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ORIGINAL ARTICLE

Prognostic implication of CD274 (PD-L1) protein expression in tumor-infiltrating immune cells for microsatellite unstable and stable colorectal cancer

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Abstract In this study, we investigated the clinical relevance of CD274 (PD-L1) protein expression by tumor cells and tumor-infiltrating immune cells in colorectal cancer (CRC). To this end, 186 microsatellite instability-high (MSI-H) and 153 microsatellite stable (MSS) CRCs were subjected to immunohistochemistry (IHC) analysis for the expression of CD274 and mismatch repair proteins. CD274 expression was evaluated in tumor cells at the center (TC) and periphery (TP), and immune cells at the center (IC) and periphery (IP) of CRC. IHC slides stained for CD3 and CD8 were scanned using an Aperio ScanScope for precise calculation of tumor-infiltrating T cell density. Additionally, samples were screened for the B-Raf (BRAF)-V600E mutation using a Cobas 4800 System and IHC. In total, $CD274^{TC}$, $CD274^{TP}$, $CD274^{IC}$, and $CD274^{IP}$ were observed in 43 (23.1%), 47 (25.3%), 107 (57.5%), and 102 (54.8%) of the MSI-H CRCs examined, and in three (2.0%), four (2.6%), 47 (30.7%), and 56 (36.6%) of the 153 MSS CRCs tested. Meanwhile, intratumoral heterogeneity of CD274 expression in tumor cells and immune cells was detected in 24 (12.9%) and 47 (25.3%) MSI-H CRCs, respectively. Notably, in both MSI-H and MSS CRC, $CD274^{IC}$ and $CD274^{IP}$ were independently associated with improved prognosis ($P < 0.05$), while *BRAF* mutation was associated with $CD274^{TP}$, poor differentiation, sporadic type, and hMLH1(−)/hMSH2(+)/hMSH6(+)/PMS2(−) in MSI-H CRC ($P < 0.006$). In conclusion, CD274 expression in tumor-infiltrating immune cells was an independent factor for improved prognosis in CRC patients. A deeper understanding of CD274 status may yield improved responses to future CRC immunotherapies.

Keywords Colorectal cancer · CD274 · PD-L1 · Tumor-infiltrating immune cells · Microsatellite instability · Prognosis

Abbreviations

AJCC	American Joint Committee on Cancer
C	Center
CI	Confidence interval
CRC	Colorectal cancer
FFPE	Formalin-fixed and paraffin-embedded
HR	Hazard ratio
IC	Immune cells at the center
IHC	Immunohistochemistry
IP	Immune cells at the periphery
LS	Lynch syndrome
MSI-H	Microsatellite instability-high
MSS	Microsatellite stable
P	Periphery
PCR	Polymerase chain reaction
PD-1	Programmed cell death 1

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PD-L1 Programmed cell death ligand 1
TC Tumor cells at the center
TP Tumor cells at the periphery

Introduction

The remarkable development of immunotherapeutics for several cancers has changed the anti-cancer therapeutic paradigm. Particularly, targeting of PDCD1 (PD-1) and its ligand, CD274 (PD-L1), has demonstrated excellent anti-tumor effects in various tumors [1, 2]. The PDCD1/CD274 checkpoint is thought to comprise a key mechanism of host immune system evasion in malignancy [3], and anti-PDCD1 antibodies have been approved by the US Food and Drug Administration (FDA) for metastatic non-small-cell lung cancer patients [4]. Interestingly, CD274 expression has primarily been observed in MSI-H CRCs, and is only rarely seen in microsatellite stable (MSS) CRCs [5, 6]. Moreover, a recent study indicated that PDCD1 blockade using drugs such as pembrolizumab comprises a specific and highly effective method for treating microsatellite instability-high (MSI-H) CRC [7]. As such, the FDA has granted a priority review to evaluate the efficacy of pembrolizumab for treating MSI-H advanced CRC.

Many studies have examined the prognostic impact of CD274 status and its predictive value in various malignancies. Recent studies showed that CD274 was expressed in several malignant tumors, where it tended to be correlated with decreased survival [1, 8]. However, the prognostic value of CD274 expression remains to be determined, as other studies have detected debatable correlations [9–11], or even no correlation [5, 6, 12], between prognosis and CD274 expression in CRC patients.

Interestingly, a previous report observed an improved response to PDCD1 blockade therapy in patients exhibiting CD274 expression on tumor-infiltrating immune cells [13]. In particular, abundant tumor-infiltrating immune cells are frequently observed in MSI-H CRC [14]. Thus, further evaluation of CD274 expression by immune cells, as well as tumor cells, is needed to facilitate CRC immunotherapy.

Frequently, certain subsets of cancer patients will show decreased responses to targeted therapies due to regional heterogeneity of target molecules, and immunotherapy seems to be no exception. CD274 expression likely has more regional heterogeneity than other mutational alterations, because the PDCD1/CD274 axis is part of a dynamic immune reaction. Indeed, certain recent studies suggest that the value of CD274 immunohistochemistry (IHC) as a predictive and prognostic marker is debatable due to frequent regional heterogeneity [2, 15]. Therefore, regional heterogeneity of CD274 expression should be evaluated in detail in CRC.

This study was conducted to investigate the clinical relevance of CD274 expression by tumor cells and immune cells in CRC, focusing primarily on the MSI-H subgroup. We also analyzed intratumoral heterogeneity and precisely determined the tumor-infiltrating lymphocyte density in CRC using an Aperio image analysis system.

Materials and methods

Patients and samples

In total, 339 CRC patients who underwent surgical resection at Seoul National University Bundang Hospital were included in our study. The patient cohort was composed of two groups: 186 MSI-H CRC patients who were surgically treated between 2003 and 2012, and for comparison, 153 consecutive MSS CRC patients who underwent surgical treatment during 2005. Patients who had received pre-operative chemotherapy or radiotherapy were excluded from the cohort. Among the MSI-H CRC patients, 104 received post-operative chemotherapy, while 74 were only treated surgically; the post-operative history for the remaining eight patients was unobtainable. Meanwhile, 107 and 46 of the MSS CRC patients received post-operative chemotherapy or surgery alone, respectively. Two pathologists (Kyu Sang Lee and Hye Seung Lee) histologically reviewed each CRC case. Clinicopathological data were obtained from hospital medical and pathologic reports. Cancer stage was determined according to the American Joint Committee on Cancer (AJCC), 7th edition. Suspected Lynch syndrome (LS) patients were selected from the MSI-H CRC cohort according to the Bethesda guideline (2004) [16]. These patients did not fulfill the diagnostic criteria for LS, as the *MMR* mutation test was not performed. Follow-up information collected included patient outcome and the interval between the date of surgical resection and the date of death by any cause or censoring (overall survival).

