Research Article

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Immunotherapy

Optimizing PD-L1 evaluation on cytological samples from advanced non-small-cell lung cancer

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Aim: Programmed cell death-ligand 1 (PD-L1) predicts response to immune checkpoint inhibitors in non-small-cell lung cancer (NSCLC) patients. Most NSCLCs are diagnosed at an advanced stage and using minimally invasive diagnostic procedures that yield small biopsies or cytological samples. **Methods:** Cytological smears and paired histological samples from 52 advanced NSCLC patients were tested for PD-L1 expression by immunocyto/histochemistry (ICC/IHC) and for *PD-L1* gene status by FISH. **Results:** *PD-L1* was overexpressed in 9/52 (17%) cytological samples and in seven (13.5%) matched biopsies. The concordance between immunocytochemistry and IHC was 92.3% (48/52; $p < 0.001$). The concordance between *PD-L1* gene status on cytology and histology was 69.2% (18/26; $p < 0.001$). No correlation between IHC and fluorescence *in situ* hybridization results was found. **Conclusion:** Our data support the feasibility and reliability of PD-L1 protein and *PD-L1* gene assessment on direct cytological smears from NSCLC patients whenever histological sample are inadequate.

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Approximately 70% of patients with non-small-cell lung cancer (NSCLC) present with a locally advanced or metastatic disease at first diagnosis and, therefore, they are excluded from surgical treatment. In these cases, the diagnosis is performed on transbronchial biopsy (TBB) samples and/or cytology smears obtained by endobronchial ultrasound-transbronchial needle aspiration (EBUS-TBNA), an emerging, minimally invasive procedure for sampling mediastinal and hilar lymph nodes [1]. Both techniques provide only a small amount of cells and, in some cases, the cytology smears may represent the only source of material for the pathological diagnosis and for the molecular and immunohistochemical tests required to address the choice of therapy.

Recently, the tissue expression of programmed cell death-ligand 1 (PD-L1 or CD274), either on tumor cells or infiltrating tumor lymphocytes has been suggested as a marker of response to immune blockade treatment strategies. Antibodies against PD-L1 and its receptor programmed cell death-1 (PD-1) [2], such as nivolumab, pembrolizumab (anti-PD-1) and atezolizumab (anti-PD-L1) are the new standard of care in advanced NSCLC treatment [3] and they are administered following PD-L1 testing.

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Patients whose tumors lack driver oncogene mutations (i.e., EGFR mutations or ALK rearrangements) and express PD-L1 on at least 50% of tumor cells are eligible for single-agent pembrolizumab treatment at first-line therapy [4]. Therefore, the determination of PD-L1 expression is mandatory to determine the correct treatment strategy for advanced NSCLC.

In some NSCLC, the expression of PD-L1 can be upregulated by amplification of the *PD-L1* gene on chromosome 9p24.1, therefore, the *PD-L1* gene copy number is a potential predictive marker to immune checkpoint blockade in alternative or association to immunostaining, although more studies are warranted to confirm this observation [5–7]. In the present study, we compared PD-L1 tissue expression by immunostaining and *PD-L1* gene copy number alterations (CNAs) tested by FISH on cytological smears and matched histological specimens obtained in routine practice. We aimed to evaluate whether cytology specimens can be used to assess PD-L1 status in a diagnostic clinical setting.

Materials & methods

Patients

Our series included 52 cytological smears obtained from primary or metastatic lesions of patients with a diagnosis of NSCLC attending the Medical Oncology Unit of the University Hospital of Parma, Italy, from January 2013 to February 2019. Cytological samples were retrospectively tested for PD-L1 immunostaining based on the availability of matched formalin-fixed paraffin-embedded (FFPE) samples with known PD-L1 status. In 26 cases, slides were also available for *PD-L1* assessment by FISH on cytology and corresponding histology.

A total of 27 out cytological samples were obtained from primary lung tumors, either by computed tomography-guided transthoracic needle aspiration (nine cases) or by touch imprints (14 cases from computed tomography-needle core biopsy and four from TBB). Mediastinal or hilar lymph node metastases were sampled by conventional TBNA (11 cases) or by EBUS-TBNA (seven cases). Four supraclavicular lymph node metastases were obtained by ultrasound-guided fine needle aspiration. Three cases were pleural effusions.

The 52 FFPE samples were from primary tumors in 39 cases, from lymph node metastases in ten cases and three samples were pleural effusions. Among the 39 primary tumors, there were 12 surgical resection specimens, 10 TBBs, 15 core biopsies and two cell blocks. The 10 lymph node metastases and the three pleural effusions were included as cell blocks.

This retrospective study was performed in accordance with a protocol approved by the institutional review board/independent ethics committee of the University Hospital of Parma (Reference number 0000607 on 4 June 2019). All patients provided written informed consent for the use of samples.

Cytological diagnosis

For cytological diagnosis, the aspirated material was smeared onto glass slides, air-dried and stained with May–Grünwald–Giemsa quick stain (Bio Optica, Milano, Italy) for a rapid on-site evaluation to verify the adequacy of the samples. When possible, additional slides were stored unstained and unfixed at -20°C for immunostains, FISH assays and DNA extraction for molecular assays.

