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Assessment of the expression and response of PD-1, LAG-3, and TIM-3 after neoadjuvant radiotherapy in rectal cancer

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Many studies have verified the safety of combined radiotherapy and immune checkpoint blockades (ICBs) without the specific radiation dose or sequencing of combination. We aimed to evaluate the expression and response of PD-1, TIM-3, LAG-3 after neoadjuvant radiotherapy (NRT) and explore the possibility and optimal schedule of combining immunotherapy with radiotherapy in treating rectal cancer. Immunohistochemistry was performed to detect the expression of PD-1, TIM-3, LAG-3, CD8, and CD3. These molecules' expression was detected on the specimens of 76 rectal cancer patients following NRT and 13 of these patients before NRT. The expression of ICBs was assessed by the percentage of positive cells. The levels of PD-1 and immune cells (ICs) LAG-3 in rectal cancer increased after NRT (0% vs. 3%, p=0.043 and 5% vs. 45%, p=0.039, respectively). However, TIM-3 in ICs and tumor cells (TCs) were both decreased (80% vs. 50%, p=0.011, 90% vs. 0%, p=0.000, respectively). The LAG-3 expression was higher in patients treated with short-course RT than long-course RT (22.5% vs. 8.0%, p=0.0440 in ICs; 0% vs. 70%, p<0.001 in TCs). On the contrary, CD8 was higher after long-course RT (15% vs. 8%, p=0.0146). Interestingly, the level of ICs TIM-3 was low in > eight weeks after long-course RT (p=0.045). The expressions of PD-1, ICs TIM-3, ICs LAG-3, CD3, and CD8 were associated with the disease-free survival (DFS) in univariate analysis (p=0.036, 0.008, 0.018, 0.025, and 0.004, respectively). Adjusted by the relevant variables, PD-1 (HR 0.274; 95% CI 0.089-0.840; p=0.024) and ICs TIM-3 (HR 0.425; 95% CI 0.203-0.890; p=0.023) were independent prognostic factors of DFS in rectal cancer patients following NRT. In conclusion, we have identified that PD-1 and ICs LAG-3 presented a trend towards increased expression after NRT, supporting the ICBs and NRT combination as a potential treatment option for local advanced rectal cancer patients. The radiotherapeutic mode and timing of the treatment might significantly affect the expression of ICBs, which indicated that the sequencing and time window of ICBs immunotherapy utility might deserve a high value.

Key words: PD-1, TIM-3, LAG-3, rectal cancer, radiotherapy

Immunotherapy has become a promising anticancer strategy alongside surgery, chemotherapy, and radiotherapy [1, 2]. Colorectal cancer is not known as an immunogenic malignancy in contrast to lung cancer and melanoma. Many early-phase trials focused on immune checkpoint blockade (ICB) immunotherapy and/or combination treatments failed to bring about considerable results in colorectal cancer [3]. Tumor mutational burden is one of the emerging indicators of response to anti-PD-1 therapy, supported by the clinical effect of PD-1 checkpoint inhibition in colorectal cancer [4]. Colorectal cancer patients with mismatch repair-deficiency (dMMR) have an apparent favorable response to anti-PD-1 therapy compared to the subtype with mismatch repair-proficiency (pMMR) [4–6]. Unfortunately, dMMR occurs in few adenocarcinomas such as the colon and rectum, stomach, and small intestine, consisting of 8% of stage I to stage III and 4% of stage IV [6].

A lot of emerging targets in cancer immunotherapy are now in early phase clinical trials, such as T-cell immunoglobulin and mucin containing protein-3 (TIM-3) and lymphocyte activation gene-3 (LAG-3) [1]. TIM-3 expression in tumor-infiltrating lymphocytes (TILs) has been determined in several cancers, such as melanoma, non-Hodgkin lymphoma, gastric cancer, lung cancer, and so on [1]. LAG-3 has been observed in colon cancer, melanoma, breast cancer, lung cancer, and etcetera [1, 7]. PD-1, TIM-3, and LAG-3 are

ICBs expressed by activated T-lymphocytes and are correlated to T-cell exhaustion [1]. Preclinical cancer models suggest that inhibition of TIM-3 or LAG-3 alone exerts little reaction on antitumor response [1, 8], while co-blockade of either immune checkpoint with PD-1 pathways may improve T-cell effector functions and antineoplastic reaction [9, 10]. Yang et al. demonstrated that cancer treatment is required for the simultaneous blockade of PD-1, TIM-3, and LAG-3 in the mouse model [11]. Several early-phase clinical trials are underway to test the combinations of anti-PD-1 agents and TIM-3 or LAG-3 targeting agents [1, 9].

Immunotherapy strategies combined with radiotherapy may produce synergistic effects. Local irradiation might activate adjuvant signals and inflammatory response in that immune cells are recruited into the tumors [12]. Recent data have suggested that in the melanoma and breast cancer models, the combination of radiotherapy and anti-PD-1 therapy could induce an antigen-specific immune response [13]. In the clinical setting of non-small-cell lung cancer, patients treated with radiation treatment before anti-PD-1 treatment have had a significantly favorable outcome compared to those who have not received radiotherapy [14]. However, the actions of suppressive immune cells might be enhanced by radiotherapy and potentially constrain antitumor response. For example, CTLA-4 upregulated by radiation can effectively inactivate the co-stimulatory signal, thus causing the T-cell activation ineffective [15]. The reactions against tumor cells might be amplified if vital immunosuppressive effects were overcome.

