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Original contribution

Comparison of three FDA-approved diagnostic immunohistochemistry assays of PD-L1 in triple-negative breast carcinoma☆,☆☆

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Summary The Dako 28−8, Dako 22C3, and Ventana SP142 assays are among the approved programmed death ligand 1 (PD-L1) immunohistochemical companion/complementary diagnostics associated with cancer treatment. To address the concordance of these assays in triple-negative breast cancer (TNBC), we examined PD-L1 expression in 98 TNBC tumors and compared the positive rates using the three assays and three scoring methods: immune cell (IC), tumor cell (TC), and combined tumor cell and immune cell (TCIC) (an equivalent to combined positive score, or CPS). The positive rate for PD-L1 expression with a 1% cutoff was highest with 28−8, followed by the 22C3. These two assays demonstrated almost perfect or substantial agreement in all three scores. There was less agreement between SP142 and the other assays. Using the IC score or the TCIC score at a 1% cutoff (CPS 1), 4% of tumors were positive for PD-L1 with SP142 but negative with the other assays. Using SP142 with a 1% cutoff as a reference, the optimal cutoff for best agreement was at 1% for IC, 30% for TC, and 2% for TCIC (CPS 2) with the other two assays. A 2% cutoff for the 22C3 TCIC (CPS 2) yielded the best agreement with SP142 1% IC cutoff (kappa 0.65). Our study showed the lowest positive rate with SP142 among the three assays. However, the other two assays were not able to identify all tumors

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that would test positive with SP142 using IC or TCIC/CPS. It is unlikely to achieve high agreement between SP142 and the other two assays by changing the analytical cutoffs.
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1. Introduction

Immune checkpoint inhibitors targeting the programmed death 1 (PD-1)/programmed death ligand 1 (PD-L1) pathway have been used to treat various cancer types in recent years. Along with several drugs approved by the U.S. Food and Drug Administration (FDA) in this category, their corresponding diagnostic immunohistochemistry (IHC) assays were developed to detect PD-L1 expression to select patients for treatment [1−6]. However, the availability of multiple PD-L1 IHC assays has presented a challenge to pathology laboratories seeking to offer appropriate PD-L1 testing in a cost-effective fashion. Given this array of choices, it is necessary to learn the concordance of these assays and determine their interchangeability in each tumor type.

In lung cancer, the Blueprint PD-L1 IHC Comparision/Comparability Projects assessed and compared the PD-L1 IHC assays [7,8]. In the phase 2 project, five clinical trial−validated PD-L1 assays (28−8 [Dako], 22C3 [Dako], SP142 [Ventana], SP263 [Ventana], and 73−10 [Dako/Agilent]) in 81 lung cancer cases were compared in their staining of tumor cells and tumor-infiltrating immune cells. The 28−8, 22C3, SP142, and SP263 assays have been approved by the FDA. The results showed similar staining between 28-8, 22C3, and SP263, and SP142 was less sensitive in tumor cells than the other assays, which was consistent with the results of the phase 1 project. In addition, the immune cell scores for the 28−8, 22C3, and SP263 assays were similar, but there was less staining with SP142 than with the other assays. The 73−10 assay showed greater staining than the other assays in both tumor cells and immune cells. Other studies supported the findings from the Blueprint Projects in lung cancer, showing that while the 28−8 and 22C3 assays appear interchangeable from the analytical point of view, the SP142 assay has considerably lower sensitivity [9,10].

The need to select appropriate assays for PD-L1 has become more pressing in triple-negative breast cancer (TNBC) as recent clinical trials testing PD-1/PD-L1 inhibitors in the treatment of this group of tumor showed encouraging results. Pembrolizumab, a PD-1 inhibitor, has had promising response rates in PD-L1−positive advanced or metastatic TNBC and significantly increased the pathologic complete response rate in early TNBC when combined with neoadjuvant chemotherapy in the KEYNOTE-012, -086, and -522 trials [11−13]. The latter two trials, which were more recent than KEYNOTE-012, used a combined positive score (CPS) of ≥1 with the Dako 22C3 assay to define positive PD-L1 staining in TNBC. In addition, in patients with locally advanced and metastatic TNBC, the phase 3 IMpassion130 trial showed prolonged progression-free survival with the PD-L1 inhibitor atezolizumab plus nab-paclitaxel compared with the placebo plus nab-paclitaxel group, and the survival benefit was significantly higher in patients with PD-L1−positive TNBC than in patients with PD-L1−negative TNBC [14,15]. In that trial, the Ventana SP142 assay was used to evaluate PD-L1 expression, and positivity was defined as ≥1% of immune cell staining in the tumor area. So far, the Ventana SP142 assay is the only FDA-approved companion assay for atezolizumab in TNBC. Thus, it would be interesting and important to compare other assays with SP142 in TNBC to understand their concordance.

