Detection of circulating tumor cells in patients with lung cancer using metallic micro-cavity array filter: A pilot study

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Abstract. We have developed a metallic micro-cavity array filter and an automated detection system for capturing circulating tumor cells (CTCs). In this single institutional pilot study, we assessed the ability of this device to detect CTCs in patients with lung cancer at each stage. Patients diagnosed with lung cancer, undergoing planned surgery for lung cancer, or suspected of having lung cancer were recruited (40 recruited and 2 excluded). Blood samples were obtained from the patients and 3 ml whole blood was applied to the device without any preparation. The captured cells were stained to differentiate the nucleus, and determine cytokeratin and CD45 expression. Subsequently, two operators blinded to clinical information counted the number of CTCs. Sample collection was performed at the time of recruitment, before treatment and ~3 months after initial blood collection. CTC counts at recruitment were 1.4±0.4, 1.8±1.2, 1.3±0.6 and 7.4±5.1 (mean ± SE) in clinical stages I, II, III and IV, respectively. No significant difference was observed among the stages. These data indicated the ability of this device to detect CTCs at early or non-metastatic stages of lung cancer. Further research on a larger scale is needed for a more accurate assessment of the device, and research on the utility of captured cells remains a future challenge.

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Introduction

Lung cancer is the leading malignancy in terms of both incidence and mortality according to recent global cancer statistics and as such is a major health issue requiring attention (1). One major risk factor of lung cancer is smoking. However, the number of non-smoker patients with lung cancer is reported to have increased (2), which implies the existence of other unknown risk factors (1). Surgery is a key treatment modality for patients with non-metastatic lung cancer. In Japan, 20% of patients with lung cancer are diagnosed with stage IV (metastatic) lung cancer (3), requiring systemic anticancer therapy including molecular targeted agents.

Along with the development of molecular targeted therapy for lung cancer, the need for repeated genetic testing of cancer cells has increased (4). This has further increased the need for capturing samples from cancer tissues or cells. It is not easy to obtain tumor tissues or cell samples from patients with lung cancer. Therefore, less invasive methods are in demand. Blood has been targeted as a sample for capturing circulating tumor cells (CTC) or DNA for molecular testing in a less invasive manner (5). Moreover, some investigators reported that CTCs are detectable in patients with non-metastatic lung cancer, and that the detected CTCs correlate with clinical outcome (6,7). However, it is unknown whether CTCs detected in patients with metastatic and non-metastatic disease have the same biological characteristics. Establishing the biological differences of CTCs in each clinical state would facilitate understanding of the mechanism of metastasis. Thus, we have developed a metallic micro-cavity array filter and an automated CTC detection system. We had previously reported that this device can isolate significantly more CTCs from patients with metastatic lung cancer compared to methods depending on epithelial cell-adhesion molecule (EpCAM) (8). In this pilot study, we tested the device for CTC detection ability in lung cancer patients of every clinical stage, including the non-metastatic stage.

Patients and methods

Metallic micro-cavity array (MCA) filter and the automated detection system. Hitachi Chemical Co., Ltd. has developed a metallic MCA filter and an automated detection system for CTCs (Fig. 1A-C) (8-10). A total of 3 ml of whole blood was added to the reservoir in the system, and sample blood was passed through the MCA filter using a peristaltic pump at a flow rate of 600 μl/min. Washing, fixation, and permeabilization were performed automatically by the system. Captured cells were stained automatically to distinguish the nucleus (DAPI), cytokeratin (CK), and CD45 expression (fluorescent dyes and antibodies for staining for nucleus, CK, and CD45 are undisclosed because the device is still under development) (Fig. 1D). The cavity size and density are undergoing an optimization process. In Fig. 1B, a scanning electron microscope image shows an array of micro-cavities used in this series.

Patients and methods. Patients at Hitachi general hospital who were diagnosed with lung cancer, undergoing planned lung resection for lung cancer, or suspected of having lung cancer, were recruited from June 2014 to May 2015. The number of patients recruited was planned as 40 for this pilot study. Written informed consent was obtained from all participating patients. A 7-ml blood sample that included backup volume for re-examination was collected in an EDTA tube twice, namely before treatment (1st) and at approximately 3 months after the initial sample collection (2nd). Each sample was anonymized and sent to the laboratory at Hitachi Chemical Co., Ltd. Two operators at the Hitachi Chemical laboratory who were blinded to the clinical information counted the CTCs according to predetermined criteria [nucleus (+), CK (+), and CD45(-)]. Surplus samples were discarded according to the protocol. All participating patients were treated and followed up at Hitachi general hospital. Their clinical data were collected from medical records. Smoking index is an indicator of smoking history that is clinically used in Japan, and was calculated as the number of cigarettes per day multiplied by years smoking during his/her lifetime. We used the 7th edition of TNM for lung cancer classification.

