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ORIGINAL ARTICLE

# Programmed cell death ligand-1 expression in gastroenteropancreatic neuroendocrine tumors

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## Summary

***Purpose:*** *Gastroenteropancreatic tumors (GEPNETs) is a heterogeneous disease with variable clinical course. While promising therapeutic options exist for other adult cancers, there are no new molecular-based treatments developed for GEPNETs. One of the main targets of cancer immunotherapy is the Programmed Cell Death Ligand-1 (PD-L1) pathway. Our purpose was to investigate the profile of PD-L1 expression in different organs of GEPNETs and compare the conventional immunohistochemistry (IHC) with the RNA expression analysis via real time polymerase chain reaction (RT-PCR) in order to determine which patients might be appropriate for immune check point-targeted therapy.*

***Methods:*** *A total of 59 surgically or endoscopically resected GEPNET tissues were retrospectively collected. The expression of PD-L1 and mRNA was evaluated with IHC.*

***Results:*** *The expression of PD-L1 was significantly associated with the high-grade classification (p=0.012). PD-L1 mRNA expression in tumor samples appeared to be higher compared to the corresponding normal tissues. In appendix, stomach and small intestine, the expression of PD-L1 mRNA was higher in the tumor tissues compared to the respective controls. In pancreas and colon, control tissues tend to have a higher PD-L1 mRNA expression compared to tumor tissues. PD-L1 mRNA expression was higher in GEP carcinomas (p=0.0031).*

***Conclusion:*** *RT-PCR was found to be more sensitive in detecting PD-L1 expression than conventional IHC. This study may provide an important starting point and useful background information for future research about immunotherapy for appendix, stomach and small intestine neuroendocrine carcinomas.*

***Key words:*** *PD-1, PD-L1, GEPNET, GEP carcinomas, RT=PCR, immunohistochemistry*

## Introduction

Neuroendocrine tumors (NETs) are rare neoplasms that originate from neuroendocrine cells and are distributed widely in the body. Approximately one-third of NETs arise in the lungs or thymus and two-thirds arise in the gastrointestinal tract [1]. Gastroenteropancreatic tumors (GEP-NETs) are less common than gastrointestinal adenocarcinomas and epidemiological studies report an estimated prevalence of 35/100.000 [1,2]. An analysis of SEER data revealed that the incidence of

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GEPNETs increased through the years, from 1975 to 2008, probably with the improved diagnostic techniques [3-5]. Female patients are more likely to have a primary GEPNET in the stomach, whereas male patients tend to have in the small intestine [1].

GEPNET is a heterogeneous disease with a variable clinical course, in which the spectrum may range from very indolent tumors to highly aggressive carcinomas. Based on the World Health Organization (WHO) 2010 classification, NETs are classified according to Ki67 value and mitotic count. The levels of these two parameters define the final grade of the tumor [6] (Table 1). The distinction of these tumor grades should be carefully identified by an expert pathologist in order to establish the optimal treatment approach. Besides tumor grading system for classification, the treatment relies on the tumor's functional status and primary origin [7]. NETs usually show slow proliferation. However, due to their relatively indolent nature, there is often a delay in their diagnosis. Because of failure at identifying their symptoms or establishing the biochemical diagnosis, NETs are mostly diagnosed at an advanced stage. These tumors represent a unique form of cancer that differs from other neoplasias in that they can synthesize and excrete various polypeptide hormones (e.g. chromogranin A (CgA), serotonin, gastrin), which cause specific clinical syndromes [8]. Chemotherapy has not demonstrated therapeutic activity in well-differentiated NETs. Somatostatin analogues (SSAs) have widely demonstrated significant improvements in symptoms and tumor growth by a complex mechanism of action over cancer cell survival, angiogenesis, and immunomodulation [9]. Additionally, chimeric molecules against somatostatin, dopamine receptors and targeted agents such as mTOR inhibitors and sunitinib were developed. These drugs primarily stabilize the disease rather than curing it [10]. On the other hand, initial response to chemoradiotherapy in neuroendocrine carcinomas is good but not durable and the disease often recurs after a few months [11].

While promising therapeutic options for survival were identified in other adult cancers, such as breast cancer, there have been no new molecular-based treatments developed for GEPNETs. Cancer immunotherapy is one of the most popular areas progressing day by day, producing significant medical discoveries with impressive results. Recently, the role of immunity has emerged with the demonstration of a favorable prognostic impact of the presence of tumor-infiltrating lymphocytes (TILs). The immune response is based on the balance between activator and inhibitor pathways that regulate TILs activity. This balance may be deteriorated in different conditions such as cancer. An increase in the immune system inhibition may lead to tumor progression [12,13]. One of the main targets of cancer immunotherapy is the PD-1 (Programmed cell death-1)-PD-L1 (Programmed cell death ligand-1) pathway. PD-1 is a cell surface membrane protein expressed by various immune cells including T-cells. It is an immune inhibitory receptor of the CD28 family, which plays a major role in escaping from the anti-tumor immune system. It is activated by its ligands PD-L1 and PD-L2, which are expressed by antigen-presenting cells such as macrophages or B-cells. They are also expressed by nonlymphoid tissues of different organs. After binding to its ligand, PD-1 attenuates lymphocyte activation and promotes T regulatory cell growth and function in order to end the immune response. PD-L1 is expressed on various tumor types to escape from the immune system. Blocking PD-1 on TILs or blocking PD-L1 on tumor cells results in reactivation of the tumor-specific T cells. Thus, direct tumor cell elimination is initiated and then immunostimulatory cytokines such as interferon gamma (IFN-g), interleukin-2 (IL-2), and tumor necrosis factor alpha (TNF-a) are released [14-16]. The inhibition of PD-1/PD-L1 binding was shown to exhibit strong and durable tumor regression in various solid tumors. Phase 3 trials have shown that monoclonal antibodies targeting PD-L1 or PD-1 are useful in treating solid cancers such as malignant melanoma, lung carcinoma, non-Hodgkin lymphoma, renal cell carcinoma, and triple negative breast cancer [17-25]. In clinical studies, PD-L1 expression in cancer was mostly studied at protein level using immunohistochemistry (IHC). However, there

