Circulating tumor cells counts are associated with CD8+ T cell levels in programmed death-ligand 1-negative non-small cell lung cancer patients after radiotherapy
A retrospective study

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Abstract

This study aimed to explore the dynamics of circulating tumor cells (CTCs) and CD8+ T cells in stage II–III non-small cell lung cancer patients with CTCs in different programmed death-ligand 1 (PD-L1) status treated with radiotherapy and evaluate the correlation between CTCs and CD8+ T cells.

This study was a retrospective study which reviewed 69 stage II–III non-small cell lung cancer patients underwent postoperative radiotherapy and peripheral blood tests of CTCs and T lymphocyte were available before radiation, 1 week after radiation and 1 month after radiation.

In this study, 25 patients had PD-L1 positive CTCs and 44 patients had PD-L1 negative CTCs. The CTCs count was significantly decreased compared with baseline in patients with different PD-L1 status CTCs at 1 week and 1 month after radiotherapy. The proportion of CD8+ T cells was significantly increased at 1 month after radiotherapy compared with baseline in the total population (mean change, 7.24 ± 2.12; $P < .05$) and patients with PD-L1 negative CTCs (mean change, 7.17 ± 2.65; $P < .05$). One month after radiotherapy, the proportion of CD8+ T cells was negatively correlated with the CTCs count in the total population ($r = -0.255$, $P = .034$) and PD-L1 negative patients ($r = -0.330$, $P = .029$). In patients with PD-L1 negative CTCs, the CTCs count 1 week after radiotherapy (hazard ratio, 0.150 [95% confidence intervals., 0.027–0.840], $P = .031$) and the proportion of CD8+ T cells 1 month after radiotherapy (hazard ratio, 7.961 [95% confidence intervals, 1.028–61.68], $P = .047$) were independent prognostic factors for disease recurrence.

After radiotherapy, only PD-L1-negative patients had a significant increase in the CD8+ T cell levels, while it was negatively correlated with CTCs count and was an independent prognostic factors of disease recurrence.

Abbreviations: CI = confidence intervals, CK = cytokeratins, CTCs = circulating tumor cells, HR = hazard ratio, IQR = inter quartile range, NSCLC = non-small cell lung cancer, PD-L1 = programmed death-ligand 1, PFS = progression-free survival, RFS = recurrence free survival, TNT = tumor node metastasis.

Keywords: CD8+ T cells, circulating tumor cells, non-small cell lung cancer, programmed death-ligand 1, radiotherapy

1. Introduction

In early-stage and locally advanced operable non-small cell lung cancer (NSCLC), surgical resection is a mainstay of curative treatment and surgically resected NSCLC cases contributed to a large number of long-term survivors. But, even in patients with early-stage tumors, >50% of patients will relapse in 2 years after surgery.[1] Microscopic or macroscopic residual tumor at the surgical margins is an important risk factor.[2,3] Positive surgery margins may not only cause local recurrence, but also release a large number of tumor cells into the circulatory system to become

circulating tumor cells (CTCs).[4] These circulating tumor cells promote metastasis.[5,6] Therefore, radiotherapy could be applied as adjuvant considering these risk factors after surgery.[7]

In the past, it was difficult to monitor the distribution of tumor cells in the circulatory system after radiotherapy. The emergence of CTC detection technology allows us to have a glimpse. In recent years, some studies indicated CTCs count and persistence of CTCs post-treatment could be used as a prognostic or predictive biomarker for radiotherapy in NSCLC patients.[8,9] Frick et al[8] observed a downward trend in the number of CTCs after radiotherapy and suggested that persistence of CTCs post-radiotherapy was significantly associated with distant metastases.

