Prognostic significance of tumor-associated macrophages in pancreatic neuroendocrine tumors

Alejandro Francisco-Cruz[1], Nahiro Uraoka[1], Suyu Liu[2], Edwin Roger Parra Cuentas[1], Luisa Maren Solis[1], Barbara Mino[1], Arvind Dasari[3], Michael J. Overman[3], Jonathan M. Loree[5], James C. Yao[3], Ignacio Ivan Wistuba[1], Daniel M. Halperin[3], Jeannelyn S. Estrella[4]

University of Texas MD Anderson Cancer Center, Houston, TX; 1. Department of Translational Molecular Pathology, 2. Department of Biostatistics, 3. Department of GI Medical Oncology, 4. Department of Pathology 5. BC Cancer, Vancouver, BC, Canada

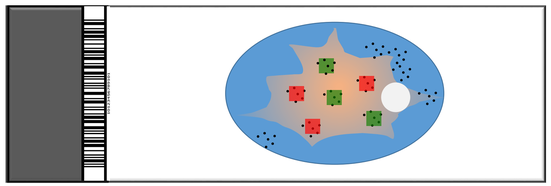
TAP TO GO BACK TO KIOSK MENU

Abstract (Click)

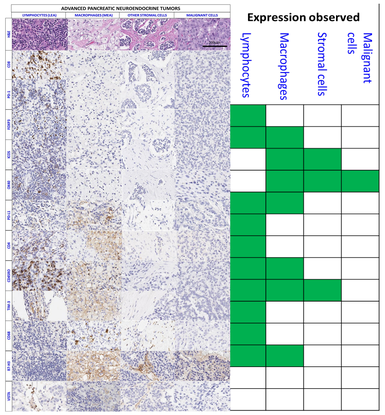
- Pancreatic Neuroendocrine Tumors (PanNETs) account for 3.6% of all NETs and 2–4% of all clinically detected pancreatic tumors and the incidence has increased considerably over the last decades.
- The diagnosis and treatment of advanced PanNETs still insufficiently effective.
- Checkpoint inhibitors have shown modest antitumor activity, and rational immunotherapy strategies have been hampered by a lack of understanding of the PanNETs immune context.

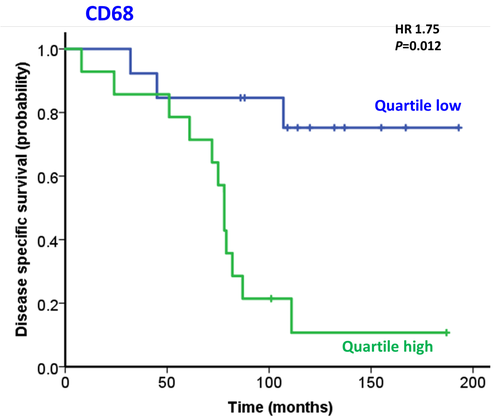
Material & Methods (Click)

- Lymphocyte-enriched areas (LEA) with high density of CD8 positive cells
- Macrophage-enriched areas (MEA) with high density of CD68 positive cells
- Whole tissue section
- Tumor area
- Tumor tissue for DNA extraction



Results (Click)





Conclusion

- TAM infiltration and TIM-3 expression were significantly correlated with inferior survival in patients with PanNETs.
- TAM depletion may therefore present an appealing and rational target for immunotherapeutic approaches in PanNETs.
- Targeting mTOR signaling and TAMs could be a good strategy for future clinical trials.
- The association of DAXX/ATRX loss of nuclear expression with an immune exhaustion phenotype opens the possibility to new therapeutic options for this group of patients.

