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Tumour CD274 (PD-L1) expression and T cells in colorectal cancer

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Use of standardised official symbols: We use HUGO (Human Genome Organisation)-approved official symbols for genes and gene products, including BRAF, CACNA1G, CD274, CDKN2A, CRABP1, FOXP3, IGF2, KRAS, MLH1, NEUROG1, PDCD1, PIK3CA, PTPRC, RUNX3, and SOCS1; all of which are described at www.genenames.org. Gene names are italicised, and gene product names are non-italicised.

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Abstract

Objective—Evidence suggests that CD274 (PD-L1, B7-H1) immune checkpoint ligand repress anti-tumour immunity through its interaction with the PDCD1 (programmed cell death 1, PD-1) receptor of T lymphocytes in various tumours. We hypothesised that tumour CD274 expression levels might be inversely associated with T-cell densities in colorectal carcinoma tissue.

Design—We evaluated tumour CD274 expression by immunohistochemistry in 823 rectal and colon cancer cases within the Nurses' Health Study and Health Professionals Follow-up Study. We conducted multivariable ordinal logistic regression analyses to examine the association of tumour CD274 expression with CD3⁺, CD8⁺, CD45RO (PTPRC)⁺, or FOXP3⁺-cell density in tumour tissue, controlling for potential confounders including tumour status of microsatellite instability (MSI), CpG island methylator phenotype, LINE-1 methylation level, and *KRAS*, *BRAF*, and *PIK3CA* mutations.

Results—CD274 expression in tumour cells or stromal cells (including immune cells) was detected in 731 (89%) or 44 (5%) cases, respectively. Tumour CD274 expression level correlated inversely with FOXP3⁺-cell density in colorectal cancer tissue (outcome) ($P_{\text{trend}}=0.0002$). For a unit increase in outcome quartile categories, multivariable odds ratio in the highest (vs. lowest) CD274 expression score was 0.22 (95% confidence interval 0.10–0.47). Tumour CD274 expression was inversely associated with MSI-high status ($P=0.001$). CD274 expression was not significantly associated with CD3⁺, CD8⁺, or CD45RO⁺-cell density, pathological lymphocytic reactions, or patient survival prognosis.

Conclusions—Tumour CD274 expression is inversely associated with FOXP3⁺ cell density in colorectal cancer tissue, suggesting a possible influence of CD274-expressing carcinoma cells on regulatory T (Treg) cells in the tumour microenvironment.

Keywords

adenocarcinoma; colorectum; immunoprevention; immunotherapy; molecular pathology

INTRODUCTION

Immunotherapy has emerged as a promising strategy to treat various types of cancers.¹ Accumulating evidence indicates that the immune checkpoint mechanism play an important role in suppressing anti-tumour T-cell-mediated immune response in the tumour microenvironment.¹ Studies have shown that therapeutic antibodies targeting the PDCD1 (programmed cell death 1, PD-1) protein and the CD274 (PDCD1 ligand 1, PD-L1, B7-H1) protein are effective in a number of cancer types.^{1–3} Emerging evidence also suggests complex roles of tumour molecular alterations and tumour-host interactions that influence response to these T-cell-based immunotherapies.^{4–6}

Colorectal carcinogenesis is not only driven by sequential genetic and epigenetic alterations of tumour cells but also influenced by tumour-host interactions.^{7–14} A strong histological lymphocytic reaction, high density of CD3⁺ T cells, and high densities of T-cell subpopulations (CD8⁺ cells, CD45RO [PTPRC]⁺ cells, and FOXP3⁺ cells) in colorectal cancer tissue have been generally associated with favourable clinical outcome, supporting a major role of T-cell-mediated immunity in repressing tumour progression.^{14–19} Studies have shown that the abundance of tumour-infiltrating T cells is associated with specific molecular features of colorectal carcinoma, including high-level microsatellite instability (MSI-high).^{413–1520} However, little is known on the complex interrelationship among tumour CD274 expression, tumour-infiltrating T cells, and major tumour molecular features. In the colorectal cancer microenvironment, CD274-expressing tumour cells may inhibit anti-tumour activity of T cells. Hence, we hypothesised that tumour CD274 expression levels might be inversely associated with T-cell densities in colorectal cancer tissue. Because any in vitro or non-human system cannot perfectly recapitulate the complexity of human tumour or immune system, analyses of tumour characteristics and immune cells in human cancer tissue are valuable.

To test our hypothesis, we analysed the two U.S.-nationwide prospective cohort studies, and examined tumour CD274 expression in relation to histological lymphocytic reaction or to CD3⁺, CD8⁺, CD45RO⁺, or FOXP3⁺ cell density in colorectal cancer tissue. Our comprehensive database including tumour immunity status and relevant clinicopathological and tumour molecular characteristics enabled us to investigate the independent association of tumour CD274 expression with T cells in tumour tissue, controlling for potential confounders.

MATERIALS AND METHODS

Study group

We utilised two independent prospective cohort studies: the Nurses' Health Study (involving 121,701 women followed since 1976) and the Health Professionals Follow-up Study (involving 51,529 men followed since 1986).^{21–23} Every two years, the participants have been sent follow-up questionnaires to update information on potential disease risk factors, and to identify newly diagnosed cancers and other diseases in themselves and their first-degree relatives. The National Death Index was used to ascertain deaths of study participants and identify unreported lethal colorectal cancer cases. Study physicians reviewed all medical

records related to colorectal cancer, extracted clinical information including the American Joint Committee on Cancer (AJCC) TNM stage, the numbers of positive and negative lymph nodes harvested, and tumour location, and determined cause of death in deceased individuals. In survival analyses, patients were followed until death or January 2012, whichever came first. We collected formalin-fixed paraffin-embedded (FFPE) tissue blocks from hospitals across the U.S. where participants with colorectal cancer had undergone tumour resection. We included both colon and rectal carcinoma cases, considering the colorectal continuum model.²⁴ We excluded polyposis syndrome cases, inflammatory bowel disease-related cancers, and cases that had received preoperative therapy. The study pathologist (S.O.) blinded to other data performed centralised pathology review, and recorded features including tumour differentiation, the extents of extracellular mucin, signet ring cells, and solid tumour areas, tumour growth pattern, and four patterns of histological lymphocytic reaction (Crohn's-like lymphoid reaction, peritumoural lymphocytic reaction, intratumoural periglandular reaction, and tumour-infiltrating lymphocytes [TIL]).²⁰ Tumour differentiation was categorised as well-moderate (>50% glandular area) or poor (<50% glandular area). On the basis of the availability of CD274 expression data, a total of 823 colorectal cancer cases diagnosed up to 2008 were included in this study. In addition, 10 cases of colorectal carcinoma were selected from the archival pathology file of the Brigham and Women's Hospital, and anonymised, to perform immunohistochemistry for CD274 in whole tissue sections. Tissue collection and analyses were approved by the human subjects committee at the Harvard T.H. Chan School of Public Health and the Brigham and Women's Hospital.