In a previous study, we have compared different scoring methods for PD-L1 IHC staining in breast cancer [16]. In the present study, the Dako 28−8 and 22C3 assays, which are widely used in many laboratories, were compared with the Ventana SP142 assay in TNBC in tumor cell staining, immune cell staining, and combined tumor and immune cell staining. The aim of this study was to provide useful information to medical oncologists and pathology laboratories in selecting the most appropriate PD-L1 testing in TNBC.

2. Materials and methods

2.1. Human breast tumor samples

This retrospective study was approved by the institutional review board of The University of Texas MD Anderson Cancer Center. We included 98 patients diagnosed with triple-negative invasive breast cancer during 2004−2016 and treated at our institution. From these patients, we used samples from surgical excision specimens of the primary tumor. The American Society of Clinical Oncology/College of American Pathologists guideline recommendations [17−19] were used as references to categorize estrogen receptor (ER), progesterone receptor (PR), and HER2 status as part of the routine pathologic evaluation. As modifications to the guideline for ER and PR, positive staining was defined as nuclear staining in at least 5% of invasive carcinoma cells because the clinical management of tumors with low expression of ER and PR is similar to that of both ER- and PR-negative tumors.

2.2. IHC for assessment of PD-L1 expression

Tissue microarrays (TMAs) were constructed from representative archival paraffin blocks of primary tumors from our Pathology files using a 1.0-mm manual tissue arrayer (Beecher Instruments, Inc.). Duplicate punches from different areas of the same tumor were obtained in 95% of the samples. Unstained tissue section 4-μm thick were prepared from the TMAs, and IHC for PD-L1 was performed using the PD-L1 28-8 pharmDx kit and the PD-L1 22C3 pharmDx kit (Dako North America Inc., Carpinteria, CA) on the Dako Autostainer Link 48, and using the PD-L1 Ventana SP142 assay kit (Roche Diagnostics, Indianapolis, IN) on the Ventana BenchMark Ultra, according to the manufacturers' instructions. Slides were counterstained with Mayer's hematoxylin. Results were evaluated with known positive and negative tissue controls. The percentage of PD-L1 expression in tumor-infiltrating immune cells (IC) was assessed as the proportion of tumor area occupied by PD-L1–positive immune cells of any intensity in any cell compartment. The percentage of PD-L1 expression in invasive tumor cells (TC) was calculated as the number of viable invasive carcinoma cells showing membranous staining of any intensity divided by the total number of viable invasive carcinoma cells. The percentage of PD-L1 expression in tumor-infiltrating immune cells and invasive tumor cells (TCIC) was calculated as the number of those cells showing PD-L1 staining (membranous staining for invasive tumor cells and any staining for immune cells) divided by the total number of invasive tumor cells. The TCIC percentage used in our study was equivalent to the CPS in the KEYNOTE trials [12,13]. For example, a TCIC of 1% was equivalent to a CPS of 1.

PD-L1 expression was evaluated by four breast pathologists, X.H., H.G., Q.D., and L.H. Difficult and discrepant cases were determined by discussion and review at multi-headed microscopes by at least two pathologists.

2.3. Statistical analysis

Statistical analysis was carried out using SAS 9.3 for Windows (SAS Institute Inc.). Cohen's kappa coefficient was used to assess the agreement between any two assays for each scoring method and was interpreted as: <0, no agreement; 0.0–0.20, slight agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; 0.81–1.00, almost perfect agreement.

3. Results

3.1. Comparison of three assays with ≥1% considered positive

The number of tumors included for analysis was 95 for the IC score, 98 for the TC score, and 96 for the TCIC score/CPS. The results with ≥1% (CPS ≥1) defined as positive using each of the three scoring methods are summarized in Fig. 1. The 28–8 assay had the highest positive rates with all scoring methods, at 36% by IC, 16% by TC, and 43% by TCIC/CPS. In contrast, the SP142 assay had the lowest positive rates: 28% by IC, 5% by TC, and 34% by TCIC/CPS. Comparing the three assays demonstrated concordant results (positive or negative) in 82% of tumors (78/95) by IC, 86% of tumors (84/98) by TC, and 79% of tumors (76/96) by TCIC/CPS. None of the tumors showed a positive TC score with SP142 unless the TC score was positive with one or both of the other two assays (Fig. 1B). However, in 4% of the tumors, the IC score and TCIC score/CPS were positive with SP142 but negative with the other assays (Fig. 1A and C). Photomicrographs of representative tumors are shown in Fig. 2.

