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ORIGINAL ARTICLE

Expression of the inhibitory B7 family molecule VISTA in human colorectal carcinoma tumors

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Abstract

Colorectal carcinoma (CRC) is one of the most common malignancies in the world. PD-1/PD-L1 inhibitors have benefited cancer patients with multiple tumor types. However, their efficacy for CRC is low and this treatment in melanoma patients results in adaptive resistance through upregulation of VISTA, another checkpoint inhibitory pathway. Thus, there is an urgent need to explore additional co-inhibitory molecular pathways such as VISTA for CRC treatment. In this study, *C10orf54* (encoding VISTA) expression was analyzed by RNA-seq data from 367 CRC patients in human cancer datasets. Moreover, 28 clinical CRC specimens were used to assess VISTA protein expression. Human cancer datasets showed that CRC tumors expressed higher levels of *C10orf54* than CD274 (encoding PD-L1). Moreover, *C10orf54* mRNA expression was significantly correlated with genes responsible for tumor immune evasion. VISTA protein expression was high in tumors compared with para-tumors and normal tissues, which is similar to PD-L1 expression. However, in contrast to PD-L1, VISTA was mainly expressed by tumor-infiltrating lymphocytes. This study is the first investigation of VISTA expression in human resected CRC tumors, and the results justify the need for future studies on the role of VISTA in anti-CRC immunity in clinical samples.

Keywords VISTA · Immune checkpoint · PD-L1 · Tumor immunity · Immunotherapy

Abbreviations

COAD	Colon adenocarcinoma
CRCL	Colorectal carcinoma
dMMR	Mismatch repair deficient
FDA	Federal Drug Agency
HR	Hazard ratio
MSI-H	Microsatellite instability high
READ	Rectum adenocarcinoma

Introduction

Colorectal carcinoma (CRC) remains the fourth most diagnosed cancer [1, 2]. CRC is the second and fifth leading cause of cancer death in the United States [3] and in China [4], respectively. The projected global burden of CRC is expected to increase by 60% to more than 2.2 million new cases and 1.1 million deaths by 2030 [5]. Furthermore, 25% of patients are diagnosed at late-stage disease and up to 40% of patients relapse after surgery, usually in the form of distant or regional metastases [6]. Currently, chemotherapy in human CRC has shown some therapeutic efficacy [7]. Because of the seminal German rectal cancer trial [8], neoadjuvant chemoradiotherapy has become the standard of care for CRC in the United States and Europe.

However, the adverse effects of radiotherapy and chemotherapy are substantial and are associated with considerable morbidity and mortality. Adverse effects, including chronic diarrhea and potential intestinal obstructions, seriously impair the quality of life for CRC patients [9]. The advent of immune checkpoint blockade has been an exciting field in cancer immunotherapy. Recently, the Federal Drug Agency (FDA) has approved pembrolizumab (anti-PD-1 inhibitor)

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for microsatellite instability high (MSI-H) or mismatch repair deficient (dMMR) solid tumors [10], including CRC. Although of considerable success in other types of cancers like melanoma and squamous cell lung cancer where anti-PD-1/PD-L1 inhibitors are approved, the efficacy of those checkpoint inhibitors in CRC is quite low [11]. This disappointing efficacy suggests the need for studying other checkpoint molecules in human CRC clinical samples and their functions in colorectal animal models.

Interestingly, the treatment of melanoma patients with PD-1/PD-L1 inhibitors results in VISTA upregulation, leading to adaptive resistance of PD-1 blockade. VISTA, also known as B7-H5, GI24, Dies1 and PD-1 homolog (PD-1H), is a type I membrane protein with an extracellular domain homologous to PD-L1 [12]. Similar to mouse VISTA, human VISTA is predominantly, if not exclusively, expressed in hematopoietic tissues or in tissues that contain significant numbers of infiltrating leukocytes [13]. Human VISTA is not expressed on B cells or NK ($CD56^{hi}$) cells, but is highly expressed on myeloid cells with a reduced expression on $CD4^+$ and $CD8^+$ T cells. Its receptor has not been discovered, but VISTA signaling exerts a suppressing effect on T cell activation [13]. Furthermore, targeting VISTA and PD-L1 simultaneously rather than individually achieves more optimal tumor-clearing therapeutic efficacy in the mouse CT26 colon cancer model [14]. These studies suggest a foundation for designing VISTA-targeted approaches alone or in combination with PD-1/PD-L1 inhibitors for CRC immunotherapy. However, few studies on VISTA expression in human cancers have been reported and the status of VISTA expression in human CRC tumors is unknown. Therefore, in this study, we take advantage of human cancer datasets and clinical resected CRC specimens to assess VISTA expression in normal human colorectal tissue and examined its expression changes in CRC. This study suggests that VISTA is a promising target for CRC immunotherapy for therapy-naïve CRC patients.

Materials and methods

Analysis of public datasets

RNA sequencing-based gene expression in colon adenocarcinoma and rectum adenocarcinoma was obtained from PEPIA for cancer Genomics [15]. Ras alternations in CRC were correlated with *C10orf54* using cBioportal dataset [16, 17]. For gene expression, the significance (p value) cutoff was 0.01.