This work was founded by Neuroendocrine Tumor Research Foundation

THE UNIVERSITY OF TEXAS MD Anderson Cancer Center
Making Cancer History®

Abstract

TAP TO GO BACK TO KIOSK MENU

- Pancreatic Neuroendocrine Tumors (PanNETs) account for 3.6% of all NETs and 2–4% of all clinically detected pancreatic tumors and the incidence has increased considerably over the last decades.[1-4]
- The diagnoses and treatment of advanced PanNETs still insufficiently effective.[5]
- The European Neuroendocrine Tumor Society (ENETS) and the World Health Organization (WHO) grading system provide the only prognostic information regarding the pathologic assessment of the malignant potential of PanNETs.[5]
- PanNETs are associated with multiple endocrine neoplasia type 1 (MEN1) in 10–20% of familiar cases and, in sporadic PanNETs, the most common somatic mutation is an inactivating mutation in MEN1 gene (44% of the cases).[6]
- Additional genes implicated in chromatin remodeling and alternative lengthening of telomeres (ALT) phenotype DAXX (death-domain– associated protein) and ATRX (a thalassemia/mental retardation syndrome X-linked) are associated with sporadic PanNETs in up to 43%.[6,7]
- Well-differentiated PanNETs have somatic mutations in genes encoding proteins involved in the mTOR signaling in 16% of the cases including genes such as PTEN (7%), TSC2 (9%), and PIK3CA (1%).[6-7]
- Everolimus targets the mTOR pathways and is approved for the treatment of adult patients with progressive PanNETs with unresectable, locally advanced or metastatic disease.[8]
- Checkpoint inhibitors have shown modest antitumor activity, and rational immunotherapy strategies have been hampered by a lack of understanding of the PanNETs immune context.

AIMS OF THE STUDY:

- To characterize intratumoral immune cell infiltrates in primary advanced PanNETs.
- To characterize key gene alterations in advanced PanNETs
- To correlate the immune-cell densities with clinical and pathological features including age, sex, stage, tumor size, histological grade, proliferation index, mutational profile status and Disease-Specific Survival.

References:

1. Zhong Q, Chen QY, Xie JW, et al. Incidence trend and conditional survival estimates of gastroenteropancreatic neuroendocrine tumors: A large population-based study. Cancer Med. 2018 Jun 5. doi: 10.1002/cam4.1598. [Epub ahead of print]
2. Fendrich V, Waldmann J, Bartsch DK, Langer P. Surgical management of pancreatic endocrine tumors. Nat Rev Clin Oncol. 2009;6:419-28.
3. Modlin IM, Lye KD, Kidd M. A 5-decade analysis of 13,715 carcinoid tumors. Cancer. 2003 Feb 15;97:934-59.
4. Dasari A, Shen C, Halperin D, et al. Trends in the Incidence, Prevalence, and Survival Outcomes in Patients With Neuroendocrine Tumors in the United States. JAMA Oncol. 2017;3:1335-1342.
5. Kondo NI, Ikeda Y. Practical management and treatment of pancreatic neuroendocrine tumors. Gland Surg. 2014;3:276-83
6. Pea A, Hruban RH, Wood LD. Genetics of pancreatic neuroendocrine tumors: implications for the clinic. Expert Rev Gastroenterol Hepatol. 2015;9:1407-19.
7. Jiao Y, Shi C, Edil BH, et al. DAXX/ATRX, MEN1, and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors. Science. 2011;331:1199–203.
8. Yao JC, Shah MH, Ito T, et al. Everolimus for advanced pancreatic neuroendocrine tumors. N Engl J Med. 2011;364:514-23.

Materials and Methods

TAP TO GO BACK TO KIOSK MENU

Selection of cases

- 53 patients with advanced PanNETs with surgical resected formalin fixed paraffin embedded (FFPE) tumors available for analysis.

Immune-cell markers

- Characterization of the tumor microenvironment focusing on tumor-infiltrating lymphocytes (TILs) and tumor-associated macrophages (TAMs) was performed using immunohistochemistry for markers listed in **table 1.**
- Selection of lymphocyte-enriched areas (LEA) and macrophage-enriched areas (MEA) for digital image analysis:
  - $1mm^2$ area with highest density of CD8 (LEA) and CD68 (MEA) IHC staining within the tumor (hotspots, n=1-5 per case) selected by a pathologist.
  - Immune-cell densities within MEA and LEA were quantitated using computer-assisted image analysis with Aperio ImageScope Version 12.3.3.7031.
  - The median of the cell density between analyzed areas per case were calculated and used for further analysis.

