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Harmonization across programmed death ligand 1 (PD-L1) assays for lung cancer by immunohistochemistry using noncontact alternating current electric field mixing

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Abstract

Background: Immune checkpoint inhibitors (ICIs) are a promising advance in the treatment of patients with lung cancer. However, each ICI has been tested with an independently designed companion diagnostic assay that is based on a unique antibody. Consequently, the different trial-validated programmed death ligand 1 (PD-L1) immunohistochemistry (IHC) assays should not be considered interchangeable. Our aim was to compare the performance of each available PD-L1 antibody for its ability to accurately measure PD-L1 expression and to investigate the possibility of harmonization across antibodies through the use of a new rapid IHC system, which uses noncontact alternating current (AC) mixing to achieve more stable staining.

Methods: First, 58 resected non-small cell lung cancer (NSCLC) specimens were stained using three PD-L1 IHC assays (28–8, SP142, and SP263) to assess the harmonization achieved with AC mixing IHC. Second, specimens from 27 patients receiving ICIs for postoperative recurrent NSCLC were stained using the same IHC method to compare the clinical performance of ICIs to PD-L1 scores. All patients received a tumor proportion score (TPS) with the 22C3 companion diagnostic test.

Results: Better staining was achieved with the new AC mixing IHC method than the conventional IHC in PD-L1-positive cases, and the interchangeability of some combinations of assays was increased in PD-L1-positive. In addition, AC mixing IHC provided more appropriate overall response rates for ICIs in all assays.

Conclusions: Stable PD-L1 IHC driven by AC mixing helped to improve TPS scoring and patient selection for ICIs through interchangeable assays.

KEYWORDS

immune checkpoint inhibitor, immunohistochemistry, lung cancer, noncontact alternating current electric field mixing, PD-L1

INTRODUCTION

Immune checkpoint inhibitors (ICIs), including programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1) inhibitors, have shown encouraging results in the treatment of patients with lung cancer. Multiple clinical studies of ICIs have evaluated the predictive value of PD-L1 expression detected with immunohistochemistry (IHC).[1–9] However, each of the ICIs evaluated in those studies was tested using a companion diagnostic assay that was

independently designed and based on a combination of a unique antibody with a custom-designed assay employing protocols, proprietary reagents, and thresholds defining elevated expression of PD-L1 protein.[7–13] The antibodies differ among these diagnostic tests, and some tests evaluate the TPS (or the percentage of stained tumor cells [TCs]), while others evaluate both stained TCs and tumor-infiltrating immune cells (ICs). In addition, the cutoff points for a positive result and scoring systems differ among the diagnostic tests. Consequently, there is confusion among clinicians about how to use PD-L1 status for their patients.

The Blueprint PD-L1 Assay Comparison Project suggested that the different trial-validated PD-L1 IHC assays should not be considered interchangeable at present and that further investigation is needed to assess the validity of using alternative PD-L1 IHC assays.[11] Recent studies indicated that the 22C3, 28–8, and SP263 assays were relatively closely aligned on TC staining, whereas the SP142 assay consistently showed fewer stained TCs.[14–16] However, these studies have several limitations, including the clinically defined cutoffs, unclear platform-specific assays, and IC staining. The one drug-one diagnostic test approach to approval of therapeutic products in stratified or selected patient populations has yet to result in PD-1/PD-L1 therapeutic agents that are either Food and Drug Administration (FDA)-approved or in late-stage development being associated with a unique anti-PD-L1 IHC assay.[11, 16, 17] In addition, an adequate IHC platform (e.g., Dako ASL48 platform or Ventana BenchMark ULTRA platform for PD-L1) may not be available in some expert pathology laboratories.[13] However, clinicians want to know whether and by how much PD-L1 IHC results differ among different assays in the real-world, whether each of the assays equally assess the amount of PD-L1 present in the tumor tissue, and whether PD-L1 IHC assays are compatible across antibodies and platforms. At present, there is no gold standard for PD-L1 evaluation that can be widely applied by pathology laboratories.

