

Original Article

Tumor infiltrating lymphocytes (TILs) before and after neoadjuvant chemoradiotherapy and its clinical utility for rectal cancer

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Abstract: Backgrounds: Radiotherapy (RT) and chemotherapy (CT) can potentiate systemic antitumor immune effect. However, immunomodulation during RT or CT and their clinical implications in rectal cancer have not been thoroughly investigated. Methods: We investigated alterations in the densities of tumor infiltrating lymphocytes (TILs) during chemoradiation and their clinical utilities in patients with rectal cancer. We analyzed 136 rectal cancer patients who underwent neoadjuvant RT, CT or chemoradiotherapy (CRT), followed by radical resection retrospectively. Pretreatment biopsy specimens and posttreatment resected specimens of all patients were immunostained for CD3 and CD8. The predictive value of TILs to neoadjuvant treatment and prognosis were examined. Results: Densities of CD3+ and CD8+TILs in posttreatment specimens after RT, CT or CRT were all significantly higher than those in pretreatment specimens. There were no significant differences between each two of these three groups. High pretreatment CD3+ and CD8+TILs were associated with good response (TRG \geq 3) after neoadjuvant treatments (P = 0.033 and 0.021). High CD3+TILs and CD8+TILs in pretreatment biopsy specimens were significantly associated with favorable disease free survival (DFS) (P = 0.010 and P = 0.022) and overall survival (OS) (P = 0.019 and P = 0.003). Conclusions: We may, thus, conclude that chemoradiation can enhance local immune response by increased TILs. High TILs densities before treatment are associated with good response to neoadjuvant chemoradiotherapy and a favorable prognosis.

Keywords: Rectal cancer, lymphocytes, inflammation, chemoradiotherapy, prognosis

Introduction

Rectal cancer is one of the most common causes of cancer death in the world [1]. Radiation therapy (RT) and fluoropyrimidine based chemotherapy (CT), before or after surgery, reduce local recurrence risk and increase sphincter preservation rate of rectal cancer [2]. Nowadays, neoadjuvant chemoradiotherapy (nCRT) combined with surgery has been a standard treatment strategy for locally advanced rectal cancer (LARC) [3]. Pathological complete response (pCR) after nCRT has been considered a strong indicator for chemoradiosensitivity and survival. However, response to nCRT ranges from none to complete [4]. Given the wide spectrum of tumor response, it is critical to find a new tool to predict the response of rectal cancer to nCRT, as well as a better surrogate to select nonresponder for an early surgery [5].

Tumor infiltrating lymphocytes (TILs) are frequently found in tumors, which may suggest that tumors trigger an immune response [6]. Many studies have reported that high abundance of TILs is associated with a favorable clinical outcome [7-9]. CD8+ cytotoxic T-lymphocytes, especially, have been found to indicate better survival in many kinds of cancer, including non-small cell lung cancer (NSCLC), colonic, esophageal, as well as urothelial cancers and melanoma [10-13]. Galon et al. [14] have established an immunoscore system that is a more valuable prognostic tool than the histopathological methods currently being used in the treatment of colorectal cancer. Chemotherapy and radiotherapy have previously been considered immunosuppressants, because lymphocytes are very sensitive to the effects of radiation and cytotoxic agents targeting tumors [15]. However, recently, more and more data

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suggest that RT and CT can potentiate systemic antitumor immune effects by causing immunogenic tumor cell death and inducing T cell response [16]. The particular mechanisms have not yet been elucidated. A number of studies have indicated an association between the presence of extensive lymphocytic infiltration and high response rates to RT or CT [17]. For rectal cancer, it has been found that density of TILs in biopsy samples before treatment can be a predictor of tumor response to CRT [18]. However, differences of immunomodulation during RT or CT and their clinical implications in rectal cancer have not been thoroughly investigated.

In this study, we examined the densities of two TIL subtypes, CD3+, a general T-lymphocyte marker, and CD8+, so far the most popular lymphocyte marker for prognosis [6] before and after CRT in rectal cancer patients to determine their clinical relevance. To clarify the respective effect of RT and CT to TILs, we investigated alterations in the densities of these TIL subsets during RT, CT or CRT and their predictive values on tumor regressions and prognosis by using pretreatment biopsy samples and posttreatment resected tissues for 136 patients with neoadjuvant treatments.

