

Original Article

Expression of STING and PD-L1 in colorectal cancer and their correlation with clinical prognosis

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Abstract: Objective: To investigate the expression of PD-L1 and STING in colorectal cancer and analyze their correlation with disease prognosis. Methods: The colorectal tissue chip was taken as the research object. Immunohistochemical methods were used to detect expression of PD-L1 and STING of 87 cases of colorectal cancer tissue and corresponding adjacent tissue in the tissue chip. Chi-square test or Fisher's test were used to analyze the relationship of expression of PD-L1 and STING and with the clinical pathological features. Kaplan-Meier curves were used to analyze the relationship between expression of PD-L1 and STING and the prognosis of colorectal cancer. Results: The positive expression rate of PD-L1 in 87 cases of colorectal cancer tissue and corresponding adjacent tissue was 79.3% and 42.5% respectively, and the difference was significant ($P < 0.05$). The positive expression rate of PD-L1 in colorectal carcinoma tissues was not related with age, gender, tumor size, differentiation, TNM clinical stage, or depth of invasion ($P > 0.05$). The positive expression rate of STING in 87 cases colorectal cancer tissues and corresponding adjacent tissue was 9.2% and 40.2% respectively and the difference was significant ($P < 0.05$). The positive expression rate of STING in colorectal carcinoma tissue was correlated with gender, age, and differentiation ($P < 0.05$), but not associated with tumor size, depth of invasion, and TNM clinical stage ($P > 0.05$). The expression rate of PD-L1 was related to the prognosis of colorectal cancer ($P=0.018$). The expression rate of STING was not correlated with prognosis of colorectal cancer ($P=0.784$). In four independent groups (PD-L1⁺STING⁺ group, PD-L1⁺STING⁻ group, PD-L1⁻STING⁺ group, PD-L1⁻STING⁻ group), there were significant differences in 5-year survival ($P=0.047 < 0.05$), the 5-year survival rate of the PD-L1⁺STING⁺ group was significantly higher than that of the other three groups. In the STING positive group, the PD-L1⁺STING⁺ group had a worse prognosis than the PD-L1⁻STING⁺ group. In the STING negative group, the PD-L1⁺STING⁻ group had a higher survival rate than the PD-L1⁻STING⁻ group. Conclusion: PD-L1 might promote the occurrence of disease and STING might play an important role in anti-tumor immunity. PD-L1 was related with the prognosis of patients with colorectal cancer but the expression of STING was not obviously associated with the prognosis. Survival rates of patients with colorectal cancer were higher in patients with PD-L1 negative expression or STING positive expression. When PD-L1 expression was negative and STING expression was positive, the 5-year survival rate of patients with colorectal cancer was highest.

Keywords: Colorectal cancer, PD-L1, STING, immunohistochemical method, prognosis

Introduction

Colorectal cancer is one of the most common malignant tumors in the world. The incidence of colorectal cancer is third and second place in male and female tumors, respectively. The estimated mortality rate is fourth and third in men's and women's tumors, respectively [1]. Cancer immunotherapy is envisioned as a new approach to achieve the purpose of controlling and

killing tumor cells by stimulating and enhancing the immune function of the body. Immunotherapy acts as a complementary therapy combined with conventional therapies such as surgery, chemotherapy, and radiotherapy, and has become a promising strategy to treat various types of cancer. The antibody targeting PD-1/PD-L1 has shown very promising efficacy in the clinic in recent years. Studies have shown that PD-1/PD-L1 can negatively regulate the

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Table 1. Expression of PD-L1 and STING in colorectal cancer and corresponding para-cancerous tissues

Groups	n	PD-L1 expression		STING expression	
		Positive	Negative	Positive	Negative
Colorectal cancer tissue	87	69	18	8	79
Para-cancerous tissue	87	37	50	35	52
<i>P</i> value		0.000		0.000	

Table 2. The relationship between PD-L1 expression and clinicopathological features in colorectal cancer

