Original Article

Lymphocyte nadir predicts tumor response and survival in locally advanced rectal cancer after neoadjuvant chemoradiotherapy: Immunologic relevance

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ABSTRACT

Background and purpose: Neoadjuvant chemoradiation (nCRT) could reduce tumor infiltrating lymphocytes. We examined absolute lymphocyte count (ALC) nadir during nCRT, pathologic response and prognosis for locally advanced rectal cancer (LARC).

Materials and methods: 102 patients with LARC (cT3–4N0, or node-positive) treated between 2010 and 2015 with nCRT followed by complete resection were analyzed. The ALC value was obtained prior to, weekly during the treatment, and one month after nCRT. Associations of ALC nadir with immune cells' infiltrations, pathologic response and survival were analyzed.

Results: Twenty-four (23.5%) and 60 (58.9%) patients achieved pathologic complete response and partial response respectively. Response rate was higher in high ALC nadir group than low nadir group (89.7% *vs.* 67.6%, $p = 0.006$). Compared to low ALC nadir group, increased tumor infiltrates of CD4+ (4% *vs.* 17.5%, $p < 0.001$), CD8+ (8% *vs.* 30%, $p < 0.001$) T cells and CD68+ macrophages (6% *vs.* 25%, $p < 0.001$) were observed in high ALC nadir group. High ALC nadir [OR = 4.32 (95% CI, 1.22–15.26), $p = 0.023$] and well differentiation [OR = 10.53 (1.87–59.36), $p = 0.008$] were associated with pathologic response. Patients with high ALC nadir yielded better DFS [HR = 0.36 (0.16–0.81), $p = 0.010$] and OS [HR = 0.24 (0.08–0.69), $p = 0.004$].

Conclusions: Higher ALC nadir during nCRT is associated with a higher rate of pathologic response and better survival for LARC patients, suggesting that ALC may be a potential stratification strategy for LARC patients.

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The current standard management for clinical stage II and III rectal cancer (cT3–4N0, or node-positive) comprises neoadjuvant chemoradiotherapy (nCRT) followed by complete resection and adjuvant chemotherapy [1]. The tumor response to nCRT has been considered as an independent predictor of survival in patients with locally advanced rectal cancer (LARC) [2,3]. The involvement of the host immune response in malignant progression as well as related anti-cancer strategies have been extensively studied but yet to be elucidated [4,5]. Immune suppression is an unintended consequence of chemo- and radio-therapy. Interestingly, recent studies have revealed that therapy-induced lymphopenia were found to be associated with poor prognosis in a variety of cancer types [6–9]. In particular, lymphocyte count in peripheral blood was implicated as a promising parameter to determine clinical outcomes in advanced rectal cancer [10]. From radiobiological point of view, lymphocytes are known to be extremely radiosensitive, and there is a concern that radiotherapy-related lymphopenia may affect responses to immunotherapy [11].

The development of lymphopenia during CRT in rectal cancer patients was found to be poor pathologic response [12,13]. Nevertheless, whether lymphocyte nadir during nCRT is capable of predicting pathologic response and survival in rectal cancer remained unknown. Herein, we hypothesized that there would be a direct link between lymphocyte nadir and poor pathologic tumor response and survival in a cohort of rectal cancer patients with nCRT followed by complete resection. The predictive significance of lymphocytes nadir levels during nCRT was also investigated.

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Materials and methods

Patients

All patients with rectal cancer who underwent nCRT followed by rectal resection with curative intent at a tertiary hospital (Guangzhou, China) between 2010 and 2015 were identified in a prospectively maintained colorectal cancer database, as previously described [41]. Patients were included in this analysis, if they had: 1) clinical T3, T4 or N+ rectal cancer, 2) at least 3 weekly documented absolute lymphocyte count (ALC) during nCRT, pre-nCRT and post-nCRT. The study complied with the principles set forth in the Declaration of Helsinki. Written informed consent was obtained from all the patients in the study. The data collection protocol was approved by the appropriate institutional review board.