The use of medical record data and patient tissue samples in this study was approved by the Institutional Review Board of Seoul National University Bundang Hospital (reference: B-1511/322-306).

Tissue array method

A tissue microarray (TMA) was constructed using representative lesions of formalin-fixed paraffin-embedded (FFPE) CRC tissues (SuperBioChips Laboratories, Seoul, South Korea) [17]. Two TMA (2 mm in diameter) single cores were placed at the tumor center and periphery.

Microsatellite instability

Microsatellite instability (MSI) was examined by fragmentation assay analysis using an automated DNA sequencer (ABI 3731 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) with the following five microsatellite markers, according to previously described methods: BAT-26, BAT-25, D5S346, D17S250, and D2S123 [18].

Immunohistochemistry

IHC analysis was performed using antibodies specific to CD274 (PD-L1, E1L3 N, 1:50; Cell Signaling Technology, Danvers, MA, USA), CD3 (1:100; Dako, Glostrup, Denmark), CD8 (1:100; Dako), B-Raf (BRAF) (Ventana, Tucson, AZ, USA), MLH1 (Ventana), MSH2 (Cell Marque, Rocklin, CA, USA), MSH6 (Cell Marque), and PMS2 (Ventana). Immunostaining was conducted using the Ventana Bench mark XT autostainer (Ventana) and the ultraView Universal DAB kit (Ventana), according to the manufacturer's recommendations. Normal colonic epithelial cells were utilized as internal negative controls. Tissues were considered CD274-positive when more than 5% of neoplastic cells showed membrane staining of any intensity (Fig. 1) [5]. Meanwhile, tissues were considered BRAF positive when at least 80% of the tumor cells exhibited moderate to strong diffuse cytoplasmic staining (Fig. 1) [19]. Mild cytoplasmic staining in neoplastic cells was considered equivocal. Lastly, tissues containing neoplastic cells that exhibited nuclear staining for the mismatch repair (MMR) proteins MLH1, MSH2, MSH6, and PMS2, respectively, were considered positive.

CD3 and CD8 IHC slides were scanned on an Aperio ScanScope (Aperio Technologies, Inc., Vista, CA, USA) at 20× magnification. For precise calculation, $CD3^+$ and $CD8^+$ tumor-infiltrating lymphocytes (TILs) were quantified with an ImageScope computerized image analysis system (Aperio Technologies) using the Nuclear v9 algorithm. A score of 2–3 indicated $CD3^+$ and $CD8^+$ T cells (Fig. 1). TIL density was calculated by dividing the percentage (%) of positive nuclei by the core area (mm^2). We arbitrarily defined the cutoff value for TIL density as the median, and thereby divided TIL density into two groups: high and low.

BRAF mutation analyses

To identify *BRAF* mutations, DNA was harvested from 40 MSI-H CRC patient tissues including 26 BRAF IHC-positive and 14 equivocal tissues, using a Cobas DNA Sample Preparation Kit (Roche, Basel, Switzerland), according to the manufacturer's instructions and as described previously [20]. Samples were then screened for the *BRAF* (V600E) mutation using a Cobas 4800 System (Roche).

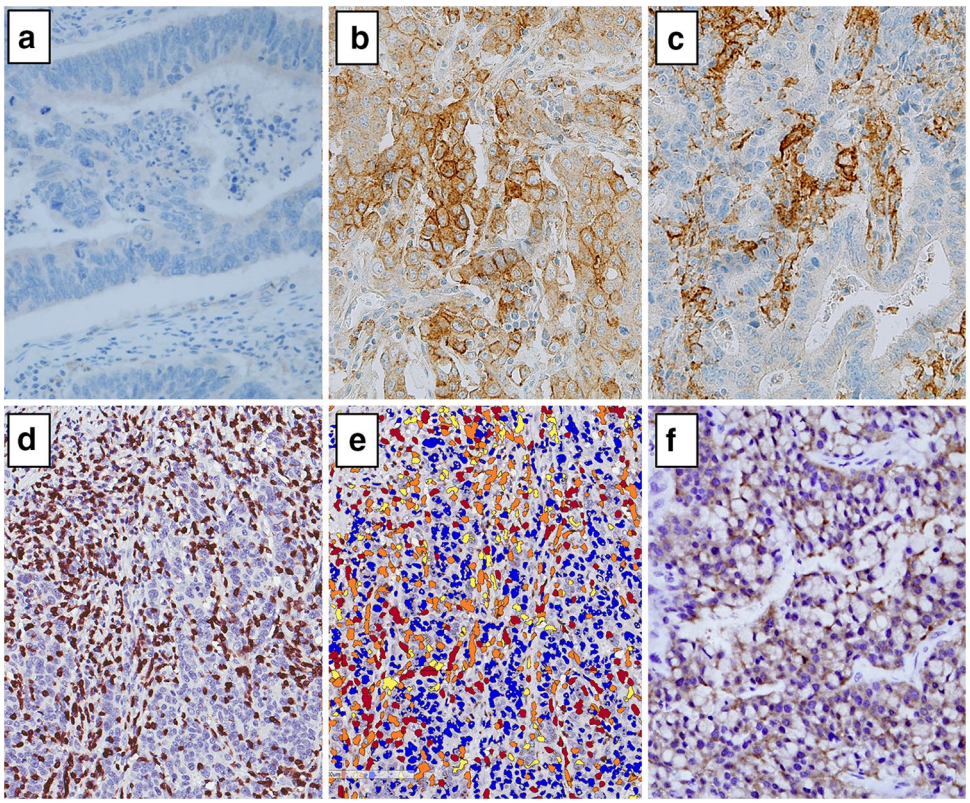

Fig. 1 Immunohistochemical staining of colorectal cancer patient samples. Images of tumor cells exhibiting **a** a lack of CD274 expression and **b** CD274 expression at the cell surface (×40). **c** CD274 expression on tumor-infiltrating immune cells (×40). **d** CD3 expression on tumor-infiltrating lymphocytes (×40). **e** Aperio image analysis using the Nuclear v9 algorithm: *blue* score 0; *yellow* score 1; *orange* score 2; *red* score 3 (×40). **f** B-Raf (BRAF) expression (×40). All immunohistochemistry images depict representative results

