SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 17: 2335-2343, 2019

Tumor microenvironment classification based on T-cell infiltration and PD-L1 in patients with mismatch repair-proficient and -deficient colorectal cancer

SHOUSHENG LIU[1,2*], PENGFEI KONG[1,2*], XIAOPAI WANG[3*], LIN YANG[1,2], CHANG JIANG[1,2], WENZHUO HE[1,2], QI QUAN[1,2], JINSHENG HUANG[1,2], QIANKUN XIE[1,2], XIAOJUN XIA[1], BEI ZHANG[1,2] and LIANGPING XIA[1,2]

[1]State Key Laboratory of Oncology in South China, Collaborative Innovation Centre for Cancer Medicine; [2]Department of The VIP Region, Sun Yat-Sen University Cancer Center, Guangzhou, Guangdong 510060; [3]Department of Pathology, Guangzhou First People's Hospital, Guangzhou Medical University, Guangzhou, Guangdong 510080, P.R. China

Received February 17, 2018; Accepted November 21, 2018

DOI: 10.3892/ol.2018.9826

Abstract. The classification of tumor microenvironments according to the presence or absence of tumor infiltrating lymphocytes (TILs) and programmed death ligand-1 (PD-L1) expression has been used to predict the efficacy of immune checkpoint inhibitor antibodies in several cancer types, not including colorectal cancer (CRC). The current study investigated the TIL/PD-L1 status of patients with CRC, particularly patients who presented as mismatch repair-proficient (pMMR) and mismatch repair-deficient (dMMR). A total of 243 patients with CRC were enrolled and defined as pMMR (121 patients) or dMMR (122 patients). Using Pearson's χ^2 test and multivariable multinomial logistic regression analysis, the associations between MMR status, TIL presence and PD-L1 expression were investigated, in addition to the association between TIL/PD-L1 status and clinicopathological features. The results demonstrated that the dMMR group more frequently exhibited TIL$^+$ (85/122 vs. 61/121) and PD-L1$^+$ (49/122 vs. 32/121) phenotypes compared with the pMMR group. PD-L1$^+$ expression was identified in 42.4% of TIL$^+$ cases in the dMMR group, while only 18.0% of TIL$^+$ cases were PD-L1$^+$ in the pMMR group. High programmed death-1 expression and dMMR status were revealed as two independent risk factors for TIL$^+$ PD-L1$^+$ status. In conclusion, compared with the pMMR group, the dMMR group was more likely to present with a TIL$^+$ PD-L1$^+$ status, which suggests that a TIL$^+$ PD-L1$^+$ tumor microenvironment may partly contribute to the improved response of dMMR patients to anti-PD-1/L1 therapy.

Correspondence to: Dr Liangping Xia or Dr Bei Zhang, State Key Laboratory of Oncology in South China, Collaborative Innovation Centre for Cancer Medicine, Sun Yat-Sen University Cancer Center, 651 Dongfeng East Road, Guangzhou, Guangdong 510060, P.R. China
E-mail: xialp@sysucc.org.cn
E-mail: zhangbei@sysucc.org.cn

*Contributed equally

Key words: colorectal cancer, mismatch repair-proficient, mismatch repair-deficient, tumor infiltrating lymphocytes, programmed death ligand-1

Introduction

Cancer immunotherapy has gained interest due to the clinical efficacy of immune checkpoint inhibitors, including antibodies targeting programmed death-1 (PD-1), programmed death ligand-1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), in a number of cancer types, including melanoma, colorectal cancer (CRC), renal cell carcinoma and non-small cell lung carcinoma (1-4). Identification of factors that could predict the curative effect of anti-PD-1/L1 therapy is a major challenge. Mismatch repair protein (MMR) status has been reported to be a primary indicator for anti-PD-1/L1 therapy in patients with CRC, as only MMR-deficient (dMMR) patients are promising candidates (2). However, to the best of our knowledge, it is unknown why only dMMR patients are sensitive to anti-PD-1/L1 therapy. In addition, only certain dMMR patients benefit from the treatment (2), which suggests that other predictive factors should also be explored.

The tumor microenvironment is a complicated system that has the dual function of promoting and inhibiting tumor growth, which therefore influences drug response. The tumor microenvironment is composed of non-tumor cells, cytokines, chemokines and the extracellular matrix. Immune cells, particularly acquired immune cells, including T and B lymphocytes, are important non-tumor cells (5). The current study primarily focused on tumor infiltrating lymphocytes (TILs), crucial acquired immune cells, and PD-L1 expressed by tumor cells, which negatively affects the function of TILs. In human melanoma, the classification of tumor microenvironments based on TIL presence and PD-L1 expression has been proposed to

predict and guide immunotherapeutic approaches (6). Among the four types of tumor microenvironments (TIL^+ $PD\text{-}L1^+$, TIL^+ $PD\text{-}L1^-$, TIL^- $PD\text{-}L1^+$ and TIL^- $PD\text{-}L1^-$), TIL^+ $PD\text{-}L1^+$ tumors exhibit the best response to checkpoint blockade and are the most inclined to benefit from single anti-PD-1/L1 agent therapy, as pre-existing intratumor T cells in these tumors are turned off by PD-L1 and reactivated by the agent (7). TIL^- $PD\text{-}L1^+$ and TIL^- $PD\text{-}L1^-$ tumors appear insensitive to single checkpoint blockade therapy due to a lack of pre-existing lymphocyte infiltrates, instead combination therapy is required to attract T cells into tumors (8-10).