Materials and methods

Patients

Since January 2000, 136 patients in Shandong cancer hospital and institute who received neoadjuvant RT, CT or CRT for locally advanced (radiological T3-T4 or N+) rectal cancer were included in this study. All patients had no evidence of distant metastases and had not received any CRT previously. The study was approved by the scientific review and ethics committee of Shandong Cancer Hospital and Institute. All patients signed informed consents.

All patients received neoadjuvant RT, CT or CRT followed by surgery. Some patients didn't receive standard neoadjuvant concomitant CRT for local advanced rectal cancer. In our study, to clarify the respect effects of radiation and chemotherapy on TILs, we analyzed some patients with neoadjuvant RT or CT retrospectively. These patients were not able to tolerate

standard nCRT because of poor performance or refusal to receive combinatory treatment. Briefly, most patients received 40-45 Gy in 25-28 fractions to the pelvis with/or without a boost of 5.4 Gy to the primary gross tumor using three-dimensional conformal irradiation or a 4-field box technique over 5 weeks. Chemotherapy was administered intravenously alone or concomitant with RT, which was single-agent or two-agents based on fluoropyrimidine. The mean interval between nCRT and surgery was 45.1 ± 5.4 days. The median follow-up period was 57 months. Two survival end-points were evaluated: disease free survival (DFS), calculated from the initiation date of treatment to the date of any evidence of local or systemic cancer recurrence; and overall survival (OS), calculated from the initiation date of treatment to the date of death or censored date of last contact.

Histopathology and tumor regression grading

Histopathological examination was performed by two independent pathologists. Tumor response was evaluated using the tumor regression grade (TRG) system established by Dworak et al. [19] as follows: Grade 0: no regression; Grade 1: minor regression, residual tumor outgrowing fibrosis; Grade 2: moderate regression, few tumor cell fibrosis in 26% to 50% of the tumor mass; Grade 3: major regression, very few tumor cells in fibrotic tissue with or without mucous substance; Grade 4: total regression, no tumor cells, only fibrotic mass. In our present study, TRG 3-4 were defined as "good response" and TRG 0-2 were defined as "poor response" [5].

Immunohistochemistry

Pretreatment biopsy specimens and posttreatment surgically resected specimens of all patients were retrieved to perform the immunohistochemistry. All 4- μ m thin sections were deparaffinized, rehydrated and then exposed to antigen retrieval system, which was performed under high pressure for 2 minutes. The sections were stained with primary specific CD3 antibody (clone ZM-0417, ready-to-use; Beijing Zhongshan Golden Bridge Biotechnology Company, Beijing, China) and specific CD8 antibody (clone ZA-0508, ready-to-use; Beijing Zhongshan Golden Bridge Biotechnology Com-

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Table 1. Clinicopathological characteristics of 136 patients with rectal cancer

Clinicopathological parameters	Cases (%)
Age	
< 65	64 (47)
≥ 65	72 (53)
Sex	
Male	75 (55)
Female	61 (45)
Histology	
Differentiated	78 (57)
Undifferentiated	58 (43)
White cell count	
< 4 (×10 ⁹ /L)	41 (30)
4-8 (×10 ⁹ /L)	58 (42)
> 8 (×10 ⁹ /L)	37 (28)
cT	
3	75 (55)
4	61 (45)
cN	
Negative	44 (32)
Positive	92 (68)
Neoadjuvant treatment	
Radiotherapy	40 (30)
Chemotherapy	33 (24)
Chemoradiotherapy	63 (46)
TGR	
Good response	64 (47)
Poor response	72 (53)

pany, Beijing, China) in humidified chamber at 37°C for 60 minutes. Reactive sites were identified by exposure to a secondary goat anti-rabbit antibody (Beijing Zhongshan Golden Bridge Biotechnology Company, Beijing, China) incubated at 37°C for 15 minutes. CD3 and CD8 staining was then visualized by using diaminobenzidine (DAB) and then counterstained in hematoxylin. Finally, sections were dehydrated and mounted.

Evaluations of CD3+TILs and CD8+TILs were carried out by two independent pathologists who were blinded to clinicopathologic information. The densities of CD3+TILs or CD8+TILs were defined as the percentage of tumor stroma containing positive CD3+T lymphocytes or CD8+ T lymphocytes in a manner similar to that of a previous publication [20]. To sub-divide the patients and analyze the predictive effects of TILs, densities of CD3+TILs or CD8+TILs were

graded as either high or low according to the mean values for the analyses presented [20].

