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Heterogeneous Expression of Programmed Death Receptor-ligand 1 on Circulating Tumor Cells in Patients With Lung Cancer

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Abstract

Circulating tumor cells (CTCs) and their programmed death receptor-ligand 1 (PD-L1) expression in patients with lung cancer were detected using a microcavity array system. PD-L1 expression was detected in 73% of patients who harbored CTCs. The proportion of PD-L1-positive CTCs ranged from 3% to 100%, suggesting intra-patient heterogeneity. No correlation on PD-L1 expression was observed between tumor tissues and CTCs.

Background: Blockade of the programmed death receptor-1 (PD-1) pathway is effective against solid tumors including lung cancer. PD-ligand 1 (PD-L1) expression on tumor tissue serves as a predictive biomarker for the efficacy of PD-1 pathway blockade. Here, we evaluated the expression of PD-L1 on circulating tumor cells (CTCs) in patients with lung cancer. **Materials and Methods:** Peripheral whole blood (3 mL) was collected from patients, and CTCs and PD-L1 expression were detected using a microcavity array (MCA) system. Immunohistochemistry for PD-L1 detection was also performed using matched tumor tissues. **Results:** Sixty-seven patients with lung cancer were enrolled in the study between July 2015 and April 2016 at Wakayama Medical University Hospital. The characteristics of the patients were as follows: median age, 71 years (range, 39-86 years); male, 72%; stage II to III/IV, 14%/85%; non–small-cell lung cancer/small-cell lung cancer/other, 73%/21%/6%. CTCs were detected in 66 of 67 patients (median, 19; range, 0-115), and more than 5 CTCs were detected in 78% of patients. PD-L1-expressing CTCs were detected in 73% of patients, and the proportion score of PD-L1-expressing CTCs ranged from 3% to 100%, suggesting intra-patient heterogeneity of PD-L1 expression on CTCs. Tumor tissues were available from 27 patients and were immunostained for PD-L1, and no correlation was observed between tumor tissues and CTCs based on the proportion score ($R^2 = 0.0103$). **Conclusion:** PD-L1 expression was detectable on CTCs in patients with lung cancer, and intra-patient heterogeneity was observed. No correlation was observed between PD-L1 expression in tumor tissues and CTCs.

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Keywords: Immune checkpoint inhibitors, Liquid biopsy, Noninvasive diagnostics, Precision medicine, Tumor heterogeneity

Introduction

Lung cancer is a leading cause of cancer-related deaths worldwide,[1] and it is often diagnosed at an advanced stage. Major progress has been made in molecular targeted therapies for advanced lung cancers during the past couple of decades,[2,3] and blockade of the programmed death receptor-1 (PD-1)/PD-ligand 1 (PD-L1) pathway has been shown to be effective against lung cancer and other solid malignancies.[4] PD-L1 expression on tumor tissue has the potential to be a predictive biomarker for the efficacy of PD-1 pathway blockade.[5] However, the detection and evaluation of PD-L1 expression remain challenging owing to its dynamic and unstable characteristics. It is known that a small subset of patients even without PD-L1 expression in their tumor tissues benefit from PD-1/PD-L1 blockade, and it has been speculated that this is owing

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to tumor heterogeneity and the lack of real-time detection of PD-L1 expression. Thus, predictive biomarkers to select those PD-L1-negative patients who are likely to benefit from PD-1/PD-L1 blockade are critically needed.

Circulating tumor cells (CTCs) have been observed in various solid tumors including lung cancers and can provide useful prognostic information regarding survival.[6-8] Moreover, it has been reported that CTCs could potentially serve as an alternative to tumor tissue as a source of material for the detection of genetic alterations and the expression of therapeutic targets.[9] This approach of analyzing CTCs for diagnostic or prognostic purposes is termed a "liquid biopsy," owing to its minimal invasiveness. We previously reported that more CTCs were detectable with a high specificity using a novel sensitive microcavity array (MCA) system in patients with lung cancer among whom it is difficult to detect CTCs using the United States Food and Drug Administration-approved CELLSEARCH system.[10,11] We have recently developed an automated MCA system for potential clinical application and obtained a similar sensitivity and specificity to those of a manually operated MCA system.[12]

In this study, we evaluated PD-L1 expression on CTCs in patients with lung cancer and investigated the correlation with its expression in tumor tissues. PD-L1 detection on CTCs may have the potential to complement tissue-based diagnosis or identify more patients who are eligible for anti-PD-1/PD-L1 therapy.

Materials and Methods

Cell Lines and Culture

The lung cancer cell lines NCI-H820, NCI-H441, A549, and NCI-H23 were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and were cultured in Roswell Park Memorial Institute 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Gibco, Grand Island, NY) under humidified 5% CO_2/95% air at 37° C.