The present study protocol was approved by The Institutional Review Board of Ibaraki Hospital Headquarters at Hitachi, Ltd. (approval no. 2014-64) and written informed consent was obtained from all participants.

Statistical analysis. Continuous variables are presented as mean ± SE and were tested using Shapiro-Wilk tests for normal distribution. If the variable followed normal distribution, mean CTC counts in each clinicopathological factor were compared using an independent t-test. Otherwise, Mann-Whitney U tests were applied. For nominal variables, χ^2 test or Fisher's exact test was applied. For correlation analyses, we applied both Pearson's and Spearman's rank correlation analysis between CTC counts or change in CTC counts and clinicopathological factors. Survival time was calculated using the Kaplan-Meier method and compared using a log-rank test. $P<0.05$ was considered to indicate a statistically significant difference. We used SPSS version 24 (SPSS, Inc.) for all statistical analyses.

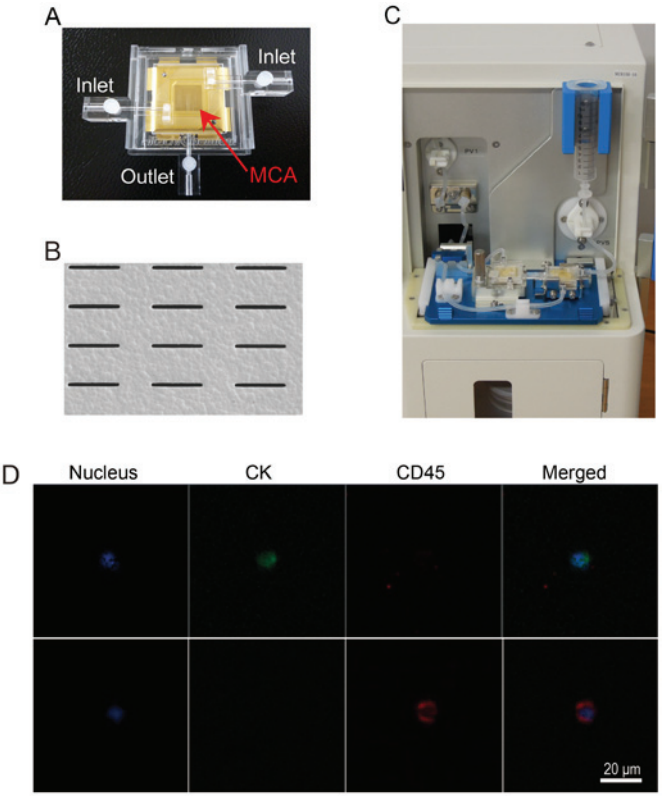

Figure 1. Circulating tumor cell detecting system and images of detected cells. (A) Image of a MCA filter cartilage. An MCA cartridge is mounted as indicated by the arrow. (B) Scanning electron microscope image of an MCA filter. Cavity width is 8 μm. (C) Image of the automated device. (D) Representative fluorescent images of circulating tumor cells (upper panel: Nucleus- and CK-positive, and CD45-negative) and hematologic cells (lower panel: Nucleus- and CD45-positive, and CK-negative). MCA, micro-cavity array; CK, cytokeratin.

Results

Patient characteristics. Of the 40 recruited patients, two cases were excluded as lung cancer was not histologically proven in one case, whereas the other case was diagnosed as metastatic lung tumor and recurrence from a prior resected lung cancer. Therefore, we analyzed 38 cases. Their clinicopathological characteristics are summarized in Table I. The mean age of the patients was 69.9 years (48-82), and they included 27 males and 11 females. Adenocarcinoma was the dominant histological type, including 1 small cell carcinoma. Considering clinical stages, the number of patients in stages I, II, III, and IV was 19, 6, 6 and 7, respectively. As for the treatment modality, surgery was dominant (26 cases). Tyrosine kinase inhibitors were used in 3 cases for EGFR mutation and in one for ALK rearrangement.

