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ORIGINAL RESEARCH

M2-Type Macrophages and Cancer-Associated Fibroblasts Combine to Promote Colorectal Cancer Liver Metastases

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Purpose: This research explored the association between CD163-labeled M2-type macrophages and cancer-associated fibroblasts (CAFs) in the tumor microenvironment (TME) of 38 colorectal cancer (CRC) liver metastases. In addition, we investigated the correlation differences between M2-type macrophages and CAFs in the tumor microenvironments of 38 primary colorectal cancer patients with confirmed liver metastases and 946 colorectal cancer patients, as well as possible mechanisms of action between the two cells.

Methods: The Immunohistochemistry (IHC) method was applied to detect the expression levels of M2-type macrophages and CAFs in the tissues of 984 cases of CRC and to analyze the correlation between M2-type macrophages and CAFs in colorectal cancer tissues. The IHC method was also applied to detect the expression levels of M2-type macrophages and CAFs in the liver metastases of 38 cases of CRC in the experimental group and to analyze the correlation between the two cells in liver metastases.

Results: 1. M2-type macrophages and CAFs expression were significantly higher in 38 primary colorectal cancer patients compared to 946 controls, and the expression of M2-type macrophages was significantly positively correlated with CAFs. 2. In 984 CRC cases, M2-type macrophages and CAFs expression levels were significantly higher in the cancer tissues than in the paired paracancerous tissues. 3. The expression levels of M2-type macrophages and CAFs in primary colorectal cancer were significantly higher in the experimental group than in colorectal cancer tissues without distant metastasis.

Conclusion: M2-type macrophages and CAFs are involved in the development of the colorectal cancer tumor microenvironment, and their interaction influences the initiation and progression of liver metastasis in colorectal cancer. It may provide new clinical ideas for early diagnosis of CRC liver metastases and searching for immune targets.

Keywords: M2-type macrophages, cancer-associated fibroblasts, colorectal cancer, liver metastasis

Introduction

Colorectal cancer is the third most common cancer type globally and the second leading cause of cancer-related deaths.[1] Extensive research has revealed that the TME is composed of an extracellular matrix and a variety of cell types,[2] immune cells, including tumor-associated macrophages (TAMs), and stromal cells such as CAFs, endothelial cells, and others.[3] CRC tissues coexist with immune cells and stroma in a complex TME,[4] significantly impacting colorectal cancer progression and metastasis due to various cellular interactions.[5,6]

TAMs can be divided into two distinct types, M1 and M2. Among them, M2-type macrophages mainly exhibit immunosuppressive and tumor-promoting effects. The expression of IL-4, IL-13, transforming growth factor-β (TGF-β), and IL-10 has been linked to increased tumor differentiation, lymph node metastasis, and hepatic metastatic ability.[7] CD163 is considered to be the predominant specific marker for M2-type macrophages.[8] M2-type macrophages can

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promote angiogenesis and extracellular matrix degradation by enhancing epithelial-mesenchymal transition (EMT) and secretion of vascular endothelial growth factor (VEGF) for cancer metastasis.[9,10]

Fibroblasts play a crucial role[11] in the TME and are the most active cell types in the stroma.[12] Fibroblasts can be divided into CAFs and myofibroblasts. α-Smooth muscle actin (α-SMA) and fibroblast-specific protein 1 (FSP-1) are the most common markers of CAF.[13] It has been shown that CAFs in CRC express higher levels of α-SMA,[14] and the combined detection of several markers can maximize the screening of CAFs.[15] CAFs is frequently shown to promote tumor growth and progression, favorably influencing the establishment of various tumor-specific mechanisms.[16]

M2-type macrophages can interact with CAFs and are associated with cell counts. The interrelationship between M2-type macrophages and CAFs has been confirmed in studies such as pancreatic cancer[17] and ovarian cancer[18] and could serve as a combinatorial predictor of disease progression in patients with CRC in colorectal cancer.[19] Our study analyzed the relationship between the number of M2-type macrophages and CAFs in liver metastases and primary foci in 38 CRC patients with liver metastases by IHC methods. Combining the quantitative relationship between M2-type macrophages and CAFs in the samples of 946 CRC patients without liver metastasis, we analyzed the correlation between the two cells in liver metastasis of colorectal cancer to provide a new diagnostic and therapeutic idea for liver metastasis of CRC.

Materials and Methods

A total of 1158 patients with colorectal malignant tumors diagnosed in the Second Ward of Colorectal Surgery, The First Affiliated Hospital of Jinzhou Medical University from January 2017 to July 2023, were collected, and 984 patients passed the inclusion and exclusion criteria. The primary clinical data and postoperative pathological tissue specimens of these 984 patients were divided into the experimental and control groups for comparison. Among them, 38 samples with a confirmed diagnosis of colorectal cancer liver metastasis and surgical treatment were defined as the experimental group (Table 1); 946 cases of Stage I, II, and III patients who with a confirmed diagnosis of colorectal cancer, and excluded liver metastasis by abdominal enhanced CT examination were defined as the control group (Table 2). Experimental group

Table 1 Clinical Characteristics of 38 CRC Patients in the Experimental Group (According to Primary Foci)

Clinical Information	n=38	%
Age (years)		
<65	16	42.11
≥65	22	57.89
Gender		
Male	29	76.32
Female	9	23.68
Histologic type		
Tubular adenocarcinoma	34	89.47
Mucinous carcinoma	4	10.53
Infiltration depth		
T3	18	47.37
T4	20	52.63
Pathological type		
Ulcer type	32	84.21
Exogenous type	6	15.79
Tumor maximum diameter (cm)		
<5	21	55.26
≥5	17	44.74
Differentiation degree		
Moderate and High	31	81.58
Low	7	18.42

(*Continued*)

Table 1 (Continued).

Clinical Information	n=38	%
Vascular invasion		
Present	26	68.42
Absent	12	31.58
Nerve invasion		
Present	16	42.11
Absent	22	57.89
Lymph node metastases		
Present	27	71.05
Absent	11	28.95

Table 2 Clinical Characteristics of 946 CRC Patients in the Control Group

Clinical Information	n=946	%
Age (years)		
<65	428	45.24
≥65	518	54.76
Gender		
Male	544	57.51
Female	402	42.49
Histologic type		
Tubular adenocarcinoma	892	94.29
Mucinous carcinoma	54	5.71
TNM staging		
T1+T2	161	17.02
T3+T4	785	82.98
Pathological type		
Ulcer type	740	78.22
Exogenous type	206	21.78
Tumor maximum diameter (cm)		
<5	469	49.58
≥5	477	50.42
Differentiation degree		
Moderate and High	753	79.60
Low	193	20.40
Vascular invasion		
Present	240	25.37
Absent	706	74.63
Nerve invasion		
Present	150	15.86
Absent	796	84.14
Lymph node metastases		
Present	314	33.19
Absent	632	66.81

patients with postoperative pathological tissue specimens of metastatic foci were divided into liver metastasis group and liver metastasis paracancerous group; primary foci were divided into two groups: colorectal cancer group and paracancerous tissue group. In the control group, Postoperative pathological tissue specimens were divided into colorectal cancer and the paracancerous tissue group.

Experimental Methods

Pathology samples: Cancer tissue was selected from the non-necrotic area in the center of the cancer lesion, and paracancerous tissue was selected from an area 3–5 cm from the cancer margin.