In addition to directly killing tumor cells, radiotherapy can also induce local and systemic immune responses through different mechanisms.[10] Specifically, radiotherapy promotes the release of tumor antigens and cytokines to recruit immune cells into the tumor microenvironment and activate cytotoxic T-cell to kill tumors.[11] Cytotoxic T cells expressing cell-surface CD8 are the most powerful effectors in the anticancer immune response.[12] CD8+ T cells recognize tumor antigens presented in concert with Major histocompatibility complex class I molecules and are activated. Upon activation, CD8+ T cells effectively lyse tumor cells in their vicinity.[13] Rutkowski et al[14] studied the changes in systemic immune response after Stereotactic Body Radiation Therapy and observed an increase in the proportion of total CD8 + T cells. A higher post-treatment cytotoxic CD8+ T cells level was predictive of better progression-free survival (PFS) in NSCLC patients receiving stereotactic ablative body radiotherapy.[15]

The immune escape theory points out that the high expression of programmed death-ligand 1 (PD-L1) in tumor cells affects the killing effect of immune system on tumor.[16] The PD-L1 state of tumor cell may affect radiotherapy-mediated immunomodulation and the clinical evidence in this aspect still needs to be accumulated.

Radiotherapy affects both CD8+ T cells and CTCs. The decrease of CTCs may be partly contributed by radiotherapy-activated CD8+ T cells. And the PD-L1 status of CTCs may affect the activation of CD8+ T cells by radiotherapy. However, the activation of CD8+ T cells and the reduction of CTCs by radiotherapy in patients with different PD-L1 status are unknown, and few studies linked changes in the CTCs count after radiotherapy with the level of CD8+ T cells. This study aimed to explore the dynamics of CTCs and CD8+ T cells in stage II–III NSCLC patients with CTCs in different PD-L1 status treated with radiotherapy and evaluate the correlation between CTCs and CD8+ T cells.

2. Methods

2.1. Study design and patients

This study was a retrospective study which reviewed 69 NSCLC patients underwent postoperative radiotherapy at Shandong Provincial Chest Hospital and the Third Affiliated Hospital of Shandong First Medical University between August 2019 and October 2020. Eligible patients were aged 18 to 75 years, histologically confirmed NSCLC, stage II–III disease according to the tumor node metastasis (TNM) staging system, and underwent postoperative radiotherapy. All patients' peripheral blood tests of CTCs and T lymphocyte were available before and after radiotherapy. All patients obtained complete demographic data and clinicopathological variables, including sex, age, tumor size, TNM stage, smoking history. Patients with evidence of the

second primary tumor and other serious diseases which might affect the prognosis were excluded. The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Third Affiliated Hospital of Shandong First Medical University ethics committee (NO. FY2020022) and individual consent for this retrospective analysis was waived.

2.2. Treatment and collection of blood samples

After surgery, patients were treated with conventional fractionation (2 Gy fractions). The mediastinal target volume was defined according to clinical guidelines and irradiated up to a total dose of 60 Gy. Patients received or did not receive concurrent or sequential chemotherapy according to the doctor's decision. Patients were followed up regularly from the date of diagnosis to October 2020, or the date of death. Peripheral blood samples (1 tube, typically 15 mL) were obtained from patients to assess for CTCs and T lymphocyte. Patients were assayed before radiation, 1 week after radiation and 1 month after radiation.

2.3. Circulating tumor cells assay

CTCs were isolated via optimized CTC enrichment technique implemented using CD45 antibody. First, erythrocytes were removed by carrying out red blood cell lysis and CD45+ leukocytes were depleted using magnetic bead separation method. CTCs were enriched by passing them through calibrated membrane filters having 8-μm-diameter pores. Detection and classification of CTCs used multiple epithelial markers, including cytokeratins (CK) 8, 18, and 19, epithelial cell adhesion molecule, vimentin, and twist. The PD-L1 mRNA expression level in CTCs was detected by RNA-ISH. The results were analyzed using a fluorescence microscope. PD-L1 expression was calculated by the following method: PD-L1 = 0 indicated negative expression; PD-L1 > 1 indicated positive expression.

