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Blood Lymphocytes as a Prognostic Factor for Stage III Non-Small Cell Lung Cancer with Concurrent Chemoradiation

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We aimed to identify blood lymphocytes as a prognostic factor for survival in patients with locally advanced stage III non-small cell lung cancer (NSCLC) treated with concurrent chemoradiotherapy (CCRT). This is a secondary study of 196 patients enrolled in the Korean Radiation Oncology Group 0903 phase III clinical trial to evaluate the prognostic significance of circulating blood lymphocyte levels. The median total lymphocyte count (TLC) reduction ratio during CCRT was 0.74 (range: 0.29-0.97). In multivariate analysis, patient age (p=0.014) and gross tumor volume (GTV, p=0.031) were significant factors associated with overall survival, while TLC reduction (p=0.018) and pretreatment neutrophil-to-lymphocyte ratio (NLR; p=0.010) were associated with progression-free survival (PFS). In multivariate logistic regression analysis, pretreatment NLR, GTV, and heart V20 were significantly associated with TLC reduction. Immunohistochemical analysis of programmed death ligand 1 and CD8 expression on T cells was performed on 84 patients. CD8 expression was not significantly associated with the pretreatment lymphocyte count (p=0.673), and PDL1 expression was not significantly associated with OS or PFS. Univariate analysis revealed that high CD8 expression in TILs was associated with favorable OS and was significantly associated with favorable PFS (p=0.032). TLC reduction during CCRT is a significant prognostic factor for PFS, and heart V20 is significantly associated with TLC reduction. Thus, in the era of immunotherapy, constraining the volume of the radiation dose to the whole heart must be prioritized for the better survival outcomes.

Key Words: *Lymphocytes; Tumor-infiltrating Lymphocytes; Chemoradiotherapy; Non-Small-Cell Lung Carcinoma*

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INTRODUCTION

Immune surveillance plays an important role in the pathogenesis and progression of non-small cell lung cancer (NSCLC). The failure of host immune surveillance mechanisms, in which lymphocytes play a pivotal role, is a key step in the early stages of tumor development.[1] The outcome of cancer-immune interactions —"cancer immunogram"— is based on a number of largely unrelated parameters, such as tumor "foreignness" and T-cell inhibitory mechanisms, and the proposed cancer immunogram assumes that T-cell activity is the ultimate effector mechanism in human tumors.[2] Among the parameters constituting a reasonable framework for building such an immunogram, targetable biomarkers for immunotherapy are the lymphocyte count, intratumoral T-cell infiltration, and the presence of T-cell checkpoints, such as cytotoxic T lymphocyte-associated protein 4 (CTLA4) and programmed cell death protein 1 (PD1) – known as immune checkpoints. Immune checkpoint inhibitors (ICIs) are approved for the treatment of advanced NSCLC.[3,4]

Lymphocytes are highly sensitive to radiotherapy (RT) and a reduction in total blood lymphocyte count is a com-

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mon consequence of irradiation.[5] Reduced absolute lymphocyte count (ALC) and an elevated neutrophil-to-lymphocyte ratio (NLR) are independent negative prognostic factors for survival in many malignancies.[6-10] The occurrence of radiation-associated lymphopenia (RAL) is dependent on the field size, fraction number, and treatment duration.[11,12] In particular, exposure of immune-related organs, such as the lungs and heart, is associated with immunosuppression during treatment, resulting in worse patient outcomes.[8,13] Moreover, severe lymphopenia at the onset of immunotherapy is associated with poor survival in patients treated with ICIs.[14] In clinical settings, following the concurrent administration of RT and chemotherapy, RAL at the onset of ICI therapy was found to be associated with increased mortality. The authors explained that the effector activity of ICIs relies on cytotoxic T lymphocytes, and that RAL might negate the activity of ICIs.

Therefore, in the present study, we aimed to identify blood or tumor-infiltrating lymphocytes as a prognostic factor for survival and identify any risk factors affecting clinically significant RAL in those patients whose group underwent concurrent chemoradiotherapy.

MATERIALS AND METHODS

1. Patients

The current study was a secondary analysis of patients enrolled in the Korean Radiation Oncology Group (KROG) 0903 phase III prospective randomized multicenter clinical trial. Details on the patient eligibility criteria and treatment scheme for the KROG 0903 trial are reported in the primary outcome manuscript.[15] For the present study, we included patient data from only one participating institute. The study was approved by the institutional review board of Chonnam National University Hwasun Hospital (CNUHH-2010-010) and was performed in accordance with the Declaration of Helsinki and the International Conference on Harmonization/Good Clinical Practice. Patient enrollment began after obtaining approval from institutional review board. All patients provided written informed consent.

2. Dosimetric analysis and hematologic evaluation

Treatment plans were generated with lung heterogeneity correction using Eclipse version 8.1 (Varian Medical Systems, Palo Alto, CA, USA). For treatment planning, the dosimetric constraints for normal tissues included the lungs, heart, and esophagus. The esophageal contour began at the level of the cricoid cartilage and extended to the gastroesophageal junction. The lung contours included the air-inflated lung parenchyma, excluding fluid and atelectasis on CT scans. Heart contours were reviewed and recontoured as necessary according to the RTOG 0617 secondary analysis atlas.[16] The relative percent volumes of the lung and heart receiving at least 2, 5, 10, 12.5, 15, 20, 30, 40, 50, and 60 Gy, and the mean doses were identified. There was a strong correlation between clinical factors, particularly among the dose-volume histogram parameters. To avoid multicollinearity, the variance inflation factor or the Pearson's correlation coefficient was calculated. Finally, we chose lung V2, V20, V50, and V60 and heart V2, V20, V50, and V60. None of the variables had a variance inflation factor of >10 or a Pearson correlation coefficient of ≥ 0.8.

