

Association between the preoperative neutrophil-to-lymphocyte ratio and tertiary lymphoid structures surrounding tumor in gastric cancer

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Abstract. The neutrophil-to-lymphocyte ratio (NLR) has been reported to be associated with a poor prognosis in various types of cancer. We previously reported that an antitumor immune response was induced by tertiary lymphoid structures (TLSs) surrounding tumor, and increased TLS was an independent prognostic factor in patients with gastric cancer. The present study examined the stratification based on the correlation between the preoperative NLR and TLS density in gastric cancer. A total of 199 patients who underwent surgery for stage Ib-IV gastric cancer were included in the study. Receiver operating characteristic curve analysis was used to determine the appropriate cut-off values of the preoperative NLR and the TLS density. The prognostic factors were evaluated in a multivariate analysis. The median NLR was 2.18 (mean \pm SD, 2.7 \pm 2.04). A total of 91 patients with an NLR \geq 2.33 was classified into the high NLR group. The overall survival was significantly improved in patients with a low NLR than in those with a high NLR. Additionally, the low NLR group tended to have a high TLS density. The multivariate analysis indicated that the preoperative NLR and TLS density were independent risk factors. When the patients were classified into the high and low NLR and TLS groups and the survival rates were compared, the prognosis was significantly improved in the low NLR and high TLS group than in the other groups. The preoperative NLR may be associated with the presence of TLSs surrounding the tumor, and the combination of NLR and TLS may be useful for the stratification of patient prognosis. The present results suggested that the NLR and TLS

density may be surrogate markers for immunotherapy against gastric cancer.

Introduction

Immune checkpoint blockade (ICB) has proven effective for prolonging the prognosis in gastric cancer (1). The main mechanism of action in ICB therapy is the anti-tumor immune response of tumor-infiltrating lymphocytes (TILs) against cancer cells. Many studies have reported that TILs are associated with a good prognosis (2-6). Meanwhile, several studies have reported that tertiary lymphoid structures (TLSs) are associated with favorable clinical outcomes in patients with various types of cancer, including lung, gastric, colorectal and breast cancer (7-10), and we previously reported that TLSs are positively associated with TILs (11). Moreover, recent studies have shown that TLSs might play an important role in sustaining the anti-tumor immune response to ICB therapy (12,13). TLSs are transient ectopic lymphoid organizations that are detected in the invasive margin of the tumor and/or in the stroma of most cancers and display an overall organization similar to that observed in secondary lymphoid organs, such as the lymph nodes (14). TLSs are composed of B-cell follicles, T-cell zones, follicular dendritic cells and high endothelial venules (15,16).

On the other hand, as the tumor grows, non-specific inflammatory responses caused by cancer cells or surrounding tissue tend to increase the peripheral blood neutrophil count and reduce the lymphocyte count (17). Thus, systemic inflammatory responses, including the high neutrophil-to-lymphocyte ratio (NLR), are related to tumor development and progression (18) and have been shown to be associated with outcomes in patients with various malignancies, including esophageal squamous cell carcinoma, gastric cancer, colorectal cancer, and hepatocellular carcinoma (19-23). The NLR could also be useful in the diagnosis of thyroiditis (24) and as an indicator to differentiate malignant from benign thyroid nodules in the preoperative period (25). In addition, it could be associated with glucose control and correlated with the HbA1c level in type 2 diabetes mellitus (26,27), and it could serve as a diagnostic tool for various other inflammatory conditions, such as

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ulcerative colitis, irritable bowel syndrome, and nonalcoholic fatty liver disease (28-30).

According to the above reasons, the NLR can be a useful biomarker for various cancers, and TLSs have a vital role in the anti-tumor immune response, such as in the prevention of tumor progression by increasing the numbers of TILs, and may be an independent prognostic marker in various cancers. Therefore, we hypothesized that the NLR, an indicator of the systemic inflammatory response, might reflect TLSs in the tumor microenvironment, and investigated the association between the preoperative NLR and clinicopathological features and their relevance to the TLS density surrounding the tumor in gastric cancer.