Hematoxylin-eosin staining and immunohistochemistry

Hematoxylin-eosin staining and immunohistochemistry were performed on resected samples. Immunohistochemistry was performed to evaluate the expression of Ki67 and infiltrates of T cells (CD4+, CD8+). Macrophages were stained using CD68 antibody. Ki67 is a nuclear protein and known as a good marker for cycling cell and tumor proliferation, because of its presence in all cell cycle phases except for the G0 and early G1 phase [42]. Formalin-fixed paraffin-embedded specimens from analyzed patients were cut in 4 μm serial sections and transferred to glass slides. The slides were deparaffinized with xylene, rehydrated with ethanol, washed and subjected to microwave retrieval in a citrate buffer. Sections were then immersed in 3% hydrogen peroxide to block endogenous peroxidase activity. Protein Block Serum Free (Dako, 60 min at room temperature) was used to reduce non-specific staining. Then, sections were further incubated with primary antibodies followed by incubation with peroxidase-conjugated anti-mouse secondary antibody (Ki67, 1:100, MIB-1, Dako; CD4, 1:400, ab133616, Abcam; CD8+, 1:100, ab4055, Abcam; CD68, 1:300, ab201340, Abcam). Expression was visualized using 1 mg/ml 3,3′-diaminobenzidine and counterstained with hematoxylin. Percentage positivity (continuous scale) in tumor area was scored in ten random fields and the mean percentage positivity was used to access the protein expression.

Scoring pathologic response

The seventh edition manual of the American Joint Committee on Cancer (AJCC) introduced a scoring system measuring nCRT response, ranging from 0 to 3, which has been shown to be superior to previously used systems [43]. In this analysis, pathologic responses to nCRT were scored using the criteria developed by the AJCC, defined as follows: 0 (complete response), no viable cancer cells; 1 (marked response), single or small groups of cancer cells; 2 (moderate response), residual cancer outgrown by fibrosis; and 3 (poor or no response), minimal or no tumor kill, extensive residual cancer, or tumor progress. Tumor regression grades of 0–2 were considered as pathologic response, and the remaining was taken as without pathologic response.
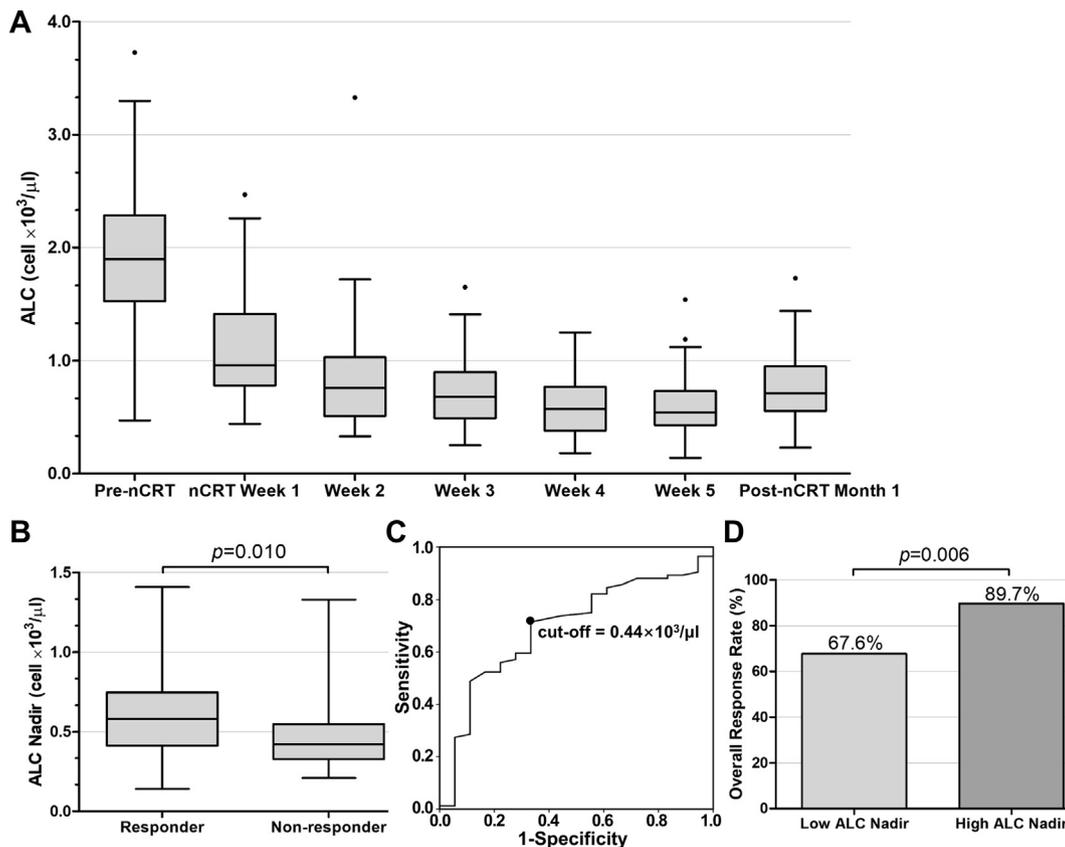
Fig. 1. (A) Distribution of ALC among analyzed patients weekly during neoadjuvant chemoradiotherapy. (B) ALC nadir for analyzed patients grouped by pathologic response. Mean Nadir of patients with pathologic response to nCRT was significantly higher than those without response. (C) Receiver operating characteristic (ROC) curve to evaluate the performance of ALC nadir for identifying patients with response to nCRT. An optimal ALC nadir cut-off ($0.44 \times 10^3/\mu l$) was determined based on a maximal Youden's Index to divide all patients into high ALC nadir and low ALC nadir groups. (D) Overall response rate for analyzed patients grouped by ALC nadir. Patients with high level of ALC nadir yielded a high overall response rate than those with low LAC nadir.

