Combined prognostic value of CD274 (PD-L1)/PDCD1 (PD-1) expression and immune cell infiltration in colorectal cancer as per mismatch repair status

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Abstract

The CD274 (programmed cell death ligand-1, PD-L1)/PDCD1 (programmed cell death-1, PD-1) pathway is crucial suppressor of the cytotoxic immune response. Antibodies targeting CD274 or PDCD1 have been revealed to be effective in several malignancies. In colorectal cancer, the response to CD274/PDCD1 blockage is associated with microsatellite instability. However, the value of CD274/PDCD1 for predicting response to treatment or survival benefit is still unclear. The aims of the study were (1) to clarify differences in immune microenvironment and expression of checkpoint proteins (CD274/PDCD1) in DNA mismatch repair-proficient, mismatch repair-deficient, and hereditary Lynch syndrome-associated colorectal cancer, and (2) to assess the prognostic value of these factors and their combinations. Ninety-four mismatch repair-deficient colorectal cancers, 100 age, sex, and AJCC/UICC stage-matched mismatch repair-proficient colorectal cancers, and 48 Lynch syndrome-associated colorectal cancers were analyzed. Using whole section samples, detailed analysis of immune cell score, PDCD1, and CD274 expression was performed. Overlapping of CD274 expression in tumor and immune cells was almost complete (95%). Immune cell score and CD274/PDCD1 positivity were significantly more frequent in mismatch repair-deficient than in mismatch repair-proficient colorectal cancers (70% vs. 41% (high immune cell score); 81% vs. 49% (PDCD1^{high}), 23% vs. 1% (CD274 on tumor cells) and 68% vs. 30% (CD274 on immune cells), $P < 0.001$), and were associated strongly with each other. Although the independent impact of immune cell score, PDCD1, and CD274 on immune cells was moderate, the immunoprofile parameter combining the above three factors appeared to be a strong independent prognostic factor for disease-specific survival and overall survival ($P = 0.001$) and had suggestive impact on disease-free survival ($P = 0.011$). Our results encourage the use of immune cell score analysis together with PDCD1 and CD274 detection to improve the prognostic evaluation of colorectal cancer patients. Particularly, the analyses from whole tissue sections are encouraged to allow reliable and cell-specific analyses of CD274 expression.

Introduction

Immune blockade targeting CD274 (programmed cell death ligand-1, PD-L1)/PDCD1 (programmed cell death, PD-1) is

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a promising therapeutic strategy for various malignancies [1, 2]. The CD274/PDCD1 pathway normally serves as a negative regulator of T cells by preventing overstimulation of physiological immune responses to produce tolerance. Overexpression of CD274 is commonly observed in malignant cells and represents one mechanism for cancer cell immune evasion. The expression of CD274 can also be driven by constitutive oncogenic signaling pathways (e.g., enhanced expression of CD274 on glioblastomas when *PTEN* is silenced), although this mechanism is less frequently encountered [3]. Upregulation of CD274 on cancer cells and/or on myeloid cells together with tumor-infiltrating lymphocytes expressing PDCD1 forms a basis for immune checkpoint blockade; this blockade occurs when the interaction between these proteins is blocked by antibodies, which in turn leads to restored T-cell activity. In colorectal cancers, the efficacy of CD274/PDCD1 treatment has been shown in tumors with microsatellite instability [4–9].

Microsatellite instability arises from deficient DNA mismatch repair function and results in increased mutational burden and increased expression of various neoantigens. Microsatellite instability has been detected in approximately 15% of colorectal cancers [10]. The sporadic microsatellite-unstable phenotype usually results from epigenetic silencing of the *MLH1* gene, whereas germline mutations of one of the mismatch repair genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) is typical in hereditary Lynch syndrome [11–13]. In general, patients with microsatellite-unstable tumors have a more favorable prognosis than do patients with microsatellite-stable tumors, which has been explained by strong cytotoxic lymphocyte infiltrates resulting from high mutational burden and neoantigen load [14–17]. Neoantigen load seems to correlate with the number of tumor-infiltrating lymphocytes in microsatellite-unstable colorectal cancer [18, 19], as well as in hypermutated microsatellite-stable colorectal cancer [20, 21]. Several immune checkpoint-related proteins have also been shown to be overexpressed in microsatellite-unstable tumors compared with microsatellite-stable tumors, most probably as an adaptive resistance to immune response [22]; this aspect may help explain the observed response to checkpoint blockade in microsatellite-unstable colorectal cancer [6, 8, 9].

However, it is somewhat unclear as to how to identify colorectal cancer patients who will benefit from CD274/PDCD1 treatments. Patients with tumors expressing CD274 seem to have higher response rates, but the lack of CD274 does not always exclude effective treatment [23]. In addition, pre-existing antitumor immune response is regarded as essential for successful CD274/PDCD1 pathway blockade [3]. Although microsatellite-unstable colorectal cancers usually have abundant lymphocytes, microsatellite-unstable status as such seems to be an insufficient predictor of CD274/PDCD1 blockage, with the observed response rate of 40% or less [6, 8]. Multifactorial biomarker panels

including tumor mutational burden, CD8⁺ T-cell density, and CD274 expression are suggested to have more predictive value [24] and therefore, classification of tumors into groups based on their CD274 status and the existence of tumor-infiltrating lymphocytes has been proposed to predict treatment response [25, 26].

Regarding the overall outcome in colorectal cancer patients, the abundance of tumor-infiltrating lymphocytes has significant prognostic value regardless of tumor mismatch repair status both in local and advanced colorectal cancer [27–31]. Immunoscore® is a quantitative classification system that describes cytotoxic immune reaction, and has been launched in conjunction with a diagnostic assay to guide treatment strategies for colorectal cancer patients, and to improve the prognostic impact of tumor, node and metastases classification [32, 33]. In contrast to tumor-infiltrating lymphocytes, assessment of the CD274/PDCD1 axis is more complicated because it involves various immunohistochemical assays and evaluation methods. As a result, the prognostic value of CD274/PDCD1 is yet unclear [34–39].

With these considerations, we aimed to make an in-depth whole tumor section analysis of PDCD1 and CD274 expression, and of immune cell infiltration; we further sought to clarify the differences in three different colorectal cancer types, i.e., mismatch repair-proficient, mismatch repair-deficient, and Lynch syndrome-associated colorectal cancers. Furthermore, we assessed the prognostic value of these factors and their combinations.

Materials and methods

Tumor sampling

Our study material was obtained as part of a previously described study by Seppälä et al. [40], which included 1088 colorectal cancer patients who underwent surgery at the Central Hospital of Central Finland in Jyväskylä during the years 2000–2010. A total of 94 mismatch repair-deficient tumors, as well as equal-sized age-, sex-, and The American Joint Committee on Cancer/Union Internationale Contre le Cancer (AJCC/UICC) stage-matched mismatch repair-proficient controls ($n = 100$) were included in our study. Colorectal cancer samples from Lynch syndrome patients ($n = 48$) collected by two leading Lynch syndrome research centers in Finland (Helsinki University and Jyväskylä Central Hospitals) were also included in the study. All Lynch syndrome patients were confirmed carriers of a pathogenic germline variant of *MLH1*, *MSH2*, or *MSH6*. These were surgical samples of tumors obtained within a period from 1983 to 2012. Due to the heterogeneity of the Lynch syndrome group and since it included only one

cancer-related death during follow-up, Lynch syndrome tumors were not included in the survival analyses.