There are still some crucial caveats that remain regarding the radio-immunotherapy combination despite early promising data [16]. Many studies have verified the safety of combined radiotherapy and ICBs without the specific radiation dose or sequencing of combination [17]. However, the timing and sequencing of the combination might affect the treatment efficacy [16]. Optimal sequencing of combined treatment may also be based on the immunomodulating drugs utilized [17]. Thus, an appropriate time window and dose/fraction of radiation in combined treatment need to be highlighted. In the present study, we examined the PD-1, LAG-3, and TIM-3 in rectal cancer patients with neoadjuvant radiotherapy (NRT). We aimed to evaluate the variation in expression of PD-1, LAG-3, and TIM-3 after NRT and compared the difference of those ICBs between long-course radiotherapy (LCRT) and short-course radiotherapy (SCRT). And we tried to explore the possibility and optimal schedule of combining immunotherapy with radiotherapy in treating rectal cancer.

Patients and methods

Patients. This retrospective study was reviewed and approved by the ethics committee of Fujian Cancer Hospital (No. KT2018-008-01). Patients were not required to give informed consent to the study because the analysis used anonymous clinical data. Tissue samples of rectal cancer were collected from patients who received NRT at our hospital between January 2012 and December 2015. All patients were confirmed with rectal adenocarcinoma by pathologists. We opted for a cohort of 80 patients with Karnofsky scores >70 and excluded patients diagnosed with more than one malignancy or immune disorder. We also excluded patients without complete data. Specimens from 4 cases were in poor condition. Finally, 76 patients were retrospectively analyzed in this study. Of these, the tissue samples of 13 patients before NRT were available.

The radiation treatment was either SCRT with a total dose of 25 Gy in five fractions or LCRT that delivered a total dose of 45–50.4 Gy in 25–28 fractions. Capecitabine (825 mg/m^2) twice daily was delivered concurrently with LCRT. After the NRT, total mesorectal excision was performed in all patients at 5–13 weeks after the LCRT or within 2 weeks (median 1 week) after the SCRT was delivered. 5-fluorouracil (5-FU)-based chemotherapy was performed following total mesorectal excision.

Immunohistochemistry. Tumor tissue sections of 4 μm thickness were prepared from formalin-fixed and paraffin-embedded specimens, two slides for each patient. Xylene and graded alcohols were used to deparaffinize the slides. For antigen retrieval, the tissue sections were boiled in EDTA, pH 8.0, for 10 min, and air-cooled for 20 min at room temperature. The slides were probed with primary antibody at 4°C overnight or 37°C for 1 h and incubated at room temperature for 30 minutes, and rinsed with PBS/0.05% Tween in between the steps. Primary antibodies included rabbit anti-human PD-1/CD279 monoclonal antibody (E18662, 1:100; Spring Bioscience, Pleasanton, CA, USA), TIM-3 antibody (2E2) (NBP2-45933, 1:800; Novus Biologicals, Laura, USA), anti-Lymphocyte Activation Gene 3 antibody [EPR4392 (2)] (ab180187, 1:800; Abcam, Southampton, UK), anti-CD8 antibody (ab4055, 1:200, Abcam, Southampton, UK), anti-CD3 antibody [SP7] (ab16669, 1:100; Abcam, Southampton, UK) and were diluted in antibody diluent based on the instructions and pre-experimental results.

The level of protein expression was quantified according to the percentage of positive cells. The optimized cutoff points of protein expression were determined by the Cutoff Finder [18] (http://molpath.charite.de/cutoff/load.jsp). Optimizing the split significance in the Kaplan-Meier plot is the straightforward method to determine the prognostic cutoff point. In our evaluation, immune cells (ICs) refer to lymphocytes surrounding tumor cells (TCs) in samples. Image-Pro Plus version 6.0 was used to detect the results of immunohistochemistry. The scoring and quantification of protein expressions were also independently evaluated by two experienced pathologists blinded to any clinical data. The consensus between the two pathologists resolved discrepancies.

Follow-up. All patients were evaluated clinically every 3 months within the first 2 years, every 6 months during the first 5 years, and annually thereafter. The endpoints in this

study were disease-free survival (DFS) and overall survival (OS). All endpoints were counted from the date of surgical resection. The time to all-cause death or censored at the last follow-up was defined as OS; the time interval to local recurrence, distant failure, death, or censored at the last follow-up was defined as DFS. Local recurrence and distant failure were diagnosed either pathologically or radiologically. At the time of the last follow-up, patients were censored if no event had occurred.

Statistical analysis. The proteins' expressions pre- and post-NRT were compared using paired t-test. The correlation between protein expression and NRT pattern as well as surgery interval was performed by Mann-Whitney U test analysis. The Kaplan-Meier method and Cox regression model were conducted for survival curve analysis and prognostic relevance of variables. p values in all the statistical tests were two-sided, and $p<0.05$ was regarded as significant. SPSS Statistics version 22.0 (IBM Corporation, Armonk, NY, USA) was used in these analyses.

Results

Patient characteristics. The characteristics of the 76 patients included in the study cohort are shown in Table 1. The median follow-up time was 29.0 months (range, 2–59 months). The patients were composed of 49 (64.5%) males and 27 (35.5%) females. The median age of the patients in the cohort was 53 years (range, 19–74 years). Twenty-eight patients were clinical TNM stage II and remaining stage III. Clinical T-stages 2, 3, and 4 (cT2/3/4) were 4 (5.3%), 41 (53.9%), and 31 (40.8%), respectively. After the NRT, patients with pathological T stage (pT) 0–II were 21 (27.6%), and stage III–IV were 55 (72.4%). In all, 38 (50.0%) patients presented pathological lymph node-positive disease (pN+); 16 (21.1%) patients were identified as low grade and 60 (78.9%) as intermediate grades. Patients receiving LCRT and SCRT were 40 (52.6%) and 36 (47.4%), respectively.

