RESEARCH

Prognostic value of PD-L1 expression in bronchopulmonary neuroendocrine tumours

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Abstract

Programmed death protein 1 (PD-1) and its ligand, PD-L1, have emerged as promising therapeutic targets for many types of cancer that overexpress PD-L1. However, data on PD-L1 expression levels in bronchopulmonary neuroendocrine neoplasms (BP-NEN) are limited and contradictory. In the present study, a total of 298 archived, formalin-fixed, paraffin-embedded BP-NEN samples from 97 patients diagnosed with typical carcinoid (TC), atypical carcinoid (AC), small cell lung cancer (SCLC), or large cell neuroendocrine carcinoma of the lung (LCNEC) were evaluated for PD-L1 expression by immunohistochemistry using the highly sensitive monoclonal anti-PD-L1 antibody 73-10. PD-L1 expression levels were semiquantitatively estimated by tumour grading. Of the 298 BP-NEN samples, 85% were positive for PD-L1 expression. PD-L1 immunostaining predominantly localized to the plasma membrane of both tumour cells and tumour-infiltrating immune cells. SCLC and LCNEC exhibited significantly higher PD-L1 expression levels than TC or AC. PD-L1 expression levels were also higher in patients with lymph node or distant metastases, in patients who smoked, and in patients who died during the follow-up period. Moreover, PD-L1 expression levels correlated positively with tumour grading, Ki-67 index and the expression of the chemokine receptor CXCR4 and negatively with the levels of somatostatin receptor 1 and chromogranin A. High tumour PD-L1 levels were associated with poor patient outcomes. In conclusion, PD-L1 expression is common in BP-NEN, increases with malignancy, and is associated with poor prognosis. Therefore, targeting the PD-1/PD-L1 axis could be a promising strategy for treating BP-NEN. PD-L1 may also represent a useful prognostic biomarker for this tumour entity.

Key Words

- PD-1
- PD-L1
- antibody
- immunohistochemistry
- lung cancer
- neuroendocrine tumour
- carcinoid
- small cell lung cancer
- large cell neuroendocrine carcinoma

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Introduction

Neuroendocrine neoplasms (NEN) comprise a heterogeneous group of tumours arising from the neuroendocrine system, which is widely distributed throughout the body. Consequently, NEN can develop in almost every organ, though they are most often found in the gastrointestinal tract or the lung. Bronchopulmonary NEN (BP-NEN) are classified into low-grade, well-differentiated typical carcinoids (TC), intermediate-grade atypical carcinoids (AC) and high-grade, poorly differentiated, highly malignant small cell lung cancer (SCLC) and large cell neuroendocrine carcinomas of the lung (LCNEC) (1, 2). Despite several advances in diagnostics and therapy during the past decades, the prognosis of SCLC and LCNEC remains very poor (3, 4), and treatment options for non-resectable or metastatic TC or AC are limited (1). Therefore, there is a pressing need for new therapeutic options for these tumour entities.

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Recently, cancer-directed immunotherapy targeting the programmed death protein 1 (PD-1) and its ligand, programmed death ligand 1 (PD-L1), has emerged as a promising treatment strategy for several tumour types. PD-1 is expressed on B and T lymphocytes, as well as on myeloid cells, and acts as a costimulatory factor leading to a downregulation of T-cell activation. Meanwhile, PD-L1 is often overexpressed in neoplastic tissues, where it promotes tumour evasion of the immune system (5, 6, 7). Several anti-PD-1 and anti-PD-L1 antibodies have been developed, such as pembrolizumab, nivolumab, atezolizumab, durvalumab, and avelumab, and some of these are already approved for treating non-small cell lung cancer (NSCLC), head and neck cancer, oesophagus, gut, colorectal or renal clear cell carcinoma, bladder cancer, melanoma, Merkel cell carcinoma, or Hodgkin lymphoma (5, 6, 7, 8). A series of trials examining the efficacy of these antibodies for SCLC, both alone and in combination with other drugs, have shown promising results (9), but similar studies have yet to be conducted for other BP-NEN entities.

Currently, the best approach for predicting tumour response to anti-PD-1 or anti-PD-L1 therapy is determining the PD-L1 status of the tumour by immunohistochemistry (10). However, PD-L1 expression in BP-NEN was mostly determined in SCLC only, and the expression levels reported show a high variability between the different studies (11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30). Moreover, there are contradictory results regarding the prognostic significance of PD-L1 expression for anti-PD-1 or anti-PD-L1 treatment efficacy in naïve BP-NEN patients (11, 15, 17, 18, 19, 20, 23, 25, 26, 28). In light of the these current limited and contradictory data, the present investigation aimed to evaluate PD-L1 expression in a large panel of bronchopulmonary neuroendocrine neoplasms comprising TC, AC, SCLC and LCNEC by immunohistochemistry using the monoclonal rabbit anti-PD-L1 antibody 73-10 and to re-examine the potential for PD-L1 as a prognostic biomarker for patients with BP-NEN.

The rabbit MAB 73-10 was selected based on published data (31) and the results of a pilot study showing that 73-10 provides better sensitivity than other commonly used anti-PD-L1 antibodies.