We reviewed hospital records to extract information regarding treatment and tumor recurrence. Causes of death were reviewed and updated in December 2015 from the Finnish Cause of Death Registry. Macroscopic examination of tumor tissue was performed by an experienced histopathologist following AJCC guidelines (5th and 6th edition). Slides were reviewed according to AJCC 8th edition to verify perineural and lymphovascular invasion rate, tumor grading, as well as tumor budding. Tumor samples were graded (grades 1–4) on the basis of the percentage of glandular formation according to the traditional World Health Organization (WHO) recommendation [41] without considering mismatch repair status. Mucinous adenocarcinoma and signet ring cell carcinoma were considered poorly differentiated (grade 3) and medullary carcinoma was considered undifferentiated (grade 4). A 6-month course of adjuvant postoperative chemotherapy with either 5-fluorouracil and oral folinic acid, oral capecitabine with or without oxaliplatin, or 5-fluorouracil, folinic acid and oxaliplatin was prescribed to medically fit patients having stage III tumors or high-risk stage II disease (T4 or T3 with perineural or lymphovascular invasion).

Immunohistochemical analyses

Mismatch repair status was determined by immunohistochemical analysis for the expression of MLH1, PMS2, MSH2, and MSH6 as described previously [40].

To select the PDCD1 and CD274 antibodies for present study, a validation was performed with clones NAT105 (Dianova GmbH, Hamburg, Germany), F07 (Sino Biological Inc., Beijing, China), and SP269 (Spring Bioscience, Pleasanton, CA, USA) for PDCD1, and clones E1L3N (Cell Signaling Technology, Danvers, MA, USA), SP142 (Spring Bioscience, Pleasanton, CA, USA), 015 (Sino Biological Inc., Beijing, China), and 28–8 (Abcam, Cambridge, UK) for CD274. For validation, a tissue microarray was constructed using formalin-fixed paraffin-embedded tissues of five tumor types known to express CD274 (advanced melanoma, non-small cell lung cancer, Hodgkin's lymphoma, renal cell carcinoma, and bladder cancer). Three tissue microarray cores (0.6 mm in diameter) were extracted from 10 representative tumor blocks of each tumor type. Staining protocols were optimized for each antibody (data not shown) before comparison. Positive control tissues for PDCD1 and CD274 immunohistochemistry were normal tonsil and placenta, respectively. All immunohistochemical staining procedures were conducted with an automated staining platform (BOND-III, Leica Biosystems). The slides were observed with a light microscope and were further scanned with a NanoZoomer-XR (Hamamatsu Photonics) at $\times 40$ magnification.

Cohorts of 100 mismatch repair-proficient, 94 mismatch repair-deficient, and 48 Lynch syndrome-associated colorectal cancer tumor samples were used for in-depth analysis of PDCD1 and CD274 expression together with immune response analysis. Formalin-fixed paraffin-embedded tissue sections of 3 μ m thickness were used for immunohistochemical analyses. Staining for PDCD1 and CD274 was conducted with anti-PDCD1 (SP269, 1:50; Spring Bioscience) and anti-CD274 (E1L3N, 1:100; Cell Signaling Technology) antibodies, using a BOND-III stainer (Leica Biosystems). Staining for CD3 and CD8 was conducted with anti-CD3 (LN 10, 1:50; Novocastra) and anti-CD8 (SP16, 1:100; Thermo Scientific) antibodies, using a Lab Vision Autostainer 480 (ImmunoVision Technologies Inc.). Signal visualization was done by diaminobenzidine and sections were counterstained with haematoxylin. Slides were scanned with a NanoZoomer-XR (Hamamatsu Photonics) at $\times 20$ magnification.

To specify the cell types (tumor cells, B/T lymphocytes, and macrophages) expressing PDCD1 and CD274, a multilabel immunofluorescent analysis was performed with five representative mismatch repair-proficient and mismatch repair-deficient colorectal cancer samples. For parallel immunofluorescent staining, primary antibodies against CK18 (E43 1–1, AlexaFluor[®] 488, Abcam), CD163 (10D6, Thermo Scientific), CD3, PDCD1, and CD274 (previously mentioned) were used together with secondary antibodies (anti-mouse AlexaFluor[®] 647 (Thermo Scientific) and anti-rabbit AlexaFluor[®] 568 (Thermo Scientific), in conjunction with a 4'-diamidino-2-phenylindole nuclear counterstain. Sections were imaged on the Olympus ScanR Platform at $\times 20$ magnification. Single-cell nuclear and cytoplasmic fluorescent intensities were calculated using the Olympus ScanR Analysis Software.

Scoring

CD274 expression was evaluated on tumor cells and tumor-infiltrating immune cells throughout the tumor center and the invasive front. Both the number of stained cells (percent of either tumor cells and/or immune cells stained for CD274) and the staining intensity were scored. Tumor samples were defined as CD274 positive (CD274^{pos}) when $> 5\%$ of the tumor cells and/or tumor-infiltrating immune cells were positive for CD274 with moderate or strong intensity. The CD274 staining intensity was scored using a scale of 0–3 as follows: 0 for no staining, 1 for faint staining, 2 for moderate staining, and 3 for strong staining. The number of PDCD1-positive (PDCD1+) tumor-infiltrating lymphocytes (per 1 mm²) were calculated using QuPath [42]. The mean calculated area was 32.0 mm² per slide. The area needed for obtaining a reliable mean value of PDCD1-positive cells was adjusted according to the

heterogeneity of the number of PDCD1-positive cells in the current slide. The samples were divided into two groups (PDCD1^{low} and PDCD1^{high}) based on calculated values for PDCD1-positive lymphocytes. To determine the cut-off value for PDCD1 positivity (55 cells/mm²) with optimal sensitivity and specificity, we used receiver operating characteristic curves drawn in relation to disease-specific 5-year mortality.

CD8- and CD3-stained whole section samples were digitally scanned. Representative areas from the tumor center and the invasion front were selected and positively stained CD8 and CD3 lymphocytes were enumerated by using QuPath [42]. The width of the invasive front analyzed for immune cell score was considered to span 360 μ m into the tumor and 360 μ m into the healthy tissue from the visible tissue frontier as proposed by Hermitte [43]. The invasive front was selected manually using an annotation brush with a diameter of 720 μ m. The mean analyzed areas from CD3- and CD8-stained sections were 11.5 mm² and 11.3 mm², respectively, for the tumor center, and 9.6 mm² and 9.3 mm², respectively, for the invasion front. Cut-off values for immune cell score were selected from receiver operating characteristic curves (327 for CD3⁺ and 148 for CD8⁺ in the tumor center; 814 for CD3⁺ and 411 for CD8⁺ in the invasive front). The immune cell score was formulated following the example of Galon et al. [44], as presented in our previous study [31]. Patients were divided into low immune cell score (scores 0–2) and high immune cell score (scores 3–4) groups for further analysis.

Combining patients based on data on tumor-infiltrating lymphocytes and CD274 expression into four distinctive groups has been proposed earlier [25, 26]. Accordingly, we classified the tumors into four groups according to immune cell score status and CD274 positivity. The subgroups were as follows: high immune cell score/CD274^{pos} (adaptive immune resistance), low immune cell score/CD274^{neg} (immunological ignorance), low immune cell score/CD274^{pos} (intrinsic induction), and high immune cell score/CD274^{neg} (tolerance). Furthermore, immune cell score and PDCD1 were combined and designated as low/high immune cell score and PDCD1^{low}/PDCD1^{high}, to define the following subgroups: high immune cell score/PDCD1^{high}, low immune cell score/PDCD1^{low}, low immune cell score/PDCD1^{high}, and high immune cell score/PDCD1^{low}. Additionally, four subgroups were formed according to CD274^{neg}/CD274^{pos} and PDCD1^{low}/PDCD1^{high} designations.

Three of the above immunologic features (immune cell score, CD274 on immune cells, and PDCD1) were dichotomized so that patients were assigned a score of 0 for low/negative and 1 for high/positive features. Consequently, patients were assigned a composite immunoprofile scoring with a range of 0–3.