2.4. CD8+ T cells assay

Five specific monoclonal antibodies against CD3 (APC), CD4 (FITC), CD8 (APC), CD25 (PE), Foxp3 (APC) were used to differentiate lymphocyte subsets. At beginning, 100 μL peripheral blood was mixed with the above monoclonal antibodies and incubated at room temperature for 15 minutes in the dark. FACS lysing solution (BD Biosciences, San Jose, CA) was used to lyse red blood cells in the mix and then washed twice with phosphate buffered saline. After that, flow cytometry was used to analysis the residual white blood cells and the proportions of CD8+ T cells were calculated by FlowJo Version 10 data analysis software (FlowJo, Ashland, OR).

Lymphocyte subpopulations were identified as follow: CD3+ T cells (CD3+CD19-), CD4+ T cells (CD3+CD4+CD8-), CD8+ T cells (CD3+CD8+CD4-), and CD4+CD25+Foxp3+ Treg cells. Lymphocyte subsets were determined by the percentages of total lymphocytes.

2.5. Statistical analyses

All statistical analyses were performed using SPSS 21.0 statistical software (IBM Corp., Armonk, NY). Demographic data, outcome data, and other clinical parameters were presented as

the proportions for categorical variables and as the median (inter quartile range) for continuous variables. Categorical variables categorical variables were compared using the chi-squared test or Fisher exact test, as appropriate. Continuous variables were tested using an independent samples *t* test or Mann–Whitney *U* test. Repeated measures analysis of variance was used to evaluate the mean change of CTCs and CD8+ T cell. We used the median as a threshold to define high-level group and low-level group. Recurrence free survival (RFS) was calculated from the start date of postoperative radiotherapy to the first occurrence of disease recurrence (local, nodal, or distant disease). Patients who did not recurrence by the last follow-up date were censored. RFS were estimated using the Kaplan–Meier method and compared by the log-rank test. The multivariate Cox proportional hazards models were performed to estimate hazard ratio (HRs) and 95% confidence intervals (CI), adjusted for clinical stage, histology, smoking history, and resected margins. Linear correlations were based on the Spearman correlation coefficient. Two-sided *P* values <.05 were considered statistically significant.

3. Results

3.1. Patient characteristics

The demographic characteristics of 69 NSCLC patients were shown in Table 1. Among these patients, the median age was 62 (inter quartile range, 55, 67); 53 patients were men and 16 patients were women; 50 patients had smoking history; 47 patients were stage III and 22 were stage II; 52 patients had negative resected margins; 30 patients received concurrent or sequential chemotherapy; 40 patients had the histological subtype of adenocarcinoma; and 25 patients had the histological subtype of squamous cell carcinoma. Twenty-five patients had PD-L1 positive CTCs.

3.2. CTCs and CD8+ T cells dynamics

The dynamics of CTCs and CD8+ T cells are summarized in Table 2. The CTCs count was significantly decreased compared with baseline in the total population at 1 week after radiotherapy (mean change, −9.49±2.14; *P*<.05) and 1 month after radiotherapy (mean change, −20.29±3.12; *P*<.05). Patients with CTCs in different PD-L1 status also had significantly reduced CTCs counts at 1 week after radiotherapy and 1 month

Table 1

Characteristics of patients before radiotherapy.

Variable	Total (n=69)
Age, median (IQR)	62 (55–67)
Gender, n (%)	
Male	53 (76.8)
Female	16 (23.2)
Smoking status, n (%)	
Never	19 (27.5)
Former	45 (65.2)
Current	5 (6.2)
Tumor size, cm, median (IQR)	3.5 (2.5–5.2)
Histology, n (%)	
Adenocarcinoma	40 (58%)
Squamous cell	25 (36.2)
Others	4 (5.8)
T stage, n (%)	
T1	26 (37.7)
T2	18 (26.1)
T3	17 (24.6)
T4	8 (11.6)
Clinical stage, n (%)	
II	22 (31.9)
III	47 (68.1)
Resected margins, n (%)	
Positive	17 (24.6)
Negative	52 (75.4)
Concurrent or sequential chemotherapy, n (%)	
Yes	30 (43.5)
No	39 (56.5)
PD-L1 status of CTCs, n (%)	
Positive	25 (36.2)
Negative	44 (63.8)

CTCs = circulating tumor cells, IQR = interquartile range, PD-L1 = programmed death-ligand 1.

