Validation of the immunohistochemical expression of programmed death ligand 1 (PD-L1) on cytological smears in advanced non small cell lung cancer

Validation of the immunohistochemical expression of Programmed Death Ligand 1 (PD-L1) on cytological smears in advanced Non Small Cell Lung Cancer

Elisa Capizzi [1], Costantino Ricci [1], Francesca Giunchi [1], Stefano Zagnoni [1], Claudio Ceccarelli [1], Begoña Urrios Álvarez Gómez [1], Laura Casolari [2], Francesco Gelsomino[2], Rocco Trisolini [3], Michelangelo Fiorentino [1*], Andrea Ardizzoni [2]

[1] Laboratory of Oncologic Molecular Pathology
[2] Department of Thoracic Endoscopy
[3] Department of Medical Oncology
S.Orsola-Malpighi Teaching Hospital, University of Bologna, Via Massarenti 9, 40138, Bologna, Italy

[*]Correspondence to:
Michelangelo Fiorentino, MD, PhD
Laboratory of Oncologic Molecular Pathology
S.Orsola-Malpighi Hospital
Addarii Institute of Oncology
Viale Ercolani 4/2
40138, Bologna
Italy
Tel +390512144556
Fax +390516363782
michelangelo.fiorentino@unibo.it

Abstract

Introduction. The assessment of PD-L1 expression by immunohistochemistry is mandatory for the administration as first-line therapy of the anti PD-1 check-point inhibitor Pembrolizumab in patients with advanced non-small-cell lung cancer (NSCLC). Currently, only formalin-fixed paraffin-embedded samples are acceptable for PD-L1 immunostaining with the anti-PD-L1 antibodies 22-C3 and SP263. We investigated retrospectively the accuracy of the anti PD-L1 antibodies 22-C3, 28-28, SP263 in 50 paired histological samples and cytological smears of NSCLC patients. **Results**. The accuracy of the three antibodies for the detection of PD-L1 in

histological samples was higher for the antibody SP263 (AUC/ROC =1) compared to the clones 28-8 (AUC/ROC =,991) and 22-C3 (AUC/ROC =,942). The overall concordance between histological samples and cytological smears using the SP263 clone was moderate (kappa =0,364). However when the cyto-histological concordance was calculated using just the <50% vs ≥50% cut-off the agreement (*kappa* =0.626) was good. The accuracy of the antibody SP263 in cytological smears was good (AUC/ROC =,921). A fluorescent in situ hybridization analysis on 10 histological cases positive for PD-L1 at immunohistochemistry showed amplification of the *CD274* gene only in one case. **Conclusions**. Immunocytochemical staining for PD-L1 in diagnostic cytological smears of NSCLC is feasible and applicable at least using the >50% cancer cell cut-off. The three antibodies SP263, 22-C3 and 28-8 are all suitable for the diagnostic detection of PD-L1 on tissue sections with a superiority of the SP263 clone. The implementation of PD-L1 immunocytochemistry on cytological smears will likely expand the pool of NSCLC patients candidate to first-line immunotherapy.

Introduction

The assessment of PD-L1 expression by immunohistochemistry (IHC) is mandatory for the treatment as first-line therapy with the anti PD-1 check-point inhibitor Pembrolizumab in patients with advanced non small-cell lung cancer (NSCLC). [1] The IHC cut-offs for the administration of Pembrolizumab in first- or second-line of therapy derive from the registration studies KEYNOTE-024 and 010 and have been stated as ≥50% PD-L1+ cells and ≥1% for first and second-line treatment, respectively. Several antibodies have been developed as companion diagnostics for the IHC predictive detection of PD-L1 in NSCLC tissue samples. A recent IHC comparative methodological study concluded that three anti-PD-L1 clones were comparable and therefore suitable for IHC detection of PD-L1 in view of the treatment with Pembrolizumab. [2] In particular, the clones named 22-C3, 28-8 and SP263 produced by different brands were selected as potential predictive reagents. Among these antibodies only the clones 22-C3 and SP263 have been finally certified for in vitro diagnostics on proprietary platforms for automated IHC. The guidelines for interpretation of PD-L1 staining issued by the manufacturers of these two companion diagnostics include the evaluation of immunostaining only in the cell-membranes, in at least 100 cancer cells.[3-4]

The pathological diagnosis and the clinical staging of advanced NSCLC are based preferably on histological tissues samples obtained by means of ultra-sound guided bronchoscopy (EBUS) or trans-thoracic TC-guided needle biopsies. [5] Unfortunately, the success rate of EBUS to obtain tissue fragments is highly variable and in many instances the diagnostic material is just

cytological. [6-8] Cytological samples can be processed as clots or pellets to obtain formalin-fixed paraffin embedded (FFPE) blocks. However, in approximately 20-30 % of cases, the only available diagnostic material is a cytological smear.

PD-L1 testing with companion diagnostics is currently certified only on tissue biopsy fragments or on other liquid cytology-derived FFPE materials such as clots or pellets, but not on cytological smears. [9,10]

Here we report on a retrospective comparative study for the validation of the three anti PD-L1 antibodies 22-C3, SP263 and 28-8 on paired histological and smeared cytological specimens of 50 patients with advanced NSCLC.