CTC Detection

CTCs were captured and immunostained using an automated MCA system (Hitachi Chemical Co, Ltd, Chikusei, Japan).[10-12] After tumor cells were recovered, an image of the entire cell array area was obtained using a fluorescence microscope (Axio Imager M2m; Carl Zeiss, Oberkochen, Germany) integrated with a 10× objective lens and a computer-operated motorized stage, a digital camera (AxioCam 503 mono; Carl Zeiss, Oberkochen, Germany), and ZEN image acquisition software (Carl Zeiss) (Supplemental Figure 1). CTCs were defined as those positive for 4′,6-diamidino-2-phenylindole and cytokeratin and negative for CD45. PD-L1 expression on CTCs was evaluated by the additional process of PD-L1 immunocytochemistry (antibody clone 28-8, Abcam, Cambridge, MA), and CTCs with fluorescence intensities over 350 in 80% of cell surface area or more were regarded as PD-L1-positive.

Spike-in Experiments

One hundred cells of each cell line were spiked into 3 mL of peripheral blood from healthy volunteers. Samples were processed using an MCA system for CTC detection and PD-L1 staining. Blood samples were collected from healthy volunteers who consented to donation. This study was approved by the independent institutional review board of Wakayama Medical University, and written informed consent was obtained from all the donors.

Patients

Before the initiation of any treatment, 3 mL of peripheral blood and tumor tissues were collected from patients who were pathologically diagnosed with lung cancer at Wakayama Medical University Hospital between July 2015 and April 2016 (Table 1). Samples were collected in a collection tube containing ethylenediaminetetraacetic acid (BD Vacutainer; BD Biosciences, Franklin Lakes, NJ) to prevent coagulation and processed within 3 hours after blood draw. This study was approved by the institutional review board and written informed consent was obtained from all patients. This study has been registered with University Medical Hospital Information Network (UMIN) Clinical Trials Registry under the identifier UMIN000021712.

PD-L1 Immunohistochemistry

Tumor tissues were obtained from 33 of the 67 patients enrolled into the study, and 27 samples were suitable for evaluation. Formalin-fixed paraffin-embedded specimens were sectioned to a thickness of 3 μm and immunostained with anti-PD-L1 antibody (Abcam, clone 28-8). Immunohistochemistry was performed at Pathology Institute (Toyama, Japan). PD-L1 positivity was defined and classified according to its expression and confirmed by pathologists. Microscopic images were processed, and PD-L1-positive cells were counted using WinROOF (Mitani Corporation, Fukui, Japan). PD-L1 positivity was evaluated according to the proportion score (PS), which was defined as the percentage of cells showing positive staining regardless of intensity. The H-score was calculated based on both the percentage of positive membrane staining and the staining intensity. The staining intensity was graded on a 4-point scale, as follows: 0 = no staining, 1 = weak, 2 = moderate, and 3 = strong. One hundred cells were counted to calculate the H-score, and its potential range is 0 to 300.

Statistical Analysis

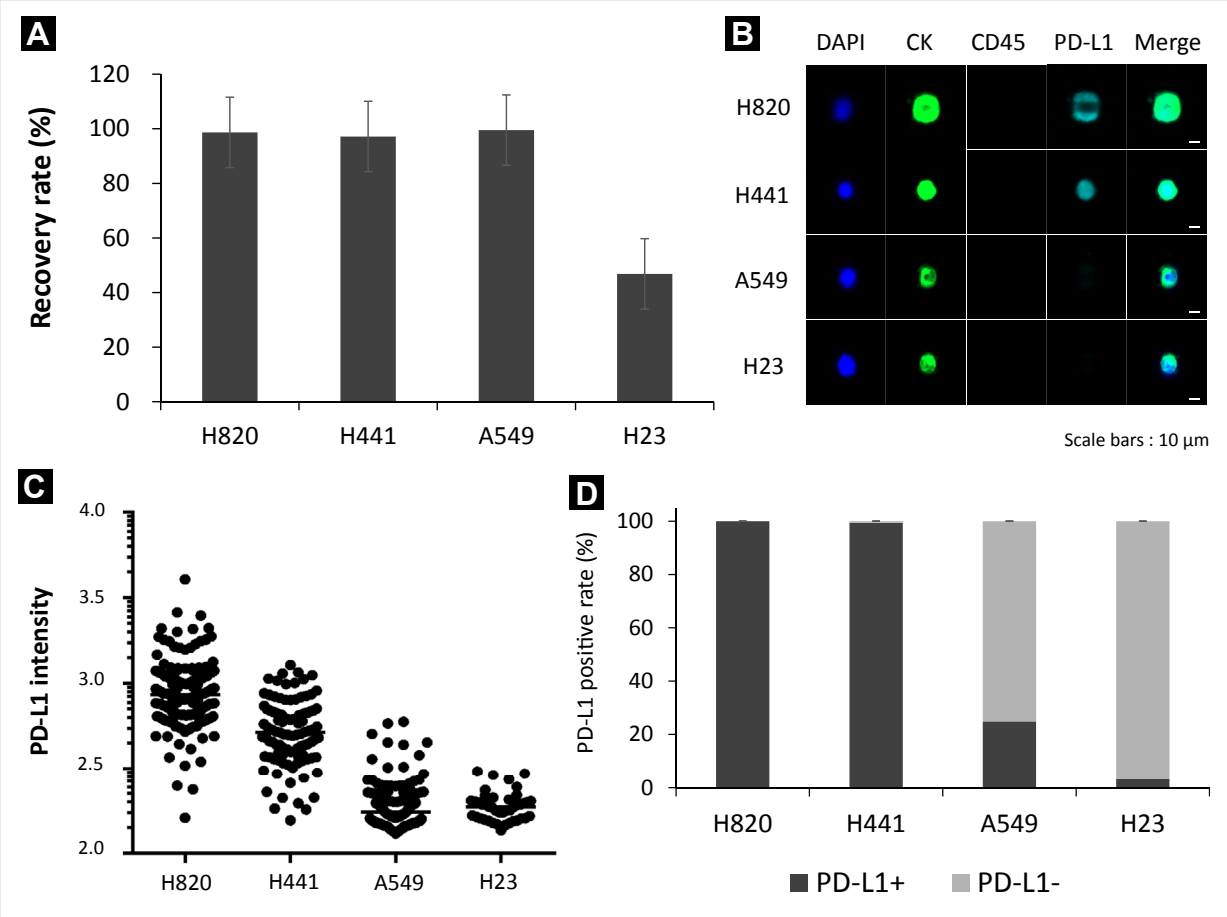
Statistical analyses were performed using Prism 6 (GraphPad Software, Inc, San Diego, CA). The significance level was set at $P < .05$.

