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.

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).

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.

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

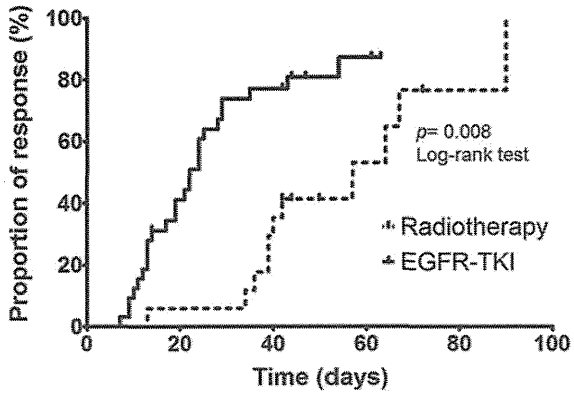

Figure 2. *Kaplan-Meier plots showing the time-to-rtial response (49 patients). Median time-to-response: radiotherapy=57 days, EGFR-TKI=22 days. EGFR-TKI: Epidermal growth factor receptor - tyrosine kinase inhibitor.*

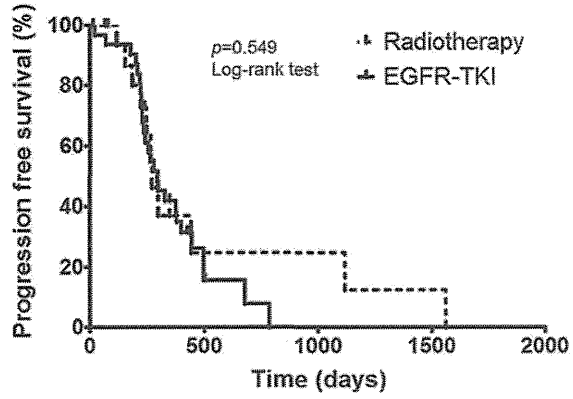

Figure 4. *Kaplan-Meier plots showing progression-free survival (PFS) (49 patients) according to the treatment modality. Median progression-free survival time: radiotherapy=273 days, EGFR-TKI=295 days.*

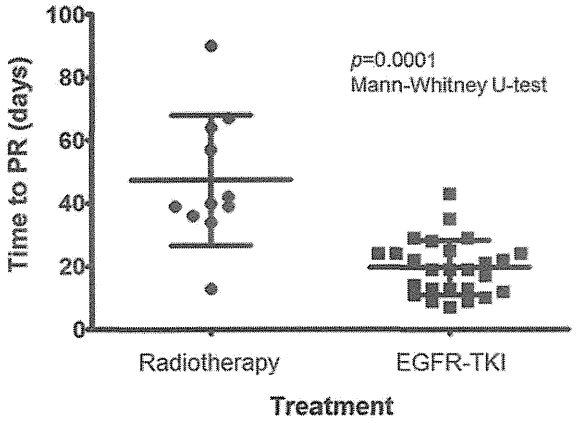

Figure 3. *Comparison of the time-to-partial response (PR) between patients treated with radiotherapy and those treated with EGFR-TKIs among patients with PR, as measured on chest radiography. Median time-to-PR: radiotherapy=40 days, EGFR-TKI =20 days.*

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

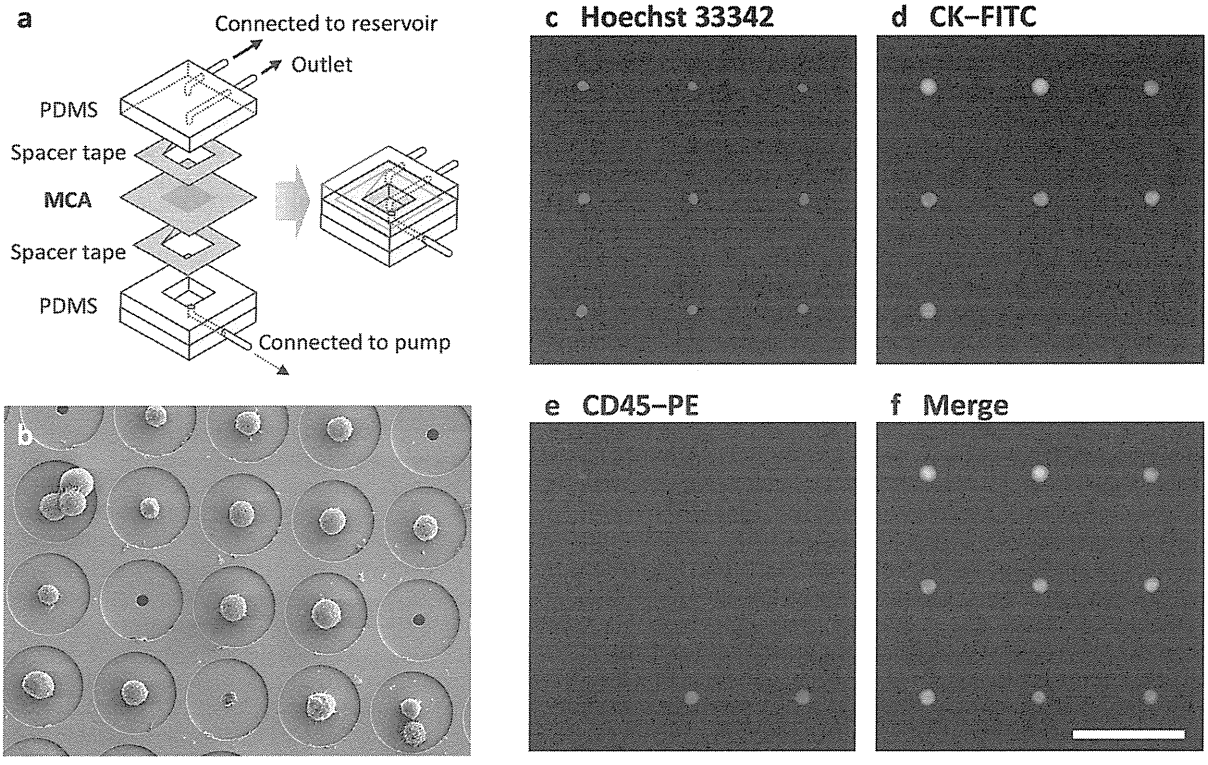

Figure 1. MCA system for size-based isolation of CTCs. (a) Schematic diagram of the structure of the MCA system. (b) Scanning electron microscope image of a cultured tumor cell line trapped on the MCA system. (c–f) Cells isolated from SCLC patient blood stained with Hoechst 33342 (c) and fluorescent-labeled 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.
doi:10.1371/journal.pone.0067466.t001