Patients' population

We retrospectively enrolled 50 patients who received the diagnosis of advanced NSCLC at the Services of Thoracic Endoscopy and Pathology of the S.Orsola-Malpighi teaching Hospital in Bologna between May 2014 and September 2017. The 50 patients were selected from a pool of 365 consecutive subjects according to the following eligibility criteria: *i*) availability of at least one positive histological biopsy sample and at least one positive cytological smear with given diagnosis of NSCLC; *ii*) availability of at least 100 cancer cells in each sample; *iii*) procurement of the two sample pairs 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 ≥18; *vi*) signed informed consent of the patient. The mean age of the 50 enrolled patients 70.6 years (49 to 85), 30 were males and 20 females. Twelve cases (24%) were NSCLC with squamous and 38 (76%) with adenocarcinoma histology. The study was approved by the internal review board of the S.Orsola-Malpighi Hospital with study number 39/2017/U.Tess, on March 14th, 2017.

Methods

Tissue specimens were fixed in 4% buffered formalin for 8-24 hours and embedded in paraffin. Three µm tissue sections were freshly cut from each formalin-fixed paraffin embedded (FFPE) block before starting immunohistochemistry.

Cytological smears obtained from aspirates of tumor nodules or metastatic lymph-nodes were promptly fixed with MicroFix spray (Diapath, Italy), hydrated, stained either with the May Grünwald-Giemsa or the Papanicolau procedure, de-hydrated to absolute ethanol, cleared in Xilene and finally mounted with cover-slides. Stained smears selected for the study were left in a Xilene bath for 48 hours to allow gentle slip-off of cover-slides, then re-hydrated through graded alcohols, destained in 1% HCl in 70° ethanol and rinsed in PBS before starting immunocytochemistry.

Immunohistochemistry and immunocytochemistry were performed on the automated Ventana BenchMark ULTRA System (Ventana Medical System, Tucson, AZ) using the OptiView DAB IHC Detection Kit (Ventana). Immounohistochemistry on FFPE samples was performed by using three different anti-PD-L1 antibodies: *i)* rabbit monoclonal anti-PD-L1(clone 28-8, Abcam); *ii)* mouse monoclonal anti-human PD-L1 (clone 22-C3 PharmDX, Dako/Agilent); *iii)* rabbit monoclonal anti-PD-L1 (clone SP263, Ventana). Immunocytochemistry for PD-L1 on the single available cytological smears was carried out only with the SP263 antibody: this clone was selected since it displayed the best sensitivity and specificity at the comparative analysis on the histological specimens (see Results section).

The immunostaining protocols included slight differences in time of pre-treatment with CC1 solution (Ventana) and in incubation time among the three PD-L1 antibodies and between cytology and histology samples as follows: *i)* clone 28-8: retrieval CC1 32', incubation 32' at 37°C, dilution 1:400; *ii)* clone 22-C3: retrieval CC1 48', incubation 32' 37°C, dilution 1:200; *iii)* clone SP263: retrieval CC1 56' (for istological samples) or 32' (for cytological samples), incubation 16' 37°C, prediluited.

PD-L1 positive and negative controls were run concurrently for each case. In order to highlight the presence of tumor-infiltrating macrophages that frequently overexpress PD-L1, a double staining with anti-PD-L1 (SP263) and anti-CD68 (KP-1, prediluited, Ventana) was adopted on tissue samples. The double immunostaining protocol included a retrieval step with CC1 56', a first incubation with SP263 antibody for 16' at 37°C followed by DAB staining (OptiView DAB IHC Detection Kit, Ventana), and a second incubation with anti-CD68 antibody for 32' at 36°C followed by AP RED staining (ultraView Universal Alkaline Phosphatase Red Detection Kit, Ventana). Prior to application , the double staining has been validated in a limited number of cases (data not shown) through a comparison with the single staining without finding relevant discrepancies in terms of number of positive cancer cells.

In both histological and cytological samples PD-L1 expression was evaluated only in cancer cells and at least 100 tumor cells per samples were scored. Viable cancer cells were considered positive in presence of membrane staining of any intensity, while cytoplasmic staining was considered positive only if there was concomitant membrane staining.

Statistical analyses were performed using the software SPSS, version 22.0. Concordance analyses among the three clones (28-8, 22-C3 and SP263) were carried out using as reference standard (gold standard) the clone that turned out with the highest positivity rate for each sample. The kappa coefficient of concordance was calculated and the agreement was considered weak for kappa values within 0,21-0,40; moderate for values 0,41-0,60; and good for values 0,61-0,99. For

statistical analysis PD-L1 expression was categorized in 3 score groups according to the following cut-offs*: i)* score 0, if PD-L1 immunostaining was absent or present in less than 1% of tumor cells; *ii)* score 1, if PD-L1 immunostaining was present in at least 1% of tumor cells but less than 50%; *iii)* score 2, if PD-L1 immunostaining was present in more than 50% of tumor cells.

To determine values of sensitivity and specificity of the three PD-L1 clones in histological and cytological samples (using expression cut-off ≥50%) a Receiver Operating Characteristic (ROC) curve was constructed. Values were considered statistically significant when $P<0.05$.

Results

At least 100 cancer cells were found in the three freshly sections of all the 50 selected histological samples (Fig. 1A). In all cases the three antibodies 28-8, 22-C3, and SP263 stained both the cancer and the inflammatory cells (Fig. 1 B,C,D).