PD-1, TIM-3, and LAG-3 scoring. Immunohistochemical staining detected TIM-3 and LAG-3 localization both in the ICs and TCs, while PD-1 was observed in ICs (Figures A1, A2, B1, B2, C1, C2, D1, D2). The relationship between protein expression levels after NRT and DFS was evaluated based on the appointed cutoff point. The optimal cutoff of PD-1 was determined as 5.5% with hazard ratio (HR) 0.32; range 0.11–0.92 (p=0.027). The optimal cutoff of ICs TIM-3 and ICs LAG-3 were 55% (HR 0.37; range 0.18–0.76; p=0.005) and 27.5% (HR 0.38; range 0.17–0.84; p=0.014), respectively. Additionally, 13.5% and 12.5% were the cutoff points of CD8 and CD3 (HR 0.34; range 0.16–0.7; p=0.0021 vs. HR 0.42; range 0.2–0.89; p=0.019). However, the optimized cutoff points of TIM-3 (45%, HR 0.74; range 0.38–1.44; p=0.38) and LAG-3 (12.5%, HR 0.73; range 0.36–1.46; p=0.37) in TCs could not be determined based on DFS (Supplementary Figure S1).

PD-1, TIM-3, and LAG-3 expression in rectal cancer. A total of 61 (80.3%) patients were interpretable for a low PD-1 expression. TIM-3 was found to have high ICs expression in 44.7% and high TCs expression in 42.1% of the patients. The

Table 1. Patient characteristics.

Characteristics		Data, n (%)
Sex	Male / Female	49 (64.5) / 27 (35.5)
Age	≤60 / >60	56 (73.7) / 20 (26.3)
cT stage	cT2 / cT3 / cT4	4 (5.3) / 41 (53.9) / 31 (40.8)
cN stage	cN0 / cN1 / cN2	28 (36.8) / 38 (50.0) / 10 (13.2)
Clinical TNM stage	II/III	28 (36.8) / 48 (63.2)
ypT stage	pT0+1+2 / pT3+4	21 (27.6) / 55 (72.4)
ypN stage	pN– / pN+	38 (50.0) / 38 (50.0)
Distant to anal verge (cm)	≤5 / 5~10 / >10	49 (64.5) / 25 (32.9) / 2 (2.6)
Gross type	Ulcerative/Protruding/Infiltrating and other type	58 (76.3) / 16 (21.1) / 2 (2.6)
Pathological differentiation	Low grade / Intermediate grade	16 (21.1) / 60 (78.9)
Venous invasion	+/–	15 (19.7) / 61 (80.3)
Carcinoma nodules	+/–	16 (21.1) / 60 (78.9)
Neural invasion	+/–	14 (18.4) / 62 (81.6)
NRT	LCRT/SCRT	40 (52.6) / 36 (47.4)
Post-CT	Yes/No	23 (30.3) / 53 (69.7)
Surgical procedure	APR / LAR	43 (56.6) / 33 (43.4)
TRG	TRG1+2+3 / TRG4+5	45 (59.2) / 31 (40.8)
Relapses	Yes/No	31 (40.8) / 45 (59.2)
Deaths	Yes/No	14 (18.4) / 62 (81.6)

Abbreviations: NRT-neoadjuvant radiotherapy; LCRT-long-course radiotherapy; SCRT-short-course radiotherapy; post-CT-postoperative chemotherapy; APR-abdominoperineal resection; LAR-low anterior resection; TRG-tumor regression grade