Genomic analysis

- To characterize the molecular alterations of PanNETs, we performed genomic analysis of DNA extracted from 47 macrodissected FFPE containing viable malignant cells using high depth targeted exome sequencing (T200 Platform) for genes MEN1, SETD2, MUTYH, CHEK2, BRCA2, ALT, DAXX, ATRX, PTEN, TSC1 and TSC2.

Statistics

- Time-to-event analyses were performed with the Kaplan-Meier method. Cox regression was used to evaluate the relationship between each cell type and survival from initial diagnosis. Differences between cell densities and groups of study was performed by two-tailed t-test.

Table 1. Immune-cell markers and antibodies used in the study

Type of marker	Marker	Clone of antibody	Expressing cells
Lymphocyte marker	CD8	EPR4877(2) Abcam	Cytotoxic T-cells
	CD4	4B12 Leica Biosystems	T-helper cells and other inflammatory cells
	CD45RO	UCHL1 (M) Leica Biosystems	Subsets of memory T-cells and other inflammatory cells
	FOXP3	D2W8E CST	T-regulatory cells and other activated T-cells
Lymphocyte co-stimulatory markers	ICOS	D1K2T CST	Activated T-cell
	OX40	ACT-35 (M) eBioscience	Activated T-cell
Lymphocyte co-inhibitory markers (immunecheckpoints)	PD-1	EPR4877(2) Abcam	Antigen experienced T-lymphocytes
	LAG3	D2G4O CST	Co-inhibited T-lymphocytes
	TIM-3	D5D5R CST	Co-inhibited T-lymphocytes
	B7-H3	D9M2L CST	Co-inhibitory antigen-presenting cell
	B7-H4	D1M8I CST	Co-inhibitory antigen-presenting cell
	PD-L1	E1L3N CST	Co-inhibitory antigen-presenting cell
	VISTA	D1L2G CST	Co-inhibitory antigen-presenting cell
Macrophage marker	CD68	PG-M1 (M) DAKO	Macrophages and fibroblasts

Figure 1. Selection of regions of interest with high density of immune-cells

Lymphocyte-enriched areas (LEA) with high density of CD8 positive cells

Macrophage-enriched areas (MEA) with high density of CD68 positive cells

Whole tissue section

Tumor area

Tumor tissue for DNA extraction

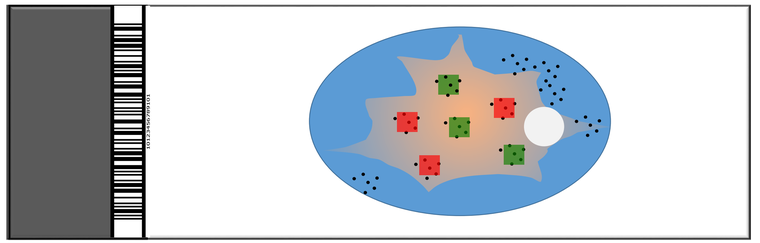


Table 2. Key genes associated with PanNET development

MEN1
SETD2

DNA Damage Repair
MUTYH
CHEK2
BRCA2

Alternative Lengthening of Telomeres phenotype
DAXX
ATRX

mTOR Pathway
PTEN
TSC1
TSC2

TAP TO GO BACK TO KIOSK MENU

Results

Clinicopathological features and immune-cell infiltrates

Table 3. Clinical and pathological data

Feature	Number (range)
Cases	53
Age (mean)	56 (29-81)
Gender	
Male	30
Female	23
Tumor size (mean)	6.28 cm (1.1-25)
Pathologic Tumor Stage (AJCC 7th Ed.)	
pT1	4
pT2	15
pT3	34
Lymph node metastasis	
No	17
Yes	36
Liver metastasis	37
Ki67 index (mean)	6.1 (0-20)
WHO 2010	
Grade 1	23
Grade 2	30