Statistical analysis

Categorical data were compared using the Pearson's chi-square test. The Kaplan–Meier method was used to calculate disease-free survival, disease-specific survival, and overall survival, and the log-rank test was used to compare differences. Survival times for disease-specific survival and overall survival were calculated from the date of surgery to the time of death or the end of follow-up. For disease-free survival, the endpoint was the date of cancer recurrence after radical surgery or the end of follow-up. Death within 30 days following surgery was considered postoperative, and a metastasis within 6 months after surgery was considered synchronous. Univariable and multivariable Cox proportional hazards regression models were used to analyze prognostic factors for disease-free, disease-specific, and overall survival. Only variables with a *P*-value of < 0.20 in univariable analysis were included in the multivariable analysis with age, sex, and mismatch repair status. Among AJCC/UICC stage I patients there were no cancer deaths. Therefore, these patients were excluded from multivariable analysis for disease-specific survival. *P*-values of < 0.005 were considered significant and *P*-values between 0.05 and 0.005 were defined as suggestive [45]. Statistical analysis was performed using IBM SPSS Statistics (version 23.0; SPSS Inc., Chicago, IL, USA).

Results

Patient demographics

A total of 242 colorectal cancer specimens were included in this study. The clinicopathological characteristics of the patients with mismatch repair-proficient (*n* = 100), mismatch repair-deficient (*n* = 94), and Lynch syndrome tumors are presented in Table 1. The median follow-up time was 7.0 years (interquartile range, 3–10 years). There were no statistically significant differences in age, sex, The American Society of Anesthesiologists (ASA) class, AJCC/UICC stage, and perineural/lymphovascular invasion between the mismatch repair-proficient and mismatch repair-deficient groups. Proximal location and poor tumor differentiation were seen more often in the mismatch repair-deficient group (*P* < 0.001). In mismatch repair-deficient tumors, mucinous histology was more frequent (*P* = 0.003) and tumor budding was less frequent (*P* = 0.001) than in mismatch repair-proficient tumors. In mismatch repair-deficient tumors, four signet ring cell carcinomas and four medullary carcinomas were found. Lynch syndrome tumors differed from sporadic instable colorectal cancers according to age, sex, tumor differentiation, and tumor location (*P* < 0.001).

Table 1 Patient demographics

	MMR-proficient (<i>N</i> = 100)	MMR-deficient (<i>N</i> = 94)	Lynch syndrome (<i>N</i> = 48)	P-value	
				MMR-P vs. MMR-D	MMR-D vs. Lynch
Age					
≤75	53	44 (47)	47 (98)	0.389	<0.001
>75	47	50 (53)	1 (2)		
Sex					
Male	36	26 (28)	28 (58)	0.213	<0.001
Female	64	68 (72)	20 (42)		
ASA class					
1	5	5 (5)	N/A	0.911	N/A
2	36	30 (32)			
3	46	42 (45)			
4	13	15 (16)			
AJCC/UICC stage					
I	14	17 (18)	15 (33)	0.404	0.107
II	46	47 (50)	20 (44)		
III	36	24 (26)	11 (24)		
IV	4	6 (6)	0 (0)		
Perineural invasion					
No	88	87 (93)	48 (100)	0.286	0.309
Yes	12	7 (7)	0 (0)		
Lymphovascular invasion					
No	51	52 (55)	27 (56)	0.547	0.988
Yes	49	42 (45)	21 (44)		
Tumor grade					
1	36	19 (20)	0 (0)	<0.001	0.001
2	48	32 (34)	12 (25)		
3	16	40 (43)	34 (71)		
4	0	3 (3)	2 (4)		
Mucinous histology					
0–49%	94	75 (80)	37 (77)	0.003	0.709
50%–100%	6	19 (20)	11 (23)		
Budding					
No	55	72 (77)	38 (79)	0.001	0.812
Yes	45	21 (23)	10 (21)		
Tumor location					
Proximal	50	75 (80)	28 (58)	<0.001	<0.001
Distal	34	16 (17)	6 (13)		
Rectum	16	3 (3)	14 (29)		
Adjuvant chemotherapy					
No	61	65 (69)	N/A	0.234	N/A
Yes	39	29 (31)			

Values are no. (%) unless otherwise stated

MMR mismatch repair, MMR-P mismatch repair-proficient, MMR-D mismatch repair-deficient, ASA The American Society of Anesthesiologists, AJCC The American Joint Committee on Cancer, UICC Union for International Cancer Control, N/A not available

Immune contexture of mismatch repair-proficient, sporadic mismatch repair-deficient, and Lynch syndrome tumors

Both CD274 tumor cell positivity and immune cell positivity were more frequently seen in mismatch repair-deficient tumors ($P < 0.001$, Table 2) as compared with

mismatch repair-proficient colorectal cancers. CD274 on tumor cells was observed in 1% of mismatch repair-proficient, 23% of mismatch repair-deficient, and 6% of Lynch syndrome tumors; CD274 on immune cells was detected in 30% of mismatch repair-proficient, 68% of mismatch repair-deficient, and 63% of Lynch syndrome tumors (Table 2). CD274 on tumor cells was suggestively less frequent in Lynch syndrome tumors compared with that in sporadic instable colorectal cancers ($P = 0.016$). Co-expression of CD274 on tumor and immune cells was seen in almost all cases (95%). Two different staining patterns of CD274 expression were seen: marginal (Figs. 1a–c) and diffuse (Figs. 1b–d); the diffuse pattern was suggestively more common in sporadic mismatch repair-deficient colorectal cancers compared with mismatch repair-proficient colorectal cancers ($P = 0.041$), and significantly more common compared with Lynch syndrome-associated tumors ($P = 0.002$).

Mismatch repair-proficient and mismatch repair-deficient groups differed significantly according to immune cell score/CD274 status ($P < 0.001$). The proportions of high immune cell score/CD274^{pos}, low immune cell score/CD274^{pos}, high immune cell score/CD274^{neg}, and low immune cell score/CD274^{neg} were 54%, 5%, 16%, and 20%, respectively, in the mismatch repair-deficient group, and were 16%, 7%, 25%, and 52%, respectively, in the mismatch repair-proficient group (Table 2). In Lynch syndrome tumors, the proportions did not differ significantly from those in sporadic mismatch repair-deficient tumors, at 44%, 8%, 29%, and 19% for high immune cell score/CD274^{pos}, low immune cell score/CD274^{pos}, high immune cell score/CD274^{neg}, and low immune cell score/CD274^{neg}, respectively ($P = 0.286$) (Table 2). Classification into immune cell score/PDCD1 and CD274/PDCD1 subgroups and their distributions are also shown in Table 2.

The immune cell score was higher in mismatch repair-deficient compared with that in mismatch repair-proficient colorectal cancer ($P < 0.001$) and no differences between sporadic mismatch repair-deficient and Lynch syndrome tumors were seen (Table 2). However, mismatch repair-deficient tumors contained more PDCD1-positive T lymphocytes compared with both mismatch repair-proficient and Lynch syndrome tumors ($P < 0.001$ and 0.012, respectively, Table 2).

To verify the cell types expressing CD274, a multilabel immunofluorescent analysis was performed for five representative samples from mismatch repair-deficient and mismatch repair-proficient groups showing strong immunohistochemical CD274 staining. Among the cell types analyzed (tumor cells, T cells, and macrophages), CD274 expression was found solely on tumor cells and macrophages. No discernible CD274 expression was seen on T cells (Fig. 2).

