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The Prognostic Value of Programmed Death-ligand 1 (PD-L1) in Patients who Received Neoadjuvant Chemoradiation Therapy Followed by Surgery for Locally Advanced Non-small Cell Lung Cancer

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Abstract. *Background/Aim: We analyzed the prognostic efficacy of programmed death-ligand 1 (PD-L1) in locally advanced non-small cell lung cancer (LA-NSCLC) patients. Patients and Methods: During 2005-2016, 211 patients underwent neoadjuvant concurrent chemoradiation therapy (CCRT) followed by surgical resection for LA-NSCLC at Asan Medical Center. PD-L1 expression and CD8+ tumor-infiltrating lymphocytes (TIL) were measured pre- and post-neoadjuvant CCRT and analyzed using immuno-histochemical staining. Results: In total, 39 patients were enrolled. Overall survival (OS) and disease-free survival (DFS) were significantly longer in patients with increased PD-L1 expression and increased CD8+ TIL density post-neoadjuvant CCRT. Univariate Cox regression analysis confirmed that increased levels of PD-L1 and increased CD8+ TIL density were prognostic factors for OS and DFS. Multivariate Cox regression analysis confirmed that increased levels of PD-L1 was a prognostic factor for OS and increased CD8+ TIL density for DFS. Conclusion: Relative changes in PD-L1 expression post-neoadjuvant CCRT can be utilized to predict the prognosis of LA-NSCLC patients.*

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Key Words: Non-small cell lung cancer, programmed death ligand-1, tumor-infiltrating lymphocytes; neoadjuvant therapy, prognosis.

Though it is not the most common in occurrence rate, lung cancer poses the highest mortality burden among all cancers. The five-year survival rate of non-small cell lung cancer (NSCLC) which accounts for 84 percent of lung cancer is 23 percent (1). Many efforts are being made to improve the prognosis of this dreadful disease. Clinical trials on new therapies, such as targeted therapy and immunotherapy, are being conducted and early results are promising so far. However, currently, a tri-modality approach of neoadjuvant concurrent chemoradiation therapy (CCRT) followed by surgical resection is the standard therapy for the treatment of patients with locally advanced disease (2, 3).

Research on biomarkers for predicting prognosis is also actively being conducted (4). However, the complexity of the immune mechanisms in the tumor environment and the heterogeneity of advanced lung cancer makes it difficult to predict the prognosis of patients. Thus, there is still no valid biomarker for LA-NSCLC, not only for new therapies but also for neoadjuvant CCRT.

Programmed death ligand 1 (PD-L1) is an immune checkpoint protein that suppresses immune function (5). Because cancer cells also use this mechanism to avoid the host immune system, interest in the role of PD-L1 is increasing and expected to shift the paradigm for lung cancer therapy (6). Indeed, early clinical trials of blocking the PD-1/PD-L1 pathway show favorable outcomes and the expression of PD-L1 is expected to help predict the outcome of immunotherapy (7-12). The PD-1/PD-L1 pathway clearly plays an important role in the tumor microenvironment, but there is still much to know about how to use it. A better understanding of PD-L1 helps to improve the survival rate of NSCLC.

Therefore, we ought to analyze the prognostic efficacy of PD-L1 in locally advanced non-small cell lung cancer patients who had neoadjuvant CCRT followed by curative resection surgery.