Table 4. Cell density of immune-cell markers by immunohistochemistry in advanced PanNETs

Immune-cell marker	Median of cell density (cells/mm²)	Mean of cell density (cells/mm²)	SD	Expression observed			
				Lymphocytes	Macrophages	Stromal cells	Malignant cells
CD8	182	87	312	■			
CD4	226	78	573	■	■		
CD68	68	44	69		■	■	
B7-H3	103	29	180		■	■	■
CD45RO	97	15	336	■	■		
PD-1	42	8	113	■			
FOXP3	17	8	44	■			
TIM3	23	5	62	■	■		
VISTA	5	2	8	■	■	■	
ICOS	3	0	10	■			
OX40	2	0	4	■			
PD-L1	1	0	3	■	■		
LAG3	0	0	0				
B7-H4	0	0	0				

PanNETs are characterized by differential expression of immune-cell markers on lymphocytes, macrophages and other stromal cells.

TAP TO GO BACK TO KIOSK MENU

Results

Characterization of immune-cell phenotypes on LEA and MEA and association with clinico-pathological features

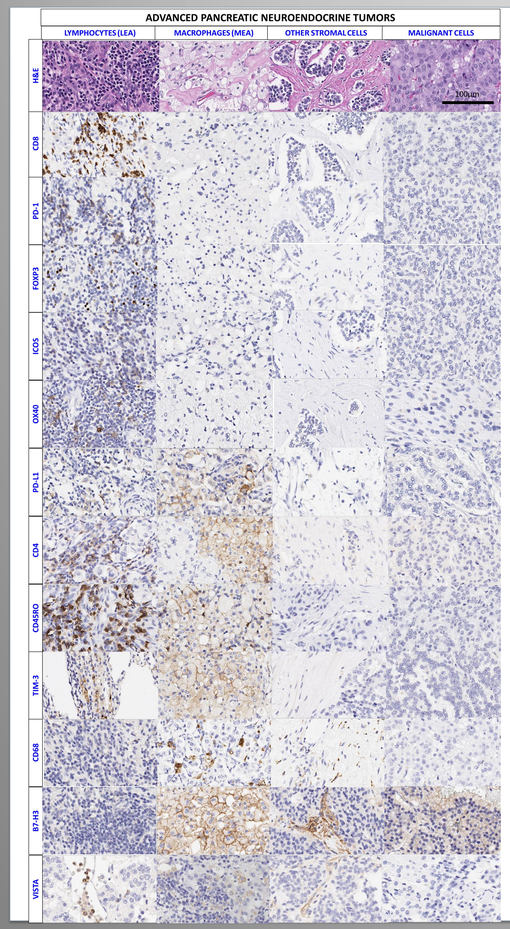


Figure 2. Density of immune-cell markers in advanced PanNETs and patterns of expression

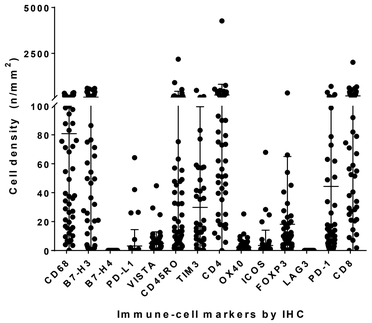
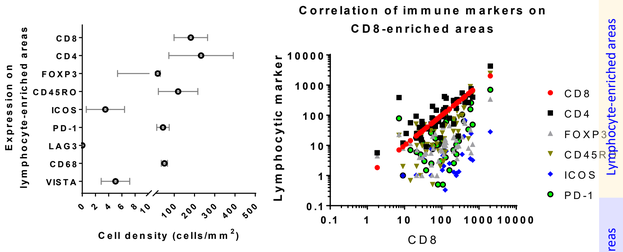
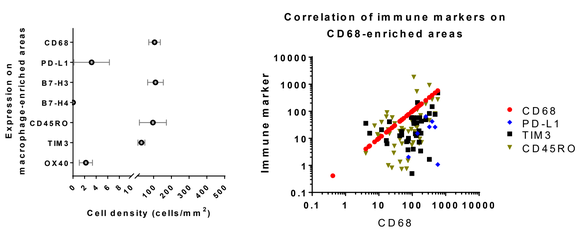


