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Brightfield Multiplex Immunohistochemistry Assay for PD-L1 Evaluation in Challenging Melanoma Samples

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Abstract: Targeting the PD1/PD-L1 immune checkpoint pathway has rapidly become a therapeutic strategy for melanoma patients. Indeed, the quantification of PD-L1 expression by immunohistochemistry (IHC) in melanoma samples is already required, in some contexts, to allow access to anti-PD-1/PD-L1 immunotherapy. Despite a rising demand for PD-L1 testing, paralleling increasing cumulative experience in its assessment and quantification, it is fair to recognize that PD-L1 evaluation in melanoma samples still presents some critical issues. The aim of this technical report is to develop and validate a multiplex double staining protocol for PD-L1/SOX10 in Ventana Benchmark Ultra for routine practice. Our results show that double labeling provides the necessary tools to identify PD-L1$^+$ melanoma cells clearly. The simultaneous visualization of 2 different proteins targets allows the topographical relationship between the 2 labeling to be evaluated within the context of the tissue morphology. Future studies are needed to test this technique's real-world applicability and effectiveness in implementing interpathologist agreement.

Received for publication May 22, 2024; accepted July 16, 2024.

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D.M. and A.S.C.: performed study concept and design, results interpretation, and review and revision of the paper. F.U. and L.T.: wrote the first draft of the manuscript. F.N., S.S., D.D.G., G.B., and P.A.: provided data collection and annotation. F.U., S.S., D.D.G., and G.B.: carried out the experiments with D.M. supervision. All authors read and approved the final paper.

This work was funded by the Fondazione AIRC "Programma di ricerca 5 per Mille 2018—ID#21073", Regione Toscana "Bando Ricerca Covid 19 Toscana", and The Tuscany Health Ecosystem grant ECS_00000017.

The authors declare no conflict of interest.

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DOI: 10.1097/PAI.0000000000001219

Key Words: melanoma, PD-L1, SOX10, brightfield IHC, multiplex IHC

(*Appl Immunohistochem Mol Morphol* 2024;32:389–394)

Melanoma is the deadliest among skin cancers and one of the strongest contributors to cancer-related mortality.[1] Despite very favorable outcomes for early-stage disease, more advanced cases of melanoma show inferior survival, only recently improved by target therapies and immune checkpoint inhibitors (ICIs), with excellent results in many patients.[2] Several studies have investigated biomarkers that predict response to immunotherapy in melanoma patients.[3]

PD-L1 is among the more promising ones, but its applicability is still debated due to the many issues surrounding its evaluation methods. In recent years, notwithstanding a rising need for PD-L1 testing paralleling increasing cumulative experience in its assessment and quantification, it is fair to recognize that PD-L1 evaluation in melanoma samples by immunohistochemistry (IHC) still presents some critical issues.

PD-L1 is a highly subjective, heterogeneous, dynamic, inducible marker influenced by several microenvironmental factors.[4–6] PD-L1 can be constitutively expressed through an oncogene-addicted process. Still, inducible expression may result from interferon-gamma (IFN-γ) produced by CD8$^+$ lymphocytes.[7–9] Thus, PD-L1 expression in mononuclear immune cells such as lymphocytes, macrophages, and dendritic cells in the tumor microenvironment (TME), particularly at the peritumoral location, may obscure an accurate estimation of the Tumor Proportion Score (TPS).[9] The debate around PD-L1's localization is very relevant, as recent evidence starts to suggest that the biological implications of its expression, and therefore its predictive and prognostic impact, are highly dependent on the cellular compartment in which it is expressed, in addition to its spatial distribution concerning other immune markers.[10]

Regardless of its variable expression, many additional histopathologic features can impact the evaluation of the labeling. For instance, moderate to intense melanin pigment can obscure PD-L1's stain, and real-world failure

rates (uninterpretable IHC) due to high pigmentation have been reported in 13.5% of melanoma cases.[11] Likely, cytoplasmic melanin may also masquerade subtle differences in the better demarcation of cell membranes by certain antibodies, and the potential utility of red chromogen, such as Fast Red, has been advocated but not universally adopted. A relevant consideration is that samples with weaker PD-L1 signal are particularly challenging.[12]