It is a well-known fact that electro-osmotic vortices are induced noncontact mixing when an alternating-current (AC) electric-field is applied to a solution. We have developed a rapid (and stable)-IHC system that makes use of an AC electric field to facilitate the antigen–antibody reaction.[18–22] Using this device, the antibody within microdroplets is mixed and stirred as the voltage is switched on and off at specific intervals, which increases the opportunity for contact between the antigen and antibody (AC mixing). In an earlier report on breast cancer,[22] we showed that reagent-saving AC mixing IHC achieved stable staining and accurately diagnosed the molecular status, even when reagent concentrations varied. Although the AC mixing IHC method is still under development, we anticipate that this technique will be applicable for the purpose of more stable and accurate IHC staining, regardless of the type of antibody.

The aim of the present study was to compare the performance of each available PD-L1 antibody for its ability to enable accurate measurement of PD-L1 expression in lung cancer, and to investigate the possibility of harmonization across antibodies with a new rapid IHC system that enables more stable staining through AC mixing.

METHODS

Patients

All experimental protocols were approved by the institutional review board at Akita University Hospital (approval number: 896, 929 & 2455). All samples were collected under IRB Protocol No. 2455, which allows collection of tissue with consent or waiver of consent when no personalized health information is required, as was the case for this study.

For the first study, 58 pathological stage (p-stage) I–III non-small cell lung cancer (NSCLC) patients who underwent radical lung cancer surgery (lobectomy/segmentectomy plus systemic hilar and mediastinal lymph node dissection) between January 2018 and November 2018 were deemed eligible for assessing harmonization across PD-L1 antibodies using AC mixing IHC. The patient clinical characteristics are listed in Table 1. In addition, for a second study, 27 postoperative recurrent patients who received ICIs between June 2017 and February 2019 were deemed eligible for comparison of the clinical performances as the predictor responding to ICI therapy. The patient clinical characteristics are listed in Table 2. TPSs from PD-L1 companion diagnostics were obtained for all patients in both studies. All surgical specimens were collected from chemotherapy-naïve patients.

Standard immunohistochemistry

Using standard histological techniques, all surgical specimens were fixed in 10% formalin, embedded in paraffin, cut at 4 μm, transferred to slides, and stained using hematoxylin and eosin staining and IHC. As the standard PD-L1 assay,

TABLE 1 Characteristics of lung cancer patients in the interchangeability study of programmed death ligand 1 (PD-L1) immunohistochemistry (IHC) staining

Characteristic		Characteristic	
Patients, n	58	p-stage	
Age, years	66 (43–85)	0	4
Sex, n		IA1	16
Male	36	IA2	11
Female	22	IA3	9
Histology, n		IB	8
Adenocarcinoma	49	IIA	1
Squamous	9	IIB	3
Other histology	0	IIIA	4
		IIIB	2

TABLE 2 Characteristics of patients with postoperative recurrence in the comparative study of the immune checkpoint inhibitor (ICI) response rate

Characteristic		Characteristic	
Patients, n	27	p-stage	
Age, years	70 (55–83)	0	0
Sex, n		IA1	0
Male	23	IA2	2
Female	4	IA3	1
Histology, n		IB	6
Adenocarcinoma	20	IIA	3
Squamous	6	IIB	3
Other histology	1	IIIA	9
		IIIB	3
Recurrent site, n			
Lung	9	Lymph node	8
Liver	1	Others	4
Bone	1		
Adrenal gland	0		
Brain	4		

all formalin-fixed, paraffin embedded (FFPE) samples were sent to an external contract laboratory (SRL), which employed the 22C3 assay on the Dako ASL48 platform (Agilent Technologies). Standard PD-L1 IHC was performed to decide whether to clinically treat with ICI therapy. These results were graded as TPS PD-L1 ≥50%, 1%–49% or <1%. The other three PD-L1 assays (28–8, SP263, and SP142) were used for manual staining through incubation at room temperature or AC mixing. All sections were treated with antigen retrieval reagent (pH > 9, 415 201, Nichirei Biosciences Inc.) at 98°C for 40 min in a water bath. For the IHC, three anti-PD-L1 antibodies were used as the primary antibodies at the appropriate dilutions, and the incubation time was 2 h. SignalStain Boost IHC Detection Reagent (HRP, Rabbit, #8114, Cell Signal Technology, Inc.) was used as the secondary antibody.