Figure 3. Immune-cell markers evaluated in lymphocyte-enriched areas (LEA)



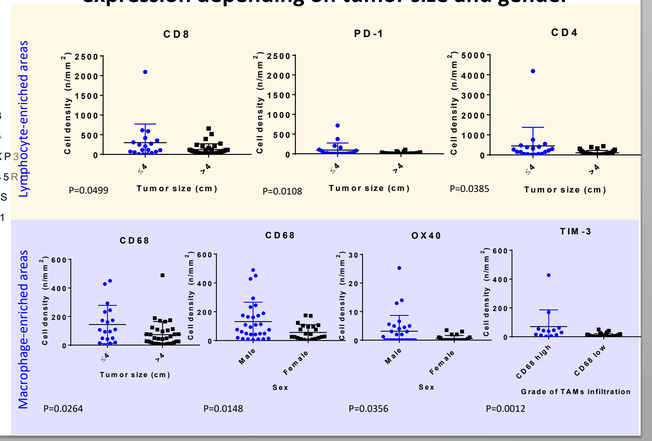
	CD8 vs. CD4	CD8 vs. FOXP3	CD8 vs. CD45RO	CD8 vs. ICOS	CD8 vs. PD-1
Spearman r	0.7444	0.5005	0.6972	0.5066	0.5613
P value	<0.0001	0.0001	<0.0001	<0.0001	<0.0001
95% CI	0.59 to 0.8463	0.2612 to 0.6818	0.5222 to 0.8159	0.2689 to 0.6862	0.3382 to 0.7246

Figure 4. Immune-cell markers evaluated in macrophage-enriched areas (MEA)



	CD68 vs. TIM3	CD68 vs. CD45RO	CD68 vs. PD-L1
Spearman r	0.6072	0.4342	0.4877
P value	<0.0001	0.0009	0.0002
95% confidence interval	0.4008 to 0.7549	0.2285 to 0.6597	0.2479 to 0.6712

Figure 5. immune-cell markers showed differential expression depending on tumor size and gender



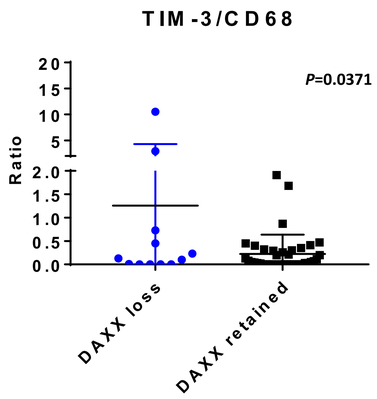
- Most of the immune cell markers were positive on TILs and TAMs.
- The association of ICOS, PD-1, CD4 and FOXP3 to CD8 within LEA suggests an exhausted phenotype of cytotoxic T-cells.
- The association of TIM-3, CD45RO and PD-L1 to CD68 within MEA suggests a collaboration of TILs and TAMs and/or co-expression of these markers on TAMs.
- Tumors smaller than 4cm showed higher TILs and TAMs infiltration associated with higher PD-1 expression.
- Males showed higher TAMs infiltration and lymphocyte activation than females.

TAP TO GO BACK TO KIOSK MENU

Results

Association between immune-cell markers and DAXX/ATRX immunohistochemistry

Figure 6. DAXX loss of nuclear expression may be associated with a higher expression of TIM-3 and FOXP3