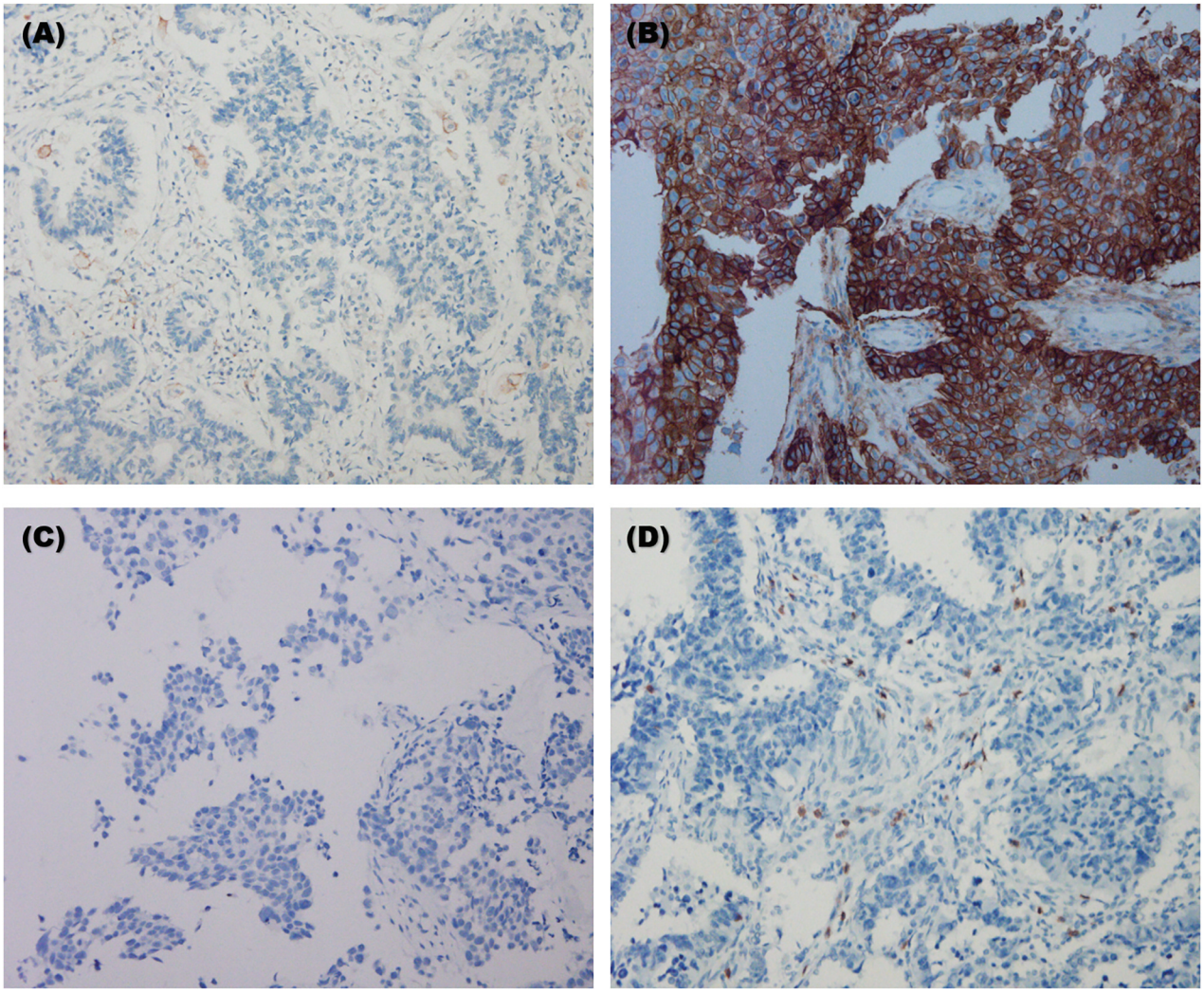

Figure 1. *Examples of immunohistochemical staining showing negative expression of PD-L1 (A), positive expression of PD-L1 (B), negative expression of CD8+ TIL (C) and positive expression of CD8+ TIL. Samples (A) and (B), (C) and (D) are paired specimen of the same patient obtained before and after neoadjuvant CCRT.*

Patients and Methods

Patients. From 2005 to 2016, 211 patients had surgical resection after neoadjuvant CCRT due to LA-NSCLC in Asan medical center. Among those, patients who had not sufficient specimen for histopathologic evaluation before or after neoadjuvant CCRT were excluded. Specimens before CCRT were obtained from biopsy for diagnosis. And paired second specimens were gained from resected lung parenchyma. We obtained whole tissue sections rather than tissue microarrays to reduce the bias that could come from differences in the method of obtaining samples before and after neoadjuvant CCRT. Patients who could not achieve curative surgical resection were excluded. And patients who had complete remission from neoadjuvant CCRT were also excluded from this study.

Baseline characteristics, clinical information and pathological features were obtained retrospectively using an electronic medical record system. This study was approved by the institutional review boards of Asan Medical Center and University of Ulsan College of Medicine.

Immunohistochemical analysis. Immunohistochemical analysis was performed with formalin-fixed, paraffin-embedded whole tissue sections. PD-L1 was detected using rabbit monoclonal anti-PD-L1 antibody (SP263, Ventana medical systems Inc., Tucson, AZ, USA) and CD8+ was detected using mouse monoclonal antibody (C8/144B, 1:400, Cell Marque, Rocklin, California, USA). The sections were stained using automated staining platform of a BenchMark XT (Ventana medical systems Inc). Visualization of antibodies was processed with OptiView DAB IHC detection kit (Ventana medical systems Inc). The expression of PD-L1 and the density of CD8+ tumor infiltrating lymphocyte (TIL) before and after neoadjuvant CCRT were evaluated and analyzed (Figure 1). If the antibodies were detected in the membrane and/or cytoplasm, the

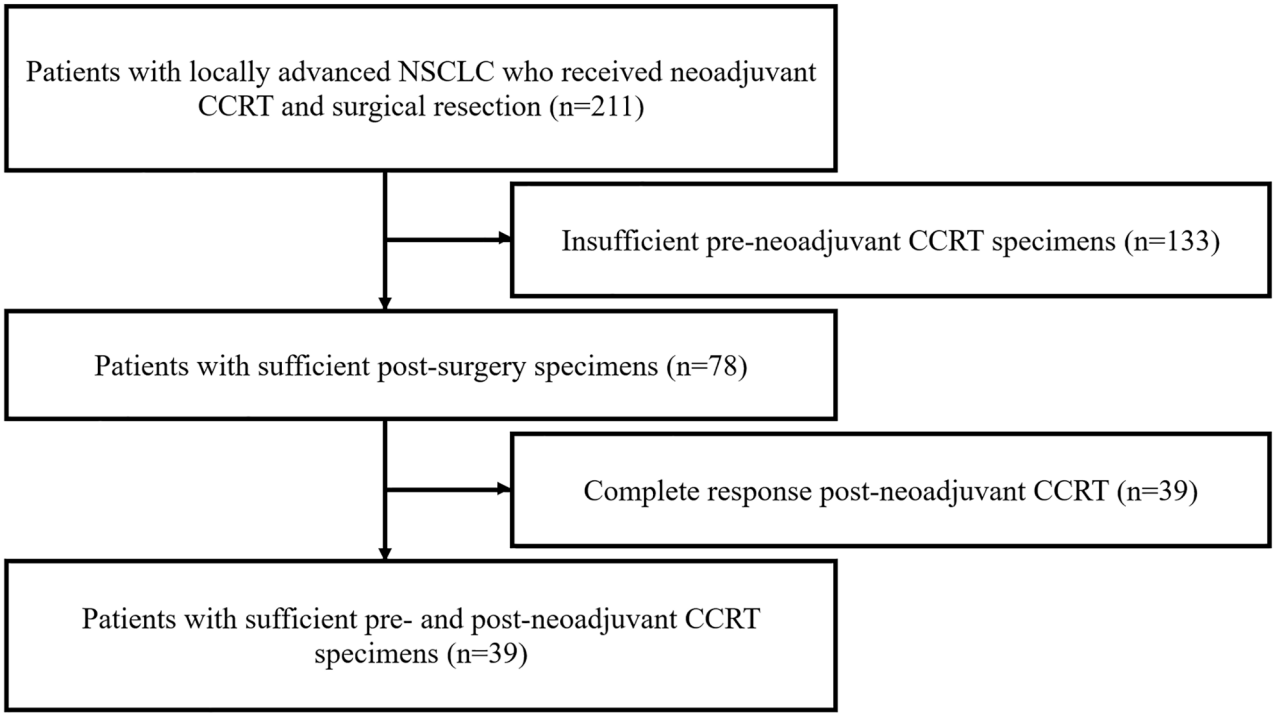

Figure 2. *Patient inclusion and exclusion criteria. NSCLC: Non-small cell lung cancer; CCRT: concurrent chemoradiation therapy.*

expression of PD-L1 was considered positive. The density of CD8+ TIL was assessed by the count of positively stained CD8+ lymphocytes within the peritumoral stroma.

