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Tumor-associated Macrophages and Neuroendocrine Differentiation Decrease the Efficacy of Bevacizumab Plus Chemotherapy in Patients With Advanced Colorectal Cancer

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Abstract

No predictive marker is available for antiangiogenic treatment modalities in routine practice. The present study included a group of patients with advanced colorectal cancer, including 123 consecutive patients treated with bevacizumab plus chemotherapy, to determine the predictive and prognostic role of tumor-associated macrophages (TAMs) and neuroendocrine differentiation (NED) of primary tumor tissue. High TAM infiltration and the presence of NED in tumor tissue were predictors for poor survival.

Background: In the present study, we investigated the prognostic and predictive role of neuroendocrine differentiation (NED) and tumor-associated macrophage (TAM) infiltration in tumor tissue from patients with advanced colorectal cancer who had received bevacizumab plus chemotherapy. **Patients and Methods:** A total of 123 consecutive patients with advanced colorectal cancer who had received bevacizumab plus irinotecan/oxaliplatin-based combination chemotherapy were included in the present study. In addition to the clinicopathologic parameters, the presence of NED and the level of TAM infiltration were studied as covariates for survival analysis. **Results:** The median patient age was 57 years (range, 30-76 years). The chemotherapy backbone was FOLFIRI (folinic acid, 5-fluorouracil, irinotecan) for 75% of the patients. Univariate analysis showed that only NED and TAM infiltration were significant predictive factors for progression-free survival. Left-sided tumors and low TAM infiltration were favorable factors for overall survival on univariate analysis. However, the TAM level was the only independent prognostic factor for overall survival (hazard ratio, 0.301; 95% confidence interval, 0.102-0.892). **Conclusion:** Our results suggest that increased TAM infiltration in tumor tissue and NED could decrease the efficacy of bevacizumab plus combination chemotherapy in patients with advanced colorectal cancer. TAM infiltration in the tumor tissue could be used as a biomarker in patients with advanced colorectal cancer receiving bevacizumab plus chemotherapy.

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Keywords: Bevacizumab, Biomarker, Colorectal cancer, Neuroendocrine differentiation, Tumor-associated macrophages

Introduction

Colorectal cancer (CRC) is the second most common cancer in women and the third most common cancer in men. Also, ~50% to 60% of the patients will eventually develop metastasis.[1,2] The median survival time of patients with advanced CRC has been ~2 years.[3] Antiangiogenic drugs, including bevacizumab and epidermal growth factor receptor (EGFR)-targeting monoclonal antibodies, have been routinely used in combination with either irinotecan- or oxaliplatin-based chemotherapy backbone regimens.[3]

The tumor grade and histologic type are well-known tumor characteristics related to prognosis in patients with colorectal cancer. Mutations of RAS and RAF oncogenes predict for the efficacy of anti-EGFR monoclonal antibodies in patients with colorectal cancer.[4,5] However, no predictive marker for antiangiogenic treatments has yet been defined. The serum levels of angiogenic factors, including vascular endothelial growth factor (VEGF), angiopoietin

