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The Expression of Immune Checkpoint Receptors and Ligands in the Colorectal Cancer Tumor Microenvironment

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Abstract. *Background/Aim: The limited efficacy of immune checkpoint inhibitors in colorectal cancer (CRC) is likely due to immunosuppressive mechanisms including T cell exhaustion caused by inhibitory immune checkpoints in the tumor microenvironment. Materials and Methods: We investigated the expression status of the inhibitory immune checkpoint receptors on tumor-infiltrating T cells and their ligands on tumor cells by flow cytometry and immunohistochemistry, using surgically-resected specimens of CRC. Results: Flow cytometry analysis indicated that TIM-3, TIGIT, and PD-1 were expressed on tumor-infiltrating CD4+ (8.3%, 56.0%, 26.1%) and CD8+ T cells (8.2%, 51.6%, 23.5%), and CRC cells abundantly expressed PD-L1, CEACAM-1, and CD155 (2.2%, 77.0%, 46.8%). Immunohistochemical analysis revealed that the tumor proportional score of PD-L1, CEACAM-1, and CD155 was 42.4%, 54.2%, and 52.1%, respectively. Conclusion: PD-1, TIM-3, and TIGIT axes may reduce T cell function in the CRC tumor microenvironment.*

The treatment of colorectal cancer (CRC) has been remarkably advanced in recent years; however, CRC is still the third most prevalent cancer and the second leading cause of cancer-related deaths worldwide, according to the GLOBOCAN 2018 database (1). Immune checkpoint inhibitors (ICIs) targeting the programmed cell death protein-1 (PD-1) axis have recently become standard therapy in patients with metastatic CRC exhibiting microsatellite instability (MSI)-high (2-6); however, the population of MSI-high CRC patients accounts for approximately 4 to 5% of metastatic CRC patients (7-9).

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It is well-known that immunotherapy targeting the PD-1 axis is ineffective in patients with MSI-low and microsatellite stable (MSS) CRC (10, 11). Furthermore, the response rate to the ICIs ranges from 30 to 60% in patients with MSI-high CRC, even though MSI-high CRC is an excellent target for immunotherapy with ICIs (2-5). Its limited efficacy is likely due to immunosuppressive mechanisms in the CRC tumor microenvironment, and the T cell exhaustion caused by the inhibitory immune checkpoints is a major immunosuppressive mechanism (12, 13).

The most promising inhibitory immune checkpoint receptors for cancer immunotherapy are cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), PD-1, T-cell immunoglobulin-3 (TIM-3), lymphocyte activation gene 3 (LAG-3), and T-cell immunoglobulin and ITIM domain (TIGIT). These receptors are expressed on immune cells including activated T cells, whereas their ligands are expressed in immune cells and tumor cells (12, 14). CTLA-4 mainly inhibits immune cells in lymph nodes, and its ligands are expressed only on antigen-presenting cells. However, the other receptors that regulate immune cells, especially exhausted effector T cells around the tumor, and their ligands are expressed on tumor cells as well as antigen-presenting cells (12, 14). Currently, the combination of anti-PD-1 monoclonal antibody (mAb) with ICIs targeting TIM-3, LAG-3, and TIGIT in advanced solid tumors including CRC are being explored in clinical trials (NCT03446040, NCT02966548 and NCT02964013). To improve the efficacy of immunotherapy with ICIs in CRC including MSI-high, MSI-low and MSS, it is crucial to reveal the expression status of these inhibitory immune checkpoint receptors on T cells and their ligands on tumor cells in the tumor microenvironment. Although some reports have shown evidence of expression of the inhibitory immune checkpoint receptors and ligands, the expression status in the tumor microenvironment of CRC patients has not yet been elucidated in detail (15-17). Therefore, in this study, we evaluated the expression of PD-1, TIM-3, LAG-3, and TIGIT on tumor-infiltrating T cells and the expression of their ligands, including programmed death

ligand-1 (PD-L1), PD-L2, carcinoembryonic antigen-related adhesion molecule-1 (CEACAM-1), CD155, and lymph node sinusoidal endothelial cell C-type lectin (LSECtin), on tumor cells in CRC using freshly isolated clinical samples and formalin-fixed paraffin-embedded tissue samples from the same patient. We also evaluated the correlation between the expression of inhibitory receptors and between that of their ligands by interrogating The Cancer Genome Atlas (TCGA) colorectal adenocarcinoma dataset.

Materials and Methods

Patients and clinical samples. The inclusion criteria were patients who underwent surgery for CRC at Fukushima Medical University Hospital between April 2019 and July 2020. Since the preoperative application of a self-expanding metal stent induces local inflammation and we needed 1-2 cm^3 samples for flow cytometry analysis, the exclusion criteria were preoperative stent application and a tumor of which diameter was 3 cm or less (18). Clinical samples, including fleshly surgically-resected samples and formalin-fixed paraffin-embedded whole tissue samples, from all enrolled patients were subjected to flow cytometry and immunohistochemistry (IHC) staining. Surgically-resected tissues of normal mucosa, tumor, and lymph node were digested as previously described, and the collected cells from these samples were used for flow cytometric analysis (18).

Ethics approval and consent to participate. This study was conducted in accordance with the ethical principles of the 1964 Declaration of Helsinki and its later amendments, and was approved by the Institutional Research Ethics Committee at Fukushima Medical University School of Medicine (Reference Nos. 2289 and 29316). Written informed consent was obtained from all patients included in this study for the use of their specimens and clinical data for research and publication prior to collecting the specimens at Fukushima Medical University Hospital.

