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Original Article

Predictive value of the tumor-infiltrating neutrophil-to-lymphocyte ratio in patients with colorectal cancer

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Abstract: Tumor-infiltrating T lymphocytes have been reported to be involved in clinical outcome in colorectal cancer. However, whether tumor-infiltrating neutrophil-to-lymphocyte ratio (NLR) could potentially serve as a prognostic marker in colorectal cancer (CRC) is unclear. In present study, tumor-infiltrating CD66b and CD3 in paired intratumoral and peritumoral tissues were evaluated by immunohistochemistry (IHC) among a cohort of 210 CRC patients. The density of intratumoral $CD66b^+$ neutrophils and $CD3^+$ lymphocytes were significantly lower than those of peritumoral areas. Univariate analyses indicated that tumor-infiltrating $CD66b^+$ neutrophils, $CD3^+$ lymphocytes and NLR ratio in both intratumoral and peritumoral were significantly associated with decreased disease-free survival (DFS) and overall survival (OS), as well as other clinical parameters such as differentiation status, depth of invasion, lymphatic metastasis and TNM stage. Moreover, differentiation status, TNM stage and peritumoral NLR were significant correlated with DFS and OS according to multivariate analysis High NLR in both intratumoral and peritumoral were significantly correlated with lymphatic metastasis and TNM stage. Sub-group analysis of the pTNM stage II patients, higher tumor-infiltrating NLR was associated with shorter survival time. Peritumoral NLR was an independent prognostic factor with a higher hazard ratio. These results defined tumor-infiltrating neutrophil-to-lymphocyte ratio as a novel prognostic marker for CRC.

Keywords: Tumor-infiltrating $CD66b^+$ neutrophils, $CD3^+$ lymphocytes, NLR, colorectal cancer

Introduction

Colorectal carcinoma (CRC) is the third most common malignancy worldwide, leading to approximately 1.4 million new cases and approximately halfthis number of deaths per year as itoften presents a high incidence and a dismal prognosis [1]. Until now, pathological tumor-node-metastasis (pTNM) classification has remainedthe most important factor for predicting the prognosis and adjuvant therapy of CRC patients [2]. Despite the prognostic power of the pTNM staging system, predicting the outcome of patients is still imprecise. The progression of advanced stage cancer can remain stable for many years, and partial regression of large metastatic lesions can also occur spontaneously. Therefore, it is of great importance to seek optimal biomarkers for predicting the clinical outcome.

During carcinogenesis, tumor cells interact with a complex microenvironment. Immune cells and inflammatory cells are important components of the tumor microenvironment [3]. Neutrophils are the most abundant leukocytes, initially recognized as short-lived effector cells providing the first line of defense against invading microorganisms [4]. Many types of immune cells scattered in the tumor microenvironment are capable of secreting neutrophil chemokines and cytokines, and neutrophils recruited to tumor sites seem to enhance cancer cells migration and invasion [5]. Accumulating evidence indicates that neutrophils in thetumor tissue are a factor in poor prognosis [6-8].

Time to recurrence and overall survival times could be governed largely by the state of the local adaptive immune reaction [9]. Increasing evidence is showing that the type, density, and

Table 1. Correlation of tumor-infiltrating NLR with clinicopathological characteristics

Variable	Total (N = 210)	Intratumoral NLR			Peritumoral NLR		
		Low	High	*P*-value	Low	High	*P*-value
Gender							
Female	84	43	41	0.612	46	38	0.114
Male	126	60	66		55	71	
Age (years)							
≥56	118	58	60	0.973	57	61	0.945
<56	92	45	47		44	48	
Tumor location							
Colon	122	60	62	0.964	57	65	0.639
Rectum	88	43	45		44	44	
Differential grade							
Well	71	40	31	0.027*	37	34	0.213
Middle	102	52	50		51	51	
Poor	37	11	26		13	24	
Depth of invasion							
T1	10	6	4	0.692	6	4	0.205
T2	18	9	9		12	6	
T3	150	75	75		71	79	
T4	32	13	19		12	20	
Lymph node metastasis							
N0	123	69	54	0.015*	67	56	0.028*
NI+N2	87	34	53		34	53	
pTNM stage							
I	23	13	10	0.015*	15	8	0.035*
II	95	56	39		51	44	
III	68	27	41		28	40	
IV	24	7	17		7	17	