Evaluation of *C10orf54* expression

Total RNA was extracted in resected tumors and normal tissues from 32 CRC patients. 1 mL of Trizol was added into 50–100 mg tissues followed by addition of 200 µL chloroform. After centrifugation, aqueous phase was transferred to a new tube with 0.5 mL of isopropanol. Pellet was washed with 1 mL of 75% ethanol and then suspended with TE buffer. *C10orf54* expression was assessed by real-time PCR using a Cyber green based kit (Bio-Rad, CA, USA) with the primers: Forward 5′-ATTCCCTGTATGTCTGTC CCG-3′; Reverse 5′-CTGCGGTACCACGTCTTGTAG-3′. *C10orf54* expression was normalized to housing-keeping gene *GAPDH* expression. *GAPDH* Forward 5′-TGCACC ACCAACTGCTTAGC-3′, *GAPDH* Reverse 5′-GGCATG GACTGTGGTCATGAG-3′.

Antibodies for immunofluorescence and flow cytometry

The primary antibodies for immunofluorescence assay are mouse anti-CD45 (2D1), rabbit anti-CD45 (polyclonal), mouse anti-VISTA (730B04), rabbit anti-PD-L1 (SP142) and goat anti-CD68 (M-20). The secondary antibodies are fluorescence-conjugated AffiniPure F(ab')2 Fragment donkey anti-rabbit/mouse/goat immunoglobulin. The following fluorescence-conjugated anti-human antibodies were used for flow cytometric analysis: anti-CD45 (HI30), anti-HLA-DR (L243), anti-CD11c (3.9), anti-CD123 (7G3), anti-CD14 (MϕP9), anti-VISTA (730,804) and anti-PD-L1 (MIH1).

Immunofluorescence

All fresh samples were snapped in liquid nitrogen and embedded in OCT. Afterwards, 4–8 µm thick cryostat sections were cut. After blocking with 10% normal donkey serum, each slide was incubated with the primary antibodies against PD-L1, VISTA, CD45 and CD68 at 4 °C overnight. AffiniPure F(ab')2 Fragment donkey anti-rabbit/mouse/goat immunoglobulin was used as the secondary antibody. Images were obtained by a fluorescence microscope (Zeiss LSM780). The quantification analysis was performed by ImageJ software.

Isolation of peripheral blood mononuclear cells (PBMCs) and tissue-infiltrating leukocytes

Blood from CRC patients was drawn into heparinized tubes and centrifuged on Ficoll-Hypaque gradients (GE Healthcare Life Sciences, Philadelphia, PA). Fresh normal colorectum, paratumors and tumors from CRC patients were

digested in RPMI-1640 medium supplemented with 0.5 mg/mL Collagenase Type IV (Gibco, Grand Island, NY), 10% FBS plus 10U/mL DNase I and isolation of tissue/tumor-infiltrating leukocytes was done according to the method described earlier [18].

FACS cell surface staining

Mononuclear cells isolated above and PBMCs were washed with PBS and 2% FCS (Gibco, Grand Island, NY), and then Fc blocking reagent was added followed by a wash with PBS and 2% FCS. Cells were then incubated for 30 min on ice with CD45 PerCP Cy5.5, Lineage FITC, HLA-DR AF700, CD11c BV605, CD123 PE-CF594, CD14 APC Cy7, CD15 PE-Cy5, VISTA BV421, PD-L1 APC and live/dead fixable aqua dye, and then washed twice with PBS and 2% FCS. The cells were stored at 4 °C until acquired by FACS Fortessa instruments (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (Version 10.0.8, Tree Star Inc., Ashland, Or).

Statistical analysis

The statistical data analysis was performed using GraphPad Prism 6 statistical package. Student's *t* test and Wilcoxon test were used for two-group analysis. *P* values less than 0.05 were considered to be statistically significant.

Results

C10orf54 is expressed in colon adenocarcinoma tumors as well as in rectum adenocarcinoma tumors

The expression and impact of VISTA (encoded by *C10orf54*) in human CRCs remain unclear. To fill this gap of information, we first took advantage of GEPIA, a web server for cancer and normal gene expression profiling [15]. Interestingly, we found that tumors from patients with colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ) expressed similar levels of *C10orf54* (Fig. 1a, upper panel). However, the expression levels of *C10orf54* were significantly reduced in CRC tumors (COAD and READ) compared to normal controls. Level of PD-L1 expression in/on tumor cells is regarded as a predictive biomarker in cancer immunotherapy with anti-PD-1/PD-L1 blockade [19], so the expression of *CD274* (encoding PD-L1) in CRC tumors and normal controls was also analyzed. Figure 1a (bottom panel) shows that there was no significant difference in *CD274* expression between CRC tumors and normal controls. We also collected specimens from 32 treatment-naïve CRC patients who fulfilled the study criteria. The clinical and pathological characteristics of the patients are summarized in Table 1. Figure 1b showed that the 32 CRC patients had a trend of higher *C10orf54* expression levels in normal tissues than in tumors, which was consistent with Fig. 1a. In addition, we compared B7/CD28 family gene expressions in this dataset. As shown in Fig. 1c, *CD276* (encoding B7-H3) and *C10orf54* were highly expressed in both COAD and READ. Compared with those two genes, the expression level of *CD274* was low.

