High Mast Cell Density Predicts a Favorable Prognosis in Patients with Pancreatic Neuroendocrine Neoplasms

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Keywords

Mast cell · Pancreatic neuroendocrine neoplasm · Tumor-infiltrating immune cells · Tumor microenvironment · Prognosis

Abstract

Introduction: Mast cells are involved in allergic diseases, immune regulation, and tumor microenvironment modulation, with both pro- and anti-tumorigenic functions, and could serve as a prognostic factor in various cancers. However, their potential role in pancreatic neuroendocrine neoplasms (PanNENs) is largely unknown. Here, our aim was to investigate the presence of mast cells in PanNENs and evaluate their association with clinicopathological parameters and other common tumor-infiltrating immune cells. **Methods:** Tissue microarrays containing PanNEN samples from 187 patients were constructed and stained immunohistochemically for CD117, CD15, CD68, CD3, CD4, and CD8. Immune cells were counted from four high-power fields (HPFs; $\times 400$) at maximal concentrations, and the mean counts were calculated per HPF. The cutoff values were set by X-tile. **Results:** The median (interquartile range) counts of CD117⁺ mast cells, CD15⁺ neutrophils, CD68⁺ macrophages, CD3⁺ T

cells, and CD4⁺ T cells were 3.5 (2.0–6.0), 3.0 (1.3–6), 3.8 (2.5–5.8), 13 (8.0–24.0), and 2.0 (1.0–4.0)/HPF, respectively. CD8⁺ T cells were not detected. The cutoff values for these immune cells were 1.5/HPF, 6/HPF, 4.8/HPF, 32.5/HPF, and 2/HPF, respectively. Low mast cell density was correlated with higher grades, noninsulinoma, and advanced stages. Moreover, high mast cell infiltration was associated with elevated CD4⁺ T cell and CD15⁺ neutrophil counts. Multivariate analysis revealed that high mast cell density was an independent predictor of prolonged progression-free survival in the entire cohort; in pancreatic neuroendocrine tumors; and in intermediate-grade, noninsulinoma, and advanced stage subgroups. **Conclusions:** These findings suggest a protective role of mast cells in PanNENs.

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Introduction

Pancreatic neuroendocrine neoplasms (PanNENs) are a group of heterogeneous neoplasms arising from neuroendocrine cells of the endocrine pancreas or from precursor cells of the exocrine pancreas [1]. PanNEN is a rare type of neoplasm, the incidence of which was only 0.48

per 100,000 individuals between 2000 and 2012 in the USA. However, an increasing number of patients have been diagnosed with PanNEN over the past 4 decades [2]. The current tumor grading system, as proposed by the World Health Organization (WHO), classifies low-grade pancreatic neuroendocrine tumors (PanNETs) as neoplasms with a Ki-67 index less than 3% or a mitotic rate less than 2 mitoses/2 mm², intermediate-grade PanNETs as neoplasms with Ki-67 indices between 3% and 20% or mitotic rates between 2 and 20 mitoses/2 mm², and high-grade PanNENs, including well-differentiated high-grade PanNETs and poorly differentiated pancreatic neuroendocrine carcinomas (PanNECs), as neoplasms with Ki-67 indices greater than 20% or mitotic rates greater than 20 mitoses/2 mm² [3]. Even though the WHO grading system is a practicable risk stratification method, the clinical courses of patients with PanNEN are difficult to predict accurately [4, 5]. Therefore, there remains an urgent need to identify novel prognostic indicators for such patients.

Recent advances in immunotherapy have ignited interest in the exploration of the tumor microenvironment-associated immune landscape [6–8]. In terms of PanNEN, the number of investigations on tumor-infiltrating immune cells is increasing but remain limited; such studies have explored dendritic cells, neutrophils, macrophages, natural killer cells, CD3⁺ T cells, CD45RO⁺ memory T cells, Foxp3⁺ regulatory T cells, CD8⁺ cytotoxic T cells, CD4⁺ helper T cells, and CD20⁺ B cells [9–13]. Among these immune cells, only macrophages and neutrophils are potential prognostic biomarkers, although the data remain inconsistent among studies [11–13]. Moreover, the correlation between blood- and tumor-infiltrating immune cells remains unclear [13].

