Lung Cancer 120 (2018) 108–112

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Short communication

PD-L1 expression in circulating tumor cells of advanced non-small cell lung cancer patients treated with nivolumab☆

Nicolas Guibert[a,b,d,*], Myriam Delaunay[a,b,d], Amélie Lusque[e], Nadia Boubekeur[b,c], Isabelle Rouquette[f], Estelle Clermont[f], Jean Mourlanette[a], Sandrine Gouin[a], Inge Dormoy[a], Gilles Favre[b,c,d], Julien Mazieres[a,b,d], Anne Pradines[b,c]

[a] Thoracic Oncology Department, Larrey Hospital, University Hospital of Toulouse, France
[b] Inserm, Centre de Recherche en Cancérologie de Toulouse, CRCT UMR-1037, Toulouse, France
[c] Laboratoire de Biologie Médicale Oncologique, Institut Universitaire du Cancer de Toulouse, France
[d] University of Toulouse III (Paul Sabatier), Toulouse, France
[e] Cellule Biostatistique, Bureau des Essais Cliniques, Institut Claudius Regaud, IUCT-Oncopole, Toulouse, France
[f] Pathology Department, IUCT-Oncopole, Toulouse, France

ARTICLE INFO

Keywords:
Non-small-cell lung cancer
PD-L1 expression
Circulating tumor cells
Monitoring of response
Immunotherapy
Anti-PD1

ABSTRACT

Background: Inhibitors of the PD-1/PD-L1 immune checkpoint have become a standard of care in non-small cell lung cancer (NSCLC). Patient selection, currently based on PD-L1 expression on tumor tissue, is limited by its temporal and spatial heterogeneity. We hypothesized that liquid biopsy with PD-L1 analysis on circulating tumor cells (CTCs) might overcome this limitation.

Methods: Blood samples were prospectively collected from patients with advanced NSCLC before nivolumab treatment and at the time of progression. CTCs were isolated using a cell size-based technology. PD-L1 expression was assessed by immunofluorescence on CTCs and immunohistochemistry on tissue biopsies.

Results: 113 specimens from 96 patients were collected. Baseline PD-L1 expression could be assessed on 72% and 93% of tissue and CTC, respectively. CTCs were more frequently found to be PD-L1 positive than tissue (83% vs. 41%) and no correlation was observed between tissue and CTC PD-L1 expression ($r = 0.04$, $p = 0.77$). Pre-treatment high CTC number was associated with increased risk of death and progression (HR1.06, $p = 0.03$ for OS; HR1.05, $p = 0.02$ for PFS). The presence of pre-treatment PD-L1$^+$CTC was not significantly correlated with outcomes but a higher baseline PD-L1$^+$ CTC number ($\geq 1\%$) was observed in the "non-responders" group (PFS < 6 months) ($p = 0.04$) and PD-L1$^+$CTC were seen in all patients at progression.

Conclusion: Assessment of PD-L1 expression in CTCs is feasible and CTCs are more often positive than in tissue. Pre-treatment PD-L1$^+$CTCs are associated with bad prognosis in patients treated with PD-1 inhibitors.

1. Introduction

Immunotherapy is proving to be an effective approach in NSCLC. US and EU regulatory agencies have approved antibodies targeting the PD-L1/PD-1 axis in second and, more recently, front-line settings [1]. However, only a subset of patients exhibits durable responses, underlying the need for biomarkers.

PD-L1 tumor expression is, along with tumor mutational burden [2], the most established predictive biomarker of response to these drugs [1,3]. However, nearly 10% of patients whose tumor does not express PD-L1 will eventually benefit from immune checkpoint inhibitors (ICI), while a large number of tumors that express PD-L1 do not respond [3]. PD-L1 expression is usually assessed on archived tissue, which could explain part of these discrepancies since PD-L1 is a dynamic biomarker that can be induced by targeted therapy, chemotherapy or radiation therapy [4–6]. Re-biopsy would expose patients to the risk of complications and delayed results. Moreover, these small samples are sometimes inadequate for PD-L1 analysis due to tumor heterogeneity [7]. While tissue only offers a snapshot of PD-L1 expression at a given time and location, liquid biopsies have the ability to dynamically and non-invasively interrogate the whole molecular landscape of tumors. More specifically, circulating tumor cells (CTCs) might represent a substrate for analysis of PD-L1 expression. This approach has been reported in NSCLC [9–11], but the concordance with tissue and the correlation

☆ Portions of these data have been presented as a poster at the WCLC Annual Meeting 2017.