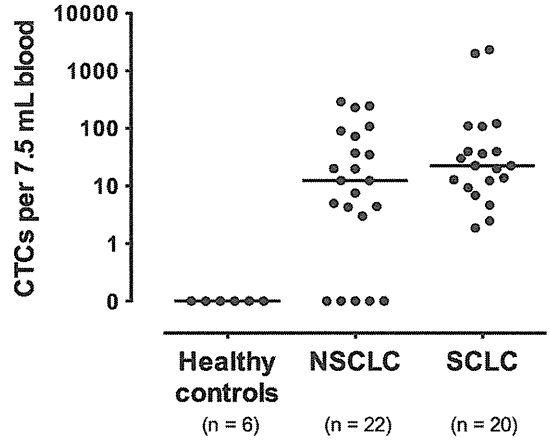

Figure 2. CTC count using the MCA system. CTC count/7.5 mL blood is shown for 6 healthy donors, 22 NSCLC patients and 20 SCLC patients.
doi:10.1371/journal.pone.0067466.g002

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

and the MCA system, 1 was identified as CTC positive by the CellSearch system only, and 9 were identified as CTC positive by the MCA system only. Considering the results obtained by both systems together, 18 (82%) of the NSCLC patients were identified as CTC positive. Analysis of these findings revealed that a significantly greater number of NSCLC patients were identified as CTC positive by the MCA system (median cell count 13, range 0–291 cells/7.5 mL; Figure 2) than by the CellSearch system (median cell count 0, range 0–37 cells/7.5 mL), demonstrating the statistical superiority of the MCA system in CTC enumeration ($p = 0.0015$, Wilcoxon test; Table 3).

In contrast, 20 of the 20 (100%) SCLC patients were identified as CTC positive using the MCA system versus 12 of the 21 (57%) patients using the CellSearch system. The median CTC count was found to be 2 cells/7.5 mL (range 0–325) using the CellSearch system and 23 cells/7.5 mL (range 2–2329) using the MCA system (Figure 2). Although not reaching a level of statistical significance, the detection sensitivity of the MCA system in CTC enumeration showed a trend toward being greater than that of the CellSearch system ($p = 0.2888$, Wilcoxon test; Table 3). For each outcome, agreement between the test results of the systems was assessed by Bland–Altman plots [33]. In the analysis of agreement regarding CTC enumeration in NSCLC patients, the mean difference was 50.1 (95% CI, range 11.1–89.1), with the limits of agreement ranging from −125.8 to 226.0. The MCA system yielded disproportionally higher CTC counts at higher mean values compared to The CellSearch system (Figure S2a). In contrast, in the analysis of agreement regarding CTC enumeration in SCLC patients, the mean difference was 202.6 (95% CI, range −116.7–521.9), with the limits of agreement ranging from −1162.0 to 1567.2. Unlike with the analysis of NSCLC blood samples, no bias was observed between the systems in the analysis of SCLC samples except for subjects with extremely high CTC titer (Figure S2b). Statistical analysis also revealed no association between site of metastasis and the CTC count of lung cancer patients using either system (data not shown).

Table 2. Patient characteristics.

		NSCLC	SCLC
No. of patients		**22**	**21**
Gender	Male	10	18
	Female	12	3
Median age		68	73
	(Range)	(36–77)	(53–83)
Smoking	Smoker	16	21
	Never-smoker	6	-
ECOG-PS	0–1	16	13
	2–4	6	8
No. of organs with metastasis	Median	2	2
	(Range)	(1–6)	(1–5)
Metastasis	Brain	9	10
	Bone	8	4
	Liver	6	6
Histology	Adenocarcinoma	14	–
	Squamous	3	–
	Others	5	–
	SCLC	–	21

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Table 3. Comparison of CTC enumeration by the CellSearch system and the MCA system.

Sample ID		CellSearch CTC (cells/7.5 mL)	MCA CTC (cells/7.5 mL)
NSCLC	1	0	0
	2	0	0
	3	9	0
	4	0	0
	5	0	5
	6	0	8
	7	2	90
	8	0	13
	9	0	13
	10	0	3
	11	1	35
	12	37	20
	13	2	246
	14	18	108
	15	0	73
	16	10	231
	17	19	20
	18	1	4
	19	0	0
	20	0	4
	21	0	291
	22	0	38
SCLC	23	200	20
	24	189	30
	25	0	13
	26	0	9
	27	0	40
	28	0	7
	29	33	23
	30	2	14
	31	3	122
	32	18	2
	33	1	2329
	34	1	2021
	35	4	13
	36	15	5
	37	325	40
	38	2	–
	39	13	36
	40	110	110
	41	0	3
	42	0	23
	43	0	109

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Morphologic Features of CTCs Isolated using the MCA System

CTCs were counted, identified as being cytokeratin positive and CD45 negative, and as having a visible nucleus on the basis of analysis of fluorescent images. As can be observed in Figure 3, which shows a representative gallery of CTCs identified by image analysis, CTCs are larger than the surrounding leukocytes and often appear in clusters, defined here as contiguous groupings of cells containing 3 or more nuclei. Figure 4 shows a solitary CTC and a CTC cluster detected in one SCLC patient using the MCA system. Using the MCA system, CTC clusters were observed in 2 of the 22 NSCLC patients (Patient No. 13 and 21) and 4 of the 21 SCLC patients (Patient No. 31, 33, 34, and 43). May-Grünwald–Giemsa staining of the CTCs isolated using the MCA system revealed that they are characterized by a high N/C ratio, nuclear molding, and morphological similarity to primary tumor cells.