PanNETs associated with hormone oversecretion syndromes (e.g., hyperinsulinemia and Zollinger-Ellison syndrome) are diagnosed as functioning PanNETs, whereas those without any of these clinical syndromes are diagnosed as nonfunctioning PanNETs [14]. Meanwhile, the genetic characteristics and prognoses of insulinomas are distinct from those of nonfunctioning PanNETs and other functioning PanNETs [15]. Tumor-infiltrating immune cells are the basis of immunotherapy [16]. Sufficient targeting immune cells are essential for the administration of immunotherapy. For example, a high tumor-infiltrating lymphocyte density was confirmed to be associated with a higher efficacy of PD-1-/PD-L1-based immunotherapy, which reactivates T-cell-based antitumor immunity [17]. Moreover, ongoing immunotherapeutic strategies targeting chemokine (C-C motif) receptor 2 and colony-stimulating factor 1 receptor [18, 19]

also require sufficient tumor-associated macrophages. However, it is largely unknown whether there are differences in terms of tumor-infiltrating immune cells in functioning PanNETs versus nonfunctioning PanNETs, as well as in insulinomas versus noninsulinoma, and the prognostic values of immune cells in these subgroups have not yet been demonstrated. Therefore, acquiring a better understanding of the immune infiltrate profiles of these subgroups might help to develop novel and personalized immune cell-targeted therapeutic strategies in a cost-effective manner based on the functionality of PanNETs.

Mast cells, which originate from the bone marrow, are innate immune cells that have a variety of roles in anaphylactic diseases [20]; they also regulate the adaptive immune response [21] and modulate tumorigenesis [22]. In terms of the latter, mast cells have been reported to be either pro-tumorigenic (e.g., in thyroid cancer and primary cutaneous lymphoma) [23, 24] or anti-tumorigenic (e.g., in breast and prostate cancers) [25–27]. Soucek et al. [28, 29] demonstrated the importance of mast cells in the development and maintenance of pancreatic islet tumors in a transgenic mouse model. However, the prognostic significance of mast cells in patients with PanNEN has not been explored.

To increase our understanding of tumor-infiltrating immune cells in PanNENs, we performed immunohistochemical staining of CD117⁺ mast cells and CD68⁺ macrophages; CD15⁺ neutrophils; and CD3⁺, CD4⁺, and CD8⁺ T cells to investigate the associations between these common immune cells and clinicopathological parameters, as well as blood immune cells, and to determine their prognostic importance. Our study aimed to determine whether mast cells have prognostic value in patients with PanNENs.

Materials and Methods

Patients and Specimens

One hundred and eighty-seven patients with primary PanNENs (PanNETs or PanNECs) who underwent surgical resection between 2004 and 2019 at Peking Union Medical College Hospital (PUMCH) with available samples were included consecutively in this study. The clinicopathological characteristics of the 187 patients are summarized in Table 1. All patients' histopathological slides were retrieved, scrutinized, and recategorized histologically by a pathologist (S.N.Y.) if any discordance with the WHO classification was observed [3]. After careful review of hematoxylin-eosin slides, representative areas of the neoplasm tissues were marked, and the corresponding formalin-fixed paraffin-embedded blocks were sampled for tissue microarray (TMA) construc-

tion. The diameter of each sample core was 2 mm, as previously described by Zong et al. [30]. To further investigate the distribution of immune cells in PanNENs and the normal pancreas, accessible normal pancreatic tissues from 23 of the 187 patients were also sampled during the TMA construction process. Clinicopathological data such as age, sex, time of surgery, surgical procedure, preoperative and/or postoperative treatments, primary tumor location, tumor size, functional status, number of examined lymph nodes, number of positive lymph nodes, metastasis status, proliferation activity (Ki-67 index and mitotic rate), WHO grade, lymphovascular invasion (LVI), and perineural invasion (PNI) were documented. Routine blood test results (including counts of white blood cells, lymphocytes, monocytes, neutrophils, eosinophils, basophils, and platelets) were obtained within 7 days before surgery. The American Joint Committee on Cancer (AJCC) 8th edition stages were classified according to the data we collected. Survival information was retrospectively extracted from the electronic medical record system and confirmed by telephone interviews. Noninsulinoma was defined as PanNETs excluding insulinoma (including nonfunctioning PanNETs and other functioning PanNETs such as gastrinoma, vasoactive intestinal polypeptide-secreting tumor, and glucagonoma). Our current study was approved by the Institutional Review Board of PUMCH (approval no. S-K1593) and complied with the Helsinki Declaration. The PUMCH Ethics Committee approved this retrospective study and formally waived the requirement for written consent.

