PD-L1 expression testing in non-small cell lung cancer

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Abstract: In recent years, immunotherapy has revolutionized and changed the standard of care in patients with advanced non-small cell lung cancer (NSCLC). Immune checkpoint inhibitors, fundamentally those that act by blocking the programmed cell death receptor-1 (PD-1) and its ligand the programmed cell death ligand-1 (PD-L1) have emerged as novel treatment strategies in NSCLC, demonstrating undoubted superiority over chemotherapy in terms of efficacy. Several of these immune checkpoint modulators have recently gained regulatory approval for the treatment of advanced NSCLC, such as nivolumab, atezolizumab and pembrolizumab in first-line (only the latter) and second-line settings, and more recently, durvalumab as maintenance after chemoradiotherapy in locally advanced disease. There is consensus that PD-L1 expression on tumor cells predicts responsiveness to PD-1 inhibitors in several tumor types. Hence PD-L1 expression evaluated by immunohistochemistry (IHC) is currently used as a clinical decision-making tool to support the use of checkpoint inhibitors in NSCLC patients. However, the value of PD-L1 as the ‘definitive’ biomarker is controversial as its testing is puzzled by multiple unsolved issues such as the use of different staining platforms and antibodies, the type of cells in which PD-L1 is assessed (tumor versus immune cells), thresholds used for PD-L1-positivity, or the source and timing for sample collection. Therefore, newer biomarkers such as tumor mutation burden and neoantigens as well as biomarkers reflecting host environment (microbiome) or tumor inflamed microenvironment (gene expression signatures) are being explored as more reliable and accurate alternatives to IHC for guiding treatment selection with checkpoint inhibitors in NSCLC.

Keywords: immunotherapy, immunohistochemistry (IHC), non-small cell lung cancer (NSCLC), PD-L1 expression testing, predictive biomarker

Introduction

The immune system plays an important dual role in cancer by a dynamic process called immunoediting.1,2 Most of the time, the innate and adaptive immune responses constrain tumor growth and destroy cancer cells in the so called ‘elimination’ phase or immunosurveillance. However, tumors can enter into an ‘escape’ phase through several mechanisms that confer a characteristic local immune suppression state by recruiting immunosuppressive cells, producing immunosuppressive cytokines, developing defects in tumor antigen presentation to T-cells or by expressing negative costimulatory molecules also called T-cell checkpoint regulators, such as the cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), programmed cell death-1 (PD-1) and programmed cell death ligand-1 (PD-L1).1 By doing so, tumors can disrupt the normal immunity in favor of their progression. Loss of heterozygosity in human leukocyte antigen, present in almost 40% of early-stage non-small cell lung cancer (NSCLC), is a hallmark of immune escape in NSCLC evolution allowing for high subclonal neoantigen burden, apolipoprotein B mRNA editing catalytic polypeptide-like cytidine deaminase (APOBEC)-mediated mutagenesis, upregulation of cytolytic activity and expression of PD-L1.3

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One of the most developed therapeutic strategies to overcome the immune evasion of tumors is the reactivation of T-cell mediated antitumor activity by modulating the immune checkpoint ligand–receptor interactions that regulate T-cell signaling. In a normal state, these immune checkpoint receptors serve to limit and extend the duration of a response on T-cell activation and prevent damage to normal tissue, providing a natural counterbalance to immune activation. Indeed, pharmacological design of antibodies directed against CTLA-4, PD-1 and PD-L1 pathways has already demonstrated efficacy in several trials among different tumor types and expression of PD-L1 in tumor tissue is currently harnessed to select patients for PD-(L)1 blockade therapies.

Herein, we aim to review the main questions concerning immunohistochemical evaluation of PD-L1 as a biomarker in NSCLC, overview the most practical issues regarding its use as a predictive diagnostic assay in the clinic and to go over other novel predictive biomarkers of response to immune checkpoint inhibitors beyond PD-L1.

**PD-L1 as predictive biomarker in NSCLC**

PD-L1 is a type 1 transmembrane protein (B7-H1) that belongs to the B7 ligands family and may be expressed both on hematopoietic cells (dendritic cells, macrophages, mast cells, T-cells and B-lymphocytes) and nonhematopoietic cells, including endothelial, epithelial and tumor cells. Expression of PD-L1 on tumor cells promotes down-regulation and self tolerance of the immune system from rejecting the tumor by suppressing T-cell inflammatory activity through binding to the regulatory T-cell receptor, PD-1. Among ligands belonging to the B7 family, PD-L1 is the principal membrane inhibitory ligand and the most studied so far in NSCLC.

Expression of PD-L1 by immunohistochemistry (IHC) staining has been the core strategy to select NSCLC patients for PD-(L)1 inhibitors. It is currently known that PD-L1 expression by IHC can enrich for PD-(L)1 blockade efficacy and overexpression has been associated with higher response rates and better outcomes to several checkpoint inhibitors.

In advanced NSCLC, the percentage of PD-L1 positive patients [Tumor Proportion Score (TPS) ≥1%] is found in around half of samples evaluated for PD-L1. However, differences in PD-L1 expression have been observed in the context of specific phase III clinical trials [53% (119/225) in CheckMate-017 trial and 54% (246/455) in CheckMate-057 trial with nivolumab; 66% (1475/2222) in KEYNOTE-010 trial with pembrolizumab and 54% (463/850) in atezolizumab OAK trial]. Approximately a third of patients have high PD-L1 expression levels (TPS ≥50%), but likewise, differences across studies have been detected [40% (214/541) in CheckMate-026 trial, 30.2% (500/1653) in KEYNOTE 024 trial and 16% (137/850) in OAK trial respectively]. Changes in the percentage of positive and high PD-L1 patients detected in these trials could be explained by the different antibody clones used and the variability across patient subpopulations.