Discussion

ISET systems have been found to have higher CTC detection sensitivity than the CellSearch system in several cancers, including NSCLC [17,22] However, the pores of ISET filters, which are made of polycarbonate by track etching, are randomly placed within the systems at a nonuniform density. Unlike such track-etched polycarbonate filters, the size, geometry, and density of the microcavities in the MCA system assessed in the current study are precisely controlled to achieve specific cell separation according to differences in cellular size and deformability. Aligning cells on the MCA not only eases cell imaging by allowing for the scanning of specified areas with an automated fluorescence microscope but also enables reduction in the labor required for CTC counting [29,31]. As such, the MCA system provides a platform for the use of high-throughput imaging technologies that provide more rapid and less expensive data collection as well as CTC enumeration and advanced analysis of molecular phenomena, including fluorescence in situ hybridization for detection of tumor-specific genomic changes. Furthermore, the MCA is integrated with a miniaturized device so that enrichment of CTCs from blood, as well as staining and washing in the microfluidic assay, can be performed within one integrated device.

In the present study, CTCs isolated on the MCA were successfully stained with fluorescent-labeled antibodies that target tumor cell markers, and staining and washing were found to have little or no effect on the retention of tumor cells on the microcavities. Due to its very small size, the MCA system is portable, which, by enabling point-of-care CTC counting, eliminates the need to ship blood for testing under unfavorable shipment conditions and expedites clinical decision-making. These features, in addition to our recently developed procedure for isolating single cells from the MCA using microcapillaries, allow tumor cells to be recovered from the MCA for subsequent molecular analysis of CTCs [29].

In this blind comparison of use of the MCA system to that of the conventional CellSearch System for CTC enumeration in lung cancer patients, the MCA system was found capable of isolating various lung cancer cell lines spiked within whole blood at high levels of efficiency. However, the MCA system performed isolation of SCLC cell lines slightly less efficiently compared to that of NSCLC cell lines, indicating that small (<8 μm in diameter) cells of the SCLC cell lines might pass through the microcavities during blood filtration. In a previous study [31], we found that breast (MCF-7 and Hs578T), gastric (AGS and SNU-1), and colon (SW620) tumor cells lines that include EpCAM-negative tumor cells could be successfully recovered using the MCA system with greater than 80% efficiency. However, we also found that the efficiency of recovery of small cells (average diameter 11.6 μm) of the tumor cell line SW620 to be slightly less than that of other cell lines, as we did of the SCLC cell lines examined in this study.

The MCA system assessed in the present study was found to possess a higher detection sensitivity than the CellSearch system in NSCLC CTC enumeration, suggesting the superiority of size- and deformability-based isolation techniques compared to immuno-magnetic-based techniques. The poor sensitivity of CellSearch has been attributed to the low EpCAM expression in advanced NSCLC. However, one of the NSCLC patients assessed in the present study was found to be CTC positive using the CellSearch system but CTC negative using the MCA system, indicating that

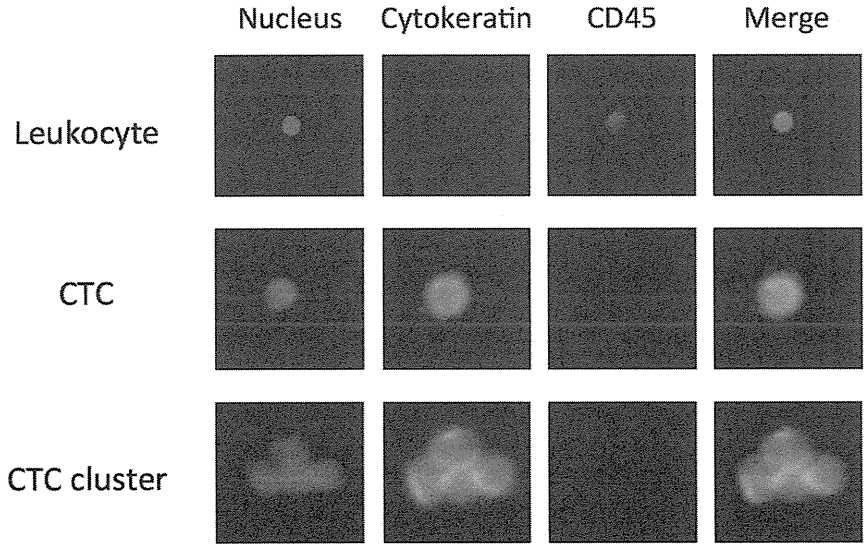

Figure 3. Gallery of cells captured on the MCA from blood of advanced lung cancer patients. Cells were stained with Hoechst 33342, FITC-labeled anti-cytokeratin antibody, and PE-labeled anti-CD45 antibody.
doi:10.1371/journal.pone.0067466.g003

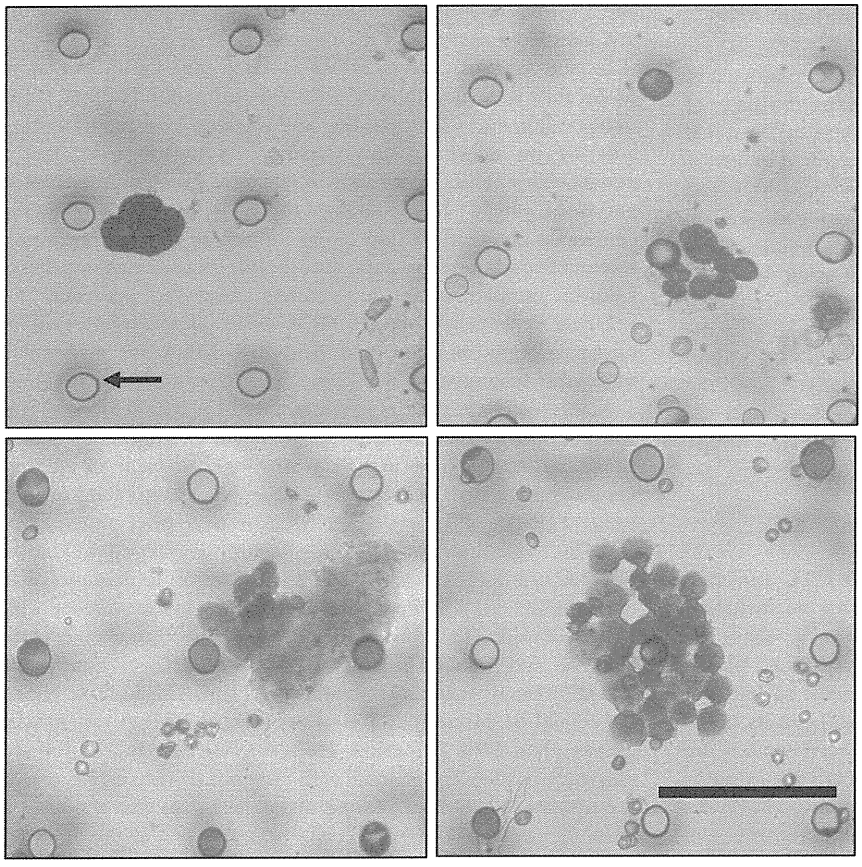

Figure 4. Gallery of CTCs captured on a transparent MCA from SCLC patient blood. May-Grünwald–Giemsa-stained cells showed a high nucleus–cytoplasm ratio and nuclear molding (×40). Black arrow indicates 9-μm microcavity. Scale bar = 60 μm.
doi:10.1371/journal.pone.0067466.g004

changes in EpCAM expression cannot solely account for the differences found between the two systems in NSCLC enumeration.

