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Original Article

CD68-positive tumor-associated macrophages predicts the survival of patients with stage I colorectal cancer

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Abstract: Current risk stratification for patients with stage I colorectal cancer is imperfect. The aim of this study is to determine whether CD68(+) tumor-associated macrophages (TAMs) is a predictor of the outcomes in patients with stage I colorectal cancer that undergo curative surgery. One hundred eighty-nine patients with stage I colorectal cancer that underwent surgical resection between August 2005 and December 2009 were retrospectively reviewed. TAMs were detected based on immunohistochemical staining of CD68. The optimum thresholds for CD68 expression was based on the maximal 2 value of the log-rank test for disease free survival (DFS). 5-year DFS and cause-specific survival (CSS) were compared between patients with low CD68(+) TAMs and those with high CD68(+) TAMs. The 5-year DFS and CSS were lower in patients with high CD68(+) TAMs than in those with low CD68(+) TAMs (87.6% vs. 92.5%, P=0.008; 90.1% vs. 94.2%, P=0.011). Cox multivariate analysis demonstrated that CD68(+) TAMs were independently associated with DFS (HR, 4.308; 95% CI, 1.296-17.524; P=0.008) and CSS (HR, 5.294; 95% CI, 1.021-35.437; P=0.012) in patients with stage I colorectal cancer. In conclusion, CD68(+) TAMs in tumor tissue is a prognostic factor predicting DFS and CSS in patients with stage I colorectal cancer that underwent curative surgery.

Keywords: Tumor associated macrophages, CD68, colorectal cancer, prognosis

Introduction

Surgery is the main treatment for stage I colorectal cancer and complete removal of tumor mass provides the best chance for cure. Although most patients with stage I colorectal cancer have long survival, the chance of local and distant recurrences still exists [1, 2]. There are few reports on the predictive factors associated with prognosis for stage I colorectal cancers.

The clinical evidence regarding the relationship between TAMs and tumor progression is tumor type-dependent. TAMs might promote tumor progression by the induction of chronic inflammation, matrix remodeling, tumor invasion, extravasation, angiogenesis, and seeding at distant sites [3]. On the other hand, the recruitment of TAMs contributes to the development of adaptive immune response against cancer [4, 5]. Current knowledge of the prognostic significance of TAMs in stage I colorectal cancer is limited. The aim of this study was to investigate the relationship between CD68(+) TAMs and clinicopathological variables of stage I colorectal cancer and evaluate the prognostic role of CD68(+) TAMs for stage I colorectal cancer patients after surgical resection.

Patients and methods

The medical records of 189 consecutive patients who were confirmed to be in stage I after curative surgical resection for colorectal cancers at our hospital between August 2005 and December 2009 were retrospectively reviewed. The study was approved by Ethics Committee of the First Affiliated Hospital of Liaoning Medical University, and written informed consent was given by all participants. We excluded patients who received preoperative chemoradiation therapy, those with inflammatory conditions or with a history of other primary cancers. The data analyzed included the age at diagnosis, gender, primary tumor site, tumor T stage, histological grade. Staging was performed according to the tumor-node-metas-

Table 1. Clinicopathological characteristics of patients

Variables	No.
Age	
<45 y	85
≥45 y	104
Gender	
Female	91
Male	98
Location	
Colon	103
Rectum	86
Tumor differentiation	
Well	78
Moderate	84
Poor	27
Tumor size	
<5	103
≥5	86
Depth of invasion	
T1	105
T2	84
Lymphatic invasion	
No	112
Yes	77
Venous invasion	
No	109
Yes	80
Number of retrieved LN	
<12	44
≥12	145
Preoperative CEA	
≥5	120
<5	69
CD68 IHC expression	
≤11.2% ($CD68^{low}$)	158
>11.2% ($CD68^{high}$)	31

tasis (TNM) classification of the American Joint Committee on Cancer (AJCC, 7th edition). Patients were scheduled to visit the outpatient clinic every 3-6 months for the first 2 years after surgery, every 6 months for the next 3 years, and every year thereafter. Physical examinations and serum carcinoembryonic antigen assay were performed at each visit. Chest X-ray, abdominopelvic computed tomography scan, and colonoscopy were performed annually. Positron emission tomography was performed for the suspicion of recurrence. Recurrence was detected by a combination of imaging and serum CEA level and confirmed by pathologic examinations. Median follow-up period was 72.0 months (range 10-125 months). Disease free survival (DFS) and cause-specific survival (CSS) were calculated for all patients from the date of surgery until recurrence and death from colorectal cancer.