Immunohistochemistry

We constructed tissue microarray (TMA) from colorectal cancer blocks,²⁵ and conducted immunohistochemistry (IHC). Immunohistochemical analyses were performed for CD3, CD8, CD45RO, and FOXP3, as previously described.¹⁵ We used an automated scanning microscope and the Ariol image analysis system (Genetix, San Jose, CA, USA) to measure CD3⁺, CD8⁺, CD45RO⁺, and FOXP3⁺ cell densities in tumour tissue. We evaluated up to four TMA cores from each tumour, and calculated the average density (cells/mm²) of each T-cell population.¹⁵ Immunohistochemical method for PDCD1 (PD-1) is described in supplementary material.

For CD274 (PD-L1) IHC, tissue sections were deparaffinised, rehydrated, and heated in a microwave for 15 minutes in Antigen Retrieval Citra Solution, pH 6 (BioGenex Laboratories, San Ramon, CA, USA). Sections were incubated with Dual Endogenous Enzyme Block (Dako, Glostrup, Denmark), then were treated with Protein Block Serum-Free (Dako). Slides were incubated for 16 hours at 4 °C with a mouse monoclonal anti-CD274 antibody (Clone MIH1, eBioscience, San Diego, CA, USA; dilution, 1:50). The primary antibody was visualised using EnVision+ System-HRP (Dako) for 30 minutes with diaminobenzidine, and counterstained with hematoxylin. Sections processed with replacement of primary antibody by Mouse IgG1 K Isotype Control (Clone P3.6.2.8.1, eBioscience) were used as a negative control. Immunohistochemical expression for CD274 was interpreted by a pathologist (Y.M.) unaware of other data. Tumour CD274 expression was evaluated based on immunostaining in the cytoplasm and membrane of tumour cells.

Cytoplasmic expression level (intensity) was scored as 0 (absent), 1 (weak), 2 (moderate), or 3 (strong), and membrane expression level was scored as 0 (absent) or 1 (present; if distinct membrane staining above cytoplasmic staining level existed) (figure 1A–E). If staining difference was observed across multiple TMA tumour cores in each case, intensity of predominant staining pattern in tumour component was recorded. The overall tumour CD274 expression score was the sum of the cytoplasmic and membrane scores, ranging from 0 to 4. We scored CD274 expression of stromal cells including immune cells, based on the presence or absence of distinct cytoplasmic and/or membrane staining in stromal cells. Immune cells, including lymphocytes and macrophages, in the lymphoid tissue within normal colorectal tissue cores served as positive controls (figure 1F). A random sample of 148 tumours was examined by a second pathologist (A.S.). The agreement between the two observers for the tumour CD274 expression score was good with a weighted κ of 0.65 (95% confidence interval [CI] 0.57–0.73). In addition, to assess intratumoural heterogeneity of CD274 expression, we performed CD274 immunohistochemistry using whole tissue sections from the 10 selected colorectal carcinoma cases, and did not observe considerable intratumoural heterogeneity in any of the cases in terms of tumour CD274 expression (supplementary figure 1).

Analyses of MSI, DNA methylation, and *KRAS*, *BRAF*, and *PIK3CA* mutations

DNA was extracted from archival colorectal cancer tissue blocks. MSI status was analysed with the use of 10 microsatellite markers (BAT25, BAT26, BAT40, D2S123, D5S346, D17S250, D18S55, D18S56, D18S67, and D18S487), as previously described.²²²⁶ We defined MSI-high as the presence of instability in $\geq 30\%$ of the markers, and MSI-low/microsatellite stability (MSS) as instability in $< 30\%$ of the markers.²²²⁶ Methylation analyses of long interspersed nucleotide element-1 (LINE-1) and eight promoters specific for CpG island methylator phenotype (CIMP) (*CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, and *SOCS1*) were performed.²²²⁷ CIMP-high, CIMP-low, and CIMP-negative were defined as $\geq 6/8$, $1/8$ – $5/8$, and $0/8$ methylated promoters, respectively, according to the previously-established criteria.²²²⁷ PCR reaction and pyrosequencing were performed for *BRAF* (codon 600),²⁶ *KRAS* (codons 12, 13, 61, and 146),²⁸ and *PIK3CA* (exons 9 and 20).²²²⁹

Analysis of the amount of *Fusobacterium nucleatum* (*F. nucleatum*) DNA

We extracted DNA from colorectal carcinoma FFPE tissue sections, and performed a quantitative PCR assay to measure the amount of tissue *F. nucleatum* DNA.^{30, 31} We categorised colorectal carcinoma cases with detectable *F. nucleatum* DNA as low or high in relation to the median cut point amount of *F. nucleatum* DNA.^{30, 31}

Statistical analysis

All statistical analyses were conducted using SAS (version 9.3, SAS Institute, Cary, NC, USA) and all *P* values were two-sided. Our primary hypothesis testing was an assessment of the association of the tumour CD274 expression score (an ordinal predictor variable) with each of (T) lymphocyte variables as outcome variables (CD3⁺, CD8⁺, CD45RO⁺, and FOXP3⁺ cell densities in colorectal cancer tissue, and the four histological lymphocytic reaction patterns). Because we examined the eight outcome variables, we adjusted two-sided

α level to 0.006 (=0.05/8) by simple Bonferroni correction. All other analyses, including evaluation of individual odds ratio (OR) estimates, represented secondary analyses. In those secondary analyses, in view of multiple comparisons, we interpreted our data cautiously, in addition to the use of the adjusted α level of 0.006.

We performed multivariable logistic regression analysis to control for potential confounders. The multivariable model initially included age (continuous), sex, year of diagnosis (continuous), family history of colorectal cancer in a first-degree relative (present vs. absent), tumour location (proximal colon vs. distal colon vs. rectum), MSI status (MSI-high vs. MSI-low/MSS), CIMP status (high vs. low/negative), *BRAF* mutation (mutant vs. wild-type), *KRAS* mutation (mutant vs. wild-type), *PIK3CA* mutation (mutant vs. wild-type), and LINE-1 methylation level (continuous). A backward elimination with a threshold of $P=0.05$ was used to select variables in the final model. For cases with missing information in any of the categorical variables, we included those cases in the majority category of a given covariate. We assessed the proportional odds assumption in the ordinal logistic regression model, which was generally satisfied for the T-cell ordinal outcome variables ($P>0.05$), but not for three of the four patterns (peritumoural lymphocytic reaction, intratumoural periglandular reaction, and tumour-infiltrating lymphocytes) of histological lymphocytic reaction ordinal variables ($P\leq 0.032$). Hence, we used the binary histological lymphocytic reaction variables as outcomes for logistic regression analysis.