Table 2 The immune contexture of mismatch repair-proficient, sporadic mismatch repair-deficient, and Lynch syndrome-associated tumors

	MMR- proficient (<i>N</i> = 100)	MMR- deficient (<i>N</i> = 94)	Lynch syndrome (<i>N</i> = 48)	Total (<i>N</i> = 242)	<i>P</i> -value	
					MMR-P vs. MMR-D	MMR-D vs. Lynch
CD274 on tumor cells						
Negative	99	72 (77)	45 (94)	216 (89)	<0.001	0.016
Positive	1	22 (23)	3 (6)	26 (11)		
CD274 on immune cells						
Negative	70	30 (32)	18 (37)	118 (49)	<0.001	0.394
Positive	30	64 (68)	30 (63)	124 (51)		
PDCD1						
Low	51	15 (16)	17 (35)	82 (34)	<0.001	0.012
High	49	76 (81)	31 (65)	156 (64)		
Immune cell score						
0	31	12 (13)	3 (6)	46 (19)	<0.001	0.519
1	17	4 (4)	5 (10)	24 (10)		
2	13	8 (9)	5 (10)	26 (11)		
3	17	13 (14)	7 (15)	37 (15)		
4	24	53 (56)	28 (58)	105 (43)		
ICS/CD274 ^{IC}						
ICS ^{high} /CD274 ^{high}	16	51 (54)	21 (44)	88 (36)	<0.001	0.286
ICS ^{low} /CD274 ^{high}	7	5 (5)	4 (8)	16 (7)		
ICS ^{high} /CD274 ^{low}	25	15 (16)	14 (29)	54 (22)		
ICS ^{low} /CD274 ^{low}	52	19 (20)	9 (19)	80 (33)		
ICS/PDCD1						
ICS ^{high} /PDCD1 ^{high}	28	62 (66)	28 (58)	118 (49)	<0.001	0.025
ICS ^{low} /PDCD1 ^{high}	21	12 (13)	3 (6)	36 (15)		
ICS ^{high} /PDCD1 ^{low}	13	3 (3)	7 (15)	23 (10)		
ICS ^{low} /PDCD1 ^{low}	38	10 (11)	1 (2)	57 (24)		
CD274/PDCD1						
CD274 ^{high} /PDCD1 ^{high}	16	51 (54)	20 (42)	87 (36)	<0.001	0.090
CD274 ^{low} /PDCD1 ^{high}	33	25 (27)	11 (23)	69 (29)		
CD274 ^{high} /PDCD1 ^{low}	7	5 (5)	5 (10)	17 (7)		
CD274 ^{low} /PDCD1 ^{low}	44	10 (11)	12 (25)	65 (27)		
Immunoprofile						
0	33	8 (9)	7 (15)	47 (19)	<0.001	0.295
1	35	11 (12)	10 (21)	56 (23)		
2	18	20 (21)	12 (25)	50 (21)		
3	14	48 (51)	19 (40)	81 (33)		

Values are no. (%) unless otherwise stated

MMR mismatch repair, MMR-P mismatch repair-proficient, MMR-D mismatch repair-deficient, CD274 programmed cell death ligand-1, IC immune cells, PDCD1 programmed cell death protein 1, ICS immune cell score

Association between CD274/PDCD1 expression and clinicopathologic parameters in mismatch repair-proficient and mismatch repair-deficient tumors

In addition to a strong association with mismatch repair deficiency ($P < 0.001$), CD274 on immune cells, CD274 on

tumor cells, and high frequency of PDCD1-positive T lymphocytes were associated significantly with a high immune cell score (all $P < 0.001$, Table 3). Furthermore, CD274 on immune cells and PDCD1^{high} statuses were suggestively associated with lower overall AJCC ($P = 0.010$ and 0.007 , respectively). CD274 on tumor cells was

Fig. 1 Hematoxylin and eosin (H&E) staining and immunohistochemical staining of CD274, PDCD1, CD3, and CD8 in colorectal adenocarcinomas. Two different staining patterns of CD274 expression are shown: marginal (**a**) and diffuse (**b**). $\times 20$ magnification shows CD274^{pos} immune cells surrounding the tumor area (**c**), whereas both

tumor and immune cells are CD274^{pos} in diffuse staining pattern (**d**) with corresponding H&E staining (**e** and **f**, respectively). Figures **g–j** show representative immunostaining of CD3 (**g**), CD8 (**h**), and PDCD1 (**i**) and corresponding H&E staining (**j**)

Fig. 2 Colocalization of CD274 with immune and tumor cell markers by immunofluorescence. CD274 staining in red, markers of immune cells in green (CD3 for T cells and CD163 for macrophages) and marker of tumor cells in blue (CK18). Upper panel shows the expression of CD274 in tumor cells (indicated with arrowheads). No

CD274 expression is seen in T cells (indicated with arrows). Lower panel shows the expression of CD274 in macrophages (indicated with arrows) as well as in tumor cells (indicated with arrowheads). 20× objective

associated significantly and CD274 on immune cells was associated suggestively with poor differentiation (73% grades 3–4, $P < 0.001$ and 41% grades 3–4, $P = 0.027$, respectively). CD274 on immune cells was also associated significantly and PDCDI was associated suggestively with tumor budding ($P = 0.002$ and 0.015 , respectively) and proximal tumor location ($P = 0.001$ and 0.048 , respectively, Table 3). Immunoprofile, the combination of immune cell score, PDCDI and CD274 on immune cells, did not have statistically significant associations with any of the conventional clinicopathological features in mismatch repair-proficient tumors (Table 4).

Prognostic impact of CD274/PDCDI expression and immune cell infiltration

Survival analyses according to clinicopathological parameters and immune contexture are shown in Table 5. A positive prognostic impact of immune contexture was seen both in mismatch repair-proficient and in mismatch repair-deficient colorectal cancer. In mismatch repair-deficient patients, immune cell score was significantly prognostic for 5-year disease-free, disease-specific, and overall survival (all $P < 0.001$). For immune cell scores 0–1, 2–3, and 4, disease-free survival was 62%, 95%, and 95%, respectively, disease-specific survival was 48%, 89%, and 94%, respectively, and overall survival was 25%, 67%, and 74%, respectively. In mismatch repair-proficient patients, immune cell score was suggestively prognostic only for disease-free survival ($P = 0.010$). CD274 on tumor cells did not affect survival of either mismatch repair-proficient or mismatch repair-deficient

patients, whereas CD274 on immune cells had a suggestively prognostic impact on disease-specific and overall survival both in mismatch repair-proficient ($P = 0.006$ and 0.011 , respectively) and mismatch repair-deficient ($P = 0.048$ and 0.029 , respectively) patients. PDCDI^{high} improved 5-year disease-free, disease-specific, and overall survival significantly (all $P < 0.005$) in mismatch repair-deficient patients. In mismatch repair-proficient patients, PDCDI^{high} impacted significantly on disease-free survival ($P = 0.002$ and 0.004 , respectively) and suggestively on overall survival ($P = 0.010$). Furthermore, perineural invasion and tumor budding had a negative impact on disease-free, disease-specific, and overall survival ($P \leq 0.001$ and $P \leq 0.005$, respectively) in mismatch repair-proficient patients.

Our study shows that combining immune cell score, CD274 on immune cells, and PDCDI into a single variable termed as immunoprofile more efficiently separates patients with better and worse prognosis than do any of the single immune contexture factors alone. Mismatch repair-deficient tumors with the highest immunoprofile had significantly improved 5-year disease-free, disease-specific, and overall survival (94%, 98%, and 77%, respectively); mismatch repair-deficient tumors with the lowest immunoprofile had a 5-year disease-free survival of 60%, a disease-specific survival of 47%, and an overall survival of 17% ($P < 0.001$). In mismatch repair-proficient colorectal cancer patients, immunoprofile was significantly prognostic for 5-year disease-specific survival (immunoprofile 3 100%, immunoprofile 0 54%, $P = 0.004$) and was suggestively prognostic for disease-free and overall survival ($P = 0.010$ and $P = 0.006$, respectively, Table 5).