C10orf54 in CRC tumors is correlated with genes responsible for tumor immune escape

By analyzing RNA-seq data in human CRC tumors, we found no significant correlation between *C10orf54* expression in CRC tumors and patient disease-free survival as well as overall survival, although patients with high *C10orf54* expression had a high hazard ratio (HR = 1.1) for relapsing (Fig. 2a). However, Fig. 2b showed that there was a significantly positive correlation between *C10orf54* expression and the expression of some other checkpoint molecules, such as *TIGIT, HAVGR2, BTLA, CD274* and *PDCD1*. Moreover, anti-inflammatory molecules *TGFb1* and *foxp3* also correlated with *C10orf54* expression in CRC tumors. More interestingly, there was a significant correlation between the expression of *C10orf54* and M2 macrophage signature genes *(CD14, CD68* and *CD163)*. However, Fig. 2c showed that *C10orf54* expression was negatively correlated with mutated *kras* associated with tumor proliferation. Taken together, these imply that VISTA might contribute to CRC immune escape and could be a useful target for CRC immunotherapy.

VISTA is highly expressed in CRC tumors compared with para-tumors and normal tissues

Next we evaluated VISTA expression in CRC tumors at the protein level. To test whether immune cells infiltrated into CRC tumors express VISTA, we performed immunofluorescence staining on clinical resected CRC tumors with anti-CD45, anti-VISTA and anti-CD68. As shown in Fig. 3a, in tumor section the majority of VISTA-expressing cells was CD45 positive. In line with previous studies showing that VISTA is predominantly expressed by myeloid cells, our study showed that tumor infiltrating macrophages ($CD45^+CD68^+$) expressed VISTA. However, CD45 negative cells as indicated by the red arrows also expressed VISTA. In order to test whether those VISTA-expressing CD45 negative cells were tumor cells, we stained sequential tumor sections with anti-VISTA and pan-cytokeratin, respectively. Indeed, some pan-cytokeratin-expressing cells were VISTA positive (Fig. 3b), implying that CRC tumor cells could also express VISTA. However, we did not observe VISTA expression on CRC cell line SW620 (data not shown). In the para-tumor (tumor-adjacent) and normal sections, $VISTA^+$

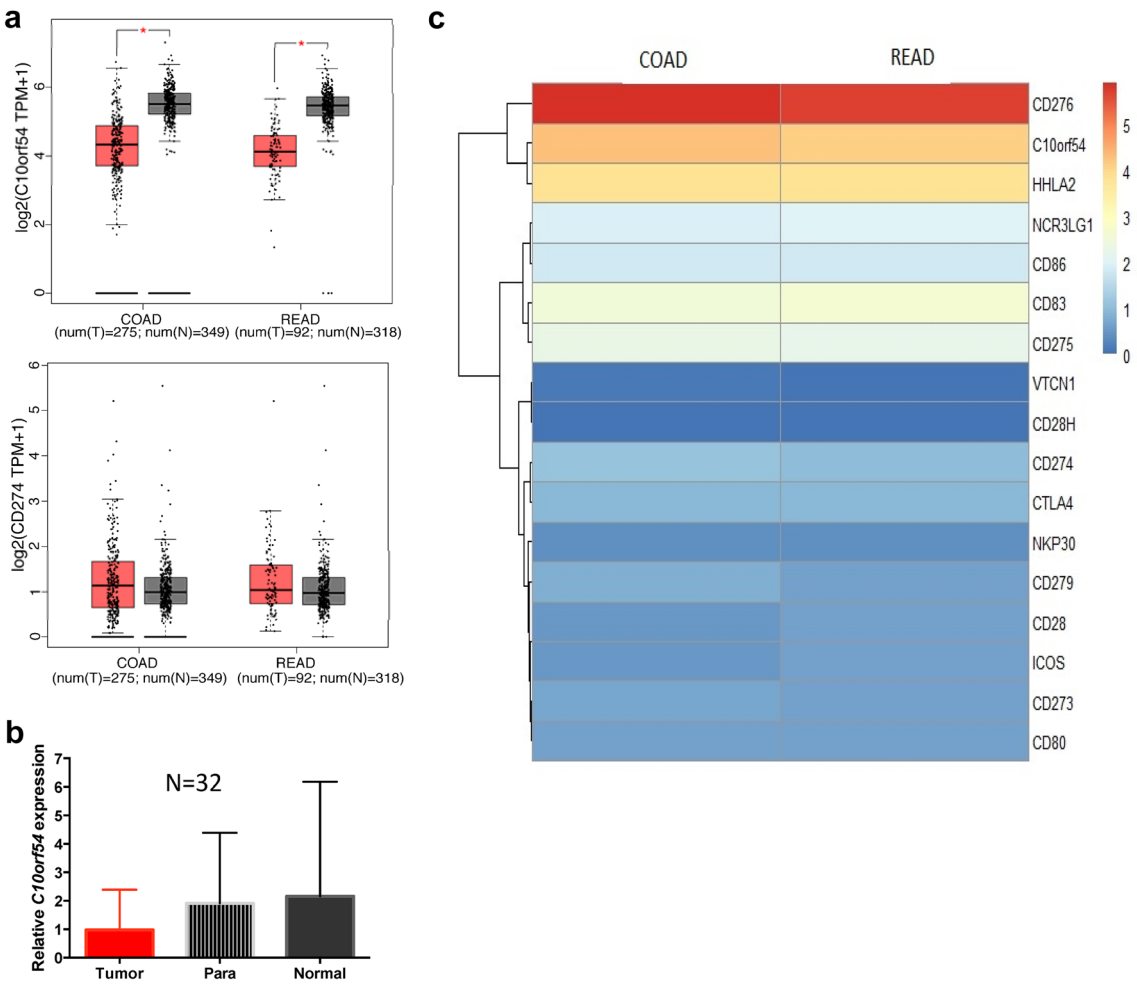

Fig. 1 *C10orf54* is expressed by colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ). **a** The expression levels of *C10orf54* (upper panel) and *CD274* (bottom panel) in human CRC tumors and normal controls. *P* value cutoff is 0.01. **b** RT-PCR analysis of *C10orf54* expression in 32 therapy-naïve CRC patients. **c** Heatmap of the scaled log2-fold change of B7/CD28 family gene mRNA expression in CRC tumors. TPM: transcripts per million

cells could be observed and most of them were $CD45^+$ cells. However, the expression levels of VISTA in para-tumors and normal sections were lower than those in tumor sections (Fig. 3c), an observation not consistent with gene expression levels in tumor and normal tissues (Fig. 1a, b), suggesting that translation of VISTA protein in normal tissues might be restrictively regulated at post-transcript levels or translation levels.