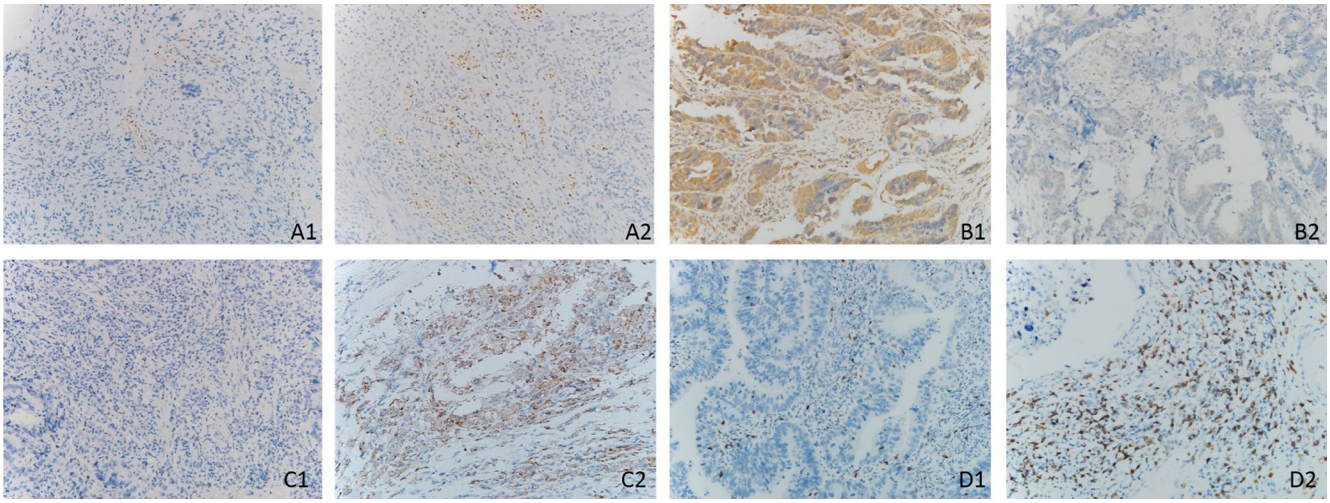

Figure 1. IHC staining and comparison of immune infiltrating in a pre- and post-treatment rectal cancer (magnification 200×). A) ICs PD-1 expression in pre- (A1) and post-treatment (A2); B) ICs and TCs TIM-3 expression in pre- (B1) and post-treatment (B2); C) ICs and TCs LAG-3 expression in pre- (C1) and post-treatment (C2); D) ICs CD8 positive in pre- (D1) and post-treatment (D2). Increased PD-1, LAG-3, and CD8 positive cells were seen in the post-treatment specimen; decreased TIM-3 positive cells were seen in the post-treatment specimen.

Table 2. PD-1, TIM-3, and LAG-3 expression in rectal cancer.

Proteins	Post-NRT (n=76)		13 patients		p-value
			Pre-NRT	Post-NRT	
PD-1	<5.5% / >5.5%	61/15	12/1	6/7	0.043
IC TIM-3	<55% / >55%	42/34	3/10	8/5	0.011
TC TIM-3	<45% / >45%	44/32	8/5	8/5	0.000
IC LAG-3	<27.5% / >27.5%	50/26	11/2	5/8	0.039
TC LAG-3	<12.5% / >12.5%	48/28	9/4	5/8	0.060
CD8	<13.5% / >13.5%	41/35	7/6	5/8	0.343
CD3	<12.5% / >12.5%	14/62	2/11	2/11	0.321

Abbreviations: post-NRT-postoperative neoadjuvant radiotherapy; pre-NRT-preoperative neoadjuvant radiotherapy; PD-1-programmed cell death 1; TIM-3-T-cell membrane protein 3; LAG-3-lymphocyte-activation gene 3; TC-tumor cell; IC-immune cell

patients with high ICs LAG-3 and TCs LAG-3 expressions were 34.2% and 36.8%, respectively. Increased expressions of CD8 and CD3 were found in 40.1% and 81.6% of patients. All the above results were derived from post-treatment specimens.

The pre-treatment specimens from the 13 patients were also analyzed. Low PD-1 expression was revealed in 12 cases. Patients detected with high TIM-3 expressions in ICs and TCs were 10 and 5, with high expressions of LAG-3 in ICs and TCs were 2 and 4, respectively. High expressions of CD8 and CD3 were found in 6 and 11 patients. The expressions of these proteins are summarized in Table 2.

The 13 matched rectal cancer samples before and after treatment were compared in our analysis (Figures 2A–2D). PD-1 and ICs LAG-3 were upregulated by NRT (median PP 0% vs. 3%, p=0.043 and 5% vs. 45%, p=0.039, respectively), while TIM-3 expressions were downregulated both in ICs and TCs after treatment (median PP 80% vs. 50%, p=0.011, 90% vs. 0%, p=0.000, respectively). However, TCs LAG-3, CD8, and CD3 had no significant changes (p>0.05) (Table 3).

The correlation between protein expression and NRT procedure. The median PP of ICs LAG-3 and CD8 expression was 15% (range 0–80%) and 10% (range 0–80%), respectively. The ICs and TCs LAG-3 expression was higher in patients treated with SCRT versus LCRT (22.5% vs. 8.0%, p=0.044; 0% vs. 70%, p=0.001, respectively). TIM-3 expression in TCs was also high in SCRT group (0% vs. 80%, p=0.007). On the contrary, CD8 was higher in patients receiving LCRT (15% vs. 8%, p=0.014). No difference was observed in other proteins (Supplementary Table S1).

The correlation between protein expression and surgery interval. A cutoff point of eight weeks from the completion of radiotherapy to the surgery was used to assess the relationship between protein expression and surgery interval in the LCRT patients. Patients with ≤8 weeks intervals were 26 (26/40), and the rest were >8 weeks. ICs TIM-3 was related

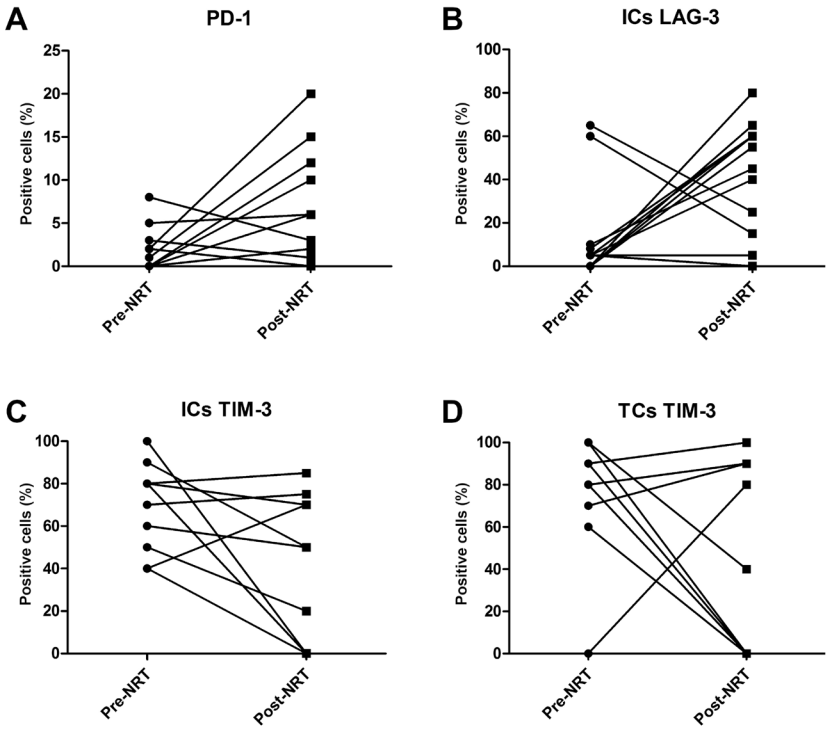

Figure 2. Quantification of the immune infiltrate changes following neoadjuvant treatment of rectal cancer in paired samples by the patient. A) PD-1 positive immune cells; B) LAG-3 positive immune cells; C) TIM-3 positive tumor cells; D) TIM-3 positive immune cells.