Results

Establishment of PD-L1 Expression Detection

We used spike-in models for the establishment of PD-L1 expression detection. The recovery rate of each cell line by the MCA system is shown in Figure 1A. The results were consistent with our previous report,[12] indicating that sensitivity is dependent on cell size with this type of filtration system. Representative results of PD-L1 staining for each cell line are shown in Figure 1B. PD-L1 expression was detected in H820 and H441 cells, which have previously been known to express PD-L1, whereas it was not detected in A549 cells and H23 cells, which have been reported to lack PD-L1 expression. Interestingly, even the PD-L1-expressing cell lines H820 and H441 manifested heterogeneous levels of PD-L1 expression, with very few of those cells expressing an undetectable level of PD-L1 (Figure 1C), suggesting that PD-L1 has a complex expression pattern. Even among A549 and H23 cells with

Figure 1 Establishment of a Method to Detect PD-L1 Expression on Circulating Tumor Cells Using Spike-in Models. Recovery Rate of Spiked Cells by a Microcavity Array System (A); Representative Staining Images of Detected Cancer Cells (B); Heterogeneous Expression Levels of PD-L1 in Each Cell Line (C); Ratio of PD-L1-positive to -negative Cells in Each Cell Line (D)



Abbreviation: PD-L1 = programmed death receptor ligand 1.

a low or null PD-L1 expression, a small subset of the cells exhibited positivity for PD-L1 expression (Figure 1D).

Evaluation of PD-1 Expression on CTCs in Patients With Lung Cancer

Sixty-seven patients with lung cancer were enrolled in the study between July 2015 and April 2016 at Wakayama Medical University Hospital. The demographics of the 67 patients included in the study are shown in Table 1 as follows: median age, 71 years (range, 39-86 years); male, 72%; stage II to III/IV, 14%/85%; non−small-cell lung cancer (NSCLC)/small-cell lung cancer/other, 73%/21%/6%. The CTC count and PD-L1 expression on CTCs in enrolled patients were analyzed using the MCA system. CTCs were detectable in most patients using the MCA system as we previously reported,[10-12] and the CTC counts of all patients are shown in Figure 2A. CTCs were detected in 66 of 67 patients (median, 19; range, 0-115), and more than 5 CTCs were detected in 78% of patients. PD-L1 expression was detectable using the MCA system as shown in Figure 2B. PD-L1-expressing CTCs were detected in 73% of the patients with lung cancer, and the PS of PD-L1-expressing CTCs ranged from 3% to 100% among patients harboring PD-L1-positive CTCs (Figure 2C), suggesting that there is an intrapatient heterogeneity of PD-L1 expression on CTCs in patients with lung cancer. Among the clinicopathologic analyses performed, the *EGFR* mutation status correlated with the CTC count and PD-L1 positivity. Significantly more CTCs were detected in patients with adenocarcinoma with *EGFR* mutations than in those without *EGFR* mutations ($P < .05$) (Figure 2D). In addition, significantly more PD-L1-expressing CTCs were detected in patients with adenocarcinoma without *EGFR* mutations than in those with *EGFR* mutations ($P < .05$) (Figure 2E).