TIL^+ $PD\text{-}L1^-$ tumors contain TILs, but no PD-L1 expression; therefore, without evident adaptive resistance, TIL^+ $PD\text{-}L1^-$ tumors may not be suitable for anti-PD-1/L1 therapy (11).

To the best of our knowledge, the typical TIL/PD-L1 status of patients with CRC has not previously been investigated. The current study enrolled 243 patients with CRC who were defined as pMMR or dMMR. The associations between MMR status, TIL expression and PD-L1 expression were investigated. In addition, the associations between TIL/PD-L1 status and clinicopathological features, particularly MMR status, were investigated to evaluate whether TIL/PD-L1 status could provide an explanation for the improved response of dMMR patients to anti-PD-1/L1 therapy and determine whether this classification method is suitable for efficacy prediction.

Patients and methods

Patients and specimens. Pathological specimens of CRC tissue were collected from 243 patients with CRC (121 pMMR patients and 122 dMMR patients) of stage I to IV who underwent primary surgery between March 2009 and December 2016 at Sun Yat-Sen University Cancer Center (Guangzhou, China). The obtained specimens were fixed in 10% formalin for 24 h at room temperature and embedded in paraffin for further use. Written informed consent was obtained from every patient. The study was performed according to the principles of the Declaration of Helsinki and was approved by the Research Ethics Committee of Sun Yat-Sen University (Gaungzhou, China).

Estimation of TILs. T-lymphocyte density within the cancer cell nests and at the invasive margin, which denoted the interface between the invading edge area of the tumor and the host stroma, was identified and estimated using a hematoxylin and eosin (H&E) staining method. Briefly, tumor samples were paraffin-embedded and sliced into 4-μm sections. Slides were deparaffinized in xylene for 10 min three times and rehydrated in a descending alcohol series (100, 95, 85 and 75%) for 5 min each at 25˚C. Subsequently, the samples were stained with H&E (cat no. 468802128; POCH S.A., Gliwice, Poland) at room temperature for 20 min. The sections were then observed with a light microscope (magnification, x200). The intensity of the lymphocyte infiltrate was scored as follows: Score 0, none; score 1, weak, rare lymphocytes; score 2, moderate, focal infiltration; and score 3, severe, diffuse infiltration (12). Populations with scores of 0 and 1 were defined as TIL^-, and those with scores of 2 and 3 were defined as TIL^+.

Immunohistochemistry and scoring. The immunohistochemistry experiment was conducted as previously described (13). To detect MMR proteins, mouse monoclonal antibodies against MutL homolog 1 (MLH1) (cat. no. ES05; 1:500), MutS homolog 2 (MSH2) (cat. no. FE11; 1:500), MutS homolog 6 (MSH6; cat. no. EP49; 1:500) and PMS1 homolog 2 (PMS2; cat. no. EP51; 1:500) (all from Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) were used. Rabbit monoclonal antibodies against PD-L1 (cat. no. 13684; 1:200; Cell Signaling Technology, Inc., Danvers, MA, USA) and PD-1 (cat. no. ab137132; 1:500; Abcam, Cambridge, UK) were also used to evaluate the expression of PD-L1 and PD-1. Samples embedded in paraffin were cut into 4-μm sections, deparaffinized in xylene, rehydrated through graded ethanol and dipped in 0.3% hydrogen peroxide for >15 min to inactivate the endogenous peroxidase. The slides were then processed for antigen retrieval with high pressure cooking at 120˚C for ~10 min in citrate antigen retrieval solution, followed by incubation with primary antibody at 4˚C overnight. Following three washes with PBS, the slides were subsequently co-incubated with goat anti-rabbit or anti-mouse biotin-linked secondary antibodies for 30 min at 37˚C (cat. no. SP-9000; 1:2,000; OriGene Technologies, Inc., Rockville MD, USA), counterstained with hematoxylin for 15 min at room temperature for color reaction and ultimately fixed in mounting media. The sections were observed with a light microscope (magnification, x200). Staining without primary antibody served as a negative control. Each slide was examined by three pathologists.

Tumors expressing all four proteins, MLH1, MSH2, PMS2 and MSH6, were defined as pMMR and all other tumors were considered to be dMMR. The expression of PD-L1 in tumor cells was divided into two groups: <5% positive cells was considered as negative and ≥5% was considered as positive (3,14). PD-1 expression in TILs was scored as follows: Score 0, none (0% of lymphocytes); score 1, isolated (<5% of lymphocytes); score 2, moderate (5-50% of lymphocytes; and 3, severe (>50% of lymphocytes. Scores of 0 and 1 were considered as low expression and score of 2 and3 were considered as high expression (7,15).

