Expression of M2 Macrophages and Regulatory T Cells in Colorectal Cancer and Their Correlation with Lymph Node Metastasis

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Abstract

Background

Colorectal cancer (CRC) is among the most common malignancies worldwide. M2 macrophages and regulatory T cells (Tregs) are immunosuppressive cells that can promote tumor progression via inhibiting anti-tumor immunities. However, the significance and correlation of the two types of cells in colorectal cancer are still inconclusive. The purpose of this study is to detect the number of M2 macrophages and Tregs in colorectal cancer and lymph nodes and to explore the clinical and pathological significance of their existence.

Methods

The pathologic specimens and clinical data of 197 patients with Colorectal cancer after radical resection were collected. Immunohistochemical methods were used to detect the expression of M2 macrophages and Tregs in colorectal cancer tissues, adjacent tissues, and lymph node tissues in each group.

Results

Compared with adjacent tissues and non-metastatic lymph node tissues, M2 macrophages and Tregs not only increased significantly in cancer tissues and metastatic lymph node tissues ($P < 0.001$), but also M2 macrophages in non-metastatic lymph node tissues adjacent to cancer tissues the number of phages expressed also increased significantly ($P < 0.05$). In addition, there was a positive correlation between the number of cancer tissues and lymph nodes ($P < 0.001$).

Conclusion

M2 macrophages are involved in the formation of lymph node immunosuppressive environment and promote the development of CRC and lymph node metastasis together with Tregs. Upregulation of M2 macrophages and Tregs expression is a prognostic marker for monitoring the condition of colorectal cancer and judging the prognosis.

Introduction

Colorectal cancer is one of the most common diagnosed malignancies in the world, which is the fourth leading cause of cancer-related death[1]. In the pathogenesis of CRC, immune factors are important factors involved in the occurrence and development of tumors, including the body's tumor immunity and immune surveillance, immune escape effects, etc. Among the many factors that affect the prognosis of CRC, the microenvironment of colorectal cancer and various immune cells in the lymph nodes play a vital role.

Studies have shown that immunosuppressive cells, especially M2 macrophages and Tregs, can promote tumor development[2–6]. M2 macrophages and Tregs play an important immunomodulatory role in the

promotion of CRC. Studies have shown that M2 macrophages have the ability to induce the formation of Tregs [7], but whether the relationship between the two affects the progression of CRC and lymph node metastasis is still unknown.

We used immunohistochemical methods to detect the expression of M2 macrophages and Tregs in the tissues and lymph nodes of 197 colorectal cancer patients, and then analyzed the correlation between the two, and further explored the mechanism of colorectal cancer invasion and metastasis, so as to better monitor the progress of CRC And prognosis.

1. Materials And Methods

1.1 Clinical data

The clinical data and postoperative paraffin specimens of patients undergoing radical resection of colorectal cancer in the Department of Colorectal Surgery of the First Affiliated Hospital of Jinzhou Medical University from March 2020 to December 2020 were collected. After inclusion and exclusion, a total of 197 cases were collected. The detailed clinical data of all patients are shown in Table 1. Divide the colorectal cancer patients' specimens into two groups of cancer tissues and adjacent tissues (at least 2cm from the edge of the tumor); The lymph node tissue is divided into three groups according to the presence or absence of metastatic lymph nodes: In group A, one lymph node from each patient was randomly selected from the I and II cases, a total of 108 cases; In group B, one lymph node metastasized from each patient was randomly selected from the Ⅱ and Ⅲ cases, a total of 89 cases; In group C, one non-metastatic lymph node in each patient was randomly selected from the Ⅱ and Ⅲ cases, a total of 89 cases. Selection criteria: ⅠPrimary colorectal cancer; ⅡDiagnosed in our hospital and received surgical treatment for the first time; ⅢAgree to participate in this study. Exclusion criteria: Ⅰ>Patients over 86 years old; ⅡYoung patients with genetic background of the confirmed disease (familial adenomatous polyposis and hereditary non-polyposis CRC); ⅢPatients treated for autoimmune and rheumatoid diseases have received chemotherapy or radiotherapy after diagnosis; ⅣReceived immune targeted therapy after diagnosis; ⅤMalignant tumors of the intestines in multiple locations or combined with malignant tumors of other systems. The clinical staging is in accordance with the 2017 American Cancer Council (AJCC) staging standard. (Eighth Edition) This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Jinzhou Medical University and is for research use only (trial number: KYLL202090). All patients signed an informed consent form.