Materials and methods

Tumour specimens

The pilot study was conducted using a panel of 43 formalin-fixed, paraffin-embedded surgically resected tumour samples from 43 patients diagnosed with either adenocarcinoma of the lung (ADC) ($n=22$) or squamous cell carcinoma of the lung (SQC) ($n=21$).

The main study included a total of 298 archived, formalin-fixed, paraffin-embedded tumour samples from 97 patients with BP-NEN. Of the 97 patients, 24 were diagnosed with TC, 27 with AC, 38 with SCLC, and 8 with LCNEC (Table 1). The number of samples obtained from a single patient ranged from 1 to 18 (1 sample from 38 patients, 2 from 16 patients, 3 from 17 patients, 4 from 8 patients, 5 from 5 patients, 6 from 4 patients, 7 from 2 patients, 8 from 2 patients, 9 from 2 patients, 14 from 1 patient, 16 from 1 patient, and 18 from 1 patient). A total of 208 samples were from primary tumours and 69 were from metastases; no respective information was available for 21 samples. The vast majority of the main study samples were resected specimens, but also 15 biopsies were included. All samples for the pilot and main studies were provided by the Institute of Pathology and Cytology Bad Berka, Bad Berka, Germany and had been resected between 1998 and 2014 at the Department of Thoracic and Vascular Surgery, Zentralklinik Bad Berka, Bad Berka, Germany. All procedures performed in this study involving human participants were in accordance with

Table 1 Patient and tumour characteristics.

	TC	AC	SCLC	LCNEC	Total
Total no.	24	27	38	8	97
Sex (number)					
Male	7	13	24	3	47
Female	17	14	13	5	49
Unknown	0	0	1	0	1
Age (years)					
mean	59.9	59.2	59.6	61.4	59.7
median	62.8	62.1	60.2	59.1	61.3
Survival (months)					
Mean	78.3	78.6	29.1	6.7	58.3
Median	77.2	89.2	15.8	6.7	54.2
T (*n*)[a] (number)					
1	11	7	3	2	23
2	4	7	5	3	19
3	0	0	1	2	3
4	0	1	2	0	3
Unknown	9	12	27	1	49
N (*n*)[a] (number)					
0	20	15	5	3	43
1	1	5	9	3	18
Unknown	3	7	24	2	36
M (*n*)[a] (number)					
0	15	12	5	4	36
1	1	5	4	2	12
Unknown	8	10	29	2	49

[a]The majority of the SCLC cases have been classified as limited or extensive disease and not according to the TNM classification.

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both the ethical standards of the institutional or national research committee and the 1964 Helsinki declaration and its later amendments. Permission for this retrospective analysis was obtained from the local ethics committee (Ethikkommission der Landesärztekammer Thüringen). For this type of study, formal consent was not required. All data were recorded and analysed anonymously.

Antibodies and immunohistochemistry

The primary antibodies used in this study, along with information regarding antibody type, epitope recognized, supplier, and concentration used, are listed in Table 2. Antibody concentrations and methods of antigen retrieval were selected according to the manufacturer's instructions. Higher concentrations of the antibodies, as well as alternative antigen retrieval methods, were tested but did not lead to better staining results.

From the paraffin blocks, 4-µm sections were prepared and floated onto positively charged slides. Immunostaining was performed by an indirect peroxidase labelling method (32). For staining of PD-L1, sections were dewaxed, microwaved in Tris-EDTA buffer, pH 9.0 (clones 73-10 and SP142) or in universal heat-induced epitope retrieval reagent (ab208572, Abcam, Cambridge, MA, USA) (clone 28-8) for 16 min at 600 W and incubated with primary anti-PD-L1 antibody overnight at 4°C. To stain somatostatin receptors (SST) 1, 2, 3, 4, and 5, CXC motif chemokine receptor 4 (CXCR4), chromogranin A (CgA), and the proliferation marker Ki-67, the same general procedure was followed but antigen retrieval was performed using 10 mM citric acid (pH 6.0). Following staining with primary antibody, samples were incubated with biotinylated anti-rabbit or anti-mouse IgG secondary antibody, followed by peroxidase-conjugated avidin (Vector ABC 'Elite' kit; Vector Laboratories, Burlingame, CA, USA) according to manufacturer's instructions. Binding of the primary antibodies was visualized using 3-amino-9-ethylcarbazole in acetate buffer (BioGenex, San Ramon, CA, USA). Sections were rinsed, counterstained with Mayer's haematoxylin, and mounted in Vectamount mounting medium (Vector Laboratories, Burlingame, CA). Samples of human placenta or tonsils were used as positive immunohistochemical controls. For negative controls, the primary antibodies were either omitted or replaced by normal rabbit or mouse serum (ab7487, ab7486; Abcam).