Immunocyto/histochemistry

Fresh cytological smears were fixed in methanol-acetone (2:1). Immunocyto/histochemical assays were performed on the automated Ventana BenchMark ULTRA System (Ventana Medical System, AZ, USA) using the prediluted monoclonal anti-PDL1 antibody (clone SP263, Ventana) and the OptiView DAB IHC Detection Kit (Ventana). PD-L1 positive and negative controls were also tested for each run. PD-L1 expression was evaluated on the entire slide excluding areas of necrosis or poor conservation. At least 100 tumor cells per sample were counted. Cancer cells were considered positive based on a partial/complete membrane staining of any intensity. Samples were evaluated independently by two observers. The cut-off value for PD-L1 positivity was set at ≥50% positive tumor cells [3,4].

FISH on cytological smears

Unstained cytological smears were fixed in methanol-acetic acid (3:1) and incubated for 5 min at 37°C in 20 μg/ml proteinase K. After alcohol dehydration, the slides were processed by FISH using 10 μl of a Spectrum Orange fluorophore-labeled α-satellite DNA probe for chromosome 9 and Spectrum Green fluorophore-labeled DNA probe for CD274 (*PD-L1*) and PDCD1LG2 (*PD-L2*) genes (ZytoLight SPEC CD274, PDCD1LG2/CEN9 Dual Color Probe, ZytoVision, Bremerhaven, Germany).

Samples were denaturated at 67°C for 5 min and incubated overnight at 37°C using a Hybrite denaturation/hybridization system for FISH (Abbott-Vysis, Wiesbaden, Germany). The following day, slides were incubated in wash buffer (0.3% NP-40, 2 × saline–sodium citrate buffer, pH 7.0–7.5) at 73°C for 2 min, air-dried in the dark and counterstained with 4′,6-diamidino-2′-phenylindole (DAPI) (Abbott-Vysis). FISH images were processed utilizing a Nikon Ni-U (Nikon Corporation, Tokyo, Japan) fluorescence microscope equipped with a 100-W mercury lamp. Separate narrow band pass filters for the detection of spectrum green, orange and DAPI were used. Separate image acquisitions were made on each field for orange and green signals at five different levels for each cell. Only distinct fluorescent signals distributed at different levels of the cell were scored positive. Two independent observers visually scored at least 50 evaluable tumor cells for each case. Results were expressed as ratio of the number of copies of *PD-L1* to the number of chromosome 9 signals. A ratio ≥2 indicated amplification. Cases showing two PD-L1 signals and two centromeric signals per cell were classified as diploid. Polysomy was defined as presence of ≥2.5 green signals per nucleus independently from the orange signals. Samples with fewer *PD-L1* copies than centromere 9 copies were classified as deleted (ratio ≤ 0.8).

FISH on paraffin sections

FFPE tissue samples were cut into 4-μm-thick sections and incubated overnight at 56°C. Deparaffinization, pretreatment, enzyme digestion, and fixation of slides were performed using the Vysis paraffin pretreatment kit (Abbot-Vysis) according to the manufacturer's recommended protocol. For hybridization, 10 μl of the same *PD-L1-PD-L2* probe (ZytoVision) used for FISH cytology were applied to tissue sections that were denaturated at 72°C for 2 mins and incubated overnight at 37°C in the HYBrite system (Abbot-Vysis). The slides were then washed at 72°C for 2 min and counterstained with DAPI. Control slides (Abbot-Vysis) were included in each assay run. For each specimen, at least 50 cells were scored for both *PD-L1* and chromosome 9 signals by using the same image analysis system used for cytological samples. For defining *PD-L1* status the same criteria used for cytological samples were applied.

Statistical analysis

Statistical analysis was performed with the package IBM-SPSS v.22. Concordance among cytological and histological methods was reported as absolute percent agreement and tested by Cohen's K. Associations were reported by Cramer's V and contingency coefficient.

All the values were considered statistically significant for $p < 0.05$.

Results

The main characteristics of the 52 NSCLC patients are summarized in Table 1. 36 patients were male and 16 were female (median age 71 years, range 41 to 88 years). The histotype distribution was as follows: 41 (79%) adenocarcinomas, eight (15%) squamous cell carcinomas, two (4%) large cell and one (2%) pleomorphic carcinomas. The histological diagnoses were performed according to the 2015 World Health Organization Classification of Lung Tumors [8].

In 40 of 52 (77%) cases, the site of sampling was the same for cytology and histology (27 lungs, seven mediastinal or hilar lymph nodes, three sovraclavear lymph nodes and three pleural effusions). In 12 cases, the cytological diagnosis was performed on a lymph node metastasis (11 mediastinal or hilar and one sovraclavear lymph nodes) while the histological diagnosis was from a lung lesion.

