ORIGINAL ARTICLE

Detection of PD-L1 in circulating tumor cells and white blood cells from patients with advanced non-small-cell lung cancer

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Background: Expression of PD-L1 in tumor cells and tumor-infiltrating immune cells has been associated with improved efficacy to anti-PD-1/PD-L1 inhibitors in patients with advanced-stage non-small-cell lung cancer (NSCLC) and emerged as a potential biomarker for the selection of patients to cancer immunotherapies. We investigated the utility of circulating tumor cells (CTCs) and circulating white blood cells (WBCs) as a noninvasive method to evaluate PD-L1 status in advanced NSCLC patients.

Patients and methods: CTCs and circulating WBCs were enriched from peripheral blood samples (ISET® platform; Rarecells) from 106 NSCLC patients. PD-L1 expression on ISET filters and matched-tumor tissue was evaluated by automated immunostaining (SP142 antibody; Ventana), and quantified in tumor cells and WBCs.

Results: CTCs were detected in 80 (75%) patients, with levels ranging from 2 to 256 CTCs/4 ml, and median of 60 CTCs/4 ml. Among 71 evaluable samples with matched-tissue and CTCs, 6 patients (8%) showed ≥1 PD-L1-positive CTCs and 11 patients (15%) showed ≥1% PD-L1-positive tumor cells in tumor tissue with 93% concordance between tissue and CTCs (sensitivity = 55%; specificity = 100%). From 74 samples with matched-tissue and circulating WBCs, 40 patients (54%) showed ≥1% PD-L1-positive immune infiltrates in tumor tissue and 39 patients (53%) showed ≥1% PD-L1 positive in circulating WBCs, with 80% concordance between blood and tissue (sensitivity = 82%; specificity = 79%). We found a trend for worse survival in patients receiving first-line cisplatin-based chemotherapy treatments, whose tumors express PD-L1 in CTCs or immune cells (progression-free and overall survival), similar to the effects of PD-L1 expression in matched-patient tumors.

Conclusions: These results demonstrated that PD-L1 status in CTCs and circulating WBCs correlate with PD-L1 status in tumor tissue, revealing the potential of CTCs assessment as a noninvasive real-time biopsy to evaluate PD-L1 expression in patients with advanced-stage NSCLC.

Key words: PD-L1 expression, circulating tumor cells, immune cells, NSCLC, immunocytochemistry, immunotherapy

Introduction

The past decade has seen major advances in the understanding of non-small-cell lung cancer (NSCLC) molecular and cellular pathogenesis. Some NSCLC tumors are infiltrated by cytotoxic leukocytes, which, together with cancer cells, inhibit the host antitumor immune response through the expression of inhibitory immune checkpoints [1]. Among those, PD-1 and its ligand PD-L1 have been highlighted as key molecules by suppressing anticancer immunity [2]. Expression studies in NSCLC report a prevalence of 13%–70% PD-L1 positivity in tumor cells (TCs),

and 26%–62% PD-L1 positivity in tumor-infiltrating immune cells (ICs); these broad ranges depend, in part, on the method and threshold used for evaluation [3, 4].

Antibodies directed against the PD-L1/PD-1 axis of immune regulation have shown remarkable efficacy in several indications and are now approved in a number of cancer types, including advanced NSCLC [5, 6]. Current efforts are focused on trials of single agents and combinations in various diseases and settings, as well as on identifying robust predictive biomarkers of response to these agents.

In patients with previously treated NSCLC, PD-L1 expression detected by immunohistochemistry (IHC) from pretreatment tumor samples correlated with improved benefit to the PD-L1 inhibitor atezolizumab [7]. However, tissue heterogeneity, development of new metastatic sites, and dynamic changes in PD-L1 expression may impact the status of PD-L1 during disease evolution and treatment [8, 9]. Circulating tumor cells (CTCs) represent an accessible, noninvasive surrogate sample that could provide a snapshot of PD-L1 status in NSCLC patients. CTCs would be particularly useful in vulnerable NSCLC patients for whom tissue biopsies are inaccessible or extremely difficult to perform and to repeat longitudinally [10]. Selection of a CTC capture methodology should take into account the sensitivity of the isolation technology, the specificity in the diagnosis of circulating cells with malignant features, and the suitability for circulating microenvironment characterization. CTC analysis by a method that combines size-based filtration with cytopathological evaluation (ISET® technology) is currently being investigated as a predictive biomarker for targeted therapy in clinical trials of NSCLC patients (e.g., NCT02372448, NCT02827344, and NCT02554448).