In the histological samples the antibody 28-8 stained 12 cases (24%) with ≥50% cancer cells; 19 (38%) with ≥1% - <50% cancer cells while in 19 (38%) the staining was negative. The staining with the 22-C3 antibody revealed 10 cases (20%) with ≥50% cancer cells, 17 (34%) with ≥1% - <50% cancer cells, while 23 (46%) were negative. Finally, the double staining with the anti-PD-L1 SP263 and the anti CD68 showed in 15 cases (30%) ≥50% PD-L1 positive cancer cells, in 20 cases (40%) ≥1% - <50% cancer cells, while 15 (30%) turned out negative.

The comparison of the three anti PD-L1 clones against the gold standard in histological samples is summarized in Table 1. The *kappa* coefficients of concordance with the gold standard turned out 0,554 (moderate), 0,698 (moderate), 0,908 (good) respectively for the clones 22-C3, 28-8 and SP263.

Description of PD-L1 immunoreactivity is depicted in Table 4. At least 100 cancer cells were found in 49 out of the 50 selected cytological smears. The 49 cytological smears were stained with the clone SP263 that turned out the most reliable in the concordance study on the histological samples. No difference in staining were found using smears stained with the May Grünwald-Giemsa or the Papanicolau procedure. The SP263 clone stained both cancer and inflammatory cells (Fig. 1E and 2E). Eleven cases (22%) showed immunostaining for PD-L1 in ≥50% cancer cells, 12 cases (24%) in ≥1% - <50% cancer cells while 26 cases (52%) turned out negative (<1% cancer cells). Several staining artifacts were encountered in immune-cytochemistry. In particular, the presence of non-specific brown blurring in multi-layered cell placards as well as non-specific nuclear staining in some cells (data not shown). All these artifactual stainings were considered negative.

The cyto-histological comparison for PD-L1 staining (clone SP263) in the same cases revealed major discrepancies in 5 out of 49 cases. Three of these cases showed a staining in <1% cells in the histological samples while >50% in the cytological counterparts (Figure 2 B-E). By contrast, in two cases the staining in the histological samples was >50% and <1% in the cytological counterparts (Figure 3 B-E). The remaining 44 cases showed no or minor staining discrepancies in the histological and the cytological specimens. On the basis of these data the overall cyto-histological comparison using the SP263 clone was moderate (kappa = 0,364; Table 2). However when the cyto-histological concordance was calculated using just the <50% vs ≥50% cut-off the agreement (*kappa* = 0.626) was good (Table 3). The accuracy of the three antibodies for the detection of PD-L1 in histological samples was perfect for the antibody SP263 (AUC/ROC =1) compared to the other two antibodies 28-8 (AUC/ROC =,991) and 22-C3 (AUC/ROC =,942). The accuracy of the antibody SP263 in cytological smears was good (AUC/ROC =,921) (Table 4).

Discussion

The immunohistochemical assessment of PD-L1 expression has become a standard-of-care front-line diagnostic procedure for administering immunotherapy to patients with advanced NSCLC. Currently, NSCLC patients may undergo first-line or second-line therapy with Pembrolizumab according to the cut-offs of PD-L1 cancer positive cells of ≥50% or >1%, respectively. The recent Blueprint comparative study revealed that the antibodies SP263, 22-C3 and 28-8 were the best reagents for the immunohistochemical detection of PD-L1 in archival tissues samples.[11] A recent comparative study reported the concordance between cell blocks and cytological smears of the same cases for the immunostaining with the 22-C3 anti PD-L1 antibody. [12] Our study confirms that, by using the two cut-offs of >1% and >50% ,the three clones SP263, 22-C3 and 28-8 are comparable with a superiority of the SP263 antibody (*kappa* =0,908; AUC/ROC =1) over the others. Currently, only the antibodies SP263 and 22-C3 possess the European Certification for in vitro diagnostics (CE-IVD). Discrepancies even between these two antibodies have been recently pointed out using the two cut-offs of >1% and >50%. [13] We demonstrated that the clone 28-8, that is not currently CE-IVD-certified for the diagnostic assessment of PD-L1, has reached in our series a better concordance compared to the 22-C3 clone (kappa 0,698 Vs 0,554) that was used for the confirmation study of Pembrolizumab. As a first conclusion of our study we can state that all the three antibodies SP263, 22-C3 and 28-8 are suitable for the diagnostic immunohistochemical detection of PD-L1. The clone 28-8 that we have used has a limited diagnostic use as it is and it could be utilized diagnostic laboratories only after through validation with strict internal and external quality controls. As and addition cost assessment evaluation it is interesting to point out

that the two clones SP263 and 22-C3 come as kits including the antibody and the development reagents, can be used interchangeably on cross automated staining platforms of the two producers, and bear similar costs. Conversely, the 28-8 clone is not IVD-certified, it can be purchased concentrated as a single reagent, it can be applied to any staining platform and its costs are less than half compared to the certified clones.