* Corresponding author at: Thoracic Oncology Unit, Respiratory Disease Department, Hôpital Larrey, CHU Toulouse, Chemin d Pouvourville, 31059, Toulouse Cedex, France.
E-mail address: guibert.n@chu-toulouse.fr (N. Guibert).

https://doi.org/10.1016/j.lungcan.2018.04.001
Received 16 February 2018; Received in revised form 28 March 2018; Accepted 1 April 2018

with response to PD-1 inhibitors need further investigation.

2. Material and methods

2.1. Patients and samples

All consecutive patients with advanced metastatic NSCLC who relapsed after chemotherapy and planned to receive nivolumab were included. All patients gave their informed consent to participate in this study (NCT02827344).

2.2. Tissue analysis

PD-L1 expression on tissue was assessed by immunohistochemistry (IHC) using an anti-PD-L1 rabbit monoclonal antibody (clone E1L3N, Cell signalling Technology) blinded to CTCs analysis.

2.3. CTCs analysis

Median time between tissue biopsy and pre-treatment blood collection was 7.8 months (n = 69). Blood specimen were obtained less than one month after tissue biopsy in 3 patients (5.7%); 1–12 months after in 37 patients (69.8%) and more than one year after in 13 patients (24.5%). Blood samples (10 ml) were collected and CTC were captured by ISET technology within the next 4 h. Five spots (1 ml of blood per spot) for each patient were subjected to IF staining for PD-L1 with a rabbit mAb (clone D8T4X, Cell signalling Technology) and CD45 (clone MEM-28, Abcam); blinded to tissue analysis. CTCs were defined as DAPI+/CD45- cells with cytomorphometric malignant features (size, shape, mononuclear, nucleus to cytoplasm ratio) [8] (Fig. 1**A**). H827 and H23 cell lines were selected as positive and negative controls, respectively, among several lung cancer cell lines, based upon their PD-L1 expression analysed by western blot (Sup. Fig. 1A). The specificity of the D8T4X rabbit mAb was evaluated on H23 and H827 cells spiked into healthy volunteer blood and enriched on ISET filters (Sup. Fig. 1B).

2.4. Statistical analysis

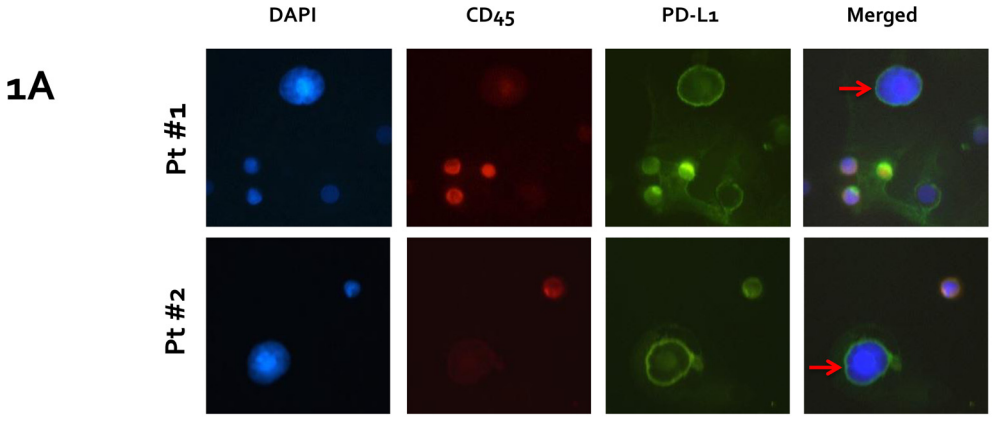
The data were summarized by frequency and percentage for qualitative variables and by median and range for continuous variables. Comparisons were assessed using chi-squared test and Fisher exact test for qualitative variables and Kruskal-Wallis test for continuous variables. Correlation was calculated using Spearman coefficient. All survival times were calculated from the initiation of immunotherapy and estimated by the Kaplan-Meier method with 95% confidence intervals (95%CI). Univariate analysis was performed using the logrank test for qualitative variables and Cox proportional hazards model for continuous variables.

