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Prognostic impact of circulating tumor cells detected with the microfluidic "universal CTC-chip" for primary lung cancer

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Abstract

Detecting rare circulating tumor cells (CTCs) in the bloodstream is extremely challenging. We had previously developed a novel polymeric microfluidic device, "CTC-chip," for capturing CTCs and have shown high capture efficiency in lung cancer cell lines by conjugating Abs against epithelial cell adhesion molecules (EpCAM). This study aimed to optimize the EpCAM-chip and clarify the prognostic impact of CTCs in lung cancer patients. Of 123 patients with pathologically proven lung cancer, both progression-free survival ($P = .037$) and cancer-specific survival ($P = .0041$) were predominantly poor when CTCs were detected before treatment. After classification into surgical and chemotherapy groups, progression-free survival was worse in CTC-positive patients in both groups (surgery, $P = .115$; chemotherapy, $P = .012$), indicating that the detection of baseline CTCs is a risk factor for recurrence and progression. Furthermore, we recovered captured CTCs using micromanipulators and undertook mutation analysis using PCR. Thus, the EpCAM-chip is a highly sensitive system for detecting CTCs that contributes to the prediction of recurrence and progression and enables genetic analysis of captured CTCs, which could open new diagnostic, therapeutic, and prognostic options for lung cancer patients.

KEYWORDS
circulating tumor cell, CTC-chip, EpCAM, lung cancer, polymeric microfluidic device

1 | INTRODUCTION

Metastatic spread of cancer is one of the leading causes of cancer-associated morbidity and mortality.[1] Circulating tumor cells form the very basis of this process, and the detection and molecular biological analysis of CTCs could help enhance the diagnosis and treatment of cancer patients.[2-4] However, CTCs are rare in the bloodstream and the detection of CTC contamination in many

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normal hematologic cells has been elusive.[5-7] In various CTC isolation technologies, most of the data pertaining to the clinical utility of CTCs was collected utilizing CellSearch (Veridex), the only FDA-approved technology for CTC-enumeration for breast, colon, and prostate cancers.[8] CellSearch is a semiautomated CTC-capture system that uses a ferrofluid coated with an Ab against EpCAM, and has showed clinical relevance in many types of epithelial cancers.[9-13] Our previous study using CellSearch showed that CTCs could be a useful surrogate marker of distant metastasis in primary lung cancer, but also indicated the need for a more sensitive system.[14] A microfluidic system, called a "CTC-chip," could capture CTCs with Abs attached to the surface of numerous microposts within a microflow chamber, and the CTC-chip conjugated with anti-EpCAM Ab showed high sensitivity in the detection of CTCs.[15,16] Based on this conventional CTC-chip, we developed a novel polymeric microfluidic device CTC-chip with lower cost, higher durability, and improved transparency.[17] Furthermore, the most important advantage of our novel CTC-chip is its capability of capturing various types of CTCs by arbitrarily coating antibody against expressed in the targeted CTC, which we named "universal CTC-chip".[18] We have reported highly sensitive capture of lung cancer and MPM cell lines using this universal CTC-chip coated with the appropriate Abs (lung cancer, anti-EpCAM Ab;[18] MPM, anti-podoplanin Ab[18-20]), and have subsequently investigated clinical cases. In the current study, based on accumulated clinical cases, we confirmed the superiority of the cell capture efficiency of the CTC-chip compared to CellSearch, and clarified the prognostic impact of CTCs in lung cancer patients. Furthermore, as the biggest concern of the CTC-chip was whether the cells captured with the CTC-chip were true CTCs, we validated the cells by capturing them with a micromanipulator and carrying out genetic analysis.

2 | MATERIALS AND METHODS

This research was carried out with the approval of the Ethics Committee of the University of Occupational and Environmental Health, Japan (approval no. H26-15). The patients and healthy volunteers provided formal written informed consent to participate in this study.

2.1 | Cell lines

Four human lung cancer cell lines were used in this study. PC9 (Riken BioResource Research Center), NCI-H441 (ATCC), and NCI-H1975 (ATCC) were cultured in RPMI-1640 medium (Wako Pure Chemical Industries) supplemented with 10% FBS (Thermo Fisher Scientific) at 37°C and 5% CO_2. A549 (ATCC) was cultured in EMEM (Wako Pure Chemical Industries) supplemented with 10% FBS and 1% nonessential amino acids (Wako Pure Chemical Industries) at 37°C and 5% CO_2.

2.2 | Flow cytometry analysis

To analyze the EpCAM expression, cell lines were incubated with anti-EpCAM Ab (1:100 dilution, clone HEA125; Santa Cruz Biotechnology) for 1 hour at room temperature, and were subsequently incubated with goat anti-mouse IgG Ab conjugated with fluorescein isothiocyanate (1:20 dilution; BD Biosciences). Flow cytometry analysis was carried out using an EC800 Cell Analyzer (Sony Biotechnology), and the data were analyzed using FlowJo software (Becton, Dickinson and Company). Mean fluorescence intensity was calculated as the ratio of positive control to negative control.

