PD-L1 Expression in Esophageal Squamous Cell Carcinoma: A Comparative Analysis of 3 Different Antibodies

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Research

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Abstract

**Background:** Several studies have assessed the comparability of various immunohistochemical assays for programmed cell death 1 ligand 1 (PD-L1) expression in different kinds of tumours. However, there is a lack of relevant research on the detection efficacy of different PD-L1 clones in esophageal squamous cell carcinoma (ESCC). This study was performed to compare the PD-L1 expression results of three PD-L1 antibodies to assess their effectiveness.

**Methods:** Three hundred and twenty-four cases of ESCC tissues in our medical institution were used to prepare tissue microarrays (TMAs). TMAs were stained with three validated assays (Ventana’s SP263 and Dako’s 28-8 and 22C3). Then, pathologists scored each tissue according to PD-L1 staining.

**Results:** We found very high concordance when comparing 28-8 with SP263 at both the 1% (95.7%) and 50% (99.1%) cutoffs. Low overall concordance was found between 28-8 and 22C3 at the 1% cutoff (85.5%). Moreover, sensitivities of 91.2% and 87.5% were found for 28-8 compared with SP263 at the 1% and 50% cutoffs, respectively. Sensitivities of only 75.6% and 50% were found for 28-8 compared with 22C3 at the 1% and 50% cutoffs, respectively, which may result in more false negatives.

**Conclusions:** We demonstrate that the PD-L1 SP263 shows high levels of overall agreement with 28-8 and relatively high sensitivity, with a low false negative rate. Thus, SP263 seems to be a reliable assay for PD-L1 testing in ESCC when considering pembrolizumab as immunotherapy.

Introduction

As one of the most aggressive and lethal malignant tumours, the 5-year survival rate of oesophageal cancer is less than 20% (1). From 2008 to 2012, the age-standardized incidence rate by world standard population (ASIRW) was 14.80/100,000, and the crude mortality rate of oesophageal cancer was 17.19/100,000 (2). There are two main pathological subtypes of oesophageal cancer: oesophageal squamous cell carcinoma (ESCC) and oesophageal adenocarcinoma (EAC) (1). In China, more than 95% of oesophageal cancer is ESCC. At present, some types of solid tumours, including lung cancer (3, 4), melanoma (5), breast cancer (6), and hepatocellular carcinoma (7), have been found to bring forth an immunosuppressive tumour microenvironment by expressing programmed cell death 1 ligand 1 (PD-L1), thus avoiding T cell-mediated cytolysis. Encouragingly, cancer immunotherapy has entered a new era recently with the discovery of drugs that interfere with specific immune checkpoints. Moreover, due to the good effect of immunotherapy in squamous cell carcinoma, it may be a new strategy for ESCC treatment in the future (8).

Programmed death 1 (PD1, CD279) regulates immune tolerance by inhibiting the activation of T or B cells (9). As one of the ligands of PD-1, PD-L1 is widely expressed in solid tumours. Therefore, the expression of PD-L1 was identified as a predictive diagnostic marker for the selection of patients who might benefit from anti-programmed cell death 1 (PD-1) axis drugs, such as nivolumab, pembrolizumab, atezolizumab and durvalumab (10-13). In addition, preliminary clinical data show that the histological response rate,
progression-free survival (PFS) and overall survival (OS) of patients with high levels of PD-L1 expression detected by immunohistochemistry (IHC) have improved (3, 14, 15). Therefore, the expression of PD-L1 may be a predictive biomarker for anti-PD-1/PD-L1 therapy (16). At present, there are many qualitative detection antibodies against PD-L1 that can be used to evaluate the expression of PD-L1. The only companion diagnostic test approved by the Food and Drug Administration (FDA) for pembrolizumab in non-small cell lung cancer (NSCLC) is the PD-L1 IHC 22C3 PharmDx kit (Agilent Technologies, Inc.), which was developed for testing on the Dako Autostainer (17). Another antibody developed for the Ventana BenchMark platform, namely, the SP263 PharmDx assay, was approved by the Conformité Européene in treatment decisions for NSCLC (18, 19). Previous studies have shown discrepancies in identifying PD-L1-positive results between SP263 and 22C3, which may lead to diagnostic bias in the selection of patients who should receive immunotherapy (20, 21). In current studies of immunotherapy for ESCC, there is no widely accepted antibody for the detection of PD-L1, which affects the consistency of these studies to a certain extent. Therefore, comparing the analytical performance and comparability of the two methods in ESCC is of great significance for the standardization of immunotherapy for ESCC.