**PD-L1 diagnostics tests**

Evaluation of PD-L1 expression by IHC has overcome a cumbersome process for pathologists and oncologists, not because of the complexity of the technique itself, but for the singularity of the co-development of an assay together with a specific checkpoint inhibitor. Each of these assays uses its own PD-L1 antibody, platform, custom reagents and scoring criteria to calculate TPS on tumor cells. Some tests have been labeled as ‘companion’ diagnostic as they are a prerequisite for receiving a drug prescription, whereas others are only ‘complementary’ this is to say not required but of aid for the use of the associated drug (Table 2).

Currently five clones are being used for PD-L1 IHC testing: 22C3, 28-8, SP142, SP263 and 73-10. In the majority, staining is evaluated on the cell membrane as it is presumed that the PD-(L)1 axis is only functional when it ligrates a counter-receptor. Herein we summarize the main characteristics of the available commercial assays specific for PD-L1 testing in lung cancer.
Table 1. Outcomes of PD-1/PD-L1 axis inhibitors in NSCLC phase III clinical trials based on PD-L1 testing.

| Immune checkpoint inhibitor | PD-L1 analysis | Stratification by PD-L1 | PD-L1 cutoffs for outcome | Outcome |
|-----------------------------|----------------|-------------------------|--------------------------|---------|
|                             | PD-L1          |                          | RR           | PFS  | OS          |
|                             | analysisa      |                          |              |      |             |
|                             |                |                          |               |      |             |
| First line (compared with standard first-line chemotherapy) | | | | |
| Nivolumab (Checkmate-026)15 | 28–8 mAb       | No                       | ≥5%          | 26% versus 33% | 4.2 m versus 5.9 m (HR 1.15) | 14.4 m versus 13.2 m (HR 1.02) |
|                            | retrospective  |                          | ≥50%         | 34% versus 39% | 5.4 m versus 5.8 m (HR 1.07) | 15.9 m versus 13.9 m (HR 0.90) |
| Pembrolizumab (KEYNOTE-024)16 | 22C3 mAb       | No                       | ≥50%         | 44.8% versus 27.8% | 10.3 m versus 6 m (HR 0.50) | Not reported (HR 0.60) |
| Second line (compared with docetaxel) | | | | |
| Nivolumab (Checkmate-017)14 | 28–8 mAb       | No                       | Negative and positive | 20% versus 9% | 3.5 m versus 2.8 m (HR 0.62) | 9.2 m versus 6 m (HR 0.59) |
|                            | retrospective  |                          | <1%          | 17% versus 10% | 3.1 m versus 3 m (HR 0.66) | 8.7 m versus 5.9 m (HR 0.58) |
|                            |                |                          | >1%          | 17% versus 11% | 3.3 m versus 2.8 m (HR 0.67) | 9.3 m versus 7.2 m (HR 0.69) |
|                            |                |                          | <5%          | 15% versus 12% | 2.2 m versus 2.9 m (HR 0.75) | 8.5 m versus 6.1 m (HR 0.70) |
|                            |                |                          | ≥5%          | 21% versus 8%  | 4.8 m versus 3.1 m (HR 0.54) | 10 m versus 6.4 m (HR 0.53) |
|                            |                |                          | <10%         | 16% versus 11% | 2.3 m versus 2.8 m (HR 0.70) | 8.2 m versus 6.1 m (HR 0.70) |
|                            |                |                          | ≥10%         | 19% versus 9%  | 3.7 m versus 3.3 m (HR 0.58) | 11 m versus 7.1 m (HR 0.50) |
| Nivolumab (Checkmate-057)17 | 28–8 mAb       | No                       | Negative and positive | 19% versus 12% | 2.3 m versus 4.2 m (HR 0.92) | 12.2 m versus 9.4 m (HR 0.73) |
|                            | retrospective  |                          | <1%          | 9% versus 15%  | 2.1 m versus 3.6 m (HR 1.19) | 10.5 m versus 10.1 m (HR 0.87) |
|                            |                |                          | ≥1%          | 31% versus 12% | 4.2 m versus 4.5 m (HR 0.70) | 17.7 m versus 9 m (HR 0.58) |
|                            |                |                          | <5%          | 10% versus 14% | 2.1 m versus 4.2 m (HR 1.31) | 9.8 m versus 10.1 m (HR 0.96) |
|                            |                |                          | ≥5%          | 36% versus 13% | 5 m versus 3.8 m (HR 0.54) | 19.4 m versus 8.1 m (HR 0.43) |
|                            |                |                          | <10%         | 11% versus 14% | 2.1 m versus 4.2 m (HR 1.24) | 9.9 m versus 10.3 m (HR 0.96) |
|                            |                |                          | ≥10%         | 37% versus 13% | 5 m versus 3.7 m (HR 0.52) | 19.9 m versus 8 m (HR 0.40) |
| Pembrolizumab (KEYNOTE-010b)18 | 22C3 mAb       | Yes                      | ≥1%          | 18% versus 9%  | 3.9 m versus 4 m (HR 0.88) | 10.4 m versus 8.5 m (HR 0.71) |
|                            | prospective    |                          | ≥50%         | 30% versus 8%  | 5 m versus 4.1 m (HR 0.59) | 14.9 m versus 8.2 m (HR 0.54) |