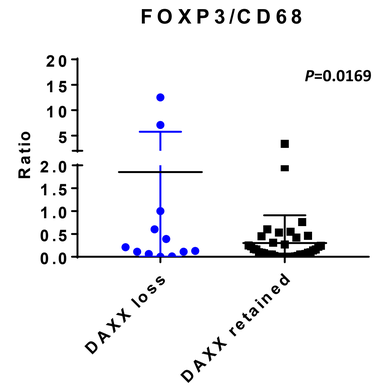
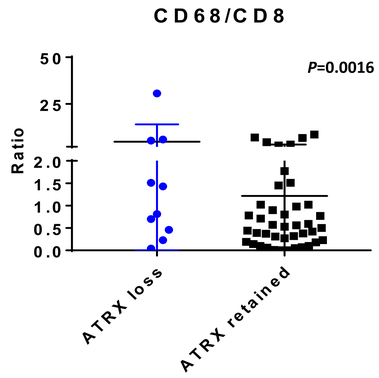
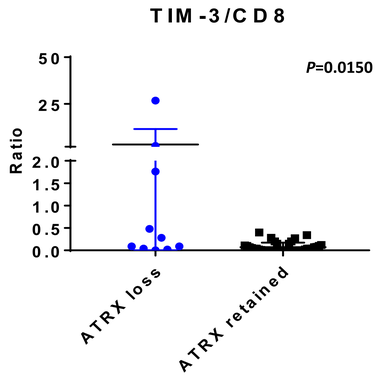
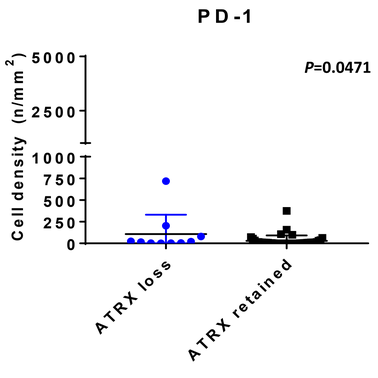
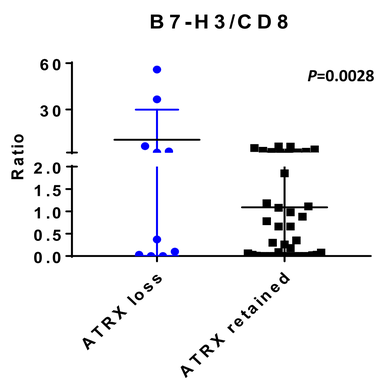


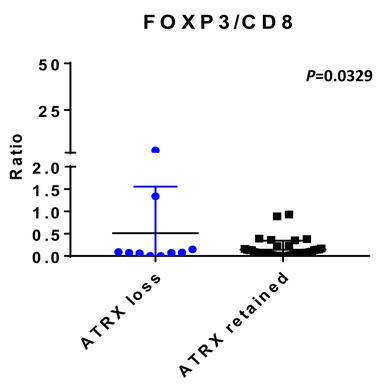
Figure 7. ATRX loss of nuclear expression may be associated with a memory and exhausted phenotype and higher macrophage infiltration

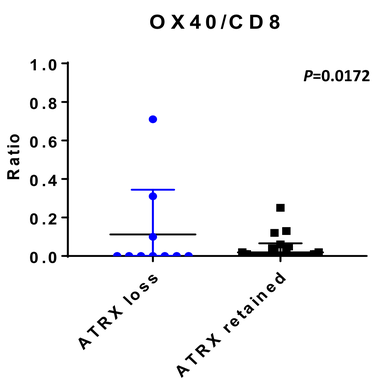












Results

TAP TO GO BACK TO KIOSK MENU

Prognostic significance of TAMs, ICOS, TIM-3, OX40 in PanNETs

Figure 8. High density of macrophages and TIM-3 signature portends poor prognosis