Here, we investigated the utility of CTCs as surrogates for the tumor's PD-L1 status by evaluating the prevalence of PD-L1 expression in blood samples using the ISET platform, and compared PD-L1 expression in both CTCs and circulating white blood cells (WBCs) and matched tumor tissue in a cohort of 80 advanced-stage NSCLC patients.

Patients and methods

Patients and samples

Tumor tissue and matched blood samples were collected simultaneously from 106 patients with histologically confirmed advanced-stage NSCLC. Patients were treated at the Department of Pulmonary Medicine and Oncology (Pasteur Hospital, Nice, France) from January 2008 to December 2013. After CTC detection and enumeration, we selected 80 patients positive for CTCs for further analysis; 20 patients presented initially with metastatic disease, and 60 patients presented with early-stage but developed metastasis during treatment or follow-up. Blood samples were collected before surgery at the time when metastatic disease was confirmed (Supplementary Material, available at Annals of Oncology online). The main clinical and histopathological parameters of the 80 patients included in the study were previously described (supplementary Table S1, available at Annals of Oncology online) [11].

PD-L1 IHC on whole tumor tissue

PD-L1 IHC was performed on the BenchMark ULTRA instrument (Ventana Medical Systems, Tucson, AZ) [12]. Briefly, 3-μm freshly cut

formalin-fixed paraffin-embedded (FFPE) tissue sections were mounted on positively charged slides and stained with PD-L1 monoclonal antibody (clone SP142, pre-diluted; Ventana), according to the manufacturer's recommendations. The OptiView DAB immunohistochemistry Detection Kit (Ventana) was used for visualization. Positive (on-slide tonsil tissue) and negative controls (buffer, no primary antibody) were tested in each run. PD-L1 status was scored in TCs (TC0, 1, 2, 3) and tumor-infiltrating ICs (IC0, 1, 2, 3) as previously described for the POPLAR study (Supplementary Material, available at Annals of Oncology online) [7].

PD-L1 expression on ISET filters

CTC enrichment on the ISET platform (Rarecells, Paris, France) was performed according to the manufacturer's recommendations (Supplementary Material, available at Annals of Oncology online). The filters were analyzed for the presence of circulating non-hematological cells with malignant (CNHC-MF) or uncertain (CNHC-UMF) features, as previously reported (supplementary Figure S1, available at Annals of Oncology online) [13, 14]. Samples that presented ≥1 CNHC-MF and/or CNHC-UMF were further tested for PD-L1 expression by immunocytochemistry (ICC) on three unstained filter spots, as follows: after 2 min of rehydration with Reaction Buffer 10× (catalog#950-300; Ventana), filters were placed on positively charged glass slides in the BenchMark ULTRA autostainer (Ventana) and followed the PD-L1 staining protocol as for IHC.

The PD-L1 ICC analysis assessed the membranous and cytoplasmic expression of PD-L1 and scored the percentage of CTCs and WBCs expressing PD-L1 (Supplementary Material, available at Annals of Oncology online). Results from blood samples and matched-tumor tissue were blinded until study completion.

Statistical analysis

Concordance between the PD-L1 expression in tissue and matched CTCs was determined with the Bland–Altman agreement plots, by calculating sensitivity, specificity, positive (PPV) and negative predictive values (NPV), and by computation of intra-class correlation coefficients (ICCC). PD-L1 status in tumor tissue and blood samples were compared with patients' clinicopathological variables (age, gender, smoking status, and tumor size), using the χ^2 analysis or the Mann–Whitney test when applicable. Primary end points for outcome analysis were progression-free survival (PFS) and overall survival (OS). Survival plots were generated using GraphPad Prism software (San Diego, CA), and multivariate Cox regression analysis was done using JMP software.

