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Co-expression of PD-1 with TIGIT or PD-1 with TIM-3 on tumor-infiltrating CD8$^+$ T cells showed synergistic effects on improved disease-free survival in treatment-naïve CRC patients

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ABSTRACT

Immune checkpoints (ICs) are highly expressed on tumor-infiltrating immune cells (TIICs) in different malignancies, including colorectal cancer (CRC). T cells play crucial roles in shaping CRC, and their presence in the tumor microenvironment (TME) has proven to be one of the best predictors of clinical outcomes. A crucial component of the immune system is cytotoxic CD8$^+$ T cells (CTLs), which play decisive roles in the prognosis of CRC. In this study, we investigated associations of immune checkpoints expressed on tumor-infiltrating CD8$^+$ T cells with disease-free survival (DFS) in 45 naïve-treatment CRC patients. First, we examined the associations of single ICs, and found that CRC patients with higher levels of T-cell immunoglobulin and ITIM-domain (TIGIT), T-cell immunoglobulin and mucin domain-3 (TIM-3) and programmed cell death-1 (PD-1) CD8$^+$ T cells tended to have longer DFS. Interestingly, when PD-1 expression was combined with other ICs, there were more evident and stronger associations between higher levels of PD-1$^+$ with TIGIT$^+$ or PD-1$^+$ with TIM-3$^+$ tumor-infiltrating CD8$^+$ T cells and longer DFS. Our findings for TIGIT were validated in The Cancer Genome Atlas (TCGA) CRC dataset. This study is the first to report on the association of co-expression of PD-1 with TIGIT and PD-1 with TIM-3 in CD8$^+$ T cells and improved DFS in treatment-naïve CRC patients. This work highlights the significance of immune checkpoint expression on tumor-infiltrating CD8$^+$ T cells as critical predictive biomarkers, especially when co-expression of different ICs is considered.

1. Introduction

Colorectal cancer (CRC) is the second leading cause of death and the third most common malignancy in men and women worldwide [1]. CRC is genetically and molecularly heterogeneous, which has significant implications for the efficacy of immunotherapy. CRC patients with microsatellite instability-high (MSI-H)/deficient mismatch repair (dMMR) tumors have better prognosis, survival, and response to immunotherapy than patients with microsatellite stable (MSS)/microsatellite instability-low (MSI-L) tumors [2–4]. MSI-H CRC patients have significantly higher survival rate due to increased tumor-infiltrating lymphocytes (TILs) recruitment within tumor tissues, including activated cytotoxic CD8$^+$ T lymphocytes (CTLs), macrophages, CD4$^+$ T cells and other immune cells [5–7]. Many studies reported that increased density of CD8$^+$ TILs were associated with longer overall survival (OS) and disease-free survival (DFS) in CRC patients, suggesting that TILs could be used as indicators of

Abbreviations: CRC, Colorectal cancer; CD, Cluster of differentiation; DFS, Disease-free survival; dMMR, Deficient mismatch repair; ICs, Immune checkpoints; LAG-3, Lymphocyte-activation gene-3; MSI-H, Microsatellite Instability-High; MSS, Microsatellite stable; MSI-L, Microsatellite instability-low; NT, Normal tissue; NILs, Normal tissue-infiltrating lymphocytes; PD-1, Programmed cell death-1; TT, Tumor tissue; TME, Tumor microenvironment; TILs, Tumor-infiltrating lymphocytes; TIICs, Tumor-infiltrating immune cells; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; TIM-3, T-cell immunoglobulin and mucin domain-3.

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improved prognosis [8,9].

Immune checkpoints (ICs) have key roles in maintaining immune homeostasis through fine-tuning the extent of immune enhancing and prevention of autoimmunity [10]. ICs expressed on T cells lead to the alteration of immune responses by modulating T-cell activation pathways [11]. They have emerged as critical targets for the elicitation of potent therapeutic anti-tumor immune responses, mainly through the inactivation of inhibitory immune receptors within the TME [12].

In our recent study, we have reported that ICs, including T cell immunoreceptor with Ig and ITIM-domains (TIGIT), T-cell immunoglobulin and mucin domain-3 (TIM-3), programmed cell death-1 (PD-1), and inducible T cell costimulatory (ICOS), were significantly overexpressed on $CD8^+$ TILs, compared to normal colon tissues [13]. Additionally, we found that CRC patients at early TNM stages (stage I and II) had significantly higher levels of $PD\text{-}1^+$, $TIM\text{-}3^+$ and $TIGIT^+$ tumor-infiltrating $CD8^+$ T cells, compared to patients at advanced TNM stages (stages III and IV) [13]. Interestingly, patients with MSI-H tumors had higher levels of ICs expressed on $CD8^+$ and $CD4^+$ T cells than patients with MSS tumors [13]. In this study, we took our findings further and investigated the association of different immune checkpoints including TIGIT, TIM-3, LAG-3, and PD-1 expressed on $CD8^+$ TILs with DFS of 45 treatment-naïve CRC patients.

2. Materials and methods

2.1. Patients and samples

This study was conducted in accordance with ethical approval (protocol no. MRC-02-18-012) from the Medical Research Center, Hamad Medical Corporation, Doha, Qatar. All patients gave their informed consent before any sample collection. Tumor tissues (TT) and corresponding normal tissues (NT), as identified by the pathologist, were collected from fifty CRC patients at all TNM stages (stage I to stage IV). All CRC patients were treatment naïve and they underwent surgical resection without any neoadjuvant chemotherapy before the operation and collection of patients samples. Forty-five patients were eligible and included in the DFS analyses in this study. Clinical and pathological characteristics of the patients are described in Table 1.

