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The Impact of Tumor-associated Macrophages on Chemoresistance *via* Angiogenesis in Colorectal Cancer

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Abstract. *Background/Aim: The tumor microenvironment plays an important role in tumor progression. Tumor-associated macrophages (TAMs) have been reported to promote proliferation, invasion, metastasis, angiogenesis, and immunosuppression. Furthermore, angiogenesis has been reported to induce chemoresistance due to the inefficient distribution of drugs to cancer cells. However, the impact of TAMs on chemoresistance via angiogenesis in colorectal cancer (CRC) remains unclear. The aim of the study was to evaluate the impact of TAMs on the chemotherapeutic outcome in CRC. Patients and Methods: We enrolled 54 patients who underwent chemotherapy for unresectable metastatic CRC after resection of the primary tumor. We evaluated the density of TAMs and the degree of angiogenesis by immunohistochemistry and then explored the correlation between the density of TAMs and chemotherapeutic outcome. Furthermore, we assessed any correlation between the density of TAMs and that of neovascularity. Results: The high-TAMs group had a significantly worse progression-free survival (p=0.0006) and a poorer response rate (p=0.0274) than the low-TAMs group. In addition, a positive correlation was observed between the density of TAMs and the degree of neovascularity (r=0.665, p=0.0004). Conclusion: TAMs were shown to promote chemoresistance via angiogenesis in CRC.*

The tumor microenvironment has been recognized to play a crucial role in cancer progression (1, 2). Most tumor-infiltrating macrophages (TAMs), which are immune cells present in the cancer microenvironment, have been reported to be differentiated into the M2 type, which relates to malignant transformation of colorectal adenoma (3) and promotes cancer progression *via* proliferation, invasion, metastasis, angiogenesis and suppression of anticancer immunity (4-8). Furthermore, angiogenesis induced by TAMs has been reported to be associated not only with cancer progression, but also resistance to chemotherapy (9, 10). These results have been proven in basic research using experimental animal models (8, 11), but few studies have verified whether or not similar results can be obtained in the clinical setting.

The present study examined the correlation between the density of TAMs and the chemotherapeutic outcomes and evaluated the correlation between the density of TAMs and angiogenesis in clinical samples.

Patients and Methods

Patients

Group I. To evaluate the correlation between the density of TAMs and the chemotherapeutic outcome, we retrospectively reviewed a database of 54 patients with stage IV colorectal cancer (CRC) who underwent palliative combination chemotherapy for unresectable metastatic tumors after resection of the primary tumor at the Department of Surgical Oncology of the Osaka City University between 2007 and 2014.

Group II. In addition to the above, to compare the differences in the cancer microenvironment between the primary tumor and the metastatic tumor, we retrospectively reviewed a database of 24 patients with stage IV CRC who underwent concurrent resection of the primary tumor and the metastatic liver tumor. Patients who underwent preoperative therapy, such as chemotherapy and radiotherapy, were excluded from this study.

This retrospective study was approved by the Ethics Committee of Osaka City University (approval number: 3853) and conducted in accordance with the Declaration of Helsinki. All patients provided their written informed consent.

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Key Words: Tumor-associated macrophages, angiogenesis, chemoresistance, neovascularity, colorectal cancer.