Results

CTCs detected by blood sample filtration

Peripheral blood samples were collected from 106 patients with advanced stage III/IV lung adenocarcinoma. Ninety-nine patients (93%) were chemotherapy naive, and 7 (7%) had neoadjuvant chemotherapy. CTCs were detected in 80 of 106 (75%) patient samples. Seventy-nine of the 80 CTC-positive samples exhibited more than 5 CTCs per 4 ml blood, with a median of 60 CTCs per 4 ml (range: 2–256 CTCs/4 ml; supplementary Figure S1, available at Annals of Oncology online).

PD-L1 expression in blood samples correlated with PD-L1 status in patient-matched tumor tissue

The ICC assay to evaluate PD-L1 expression on CTCs was developed using a panel of human lung carcinoma cell lines with
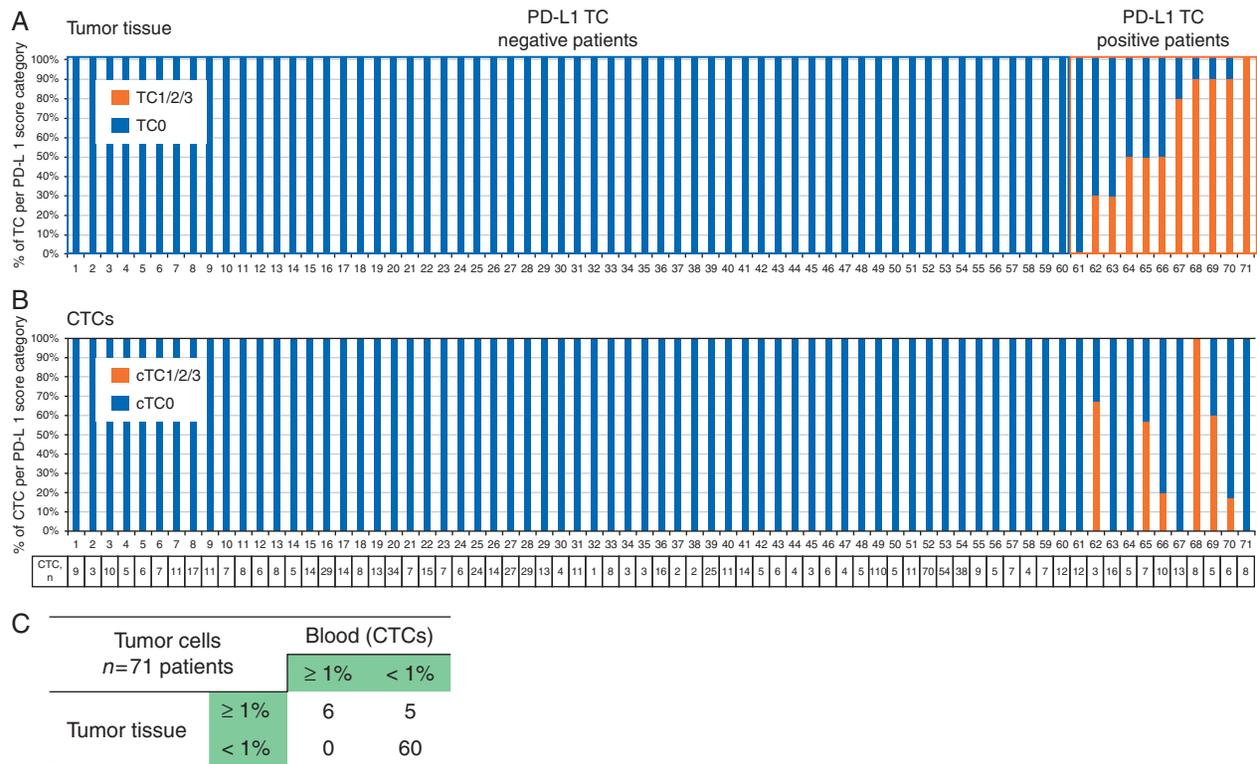
Figure 1. Distribution of PD-L1 expression in tumor cells from tissue and circulation. (A) PD-L1 expression in TCs in tissue from 71 NSCLC patients. Grey bars, percentage of TCs with no PD-L1 expression; orange bars, percentage of TCs exhibiting PD-L1 expression; classification as TC0, 1, 2, or 3 as described in Patients and methods section. (B) PD-L1 expression in CTCs from the same 71 patients analyzed in tissue. Grey, no PD-L1 expression; orange, positive PD-L1 expression; classification as cTC0, 1, 2, or 3 as described in Patients and methods section. (C) Concordance of PD-L1 expression in TCs from tumor tissue and corresponding CTCs. NSCLC, non-small-cell lung cancer; CTCs, circulating tumor cells.