Immunohistochemistry

The immunohistochemical methods used in this study were described previously [31, 32]. Briefly, immunostaining of CD3, CD4, CD8, CD15, CD68, and CD117 was performed on serial TMA slices (4 µm). Details of the antibodies are shown in online supplementary Table S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000521651). Tonsillar tissues treated with primary antibodies were used as positive controls; normal pancreatic tissues were used as internal controls, whereas tissues not treated with isotype-matched antibodies were used as negative controls. All slides were stained automatically using an immunostaining instrument (BOND-III; Leica Biosystems), as per the manufacturer's instructions.

Immunohistochemical Evaluation

The aforementioned immune markers were scored only when there were sufficient neoplasm tissues, and they were also assessed in normal pancreatic tissues using the same method. In brief, two investigators (S.W.M. and X.L.C.) who were blinded to the patients' clinicopathological characteristics and outcomes (death or progression) evaluated all slides. Any discrepancy was resolved by consensus between these two investigators.

Immune cells were counted manually from four high-power fields (HPFs) of a maximal density at ×400 magnification, and the mean counts of the corresponding cells per HPF were calculated for each sample; this was similar to the method used by Cai et al. [12]. The cutoff values for CD3⁺, CD4⁺, and CD8⁺ T cells; CD15⁺ neutrophils; CD68⁺ macrophages; and CD117⁺ mast cells were determined using X-tile (Yale University, New Haven, CT, USA). Tumor-infiltrating CD3⁺, CD4⁺, CD15⁺ neutrophils; CD68⁺ macrophages; and CD117⁺ mast cells were considered high when the values were equal to or greater than 32.5/HPF, 2/HPF, 6/HPF, 4.8/HPF, and 1.5/HPF, respectively.

Table 1. Clinicopathological characteristics of patients with Pan-NENs (N = 187)

Variables	Median (IQR) or N (%)
Mean age (IQR), years	50 (41–59)
Sex, n (%)	
Female	92 (49)
Male	95 (51)
Functional, n (%) (N = 179)	
No	87 (49)
Yes ^a	92 (51)
Functional, n (%) (N = 179)	
Insulinoma	77 (43)
Noninsulinoma	102 (57)
Primary pancreatic sites, n (%)	
Head/neck	70 (36)
Body/tail	115 (62)
Multiple sites	2 (2)
Surgical procedure, n (%)	
Distal pancreatectomy	89 (48)
Pancreaticoduodenectomy	30 (16)
Enucleation	53 (28)
Segmental pancreatectomy	8 (4)
Subtotal/total pancreatectomy	5 (3)
Unknown	2 (1)
Synchronous metastasis, n (%)	
No	138 (74)
Yes	49 (26)
LVI, n (%)	
Absent	171 (91.4)
Present	16 (8.6)
PNI, n (%)	
Absent	175 (93.6)
Present	12 (6.4)
WHO grade, n (%)	
Low	79 (42.3)
Intermediate	98 (52.4)
High ^b	10 (5.3)
AJCC stage, n (%)	
I	58 (31)
II	81 (43)
III	20 (11)
IV	28 (15)
Median tumor size (IQR), cm	2.5 (1.5–4)
Median Ki-67 index (IQR), %	3 (1–5)
Median mitotic rate (IQR), /10 HPF	1 (1–2)
Median number of positive nodes (IQR)	3 (2–5.25)
Median number of resected nodes (IQR)	2 (0–8)
Median number of WBCs (IQR), ×10 ⁹ /L	5.47 (4.48–7.14)
Median number of lymphocytes (IQR), ×10 ⁹ /L	1.67 (1.33–2.02)
Median number of monocytes (IQR), ×10 ⁹ /L	0.33 (0.25–0.40)
Median number of neutrophils (IQR), ×10 ⁹ /L	3.24 (2.30–4.22)
Median number of eosinophils (IQR), ×10 ⁹ /L	0.11 (0.07–0.17)
Median number of basophils (IQR), ×10 ⁹ /L	0.02 (0.02–0.03)
Median number of platelets (IQR), ×10 ⁹ /L	220 (180.50–256.75)

^aIncluding 77 insulinomas, 7 vasoactive intestinal polypeptide-secreting tumors, 3 gastrinomas, 3 glucagonomas, and 2 adrenocorticotrophic-hormone-producing tumors. ^bIncluding 2 neuroendocrine grade 3 tumors and 8 neuroendocrine carcinomas.