Statistical analysis of the data. All statistical analyses were performed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). Pearson's χ^2 test was used to assess the association between categorical variables. Multivariable multinomial logistic regression analysis was used for multivariate analysis to predict the odds ratio (OR) of individual factors for TIL^+ $PD\text{-}L1^+$ status. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. The clinicopathological characteristics of all 243 patients are presented in Table I. Patients were selected according to pMMR (121 patients) and dMMR (122 patients) status, including 189 patients with colon cancer (left, 90 patients; right, 99 patients) and 54 patients with rectum cancer. Table II presents the frequency of aberrant MMR protein expression in dMMR patients. The median age at the time of diagnosis was 60 years (range, 22-84 years),

Table I. Association analysis between MMR status and clinicopathological features using a χ^2 test.

Characteristic	pMMR, n (%)	dMMR, n (%)	C	P-value
Age at diagnosis, years			0.190	0.003
<60	54 (44.6)	78 (63.9)		
≥60	67 (55.4)	44 (36.1)		
Sex				0.22
Male	66 (54.5)	76 (62.3)		
Female	55 (45.5)	46 (37.7)		
Primary tumor site			0.18	0.017
Left colon	55 (45.5)	35 (28.7)		
Right colon	40 (33.1)	59 (48.4)		
Rectum	26 (21.5)	28 (23.0)		
Stage[a]				0.514
I	22 (18.2)	26 (21.3)		
II	73 (60.3)	62 (50.8)		
III	20 (16.5)	26 (21.3)		
IV	6 (5.0)	8 (6.6)		
Tumor stage			0.204	0.033
T1	6 (5.0)	5 (3.1)		
T2	21 (17.4)	24 (14.7)		
T3	82 (67.8)	63 (51.6)		
T4a	9 (7.4)	22 (18.0)		
T4b	3 (2.5)	8 (6.6)		
Node stage				0.458
Negative	97 (80.2)	93 (76.2)		
Positive	24 (19.8)	29 (23.8)		
Tumor histological grade			0.186	0.033
Well-differentiated	1 (0.8)	1 (0.8)		
Moderately differentiated	80 (66.1)	62 (50.8)		
Poorly differentiated	19 (15.7)	18 (14.8)		
Mucinous	21 (17.4)	41 (33.6)		
Vascular invasion			0.149	0.019
No	106 (87.6)	117 (95.9)		
Yes	15 (12.4)	5 (4.1)		
Nerve invasion			0.179	0.005
No	100 (82.6)	115 (94.3)		
Yes	21 (17.4)	7 (5.7)		
NLR[b]				0.699
Low (≤5)	109 (91.6)	110 (90.2)		
High (>5)	10 (8.4)	12 (9.8)		
CRP, mg/l			0.201	0.001
Low (≤5)	85 (72.0)	63 (52.1)		
High (>5)	33 (28.0)	58 (47.9)		
PD-1 expression				0.805
Low	43 (44.8)	40 (43.0)		
High	53 (55.2)	53 (57.0)		
TIL expression			0.193	0.002
TIL^-	60 (49.6)	37 (30.3)		
TIL^+	61 (50.4)	85 (69.7)		
PD-L1 expression			0.144	0.023
$PD\text{-}L1^-$	89 (73.6)	73 (59.8)		
$PD\text{-}L1^+$	32 (26.4)	49 (40.2)		

[a]According to the 7th edition of the American Joint Committee on Cancer staging system. [b]NLR data was missing for 2 patients. C, contingency coefficient; MMR, mismatch repair; pMMR, MMR-proficient; dMMR, MMR-deficient; NLR, neutrophil-lymphocyte ratio; CRP, C-reactive protein; PD-1, programmed death-1; TIL, tumor-infiltrating lymphocyte; PD-L1, programmed death ligand-1.

Table II. Aberrant protein expression in MMR-deficient patients.

Aberrant protein expression	Cases, n (%)
MMR (1)	44 (36.1)
MLH1	9 (7.4)
MSH2	4 (3.3)
MSH6	25 (20.5)
PMS2	6 (4.9)
MMR (2)	68 (55.7)
MMR (3)	7 (5.7)
MMR (4)	3 (2.5)

MMR (1), negative expression of 1 MMR protein; MMR (2), concurrent negative expression of 2 MMR proteins; MMR (3), concurrent negative expression of 3 MMR proteins; MMR (4), concurrent negative expression of 4 MMR proteins. MMR, mismatch repair; MLH1, MutL homolog 1; MSH2, MutS homolog 2; MSH6, MutS homolog 6; PMS2, PMS1 homolog 2.

and 58.4% of patients were male and 41.6% were female. According to the American Joint Committee on Cancer (AJCC) 7th edition TNM staging system, pathologically confirmed stage I-IV disease was identified in 48 (19.8%), 135 (55.6%), 46 (18.9%) and 14 (5.8%) patients respectively. Moderately differentiated adenocarcinoma accounted for 58.4% of cases and well-differentiated adenocarcinoma accounted for 0.8% of all cases. A total of 20 (8.2%) and 28 (11.5%) patients exhibited vascular and nerve invasion, respectively.