Table 3 Clinicopathological and molecular characteristics according to programmed cell death ligand-1 (CD274) and programmed cell death protein 1 (PDCD1) expression

	CD274 on immune cells			CD274 on tumor cells			PDCD1		
	Negative (<i>n</i> = 115)	Positive (<i>n</i> = 79)	<i>P</i> -value	Negative (<i>n</i> = 172)	Positive (<i>n</i> = 22)	<i>P</i> -value	Low (<i>n</i> = 65)	High (<i>n</i> = 125)	<i>P</i> -value
Age									
≤75	55 (48)	42 (53)	0.465	89 (52)	8 (36)	0.174	32 (49)	65 (52)	0.717
>75	60 (52)	37 (47)		83 (48)	14 (64)		33 (51)	60 (48)	
Sex									
Male	36 (31)	26 (33)	0.814	58 (34)	4 (18)	0.141	21 (32)	40 (32)	0.966
Female	79 (69)	53 (67)		114 (66)	18 (82)		44 (68)	85 (68)	
ASA									
1	4 (3)	6 (8)	0.066	10 (6)	0 (0)	0.582	2 (3)	8 (6)	0.603
2	32 (28)	34 (43)		57 (33)	9 (41)		21 (32)	45 (36)	
3	59 (51)	29 (37)		79 (46)	9 (41)		33 (5)	52 (42)	
4	18 (16)	10 (13)		24 (14)	4 (18)		9 (14)	18 (14)	
AJCC/UICC stage									
I	20 (17)	11 (14)	0.010	30 (17)	1 (5)	0.102	6 (9)	24 (19)	0.007
II	44 (38)	49 (62)		78 (45)	15 (68)		26 (40)	65 (52)	
III	44 (38)	16 (20)		56 (33)	4 (18)		26 (40)	33 (26)	
IV	7 (6)	3 (4)		8 (5)	2 (9)		7 (11)	3 (2)	
Perineural invasion									
No	100 (87)	75 (95)	0.066	154 (90)	21 (95)	0.379	55 (85)	116 (93)	0.074
Yes	15 (13)	4 (5)		18 (10)	1 (5)		10 (15)	9 (7)	
Lymphovascular invasion									
No	60 (52)	43 (54)	0.757	91 (53)	12 (55)	0.885	10 (48)	34 (55)	0.325
Yes	55 (48)	36 (46)		81 (47)	10 (45)		34 (52)	56 (45)	
Tumor grade									
1	56 (31)	19 (24)	0.027	54 (31)	1 (5)	<0.001	22 (34)	33 (26)	0.061
2	52 (45)	28 (35)		75 (44)	5 (23)		22 (48)	46 (37)	
3	27 (23)	29 (37)		43 (25)	13 (59)		12 (18)	43 (34)	
4	0 (0)	3 (4)		0 (0)	3 (14)		0 (0)	3 (2)	
Mucinous histology									
0–49%	97 (85)	70 (89)	0.481	148 (86)	19 (86)	0.981	57 (89)	107 (86)	0.506
50–100%	17 (15)	9 (11)		23 (13)	19 (14)		7 (11)	18 (14)	
Tumor budding									
No	64 (57)	62 (78)	0.002	108 (63)	18 (82)	0.089	34 (53)	88 (71)	0.015
Yes	64 (43)	17 (22)		62 (36)	4 (18)		30 (47)	36 (29)	
MMR status									
MMR-proficient	77 (67)	23 (29)	<0.001	99 (58)	1 (5)	<0.001	50 (77)	49 (39)	<0.001
MMR-deficient	38 (33)	56 (71)		73 (42)	21 (95)		15 (23)	76 (61)	
Immune cell score									
0–1	54 (47)	8 (10)	<0.001	62 (36)	0 (0)	<0.001	35 (54)	24 (19)	<0.001
2–3	37 (32)	14 (18)		48 (28)	3 (14)		20 (31)	31 (25)	
4	20 (17)	57 (72)		59 (34)	18 (82)		8 (12)	68 (54)	
CD274 on immune cells									
Negative				114 (66)	1 (5)	<0.001	53 (82)	58 (46)	<0.001

Table 3 (continued)

	CD274 on immune cells			CD274 on tumor cells			PDCD1		
	Negative (n = 115)	Positive (n = 79)	P-value	Negative (n = 172)	Positive (n = 22)	P-value	Low (n = 65)	High (n = 125)	P-value
CD274 on immune cells									
Positive				58 (34)	21 (95)		12 (18)	67 (54)	
CD274 on tumor cells									
Negative	114 (99)	58 (73)	< 0.001				64 (98)	104 (83)	0.002
Positive	1 (1)	21 (27)					1 (2)	21 (17)	
PDCD1									
Low	53 (46)	12 (15)	< 0.001	64 (37)	1 (5)	0.002			
High	58 (50)	67 (85)		104 (60)	21 (95)				
Tumor location									
Proximal colon	65 (57)	60 (76)	0.001	105 (61)	20 (91)	0.020	36 (55)	86 (69)	0.048
Distal colon	32 (28)	18 (23)		48 (28)	2 (9)		18 (28)	31 (25)	
Rectum	18 (16)	1 (1)		19 (11)	0 (0)		11 (17)	31 (6)	

Values are no. (%) unless otherwise stated

ASA The American Society of Anesthesiologists, AJCC The American Joint Committee on Cancer, UICC Union for International Cancer Control, MMR mismatch repair, PDCD1 programmed cell death protein 1, CD274 programmed cell death ligand-1

Multivariable analysis with the Cox proportional hazards model on separate immune parameters revealed that PDCD1^{high} was an independently significant prognostic factor for disease-specific survival ($P = 0.004$) and was suggestive for disease-free and overall survival ($P = 0.029$ and 0.007 , respectively, Table 6a). Additional independent prognostic factors were age for overall survival and AJCC/UICC stage for disease-specific and overall survival ($P < 0.001$). In the multivariable model where the immune parameters immune cell score, PDCD1, and CD274 on immune cells were combined, the immunoprofile was an independent prognostic factor for disease-specific and overall survival ($P = 0.001$) and was suggestive also for disease-free survival ($P = 0.011$, Table 6b). With an immunoprofile score of 3 used as the reference, the hazard ratio (HR) for an immunoprofile score of 0 for disease-free survival was 13.01 (95% confidence interval (CI), 2.31–73.32, $P = 0.004$), for disease-specific survival it was 39.13 (95% CI, 4.49–340.72, $P = 0.001$) and for overall survival it was 3.82 (95% CI, 1.90–7.69, $P < 0.001$).

Discussion

This study represents an in-depth analysis of PDCD1 and CD274 expression and immune cell infiltration together with the prognostic value of these factors in a series of mismatch repair-proficient, mismatch repair-deficient, and Lynch syndrome-associated colorectal cancers. Our results showed that immune cell infiltration and CD274/PDCD1

positivity are significantly more frequent in mismatch repair-deficient than in mismatch repair-proficient colorectal cancer, and that these factors are strongly associated with each other. In addition to tumor cells, CD274 expression was induced in inflammatory cells. CD274 positivity, either on tumor cells or on immune cells was significantly associated with high levels of cytotoxic lymphocytes in the tumor microenvironment. However, only patients with CD274 on immune cells had suggestively favorable 5-year disease-specific and overall survival despite mismatch repair status. This suggests that although considered inhibitory, PDL1 expression in immune cells is an indicator of enhanced immune activation and acts as a balancing factor of inflammatory reactions as it does during normal tolerance formation. Furthermore, the immunoprofile parameter, derived as a combination of immune cell score, PDCD1, and CD274 on immune cells, was a strong independent prognostic factor for disease-specific and overall survival, and was suggestive for disease-free survival.

Clinicopathological features analyzed in our study correspond well with those from previous studies. CD274 positivity, as well as PDCD1 density tend to associate with proximal tumor location and poor differentiation [36, 39], and these features are commonly seen in mismatch repair-deficient colorectal adenocarcinomas. In turn, tumors with high budding are more likely mismatch repair-proficient tumors [46] and show a low frequency of CD274 and PDCD1 positivity [36, 39].