Rapid and stable immunohistochemistry by AC mixing

For AC mixing IHC, we used the previously described Histo-Teq R-IHC (Figure S1).[18] The theory behind AC electric field mixing has previously been described in detail.[18–22] Concisely, we used a device to apply a high-voltage (4.5 kV, offset 2.4 kV), low-frequency (25 Hz) AC electric field to the sections. The antibodies within microdroplets were mixed as the voltage is switched on and off at regular intervals by the device. To compare the staining stability and comparability between conventional IHC and the new AC-mixing IHC, sections were separately incubated with each of three anti-PD-L1 monoclonal antibodies at the appropriate dilutions while AC mixing for 2 h at 37°C. The antibody dilution, incubation time, and temperature were all the same as in the conventional IHC. Table S1 and Figure 1 summarize the procedures for the conventional and AC mixing IHC.

PD-L1 scoring

The stained slides were all diagnosed by board-certified pathologists at Akita University Hospital. The pathologists calculated the TPS and the TC/IC score. Following the 28–8 and 22C3 Dako pharmDx assay interpretation guides,[23, 24] TPSs were calculated for each antibody and assigned to three aggregated levels: <1%, 1%–49% or ≥50%.[17, 23, 24] PD-L1 expression in TCs was assessed as the proportion of TCs showing membrane staining of any intensity; expression in ICs was assessed as the proportion of tumor area occupied by PD-L1-positive ICs of any intensity.[16, 25] TC/IC scores were calculated using a stepwise approach in which TCs and ICs were first assigned scores of 0, 1, 2 or 3.[17]

Statistical analysis

The data were analyzed by interchanging various cutoffs on each set of slides stained using the different assays by four PD-L1 antibodies. Statistical analyses were performed using JMP IN 14.2.0 software (SAS Institute). Best fit curves data were fitted in GraphPad using the Sigmoidal 4PL option. This fitting procedure enabled us to statistically compare curves for top and bottom values as well as for the IC50 and Hill slope.

RESULTS

Between January 2018 and February 2018, 58 FFPE samples from p-stage I–III NSCLC patients who had undergone radical lung cancer surgery and had PD-L1 companion diagnoses were eligible for the first study of harmonization across PD-L1 antibodies with AC mixing IHC (Table 1). In all PD-L1 IHCs, 50 AC mixing IHC cases were improved TPS than conventional IHCs using each PD-L1 antibody, and four PD-L1 ≥1%-positive cases were increased by AC mixing in PD-L1 <1% 22C3 IHC samples. AC mixing IHC stained as well as or better than conventional IHC in nearly all PD-L1 ≥1%-positive cases (Figure 1). Moreover, the PD-L1 ≥50%-positive cases in all four assays were similarly stained, whether using conventional or AC mixing IHC. Among the four assays, the staining achieved with SP142/AC mixing was lighter in intensity than the other assays.

Frequency distribution graphs showing the percentages of stained cells contributing to the TPS were generated for each assay (Figure 2). When assessing the TPS for PD-L1-staining among 22C3 PD-L1 ≥1% (AC mixing)-positive patients, nonlinear best fit curves calculated using a sigmoidal 4PL method showed one curve for each PD-L1