In a previous study, we used 28-8 (Abcam, Cambridge, United Kingdom) as an antibody to detect the expression of PD-L1 in 233 patients with ESCC and to explore the relationship between the expression of PD-L1 and patient prognosis (22). As a widely used antibody, the 28-8 antibody is used for commercial testing and laboratory research. Here, in this study, we compared the PD-L1 expression results of IHC 28-8 with those of Dako’s 22C3 and Ventana’s SP263 to assess their effectiveness as a screening tool in the diagnostic routine of ESCC.

**Methods**

**Patients**

A total of 324 consecutive patients who underwent curative oesophagectomy with R0 resection for histologically verified ESCC between December 2005 and June 2013 at the National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences were included in the present study. Patients who received preoperative chemotherapy/radiotherapy and with distant metastasis were excluded. All tissue specimens used in this study were obtained from the biobank, and all 324 patients provided informed consent before surgery. The patients’ clinical parameters, including age, sex, smoking status, tumour location, tumour differentiation, T stage, lymph node metastasis and tumour-node-metastasis (TNM) stage, were recorded. The pathological classification of the primary tumour and the degree of lymph node metastasis were assessed according to the American Joint Committee on Cancer staging manual (eighth edition) (23).

**Tissue microarrays (TMAs)**

All ESCC tissue samples were stained with haematoxylin and eosin (H&E) and confirmed by two pathologists independently. All 324 samples of a respective tumor region were extracted from areas harboring a high tumor/stroma ratio. A series of 4-μm-thick sections were cut and
transferred to adhesive slides according to the manufacturer’s instructions.

**IHC and evaluation**

The TMAs were stained with three validated assays according to the manufacturer’s instructions: staining for clones 22C3 and 28-8 (Abcam, ab205921) was performed on the Dako Autostainer Link 48 platform. Staining for SP263 was performed on the Ventana Benchmark Ultra platform.

Two pathologists who were blinded to the clinical data assessed the samples independently, and disagreements were resolved by a third experienced pathologist. Tumours positive for PD-L1 had a combined positive score (CPS) of 10 or more (24). In the present study, for the CPS, the results were divided into 3 groups: those with zero staining were considered “negative,” those with 1%-49% positive cells were considered “low positive,” and those with 50%-100% positive cells were considered “high positive.” In addition, a 4-category group was evaluated using 0%, 1%-9%, 10%-49%, and 50%-100% positive cells.

**Statistical analysis**

Statistical analyses were performed using SPSS 18.0 software (IBM Corporation, Armonk, NY). McNemar’s test was used to compare differences between the agreement proportions in different clones. The overall percent agreement (OPA), positive predictive value (PPV), negative predictive value (NPV), and area under the curve (AUC) were calculated to evaluate the clinical performance of the assays.

**Results**

A total of 324 patients with ESCC were included in the current study. Patient characteristics are summarized in Table 1. The median age of the patients was 59 years (34-78 years), and 23.8% were women.

Figure 1 illustrates the immunohistochemical staining in a representative ESCC sample from the TMAs. Different staining scores for the same tumour were compared, with relatively high inter-observational consistency (agreement higher than 90%). When the staining score of the same tissue was inconsistent, it was re-assessed by a more experienced pathologist.

