



Research Article

Differences in Co-Expression of T Cell Co-Inhibitory and Co-Stimulatory Molecules with PD-1 Across Different Human Cancers

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Citation: Tarhini AA, Hedges D, Tan AC, Rodriguez P, Sukrithan V, et al. (2024) Differences in Co-Expression of T Cell Co-Inhibitory and Co-Stimulatory Molecules with PD-1 Across Different Human Cancers. J Oncol Res Ther 9: 10224. DOI: 10.29011/2574-710X.10224.

Received Date: 30 May, 2024; **Accepted Date:** 06 June, 2024; **Published Date:** 10 June, 2024

Abstract

Purpose: The promise of immune checkpoint inhibitor (ICI) therapy underlines the importance of comprehensively investigating the rationale for combinations with diverse immune modulators across different cancer types. Given the progress made with PD1 blockade to date, we examined mRNA co-expression levels of PD-1 with 13 immune checkpoints, including co-inhibitory receptors (LAG3, CTLA4, PD-L1, TIGIT, TIM3, VISTA, BTLA) and co-stimulatory molecules (CD28, OX40, GITR, CD137, CD27, HVEM), using RNA-Seq by Expectation-Maximization (RSEM). **Methods:** We analyzed real-world clinical and transcriptomic data from the Total Cancer Care Protocol (NCT03977402) and Avatar® project of patients with cancer treated within the Oncology Research Information Exchange Network (ORIEN) network. Using anti-PD1 as a backbone, we intended to investigate the rationale for combinations in different cancers. Pearson's R coefficients and associated P-values were calculated using SciPy 1.7.0. **Results:** The co-expression of PD1 with 13 immune checkpoints and PD-L1 varies across selected malignancies included. In cutaneous melanoma, PD1 expression correlated significantly with four co-inhibitory receptors (LAG3, TIM3, TIGIT, VISTA) and one co-stimulatory molecule (CD137). In urothelial carcinoma, PD1 expression significantly correlated with four co-inhibitory (TIGIT, CTLA4, LAG3, VISTA) and four co-stimulatory (OX40, CD27, CD137, HVEM) molecules. In pancreatic adenocarcinoma, only CD28 showed a significant correlation with PD1 expression. No significant correlations with PD1 expression were found in the ovarian cancer cohort. Notably, melanoma and urothelial carcinoma exhibited a dominant co-expression of co-inhibitory molecules with PD1, indicative of exhausted T cells, in contrast to the co-stimulatory molecule dominance in ovarian and pancreatic cancers, suggesting less differentiated T cells. **Conclusions:** Our findings highlight the potential for diverse combination strategies in immunotherapy, particularly with PD1 blockade, across various cancers.

Keywords: Checkpoints; Co-Inhibitory; Co-Signaling; Co-Stimulatory; PD-1 Co-Expression; Transcriptomics

Trial Registration: NCT03977402

Introduction

Cutaneous melanoma, ovarian cancer, pancreatic adenocarcinoma, and urothelial carcinoma rank among the top 10 prevalent cancers for estimated new cancer cases in the United States in 2023. The five-year survival rates for these cancers vary dramatically, with cutaneous melanoma and urothelial carcinoma tending to have superior outcomes, which can be partly explained by their likelihood of response to immunotherapy [1]. Targeting immune checkpoints, cytotoxic T-lymphocyte antigen-4 (CTLA-4), and programmed death/ligand 1 (PD-1/PD-L1) has made a significant impact in the treatment of melanoma and urothelial carcinoma with limited activity seen in ovarian cancer, pancreatic adenocarcinoma [2]. These differences can be explained in part by the unique characteristics of the tumor microenvironment (TME) that are crucial in the oncogenesis and immune resistance of these cancer types [3]. Particularly, specifics of the TME may inform the tumor's capacity to evade immune surveillance through immunosuppressive cellular elements and cytokines and the expression of modulatory immune checkpoint molecules [4].

Checkpoint molecules are cell-surface molecules that transmit signals to T cells to either enhance (stimulatory checkpoint proteins) or suppress (inhibitory checkpoint proteins) T cell receptor (TCR) signaling. Among stimulatory checkpoint proteins are tumor necrosis factor family molecules, CD28, CD40, OX40, and CD27,

while CTLA-4, lymphocyte-activation gene 3 (LAG3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), V-domain Ig suppressor of T cell activation (VISTA), T-cell immunoglobulin and mucin-domain containing-3 (TIM3), molecules B and T Lymphocyte Associated (BTLA) are among inhibitory checkpoint molecules [5].

T cell co-signalling receptors, responsive to the specific tissue environment, interact with cells through their ligands or counter-receptors. PD-1, a member of the immunoglobulin superfamily and present in T, B, and myeloid cells, plays a crucial role in regulating T cell activation during inflammatory responses and preventing autoimmunity [6-12]. Specifically, chronic PD-1 expression following T cell activation can lead to the inhibition of kinases involved in T cell activation and lead to exhaustion [11, 13]. This mechanism significantly contributes to immune resistance within the TME [14-16]. Therefore, PD-1 expression indicates an active host anti-tumor immune response in this context, although recent studies have indicated variable patterns of PD-1 expression and function across different cancer types [17-19].

