

Comparative study of the PD-L1 expression and CD8+ tumor-infiltrating lymphocyte between surgically resected and matched re-biopsy specimens in recurrent non-small cell lung cancer

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Introduction: Numerous studies conducted until date have reported that the chemotherapy regimen could affect the programmed cell death ligand 1 (PD-L1) expression status in patients with non-small cell lung cancer (NSCLC).

Materials and methods: A total of 36 NSCLC patients for whom both the surgically resected specimens of the primary tumors and re-biopsy specimens of the recurrent tumors were available were enrolled in this study. The PD-L1 expression status and tumor-infiltrating CD8-positive T lymphocytes (CD8+TILs) count were measured in paired samples by immunohistochemistry. The concordance rate in the tumor immune microenvironment (TIME) classification based on the PD-L1 expression status and CD8+TILs count was analyzed.

Results: While the PD-L1 expression levels were similar between the surgical and re-biopsy specimens in 77.8% of cases, in 16.7% of cases, the expression levels were higher in the re-biopsy specimens. When the analysis was confined to patients who had received platinum-based chemotherapy, the percentage increased to 42.9%. The TIME classification changed in the re-biopsy specimens as compared to the surgical specimens in one-third of the patients, especially in those who had received chemotherapy previously. The TIME classification in the re-biopsy specimens more closely resembled that in the metastatic lymph nodes as compared to that in the primary tumor.

Conclusion: In patients with recurrent NSCLC, especially those who have received chemotherapy previously, a recent re-biopsy sample is required to determine whether PD-1/PD-L1 inhibitors should be used for treatment or not.

Keywords: non-small cell lung cancer, NSCLC, programmed cell death ligand 1 (PD-L1), tumor-infiltrating T lymphocytes, tumor immunomicroenvironment (TIME), re-biopsy

Introduction

Lung cancer is a major cause of death in many developed countries. Although surgery is a major component of treatment for localized and resectable lung cancer, patients often develop local and/or distant recurrence after curative operation.^{1,2} Several biomarkers have now been reported as predictors of survival and recurrence in patients with non-small cell lung cancer (NSCLC). Especially, immunological biomarkers in the tumor microenvironment are useful prognostic predictors, in addition to being promising targets for novel therapeutic approaches.³

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The interaction between programmed cell death-1 (PD-1) and PD-1 ligand 1 (PD-L1) inhibits T-cell activation and proliferation.^{4,5} Several previous studies have reported that PD-L1 overexpression is a predictor of a poor clinical outcome in patients with NSCLC.^{6–8} On the other hand, PD-L1 overexpression has been shown to be correlated with an improved response to treatment with PD-1/PD-L1 inhibitors.^{9–12} Meanwhile, several previous studies have indicated that the presence of tumor-infiltrating CD8-positive T lymphocytes (CD8+TILs) is associated with a longer survival period in patients with colorectal, ovarian, and breast cancer.^{13–15} CD8+TIL count is a candidate biomarker for tumor-associated immune response, and most previous studies of CD8+TILs in NSCLC have reported an association of a high count with a favorable outcome.^{16–18}

Recently, we studied the PD-L1 expression status and CD8+TIL count in the tumors of 170 lung adenocarcinoma patients who had undergone pulmonary resection as the initial treatment.¹⁹ We divided them into four subgroups according to the PD-L1 expression status and CD8+TIL count in the tumors and reported that the outcomes of patients with low PD-L1 expression levels and high CD8+TIL counts in the tumor were significantly better than those of patients with high PD-L1 expression levels and low CD8+TIL counts in the tumors. In the current study, we evaluated the concordance rate of the tumor immune microenvironment (TIME) classification based on the PD-L1 expression status and CD8+TIL count between surgically resected specimens and matched re-biopsy specimens in patients with recurrent NSCLC.

Materials and methods

Patients and specimens

We retrospectively collected and reviewed the medical records of patients with histologically confirmed, recurrent NSCLC who had undergone curative resection between 2010 and 2016 at our hospital. Among these patients, patients whose surgical specimens and re-biopsy specimens after recurrence were available were enrolled in this study. Written informed consent for study of the excised tissue samples was obtained from each patient. This study was conducted with the approval of the Ethics Committee of Kawasaki Medical School (No.2798: Approved on September 30, 2017). The study was conducted according to the criteria set by the Declaration of Helsinki.

