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Original Research

Evaluation of PD-L1 expression in a large set of gastroenteropancreatic neuroendocrine tumours and correlation with clinicopathological data

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ABSTRACT

Background: Targeting programmed death protein 1 (PD-1) or its ligand PD-L1 is a promising therapeutic approach for many types of cancer in which PD-L1 is overexpressed. However, data on PD-L1 expression levels in gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) are limited and contradictory.
Methods: We evaluated PD-L1 expression in 457 archived, formalin-fixed, paraffin-embedded GEP-NEN samples from 175 patients by immunohistochemistry using the highly sensitive monoclonal anti-PD-L1 antibody 73-10. The immunostaining was semiquantitatively evaluated using a 12-point immunoreactivity score (IRS) taking both PD-L1-positive tumour cells and immune cells into account. Tumour samples with an IRS $\geq$ 3 were considered PD-L1-positive. Results were correlated with clinicopathological data and with the expression of several typical markers and receptors for neuroendocrine tumours.
Results: Of the GEP-NEN samples, 73% were PD-L1-positive. The median IRS value across all samples was 4.0, corresponding to low expression. PD-L1 immunostaining was predominantly localised at the plasma membrane of the tumour cells. Positive correlations were observed between PD-L1 expression and tumour grading or Ki-67 index, between PD-L1 expression and the expression of chromogranin A, and between PD-L1 expression and the expression of each of the five somatostatin receptors. PD-L1 expression was lower in tumours with lymph node metastases at diagnosis than in those without regional metastasis and lower in high-stage than in earlier-stage tumours. No association was noted between PD-L1 expression and patient survival.
Conclusions: PD-L1 expression is common in GEP-NENs and increases with malignancy. Therefore, especially in high-grade GEP-NENs, targeting the PD-1/PD-L1 axis could be a promising additional therapeutic strategy.

Introduction

Neuroendocrine neoplasms (NENs) are a heterogeneous group of tumours arising from neuroendocrine cells. Because these cells are widely distributed throughout the body, NENs can develop in almost every organ, but they are most frequently found in the gastrointestinal tract, pancreas, or lung. With an incidence of 2.5–7 per 100,000 people per year, NENs are rare malignancies; however, the incidence of all NENs and, specifically, of gastroenteropancreatic NENs (GEP-NENs) has increased sharply in recent decades [1–5]. Ranging from indolent tumours to aggressive carcinomas, GEP-NENs have a variable clinical course. Based on histopathological criteria, mitotic rate, and Ki-67 index, GEP-NENs are graded as well-differentiated G1 and G2 tumours (NETs) or poorly differentiated G3 carcinomas (G3-GEP-NECs) [4,6]. Although most GEP-NENs are low-grade tumours, diagnosis is often delayed because the symptoms are unspecific. Hence, GEP-NENs are mostly diagnosed at an advanced stage by which point 40–95% of tumours have metastasised [7]. Despite recent advances in diagnostics and therapy, treatment options for nonresectable, metastatic, or aggressive G3-GEP-NECs are still limited. G3-GEP-NEC patients are usually treated with platinum-based chemotherapies. Initial response to these therapies is good but short-lived and the disease often recurs after a few months.

Abbreviations: BP-NEN, bronchopulmonary neuroendocrine neoplasms; CgA, chromogranin A; CXCR4, CXC motif chemokine receptor 4; GEP-NEN, gastroenteropancreatic neuroendocrine neoplasms; IRS, immunoreactivity score; NEC, neuroendocrine carcinoma; NEN, neuroendocrine neoplasm; NSCLC, non-small cell lung cancer; PD-1, programmed death protein 1; PD-L1, programmed death ligand 1; rsp, correlation coefficient (Spearman); SST, somatostatin receptor.

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Ultimately, the rates of grade 3 and grade 4 toxicity of these chemotherapies are high, the median overall survival of these patients is only 10–19 months, and there is no standard second-line therapy [8]. Therefore, novel treatment strategies for these tumours are urgently needed.