Historically, the expression of co-signaling receptors has not been explored widely in cancer. In two studies, prior research primarily focused on the B7 family as co-stimulatory and co-inhibitory molecules correlated with T cells. In 1998, Chong et al. [20] demonstrated that the *in vivo* production of B7 could augment systemic immunity against immunogenic tumors such as melanoma. Later, in 2011, Binfeng Lu and colleagues [21] found that inhibitory B7 molecules such as B7-H1 and B7-H4 in esophageal and gastric cancer tissue were associated with adverse

clinical features and reduced survival.

Immune checkpoint inhibitors (ICIs) have been proven to be the most efficient therapy for a number of locally advanced or metastatic malignancies, consistently yielding prolonged favorable outcomes whether administered as monotherapies or combined with other treatments. Nevertheless, a considerable subset of patients demonstrate suboptimal responses, and some experience early disease progression resulting from a variety of factors that promote tumor immune resistance [22]. Beyond PD-1, additional immune checkpoints are pivotal in facilitating immune activation or inhibition. The recent approval of relatlimab, an anti-LAG-3 ICI, in combination with nivolumab for treating metastatic melanoma, emphasizes the value of continued extensive investigations into co-signaling receptors. This development highlights the significance of LAG3 inhibition in immunotherapy and as a candidate for future immunotherapeutic combinations along with PD1 [23]. Therefore, in an effort to explore candidate targets for combinatorial approaches along with PD1, we investigated the co-expression of co-inhibitory (BTLA, CTLA-4, PD-L1, LAG3, TIGIT, TIM3, VISTA) and co-stimulatory molecules (CD28, CD27, CD137, Glucocorticoid-Induced TNFR-Related protein (GITR), Herpesvirus Entry Mediator (HVEM) (TNFRSF14), OX40) with PD1 across four human cancer types that vary by the immunogenicity of the TME and the likelihood of response to immunotherapy.

Materials and Methods

Patient Cohort and Data Collection

The study utilized a combination of real-world clinical and gene expression profiles retrospectively collected under the Total Cancer Care® (TCC) Protocol (NCT03977402) and the Avatar® project within the Oncology Research Information Exchange Network (ORIEN) comprising 18 cancer centers in collaboration [24, 25]. The dataset focuses on cancer patients aged 18 and older who had never received any cancer-related medications or radiation, using the complete history available at the time of specimen collection. The protocol included the acquisition of tumor, blood, and/or fluid specimens collected as a part of the routine clinical care. Data curation spanned from the point of patient registration in ORIEN to the initiation of this study. All participants provided written consent for the genetic analysis of their germline and tumor cells

and the ongoing collection of their clinical information. The study was conducted according to the ethical standards of the Declaration of Helsinki and with approval of the Institutional Review Board (IRB) from each center.

RNA Sequencing and Acquisition

RNA sequencing was conducted to analyze gene expression relevant to our research focus, following protocols, as detailed in a previously published white paper at (<https://www.asterinsights.com/white-paper/renal-cell-carcinoma-rwd-data/>). The RNA expression data were retrieved from the ORIEN database, involving numerous FASTQ file downloads for additional analysis.

RNA Gene Expression Quantification

The quantification of gene expression entailed several technical stages. Initially, the Bbduk software (version 38.96) was utilized for trimming adapter sequences from RNA-seq reads, available at "<https://sourceforge.net/projects/bbmap/>" [26]. The alignment of these reads was against the human reference genome (CRCH38/hg38) using the STAR software (version 2.7.3a), which can be found at "<https://github.com/alexdobin/STAR>" [27]. The quality of RNA data was assessed using RNA-Seq Quality Control (RNA-SeQC) software (version 2.3.2), available at "<https://github.com/getzlab/rnaseqc>" [28]. Gene expression levels were then quantified in terms of Transcripts Per Million (TPM) based on the alignment with the GeneCode build version 32 reference annotation, employing the RNA-Seq by Expectation Maximization (RSEM) software (version 1.3.1) at "<https://github.com/deweylab/RSEM>" [29]. Subsequently, the TPM values were logarithmically transformed after adding +1 to convert them to linear scaling in Log2 (TPM+1). Batch variations were corrected using the ComBat function within the sva package (version 3.34.0) at "<https://doi.org/doi:10.18129/B9.bioc.sva>" [30].

Gene Selection and Data Extraction

Individual sample TPM values were collected into a single master table, where rows represent genes and columns indicating RNA-Seq identifiers (SLIDs). Subsequently, expression values of 13 key co-signaling genes were extracted using the "Linux grep" command to produce a smaller table for downstream data mining. The complete list of co-signaling proteins and their genes is summarized in Table 1.

Table 1: Selected co-inhibitory and co-stimulatory immune checkpoint molecules and their corresponding genes.

Immune Checkpoint Molecule	Gene	T cell Signaling
PD-1	PD-1	Co-inhibitory
BTLA	BTLA	Co-inhibitory
CTLA-4	CTLA-4	Co-inhibitory
LAG3	LAG3	Co-inhibitory
PD-L1	PD-L1	Co-inhibitory
TIGIT	TIGIT	Co-inhibitory
TIM3	HAVCR2	Co-inhibitory
VISTA	VSIR	Co-inhibitory
CD27	CD27	Co-stimulatory
CD28	CD28	Co-stimulatory
CD137	TNFRSF9	Co-stimulatory
GITR	TNFRSF18	Co-stimulatory
HVEM	TNFRSF14	Co-stimulatory
OX40	TNFRSF4	Co-stimulatory

BTLA; B and T lymphocyte associated, CD; cluster of differentiation, CTLA-4; cytotoxic T-lymphocyte associated protein 4, GITR; glucocorticoid-induced TNFR-related protein, HAVCR2; hepatitis A virus cellular receptor 2, HVEM; herpesvirus entry mediator, LAG3; lymphocyte-activation gene 3, OX40; OX40 receptor, PD-1; programmed death-ligand 1, PD-L1; programmed death-ligand 1, TIGIT; T cell immunoreceptor with Ig and ITIM domains, TIM3; T-cell immunoglobulin and mucin-domain containing-3, TNFRSF4/9/14/18; TNF Receptor Superfamily Member 4/9/14/18, VISTA; V-domain Ig suppressor of T cell activation, VSIR; V-set immunoregulatory receptor

Study Outcome

The primary study outcome involved analyzing mRNA co-expression levels of PD-1 with immune checkpoints, utilizing RSEM to elucidate their roles in various cancers.