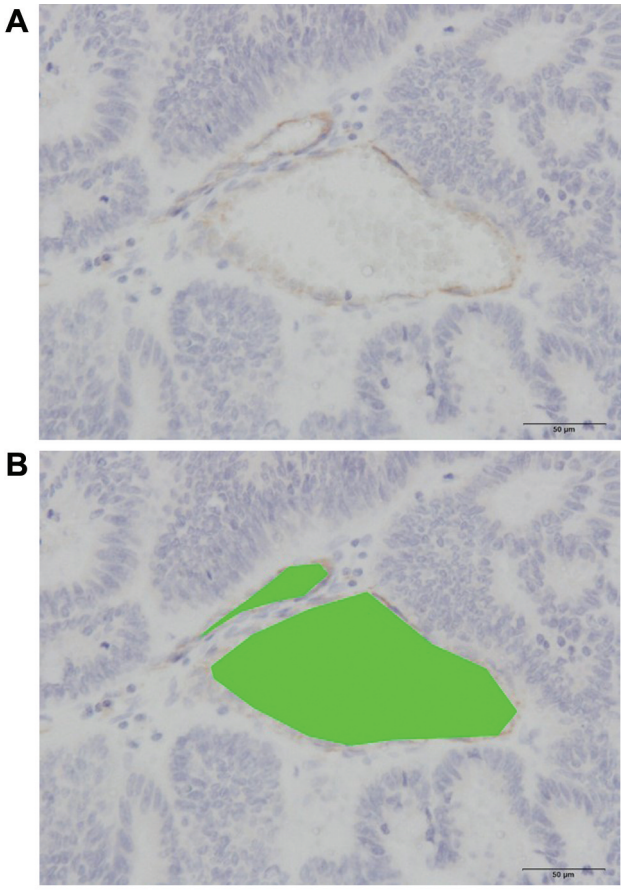

Figure 1. *Immunohistochemical detection of CD31, a vascular endothelial cell-specific marker (×200) (A). The area filled with green color is the vessel lumen (B).*

Immunohistochemistry. CD31 has been used as a specific marker to identify vascular endothelial cells (12, 13), and CD163 has been used as a specific marker to identify M2 macrophages (8, 14, 15). Surgically resected specimens were retrieved to perform immunohistochemistry. Sections 4 μm in thickness were deparaffined and rehydrated. The sections were then subjected to endogenous peroxidase blocking in 1% H_2O_2 solution in methanol for 15 min. Antigen retrieval was performed by autoclaving the sections at 105˚C for 10 min in Dako Target Retrieval Solution (Dako, Glostrup, Denmark). Serum blocking was performed with 10% normal rabbit serum for 10 min. After H_2O_2 and serum blocking, the slides were incubated with primary mouse monoclonal anti-CD31 antibody (1:40 dilution; Dako)/anti-CD163 antibody (1:200 dilution; Leica Biosystems, Newcastle Upon Tyne, UK) at room temperature for 1 h. The secondary antibody was biotin-labeled rabbit anti-mouse IgG (1:500; Nichirei, Tokyo, Japan). Detection was performed with a DAB kit (Histofine simple stain kit; Nichirei). The sections were counterstained with hematoxylin.

Evaluation of the density of microvessels. The morphometric analysis of microvessels was carried out on the immunohistochemical stained

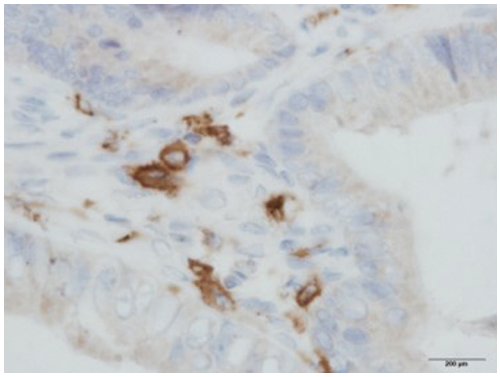

Figure 2. *Immunohistochemical detection of CD163, an M2 macrophage-specific marker (×400).*

sections using "Image-J" (National Institutes of Health, Bethesda, MD, USA), an automated imaging software program. In tumor sections, 5 randomly selected fields at the invasive margin were selected with a light microscope at a magnification of 200×. The average area and number of microvessels obtained in five different fields was used to determine the density of microvessels for the data analysis according to the methods described in previous reports (12, 16-18) (Figure 1).

Evaluation of the density of TAMs. The number of immunoreactive macrophages at the invasive margin was counted with a light microscope in a randomly selected field at a magnification of 400× (Figure 2). The average number obtained in five different areas was used for the data analysis.