The peripheral blood count was analyzed before, during, and after concurrent chemoradiotherapy (CCRT). Complete blood count data, including the white blood cell (WBC) count, absolute lymphocyte count (ALC), monocyte count, and absolute neutrophil count (ANC), were collected within the 2 weeks before initiating RT, weekly during CCRT, and randomly whenever patients were followed up after completing RT. The nadir blood count was the lowest during CCRT. The NLR was calculated by dividing the ANC by the ALC. Clinically significant lymphopenia was defined as an ALC nadir of <500 cells/μL during CCRT. Total lymphocyte count (TLC) reduction was defined as the ratio of baseline TLC minus nadir TLC during CCRT to baseline TLC.

3. Immunohistochemistry for programmed death-ligand 1 (PDL1) and CD8

Immunohistochemistry was performed using formaldehyde-fixed paraffin-embedded (FFPE) tissue specimens obtained on the first diagnostic biopsy of 84 patients. Immunohistochemical staining was performed with 5-μm entire standard tissue sections of FFPE tumor samples. To detect PDL1, a pre-diluted PDL1 rabbit monoclonal antibody (SP263; Ventana Medical Systems, Oro Valley, Arizona, USA) was used as the primary antibody, with staining performed on a Ventana Benchmark Ultra autostainer using an UltraView diaminobenzidine kit (Ventana Medical Systems, Oro Valley, AZ, USA). Immunohistochemical staining for CD8 expression on T cells was performed using an anti-CD8 mouse monoclonal antibody (clone 144 B, diluted 1:100; Abcam, Cambridge, UK) as the primary antibody, with staining performed using the BondMax Leica autostainer.

1) Assessment of PDL1 expression: PDL1 expression in the tumor cells was quantitatively measured using an established immunohistochemical assay (Ventana SP 263). The PDL1 tumor proportion score (TPS), which is the percentage of tumor cells showing partial or complete membrane staining, was calculated. PDL1 expression in $\geq 1\%$ of tumor cells was considered positive. We divided patients into PDL1-positive and PDL1-negative groups (Figs. 1A and 1B).

2) Assessment of CD8 expression on tumor-infiltrating lymphocytes (TILs): CD8 expression on lymphocytes was reported as the proportion of positive cells among all nucleated cells in the adjacent stromal compartment of the tumor nests. Scoring was recorded as the count of TILs with CD8 expression, which was classified as a low count ($<30\%$) or a high count ($\geq 30\%$) (Figs. 1C and 1D).

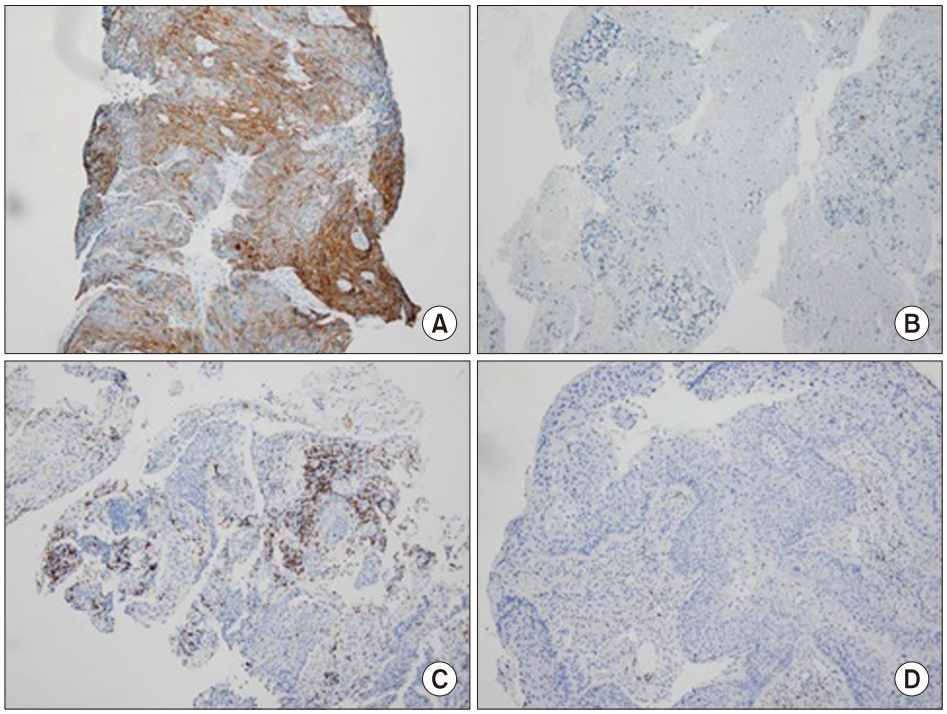

Fig. 1. Immunohistochemical (IHC) staining for programmed cell death-ligand 1 (PDL1), (A) positive and (B) negative staining. Immunohistochemical (IHC) staining for $CD8^+$ tumor infiltrating lymphocyte (TIL), (C) high group ($\geq$30%) and (D) low group ($<$30%).

4. Follow-up and statistical analyses

The patients were evaluated weekly and clinically indicated during treatment, and the protocol was continued as needed. After the completion of CCRT, patients were followed-up using CT scans after 1 month and then at 3-month intervals for the first 1-3 years, and then every 6 after 3 years thereafter until death. Acute and late toxicities were graded using the Common Terminology Criteria for Adverse Events (CTCAE), version 3.0. Acute toxicities were defined as those that occurred within 90 days of treatment initiation, while late toxicities were defined as those that occurred thereafter. The maximum toxicity grade was chosen to represent the final toxicity grade among the acute toxicities during radiotherapy and late toxicities at follow-up visits. Progression-free survival (PFS) was defined as the period between the start of CCRT and tumor progression or death in the absence of disease progression. Disease progression within the initial radiation field, which included the primary tumor and the involved lymph nodes, was defined as local failure; distant failure was defined as recurrence outside the radiation field; and elective nodal failure is defined as an uninvolved nodal failure outside the initial irradiation field.

