# An Immunohistochemical Analysis of PD-L1 Protein Expression in Surgically Resected Small Cell Lung Cancer Using Different Antibodies and Criteria

KAZUKI TAKADA[1,2], GOUJI TOYOKAWA[1], TATSURO OKAMOTO[1], TAKAKI AKAMINE[1], SHINKICHI TAKAMORI[1], MASAKAZU KATSURA[1], TAKATOSHI FUJISHITA[1], FUMIHIRO SHOJI[1], YOSHINAO ODA[2] and YOSHIHIKO MAEHARA[1]

*Departments of [1]Surgery and Science, [2]Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan*

**Abstract.** *Background: Programmed cell death ligand 1 (PD-L1) expression in lung cancer appears to be important in immunotherapy, as its expression can predict responses to programmed cell death-1 (PD-1)-blocking antibodies. However, a definitive antibody and cut-off value for PD-L1 expression are urgently needed. Materials and Methods: The PD-L1 expression in 40 surgically resected small cell lung cancer (SCLC) specimens was evaluated by immunohistochemistry (IHC) with three different antibodies: clones E1L3N, 28-8, and SP142, and using three different evaluations: the Allred score, 1% cut-off, and 5% cut-off. Results: The percentage of tumors with positive PD-L1 expression was inconsistent in the IHC evaluations using the Allred score and 1% cut-off. However, the IHC evaluations using the 5% cut-off showed similar rates of expression using the three different antibodies. Conclusion: The results of this study provided detailed evidence on the frequency of PD-L1 expression in surgically resected SCLC, which may be a useful reference for identifying patients with PD-L1-expressing SCLC.*

Lung cancer is the leading cause of cancer-related death worldwide (1). Small cell lung cancer (SCLC) is a devastating histological subtype of lung cancer that is sensitive to chemotherapy and radiotherapy. However, despite good responses to these treatments, relapses are frequently observed, and the prognosis of SCLC remains poor. Therefore, new strategies, such as immunotherapy, are urgently needed to improve the outcome.

Protein expression of programmed cell death ligand 1 (PD-L1) is expected to be a predictive biomarker for responses to programmed cell death-1 (PD-1) antibodies in lung cancer, specifically non SCLC (2, 3), and a subset analysis of CheckMate-057 showed close correlation between PD-L1 protein expression and efficacy of PD-1 antibodies (4). Therefore, it seems important to examine PD-L1 protein expression in treatment with immunotherapy; however, the definitive antibody and criteria for PD-L1 protein expression have yet to be established. Recently, McLaughllin *et al.* reported that discordance of PD-L1 protein expression was observed even for the same sample using different antibodies (5). Therefore, issues regarding definitive antibodies and criteria should be urgently addressed to identify patients who would benefit from immunotherapy.

The identification of patients with SCLC expressing PD-L1 protein will be important when immune checkpoint inhibition becomes recognized as a standard treatment option for SCLC. Several recent studies have evaluated the PD-L1 protein expression in patients with SCLC, and we also examined its expression in surgically resected SCLC specimens (unpublished results), as summarized in Table I (6-8). However, the PD-L1 protein expression reported in these studies ranges widely, possibly due to the use of different antibodies and cut-off values in each study and the non-surgical biopsied specimens examined. A standardized quantitative assay for immunohistochemistry (IHC) of PD-L1 is, therefore, necessary for the accurate identification of patients with SCLC who can be successfully treated with antibodies to PD-1.

In this translational study, three different antibodies (clones E1L3N, 28-8, and SP142) and cut-off values (the Allred score, 1% cut-off, and 5% cut-off) were used to examine the PD-L1 protein expression in surgically resected SCLC.

*Correspondence to:* Gouji Toyokawa, MD, Ph.D., Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, 812-8582 Fukuoka, Japan. Tel.: +81 926425466, Fax: +81 926425482, e-mail: gouji104kawa@gmail.com

*Key Words:* PD-L1, immunohistochemistry, small cell lung cancer.

