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Original Article

TIM-3 transcriptomic landscape with clinical and immunomic correlates in cancer

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Abstract: TIM-3, an inhibitory checkpoint receptor, may invoke anti-PD-1/anti-PD-L1 immune checkpoint inhibitor (ICI) resistance. The predictive impact of TIM-3 RNA expression in various advanced solid tumors among patients treated with ICIs is yet to be determined, and their prognostic significance also remains unexplored. We investigated TIM-3 transcriptomic expression and clinical outcomes. We examined TIM-3 RNA expression data through the OmniSeq database. TIM-3 transcriptomic patterns were calibrated against a reference population (735 tumors), adjusted to internal housekeeping genes, and calculated as percentiles. Overall, 514 patients (31 cancer types; 489 patients with advanced/metastatic disease and clinical annotation) were assessed. Ninety tumors (17.5% of 514) had high (≥75th percentile RNA rank) TIM-3 expression. Pancreatic cancer had the greatest proportion of TIM-3 high expressors (36% of 55 patients). Still, there was variability within cancer types with, for instance, 12.7% of pancreatic cancers harboring low TIM-3 (<25th percentile) levels. High TIM-3 expression independently and significantly correlated with high PD-L2 RNA expression (odds ratio (OR) 9.63, 95% confidence interval (CI) 4.91-19.4, P<0.001) and high VISTA RNA expression (OR 2.71, 95% CI 1.43-5.13, P=0.002), all in multivariate analysis. High TIM-3 RNA did not correlate with overall survival (OS) from time of metastatic disease in the 272 patients who never received ICIs, suggesting that it is not a prognostic factor. However, high TIM-3 expression predicted longer median OS (but not progression-free survival) in 217 ICI-treated patients (P=0.0033; median OS, 2.84 versus 1.21 years (high versus not-high TIM-3)), albeit not retained in multivariable analysis. In summary, TIM-3 RNA expression was variable between and within malignancies, and high levels associated with high PD-L2 and VISTA checkpoints and with pancreatic cancer. Individual tumor immunomic assessment and co-targeting co-expressed checkpoints merits exploration in prospective trials as part of a precision immunotherapy strategy.

Keywords: TIM-3, immune-checkpoint inhibitors, solid tumors, RNA expression

Introduction

TIM-3 (T-cell immunoglobulin and mucin domain-containing protein 3) also known as HAVCR2 (Hepatitis A virus cellular receptor 2), a member of the T-cell immunoglobulin and mucin domain (TIM) family of proteins is a receptor expressed by various cells of the immune system, including activated T-cells, T helper type 1 (Th1) cells, regulatory T-cells (Tregs), natural killer (NK) cells, and dendritic cells (DCs) [1-4].

TIM-3 acts as a co-inhibitory receptor. Upon combining with ligands such as galectin-9 and CEACAM-1 (carcinoembryonic antigen-related cell adhesion molecule 1), the intracellular tyrosine signaling motifs (Y256 and Y263) in the

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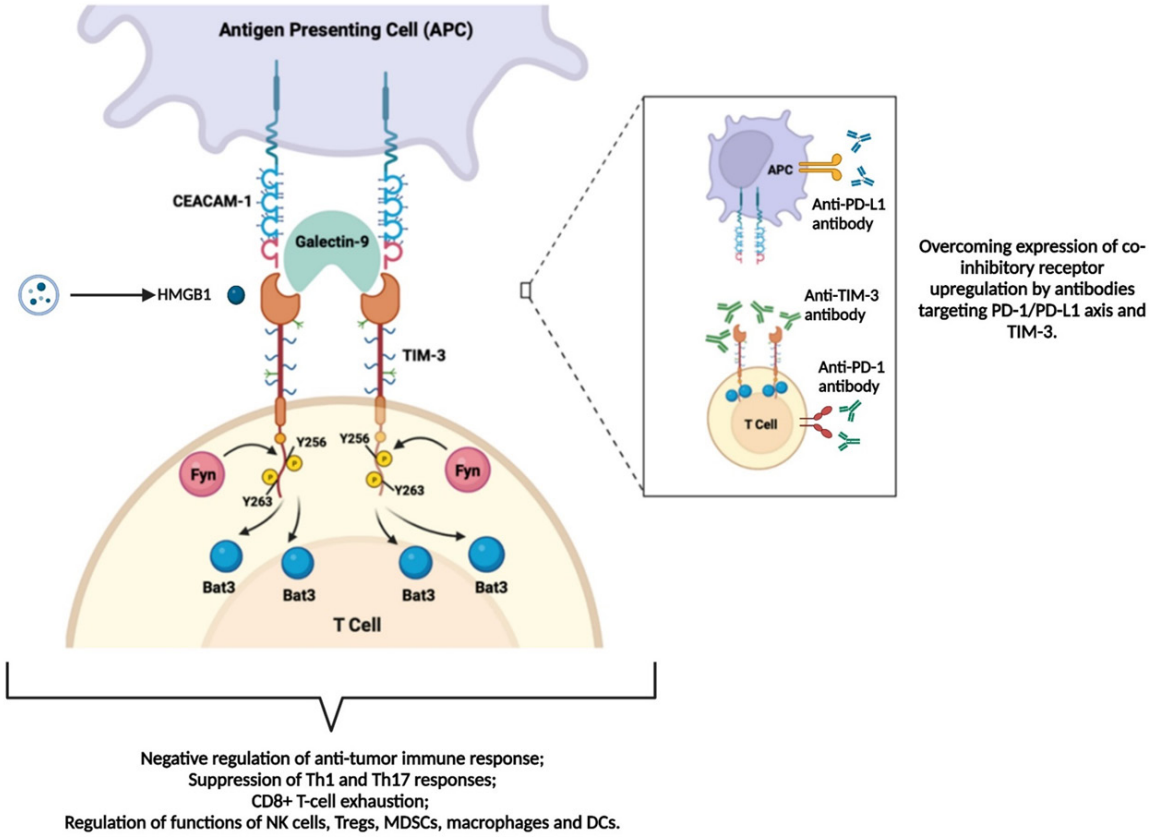

