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The prognostic value of combining the CD8+ lymphocyte density and the circulating lymphocyte ratio in circumferential resection margin biopsy in rectal cancer

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Abstract

Background: A positive circumferential resection margin (CRM) may result in local recurrence (LR), but the significance remains controversial. We attempted to utilize the lymphocyte ratio (LYMR), neutrophil–lymphocyte ratio (NLR), tumor-infiltrating lymphocyte (TIL) count, and their combinations (TIL–LYMR/TIL–NLR) in predicting LR after rectal resection.

Methods: Patients with rectal cancer who underwent curative resection between January 2016 and December 2018 were enrolled. Biopsy samples and data from the blood tests of 124 patients with rectal cancer who underwent curative resection were retrospectively obtained. Patients were divided into 2 groups: LR group and non-local recurrence (nLR) group. CD8+TILs were immunostained using an antibody against CD8. The density of TILs was defined as the number of positive CD8 lymphocytes per square millimeter and was then graded as either high or low (cutoff = 80/mm^2). The count of LYMR and NLR was also graded as either high or low. The associations between TILs, LYMR, NLR, and their combinations (TIL–LYMR/TIL–NLR) were evaluated.

Results: With a median follow-up of 24.4 months, TIL–LYMR showed a positive correlation with LR ($P = .001$), but not with the CD8+TIL count ($P = .215$) or TIL-NLR count ($P = .638$). Among inflammatory and immune markers variables, univariate analysis revealed that gender, CD8+TIL count, and TIL-NLR count were associated with anastomotic leakage ($P = .001$, $P = .014$, and $P = .036$, respectively). In multivariate analysis, TIL–LYMR remained an independent predictor of LR (OR = 8.918, CI = 1.124–70.747, $P = .038$). We also showed that gender associated with anastomotic leakage in rectal cancer (OR 5.429; 95% CI 1.885–15.637; $P = .002$).

Conclusion: In this study, our data indicate that absence of CD8+T-cell infiltration in CRM may influence LR. These parameters may help identify LR provide additional information for therapeutic decision-making.

Abbreviations: CRC = colorectal cancer, CRM = circumferential resection margin, LR = local recurrence, LYMR = lymphocyte ratio, NLR = neutrophil–lymphocyte ratio, nLR= non-local recurrence, TIL = tumor-infiltrating lymphocyte.

Keywords: circumferential resection margin, local recurrence, lymphocyte ratio, neutrophil–lymphocyte ratio, rectal cancer, tumor-infiltrating lymphocytes

1. Introduction

Colorectal cancer (CRC) is a major cause of cancer mortality and morbidity, and it has been reported that cancer of rectum accounts for 1 in 3 CRC.[1] The circumferential resection margin (CRM), the distance from the tumor to the mesorectal fascia, is a prognostic factor for local recurrence (LR) in rectal cancer.[2–4] However, rectal cancer surgery is demanding in both technique and experience, as reflected by a higher postoperative recurrence rate than that after colon cancer surgery, due to the higher frequency of narrow pelvic anatomy, male sex, bulky mesorectum, and high body mass index.[5–7] The surgical treatment of locally advanced rectal cancer with negative surgical margins can enhance the chance of sphincter-preserving surgery, decrease LR, and improve survival. Although total mesorectal excision, which consists of the removal of the mesorectum en bloc along with the fascia recti propria, has attracted attention, LR remains a major problem, with rates between 7.6% and 11.3%.[8,9] Therefore, it is necessary to identify patients at greatest risk of worse outcomes by using clinical, inflammatory, or molecular biomarkers.[10–12]

Recently, it has been noted that the innate immune/inflammatory response is a prognostic factor of rectal cancer, and reflects the incidence of LR originating from residual tumor on the anastomosis after the initial resection.[13,14] Moreover, we have demonstrated that inflammation is involved in the development and progression of malignant tumors, including CRC.[15] An inflammatory microenvironment can be achieved by promoting angiogenesis and metastasis, subverting adaptive immune responses that inhibit or stimulate an active response to tumor cells.[16]

Until now, it has not been possible to identify a correlation between tumor infiltration by immune cells and CRM involve-