NLR: neutrophil-to-lymphocyte ratio; *P<0.05.

location of immune cells in colorectal cancer could provide superior prognostic factors and be independent of the criteria related to the anatomic extent of the tumors [10]. Tumor-infiltrating lymphocytes have been proven to inhibit tumor growth and are associated with improved prognoses in non-small-cell lung carcinoma [11]. Mice with T-cell deficiencies tend to develop malignancy [12]. The immune system protects the host in control of tumor development through immune-surveillance mechanisms [13]. The presence of tumor-infiltrating T lymphocytes was shown to correlate with a favorable prognosis in patients with CRC [14].

The biological behaviors of cancer could also be affected by the innate immune system through inflammation-dependent mechanisms in previous studies [15]. An elevation in peripheral blood neutrophil-to-lymphocyte ratio is considered as a marker of systemic inflammatory response, which predisposes the tumor to proliferate and metastasize through the inhibition of apoptosis and promotion of angiogenesis [16, 17]. In tumor tissues, intratumoral $CD66b^+$ neutrophil-to-$CD8^+$ T cell ratio was an independent prognostic factor for a high rate of disease recurrence and poor prognosis in patients with some Cancers following resection [18].

However, until now, the impact of the tumor-infiltrating neutrophil-to-lymphocyte ratio (NLR) remains elusive in CRC. In this study, we investigated the prognostic significance of the tumor-infiltrating $CD66b^+$ neutrophils, $CD3^+$ lymphocyte and neutrophil-to-$CD3^+$ lymphocyte ratio for CRC.

Material and methods

Patients and specimens

A total of 210 CRC samples (collected from January 2005 and April 2006) were obtained from the First Affiliated Hospital of Anhui Medical University (Hefei, China). The pathology of each patient was confirmed by pathologist, and patients did not receive any anticancer therapy before surgery. Individuals with an autoimmune disease, HIV or syphilis were excluded. The patients were staged according to the criteria of the seventh edition of the pTNM classification of the American Joint Committee on Cancer stage (AJCC, 7th edition). Clinicopathologic parameters were collected and were shown in **Table 1**. The data were censored at the last follow-up for patients without recurrence or death. Disease-free survival time (DFS) was defined as the interval between the date of surgery to the date of first evidence of

