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Preoperative Neutrophil-to-lymphocyte Ratio Predicts Tumor-infiltrating CD8+ T Cells in Biliary Tract Cancer

RYOTA TANAKA[1], KENJIRO KIMURA[2], SHINPEI EGUCHI[2], JUN TAUCHI[2], MASATUNE SHIBUTANI[1], HIROJI SHINKAWA[2], GO OHIRA[2], SADAAKI YAMAZOE[2], SHOGO TANAKA[2], RYOSUKE AMANO[2], HIROAKI TANAKA[1], MASAKAZU YASHIRO[1,3,4], SHOJI KUBO[2] and MASAICHI OHIRA[1]

[1]*Department of Gastroenterological Surgery, Osaka City University Graduate School of Medicine, Osaka, Japan;*
[2]*Department of Hepato-Biliary-Pancreatic Surgery, Osaka City University Graduate School of Medicine, Osaka, Japan;*
[3]*Molecular Oncology and Therapeutics, Osaka City University Graduate School of Medicine, Osaka, Japan;*
[4]*Cancer Center for Translational Research, Osaka City University Graduate School of Medicine, Osaka, Japan*

Abstract. *Background/Aim: This study evaluated the prognostic significance of preoperative neutrophil-to-lymphocyte ratio (NLR) and CD8+ tumor-infiltrating lymphocytes (TILs), and whether preoperative NLR was associated with CD8+ TILs in biliary tract cancers (BTCs). Patients and Methods: A total of 154 patients with BTCs who underwent surgery were enrolled in this study. We obtained neutrophil and lymphocyte counts, and calculated NLR from preoperative peripheral blood samples. CD8+ TILs were identified by immunohistochemical staining. Results: The overall survival (OS) and recurrence-free survival (RFS) of patients with high NLR were shorter than those with low NLR. The OS and RFS of patients with high CD8+ TILs were longer than those with low CD8+ TILs. Preoperative NLR and CD8+ TILs were negatively correlated. Conclusion: NLR and CD8+ TILs were associated with OS and RFS in BTCs. NLR can predict CD8+ TILs infiltrating the cancer microenvironment.*

Bile duct cancers (BTCs) are diverse biliary epithelial tumors involving the intrahepatic, perihilar, and distal biliary tree (1). BTCs are aggressive tumors, and most patients have an advanced-stage disease at diagnosis (2). Surgical treatment is adequate for all subtypes, but only one third of patients (approximately 35%) have an early-stage disease that can be treated with surgical curative resection (2). For patients with advanced-stage or unresectable cholangiocarcinoma, there are few effective systemic therapies. The median overall survival of patients treated with current standard-of-care chemotherapy regimens including cisplatin and gemcitabine is less than a year (3).

The inflammatory reaction plays an important role in carcinogenesis and cancer progression (4). Both systemic inflammation and local immune response reflect the prognosis in many solid cancers (5). Cross-talk between systemic and local immune responses is suspected during malignancy development (6). It has been reported that high neutrophil-to-lymphocyte ratio (NLR) in systemic blood flow is prognostic and therapeutic outcome for various types of cancer (7-9). Furthermore, tumor-infiltrating lymphocytes (TILs), especially CD8+ T cells, have been shown to play a major role in antitumor immunity in the cancer microenvironment (10, 11). TILs correlate with the therapeutic outcome and survival of patients with various types of cancer, including colorectal, breast, and gastric cancers (12-14). Recent research has revealed a correlation between preoperative NLR and TILs (15), but there are few reports regarding BTCs (8).

The purpose of this study was to assess the prognostic significance of preoperative NLR and CD8+ TILs and evaluate whether preoperative NLR was associated with CD8+ TILs in BTCs.