Concordance Between PD-L1 Expression on CTCs and That in Tumor Tissues

We obtained tumor tissues from 33 of 67 patients, and PD-L1 immunohistochemistry was performed. Twenty-seven of the tissue samples were suitable for further analysis. Representative pictures of PD-L1 immunostaining are shown in Figure 3A. The PS rates of

Table 1 Patient Characteristics (n = 67)

Characteristics	N (%)
No. patients	67
Age, y	
Median	71
Range	39-86
Gender	
Male	48 (72)
Female	19 (28)
Smoking status	
Pack-year ≥ 30	35 (52)
Pack-year < 30	15 (22)
Never-smoker	17 (25)
Histologic type	
Adenocarcinoma	38 (57)
EGFR mutated	14 (37)
ALK rearrangement	2 (5)
Squamous cell carcinoma	11 (16)
Small cell	14 (21)
Others	4 (6)
Stage	
II	1 (1)
III	9 (13)
IV	57 (85)
Previous treatment	
0	35 (52)
1	16 (24)
2	5 (7)
3	3 (4)
4	2 (3)
5	1 (1)
Performance status	
0	14 (21)
1	41 (61)
2	9 (13)
3	3 (4)

PD-1-expressing tumor cells among the samples were as follows: < 1% in 7 samples, 1% to 49% in 11 samples, and ≥ 50% in 9 samples. PD-L1 positivity was also evaluated according to the H-score on a scale of 0 to 300. No positive correlation was observed between PD-L1 expression in tumor tissues and CTCs based on either the PS (R^2 = 0.0034) (Figure 3B) or the H-score (R^2 = 0.0081) (Figure 3C).

Although no correlation was observed between tumor tissues and CTCs regarding PD-L1 expression, some intriguing cases were identified. Three adenocarcinoma cases with PD-L1-positive tumor tissues did not harbor any PD-L1-expressing CTCs. Conversely, 3 other adenocarcinoma cases with PD-L1-negative tumor tissues harbored PD-L1-expressing CTCs, demonstrating inter-patient heterogeneity regarding the concordance between PD-L1 expression in tumor tissues and CTCs. Representative cases are shown in Figures 4A and 4B, respectively. It is also noteworthy that the patients with small-cell lung cancer had perfect concordance in terms of PD-L1 positivity between PD-L1 expression in tumor tissues and CTCs, suggesting that the rate of such concordance may differ among lung cancer tumor types (see Supplemental Table 1 in the online version). However, the sample size for each tumor type was small, and this needs to be further evaluated.