Staining of PD-L1, SSTs, CXCR4, and CgA in the tumour sections was scored using the semiquantitative immunoreactivity score (IRS) according to Remmele and Stegner (33), taking into account both membranous and cytoplasmic staining of tumour cells, tumour-infiltrating immune cells, and stromal cells. The IRS was chosen because it has proven itself in the evaluation of membrane receptors and has also been shown to have the best correlation with qRT-PCR data compared to other scores (34). To determine the IRS, the percentage of positive cells quantified in five gradations (no positive cells (0), <10% positive cells (1), 10–50% positive cells (2), 51–80% positive cells (3), and >80% positive cells (4)) was multiplied by the staining intensity quantified in four gradations (no staining (0), mild staining (1), moderate staining (2), and strong staining (3)), resulting in IRS ranging from 0 to 12. All immunohistochemical stainings were evaluated by two independent blinded investigators (ER, AL). For discrepant scores, final decisions were achieved by consensus. Tumour samples with an average IRS $\geq$ 3 for a given receptor or marker were considered positive for that receptor or marker. IRS was classified as follows: 0–2, negative/no expression; 3–5, low expression;

Table 2 Primary antibodies used for immunohistochemical staining.

Antibody	Clone	Type	Epitope (amino acids)	Supplier	Dilution or concentration
PD-L1	73-10	Rabbit monoclonal	250–290	Abcam	1:1000
PD-L1	SP142	Rabbit monoclonal	250–290	Abcam	1:100
PD-L1	28-8	Rabbit monoclonal	19–239	Abcam	2 µg/mL
SST1	UMB-7	Rabbit monoclonal	377–391	Abcam	1:25
SST2	UMB-1	Rabbit monoclonal	335–369	Abcam	1:10
SST3	UMB-5	Rabbit monoclonal	398–418	Abcam	1:20
SST4	N/A	Rabbit polyclonal (4802)	366–388	Gramsch, Schwabhausen, Germany	0.1 µg/mL
SST5	UMB-4	Rabbit monoclonal	344–364	Abcam	1:10
CXCR4	UMB-2	Rabbit monoclonal	338–359	Abcam	1:2
Ki-67	MIB-1	Mouse monoclonal	N/A	Dako	1:75
CgA	LK2H10	Mouse monoclonal	N/A	BioLogo, Kronshagen, Germany	1:50

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6–8, moderate expression; 9–12, strong expression. Ki-67 staining was quantified as the percentage of positively staining nuclei in a sample (Ki-67 index). For patients from whom there were multiple samples, arithmetic means were calculated from the IRS or Ki-67 indices of all slides for that patient, regardless of whether the samples were from primary tumours or metastases (per patient analysis).

Statistical analyses

IBM SPSS statistics software, version 25.0 (Armonk, NY, USA), was used for statistical analyses. Because the data were not normally distributed, as determined by a Kolmogorov–Smirnov test, the Mann–Whitney test, Kruskal–Wallis test, chi-square test, Kendall's τ-b test, and Spearman's rank correlation test were used. For survival analyses, the Kaplan–Meier method with log-rank or Breslow test was used. A P value ≤ 0.05 was considered indicative of statistical significance.

Results

Pilot study to evaluate anti-PD-L1 antibody sensitivity and specificity

To determine the best antibody for evaluating PD-L1 expression levels, a pilot study was conducted to compare the sensitivity and specificity of three of the most commonly used anti-PD-L1 antibodies, namely 73-10 (ab228415), SP142 (ab228462), and 28-8 (ab205921). Antibodies 73-10 and SP142 both recognize amino acids 250 to 290 (C-terminus) of PD-L1, whereas 28-8 recognizes amino acids 19 to 239 (extracellular domain) of PD-L1 (Table 2). Two other commonly used antibody clones, 22C3 and SP263, were not tested because previous studies demonstrated these to produce staining comparable to that with 28-8 (31, 35, 36, 37, 38, 39). The pilot study was conducted on a panel of formalin-fixed, paraffin-embedded NSCLC (ADC and SQC) samples, because several studies comparing different diagnostic anti-PD-L1 antibodies already exist in NSCLC, thus providing a basis for comparison (31, 35, 36, 37, 38, 39, 40, 41). Representative examples of the staining patterns obtained with the antibodies 73-10, SP142, and 28-8 in serial sections of ACD and SQC are shown in Fig. 1. The immunostainings clearly revealed that, although all three clones yielded similar staining patterns, 73-10 exhibited the best sensitivity, followed by SP142; by far the worst staining was achieved with 28-8. Furthermore, whereas 73-10 and SP142 predominantly stained the tumour cell membrane, 28-8 staining was mainly cytoplasmic. Based on these results, in combination with the results of the Blueprint phase 2 project, which also demonstrated better sensitivity of 73-10 compared to SP142 or 28-8 (31), antibody 73-10 was selected for the subsequent immunohistochemical studies of BP-NEN.