known PD-L1 expression levels (supplementary Figure S2, available at *Annals of Oncology* online). Because the SP142 clone has been previously used to assess PD-L1 expression in NSCLC tumor tissue in the POPLAR and BIRCH studies [7, 15], we selected the SP142 ICC assay to evaluate PD-L1 expression in CTCs. PD-L1 staining was assessed in 71 samples that showed >1 CTC (median, 26 CTCs; range: 1–110). Nine patients with CTCs on MGG colored spots did not show evaluable CTCs on the PD-L1 stained spots. Six of the 71 (8%) patients showed more than 1% PD-L1-positive CTCs (Figure 1). PD-L1 staining in circulating WBCs was evaluable in 74 samples, with 39 samples exhibiting PD-L1 expression on >1% circulating WBCs (Figure 2).

Applying the POPLAR score on patient-matched FFPE tumor samples [7], PD-L1 expression in TCs was positive (TC1/2/3) in 11 of 71 (15%) samples (Figure 1) and PD-L1 expression in tumor-infiltrating ICs was positive (IC1/2/3) in 40 of 74 (54%) samples (Figure 2).

Comparison of PD-L1 expression in CTCs and matched tumor tissue revealed concordant PD-L1 classification in 66 of the 71 (93%) patients evaluated for the TC score (Figures 1C and 3). Concordant classification was observed in 59 of the 74 (80%) patients evaluated for the IC score, 32 of which were PD-L1 positive and 27 were PD-L1 negative in tissue and blood (Figures 2C and 3). The ICCC was between 0.543 and 0.793 (ICCC = 0.688) based on TC score, and between 0.424 and 0.723 (ICCC = 0.594) based on IC score (supplementary Figure S3, available at *Annals of Oncology* online).

Discordant PD-L1 classification from tumor was observed in five patients, all of which were PD-L1 positive in tumor tissue [one with 1% PD-L1 expression (TC1), one with 30% PD-L1 expression (TC2), and three with ≥50% PD-L1 expression (TC3)], and negative in CTCs (Figures 1C and 4; sensitivity = 55%; specificity = 100%; PPV = 97%; NPV = 81%). Discordant PD-L1 classification in ICs was observed in 15 patients: 8 patients with positive PD-L1 expression in tumor-infiltrating ICs and negative in circulating WBCs and 7 patients with positive PD-L1 expression in circulating WBCs but not in tumor-infiltrating ICs (Figures 2C and 4; sensitivity = 80%; specificity = 79%; PPV = 92%; NPV = 78%).

Correlation of CTC enumeration and PD-L1 expression with clinicopathological variables and clinical outcome

Our study confirmed a significant correlation between CTC enumeration and worse PFS [HR = 2.725 (1.607–4.621), P = 0.0002; supplementary Figure S5, available at *Annals of Oncology* online] in stage IIIB/IV NSCLC patients (Supplementary Material, available at *Annals of Oncology* online).

There was no relationship between PD-L1 expression on tumor tissue and clinicopathological variables of patients (age, P = 0.234; gender, P = 0.542; smoking status, P = 0.086; tumor size, P = 0.126).