2.3 | Preparation of CTC-chip and evaluation of cell-capture efficiency in lung cancer cell lines

Preparation of the CTC-chip and evaluation of cell capture efficiency were carried out as described previously.[18] The CTC-chip was first incubated with goat anti-mouse IgG (SouthernBiotech) as the base Ab overnight at 4°C and then with mouse anti-human EpCAM Ab as the capture Ab at room temperature for 1 hour ("EpCAM-chip"). For the evaluation of cell-capture efficiency, 1 mL (100 cells/mL) of blood collected from healthy volunteers was mixed with tumor cells labeled using the CellTrace CSFE Cell Growth Kit (Thermo Fisher Scientific). The sample was then applied to the CTC-chip at a constant flow rate (1.0 mL/h) and monitored using a fluorescence microscope (CKX41; Olympus). The total number of cells applied to the CTC-chip (N-total) was determined by counting the cells that passed through the inlet of the CTC-chip, and the number of captured cells (N-captured) was determined by counting the cells that remained on the CTC-chip. Cell-capture efficiency was calculated as the N-captured / N-total ratio, and the average and standard errors were calculated for each sample. The blood collection tube used for the CTC-chip was a BD Vacutainer EDTA-2K (Becton, Dickinson and Company), and each experiment was carried out in triplicate.

2.4 | Clinical evaluation of CTCs in lung cancer patients

Peripheral blood was sampled from patients with a pathological diagnosis of lung cancer, and 1 mL blood was applied to the EpCAM-chip. Cells captured on CTC-chips were incubated with primary Abs, rabbit anti-CK Ab (ab9377; Abcam) and rat anti-CD45 Ab (clone YTH24.5; Abcam), for 1 hour at room temperature, followed by incubation for 30 minutes at room temperature with a secondary Ab, Alexa Fluor 594 anti-rabbit IgG Ab (Thermo Fisher Scientific) and Alexa Fluor 488 anti-rat IgG Ab (Thermo Fisher Scientific) containing 1 μg/mL Hoechst 33 342 (Cell Signaling Technology). Cells with round-to-oval morphology, Hoechst 33342-positive nuclei, CK-positive staining in the cytoplasm, and CD45-negative staining were identified as CTCs. Circulating tumor cell identification was independently examined by two of the investigators without any

knowledge of the clinical data. In case of discrepancy between the two investigators, a consensus was reached by simultaneous examination. For CTC detection in the CellSearch system to compare the sensitivity, CTCs were quantitatively evaluated in 7.5 mL blood per standard manufacturer protocol. The number of CTCs in 1 mL blood was indicated as the CTC count for the CTC-chip and that in 7.5 mL blood was indicated as the CTC count for the CellSearch system. Additionally, to evaluate nonspecific detection, peripheral blood samples were collected from 10 healthy individuals and used to detect CTCs in the same manner.

2.5 | Genetic analysis for validation of captured cells with EpCAM-chip

For the validation of captured cells with the EpCAM-chip, 1 mL (100 cells/mL) of blood collected from healthy volunteers containing PC9 (*EGFR* exon 19 deletion), NCI-H441 (*KRAS* G12V mutation), and NCI-H1975 (*EGFR* exon 21 L858R and exon 20 T790M mutations) was applied to the CTC-chip system in the above method. After tumor cell identification, captured single cells were collected into 200-μL tubes (including 2 μL PBS) by micromanipulator M-152 (Narishige Group), and was submitted for WGA using the PicoPLEX Single Cell WGA Kit version 3 (Takara Bio) according to the manufacturer's instructions, in order to obtain a sample suitable for sequencing analysis. Mutational analysis of *EGFR* exons 19 and 21 and *KRAS* codon 12 was based on our previous reports.[21] Polymerase chain reaction was carried out by using Taq polymerase (Takara Taq; Takara Bio) and sequences of the primers are as follows: *EGFR* exon 19, 5′-GTCTTCCTTCTCTCTCTGTCATAG-3′ (sense) and 5′-CCACACAGCAAAGCAGAAACTCAC-3′ (antisense); *EGFR* exon 21, 5′-TCAAGATCACAGATTTTGGGCG-3′ (sense) and 5′-CATCCTCCCCTGCATGTGTTAAAC-3′ (antisense); and *KRAS* codon 12, 5′-AAACTTGTGGTAGTTGGACCT-3′ (sense) and 5′-CTATTGTTGGATCATATTCG-3′ (antisense).

A fraction of *KRAS* PCR product was subsequently digested with *Bst*NI (New England Biolabs) to identify codon 12 mutation. All PCR reactions were carried out on the GeneAmp PCR System 9700 (Thermo Fisher Scientific). Amplification of the intended fragments was confirmed by electrophoresis on 3.0% agarose gel (Nippon Gene Co., Ltd.) containing 0.5 μg/mL ethidium bromide (Genesee Scientific).