Immunocyto/histochemistry

The detailed results of PDL1 expression and *PDL1* gene status on cytology and paired histology in each NSCLC sample are shown in Table 2. Nine of 52 (17%) cytological samples (four lung lesions, four mediastinal/hilar and one sovraclavear lymph nodes) showed PD-L1 specific membrane staining in ≥50% of cells (Figures 1A & 2A). Likewise, nine of the 52 (17%) histological samples (six lung lesions, two mediastinal/hilar and one sovraclavear lymph nodes) were PD-L1-positive (Figures 1B & 3B). Comparing the results obtained on cytology and corresponding histology, 41 (79%) cases were PD-L1-negative, seven (13%) cases were PD-L1-positive and four cases were discordant. Two discordant cases were positive on histology and negative on cytology and two cases vice versa (Table 2, case n° 7, 16, 17, 23). Representative examples of discordant cases are shown in Figures 2 and 3. Based on a PD-L1 cut-off value of ≥50% positive cells, the concordance of PD-L1 status between cytology and histology samples was 92.3% (48/52), Cohen's K = 0.731 ($p < 0.001$) (Table 3). For a cut-off value of ≥1%

Table 1. Patient characteristics.

	Number of patients (n = 52)	%
Gender		
Males	36	69
Females	16	31
Age (year)		
Median (range)	71 (41–88)	
Histotype		
Adenocarcinoma	41	79
Squamous cell CA	8	15
Large cell CA	2	4
Pleomorphic CA	1	2
Cytology site of lesion		
Lung	27	52
Mediastinal/hilar LN	18	35
Sovraclavear LN	4	7
Pleural effusion	3	6
Cytology procedure		
CT-TTNA	9	17
Touch-imprint	18	35
TBNA	11	21
EBUS-TBNA	7	13
US-FNA	4	8
Pleural effusion	3	6
Histology site of lesion		
Lung	39	75
Mediastinal/hilar LN	7	13
Sovraclavear LN	3	6
Pleural effusion	3	6
Histology procedure		
Surgical resection	12	23
TBB	10	19
Core biopsy	15	29
Cell block	15	29

CA: Carcinoma; CT-TTNA: Computed tomography-guided transthoracic needle aspiration; EBUS-TBNA: Endobronchial ultrasound-transbronchial needle aspiration; LN: Lymph node; TBB: Transbronchial biopsy; TBNA: Transbronchial needle aspiration; US-FNA: Ultrasound-guided fine needle aspiration.

PD-L1-positive cells, the cytohistological agreement was 76.9% (40/52), Cohen's K = 0.545 ($p < 0.001$). The agreement for paired samples from different sites (lung lesions vs lymph nodes) was 91.6% (11/12).

FISH

PD-L1 FISH was evaluable in 33 of the 52 (63%) cytological samples (Table 2). Of the 19 unassessable cases, 12 were run out of slides, five were technically unsatisfactory and two smears had an insufficient number of cells. FISH results on FFPE sections were obtained in 26 of the 33 cases evaluated on cytology; in four cases, the paraffin blocks run out and in three cases the hybridization was not adequate.

Of the 33 cytological samples evaluable for *PD-L1* FISH, two cases (6.1%) were amplified, 11 were diploid (33.3%), nine (27.3%) polysomic and 11 (33.3%) were deleted. Only one polysomic case showed four green and four orange signals per nucleus while the others showed a lower level of polysomy with <4 signals. Representative images of *PD-L1* FISH are shown in Figure 4.

Among the 26 histological samples, *PD-L1* amplification was found in two (8%) cases. A total of 11 of the 26 (42%) FFPE samples were diploid, five (19%) polysomic and eight (31%) were deleted. An example of a lymph node metastasis showing *PD-L1* amplification is shown in Figure 1C.

Table 2. PD-L1 status evaluated by immunocyto/istochemistry and *PD-L1* gene status evaluated by FISH in cytological and histological samples of non-small-cell lung cancer.

