RESEARCH ARTICLE

Correlations of Tumor-associated Macrophage Subtypes with Liver Metastases of Colorectal Cancer

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Abstract

Objective: This work aimed to investigate the correlations of tumor-associated macrophages (TAMs) and their subtypes M1 and M2 with liver metastasis of colorectal cancer, and provide useful references for seeking predictors of liver metastasis and studying mechanisms. Methods: 120 patients with colorectal cancer from 2000 to 2009 were divided into low, middle and high liver metastasis groups (group A, B and C, respectively). S-P immunohistochemical staining and microscopic observation were conducted to compare expression in CD68-positive cells (TAMs), CD80-positive cells (M1) and CD163-positive cells (M2) in three groups. Correlations of TAMs, M1, M2, and M2/M1 ratio with clinical and pathological parameters were analyzed. Results: With increase of liver metastatic ability, the number of TAMs decreased gradually, with no significant difference between any two of the three groups ($P > 0.05$), while the numbers of M1 and M2 were significantly decreased and increased, respectively, with significant difference between any two of three groups ($P < 0.05$ or $P < 0.01$). In addition, the M2/M1 ratio increased with increase of liver metastatic ability ($P < 0.01$). There was no statistical significance of correlation of TAMs with each clinical and pathological parameter. M1 was negatively related with lymphatic metastasis and liver metastatic ability. M2 was positively correlated with preoperative CEA level, lymphatic metastasis, tumor differentiation degree and liver metastatic ability. The same was the case for the M2/M1 ratio. Conclusions: Effects of TAMs on liver metastasis of colorectal cancer do not depend on the total number of TAMs, but on the number and proportion of functional subtypes M1 and M2. M2 number and M2/M1 ratio are more accurate predictors for liver metastasis of colorectal cancer.

Keywords: Liver metastasis - colorectal cancer - tumor-associated macrophages - correlation

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Introduction

Colorectal cancer is one of common malignancies in the world. The incidence of colorectal cancer ranks 3rd among malignancies, and the mortality is only inferior to that of lung cancer, gastric cancer and liver cancer (Parkin et al., 2005). Distant metastasis is the main cause of death in patients with colorectal cancer, in which liver metastasis is the most common. The liver metastasis rate of colorectal cancer is up to 20-70% (Penna and Nordlinger, 2002), in which 1/3 is with simple liver metastasis (Kemeny, 2006). Therefore, to further study the mechanism of liver metastasis of colorectal cancer, and seek more accurate predictors of liver metastasis are particularly important for establishment of more targeted and individualized prevention, treatment and follow-up measures. Traditional clinical and pathological indicators for prognosis of colorectal cancer include tumor invasion (Wong et al., 2008), vascular invasion (Talbot et al., 1980) and lymphatic metastasis (Adachi et al., 1999), but their ultimate significances are not obvious (Hu et al., 2009). A lot of evidences indicate that, tumor-associated macrophages (TAMs) are related to early metastasis in many primary tumors, and can be used as the evaluation indicators (Leek et al., 1996; Coussens and Werb, 2002). Although it is confirmed that, TAMs and related functional subtypes M1 and M2 are associated with the progression and metastasis in many tumors, their roles in liver metastasis of colorectal carcinoma still need to be further confirmed. In most studies, the sample size is small, with no involvement of functional subtypes of TAMs. This will cause insufficiency of study depth, leading to disunity of conclusions. In this study, the number and proportion of TAMs, M1 and M2 macrophages in colorectal cancer with larger-size samples were studied, and the correlations of TAMs with liver metastasis of colorectal cancer was further investigated. The objective is to provide useful references for seeking predictors of liver metastasis of colorectal cancer and studying its mechanism.