Among previous studies, we observed poor standardization with the use of anti-PD-L1 antibodies, owing to the use of several immunohistochemistry platforms and PD-L1 clones (including 5H1, SP142, SP263, 28-8, and 22C3),[13–16] different cutoffs for positivity, cellular sublocalization of the stain (membranous, cell surface, cytoplasmatic),[17] cellular compartment considered (immune cells and melanoma cells)[18] and preanalytical variables. Some of these variables may impact the reported intratumoral (spatial and temporal) heterogeneity.[19]

The aim of this technical report is to validate a multiplex double staining protocol for PD-L1/SOX10 using the clone SP263 in the Ventana Benchmark Ultra platform and the effectiveness and applicability of this new brightfield multiplex IHC protocol in routine practice.

MATERIALS AND METHODS

Tissue Samples

Formalin-fixed paraffin-embedded (FFPE) melanoma brain metastasis (MBM) tissues samples (n = 10) were retrospectively selected. Tissue sections, 3μm in thickness, were obtained from paraffin blocks retrospectively selected from the Archive of the Section of Pathology, Department of Health Sciences, University of Florence, Florence, Italy. A lymph node melanoma metastases sample has been used as an internal control to validate the RED chromogen singleplex stain protocol. The clinicopathologic features and treatments of the case-cohort are detailed in Table 1.

Ethics Approval

The use of formalin-fixed and paraffin-embedded (FFPE) samples of human tissue was approved by the Local Ethics Committee "Comitato Etico Regione Toscana-Area Vasta Centro (CEAVC)" (13676_bio; 22156_bio). This study was performed in accordance with the Declaration of Helsinki.

Immunohistochemistry

Immunohistochemistry was performed on Ventana automated stainer BenchMark ULTRA. A measure of 3μm sections were deparaffinized in EZ prep (#950-102; Ventana), and antigen retrieval was achieved by incubation with cell-conditioning solution 1 (#950-124; Ventana), a Tris ethylenediaminetetraacetic acid-based buffer (pH 8.2) both for singleplex and multiplex IHC.

Singleplex IHC

Sections were incubated with the following primary antibodies: anti-CD4 (#790-4423, rabbit monoclonal, clone SP35, ready to use; Ventana Medical System, Tucson, AZ), anti-CD8 (#790-4460, rabbit monoclonal, clone SP57, ready to use; Ventana Medical System), anti-FoxP3 (#14-477-82, mouse monoclonal, clone 236A/E7; Invitrogen, USA), anti-CD68 (#PDM065, mouse monoclonal, ready to use, clone PGM1; Diagnostics BioSystem, USA), anti-CD163 (#760-4437, clone MRQ-26, mouse monoclonal, ready to use; Ventana Medical System), and anti-PD-L1 (741-4905, rabbit monoclonal, clone SP263, ready to use; Ventana Medical Systems). The signal was developed with the UltraView Universal RED detection kit (#760-501; Ventana Medical Systems), sections were counterstained with eematoxylin (#760-2021, ready to use; Ventana Medical Systems).

PD-L1/SOX10 Multiplex IHC

Sections were incubated with the following primary antibodies: anti-SOX10 (#760-4968, rabbit monoclonal, clone SP267, ready to use; Ventana Medical Systems) and anti-PD-L1 (#741-4905, rabbit monoclonal, clone SP263, ready to use; Ventana Medical Systems). For double labeling, each denaturation step was done by treating the slides with Reaction buffer (#950-300; Ventana Medical Systems) for 8 minutes, at 95°C. For SOX10 chromogenic