Statistical analyses

Categorical variables were compared using the Chi square or Fisher's exact test, as appropriate. The correlation between TIL density and CD274 expression was analyzed by determining Pearson's correlation coefficients. The McNemar test was used to determine intratumoral CD274 expression heterogeneity. The association between survival and CD274 expression was evaluated using Kaplan–Meier curves with the log-rank test and Cox's proportional hazards model. A threshold of $P < 0.05$ was considered statistically significant. To prevent inflation of type I error, data were subjected to multiple testing correction by Bonferroni adjustment [21]. The adjusting P values in Tables 1, 2, and 3 were 0.003, 0.004, and 0.006, respectively. IBM SPSS statistics version 21 (IBM, Armonk, NY, USA) was utilized for all statistical analyses.

Results

Frequency and clinicopathological features of CD274 expression in MSI-H and MSS CRC patients

We investigated CD274 expression in CRC in four different tissue lesions and cell types: tumor cells at the center (TC) and at the periphery (TP), and immune cells at the center (IC) and at the periphery (IP). CD274-expressing immune cells consisted primarily of macrophages, plasma cells, and lymphocytes. In total, $CD274^{TC}$ was observed in 43 (23.1%) of the 186 MSI-H samples, while $CD274^{TP}$ was observed in 47 (25.3%) samples, $CD274^{IC}$ in 107 (57.5%) samples, and $CD274^{IP}$ in 102 (54.8%) samples. Table 1 summarizes the correlations detected between clinicopathological features and CD274 expression in MSI-H CRCs. CD274 expression in tumor cells at both the center and periphery tended to be associated with old age, high grade of histologic differentiation, non-mucinous type, lymphatic invasion, and $CD3^+$ and $CD8^+$ T cell infiltration, in accordance with the previous studies ($P < 0.003$) [5, 6]. Meanwhile, CD274 expression on tumor-infiltrating immune cells at the center and periphery tended to be associated with $CD3^+$ and $CD8^+$ TIL infiltration ($P < 0.003$).

In contrast to MSI-H tissues, CD274 expression on tumor cells was rarely observed in the 153 MSS CRC tissues examined; $CD274^{TC}$ and $CD274^{TP}$ were observed in only three (2.0%) and four (2.6%) cases, respectively. In contrast, CD274 expression was frequently observed on immune cells, with $CD274^{IC}$ and $CD274^{IP}$ being observed in 47 (30.7%) and 56 (36.6%) cases, respectively. The correlations detected between clinicopathological features and CD274 expression on tumor-infiltrating immune cells of MSS CRCs are summarized in Table 2. Notably, $CD274^{IP}$ was associated with the absence of metastasis of MSS CRC ($P < 0.004$).

Correlation between peripheral CD274 expression and TIL density in MSI-H CRC patients

We investigated the correlation between CD274 expression and TIL density at the tumor periphery in the 186 MSI-H CRC patients. The density (%/mm^2) of $CD3^+$ TILs was higher [median, interquartile range (IQR): 422, 196–685] than that of $CD8^+$ TILs [median, IQR: 125, 65.6–242]. $CD274^{TP}$ expression exhibited a moderate positive correlated with $CD3^+$ (ρ, 0.538; $P < 0.001$) and $CD8^+$ (ρ, 0.546; $P < 0.001$) TILs, according to Dancey and Reidy's categorization method (2004) [22]. Meanwhile, $CD274^{IP}$ expression was moderately correlated with $CD3^+$ (ρ, 0.438; $P < 0.001$), but weakly correlated with $CD8^+$ TILs (0.365; $P < 0.001$).

Intratumoral heterogeneity of CD274 expression in MSI-H and MSS CRC patients

All 339 CRC cases were screened for CD274 expression at the tumor center and periphery to evaluate intratumoral heterogeneity (Supplementary Table 1). Intratumoral heterogeneity of CD274 expression was not uncommon in CRC. Among the 186 MSI-H CRC cases, discordance between $CD274^{TC}$ and $CD274^{TP}$ was observed in 24 (12.9%) cases, and discordance between $CD274^{IC}$ and $CD274^{IP}$ was observed in 47 (25.3%) cases. In the MSS CRC cohort, evaluation of intratumoral heterogeneity of CD274 expression of tumor cells was meaningless due to the low incidence of positivity. However, discordance between $CD274^{IC}$ and $CD274^{IP}$ was found in 37 (24.1%) out of 153 MSS CRC cases. The difference in intratumoral heterogeneity of CD274 expression between MSI-H and MSS CRC was not significant.

Prognostic impact of CD274 expression in MSI-H and MSS CRC patients

In MSI-H CRC, $CD274^{TC}$ and $CD274^{TP}$ were not associated with survival (Supplementary fig. 1; $P > 0.05$). Conversely, $CD274^{IC}$ and $CD274^{IP}$ were significantly associated with improved survival (Fig. 2; $P = 0.003$ and $P = 0.005$, respectively). However, the density of $CD3^+$ and $CD8^+$ TILs did not correlate with patient survival (Supplementary fig. 1; $P > 0.05$). As mentioned above, survival analysis of CD274 expression of tumor cells in the MSS cohort was meaningless due to a low incidence of positivity. However, $CD274^{IC}$ and $CD274^{IP}$ were significantly

Table 1 Correlation between clinicopathological factors and CD274 expression in 186 MSI-H CRC patients

