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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ABSTRACT

**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 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 non-invasive 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/4ml, and median of 60 CTCs/4ml. 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 circulating tumor or immune cells (PFS and OS), 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 non-invasive 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
Key message
This proof-of-concept study shows that PD-L1 status in circulating tumor cells captured with the ISET-technology correlates with PD-L1 status in tumor tissue, and suggests that ISET-enriched circulating cells could be used as a sensitive and specific diagnostic testing of PD-L1 for immunotherapy stratification and/or real-time monitoring of treatment response in patients with advanced NSCLC.
INTRODUCTION

The past decade has seen major advances in the understanding of 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% to 70% PD-L1 positivity in tumor cells (TCs), and 26% to 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 pre-treatment 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, non-invasive 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, 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). The main clinical and histopathological parameters of the 80 patients included in the study were previously described (Supplementary Table S1) [11].

PD-L1 immunohistochemistry on whole tumor tissue

PD-L1 Immunohistochemistry (IHC) was performed on the BenchMark ULTRA instrument (Ventana Medical Systems, Tucson, AZ, USA) [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) [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). 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 Fig. S1, Supplementary Material) [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 minutes of rehydration with Reaction Buffer 10x (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). 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 and negative predictive values (PPV, NPV), and by computation of intra-class correlation coefficients (ICC). PD-L1 status in tumor tissue and blood samples were compared with patients' clinicopathological variables (age, gender, smoking status, and tumor size), using the χ² 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. 99 patients (93%) were chemotherapy naïve, and 7 (7%) had neoadjuvant chemotherapy. CTCs were detected in 80 of 106 (75%) patient samples. 79 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/4ml; Supplementary Fig. S1, Supplementary Material).

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 known PD-L1 expression levels (Supplementary Fig. S2, Supplementary Material). 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 more than one CTC (median, 26 CTCs; range: 1-110). 9 patients with CTCs on MGG colored spots, did not show evaluable CTCs on the PD-L1 stained spots. 6 of the 71 (8%) patients showed more than 1% PD-L1 positive CTCs (Fig. 1). PD-L1 staining in circulating WBCs was evaluable in 74 samples, with 39 samples exhibiting PD-L1 expression on more than 1% circulating WBCs (Fig. 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 (Fig. 1), and PD-L1 expression in tumor-infiltrating ICs was positive (IC1/2/3) in 40 of 74 (54%) samples (Fig. 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 (Fig. 1C, Fig. 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 (Fig. 2C, Fig. 3). The ICC was between 0.543 and 0.793 (ICC=0.688) based on TC score, and between 0.424 and 0.723 (ICC=0.594) based on IC score (Supplementary Fig. S3).

Discordant PD-L1 classification from tumor was observed in 5 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 (Fig. 1C; Fig. 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 (Fig. 2C; Fig. 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-value=0.0002; Supplementary Fig. S5] in stages IIIb/IV NSCLC patients (Supplementary Material).

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

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). 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, Fig. 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, 3.47], 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 (Fig. 5B and Supplementary Fig. S4).

**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 tumor cells. 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 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 for 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 anti-tumor 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.

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Conflicts of Interest

The other authors have no conflict of interest to disclose.
References


Legend to Figures

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 Methods. 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 Methods. C. Concordance of PD-L1 expression in TCs from tumor tissue and corresponding CTCs.

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 Methods). 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 cI0, 1, 2 or 3 as described in Methods. C. Concordance of PD-L1 expression in ICs from tumor tissue and corresponding blood samples.

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 x400), and concordant positive PD-L1 staining in CTCs (cTC3) and circulating WBCs (cIC3) (right panel, Original magnification x1000; 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 x400), and concordant negative PD-L1 in CTCs (cTC0) and positive PD-L1 labeling in circulating WBCs (cIC3) (right panel, Original magnification x1000; bar, 8 µm). C. Patient with negative PD-L1 staining in tumor tissue (TC0, IC0) (left panel, Original magnification x400), and negative PD-L1 staining in both CTCs (cTC0) and circulating WBCs (cIC0) (right panel, Original magnification x1000; bar, 8 µm).
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 x100) and corresponding ISET filters demonstrating negative PD-L1 staining in CTCs (cTC0; left-upper panel, 2-picture gallery with negative stained CTCs; Original magnification x1000; bar, 8 µm), but positive PD-L1 staining in circulating WBCs (cIC2) (left-lower panel, Original magnification x1000; bar, 8 µm). B. Strong PD-L1 expression in tumor tissue (TC3, IC3; left panel, Original magnification x400) and corresponding ISET filters demonstrating negative PD-L1 staining in CTCs (cTC0; left-upper panel, 2-picture gallery with negative stained CTCs; Original magnification x1000; bar, 8 µm), but positive PD-L1 staining in circulating WBCs (cIC3) (left-lower panel, 2-picture gallery with positive stained circulating WBCs, Original magnification x1000; bar, 8 µm).

