smoking status. Univariate analysis showed that smoking history was significantly associated with developing ILD (odds ratio 0.18; 95% confidence interval (CI): 0.05–0.74; $P = 0.01$). This accounted for 10.9% (95% CI: 5.4–20.9%) of the incidence rate of ILD among smokers, versus 2.2% (95% CI: 0.8–6.3%) among never-smokers.

Figure 1 shows a Kaplan–Meier curve of the cumulative incidence rate of ILD. Among the overall population, the cumulative incidence rate in the first 4 weeks, 5th–8th weeks and 9th–12th weeks was 1.5% (95% CI: 0.5–4.3%), 1.5% (95% CI: 0.5–4.4%) and 0.5% (95% CI: 0.1–2.9%), respectively. Smoking status was associated with the timing of the onset of ILD . Between smokers and never-smokers, the cumulative incidence rate of ILD in the first 4 weeks was significantly different (4.7 versus 0%, $P = 0.03$), whereas that in the other periods (5th–8th weeks and 9th–12th weeks) was similar (Fig. 1). Three of 10 patients developed ILD after 8 weeks of gefitinib administration (days 135, 171 and 190, respectively).

Most of the patients who developed severe ILD (Gr $\geq$ 3) were given steroid therapy. One patient was treated with an immunosuppressive agent (cyclosporine). Non-invasive positive pressure ventilation was used in one patient (No. 10) but unfortunately this patient died.

DISCUSSION

Three large studies of ILD associated with EGFR-TKI have been conducted in Japan (Table 3). Ando et al. (8) performed a retrospective study including 1976 NSCLC patients treated with gefitinib and found an incidence rate of 3.5% and mortality rate of 1.6%. In a prospective cohort and nested-case control study by Kudoh et al. (9), cumulative incidence rates during 12 weeks of treatment were 4.0%. They also mentioned that the risk of developing ILD was higher

Table 2. Clinical characteristics of 10 patients who developed ILD in the gefitinib arm

No.	Age	Sex	Smoking index (BI)	PS	Stage	Site of *EGFR* mutation	Onset day from EGFR-TKI	ILD (CTCAE grade)	Outcome
1	69	M	800	0	r	Exon 19	48	1	Improved
2	57	F	0	1	4	Exon 19	70	1	Improved
3	60	M	860	1	4	Exon 21	15	1	Improved
4	56	F	370	1	4	Exon 19	14	1	Improved
5	71	F	0	1	4	Exon 21	171	2	Improved
6	57	M	740	0	r	Exon 19	25	3	Improved
7	68	M	1075	0	4	Exon 21	190	3	Improved
8	75	M	525	1	4	Exon 21	53	3	Improved
9	65	M	1320	0	r	Exon 19	135	5	Died
10	60	F	0	1	4	Exon 21	32	5	Died

BI, Brinkman Index; PS, Eastern Cooperative Oncology Group performance status; EGFR-TKI, EGFR-tyrosine kinase inhibitor; CTCAE, Common Terminology Criteria for Adverse Events; M, male; F, female.

with gefitinib than with chemotherapy (the odds ratio was 3.2). With regard to erlotinib, Nakagawa et al. (10) conducted a post-marketing survey in Japan and reported that 158 of 3488 patients were confirmed to have ILD (any grade, 4.5%), with a mortality rate of 1.6%. These studies suggested that male gender, smoking history, poor PS, pre-existing lung disorder and prior history of chemotherapy were risk factors of ILD. However, none of the three studies mentioned *EGFR* mutation status.

To our knowledge, ours is the first study to describe the clinical characteristics of ILD associated with gefitinib limited to *EGFR*-mutated patients. Similar to Kudoh's report, ILD was relatively more common in the gefitinib arm than in the chemotherapy arm. The incidence rate of ILD associated with gefitinib was as high as 5% with a mortality rate of 2.5%, even though our analysis contained a high proportion of patients from low-risk groups (female, non-smokers with good PS).

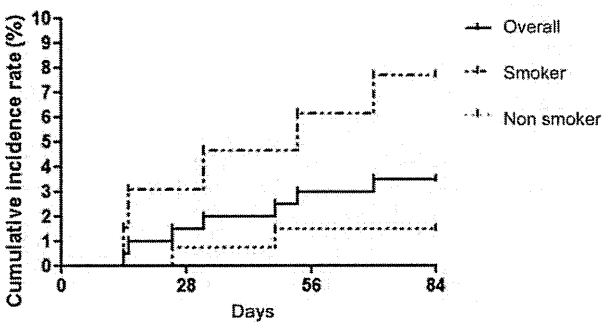

Figure 1. Cumulative incidence rate of interstitial lung disease associated with gefitinib. Kaplan–Meier-estimated cumulative incidence rate of interstitial lung disease in patients who were allocated to the gefitinib arm in WJTOG 3405 and NEJ 002 trial (overall population ($n = 201$), bold line; smoker ($n = 64$), dashed line; non-smoker ($n = 137$), dotted line).

Similarly to the previous studies, our analysis showed that smoking history was highly associated with developing ILD associated with gefitinib (odds ratio 0.18). Smoking induces airway epithelial damage, and lung injury could be prolonged and worsened by gefitinib in a preclinical model (16). Most of the other risk factors were excluded at the time of registration, because enrolled patients were required to be chemo-naïve, with a PS of 0–1, and confirmed not to have pulmonary fibrosis. Therefore, we should pay more attention to smoking status even if the patient has *EGFR* mutation. In terms of the timing of the onset of ILD, smoking history seemed to be an important factor. Between smokers and never-smokers, the cumulative incidence rate of ILD in the first 4 weeks was significantly different (4.7 versus 0%, $P = 0.03$). Previous studies stated that ILD occurred most commonly in the first 4 weeks (median: 23–31 days) and 60% of participants were smokers. So, despite the small subset analysis in the present study, the higher incidence rate observed in the first 4 weeks among smokers is noteworthy.

Another point is that three of 10 patients developed ILD after several months of gefitinib treatment. With erlotinib, it was reported that ILD occurred at the rate of 0.11 per 100 patient-weeks after 8 weeks of treatment. It is not clear whether the mechanism of ILD varies over time from its onset; further investigation on late-onset ILD is needed.

