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ORIGINAL ARTICLE

Comparable immunoreactivity rates of PD-L1 in archival and recent specimens from non-small cell lung cancer

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Abstract

Background: Molecular targeted therapy including the use of monoclonal antibodies directed against the immune checkpoints PD-L1 and PD-1 receptor have remarkably improved the therapeutic response and survival of cancer patients. The tumor expression level of PD-L1 can predict the response rate to checkpoint inhibitors. We evaluated whether the time interval between tumor tissue sampling/paraffinization and immunohistochemistry affects the staining level of PD-L1 in non-small cell lung cancer (NSCLC).

Methods: This study comprised 137 patients with NSCLC. Tumors were stained with 22C3 or 28-8 antibodies.

Results: There was a significant correlation between the immunoreactivity rate of tumor tissues obtained using 22C3 and 28-8 clones. No statistical difference in immunoreactivity between archival and recent samples stained either with 22C3 or 28-8 antibodies was observed. The immunoreactivity rate achieved with 22C3 or 28-8 antibodies significantly correlated with tumor histological type and size, but not with specimen storage time, age, gender, smoking history, clinical stage, or lymph node metastasis.

Conclusion: In brief, the results of this study show that the time interval between tissue sampling/paraffinization and immunohistochemical analysis has no influence on the immunoreactivity rate of PD-L1 in NSCLC.

Introduction

Among malignant tumors, lung cancer is the leading cause of death, with an estimated 1.59 million deaths globally per year.[1,2] The five-year survival rate is approximately 15% among all patients and < 4% in patients with metastatic disease.[3,4] Histologically, approximately 85% of lung carcinomas are non-small cell lung cancer (NSCLC), including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.[5] The therapeutic efficacy of canonical cytotoxic drugs in advanced stages of lung cancer is limited.[6] Tyrosine kinase inhibitors have shown better therapeutic performance in a subgroup of tumors with oncogenic driver mutations.[7] However, the majority of lung carcinomas harbor no targetable genetic aberrations.[3] A promising approach for the management of lung cancer is the use of immune checkpoint inhibitors targeting PD-1 and PD-L1.[8] PD-1 is a receptor present on the surface of T cells that downregulates activated T cells when it binds to its ligands, PD-L1 and PD-L2.[9]

Many malignant tumors express high levels of PD-L1 that enable them to evade immune recognition by inducing anergy of cytotoxic T cells.[10] The significant correlation between the increased surface expression of PD-L1 and PD-L2 on tumor cells and poor prognosis of patients with cancer, including NSCLC, highlights the immune

checkpoint molecules as key targets for immunotherapy of cancer.[11–14] Indeed, monoclonal antibodies against PD-1/PD-L1 or checkpoint inhibitors currently used in clinical practice significantly prolong the survival of patients with malignancy.[15,16] Immune checkpoint molecules are also used as biomarkers.[17] The expression of PD-L1 on cancer cells can predict the therapeutic response to PD-L1 inhibitors, and various commercial immunohistochemical PD-L1 assays are currently available.[17,18] Positive staining of PD-L1 on tumor cells is required for the clinical indication of checkpoint inhibitors, thus rigorous assay standardization and awareness of factors that may affect PD-L1 expression are critical for reliability of the assay.[18,19]

The question we posed in this study is whether the time between tumor specimen sampling/paraffinization and immunohistochemical analysis affects the immunoreactivity of PD-L1 in tumor tissues from NSCLC patients. To address this question, we compared the PD-L1 expression level between archival and recent specimens taken from a heterogeneous population of NSCLC patients.

Methods

This study comprised 137 patients with lung cancer who consulted, underwent diagnostic procedures, and were treated from March to August 2017 at the Respiratory Center of Matsusaka Municipal Hospital. The characteristics and therapeutic history of the patients were retrospectively obtained from medical records. Table 1 describes the demography data and therapeutic history. Smoking status was defined as (former or current) smoker and never smoker. Tissue samples for diagnosis were collected during routine clinical practice. Sampling methods included surgery (n = 34), biopsy guided by bronchoscopy (n = 60) or computed tomography (CT, n = 24), and biopsy of metastatic lymph nodes (n = 6) or pleura (n = 4). There was no record of the sampling procedure in nine cases. Pathological tumor staging was performed using the eighth edition American Joint Committee on Cancer Cancer Staging Manual.[20] For statistical purposes, the number after T of the tumor node metastasis (TNM) classification was taken as the tumor size. The institutional review board of Matsusaka Municipal Hospital approved the study protocol (Approval No. J-4-170327-3, March 2017).