Quantification of CD68 density

Tissue microarrays were constructed using duplicate 1.5-mm-diameter cores of formalin-fixed, paraffin-embedded tumor tissue. Immunohistochemical staining was performed using a multimer detection kit (UltraView Universal DAB). Immunostained slides were scanned by Aperio ScanScope XT at 20 magnification. CD68 staining was analyzed using the Positive Pixel Count algorithm with the Aperio Image Scope (Version 11) viewer. Every core of tissue on the microarrays was checked by a pathologist to ensure that computer image analysis was performed correctly. Visual scoring was performed by estimating percentages of CD68 positive cells in relation to total cells in the tissues.

Statistics analysis

Pearson's χ^2 or Fisher's exact test was used to assess differences in the clinicopathological features. Continuous data were compared by Student's t test. Kaplan-Meier survival analysis was used to estimate DFS and CSS. Differences between survival curves for each variable were analyzed by using the log-rank test. Multivariate analysis was performed using the Cox regression model with a stepwise forward method to derive the final model of the variables. The statistical software X-tile (Version 3.6.1) was used to determine the thresholds for CD68 expression, by selecting the maximal 2 values of the log-rank test for DFS, designated as low and high risk. These thresholds were then carried forward and tested in the independent validation cohort. Statistical analysis was performed with SPSS software (Version 14.0), $P<0.05$ was considered significant.

Results

Clinicopathological characteristics of patients

Table 1 shows the characteristics of patients who underwent curative resection for stage I colorectal cancer. The 189 patients included

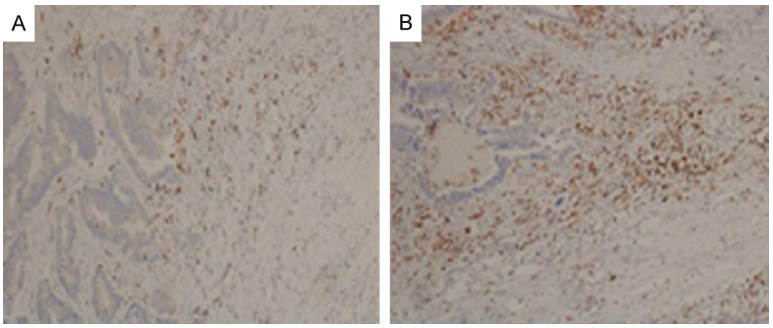

Figure 1. Representative staining of CD68(+) TAMs in stage 1 colon cancer patients. Different grades of macrophage infiltration in the tumor tissue were examined with immunohistochemical assay of CD68. A. Low CD68 staining; B. High CD68 staining. 100× magnification.