Patients and Methods

Patient population and tissue samples. Clinical data and formalin fixed paraffin-embedded (FFPE) tissues were obtained from 154 patients who underwent resection for BTCs at our institution between 2001 and 2017. BTCs include intrahepatic, perihilar, and distal bile duct cancer, gallbladder cancer, and ampullary cancer. None of the patients underwent preoperative radiotherapy or chemotherapy. Pathological findings were retrospectively evaluated following the Japanese classification of biliary tract cancers, third edition (16). The TMN classification was reclassified following the American Joint Committee on Cancer system, eighth edition (17). After surgery, the patients were followed at 3- to 6-month intervals

Correspondence to: Kenjiro Kimura, Department of Hepato-Biliary-Pancreatic Surgery, Osaka City University Graduate School of Medicine, 1-4-3 Asahimachi, Abenoku, Osaka, 545-8585, Japan. Tel: +81 666453838, Fax: +81 666466450, e-mail: kenjiro@med.osaka-cu.ac.jp

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by tumor marker and enhanced computed tomography. Recurrence-free survival (RFS) and overall survival (OS) are defined as the time from surgery to recurrence or death. This study conforms to the Declaration of Helsinki, and was approved by the Osaka City University Ethics Committee (approval number 924). Written informed consent was obtained from each patient.

Blood inflammatory biomarkers for biliary tract cancers. Peripheral blood samples were obtained preoperatively. NLR was calculated from blood samples, retrospectively. Antibiotics were provided and/or biliary drainage was performed in cases of cholangitis or cholecystitis. A surgical resection was performed after the inflammation subsided. The data from were evaluated within two weeks of the operation when the inflammatory response was the lowest. Patients were divided into high or low preoperative NLR, based on the median preoperative NLR value.

Immunohistochemical staining. Then, the tumor-infiltrating lymphocytes were examined by immunohistochemical staining. Sections with a thickness of 4 μm were obtained from paraffin-embedded tissue blocks. The sections were deparaffinized and heated by an autoclave with Target Retrieval Solution (Dako, Carpinteria, CA, USA) for 10 min at 105˚C. After blocking endogenous peroxidase activity, the samples were incubated overnight with a primary monoclonal antibody against anti-human CD8 (1:50, Dako, Carpinteria, CA, USA) at 4˚C. Biotinylated IgG incubation was performed for 10 min at room temperature. The immunohistochemical reaction was performed in a streptavidin-peroxidase reagent, and counterstaining was performed with Mayer's hematoxylin. The immunohistochemical evaluation was performed independently by RT and KK. The infiltration of $CD8^+$ T cells around the tumor cells was evaluated with a microscope in three randomly selected fields at a magnification of ×400 and the average was calculated.

Statistical analysis. Continuous variables were compared using the Mann-Whitney *U*-test. Categorical variables were compared using the chi-square or Fisher exact tests, as appropriate. Cox proportional hazard regression analyses were performed to identify prognostic predictors. The OS and RFS rates were estimated by the Kaplan–Meier method, and survival curves were compared using the log-rank test. Groups were considered to be significantly different at $p<0.05$. All tests were performed using JMP software version 13 (SAS Institute, Cary, NC, USA).

Results

The clinicopathological characteristics of patients with biliary tract cancers with high and low NLR. The clinicopathological characteristics of the 154 cases resected for BTCs are listed in Table I. The median age of all patients was 69 years old. Half of the patients were narrowly defined as having bile duct cancers. The median NLR was 2.3 (range=0.7-18). Patients were divided into high and low NLR groups, based on the median NLR. There were statistically significant differences between high and low NLR patients in only the lymph node metastasis (Table II). High NLR was associated with lymph node metastasis ($p=0.004$).

Association between NLR and survival outcomes. In the total patient population, patients with low NLR (≤2.3) showed longer OS and RFS. The low NLR group showed statistically significant ($p=0.004$) superior OS (median OS, not reached; 5-year survival rate, 51.9 %) compared to high NLR group (median OS: 27.3 months; 5-year survival rate: 28.1 %). Similarly, patients with low NLR showed statistically significant ($p=0.01$) superior RFS (median RFS: not reached; 5-year RFS rate: 41.9 %) compared to high NLR group (median RFS: 18.5 months; 5-year RFS rate: 17.4 %) (Figure 1).

The clinicopathological characteristics of $CD8^+$ TILs. The median number of infiltrating $CD8^+$ TILs was 40 (range=0-216). All patients were divided into the high infiltrating group of $CD8^+$ T cells (high $CD8^+$ TILs; n=79) and the low infiltrating group of $CD8^+$ T cells (low $CD8^+$ TILs; n=75), based on the median number of $CD8^+$ TILs. Figures 2a and b show high and low $CD8^+$ TILs. High $CD8^+$ TILs were statistically significantly associated with the Union for International Cancer Control (UICC) stage ($p=0.04$) (Table III).