1. Paraffin sections were deparaffinized in a thermostat. They were placed sequentially in xylene I for 5 minutes, xylene II for 5 minutes, anhydrous ethanol I for 5 minutes, 95% alcohol for 5 minutes, and 75% alcohol for 5 minutes.
2. Warm up the EDTA Antigen Retrieval Solution PH=8.0 (ZLI-9072, ZSGB-Bio, Beijing, China) in a water bath within an autoclave until it reaches the boiling point, then transfer the sections into the autoclave and proceed with heating for 10 minutes. Remove the heat source, remove the sections, cool to room temperature in a bucket of cool water, and circle the location of the tissues. Rinse with PBS 3 times, each time at an interval of 3 minutes.
3. Shake off PBS, add endogenous Peroxidase-blocking Solution (KIT-9710, Maixin-Bio, Fuzhou, China), and incubate at room temperature for 10 min. Wash with PBS three times, and leave for 3 min each time. Wash with PBS 3 times and incubate for 3 minutes each time. Discard PBS, incorporate a non-specific staining blocker (KIT-9710, Maixin-Bio, Fuzhou, China) gradually, and allow to incubate for 10 minutes at ambient temperature.
4. shake off the residual reagent and add the Anti-CD163 antibody (1:500, ab182422, Abcam, Cambridge, UK), Anti-alpha smooth muscle Actin antibody (1:500, ab7817, Abcam, Cambridge, UK), and Anti-S100A4 antibody (1:2000, Ab197896, Abcam, Cambridge, UK) dropwise, respectively, and incubate at 4°C for 16 hours.
5. After incubation, leave room temperature for 30 minutes to return to room temperature. Wash with PBS 3 times, 3 minutes apart. Wash with PBS 3 times at 3-minute intervals. Remove the PBS, add Biotin-Labeled Goat Anti-Rabbit/Mouse IgG polymer (KIT-9710, Maixin-Bio, Fuzhou, China), and incubate for 10 minutes at room temperature. Wash again with PBS 3 times, each time at 3-minute intervals. After removing the PBS, Streptomyces antibiotic protein-peroxidase was gradually introduced and incubated for 10 minutes at ambient temperature.
6. Wash with PBS for 3x3min, remove PBS, add DAB (DAB-0031, Maixin-Bio, Fuzhou, China) color development solution dropwise, and leave for 5min at room temperature.
7. After washing with PBS 3 times at 3-minute intervals, remove PBS, add DAB color development solution dropwise, and leave for 5 minutes at room temperature.
8. Place the paraffin sections in the following experimental reagents in order: 75% alcohol for 5 minutes, 95% alcohol for 5 minutes, anhydrous ethanol for 5 minutes, xylene I for 5 minutes, and xylene II for 5 minutes. After the task is finished, the resin is sealed neutrally.

Judgment of Staining Results

Five 400x field of view images were randomly intercepted under the microscope from the immunohistochemically stained sections. Image J was applied to determine the positive cells, and two pathologists scored the counts under double-blind conditions. The average of the counting scores from the two pathologists was the final score counted for statistical purposes.

Known positive results were used as positive controls, and PBS was used instead of primary antibody as negative control. Positive staining was determined when yellow or brown material appeared within the cell membrane or cytoplasm of M2-type macrophages or CAF; the average number of cells staining positive for CD163 antibody in five fields of view was calculated as the final score.

The average value of the staining index of 5 visual fields of CAFs stained by α-SMA and FSP-1 antibody was used as the final score. Staining index = staining intensity x proportion of positive cells. Percentage of positive cells: Score 0, 0–10%; Score 1, 11–25%; Score 2, 26–40%; Score 3, 41–75%; Score 4, 76–100%. Staining intensity:0 points, basically no coloration; 1 point, light yellow; 2 points, dark yellow; 3 points, brown.

The final scores are all bounded by the median, with greater than or equal to that bound being a high expression and less than a low expression.

Data were analyzed using SPSS 27.0 software and GraphPad Prism 9. The measured results were compared by independent sample *t*-test and non-parametric test. Counting data was analyzed with the χ^2-test and compared with Spearman correlation and linear regression analyses. The disparities were deemed statistically significant when the P value was less than 0.05.

Results

Expression of CD163, α-SMA and FSP-1 in Different Colorectal Tissues

According to the results of IHC experiments, CD163 was mainly expressed in the cell membrane and cytoplasm of M2-type macrophages in the tumor stroma. In the experimental group, brown and dark yellow coloration was predominant in colorectal cancer primary foci. In contrast, in the control group, dark yellow coloration was dominant in colorectal cancer tissues and was less in number than in the experimental group.

α-SMA and FSP-1 are predominantly expressed in CAFs' cytoplasm and cell membrane. In the experimental group, the primary foci of colorectal cancer with liver metastasis showed dark yellow or brown color. In contrast, the expression in colorectal cancer tissues without liver metastasis in the control group was more frequent in dark yellow. In quantity, the experimental group was significantly more than the control group.

We experimentally found that when liver metastasis of colorectal cancer occurred, the increase in the expression number of CD163 with α-SMA and FSP-1 was more pronounced, suggesting that it was closely related to liver metastasis of colorectal cancer (Figures 1 and 2).

Expression of CD163, α-SMA and FSP-1 in Liver Metastasis Tissues

CD163 was expressed in liver metastases in dark yellow or brown color, whereas its paired paracancerous tissues stained in smaller amounts and mainly were light yellow. Meanwhile, liver metastasis tissues in the experimental group were stained by α-SMA and FSP-1 in a larger area and dark yellow or brown color. In contrast, in the paracancerous tissues paired with them, the staining size was smaller and in light yellow color or no staining (Figure 3).