2.2. Cell staining and flow cytometric analyses

Cells were isolated from TT and NT by mechanical disaggregation, as we have previously described [14]. Flow cytometric immunephenotyping, and analyses were done as per our previous methods and protocols [13]. In brief, the isolated cells were washed with PBS and re-suspended in flow cytometry staining buffer, and FcR Blocking Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) was added to block Fc receptors. Cells were then stained with the viability dye 7-Aminoactinomycin D (7-AAD; BioLegend, San Diego, CA, USA) and monoclonal antibodies against different surface markers. These markers included CD3, CD8, PD-1, TIM-3, TIGIT and LAG-3. The used monoclonal antibodies were purchased from BD Biosciences, BioLegend and eBioscience; details of these antibodies were described in Toor et al. and Al-Mterin et al. [13,15]. Following staining, live cells were gated by exclusion of 7-ADD. Fluorescence minus one (FMO) and isotype controls were employed for staining validation and data interpretation. Samples were analyzed on a BD LSRFortessa X-20 flow cytometer using BD FACSDiva™ software (BD Biosciences). Then data were analyzed by using FlowJo V10 software (FlowJo, Ashland, OR, USA). The flow cytometric plots used in this analysis have already been shown in Fig. 3 in Toor et al. [13].

Table 1
Clinical and pathological characteristics of the CRC cohort.

	CRC patients
Number	45
Median age [range]	56 [18–79]
Gender [Male:Female]	30:15
TNM stage	
I	4
II	20
III	14
IV	7
Tumor histological grade	
G2 (Moderately differentiated)	41
G3 (Poorly differentiated)	4
MSI-H/dMMR	8
Loss of nuclear expression for MLH1 & PMS2	7
Loss of nuclear expression of MSH2	1

2.3. TCGA analysis

Transcriptome profiling data and clinical information of colorectal cancer patients were acquired from The Cancer Genome Atlas (TCGA) using the TCGAbiolinks package in R. Data filtration and normalization were performed with limma and edgeR packages. Primary solid tumor samples were chosen for further analysis. DFS data were extracted from cBioPortal (https://www.cbioportal.org), and data preparation and cleaning were done in R. OS data were obtained from clinical information downloaded from TCGA. Primary solid tumor samples based on the median of gene expression were grouped into high-expressed and low-expressed samples for targeted genes. The prognostic difference, including OS and DFS, between high-expressed and low-expressed groups, was calculated utilizing Survival packages in R. Kaplan-Meier method was applied to estimate survival probability for groups, and the Survminer package was used to plot the results.

2.4. Statistical analyses

Statistical analyses were done by using GraphPad Prism 9 software (GraphPad Software, California, USA). Shapiro-Wilk test was used for evaluating the normality of datasets. Cell subsets were categorized as high or low groups if they were less than or more than the median for non-normally distributed data and less than or more than the mean value for normally distributed data. Kaplan-Meier method was used for comparing DFS between low and high frequency groups, and P values for PFS curves were calculated by using the log-rank test. Paired/unpaired t tests were performed based on distribution of data and the normality of datasets, for comparisons within and between groups, respectively. Normalized values were analyzed using Pearson's correlation test, while Spearman's rank correlation test was used for analyzing the samples that don't give normal distributed. Statistical significance was determined by P values of less than or equal 0.05.

3. Results

3.1. Association of immune checkpoint-expressing $CD8^+$ TILs with DFS

Multiple studies have found that several IC molecules are expressed on TILs in many malignancies, including CRC, and they have a significant role in tumor progression [16–18]. We have recently reported that co-inhibitory/stimulatory immune checkpoints including PD-1, TIM-3, TIGIT, LAG-3 and ICOS were significantly overexpressed in $CD8^+$ TILs of CRC patients [13,19]. Expression of ICs on TILs was shown to be associated with prognosis of different cancer patients [20–23]. We have recently shown that high levels of TIM-3 in circulating and tumor-infiltrating $CD8^+$ T cells were associated with better DFS [19]. In this study, we extended our findings in a different and larger cohort of CRC patients, and investigated associations of different IC-expressing $CD8^+$ TILs with DFS; NILs were used as controls. Forty-five CRC patients were divided into two groups as above and below median/mean levels of these cells ($PD\text{-}1^+$: TILs (median 16.7), NILs (median 1.5); $TIM\text{-}3^+$: TILs (median 13.8), NILs (median 2.5); $TIGIT^+$: (mean 29.4), NILs (median

24.0); LAG-3$^+$: TILs (median 1.9), NILs (median 2.2). Of note, CD8$^+$ TILs expressed higher levels of PD-1, TIM-3 and TIGIT than CD8$^+$ NILs. Patients with higher levels of PD-1, TIM-3 and TIGIT in CD8$^+$ TILs tended to have better DFS; however, these differences did not reach statistical significance (Fig. 1A, B and C). On the contrary, patients with high frequencies of LAG-3$^+$ CD8$^+$ TILs tended to have shorter DFS, but again without any significant difference (Fig. 1D). Of note, this trend was also observed in NILs (Fig. 1D). The lack of statistical significance in these data could be attributed to the limited sample size of our study cohort; however, the trends highlight the importance of these ICs for DFS in CRC patients.