Patients were divided into two groups if categorical comparison was needed. For analysis, in either before or after neoadjuvant CCRT, the cutoff value for high and low was determined by the median number of PD-L1 and CD8+. To analyze the relative changes before and after neoadjuvant CCRT, we divided patients into an 'increase' or 'not increase' group based on the proportion of the expression. Proportions of positive expression in tumor cells were calculated in paired specimens of before and after neoadjuvant CCRT. If the proportion of the expression had increased after neoadjuvant CCRT, we consider it 'increase'. We regarded as 'not increase' if the proportion of the expression had not changed or decreased. Two experienced pulmonary pathologists (JS, SJ) independently performed analysis and the mean value was recorded for each expression.

Statistical analysis. We compared the continuous variables with Mann–Whitney test, categorical variables with Fisher's exact test and ordered categorical variables with linear-by-linear test. Spearman rank correlation test was used to analyze the correlation between the continuous variables. Overall survival and disease-free survival were determined using Kaplan–Meier curves and the difference was analyzed with log-rank test. Cox proportional hazard models were used to analyze the outcomes related to survival. *p*-Values were derived from two-tailed test and considered significant when the values were equal or less than 0.05. Statistical analysis was performed using R software, version 3.6.2 (R Foundation, Vienna, Austria) and IBM SPSS Statistics™ version 25.0 (IBM Corporation, Armonk, NY, USA).

Results

Patient characteristics. A total of 39 patients were enrolled in this study (Figure 2). Baseline characteristics of patients are presented in Table I. Mean age was 57 years (range=54-62 years) and 79% (31/39) of patients were male. Histologic types of lung cancer were adenocarcinoma (20/39, 51%), squamous cell carcinoma (16/39 41%) and other subtypes (3/39, 8%). More than half (21/39, 54%) were stage III A with TNM staging 7th edition, followed by III B (12/39, 31%) and II B (6/39, 15%). Most of patients (36/39, 92%) had Paclitaxel plus Cisplatin chemotherapy with 45Gy radiotherapy while three patients had other combinations of chemotherapy. The ypTNM stage in 21 patients (54%), had been down-regulated after neoadjuvant CCRT. Median time interval from neoadjuvant CCRT to surgical resection was 70 days (range=66-70 days).

Expression of PD-L1 and CD8+ TIL before and after neoadjuvant CCRT. The median expression of PD-L1 before neoadjuvant CCRT was 34.6%, whereas after neoadjuvant CCRT was 38.3% (Figure 3). There was no statistically significant difference between the proportion of PD-L1 expression before and after neoadjuvant CCRT (p=0.460). However, the median expression of CD8+ TIL after

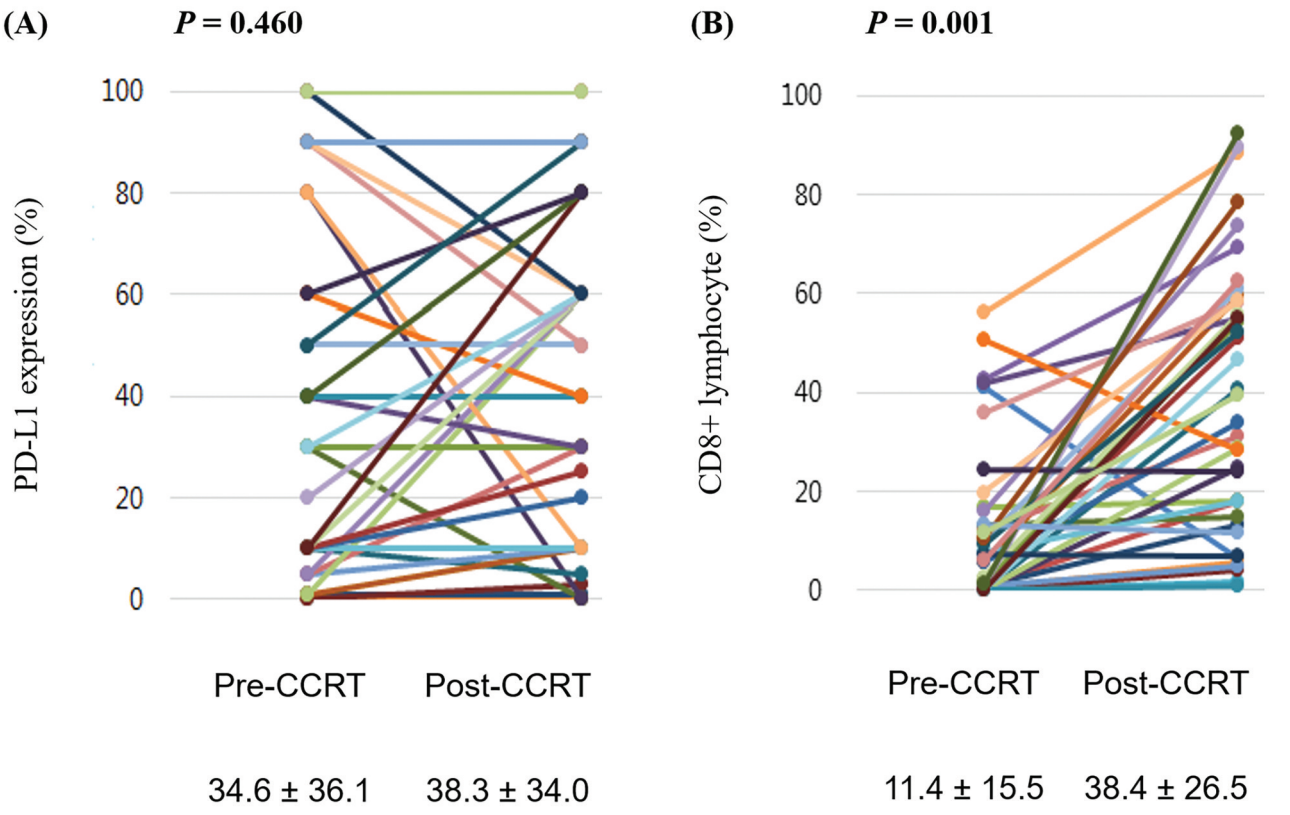

Figure 3. *Changes in programmed death-ligand 1 (PD-L1) positivity (A) and CD8+ tumor-infiltrating lymphocyte (TIL) density (B) expressed as percentage values, pre- and post-neoadjuvant concurrent chemoradiation therapy (CCRT).*

neoadjuvant CCRT was significantly increased from 11.4% to 38.4% (p=0.001). The pre-neoadjuvant CCRT PD-L1 positivity levels did not correlate with the pre-neoadjuvant CCRT CD8+ TIL density values (p=0.090) (Figure 4). However, the post-neoadjuvant CCRT PD-L1 positivity levels correlated positively with the post-neoadjuvant CCRT CD8+ TIL density values (p=0.008).