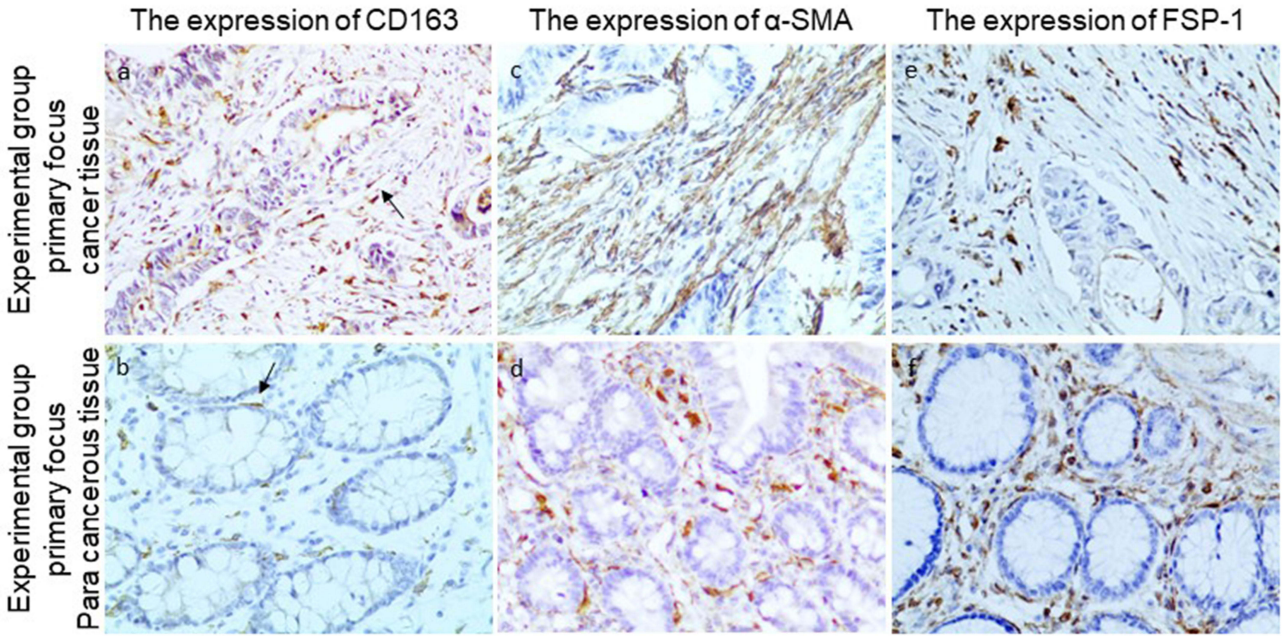

Figure 1 Representative immunohistochemical staining of CD163, α-SMA, and FSP-1 in the experimental group's primary focal cancer and paracancerous tissues (200x field). (**a**) Expression of CD163 in primary focus cancer tissues of the experimental group (Arrows mark M2-type macrophages). (**b**) Expression of CD163 in paracarcinoma tissues of primary foci in the experimental group (Arrows mark M2-type macrophages). (**c**) Expression of α-SMA in primary focal cancer tissues of the experimental group. (**d**) Expression of α-SMA in primary foci of paracancerous tissues in the experimental group. (**e**) Expression of FSP-1 in primary foci cancer tissues of the experimental group. (**f**) Expression of FSP-1 in primary foci of paracarcinoma tissues in the experimental group.

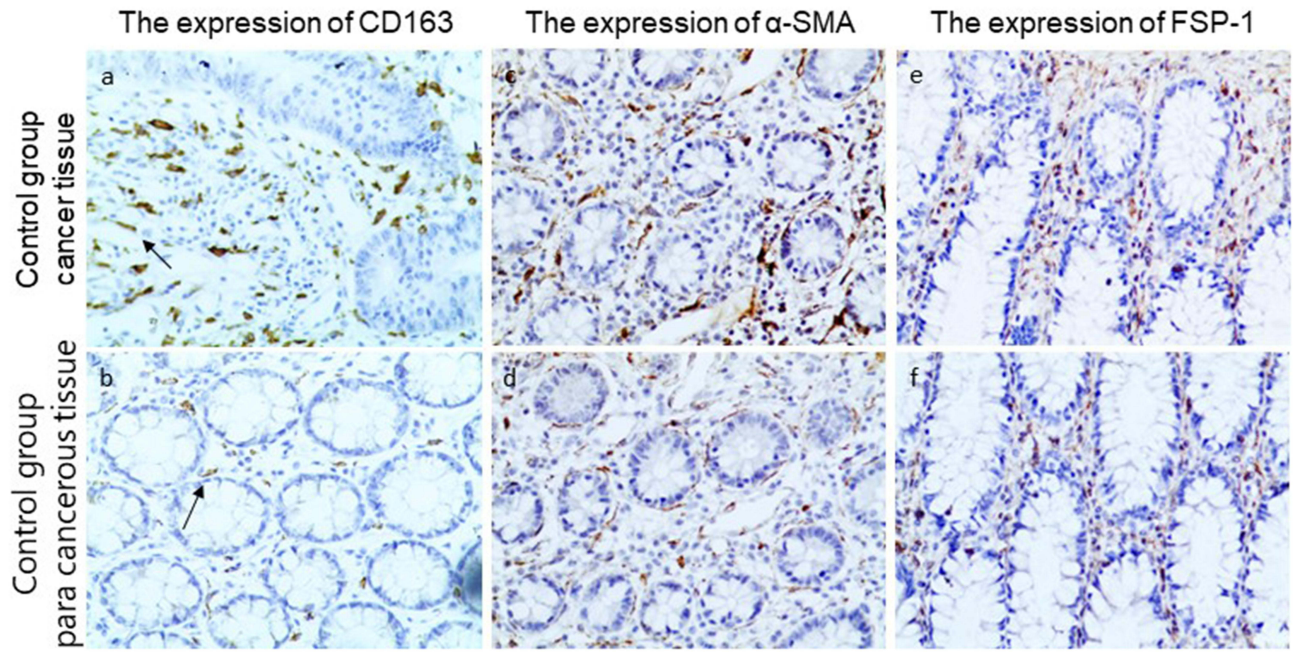

Figure 2 Representative immunohistochemical staining of CD163, α-SMA, and FSP-1 in control cancer tissues and paracancerous tissues (200x field). (**a**) CD163 expression in control CRC tissues (Arrows mark M2-type macrophages). (**b**) CD163 expression in control CRC paracancer tissues (Arrows mark M2-type macrophages). (**c**) Expression of α-SMA in control CRC tissues. (**d**) Expression of α-SMA in control CRC paracancer tissues. (**e**) Expression of FSP-1 in accurate control CRC tissues. (**f**) Expression of FSP-1 in control CRC paracancer tissues.