3.2. Higher levels of PD1$^+$TIGIT$^+$ and PD1$^+$TIM-3$^+$ CD8$^+$TILs are significantly associated with improved DFS

Single immune checkpoint expressions showed trends but without statistical significance. Therefore, we opted to investigate the significance of a combination of immune checkpoints expressed on tumor-infiltrating CD8$^+$ T cells as more sensitive predictive biomarkers. Specifically, we investigated whether co-expression of PD-1 with other ICs on CD8$^+$ TILs can be associated with DFS. Interestingly, we found that high levels of PD1$^+$TIGIT$^+$ and PD1$^+$TIM-3$^+$ CD8$^+$ TILs were significantly associated with longer DFS, compared to lower frequencies of these ICs-expressing CD8$^+$ TILs (Fig. 2A and B). These associations were not observed in NILs, confirming that the synergistic effects in TILs are tumor-specific. In contrast, there were no significant associations between levels of PD1$^+$LAG-3$^+$ in CD8$^+$ T cells in TILs and NILs with DFS (Fig. 2C).

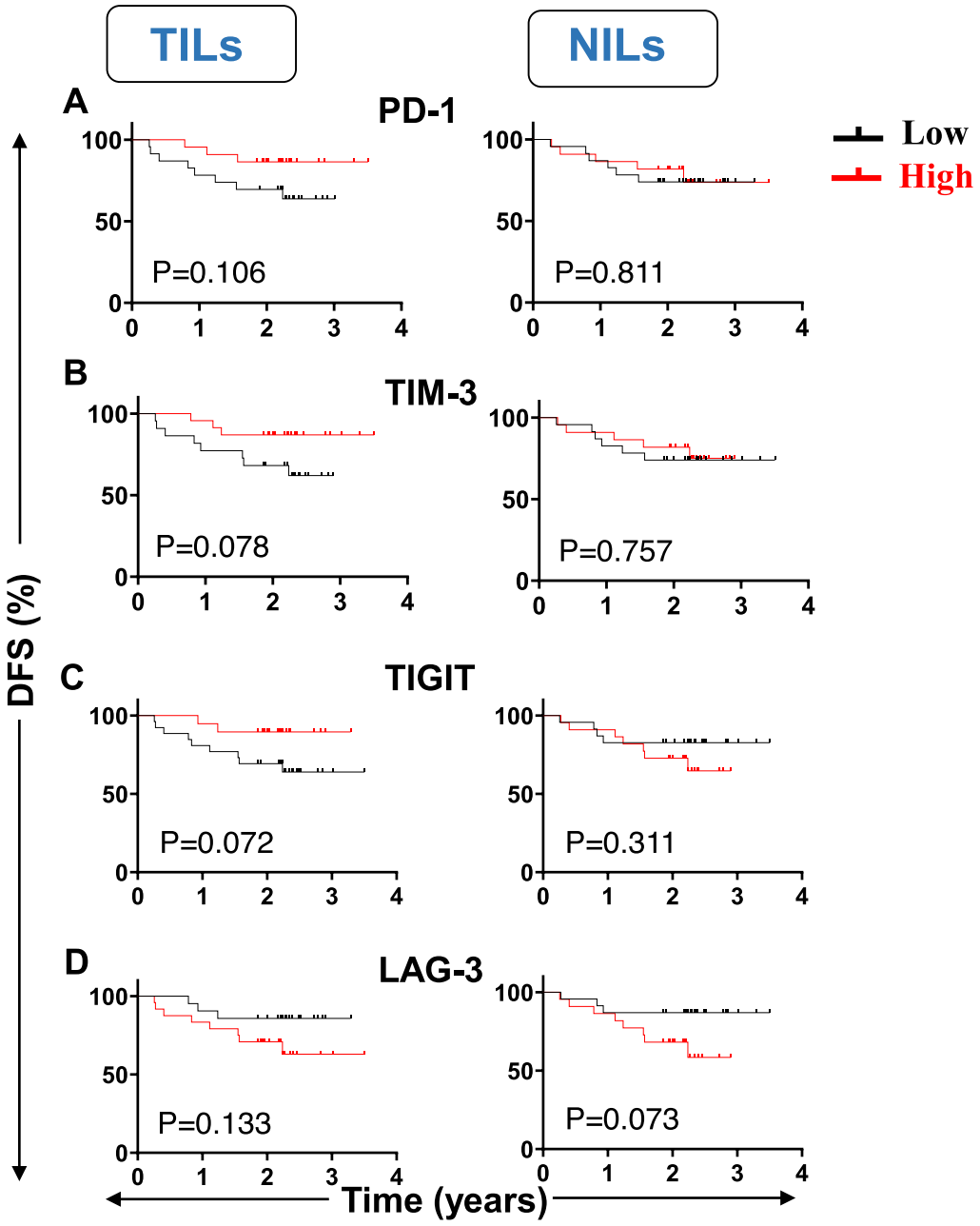

Fig. 1. Kaplan–Meier curves of DFS based on levels of different expression of ICs in TILs and NILs. Patients with high levels of PD-1$^+$ (**A**), TIM-3$^+$ (**B**), TIGIT$^+$ (**C**), and LAG-3$^+$ (**D**) in CD8$^+$ T cells were compared with patients with low levels of these cells.