Statistical Analysis

Pearson's correlation analysis was applied to assess the correlation between PD-1 mRNA expression and co-signaling molecules mRNA expression. A Person's correlation coefficient (r) >0.5 and a P-value (p) $< 1 \times 10^{-10}$ were established as criteria for significance. Statistical analysis was conducted using SciPy 1.7.0 software.

Results

Patients Characteristics

As shown in Table 2, 1892 patients were included in our analysis, including 232 with cutaneous melanoma, 664 with ovarian cancer, 647 with pancreatic adenocarcinoma, and 349 patients

with urothelial cancer. The mean and standard deviation (SD) of age for the total cohort was 62 ± 13 years. Excluding ovarian cancer, the majority of patients were male in the other cancer types. Approximately, more than 90% of participants were non-Hispanic white in all cancer types. Cancer stage III at diagnosis was the highest among other stages in all cancer types, except in pancreatic adenocarcinoma where around half of the participants were first diagnosed as stage II. Within the ovarian cancer cohort included in our analysis, the majority of cases were of the epithelial subtype, constituting 78% ($n=515$) and mixed epithelial and mesenchymal at 17% ($n=114$), while the remaining 5% ($n=35$) included sex-cord stromal, germ cell and other miscellaneous types. Within the epithelial subtype, serous carcinoma was the most prevalent epithelial sub-classification, accounting for 75% ($n=387$) of epithelial cases and 58% of all ovarian cancer cases. Other epithelial subtypes included endometrioid (9%, $n=48$), clear cell (5%, $n=24$), and mucinous carcinomas (3%, $n=14$), with the remaining categorized as other carcinomas (8%, $n=40$).

Table 2: Patients' Demographics and Characteristics.

Characteristic	Total (N= 1892)	Cutaneous melanoma (N=232)	Ovarian cancer (N= 664)	Pancreatic adenocarcinoma (N= 647)	Urothelial carcinoma (N= 349)
Age in years					
Mean ± SD	62 ± 13	59 ± 14	59 ± 13	63 ± 13	68 ± 11
Sex, n (%)					
Female	1141 (60.3)	89 (38.4)	664 (100)	301 (46.5)	87 (24.9)
Male	751 (39.7)	143 (61.6)	0 (0)	346 (53.5)	262 (75.1)
Ethnicity, n (%)					
Hispanic	94 (5.0)	9 (3.9)	39 (5.9)	32 (4.9)	14 (4.0)
Non-Hispanic	1749 (92.4)	217 (93.5)	618 (93.1)	603 (93.2)	311 (89.1)
Unknown	49 (2.6)	6 (2.6)	7 (1.1)	12 (1.9)	24 (6.9)
Race, n (%)					
African American	55 (2.9)	1 (0.4)	21 (3.2)	23 (3.6)	10 (2.9)
American Indian or Alaska Native	11 (0.6)	1 (0.4)	8 (1.2)	2 (0.3)	0 (0)
Asian	19 (1.0)	0 (0)	9 (1.4)	7 (1.1)	3 (0.9)
Native Hawaiian or Other Pacific Islander	2 (0.1)	0 (0)	2 (0.3)	0 (0)	0 (0)
White	1757 (92.9)	226 (97.4)	610 (91.9)	601 (92.9)	320 (91.7)
Other	24 (1.3)	1 (0.4)	6 (0.9)	7 (1.1)	10 (2.9)
Unknown	24 (1.3)	3 (1.3)	8 (1.2)	7 (1.1)	6 (1.7)
Cancer stage at initial diagnosis, n (%)					
Stage I	282 (14.9)	25 (10.8)	97 (14.6)	117 (18.1)	43 (12.3)
Stage II	527 (27.9)	48 (20.7)	74 (11.1)	350 (54.1)	55 (15.8)
Stage III	526 (27.8)	73 (31.5)	292 (44.0)	47 (7.3)	114 (32.7)
Stage IV	324 (17.1)	43 (18.5)	129 (19.4)	77 (11.9)	75 (21.5)
Unknown	193 (10.2)	43 (18.5)	72 (10.8)	36 (5.6)	42 (12.0)
Performance status (ECOG), n (%)					
0	386 (20.4)	39 (16.8)	140 (21.1)	141 (21.8)	66 (18.9)
1	282 (14.9)	11 (4.7)	117 (17.6)	114 (17.6)	40 (11.5)
2	48 (2.5)	4 (1.7)	22 (3.3)	12 (1.9)	10 (2.9)
3	8 (0.4)	1 (0.4)	4 (0.6)	1 (0.2)	2 (0.6)
Unknown	1168 (61.7)	177 (76.3)	381 (57.4)	379 (58.6)	231 (66.2)

N; number of patients, SD; standard deviation

Overview of Analyzed Data

The overall assessment of the correlational analysis results in Table 3 suggests that the PD-1 co-expression with multiple co-inhibitory and co-stimulatory molecules had similar strength and direction in melanoma and urothelial cancers. Conversely, pancreatic adenocarcinoma exhibited a limited co-expression of other target molecules, while ovarian cancer stands out with a lack of any significant correlation.