PD-L1 is expressed mainly by $CD45^-$ cells in CRC specimens

To compare VISTA expression and PD-L1 expression in CRC specimens, we also measured PD-L1 expression by immunofluorescence. Unlike the observation of VISTA expression by both $CD45^+$ and tumor cells, PD-L1 was predominantly expressed by CD45 negative cells (Fig. 4a). However, similar to VISTA protein expression, the highest expression level of PD-L1 protein was detected in tumor sections compared to para-tumor and normal sections (Fig. 4b).

VISTA is detected on all tested subsets of myeloid cells in the tumors

Given that VISTA is mainly expressed by tumor-infiltrating lymphocytes (TILs), we sought to identify which subsets of myeloid cells express it. TILs were isolated by enzymatic digestion and then stained with different subset surface markers. PBMCs were also isolated from blood as a control. 9 out of 14 CRC specimens were found to express VISTA on TILs.

Table 1 Clinical and pathological characteristics of the CRC patients

Patient No.	Gender	Age	Tumor location	TNM stage	Tumor stage	Tumor No.	Tumor size
P#1	Female	65	Sigmoid	T3N0M0	IIA	1	3.5
P#2	Female	55	Colon	T3N1bM0	IIIB	1	2.3
P#3	Male	59	Sigmoid	T3N0M0	IIA	1	4.8
P#4	Male	77	Right colon	T3N1aM0	IIIB	1	3.0
P#5	Female	61	Rectum	T3N0M0	IIA	1	6.0
P#6	Female	70	Rectum	T3N0M0	IIA	1	6.0
P#7	Female	49	Right colon	T3N0M0	IIA	1	4.0
P#8	Male	66	Rectum	T3N0M0	IIA	1	3.0
P#9	Male	66	Rectum	T3N1bM0	IIIB	1	4.5
P#10	Male	58	Rectum	T2N0M0	I	1	1.6
P#11	Female	81	Right colon	T3N1bM0	IIIB	1	4.0
P#12	Male	64	Rectum	T3N2bM0	IIIC	1	3.5
P#13	Male	66	Right colon	T3N1bM0	IIIB	1	4.3
P#14	Male	53	Sigmoid	T3N0M0	IIA	1	3.7
P#15	Male	61	Right colon	T2N0M0	I	1	3.5
P#16	Male	69	Transverse colon	T3N0M1	IVA	1	1.5
P#17	Female	51	Right colon	T3N0M0	IIA	1	5.0
P#18	Female	73	Right colon + transverse colon	T4aN0M0	IIB	2	6.5
P#19	Female	60	Sigmoid	T3N0M0	IIA	1	4.5
P#20	Male	74	Rectum	T3N0M0	IIA	1	4.3
P#21	Male	84	Rectum	T1N0M0	I	1	6.5
P#22	Female	74	Right colon	T3N2bM0	IIIC	1	3.0
P#23	Female	60	Right colon	T3N1bM0	IIIB	1	2.5
P#24	Female	83	Right colon	T3N1bM0	IIIB	1	1.8
P#25	Male	74	Right colon	T3N0M0	IIIB	1	6.0
P#26	Male	56	Rectum	T3N1M0	IIIC	1	2.0
P#27	Female	77	Left colon	T2N0M0	I	1	2.0
P#28	Male	68	Right colon	T3N2aM0	IIIB	1	3.5
P#29	Male	78	Right colon	T2N0M0	I	1	6.5
P#30	Male	56	Rectum	T3N0M0	IIA	1	8.0
P#31	Male	46	Sigmoid	T2N0M0	I	1	2.7
P#32	Male	74	Rectum	T3N1aM0	IIIB	1	3.2

As shown in Fig. 5a, peripheral VISTA is mainly expressed by monocytic MDSCs (mMDSCs, $CD45^+HLA\text{-}DR^-CD14^+$) and monocytes ($CD45^+HLA\text{-}DR^+CD14^+$), while intratumoral VISTA could be detected on almost all the tested subsets of myeloid cells. Comparing different subsets of myeloid cells in the blood and tumors, we found that higher levels of VISTA expression on all subsets in the tumors than in the blood (Fig. 5b), suggesting that tumor microenvironment (TME) contributes to VISTA expression, which in turn could promote tumor escape from anti-tumor immunity. Unfortunately, there were few myeloid cells infiltrating into normal tissues, so we could not detect VISTA expression in normal colorectal tissue samples.