Association between TILs and survival outcomes. In the total patient population, patients with high $CD8^+$ TILs showed longer OS. The high $CD8^+$ TILs group showed statistically significant ($p=0.04$) superior OS (median OS: 51.1 months; 5-year survival rate: 46.2 %) compared to the low $CD8^+$ TILs group (median OS: 34.5 months; 5-year survival rate: 32.5 %) (Figure 2c). Similarly, patients with high $CD8^+$ TILs showed statistically significant ($p=0.02$) superior RFS (median RFS: 45.9 months; 5-year RFS rate: 39.9%) compared to patients with low $CD8^+$ TILs (median RFS: 14.7 months; 5-year RFS rate: 19.3 %) (Figure 2d).

Correlation between preoperative NLR and $CD8^+$ TILs infiltrating in the local tumor microenvironment. We explored the correlation of $CD8^+$ TILs with preoperative NLR. A scatter chart of $CD8^+$ TILs and preoperative NLR revealed a negative correlation ($p=0.0001$, r=0.033) (Figure 3a). The patients were divided into two groups based on the median preoperative NLR value (2.3, range=0.7-18). Patients with high NLR had a significantly lower average number of $CD8^+$ TILs as compared to patients with low NLR ($p=0.02$) (Figure 3b). Preoperative NLR was associated with $CD8^+$ TILs infiltrating in the cancer microenvironment.

Discussion

This study revealed that preoperative NLR was a prognostic factor for patients with BTCs. The OS and RFS were significantly shorter in patients with high NLR than in those with low NLR. We observed that low NLR was correlated with high levels of tumor-infiltrating $CD8^+$ T cells. Recent reports have indicated that infiltrating immune cells in the

Table I. *Clinicopathological characteristics of 154 patients with BTCs.*

	Number
Gender	
Men	86
Women	68
Age, median (range)	69 (43-87)
Differentiated	
Differentiated	123
Undifferentiated	14
Other	17
Location of cancer	
Peripheral and distal bile duct	67
Intrahepatic bile duct	24
Gallbladder	27
Ampullary	36
T category	
pT0	18
pT1	18
pT2	47
pT3	58
pT4	13
Lymph node metastasis	
Absent	98
Present	49
Distant metastasis	
Absent	136
Present	11
Lymphatic invasion	
Absent	60
Present	64
Vascular invasion	
Absent	21
Present	103
Neural invasion	
Absent	58
Present	62
UICC stage	
0	18
1	36
2	64
3	14
4	22
Serum CEA level, ng/ml, median (range)	2.6 (0-86.5)
Serum CA19-9 level, U/ml, median (range)	29 (0-45,152)
Recurrence	
Yes	76
No	78
Outcome	
Death	70
Alive	84
Recurrence free survival, days, median (range)	521 (0-4,160)
Overall survival, days, median (range)	778 (9-4,157)
NLR, median (range)	2.3 (0.7-18)
CD8+ TILs, median (range)	40 (0-216)

BTCs: Bile tract cancers; UICC: Union for International Cancer Control; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9.

Table II. *Correlation between clinicopathological features and NLR in 154 patients with BTCs.*

	High NLR n=75	Low NLR n=79	*p*-Value
Gender			
Men	38	48	0.2
Women	37	31	
Age, median (range)	69 (43-87)	69 (43-86)	0.27
T category			
pT1-2	36	45	0.32
pT3-4	39	34	
Lymph node metastasis			
Absent	43	62	0.007*
Present	32	17	
Distant metastasis			
Absent	68	75	0.32
Present	7	4	
Lymphatic invasion			
Absent	26	34	0.45
Present	32	32	
Vascular invasion			
Absent	50	50	0.38
Present	8	13	
Neural invasion			
Absent	27	31	0.84
Present	30	32	
UICC stage			
≤2	53	59	0.67
>2	22	20	
Serum CEA level			
<5 ng/ml	56	63	0.79
≥5 ng/ml	12	12	
Serum CA19-9 level			
<37 U/ml	40	40	0.78
≥37 U/ml	31	34	

*$p<0.05$. NLR: Neutrophil-to-lymphocyte ratio; BTCs: bile tract cancers; UICC: Union for International Cancer Control, CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9.

cancer microenvironment affect the therapeutic response and prognosis in various cancers (12-14). In the cancer microenvironment, $CD8^+$ T cells have a direct cell-killing effect and play an important role in the anti-cancer immune reactions (18, 19). Our findings suggest that preoperative NLR may predict the tumor infiltrating $CD8^+$ T cells.