	Site of sampling		PD-L1 expression (%)		*PD-L1* gene status		Histotype
	Cytology	*Histology*	*Cytology*	*Histology*	*Cytology*	*Histology*	
1	Lung	Lung	<1	<1	Polisomic	Diploid	ADC
2	Mediastinal/hilar LN	Lung	>50	60	Deleted	Deleted	SCC
3	Lung	Lung	>50	90	Deleted	Diploid	ADC
4	Lung	Lung	<1	3	Diploid	Diploid	ADC
5	Lung	Lung	<1	10	Deleted	Diploid	ADC
6	Mediastinal/hilar LN	Lung	<1	<1	Polisomic	NE	ADC
7	Lung	Lung	>50	3	Diploid	Diploid	ADC
8	Lung	Lung	<1	<1	Deleted	NE	ADC
9	Lung	Lung	<1	<1	Diploid	Diploid	ADC
10	Lung	Lung	<1	<1	Polisomic	Polisomic	ADC
11	Mediastinal/hilar LN	Lung	<1	<1	Diploid	Diploid	ADC
12	Mediastinal/hilar LN	Lung	<1	<1	Amplified	Amplified	ADC
13	Sovraclavear LN	Sovraclavear LN	>50	80	Inadequate	Not assessed	ADC
14	Mediastinal/hilar LN	Mediastinal/hilar LN	<1	3	Diploid	FFPE block run out	ADC
15	Mediastinal/hilar LN	Lung	>50	80	Amplified	Amplified	ADC
16	Mediastinal/hilar LN	Mediastinal/hilar LN	<1	55	Deleted	Polisomic	Large cell
17	Mediastinal/hilar LN	Lung	>50	<1	Polisomic	Polisomic	ADC
18	Mediastinal/hilar LN	Lung	1	30	Diploid	Diploid	ADC
19	Mediastinal/hilar LN	Lung	<1	<1	NE	Not assessed	ADC
20	Lung	Lung	<1	<1	Deleted	Deleted	ADC
21	Lung	Lung	>50	90	Deleted	Deleted	ADC
22	Lung	Lung	<1	<1	Diploid	Deleted	ADC
23	Lung	Lung	<50	70	Run out	Not assessed	ADC
24	Sovraclavear LN	Sovraclavear LN	<50	35	Diploid	Polisomic	ADC
25	Lung	Lung	>50	60	Run out	Not assessed	SCC
26	Lung	Lung	<50	<1	Run out	Not assessed	SCC
27	Lung	Lung	<1	<1	Run out	Not assessed	ADC
28	Mediastinal/hilar LN	Lung	<1	<1	Run out	Not assessed	ADC
29	Lung	Lung	<1	5	Run out	Not assessed	ADC
30	Lung	Lung	<1	<1	Run out	Not assessed	ADC
31	Lung	Lung	<1	3	Inadequate	Not assessed	ADC
32	Mediastinal/hilar LN	Lung	<1	3	Deleted	Deleted	ADC
33	Pleural effusion	Pleural effusion	<50	5	Diploid	FFPE block run out	ADC
34	Pleural effusion	Pleural effusion	<1	<1	NE	Not assessed	ADC
35	Sovraclavear LN	Lung	<50	30	Run out	Not assessed	ADC
36	Mediastinal/hilar LN	Mediastinal/hilar LN	<1	<1	Deleted	Diploid	ADC
37	Lung	Lung	<50	3	Run out	Not assessed	SCC
38	Lung	Lung	<1	<1	Run out	Not assessed	Pleomorphic CA
39	Lung	Lung	<1	10	Run out	Not assessed	ADC
40	Lung	Lung	<50	20	Deleted	Deleted	SCC
41	Lung	Lung	<50	25	Polisomic	Polisomic	ADC
42	Lung	Lung	0	<1	Diploid	Diploid	SCC
43	Mediastinal/hilar LN	Lung	<50	1	Diploid	Diploid	SCC
44	Pleural effusion	Pleural effusion	<50	3	Polisomic	NE	ADC
45	Mediastinal/hilar LN	Mediastinal/hilar LN	0	<1	Run out	Not assessed	ADC

ADC: Adenocarcinoma; CA: Carcinoma; FFPE: Formalin-fixed, paraffin-embedded; Inadequate: Not evaluable for scanty material; LN: Lymph node; NE: Not evaluable for lack of hybridization; SCC: Squamous cell carcinoma.

Table 2. PD-L1 status evaluated by immunocyto/istochemistry and *PD-L1* gene status evaluated by FISH in cytological and histological samples of non-small-cell lung cancer (cont.).

	Site of sampling		PD-L1 expression (%)		*PD-L1* gene status		Histotype
	Cytology	*Histology*	*Cytology*	*Histology*	*Cytology*	*Histology*	
46	Lung	Lung	<50	1	Deleted	Deleted	ADC
47	Mediastinal/hilar LN	Mediastinal/hilar LN	>50	70	Polisomic	FFPE block run out	ADC
48	Lung	Lung	1	<1	NE	Not assessed	ADC
49	Lung	Lung	1	1	Polisomic	Deleted	Large cell
50	Mediastinal/hilar LN	Mediastinal/hilar LN	0	1	Polisomic	FFPE block run out	SCC
51	Mediastinal/hilar LN	Mediastinal/hilar LN	0	<1	NE	Not assessed	ADC
52	Sovraclavear LN	Sovraclavear LN	0	<1	NE	Not assessed	ADC

ADC: Adenocarcinoma; CA: Carcinoma; FFPE: Formalin-fixed, paraffin-embedded; Inadequate: Not evaluable for scanty material; LN: Lymph node; NE: Not evaluable for lack of hybridization; SCC: Squamous cell carcinoma.