2.6 | Statistical analysis

Differences in categorical variables were evaluated using Pearson's χ^2 test or Fisher's exact test, as appropriate. Differences in continuous variables were evaluated using a *t* test (Student's *t* test or Welch's *t* test) for normally distributed data and the Mann-Whitney *U* test for nonnormally distributed data. Cancer-specific survival was calculated from the date of the CTC test until cancer-specific mortality or the last follow-up. Progression-free survival was calculated from the

date of the CTC test until recurrence, mortality, or the last follow-up. The Kaplan-Meier method was used to estimate CSS and PFS, and survival differences were compared between the two groups using the log-rank test. The analysis of prognostic factors was undertaken using the Cox proportional hazards regression model. A *P* value of less than .05 was considered statistically significant. All statistical analyses were carried out using SPSS (version 27.0; IBM).

3 | RESULTS

3.1 | Optimizing EpCAM-chip and cell-capture efficiency in lung cancer cell lines

In the previous study, with a base Ab concentration of 20 μg/mL and a capture Ab concentration of 20 μg/mL, PC9 cells spiked in blood were captured with an average capture rate of 88%.[18] When the concentration of the base Ab was increased to 200 μg/mL to achieve a higher capture rate, PC9 cells were captured at approximately 100% (*P* = .022; Figure S1A), and we adopted this condition for further experiments. NCI-H441 cells with positive EpCAM expression were effectively captured with the optimized EpCAM-chip (average capture efficiency, 88.8%). In contrast, A549 cells with low EpCAM expression were not effectively captured with the EpCAM-chip (average capture efficiency, 13.4%) (Figure S1B).

3.2 | Comparison of CTC detection sensitivity of EpCAM-chip and CellSearch in lung cancer patients

Overall, 31 peripheral blood samples drawn from individual lung cancer patients were subjected to quantitative analyses for CTCs using the EpCAM-chip (Figure S2) and CellSearch system. Patients' characteristics and distribution of CTC count with the EpCAM-chip and CellSearch system for each sample are summarized in Table 1 and Figure 1A. Circulating tumor cells were detected in 58.1% samples (18/31 patients) with the EpCAM-chip and in 25.8% samples (8/31 patients) with the CellSearch platform. Median CTC counts with the EpCAM-chip and CellSearch system were 1/1 mL (range, 0-11) and 0/7.5 mL (range, 0-19), respectively, and average CTC counts were 1.88/1 mL and 1.74/7.5 mL, respectively. Despite no significant difference being observed between the two devices (*P* = .257), the results suggested that the EpCAM-chip has better performance in CTC detection. In 10 healthy participants, no cells were detected, and there was no obvious nonspecific detection (Figure 1B).

3.3 | Clinical impact of CTCs detected with EpCAM-chip in lung cancer patients

To evaluate the clinical implications of CTCs detected using the EpCAM-chip, we further analyzed additional blood samples drawn from a total of 123 lung cancer patients prior to treatment,

TABLE 1 Characteristics of 31 patients with lung cancer

Characteristics	Patients (n = 31)
Age (y); median (range)	69.0 (55-91)
Sex	
Male	19 (61.3)
Female	12 (38.7)
TNM stage	
IA/IB	2 (6.5)/7 (22.6)
IIA/IIB	2 (6.5)/1 (3.2)
IIIA/IIIB	9 (29.0)/2 (6.5)
IV	8 (25.8)
Histology	
Adenocarcinoma	16 (51.6)
Squamous cell carcinoma	12 (38.7)
Others	3 (9.7)

Note: Data are shown as n (%) unless otherwise indicated.

FIGURE 1 A, Distribution of circulating tumor cells (CTCs) detected using the CellSearch system and epithelial cell adhesion molecule (EpCAM)-chip according to their count in 7.5 mL and 1 mL peripheral blood collected from lung cancer patients. B, Distribution of CTCs detected using the EpCAM-chip according to their count in 1 mL peripheral blood collected from lung cancer patients and healthy participants

