Percentage of Natural Killer (NK) Cells in Peripheral Blood Is Associated with Prognosis in Patients with Gastric Cancer: A Retrospective Study from a Single Center

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Natural killer (NK) cells are important for the prognosis of multiple cancers, but their prognostic value remains to be evaluated in patients with gastric cancer. Thus, this retrospective study was conducted at a single center to investigate the association between percentage of NK cells in the peripheral blood and prognosis in patients with gastric cancer.

Material/Methods: The data of 180 gastric cancer patients were collected. Univariate and multivariate Cox regression models were applied to screen candidate prognostic factors. A time-dependent receiver operating characteristic curve was employed to evaluate the ability of NK cells as a prognostic marker. Furthermore, we determined the correlation between the NK cells percentage and other parameters and their clinical significance.

Results: Patients with a higher percentage of NK cells survived longer than those with a lower percentage of NK cells. Cox analysis revealed that NK cells could be used as an independent indicator for patients with gastric cancer. The percentage of NK cells was positively correlated with lymphocyte count and albumin, but was negatively correlated with CA125 and neutrophil-lymphocyte ratio. The area under the curve for NK cells in predicting the 5-year survival rate for gastric cancer was 0.792. This increased to 0.830 upon combining NK cells with neutrophil-lymphocyte ratio. Patients at early T, N, and clinical stages possessed a significantly higher percentage of NK cells compared to those at advanced T, N, and clinical stages of gastric cancer.

Conclusions: Our results suggest that a higher percentage of NK cells predicts is associated with longer survival of gastric cancer patients and could serve as an independent prognostic biomarker.

MeSH Keywords: Biological Markers • Natural Killer T-Cells • Prognosis • Stomach Neoplasms

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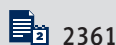
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Background

Gastric cancer remains one of the most common malignant tumors worldwide, especially at later stages, with a high mortality rate [1]. Despite the significant advancements in therapeutic approaches, the cure and prognosis of gastric cancer patients remain poor [2]. Currently, conventional tumor biomarkers, including CA153, CA199, and CA125, are used to assess the diagnosis and prognosis of gastric cancer; however, these biomarkers exhibit low sensitivity and specificity and are poor markers for the prognosis of cancer [3,4]. Therefore, efforts are being made to identify effective biomarkers for gastric cancer; studies have reported some biomarkers in the peripheral blood, such as neutrophil-lymphocyte ratio (NLR) [5] and platelet-lymphocyte ratio (PLR) [6].

Immune cells, including T lymphocytes, B lymphocytes, and natural killer (NK) cells, contribute to the host antitumor immune response. The percentage concentration of these immune cells is crucial for the pathogenesis of certain cancers [7–9]. NK cells are good markers for the prognosis of cancers. Tang et al. [10] showed that NK cells in the blood were an independent predictor of survival in CRC patients. A recent study reported that low NK cell counts in the circulation or tumor tissue before treatment were associated with poorer outcomes in patients with follicular lymphoma and diffuse large B cell lymphoma undergoing antibody-based therapy [11]. Similar results were observed in patients with mantle cell lymphoma [12] and acute lymphoblastic leukemia [13]. In addition, the above-mentioned biomarkers are nonspecific, and the clinical significance and utility of these biomarkers are still unclear at best, as many of them are statistically but not clinically significant [14].

Pervious research has reported that higher percentages of NK cells lead to better prognosis in gastric cancer patients [15,16], suggesting the potential of NK cells in prognosis. However, the sample size was small, which reduced the robustness of the results. Moreover, there is no evidence for the importance of NK cells in gastric cancer patients undergoing radical surgery and adjuvant chemotherapy. Therefore, this retrospective study was conducted at a single center to investigate the association between percentage of NK cells in the peripheral blood and prognosis in patients with gastric cancer.

Material and Methods

Collection of gastric cancer patients

In this single-center study, we retrospectively assessed all patients who underwent surgery between January 2014 and December 2016 from the electronic medical record system in our hospital. The sample size was determined using the powerandsamplesize online tool (*http://powerandsamplesize.com/*). By using the tool, the type I error rate (α) set as 0.05, the power value (1-β) set as 0.8, and the hazard ratio was set as 2, and we obtained a sample size of 82. Inclusion criteria were: (1) histologically confirmed adenocarcinoma of the stomach; (2) underwent D2 gastrectomy with R0 resection; and (3) adjuvant chemotherapy using 5-fluorouracil. Patients were excluded based on the following criteria: (1) autoimmune, cardiovascular, severe liver and kidney, hematological, and infectious diseases; (2) incomplete or inaccurate medical records; and (3) follow-up time of less than 3 months. This study complied with the 2008 Declaration of Helsinki guidelines, and was approved by the Hospital Ethics Committee.