Materials and Methods

Clinical samples and grouping

120 patients with colorectal cancer in Tianjin Medical

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Table 1. Clinical and Pathological Parameters of Patients

Clinical and pathological features		Group A	Group B	Group C	*P*
Age (Mean±SD, years)		63.45±8.74	61.98±10.65	63.35±9.22	0.774
Male (n, %)		25 (62.5%)	27(67.5%)	26(65.0%)	0.896
Preoperative CEA (median, range, ng/mL)		11.09	17.65	31.1	0.806
		(2.70-243.05)	(4.30-190.00)	(3.25-262.50)	
T staging (n, %)	T1 or T2	2 (5.0%)	1 (2.5%)	0(0%)	0.359
	T3 or T4	38 (95.0%)	39 (97.5%)	40(100%)	
Lymphatic metastasis (n, %)	N1	22 (55.0%)	25(62.5%)	18(45.0%)	0.289
	N2	18 (45.0%)	15 (37.5%)	22(55.%)	
Liver metastases	Yes	0	0	40	0
	No	40	40	0	
Primary lesion location	Right hemicolon	12	11	10	0.87
	Left hemicolon	10	9	13	
	Rectum	18	20	17	
Degree of tumor differentiation	High	4	3	4	0.963
	Middle	32	31	31	
	Low	4	6	5	

University Cancer Institute and Hospital from 2000 to 2009 were divided into group A (low liver metastasis group, without recurrence or metastasis within 2 years after surgery for stage III colorectal cancer), group B (middle liver metastasis group, with liver metastasis within 2 years after surgery for stage III colorectal cancer) and group C (high liver metastasis group, with simultaneous liver metastasis), 40 patients in each group. Inclusion criteria were as follows: (1) Patients had received the first radical tumor resection in our hospital, and colorectal cancer was confirmed by histopathology. (2) Patients' ages were 18-75 years old. (3) The preoperative and postoperative follow-up data were complete, with adequate specimens. (4) No preoperative chemoradiation, intervention or ablation. (5) The amount of intraoperative bleeding and postoperative blood transfusion were less than 800 mL, respectively. (6) No postoperative complications such as infection or organ failure which caused death or influenced the prognosis. (7) No history of diabetes, autoimmune diseases, or taking immunosuppressive drugs. (8) The specimens were treated by routine dehydration, formalin fixation, and paraffin embedding. The primary lesion of tumor was staged according to UICC TNM staging system (Edition 6), with pathological grading according to WHO grading system. Continuous paraffin sections were prepared using conventional methods, and the thickness of section was 4 μm.

S-P immunohistochemical staining

Paraffin sections were treated by dewaxing, hydration, washing with PBS (5 minutes for 3 times), and antigen retrieval. Normal goat serum was used as a blocking solution. Primary antibody mouse anti-human CD163 monoclonal antibody (Abcam Inc., USA), mouse anti-human CD68 monoclonal antibody and mouse anti-human CD80 monoclonal antibody (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., China) were added for incubation, respectively, followed by addition of corresponding second antibodies. After incubation at room temperature by adding horseradish peroxidase labeled streptavidin, DAB liquid was added, followed by counterstaining with hematoxylin for 2 minutes. After mounting with neutral resin, the microscopic observation and cell counting were performed.

Determination of results

Staining results were determined as follows: Pale yellow to brown granules appeared in cytoplasm, with definite location and obvious staining. The section with more than 20% of stained cells was regarded as positive. Counting methods of TAMs were as follows: CD68TFHotspot area (area where the expression level of CD68-positive cells was higher than average level) was found under microscope with low magnification (100 ×), and number of cells in CD68TFHotspot area was counted under microscope with high magnification (400 ×). The average value of 6 cancerous tissue fields was regarded as the number of TAMs. CD80-and CD163-positive cells (M1 and M2, respectively) in corresponding area were counted using the same method, and the M2/M1 ratio of each sample was calculated.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 statistical software. Chi-square test was performed for comparing rates between different groups. t test and single factor ANOVA were conducted for comparing measurement data meeting normal distribution and homoscedasticity, respectively. Rank-sum test was used to comparing other data, and the correlations of TAMs, M1, M2 and M2/M1 with clinical and pathological parameters were analyzed by Spearman test. $P < 0.05$ was considered as statistically significant.

Results

Clinical and pathological parameters of patients

As shown in Table 1, there was no significant difference of age, gender, preoperative carcinoembryonic antigen (CEA) level, primary lesion location, T staging, lymphatic metastasis, and degree of tumor differentiation among three groups, respectively. The basic conditions in each group had comparability.