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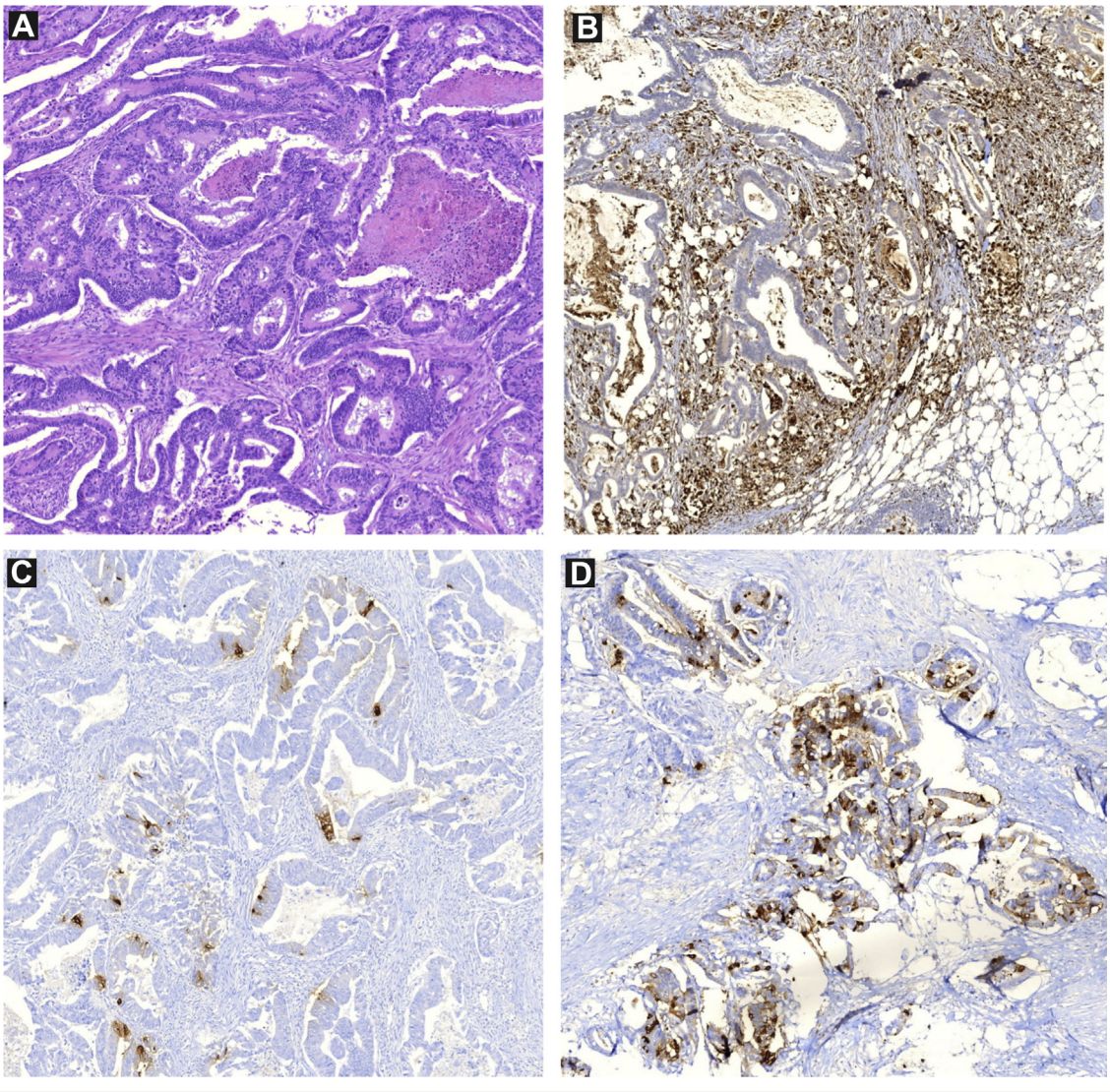
Figure 1 Examples of Colon Adenocarcinoma Sections With (A) Hematoxylin and Eosin Staining (Original Magnification ×200), (B) Increased Numbers of Tumor-associated Macrophages (+4; CD68; Original Magnification ×200), (C) Synaptophysin Staining of Tumor Cells (+2; Original Magnification ×200), and (D) Chromogranin A Staining of Tumor Cells (+3; Original Magnification ×200)



2, placental growth factor, and soluble VEGF receptor, a number of circulating endothelial progenitor cells, and various genetic markers, including polymorphisms of proangiogenic factors and microRNAs, have been studied as prognostic and predictive biomarkers for colorectal cancer.[5,6] However, none was found to be consistently useful in the clinic.