Table 3: Co-expression of PD1 with selected co-inhibitory and co-stimulatory molecules

Molecule	Cutaneous melanoma (N 235)		Ovarian cancer (N= 725)		Pancreatic adenocarcinoma (N= 668)		Urothelial carcinoma (N= 360)	
	Pearson <i>r</i> *	<i>P</i> -value*	Pearson <i>r</i> *	<i>P</i> -value*	Pearson <i>r</i> *	<i>P</i> -value*	Pearson <i>r</i> *	<i>P</i> -value*
BTLA	0.116	7.60E-02	0.107	3.77E-03	0.341	1.35E-19	0.376	2.78E-15
CD27	0.559	1.12E-20	0.217	3.78E-09	0.42	6.71E-30	0.526	5.43E-27
CD28	0.197	2.43E-03	0.107	4.04E-03	0.524	2.22E-48	0.472	2.24E-21
CD137	0.583	7.87E-23	0.105	4.78E-03	0.283	1.00E-13	0.516	7.37E-26
CTLA-4	0.3	5.85E-14	0.105	4.52E-03	0.309	3.31E-16	0.67	6.33E-37
GITIR	0.325	3.63E-06	0.055	1.38E+01	0.296	5.37E-15	0.376	1.51E+13
HVEM	0.178	6.33E-03	0.071	5.64E-02	0.313	1.29E-16	0.506	7.74E-25
LAG3	0.646	3.87E-29	0.089	1.66E-02	0.193	4.69E-07	0.602	6.33E-37
OX40	0.134	3.99E-02	0.052	1.63E-01	0.236	6.74E-10	0.544	4.08E-29
PD-1	1	-	1	-	1	-	1	-
PD-L1	0.164	1.18E-02	0.66	7.55E-02	0.412	2.11E-30	0.186	3.92E-04
TIGIT	0.524	5.40E-18	0.109	3.40E-03	0.425	1.00E-30	0.679	5.04E-50
TIM3	0.605	3.87E-29	0.09	1.56E-02	0.372	2.42E-23	0.458	4.28E-20
VISTA	0.464	5.85E-14	0.125	7.45E-04	0.384	6.11E-25	0.526	5.85E-27

* Person's correlation coefficient $r > 0.05$ and a P -value $< 1E-10$ were considered significant correlation. BTLA; B and T lymphocyte associated, CD; cluster of differentiation, CTLA-4; cytotoxic T-lymphocyte associated protein 4, GITIR; glucocorticoid-induced TNFR-related protein, HVEM; herpesvirus entry mediator, LAG3; lymphocyte-activation gene 3, N; number of samples analyzed, OX40; OX40 receptor, PD-1; programmed death-1, PD-L1; programmed death-ligand 1, TIGIT; T cell immunoreceptor with Ig and ITIM domains, TIM3; T-cell immunoglobulin and mucin-domain containing-3, VISTA; V-domain Ig suppressor of T cell activation

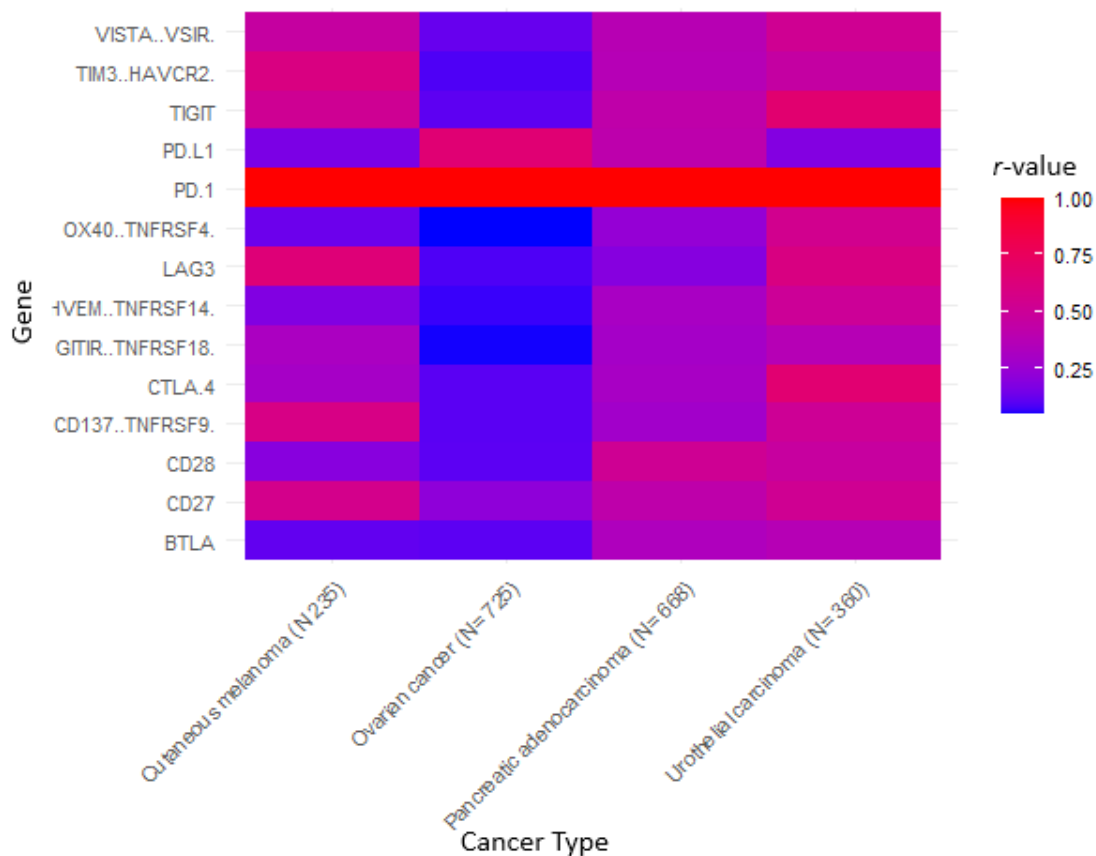
Cancer-Specific PD1 Coexpression Findings