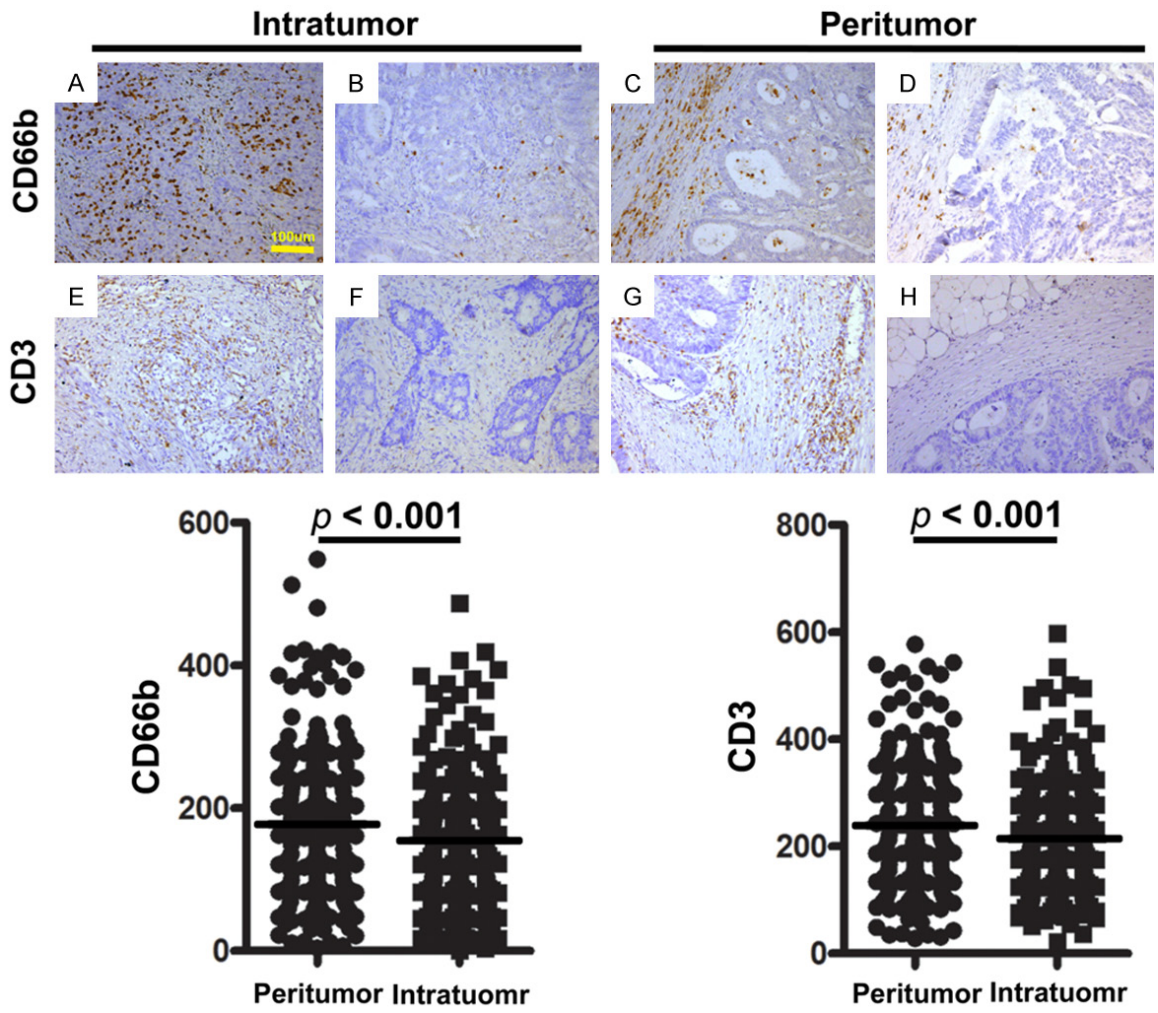

Figure 1. Representative examples of immunostaining and the number of CD66b and CD3. (A) Representative examples of immunostaining of $CD66b^+$ neutrophils (A-D) and $CD3^+$ lymphocytes (E-H) (200×). Different levels of $CD66b^+$ neutrophil infiltration can be observed in the intratumoral and peritumoral regions: high density (A, C) and low density (B, D). While different levels of $CD3^+$ lymphocyte infiltration can be observed in the intratumoral and peritumoral regions: high density (E, G) and low density (F, H). (B) Statistical analysis of the number of $CD66b^+$ neutrophils and $CD3^+$ lymphocytes in intratumoral and paired peritumoral tissues of CRC patients.

relapse or death. Overall survival (OS) was measured between the time of surgery to death or the last follow-up for surviving patients [19]. The study was approved by the human Ethics Committee of the First Affiliated Hospital of Anhui Medical University (Hefei, China), and informed consent was obtained from all patients.

Immunohistochemistry (IHC)

All samples were fixed in formalin and then embedded in paraffin. Tissue blocks were sectioned at 4 μm and mounted on glass slides. The primary antibodies were against CD66b (clone G10F5, diluted at 1:400, BD Biosciences, San Jose, CA, USA) and CD3 (polyclonal rabbit, diluted at 1:400, Abcam, Cambridge, UK). Deparaffinization, epitope retrieval and IHC were performed according to the manufacturer's protocol. Sections were dried for 60 min at 60°C and afterwards deparaffinized in xylene and rehydrated through a gradient ethanol series. Antigens were retrieved using a microwave oven in 0.01 M citrate buffer (PH 6.0). The sections were incubated in primary antibody overnight at 4°C. Horseradish peroxidase (HRP) and diaminobenzidine (DAB) were used according to the manufacturer's instructions

(DakoCytomation). Finally, the sections were stained with hematoxylin and mounted.