The theragnostic immunohistochemical assessment of PD-L1 has been validated in tissues samples and in pellets and clots from cytological samples treated as FFPE materials. [9,10] With the present study we provide one of the early evidences that cytological smears can be successfully used for the detection of PD-L1 immunoreactivity at least using the cut-off of 50%. In our series, we have found only five major discrepant cases between histological and cytological immunostaining. Three cases turned out negative (<1%) in the cytological smear but >50% positive cells in the histological samples. In two of these cases the smear contained a limited number of cancer cells, slightly over one hundred, and we ascribe the discrepancy to this limitation. Another case was negative in the cytological smear while the immunoreactivity in the histological sample (surgical specimen with a 7 cm. in diameter cancer nodule) was heterogeneous with positive tumor areas intermingled with very negative ones. We explain this discrepant case with tumor heterogeneity and sampling of the cytological aspirate falling into a negative PD-L1 tumor area. Heterogeneous PD-L1 expression throughout tumor masses is a relevant bias that has been recently pointed out and cannot be unfortunately ruled out completely in NSCLC diagnostic specimens. [14] Conversely, we have found two cases with PD-L1 positive smears in >50% cancer cells and negative histological samples. In one of the two cases we suspect a heterogeneous expression of PD-L1 as above since the cancer nodule as well. In a second discrepant case, we pose the potential issue of PD-L1 antigen degradation over the time since the histological case dated from 2014. We have previously reported on the fading with time of PD-L1 immunoreactivity in NSCLC tissues even after one year of storage of tissue paraffin blocks in histology archives. [14] The 10% rate of major discrepancies surely implies a serious shift in treatment decision. However, the tumor heterogeneity bias affects most of the predictive biological test in NSCLC because of the limited amount of diagnostic material in biopsy compared to the entire tumor masses.

In our results, the cyto-histological comparison for the detection of PD-L1 with the SP263 antibody was good (kappa =0,626) only when we applied the >50% cut-off. When we run the same analysis including also the >1% cut-off the concordance was weak (kappa =0,364). We can explain this flaw with the poor reproducibility of the pathological semi-quantitative evaluation of immunostaining using the >1% cut-off. This poor reproducibility of scoring at low rates of positive cells can be further worsened by the presence of staining artifacts that we found frequently in the

cytological smears. The slides of the present study have been read by two pathologists with a multi-headed microscope in a non-blinded manner and this represent a study limitation. An inter-rater concordance study on the scoring of PD-L1 on cytological smears was outside the scope of the present study but is required to define the suitability of cytological smears for the use of the >1% cut-off. In the meantime, we feel that the applicability of PD-L1 immunostaining to cytological smears at least using the >50% cut-off represents a significant step beyond compared to the current status for the first-line treatment with immunotherapy of NSCLC patients.

Immunocytochemical staining for PD-L1 in diagnostic cytological smears of NSCLC is feasible and applicable at least using the >50% cancer cell cut-off. This technical progress can potentially lead to an increase in the number of patients candidate to first-line treatment with immune check-point inhibitors. A prospective study is currently ongoing at our institution to validate our retrospective observation.

Acknowledgements
This study was funded by Associazione Italiana per la Ricerca sul Cancro (AIRC) with an Investigator Grant - IG 2016, code 19026 to Andrea Ardizzoni.

References

1. Reck M, Rodríguez-Abreu D, Robinson AG, et al. Pembrolizumab versus Chemotherapy for PD-L1-Positive Non-Small-Cell Lung Cancer. N Engl J Med. 2016; 375(19):1823-1833.
2. Tsao MS, Kerr KM, Kockx M, et al. PD-L1 immunohistochemistry comparability study in real-life clinical samples: results of Blueprint phase 2 project. J Thorac Oncol. 2018; doi: 10.1016/j.jtho.2018.05.013.
3. Dolled-Filhart M, Roach C, Toland G et al. Development of a Companion Diagnostic for Pembrolizumab in Non-Small Cell Lung Cancer Using Immunohistochemistry for Programmed Death Ligand-1. Arch Pathol Lab Med. 2016; DOI:10.5858/arpa.2015-0542-OA.
4. Rebelatto MC, Midha A, Mistry A, et al. Development of a programmed cell death ligand-1 immunohistochemical assay validated for analysis of non-small cell lung cancer and head and neck squamous cell carcinoma. Diagn Pathol. 2016 Oct 8;11(1):95.
5. Van der Heijden EH, Casal RF, Trisolini R, et al. World Association for Bronchology and Interventional Pulmonology, Task Force on Specimen Guidelines. Guideline for the acquisition and preparation of conventional and endobronchial ultrasound-guided transbronchial needle aspiration specimens for the diagnosis and molecular testing of patients with known or suspected lung cancer. Respiration. 2014;88(6):500-17.
6. Kinoshita T, Ujiie H, Schwock J, et al. Clinical evaluation of the utility of a flexible 19-gauge EBUS-TBNA needle. J Thorac Dis. 2018;10(4):2388-2396.
7. Folch E, Yamaguchi N, VanderLaan PA, et al. Adequacy of lymph node transbronchial needle aspirates using convex probe endobronchial ultrasound for multiple tumor genotyping techniques in non-small-cell lung cancer. J Thorac Oncol. 2013;8(11):1438-1444.
8. Trisolini R, Natali F, Ferrari M, Livi V, Paioli D, Romagnoli M, Cancellieri A. Endobronchial ultrasound-guided transbronchial needle aspiration with the flexible 19-gauge needle. Clin Respir J. 2018;12(4):1725-1731.
9. Wang H, Agulnik J, Kasymjanova G, et al. Cytology cell blocks are suitable for immunohistochemical testing for PD-L1 in lung cancer. Ann Oncol. 2018 Jun 1;29(6):1417-1422.
10. Lozano MD, Echeveste JI, Abengozar M, et al. Cytology Smears in the Era of Molecular Biomarkers in Non-Small Cell Lung Cancer: Doing More With Less. Arch Pathol Lab Med. 2018 Mar;142 (3):291-298.
11. Hirsch FR, McElhinny A, Stanforth D, Ranger-Moore J, et al. PD-L1 Immunohistochemistry Assays for Lung Cancer: Results from Phase 1 of the Blueprint PD-L1 IHC Assay Comparison Project. *J Thorac Oncol*. 2017; 12(2):208-222.
12. Noll B, Wang WL, Gong Y, et al. Programmed death ligand 1 testing in non-small cell lung carcinoma cytology cell block and aspirate smear preparations. Cancer Cytopathol. 2018 May;126(5):342-352.
13. Munari E, Rossi G, Zamboni G, Lunardi G, Marconi M, Sommaggio M, Netto GJ, Hoque MO, Brunelli M, Martignoni G, Haffner MC, Moretta F, Pegoraro MC, Cavazza A, Samogin G, Furlan V, Mariotti FR, Vacca P, Moretta L, Bogina G. PD-L1 Assays 22-C3 and SP263 are Not Interchangeable in Non-Small Cell Lung Cancer When Considering Clinically Relevant Cutoffs: An Interclone Evaluation by Differently Trained Pathologists. Am J Surg Pathol. 2018 Jun 12. doi: 10.1097/PAS.0000000000001105. [Epub ahead of print] PubMed PMID: 29901568.
14. Munari E, Zamboni G, Lunardi G, Marchionni L, Marconi M, Sommaggio M, Brunelli M, Martignoni G, Netto GJ, Hoque MO, Moretta F, Mingari MC, Pegoraro MC, Inno A, Paiano S, Terzi A, Cavazza A, Rossi G, Mariotti FR, Vacca P, Moretta L, Bogina G. PD-L1 Expression Heterogeneity in Non-Small Cell Lung Cancer: Defining Criteria for Harmonization between Biopsy Specimens and Whole Sections. J Thorac Oncol. 2018 Aug;13(8):1113-1120. doi: 10.1016/j.jtho.2018.04.017. Epub 2018 Apr 25. PubMed PMID: 29704674.
15. Giunchi F, Degiovanni A, Daddi N, Trisolini R, Dell'Amore A, Agostinelli C, Ardizzoni A, Fiorentino M. Fading With Time of PD-L1 Immunoreactivity in Non-Small Cells Lung Cancer Tissues: A Methodological Study. Appl Immunohistochem Mol Morphol. 2016 Oct 31.