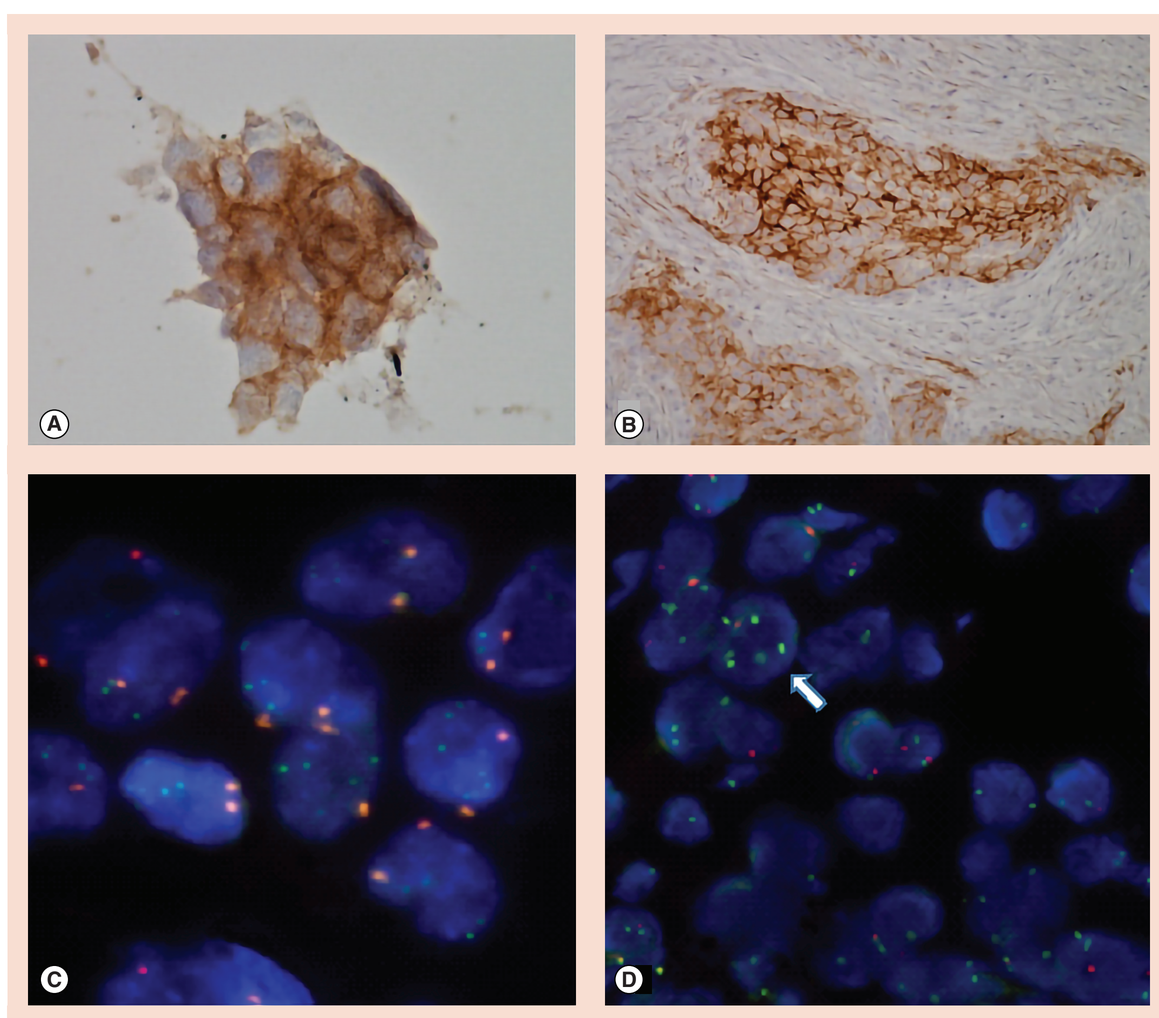

Figure 1. Example of PD-L1 expression and *PD-L1* gene status evaluated on cytological and histological samples from a non-small-cell lung cancer patient. Representative example of a case (n°15) immunostained for PD-L1 (clone SP263, Ventana) showing >50% of positive membrane staining on cytological smear obtained by endobronchial ultrasound-transbronchial needle aspiration from a lymph node metastasis **(A)** (40×). The lung lesion sampled by transbronchial biopsy showed 80% of positive cells **(B)** (20×). **(C)** *PD-L1* FISH assay on cytology. The number of green signals relative to PD-L1 gene copy number is suggestive of *PD-L1* amplification (DAPI stain, ×1250). **(D)** *PD-L1* FISH assay on histology showing a lower number of amplified cells compared with concomitant cytology. The arrow indicates two amplified cells.