To assess associations between tumour CD274 expression level and other categorical variables (except for the extents of signet ring cells and solid tumour areas, for which Fisher's exact test was performed), the chi-square test was performed. To compare mean age and mean LINE-1 methylation levels, an analysis of variance assuming equal variances was performed. All of those cross-sectional analyses for clinical, pathological, and molecular associations were secondary exploratory analyses, with adjusted two-sided α level of 0.002 (=0.05/22) for multiple hypothesis testing.

Deaths from causes other than colorectal cancer were censored in colorectal cancer-specific mortality analyses. We performed Kaplan-Meier analysis, and log-rank test was used to compare survival between patient groups. To adjust for confounding, we used Cox proportional hazards regression models, and calculated hazard ratio for mortality. The multivariable models initially included disease stage (I/II vs. III/IV/unknown) and the same set of variables as in multivariable logistic regression analysis. Variables were selected in a final multivariable model, using backward elimination with a threshold of $P=0.05$. To limit the degrees of freedom of the models, with missing information in any of the categorical covariates (tumour location [0.5%], MSI status [2.9%], CIMP status [8.3%], LINE-1 methylation level [2.8%], *BRAF* mutation [2.5%], *KRAS* mutation [2.9%], and *PIK3CA* mutation [8.7%]), we included those cases in the majority category of a given covariate. We confirmed that excluding the cases with missing information in any of the covariates did not substantially alter results (data not shown). We tested statistical interaction by the Wald test on the cross-product term of tumour CD274 expression score (ordinal categories ranging from 0 to 4) and each T-cell density variable (ordinal quartile categories) in a Cox proportional hazards regression model.

RESULTS

We examined expression levels of the CD274 (PD-L1) protein in 823 cases of colorectal carcinoma within the two U.S.-nationwide prospective cohort studies. We scored tumour CD274 expression levels in cytoplasm (intensity, ranging from 0 to 3) and membrane (absent [0] or present [1] if distinct membrane staining above cytoplasmic staining level existed) (figure 1 and supplementary table 1). We used the sum of the cytoplasmic and membrane scores (ranging from 0 to 4) in each case for further analyses. Among the 823 colorectal cancer cases, 92 (11%), 234 (28%), 216 (26%), 238 (29%), and 43 (5%) tumours had tumour CD274 expression score of 0, 1, 2, 3, and 4, respectively.

In 44 (5%) of the 823 colorectal cancer cases, CD274 expression was detectable in stromal cells including lymphocytes and macrophages in tumour stroma. There was no appreciable or significant association between the presence of CD274-expressing stromal cells and any of the clinical, pathological, and tumour molecular features examined (supplementary table 2).

Clinical, pathological, and molecular characteristics according to the tumour CD274 expression score in colorectal cancer are summarised in table 1 (supplementary table 3 with 5 ordinal categories of tumour CD274 expression variable). We observed inverse associations of tumour CD274 expression with tumour MSI status ($P=0.001$) and the extent of extracellular mucin ($P<0.0001$). The tumour CD274 expression level was not significantly associated with any of the other characteristics examined ($P\geq 0.003$; with the adjusted α level of 0.002 for multiple hypothesis testing) (table 1).

Table 2 shows the distribution of colorectal carcinoma cases according to the tumour CD274 expression score, T-cell densities, and histological lymphocytic reaction patterns. Tumour CD274 expression score was inversely correlated with FOXP3⁺ cell density ($P<0.0001$, by Spearman correlation test) and tumour-infiltrating lymphocytes (TIL) ($P=0.006$, by Spearman correlation test) with the adjusted α level of 0.006. In our primary hypothesis testing, we conducted logistic regression analyses to assess the associations of the tumour CD274 expression score (an ordinal predictor variable) with CD3⁺, CD8⁺, CD45RO⁺, or FOXP3⁺ cell density (an ordinal quartile outcome variable) in colorectal cancer tissue (table 3, supplementary table 4). Tumour CD274 expression score was inversely associated with FOXP3⁺ cell density in both univariable and multivariable analyses ($P_{\text{trend}}\leq 0.0002$). For a unit increase in quartile categories of FOXP3⁺ cell density, the multivariable odds ratio in the highest tumour CD274 expression score compared to the lowest score was 0.22 (95% CI 0.10–0.47). The tumour CD274 expression score was not significantly associated with CD3⁺, CD8⁺, or CD45RO⁺ cell density (all $P_{\text{trend}}\geq 0.007$, with adjusted α level of 0.006). Similar results were observed when we used binary T-cell density variables (supplementary tables 5 and 6). We also examined the relationship of tumour CD274 expression levels with histological lymphocytic reaction (a binary outcome variable) (table 4). Although tumour CD274 expression was inversely associated with tumour-infiltrating lymphocytes (TIL) in univariable analysis ($P_{\text{trend}}=0.004$), the association was not significant in multivariable analysis ($P_{\text{trend}}=0.049$) with the adjusted α level of 0.006. The tumour CD274 expression

score was not significantly associated with any of the other histological lymphocytic reaction patterns.

We assessed PDCD1 (PD-1) expression by immunohistochemistry in 793 cases of colorectal carcinoma, and semiquantitatively scored PDCD1⁺ cell density in tumour tissue. Among the 793 cases, PDCD1⁺ cell density was scored as absent, very low, low, intermediate, and high, in 230 (29%), 194 (24%), 140 (18%), 102 (13%), and 127 (16%) cases, respectively (supplementary figure 2). Clinicopathological and molecular characteristics according to PDCD1⁺ cell density in colorectal cancer are summarised in supplementary table 7.

As exploratory analyses to assess a prognostic role of CD274 expression in colorectal cancer, we conducted Kaplan-Meier analysis and Cox proportional hazards regression analysis, and did not observe significant association of tumour CD274 expression score with colorectal cancer-specific or overall mortality (table 5 and supplementary figure 3). There was no significant association between CD274 expression in stromal cells and colorectal cancer mortality (supplementary table 8 and supplementary figure 4). We further examined whether prognostic associations of T-cell densities in tumour might be modified by tumour CD274 expression status, and did not observe significant interaction between tumour CD274 expression and T cell (CD3⁺, CD8⁺, CD45RO⁺, or FOXP3⁺ cell) density ($P_{\text{interaction}} > 0.10$) (supplementary table 9).

DISCUSSION

We conducted this study to test the hypothesis that tumour CD274 (PD-L1) expression levels might be inversely associated with T-cell densities in colorectal cancer. We found that higher tumour CD274 expression was independently associated with lower density of FOXP3⁺ cells in human colorectal carcinoma tissue, after controlling for potential confounders, including MSI status, CIMP status, and LINE-1 methylation level; these tumour molecular features have been associated with histological lymphocytic reaction and tumour infiltrating T cells in colorectal cancer.^{413–1520} Evidence from our current study suggests a possible role of CD274 expression of tumour cells in regulating host immunity in colorectal cancer microenvironment.