Flow cytometry and gating methods. The cells collected from fleshly isolated clinical samples (n=20) were stained according to the manufacturer's protocol for each antibody. The following antibodies were purchased from BD Biosciences (San Jose, CA, USA): APC-H7 conjugated mouse anti-human CD3 mAb (cat. no. 560275), Alexa Fluor®488 conjugated mouse anti-human CD4 mAb (cat. no. 557695), BV421 conjugated mouse anti-human CD8 mAb (cat. no. 562429), Alexa Fluor® 647 conjugated mouse anti-human CD223 (LAG-3) antibody (cat. no. 565717), PerCP-Cy™ 5.5 conjugated mouse anti-human CD279 (PD-1) mAb (cat. no. 561273), and PE conjugated mouse anti-human CD366 (TIM-3) mAb (cat. no. 565570). The following antibodies were purchased from BioLegend (San Diego, CA, USA): PE/Cyanine7 conjugated mouse anti-human TIGIT mAb (cat. no. 372713), APC/Cy7 conjugated mouse anti-human CD31 mAb (cat. no. 303119), PerCP/Cy5.5 conjugated mouse anti-human CD66a/c/e (CEACAM-1, CEACAM-5 and CEACAM-6,) mAb (cat. no. 342311), and PE/Cy7 conjugated mouse anti-human CD155 mAb (cat. no. 337613). APC conjugated mouse anti-human CD273 (PD-L2) mAb (cat. no. 17-5888-42), PE conjugated mouse anti-human CD274 (PD-L1) mAb (cat. no. 12-5983-42), and Alexa Fluor 488 conjugated mouse anti-human CD326 (EpCAM) mAb (cat. no. 53-8326-41) were purchased from Invitrogen™ (Waltham, MA, USA), and Alexa Fluor® 405-conjugated mouse anti-human LSECtin mAb (cat. no. FAB2947V) was obtained from R&D SYSTEMS (Minneapolis, MN, USA). Unstained samples served as negative controls.

For analysis of the immune checkpoint receptors on lymphocytes, we first gated the population of lymphocytes in a setting with forward scatter and side scatter. Following the gating of lymphocytes, a second gate was set on CD3-positive cells. Under these two gates, the quadrant was made with CD4 and CD8 to detect CD4+ and CD8+ T cells. The expression of the immune checkpoint receptors, including PD-1, TIM-3, LAG-3, and TIGIT on CD4+ and CD8+ T cells, was evaluated (Figure 1). For analysis of the immune checkpoint ligands on the tumor cells, we first gated the population of large cells in a setting with forward scatter and side scatter. Following the gating of large cells, the quadrant was made with CD31 and EpCAM-1, and CD31-negative and EpCAM-1-positive cells were used as tumor cells in this study (19). The expression of the immune checkpoint ligands including PD-L1, PD-L2, CEACAM-1, LSECtin, and CD155 on the tumor cells was evaluated (Figure 2). All stainings were measured using a BD FACSCanto II flow cytometer (BD Biosciences), and data were analyzed using FlowJo software, version 10.3.0 (Ashland, OR, USA).

IHC staining. We used 20 formalin-fixed paraffin-embedded whole tissue samples of CRC. IHC was performed as previously described using the following primary antibodies: rabbit anti-PD-L1 mAb (1:400; cat. no. 13684; Cell Signaling Technology; Danvers, MA, USA), rabbit anti-PD-L2 mAb (1:200; cat. no. 82723; Cell Signaling Technology), rabbit anti-CD155 mAb (1:200; cat. no. 81254; Cell Signaling Technology), rabbit anti-CEACAM-1 mAb (1:500; cat. no. ab243889; Abcam; Cambridge, UK), and rabbit anti-LSECtin mAb (1:200; cat. no. ab181196; Abcam) (20, 21). Assessment of IHC staining was performed by three independent observers (PN, SN, and KS), who were blinded to the clinical data. The expression of PD-L1, PD-L2, CEACAM-1, CD155, and LSECtin was evaluated using the tumor proportional score (TPS) that measured membrane staining of the tumor cells; ≥1% was defined as positive and <1% was defined as negative (22).

TCGA dataset analysis. The mRNA expression z-scores of genes (RNA Seq V2 RSEM) were retrieved from the TCGA colorectal adenocarcinoma dataset (PanCancer Atlas, n=592) through cBioPortal (23, 24). In this study, we evaluated the mRNA expression levels of PD-1 (*PDCD1*), TIM-3 (*HAVCR2*), LAG-3 (*LAG3*), TIGIT (*TIGIT*), PD-L1 (*CD274*), PD-L2 (*PDCD1LG2*), CEACAM-1 (*CEACAM1*), CD155 (*PVR*), and LSECtin (*CLEC4G*).

Statistics. Comparison of multiple groups was performed with one-way analysis of variance followed by a Tukey's post hoc test. Correlation analysis was performed with Pearson's product-moment correlation coefficient and significance was calculated with Fisher's exact test. All error bars represent mean±standard deviation. *p*-Values <0.05 were considered statistically significant. GraphPad Prism 6 software (GraphPad Software Incorporation, La Jolla, CA, USA) was used for statistical analysis.