The tumor-associated macrophage (TAM) is one of the major components of the tumor microenvironment and a major driver leading to tumor progression. TAMs have been shown to secrete VEGF and have been associated with angiogenesis.[7] Recently, reported studies have shown that the immune cells infiltrating the tumors might be linked to the prognosis in various cancers.[8] However, these studies have yielded conflicting results. TAM infiltration has been reported as a poor prognostic factor in breast cancer, glioblastoma, cervical cancer, and bladder cancer and as a good prognostic factor in gastric cancer, irrespective of the treatment modalities.[9]

Neuroendocrine differentiation (NED) of the tumor has also been linked to a poor prognosis in various solid tumors.[10-13] Likewise, in a few studies investigating the prognostic role of NED in patients with colorectal cancer, a shorter survival time was found for patients with NED detected by synaptophysin and chromogranin A staining.[14,15] A significant relationship between NED and vascularization in prostate adenocarcinoma has been reported.[16] However, to the best of our knowledge, no study has investigated the relationship between treatment and NED. In a recently reported case, a partial response to bevacizumab plus FOLFOX4 (folinic acid, 5-fluorouracil, oxaliplatin) treatment was

Table 1 Patient Characteristics

Parameter	n (%)
Gender	
Female	44
Male	79
Age, y	
Median	57
Range	30-76
Age group	
≤ 50 y	34
> 50 y	89
Primary tumor localization	
Right colon	38
Left colon	85
Disease status	
Metastatic	90
Recurrent	33
Metastasis site	
Liver	103
Lung	44
Peritoneum	25
Adrenal gland	11
Central nervous system	9
Bone	9
Spleen	2
Pancreas	2
KRAS mutation status	
Mutant	58
Wild type	49
Unknown	16
CEA level at diagnosis	
High	76
Normal	47
Bevacizumab setting	
First line	44 (35.8)
Second line	73 (59.3)
Third line	6 (4.9)
Chemotherapy backbone	
Oxaliplatin-based	23 (18.7)
Irinotecan-based	92 (74.8)
FOLFIRINOX	1 (0.8)
Fluoropyrimidine	7 (5.7)

Abbreviations: CEA = carcinoembryonic antigen; FOLFIRINOX = folinic acid, 5-fluorouracil, leucovorin, irinotecan, oxaliplatin.

Table 2 Treatment Outcomes

Parameter	n (%)
Bevacizumab cycles	
Median	6.5
Range	1-18
Response	
Complete response	1 (0.8)
Partial response	32 (26.0)
Stable disease	54 (43.9)
Progression	36 (29.3)
Response duration, mo	
Median ± SEM	5.3 ± 0.8
95% CI	3.7-6.8
PFS, mo	
Median ± SEM	7.1 ± 0.6
95% CI	6.0-8.4
OS, mo	
Median ± SEM	15.1 ± 1.6
95% CI	12.0-18.1

Abbreviations: CI = confidence interval; OS = overall survival; PFS = progression-free survival; SEM = standard error of mean.

reported in a patient with stage IV colon neuroendocrine carcinoma who had had no first-line chemotherapy response.[17]

Although tremendous efforts have been made to identify and validate predictive biomarkers of clinical benefit and to stratify patients to treatment with the currently available antiangiogenic drugs, this remains one of the major priorities in clinical oncology. In the present study, we investigated the predictive and prognostic role of NED and TAM infiltration in the tumor tissue of patients with advanced CRC receiving the combination of bevacizumab plus chemotherapy.