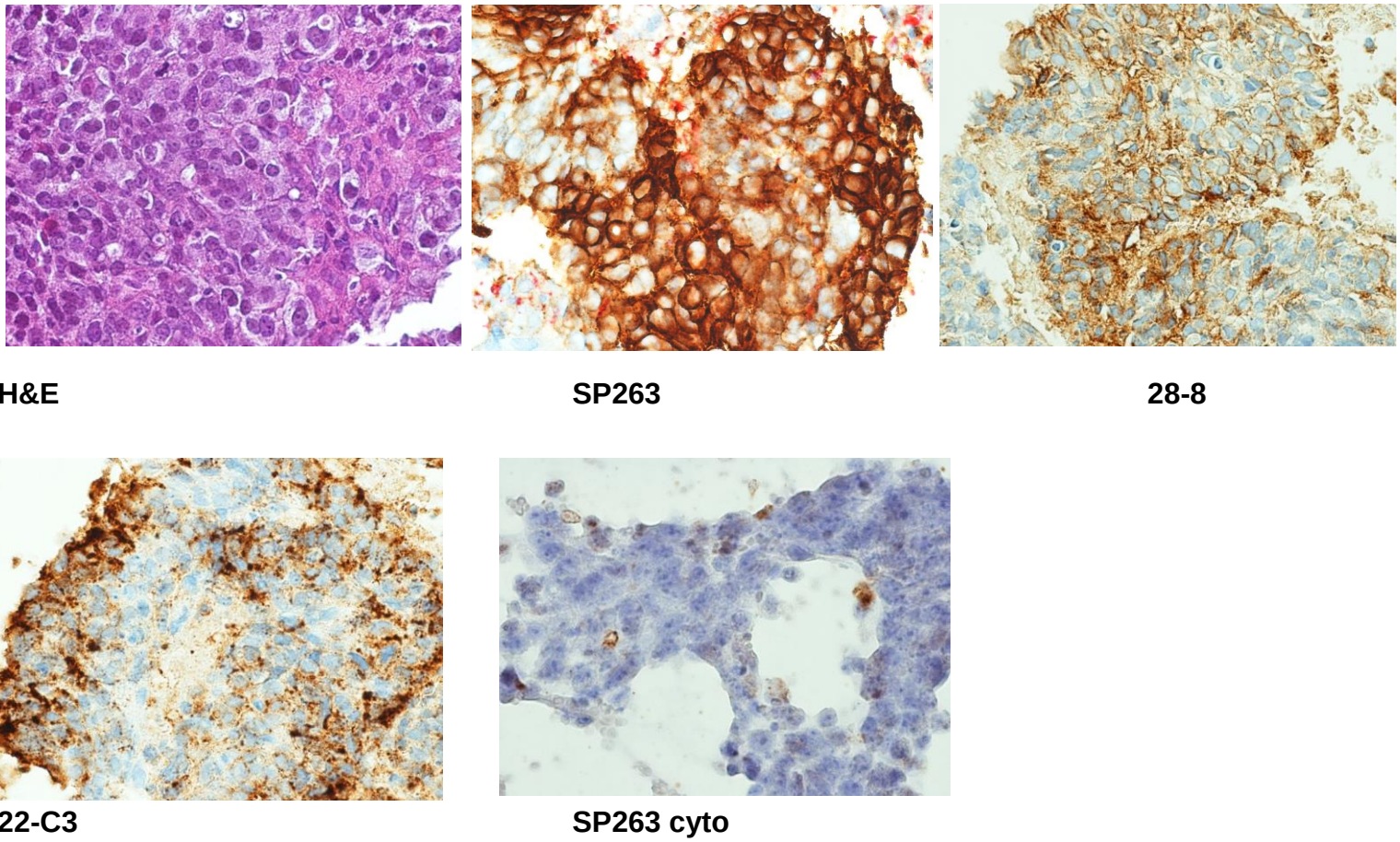


Figure 1 Immunohistochemical, immunocytochemical analysis of PD-L1 in serial histological sections and paired cytological smear of lung adenocarcinoma. (A) Hematoxilyn-eosin (X60). (B) High expression of PD-L1 (60% cancer cells) in the histological specimen with the double immunostaining with SP263 (brown) and CD68 (red) (X60). (C) Low expression of PD-L1 (30% cancer cells) on a serial section using the clone 28-8 (X60). (D) Intermediate expression of PD-L1 (50% cancer cells) using the clone 22-C3 (X60). (E) High expression of PD-L1 (60% cancer cells) in the paired cytological smear using the clone SP263 (X60).