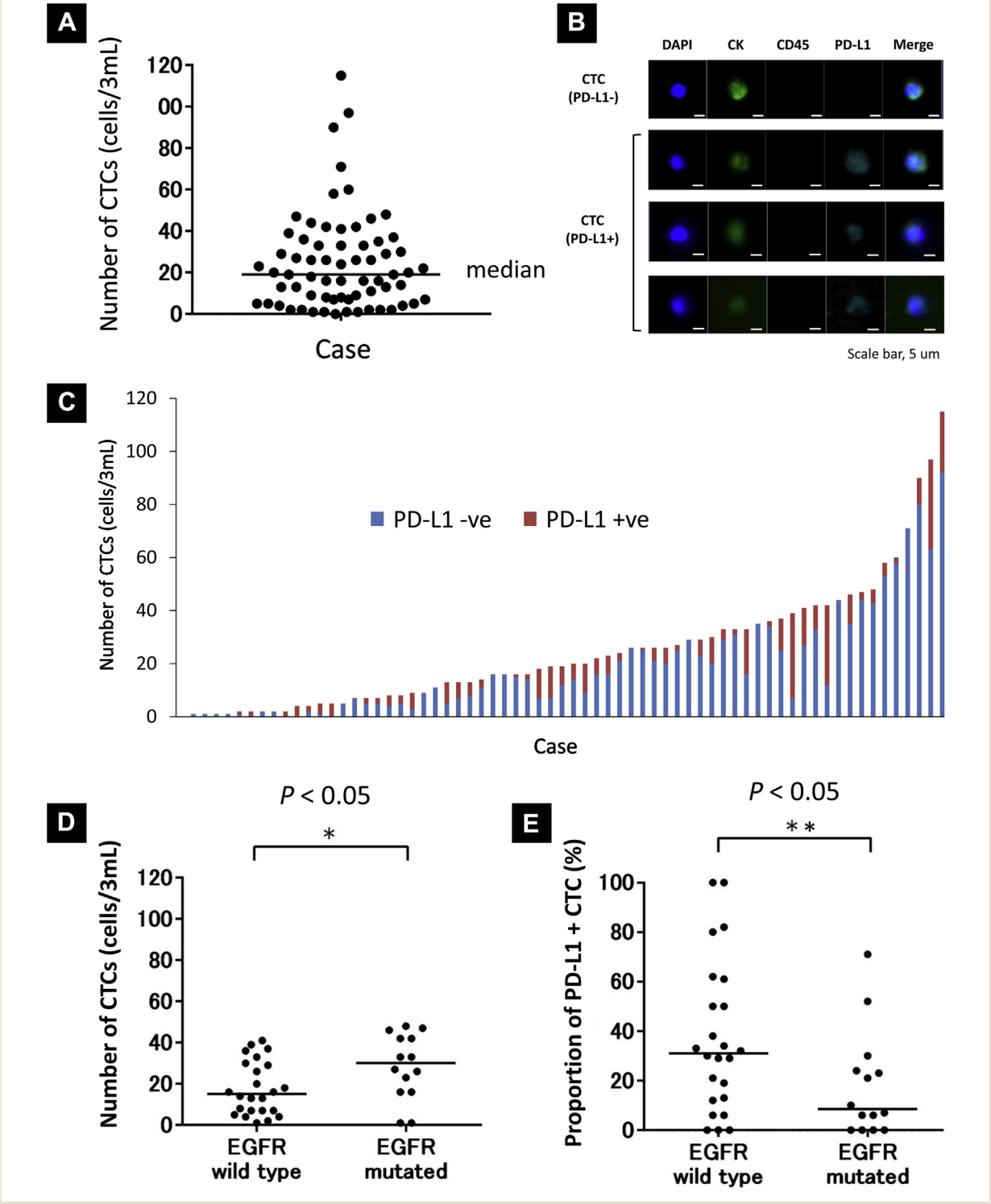
Discussion

In this study, we established a method for staining PD-L1 on CTCs. Using this approach, we detected the expression of PD-L1 on CTCs and evaluated the correlation with its expression in tumor tissues among patients with lung cancer. The expression of PD-L1 in tumor tissues represents a crucial predictive biomarker for anti-PD-1/PD-L1 therapy in patients with lung cancer. Patients with a higher expression of PD-L1 in their tumor tissues are more likely to benefit clinically from anti-PD-1/PD-L1 therapy.[5] In the clinical setting, a PD-L1 expression rate of 50% or more in tumor tissues is required to initiate first-line pembrolizumab treatment for NSCLC.[13] Even for second-line or later anti-PD-1/PD-L1 therapy, patients with PD-L1 positivity are expected to benefit more than those without PD-L1 positivity.[14]

However, major concerns remain about the reliability of PD-L1 immunohistochemistry using tumor biopsy specimens owing to tumor heterogeneity and the timing of tissue acquisition. A single small tumor biopsy may not represent the characteristics of the whole tumor. Furthermore, a specimen obtained at diagnosis may not reflect the molecular and/or biological features of the tumor at later phases, especially after systemic treatments with chemotherapeutic agents. Lung cancer is one of the most difficult types of cancer in which to perform a tumor biopsy, and performing a molecular diagnosis with tumor re-biopsy remains challenging.[15] The era of precision medicine has propelled the concept of the liquid biopsy into the spotlight, and mutation detection using circulating tumor DNA instead of tumor tissue DNA has been approved as a companion diagnostic method for EGFR tyrosine kinase inhibitors.[16] This represents a giant leap in clinical practice from tissue-based diagnosis to blood-based diagnosis. CTCs can be also utilized for this type of diagnosis, especially for cancers that cannot be diagnosed based on circulating tumor DNA.

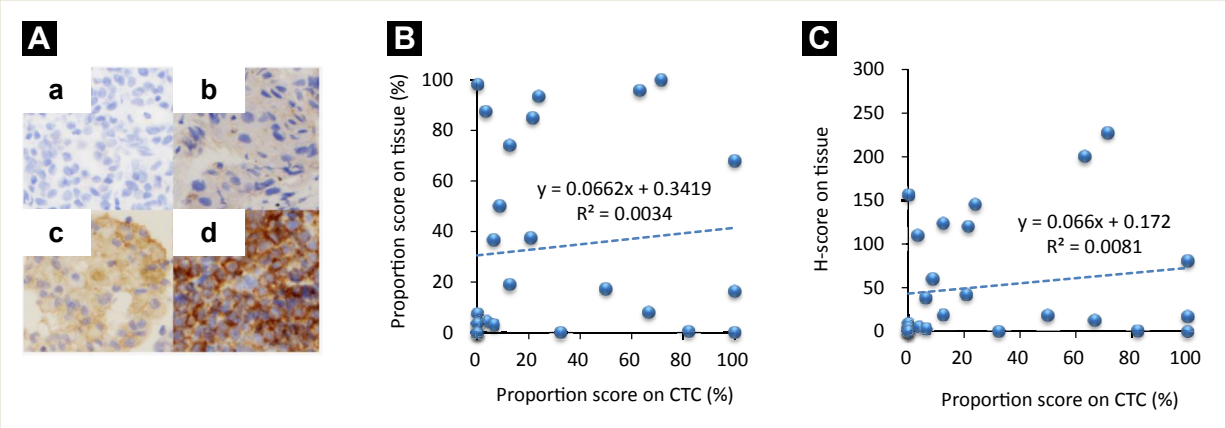
In this study, we accessed the potential of CTCs as a surrogate specimen for tumor tissue that can be used for PD-L1 evaluation. We have previously reported that the CELLSEARCH system may not be optimized for CTC detection in patients with NSCLC, and we employed an automated MCA system in the present work. We successfully detected PD-L1-positive CTCs in the patients and found that PD-L1 positivity varied among CTCs from each patient. It is no surprise that intra-patient heterogeneity of PD-L1 expression on CTCs was observed in this study, considering that heterogeneous PD-L1 expression was found even in our preclinical study using monoclonal cancer cell lines (Figure 1C). CTCs that migrate from the primary tumor and enter the blood circulation may have different biological properties than those that remain at the primary site. Therefore, the significance of PD-L1-expressing tumor cells may differ between the tumor tissue and CTCs, and the clinical significance of PD-L1-positive CTCs remains to be elucidated.