	Total no. of cases	$CD274^{TC}$			$CD274^{TP}$			$CD274^{IC}$			$CD274^{IP}$		
		Negative	Positive	*P* value	Negative	Positive	*P* value	Negative	Positive	*P* value	Negative	Positive	*P* value
Age													
Mean	186	58.1	65.6	0.003	57.6	66.4	<0.001	59.7	59.9	0.899	59.8	59.8	0.997
Sex													
Male	86 (46.2%)	74 (51.7%)	12 (27.9%)	0.006	70 (50.4%)	16 (34.0%)	0.049	35 (44.3%)	51 (47.7%)	0.650	40 (47.6%)	46 (45.1%)	0.731
Female	100 (53.8%)	69 (48.3%)	31 (72.1%)		69 (49.6%)	31 (66.0%)		44 (55.7%)	56 (52.3%)		44 (52.4%)	56 (54.9%)	
Location													
Cecum to D-colon	154 (83.2%)	114 (80.3%)	40 (93.0%	0.050	112 (80.6%	42 (91.3%)	0.091	66 (83.5%)	88 (83.0%)	0.925	69 (82.1%)	85 (84.2%)	0.715
Rectosimoid colon	31 (16.8%)	28 (19.7%)	3 (7.0%)		27 (19.4%)	5 (8.7%)		13 (16.5%)	18 (17.0%)		15 (17.9%)	16 (15.8%)	
Hereditary vs. sporadic													
Sporadic	132 (71.0%)	96 (67.1%)	36 (83.7%)	0.036	91 (65.5%)	41 (87.2%)	0.004	55 (69.6%)	77 (72.0%)	0.728	59 (70.2%)	73 (71.6%)	0.842
Suspected LS	54 (29.0%)	47 (32.9%)	7 (16.3%)		48 (34.5%)	6 (12.8%)		24 (30.4%)	30 (28.0%)		25 (29.8%)	29 (28.4%)	
Differentiation													
Low grade	143 (76.9%)	125 (87.4%)	18 (41.9%)	<0.001	123 (88.5%)	20 (42.6%)	<0.001	59 (74.7%)	84 (78.5%)	0.541	64 (76.2%)	79 (77.5%)	0.839
High grade	43 (23.1%)	18 (12.6%)	25 (58.1%)		16 (11.5%)	27 (57.4%)		20 (25.3%)	23 (21.5%)		20 (23.8%)	23 (22.5%)	
Mucinous component													
Absent	117 (62.9%)	84 (58.7%)	33 (76.7%)	0.032	79 (56.8%)	38 (80.9%)	0.003	43 (54.4%)	74 (69.2%)	0.040	48 (57.1%)	69 (67.6%)	0.140
Present	69 (37.1%)	59 (41.3%)	10 (23.3%)		60 (43.2%)	9 (19.1%)		36 (45.6%)	33 (30.8%)		36 (42.9%)	33 (32.4%)	
Tumor border													
Expending	71 (38.2%)	61 (42.7%)	10 (23.3%)	0.022	54 (38.8%)	17 (36.2%)	0.744	22 (27.8%)	49 (45.8%)	0.013	24 (28.6%)	47 (46.1%)	0.014
Infiltrating	115 (61.8%)	82 (57.3%)	33 (76.7%)		85 (61.2%)	30 (63.8%)		57 (72.2%)	58 (54.2%)		60 (71.4%)	55 (53.9%)	
T stage													
1–3	161 (86.6%)	127 (88.8%)	34 (79.1%)	0.101	121 (87.1%)	40 (85.1%)	0.736	66 (83.5%)	95 (88.8%)	0.300	68 (81.0%)	93 (91.2%)	0.042
4	25 (13.4%)	16 (11.2%)	9 (20.9%)		18 (12.9%)	7 (14.9%)		13 (16.5%)	12 (11.2%)		16 (19.0%)	9 (8.8%)	
N stage													
0	165 (89.2%)	128 (90.1%)	37 (86.0%)	0.449	122 (88.4%)	43 (91.5%)	0.557	65 (83.3%)	100 (93.5%)	0.029	70 (84.3%)	95 (93.1%)	0.055
1–2	20 (10.8%)	14 (9.9%)	6 (14.0%)		16 (11.6%)	4 (8.5%)		13 (16.7%)	7 (6.5%)		13 (15.7%)	7 (6.9%)	
M stage													
0	177 (95.2%)	137 (95.8%)	40 (93.0%)	0.456	133 (95.7%)	44 (93.6%)	0.568	71 (89.9%)	106 (99.1%)	0.004	76 (90.5%)	101 (99.0%)	0.007
1	9 (4.8%)	6 (4.2%)	3 (7.0%)		6 (4.3%)	3 (6.4%)		8 (10.1%)	1 (0.9%)		8 (9.5%)	1 (1.0%)	
pTMN stage													
I–II	121 (65.4%)	98 (69.0%)	23 (53.5%)	0.061	92 (66.7%)	29 (61.7%)	0.537	44 (55.7%)	77 (72.6%)	0.017	49 (58.3%)	72 (71.3%)	0.065
III–IV	64 (34.6%)	44 (31.0%)	20 (46.5%)		46 (33.3%)	18 (38.3%)		35 (44.3%)	29 (27.4%)		35 (41.7%)	29 (28.7%)	
Venous invasion													
Absent	177 (95.2%)	136 (95.1%)	41 (95.3%)	0.948	132 (95.0%)	45 (95.7%)	0.829	74 (93.7%)	103 (96.3%)	0.416	78 (92.9%)	99 (97.1%)	0.184