Statistical analysis

Demographic and clinicopathologic characteristics and oncologic outcomes were collected and analyzed. The ALC (cells $\times 10^3$/µl) was examined prior to, weekly during the treatment, or one month after nCRT, and the results were plotted to show the cell count trends from beginning to the end of neoadjuvant therapy. The ALC nadir is defined as the minimum cell count during the nCRT. The optimal cut-off of ALC nadir was determined based on a receiver operating characteristic (ROC) curve by evaluating the performance of ALC nadir to identify patient with response to nCRT (Fig. 1C). The cut-off (0.44×10^3/µl) of ALC nadir was determined as a threshold, based on a maximal Youden's Index (equal to Sensitivity + Specificity−1), to divide all patients into two groups and the corresponding overall response (i.e., tumor regression grade 0–2) rates were assessed. Accordingly, ALC nadir $\geq 0.44 \times 10^3$/µl was defined as "high" and $<0.44 \times 10^3$/µl as "low" ALC nadir. Data are presented as mean ± standard deviation for continuous variable (for those with non-normal distribution, median and range are shown) and as number (%) for categorical variables. Mann–Whitney's *U* test was used to compare ALC nadir and protein expression. Pearson's chi-square test was used for comparison of overall response rates by the ALC nadir value. The predictors of pathologic response were accessed by uni- and multivariate analyses using logistic regression. Variables were initially entered into the multivariable model based on statistical ($p < 0.10$) or clinical significance and an "Enter" method was used in the final model. Disease-free survival (DFS) and overall survival (OS) were estimated from the time of surgery using the Kaplan–Meier method and differences were assessed with the log-rank test. Cox regression model was used for survival-related factor analysis. All statistical analyses were performed using SPSS Statistics for Windows, Version 20.0 (IBM, Armonk, NY, USA). A 2-tailed *p* value <0.05 was considered statistically significant.

Results

Patient characteristics

Baseline characteristics of analyzed patients are listed in Table 1. A total of 102 consecutive patients met the inclusion criteria and were included for further analysis. The mean age at diagnosis was 52.3 years and 77.5% of the patients were male. The majority of the tumors was villous or tubular adenocarcinoma (86.3%) and was with well or moderately differentiation (87.3%). Sixty-six percent were located in the lower rectum. The clinical stage upon presentation was stage II in 34.3% and stage III in 65.7% of cases. After nCRT, 24 patients (23.5%) achieved pathologic complete response (score 0), whereas scores 1, 2, and 3 were assigned to 39 (38.3%), 21 (20.6%), and 18 (17.6%) patients, respectively. Most patients received an R0 resection (96.1%). After operation, 87.3% received adjuvant chemotherapy. The median follow-up was 31 months (range 1–88 months).

*Lymphocyte counts during nCRT and pathologic response*

Variation of ALC before, during and after nCRT is shown in Fig. 1A. During nCRT, the ALC in peripheral blood decreased weekly and generally maintained a steady level by the end of nCRT. The median baseline pre-nCRT ALC was 1.90×10^3/µl, then declined to 0.96, 0.76, 0.68, 0.57 and 0.54×10^3/µl from the first week onwards. On the first month after nCRT, a recovery trend of ALC was observed (median 0.71×10^3/µl). The baseline ALC before nCRT was similar for all patients (mean ALC, responder 2.02×10^3/µl *vs.* non-responder 1.81×10^3/µl, $p = 0.226$), however, the ALC nadir during nCRT was significantly higher in patients with

pathologic response to nCRT, compared with those without response (mean nadir, responder 0.60×10^3/µl *vs.* non-responder 0.47×10^3/µl, $p = 0.010$) (Fig. 1B). ROC analysis was performed to determine an optimal cut-off of ALC nadir to classify patients based on their responses to nCRT (Fig. 1C). Patients were further divided into two group based on their ALC nadirs, by a threshold of 0.44×10^3/µl. A higher response rate of 89.7% was also observed in the group with high ALC nadir level, compared with a rate of 67.6% in those with low ALC nadir ($p = 0.006$) (Fig. 1D). For MRI scans, remarkable tumor regression was also observed in patients with high ALC nadir level, compared with those with low one based on individual qualitative observation by clinician (Fig. 2C). Intriguingly, tumors resected from patients maintaining higher ALC nadir during nCRT showed relatively lower Ki67 expression with less proliferation capacity (median, high vs. low: 39.5% *vs.* 75%, $p = 0.002$) (Fig. 2A and B).