Patients and Methods

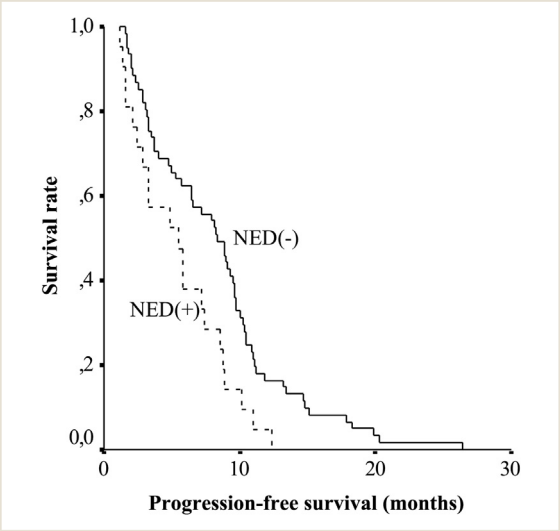
Patients

After the Clinical Research Ethics Committee of Ankara University had approved the study protocol (approval no. 17-493-15), 123 consecutive patients with metastatic CRC who had met the eligibility criteria were included in the present study. The inclusion criteria were age ≥ 18 years; Eastern Cooperative Oncology Group performance status ≤ 2; treatment with ≥ 2 months of bevacizumab (4 cycles every 2 weeks) plus either irinotecan-based or oxaliplatin-based or fluoropyrimidine chemotherapy; measurable disease; and adequate paraffin-embedded tumor tissue from surgically resected specimens from either primary tumor or metastases for histopathological evaluation. The treatment response was evaluated using the Response Evaluation Criteria In Solid Tumors at every 2 months of treatment. The responses were reassessed centrally. The disease control rate was defined as the proportion of subjects with a complete response, partial response, or stable disease 8 weeks after the start of bevacizumab.

The survival times were calculated from the start of bevacizumab until disease progression or death from any cause for progression-free survival (PFS) and until death from any cause for overall survival (OS). The treatment outcomes were stratified by the treatment line setting, including bevacizumab.

Clinicopathologic parameters, including age, performance status, tumor location (right vs. left colon), pretreatment carcinoembryonic antigen (CEA) level determined 2 weeks before the first bevacizumab administration, KRAS mutation status, tumor grade, and histologic subtype (mucinous vs. nonmucinous) were used as covariates. The histopathologic parameters, including tumor grade

Figure 2 Progression-free Survival Curves According to the Presence of Neuroendocrine Differentiation (NED) in Primary Tumor Tissue. Patients NED$^-$ Disease had 50% Longer Progression-free Survival Than Those With NED$^+$ Disease (P = .0085)



and histologic subtype, were reevaluated from the archived material of the patients. Immunohistochemical analysis of chromogranin A (Chr-A) and synaptophysin (SYNP) was evaluated for the presence of NED and CD68 was evaluated to determine the level of TAM infiltration.

Immunohistochemical Studies

Sections taken from paraffin-embedded blocks were examined for both SYNP and Chr-A for NED and CD68 staining for TAM using light microscopy. Routine hematoxylin-eosin preparations obtained from the paraffin-embedded blocks of the materials were reevaluated for histologic subtype and tumor grade (Figure 1A). For histopathological analysis, for tumors $<$ 2 cm in diameter, all the tumor tissue was assessed. For the tumors $>$ 2 cm, $\geq$ 5 paraffin blocks were assessed. Immunohistochemical study was performed from the most representative tumor-bearing paraffin-embedded blocks. Sections 4-µm thick were taken for analysis. The immunohistochemical studies were performed with immunohistochemical staining using the Ventana automatic dyeing device. Anti−Chr-A (Ventana clone, LK2H10; Ventana Medical Systems Inc, Basel, Switzerland), anti-SYNP (Cell Marque clone, MRQ-40; Cell Marque, Rocklin, CA), and anti-CD68 (Ventana clone, KP-1; Ventana Medical Systems Inc.) primary antibodies were used.

Evaluation of Immunohistochemical Staining

CD68$^+$ TAMs were estimated by counting the number of CD68$^+$ TAMs in each of the 3 tissue cores from each patient tumor sample, and the mean of 3 counts was recorded. Two experienced pathologists evaluated the tissue sections in a blinded manner using light microscopy. CD68$^+$ TAMs were estimated by counting the number of CD68$^+$ macrophages in the stroma of the tumor in the 3 tissue cores from each patient's tumor sample.