Data extraction

The potential candidates for indicators for the prognosis of gastric cancers were extracted, including sex, age, preoperative blood counts, tumor biomarkers (including CA153, CA199, CA125, and CEA), immune cell counts (NK cells, T lymphocytes, and B lymphocytes), and inflammatory markers, such as C-reactive protein (CRP) and high-sensitivity CRP (hsCRP). NLR and PLR were calculated. Details of the tumor location, degree of differentiation, and TNM staging (American Joint Committee on Cancer criteria, 8th Edition) [10] were also collected. All the patients were followed up after treatment until December 2016 or death. Overall survival (OS) was defined as the date from treatment to the date of last follow-up or death.

Collection of blood samples

Fresh blood samples were collected prior to surgery. Venous blood (10 mL) was collected from each patient in the morning to determine fasting levels. Samples (5 mL) were placed in a water bath at 37°C for 30 min and centrifuged at 1400×g. Subsequently, the supernatants were collected and stored at –20°C until analysis. The remaining 5 mL of blood was transferred to collection tubes containing the general anti-coagulator EDTA-K3, and the samples were prepared for flow cytometry within 30 min. To ensure maximum viability, stained cells were analyzed promptly to detect the lymphocyte subsets.

Flow cytometry for T and B lymphocytes and NK cells

Lymphocytes were gated according to the CD45/side scatter dot plots. T cell subsets were defined as CD3$^+$/CD4$^+$, and CD3$^+$/CD8$^+$, B cells were defined as CD19$^+$, and NK cells were CD3$^-$/CD16$^+$/CD56$^+$. Flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) determination for the cells was performed as previously described [17]. Briefly, blood cells were lysed with the red blood cell lysis buffer, then the samples were centrifuged and supernatants were discarded and heavy precipitates

were retained. Membrane-labeled monoclonal antibodies were added to the precipitates and incubated at 4°C for 30 min.

Statistical Analysis

SPSS 19.0 and R language (version 3.6.1) were used to conduct the statistical analyses. The normal distribution of NK cell number was confirmed by histogram prior to analysis. Comparison between the NK cells within groups was the continuous parameter that was evaluated using the *t* test or Mann-Whitney U test, as needed. Comparison of the NK cells between 3–4 groups differing in tumor location and grade was performed using ANOVA. Correlations between NK cells and other variables were determined using Pearson analysis. Kaplan-Meier analysis and log-rank test were used to compare the OS in different groups. A Cox regression model was established to identify the risk factors associated with OS by univariate and multivariate analyses, respectively. The proportional hazards assumption was checked using statistical tests based on the scaled Schoenfeld residuals; *P* value >0.05 means that the Cox regression model meets the proportional hazards, meaning that the ratio of the hazards for any 2 individuals is constant over time. Because there were more male patients than female patients, sex-stratified models were created, and we also considered the unbalanced numbers between males and females. The optimal cut-off of NK cells was selected using the survminer package (version 0.4.6; *http://cran.r-project.org/*). The significance of NK cells for prognosis was determined by time-dependent receiver operating characteristic (time-ROC) curves and the area under the curve (AUC) using the R packages of "timeROC" [18], and the comparison of AUC values was also performed. *P* values of <0.05 were considered statistically significant.

Results

Patient characteristics

After filtering out the incomplete records, the data from 180 gastric cancer patients were selected for this study. The cohort comprised 111 male and 69 female patients with the mean age of 57.56±12.34 years. The follow-up appointments were conducted at 12–58 months (median: 27 months); 81 patients died during the follow-up. The clinical pathological characteristics are listed in **Table 1**.

NK cells as a risk factor for prognosis in gastric cancer patients

Based on the histogram, the distribution of NK cells was normal (**Supplementary Figure 1**). We included age, lymphocyte count, albumin, tumor biomarkers, NK cells, CRP, hsCRP, N

Table 1. Clinical pathological characteristic of gastric cancer patients.

