Association of Serum and Intratumoral Cytokine Profiles with Tumor Stage and Neutrophil Lymphocyte Ratio in Colorectal Cancer

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Abstract. *Background: We examined cytokine profiles and evaluated the association between cytokine levels, pathological stages, and neutrophil/lymphocyte ratio (NLR). Materials and Methods: Patients with colorectal cancer (n=20, TNM stage I, II, and III) were enrolled. Levels of nine cytokines [interleukin (IL)-4,-6, -8, -10, -12, tumor necrosis factor-alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ), and vascular endothelial growth factor (VEGF)] were measured in serum, normal mucosa, and tumor using the Bio-Plex® cytokine assay. Results: The mean IL8, GM-CSF and VEGF levels were higher in tumors, whereas the mean IL6 level was higher in serum. Cytokine levels correlated with TNM stage (IL6 and IL8 in serum, and IL8 and VEGF in tumor) and with NLR (IL6 and IL8 in serum, and IL8 in tumor). Conclusion: Different cytokine profiles were observed in serum, normal mucosa, and tumor tissue. The elevation of specific cytokines in sera or tumors reflects features of advanced disease.*

Inflammation is closely associated with cancer development and progression. In addition to the pathological TNM stage, both systemic and local inflammatory responses between the tumors and the host have been suggested as significant prognostic factors. Elevated systemic inflammatory response, as determined by neutrophil/lymphocyte ratio (NLR), or modified Glasgow prognostic score (mGPS) based on C-reactive protein and albumin levels, has been shown to be a risk factor for inferior survival in colorectal cancer (1, 2). On the other hand, local inflammatory reaction induced by inflammatory cell infiltration around the tumor has been reported to be a marker for favorable prognosis (3, 4).

Within the tumor microenvironment, colorectal carcinomas are infiltrated by immune cells such as neutrophils, T- and B-lymphocytes, dendritic cells, macrophages, natural killer cells, and mast cells (5). These cells produce cytokines, chemokines and inflammatory mediators. Cytokines modulate immune responses between the infiltrating inflammatory cells and the primary tumor. Given that serum cytokine levels reflect a systemic inflammatory reaction, cytokine levels in normal mucosa and tumor tissue can reveal a localized inflammatory reaction to a tumor. Indeed, it has been reported that serum levels of pro-tumorigenic cytokines are higher in colorectal malignancies than in benign disease, and correlate well with TNM stage (6, 7). However, there have been few studies investigating both serum and intratumoral cytokine profiles in colorectal cancer. In addition, it is still not very well defined whether serum cytokine levels can reflect a systemic inflammatory reaction or whether cytokine levels in normal mucosa and tumor tissue can reveal a localized inflammatory reaction within the tumor microenvironment.

Thus, we examined cytokine profiles in the serum, normal mucosa, and tumor. We also investigated the correlation of cytokine levels of serum, normal mucosa, and tumor with pathological tumor features and markers of such as NLR. A multiplex assay was performed to evaluate nine cytokines [interleukin (IL)-4,-6, -8, -10, -12, tumor necrosis factor-alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ), and vascular endothelial growth factor (VEGF)] using serum, normal mucosa, and tumor tissue.

Materials and Methods

Patients. A total of 25 patients who underwent major colorectal surgery with curative intent for stage I, II, or III colorectal cancer between June 2012 and October 2012 were prospectively enrolled

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in this study. Five patients were excluded due to the unavailability of paired tissue samples from them. Accordingly, the data of 20 patients were analyzed. This study was registered at ClinicalTrials.gov (NCT01629524). Patients were excluded if they had received neoadjuvant chemoradiation therapy or had inflammatory bowel disease. This study was approved by the Institutional Review Board of Yonsei University Wonju College of Medicine (No. 2012-08).

Selection of cytokines. A total of nine cytokines were selected: three cytokines of T-helper cell type 1 (IL12, IFN-γ, and GM-CSF), one cytokine of T-helper cell type 2 (IL4), three proinflammatory cytokines (TNF-α, IL6, and IL8), one anti-inflammatory cytokine (IL10), and one angiogenic cytokine (VEGF) (8-16).