Regarding the comparison between 28-8 and SP263, at the 1% cutoff, 78.4% and 79% of patients were negative (CPS <1%), respectively; 18.8% and 18.5% had CPS values of 1-49%, respectively; and 2.8% and 2.5% were strongly positive (CPS ≥50%), respectively. The actual numbers of cases for the comparison between 28-8 and SP263 are shown in Table 3 according to the thresholds < 1%, 1-49% and ≥50%. The OPA between 28-8 and SP263 was 95.7% at the 1% cutoff and 99.1% at the 50% cutoff (Table 2). At the 1% cutoff, Table 2 shows the sensitivity and specificity (91.2% and 96.9%, respectively), with a PPV and NPV of 88.6% and 97.6%, respectively, and an AUC of 0.931. At the 50% cutoff, the sensitivity and specificity were 87.5% and 99.4%, respectively, and the AUC was 0.872. The analytical comparison of CPS values was performed in each case after analysis with these two assays (Figure 2).
The actual numbers of concordance cases for the 28-8 and 22C3 assays are shown in Table 5. At the 1% cutoff, the OPA was 85.5%, with a difference in proportion of 14.5 (Table 4). The sensitivity and specificity were 75.6% and 87.1%, respectively, with a PPV and NPV of 48.6% and 95.7%, respectively, and an AUC of 0.719. At the 50% cutoff, the OPA was 97.2%, with a sensitivity of 50% and a specificity of 98.1%. The PPV and NPV were 33.3% and 99%, respectively, and the AUC was 0.662.

Overall, we found that 28-8 and SP263 IHC staining scores were highly consistent, with strong and intense membrane staining. For 22C3, the tumour cell (TC) scores tended to be lower than those of the other two assays, and the IHC staining tended to be weaker. The analytical comparison of CPS values was also performed in each case after analysis with these two assays (Figure 3).

**Discussion**

To the best of our knowledge, this is the first study performed to assess the concordance of the PD-L1 IHC 28-8 assay with the SP263 and 22C3 assays in the TMA of ESCC. A previous study of NSCLC suggested that the overall agreement between the SP263 clone and the PD-L1 clone E1L3N was higher than that between the 22C3 clone and the PD-L1 clone E1L3N (25). In our study, similar results were observed in the TC scores of ESCC when comparing 28-8 with 22C3 or SP263 at 1% and 50% cutoffs. Thus, the detection of PD-L1 expression with the SP263 assay is more sensitive than with the others and may further reduce the number of patients misclassified as PD-L1 negative.

At present, increasing evidence shows the effectiveness of immunotherapy in the treatment of oesophageal cancer. In the metastatic setting, phase II clinical trials have shown that the objective response rate for PD-L1-positive oesophageal cancer ranged from 14% to 17% (26, 27). Data from the KEYNOTE-181 trial in Japan demonstrated that OS was superior in the pembrolizumab arm in patients whose tumours had a PD-L1 CPS of ≥10 (28). Although there was no statistically significant difference, patients with ESCC had a better response to pembrolizumab than those with EAC. More ongoing studies will help determine whether ESCC is more sensitive to immunotherapy than EAC. However, it cannot be ignored that some patients with tumours negative for PD-L1 also show a clinical response when treated with PD1/PD-L1 inhibitors (29). The strikingly heterogeneous PD-L1 expression in different PD-L1 IHC assays of the same tumour tissue may lead to the misclassification of patients who could be treated with PD1/PD-L1 inhibitors (30). In this regard, we attempted to assess the correlation and differences between the expression scores obtained by the SP263 and 22C3 assays. Medical centres seldom have two or more automatic detection systems, so it is difficult to run two different staining platforms simultaneously on limited samples. In addition, different antibodies have different antigen epitopes, which affect the consistency of IHC staining intensity. The selection of an appropriate antibody for PD-L1 detection has important clinical significance because biopsy specimens are often limited. A previous study showed high discordance between small biopsy specimens and surgical resection specimens, even with the same antibody clone (31). Therefore, antibodies with high sensitivity used for PD-L1 detection may be beneficial in reducing false negatives in small biopsy specimens.
In contrast to the U.S. FDA-approved 22C3 antibody clone as a companion diagnostic IHC assay for using pembrolizumab and Conformité Européenne-marked Ventana's SP263, which is used to make treatment decisions in NSCLC for both nivolumab and pembrolizumab, the 28-8 Dako antibody was approved as a diagnostic to nivolumab (10, 32-34). The technical equivalency and predictive equivalency of PD-L1 IHC assays, including the Dako 22-C3 and 28-8, Ventana SP263 and SP142 assays, has been tested widely in NSCLC (35). However, for the immunotherapy of oesophageal cancer, there is still no unified companion diagnostic criteria or recognized PD-L1 IHC assay. Moreover, considering the low sensitivity of the SP142 assay for determining the tumour proportion score on TCs and the fact that SP142 is not commercially available, it was not included in our study (36, 37).