Notably, in cutaneous melanoma, there was moderately positive significant coexpression of co-inhibitory molecules LAG3 ($r = 0.65$; $p = 3.87E-29$), TIGIT ($r = 0.52$; $p = 5.40E-18$), and TIM3 ($r = 0.61$; $p = 7.66E-25$), and co-stimulatory molecules CD27 ($r = 0.56$; $p = 1.12E-20$) and CD137 ($r = 0.58$; $p = 7.87E-23$) with PD1 expression. To some extent, a similar pattern was observed in urothelial carcinoma. Specifically, PD-1 co-expression was significantly correlated with the expression of co-inhibitory molecules CTLA-4 ($r = 0.67$; $p = 3.10E-48$), LAG3 ($r = 0.60$; $p = 6.33E-37$), TIGIT ($r = 0.68$; $p = 6.33E-37$), and VISTA ($r = 0.53$; $p = 5.85E-27$), as well as with co-stimulatory molecules CD27 ($r = 0.53$; $p = 5.43E-27$), CD137 ($r = 0.52$; $p = 7.37E-26$), HVEM ($r = 0.51$; $p = 7.74E-25$), and OX40 ($r = 0.54$; $p = 4.08E-29$). In pancreatic adenocarcinoma, only CD28 ($r = 0.52$; $p = 2.22E-48$) as a co-stimulatory molecule showed a significant, moderate positive correlation with PD1 expression. No significant correlation was observed between the expression of any investigated immune checkpoints and the PD1 expression in ovarian cancer.

Comparative Analysis

What emerges from the results reported here is that the co-inhibitory molecule TIGIT had the strongest association with PD-1 co-expression in urothelial cancer patients. Not only that, but also, urothelial carcinoma was the top cancer type with a considerable number of checkpoint molecules (eight molecules) that showed a significant expression association with PD-1 expression, evenly distributed between co-inhibitory and co-stimulatory molecules (four per each group). In melanoma, as another immunogenic tumor, we noticed that five checkpoint molecules, three co-inhibitory (LAG3, TIM3, TIGIT) and two co-stimulatory (CD137, CD27) demonstrated significant expression association with PD-1 expression. In contrast, pancreatic adenocarcinoma and ovarian cancer demonstrated either weak correlations or failed to reach the threshold for statistical significance for any of the tested immune checkpoints along with PD1 (Figure 1).

Figure 1: Heatmap of PD-1 gene co-expression with gene expression of co-inhibitory and co-stimulatory molecules in four Cancer Types.



Each cell represents the Pearson correlation coefficient (r) value between PD-1 and specific co-inhibitory or co-stimulatory genes. Gene names and molecule names (if the names are different) are displayed on the vertical axis (y-axis) if they are, and cancer types are listed on the horizontal axis (x-axis). Color intensity reflects the correlation level, with red indicating a higher correlation and blue indicating a lower correlation. BTLA; B and T lymphocyte associated, CD; cluster of differentiation, CTLA-4; cytotoxic T-lymphocyte associated protein 4, GITIR; glucocorticoid-induced TNFR-related protein, HAVCR2; hepatitis A virus cellular receptor 2, HVEM; herpesvirus entry mediator, LAG3; lymphocyte-activation gene 3, OX40; OX40 receptor, PD-L1; programmed death-ligand 1, TIGIT; T cell immunoreceptor with Ig and ITIM domains, TIM3; T-cell immunoglobulin and mucin-domain containing-3, TNFRSF4/9/14/18; TNF Receptor Superfamily Member 4/9/14/18, VISTA; V-domain Ig suppressor of T cell activation, VSIR; V-set immunoregulatory receptor.

Discussion

Co-signaling molecules are pivotal in modulating T cell activation, guiding their differentiation, orchestrating effector functions, and influencing T cell survival. These molecules are often strategically positioned alongside TCR molecules at the immunological synapse, becoming activated following the TCR's interaction with specific peptide-major histocompatibility complex (MHC) complexes presented by antigen-presenting cells (APCs). In an appropriate interaction with TCR signaling, they either amplify or attenuate T cell activation and subsequent responses. This complicated interaction results in a unique expression of co-stimulatory and co-inhibitory molecules, marked by overlapping yet distinct spatiotemporal patterns [31]. Our understanding of individual co-signaling molecules has significantly deepened, especially regarding their role in various T cell response phases. However, comprehending how these multiple pathways synergize remains an area of limited insight. The fundamental mechanics of T cell co-signaling are critical for developing advanced co-signaling-based immunotherapies. PD-1 expression, in particular, has been validated as an essential and central target when it comes to immune modulator therapies for cancer and an important backbone for future combinatorial strategies [32]. This study was designed to explore the relationship between PD-1 expression and the co-expression of T cell co-signaling molecules, a relationship pivotal to refining co-signaling immunotherapy strategies. For this purpose, we analyzed the mRNA expression patterns of PD-1 and its co-expression with various co-inhibitory and co-stimulatory molecules, utilizing Pearson's correlation coefficient as our analytical tool.