Association between MMR status, TILs, PD-L1 expression and other clinicopathological features. Representative images obtained to determine TIL presence and PD-L1 expression are presented in Fig. 1. The presence of TILs was classified as positive or negative according to H&E staining. The degree of PD-L1 expression was based on IHC (negative, <5% positive tumor cells; positive, ≥5% positive tumor cells). The associations of MMR status with TIL presence and PD-L1 expression are presented in Table I. The dMMR group exhibited a significantly higher frequency of TIL^+ (85/122 vs. 61/121) and $PD\text{-}L1^+$ expression (49/122 vs. 32/121) compared with the pMMR group ($P<0.05$). Other clinicopathological characteristics, including age, primary tumor site, tumor stage, histological grade, C-reactive protein (CRP) level, vascular invasion and nerve invasion were also significantly associated with MMR status ($P<0.05$).

Association of TILs with PD-L1 expressed by CRC cells. The association between TIL frequency and PD-L1 expression by CRC cells was investigated in the pMMR and dMMR groups. As presented in Table III, $PD\text{-}L1^+$ expression was identified in 42.4% of TIL^+ cases in the dMMR group, while $PD\text{-}L1^+$ expression was only identified in 18.0% of TIL^+ cases in the pMMR group. A significant association was revealed between the presence of TILs and PD-L1 expression in the pMMR group ($P<0.05$), but not in the dMMR group ($P>0.05$).

Association between TIL/PD-L1 status and clinicopathological features. A total of 4 distinct groups were determined according to the presence of TILs and PD-L1: The presence of TILs and PD-L1 (TIL^+ $PD\text{-}L1^+$), the presence of TILs without PD-L1 (TIL^+ $PD\text{-}L1^-$), PD-L1 expression without TILs (TIL^- $PD\text{-}L1^+$) and the absence of TILs and PD-L1 expression (TIL^- $PD\text{-}L1^-$). The current study investigated whether TIL/PD-L1 status was associated with other clinicopathological features, particularly MMR status. As presented in Table IV, TIL/PD-L1 status was significantly associated with AJCC stage, tumor stage, node stage, tumor histological grade, nerve invasion, neutrophil lymphocyte ratio, CRP level, PD-1 expression and MMR status ($P<0.05$). These significantly associated features were then further analyzed by multivariable multinomial logistic regression analysis (Table V). The results demonstrated that PD-1 expression and MMR status were significantly associated with TIL/PD-L1 status. Taking TIL^+ $PD\text{-}L1^+$ as a reference, low PD-1 expression compared with high expression was associated with a higher likelihood of presenting as TIL^- $PD\text{-}L1^-$ [OR, 10.473; 95% confidence interval (CI), 3.005-36.503; $P<0.001$] and TIL^+ $PD\text{-}L1^-$ (OR, 3.443; 95% CI, 1.145-10.352; $P=0.028$). pMMR status compared with dMMR status was associated with an increased likelihood of being TIL^- $PD\text{-}L1^-$ (OR, 11.536; 95% CI, 3.223-41.290; $P<0.001$), TIL^- $PD\text{-}L1^+$ (OR, 14.523; 95% CI, 3.358-62.803; $P<0.001$) and TIL^+ $PD\text{-}L1^-$ (OR 4.718; 95% CI, 1.607-13.848; $P=0.005$). Compared with the pMMR group, the dMMR group was more likely to present with a TIL^+ $PD\text{-}L1^+$ status (76.6 vs. 23.4%; Table IV).

Discussion

Classification of tumor microenvironments according to the presence of TILs and PD-L1 expression has been established in certain cancer types, particularly human melanoma, and applied to predict the effect of anti-PD-1/L1 and select the optimum immunotherapy strategies (6,11). However, to the best of our knowledge, the occurrence of the four types of immune microenvironments in CRC and their association with MMR status remains to be investigated. The current study performed this investigation and revealed a difference in TIL/PD-L1 status between pMMR and dMMR groups, with dMMR patients more likely to exhibit a TIL^+ $PD\text{-}L1^+$ tumor microenvironment.

Compared with the pMMR group, the dMMR group more frequently exhibited a TIL^+ $PD\text{-}L1^+$ phenotype. Previous studies have also demonstrated that tumors from dMMR patients contain a greater TIL density and higher PD-L1 expression compared with tumors from pMMR patients (16-19). The extent of DNA mutation directly or indirectly correlates with the strength of immunogenicity in tumors (20); therefore, the higher density of TILs in dMMR tumors may be due to the accumulation of frameshift mutations and the production of neo-antigens, which activates the immune system of the host (21,22).

TILs upregulate PD-L1 expression through the release of interferon-γ, which mediates an adaptive immune-resistance mechanism by inhibiting local effector T cell activity (6,23-25). This suggests that PD-L1 expression is an adaptive approach for tumor escape from cytokine-mediated T-cell killing. In

Table III. Association analysis between TILs and PD-L1 expression in colorectal cancer cells.

	TIL^+, n (%)		TIL^-, n (%)			
MMR status	$PD\text{-}L1^+$	$PD\text{-}L1^-$	$PD\text{-}L1^+$	$PD\text{-}L1^-$	C	P-value
pMMR	11 (18.0)	50 (82.0)	21 (35.0)	39 (65.0)	0.189	0.034
dMMR	36 (42.4)	49 (57.6)	13 (35.1)	24 (64.9)		0.455
All	47 (32.2)	99 (67.8)	34 (35.1)	63 (64.9)		0.643

MMR, mismatch repair; pMMR, MMR-proficient; dMMR, MMR-deficient; C, contingency coefficient; TIL, tumor-infiltrating lymphocyte; PD-L1, programmed death ligand-1.

