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Reliability of programmed death ligand 1 (PD-L1) tumor proportion score (TPS) on cytological smears in advanced non-small cell lung cancer: a prospective validation study

Costantino Ricci, Elisa Capizzi, Francesca Giunchi, Laura Casolari, Francesco Gelsomino, Karim Rihawi, Filippo Natali, Vanina Livi, Rocco Trisolini, Michelangelo Fiorentino and Andrea Ardizzoni

Abstract

Introduction: Programmed death-ligand 1 (PD-L1) immunohistochemistry (IHC) assessment is mandatory for the single agent pembrolizumab treatment of patients with advanced non-small cell lung cancer (NSCLC). PD-L1 testing has been validated and is currently certified only on formalin-fixed paraffin-embedded materials but not on cytological smears. Unfortunately, a significant proportion of patients, having only cytological material available, cannot be tested for PD-L1 and treated with pembrolizumab. In this study, we aimed to validate PD-L1 IHC on cytological smears prospectively by comparing clone SP263 staining in 150 paired histological samples and cytological smears of NSCLC patients.

Methods: We prospectively enrolled 150 consecutive advanced NSCLC patients. The clone SP263 was selected as, in a previous study of our group, it showed higher accuracy compared with clones 28-8 and 22-C3, with good cyto-histological agreement using a cut-off of 50%. For cyto-histological concordance, we calculated the kappa coefficient using two different cut-offs according to the percentage of PD-L1 positive neoplastic cells (<1%, 1–49% and ⩾50%; <50%, ⩾50%).

Results: The overall agreement between histological samples and cytological smears was moderate (kappa = 0.537). However, when the cyto-histological concordance was calculated using the cut-off of 50%, the agreement was good (kappa = 0.740). With the same cut-off, and assuming as gold-standard the results on formalin-fixed paraffin-embedded materials, PD-L1 evaluation on smears showed specificity and negative predictive values of 98.1% and 93.9%, respectively.

Conclusion: Cytological smears can be used in routine clinical practice for PD-L1 assessment with a cut-off of 50%, expanding the potential pool of NSCLC patients as candidates for first-line single agent pembrolizumab therapy.

Keywords: cytological smears, immunohistochemistry, non-small cell lung cancer, PD-L1

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Correspondence to:
Michelangelo Fiorentino
Department of Pathology, Maggiore Hospital, Via dell'Ospedale 8, Bologna, 40132, Italy

Department of Specialistic, Diagnostic and Experimental Medicine, University of Bologna, Bologna, Italy
michelangelo.fiorentino@unibo.it

Costantino Ricci
Department of Pathology Maggiore Hospital , Bologna, Italy
Francesca Giunchi
Department of Pathology, Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italia

Elisa Capizzi
Department of Pathology, Department of Specialistic, Diagnostic and Experimental Medicine, University of Bologna, Bologna, Italy

Laura Casolari
Andrea Ardizzoni
Department of Medical Oncology,

Francesco Gelsomino
Department of Medical Oncology, Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italy

Karim Rihawi
Department of Specialistic, Diagnostic and Experimental Medicine, University of Bologna, Bologna, Italy

Filippo Natali
Department of Interventional Pulmunology , Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italy
Vanina Livi
Rocco Trisolini
Unit of Interventional Pulmonology, Agostino Gemelli Hospital, Largo Agostino Gemelli, Roma, Italy

Introduction

Programmed death-ligand 1 (PD-L1) immunohistochemistry (IHC) assessment is recommended in baseline molecular screening of patients with advanced non-small-cell lung cancer (NSCLC) for programming optimal medical treatment strategy.[1,2] Different anti-PD-L1 clones (Dako 22C3 pharmDx and Ventana SP263), nivolumab (Dako 28–8 pharmDx) (Ventana SP142) have been approved by the United States Food and Drug Administration (FDA) for detection of PD-L1 in advanced NSCLC patients.[3–5] Although