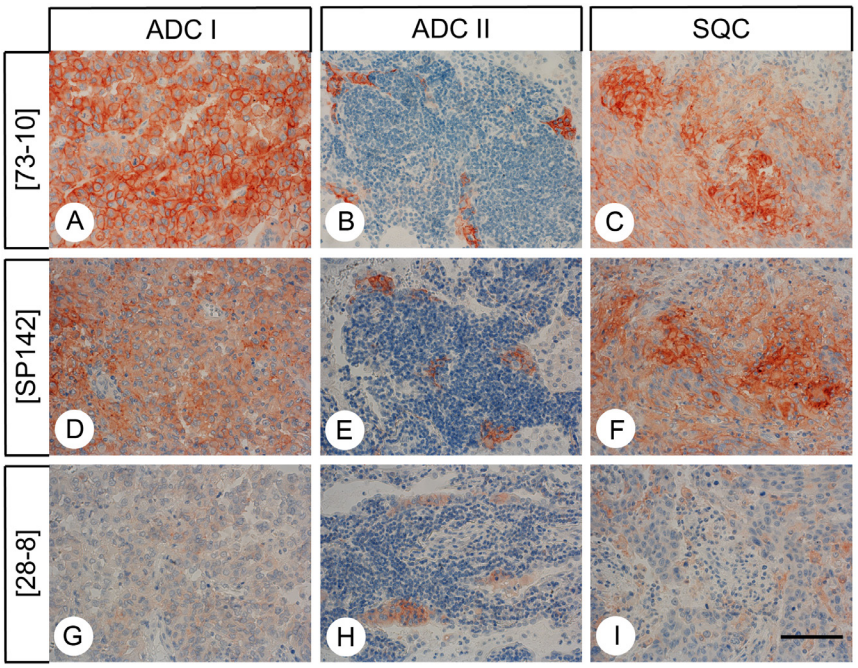

Figure 1
Representative examples of staining patterns obtained using the anti-PD-L1 antibodies 73-10, SP142, and 28-8 in serial sections of ADC and SQC tumours. Immunohistochemistry (red-brown colour), counterstaining with haematoxylin. Scale bar: 100 µm.

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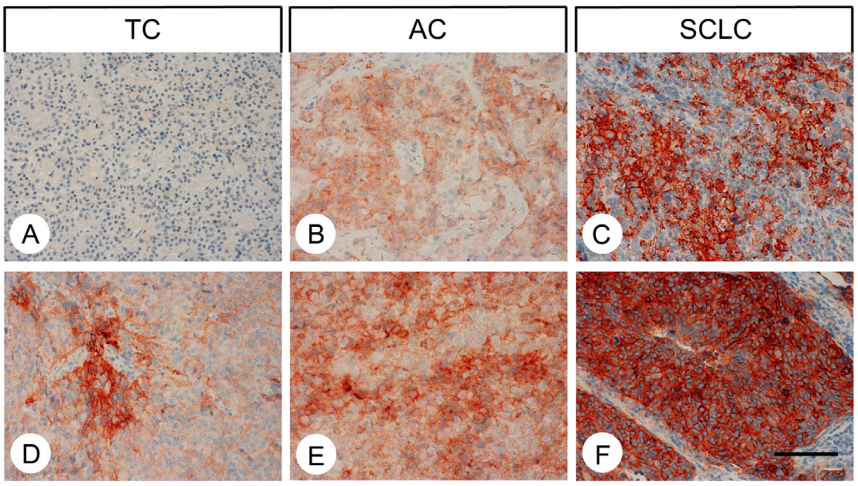

Figure 2
Representative examples of 73-10 staining patterns in TC, AC, and SCLC tumours. Immunohistochemistry (red-brown colour), counterstaining with haematoxylin. Scale bar: 100 µm.

PD-L1 expression in BP-NEN

Patient characteristics

The clinicopathological characteristics of the 97 BP-NEN patients included in this study are depicted in Table 1. In addition to the clinicopathological data, smoking status was reported for 41 of the 97 patients. Of these, 25 were smokers and 16 were non-smokers.

PD-L1 expression patterns

Figure 2 shows representative images of TC, AC, and SCLC samples stained with the anti-PD-L1 antibody 73-10. Figure 3A and B depict the expression intensity as well as the number of samples showing no, low, moderate or strong PD-L1 expression for all four BP-NEN entities. The distribution of the IRS values across all tumours is visualized in Fig. 3C. In all cases, PD-L1-immunostaining was predominantly localized at the plasma membrane of the tumour cells. Infiltrating immune cells and, if present, alveolar macrophages in the surrounding normal lung tissue also exhibited strong PD-L1 positivity. There was marked variation in PD-L1 expression levels between individual patients with the same tumour entity, as indicated by the length of the respective boxes and whiskers in Fig. 3A, and also between different samples from the same tumour and within one tumour slide, as can be seen in Fig. 2D.

Across all four tumour entities, 82 of the 97 tumours were PD-L1 positive (IRS ≥ 3). The median IRS was 6.0 (mean ± S.E.M.: 5.67 ± 0.28), corresponding to moderate expression, which matches the most frequent IRS (Fig. 3C). However, PD-L1 expression was significantly higher in SCLC and LCNEC than in TC and AC (Kruskal–Wallis test: $P=0.001$; Mann–Whitney test: TC vs SCLC, $P=0.001$; TC vs LCNEC, $P=0.001$; AC vs SCLC, $P=0.045$; AC vs LCNEC, $P=0.056$) (Fig. 3A). Additionally, the percentage of PD-L1-positive cases (IRS ≥ 3) was significantly higher in AC than in TC (χ^2 test: TC vs AC, $P=0.005$) and significantly higher in SCLC than in TC or AC (χ^2 test: TC vs SCLC, $P<0.001$; AC vs SCLC, $P=0.003$) (Fig. 3B).