Table 1 Characteristic of the study subjects

Variables	Number of patients
Total number of patients	137
Mean age (years)	74.08 ± 9.32
Gender	
Men	92
Women	45
Smoking history	
Never smoker	42
Smoker (former + current)	95
Specimen	
Archival sample	28
Fresh sample	109
Tumor stage	
I	49
II	8
III	29
IV	51
Histologic subtype	
Adenocarcinoma	89
Squamous cell carcinoma	45
Adenosquamous carcinoma	2
Other	1
PD-L1 expression level	
With 22C3 clone	
No-stain group (≤ 1%)	67
Low-stain group (1–49%)	43
High-stain group (≥ 50%)	27
With 28–8 clone	
No-stain group (≤ 1%)	72
Low-stain group (1–9%)	26
High-stain group (≥ 10%)	39
Previous treatment	
Platinum-based doublet chemotherapy	28
Platinum-based doublet chemotherapy + nivolumab (2nd line)	17
Platinum-based doublet chemotherapy + pembrolizumab (2nd line)	6
Platinum-based doublet chemotherapy + bevacizumab	12
EGFR-TKI	11
Pembrolizumab	3
Uracil + Tegafur	3
Surgery	34
Others	23

TKI, tyrosine kinase inhibitor.

Immunohistochemistry of PD-L1

Formalin-fixed, paraffin-embedded tumor samples were used for staining PD-L1 expression. The expression of PD-L1 in tumor tissues was stained at a specialized Laboratory at the LSI Medience Corporation (Chiyoda-ku, Tokyo, Japan) by immunohistochemistry (IHC) using the PD-L1 IHC 22C3 pharmDx and the PD-L1 IHC 28-8 pharmDx (Dako, Carpinteria, CA, USA) companion diagnostic tests.

The laboratory report of the immunoreactivity rate on tumor tissue was converted to continuous variables to assess correlations and differences between groups. The specimens were categorized into three groups according to the immunoreactivity rate: no-stain group for both 22C3 and 28-8 clones, low-stain group for 22C3 (≥ 1–49%) and 28-8 (1–10%) clones, and high-stain group for 22C3 (≥ 50%) and 28-8 (≥ 10%) clones. In addition, the tissue

specimens were categorized into archival samples (staining performed ≥ 6 months of collection/paraffinization) and recent samples (staining performed < 6 months of collection/paraffinization). In the KEYNOTE-010 study, archival samples included specimens collected in a median time of 250 days (8.3 months) and fresh samples were those collected in a median time of 11 days. In the present study, the number of specimens collected ≥ 7 months before PD-L1 assessment was very small, therefore, 6 months was used as the period of time to allocate the specimens into archival or recent samples.

Statistical analysis

All data are expressed as the mean ± standard deviation of the mean (SD). The difference between tumor immunoreactivity rates was assessed by Fisher's exact test. All PD-L1 staining proportions were considered as continuous variables to evaluate the difference in immunoreactivity rates between 22C3 and 28-8 antibody clones, archival and recent specimens, tumor histological types, tumor size, and to assess the relationship between parameters. Differences between the two groups were evaluated by Student's *t* or Mann–Whitney *U* test depending on whether the samples had a normal or skewed distribution. We used the Spearman correlation to assess the relationship between variables. Prism version 7 (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis. A P value < 0.05 was considered statistically significant.

Results

Demography data

There was a significant difference in age and lung cancer clinical stage between patients with archival and recent specimens (Table 2). There were no significant statistical differences in gender, smoking history, tumor histological type, tumor size, lymph node metastasis, or immunoreactivity rates using 22C3 or 28-8 clones between patients with archival and recent specimens (Table 2).

Correlation between the immunoreactivity rates obtained using 22C3 and 28-8 antibodies

The positive immunoreactivity rates obtained using 22C3 and 28-8 clones were significantly correlated among all patients and their mean immunoreactivity rates were not significantly different (Fig 1).