The CTC detection rate using the CellSearch system in SCLC patients was 67%, considerably higher than that in NSCLC patients and consistent with that found in previous studies [7,34–36]. Although the MCA system does not rely on EpCAM expression, which circulating SCLC cells have been reported to show high levels [37], in performing CTC isolation, its use was found to yield a high detection rate, indicating that it could be utilized for CTC detection in not only NSCLC but also SCLC patients. Nevertheless, the CTC counts of several patients were higher when analyzed using the CellSearch System compared to the MCA system, indicating that some small tumor cells in patient blood might flow through the microcavities, as described above. Previous research has suggested that immunomagnetic separation techniques lack the capacity to isolate large clusters, whereas use of size-based separation techniques leads to loss of small CTCs [17]. To address these problems, the shape of the microcavities in the MCA was modified to improve their efficiency in isolating small cells from tumor cells in whole blood in our recent study [38].

Observation of CTC clusters has been reported in various cancers, including lung cancer [23,24,39–42]. It is hypothesized that forming in clusters provides CTC cells with advantages over remaining solitary in terms of survival, proliferative capacity, and ability to form micrometastases. In this study, CTC clusters were isolated from both NSCLC and SCLC patients using the MCA system. Interestingly, the CTC-positive clusters were identified as having a small number of CTC cells by the CellSearch system but a large number by the MCA system. One reason why several SCLC patients were found to have a large CTC count when assessed by the MCA system may be that this system enables isolation of larger CTC clusters that cannot be isolated by immunomagnetic separation. Examination of this hypothesis requires further detailed analysis of the characteristics of CTC clusters, such as expression of epithelial markers and the presence of apoptotic cells within CTC clusters, which could be performed using the MCA system.

In conclusion, our results suggest that the MCA system is potentially superior to the CellSearch system in the CTC detection of lung cancer patients, with the former found capable of isolating significantly more CTCs and CTC clusters than the latter. The major limitation of this study was its examination of a small sample of patients with only one type of cancer. Further studies should thus examine larger cohorts of patients with various types of cancers to assess whether the MCA system is a more appropriate tool for CTC enumeration and characterization of metastatic tumors in patients with cancers other than lung cancer compared to other systems. We are currently planning the development of an automated MCA system that achieves robust, reliable, and reproducible sample processing for validation study using large cohorts of patients presenting at multiple institutes to assess the prognostic utility of CTC count in cancer patients.

Supporting Information

Figure S1 Comparison of cell recovery rate using the microcavity array (MCA) system and an isolation by size of epithelial tumor cell (ISET) filter. Non-small cell lung cancer cell line NCI-H358 was spiked into whole blood at a volume of 100 cells/mL to perform 3 separate tests of circulating cancer cell recovery using an MCA (pore size = 8 μm) and a track-etched polycarbonate ISET filter (pore size = 8 μm; Nucleopore).
(TIFF)

Figure S2 Bland–Altman plots of agreement between circulating tumor cell (CTC) test results obtained for non-small cell lung cancer (NSCLC; a) and small cell lung cancer (SCLC; b) patients using the CellSearch and microcavity array (MCA) systems. The solid horizontal line represents the mean difference and the dashed lines the limits of agreement (mean difference +/−2SD). In NSCLC, the mean difference was 50.1 (95%CI, 11.1 to 89.1), limits of agreement (-125.8 to 226.0) with the difference between systems becoming disproportionately greater with higher average CTC-count. In SCLC, the mean difference was 202.6 (95%CI, −116.7 to 521.9), limits of agreement (-1162.0 to 1567.2) with no bias observed between systems except for subjects with extremely high titer of CTCs.
(TIFF)

Table S1 Evaluation of sensitivity of microcavity array (MCA) system for circulating tumor cell (CTC) detection. Sensitivity testing was performed using artificial samples created by adding 1 and 3 cultured NCI-H358 cells to healthy donor blood samples. Individual cells were selected by micropipette under direct visualization, spiked into 7.5 mL aliquots of blood, and the resulting blood samples processed using the MCA system in 3 separate tests.
(DOC)

Author Contributions

Conceived and designed the experiments: MH H. Kenmotsu YK T. Yoshino TN TM NY. Performed the experiments: MH T. Yoshikawa RW SO. Analyzed the data: MH H. Kenmotsu YK T. Yoshikawa TN RW SO KM NY. Contributed reagents/materials/analysis tools: H. Kenmotsu TN T. Takahashi HM YN AT TS AO HA H. Kanbara KY NY. Wrote the paper: MH H. Kenmotsu YK T. Yoshino T. Yoshikawa TN T. Tanaka TM NY.

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Outcome of platinum-based chemotherapy for non-small-cell lung cancer patients with pleural dissemination detected during surgery

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Abstract. Pleural dissemination detected by computed tomography (CT) is considered to be unfavorable for patients with non-small-cell lung cancer (NSCLC). However, the prognosis of NSCLC patients who are diagnosed with pleural dissemination at the time of surgery has yet to be adequately elucidated. To assess the outcomes of platinum-based chemotherapy in NSCLC patients in whom pleural dissemination was detected during exploratory thoracotomy with or without a videoscope, the clinical records of NSCLC patients who were admitted to Shizuoka Cancer Center between September, 2002 and April, 2009 were reviewed. A total of 19 patients were included in this study, 12 males and 7 females, with a median age of 65 years. All patients were diagnosed with adenocarcinoma and 6 were epidermal growth factor receptor (EGFR) gene mutation-positive. The median number of treatment cycles of first-line platinum-based chemotherapy was 4 (range, 1-6 cycles) and the objective response rate was 21% [95% confidence interval (CI): 8.5-43]. The median progression-free and overall survival were 10.4 (95% CI: 6.3-18.4) and 50.5 months (95% CI: 32.5-98.0), respectively. Of the 18 patients with reported disease progression, 9 (50%) developed locoregional tumor progression. In conclusion, NSCLC patients in whom pleural dissemination is detected during surgery tend to have a favorable prognosis for survival. Systemic chemotherapy and additional local treatment may improve their clinical outcomes.