Several previous studies have provided data on the expression patterns and prognostic relevance of

Table 4 Association of immunoprofile to other clinicopathological variables in mismatch repair-proficient tumors

	Immunoprofile 0		Immunoprofile 1		Immunoprofile 2		Immunoprofile 3		<i>P</i> -value
	<i>N</i>	%	<i>N</i>	%	<i>N</i>	%	<i>N</i>	%	
Age									
≤75	12	36	23	66	9	50	9	64	0.081
>75	21	64	12	34	9	50	5	36	
Sex									
Male	9	27	13	37	6	33	8	57	0.274
Female	24	73	22	63	12	67	6	43	
ASA class									
1	1	3	1	3	1	6	2	14	0.355
2	7	21	14	40	9	50	6	43	
3	19	58	16	46	7	39	4	29	
4	6	18	4	11	1	6	2	14	
Tumor location									
Proximal colon	16	49	14	40	9	50	11	79	0.283
Distal colon	10	30	15	43	6	33	3	21	
Rectum	7	21	6	17	3	17	0	0	
AJCC/UICC stage									
I	3	9	3	9	6	33	14	14	0.253
II	15	46	15	43	7	39	64	46	
III	13	39	16	46	4	22	21	36	
IV	2	6	1	3	1	6	0	0	
Perineural invasion									
No	28	85	28	80	18	100	14	100	0.079
Yes	5	15	7	20	0	0	0	0	
Lymphovascular invasion									
No	18	55	17	49	7	39	9	64	0.514
Yes	15	45	18	51	11	61	5	36	
Tumor budding									
No	12	36	19	56	11	61	12	86	0.017
Yes	21	64	15	44	7	39	2	14	
Tumor grade									
1	13	39	12	34	5	28	6	43	0.200
2	14	42	17	48	13	72	4	29	
3	6	18	6	17	0	0	4	29	
4	0	0	0	0	0	0	0	0	
Mucinous tumor									
0–49%	31	94	30	88	18	100	14	100	0.261
50–100%	2	6	44	12	0	0	0	0	

ASA The American Society of Anesthesiologists, AJCC The American Joint Committee on Cancer, UICC Union for International Cancer Control

CD274/PDCD1 in colorectal cancer [34–39], but the results have been variable and discordant. As the CD274/PDCD1 axis is involved in dynamic immune reactions, heterogeneity of immunohistochemical staining of CD274 within and between tumors is presumable, as has been shown for instance in non-small cell lung cancer, melanoma [47, 48], and recently also in colorectal cancer [35]. This can explain the variability of the results seen in studies using tissue

microarrays or small biopsies for immunohistochemical analysis. To overcome this issue, we analyzed the whole section of a representative tumor block in each case. In addition, discrepant results between different studies are probably explained by differences in the CD274 antibodies used. Our decision to use clone E1L3N was based on careful validation of antibodies available. Since then, validation of commonly used antibodies has increased

Table 5 Survival according to clinicopathological variables in mismatch repair-proficient and mismatch repair-deficient tumors

MMR status	Disease-free survival						Disease-specific survival						Overall survival					
	N		5-year survival		P-value		N		5-year survival		P-value		N		5-year survival		P-value	
	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D
Age																		
≤75	49	40	81%	92%	0.100	0.669	53	44	92%	86%	<0.001	0.628	53	44	89%	73%	<0.001	0.012
>75	39	43	57%	88%			47	50	56%	81%			47	50	43%	54%		
Sex																		
Male	31	23	84%	100%	0.074	0.107	36	26	82%	92%	0.246	0.306	36	26	75%	54%	0.352	0.148
Female	57	60	64%	87%			64	68	72%	81%			64	68	63%	66%		
ASA class																		
1	5	4	100%	100%	0.050	0.602	5	5	100%	80%	0.002	0.877	5	5	100%	80%	<0.001	0.399
2	34	26	77%	86%			36	30	89%	82%			36	30	86%	67%		
3	40	38	69%	89%			46	42	72%	82%			46	42	63%	62%		
4	9	13	33%	100%			13	15	33%	92%			13	15	15%	53%		
Tumor location																		
Proximal colon	43	68	74%	94%	0.332	0.095	50	75	71%	86%	0.438	0.294	50	75	60%	63%	0.357	0.840
Distal colon	30	12	77%	80%			34	16	81%	73%			34	16	71%	63%		
Rectum	15	3	53%	67%			16	3	81%	67%			16	3	81%	67%		
AJCC/UICC stage																		
I	13	16	100%	93%	0.050	0.130	14	17	100%	100%	<0.001	<0.001	14	17	93%	53%	<0.001	<0.001
II	42	44	68%	95%			46	47	79%	95%			46	47	72%	75%		
III	33	23	64%	82%			36	24	69%	73%			36	24	58%	63%		
IV	0	0	0%	0%			4	6	0%	0%			4	6	0%	0%		
Perineural invasion																		
No	80	77	76%	91%	<0.001	0.518	88	87	79%	85%	0.001	0.472	88	87	72%	62%	<0.001	0.719
Yes	8	6	25%	83%			12	7	44%	71%			12	7	33%	71%		
Lymphovascular invasion																		
No	46	47	75%	93%	0.222	0.377	51	52	83%	87%	0.053	0.208	51	52	71%	69%	0.603	0.022
Yes	42	26	66%	86%			49	42	69%	79%			49	42	63%	55%		
Tumor grade																		
1	35	18	67%	100%	0.903	0.465	36	19	76%	100%	0.988	0.075	36	19	69%	63%	0.578	0.308
2	41	30	73%	86%			48	32	73%	86%			48	32	67%	69%		
3	12	33	75%	88%			16	40	85%	73%			16	40	63%	60%		
4	0	2	100%	100%			0	3	100%	100%			0	3	100%	33%		
Mucinous histology																		
0–49 %	82	66	71%	90%	0.765	0.746	93	75	76%	85%	0.839	0.414	93	75	68%	67%	0.783	0.345
50–100%	5	17	80%	92%			6	19	83%	74%			6	19	67%	47%		
Tumor budding																		
No	49	63	83%	93%	0.005	0.208	54	72	88%	83%	0.001	0.939	54	72	80%	64%	0.001	0.703
Yes	38	19	57%	80%			45	21	62%	84%			45	21	53%	57%		
Immune cell score																		
0–1	39	12	58%	62%	0.010	0.001	46	16	66%	48%	0.054	<0.001	46	16	57%	25%	0.053	<0.001
2–3	26	20	68%	95%			30	21	78%	89%			30	21	67%	67%		
4	23	49	96%	95%			24	53	92%	94%			24	53	88%	74%		
CD274 on tumor cells																		
No	87	66	71%	90%	0.556	0.698	99	73	76%	83%	0.568	0.896	99	73	67%	62%	0.524	0.924
Yes	1	17	100%	93%			1	21	100%	82%			1	21	100%	67%		

Table 5 (continued)

MMR status	Disease-free survival						Disease-specific survival						Overall survival					
	N		5-year survival		P-value		N		5-year survival		P-value		N		5-year survival		P-value	
	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D
CD274 on immune cells																		
No	65	33	66%	84%	0.063	0.063	77	38	70%	74%	0.006	0.048	77	38	60%	50%	0.011	0.029
Yes	23	50	86%	95%			23	56	96%	90%			23	56	91%	71%		
PDCD1-positive cells																		
Low	42	10	54%	70%	0.002	0.002	50	15	63%	44%	0.004	<0.001	50	15	50%	20%	0.008	<0.001
High	46	70	87%	95%			49	76	89%	92%			49	76	86%	72%		
Immunoprofile																		
0	25	10	50%	60%	0.010	<0.001	32	12	54%	47%	0.004	<0.001	32	12	39%	17%	0.006	0.001
1	33	10	67%	89%			35	14	79%	66%			35	14	74%	50%		
2	16	17	88%	100%			18	20	83%	84%			18	20	83%	65%		
3	14	46	100%	94%			14	48	100%	98%			14	48	93%	77%		

MMR mismatch repair, P mismatch repair-proficient, D mismatch repair-deficient, ASA The American Society of Anesthesiologists, AJCC The American Joint Committee on Cancer, UICC Union for International Cancer Control, CD274 programmed cell death ligand-1, PDCD1 programmed cell death protein 1

enormously; E1L3N is regarded as a reliable antibody and the results obtained with this antibody have been considered comparable to those obtained using the Food and Drug Administration-approved drug-specific assays [49, 50]. Further discrepancy arises from the definition of the threshold value for positive CD274 labeling. There is no consensus of what is the appropriate level of CD274 expression to separate a positive sample from a negative one. Among reported studies, CD274 positivity cut-off values vary from 1 to 50% [51] and companion diagnostic assays include different cut-offs for defining positivity [52]. In our study, a tumor sample was defined as CD274 positive when >5% of either tumor cells or immune cells was positive for CD274 with a moderate or strong intensity. By using the same cut-off, we obtained comparable results with previous studies on colorectal cancer using the same antibody clone (E1L3N) [34–36, 39].