several comparative studies showed 22-C3, 28-8 and SP263 to have comparable detection performance, only 22-C3 and SP263 have been certified for *in vitro* diagnostics on automated platforms, and these are now companion diagnostics mandatory to establish eligibility for single-agent pembrolizumab therapy.[6,7] The majority of NSCLC patients are diagnosed at an advanced stage, with diagnosis and staging preferably based on cyto-histological samples obtained by transthoracic computed tomography (CT)-guided needle biopsies or ultrasound endobronchial (EBUS) bronchoscopy.[8] Unfortunately, the success rate of these two diagnostic techniques is variable and, in a large percentage (30%–50%) of patients, only cytological material can be obtained.[9–11] Cytological samples can often be processed to obtain formalin-fixed paraffin-embedded (FFPE) material suitable for PD-L1 evaluation.[12–19] However, about 20–30% of advanced NSCLC patients are diagnosed exclusively on cytological smears.[20] As PD-L1 testing is currently certified on FFPE materials (histological samples and cytological samples as clots or pellets) but not on cytological smears, a significant proportion of advanced NSCLC patients cannot be tested for PD-L1 expression and are precluded from first-line single-agent immune checkpoint inhibitor treatment.[12–19,21] For this reason, expanding the evaluation of PD-L1 to cytological smears is essential to increase the number of patients who may benefit from this revolutionary treatment. In previous work by our group, we found that clone SP263 has the best sensitivity and specificity compared with clones 22-C3 and 28-8 on histological specimens, with good cyto-histological concordance (kappa = 0.626) using the cut-off of 50% of positive neoplastic cells.[21] Here, we report the results of a prospective validation study of anti-PD-L1 SP263 IHC assessment on paired histological and cytological smears of 150 NSCLC patients.

Material and methods

Ethics statement

All clinical investigations were conducted according to the principles of the Declaration of Helsinki. The study was approved by the internal review board of the S. Orsola-Malpighi Hospital with study number 39/2017/U.Tess, on 14 March 2017. All information regarding the human material used in this study has been managed using anonymous numerical codes. All participants provided written informed consent for participation in the study.

Patient population

We prospectively enrolled 150 patients who received a diagnosis of advanced NSCLC at the Services of Interventional Pulmonology and Pathology of the S. Orsola-Malpighi and Maggiore Hospitals in Bologna between October 2017 and June 2019. The specimens were obtained from transthoracic (CT or ultrasound-guided) needle aspiration biopsies, or from needle aspiration procedures performed during endosonograhy [endobronchial ultrasound (EBUS); endoscopic ultrasound (EUS)] or guided-bronchoscopy. The 150 patients were selected from a pool of 1055 consecutive subjects according to the following inclusion and exclusion criteria.

Inclusion criteria. (i) Availability of at least one positive histological sample and one positive cytological smear with a given diagnosis of advanced NSCLC; (ii) availability of at least 100 cancer cells in each sample; (iii) procurement of two sample pairs from the same site (lung and/or lymph node) taken at the same time or with a maximum time interval of 90 days; (iv) first diagnosis of NSCLC and no previous systemic treatment; (v) age $\geqslant$18; (vi) patient's signed informed consent.

Exclusion criteria. (i) No availability of cyto-histological material simultaneously collected and suitable for comparative evaluation; (ii) lack of previously mentioned inclusion criteria (age $<$ 18, $<$100 cancer cells in one of the samples, no signed informed consent).

A total of 905 patients were excluded, mostly due to the unavailability of cyto-histological samples simultaneously collected (510) or the absence of at least 100 viable tumor cells in both samples (303). The remaining 92 patients were excluded due to a lack of other criteria (samples obtained from the same site with a maximum time interval of 90 days, first diagnosis with no previous treatment and signed informed consent).