Our analysis has several limitations. First, this was an investigator-dependent analysis. Most of the ILD cases were diagnosed by clinical manifestations and a chest CT. Bronchoalveolar lavage was recommended in the protocols, but actually done in only one case. As acute exacerbation of ILD after bronchoscopy has been reported (15), this may be acceptable. In our analysis, all patients were assessed by chest CT every 2 months, and severe cases were confirmed by independent, multidisciplinary committees. Secondly, this analysis was done with a small sample size due to the population and rarity of incidence.

Table 3. ILD associated with EGFR-TKI in Japanese patients: pivotal studies and ours

	Ando et al. (8)	Kudoh et al. (9)	Nakagawa et al. (10)	Present data
Study design	Retrospective	Prospective	Retrospective	Retrospective
No. of patients	1976	1482	3488	201
Type of EGFR-TKI	Gefitinib	Gefitinib	Erlotinib	Gefitinib
Patient selection by *EGFR* mutation status	No	No	No	Yes
ILD (any Grade; %)	70 (3.5)	59 (4.0)	158 (4.5)	10 (5.0)
ILD (Grade 5; %)	31 (1.6)	25 (1.7)	55 (1.6)	2 (1.0)
Risk factors of ILD	Smoking Pre-existing lung disorder Male	Smoking Pre-existing lung disorder Poor PS Elderly Cardiac disease	Smoking Pre-existing lung disorder Poor PS Lung infection	Smoking

In conclusion, the incidence of ILD associated with gefitinib among *EGFR*-mutated patients was not different from that in previous reports. Smoking history was highly associated with developing ILD. In addition, a substantial number of patients developed ILD after several months of gefitinib treatment.

Acknowledgements

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Conflict of interest statement

A.I., K.N. and N.Y. have received honoraria from Astra Zeneca. T.M. has received honoraria from Astra Zeneca and Chugai. T.N. has received honoraria from Chugai. Y.N. has received honoraria and research grants from Chugai. All other authors declare no conflicts of interest.

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Comparison of the Time-to-response Between Radiotherapy and Epidermal Growth Factor Receptor - Tyrosine Kinase Inhibitors for Advanced Non-small Cell Lung Cancer with *EGFR* Mutation

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Abstract. *Background: Patients harboring sensitive epidermal growth factor receptor (EGFR) mutations show a dramatic response to treatment with EGFR tyrosine kinase inhibitors (TKIs). However, there have been no clinical reports in lung cancer patients that compare the time-to-response between radiotherapy and EGFR-TKIs. Patients and Methods: We reviewed 17 and 32 consecutive patients with inoperable stage III/IV NSCLC who harbored sensitive EGFR mutations and who were treated with thoracic radiotherapy with or without chemotherapy and EGFR-TKIs, respectively. Results: There were statistically significant differences in time-to-partial response (PR) with regard to the treatment modalities (radiotherapy vs. EGFR-TKIs, median 57 days vs. 22 days, log-rank test, p=0.008). Conclusion: EGFR-TKIs elicit tumor shrinkage earlier than does radiotherapy in patients with a sensitive EGFR mutation, suggesting that EGFR-TKIs may be useful for early symptom improvement in these patients.*

Lung cancer is the most common cause of cancer-related mortality worldwide. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers (1). In patients with advanced lung cancer, improvements in the quality of life and disease-related symptoms are key treatment goals. Oncological emergencies arise most commonly in patients who have advanced or metastatic disease. Many of these patients develop symptoms associated with their intrathoracic disease that are directly life threatening or can affect their quality of life.

As for the patients with advanced lung cancer, various painful symptoms often develop. These painful symptoms are often due to various causes such as superior vena cava syndrome (SVCs), venous obstruction and airway obstruction and can be relieved by reducing the size of their tumor. Prompt effects of tumor reduction often lead to palliation. Radiotherapy and chemoradiotherapy have been empirically used for reducing the size of tumor. If such treatment is ineffective, therapy directed at the underlying cause should be considered. Symptoms do not usually show rapid improvement if the tumor is unresponsive. Under these circumstances, symptom relief correlates with the magnitude of tumor response (2, 3).

Gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), is an one of the options for first-line treatment for patients with NSCLC who harbor sensitive *EGFR* mutations based on the findings of previous clinical trials (4-6). Patients with sensitive *EGFR* mutations responded dramatically to gefitinib (as shown in Figure 1), demonstrating symptom improvement that correlated with radiographic tumor shrinkage in most cases. Although prompt response is important for patients with oncological emergencies, such as SVC or airway obstruction, the best treatment modality for clinical practice has not yet been established.

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Key Words: Epidermal growth factor receptor tyrosine kinase inhibitor, gefitinib, erlotinib, non-small cell lung cancer; radiotherapy.

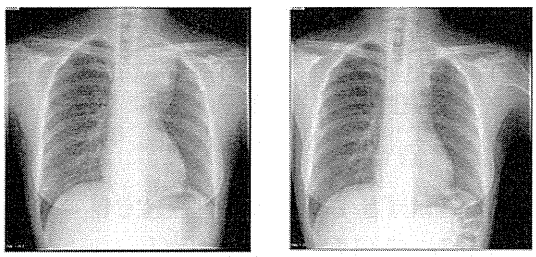

Figure 1. *Case of response to Epidermal Growth Factor Receptor - Tyrosine Kinase Inhibitor (gefitinib). A 57-year-old woman with an exon 19 deletion. Time-to-partial response, 10 days.*

Despite the correlation of symptom improvement with tumor response in patients with NSCLC receiving gefitinib (2), there have been no clinical reports comparing the time-to-response between radiotherapy and EGFR-TKIs. Therefore, this retrospective study was conducted to compare and clarify the efficacy of radiotherapy compaired to EGFR-TKIs for patients with advanced NSCLC harboring sensitive *EGFR* mutations.

Materials and Methods

Patients. The eligibility criteria in this study were as follows: histologically or cytologically proven NSCLC; unresectable stage III/IV disease or recurrent disease after surgery; a tumor that harbors an *EGFR* mutation known to be associated with drug sensitivity (exon 18 G719X, exon 19 deletion, and exon 21 L858R); continuous treatment with an EGFR-TKI or radiotherapy (with or without chemotherapy); age ≥20 years; and measurable disease by chest radiography according to the response evaluation criteria in solid tumors (RECIST) ver 1.1 (7). For patients who were treated with radiotherapy and EGFR-TKIs, the first treatment employed was applicable to evaluation. Initial EGFR-TKI therapy was limited to those patients who were receiving first- or second-line chemotherapy. Based on these criteria, we reviewed 17 and 32 consecutive patients with inoperable stage III/IV NSCLC who were treated with thoracic radiotherapy, with or without chemotherapy, and who were treated with EGFR-TKIs (gefitinib or erlotinib), respectively, at Shizuoka Cancer Center between September 2002 and June 2011. The study protocol was approved by the Institutional Review Board of Shizuoka Cancer Center (#.24-J46-24-1-3).