Table 1
Patient clinical data characteristics

Clinicopathological parameters	Group A (☐ + ☐) (n = 108)(%)	Group B or C (☐ + ☐) (n=(89)(%)
age		
≥ 65	65(60)	48(54)
< 65	43(40)	41(46)
gender		
male	70(65)	48(54)
Female	38(35)	41(46)
Tumor site		
colon	58(54)	42(47)
rectum	50(46)	47(53)
TNM staging		
☐	25(23)	
☐	83(77)	
☐		64(82)
☐		25(18)
N stage		
N0	108(100)	
N1		60(67)
N2		29(33)
Differentiation		
Medium + well differentiated	93(86)	61(69)
Poorly differentiated	15(14)	28(31)
Tumor diameter (cm)		
≥ 5	63(58)	40(45)
<5	45(42)	49(55)
Distant metastasis		
M ₀	108(100)	64(72)

1.2 Immunohistochemical analysis

Using immunohistochemistry streptomyces antibiotic protein-peroxidase link (SP) method: All specimens were fixed in formalin, embedded in paraffin, and cut into 4 μ m thick sections. Place the section on a microscope slide, deparaffinize with xylene and hydrate with a series of graded ethanol, and perform antigen retrieval with ethylenediaminetetraacetic acid buffer (pH 9.0) at sub-boiling temperature for 20 minutes. Tissue sections were incubated with endogenous peroxidase blocker for 10 minutes at room temperature, and blocked with goat serum (Cat. No. KIT-9710; Maixin Biotechnology, Fuzhou, China) for 30 minutes. Wash the sections with phosphate buffered saline (PBS) 3 times, then add anti-CD163 (1:500, ab182422, Abcam), anti-Foxp3 (1:500, ab20034, Abcam) and incubate overnight at 4°C, then add biotin-labeled IgG Polymer and streptavidin-peroxidase (Cat. No. KIT-9710; Maixin Bio), Finally, the sections were developed using the Diaminobenzidine (DAB) kit (catalog number DAB-0031; Maixin-Bio) and counterstained with hematoxylin.

Use optical biomicroscopy (Olympus BX43; Japan) to determine the density of M2 macrophages and Tregs in colorectal cancer and lymph nodes. The density of CD163 and Foxp3 was determined by two pathologists without knowing the patient's clinicopathological data. First, at low power ($\times 200$), randomly select 5 areas with M2 macrophages and Tregs infiltrated, and then calculate the number of M2 type macrophages and Tregs (per mm²) at high power ($\times 400$), and Calculate the average number of M2 macrophages and Tregs. The expression of Tregs in lymph nodes is expressed as a percentage.

1.3 Statistical analysis

Use SPSS26.0 version software program and GraphPad Prism 8 for data analysis, The independent sample t test was used to evaluate the correlation between the expression number of M2 macrophages and Tregs and the pathological characteristics of the tumor, the Mann-Whitney U test was used to analyze and compare the expression differences between groups, and the Spearman correlation analysis was used to evaluate the expression of M2 macrophages and The relationship between Tregs and the correlation between the two and tumor markers. For the above results, $P < 0.05$ is considered to be statistically significant.