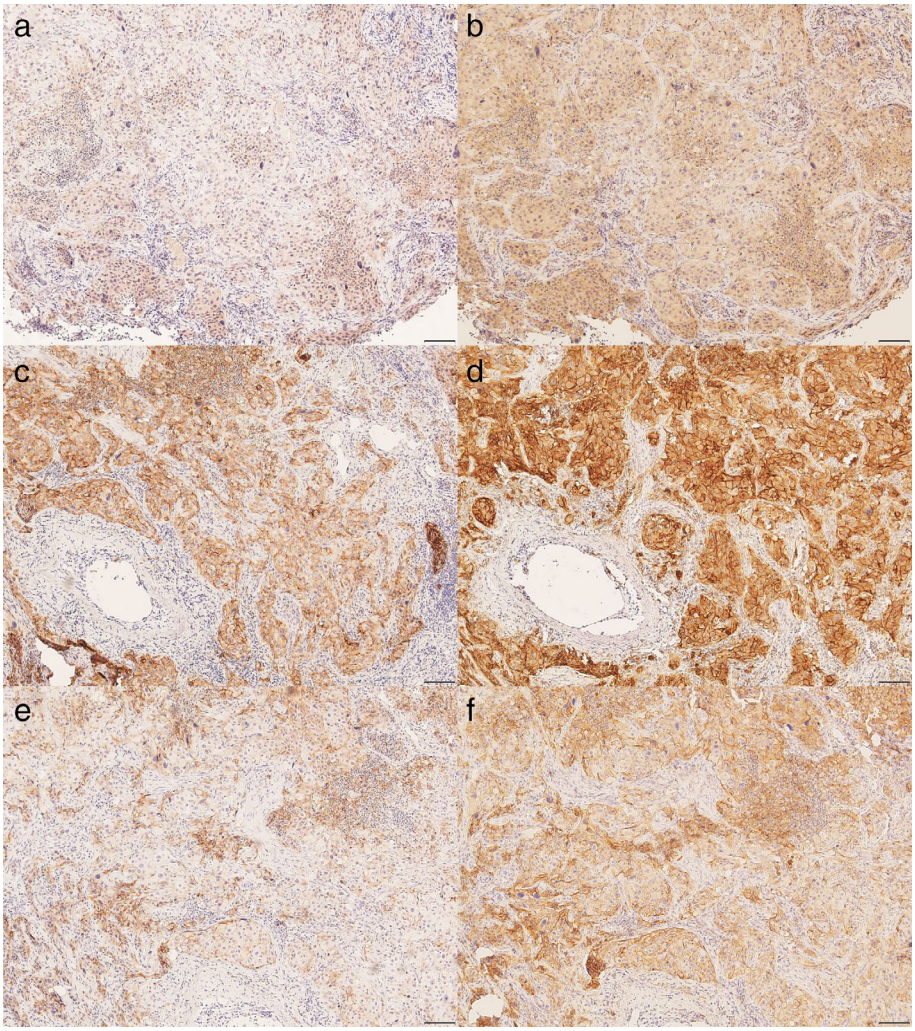

FIGURE 1 Staining pattern of programmed death ligand 1 (PD-L1) immunohistochemistry (IHC) in lung cancer. (a) 28–8, Conventional or (b) alternating current (AC) mixing IHC. (c) SP263, Conventional or (d) AC mixing IHC. (e) SP142, Conventional or (f) AC mixing IHC

antibody (n = 40, extra sum-of squares F test, F (DFn, DFd) = 1.438 (12, 144), p = 0.1551). When using AC mixing IHC, 31 PD-L1-positive cases showed greater staining than conventional IHC. Table 3 summarizes the PD-L1 scores for all 58 samples determined using conventional IHC and AC mixing IHC.

To assess the agreement among the four PD-L1 IHC assays, Venn diagrams were generated from the 58 lung cancer patients. Figure 3(a) (conventional IHC) and 3C (AC mixing) were generated for TPS >1%, whereas Figure 3(b) (conventional IHC) and 3D (AC mixing) were generated using algorithms selected for each specific staining assay. The data show comparisons of cases allocated as above or below the clinical assay threshold (Figure 3(a)/(c); common cutoff TPS 1% in all assays, or Figure 3(b)/3D; TPS >1% on 22C3 and 28–8, TPS >25% on SP263, and TC1/IC1 on SP142).

When using the conventional IHC protocol, 21 of the 58 cases (36.2%) were above the cutoff of TPS 1% in all assays (Figure 3(a)), which means that for those cases clinical PD-L1 positivity would be consistent, irrespective of the assay used. The remaining 23 cases (39.7%) above the TPS 1% cutoff showed a lack of consistency between the clinical levels of PD-L1 expression. Fourteen of 58 (24.1%) samples were determined to be below the TPS 1% cutoff, irrespective of the assay used. In addition, 10 of the 58 cases (17.2%) were above the specific cutoffs utilized for each of the four assays (Figure 3(b)), and the remaining 33 cases (56.9%) above the specific cutoffs lacked consistency. Fifteen of the 58 (25.9%) samples were determined to be below the specific cutoffs, irrespective of the assay used.