Results

Co-expression of immune inhibitory receptors on T cells in CRC patients. The characteristics of patients and tumors are presented in Table I (Stage I; 7 cases, Stage II; 6 cases, Stage III; 6 cases, Stage IV; 1 case). Clinical samples from these patients were subjected to flow cytometry and IHC staining to evaluate the expression of each immune inhibitory

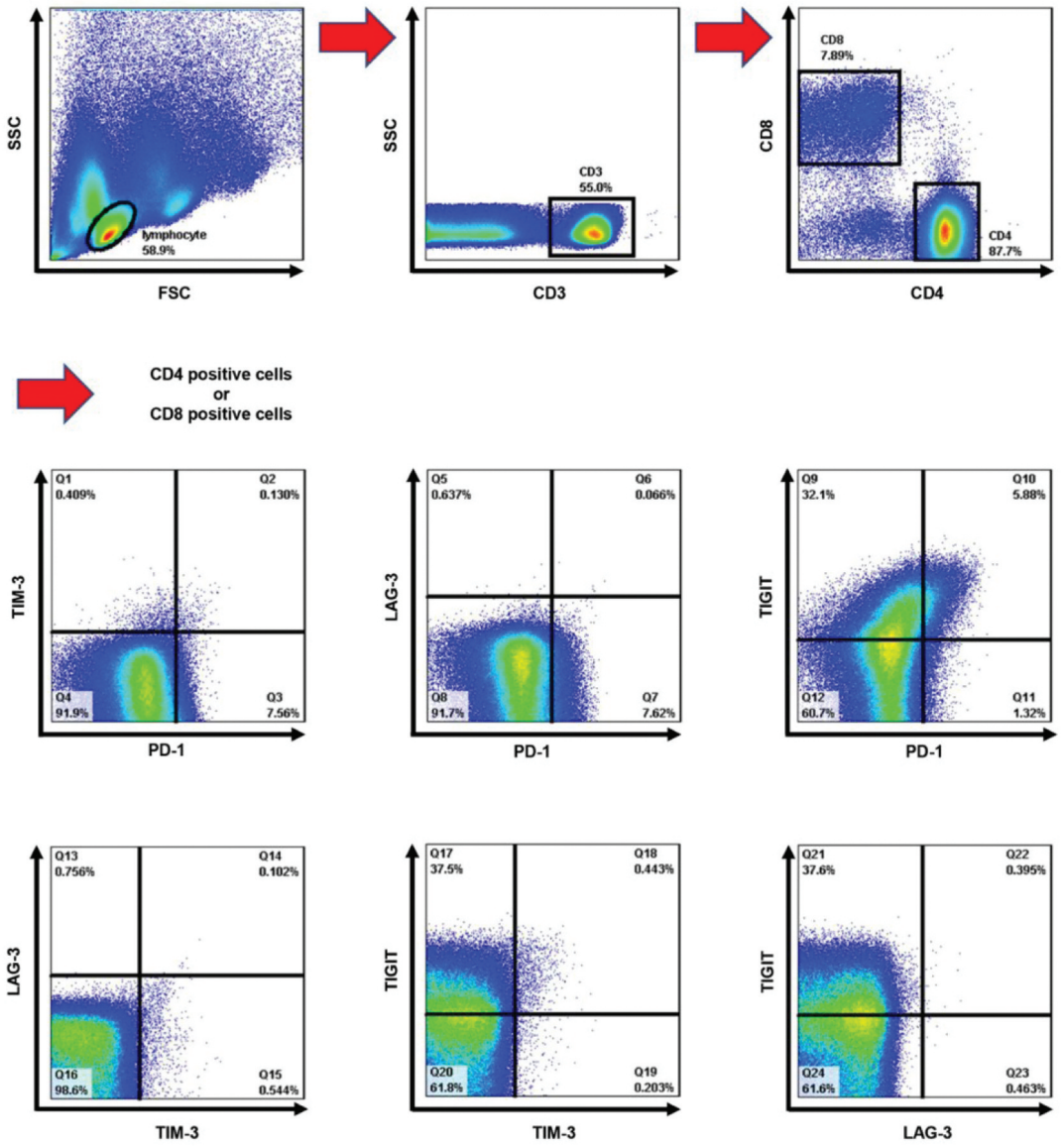

Figure 1. *The gating method for flow cytometry of cells collected from freshly resected surgical specimens to evaluate the expression of the immune checkpoint receptors on CD4+ and CD8+ T cells.*

receptor (PD-1, TIM-3, LAG-3, and TIGIT) and ligand (PD-L1, PD-L2, CEACAM-1, CD155, and LSECtin).

At first, we evaluated the frequency of expression of immune inhibitory receptors including PD-1, TIM-3, LAG-3, and TIGIT on CD4+ and CD8+ T cells in the normal mucosa (Normal), tumor (Tumor), and lymph node (LN) by flow cytometry using freshly resected surgical specimens. The gating method to detect each immune inhibitory receptor on T cells is presented in Figure 1. The frequency of PD-1 and TIGIT expression on CD4+ T cells was significantly higher in the Tumor than in the Normal and LN (PD-1 Normal 7.3%, Tumor 26.1%, LN 11.6%; TIGIT Normal 31.0%, Tumor 56.0%, LN 30.0%) (Figure 3A). Regarding CD8+ T cells, the frequency of PD-1 expression was significantly higher in the Tumor than in the Normal (PD-1 Normal 4.2%, Tumor 23.5%, LN 15.9%) (Figure 3A). Although there was no significance, the frequency of TIM-3 expression on both CD4+ and CD8+ T cells was higher in the Tumor than in the Normal and LN (CD4+ Normal 5.3%, Tumor 8.3%, LN 1.9%; CD8+ Normal 5.4%, Tumor 8.2%, LN 1.7%) (Figure 3A). The frequency of TIGIT expression on both CD4+ and CD8+ T cells was obviously higher than the other receptors (CD8+ Normal 45.1%, Tumor 51.6%, LN 39.8%) (Figure 3A).