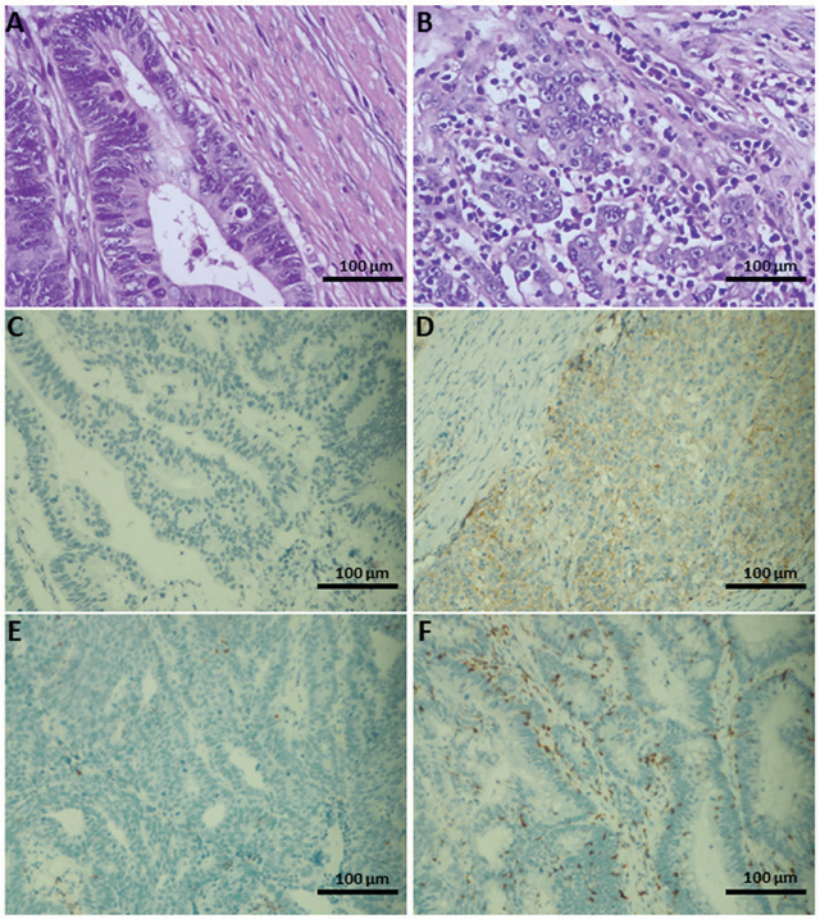

Figure 1. Representative expression of TILs, PD-L1 and PD-1. (A) Absence and (B) presence of TILs at the central area of the tumor and the invasive margin. The presence of TILs was determined by hematoxylin and eosin staining. (C) Negative and (D) positive PD-L1 expression in tumor cells. (E) Low and (F) high expression of PD-1 by TILs. PD-L1 and PD-1 expression was determined by immunohistochemistry. Magnification, x200. TIL, tumor-infiltrating lymphocyte; PD-L1, programmed death ligand-1; PD-1, programmed death-1.

accordance with previous studies, the current study revealed that $PD\text{-}L1^+$ expression was significantly higher in the TIL^+ dMMR group compared with that in the TIL^+ pMMR group, which indicated that TILs may induce the expression of PD-L1 in the dMMR group. In addition, a negative association was identified between TILs and PD-L1 expression in the pMMR group, implying a complexity in the immune tolerance mechanism in this group that cannot be explained by the current study.

Previous studies have divided the tumor immune microenvironments into 4 groups namely $TIL^+PD\text{-}L1^+$, TIL^+ $PD\text{-}L1^-$, TIL^- $PD\text{-}L1^+$ and TIL^- $PD\text{-}L1^-$, according to PD-L1 expression and the presence or absence of TILs. The association of TILs with PD-L1 expression is considered to be more valuable for predicting the response to anti-PD-1/L1 therapy compared with TILs or PD-L1 expression alone (26). In TIL^+ $PD\text{-}L1^+$ tumors, a sufficient number of T cells exist inside the tumor that can induce adaptive expression of PD-L1, which in turn suppresses the function of T cells and may support an effective response to PD-1/L1 blockade therapy (6,7). TIL^+ $PD\text{-}L1^-$ tumors account for ~20% of melanoma cases and contain TILs, but no PD-L1 expression, which indicates a lack of adaptive resistance and

Table IV. Association analysis of TIL/PD-L1 status with clinicopathological features using a χ^2 test.