**Table 1.** World Health Organization Classification of neuroendocrine tumours

| | *Neuroendocrine tumours* | | *Neuroendocrine carcinomas* |
|---|---|---|---|
| Grade | G1 | G2 | G3 |
| Ki67 index (%)[1] | ≤2 | 3-20 | >20 |
| Mitotic count[2] | <2 | 3-20 | >20 |

[1]MBI1 antibody; % of 2000 tumor cells in areas of highest nuclear labeling. [2]10 high power field=2mm$^2$, at least 40 fields (at 40x magnification) evaluated in the areas of highest mitotic density

are some limitations in PD-L1 IHC standardization that may end up with discordant results. Previous studies revealed that patients with IHC-positive tumors may not respond [26,27]. As a result, discordant results have been reported across the studies, particularly in prognostic ones. The main reasons for these differences are some limitations about standardization of the PD-L1 IHC. There are many PD-L1 antibodies that lack specificity and reproducibility [28,29]. The optimal positivity cut-off is not defined and the staining interpretation is influenced by subjectivity. These limitations have led to the use of alternative methods such as real time-polymerase chain reaction (RT-PCR) based on mRNA analysis. Many studies reported that there is a strong and positive association between protein and mRNA expression. Therefore, our analysis relied on mRNA expression measured by RT-PCR as well as IHC [29-31].

Because of the broad-acting role of IFNs in NETs, we aimed to investigate new immunological agents against inhibitory signals, such as PDL-1 and also analyze the profile of PD-L1 expression in different organs of GEPNETs, comparing the conventional IHC with the RNA expression analysis via RT-PCR in order to determine which patients might be appropriate for immune check point-targeted therapy [32].

## Methods

*Ethics*

This study was approved by the Manisa Celal Bayar University Hospital's Institutional Review Board.

*Patients and samples*

Patients with a histologic diagnosis of GEPNETs were collected retrospectively through the files of the Departments of Pathology from Izmir Tepecik Training and Research Hospital, Manisa Celal Bayar University Training and Research Hospital, Izmir Ege University Training and Research Hospital, and Aydin Ataturk State Hospital (Turkey) between March 2007 and February 2017. A total of 59 surgically or endoscopically resected GEPNET tissue blocks were available. The following clinicopathological characteristics were collected for all 59 patients: age, gender, primary site and tumor grade according to the 2010 WHO classification. Patients between 18 and 86 years were included in the study. This retrospective study was performed with the approval of the Ethics Committee of Celal Bayar University, in compliance with the ethical standards. Since the study was retrospective, no approval form was obtained from the patients. Briefly, formalin-fixed, paraffin-embedded tissue blocks from GEPNET were obtained. Tissue cylinders with a 0.6 mm diameter were punched from representative tissue areas of each donor tissue and brought on to a recipient paraffin block. Each tissue microarray (TMA) spot included at least 50% tumor cells.

**Table 2.** Correlations of PD-L1 expression with clinicopathological features

| *Patients characteristics* | *N (%)* | *PD-L1 expression positive IHC* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Tumor* | | *Stroma* | | *Total* | |
| | | N positive | p | N positive | p | N positive | p |
| Sex | | | 0.157 | | 0.1492 | | 0.652 |
| Male | 30 (50.8) | 2 | | 1 | | 3 | |
| Female | 29 (49.2) | 0 | | 4 | | 4 | |
| Total | 59 (100) | 2 | | 5 | | 7 | |
| Site | | | 0.611 | | 0.299 | | 0.542 |
| Stomach | 23 (39) | 1 | | 3 | | 4 | |
| Appendix | 17 (29) | 0 | | 1 | | 1 | |
| Small Intestine | 11 (18.6) | 0 | | 1 | | 1 | |
| Pancreas | 6 (10.1) | 1 | | 0 | | 1 | |
| Rectum | 2 (3.3) | 0 | | 0 | | 0 | |
| WHO 2010 | | | 0.019 | | 0.169 | | 0.012 |
| G1 | 27 (45.8) | 0 | | 1 | | 1 | |
| G2 | 20 (33.9) | 0 | | 2 | | 2 | |
| G3 | 12 (20.3) | 2 | | 2 | | 4 | |
| Primary site | | | 0.602 | | 0.652 | | 0.817 |
| Foregut | 30 (50.8) | 0 | | 3 | | 3 | |
| Midgut | 27 (45.8) | 2 | | 2 | | 4 | |
| Hindgut | 2 (3.4) | 0 | | 0 | | 0 | |

N: number of patients, PD-L1: Programmed death ligand-1,IHC: immunohistochemistry, WHO: World Health Organization

*Immunohistochemistry*

Immunohistochemical staining for the PD-L1 protein was performed using sections prepared from formalin-fixed diagnostic samples. In addition, serial 4 to 5 tissue sections were stored in special tubes for molecular tests. Briefly, 4-µm sections were deparaffinized in xylene and rehydrated through a graded ethanol series to distilled water. After processing via routine procedures, the sections were incubated at room temperature during an hour with rabbit anti-PD-L1 antibody (abcam, ab-205921-pd-l1, RabMabAB, clone 28-8) diluted 1:100 in blocking solution, followed by incubation with a horse radish peroxidase-conjugated secondary antibody (EnV FLEX HRP, DAKO) according to the manufacturer's instructions. After washing, color was developed by incubation in 3, 3'-diaminobenzidine tetrahydrochloride, followed by hematoxylin counter staining.