Table I. *Previous studies examining programmed cell death-ligand 1 protein expression in small cell lung cancer.*

| Author (Add reference to list) | Samples | Antibody | IHC evaluation, % (n/N) |
|---|---|---|---|
| Schultheis *et al*. (6) | 94 | 5H1 (Non-commercical) | Membranous |
| | Resection 51, biopsy 43 | | Allred score |
| | | E1L3N (Cell Signaling Technology) | TCs: 0% (0/92) |
| | Tissue microarrays | | ICs: 18.5% (17/92) |
| Ott *et al*. (7) | 147 | 22C3 (Non-commercial) | Membranous |
| | | | Cut-off ≥1% |
| | Unclear | | TCs/ICs: 28.6% (42/147) |
| Ishii *et al*. (8) | 102 | EPR1161 (Abcam) | Membranous/cytoplasmatic |
| | Biopsy in most cases | | Cut-off ≥5% |
| | | | TCs: 71.6% (73/102) |
| Toyokawa *et al*.* | 40 | SP142 (Ventana) | Membranous |
| | | | Cut-off ≥5% |
| | Resection in all cases | | TCs: 15% (6/40), ICs: 40% (16/40) |
| | | | TCs/ICs: 45% (18/40) |

IHC: Immunohistochemistry, TCs: tumor cells, ICs: tumor-infiltrating immune cells. *Unpublished data.

## Materials and Methods

*Materials*. We retrospectively screened 62 patients who underwent surgical resection of SCLC between April 1974 and August 2015 at the Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University. Of these 62 patients, the 40 with specimens available for the IHC analysis of PD-L1 expression were included in this study. This study was approved by our Institutional Review Board (Kyushu University, IRB No. 27-435).

*Immunohistochemical analysis*. The PD-L1 IHC analysis was performed using three different antibodies, as follows: clone E1L3N (dilution 1:200; Cell Signaling Technology, Cambridge, UK), clone 28-8 (dilution 1:450; Abcam, Cambridge, UK), and clone SP142 (dilution 1:100; Spring Bioscience, Ventana, Tucson, AZ, USA). All three of these antibodies are commercially available rabbit monoclonal antibodies and yield similar staining patterns (Figure 1).

Immunohistochemistry was performed using formalin-fixed tissue sections. Sections were cut (4 μm thickness), dewaxed with xylene, and rehydrated through a graded series of ethanol. After inhibition of endogenous peroxidase activity for 30 min with 3% $H_2O_2$ in methanol, the sections were pretreated with Target Retrieval Solution (Dako, Glostrup, Denmark) in a decloaking chamber at 110˚C for 15 min (clones 28-8 and SP142) or with EDTA in a microwave at 100˚C for 20 min (clone E1L3N) and then incubated with monoclonal antibodies at 4˚C overnight. The immune complex was detected with a DAKO EnVision Detection System (Dako). The sections were finally reacted in 3,3'-diaminobenzidine, counterstained with hematoxylin, and mounted. Tumor cells (TCs) and tumor-infiltrating immune cells (ICs) such as macrophages exhibit membranous staining for PD-L1, which was evaluated as positive staining. Cytoplasmatic staining was considered nonspecific.

The proportions of PD-L1-positive TCs and ICs were estimated as the number of stained TCs and ICs divided by the total numbers of TCs and ICs, respectively. They were estimated in whole sections independently by three investigators (K.T., M.K. and G.T.). If the independent assessments did not agree, the slides were reviewed by all three investigators together to achieve consensus. The consensus judgments were adopted as the final results. Sections of human placenta were used as positive controls. All of the IHC analyses were evaluated according to three different criteria, as follows: Firstly, we scored the PD-L1 protein expression using the Allred score, with the intensity subdivided into four categories (range: 0-3), the proportion of PD-L1-positive cells subdivided into six categories (range: 0-5), and both values added to yield the Allred score (range: 0-8) (9). The cut-off value was defined as the median of these scores. Secondly, cases with fewer than 1% stained cells were considered negative (1% cut-off). Thirdly, cases with fewer than 5% stained cells were considered negative (5% cut-off).