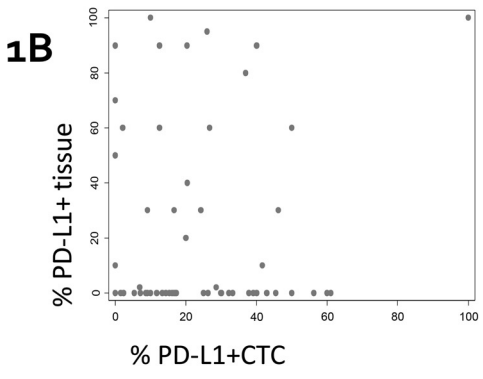
Tests were two-sided and p-values < 0.05 were considered significant. Statistical analyses were conducted using Stata®, version 13.

3. Results

3.1. Patient cohort characteristics

The clinical and pathological characteristics of the patient population are detailed in Sup. Table 1. Blood specimens were collected pre-treatment (n = 96, all treated with nivolumab) and at progression (n = 24).





1C

Baseline PD-L1 expression on Tissue (IHC) (n=69)			Baseline PD-L1 expression on CTCs (IF) (n=89)		
% PD-L1+ tissue	n	%	% PD-L1+ CTC	n	%
0%	41	59%	0%	15	17%
≥1%	28	41%	≥1%	74	83%
≥5%	25	36%	≥5%	71	80%
≥50%	16	23%	≥10%	61	68%

Correlation Tissues/CTCs				
	PD-L1 tissue (%)			
PD-L1+CTC (%)	0%	≥1%	ND	Total
0%	8	5	2	15
≥1%	31	22	21	74
ND	2	1	4	7
Total	41	28	27	96

Fig. 1. 1A: Analysis of PD-L1 on CTCs. Example of 2 cases with strong membranous PD-L1 staining on CTC (red arrow). **1B**: Correlation study between PD-L1 expression on tissue and CTC. **1C**: Correlation between tissue and CTCs PD-L1 expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. PD-L1 expression on CTCs and correlation with tissue

All 96 patients had matched tissue available, but only 72% (n = 69) had enough material for PD-L1 testing. Staining for PD-L1 in tissue was seen in 28 of 69 patients (40.6%). The technique used to obtain tissue was known in 53/69 patients. PD-L1 analyses were performed on small biopsies in 69.9% of cases (64.2% obtained by bronchoscopy, 5.7% obtained using CT-guided biopsies) and on surgical resection specimen in 30.2%.

CTCs were detected in 93% samples at baseline (n = 89/96). At baseline, median number of CTCs/7.5 ml was 30 (range 2–242) and the median proportion of CTCs expressing PD-L1 was 17.2% (range 0–100). 83% of patients expressed PD-L1 on, at least, 1% of CTCs. Detailed data for tissue and CTC and tissue analyses using different PD-L1 thresholds are presented in Fig. 1**C**.

No correlation between pre-treatment PD-L1 expression on archived tissue and CTCs was observed (r = 0.04, p = 0.77, Fig. 1**B**). The concordance rate was 45.4% (n = 30/66). Of the 36 discordant cases, 31 were negative on tissue but positive on CTC, and 5 tested positive on tissue but not in CTC (Fig. 1**C**). Of the 30 concordant cases, 8 were negative and 22 positive on both.

3.3. Association between PD-L1 expression in tissue and CTCs and response to PD-1 inhibitors

Since no cut-off has been validated in the metastatic setting, the median CTC number at baseline was chosen as cut-off (30/7.5 ml). Patients with high CTC count (n = 43/89) experienced worse outcomes compared to those with a low CTC load (PFS: HR 2.44[1.46–4.07], p = 0.0004; OS: HR 2.37[1.22–4.60], p = 0.0088) (Fig. 2**A** and **B**). Similar results (OS: HR 2 [1.04–3.84], p = 0.03; PFS: HR 2.09[1.25–3.5], p = 0.0039) were observed using 50/7.5 ml as cutoff (a cut-off previously used by Hofman V. et al. in a preoperative setting [12]). Responders (defined by PFS > 6 months) had a lower baseline CTC count (p < 0.0001) (Fig. 2**E**).

The presence of PD-L1$^+$CTCs had no significant impact on PFS (≥1% threshold: HR 1.21[0.64–2.27], p = 0.55; ≥5%: HR 1.05[0.59–1.88], p = 0.86; ≥10%: HR 0.75[0.45–1.25], p = 0.27) or OS (≥1% threshold: HR 1.06[0.46–2.42], p = 0.89; ≥5%: HR 1.38[0.60–3.17], p = 0.44; ≥10%: HR 0.95 [0.48–1.88], p = 0.89) (Fig. 2**C** and **D**), even in subgroup analyses according to CTC count (≤30 and > 30). However, patients with PD-L1$^+$CTCs (≥1%) at baseline were more frequently non-responders compared to patients who had CTC PD-L1 negative (n = 47/69 (68%) vs. 6/15 (40%), p = 0.04, Fig. 2**F**).