Characteristic	TIL^+ $PD\text{-}L1^{+}$ [b]	TIL^+ $PD\text{-}L1^{-}$ [b]	TIL^- $PD\text{-}L1^{+}$ [b]	TIL^- $PD\text{-}L1^{-}$ [b]	C	P-value
Age at diagnosis, years						0.751
<60	28 (59.6)	51 (51.5)	20 (58.8)	33 (52.4)		
≥60	19 (40.4)	48 (48.5)	14 (41.2)	30 (47.6)		
Sex						0.271
Male	23 (48.9)	63 (63.6)	22 (64.7)	34 (54.0)		
Female	24 (51.1)	36 (36.4)	12 (35.3)	29 (46.0)		
Primary tumor site						0.297
Left colon	11 (23.4)	37 (37.4)	14 (41.2)	28 (44.4)		
Right colon	26 (55.3)	37 (37.4)	13 (38.2)	23 (36.5)		
Rectum	10 (21.3)	25 (25.3)	7 (20.6)	12 (19.0)		
Stage[a]					0.389	<0.001
I	13 (27.7)	33 (33.3)	1 (2.9)	1 (1.6)		
II	29 (61.7)	47 (47.5)	20 (58.8)	39 (61.9)		
III	4 (8.5)	17 (17.2)	8 (23.5)	17 (27.0)		
IV	1 (2.1)	2 (2.0)	5 (14.7)	6 (9.5)		
Tumor stage					0.396	<0.001
T1	2 (4.3)	9 (9.1)	0 (0.0)	0 (0.0)		
T2	12 (25.6)	30 (30.3)	1 (2.9)	2 (3.2)		
T3	27 (57.4)	51 (51.5)	24 (70.6)	43 (68.3)		
T4a	5 (10.6)	8 (8.1)	6 (17.6)	12 (19.0)		
T4b	1 (2.1)	1 (1.0)	3 (8.8)	6 (9.5)		
Node stage					0.227	0.004
Negative	43 (91.5)	82 (82.8)	23 (67.6)	42 (66.7)		
Positive	4 (8.5)	17 (17.2)	11 (32.4)	21 (33.3)		
Tumor histological grade					0.279	0.015
Well-differentiated	0 (0.0)	2 (2.0)	0 (0.0)	0 (0.0)		
Moderately differentiated	30 (63.8)	65 (65.7)	14 (41.2)	33 (52.4)		
Poorly differentiated	5 (10.6)	18 (18.2)	8 (23.5)	6 (9.5)		
Mucinous	12 (25.5)	14 (14.1)	12 (35.3)	24 (38.1)		
Vascular invasion						0.166
No	46 (97.9)	92 (92.9)	29 (85.3)	56 (88.9)		
Yes	1 (2.1)	7 (7.1)	5 (14.7)	7 (11.1)		
Nerve invasion					0.256	0.001
No	45 (95.7)	94 (94.9)	28 (82.4)	48 (76.2)		
Yes	2 (4.3)	5 (5.1)	6 (17.6)	15 (23.8)		
NLR					0.235	0.003
Low (≤5)	41 (87.2)	98 (99.0)	27 (81.8)	53 (85.5)		
High (>5)	6 (12.8)	1 (1.0)	6 (18.2%)	9 (14.5)		
CRP, mg/l					0.311	<0.001
Low (≤5)	23 (50.0)	80 (80.8)	15 (48.4)	30 (47.6)		
High (>5)	23 (50.0)	19 (19.2)	16 (51.6)	33 (52.4)		
PD-1 expression					0.315	<0.001
Low	7 (18.9)	33 (43.4)	9 (36.0)	34 (66.7)		
High	30 (81.1)	43 (56.6)	16 (64.0)	17 (33.3)		
MMR status					0.268	<0.001
pMMR	11 (23.4)	50 (50.5)	21 (61.8)	39 (61.9)		
dMMR	36 (76.6)	49 (49.5)	13 (38.2)	24 (38.1)		

[a]According to the 7th edition of the American Joint Committee on Cancer staging system. [b]n (%). C, contingency coefficient; MMR, mismatch repair; pMMR, MMR-proficient; dMMR, MMR-deficient; NLR, neutrophil-lymphocyte ratio; CRP, C-reactive protein; PD-1, programmed death-1; TIL, tumor-infiltrating lymphocyte; PD-L1, programmed death ligand-1.

Table V. Multivariate multinomial logistic regression analysis of risk factors for TIL^+ $PD\text{-}L1^+$ status.

	TIL^- $PD\text{-}L1^-$		TIL^- $PD\text{-}L1^+$		TIL^+ PD-L1-	
Factor	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Stage (reference, stage IV)		All >0.05		All >0.05	All P>0.05	All >0.05
Tumor stage (reference, T4b)		All >0.05		All >0.05	All P>0.05	All >0.05
Negative lymph node metastasis (reference, positive)	1.889 (0.030-120.105)	0.764	1.072 (0.014-83.342)	0.975	21.414 (0.093-4946.861)	0.764
Tumor histological grade (reference, mucinous)		All >0.05		All >0.05		All >0.05
No nerve invasion (reference, nerve invasion)	6.533 (0.935-45.646)	0.058	1.058 (0.100-11.239)	0.963	0.417 (0.036-4.826)	0.484
Low NLR level (reference, high NLR level)	0.677 (0.100-4.569)	0.689	0.588 (0.082-4.214)	0.598	N/A	
Low CRP level (reference, high CRP level)	0.735 (0.209-2.585)	0.631	1.409 (0.343-5.786)	0.634	2.697 (0.871-8.351)	0.085
Low PD-1 expression (reference, high expression)	10.473 (3.005-36.503)	<0.001	2.017 (0.490-8.295)	0.331	3.443 (1.145-10.352)	0.028
pMMR (reference, dMMR)	11.536 (3.223-41.290)	<0.001	14.523 (3.358-62.803)	<0.001	4.718 (1.607-13.848)	0.005