Table 1
Baseline characteristics in LARC with nCRT.

Characteristic	No. of patients ($n = 102$)
Mean age at diagnosis, yrs. (SD)	52.3 (12.7)
Gender	
Male	79 (77.5%)
Female	23 (22.5%)
Comorbidity	
CAD, *n* (%)	18 (13.7%)
DM, *n* (%)	4 (3.9%)
Current smoker, *n* (%)	27 (26.5%)
Tumor size (cm), mean (SD)	2.9 (1.8)
Tumor location	
Upper/mid	35 (34.3%)
Lower	67 (65.7%)
Clinical TNM stage	
II	35 (34.3%)
III	67 (65.7%)
Clinical T stage	
T2	5 (4.9%)
T3	31 (30.4%)
T4	66 (64.7%)
Clinical N stage	
N0	35 (34.3%)
N1	46 (45.1%)
N2	21 (20.6%)
Tumor histology	
Villous/tubular adenocarcinoma	88 (86.3%)
Mucinous adenocarcinoma	14 (13.7%)
Tumor differentiation	
Well-moderate	89 (87.3%)
Poor	13 (12.7%)
Radiation dose (Gy), mean (SD)	48.0 (5.0)
Concurrent chemo regimen	
Xelox	16 (15.7%)
Folfox	28 (27.5%)
Xeloda	58 (56.9%)
Tumor regression grade	
0 (complete response)	24 (23.5%)
1 (marked response)	39 (38.3%)
2 (moderate response)	21 (20.6%)
3 (no or poor response)	18 (17.6%)
R1 margin	4 (3.9%)
Adjuvant chemo	
Yes	89 (87.3%)
No	13 (12.7%)
Follow-up duration (month), median (range)	31 (1–88)

Abbreviations: SD = standard deviation, CAD = coronary artery disease, DM = diabetes mellitus.
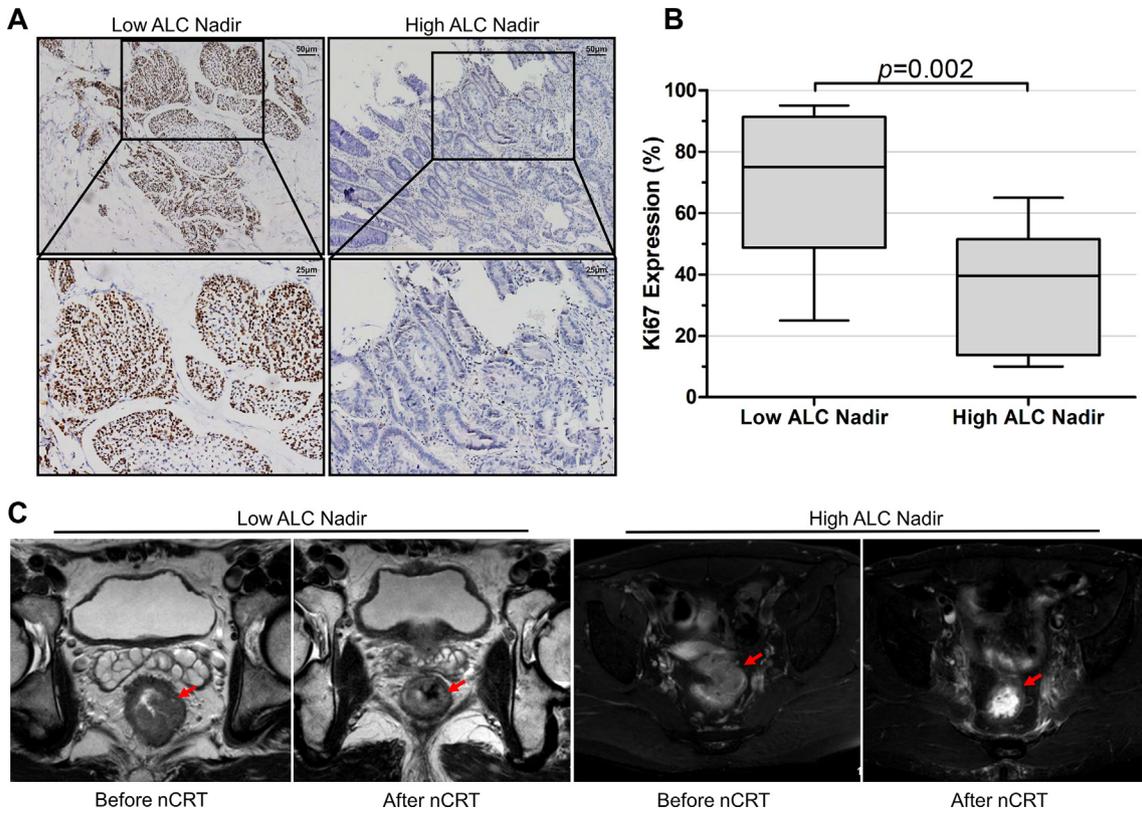
Fig. 2. (A) Representative micrographs of immunostaining for Ki-67 expression in samples after therapy. (B) Boxplot for Ki-67 expression in patients with low ALC nadir [75% (25–95%)] and with high ALC nadir [39.5% (10–65%)], *p* = 0.002. (C) Representative MRI scans of tumor before and after neoadjuvant chemoradiotherapy.