Survival rates were calculated using the Kaplan–Meier method and compared using log-rank statistics. The forward conditional Cox regression model was used for the multivariate analysis. Multivariate logistic regression was used to analyze the correlation between TLC reduction and dosimetric parameters. Maxstat, the maximum chi-square method in R 2.13.0 (R Development Core Team, Vienna, Austria, http://www.R-project.org), was used to identify the optimal cutting points. Statistical analyses were performed using SPSS, version 21 (SPSS Inc., Chicago, IL, USA), and p-values $<$ 0.05 were considered statistically significant.

RESULTS

1. Patient characteristics

The clinical characteristics of the 196 patients are presented in Table 1. A total of 183 patients (93.4%) were men, with an age range of 40 to 75 years (median: 66 years). Among them, 146 patients (74.5%) had squamous cell carcinoma, with stage IIIA and IIIB tumors constituting 71.9% and 28.1% of the cases, respectively. FDG-PET was performed for all patients. The median follow-up period for all patients was 23 months (range: 2-99 months). The median follow-up time was 40 months for the surviving patients.

2. Survival analysis and prognostic factor determination

The median overall survival (OS) of all 196 patients was 29 months, and the 2-year and 5-year OS rates were 55.9% and 29.8%, respectively. The 2-year and 5-year PFS rates were 32.3% and 25.3%, respectively, and with median PFS of 12 months. The 2-year and 5-year actuarial local control rates were 54.3% and 48.1%, respectively, and the elective nodal failure rate was 6.1% (observed in 12 of 196 patients). The factors associated with OS and PFS in univariate analysis are summarized in Table 2. In the multivariate analysis, age and GTV were identified as significant factors associated with OS, while TLC reduction and pretreatment NLR were associated with PFS (Table 3 and Fig. 2). Both local recurrence-free survival (LRFS) and distant recurrence-free survival (DRFS) were significantly associated with

TABLE 1. Patients character (n=196)

Character	All patients (median)
Age, years	40-75 (66)
Gender	
Male	183 (93.4%)
Female	13 (6.6%)
ECOG PS	
0	25 (12.8%)
1	171 (87.2%)
Weight loss (%)	0-19 (0)
Smoking History	
Never	16 (8.2%)
Ex-smoker	53 (27.0%)
Current smoker (<1 y quit)	127 (64.8%)
Histology	
SqCC	146 (74.5%)
Non-SqCC	50 (25.5%)
Clinical stage	
IIIA	141 (71.9%)
IIIB	55 (28.1%)
GTV (cm^3)	20.2-869.0 (154.65)
Hematologic parameters (cells/μL)	
Pre-treatment WBC	3400-32800 (8700)
Pre-treatment ALC	860-4640 (2130)
Pre-treatment ANC	1039-9760 (4985)
Pre-treatment monocyte	150-1870 (680)
Pre-treatment NLR	0.22-8.95 (2.33)
DLCO (%)	27-193 (89)
RT duration (day)	27-56 (41)
Chemotherapy cycle	4-6 (6)

ECOG PS: Eastern Cooperative Oncology Group Performance Status, SqCC: squamous cell carcinoma, WBC: white blood cell, ALC: absolute lymphocyte count, ANC: absolute neutrophil count, NLR: neutrophil-to-lymphocyte ratio, FEV_1: forced expiratory volume in one second, DLCO: diffusing capacity for carbon monoxide.

TLC reduction during CCRT. In contrast, the GTV was significantly associated with LRFS, and the pretreatment NLR was associated with DRFS.

3. Association between peripheral blood cells and dosimetric parameters

The median WBC, lymphocyte, neutrophil, and monocyte counts before CCRT were 8700 cells/μL (range: 3400-32800 cells/μL), 2130 cells/μL (range: 860-4640 cells/μL), 4985 cells/μL (range: 1039-9760 cells/μL), and 680 cells/μL (range: 150-1870 cells/μL), respectively (Table 1). Lymphocytes decreased more markedly than other blood cell counts immediately after the second week of CCRT (Fig. 3). From weeks 2 to 6, the median ALC was 1520 cells/μL, 1060 cells/μL, 795 cells/μL, 675 cells/μL, and 615 cells/μL, respectively. The median ALC nadir during CCRT was 555 cells/μL (range: 50-1930 cells/μL); the difference between the ALC before CCRT and nadir was 1520 cells/μL (range: 440-3640 cells/μL); and the median TLC reduction was 0.74 (range: 0.29-0.97). Lymphopenia was the most common hematologic toxicity grade ≥3 during CCRT (Table 4).

In multivariate logistic regression analysis, the pretreatment NLR, GTV, and heart V20 were significantly associated with TLC reduction (Table 5). Patient age, treatment duration, and RT technique were not significant factors in the multivariate analysis. Lung V2, lung V20, and heart V2 doses were significantly associated with TLC reduction in univariate analysis, although the significance was not retained in multivariate analysis.