Correlation between PD-L1 increment and clinicopathologic characteristics. Fifteen out of 39 patients showed increased expression of PD-L1 (increase (+) group) while 24 patients did not show a change or a decrease (increase (–) group) after neoadjuvant CCRT. We compared the characteristics of PD-L1 increase (+) and (–) group and the results are shown in Table II. There was no significant difference between the two groups in age, sex, histology, ypTNM stage, regimen of CCRT, and number of down staged patients. The ratio of increased density of CD8+ TIL after neoadjuvant CCRT was not different between the two groups (p=0.374).

Table I. *Patient characteristics*.

Patient characteristics	Total (%) (N=39)
Age (years)	57 (54-62)
Gender (male)	31 (79%)
Histology	
Adenocarcinoma	20 (51%)
Squamous cell carcinoma	16 (41%)
Others[i]	3 (8%)
Stage	
II B	6 (15%)
III A	21 (54%)
III B	12 (31%)
Chemo-radiotherapy regimen	
Paclitaxel plus Cisplatin	36 (92%)
Others[ii]	3 (8%)
Radiotherapy dose	
45 Gy	39 (100%)

[i]Adenosquamous in 2 patients, large cell neuroendocrine tumor in 1 patient; [ii]Etoposide plus carboplatin, Gemcitabine plus oxaliplatin, Pemetrexed in 1 patient, each.

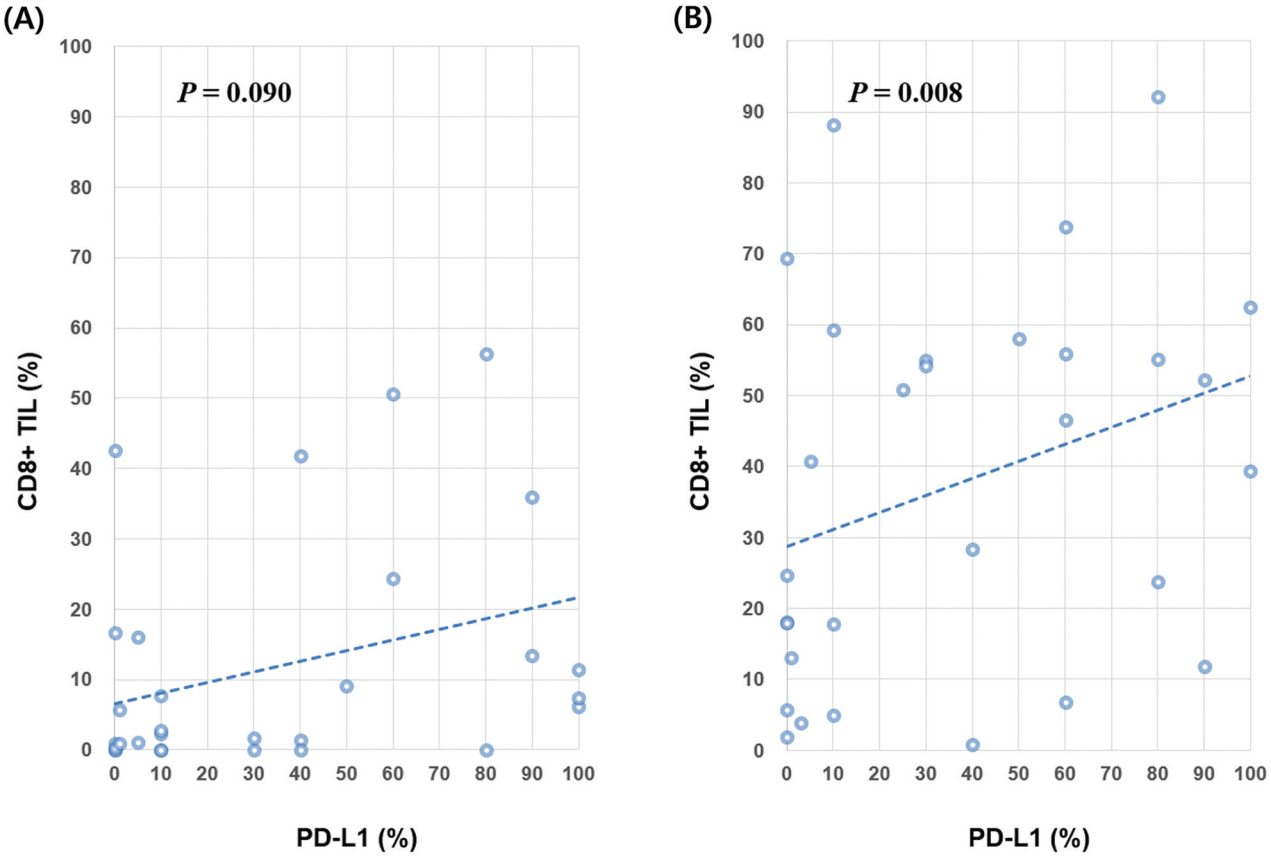

Figure 4. *Correlation between programmed death-ligand 1 (PD-L1) positivity and CD8+ tumor-infiltrating lymphocyte (TIL) density expressed as percentage values, pre- (A) and post- (B) neoadjuvant concurrent chemoradiation therapy (CCRT).*

Survival analysis. Overall survival (OS) and disease-free survival (DFS) were significantly higher in patients with increased PD-L1 expression (p=0.016, 0.022, respectively) (Figure 5). The same tendency was observed in patients who showed increased density of CD8+ TIL, with the p-value of 0.004 in OS and 0.001 in DFS.