Recently, cancer-directed immunotherapy, specifically involving drugs targeting the programmed death protein 1 (PD-1) and its ligand programmed death ligand 1 (PD-L1), has emerged as a promising treatment approach in several tumour entities. PD-1 is expressed on B and T lymphocytes, as well as on myeloid cells, and binding of PD-L1 to PD-1 inactivates and downregulates T-cells. PD-L1 is often overexpressed in neoplastic tissues as a mechanism to evade the immune response [9–11]. Several anti-PD-1 antibodies such as cemiplimab, dostarlimab, nivolumab, and pembrolizumab and anti-PD-L1 antibodies such as atezolizumab, avelumab, and durvalumab have been approved, and many other inhibitors are under development, for the treatment of cancers including non-small cell lung cancer (NSCLC); head and neck squamous cell carcinoma; oesophagus, gut, colorectal, and renal clear cell carcinoma; bladder cancer; melanoma; Merkel cell carcinoma; and Hodgkin's lymphoma [9–12]. Trials examining the efficacy of these antibodies for GEP-NENs either alone or in combination with other therapies, however, have produced mixed results [13–23]. The inconsistencies may be due to the small numbers of patients included in the trials or to the substantial differences between studies in grading, localisation, and other clinicopathological characteristics of the tumours. Determination of the PD-L1 status of a tumour by immunohistochemistry is currently considered the best approach to predict its response to an anti-PD-1 or anti-PD-L1 therapy [24–26]. However, data on PD-L1 expression in GEP-NENs are limited, and reported PD-L1 positivity rates vary between 6.1% and 75% among studies [27–43]. Moreover, results regarding the impact of PD-L1 expression on patient prognosis are contradictory [27,28,31,33–35,39–42], and often no correlation is observed between response to anti-PD-1/PD-L1 treatment and PD-L1 expression of the GEP-NENs [16,18,19], suggesting that PD-L1 expression in the tumours might be underestimated by the diagnostic antibodies used.

In light of these contradictory data, the present investigation aimed to re-evaluate PD-L1 expression in a large panel of GEP-NENs comprising 457 whole-block samples from 175 patients by immunohistochemistry using the highly sensitive monoclonal rabbit anti-PD-L1 antibody 73–10 and to correlate the expression data with clinicopathological parameters. The antibody 73–10 was selected based on data from the "Blueprint phase 2 project" in NSCLC [44] and on our own findings that 73–10 provides distinctly better sensitivity than other commonly used anti-PD-L1 antibodies in NSCLC and bronchopulmonary NENs (BP-NENs) [45]. To the best of our knowledge, this is the first study to use 73–10 to evaluate GEP-NENs and the largest study of PD-L1 expression in GEP-NENs using whole-block samples.

Materials and methods

Tumour specimens

This investigation included 457 archived formalin-fixed, paraffin-embedded tumour samples from 175 patients (71 × 1, 35 × 2, 27 × 3, 24 × 4, 8 × 5, 4 × 6, 1 × 7, 1 × 8, 1 × 9, 1 × 11, 1 × 12, and 2 × 14 samples per patient) with histologically verified GEP-NENs (209 samples from primary tumours, 240 samples from metastases; for 8 samples, no respective information was available). Of the tumours, 18 (10.3%) originated from the stomach, 16 (9.1%) from the duodenum/jejunum, 59 (33.7%) from the ileum, 5 (2.9%) from the appendix, 12 (6.9%) from the colon, 16 (9.1%) from the rectum, and 49 (28.0%) from the pancreas. The clinicopathological characteristics of the patients and tumours are described in Supplemental Tables 1 and 2. All data were recorded and analysed anonymously. The samples mainly comprised resected specimens but also included several biopsies. All samples were provided by the Laboratory of Pathology and Cytology Bad Berka, Bad Berka, Germany, and had been resected between 2004 and 2015 at the Department of General and Visceral Surgery, Zentralklinik Bad Berka, Bad Berka, Germany. All procedures in this study involving human participants were performed in accordance with both the ethical standards of the institutional or national research committee and the 1964 Helsinki declaration and its later amendments. Permission was gained from the local ethics committee (Ethikkommission der Landesärztekammer Thüringen) for this retrospective analysis. Informed consent for the use of tissue samples for scientific purposes was obtained from all individual participants included in the study when entering the Theranostic Research Center, Zentralklinik Bad Berka, Bad Berka, Germany, and the Department of General and Visceral Surgery, Zentralklinik Bad Berka, Bad Berka, Germany. All data were analysed anonymously.

Immunohistochemistry

From the paraffin blocks, 4-µm sections were prepared and floated onto positively charged slides. Immunostaining was performed by an indirect peroxidase labelling method as described previously [46]. Briefly, sections were dewaxed, rehydrated in a descending ethanol series during which endogenous peroxidase was blocked by incubation in 0.3% H_2O_2 in methanol for 45 min, microwaved in Tris-EDTA buffer, pH 9.0, for 16 min at 600 W, and incubated with the primary anti-PD-L1 antibody (rabbit monoclonal, clone 73–10, dilution: 1:1000; Abcam, Cambridge, MA, USA) overnight at 4 °C. Samples were then incubated with biotinylated anti-rabbit IgG secondary antibody followed by peroxidase-conjugated avidin (Vector ABC "Elite" kit; Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Binding of the primary antibody was visualised 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 serum (ab7487; Abcam, Cambridge, MA, USA). At least five positive and five negative controls were included in each staining run (comprising approximately 100 samples).

