RESEARCH

Immune checkpoint markers in gastroenteropancreatic neuroendocrine neoplasia

Florian Bösch^{1,2}, Katharina Brüwer^{1,2}, Annelore Altendorf-Hofmann³, Christoph J Auernhammer^{2,4}, Christine Spitzweg^{2,4}, C Benedikt Westphalen^{2,5}, Stefan Boeck^{2,5}, Gabriele Schubert-Fritschle^{2,6}, Jens Werner^{1,2}, Volker Heinemann^{2,5}, Thomas Kirchner^{2,7}, Martin Angele^{1,2} and Thomas Knösel^{2,7}

¹Department of General, Visceral, and Transplant Surgery, Ludwig-Maximilians-University Munich, Munich, Germany

²Interdisciplinary Center of Neuroendocrine Tumors of the GastroEnteroPancreatic System, Ludwig-Maximilians-University of Munich, Munich, Germany

³Department of General, Visceral und Vascular Surgery, Friedrich-Schiller University, Jena, Germany

⁴Department of Internal Medicine 4, Ludwig-Maximilians-University Munich, Munich, Germany

⁵Department of Medicine 3 and Comprehensive Cancer Center, Ludwig-Maximilians-University Munich, Munich, Germany

⁶Munich Cancer Registry (MCR) of the Munich Tumour Centre (TZM), Institute for Medical Information Processing, Biometry, and Epidemiology (IBE), Ludwig-Maximilians-University Munich, Munich, Germany

⁷Institute of Pathology, Ludwig-Maximilians-University, Munich, Germany

Correspondence should be addressed to T Knösel: Thomas.Knoesel@med.uni-muenchen.de

Abstract

Cancer immunotherapy has evolved major breakthroughs in the last years. The cell-surface receptor programmed death-1 (PD-1) and its ligand, programmed death ligand-1 (PD-L1), have been detected in various cancer types. However, the analysis on gastroenteropancreatic neoplasia (GEP-NENs) is limited. Therefore, the aim of this study was to characterize GEP-NENs with regard to PD-1/PD-L1 pathway and tumor-infiltrating lymphocytes (TILs). On protein level, we examined TILs, PD-1 and PD-L1 expression in tumor tissue of 244 GEP-NENs using immunohistochemistry. Expression levels were correlated with clinicopathological parameters including long-term survival in an observational study. In total, 244 patients could be included. Most of the patients had a NEN of the small intestine (52.5%) or the pancreas (29.5%). All tumors could be graded by their morphology and Ki67 index, with 57.8% G1, 34% G2 and 8.2% G3 tumors. High TILs (19.6%) and high PD-1 (16.1%) expression showed a significant correlation with shorter patient survival ($P < 0.05$) and with a higher grading. Furthermore, expression of PD-L1 (8.7%) showed a trend to shorter patient survival. High TILs and PD-1 expression are significantly associated with shorter patient survival and higher grading in GEP-NENs. PD-L1 expression showed a trend to shorter patient survival. Immunotherapy might be a promising therapeutic approach in GEP-NENs especially in tumors with high TILs.

Key Words

- ▶ checkpoint inhibitors
- ▶ neuroendocrine tumor
- ▶ PD-L1
- ▶ TIL
- ▶ microarray

Endocrine-Related Cancer
(2019) **26**, 293–301

Introduction

Neuroendocrine neoplasia (NENs) are a heterogeneous group of tumors, which can arise from neuroendocrine cells throughout the body. These tumors have an increasing incidence of 2.5–5/100,000 people per year

(Kloppel & Anlauf 2005, Lawrence *et al.* 2011). Thus, NENs are nowadays as frequent as testicular tumors, Hodgkin's disease, gliomas and multiple myeloma (Modlin *et al.* 2008). Gastroenteropancreatic NENs (GEP-NENs) represent

the preponderance with primaries localized in the foregut, the midgut and the hindgut (Williams & Sandler 1963). Well-differentiated GEP-NENs are slow-growing tumors and remain silent for years until they get diagnosed for metastases. Although most of these neoplasia are low-grade tumors, 40–95% are metastasized at initial diagnosis (Saxena *et al.* 2011). GEP-NENs are graded into three groups based on the morphology of the tumor cells (well/poorly differentiated) and the mitotic rate and/or the proliferative index (Rindi *et al.* 2007). Treatment strategies are highly influenced by grading. The increasing clinical impact of this tumor entity is further emphasized by the fact that treatment strategies underwent only minor changes over the last decades. Therefore, novel diagnostic tools are required to enable improved risk stratification and thereby allow individualized tailor-made therapy.

Cancer immunotherapy has evolved over the last years as a scientific field with major breakthroughs in tumor-targeted therapy. Cancer immunotherapy, which enables immune cells to attack cancer cells, reached outstanding results and long-term survival in patients with melanoma, non-small cell lung cancer or bladder cancer (Powles *et al.* 2014, Taube *et al.* 2014, Farkona *et al.* 2016). Key players of immunotherapy are programmed death-1 (PD-1) and its ligand, programmed death ligand-1 (PD-L1), which influence the host immune response to cancer. PD-1 is expressed on immune cells (B cells, T cells, myeloid cells) and is overexpressed on TILs. PD-L1 is expressed on many cancer cells. Binding of PD-L1 to PD-1 leads to an inactivation and downregulation of T cells in the tumor microenvironment. Thus, PD-L1 provides tumor cells a means of escape from the immune response, subsequently enabling tumor progression and metastases (Ribas 2012, Zitvogel & Kroemer 2012, Postow *et al.* 2015). In this respect, PD-1 and PD-L1 are key targets of cancer immunotherapy. Immune checkpoint inhibitors block these inhibitory signals and T cells are activated and attack the cancer (Sullivan & Flaherty 2015).