The CD68$^+$ TAMs were scored as follows: +1, $<$ 25% staining in the stroma around the tumor; +2, 25% to 49% staining; +3, 50% to 74% staining; and +4, $\geq$ 75% staining of stromal cells (Figure 1B). A score of +2 or less with anti-CD68 on immunohistochemistry was regarded as low TAM infiltration and +3 or +4 staining as high TAM infiltration.

Chr-A$^+$ and SYNP$^+$ cells were quantified by counting $\geq$ 500 cells from areas in which the most active dye was present. All preparations showed normal mucosa, and nerve cells stained with SYNP and Chr-A were taken as positive control. Chr-A and SYNP stains were scored as follows: 0, no staining; +1, $<$ 2% staining in the tumor cell; +2, 2% to 9% staining in the tumor cells (SYNP; Figure 1C); and +3, 10% to 29% of the tumor cells were positive (Chr-A; Figure 1D). The patients with staining with either SYNP or Chr-A of $\geq$ 2% were regarded as having NED$^+$ disease.[12,13]

Statistical Analysis

Statistical analysis was performed using the SPSS, version 10.0, for Windows software (IBM Corp, Armonk, NY). The Student t test, χ^2 test, and the Pearson correlation test were used to analyze the patients' clinicopathologic parameters. Univariate survival analyses were performed using the log-rank test. Multivariate survival analyses were performed using Cox regression analysis. Survival analyses were performed using the Kaplan-Meier method. All the P values were derived from 2-tailed tests.

Results

The data from 123 patients who had met the inclusion criteria were analyzed for treatment outcomes. The median age was 57 years (range, 30-76 years). The patient characteristics are listed in Table 1. Most of the patients were men (64%). The vast majority of the patients had sporadic colorectal cancer, and only 5 patients had hereditary nonpolyposis colorectal cancer. The tumor location of the patients was mostly in the left colon (69%). Of the 123 patients, 73% had had metastatic disease at diagnosis. The liver was the leading metastatic site (83.7%); however, 86.2% of the patients had $>$ 1 metastatic region. Of the 123 patients, 26% had NED (25.3% of the patients with sporadic colorectal cancer vs. 40% of the patients with nonsporadic colorectal cancer [2 of 5 patients]). Immunohistochemical analyses were performed for 12 patients from the paraffin-embedded blocks prepared from the surgical specimens after first-line chemotherapy without bevacizumab. However, for the remaining patients, tumor tissues taken before treatment were used for immunohistochemical analysis. The NED rate in the tumor tissues sampled after first-line chemotherapy (26.7%) was similar to that in those sampled before treatment (26.0%). Likewise, no significant difference in the TAM infiltration levels was found between the tumors sampled before and after first-line treatment (P = .450). Although not statistically significant, a weak correlation was found between NED and poor tumor grade (P = .127). Likewise, a slight, but not significant, reverse correlation was found between NED and the CEA level (P = .147). We found no correlations between the NED rate and lymph node involvement (P = .636), right versus left colon location (P = .501), or metastatic site (P = .656). Also, no significant correlations were found between the TAM infiltration level and the clinicopathologic features studied.