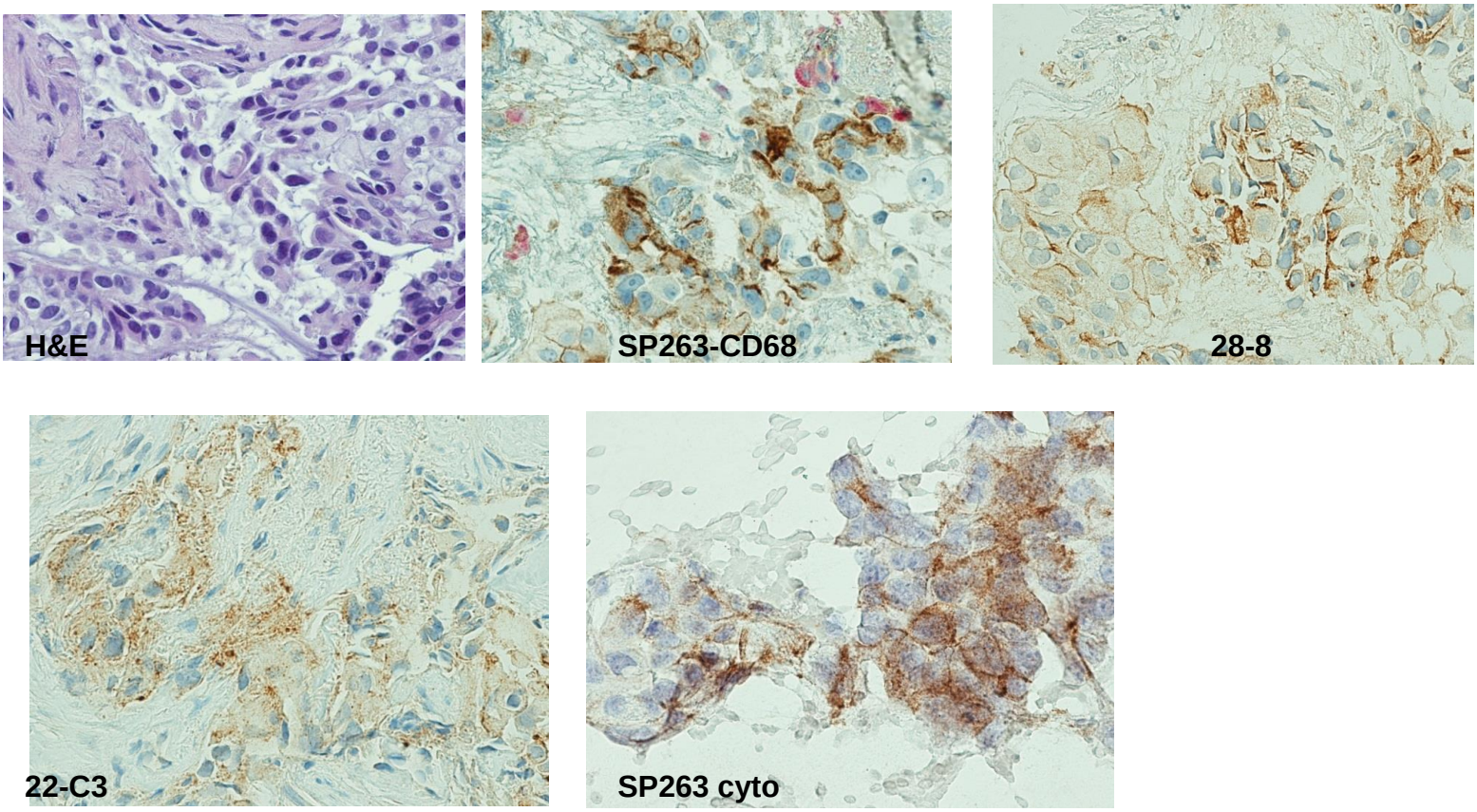


Figure 2 Discrepant negative immunohistochemical analysis with positive immunocytochemistry for PD-L1 in serial histological sections and paired cytological smear of lung adenocarcinoma. (A) Hematoxilyn-eosin (X60). (B) Very low expression of PD-L1 (1% cancer cells) in the histological specimen with the double immunostaining with SP263 (brown) and CD68 (red) (X60). (C) Absent expression of PD-L1 (0% cancer cells) using both the 28-8 and the 22-C3 (D) clones (X60). (E) High expression of PD-L1 (60% cancer cells) in the paired cytological smear using the clone SP263 (X60).