A growing body of evidence recently suggests a possible importance of the aforementioned immune checkpoints in NENs. However, most studies were conducted on pulmonary NENs or with small collectives (Kim *et al.* 2016, Cavalcanti *et al.* 2017, Roberts *et al.* 2017, Tsuruoka *et al.* 2017). Nonetheless, new biomarkers to stratify GEP-NENs are needed. Therefore, the aim of this study was to analyze a large and well-characterized collective of GEP-NENs with regard to the expression of PD-1, PD-L1 and TILs.

Materials and methods

Patient selection

An exploratory retrospective cohort study design was chosen to address the research question. Formalin-fixed paraffin-embedded tumor specimens from 244 patients diagnosed with GEP-NENs were retrieved from the Institute of Pathology of the Ludwig-Maximilians-University, Munich from the year 1995 to 2013. Data on clinical parameters were extracted from the pathologists' original reports and a prospective led database. The tumors were reclassified according to the actual 2017 WHO classification system. The assessed variables refer to demographic data, the primary tumor characteristics, histopathology and grading of tumors and overall survival. The Internal Review Board and the Ethical Review Committee of the Ludwig-Maximilians-University Munich approved the protocol of the study (grant no. 18–177). Informed consent was not required since this is a retrospective study evaluating non-reversible anonymized pathological specimens. Thus, these data do not make it possible to deduce the identities of the study participants.

Tissue microarrays and immunohistochemistry

A total of 244 tumor samples (primary tumor, if available or liver metastasis) on tissue microarrays (TMAs) with a diameter of 0.6 mm were acquired from the formalin-fixed paraffin-embedded (FFPE) tissue (Fig. 1A and B) as previously described (Kampmann *et al.* 2015). For the immunohistochemical detection of the PD-1 and PD-L1 proteins, commercially available and validated monoclonal antibodies (PD-1, dilution 1:80; clone NAT105, Cell Mark, MEDAC, Wedel, Germany; PD-L1, dilution 1:100; E1L3N, Cell Signaling Technology) were used (Konishi *et al.* 2004, Wimberly *et al.* 2015). Staining was performed on a Ventana Benchmark XT Autostainer with the XT ultraView DAB Kit (Ventana Medical Systems, Tucson, AZ). All slides were counterstained with hematoxylin (Vector). To exclude unspecific staining, system controls were included. Tonsillar tissue served as a positive control for immunohistochemistry (IHC).

Immunostaining of cells was evaluated and scored semi-quantitatively (0, negative; 1, weakly positive; 2, moderately positive; 3, strongly positive). PD-1-expressing TILs were counted manually and categorized semi-quantitatively per punch into low (<3 positive lymphocytes) and into high (≥3 positive lymphocytes)

Figure 1

Examples of (A) well-differentiated neuroendocrine tumor (NET, G1), (B) poorly differentiated neuroendocrine carcinoma (NEC, G3) with high TILs and areas of necrosis (10× magnification), (C) high PD-1-positive lymphocytes in specimen with high TILs (40× magnification) and (D) high PD-L1 staining (score 3) in tumor cells within a specimen with high TILs.

(Fig. 1C). According to Katz *et al.*, the cut-off value was based on the median score of TILs (Katz *et al.* 2010). High/positive expression of PD-L1 was defined as membranous staining of >1% of the tumor cells (Fig. 1D) (Straub *et al.* 2016). TILs were categorized semi-quantitatively into low (<3 TILs) and high (≥ 3 TILs) lymphocytes per hematoxylin and eosin-stained TMA punch (Fig. 1B). The identification of lymphocytes was performed microscopically and counted manually. A patient was excluded from a specific analysis if the specific staining failed. An experienced pathologist (T K) with special expertise in GEP-NEN pathology and a second observer (F B) carried out all immunohistochemical and pathologic evaluations independently and blinded. In the case of discrepancy, the slides were reevaluated under a multiheaded microscope and consensus reached.

Statistical analysis

Follow-up was done in outpatient clinics or by contacting the registration office. Statistical analyses were performed by using SPSS 22.0 (IBM Corp.) software. Univariate analysis was carried out by using chi-squared test for categorical parameters (e.g., grading, presence of distant metastases); *P* values lower than 0.05 are considered significant. Observed survival was calculated from the date of diagnosis. Mean survival times along with their 95% CIs and Kaplan–Meier survival statistics were calculated for the entire sample using log-rank tests. Significant and

independent predictors of observed survival were identified by Cox proportional hazard analysis. The stepwise procedure was set to a threshold of 0.05.

Results

General clinicopathological characteristics

The baseline characteristics of the investigated cohort are summarized in Table 1. In total, 244 patients were analyzed. Mean age was 60 years (18–92 years), 107 patients (43.9%) were female and 137 patients (56.1%) were male ($P > 0.05$). The most common primary tumor origin was the small intestine (52.5%), followed by the pancreas (29.5%). All tumors could be graded by their morphology and Ki67 index. A majority of 141 patients (57.8%) had a G1 tumor, compared to 83 patients (34%) and 20 patients (8.2%) with a G2 or a G3 tumor, respectively. Comparable to the broad diversification of grading, the primary tumors were T1 in 19.2%, T2 in 22.4%, T3 in 43.5% and T4 in 15% cases.