Table 2 Characteristics of subjects with archival and recent specimens

Variables	Archival specimens	Recent specimens	*P*
Number of patients	28	109	
Age (range) (years)	71.1 ± 7.4	74.8 ± 9.6	0.03
Gender			0.224
Men	21	71	
Women	7	38	
Smoking history			0.491
Never smoker	8	34	
Former smoker	20	75	
Stage			0.005
I	3	46	
II	1	7	
III	11	18	
IV	13	38	
Histologic subtype			0.888
Adenocarcinoma	17	70	
Squamous cell carcinoma	10	34	
Other	1	5	
Tumor size			0.08
1 (+Tis, +Tmi)	5	46	
2	14	32	
3	4	13	
4	5	18	
Lymph node			0.263
N0	11	58	
N1	3	9	
N2	10	21	
N3	4	21	
PD-L1 expression level			0.415
With 22C3 antibody			
No-stain group (≤ 1%)	12	55	
Low-stain group (1–49%)	8	35	
High-group (≥ 50%)	8	19	
With 28–8 antibody			0.650
No-stain group (≤ 1%)	12	60	
Low-stain group (1–9%)	6	18	
High-stain group (≥ 10%)	8	31	

No significant difference in immunoreactivity rates between archival and recent samples

To clarify whether the time from tissue sampling/paraffinization to IHC analysis affects the expression level with the antibodies, we compared the immunoreactivity rates between archival and recent samples. There were no statistical differences between the immunoreactivity rates of archival and recent samples stained with either 22C3 or 28-8 clones (Fig 2).

Effect of histological type and T factor on immunoreactivity rate

The expression levels with 22C3 or 28-8 clones were significantly higher in patients with squamous cell carcinoma

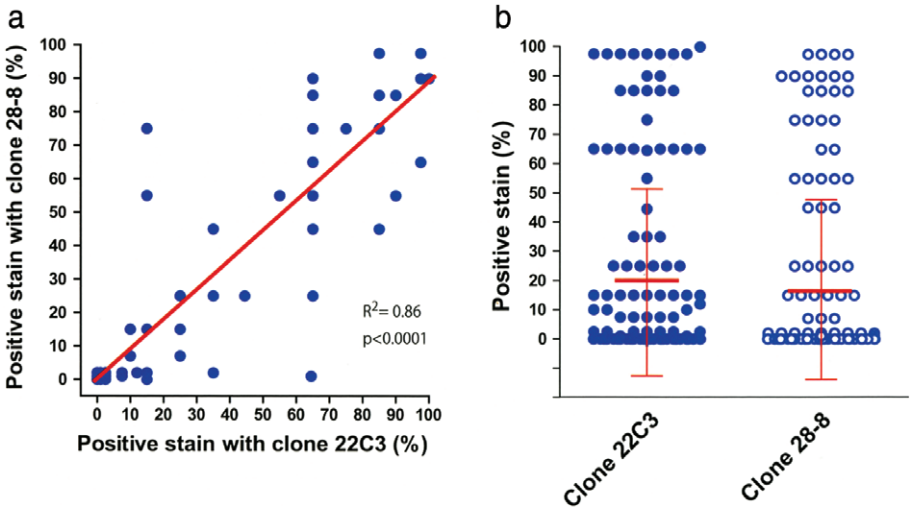

Figure 1 Correlation between the immunoreactivity rates using 22C3 and 28–8 clones. The immunoreactivity rates for each antibody were used as continuous variables for statistical analysis. The figure depicts the Spearman correlation coefficients. Wide bars indicate the mean values and narrow bars indicate the standard deviation of the mean.