(Continued)
### Table 1. (Continued)

| Immune checkpoint inhibitor | PD-L1 analysis | Stratification by PD-L1 | PD-L1 cutoffs for outcome | Outcome |
|-----------------------------|----------------|-------------------------|--------------------------|---------|
|                             |                | RR                      | PFS                      | OS      |
| **Atezolizumab (OAK)**      | SP142 mAb      | Yes                     | 14% versus 13%           | 2.8 m versus 4 m (HR 0.95) | 13.8 m versus 9.6 m (HR 0.73) |
|                             | prospective    | TC1/2/3 or IC1/2/3      | 18% versus 16%           | 2.8 m versus 4.1 m (HR 0.91) | 15.7 m versus 10.3 m (HR 0.74) |
|                             |                | TC2/3 or IC2/3          | 22.5% versus 12.5%       | 4.1 m versus 3.6 m (HR 0.76) | 16.3 m versus 10.8 m (HR 0.67) |
|                             |                | TC3 or IC3              | 31% versus 11%           | 4.2 m versus 3.3 m (HR 0.63) | 20.5 m versus 8.9 m (HR 0.41) |
|                             |                | TC0 and IC0             | 8% versus 11%            | 2.6 m versus 4 m (HR 1)     | 12.6 m versus 8.9 m (HR 0.75) |

**Stage III NSCLC (compared with placebo)**

| Durvalumab (Pacific) | SP263 mAb      | No                      | Negative and positive   | 28.4% versus 16% | 16.8 m versus 5.6 m (HR 0.52) | Not reported |
|----------------------|----------------|-------------------------|-------------------------|-----------------|--------------------------------|--------------|
|                      | prospective    | TC1/2/3 or IC1/2/3      | 18% versus 16%           | 2.8 m versus 4.1 m (HR 0.91) | 15.7 m versus 10.3 m (HR 0.74) |
|                      |                | TC2/3 or IC2/3          | 22.5% versus 12.5%       | 4.1 m versus 3.6 m (HR 0.76) | 16.3 m versus 10.8 m (HR 0.67) |
|                      |                | TC3 or IC3              | 31% versus 11%           | 4.2 m versus 3.3 m (HR 0.63) | 20.5 m versus 8.9 m (HR 0.41) |
|                      |                | TC0 and IC0             | 8% versus 11%            | 2.6 m versus 4 m (HR 1)     | 12.6 m versus 8.9 m (HR 0.75) |

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*a* In all these studies PD-L1 analysis were made in fresh or archival FFPE biopsies.

*b* In the KEYNOTE-010 study we only reported data of the cohort treated at the approved dose of 2 mg/kg of pembrolizumab.

HR, hazard ratio; IC, immune cell; m, months; mAb, monoclonal antibody; NSCLC, non-small cell lung cancer; OS, overall survival; PD-1, programmed cell death receptor-1; PD-L1, programmed cell death ligand-1; PFS, progression-free survival; RR, objective response rate; TC, tumor cell; TC0 or IC0, PD-L1 expression on 10% or more of tumor-infiltrating immune cells; TC1/2/3, PD-L1 expression on 1% to 10% of tumor cells or tumor-infiltrating immune cells, respectively; TC 1/2/3 or IC 1/2/3, PD-L1 expression on less than 1% of tumor cells or tumor-infiltrating immune cells, respectively; TC 2/3 or IC 2/3, PD-L1 expression on 5% or more of tumor cells or tumor-infiltrating immune cells, respectively; IC3, PD-L1 expression on 10% or more of tumor-infiltrating immune cells; TC3, PD-L1 expression on 50% or more of tumor cells.
Table 2. Summary of PD-L1 monoclonal antibodies and technical aspects for evaluation and agencies’ approvals in NSCLC.

| PD-L1 mAb clone | Ab host species | Automated platform | Checkpoint inhibitor (target) | PD-L1 scoring | Definition of positivity (cutoffs) | FDA status | EMA status | Indication | Cutoffs for indications |
|------------------|----------------|--------------------|-------------------------------|----------------|------------------------------------|------------|------------|-------------|------------------------|
| 22C3             | Mouse          | Dako (Autostainer Link 48) | Pembrolizumab (PD-1)          | TC             | TC ≥1% (minimum of 100 TC)          | Companion  | CE mark    | Second and first-line NSCLC | ≥1% second line |
|                  |                |                    |                               |                |                                    |            |            |                          | ≥50% first line     |
| 28-8             | Rabbit         | Dako (Autostainer Link 48) | Nivolumab (PD-1)              | TC             | TC ≥1% (minimum of 100 TC)          | Complementary | CE mark   | Second-line NSCLC       | All comers            |
| SP142            | Rabbit         | Ventana (BenchMark ULTRA) | Atezolizumab (PD-L1)          | TC, IC         | TC ≥50% or IC ≥10% (minimum of 50 TC with associated stroma) | Complementary | CE mark   | Second-line NSCLC       | All comers            |
| SP263            | Rabbit         | Ventana (BenchMark ULTRA) | Durvalumab (PD-L1)            | TC             | TC ≥25% (minimum of 100 TC)         | FDA approval only for urothelial carcinoma | CE mark for nivolumab and pembrolizumab in NSCLC and durvalumab in urothelial carcinoma | Locally advanced NSCLC | All comers            |
| 73-10            | Rabbit         | Dako               | Avelumab (PD-L1)              | TC             | TC ≥1% (minimum cells not defined) | FDA approval | NA         | NA          | NA                     |

Ab, antibody; CE, European Conformity (CE)-marked; Companion, provides information that is essential for the effective use of a corresponding drug or biological product, within approved label; Complementary, provides additional information about how a drug might be used, but not required; EMA, European Medicines Agency; FDA, United States Food and Drug Administration; IC, immune cell; mAb, monoclonal antibody; NA, not available; NSCLC, non-small cell lung cancer; PD-1, programmed cell death receptor-1; PD-L1, programmed cell death ligand-1; TC, tumor cell.
to consider a sample valid for its evaluation in formalin-fixed paraffin-embedded (FFPE) tissue using the monoclonal mouse [immunoglobulin (Ig)G1] clone 22C3 and PD-L1 positivity criteria is defined when a membrane staining (partial or complete) of tumor cells $\geq 1\%$ is observed.