TABLE 1. Summary of the Cohort's Baseline Clinicopathologic Features

Case	Age	Sex	Localization	EOR	Number of BM	ECM	BRAF	mol-GPA score	pKPS	Adjuvant treatment	Outcome	OS (mo)
1	50	F	Left parietal	GTR	Single	No	Wildtype	3	80	RT+TMZ	NED	80
2	39	F	Right occipital	GTR	Single	No	p.V600E	4	100	RT+Vemurafenib	DOD	69
3	57	F	Multicentric	STR	Multiple	No	p.V600E	2	70	RT+TMZ	DOD	11
4	50	M	Right frontal	GTR	Multiple	No	Wildtype	2.5	90	RT	DOD	9
5	74	F	Multicentric	STR	Multiple	No	p.V600E	1.5	60	TMZ	DOD	4
6	54	F	Left frontal	GTR	Single	Liver	p.V600E	3	100	RT+Vemurafenib	DOD	80
7	50	F	Left frontal	GTR	Multiple	Lung	Wildtype	1	80	RT+TMZ	DOD	5
8	65	M	Right temporal	GTR	Single	Nodal	Wildtype	2	80	RT	DOD	18
9	70	M	Cerebellum	GTR	Single	Lung, liver	Wildtype	0	60	Nivolumab	DOD	2
10	20	M	Left frontal	GTR	Single	Lung, bone	p.V600K	2.5	80	Nivolumab	DOD	4

BM indicates brain metastases; DOD, dead of disease; ECM, extracranial metastases; EOR, extent of resection; F, female; GTR, gross total resection; M, male; mol-GPA, molecular-graded prognostic assessment; NED, no evidence of disease; OS, overall survival; pKPS, postoperative Karnofsky Performance Status; RT, radiotherapy; STR, subtotal resection; TMZ, temozolomide.

detection, OptiView DAB IHC Detection Kit (#760-700; Ventana Medical Systems) was used. For PD-L1 chromogenic detection, the UltraView Universal RED detection kit (#760-501; Ventana Medical Systems Tucson) was used. Finally, sections were counterstained with hematoxylin (#760-2021, ready to use; Ventana Medical Systems).

RESULTS

Comparison Between DAB and RED Chromogens

We first validated singleplex staining with RED chromogen for PD-L1 in the Ventana Benchmark Ultra platform. To investigate RED chromogen performance, IHC staining with PD-L1 was performed with the 2 different chromogens available in routine diagnostic, RED and DAB, which is currently considered the gold standard for singleplex staining, as shown in Figure 1. DAB remains one of the most used chromogens for IHC, maintaining a high level of contrast and clearness (Fig. 1B). Indeed, it exhibits many desirable features, including that DAB precipitates are virtually insoluble in aqueous and organic solvents. In contrast, as shown in Figure 1C, RED chromogen guarantees a good contrast and brightness, resulting in IHC stains whose visualization is comparable with those employing DAB but that provides a better contrast with melanic pigmentation, significantly reducing, in our assessment, the risk of improper interpretations in pigmented lesions.

Validation of Double-Labeling PD-L1/SOX10 Protocol

Starting from validated singleplex staining protocols routinely used, we first assessed whether these singleplex IHC methods could be combined; in particular, we combined SOX10 singleplex protocol with DAB chromogen with PD-L1 singleplex protocol with RED chromogen (Fig. 2). Since these antigens are present on different cell compartments, that is, the cell membrane for PD-L1 and the nucleus for SOX10, it is recommended to start the multiplex sequence with the antibody directed against nuclear antigens, then proceed with the one against the membrane. According to our experience, this sequencing allows for better development of both signals, without one masking the other.[20]

As shown in Figure 2C, multiplex IHC (Fig. 2C) provides many advantages over singleplex IHC (Fig. 2B). Double labeling provides the tools necessary to identify PD-L1$^+$ melanoma cells (Fig. 2C) clearly. The simultaneous visualization of 2 different molecular targets allows the topographical relationship between the 2 labeling to be evaluated within the context of the tissue morphology.