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Key words: non-small-cell lung cancer, pleural dissemination, surgery, unresectable, chemotherapy

Introduction

In early-stage non-small-cell lung cancer (NSCLC), surgical resection is the core of curative treatment. However, unexpected pleural dissemination is occasionally detected during surgery. According to TNM revisions (7th edition) and new stage groupings proposed by the International Association for the Study of Lung Cancer (IASLC), patients with pleural dissemination are classified as stage IV (1,2). Therefore, when malignant pleuritis is identified during thoracotomy, resection of the primary tumor is considered to be a contraindication and the thorax is closed without performing resection (1-5). In the majority of cases, these patients are administered systemic chemotherapy. Previous studies on platinum-based chemotherapy in patients with advanced NSCLC reported that the median progression-free survival (PFS) and overall survival (OS) were 3.1-4.8 and 11.4-15.2 months, respectively (6-8). However, there is little available data on systemic chemotherapy and prognosis for NSCLC patients with pleural dissemination detected during surgery, compared to patients who are preoperatively diagnosed as stage IV by thorough examination. The aim of this study was to assess the outcome of platinum-based chemotherapy in NSCLC patients with pleural dissemination detected during surgery who did not undergo any form of resection.

Patients and methods

Study population. The subjects of this study were non-resected NSCLC patients with pleural dissemination detected during thoracotomy. Patient selection was performed based on the clinical records of NSCLC patients who underwent thoracotomy with or without videoscope at Shizuoka Cancer Center between September, 2002 and April, 2009. Ethics approval for this study was obtained by the Institutional Review Board. Written informed consent was obtained from the patients.

Of the 681 patients who underwent thoracotomy, pleural dissemination was identified in 31 patients during exploratory thoracotomy. Of these 31 patients who did not undergo any form of resection, 19 received platinum-doublet chemotherapy. The

19 patients underwent preoperative workup, including chest X-ray, bronchoscopy, computed tomography (CT) scans of the chest and spirometry. A thorough search for distant metastases was also conducted, including magnetic resonance imaging of the brain in 17 patients (89%) and positron emission tomography scans in 18 patients (95%), prior to exploratory thoracotomy. The epidermal growth factor receptor (EGFR) gene status was assessed by the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp-based method (9).

Statistical analysis. Survival was defined as the time from surgery until death from any cause. The survival curves of the patients were calculated using the Kaplan-Meier method and statistical evaluation was performed by means of a log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patients. A total of 19 patients whose pleural disseminated nodules were identified during surgery were included in this study. The patient characteristics are listed in Table I. The patients comprised 12 men and 7 women with a median age of 65 years (range, 48-80 years). All patients had histologically confirmed adenocarcinoma. The EGFR gene status was evaluated in 10 patients (53%), of whom 6 were mutant, 5 had exon 19 deletions and 1 had an exon 21 point mutation (L858R). Reclassification was performed according to the TNM revisions (7th edition) and the new stage groupings proposed by IASLC (1). No lymph node metastasis (N0) was diagnosed in 18 patients (95%) and N1 disease was diagnosed in 1 patient (5%). Pleural dissemination was detected in all the included patients and was confirmed by pleural biopsy during surgery. Positive pleural lavage cytology was diagnosed in 16 patients (84%). All patients were diagnosed as stage IV and did not undergo any form of resection.

Systemic chemotherapy. Patients were administered systemic chemotherapy following exploratory thoracotomy. The median time between surgery and systemic chemotherapy initiation was 17 days (range, 7-50 days). The chemotherapy regimens were as follows: 13 patients received carboplatin and paclitaxel, 2 received carboplatin and nab-paclitaxel, 1 received carboplatin and gemcitabine, 1 received carboplatin and paclitaxel plus bevacizumab, 1 received cisplatin and pemetrexed plus axitinib and 1 received cisplatin and gemcitabine. The median number of treatment cycles of first-line platinum-based chemotherapy was 4 (range, 1-6 cycles). The chemotherapy response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 (10). The overall response rate (ORR) was 21% (95% CI: 8.5-43), stable disease (SD) was observed in 69% of the patients, progressive disease (PD) in 5% and the remaining 5% of patients were not evaluable. There were no treatment-related mortalities.

Survival. The median PFS and OS were 10.4 (95% CI: 6.3-18.4) and 50.5 months (95% CI: 32.5-98.0), respectively (Fig. 1), and the 2- and 5-year survival rates were 84 and 37%, respectively. Following first-line platinum-based chemotherapy, disease progression was observed in 18 of the 19 patients. One patient has remained alive without disease progression for ~56 months; this patient is continuing bevacizumab as maintenance therapy following 6 cycles of carboplatin and paclitaxel plus bevacizumab as first-line chemotherapy. The sites of initial failure are shown in Table II. Of the 18 patients with disease progression, 9 (50%) developed locoregional tumor progression. The remaining 9 patients developed distant tumor progression and the majority had pleural disease progression.

In the EGFR-mutant group, the median PFS was 19.6 months (95% CI: 6.3-60.2). The median OS was not reached due to the limited number of mortalities. The median follow-up time was 55.3 months (range, 40.2-73.6 months) and the 2- and 5-year survival rates were 100 and 80%, respectively. In the EGFR wild-type or unknown group, the median PFS and OS were 9.8 (95% CI: 4.2-14.9) and 33.9 months (95% CI: 17.3-55.1), respectively, and the 2- and 5-year survival

Table I. Patient characteristics.