after radiotherapy. The proportion of CD8+ T cells was significantly increased at 1 month after radiotherapy compared with baseline in the total population (mean change, 7.24±2.12; *P*<.05) and patients with PD-L1 negative CTCs (mean change, 7.17±2.65; *P*<.05). In patients with PD-L1 positive CTCs, no significant increase in the proportion of CD8+ T cells was found at 1 week after radiotherapy and 1 month after radiotherapy compared with baseline. After adjusting the baseline data, there was no significant difference in CTCs counts between patients

Table 2

Dynamic of CTCs and CD8+ T cell.

	Total	PD-L1 positive	PD-L1 negative
Baseline			
The proportion of CD8+ T cell, median (IQR)	39.83 (30.74, 45.18)	36.13 (29.06, 42.36)	42.08 (34.23, 47.91)
CTCs counts, median (IQR)	18.3 (8.20, 41.9)	17.00 (7.90, 48.80)	18.35 (8.88, 37.53)
One week after radiotherapy			
The proportion of CD8+ T cell, median (IQR)	43.34 (38.46, 46.19)	43.27 (40.56, 47.16)	43.38 (37.35, 46.08)
Mean change from baseline in proportion of CD8+ T cell at 1 week, mean±SE	2.49±2.24	7.03±2.79	−2.06±2.98
CTCs counts, median (IQR)	9.3 (5.15, 26.05)	8.10 (3.20, 25.05)	9.35 (5.85, 26.27)
Mean change from baseline in CTCs counts at 1 week, mean±SE	−9.49±2.14[*]	−11.07±2.80[*]	−7.91±2.80[*]
One month after radiotherapy			
The proportion of CD8+ T cell, median (IQR)	51.25 (37.62, 57.67)	49.80 (31.78, 53.40)	53.61 (42.58, 60.36)
Mean change from baseline in proportion of CD8+ T cell at 1 month, mean±SE	7.24±2.12[*]	7.31±3.10	7.17±2.65[*]
CTCs counts, median (IQR)	1.9 (0.50, 10.15)	2.10 (0, 10.35)	1.85 (0.50, 9.98)
Mean change from baseline in CTCs counts at 1 month, mean±SE	−20.29±3.12[*]	−22.20±4.75[*]	−18.38±3.84[*]

CTCs = circulating tumor cells, IQR = interquartile range, SE = standard error.
[*] *P*<.05.

Table 3

Correlations of CD8+ T cell and CTCs before and after radiotherapy.

	Total		PD-L1 positive		PD-L1 negative	
	r	*P* value	*r*	*P* value	*r*	*P* value
One week after radiotherapy						
The proportion of CD8+ T cell						
CTCs counts	−0.136	.266	−0.388	.056	0.048	.758
One month after radiotherapy						
The proportion of CD8+ T cell						
CTCs counts	−0.255	.034	−0.240	.247	−0.330	.029

CTCs = circulating tumor cells, PD-L1 = programmed death-ligand 1.

with CTCs in different PD-L1 status after radiotherapy (at 1 week, $P=.535$; at 1 month, $P=.536$). Similarly, no significant difference in the proportion of CD8+ T cells was found between patients with CTCs in different PD-L1 status after radiotherapy (at 1 week, $P=.969$; at 1 month, $P=.279$).

One month after radiotherapy, the proportion of CD8+ T cells was negatively correlated with the CTCs count in ($r=-0.255$, $P=.034$) and patients with PD-L1 negative CTCs ($r=-0.330$, $P=.029$, Table 3).

3.3. Kaplan–Meier estimates

In the total population, the count of CTCs before radiotherapy ($P=.818$, Fig. 1A) and the count of CTCs 1 week after radiotherapy ($P=.145$, Fig. 1B) were not significantly associated with disease recurrence. Patients with the count of CTCs ≤1.9 1 month after radiotherapy had significant longer RFS than that with the count of CTCs >1.9 (not reach vs 8.75 months [95% CI, 5.56–11.95], $P=.013$, Fig. 1C). The proportion of CD8+ T cells >39.83 was not significantly associated with disease recurrence before radiotherapy ($P=.172$, Fig. 1D) and 1 week after

radiotherapy ($P=.410$, Fig. 1E). One month after radiotherapy, patients with the proportion of CD8+ T cells >51.25 had longer RFS than patients with the proportion of CD8+ T cells <51.25 ($P=.048$, Fig. 1F).