Staining of PD-L1 in the tumour sections was scored using the semiquantitative immunoreactivity score (IRS) according to Remmele and Stegner [47], taking into account both membranous and cytoplasmic staining of tumour cells, tumour-infiltrating immune cells, and stromal cells. The IRS was chosen because its use has proven itself in the evaluation of membrane proteins and has also been shown to have the best correlation with reverse transcription–quantitative PCR (qRT-PCR) data compared to other scores [48]. To determine the IRS, each sample was assigned a value from 0 to 4 representing the percentage of positive cells (0, no positive cells; 1, <10% positive cells; 2, 10–50% positive cells; 3, 51–80% positive cells; or 4, >80% positive cells) and a value from 0 to 3 representing the staining intensity (0, no staining; 1, mild staining; 2, moderate staining; or 3, strong staining), which were multiplied to produce an IRS 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 were considered PD-L1-positive. IRS values were classified as follows: 0–2, negative/no expression; 3–5, low expression; 6–8, moderate expression; 9–12, strong expression. For patients with multiple samples, arithmetic means were calculated from the IRS values 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, Mann–Whitney test, Kruskal–Wallis test, χ^2 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 applied. A p value ≤ 0.05 was considered indicative of statistical significance.

Results

PD-L1 expression patterns

Fig. 1 shows representative images of GEP-NEN samples stained with the anti-PD-L1 antibody 73–10. Fig. 2A and B depict the IRS values and the number of samples showing no, low, moderate, or strong PD-L1 expression for GEP-NENs based on the origin of the primary tumour. The distribution of the IRS values across all tumours is visualised in Fig. 2C. PD-L1 immunostaining was predominantly localised at the plasma membrane of the tumour cells. Infiltrating immune cells also exhibited strong PD-L1 positivity. There was a 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. 2A, but also between different samples from the same tumour and within one tumour slide.

Across all GEP-NENs, 73% were PD-L1-positive (IRS $\geq$ 3). The median IRS, however, was only 4.0 (mean ± S.E.M.: 4.02 ± 0.17), reflecting low expression overall, and matched the most frequently observed IRS (Fig. 2C). PD-L1 expression differed significantly among tumours of different origin (Kruskal–Wallis test: $p < 0.001$; Fig. 2A). Tumours derived from the stomach (median IRS value: 6.0; mean IRS value: 5.5) showed significantly higher PD-L1 expression than did those originating from the duodenum/jejunum (median IRS value: 4.0; mean IRS value: 3.6; Mann–Whitney test: $p = 0.015$), ileum (median IRS value: 2.8; mean IRS value: 2.7; Mann–Whitney test: $p < 0.001$), appendix (median and mean IRS values: 2.0; Mann–Whitney test: $p = 0.009$), or colon (median and mean IRS values: 4.0; Mann–Whitney test: $p = 0.030$). Similarly, tumours originating from the rectum (median IRS value: 6.0, mean IRS value: 5.6) displayed significantly higher IRS values compared with those derived from the duodenum/jejunum (Mann–Whitney test: $p = 0.030$), ileum (Mann–Whitney test: $p < 0.001$), appendix (Mann–Whitney test: $p = 0.011$), or colon (Mann–Whitney test: $p = 0.037$). Pancreatic neoplasms (median IRS value: 4.5; mean IRS value 4.8) had significantly higher IRS values than did tumours originating from the ileum (Mann–Whitney test: $p < 0.001$) or appendix (Mann–Whitney test: $p = 0.012$). Finally, tumours derived from the duodenum/jejunum (Mann–Whitney test: $p = 0.040$) or colon (Mann–Whitney test: $p = 0.023$) showed significantly higher PD-L1 expression than did those derived from the ileum. A similar picture emerged when analysing the percentage of PD-L1-positive cases (IRS $\geq$ 3) (χ^2 test: $p < 0.001$). Tumours derived from the rectum were PD-L1-positive in 100% of cases, followed by those from the stomach (88.2%), pancreas (85.7%), duodenum/jejunum (81.3%), colon (75.0%), ileum (50.0%), and appendix (40.0%) (Fig. 2B).