Figure 2 CTC Enumeration and PD-L1 Detection in Patient Samples. CTC Counts in 67 patients With Lung Cancer (Median 19; Range, 0 −115) (A); Representative Pictures of PD-L1-positive and -negative CTCs Observed in Patient Samples (B); Proportions of PD-L1-positive and -negative CTCs in Each Patient Sample (C); More CTCs Were Detected in EGFR-mutated Adenocarcinoma (n = 24) Than EGFR-mutated Ones (n = 14) (Median, 15 and 30; $P = .039$) (D); Significantly More PD-L1-expressing CTCs Were Detected in Patients Without EGFR Mutations Than Those With EGFR Mutations ($P = .0433$) (E)



Abbreviations: CTCs = circulating tumor cells; PD-L1 = programmed death receptor ligand 1.

Figure 3 Comparison of PD-L1 Positivity Between Tumor Tissues and CTCs. Representative Staining Results From PD-L1 Immunohistochemistry (A). Comparison of PD-L1 Positivity Between Tumor Tissues and CTCs Based on the Tumor Proportion Score (B) and H-score (C)



Abbreviations: CTCs = circulating tumor cells; PD-L1 = programmed death receptor ligand 1.

There are previous reports that addressed PD-L1 detection on CTCs in patients with lung cancer. Using the CELLSEARCH system and ISET filtration platform, it has been reported that PD-L1-positive CTCs were detected in 8% to 95% of CTCs.[17-20] The differences in the PD-L1 positivity rate may result from several reasons, such as different platforms for CTC detection, different antibodies for PD-L1 staining, and different patient populations at the different time points. Ilie et al reported that concordance rate for PD-L1 positivity between tissue and CTCs was 93%,[18] which is different from our findings. They found that very few CTCs were PD-L1 positive (8%), and the majority of cases were negative in both CTCs and tissues, resulting in higher concordance. This may be affected by the difference in anti-PD-L1 antibodies used for the 2 studies. Clones 28-8 used in our study and SP142 used in theirs are known to show considerably different staining intensities.[21] Another report using the ISET filtration system showed a different PD-L1 positivity rate (53%), and this may also result from the usage of a different antibody (clone EH 12.2H7).[20]