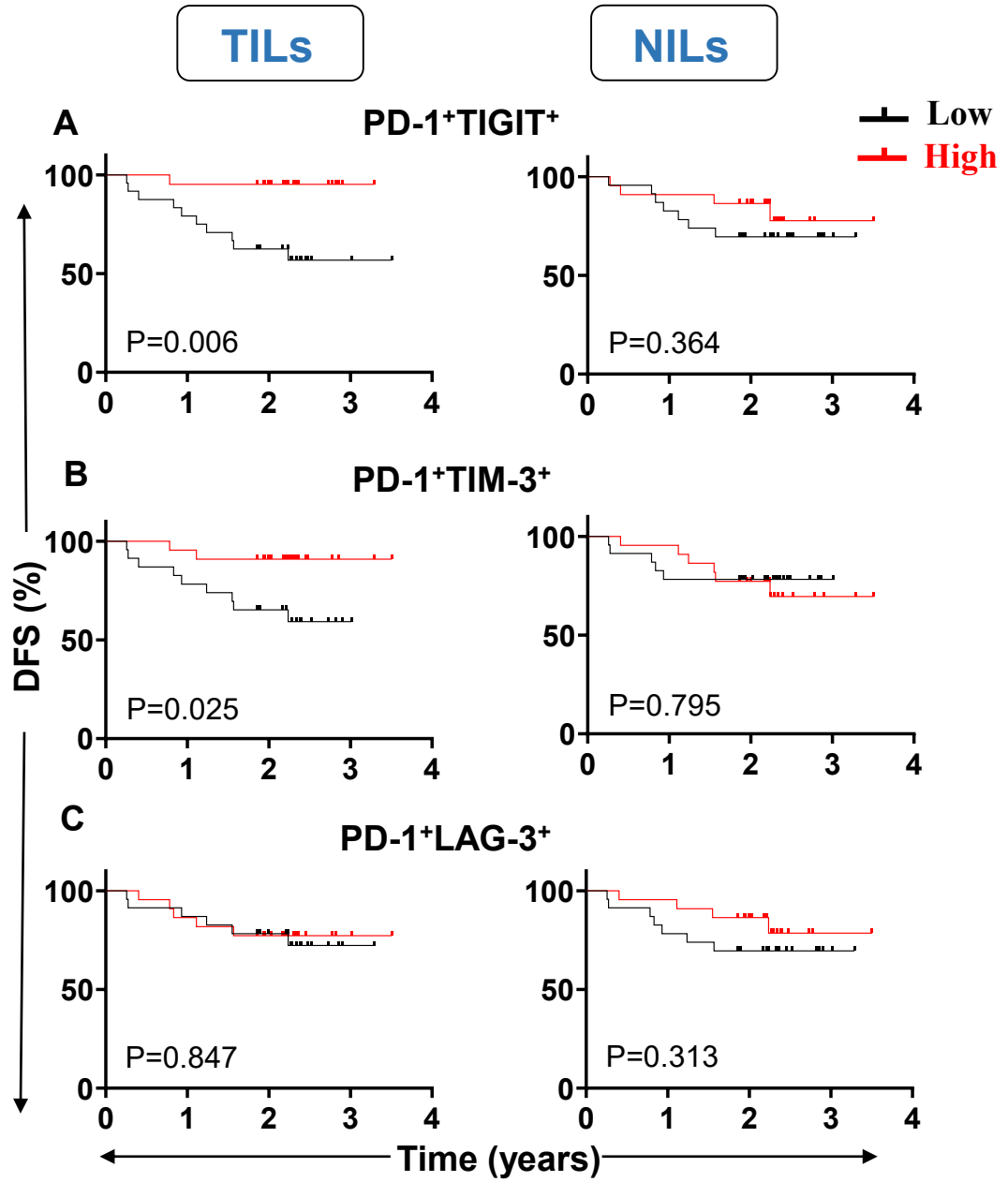

Fig. 2. Kaplan–Meier curves of DFS based on PD-1 co-expression with other ICs in TILs and NILs. Patients with high levels of PD-1^{+}TIGIT^{+} (**A**), PD-1^{+}TIM-3^{+} (**B**), and PD-1^{+}LAG^{+} (**C**) in CD8^{+} T cells were compared with patients with low frequencies of these cells.

3.3. Validation of immune checkpoint association with DFS and OS in TCGA dataset

TCGA database was used in order to validate our findings in a large cohort of patients. TCGA cohort contained 560 and 642 CRC patients for DFS and OS analyses, respectively. Patients were divided into groups based on high or low (above or below median) expressions of single immune checkpoint genes including PD-1, TIGIT, and TIM-3. Additionally, CRC patients were divided into groups based on high or low (above or below median) co-expression of two immune checkpoint genes. These groups included PD-1^{hi}TIGIT^{hi}, PD-1^{low}TIGIT^{low}, PD-1^{hi}TIM-3^{hi} and PD-1^{low}TIM-3^{low}. We then investigated the associations between expression levels of single or double genes and DFS or OS for this cohort of patients (Fig. 3). Our analysis revealed that TIGIT expression was significantly associated with DFS and OS (Fig. 3A). CRC patients with high expression level of TIGIT gene showed significantly longer DFS and OS than patients with low TIGIT expression level [median survival for DFS: 9.078 years (high group) versus 5.256 years (low group); median survival for OS: 8.329 years (high group) versus 5.484 years (low group)] (Fig. 3A). For PD-1 and TIM-3, there were no differences in DFS and OS between the high and low groups (Fig. 3B, C). As we found that high levels of PD1^{+}TIGIT^{+} and PD1^{+}TIM-3^{+} CD8^{+} TILs were significantly associated with longer DFS, we opted to check such associations in a large cohort of TCGA CRC dataset. In agreement with our findings, we found that high co-expression of PD-1 with TIGIT genes was significantly associated with longer DFS, and there was a trend towards improved OS [median survival for DFS: 9.078 years (high group) versus 5.256 years (low group); median survival for OS: 8.329 years (high group) versus 5.229 years (low group)] (Fig. 3D). However, co-expression of PD-1 with TIM-3 was not associated with DFS or OS (Fig. 3E). Altogether, our data and the TCGA validation highlight the significance of TIGIT expression alone or with PD-1 and the association