Pairwise comparison of the assays using the kappa coefficient showed higher agreement between 28-8 and 22C3 compared with that between SP142 and either of the other assays with each scoring method (Table 1). Comparison of SP142 and each of the other assays showed substantial agreement when using the IC and TCIC/CPS scores but only low moderate to fair agreement when using the TC score.

3.2. Identification of cutoff values to improve agreement between SP142 and the other assays

To find the cutoff values at which the 28–8 and 22C3 assays would show best agreement with the SP142 assay with the 1% cutoff, we applied a wide range of cutoff values to the results of the 28–8 assay and the 22C3 assay, and pairwise comparison was conducted using the SP142 assay results with a 1% cutoff as the reference, for each scoring method. As shown in Fig. 3, for 28–8, the highest agreement was reached when a cutoff of 1% was chosen for IC, a cutoff of 30% was chosen for TC, and a cutoff of 2% was chosen for TCIC (CPS 2), with kappa coefficients of 0.64, 0.60, and 0.61, respectively (Fig. 3A, C, E). Of note, the kappa coefficient for TCIC was almost identical between cutoffs of 1%, 2%, and 4% (CPS 1, 2, and 4, respectively); the difference was less than 0.01. For 22C3, the highest agreement was reached at the same cutoffs: 1% for IC, 30% for TC, and 2% for TCIC (CPS 2), with kappa coefficients of 0.65, 0.71, and 0.72, respectively (Fig. 3B, D, F). Again, the difference in the kappa coefficient between cutoffs of 1% and 2% for TCIC (CPS 1 and 2) was small (0.70 vs. 0.72). Obviously, the biggest improvement in agreement between SP142 and the other two assays was demonstrated in the TC score. When we increased the TC cutoff from 1% to 30%, the kappa coefficient increased from 0.43 to 0.60 for the comparison of 28–8 and SP142, and from 0.40 to 0.71 for comparison of 22C3 and SP142, with a 1% SP142 TC cutoff as the reference. The

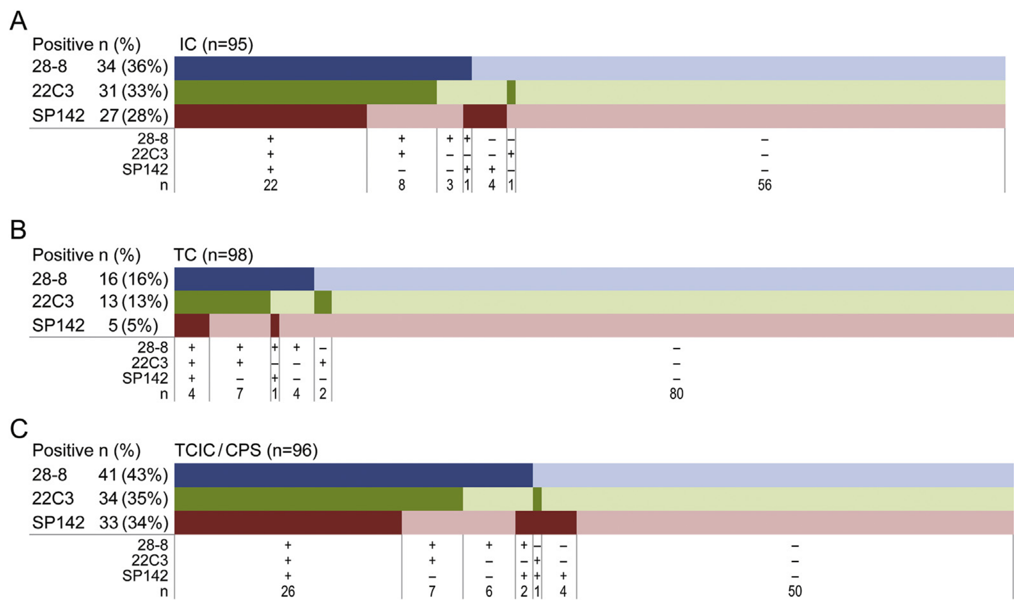

Fig. 1 Summary of staining results and comparison of the three PD-L1 assays using a 1% cutoff. (A) immune cell (IC) score. (B) tumor cell (TC) score. (C) combined tumor cell and immune cell (TCIC) score/combined positive score (CPS). The darker color represents positive cases, and the lighter color represents negative cases. The positive case numbers and positive rates with each assay are on the left. The numbers of cases in categories defined by shared results across all three assays are below the bar graphs. PD-L1, programmed death ligand 1.(For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