In agreement with the study of Hendry et al., the TC scores of the 28-8 assay were similar to those of 22C3 but slightly lower than those of SP263 (35), which may be explained by the high-intensity staining of SP263 on the cell membrane. Most previous studies have shown a high correlation between the 22C3 and SP263 assays (36-38) (except for the studies of Munari and Hendry (25, 35)). Consistent with the latter view, we found that 22C3 showed a lower correlation than 28-8, with a sensitivity of 50% at the cutoff of 1%. The precise reason for this discrepancy is not yet clear. However, considering that there is no similar study in oesophageal cancer at present, this discrepancy may be caused by the tumour heterogeneity of oesophageal cancer and lung cancer. In addition, the inconsistency between the SP263 and 22C3 assays, according to Munari et al's results, is more common in small biopsy samples (25). More clinical trials are needed to further verify the relationship between the response rate of immunosuppressive agents and differences in these assays in patients with oesophageal cancer to identify an economical and efficient antibody for PD-L1 detection.

There were some limitations to this study. First, in the present study, the expression of PD-L1 was affected by the use of small cores rather than whole sections, according to a previous study (38). It is worth noting that tumours may heterogeneously express PD-L1, which may lead to an overestimation or underestimation of the true PD-L1 levels. Second, this study was a retrospective, single-centre study, and there is potential for selection bias. Therefore, our results would be best answered in the context of a multicentre, prospective study with a large sample size. Although our results demonstrate that the 28-8 assay shows high agreement with the SP263 assay and low levels of overall agreement with the 22C3 assay, the efficacy of these antibodies in detecting the expression of PD-L1 for the purposes of guiding therapy with PD1/PD-L1 inhibitors in ESCC still needs more research.

**Conclusions**

In summary, the PD-L1 clone SP263 exhibits relatively high sensitivity and specificity compared with clone 28-8 at both cutoffs of 1% and 50%. Thus, SP263 seems to be a reliable assay for PD-L1 testing in ESCC. However, evaluating the clinical validity of these assays is another essential aspect that cannot be ignored. A more comprehensive analysis of the potential immune microenvironment is needed in the future.
Abbreviations

ESCC (esophageal squamous cell carcinoma); PD-1 (programmed cell death receptor 1); PD-L1 (programmed cell death ligand 1); IRS (immunoreactivity scoring system); TCs (tumor cells); OS (overall survival); DFS (disease-free survival); CI (confidence interval); HR (hazard ratio); TMA (tissue microarray); NSCLC (non-small cell lung cancer); FFPE (formalin-fixed paraffin-embedded); H&E (hematoxylin and eosin).

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the ethics committee of National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (191137-1921). The requirement of patients’ informed consent was waived owing to the retrospective nature of the study.

Consent for publication

The requirement of patients’ informed consent was waived owing to the retrospective nature of the study.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

Funding

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Authors' contributions

SGG, JMY and WG designed the study. LG, YJ and WG wrote the draft manuscript; WG, YJ, HH, PS and BQ performed the data analysis. LG, XMX and JMY performed the immunochemistry and evaluation work. All authors approved the final manuscript.