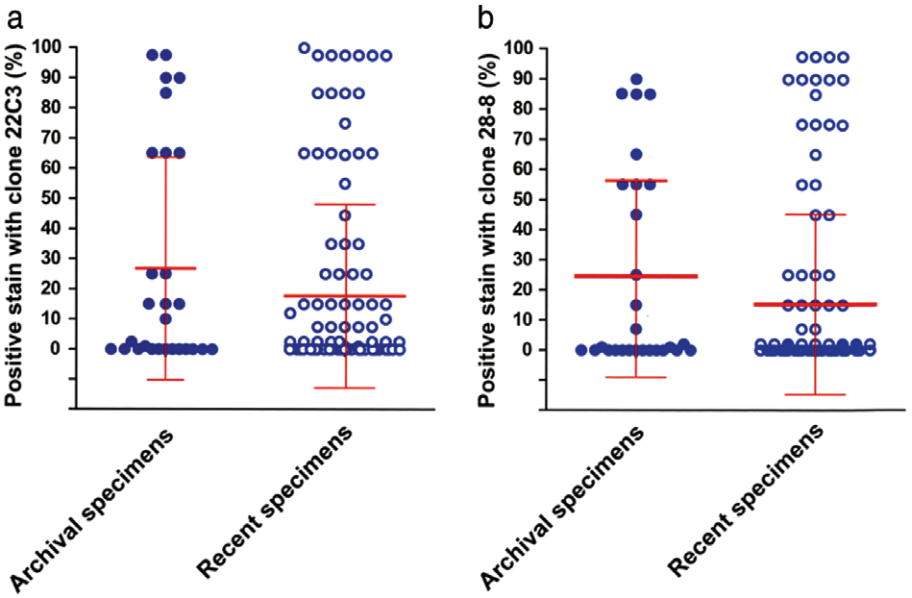

Figure 2 There were no significant differences in immunoreactivity rates between archival and recent samples. The immunoreactivity rates for each antibody were used as continuous variables for statistical analysis. Wide bars indicate mean values and narrow bars indicate the standard deviation of the mean.

than adenocarcinoma (Fig 3). The tumor specimens from patients with T2 tumors showed a significantly high immunoreactivity rate with 22C3 compared to patients with T1, while patients with T3 showed a significantly high immunoreactivity rate with 28-8 compared to patients with T1 (Fig 4).

Correlation of stain rate with clinical parameters

The expression level achieved using both 22C3 and 28-8 clones was significantly correlated with tumor histological type and size, but showed no significant correlation with the time interval between tissue sampling/paraffinization to immunohistochemistry analysis or with age, gender, smoking history, clinical stage, or lymph node metastasis (Table 3).

Discussion

Recent clinical trials have proven the therapeutic efficacy of checkpoint inhibitors.[15] As second-line therapy, two PD-1 inhibitors (nivolumab, pembrolizumab) and one PD-L1 inhibitor (atezolizumab) significantly ameliorate the response rate and overall survival of NSCLC patients in comparison with standard chemotherapy.[21–24] In addition, the improvement in survival after pembrolizumab administration is superior to standard chemotherapy, even as first-line therapy.[25] The survival benefit achieved with this targeted immunotherapy has led to a dramatic global change

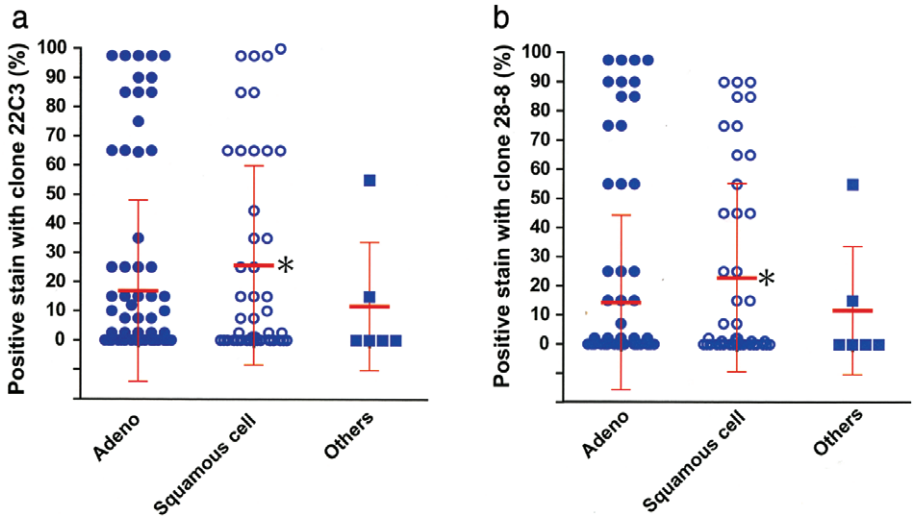

Figure 3 Effect of histological type on immunoreactivity rate. The immunoreactivity rates for each antibody were used as continuous variables for statistical analysis. Wide bars indicate the mean values and narrow bars indicate the standard deviation of the mean. **P* < 0.05 versus adenocarcinoma.