improvement was owing to 5 more tumors being categorized as negative with 28−8 and 7 more tumors being categorized as negative with 22C3 at the 30% cutoff than at the 1% cutoff (Fig. 3G).

Because cutoffs of 1% in IC with the SP142 assay and CPS 1 with the 22C3 assay have been applied in anti−PD-1/PD-L1 clinical trials in TNBC [12−14], the agreement between the 22C3 TCIC/CPS at various cutoffs and the SP142 IC at a cutoff of 1% was further explored in our cohort to find a cutoff for 22C3 TCIC/CPS that would yield the best kappa coefficient between the two assays. As shown in Fig. 4A, the highest kappa coefficient, 0.65, was reached at a 2% cutoff for the 22C3 TCIC (CPS 2), with the 1% cutoff for the SP142 IC as the reference - a mild improvement compared with a 1% cutoff for the 22C3 TCIC (CPS 1), which yielded a kappa coefficient of 0.59. In this comparison, the positive rate using the 2% cutoff for the 22C3 TCIC (CPS 2) was 33% (31/95); three more tumors were categorized as negative with the 2% cutoff than with the 1% cutoff (Fig. 4B). Of note, 5% (5/95) of the tumors were positive with SP142 at the 1% cutoff for IC but negative with 22C3 at either a 1% or a 2% cutoff for TCIC (CPS 1 or CPS 2).

4. Discussion

In this cohort of 98 patients with TNBC, when a 1% cutoff was used, the highest positive rate was observed with the 28−8 assay, followed by 22C3 and then SP142, regardless of scoring method. On pairwise comparison, 28−8 and 22C3 assays reached almost perfect agreement (kappa coefficient 0.88) for IC and highly substantial agreement for TC and TCIC/CPS. In contrast, the SP142 assay had worse agreement with the other two assays, especially when using TC, where the agreement with 28−8 was moderate (0.43) and the agreement with 22C3 was fair (0.40). The concordance rates for TC (positive or negative) between SP142 and the other assays appeared reasonable (89% between SP142 and 28−8 and 90% between SP142 and 22C3, Table 1), but those numbers were largely driven by the high proportion of negative cases. The high similarity between the 28−8 and 22C3 assays, as well as the lower positive rate with SP142 in our TNBC cohort was consistent with the findings in the Blueprint studies and other studies in lung cancer [7−10].

The SP142 assay remains the only clinically validated assay for selecting patients with metastatic TNBC for treatment with atezolizumab plus nab-paclitaxel. Two IMpassion130 substudies, both currently in abstract forms, have explored the analytical concordance between the 22C3 and SP142 assays. One substudy, using the 1% IC cutoff for SP142 and the CPS 1 cutoff for 22C3, showed that 45% of patients with TNBC had positive PD-L1 expression with both assays, 36% had positive expression with 22C3 and negative expression with SP142, 1% had negative expression with 22C3 and positive expression with SP142, and 18% had negative expression with both assays [20]. Thus, the concordance rate (positive and negative) was only 63%. Our cohort showed a higher concordance for an equivalent comparison between 22C3 using a 1% TCIC/CPS 1 cutoff and SP142 using a 1% IC cutoff (82%, or 78/95, Fig. 4B), attributable to a higher proportion of negative