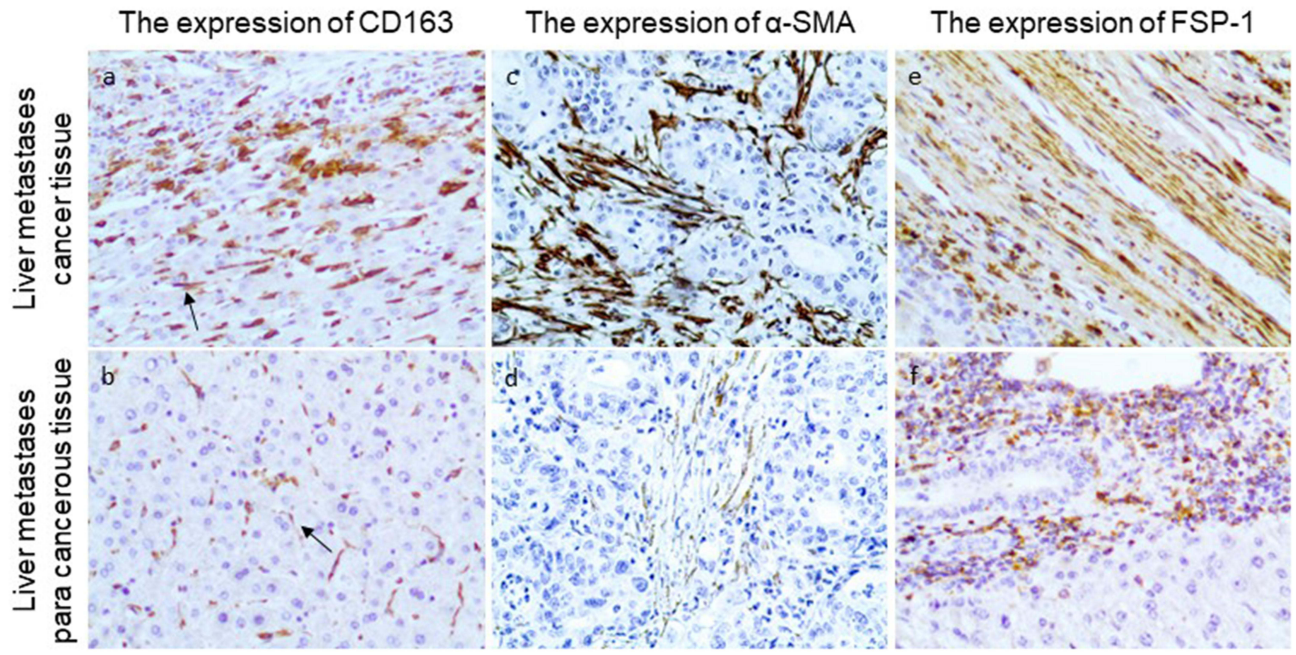

Figure 3 Representative immunohistochemical staining of CD163, α-SMA, and FSP-1 in cancer tissues and paracancerous tissues of liver metastases in the experimental group (200x field of view). (**a**) CD163 expression in liver metastases cancer tissues of the experimental group (Arrows mark M2-type macrophages). (**b**) Expression of CD163 in paracancerous tissues of liver metastases in the experimental group (Arrows mark M2-type macrophages). (**c**) Expression of α-SMA in cancerous tissues of liver metastases in the experimental group. (**d**) Expression of α-SMA in paracancerous tissues of liver metastases in the experimental group. (**e**) Expression of FSP-1 in cancerous tissues of liver metastases in the experimental group. (**f**) Expression of FSP-1 in the paracancerous tissues of liver metastases in the experimental group.

Statistical Analysis of the Differences in the Expression of CD163, α-SMA, and FSP-1 in Different Colorectal Cancer Tissues

1. Independent samples *t*-test was applied to analyze the differences in the expression of CD163 (t=13.661 P=0.001), α-SMA (t=16.449 P=0.001), and FSP-1 (t=12.993 P=0.001) in primary foci colorectal cancer tissues and paracancerous tissues of the experimental group. All differences were statistically significant (Figure 4).
2. Non-parametric tests were applied to analyze the differences in the expression of CD163 (Z=−29.201 P=0.001), α-SMA (Z=−23.965 P=0.001) and FSP-1 (Z=−19.998 P=0.001) in CRC tissues and paracancerous tissues in the control group. All differences were statistically significant (Figure 5).
3. Non-parametric tests were applied to analyze the differences in the expression of CD163 (Z=−5.221 P=0.001), α-SMA (Z=−6.463 P=0.001) 和FSP-1 (Z=−5.272 P=0.001) in the primary foci colorectal cancer tissues of the experimental group and the control colorectal cancer tissues. Analysis of the results showed that all the increases in the experimental group were significantly more than those in the control group, and the differences were statistically significant (Figure 6).

Statistical Analysis of the Differences in the Expression of CD163, α-SMA, and FSP-1 in Colorectal Cancer Liver Metastasis Tissues

Independent samples *t*-test was applied to analyze the difference in the expression of CD163 (t=13.241 P=0.001), α-SMA (t=20.592 P=0.001), and FSP-1 (t=13.771 P=0.001) in liver metastasis tissues versus paracancerous tissues of experimental groups. The expression of CD163, α-SMA, and FSP-1 in liver metastasis tissues was significantly more than that in paracancerous tissues, and the differences were all statistically significant (Figure 7).

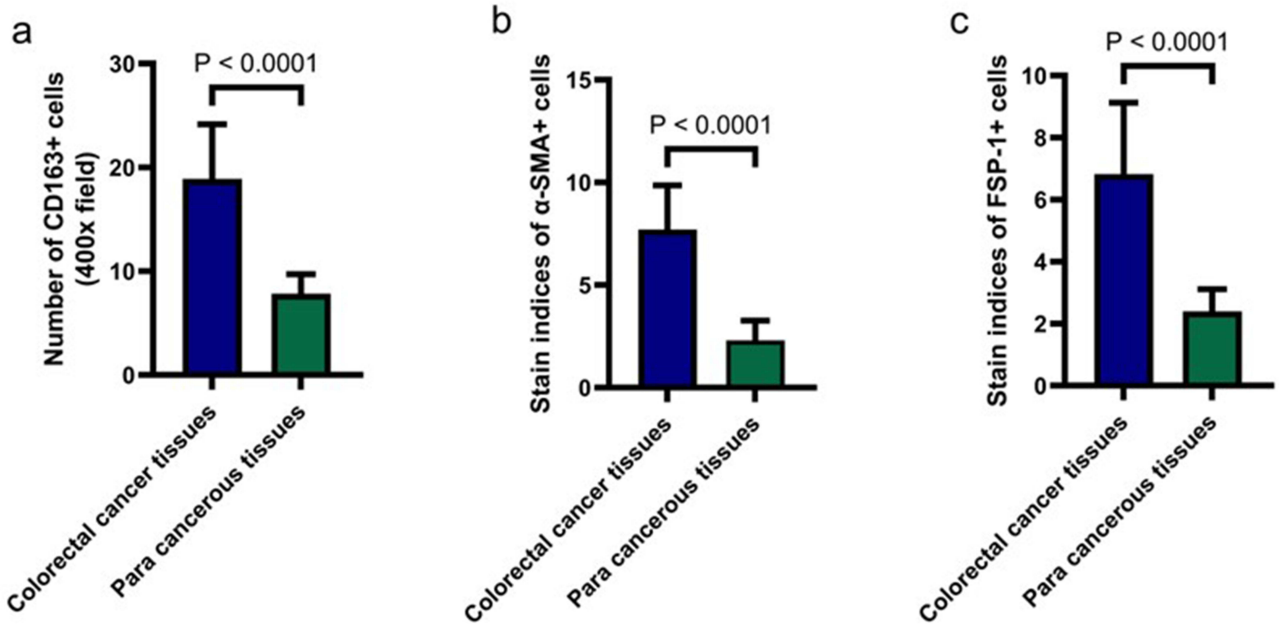

Figure 4 The expression levels of CD163, α-SMA, and FSP-1 were different in paracancerous and cancerous tissues of the primary foci in the experimental groups. (**a**) The number of CD163 in CRC tissues matched paracancerous tissues. (**b**) The staining indices of α-SMA in CRC tissues matched paracancerous tissues. (**c**) The staining indices of FSP-1 in CRC tissues matched paracancerous tissues.