Characteristics	Values
Age, years [median (range)]	65 (48-80)
Gender	
Male	12 (63%)
Female	7 (37%)
ECOG performance status	
0	17 (89%)
1	2 (11%)
Histology	
Adenocarcinoma	19 (100%)
EGFR gene status	
Mutant	
Exon 19 deletions	5 (27%)
Exon 21 L858R	1 (5%)
Wild-type	4 (21%)
Unknown	9 (47%)
Smoking history	
Yes	11 (58%)
No	8 (42%)
Clinical T factor	
T1a	1 (5%)
T1b	7 (37%)
T2a	8 (42%)
T3	3 (16%)
Clinical N factor	
N0	18 (95%)
N1	1 (5%)
Pleural lavage cytology	
Positive	16 (84%)
Negative	1 (5%)
Not estimated	2 (11%)

ECOG, Eastern Cooperative Oncology Group; EGFR, epidermal growth factor receptor.

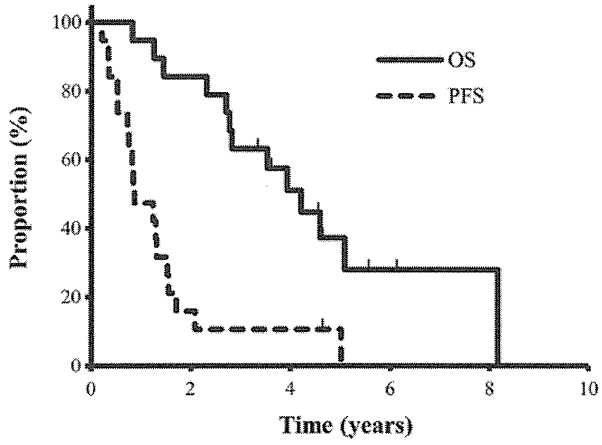

Figure 1. Kaplan-Meier curves of progression-free survival (PFS) and overall survival (OS). The median PFS and OS were 10.4 and 50.5 months, respectively.

rates were 84 and 37%, respectively. The patients positive for EGFR mutations appeared to exhibit a significantly prolonged PFS (P=0.02; HR=0.27; 95% CI: 0.07-0.81; 18 events) and OS (P=0.02; HR=0.13; 95% CI: 0.01-0.68; 13 events) compared to the patients with EGFR wild-type or unknown gene status.

Subsequent chemotherapy. The 18 patients who exhibited disease progression following first-line chemotherapy were administered systemic chemotherapy. Second-line chemotherapy regimens are listed in Table III. The 5 patients with EGFR mutations were administered anti-EGFR therapy as second-line chemotherapy. The EGFR gene status was wild-type in 5 patients and unknown in the remaining 8 patients. The second-line chemotherapy regimens were EGFR-tyrosine kinase inhibitors (EGFR-TKIs) in 5, single-agent chemotherapy in 6 and doublet chemotherapy in 2 patients.

Table II. Sites of initial failure.

Sites	No. (%)
Local	9 (50)
Distant	9 (50)
Pleural dissemination	7 (39)
Lung	4 (22)
Bone	2 (11)

Table III. Second-line chemotherapy.

	EGFR gene status	
	Mutant (n=5)	Wild-type or unknown (n=13)
Anti-EGFR therapy		
Gefitinib	1	5
Erlotinib	1	
Other	3	
Docetaxel		4
Pemetrexed		2
Cisplatin + Pemetrexed		1
Docetaxel + Aflibercept		1

EGFR, epidermal growth factor receptor.

Discussion

In this study, the prognosis for NSCLC patients who received platinum-based chemotherapy following detection of pleural dissemination during surgery was significantly better compared to that of general stage IV NSCLC patients. Previous studies reviewed the N status and histological characteristics as prognostic indicators for patients with pleural dissemination identified during surgery and suggested that N0 status was a good prognostic indicator, reporting a 5-year survival rate of 24-35% (11-14). In this study, 18 of the 19 patients (95%) were classified as N0. Thus, the N factor may be an indicator of prolonged survival. As regards histological characteristics, Mordant *et al* (11) analyzed 27 NSCLC patients with pleural malignant disease detected during thoracotomy (21 adenocarcinomas and 6 other types of NSCLC) and reported that the histological characteristics were not significantly associated with overall survival (P=0.40). All patients included in the present study had adenocarcinomas. Therefore, whether histological characteristics had a tendency towards improved survival could not be determined.

Pleural lavage cytology is also considered to be an important prognostic factor. According to the International Pleural Lavage Cytology Collaborators, a positive pleural lavage cytology result is an independent predictor of poor survival (15). It was stated that the effect on the survival of patients with positive pleural lavage cytology justified upstaging patients by one T category. In this study, pleural lavage cytology was performed in 17 patients (89%) and although only 1 patient was negative and the remaining 16 patients were positive for malignancy, the prognosis was better compared to the M1a designation of the IASLC proposals for stage grouping in the 7th edition of TNM on lung cancer (1).

With regards to the EGFR gene status, the consensus is that EGFR gene mutation is a strong predictor of a better outcome with EGFR-TKIs (16-19). Previous studies on NSCLC patients harboring EGFR mutations reported that the median PFS and OS were 9.6-10.8 and 30.5-35.5 months, respectively, with an ORR of 62.1-73.7% in patients receiving gefitinib as first-line chemotherapy, whereas the median PFS and OS were 5.4-6.6 and 23.6-38.8 months, respectively, with an ORR of 30.7-32.2%, in patients receiving platinum-based chemotherapy as first-line chemotherapy (16,17). In this study, in the EGFR mutant group, platinum-based chemotherapy was administered rather than EGFR-TKIs and the median PFS was 19.6 months. The median OS was not achieved (the median follow-up time was 55.3 months) and the ORR was 33%. The EGFR wild-type or gene status-unknown patients in this study also exhibited a longer OS compared to that previously reported (6).