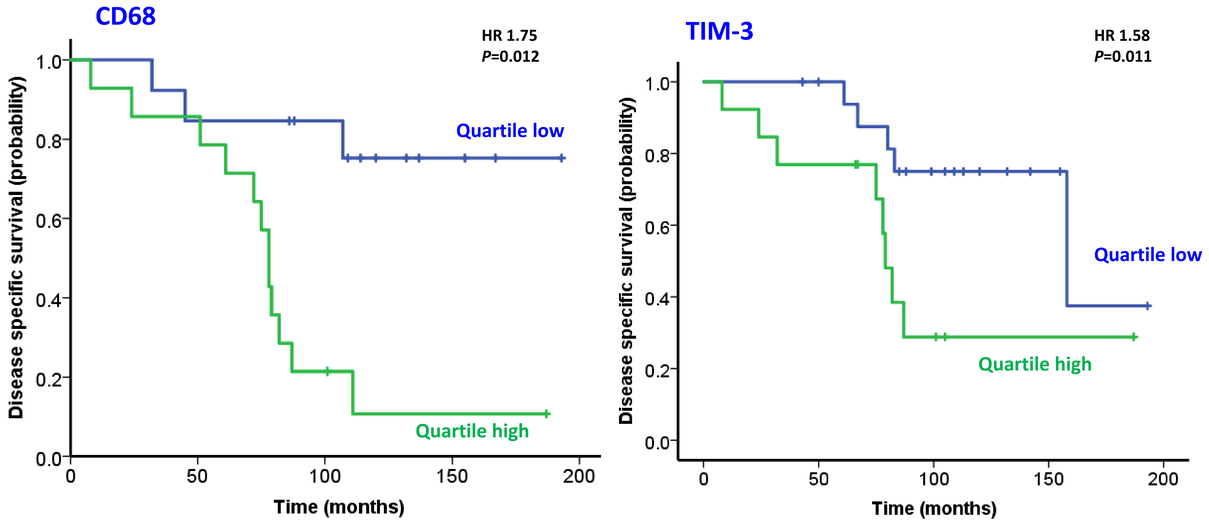
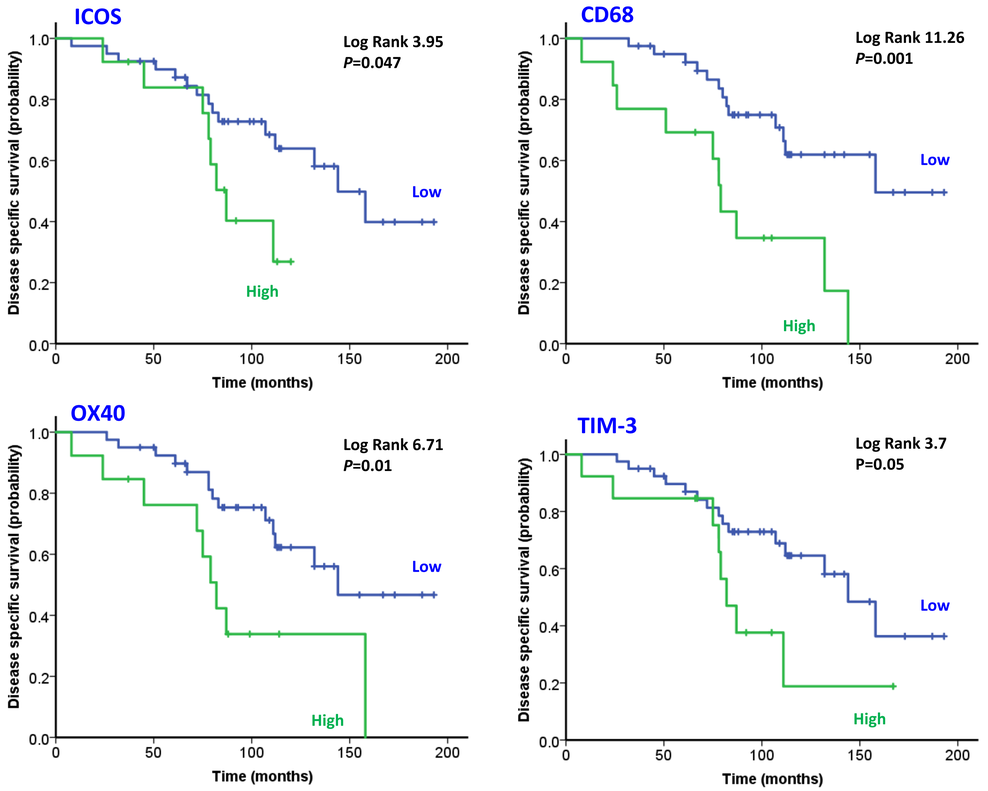