Evaluation of IHC

The expression of CD66b and CD3 were evaluated under light microscopy (model DM IRB; Leica, 200× magnification) by two pathologist-srespectively. The number of tumor-infiltrating-leukocytesper low power fields (×200) was estimated using a stereological counting technique (CAST software). The sections were screened at the five most representative fields in tumor-infiltrating areas for $CD66b^+$ neutrophils, and $CD3^+$ lymphocytes were counted. The mean of the 5 fields of vision were assessed per tumor section. Mean values were used as a cut-off in subsequent analyses unless specified. The tumor-infiltrating NLR was calculated according to the method of the immune imbalance of the local microenvironment [20]. NLR was categorized as either ≥ or <0.65.

Statistics

Statistical analyses were performed using the SPSS statistical software package (version 19.0, IBM). Correlations between clinicopathologic features and IHC parameters were analyzed by the chi-square test (for categorical variables) and Student t test (for continuous variables). Associations between continuous variables were examined by calculating Pearson's correlation coefficient. OS and DFS were calculated using the Kaplan-Meier method, and survival was measured in months from the resection to either recurrence or the last review. Univariate and multivariate survival analysis was carried out with the Cox proportional hazards regression model and log-rank test. All statistical analyses were two-sided, and only *P* values <0.05 were considered as statistically significant.

Results

Analysis of IHC parameters

The following antigens were as markers for detecting different cell types: CD66b for neutrophils, and CD3 for total T lymphocytes (**Figure 1**). The mean ± standard deviation (SD) of neutrophils in the intratumor and peritumorwere 154.9±95.6 (range 0-487 cells/field) and 177.4±105.6 (range 4-549 cells/field), and the SD number of $CD3^+$ lymphocytes in the intratumor and peritumor were 214.2±108.4 (range 22-598 cells/field) and 239.8±119.0 (range 28-577 cells/field). The density of both intratumoral neutrophils and $CD3^+$ lymphocytes were significantly lower than those of peritumoral areas (P<0.001 for both; **Figure 1**). The density of the intratumoral neutrophils was not significantly associated with that of the intratumoral $CD3^+$ lymphocytes (r = -0.015, P = 0.832), and there were no correlations between the density of either peritumoral neutrophils or $CD3^+$ lymphocytes (r = -0.106, P = 0.124). The density of intratumoral neutrophils was correlated with that of peritumoral neutrophils (r = 0.877, P< 0.001), and the density of intratumoral $CD3^+$ lymphocytes was significantly correlated with that of peritumoral $CD3^+$ lymphocytes (r = 0.903, P<0.001).

Correlations between IHC parameters and clinicopathological features

We next analyzed the correlations between the clinicopathological variables of CRC. The results demonstrated that the tumor-infiltrating neutrophils had no association with any of the clinicopathological parameters. The expression ofthe intratumoral $CD3^+$ lymphocytes was significantly associated with lymph node metastasis (P = 0.016) and advanced pTNM stages (P = 0.025). Furthermore, the increased peritumoral $CD3^+$ lymphocytes were correlated with an advanced depth of invasion (P = 0.045), lymph node metastasis (P = 0.013) and an advanced pTNM stage (P = 0.006). The results also showed that increased tumor-infiltrating NLR were associated with tumor lymph node metastasis and advanced pTNM stage (P<0.05). In addition, decreased intratumoral NLR was more likely to be well-correlated with the tumor differential grade (P = 0.027) (**Table 1**).