Figure 5. Correlation between PD-L1 expression in blood samples filtered by ISET and progression-free survival (PFS) or overall survival (OS) in 80 advanced-stages 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.
Legend to Supplementary Table and Figures

Supplementary Table S1. Clinical and pathological characteristics of 80 patients with advanced-stage NSCLC included in this study.

Supplementary Fig. S1. A. Example of an isolated CTC with malignant cytomorphological features (Original magnification x1000; bar, 10 µm). B. Circulating tumor cluster composed of more than 20 CTCs with malignant cytomorphological features (Original magnification x1000; bar, 8 µm).

Supplementary Fig. S2. Development of PD-L1 assays on blood samples. Cultured cell lines were spiked into healthy smoking donors' blood and isolated on ISET filters. PD-L1 expression was assessed on ISET filters by immunocytochemistry with various commercialized anti-PD-L1 antibodies (clone and supplier are indicated on the left). All experiments were repeated three times and the figures show representative results.

Supplementary Fig. S3. Bland-Altman plots describing the PD-L1 inter-sample relationship according to PD-L1 expression in tumor and immune cells. A. PD-L1 TC status versus PD-L1 in CTCs. B. PD-L1 IC status versus PD-L1 in immune circulating cells.

Supplementary Fig. S4. Correlation between PD-L1 expression in tumor tissue and progression-free survival (PFS) or overall survival (OS) in advanced-stage NSCLC patients. A. PFS curve according to PD-L1 expression in tumor cells and immune cells (POPLAR TC/IC score), B. OS curve according to PD-L1 expression in tumor cells and immune cells (POPLAR TC/IC score), C. PFS curve according to PD-L1 expression in tumor cells (POPLAR TC score), D. OS curve according to PD-L1 expression in tumor cells (POPLAR TC score). Table, median survival rates and multivariate HR adjusted for disease stage for PFS and OS for subgroups of PDL-1 expression in tumor tissue and in tumor tissue/infiltrating immune cells.
**Supplementary Fig. S5.** Correlation between CTC enumeration by ISET and progression-free survival (PFS) in NSCLC patients included in the study.
Figure 1

A) Tumor tissue

B) CTCs

C) Tumor cells | Blood (CTCs) | ≥ 1% | < 1%
--- | --- | --- | ---
n=71 patients | 6 | 5 | 0 | 60
Tumor tissue | 1% | 6 | 5 | 0 | 60

275x190mm (300 x 300 DPI)
Figure 2

A  Tumor tissue

B  Immune circulating cells

C  Immune cells
   n=4 patients

   Blood (WBCs)
   \n   \n   \n   \n   ≥ 1%  < 1%

   Tumor tissue  32  8
   < 1%        7  27

275x190mm (300 x 300 DPI)
275x190mm (300 x 300 DPI)
Figure 3B

275x190mm (300 x 300 DPI)
Figure 3C

275x190mm (300 x 300 DPI)
Figure 4

A

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B

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Figure 5

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275x190mm (300 x 300 DPI)
SUPPLEMENTARY MATERIAL

PATIENTS AND METHODS

Patients and samples
Among the 80 patients positive for CTCs, 34 were treated with cisplatin/pemetrexed, 22 were treated with cisplatin/docetaxel, 16 were treated with cisplatin/gemcitabine, and 3 were treated with erlotinib. None of these patients were treated with anti-PD-1/PD-L1 inhibitors.

The institutional review board and local ethics committee of the University of Nice Sophia Antipolis approved this study. Informed written consent was obtained from all patients.

PD-L1 immunohistochemistry on whole tumor tissue
PD-L1 expression was scored by IHC on tumor cells (as percentage of PD-L1-expressing tumor cells TC3≥50%, TC2≥5% and <50%, TC1≥1% and <5%, and TC0<1%) and tumor-infiltrating immune cells (as percentage of tumor area: IC3≥10%, IC2≥5% and <10%, IC1≥1% and <5%, and IC0<1%). Scoring was blindly assessed by 3 trained pathologists (MI, VH, and PH), with an overall concordance of 97% for the TC score and 85% for the IC score. When a discrepancy between the three pathologists was noted, the slides were commonly reviewed in order to obtain a consensus.