The foremost observation from our analysis elucidates that the co-expression patterns of PD-1 with both co-inhibitory and co-stimulatory molecules exhibit considerable heterogeneity across different cancer types. Notably, the coexpression of co-inhibitory molecules demonstrated a dominant presence in melanoma, while that of co-stimulatory molecules was more pronounced in urothelial cancer. In contrast, pancreatic adenocarcinoma presented a more restricted co-expression profile, characterized by a significant correlation of PD1 expression solely with CD28. Intriguingly, ovarian cancer displayed no notable correlations in this regard. This finding highlights the involved and diverse nature of immune responses across various cancer forms, thereby accentuating the critical need for tailored immunotherapy approaches specific to each cancer type. Furthermore, our study reveals a heightened state of immune activation or exhaustion, a characteristic prominently observed in both melanoma and urothelial carcinoma, which are current targets of immunotherapeutic interventions. This discussion will cover more profoundly the distinctions of our findings, mainly focusing on the roles and implications of immune-inhibitory and immuno-stimulatory molecules within the

context of these malignancies.

Our investigation revealed a notable correlation in the expression levels of LAG3 and PD-1 in both melanoma and urothelial carcinoma. LAG-3 functions as a surface receptor on activated T cells, primarily binding to MHC-II, although other ligands have been identified as well. This significant correlation could be attributed to the fact that both LAG-3 and PD-1 are co-inhibitory molecules, often indicative of T-cell exhaustion. Prolonged exposure of T cells to antigens, such as in cancer, leads to the activation of various inhibitory receptors, including LAG-3 and PD-1 [33]. These results are consistent with findings suggesting that concurrent blockage of PD-1 and LAG3 can synergistically reverse T-cell exhaustion [34, 35]. In a clinical context, this understanding has been applied in the recent approval of a combination therapy comprising anti-LAG3 relatlimab and anti-PD-1 nivolumab, specifically for the treatment of patients with metastatic melanoma [23].

Another important finding from our study is the moderate positive correlation between TIGIT and PD-1. TIGIT, a transmembrane protein receptor, operates as an immunological checkpoint on T and natural killer (NK) cells, facilitated by its cytoplasmic tail containing two immunoreceptor tyrosine-based inhibitory motifs (ITIM). The receptor's primary ligands, CD155 and CD112, are predominantly expressed on APCs, with nectin-2 emerging as a more recent ligand [36, 37]. This correlation aligns with prior observations where TIGIT expression was linked with PD-1 in various contexts, including mouse tumor models and human non-small cell lung cancer (NSCLC) and colon cancer [38]. This association is further corroborated by findings from Chauvin et al., who reported co-expression of TIGIT and PD-1 in advanced melanoma tumors [39]. The therapeutic potential of targeting TIGIT is emphasized by a recent phase I trial, where Tiragolumab, an anti-TIGIT humanized IgG1 monoclonal antibody inhibiting TIGIT-CD155 interaction, showed promising efficacy in patients with immunotherapy-naïve NSCLC and esophageal cancer [40].

The phase III KEYVIBE-010 study (NCT05665595), a randomized, double-blind trial, aimed to assess the effectiveness and safety of vibostolimab (Anti-TIGIT) + pembrolizumab (Anti-PD1) (MK-7684A) compared to pembrolizumab monotherapy as adjuvant therapy in patients with resected high-risk stage IIB-IV melanoma.

This study's findings reveal a moderate correlation between TIM-3 and PD-1 expression, specifically in melanoma. TIM-3, recognized as an immunoinhibitory molecule, was first identified on CD4+ Th1 (helper) and CD8+ Tc1 (cytotoxic) T-cells, and its presence was later confirmed on various other cell types [41-46]. The molecule's primary ligand, galectin-9, initiates the apoptosis of effector T-cells [47, 48]. TIM-3 expression is a marker of T cell

exhaustion, frequently observed in chronic infections [49]. This study's findings are in line with those reported by Fourcade et al. [50], who noted a similar upregulation of TIM-3 in conjunction with PD-1 expression in advanced melanoma patients. This correlation may shed light on potential efficacy observed in preclinical and in vivo models when simultaneously targeting both PD-1 and TIM-3 pathways, as opposed to targeting each pathway individually, in the treatment of malignant tumors [50-53].

In this study, a remarkable correlation was identified between VISTA expression and PD-1 in urothelial carcinoma. VISTA, serving both as a ligand and a receptor on T cells, plays a pivotal role in inhibiting T cell proliferation and fostering the transformation of naive T cells into regulatory T cells [54]. The observed positive correlation can potentially be attributed to VISTA's structural resemblance to the B7 superfamily, positioning it as a PD1 homolog [55, 56]. The co-expression of PD-1 and VISTA in urothelial carcinoma represents a novel area of research. While the precise nature of this relationship remains challenging to elucidate, it could be linked to the strong association between PD-L1, the primary ligand of PD-1, and VISTA. A recent 2022 study proposed that a combination therapy targeting VISTA and PD-L1 might offer a new avenue for enhancing immunosuppression in cancer treatments [57].