Survival analysis

At the study end-point, 90 patients (42.9%) had recurrence, and 111 patients (52.9%) died. The estimated 1-, 3-, 5-year DFS and OS rates were 75.2%, 93.3%, and 52.9%, 68.1%, and 41.4%, 56.7%, respectively. The median DFS was 53 months (range, 2 to 127 months), and the median OS was 63 months (range, 4 to 127 months) for the patients.

To evaluate the whether tumor-infiltrating neutrophils, $CD3^+$ lymphocytes, NLR and other clinicopathological parameters were associated

Table 2. Univariate and multivariate analysis of factors associated with disease-free survival (DFS) and overall survival (OS) for CRC

	DFS		OS	
Variable	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Univariate analysis				
Age	1.167 (0.804-1.695)	0.417	1.173 (0.804-1.712)	0.408
Gender	0.942 (0.647-1.371)	0.753	0.776 (0.527-1.145)	0.201
Tumor location	0.930 (0.642-1.347)	0.702	0.952 (0.654-1.386)	0.798
Differentiation status	2.014 (1.314-3.088)	0.001*	2.165 (1.386-3.382)	0.001*
Depth of invasion	1.902 (1.021-3.544)	0.043*	2.313 (1.169-4.576)	0.016*
Lymph node metastasis	1.911 (1.320-2.766)	0.001*	2.855 (1.954-4.173)	<0.001*
TNM Stage	2.074 (1.432-3.004)	<0.001*	3.380 (2.295-4.978)	<0.001*
Intratumoral neutrophils	1.697 (1.173-2.453)	0.005*	1.699 (1.167-2.473)	0.006*
Peritumoral neutrophils	1.478 (1.023-2.135)	0.037*	1.512 (1.041-2.197)	0.030*
Intratumoral $CD3^+$ lymphocytes	0.689 (0.473-1.002)	0.051	0.607 (0.413-0.894)	0.011*
Peritumoral $CD3^+$ lymphocytes	0.616 (0.425-0.895)	0.011*	0.551 (0.376-0.807)	0.002*
Intratumoral NLR	2.138 (1.468-3.114)	<0.001*	2.517 (1.703-3.720)	<0.001*
Peritumoral NLR	2.352 (1.606-3.444)	<0.001*	2.606 (1.756-3.869)	<0.001*
Multivariate analysis				
Differentiation status	1.845 (1.178-2.888)	0.007*	1.834 (1.146-2.936)	0.012*
Stage	4.684 (1.381-15.888)	0.013*	10.522 (3.830-28.907)	<0.001*
Peritumoral NLR	2.291 (1.211-4.332)	0.011*	2.056 (1.081-3.911)	0.028*

CI = confidence interval; HR = hazard ratio; *P<0.05.

with prognosis, univariate and multivariate analyses were carried on to assessed their prognostic value. In univariate analysis, decreased tumor-infiltrating $CD3^+$ lymphocytes, increased neutrophils and NLR were significantly associated with poor DFS and OS (P<0.05 for both, **Table 2**), except for the DFS of intratumoral $CD3^+$ lymphocytes (P = 0.051, **Table 2**). Positive lymph node metastasis, advanced differential grade, depth of invasion and pTNM stages were significantly associated with poor FDS and OS (P<0.05, **Table 2**). Multivariate analysis together with conventional clinicopathological variables that had been found to be significant by univariate analysis revealed that increased peritumoral NLR, advanced differential status and pTNM stages were independent prognostics for OS and DFS with higher hazard ratio values (**Table 2**). Moreover, positive lymph node metastasis was independently associated with the decreased OS.

Kaplan-Meier survival analyses revealed that decreased tumor-infiltrating $CD3^+$ lymphocytes, increased intratumoral neutrophils and NLR were all significantly associated with both DFS and OS (**Figure 2**). In the sub-group analysis of the pTNM stage II patients, a high tumor-infiltrating NLR was associated with shorter survival time (**Figure 3**).