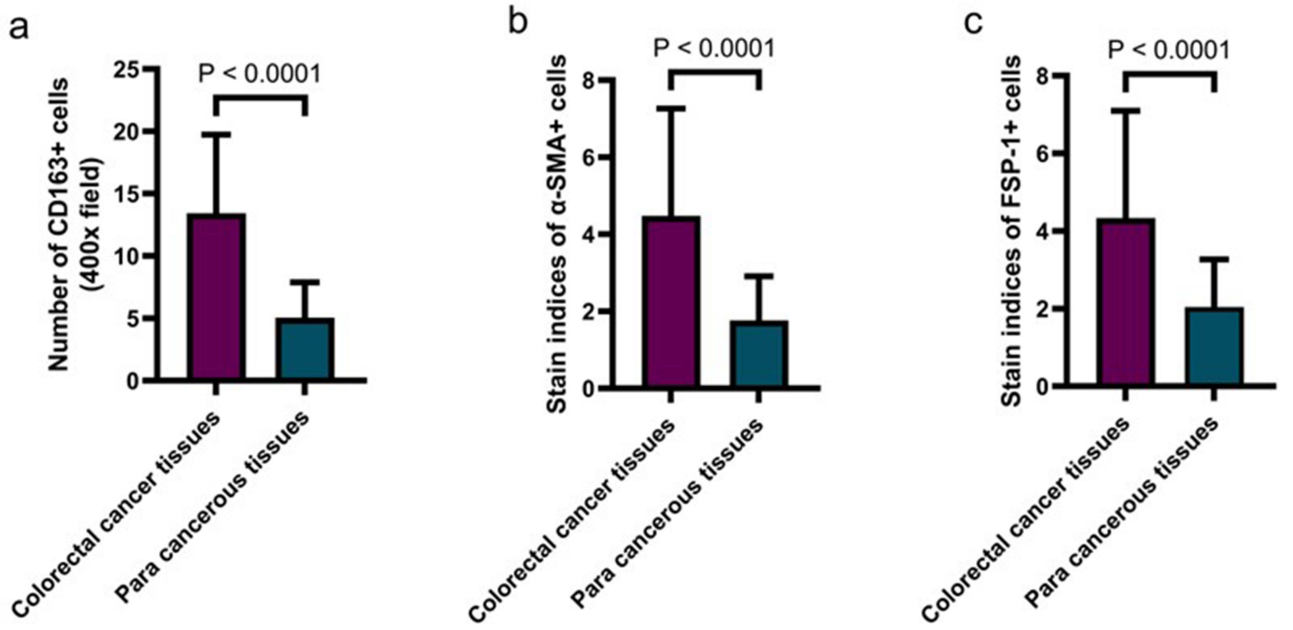

Figure 5 The expression levels of CD163, α-SMA, and FSP-1 were different in paracancerous and cancerous tissues in the control groups. (**a**) The number of CD163 in CRC tissues matched paracancerous tissues. (**b**) The staining indices of α-SMA in CRC tissues matched paracancerous tissues. (**c**) The staining indices of FSP-1 in CRC tissues matched paracancerous tissues.

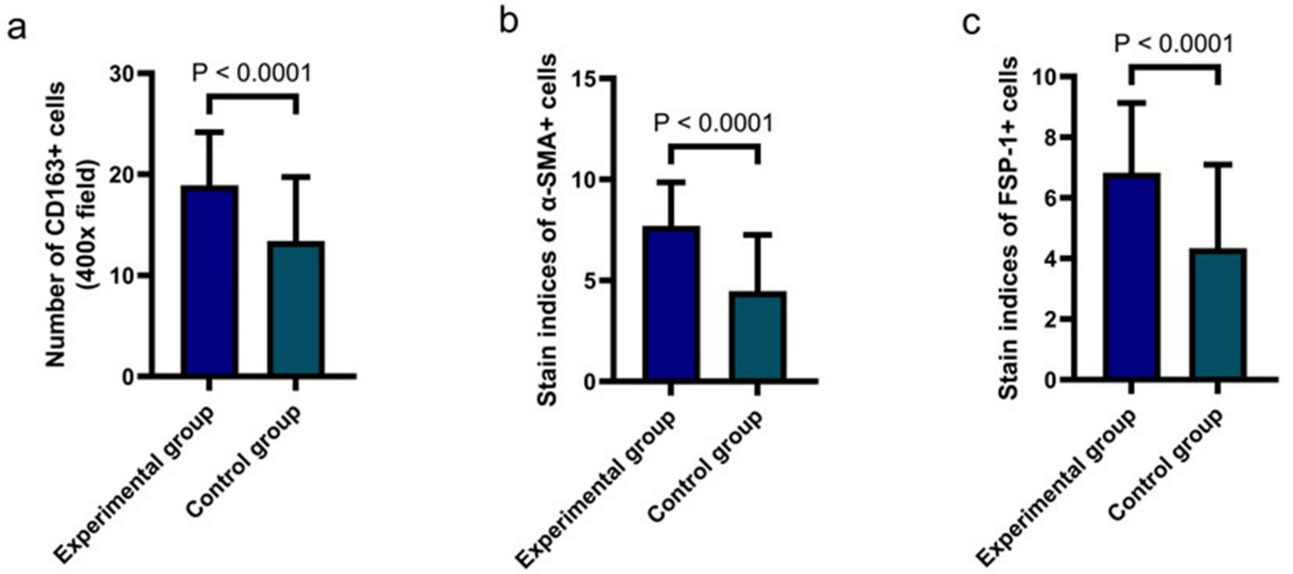

Figure 6 The expression levels of CD163, α-SMA, and FSP-1 were different in the experimental and control groups. (**a**) The number of CD163 in experimental groups matched control groups. (**b**) The staining indices of α-SMA in experimental groups matched control groups. (**c**) The staining indices of FSP-1 in experimental groups matched control groups.

Relationship Between the Expression Levels of CD163 and α-SMA, FSP-1 in Colorectal Cancer Tissues of Experimental and Control Groups Concerning Clinicopathological Parameters

The χ^2-test was applied to compare the relationship between the expression levels of CD163 and α-SMA, FSP-1, and clinicopathological features and then to investigate the possible role of M2-type macrophages and CAFs in colorectal cancer liver metastasis. The results were as follows:

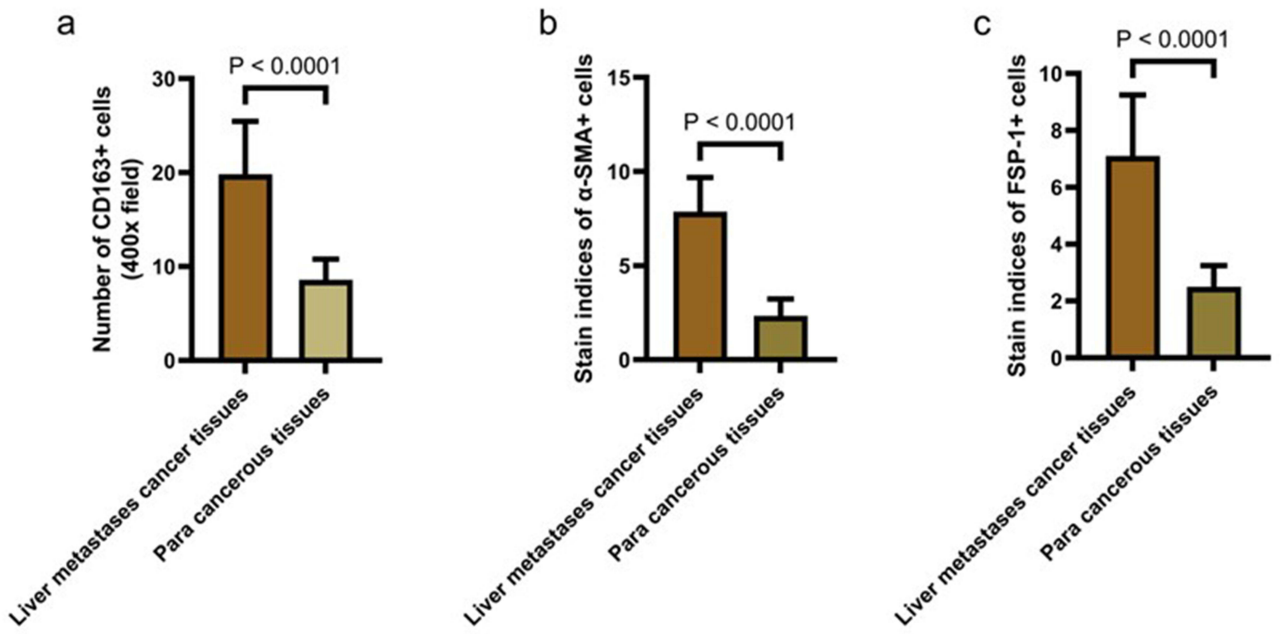

Figure 7 The expression levels of CD163, α-SMA, and FSP-1 were different in paracancerous and cancerous tissues of liver metastases in the experimental group. (**a**) The number of CD163 in liver metastases cancer tissues matched paracancerous tissues. (**b**) The staining indices of α-SMA in liver metastases cancer tissues matched paracancerous tissues. (**c**) The staining indices of FSP-1 in liver metastases cancer tissues matched paracancerous tissues.