Specimens and PD-L1 IHC staining

Tissue specimens were fixed in 4% buffered formalin for 8–24 h and embedded in paraffin. A 3-μm tissue section was freshly cut from each FFPE block for PD-L1 staining before starting

the immunohistochemical characterization of the tumor (TTF-1 and p40). Cytological smears were either fixed promptly with 95° ethanol or with the isopropanol-based MicroFix fixation spray (Diapath, Martinengo, Italy), and hydrated. Cytological specimens were divided to obtain one slide for the May Grünwald-Giemsa rapid procedure and one for the Papanicolau definitive staining, de-hydrated to absolute ethanol, cleared in xylene, and finally mounted with cover-slides. Stained smears selected for the study were left in a xylene bath for 48 h to allow gentle slip-off of cover-slides, then rehydrated through graded alcohols, de-stained in 1% HCl in 70° ethanol and rinsed in PBS before starting IHC. IHC and immunocytochemistry were carried out using the anti-PD-L1 clone SP263 (Ventana Medical System, Tucson, AZ, USA) and the OptiView DAB IHC Detection Kit (Ventana) on the automated Ventana BenchMark ULTRA System (Ventana). Clone SP263 was selected since in the retrospective analysis it showed higher accuracy compared with clones 28-8 and 22-C3 on histological samples, with a good cyto-histological concordance (kappa = 0.626) using the cut-off of 50%.[22] The immunostaining protocols required different pre-treatment time with CC1 solution (Ventana) for histological and cytological samples: retrieval CC1 was 56 min for histological and 32 min for cytological samples. Conversely, the incubation time was the same for both histology and cytology: 16 min 37°C, pre-diluted. PD-L1 positive and negative controls were performed simultaneously for each case. Tumor-infiltrating macrophages could overexpress PD-L1 and interfere with correct evaluation of PD-L1 expression by tumor cells. For this reason, double staining with anti-PD-L1 (SP263) and anti-CD68 (KP-1, prediluted, Ventana) was adopted on FFPE samples. The double immunostaining protocol included a retrieval step with CC1 56 min , the first incubation with SP263 antibody for 16 min at 37°C followed by DAB (3,3'-diaminobenzidine) staining (OptiView DAB IHC Detection Kit, Ventana), and a second incubation with anti-CD68 antibody for 32 min at 36°C followed by alkaline phosphatase (AP) RED staining (ultraView Universal Alkaline Phosphatase Red Detection Kit, Ventana). The double immunostaining has already been used in the retrospective analysis and previously validated in a limited number of cases without significant discrepancies compared with the single staining (data not shown).[22] According to the guidelines for PD-L1 interpretation by the manufacturers of clone SP263, immunostaining was evaluated only in the cell membranes, in at least 100 viable neoplastic cells per sample.[5,6] Samples with less than 100 viable neoplastic cells were excluded (see *Inclusion criteria*). Neoplastic cells were judged positive with a membrane staining of any intensity, while cytoplasmic and/or nuclear staining were assessed as positive only with a coexistent membrane staining. The slides of the present study have been read by three pathologists with a multiheaded microscope in a non-blinded manner. In case of a discrepancy among the evaluators, the case was discussed until agreement was reached.

Statistical analyses

Statistical analyses were performed using the software SPSS, version 22.0 (SPSS, Chicago, IL. USA). For cyto-histological agreement, we calculated the kappa coefficient of concordance. The agreement was considered weak for kappa values within 0.2–0.40; moderate for values 0.41–0.60; and good for values 0.61–0.99. For statistical analyses, we adopted two different cut-off systems according to the percentage of PD-L1 positive neoplastic cells, one with three score groups (score 0: 0% and/or $<1\%$, score 1: 1–49%, score 2: $\geqslant 50\%$) and one with two score groups (score 0: $<50\%$, score 1: $\geqslant 50\%$). To determine values of sensibility (SS), specificity (SP), diagnostic accuracy (AD), positive predictive value (PPV) and negative predictive value (NPV) of the clone SP263 on cytology, we assumed as the reference standard (gold standard) the PD-L1 values on histology (using only the cut-off system with 2 score groups, $<50\%$ and $\geqslant 50\%$). The power calculation of 150 cases was based on the following asymptotic normal distribution theory. If the study is to have 80% power against the hypotheses that sensitivity and specificity are $\geqslant 80\%$, a total of 43 cases (PD-L1 $\geqslant 50\%$) and 43 controls (PD-L1 $< 50\%$) will be required. Assuming that the prevalence of PD-L1 expression level $>50\%$ is about 30%, a total of at least 145 samples could be evaluated to detect 43 cases.