Discussion

Solid tumors are commonly composed of various immune cells, including T and B lymphocytes, natural killer cells, macrophages, and neutrophils. Adaptive immunity and immunosuppression always show different expression levels in tumors, and many immune genes are related to inflammation. The interplay between immune cells determines the promotion or restraint of tumor progression, angiogenesis, and metastasis, and the immune context of the primary tumors is an essential prognostic factor for patients' DFS and OS [21]. In the present study, we analyze the distribution of tumor-infiltrating $CD66b^+$ neutrophils and $CD3^+$ lymphocytes together in CRC patients following resection by immunohistochemistry. We found that the patients with high tumor-infiltrating neutrophils had a significantly short DFS and OS compared with the low subgroups.

Neutrophils constitute an important component of the leukocytes infiltrating the tumor

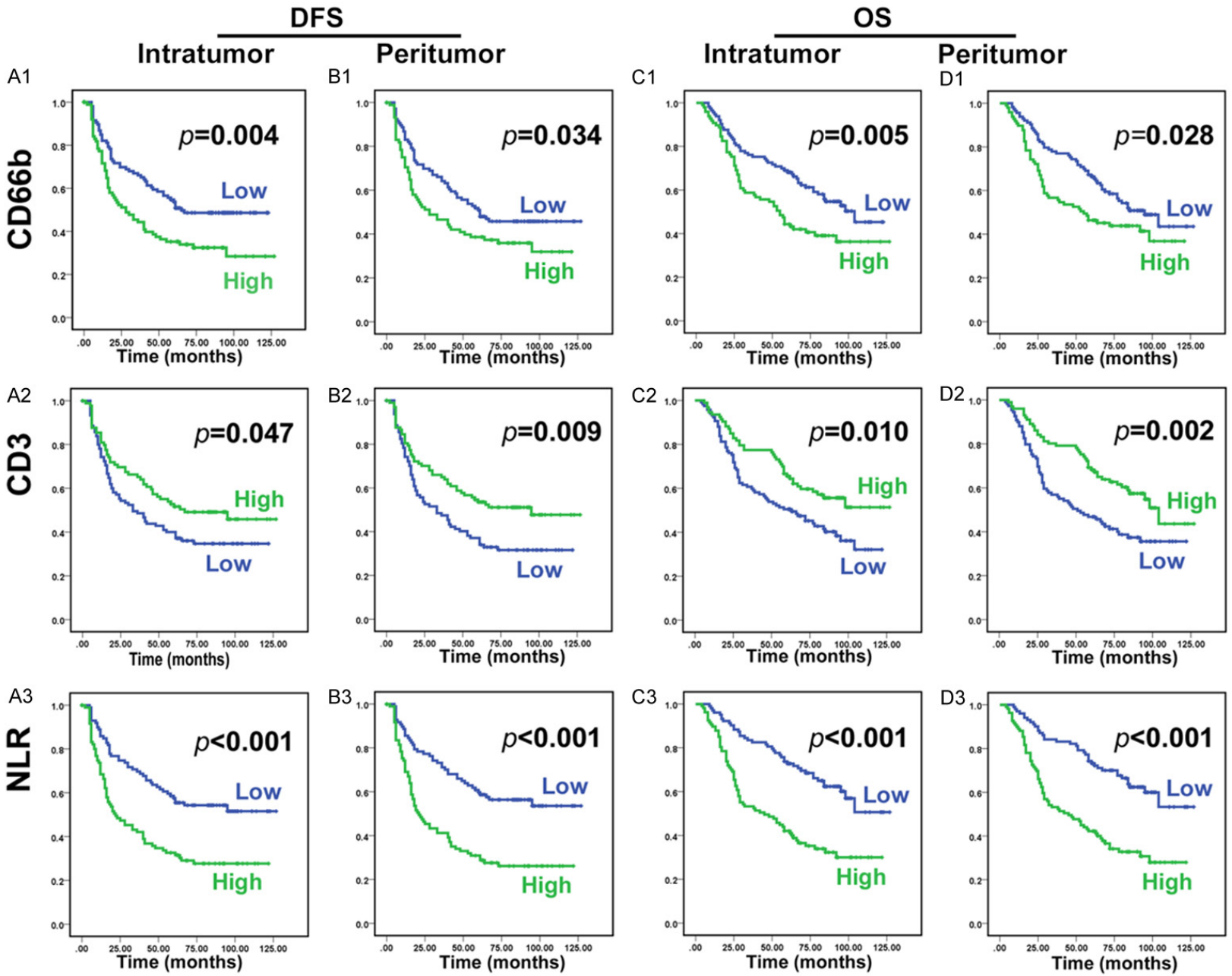
DFS
OS
Intratumor
Peritumor
Intratumor
Peritumor
A1
B1
C1
D1
CD66b
p=0.004
p=0.034
p=0.005
p=0.028
Low
High
A2
B2
C2
D2
CD3
p=0.047
p=0.009
p=0.010
p=0.002
High
Low
A3
B3
C3
D3
NLR
p<0.001
p<0.001
p<0.001
p<0.001
Low
High
Time (months)