4. Toxicity analysis

The overall incidence rates of radiation pneumonitis and esophagitis are shown in Table 4. Clinically significant radiation pneumonitis and esophagitis of grade ≥3 were observed in ten patients (5.1%) and seven patients (3.6%), respectively. On pretreatment CT scans, nine patients (4.6%) had interstitial lung disease. On univariate analysis, older age, lower lobe interstitial lung disease, and low diffusing capacity for carbon monoxide were significantly associated with grade ≥3 radiation pneumonitis (Supplementary Table 1). Dosimetric analysis showed that MLD, V5, V10, V20, and V30 of the whole lung were significantly associated with grade ≥3 radiation pneumonitis (Supplementary Table 2). Dosimetric parameters were not significantly associated with grade ≥3 radiation esophagitis, but there was a trend toward a higher radiation dose in patients with severe grade ≥3 radiation esophagitis (Supplementary Table 3). After CCRT, 22 patients (11.2%) developed heart disease. Eleven patients had heart problems before treatment, including nine with ischemic heart disease and two with arrhythmia. Most cardiac events occurred within 3 years of CCRT, and there was no significant association between clinical factors and cardiac events after CCRT. Maximum doses to the entire heart and left ventricle were significantly associated with cardiac toxicity after CCRT (Supplementary Table 4).

5. Subgroup analysis for PD-L1 and CD8 TIL expression

For PDL1 and CD8 analyses, adequate histological tissues containing abundant tumor cells were obtained from 84 patients. The relationship between PDL1 expression and patient characteristics is shown in Table 6. No significant correlation was found between PDL1 expression and patient characteristics. Patients with more advanced disease stages showed a tendency toward PDL1 expression (p=0.070). No correlation was found between PDL1 and CD8 expression. CD8 expression was not significantly associated with the pretreatment lymphocyte count (p=0.673), and PDL1 expression was not significantly associated with OS or PFS (Figs. 4A and 4C). Univariate analysis revealed that high CD8 expression in TILs was associated with favorable OS (Fig. 4B, p=0.068) and was significantly associated with favorable PFS (Fig. 4D, p=0.032).

DISCUSSION

Platinum-based doublet concurrent chemoradiotherapy

TABLE 2. Univariate analysis with treatment outcome

Variable	No (%)	OS		PFS		LRFS		DRFS	
		MS (months)	p value	MS (months)	p value	MS (months)	p value	MS (months)	p value
Age (year)									
≤65	91 (46.4)	39		13		21		22	
>65	105 (53.6)	22	0.014	11	0.041	14	0.028	15	0.024
Clinical stage									
IIIA	141 (71.9)	33		13		15		17	
IIIB	55 (28.1)	27	0.592	10	0.473	14	0.625	17	0.527
Gender									
Male	183 (93.4)	29		12		15		17	
Female	13 (6.6)	36	0.532	11	0.233	14	0.950	16	0.489
ECOG PS									
0	25 (12.8)	43		13		14		14	
1	171 (87.2)	28	0.124	12	0.336	15	0.364	17	0.341
Weight loss									
≤5%	140 (71.4)	34		13		17		17	
>5%	56 (28.6)	20	0.509	8	0.382	12	0.446	11	0.500
Current smoking									
No	69 (35.2)	29		12		18		18	
Yes (quit <1 y)	127 (64.8)	29	0.813	13	0.595	14	0.997	16	0.740
Pre NLR									
≤2.63	119 (60.7)	38		13		16		18	
>2.63	77 (39.3)	28	0.194	11	0.047	14	0.346	12	0.013
TLC reduction									
≤0.78	127 (64.8)	37		14		18		19	
>0.78	69 (35.2)	20	0.018	8	0.014	11	0.008	11	0.018
Nadir ALC (cell/μL)									
≤500	113 (57.7)	36		13		18		19	
<500	83 (42.3)	24	0.031	10	0.075	12	0.029	12	0.045
NLR during CCRT									
≤9.25	163 (83.2)	35		13		17		17	
>9.25	33 (16.8)	17	0.039	8	0.020	8	0.012	9	0.033
GTV (cc)									
≤165	104 (53.1)	38		14		22		22	
>165	92 (46.9)	19	0.032	10	0.022	11	0.010	12	0.088
RT duration (days)									
≤42	128 (65.3)	35		13		14		18	
>43	68 (34.7)	21	0.300	11	0.388	14	0.374	14	0.245
RT technique									
3D CRT only	87 (44.4)	28		11		14		16	
IMRT*	109 (55.6)	36	0.867	13	0.567	16	0.728	18	0.804

*Patients had hybrid 3D CRT (32 patients) & IMRT (77 patients). 3D CRT: three dimensional conformal radiotherapy, ECOG PS: Eastern Cooperative Oncology Group Performance Status, NLR: neutrophil to lymphocyte ratio, TLC reduction: ratio of baseline total lymphocyte count (TLC) minus nadir TLC during concurrent chemoradiation to baseline TLC, Nadir ALC: lowest lymphocyte, GTV: gross tumor volume, OS: overall survival, PFS: progression free survival, LRFS: local recurrence free survival, DRFS: distant recurrence free survival.

(CCRT) followed by durvalumab has been the standard treatment for eligible patients with locally advanced unresectable NSCLC, with good performance and minimal weight loss.[17] Suboptimal primary tumor control has led to radiation dose escalation studies aimed at achieving better treatment outcomes. RTOG 0617 was a landmark study that compared high-dose RT (74 Gy) and standard-dose RT (60 Gy) with or without cetuximab in patients with IIIA or IIIIB NSCLC.[18] The median OS was 28.7 months for patients who received standard-dose radiotherapy and 20.3 months for those who received high-dose radiotherapy (p=0.004). Unexpectedly, there were more treatment-related deaths among patients treated with high-dose RT.