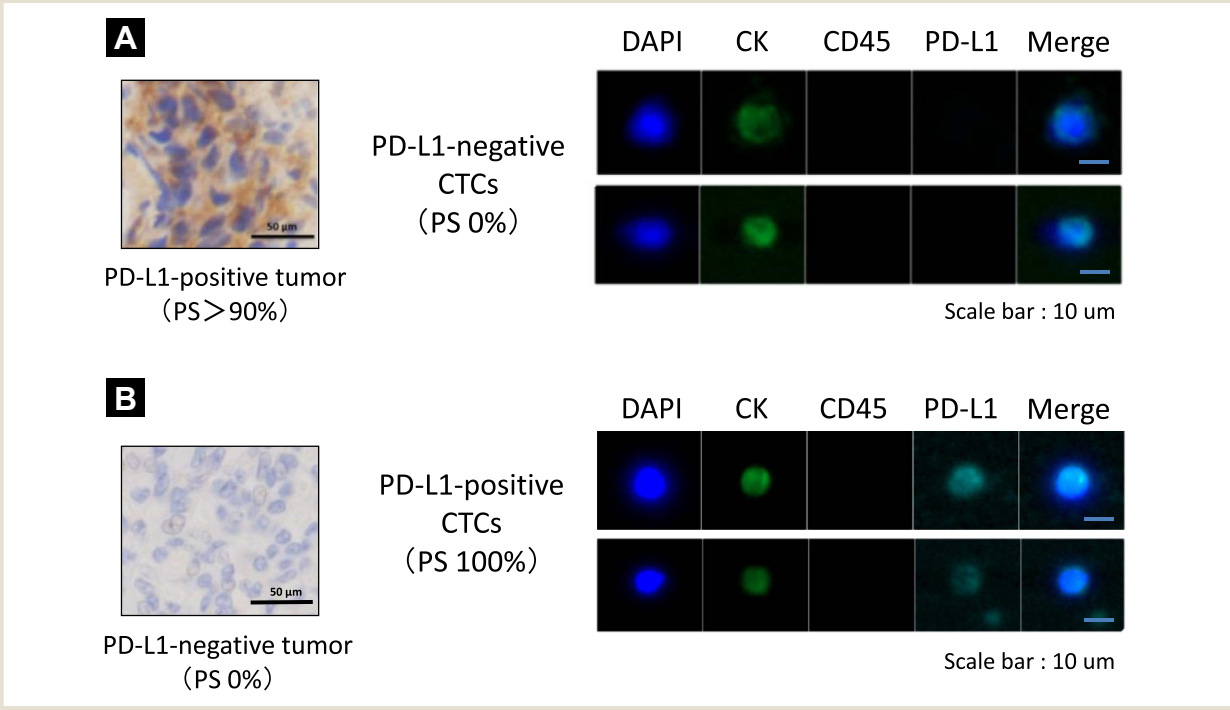
A few studies have been conducted to evaluate the predictive significance of PD-L1-positive CTCs for the treatment with immune checkpoint inhibitors in patients with lung cancer. Nicolazzo et al assessed the predictive significance of PD-L1-positive CTCs for treatment with nivolumab and reported that baseline PD-L1-positive CTCs at least did not correlate with the clinical benefit, although they reported the significance of longitudinal monitoring.[17] Guibert et al also reported that the presence of pretreatment PD-L1-positive CTCs was not significantly correlated with outcomes.[19] We are currently performing a clinical study to determine whether PD-L1 detection on CTCs can be used as a predictive biomarker for the efficacy of immune checkpoint inhibitors along with the collection of tumor tissues at the same time as the blood draw to enable direct comparison between tumor tissues and CTCs (UMIN000024414).

We did not observe any reliable concordance between PD-L1 expression in CTCs and tumor tissues in this study. This may be caused by the timing of sample acquisition. Most of the tumor tissues were obtained at the point of diagnosis, and they may not reflect the state of tumor tissues at the time of the blood draw for CTC evaluation. Interestingly, a few cases showed total discrepancies between PD-L1 expression in tumor tissue and CTCs. It is well known that a subset of patients without PD-L1 expression in their tumor tissue benefit from PD-1 blockade, and the analysis of PD-L1 expression on CTCs could be a potential tool to identify those who are likely to benefit. There are ongoing studies to address a similar clinical topic in the field of breast cancer research. It has been reported that a subset of patients with breast cancer without human epidermal growth factor receptor 2 (HER2) expression in their tumor tissue harbor HER2-positive CTCs.[22] The DETCT III clinical trial is currently underway to investigate whether those patients benefit from the addition of anti-HER2 therapy alongside conventional chemotherapy (NCT0161911).

We detected significantly more PD-L1-positive CTCs in patients with adenocarcinoma without *EGFR* mutations than those with *EGFR* mutations. Azuma et al previously reported that a high expression of PD-L1 was associated with the presence of *EGFR* mutations in surgically resected NSCLC tissue.[23] On the other hand, Takada et al reported that PD-L1 expression was significantly associated with wild-type *EGFR* in surgically resected lung adenocarcinomas.[24] Considering the biological context, it is plausible that the PD-L1 statuses of surgically resected tissue and CTCs may differ, and the details of crosstalk between the EGFR and PD-L1 signaling pathways in CTCs remain elusive, and further investigation is warranted.

This study has several limitations. The sample size is limited, and it is difficult to draw conclusions regarding the clinical utility of detecting PD-L1 expression on CTCs. Tumor tissues were obtained from only one-half of the enrolled patients, and the timing of sample acquisition varied. We will address those issues in the currently ongoing study mentioned above.

Figure 4 Discrepancy Between Tumor Tissues and CTCs. Representative Cases of Patients With PD-L1-positive Tumor Tissue and PD-L1-negative CTCs (A) and Patients With PD-L1-negative Tumor Tissue and PD-L1-positive CTCs (B)



Abbreviations: CTCs = circulating tumor cells; PD-L1 = programmed death receptor ligand 1.

Conclusions

We established a method for staining PD-L1 on CTCs and compared its expression between CTCs and tumor tissues. This is the first report to reveal the heterogeneous expression of PD-L1 among CTCs and directly compare its expression between tumor tissues and CTCs in patients with lung cancer. This approach should be facilitated to elucidate the biology of PD-L1-expressing CTCs and promote the development of anti-PD-1/PD-L1 personalized medicine in lung cancer.