Figure 2. Distribution of PD-L1 expression in immune cells from tissue and circulation. (A) PD-L1 expression in tumor-infiltrating ICs in tissue from 74 NSCLC patients. Grey bars, percentage of tumor-infiltrating ICs negative for PD-L1 expression (IC0); orange bars, percentage of tumor-infiltrating ICs positive for PD-L1 expression (IC1, IC2, or IC3 as described in Patients and methods section). (B) PD-L1 expression in circulating WBCs from the same patients analyzed in tissue. Grey, no PD-L1 expression; orange, positive PD-L1 expression; classification as cl0, 1, 2, or 3 as described in Patients and methods section. (C) Concordance of PD-L1 expression in ICs from tumor tissue and corresponding blood samples. NSCLC, non-small-cell lung cancer; ICs, immune cells; WBCs, white blood cells.
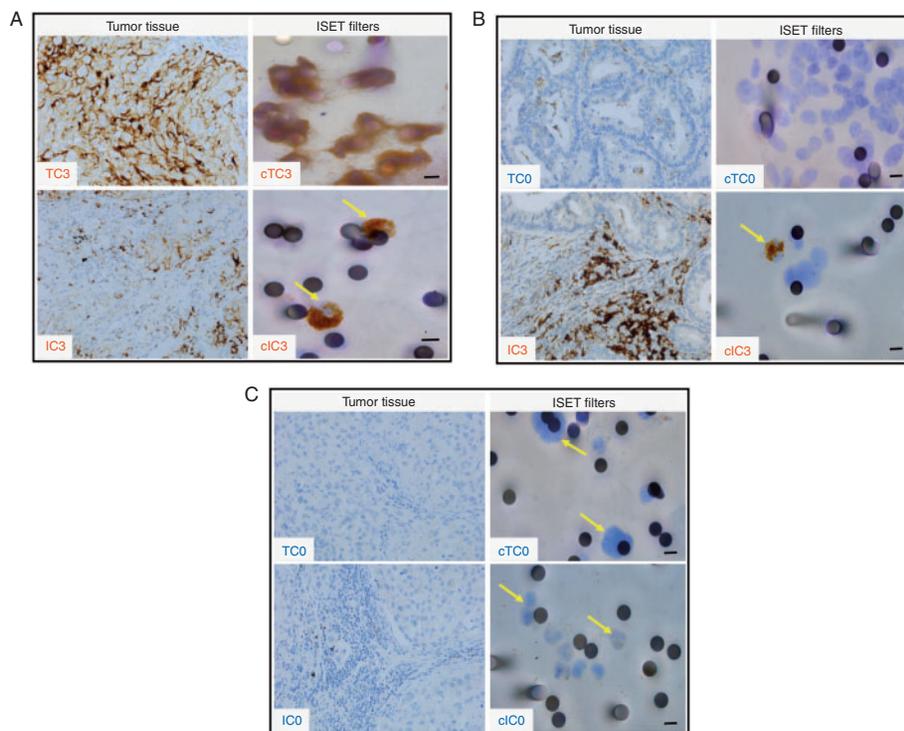