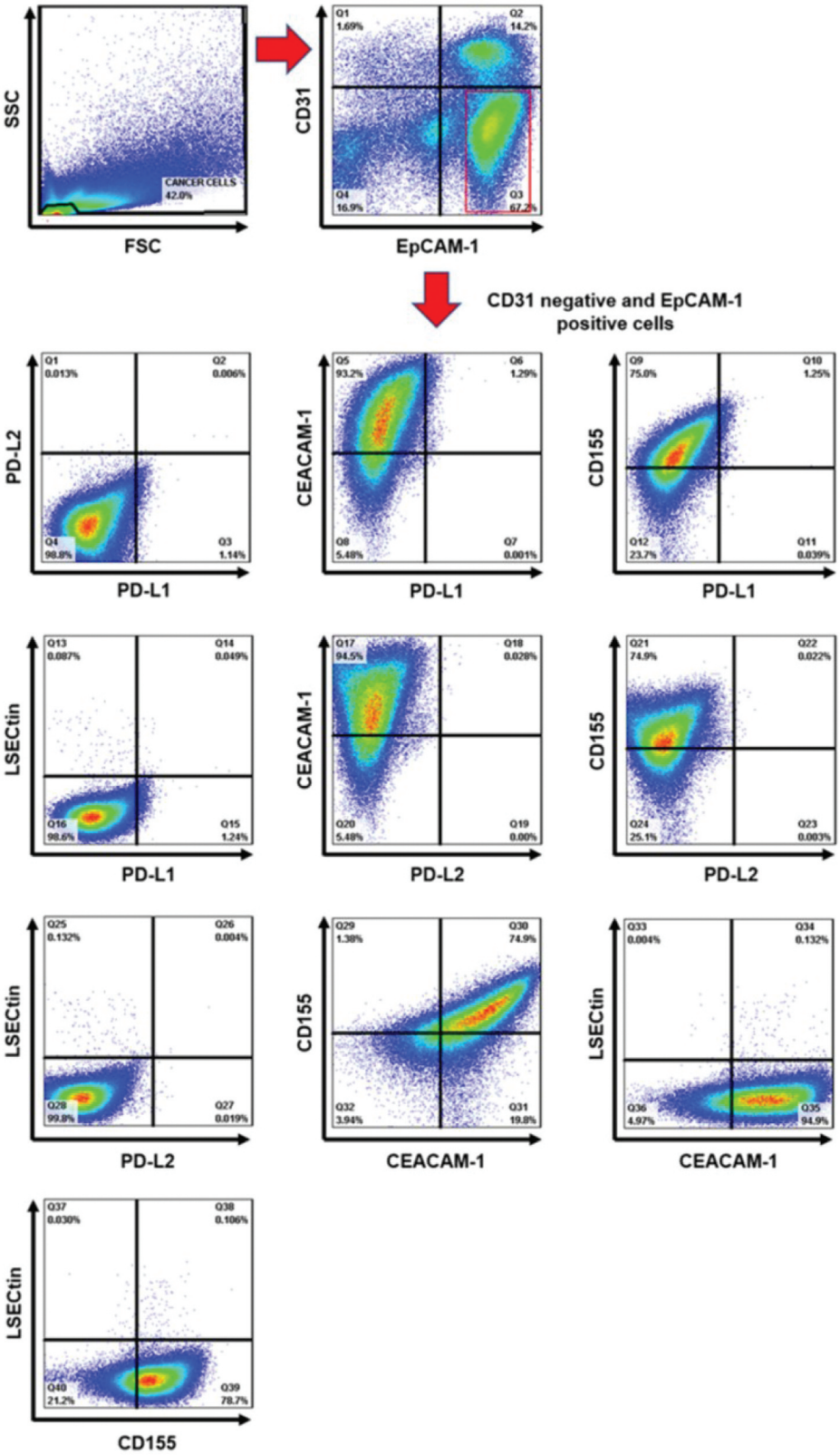

Figure 2. *The gating method for flow cytometry of cells collected from freshly resected surgical specimens to evaluate the expression of the immune checkpoint ligands on CRC cells.*

Table I. *Patient and tumor characteristics*.

No	Age	Gender	Diagnosis	T	N	M	Stage	Pathology
1	88	M	S	3	1a	0	IIIb	tub 2
2	74	M	A	4a	1a	0	IIIb	por 1
3	78	F	T	4a	0	0	IIb	tub 1>pap/muc
4	63	M	R	3a	0	0	IIa	tub 1>tub 2
5	78	M	R	2	0	0	I	tub 2> tub1
6	60	F	S	2	0	0	I	tub1-tub2
7	57	M	R	2	0	0	I	tub 2
8	57	M	T	3	1b	0	IIIb	tub 2>pap
9	76	M	R	1b	0	0	I	tub 2
10	81	M	R	3	0	0	IIa	tub 2> tub1
11	75	M	R	1b	0	0	I	tub1-tub2
12	63	F	S	4a	1b	0	IIIb	tub 2> tub1
13	69	F	T	1b	0	0	I	tub 1
14	66	F	S	4a	0	0	IIb	tub 2> tub1
15	62	M	R	4a	2a	1a	IVa	tub 2>muc> tub1
16	68	M	A	3	0	0	IIa	tub 2
17	72	M	A	4a	0	0	IIb	tub 1>tub 2
18	52	M	S	3	1b	0	IIIb	muc>tub2
19	75	F	R	1b	0	0	I	tub 2
20	74	F	A	3	1a	0	IIIb	tub 2

C: Cecum; A: ascending colon; T: transverse colon; D: descending colon; S: sigmoid colon; R: rectum. Tumor (T), lymph node metastasis (N), distant metastasis (M), and stage were determined according to the Japanese Classification of Colorectal, Appendiceal and Anal Carcinoma (9^{th} edition).