Fig. 1. Tumor-infiltrating innate immune cells in resected primary PanNEN formalin-fixed and paraffin-embedded samples using immunohistochemically stained TMAs. High (≥ 1.5 /HPF) CD117⁺ mast cell density in a low-grade nonfunctioning PanNET sample (a); low (< 1.5 /HPF) CD117⁺ mast cell density in a high-grade insulinoma sample (b); (c) high (≥ 6 /HPF) CD15⁺ neutrophil density in

an intermediate-grade insulinoma sample (c); low (< 6 /HPF) CD15⁺ neutrophil density in an intermediate-grade insulinoma sample (d); high (≥ 4.8 /HPF) CD68⁺ macrophage density in a high-grade insulinoma sample (e); and low (< 4.8 /HPF) CD68⁺ macrophage density in a low-grade vasoactive intestinal polypeptide-secreting tumor sample (original magnifications, $\times 400$; scale bar, 50 μ m) (f).

Statistical Analysis

Continuous and categorical variables are presented as medians with interquartile ranges (IQRs; first quartiles–third quartiles) and frequencies with percentages, respectively. Relative risk was determined by calculating the hazard ratio and corresponding 95% confidence interval. The Mann-Whitney U-test or Kruskal-Wallis test was undertaken for comparisons of quantitative variables among groups, whereas Pearson's χ^2 or Fisher's exact test was performed to compare qualitative variables. Correlations between quantitative variables were determined using Spearman's ρ .

Progression-free survival (PFS) and overall survival (OS) were calculated from the date of surgery to the date of progression or death from any cause, respectively, or to the date of the last follow-up. The final data collection date was December 29, 2020. Kaplan-Meier plots were generated using Prism 8.0.2 software (GraphPad Software Inc., San Diego, CA, USA). Univariate

and multivariate analyses were performed using Cox proportional hazards regression (forward: LR method). After excluding 7 patients who were lost to follow-up and one who died of postoperative infection and acute hepatic failure, 179 patients (96%) were subjected to survival analysis. No significant difference in clinicopathological data and immune infiltrates was observed between the included and excluded patients. To investigate the prognostic value of tumor-infiltrating immune cells in different clinically relevant subgroups, patients included in survival analysis were divided into different subgroups. The flow diagram for subgrouping and sample sizes of each subgroup are summarized in online supplementary Figure S1.

A p value less than 0.05 was considered statistically significant, and all statistical tests used were two-tailed. Data were analyzed using Statistical Product and Service Solutions statistical software for Windows, version 21.0 (IBM Corp., Armonk, NY, USA).

Fig. 2. Tumor-infiltrating T lymphocytes in resected primary PanNEN formalin-fixed and paraffin-embedded samples using immunohistochemically stained TMAs. High (≥ 32.5 /HPF) CD3⁺ T-cell density in a high-grade PanNEC sample (**a**); low (< 32.5 /HPF) CD3⁺ T-cell density in an intermediate-grade nonfunc-

tioning PanNET sample (**b**); high (≥ 2 /HPF) CD4⁺ T-cell density in an intermediate-grade nonfunctioning PanNET (**c**); and low (< 2 /HPF) CD4⁺ T-cell density in an intermediate-grade nonfunctioning PanNET (original magnifications, $\times 400$; scale bar, 50 μ m) (**d**).