Furthermore, in melanoma samples in which the inflammatory infiltrate is strongly present, as shown in Figure 3, especially in nodal involvement and distant metastases (panels A to F), this new double-labeling protocol allowed to clearly distinguish PD-L1$^+$ melanoma cells to PD-L1$^+$ immune cells, as can be seen in Figures 3G and H, which illustrates how strong PD-L1 membranous expression in histiocytes and lymphocytes might lead to an overestimate of the TPS score, in this metastatic melanoma sample.

DISCUSSION

In this technical report, we validated for the first time a multiplex double-labeling PD-L1 (clone SP263)/SOX10 (clone SP267) protocol in Ventana Benchmark Ultra for the evaluation of PD-L1 IHC staining in challenging melanoma cases.

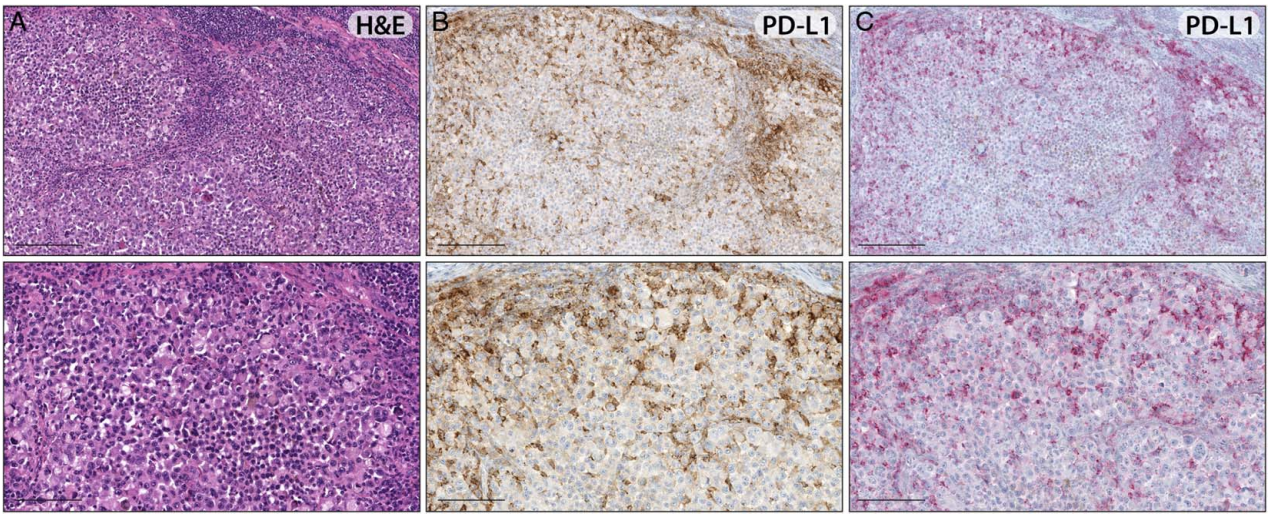

FIGURE 1. Representative images of hematoxylin and eosin staining of lymph node melanoma metastasis (A). Representative images of PD-L1 labeling stained with two different chromogens DAB (B) and RED (C) chromogens. The IHC stains, differing only in the chromogen used, are here clearly shown to be entirely comparable in this melanoma sample. Magnification: ×100 and ×200 (scale bar: 100 and 25 μm).