1. The expression level CD163 antibody-screened M2-type macrophages in colorectal cancer tissues was independent of tumor pathology type, TNM stage, infiltration depth, maximum tumor diameter, differentiation, presence of bile duct embolism, presence of nerve invasion, presence of lymph node metastasis, and presence of hepatic metastasis, regardless of age, sex, and histological type.
2. The expression levels of CAFs in colorectal cancer tissues co-screened by α-SMA antibody and FSP-1 antibody were correlated with the patient's gender, tumor pathology type, TNM stage, depth of infiltration, maximal diameter of the tumor, degree of differentiation, the presence or absence of cholangiocarcinoma embolus, the presence or absence of neural invasion, lymph node metastasis, and hepatic metastasis. They were not correlated with age, gender, or histology type.

Relationship between the expression of CD163, α-SMA, and FSP-1 and clinicopathologic parameters in colorectal cancer tissues of experimental and control groups (Table 3).

Correlation of CD163, α-SMA, and FSP-1 Expression Levels in Colorectal Cancer Tissues

Spearman correlation analysis and linear regression equations were applied to compare CD163, α-SMA, and FSP-1 expression levels (Figure 8). The findings indicated a strong link between M2-type macrophages and the amount of CAFs (Tables 4 and 5).

Correlation of CD163, α-SMA, and FSP-1 Expression Levels in Colorectal Cancer Liver Metastasis Tissues

CD163, α-SMA, and FSP-1 expression levels were compared using Spearman correlation analysis and linear regression equations (Figure 9). The data indicated a strong link between M2-type macrophages and the amount of CAFs (Tables 6 and 7).

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Table 3 Relationship Between the Expression of CD163, α-SMA, and FSP-1 and Clinicopathologic Characteristics

Clinicopathological Characteristics	N (%) = 984	CD163				α-SMA				FSP-1			
		Low Expression	High Expression	χ^2	P-value	Low Expression	High Expression	χ^2	P-value	Low Expression	High Expression	χ^2	P-value
Age (years)				0.883	0.347			0.023	0.878			0.026	0.871
<65	444	237	207			225	219			235	209		
≥65	540	272	268			271	269			283	257		
Gender				0.113	0.737			0.636	0.425			0.319	0.572
Male	573	299	274			295	278			306	267		
Female	411	210	201			201	210			212	199		
Pathological type				63.458	<0.001			19.042	<0.001			33.730	<0.001
Exogenous type	212	161	51			135	77			149	63		
Ulcer type	772	348	424			361	411			369	403		
Histologic type				1.836	0.175			0.004	0.949			0.021	0.885
Mucinous carcinoma	58	25	33			29	29			30	28		
Tubular adenocarcinoma	926	484	442			467	459			488	438		
TNM staging				113.287	<0.001			49.562	<0.001			60.982	<0.001
T1+T2	161	145	16			122	39			130	31		
T3+T4	823	364	459			374	449			388	435		
Tumor maximum diameter (cm)				10.615	<0.001			14.643	<0.001			10.234	<0.001
<5	490	279	211			277	213			283	207		
≥5	494	230	264			219	275			235	259		
Differentiation degree				17.589	<0.001			18.048	<0.001			5.881	0.015
Moderate and High	784	432	352			422	362			428	356		
Low	200	77	123			74	126			90	110		
Vascular invasion				134.026	<0.001			152.720	<0.001			116.336	<0.001
Present	266	57	209			48	218			65	201		
Absent	718	452	266			448	270			453	265		

Nerve invasion				46.127	<0.001			34.854	<0.001			36.398	<0.001
Present	166	46	120			49	117			52	114		
Absent	818	463	355			447	371			466	352		
Lymph node metastases				256.185	<0.001			307.543	<0.001			227.870	<0.001
Present	341	57	284			41	300			67	274		
Absent	643	452	191			455	188			451	192		
Liver metastases				12.449	<0.001			28.576	<0.001			21.533	<0.001
Present	38	9	29			3	35			6	32		
Absent	946	500	446			493	453			512	434		

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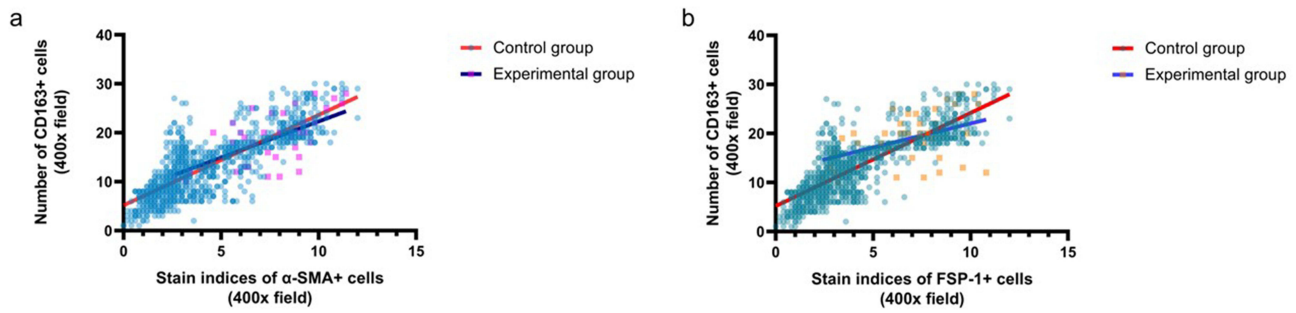

Figure 8 Correlation of CD163, α-SMA, and FSP-1 expression levels in colorectal cancer tissues. (**a**) Correlation between the Number of CD163+ cells and the stain indices of α-SMA+ cells. (**b**) Correlation between the Number of CD163+ cells and the stain indices of FSP-1+ cells.

Correlation Analysis of the Number of M2-Type Macrophages and CAFs with Preoperative Tumor Markers in Colorectal Cancer Tissues

To investigate the correlation between the number of M2-type macrophages and CAFs in colorectal cancer tissues and the expression levels of preoperative tumor markers CEA, CA19-9, and CA72-4, we still analyzed them using Spearman correlation analysis. M2-type macrophages and CAFs were correlated with preoperative CEA, CA19-9, and CA72-4 expression. The number of M2-type macrophages was significantly and positively correlated with the preoperative expression levels of CEA and CA19-9 (Table 8).