Parameters	Cases	PD-L1 expression		Positive rate (%)	<i>P</i> value
		Positive	Negative		
Gender					0.798
Male	46	36	10	78.3	
Female	41	33	8	80.5	
Age					0.863
≤ 60	28	23	5	82.1	
> 60	59	46	13	77.9	
Tumor size					0.209
≤ 5	50	42	8	84	
> 5	37	27	10	72.9	
Differentiation					0.029
High	21	13	8	61.9	
Medium	52	35	17	67.3	
Low	14	10	4	71.4	
Lymph node metastasis					0.261
Yes	29	21	8	72.4	
No	58	48	10	82.8	
TNM stage					0.993
I	10	7	3	70	
II	49	39	10	79.6	
III	28	25	3	89.3	
Depth of invasion					0.375
T1-T2	10	6	4	60	
T3-T4	77	60	17	77.9	

immune response by inhibiting activation and proliferation of T cells, and it also can participate in regulating immune tolerance, microbial infection, and tumor cell immune escape [2].

STING (stimulator of interferon genes), which mediates cytosolic DNA-induced signaling events, has recently been implicated as a mediator of processes in cancer immunotherapy. Exogenous or endogenous DNA can lead to STING dimerization, activation of Tank binding kinase 1 (TBK1), activation of interferon regulatory factor 3 (interferon regulatory 3 factor,

IRF3) and nuclear factor kappa B (nuclear factor kappa-B kinase, NF-κB), and eventually generation of interferon [3, 4]. It is known that interferon is widely used in cancer treatment because it has the effect of regulating immune function and therefore inhibiting tumor cell proliferation. Recent studies have found that IFN can induce expression of PD-L1 in ovarian cancer, hepatoma, gastric cancer, and so on [5-7]. Moreover, it has been found that downregulation of STING could decrease cisplatin induced upregulation of PD-L1 in breast cancer [8], and the STING signaling pathway is essential for the anti-tumor efficacy of PD-L1 antibody blockage [9]. Furthermore, the combination of a STING agonist and PD-1 pathway blockage could reduce the tumor growth in tolerized mice [10], therefore, STING and PD-L1 have important interactions that control the cancer prognosis. However, few studies have provided information regarding a correlation of PD-L1 and STING expression with clinicopathological factors and disease prognosis in colorectal cancer. This study aims to analyze the expression of PD-L1 and STING in colorectal cancer, and compare the clinical pathological features to investigate their expression in colorectal carcinoma and its relation with prognosis in colorectal cancer, which might provide some

insights for the immunotherapy of colorectal cancer in the near future.

Materials and methods

Materials and agents

The PD-L1 monoclonal antibody purchased from the Cell Signaling Technology Company (USA), the STING monoclonal antibody purchased from the Proteintech Company (China). The Goat anti-rabbit antibody and Diaminobenzidine (DAB) reagent purchased from the

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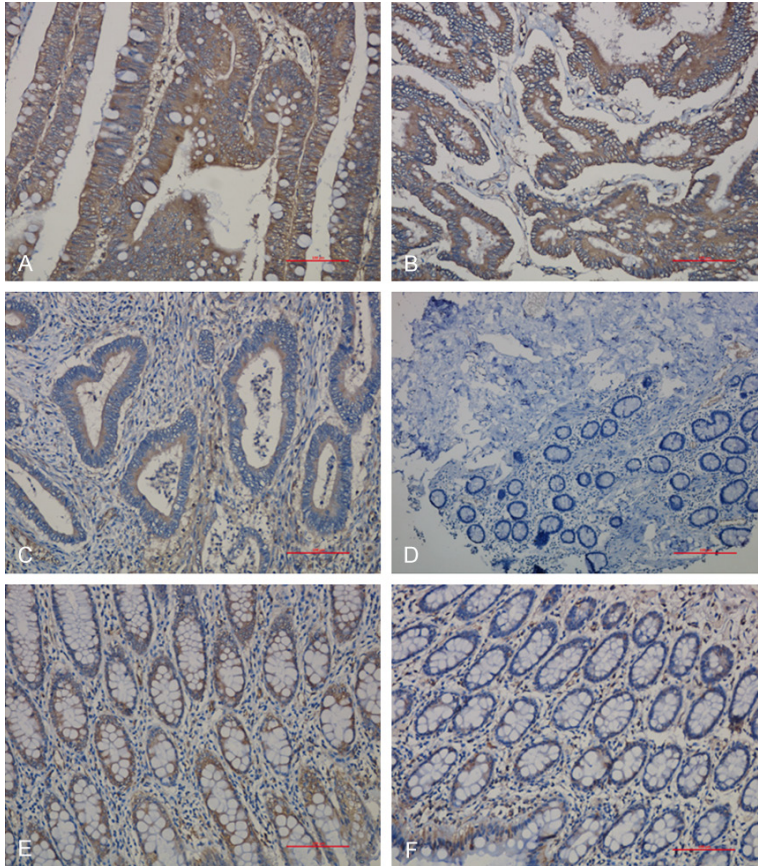