**PD-L1 IHC 28-8 pharmDx assay**

The Dako 28-8 pharmDx (Agilent Technologies/Dako), is a qualitative PD-L1 immunohistochemical assay that gained US FDA approval as a ‘complementary’ diagnostic test as well as the European Conformity (CE)-mark certification for the use of nivolumab in the second line of advanced NSCLC. Clone PD-L1 28-8 (ab205921; Abcam, Cambridge, UK) is a monoclonal rabbit anti-human antibody, which binds to the extracellular domain of human PD-L1. As stated previously, a minimum of 100 tumor cells are needed to address PD-L1 in FFPE tissue samples and positivity is defined at TPS equal or greater than 1%.

**PD-L1 IHC SP142 assay**

The Ventana SP142 assay (Ventana Medical Systems Inc., Tucson, AZ, USA) is a qualitative IHC assay that uses a rabbit monoclonal anti-PD-L1 clone which binds the intracellular domain of the protein. This test is CE-marked and has been approved by the US FDA as ‘complementary’ diagnostic tool for atezolizumab treatment in patients with metastatic NSCLC whose disease progressed during or following platinum-containing chemotherapy, as well as in patients with advanced urothelial carcinoma. Evaluation in FFPE samples requires at least 50 viable tumor cells. Tumor-associated stroma is not required for tumor cells scoring but it is essential for scoring tumor-infiltrating immune cells (ICs). Unlike the other assays, the scoring algorithm is based on either the percentage of PD-L1 expressing tumor cells or IC of any intensity. PD-L1 expression in $\geq 50\%$ tumor cells or $\geq 10\%$ ICs may be associated with enhanced overall survival (OS) from atezolizumab.

**PD-L1 IHC SP263 assay**

PD-L1 clone SP263 is a rabbit monoclonal primary antibody that binds to a transmembrane glycoprotein corresponding to amino acids 284-290 of PD-L1 protein. The Ventana SP263 assay (Ventana Medical Systems Inc.) is intended to assess PD-L1 expression in FFPE tissue and gained the CE-In Vitro Device certification and US FDA ‘complementary’ test designation for the identification of patients with locally advanced or metastatic urothelial carcinoma most likely to benefit from durvalumab. For durvalumab treatment, PD-L1 cell positivity is considered when plasma membrane protein staining at any intensity is observed in at least 25% of tumor cells. A minimum of 100 tumor cells are required to determine the TPS. Recently this test has gained CE-mark, not US FDA, label expansion to inform treatment decisions in NSCLC patients being considered for pembrolizumab and nivolumab based on the results of a comparison study with other currently available PD-L1 assays (22C3 and 28-8).

**PD-L1 IHC 73-10 assay**

The Dako 73-10 assay, the fifth PD-L1 US FDA-approved diagnostic test, was co-developed and commercialized to support the use of avelumab therapy. Clone PD-L1 73-10 is a monoclonal rabbit antibody property of Merck (KGaA, Darmstadt, Germany) optimized to detect PD-L1 expression in FFPE samples. Although a cutoff has not been definitely determined, the predefined PD-L1 positivity is considered when a complete circumferential or partial linear plasma membrane staining is observed at any intensity in at least 1% of tumor cells. Since this assay is still in development, the minimum of viable tumor cells required to determine the TPS is still undefined.

**Preanalytical considerations and tissue preparation for PD-L1 testing**

General recommendations for IHC, including PD-L1 antibodies, are to perform the technique on FFPE freshly cut tissue sections at a thickness of 3–5 μM and mounted on positively charged slides. For each PD-L1 staining section it is advised to process a positive control to ensure the reliability of the PD-L1 expression since variations have been observed as a function of the preanalytical process (such as time to fixation, fixation time and sample processing), although there are not much data currently available on the reproducibility of PD-L1 IHC assays based on these preanalytical variable factors. A sample over-fixation can be the reason for an inadequate antibody penetration impairing final reading. Therefore, time to sample fixation must be
reduced to the minimum possible (optimally under 30 min) followed by a 10% neutral formalin buffering for at least 6–48 h in biopsies and 24–48 h in resection samples. Long-term storage of cut tissue sections and of tissue blocks should be also avoided to ensure the quality of the PD-L1 expression. Different studies have reported that PD-L1 expression fades with the age of the specimens used for analyses, particularly in tissue blocks older than three years and even older than one year.

Comparison and standardization of different PD-L1 IHC platforms

As outlined before, several reproducible PD-L1 assays have been developed for each of the immune-inhibitors (Table 2). This is leading to the paradoxical situation where the pathologists must select between different antibodies and assay conditions according to the prescription of a drug, rather than focusing on the accuracy of the technique itself. In the effort to overcome this limitation, and reach harmonization between the assays, several initiatives are ongoing to validate their reproducibility and improve standardization for IHC scoring (Table 3).