The staining was independently assessed by three pathologists using a semi-quantitative scale that ranged from 0 to 100% for the proportion of PD-L1-positive cancer and inflammatory cells.

*RNA isolation and generation of cDNA*

Total RNA was isolated from tumor-rich areas of formalin-fixed paraffin-embedded (FFPE) tissue blocks using the Invitrogen RNA FFPE Kit reagents following the manufacturer's standard protocols. RNA concentration and purity were determined via spectrophotometry. Around 1 µg of RNA was reverse transcribed using high capacity RNA to cDNA kit (Applied Biosystems) according to the manufacturer's instructions and used in RT-PCR reactions.

*RNA quantification via RT-PCR*

The cDNA was subjected to quantitative PCR analysis with PD-L1-specific primers (Taqman Gene Expression Assay from Applied Biosystems) and quantifications were performed according to the manufacturers' instructions (Applied Biosystems). Relative abundance of mRNA was obtained by normalization to actin mRNA levels.

*Statistics*

All the statistical analyses for the quantitative RT-PCR were performed using statistical software. Namely, unpaired t-test with Welch's correction for column analysis, Fisher's exact test for contingency test, 1-way ANOVA test and the graphs regarding the PDL-1 mRNA expression were performed using Prism 5 software (GraphPad Software, Inc., San Diego, USA). Significant differences are shown by asterisks indicating *p<0.05, **p<0.005, ***p<0.0005. Error bars in Figures represent standard error of the mean (SEM).

## Results

*Patient characteristics*

Fifty nine patients, who were pathologically diagnosed with GEPNET in Tepecik Training and Research Hospital, Ege University Training and Research Hospital, Celal Bayar University Training and Research Hospital and Aydin Ataturk Government Hospital between March 2007 and February 2017, were analyzed for PD-L1 expression in this study. The characteristics of the patients are summarized in Table 2. Thirty (50.8%) patients were male with median age 54 years. According to WHO classification, 27 patients had grade 1 and 20 patients had grade 2 NET. Twelve patients had grade 3 neuroendocrine carcinoma (NEC). The most fre-

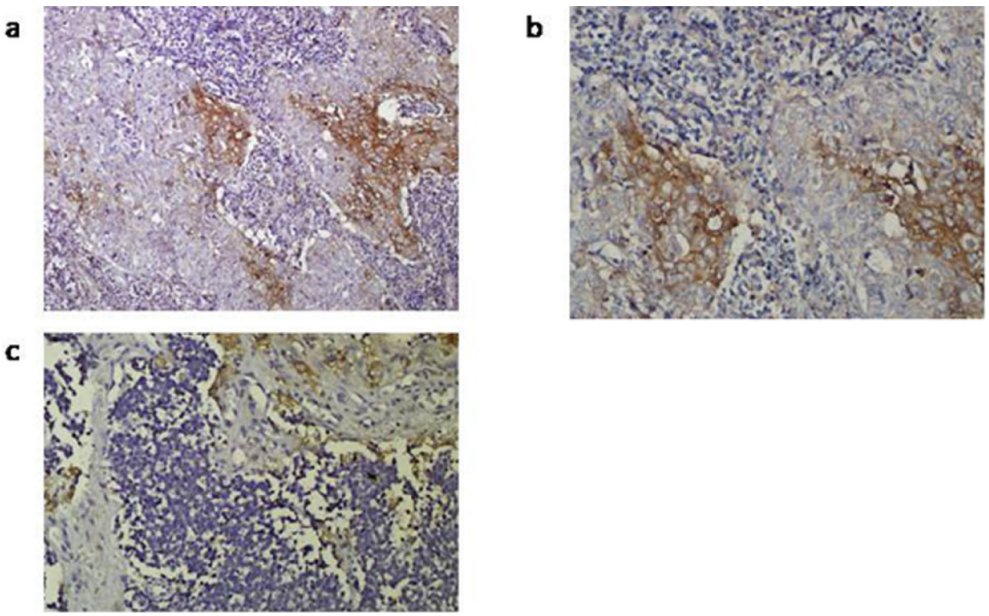


**Figure 1.** Immunohistochemical staining for programmed death-1 ligand-1 (PD-L1) in GEP-NETs. **a, b:** Representative immunohistochemical staining showing positive cells in tumor stroma. **c:** Respresentative immunohistochemical showing PD-L1 positive cells in adjacent stroma.

quent anatomic locations were stomach (39%) and appendix (29%). Primary sites included 30 foregut-derived, 27 midgut-derived, and 2 hindgut-derived GEPNETs.

*Correlation between PD-L1 expression and clinicopathological variables*

Two of the tested GEPNET samples were stained positive for PD-L1 in tumor tissues and 5 of the cases showed PD-L1 positive cells in the adjacent stroma (Figure 1). The expression of PD-L1 was significantly associated with high-grade WHO classification (p=0.012) but not with gender, primary site, or origin (Table 2).