CTLA-4 emerged as another immunoinhibitory molecule in our study, displaying a co-expression pattern with PD-1. Considered the godfather of immune checkpoints, CTLA-4 was the first clinically targeted clinical immune checkpoint receptor. It predominantly controls the initial phase of T cell activation, while PD-1 is more involved in regulating T cells within tissues and tumors [58]. The observed association between PD-1 and CTLA-4 expression might be partially attributed to the PD-1 receptor's classification within the CD28/CTLA-4 family [6]. This study's findings mirror those of the previous research, which observed parallel expression levels of CTLA-4 and PD-1 in tumor tissues, markedly exceeding those in normal tissues in patients with urothelial carcinoma [59]. Our results support the rationale for combining anti-CTLA4 (ipilimumab) with anti-PD1 (nivolumab) in urothelial carcinoma. This has been demonstrated clinically in the checkmate 032 phase I/II multicenter trial, where nivolumab 3 mg/kg plus ipilimumab 1 mg/kg every three weeks for four doses, followed by nivolumab monotherapy 3 mg/kg every two weeks showed a better objective response rate than nivolumab 3 mg/kg monotherapy every two weeks at a median follow-up of 22 months (38% vs. 26.9%) [60].

In our study, we observed a significant correlation between the expressions of CD27 and CD137, both co-stimulatory molecules, along with PD-1 expression in melanoma and urothelial carcinoma patients. These receptors are part of the tumor necrosis factor (TNF) receptor superfamily, playing crucial roles in immune response

modulation. CD27, exclusively located on lymphocytes, enhances immune function and aids in the differentiation of effector T cells [61-63]. CD137, on the other hand, is found in a broader range of immune cells, including T cells, B cells, NK cells, monocytes, and neutrophils. The activation and proliferation of these cells, mediated by CD137, contribute to augmenting the efficacy of anti-tumor therapies [64, 65]. Current early-phase clinical trials are exploring the potential of varilumab, a CD27 agonist, and utomilumab, a CD137 agonist, either as monotherapy treatments or in combination with anti-PD-1 drugs [49, 66]. A notable example is the ongoing clinical research involving CDX-527, a bispecific antibody. This antibody has demonstrated synergistic effects in mice models, effectively inhibiting PD-1 signaling while robustly triggering CD27-mediated T-cell co-stimulation via PD-L1 cross-linking [67]. This supports our finding of the positive association between CD27 and PD-1. In terms of CD137, our results align with existing studies that have identified a concurrent expression of CD137 and PD-1 on CD8+ tumor-infiltrating lymphocyte (TIL) subpopulations in melanoma [68]. Further validating the interconnection between these co-stimulatory pathways and their role in cancer immunotherapy.

Another notable finding in urothelial carcinoma is that OX40 expression directly correlates with PD-1 expression. OX40, also known as CD134 and a member of the TNF receptor superfamily, is a crucial player in T-cell activation. Its upregulation has been linked to enhanced patient outcomes [69, 70]. This particular association between OX40 and PD-1 in our study presents a contrast to the findings of Zhu et al. (2021) [71]. While Zhu et al. did not specifically focus on the direct co-expression relationship between PD-1 and OX40, their analysis of CD8+ T cells in bladder cancer patients indicated notable differences in the expression levels of CD8+ PD-1+ and CD8+ OX40+ cells. This divergence points out the complexity of immune responses in urothelial carcinoma and highlights the potential for immunotherapeutic targeting based on specific co-expression patterns.

The exploration of urothelial carcinoma in this study uncovered a significant correlation between HVEM, a co-stimulatory molecule, and PD-1 co-expression. HVEM also referred to as TNFRSF14, is a multifaceted TNFR superfamily protein capable of binding to both co-stimulatory and co-inhibitory receptors [72]. This particular correlation between HVEM and PD-1 is a novel discovery, as such an association has not been previously reported in any cancer type, either directly or indirectly.

In our analysis of pancreatic adenocarcinoma, only the expression of CD28 demonstrated a moderate positive correlation with PD-1 expression. This correlation might be related in part to PD-1+ CD8 T cells, which proliferate in response to PD-1 therapy, and express CD28 [73-75].

This research is distinguished by its extensive examination of PD-1 co-expression with a wide array of co-inhibitory and co-stimulatory molecules across four cancer types that are known to vary in terms of the immunogenicity of the TME and the likelihood of response to PD1 blockade. The use of a substantial and real-world clinical and transcriptomic dataset from the Total Cancer Care Protocol and Avatar® project that is active across 18 cancer centers within the U.S. allowed for the analysis of a diverse and robust patient population. This analysis facilitated a deeper understanding of the TME in different cancers, significantly broadening the applicability of our findings. However, the study's retrospective design and reliance on pre-existing data present certain limitations. This approach may not fully capture the complete spectrum of patient demographics or the diversity of cancer subtypes.

Moreover, the primary focus on transcriptomic data, while valuable, overlooks potential post-transcriptional modifications and protein-level interactions that are crucial in cancer development and progression. Consequently, these findings underline the need for future research, both preclinical and prospective clinical studies that can validate and build upon these results. Such studies should aim to determine the clinical impact of immunotherapies designed around specific co-expression patterns. Furthermore, while we used PD1 as a backbone in this study, a more comprehensive analysis looking for dominant immune checkpoints independent of PD1 in certain malignancies can be conducted. An immersed exploration into post-transcriptional mechanisms and protein interactions within the TME could yield further insights. Additionally, expanding the research to encompass a wider variety of cancers and a more diverse patient demographic would enhance the relevance and impact of the findings, paving the way for more personalized and effective cancer treatments.