It is noteworthy that PD-L1 expression in tissue was not, in our population, associated with outcomes (≥1%: HR 0.64, p = 0.15; ≥5%: HR 0.81, p = 0.49; ≥50%: HR 0.77, p = 0.47 for PFS) (Sup. Fig. 2).

3.4. PD-L1$^+$ CTC at progression

Specimens at the time of progression were available for 24 patients, of which 23 had CTCs. Median CTC number was higher at progression compared to pre-treatment (52.5/7.5 ml (7.5–228) vs. 30/7.5 ml pre-treatment, p = 0.03). All patients with CTCs had PD-L1$^+$CTC at resistance (n = 23/23), of whom 83% (n = 19/23) had more than 10% of CTCs stained (vs. 68% pre-treatment).

4. Discussion

We herein confirmed in a large population the feasibility of the non-invasive analysis of PD-L1 expression through CTCs in advanced NSCLC. PD-L1 expression on CTCs could be performed in 93% of cases, compared to 72% in tissue, underlying the limitations of small samples, which are often exhausted after the diagnosis steps. This approach has already been reported in NSCLC, using various devices [9–11]. Nicolazzo et al. monitored CTCs using a technology based on epithelial marker expression during nivolumab treatment and the prevalence of PD-L1$^+$CTCs at baseline was very high (100%). The expression of PD-L1 by either circulating immune or tumor cells was found to be associated with poor prognosis in another cohort of patients not treated by ICI [10]. Nevertheless, the impact of circulating PD-L1 stained immune cells is unclear, since they are not necessarily issued from the tumor microenvironment. Finally, Ilie et al. reported recently for the first time a concordance with matched tissue using the ISET platform but PD-L1 was performed by IHC using the Ventana SP142 antibody (which constantly stains fewer tumor cells [13]). The rate of PD-L1 staining was low: 8% (n = 6/71) on CTCs and 15% (n = 11/71) on tissue [11]. If a blood-based detection of PD-L1 tumor expression is to enter routine clinical use, standardizing the assay will be required, like in tissue [14].

Besides the fact that we used different antibodies in the two approaches, the absence of correlation between tissue and blood analyses in our study can be partially explained by: *i)* the temporal heterogeneity, since archival tissue was used for this study, with a median interval time between the biopsy and the blood draw of 7.8 months (with only 3 patients having a blood draw less than one month after tissue biopsy) and *ii)* the spatial tumour heterogeneity, which was previously reported as a major limitation of tissue analysis in this purpose. The use of small biopsies underrates the proportion of PD-L1 positive tumors compared to surgically resected specimens [7]. This explains the high rate of patients that tested positive for CTCs in our study, in agreement with previous findings [9] and to what is observed on larger, surgical specimens (74% [7]).

Our survival analysis confirmed the prognostic value of the CTC load among a population of patients treated with ICI. Moreover, our data are in agreement with the known detrimental effect of PD-L1 expression on survival [14] and with what was observed previously [9]. One explanation might rely on the detrimental effect of CTCs, suggesting more extended disease regardless of PD-L1 expression. The presence of high numbers of CTCs probably raises the probability of finding PD-L1$^+$CTCs. However, subgroup analyses according to CTC load didn't change the results. Also, the difference in outcomes may be related to an overall prognostic effect of PD-L1$^+$CTC more than a direct impact of these cells on response to nivolumab. Nicolazzo et al. studied the relationship between PD-L1 expression on CTCs and response to ICI, though a limited number of patients and the fact that all patients harboured PD-L1$^+$CTCs, hampered the analysis of its correlation with outcomes [9]. The persistence of PD-L1$^+$CTCs at 6 months in 5/10 patients was associated with a progression of disease [9]. In our study PD-L1$^+$CTCs were detected in all patients at progression, suggesting the role of this cell population in acquired resistance to ICI. The high prevalence of PD-L1$^+$CTC, and their discordant value compared to tissue suggest a complementary role, and highlight the need to better characterise the functional role of these cells.

In conclusion, PD-L1 analysis on CTCs is highly feasible. Pre-treatment high CTC load are associated with poor outcomes but PD-L1 expression on CTC has no significant prognostic impact. Further studies are needed to assess the role of liquid biopsy in immuno-oncology, including other circulating markers.