Next, we evaluated the frequency of co-expression of immune inhibitory receptors on T cells: PD-1 and TIM-3, PD-1 and LAG-3, PD-1 and TIGIT, TIM-3 and LAG-3, TIM-3 and TIGIT, LAG-3 and TIGIT. Although the frequency of each co-expression combination was not high, the frequencies of CD4+ T cells expressing PD-1+/TIM-3+ (Normal 1.2%, Tumor 4.0%, LN 0.2%), PD-1+/TIGIT+ (Normal 3.9%, Tumor 17.2%, LN 7.1%), and TIM-3+/TIGIT+ (Normal 5.2%, Tumor 8.0%, LN 1.1%), and those of CD8+ T cells expressing PD-1+/TIM-3+ (Normal 0.1%, Tumor 5.2%, LN 0.3%), PD-1+/TIGIT+ (Normal 2.0%, Tumor 14.3%, LN 10.6%), and TIM-3+/TIGIT+ (Normal 3.6%, Tumor 6.9%, LN 0.6%) were significantly higher in the Tumor than in the Normal and/or LN (Figure 3B).

In the analysis of the TCGA colorectal adenocarcinoma dataset, a significant positive correlation was noted between mRNA expression of PD-1 and TIM-3, LAG-3, or TIGIT, and between TIM-3 and LAG-3 or TIGIT, and between TIGIT and LAG-3 (Figure 3C).

Co-expression of immune inhibitory ligands on CRC cells. We evaluated the frequency of expression of immune inhibitory ligands including PD-L1, PD-L2, CEACAM-1, CD155, and LSECtin on CRC cells by flow cytometry using the freshly resected surgical specimens. The gating method to detect each immune inhibitory ligand on CRC cells is presented in Figure 2. The frequency of each ligand expression on CRC cells is shown in Figure 4A. The frequency of expression of CEACAM-1 (77.0±16.3%, ligand for TIM-3) and CD155 (46.8±27.2%, ligand for TIGIT) was obviously higher compared to that of PD-L1 (2.2±4.3%, ligand for PD-1), PD-L2 (0.2±0.4%, ligand for PD-1) and LSECtin (5.1±5.8%, ligand for LAG-3) (Figure 4A). The co-expression status of the immune inhibitory ligands on CRC cells is shown in Figure 4B. All patients simultaneously expressed several ligands on CRC cells, even though the expression level of each ligand was different in each patient (Figure 4B). Furthermore, a positive correlation was also noted between mRNA expression of PD-L1 and PD-L2 or LSECtin, between PD-L2 and LSECtin, and between CEACAM-1 and CD155 in the TCGA colorectal adenocarcinoma dataset (Figure 4C).

Subsequently, we evaluated the frequency of ligand expression on CRC cells by IHC staining using formalin-fixed paraffin-embedded whole tissue samples. Representative images of the IHC staining are shown in Figure 5A. The expression of the ligands was evaluated by TPS for membrane staining. The TPS of each ligand is shown in Figure 5B. The TPS of PD-L1 (42.4±28.9%), CEACAM-1 (54.2±25.5%), and CD155 (52.1±22.7%) was remarkably higher than that of PD-L2 (6.7±9.6%) and LSECtin (no staining) (Figure 5B). The IHC staining also revealed that all patients simultaneously expressed several ligands on CRC cells (Figure 5C). Interestingly, there was heterogeneity of expression of these ligands in each patient (Figure 5D).