Figure 1. PD-L1 expression in colorectal cancer and its adjacent tissue. A: PD-L1 positive expression (+++); B: PD-L1 positive expression (++); C: PD-L1 positive expression (+); D: PD-L1 negative expression; E: PD-L1 positive expression in normal adjacent tissue; F: PD-L1 negative expression in normal adjacent tissue. (Magnification: 200 \times).

Shanghai Genetech Biotech Co., Ltd (China). Other reagents used were obtained from the Shanghai Beyotime Biotech Co., Ltd (China).

The patients' tissue chip of colorectal cancer was purchased from Shanghai Outdo Biotech Co., Ltd (Product number: HCol-Ade180Sur-07), all patients did not receive any treatment before operation, and had complete clinical data. The chip included 87 colorectal cancer tissues and its paired adjacent tissue. The detailed clinical information of colorectal cancer patients were shown in **Table 2**.

Immunohistochemistry

To retrieve antigen, the purchased tissue chips were routinely dewaxed, rehydrated, and immunohistochemistry was performed in accordance with the standard operation process.

The anti-human antibody PD-L1 (1:500 dilution) and STING (1:500 dilution) were used as primary antibody, and HRP-labeled anti-mouse antibody used as secondary antibody to incubate with the chips and Diaminobenzidine (DAB) reagent was used to visualize the expression of PD-L1 and STING on the colorectal cancer tissue. PBS was used as a negative control. A double blind method was used for the determination of positive staining results, and 2 senior pathologists read the film independently. Expression of PD-L1 and STING was mainly located in the cytoplasm and cell membrane, and the yellow or yellow brown granules were indicated as positive expression. Five visual fields from different areas of each specimen were chosen randomly.

PD-L1 positivity was evaluated by modified H-Score [11], a combination of staining intensity and percentage of tumor cell staining. The score of PD-L1 expression was in two steps, first, the PD-L1 slices were scored according to the percentage of positive cells: < 5% was 0, 5% to 25% was 1, 26% to 50% was 2, 51% to 75% was 3, and > 75% was 4. At the same time, the staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong) based on membranous localization. After that, the final score of each slice was calculated by multiplying the score of which based on the percentage of positive cells in first step and the score of staining intensity. Therefore, the modified H-scores ranged from 0 to 12. Based on the above score, the PD-L1 expression was divided into 4 groups: Negative (-): score 0~1; weak positive (+): score 2~4; medium positive (++) : score 5~8; strong positive (+++): score 9~12. The '+~+++' was defined as positive. For STING positivity, the positive/negative was considered as staining with/without yellow (or yellow brown) granules (**Figures 1 and 2**).