Correlations with clinical data

There was no correlation between PD-L1 expression and patient age and no difference in PD-L1 expression levels between males and females or between patients who were still alive at the end of the follow-up period and those who had died before. Furthermore, PD-L1 expression did not vary with tumour functionality (i.e, presence of symptoms like rashes, diarrhoea, stomach ulcers, hypo- or hyperglycaemia), tumour size, or the presence of distant metastases at diagnosis. No difference in PD-L1 IRS values was noted between primary tumour samples and metastases. However, there was a significant difference in tumour PD-L1 expression between patients with or without lymph node metastases at diagnosis, with lower IRS values in tumours that had already metastasised regionally (mean IRS values ± S.E.M.: no lymph node

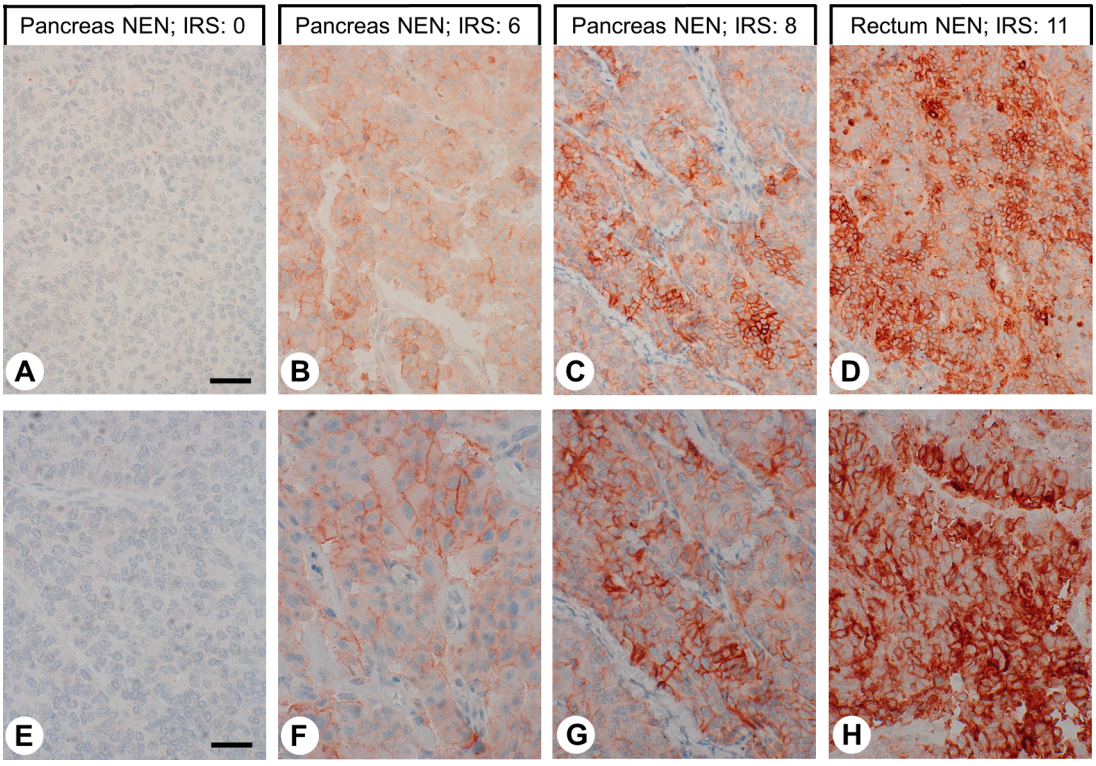

Fig. 1. Representative staining patterns of different intensities obtained in gastroenteropancreatic neuroendocrine neoplasms (NENs) using the anti-PD-L1 antibody 73-10. Immunohistochemistry (red-brown colour), counterstaining with haematoxylin. IRS, immunoreactivity score. Scale bar: (A-D) 100 μm, (E-H) 30 μm.