Discussion

Although VISTA is a novel immune checkpoint molecule, the relevance of this molecule in clinical resected tumors has rarely been reported. So far VISTA expression has been documented only on human oral squamous cell carcinoma [20] and gastric cancer [21]. This study is the first investigation of VISTA expression in clinical resected CRC tumors. In the context of CRC, similar to PD-L1, VISTA is expressed in normal colorectal tissues, para-tumor and tumors, with the highest expression level in the tumors. However, unlike intratumoral PD-L1, which is mostly expressed by CD45 negative cells, intratumoral VISTA is predominantly

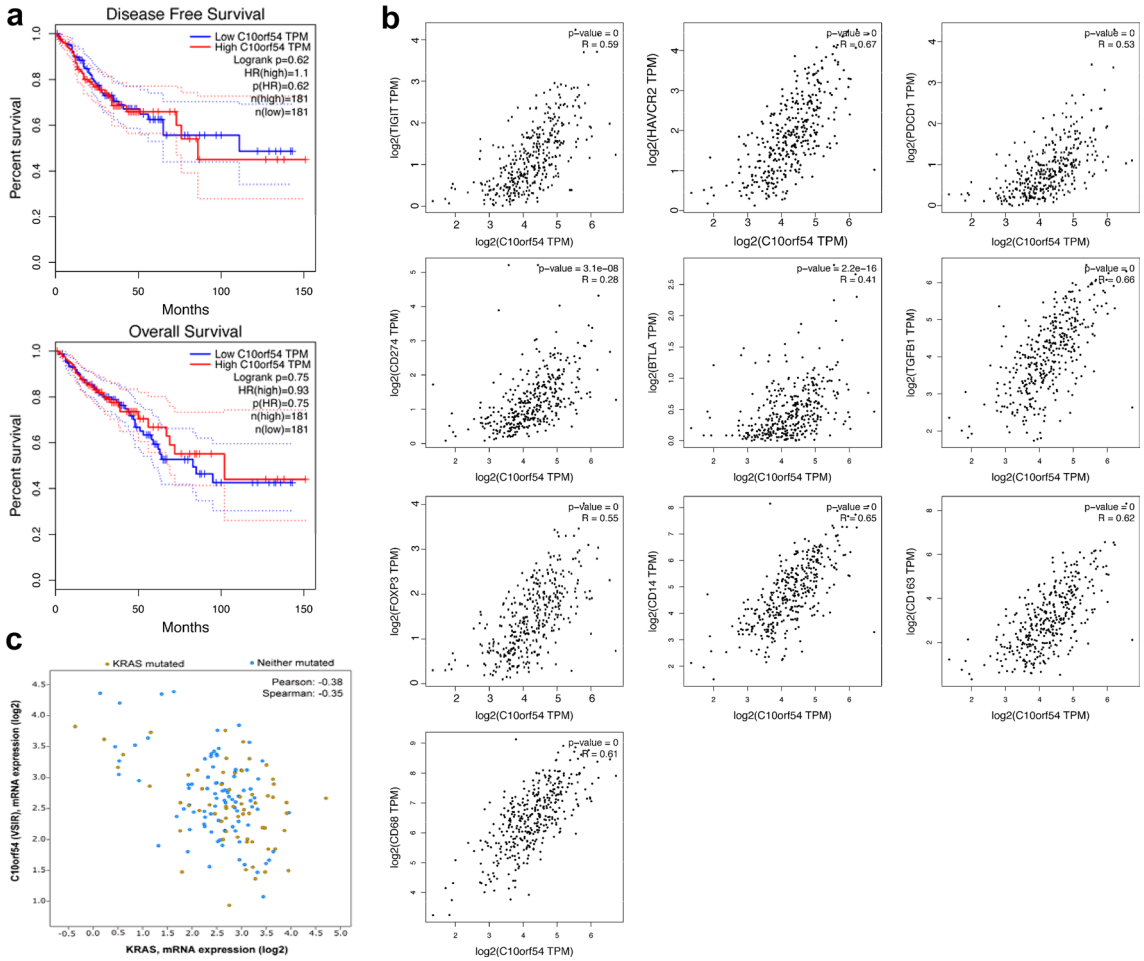

Fig. 2 *C10orf54* is positively correlated with genes responsible for tumor immune escape. **a** Correlations of *C10orf54* mRNA expression with disease-fee survival (upper panel) and overall survival (bottom panel) in CRC patients. **b** Correlations of *C10orf54* with *HAVCR2, TIGIT, CD274, PDCD1, BTLA, TGFb1, foxp3, CD14, CD68* and *CD163* mRNA expressed in CRC tumors. **c** Correlation of *C10orf54* expression with *k-ras* mutation. *HR* hazard ratio

expressed by myeloid cells, such as macrophage, mDCs and mMDSCs. In a mouse colorectal model, Juneja *et al*. [22] recently showed that PD-L1 on MC38 colorectal adenocarcinoma cells is sufficient to suppress anti-tumor immunity, as deletion of PD-L1 on highly immunogenic MC38 tumor cells, but not on host cells, allows effective anti-tumor immunity. Thus, the inhibitors of PD-L1 and VISTA target tumor cells and antigen-presenting cells, respectively (Fig. 5c).

A previous study showed that tumor cells, such as melanoma and bladder cancer cells, do not express mouse VISTA [23]. Recently, Christine *et al*. reported that gastric cancer cells expressed VISTA [21] and Wu *et al*. showed that VISTA was expressed by primary oral squamous cell carcinoma [20]. Moreover, this study is the first to demonstrate that a distinct cytoplasmic VISTA expression in tumor cells was observed in some patients. These data suggest that only certain subtypes of tumor cells express VISTA. The possible immune regulatory function of VISTA on tumor cells urgently needs to be investigated. In this study, VISTA expression did not correlate with tumor size, gender, age, tumor stage (T-category), lymph node metastases (N-category) or distant metastases (M-category). This finding could be due to a small sample size, so a larger cohort of CRC patients needs to be tested to address this important point.