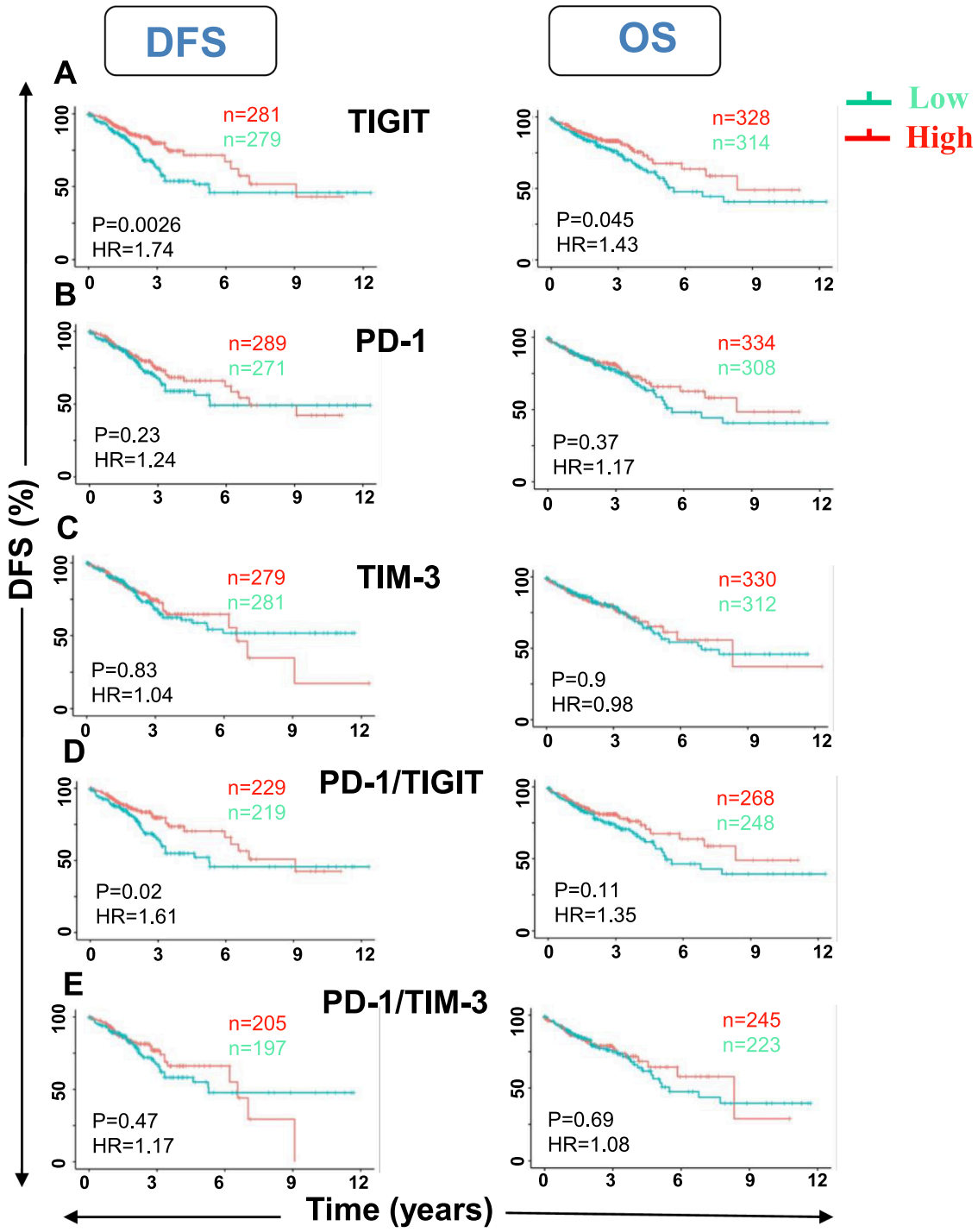

Fig. 3. Kaplan–Meier curves of DFS and OS based on levels of different expression levels of ICs genes in bulk tumors of TCGA CRC dataset. Patients with high gene expressions of TIGIT (**A**), PD-1 (**B**), TIM-3 (**C**), PD-1 with TIGIT (**D**), and PD-1 with TIM-3 **(E)** in tumor tissues were compared with patients with low levels of these genes. P values, hazard ratios (HR) and number of patients in each subgroup are shown on plots.

with improved clinical outcome of CRC patients.

Although our investigations are in $CD8^+$ T cells and the TCGA data are derived from bulk tumors, our findings for TIGIT was validated in the TCGA database. This could be because TIGIT is mainly expressed on T cells [24]. However, our findings for TIM-3 could not be validated in the TCGA. This could be attributed to the expression of TIM-3 in different immune cell subsets such as T cells, antigen-presenting cells and monocytic myeloid cells, which could play different roles in cancer progression [25]. Our previous work showed that TIM-3 expression in T cells is associated with better prognoses, while its expression in antigen-presenting cells could be associated with bad prognoses in CRC patients [25].