Patients and methods

Patients. This retrospective study included all 199 consecutive patients with stage IB-IV gastric cancer who had undergone initial surgical resection without preoperative chemotherapy or radiotherapy between 2007 and 2010 at Osaka City University Hospital, Japan. Patients with stage IA disease were excluded from this study because the tumor had been resected by Endoscopic mucosal resection (EMR) or because of the small size of the tumor, which makes it difficult to assess TLSs. All patients were followed up regularly until April 2015 or until their death. Follow-up examinations were scheduled for every three months for the first two years, every six months during the third to fourth years, and annually thereafter. The median follow-up period after surgery was 49 (1-92) months. Overall survival (OS) was defined as the time between the date of surgery and death, and disease-free survival (DFS) was defined as the time between the date of surgery and recurrence. This study was approved by the Osaka City University Ethics Committee. Informed consent was obtained from all patients.

Data collection. Clinicopathological information was extracted retrospectively from hospital data. The patient data included age, sex, smoking history, tumor staging (TNM), histological type, lymphatic invasion, venous invasion, preoperative NLR and TLS density. Pathological TNM staging was recorded for all patients based on the UICC TNM classification, 7th edition. The preoperative NLR, which is calculated by dividing the absolute neutrophil count by the absolute lymphocyte count, was determined based on routine test results from peripheral blood samples that were collected within two weeks before the operation. In cases with multiple blood samples, the sample from the first hospital visit was used to calculate the NLR. Then, to examine the impact of the preoperative NLR on the clinicopathological features, we divided the patients into 2 groups according to the NLR, and compared the low and high NLR groups.

Immunohistochemistry. The centers of TLSs surrounding tumor were located in B cells that formed clusters. We therefore counted B cell clusters as TLSs, as we previously reported (11). Immunohistochemistry was performed on 4- μ m thick sections from formalin-fixed paraffin-embedded (FFPE) tumor blocks, which were obtained from patients with gastric cancer and fixed with 10% formalin at room temperature

for 6-48 h. After incubation at 60°C for 10 min, the sections were deparaffinized using xylene and rehydrated in a graded ethanol series (70, 80, 90 and 100%) for 3 min each time, twice. Endogenous peroxidase activity was blocked with absolute methanol containing 3% hydrogen peroxide at room temperature for 15 min. After washing the sections in PBS, they were microwaved for 10 min to achieve antigen retrieval. Non-specific binding was blocked using the non-specific staining blocking reagent, Target Retrieval Solution (Dako; Agilent Technologies, Inc.), which was diluted 10 times with sterile distilled water, and the samples incubated at 95°C for 45 min. The sections were subsequently incubated with the primary antibody overnight at 4°C, following which, they were incubated with the secondary antibody, histofine reagent (pre-diluted; Nichirei Biosciences, Inc.) at room temperature for 10 min, and the signal was visualized using 3-3'-diaminobenzidine, and finally counter-stained with hematoxylin at room temperature for 20 sec before mounting. The primary antibodies used for the immunohistochemical analyses were mouse anti-CD20 for B cells (clone L26; pre-diluted; Dako; Agilent Technologies, Inc.). The primary antibodies were diluted with 5% BSA (Sigma-Aldrich, Inc.; Merck KGaA) in PBS. Then, we measured the area (mm²) of CD20-positive cells and calculated the CD20-positive area (%) of each field using the ImageJ software program (version 15.1; National Institutes of Health). The CD20⁺ B cell density was determined as the mean CD20-positive area in three fields. Fig. 1 shows TLS-high and TLS-low images in one low-power field of view.

Statistical analysis. All statistical analyses were performed using the JMP software program (version 13; SAS Institute, Inc.). The receiver operating characteristic (ROC) curve and area under the ROC curve were used to select the best cut-off values for the preoperative NLR and TLS density. Categorical variables were compared using the chi-squared test. Correlation analysis was performed using Pearson's correlation analysis. The Kaplan-Meier method and log-rank test were used to compare survival curves. Univariate and multivariate analyses were performed using a Cox proportional hazards regression model. P-values of <0.05 were considered to indicate statistical significance.