Correlations with clinical data

There was no correlation between PD-L1 expression and patient age, but expression levels were generally higher in males than in females (mean IRS ± S.E.M.: male patients, 6.05 ± 0.37; female patients, 5.27 ± 0.43; Mann–Whitney test: $P=0.099$). Male patients also had worse outcomes than female patients (mean overall survival (months) ± S.E.M.: male patients, 45.81 ± 6.36; female patients, 68.83 ± 6.33; Mann–Whitney test: $P=0.028$; Kaplan–Meier survival analysis: log-rank test: $P=0.079$). PD-L1 expression did not vary with tumour size. However, there was a significant difference in tumour PD-L1 expression between patients with or without lymph node metastases at diagnosis, with higher IRS in tumours that had already metastasized regionally (mean IRS ± S.E.M.: no lymph node metastases, 4.52 ± 0.38; with lymph node metastases, 6.69 ± 0.52; Mann–Whitney test: $P=0.003$; Fig. 4A). PD-L1 levels were also higher in tumours from patients who presented with distant metastases at diagnosis, although this difference was not statistically significant (mean IRS ± S.E.M.: no distant metastases, 4.80 ± 0.48; with distant metastases, 5.80 ± 0.74; Mann–Whitney test: $P=0.196$; Fig. 4B). Smokers had higher tumour PD-L1 levels compared to non-smokers (mean IRS ± S.E.M.: non-smokers, 3.82 ± 0.64; smokers, 5.22 ± 0.55; Mann–Whitney test: $P=0.051$; Fig. 4C), and PD-L1 expression levels were significantly higher in patients who died than in patients who were still alive

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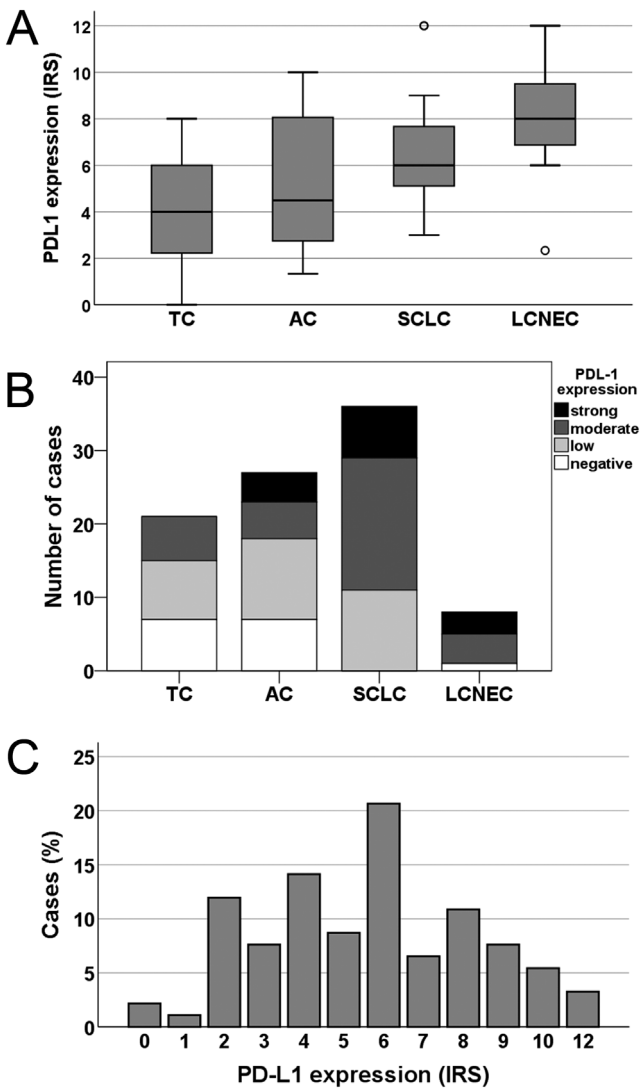

Figure 3
PD-L1 expression in TC, AC, SCLC, and LCNEC tumours. (A) Box plots of PD-L1 expression levels (IRS) of BP-NEN tumours of each type, including those that were PD-L1-negative. Plots depict median values, upper and lower quartiles, minimum and maximum values, and outliers. For outliers, circles indicate mild outliers (1.5–3 IQR [interquartile range] away from the nearest quartile), and asterisks indicate extreme outliers (>3 IQR away from the nearest quartile). (B) Number of tumours showing no (negative; IRS = 0–2), low (IRS = 3–5), moderate (IRS = 6–8) or strong (IRS = 9–12) PD-L1 expression. (C) Distribution of PD-L1 expression levels (IRS) across all BP-NEN tumours.

at the end of the follow-up period (mean IRS ± S.E.M.: alive, 5.16 ± 0.34; deceased, 6.43 ± 0.49; Mann–Whitney test: $P=0.042$; Fig. 4D). Furthermore, a significant positive correlation was observed between PD-L1 expression and tumour grade (Kendall's τ-b=0.358; $P < 0.001$; Kruskal–Wallis test: $P < 0.001$), with significantly higher IRS in G3-tumours compared to G1- or G2-tumours (mean IRS ± S.E.M.; G1-tumour, 4.08 ± 0.51; G2-tumour, 5.08 ± 0.69; G3-tumour, 6.75 ± 0.33); Mann–Whitney test: G1- vs G3-tumours, $P < 0.001$; G2- vs G3-tumours, $P=0.014$).