3.4. Correlations between frequencies of PD-1$^+$ and other IC-expressing CD8$^+$ T cells in TILs and NILs in CRC patients

We have recently determined the correlations between different $CD4^+$ Treg/T cell subsets with immune checkpoints in CRC patients with early and advanced stages [26,27]. In this study, we determined synergistic effects for co-expression of PD-1 and other ICs on DFS; therefore,

we investigated correlations between PD-1 expression and other ICs. We identified the correlations between frequencies of $CD8^+PD\text{-}1^+$ T cells with $CD8^+TIM\text{-}3^+$ or $CD8^+TIGIT^+$ or $CD8^+LAG\text{-}3^+$ T cells in TILs and NILs (Fig. 4). There were moderate correlations between frequency of $PD\text{-}1^+$ cells with $TIGIT^+$ $CD8^+$ TILs (correlation coefficient r = 0.311, P = 0.038 [TILs] (Fig. 4A)). Interestingly, there was a strong correlation between frequency of $CD8^+PD\text{-}1^+$ T cells with $CD8^+TIM\text{-}3^+$ in TILs, but not in NILs (correlation coefficient r = 0.794, P < 0.0001[TILs]; r = 0.068, P = 0.655 [NILs] (Fig. 4B)). No significant correlations were observed between frequency of $CD8^+PD\text{-}1^+$ T cells and $CD8^+LAG\text{-}3^+$ T cells in TILs and NILs (correlation coefficient r = 0.096, P = 0.531 [TILs]; r = -0.267, P = 0.076 [NILs] (Fig. 4C)). These findings confirm the strong correlation between expression of PD-1 and other ICs, especially TIM-3.

3.5. Levels of $PD\text{-}1^+TIGIT^+$, $PD\text{-}1^+TIM\text{-}3^+$, and $PD\text{-}1^+LAG\text{-}3^+$ $CD8^+$ TILs in CRC patients with MSI-H versus MSI-L

We have recently reported that patients with mismatch-repair deficiency/microsatellite instability-high tumors (MSI-H) had higher levels of IC-expressing T cells than patients with proficient MMR and microsatellite stable tumors (MSI-L/MSS) [13]. With regards to $CD8^+$ TILs, we found that levels of $TIM\text{-}3^+$ cells, but not other ICs-expressing $CD8^+$ TILs, were significantly higher in MSI-H than MSI-L tumors [13]. In this study, we investigated differences in levels of $CD8^+$ TILs co-expressing two ICs between MSI-H and MSI-L patients. Seven CRC patients (15.5%) out of 44 patients had MSI-H tumors. We found that patients with MSI-H tumors have higher levels of $PD\text{-}1^+TIGIT^+$ and $PD\text{-}1^+TIM\text{-}3^+$ $CD8^+$ T cells, compared to patients with MSI-L ($PD\text{-}1^+TIGIT^+$: mean ± SEM; MSI-H 11.83 ± 3.34 vs. MSI-L 9.39 ± 1.98, P = 0.071; $PD\text{-}1^+TIM\text{-}3^+$: mean ± SEM; MSI-H 12.97 ± 3.54 vs. MSI-L 9.82 ± 1.90, P = 0.089) (Fig. 5A and B). However, the difference did not reach statistical significance, which could be due to small sample size. Additionally, there was no difference in $PD\text{-}1^+LAG\text{-}3^+$ $CD8^+$ TILs between patients with MSI-H and MSI-L tumors (Fig. 5C).

4. Discussion

Changes in T-cell subsets such as location, levels, or even biological function elements within the TME, have an influence on tumor outcomes, which occur through tumor progression or tumor regression [28]. Vitorino et al., reported that a high level of tumor-infiltrating lymphocytes was associated with better survival in stages II and III CRC patients [29]. Tumor-infiltrating immune cells (TIICs) including