Statistical analyses. The progression-free survival was defined as the time from initiation of first-line chemotherapy to disease progression, death due to any cause or last follow-up. Survival curves were made using the Kaplan-Meier method. Differences in survival curves were assessed using the log-rank test. Response evaluations by computed tomography were performed according to the Response Evaluation Criteria in Solid Tumors (19). Disease control was defined as a complete or partial response or stable disease. The association between the density of TAMs and that of microvessels was evaluated by the Spearman's rank correlation coefficient. Similarly, the association between the density of TAMs in the primary tumor and that in the metastatic tumor was also evaluated by Spearman's rank correlation coefficient. All of the statistical analyses were conducted using the JMP software program, ver. 13.0.0 (SAS Institute Inc., Cary, NC, USA). $p<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics

Group I. The characteristics of patients with stage IV CRC who underwent palliative combination chemotherapy for unresectable metastatic tumor are listed in Table I. A total of 26 men and 28 women were included. The median age of patients was 63 years old (range= 40-83 years old). The most

Table I. *Characteristics of Group I patients*.

Gender (n)	
Men	26
Women	28
Age (years)	
Median (range)	63 (40-83)
Location of the primary tumor (n)	
Colon	35
Rectum	19
Tumor depth (n)	
T1-3	20
T4	34
Histological type (n)	
Well-, Moderately differentiated	50
Poorly differentiated, Mucinous	4
Liver metastasis (n)	
Negative	15
Positive	39
Peritoneal dissemination (n)	
Negative	43
Positive	11
Lung metastasis (n)	
Negative	33
Positive	21
Number of metastatic organs (n)	
1	30
≥2	24
Regimen of first-line chemotherapy (n)	
mFOLFOX6	31
CapeOX	14
FOLFIRI	8
SOX	1
Molecular-targeted therapy (n)	
Bevacizumab	21
Cetuximab	6
Panitumumab	2
None	25
The density of TAMs (/HPF)	
Median (range)	10.4 (3.2-28.0)

FOLFOX: 5-Fluorouracil+leucovorin+oxaliplatin; CapeOX: capecitabine+oxaliplatin; FOLFIRI: 5-fluorouracil+leucovorin+irinotecan; SOX: S-1+oxaliplatin; TAMs: tumor-associated macrophages.

common metastatic organ was the liver. Twenty-four patients (44.4%) had multiple metastatic organs. Forty-six patients (85.2%) underwent oxaliplatin-based combination chemotherapy, and 8 (14.8%) underwent irinotecan-based combination chemotherapy as first-line chemotherapy.

Group II. The characteristics of patients who underwent concurrent resection of the primary tumor and the metastatic liver tumor are listed in Table II. A total of 13 men and 11 women were included. The median age of patients was 67 years old (range=33-78 years old). Twenty-three patients (95.8%) had well- or moderately differentiated adenocarcinoma. The median number of metastatic liver tumors was 1 (range=1-4).

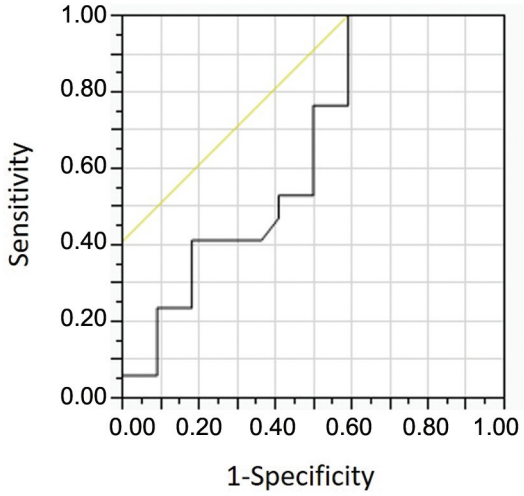

Figure 3. *A receiver operating characteristic curve analysis of the density of tumor-associated macrophages (TAMs). Area under curve=0.6484, 95% confidence interval=0.475-0.822, p=0.116.*

Classification according to the density of TAMs. We used the density of TAMs, which was a continuous variable, as the test variable and the objective response rate as the state variable. A receiver operating characteristic (ROC) curve analysis of the density of TAMs suggested the appropriate cut-off value was 14.9 with an area under the curve of 0.6484 (95% CI=0.475-0.822) (Figure 3). Thus, 14.9 was adopted as the cut-off value of the density of TAMs (sensitivity: 100%, specificity: 40.9%), and patients were classified into the high-TAMs group and the low-TAMs group based on this value.

Correlations between the density of TAMs and clinicopathological factors. There was no marked relationship between the density of TAMs and the clinicopathological factors (Table III).