Two previous studies have reported that tumour CD274 expression levels are positively associated with expansion of FOXP3⁺ regulatory T cells in surgically resected colorectal carcinoma tissue,³²³³ which is not consistent with our current data. However, these previous studies were severely limited by their small sample sizes (n=33 and n=56), in contrast to our much larger sample size.

Colorectal cancers are a heterogeneous group of diseases that result from the accumulation of differing sets of genomic and epigenomic alterations and influenced by tumour-host interactions.^{34–37} The analyses of host immunity against human cancer are increasingly important in cancer research and clinical practice.³⁸³⁹ High densities of CD3⁺, CD8⁺, and CD45RO⁺ cells in colorectal cancer tissue have been associated with better patient survival,^{14–19} suggesting the densities of these T cells assessed by immunohistochemistry as a measure of anti-tumour T-cell-mediated immune response to colorectal tumours.

High FOXP3⁺ cell density has been generally associated with favourable outcome of patients with colorectal cancer, although functional roles of FOXP3⁺ regulatory T cells in various types of cancers remain poorly understood.^{1417–19} FOXP3⁺ regulatory T cells, which have been considered as an immunosuppressive subset of T lymphocytes, are functionally and phenotypically diverse, with multiple possible origins and various functional profiles.⁴⁰ An enhanced infiltration of FOXP3⁺ cells has been detected in MSI-high colorectal cancer, which is a favourable tumour molecular subtype.¹⁵ Accumulating evidence indicates that function of FOXP3⁺ regulatory T cells can be tailored for differing immune milieu and contexts, and that their roles for cancer progression (tumour-promoting or tumour-suppressive roles) appear to depend on tumour site and progression stage, probably reflecting alterations of the tumour microenvironment.¹⁸⁴⁰ Ladoire et al. have proposed that the association of FOXP3⁺ cells in colorectal cancer tissue with better outcome reflects their capacity of repressing tumour-promoting inflammatory responses to gut microbiota.⁴¹ It is possible that FOXP⁺ regulatory T cells may have a role in suppressing tumour progression via regulating tumour-promoting inflammation in the colorectal cancer microenvironment.

We observed CD274 expression in the cytoplasm and membrane of colorectal cancer cells, as many other investigators reported,^{3, 32, 33, 42–44} with the use of anti-CD274 antibody clone that was referenced and validated in a number of studies for various tumour types.^{45–48} However, lack of widely-accepted immunohistochemical method for FFPE tissue has been a considerable challenge in the assessment of cellular CD274 expression.⁴⁵⁴⁹ CD274 expression on the cell surface membrane is likely important for binding to its receptor PDCD1 (PD-1). However, membrane expression was considerably masked in cells with high-level cytoplasmic expression, in contrast to cells with no cytoplasmic expression. Hence, we used both membrane and cytoplasmic expression levels to calculate the tumour CD274 expression score. To assess replicability of our immunohistochemical assessments, we conducted blinded and independent assessment of CD274 expression by the two pathologists, which yielded reasonably good interobserver agreement (a weighted κ of 0.65). Any random misclassification of tumour CD274 expression score would have driven our results towards the null hypothesis. However, despite limitations of immunohistochemical evaluation for CD274 expression, we were able to observe significant inverse association of tumour CD274 expression with FOXP3⁺ cell density in colorectal cancer tissues.

Our results suggesting a potential inverse association between tumour MSI-high status and tumour CD274 expression level are intriguing. In contrast, the previous study described that tumour CD274 expression was detected in 5 of 15 MSI-high colorectal cancers and only 1 of 11 MSS cancers;³ however, statistical power was severely limited. Another report showed that strong tumour CD274 expression was observed in 433 (36%) of 1197 MSS colorectal cancers, and in 62 (29%) of 223 MSI-high cancers ($P=0.040$ by the chi-square test),⁴² consistent with our findings. Another study reported that MSI-high colorectal cancers harboured a larger number of CD274-expressing myeloid cells in tumour tissue than MSS cancers.⁴ In our current study, we did not observe significant association of the presence of CD274-expressing stromal cells with MSI status in colorectal cancers; however, we found a limited proportion of cases with detectable CD274-expressing stromal cells (5%). Although Herbst et al. have underscored the importance of CD274-expressing myeloid cells rather than CD274-expressing tumour cells in the prediction of response to PDCD1 (PD-1)

blockade therapy,⁵⁰ the relative importance of CD274 expression in stromal cells versus that in tumour cells remains to be clarified.¹ Further studies are needed to determine the possible association of tumour MSI status with immune checkpoint molecules in tumour cells and stromal cells.

We did not observe significant association of tumour CD274 expression levels with colorectal cancer mortality. Previous studies^{42–45} have reported conflicting results in terms of the association between tumour CD274 expression and clinical outcome in colorectal cancer, possibly due to differences in study populations, designs and methods.

We observed significant inverse association of tumour CD274 expression with FOXP3⁺ cell density but not with CD3⁺, CD8⁺, or CD45RO⁺ cell density, suggesting that influences of tumour CD274 expression on T-cell densities vary by different T-cell subpopulations.

One limitation of the current study is its cross-sectional design. Hence, we cannot exclude a possibility of reverse causation. It is possible that effects of T cells on tumour cells might change tumour CD274 expression levels. However, our specific hypothesis was based on several lines of experimental evidence indicating that tumour CD274 expression suppresses T-cell-mediated immune response against tumour.^{1–6} We recognise the limitations in evaluating T cells in human colorectal cancer tissue. We evaluated the well-characterised T-cell markers such as CD3, CD8, CD45RO, and FOXP3 with the use of TMA immunohistochemistry and computer-assisted image analysis to objectively quantify the density of T cells in a large number of cases. In addition, we evaluated up to four tissue cores from each tumour, considering spacial heterogeneity of immune infiltrates. Lack of data on postoperative treatment was another limitation. However, distributions of chemotherapy use and its regimen would unlikely substantially differ according to tumour CD274 expression levels as these data were not available for treatment decision making. Lastly, we admit the limitation of TMA-based assessment, although we did not observe considerable intratumoural heterogeneity in terms of tumour CD274 expression in our validation study using the whole tissue sections. Previous studies suggest that CD274 expression has been prominent (especially in myeloid cells) at the tumour margin of colorectal cancer tissue.³⁴ Considering spacial tumour heterogeneity, when constructing TMA blocks, we punched tumour area, including tumour centre and tumour margin, to select up to four tumour tissue cores from each tumour. Potential misclassification of tumours in terms of CD274 expression due to intratumoural heterogeneity would be expected to be distributed nearly at random, and hence would have driven our results towards the null hypothesis.