Results

The association of the preoperative NLR with the clinicopathological features. The median and mean values of the preoperative NLR were 2.18 and 2.7, respectively, with a standard deviation (SD) of 2.04 and a range of 0.59-15.17. The ROC analysis showed that the optimal cut-off value of the preoperative NLR was 2.33 (area under the curve [AUC] 0.625) (Fig. 2A). Based on the cutoff value, the diagnostic sensitivity and specificity were 66.7 and 61.5%, respectively. This value was then used to divide the patients into 2 groups: The low NLR group (<2.33; n=108) and the high NLR group (\geq 2.33; n=91). There were no significant differences in age, sex, smoking history, T category, N category, TNM stage, histological type or incidence of venous invasion between the groups (Table I). The high NLR group had a higher incidence of lymphatic invasion than the low NLR group. The overall survival of the high NLR group was significantly worse than

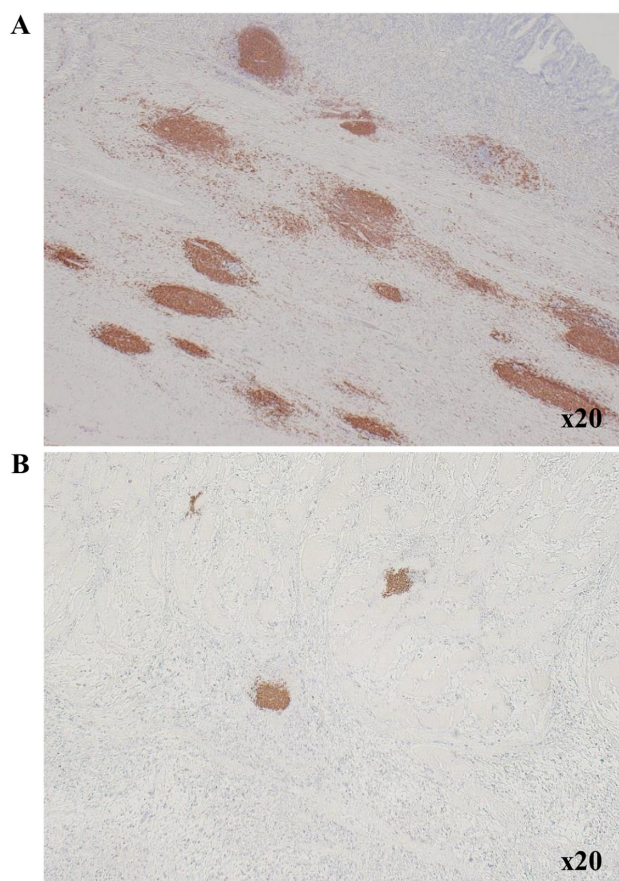


Figure 1. Immunohistochemistry showing TLS surrounding the primary tumor in gastric cancer. (A) TLS high and (B) TLS low (magnification, x20). TLS, tertiary lymphoid structure.

that of the low NLR group (Fig. 3). The 5-year survival rate was 66.9% in the low NLR group and 39.5% in the high NLR group.

The association of the TLS with the NLR and the impact on survival. The median TLS density was 1.78 (average 2.96 ± 2.72). The ROC analysis showed that the optimal cut-off value of the TLS density was 2.00 (AUC 0.619) (Fig. 2B). Based on the cutoff value, the diagnostic sensitivity and specificity were 56.5 and 63.7%, respectively. A scatter chart plotting the preoperative NLR and TLS density revealed a marginal negative association; however, this association did not reach statistical significance ($P=0.0084$, $R=-0.1864$) (Fig. 4). The comparison of the 2 groups revealed that 66% of the high NLR group had fewer TLSs, and 58% of the low NLR group had more TLSs (Table II). Regarding the survival curves according to the combination of the preoperative NLR and TLS density, the OS of the low NLR/high TLS density groups was significantly better than that of the other groups (Fig. 5). In an analysis according to stage, the high NLR/low TLS group also had a significantly worse prognosis than the low NLR/high TLS group (Fig. 6).