The first of its kind was the Blueprint PD-L1 IHC assay project, aimed to compare the analytical performance of four PD-L1 validated assays (22C3, 28-8, SP142, SP263) in 39 NSCLC samples assessed by three independent readers. A total of three (22C3, 28-8 and SP263) out of four assays were analytically similar for TPS but inter-observer concordance for IC staining was poor. The NCCN study by Rimm and colleagues compared the performance of four different antibodies (22C3, 28-8, SP142, E1L3N) in 90 resected NSCLC. The study showed that three of the four assays (22C3, 28-8 and E1L3N) evaluated by 13 independent readers were interchangeable, whereas the SP142 assay was associated with a lower score. Later on, three other prospective studies by Scheel and colleagues, Fujimoto and colleagues and Hendry and colleagues tested in 15, 40 and 368 specimens respectively, the reliability of measuring PD-L1 protein expression by comparing four different clones (22C3, 28-8, SP142, SP263). In the German harmonization study by Scheel and colleagues, although scoring of tumor cells was reproducible and no differences inter-observers were noticed for all assays; staining patterns observed were not similar in all situations. On the other hand, the scoring of ICs yielded low concordance levels. Fujimoto and colleagues observed only an equivalent performance between 22C3, 28-8 and SP263 assays, whereas the work by Hendry and colleagues concluded that apart from 22C3 and 28-8, the SP142 and SP263 assays cannot be used interchangeably in clinical practice. As an additional analysis, the IC scoring was also assessed and a poor concordance was observed.

The Italian harmonization study reported an excellent agreement concordance of 0.99 (95% confidence interval: 0.96–1) between 22C3 pharmDx and SP263 assay at a cutoff of ≥50. In a larger comparative study (n = 500 FFPE archival NSCLC specimens), Ratcliffe and colleagues compared three antibody clones (22C3, 28-8, SP263) and found high analytical concordance (percentage agreement >90%) among the three commercially available assays at multiple expression cutoff and inter-observer, expanding indications of the Ventana SP263 assay to identify patients eligible for treatment with pembrolizumab or nivolumab. The Swedish harmonization study by Brunnström and colleagues compared four PD-L1 antibody clones (22C3 from two different vendors, 28-8, SP142, and SP263) and investigated interrater variation among pathologists, concluding that inter-pathologist variability is higher than assay variability. A better agreement was obtained between 22C3, 28-8 and SP263 antibodies and among pathologists at a cutoff of TPS ≥50.

In order to determine whether laboratory developed tests (LDTs) with different automated staining platforms could achieve an analytical performance close to the validated ones, the multicentric French harmonization study evaluated PD-L1 IHC status on 41 NSCLC samples using different PD-L1 clones (22C3, 28-8, SP142, E1L3N) performed either with Ventana BenchMark Ultra, Bond (Leica Biosystems) or Autostainer Link 48 (Dako) and found high concordance for three of the clones (22C3, 28-8 and SP263) in tumor cells above ≥50% threshold and IC staining. Røge and colleagues successfully developed and validated LDT protocols (Ventana, Leica Biosystems, Dako) with clone 22C3 in 77 specimens of NSCLC providing an almost identical result to that of pharmDx Assay. Ilie and colleagues, by using the same clone and platforms showed almost 100% concordance for LDT protocols.
| Study                                           | Samples | Monoclonal Ab          | Platforms       | Readers | Results                                                                                                                                                                                                 |
|------------------------------------------------|---------|------------------------|-----------------|---------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| IASLC BLUEPRINT phase I study Hirsch et al.    | 39      | 22C3, 28-8, SP142, SP263 | Dako, Ventana   | 3       | 3 [22C3, 28-8 and SP263] of 4 assays similar for TC staining. SP142 lower score. IC scored poor                                                                                                         |
| NCCN study Rimm et al.                         | 90      | 22C3, 28-8, SP142, E1L3N | Dako, Ventana, Leica | 13      | 3 [22C3, 28-8 and E1L3N] of 4 assays appear to be interchangeable from analytical perspective. SP142 lower score                                                                 |
| German harmonization study Scheel et al.       | 15      | 22C3, 28-8, SP142, SP263 | Dako, Ventana   | 9       | Scoring on TC reproducible for all assays. Variability in staining patterns                                                                                                                                |
| Japan harmonization study Fujimoto et al.      | 40      | 22C3, 28-8, SP142, SP263 | Dako, Ventana   | 4       | 3 [22C3, 28-8 and SP263] of 4 assays show equivalent predictive performance. SP142 lower score                                                                                                          |
| Australian harmonization study Hendry et al.   | 368     | 22C3, 28-8, SP142, SP263 | Dako, Ventana   | 1       | 2 [22C3 and 28-8] of 4 assays are comparable enough to use interchangeably. Agreement between IC scores were poor                                                                                       |
| Italian harmonization study Marchetti et al.   | 100     | 22C3, SP263            | Dako, Ventana   | 4 centers | 22C3 pharmDx and the Ventana SP263 assays could be used interchangeably. Excellent agreement at a cutoff of ≥50%                                                                                      |
| AZ500 study Ratcliffe et al.                   | 500     | 22C3, 28-8, SP263      | Dako, Ventana   | 2       | High analytical correlation among the 3 commercially available assays and multiple cutoffs                                                                                                             |
| Swedish harmonization study Brunnström et al.  | 55      | 28-8 from two different vendors, 22C3, SP142, SP263 | Dako, Ventana | 7       | Good concordance between 22C3 (from two different vendors), 28-8 and SP263. SP142 presented the highest deviation from the reference scores. Better agreement at a cutoff of ≥50%                                      |
| French harmonization study Adam et al.         | 41      | 22C3, 28-8, SP142, SP263, E1L3N | Dako, Ventana, Leica | 7 centers | 22C3, 28-8 and SP263 assays gave highly concordant TC results for ≥50% threshold                                                                                                                        |
| 22C3 PD-L1 harmonization study Røge et al.     | 77      | 22C3                   | Dako, Ventana, Leica | 3       | LDT protocols provide an almost identical result to the 22C3 pharmDx kit                                                                                                                                |
LDT protocols on Dako and Ventana platforms showed an almost 100% concordance on Ventana’s platform.