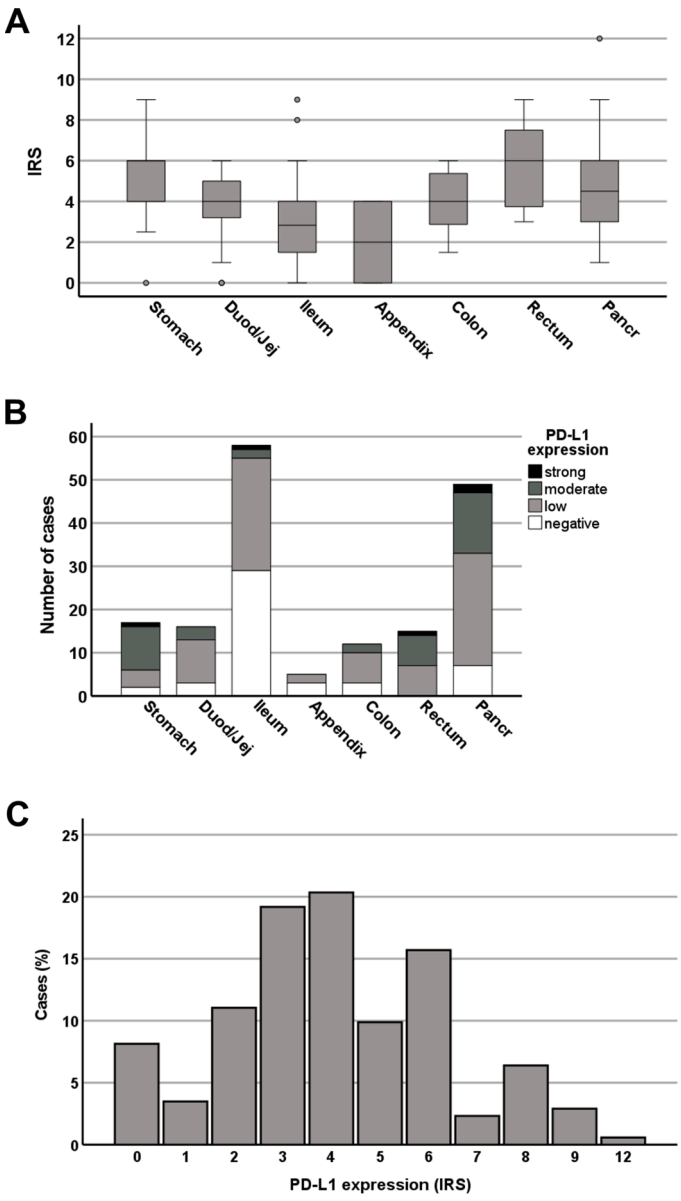

Fig. 2. PD-L1 expression in gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) by site of origin of the primary tumour. (A) Box plots of PD-L1 expression levels of the tumours, including those that were PD-L1-negative. Plots depict median values, upper and lower quartiles, minimum and maximum values, and outliers. For outliers, circles indicate mild outliers (1.5–3 interquartile range [IQR] from the nearest quartile), and asterisks indicate extreme outliers (>3 IQR from the nearest quartile). For significance levels regarding differences in IRS values between different sites of origin see Results. (B) Number of tumours showing no (negative; immunoreactivity score [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 values) across all GEP-NEN tumours. Duod, duodenum; Jej, jejunum; Pancr, pancreas. $n = 175$.

metastases, 4.52 ± 0.31; with lymph node metastases, 3.47 ± 0.21; Mann–Whitney test: $p = 0.009$). Correspondingly, PD-L1 expression levels declined with increasing tumour stage (mean IRS values ± S.E.M.: stage 1, 5.03 ± 0.48 ($n = 22$); stage 2, 4.28 ± 0.60 ($n = 27$); stage 3, 2.50 ± 0.37 ($n = 53$); stage 4, 3.89 ± 0.21 ($n = 21$); Kruskal–Wallis test: $p < 0.001$; pairwise Mann–Whitney tests: stage 1 vs. stage 3, $p < 0.001$; stage 1 vs. stage 4, $p = 0.016$, stage 2 vs. stage 3, $p = 0.023$; Fig. 3A). By contrast, a significant positive correlation was observed between PD-L1 expression and tumour grade (Kendall's τ-b: 0.243; $p < 0.001$; Kruskal–Wallis test: $p < 0.001$), with significantly higher IRS values in G3