especially surgery (n = 71, including postinduction therapy [n = 3]) or chemotherapy (n = 52, including more than second-line therapy [n = 8]) (Figure 2). Patients' characteristics are described in Table 2. Circulating tumor cells were detected in 58 patients (47.2%), and the median and average CTC counts were 0 and 1.73 (range, 0-29), respectively, in the overall population. Circulating tumor cell positivity according to the stage was as follows: 26.5% (9/34) in stage I, 56.3% (9/16) in stage II, 50.0% (12/24) in stage III, and 57.1% (28/49) in stage IV. The detection rate of CTCs was significantly higher in chemotherapy group patients than in surgery group patients (P = .0062). These results suggested that CTCs are more easily detected in inoperable patients due to lymph node metastasis or distant metastasis, and that CTCs might contribute to tumor progression. The survival analysis is presented in Figure 3. The median follow-up was 984 (range, 13-1684) days. The 1-year/3-year CSS rates for the CTC-positive and CTC-negative groups were 69.8%/60.3% and 90.1%/72.3%, respectively. The 1-year/3-year PFS rates for the CTC-positive and CTC-negative groups were 39.5%/23.9% and 68.5%/50.5%, respectively. Both CSS (P = .037) and PFS (P = .0041) were significantly better in the CTC-negative group (Figure 3A). Patient characteristics for the surgical group, excluding postinduction therapy, (n = 68) are described in Table S1. Circulating tumor cells were detected in 24 patients (35.3%), and the median and average CTC counts were 0 and 1.53 (range, 0-29), respectively. In the CTC-negative group, 54.5% patients were stage I, whereas 37.5% were stage I patients in the CTC-positive group. The 1-year/3-year CSS rates for the CTC-positive and CTC-negative groups were 91.3%/95.1% and 81.9%/80.7%, respectively. The 1-year/3-year PFS rates for the CTC-positive and CTC-negative groups were 69.9%/38.6% and 75.6%/60.7%, respectively. Cancer-specific survival was similar between the CTC-positive and CTC-negative groups (P = .841); PFS tended to be better in the CTC-negative group, although the difference was not statistically significant (P = .115) (Figure 3B). Patient characteristics (n = 52) for the chemotherapy group are
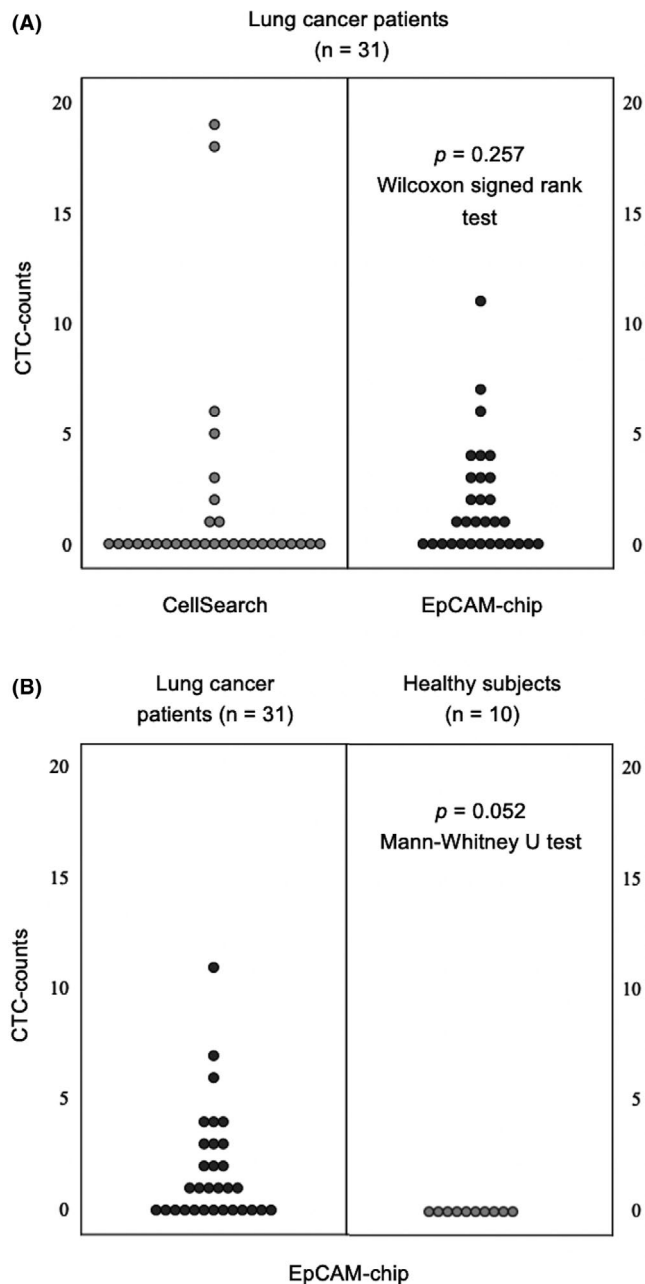
described in Table S2. Circulating tumor cells were detected in 32 patients (61.5%), and the median and average CTC counts were 1 and 1.88 (range, 0-12), respectively. The 1-year/3-year CSS rates for the CTC-positive and CTC-negative groups were 79.4%/50.0% and 59.7%/39.4%, respectively. The 1-year/3-year PFS rates for the CTC-positive and CTC-negative groups were 52.9%/27.5% and 13.3%/6.7%, respectively. In the CTC-positive group, CSS (P = .060) and PFS (P = .012) were associated with poor prognosis, and the difference in PFS was statistically significant (Figure 3C). Furthermore,
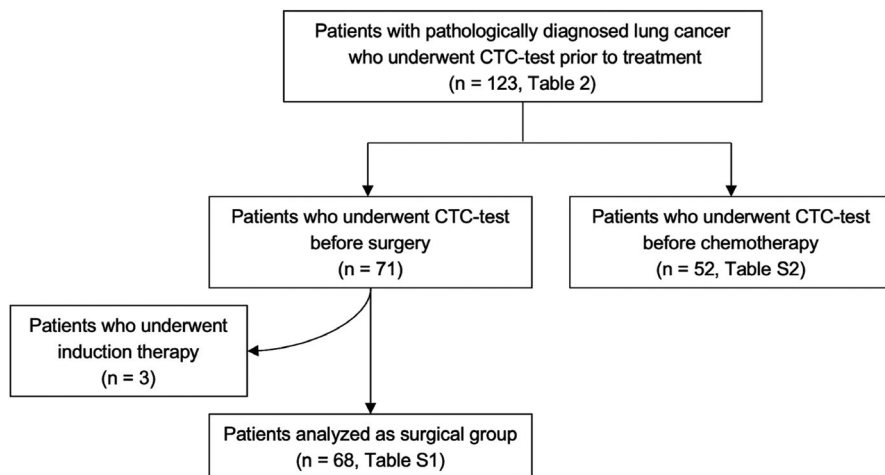
FIGURE 2 CONSORT diagram of the study. CTC, circulating tumor cell