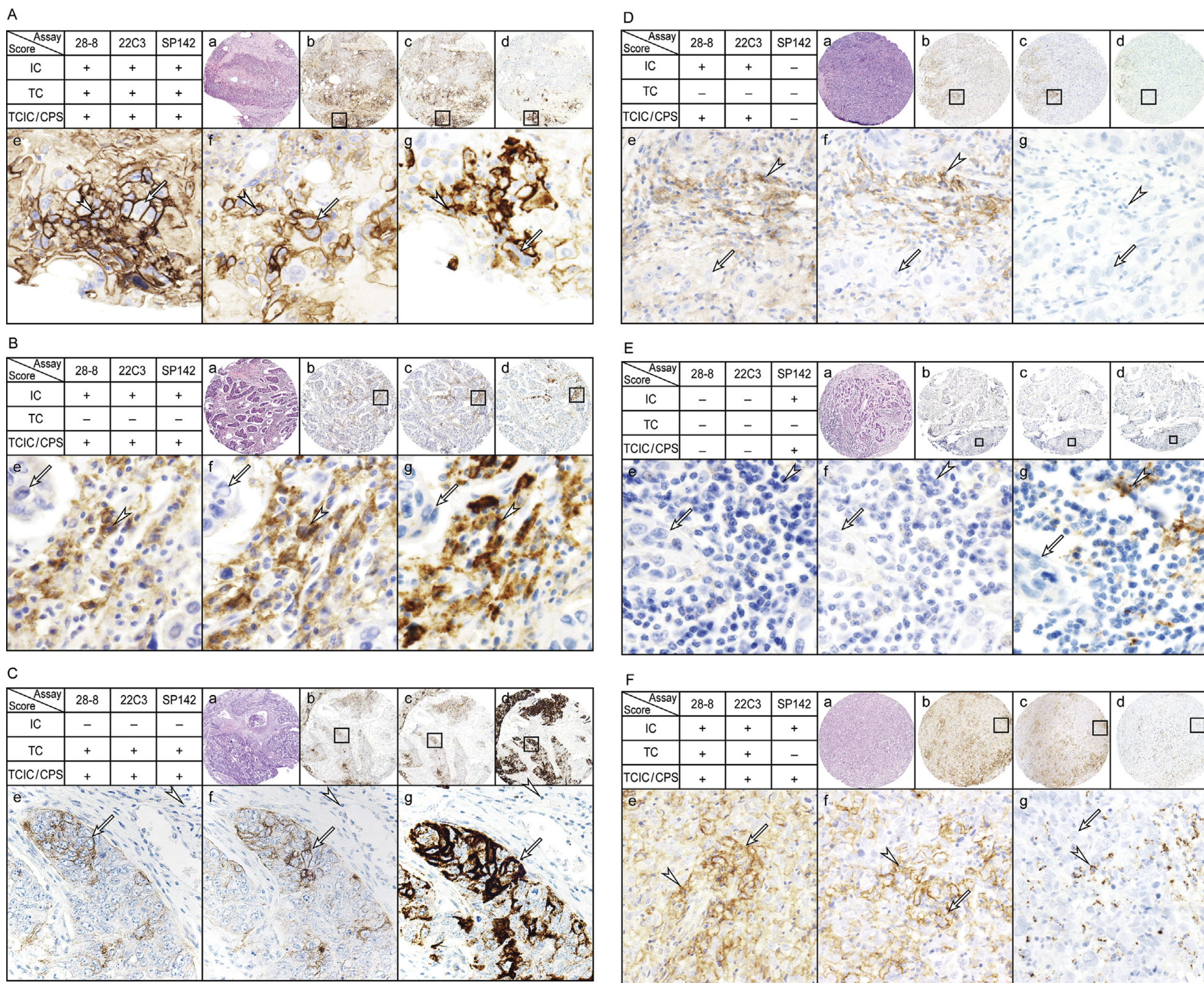

Fig. 2 Photomicrographs of representative cases. A-F, each panel represents one case. The staining results of each case with each assay and scoring method are depicted in the upper left table in each panel. a, hematoxylin and eosin stain; b-d, PD-L1 stains using 28−8, 22C3, and SP142, respectively; e-g, higher-magnification images of the boxed areas in b-d, respectively. Arrow, tumor cells; arrowhead, immune cells. Original magnification: a-d, × 40; e-g in 2 A-2D, 2 F, × 200; e-g in 2 E, × 400. PD-L1, programmed death ligand 1.

cases. Interestingly, in that previous study, even though the positive cases identified by 22C3 encompassed almost all positive cases identified by SP142, 1% of cases were positive only with SP142. Similarly, in our present study, 5 of 95 (5%) cases were positive with SP142 and negative with 22C3 using the IC score with a 1% cutoff (Fig. 1A), and the same number of cases were positive with SP142 using 1% IC and negative with 22C3 using TCIC at either a 1% or a 2% cutoff (CPS 1 or 2) (Fig. 4B), suggesting that despite its higher sensitivity, the 22C3 assay is not able to select all patients who would test positive with SP142.