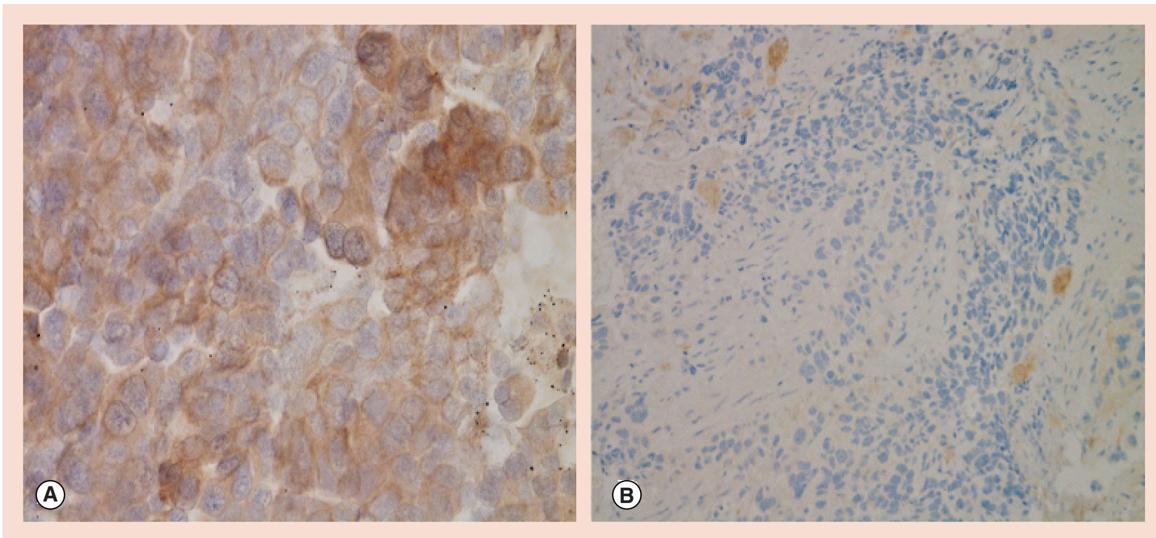

Figure 2. Discordance between PD-L1 expression evaluated on cytology and histology from the same non-small-cell lung cancer patient. (A) Positive immunostaining (>50% PD-L1-positive cells) of a right paratracheal lymph node sampled by transbronchial needle aspiration (40×) and **(B)** absent PD-L1 expression in the lobar bronchus sampled by transbronchial biopsy (20×) (Case n°17).

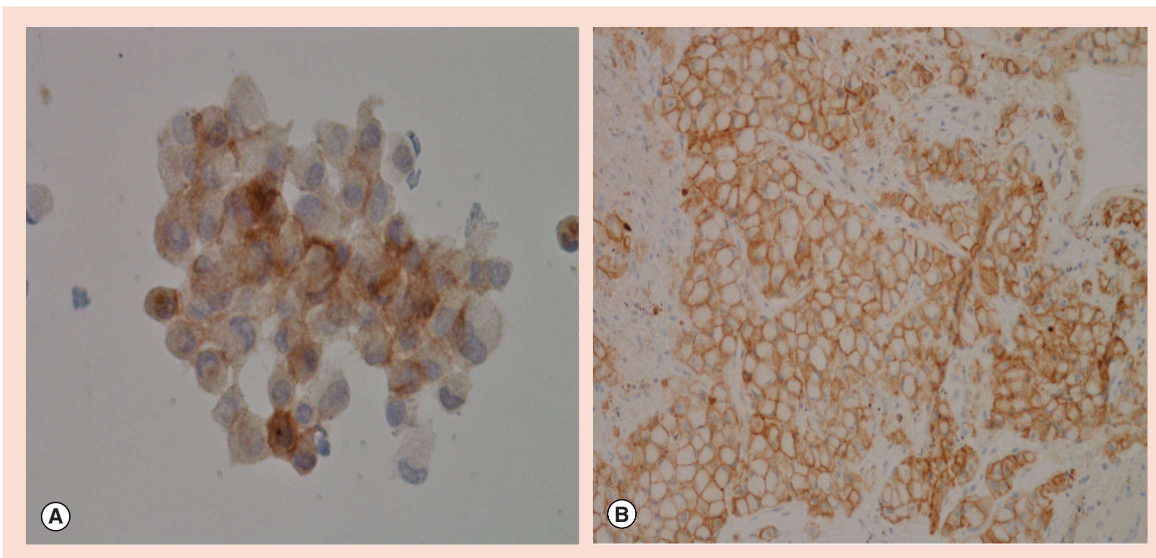

Figure 3. Example of PD-L1 immunostaining on touch imprint and paired transbronchial biopsy. Images of a non-small-cell lung cancer with several signet ring cells. **(A)** Touch imprint from the transbronchial biopsy: only a cluster of immunostained cells is shown while prevalent isolated cells were negative (40×). **(B)** Positive immunostaining of formalin-fixed, paraffin-embedded sample (20×).

Table 3. Comparison between PD-L1 expression evaluated on cytological smears and formalin-fixed, paraffin-embedded samples.

Cytological smears	FFPE samples		
	<50%	≥50%	Total
<50%	41	2	43
≥50%	2	7	9
Total	43	9	52

$p < 0.001$.
FFPE: Formalin-fixed, paraffin-embedded.