Figure 1. TIM-3 signaling in T-cells. The figure shows TIM-3 functions as a co-inhibitory receptor by engaging with ligands galectin-9 and CEACAM-1 resulting in phosphorylation of intracellular tyrosine motifs (Y256, Y263). The subsequent release of bat3 and recruitment of Fyn protein leads to T-cell exhaustion, anergy, and apoptosis. Anti-TIM-3 antibodies thus provide a therapeutic approach to overcome the effects of TIM-3. Abbreviations: Bat 3, HLA-B associated transcript 3; CEACAM-1, Carcinoembryonic antigen-related cell adhesion molecule 1; DCs, dendritic cells; Fyn, SH2 (Src homology 2) domain-containing protein; HMGB1, High mobility group box 1 protein; MDSCs, myeloid derived stem cells; NK, natural killer cells; PD-1, Programmed death receptor-1; PD-L1, Programmed death ligand-1; TCR, T-cell receptor; Th1, T helper 1 cells; Th17, T helper 17 cells; TIM-3, T-cell immunoglobulin and mucin domain-containing protein 3; Tregs, regulatory T-cells; Y256 and Y263, intracellular tyrosine signaling. Figure created with BioRender.com.

cytoplasmic tail of TIM-3 become phosphorylated and release the HLA-B associated transcript 3 (Bat3) (**Figure 1**). This allows for recruitment of SH2 (Src homology 2) domain-containing protein Fyn, resulting in the disruption of immune synapse between the T-cell and antigen presenting cell. This leads to cell anergy, T-cell exhaustion and apoptosis [5, 6]. Similarly, TIM-3 is also constitutively expressed on NK cells and DCs and plays a role in cancers [2, 7].

The synergistic effect of coinhibitory receptors TIM-3, LAG-3 (lymphocyte-activating gene 3), and TIGIT (T-cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibitory motif domains) in regulating T-cell function has been extensively studied and has been shown to play a critical role in controlling the immune responses and promoting immune tolerance in cancers [8, 9]. Studies have also shown that TIM-3 is upregulated on exhausted T-cell in various tumors and that blocking TIM-3 signaling can restore T-cell function and enhance antitumor activity [10].

TIM-3 expression is also recognized as a prognostic marker and a potential predictor of poor outcome after anti-PD-1/PD-L1 (anti-programmed death receptor-1/anti-programmed death ligand-1) therapy in some of those cancers [11, 12].

Anti-PD-1/anti-PD-L1 immune check point inhibitors (ICIs) have shown remarkable results in

treating various tumors including but not limited to melanoma, head and neck cancers, non-small cell lung cancers, renal cell carcinoma, colorectal cancer, hepatocellular carcinoma, urothelial cancer, endometrial cancer among several other tumor types [13-17]. While these checkpoint inhibitors produce variable survival benefits in different cancer types, the overall response rates across cancers remain around 15-20% [18]. Importantly, tumors can evade the immune response by upregulating the expression of TIM-3 and this has been associated with resistance to anti-PD-1/anti-PD-L1 ICIs [19].

Currently, there are several clinical trials evaluating the clinical efficacy of anti-TIM-3 targeted therapies, either alone or in combination with other immune check point inhibitors. The reduced efficacy of immunotherapy in cancers such as pancreatic cancer can be partly attributed to the immunosuppressive tumor microenvironment [20-22]. Thus it is important to assess the prognostic and predictive potential of primary resistance mechanisms to anti-PD-1/anti-PD-L1 inhibitors.

In this study, we investigated the RNA (ribonucleic acid) expression level of TIM-3 in patients with solid tumors and its correlation with other immunoregulatory molecules as well as with outcome.

Methods

Patients

We conducted an analysis of RNA expression level of TIM-3 in a cohort of 514 patients with cancer (including 489 patients with advanced/metastatic disease that had adequate clinically annotated data) treated at the University of California San Diego (UCSD) Moores Cancer Center for Personalized Therapy, using an immune profiling assay at OmniSeq (https://www.omniseq.com/), a Clinical Laboratory Improvement Amendments (CLIA)-licensed and College of American Pathologist (CAP)-accredited clinical laboratory. Data were collected on the patients' age, sex, cancer type, microsatellite instability (MSI) status, tumor mutational burden (TMB), and PD-L1 IHC (immunohistochemistry). This database has been previously described [12, 23-26]. Designated variables and histologies are according to the most recent review as of September 2023. The current analysis utilized the sample taken at an earlier timepoint if a patient had multiple samples collected on different days. The study was conducted in compliance with the guidelines and regulations set by the UCSD Institutional Review Board Study of Personalized Cancer Therapy to Determine Response and Toxicity, UCSD_PREDICT, NCT02478931 and for investigational interventions for which patients gave informed consent.

Tissue collection and analysis of immune expression

The tumors were obtained as formalin-fixed, paraffin-embedded (FFPE) samples after tissue collection and evaluated with RNA transcriptome sequencing at OmniSeq utilizing a clinically validated 395-gene expression panel relating to the anticancer immune response as previously described [27]. With some modifications applied to the manufacturer's instructions, the RNA and DNA co-extraction from FFPE was conducted using the truXTRAC FFPE extraction kit from Covaris, Inc. (Woburn, MA). The purified RNA was dissolved in 50 µL of water and the yield was determined using Quant-iT RNA HS assay from Thermo Fisher Scientific (Waltham, MA). 10 ng (nanogram) RNA was used as sample input for library preparation using the Oncomine Immune Research Response Assay (Thermo Fisher Scientific, Waltham, MA). Following sequencing on an Ion Torrent S5XL system (Thermo Fisher Scientific, Waltham, MA), Torrent Suite's plugin immuneResponseRNA (v5.2.0.0) was used for generating absolute reads for the measurement of RNA expression.

The transcript abundance was normalized based on a reference group of 735 tumors including 35 histologies and then ranked on a scale of 0 to 100 percentile. TIM-3 and other checkpoint marker's expression profiles were stratified into three groups, "High" (75-100 percentile RNA rank), "Moderate" (25-74 percentile RNA rank), and "Low" (0-24 percentile RNA rank), based on their rank values.