Ventana’s 22C3 protocols obtained similar results than those using the Dako 22C3 staining platform.

22C3, 28-8 and SP263 are comparable, SP142 detects less, while 73-10 stains more PD-L1 positive TCs.

Scoring of TC PD-L1 expression by pathologists on tissue samples shows strong reliability whereas scoring of PD-L1 on ICs and cytology samples may have lower reliability.

PD-L1 staining can be performed on cytological samples. A good correlation was found between 22C3 and 28-8 assays, whether applied to histological or cytological cell blocks.

**Table 3.** (Continued)

| Study | Samples | Monoclonal Ab | Platforms | Readers | Results |
|-------|---------|--------------|-----------|---------|---------|
| 22C3 PD-L1 harmonization study Ilie et al. | 120 | 22C3 | Dako, Ventana, Leica | 3 | LDT protocols on Dako and Ventana platforms showed an almost 100% concordance |
| 22C3 PD-L1 harmonization study on Ventana’s platform Neuman et al. | 41 | 22C3 | Dako, Ventana | 2 | Ventana’s 22C3 protocols obtained similar results than those using the Dako 22C3 staining platform |
| IASLC BLUEPRINT phase II Tsao et al. | 81 (different sample and histological types) | 22C3, 28-8, SP142, SP263, 73-10 | Dako, Ventana | 24 | 22C3, 28-8 and SP263 are comparable, SP142 detects less, while 73-10 stains more PD-L1 positive TCs. Scoring of TC PD-L1 expression by pathologists on tissue samples shows strong reliability whereas scoring of PD-L1 on ICs and cytology samples may have lower reliability |
| Cytologic and histologic PD-L1 comparison study Skov et al. | 86 (paired samples from histological and cytological materials) | 22C3, 28-8 | Dako | 1 | PD-L1 staining can be performed on cytological samples. A good correlation was found between 22C3 and 28-8 assays, whether applied to histological or cytological cell blocks |

Ab: antibody; IC: immune cell; LDT: laboratory developed test; NCCN: National Comprehensive Cancer Network; PD-L1, programmed cell death ligand-1; TC: tumor cell.
developed on Ventana and Dako platforms and a very high inter-pathologist concordance, whereas the harmonization study on Ventana's platform using 22C3 by Neuman and colleagues found similar results than using Dako platforms (87.8% and 85.3% concordance with the Ventana Ultraview kit and Ventana Optiview kit respectively) as well as a high inter-observer and intra-observer agreement.

Recently, preliminary results of the Blueprint phase II project have been reported. The study aims to validate assay comparability results observed in the Blueprint phase I but in a larger cohort of 81 specimens, using a larger panel of readers and to evaluate heterogeneity of PD-L1 scores in ‘real-life’ lung cancer specimens such as core needle biopsies or fine needle aspiration cytologies prepared from the same resected tumor, comparing five PD-L1 IHC assays (22C3, 28-8, SP142, SP263 and 73-10). The results obtained so far demonstrate high reliability to PD-L1 TPS between digital pathology versus glass slides, a poor concordance of IC staining between assays and a comparable equivalence among all pathologists.

**Practical aspects of PD-L1 expression testing in the clinic**

Current guidelines for advanced NSCLC have recently incorporated immune checkpoint therapies in their treatment algorithms and therefore, PD-L1 biomarker testing is today a requirement in the initial molecular workup of NSCLC.

**Selection of anti-PD-L1 clones and thresholds for treatment indications**

Nivolumab and pembrolizumab were the first antibodies targeting PD-1 that gained US FDA and European Medicines Agency (EMA) approval for the treatment of advanced NSCLC after demonstration of statistically significant improvement in OS as compared with standard chemotherapy with docetaxel. Later on, in October 2016 and September 2017, atezolizumab was approved by the US FDA and EMA respectively for the same indication, being the first anti-PD-L1 therapy approved for the treatment of NSCLC. In a first-line setting, pembrolizumab has been approved by both agencies, the US FDA and EMA, based on a significant improvement in progression-free survival (PFS) and OS compared with platinum-based chemotherapy, and more recently, maintenance treatment for one year with durvalumab, an anti-PD-L1 checkpoint inhibitor, has granted breakthrough therapy designation by the US FDA to treat locally advanced unresectable NSCLC patients after chemoradiotherapy. Specific thresholds requisitions for PD-L1 expression have been established for each drug prescription in which they were co-developed (Table 2). While a minimum of 1% PD-L1 expression is required for pembrolizumab treatment in second-line treatment, no restriction based on any threshold is required for nivolumab or atezolizumab. In the first line, pembrolizumab is only indicated in those tumors with strong PD-L1 expression higher or equal to 50% whereas no selection based on PD-L1 expression on tumor cells is required for durvalumab treatment after chemoradiotherapy in locally advanced unresectable NSCLC.

**Incorporating PD-L1 in the molecular diagnostic workup of NSCLC**

The optimal sequential approach for PD-L1 biomarker testing in advanced NSCLC is not yet well defined. In squamous NSCLC, where no other biomarkers have been identified for treatment selection, PD-L1 testing might be straightforward in terms of sample disposition. On the contrary, in nonsquamous tumors, assessment of PD-L1 might be an issue in small tumor biopsies when several other relevant biomarkers such as EGFR, ALK, ROS1 or BRAF are also required to define treatment selection. There is consistent data suggesting that oncogenic-driven tumors might have lower response to PD-(L)1 inhibitors compared with the wild-type population and currently there is no approved indication, nor PD-L1 testing recommendation, for immunotherapy in this setting. Whether PD-L1 testing in nonsquamous tumors has to be restricted only after excluding other relevant oncogenic biomarkers, that is to say in a sequential approach, is certainly an issue that needs to be addressed individually in each center by defining customized tissue-management workflows in order to optimize the use of biological samples without impairing the time for treatment initiation.