A secondary study of the RTOG 0617 trial evaluated host immune function and tumor control.[19] The effective dose to immune cells was modeled using the RT fractions and doses to the lungs, heart, and whole body. The 2-year OS of patients with a high effective dose to immune cells was

TABLE 3. Multivariate analysis by prognostic factor

Variable	Prognostic factor	HR (95% CI)	p value
OS	Age (year), ≤65 vs. >65	1.579 (1.097-2.274)	0.014
	GTV (cc), ≤165 vs. >165	1.482 (1.036-2.119)	0.031
PFS	TLC reduction, ≤0.78 vs. >0.78	1.510 (1.075-2.121)	0.018
	Pre-treatment NLR ≤2.63 vs. >2.63	1.561 (1.110-2.197)	0.010
LRFS	TLC reduction, ≤0.78 vs. >0.78	1.505 (1.060-2.136)	0.022
	GTV (cc), ≤165 vs. >165	1.467 (1.041-2.068)	0.029
DRFS	Pre-treatment NLR ≤2.63 vs. >2.63	1.578 (1.135-2.244)	0.007
	TLC reduction, ≤0.78 vs. >0.78	1.578 (1.118-2.228)	0.010

OS: overall survival, PFS: progression free survival, LRFS: local recurrence free survival, DRFS: distant recurrence free survival, GTV: gross tumor volume, TLC reduction: ratio of baseline total lymphocyte count (TLC) minus nadir TLC during concurrent chemoradiation to baseline TLC, NLR: neutrophil to lymphocyte ratio, HR: hazard ratio.

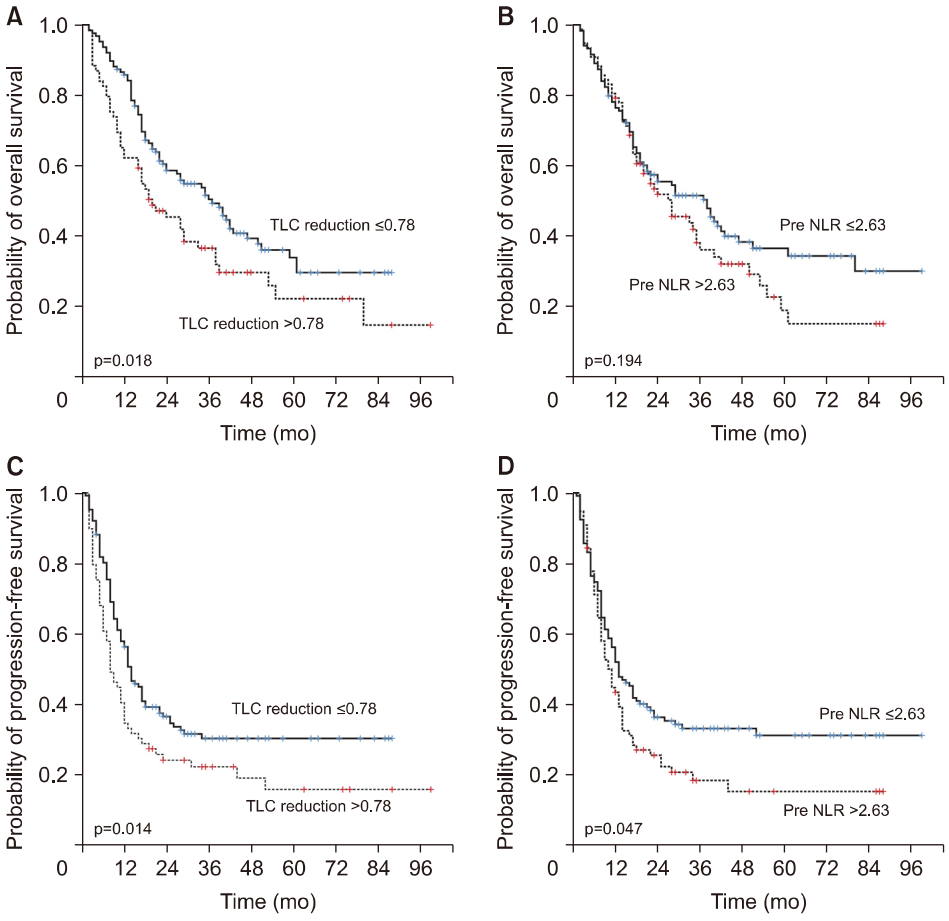

FIG. 2. Overall survival according to (A) total lymphocyte counts (TLC) reduction (≤0.78 vs. >0.78) and (B) pretreatment neutrophil to lymphocyte ratio (NLR) (≤2.63 vs. >2.63). Progression-free survival according to (C) TLC reduction (≤0.78 vs. >0.78) and (D) pretreatment NLR (≤2.63 vs. >2.63).

poor. Immunotoxicity associated with RT has been shown to be a predictive factor for treatment outcomes. The immunosuppressive effects of RT include the inactivation of lymphocytes, recruitment of MDSCs and Treg lymphocytes, M2 polarization of macrophages, secretion of TGF-β, and induction of PDL1 expression on tumor cells.[20]

Lymphocytes are highly sensitive to radiation; indeed, the LD50 of lymphocytes (lethal dose required to reduce the surviving fraction of lymphocytes by 50%) is approximately 2 Gy, and the LD90 is 3 Gy.[21] TLC remains stable during neoadjuvant chemotherapy, grade III-IV lymphocyte reduction was observed in nearly half of the patients 2 months after the initiation of radiation.[6] Treatment-related lymphopenia is more likely to be radiation-related than chemotherapy-related. Our study showed that lymphopenia was the most common grade ≥3 hematologic toxicity during CCRT and lymphocytes decreased more markedly than the other blood cells immediately after CCRT. The median TLC reduction ratio was 0.74, reassuring that radiation could induce profound lymphopenia.