Pleural dissemination indicates the systemic spread of cancer. Systemic chemotherapy is indicated for patients

with pleural dissemination detected during surgery, which is classified as stage IV with M1a disease. Chemotherapy may suppress tumor progression or micrometastasis and contribute to the improvement of survival rates. As regards local treatment, previous studies supported surgery as an option for NSCLC patients with malignant pleuritis detected during thoracotomy, suggesting that it was beneficial to survival (11,12,14,20-23). Furthermore, Mordant *et al* (11) reported a long-term survival of 16% and suggested that identification of clinical T1-2N0 NSCLC with previously undiagnosed pleural malignant metastatic disease may justify surgery as part of a multimodality treatment. In this study, all the patients were administered systemic chemotherapy, but none underwent resection. One patient underwent thoracic radiotherapy [stereotactic radiotherapy (SRT)] following platinum-doublet chemotherapy. That patient, whose EGFR gene status was mutant, was administered carboplatin and paclitaxel as first-line chemotherapy and the best overall response was SD. The tumor size remained unchanged for 8 months after the last administration of chemotherapy and SRT were performed as a curative attempt. The PFS of the patient was 60.2 months, whereas the median PFS was 10.1 months (95% CI: 6.3-15.7) in the remaining 17 patients. Of the 18 patients, 9 developed initial failure as local progression. A multimodality treatment centered on systemic chemotherapy following local treatment, such as limited resection or radiation, may improve the prognosis of such patients.

In conclusion, platinum-based chemotherapy for NSCLC patients with pleural dissemination detected during surgery demonstrated a favorable prognosis for survival. It is recommended that NSCLC patients with previously undiagnosed pleural dissemination are classified as a special group, for whom systemic chemotherapy followed by local treatment may improve clinical outcomes. Additional multimodality treatment trials are required in this population.

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PHASE II STUDIES

A phase II study of cisplatin /S-1 in patients with carcinomas of unknown primary site

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Summary *Background* Carcinomas of unknown primary site (CUPs) are heterogeneous tumors associated with a poor prognosis. This phase II trial was designed to evaluate the efficacy and safety of a novel combination chemotherapy of S-1 and cisplatin (CDDP) in patients with CUP. *Patients and Methods* Patients with previously untreated CUPs were eligible for this trial. The treatment schedule consisted of oral S-1 (40 mg/m^2) twice a day on days 1–21, and intravenous CDDP (60 mg/m^2) on day 8. This schedule was repeated every 5 weeks. *Results* A total of 46 patients were enrolled. The overall response rate and the disease control rate were 41.3 % and 80.4 %, respectively. The median overall survival time was 17.4 months. Grade 3/4 neutropenia, thrombocytopenia, and febrile neutropenia occurred in 28.3 %, 13.0 %, and 2.2 % of the patients, respectively. *Conclusion* CDDP plus S-1 combination chemotherapy is well tolerated and active first-line empiric therapies for patients with CUP.

Key words Cisplatin · S-1 · Chemotherapy · Carcinoma of unknown primary site

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Introduction

Carcinomas of unknown primary site (CUPs) represent a group of heterogeneous tumors that has no identifiable origin. Despite advances in tumor pathology and imaging techniques, such as positron emission tomography, CUPs account for about 5 % of all cancers [1]. Several clinicopathological subsets with favorable prognosis have been identified. However, most patients do not fit into any of these subsets. Because the prognosis of CUP is generally poor, with a median overall survival time (OS) of 6–13 months, the benefit of chemotherapy compared with best supportive care is still unclear [2]. During the past 3 decades, some phase II trials of platinum-based combination regimens containing newer cytotoxic agents (taxanes, gemcitabine, and irinotecan) resulted in response rates of 30 %–40 % and median survivals of 8–11 months [3–9].

S-1 is a new oral fluoropyrimidine agent designed to enhance antitumor activity and to reduce gastrointestinal toxicity through the combined use of an oral fluoropyrimidine agent (tegafur), a dihydrophyrimidine dehydrogenase inhibitor (5-chloro-2,4-dihydroxypyridine), and an orotate phosphoribosyl transferase inhibitor [10]. Treatment with the single agent S-1 results in response rates of 49 % in advanced gastric cancer patients [11] and 35 % in metastatic colorectal cancer patients [12]. Good results have also been reported in breast cancer, lung cancer, pancreatic cancer [13–15], and head and neck cancer. Thus, S-1 has a broad spectrum of clinical activity in

solid tumors. Moreover, preclinical studies showed a synergistic effect between S-1 and cisplatin (CDDP) [16]. In the hopes of developing a more safe and effective therapy, we conducted a phase II study of this novel combination chemotherapy of CDDP and S-1 in patients with CUP.

Patients and methods

This nonrandomized phase II trial was initiated in October 2005 and performed at multiple institutions.

Patient eligibility Patients eligible for this trial were required to have a previously untreated, histologically or cytologically documented CUP. For purposes of this trial, patients were considered to have CUP if no primary site was evident after an evaluation including medical history; physical examination; complete blood counts; chemistry profile; computed tomography (CT) scan of the chest, abdomen, and pelvis; and directed radiologic or endoscopic workup of symptomatic areas. Histological examination which includes standard immunohistochemistry (IHC) inspection for diagnosis as CUP was required. Additional eligibility criteria included measurable disease, Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2, age of 20 years or older, adequate hematologic, renal, and hepatic functions. Patients known to have good prognostic features were excluded. These subsets included: 1) patients with a single metastasis; 2) women with adenocarcinoma isolated to axillary lymph nodes; 3) patients with squamous carcinoma limited to cervical or inguinal nodes; 4) patients with features of neuroendocrine or extragonadal germ cell tumor; 5) men with high levels of serum prostate-specific antigen (PSA) or positive for PSA by immunohistochemistry; and 6) women with peritoneal carcinomatosis. Patients were excluded if they had symptomatic brain metastases. Additional exclusion criteria included concomitant serious diseases and pregnant or lactating females. Written informed consent was obtained from all patients, and the protocol was approved by the institutional ethics committee of each of the participating institutions.

Treatment S-1 was administered orally, 40 mg/m^2 twice a day after meals between days 1 and 21. CDDP (60 mg/m^2) was administered intravenously on day 8 when patients were hydrated with enough infusion. The oral dose of S-1 for each patient was assigned based on the body surface area. The 3 doses administered were 40 mg (body surface area$<$1.25 m^2), 50 mg (1.25 m$^2\leq$body surface area$<$1.50 m^2), and 60 mg (body surface area$\geq$1.50 m^2). Supportive care, which included adequate hydration and antiemetics, was provided at the discretion of the patient's physician and respective institution. If laboratory variables were not met eligibility criteria for the CDDP administration on day 8, administration of CDDP were withheld until the abnormality had resolved. If there was no resolution of abnormality within day15, the patient skipped CDDP administration. The treatment regimen was repeated every 5 weeks for at least 3 cycles unless disease progression or unacceptable toxicity occurred. The doses of S-1 were reduced in the event of any of the following toxicities during the previous treatment cycle: grade 4 hematological toxicity, or grade 3 or higher nonhematological toxicity. For the subsequent treatment courses, S-1 was reduced from 60, 50, or 40 mg twice daily to 50, 40, or 25 mg twice daily, respectively. If a rest period of more than 28 days was required, then the patient stopped the protocol treatment.