to surgery interval in the LCRT cohort through the Mann-Whitney U test for the nonparametric test of the independent samples (p=0.045). ICs TIM-3 had low expression in patients with >8 weeks interval compared with ≤8 weeks interval (median PP, 22.5% vs. 60%) (Figure 3). No correlations between surgery interval and other proteins were found in our study (p>0.05). The SCRT cohort was not analyzed because of the short interval.

High PD-1 and ICs TIM-3 levels were associated with favorable DFS. In all patients who underwent NRT, the expressions of PD-1, ICs TIM-3, ICs LAG-3, CD3, and CD8 were associated with the DFS in univariate analysis (p=0.036, 0.008, 0.018, 0.025, and 0.004, respectively) (Figures 4A–4E). Disease progression was frequent in patients with low levels of PD-1, with a median time of 27.0 months (95% CI 24.9–35.0 months) vs. 46.6 months (95% CI 36.3–56.9 months) in patients with high PD-1 staining (Figure 2A). Compared with low levels, high staining of ICs TIM-3 was associated with longer DFS (28.1 months; 95% CI 21.6–34.5 vs. 39.5 months; 95% CI 33.4–45.5) (Figure 2B). With the forward likelihood ratio method, the Cox regression model was operated to examine the prognostic relevance of variables. Adjusted by the relevant variables, PD-1 (HR 0.274; 95% CI 0.089–0.840; p=0.024) and ICs TIM-3 (HR 0.425; 95% CI 0.203–0.890; p=0.023) were independent prognostic factors of DFS in rectal cancer patients following NRT (Table 4).

Table 3. Median PD-1, TIM-3, LAG-3, CD8, CD3 positive cells pre- and post- neoadjuvant radiotherapy.

	pre-NRT	post-NRT	p-value
	median of positive cells (range) (%)	median of positive cells (range) (%)	
PD-1	0 (0–8)	3 (0–20)	0.043
IC TIM-3	80 (40–100)	50 (0–85)	0.011
TC TIM-3	90 (0–100)	0 (0–100)	<0.001
IC LAG-3	5 (0–65)	45 (0–80)	0.039
TC LAG-3	5 (0–90)	60 (0–100)	0.060
CD8	10 (1–60)	15 (0–70)	0.343
CD3	40 (5–90)	35 (0–90)	0.321

Abbreviations: post-NRT – postoperative neoadjuvant radiotherapy; pre-NRT – preoperative neoadjuvant radiotherapy; PD-1 – programmed cell death 1; TIM-3 – T-cell membrane protein 3; LAG-3 – lymphocyte activation gene 3; TC – tumor cell; IC – immune cell

The proteins detected in our study were not correlated with overall survival (OS) (Supplementary Table S2).

Discussion

In this study, we focused on the presence of PD-1, LAG-3, and TIM-3 in rectal cancer patients who received NRT. We demonstrated a trend towards the increased density of ICBs as represented by PD-1 and ICs LAG-3 after NRT. Moreover,

the expressions of LAG-3 and CD8 were different between LCRT and SCRT, and ICs TIM-3 showed low expression in patients with >8 weeks surgery time interval after LCRT. In addition, we found that high expressions of PD-1, ICs TIM-3, and LAG-3 were correlated with favorable DFS.

Synergistic effects may occur after the combination of radiotherapy and immunotherapy and have been analyzed in some cancers such as lung cancer [19]. Three effects of radiotherapy may be employed in the combined therapy: tumor burden reduction, tumor-stromal cell modification, and antitumor response potentiation via exposing neoantigen [20]. A trend towards increased expression of PD-1 and ICs

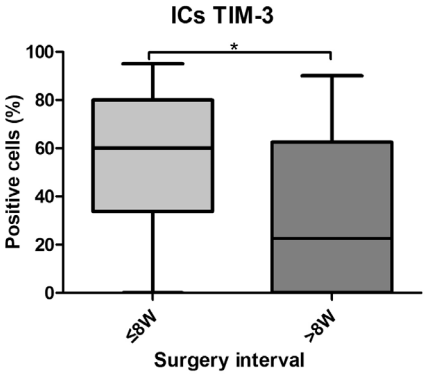

Figure 3. Box plot of ICs TIM-3 expression between ≤8 weeks and >8 weeks surgery interval in rectal patients with LCRT.

LAG-3 in rectal cancer following treatment was observed by comparing the matched pre- and post-treatment specimens in our analysis. After chemoradiotherapy, esophageal adenocarcinoma also demonstrated upregulation of multiple ICBs, including LAG3, PD-L1, TIM3, and so on [21]. This observation suggests the potential feasibility of combining PD-1 or LAG-3 checkpoint inhibition with radiotherapy. Combinations of immunotherapies may enhance response rates, and the duration of response is also improved via antitumor immunological memory stimulation [20]. However, we found that TIM-3 was downregulated by the NRT both in TCs and ICs. The explanation might be the heterogeneity of different ICBs and tumors and sensitivity difference induced by NRT. The mechanism should be explored in further study.