Samples. Patient's preoperative serum, normal colonic mucosa, and tumor tissue were used for cytokine assays. Peripheral blood (2 ml) was taken one day before surgery. Serum was separated from the blood by centrifugation and stored at −80˚C. Tissue specimens from colorectal cancer were obtained during standard surgical procedures. Normal colonic mucosa 5 cm proximal from the main tumor, as well as tumor tissue, were obtained by a pathologist and stored at −80˚C. Before quantification of cytokines, tissue samples were minced into a fine pulp and protein was extracted using a Qiagen stainless steel bead kit (Valencia, CA, USA).

Bio-Plex® cytokine assay. We used a fluorescent bead-based detection assay, which is a highly sensitive method for profiling multiple cytokines (17). The fluorescent bead-based detection assay has an advantage over conventional enzyme-linked immunosorbent assay (ELISA), which enables analysis of a number of analytes simultaneously. Cytokine levels were measured using a Multiplex kit (Bio-Rad, San Diego, CA, USA) according to the manufacturer's protocol. Standard curves for each cytokine were generated using the reference concentrations provided in the kit. The plate was run on a Luminex 200 Bio-Plex Instrument (Bio-Rad, Hercules, CA, USA). Raw fluorescence data were analyzed with software using the 5-parameter logistic method. Detection range was from 2 to 32,000 pg/ml. Detailed procedures of the Bio-Plex® Suspension Array System have been described elsewhere (18).

Makers of systemic inflammation. The mGPS was calculated as follows: score 0, C-reactive protein ≤10 mg/l; score 1, C-reactive protein >10 mg/l and albumin ≥35 g/l; score 2, C-reactive protein >10 mg/l and albumin <35 g/l (1). NLR was calculated as the neutrophil count divided by the lymphocyte count using preoperative blood test results. An NLR ≥3 was considered elevated.

Surgery, adjuvant therapy, and pathological examination. All surgeries were performed by two colorectal surgeons. A standardized pathological examination was performed, and histopathological data were recorded according to the American Joint Committee on Cancer (AJCC) staging system (19).

Statistical analysis. All statistical analyses were performed using IBM SPSS Statistics for Windows, version 20.0 (IBM, Armonk, NY, USA). Mann–Whitney *U*-test and Kruskal–Wallis test were used for comparison of continuous variables and Chi-squared tests (Fisher's exact tests) for comparison of categorical variables. For the construction of scatter plots, GraphPad Prism software version 6.02 (GraphPad, San Diego, CA, USA) was used. A *p*-value of less than 0.05 was considered to be statistically significant.

Table I. *Patients' characteristics*.

	N (%) or median (IQR)
Age (years)	67 (59-73)
Gender	
Male	14 (70)
Female	6 (30)
BMI (kg/m^2)	24 (22-25)
Tumor location	
Colon	18 (90)
Rectum	2 (10)
pTNM	
I	3 (15)
II	9 (45)
III	8 (40)
Histological grade	
WD, MD	17 (85)
PD, mucinous	3 (15)
NLR	
≤3	12 (60)
>3	8 (40)
mGPS	
0, 1	15 (75)
2	5 (25)
Tumor size (cm)	
≤4	9 (45)
>4	11 (55)
CEA (ng/ml)	3.2 (2.1-3.8)

IQR, Interquartile range; BMI, body mass index; TNM, tumor node metastasis; WD, well-differentiated; MD, moderately-differentiated; PD, poorly-differentiated; NLR, neutrophil:lymphocyte ratio; mGPS, modified Glasgow prognostic score; CEA, carcinoembryonic antigen.

Results

Patients' characteristics. The mean age was 67 years, and there fourteen males and six females were included. Eighteen patients had colon cancer and two had rectal cancer. There were three cases of TNM I, nine cases of TNM II, and eight cases of TNM III disease. Detailed patients' characteristics are presented in Table I.

Cytokine profiles according to the type of sample. A total of nine cytokines were evaluated in this study. Five cytokines (IL4 IL10, IL12, IFN-γ, and TNF-α) were excluded because 14 (70%) or more of the values were below the quantifiable limit (<2 pg/ml) in serum, normal mucosa, or tumor tissue.