Correlation between TILs, PD-1 and PD-L1 expression and grading

High TILs (≥ 3 lymphocytes per TMA-spot) could be evaluated in 47 cases (19.6%). High PD-1 expression in TILs was seen in 35 samples (16.1%), and 20 samples (8.7%) were highly positive for PD-L1. As mentioned above, tumor grading was available for the entire cohort

Table 1 Baseline characteristics in 244 patients with gastroenteropancreatic neuroendocrine neoplasia.

Patient characteristics	n	%
Gender		
Male	137	56.1
Female	107	43.9
Age		
<60 years	117	48
≥60 years	127	52
Localization		
Esophagus/stomach	17	7
Small intestine	128	52.5
Colon	18	7.4
Rectum	8	3.3
Papilla Vateri	1	0.4
Pancreas	72	29.5
Primary tumor		
pT1	41	16.8
pT2	48	19.7
pT3	93	38.1
pT4	32	13.1
Unknown	30	12.2
Tumor grading		
Well-differentiated G1	141	57.8
Well-differentiated G2	83	34
Poorly differentiated G3	20	8.2
Lymph node metastases		
Yes	56	23
No	134	54.9
Unknown	54	22.1
Angioinvasion		
Yes	17	7
No	93	38.1
Unknown	134	54.9
Lymphovascular infiltration		
Yes	57	23.4
No	62	25.4
Unknown	125	51.2
Distant metastases		
Yes	104	42.6
No	136	55.7
Unknown	4	1.6

consisting of 244 patients. But immunohistochemistry for TILs, PD-1 and PD-L1 was not conclusive for every patient. TILs could be analyzed in 229 samples (93.9%) and PD-1 and PD-L1 each in 215 (88.1%) cases. There was a highly significant correlation between high TILs and a higher grading ($P<0.001$). Fifty percent ($n=9$) of G3 tumors had high TILs compared to only 17.1% ($n=38$) of G1/2 tumors ($P<0.001$). Furthermore, PD-1 positivity was significantly associated with a higher tumor grading. Thus, 53.8% ($n=7$) of G3 tumors were PD-1 positive compared to only 13.7% ($n=28$) in G1/2 tumors ($P<0.001$). However, there was no significant correlation between PD-L1 expression and grading ($P>0.05$). G3 cases were PD-L1 positive in 16.7% (Table 2).

Table 2 Correlation of TILs and of the expression of PD-1 and of PD-L1.

	TIL n (%)	PD-1 n (%)	PD-L1 n (%)
G1/2	38/222 (17.1)*	28/204 (13.7)**	17/213 (8)***
G3	9/18 (50)*	7/13 (53.8)**	3/18 (16.7)***
PD-L1	9/42 (21.4)*	7/35 (20)***	

* $P=0.002$; ** $P=0.001$; *** $P>0.05$.

Survival analyses with respect to TILs, PD-1 and PD-L1

High TIL tumors showed a shorter patient survival. A highly significant prolonged mean overall survival of 53.9 months (CI 95%: 51.7; 56.1 months) was reached if TILs were low, compared to 39.4 months (CI 95%: 32.2; 46.6 months) in patients with higher TILs ($P<0.001$) (Fig. 2). Concerning the overall survival rate, there is a correlation between grading and TILs. High TIL patients showed a significantly decreased overall survival rate in well-differentiated patients (Fig. 3). Patients with high TILs had a mean overall survival of 46.4 months (CI 95%: 93.6; 53.3 months) compared to 55 months (CI 95%: 53; 57 months) in patients with low TILs ($P<0.05$). Patients with G3 tumors and high TILs had a deteriorated survival rate of 8.8 months (CI 95%: 3.9; 13.6 months) compared to 30.3 months (CI 95%: 12.2; 48.4 months) ($P>0.05$).

Moreover, statistical analysis demonstrated a significant association of PD-1 positivity and inferior overall survival rates ($P<0.05$). Patients with PD-1 negative tumors had a mean overall survival of 53.8 months (CI 95%: 51.6; 56.1 months) compared to a mean overall survival of 44.5 months (CI 95%: 36.2; 52.7 months) if the tumor was PD-1 positive (Fig. 4). High expression of PD-L1 tumor cells showed a trend to shorter survival (Fig. 5). The mean overall survival of PD-L1-negative tumors reached 51.9 months (CI 95%: 49.5; 54.4 months) compared to only 46 months (CI 95%: 35.4; 56.6 months)

Figure 2

Kaplan-Meier overall survival curves for patients divided into levels of tumor-infiltrating lymphocytes (TIL) ($P<0.05$).

Figure 3

Kaplan–Meier overall survival curves for patients with G1/2 (A) ($P < 0.05$) and G3 (B) tumors ($P > 0.05$) divided into levels of tumor-infiltrating lymphocytes (TIL).

in the case of PD-L1 positivity ($P > 0.05$). However, PD-1 and PD-L1 expression levels had no prognostic relevance in well- and poorly differentiated tumors. Although there was a visible trend, the difference in overall survival did not reach statistical significance.

Patients were classified into pancreas and non-pancreas NEN according to the site of the primary tumor and were evaluated for TILs and the expression of PD-1 and PD-L1. This analysis revealed that pancreas NEN had a higher rate of high PD-1 (23.5%) and high PD-L1 (17.5%) expression

Figure 4

Kaplan–Meier overall survival curves for patients divided into PD-1 expression ($P < 0.05$).