RT might induce the upregulation of some ICBs, while the magnitude and kinetics of the induction might depend on dose and fractionation. So, the choice of radiotherapeutic modalities of their combinations will be crucial. The two RT strategies delivered in rectal cancer patients were used to compare the ICBs expression in different fractions and doses. Our study showed the expression difference of LAG-3 and CD8 between the LCRT and SCRT, suggesting that some ICBs and TILs might be modified by the treatment regimens. In accordance with these results, PD-L1 expression in TCs was mostly positive in the SCRT subgroup in our previous study [22]. Tumor local inflammation might be induced by the radiotherapeutic dose and fractionation, also the chemotherapy alongside the LCRT was a consideration. The

Table 4. Impact of protein expressions on the DFS of patients with rectal cancer.

Variables		DFS					
		Univariate			Multivariate		
		HR	95% CI	p-value	HR	95% CI	p-value
Sex	Male/Female	0.751	0.369–1.527	0.429			
Age	≤60 / >60	0.870	0.378–2.003	0.743			
cT	cT2+3 / cT4	1.093	0.572–2.089	0.788			
cN	cN0 / cN1+2	1.566	0.777–3.155	0.210			
cTNM stage	II/III	1.491	0.739–3.008	0.265			
pT	pT0+1+2 / pT3+4	1.883	0.821–4.316	0.135			
pN	pN – / pN+	3.057	1.511–6.182	0.002	2.157	0.882–5.280	0.092*
Pathological differentiation	Low grade / Intermediate grade	1.708	1.259–2.535	0.001	1.778	1.236–2.558	0.002
Venous invasion	+/ –	0.560	0.392–0.800	0.001	0.671	0.467–0.965	0.032
Neural invasion	+/ –	0.801	0.537–1.193	0.275			
Carcinoma nodules	+/ –	0.922	0.621–1.369	0.686			
TRG	TRG1+2+3 / TRG4+5	1.894	0.989–3.630	0.054	1.741	0.831–3.646	0.142*
PD-1	<5.5% / >5.5%	0.324	0.113–0.929	0.036	0.274	0.089–0.840	0.024
IC TIM-3	<55% / >55%	0.370	0.178–0.768	0.008	0.425	0.203–0.890	0.023
TC TIM-3	<45% / >45%	0.748	0.385–1.453	0.391			
IC LAG-3	<27.5% / >27.5%	0.381	0.172–0.846	0.018	0.725	0.283–1.862	0.504*
TC LAG-3	<12.5% / >12.5%	0.732	0.365–1.467	0.379			
CD3	<12.5% / >12.5%	0.430	0.206–0.899	0.025	0.486	0.201–1.179	0.111*
CD8	<13.5% / >13.5%	0.334	0.160–0.699	0.004	1.207	0.497–3.243	0.618*

Note: *the enter method of logistic regression was used. Abbreviations: TRG – tumor regression grade; PD-1 – programmed cell death 1; TIM-3 – T-cell membrane protein 3; LAG-3 – lymphocyte activation gene 3; TC – tumor cell; IC – immune cell

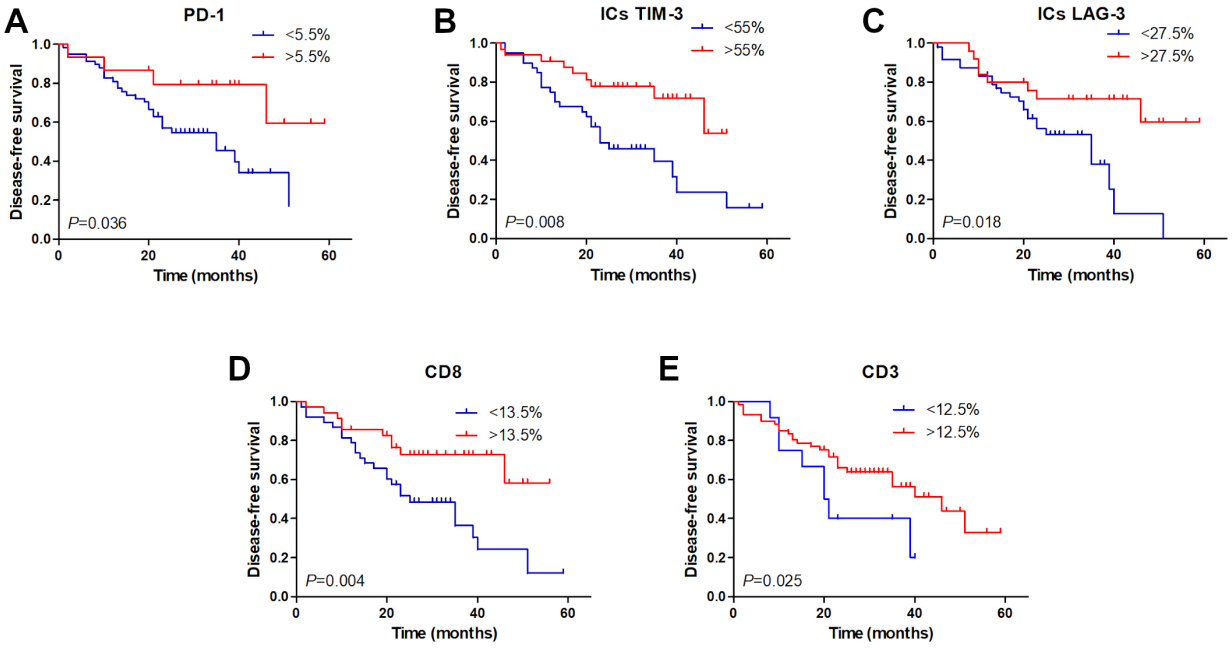

Figure 4. Kaplan-Meier analysis of disease-free survival (DFS). The following were significantly associated with DFS: A) positive expression of PD-1; B) high expression of TIM-3 in immune cells; C) high expression of LAG-3 in immune cells; D) high density of CD8 positive tumor-infiltrating lymphocytes; E) high density of CD3 positive immune cells.