Strengths of our current study include the use of our molecular pathological epidemiology⁵¹⁵² database of a large number of colorectal cancer cases in the two U.S.-nationwide prospective cohort studies. This population-based colorectal cancer database enabled us to rigorously examine the association of tumour CD274 expression with histological lymphocytic reaction or with the density of T cells, controlling for potential confounders. In addition, our colorectal cancer specimens were derived from a large number of hospitals in diverse settings across the U.S. (but not based on a limited number of hospitals), which increase the generalisability of our findings.

In conclusion, our current study has shown that tumour CD274 expression level is inversely associated with the density of FOXP3⁺ lymphocytes in colorectal carcinoma tissue. Upon validation, our human population data suggest an influence of tumour CD274 expression on regulatory T cells, and can inform further mechanistic studies to elucidate potential interactive roles of the immune checkpoint pathway and host immunity in colorectal carcinogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AJCC	American Joint Committee on Cancer
CI	confidence interval
CIMP	CpG island methylator phenotype
FFPE	formalin-fixed paraffin-embedded
LINE-1	long interspersed nucleotide element-1
MSI	microsatellite instability
MSS	microsatellite stable
TIL	tumour-infiltrating lymphocyte
TMA	tissue microarray

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Significance of this study

What is already known about this subject?

- The immune checkpoint PDCD1 (PD-1) pathway-targeted immunotherapy has emerged as a promising therapeutic strategy in various tumour types.
- Tumour CD274 (PD-L1) expression appears to suppress T-cell-mediated immune response against tumour through its interaction with T-cell co-receptor PDCD1.
- High densities of various types of T cells in colorectal carcinoma tissue are associated with better clinical outcome.

What are the new findings?

- Tumour CD274 expression level is inversely associated with the density of FOXP3⁺ lymphocytes in colorectal cancer tissue, independent of potential confounders including tumour molecular status of microsatellite instability (MSI), CpG island methylator phenotype (CIMP), LINE-1 methylation level, and *KRAS*, *BRAF*, and *PIK3CA* mutations.
- Tumour CD274 expression was inversely associated with high-level MSI status in colorectal cancer.
- Tumour CD274 expression level is not significantly associated with the density of CD3⁺, CD8⁺, or CD45RO⁺ cells in colorectal cancer tissue.

How might it impact on clinical practice in the foreseeable future?

- Our human population-based data suggest a possible influence of tumour CD274 expression on regulatory T cells in the tumour immune microenvironment. Hence, our current study can likely inform translational research on the development of immunotherapy strategies against colorectal cancer.

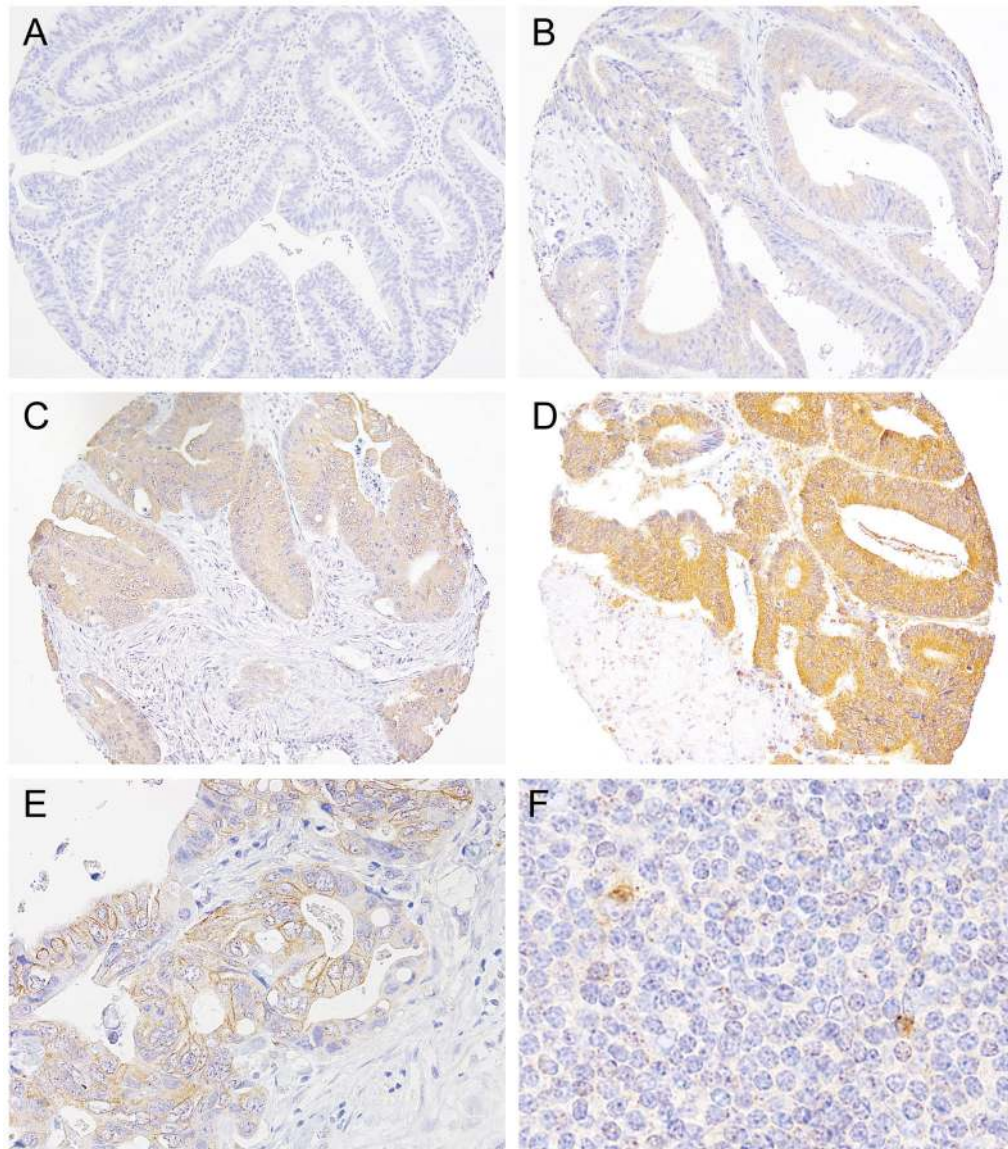


Figure 1.