Statistical analysis

The associations of TILs with clinicopathologic parameters (age, sex, cT, cN and differentiation), including the TRG, were assessed using the chi-squared test. Comparisons of TILs between pretreatment biopsy specimens and posttreatment resected specimens were performed by unpaired t-tests. Patient survival curves were generated by the Kaplan-Meier method and compared by log-rank tests. All the statistical tests were two sided, and considered significant when $P \leq 0.05$. Statistical analyses were performed using SPSS_ version 19.0 (IBM SPSS, Chicago, Illinois, USA).

Results

Patient characteristics

A total of 136 patients who underwent neoadjuvant RT (30%), CT (24%) or CRT (46%) and potentially curative resection of rectal cancer were included. There were 75 male and 61 female with a median age of 62 years old. Among the 136 patients, 75 were assessed as clinical T3 (cT3) and 61 as clinical T4 (cT4). Clinical lymph node negative (cLN-) and positive (cLN+) were identified in 44 and 92 patients, respectively. Additionally, 78 patients had differentiated histology and 58 patients had undifferentiated histology. According to the TRG system, there were 64 (47%) patients with good response and 72 (53%) patients with poor response (**Table 1**).

Relationships between TILs in pretreatment biopsy specimens and clinical-pathological characteristics

As shown in **Figure 1**, CD3 and CD8 were clearly stained in the cell membrane of interstitial infiltrates. The average value of CD3+TILs and CD8+TILs in 136 pretreatment biopsy specimens was 30% and 15%, respectively. The CD8+TILs in specimens with cN-tumors were significantly more than those in specimens with cN+ patients (68.2% vs. 48.9%, $P = 0.035$). Patients with high CD3+TILs seemed to have better N stage; however, the associations were not statistically significant. CD3+ and CD8+TILs

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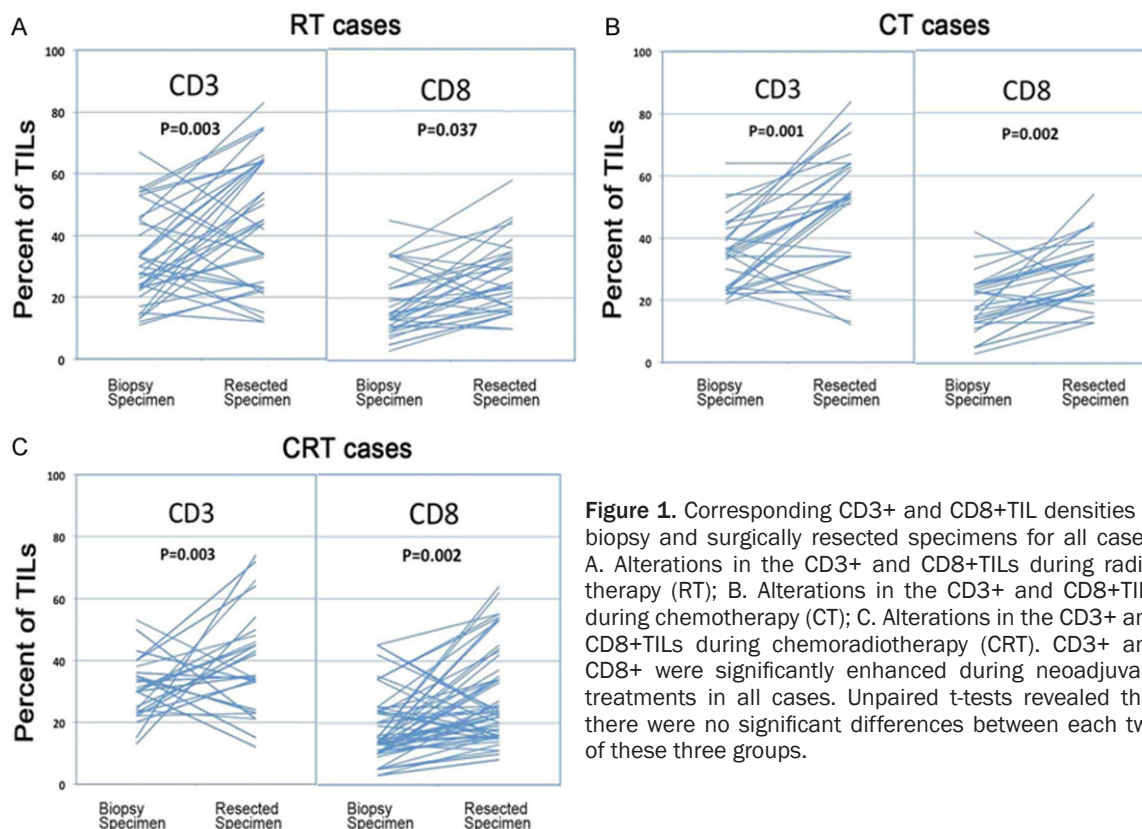