Genomic DNA was extracted from tumor samples, and *EGFR* mutations in exons 18-21 were analyzed as described previously (8, 9).

Medical records and films were cross-reviewed by two principal investigators. To test interobserver variability, each finding was reassessed by the same investigators after completion of the first assessment. The time interval between the first and second assessments was at least four weeks. Intraobserver variability was also determined by comparing the values of the first measurements of each of the two investigators.

Treatment methods. Radiotherapy: Patients had disease at clinical stage III/IV and received definitive thoracic radiotherapy with or without chemotherapy. Six patients were also treated with combination chemotherapy of cisplatin plus vinorelbine, five patients with cisplatin plus S-1, two patients with carboplatin alone, and two patients with other regimens. Two patients received radiotherapy alone. The prescribed dose was over 60 Gy in 30 fractions. It was ensured that the normal lung volume receiving more than 20 Gy (V20) was equal to or less than 35% of the total lung volume. The maximal dose to the spinal cord did not exceed 45 Gy at any level. All patients were required to undergo chest computed tomography (CT) to facilitate treatment planning.

EGFR-TKIs: Patients received gefitinib (250 mg, orally, once daily) or erlotinib (150 mg, orally, once daily). EGFR-TKIs were continued until disease progression, the appearance of intolerable toxicity, or withdrawal of consent. All patients were EGFR-TKI-naive.

Response evaluation: Patients were evaluated to determine the disease stage before the start of the treatment and at the time of disease progression or relapse. Disease stage was determined according to complete medical history and a physical examination, including chest radiography, CT of the chest and abdomen, magnetic resonance imaging (MRI) of the head, and additional staging procedures such as bone scintigraphy and positron-emission tomography (PET). Radiographic tumor response was evaluated according to RECIST ver. 1.1 (7), and assessments were performed almost weekly using chest radiography from treatment initiation to the end of the first month. In the second month, chest radiography was performed almost fortnightly. After the third month, chest radiography was performed on the basis of the judgment of the physician. Tumor lesions were accurately measured in at least one dimension (longest diameter) and considered positive for a minimum size of 20 mm by chest radiography. The tumor response was evaluated and classified as follows: complete response (CR), disappearance of all target lesions; partial response (PR), at least a 30% decrease in the sum of diameters of target lesions, with the baseline sum diameters as reference; progressive disease (PD), at least a 20% increase in the sum of diameters of target lesions, with the smallest sum during the study as reference (this included the baseline sum if that was the smallest during the study); and stable disease (SD), neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, with the smallest sum of diameters during the study as reference. The time to PR was calculated from the date of starting radiotherapy or administration of the first dose of EGFR-TKI to the date of the occurrence of a PR as confirmed using chest radiography. Progression-free survival (PFS) was calculated from the starting date of treatment to the date of PD or the date of occurrence of death from any cause.

Statistical analyses. To evaluate the differences in the treatment response, the Fisher's exact test was used. A Mann-Whitney *U*-test was used to compare the mean values of the variables of the groups studied. Survival curves were plotted using the Kaplan Meier technique and a log-rank test comparison was performed. In the case of SD or PD, cases were censored at the time of confirmation using chest radiography. PFS was censored at the date of the last visit for those patients who were alive without documented PD. PFS was compared using the log-rank test according to the treatment modality (radiotherapy *vs.* EGFR-TKI). A p-value of 0.05 or less was considered significant for all tests. Statistical analyses were performed using the GraphPad Prism version 5.0 software program for Windows (GraphPad Software, San Diego, CA, USA).

Table I. *The baseline characteristics of patients.*

	Therapy	
	Radiotherapy	EGFR-TKI
Total	17	32
Gender		
Male/female	10/7	10/21
Median age at treatment (years)	71 (58-80)	62 (33-76)
Performance status		
0/1/2/3/4	12/5/0/0/0	13/15/2/1/1
Smoking status		
Never/former/current	7/8/2	23/8/1
Histology		
Ad/Sq/other	17/0/0	32/0/0
Stage		
IIIA/IIIB/IV	12/4/1	0/0/32
Median tumor size (mm)	38 (22-61)	37 (23-55)
<30/30-50/50-70/>70 (mm)	3/11/3/0	8/22/2/0
Previous number of chemotherapies		
0/1	17/0	12/20
Mutation status		
Exon 19 del/exon 21 L858R/other	7/8/2*	18/12/2**

Ad: Adenocarcinoma; EGFR-TKI: epidermal growth factor receptor - tyrosine kinase Inhibitor; Sq: squamous cell carcinoma. *exon18 G719X **exon19 deletion + exon21 L858R.

Table II. *Treatment efficacy.*

	Therapy, n (%)	
	Radiotherapy (n=17)	EGFR-TKI (n=32)
CR	0 (0)	0 (0)
PR	11 (64.7)	26 (81.3)
SD	6 (35.3)	5 (15.7)
PD	0 (0)	1 (3)
Response rate (%)	64.7	81.3 p=0.296
Disease control rate* (%)	100	97

CR: Complete response; PR: partial response; SD: stable disease; PD: progressive disease. *CR+PR+SD.

Results

Patients' characteristics and treatment methods. We reviewed the clinical records of consecutive patients with, unresectable, locally advanced lung cancer, harboring an *EGFR* mutation known to be associated with drug sensitivity, who had received radiotherapy with a prescribed dose of ≥60 Gy. Twenty-four patients were identified. Out of 24 patients, 17 had measurable lesions on chest radiography (radiotherapy group). One hundred and thirty-eight patients harboring a sensitive *EGFR* mutation continuously received EGFR-TKIs as first- or second-line treatment. Out of these 138 patients, 32 had measurable lesions on chest radiography (EGFR-TKI group). The baseline characteristics of patients are summarized in Table I. The tumor type in all patients was adenocarcinoma. Among the 17 patients in the radiotherapy group, 12, 4, and one patients had disease of stage IIIA, IIIB, and IV, respectively. All 32 patients in the EGFR-TKI group had disease stage IV. With regard to *EGFR* mutation status, seven and eight patients in the radiotherapy group had an exon 19 deletion and exon 21 L858R mutation, respectively. In the EGFR-TKI group, 18 and 12 patients had an exon 19 deletion and exon 21 L858R mutation, respectively.