Clinical Practice Points

- Blockade of the PD-1 pathway is effective against solid tumors including lung cancer. PD-L1 expression on tumor tissue serves as a predictive biomarker for the efficacy of PD-1 pathway blockade. PD-L1 expression status on CTCs and its correlation with that on tumor tissues in patients with lung cancer remain elusive.
- PD-L1 expression was detectable on CTCs, and the proportion of PD-L1-positive CTCs ranged from 3% to 100%, suggesting intra-patient heterogeneity. Direct comparison of PD-L1 expression was performed between tumor tissues and CTCs in patients with lung cancer. However, no correlation on PD-L1 expression was observed between tumor tissues and CTCs.
- CTCs may have a potential to be utilized for treatment decisions to predict the clinical benefit from anti-PD-1 therapy in lung cancer. The biology of PD-L1-expressing CTCs should be further investigated to promote the development of anti-PD-1/PD-L1 personalized medicine and clarify its clinical significance in lung cancer.

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Disclosure

Y. Koh received research funding from Daiichi Sankyo (TaNeDS). S. Yagi, M. Higuchi, and H. Kanbara are employees of Hitachi Chemical Co, Ltd. The remaining authors have stated that they have no conflicts of interest.

Supplemental Data

Supplemental figure and table accompanying this article can be found in the online version at https://doi.org/10.1016/j.cllc.2019.03.004.

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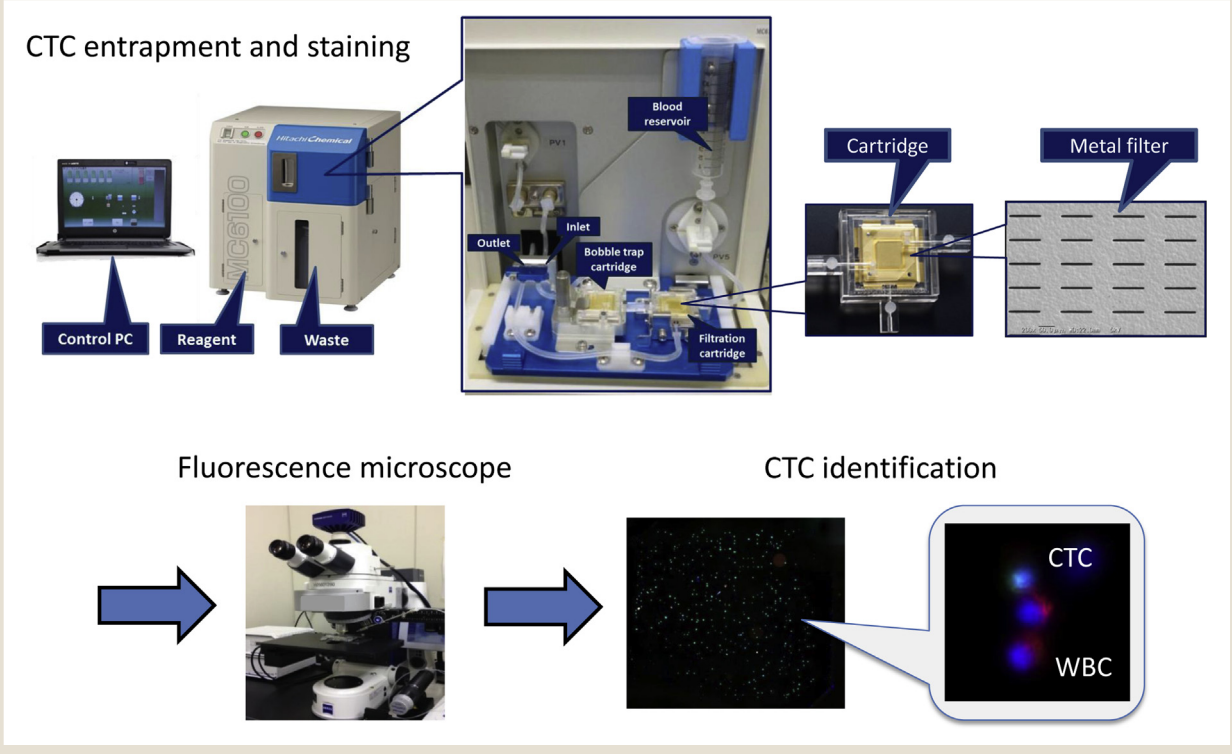
Supplemental Data

Supplemental Table 1 Comparison of PD-L1 Positivity Between Tumor Tissues and CTCs in Patients With Small-cell Lung Cancer (N = 5)

Case	PD-L1-positive CTCs/Total CTCs, n (%)	Tissue IHC	
		TPS (%)	H-score
No. 35	6/9 (67)	8	13
No. 36	0/2 (0)	0	0
No. 40	3/24 (13)	19	19
No. 52	1/26 (4)	4	5
No. 62	6/29 (21)	38	42

Abbreviations: CTCs = circulating tumor cells; IHC = immunohistochemistry; PD-L1 = programmed death receptor-ligand 1; TPS = tumor proportion score.

Supplemental Figure 1 Automated Microcavity Array System



Abbreviations: CTC = circulating tumor cell; WBC = white blood cell.