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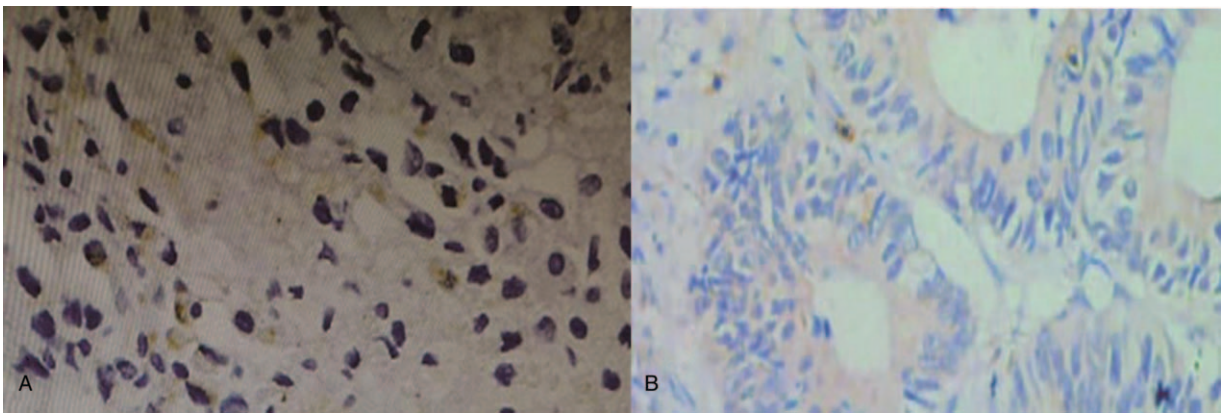

Figure 1. Immunoreactive tumor-infiltrating lymphocytes determined at the invasive margin in resected specimens of locally advanced rectal cancer. Magnification, ×400. (A) High density of CD8+ TILs in biopsy samples in circumferential resection margin sections. (B) Low density of CD8+ TILs in biopsy samples in circumferential resection margin section. Magnification, ×400. CRM = circumferential resection margin, TIL = tumor-infiltrating lymphocyte.

ment in rectal cancer. The primary objective of this study was to determine markers associated with LR. We stained for several markers of the innate and adaptive immune response and analyzed the local immune status, as measured by the CD8 + TIL (tumor-infiltrating lymphocyte) count, and host immune status, as measured by the neutrophil–lymphocyte ratio (NLR) and lymphocyte ratio (LYMR), to determine predictors of the tumor response.

2. Patients and materials

The medical records of patients who underwent surgical resection for rectal cancer at the Hongqi Affiliated Hospital Mudanjiang Medical University from January 2016 to December 2018 with a diagnosis of rectal cancer were retrieved. All patients provided pretreatment biopsy samples. The inclusion criteria included: histologically confirmed rectal adenocarcinoma; T3–4 or N+ initial disease; disease located within 15 cm of the anal verge; and blood test within 2 weeks after surgery and LYMR could be calculated. The exclusion criteria were as follows: neoadjuvant chemoradiotherapy or radical surgery; metastatic disease; presence of infection; age younger than 18 years or older than 85 years, and death within 1 month postoperatively. The study was performed with approval from the institutional research ethics committee of Hongqi Affiliated Hospital Mudanjiang Medical University. Written consent forms notifying the use of specimens and publication of the results were obtained upon admission.

2.1. Immunohistochemical method

The distribution and density of CD8 + lymphocytes in biopsy samples of primary rectal tumors were evaluated by immunohistochemical staining using affinity-purified mouse monoclonal antibodies against CD8 (ZA0508; ZSGB-BIO, Beijing, China). Standard streptavidin-peroxidase procedures were used for immunohistochemistry. Briefly, 4-μm-thick sections were deparaffinized and hydrated through a graded series of ethanol. After rinsing in phosphate-buffered saline (PBS), endogenous peroxidase activity was inhibited by incubation with 3% H_2O_2. Slides were boiled at 95 °C for 15 to 20 minutes in 0.01 M citrate buffer (pH 6.0), naturally cooled over 20 minutes, and washed in cold water, after which they underwent accelerated cooling to room temperature and were rinsed with PBS. The sections were incubated with normal goat serum and then incubated at 4 °C in primary antibody. Slides were incubated with a biotin-labeled secondary antibody at 37 °C and flushed with PBS. Slides were stained with DAB and counterstained with hematoxylin, then dehydrated, treated with transparentizing reagent, dried, and sealed.

Immunohistochemical evaluations were carried out by a pathologist who was blinded to the clinical information. The number of immunoreactive lymphocytes was counted under a light microscope in a randomly selected field at 400× magnification in 3 different sections. The density of TILs was defined as the number of CD8 + lymphocytes per square millimeter and was then graded as either high or low (cutoff = 80/mm^2) (Fig. 1A, B). This cutoff value yielded a minimal *P*-value in the analysis of correlation with LR and anastomotic leakage.