Response to therapy. Among the 17 patients of the radiotherapy group, eleven, six and none had PR, SD, and PD, respectively; the response rate was 64.7% and the disease control rate was 100%. In the 32 patients of the EGFR-TKI group, 26, 5, and one had PR, SD, and PD, respectively; the response rate was 81.3% and the disease control rate was 97% (Table II). The differences in the response rate between the two groups were not statistically significant (Fisher's exact test, p=0.296).

Time-to-partial response and progression-free survival. Patients treated with EGFR-TKIs had a significantly different median time to PR of 22 days compared with 57 days for patients treated with radiotherapy (radiotherapy *vs*. EGFR-TKI; log-rank test, p=0.008; Figure 2).

When limited to patients with a response, the time-to-PR was significantly shorter in the EGFR-TKI group (Mann-Whitney U-test, p=0.0001; Figure 3). The median time-to-PR was 40 days and 20 days for patients who received radiotherapy and those who received EGFR-TKIs, respectively.

Figure 4 shows the Kaplan-Meier curves for PFS. There were no statistically significant differences in PFS with regard to the treatment modality (radiotherapy *vs*. EGFR-TKIs, log-rank test, p=0.549). The median PFS values were 273 days and 295 days for patients who received radiotherapy and those who received EGFR-TKIs, respectively.

Discussion

This is the first study as far as we are aware to evaluate and compare the time-to-response between radiotherapy and EGFR-TKIs for patients with advanced NSCLC who harbor *EGFR* mutations. In the present study, we found that the time to PR was significantly shorter in patients who received EGFR-TKIs than in those treated with radiotherapy. However, no statistically significant differences in response rate or PFS were found with regard to the treatment modalities.

Several prospective clinical trials on gefitinib, and erlotinib for the treatment of patients with NSCLC and *EGFR* mutation have been conducted (10-13). These trials

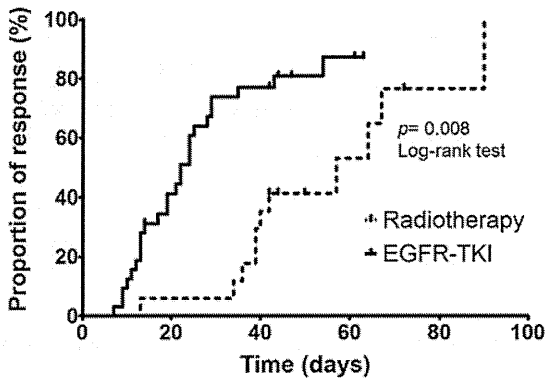

Figure 2. *Kaplan-Meier plots showing the time-to-rtial response (49 patients). Median time-to-response: radiotherapy=57 days, EGFR-TKI=22 days. EGFR-TKI: Epidermal growth factor receptor - tyrosine kinase inhibitor.*

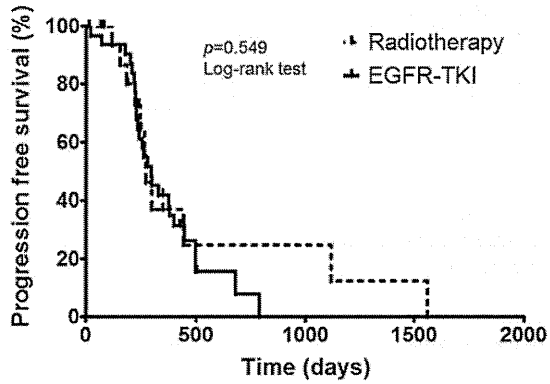

Figure 4. *Kaplan-Meier plots showing progression-free survival (PFS) (49 patients) according to the treatment modality. Median progression-free survival time: radiotherapy=273 days, EGFR-TKI=295 days.*

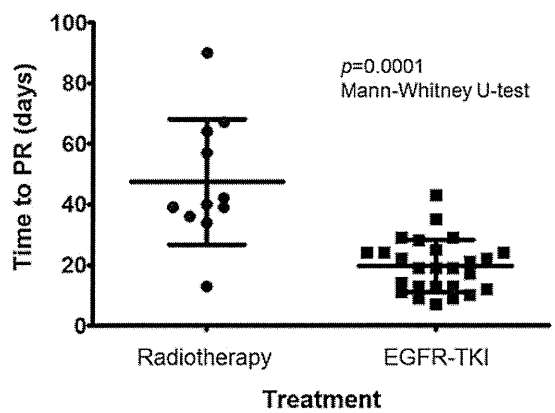

Figure 3. *Comparison of the time-to-partial response (PR) between patients treated with radiotherapy and those treated with EGFR-TKIs among patients with PR, as measured on chest radiography. Median time-to-PR: radiotherapy=40 days, EGFR-TKI =20 days.*

demonstrated radiographic response rates ranging from 75% to 90.5%. Our findings are consistent with those of previous studies showing a similar response rate for EGFR-TKI treatment in patients with *EGFR* mutations. To our knowledge, no reports have assessed tumor shrinkage time using chest radiography in patients with NSCLC treated with EGFR-TKIs. However, there are some reports concerning symptom improvement in patients treated with EGFR-TKIs (2, 14-17). For example, median time to improvement with gefitinib was eight days in patients with EGFR mutation-positive tumors (14). Cella *et al.* reported that symptom improvement was rapid; the median time to symptomatic relief was less than two weeks (2). Other studies reported that symptomatic improvement was observed in approximately 40% of patients within three weeks (15, 16). This may support our findings that patients treated with EGFR-TKIs had a median time-to-PR of 22 days. These results suggest that most of the observed improvement in symptoms is correlated with radiographic response.

There are few reports discussing the time-to-response following radiotherapy. Time to a 30% reduction in tumor burden was approximately 40 days in patients with lung adenocarcinoma who received radiotherapy (18), which is consistent with our findings of the median time-to-PR being 40 days in the radiotherapy group. In our study, 15 patients received radiotherapy with chemotherapy. Although one report suggests that a combination of radiotherapy and chemotherapy does not synergistically improve symptomatic relief compared with radiotherapy alone (18), there are no published data comparing radiotherapy and chemoradiotherapy with regard to the assessment of time to response. At any rate, the time-to-PR was significantly shorter in patients who received EGFR-TKIs than in those treated with radiotherapy with/without chemotherapy.