Lymphocyte counts during nCRT and immune infiltrates

The infiltration of immune cells was further examined for human specimen. Increased CD4+ T helper cells' infiltration in tumor was observed from patients with high ALC nadir level during nCRT, compared with those with low ALC nadir (low vs. high: 4% *vs.* 17.5%, *p* < 0.001). Similar results were found for infiltrates of CD8+ cyto-toxic T cells (low vs. high: 8% *vs.* 27.5%, *p* < 0.001). In parallel, enhanced infiltrates of CD68+ microphages were also found in tumors from those maintaining high ALC nadir (low *vs.* high: 6% *vs.* 25%, *p* < 0.001). In aspect of histological differentiation, tumors from patients with high ALC nadir level tended to be with well differenti-ation and those with low ALC nadir differentiated worse (Fig. 3).
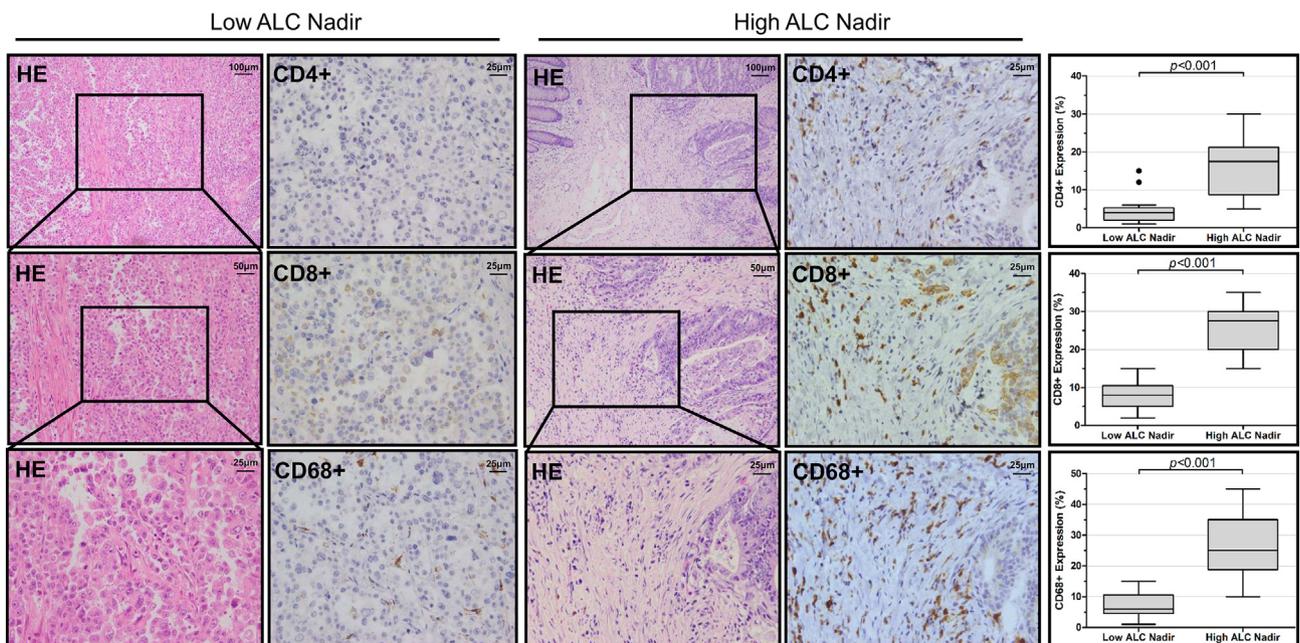
Fig. 3. Representative images of HE and immunohistochemical stainings for resected tumor specimen between low- and high-ALC nadir. Subpopulation of infiltrated immune cells including CD4+ T-cells, CD8+ T-cells and CD68+ macrophages were analyzed.