By contrast, when using the AC mixing IHC protocol, 23 of the 58 cases (39.7%) were above the TPS 1% cutoff in all assays (Figure 3(c)), while the remaining 21 cases (36.2%) lacked consistency. Fourteen of the 58 (24.1%) samples were determined to be below the TPS 1% cutoff, irrespective of the assay used. In addition, 12 of the 58 cases (20.7%) were above the specific cutoffs utilized for each of the four assays (Figure 3(d)), and the remaining 32 cases (55.2%) above the specific cutoffs lacked consistency. Fourteen of the 58 (24.1%) samples were determined to be below the specific cutoffs, irrespective of the assay used. The number of PD-L1-positive interchangeable cases was higher with AC mixing IHC in some combination of assays.

Between June 2017 and February 2019, 27 postoperative recurrent patients who received ICIs were eligible for studies evaluating the clinical performance of PD-L1 assays as

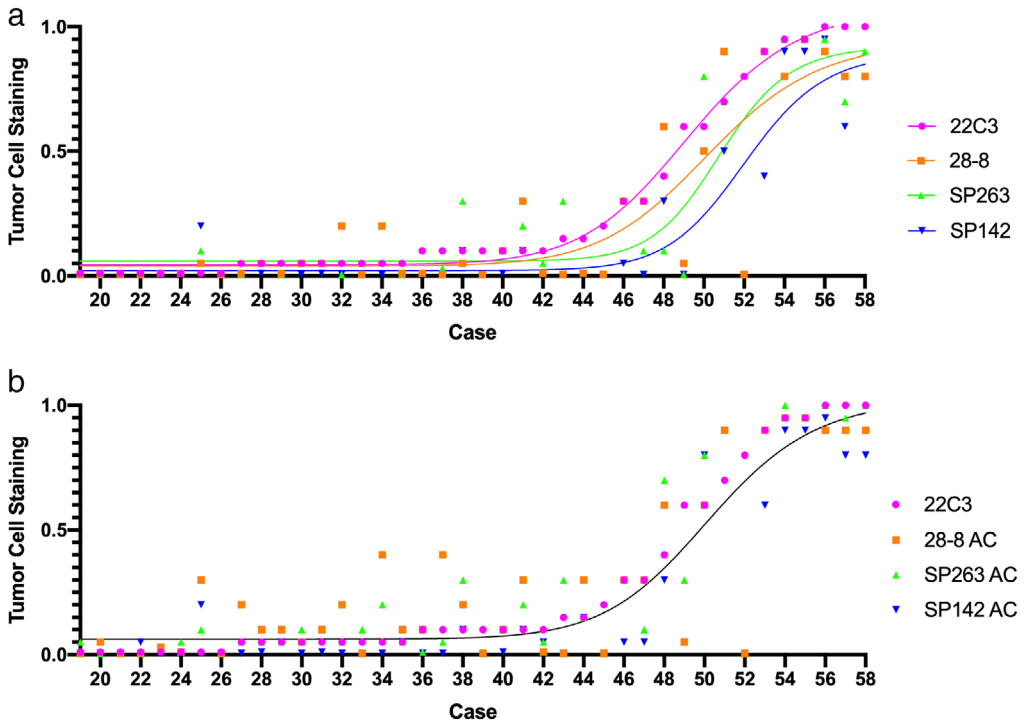

FIGURE 2 Comparison of the percentage of tumor cell staining for each programmed death ligand 1 (PD-L1) assay (the 22C3, 28–8, SP263, and SP142 assays), by case, with/without alternating current (AC) mixing. (a) Conventional immunohistochemistry (IHC) (—●—) 22C3, (—■—) 28-8, (—▲—) SP263, (—▼—) SP142. (b) AC mixing IHC (●) 22C3, (■) 28-8 AC, (▲) SP263 AC, (▼) SP142 AC. The standard IHC is the 22C3 phamDx IHC on Autostainer Link 48 platform in both figures. Best fit colored curves enable comparison of score ranges between the four assays. When assessing PD-L1 TPS in the cases of 22C3 PD-L1 >1%-positive patients using AC mixing, the nonlinear best fit curve calculated using the sigmoidal 4PL method showed one curve for all four PD-L1 antibodies (extra sum-of squares F test, $p = 0.2552$)

TABLE 3 Assay comparison: Programmed death ligand 1 (PD-L1) scoring by each PD-L1 antibody with or without alternating current (AC) mixing