There were significant differences in IL6, IL8, GM-CSF, and VEGF levels depending on the type of samples, *i.e.* serum, normal mucosa, and tumor. Mean IL8, GM-CSF, and VEGF levels were higher in tumor compared to those in

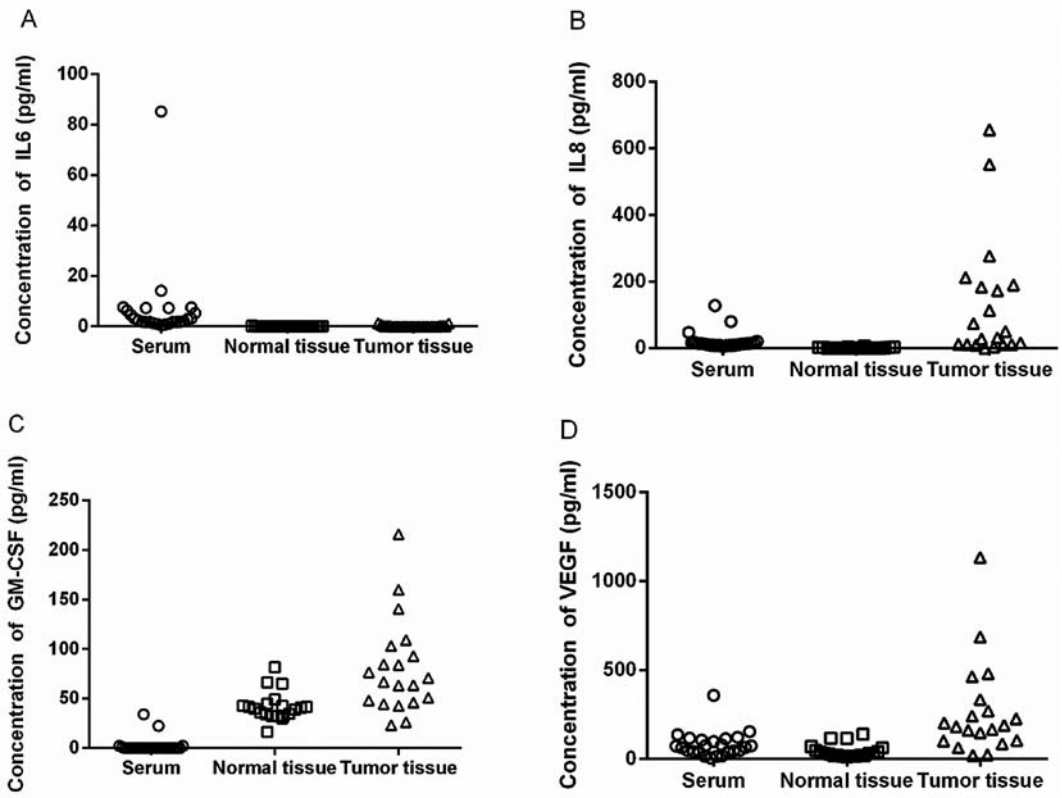

Figure 1. *Levels of cytokines in serum, normal colon mucosa, and tumor tissue. Scatter plots showing the distribution of levels (pg/ml) of four cytokines. A: Interleukin (IL)-6; B: IL8; C: granulocyte-macrophage colony-stimulating factor (GM-CSF); D: vascular endothelial growth factor (VEGF).*

serum or normal mucosa, whereas the mean IL6 level was higher in the serum compared to that in the normal mucosa or tumor (Figure 1) (Table II).

Correlation of cytokine levels with pathological features. Higher serum IL6 ($p<0.001$) and IL8 levels ($p=0.001$) correlated with TNM III. Higher intratumoral IL8 ($p=0.002$) and VEGF ($p=0.01$) correlated with TNM III. Higher serum IL6 ($p=0.01$) also correlated with poor or mucinous histology. Higher serum ($p=0.02$) and intratumoral IL8 levels ($p=0.01$) correlated with larger tumor size (Table III).

Correlation of cytokine levels with systemic inflammation marker. Higher serum IL6 ($p=0.01$) and IL8 levels ($p=0.01$) correlated with elevated NLR. Higher intratumoral IL8 levels correlated with elevated NLR ($p<0.001$). Higher intratumoral IL8 levels also correlated with greater mGPS ($p=0.03$) (Table IV).

Discussion

The major finding of this study is that cytokine profiles differ between serum and tumor. Cytokine levels correlated with pathological TNM stage (IL6 and IL8 in serum, and IL8 and VEGF in tumor) and with the marker of systemic inflammation, NLR (IL6 and IL8 in serum, and IL8 in tumor).

Cytokines have pleiotropic functions and interact with each other. Examination of multiple cytokines is useful because underlying immune mechanisms of colorectal cancer are complex and it is difficult to predict which single cytokine might be the most informative (17).