CTC counts in each clinical stage. CTC counts were 1.4±0.4, 1.8±1.2, 1.3±0.6 and 7.4±5.1 in clinical stages I, II, III, and IV, respectively. Detection rates (defined as CTC counts of one or more) of each clinical stage were: 63.2% (I), 33.3% (II), 66.7% (III), and 71.4% (IV) (Fig .2). The ratios of CTC counts=0 in each clinical stage were: 36.8% (I), 66.7 % (II), 33.3% (III), and 28.6% (IV). Two-group comparisons between each stage or between each possible combination, such as stage I and other stages, stage I/II and III/IV, stage IV and other stages,

Table I. Patient characteristics.

Characteristic	Result
Age	69.9 (48-82)
Sex, M:F	27:11
Histology	
AD	32
SQ	4
SM	1
NSCLC	1
Clinical stage	
IA	15
IB	4
IIA	3
IIB	3
IIIA	4
IIIB	2
IV	7
Treatment modalities	
Surgery	26
Radiotherapy	1
Chemotherapy	7
TKI	4

AD, adenocarcinoma; SQ, squamous cell carcinoma; SM, small cell carcinoma; NSCLC, non-small cell lung cancer; TKI, tyrosine kinase inhibitor; M, male; F, female.

using Mann-Whitney U tests showed no statistically significant difference.

Comparison of CTCs and clinicopathological factors. We compared CTC counts and clinicopathological factors and summarized them in Table II. Sex, histological type, smoking index, presence or absence of interstitial pneumonitis, clinical stage, and carcinoembryonic antigen (CEA) levels were analyzed. None of the clinicopathological factors showed a statistically significant difference (Mann-Whitney U test). In this study, we did not define a specific number of CTCs as positive. If one or more CTCs were defined as positive, we could compare CTC numbers with clinicopathological factors as means of continuous variables (age, smoking index, WBC, lymphocyte counts, CRP, CEA, Cyfra, and c-tumor size) and as frequency of nominal variables (sex, presence or absence of interstitial pneumonitis, adenocarcinoma or other than adenocarcinoma, stage I and more than II, and stage IV and others). A significant difference was observed only in the smoking index. The CTC-positive group showed a smaller smoking index than the negative group (382±485 vs. 892±769, P=0.018).

Correlation analyses between CTC counts and clinical factors. We performed correlation analyses between changes in CTC counts based on time and clinical factors. Changes in CEA, WBC, lymphocyte, and CRP did not show a correlation with

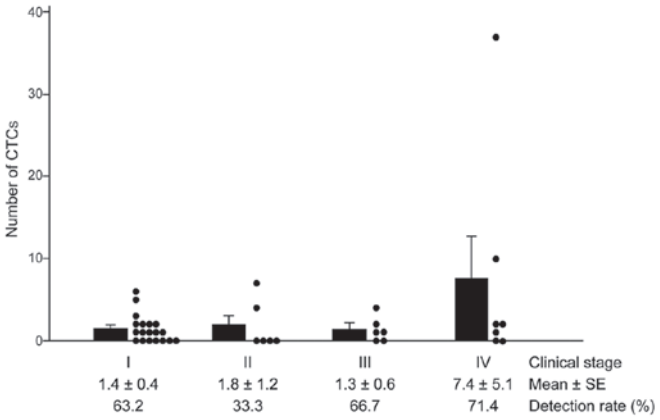

Figure 2. Number of circulating tumor cells in each clinical stage is shown as bar and dot plots. Error bars indicate SE. CTCs, circulating tumor cells.

differences in CTC counts in both Pearson's and Spearman's rank correlation analyses (Table III).