Analysis of variables

PD-L1 expression level was measured using Dako PD-L1 IHC 22C3 pharmDx assay (Dako

North America, Inc., Carpinteria, California, USA).

For tumor mutational burden (TMB), genomic DNA from qualified FFPE tumors containing more than 30% malignant nuclei was evaluated with a 10 ng input for library preparation using the Comprehensive Cancer Panel (Thermo Fisher Scientific, Waltham, MA) as described [27]. The enrichment and preparation of templates were performed with the Ion Chef system with sequencing on the Ion S5XL 540 chip (Thermo Fisher Scientific, Waltham, MA). After eliminating germline variations, synonymous variations, insertions/deletions, and single nucleotide variants with a variant allele frequency (VAF) of less than 5%, TMB was calculated as the number of suitable mutations per megabase of sequence (mut/Mb).

Microsatellite instability (MSI) was assessed in genomic DNA from qualified FFPE tumors containing greater than 20% neoplastic nuclei. The MSI next-generation sequencing (NGS) assay evaluates 29 homopolymer loci, including BAT-25 and BAT-26, through sequencing 20 ng of tumor DNA using a MiSeq sequencer (Illumina, San Diego, CA). The MSI-NGS Caller, a component of the assay, makes MSI calls ("unstable", "stable" or "inconclusive") based on a comparison of the tumor homopolymer repeat profile of a sample to a predefined normal allele distribution at each locus, without requiring a matching normal DNA sample as described [28].

Data analysis and outcomes

Descriptive statistics were used to summarize patient characteristics. To investigate the association between high RNA expression of TIM-3 and pancreatic cancer, we performed univariate expression analysis. Multivariable logistic regression was also performed with variables that had *p* values <0.2 in the univariate analysis.

Survival analysis was performed using the Kaplan-Meier method. To determine prognostic indicators, the overall survival (OS) of patients was measured as the time elapsed from the date of diagnosis of metastatic or locally advanced disease to the date of their last recorded follow-up or death. To determine indicators predictive of therapeutic outcome, for the subgroup of patients who received immunotherapy, OS was calculated from their start date of the treatment to their last follow-up or death, and progression-free survival (PFS) was determined from the date of initiation of immunotherapy to either the earliest occurrence of clinical or radiological disease progression or death. Patients without progression at last follow up were censored for PFS at that time; patients still alive at last follow up were censored for OS at that time.

TIM-3 expression level in patients was stratified into two groups: high (≥75th percentile RNA rank) and moderate/low (<75th percentile RNA rank). The survival of these groups was then compared using log-rank test. A multivariate Cox proportional hazard model was used to identify factors that were associated with OS and PFS. The relationship between TIM-3 levels and response to treatment was analyzed using chi-square tests and Student's t tests.

The statistical analyses were conducted using R 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria), and a significance level of P≤0.05 was used.

Results

Patient characteristics

In our cohort of 514 patients, 310 were women (60%), and median age was 61 years; 489 patients had advanced/metastatic disease and survival/outcome data. The most common tumor types examined were colorectal (N=140 patients), pancreatic (N=55), breast (N=49) and ovarian (N=43) (**Table 1**). Overall, 217 patients had treatment with immune checkpoint blockade during the course of their disease (Supplementary Figure 1, flow chart). Only 2 of 217 patients received anti-CTLA-4 as a single therapy; the other patients received anti-PD-1/PD-L1-based regimens.

TIM-3 transcript levels were variable between and within tumor types, with pancreatic cancer having the highest proportion of high TIM-3 levels

Ninety tumors (17.5% of 514) had high (≥75th percentile RNA rank) TIM-3 expression; 424 had medium/low (<75th percentile RNA rank) TIM-3 expression level (**Figure 2**). Pancreatic cancer had the highest proportion of TIM-3

Table 1. Patient characteristics, immunotherapy biomarkers and cancer types in 514 patients with high (≥75th percentile RNA rank) and moderate/low (<75th percentile RNA rank) TIM-3 expression

Variable	Condition N	Proportion of patients with high TIM-3 expression (N=90)	Univariable analysis		Multivariable analysis*	
			Odds Ratio (95% CI)	*P*-value	Odds Ratio (95% CI)	*P*-value
Age (years)	≥61	16% [42/256]	0.86 (0.54-1.35)	0.512		
	<61	19% [48/258]	-			
Gender	Male	16% [33/204]	0.86 (0.53-1.36)	0.519		
	Female	18% [57/310]	-			
BTLA	≥75	41% [39/96]	4.92 (2.98-8.14)	<0.001	0.98 (0.43-2.16)	0.959
	<75	12% [51/418]	-		-	
CTLA-4	≥75	45% [39/87]	5.99 (3.58-10.0)	<0.001	1.75 (0.78-3.88)	0.172
	<75	12% [51/427]	-		-	
LAG-3	≥75	32% [37/116]	3.05 (1.87-4.95)	<0.001	0.84 (0.37-1.84)	0.679
	<75	13% [53/398]	-		-	
PD-L1	≥75	39% [26/67]	3.79 (2.16-6.61)	<0.001	0.91 (0.36-2.14)	0.826
	<75	14% [64/447]	-		-	
PD-L2	≥75	55% [55/100]	13.2 (7.89-22.6)	<0.001	9.63 (4.91-19.4)	<0.001 (High TIM-3 is more common in tumors with high PD-L2)
	<75	8% [35/414]	-		-	
TIGIT	≥75	45% [45/99]	6.85 (4.16-11.4)	<0.001	2.12 (0.90-4.90)	0.081
	<75	11% [45/415]	-		-	
TNFRSF14	≥75	25% [27/106]	1.87 (1.11-3.10)	0.017	1.17 (0.58-2.33)	0.651
	<75	15% [63/408]	-		-	
VISTA	≥75	35% [58/166]	5.30 (3.29-8.68)	<0.001	2.71 (1.43-5.13)	0.002 (High TIM-3 is more common with high VISTA)
	<75	9% [32/348]	-		-	
Pancreatic cancer	Yes	36% [20/55]	3.18 (1.71-5.78)	<0.001	4.04 (1.75-9.36)	0.001 (High TIM-3 is more common in pancreatic cancer than in other cancers)
	No	15% [70/459]	-		-	
Small intestine cancer	Yes	25% [3/12]	1.59 (0.35-5.45)	0.493		
	No	17% [87/502]	-			
Breast cancer	Yes	24% [12/49]	1.61 (0.77-3.14)	0.180	1.85 (0.65-5.02)	0.235
	No	17% [78/465]	-		-	
Lung cancer	Yes	20% [4/20]	1.19 (0.33-3.33)	0.765		
	No	17% [86/494]	-			
Colorectal cancer	Yes	14% [19/140]	0.67 (0.38-1.14)	0.153	1.11 (0.53-2.30)	0.775
	No	19% [71/374]	-		-	
Ovarian cancer	Yes	12% [5/43]	0.60 (0.20-1.43)	0.294		
	No	18% [85/471]	-			
Esophageal cancer	Yes	0% [0/17]	0	0.979		
	No	18% [90/497]	-			