Firstly, we examined cytokine profiles in serum, normal mucosa, and tumor tissue. We observed that the mean cytokine concentrations differed among the different types of samples. IL8, GM-CSF, and VEGF levels in tumor tissue were higher than those in serum or normal mucosa. The cause of this finding could be that cytokines act in a paracrine manner, close to the site of inflammatory reactions (20). If an antitumor immune response is initiated, cytokine secretion by immune cells within the primary tumor could cause higher cytokine concentrations in the tumor tissue. Interestingly, the IL6 level in serum was higher than that in normal mucosa or tumor tissue. IL6 is produced by macrophages, endothelial cells, and T-cells. The mechanism for the increased serum IL6 levels is unclear. Ramsey *et al*. found that preoperative IL6

Table II. *Cytokine profile [median (IQR) value] according to the type of sample.*

Cytokine (pg/ml)	Sample type (n=20)			*p*-Value*
	Serum	Normal mucosa	Tumor tissue	
IL4	0 (0)	0 (0)	0 (0)	-
IL6	2.3 (1.7-6.3)	0 (0)	0 (0-0.2)	<0.001
IL8	12.0 (8.4-15.8)	0.2 (0-2.8)	41.0 (11.5-187.0)	<0.001
IL10	0 (0)	0 (0)	0 (0)	-
IL12	0 (0)	0 (0)	0 (0)	-
GM-CSF	0 (0)	40.2 (33.0-43.7)	68.8 (47.2-98.1)	<0.001
IFN-γ	0 (0)	0 (0)	0 (0-8.3)	-
TNF-α	0 (0)	0 (0)	0 (0)	-
VEGF	69.5 (41.1-102.5)	30.8 (19.7-55.8)	185.6 (102.9-302.3)	<0.001

IQR, Interquartile range; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor; *Kruskall–Wallis test.

levels did not return to normal after curative resection of renal cancer. The authors pointed-out that tumor tissue is not the sole source of IL6 (21). In addition to tumor origin, an *IL6* gene polymorphism has been suggested (22), and IL6 may be distributed systemically rather than locally after initial tumor development. In the present study, IL4, IL10, IL12, IFN-γ, and TNF-α levels were out of the detection range in most cases among serum, normal mucosa, and tumor tissues. One possible explanation is that cytokine responses to injury are different between acute and chronic inflammation. Acute inflammatory responses to injury lead to abrupt increases in leukocyte counts and cytokine levels. In contrast, chronic inflammation is characterized by humoral and cell-mediated immune responses to the presenting antigen. Cancer is one such chronic inflammatory condition. Under low levels of chronic stimuli, immune cells may express low levels of these cytokines. For instance, TNF-α is produced by activated macrophages, has a function in acute inflammatory diseases, and has tumor-promoting effects (23). In this study, TNF-α was below the limit of detection (<2 pg/ml) in all patients. Similarly, Chung *et al*. found that TNF-α was not detectable in patients with colorectal cancer (13). We believe that TNF-α levels may be high in cases of microbial infection, however, under low level chronic stimuli such as malignancy, TNF-α production may be low. In addition, we did not include stage IV disease in this study, and it is possible that late-stage colorectal cancer may express higher levels of cytokines.

Secondly, we evaluated the association between cytokine levels and clinicopathological features. IL6 and IL8 are well-known pro-inflammatory cytokines. Esfandi *et al*. measured IL6 in serum and colorectal cancer tissue using an ELISA method (24). The authors found that serum IL6 levels correlated with tumor IL6 levels, and that the serum and tumor IL6 levels were significantly higher in stage IV disease. Recently, Kantola *et al*. investigated serum cytokine profiles in patients with colorectal cancer using multiplex assays (6). The authors found that serum IL6 and IL8 levels were associated with histological grade, and that high IL8 was related to lymph node metastasis. In this study, serum IL6 and IL8 levels, and tumor IL8 and VEGF levels were all higher in TNM stage III. Our findings support the hypothesis that proinflammatory cytokines such as IL6 and IL8 may influence tumor stage and tumor-promoting activity (13-15). In this study, serum GM-CSF was wholly undetectable, and GM-CSF levels in normal mucosa and tumor showed no correlation with clinicopathological parameters. Ueda *et al*. observed that serum GM-CSF levels increased slightly in patients with colorectal cancer but there were no significant relationships between serum GM-CSF and clinicopathological variables. (14). GM-CSF is involved in acute inflammation and protective immunity, mainly by stimulating tumor antigen presentation by recruited dendritic cells and macrophages (25). In contrast, Takeda *et al*. suggested that GM-CSF mRNA expression in tumor cells was associated with increased metastatic activity in a murine model (26). Elevation of tumor GM-CSF levels in the present study may reflect an enhanced antitumor immune response; however, its exact effect on the tumor microenvironment needs to be further elucidated. VEGF is produced by fibroblasts, macrophages, neutrophils, endothelial cells, and T-cells, and influences tumor neovascularization and metastasis (16). VEGF is more highly expressed in tumors than in distant colonic mucosa (27), as was found in our study. De Vita *et al*. demonstrated that serum VEGF level is associated with Dukes' stage (28). In the present study, serum VEGF was higher in patients with TNM stage III disease, but this did not reach statistical significance (p=0.07). Tumor VEGF levels, however, showed a significant correlation with TNM stage.