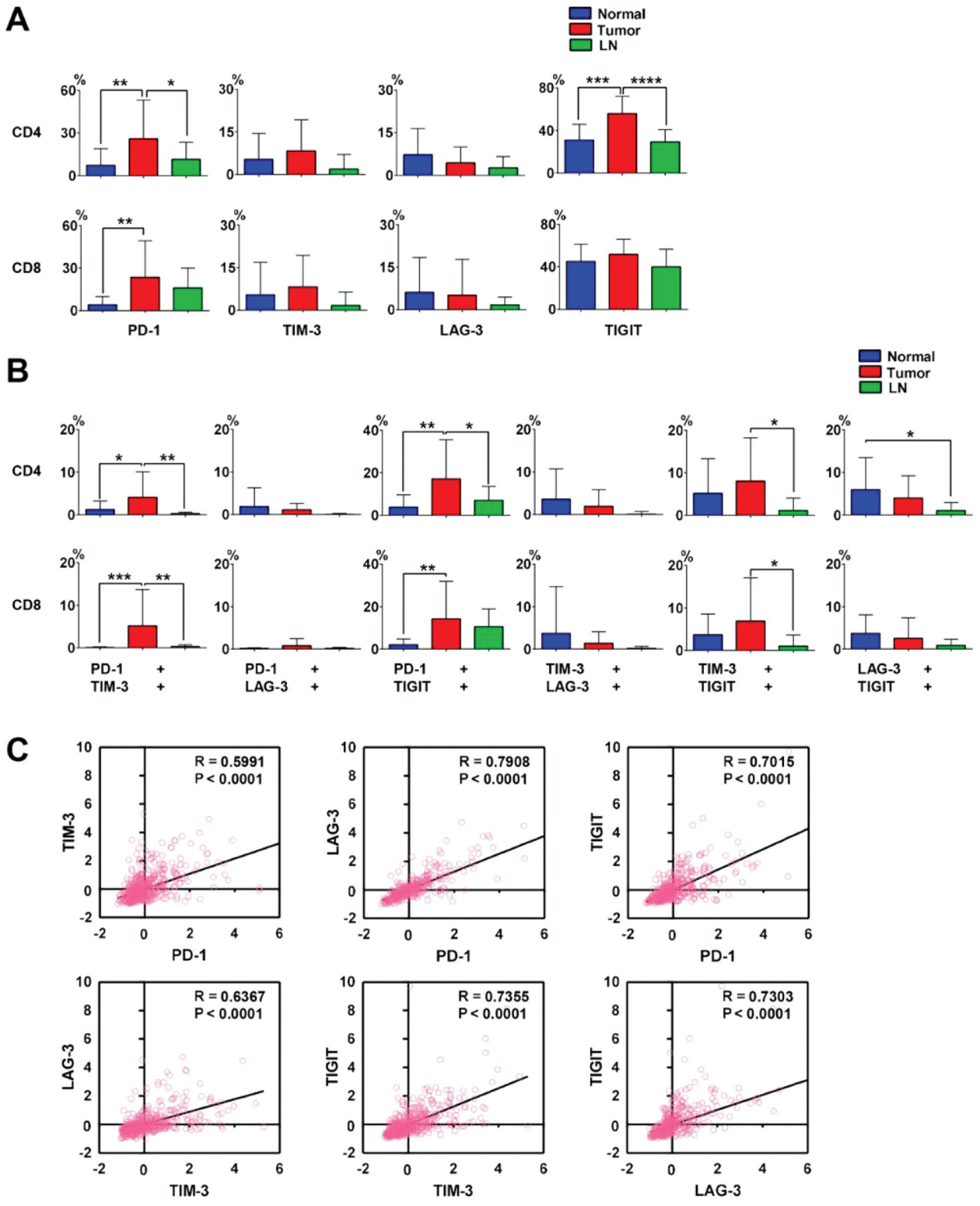

Figure 3. *The expression of immune checkpoint receptors on T cells. CD4+ and CD8+ T cells were collected from freshly resected surgical specimens including normal mucosa (Normal), tumor (Tumor), and lymph node (LN). The expression of the immune checkpoint receptors on T cells was evaluated by flow cytometry using fresh samples and by analysis of the TCGA colorectal adenocarcinoma dataset. (A) The frequency of CD4+ and CD8+ T cells expressing PD-1, TIM-3, LAG-3, or TIGIT. (B) The frequency of T cells that co-expressed immune checkpoint receptors. (C) The correlation between mRNA expressions of two immune checkpoint receptors in the TCGA colorectal adenocarcinoma dataset. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

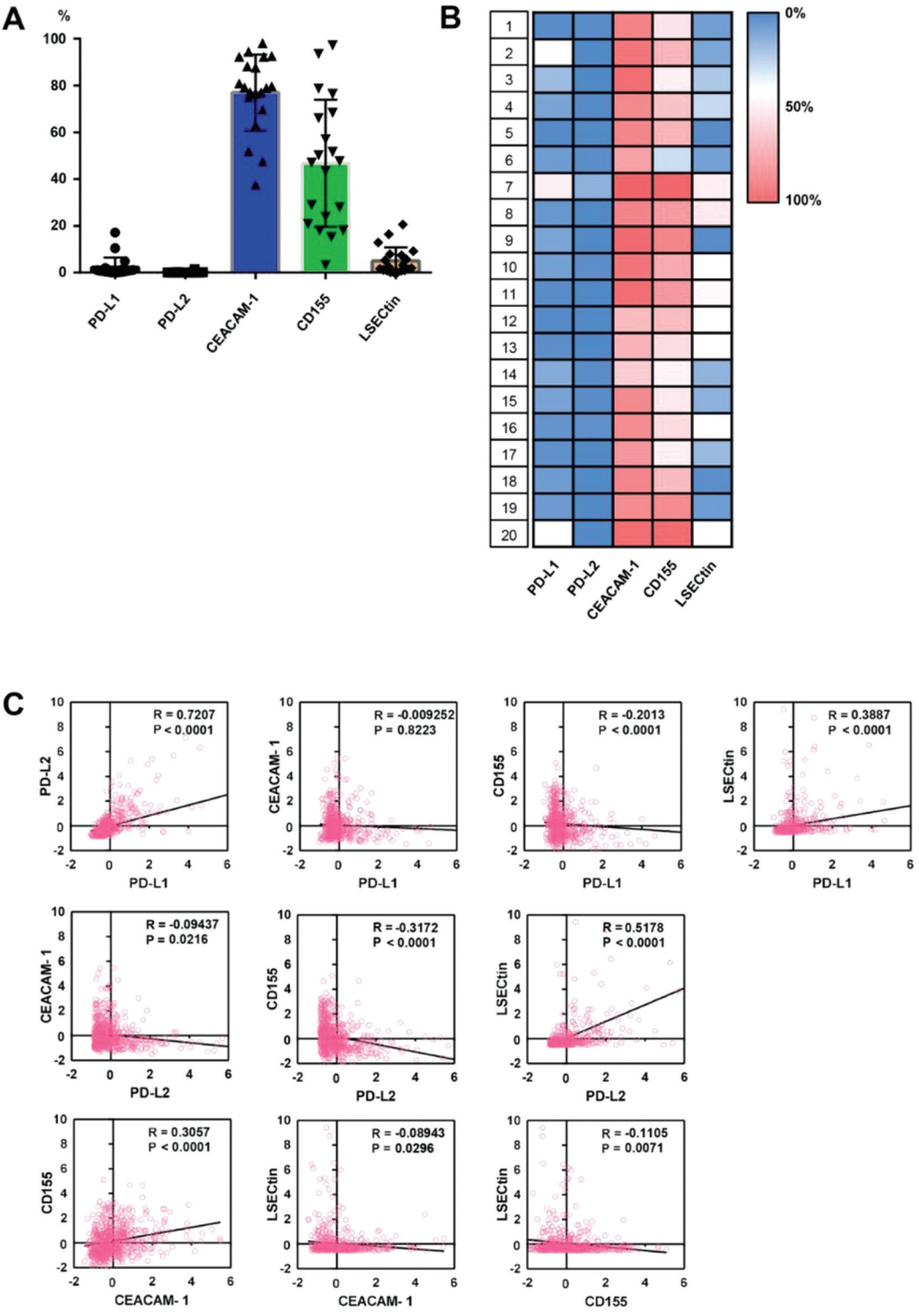

Figure 4. *The expression of immune checkpoint ligands on CRC cells. CRC cells were collected from freshly resected surgical specimens, and the immune checkpoint ligands on CRC cells were evaluated by flow cytometry. (A) The frequency of CRC cells expressing PD-L1, PD-L2, CEACAM-1, CD155, or LSECtin. (B) The heatmap showing the frequency of each immune checkpoint ligand on CRC cells in each patient. (C) The correlation between mRNA expressions of two immune checkpoint ligands in the TCGA colorectal adenocarcinoma dataset.*