2. Results

2.1 The relationship between the number of M2 macrophages and Tregs expression and the clinical characteristics of tumors

In order to clarify the infiltration characteristics of M2 macrophages and Tregs cells in colorectal cancer, we performed statistical analysis on the number of M2 macrophages and Tregs and clinicopathological parameters in 197 cases of colorectal cancer tissues (see Table 2) .It was found that the increase in the

expression of CD163 + M2 and Foxp3 + tregs was related to the TNM staging, depth of invasion, lymph node metastasis, vascular tumor thrombosis, nerve invasion and distant metastasis of colorectal cancer, and the difference was statistically significant ($p < 0.05$); It has nothing to do with the patient's age, gender, tumor diameter, tumor location and degree of differentiation, and the difference is not statistically significant ($p > 0.05$).

Table 2
Clinicopathological characteristics of 197 patients with nodal cancer

Clinicopathological parameters	cases	CD163 + M2		Foxp3 + Tregs	
		(mean/HP)	P	(mean/HP)	P
gender			0.166		0.760
male	118	18.45 ± 5.85		23.04 ± 6.89	
Female	79	19.67 ± 6.33		22.72 ± 7.67	
age			0.156		0.544
≥ 65	118	19.44 ± 6.15		23.17 ± 7.09	
<65	79	18.19 ± 5.89		22.53 ± 7.38	
Tumor diameter (cm)			0.623		0.691
≥ 5	103	19.15 ± 5.17		22.72 ± 7.30	
<5	94	18.71 ± 6.93		23.13 ± 7.13	
Tumor site			0.541		0.927
colon	100	19.20 ± 5.88		22.96 ± 7.46	
rectum	97	18.67 ± 6.26		22.87 ± 6.65	
Lymph node metastasis			< 0.001		< 0.001
Have	89	22.57 ± 4.76		26.73 ± 6.06	
no	108	15.94 ± 5.36		19.77 ± 6.53	
TNM staging			< 0.001		< 0.001
Ⅰ-Ⅱ	108	15.94 ± 5.36		19.77 ± 6.53	
Ⅲ-Ⅳ	89	22.57 ± 4.76		26.73 ± 6.06	
Differentiation			0.540		0.254
Medium + well differentiated	154	18.80 ± 5.98		22.60 ± 7.19	
Poorly differentiated	43	19.44 ± 6.39		24.02 ± 7.19	
Infiltration depth			< 0.001		0.021
T1 + T2	26	10.27 ± 4.70		19.88 ± 5.83	
T3 + T4	171	20.26 ± 5.07		23.37 ± 7.29	
Vascular tumor thrombus			< 0.001		< 0.001

Clinicopathological parameters	cases	CD163 + M2		Foxp3 + Tregs	
		(mean/HP)	P	(mean/HP)	P
Have	85	22.48 ± 4.86		26.00 ± 6.47	
no	112	16.25 ± 5.49		20.57 ± 6.86	
Nerve invasion			< 0.001		< 0.001
Have	79	22.05 ± 5.37		26.06 ± 6.67	
no	118	16.86 ± 5.61		20.81 ± 6.79	
Distant metastasis			< 0.001		< 0.001
M0	172	18.02 ± 5.70		21.76 ± 6.55	
M1	25	25.28 ± 4.61		30.84 ± 6.56	

2.2 CD163 and Foxp3 immunohistochemistry results in each tissue

The main feature of CD163 is that yellow or brown medium particles appear on the membrane of M2 macrophages, while the staining in the cell matrix is weak (Fig. 1A-1D) ;The main feature of Foxp3 is the appearance of brown-yellow particles on the nucleus of Treg cells, which are distributed in various tissues of CRC patients (Fig. 1E-1H). Cancer tissue (Fig. 1B, 1F) stained significantly more than adjacent tissues (Fig. 1A, 1E), and metastatic lymph node tissue (Fig. 1C, 1G) stained significantly more than non-metastatic tissue (Fig. 1D, 1H)