Joseph et al.[9] demonstrated that lymphopenia is associated with poor survival in patients with lung cancer. In

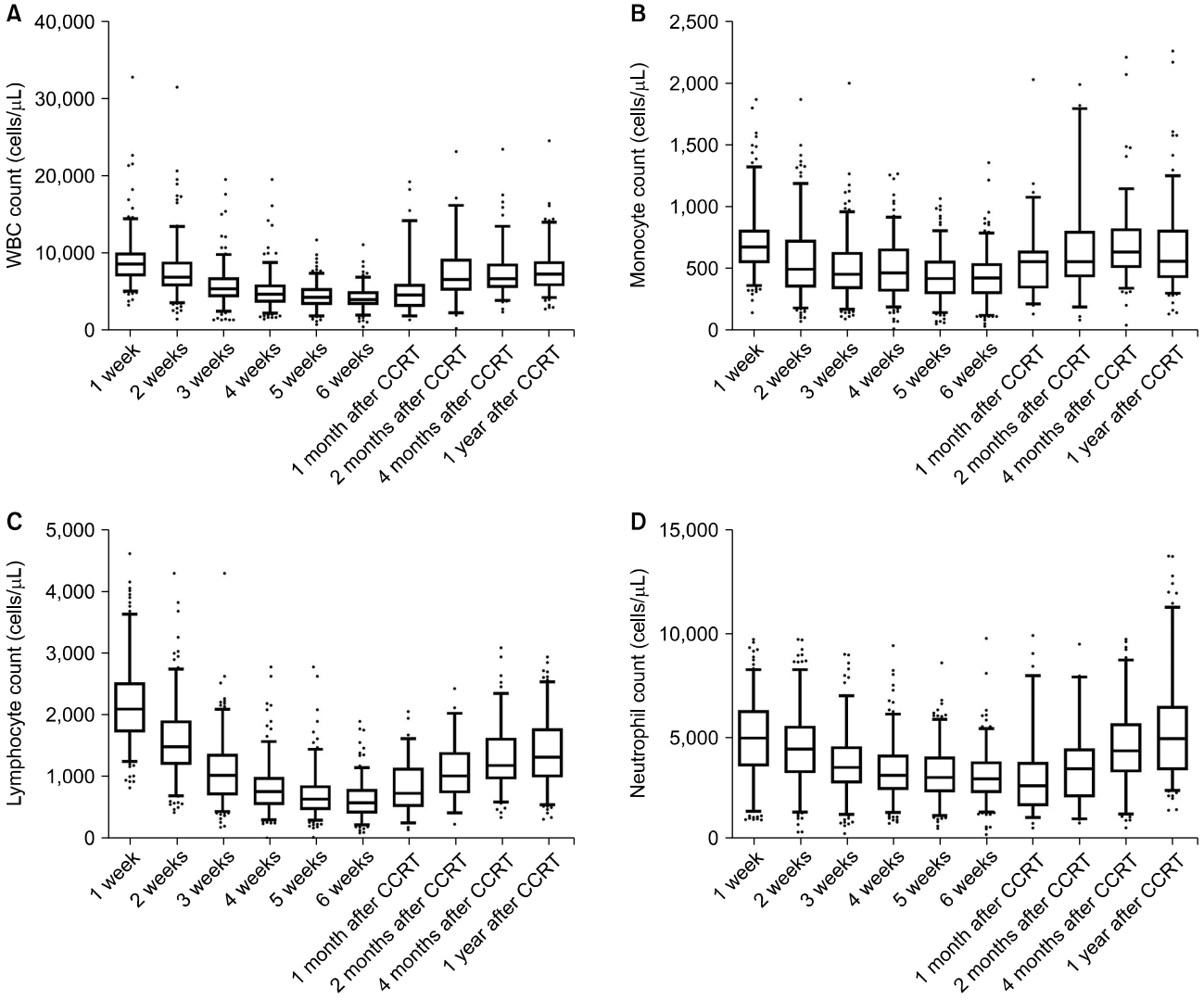

FIG. 3. Changes in total blood counts over time in individual (A) white blood cells (WBCs), (B) monocytes, (C) lymphocytes, and (D) neutrophils.

TABLE 4. Hematologic and non-hematologic toxicity during treatment

Adverse event (n, %)	G0-1	G2	G3	G4	G5
Hematologic toxicity					
Leukopenia	152 (77.6)	27 (13.8)	15 (7.7)	2 (1.0)	0 (0.0)
Neutropenia	147 (75.0)	34 (17.3)	11 (5.6)	4 (2.0)	0 (0.0)
Thrombocytopenia	194 (99.0)	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)
Lymphopenia	43 (21.9)	70 (35.7)	79 (40.3)	4 (2.0)	0 (0.0)
Non-hematologic toxicity					
Radiation pneumonitis	171 (87.2)	15 (7.7)	4 (2.0)	0 (0.0)	6 (3.1)
Radiation esophagitis	142 (72.5)	47 (24.0)	4 (2.0)	2 (1.0)	1 (0.5)

multivariate analysis, low post-treatment ALC, high pre-treatment ANC, and high PTV integral dose were associated with poor survival. The authors explained that a high pre-treatment ANC indicates cancer-induced inflammation and is useful for predicting aggressive tumor biology. Neutrophils promote tumor growth by inducing tumor growth and angiogenesis.[22] The current study demonstrated that TLC reduction was significantly associated with PFS, and that the pretreatment NLR was significantly associated with DRFS. In many studies, a high NLR has been recognized as a poor prognostic indicator of solid cancers.[7,10]

Tang et al.[13] sought to determine factors associated with lymphopenia in patients receiving definitive radiotherapy