Survival analyses related to CTC counts and changes in CTC counts. During the follow-up period, 12 deaths were recorded, of which 11 resulted from lung cancer and one from acute myeloid leukemia. The median follow-up period in survivors was 36 months. The 3-year survival rate for all patients was 68% and did not reach 50%, and therefore the median survival time was not calculated. We divided cases into two groups according to CTC counts or changes in CTC counts: 1st CTC=0 and >0; 1st CTC=0 or 1 and >1; 1st CTC=0 to 2 and >2; 1st CTC=0 to 3 and >3; 1st CTC=0 to 4 and >4; 1st CTC=0 to 5 and >5; CTC decreased group and other than decreased group; and CTC increased group and other than increased group. We compared the survival of each group and none of the analyses showed any significant difference. Because the cohort of this pilot study included various treatment modalities, we further analyzed only non-surgical cases (n=12). Analyses between 1st CTC=0 and >0; 1st CTC=0 or 1 and >1; 1st CTC=0 to 2 and >2; 1st CTC=0 to 3 and >3; CTC decreased group and other than decreased group; and CTC increased group and other than increased group; 2nd CTC=0 and >0; 2nd CTC=0 or 1 and >1 showed no significant differences. In contrast, cases of 2nd CTC >2 showed significantly worse survival that those with 2nd CTC=0 to 2 (P=0.043, Fig. 3).

Discussion

In this study, we tried to assess the ability to detect CTC at each clinical stage of lung cancer using the metallic micro-cavity array filter developed by Hitachi Chemical. Although CTC counts at each clinical stage did not show any significant differences, our data show that CTCs were detected in patients with non-metastatic lung cancer, which indicates the possible utility of this device in early stage lung cancer. CTC studies targeting non-metastatic lung cancer are limited and the CTC detection rate (positive rate) varies (6,7,11-13). As we did not confirm whether the cells captured on our device are cancer cells using other methods such as morphological examination under white light in all cases, we did not define the number of CTCs as positive in this study. If we could define one or

Table II. Comparison between CTC counts and clinical factors.

Clinical factor	CTC counts (mean ± SE)	P-value
Sex		
Male (n=27)	1.74±0.49	0.505
Female (n=11)	4.64±3.27	
Histology		
AD (n=32)	2.69±1.15	0.422
Non-AD (n=6)	2.00±1.63	
Smoking index[a]		
≥600 (n=17)	2.06±0.74	0.685
<600 (n=20)	3.00±1.72	
0 (n=13)	4.23±2.76	0.429
Other than 0 (n=24)	1.71±0.52	
IP		
Present (n=5)	3.20±2.06	0.967
Absent (n=33)	2.48±1.12	
Clinical stage		
I (n=19)	1.42±0.39	0.795
II or more (n=19)	3.74±1.95	
I and II (n=25)	1.52±0.41	0.447
III and IV (n=13)	4.62±2.80	
IV (7)	7.43±5.10	0.299
Other than IV (n=31)	1.48±0.34	
CEA		
≥3.5 (n=21)	3.67±1.77	0.622
<3.5 (n=17)	1.24±0.34	

[a]Calculated by the number of cigarettes per day multiplied by number of years smoking in his/her lifetime. The smoking history of one case could not be obtained from medical records. AD, adenocarcinoma; IP, interstitial pneumonitis; CEA, carcinoembryonic antigen; CTC, circulating tumor cell.

Table III. Correlation between changes in circulating tumor cell counts and clinical factors.

Difference in clinical factors with time	r	P-value	r_s	P-value
Δ CEA (ng/ml)	0.020	0.905	0.056	0.743
Δ WBC (μl)	-0.082	0.624	-0.159	0.339
Δ lymphocyte (μl)	-0.051	0.821	0.089	0.692
Δ CRP (mg/dl)	0.102	0.544	0.139	0.407

Δ, difference between the initial value and that measured ~3 months later; CEA, carcinoembryonic antigen; WBC, white blood cell counts; CRP, C reactive protein.

more CTCs as positive, the positive rate in clinical stage I would be 63.2%, which is comparable to that reported for a size-based device (7,12). Regarding the specificity of captured cells, we washed the filter and stained the captured cells with