Variables	Values
Age (years)	57.56±12.34
Gender (Male/Female)	111/69
White blood cell (×10⁹/L)	6.51±2.19
Hemoglobin (g/L)	123.42±86.10
Albumin (g/L)	38.92±6.06
Platelet (×10⁹/L)	300.25±101.25
Neutrophils (×10⁹/L)	5.09±14.66
Lymphocyte (×10⁹/L)	1.87±1.81
CEA (ng/L)	25.06±123.34
CA125 (U/ml)	42.38±127.59
CA153 (U/ml)	12.44±13.03
CA199 (U/ml)	100.46±246.17
T cells (%)	62.68±14.18
B cells (%)	9.30±6.55
NK cells (%)	13.16±10.29
CRP (mg/L)	8.38±19.58
hsCRP (mg/L)	1.75±2.36
NLR	3.02±4.95
PLR	201.60±129.02
Location (antrum/body/cardiac/fundus)	133/21/18/8
Differentiation (low/middle/high)	116/58/5
Lauren (diffuse/intestinal)*	21/7
T stage (T1/T2/T3/T4)	11/30/21/118
N stage (N0/N1/N2/N3/NX)	38/46/40/32/24
M stage (M0/M1)	137/43
Stage (I/II/III/IV)	11/33/93/43
Follow-up (months)	27(12–41)

NK – natural killer; CEA – carcinoembryonic antigen; CRP – C-reactive protein; hsCRP – high-sensitivity C-reactive; NLR – neutrophil-lymphocyte ratio; PLR – platelet-lymphocyte ratio. * Only some patients had Lauren classification data.

stage, M stage, and NLR data from the pathological parameters in the univariate Cox regression models. These parameters were subsequently analyzed using a multivariate Cox regression model. The proportional hazards assumption showed that differences were not statistically significant (*P*=0.084), suggesting that this model met the proportional hazards assumption. We observed that the percentages of lymphocytes, NK cells, CA199, and hsCRP and M stages were correlated with the prognosis of patients with gastric cancer (**Table 2**). The C-index of the Cox regression models was 0.672. To determine whether sex is associated with the prognostic ability

Table 2. Risk factors for the prognosis in gastric cancer patients, with N0 and M0 as reference groups.

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
Age	1.02 (1.00–1.04)	0.019	1.01 (0.99–1.04)	0.144
Lymphocyte	0.61 (0.42–0.88)	0.009	**0.95 (0.92–0.99)**	**0.019**
Albumin	0.94 (0.91–0.96)	<0.01	1.01 (0.99–1.03)	0.466
CEA	1.00 (1.00–1.03)	<0.01	1.01 (0.93–1.07)	0.405
CA125	1.00 (1.00–1.05)	<0.01	1.02 (1.00–1.04)	0.180
CA153	1.02 (1.01–1.06)	<0.01	0.99 (0.97–1.02)	0.901
CA199	1.00 (1.00–1.02)	<0.01	**1.01 (1.00–1.02)**	**0.004**
NK	0.92 (0.89–0.95)	<0.01	**0.92 (0.87–0.96)**	**0.007**
CRP	1.01 (1.07–1.02)	<0.01	1.00 (0.98–1.02)	0.863
hsCRP	1.16 (1.09–1.24)	<0.01	**1.25 (1.05–1.49)**	**0.009**
NLR	1.02 (1.06–1.05)	0.013	**1.17 (1.03–1.32)**	**0.012**
N stage				
N0	–		–	
N1	0.92 (0.56–1.50)	0.740	1.03 (0.61–1.76)	0.889
N2	1.69 (1.07–2.66)	0.022	1.15 (0.67–1.98)	0.599
N3	1.75 (1.05–2.75)	0.029	2.63 (1.55–4.46)	0.000
M stage				
M0	–		–	
M1	4.89 (3.08–7.77)	<0.01	**4.41 (2.61–7.46)**	**<0.01**

Table 3. Correlations between the percentage of NK cells and the other indicators.

NK cells percentage	r value	P value
Lymphocyte	0.318	<0.001
Albumin	0.331	<0.001
CA125	−0.161	0.045
NLR	−0.209	0.009

of the above indexes, we included interactions between NK cell percentages and sex in the model and assessed whether the interaction was significant. As **Supplementary Table 1** shows, sex did not significantly affect the prognostic ability of the above indexes.