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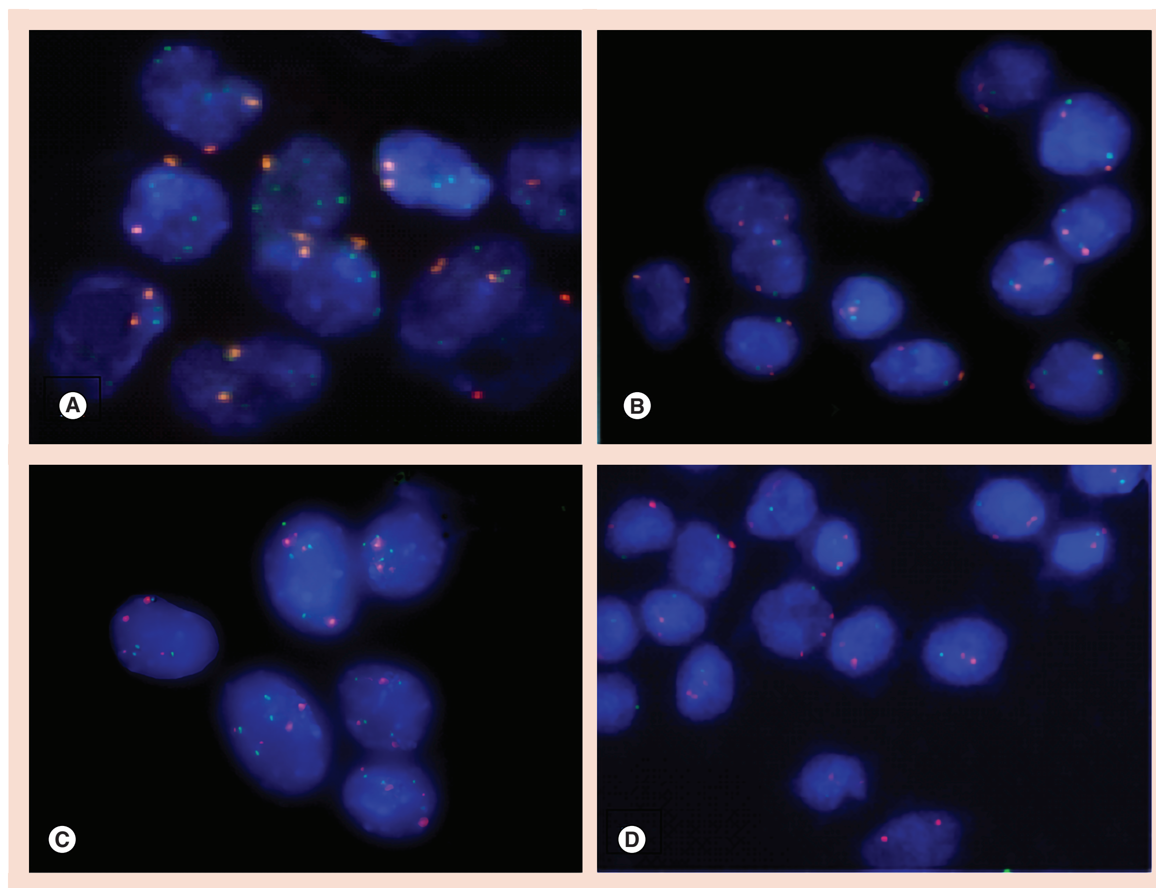

Figure 4. Images of PD-L1 gene status evaluated by FISH on cytological smears from non-small-cell lung cancer patients. Green signals refer to PD-L1 gene locus and orange signals to the centromeric region of chromosome 9. **(A)** Amplification; **(B)** disomy; **(C)** polisomy and **(D)** deletion (4′,6-diamidino-2′-phenylindole [DAPI] stain, original magnification: ×1250).

Table 4. Correlation between *PD-L1* status evaluated by FISH on cytological and histological samples from non-small-cell lung cancer patients.

PD-L1 cytology	*PD-L1* histology			
	Amplification	Disomy	Polisomy	Deletion
Amplification	2	0	0	0
Disomy	0	7	1	1
Polisomy	0	1	3	1
Deletion	0	3	1	6

$p < 0.001$.

A total of 15 of 26 (58%) cases evaluated on both cytology and histology had *PD-L1* assessed on the same primary lung lesion and three cases on the same lymph node metastasis; in eight cases the comparison was between FFPE sample from the primary tumor and cytology from mediastinal or hilar lymph node metastasis. The concordance of *PD-L1* FISH results between cytology and paired histology was 69% (18/26) (Cohen's K = 0.556; $p < 0.001$) (Table 4). No concordance between PD-L1 expression and *PD-L1* gene status was found either on cytological smears or FFPE samples.

Discussion

EBUS-TBNA and transthoracic needle aspiration have recently emerged as safe and minimally invasive techniques for collecting tumor cells from lung cancer patients. It has also been suggested that EBUS-TBNA may represent a robust method to obtain tissue specimens for the study of PD-L1 expression [9–11].

Immunohistochemistry is currently the accepted technique to evaluate PD-L1 expression in clinical trials. However, not always FFPE biopsies and cell blocks can be obtained or provide enough material for diagnosis and tumor typing given the rising number of variables required for diagnostic, prognostic and predictive purposes. In these cases, cytological material, such as direct smears and imprints, may represent a valuable source of tumor cells. Moreover, the opportunity to evaluate fresh unfixed and unstained smears may overcome the technical variability due to various fixative and destaining protocols employed by different laboratories.

Several papers have favorably compared the evaluation of PD-L1 status on cytology cell blocks and corresponding biopsies in NSCLC [9–13], but only recently the feasibility of PD-L1 testing on archival stained smears has been demonstrated. Munari *et al.* [14] found an overall agreement of 91% in a series of 50 NSCLC immunostained for PD-L1 on whole tumor sections and on smears obtained by FNA directly from the surgical specimen. The authors conclude that their observation should be validated on a cohort of preoperative FNA cell smears. In two studies performed on Papanicolaou stained smears, both Noll [15] *et al.* and Lozano *et al.* [16] showed 96% and 97% of concordance, respectively, between PD-L1 staining on cytology and corresponding histology specimens. Capizzi *et al.* [17], on an analogous series of 49 cases found 86% of concordance using the SP263 antibody. All these studies used a cut-off value of ≥50% positive cells to define PD-L1 status. However, up to now, current recommendations do not support the use of PD-L1 stains on cytology smears in routine practice because of lack of validation.