In agreement with our study, Lee et al. [35] showed that CD274 expression in immune cells was associated with improved prognosis in both microsatellite-unstable and microsatellite-stable colorectal cancer, but was independent of high density of tumor-infiltrating lymphocytes. In contrast, our study showed that the prognostic impact of CD274 expression on immune cells is strongly enhanced when combined with a high immune cell score and PDCD1 positivity, which further indicates that an active immune microenvironment is counterbalanced by immune inhibition. In turn, CD274 on tumor cells did not show any prognostic impact on survival outcome in accordance with Lee's study [35]; however, this was in contrast to other studies, which showed an association of CD274 on tumor

cells with worse survival [34, 36, 39]. However, all our CD274 tumor-positive patients, except for one, were simultaneously expressing CD274 on immune cells. The observed intense overlapping may have resulted from extensive analyses of whole tissue sections. The common intratumoral heterogeneity of CD274 expression in tumor and immune cells in colorectal cancer may explain the discrepancy between results from different studies.

According to the cancer immunoediting theory [53], immune evasion includes three phases, as follows: elimination, equilibrium, and escape. The expression of CD274 on tumor cells is regarded as a prominent mechanism devised to escape antitumor response. Thus, logically, CD274 tumor positivity could then mark worse prognosis. Potentially, the lack of impact on survival in our study may be due to the limited number of tumor-positive CD274 samples, although the number of CD274-positive tumor samples was also small in other studies [34, 35, 39]. This suggests that the PD1/PDL1 checkpoint mechanism is not the major escape mechanism for tumor cells in colorectal cancer, which could also explain the limited impact of anti-PDL1/PD1 treatment [6, 8]. There is evidence suggesting that local overproduction of prostaglandins such as PG2, promotes the presence of immunosuppressive and regulatory immune cells, allowing host immune evasion of tumor cells [54]. Reduction of prostaglandin production by inhibiting cyclooxygenase enzymes (COX-1 and COX-2) with aspirin therapy may reverse these effects and enhance T-cell-mediated immune response against tumor cells, although tumors with a high level of CD274 expression seem to develop aspirin resistance. Prostaglandin inhibition

Table 6a Multivariable analysis using Cox proportional hazards model with various immune parameters

	Univariable analysis	Disease-free survival (<i>n</i> = 161)		Disease-specific survival (<i>n</i> = 147)		Overall survival (<i>n</i> = 184)	
	<i>P</i>	HR (95% CI)	<i>P</i>	HR (95% CI)	<i>P</i>	HR (95% CI)	<i>P</i>
Age	DFS: 0.041						
<75	DSS: 0.002	1	0.118	1	0.015	1	<0.001
>75	OS: <0.001	1.94 (0.84–4.47)		3.25 (1.25–8.45)		3.49 (2.06–5.90)	
Sex	DFS: 0.052						
Male	DSS: 0.198	0.37 (0.13–1.03)	0.056	0.48 (0.18–1.28)	0.143	1.72 (1.03–2.88)	0.038
Female	OS: 0.850	1		1		1	
ASA class							
1	DFS: 0.864	Excluded	1	0.872	1	0.031	
2	DSS: 0.160		0.66 (0.08–5.76)		2.73 (0.35–20.99)		
3	OS: <0.001		0.91 (0.11–7.73)		4.16 (0.55–31.38)		
4			0.66 (0.07–6.44)		7.03 (0.90–54.87)		
Tumor location							
Proximal	DFS: 0.021	1	0.607	Excluded			
Distal	DSS: 0.778	1.39 (0.48–4.01)					
Rectum	OS: 0.357	1.70 (0.59–4.87)					
AJCC/UICC stage:							
I	DFS: 0.028	1	0.228	Excluded	<0.001	1	<0.001
II	DSS: <0.001	4.28 (0.50–36.38)		1		0.66 (0.34–1.29)	
III	OS: <0.001	6.31 (0.70–56.47)		1.41 (0.62–3.19)		0.77 (0.38–1.56)	
IV		No cases		31.91 (10.23–99.57)		4.70 (1.76–12.50)	
Perineural invasion	DFS: 0.002						
No	DSS: 0.007	1	0.050	1	0.084	1	0.058
Yes	OS: 0.004	2.88 (1.00–8.29)		2.59 (0.88–7.60)		2.00 (0.98–4.08)	
Lymphovascular invasion	DFS: 0.117		0.327		0.075		0.927
No	DSS: 0.017	1		1		1	
Yes	OS: 0.057	1.38 (0.59–4.87)		2.21 (0.92–5.31)		0.98 (0.60–1.58)	
Tumor budding	DFS: 0.001						
No	DSS: 0.005	1	0.588	1	0.762	1	0.155
Yes	OS: 0.007	1.30 (0.50–3.36)		0.86 (0.34–2.22)		1.43 (0.87–2.35)	
MMR status	DFS: 0.005						
MMR-proficient	DSS: 0.127	1.03 (0.31–3.43)	0.959	0.70 (0.26–1.91)	0.485	0.49 (0.28–0.86)	0.013
MMR-deficient	OS: 0.638	1		1		1	
Immune cell score							
0–1	DFS: <0.001	5.76 (1.30–25.44)		3.74 (1.13–12.38)		1.93 (1.00–3.73)	
2–3	DSS: <0.001	3.59 (0.78–16.51)		2.91 (0.83–10.18)		1.66 (0.90–3.05)	
4	OS: <0.001	1	0.062	1	0.097	1	0.132
CD274 on immune cells	DFS: 0.002						
Low	DSS: 0.001	1.20 (0.35–4.04)	0.77	2.14 (0.64–7.20)	0.218	1.18 (0.63–2.23)	0.602
High	OS: 0.004	1	3	1		1	
PDCD1	DFS: <0.001						
Low	DSS: <0.001	2.83 (1.11–7.24)	0.029	3.65 (1.50–8.87)	0.004	2.05 (1.21–3.47)	0.007
High	OS: 0.001	1		1		1	

Univariate analyses were performed with the following reference categories: 75 years or under, female sex, ASA class 1, proximal tumor location, AJCC/UICC stage I (or in DSS stage II), no perineural invasion, no lymphovascular invasion, no tumor budding, mismatch repair deficiency, immune cell score 4, CD274 on immune cells and high PDCD1. Only variables with a *P*-value of <0.20 in univariable analysis were included in the multivariable analysis together with age and sex. Mismatch repair status was included also in overall survival analysis because of special interest in its impact. There were 161 cases available for the analysis of disease-free survival, 147 for disease-specific survival, and 184 for overall survival. Twenty-three patients were excluded from disease-free survival analysis due to either existing metastasis or non-radical surgery, and 3 patients were censored before the earliest event in a stratum. Patients with AJCC/UICC stage I disease (*n* = 31) were discarded from disease-specific survival analysis because there was no disease-specific mortality and nine patients were censored before the earliest event in a stratum. Data pertaining to ASA class were missing for two, tumor budding was missing for 2, PDCD1 were missing for three, and immune cell score were missing for four patients

HR hazard ratio, *CI* confidence interval, *ASA* The American Society of Anesthesiologists, *AJCC* The American Joint Committee on Cancer, *UICC*, Union for International Cancer Control, *PDCD1* programmed cell death protein 1, *MMR* mismatch repair, *DFS* disease-free survival; *DSS* disease-specific survival, *OS* overall survival

Table 6b Multivariable analysis using the Cox proportional hazards model with the parameter immunoprofile (a parameter combining immune cell score, programmed cell death protein 1 (PDCD1), and programmed cell death ligand-1 (CD274) on immune cells)