Tumour CD274 expression in colorectal cancer. Tumour CD274 expression was evaluated based on immunostaining in the cytoplasm and membrane of tumour cells. Cytoplasmic expression level was scored as 0 (A), 1 (B), 2 (C), or 3 (D), according to cytoplasmic intensity. (E) Membrane CD274 expression in tumour cells. Membrane expression level was scored as 0 (absent) or 1 (present; if distinct membrane staining above cytoplasmic staining level existed). (F) Immune cells, including lymphocytes and macrophages, in the lymphoid tissue within normal colorectal tissue cores served as positive controls.

Clinical, pathological, and molecular features according to the tumour CD274 expression score in 823 colorectal cancer cases

Table 1

Characteristic*	Total No. (n=823)	Tumour CD274 expression score			P value [†]
		0 (n=92)	1/2 (n=450)	3/4 (n=281)	
Mean age±SD (yr)	69.1±9.0	69.9±9.5	69.6±8.9	68.1±8.9	0.06
Sex					0.11
Men	365 (44%)	45 (49%)	209 (46%)	111 (39%)	
Women	458 (56%)	47 (51%)	241 (54%)	170 (61%)	
Year of diagnosis					0.003
Prior to 1999	407 (50%)	41 (45%)	204 (46%)	162 (58%)	
1999 to 2008	408 (50%)	50 (55%)	242 (54%)	116 (42%)	
Family history of colorectal cancer in a first-degree relative					0.86
Absent	642 (79%)	73 (80%)	354 (80%)	215 (78%)	
Present	168 (21%)	18 (20%)	90 (20%)	60 (22%)	
Tumour location					0.06
Proximal colon	412 (50%)	42 (46%)	233 (52%)	137 (49%)	
Distal colon	242 (30%)	22 (24%)	127 (29%)	93 (33%)	
Rectum	163 (20%)	28 (30%)	85 (19%)	50 (18%)	
pT stage					0.034
pT1	74 (10%)	16 (19%)	43 (10%)	15 (6%)	
pT2	150 (20%)	17 (20%)	82 (20%)	51 (20%)	
pT3	496 (65%)	47 (55%)	272 (65%)	177 (70%)	
PT4	38 (5%)	5 (6%)	22 (5%)	11 (4%)	
pN stage					0.13
pN0	450 (61%)	52 (67%)	259 (64%)	139 (56%)	
pN1	180 (25%)	16 (21%)	89 (22%)	75 (30%)	
pN2	102 (14%)	10 (13%)	59 (15%)	33 (13%)	
M stage					0.17
M0	641 (84%)	73 (91%)	351 (84%)	217 (83%)	
M1	118 (16%)	7 (9%)	65 (16%)	46 (17%)	
Disease stage					0.12

Characteristic*	Total No. (n=823)	Tumour CD274 expression score				P value†
		0 (n=92)	1/2 (n=450)	3/4 (n=281)		
I	175 (23%)	23 (29%)	99 (24%)	53 (20%)		
II	245 (32%)	27 (34%)	143 (34%)	75 (29%)		
III	221 (29%)	23 (29%)	109 (26%)	89 (34%)		
IV	118 (16%)	7 (9%)	65 (16%)	46 (17%)	0.14	
No. of negative lymph nodes						
0–4	159 (23%)	20 (27%)	86 (23%)	53 (23%)		
5–8	156 (23%)	12 (16%)	87 (23%)	57 (25%)		
9–14	178 (26%)	24 (32%)	86 (23%)	68 (30%)		
≥15	185 (27%)	19 (25%)	115 (31%)	51 (22%)	0.22	
Tumour differentiation						
Well to moderate	744 (91%)	78 (86%)	409 (91%)	257 (91%)		
Poor	77 (9%)	13 (14%)	40 (9%)	24 (9%)	<0.0001	
Extent of extracellular mucin						
0%	482 (60%)	45 (51%)	249 (56%)	188 (68%)		
1–50%	238 (29%)	22 (25%)	144 (32%)	72 (26%)		
≥50%	90 (11%)	22 (25%)	51 (11%)	17 (6%)	0.004	
Extent of signet ring cells						
0%	714 (88%)	73 (83%)	386 (87%)	255 (92%)		
1–50%	87 (11%)	11 (13%)	54 (12%)	22 (8%)		
≥50%	8 (1%)	4 (5%)	4 (1%)	0 (0%)	0.06	
Extent of solid tumour areas						
0%	583 (83%)	61 (80%)	308 (80%)	214 (88%)		
1–50%	96 (14%)	10 (13%)	63 (16%)	23 (9%)		
≥50%	27 (4%)	5 (7%)	15 (4%)	7 (3%)	0.11	
Tumour growth pattern						
Expansile	216 (29%)	33 (41%)	118 (29%)	65 (26%)		
Intermediate	413 (56%)	38 (48%)	231 (56%)	144 (57%)		
Infiltrative	113 (15%)	9 (11%)	61 (15%)	43 (17%)	0.001	
MSI status						
MSI-low/MSS	663 (83%)	64 (70%)	358 (83%)	241 (87%)		

Characteristic*	Tumour CD274 expression score				P value [†]
	Total No. (n=823)	0 (n=92)	1/2 (n=450)	3/4 (n=281)	
MSI-high	136 (17%)	27 (30%)	74 (17%)	35 (13%)	
CIMP status					0.038
Low/negative	622 (83%)	62 (76%)	330 (81%)	230 (87%)	
High	131 (17%)	20 (24%)	76 (19%)	35 (13%)	
<i>BRAF</i> mutation					0.86
Wild-type	683 (85%)	77 (84%)	368 (85%)	238 (86%)	
Mutant	120 (15%)	15 (16%)	66 (15%)	39 (14%)	
<i>KRAS</i> mutation					0.11
Wild-type	475 (59%)	64 (70%)	253 (58%)	158 (58%)	
Mutant	324 (41%)	28 (30%)	181 (42%)	115 (42%)	
<i>PIK3CA</i> mutation					0.32
Wild-type	641 (85%)	74 (86%)	359 (87%)	208 (83%)	
Mutant	111 (15%)	12 (14%)	55 (13%)	44 (17%)	
Mean LINE-1 methylation level \pm SD (%)	62.3 \pm 9.7	63.6 \pm 9.4	62.7 \pm 10.0	61.1 \pm 9.2	0.035
<i>Fusobacterium nucleatum</i> DNA					0.041
Negative	574 (87%)	60 (77%)	306 (86%)	208 (90%)	
Low	45 (7%)	9 (12%)	23 (6%)	13 (6%)	
High	44 (7%)	9 (12%)	26 (7%)	9 (4%)	

Abbreviations: CIMP, CpG island methylator phenotype; LINE-1, long interspersed nucleotide element-1; MSI, microsatellite instability; MSS, microsatellite stable; SD, standard deviation.

* Percentage indicates the proportion of cases with a specific clinical, pathological, or molecular feature in colorectal cancer cases with each tumour CD274 expression level. There were cases that had missing values for any of the characteristics except for age and sex.