In the last decade, NLR has been widely recognized as an attractive proxy instead of the systemic inflammatory status. Several studies have suggested that high NLR is a poor prognostic marker. Neutrophils activate proangiogenic factors, including vascular endothelial growth factor, or inflammatory cytokines, such as IL-1β (8, 20, 21). Lymphocytes are critically involved in the innate immunity and the adaptive immune response, so that they can kill tumor cells by cytotoxic cell death and cytokine secretion (22). Also, neutrophils suppress

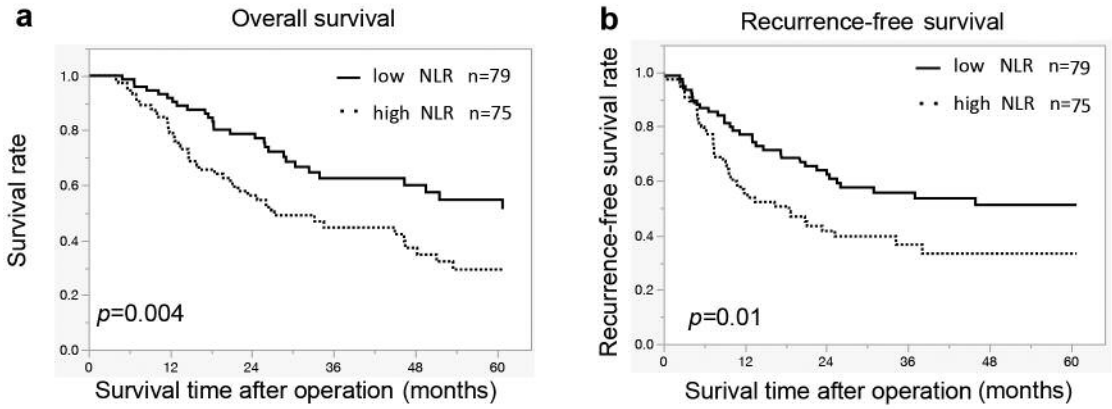

Figure 1. *Overall survival and recurrence free survival according to NLR. A Kaplan–Meier survival curve indicates that the patients with high NLR had a significantly shorter overall survival (a) and recurrence-free survival (b) as compared to those with low NLR. The overall survival and recurrence-free survival of patients with high NLR were significantly shorter compared with patients with low NLR.*