Predictors of tumor regression

The association of clinicopathologic and treatment characteristics with pathologic response to nCRT shows in Table 2. In univariate analysis, a high level of ALC nadir (Odd ratio, OR = 4.17, 95% CI 1.44–12.05, *p* = 0.008), tumor size < 3 cm (OR = 3.27, 95% CI 1.11–9.62, *p* = 0.032) and well or moderate tumor differentiation (OR = 5.50, 95% CI 1.58–19.17, *p* = 0.007) were significant factors associated with improved tumor regression. In multivariate analysis, a high level of ALC nadir and well or moderate tumor differentiation were identified as independent favorable factors for pathologic response (OR = 4.32, 95% CI 1.22–15.26, *p* = 0.023; OR = 10.53, 95% CI 1.87–59.36, *p* = 0.008, respectively). Age, gender, comorbidity, smoking history, clinical T and N stage, histology type, tumor location and radiation dose were not significantly associated with tumor regression.

High ALC nadir is associated with improved survival in responders

During follow-up, there were a total of 24 patients' occurring events with a median DFS time of 64.2 (95% CI 57.0–71.4) months.
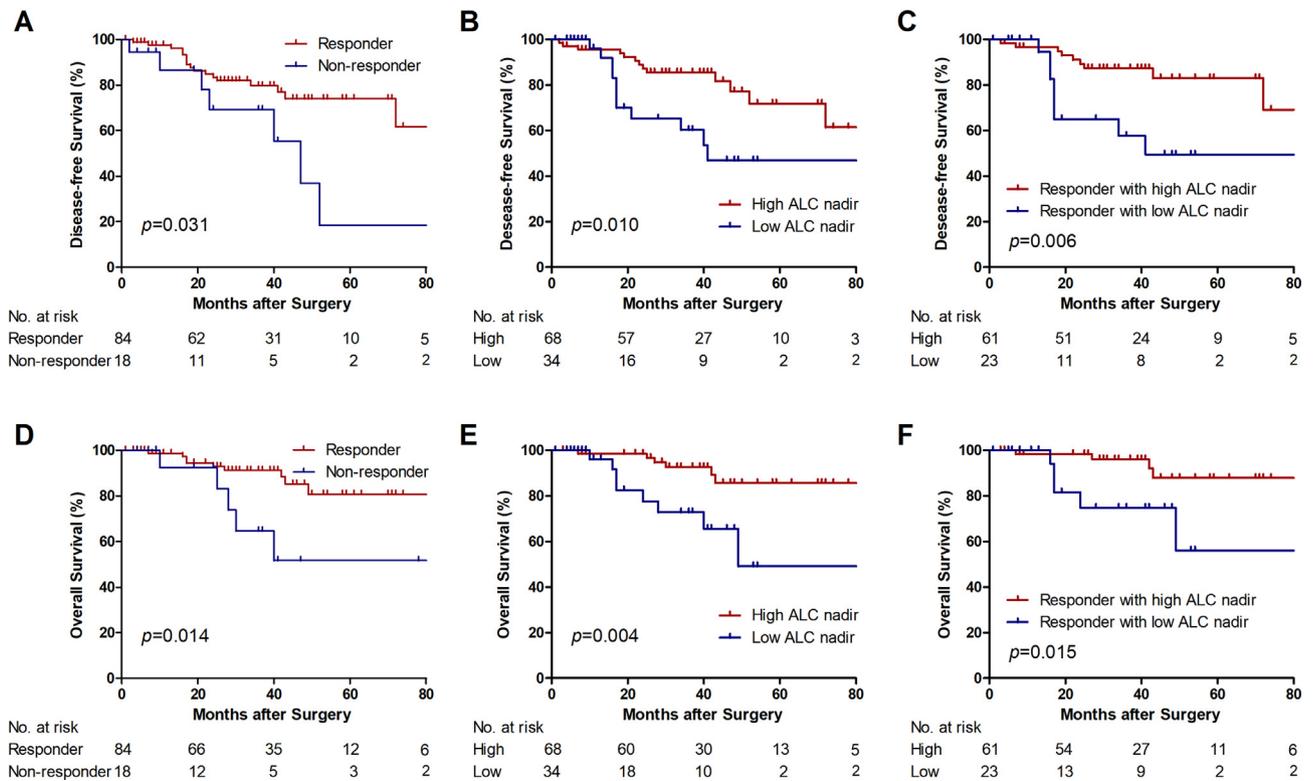
Patients with pathologic response yield a better DFS than those non-responders (Hazard ratio, HR = 0.39, 95% CI 0.16–0.95, *p* = 0.031); the median DFS time of pathologic non-responders was 47.0 (95% CI 33.2–60.79) months and was not reached in response group (Fig. 4A). The mean OS time in response and non-response groups was 75.8 (69.6–82.1) and 59.1 (40.2–77.9) months respectively, with a significant difference (HR = 0.28, 95% CI 0.09–0.83, *p* = 0.014) (Fig. 4D). Patients with high level of ALC nadir during nCRT yielded a better DFS than those with low ALC nadir; the mean DFS time was 69.2 (61.2–77.2) and 41.3 (32.7–49.8) months, respectively (HR = 0.36, 95% CI 0.16–0.81, *p* = 0.010) (Fig. 4B). Similar to DFS, the mean OS time was 80.1 (74.0–86.1) and 46.1 (37.9–54.2) months, respectively (HR = 0.24, 95% CI 0.08–0.69, *p* = 0.004) (Fig. 4E). Notably, among responders, patients with high level of ALC nadir during nCRT had a longer mean DFS time of 73.5 (95% CI 66.0–80.9) months versus a mean of 41.6 (31.7–51.6) months for those with low level of ALC nadir (HR= 0.27, 95% CI 0.10–0.73, *p* = 0.006) (Fig. 4C), and also yielded a better OS than those with low ALC nadir (HR = 0.22, 95% CI 0.06–0.84, *p* = 0.015) (Fig. 4F).