Table 3 Factors Affecting PFS and OS on Univariate Analysis

Parameter	Median PFS (95% CI)	*P* Value	Medan OS (95% CI)	*P* Value
Primary tumor location		.3702		.0944
Right colon	6.7 ± 0.5 (5.7-7.6)		11.7 ± 1.7 (8.4-14.9)	
Left colon	7.9 ± 0.7 (6.5-9.2)		16.2 ± 2.0 (12.3-20.1)	
Age		.5978		.7555
≤ 50 y	7.2 ± 1.7 (5.9-10.4)		16.2 ± 4.6 (7.1-25.3)	
> 50 y	7.4 ± 0.8 (5.9-9.0)		14.8 ± 1.2 (12.4-17.2)	
Disease status		.7148		.6283
Metastatic	7.2 ± 0.7 (5.9-8.5)		13.4 ± 1.6 (10.4-16.5)	
Recurrent	7.4 ± 1.0 (5.5-9.4)		17.9 ± 3.2 (1.7-24.0)	
Tumor grade		.2673		.1048
1	6.6 ± 1.0 (4.7-8.6)		15.1 ± 8.7 (0-28.6)	
2	7.4 ± 1.2 (5.1-9.7)		16.2 ± 1.8 (12.6-19.8)	
3	7.1 ± 0.9 (5.2-9.0)		11.4 ± 1.5 (8.5-14.3)	
CEA level, ng/mL		.4973		.9580
≤ 5.0	7.1 ± 0.5 (6.2-8.1)		15.2 ± 4.5 (6.4-24.0)	
> 5.0	7.1 ± 1.4 (4.4-9.8)		13.8 ± 1.5 (10.8-16.8)	
KRAS status		.3335		.4096
Mutant	8.0 ± 0.8 (6.5-9.5)		16.2 ± 1.4 (13.4-19.0)	
Wild type	7.1 ± 1.7 (3.8-10.4)		12.4 ± 2.0 (8.4-16.3)	
Chemotherapy		.7876		.9639
Oxaliplatin-based	7.4 ± 0.7 (6.1-8.8)		14.8 ± 2.8 (9.3-20.4)	
Irinotecan based	7.9 ± 0.7 (6.5-9.4)		15.4 ± 1.7 (12.0-18.7)	
NED		.0085		.5474
Negative	8.3 ± 1.0 (6.3-10.9)		15.4 ± 2.0 (11.4-19.4)	
Positive	5.5 ± 1.4 (2.7-8.3)		10.6 ± 2.4 (5.9-15.4)	
TAM infiltration		.0272		.0076
Low	9.3 ± 1.8 (5.8-12.8)		26.7 ± 8.8 (9.5-43.9)	
High	6.5 ± 1.2 (4.9-8.8)		14.1 ± 1.7 (10.8-17.4)	

Abbreviations: CEA = carcinoembryonic antigen; CI = confidence interval; NED = neuroendocrine differentiation; TAM = tumor-associated macrophage.

Bevacizumab was given at a dose of 5 mg/kg every 2 weeks. Only 35.8% of the patients were given bevacizumab as the first-line setting. The remaining patients had mainly received bevacizumab in the second line combined with the chemotherapy backbone. The chemotherapy backbone administered concurrently with bevacizumab was mainly the FOLFIRI (folinic acid, 5-fluorouracil, irinotecan) regimen (74.8%). Almost 45% of the patients had KRAS wild type (Table 1).

The treatment outcomes are listed in Table 2. The median follow-up duration for the patients was 47.4 months. The median number of bevacizumab cycles was 6.5. The overall response rate was 26.8%, with only 1 patient having a complete response. However, the disease control rate was 70.7% (Table 2). The median PFS duration for the patients was 7.1 ± 0.6 months (95% confidence interval [CI], 6.0-8.4), and the median OS duration was 15.1 ± 1.6 months (95% CI, 12.0-18.1; Table 2).