Head and neck cancer	Yes	17% [2/12]	0.94 (0.14-3.65)	0.938		
	No	18% [88/502]	-			
Neuroendocrine tumor	Yes	7% [1/15]	0.88 (0.20-2.71)	0.841		
	No	18% [89/499]	-			
Sarcoma	Yes	13% [3/24]	0.33 (0.02-1.67)	0.286		
	No	18% [87/490]	-			
Stomach cancer	Yes	8% [2/25]	0.66 (0.15-1.97)	0.511		
	No	18% [88/489]	-			
Unknown primary cancer	Yes	15% [2/13]	0.40 (0.06-1.37)	0.215		
	No	18% [88/501]	-			
Uterine cancer	Yes	13% [3/24]	0.85 (0.13-3.25)	0.838		
	No	18% [87/490]	-			
TMB (muts/Mb)	≥10	18% [6/33]	1.27 (0.46-3.02)	0.610		
	<10	15% [62/417]	-			
PD-L1 IHC	Positive (≥1%)	19% [30/156]	1.18 (0.72-1.90)	0.507		
	Negative	17% [60/357]	-			
MSI	High	33% [5/15]	2.41 (0.73-6.97)	0.118	1.86 (0.41-7.54)	0.402
	Not high	17% [80/465]	-			

*In the table, values with *p* values <0.2 in univariate analysis were selected for multivariate analysis; in the Forest plot below the table, all variables were selected for multivariate analysis. Abbreviations: BTLA, B and T Lymphocyte Attenuator; CI, confidence interval; CTLA-4, Cytotoxic T-Lymphocyte Antigen 4; IHC, immunohistochemistry; LAG-3, Lymphocyte Activation Gene 3; MSI, microsatellite instability; mut/Mb, mutation per megabase of DNA; PD-L1, Programmed Death Ligand 1; PD-L2, Programmed Death Ligand 2; TIGIT, T-cell immunoglobulin and ITIM domain; TIM-3, T-cell immunoglobulin and mucin domain-containing protein 3; TMB, tumor mutational burden; TNFRSF14, Tumor Necrosis Factor Receptor Superfamily Member 14; VISTA, V-domain Ig Suppressor of T cell Activation.

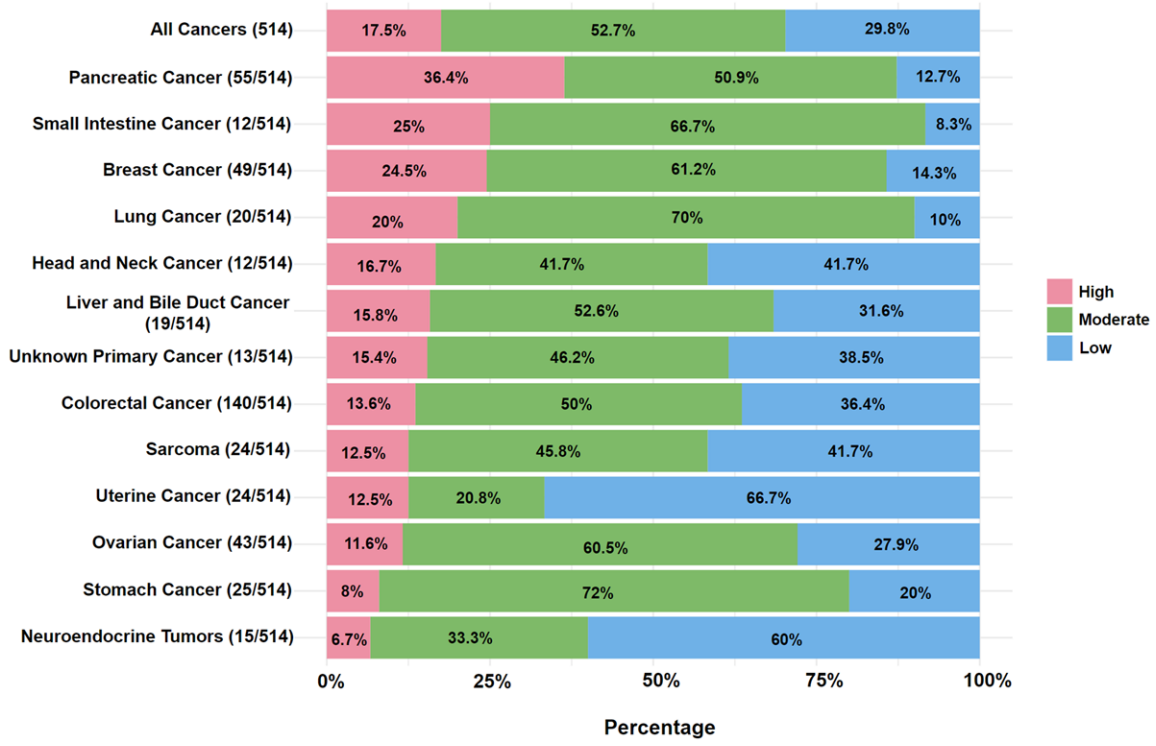

Figure 2. Proportion of patients with "High" (75-100 percentile RNA rank), "Moderate" (25-74 percentile RNA rank), and "Low" (0-24 percentile RNA rank) RNA expression of TIM-3 RNA stratified by cancer types. The designated variables and histologies are based according to the latest review conducted as of September 2023.

high expressors (36% of 55 patients); neuroendocrine tumors had the lowest proportion of high TIM-3 RNA expressors (7% of 15 patients). High TIM-3 expression was independently and significantly correlated with pancreatic cancer (multivariate odds ratio (OR) 95% confidence interval (CI) =4.04 (1.75-9.36) (P=0.001)) (**Table 1**). Still, there was variability within cancer types with, for instance, 12.7% of pancreatic cancers expressing low TIM-3 levels (<25th percentile rank) (**Figure 2**).