Figure 1. Corresponding CD3+ and CD8+TIL densities in biopsy and surgically resected specimens for all cases. A. Alterations in the CD3+ and CD8+TILs during radiotherapy (RT); B. Alterations in the CD3+ and CD8+TILs during chemotherapy (CT); C. Alterations in the CD3+ and CD8+TILs during chemoradiotherapy (CRT). CD3+ and CD8+ were significantly enhanced during neoadjuvant treatments in all cases. Unpaired t-tests revealed that there were no significant differences between each two of these three groups.

Table 2. Relationships of TILs in pretreatment biopsy specimens and clinicopathological characteristics in patients with rectal cancer

Clinicopathological parameters	CD3		P value	CD8		P value
	High	Low		High	Low	
Age						
<65	37	27	0.453	36	28	0.807
≥ 65	37	35		39	33	
Sex						
Male	42	33	0.680	46	29	0.108
Female	32	29		29	32	
Histology						
Differentiated	43	35	0.846	38	40	0.966
Undifferentiated	31	27		30	28	
White cell count						
<4 (×10 ⁹ /L)	25	16	0.541	24	17	0.419
4-8 (×10 ⁹ /L)	31	27		34	24	
> 8 (×10 ⁹ /L)	18	19		17	20	
cT						
3	44	31	0.269	46	29	0.108
4	30	31		29	32	
cN						
Negative	29	15	0.063	30	14	0.035*
Positive	45	47		45	47	

*Statistically significant.

expressions were not correlated with age, sex, histology, white blood cells (WBCs) or T stage (**Table 2**).

Associations of TILs between biopsy and resected specimens

After neoadjuvant RT, CT or CRT, densities of CD3+ and CD8+ T lymphocytes were all significantly higher than those before treatment (RT cases: (average CD3+: 33% vs. 45% P = 0.003; CD8+: average 21% vs. 27% P = 0.037); CT cases: (CD3+: average 34% vs. 48% P = 0.001; CD8+: average 19% vs. 28% P = 0.002); CRT cases: (CD3+: average 31% vs. 39% P = 0.003; CD8+: average 18% vs. 25% P = 0.002). In **Figure 2A-C**, most of the lines showed an upslope. To show the differences among RT, CT and CRT cases, the increases of CD3+

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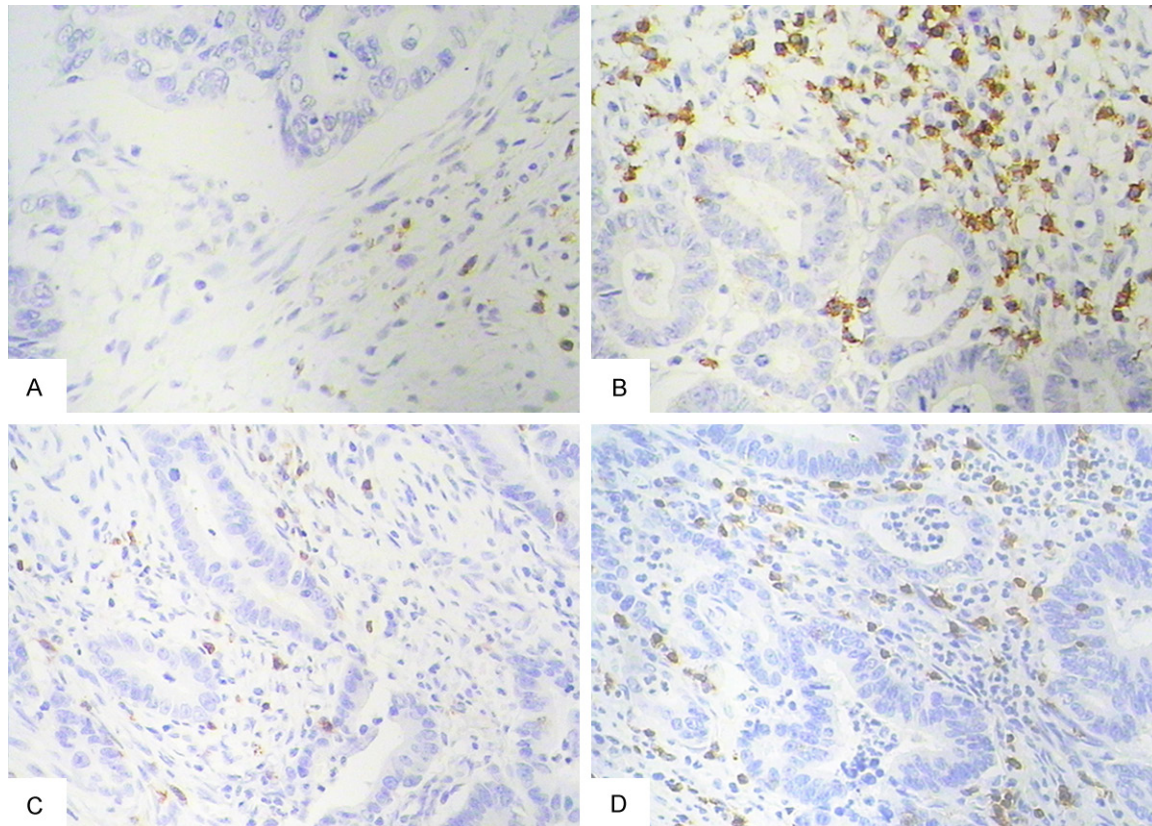