The TNM stage was determined according to the revised criteria published in 2009 (version 7; TNM-7), and the

histological diagnoses of the tumors were based on the WHO classification published in 2015.^{20,21} Selection of the post-operative adjuvant chemotherapy regimen was based on consensus among the members of institution's cancer board or on enrollment in a clinical trial. In practice, oral tegafur was selected for patients with stage I disease (T1bN0M0 and T2N0M0), and platinum-based chemotherapy was selected for patients with stage II or stage IIIA disease.

Immunohistochemical analysis and assessment

Four-micrometer-thick sequential histologic sections were prepared from representative formalin-fixed, paraffin-embedded tumor blocks. Immunohistochemical (IHC) analysis was performed using an automated immunostainer (Nexes; Ventana, Tucson, AZ, USA). The following primary antibodies were used according to the manufacturer's instructions and according to a previously described protocol: a mouse monoclonal anti-PD-L1 antibody (1: 50, clone 28–8; ab205921; Abcam, Cambridge, MA, USA) and CD8 (1:50, clone C8/144B, Dako; Agilent Technologies, Inc., Santa Clara, CA, USA).^{10,16}

The expression levels of each marker protein were examined and evaluated according to a previously reported original protocol. PD-L1 expression was categorized as positive when staining of the tumor-cell membrane (at any intensity) was present. PD-L1 high expression was observed at pre-specified expression levels of 5% of all cells in a section that included at least 100 evaluable tumor cells.¹⁰ To evaluate the CD8+TIL count, 10 digital high-power field images of the tumor area were selected, and the absolute number of CD8+TILs in these images was determined.¹⁶

Tumor immune microenvironment classification

The TIME classification into four groups based on the PD-L1 expression status and CD8+TIL count has been previously proposed, as follows: type-I (high PD-L1 expression with high CD8+TIL count), type-II (low PD-L1 expression with low CD8+TIL count), type-III (high PD-L1 expression with low CD8+TIL count), and type-IV (low PD-L1 expression with high CD8+TIL count).^{19,22}

Statistical analysis

All the statistical analyses were performed using the SPSS statistical software package (version 23.0; SPSS, Chicago, IL, USA). The Chi-square test or Fisher's exact test was

performed to compare the changes of TIME classification in the patients. In all cases, $p < 0.05$ was considered as denoting statistical significance. The prognostic evaluation was performed based on the overall survival (OS).

Results

A total of 36 eligible patients with recurrent NSCLC were included in the present study (Table 1). The mean age at surgery was 66.4 years (range, 44–81 years). The histologic subtype was adenocarcinoma in 27 patients, squamous cell carcinoma in 4 patients, and the other type of NSCLC in 5 patients. The TNM-7 cancer stages were IA, IB, IIA, IIB, and IIIA in 13, 9, 4, 2, and 8 patients, respectively. The median OS from surgery was 55.2 months and the 3-year OS rate was 76.8%.

Table 1 Characteristics of the patients enrolled in this study (n=36)

	n	%
Age		
≥70	16	44.4
<70	20	55.6
Sex		
Male	22	61.2
Female	14	38.8
Histological subtype		
Adenocarcinoma	27	75.0
Squamous cell carcinoma	4	11.1
Other*	5	13.9
Pathological nodal status		
N0	26	72.2
N1	5	13.9
N2	5	13.9
Pathological stage (TNM-7)		
IA	13	36.1
IB	9	25.0
IIA	4	11.1
IIB	2	5.6
IIIA	8	22.2
EGFR mutation status		
Positive**	10	27.8
Negative	24	66.7
Unknown	2	5.5

Notes: *Other histological types: adenosquamous carcinoma, 2; large-cell carcinoma, 2; pleomorphic carcinoma, 1. **EGFR mutation status: Ex18 G719X, 1; Ex19 Del, 5; Ex21 L858R, 4.

Characteristics of the re-biopsy samples

Characteristics of the post-recurrence treatments and of the re-biopsy samples are summarized in Table 2. Of the re-biopsies, 47.2% (n=17) were collected from the lungs or bronchi and 52.8% (n=19) from metastatic sites. Metastatic organs included the lymph node (n=5), chest wall or pleura (n=4), adrenal glands (n=3), bone (n=3), GI tract (n=1), and soft tissue (n=1). Samples were obtained by surgery in 21 patients (58.3%) and by biopsy in 15 patients (41.7%). The median interval from surgery to the re-biopsy was 32.4 months (range, 4.9–114.5), with 80.6% of the paired samples collected >24 months apart. During the interval between the initial surgery and re-biopsy, 36.1% (n=13) of the patients received no treatment (Group A), while 63.9% of patients received chemotherapy or epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) therapy. The most common chemotherapy regimens used