Table II. *The percentages of tumors with positive expression of programmed cell death-ligand 1 protein.*

| | | Antibody clone | | |
|---|---|---|---|---|
| Method | Cells | E1L3N | 28-8 | SP142 |
| Allred score | TCs | 22.50% | 27.50% | 35% |
| | ICs | 42.50% | 42.50% | 50% |
| | TCs/ICs | 50% | 47.50% | 60% |
| ≥1% | TCs | 20% | 27.50% | 32.50% |
| | ICs | 40% | 42.50% | 52.50% |
| | TCs/ICs | 45% | 47.50% | 57.50% |
| ≥5% | TCs | 15% | 15% | 15% |
| | ICs | 37.50% | 37.50% | 40% |
| | TCs/ICs | 42.50% | 42.50% | 45% |

TCs: Tumor cells, ICs: tumor-infiltrating immune cells, TCs/ICs: at least one of TCs and ICs.

## Results

*PD-L1 protein expression in SCLC*. Immunohistochemical staining for PD-L1 was observed in the membrane of TCs and ICs, and representative PD-L1 staining patterns are shown in Figure 1. Table II shows the percentages of tumors with

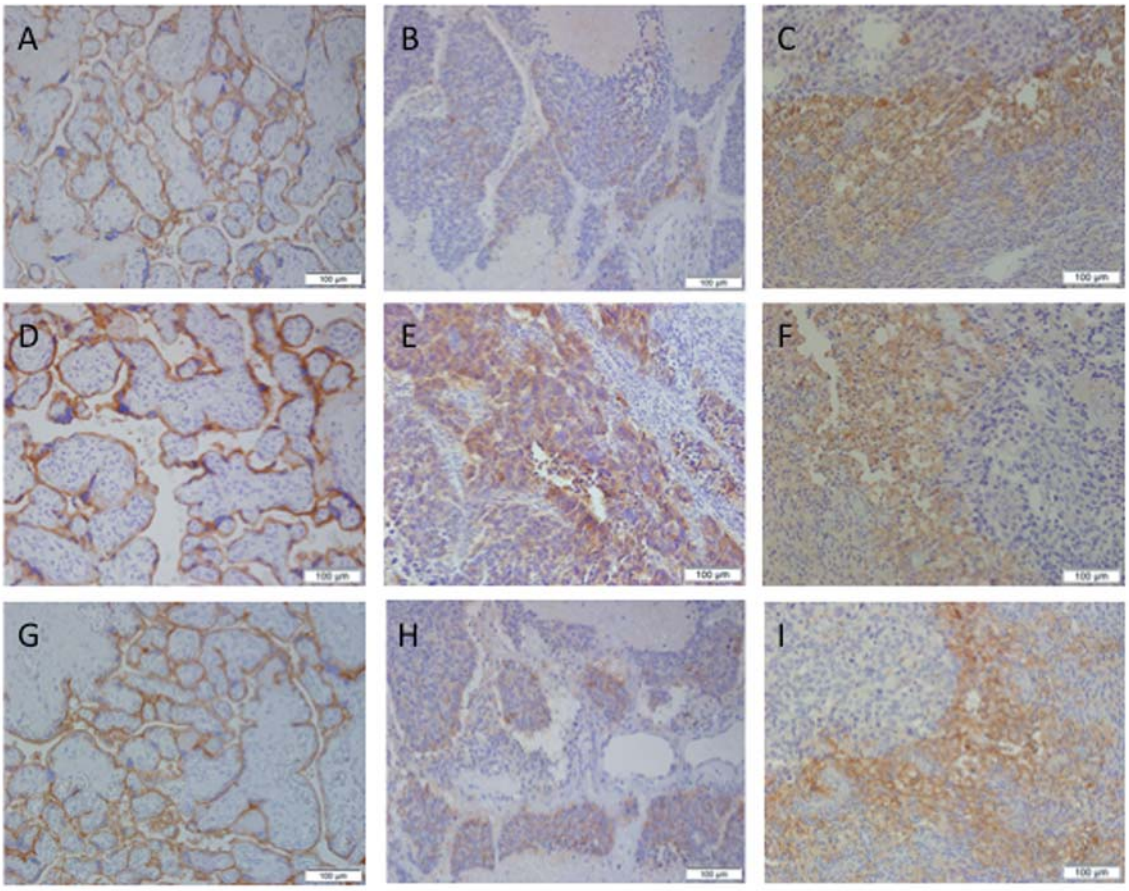


Figure 1. *The findings for immunohistochemical staining of programmed cell death-ligand 1 (PD-L1) using antibody clone E1L3N (A-C), clone 28-8 (D-F), and clone SP142 (G-I). Representative images of the human placenta tissue (positive control; A, D, G), PD-L1-positive tumor cells (B, E, H), and PD-L1-positive tumor-infiltrating immune cells (C, F, I) are shown. Scale bar: 100 μm.*

positive PD-L1 protein expression in this study. The percentage of tumors with positive PD-L1 protein expression varied widely in the IHC evaluations using the Allred score: TCs: 22.5-35%; ICs: 42.5-50%; TCs/ICs: 50-60%. The rate of expression was also inconsistent when using the 1% cut-off: TCs: 20-32.5%; ICs: 40-52.5%; TCs/ICs: 45-57.5%. However, the IHC evaluation using the 5% cut-off showed similar rates of expression in each type of cell using the three different antibodies: TCs: 15%, ICs: 37.5-40%, TCs/ICs: 42.5-45%.

## Discussion

Immunotherapy is expected to become a novel treatment option for SCLC, and phase I/II studies KEYNOTE-028 and CheckMate-032 are ongoing (7, 10). Specifically, KEYNOTE-028 is investigating the efficacy of immunotherapy for SCLC patients expressing PD-L1 protein (7). If PD-1 monoclonal antibodies become the standard of care for SCLC, identifying patients with PD-L1 protein expression will be crucial.

As shown in Table I, previous studies have used a number of different antibodies and criteria for assessing PD-L1 protein expression, and importantly, almost all specimens examined in these studies were obtained from biopsy, which can lead to inconsistent PD-L1 protein expression in SCLC (6-8). As such, the frequency of PD-L1 protein expression in patients with SCLC remains unclear. We, therefore, retrospectively examined the PD-L1 protein expression in surgically resected SCLC specimens using the three different antibodies and three different criteria used in the previous studies (clone 28-8 was used instead of clone EPR1161) and compared the results (6-8).