In conclusion, melanoma and urothelial carcinoma as more immunogenic tumors reflected a PD-1 plus an immunoinhibitory dominant phenotype. The less immunogenic ovarian and pancreatic carcinomas reflected a trend toward a PD-1 plus an immunostimulatory phenotype. The dominance of the co-expression of co-inhibitory molecules in melanoma and to a certain extent in urothelial carcinoma may reflect the prevalence of late exhausted T cells, as compared to less differentiated T cells in ovarian and pancreatic carcinomas. Further validation of our findings is warranted as these may inform potential combination strategies suggested that may be effective in the tested malignancies. Future directions should focus on preclinical and clinical testing in an effort to validate these findings. This is in addition to expanding this research to include a broader range of cancers and exploring the functional impact of these co-expression patterns within the TME.

Acknowledgments

Author Contributions: Conceptualization, Ahmad Tarhini; Data curation, Ahmad Tarhini, Dale Hedges, Aik Choon Tan, Paulo Rodriguez, Vineeth K. Sukrithan, Aakrosh Ratan, Martin McCarter, John Carpten, Howard Colman, Alexandra Ikeguchi, Igor Puzanov, Susanne Arnold, Michelle Churchman, Patrick Hwu, Jose Conejo-Garcia, William Dalton, George Weiner and Islam Eljilany; Formal analysis, Ahmad Tarhini and Dale Hedges; Funding acquisition, Ahmad Tarhini; Investigation, Ahmad Tarhini and Islam Eljilany; Methodology, Ahmad Tarhini and Dale Hedges; Project administration, Ahmad Tarhini; Resources, Ahmad Tarhini; Supervision, Ahmad Tarhini; Writing – original draft, Ahmad Tarhini and Islam Eljilany; Writing – review & editing, Ahmad Tarhini, Dale Hedges, Aik Choon Tan, Paulo Rodriguez, Vineeth K. Sukrithan, Aakrosh Ratan, Martin McCarter, John Carpten, Howard Colman, Alexandra Ikeguchi, Igor Puzanov, Susanne Arnold, Michelle Churchman, Patrick Hwu, Jose Conejo-Garcia, William Dalton, George Weiner and Islam Eljilany.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (IRB; IRB# Pro00014441) of the Total Cancer Care Protocol (NCT03977402) and Avatar® project.

Informed Consent Statement: Written informed consent was obtained at the participating institutions from all subjects involved in the original study protocol within which this project was nested.

Data Availability Statement: All results relevant to this study are included in the article. Data are available upon reasonable requests to the corresponding author.

Conflicts of Interest: I.E., D.H., M.L.C., J.C., A.P.I., W.S.D., G.J.W., and P.H. declare no conflicts of interest. V.S. declares receiving research funding from Eli Lilly and Company and Crinetics Pharmaceuticals. Consulting for GE Health. Advisory Board member of Exelixis. Receiving speaking fees from Lantheus Pharmaceuticals. M.M. declares contracted research grants with Merck, Taiho, and the National Comprehensive Cancer Network. IP declares Program Leader (1.2 cal months) for NIH/NCI Cancer Center Support Grant (P30CA016056); consulting with Nouscom, Iovance, Nektar, and Regeneron; stock owner with Ideaya, Inc. H.C. declares advisory board/consultant: Best Doctors/Teladoc, Orbus Therapeutics, Bristol Meyers Squibb, Regeneron, Novocure, and PPD/Chimerix; research funding (site PI/institutional contract): Orbus, GCAR, Array BioPharma, Karyopharm Therapeutics, Nuvation Bio, Bayer, Bristol Meyer Squib, and Sumitomo Dainippon Pharma. To date, S.M.A. is a member of ASCO COI. I.M.E.N. is on the scientific advisory board of Endectra, L.L., acts as deputy editor for the Journal of Medical Physics, and receives

funding from NIH and DoD. J.R.C.G has stock options in Compass Therapeutics, Anixa Biosciences and Alloy Therapeutics; receives consulting fees from Alloy Therapeutics; has intellectual property with Compass Therapeutics and Anixa Biosciences; receives licensing fees from Anixa Biosciences; and is co-founder of Cellep Therapeutics. A.A.T. declares contracted research grants with institution from Bristol Myers Squibb, Genentech-Roche, Regeneron, Sanofi-Genzyme, Nektar, Clinigen, Merck, Acrotech, Pfizer, Checkmate, OncoSec, Scholar Rock, InflaRx GmbH, Agenus; personal consultant/advisory board fees from Bristol Myers Squibb, Merck, Eisai, Instil Bio, Clinigen, Regeneron, Sanofi-Genzyme, Novartis, Partner Therapeutics, Genentech/Roche, BioNTech, Concert AI, AstraZeneca outside the submitted work.

Funding: This work was supported by an ORIEN FOUNDATION NOVA (New Oncologic Visionary Award) Grant and Community Foundation of Tampa Bay: 69-21295-01-01 (PI, A. Tarhini). Also, partly supported by Moffitt Cancer Center National Institute of Health (NIH) and National Cancer Institute (NCI) Center Core Grant (P30) 5P30CA076292-22. AT

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