PD-L1 and STING expression in colorectal cancer

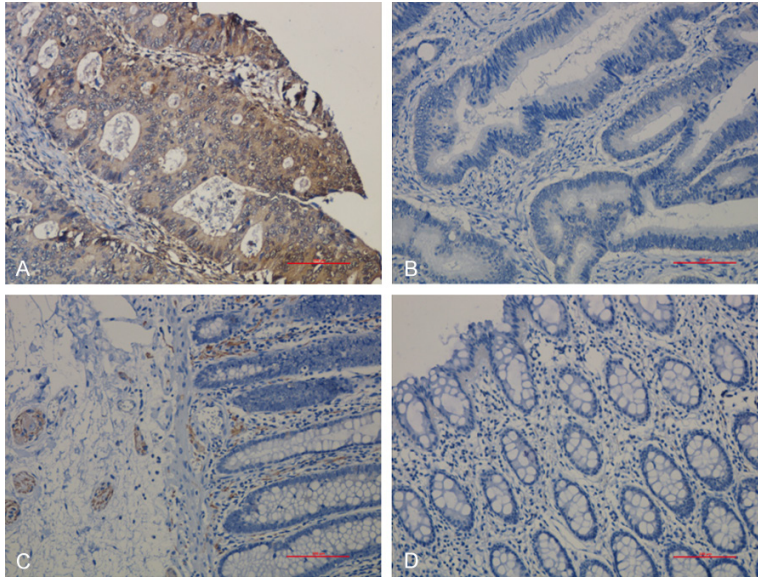


Figure 2. STING expression in colorectal cancer and its adjacent tissue. A: STING positive expression; B: STING negative expression; C: STING positive expression in normal adjacent tissue; D: STING negative expression in normal adjacent tissue. (Magnification: 200 \times).

Statistical method

SPSS 13 statistical software was used for data analysis. Chi square test or Fisher's exact test were used for comparison between groups. Kaplan-Meier method and parallel log-rank test were used to compare survival status. $P < 0.05$ was considered as statistically significant difference.

Results

PD-L1 expression in colorectal cancer tissue and its adjacent tissue

In 87 cases of colorectal cancer, 69 cases were positive for PD-L1 expression at a rate of 79.3%. In the corresponding adjacent tissues, PD-L1 expression was positive in 37 cases, and the positive rate was 42.5%. There was a significant difference between the colorectal cancer tissue and its adjacent tissue on PD-L1 expression ($P < 0.05$). The data are summarized in **Table 1**.

STING expression in colorectal cancer tissue and its adjacent tissue

In 87 colorectal cancer tissues, 8 cases had positive expression of STING with the positive rate being 9.2%. However, there were 35 cases

with positive expression in the adjacent tissues, which was significantly higher than that in the corresponding colorectal cancer tissues ($P < 0.05$), as shown in **Table 1**.

The relationship between PD-L1 expression and clinicopathological features in colorectal cancer

In 87 cases of colorectal carcinoma, the positive rate in low differentiation group was higher than that of medium and high differentiation group (71.4% vs. 67.3% vs. 61.9%). For TNM clinical stage (I, II, III), the late of clinical stage, the higher expression of PD-L1 (I: 70%, II: 79.6%, III: 89.3%). Considering the factor of tumor invasion depth, it was

found that positive rate in T3-T4 group was higher than that of T1-T2 group (77.9% vs. 60%). However, it was found that there was no statistical significance between them, which indicated the no correlation of the PD-L1 protein expression with the differentiation, clinical stage, and depth of tumor invasion. Also, it was found that PD-L1 expression was unrelated with the patients' age, gender, tumor size, and whether they had lymph node metastasis or not ($P > 0.05$). (Data are summarized in **Table 2**).

The relationship between STING expression and clinicopathological features in colorectal cancer

In 87 cases of colorectal carcinoma, the positive ratio of STING expression in high differentiation group was higher than that of medium and low differentiation group (38.1% vs. 11.5% vs. 14.3%), and it was also found that the positive rate of STING in women was higher than that of men (17.1% vs. 2.17%) with the difference being significant ($P < 0.05$). Also, the positive rate in age less than 60 was higher than that age larger than 60, all of which illustrated the positive expression of STING was related with the tumor differentiation status, gender,

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Table 3. The relationship between STING expression and clinicopathological features in colorectal cancer

Parameters	Cases	STING expression		Positive rate (%)	P value
		Positive	Negative		
Gender					0.016
Male	46	1	45	2.17	
Female	41	7	34	17.1	
Age					0.007
≤ 60	28	6	22	21.4	
> 60	59	2	57	3.4	
Tumor size					0.051
≤ 5	50	2	48	4	
> 5	37	6	31	16.2	
Differentiation					0.234
High	21	8	13	38.1	
Medium	52	6	46	11.5	
Low	14	2	12	14.3	
Lymph node metastasis					0.6
Yes	29	2	27	6.8	
No	58	6	52	10.3	
TNM stage					0.449
I	10	2	8	20	
II	49	4	45	8.2	
III	28	2	26	7.1	
Depth of invasion					0.209
T1-T2	10	2	8	20	
T3-T4	77	6	71	7.8	

and age. For TNM clinical stage (I, II, III), the later of clinical stage, the low expression of STING (20% vs. 8.2% vs. 7.1%). Even though the positive ratio of STING in T1-T2 group was higher than that of T3-T4 group (20% vs. 7.8%), there was no significant difference between them ($P > 0.05$), which suggested that the positive expression of STING was unrelated with patients' clinical TNM stage, tumor invasion depth. Moreover, the positive expression of STING was also unrelated with tumor size and whether lymph node metastasis was present or not ($P > 0.05$), as shown in **Table 3**.