Figure 2. Chemoradiotherapy (CRT)-induced alterations in the microscopic appearance of rectal cancer tissues stained for CD3+ and CD8+ from the same patient. A. CD3+TILs in a pretreatment biopsy specimen; B. Enhanced CD3+TILs in a post-CRT resected specimen; C. CD8+TILs in a pretreatment biopsy specimen; D. Enhanced CD8+TILs in a post-CRT resected specimen (magnification for panels a and b: $\times 200$).

Table 3. Correlations between clinicopathological parameters and tumor response to neoadjuvant treatment

Clinicopathological Parameters	Good response
High vs. Low CD3+TILs	55.4% vs. 37.1%; P = 0.033*
High vs. Low CD8+TILs	56% vs. 36.1%; P = 0.021*
Age < 65 vs. age \geq 65	45.3% vs. 48.6%; P = 0.700
Male vs. Female gender	46.6% vs. 47.5%; P = 0.919
Differentiated vs. Undifferentiated history	53.4% vs. 42.3%; P = 0.198
cT3 vs. cT4 stage	54.6% vs. 37.7%; P = 0.049*
Node negative vs. Node positive	65.9% vs. 38.0%; P = 0.002*

*Statistically significant.

and CD8+TILs in biopsy and resected specimens were compared. Unpaired t-tests revealed that there were no significant differences between each two of these three groups (RT vs. CT vs. CRT: 6.6% vs. 8.6% vs. 8.4% for CD3; 11.0% vs. 6.7% vs. 9.2% for CD8; all $P > 0.05$), which suggested that there were no differences in the abilities to enhance TILs by RT, CT or

CRT in rectal cancer between the groups. Representative microscopic appearances are shown in **Figure 1**.

Predictive values to neoadjuvant treatment of TILs

As shown in **Table 3**, patients who had tumors with cT3 and cN- were more likely to achieve good tumor regression ($P = 0.049$ and 0.002 , respectively).

Statistical analyses revealed that pretreatment with CD3+TILs and CD8+TILs were associated with good response ($TRG \geq 3$) after neoadjuvant treatment. There were 55.4% patients with high pretreatment CD3+TILs and 37.1% patients with low pretreatment CD3+TILs who achieved good responses ($P = 0.033$). Patients with high CD8+TILs had better responses to neoadjuvant treatment than

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Table 4. Correlations between clinicopathological parameters and survival in patients with rectal cancer