Table 2 Characteristics of the re-biopsy specimens

Variable	n
Tumor site	
Lung	17
Lymph node	5
Pleura + chest wall	4
Bone	3
Adrenal gland	3
GI tract	3
Soft tissue	1
Sample type	
Biopsy	15
Surgical	21
Time interval between sample collection, mo	
<6	2
6–11.9	5
12–23.9	8
24–35.9	5
36–47.9	7
48–59.9	7
>60	2
Treatment between sample collection	
No (Group A)	13
Yes	
Oral tegafur (Group B)	12
Platinum-based chemotherapy (Group C)	7
EGFR-TKI (Group D)	4

were oral tegafur (n=12, Group B), platinum-based chemotherapy (n=7, Group C), and EGFR-TKI (n=4, Group D). None of the patients was treated with PD-1/PD-L1 inhibitors.

Comparison of PD-L1 expression and CD8+TIL status among paired samples

Representative sections showing PD-L1 expression and CD8+TILs are depicted in Figure 1. The PD-L1 expression levels were similar between the primary tumor and re-biopsy specimens in 28 patients (77.8%), whereas they were higher in the re-biopsy specimens in 6 patients (16.7%) and lower in the re-biopsy specimens in 2 patients (5.5%). Among the 7 patients who had received platinum-based chemotherapy (Group C), the PD-L1 expression level was higher in the re-biopsy specimens in 3 patients (42.9%). On the other hand, the CD8+TIL count did not differ between the primary tumor and re-biopsy specimens in 26 patients (72.2%), whereas it was higher in the re-biopsy specimens in 3 patients (8.3%) and lower in the re-biopsy specimens in 7 patients (19.5%). In the patients who had received oral tegafur chemotherapy (Group B), the CD8+TIL count was lower in the re-biopsy specimens in 4 patients (36.4%). (Table 3)

Changes of TIM classification

The TIME classification remained unchanged between the specimens in 24 cases (66.7%), whereas it changed in the re-biopsy specimens as compared to specimens of the primary tumors in 12 (33.3%) patients. The TIME classification changed in 23.1% in Group A, in 41.7% in Group B, in 57.1% in Group C, and in 50.0% in Group D (Figure 2A). Although there was a tendency toward a higher rate of change in Group C, the differences among the groups were not statistically significant.

Heterogeneity of the TIM classification among the primary tumors, metastatic lymph node, and re-biopsy specimens

Of the 36 eligible patients enrolled in this study, 10 had lymph node metastasis at the time of the surgery (Table 1). We next compared the TIME classification among the primary tumor, one representative metastatic lymph node (the largest metastatic lymph node), and a re-biopsy specimen in each case. Comparisons of paired primary tumor and metastatic lymph node specimens showed that the TIME classification was the same in 7 patients (70.0%), but discordant in 3 patients (30.0%) (Figure 2B). The discordance between primary tumor and re-biopsy specimen was shown in 7

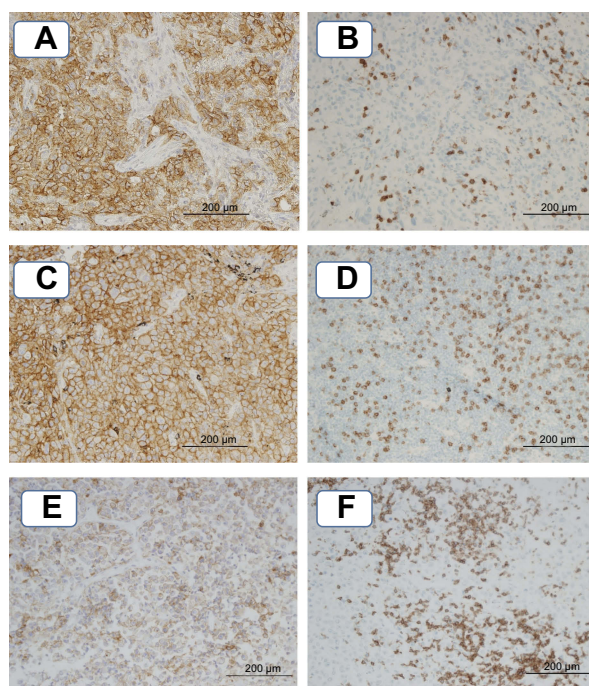


Figure 1 Representative images of IHC staining. (A) PD-L1 expression status in the primary tumor (TPS \geq 50), (B) CD8+TIL count of the primary tumor (score 45), (C) PD-L1 expression status in the metastatic lymph node (TPS \geq 50), (D) CD8+TIL count in the metastatic lymph node (score 150), (E) PD-L1 expression status in the re-biopsy specimen (26 \leq TPS<50), (F) CD8+TIL count in the re-biopsy specimen (score 230). **Abbreviation:** TPS, tumor proportion score.