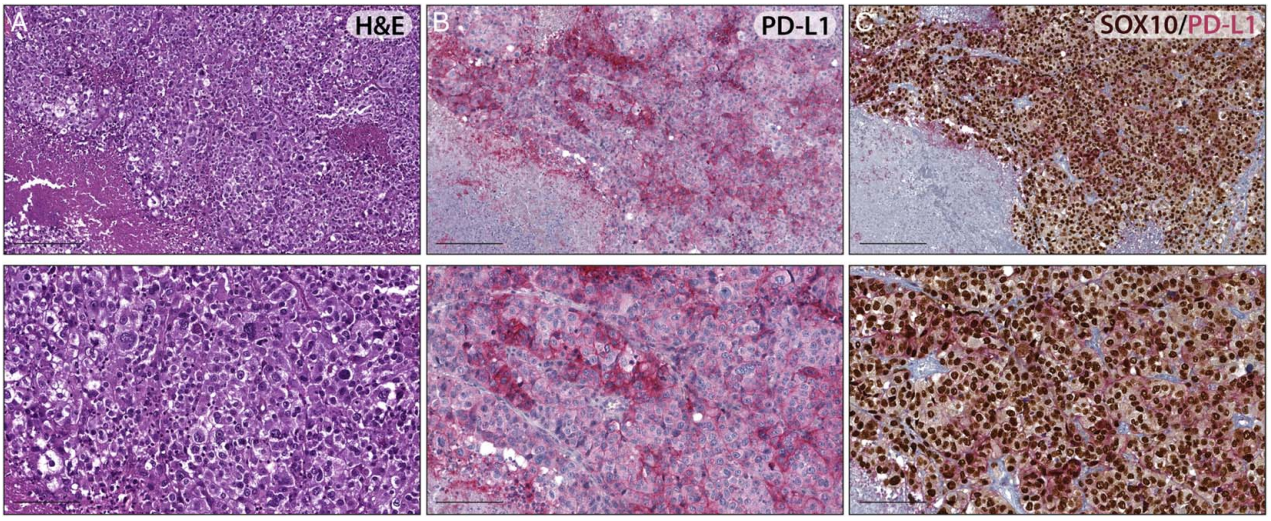

FIGURE 2. Representative images of hematoxylin and eosin staining of brain melanoma metastasis (A). Representative images of PD-L1 labeling stained with RED chromogen (B) and with the double labeling protocol for SOX10 (DAB) and PD-L1 (RED) (C). PD-L1^{+} reactive astrocytes and microglia within the tumor mass could be misinterpreted as positive uptake of PD-L1. We faced this pitfall by developing a double staining protocol with PD-L1/SOX10 that strongly helped to better detect PD-L1 expression in melanoma cells only. SOX10 negative areas can be excluded with confidence from the TPS evaluation. Magnification: ×100 and ×200 (scale bar: 100 and 25 μm). full color online

Scoring PD-L1 in melanoma could be difficult for its variable and widespread expression, especially in cases with a low TPS, in which tumor cells show complete or incomplete membranous immunoreactivity of low intensity and in highly pigmentation.

We showed that these techniques produce an immunohistochemical stain that could help pathologists to provide a better interpretation of PD-L1, compared with the singleplex standard alone. Assay validation studies are beneficial in the context of PD-L1's evaluation as, because of the many issues we introduced, it tends to have very high discordance rates between different clones, platforms, laboratories, and individual pathologists, hindering its usefulness.[16,21]

A recent study focused on the intrapathologist reproducibility of PD-L1 scoring and showed the highest disagreement in melanoma samples, with an intraclass correlation coefficient (ICC) of 0.08 and 0.20 for TPS and CPS, respectively,[21] which was improved using a double-labeling (SOX10 and PD-L1) technique, reaching an "excellent agreement" for the TPS score. Our work is meant to strengthen these results, validating this multiplex technique in Ventana Benchmark Ultra with PD-L1 SP263 clone. Furthermore, we argue that the use of the RED chromogen for PD-L1 in melanoma improves the readability of the stain.