Figure 9. High density of macrophages and activated lymphocytes with TIM-3 signature portend poor prognosis



TAP TO GO BACK TO KIOSK MENU

Results

Mutational profile of primary advanced PanNETs and survival

Table 5. Mutation profile of key cancer genes in advanced PanNETs (n=47)

Gene	Wild-type	Mutated	Mutation Rate
MEN1	30	17	36%
SETD2	46	1	2%
DNA Damage Repair	42	5	11%
MUTYH	45	2	4%
CHEK2	44	3	6%
BRCA2	45	2	4%
Alternative Lengthening of Telomeres phenotype	28	19	40%
DAXX	35	12	26%
ATRX	39	8	17%
mTOR Pathway	37	10	21%
PTEN	44	3	6%
TSC1	46	1	2%
TSC2	40	7	15%

Table 6. mTOR pathway mutations may be associated with macrophage infiltration in MEA

	***mTOR* wild-type**	***mTOR* mutated**	**Total**
$CD68^{low}$	10	1	11
$CD68^{high}$	6	6	12
Total	16	7	23
	***mTOR* wild-type**	***mTOR* mutated**	**Total**
$TIM\text{-}3^{low}$	14	2	16
$TIM\text{-}3^{high}$	10	2	12
Total	24	4	28

Likelihood Ratio = 4.9, *P*=0.026

Figure 10. PTEN mutation is related to macrophage infiltration

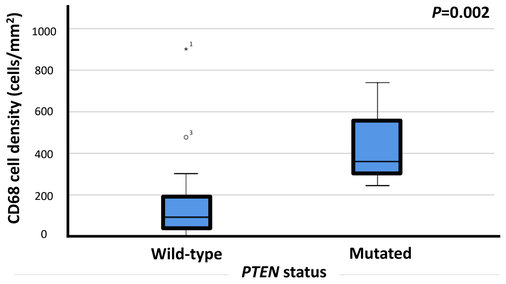
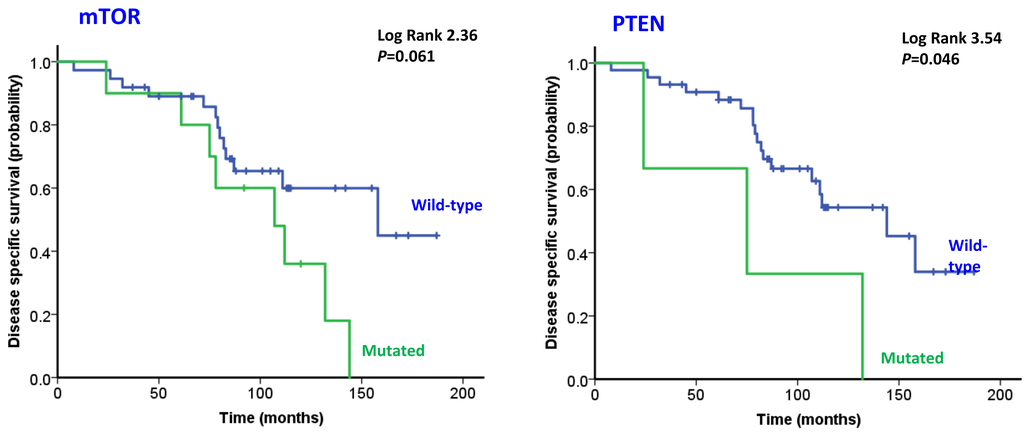


Figure 11. PTEN mutation portends poor survival, with a non-significant trend towards association of poor survival with mTOR pathway mutations



The mutational profile on these tumors showed an ALT phenotype and mTOR pathway alteration in more than 50% of cases.
mTOR pathway mutations may be associated with higher macrophage infiltration and poor survival.