*PD-L1 mRNA expression in GEPNET*

In order to determine the PD-L1 mRNA expression in different tumors and control tissues, we prepared total cDNA from paraffin embedded tissue samples. mRNA expression of PD-L1 gene was determined by quantitative RT-PCR and the expression levels were normalized to endogenous mRNA levels of the actin gene. The expression of PDL-1 mRNA in different control and tumor tissues are shown in Figure 2. Mean and standard error of the mean (SEM) of each group was indicated by long horizontal bar and black vertical bars, respectively. Average PD-L1 mRNA expression in tumor samples appeared to be higher compared to that in the corresponding normal tissues. However, it is apparent from the graph that PD-L1 expression demonstrated high level of variation among different tissue samples, ranging from very low expression levels to very high.

In appendix, stomach and small intestine, the expression of PD-L1 mRNA was higher in the tumor tissues compared to that in the respective controls. In pancreas and colon, control tissues tend to have a higher PD-L1 mRNA expression compared to tumor tissues. However, the sample size for pancreas and colon tissues was not large enough to perform statistical analysis. Therefore, we only included appendix, stomach and small intestine in our analysis.

We combined the results from three different tumor tissues (appendix, stomach, small intestine) in order to determine whether the relatively higher PD-L1 expression in tumor tissues compared to that in normal tissues was statistically significant (Figure 3a). Mean PD-L1 expression in the combination of all three groups was 1464-fold of actin mRNA in tumor tissues (n=24) vs. 8.59-fold of actin mRNA in normal tissues (n=13). The difference, however, was not statistically significant according to the unpaired t-test with Welch's correction (p=0.104) (Figure 3a). It is possible that very high variation of PD-L1 expression and high SEM (SEM=1123 in tumor samples) were the reasons for the high p values. Fisher's exact test, nevertheless, confirmed that the frequency of tissues with high PD-L1 expression was significantly higher in tumor tissues (19 of 24 samples) than that in the

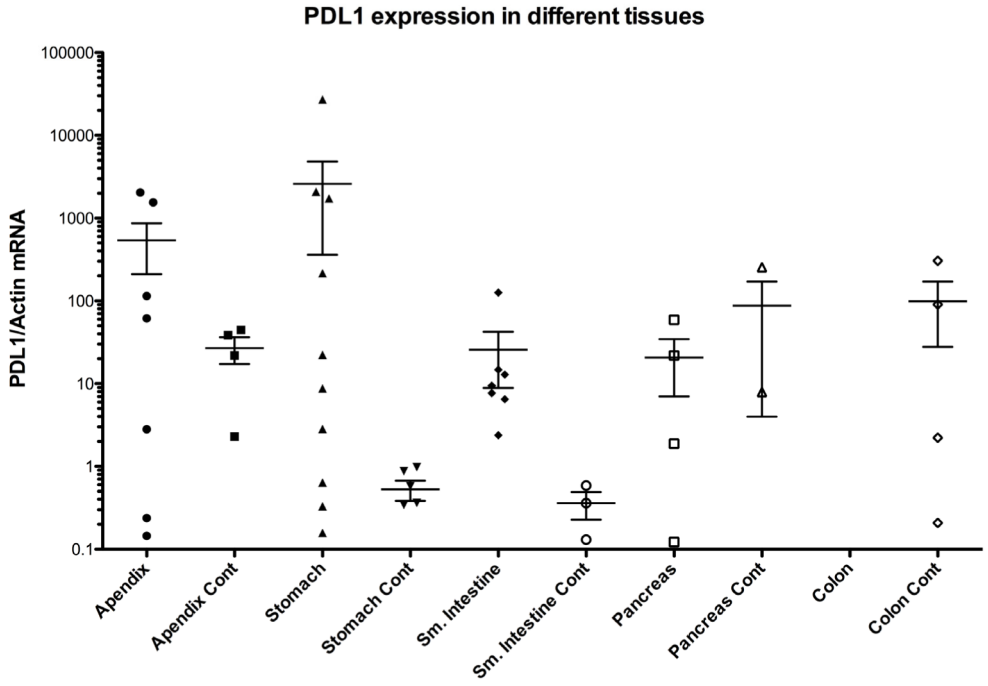


**Figure 2.** The Figure shows programmed death-1 ligand-1 (PD-L1) mRNA expression in tumor vs. control tissues (p>0.05).

normal tissue samples (4 out of 9 samples), with p=0.0083 (Figure 3b). Odds ratio of the association was 8.55 (95% confidence interval 1.841 to 39.72). Interestingly, all the four normal tissue samples with high PD-L1 expression were from appendiceal tissue, suggesting that basal expression levels of PD-L1 mRNA may be higher in appendiceal tissue.

Next, we compared the average PD-L1 expression in appendiceal tumors relative to normal appendiceal tissue samples using unpaired t-test with Welch's correction (Figure 4a). Although the mean relative expression levels were higher in tumor tissues compared to those in controls (540.7-fold in tumors vs. 26.8-fold in controls), the difference was