There is no complete agreement on the ideal scoring methods to employ for PD-L1, whether ones that consider the tumor cells (TCs) only, such as the TPS, or others that include immune cells (ICs) also, such as the MELscore[22] or CPS. For instance, in Mercier's study, reproducibility was higher for the TPS score, but Darmon-Novello and colleagues argued that using MELscore led to a higher concordance, as measured with a kappa coefficient. As for these scores' clinical relevance, much evidence supports the importance of PD-L1's expression on TCs specifically.[23]

These issues have been reported for non–small-cell lung cancer (NSCLC) as well as melanoma. Several studies have observed that the TPS has a higher interpathologist concordance[24] and that the staining on ICs itself is more variable between different assays.[24,25] Considering these results, it appears that, in general, scores that include the staining on ICs tend to be less reproducible than those that only account for TCs. Therefore, tools that provide help in accurately detecting the cell compartment in which PD-L1 is expressed are sorely needed.

A universally accepted and reproducible IHC protocol for PD-L1 would provide the ideal base for its implementation as a predictive marker. Immunotherapies targeting immune checkpoint receptors such as programmed death-1 (PD-1), programmed death ligand 1 (PD-L1), or cytotoxic T-lymphocyte antigen-4 (CTLA-4) have recently revolutionized the treatment and achieved unprecedented benefit in survival of advanced melanoma patients.[23,26] Based on the efficacy results of the phase III CheckMate 067 trial, nivolumab in combination with ipilimumab, is one of the first-line standard options for advanced melanoma.[23] Combinations of these checkpoint therapies with other agents are now being explored to improve outcomes and enhance benefit-risk profiles of treatment and it is crucial to identify predictive, reliable biomarkers to improve patient selection.

In the checkmate 067 phase III trial, Wolchok et al[23] showed a significant difference in response to different ICIs based on PD-L1 evaluation in melanoma tissue