Correlations with other tumour markers

In addition to staining for PD-L1, all BP-NEN samples were stained for SST 1–5, CXCR4, CgA, and the proliferation marker Ki-67, and expression levels were assessed for correlations with PD-L1 levels. There was a highly significant negative association between PD-L1 levels and both SST1 and CgA expression; whereas a strong positive correlation between PD-L1 levels and both CXCR4 expression and Ki-67 index was noted (Table 3).

Separately, SST1 expression positively correlated with SST5 and CgA expression but showed a negative interrelationship with CXCR4 expression and Ki-67 index. SST3 expression positively correlated with SST5 expression, whereas SST5 expression negatively correlated with Ki-67 index. There was also a negative interrelationship between CXCR4 and CgA expression, whereas a strong positive correlation between CXCR4 expression and Ki-67 index was observed. Accordingly, CgA expression showed a negative association with Ki-67 index (Table 3).

Kaplan–Meier survival analysis was performed, using the overall median IRS of 6.0 as the cut-off between groups. This analysis revealed that patients with PD-L1 IRS ≥ 6.0 tended to have a worse prognosis than patients with IRS < 6.0 (Breslow test: $P=0.051$; Fig. 5). Multivariate analysis, including IRS for PD-L1, SST 1–5, CXCR4, and CgA, Ki-67 index, and patient age, revealed that SST1, CXCR4, Ki-67, and PD-L1 were independent prognostic factors.

PD-L1 expression in the NSCLC samples from the pilot study

The IRS of the 43 NSCLC samples from the pilot study stained with 73-10 (median: 2.50; mean ± S.E.M.: 3.53 ± 0.37) were slightly lower than those of the BP-NEN samples. Additionally, only 21 of the 43 NSCLC tumours were PD-L1 positive (IRS ≥ 3). PD-L1 expression was significantly higher in SQC than in ADC (median IRS: 4.00 vs 2.13; mean IRS ± S.E.M.: 4.46 ± 0.56 vs 2.63 ± 0.41; Mann–Whitney test, $P=0.009$), and the percentage of PD-L1-positive cases of SQC also significantly exceeded that of ADC (14/21 cases vs 7/22 cases; χ^2 test, $P=0.034$).

For all NSCLC samples taken together, PD-L1 levels were significantly correlated with both CXCR4 expression ($r_{sp}=0.426$; $P=0.004$) and Ki-67 index ($r_{sp}=0.520$;

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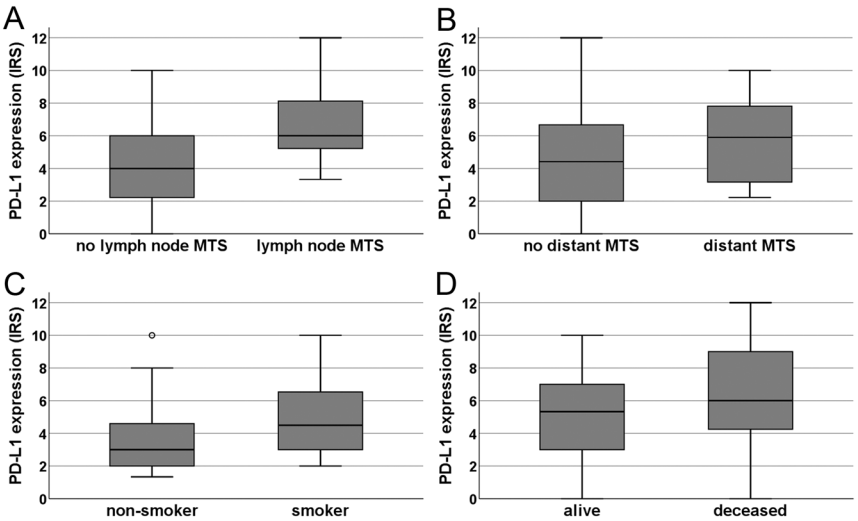

Figure 4
PD-L1 expression in BP-NEN tumours in dependence on (A) the presence of lymph node metastases (Mann–Whitney test: $P = 0.003$), (B) the presence of distant metastases ($P = 0.196$), (C) smoking status ($P = 0.051$), and (D) survival status ($P = 0.042$). Plots depict median values, upper and lower quartiles, minimum and maximum values, and outliers. For outliers, circles indicate mild outliers (1.5–3 IQR away from the nearest quartile) and asterisks indicate extreme outliers (>3 IQR away from the nearest quartile).

$P < 0.001$). Similar results were obtained for SQC samples alone (PD-L1 vs CXCR4: $r_{sp} = 0.634$; $P = 0.002$; PD-L1 vs Ki-67: $r_{sp} = 0.497$; $P = 0.022$), whereas no correlations were identified for ADC samples alone. However, regardless if the SQC and ADC samples were analysed collectively or separately, no correlation was found between PD-L1 expression and any of the following: patient age, gender, smoking status, survival time, tumour size, lymph node or distant metastases, and SST expression. Accordingly, the PD-L1 status of the tumour had no significant impact on overall patient outcome.