Preclinical studies have shown that NSCLC cells harboring *EGFR* mutations have a predominantly radiosensitive phenotype associated with a delay in the repair of radiation-induced DNA damage, defective radiation-induced arrest of DNA synthesis or mitosis, and a pronounced increase in the frequency of radiation-induced apoptosis (19). Few studies report clinical trials on radiotherapy for treatment of NSCLC with *EGFR* mutations (20-22). A previous study reported that patients with EGFR-mutant locally advanced NSCLC achieve better locoregional tumor control after thoracic radiotherapy and chemotherapy than patients with wild-type *EGFR* tumors. However, it is unclear whether radiotherapy has an advantage in patients with TKI-sensitive *EGFR* mutations. Furthermore, no clinical

reports have compared radiotherapy with EGFR-TKIs to assess tumor response.

With regard to reducing symptoms and tumor shrinkage, prompt treatment can lead to a markedly improved quality of life for patients with SVCs or airway obstruction. Our study suggests that the administration of EGFR-TKIs is more useful for tumor shrinkage than radiotherapy to rapidly improve tumor-related symptoms in patients with activating *EGFR* mutations.

This study has several limitations. Firstly, it was a retrospective analysis and the intervals between evaluations in the present study were not as closely monitored as possible in a prospective study. However, all patients were evaluated using chest radiography within similar time frames over the course of treatment, as described in the Materials and Methods section. Although evidence from randomized studies would be very valuable in the management of oncological emergencies regarding the usefulness of treatment modalities, a previous study reported that it is difficult to perform randomized studies in palliative patient groups because of a lack of accrual patient accrual (23). Secondly, the sample size was small. However, because few cases involve tumors measurable on chest radiography, it is difficult to overcome this limitation. Thirdly, although our study evaluates the time-to-response, the time to symptom improvement was not directly evaluated. However, previous studies reported that tumor response and symptom response are related in patients with advanced NSCLC (2, 17). Finally, although this study compared patients with inoperable stage III/IV NSCLC who were treated with definitive radiotherapy and with EGFR-TKIs, the number of patients with stage IV disease with *EGFR* mutations who received thoracic radiotherapy was limited. Therefore, we had no alternative but to compare definitive radiotherapy and systemic therapy with EGFR-TKIs.

In conclusion, EGFR-TKIs lead to earlier tumor shrinkage than radiotherapy in patients with activating *EGFR* mutations. The results of this study indicate that the administration of EGFR-TKIs is more useful for tumor reduction than is radiotherapy to promptly improve tumor-related symptoms in patients with activating *EGFR* mutations. Further pooling of greater numbers of patients and the completion of prospective trials are needed to define the differences in the effects of treatment modalities.

Conflicts of Interest

None of the Authors have financial or personal relationships with other people or organizations that could inappropriately influence this work.

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Size-Based Isolation of Circulating Tumor Cells in Lung Cancer Patients Using a Microcavity Array System

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Abstract

Background: Epithelial cell adhesion molecule (EpCAM)-based enumeration of circulating tumor cells (CTC) has prognostic value in patients with solid tumors, such as advanced breast, colon, and prostate cancer. However, poor sensitivity has been reported for non-small cell lung cancer (NSCLC). To address this problem, we developed a microcavity array (MCA) system integrated with a miniaturized device for CTC isolation without relying on EpCAM expression. Here, we report the results of a clinical study on CTCs of advanced lung cancer patients in which we compared the MCA system with the CellSearch system, which employs the conventional EpCAM-based method.

Methods: Paired peripheral blood samples were collected from 43 metastatic lung cancer patients to enumerate CTCs using the CellSearch system according to the manufacturer's protocol and the MCA system by immunolabeling and cytomorphological analysis. The presence of CTCs was assessed blindly and independently by both systems.

Results: CTCs were detected in 17 of 22 NSCLC patients using the MCA system versus 7 of 22 patients using the CellSearch system. On the other hand, CTCs were detected in 20 of 21 small cell lung cancer (SCLC) patients using the MCA system versus 12 of 21 patients using the CellSearch system. Significantly more CTCs in NSCLC patients were detected by the MCA system (median 13, range 0–291 cells/7.5 mL) than by the CellSearch system (median 0, range 0–37 cells/7.5 ml) demonstrating statistical superiority ($p = 0.0015$). Statistical significance was not reached in SCLC though the trend favoring the MCA system over the CellSearch system was observed ($p = 0.2888$). The MCA system also isolated CTC clusters from patients who had been identified as CTC negative using the CellSearch system.

Conclusions: The MCA system has a potential to isolate significantly more CTCs and CTC clusters in advanced lung cancer patients compared to the CellSearch system.

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Competing Interests: MH, TYoshino, HKanbara, and TM have applied for patents related to the MCA system. HKanbara is employed by Hitachi Chemical Co., Ltd. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Lung cancer is the leading cause of cancer-related death in most industrialized countries. Small cell lung cancer (SCLC) accounts for approximately 15% of lung cancer cases, and non-small cell lung cancer (NSCLC), which includes adenocarcinoma (ADC) and squamous cell carcinoma (SCC), accounts for 85% of lung cancer cases. It has recently been shown that identification of NSCLC patients by detection of genetic aberrations, specifically *EGFR*-activating mutations and the *EML4-ALK* fusion gene, allows for better prediction of response to EGFR tyrosine kinase inhibitors and ALK inhibitors, respectively [1,2]. Despite advances in prevention and treatment, NSCLC patients are often diagnosed at an advanced stage and have a poor prognosis due to the disease's tendency toward distant metastasis, the primary cause of mortality among NSCLC patients. Characterized by aggressive tumor growth and often presenting with metastases in the regional nodes and distant organs, SCLC is initially highly sensitive to chemotherapy but tends to acquire chemoresistance, leading to inevitable relapse.