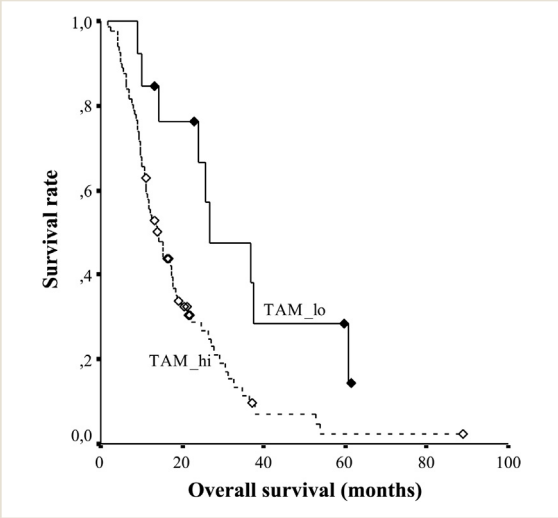
The covariates for survival analysis were the clinicopathologic features (ie, age, gender, primary tumor location, metastasis at diagnosis, KRAS mutation, tumor grade), the pretreatment CEA level, NED, and TAM. We found only the lack of NED and lower TAM infiltration were significant factors for PFS on univariate analysis. Although the median PFS was 8.3 ± 1.0 months for patients with NED^- disease, it was 5.5 ± 1.4 months for those with NED^+ cancer (Figure 2, Table 3). Left-sidedness cancer (P = .0944) and low levels of TAM infiltration (P = .0074; Figure 3) were favorable factors for OS on univariate analysis (Table 3).

Multivariate analysis showed only NED as an independent factor for PFS for our patients (hazard ratio [HR], 0.542; 95% CI, 0.300-0.979). However, the TAM level was the only independent factor predictive for OS on multivariate analysis. The patients with lower TAM infiltration survived ~2 times longer than those with higher TAM infiltration (26.7 ± 8.8 vs. 14.1 ± 1.7 months, respectively; HR, 0.301; 95% CI, 0.102-0.892; Figure 3).

Discussion

The addition of bevacizumab to standard chemotherapy regimens (irinotecan- or oxaliplatin-based combinations or fluoropyrimidines) for patients with advanced CRC has improved OS. The median survival time obtained with the addition of bevacizumab to either FOLFIRI or FOLFOX regimens in the first-line setting has usually been 20 to 22 months.[3] In contrast, in the trials using bevacizumab

Figure 3 Overall Survival Curves According to Tumor-associated Macrophage (TAM) Intensity in Tumor Microenvironment. Patients With Low TAM Infiltration in the Tumor Microenvironment (TAM_lo) Survived 2 Times Longer Than Those With High TAM Infiltration (TAM_hi; $P = .0076$)



in the second line, the median survival time was ~15 to 18 months.[18-20] Most patients in our study had received bevacizumab in the second line. In our study, PFS was 7.1 ± 0.6 months and OS was 15.1 ± 1.6 months, compatible with the data from studies reporting on second-line therapy.

Although RAS and BRAF mutations and right-sided cancer are poor predictive markers for anti-EGFR monoclonal antibodies in patients with advanced CRC, no predictive markers are available for antiangiogenic treatment, including bevacizumab. Prager et al[21] have shown that CEA could be a predictive marker for CRC treated with bevacizumab. They reported that the treatment response to bevacizumab for those with a high CEA level was worse than that for the group with a normal CEA level.[21] In our study, age, primary tumor localization, CEA level, tumor grade, and KRAS mutation status were examined as clinicopathologic parameters for bevacizumab outcomes. Although not statistically significant, tumor localization was the only conventional clinicopathologic variable that affected survival on univariate analysis of patients receiving bevacizumab. The patients with left-sided tumors had prolonged OS compared with those with right-sided primary tumors (Table 3).