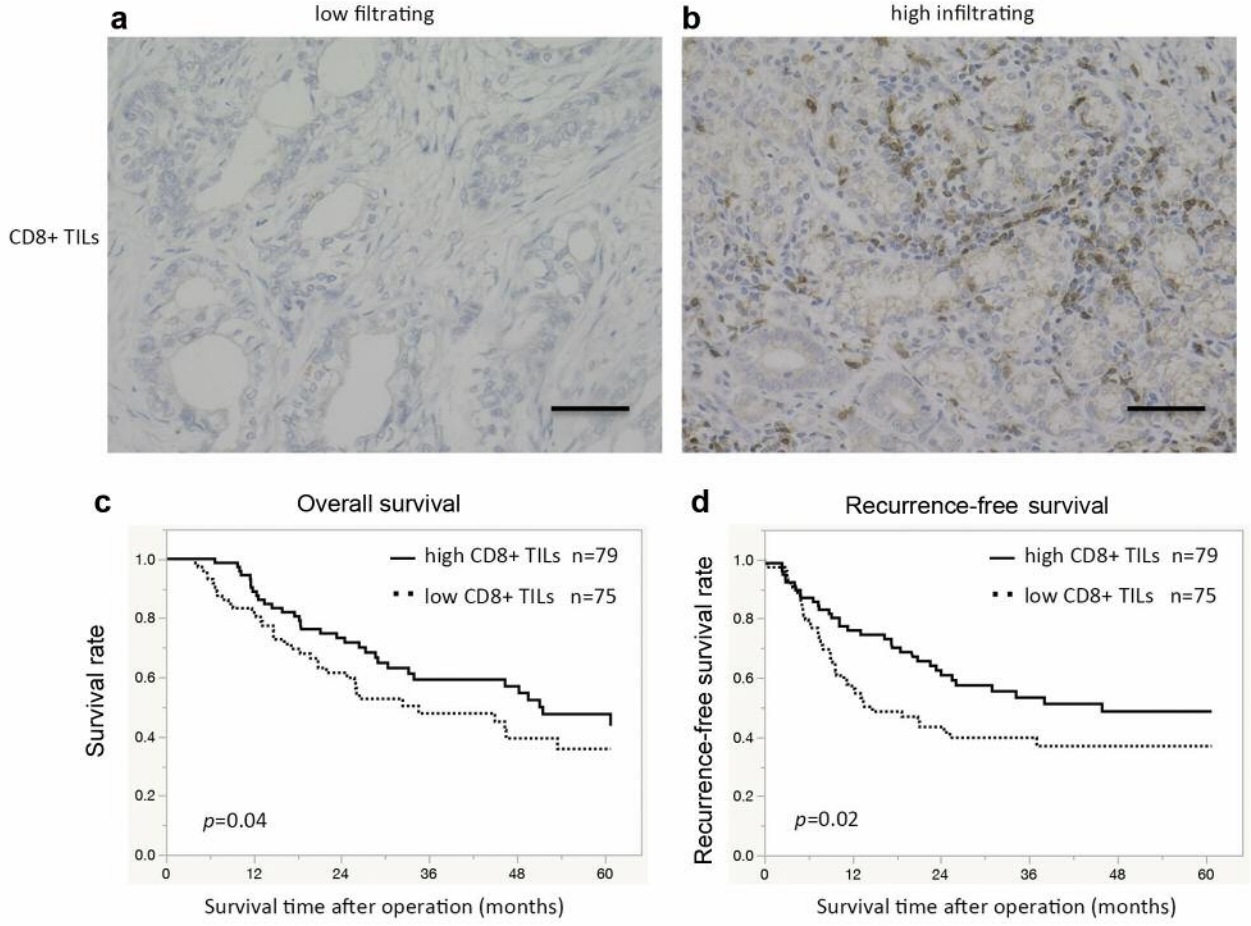

Figure 2. *Immunohistochemical staining of TILs. Microscopic images are shown as follows: a: low CD8+ TILs and b: high CD8+ TILs. The original magnification was 400-fold, and the scale bar is 50 μm. CD8+ TILs around the cancer cells are stained brown, and cancer cells are not stained. (c) and (d) Overall survival and recurrence-free survival, according to CD8+ TILs. The Kaplan–Meier survival curve indicates that the patients with high CD8+ TILs have significantly longer overall survival (c) and recurrence-free survival (d) as compared to those with low CD8+ TILs.*

Table III. *Correlation between clinicopathological features and infiltrating CD8+ TILs in patients with BTCs.*

	High CD8+ TILs n=79	Low CD8+ TILs n=75	*p*-Value
Gender			
Men	50	36	0.06
Women	29	39	
Age, median (range)	68 (43-86)	71 (51-87)	0.81
T category			
pT1-2	43	38	0.74
pT3-4	36	37	
Lymph node metastasis			
Absent	51	49	0.56
Present	23	26	
Distant metastasis			
Absent	73	70	0.79
Present	6	5	
Lymphatic invasion			
Absent	35	25	0.55
Present	34	30	
Vascular invasion			
Absent	61	42	0.08
Present	8	13	
Neural invasion			
Absent	36	22	0.24
Present	32	30	
UICC stage			
≤2	63	49	0.04*
>2	16	26	
Serum CEA level			
<5 ng/ml	66	53	0.22
≥5 ng/ml	10	14	
Serum CA19-9 level			
<37 U/ml	42	38	0.87
≥37 U/ml	35	39	

*$p<0.05$. UICC: Union for International Cancer Control; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9.