Parameters	5-year DFS (%)	P value	5-year OS (%)	P value
Age				
<65	59%	0.149	71%	0.238
≥ 65	43%		62%	
Sex				
Male	56%	0.160	70%	0.302
Female	44%		62%	
Histology				
Differentiated	51%	0.711	69%	0.622
Undifferentiated	50%		65%	
White cell count				
<4 (×10 ⁹ /L)	43%	0.390	58%	0.323
4-8 (×10 ⁹ /L)	51%		72%	
> 8 (×10 ⁹ /L)	56%		67%	
CT				
3	56%	0.033*	76%	0.008*
4	44%		55%	
CN				
Negative	65%	0.012*	81%	0.014*
Positive	43%		59%	
Neo-adjuvant therapy				
Chemotherapy alone	36%	0.023*	70%	0.128
Radiotherapy alone	45%		48%	
Chemo-radiotherapy	61%		74%	
TRG				
Good response	64%	0.001*	81%	0.001*
Poor response	38%		54%	
CD3+TILs				
High	59%	0.010*	75%	0.019*
Low	40%		56%	
CD8+				
High	61%	0.002*	77%	0.003*
Low	37%		54%	

*Statistically significant.

patients with low CD8+TILs (56% vs. 36.1%; P = 0.021).

Prognostic values of TILs

The median follow-up period for the survivors in the study was 57 months (range 23-111). The prognostic values of host characteristics, including TILs, are shown in **Table 4**. High CD3+TILs and CD8+TILs in pretreatment biopsy specimens were significantly associated with better prognosis. The 5y-DFS of patients with high pretreatment CD3+TILs was significantly higher

than that of patients with low CD3+TILs (59% vs. 40%, P = 0.010). Patients with high pretreatment CD3+TILs had significantly better 5y-OS than patients with low CD3+TILs (75% vs. 56%, P = 0.019) (**Figure 3A**). The 5y-DFS and 5y-OS of patients with high pretreatment CD8+TILs were significantly longer than those of patients with low CD8+TILs (P = 0.002, P = 0.003) (**Figure 3B**). As shown in **Table 4**, T stage, N stage, neoadjuvant regimens and TRG were associated with DFS (P = 0.033, 0.012, 0.023, and 0.001, respectively). T stage, N stage and TRG were sensitive predictors for OS (P = 0.008, 0.014, and 0.001, respectively). Compared with neoadjuvant RT or CT, patients in neoadjuvant CRT group had longer OS, but the difference was not statistically significant (P = 0.128).

Discussion

The results of this study demonstrated that local antitumor immunity was enhanced by increased CD3+ and CD8+TILs after neoadjuvant RT, CT or CRT in rectal cancer. These findings were in accordance with the results of previous study, which suggested that TILs were increased after CRT [21]. To the best of our knowledge, this is the first study to evaluate the respective effect of RT, CT and CRT on the numbers and compositions of TIL subsets. The data indicated that CD3+ and CD8+TILs in resected specimens after treatment were significantly higher than those in biopsy specimens in all cases (RT, CT and CRT). The evolution of a local immune response was possibly triggered by the RT- or CT-mediated release of tumor antigens and by chemotaxis toward certain mediators such as proinflammatory cytokine interferon- γ . Benjamin et al. have summarized that the necrotic tumor cell death is induced by multimodal treatments and finally