Table 2. Distribution of immune cells in PanNENs and in normal pancreatic tissues

Cell type	PanNEN (median [IQR]/HPF)	Normal pancreas (median [IQR]/HPF)	<i>p</i> value
CD117 ⁺ mast cell	3.5 (2–6)	2.3 (1–4.8)	0.035
CD15 ⁺ neutrophil	3 (1.3–6)	2.5 (1.3–3.8)	0.274
CD68 ⁺ macrophage	3.8 (2.5–5.8)	2.6 (1–4.4)	0.036
CD3 ⁺ T cell	13 (8–24)	13 (5–23.8)	0.661
CD4 ⁺ T cell	2 (1–4)	1.8 (0.8–4)	0.623

Results

Tumor-Infiltrating Immune Cells in PanNENs

Immune cell infiltration was detected in all neoplasm samples, and representative immunohistochemical staining images of CD117⁺ mast cells, CD68⁺ macrophages, CD15⁺ neutrophils, CD3⁺, and CD4⁺ T cells are presented (shown in Fig. 1, 2). The median counts of these cells in PanNENs and normal pancreatic tissues are shown in Table 2 and online supplementary Figure S2. T cells labeled by CD3 were the most abundant immune cells in PanNENs, whereas CD8⁺ T cells were not observed in our cohort of samples. The median count of mast cells was 3.5/HPF (IQR: 2.0–6.0/HPF), and high (≥ 1.5 /HPF) mast

cell infiltration was observed in 155 (83%) of the samples. Furthermore, a high density of CD117⁺ mast cells was correlated with high numbers of CD4⁺ T cells (Spearman's $\rho = 0.187$, $p = 0.010$) and CD15⁺ neutrophils (Spearman's $\rho = 0.184$, $p = 0.012$). Details of the associations between these immune cells are shown in online supplementary Table S2; Figure S3.

Associations between Immune Infiltrates and Clinicopathological Characteristics in PanNEN

We further compared the numbers of CD117⁺ mast cells, CD68⁺ macrophages, CD15⁺ neutrophils, and CD3⁺ and CD4⁺ T cells based on different WHO grades, PanNET versus PanNEC, the absence or presence of LVI and

Table 3. Univariate analysis of factors potentially associated with PFS

	HR (95% CI)	<i>p</i> value
WHO grade		
Low-grade	1	<0.001
Intermediate-grade	6.29 (2.43–16.28)	
High-grade	29.32 (8.64–99.47)	
Ki-67 index		
<3%	1	<0.001
≥3%	7.30 (3.05–17.46)	
Mitotic rate		
<2/10 HPF	1	<0.001
≥2/10 HPF	6.21 (2.74–14.06)	
LVI		
Absent	1	<0.001
Present	4.37 (2.08–9.18)	
PNI		
Absent	1	0.022
Present	3.01 (1.18–7.69)	
Tumor stage		
T1/T2	1	<0.001
T3/T4	4.28 (2.33–7.88)	
Lymph node metastasis		
Absent	1	<0.001
Present	5.85 (3.18–10.73)	
Distant metastasis		
Absent	1	<0.001
Present	16.01 (8.47–30.27)	
AJCC stage		
I/II	1	<0.001
III/IV	17.19 (8.17–36.14)	
CD117 ⁺ mast cell		
<1.5/HPF	1	0.003
≥1.5/HPF	0.36 (0.18–0.72)	
CD15 ⁺ neutrophil		
<6/HPF	1	0.095
≥6/HPF	0.37 (0.11–1.19)	
CD68 ⁺ macrophage		
<4.8/HPF	1	0.061
≥4.8/HPF	1.79 (0.98–3.27)	
CD3 ⁺ T cell		
<32.5/HPF	1	<0.001
≥32.5/HPF	2.74 (1.42–5.27)	
CD4 ⁺ T cell		
<2/HPF	1	0.897
≥2/HPF	0.96 (0.51–1.81)	

HR, hazard ratio; CI, confidence interval.

PNI, different functional statuses, T1/2 versus T3/4, the absence or presence of lymph node metastasis and distant metastasis, and AJCC stage I/II versus III/IV subgroups, and the results are summarized in online supplementary Figure S4. The number of mast cells in low-grade PanNENs (4.5, IQR: 2.25–6.75) was higher than that in inter-

mediate- or high-grade PanNENs (intermediate-grade: 3, IQR: 1.75–5, $p = 0.021$; high-grade: 2.63, IQR: 0.25–5.25, $p = 0.040$; online suppl. Fig. S4a). In contrast, the number of macrophages in low-grade PanNENs (3.25, IQR: 2.25–4.75) was lower than that in intermediate- or high-grade PanNENs (intermediate-grade: 4.25, IQR: 2.5–6.5, $p = 0.012$; high-grade: 5.88, IQR: 4.38–12.75, $p = 0.006$; online suppl. Fig. S4a), whereas patients with PanNEC had significantly higher counts of CD3⁺ T cells (38.63 vs. 13/HPF, $p = 0.004$) when compared with those in patients with PanNET (online suppl. Fig. S4b).