PD-L1 testing							
Standard 22C3		**28–8**	**28–8 AC mixing**	**SP263**	**SP263 AC mixing**	**SP142**	**SP142 AC mixing**
TPS >50%							
Patients ($n = 10$)	≥50%	8	8	8	8	7	8
	1%–49%	1	1	0	1	1	1
	<1%	1	1	2	1	2	1
TPS 1%–49%							
Patients ($n = 30$)	≥50%	1	1	0	1	0	0
	1–49%	20	21	22	24	13	15
	<1%	9	8	8	5	17	15
TPS <1%, or							
negative	≥50%	0	1	0	0	0	0
Patients ($n = 18$)	1%–49%	3	3	3	3	1	1
	<1%	15	14	15	15	17	17

Note: Table indicates the number of cases when an alternative cutoff for the 22C3 assay was used to determine the allocation of cases to clinical groups above and below the cut-point.
Abbreviations: TPS, tumor proportion score.

predictors of responsiveness to ICI therapy (Table 2). Figure 4 shows the overall response rates (ORR) to ICIs when diagnosed using any PD-L1 antibody and any cutoff, with or without AC mixing. ICI ORRs tended to be higher when diagnosed using AC mixing IHC as the validated predictor of response. The ORRs for 22C3 were 44.44% for

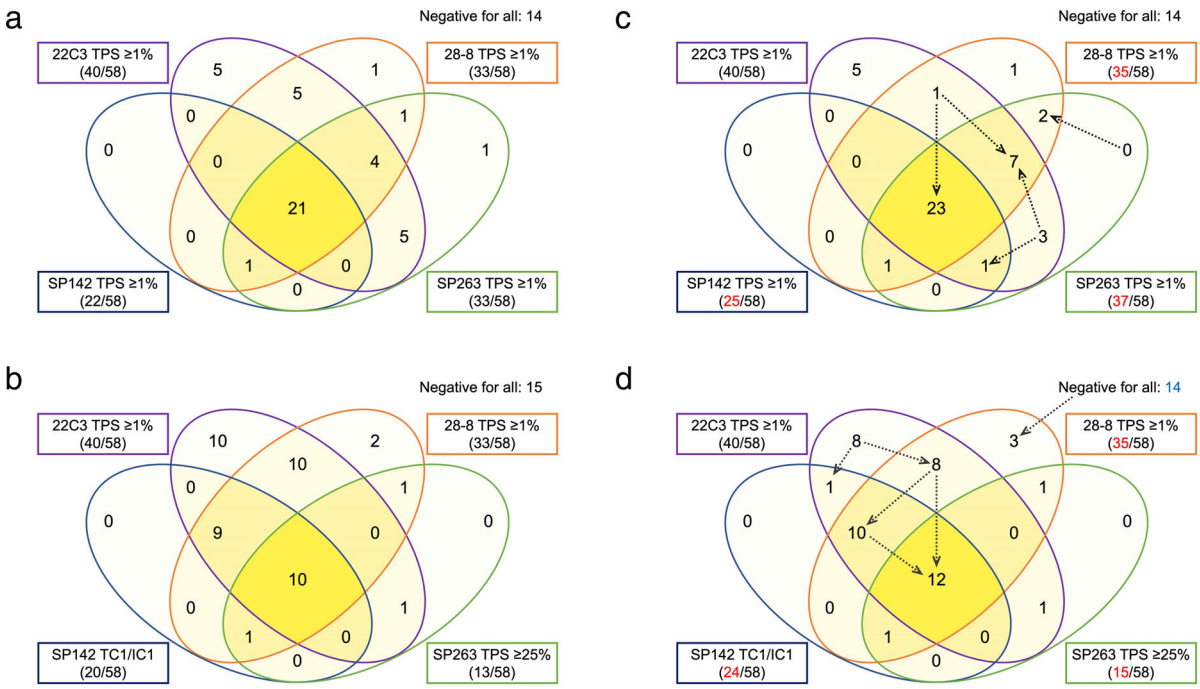

FIGURE 3 Venn diagram showing the diagnostic programmed death ligand (PD-L1) classifications with alternating current (AC) mixing. (a) Conventional immunohistochemistry (IHC) and (c) AC mixing IHC assessed based on TPS. (b) Conventional IHC and (d) AC mixing IHC assessed using the specific cutoff for each antibody. Cases comparison allocated above or below TPS 1% or the specific cutoff