TIL^+ $PD\text{-}L1^+$ status was taken as reference. OR, odds ratio; CI, confidence interval; TIL, tumor-infiltrating lymphocyte; PD-L1, programmed death ligand-1; NLR, neutrophil-lymphocyte ratio; CRP, C-reactive protein; PD-1, programmed death-1; pMMR, mismatch repair-proficient; dMMR, MMR-deficient.

suggests that PD-1/L1 blockade is ineffective in this tumor type (11). No definite immunotherapy approaches have been applied in the clinic for patients with these tumors; therefore, novel immunosuppressive strategies should be developed in the future (11). In TIL^- $PD\text{-}L1^+$ tumors, PD-L1 is expressed by tumor cells through oncogenic signaling instead of in response to TILs. It is unlikely that blocking PD-1 or PD-L1 alone is effective in this group due to a lack of T cell involvement (11). Radiotherapy or chemotherapy that induces cell death and the release of neo-antigens to induce a T-cell-mediated anti-tumor response has been used in combination with anti-PD-1 agents (8,27,28). Similar to that in TIL^- $PD\text{-}L1^+$ tumors, single checkpoint blockade agents appear to be ineffective in TIL^- $PD\text{-}L1^-$ tumors due to the lack of T-cell infiltrates. Combination therapy strategies that aim to attract T cells into tumors and prevent inhibition of T cells should be considered. CTLA-4, an inducer of numerous T-cell responses, in combination with anti-PD-1, has been confirmed to effective in a clinical trial, regardless of PD-L1 expression (9,10). Based on the recognition that TIL^+ $PD\text{-}L1^+$ tumors exhibit the best response to PD-1/L1 blockade therapy, the current study assumed that dMMR patients contain a higher proportion of TIL^+ $PD\text{-}L1^+$ tumors compared with pMMR patients. The results confirmed this hypothesis and demonstrated that dMMR status is an independent risk factor for TIL^+ $PD\text{-}L1^+$ status. This could provide immunological evidence for an improved response to anti-PD-1/L1 therapy in dMMR patients compared with that in pMMR patients.

Immune checkpoint blockade is predominantly applied for patients with stage IV CRC (2,3), therefore, the current study attempted to investigate dMMR patients with an advanced stage of CRC. However, dMMR patients account for only a small percentage of patients with stage IV disease and obtaining pathological specimens from these patients was limited due to the loss of surgical opportunities. Although some immunological evidence could be provided, the current study would be more valuable if a higher number of stage IV patients were included. The current study aimed to collect samples from multiple centers to solve this problem, however, this was difficult due to objective factors. This is a notable limitation of the present study, therefore, further investigations are required in the future.

In conclusion, the current study indicated that dMMR patients are more likely to express TILs and PD-L1, and present with a TIL^+ $PD\text{-}L1^+$ status compared with pMMR patients. Therefore, the tumor type identified by this classification method can partially explain the improved response of dMMR patients to PD-1/L1 blockade. However, the response of each tumor type to PD-1/L1 blockade requires further investigation.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Natural Science Foundation of Guangdong Province (grant no. 2017A030310337), the Natural Science Foundation of Guangdong Province (grant no. 2015A030313010), the Science and Technology Program of Guangzhou, China (grant no. 1563000305) and the National Natural Science Foundation of China (grant nos. 81272641 and 81572409).

Availability of data and materials

All data generated and analyzed during this study are included in this manuscript.

Authors' contributions

SL, PK, CJ, QQ, QX and LX designed and performed the study. XW, LY, WH and JH collected and analyzed the data. SL, BZ and XX analyzed the results and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was performed according to the principles of the Declaration of Helsinki and was approved by the Research Ethics Committee of Sun Yat-sen University (Guangzhou, China). Written informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, *et al*: Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363: 711-723, 2010.
2. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, Skora AD, Luber BS, Azad NS, Laheru D, *et al*: PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med 372: 2509-2520, 2015.
3. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, *et al*: Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 366: 2443-2454, 2012.
4. Herbst RS, Soria JC, Kowanetz M, Fine GD, Hamid O, Gordon MS, Sosman JA, McDermott DF, Powderly JD, Gettinger SN, *et al*: Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature 515: 563-567, 2014.
5. Lin WF, Lu JY, Cheng BB and Ling CQ: Progress in research on the effects of traditional Chinese medicine on the tumor microenvironment. J Integr Med 15: 282-287, 2017.
6. Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, Chen S, Klein AP, Pardoll DM, Topalian SL and Chen L: Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. Sci Transl Med 4: 127ra37, 2012.
7. Taube JM, Klein A, Brahmer JR, Xu H, Pan X, Kim JH, Chen L, Pardoll DM, Topalian SL and Anders RA: Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. Clin Cancer Res 20: 5064-5074, 2014.