Distributions of TAMs, M1 and M2

Most of CD68-positive cells (TAMs) were located

Table 2. Correlations of TAMs, M1, M2, and M2/M1 with Clinical and Pathological Parameters

Parameter	TAMS		M1		M2		M2/M1	
	Correlation coefficient	*P*	Correlation coefficient	*P*	Correlation coefficient	*P*	Correlation coefficient	*P*
Age	-0.082	0.373	-0.127	0.167	0.052	0.571	0.082	0.373
Gender	-0.063	0.498	-0.078	0.396	-0.016	0.865	0.075	0.414
Preoperative CEA	-0.07	0.448	-0.039	0.673	0.225	0.013	0.201	0.028
Lymphatic metastasis	-0.1	0.277	-0.208	0.023	0.192	0.035	0.223	0.014
Primary lesion T staging	-0.024	0.796	-0.072	0.437	0.072	0.437	0.072	0.437
Primary lesion location	-0.151	0.099	-0.134	0.146	-0.151	0.099	-0.107	0.245
Tumor differentiation degree	-0.057	0.535	-0.025	0.79	0.204	0.025	0.286	0.002
Liver metastatic ability	-0.127	0.166	-0.529	0	0.201	0.028	0.63	0

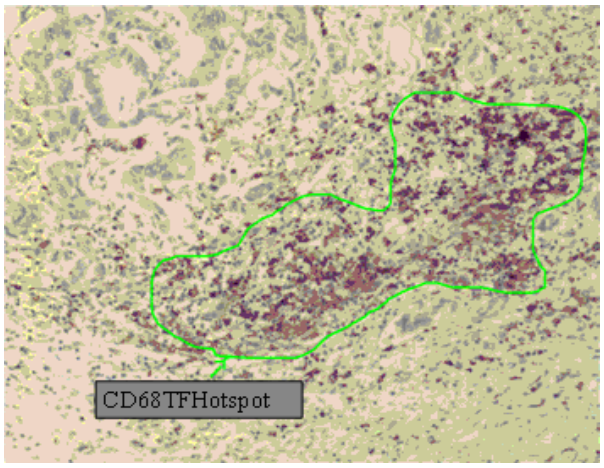

Figure 1. CD68TFHotspot of TAMs (100×)

in tumor stroma, especially in invasive front. Although the expressions of CD68-positive cells in most areas of invasive front were uniform, there are still a small part of areas where the expression level of CD68-positive cells was higher than average level (CD68TFHotspot) (Figure 1). The distributions of CD80-and CD163-positive cells (M1 and M2, respectively) were similar with TAMs, respectively.

Correlations of TAMs, M1, M2 and M2/M1 with liver metastatic ability

Figure 2 showed that, TAMs medians (ranges) in group A, B and C were 76.96 (23.83-175.17)/HP, 74.38 (20.83-204.33)/HP and 65.38 (16.33-201.58)/HP, respectively. With increase of liver metastatic ability, the number of TAMs decreased gradually, but the difference among three groups was no significant ($P > 0.05$). M1 medians (ranges) in group A, B and C were 40.55 (10.25-90.17)/HP, 30.42 (8.5-120.67)/HP and 15.75 (2.67-44.42)/HP, respectively. With increase of liver metastatic ability, the number of M1 was reduced, with significant difference between any two of three groups ($P < 0.05$ or $P < 0.01$). M2 medians (ranges) in group A, B and C were 16.00 (1.5-50.67)/HP, 22.62 /HP (19.17-80.42)/HP and 33.92 (7.17-105.83)/HP, respectively. The number of M2 increased with increase of liver metastatic ability, and the difference between any two of three groups was statistically significant ($P < 0.05$ or $P < 0.01$). Medians (ranges) of M2/M1 ratio in group A, B and C were 0.40 (0.10-1.12), 0.77 (0.19-1.73) and 2.06 (0.32-5.23), respectively. M2/M1 ratio increased with increase of liver metastatic ability, with significant difference between any two of three groups ($P < 0.01$).

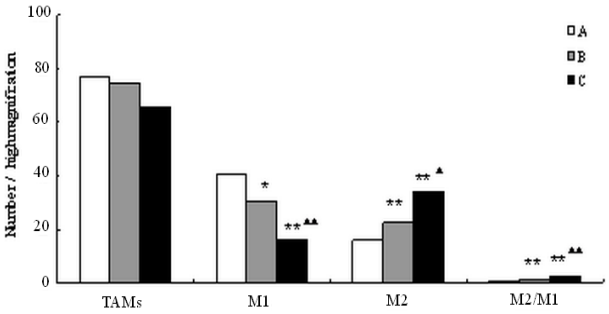

Figure 2. Counts of TAMs and Their Subtypes in Three Groups. Note: *P < 0.05 and **P < 0.01, compared with group A; ▲P < 0.05 and ▲▲P < 0.01, group C compared with group B