As shown in the figure, the immunohistochemical staining of colorectal cancer and lymph nodes; (A) adjacent tissues and (B) cancerous tissues are membrane staining of CD163, (E) adjacent tissues and (F) cancerous tissues are nuclear staining of Foxp3 ; (C) metastatic lymph nodes and (D) non-metastatic lymph nodes are membrane staining of CD163, (G) metastatic lymph nodes and (H) non-metastatic lymph nodes are nuclear staining of Foxp3. Original magnification ×400

2.3 Correlation analysis between M2 macrophages and Tregs in CRC and lymph nodes

Through comparative analysis (Fig. 1), we found that M2 type macrophages and Tregs are abundantly distributed in CRC and lymph nodes. In order to verify whether there is a certain correlation between the two in the development of colorectal cancer and lymph node metastasis, we used Spearman correlation analysis to find: (Fig. 2a and 2b) M2 type macrophages and Tregs are both present in CRC and lymph nodes. Positive correlation ($r = 0.269$, $P < 0.001$; $r = 0.541$, $p < 0.001$).

(Figure a) Correlation between M2 macrophages and Tregs in tumor tissue ($r = 0.269$, $P < 0.001$); (Figure b) Correlation between M2 macrophages and Tregs in lymph node tissue ($r = 0.541$, $P < 0.001$)

2.4 Differences in the expression of M2 macrophages and Tregs in various tissues

We observed (Figs. 3A and 3B) that the medians of M2 macrophages and Tregs in colorectal cancer tissues were 19/HP and 23/HP, respectively; significantly higher than 7/HP and 10/HP in adjacent tissues HP, ($P < 0.05$). It is worth noting that in the adjacent tissues (Fig. 3C), the median of M2 macrophages stage I+II was 5/HP lower than 9/HP in stage I+II , ($P < 0.05$); however, (Fig. 3D) The median of Tregs in stage I+II was 10/HP higher than 9/HP in stage I+II , ($P < 0.05$). In order to further analyze the prognosis of M2 macrophages and Tregs in colorectal cancer patients, we counted their lymph nodes, It was found (Figs. 3E and 3F) that the medians of M2 macrophages and Tregs in group A were 9/HP and 10/HP, respectively, significantly lower than 16/HP and 18/HP in group B, ($P < 0.05$); however; It is unclear whether the number of M2 macrophages and Tregs in the lymph nodes of patients with stage I + II is different from the number of M2 macrophages and Tregs in the non-metastatic lymph nodes of patients with stage III + IV. Therefore, we counted the M2 macrophages in group A and C, and found (Fig. 3G) that the median of M2 macrophages in group A was 9/HP lower than 10/HP in group C, ($P < 0.05$). (Fig. 3H) The median of Tregs in group A and group C were both 10/HP, ($P > 0.05$).

The Mann-Whitney U test was used to analyze the statistical differences between the two groups. (Figures A and B) M2 macrophages and Tregs expressed in colorectal cancer tissues were significantly higher than those in adjacent tissues, and the differences were statistically significant ($***P < 0.001$; $***P < 0.001$); In paracancerous tissues (Figure C) the number of M2 macrophages expressed in stage I+II was lower than that in stage I+II , (Figure D) the number of expressions of Tregs in stage I+II was higher than that in stage I+II . The difference was both There is statistical significance ($***P < 0.001$; $**P < 0.01$). (Figures E and F) The expression of M2 macrophages and Tregs in group A was significantly lower than that in group B, and the differences were statistically significant ($***P < 0.001$; $***P < 0.001$); in non-metastatic lymph nodes (Figure G) The expression of M2 macrophages in group A was lower than that in group C, and the difference was statistically significant ($*P < 0.05$). (Figure H) There was no significant difference in Tregs between group A and group C. No statistical significance ($P > 0.05$).