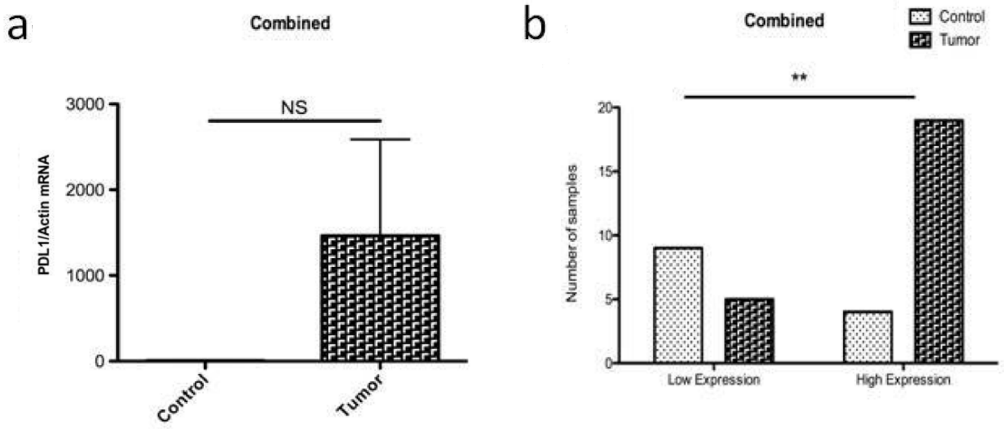


**Figure 3.** Programmed death-1 ligand-1 (PD-L1) mRNA expression in appendix, stomach and small intestine tumor vs. control tissues. **a:** Unpaired t-test with Welch's correction. NS: not statistically significant (p=0.104). **b:** Fisher's exact test, **p<0.005.

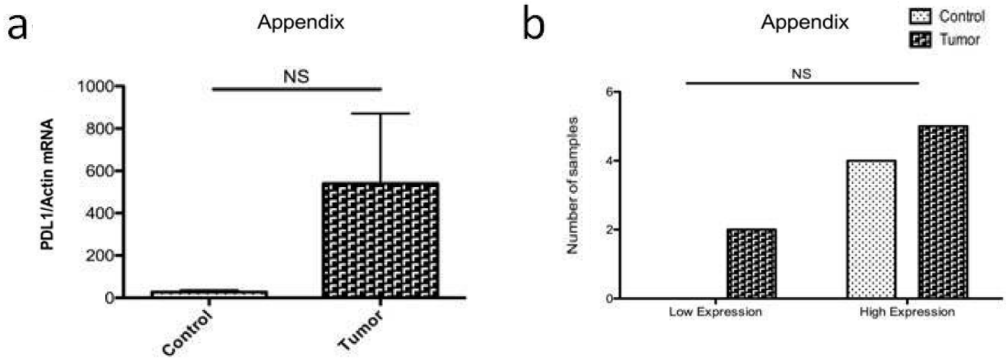


**Figure 4.** Programmed death-1 ligand-1 (PD-L1) mRNA expression in appendix tumor vs. control tissues. **a:** Unpaired t-test with Welch's correction. NS: not statistically significant (p=0.0857). **b:** Fisher's exact test. NS: not statistically significant (p=0.3818).

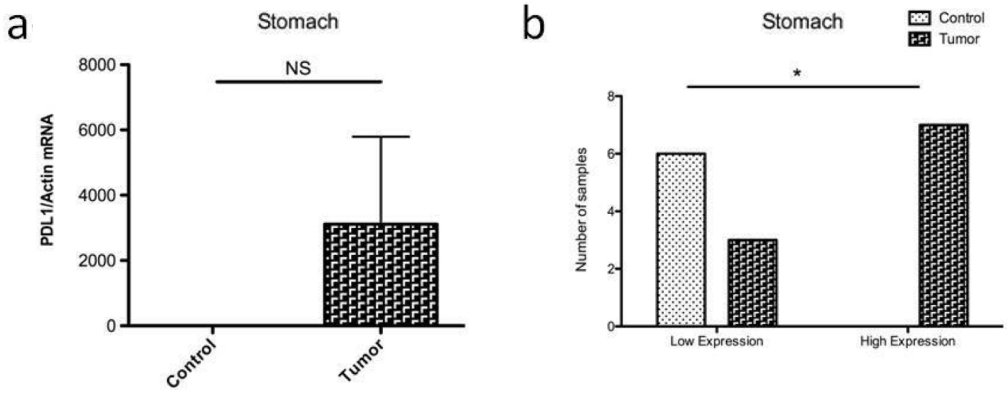


**Figure 5.** Programmed death-1 ligand-1 (PD-L1) mRNA expression in stomach tumor vs. control tissues. **a:** Unpaired t-test with Welch's correction. NS: not statistically significant (p=0.1371). **b:** Fisher's exact test, *p<0.05.

not statistically significant (p=0.0857) due to the small sample size (n=7 for tumors vs. n=4 for controls) or high variation of the PD-L1 expression. In order to determine whether the number of samples that showed very high PD-L1 expression was associated with the tumor phenotype, we performed contingency test (Fisher's exact test). To perform this analysis, we decided to use actin expression as an indicator of the strength of PD-L1 expression. Namely, samples with PDL-1 expression, which is equal to and lower than endogenous actin mRNA, were defined as "Low expression." Samples with significantly higher PD-L1 expression compared to actin mRNA were defined as "High expression" (Figure 4b). According to this analysis, the number of samples with higher PD-L1 expression was not significantly associated with any of the phenotypes (p=0.3818).

Stomach tissue samples displayed similar characteristics to appendix samples. Mean PD-L1 expression was again higher in tumor tissues compared to that in the normal tissues (3116-fold in tumors vs. 0.5299-fold in normal tissues). Nevertheless, this difference was not statistically sig-