Correlations between the density of TAMs in the primary tumor and the chemotherapeutic outcome. The distribution of the chemotherapeutic response with reference to the TAMs subgroups is shown in Table IV. A high TAMs level was associated with a significantly lower objective response rate than a low TAMs level (50.0% *vs*. 82.5%, p=0.0274). The progression-free survival was significantly worse in the high-TAMs group than in the low-TAMs group (p=0.0006) (Figure 4).

Correlations between the density of TAMs and that of microvessels. Regarding the primary tumor, the number of TAMs was significantly associated with the number of microvessels and the area occupied by the microvessels

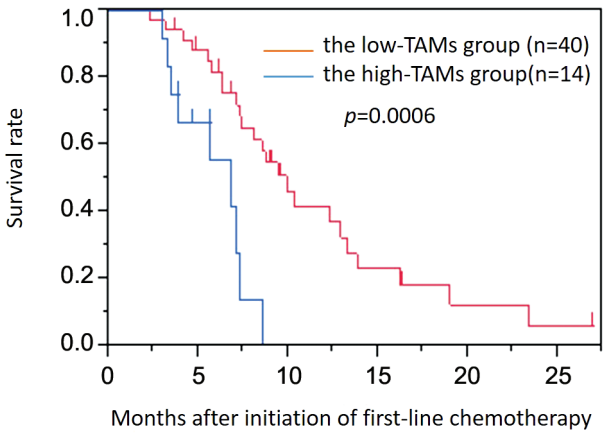

Figure 4. *The Kaplan-Meier survival curves for the progression-free survival according to the density of tumor-associated macrophages (TAMs). The high-TAMs group was associated with a poorer prognosis compared to the low-TAMs group with regard to progression-free survival (p=0.0006).*

Table II. *Characteristics of Group II patients.*

Gender (n)	
Men	13
Women	11
Age (years)	
Median (range)	67 (33-78)
Location of the primary tumor (n)	
Colon	15
Rectum	9
Tumor depth of the primary tumor (n)	
T1-3	12
T4	12
Histological type of the primary tumor (n)	
Well-, Moderately differentiated	23
Poorly differentiated, Mucinous	1
Lymphatic involvement of the primary tumor (n)	
Negative	1
Positive	23
Venous involvement of the primary tumor (n)	
Negative	10
Positive	14
Lymph node metastasis (n)	
Negative	8
Positive	16
Number of metastatic liver tumors (n)	
Median (range)	1 (1-4)

(r=0.665, p=0.0004; r=0.633, p=0.0009, respectively) (Figure 5A, B). Furthermore, regarding the metastatic liver tumor, the density of TAMs was also significantly associated with the number of microvessels and the area occupied by the microvessels (r=0.859, p<0.0001; r=0.592, p=0.0023, respectively) (Figure 5C, D).

Table III. *Correlation between the density of TAMs and clinicopathological factors.*

Factors	The density of TAMs		
	High (n=14)	Low (n=40)	*p*-Value
Gender (n)			
Men	5	21	
Women	9	19	0.276
Age (years) (n)			
<63	7	22	
≥63	7	18	0.747
Location of the primary tumor (n)			
Colon	7	28	
Rectum	7	12	0.183
Histological type (n)			
Well-, Moderately differentiated	14	36	
Poorly differentiated, Mucinous	0	4	0.113
Number of metastatic organs (n)			
1	7	23	
≥2	7	17	0.543
Peritoneal dissemination (n)			
Negative	11	32	
Positive	3	8	0.909
Molecular-targeted therapy (n)			
Without	5	20	
With	9	20	0.356

TAMs: Tumor-associated macrophages.

Table IV. *Distribution of chemotherapeutic response with reference to the TAMs subgroups.*

Response	The density of TAMs		
	High (n=14)	Low (n=40)	*p*-Value
Complete response	0	2	
Partial response	7	31	
Stable disease	3	4	
Progressive disease	4	3	
Objective response rate	50.0%	82.5%	0.0274

TAM: Tumor-associated macrophages.