antitumor immunity induced by different doses and fractionation remains elusive. The ICs LAG-3 expression was higher in the SCRT cohort, while CD8 was higher in patients receiving LCRT in our research. Similarly, preclinical evidence showed that compare to high-dose hypo-fractionated (8 Gy × 2), daily fractionated RT (2 Gy × 10) preserved tumor-infiltrating CD8 T-cell activation and accumulation [17].

Despite the fraction of radiotherapy, the sequencing and timing of combination might affect the efficacy [16]. Evidence presented that immunotherapy should be started at the beginning of the radiation for the releasement of neo-antigens, followed later by more limited T-cell epitope availability [17]. Our results showed that in the analysis of the LCRT subgroup, ICs TIM-3 indicated low expression in patients with >8 weeks compared to those with ≤8 weeks interval, suggesting that ICs TIM-3 expression might decrease with time after LCRT. In the animal model, Kelly et al. evaluated PD-L1 expression levels at different time points after radiation, and the results indicated that PD-L1 upregulation was transient [21]. Those results showed a narrow time window of ICBs immunotherapy utility in clinical practice. Most of the research focused on the sequencing of combination [17], while the appropriate time window of combination might deserve high value. The efficacy of combined treatment might not be achieved for the low expression of ICBs based on our results. More prospective analyses and fundamental researches are imperative to verify the appropriate time window to add immunotherapy to radiotherapy.

Additionally, PD-1 and ICs TIM-3 were both independent prognostic factors for DFS in our study; and high expression of ICs LAG-3, CD8, and CD3 was related to better DFS in univariate analysis. In line with our observation, it has been reported that the expression of ICBs can serve as a predictor for outcome [21, 23–25]. However, the correlation between PD-1, TIM-3 expression, and patients' outcome remained controversial in various cancers [23, 26, 27]. The reason might be the different cutoff points among the diverse analyses. More specific research is needed to establish a unified standard.

It should be noted that as retrospective research, our sample size was relatively small, especially the specimens collected before the NRT. Further analyses with a larger sample size are needed to validate the results. Prospective studies are essential to confirm the role of radiotherapy and chemotherapy in the expression of ICBs. The appropriate time window and radiotherapy strategy of combination should be identified in further analysis. Lastly, the deficiency of the detection in microsatellite instability (MSI) and MMR also restricted our further analysis.

In conclusion, we have identified that PD-1 and ICs LAG-3 presented a trend towards increased expression after NRT, supporting the ICBs and NRT combination as a potential treatment option for local advanced rectal cancer patients. Furthermore, the expression of LAG-3 and CD8 differed between LCRT and SCRT, and ICs TIM-3 had low expression in patients with >8 weeks surgery time interval after LCRT.

On the other hand, the expression of PD-1 and TIM-3 might be considered indicators of prognosis. The radiotherapeutic mode and timing of these combinations might significantly affect the expression of ICBs, which indicated the sequencing and time window of ICBs' immunotherapy utility in combination treatment might deserve a high value.

Supplementary information is available in the online version of the paper.

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Assessment of the expression and response of PD-1, LAG-3, and TIM-3 after neoadjuvant radiotherapy in rectal cancer

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Supplementary Information

Supplementary Table S1. The correlation between protein expression and NRT procedure.