The other substudy of IMpassion130 attempted to harmonize the analytical concordance between SP142 and other assays [21], and identified the optimal cutoffs for other assays that could maximize the overall percentage of agreement, using a 1% IC cutoff with SP142 as the reference standard. For 22C3, the highest overall agreement was found when a cutoff of CPS 10 was applied. However, the agreement was considered suboptimal because the positive percentage agreement actually decreased. The authors suggested that different assays may not identify the same tumor biology and could not be harmonized. In our study, different cutoffs were applied to obtain the best kappa coefficients between SP142 and the other assays, using SP142 with the 1% cutoff as the reference for each scoring method. The biggest improvement was seen in the TC score: changing the cutoff to 30% for 28−8 and 22C3 increased the kappa coefficient by 0.17 for 28−8 versus SP142 and by 0.31 for 22C3 versus SP142 (Fig. 3C and D). In contrast, when using the IC score, the kappa coefficient

Table 1 Pairwise comparisons for concordance (positive or negative) and agreement between assays with a ≥1%/CPS 1 cutoff.

Scoring method and comparison	Concordant cases, no. (%)	Kappa coefficient (95% confidence interval)	Category of agreement
IC (n = 95)			
28−8 vs. 22C3	90 (95%)	0.88 (0.78−0.98)	Almost perfect
28−8 vs. SP142	80 (84%)	0.64 (0.48−0.80)	Substantial
22C3 vs. SP142	81 (85%)	0.65 (0.49−0.82)	Substantial
TC (n = 98)			
28−8 vs. 22C3	91 (93%)	0.72 (0.52−0.91)	Substantial
28−8 vs. SP142	87 (89%)	0.43 (0.17−0.69)	Moderate
22C3 vs. SP142	88 (90%)	0.40 (0.11−0.69)	Fair
TCIC/CPS (n = 96)			
28−8 vs. 22C3	87 (91%)	0.80 (0.68−0.92)	Substantial
28−8 vs. SP142	78 (81%)	0.61 (0.45−0.77)	Substantial
22C3 vs. SP142	83 (86%)	0.70 (0.55−0.85)	Substantial

IC, immune cell; TC, tumor cell; TCIC, combined tumor cell and immune cell; CPS, combined positive score.

could not be further improved, as the best cutoff values remained at 1%. For the TCIC score/CPS, the best cutoff values shifted to 2% (CPS 2) for both 28−8 and 22C3, but with minimal improvement in the kappa coefficient. The CPS 10 cutoff that emerged in the above-mentioned study [21] is intriguing because a recent press release on the KEYNOTE-355 trial indicated that a CPS of ≥10 could identify metastatic TNBC patients with significantly improved progression-free survival on pembrolizumab plus chemotherapy compared with chemotherapy alone [22]. In our previous study [16], using the TCIC score/CPS with 22C3 assay, the positive rate of PD-L1 expression decreased from 35% to 19% in TNBC when the cutoff was changed from 1% to 10% (CPS 1 to CPS 10). In the current study, we sought the cutoff of TCIC/CPS with 22C3 that could generate the best kappa coefficient when compared with SP142 using the 1% IC cutoff, and we found that a 2% cutoff for TCIC (CPS 2) gave the highest kappa coefficient, 0.65, with a slightly decreased positive rate of 33% compared with TCIC at 1% cutoff (CPS 1) (Fig. 4B). Thus, our findings corroborate those from the IMpassion130 substudies [20,21] and indicate that it is unlikely to accomplish high agreement between SP142 and the other two assays by simply changing the cutoffs.

In contrast to the 41% rate of positivity with SP142 reported in the IMpassion130 trial [14], our cohort had 28% positive staining with SP142 using the same IC cutoff. This difference could be due to the small sample size in our cohort and interobserver variation. Our use of TMAs may also have contributed to a lower positive rate, as PD-L1 expression is often patchy in breast cancer and may not be captured in small samples. In addition, in our cohort, all patients underwent surgical resection of the primary tumor, and most did not have metastasis, in contrast to the IMpassion130 trial patients, who had metastatic or unresectable locally advanced TNBC. It is possible that this difference in patient population affected the prevalence of PD-L1 expression in the two cohorts. Small sample size and the use of TMAs are both limitations of this retrospective study. Although the study design included two different areas of each tumor to generate the TMAs, whether this limited sampling approach can represent PD-L1 expression in TNBC needs to be further validated. Such validation is warranted because it will not only address the issue of using TMAs in research studies but also shed light upon clinical use of small biopsies of primary or metastatic tumors for PD-L1 evaluation, because some of the biopsies may have similar amount of tumor tissue to that in TMAs. Another limitation of our study is the inability to assess the predictive value of the described cutoffs in anti−PD-1/PD-L1 therapy because none of the patients in the cohort was treated with anti−PD-1/PD-L1.