2.5 Correlation analysis between the number of M2 macrophages and Tregs in CRC and tumor markers

In order to analyze the influence of M2 macrophages and Tregs on the condition and prognosis of CRC patients, we used Spearman correlation analysis to evaluate the correlation between the number of M2 macrophages and Tregs and the preoperative CEA, CA199, and CA724 concentrations. as shown in Table 3, It was found that the number of M2 macrophages was positively correlated with the concentration of CEA and CA199 before surgery ($P < 0.001$; $P < 0.05$); it has nothing to do with the concentration of CA724 before surgery ($P > 0.05$). The number of Tregs is positively correlated with the preoperative CEA concentration ($P < 0.001$); it has nothing to do with the preoperative CA199 and CA724 concentrations ($P > 0.05$).

Table 3
Correlation analysis between the number of M2 macrophages, Tregs and tumor markers

Tumor markers	Number of M2 macrophages		Number of Tregs	
	Spearman correlation coefficient	P value	Spearman correlation coefficient	P value
CEA level before surgery	0.464	< 0.001	0.365	< 0.001
CA199 level before surgery	0.170	0.017	0.089	0.211
CA724 level before surgery	0.095	0.182	0.050	0.486

3. Discussion

Immune cells in the tumor microenvironment play a vital role in the occurrence and development of tumors [8]. We performed immunohistochemical staining on 197 cases of colorectal cancer tissue and surrounding lymph nodes. Mainly study the expression of M2 macrophages and Tregs in the microenvironment of colorectal cancer and the expression of each group of lymph nodes. It was found that the number of expressions of M2 macrophages and Tregs was significantly reduced compared with cancer tissues. The number of M2 macrophages of stage ≥ 4 in the adjacent tissues was significantly more than that of stage ≥ 3 , but the number of stage ≥ 4 in Tregs was less than that of stage ≥ 3 . In the lymph nodes, we found that compared with the metastatic lymph node tissue, the number of the two types of cells in the non-metastatic nodes was significantly reduced. In non-metastatic lymph nodes, the number of M2 macrophages in stage ≥ 4 was significantly higher than that in stage ≥ 3 , but there was no significant difference in the number of Tregs. In addition, our research also found that the number of expressions of these two types of cells in cancer tissues and lymph node tissues is significantly positively correlated.

Macrophages have functional and phenotypic plasticity that can be categorized into M1 and M2 macrophages[9]. M1 macrophages and M2 macrophages play opposite roles in the tumor microenvironment. The increase in the ratio of M1 to M2 in colorectal cancer is closely related to the enhancement of tumor cell invasion [10, 11]. Tumor-derived cells secrete granulocyte macrophage colony-stimulating factor, monocyte chemotactic protein, and so on. These factors are related to the non-classical activation pathway of macrophages, which promotes the differentiation of monocytes into M2 macrophages, which increases their expression in tumor tissues [12]. In our study, the expression level of M2 macrophages in tumor tissues is significantly up-regulated, and it is closely related to tumor TMN staging, lymph node metastasis and depth of invasion. These results indicated that M2 macrophages are involved in the formation of immunosuppressive tumor microenvironment, which is one of the important factors in tumor microenvironments that cause the poor prognosis of patients. Lian et al. [13] also noted

that colon cancer cells secrete EGF, which can bind to EGFR on monocytes, activate the smad-PI3K-Akt-MTOR pathway, and promote the differentiation of monocytes into M2 macrophages. The number of M2 macrophages of stage $\text{I}+\text{II}$ in the adjacent tissues is significantly more than that of stage $\text{III}+\text{IV}$. In our analysis, with the proliferation of the tumour leads to increased metabolism and uneven vascularization around it, so the lack of blood vessels in the tumor area causes tumor hypoxia. The main cellular response to hypoxia is mediated by HIF-1 α and HIF-2 α . The above factors can increase the expression of miRNAs through the PI3K/AKT/mTOR pathway and promote the polarization of M2 cells[14–16]. In the lymph node tissue, it merits our attention. The number of M2 macrophages in non-metastatic lymph nodes of stage $\text{I}+\text{II}$ was significantly higher than that of stage $\text{III}+\text{IV}$. In the lymph node tissue deserves our attention that the number of M2 macrophages in the non-metastatic lymph nodes of stage $\text{I}+\text{II}$ is significantly higher than that in stage $\text{III}+\text{IV}$. It was suggested that in patients with metastatic lymph nodes, there are more infiltration of M2 macrophages in the part where tumor metastasis has not occurred, indicating that the lymph node microenvironment has changed before metastasis, and M2 macrophages are involved, which is the same as our previous research results [17]. Therefore, we speculate that M2 macrophages play an important role in metastasis of lymph node colorectal cancer, and it indicated that poor prognosis. Tacconi C et al. [18] pointed out that VEGF-C can promote the proliferation and expansion of lymphatic vessels, thereby increasing the way for the metastatic spread of tumor cells to lymph nodes. Thus, M2 macrophages may change the tumor microenvironment and promote colorectal cancer lymph node metastasis [17, 19].