TABLE 2 Characteristics of 123 lung cancer cases according to circulating tumor cell (CTC) counts (CTC ≥ 1/CTC = 0)

Characteristic	CTC ≥ 1 (n = 58)	CTC = 0 (n = 65)	P value
Age (y); median (range)	74.0 (42-91)	73.0 (49-91)	.883
Sex			
Male	36 (62.1)	43 (66.2)	.637
Female	22 (37.9)	22 (33.8)	
Tumor laterality			
Right	32 (55.2)	41 (63.1)	.373
Left	26 (44.9)	24 (36.9)	
Histology			
Adenocarcinoma	34 (58.6)	45 (69.2)	.206
Squamous cell carcinoma	17 (29.3)	13 (20.0)	
Small cell carcinoma	3 (5.2)	4 (6.2)	
Others	4 (6.9)	3 (4.6)	
TNM stage			
IA/IB	5 (8.6)/4 (6.9)	13 (20.0)/12 (18.5)	.217
IIA/IIB	2 (3.4)/7 (12.1)	5 (7.7)/2 (3.1)	
IIIA/IIIB/IIIC	6 (10.3)/4 (6.9)/2 (3.4)	9 (13.8)/2 (3.1)/1 (1.5)	
IVA/IVB	15 (25.9)/13 (22.4)	11 (16.9)/10 (15.4)	
Classification			
Surgery	26 (44.8)	45 (69.2)	.0062
Postadjuvant therapy	2 (7.7)	1 (2.2)	
Chemotherapy	32 (55.2)	20 (30.8)	
First line	29 (90.6)	16 (80.0)	
More than second line	3 (9.4)	4 (20.0)	
CTC counts (median, average)	2, 3.67	0, 0	

Note: Data are shown as n (%) unless otherwise indicated.

in the analysis of patients with (17 patients; EGFR tyrosine kinase inhibitors, 14 patients; anaplastic lymphoma kinase inhibitors, 3 patients) and without (35 patients) the use of molecular targeted agents, there was no significant difference in CSS ($P = .567$) or PFS ($P = .973$) in patients with molecular targeted agents (Figure S3A). In patients without molecular targeted agents, both CSS ($P = .095$) and PFS ($P = .0089$) were poor in the CTC-positive group, with a significant difference in PFS (Figure S3B).