Circulating tumor cells (CTCs) are defined as tumor cells circulating in the peripheral blood of patients with metastatic cancer. When measured using the US Food and Drug Adminis-

tration (FDA)-approved CellSearch system (Veridex, Raritan, NJ, USA), the number of CTCs in peripheral blood can be used to predict the prognosis of patients with metastatic breast cancer [3], colorectal cancer [4], prostate cancer [5], NSCLC [6], and SCLC [7]. The CellSearch system enriches CTCs using magnetic beads coated with a monoclonal antibody-targeting epithelial cell marker, such as the epithelial cell-adhesion molecule (EpCAM) [8,9]. However, several studies have shown that the presence of EpCAM on tumor cells varies with tumor type [10,11]. The expression of epithelial cell markers, including EpCAM, is downregulated to increase invasiveness and metastatic potential by epithelial-to-mesenchymal transition (EMT) [12–16]. It has been suggested that the low prevalence of CTCs detected in patients with advanced NSCLC using the CellSearch system may be due to the loss of EpCAM expression [17], indicating that EpCAM-based CTC isolation methods cannot achieve stable and reproducible CTC recovery from all tumor types.

Other CTC isolation methods are mainly based on differences in the size and deformability between CTCs and hematologic cells. As tumor cells (>8 μm) are larger than leukocytes [18–21], isolation by size of epithelial tumor cells (ISET) can be achieved using filtration to separate individual cells. ISET using a polycarbonate filter, an inexpensive, user-friendly method of enriching CTCs, enables the recovery and detection of epithelial-marker-negative CTCs on the basis of size-dependent CTC isolation. In clinical tests, use of an ISET-based system has been found to achieve higher CTC detection sensitivity in patients with metastatic lung cancer compared to use of the CellSearch system [22–24].

Recently, microfabricated devices for size-based separation of tumor cells have been widely developed to enable precise and efficient enrichment of CTCs from whole blood [25–28]. These devices include a miniaturized microcavity array (MCA) system that we developed for the highly efficient entrapment of single cells by filtration based on differences in the sizes of cells [29,30]. In a previous study, we examined the application of our MCA system to the detection of spiked tumor cells from unprocessed human whole blood based on differences in the size and deformability between tumor cells and other blood cells [31]. Using our device, we were able to entrap tumor cells onto size- and geometry-controlled microcavity arrays composed of 10,000 apertures by applying negative pressure, allowing the entrapped cells to be easily enumerated and analyzed by microscopic imaging of specified areas. Furthermore, we found that use of the miniaturized device allowed for introduction of a series of reagents for detection of tumor cells through the microfluidic structure. Our results indicate that our system is a simple yet precise system for the detection of tumor cells within whole blood. To confirm and build on our previous findings, we compared the capacity and efficiency of our novel MCA system and the current gold standard CellSearch system in performing CTC detection and enumeration in whole blood samples drawn from a cohort of NSCLC and SCLC patients.

Materials and Methods

Study Design and Ethics Statement

This prospective study was conducted to evaluate CTC enumeration using the CellSearch system and the MCA system in patients with metastatic lung cancer in a blinded experiment (UMIN clinical trial registry, number UMIN000005189). The presence of CTCs was assessed individually according to their criteria before knowing any results from each other. The study inclusion criteria were diagnosis of pathologically proven lung cancer with radiologically evident metastatic lesions, i.e., histologically or cytologically confirmed metastatic NSCLC or SCLC, and enrollment at the Shizuoka Cancer Center. The institutional review boards of the Shizuoka Cancer Center approved the study protocol, and all patients provided written informed consent. From each of the 43 patients who were enrolled, among whom 22 had been diagnosed with NSCLC and 21 with SCLC, 10–15 mL of blood was collected in EDTA tubes for CTC enumeration by the MCA system in our laboratory (Shizuoka Cancer Center, Shizuoka, Japan) and 20 mL was collected in CellSave collection tubes for CTC enumeration by the CellSearch system in the laboratory of SRL Inc. (Tokyo, Japan).

Cell Culture and Labeling

HCC827, NCI-H358, NCI-H441, DMS79, NCI-H69, and NCI-H82 cell lines were purchased from the American Type Culture Collection without further testing or authentication. A549 (Riken Bioresource Center, Tsukuba, Japan) and PC-14 [32] were kindly provided by Dr. Fumiaki Koizumi (National Cancer Center, Tokyo, Japan). The A549, HCC827, NCI-H358, NCI-H441, PC-14, DMS79, NCI-H69, and NCI-H82 NSCLC and SCLC cell lines were cultured in RPMI 1640 medium containing 2 mM of L-glutamine (Sigma-Aldrich, Irvine, UK), 10% (v/v) fetal bovine serum (FBS; Invitrogen Corp., Carlsbad, CA, USA), and 1% (v/v) penicillin/streptomycin (Invitrogen Corp.) for 3–4 days at 37°C with 5% CO_2 supplementation. Immediately prior to each experiment, cells grown to confluence were trypsinized and resuspended in phosphate-buffered saline (PBS). As a measurement of tumor cell size, cell size distribution was determined using the CASY® Cell Counter+Analyzer System Model TTC (Schärfe System GmbH, Reutlingen, Germany). To evaluate device performance, the tumor cell lines were labeled with CellTracker Red CMTPX (Molecular Probes, Eugene, OR, USA), with labeling achieved by incubating the cells with a tracking dye (5 μM) for 30 min. After the cells had been pelleted by centrifugation (200 g for 5 min), the supernatant was decanted. The cells were then washed twice with PBS to remove any excess dye before being resuspended in PBS containing 2 mM EDTA and 0.5% bovine serum albumin (BSA).

Fabrication of the MCA System

The MCA system was fabricated in the same manner as previously reported [29,31]. For CTC enumeration with fluorescence microscope observation, an MCA that had been manufactured by electroforming of nickel was used. For CTC morphological analysis by Giemsa staining, a transparent MCA that had been manufactured by laser irradiation of poly(ethylene terephthalate) (PET) was used. Each of the 10,000 cavities arranged in each 100×100 array was fabricated to have a diameter of 8–9 μm at the top surface and to be 60 μm distant from the adjacent microcavity. Poly(dimethylsiloxane) (PDMS) structures were fabricated and then integrated with the MCA such that the upper substrate consisted of a microchamber, a sample inlet, and an outlet, while the lower substrate beneath the MCA contained a vacuum line to produce negative pressure, enabling cell entrapment. The CTC isolation device was constructed by assembling the MCA, while the upper and lower PDMS layers were constructed using spacer tapes (Figure 1a). The sample inlet was connected to a reservoir, while the vacuum microchannel was connected to a peristaltic pump.