In patients with PD-L1 positive CTCs, no significant difference in RFS was found in all subgroups (Fig. 2). In patients with PD-L1 negative CTCs, the subgroup with CTCs count ≤9.35 1 week after radiotherapy had significant longer RFS than that with CTCs count >9.35 ($P=.027$, Fig. 3B). And there was no significant difference in other subgroups of patients with PD-L1 negative CTCs (Fig. 3A and C–F).

3.4. Multivariable survival analyses

In the total population, the multivariate cox regression analysis adjusting for clinical stage, histology, smoking history, and resected margins, further revealed that the CTCs count 1 month after radiotherapy was independent prognostic factor for disease recurrence (HR, 0.125 [95% CI, 0.025–0.624], $P=.011$, Table 4). The proportion of CD8+ T cells 1 month after radiotherapy had a trend towards reducing recurrence, but was

Figure 1. Kaplan–Meier analysis of recurrence-free survival. Recurrence-free survival in patients with different cut-off values of circulating tumor cells before radiotherapy (A), 1 week after radiotherapy (B), and 1 month after radiotherapy (C). Recurrence-free survival in patients with different cut-off values of CD8+ T cells before radiotherapy (E), 1 week after radiotherapy (F), and 1 month after radiotherapy (G).
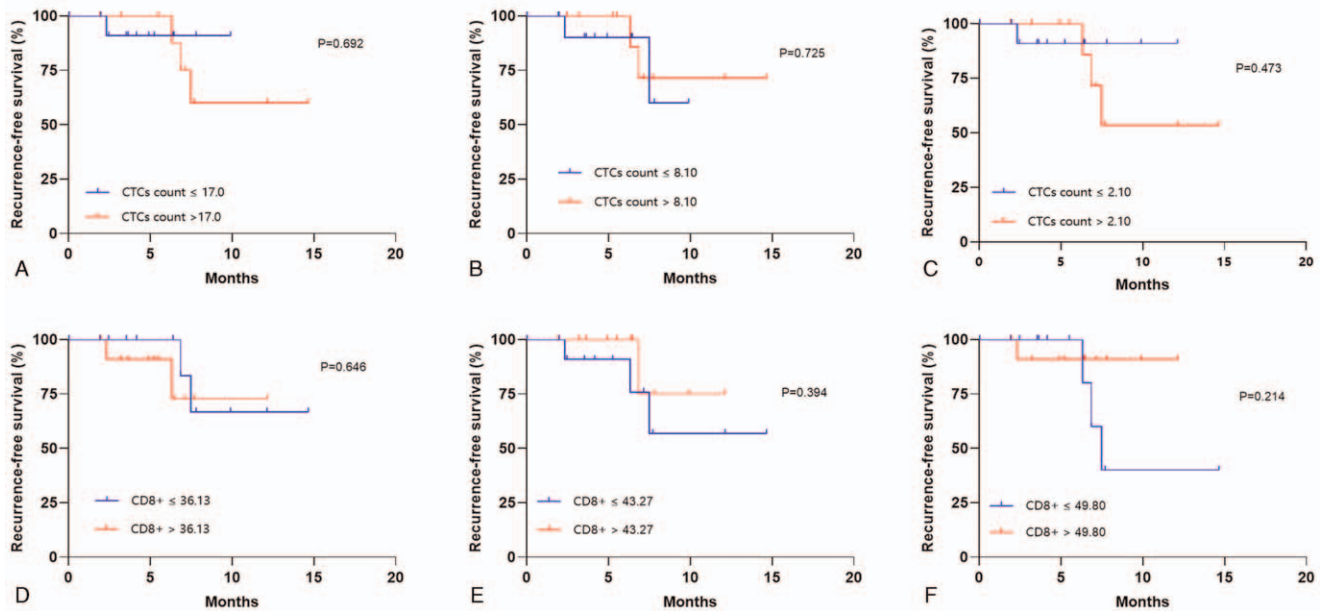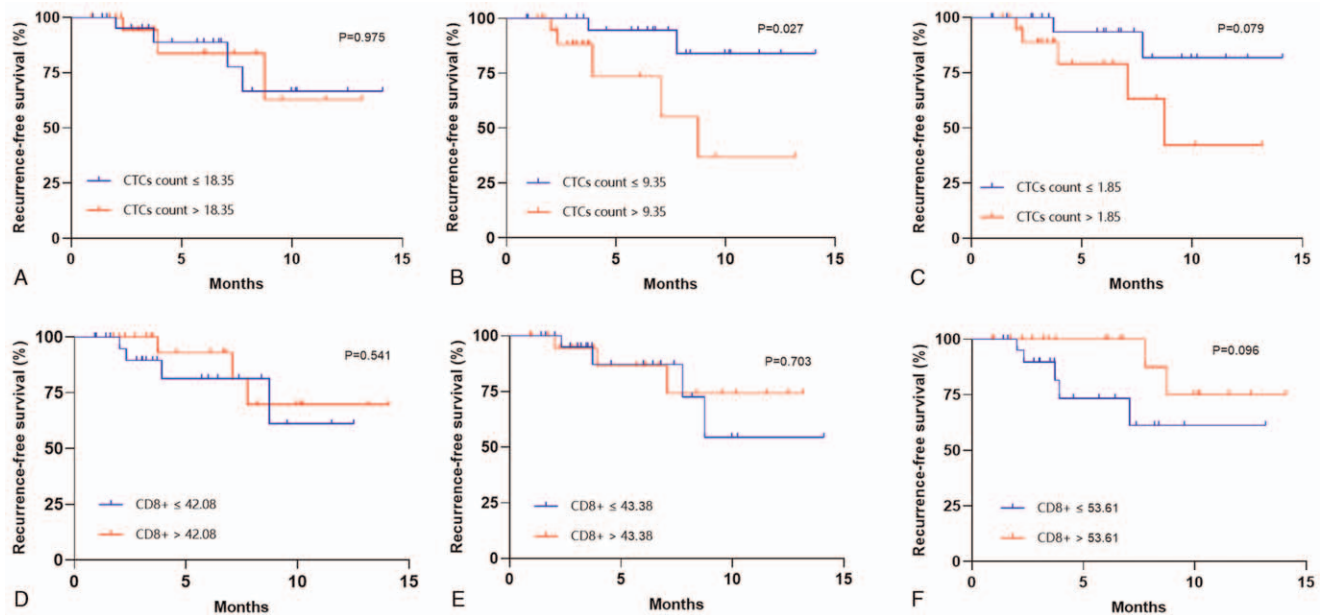
Figure 2. Kaplan–Meier analysis of recurrence-free survival in PD-L1 positive patients. Recurrence-free survival in patients with different cut-off values of circulating tumor cells before radiotherapy (A), 1 week after radiotherapy (B), and 1 month after radiotherapy (C). Recurrence-free survival in patients with different cut-off values of CD8+ T cells before radiotherapy (E), 1 week after radiotherapy (F), and 1 month after radiotherapy (G). PD-L1 = programmed death-ligand 1.

not statistically significant (HR, 3.807 [95% CI, 0.899, 16.112], $P=.069$, Table 4).

In patients with PD-L1 positive CTCs, CTCs count and the proportion of CD8+ T cells were not significantly associated with disease recurrence, after adjusting for clinical stage, histology, smoking history, and resected margins (Table 4).

In patients with PD-L1 negative CTCs, the CTCs count 1 week after radiotherapy (HR, 0.150 [95% CI, 0.027–0.840], $P=.031$)

and the proportion of CD8+ T cells 1 month after radiotherapy (HR, 7.961 [95% CI, 1.028–61.68], $P=.047$) were independent prognostic factors for disease recurrence (Table 4).