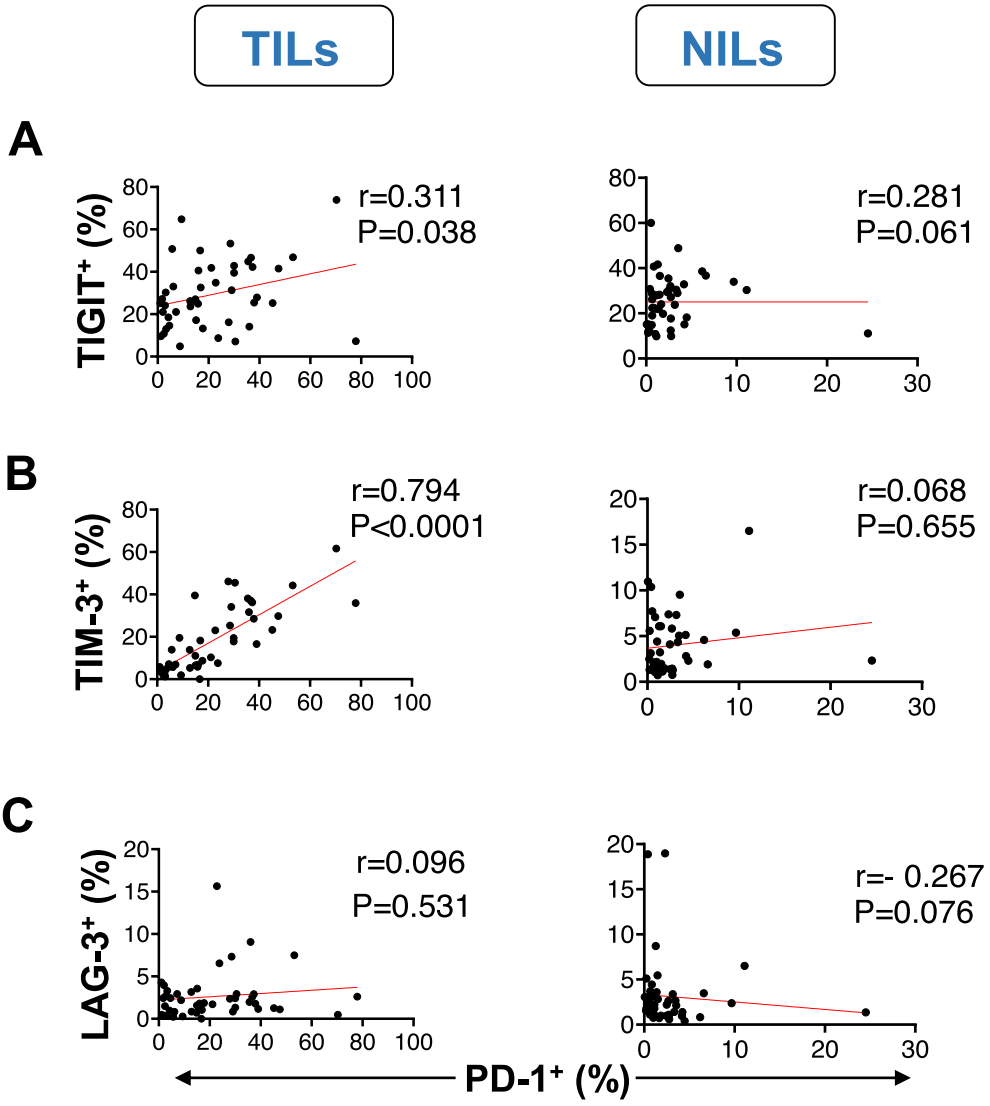

Fig. 4. Correlations between frequencies of $PD\text{-}1^+$ cells and other immune checkpoint-expressing $CD8^+$ T cells. Correlations between frequencies of $PD\text{-}1^+$ cells with $TIGIT^+$ **(A)**, $TIM\text{-}3^+$ **(B)**, and $LAG\text{-}3^+$ **(C)** in $CD8^+$ TILs and NILs in CRC patients.

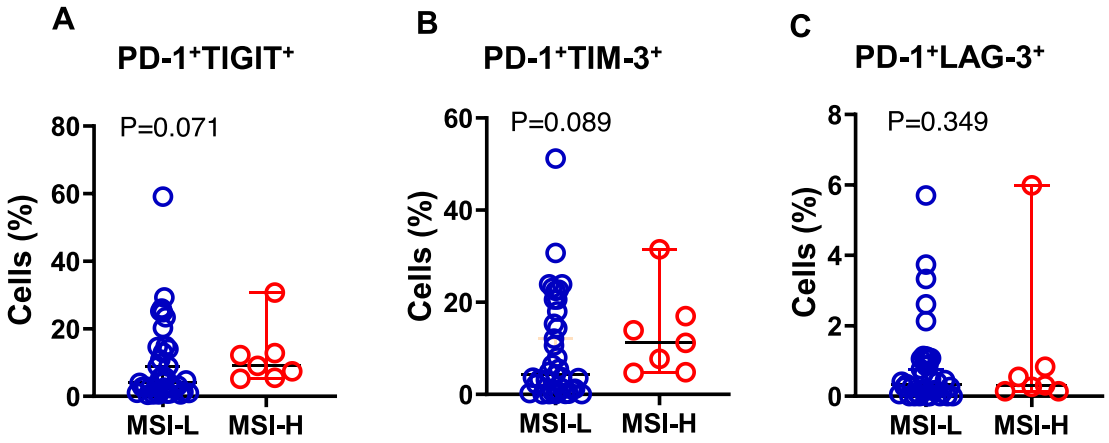

Fig. 5. Scatter plots of frequencies of different immune checkpoint co-expression in MSI-H versus MSI-L tumors. Scatter plots show PD1$^+$TIGIT$^+$ (**A**), PD-1$^+$TIM-3$^+$ (**B**) and PD-1$^+$LAG-3$^+$ (**C**) in tumor-infiltrating CD8$^+$ T cells in MSI-H and MSI-L tumors. Data represent mean ± standard error of the mean (SEM).

CD8$^+$ T cells are associated with better prognoses in different tumors such as CRCs, renal cancer, and lung cancer [30–32]. Moreover, increased infiltration of Th1 cells, CD3$^+$ T cells and CD8$^+$ T cells in the CRC TME correlated with improved DFS and OS, while decreased T cell density was correlated with worse prognosis [33,34]. Therefore, activated CD8$^+$ T cells are critical components of the tumor-immune microenvironment (TIME), and they express several cell surface molecules, including ICs such as TIGIT, TIM-3, LAG-3, and PD-1 [24,35,36].