Univariate Cox proportional hazard ratio model confirmed ypTNM stage (HR=1.44, 95%CI=1.12-1.86, p=0.005), increased PD-L1 (HR=0.36, 95%CI=0.15-0.85, p=0.020) and increased CD8+ TIL density (HR=0.31, 95%CI=0.14-0.71, p=0.006) were independent prognostic factors for OS (Figure 6). The same results were obtained regarding DFS as follow; ypTNM stage (HR=1.32, 95%CI=1.06-1.65, p=0.015), increased PD-L1 (HR=0.41, 95%CI=0.19-0.90, p=0.026) and increased CD8+ TIL density (HR=0.26, 95%CI=0.11-0.59, p=0.001).

Multivariate Cox proportional hazard ratio model confirmed that ypTNM stage (HR=1.32, 95%CI=1.01-1.73, p=0.040) and increased PD-L1 (HR=0.38, 95%CI=0.16-0.91, p=0.030) were independent prognostic factors for OS (Figure 7). Regarding DFS, increased CD8+ TIL density (HR=0.40, 95%CI=0.16-0.99, p=0.047) was identified as a significant prognostic factor. The hazard ratio of increased PD-L1 did not reach statistical significance (HR=0.46, 95%CI=0.21-1.02, p=0.058).

Subgroup analysis based on the combination of PD-L1 expression and CD8+ TIL density. Patients were divided into four groups based on the combination of PD-L1 expression and CD8+ TIL density after neoadjuvant CCRT. The four groups were as follows; Low PD-L1/Low CD8+, High PD-L1/Low CD8+, Low PD-L1/High CD8+ and High PD-L1/ High CD8+. The criteria high or low were defined based on the median values of PD-L1 expression and CD8+ TIL density. Low expression of PD-L1 with high expression of CD8+ TIL density was associated with the longest overall survival [median 69.2 months (28.8-109.6)], while high expression of PD-L1 with low expression of CD8+ TIL density were associated with worst prognosis [median 45.0 months (14.8-75.2)] (Figure 8). However, differences in survival between the groups were not statistically significant (p=0.957).

Table II. *Patient characteristics related to change in PD-L1 expression.*

Patient characteristics	N (Total=39)	PD-L1 expression		*p*-Value
		Increase (+)	Increase (–)	
Age (years)	57 (54-62)	58 (55-65)	57 (52-61)	0.204[i]
Gender (male)	31 (79%)	13 (33%)	18 (55%)	0.450[ii]
Histology				0.294[iii]
Adenocarcinoma	20 (51%)	8 (21%)	12 (31%)	
Squamous cell carcinoma	16 (41%)	7 (18%)	9 (23%)	
Others	3 (8%)	0	3 (8%)	
Stage (ypTNM)				0.374[iii]
I A	7 (18%)	3 (8%)	4 (10%)	
I B	1 (3%)	0	1 (3%)	
II A	4 (%)	3 (8%)	1 (3%)	
II B	7 (18%)	3 (8%)	4 (10%)	
III A	15 (38%)	5 (%)	10 (26%)	
III B	4 (10%)	1 (3%)	3 (8%)	
IV	1 (3%)	0	1 (3%)	
Chemo-radiotherapy regimen				0.851[iii]
Paclitaxel plus Cisplatin	36 (92%)	14 (36%)	22 (56%)	
others	3 (8%)	1 (3%)	2 (5%)	
Down staging[iv]				0.547[iii]
Yes	21 (54%)	9 (23%)	12 (31%)	
No	18 (46%)	6 (15%)	12 (31%)	
CD8+ density				0.374[iii]
Increase (+)	28 (%)	12 (31%)	16 (41%)	
Increase (–)	11 (28%)	3 (8%)	8 (21%)	

[i]Mann-Whitney test; [ii]Fisher's exact test; [iii]linear by linear association; [iv]down-regulated TNM stage after neoadjuvant CCRT.

Discussion

Our study indicates that increased expression of PD-L1 after neoadjuvant CCRT positively correlates with survival time. However, we could not find any statistically significant difference in prognosis when we divided patients into positive and negative groups of PD-L1 expression. Most of the previous studies which had found a correlation between PD-L1 and prognosis, divided patients into positive and negative groups based on specific expression ratio (*i.e.*, greater than 25%) which has not been officially established yet (13-15). Therefore, each study had different criteria for defining positivity of PD-L1, and the range of positivity for each study differed from 7.4% to 72.7%. Furthermore, some studies have argued that the expression of PD-L1 has no prognostic value, regardless of what category of positivity they used (16, 17). We also applied all possible criteria, but failed to find any significant result.

The main reason for this discrepancy is the diversity of the immunohistochemical methods used (18). There are various antibodies produced by different manufacturers, and many combinations are possible depending on which analytical system is chosen. Pointing out these issues, some studies compared the analytical methods of PD-L1 and concluded that it is hard to replace each other (19, 20).

In this study, rabbit monoclonal antibody (SP263) was used for staining PD-L1. And detection was performed with OptiView DAB IHC detection kit on a BenchMark XT automated staining platform. Currently, there is a consensus that more than 25% of the PD-L1 expression in tumor proportion score is considered positive in SP263 settings. Though it is for durvalumab use in advanced urothelial carcinoma, SP263 has been approved by the FDA as a significant method for predicting treatment effects with the criterion of more than 25% PD-L1 expression (21). When the 25% criterion was applied to our study, the positive rate of PD-L1 before and after neoadjuvant CCRT was 49% and 59%, respectively, and was not significantly correlated with overall survival (p=0.766, 0.588, respectively), nor with disease-free survival (p=0.936, 0.416, respectively). We also analyzed other combinations of positive rates of ≥1%, ≥5%, ≥10% and ≥50%. However, none of them was significantly related with prognosis. We confirmed statistical significance only when the analysis was based on the relative increment of PD-L1 before and after neoadjuvant CCRT.