Patients with LVI exhibited a higher concentration of CD3⁺ T cells (23.88 vs. 12.25/HPF, $p = 0.010$) and CD4⁺ T cells (4 vs. 2/HPF, $p = 0.003$) than those without LVI (online suppl. Fig. S4c). Similarly, a higher concentration of CD3⁺ T cells (21.88 vs. 12.5/HPF, $p = 0.021$) and CD4⁺ T cells (4 vs. 2/HPF, $p = 0.016$) was observed in patients with PNI than in those without PNI (online suppl. Fig. S4d).

No significant difference between functioning PanNETs and nonfunctioning PanNETs was detected (online suppl. Fig. S4e), but higher numbers of mast cells (4.25 vs. 3.13/HPF, $p = 0.042$) and neutrophils (3.5 vs. 2.5/HPF, $p = 0.016$) were observed in the insulinoma subgroup but not in the noninsulinoma subgroup (online suppl. Fig. S4f).

There were no statistically significant differences between different tumor stages or lymph node metastasis statuses (online suppl. Fig. S4g, h). Of note, patients with distant metastases had lower mast cell infiltration than those without distant metastases (2.25 vs. 3.75/HPF, $p = 0.017$) (online suppl. Fig. S4i), and patients with advanced-stage disease (AJCC stage III/IV) showed lower counts of mast cells (2.38 vs. 4/HPF, $p = 0.006$) and neutrophils (2.13 vs. 3.25/HPF, $p = 0.008$) than those with AJCC stage I/II disease; however, patients with AJCC stage III/IV disease had higher macrophage infiltration (4.75 vs. 3.5/HPF, $p = 0.042$; online suppl. Fig. S4j).

Additionally, elevated numbers of CD3⁺ T cells were associated with a high blood lymphocyte count in PanNEN tissues. Details of the associations between blood cell counts and immune infiltrates are shown in online supplementary Table S3; Figure S5.

Survival Analyses of Patients with PanNEN

The 5-year OS and PFS rates were 94% and 68%. The median follow-up time was 44 months (IQR: 26–66 months), and the median 5-year PFS was 40 months (IQR: 19–52 months). Moreover, none of the examined variables were found to significantly influence OS. Univariate analysis data for PFS are presented in Table 3. Kaplan-Meier curves with patients categorized by clinico-

Fig. 3. Kaplan-Meier curves of the PFS of patients with PanNENs ($N = 179$) categorized by clinicopathological parameters and tumor-infiltrating immune cell counts. PFS of patients based on WHO grade (low-grade vs. intermediate-grade vs. high-grade) (a); Ki-67 index (<3% vs. ≥3%) (b); mitotic rate (<2/HPF vs. ≥2/HPF) (c); LVI (absent vs. present) (d); PNI (absent vs. present) (e); tumor stage (T1/T2 vs. T3/T4) (f); lymph node metastasis (absent vs.

present) (g); distant metastasis (absent vs. present) (h); AJCC stage (stage I/II vs. III/IV) (i); CD117⁺ mast cell density (<1.5/HPF vs. ≥1.5/HPF) (j); CD15⁺ neutrophil density (<6/HPF vs. ≥6/HPF) (k); CD68⁺ macrophage density (<4.8/HPF vs. ≥4.8/HPF) (l); CD3⁺ T-cell density (<32.5/HPF vs. ≥32.5/HPF) (m); and CD4⁺ T-cell density (<2/HPF vs. ≥2/HPF) (n).

pathological parameters and tumor-infiltrating immune cell counts are shown in Figure 3. No significant association was found between PFS and any of CD4⁺ T cells, CD15⁺ neutrophils, and CD68⁺ macrophages, although a low number of neutrophils and high macrophage counts showed a trend toward an association with poorer survival ($p = 0.095$ and 0.061 , respectively). Meanwhile, patients with a high density of mast cells and low CD3⁺ T cells had longer PFS.