	Univariable analysis	Disease-free survival (<i>n</i> = 161)		Disease-specific survival (<i>n</i> = 147)		Overall survival (<i>n</i> = 184)	
	<i>P</i>	HR (95% CI)	<i>P</i>	HR (95% CI)	<i>P</i>	HR (95% CI)	<i>P</i>
Age	DFS: 0.041						
<75	DSS: 0.002	1	0.176	1	0.016	1	<0.001
>75	OS: <0.001	1.79 (0.77–4.18)		3.37 (1.26–9.01)		3.29 (1.95–5.55)	
Sex	DFS: 0.052						
Male	DSS: 0.198	0.39 (0.14–1.08)	0.071	0.58 (0.22–1.53)	0.271	1.83 (1.10–3.06)	0.021
Female	OS: 0.850	1		1		1	
ASA class							
1	DFS: 0.864	Excluded		1	0.960	1	0.021
2	DSS: 0.160			0.98 (0.12–8.36)		4.45 (0.59–33.38)	
3	OS: <0.001			1.24 (0.15–10.32)		3.47 (0.46–26.20)	
4				1.02 (0.11–9.64)		8.66 (1.11–67.33)	
Tumor location							
Proximal	DFS: 0.021	1	0.511	Excluded			
Distal	DSS: 0.778	1.40 (0.52–3.81)					
Rectum	OS: 0.357	1.84 (0.65–5.21)					
AJCC/UICC stage							
I	DFS: 0.028	1	0.135	Excluded	<0.001	1	<0.001
II	DSS: <0.001	6.21 (0.76–50.68)		1		0.71 (0.37–1.36)	
III	OS: <0.001	8.73 (1.02–74.77)		1.43 (0.63–3.22)		0.87 (0.44–1.73)	
IV		No cases		30.08 (9.31–97.17)		6.41 (2.41–17.00)	
Perineural invasion	DFS: 0.002						
No	DSS: 0.007	1	0.048	1	0.079	1	0.097
Yes	OS: 0.004	2.95 (1.01–8.61)		2.68 (0.89–8.02)		1.85 (0.89–3.82)	
Lymphovascular invasion	DFS: 0.117						
No	DSS: 0.017	1	0.415	1	0.080	1	0.847
Yes	OS: 0.057	1.42 (0.61–3.31)		2.18 (0.91–5.20)		0.95 (0.59–1.55)	
Tumor budding	DFS: 0.001						
No	DSS: 0.005	1	0.509	1	0.795	1	0.338
Yes	OS: 0.007	1.36 (0.54–3.43)		0.89 (0.35–2.23)		1.27 (0.78–2.09)	
MMR status	DFS: 0.005						
MMR-proficient	DSS: 0.127	1.08 (0.35–3.36)	0.890	0.79 (0.31–2.00)	0.622	0.53 (0.31–0.92)	0.024
MMR-deficient	OS: 0.638	1		1		1	
Immunoprofile							
0	DFS: <0.001	13.01 (2.31–73.32)	0.011	39.13 (4.49–340.72)	0.001	3.82 (1.90–7.69)	0.001
1	DSS: <0.001	7.71 (1.44–41.37)		22.89 (2.73–191.79)		2.18 (1.11–4.28)	
2	OS: 0.001	1.96 (0.26–14.92)		6.77 (0.74–62.00)		1.00 (0.49–2.03)	
3		1		1		1	

Univariate analyses were performed with the following reference categories: 75 years or under, female sex, ASA class 1, proximal tumor location, AJCC/UICC stage I (or in DSS stage II), no perineural invasion, no lymphovascular invasion, no tumor budding, mismatch repair deficiency and immunoprofile class 3. Only variables with a *P*-value of <0.20 in univariable analysis were included in the multivariable analysis together with age and sex. Mismatch repair status was included also in overall survival analysis because of special interest in its impact. There were 161 cases available for analysis of disease-free survival, 147 for disease-specific survival, and 184 for overall survival. Twenty-three patients were excluded from disease-free survival analysis due to either existing metastasis or non-radical surgery. Patients with AJCC/UICC stage I disease (*n* = 31) were discarded from disease-specific survival analysis because there was no disease-specific mortality and nine patients were censored before the earliest event in a stratum. Data pertaining to ASA class were missing for two, tumor budding were missing for two, and immunoprofile were missing for seven patients

HR hazard ratio, CI confidence interval, ASA The American Society of Anesthesiologists, AJCC The American Joint Committee on Cancer, UICC Union for International Cancer Control, MMR mismatch repair, DFS disease-free survival, DSS disease-specific survival, OS overall survival

combined with immune checkpoint blockade therapy is therefore suggested as a possible means to improve clinical patient outcome [55].

Remarkably, CD274 on tumor cells was less common in Lynch syndrome patients compared with sporadic mismatch

repair-deficient patients, as reported previously [39, 56]. Lynch syndrome colorectal cancer patients have exceptionally good prognosis even though they have an increased risk for metachronous tumors in various tissues [57, 58]. We did not see any difference in immune cell infiltration

between sporadic mismatch repair-deficient and Lynch syndrome patients, except for a lower density of PDCD1-positive lymphocytes in Lynch syndrome tumors. Whether the rarity of CD274 on tumor cells also impacts on the enhanced survival of Lynch syndrome colorectal cancer patients, remains to be studied.

Recent evidence has shown an association between sensitivity to immune checkpoint blockage and mismatch repair deficiency in colorectal cancer [6, 8]. However, not all microsatellite-unstable colorectal cancers respond. It is currently unknown whether there is a difference in response to CD274/PDCD1 immunotherapy between CD274 expression on tumor versus immune cells in colorectal cancer. Unfortunately, our study does not enable us to correlate the staining patterns with the response to checkpoint blockade. However, several studies on murine models have shown that the expression of CD274 in host myeloid cells is highly essential for the response to checkpoint blockade, as recently reviewed by Tang and Zheng [59]. Interestingly, Zhang et al. showed that blocking both tumor- and non-tumor-derived CD274 achieves synergy and maximizes antitumor T-cell response in mouse models [60]. Whether this applies to human remains to be clarified, as the number of treated colorectal cancer patients increases. In our study, one mismatch repair-proficient colorectal cancer case was highly divergent from the rest of the mismatch repair-proficient group and showed strong CD274 expression both in tumor and immune cells. It has been suggested that microsatellite-stable patients with increased CD274 expression and high immune cell score could also benefit from immune checkpoint blockage [61]. Recently, the first case of a microsatellite-stable colorectal cancer patient harboring amplification of both the CD274 and PD-L2 genes was reported; this patient showed a dramatic response to a checkpoint inhibitor [62]. Whether the exceptional CD274 expression in tumor cells of mismatch repair-proficient patients seen in our study reflects amplification of the above-mentioned genes, is not known. However, these observations strongly support the importance of CD274 expression in both cell types. Large cohort of patients treated with checkpoint inhibitors need to be evaluated to confirm the predictive value of CD274/PDCD1 expression on treatment response.

Overall, our study provided data indicating that although the analyzed immune microenvironment factors in colorectal cancer were more common in mismatch repair-deficient patients, the same features in mismatch repair-proficient patients were also associated with better survival. In addition to the well-known prognostic value of tumor-infiltrating T cells in colorectal cancer [27–31, 33], the variable immunoprofile, derived by combining immune cell score, PDCD1, and CD274, was a strong independent prognostic factor for survival. Although traditional tumor

classification and risk factors like AJCC/UICC stage, tumor invasion, and patient comorbidities are recognized for their prognostic value, our results support the use of the immune cell score analysis together with PDCD1 and CD274 detection as an important prognostic variable in colorectal cancer. We also encourage the analysis of whole tissue sections for a reliable and cell-specific analysis of CD274 expression.

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Compliance with ethical standards

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

Ethical aspects The study was approved by the ethical committee of the central Finland Central Hospital and the National Supervisory Authority for Welfare and Health (Valvira).

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