Regarding the prognostic factors, the univariate analysis showed that the T stage, N stage, TNM stage, histological type, lymphatic invasion, NLR and TLS density were associated with overall survival. The multivariate analysis results showed that the T stage, histological type, NLR and TLS density

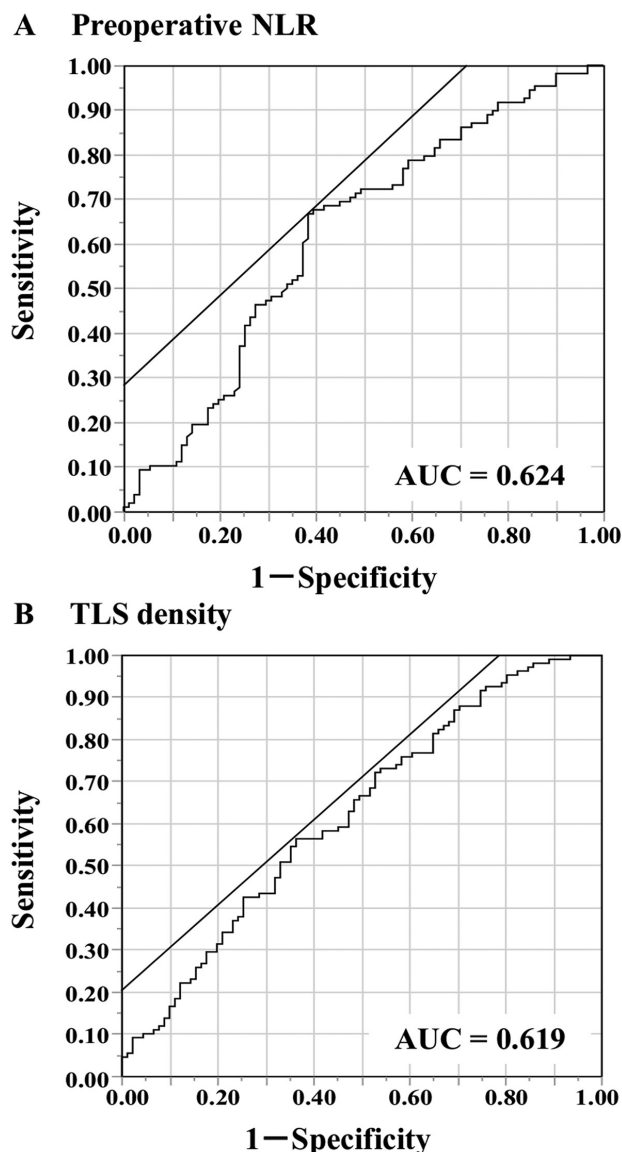


Figure 2. Receiver operating characteristic curves were used for determining the cut-off values. (A) Preoperative NLR and (B) TLS density. NLR, neutrophil-to-lymphocyte ratio; TLS, tertiary lymphoid structure; AUC, area under the curve.

were independently associated with the overall survival rate (Table III).

Discussion

In the present study, we investigated the relationships between the preoperative NLR and TLSs in the tumor. We showed that the NLR was potentially correlated with the TLS density, and both the NLR and TLSs were independent prognostic factors. Our results suggested that the systemic NLR might reflect the TLS density in the tumor microenvironment.

Tumor-associated neutrophils (TANs) exhibit plasticity and are capable of polarization into either an anti-tumorigenic 'N1' phenotype or a pro-tumorigenic 'N2' phenotype (31,32). The 'N2' phenotype produces pro-tumorigenic factors, including vascular endothelial growth factor, inflammatory mediators and matrix metalloproteinases, and promotes tumor growth

Table I. Association of the preoperative NLR with the clinicopathological characteristics of patients with gastric cancer (n=199).