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). Briefly, 10 mL of blood were processed though the ISET filter within one hour of blood collection. The ISET filter contains 10 filter-spots, each representing the filtration of 1 ml blood. Processed filters were either stored at -20°C for up to 180 days, or immediately stained with May-Grünwald Giemsa (MGG) coloration on 4 out of the 10 filter-spots, and further analyzed for the presence of circulating
non-hematological cells with malignant (CNHC-MF), uncertain (CNHC-UMF) and benign features (CNHC-BF), as previously reported (Supplementary Fig. S1 [1, 2]).

A representative example of a CNHC-MF, characterized by a large size (diameter larger than 20 µm), large and irregular nucleus and presence of nucleoli is shown in Supplementary Fig. S1. CTC enumeration was computed as the number of CNHC-MF and/or CNHC-UMF identified in the 4 ml blood-collection. Clusters of CTCs containing two or more tightly juxtaposed CNHC-MF/UMF were defined as circulating tumor microemboli (CTM).

The PD-L1 ICC analysis assessed the membranous and cytoplasmic expression of PD-L1 in CTCs identified by the same morphological criteria used for the MGG analysis: size of the nucleus, anisonucleosis, nuclear-cytoplasmic ratio, presence of clusters/CTMs, as well as on the circulating white blood cells (WBCs), such as lymphocytes, neutrophils, monocytes, and macrophages.

The PD-L1 ICC analysis assessed the membranous and cytoplasmic expression of PD-L1 in CTCs identified by the same morphological criteria used for the MGG analysis (Supplementary Material). PD-L1 status in CTCs was scored as a percentage of CTCs that show expression of PD-L1 (cTC3, ≥50%, cTC2, ≥5% and <50%, cTC1, ≥1% and <5%, and cTC0, <1% CTCs expressing PD-L1), and PD-L1 status in circulating WBC was scored as a percentage of WBCs deposited on the ISET filter that express PD-L1 (cIC3, ≥10%, cIC2, ≥5% and <10%, cIC1, ≥1% and <5%, and cIC0, <1% WBC expressing PDL-1). Results from blood samples and matched-tumor tissue were blinded until study completion.

SUPPLEMENTARY RESULTS

CTCs detected by blood sample filtration

Circulating tumor microemboli (CTMs) were observed in 75 of the 80 CTC-positive patients (94%), with a number of CTMs ranging from 1 to 23 per 4 ml sample, and a median of 5 CTMs per 4 ml blood sample (Supplementary Fig. S1).
PD-L1 expression in blood samples correlated with PD-L1 status in patient-matched tumor tissue

The immunocytochemistry (ICC) assay to evaluate PD-L1 expression on CTCs was developed using a panel of human lung carcinoma cell lines with known PD-L1 expression levels (ATCC; high PD-L1 expressing H820 cell line; and weak to negative PD-L1 expression cell lines A549, H1299, and H23). Cells spiked into healthy smoking donors’ blood were captured on ISET filters and stained for PD-L1 with various commercial antibodies. Staining conditions were optimized to result in concordant PD-L1 expression for cell-blocks (IHC) and the corresponding blood spike-ins (ICC). The Ventana’s (SP142 and SP263) and Dako’s (concentrated 22C3 and 28-8) antibodies showed similar cytoplasmic and membrane-localized staining in H820 cells, with no staining in the weak to negative cell lines, while the E1L3N clone showed non-specific staining (Supplementary Fig. S2). PD-L1 was not expressed on the circulating WBCs from the healthy smoking donors (data not shown).

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

In NSCLC patients evaluated for CTCs on ISET, a prognostic value of CTC counts was reported using a cutoff of 50 CTCs [2]. Our study confirmed a significant correlation between CTC enumeration and worse PFS [HR= 2.725 (1.607–4.621), p-value=0.0002; Supplementary Fig. S5] in stages IIIB/IV NSCLC patients. There was no relationship between PD-L1 expression on tumor tissue and clinicopathological variables of patients (age, p-value=0.234; gender, p-value=0.542; smoking status, p-value=0.086; tumor size, p-value=0.126).

At a median follow up of 29 months, 58 patients had progressed and 27 patients had died.