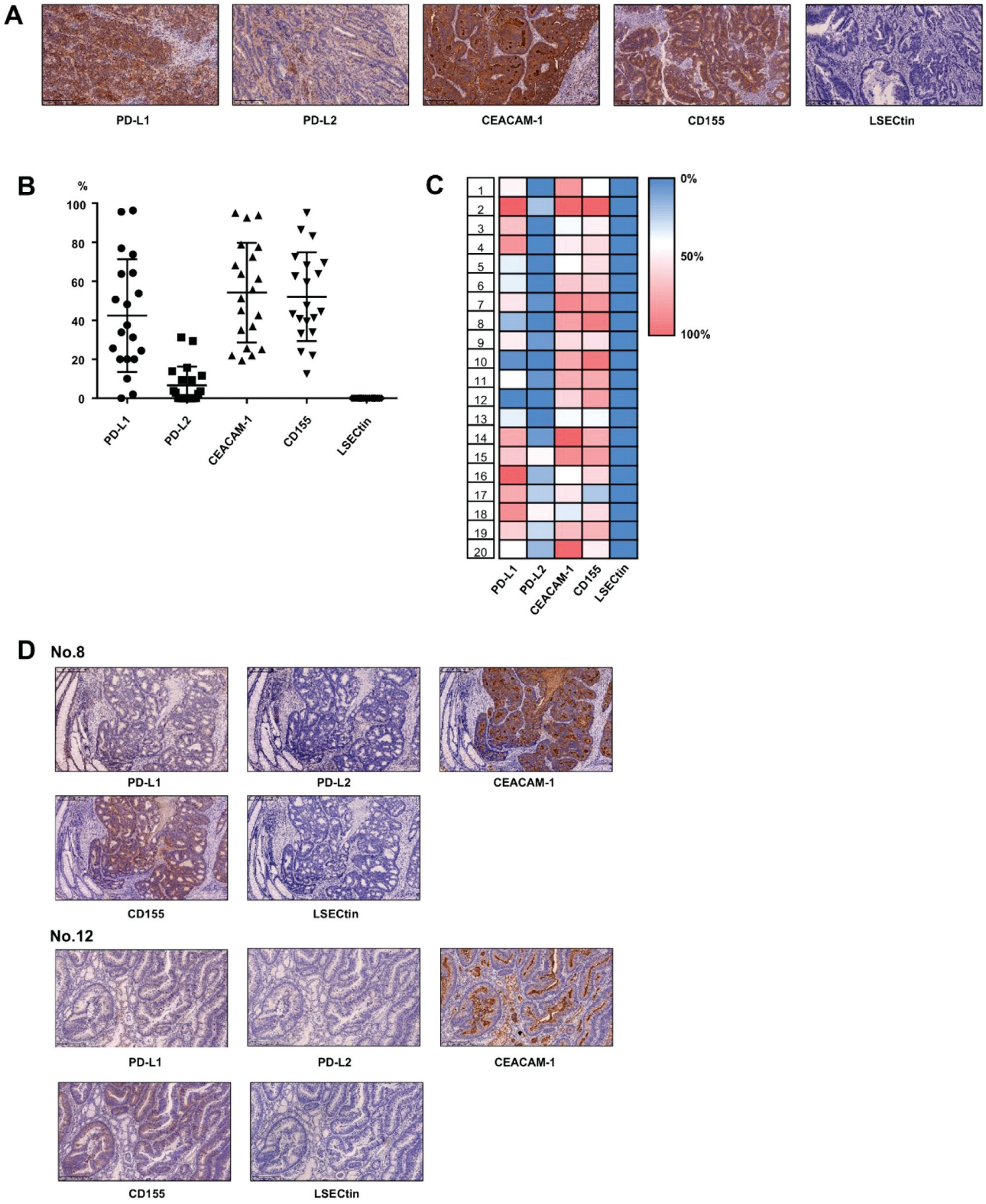

Figure 5. *The expression of the immune checkpoint ligands on CRC cells evaluated by IHC. (A) Representative IHC staining with PD-L1, PD-L2, CEACAM-1, CD155, and LSECtin. Original magnification ×10. (B) TPS of each ligand. (C) The heatmap showing the TPS of each ligand in each patient. (D) Representative IHC staining showing the heterogeneity of the expression of five ligands in two patients (No.8 case and No.12 case). Original magnification ×10.*

Discussion

In order to improve the efficacy of immunotherapy of CRC patients with ICIs, we investigated the expression status of immune checkpoint receptors and ligands in the CRC tumor microenvironment using surgically-resected specimens and the TCGA colorectal adenocarcinoma dataset. We revealed that the frequency of CD8+ T cells co-expressing PD-1/TIM-3, PD-1/TIGIT, or TIM-3/TIGIT significantly increased in the CRC tumor microenvironment, and that the CRC cells abundantly expressed PD-L1 (ligand for PD-1), CEACAM-1 (ligand for TIM-3), and CD155 (ligand for TIGIT). These results suggest that the PD-1, TIM-3, and TIGIT axes may reduce T cell function in the tumor microenvironment of CRC patients.