So far, the role of Treg cells in CRC is controversial. The main reason for our analysis is that Tregs have duality in CRC. This duality may be affected by many factors, including the role of Tregs in the occurrence and development of CRC is not clear, such as the function and distribution in the development of the disease. And changes in the number, including external factors (such as treatment) interference. In order to study the potential functions of Tregs in CRC. We analyzed the correlation between Tregs and clinicopathological characteristics, and divide CRC into early stage ($\text{I}+\text{II}$ stage) and late stage ($\text{III}+\text{IV}$ stage) for discussion. We found that Tregs in CRC are closely related to TNM staging, lymph node metastasis, and distant metastasis. TNM staging and lymph node metastasis are important indicators for judging the prognosis and survival time of tumor patients. Thus, our results indicate that Tregs infiltration is related to metastasis and poor prognosis of colorectal cancer. Macrophage-derived chemokine CCL22, which can bind to the CCR4 receptor highly expressed on the surface of FOXP3 + Tregs, thereby helping to recruit Treg cells to tumor tissues [2, 20]. It is noteworthy that we found that the number of Tregs in the late stage (stage $\text{III} + \text{IV}$) adjacent to the cancer is less than that in the early stage (stage $\text{I} + \text{II}$). We analyzed that the dysregulation of colonic inflammatory response is an important inducement for the development of CRC. The increased inflammation in adjacent tissues in the early stage of CRC promotes the continuous accumulation of Treg cells to inhibit inflammation [21, 22]. Märkl et al. [23] analyzed the specimens of 136 patients with early-stage (stage I, II) colon cancer and found that Tregs infiltrated more cancerous tissues than adjacent tissues, and high Tregs values in adjacent tissues suggested a better prognosis.

In our study, we found that there is a certain correlation between M2 macrophages and Tregs in CRC and lymph nodes. The possible explanation is that M2 macrophages secrete immune suppressive cytokines and chemokines. These cytokines and chemokines participate in the recruitment of lymphocytes and stimulate them to develop into Tregs [2, 24]. In addition, Tregs produce high levels of IL-10, IL-32 and TGF- β , which further inhibit the anti-tumor inflammatory response and stimulate M2 macrophages to increase the production of cytokines and chemokines, thereby being able to recruit additional Tregs[25]. Studies have pointed out [26] that M2 macrophages and Tregs have a synergistic effect in promoting the proliferation, tumor angiogenesis ,and metastasis of ovarian cancer. Sun et al. [27] studied 65 patients with laryngeal squamous cell carcinoma (LSCC) and found that Tregs and M2 macrophages in LSCC were positively correlated with each other, and proved that the two formed a positive feedback loop. Therefore, we speculate that M2 macrophages in CRC may have the ability to induce the formation of Tregs, which will increase the expression of Tregs in tumor tissues. The interaction between the two may change the tumor microenvironment and promote the development of CRC and lymph node metastasis. Because our experiment is relatively limited, the correlation between M2 macrophages and Tregs and its specific mechanism need to be further studied. In addition, studies have pointed out [28, 29] that CD163 can be used as a potential prognostic biomarker for CRC patients, and Foxp3 can directly affect the prognosis of CRC patients. In order to further verify the impact of the two on the monitoring and prognosis of CRC patients, our study analyzed the correlation between the levels of CEA, CA199 and CA724 before surgery and the number of M2 macrophages and Tregs. CEA level is closely related to the number of M2 macrophages and Tregs, and the correlation coefficient is higher than CA199 and CA724 levels. Therefore, we speculate that M2 macrophages and Tregs have a close relationship with CEA, which is expected to become an important observation index for monitoring the condition of colorectal cancer and judging the prognosis.