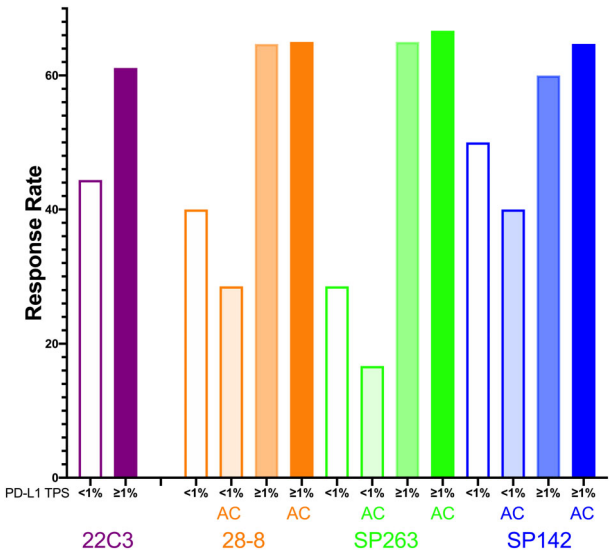

FIGURE 4 Overall response rates of immune checkpoint inhibitors (ICIs) when assessed with any PD-L1 antibody and any cutoff, with or without alternating current (AC) mixing. Immunohistochemistry with AC mixing provided more appropriate overall response rates for ICIs in all three assays. Darker color bars show the results using AC mixing (■) 22C3, (■) 28-8, (■) SP263, (■) SP142

PD-L1 TPS <1% and 61.11% for TPS ≥1%. When using AC mixing IHC, the ORRs for 28–8 decreased from 40% to 28.57% for TPS <1%, but improved from 64.71% to 65% for TPS ≥1%. ORRs for SP263 decreased from 28.57% to 16.67% for TPS <1%, but improved from 65.0% to 66.67% for TPS ≥1%. ORRs for SP142 decreased from 50% to 40% for TPS <1%, but improved from 60.0% to 64.71% for TPS ≥1%. AC mixing IHC provided more appropriate ORRs for ICIs in all three assays.

DISCUSSION

In the present study, we demonstrated that AC mixing IHC can achieve stable staining that can be used to accurately determine a PD-L1 TPS in NSCLC samples, irrespective of which PD-L1 antibody is used. Moreover, the numbers of interchangeable PD-L1-positive cases were increased in some combinations of assays when AC mixing IHC was used. Based on the of ICI ORRs in NSCLC patients with postoperative recurrence, the accuracy of PD-L1 TPS 1% and TC/IC status obtained using AC mixing IHC was greater than that obtained with conventional IHC.

The introduction of ready-to-use, highly standardized assays has led to improvement in the quality of predictive IHC, for instance in human epidermal growth factor receptor 2 testing in breast cancer.[22] By contrast, these PD-L1 assays in lung cancer are much more expensive, and reimbursement for this test is insufficient in many countries. Moreover, despite lung cancer being the most common cancer-related death cause globally, an adequate IHC platform may not be available in some expert pathology laboratories.[13] Our AC mixing IHC system is an effective procedure for obtaining very stable IHC staining, even when antibody and reagent concentrations vary.[18–22] The

advantages of this new method are its simplicity, good accuracy, and cost-effectiveness.

Our study, the Blueprint PD-L1 IHC Assay Comparison Project, and others[11, 14, 15, 26] revealed that tumor cell staining performed with 22C3, 28–8, and SP263 is fairly well aligned, though not completely interchangeable. Our results are important because they indicate that TC staining with these three PD-L1 assays may become interchangeable if AC mixing IHC is used. This would enable a single PD-L1 test to be use for ICIs in future PD-1/PD-L1 inhibitor clinical trials. However, most feasibility studies do not reflect real-world situations because experts in IHC staining and its interpretation were used in the PD-L1 assay comparisons.[11, 14, 15, 26] Not every pathology laboratory has a specific autostainer like the Dako ASL 48 and/or the Ventana BenchMark ULTRA platform (Ventana Medical Systems). Standardization, proper quality control, processing automation, laboratory accreditation, and interpretation methods all play important roles in PD-L1 testing, and the AC mixing IHC device could contribute to make stable PD-L1 reagent reaction when using any PD-L1 antibody.