Results

PD-L1 expression of the cohort

The mean age of the 150 enrolled patients was 69.9 years [range: 47–87), 92 (61.3%) males and 58 (38.7%) females]. The diagnosis was rendered according to the World Health Organization

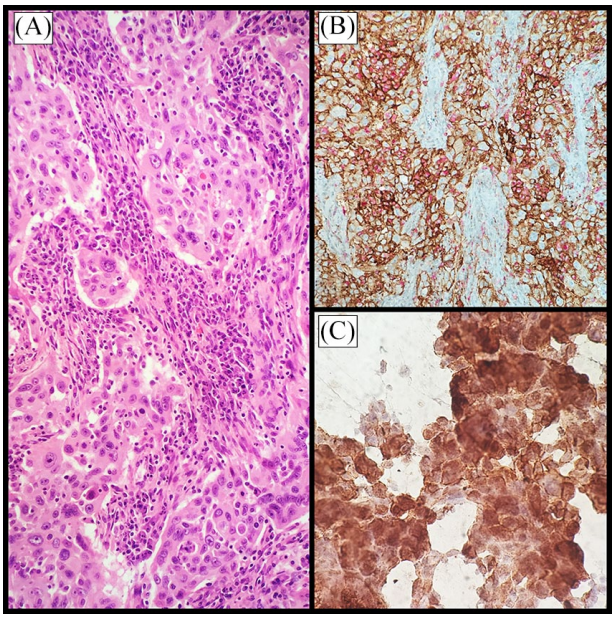

Figure 1. Case-69: Concordant positive case with high PD-L1 expression (⩾50%) in the histological sample and paired cytological smear of solid adenocarcinoma. (A) Hematoxylin-eosin (original magnification ×200). (B) High expression of PD-L1 (95% cancer cells) in the histological specimen with the double immunostaining with SP263 (brown) and CD68 (red) (original magnification ×200). (C) High expression of PD-L1 (90% cancer cells) in the paired cytological smear with scattered positive inflammatory cells (original magnification ×200). PD-L1, programmed death-ligand.

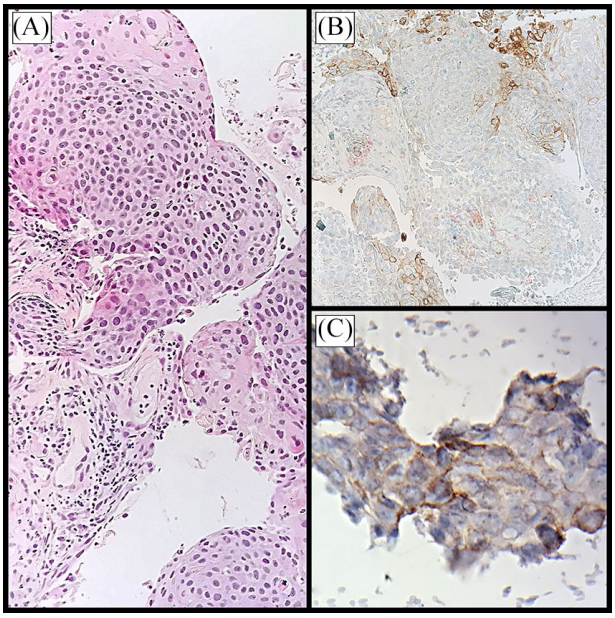

Figure 2. Case-135: Concordant case with PD-L1 expression (<1%–⩾50%) in the histological sample and paired cytological smear of squamous cell carcinoma. (A) Hematoxylin-eosin (original magnification ×200). (B) Scattered positive cells for PD-L1 (10% cancer cells) in the histological specimen with the double immunostaining with SP263 (brown) and CD68 (red) (original magnification ×100). (C) Rare positive cells for PD-L1 (2% cancer cells) in the paired cytological smear (original magnification ×200). PD-L1, programmed death-ligand.