3.4 | Genetic analysis for validation of cells captured with EpCAM-chip

The detection of cells from the CTC-chip is described in Figure 4, and the results of electrophoresis of cells captured by the CTC-chip are presented in Figure 5. The PCR products containing exon 19 revealed a 147-bp band when the allele was a WT and a shorter band (132 bp) when the allele was a deletion type. To detect a point
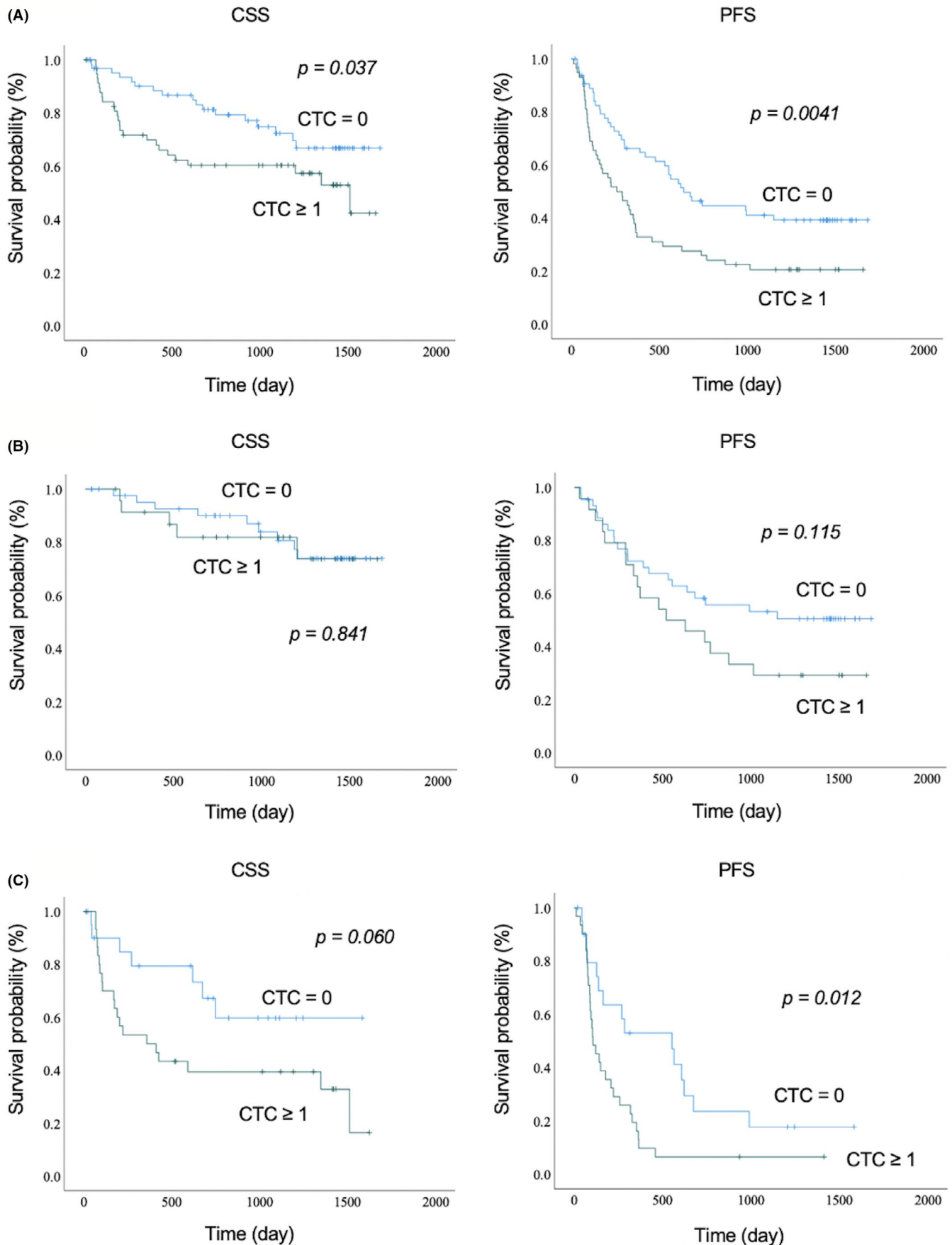
FIGURE 3 Cancer-specific survival (CSS) and progression-free survival (PFS) according to circulating tumor cell (CTC) count (CTC ≥ 1/ CTC = 0) in (A) all cases, (B) surgery patients, and (C) chemotherapy patients

(A)

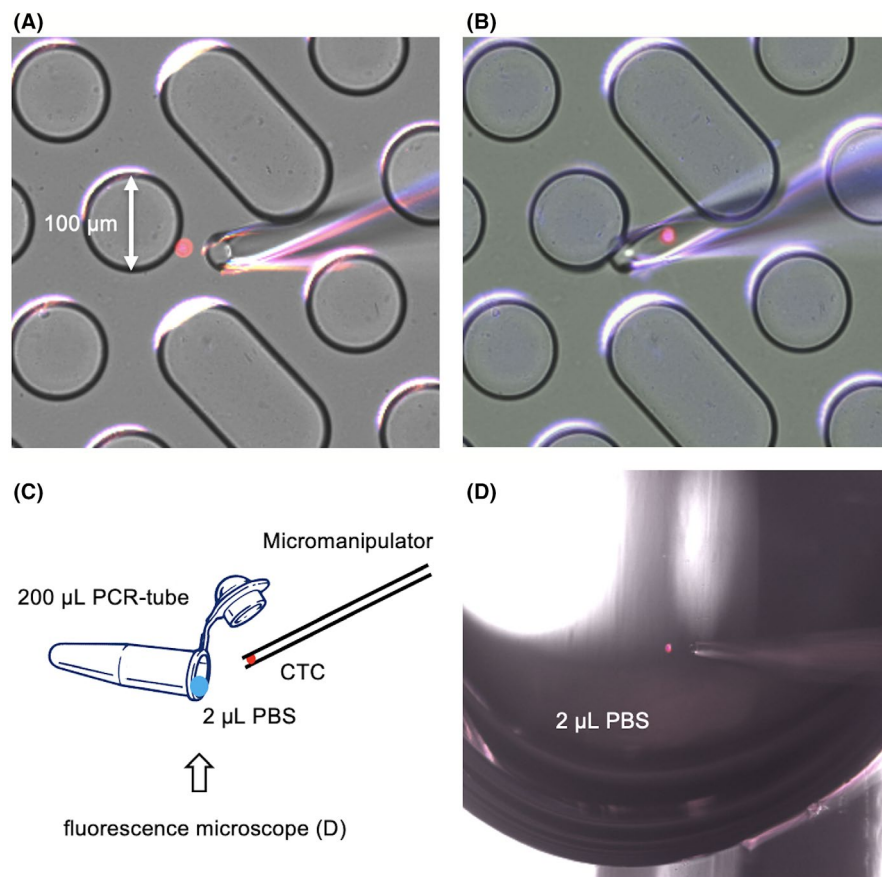**(B)**

(C)