One of the main causes of incompatibility of PD-L1 staining is probably the heterogeneous protein expression of PD-L1 observed among different lung cancer patients, as well as between primary and distant metastases, which may reflect differences in biopsy methods. In the present study, for analysis of the companion diagnosis, we used a portion of the surgically resected samples of primary tumor to make a 5 mm tumor block in addition to the FFPE blocks used for final pathological diagnosis. Surgical samples and lung biopsies can be taken from different sites within a tumor or from primary versus distant metastasis. PD-L1 IHC status may show intra- and intertumoral heterogeneity, and it is important to understand the variation in PD-L1 expression among different sample sites in order to assess their suitability for testing.[12] These disadvantages related to tumor heterogeneity are common among companion diagnostic methods, including IHC and other high-throughput molecular profiling techniques such as in situ hybridization and qPCR.

The SP 263 PD-L1 assay has received an in vitro diagnostic designation in Europe for nivolumab, pembrolizumab, or durvalumab therapy. The discrepancy of sensitivity between SP263 and 22C3 perhaps have a great clinical impact for patients with nonresectable NSCLC stage III with TPS ≥1%, who are considered for durvalumab plus chemoradiotherapy.[7, 8] In the present study, SP263 assays showed good agreement in TC staining among all three assays when AC mixing IHC was used, whereas the highest level of agreement was between the 22C3 and 28–8 assays when conventional IHC was used.[17] Although a survival benefit was observed with durvalumab, irrespective of SP 263 PD-L1 expression before chemoradiotherapy,[7, 8] SP263 PD-L1 IHC ≥25% or <25% expanded the body of evidence for third line or later treatment. The predictive role of tumor PD-L1 expression and the efficacy of mono/combination therapy with durvalumab + tremelimumab in patients with metastatic NSCLC was evaluated in the latest ARCTIC study.[9] Going forward, the ensured robust SP263 assay will be very important for deciding the treatment strategy for patients with metastatic NSCLC.

The SP142 PD-L1 assay is approved in over 85 countries as a complementary test for treatment of metastatic NSCLC previously treated with atezolizumab. As was seen in earlier studies,[11, 14, 15, 26] we observed that SP142 gave consistently lower TC and IC scores than the other three assays. At present, IC expression of PD-L1 in lung cancer is included only in the diagnostic scoring algorithm of the SP142 assay.[6] Several seminal studies have shown less agreement between readers or assays for PD-L1 staining of ICs than for TCs.[11, 14, 26] Likewise, for IC staining in the present study, although there was less nonspecific staining in the AC mixing IHC results, the agreement among the four assays was uncertain because IC staining was susceptible to variation in the pathologist's interpretation. Many factors may contribute to making harmonization of PD-L1 staining more difficult to achieve for ICs than for TCs. These differences in staining between TCs and ICs may be explained by the fact that the SP142 assay was specifically designed to stain ICs and that some of its binding epitopes are absent from the PD-L1 isoform 2.[27]

Despite its advantages, AC mixing IHC has several limitations. The important first limitation of this study is its small sample size and selection and allocation bias, which are the main pitfalls of comparison studies by histological tissue and data. The analysis and result of ICI response rate data has provided interesting results, but these results should be interpreted cautiously. Second, the 22C3 antibody is a registered in vitro diagnostic device and not a commercially available antibody, while the other three antibodies are designated for research use only, and we could not develop a reliable protocol that would allow PD-L1 IHC testing using the 22C3 antibody with AC mixing. To complete this new diagnostic system, future analysis of a larger number of NSCLC patient samples, including lung biopsy samples, which are smaller than those used here, will be required.

In conclusion, our AC mixing IHC system is an effective procedure for assaying PD-L1 in lung cancer, which supports IHC interchangeability and stable PD-L1 IHC staining with any antibody and without a specific autostainer. The goal is to use the accurate PD-L1 status to inform us of the likelihood of a patient's response to, and outcome with, ICIs. AC mixing IHC will help pathologists and clinicians when making PD-L1 companion diagnoses and deciding on the appropriate ICI procedure, without the need to consider the specific antibody and/or platform.

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CONFLICT OF INTEREST

The authors have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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