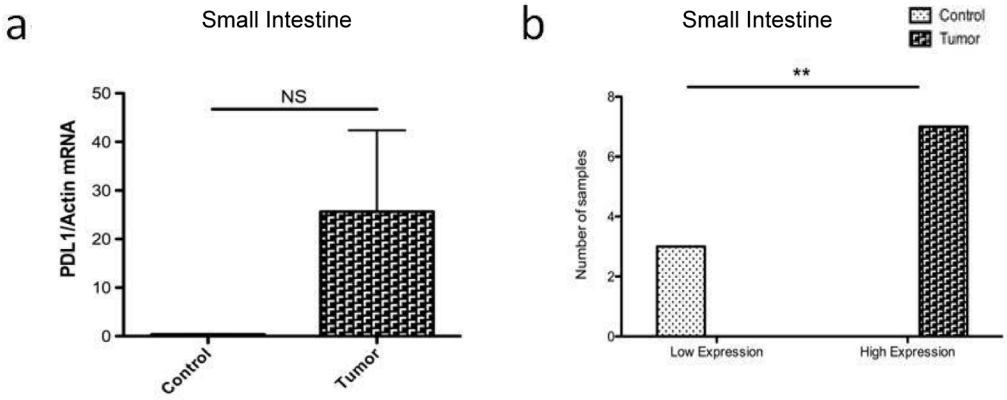


**Figure 6.** Programmed death-1 ligand-1 (PD-L1) mRNA expression in small intestine tumor vs. control tissues. **a:** Unpaired t-test with Welch's correction. NS: not statistically significant (p=0.091). **b:** Fisher's exact test, **p<0.05.

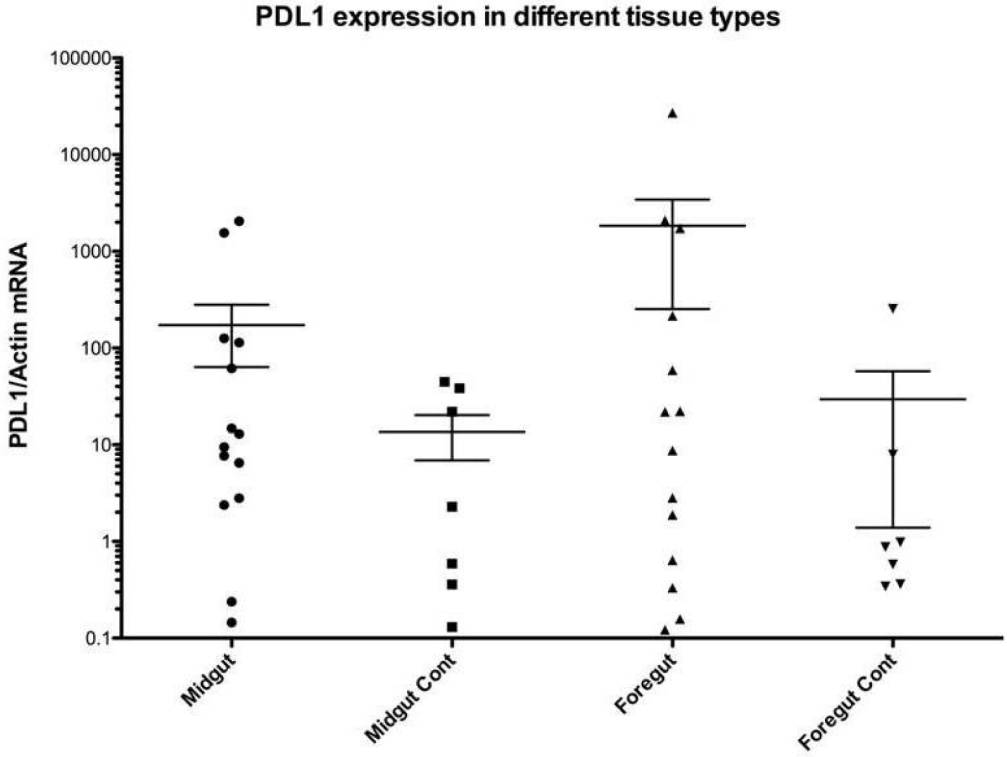


**Figure 7.** The Figure shows programmed death-1 ligand-1 (PD-L1) mRNA expression in foregut and midgut tumors and control tissues. Cont: control tissues, mRNA: messenger RNA. ■Data points midgut tumor tissues. ●Data points midgut control tissues. ▲Data points foregut tumor samples. ▼Data points foregut control tissues.