In summary, in our study of a cohort of 98 TNBC primary tumors, the positive detection rate for PD-L1 expression with a 1% cutoff using the TC, IC, and TCIC/CPS scoring methods was highest with the Dako 28−8 IHC assay, followed by the Dako 22C3 IHC assay and then the Ventana SP142 IHC assay. On pairwise comparison, the 28−8 and 22C3 assays demonstrated almost perfect or substantial agreement in the TC score, IC score, and TCIC score/CPS. There was less agreement between SP142 and the other assays, especially in the TC score. Using SP142 with a 1% cutoff as a reference, the optimal cutoff for best agreement was found to be 1% for IC, 30% for TC, and 2% for TCIC (CPS 2), with both the 28−8 and 22C3 assays. In addition, a 2% cutoff for the 22C3 TCIC (CPS 2) yielded the best agreement with SP142 at a 1% IC cutoff, with a kappa coefficient of 0.65,

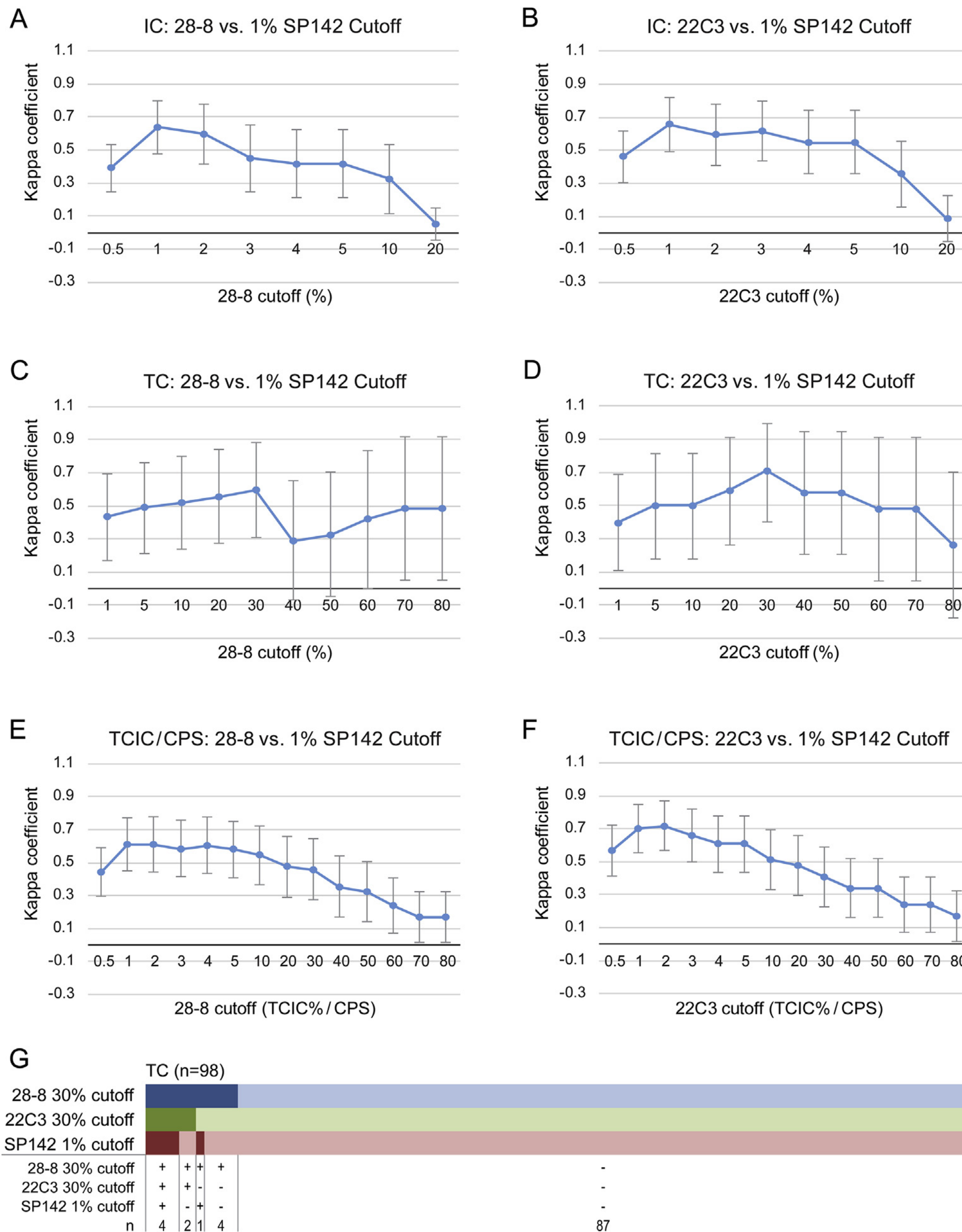

Fig. 3 Changes in kappa coefficient based on the indicated cutoff values to determine positive or negative staining with the 28−8 assay or the 22C3 assay compared with the SP142 assay at a 1% cutoff. A and B, IC score; C and D, TC score; E and F, TCIC score/CPS. The 0.5% cutoff represents tumors with staining between 0% and 1%. Error bars represent 95% confidence intervals. G, comparison of the TC score using 28−8 at a 30% cutoff, 22C3 at a 30% cutoff, and SP142 at a 1% cutoff. The darker color represents positive cases, and the lighter color represents negative cases. The numbers of cases in categories defined by shared results across all three assays are indicated below the bar graph. IC, immune cell; TCIC, combined tumor cell and immune cell; TC, tumor cell; CPS, combined positive score. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