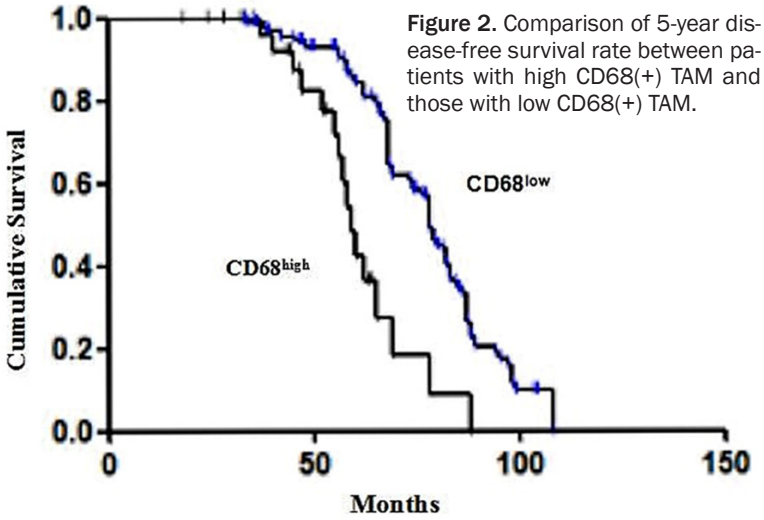

Figure 2. Comparison of 5-year disease-free survival rate between patients with high CD68(+) TAM and those with low CD68(+) TAM.

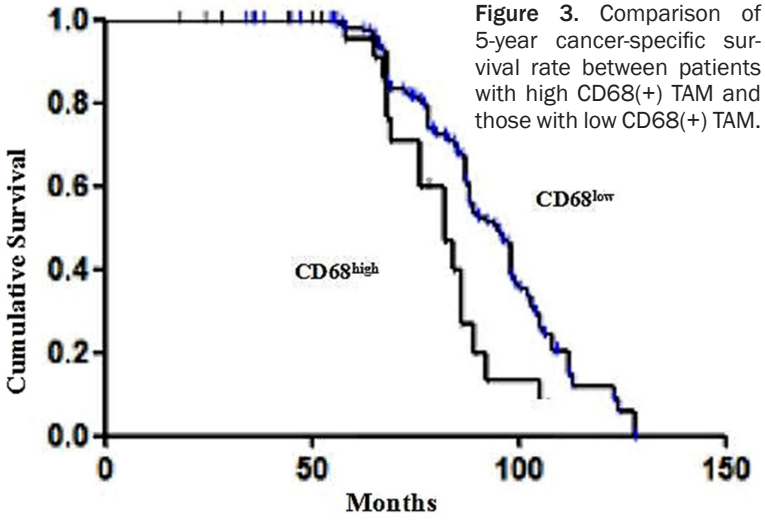

Figure 3. Comparison of 5-year cancer-specific survival rate between patients with high CD68(+) TAM and those with low CD68(+) TAM.

98 men and 91 women with a median age of 65 years (range 18-85 years). Typical staining of CD68 was shown in **Figure 1**. Using the optimum threshold of 11.2% obtained with X-tile, 31 patients (16.4%) had high CD68(+) TAMs. There was no significant difference between patients with high CD68(+) TAMs and those with low CD68(+) TAMs with respect to tumor location, tumor differentiation, tumor size, the depth of invasion, serum CEA level, and the number of retrieved lymph nodes. Of 189 patients, eight patients had recurrences with a median time to recurrence of 28 months (range 12-54 months). Among the 158 patients with low TAMs, three patients had recurrence; among the 31 patients with high TAM, five patients had recurrence. In the eight patients with recurrences, five patients had tumor related deaths.

Survival analysis of patients

The 5-year DFS rate was lower in patients with high CD68(+) TAMs compared to those with low CD68(+) TAMs (87.6% vs. 92.5%, P=0.008) (**Figure 2**). The 5-year CSS rate was also lower in patients with high CD68(+) TAMs compared to those with low CD68(+) TAMs (90.1% vs. 94.2%, P=0.011) (**Figure 3**). Univariate and multivariate analysis demonstrated that CD68(+) TAMs was independently associated with DFS and CSS in patients with stage I colorectal cancer (**Tables 2** and **3**).