(D)

FIGURE 4 Images of cells trapped in the epithelial cell adhesion molecule (EpCAM)-chip being collected using a micromanipulator. CTC, circulating tumor cell

mutation of the second base of codon 858 in exon 21, mutant allele-specific amplification was carried out, and only the DNA sample with a mutation revealed a band. Following *Bst*NI (New England Biolabs) digestion, the 94-bp product of the WT *KRAS* was cut into 74 bp and 20 bp fragments. If there is any mutation at the second position of codon 12 of *KRAS*, *Bst*NI digestion is not possible. In cell line analysis, genetic mutations in each cell line were detected in the cells captured by the CTC-chip. Therefore, subsequent examinations of clinical specimens from lung cancer patients with *EGFR* exon 19 deletions (78-year-old woman with recurrence of intrapulmonary metastasis and pleural dissemination after treatment with EGFR tyrosine kinase inhibitor osimertinib for pT4 [ipsilateral intrapulmonary metastasis] N0M1a stage 4a) revealed that the captured cells had *EGFR* exon 19 deletions (CTCs) (Figure 6).

4 | DISCUSSION

In our study, we first optimized the EpCAM-chip by increasing the concentration of the base Ab that showed better performance than the CellSearch system in clinical cases. Second, PFS was worse in CTC-positive patients in both surgical and chemotherapy cases, indicating that the detection of baseline CTCs is a risk factor for recurrence and progression of lung cancer. Finally, we determined that the cells captured with the CTC-chip were true CTCs by recovering them using a micromanipulator and undertaking genetic mutation analysis.

Enumeration of CTCs can provide prognostic information for cancer management.[9,10,22] However, it is extremely difficult to detect CTCs present in very small amounts in the blood, and even the CellSearch system, the only method approved for clinical use by the US FDA, has been suggested to have insufficient detection sensitivity.[8,12,14] Therefore, we developed a new microfluidic device, the CTC-chip, as a more sensitive detection system.[17,18] In our study, the EpCAM-chip was optimized and showed higher capture efficiency than the CellSearch system with clinical specimens. Furthermore, pretreated CTC-positive patients evaluated with the optimized EpCAM-chip had predominantly poor PFS and CSS, suggesting the clinical significance of baseline CTC count as a prognostic marker. In particular, CTCs could be useful in assessing the risk of recurrence and progression as CTC-positive patients had poor PFS even after stratification by surgery and chemotherapy. These results indicated that a CTC test could be useful in detecting and predicting the presence of minimal residual disease, which could cause tumor recurrence after complete surgical resection, and in prescribing optimal postoperative therapy. Additionally, the evaluation of risk factors for recurrence might also be useful in selecting the appropriate chemotherapy. Although there was no difference in CSS or PFS between CTC-positive and CTC-negative patients treated with molecular targeted agents, the number of cases was small at 17, and five of them (CTC ≥1, 2 cases; CTC = 0, 3 cases) were more than second-line treatment. Therefore, further studies are needed to validate these results with more accumulated cases.
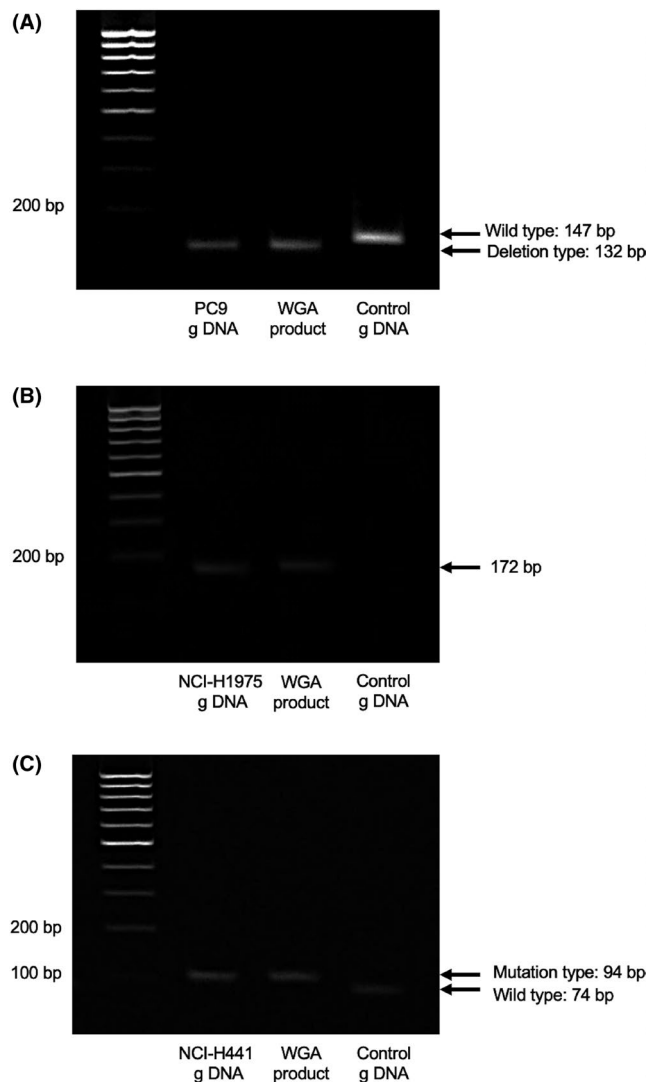
FIGURE 5 Results of electrophoresis for cells. (A) PC9 (*EGFR* exon 19 deletion), (B) NCI-H1975 (*EGFR* exon 21 L858R and exon 20 T790 M mutations), and (C) NCI-H441 (*KRAS* G12V mutation) captured using the epithelial cell adhesion molecule (EpCAM)-chip. g DNA, genomic DNA; WGA, whole-genome amplification