Characteristics	No. of patients	NLR low (n=108)	NLR high (n=91)	P-value
Age, years				
<60	39	22	17	
≥60	160	86	74	0.7649
Sex				
Male	143	78	65	
Female	56	30	26	0.9013
Smoking history				
No	122	61	61	
Yes	77	47	30	0.1279
pT category				
T1	15	11	4	
T2	48	26	22	
T3	45	29	16	
T4	91	42	49	0.0901
pN category				
N0	65	39	26	
N1-N3	134	69	65	0.2586
pStage				
Ib	38	21	17	
II	58	37	21	
III	72	39	33	
IV	31	11	20	0.0880
Histological type				
tub1, tub2, pap	82	50	32	
por, sig, muc	115	57	58	
Others ^a	2	1	1	0.2828
Lymphatic invasion				
Negative	33	24	9	
Positive	166	84	82	0.0198
Venous invasion				
Negative	144	80	64	
Positive	55	28	27	0.5563

^aAdenosquamous carcinoma and endocrine cell carcinoma. NLR, neutrophil-to-lymphocyte ratio; p, pathological; T, tumor; N, node; tub1, well differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma; pap, papillary adenocarcinoma; por, poorly differentiated adenocarcinoma; sig, signet-ring cell carcinoma; muc, mucinous adenocarcinoma.

Table II. Association between the preoperative NLR and TLS density in the tumor microenvironment.

TLS density	No. of patients	NLR low	NLR high	P-value
All cases	199	108	91	
TLS low	105	45	60	
TLS high	94	63	31	0.0006

NLR, neutrophil-to-lymphocyte ratio; TLS, tertiary lymphoid structure.

and progression (33). We previously reported that an increase in neutrophils at the tumor site was found within the primary tumor and lymph node metastasis, with a poor prognosis in patients with a high neutrophil count (34). Furthermore, we

demonstrated experimentally that neutrophils exhibited an increased PD-L1 expression when they reacted with cancer cells and exerted an immunosuppressive function, such as the suppression of T cell proliferation by TANs (35).

The NLR is a systemic inflammation marker reported that has been to be an independent prognostic factor for survival in several malignancies (36-40). Among the many reports on gastric cancer, several studies have reported that the NLR may be a useful marker not only for surgery but also for chemotherapy and metastasis (41-45). In this study, we showed that the preoperative NLR was an independent prognostic factor for overall survival in gastric cancer patients, suggesting that an elevated NLR might reflect the host immune status. We previously reported that TANs were associated with tumor progression, and that high TAN infiltration was correlated with the preoperative NLR (46), with TANs exerting an immunosuppressive function (35). On the other hand, Choi *et al* showed that, within the tumor microenvironment,

Table III. Results of the univariate and multivariate analyses of the prognostic factors of the overall survival for patients with gastric cancer.

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (<60/≥60 years)	1.273 (0.762-2.265)	0.3693	NA	NA
Sex (male/female)	1.111 (0.687-1.795)	0.6653	NA	NA
pT category (T1+T2/T3+T4)	3.153 (1.866-5.708)	<0.0001	2.7 (1.552-5.014)	0.0002
pN category (N0/N1-3)	2.423 (1.492-4.134)	0.0002	1.627 (0.979-2.844)	0.0609
pStage (Ib+II/III+IV)	3.328 (2.142-5.315)	<0.0001	NA	NA
Histological type (tub1,tub2,pap/por,sig,muc)	1.809 (1.171-2.848)	0.0071	1.569 (1.016-2.479)	0.0422
Lymphatic invasion (negative/positive)	7.629 (2.861-31.081)	<0.0001	2.901 (0.992-12.367)	0.0520
Venous invasion (negative/positive)	1.092 (0.676-1.710)	0.7102	NA	NA
NLR (low/high)	2.300 (1.516-3.531)	<0.0001	1.65 (1.068-2.579)	0.0241
TLS (high/low)	2.003 (1.314-3.104)	0.0012	2.042 (1.311-3.227)	0.0015

HR, hazard ratio; NLR, neutrophil-to-lymphocyte ratio; TLS, tertiary lymphoid structure; NA, not applicable; p, pathological; T, tumor; N, node; tub1, well differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma; pap, papillary adenocarcinoma; por, poorly differentiated adenocarcinoma; sig, signet-ring cell carcinoma; muc, mucinous adenocarcinoma.