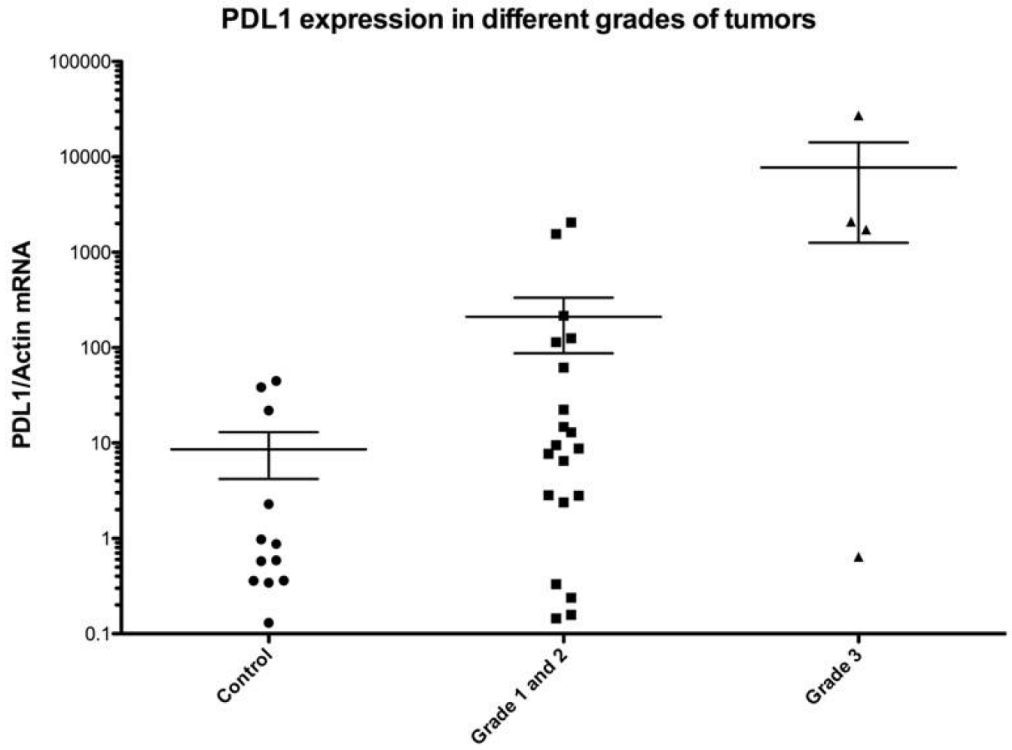


**Figure 8.** Programmed death-1 ligand-1 (PD-L1) mRNA expression in different grades of tumors and their comparisons with GEPNETs and carcinomas. One-way ANOVA test, p= 0.0031. P value implies that the 3 groups are significantly different from each other. Post test for linear trend also showed a significant positive association with a slope of 3860 (R2= 0.2551 and p=0.0013). ●Data points control group samples. ■Data points grade 1 and 2 samples. ▲Data points grade 3 samples.

nificant according to unpaired t-test with Welch's correction (p=0.1371, n=10 for tumors vs n=6 for controls) (Figure 5a). However, contingency test (Fisher's exact test) revealed that the frequency of tissues with high PD-L1 expression was significantly higher in tumors (7 out of 10 samples) than that in the normal tissue samples (0 out of 6 samples), with a p value lower than 0.05 (p=0.0105) (Figure 5b). Strength of the association was also apparent from the odds ratio of 27.86 (95% confidence interval 1.2 to 646.6).

Finally, small intestine tissue samples were consistent with the stomach tissue samples. Mean PD-L1 expression levels were higher in tumor tissues compared to those in normal tissues (25.64-fold in tumors vs. 0.3596-fold in normal tissues). The difference was not statistically significant according to the unpaired t-test with Welch's correction (p=0.091, n=7 for tumors vs n=3 for controls) (Figure 6a). Fisher's exact test again revealed that the frequency of tissues with high PD-L1 expression was significantly higher in tumors (all 7 samples) than that in the normal tissue samples (0 out of 3 samples) with p=0.0083 (Figure 6b). Odds ratio of the association was 105.0 (95% confidence interval 1.704 to 6471).

We classified the tumors according to the anatomic site as foregut (gastric, pancreas) and midgut (appendix, small intestine) and then compared the PD-L1 mRNA expression (Figure 7). Herein, both midgut and foregut tumors tend to express higher levels of PD-L1 mRNA compared to their respective control tissues. However, these differences were not statistically significant according to the unpaired t-test with Welch's correction for column analysis.

Finally, we decided to analyze the association of average PD-L1 mRNA expression with the progression of the tumorigenicity. Ki67 expression was positively correlated with the stage of cancer. NETs in our study were classified as Grade 1, 2, and 3 according to their Ki67 expression levels. In order to find an association, we categorized the tumor samples in two groups (Group of Grade 1 and 2 tumors together and (neuroendocrine tumors), Grade 3 tumors (neuroendocrine carcinomas alone). We then plotted the relative PD-L1 expression on graph together with the normal tissues (Figure 8). Mean and SEM of each group was indicated by long horizontal bar and vertical black bars, respectively. To find an association of PDL-1 expression with neuroendocrine tumors, neuroendocrine carcinomas

and control tissues we performed 1-way ANOVA test which revealed a significant association of PD-L1 expression in Grade 3 tumors compared to grade 1-2 tumors or control tissues (p=0.0031). We found that PD-L1 expression tends to be significantly higher with the progression of cancer. Post-test for linear trend also showed a significant positive association with a slope of 3860 (R2=0.2551 and p=0.0013).

## Discussion

PD-L1 expression in 59 GEPNET tissues was analyzed with two different techniques: IHC and the RT-PCR. The results showed a general increase in PD-L1 mRNA expression in GEPNET compared to the control tissues in our study (Figure 2 and 3). Our analysis showed that PD-L1 mRNA expression was more effectively detected using RT-PCR technique compared to IHC. To our knowledge, this is the first study analyzing PD-L1 expression profile in different organs of GEPNETs by applying IHC and RT-PCR methods together.

Two of the tested GEPNET samples were stained positive for PD-L1 in tumor tissues and 5 of the samples showed PD-L1 positive cells in the adjacent stroma. These IHC-stained samples showed that the PD-L1 expression was significantly associated only with grade 3 (determined by WHO classification) GEPNETs (p=0.012). The majority of the immunohistochemistry staining results turned out to be negative. The transcript levels of PD-L1 were not abundant; however, most of the tissues displayed mRNA expression. Similarly, previous studies with small cell carcinoma, non-small cell lung cancer and gastrointestinal stromal tumor samples also displayed negative immunohistochemistry. On the other hand, RNA-sequencing data showed mRNA expression, although not abundant [11,19,30].

GEPNETS results indicated that appendix, gastric and small intestine tumors expressed higher levels of PD-L1 mRNA compared to their respective control tissues (Figures 3,4,5,6). In pancreas and colon, control tissues tended to have a higher PD-L1 mRNA expression compared to tumor tissues (Figure 2). However, the sample size for the pancreas and colon tissues was not large enough to perform a statistical analysis. Therefore, we only included appendix, stomach and small intestine in our analysis.