Discussion

The tumor microenvironment (TME) is considered one of the drivers of tumor development and invasion; the complex reactions involved have essential implications for tissue homeostasis, immune monitoring, and tumor cell development and metastasis.[20] M2-type macrophages and CAFs are two essential components of the tumor microenvironment, and these two cells are closely related in the occurrence, development, and metastasis of many malignant tumors, including gastric cancer,[21] pancreatic cancer,[17] ovarian cancer,[18] and prostate cancer.[22] We found that liver metastasis of colorectal cancer significantly correlates with the expression of M2-type macrophages and CAFs through IHC experiments. Meanwhile, M2-type macrophages were highly expressed in liver metastases and significantly and positively correlated with CAFs. These findings have important implications for diagnosing and treating liver metastases in patients with CRC and determining prognosis.[19]

In the TME, macrophages tend to be the most plentiful immune system. Tumor cells can generate cytokines like IL-6, IL-10, TGF-β, and PGE2, thereby facilitating the transformation of macrophages into M2-type macrophages with pro-tumorigenic roles.[23] Polarized M2-type macrophages mainly express helper T cell type 2 (Th2) cytokines and regulatory cytokines, including IL-4, IL-13, TGF-β and IL-10.[24] M2-type macrophages secrete large amounts of cytokines such as TGF-β, vascular endothelial

Table 4 Correlation Analysis of CD163 and α-SMA Expression in CRC Tissues

		α-SMA
CD163	r	0.815
	P	0.001

Table 5 Correlation Analysis of CD163 and FSP-1 Expression in CRC Tissues

		FSP-1
CD163	r	0.829
	P	0.001

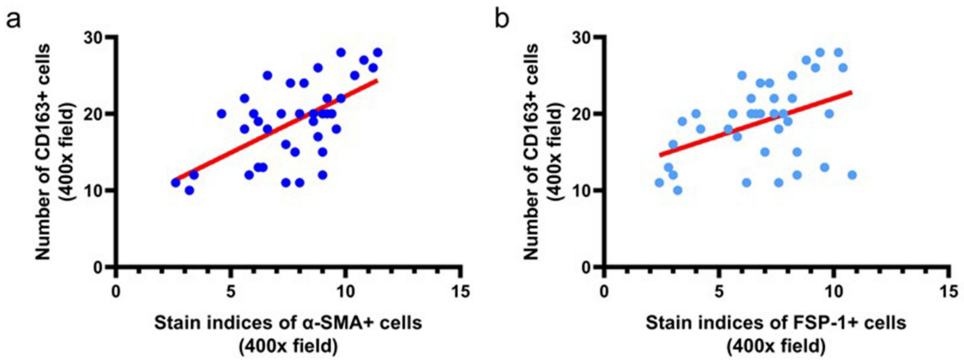

Figure 9 Correlation of CD163, α-SMA, and FSP-1 expression levels in liver metastases cancer tissues. (**a**) Correlation between the Number of CD163+ cells and the stain indices of α-SMA+ cells. (**b**) Correlation between the Number of CD163+ cells and the stain indices of FSP-1+ cells.

growth factor (VEGF), and IL-10[25] during tumor development and promote cancer metastasis by enhancing epithelial-mesenchymal transition (EMT) and angiogenesis.[26] In this study, we found that M2-type macrophage expression levels were significantly up-regulated in CRC, and the increased expression was particularly pronounced in colorectal cancer samples with liver metastases. Meanwhile, the upregulation of M2-type macrophage expression level was also closely correlated with the presence or absence of lymph node metastasis and TNM staging. These above conclusions are consistent with our previous study. Published studies have demonstrated that polarized M2-type macrophages can promote colorectal cancer liver metastasis.[27,28] In studies of other tumors, M2-type macrophages can promote distant metastasis in gastric and breast cancers by activating the protein kinase pathway.[29] In the study by El-Arabey et al on ovarian cancer, M2-type macrophages were able to enhance the invasive potential and chemoresistance of tumor cells and promote tumor cell metastasis in the peritoneum and ascites.[30] Combined with our group's previous studies on M2-type macrophages in colorectal cancer, we found that M2-type macrophages mediate lymph node metastasis in colorectal cancer[31] and can promote colorectal cancer progression by activating the TGF-β/

Table 6 Correlation Analysis of CD163 and α-SMA Expression in CRC Liver Metastases Tissue

		α-SMA
CD163	r	0.605
	P	0.001

Table 7 Correlation Analysis of CD163 and FSP-1 Expression in CRC Liver Metastases Tissue

		FSP-1
CD163	r	0.551
	P	0.001

Table 8 Correlation Analysis of the Number of M2-Type Macrophages, Staining Index of α-SMA, and Staining Index of FSP-1 with Preoperative CEA, CA19-9, and CA72-4

Preoperative Tumor Markers	**Average Number of M2 Macrophages**		**Staining Index of α-SMA**		**Staining Index of FSP-1**	
	r	**P**	**r**	**P**	**r**	**P**
CEA	0.388	<0.001	0.292	<0.001	0.101	0.009
CA19-9	0.292	<0.001	0.280	<0.001	0.161	<0.001
CA72-4	0.101	<0.001	0.265	<0.001	0.112	0.004

Smad signaling pathway.[32] Based on the above findings, combined with the fact that M2-type macrophages in the primary foci of CRC in the experimental group were significantly higher than those in the control group in the present study, we confirmed a positive correlation between M2-type macrophages and hepatic metastasis of CRC, which is in agreement with previous studies.

Activated fibroblasts in TME, usually dominated by CAFs, interact with tumor-associated immune cells and other immune components to enable cancer cells to escape immune surveillance.[33] CAFs are critical in shaping the body's immunosuppression, promoting tumor growth, progression, and metastasis, and enhancing tumor chemoresistance.[34] CAFs produce fibrillar collagen and other extracellular matrix (ECM) components that actively participate in matrix remodeling and form adhesions around the tumor, causing peritumoral fibrosis or scarring adhesions within the tissue.[2] Kai et al[35] found that tumor progression and metastasis were accelerated due to CAFs proliferation in the ECM and the production of large amounts of fibronectin, which resulted in stromal remodeling stiffness and hypoxia. Our study found that CAFs expression in CRC was significantly correlated with TNM stage, presence of liver metastasis, presence of lymph node metastasis, presence of vascular invasion, and presence of neurologic invasion. Existing studies have shown that CAFs is closely related to tumor metastasis. For example, CAFs can promote the invasiveness and peritoneal metastasis of colon cancer cells by up-regulating CPT1A;[36] in hepatocellular carcinoma (HCC), CAFs triggers the HIF1α/ZEB1 cascade reaction through the secretion of the chemokine CCL5 thereby enhancing lung metastasis;[37] in breast cancer, CAFs can promote breast cancer invasion and metastasis by secreting IL-32[38] and can accelerate distant breast cancer metastasis using FAK signaling.[39] The same occurred in ovarian cancer, where CAFs-derived POSTN promoted ovarian cancer cell invasion and metastasis by activating the PI3K/Akt pathway and inducing EMT.[40] Based on the discoveries mentioned earlier, it can be inferred that CAFs play a role in the metastasis of colorectal cancer to the liver.