Correlations between the density of TAMs in the primary tumor and that in the metastatic liver tumor. The number of TAMs in the primary tumor was significantly associated with that in the metastatic liver tumor (r=0.781, p<0.0001) (Figure 6).

Discussion

As a result of this study, it became clear that high TAMs infiltration was associated with worse chemotherapeutic

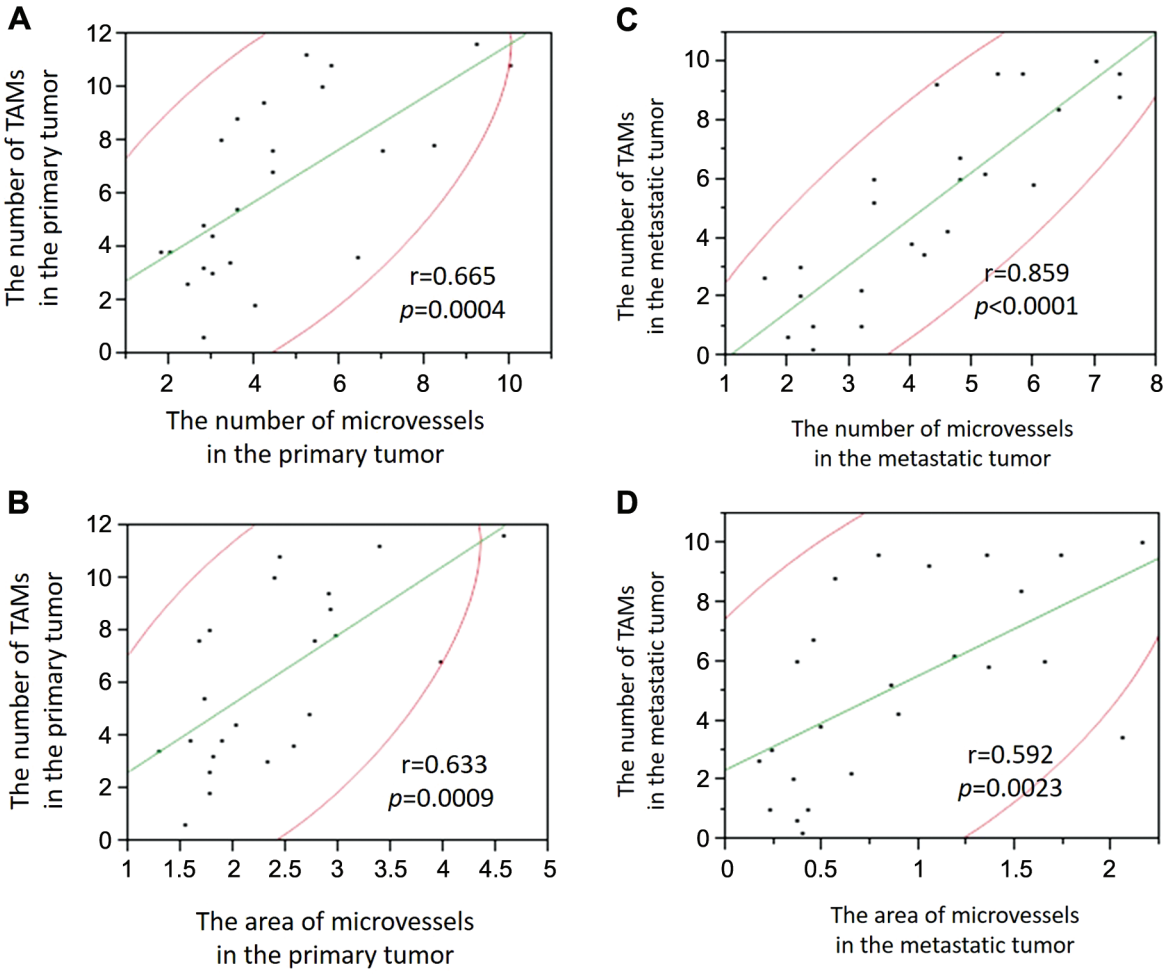

Figure 5. *Correlation between the density of tumor-associated macrophages (TAMs) and that of microvessels. (A) Correlation between the number of TAMs in the primary tumor and the number of microvessels in the primary tumor. (B) Correlation between the number of TAMs in the primary tumor and the area occupied by microvessels in the primary tumor. (C) The correlation between the number of TAMs in the metastatic liver tumor and the number of microvessels in the metastatic liver tumor. (D) The correlation between the number of TAMs in the metastatic liver tumor and the area occupied by microvessels in the metastatic liver tumor.*

outcomes than low infiltration in patients with unresectable metastatic CRC.