2.2. Evaluation of hematological factors

Blood samples were taken within 2 weeks after resection of rectal cancer at our center. None of the patient had evidence of infectious complications such as fever, chills, or headache at the time of blood withdrawal. The data from regular blood tests were assessed, including white blood cell (WBC), lymphocyte, and neutrophil counts. The NLR was calculated as the absolute neutrophil count divided by the absolute lymphocyte count, and the LYMR was calculated as the absolute lymphocyte count divided by the WBC count. The NLR was graded as either high or low (cutoff = 2.0), and the LYMR was graded as either high or low (cutoff = 3.0). The LYMR combined with the CD8 + TIL count (TIL–LYMR) was then classified into 3 groups: low (both LYMR and density of CD8 + TILs were low), mid (either LYMR or density of CD8 + TILs was low), and high (both LYMR and density of CD8 + TILs were high). The combination of the NLR with CD8 + TILs (TIL–NLR) was similarly grouped as low, mid, or high.

2.3. Statistical analysis

The clinicopathological parameters were analyzed using chi-squared test or Fisher exact test. Comparison of blood cell counts and ratios between different groups was performed using nonpaired *t* test or Mann–Whitney *U* test. Multivariate analysis

Table 1

Correlation of recurrence with clinicopathological characteristics (n = 124).

Variables	Recurrence, n, %		*P*-value
	Yes	No	
Gender			
Male	22	61	.583
Female	9	32	
Age, y			
<60	18	13	.408
≥60	46	47	
Tumor location			
Middle	23	8	.436
Low	62	31	
Surgical producer			
AR	25	60	.095
APR or Hartmann	6	34	
Tumor differentiation			
Well	7	11	.465
Moderate	17	62	
Poor	7	20	
T-stage			
1–2	20	51	.298
3	11	43	
N-Statu			
0	11	28	.578
1	20	65	

APR = abdominal perineal resection, AR = anterior resection.

using logistic regression was performed to determine independent factors impacting LR. Statistical analysis was performed using IBM SPSS Statistics (SPSS, Chicago, IL), version 24.0. A two-sided *P*-value < .05 was considered as statistically significant.

2.4. Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

2.5. Informed consent

Written informed consent was obtained from all individual participants included in the study.

3. Results

A total of 124 patients who underwent curative resection were included in this analysis, 83 patients were male (67%), and 41 patients were female (33%). The median age of the patients was 58 years (range 19–85 years). The median follow-up of all patients was 24.4 ± 12.4 months. Twenty-four (6.4%) patients experienced LR. According to the clinical outcome, patients were divided into 2 groups: LR group and non-local recurrence (nLR) group.

A comparison of the clinicopathological characteristics of 2 groups is summarized in Table 1. There were no differences in gender (*P* = .583), age (*P* = .408), tumor location (*P* = .436), surgical producer (*P* = .095), tumor difference (*P* = .465), N-statu (*P* = .578), and T-stage (*P* = .298) between the LR and nLR groups (Table 1).

Table 2

Correlations of recurrence with hematological and immunological factors (n = 124).

Variables	Recurrence, n, %		*P*-value
	Yes	No	
NLR			
<0.2	14	44	.836
≥0.2	17	49	
LYMR			
<0.3	16	47	.918
≥0.3	15	46	
CD8+			
Low	19	45	.215
High	12	48	
TIL-NLR			
LOW	8	20	.638
MID	17	53	
HIG	6	20	
TLI-LYMR			
LOW	8	56	.001
MID	19	33	
HIGH	4	4	

LYMR = lymphocyte ratio, NLR = neutrophil–lymphocyte ratio, TIL = tumor-infiltrating lymphocyte.

Regarding hematological factors, patients who achieved LR did not showed relation with the counts of LYMR and NLR (*P* = .918 and *P* = .836, respectively) (Table 2). CD8 + TILs were clearly immunostained in tumor nests using specific antibodies (Fig. 1). Counts of CD8 + TILs in recurrence patients were higher than those in nonrecurrence group, and tumors with a high density of CD8 + TILs tended to achieve better regression than tumors with a low density, although statistical significance was not reached in either case (*P* = .215 respectively). When the CD8 + TILs were combined with LYMR (TIL–LYMR), a significant difference was observed in the 2 groups (*P* = .001) (Table 2). Among inflammatory and immune markers variables, LYMR, NLR, and TIL-LYMR were not associated with anastomotic leakage (*P* = .559, *P* = .356, and *P* = .960, respectively); however, univariate analysis revealed that gender (*P* = .001); CD8 + TIL (*P* = .014) and TIL-NLR (*P* = .036) significantly affected anastomotic leakage (Table 3).

Variables with *P*-values <.1 in the univariate analysis, which only CD8 + TILs–LYMR were entered into a multivariate analysis. The results showed that when the CD8 + TIL combined with LYMR (TIL–LYMR) were remained significant of LR (odds ratio [OR] = 8.918, 95%confidence interval [CI] = 1.124–70.747, *P* = .038). When gender, age, TIL-NLR, and CD8 + TIL were included in a logistic regression model, the risk of gender was the only independent predictor of anastomotic leakage (OR = 5.429, CI = 1.885–15.637, *P* = .002). The TIL–NLR was not remained significant predictor of anastomotic leakage.