Figure 5

Kaplan–Meier overall survival curves for patients divided into PD-L1 expression ($P > 0.05$).

and of high TILs (38.5%) compared to non-pancreas NEN (Table 3). Additionally, high TIL tumors were significantly correlated with shorter overall survival in patients with pancreas and non-pancreas NEN, respectively. Patients with high TIL pancreas NEN had a mean overall survival of 36.4 months (CI 95%: 24.9; 47.9 months) compared to 53.2 months (CI 95%: 48.9; 57.6 months) in patients with low TIL pancreas NEN ($P < 0.05$). Regarding TILs, the mean overall survival times in patients with non-pancreas NEN were similar. Patients with high TIL non-pancreas NEN had a mean overall survival of 40.9 months (CI 95%: 31.8; 50 months) compared to 54.2 months (CI 95%: 51.6; 56.7 months) in patients with low TIL non-pancreas NEN ($P < 0.05$).

Regarding high PD-1 and PD-L1 expression, it was shown that the survival rates for pancreas NEN were statistically non-significant. Patients with a highly PD-1-positive pancreas NEN had a mean overall survival of 45.8 months (CI 95%: 32.1; 59.5 months) compared to 51.9 months (CI 95%: 47.3; 56.5 months) in the absence of PD-1 expression ($P > 0.05$). However, high PD-1 positivity was associated with a significantly shorter overall survival for patients with non-pancreas NEN. These patients had a mean overall survival time of 43.7 months (CI 95%: 33.4; 54.1 months) compared to 54.6 months

Table 3 Correlation of TILs and the expression of PD-1 and of PD-L1 according to the site of primary tumor. The univariate survival analysis showed a significant shorter patient survival for patients with high TIL tumors and for patients with non-pancreas tumors with a high expression of PD-1.

	High TIL <i>n</i> (%)	High PD-1 <i>n</i> (%)	High PD-L1 <i>n</i> (%)
Pancreas	20/52 (38.5)*	12/51 (23.5)	10/57 (17.5)
Non-pancreas	27/141 (19.2)*	23/131 (17.6)*	10/154 (6.5)

* $P < 0.05$.

Table 4 Cox proportional hazard regression analysis.

	P-value	Exp (B)	CI 95%
G1/2 vs G3	0.000	8.951	3.476–21.260
Age	0.001	1.04	1.015–1.065
M0 vs M1	0.024	1.88	1.088–3.248
PD-1 low vs high	0.132	1.708	0.851–3.428
PD-L1 low vs high	0.999	1.001	0.275–3.644
TIL low vs high	0.985	1.006	0.565–1.789

(CI 95%: 52; 57.1 months) in patients with PD-1-negative non-pancreas NEN ($P < 0.05$).

The mean overall survival time of patients with highly PD-L1-positive pancreas NEN was 44.1 months (CI 95%: 35; 53.2 months) and of patients with PD-L1-negative pancreas NEN 49.3 months (CI 95%: 44.2; 54.4 months) ($P > 0.05$). Also, PD-L1 expression of non-pancreas NEN was not associated with a significantly shorter patient survival (high PD-L1: 41.8 months (CI 95%: 27.5; 56 months) vs PD-L1 negative: 52.9 months (CI 95%: 50.2; 55.7 months)) ($P > 0.05$).

Furthermore, a multivariate analysis was conducted to test if immunostaining (TIL, PD-1, PD-L1) might be an independent risk factor (Table 4). This analysis confirmed the well-known prognostic markers in GEP-NEN. Thus, the presence of distant metastases, the tumor grading and the age at diagnosis were independent risk factors for survival. However, immunostaining and TILs did not reach statistical significance.

Discussion

Immune checkpoint inhibitors showed substantial beneficial effects in a series of studies in various tumors (Powles *et al.* 2014, Taube *et al.* 2014, Farkona *et al.* 2016, Auernhammer *et al.* 2018). Therefore, the aim of this study was to analyze GEP-NENs according to the amount of TILs and PD-1-positive lymphocytes and the *in situ* protein expression of PD-L1 in tumor cells. We correlated the results with clinicopathological features including the overall survival of the patients. To the best of our knowledge, this is the largest collective of GEP-NENs addressing this issue. Scarce information exists about the potential role of TILs in GEP-NENs. However, it is well known that cancer cells can escape from immunosurveillance by inactivating TILs. Cancer cells exploit the mechanisms to inactivate TILs after binding of immune checkpoint receptors (Zitvogel & Kroemer 2012, Beatty & Gladney 2015). Evidence of the prognostic relevance of TILs is ambiguous in the literature. Whereas in melanoma and breast cancers, high levels of TILs are

associated with better overall survival (Clemente *et al.* 1996, Dieci *et al.* 2015), there are contradictory results as well (Ishigami *et al.* 2003, Koide *et al.* 2004). The negative prognostic value of high levels of TILs was demonstrated in patients with non-small-cell lung cancer, where TILs were associated with a significantly higher rate of recurrence (Yan *et al.* 2017). Additionally, various studies on renal cell carcinoma further support these findings, demonstrating an association between TILs and poor prognosis (Nakano *et al.* 2001, Remark *et al.* 2013, Giraldo *et al.* 2015). In this respect, our findings that high TILs are associated with a poor outcome – irrespective of the primary tumor site – are in concordance to the aforementioned studies. The deteriorated survival rates reflect the effective cancer immune evasion. We demonstrated that a high expression of PD-L1 in tumor cells was associated with high rates of PD-1-positive lymphocytes and these with a significantly high amount of TILs. Hence, our results further support the hypothesis that in high TIL tumors more PD-1 positive lymphocytes are present and thereby tumor cells protect themselves with an upregulation of PD-L1.