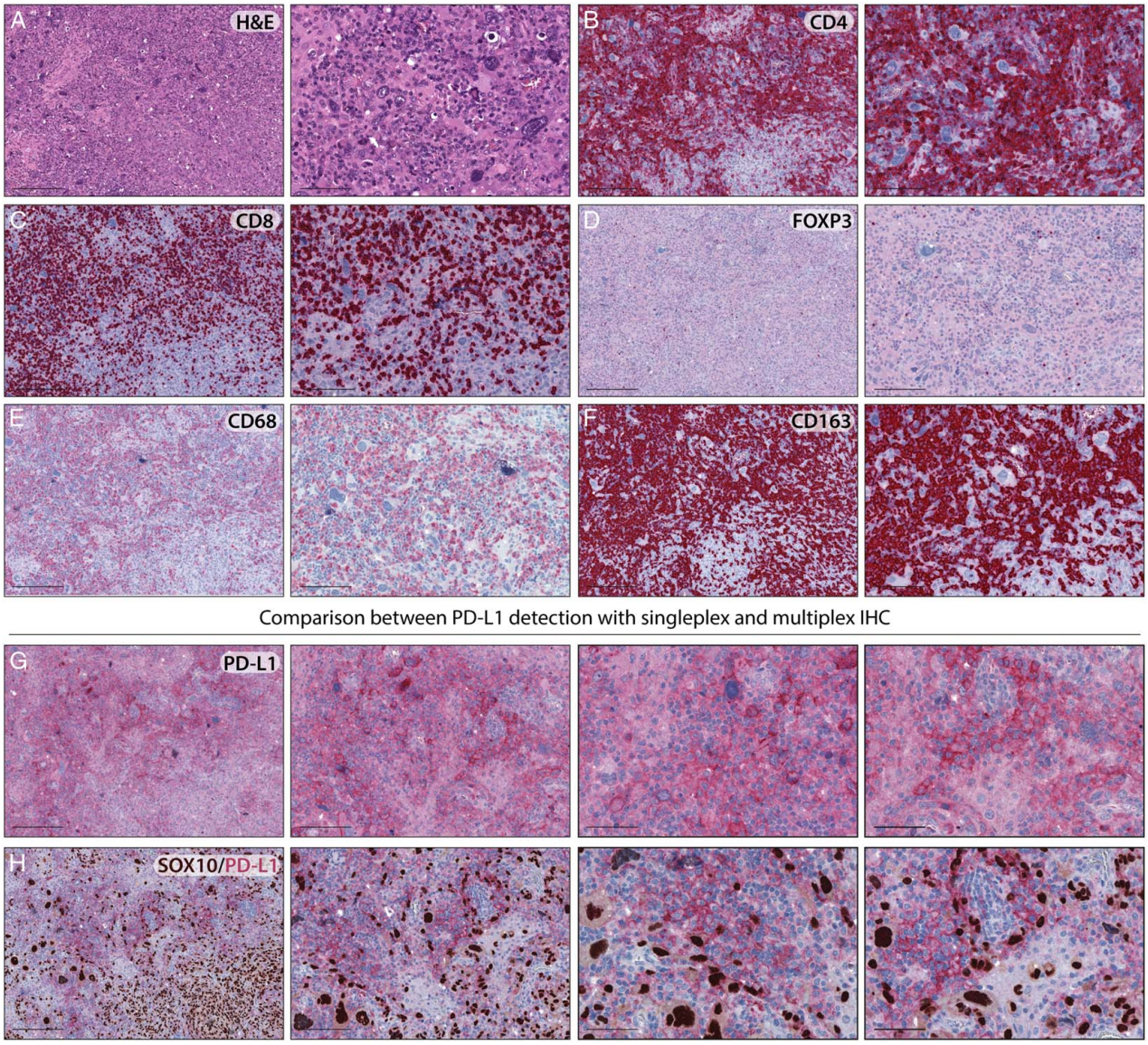

FIGURE 3. Representative images of hematoxylin and eosin staining of brain melanoma metastases (A). Representative images of CD4 (B), CD8 (C), FOXP3 (D), CD68 (E) and CD163 (F) for characterization of the tumor microenvironment. Magnification: ×100, ×200 (scale bar: 100 and 50 μm). Representative images of comparison between PD-L1 singleplex staining (G) and SOX10/PD-L1 multiplex staining (H). Comparing the Figures in (G) and (H) we can observe how the double labeling protocol can help pathologist to identify PD-L1^{+} melanoma cells, reducing the risk of TPS underestimation or overestimation in lesions with rich immune infiltrates such as these. Magnification: ×100, ×200, x400 (scale bar: 100, 50, and 25 μm). full color online

samples. In particular, melanoma patients with PD-L1 levels $<1\%$ (tested with the 28-8 pharmDx assay) showed a better prognosis in the double-agent treatment arm, with a 5-year overall survival (OS) of 50%, when compared with the single-agent arm (with 5-y OS of 36% with Nivolumab alone). In comparison, patients in the PD-L1 $\geq 1\%$ group did not show significant benefits with the addition of Ipilimumab to Nivolumab (5-y OS were 52% for the single-agent arm and 54% for the double-agent arm, respectively.[23]

Some authorities are already releasing treatment eligibility guidelines based on PD-L1's expression. For instance, considering the results published by Wolchok and colleagues in the Checkmate 067 trial, the Italian Drug Administration Agency (AIFA) authorized the use of combination immunotherapy in advanced-stage melanoma patients with PD-L1 expression $<1\%$. Moreover, the European Medicines Agency (EMA) approved the use of combination immunotherapy of nivolumab and relatlimab (anti-Lag 3) as first-line treatment for

advanced-stage melanoma in adults and adolescents 12 years of age and older with tumor cell PD-L1 expression $< 1\%$.

The main limitation of this study is that the proposed PD-L1/SOX10 multiplexing protocol can run only on the Ventana Benchmark Ultra platform, and although it is prevalent, it is not universally adopted. Moreover, if PD-L1/SOX10 multiplex IHC staining is to be implemented in the clinical setting as a companion test for melanoma, standardized protocols, and validated techniques to face the many challenges of its evaluation are a necessity in every IHC platforms available.

Furthermore, this analysis was performed on a small sample size, and future larger studies are needed to test this technique's real-world applicability and effectiveness in implementing the interpathologist agreement and reproducibility.

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