Table 1 continued

	Total no. of cases	$CD274^{TC}$			$CD274^{TP}$			$CD274^{IC}$			$CD274^{IP}$		
		Negative	Positive	*P* value	Negative	Positive	*P* value	Negative	Positive	*P* value	Negative	Positive	*P* value
Present	9 (4.8%)	7 (4.9%)	2 (4.7%)		7 (5.0%)	2 (4.3%)		5 (6.3%)	4 (3.7%)		6 (7.1%)	3 (2.9%)	
Lymphatic invasion													
Absent	124 (66.7%)	104 (72.7%)	20 (46.5%)	0.001	103 (74.1%)	21 (44.7%)	<0.001	46 (58.2%)	78 (72.9%)	0.036	54 (64.3%)	70 (68.6%)	0.532
Present	62 (33.3%)	39 (27.3%)	23 (53.5%)		36 (25.9%)	26 (55.3%)		33 (41.8%)	29 (27.1%)		30 (35.7%)	32 (31.4%)	
Perineural invasion													
Absent	166 (89.2%)	126 (88.1%)	40 (93.0%)	0.362	122 (87.8%)	44 (93.6%)	0.263	68 (86.1%)	98 (91.6%)	0.230	75 (89.3%)	91 (89.2%)	0.988
Present	20 (10.8%)	17 (11.9%)	3 (7.0%)		17 (12.2%)	3 (6.4%)		11 (13.9%)	9 (8.4%)		9 (10.7%)	11 (10.8%)	
Eosinophilic infiltration													
Minor	116 (62.4%)	91 (63.6%)	25 (58.1%)	0.514	85 (61.2%)	31 (66.0%)	0.557	51 (64.6%)	65 (60.7%)	0.596	52 (61.9%)	64 (62.7%)	0.906
Dominant	70 (37.6%)	52 (36.4%)	18 (41.9%)		54 (38.8%)	16 (34.0%)		28 (35.4%)	42 (39.3%)		32 (38.1%)	38 (37.3%)	
CD8-positive TIL													
Low	93 (50.0%)	79 (55.2%)	14 (32.6%)	0.009	83 (59.7%)	10 (21.3%)	<0.001	53 (67.1%)	40 (37.4%)	<0.001	62 (73.8%)	31 (30.4%)	<0.001
High	93 (50.0%)	64 (44.8%)	29 (67.4%)		56 (40.3%)	37 (78.7%)		26 (32.9%)	67 (62.6%)		22 (26.2%)	71 (69.6%)	
CD3-positive TIL													
Low	93 (50.0%)	80 (55.9%)	13 (30.2%)	0.003	84 (60.4%)	9 (19.1%)	<0.001	52 (65.8%)	41 (38.3%)	<0.001	63 (75.0%)	30 (29.4%)	<0.001
High	93 (50.0%)	63 (44.1%)	30 (69.8%)		55 (39.6%)	38 (80.9%)		27 (34.2%)	66 (61.7%)		21 (25.0%)	72 (70.6%)	

CRC colorectal cancer, *D* descending; high grade, poorly and undifferentiated, *IC* immune cells at the center, *IP* immune cells at the periphery; low grade, well and moderately differentiated, *LS* Lynch syndrome, *M* metastasis, *MSI-H* microsatellite instability-high, *N* lymph node, *No* number, *T* tumor, *TC* tumor cells at the center, *TIL* tumor-infiltrating lymphocyte, *TP* tumor cells at the periphery

P values are calculated using χ^2 test or Fisher's exact test

Table 2 Correlation between clinicopathological factors and CD274 expression in 153 MSS CRC patients

	Total no. of cases	$CD274^{IC}$		*P* value	$CD274^{IP}$		*P* value
		Negative	Positive		Negative	Positive	
Age							
Mean	153	64.5	63.4	0.585	63.3	65.7	0.237
Sex							
Male	79 (51.6%)	52 (49.1%)	27 (57.4%)	0.338	48 (49.5%)	31 (55.4%)	0.484
Female	74 (48.4%)	54 (50.9%)	20 (42.6%)		49 (50.5%)	25 (44.6%)	
Location							
Cecum to D-colon	51 (33.3%)	32 (30.2%)	19 (40.4%)	0.215	35 (36.1%)	16 (28.6%)	0.342
Rectosigmoid	102 (66.7%)	74 (69.8%)	28 (59.6%)		62 (63.9%)	40 (71.4%)	
Differentiation							
Low grade	143 (93.5%)	98 (92.5%)	45 (95.7%)	0.447	90 (92.8%)	53 (94.6%)	0.654
High grade	10 (6.5%)	8 (7.5%)	2 (4.3%)		7 (7.2%)	3 (5.4%)	
Tumor border							
Expending	35 (22.9%)	26 (24.5%)	9 (19.1%)	0.465	19 (19.6%)	16 (28.6%)	0.203
Infiltrating	118 (77.1%)	80 (75.5%)	38 (80.9%)		78 (80.4%)	40 (71.4%)	
T stage							
1–3	125 (81.7%)	86 (81.1%)	39 (83.0%)	0.785	75 (77.3%)	50 (89.3%)	0.065
4	28 (18.3%)	20 (18.9%)	8 (17.0%)		22 (22.7%)	6 (10.7%)	
N stage							
0	72 (47.1%)	46 (43.4%)	26 (55.3%)	0.173	39 (40.2%)	33 (58.9%)	0.025
1–2	81 (52.9%)	60 (56.6%)	21 (44.7%)		58 (59.8%)	23 (41.1%)	
M stage							
0	135 (88.2%)	91 (85.8%)	44 (93.6%)	0.169	79 (81.4%)	56 (100.0%)	0.001
1	18 (11.8%)	15 (14.2%)	3 (6.4%)		18 (18.6%)	0 (0.0%)	
pTMN stage							
I–II	71 (46.4%)	45 (42.5%)	26 (55.3%)	0.141	37 (38.1%)	34 (60.7%)	0.007
III–IV	82 (53.6%)	61 (57.5%)	21 (44.7%)		60 (61.9%)	22 (39.3%)	
Venous invasion							
Absent	128 (83.7%)	85 (80.2%)	43 (91.5%)	0.081	78 (80.4%)	50 (89.3%)	0.153
Present	25 (16.3%)	21 (19.8%)	4 (8.5%)		19 (19.6%)	6 (10.7%)	
Lymphatic invasion							
Absent	71 (46.4%)	49 (46.2%)	22 (46.8%)	0.947	44 (45.4%)	27 (48.2%)	0.733
Present	82 (53.6%)	57 (53.8%)	25 (53.2%)		53 (54.6%)	29 (51.8%)	
Perineural invasion							
Absent	102 (66.7%)	64 (60.4%)	38 (80.9%)	0.013	59 (60.8%)	43 (76.8%)	0.044
Present	51 (33.3%)	42 (39.6%)	9 (19.1%)		38 (39.2%)	13 (23.2%)	

CRC colorectal cancer, *D* descending; high grade, poorly and undifferentiated, *IC* immune cells at the center, *IP* immune cells at the periphery; low grade, well and moderately differentiated, *M* metastasis, *MSS* microsatellite stable, *N* lymph node, *T* tumor

P values are calculated using χ^2 test or Fisher's exact test

associated with improved prognosis in the MSS cohort (Fig. 2; $P = 0.014$ and $P < 0.001$, respectively).