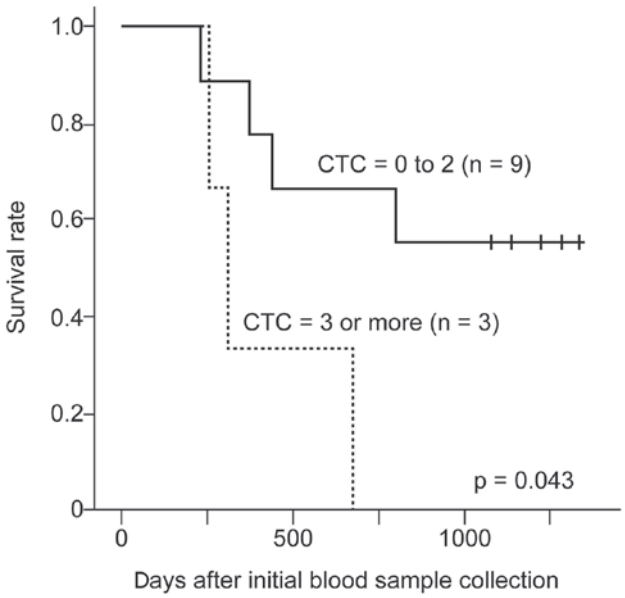

Figure 3. Kaplan-Meier curves showing overall survival after initial blood collection in cases treated non-surgically (n=12) with 2nd count of CTC=0 to 2 and CTC >2. CTC >2 was significantly associated with worse prognosis. CTC, circulating tumor cell.

Diff-quick stain kit (Sysmex) in some cases. The morphology of the captured CK-positive cells was confirmed as CTCs, and that of double positive (CK and CD45 positive) cells was determined as hematologic cells (data not shown). Recently, an earlier version of this device was tested in patients with metastatic lung cancer (8). In that investigation, non-detection rates (CTC=0) in metastatic non-small cell lung cancer and small cell lung cancer were 23 and 0%, respectively. In our series, 2 among 7 cases of metastatic non-small cell lung cancer did not show any CTCs. This non-detection rate (28.6%) might be comparable but requires further large-scale assessment. One patient with small cell lung cancer who did not show CTCs in our series was at clinical stage I.

We did not identify any clinicopathological factors associated with the number of CTCs other than smoking index. However, our results on the association between CTC counts and smoking history are inconsistent with the data presented by Dandachi *et al* (6). We speculate that our prospectively recruited series was adenocarcinoma-dominant (84% of all cases), which would confound any correlation with the smoking index. As low-dose CT screening, which is effective in finding a small peripheral ground glass nodules representing a lepidic-growth type adenocarcinoma, is being carried out since 1998 in Hitachi city (14), the histological type in our hospital might have a tendency to be adenocarcinoma-dominant.

Although 1st CTC counts and changes in CTC counts did not show any significant contribution to survival in all 38 cases and 12 non-surgical cases, cases with 2nd CTC count >2 in non-surgical cases showed significantly worse survival than those with 2nd CTC=0 to 2 (Fig. 3). Because the cohort of this study included various treatment modalities, we further selected only non-surgical cases for survival analyses. Whereas these results might indicate the possibility that CTC counts using this device after non-surgical treatment would be of prognostic value, the clinical implication and utility of CTC

counts captured by this device require further investigation with a larger number of patients.

The goal of CTC detection would then be to recognize and evaluate the clinical utility of captured CTCs. For advanced metastatic lung cancer, a possible application in genetic analyses could facilitate precision medicine (4,15). Additionally, if the cutoff number of CTC counts for post-operative recurrence were available, patients undergoing lung resection for lung cancer could avoid radiation exposure upon follow-up examination. In both settings, single-cell analysis would not be necessarily required. Our development concept of this device was for simple capture of CTCs without sample preparation that would combine clinical convenience with high throughput, and not for single-cell manipulation. The development of techniques and methods and evaluation of the utility of captured cells remain future challenges. We developed this filter and device step by step that included experiments using whole human blood spiked with cultured cancer cells and measurements of CTC in healthy controls (8-10). Because this prospective study was carried out according to a protocol determined in advance, we could not recruit further cases for more data nor add data from healthy controls. In a future study, concurrent acquisition of healthy control samples and a sufficiently large sample size should be considered.

In conclusion, this pilot study shows that the metallic micro-cavity array filter developed by Hitachi Chemical captured CTCs in patients with lung cancer even in early clinical stages.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HI wrote the manuscript. TN designed the present study. HI, TN, YY, KS, KK and SK treated and cared for the patients. HK, TO, KE, TM, SN and SY developed the micro-cavity array filter and the device. TN and YS comprehensively supervised the present study. HI, TN, YY, KS, KK, SK, HK, TO, KE, TM, SN, SY and YS interpreted the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study protocol was approved by The Institutional Review Board of Ibaraki Hospital Headquarters at Hitachi, Ltd. (approval no. 2014-64) and written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The filter and device were developed by Hitachi Chemical Co., Ltd. The authors declare that they have no competing interests.

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