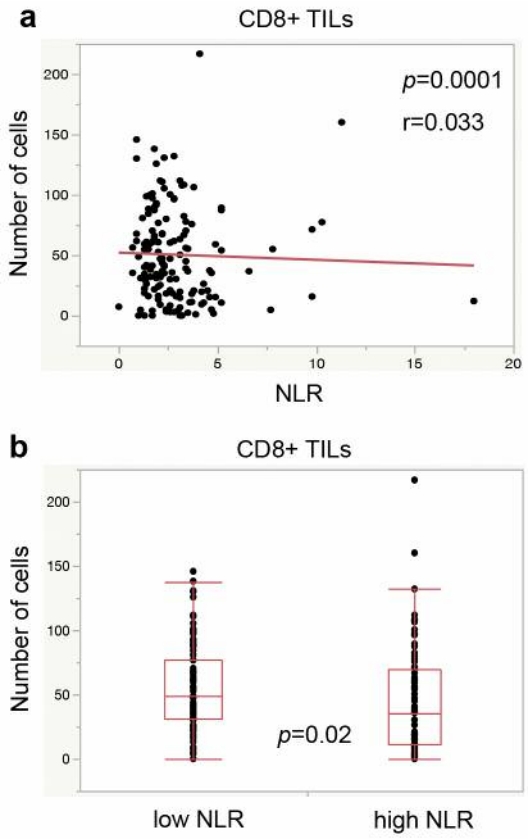

Figure 3. *Correlation between preoperative NLR and CD8+ TILs infiltrating the local tumor microenvironment. (a) Scatter chart of preoperative NLR and CD8+ TILs. There is negative correlation between preoperative NLR and CD8+. (b)The number of infiltrating TILs is compared between high and low NLR groups. Patients with high NLR had a significantly lower average number of CD8+ TILs compared to patients with low NLR.*

the cytolytic activity of lymphocytes and natural killer cells towards tumor cells (23). In our study, patients with higher NLR exhibited poorer prognosis than those with lower NLR, which is consistent with previous studies in BTCs.

CD8+ T cells are cytotoxic T lymphocytes that directly attack cancer cells and play a central role in anti-cancer immunity (24). A previous study has found that CD8+ TILs infiltrating in the cancer microenvironment were associated with long-term survival in patients with various types of cancer (24-26). Furthermore, they have also been associated with the therapeutic effect of chemotherapy and radiotherapy (26-28). In this study, high CD8+ TILs were predictive of a favorable prognosis. This indicates that CD8+ TILs in BTCs play a key role in tumor immunity in the cancer microenvironment.

The most important finding in this study was the negative correlation between the preoperative NLR and the number of CD8+ TILs. Patients with low NLR had higher number of CD8+ TILs than the patients with high NLR. The relationship between the systemic immune response and the tumor microenvironment has not been fully elucidated. Currently immune checkpoint inhibitors are being clinically applied to BTCs (29). If the systemic immune response reflects cancer microenvironment immunity in BTCs, the correlation between the systemic and local immune responses should be investigated further.

There are some limitations to this study. First, this study was retrospective and a single-center cohort. Second, it included five subtypes of biliary tract cancers, intrahepatic, perihilar, and distal bile duct cancer, gallbladder cancer, and ampullary cancer. Third, we did not analyze other immune cells by immunohistochemical staining, such as neutrophils,

macrophages, or regulatory T cells. These immune cells may also affect tumor progression in the cancer microenvironment. Fourth, we did not exclude patients with pre-operative systemic inflammation, including cholangitis and cholecystitis. As mentioned previously in the Materials and Methods section, a surgical operation was performed after the inflammation subsided in cases of cholangitis or cholecystitis. However, preoperative inflammation might affect NLR. Lastly, we set the optimal NLR value as the median NLR, 2.3. As the number of cases in this study was small and an effective cut-off value could not be calculated, the median NLR was used. In previous studies that explored the effects of NLR on prognosis in BTCs, NLR ranged from 1.93 to 3 (7, 8, 15, 30). Our cut-off value of 2.3 seems reasonable.

Conclusion

Preoperative NLR was a prognostic factor for BTCs. The OS and RFS of patients with high NLR were significantly poorer than that of patients with low NLR. We observed that low NLR correlated with high levels of tumor-infiltrating $CD8^+$ T cells. Our findings suggest that preoperative NLR as an indicator of systemic immune response can predict the tumor infiltrating $CD8^+$ T cells in the cancer microenvironment.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

Ryota Tanaka, Kenjiro Kimura and Masaichi Ohira: drafting of manuscript. Shinpei Eguchi, Jun Tauchi, Masatune Shibutani, Hiroji Shinkawa, Go Ohira, Sadaaki Yamazoe, Shogo Tanaka, Ryosuke Amano, Hiroaki Tanaka, Masakazu Yashiro, Shoji Kubo: critical revision of manuscript.

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