High TIM-3 RNA correlated with high PD-L2 and VISTA checkpoints

High TIM-3 expression independently and significantly correlated with high PD-L2 RNA expression (odds ratio (OR) 9.63, 95% confidence interval (CI) 4.91-19.4, P<0.001) and high VISTA RNA expression (OR 2.71, 95% CI 1.43-5.13, P=0.002), all in multivariate analysis, when assessed as a dichotomized variable (high RNA versus not high) (**Figure 3**). TIM-3 also correlated with PD-L2 and with VISTA when assessed as a linear variable (**Figure 4**) (TIM-3 vs. PD-L2, Pearson R^2 is 0.48 (P<0.001); TIM-3 vs. VISTA, Pearson R^2 is 0.30 (P<0.001)). High TIM-3 transcript expression did not correlate with TMB≥10 muts/Mb, MSI-H or PD-L1 IHC positivity (**Table 1**). Finally, PD-L2 and VISTA checkpoints correlated linearly with each other (Pearson's R^2, 0.27 (P<0.001)), data not shown). The analysis included the following immunomic biomarkers: BTLA (B and T Lymphocyte Attenuator), CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4), LAG-3 (Lymphocyte Activation Gene 3), PD-L1, PD-L2 (Programmed Death Ligand 2), TIGIT, TNFRSF14 (Tumor Necrosis Factor Receptor Superfamily Member 14), and VISTA (V-domain Ig Suppressor of T-cell Activation). Additional information regarding coinhibitory receptors and their corresponding ligands can be found in Supplementary Table 1.

TIM-3 RNA expression levels were not a prognostic factor for survival from the time of metastatic/advanced disease

Out of 489 patients evaluable for survival outcomes, 272 patients did not receive immunotherapy while 217 patients were treated with an immunotherapy-based regimen

Variable	Odds ratio	Estimate (95% CI)	p
BTLA high vs. not high		0.98 (0.43–2.16)	0.959
CTLA-4 high vs. not high		1.75 (0.78–3.88)	0.172
LAG-3 high vs. not high		0.84 (0.37–1.84)	0.679
PD-L1 high vs. not high		0.91 (0.36–2.14)	0.826
PD-L2 high vs. not high		9.63 (4.91–19.41)	<0.001
TIGIT high vs. not high		2.12 (0.90–4.90)	0.081
TNFRSF14 high vs. not high		1.17 (0.58–2.33)	0.651
VISTA high vs. not high		2.71 (1.43–5.13)	0.002
Pancreatic cancer vs. others		4.04 (1.75–9.36)	0.001
Breast cancer vs. others		1.85 (0.65–5.02)	0.235
Colorectal cancer vs. others		1.11 (0.53–2.30)	0.775
MSI high vs. not high		1.86 (0.41–7.54)	0.402

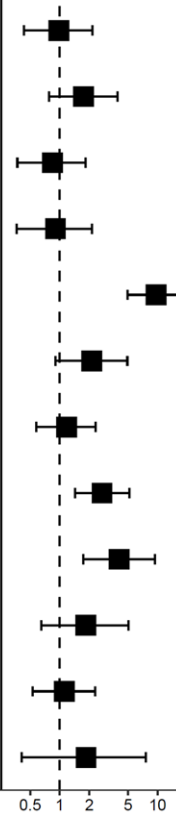

Figure 3. Forest plot for logistic regression analysis. All variables were included in the multivariate analysis. PD-L2, VISTA and pancreatic cancer correlated significantly with high TIM-3 (≥75th percentile RNA rank).

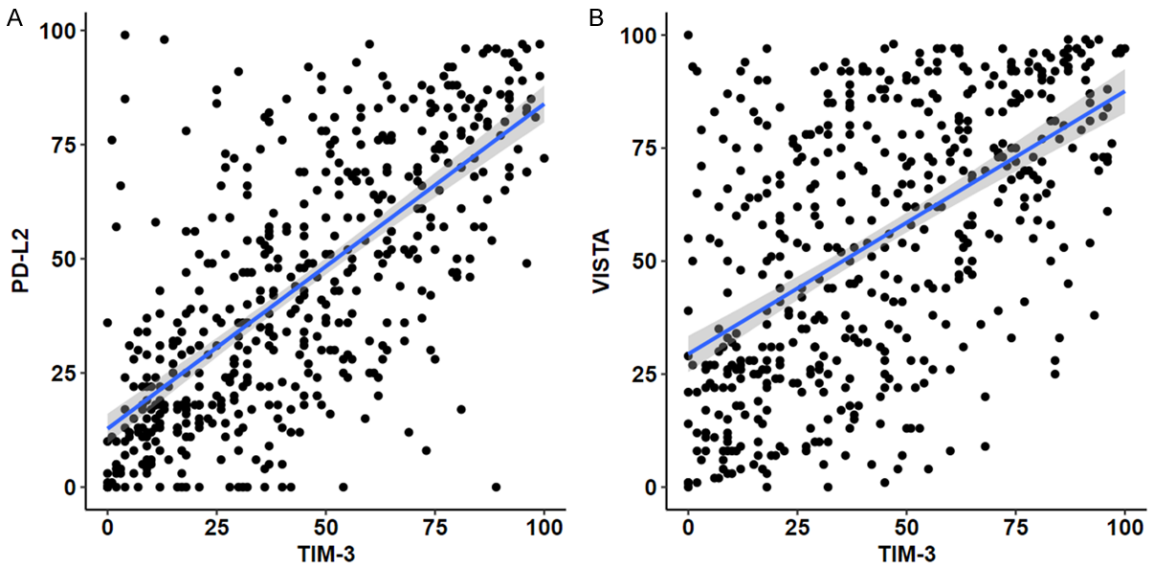

Figure 4. Scatter plots show correlation between TIM-3 and PD-L2 and VISTA RNA percentile rank score. A. TIM-3 vs. PD-L2. Pearson's R^2 is 0.48 (P<0.001). B. TIM-3 vs. VISTA. Pearson's R^2 is 0.30 (P<0.001).