Recent studies on immune checkpoint inhibitors CTLA-4 inhibitors [24] and PD-1 inhibitors [25] demonstrated increased VISTA expression in treated prostate cancer and melanoma patients, respectively, suggesting that negative immune checkpoint regulation by VISTA represents an important mechanism of acquired resistance

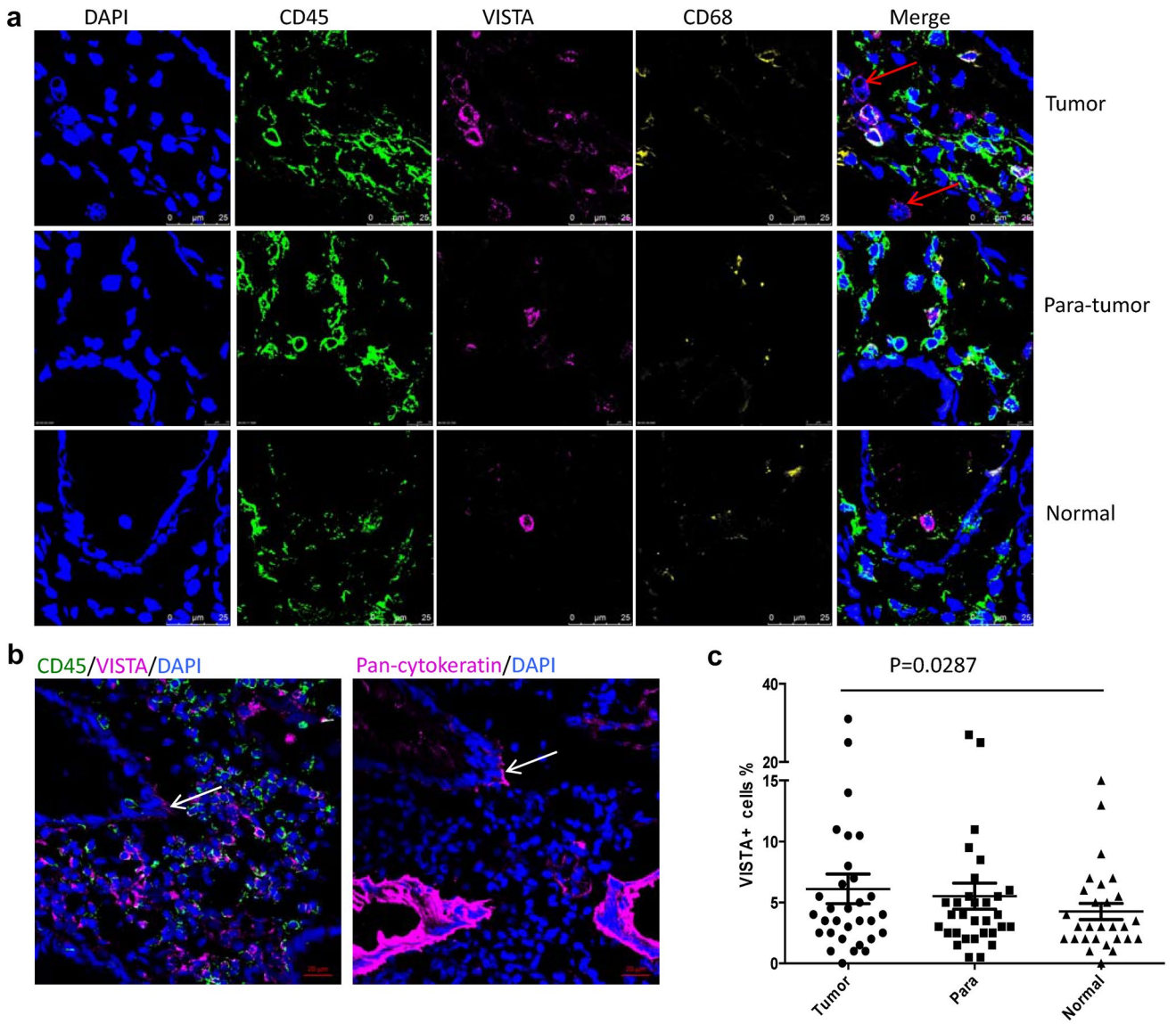

Fig. 3 Intratumoral VISTA protein is expressed by tumor-infiltrating lymphocytes. **a)** OCT-embedded tumor, para-tumor and normal tissue were cut into 5 μM slices and then stained by anti-CD45, anti-VISTA, anti-CD68 and control IgG. Sections were mounted in medium with DAPI. The majority of VISTA-expressing cells are CD45 positive and are macrophages ($CD45^{+}CD68^{+}$) expressing VISTA. The expression levels of VISTA in para-tumor and normal sections are lower than those in tumor sections. **b** Sequential sections were cut from tumor and then stained with anti-VISTA and anti-pan-cytokeratin, respectively. **c** The summary data for VISTA expression in tumors, para-tumor and normal tissues. Each dot represents data generated from one patient. *P* values were acquired by the Student's paired *t* test

in cancer patients treated with immune checkpoint inhibitors. Thus, there is an urgent need to evaluate VISTA expression in CRC patients who have received checkpoint blockade therapy to verify this hypothesis. Nonetheless, these interesting observations indicate that combined VISTA and PD-1/PD-L1 blockade may be a new CRC treatment option. Indeed, combination immunotherapy of PD-L1 and VISTA blockade in the CT26 colon cancer model led to a synergistic therapeutic effect [14]. In addition, VISTA/PD-1 double knockout mice lack overt autoimmunity, which may offer a less toxic alternative to PD-1/CTLA-4 combination therapy. Currently, one phase I trial that targets both VISTA and PD-L1/PD-L2 in solid tumors using a small molecule, CA-170 (referred to its clinicaltrials.gov identifier NCT02812875) has been started based on this possibility.