Different studies reported the overexpression of different immune checkpoints on T cells and their associations with T cell exhaustion in various cancers including CRC [37], melanoma [38], multiple myeloma [39], breast cancer [40], liver cancer [41], and acute myeloid leukemia [42,43]. Furthermore, a number of studies documented that overexpression of various ICs on T cells was associated with cancer prognoses. A recent study found that gastric cancer with positive expression of PD-1, TIM-3, and LAG-3 tended to have a better prognosis than cancer with negative expression [44]. Another study reported that expressions of PD-1 and TIM-3 on T cells were increased in multiple myeloma patients but not in the healthy controls, especially for progressive disease [45]. In our study, we found that CRC patients with higher levels of TIM-3$^+$, TIGIT$^+$, and PD-1$^+$ CD8$^+$ TILs showed improved DFS, although it was not significant. High levels of TIM-3 in tumor-infiltrating lymphocytes [46] and NK cells [47] contributed to a worse prognosis in different tumors. In contrast, Wang et al. found that increased level of TIM-3 was associated with a better prognosis in cervical cancer [48]. In addition, Al-Badran et al. found that high levels of TIM-3 and other ICs such as PD-1 and LAG-3 on stromal immune cells were correlated with a favorable prognosis and better survival rate in CRC, which may be attributed to the activation of immune responses in cancer tissues [49]. Another study found that down-regulation of TIM-3 may enhance CRC progression, which indicate that expression levels of TIM-3 is one of the most helpful predictors of clinical prognosis in CRC [50]. A recent study in CRC patients found that increased expressions of TIGIT and PD-1 were associated with better OS [51]. Another study found that PD-1 and TIGIT were upregulated in CRC patients with dMMR, which were related to TNM stage and DFS [52]. It was reported that patients with advanced TNM stage had higher expression of TIGIT and PD-1. Moreover, higher expression of TIGIT and PD-1 were found to be associated with better DFS in CRC patients with dMMR [52].

Co-expressions of different ICs have been detected in the TME of different tumors. However, studies reported on the associations of co-expression of different ICs with prognoses of cancer patients are limited. A recent study reported that co-expression of PD-L1 with TIM-3 or TIGIT were associated with worse overall survival of esophageal squamous cell carcinoma patients [53]. Other studies have found that co-expression of IC receptors was associated with poor prognoses in different types of solid tumors, including lung cancer [54], ovarian cancer [55], and renal cell carcinoma [56]. However, there are no reports on the association of IC co-expression and disease prognoses in CRC. Our study is the first to discover that CRC patients with high levels of PD-1$^+$TIGIT$^+$ and PD-1$^+$TIM-3$^+$ CD8$^+$ TILs had significantly improved DFS, compared to patients with lower levels of these cells. Additionally, a strong positive correlation was observed between frequency of CD8$^+$PD-1$^+$ TILs and CD8$^+$TIM-3$^+$ TILs, while moderate positive correlation was found between level of CD8$^+$PD-1$^+$ TILs and CD8$^+$TIGIT$^+$ TILs.

High expression of LAG-3 in T lymphocytes leads to a decline in T cell responses [57]. Therefore, LAG-3 could be an interesting target for biological therapies such as immunotherapy. Many early-phase clinical trials in various malignances investigated the therapeutic antibodies against LAG-3 [58]. In our study, we noticed that high levels of LAG-3$^+$ CD8$^+$ TILs were associated with shorter DFS, although not significant. Recent studies reported that hepatocellular carcinoma and gastric cancer with Epstein-Barr virus positivity and MLH1 mutations had shorter OS and DFS, which were correlated with LAG-3 expression [59,60]. Furthermore, co-expression of PD1$^+$LAG-3$^+$ was detected in different cancer types. For instance, in an ovarian tumor mouse model, it has been found that dual blockade of LAG-3 and PD-1 showed to enhance tumor antigen-specific CD8$^+$ T cell production of cytokines. Therefore, targeting those inhibitory receptors would enhance the antitumor functions of these CD8$^+$ T cells [61].

In our study, we found that MSI-H tumors have relatively higher levels of PD-1$^+$TIGIT$^+$ CD8$^+$ TILs compared to MSI-L tumors. It has been found that MSI-H tumors have higher response rate to ICIs than MSI-L tumors [62,63]. Another study reported that dMMR subset of CRC patients have active T-helper 1 (TH-1)/cytotoxic T cells (CTL) microenvironment, which would result in upregulation of multiple ICs including PD-1, PD-L1, CTLA-4, LAG-3, and IDO [64]. This evidence supports that MSI-H CRC patients would have better clinical prognosis, and would be more sensitive to ICI therapy than MSI-L CRC patients.

5. Conclusion

Our study showed that patients with high levels of TIGIT$^+$, TIM-3$^+$, and PD-1$^+$ CD8$^+$ TILs tended to have longer DFS, but without any statistical significance. More importantly, increased levels of PD-1$^+$TIGIT$^+$ and PD-1$^+$TIM-3$^+$ CD8$^+$ TILs in CRC patients were significantly associated with longer DFS. It is not clear whether these IC-expressing CD8$^+$ T cells are activated effector cells with anti-tumor activity or anti-inflammatory cells, which inhibit inflammation and contribute positively to CRC prognosis. This warrants further investigations. Overall, expressions of ICs on CD8$^+$ TILs in CRC patients are important predictive

biomarkers; however, investigations of multiple ICs are critical for determining more accurate prognostic significance.

Authors contributions

A.M. and E.E. wrote the manuscript. A.M., M.A.A. K.M. and E.E. analyzed the data. A.M., M.A.A. E.E. prepared the figures. G.M.C. and M. A.M. performed TCGA validation. E.E. supervision, project administration, conceptualization, funding acquisition. All authors reviewed the manuscript.

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Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of the Medical Research Center, Hamad Medical Corporation (protocol no. MRC-02-18-012).

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on a reasonable request.

Consent for publication

Informed consent for publication was obtained from all subjects involved in the study.

CRediT authorship contribution statement

Abdo Meyiah: . Ghanbar Mahmoodi Chalbatani: . Mohamed A. Al-Mterin: . Mohammad Amin Malekraeisi: . Khaled Murshed: . Eyad Elkord: Supervision, Project administration, Conceptualization, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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