As described above, antibody 28-8 results in much weaker immunosignals than 73-10. To evaluate if similar correlations with clinicopathological data can be obtained also with 28-8 or if important information will be overlooked, 28-8-staining was also graded and respective statistics were calculated. Using 28-8, the median PD-L1 IRS of the 43 NSCLC samples amounted only to 0.00 (mean ± S.E.M.:

Table 3 Correlation between expression levels of PD-L1, SST 1–5, CXCR4, Ki-67, and CgA in BP-NEN tumours.

	SST1	SST2	SST3	SST4	SST5	CXCR4	CgA	Ki-67
PD-L1								
r	**−0.355**	−0.043	0.079	−0.005	−0.201	**0.357**	**−0.449**	**0.432**
P	**0.001**	0.693	0.465	0.965	0.062	**0.001**	**<0.001**	**<0.001**
SST1								
r	—	0.026	0.053	0.161	**0.525**	**−0.258**	**0.303**	**−0.540**
P	—	0.809	0.616	0.125	**<0.001**	**0.008**	**0.004**	**<0.001**
SST2								
r	—	—	−0.015	−0.073	−0.028	−0.069	0.043	0.021
P	—	—	0.886	0.490	0.788	0.529	0.687	0.845
SST3								
r	—	—	—	0.120	**0.270**	0.087	0.001	0.014
P	—	—	—	0.254	**0.009**	0.426	0.992	0.892
SST4								
r	—	—	—	—	0.110	0.084	−0.097	0.065
P	—	—	—	—	0.295	0.440	0.368	0.538
SST5								
r	—	—	—	—	—	−0.107	0.154	**−0.303**
P	—	—	—	—	—	0.325	0.152	**0.003**
CXCR4								
r	—	—	—	—	—	—	**−0.537**	**0.628**
P	—	—	—	—	—	—	**<0.001**	**<0.001**
CgA								
r	—	—	—	—	—	—	—	**−0.659**
P	—	—	—	—	—	—	—	**<0.001**

Significant correlations ($P < 0.05$) are indicated in bold.
r, Spearman's correlation coefficient; *P*: *P* value.

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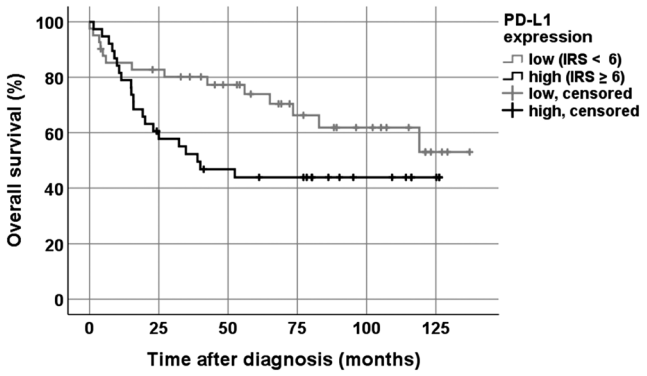

Figure 5
Overall survival of BP-NEN patients in dependence on the level of PD-L1 expression. The median IRS of 6.0 was set as the cut-off. Breslow test: $P = 0.051$.

1.09 ± 0.24). Consequently, only 8 of the 43 samples were PD-L1 positive (IRS ≥ 3). Nevertheless, a significant correlation was observed between IRS determined with 28-8 and 73-10 (r_{sp}=0.452; P=0.002). As with 73-10, also with 28-8 PD-L1 expression was slightly higher in SQC than in ADC (median IRS: 1.00 vs 0.00; mean IRS ± S.E.M.: 1.56 ± 0.37 vs 0.66 ± 0.29; Mann–Whitney test: P=0.015), but there was no difference in the percentage of positive cases (4/21 samples (SQC) vs 4/22 samples (ADC); χ^2 test: P=0.942). For all NSCLC samples taken together, a significant correlation between PD-L1 expression and Ki-67 index was observed (r_{sp}=0.413; P=0.006). However, no such correlation could be noted if SQC samples were analysed alone. There was also no correlation between PD-L1 and CXCR4 expression.

Discussion

The pilot study comparing staining of NSCLC samples with different commercially available diagnostic anti-PD-L1 antibodies clearly revealed that antibody 73-10 was more sensitive than SP142 or 28-8. These results agree with those of the Blueprint phase 2 project that compared different commercially available anti-PD-L1 antibodies for staining efficiency in NSCLC (31). However, in contrast to the Blueprint phase 2 project, and several other published studies (31, 35, 36, 38, 39, 40, 41), our pilot study indicated that SP142 yielded distinctly better staining than 28-8. The reason for this discrepancy remains unclear, but the poor 28-8 staining observed in our pilot study may be because, unlike 73-10 and SP142, which are directed against the C-terminal region of PD-L1, 28-8 was generated against the N-terminus of this protein. The N-terminus of PD-L1 is located extracellularly and, consequently, is subject to glycosylation, especially in cancer cells (42, 43). There is some evidence that this N-linked glycosylation impedes anti-PD-L1 antibody recognition of the PD-L1 N-terminus in fixed samples, thus leading to an underestimation of PD-L1 expression. Furthermore, it has been shown that enzymatic deglycosylation of PD-L1 can enhance the sensitivity and, accordingly, the predictive value of antibodies directed against the N-terminus of PD-L1 (42, 43). It has also been noted that antibodies directed against the N-terminus of PD-L1 exhibit predominantly cytoplasmic staining whereas with those binding to the intracellular C-terminal part of this protein mainly a membranous positivity is obtained (44). This may be because intracellular PD-L1 molecules are not yet glycosylated and thus, in contrast to membranous ones, binding of antibodies directed against the N-terminal region of PD-L1 is not impeded.