Sources of funding

This research was supported in part by La Ligue contre le Cancer and Toulouse University Hospital (DRCI: Direction de la Recherche Clinique et de l'Innovation).

Conflict of interest

No authors have any conflicts of interest regarding this work.

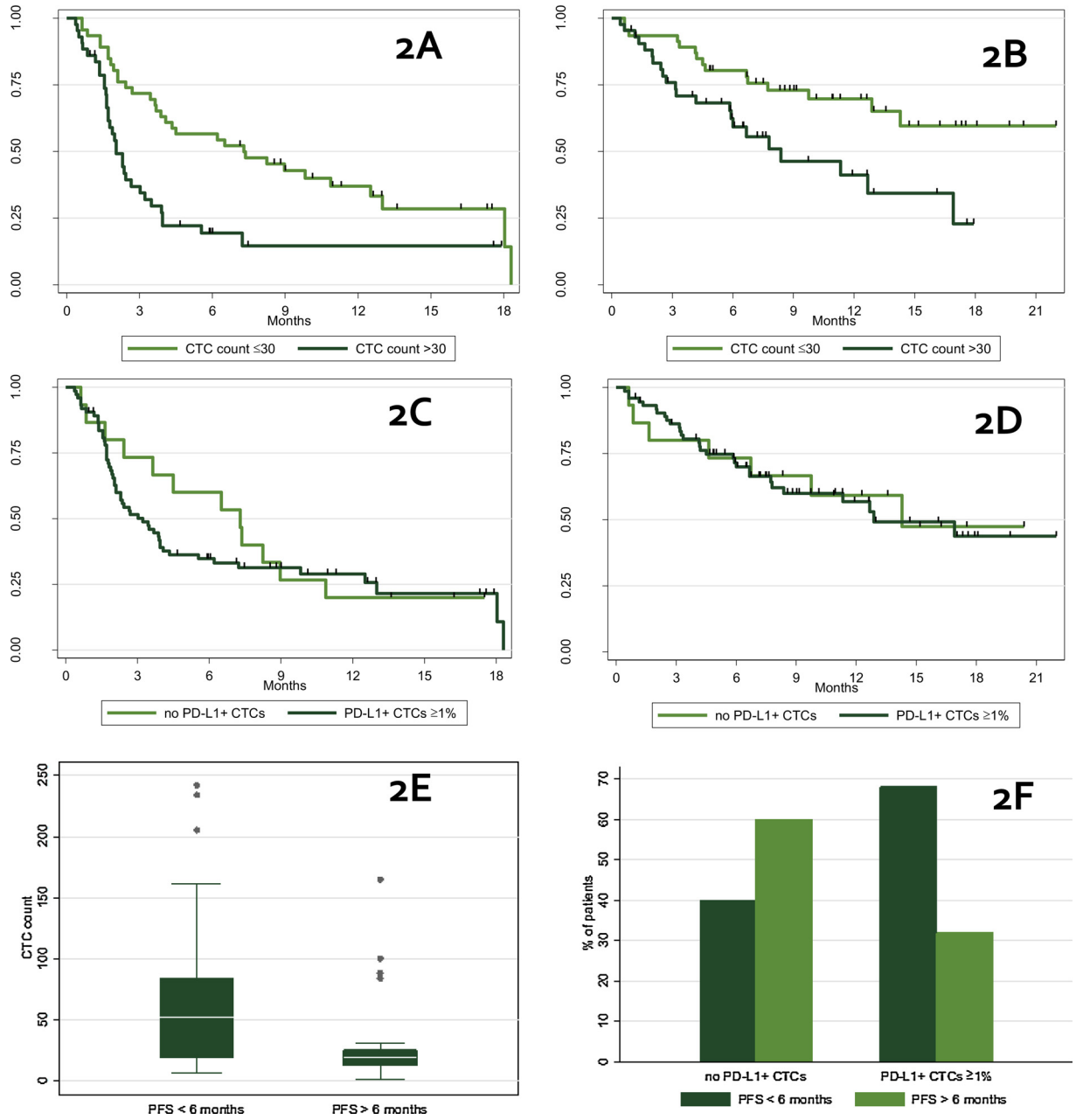


Fig. 2. Survival analyses. **2A** & **2B:** PFS (**2A**) and OS (**2B**) depending on the pre-treatment CTC load (cut-off 30 CTC/7.5 ml). **2C** & **2D:** PFS (**2C**) and OS (**2D**) depending on the presence of PD-L1 $^{+}$CTC pre-treatment. **2E**: CTCs number in "non-responders" (PFS < 6 months) and "responders" patients (PFS > 6 months). **2F**: Prevalence of PD-L1 $^{+}$CTC in "non-responders" (PFS < 6 months) and "responders" patients (PFS > 6 months).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.lungcan.2018.04.001.