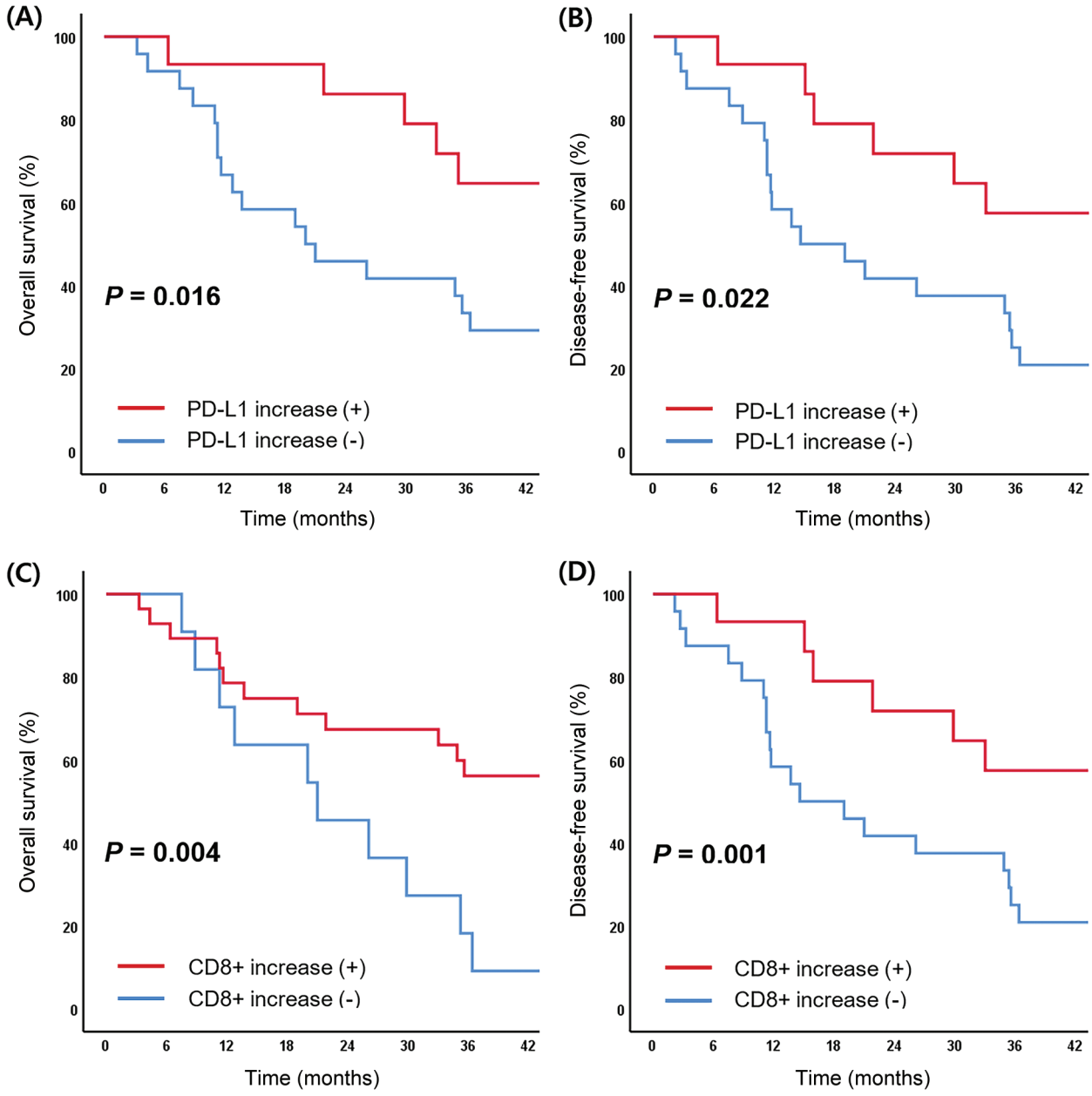

Figure 5. *Kaplan–Meier curves for overall survival of patients with increased PD-L1 and not-increased PD-L1 expression (A). Kaplan–Meier curves for disease free survival of patients with increased PD-L1 and not-increased PD-L1 expression (B). Kaplan–Meier curves for overall survival of patients with increased CD8+ and not-increased CD8+ density (C). Kaplan–Meier curves for disease free survival of patients with increased CD8+ and not-increased CD8+ density (D).*

Fujimoto *et al*. also sought to determine whether PD-L1 expression was relevant to the prediction of the outcomes of CCRTs using the difference in PD-L1 expression before and after CCRT (22). Since they used the clone 28-8 antibody (Abcam, 1:150), patients with 1% or greater PD-L1 staining of tumor cells were considered to be positive for PD-L1 expression. They could not also find a significant correlation between positive expression of PD-L1 and prognosis. Incremental-based analysis confirmed that the group with reduced expression of PD-L1 showed a higher survival rate. Their research differed from ours in several respects. First, there was negative correlation between the expression of PD-L1 and survival, whereas our study showed a positive correlation. Another difference was