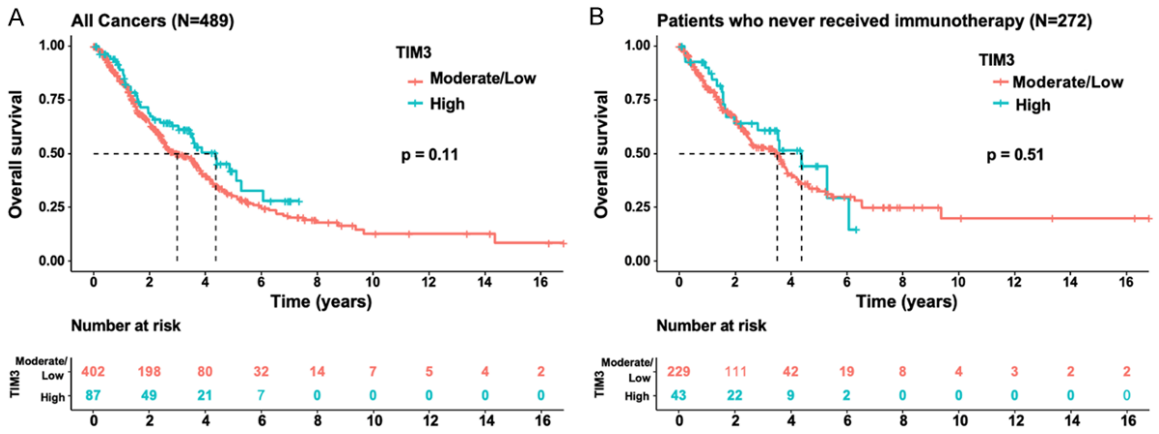

Figure 5. Kaplan-Meier analysis of TIM-3 RNA expression as a prognostic factor for outcome in cancer patients. A: Overall survival analysis for all cancer patients from date of advanced/metastatic disease to date of last follow up or death (N=489). B: Overall survival in cancer patients who never received immunotherapy from date of advanced/metastatic disease to date of last follow up or death (N=272). High TIM-3 is defined as ≥75th percentile RNA rank. High TIM-3 RNA expression level was not a prognostic factor for outcome from time of metastatic/advanced disease.

(Supplementary Figure 1). High TIM-3 RNA expression levels (≥75th percentile RNA rank) did not correlate with OS from time of metastatic disease in the complete group of 489 patients (**Figure 5A**, *p*-value 0.11) and among 272 patients who never received immunotherapy (**Figure 5B**, *p*-value 0.51). Thus, high TIM-3 expression is not a prognostic factor and does not predict a better or worse survival across cancers. Finally, we analyzed the 52 pancreatic cancer patients with survival outcome data; TIM-3 level high versus not-high had no impact on outcome from the date of advanced/metastatic disease (P=0.62) (data not shown).

High TIM-3 transcript levels correlated with overall survival, but not progression-free survival after immune checkpoint blockade in univariable analysis

TIM-3 levels and immunotherapy outcomes were assessed from the first day of the first immunotherapy treatment. Among the 217 patients treated with immunotherapy (immune checkpoint blockade) there was no significant correlation between TIM-3 expression level and PFS (**Figure 6A**, *p*-value 0.19). However, higher TIM-3 expression levels correlated with longer OS in these patients (**Figure 6B**, *p*-value 0.0033); median OS of 2.84 years in high (≥75th RNA percentile rank) TIM-3 compared to 1.21 years in moderate/low (<75th percentile RNA rank) TIM-3 expression patients. However, the correlation between high TIM-3 expression and OS was not retained in multivariable analysis (not shown). Although pancreatic cancer was associated with high TIM-3, only 16 patients received ICIs, precluding a statistical analysis of high versus not-high TIM-3 groups for outcome.

Discussion

We used transcriptomics to interrogate the TIM-3 checkpoint, an important co-inhibitory molecule for several cell types such as CD4^{+} T-cells, CD8^{+} T-cells, FoxP3^{+} Treg (forkhead box P3 positive regulatory T-cells), FoxP3^{-} Tr1 (forkhead box P3 negative type 1 regulatory) cells, NK (natural killer) cells, DCs (dendritic cells), and MDSCs (myeloid derived stem cells), as well as other immunoregulatory mediators [8]. We observed that TIM-3 expression was high (≥75th percentile RNA rank) in 17.5% of 514 advanced/metastatic malignancies, with the highest proportion of tumors having high expression being pancreatic (36% of tumor tissue samples), small intestinal (25%), breast (24%) and lung (20%) cancers. Importantly, the correlation between high TIM-3 and pancreatic cancer was significant and independent of other factors in multivariable analysis. Prior investigators have also shown high TIM-3 (by immunohistochemistry) in pancreatic cancer, supporting our observations at the transcript level [29]. Furthermore, despite the efforts to neutralize immune inhibitory receptors by a single agent or combinatorial immune check-