Table 5. Logistic regression analysis associating with TLC reduction

Variable	Univariate		Multivariate		
	OR	p value	OR	p value	95% CI
Age (year)	1.016	0.445			
Treatment duration (day)	0.999	0.984			
Pre-NLR	0.764	0.043	0.714	0.018	0.540-0.945
GTV (cm^3)	1.003	0.003	1.003	0.010	1.001-1.005
Lung V2 (%)	1.026	0.002			
Lung V20 (%)	1.077	0.006			
Lung V50 (%)	0.999	0.968			
Lung V60 (%)	0.923	0.184			
Heart V2 (%)	1.018	0.001			
Heart V20 (%)	1.026	0.001	1.025	0.002	1.009-1.040
Heart V50 (%)	1.023	0.093			
Heart V60 (%)	1.027	0.374			
Smoking (others vs. never)	1.029	0.927			
RT technique (3D vs. IMRT)	1.393	0.276			

GTV: gross tumor volume, WBC: white blood cell, ALC: absolute lymphocyte count, ANC: absolute neutrophil count.

Table 6. Relationship between PD-L1 expression, CD8 and patient characteristics

Characteristic	n (%)	PDL1 expression		p value	CD8 expressiom		p value
		No	Yes		Low	High	
Age (year)							
≤65	46 (54.8)	26	20	0.899	34	12	0.800
>65	38 (45.2)	22	16		29	9	
Clinical Stage							
IIIA	60 (71.4)	38	22	0.070	46	14	0.577
IIIB	24 (28.6)	10	14		17	7	
Gender							
Male	77 (91.7)	44	33	1.000	58	19	0.820
Female	7 (8.3)	4	3		5	2	
Smoking							
No	28 (33.3)	13	15	0.161	18	10	0.109
Yes (quit <1 y)	56 (66.7)	35	21		45	11	
Pathology							
SqCC	64 (76.2)	37	27	0.824	48	16	1.000
Non-SqCC	20 (23.8)	11	9		15	5	
Pre NLR							
≤2.63	54 (64.3)	32	22	0.599	40	10	0.066
>2.63	30 (35.7)	16	14		19	11	
GTV (cc)							
≤165	49 (58.3)	27	22	0.655	38	11	0.523
>165	35 (41.7)	21	14		25	10	
CD8							
Low	63 (75.0)	38	25	0.309			
High	21 (25.0)	10	11				

SqCC: squamous cell carcinoma, NLR: neutrophil-to-lymphocyte ratio, GTV: gross tumor volume, PDL1: programmed cell death-ligand 1.

for NSCLC. The results demonstrated that the GTV was more significantly inverse-correlated with the nadir of lymphocytes than with other WBCs, such as neutrophils and monocytes, during RT. In association with TLC reduction, pretreatment NLR, GTV, and heart V20 were significant predictive factors in the current study. To avoid multicollinearity, heart V2, V20, V50, and V60 were selected for the analysis. Multivariate linear regression analysis showed that heart V20 was more significantly associated with TLC reduction than V60. A large volume of the critical lower dose could lead to more lymphocyte destruction (i.e., a greater "low-dose bath").[13] Contreras et al.[8] dem-

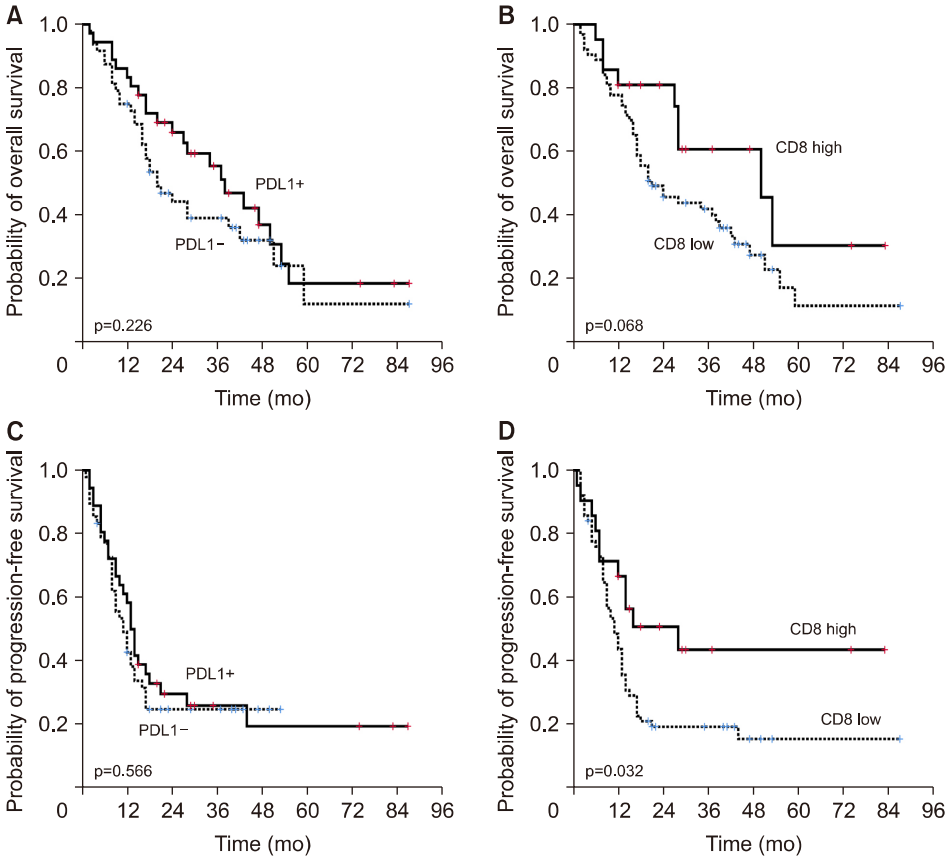

FIG. 4. Overall survival according to (A) programmed death ligand 1 (PDL1) (no vs. yes) and (B) $CD8^+$ tumor infiltrating lymphocyte (TIL) low group ($<30\%$) or high group ($\geq 30\%$). Progression-free survival according to (C) PDL1 (no vs. yes) and (D) $CD8^+$ TIL low group or high group.