Evaluation of response and toxicity All eligible patients were considered assessable for response and toxicity. The response was evaluated in accordance with the Response Evaluation Criteria in Solid Tumors [17]. The response was confirmed for at least 4 weeks (for complete response [CR] or a partial response [PR]) after it was first documented or 6 weeks (for stable disease [SD]) after the start of therapy. Progression-free survival (PFS) was defined as the time from registration until objective tumor progression or death. OS was defined as the time from registration until to death from any cause. Adverse

Table 1 Patient characteristics(N=46)

Characterisitic	No. of Patients	(%)
Median age, years (range)	63	(31–84)
Gender		
Male	25	(54.3)
Female	21	(45.7)
ECOG performance status		
0	4	(8.7)
1	37	(80.4)
2	5	(10.9)
Histology		
Adenocarcinoma	23	(50.0)
Poorly differentiated adenocarcinoma	3	(6.5)
Poorly differentiated carcinoma	3	(6.5)
Squamous carcinoma	14	(30.4)
Other	3	(6.5)
Dominant site of disease		
Lymph nodes	40	(87.0)
Only nodal disease	18	(39.1)
Bones	10	(21.7)
Lung	8	(17.4)
Liver	5	(10.9)
Adrenal	3	(6.5)
Pancreas	3	(6.5)
Others	10	(21.7)

ECOG (Eastern Cooperative Oncology Group)

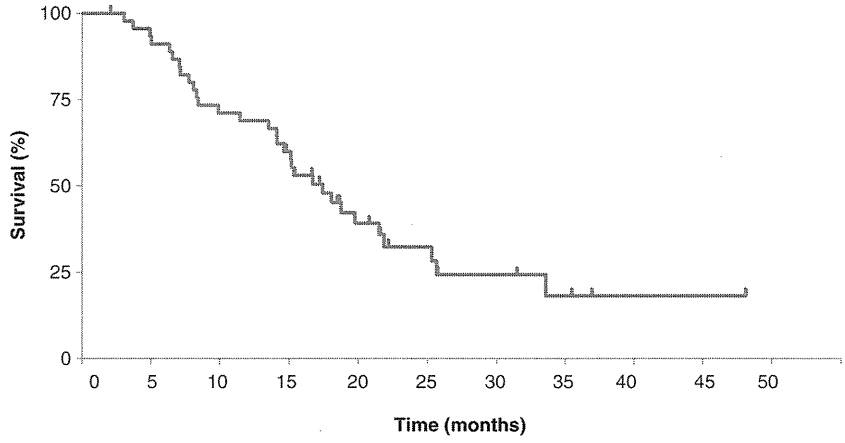

Fig. 1 Kaplan-Meier plot for overall survival (N=46)

events were graded according to National Cancer Institute Common terminology criteria for Adverse Events, version 3.0.

Statistical analysis The primary end point of this study was the response rate, and the secondary endpoints were toxicity, OS, and 1-year survival rate. In previous reports with first-line therapy of patients with CUP, the range of response rate was 25 %–55 %. Therefore, the number of patients to be enrolled in this study was calculated as 45, which was the number required to refute the assumption that the 95 % confidence interval (95 % CI) would be 25 % under conditions of α=0.05 and β=0.2, while assuming an expected response rate of 45 %. OS and PFS were estimated by the Kaplan–Meier method.

Results

Patient characteristics Between October 2005 and September 2009, 46 patients were enrolled on this clinical trial and were treated with a combination of S-1 and CDDP. The patient characteristics are summarized in Table 1. The median age was 63 years (range, 31–84 years). Most of the patients had an ECOG performance status (PS) of 0–1, and only 5 (10.9 %) patients had a PS of 2. 25 patients were male. 23 patients had adenocarcinoma, 14 had squamous cell carcinoma, 3 had poorly differentiated carcinoma, and 3 had poorly differentiated adenocarcinoma. 18 patients presented with lymph nodes metastasis only.

Treatment delivery The median number of treatment courses of the S-1 and CDDP regimen received by patients on this trial was 4 (range 1–10). 31 patients (67.4 %) received at least 3 treatment courses. 15 patients withdrew treatment prior to completing 3 courses for the following reasons: tumor progression in 11 patients, treatment-related toxicity in 1 patient, and patient's refusal in 3 patients. In accordance with the study protocol, dose reductions were necessary in 7 patients. CDDP was skipped in 6 patients because of following reasons: tumor progression in 1 patient, hematologic toxicities in 4 patients, and nausea in 1 patient.

Efficacy The overall response rate was 41.3 % (95 % CI, 27.0 to 56.8). The disease control rate (DCR; CR+PR+SD) was

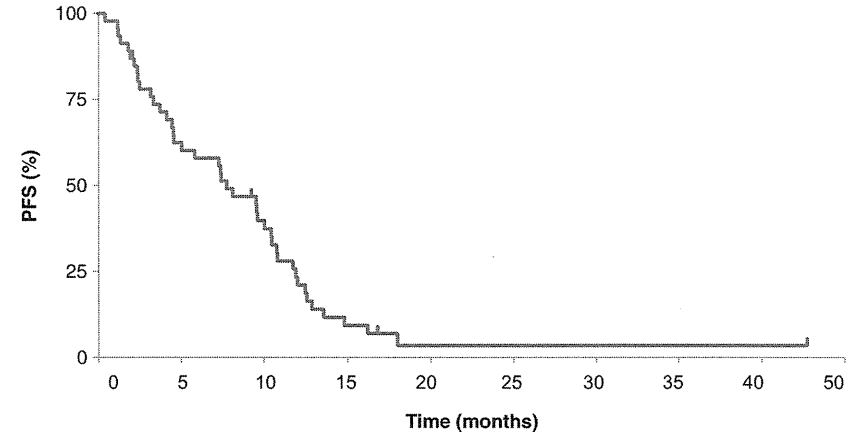

Fig. 2 Kaplan-Meier plot for progression-free survival (N=46)