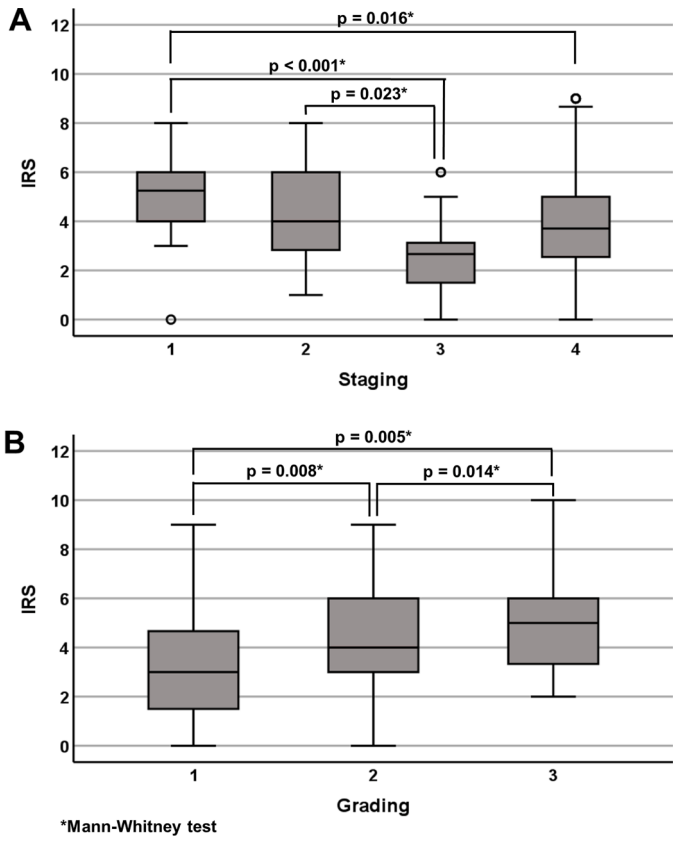

Fig. 3. PD-L1 expression in gastroenteropancreatic neuroendocrine neoplasms by (A) staging of the tumours (Kruskal–Wallis test: $p < 0.001$) and (B) grading of the tumours ($p < 0.001$). Plots depict median values, upper and lower quartiles, minimum and maximum values, and outliers. For outliers, circles indicate mild outliers (1.5–3 interquartile range [IQR] from the nearest quartile) and asterisks indicate extreme outliers (>3 IQR from the nearest quartile). Grading (G): G1: $n = 79$; G2: $n = 67$; G3: $n = 23$. Staging (S): S1: $n = 22$; S2: $n = 27$; S3: $n = 53$; S4: $n = 21$.

tumours compared to G1 or G2 tumours (mean IRS values ± S.E.M.: G1, 3.30 ± 0.25 ($n = 79$); G2, 4.37 ± 0.25 ($n = 67$); G3, 4.81 ± 0.39 ($n = 23$); Mann–Whitney test: G1 vs. G2, $p = 0.008$; G1 vs. G3, $p = 0.005$; G2 vs. G3, $p = 0.014$; Fig. 3B). Possibly because of these divergent relationships, no correlation was found between PD-L1 expression level and overall survival (Spearman correlation coefficient (rsp) = –0.133, $p = 0.364$). No differences in patient outcomes were also found in Kaplan–Meier survival analyses when using either the threshold for PD-L1 positivity (IRS ≥ 3) or the overall median IRS value (IRS ≥ 4) as the cut-off between groups (Fig. 4).

Correlations with other tumour markers

We also analysed interrelationships between PD-L1 expression and the expression of several typical markers of and receptors for neuroendocrine tumours determined using the same detection and evaluation methods in a subset of the present samples as part of a previous study [49]. We observed a positive correlation between the IRS values of PD-L1 and those of the somatostatin receptors SST1, SST2, SST3, SST4, and SST5; the neuroendocrine tumour marker chromogranin A (CgA); and the proliferation marker Ki-67. By contrast, no association was observed between the IRS values of PD-L1 and those of the chemokine receptor CXCR4 (Table 1). Furthermore, in pancreatic NENs, a positive correlation was observed with the expression of insulin (rsp = 0.295, $p = 0.044$) but not with that of glucagon or somatostatin-14/28.

Regarding serum parameters, no correlation was found with serum

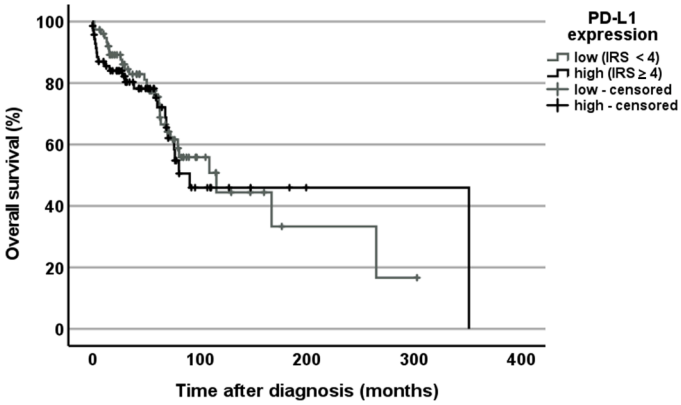

Fig. 4. Overall survival of gastroenteropancreatic neuroendocrine neoplasm patients by PD-L1 expression of their tumours. The median immunoreactivity score (IRS) of 4.0 was set as the cut-off between high and low PD-L1 expression. Log-rank test: $p = 0.799$; Breslow test: $p = 0.455$. $n = 175$.