Discussion

Patients with stage I colorectal cancer are likely to be followed up with a longer interval after surgery [6]. The prognosis of stage I colorectal cancer is excellent, but there is still

Table 2. Univariate analysis of factors associated with patient survival

Factor	No.	5-y DFS, %	*P*	5-y CSS, %	*P*
Age					
<45 y	85	93.6	.522	95.4	.417
≥45 y	104	94.5		96.1	
Gender					
Female	91	94.2	.428	95.9	.814
Male	98	92.8		93.6	
Location					
Colon	103	93.6	.315	94.3	.227
Rectum	86	95.2		96.6	
Tumor differentiation					
Well	78	92.8	.185	95.6	.128
Moderate	84	91.9		93.3	
Poor	27	90.4		91.6	
Tumor size					
<5	103	94.5	.452	96.2	.207
≥5	86	94.1		97.3	
Depth of invasion					
T1	105	93.1	.680	95.4	.426
T2	84	92.5		94.6	
Lymphatic invasion					
No	112	93.5	.306	95.1	.132
Yes	77	91.7		94.8	
Venous invasion					
No	109	92.6	.105	94.8	.210
Yes	80	91.7		93.6	
Number of retrieved LN					
<12	44	93.2	.305	97.4	.502
≥12	145	94.8		96.8	
Preoperative CEA					
≥5	120	93.2	.216	95.6	.113
<5	69	94.8		96.1	
CD68 IHC expression					
≤11.2% ($CD68^{low}$)	158	92.5	.008	94.2	.011
>11.2% ($CD68^{high}$)	31	87.6		90.1	

chance of local and distant recurrences [7, 8]. It is important to detect the recurrence as early as possible. In this study, we have shown that 4.2% of stage I colorectal cancer patients developed tumor recurrence despite radical resection. Our results are compatible with previous studies which reported recurrence rate up to 12% [9-11].

In this study we showed that pT1N0M0 tumor was not different from pT2N0M0 tumor with regard to DFS (93.1% vs. 91.5%) and CSS (93.8% vs. 92.6%), suggesting that T category is not a significant prognostic factor in stage I colorectal cancer. The depth of invasion is not likely to be associated with prognosis in stage I colorectal cancer, if radical resection is performed. Other adverse pathological factors, such as tumor differentiation, serum CEA level or the number of retrieved lymph nodes, were not associated with survival. In contrast, we found that CD68(+) TAMs was an independent prognostic factor in stage I colorectal cancer. A high abundance of CD68(+) TAM was good indicator for unfavorable patient outcome and poor long term survival in stage I colorectal cancer. Our findings suggest the prognostic significance of TAMs in stage I colorectal cancer.

In this study, we demonstrated an objective method of quantitative analysis of CD68 staining using computer imaging and established robust thresholds for CD68 expression in stage I colorectal cancer. Determination of CD68(+) TAMs in patients undergoing curative surgery for early stage colorectal cancer is a simple and inexpensive way. Identification of patients with poor prognosis may be considered clinically useful during postoperative follow-up [12-14]. Evaluation of TAMs should be considered in prospective clinical trials, and patients with increased TAMs may benefit from more intensive chemotherapy or novel agents designed to disrupt the crosstalk between tumor cells and macrophages. A rational clinical translation of these results suggests standardized utilization of TAMs as prognostic marker for patients in stage I colorectal cancer undergoing radical resection. Further studies are required to determine the underlying mechanisms associated with increased numbers of TAMs in tumor tissue and the relationship with prognosis of stage I colorectal cancer.

While we showed that CD68(+) TAMs could be a prognostic factor predicting DFS and CSS in stage I colorectal cancer patients, the cut-off value of CD68(+) TAMs needs to be defined. Further studies with larger sample size are nec-

Table 3. Multivariate analysis of disease-free and cancer-specific survival in patients

Factor	HR	95% CI	*P*
DFS			
$CD68^{high}$	4.308	1.296-17.524	.008
CSS			
$CD68^{high}$	5.294	1.0212-35.437	.012

essary to establish the prognostic value of CD68(+) TAMs in stage I colorectal cancer. The evaluation of TAMs could offer additional diagnostic modality for the selection of patients for further treatment strategies [15-17]. This could be performed routinely by histological evaluation of paraffin-embedded tumor specimens with commercially available antibodies against macrophage marker CD68.

In conclusion, we demonstrate for the first time that the presence of CD68-positive TAMs in tumor tissue was correlated with patient survival and could serve as an independent prognostic factor for stage I colorectal cancer.

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Disclosure of conflict of interest

None.

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