Furthermore, a strong correlation between a higher tumor grading and levels of high TILs is reported for the first time in NENs. Poorly differentiated GEP-NENs had the highest levels of TILs. These results indicate an immense immunological response in the presence of poorly differentiated GEP-NENs. This hypothesis was further supported by the pronounced correlation of high PD-1 expression and G3 GEP-NENs. Therefore, these findings suggest that immunotherapy might be a promising therapeutic approach in GEP-NENs.

Previous studies suggested an association between PD-L1 positivity and response to PD-1 antibodies. However, there are reports about patients with PD-L1-positive tumors, who do not respond, and patients with PD-L1-negative tumors, who respond (Campesato *et al.* 2015, Teixeira *et al.* 2015). In this respect, we could demonstrate that in total, 8.7% of tumors showed an expression of PD-L1. In a multicohort phase 1b study, the effect of pembrolizumab on patients with PD-L1-positive GEP-NENs was evaluated. The treatment with pembrolizumab led to an objective response rate of 12%; the stable disease rate was 60% (Mehnert *et al.* 2017).

With regard to overall survival rates, our data revealed a prognostic relevance for TILs, PD-1 and PD-L1 in univariate analysis. GEP-NENs with high rate of TILs and PD-1 positivity showed significantly deteriorated survival rates. The prognostic relevance of PD-L1 did not reach statistical significance. These results are in agreement with studies carried out in other malignancies

(Ishigami *et al.* 2003, Koide *et al.* 2004, Krpina *et al.* 2015). Additionally, the prognostic value of high PD-1 positivity was associated with the primary tumor site; non-pancreas NEN had a significantly shorter mean overall survival compared to pancreatic NEN. However, this difference was statistically non-significant for pancreas NEN, although a trend was obvious. Moreover, the prognostic value of high TILs was verifiable irrespective of the primary tumor site. Nonetheless, larger studies are necessary to comprehensively validate these findings. In the multivariate analysis, the well-known factors grading, age at diagnosis and status of metastases had a significant influence on overall survival. Interestingly, in cases with higher grading, higher age and metastasis, we detected a higher amount of TILs, and in these patients, after stratification, immunotherapy might be a new avenue of therapy. PD-1 is a key player expressed on T cells and mediates tumor immune evasion by interaction with its ligand PD-L1. In this respect, it has been suggested that an increase in PD-1 positivity results in immunosuppression subsequently leading to tumor progression and deteriorated survival rates. Therefore, inhibition of the PD-1/PD-L1-pathway is a powerful tool to improve the activity levels of cytotoxic T cells.

Furthermore, TILs might be a novel marker to help distinguishing between well-differentiated GEP-NENs. However, the lack of prognostic relevance in patients with G3 GEP-NENs is most probably due to the small number of patients with a G3 tumor. Additionally, the expression levels of PD-1 and PD-L1 could not help to subdivide well- and poorly differentiated tumor groups. Nonetheless, these results indicate that high TIL tumors bear an increased malignant potential. In further unselective and consecutive investigations in GEP-NENs grading, age at diagnosis and status of metastasis are very important factors and should be defined. This group should be stratified to analyze the effect of immunotherapy regarding the TILs.

Hanahan and Weinberg established the concept of tumor microenvironment and recently updated 'The Hallmarks of Cancer' of which avoiding immune destruction is one (Hanahan & Weinberg 2000, 2011). Tumors can escape from immune surveillance by downregulating the activation of T cells through immune checkpoints and thereby establish their tumor immune microenvironment. Thus, immune checkpoint inhibitors are nowadays used in the treatment of various malignancies (Powles *et al.* 2014, Taube *et al.* 2014, Farkona *et al.* 2016, Latteyer *et al.* 2016). Nonetheless, immune checkpoint inhibitors have not – yet – been

approved as treatment for GEP-NENs. However, clinical trials are recruiting, encouraging results of single-center studies have been presented and reviews on this topic have been published (Mehnert *et al.* 2017, Chauhan *et al.* 2018, Weber & Fottner 2018). Furthermore, studies revealed that the pathogenesis and therapy of NENs is dependent on the immune microenvironment, which is infiltrated by immune cells (Ryschich *et al.* 2003, Vikman *et al.* 2009, Katz *et al.* 2010). Thus, treatment with immune checkpoint inhibitors will increase the patient's immune response against GEP-NENs. PD-1 and PD-L1 expression was shown in well- and poorly differentiated intestinal and pancreatic NENs (da Silva *et al.* 2016, Kim *et al.* 2016, Lamarca *et al.* 2018). Additionally, in the present study, TILs and PD-1 were significantly associated with grading, and high expression of PD-1 and PD-L1 was seen in 16.1% and 8.7%, respectively. Additionally, high TILs were present in 19.6%. Due to these findings and the growing body of literature, treatment with immune checkpoint inhibitors represents a promising therapeutic strategy for patients with GEP-NENs.

Although we performed a retrospective analysis, this study analyzed the largest patient collective reported so far. Due to the low incidence, slow growing and long survival rates of GEP-NENs prospective studies are scarce and extremely difficult to conduct. Moreover, the topic presented is rapidly evolving, and indication for immunotherapy is constantly extended. Nonetheless, the treatment with immune checkpoint inhibitors is not yet established for GEP-NEN. This new agents will lead to increased socioeconomic costs and thus a definition of subgroups which may profit are needed. Therefore, the present findings give an overview of immune checkpoint marker expression and might help to establish treatment regimens for patients with GEP-NEN. Various antibodies for the detection of PD-L1 are commercially available, and we used the clone E1L3N from cell signaling in this study. This specific antibody targets the intracellular domain of PD-L1. It was shown recently that cytoplasmic staining responds well with the PD-L1-positive cell membrane in an FFPE tissue and its interpretation is feasible (Mahoney *et al.* 2015). Probably, E1L3N even facilitates to discriminate between responders and non-responders in melanoma patients (Kluger *et al.* 2017).