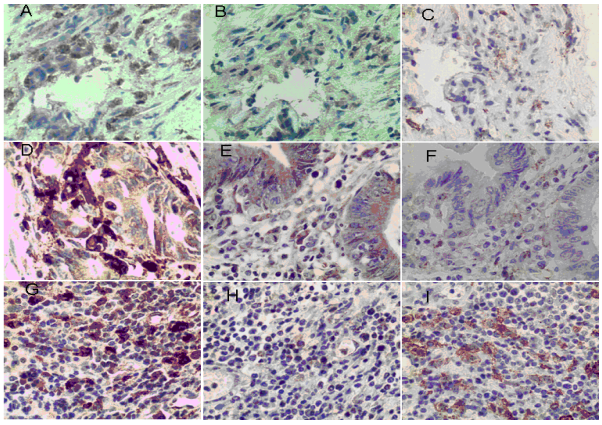

Figure 3. Observations of TAMs and Their Subtypes under Microscope (400 ×). (A, B, C: TAMs, M1 and M2 staining in group A, respectively; D, E, F: TAMs, M1 and M2 staining in group B, respectively; G, H, I: TAMs, M1 and M2 staining in group C, respectively)

Staining results showed that, in group A, number of M1 was larger than M2. In group B, the difference between numbers of M1 and M2 was not obvious. In group C, number of M2 was significantly larger than M1 (Figure 3).

Correlations of TAMs, M1, M2 and M2/M1 with clinical and pathological parameters

In order to further analyze the correlations of TAMs and their subtypes with patient age, gender, preoperative CEA level, lymphatic metastasis, primary lesion location and T staging, tumor differentiation degree and liver metastatic ability, the proportion of TAMs, M1, M2, and M2/M1 were ranked from low to high, and divided into four equal parts according to quartile for statistical analysis. +, ++, +++ and ++++ represented the range of

0-25%, 25-50%, 50-75% and 75-100%, respectively. Results were shown in Table 2. There was no statistical significance of correlation of TAMs with each parameter. M1 was negatively related with lymphatic metastasis and liver metastatic ability, respectively. M2 was positively correlated with preoperative CEA level, lymphatic metastasis, tumor differentiation degree and liver metastatic ability, respectively. It was the same with M2/M1 ratio.

Discussion

Liver metastasis is one of the major causes of death in patients with colorectal cancer. Further investigating the mechanism and seeking predictors of liver metastasis are especially urgent. Although it is confirmed that TAMs can promote the progression and metastasis of a variety of tumors, the roles of TAMs as predictors in colorectal cancer in different studies are not the same. Some studies find that, TAMs mainly exhibit anti-tumor effects in colorectal cancer. Ong et al. (2012) have investigated the colorectal cancer models and find that, TAMs can secrete chemokines and promote T cell proliferation, thus activate type 1 T cell responses and exert anti-tumor effects. Kinouchi et al. (2011) have studied 52 colorectal cancer specimens and found that, $CD14^+$ macrophages are mainly distributed in tumor invasive front, and the 5-year survival rate of patients with large number of $CD14^+$ macrophages is higher than patients with small number of $CD14^+$ macrophages. So presence of a large number of $CD14^+$ macrophages in tumor invasive front is an indicator for good prognosis.

Some studies suggest that, TAMs can promote the occurrence, development and metastasis of colorectal cancer, which is more related to the functional subtype M2. Jedinak et al. (2010) find that, there are multiple cytokines such as IL-1, IL-6 and TNF-αin in mediator containing activated macrophages, which can obviously induce the proliferation and migration of colon cancer cells. In addition, they can also activate NF-κB and induce colon cancer cells to produce VEGF, thus promoting angiogenesis and tumor progression and metastasis. As found by Pander et al. (2011), there are a large number of M2 macrophages in colorectal cancer, which promote the progression of tumor by secreting IL-10 and VEGF. Green et al. (2009) have established colorectal cancer models for studying the signaling system between colorectal cancer cells CT-26 and RAW 264.7 macrophages. They find that, CT-26 and RAW 264. 7 are attracted to each other, and with presence of CT-26, RAW 264.7 become protrusive phenotypes with high migratory ability, which are more similar with M2.