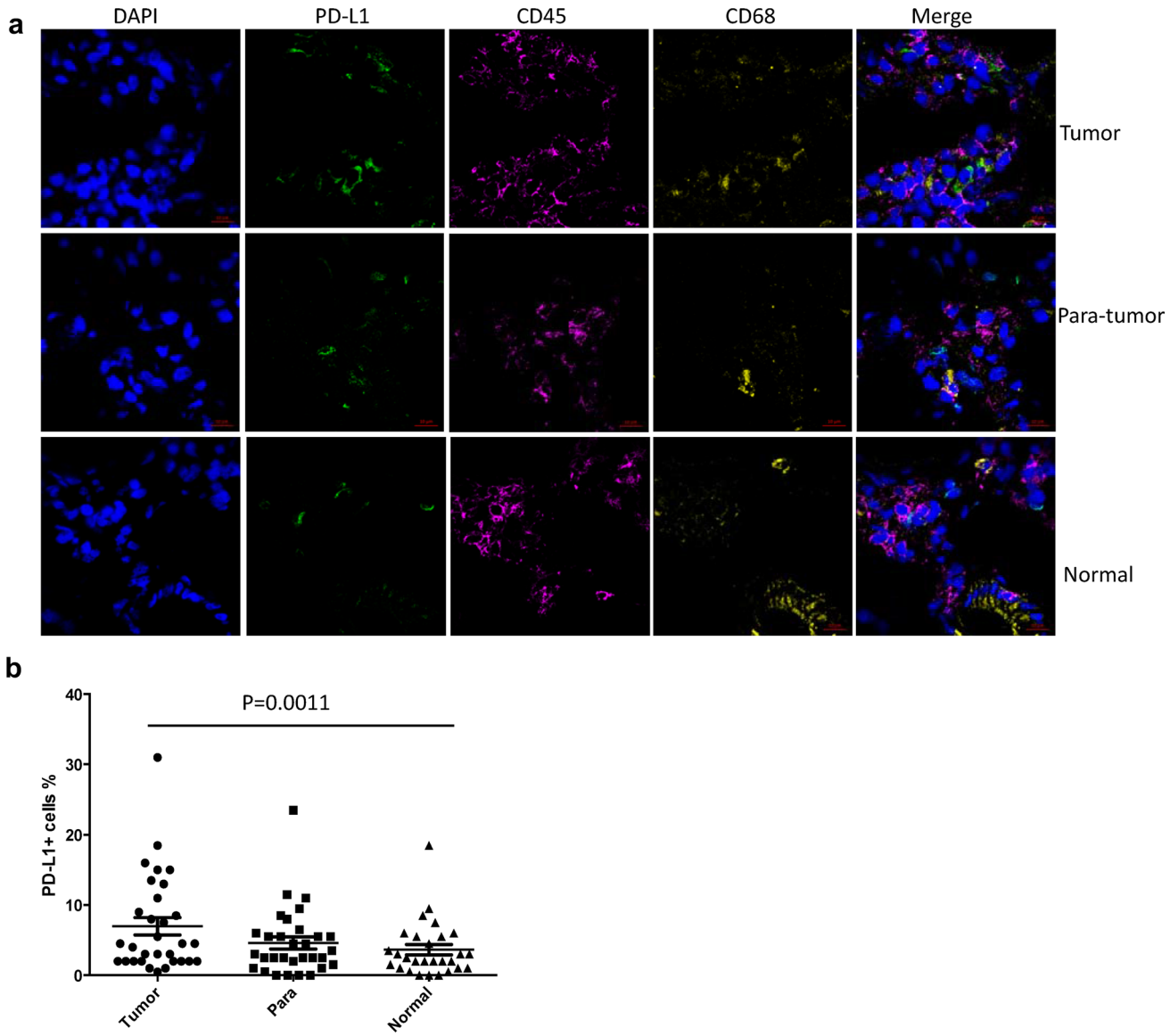

Fig. 4 Intratumoral PD-L1 protein is expressed by CD45 negative cells. **a** OCT-embedded tumor, para-tumor and normal tissue were cut into 5 μM slices and then stained by anti-CD45, anti-PD-L1, anti-CD68 and control IgG. Sections were mounted in medium with DAPI. The majority of PD-L1-expressing cells are CD45 negative. **b** The summary data about VISTA expression in tumors, para-tumor and normal tissues. The expression level of PD-L1 in normal sections was significantly lower than that in tumor section. Each dot represents data generated from one patient. *P* values were acquired by the Student's paired *t* test