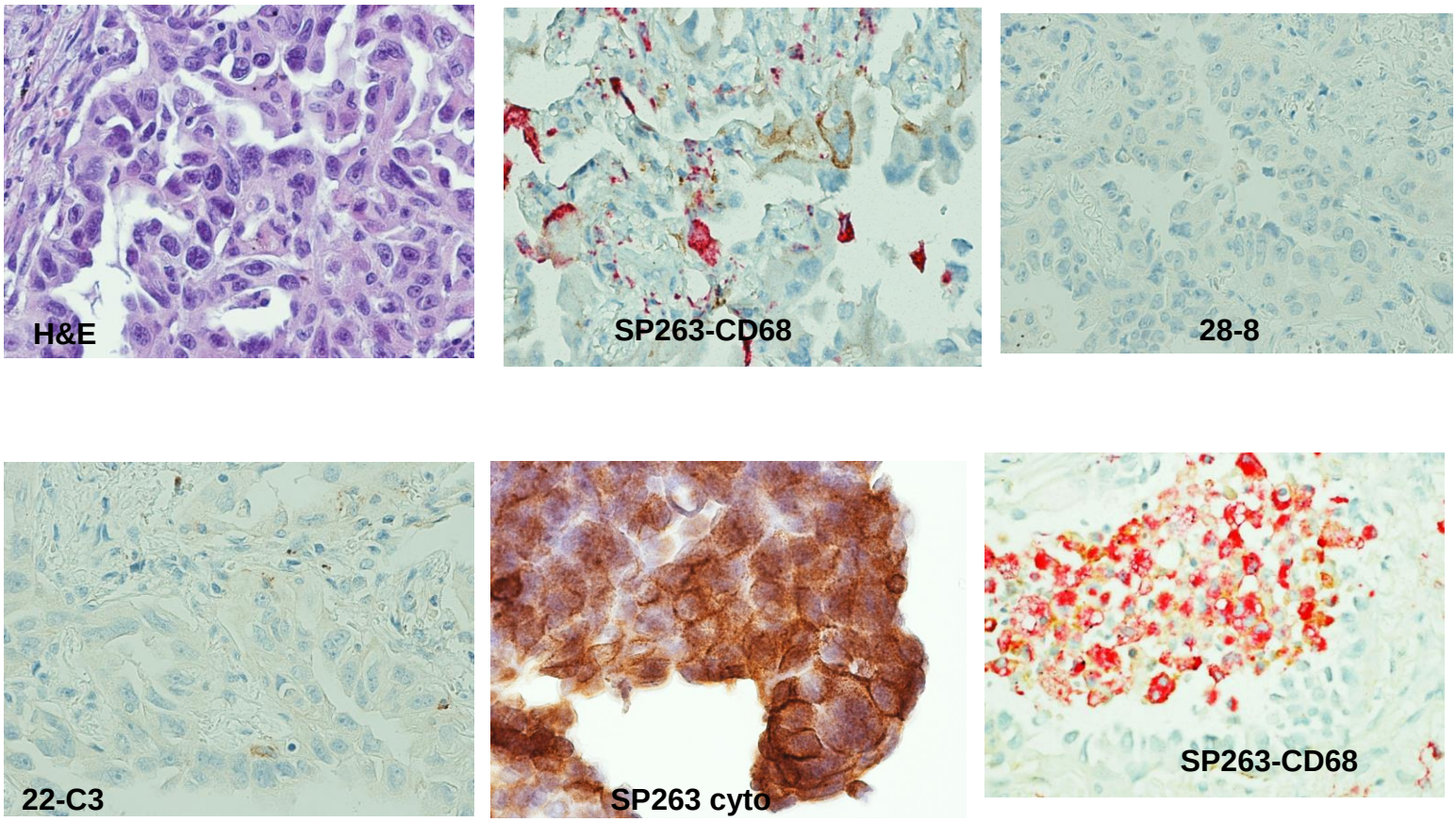


Figure 3 Discrepant positive immunohistochemical analysis with negative immunocytochemistry for PD-L1 in serial histological sections and paired cytological smear of lung squamous carcinoma. (A) Hematoxilyn-eosin (X60). (B) High expression of PD-L1 (80% cancer cells) in the histological specimen with the double immunostaining with SP263 (brown) and CD68 (red) (X60). (C) Moderate expression of PD-L1 (60% cancer cells) using the 28-8 clone and moderate expression (40% cancer cells) using the 22-C3 (D) clone (X60). (E) Negative (0% cancer cells) paired cytological smear using the clone SP263 with scattered positive inflammatory cells (X60). (F) Dual expression of PD-L1 and CD68 in macrophages but not in cancer cell with the double immunostaining with SP263 (brown) and CD68 (red) (X60).