[†]To assess associations between tumour CD274 expression level and categorical variables (except for the extents of signet ring cells and solid tumour areas, for which Fisher's exact test was performed), the chi-square test was performed. To compare mean age and mean LINE-1 methylation levels, an analysis of variance was performed. We adjusted two-sided α level to 0.002 ($=0.05/22$) by simple Bonferroni correction for multiple hypothesis testing.

Table 2

Distribution of colorectal cancer cases according to the tumour CD274 expression score, the densities of T cells, and histological lymphocytic reaction patterns

	Total No.	Tumour CD274 expression score				P value*
		0	1	2	3	
CD3⁺ cell density (n=571)						
Quartile 1 (lowest)	143 (25%)	16 (29%)	48 (31%)	32 (22%)	41 (23%)	6 (17%)
Quartile 2 (second)	142 (25%)	12 (21%)	37 (24%)	29 (20%)	52 (29%)	12 (33%)
Quartile 3 (third)	143 (25%)	11 (20%)	38 (24%)	37 (25%)	47 (27%)	10 (28%)
Quartile 4 (highest)	143 (25%)	17 (30%)	33 (21%)	48 (33%)	37 (21%)	8 (22%)
CD8⁺ cell density (n=564)						
Quartile 1 (lowest)	141 (25%)	16 (30%)	44 (29%)	29 (20%)	42 (24%)	10 (29%)
Quartile 2 (second)	141 (25%)	10 (19%)	42 (27%)	41 (28%)	43 (24%)	5 (15%)
Quartile 3 (third)	141 (25%)	15 (28%)	39 (25%)	32 (22%)	48 (27%)	7 (21%)
Quartile 4 (highest)	141 (25%)	13 (24%)	29 (19%)	44 (30%)	43 (24%)	12 (35%)
CD45RO⁺ cell density (n=577)						
Quartile 1 (lowest)	145 (25%)	22 (39%)	42 (26%)	31 (21%)	42 (24%)	8 (23%)
Quartile 2 (second)	144 (25%)	9 (16%)	47 (29%)	42 (29%)	38 (21%)	8 (23%)
Quartile 3 (third)	143 (25%)	12 (21%)	42 (26%)	31 (21%)	49 (28%)	9 (26%)
Quartile 4 (highest)	145 (25%)	13 (23%)	30 (19%)	43 (29%)	49 (28%)	10 (29%)
FOXP3⁺ cell density (n=549)						
Quartile 1 (lowest)	137 (25%)	8 (15%)	35 (23%)	33 (24%)	45 (26%)	16 (44%)
Quartile 2 (second)	137 (25%)	9 (16%)	34 (23%)	35 (26%)	52 (31%)	7 (19%)
Quartile 3 (third)	138 (25%)	13 (24%)	44 (29%)	33 (24%)	42 (25%)	6 (17%)
Quartile 4 (highest)	137 (25%)	25 (45%)	38 (25%)	36 (26%)	31 (18%)	7 (19%)
Crohn's-like lymphoid reaction (n=681)						
Absent	509 (75%)	46 (68%)	147 (75%)	136 (71%)	155 (80%)	25 (78%)
Low	126 (19%)	14 (21%)	33 (17%)	44 (23%)	31 (16%)	4 (13%)
High	46 (7%)	8 (12%)	15 (8%)	12 (6%)	8 (4%)	3 (9%)
Peritumoural lymphocytic reaction (n=808)						
Absent	122 (15%)	7 (8%)	37 (16%)	44 (21%)	28 (12%)	6 (15%)

	Total No.	Tumour CD274 expression score				P value*
		0	1	2	3	
Low	562 (70%)	63 (72%)	155 (68%)	137 (64%)	180 (76%)	27 (66%)
High	124 (15%)	18 (20%)	36 (16%)	33 (15%)	29 (12%)	8 (20%)
Intratumoural periglandular reaction (n=811)						
Absent	101 (12%)	7 (8%)	32 (14%)	38 (18%)	20 (8%)	4 (10%)
Low	614 (76%)	70 (79%)	170 (74%)	148 (69%)	194 (82%)	32 (78%)
High	97 (12%)	12 (13%)	29 (13%)	28 (13%)	23 (10%)	5 (12%)
Tumour-infiltrating lymphocytes (TIL) (n=811)						
Absent	592 (73%)	55 (62%)	163 (71%)	157 (73%)	187 (79%)	30 (73%)
Low	130 (16%)	21 (24%)	42 (18%)	32 (14%)	31 (13%)	4 (10%)
High	89 (11%)	13 (15%)	26 (11%)	25 (12%)	18 (8%)	7 (17%)

* P value was calculated by Spearman correlation test between the tumour CD274 expression score (ranging from 0 to 4) and (T) lymphocyte variables, including the densities of T cells (cells/mm²; as continuous variables) and histological lymphocytic reaction patterns (absent, low, and high; as ordinal variables). Because we assessed the eight primary (T) lymphocyte variables, we adjusted two-sided α level to 0.006 (=0.05/8) by simple Bonferroni correction.

Table 3

Ordinal logistic regression analysis to assess the association of the tumour CD274 expression score (predictor) with the density of T cells (outcome)

		Univariable OR (95% CI)	Multivariable OR (95% CI)*
Model for CD3⁺ cell density (n=571, as an ordinal outcome variable)			
Tumour CD274 expression score	0	1 (reference)	1 (reference)
	1	0.77 (0.45–1.33)	0.78 (0.45–1.35)
	2	1.34 (0.77–2.32)	1.37 (0.79–2.38)
	3	0.90 (0.53–1.54)	0.94 (0.55–1.61)
	4	1.05 (0.50–2.23)	1.07 (0.51–2.26)
	$P_{\text{trend}}^{\ddagger}$	0.60	0.51
Model for CD8⁺ cell density (n=564, as an ordinal outcome variable)			
Tumour CD274 expression score	0	1 (reference)	1 (reference)
	1	0.83 (0.48–1.45)	0.88 (0.50–1.53)
	2	1.30 (0.74–2.27)	1.49 (0.84–2.62)
	3	1.10 (0.64–1.90)	1.16 (0.67–2.03)
	4	1.32 (0.61–2.84)	1.61 (0.74–3.50)
	$P_{\text{trend}}^{\ddagger}$	0.16	0.08
Model for CD45RO⁺ cell density (n=577, as an ordinal outcome variable)			
Tumour CD274 expression score	0	1 (reference)	1 (reference)
	1	1.20 (0.70–2.08)	1.42 (0.81–2.48)
	2	1.68 (0.97–2.92)	2.09 (1.19–3.69)
	3	1.69 (0.99–2.90)	2.06 (1.18–3.59)
	4	1.72 (0.81–3.67)	1.92 (0.89–4.15)
	$P_{\text{trend}}^{\ddagger}$	0.020	0.007
Model for FOXP3⁺ cell density (n=549, as an ordinal outcome variable)			
Tumour CD274 expression score	0	1 (reference)	1 (reference)
	1	0.46 (0.26–0.82)	0.46 (0.26–0.82)
	2	0.44 (0.25–0.77)	0.39 (0.22–0.70)
	3	0.33 (0.19–0.58)	0.36 (0.21–0.63)
	4	0.21 (0.10–0.45)	0.22 (0.10–0.47)
	$P_{\text{trend}}^{\ddagger}$	<0.0001	0.0002

Abbreviations: CI, confidence interval; OR, odds ratio.