Correlation between absolute NK cell numbers and other markers

As listed in **Table 3**, analysis revealed that the percentage of NK cells was positively correlated with the levels of lymphocytes and albumin (*P*<0.05), but was negatively correlated with CA125 levels and NLR (*P*<0.001).

Prognostic value of NK cells in patients with gastric cancer

We calculated the optimal cut-off for the percentage of NK cells and found that at a cut-off of 5.07 the high and low percentages NK cells helped divide the patients into high-risk and low-risk groups based on the cut-off value (log-rank value <0.001; **Figure 1**). To determine the effect of NK cells on survival in gastric cancer patients, we calculated AUC using time-ROC curves; the results showed that the percentage of NK cells had a moderate prognostic association with the 1-, 3-, and 5-year OS, with AUC values of 0.732, 0.806, and 0.792, respectively (**Figure 2A**).

Prognostic value of NK cells combined with NLR in gastric cancer patients

NLR is an independent predictor of gastric cancer, as previous study described [5]. Here, we confirmed that NLR was also related to the survival of gastric cancer patients. Therefore, we
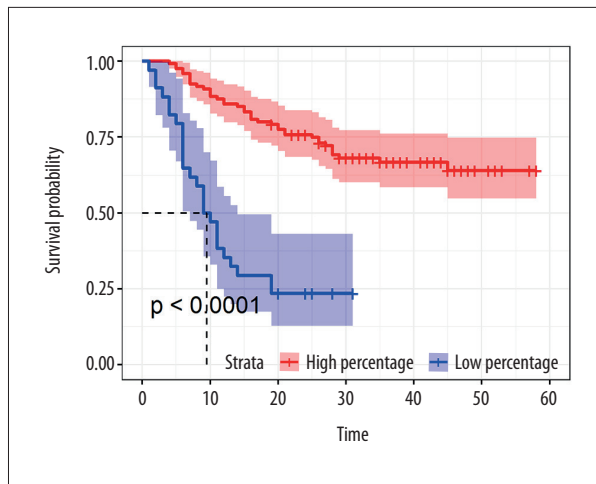
Figure 1. Kaplan-Meier curve of NK cells using the optimal cut-off value in patients with gastric cancer.

assessed whether the combination of the percentage of NK cells with NLR could improve the prognostic value. As shown in **Figure 2B**, this combination increased the prognosis of 1-, 3-, and 5-year OS, for which the AUC was 0.758, 0.823, 0.830, respectively, and the differences at all 3 timepoints were remarkable compared with NK cells alone (all *P*<0.01). These results indicated that this combination could improve the prediction of prognosis of gastric cancer patients.

Correlation between NK cells and clinical features of gastric cancer patients

To understand the correlation between percentage of NK cells and clinical features of gastric cancer, we analyzed the percentage of NK cells in patients with different clinical features.

As shown in **Table 4**, the percentage of NK cells was obviously higher in patients at early T stages (T1+T2), N stage (N0), and clinical stages (I+II) compared with those at advanced T stages (T3+T4), N stages (N1+N2+N3), and clinical stages (III+IV; *P*<0.05). There were no significant differences in patient age, sex, tumor location, histological differentiation, and M stage (*P*>0.05).

Discussion

NK cells are crucial in innate immunity; they can recognize and kill tumor cells and produce multiple cytokines to regulate adaptive immunity [19]. NK cells exert a robust protective response to reactivated pathogens [20,21]. The higher percentage of NK cells allowed treatment of gastric cancer patients to be effective. A huge decrease in the percentage of NK cells and secreted cytokines in gastric cancer patients damages the immune system [22,23]. Increasing the percentage of or activating NK cells are efficient approaches that are tolerated by the immune system to treat gastric cancer [24–26].