(WHO) classification with the following distribution of the different histotypes: 42 (28%) squamous cell carcinoma (SCC) and 108 (72%) non-squamous carcinoma (105 adenocarcinoma (ADK), and 3 pleomorphic carcinoma (PC). In all the enrolled cases, the clone SP263 stained both cancer and inflammatory cells, but double immunostaining with anti-CD68 and anti-PD-L1 SP263 helped u to discriminate neoplastic cells from macrophages (Figures 1–3). In the histological samples, double staining with anti-PD-L1 SP263 and anti CD68 showed ⩾50% PD-L1 positive tumor cells in 44 cases (29.3%), ⩾1%–<50% PD-L1 positive tumor cells in 49 cases (32.7%), while 57 cases (38%) turned out negative. Using the cut-off system with only two score groups, we obtained 44 cases (29.3%) with ⩾50% PD-L1 positive tumor cells and 106 cases (70.7%) with <50% PD-L1 positive tumor cells. In the cytological samples, as found in the retrospective study, no differences in staining were detected using smears stained with the May Grünwald-Giemsa or the Papanicolau procedure.[22] The clone SP263 stained both tumor and inflammatory cells (Figures 1–3). In the cytological smears, the staining with the anti-PD-L1 SP263 showed in ⩾50% PD-L1 positive tumor cells 33 cases (22%), ⩾1%–<50% PD-L1 positive tumor cells in 25 cases (16.7%), while 92 cases (61.3%) turned out negative. Using the cut-off system with only 2 score groups, we found 33 cases (22%) with ⩾50% PD-L1 positive tumor cells and 117 cases (78%) with <50% PD-L1 positive tumor cells. PD-L1 immunoreactivity in FFPE and cytological samples is described in Table 1. As in the retrospective study, we encountered several staining artifacts in PD-L1 staining on cytological smears (non-specific brown blurring in multi-layered cell placards and non-specific nuclear and/or cytoplasmatic without membranous staining). These staining were considered artifacts and judged negative.[22]

Concordance of PD-L1 expression in paired cyto-histological samples

The cyto-histological concordance using the system with three cut-off groups (<1%, ⩾1–<50%, ⩾50%) was moderate (kappa = 0.537; Table 1). However, when the cyto-histological concordance was calculated using only the 50% cut-off (<50% *versus* ⩾50%), the agreement was good (kappa = 0.740; Table 1). The cyto-histological comparison for SP263 staining revealed major discrepancies in 9 out of 150 cases (6%). All these cases showed staining in <1% of neoplastic cells

in the cytological smears as opposed to ⩾50% in the FFPE counterparts. No case with ⩾50% of positive neoplastic cells in the cytological smears and <1% in the FFPE counterparts was found. In the remaining 141 cases (94%), we observed complete cyto-histological concordance in 105 cases (70%) and minor cyto-histological discrepancies in 36 cases (24%). Adopting the binary cut-off (<50% and ⩾50%), and assuming as standard reference (gold-standard), the values obtained on FFPE samples, the values of SS, SP, AD, PPV, and NPV of the clone SP263 on cytological smears were 70.4%, 98.1%, 90%, 93.9%, and 88.9%, respectively.