Correlation between the expression of PD-L1 and STING in colorectal cancer and the prognosis of patients

The expression of PD-L1 in colorectal cancer was associated with prognosis of colorectal cancer ($P=0.018$), and expression of STING was not correlated with the prognosis of colorectal cancer ($P=0.784$), as shown in **Figures 3** and **4**, respectively.

The correlation of PD-L1 and STING expression with colorectal cancer patients' survival

To explore the relationship of the PD-L1 and STING with colorectal cancer survival, as shown in **Figure 5**, 87 cases of colorectal cancer samples were divided into 4 groups (PD-L1+STING+ group, PD-L1- STING- group, PD-L1- STING+ group, PD-L1+STING- group), the analyzed results showed that there were significant differences between the 4 groups ($P=0.047$, **Figure 5**), the 5 year survival rate in the PD-L1- STING+ group was significantly

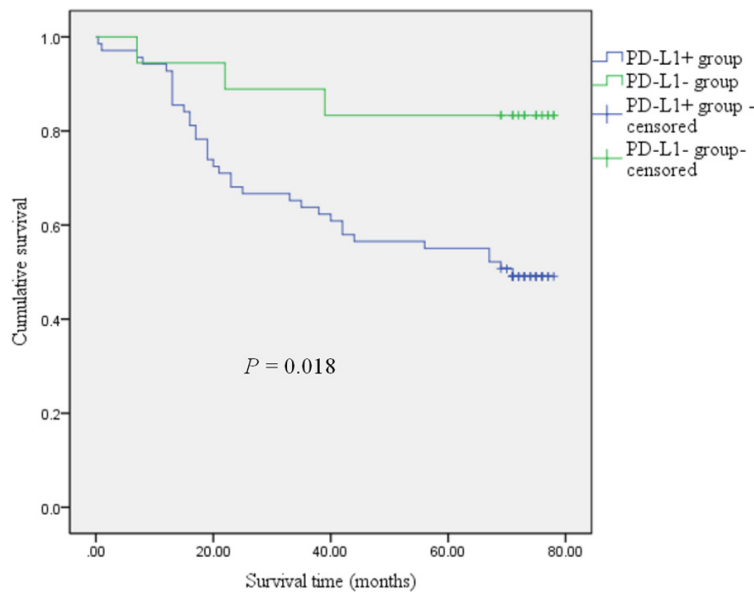


Figure 3. Kaplan-Meier curves comparing 5-year survival outcome of colorectal cancer patients with positive and negative PD-L1 expression. The log-rank test was used to calculate P value.

PD-L1 and STING expression in colorectal cancer

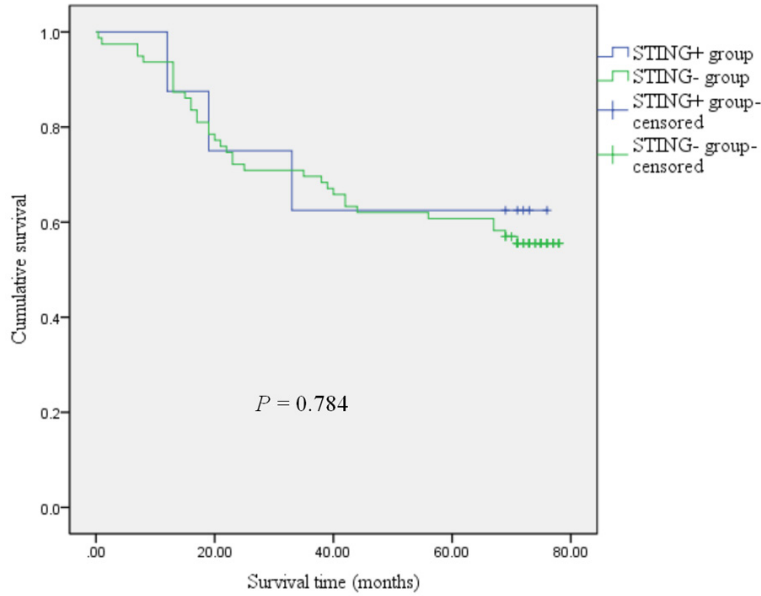


Figure 4. Kaplan-Meier curves comparing 5-year survival outcome of colorectal cancer patients with positive and negative for STING expression (log-rank test).