only slightly higher than the kappa coefficient of 0.59 when 22C3 TCIC at a 1% cutoff (CPS 1) was compared with the same reference. When PD-L1 IHC stains are evaluated in clinical settings, it can be difficult to consistently make the distinction between 1% and 2% expression levels, especially when large tissue sections are used. Thus, it is unlikely to obtain high agreement between SP142 and the other two assays by changing the analytical cutoffs. Furthermore, 5% of tumors in our cohort were positive for PD-L1 with SP142 at a 1% cutoff for the IC score, but negative with 22C3 with either the IC (with 1% cutoff) or the TCIC score (at 1% or 2% cutoff, CPS 1 or 2), suggesting that 22C3 is not able to identify all tumors that would test positive with SP142 using the IC score. Other independent studies are warranted to confirm the clinical implications of our findings.

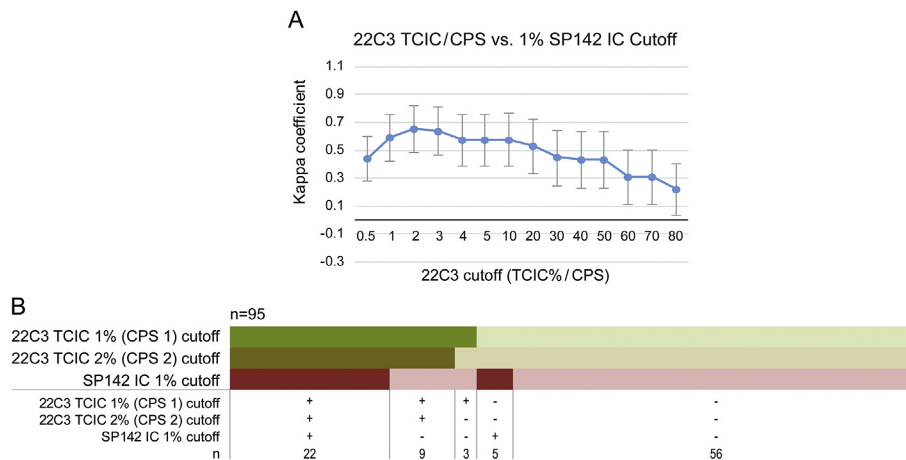

Fig. 4 Comparison between 22C3 using TCIC/CPS and SP142 using IC. (A) Changes in kappa coefficient based on the indicated cutoff values to determine positive or negative staining with 22C3 using TCIC/CPS, compared with SP142 using IC at a 1% cutoff. The 0.5% cutoff represents tumors with staining between 0% and 1%. Error bars represent 95% confidence intervals. (B) Comparison of 22C3 using TCIC at a 1% (CPS 1) cutoff, 22C3 using TCIC at a 2% (CPS 2) cutoff, and SP142 using IC at a 1% cutoff. The darker color represents positive cases, and the lighter color represents negative cases. The numbers of cases in categories defined by shared results across all three approaches are indicated below the bar graph. TCIC, combined tumor cell and immune cell; CPS, combined positive score; IC, immune cell. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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