Figure 3. Concordant PD-L1 staining in tumor tissue and corresponding ISET filters from selected NSCLC patients. (A) Patient with positive PD-L1 staining in TCs (TC3) and ICs (IC3) in tumor tissue (left panel, original magnification ×400), and concordant positive PD-L1 staining in CTCs (cTC3) and circulating WBCs (cIC3) (right panel, original magnification ×1000; bar, 8 μm). (B) Patient with negative PD-L1 staining in TCs (TC0) and positive PD-L1 staining in ICs (IC0) in tumor tissue (left panel, original magnification ×400), and concordant negative PD-L1 in CTCs (cTC0) and positive PD-L1 labeling in circulating WBCs (cIC3) (right panel, original magnification ×1000; bar, 8 μm). (C) Patient with negative PD-L1 staining in tumor tissue (TC0, IC0) (left panel, original magnification ×400), and negative PD-L1 staining in both CTCs (cTC0) and circulating WBCs (cIC0) (right panel, original magnification ×1000; bar, 8 μm). NSCLC, non-small-cell lung cancer; TCs, tumor cells; ICs, immune cells; CTCs, circulating tumor cells; WBCs, white blood cells.
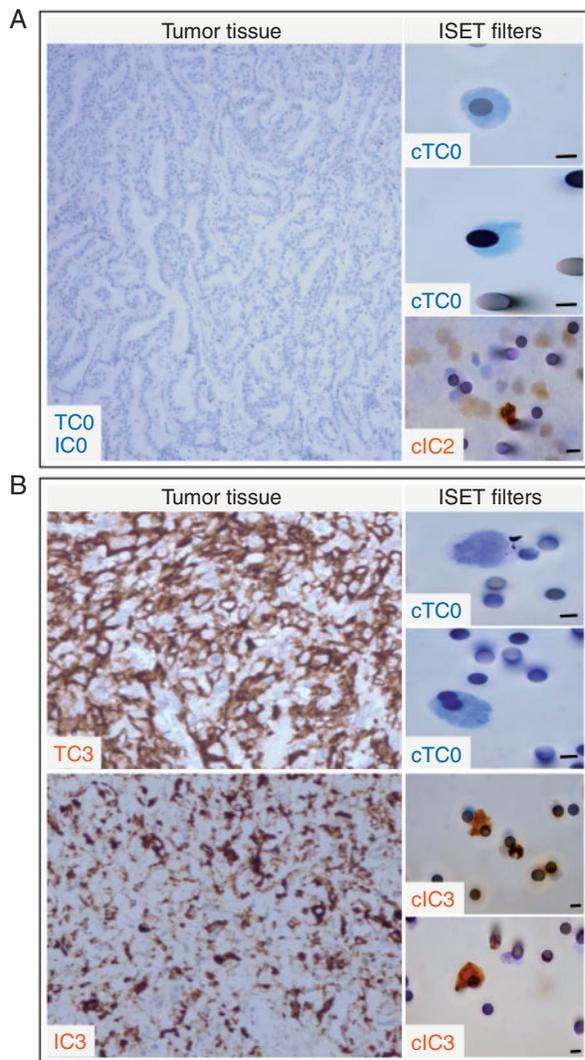
Figure 4. Discordant PD-L1 staining in tumor tissue and corresponding ISET filters from selected NSCLC patients. (A) No PD-L1 expression in tumor tissue (TC0, IC0; left panel, original magnification ×100) and corresponding ISET filters demonstrating negative PD-L1 staining in CTCs (cTC0; left-upper panel, two-picture gallery with negative stained CTCs; original magnification ×1000; bar, 8 μm), but positive PD-L1 staining in circulating WBCs (cIC2) (left-lower panel, original magnification ×1000; bar, 8 μm). (B) Strong PD-L1 expression in tumor tissue (TC3, IC3; left panel, original magnification ×400) and corresponding ISET filters demonstrating negative PD-L1 staining in CTCs (cTC0; left-upper panel, two-picture gallery with negative stained CTCs; original magnification ×1000; bar, 8 μm), but positive PD-L1 staining in circulating WBCs (cIC3) (left-lower panel, two-picture gallery with positive stained circulating WBCs, original magnification ×1000; bar, 8 μm). NSCLC, non-small-cell lung cancer; TCs, tumor cells; ICs, immune cells; CTCs, circulating tumor cells; WBCs, white blood cells.

Expression of PD-L1 on ≥1% of TCs or ICs showed a weak correlation with PFS in univariate [HR: 1.37, 95% CI (0.80–2.32)] and multivariate analysis adjusted for disease stage [HR: 1.35, 95% CI (0.79–2.28)], and a stronger, although not significant, correlation with OS [HR: 1.90, 95% CI (0.88–4.10), stage-adjusted HR: 1.91, 95% CI (0.89–4.14); Supplementary Material,

available at *Annals of Oncology* online]. A trend for longer PFS was observed in cases with PD-L1 expression in CTCs or WBCs [HR: 1.452, 95% CI (0.82–2.58), and stage-adjusted HR: 1.36, 95% CI (0.77, –2.42)], with median PFS of 18.6 months for patients with no PD-L1 expression (cTC0, cIC0) and 24.2 months for patients that expressed PD-L1 in CTCs or cICs (cTC/cIC1, 2, 3, Figure 5A). Similarly, a trend for longer OS was observed in patients expressing PD-L1 in CTCs or WBCs [HR: 1.55, 95% CI (0.7–3.470), and stage-adjusted HR: 1.42, 95% CI (0.63–3.31)], with the same median OS for cTC/cIC groups as those from patients analyzed by PD-L1 expression in tissue (Figure 5B and supplementary Figure S4, available at *Annals of Oncology* online).