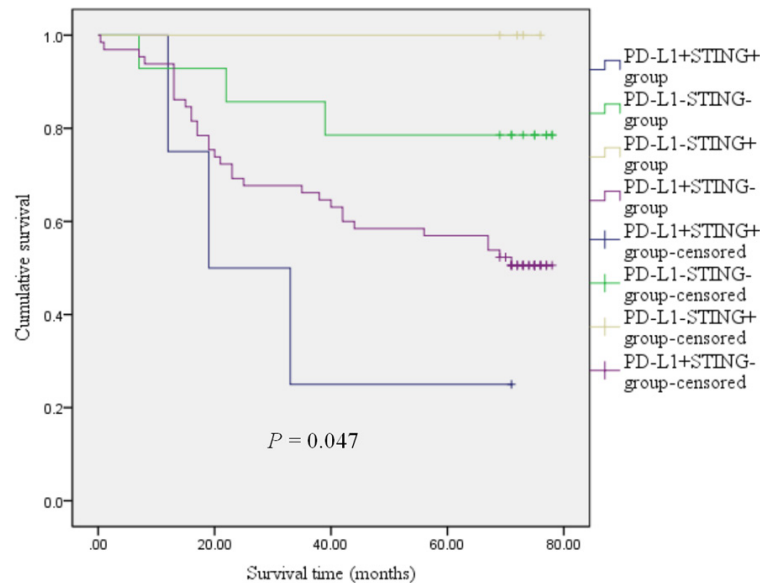


Figure 5. Kaplan-Meier survival curves of patients with colorectal cancer according to the combination status with PD-L1 and STING. The log-rank test was used to calculate *P* value.

higher than that in the other three groups (PD-L1⁺STING⁺ group, PD-L1⁻STING⁻ group, PD-L1⁺STING⁻ group). In contrast, the 5 year survival rate in the PD-L1⁺STING⁺ group was worst. In the STING positive group, the PD-L1⁺STING⁺ group had a worse prognosis than that of the PD-L1⁻STING⁺ group. In the STING negative

group, the 5 year survival rate in the PD-L1⁻STING⁻ group was higher than that in the PD-L1⁺STING⁻ group (Figure 5).

Discussion

Immunotherapy has become one of the choices for the treatment of various cancers [12]. More and more evidence suggests that the immunological checkpoint plays an important role in the anti-tumor immune response which is mediated by T cells in the tumor microenvironment [12]. Although T cells are present in almost all tumors, most cancers are not naturally identified by the immune system. Preclinical and clinical data indicated the key function of type I interferon in connecting tumor immune rejection with the innate and adaptive immune responses [13-15].

PD-L1 is a member of the B7 family which was found by Dong et al. from a placenta cDNA Library in 1999 [16]. After binding with its receptor PD-1, can mediate inhibitory signals for activation of T cells and cytokine secretion, and thus promotes immune tolerance, microbial infection, and immune escape of tumor cells. At present, the effect of PD-1/PD-L1 inhibitor on the anti-tumor efficacy has been greatly recognized [17].

Interferon stimulated gene (STING) acts as a sensor, which can recognize the exogenous and endogenous cyclic dinucleotide (CDNs), and then activates the downstream genes TBK1/IRF3 (interferon regulatory factor 3), nuclear factor κ B, and STAT6 (signal transducer and activator of transcription 6), which induce type I interferons and pro-inflammatory cytokines [18]. STING signals are critical for innate immune response. It causes

the production of type I IFN in tumor microenvironment, and promotes the production of tumor antigen specific CD8⁺ T cells via the dendritic cells, thereby playing a role in tumor inhibition [13, 18].