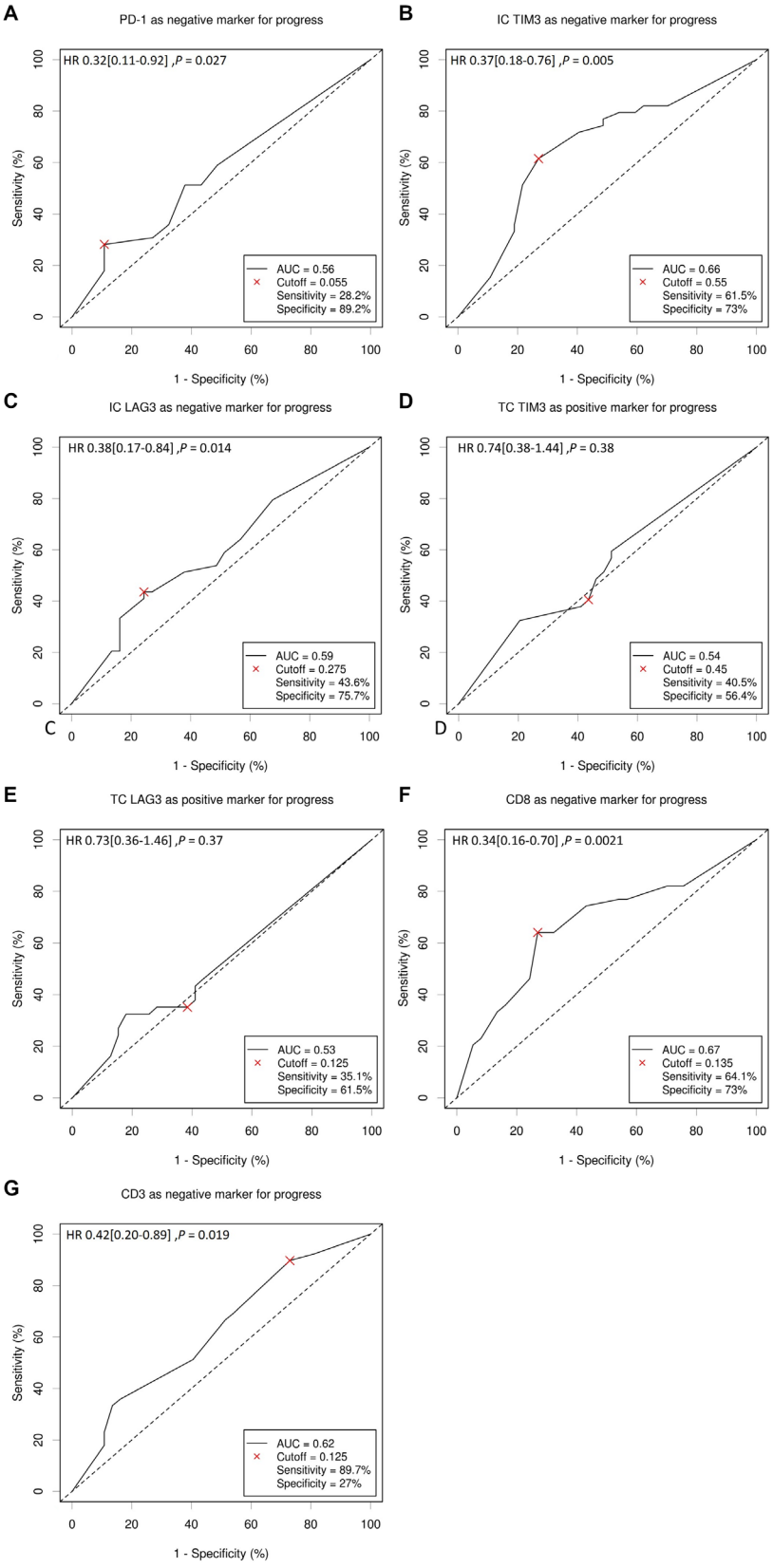
Proteins	LCRT			SCRT			p-value
	Low	High	Median PP (range) (%)	Low	High	Median PP (range) (%)	
PD-1	33	7	1 (0–20)	28	8	2.5 (0–15)	0.465
IC TIM-3	20	20	55 (0–95)	22	14	50 (0–90)	0.737
TC TIM-3	28	12	0 (0–80)	16	20	80 (0–100)	0.007
IC LAG-3	31	9	8 (0–60)	19	17	22.5 (0–80)	0.044
TC LAG-3	32	8	0 (0–100)	16	20	70 (0–100)	0.000
CD8	18	22	15 (0–80)	23	13	8 (0–70)	0.014
CD3	7	33	28(5–90)	7	29	32.5 (0–90)	0.992

Abbreviations: PD-1 – programmed cell death 1; TIM-3 – T cell membrane protein 3; LAG-3 – Lymphocyte activation gene 3; TC – tumor cell; IC – immune cell; PP – percentage of positive cells; LCRT – long-course radiotherapy; SCRT – short-course radiotherapy

Supplementary Table S2. Impact of proteins expression on the OS of patients with rectal cancer.

Variables		OS					
		Univariate			Multivariate		
		HR	95% CI	p-value	HR	95% CI	p-value
Sex	Male / Female	3.567	0.797–15.963	0.096			
Age	≤60 / >60	3.051	1.018–9.145	0.046			
cT	cT2+3 / cT4	0.194	0.043–0.871	0.032	0.194	0.040–0.943	0.042
cN	cN0 / cN1+2	1.693	0.528–5.433	0.376			
cTNM stage	II/III	1.622	0.505–5.211	0.417			
pT	pT0+1+2 / pT3+4	5.327	0.692–41.009	0.108			
pN	pN–/pN+	6.789	1.518–30.372	0.012	4.811	0.953–24.298	0.057
Pathological differentiation	Low grade / Intermediate grade	6.525	2.246–18.958	0.001	4.331	1.221–15.367	0.023
Venous invasion	+/–	0.212	0.074–0.607	0.004			
Neural invasion	+/–	1.458	0.401–5.304	0.567			
Carcinoma nodules	+/–	0.628	0.193–2.044	0.440			
TRG	TRG1+2+3 / TRG4+5	5.592	1.656–21.397	0.006	10.527	2.419–45.818	0.002
PD-1	<5.5% / >5.5%	0.565	0.124–2.565	0.459			
IC TIM-3	<55% / >55%	0.687	0.230–2.052	0.501			
TC TIM-3	<45% / >45%	0.610	0.203–1.836	0.380			
IC LAG-3	<27.5% / >27.5%	1.069	0.361–3.166	0.904			
TC LAG-3	<12.5% / > 12.5%	1.097	0.375–3.205	0.866			
CD3	<12.5% / >12.5%	0.505	0.158–1.611	0.248			
CD8	<13.5% / >13.5%	0.468	0.145–1.510	0.204			

Abbreviations: TRG – tumor regression grade; PD-1 – programmed cell death 1; TIM-3 – T cell membrane protein 3; LAG-3 – Lymphocyte activation gene 3; TC – tumor cell; IC – immune cell



Supplementary Figure S1. Detailed analysis of the optimal cutoff points about the PD-1, TIM-3, LAG-3, CD8, and CD3