Previous studies have shown the prognostic value of NK cells in several cancers. Bar et al. [27] reported that lower percentages of NK cells resulted in a weaker immune response to residual leukemia and predicted a poor outcome in acute myeloid leukemia. The percentage of NK cells is an independent indicator for prognosis of colon cancer and is significantly correlated with the lymph nodes [28]. Yang et al. [29] reported that patients with advanced pancreatic cancer possess NK cells of a unique subtype with anergic features; a high percentage of NKs predicts poor survival of these patients. A recent study reported that baseline concentration of circulating tumor cells is
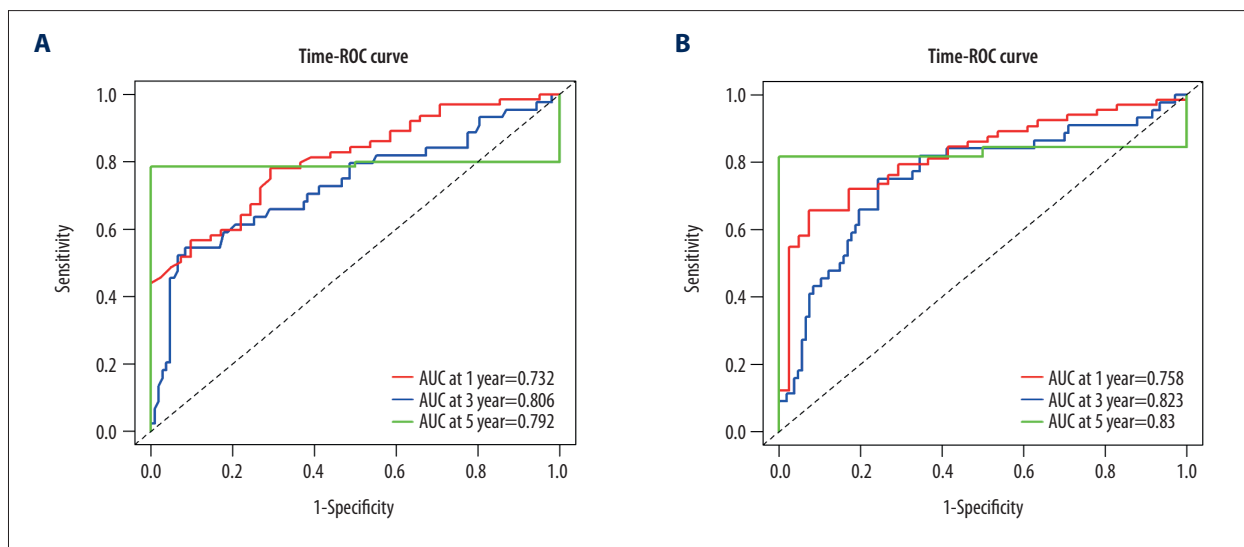

Figure 2. (**A**) Time-ROC curve of NK cells in 1-, 3-, 5- year survival in patients with gastric cancer. (**B**) Time-ROC curve of NK cells combined with NLR in 1-, 3-, 5- year survival in patients with gastric cancer.

Table 4. Association of percentage of NK cells with the clinical features of gastric cancer patients.

Variables	NK cell	P value
Age		0.803
>60 years	13.06±10.62	
≤60 years	12.65±9.51	
Gender		0.118
Male	11.90±10.45	
Female	14.41±9.11	
Location		0.580
Antrum	13.06±10.03	
Body	14.75±11.64	
Cardiac	10.53±8.72	
Fundus	10.23±8.92	
Differentiation		0.365
Low	13.87±9.92	
Middle	10.86±9.44	
High	18.47±13.75	
T stage		0.018
T1+T2	17.08±11.70	
T3+T4	11.69±9.22	
N stage		0.033
N0	16.61±12.22	
NI+N2+N3	11.73±9.03	
M stage		0.101
M0	10.30±10.42	
M1	13.59±9.81	
Clinical stage		0.010
I+II	16.87±10.95	
III+IV	11.57±9.39	

positively correlated with concentration of peripheral NK cells; a combination of the circulating tumor cells and NK cells predicts progression-free survival in triple-negative breast cancer patients without any pre-existing conditions [30].

In the present study, the percentage of NK cells was correlated with the OS of gastric cancer patients, indicating that NK cells can act as an independent biomarker. This result was inconsistent with that reported by Rosso et al. [31]. We also identified an optimal cut-off for the percentage of NK cells that allowed a clear division of patients into low- and high-risk groups. Subsequently, we analyzed the prognostic value in patients' survival. However, the prognostic value of NK cells for 1-, 3-, and 5-year survival was moderate. Therefore, we combined other biomarkers to improve the prognostic ability. As expected, combining the NK cells with NLR greatly increased the prognostic ability compared to using only NK cells, suggesting that this combination can better predict the prognosis of gastric cancer patients.