In the microenvironment of colorectal cancer, the antitumor activity of T cells could be inhibited by the PD-L1 expressed on the tumor cell. Usually, STING plays an active role in the antitumor immune via the promoting the generation of CD8⁺ T cells, however, some studies have shown that STING can induce the formation of an inhibitory tumor microenvironment, indirectly promoting tumor growth and metastasis [19, 20]. Therefore, STING play a complicated role in signaling in tumor immune responses. Recently, some studies have shown a close connection of the STING signaling pathway with PD-L1 [8-10, 21], and in our study we also found that downregulation of STING expression could decrease the expression level of PD-L1 in colon cancer (data not shown). In order to investigate the role of STING and PD-L1 in colorectal cancer, the IHC method was used to detect the STING and PD-L1 expression in 87 cases of colorectal cancer and its peri-tumor tissue. The results showed that PD-L1 was expressed in 69 cases and the positive rate was 79.3%, the positive rate of PD-L1 in peri-tumor tissue was 42.5%, which was significantly lower than that of tumor tissues. For STING expression, only 8 cases in 87 cases were positive and the positive rate was 9.2%, however, the positive rate in the peri-tumor tissue was 40.2%, which was significantly higher than that of colorectal cancer tissues ($P < 0.05$).

After that, the correlation of PD-L1 and STING expression in colorectal cancer with the clinicopathological data were analyzed. It was found that the positive expression of PD-L1 was not correlated with the patients' age, gender, tumor size, differentiation, TNM clinical stage, depth of invasion ($P > 0.05$). In contrast, the STING expression was correlated with the patients' gender, age, differentiation ($P < 0.05$), and was not correlated with tumor size, depth of invasion, and TNM clinical stage ($P > 0.05$). The STING positive expression had a linear correlation with differentiation, higher differentiation, and a higher positive expression of STING. It is known that the higher differentiation of tumor tissue usually means better prognosis, therefore, positive expression of STING might be

a potential biomarker for the evaluation of patients' prognosis in colorectal cancer.

Studies have shown that PD-1/PD-L1 (programmed cell death receptor 1/programmed cell death ligand 1) inhibitors are effective in many types of cancer [17]. Recent studies showed that mice lacking STING gene have poor responses on radiotherapy and immunotherapy, such as blocking immunosuppressant molecules, including PD-1, PD-L1, CTLA4, and CD47 [8-10, 22]. Therefore, STING and PD-L1 might have a close connection in disease progression. Survival analysis found that the expression of PD-L1 was correlated with prognosis of colorectal cancer ($P=0.018$), and expression of STING was not correlated with the prognosis of colorectal cancer ($P=0.784$). The difference between the 4 independent groups (PD-L1⁺STING⁺ group, PD-L1⁺STING⁻ group, PD-L1⁻STING⁺ group, PD-L1⁻STING⁻ group) was statistically significant ($P=0.047$), the PD-L1⁺STING⁺ group had worse prognosis than the PD-L1⁻STING⁺ group in the STING positive group, and the PD-L1⁻STING⁺ group had higher survival rate than the PD-L1⁻STING⁻ group in the PD-L1 negative group. This would provide a theoretical basis for the application of PD-L1 and STING related immunotherapy in colorectal cancer.

In summary, this study confirmed that expression of PD-L1 in colorectal cancer tissues was higher than that of adjacent tissues and that the expression of STING in colorectal cancer was lower than the corresponding adjacent tissues. The expression of PD-L1 in colorectal cancer and the clinical indicators were not correlated, suggesting that PD-L1 expression might be an independent prognostic factor. Expression of STING in colorectal cancer was related to gender, age and degree of differentiation. Therefore, PD-L1 may promote the occurrence of tumors, and STING may play an important role in anti-tumor immunity. Expression of PD-L1 correlated with the prognosis of colorectal cancer patients, with the PD-L1⁺STING⁺ group having the highest survival rate, thus providing a theoretical basis for PD-L1 and STING related immunotherapy in colorectal cancer. Furthermore, STING agonist combined with PD-1/PD-L1 inhibitors could achieve better therapeutic effects than that of single treatment. Of course, there are many inadequacies in this study, and large sample studies and

long-term follow-up are also warranted in future studies.

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Disclosure of conflict of interest

None.

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