FIGURE 6 A, Image of immunohistochemical staining of cells in the peripheral blood of lung cancer patients with *EGFR* exon 19 deletion captured using the epithelial cell adhesion molecule (EpCAM)-chip. B, Electrophoresis results of these cells. CTC, circulating tumor cell; g DNA, genomic DNA; WBC, white blood cell; WGA, whole-genome amplification

Recent studies have recognized that CTCs are useful not only in providing various information about the primary tumor and distant metastasis but also in elucidating molecular mechanisms involved in cancer evolution.[23-25] In addition to the previous information on the number of CTCs, genetic analysis of captured CTCs is expected to provide new insights, which should be validated in future prospective studies.

There are several limitations to this study. First, the detection of CTCs was essentially done only once for each patient. Monitoring CTCs during and after treatment might provide unique information for the clinical management of an individual cancer patient and allow an early induction or change in therapy before the appearance of overt metastases signals. Therefore, we are currently collecting peripheral blood samples from patients who are newly diagnosed or treated for lung cancer, which will reveal
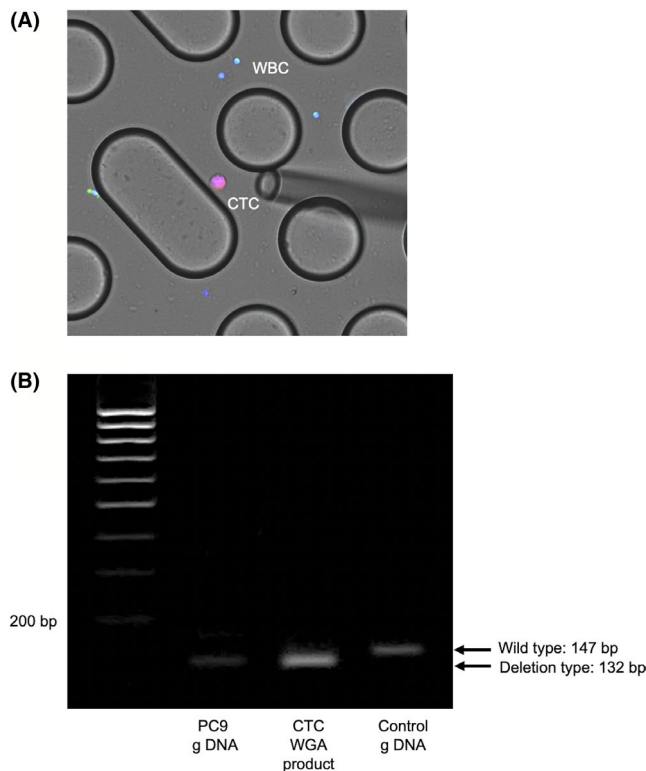
the prognostic and predictive value of our CTC detection system. Second, the molecular biological analysis of captured CTCs has not been fully performed. There is increasing evidence that the phenotype and genotype of primary and metastatic cancer cells are discordant and that the molecular analysis of CTCs isolated from peripheral blood will reveal characteristics of metastatic cancer cells.[2-4] We are currently staining captured CTCs for PD-L1 and undertaking genetic analysis using next-generation sequencers to characterize the molecular characteristics of CTCs, which will help us to evaluate treatment resistance and elucidate the mechanism of resistance. Finally, the EpCAM-chip showed more sensitive detection than the CellSearch system; however, CTCs were not detected in approximately 40% of patients with stage IV lung cancer. This result suggested that the EpCAM-chip might overlook CTCs undergoing EMT,[26,27] which is considered an important metastasis-causing cell subtype.[28,29] Therefore, we are currently integrating clinical specimens using the CTC-chip with Abs against CSV as an EMT-CTC marker,[30-33] which might be a more prognostically correlated indicator than EpCAM.

In conclusion, this study showed that the EpCAM-chip has a high performance in detecting CTCs in lung cancer patients, and the number of detected CTCs is a useful risk factor for recurrence and progression. Our current efforts to capture CTCs using CSV and elucidation of the molecular characteristics of captured CTCs

using additional staining for PD-L1 and genetic analysis will reveal the further clinical value of CTCs. We believe that the universal CTC-chip can become an indispensable tool for developing new diagnostic, therapeutic, and prognostic options for lung cancer patients.

DISCLOSURE

The authors have no conflict of interest.

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