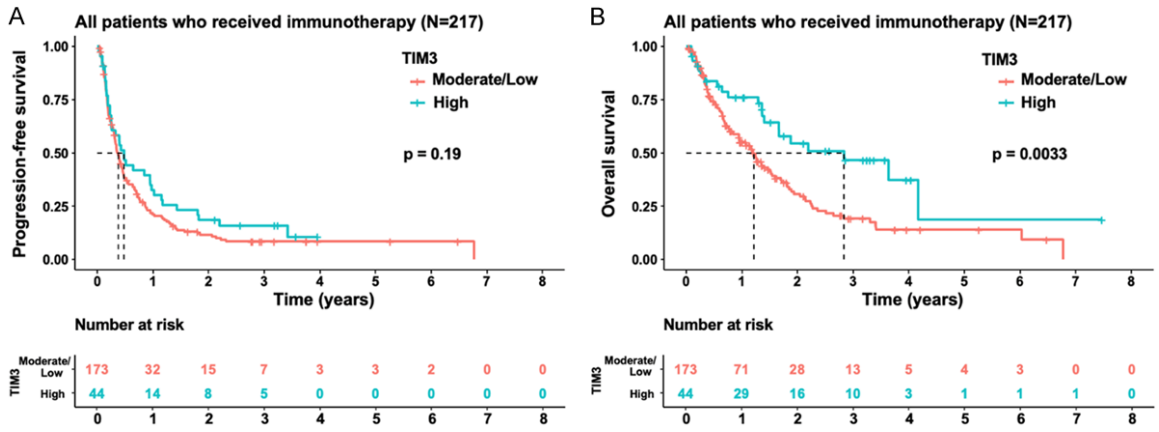

Figure 6. Kaplan-Meier analysis of progression-free survival (PFS) and overall survival (OS) in 217 cancer patients who received immunotherapy. High TIM-3 RNA expression level was predictive of a longer OS but not PFS in patients who received immune checkpoint blockade. High TIM-3 means ≥75th percentile RNA rank; moderate/low TIM-3 means <75th percentile RNA rank. A: PFS among all cancer patients who received immunotherapy from first day of first course of immunotherapy to the date of earliest progression or death from any cause. B: OS in all cancer patients who received immunotherapy from first day of first course of immunotherapy to the date of death from any cause.

point blockade, pancreatic cancer remains mainly unresponsive to these therapies [30] with only a few exceptions [31] suggesting that multiple molecular mechanisms play a role in immune evasion in pancreatic cancer. It is plausible that co-targeting with anti-TIM-3 agents might be necessary in pancreatic cancer [12]. Even so, there was variability of transcriptomic expression even within cancer types with, for instance, 12.7% of pancreatic cancers expressing low TIM-3 levels (<25th percentile rank). The individual variability is consistent with the notion that diagnostic interrogation of individual cancers is required in order to determine their immunomic profile [32].

Importantly, high TIM-3 also correlated with high levels of multiple other checkpoints, and the correlation remained significant and independent (in multivariate analysis) for the association between high TIM-3 and high expression of the checkpoints PD-L2 and VISTA. These data add support to the concept that co-targeting co-expressed checkpoints might be necessary for optimized immunotherapy and that immunogram interrogation will be critical to such efforts.

We also examined the relationship between TIM-3 outcomes, both as a prognostic factor as well as a predictive factor for immune checkpoint blockade outcome. Prior studies emphasize a complex biological role for TIM-3, and a meta-analysis showed that TIM-3 protein overexpression was correlated with poor survival of cancer patients, as well as with lymph node metastases and higher tumor grade; significant correlations between high TIM-3 expression and poor survival was especially observed for patients with non-small cell lung cancer and gastric cancer [9, 33, 34]. In another study, high CD3 and ICOS (Inducible T-cell Co-stimulator) and low TIM-3 expression predict favourable survival in resected oesophageal squamous cell carcinoma [35]. Yet, in a different meta-analysis, the results were more nuanced and a bit divergent: high TIM-3 expression was associated with poor prognosis in osteosarcoma, gastric cancer, liver cancer, esophageal cancer, and lymphoma, while no prognostic significance was detected for TIM-3 expression in lung cancer, kidney cancer, or breast cancer [36]. In our current study examining 489 patients with advanced/metastatic disease and clinical annotation, TIM-3 did not emerge as a prognostic factor, either for the full patient set or for the subgroup of 272 patients that were never exposed to immunotherapy. Moreover, TIM-3 expression also did not predict survival from advanced/metastatic disease in the 52 patients with pancreatic cancer. Differences between our study and prior studies regarding prognostic impact of TIM-3 high expressors could be due to the fact that we measured mRNA expression, while some

prior studies examined protein level (immunohistochemistry) expression, but it should also be noted that, as mentioned above, several prior studies also showed results inconsistent with each other.

Perhaps unexpectedly, high TIM-3 transcript levels did predict a better outcome after immunotherapy across cancers with an improved OS from the time of initiation of first immune checkpoint blockade treatment (*p*-value 0.0033), but the impact on PFS was not significant (*p*-value 0.19). The discordance between early tumor-based end points such as PFS and OS has been discussed in a recent publication by the FDA; the FDA noted that it has evaluated multiple trials in which a clinically important OS advantage had been demonstrated without substantive improvements in PFS or objective response rates, especially in the immunotherapy space [37]. The FDA further commented that, unlike conventional cytotoxic drugs where the relationship between early end points and OS has been more consistently observed, the unique mechanism of action of the immune checkpoint inhibitors may alter tumor growth kinetics rather than solely act via direct cytotoxicity, hence accounting for this disconnect [37-39].

Our study has several limitations. First, this investigation was conducted using data from a single institution. Additionally, certain subgroups of patients had small sample sizes, which limited our ability to detect statistical correlations in specific tumor types. Moreover, the use of immunotherapy in different treatment lines and across various cancer types introduces factors such as variations in tumor microenvironment and signaling pathways, which could influence the expression of TIM-3 and affects immunotherapy outcomes. Also, it is important to consider that TIM-3 expression might differ between primary tumors and metastatic sites [40]. Our study analyzed RNA expression of immune checkpoint receptors without information on immunostaining or annotated genomic profiles of individual tumors. We further acknowledge our limitation in RNA expression analysis at different timepoints in the patient treatment course. Finally, among our 52 pancreatic cancer patients, although TIM-3 expression was not a prognostic factor, there were not enough patients with pancreatic cancer treated with ICIs to analyze the impact of TIM-3 levels on immunotherapy outcome. This should be the subject of further analysis. A comprehensive analysis of individual tumor immune microenvironment and identification of phenotypic variations in correlation to TIM-3 expression is warranted.