Finally, we evaluated the association between cytokine levels and markers of systemic inflammation. Cytokines

modulate tumor-associated inflammation. The levels of serum cytokines, secreted by circulating immune cells, reflect systemic inflammatory reactions, and cytokine levels in normal mucosa and tumor tissue reveal localized inflammatory reactions to tumors. Serum IL6 and IL8 levels have been shown to be related to systemic inflammatory response markers such as mGPS, CRP, and NLR (6). In the present study, higher levels of serum IL6 and IL8 were associated with elevated NLR. In addition, tumor IL8 levels were associated with NLR and mGPS.

This study has limitations. This study included a small number of patients. The strengths of this study are, for one, that multiple cytokines were evaluated in the serum, normal mucosa, and tumor tissues simultaneously. In addition, serum and tissue samples for cytokine assays were prospectively obtained through standardized procedures. To the best of our knowledge, this is the first study to simultaneously evaluate cytokine profiles in the serum, normal mucosa, and tumor tissues in colorectal cancer.

In summary, different cytokine profiles were observed among serum, normal mucosa, and tumor tissue. Cytokine levels were higher in tumor than in serum, except for IL6. IL6 appears to be produced from sources other than the tumor microenvironment. Pro-inflammatory (IL6 and IL8) and angiogenic (VEGF) cytokines correlated with pathological tumor stage and a maker of systemic inflammation (NLR). These findings suggest that elevation of serum or tumor cytokine level reflects features of advanced disease. Based on our results, cytokine levels may be used as a surrogate marker for disease progression. Further studies are being conducted to clarify the impact of distinct cytokine patterns on oncological outcomes.

Table III. *Correlation of cytokine levels (pg/ml) with pathological features.*

		Median (IQR)	pTNM			Histological grade			Tumor size (cm)		
			I, II	III	*p*-Value*	WD,MD	PD, mucinous	*p*-Value*	≤4	>4	*p*-Value*
Serum		IL6	1.7 (1.0-2.0)	7.3 (5.0-7.6)	<0.001	1.9 (1.7-3.1)	7.6 (7.5-85.2)	0.01	1.7 (1.7-2.2)	4.6 (1.8-7.3)	0.1
		IL8	9.3 (8.1-11.0)	17.0 (13.6-32.6)	0.001	10.7 (8.1-14.4)	17.2 (12.7-48.0)	0.08	8.7 (8.1-12.7)	14.4 (10.7-17.2)	0.02
		GM-CSF	0 (0)	0 (0-0.5)	0.7	0 (0)	0 (0)	0.4	0 (0)	0 (0-2.2)	0.3
		VEGF	56.4 (15.0-77.2)	90.8 (57.3-134.4)	0.07	66.2 (40.9-97.7)	74.3 (48.4-358.6)	0.4	64.5 (48.4-75.4)	74.3 (40.9-114.0)	0.5
Normal mucosa		IL6	0 (0)	0 (0)	0.9	0 (0)	0 (0-0.2)	0.3	0 (0)	0 (0)	0.9
		IL8	0.2 (0-1.3)	1.4 (0-3.3)	0.5	0 (0-1.6)	3.4 (0-6.9)	0.2	0 (0-1.0)	0.4 (0-3.1)	0.6
		GM-CSF	41.1 (34.0-45.8)	36.9 (33.0-43.7)	0.6	39.7 (34.0-42.5)	40.6 (30.2-81.5)	0.9	40.6 (35.0-42.5)	39.7 (32.1-49.0)	0.9
		VEGF	30.8 (17.5-39.3)	38.0 (19.9-89.4)	0.5	30.7 (19.7-42.2)	63.1 (20.1-115.6)	0.3	30.7 (19.7-36.4)	32.5 (19.7-71.0)	0.7
Tumor		IL6	0 (0-0.2)	0.1 (0-0.2)	0.8	0 (0-0.2)	0.2 (0-0.2)	0.7	0 (0)	0.2 (0-0.7)	0.08
		IL8	12.0 (10.7-29.9)	187.0 (143.4-244.6)	0.002	27.9 (11.5-113.5)	190.4 (173.3-655.9)	0.05	11.5 (11.0-15.5)	173.3 (50.2-277.1)	0.01
		GM-CSF	68.8 (53.8-106.3)	67.5 (47.2-88.7)	0.9	70.8 (48.1-103.3)	63.2 (42.5-84.6)	0.5	63.2 (48.1-76.6)	84.1 (44.4-140.8)	0.3
		VEGF	155.0 (93.6-192.2)	398.6 (229.8-584.0)	0.01	166.3 (101.8-242.2)	270.2 (189.4-686.9)	0.2	181.8 (101.8-202.6)	270.2 (148.4-481.1)	0.2