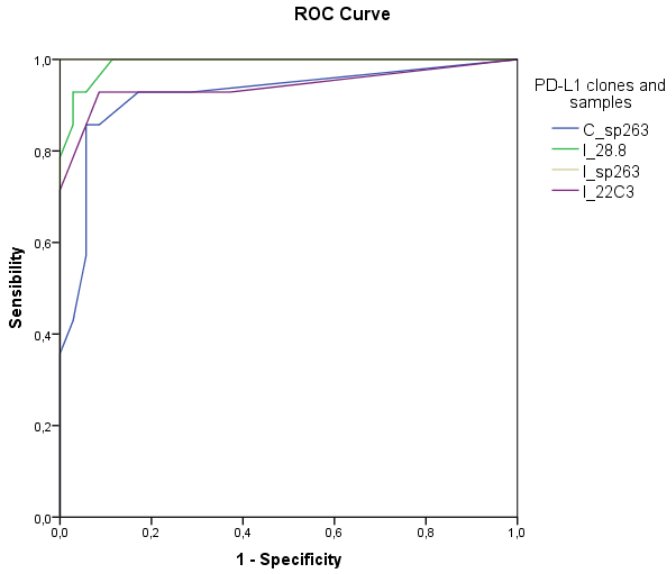
Table 1: Comparison of PD-L1 immunohistochemical expression by 3 different antibodies and gold standard.

		Gold Standard				Kappa value
		<1%	1-49%	≥50%	Total	
28-8	<1%	12	7	0	19	0.698
	1-49%	0	16	3	19	
	≥50%	0	0	12	12	
	Total	12	23	15	50	
22-C3	<1%	12	10	1	23	0.554
	1-49%	0	13	4	17	
	≥50%	0	0	10	10	
	Total	12	23	15	50	
SP263	<1%	12	3	0	15	0.908
	1-49%	0	20	0	20	
	≥50%	0	0	15	15	
	Total	12	23	15	50	

Table 2: Comparison of PD-L1 immunohistochemical expression in cytological and histological samples by sp263 antibody using different cut-off.

		Histology Samples PD-L1 (sp263) expression				Kappa value
Cytology Samples PD-L1 (sp263) expression		**<1%**	**1-49%**	**≥50%**	Total	
	<1%	13	12	1	26	0.364
	1-49%	2	6	4	12	
	≥50%	0	2	9	11	
	Total	15	20	14	49	
		<50%		**≥50%**	Total	
	<50%	33		5	38	0.626
	≥50%	2		9	11	
	Total	35		14	49	

Table 3: Receiver Operating Characteristics (ROC) curve comprising the three clones in cytological and histological specimens. The area under the curve (AUC) was almost perfect for the antibody SP263 (I_SP263) compared with the other two clones (I_28-8 and I_22-C3) in histological samples. The AUC for the clone SP263 in cytological smears (C_SP263) was smaller but still with a 0,921 value.



Area Under the Curve (AUC)	
PD-L1 clones and samples	**AUC**
C_SP263 (SP263 in cytological samples)	0.921
I_SP263 (SP263 in histological samples)	1.000
I_28-8 (28-8 in histological samples)	0.991
I_22-C3 (22-C3 in histological samples)	0.942

Table 4: Comprehensive description of PD-L1 immunoreactivity with the 3 clones in the 50 patients

		PDL1 on cytology	PDL1 expression on FFPE samples		
pts	**Histology**	**SP263**	**28-8**	**SP263**	**22-C3**
1	SCC	> 90%	80%	70%	50%
2	SCC	5%	3%	10%	0%
3	SCC	0%	0%	0%	0%
4	SCC	0%	1%	5%	3%
5	SCC	20%	15%	20%	1%
6	SCC	10%	2%	10%	10%
7	SCC	0%	0%	0%	0%
8	SCC	70%	6%	1%	5%
9	SCC	80%	80%	70%	70%
10	SCC	1%	5%	0%	30%
11	SCC	0%	80%	80%	80%
12	SCC	10%	60%	60%	70%
13	ADK	N/A	90%	70%	80%
14	ADK	60%	15%	70%	20%
15	ADK	40%	80%	60%	60%
16	ADK	60%	0%	2%	0%
17	ADK	0%	3%	5%	1%
18	ADK	10%	0%	<1%	0%
19	ADK	0%	5%	5%	10%
20	ADK	5%	0%	1%	0%
21	ADK	0%	0%	<1%	0%
22	ADK	0%	3%	10%	0%
23	ADK	80%	65%	80%	70%
24	ADK	0%	0%	0%	0%
25	ADK	80%	90%	70%	70%
26	ADK	0%	0%	0%	0%
27	ADK	10%	0%	15%	30%
28	ADK	0%	<1%	0%	2%
29	ADK	<1%	0%	5%	0%
30	ADK	40%	50%	60%	30%
31	ADK	0%	0%	0%	0%
32	ADK	0%	5%	10%	3%
33	ADK	0%	0%	<1%	0%
34	ADK	90%	60%	70%	30%
35	ADK	0%	0%	0%	0%
36	ADK	50%	60%	60%	<1%
37	ADK	0%	1%	0%	0%
38	ADK	0%	0%	0%	0%

39	ADK	60%	30%	60%	50%
40	ADK	0%	0%	0%	0%
41	ADK	30%	40%	50%	40%
42	ADK	0%	5%	7%	5%
43	ADK	1%	0%	5%	0%
44	ADK	0%	0%	5%	0%
45	ADK	0%	15%	5%	5%
46	ADK	0%	0%	0%	0%
47	ADK	0%	40%	40%	20%
48	ADK	<1%	2%	1%	<1%
49	ADK	70%	70%	80%	60%
50	ADK	<1%	20%	30%	<1%