On a series of 52 paired cytological and histological samples obtained in routine clinical practice, we found a good correlation (92%) in the assessment of PD-L1 status by immunostaining with only four discordant cases. In one discordant case, PD-L1 was positive on a TBNA sample from a paratracheal lymph node and negative on TBB from lobar bronchus. This discrepancy might be due to the different site of sampling and to intratumoral heterogeneity that has been widely documented even within a single tumor slide [18–20]. Conversely, a false negative result due to a fixation defect is unlikely given the small size of the biopsy. This case showed a low level of *PD-L1* polisomy by FISH on both cytology and histology. The second discordant case was one of the oldest samples in our series (year 2013). It was positive on cytology and negative on the surgical sample despite both the samples were obtained from the same lung lesion. In this case, a loss of antigenicity in paraffin blocks stored for a long time [21] can be hypothesized. Both cytological and histological samples showed a diploid *PD-L1* asset. The third discrepancy was observed in a NSCLC with signet ring cells. In the touch imprint obtained from the TBB, the immunoreactivity was confined to clusters of tumor cells whereas the majority of isolated tumor cells were negative. This might explain why only 30% of positive cells were scored on cytology compared with 70% on histology where signet ring cells were mostly in clusters and the cell membranes were better preserved. This case had no available slides for FISH assay. The last discordant case was negative on cytology and positive on histology even though both samples were obtained from the same lymph node metastasis. This case showed *PD-L1* deletion on cytology while histology showed *PD-L1* polysomy.

In our series, only eight samples were older than 5 years. Two of them were positive on both cytology and histology while one case showed 3% positivity on histology and >50% on cytology. We suppose that the antigenicity was better preserved on cytology specimens that were stored refrigerated than in formalin-fixed specimens.

The limited power of PD-L1 expression in predicting response to inhibitors as underlined by Califano *et al.* [22] points to the need of alternative biomarkers. Furthermore, immunohistochemistry (IHC) score can change over the course of therapy because of variations in PD-L1 expression. PD-L1, as already mentioned, often has a heterogeneous space distribution in a tumor mass. Finally, the cut-off values used to define responders and nonresponders is arbitrary as PD-L1 behaves as a continuous variable, both biologically and analytically.

A subset of NSCLC patients with *PD-L1* amplification has been recently described, suggesting that *PD-L1* gene copy number, as assessed either by FISH or PCR, might be a predictive marker alternative to IHC [5–7]. Ikeda *et al.* [5] found *PD-L1* amplification in 5.3% (5/94) of surgically resected NSCLCs, but they failed to find any correlation between PD-L1 protein expression levels and gene amplification. Goldmann *et al.* [23] found *PD-L1* amplification in 11 out of 221 (4.9%) NSCLC cases; all of them PD-L1-positive by IHC. Inoue *et al.* [6] in a large series of surgically resected NSCLC patients tested by FISH found 3.1% (20/654) cases with *PD-L1* amplification and 13.2% (84/654) with polysomy, reporting that *PD-L1* copy number increase was associated with

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PD-L1 expression. Recently, Clavè *et al.* [24], in 159 resected NSCLC patients, described 14 tumors with *PD-L1* amplification (8.8%), 12 of which with PD-L1-positive expression.

In our series, *PD-L1* amplification evaluated by FISH was observed in 2/26 (7.7%) cases on paired cytology and histology. However, one of the two amplified cases showed a lower percentage of amplified cells on histology compared with cytology. The different site and time of sampling between the two specimens may explain this discrepancy; in fact, the cytology was obtained from a metastatic lymph node, whereas the histology was from the primary tumor. Moreover, an interval of 6 months elapsed between histology and cytology, a time frame allowing for a selection of amplified cells in the lymph node.

No correlation was found between PD-L1 expression and *PD-L1* gene status on 26 paired cytological and histological samples. In particular, *PD-L1* polysomy was not associated with PD-L1 overexpression. Of the two PD-L1 amplified cases, one was PD-L1-positive by immunostains whereas the other was negative at a <1% cut-off on both cytology and histology.

Conclusion

We conclude that cytological smears represent a valid alternative for assessing PD-L1 status in NSCLC patients whenever a biopsy or a cell block are not available or do not provide enough cellular material. Direct cytological smears have also the added advantages of allowing immediate assessment of the adequacy of the sample by rapid on-site evaluation and of reducing delays and technical artefacts due to fixation or destaining methods that could alter the protein antigenicity. The ability to assess PD-L1 status by immunostains on direct cytological smears will allow the eligibility of a larger number of patients to first-line treatment with immune checkpoint inhibitors.

Summary points

- Recently, PD-L1 blockade represents the new standard of care for patients with advanced non-small-cell lung cancer and PD-L1 protein assessment by immunohistochemistry is mandatory to determine the correct treatment strategy.
- Minimally invasive diagnostic procedures not always provide sufficient cellular material for diagnosis and tumor typing. In these cases, cytology smears represent the only alternative for PD-L1 evaluation by immunocytochemistry (ICC).
- The present study was designed to assess PD-L1 expression on direct cytology smears and paired histological samples.
- Despite current recommendations not supporting the routine practice of ICC because of lack of validation, the good concordance found between PD-L1 values obtained on cytology and matched histology underline the reliability of ICC whenever histological samples are not available.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval (Local Ethical Committee of Parma). Informed consent has been obtained from the participants involved where applicable according to the Local Ethical Committee indications.

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