IL, Interleukin; GM-CSF, granulocyte-macrophage colony stimulating factor; VEGF, vascular endothelial growth factor; IQR, interquartile range; TNM, tumor node metastasis; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated; *Mann–Whitney *U*-test.

Disclosures

Young Wan Kim, Soo-Ki Kim, Cheol Su Kim, Ik Yong Kim, Mee Yon Cho and Nam Kyu Kim have no conflicts of interest or financial ties to disclose.

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Table IV. *Correlation of cytokine levels (pg/ml) with the neutrophil/lymphocyte ratio, a marker of systemic inflammation.*

		NLR			mGPS		
	Median (IQR)	≤3	>3	*p*-Value*	0, 1	2	*p*-Value*
Serum	IL6	1.7 (1.4-2.3)	6.3 (3.9-7.6)	0.01	1.9 (1.7-3.1)	7.3 (5.3-7.5)	0.07
	IL8	9.3 (8.1-12.6)	15.5 (12.7-32.6)	0.01	9.8 (8.1-15.0)	12.7 (12.7-48.0)	0.1
	GM-CSF	0 (0-0.5)	0 (0)	0.6	0 (0-0.9)	0 (0)	0.2
	VEGF	56.4 (27.8-77.2)	90.8 (57.3-134.4)	0.1	64.5 (15.3-79.0)	97.7 (74.3-107.2)	0.1
Normal mucosa	IL6	0 (0)	0 (0)	0.9	0 (0)	0 (0-0.1)	0.07
	IL8	0.2 (0-1.9)	0.8 (0-3.3)	0.6	0 (0-1.6)	3.1 (0-4.0)	0.2
	GM-CSF	38.7 (32.1-43.7)	40.2 (36.4-53.3)	0.7	41.6 (35.0-44.8)	34.0 (32.1-40.6)	0.3
	VEGF	30.8 (17.5-42.4)	34.9 (19.9-89.4)	0.5	30.7 (15.3-42.2)	63.1 (20.1-71.0)	0.3
Tumor	IL6	0 (0-0.2)	0.2 (0-0.9)	0.09	0 (0-0.2)	0.2 (0-0.2)	0.5
	IL8	12.0 (10.7-29.9)	201.2 (178.4-414.8)	<0.001	15.5 (11.0-113.5)	190.4 (183.6-212.1)	0.03
	GM-CSF	57.2 (45.3-84.7)	84.3 (67.0-150.2)	0.09	70.8 (46.2-103.3)	63.6 (63.2-84.1)	0.9
	VEGF	163.9 (102.9-214.7)	366.5 (107.0-584.0)	0.1	166.3 (85.3-242.2)	462.9 (189.4-686.9)	0.05

IL, Interleukin; GM-CSF, granulocyte-macrophage colony stimulating factor; VEGF, vascular endothelial growth factor; IQR, interquartile range; mGPS, modified Glasgow prognostic score; *Mann–Whitney *U*-test.

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