**Optimal samples for PD-L1 testing in NSCLC**

Besides the inherent variability associated to each technique and commercially available antibody, there are other important biological aspects to be considered when analyzing PD-L1 expression in...
NSCLC such as the tumor heterogeneity\textsuperscript{54–59} (Figure 1) or the dynamic expression evolving after therapies.\textsuperscript{60,61} These factors might explain in part the robust 10–20% of responses observed with PD-(L)1 inhibitors despite the absence or weak immunoreactivity for PD-L1 expression.\textsuperscript{13} However, recent data suggest a reasonable concordance between both metachronous (different time-points) and synchronous (different locations at the same time-point) specimens. In the KEYNOTE-010 trial, pembrolizumab provided benefit compared with docetaxel irrespective of whether archival or new tumor samples were used to assess PD-L1 expression [(hazard ratio (HR) 0.81 and 0.86 respectively).\textsuperscript{18} Likewise, in the FIR study with atezolizumab, high agreement of PD-L1 expression was observed between paired archival and fresh tumor samples at TPS ≥50% or IC ≥10% cutoffs.\textsuperscript{62} In one of the largest cohorts (n = 4784) of patients with advanced NSCLC screened for PD-L1 in pembrolizumab KEYNOTE-001, -010, and -024 trials, the prevalence of PD-L1 expression was similar across prior lines of therapy and different disease characteristics examined.\textsuperscript{63} On the other hand, there is reasonable concordance between PD-L1 expression among different FFPE samples from the same tumor indicating that staining of one block might be enough to capture the entire tumor heterogeneity.\textsuperscript{54,65}

Analytical interpretation and reading of PD-L1 staining on tiny tumor samples can be often misleading and result in a false negative interpretation.\textsuperscript{32,55,66} Thus, defining the minimum number of viable tumor cells before testing is a crucial aspect for interpretation. Cytology specimens can be excellent material for PD-L1 testing. However, none of the assays are advocated for its use as they have not been technically validated for cytology specimens yet and have never been used in clinical trials. Nonetheless, there is growing data suggesting that PD-L1 IHC testing and quantification on non-FFPE samples such as smears, block sections or liquid based cytologies, might be as well feasible and comparable to those obtained from

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**Figure 1.** Representative examples of different NSCLC cases stained with anti-PD-L1 22C3 antibody [Dako pharmDx Assay]. (a) FFPE-biopsy section with strongly membranous PD-L1 staining on both tumor and stromal cells. (b) Positive membranous PD-L1 staining on a cytology specimen. Microscope images captured at ×10 and ×20 magnifications. FFPE, formalin-fixed paraffin-embedded; NSCLC, non-small cell lung cancer; PD-L1, programmed cell death ligand-1.
biopsy specimens\textsuperscript{49,67} (Figure 2). This is of particular relevance as cytology is the only source of material for diagnose in more than one third of the patients with advanced NSCLC. Another challenging issue for cytology samples is the lack of a validated scoring algorithm. There is currently not enough data available to guide how cytology specimens must be scored or whether cutoffs for positivity should be similar to those used on histologic material. Bearing in mind that a minimum of 100 tumor cells are required in the majority of PD-L1 assays, a significant number of cytology preparations would fall far below this threshold and result nonassessable. A clinical trial to assess the feasibility of PD-L1 expression testing on cytological samples as a surrogate of tissue specimens is currently ongoing [ClinicalTrials.gov identifier: NCT03092739].

In practical terms, the use of various types of tumor samples including fresh or archival FFPE biopsies as well as diverse FFPE samples obtained from primary or metastatic sites of the same tumor might be adequate in NSCLC to identify the best candidates for anti-PD-(L)1 therapeutic strategies. However, bearing in mind the intratumor heterogeneity and that small samples can miss to capture the entire immune contexture of the tumor, an effort should be made to select for each tumor those specimens with higher tumor representation and preferably those samples obtained after systemic treatments or radiotherapy shortly before immune checkpoint inhibitors administration to ensure a robust and reliable PD-L1 result.

**Future prospects for immunotherapy**

Understanding the molecular causes of response to immunotherapy in lung cancer and other tumors is currently one of the most important fields of research in oncology. Therefore, several other biomarkers beyond PD-L1 are being evaluated to predict better outcomes to immunotherapy.

Lung cancer has a very high rate of somatic mutations when compared with other tumors, with 8.7 mutations per megabase in adenocarcinomas (ADCs) and 9.7 in squamous cell carcinomas (SqCCs).\textsuperscript{68} However, mutation load is 10-fold higher in smokers than in never-smokers\textsuperscript{69,70} which correlates with the consistently lower tumor mutation burden (TMB) observed in NSCLC harboring known oncogenic drivers such as \textit{EGFR}, \textit{ALK}, \textit{ROS1}, \textit{BRAF-V600E}, \textit{MET} exon 14 skipping mutation with the exception of \textit{BRAF non-V600E} and \textit{KRAS} mutant tumors.\textsuperscript{71} Recently mismatch repair deficiency, which is characterized by having high rate of somatic mutations and neoantigens has been also associated to immune checkpoint blockade response regardless of the cancers’ tissue of origin.\textsuperscript{72}