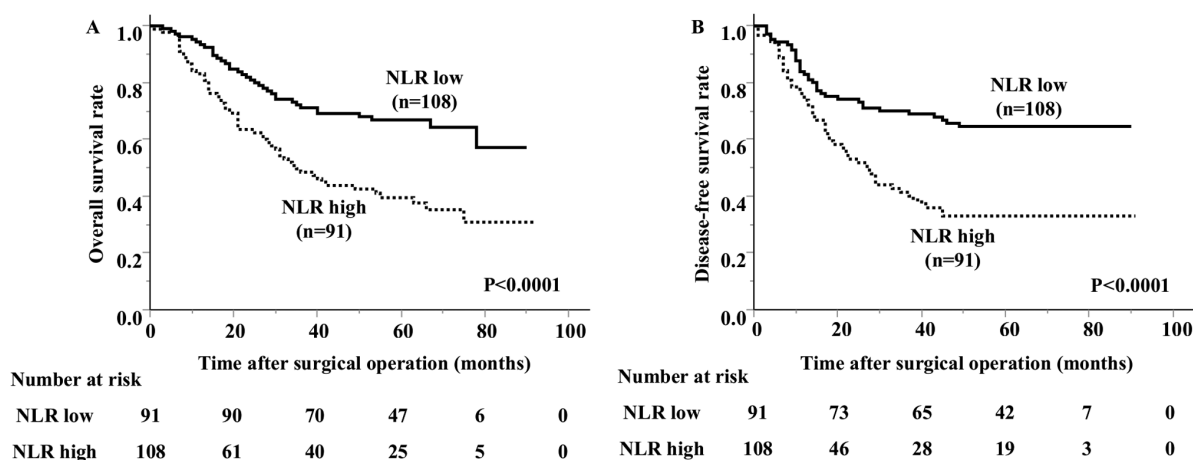


Figure 3. Kaplan-Meier survival curves stratified according to preoperative NLR levels. (A) Overall survival and (B) disease-free survival. NLR, neutrophil-to-lymphocyte ratio.

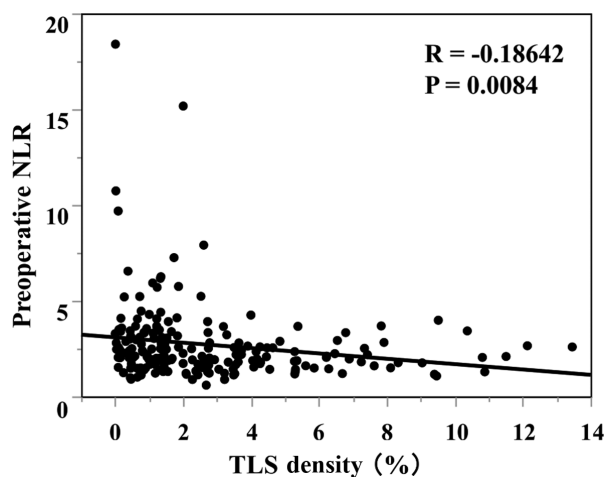


Figure 4. Correlation between the preoperative NLR and TLS density in the tumor microenvironment. NLR, neutrophil-to-lymphocyte ratio; TLS, tertiary lymphoid structure.

the NLR was associated with the density of CD4⁺ T cells, supporting the prognostic value of systemic inflammation in gastric cancer (47). Tanaka *et al* also showed that in biliary tract cancer, the preoperative NLR was negatively correlated with CD8⁺ TILs, and that it may predict CD8⁺ TILs infiltrating in the tumor microenvironment (48). Furthermore, it has been reported that the high pre-treatment NLR was significantly associated with high neutrophil infiltration and low CD3⁺ T cell infiltration into tumors in patients with glioblastoma (49), and that the preoperative NLR might originate from proinflammatory conditions such as tumor necrosis or absence of TILs in hepatocellular carcinoma (50).