Notably, multivariate Cox proportional hazards analysis indicated that CD274 expression on tumor-infiltrating immune cells independently predicted improved prognosis in both MSI-H and MSS CRC cohorts (Table 3).

Detection of the BRAF-V600E mutation in MSI-H CRC patients

We investigated BRAF IHC status in 186 MSI-H CRC cases. To validate BRAF-V600E IHC, 26 IHC-positive and 14 IHC-equivocal cases were subjected to *BRAF* (V600E)

Table 3 Multivariate Cox proportional hazard models for predictors of overall survival

Factors	Univariate survival analysis		Multivariate survival analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
MSI-H cohort				
$CD274^{IC}$ expression	0.211 (0.069–0.649)	0.007	0.241 (0.071–0.824)	0.023
Age	1.04 (1.01–1.09)	0.019	1.05 (1.00–1.10)	0.032
Tumor border (infiltrative vs. expending)	11.5 (1.52–86.8)	0.018	3.93 (0.478–32.4)	NS (0.203)
Perineural invasion	5.07 (1.87–13.7)	0.001	3.734 (1.17–11.9)	0.026
T stage (1–3 vs. 4)	6.84 (2.59–18.1)	<0.001	1.84 (0.572–5.91)	NS (0.307)
N stage (0 vs. 1, 2)	6.23 (2.26–17.2)	<0.001	2.97 (0.974–9.05)	NS (0.056)
M stage (0 vs. 1)	11.6 (3.22–42.9)	<0.001	3.03 (0.642–14.3)	NS (0.161)
$CD274^{IP}$ expression	0.232 (0.076–0.712)	0.011	0.274 (0.081–0.921)	0.036
Age	1.05 (1.01–1.09)	0.019	1.04 (0.999–1.09)	NS (0.056)
Tumor border (infiltrative vs. expending)	11.5 (1.52–86.8)	0.018	3.87 (0.470–31.8)	NS (0.209)
Perineural invasion	5.07 (1.87–13.7)	0.001	3.40 (1.11–10.4)	0.032
T stage (1–3 vs. 4)	6.84 (2.59–18.1)	<0.001	1.79 (0.558–5.72)	NS (0.328)
N stage (0 vs. 1, 2)	6.23 (2.26–17.2)	<0.001	3.41 (1.04–11.2)	0.043
M stage (0 vs. 1)	11.6 (3.22–42.9)	<0.001	3.40 (0.731–15.8)	NS (0.119)
MSS cohort				
$CD274^{IC}$ expression	0.355 (0.150–0.844)	0.019	0.408 (0.169–0.985)	0.046
Lymphatic invasion	3.95 (1.89–8.25)	<0.001	1.96 (0.852–4.51)	NS (0.113)
Venous invasion	4.54 (2.44–8.48)	<0.001	1.33 (0.583–3.05)	NS (0.495)
Differentiation (low vs. high grade)	6.31 (2.76–14.4)	<0.001	3.67 (1.48–9.13)	0.005
T stage (1–3 vs. 4)	4.24 (2.30–7.82)	<0.001	1.98 (0.943–4.14)	NS (0.071)
N stage (0 vs. 1, 2)	4.20 (2.01–8.79)	<0.001	2.09 (0.919–4.74)	NS (0.079)
M stage (0 vs. 1)	6.87 (3.58–13.2)	<0.001	3.06 (1.39–6.71)	0.005
$CD274^{IP}$ expression	0.211 (0.083–0.537)	0.001	0.290 (0.109–0.771)	0.013
Lymphatic invasion	3.95 (1.89–8.25)	<0.001	2.12 (0.921–4.69)	NS (0.077)
Venous invasion	4.54 (2.44–8.48)	<0.001	1.59 (0.697–3.62)	NS (0.271)
Differentiation (low vs. high grade)	6.31 (2.76–14.4)	<0.001	3.82 (1.53–9.54)	0.004
T stage (1–3 vs. 4)	4.24 (2.30–7.82)	<0.001	1.92 (0.945–3.92)	NS (0.071)
N stage (0 vs. 1, 2)	4.20 (2.01–8.79)	<0.001	1.96 (0.861–4.48)	NS (0.109)
M stage (0 vs. 1)	6.87 (3.58–13.2)	<0.001	2.08 (0.919–4.69)	NS (0.079)

CRC colorectal cancer; high grade, poorly and undifferentiated, *HR* hazard ratio, *IC* immune cells at the center, *IP* immune cells at the periphery; low grade, well and moderately differentiated, *M* metastasis, *MSI-H* microsatellite instability-high, *MSS* microsatellite stable, *N* lymph node, *T* tumor, *TC* tumor cells at the center, *TP* tumor cells at the periphery

mutation analysis using the Cobas 4800 System. Each of the 26 BRAF IHC-positive cases, but none of the IHC-equivocal cases, contained the V600E mutation. Notably, *BRAF* mutation was associated with $CD274^{TP}$, distinguishing expression pattern of MMR proteins [hMLH1(−)/hMSH2(+)/hMSH6(+)/PMS2(−)], sporadic type, and high grade of histologic differentiation ($P < 0.006$), in agreement with previous studies (Table 4) [23, 24]. While *BRAF* mutation did not predict overall survival in MSI-H CRC patients ($P = 0.987$; Supplementary Figure 1), this mutation was significantly associated with worse prognosis in a subgroup of patients who had received post-operative chemotherapy ($P = 0.041$; Supplementary Figure 1). As such, *BRAF* mutation seems to be associated with worse prognoses only at the later stages of MSI-H CRC, which is an indication for post-operative chemotherapy.