Table 2
Uni- and multivariate analyses of factors associated with pathologic response.

Characteristic	Univariate			Multivariate		
	OR	95% CI	*p*	OR	95% CI	*p*
ALC nadir						
Low	1			1		
High	4.17	1.44–12.05	0.008	4.32	1.22–15.26	0.023
Age ≥ 65 yrs.						
Yes	1					
No	0.22	0.03–1.73	0.149			
Gender						
Female	1					
Male	0.38	0.08–1.77	0.215			
Comorbidity						
Yes	1					
No	0.92	0.24–3.58	0.904			
Current smoker						
Yes	1					
No	1.08	0.35–3.39	0.890			
Tumor size ≥ 3 cm						
Yes	1			1		
No	3.27	1.11–9.62	0.032	3.34	0.98–11.39	0.054
Tumor location						
Upper/mid	1					
Lower	1.27	0.45–3.64	0.653			
Clinical T stage						
T2	1			1		
T3	1.69	0.15–19.2	0.673	2.00	0.09–46.72	0.667
T4	1.02	0.11–9.90	0.987	0.87	0.04–17.86	0.927
Clinical N stage						
N0	1			1		
N1	1.39	0.44–4.42	0.574	1.64	0.39–6.80	0.498
N2	1.06	0.27–4.17	0.931	2.07	0.37–11.44	0.406
Histology						
Villous/tubular adenocarcinoma	1					
Mucinous adenocarcinoma	0.75	0.19–3.03	0.690			
Tumor differentiation						
Poor	1			1		
Well-moderate	5.50	1.58–19.17	0.007	10.53	1.87–59.36	0.008
RT dose > 50 Gy						
No	1					
Yes	0.20	0.01–3.42	0.268			

Abbreviations: OR = odds ratio, CI = confidence interval, ALC = absolute lymphocyte count, RT = radiotherapy.

Fig. 4. Kaplan–Meier's estimates of disease-free and overall survival for analyzed patients stratified by pathologic response (A and D) and the level of ALC nadir (B and E), and for patients with pathologic response by the level of ALC nadir (C and F).

Discussion

Pathologic complete response (pCR) after neoadjuvant therapy in LARC is an independent predictor for overall survival and decreased local recurrence [14]. Patients with LARC obtained a complete clinical response after nCRT may be managed by a "watchful waiting" nonsurgical strategy [15]. Radiotherapy is considered to have potential immunomodulatory properties in additional to tumor cell killing effect [16,17]. Lymphocytes are known to be exquisitely sensitive to ionizing radiation, and the direct effect of radiation treatment on tumor infiltrating lymphocytes is generally cytocidal [18]. Cytotoxic T lymphocytes are general known as the active role of adaptive cellular immunity against tumor cells [19]. In line with this, an increased infiltration level of T cells (CD4+, CD8+) and macrophages (CD68+) were observed in tumors with high ALC nadir by immunohistochemistry, in comparison with low ALC nadir group. A number of reports have indicated that tumor infiltrating lymphocyte predict response to neoadjuvant chemotherapy in rectal cancer [20]. Furthermore, several studies have also demonstrated that the ALC level during nCRT is associated with tumor response in rectal cancer [5,13,21]. Heo et al. concluded that maintaining a high ALC of $>0.35 \times 10^3/\mu l$ during the fourth week of CRT could yield a better pathologic response in rectal cancer [13]. In a similar study by Kitayama et al., sufficient circulating lymphocytes as well as small tumor size and localized circumferential extent were identified as favorable factors for pCR in rectal cancer [21]. Also, Choi et al. claimed that pretreatment baseline level of circulating lymphocytes was associated pathologic response in rectal cancer [5]. The definition of the threshold of ALC varied across these studies.