In our pilot study, 73-10 staining indicated that 21 of the 43 NSCLC tumours were PD-L1 positive (IRS ≥ 3), whereas only 8 positive cases were found with 28-8. According to the literature, approximately 20–40% of unselected NSCLC patients benefit from anti-PD-1 or anti-PD-L1 therapy, although only some of these patients had PD-L1-positive tumours according to diagnostic assays. This clearly points to an underestimation of PD-L1-positive cases using current diagnostic assays, which in most cases include antibodies 28-8, 22C3 (which is also directed against the N-terminal region of PD-L1), and SP142 (8, 45). Furthermore, our data indicate that with the less sensitive antibody 28-8 some important relationships with clinical data may be overlooked.

Based on the results of our pilot study and the Blueprint phase 2 project, we selected antibody 73-10 for immunohistochemical analysis of BP-NEN samples. To the best of our knowledge, the present study is the first to use 73-10 in this tumour entity. Across all four BP-NEN entities, 85% of the tumours were PD-L1 positive. Prior investigations on PD-L1 expression in BP-NEN have been mostly limited to SCLC and have reported highly divergent results, ranging from 0% to 82.8% positivity for PD-L1 (11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30). Possible explanations for these discrepancies include the following: use of different antibodies with different sensitivities; use of different detection systems; and use of diverse rating methods and dissimilar cut-off values for assigning tumour positivity. Furthermore, many of these studies were conducted on tissue microarrays or (predominantly) on biopsies (11, 12, 13, 14, 16, 18, 20, 22, 23, 24, 26, 27, 30), which may not be representative of the entire tumour and, therefore, may

result in an incorrect rating. In the present investigation, a distinct heterogeneity in PD-L1 expression within the BP-NEN samples was observed. Heterogeneity of PD-L1 expression has been described previously, both for BP-NEN (17) and for other tumour entities such as NSCLC and melanoma (46, 47, 48, 49, 50, 51). Notably, this heterogeneity has been demonstrated to result in an underestimation of PD-L1 expression in biopsies compared to resection specimens (48).

The large variability in PD-L1 positivity rates reported for BP-NEN could explain why the few available data on the impact of PD-L1 expression on BP-NEN prognosis are also highly contradictory. While some authors report better survival of SCLC patients with higher PD-L1 expression levels (11, 17, 18, 20, 23, 25, 26), in other studies the opposite was shown (15, 19, 28). In the present investigation, high PD-L1 expression was associated with poor patient outcomes. Additionally, PD-L1 expression was higher in male patients (who had worse outcomes than females), in smokers, in patients with metastatic disease, and in patients who had died during the follow-up period. These results were further corroborated by the fact that PD-L1 expression significantly correlated with tumour grade and with the expression of the proliferation marker Ki-67. A positive interrelationship was also found between PD-L1 expression levels and those of the chemokine receptor CXCR4. In BP-NEN, as well as other tumour entities, CXCR4 expression has been shown to increase with tumour malignancy and to be associated with poor prognosis (32). Meanwhile, a significant negative correlation was found between PD-L1 expression and that of both the neuroendocrine tumour marker CgA and SST1, the latter of which was recently shown to be associated with lower BP-NEN malignancy and with favourable patient outcomes (52).

Conclusions

Using the highly sensitive anti-PD-L1 antibody 73-10, the present study indicates that PD-L1 expression is common in BP-NEN, increases with malignancy, and is associated with poor patient outcomes. Therefore, PD-L1 expression levels may be of prognostic relevance in BP-NEN patients, and targeting the PD-1/PD-L1 axis may represent a promising therapeutic strategy. Given the highly discordant data in the literature, further studies on PD-L1 expression in BP-NEN using highly sensitive antibodies (e.g. 73-10), strict standardization of the staining protocol, a uniform scoring system, and identical staining thresholds are warranted.

Declaration of interest

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Author contribution statement

Amelie Lupp and Daniel Kaemmerer conceived and designed the experiments. Daniel Kaemmerer and Jörg Sänger provided the tumour samples. Daniel Kaemmerer provided the clinical data. Erik Rösner, Elisa Neubauer and Amelie Lupp performed the experiments. Erik Rösner and Amelie Lupp analysed the data. Amelie Lupp interpreted the data. Amelie Lupp wrote the paper. Erik Rösner, Daniel Kaemmerer, Elisa Neubauer, Jörg Sänger and Amelie Lupp critically revised the manuscript.

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