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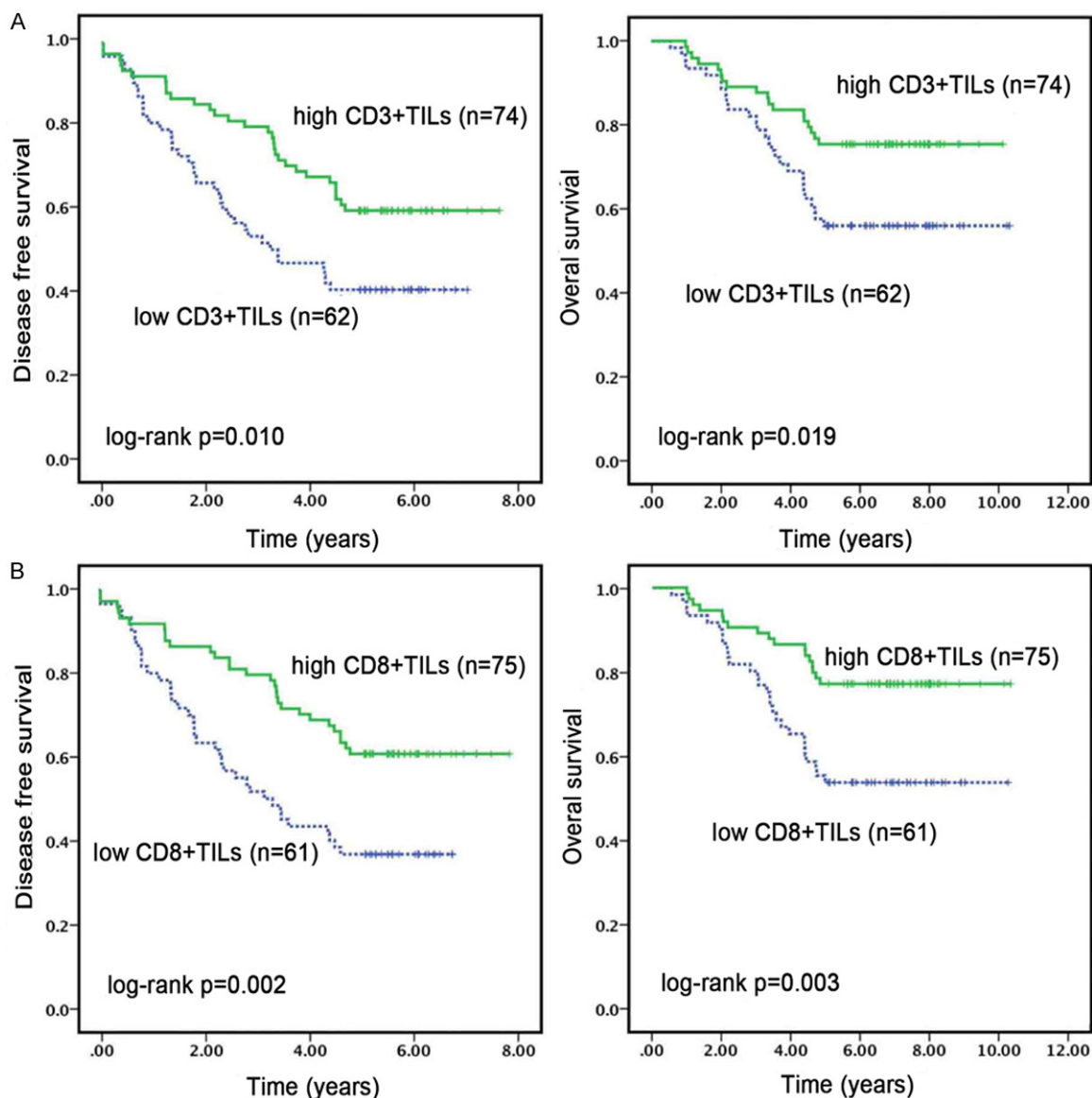


Figure 3. Kaplan-Meier analyses of DFS and OS according to CD3+TILs and CD8+TILs in pretreatment biopsy specimens. Patients with high CD3+TILs (A) and CD8+TILs (B) achieved better DFS and OS than patients with low CD3+ and CD8+TILs.

leads to antitumor immunity [22]. According to the data obtained from multiple cancer models, some of the effects of ionizing radiation and chemotherapy contribute to systemic antitumor immunity [23]. A recent example of the abscopal effect in a patient with melanoma treated with ipilimumab and radiotherapy supports this view [24]. Our results of increased TILs during treatment may provide evidences supporting the role of radiotherapy and chemotherapy as immune adjuvants.

Certainly, each tumor treatment modality has its advantages and disadvantages when viewed

separately. Improved RT techniques allow a maximum local tumor control by concomitantly minimizing the normal tissue side actions, but do not primarily result in systemic antitumor responses. Low dose CT is beneficial for shifting the tumor microenvironment into an activating one, but may not have the sufficient cytotoxicity to kill the tumor cells. These considerations are highly relevant to decisions on the best approaches to deliver chemoradiation and to integrate it with other more systemic therapies, such as immunotherapy. In this study, there were no differences in the ability to enhance TILs in rectal cancer between RT, CT

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or CRT. It is known that patients with LARC have better local control after nCRT than those with RT or CT monotherapy [3]. Besides, according to our results, local antitumor immunity was also enhanced after CRT. It demonstrated that nCRT, as the current standard treatment of LARC, was still immune-active and higher doses may be required to suppress the antitumor immunity.