Discussion

We evaluated the utility of blood samples to assess the PD-L1 status in advanced NSCLC patients. We used the ISET platform for direct enrichment of CTCs and developed a PD-L1 staining methodology using an antibody and similar scoring algorithms as previously used on tissue [7]. The ability of ISET to detect CTCs in a large proportion (75%) of NSCLC blood samples, and the prior experience of ICC assays on this platform [16], supported our choice of ISET to pursue PD-L1 assessment in CTCs. We found a good correlation between PD-L1 expression in tumor tissue and in CTCs.

Efforts have been made to investigate the performance of CTCs as surrogate to assess PD-L1 expression into the bloodstream of several tumor types, including NSCLC [17–19]. These studies were performed in small-sized populations and used indirect approaches to capture CTCs based on EpCAM/CK selection and CD45-negative depletion enrichment (e.g. CellSearch®; Cytospin), which are prone to false negative and positive results, raising concerns over their specificity and sensitivity [10, 17, 18]. PD-L1 expression in non-epithelial CTCs has been described in NSCLC [18, 20, 21]. Thus, a technology that allows accurate identification of CTCs (including CTCs undergoing epithelial mesenchymal transition, and excluding early hematopoietic and myeloid cells with low CD45 expression), and employs PD-L1 antibodies consistent with clinical trials [17–19], will likely provide a rigorous and convenient evaluation of PD-L1 in CTCs.

In our study, concordant PD-L1 expression in CTCs and tumor tissue was observed in 93% of patients, with 5 discordant patients, all of which had PD-L1-negative CTCs and PD-L1-positive tumor. In these cases, CTCs might have lost PD-L1 expression into the bloodstream. We can rule out a time-dependent effect, since blood collection to processing times were consistent in all samples analyzed. PD-L1 expression was observed in circulating WBCs from the same patients, suggesting that loss of antigenicity due to technical constrains is unlikely. Since CTCs arise from the tumor bulk, it is conceivable that, under evolutionary pressure, they might share some immune escape mechanisms inherent to TCs. While PD-L1 might represent one of the mechanisms that CTCs use to survive immune system attack, other molecules may be engaged [17, 22].

A bonus feature of the ISET PD-L1 assay is the ability to detect PD-L1 in a subpopulation of circulating WBCs (>8 μm in diameter), with 73% concordance between PD-L1 expression in tumor-infiltrating ICs and circulating WBCs. Clinical evidence to date suggests that checkpoint inhibitors act by reinvigorating
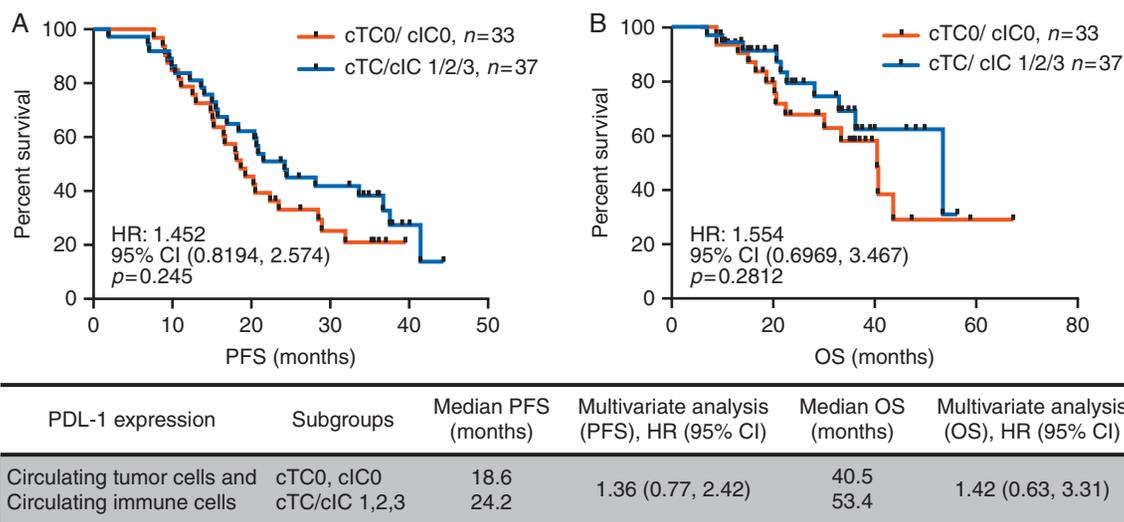
Figure 5. Correlation between PD-L1 expressions in blood samples filtered by ISET and progression-free survival (PFS) or overall survival (OS) in 80 advanced-stage NSCLC patients. (A) PFS curve according to PD-L1 expression in CTCs and circulating WBCs (cTC/IC score). (B) OS curve according to PD-L1 expression in CTCs and circulating WBCs (cTC/IC score). Table: median survival rates and multivariate HR adjusted for disease stage for PFS and OS. NSCLC, non-small-cell lung cancer; TCs, tumor cells; ICs, immune cells; CTCs, circulating tumor cells; WBCs, white blood cells.