According to multivariate Cox regression analysis, WHO grade, LVI, AJCC stage, and mast cell counts were

independent predictors of PFS (Table 4). We further conducted subgroup analyses to investigate the prognostic value of CD117⁺ mast cells, as well as other immune cells, in the clinically relevant subgroups using multivariate Cox regression analysis. Results of mast cell density stratified by clinically important subgroups are shown in online supplementary Table S4; Figure S6. Intriguingly, we found that high mast cell density remained an independent protective indicator in intermediate-grade, PanNET, noninsulinoma, and AJCC stage III/IV subgroups, whereas other immune cells, except CD3⁺ T cells (hazard

Table 4. Multivariate analysis of factors potentially associated with PFS

	HR (95% CI)	p value
WHO grade		<0.001
Low-grade	1	
Intermediate-grade	2.64 (0.98–7.11)	0.055
High-grade	20.42 (5.73–72.80)	<0.001
PNI		
Absent	1	0.003
Present	4.84 (1.71–13.68)	
AJCC stage		
I/II	1	<0.001
III/IV	15.28 (6.93–33.66)	
CD117 ⁺ mast cell		
<1.5/HPF	1	<0.001
≥1.5/HPF	0.26 (0.13–0.55)	

HR, hazard ratio; CI, confidence interval.

ratio: 2.76, 95% confidence interval: 1.01–7.52, $p = 0.048$), were not found to have independent prognostic significance in any of the subgroups, based on multivariate Cox regression analysis of patients with nonfunctioning PanNET (online suppl. Fig. S7).

Discussion/Conclusion

Although recent studies of PanNEN based on both animal models and human tissues have shown that tumor-infiltrating immune cells (e.g., tumor-associated macrophages, neutrophils, and CD3⁺ programmed cell death protein 1⁺ T cells) might play an important role in the pathogenesis of this disease and serve as prognostic biomarkers [10–13], the limitations of these studies are obvious. Most of the previous studies either investigated tumor-infiltrating immune cells in a group (e.g., nonfunctioning PanNETs) with a small number of patients or included heterogeneous neuroendocrine neoplasms from other sites, which potentially limits their clinical application. Therefore, to improve our understanding of the role of tumor-infiltrating immune cells in PanNENs, a comprehensive analysis of six common immune cells in 187 patients with PanNEN was conducted.

Intriguingly, we found that mast cells were the most informative prognostic indicator among these six immune cells, independent of the WHO grade and AJCC stage. Accumulating evidence indicates that the prognostic significance and the plastic pro- or anti-tumorigenic

function of mast cells are tumor-dependent [22–27]. Soucek et al. [28, 29] first noted the importance of mast cells in a *pIns-mycER^{TAM};RIP7-bcl-x_L* pancreatic islet tumor model, where inhibiting mast cell degranulation or creating a mast cell-deficient mouse model induced hypoxia and apoptosis in tumor and endothelial cells, indicating a pro-tumorigenic role of mast cells. Although Soucek et al. [28] confirmed the presence of CD117⁺ mast cells in human insulinomas, the prognostic significance of these cells with respect to PanNEN was still largely unclear. Furthermore, CD117⁺ mast cells were not detected in the tumor islets of *pIns-mycER^{TAM};RIP7-bcl-x_L* transgenic mice [29]. Moreover, the authors acknowledged that the degranulation inhibitors and mast cell deficiency in their transgenic animals might have produced off-target effects on other cells [28, 29]. In contrast, we highlighted the potentially anti-tumorigenic role of mast cells in PanNENs, which could be explained as follows: (i) endogenous peroxidase and reactive oxidative species released by mast cells might be tumoricidal [33, 34]; (ii) cytokines (such as tumor necrosis factor) that are required for mast cell-dependent T-cell activation can be produced by mast cells [35, 36]; (iii) mast cells can enhance the migration, maturation, antigen uptake, and presentation capabilities of dendritic cells via the secretion of histamine, mast cell-derived exosomes, and granules, as well as the transmission of antigen-IgE-FcεRI complexes [21]; and (iv) mast cells can serve as antigen-presenting cells and further activate cytotoxic CD8⁺ T cells [37]. Another possible explanation for this prognostic importance of CD117⁺ mast cells is that the correlation between a high density of CD117⁺ mast cells and prolonged PFS reflects the biological behavior of PanNEN rather than an active antitumor role for them. There were significantly more mast cells in low-grade PanNENs than in their intermediate- or high-grade counterparts. If the number of mast cells was relatively constant, then, in fast-growing intermediate- or high-grade PanNENs, the density of mast cells could be “diluted”, and the WHO grade could be a confounder. Therefore, we conducted multivariate Cox regression analysis and stratified (subgroup) analyses to control for confounding factors. Moreover, the results of our study based on these analyses did not support such hypotheses based on the following: (i) mast cell density was a prognostic indicator independent of WHO grade in multivariate Cox regression analysis and (ii) mast cell density remained an independent prognostic factor in the intermediate-grade PanNENs subgroup, and high mast cell density tended to be associated with longer PFS in low- and high-grade PanNEN subgroups.