Figure 2. Kaplan-Meier analysis of tumor-infiltrating CD66b$^+$ neutrophils and CD3$^+$ lymphocytes in CRC patients. Decreased tumor-infiltrating CD66b$^+$ neutrophils (A1, B1, C1 and D1) and NLR (A3, B3, C3 and D3) were significantly associated with prolonged RFS and OS. Increased tumor-infiltrating CD3$^+$ lymphocytes (A2, B2, C2 and D2) were significantly associated with prolonged DFS and OS. Differences in DFS and OS were analyzed with the log-rank test.

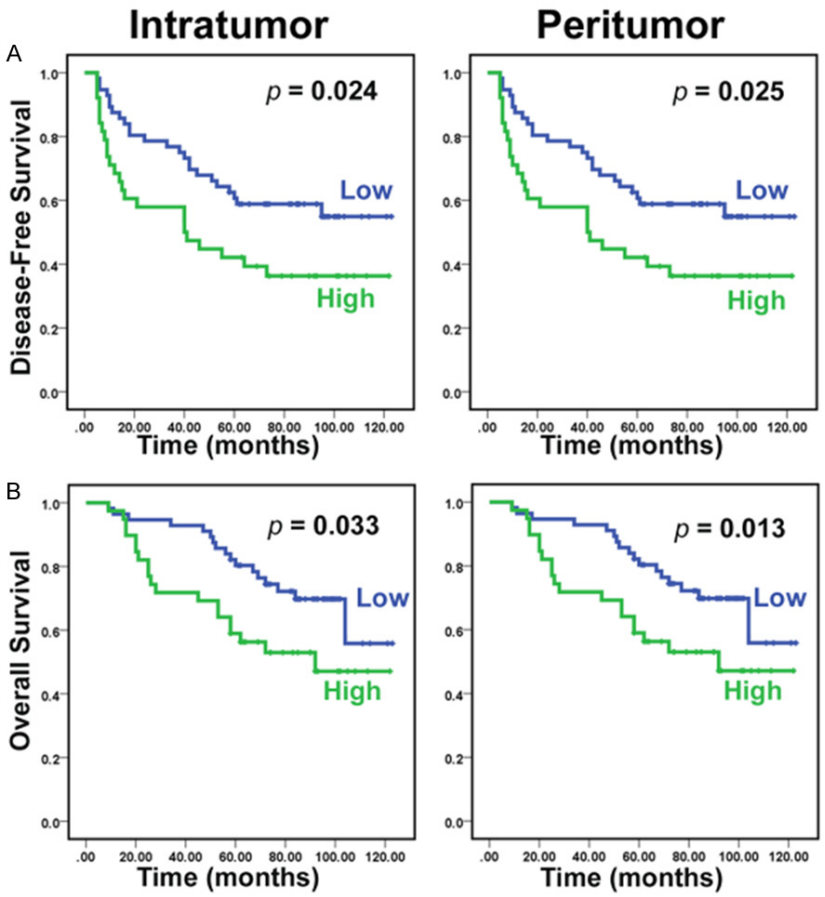

Figure 3. The cumulative OS and DFS were calculated using the Kaplan-Meier method in the sub-group analysis of pTNM stage II patients. A. The correlation between intratumoral/peritumoralNLR and DFS. B. The correlation between intratumoral/peritumoral NLR and OS.