preexisting antitumor T-cell responses and are most effective in inflamed tumors characterized by PD-L1 expression, high density of ICs, or presence of a strong IFN-γ cytolytic T-cell signature [3, 7, 23]. However, further investigations have to evaluate the clinical significance of PD-L1 expression on circulating WBCs.

Efforts have been focused on analyses in whole blood for the identification of predictive biomarkers for checkpoint inhibitors, but their clinical relevance remains unclear [24]. Serum or plasma markers have been investigated, with limited correlations between monitoring and antitumor activity [25]. The information provided on ISET is essentially different from the detection of shed PD-L1 in the blood, which gives no information on its cellular source [19]. It would be of interest to develop ISET-ICC assays in combination with multicolor flow cytometry to better characterize the immune 'liquid microenvironment'.

The prognostic value of PD-L1 in NSCLC patients has been confounded by the use of various PD-L1 primary antibodies, thresholds, patient populations with different clinico-biological parameters, and selection of samples [26, 27]. Several studies reported that PD-L1 expression may be associated with either shorter or longer survival in NSCLC patients [27–29]. In this study, PD-L1 expression in TCs and tumor-infiltrating ICs shows a similar prognostic trend as PD-L1 expression in CTCs and WBCs. However, further investigations are warranted to evaluate the clinical significance of PD-L1 expression on CTCs and/or WBCs.

Patient stratification based on PD-L1 levels on both CTCs and circulating WBCs may be an appropriate model to tailor PD-1/PD-L1 immunotherapy in advanced NSCLC patients. It is likely that the prognostic and predictive significance relates not to single markers of tumor or immune signals but to the overall balance of the host antitumor immune response and tumor-mediated immunosuppression.

Our study has several limitations. The PD-L1 status was assessed by the SP142 antibody which behaves differently from

the other clones [30]. Although we tested different antibodies on cancer cell lines, it is necessary to extend the application of CTC-based PD-L1 assay to the other PD-L1 clones in NSCLC patients. Moreover, while PD-L1 membranous staining of TCs is becoming a standard for interpretation [30], some CTCs showed cytoplasmic staining with or without membrane staining. This cellular pattern was also observed in cell lines spiked into blood and stained with the antibodies used for assay development. The significance of this pattern in CTCs requires elucidation.

In conclusion, we showed that PD-L1 status in the ISET-captured 'liquid microenvironment' correlates with PD-L1 status in tumor tissue advanced NSCLC patients. This study provides a proof-of-concept for the potential use of ISET-enriched CTCs and circulating WBCs as a surrogate for the evaluation of PD-L1 expression for immunotherapy stratification and/or real-time monitoring in patients with advanced NSCLC.

Disclosure

The authors have no conflicts of interest.

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