Additionally, we observed weak but significant correlations between mast cells and CD4⁺ T cells, as well as CD15⁺ neutrophils; these might be attributable to tumor necrosis factor, CCL3/4 (which are capable of recruiting T cells), CXCL1/2 (which are responsible for neutrophil recruitment), and OX40 ligand (which can promote T-cell proliferation via OX40-OX40 ligand interactions) [38–41]. Such correlations and interactions could partially explain why CD3⁺ T cells, CD4⁺ T cells, and CD15⁺ neutrophils were not found to be an independent prognostic indicator in the current study. Hence, the complex interactions between mast cells and other immune cells in PanNENs still require additional investigation.

We also analyzed CD3⁺, CD4⁺, and CD8⁺ T cells, as well as CD15⁺ neutrophils and CD68⁺ macrophages, in PanNENs. CD3⁺ T cells were significantly more abundant in PanNEC and were associated with a shorter PFS; this finding was consistent with those of Takahashi et al. [10] and Milione et al. [42]. Although no statistically significant associations between other infiltrating immune cell counts and prognosis were found, high macrophage density approached significance and was more prevalent in patients with adverse prognoses in our cohort; this was consistent with previous studies of patients with PanNETs [11, 12]. The discrepancy between our study and previous investigations of tumor-infiltrating immune cells in patients with PanNET might be explained by the differences between cohorts (i.e., PanNENs vs. PanNETs vs. only nonfunctioning PanNETs), as well as the different evaluation methods used. Moreover, mast cells were not investigated in these studies. Therefore, full-scale investigations into tumor-infiltrating immune cells in PanNENs remain an urgent need.

This study had some limitations. First, given its retrospective nature and the lack of a validation cohort, in addition to the fact that patients were from a single center, the generalizability of our results might be limited. However, we were still able to investigate common tumor-infiltrating immune cells in a rare type of neoplasm (i.e., PanNEN), with a relatively large sample size. Second, to reduce the influence of intra-tumoral heterogeneity on our results as much as possible by using TMAs that were 2 mm in diameter were constructed to include greater areas, and immune cells were counted from four HPFs. Third, the mechanisms underlining the interactions between PanNEN and immune cells were not examined, owing to the paucity of human PanNEN cell lines and the difficulty of establishing PanNEN organoids [43].

In summary, our comprehensive analysis of common tumor-infiltrating immune cells in patients with PanNEN underscored the potentially protective role of mast

cells. Low mast cell counts can potentially be used to identify patients with PanNEN who have a high risk of progression.

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Statement of Ethics

Our study was conducted in accordance with the guidelines set forth by the Declaration of Helsinki and was approved by the Institutional Review Board of PUMCH (approval no. S-K1593). Given the retrospective study design and analysis of clinical data, the requirement for written consent was formally waived by the PUMCH Ethics Committee.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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Author Contributions

Jie Chen and Shuangni Yu made substantial contributions to the conception, design, and critical revision of the manuscript. Shengwei Mo, Liju Zong, and Xianlong Chen developed the methodology for immunostaining and evaluated the immunohistochemical data. Shengwei Mo, Liju Zong, Xianlong Chen, Shuangni Yu, Xiaoyan Chang, Zhaohui Lu, and Shuangni Yu made substantial contributions to sample collection, as well as TMA construction. Shengwei Mo made substantial contributions to data acquisition. Shengwei Mo and Xianlong Chen interpreted the data, performed statistical analysis, and drafted the manuscript. All the authors read and approved the final manuscript.

Data Availability Statement

The data used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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