CTC Enumeration using the MCA System

Human blood samples were collected in a collection tube with EDTA to prevent coagulation and used within 2 h. The average

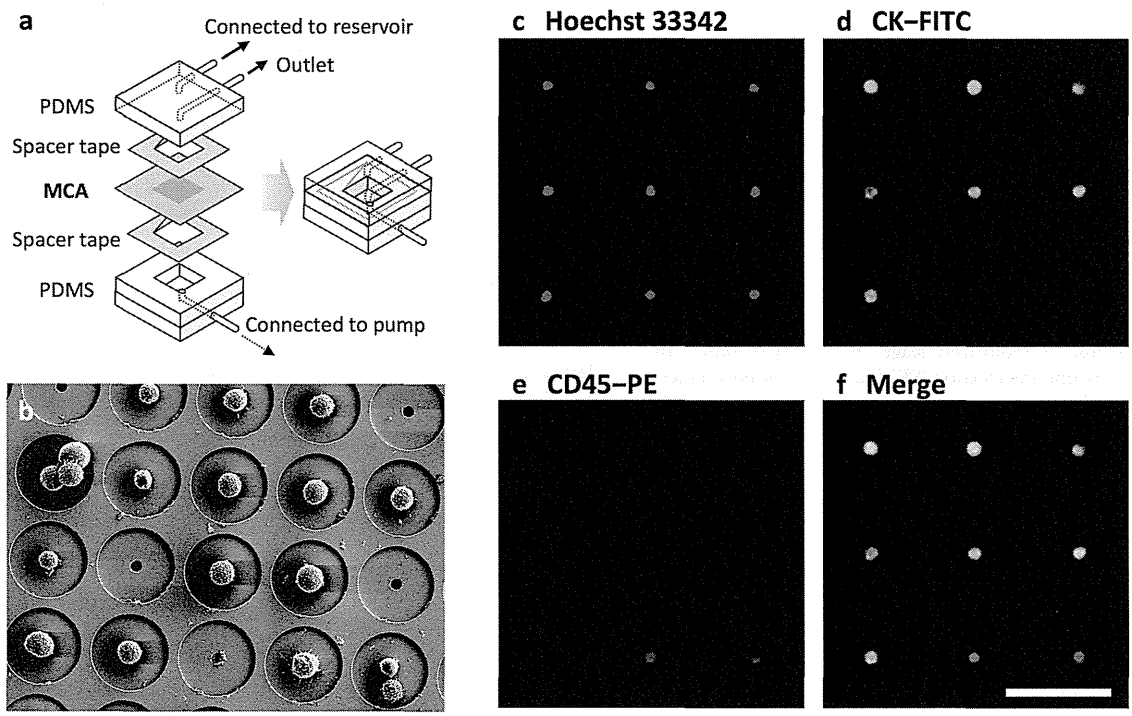

Figure 1. MCA system for size-based isolation of CTCs. (a) Schematic diagram of the structure of the MCA system. (b) Scanning electron microscope image of a cultured tumor cell line trapped on the MCA system. (c–f) Cells isolated from SCLC patient blood stained with Hoechst 33342 (c) and fluorescent-labeled antibodies that target cytokeratin (d) and CD45 (e). Merging of the images (f) allowed for identification of CTCs and hematologic cells. Scale bar = 60 μm.
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volume of blood analyzed was 4.0 mL per sample (range, 3.0–7.5 mL). All CTC enumeration using the MCA system was performed without knowledge of patient clinical status in the laboratory of the Shizuoka Cancer Center Research Institute. After introduction of blood samples into the reservoir, negative pressure was applied to a cell suspension using a peristaltic pump connected to a vacuum line, allowing the sample to be passed through the microcavities at a flow rate of 200 μL/min. To remove any blood cells remaining on the array, PBS containing 2 mM EDTA and 0.5% BSA (1 mL) was introduced into the reservoir and passed through the microcavities at a flow rate of 200 μL/min for 5 min.

To stain the CTCs with anti-pancytokeratin antibody, trapped cells were fixed by flowing 400 μL of 1% paraformaldehyde (PFA) in PBS through the MCA at a flow rate of 20 μL/min for 20 min. After washing with 100 μL of PBS, the cells were treated with 300 μL of 0.2% Triton X-100 in PBS at a flow rate of 20 μL/min for 15 min. After permeabilization, cells were treated with 3% BSA in PBS at a flow rate of 20 μL/min for 30 min. To identify CTCs and leukocytes, 600 μL of cell-staining solution containing 1 μg/mL of Hoechst 33342 (Molecular Probes); a cocktail of anti-pancytokeratin antibodies (Alexa488-AE1/AE3 (1:100 dilution; eBioscience, San Diego, CA, USA) and FITC-CK3-6H5 (1:60 dilution; Miltenyi Biotec, Auburn, California CA USA); and PE-labeled anti-CD45 antibody (1:120 dilution; BD Biosciences, San Jose, CA, USA) was flowed through the microcavities at a flow rate of 20 μL/min for 30 min. Finally, the array was washed with 400 μL of PBS containing 2 mM of EDTA and 0.5% BSA to remove any excess dye. After recovery of tumor cells, an image of the entire cell array area was obtained using a fluorescence microscope (BX61; Olympus Corporation, Tokyo, Japan) integrated with a 10× objective lens and a computer-operated motorized stage; WU, NIBA, and WIG filter sets; a cooled digital camera (DP-70; Olympus Corporation); and Lumina Vision acquisition software (Mitani Corporation, Tokyo, Japan).

In clinical trials, an entire image of the cell array area had been obtained using a fluorescence microscope (Axio Imager Z1; Carl Zeiss, Oberkochen, Germany) integrated with a 10× or 20× objective lens and a computer-operated motorized stage; WU, FITC, and Texas Red filter sets; a digital camera (AxioCam HRc; Carl Zeiss); and AxioVision acquisition software (Carl Zeiss). Subsequently, image analysis had been performed and objects that satisfied predetermined criteria had been counted. Fluorescent intensities and morphometric characteristics, such as cell size, shape, and nuclear size, were considered when performing CTC identification and non-tumor cell exclusion, with cells characterized by a round to oval morphology and a visible nucleus (i.e., as Hoechst-33342 positive) that were positive for cytokeratin and negative for CD45 identified as CTCs. Isolated CTCs on the transparent MCA were also stained using a May-Grünwald–Giemsa (MGG) staining method consisting of fixation with 4% PFA, undiluted May-Grünwald stain for 2 min, May-Grünwald stain diluted 50% in PBS for 1 min, and Giemsa stain for 18 min, followed by rinsing with PBS for 1 min.