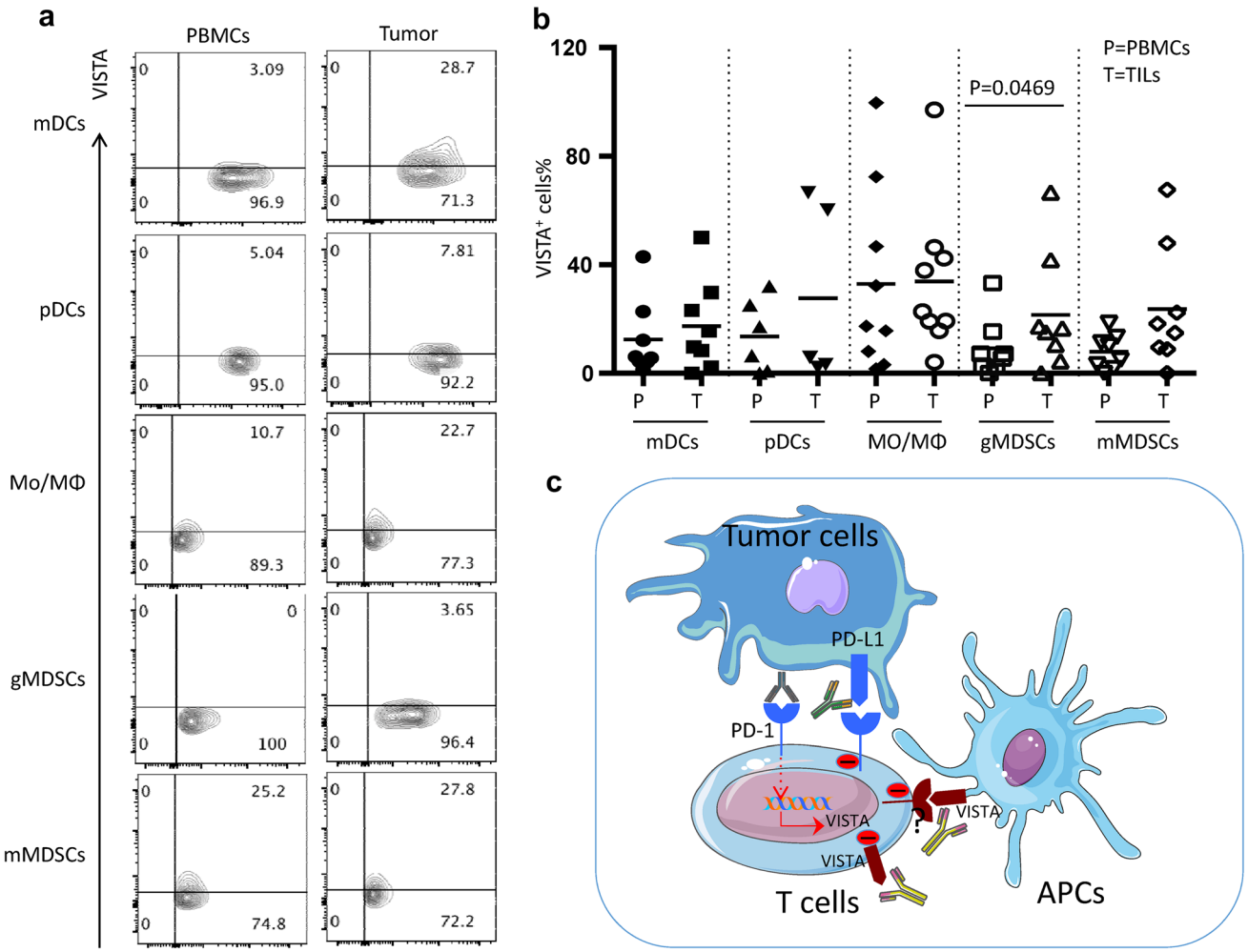

Fig. 5 Unlike PD-L1, VISTA protein is expressed by different myeloid cell subsets in CRC tumors. **a** Tumor-infiltrating lymphocytes were isolated by enzymatic digest and then stained with surface markers of different myeloid cell subsets. PBMCs were also isolated from blood by Ficoll-Hypaque gradient centrifugation. Peripheral VISTA was mainly expressed by monocytic MDSCs and monocytes while intra-tumoral VISTA could be detected on all the tested subsets of myeloid cells from this patient, except gMDSCs. **b** Summary data about VISTA expression in peripheral blood and tumors. Each dot represents data generated from one patient. *P* values were acquired by the Wilcoxon test. **c** VISTA and PD-L1 are non-redundant immune checkpoint molecules. Tumor-expressed PD-L1 can interact with PD-1 on T cells that results in inhibition, whereas VISTA can function as a co-inhibitory ligand expressed on APCs and as a co-inhibitory receptor expressed on T cells within tumor microenvironments. Antibodies interfering with PD-L1/PD-1 interaction upregulate VISTA expression. Combinatorial blockade of PD-L1/PD-1 and VISTA might lead to enhanced therapeutic efficacy

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Author contributions Shan Xie and Wenjuan Zang performed most of the experiments; Jia Huang, Qin Qiao, Shanjuan Hong and Haidong Tan performed some of the experiments or collected clinical specimens; Zhiying Yang supervised clinical specimens; Chen Dong and Ling Ni designed and supervised the study; Ling Ni wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest to disclose.

Ethics statement All procedures in this study were approved by the institutional review board at Tsinghua University and were performed in accordance with the institutional guidelines. All patients' data were anonymized before study inclusion. Fresh specimens and matched blood of CRC patients without any therapies were obtained from the China-Japan Friendship Hospital in Beijing.

Informed consent Informed consent was obtained from all individual participants included in the study.

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