In conclusion, TIM-3 RNA expression was associated with improved OS but not PFS in univariable analysis of patients treated with immunotherapy. This effect was not a prognostic one since there was no correlation between TIM-3 levels and OS in patients who never received immune checkpoint blockade. High TIM-3 expression was most frequent among pancreatic cancer patients but varied between and within tumor types. Perhaps importantly, high TIM-3 levels correlated with high levels of other checkpoints, and the correlation with PD-L2 and VISTA was retained as significant and independent in multivariate analysis. The latter observation may explain why the predictive effect of TIM-3 on OS after immunotherapy was significant only in univariate and not multivariate analysis. It is plausible that, in order to enable a precision immunotherapy approach, immunogram interrogation is necessary for each patient's tumor in order to co-target checkpoints such as TIM-3 and PD-L2 or TIM-3 and VISTA, which may be co-expressed in some cancers and could lead to resistance to anti-PD-1/PD-L1 agents [41]. Further elucidation of the role of TIM-3 and other checkpoints as a biomarker, particularly among a larger cohort of pancreatic cancer patients, can be achieved through prospective clinical trials that include TIM-3 expression as a criterion for enrollment eligibility and for correlative analysis.

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Disclosure of conflict of interest

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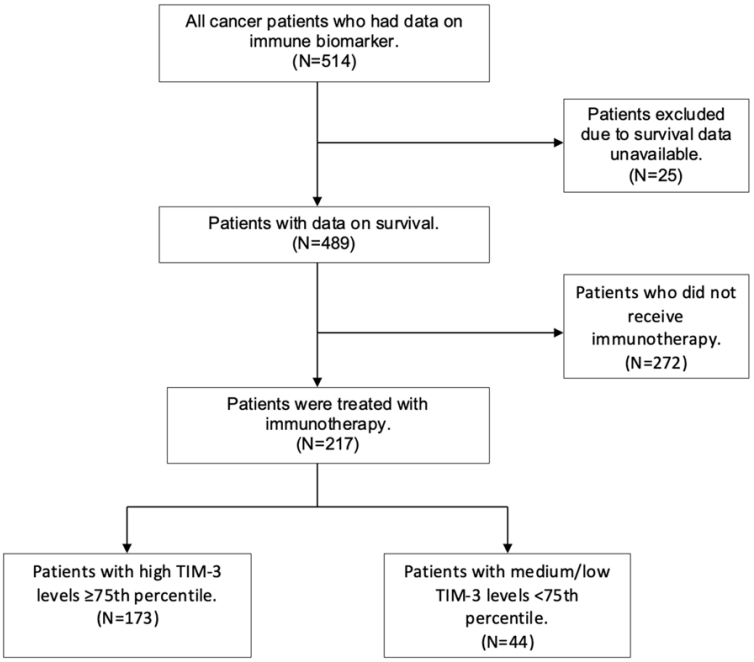

Supplementary Figure 1. Patient flow diagram. N, number of patients; TIM-3, T-cell immunoglobulin and mucin domain-containing protein 3.

Supplementary Table 1. Immune checkpoint inhibitory receptors

Immune checkpoint protein	Other Names	Class	Mechanism	Major Ligands
BTLA	CD272, BTLA1	Immunoglobulin superfamily	Inhibitory receptor	HVEM
CTLA-4	CD152	Immunoglobulin superfamily	Inhibitory receptor	B7 family ligands: CD80, CD86
LAG-3	CD223	Immunoglobulin superfamily	Inhibitory receptor	Class II MHC, FGL-1
PD-1	CD279	Immunoglobulin superfamily	Inhibitory receptor	B7 family ligands: PD-L1 (B7-H1 or CD274), PD-L2 (B7-DC, CD273)
TIGIT	WUCAM, VSTM3, VSIG9	PVR family, Immunoglobulin superfamily	Inhibitory receptor	CD155 (PVR, Necl-5), CD112 (PVRL2, Nectin-2)
TIM-3	CD366, HAVCR2	TIM family, Immunoglobulin superfamily	Inhibitory receptor	Galectin-9, HMGB1, CEACAM1, phosphatidyl serine (PtdSer)
TNFRSF14	CD270, HVEM, LIGHTR	TNF receptor superfamily	Receptor for inhibitory signaling pathways	TNFSF14/LIGHT, homotrimeric lymphotoxin-alpha, BTLA, CD160
VISTA	B7-H5, VSIR	B7 family ligands, Immunoglobulin superfamily	Inhibitory receptor	VSIG3, PSGL-1

Abbreviations: BTLA, B and T Lymphocyte Attenuator; CD, cluster of differentiation; CEACAM-1, Carcinoembryonic antigen-related cell adhesion molecule 1; CTLA-4, Cytotoxic T-Lymphocyte Antigen 4; FGL-1, fibrinogen-like protein 1; HAVCR2, hepatitis A virus cellular receptor 2; HMGB1, high mobility group protein B1; HVEM, Herpesvirus entry mediator; LIGHT, homologous to lymphotoxin, exhibits inducible expression and competes with HSV glycoprotein D for binding to herpesvirus entry mediator, a receptor expressed on T lymphocytes; LAG-3, Lymphocyte Activation Gene 3; MHC, major histocompatibility complex; Necl-5, Nectin like molecule 5; PD-1, Programmed Death Receptor 1; PSGL-1, P-selectin glycoprotein ligand 1; PVR, poliovirus receptor; PVRL2, Poliovirus receptor-related 2; TIGIT, T-cell immunoglobulin and ITIM domain; TNFRSF14, Tumor Necrosis Factor Receptor Superfamily Member 14; TIM-3, T-cell immunoglobulin and mucin domain-containing protein 3; VISTA, V-domain Ig Suppressor of T-cell Activation; VSIG, V-set and immunoglobulin domain containing protein; VSIR, V-set immunoregulatory receptor; VSTM, V-set and transmembrane containing protein.