CTC Enumeration using the CellSearch System

Whole blood samples were maintained at room temperature, mailed overnight to the laboratory of SRL Inc., and processed within 96 h of collection. All CTC evaluations were performed without knowledge of patient clinical status in the laboratory and

the results were reported quantitatively as the number of CTCs/7.5 mL of blood. CTCs were defined as EpCAM-isolated intact cells showing positive staining for cytokeratin and negative staining for CD45. In accordance with previous evaluations of the CellSearch system [8], a patient was considered CTC positive if ≥2 CTCs/7.5 mL of blood were detected in the patient's sample.

Results

CTC Isolation and Image Analysis using the MCA System

Isolation and staining of the tumor cells from whole blood was completed within 120–180 min, and image scanning of the MCA was performed at 3 fluorescence wavelengths using a 10× or 20× objective lens and a motorized stage. Figure 1b–f shows the scanning electroscope microscopy (SEM) and fluorescence images of the stained cells that were recovered on the MCA. As can be observed, solitary cells and cell clusters were individually trapped and retained on the microcavities that could be easily enumerated. Recovered cells that had a round to oval morphology and a visible nucleus (i.e., were Hoechst 33342 positive) and were positive for pancytokeratin and negative for CD45 were identified as tumor cells, while CD45-positive cells were identified as contaminating normal hematologic cells. The images reveal the existence of a distinct immunophenotype of epithelial cell marker-positive tumor cells. Although a number of leukocytes were retained on the array, tumor-cell enumeration was relatively facile because individual cells had been trapped on the precisely aligned microcavities.

Sensitivity of the MCA System in CTC Detection of Lung Cancer Cell Lines

In our previous study, varying numbers of cells of the lung cancer cell line NCI-H358 were spiked into blood, and tumor cell isolation was evaluated using our MCA system [31]. The calculated detection efficiency was constant and over 90% when 10–100 tumor cells were present per milliliter of blood. In this study, in order to evaluate the recovery efficiency of various lung cancer cell lines using the MCA system, 100 cells of each of 8 lung cancer cell lines (A549, HCC-827, NCI-H358, NCI-H441, PC-14, DMS-72, NCI-H69, and NCI-H82) were spiked into healthy donor blood samples and then processed by MCA assay. Table 1 shows the average recovery efficiency and typical diameter of the cell lines. As can be observed, a high recovery rate was obtained, regardless of tumor type, ranging from 68% to 100% in the cell line spike-in experiments. Most of the recovered cells were viable and able to proliferate even after undergoing the isolation process, suggesting the potential for further biological and molecular analysis of CTCs.

Next, in order to evaluate the specificity and sensitivity of CTCs detection, the sensitivity tests were performed on artificial samples prepared by adding 1 and 3 cultured NCI-H358 cells to healthy donor blood samples, as previously reported by Vona et al. [20]. One and 3 cultured NCI-H358 cells were spiked into separate 7.5 mL aliquots of blood. These 7.5 mL blood samples were processed with the MCA system in 3 independent tests (Table S1). The results demonstrated a sensitivity threshold for MCA system close to 1 tumor cell per 7.5 mL of blood. In addition, CTCs were not detectable from 6 healthy donor bloods using the MCA system (Figure 2). Therefore, a patient was considered CTC positive if ≥1 CTCs per 7.5 mL of blood was detected by the MCA system.

In addition, the tumor cell recovery efficiency of the MCA system was compared with that of ISET system (Figure S1). In this comparison, 100 cells of NSCLC cell line NCI-H358 was spiked into healthy donor blood samples and then processed by the MCA system and a track-etched polycarbonate 8-μm pore membrane (Nucleopore; Whatman Ltd., Kent, UK). The results revealed the recovery rate using the MCA system (100% ±5%) to be significantly higher than that using the ISET system (91% ±2%) ($p<0.05$, t-test), indicating that use of the MCA system enables CTC isolation with an efficiency equivalent to or greater than that of the ISET system.

Table 1. CTC recovery efficiency and average cell diameter.

Cell line	Origin	Average cell diameter (μm)	Recovery efficiency (%)
A549	NSCLC	17.3	98±3
HCC827	NSCLC	19.6	99±6
NCI-H358	NSCLC	18.1	100±6
NCI-H441	NSCLC	20.6	98±8
PC-14	NSCLC	19.5	97±2
DMS79	SCLC	14.1	76±1
NCI-H69	SCLC	12.5	68±2
NCI-H82	SCLC	13.5	80±4

Cells were spiked into 1 mL of normal blood and recovered using the MCA system.
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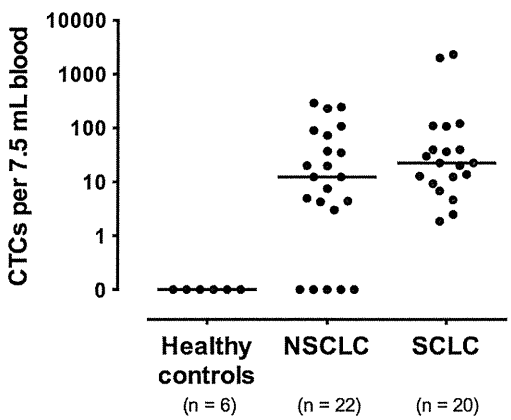

Figure 2. CTC count using the MCA system. CTC count/7.5 mL blood is shown for 6 healthy donors, 22 NSCLC patients and 20 SCLC patients.
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CTC Enumeration using the CellSearch System and the MCA System

To conduct blind comparison of the detection sensitivity of the CellSearch and MCA systems, blood samples were collected from 22 metastatic NSCLC and 21 SCLC patients between April 2011 and February 2012 and analyzed for determination of the number of patients identified as CTC positive by each system (Table 2). Of these samples, 1 sample collected from 1 SCLC patient was not evaluated by the MCA system because an insufficient volume of blood had been collected for processing by both systems. As a result, 17 of the 22 (77%) NSCLC patients were identified as CTC positive using the MCA system but only 7 of the 22 (32%) NSCLC patients using the CellSearch system (Table 3). Of these patients, 8 were identified as CTC positive by both the CellSearch system