4. Discussion

In this study, we showed that the density of CD8 + TILs in the CRM combined with the circulating LYMR (TIL–LYMR) was a significant difference between the LR group and nLR group, and the density of CD8 + TILs in the CRM combined with the NLR (TIL–NLR) was associated with anastomotic leakage in rectal cancer. Patients with a low CD8 + TIL density were likely to increases the risks of both LR and anastomotic leakage. In

Table 3

Correlations of anastomotic leakage with hematological and immunological factors (n = 124).

	Variables anastomotic leakage, n, %		
	Yes	No	*P*-value
NLR			
<0.2	15	69	.356
≥0.2	10	30	
LYMR			
<0.3	13	47	.059
≥0.3	12	54	
CD8+			
Low	15	36	.033
High	10	63	
TIL-NLR			
LOW	11	15	.014
MID	9	50	
HIG	5	34	
TLI-LYMR			
LOW	8	33	.960
MID	10	36	
HIGH	7	30	

LYMR = lymphocyte ratio, NLR = neutrophil–lymphocyte ratio, TIL = tumor-infiltrating lymphocyte.

contrast, although circumferential margin positivity was found to be risk for metastasis and survival in rectal cancer, CRM involvement did not predictive LR rate, due to the development of distant disease instead of local failure.[3,17] To our knowledge, this study is the first to demonstrate the LYMR, NLR, and CD8 + TILs are predictive of LR in rectal cancer. Also in our study, the new indicator is thought to be a simple and useful parameter to determine the inflammation response and host immune status of patients.

The tumor microenvironment may represent a mechanism for the presentation of tumor antigen to T cells, and many inflammatory cells are involved in the process of tumor development.[18–20] In addition, a recent study found that the infiltration of T cells in the tumor as part of the immune response, and the immune cell to predict cancer-special survival based on lymphocyte scattered in different areas of the tumor.[21,22] Infiltration by CD8 + T cells is strongly associated with favorable clinical outcome in CRC patients and has been shown to predict patient survival more efficiently than histopathological staging.[23] It revealed that intratumoral T cell infiltration has been determined to be an important predictor of outcome in rectal cancer. Immune cells have a crucial role in host antitumor immunity suppression and tumor cell migration and invasion. In our study, although a lower density of CD8 + TILs was observed in the LR group rather than in the nLR group, the difference was not statistically significant ($P = .215$). However, our results need to be further confirmed by a large number of sample sizes and uniform nCRT protocols study to provide a better conclusion.

Recent data[24–26] have expanded on the concept that the release of inflammatory cytokines and growth factors lead to intra-abdominal sepsis and sepsis induced immunosuppression could alter the activity of immune system against inflammation. The levels of inflammation cell, such as NLR and LYMR, is examined as a function of oncologic outcome of rectal cancer. Patients with a decreased LYMR and an elevated NLR has been suggested as predictors of poor tumor response in previous studies.[27] In our study, we found that the NLR and LYMR did not predict LR was observed in univariate analysis ($P = .836$ and $P = .918$, respectively). However, when the CD8 + TILs was combined with LYMR showed a positive association with LR ($P = .001$); here, we also showed that TIL–LYMR remained an independent factor in the multivariate analysis ($P = .030$). This finding suggests that the combination of the LYMR and CD8 + TILs might perform better than either alone in predicting the tumor response after rectal cancer resection. It was also previously believed that tumor cell migrates out of the tumor to present the antigen to T cells and release inflammatory cytokines and grow factors.

The presence of cancer cells at the suture-line may associate with LR. Therefore, it is reasonable to predict that the presence cancer cells inside the CRM may result in LR at the double-stapling anastomosis.[28] It has also been shown that anastomotic leakage is associated with a greater risk of LR.[29] When anastomotic leakage occurs, it may lead to intraluminal tumor cells through the leakage site. Moreover, Salvans et al[30] noted that an astomotic leakage may stimulate proliferation, migration, and invasion capacities of residual tumor cells. Here, we showed that the NLR combined with CD8 + TIL density was associated with anastomotic leakage in rectal cancer ($P = .014$).

There are some limitations to this study. First, the sample size of our study was small, these data are from a single institution and thus require validation. Therefore, our results need to be further confirmed by a large number of sample sizes and uniform nCRT protocols study better conclusion. Second, circulating lymphocytes and neutrophils can be affected by infectious or cardiovascular diseases.[31,32] Third, the define of LYMR, NLR may reflect the different approaches used when determining cutoff values. In sum, the inflammatory and immune response are of considerable importance in the relationship between the tumor and outcome in patients with cancer.

Author contributions

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Formal analysis: Shengchao Zhao.
Project administration: Bo Wu.
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