In conclusion, to the best of our knowledge, this is the first study in a large well-characterized tumor collective showing that high TIL and PD-1 expression are significantly associated with shorter patient survival and higher grading in GEP-NENs. PD-L1 expression showed

a trend to shorter patient survival. Immunotherapy might be a promising therapeutic approach in GEP-NENs especially in G3 NENs and tumors with high TILs.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

References

- Auernhammer CJ, Spitzweg C, Angele MK, Boeck S, Grossman A, Nolting S, Ilhan H, Knosel T, Mayerle J, Reincke M, *et al.* 2018 Advanced neuroendocrine tumours of the small intestine and pancreas: clinical developments, controversies, and future strategies. *Lancet Diabetes and Endocrinology* **6** 404–415. ([https://doi.org/10.1016/S2213-8587\(17\)30401-1](https://doi.org/10.1016/S2213-8587(17)30401-1))
- Beatty GL & Gladney WL 2015 Immune escape mechanisms as a guide for cancer immunotherapy. *Clinical Cancer Research* **21** 687–692. (<https://doi.org/10.1158/1078-0432.CCR-14-1860>)
- Campeato LF, Barroso-Sousa R, Jimenez L, Correa BR, Sabbaga J, Hoff PM, Reis LF, Galante PA & Camargo AA 2015 Comprehensive cancer-gene panels can be used to estimate mutational load and predict clinical benefit to PD-1 blockade in clinical practice. *Oncotarget* **6** 34221–34227. (<https://doi.org/10.18632/oncotarget.5950>)
- Cavalcanti E, Armentano R, Valentini AM, Chieppa M & Caruso ML 2017 Role of PD-L1 expression as a biomarker for GEP neuroendocrine neoplasm grading. *Cell Death and Disease* **8** e3004. (<https://doi.org/10.1038/cddis.2017.401>)
- Chauhan A, Horn M, Magee G, Hodges K, Evers M, Arnold S & Anthony L 2018 Immune checkpoint inhibitors in neuroendocrine tumors: a single institution experience with review of literature. *Oncotarget* **9** 8801–8809. (<https://doi.org/10.18632/oncotarget.23753>)
- Clemente CG, Mihm MC Jr, Bufalino R, Zurrida S, Collini P & Cascinelli N 1996 Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer* **77** 1303–1310. ([https://doi.org/10.1002/\(SICI\)1097-0142\(19960401\)77:7<1303::AID-CNCR12>3.0.CO;2-5](https://doi.org/10.1002/(SICI)1097-0142(19960401)77:7<1303::AID-CNCR12>3.0.CO;2-5))
- da Silva A, Qian Z, Zhang S, Masugi Y, Rodig S, Brais L, Dutton T, Chan J, Hodi S, Ogino S, *et al.* 2016 Immune checkpoint markers and immune response in well differentiated neuroendocrine tumors (NET) of the small intestine and pancreas. In *NANETS Symposium abstract B03*. (available at: <https://nanets.net/abstracts-archive/2016>)
- Dieci MV, Mathieu MC, Guarneri V, Conte P, Delaloge S, Andre F & Goubar A 2015 Prognostic and predictive value of tumor-infiltrating lymphocytes in two phase III randomized adjuvant breast cancer trials. *Annals of Oncology* **26** 1698–1704. (<https://doi.org/10.1093/annonc/mdv239>)
- Farkona S, Diamandis EP & Blasutig IM 2016 Cancer immunotherapy: the beginning of the end of cancer? *BMC Medicine* **14** 73. (<https://doi.org/10.1186/s12916-016-0623-5>)
- Giraldo NA, Becht E, Pages F, Skliris G, Verkarre V, Vano Y, Mejean A, Saint-Aubert N, Lacroix L, Natario I, *et al.* 2015 Orchestration and prognostic significance of immune checkpoints in the microenvironment of primary and metastatic renal cell cancer. *Clinical Cancer Research* **21** 3031–3040. (<https://doi.org/10.1158/1078-0432.CCR-14-2926>)
- Hanahan D & Weinberg RA 2000 The hallmarks of cancer. *Cell* **100** 57–70. ([https://doi.org/10.1016/S0092-8674\(00\)81683-9](https://doi.org/10.1016/S0092-8674(00)81683-9))
- Hanahan D & Weinberg RA 2011 Hallmarks of cancer: the next generation. *Cell* **144** 646–674. (<https://doi.org/10.1016/j.cell.2011.02.013>)
- Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Okumura H, Matsumoto M, Miyazono F, Hokita S & Aikou T 2003 Tumor-associated macrophage (TAM) infiltration in gastric cancer. *Anticancer Research* **23** 4079–4083.
- Kampmann E, Altendorf-Hofmann A, Gibis S, Lindner LH, Issels R, Kirchner T & Knosel T 2015 VEGFR2 predicts decreased patients survival in soft tissue sarcomas. *Pathology – Research and Practice* **211** 726–730. (<https://doi.org/10.1016/j.prp.2015.04.015>)
- Katz SC, Donkor C, Glasgow K, Pillarisetty VG, Gonen M, Espat NJ, Klimstra DS, D'Angelica MI, Allen PJ, Jarnagin W, *et al.* 2010 T cell infiltrate and outcome following resection of intermediate-grade primary neuroendocrine tumours and liver metastases. *HPB* **12** 674–683. (<https://doi.org/10.1111/j.1477-2574.2010.00231.x>)
- Kim ST, Ha SY, Lee S, Ahn S, Lee J, Park SH, Park JO, Lim HY, Kang WK, Kim KM, *et al.* 2016 The impact of PD-L1 expression in patients with metastatic GEP-NETs. *Journal of Cancer* **7** 484–489. (<https://doi.org/10.7150/jca.13711>)
- Kloppel G & Anlauf M 2005 Epidemiology, tumour biology and histopathological classification of neuroendocrine tumours of the gastrointestinal tract. *Best Practice and Research: Clinical Gastroenterology* **19** 507–517. (<https://doi.org/10.1016/j.bpg.2005.02.010>)
- Kluger HM, Zito CR, Turcu G, Baine MK, Zhang H, Adeniran A, Sznol M, Rimm DL, Kluger Y, Chen L, *et al.* 2017 PD-L1 studies Across tumor types, its differential expression and predictive value in patients treated with immune checkpoint inhibitors. *Clinical Cancer Research* **23** 4270–4279. (<https://doi.org/10.1158/1078-0432.CCR-16-3146>)
- Koide N, Nishio A, Sato T, Sugiyama A & Miyagawa S 2004 Significance of macrophage chemoattractant protein-1 expression and macrophage infiltration in squamous cell carcinoma of the esophagus. *American Journal of Gastroenterology* **99** 1667–1674. (<https://doi.org/10.1111/j.1572-0241.2004.30733.x>)
- Konishi J, Yamazaki K, Azuma M, Kinoshita I, Dosaka-Akita H & Nishimura M 2004 B7-H1 expression on non-small cell lung cancer cells and its relationship with tumor-infiltrating lymphocytes and their PD-1 expression. *Clinical Cancer Research* **10** 5094–5100. (<https://doi.org/10.1158/1078-0432.CCR-04-0428>)
- Krpina K, Babarovic E & Jonjic N 2015 Correlation of tumor-infiltrating lymphocytes with bladder cancer recurrence in patients with solitary low-grade urothelial carcinoma. *Virchows Archiv* **467** 443–448. (<https://doi.org/10.1007/s00428-015-1808-6>)
- Lamarca A, Nonaka D, Breitwieser W, Ashton G, Barriuso J, McNamara MG, Moghadam S, Rogan J, Mansoor W, Hubner RA, *et al.* 2018 PD-L1 expression and presence of TILs in small intestinal neuroendocrine tumours. *Oncotarget* **9** 14922–14938. (<https://doi.org/10.18632/oncotarget.24464>)
- Lattayer S, Tiedje V, Schilling B & Fuhrer D 2016 Perspectives for immunotherapy in endocrine cancer. *Endocrine-Related Cancer* **23** R469–R484. (<https://doi.org/10.1530/ERC-16-0169>)
- Lawrence B, Gustafsson BI, Chan A, Svejda B, Kidd M & Modlin IM 2011 The epidemiology of gastroenteropancreatic neuroendocrine tumors. *Endocrinology and Metabolism Clinics of North America* **40** 1–18, vii. (<https://doi.org/10.1016/j.ecl.2010.12.005>)
- Mahoney KM, Sun H, Liao X, Hua P, Callea M, Greenfield EA, Hodi FS, Sharpe AH, Signoretti S, Rodig SJ, *et al.* 2015 PD-L1 antibodies to its cytoplasmic domain most clearly delineate cell membranes in immunohistochemical staining of tumor cells. *Cancer Immunology Research* **3** 1308–1315. (<https://doi.org/10.1158/2326-6066.CIR-15-0116>)