Expression of PD-L1 on ≥1% of TCs or ICs (TC1/2/3 or IC1/2/3) 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]). Median survival times were 18.1 and 22.4 months (PFS), and 40.5 and 53.4 months (OS) for TC0 and IC0 vs. TC1/2/3 or IC1/2/3.
IC1/2/3 sub-groups, respectively (Supplementary Fig. S4). 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 tumor cells or immune cells in circulation (cTC/cIC1,2,3, Fig. 5A). Similarly, a trend for longer OS was observed in patients expressing PD-L1 in circulating cells (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 (Fig. 5B and Supplementary Fig. S4).

References


### Supplementary Table S1

<table>
<thead>
<tr>
<th>Patients demographics (n = 80)</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>65 (41–86)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>54 (68%)</td>
</tr>
<tr>
<td>Female</td>
<td>26 (32%)</td>
</tr>
<tr>
<td><strong>Tobacco Use History</strong></td>
<td></td>
</tr>
<tr>
<td>Former or current smokers</td>
<td>67 (84%)</td>
</tr>
<tr>
<td>Never smoked</td>
<td>13 (16%)</td>
</tr>
<tr>
<td><strong>Histological cell type</strong></td>
<td></td>
</tr>
<tr>
<td>Invasive adenocarcinoma</td>
<td>63 (79%)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>9 (11%)</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>3 (4%)</td>
</tr>
<tr>
<td>Sarcomatoid carcinoma</td>
<td>2 (2%)</td>
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<tr>
<td>Adenosquamous carcinoma</td>
<td>3 (4%)</td>
</tr>
<tr>
<td><strong>Tumor site sampling</strong></td>
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<tr>
<td>Primary</td>
<td>60 (75%)</td>
</tr>
<tr>
<td>Metastatic</td>
<td>20 (25%)</td>
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<tr>
<td><strong>Median tumor size (range) cm</strong></td>
<td>2.5 (1–16)</td>
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<tr>
<td><strong>pTNM stage</strong></td>
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<tr>
<td>IIA</td>
<td>33 (41%)</td>
</tr>
<tr>
<td>IIIB</td>
<td>14 (18%)</td>
</tr>
<tr>
<td>IV</td>
<td>33 (41%)</td>
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<tr>
<td><strong>Differentiation grade</strong></td>
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<tr>
<td>Well</td>
<td>17 (21%)</td>
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<tr>
<td>Moderate</td>
<td>19 (11%)</td>
</tr>
<tr>
<td>Poor</td>
<td>54 (68%)</td>
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</tbody>
</table>

Abbreviations: TNM = tumor node metastasis.
Supplementary Figure S1

190x275mm (300 x 300 DPI)
Supplementary Figure S2

<table>
<thead>
<tr>
<th>Antibody</th>
<th>H23</th>
<th>H1299</th>
<th>A549</th>
<th>H820</th>
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<tbody>
<tr>
<td>SP142 (Ventana)</td>
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<td>SP263 (Ventana)</td>
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<td>22C3 (Dako)</td>
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<td>28-8 (Dako)</td>
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<td>E11.3N (Cell Signaling)</td>
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275x190mm (300 x 300 DPI)
## Supplementary Figure S4

<table>
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<tr>
<th>POL-1 expression</th>
<th>Subgroups</th>
<th>Median PFS (months)</th>
<th>Multivariate analysis (PFS), HR (95% CI)</th>
<th>Median OS (months)</th>
<th>Multivariate analysis (OS), HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor Tissue and Infiltrating Immune Cells</td>
<td>TCO, IC0</td>
<td>18.1</td>
<td>1.35 (0.79, 2.28)</td>
<td>40.5</td>
<td>1.91 (0.89, 4.14)</td>
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<tr>
<td>TCO, TC1, TC2, TC3</td>
<td>22.4</td>
<td></td>
<td></td>
<td>53.4</td>
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<td>TCO, IC0, n=33</td>
<td>IC/TC1, 2, 3, n=47</td>
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<tr>
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<td>IC/TC1, 2, 3, n=47</td>
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<tr>
<td>TCO, n=64</td>
<td>TC1, 2, 3, n=16</td>
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<tr>
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<td>TC1, 2, 3, n=16</td>
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<table>
<thead>
<tr>
<th>Percent survival</th>
<th>PFS (months)</th>
<th>OS (months)</th>
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<tr>
<td>HR: 1.38</td>
<td>95% CI (0.53, 4.72)</td>
<td>p=0.0020</td>
</tr>
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<td>HR: 1.38</td>
<td>95% CI (0.53, 4.72)</td>
<td>p=0.0020</td>
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</table>

275x190mm (300 x 300 DPI)
Supplementary Figure S5

![Supplementary Figure S5](image)

254x190mm (300 x 300 DPI)