Acknowledgements
References


Table 1 Distribution of clinical parameters in 324 esophageal squamous cell carcinoma patients

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>n(%) (N=324)</th>
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<tbody>
<tr>
<td>Age at diagnosis (year)</td>
<td>Mean (SD)</td>
</tr>
<tr>
<td></td>
<td>59.06 (9.05)</td>
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<tr>
<td>Gender</td>
<td></td>
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<tr>
<td>Male</td>
<td>247 (76.2)</td>
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<tr>
<td>Female</td>
<td>77 (23.8)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>121 (37.3)</td>
</tr>
<tr>
<td>Ever</td>
<td>203 (62.7)</td>
</tr>
<tr>
<td>Location</td>
<td></td>
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<tr>
<td>Up</td>
<td>65 (20.1)</td>
</tr>
<tr>
<td>Middle</td>
<td>168 (51.9)</td>
</tr>
<tr>
<td>Low</td>
<td>91 (28.1)</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>90 (27.8)</td>
</tr>
<tr>
<td>Moderate</td>
<td>173 (53.4)</td>
</tr>
<tr>
<td>Poor</td>
<td>61 (18.8)</td>
</tr>
<tr>
<td>T stage</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>10 (3.1)</td>
</tr>
<tr>
<td>T2</td>
<td>44 (13.6)</td>
</tr>
<tr>
<td>T3</td>
<td>230 (71.0)</td>
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<tr>
<td>T4</td>
<td>40 (12.3)</td>
</tr>
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<td>Lymph node metastasis</td>
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<tr>
<td>N0</td>
<td>190 (58.6)</td>
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<tr>
<td>N+</td>
<td>134 (41.4)</td>
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<td>TNM stage</td>
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<tr>
<td>I</td>
<td>9 (2.8)</td>
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<tr>
<td>II</td>
<td>169 (52.2)</td>
</tr>
<tr>
<td>III</td>
<td>146 (45.1)</td>
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</table>

Table 2 Comparison between 28-8 and SP263

<table>
<thead>
<tr>
<th></th>
<th>28-8 VS SP263</th>
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<tbody>
<tr>
<td></td>
<td>Agreemen</td>
</tr>
<tr>
<td>CPS&gt;=1%</td>
<td>95.7</td>
</tr>
<tr>
<td>CPS&gt;=50%</td>
<td>99.1</td>
</tr>
</tbody>
</table>

Table 3 Actual number of cases for the comparison between 28-8 and SP263 according to the thresholds < 1%, 1-49% and ≥ 50%
Table 4 Comparison between 28-8 and assay 22C3

<table>
<thead>
<tr>
<th>28-8 VS 22C3</th>
<th>Agreement</th>
<th>DP</th>
<th>P value</th>
<th>Sen (%)</th>
<th>Spec (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>AUC</th>
</tr>
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<tr>
<td></td>
<td>CPS&gt;=1%</td>
<td>85.5</td>
<td>0.011</td>
<td>75.6</td>
<td>87.1</td>
<td>48.6</td>
<td>95.7</td>
<td>0.719</td>
</tr>
<tr>
<td></td>
<td>CPS&gt;=50%</td>
<td>97.2</td>
<td>0.434</td>
<td>50</td>
<td>98.1</td>
<td>33.3</td>
<td>99</td>
<td>0.662</td>
</tr>
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</table>

Table 5: actual number of cases for the comparison between E1L3N and 22C3 according to the thresholds < 1%, 1-49% and ≥ 50%

<table>
<thead>
<tr>
<th>28-8</th>
<th>22C3</th>
<th>&lt;1%</th>
<th>1-49%</th>
<th>&gt;=50%</th>
<th>Total</th>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1%</td>
<td>243</td>
<td>34</td>
<td>2</td>
<td>279</td>
<td></td>
</tr>
<tr>
<td>1-49%</td>
<td>10</td>
<td>25</td>
<td>4</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>&gt;=50%</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td></td>
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<tr>
<td>Total</td>
<td>254</td>
<td>61</td>
<td>9</td>
<td>324</td>
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</table>

Figures
The immunohistochemical staining in a representative ESCC sample from the TMAs. Different staining scores for the same tumour were compared, with relatively high inter-observational consistency (agreement higher than 90%). When the staining score of the same tissue was inconsistent, it was re-assessed by a more experienced pathologist.
Figure 2

The analytical comparison of CPS values was performed in each case after analysis with these two assays
Figure 3

The analytical comparison of CPS values was also performed in each case after analysis with these two assays.