Whether the ALC nadir during neoadjuvant treatment predicts pathological response and survival in rectal cancer was unclear. In this context, we identified a 23.5% of pCR rate, in agreement with most of previous reports ranging from 12% to 38% [14,22–

25]. Further analysis demonstrated that the lymphocyte nadir during nCRT decreased dramatically and a high level of lymphocyte nadir during nCRT is associated with improved pathological response and better survival in LARC. Multivariate analysis showed high ALC nadir was significantly associated with OS (HR = 0.24, 95% CI 0.08–0.69, $p = 0.008$) and DFS (HR = 0.41, 95%CI: 0.18–0.94, $p = 0.036$), indicating ALC nadir during nCRT was a robust, independent predictor of survival (see Supplementary file Table S1). Furthermore, we also found that in LARC, a higher level of ALC nadir and well-moderate differentiation were associated with pathologic response, but the ALC nadir threshold of $0.44 \times 10^3/\mu l$ defined in our study was inconsistent with that found previously. Our finding that poor tumor differentiation was associated with unresponsiveness to nCRT was consistent with previous reports [26–28].

Regarding the immunologic balance, it has been known that chemotherapy, radiation therapy and chemoradiotherapy develop a pro-inflammatory tumor microenvironment, allowing the activation of tumor specific adaptive immunity [16,29]. In addition to the effect of radiation therapy as an immune adjuvant [30–32], recent preliminary results supported combining PD-1/PD-L1 inhibitors with chemotherapy or chemoradiotherapy [33,34]. A higher level of ALC nadir was associated with improved survival in responders with LARC after nCRT in our study, suggesting the potential for a combined strategy of cytotoxic treatment and immune checkpoint inhibitors.

Radiation dose is one of the most critical factors in tumor local control. A number of studies have shown the impact of radiation dose dependent pCR rate to neoadjuvant therapy [35]. It suggested that the patients who received 40 Gy, 46 Gy, or 50 Gy in 2 Gy/fraction with continuous infusion 5-FU, the rates of pCR was 18%, 23%, and 33% respectively at the three dose levels [36]. One-hundred and three patients with advanced distal rectal cancer were randomized to continuous venous infusion 5-FU plus hyperfractionated radiation treatment of 55.2 to 60 Gy (1.2 Gy twice a

day) versus continuous venous infusion 5-FU and irinotecan with conventional fractionation radiation of 50 to 54 Gy (1.8 Gy per fraction). The response rate between the two arms was similar with a pCR rate of 28%, higher than in other studies [37]. In our study, the prescribed radiotherapy dose was 48.0 ± 5.0 Gy (2 Gy per fraction) and was adopted in LARC patients (clinical T3, T4, or N positive by MRI and EUS), the rate of pCR was 23.5%, noninferior to above mentioned reports.

It is also of importance to note the different prognostic ability of nCRT response between ypII and ypIII tumors. Karagkounis et al. found that patients with poor response to nCRT and pathologic stage III rectal cancer are at high risk for adverse oncological outcomes [38]. We also found a significant prognostic value of responder to nCRT in LARC, in agreement with previous reports [39,40], but no significant difference between ypII and ypIII stages.

Our study has several limitations. First, its retrospective design has the weakness of being observational or non-experimental in nature. For example, variables such as infections or medications that could potentially influence circulating lymphocyte count failed to be fully considered. Second, the potential variability of ALC value could not be well studied in present study, despite using a relative stable parameter–ALC nadir level. Third, because of lack of a standard tool to quantify tumor regression by MRI scans at clinics, it is hard to perform statistical comparisons of tumor regression confirmed by MRI scans after nCRT between high and low ALC nadir groups. Lastly, although we identified a threshold of ALC nadir for prediction of pathologic response and survival, the optimal threshold needs to be validated on a well-designed multicenter trial.

In conclusion, our study revealed a strong link between radiation-induced lymphopenia, pathological response and survival. Several factors such as the tumor differentiation status, immune cells infiltration level could have potential impact on pathological response following nCRT. Our findings add to the evidence supporting the importance of an intact immune system for optimal oncologic outcomes. This has important potential implications for potential therapeutic strategies that lessen the impact of standard therapies on the ALC or that enhance host immunity.

Conflict of interest statement

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.radonc.2018.12.001.

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