TAMs promote angiogenesis by producing vascular endothelial growth factor (VEGF) (20). Newly formed tumor blood vessels increased by angiogenesis differ from normal microvessels in that they have structural anomalies of the vessel wall, such as large gaps between endothelial cells, defects in pericyte coverage and discontinuous basement membranes (21, 22). Such structural abnormalities of the vessel wall increase the hyperpermeability of the tumor vessels, resulting in poor drug delivery to cancer cells. Furthermore, inadequate drug distribution diminishes the efficacy of chemotherapy. Based on these mechanisms, the chemotherapeutic effect is expected to decrease as the degree of TAMs infiltration increases. Although this relationship has been reported in

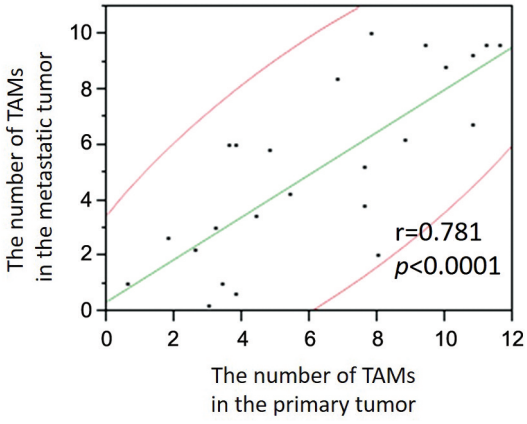

Figure 6. *Correlation between the density of tumor-associated macrophages (TAMs) in the primary tumor and that in the metastatic liver tumor. A significant correlation was observed (r=0.781, p<0.0001).*

basic research, it has rarely been verified using clinical samples. The significance of the present study is that it clinically shows that TAMs, as well as cancer cells, may promote chemoresistance *via* angiogenesis.

In this study, we examined the correlation between the density of TAMs in the primary tumor and the therapeutic outcomes. However, the relationship between the density of TAMs in the metastatic tumor, which was the target of treatment, and the chemotherapeutic outcome has not been investigated. Therefore, we examined the relationship between the density of TAMs in the primary tumor and that in the metastatic tumor, and a strong correlation between them became clear. Based on this result, the density of TAMs in the primary tumor was revealed to be a surrogate marker of the density of TAMs in the metastatic tumor. Because it is difficult to obtain a tissue sample of metastatic tumors in clinical practice, the findings revealed in this study were extremely significant.

Several limitations associated with the present study warrant mentioning. First, the current study was a retrospective study with a small cohort in a single center. Second, in addition to the TAMs, there are other factors, such as cancer cells, which promote angiogenesis by producing VEGF (23, 24). Third, in this study, the density of microvessels was measured as a surrogate marker of neovascularization, according to previous reports (11, 15-17), when angiogenesis was evaluated. However, it was difficult to determine whether or not the microvessels we counted were normal vessels that had originally existed or neovascularity that had newly developed in cancer microenvironment. In some studies, angiogenesis was assessed by the expression of Nestin or Ki-67 in vascular endothelial cells to strictly assess the neovascularity (25, 26).

The results of this study suggested that the TAMs may be the target of treatment. New drugs that block the differentiation of macrophages into the M2 type are being developed at the basic research level. In the future, these agents may be able to suppress angiogenesis and enhance the efficacy of chemotherapy.

In conclusion, TAMs were revealed to promote chemoresistance *via* angiogenesis in CRC.

Conflicts of Interest

The Authors declare no conflicts of interest in association with the present study.

Authors' Contributions

MS, SN and KM designed the study, performed the statistical analysis and drafted the manuscript. HN collected the clinical data and revised the manuscript critically. SK, KH and MO designed the study and critically reviewed the manuscript. All Authors read and approved the final manuscript.

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