[22], and have long been recognized as a first-line defender against foreign pathogens. However, the exact role of neutrophils in human tumors remains unknown. There is evidence in support of the conclusion that neutrophils play an important role in reacting against the host in cancer [23]. Neutrophils have been proved to mediate the initial angiogenic switch via the production of matrix metallo proteinase-8 and matrix metallo proteinase-9 [24]. By releasing prestored proteases, neutrophils actively remodel the extracellular matrix and accelerate angiogenesis, which could promote the invasion and metastasis of cancer cells [25]. However, neutrophils have protumorigenic or antitumorigenic functions depending on the presence of TGF-β in the tumor microenvironment [26]. We found that higher CD66b$^+$ neutrophils were expression in the peritumoral area, which may reflect the important function of neutrophils in promoting the invasion and metastasis of tumors and should be further studied.

Immunohistochemistry was used to investigate the total T lymphocytes (CD3) in our study. We found that patients with high levels of tumor-infiltrating CD3$^+$ lymphocytesin their tumors were atearlierp TNM stages and were lymph nodenegative. Patients with low tumor-infiltrating CD3$^+$ lymphocyteshad anunfavorable prognosis. In addition, the decreased peritumoral CD3$^+$ lymphocytes were correlated with an advanced depth of invasion. These results indicated that tumor-infiltrating T lymphocytes may inhibit CRC progression and were consistent with reports in other researches about CRC [10].

Recently, great interest has been generated in illuminating the role of cancer inflammation cells in predicting the prognosis of malignancies. However, tumor-associated T lymphocytes and neutrophils do not act on each other directly, instead, they cooperate and determine the progress of cancer together [26]. Elevated blood total white cells can be a useful addition to the currently established prognostic markers of the systemic immune response [27]. In

recent studies, the peripheral neutrophil-to-lymphocyte ratio has been found to be correlated with disease progression ingastric cancer [17]. The tumor-infiltrating NLR had been reported as a marker of the systemic inflammatory response, could predict clinical outcomes in patients with metastatic CRCs [28]. The question, therefore, arises as to whether the tumor-infiltrating NLR is more advantageous than the use of one of the two parameters alone. Up until now, the prognostic value of tumor-infiltrating NLR in CRCs is unclear. The results of the present study show clearly that increased tumor-infiltrating NLR is associated with lymph node metastasis and a trend towards advancedp TNM stages. Notably, we found that tumor-infiltrating NLR had a persistently superior prognostic value over the neutrophil and $CD3^+$ lymphocytes alone; it could better predict the outcome in terms of the minimum *P* values.

In this study, we founded the peritumoral NLR was an independent prognostic factor in CRCs. This finding was in accordance with results from peripheral neutrophil-lymphocyte ratio [29]. In the sub-group analysis of pTNM stage II patients, a high tumor-infiltrating NLR was linked to a short survival time (**Figure 3**). Therefore, pTNM classification together with tumor-infiltrating NLR exerted a better prediction effect. Efforts to target of this work might be a strategy in treating patients with CRC.

However, our study has several limitations. First, clinical association studies cannot prove causal relationships. Therefore, further studies about the functional link between tumor-infiltrating neutrophils and $CD3^+$ lymphocytes and tumor progress are warranted. Second, data about the peripheral NLR were not collected in CRC patients. Nevertheless, we found that peritumoral NLR was an independent prognostic factor with a high hazard ratio in patients with CRC.

In conclusion, increased tumor-infiltrating $CD66b^+$ neutrophils and NLR as well as decreased $CD3^+$ lymphocytes implied a poor prognosis in patients with CRC following resection. The peritumoral NLR was an independent prognosticator with a higher hazard ratio, which may substantially aid in the identification of high-risk patients with CRC following resection, especially for early stage II CRC patients.

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Disclosureof conflict of interest

None.

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