onstrated a relationship between lymphopenia and increased heart dose. Most patients (n=310, 77%) underwent CCRT, and male sex, RT alone, percentage of the heart receiving ≥ 50 Gy, and a higher NLR at 4 months were found to be associated with reduced OS in multivariate analysis. In subgroup analyses of patients with stage III disease treated with CCRT, heart V50 $>25\%$ was associated with an elevated NLR at 4 months after RT on multivariable logistic regression analysis.

When lung cancer is treated with RT, the lungs, heart, great vessel, and bone marrow are affected. We confirmed that RAL during CCRT was immediately apparent within 2-3 weeks of treatment. Nadir ALC during CCRT was significantly associated with OS, LRFS, and DRFS in the current study, although the significance disappeared in the multivariate analysis. In contrast, the NLR during CCRT was significantly associated with survival outcomes only on univariate analysis, whereas the pretreatment NLR was significantly associated with PFS and DRFS on multivariate analysis. Hence, we hypothesized that the patient's immune status before treatment might be more important than that during or after treatment in predicting treatment outcomes.

High PDL1 expression predicts the response to pembrolizumab in the primary treatment of advanced NSCLC.[23] In a meta-analysis, PDL1 expression was associated with sex, smoking status, histology, differentiation, tumor size, lymph node metastasis, TNM stage, and EGFR mutation.[24] However, our data did not show a significant relationship between PDL1 expression and the aforementioned clinical parameters, except that patients with more advanced stage IIIB tumors tended to express PDL1. PDL1 does not appear to be a prognostic factor in patients with locally advanced NSCLC who have undergone CCRT alone.[25] Moreover, our data did not reveal a relationship between PDL1 expression and survival outcomes; however, changes in PDL1 expression after CCRT have been shown to be associated with the prognosis of patients with locally advanced NSCLC.[26] Indeed, the OS of patients with locally advanced NSCLC and increased PDL1 expression after CCRT was poorer than that of patients with decreased or unchanged PDL1 expression. Gong et al.[27] showed that PDL1 expression increased after conventional fractionated radiation. Patients with negative PDL1 expression showed significantly higher objective responses and disease control rates than those with positive PDL1 expression. This study demonstrated the possibility that radiotherapy plus anti-PDL1 antibody synergistically enhances antitumor immunity.

TILs are significantly associated with treatment outcomes in NSCLC and survival after therapy.[28] Tokito et al.[25] demonstrated that the density of $CD8^+$ TILs was an independent and significant predictive factor for PFS and OS in patients with locally advanced NSCLC who underwent CCRT. In the current study, $CD8^+$ TILs were significantly associated with PFS and marginally associated with OS. $CD8^+$ T cells are also significant predictors of OS and dis-

ease-free survival in early stage NSCLC.[29] However, in the present study, no relationship was observed between $CD8^+$ TILs and peripheral circulating lymphocytes, and no correlation was noted between $CD8^+$ expression and pretreatment lymphocyte counts.

Previous studies have suggested that RAL may reduce the effectiveness of immunotherapy.[14,30] Pike et al.[14] demonstrated the effect of radiation on lymphocyte counts and the survival of patients with metastatic cancer receiving a PD1 ICI. Severe lymphopenia at the time of ICI treatment initiation was associated with decreased survival. In a retrospective study of lymphopenia in patients receiving immunotherapy for NSCLC, radiation was a significant risk factor for peri-immunotherapy lymphopenia in multivariate logistic regression analysis.[30] Peri-immunotherapy lymphopenia was a significant prognostic factor of both PFS and OS. Hence, greater efforts are needed to determine the optimal radiation technique that preserves ALC during radiotherapy in the era of standard immunotherapies, such as durvalumab, following CCRT in patients with unresectable NSCLC. Unfortunately, our data did not confirm a survival difference when the radiation techniques were considered. Patients treated with partial or full IMRT showed a trend towards better survival outcomes than those treated with 3D-CRT alone, albeit without statistical significance.

As a secondary study spin off from the well-designed clinical trial, we need to consider some possible limitations interpreting the results. First, we included only one hospital's data to minimize the bias from the different standardization of laboratory interpretations in each participating institution. Second, although serial hematological parameters could be obtained faithfully, data on PDL1 and $CD8^+$ TILs were obtained only from patients with available tissue specimens, and the post-radiation changes in these parameters could not be performed. Third, we did not scrutinize the effects of immunotherapy as a salvage therapy after tumor recurrence, which might affect overall survival. However, it would be unlikely that salvage immunotherapy had a significant impact on the results because most patients in this study were enrolled before the era of immunotherapy.

In conclusion, TLC reduction during CCRT and pretreatment NLR are significant prognostic factors for PFS. In subgroup analysis, $CD8^+$ TILs were significantly associated with PFS and marginally significantly associated with OS. Both LRFS and DRFS were significantly dependent on TLC reduction during CCRT, whereas DRFS was associated with pretreatment NLR. TLC reduction during CCRT is closely associated with GTV, pretreatment NLR, and heart V20. Thus, efforts are needed to reduce TLC by constraining the volume of the radiation dose to the entire heart. Further studies should focus on developing precise RT techniques to overcome RAL.

CONFLICT OF INTEREST STATEMENT

None declared.

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