In recent years, the correlation between M2-type macrophages and CAFs in CRC has gradually attracted attention. Some research has shown that tumor cells promote tumor growth and metastasis by secreting large amounts of TGF-β[23] and regulating cellular immunity.[41] TGF-β can promote the conversion of TAM to an M2-type phenotype, including through SNAIL upregulation,[42] resulting in a substantial increase in the number of M2-type macrophages expressed in the TME. These polarized M2-type macrophages can secrete more TGF-β.[25] Under pathological conditions, overexpressed TGF-β triggers the deposition of EMT and ECM, which contributes to the transformation of fibroblasts into CAFs, leading to fibrotic diseases and cancer.[43] These studies indicated that M2-type macrophages are involved in the differentiation process of CAFs. Chen et al[44] in their study of HCC, found that hepatocellular carcinoma cells and CAFs could induce the polarization of TAMs toward M2-type macrophages by up-regulating the level of mRNA expression of CD163 and CD206. At the same time, CAFs are the leading producers of C-X-C motif chemokine ligand 12 (CXCL12) in the TME, and CXCL12 expression, in turn, stimulates M2 macrophage migration.[45] Cho et al[46] found that CAFs in an in situ syngeneic colorectal cancer mouse model increased the levels of IL6 and GM-CSF, which promoted the conversion of TAM to M2-type macrophages. Based on Mayer et al.[3] CAFs is equally involved in TAM to M2-type conversion and M2-type macrophage recruitment. We analyzed that a positive feedback mechanism may be between M2-type macrophages and CAFs in TME, which promotes tumor development (Figure 10). In the CAFs-induced

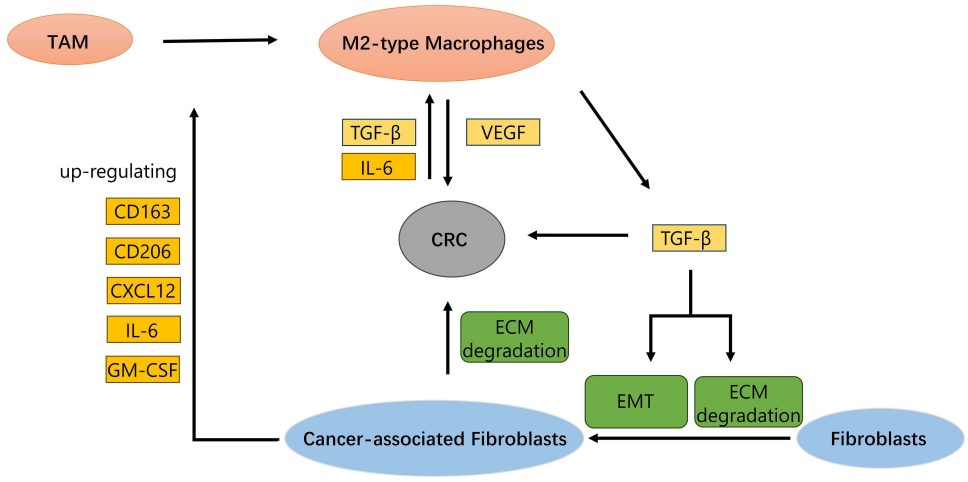

Figure 10 A possible mechanistic pathway of M2-type macrophages and Cancer-associated fibroblasts.

hypoxic environment of TME,[36] M2-type macrophages and CAFs work together to cause ECM stiffness and degradation;[47] the pathways formed in the TME as a result of ECM degradation are more conducive to tumor cell infiltration and metastasis. Our experiments showed that M2-type macrophages and CAFs expression levels were significantly higher in CRC tissues than in paracancerous tissues, and both were positively correlated. Thus, interactions between M2-type macrophages and CAFs together promote CRC progression. We conclude that the interaction between M2-type macrophages and CAFs jointly promotes CRC progression. In addition, we found that the expression levels of M2-type macrophages and CAFs were significantly higher in the experimental group compared to the control group. It is reasonable to hypothesize that the interaction between M2-type macrophages and CAFs promotes the progression of CRC and liver metastasis.

Monitoring tumor markers is valuable for early screening and determining the prognosis of colorectal cancer.[48] Carcinoembryonic antigen (CEA) protects metastatic cancer cells from immune surveillance and promotes the expression of adhesion molecules, facilitating liver metastasis in colorectal cancer.[49] In colorectal cancer, Glycated antigen 19–9 (CA19-9), similar to CEA, can indicate lymph node and liver metastasis.[50] Previous studies from our group have shown[31,51] that preoperative CEA, CA19-9, and glycan antigen 72–4 (CA72-4) are strongly associated with colorectal cancer severity and lymph node metastasis. In summary, we concluded that the preoperative expression levels of CEA, CA19-9, and CA72-4 were highly correlated with the increased expression of M2-type macrophages with CAFs in colorectal cancer tissues and were suggestive of a poor prognosis, such as CRC liver metastasis.

In our study of liver metastases in CRC, we found that the expression of M2-type macrophages and CAFs was significantly higher in liver metastases than in paracancerous tissues, and there was also a specific positive correlation between the two cells. Following the research conducted by Tan et al.[52] TGF-β1 still acts as a critical signal mediating the differentiation of hepatic stellate cells into CAFs and plays a vital role in colorectal cancer liver metastasis. Tu et al,[27] in an established mouse model of CRC liver metastasis, observed that the number of polarized M2-type macrophages was more markedly increased in the hepatic metastasis than in the primary focus. As M2-type macrophages continue to infiltrate CRC liver metastases, they continue to evolve in a direction that favors the promotion of growth infiltration of CRC liver metastases.[53] We concluded that the interaction between M2-type macrophages and CAFs in CRC liver metastases is consistent with the roles in CRC primary foci and contributes to liver metastasis of CRC.

The results of our research indicated that M2-type macrophages and CAFs were significantly increased in CRC, and there was a strong positive association between the two, implying that M2-type macrophages and CAFs have a combined effect in CRC. The expression of M2-type macrophages and CAFs in the primary foci of the experimental group was significantly higher than that of the control group, and there was also a significant positive correlation between the two, suggesting that M2-type macrophages and CAFs jointly promoted the liver metastasis of CRC.

Conclusion

M2-type macrophages and CAFs are essential participants in CRC liver metastasis. Both cells are jointly involved in forming the tumor microenvironment in colorectal cancer. Their interactive relationship may influence the occurrence and development of liver metastasis from colorectal cancer. They are increased and correlated with M2-type macrophages and CAFs expression in colorectal cancer liver metastases. Exploring the connection between M2-type macrophages and CAFs interactions could open up novel clinical possibilities for early detection of CRC, identifying immune targets, and anticipating long-term prognosis.

Abbreviations

CRC, colorectal carcinoma; CAFs, cancer-associated fibroblasts; TME, tumor microenvironment; IHC, immunohistochemical; TAMs, tumor-associated macrophages; TGF-β, transforming growth factor-β; EMT, epithelial-mesenchymal-transition; VEGF, vascular endothelial growth factor; α-SMA, α-Smooth muscle actin; FSP-1, fibroblast-specific protein 1; CXCL12, C-X-C motif chemokine ligand 12; CEA, Carcinoembryonic antigen; CA19-9, Glycated antigen 19-9; CA72-4, glycan antigen 72-4.

Data Sharing Statement

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

Statement of Ethics

The samples were obtained from the First Affiliated Hospital of Jinzhou Medical University, Jinzhou, China. The study was conducted following the Ethics Committee of the First Affiliated Hospital of Jinzhou Medical University(2023103) and the 1964 Helsinki Declaration. Informed consent was obtained from all participants included in the study. The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors have declared that no competing interest exists.

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