- Mehnert JM, Rugo HS, O'Neil BH, Santoro A, Schellens JHM, Cohen RB, Doi T, Ott PA, Pishvaian MJ, Puzanov I, *et al.* 2017 Pembrolizumab for patients with PD-L1-positive advanced carcinoid or pancreatic neuroendocrine tumors: results from the KEYNOTE-028 study. *Annals of Oncology* **28** v142–v157.
- Modlin IM, Oberg K, Chung DC, Jensen RT, de Herder WW, Thakker RV, Caplin M, Delle Fave G, Kaltsas GA, Krenning EP, *et al.* 2008 Gastroenteropancreatic neuroendocrine tumours. *Lancet Oncology* **9** 61–72. ([https://doi.org/10.1016/S1470-2045\(07\)70410-2](https://doi.org/10.1016/S1470-2045(07)70410-2))
- Nakano O, Sato M, Naito Y, Suzuki K, Orikasa S, Aizawa M, Suzuki Y, Shintaku I, Nagura H & Ohtani H 2001 Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. *Cancer Research* **61** 5132–5136.
- Postow MA, Callahan MK & Wolchok JD 2015 Immune checkpoint blockade in cancer therapy. *Journal of Clinical Oncology* **33** 1974–1982. (<https://doi.org/10.1200/JCO.2014.59.4358>)
- Powles T, Eder JP, Fine GD, Braithel FS, Loriaut Y, Cruz C, Bellmunt J, Burris HA, Petrylak DP, Teng SL, *et al.* 2014 MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. *Nature* **515** 558–562. (<https://doi.org/10.1038/nature13904>)
- Remark R, Alifano M, Cremer I, Lupo A, Dieu-Nosjean MC, Riquet M, Crozet L, Ouakrim H, Goc J, Cazes A, *et al.* 2013 Characteristics and clinical impacts of the immune environments in colorectal and renal cell carcinoma lung metastases: influence of tumor origin. *Clinical Cancer Research* **19** 4079–4091. (<https://doi.org/10.1158/1078-0432.CCR-12-3847>)
- Ribas A 2012 Tumor immunotherapy directed at PD-1. *New England Journal of Medicine* **366** 2517–2519. (<https://doi.org/10.1056/NEJMe1205943>)
- Rindi G, Kloppel G, Couvelard A, Komminoth P, Korner M, Lopes JM, McNicol AM, Nilsson O, Perren A, Scarpa A, *et al.* 2007 TNM staging of midgut and hindgut (neuro) endocrine tumors: a consensus proposal including a grading system. *Virchows Archiv* **451** 757–762. (<https://doi.org/10.1007/s00428-007-0452-1>)
- Roberts JA, Gonzalez RS, Das S, Berlin J & Shi C 2017 Expression of PD-1 and PD-L1 in poorly differentiated neuroendocrine carcinomas of the digestive system: a potential target for anti-PD-1/PD-L1 therapy. *Human Pathology* **70** 49–54. (<https://doi.org/10.1016/j.humpath.2017.10.003>)
- Ryschich E, Autschbach F, Eisold S, Klar E, Buchler MW & Schmidt J 2003 Expression of HLA class I/II antigens and T cell immune response in human neuroendocrine tumors of the pancreas. *Tissue Antigens* **62** 48–54. (<https://doi.org/10.1034/j.1399-0039.2003.00075.x>)
- Saxena A, Chua TC, Sarkar A, Chu F, Liauw W, Zhao J & Morris DL 2011 Progression and survival results after radical hepatic metastasectomy of indolent advanced neuroendocrine neoplasms (NENs) supports an aggressive surgical approach. *Surgery* **149** 209–220. (<https://doi.org/10.1016/j.surg.2010.06.008>)
- Straub M, Drecoll E, Pfarr N, Weichert W, Langer R, Hapfelmeier A, Gotz C, Wolff KD, Kolk A & Specht K 2016 CD274/PD-L1 gene amplification and PD-L1 protein expression are common events in squamous cell carcinoma of the oral cavity. *Oncotarget* **7** 12024–12034. (<https://doi.org/10.18632/oncotarget.7593>)
- Sullivan RJ & Flaherty KT 2015 Immunotherapy: anti-PD-1 therapies—a new first-line option in advanced melanoma. *Nature Reviews Clinical Oncology* **12** 625–626. (<https://doi.org/10.1038/nrclinonc.2015.170>)
- Taube JM, Klein A, Brahmer JR, Xu H, Pan X, Kim JH, Chen L, Pardoll DM, Topalian SL & Anders RA 2014 Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. *Clinical Cancer Research* **20** 5064–5074. (<https://doi.org/10.1158/1078-0432.CCR-13-3271>)
- Teixido C, Karachaliou N, Gonzalez-Cao M, Morales-Espinosa D & Rosell R 2015 Assays for predicting and monitoring responses to lung cancer immunotherapy. *Cancer Biology and Medicine* **12** 87–95. (<https://doi.org/10.7497/j.issn.2095-3941.2015.0019>)
- Tsuruoka K, Horinouchi H, Goto Y, Kanda S, Fujiwara Y, Nokihara H, Yamamoto N, Asakura K, Nakagawa K, Sakurai H, *et al.* 2017 PD-L1 expression in neuroendocrine tumors of the lung. *Lung Cancer* **108** 115–120. (<https://doi.org/10.1016/j.lungcan.2017.03.006>)
- Vikman S, Sommaggio R, De La Torre M, Oberg K, Essand M, Giandomenico V, Loskog A & Totterman TH 2009 Midgut carcinoid patients display increased numbers of regulatory T cells in peripheral blood with infiltration into tumor tissue. *Acta Oncologica* **48** 391–400. (<https://doi.org/10.1080/02841860802438495>)
- Weber MM & Fottner C 2018 Immune checkpoint inhibitors in the treatment of patients with neuroendocrine neoplasia. *Oncology Research and Treatment* **41** 306–312. (<https://doi.org/10.1159/000488996>)
- Williams ED & Sandler M 1963 The classification of carcinoid tumors. *Lancet* **1** 238–239. ([https://doi.org/10.1016/S0140-6736\(63\)90951-6](https://doi.org/10.1016/S0140-6736(63)90951-6))
- Wimberly H, Brown JR, Schalper K, Haack H, Silver MR, Nixon C, Bossuyt V, Pusztai L, Lannin DR & Rimm DL 2015 PD-L1 expression correlates with tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy in breast cancer. *Cancer Immunology Research* **3** 326–332. (<https://doi.org/10.1158/2326-6066.CIR-14-0133>)
- Yan X, Jiao SC, Zhang GQ, Guan Y & Wang JL 2017 Tumor-associated immune factors are associated with recurrence and metastasis in non-small cell lung cancer. *Cancer Gene Therapy* **24** 57–63. (<https://doi.org/10.1038/cgt.2016.40>)
- Zitvogel L & Kroemer G 2012 Targeting PD-1/PD-L1 interactions for cancer immunotherapy. *Oncoimmunology* **1** 1223–1225. (<https://doi.org/10.4161/onci.21335>)

Received in final form 27 December 2018

Accepted 3 January 2019

Accepted Preprint published online 3 January 2019