CgA levels, but we observed a negative correlation with serum serotonin values (rsp = –0.373, $p = 0.008$).

Discussion

In our study, 73% of GEP-NEN tumours were PD-L1-positive, including 58.2% of G1 tumours, 83.1% of G2 tumours, and 86.4% of G3 tumours. However, the overall median IRS value in GEP-NENs was only 4.0, representing low expression. Hence, PD-L1 expression in GEP-NENs is lower than we recently reported with the same antibody in BP-NENs (85% of tumours were PD-L1-positive with a median IRS of 6.0, representing moderate expression; [45]). The percentage of PD-L1-positive GEP-NEN cases varies between 6.1% and 75% in previous reports [27–43]. A possible explanation for the discrepancies might be the use of different antibodies with dissimilar specificity, different sensitivity, and diverse target epitopes at the N or C terminus of the protein between studies [25]. This view is supported by a direct comparison of the staining results of three different PD-L1 antibodies in GEP-NENs [33]. In that study, the antibody SP142 yielded PD-L1 positivity in 6% of the tumour samples evaluated, whereas with the antibodies 28-8 and 22C3 no staining was observed. Another reason for the discrepant results might be the use of different staining methods, detection systems, rating methods, or cut-off values for assessing tumour positivity between investigations. We used the IRS to evaluate the PD-L1 expression levels in the GEP-NEN tissues, taking both the frequency and the intensity of expression into account because in this way the expression level can be determined most precisely. Only samples displaying an IRS ≥3 were considered positive for PD-L1 expression. Some previous studies did not describe whether the staining frequency and intensity were both measured or which rating method was used. In many other studies a tumour was already evaluated as PD-L1-positive if ≥1% of the tumour cells were stained [28,30,34,41–43]. Finally, some studies were conducted on tissue microarrays [34,37], which might not be representative of the entire tumour and, therefore, might result in an incorrect rating [25]. We observed distinct heterogeneity in PD-L1 expression within the GEP-NEN samples, which has been described before for GEP-NENs [36], BP-NENs [45,50], and other tumour entities such as NSCLC and melanoma [51–56]. This heterogeneity can result in underestimation of PD-L1 expression in biopsies compared to resection specimens [53]. For receptors and markers, at least moderate expression (i.e., an IRS value of 6) is generally assumed to be necessary for their clinical utility as target structures. In our study, 25% of GEP-NEN tumours met this criterion for PD-L1, which is consistent with the percentage of responders in most studies of PD-L1 therapy [13–15,17–20,22,23].

The large variability in PD-L1 positivity rates reported for GEP-NENs might explain the contradicting data in the literature regarding the impact of PD-L1 expression on prognosis in GEP-NEN patients. While some studies demonstrated better survival of GEP-NEN patients with higher PD-L1 expression levels [27], others found no correlation with patient survival [31,33,35,40] or even the opposite trend [28,34,39,41,42]. In the present investigation, no correlation between PD-L1 expression and patient survival was noted. We also observed no difference in PD-L1 expression between patients who died and those who were still alive at the end of the observation period, although there was a significant increase in PD-L1 expression with increasing Ki-67 index or tumour grading. A correlation between PD-L1 expression and Ki-67 expression and/or grading in GEP-NENs has been reported in most published studies [28,29,32,36–38,41,43]. This association also corresponds well with the observation of better response to anti-PD-L1 therapy in patients with G3 tumours than in those with G1 or G2 tumours in some clinical trials [13,17,18,20]. In the present study, IRS values were lower in tumours that had already metastasised to the lymph nodes than in those without lymph node metastases at diagnosis. In the literature, in contrast, either no association [27,29,31,32,35,39], or a positive correlation between PD-L1 expression and lymph node metastatic status was reported [38].

In the present study, no associations between PD-L1 expression and clinicopathological data such as patient age, gender, tumour size, presence of distant metastases, staging, or functionality were noted. These observations are in concordance with published data [27–29,31–33,35–39,42], as well as with a meta-analysis of PD-L1 expression in

Table 1
Correlations between the expression intensities of PD-L1, the different SSTs, the CXCR4, Chromogranin A (CgA) and Ki-67 (data were calculated with the mean receptor IRS values of each patient; $n = 179$). Significant correlations ($p < 0.05$) are marked in bold; r: correlation coefficient (Spearman); p: p value.