Table 3 PD-L1 expression status and CD8+TIL count in re-biopsy specimens as compared to the primary tumors

	Group				
	All	A	B	C	D
PD-L1					
Higher	6	1	1	3	1
Equivalent	28	12	10	4	2
Lower	2	0	1	0	1
CD8+TIL count					
Higher	3	1	1	1	0
Equivalent	26	10	7	5	4
Lower	7	2	4	1	0

Notes: Group A: no treatment, Group B: oral tegafur, Group C: platinum-based chemotherapy, Group D: EGFR-TKI.

patients (70.0%); furthermore, discordance between the metastatic lymph node and re-biopsy specimens was observed in 4 patients (40.0%). Thus, the TIME classification of the re-biopsy specimens more closely resembled that of the metastatic lymph nodes as compared to that of the primary tumor.

Discussion

Recently, several studies have attempted to identify predictive biomarkers to help identify populations that are likely to respond to immunotherapy to PD-1/PD-L1 inhibitor, such as 1) PD-L1 expression status, 2) count of TILs, 3) mismatch-repair (MMR) protein expression status, tumor mutation and neoantigen burdens, 4) oncogene mutations, 5) radiographic markers, and 6) clinical pathological features.²³

In 2017, we divided lung adenocarcinoma patients into four different TIME subgroups based on the PD-L1 expression status and CD8+TIL count, in an attempt to ascertain the prognosis and select the appropriate treatment.¹⁹ In this study, we used paired tumor samples from the same patients collected at different times. The purpose of this study was to compare the TIME classification based on the PD-L1 expression status and CD8+TIL count between the primary sites and recurrence sites. Until date, there are few reports on the changes in the PD-L1 expression status between surgically resected specimens of the primary tumors and paired re-biopsy specimens of the recurrent tumors. Another purpose of this study was to determine whether the chemotherapy regimen affects the TIME classification.

In this study, the PD-L1 expression level in the re-biopsy specimen was higher as compared to that in the

primary tumors in 17% of patients. However, when this analysis was confined to patients who had received platinum-based chemotherapy, the percentage of cases in which the PD-L1 expression increased in the re-biopsy specimens increased from 17% to 43%. On the other hand, the CD8+TIL count decreased in the re-biopsy specimens in 20% of cases. However, when the analysis included only patients who had received oral tegafur, this percentage increased to 36%. The TIME classification changed from the primary tumor to re-biopsy specimens in one-third of patients. When this analysis included only patients who had received chemotherapy, the percentage increased to >40%.

The discordance rate of the PD-L1 expression status between the paired samples was 22.2%. This could be attributable to either heterogeneity of the TIME within tumors or change of the tumor biology by chemotherapy. The discordance rate of PD-L1 expression between paired samples obtained at difference time-points observed in the present study is consistent with that reported from other studies.^{24–28} On the other hand, the current study revealed that the TIME classification in re-biopsy specimens more closely resembled that of the metastatic lymph nodes as compared to that of the primary tumors. These results suggest that in case re-biopsy proves difficult, it might be better to refer to the biological status of the metastatic lymph nodes than that of the primary tumor for determining the most appropriate chemotherapy regimen.

Oral tegafur (UFT and S1) is often selected as adjuvant chemotherapy in patients with various cancers, including gastric, lung, and pancreatic cancer. Postoperative adjuvant chemotherapy with UFT is the standard treatment for stage I NSCLC patients in Japan.^{29,30} Until date, there are few reports on the changes of the PD-L1 expression levels or CD8+TIL count after treatment with oral tegafur. In an in-vitro study of 5-FU treatment, Van Der Kraak et al reported that the PD-L1 expression increased following the treatment in gastrointestinal cancer cell lines.³¹ In our study, PD-L1 expression in the re-biopsy specimen increased in only one patient who had received oral tegafur, although the CD8+TIL count decreased in 33% of patients. Considering these results, we concluded that oral tegafur does not upregulate PD-L1 expression.