References

[1] M. Reck, D. Rodríguez-Abreu, A.G. Robinson, R. Hui, T. Csőszi, A. Fülöp, et al., Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer, N. Engl. J. Med. 375 (19) (2016) 1823–1833.
[2] N.A. Rizvi, M.D. Hellmann, A. Snyder, P. Kvistborg, V. Makarov, J.J. Havel, et al., Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer, Science 348 (2015) 124–128.
[3] M. Khunger, A.V. Hernandez, V. Pasupuleti, S. Rakshit, N.A. Pennell, J. Stevenson, et al., Programmed cell death 1 (PD-1) ligand (PD-L1) expression in solid tumors as a predictive biomarker of benefit from PD-1/PD-L1 axis inhibitors: a systematic review and meta-analysis, JCO Precis. Oncol. 1 (2017) 1–15.
[4] J.J. Han, D.-W. Kim, J. Koh, B. Keam, T.M. Kim, Y.K. Jeon, et al., Change in PD-L1 expression after acquiring resistance to gefitinib in EGFR-mutant non-small-cell lung cancer, Clin. Lung Cancer 17 (4) (2016) 263–270 e2.
[5] X. Gong, X. Li, C. Zhou, 81P Radiation resistance induced immunity evasion by evoking PD-L1 expression, J. Thorac. Oncol. 11 (Suppl. 4) (2016) S89–S90.
[6] S.H. Lim, M. Hong, S. Ahn, Y.-L. Choi, K.-M. Kim, D. Oh, et al., Changes in tumour expression of programmed death-ligand 1 after neoadjuvant concurrent chemoradiotherapy in patients with squamous oesophageal cancer, Eur. J. Cancer 52 (2016) 1–9.
[7] M. Ilie, E. Long-Mira, C. Bence, C. Butori, S. Lassalle, L. Bouhlel, et al., Comparative study of the PD-L1 status between surgically resected specimens and matched biopsies of NSCLC patients reveal major discordances: a potential issue for anti-PD-L1 therapeutic strategies, Ann. Oncol. 27 (1) (2016) 147–153.
[8] V. Hofman, E. Long, M. Ilie, C. Bonnetaud, J.M. Vignaud, J.F. Fléjou, et al., Morphological analysis of circulating tumour cells in patients undergoing surgery for non-small cell lung carcinoma using the isolation by size of epithelial tumour cell (ISET) method, Cytopathology 23 (1) (2012) 30–38.
[9] C. Nicolazzo, C. Raimondi, M. Mancini, et al., Monitoring PD-L1 positive circulating tumor cells in non-small cell lung cancer patients treated with the PD-1 inhibitor Nivolumab, Sci. Rep. 6 (2016) 31726.
[10] D.J. Boffa, R.P. Graf, M.C. Salazar, et al., Cellular expression of PD-L1 in the

peripheral blood of lung cancer patients is associated with worse survival, Cancer Epidemiol. Biomarkers Prev. 26 (7) (2017) 1139–1145.
[11] M. Ilié, E. Szafer-Glusman, V. Hofman, et al., Detection of PD -L1 in circulating tumor cells and white blood cells from patients with advanced non-small cell lung cancer, Ann. Oncol. 29 (1) (2017) 193–199 0923-7534.
[12] V. Hofman, C. Bonnetaud, M.I. Ilie, et al., Preoperative circulating tumor cell detection using the isolation by size of epithelial tumor cell method for patients with lung cancer is a new prognostic biomarker, Clin. Cancer Res. 17 (2011) 827–835.
[13] F.R. Hirsch, A. McElhinny, D. Stanforth, et al., PD-L1 immunohistochemistry assays for lung cancer: results from phase 1 of the blueprint PD-L1 IHC assay comparison project, J. Thorac. Oncol. 12 (2) (2017) 208–222.
[14] P. Wu, D. Wu, L. Li, et al., PD-L1 and survival in solid tumors: a meta-analysis, PLoS One 10 (6) (2015) e0131403.