Although previous studies have reported the prognostic utility of the percentage of NK cells in patients with gastric cancer, the results obtained were inconsistent. Pernot et al. [32] showed that gastric cancer patients with a high percentage of circulating NK cells exhibit better OS than patients with a low percentage. Similarly, Rosso et al. [31] showed that the NK cell percentage in tissues acted as an independent prognostic predictor for gastric cancer patients. In contrast, Yu et al. [14] observed that the circulating NK cells were increased in gastric cancer patients compared to those in healthy controls; however, the percentage of NK cells did not correlate with the survival of patients. Akagi et al. [33] have shown similar results. Thus, the correlation between the percentage of NK cells and survival of patients with gastric cancer remains unclear. The present study provides evidence that the percentage of NK cells is correlated with the survival of gastric cancer patients.

The strength of our study was the relatively large cohort with data from extended follow-up appointments. However, there are some limitations to this study. First, the data originated from patients at a single-center setting, thereby decreasing the generalizability of our findings. Second, the design of this study was of a retrospective nature, which could have introduced bias and reduced the reliability of the results. Third, owing to the limited data, some well-known risk factors for gastric cancer, such as *Helicobacter pylori* and genetic factors, were not included in the analysis, which might have affected the accuracy of the results. Fourth, due to lack of data, we could not compare the differences in baseline characteristics (including tumor grade and past medical history). Therefore, using data from a larger and well-defined prospective cohort will help confirm our findings.

Conclusions

This study demonstrated that the percentage of peripheral NK cells is correlated with the survival of gastric cancer patients, and could be a promising prognostic biomarker. However, these results need further validation owing to the study limitations.

Supplementary Data

Normal distribution
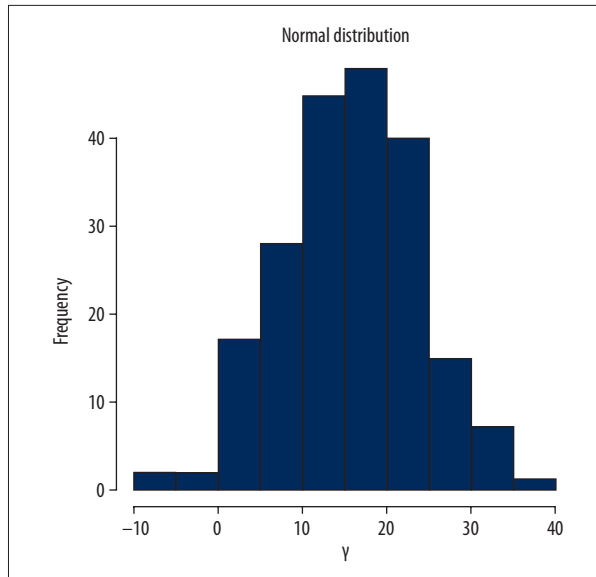
Supplementary Figure 1. Distribution of NK cell perecntage.

Supplementary Table 1. Multivariate Cox regression analysis of variables in prognosis of gastric cancer after stratification by sex.

	P value	HR (95%CI)
Age	0.103	1.48 (0.92–2.38)
Lymphocyte	0.022	1.02 (1.00–1.04)
Albumin	0.318	0.97 (0.91–1.02)
CEA	0.505	0.99 (0.99–1.01)
CA125	0.192	1.01 (0.99–1.04)
CA153	0.477	1.09 (0.98–1.03)
CA199	<0.01	1.01 (1.00–1.02)
NK	0.005	0.96 (0.91–0.98)
CRP	0.419	0.98 (0.96–1.01)
hsCRP	0.002	1.27 (1.09–1.47)
NLR	0.122	1.04 (0.98–1.01)

	P value	HR (95%CI)
T stage		
T1	–	–
T2	0.996	0.12 (0.02–1.03)
T3	0.577	1.33 (0.48–3.65)
T4	0.201	0.62 (0.29–1.29)
N stage	–	–
N0	0.019	0.15 (0.03–0.73)
N1	0.840	0.92 (0.44–2.08)
N2	0.385	0.77 (0.37–1.46)
N3	0.673	1.19 (0.56–2.77)
M stage		
M0	–	–
M1	<0.01	0.28 (0.148–0.56)

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