*The multivariable ordinal logistic regression analysis model initially included age, sex, year of diagnosis, family history of colorectal carcinoma in any parent or sibling, tumour location, microsatellite instability, CpG island methylator phenotype, *KRAS*, *BRAF*, and *PIK3CA* mutations, and LINE-1 methylation level. A backward elimination with a threshold of $P=0.05$ was used to select variables in the final models.

$\ddagger P_{\text{trend}}$ value was calculated by the linear trend across the ordinal categories of the tumour CD274 expression score (0 to 4, as an ordinal predictor variable) in the ordinal logistic regression model for the density of CD3⁺ cells, CD8⁺ cells, CD45RO⁺ cells, or FOXP3⁺ cells (an ordinal quartile outcome variable). Because we assessed eight primary outcome variables, we adjusted two-sided α level to 0.006 (=0.05/8) by simple Bonferroni correction.

Table 4

Logistic regression analysis to assess the association of the tumour CD274 expression score (predictor) with histological lymphocytic reaction (outcome)

		Univariable OR (95% CI)	Multivariable OR (95% CI) [‡]
Model for Crohn's-like lymphoid reaction (n=681, as a binary outcome variable[*])			
Tumour CD274 expression score	0	1 (reference)	1 (reference)
	1	0.68 (0.37–1.25)	0.79 (0.40–1.57)
	2	0.86 (0.47–1.56)	1.06 (0.54–2.09)
	3	0.53 (0.28–0.98)	0.65 (0.32–1.31)
	4	0.59 (0.22–1.56)	0.68 (0.23–1.98)
	$P_{\text{trend}}^{\ddagger}$	0.09	0.30
Model for peritumoural lymphocytic reaction (n=808, as a binary outcome variable[*])			
Tumour CD274 expression score	0	1 (reference)	1 (reference)
	1	0.45 (0.19–1.04)	0.43 (0.18–1.03)
	2	0.33 (0.14–0.77)	0.35 (0.15–0.82)
	3	0.65 (0.27–1.54)	0.58 (0.24–1.42)
	4	0.50 (0.16–1.61)	0.43 (0.13–1.40)
	$P_{\text{trend}}^{\ddagger}$	0.87	0.59
Model for intratumoural periglandular reaction (n=812, as a binary outcome variable[*])			
Tumour CD274 expression score	0	1 (reference)	1 (reference)
	1	0.53 (0.23–1.25)	0.54 (0.22–1.28)
	2	0.40 (0.17–0.92)	0.42 (0.18–0.99)
	3	0.93 (0.38–2.27)	0.88 (0.35–2.19)
	4	0.79 (0.22–2.86)	0.71 (0.19–2.62)
	$P_{\text{trend}}^{\ddagger}$	0.51	0.69
Model for tumour-infiltrating lymphocytes (n=811, as a binary outcome variable[*])			
Tumour CD274 expression score	0	1 (reference)	1 (reference)
	1	0.68 (0.40–1.13)	0.78 (0.42–1.43)
	2	0.59 (0.35–0.99)	0.66 (0.36–1.24)
	3	0.42 (0.25–0.72)	0.54 (0.29–1.01)
	4	0.59 (0.26–1.34)	0.64 (0.25–1.65)
	$P_{\text{trend}}^{\ddagger}$	0.004	0.049

Abbreviations: CI, confidence interval; OR, odds ratio.

^{*} Since the proportional odds assumption was not satisfied in the ordinal logistic regression model, we used the binary logistic regression model to assess the independent association of the tumour CD274 expression score with each histological lymphocytic reaction pattern.

[‡] The multivariable logistic regression analysis model initially included age, sex, year of diagnosis, family history of colorectal carcinoma in any parent or sibling, tumour location, microsatellite instability, CpG island methylator phenotype, *KRAS*, *BRAF*, and *PIK3CA* mutations, and LINE-1 methylation level. A backward elimination with a threshold of $P=0.05$ was used to select variables in the final models.

[‡] P_{trend} value was calculated by the linear trend across the ordinal categories of the tumour CD274 expression score (0 to 4, as an ordinal predictor variable) in the binary logistic regression model for each histological lymphocytic reaction pattern (a binary outcome variable [absent vs. low/high]). Because we assessed eight primary outcome variables, we adjusted two-sided α level to 0.006 ($=0.05/8$) by simple Bonferroni correction.

Table 5

Tumour CD274 expression and colorectal cancer patient mortality

Tumour CD274 expression score	Total No.	Colorectal cancer-specific mortality			Overall mortality		
		No. of events	Univariable HR (95% CI)	Multivariable HR (95% CI)*	No. of events	Univariable HR (95% CI)	Multivariable HR (95% CI)*
0	91	24	1 (reference)	1 (reference)	47	1 (reference)	1 (reference)
1	230	70	1.18 (0.74–1.87)	1.00 (0.63–1.60)	121	1.00 (0.71–1.40)	0.89 (0.63–1.25)
2	216	61	1.10 (0.68–1.76)	1.00 (0.62–1.60)	117	1.08 (0.77–1.51)	1.02 (0.72–1.43)
3	235	79	1.28 (0.81–2.03)	0.95 (0.60–1.52)	139	1.06 (0.76–1.48)	0.96 (0.68–1.34)
4	43	12	1.06 (0.53–2.12)	0.64 (0.32–1.28)	26	1.03 (0.64–1.66)	0.73 (0.45–1.19)
$P_{\text{trend}}^{\ddagger}$			0.49	0.30		0.65	0.66

Abbreviations: CI, confidence interval; HR, hazard risk.

* The multivariable Cox regression model initially included age, sex, year of diagnosis, family history of colorectal carcinoma in any parent or sibling, tumour location, disease stage, microsatellite instability, CpG island methylator phenotype, *KRAS*, *BRAF*, and *PIK3CA* mutations, and LINE-1 methylation level. A backward stepwise elimination with a threshold of $P=0.05$ was used to select variables in the final models.

\ddagger P_{trend} value was calculated across the ordinal categories of tumour CD274 expression score as a continuous variable in the Cox regression model.