TIM-3 is expressed on activated CD8 T cells as well as CD4 Th1 T cells, Treg cells, Th17 cells, and other innate immune cells, and binds to CEACAM-1, galectin-9, phosphatidylserine, and high mobility group box1 protein (25, 26). The ligands for TIM-3 are expressed on a variety of cell types including cancer cells (25), and we confirmed that CEACAM-1 is expressed on CRC cells (Figure 4 and Figure 5). It has been reported that TIM-3 regulates the proliferation and cytokine release by CD4 Th1 T cells, is refractory to induction of antigen-specific tolerance (27, 28), and is involved in CD8 T cells exhaustion (29-31). We also showed that the frequency of TIM-3 expression on CD4+ and CD8+ T cells is higher in the Tumor than in the Normal and LN, although there was no significance (Figure 3A). Furthermore, we recently reported that an anti-TIM-3 mAb enhanced the cytotoxicity of the tumor-antigen specific CTL clone against gastric cancer cells expressing ligands for TIM-3 (32). Since the function of activated CD8 T cells, as well as CD4 T cells, could be reduced in the CRC tumor microenvironment, it is important to inhibit the TIM-3 axis in both CD4 and CD8 T cells to increase the efficacy of immunotherapy for patients with CRC.

Im *et al*. reported that the subset of CD8 T cells expressing PD-1 and TIM-3 was the more terminally differentiated phenotype, and was present in both lymphoid and non-lymphoid organs, but the subset of CD8 T cells expressing PD-1 without TIM-3 was present in lymphoid tissues (33). Our results reconfirmed that the subset of CD8+ T cells expressing PD-1 and TIM-3 was significantly higher in the Tumor than in the Normal and LN in patients with CRC (Normal 0.1%, Tumor 5.2%, LN 0.3%) (Figure 3B). Furthermore, it has been reported that co-expression of PD-1 and TIM-3 facilitates T cell exhaustion and leads to tumoral immune escape, and anti-TIM-3 or anti-PD-L1 mAb alone often has a weaker effect on interferon-γ production relative to the effect of anti-TIM-3 plus anti-PD-L1 mAb (29, 30). Therefore, a rational approach to improve the efficacy of anti-PD-1 mAb includes its combination with anti-TIM-3 mAb. The anti-PD-1 mAb in combination with ICI targeting TIM-3 is being explored in clinical trials such as NCT02817633 (AMBER study) and NCT03446040.

In this study, we found that the frequency of CD4+ and CD8+ T cells expressing TIGIT was very high in the tumor microenvironment and that most tumor cells expressed CD155 in CRC patients (Figure 3, Figure 4 and Figure 5). The TIGIT axis suppresses T cell activation and regulates anti-tumor and anti-viral CD8 T cell effector function (34, 35). Recently, Inosume *et al*. reported that overexpression of CD155 on melanoma cells suppressed the activation of melanoma-specific T cells via TIGIT (36). In addition, Liu *et al*. reported that CD8 T cells expressing TIGIT correlated with poor prognosis in patients with advanced bladder cancer (37). These findings render the TIGIT as PD-1 and TIM-3 axes as potential candidates for checkpoint blockade in CRC patients as well. Furthermore, Takimoto *et al*. reported that the immune-cell therapy using T lymphocytes in combination with capecitabine-including regimens (Cap) provided a survival benefit in patients with advanced CRC (38). Our results suggest that the ICIs targeting for PD-1, Tim-3, and TIGIT axes may enhance the efficacy of the immune-cell therapy with Cap in these patients.

There was a discrepancy in the ligand expression results between flow cytometry (Figure 4) and IHC (Figure 5) in each patient in our study. In particular, regarding the expression of PD-L1 and LSECtin, PD-L1 was detected by IHC but not flow cytometry whereas LSECtin usually was detected by flow cytometry but not IHC. This may be due to the difference in sensitivity of the antibodies in each assay. Expression of other ligands, including CEACAM-1 and CD155, was detected by both assays, even when the expression levels were different in each patient (Figure 4 and Figure 5). Another reason for the discrepancy in the PD-L1 expression results was the difference in the evaluated area in the two assays, because we used formalin-fixed paraffin-embedded whole tissue samples for IHC and 1-2 cm^3 samples from freshly-isolated clinical specimens for flow cytometry. This means that we evaluated each ligand expression on whole tissue sections by IHC and in a small area of the tumor by flow cytometry. On the other hand, it is generally accepted that quantification and identification of each cell type is more accurate by flow cytometry than by IHC. Moreover, it was noted in this study that heterogeneity is a critical issue when evaluating expression of these ligands, especially of PD-L1 (Figure 5D).

In conclusion, PD-1, TIM-3, and TIGIT were expressed on tumor-infiltrating CD4+ and CD8+ T cells, and their ligands were expressed on tumor cells in patients with CRC. Therefore, the PD-1, TIM-3, and TIGIT axes may reduce T cell function in the CRC tumor microenvironment.

Conflicts of Interest

The Authors declare that they have no competing interests in relation to this study.

Authors' Contributions

KM and KK contributed to the study conception and design. PN, SN, and MI contributed to the acquisition of data by flow cytometry. PN, SN, and KS performed and evaluated the IHC staining. PN, KM, SN, HO, MI, AKTM, KS, HO, SF, WS, MS, ZS, TM, and KK performed the analysis and interpretation of data. PN, KM, and KK drafted the manuscript. All Authors read and approved the final manuscript.

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