Platinum-based chemotherapy is often selected as first-line chemotherapy in patients with advanced NSCLC and as adjuvant chemotherapy in resected stage II or IIIA NSCLC patients.^{32–34} In a study of ovarian cancer, increase of the PD-L1 expression and

A

Group A	Histological type	Primary tumor	Re-biopsy
1	AD(Sol)	I	I
2	AD(Lep)	I	I
3	AD(Sol)	I	I
4	SQ	I	I
5	AD(Pap)	I	I
6	SQ	I	I
7	AD(Aci)	I	I
8	AD(Lep)	I	III
9	SQ	III	III
10	AD(Sol)	III	III
11	AD(Aci)	IV	II
12	AD(Aci)	IV	I
13	AD(Pap)	II	II

Group C	Histological type	Primary tumor	Re-biopsy
1	AD(Pap)	I	I
2	AD(Pap)	III	III
3	AS	IV	II
4	Large	II	I
5	AS	II	III
6	AD(Pap)	II	III
7	Large	II	II

Group B	Histological type	Primary tumor	Re-biopsy
1	AD(Pap)	I	I
2	AD(Sol)	I	I
3	AD(Pap)	I	I
4	AD(Sol)	I	III
5	AD(Aci)	I	III
6	SQ	I	II
7	AD(Pap)	III	III
8	AD(Pap)	III	III
9	Pleomorphic	IV	I
10	AD(Pap)	IV	IV
11	AD(Lep)	IV	IV
12	AD(Lep)	IV	II

Group D	Histological type	Primary tumor	Re-biopsy
1	AD(Aci)	III	II
2	AD(Pap)	II	III
3	AD(Aci)	II	II
4	AD(Aci)	II	II

B

	Group	Histological type	Primary tumor	Metastatic LN	Re-biopsy
1	A	AD	IV	IV	II
2	B	AD	I	III	III
3	B	AD	III	III	III
4	C	AD	I	I	I
5	C	AS	IV	II	II
6	C	AS	II	III	III
7	C	AD	II	II	III
8	D	AD	III	III	II
9	D	AD	II	II	III
10	D	AD	II	II	II

Figure 2 (A) Change of the tumor immune microenvironment classification, **(B)** heterogeneity of the tumor immune microenvironment classification Group A: patients received no treatment, Group B: patients received oral tegafur chemotherapy, Group C: patients received platinum-based chemotherapy, Group D: patients received EGFR-TKI. **Abbreviations:** AD, adenocarcinoma; SQ, squamous cell carcinoma; AS, adenosquamous carcinoma; Lep, lepidic; Aci, acinar; Pap, papillary; Sol, solid; LN, lymph node.

TILs was noted following neoadjuvant chemotherapy (mainly carboplatin and paclitaxel).³⁵ In the case of head and neck squamous cell carcinoma, alteration of PD-L1 expression was noted during treatment,

particularly following cisplatin-containing chemotherapy, in baseline PD-L1-negative patients.³⁶ In NSCLC cell lines, cisplatin upregulated PD-L1 expression, and the enhancement of PD-L1 expression in the cancer cell

lines occurred in a drug dose-dependent manner, and the depletion of PD-L1 significantly reduced cisplatin resistance.³⁷ Considering these results, it may be surmised that platinum-based chemotherapy upregulates PD-L1 expression. Our finding of increase of the PD-L1 expression in the re-biopsy in 43% of patients who had received platinum-based chemotherapy was consistent with these reports.

EGFR-TKIs are often selected as first-line chemotherapy in advanced NSCLC patients with tumors harboring mutated EGFR.^{38,39} In a previous study, the EGFR-TKI gefitinib reduced the tumor PD-L1 expression via inhibiting NF- κ B, in EGFR-mutant NSCLC, both in vitro and in vivo.⁴⁰ However, in our study, the re-biopsy specimens showed a decrease of the tumor PD-L1 expression in only 1 of 4 patients treated with EGFR-TKIs.

Our study had several limitations. First, the number of patients enrolled in the study was small, reflecting the difficulties in obtaining an appropriate number of paired specimens for such studies. Second, for IHC analyses of PD-L1 expression and CD8+TILs, we used relatively standardized scoring systems. However, different PD-L1 scoring protocols have been used to determine the appropriateness of treatment with each of the PD-1/PD-L1 inhibitors available in the market. Although a standardized stromal CD8+TIL scoring system is currently available, there is as yet no standardized CD8+TIL scoring protocol for lung cancer.

Conclusion

Comparisons of paired specimens of the primary tumor and re-biopsy specimens showed that the TIME classification changed from the primary to the recurrent tumor in one-third of the patients, especially in patients who had received tegafur or platinum-based chemotherapy. Our study results suggest that a recent re-biopsy sample is required in patients with recurrent NSCLC, especially those who have previously received chemotherapy, to determine whether PD-1/PD-L1 inhibitors should be used for treatment.

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Disclosure

The authors report no conflicts of interest in this work.

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