ICB to unleash an antitumor immune response results in a durable effect in gastric cancer (51). However, because some patients do not respond to ICB therapy, case selection will be necessary in the future. Increased local antitumor immune mechanisms, or TILs, are thought to be hot tumors and are more responsive to ICB therapy. An analysis of samples from

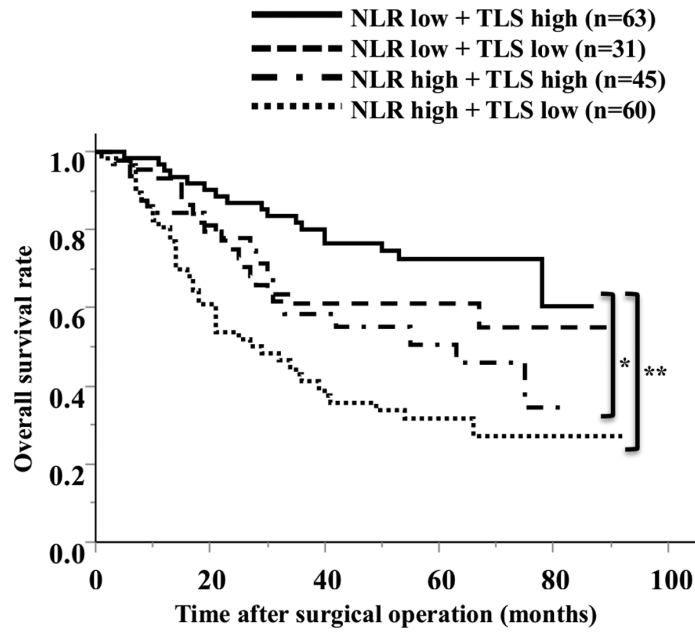


Figure 5. Overall survival rates according to the combination of the preoperative NLR and TLS density. *P=0.0102; **P<0.001. NLR, neutrophil-to-lymphocyte ratio; TLS, tertiary lymphoid structure.