In addition to the conventional pathological parameters, NED has also been studied as a prognostic factor for various solid tumors.[10-13] However, tumor heterogeneity and sampling errors of tumor tissue could have caused some limitations in the evaluation of NED. To decrease those limitations, we used surgically resected specimens and assessed all the tumor tissue for tumors < 2 cm in diameter and ≥ 5 paraffin blocks for the tumors > 2 cm in diameter. Although the number of tumor samples taken after first-line chemotherapy was small (~10%) in the present study, the treatment effect on tumor tissue could have been another limitation. The prognostic role of NED in CRCs is not clear. In a study of 53 cases reported by Suresh et al,[13] they could not find a positive or negative effect for NED on prognosis. In another study, NED was detected extensively in stage II poorly differentiated CRC cases and was found to be a poor prognostic factor.[14] However, the relationship between NED and bevacizumab activity was not investigated in these studies. To the best of our knowledge, no reported studies have evaluated the response of NED to bevacizumab in patients with CRC. However, a few studies, mainly case reports, have investigated the response to bevacizumab in patients with neuroendocrine tumors. In one case report, a partial response was obtained after treatment with FOLFOX plus bevacizumab in one patient with a diagnosis of colon neuroendocrine carcinoma with progressive failure after first-line chemotherapy.[17] In another case report by Kusakabe et al,[22] FOLFOX plus bevacizumab failed in one patient with metastatic mixed adenoneuroendocrine carcinoma of the colon. In the present study, we found that the median PFS for patients with NED^{+} disease was significantly shorter than that for those with NED^{-} disease (5.5 months vs. 8.3 months; Figure 2). Accordingly, NED was found to be an independent factor for PFS in patients receiving chemotherapy plus bevacizumab (HR, 0.542; 95% CI, 0.300-0.0979). Although not statistically significant, a 50% prolongation in OS for patients with NED^{-} disease was found (Table 3).

TAMs in the tumor microenvironment have usually correlated with a poor prognosis.[23] However, a limited number of trials have studied the role of TAMs in CRC. In a study of 44 colon carcinoma biopsy specimens and 15 polypectomy materials investigating macrophage infiltration and microvessels with CD68 and F-VIII staining, macrophage infiltration was significantly greater in the malignant cases than in the benign polyps. However, a correlation was found between high TAM infiltration and advanced stage, hypervascularity, and TNM stage.[24] To the best of our knowledge, no trial has evaluated the role of TAMs in patients receiving bevacizumab. In the present study, we found a significant correlation between TAMs in the tumor microenvironment and the prognosis of the patients with advanced CRC receiving bevacizumab plus chemotherapy. The high number of patients (>50%) given bevacizumab in the second-line setting is an important limitation of the present study. The significantly longer PFS and OS seen in patients with low TAM infiltration suggests the potential use of TAM as a biomarker in patients with advanced CRC receiving bevacizumab (Table 3, Figure 3). However, testing TAMs in a homogenous group of patients with CRC receiving bevacizumab in the first-line setting could further clarify the predictive and prognostic role of TAMs.

Recently, the emerging role of programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1) checkpoint interaction in TAMs has raised the potential use of anti−PD-1 and anti−PD-L1 inhibitor drugs in patients with high TAM infiltration.[25,26] The poor outcome of patients with increased TAM infiltration in the present study could provide a clue for the use of immune checkpoint inhibitors in this group of patients.

Conclusions

We found that the patients with advanced CRC and high TAM infiltration or NED had significantly shorter PFS (Table 3). Likewise, NED was an independent predictor for PFS of patients with

advanced CRC receiving bevacizumab plus chemotherapy. High TAM infiltration in the primary tumor tissue significantly decreased the OS duration for patients with advanced CRC.

Clinical Practice Points

- We have reported the results from a prospective study that investigated the predictive and prognostic role of TAMs and NED in patients with advanced CRC.
- To the best of our knowledge, the present study is the first to investigate the role of TAMs and NED in patients with advanced CRC treated with bevacizumab.
- Our results suggest that TAM infiltration in the tumor tissue could be used as predictive marker of the efficacy of bevacizumab plus combination chemotherapy in patients with advanced CRC.
- The shorter duration of PFS and OS in patients with high TAM infiltration could be improved by the combination of checkpoint inhibitors and bevacizumab.
- We propose the investigation of the combination of checkpoint inhibitors, including anti-CD47, anti−PD-1, and anti−PD-L1 monoclonal antibodies and bevacizumab in patients with advanced CRC and high TAM infiltration in a clinical trial.

Acknowledgments

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