		SST1	SST2	SST3	SST4	SST5	CXCR4	CgA	Ki-67
PD-L1	**r**	**0.242**	**0.211**	**0.234**	**0.400**	**0.284**	0.115	**0.256**	**0.352**
	p	**0.001**	**0.005**	**0.002**	**<0.001**	**<0.001**	0.129	**<0.001**	**<0.001**
SST1	**r**		0.038	**0.331**	**0.211**	**0.405**	**0.217**	-0.004	**0.170**
	p		0.617	**<0.001**	**0.005**	**<0.001**	**0.004**	0.960	**0.026**
SST2	**r**			**0.242**	0.094	0.137	-0.039	**0.308**	0.091
	p			**0.001**	0.213	0.069	0.608	**<0.001**	0.236
SST3	**r**				**0.162**	**0.306**	0.091	**0.250**	0.030
	p				**0.030**	**<0.001**	0.229	**<0.001**	0.700
SST4	**r**					**0.193**	**0.151**	**0.192**	**0.269**
	p					**0.010**	**0.045**	**0.011**	**<0.001**
SST5	**r**						**0.201**	-0.088	**0.259**
	p						**0.007**	0.251	**<0.001**
CXCR4	**r**							**-0.167**	**0.296**
	p							**0.031**	**<0.001**
CgA	**r**								-0.119
	p								0.124

GEP-NENs [57]. No difference in PD-L1 expression between primary tumours and metastases was detected, which also corresponds with the literature [31].

Between the different primary tumour origins, however, variations in PD-L1 expression were observed in the present investigation, with the highest median IRS values observed in tumours from the stomach, rectum, or pancreas, followed by those from the duodenum or colon. In the literature, higher PD-L1 expression was reported in pancreas NENs compared with non-pancreas NENs [34] and in duodenal NENs compared with jejunal or ileal tumours [35], which corresponds well with our findings. In contrast, other studies described comparable PD-L1 expression across different sites of origin [28,31,36,37,42]. Additional investigations with larger case numbers, especially for the rare GEP-NEN sites of origin, are necessary to explore this variability.

Regarding interrelationships between PD-L1 expression and that of several typical markers of and receptors for neuroendocrine tumours, we observed positive correlations between the IRS values of PD-L1 and those of the somatostatin receptors SST1, SST2, SST3, SST4, and SST5; the neuroendocrine tumour marker CgA; and the proliferation marker Ki-67. No association was observed between the IRS values of PD-L1 and those of the chemokine receptor CXCR4. Regarding serum parameters, no correlation was found between tumour PD-L1 expression and serum CgA levels, but there was a negative interrelationship between PD-L1 expression and serum serotonin values. In the literature, no correlation was demonstrated between PD-L1 and CgA expression [38] or between tumour PD-L1 expression and CgA or 5-hydroxyindoleacetic acid levels in serum or urine [31]. Whereas Ki-67 and CXCR4 expression in literature have been shown to increase with increasing malignancy of GEP-NEN tumours and to be associated with poor prognosis, the expression of SST2, SST5, and CgA has been demonstrated to decrease with increasing malignancy of the neoplasms and to be associated with favourable patient outcomes [49,58]. Our in part contradictory findings regarding the correlations between PD-L1 expression levels and staging or grading and the expression of the investigated tumour markers indicate that PD-L1 may be differently involved in tumour spread and dedifferentiation. This assumption is supported by the lack of association between PD-L1 expression and patient survival.

Conclusions

Using the highly sensitive antibody 73–10, the present study indicates that PD-L1 expression is common in GEP-NENs and increases with histological grading. Therefore, targeting the PD-1/PD-L1 axis may represent a promising therapeutic strategy, specifically in G3 tumours. Given the discordant data in the literature, further studies of PD-L1 expression in GEP-NENs using highly sensitive antibodies (e.g., 73–10) and a scoring system taking both the staining intensity and the percentage of PD-L1-positive cells into account are strongly needed.

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CRediT authorship contribution statement

Erik Rösner: Investigation, Writing – review & editing. **Daniel Kaemmerer:** Conceptualization, Resources, Writing – review & editing. **Jörg Sänger:** Resources, Writing – review & editing. **Amelie Lupp:** Conceptualization, Project administration, Supervision, Methodology, Investigation, Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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Supplementary materials

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