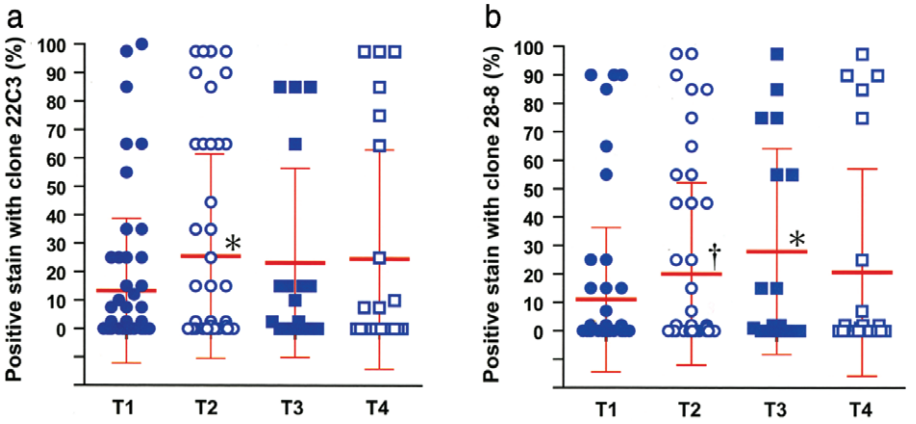

Figure 4 Effect of T factor on immunoreactivity rate. The immunoreactivity rates for each antibody were used as continuous variables for statistical analysis. Wide bars indicate the mean values and narrow bars indicate the standard deviation of the mean. †*P* = 0.05 versus T1. **P* < 0.05 versus T1.

Table 3 Correlation coefficients of immunoreactivity rate with clinical parameters

	Staining with 22C3 clone (%)		Staining with 28-8 clone	
(%)	R values	*P*	R values	*P*
Age	0.118	0.084	0.075	0.191
Gender	0.122	0.077	0.088	0.151
Smoking	0.116	0.086	0.090	0.147
Days before staining	0.056	0.256	0.016	0.423
Histology	0.179	0.017	0.178	0.018
Stage	0.076	0.188	0.090	0.145
Tumor size†	0.158	0.031	0.210	0.006
Lymph node metastasis	−0.000	0.476	−0.003	0.482

†The number after T of the tumor node metastasis classification was taken as tumor size. R calculated by Spearman correlation.

in guidelines for the clinical management of NSCLC patients. The indication of checkpoint inhibitors in clinical practice requires the positive staining of PD-L1 on tumor tissues by IHC.[19] Therefore, the appropriate identification of eligible patients for anti-PD-1 or anti-PD-L1 therapy requires a reliable evaluation of the expression of checkpoint molecules on tumor tissues. To date, several studies have shown that multiple factors can affect the reported expression level or IHC status of PD-L1 on malignant tumors, including tumor heterogeneity, histological type, tumor or specimen size, tissue source (metastatic or primary tumor), antibody clones, cutoff expression, pathologist interpretation, assay variability, or sampling error.[18,19,26,27] Consistent with the results of some prior studies, our reported immunoreactivity rate of PD-L1 was significantly higher in squamous cell carcinoma or in large sized tumors than in adenocarcinomas or small sized tumors, and there was good concordance between the immunoreactivity rates yielded by staining with 22C3 and 28-8 antibody clones.

In addition to factors described above, in clinical practice, another factor that may potentially affect the staining level of PD-L1 in tumor specimens is the time from tissue

fixation/paraffin embedding to IHC. This particular situation may occur in hospitals where the samples are transferred to a distant laboratory to perform IHC. Giunchi *et al.* reported that the expression level of PD-L1 faded in paraffin-embedded NSCLC tissues stored for more than a year.[28] Further, Herbst *et al.* reported that the tumor proportion score for PD-L1 was superior in fresh samples compared to archival samples, although patient survival was not related to the time of sample collection.[29] Herein, we attempted to define a "safe storage time" for a validated evaluation of the expression level of PD-L1 by IHC. We retrospectively collected paraffin-embedded tissues sampled from NSCLC patients at different time points and assessed their PD-L1 expression level at a specialized laboratory. The results revealed no significant correlation between the immunoreactivity rate of PD-L1 and the storage time of the samples, and arbitrary categorization of the tumor specimens into archival (≥ 6 month old) and recent (< 6 month old) samples disclosed no significant difference. These findings suggest that immunohistochemical staining of PD-L1 is feasible and has clinical significance even in specimen blocks older than six months.

Limitations of the current study include the small number of patients, the retrospective nature of the study, the different kinds of methods used to obtain the specimens, and the comparative evaluation between archival and recent samples using specimens collected from different groups of patients.

In conclusion, this study is the first to demonstrate that the time interval between tissue sampling/paraffinization to IHC analysis has no influence on the expression level of PD-L1 in NSCLC.

Disclosure

No authors report any conflict of interest.

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