High TMB is emerging as a key biomarker of sensitivity to immune checkpoint inhibitors across all lung cancers. Data regarding the potential of TMB has been retrospectively assessed in several clinical trials with PD-(L)1 inhibitors. By using whole-exome sequencing of NSCLC patients treated with pembrolizumab, higher nonsynonymous mutation load was associated with improved objective response, durable clinical benefit, and PFS.\textsuperscript{73} Efficacy also correlated with the molecular smoking signature, higher neoantigen burden, and DNA repair pathway mutations. In the phase III CheckMate-026 trial with nivolumab in chemonaive NSCLC patients, PFS was longer in patients treated with nivolumab and high TMB regardless of PD-L1 status (HR = 0.62), albeit numbers were too small to drive conclusions.\textsuperscript{70} In an exploratory analysis of the CheckMate-032, small-cell lung cancer patients with high TMB had improved overall response rate, PFS, and OS compared with low/medium TMB for both nivolumab monotherapy and nivolumab plus ipilimumab, being the first study to evaluate the impact of TMB on outcomes with combination
In 153 patients treated with pembrolizumab in the KEYNOTE-028 and KEYNOTE-012 clinical trials, increasing mutational and neoantigen load as well as T-cell inflamed genetic signature were significantly associated with higher responses and longer PFS to pembrolizumab above cutoffs. More recently, TMB has been associated with improved PFS and OS with atezolizumab in first and second-line NSCLC patients enrolled in three phase II trials (POPLAR, BIRCH and FIR) suggesting that TMB may be an independent predictor of improved responsiveness to atezolizumab in advanced NSCLC. One of the major issues about TMB testing is the absence of a standardized cutoff, variable number of exome sequences reads, total genes included and the use of several platforms and panels for exome sequencing. Therefore, optimization of TMB cutoff and prospective validation is warranted in lung cancer.

Both, lung ADCs and SqCCs NSCLC tumors have distinct patterns of somatic genome alterations but share characteristic mutational signatures mainly related to smoking exposure and APOBEC, a key molecular driver inducing mutations in multiple human cancers. These specific mutational signatures have been correlated with the expression of PD-L1 and a T-cell inflamed signature in head and neck carcinomas suggesting that a specific mutational signature might also serve as a biomarker for immune checkpoint inhibition in cancer.

Still, not only quantity but also quality of mutations is crucial to predict the immune response. The immunogenicity of a mutant peptide depends on its affinity for binding major histocompatibility complex class I ligands so that it can be presented and recognized by cytotoxic T-lymphocytes. Neoantigen load does not differ between ever-smokers from lung ADCs and SqCCs tumors but is significantly lower in lung ADCs from never-smokers. Neoantigen intratumor heterogeneity as well as clonal neoantigens, which are associated with a higher PD-L1 expression and smoking signatures, elicit T-cell immunoreactivity and influence the response of lung cancer patients to immune checkpoint inhibitors.

Capturing the stromal immune compartment by transcriptome analysis and expression profiles may also provide new insights into the molecular features associated with clinical response to checkpoint inhibitors. To date, no genetic immune signatures have been identified for lung cancer. In the POPLAR trial, patients with pre-existing immunity, defined as high T-effector (Teff) interferon gamma (IFNG)-associated gene expression, had an improved OS with atezolizumab (Teff/IFNG high HR 0.43 versus low Teff/IFNG low HR 1.10).

Currently blood-based biomarkers of immune response are also being explored as a surrogate source of information alternative to tissue samples. The first data on a blood-based TMB test (bTMB) to measure TMB were recently presented in a large number of plasma samples (n = 794) from two pivotal trials with atezolizumab (phase II POPLAR and phase III OAK) and found that high bTMB was associated with a longer PFS in patients treated with atezolizumab. A circulating tumor DNA-TMB validation study (B-F1RST) is underway to prospectively explore the efficacy and safety of multiple targeted therapies in NSCLC patients using Foundation ACT (FACT) platform [ClinicalTrials.gov identifier: NCT03178552]. By using this blood-based testing approach, it may be possible to extend TMB testing to a broad number of patients, including those who are unable to undergo an invasive tumor biopsy. New evidence shows that primary resistance to immune checkpoint inhibitors can be due to abnormal gut microbiome composition. Fecal microbiota transplantation from cancer patients who responded to checkpoint inhibitors into germ-free or antibiotic-treated mice ameliorates the antitumor effects of PD-1 blockade and metabolomic whole genome shotgun sequencing demonstrates correlations between clinical responses and the relative abundance in patient’s stools of Akkermansia muciniphila, a mucin-degrading bacterium of the human intestine, offering novel avenues for manipulating the gut ecosystem.

Perspectives and conclusions
Immunotherapy has already established a firm foothold in the landscape of NSCLC treatment. Therapeutic blockade of immune checkpoint regulators, mainly those that focus on the PD-(L)1 axis, has demonstrated improved clinical outcomes in lung cancer patients by restoring T-cell responses, hence host immunity against tumors. Even so, identifying which patients are going to derive most benefit from these agents is an issue that has still to be resolved. Despite its
inherent analytic and predictive limitations, PD-L1 protein expression testing remains presently the biomarker of choice to inform clinical decision-making on treatment with immune checkpoint inhibitors. In an effort for harmonization, several cross-validation studies between platforms are ongoing and will be key to achieve standardization between IHC assays. Meanwhile, prospective studies assessing cytology specimens as an alternative to tissue samples for PD-L1 testing as well as incorporating novel and more precise immune predictive biomarkers such as TMB are warranted to validate them as reliable predictors of response to immunotherapy.

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