PD-L1 expression is associated with the poor prognosis of breast cancer [12,13], non-small cell lung cancer [19,20], gastric cancer [34,35], and also for some other type of cancers. Tamura et al. found that high expression of PD-L1 was observed in 29.6% gastric carcinoma patients. In their analysis, PD-L1 expression was associated with worse overall survival and PD-L1 was an independent prognostic factor for patients with stage II/III gastric cancer [34]. In a meta-analysis of gastric cancers enrolling 10 studies, PD-L1 positive patients had significantly worse survival than PD-L1 negative patients [36]. In contrast, univariate and multivariate results indicated a significant and moderate correlation between high expression of PD-L1 and good prognosis in colon adenocarcinomas. Strong PD-L1 expression in mismatch repair (MMR) proficient colorectal cancer was found to be associated with a significantly improved 5-year survival. However, overexpression of PD-L1 in tumor cells was not associated with an improved survival in MMR deficient colorectal cancer [37]. Additionally, in a study of pancreatic adenocarcinoma, it was found that 81% of tumors did not show PD-L1 mRNA upregulation. PD-L1 overexpression was associated with shorter disease-free survival and overall survival in multivariate analyses in pancreatic adenocarcinoma [38]. Therefore, our results are consistent with these previous findings reporting that the tumor tissues of appendix, stomach and small intestine tend to display higher levels of PD-L1 mRNA compared to the respective control tissues (Figures 3,4,5,6).

We showed that midgut and foregut tumors express higher levels of PD-L1 mRNA compared to respective controls, although not statistically significant (Figure 7). A recent study by Kim et al. in 2016 compared foregut and hindgut GEPNETs and the expression of PD-L1 was not dound to be significantly associated with the primary site [40]. Some studies demonstrated that midgut carcinoids seem to be more sensitive to immunotherapies [40,41].

We think that the causes of higher PD-L1 mRNA expression in the control tissues of pancreas and colon (hindgut tumors) may be related to the immune microenvironment. In tumor immune microenvironment, TILs have been shown to inhibit tumor growth in a variety of solid tumors and a high frequency of TILs is associated with improved prognosis [42]. Tumor immune microenvironment varies according to the tumor type [43]. Increased levels of CD3+, CD4+ and CD8+ TILs and CD20+ B cells were associated with better outcome, but regulatory T-cells or myeloid-derived suppressor cells play a significant role in suppressing antitumor response and affect prognosis [43,44]. In 2016 Birnbaum et al. analyzed 2,405 genes in pancreas cancer tissues and found that PD-L1 expression and the probability of activation of immune-related pathways (IFNα, IFNγ) were lower in pancreatic

carcinomas than in those with breast cancers and GISTs. FOXP3, which is the transcription factor for Tregs, and its effective cytokine IL10, were also upregulated in the PD-L1 positive pancreatic cancers. Additionally, in this study, many genes that are related to antigen processing and presentation of exogenous peptide antigen via MHC class-I, were not present in the pancreatic signature [38]. In 2013 Raoul et al. found that PD-L1 expression in infiltrating CD8+ lymphocytes is extremely limited in both colorectal cancers and normal colon mucosa [37]. In addition, another study showed that FOXP3 positive Tregs cells were more common in tumor tissues compared to normal colon mucosa tissues [45]. As a result, our finding that the PD-L1 mRNA expression is higher in pancreas and colon control tissues could be due to the immune microenvironment. In these organs, immune system inhibition is more pronounced than in other organs. Their molecular profiles of the immune microenvironment, the composition of TILs, and the immune-related pathways may be different from the other organs. Therefore, this hypothesis may be investigated in future studies for novel immunotherapies.

Comparison of GEPNETs (Grade 1+2) and neuroendocrine carcinomas (Grade 3) showed that PD-L1 mRNA expression in neuroendocrine carcinomas was significantly higher than that in NETs (Figure 8). In a recent study, 32 patients with metastatic GEPNET were analyzed via IHC. This study revealed that the expression of PD-L1 was associated with higher tumor grade (grade 3) in metastatic GEPNETs. PD-L1 expression had both predictive and prognostic value for survival of patients with metastatic GEPNETs [39]. In the aforementioned study, PD-L1 expression was not accurately compared to different locations of primary GEPNETs due to the few numbers of the tissues. IHC was the only method used and the organs expressing high levels of PD-L1 could not be detected [39].

IFN, which is used in immunotherapy, affects immunomodulation by activating T lymphocytes. Due to the broad effective roles of IFN in NETs, it is used in the treatment of these tumors. Since neuroendocrine carcinomas are more aggressive compared to NETs, they are known to be more immunogenic and sensitive to interferon therapy [41]. Therefore, PD-L1 expression is expected to be higher in Grade 3 tumors, as we observed in our study. Based on these findings, gastroenteropancreatic neuroendocrine carcinomas may also respond better to targeted therapy.

In conclusion, this study showed that PD-L1 mRNA expression is heterogeneous in GEPNETs and associated with higher tumor grade. PD-L1 mRNA expression in GEP neuroendocrine carcinomas is significantly higher when both IHC and RT-PCR were used. RT-PCR was found to be more sensitive in detecting PD-L1 expression than conventional IHC. We analyzed the expression profile of PD-L1 in different organs of GEPNETs. Appendix, stomach and small intestine GEPNETs express higher levels of PD-L1 mRNA compared to control tissues. This study may provide a good starting point and valuable background for future research about immunotherapy for appendix, stomach and small intestine neuroendocrine carcinomas.

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## Conflict of interests

The authors declare no conflict of interests.

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