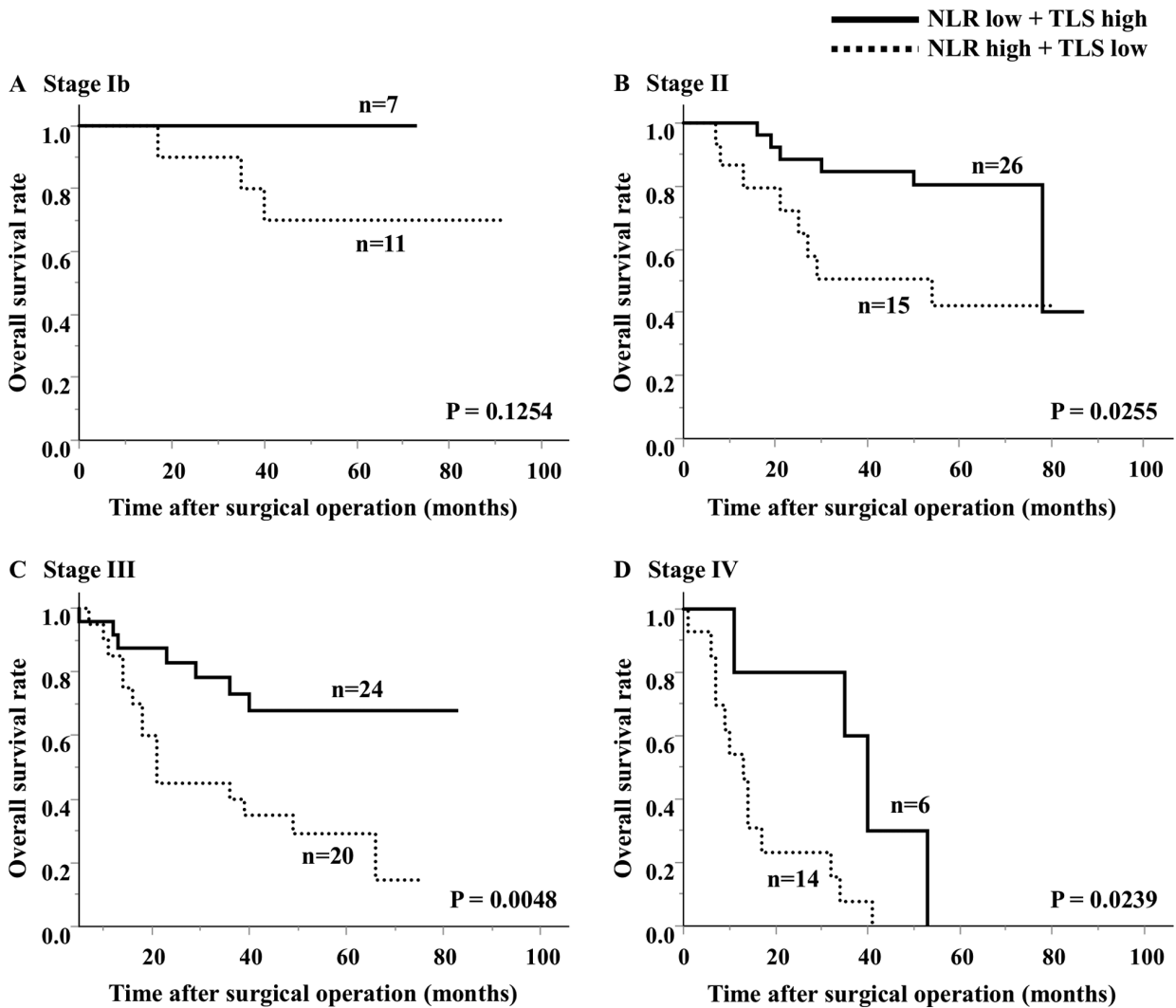


Figure 6. Analysis of the overall survival rates at different tumor stages according to the combination of the preoperative NLR and TLS density. (A) Stage Ib, (B) stage II, (C) stage III and (D) stage IV. NLR, neutrophil-to-lymphocyte ratio; TLS, tertiary lymphoid structure.

clinical trials of ICB-treated malignant melanoma and renal-cell carcinoma cases reported that the maturity of B cells in TLSs was associated with the treatment response (12). We previously reported the presence of TLSs in gastric cancer tissue, the correlation of peri-tumor TLSs with TILs, and the favorable prognosis of TLSs observed more frequently in patients with gastric cancer (11). In addition, we showed that an analysis of B cells in TLSs was able to induce CTLs in TLSs and TLSs may be involved in cellular immunity (52). TLSs can also be associated with humoral immunity (53). These results suggest that TLSs plays an important role in the induction of local tumor immunity. Moreover, in this study, we suggested a potential association between the NLR and TLSs in the tumor microenvironment.

The present findings suggest that the NLR may be useful for evaluating the immunologic status of the tumor microenvironment. Importantly, the NLR can be easily calculated from peripheral blood counts, eliminating the need for invasive procedures, such as a tissue biopsy, to evaluate the tumor microenvironment. Thus, the NLR in the peripheral blood might serve as an easy and useful marker for evaluating the immunoreactivity in the tumor microenvironment.

The present study was associated with some limitations. First, this was a retrospective, single-center study with a relatively small number of patients. Thus, the results may be biased. Second, the correlation between the preoperative NLR and TLS density did not reach statistical significance. Further studies, including prospective studies with a larger number of patients, should be performed to confirm our findings.

In conclusion, the preoperative NLR appears to be correlated with the TLS density in the primary tumor and to be a useful prognostic factor. Our results suggested that the local immune response might be related to systemic neutrophilic induction. Therefore, the preoperative NLR and TLSs surrounding the tumor may be a predictive biomarker with applications in cancer immunotherapy against gastric cancer.

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Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Authors' contributions

YY acquired, analyzed and interpreted the data, confirmed the authenticity of the data and drafted the manuscript. HT made substantial contributions to the conception and design of the study, interpreted the data, confirmed the authenticity of the data and revised the manuscript critically. CS, TM, SD,

MY, TTA, TTo, SL and KM acquired and analyzed the data. KH and MO contributed to the conception and design of the study, and revised the manuscript critically. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures after 2013 were approved by the Osaka City University Ethics Committee (approval no. 3138; Osaka, Japan), and all patients provided written informed consent for the collection and analysis of the specimens. All patients who were managed prior to 2013 provided their written informed consent for sample collection and were allowed to withdraw from the study by signing an opt-out form approved by the Osaka City University Ethics Committee (approval no. 4092).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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