4. Discussion

In this study, 69 patients with stage II–III NSCLC treated with radiotherapy were retrospectively analyzed. The CTCs count was

Figure 3. Kaplan–Meier analysis of recurrence-free survival in PD-L1 negative patients. Recurrence-free survival in patients with different cut-off values of circulating tumor cells before radiotherapy (A), 1 week after radiotherapy (B), and 1 month after radiotherapy (C). Recurrence-free survival in patients with different cut-off values of CD8+ T cells before radiotherapy (E), 1 week after radiotherapy (F), and 1 month after radiotherapy (G). PD-L1 = programmed death-ligand 1.

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radiotherapy leading to appropriate CD8 + T cells priming. We also found that the proportion of CD8+ T cells of patients with PD-L1 negative CTCs was negatively correlated with CTCs count at 1 month after radiotherapy, but no correlation was found in patients with PD-L1 positive CTCs. Combined with our previous results, this further indicates that CD8+ T cells were not activated enough to kill tumor cells in PD-L1 positive patients.

CTCs as a prognostic indicator of non-small cell lung cancer has been widely confirmed,[28,29] and could predict prognosis following target therapy and chemotherapy.[30,31] CTCs also has a suggestive effect on the recurrence of NSCLC patients after radiotherapy. The continuous detectability of CTCs after radiotherapy was associated with distant failure ($P = .04$), and there was a trend of increasing regional ($P = .08$) and local failure ($P = .16$).[8] In the present study, the CTCs count 1 month after radiotherapy was independent prognostic factor for disease recurrence in the total population and patients with PD-L1 negative CTCs.

In univariate analysis, we found that patients with the higher proportion of CD8+ T cells 1 month after radiotherapy had significantly longer RFS, but no statistical significance was observed in the multivariate analysis. However, in patients with negative CTCs, the proportion of CD8+ T cells 1 month after radiotherapy was independent prognostic factors for disease recurrence. This indicates that the activation of immune system by radiotherapy may benefit patients after excluding the interference of PD-L1. In recent studies, the relationship between CD8+ T cells and patients' prognosis was still uncertain. Zheng et al,[15] reported that an elevated posttreatment cytotoxic CD8+ T cells level was an independent prognostic factor for longer PFS (HR, 1.16; 95% CI, 1.01–1.28; $P = .01$). An et al [32] did not find a relationship between the proportion of CD8+ T cells and PFS, but found that the %divided of CD8+ T cells was an independent predictor for PFS (HR, 4.342, 95% CI 1.324–14.245; $P = .015$). Liu et al[33] suggested that the pre-radiotherapy CD8+CD28+ T cell count could independently predict tumor response 1 month after radiotherapy (OR 0.19, 95% CI 0.04–0.90; $P = .037$). From these studies' point of view, the evidence of the relationship between CD8 + T cells and the prognosis of radiotherapy still need to be accumulated.

This study has some limitations. This is a retrospective study and some data have not been able to collect so that more in-depth analysis is not able to be conducted. The sample size of this study is small. The follow-up time of the patients was relatively short, only a small number of disease recurrence events were observed, no death events were observed, and the overall survival could not be evaluated. Therefore, a prospective study with long-term follow-up is needed.

5. Conclusion

After radiotherapy, CTCs were significantly reduced in patients with different PD-L1 status, but CD8+ T cell was only significantly increased in PD-L1 negative patients, but not in PD-L1 positive patients. Furthermore, the proportion of CD8+ T cells was negatively correlated with the CTCs count and it could independently predict disease recurrence in patients PD-L1 negative CTCs after radiotherapy. Therefore, for PD-L1 positive early-stage NSCLC patients, the combination regimen of radiotherapy and immune checkpoint inhibitors may be a choice to improve the immune activation effect.

Author contributions

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Methodology: Qingyun Liu, Chaoren Zhao.
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Writing – review & editing: Penghui Jiang, Dawei Liu.

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