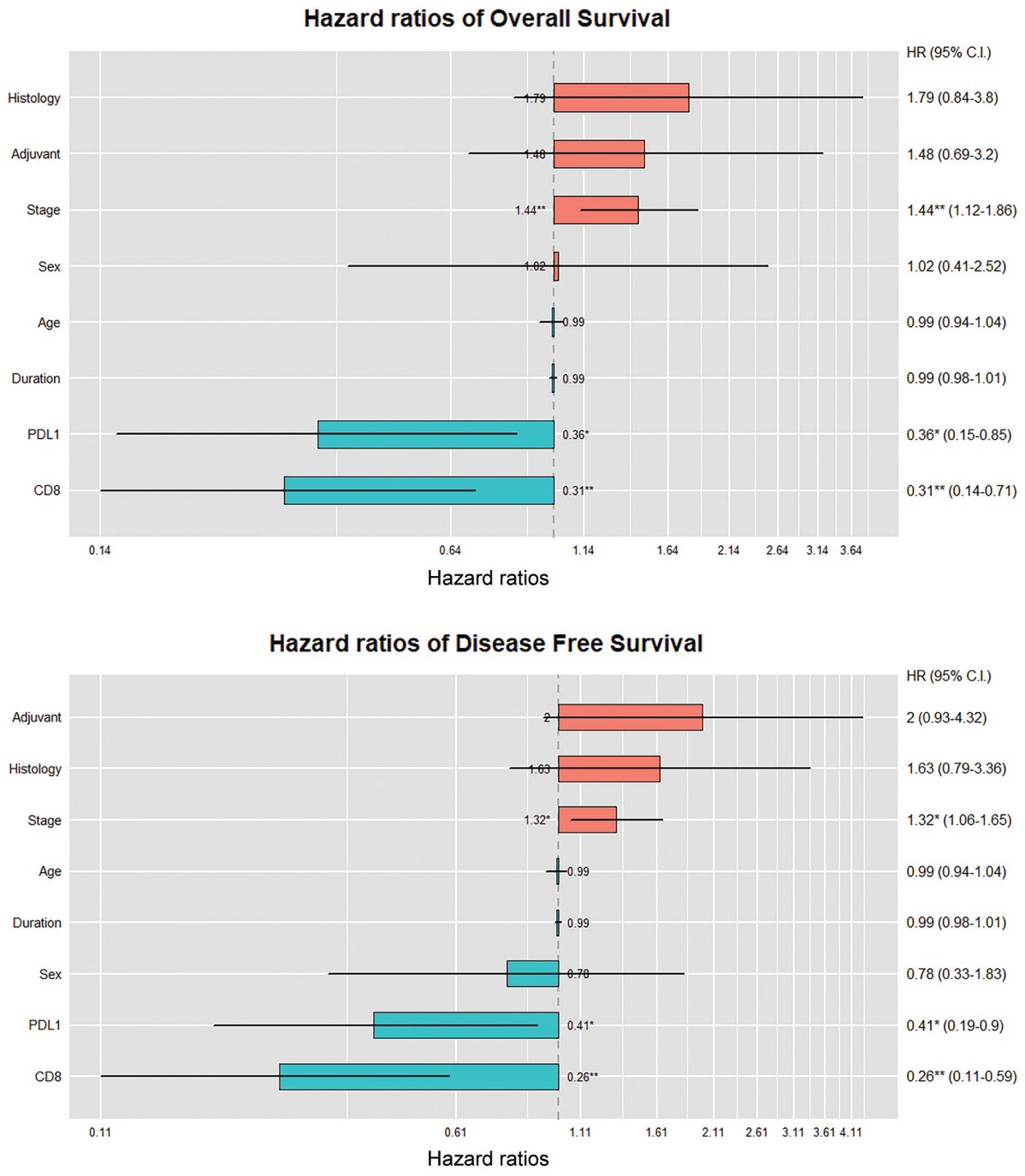

Figure 6. *Univariate analysis of clinicopathologic factors associated with overall survival (A) and disease-free survival (B).*

that no significant association was found between CD8+ TIL density and prognosis, which revealed significant in our study.

Our study demonstrated that the increase in CD8+ TIL density after neoadjuvant CCRT is a positive prognostic factor. Like PD-L1, studies on CD8+ are also controversial regarding its correlation with prognosis (23, 24). However, the prevailing theory so far is that there is a positive correlation between CD8+ TIL and prognosis because CD8+ T cells play a key role in cell-mediated immunity (25, 26).

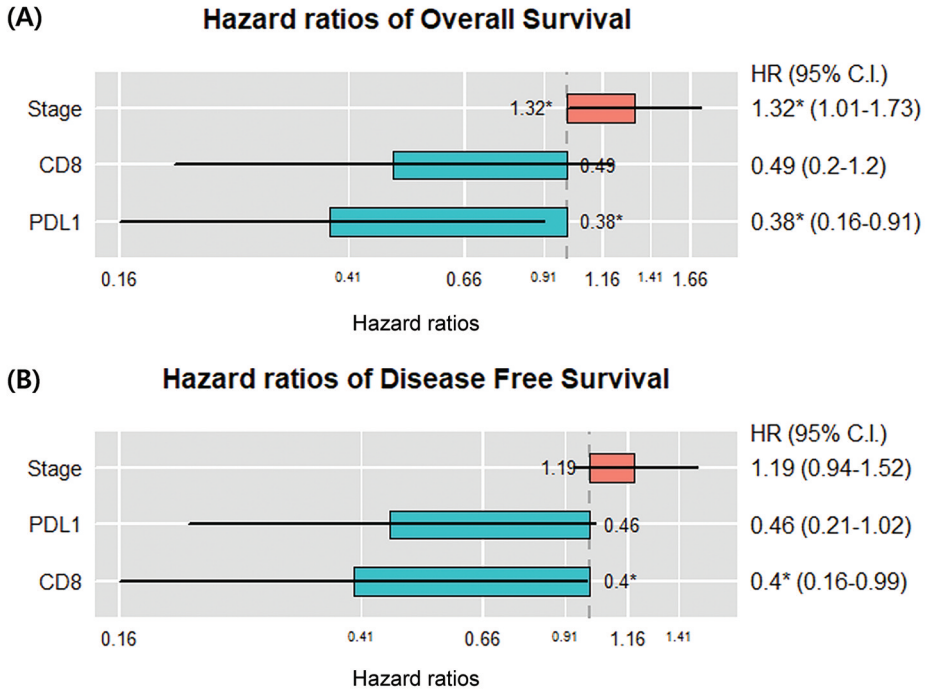

Figure 7. *Multivariate analysis of clinicopathologic factors associated with overall survival (A) and disease-free survival (B).*