In summary, M2 macrophages participate in the formation of lymph node immunosuppressive environment and may promote the development of CRC and lymph node metastasis together with Tregs. Our results provide some insights into the role of M2 macrophages and Tregs in colorectal cancer and its lymph node metastasis. M2 macrophages and Tregs are up-regulated in CRC, which is expected to be an effective indicator for monitoring the condition of CRC and judging the prognosis.

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the Ethics Committee of the First Affiliated Hospital of Jinzhou Medical University and the 1964 Helsinki Declaration. Informed consent was obtained from all participants included in the study.

Consent for publication

Not applicable

Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request. Inquiries for data access may be sent to the following e-mail address: shifengqiao2020@163.com.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

Y-LC, X-QM, C-YY and YW performed the experiments. Y-LC, Y-PW and J-HL participated in collecting the data and drafted the manuscript. YS, X-YS and YF contributed to the statistical analysis and manuscript writing. S-FQ conceived the present study and helped revise the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

The main feature of CD163 is that yellow or brown medium particles appear on the membrane of M2 macrophages, while the staining in the cell matrix is weak (Figure 1A-1D) ;The main feature of Foxp3 is the appearance of brown-yellow particles on the nucleus of Treg cells, which are distributed in various tissues of CRC patients (Figure 1E-1H). Cancer tissue (Figure 1B, 1F) stained significantly more than

adjacent tissues (Figure 1A, 1E), and metastatic lymph node tissue (Figure 1C, 1G) stained significantly more than non-metastatic tissue (Figure 1D, 1H)

a

b

Figure 2

In order to verify whether there is a certain correlation between the two in the development of colorectal cancer and lymph node metastasis, we used Spearman correlation analysis to find: (Figure 2a and 2b) M2 type macrophages and Tregs are both present in CRC and lymph nodes. Positive correlation ($r=0.269$, $P<0.001$; $r=0.541$, $p<0.001$).

Figure 3

We observed (Figures 3A and 3B) that the medians of M2 macrophages and Tregs in colorectal cancer tissues were 19/HP and 23/HP, respectively; significantly higher than 7/HP and 10/HP in adjacent tissues HP, ($P<0.05$). It is worth noting that in the adjacent tissues (Figure 3C), the median of M2 macrophages stage I+II was 5/HP lower than 9/HP in stage III+IV, ($P<0.05$); however, (Figure 3D) The median of Tregs in stage I+II was 10/HP higher than 9/HP in stage III+IV, ($P<0.05$). In order to further analyze the prognosis of M2 macrophages and Tregs in colorectal cancer patients, we counted their lymph nodes, It was found (Figures 3E and 3F) that the medians of M2 macrophages and Tregs in group A were 9/HP and 10/HP, respectively, significantly lower than 16/HP and 18/HP in group B, ($P<0.05$); however; It is unclear whether the number of M2 macrophages and Tregs in the lymph nodes of patients with stage I+II is different from the number of M2 macrophages and Tregs in the non-metastatic lymph nodes of patients with stage III+IV. Therefore, we counted the M2 macrophages in group A and C, and found (Figure 3G) that the median of M2 macrophages in group A was 9/HP lower than 10/HP in group C, ($P<0.05$). (Figure 3H) The median of Tregs in group A and group C were both 10/HP, ($P>0.05$).