Discussion

The high density of TILs that is commonly observed in MSI-H CRCs [14] suggests that these cancers vigorously induce a host immune reaction, which could be due to the higher mutational burden of these tumors; next-generation sequencing (NGS) studies have reported that MSI-H CRCs contain 10–50 times more mutations than

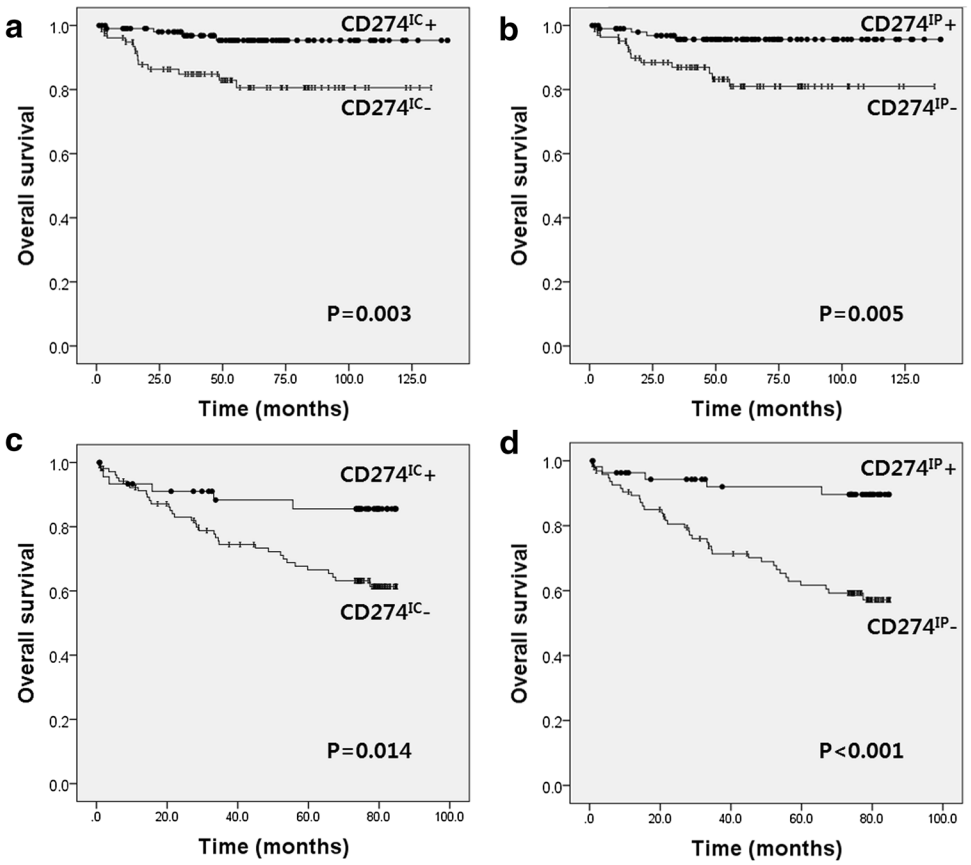

Fig. 2 Kaplan–Meier survival curves illustrating the prognostic effect of CD274 expression on tumor-infiltrating immune cells in colorectal cancer (CRC). CD274 expression on tumor-infiltrating immune cells at **a** the tumor center and **b** the tumor periphery in microsatellite instability-high CRC tissues. CD274 expression on tumor-infiltrating immune cells at **c** the tumor center and **d** the tumor periphery of microsatellite stable CRC tissues

MSS CRCs [25]. These molecular alterations produce abnormal neoantigens, which have the potential to result in increased numbers of TILs. Giannakis et al. showed that increased neoantigen load was positively correlated with TILs density and improved prognosis in CRC [26]. Actually, these data indicate that neoantigen load is more significant than MSI in clinical implication. Moreover, a recent study demonstrated that MSI-H CRCs show stronger expression of immune-regulation gene clusters than MSS CRCs [27, 28]. These clusters predominantly consist of gene-related T-helper 1 (Th1) and immune checkpoint receptors including PDCD1, CD274, CTLA-4, and LAG-3 [28, 29]. Notably, elevated expression of immune checkpoint molecules can create an immune-suppressive microenvironment [30], which could yield improved responses to immune checkpoint blockade.

The relationship between CD274 expression on tumor cells and prognosis in CRC is highly variable and controversial. While Droeser et al. demonstrated that CD274 expression was associated with better prognosis in MMR-proficient CRC [9], another study reported that the occurrence of CD274 expression on tumor cells with PDCD1 expression on TILs resulted in a worse CRC prognosis [11]. In the current study, CD274 expression on tumor cells was associated with a tendency toward a favorable prognostic value; however, this result had limited significance (Supplementary fig. 1) [6].

Interestingly, CD274 positivity was detected in >50% of tumor-infiltrating immune cells in MSI-H CRCs and >30% of those in MSS CRCs. CD274 expression on tumor and immune cells is thought to have distinct implications. However, a few other studies have also examined the clinicopathological implications of CD274 expression on immune cells. CD274 expression on immune cells shows a tendency towards decreased survival in gastric and uterine cervix adenocarcinomas [31, 32]. On the contrary, CD274 expression on immune cells was associated with improved survival in advanced urothelial carcinoma and spinal chordoma [33, 34]. Notably, our data indicated that CD274 expression on tumor-infiltrating immune cells was an independent factor for improved prognosis in both MSI-H and MSS CRC. Interestingly, in contrast to our data, Wang et al. showed that CD274 positivity on immune cells signifies worse prognoses in consecutive CRC [10]. However, a recent study supported our results that CD274 expression on immune cells results in improved survival of patients with stage IIIb CRC [35]. Similarly, another study also demonstrated that CD274 expression on immune cells

Table 4 The correlation between clinicopathological factors and BRAF mutation in 186 MSI-H CRC patients