Discussion

Immunohistochemical evaluation of PD-L1 expression is currently mandatory to administer single-agent pembrolizumab in advanced NSCLC patients. Specifically, these patients are eligible for first-line or second-line pembrolizumab therapy based on PD-L1 tumor proportion score (TPS) cut-off values of ⩾50% or ⩾1, respectively.[1,2] However, recently, KN 407 and 189 studies have shown that pembrolizumab in combination with platinum-based chemotherapy is superior to chemotherapy alone regardless of PD-L1 expression in first-line treatment of both squamous and non-squamous advanced NSCLC.[23,24] Based on these results, the need for PD-L1 assessment seems now to be less compelling if a patient is possibly a candidate for chemo-immunotherapy combination. However, the evaluation of PD-L1 remains mandatory for the administration of single agent pembrolizumab, especially as first-line treatment for those patients who are not eligible for chemotherapy.[25,26] The immunohistochemical assessment of PD-L1 has been validated in histological (biopsies and surgical specimens) and cytological samples treated as FFPE materials (pellets and clots).[12–19,21] However, in 20–30% of patients, the only available diagnostic material is a cytological smear, thereby precluding PD-L1 evaluation and pembrolizumab therapy.[20] In a previous retrospective work by our group, we found as the anti-PD-L1 clone SP263 had good cyto-histological concordance, with a cut-off of 50% of positive neoplastic cells (kappa = 0.626).[22] In the present study, we validated these results prospectively, providing additional evidence that cytological smears could be used successfully for the immunohistochemical assessment of PD-L1 with the TPS cut-off value of 50% (kappa = 0.740). Although previous studies have already highlighted a good cyto-histological agreement using cytological smears, these results are difficult to compare (different clones and cut-offs) and to apply to clinical practice (small case series and/or retrospective studies).[12,13,22,27,28] Our study has a larger sample size and is the first with prospective case enrollment. Besides, PD-L1 staining was carried out on routinely collected cyto-histological material, a very short time after paraffin embedding. We have previously reported that PD-L1 immunoreactivity fades with time in NSCLC tissue blocks and therefore PD-L1 should be assessed carefully in tissue blocks older than 1 year.[29] In previous retrospective studies on the cyto-histological agreement, the evaluation of PD-L1 was performed also on archival tissues older than 1 year.[12–15,17–19,22,27,28] As a consequence, it is not surprising that in some of these studies the number of cases with PD-L1 ⩾50% was lower than expected.[13,14,28,30] In our opinion, these data must be taken into account in the interpretation of the results, since they were obtained from case series that do not reflect the real distribution of PD-L1 positivity in NSCLC patients. On the contrary,

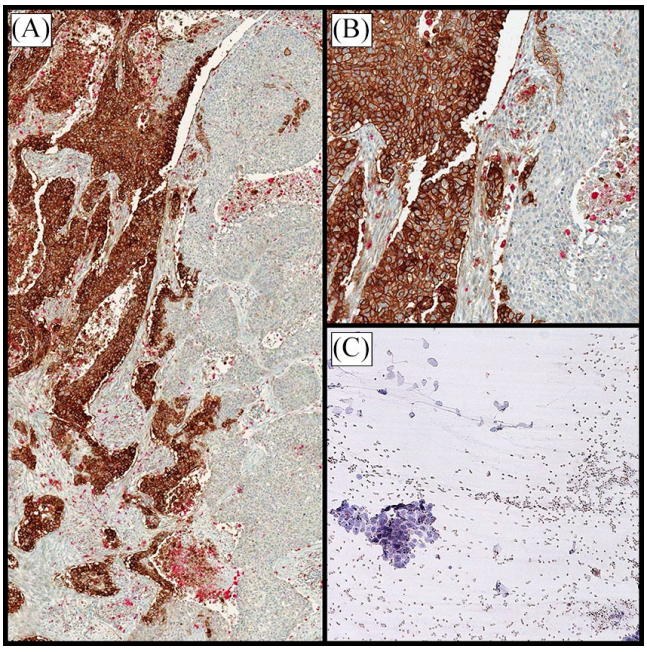

Figure 3. Case-40: Discordant case with high but heterogeneous PD-L1 expression (⩾50%) in the histological sample and no PD-L1 expression (<1%) in paired cytological smear of squamous cell carcinoma. (A–B) High and heterogeneous expression of PD-L1 (50% cancer cells) in the histological specimen with the double immunostaining with SP263 (brown) and CD68 (red) (A: original magnification ×50; B: original magnification ×100). (C) Cancer cells negative for PD-L1 in the paired cytological smear (original magnification ×200).
PD-L1, programmed death-ligand.