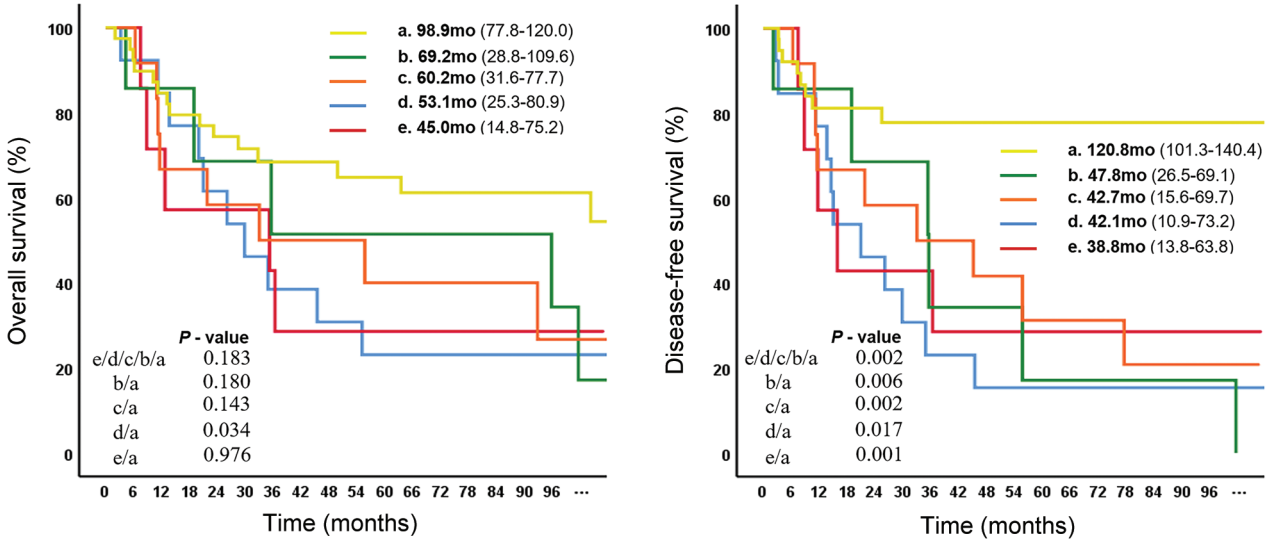

Figure 8. *Kaplan–Meier curves for overall survival of four groups based on the combination of PD-L1 expression and CD8+ TIL density after neoadjuvant CCRT. Median overall survival and range in months are indicated.*

Therefore, efforts are underway to verify its validity as a biomarker by analyzing the combination of PD-L1 and CD8+ and several studies have shown that PD-L1 with CD8+ predicts prognosis (15, 27).

Lin *et al*. analyzed the prognosis of 1,013 NSCLC patients using the combination of CD8+ and PD-L1. They could not find any significant correlation between PD-L1 or PD-1 mRNA expression and prognosis (28). However, the analysis

of the combination of CD 8+ with PD-L1 mRNA expression, confirmed a significant correlation with prognosis. The CD8+ TIL density and PD-L1 mRNA expression were divided into high and low, respectively, and these were classified into 4 groups. Among those four groups, the low PD-L1 expression/high CD8+ TIL infiltration group showed the best prognostic outcome while high PD-L1 expression/low CD8+ TIL infiltration group showed the worst (p=0.003). Other studies have also examined the impact of the combination of PD-L1 and CD8+, and the results were controversial regarding whether PD-L1 had a positive or negative correlation with prognosis (29-31). In this study, 'low PD-L1 expression/high CD8+ TIL infiltration' group showed the longest median overall survival after neoadjuvant therapy, but the difference was not statistically significant (p=0.957).

Many studies have shown that an increase in CD8+ is associated with a good prognosis, but there is still little information about the association with PD-L1. First, it is not yet clear whether CD8+ affects the expression of PD-L1 or not, and if it does, we also have to consider whether the effect is direct or indirect, positive or negative. Interferon-gamma (IFN-γ), for example, is one of the cytokines produced by CD8+ and elevated IFN-γ is commonly related with enhanced anti-tumor response. However, an increase in IFN-γ can also promote PD-L1 expression by the tumor to escape an immune reaction (32). Also, sustained exposure to increased IFN-γ can cause exhaustion of CD8+, consequently resulting in tumor progression (33). Therefore, CD8+, IFN-γ and PD-L1 can be considered to have a mutual relationship, not a causal. How these affect prognosis might vary greatly depending on circumstances.

In addition to these complex causalities, the presence of molecular alterations in EFGR, KRAS and the expression of *MET* gene are thought to be related to PD-L1 expression (34, 35). And recent papers suggest that the expression of STK1/LKB1 also affects the expression of PD-L1 by enhancing stimulator of interferon genes (STING) (36, 37). The cancer-immunity cycle activated by STING releases immune inflammatory cytokines and they stimulate the expression of PD-L1 (38, 39).

The discordant results of PD-L1 as a biomarker imply the existence of more multiple complex pathways. Thus, it is not easy to predict the prognosis by reading the positivity for PD-L1 with a single measurement based on a specific value. Therefore, until we have a better understanding of the immune mechanisms, efforts to find alternatives to the use of PD-L1 expression for predicting prognosis are needed.

Our study has several limitations. First, the cohort was relatively small and patients were heterogeneous with advanced stage disease. A large cohort study is required to correct the predicted variables and increase statistical significance. Second, the methods of obtaining samples before and after neoadjuvant CCRT were different (biopsy *vs*. resection). Some studies have pointed out that method and time interval of biopsy can also result in discrepancies (40, 41). However, we used whole tissue sections rather than tissue microarrays to minimize statistical bias. Also, the time interval from neoadjuvant CCRT to surgical resection was as short as a median of 70 days. Third it was a single center study of a single nation. Since there might also be ethnic differences in the expression of PD-L1, a multicenter, multi-racial study is needed for validation (42).

Conclusion

The expression of PD-L1 could be a potential biomarker even for a heterogeneous group of NSCLC patients who require neoadjuvant CCRT. However, it is part of a dynamic and complex immune mechanism involving multiple mediators, and standardization and optimization are still needed. Utilizing the relative variation of PD-L1 expression before and after treatment may help predict the prognosis of LA NSCLC patients until the immune mechanism is better understood.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

Conceptualization: Lee G-D, Kim H-R; formal analysis: Chung B, funding acquisition: Lee G-D, investigation, methodology: Song J-S, Jang S-J; supervision: Song J-S, Jang S-J, Kim H-R, validation: Kim H-R, writing original draft: Lee G-D, Chung B, original draft review and editing: Kim H-R, Lee G-D, Chung B.

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