We also found that CD3+ and CD8+TILs in pretreatment specimens were associated with good responses after neoadjuvant RT, CT and CRT, which indicated that tumors attracting T cells were more sensitive to chemoradiation. Our results were consistent with the results of other reports in the literature [18, 21]. Basic research studies have demonstrated that RT or CT is more efficient in immunocompetent mice, and that immunosuppression compromised CRT efficacy [25, 26]. Growing evidence has indicated that radiation exerts its antitumor activity not only by inducing an irreversible DNA damage, but also by promoting tumor-specific immune response [27]. In addition, preclinical studies have demonstrated that cytotoxic agents induce immune response against tumor cells, which significantly contributes to their antitumor effects [28]. Chemoradiation may promote antitumor effects by further inducing the expression of tumor associated antigens in tumors with a higher number of TILs, because these tumors are originally immunogenic. Our results provided further evidence for a mechanical linkage between tumor response to chemoradiation and host immunity. Given the predictive effect of pretreatment local immunity for chemoradiotherapy, it may indicate chemoradiosensitivity and help to determine whether to undergo additional CRT or have surgery early. In addition, in our results, CD8+ seemed to be a more efficient predictor for nCRT than CD3+. The potential reason may be that CD8+cytotoxic T lymphocytes are directly capable of killing tumor cells; while CD3+ represents the total general T lymphocytes, but not directly target tumor cells. The capability of chemoradiation to kill tumor cells is more related to the CD8+ than the CD3+ subtype. Further studies are required to elucidate the detailed mechanisms.

A large number of studies have assessed the prognostic value of TILs in colorectal cancer [8, 14, 18, 21, 29]. Our results demonstrated that CD3+TILs and CD8+TIL subtypes in pretreat-

ment biopsy specimens were significantly associated with DFS and OS. The results were consistent with those of previous studies. Richards et al. have demonstrated that CD3+TIL at the invasive margin and CD8+TIL in the cancer cell nests are strong predictor of survival in colorectal cancer [29]. Shinto et al. have found that post-CRT CD8+ lymphocytes in tumor stroma are independent prognostic factors for rectal cancer [21]. It is speculated that intratumoral T cells can attenuate the metastatic potential of tumor cells by modifying tumor stroma or tumor cells. This suggests that the state of the local adaptive immune response partly determines the time of recurrence and overall survival time. However, T-cell activation is the result of a balance between positive and negative signals. The modulation of signaling via costimulatory or coinhibitory receptors on T cells has proven to be a potent way to affect antitumor immune responses [30]. Ipilimumab, an antibody that blocks the coinhibitory receptor cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), has been approved by FDA for the treatment of unresectable or metastatic melanoma. Other agents that target a coinhibitory receptor, programmed death 1 (PD-1), and its ligand, PD-L1, are in clinical development [30]. With these inhibitory checkpoints, the effect may be more significant when TILs and these checkpoints are used in combination as predictors of prognosis.

There were some limitations of this study. First, single RT or CT before surgery was not standard treatment for local advanced rectal cancer. In our study, to distinguish the respect effect of radiotherapy and chemotherapy on TILs, we analyzed some patients with neoadjuvant RT or CT retrospectively. These patients were not able to tolerate standard neoadjuvant CRT because of poor performance or their decision to refuse to receive combinatory treatment. Secondly, we chose to assess the density of T-cell infiltration by observers rather than utilizing automated imaging software, which may result in variability. However, automated imaging software distinguishes cells by different depths of color without considering the shapes, features and positions. In contrast, in the present study, evaluation of T-cell density was carried out by two independent investigators blinded to clinicopathologic information. They could give a broader examination of full sections, avoiding ecrotic areas and without missing

tumor compartments. In view of the heterogeneity of T-cell density within different single sections, we believe that this technique shows more advantage than analysis performed with automated imaging software. Finally, we did not analyze TILs in different areas of the tumor microenvironment, such as invasive margin and cancer cell nests. However, previous studies have suggested that inflammatory cells in all areas of the tumor microenvironment may be important indicators of prognosis, and the density of infiltration is prior to the type and location of inflammatory cells [29, 31, 32].

To the best of our knowledge, this is the first study demonstrating that RT, CT and CRT can all enhance local immune response by increased TILs. Moreover, there was no significant difference among these three cases. Our results provided further evidences for a mechanical linkage between tumor response to chemoradiotherapy and host immunity. It may be a new potential tool to predict the response of rectal cancer to nCRT, as well as a better surrogate to select nonresponders for an early surgery. In addition, recent preclinical studies have suggested that the combination of chemoradiation and immune therapies shows significantly better control of tumor than single treatment, but some subpopulations fail to respond. The local tumor immunity may be an effective predictor for these combinations, but the correlation or alteration in the number of tumor infiltrating and circulating T lymphocytes remains to be clarified. These information may provide a more convenient and efficient tool to monitor treatment effects in the future.

Disclosure of conflict of interest

None.

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