Other studies find that, TAMs have double functions in colorectal cancer. As found by Algars et al. (2011), the number of peritumoral M2 in colorectal cancer are positively correlated to survival time, but in stage IV colorectal cancer, it is negatively related to disease-free survival time. In addition, distant tumor metastasis and recurrence are easier to appear in patients with high proportion of intratumoral M2 than patients with low proportion. So it is believed that, the role of TAMs on colorectal cancer depends on TAMs type, location in tumor and tumor staging. Imano et al. (2011) have studied 41 cases of stage II and stage III colorectal cancer specimens and find that, TAMs in center area of colorectal cancer can promote the liver metastasis, while TAMs in tumor invasive front can inhibit the liver metastasis. Zhou et al. (2010) have investigated TAMs subtypes in colon cancer and find that, TAMs can simultaneously express the markers of M1 and M2. So TAMs are the combination of M1 and M2, but not single M1 or M2. Multivariate and univariate analysis find that, the phenotypic mixing density of TAMs is an independent factor for prognosis in stage IIIB colorectal cancer.

Reasons for above different conclusions may be as follows: (1) The sample size in most studies is small. (2) All cases of colorectal cancer were enrolled in general statistics, without fine grouping of liver metastatic ability for comparison. Different proportion of cases with high and low liver metastatic ability will lead to different conclusions. (3) Majority of studies only involve the total number of macrophages (M1+M2), without differentiating M1 and M2 which have opposite effects. In different samples with the same total number of M1 and M2 which have different proportions, the effects of macrophages on liver metastasis of colorectal cancer are not the same.

In this study, in order to make up above deficiencies, 120 cases of colorectal cancer specimens with larger sample size were selected for research. In addition, the liver metastatic ability is finely grouped, and TAMs subtype M1 and M2 are studied. Results show that, with increase of liver metastatic ability, the number of TAMs does not significantly change. This indicates that, the number of TAMs is not very sensitive to liver metastatic ability. With increase of liver metastatic ability, numbers of M1 and M2 amount obviously change, respectively, with statistical difference among three groups, indicating that both M1 and M2 are more sensitive to liver metastatic ability than TAMs. Due to individual differences, the absolute number of M1 and M2 are obvious difference. Therefore, it is a big problem to determine liver metastatic ability of colorectal cancer only according to number of single M1 and M2. This is confirmed by results in this study that, M2/M1 ratio is most sensitive to change in liver metastatic ability, with significant difference among three groups. Therefore, it will be more accurate to use TAMs, M1 and M2 number and M2/M1 ratio for evaluating the liver metastatic ability of colorectal cancer.

As found in this study, there no correlation of TAMs with each clinical and pathological parameter. M1 is negatively correlated with liver metastatic ability and lymphatic metastasis, respectively, which has proved the anti-tumor property of M1. Ma et al. (2010) have studied non-small cell lung cancer and find that, M1 is an independent factor for good prognosis. Notably, M2 is almost significantly correlated with all parameters related to tumor invasiveness such as preoperative CEA level, lymphatic metastasis, tumor differentiation and liver metastatic ability. CEA is a common tumor marker, and is closely related to liver metastasis of colorectal cancer (Leskoviku et al., 1992; Thomas et al., 1995; Hatate et al., 2008). Aarons et al. (2007) find that, CEA

can act on the receptor in macrophages, and stimulate macrophages to secrete cytokines and endothelial cell adhesion molecules, thus promoting the liver metastasis in colorectal cancer. Recent study of Rolny et al. (2011) finds that, host-derived histidine-rich glycoprotein can down-regulate the expression of placenta growth factor, and promote the differentiation of M2 to M1, thus reducing the tumor growth and metastasis. This has also confirmed the tumor promoting effect of M2. Takai et al. (2009) find that, M2 is associated with the progression and metastasis of hepatocellular carcinoma. These are consistent with results of this study that, M2 plays a very important role in tumor metastasis. In addition, preoperative CEA is positively correlated to M2, indicating that CEA may be associated with M2 type differentiation of macrophages.

In conclusions, effects of TAMs on liver metastasis of colorectal cancer do not depend on the total number of TAMs, but on the number and proportion of the functional subtype M1 and M2. M2 number and M2/M1 ratio are more accurate predictors for liver metastasis of colorectal cancer.

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