8. Kalbasi A, June CH, Haas N and Vapiwala N: Radiation and immunotherapy: A synergistic combination. J Clin Invest 123: 2756-2763, 2013.
9. Huang RR, Jalil J, Economou JS, Chmielowski B, Koya RC, Mok S, Sazegar H, Seja E, Villanueva A, Gomez-Navarro J, *et al*: CTLA4 blockade induces frequent tumor infiltration by activated lymphocytes regardless of clinical responses in humans. Clin Cancer Res 17: 4101-4109, 2011.
10. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, Segal NH, Ariyan CE, Gordon RA, Reed K, *et al*: Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 369: 122-133, 2013.
11. Teng MW, Ngiow SF, Ribas A and Smyth MJ: Classifying cancers based on t-cell infiltration and PD-L1. Cancer Res 75: 2139-2145, 2015.
12. Klintrup K, Mäkinen JM, Kauppila S, Väre PO, Melkko J, Tuominen H, Tuppurainen K, Mäkelä J, Karttunen TJ and Mäkinen MJ: Inflammation and prognosis in colorectal cancer. Eur J Cancer 41: 2645-2654, 2005.
13. Shen SJ, Zhang YH, Gu XX, Jiang SJ and Xu LJ: Yangfei Kongliu Formula, a compound Chinese herbal medicine, combined with cisplatin, inhibits growth of lung cancer cells through transforming growth factor-β1 signaling pathway. J Integr Med 15: 242-251, 2017.
14. Droeser RA, Hirt C, Viehl CT, Frey DM, Nebiker C, Huber X, Zlobec I, Eppenberger-Castori S, Tzankov A, Rosso R, *et al*: Clinical impact of programmed cell death ligand 1 expression in colorectal cancer. Eur J Cancer 49: 2233-2242, 2013.
15. Zhang Y, Sun Z, Mao X, Wu H, Luo F, Wu X, Zhou L, Qin J, Zhao L and Bai C: Impact of mismatch-repair deficiency on the colorectal cancer immune microenvironment. Oncotarget 8: 85526-85536, 2017.
16. Park JH, Powell AG, Roxburgh CS, Horgan PG, McMillan DC and Edwards J: Mismatch repair status in patients with primary operable colorectal cancer: Associations with the local and systemic tumour environment. Br J Cancer 114: 562-570, 2016.
17. Smyrk TC, Watson P, Kaul K and Lynch HT: Tumor-infiltrating lymphocytes are a marker for microsatellite instability in colorectal carcinoma. Cancer 91: 2417-2422, 2001.
18. Inaguma S, Lasota J, Felisiak-Golabek A, Kowalik A, Wang Z, Zieba S, Kalisz J, Ikeda H and Miettinen M: Histopathological and genotypic characterization of metastatic colorectal carcinoma with PD-L1 (CD274)-expression: Possible roles of tumour micro environmental factors for CD274 expression. J Pathol Clin Res 3: 268-278, 2017.
19. Rosenbaum MW, Bledsoe JR, Morales-Oyarvide V, Huynh TG and Mino-Kenudson M: PD-L1 expression in colorectal cancer is associated with microsatellite instability, BRAF mutation, medullary morphology and cytotoxic tumor-infiltrating lymphocytes. Mod Pathol 29: 1104-1112, 2016.
20. Chen DS, Irving BA and Hodi FS: Molecular pathways: Next-generation immunotherapy-inhibiting programmed death-ligand 1 and programmed death-1. Clin Cancer Res 18: 6580-6587, 2012.
21. Woerner SM, Gebert J, Yuan YP, Sutter C, Ridder R, Bork P and von Knebel Doeberitz M: Systematic identification of genes with coding microsatellites mutated in DNA mismatch repair-deficient cancer cells. Int J Cancer 93: 12-19, 2001.
22. Angelova M, Charoentong P, Hackl H, Fischer ML, Snajder R, Krogsdam AM, Waldner MJ, Bindea G, Mlecnik B, Galon J and Trajanoski Z: Characterization of the immunophenotypes and antigenomes of colorectal cancers reveals distinct tumor escape mechanisms and novel targets for immunotherapy. Genome Biol 16: 64, 2015.
23. Dong H, Zhu G, Tamada K and Chen L: B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. Nat Med 5: 1365-1369, 1999.
24. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, *et al*: Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 192: 1027-1034, 2000.
25. Tseng SY, Otsuji M, Gorski K, Huang X, Slansky JE, Pai SI, Shalabi A, Shin T, Pardoll DM and Tsuchiya H: B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. J Exp Med 193: 839-846, 2001.
26. Sznol M and Chen L: Antagonist antibodies to PD-1 and B7-H1 (PD-L1) in the treatment of advanced human cancer. Clin Cancer Res 19: 1021-1034, 2013.
27. Aboudaram A, Modesto A, Chaltiel L, Gomez-Roca C, Boulinguez S, Sibaud V, Delord JP, Chira C, Delannes M, Moyal E and Meyer N: Concurrent radiotherapy for patients with metastatic melanoma and receiving anti-programmed-death 1 therapy: A safe and effective combination. Melanoma Res 27: 485-491, 2017.
28. Karaca B, Yayla G, Erdem M and Gürler T: Electrochemotherapy with anti-PD-1 treatment induced durable complete response in heavily pretreated metastatic melanoma patient. Anticancer Drugs: Dec 21, 2017 (Epub ahead of print). doi: 10.1097/CAD.0000000000000580.