Table 1. Comparison of PD-L1 immunohistochemical expression in cytological and histological samples by sp263 antibody using different cut-off.

FFPE samples PD-L1 (SP263) expression						Kappa value
Cytology samples PD-L1 (SP263) expression		<1%	1–49%	⩾50%	Total	
	<1%	55	28	9	92	0.537
	1–49%	2	19	4	25	
	⩾50%	0	2	31	33	
	Total	57	49	44	150	
			<50%	⩾50%	Total	
		<50%	104	13	117	0.720
		⩾50%	2	31	33	
		Total	106	44	150	

FFPE, formalin-fixed paraffin-embedded; PD-L1, programmed death-ligand.

the percentage of patients with PD-L1 ⩾50% in our case series was 29.3% (44/150), in line with that expected in advanced NSCLC patients.[31] In our series, we found nine major discrepant cases with ⩾50% positive cells in the histological samples but negative (<1%) in the paired cytological smears. All these cases showed a heterogeneous immunoreactivity in the histological samples, with positive areas intermixed with negative ones (Figure 3). Therefore, discrepant cases can likely be explained by heterogeneous PD-L1 expression in NSCLC and cytological sampling falling into a negative PD-L1 tumor area. Tumor heterogeneity is a known bias of all predictive tests of response to therapy in NSCLC that cannot be ruled out and must be considered in the therapeutic evaluation of these patients.[32–34] It is a matter of note that, from the perspective of using cytological smears in the absence of FFPE material, the majority of discrepancies found in our study would not have modified treatment strategy in these patients. Importantly, we did not find cases with ⩾50% positive cells in the smears but negative (<1%) in the paired histological samples, where a major discrepancy could result in inappropriate use of pembrolizumab in a PD-L1 negative patient.[22] Besides, we found a very high specificity for SP263 clone in cytology (98%), which emphasizes the low probability that a negative patient (PD-L1 < 50%) could be identified erroneously as positive (⩾50%) on cytology. In our case series, only two cases showed staining in ⩾50% of neoplastic cells in the cytological smear and in ⩾1%–<50% in the FFPE counterparts, a minor discrepancy that should not have a significant negative impact on the appropriate first-line use of single-agent pembrolizumab therapy. In practice, in a patient without histological material available, a cytological smear with ⩾50% PD-L1 TPS, would not mandate a re-biopsy for confirmation on histological material. Overall, we found that cyto-histological concordance with the SP263 clone was good (kappa = 0.740) with the ⩾50% cut-off but turned out moderate (kappa = 0.537) including also the >1% cut-off. We explained this lower agreement with the low reproducibility of the semi-quantitative scoring of PD-L1 with the >1% cut-off and the presence of staining artifacts in the cytological smears. A limitation of the present study was the reading of the slides by three pathologists with a multi-headed microscope in a non-blinded manner. Therefore, a concordance study on the evaluation of PD-L1 on smears is mandatory to establish their suitability with a >1% cut-off. In conclusion, we provide convincing evidence that cytological smears can be used successfully in routine clinical practice for the detection of PD-L1. Staining for PD-L1 in cytological smears of NSCLC is suitable with a TPS cut-off ⩾50%, with a low rate of major discrepancies that would not affect an appropriate use of single-agent pembrolizumab in first-line treatment of advanced NSCLC. Our study confirms that cytological smears could be used as a surrogate for

PD-L1 assessment in the absence of FFPE samples, leading to a significant increase in the number of patients testable for PD-L1 and possibly eligible for single-agent pembrolizumab therapy.

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Conflict of interest statement

Andrea Ardizzoni, Honoraria (self) for advisory board participation: BMS, MSD, ROCHE, Astra Zeneca, Eli-Lilly-Research Grants to my Institution: Celgene, BMS, Ipsen, Roche. All the other authors declare no conflict of interest.

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ORCID iD

Michelangelo Fiorentino https://orcid.org/0000-0002-1749-150X

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