	Total no. of cases	*BRAF* mutation		
		Negative	Positive	*P* value
$CD274^{TC}$				
Negative	143	128 (80.0%)	15 (57.7%)	0.012
Positive	43	32 (20.0%)	11 (42.3%)	
$CD274^{TP}$				
Negative	139	126 (78.8%)	13 (50.0%)	0.002
Positive	47	34 (21.3%)	13 (50.0%)	
$CD274^{IC}$				
Negative	79	69 (43.1%)	10 (38.5%)	0.655
Positive	107	91 (56.9%)	16 (61.5%)	
$CD274^{IP}$				
Negative	84	75 (46.9%)	9 (34.6%)	0.244
Positive	102	85 (53.1%)	17 (65.4%)	
CD8-positive TIL				
Low	93	84 (52.5%)	9 (34.6%)	0.091
High	93	76 (47.5%)	17 (65.4%)	
CD3-positive TIL				
Low	93	84 (52.5%)	9 (34.6%)	0.091
High	93	76 (47.5%)	17 (65.4%)	
MMR protein (hMLH1/hMSH2/hMSH6/PMS2)				
(+/+/+/+)	9	9 (5.6%)	0 (0.0%)	0.001
(−/+/+/−)	113	87 (54.4%)	26 (100.0%)	
(+/−/−/+)	41	41 (25.6%)	0 (0.0%)	
(+/+/−/+)	8	8 (5.0%)	0 (0.0%)	
(+/+/+/−)	15	15 (9.4%)	0 (0.0%)	
Hereditary vs. sporadic				
Sporadic	132	106 (66.3%)	26 (100.0%)	<0.001
Suspicious LS	54	54 (33.8%)	0 (0.0%)	
Differentiation				
Low grade	143	129 (80.6%)	14 (53.8%)	0.003
High grade	43	31 (19.4%)	12 (46.2%)	

CRC colorectal cancer; high grade, poorly and undifferentiated, *IC* immune cells at the center, *IP* immune cells at the periphery; low grade, well and moderately differentiated, *LS* Lynch syndrome, *MMR* mismatch repair, *MSI-H* microsatellite instability-high, *No* number, *TC* tumor cells at the center, *TP* tumor cells at the periphery

P values are calculated using χ^2 test or Fisher's exact test

showed a tendency toward improved survival in MSI-H CRCs, but this result was not statistically significant [6]. Moreover, this latter study suggested that favorable prognoses resulting from CD274 expression on immune cells was considered an effect of a high density of tumor-infiltrating T cells [6]. To rule out the causes of the favorable prognosis suggested by other groups, we precisely counted and analyzed tumor-infiltrating $CD3^+$ and $CD8^+$ T cells using an Aperio image analysis system, instead of the traditional method of counting by eye. Despite these elaborative analyses, the presence of $CD3^+$ and $CD8^+$ TILs was not significantly associated with prognosis for any of the cutoff values tested. These results support the conclusion that the prognostic value of the CD274 expression on immune cells was not a reflection of high TIL density.

CD274 has been shown to be expressed on macrophages, dendritic cells, T and B lymphocytes, and to protect tissues from excessive immune reaction [36]. While the mechanism by which expression of CD274 on TILs leads to improved patient prognoses remains unclear, there are several possible hypotheses. First, it is conceivable that active anti-tumor immune reactions could enable the positive selection of tumor cells exhibiting mutations in genes encoding human leukocyte antigen (HLA) and/or antigen-processing machinery (APM) [26]. In this case, expression of CD274 on TILs could relieve the anti-tumor immune reaction, leading to reduced numbers of such mutant tumor cells and thereby to better patient outcomes. Alternatively, Di Caro et al. suggested that chemotherapeutic effects on tumor cells seem to be enhanced under immune-escape conditions [37]. CD274 expression on TILs could, therefore, have enhanced the chemotherapeutic responsiveness of tumor cells by promoting an immune-escape condition in CRC. Another contributing factor for favorable prognosis is interferon (IFN)-r-producing tumor-infiltrating T cells. Droeser et al. demonstrated that CD274 mRNA expression was significantly correlated with IFN-γ gene expression in CRC specimens [9], and that IFN-γ might play a role in the tumor surveillance and cytotoxic anti-tumor function. However, further studies are necessary to clarify this possibility.

Recently, several studies detected regional heterogeneity of CD274 expression in various cancers, including lung cancer, melanoma, and renal cell carcinoma [15, 38, 39]. These data suggest that we should be careful in evaluating CD274 expression in routine small biopsies due to the potential for false-negative results. To the best of our knowledge, our study is the first to evaluate intratumoral heterogeneity (central and peripheral portions of the primary tumor) in CD274 expression in CRCs; our data indicate that such heterogeneity is common in resected CRC specimens (Supplementary Table 1), which are more reliable than biopsy specimens. A possible reason for this regional discordance is that various immune reactions might further affect the peripheral portion of tumors than the central portion. Additionally, hypoxic conditions in the central portion might induce intratumoral heterogeneity of CD274 expression. Several studies suggested that hypoxia can promote the expression of CD274 on tumor cells and TILs via hypoxia-inducible factor-1α (HIF-1α) up-regulation [40, 41].

Recent molecular studies indicated that CRC is a heterogeneous disease, arising from several genetic pathways. These pathways are crucial in determining patient

prognosis and treatment [42, 43]. In non-small cell lung cancer, recent studies indicated that the genomic landscape determined patient response to PDCD1 blockade therapy [44], and that CD274 expression was associated with *EGFR* mutations [45]. Our study and others demonstrated that mutation of *BRAF* is significantly associated with CD274 expression in CRC tumor cells, but not in immune cells [5, 6]. Another recent study suggested that CD274 positivity is associated with the serrated pathway and stem cell features in CRC [24]. Thus, the diverse genetic alterations that correlate with CD274 expression in CRC should be further investigated.

To conclude, our study comprehensively evaluated CD274 expression in MSI-H CRCs, as well as in MSS CRCs. Notably, we found that CD274 expression on tumor-infiltrating immune cells was an independent predictive factor for improved prognosis in both MSI-H and MSS CRCs. Our findings indicate that the CD274 status may be helpful in predicting CRC patient outcomes. Moreover, our results indicate that discordance of CD274 expression between the central and peripheral portions of CRC tumors is not uncommon. Thus, evaluation of various tumor portions is recommended to enhance the validity of CD274 expression results. Further investigation of the mechanism underlying CD274 expression in immune cells as well as the predictive and prognostic role of this protein in CRC is needed.

Compliance with ethical standards

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Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent For this type of study formal consent is not required.

Conflict of interest The authors declare that they have no conflict of interest.

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