In the present study, the percentages of tumors with positive PD-L1 protein expression varied widely in the IHC evaluations using the Allred score and 1% cut-off; however, the rates of expression obtained using the 5% cut-off were similar among the three different antibodies. The strengths of the study are as follows: firstly, we conducted detailed expression analyses using different antibodies and cut-off values; secondly, we used surgically resected specimens rather than biopsied ones; thirdly,

we performed detailed evaluations not only for TCs, but also for ICs and TCs and ICs together.

Given that the antibody for clone 22C3 is non-commercial, we did not conduct companion diagnostics in this study; however, we did conduct complementary diagnostics using clone 28-8. Using a cut-off value of 5%, the percentages of tumors with positive PD-L1 protein expression were almost the same for the three different antibodies. However, using other criteria, the PD-L1 protein expression varied widely, suggesting the potential for overdiagnosis or underdiagnosis. Further studies are necessary to establish the definitive cut-off value for PD-L1 protein expression.

In the previous studies, almost all samples were derived from non-surgical patients (6-8). A phase I study showed that the percentage of tumors with positive PD-L1 protein expression was 28.6%; however, the percentage in the present study, using the same antibody and criteria, was 47.5% (7). This difference may be due to our using surgical samples, *versus* the non-surgical ones used in the phase I study. Since all samples examined in our study were from surgical patients, we believe that our results more closely reflect the true frequency of PD-L1 protein expression in SCLC, which will help identify patients with PD-L1-expressing SCLC who might benefit from immunotherapy.

Because PD-L1 protein is expressed on the surface of not only TCs but also ICs, the PD-L1 protein expression was analyzed in both TCs and ICs in this study (11). At present, it is unclear whether PD-L1-expressing TCs, ICs, or both are the most appropriate cell type to examine for a response to PD-1 antibody. Therefore, the correlation of PD-L1 protein expression in TCs, ICs, and both with the outcome of patients treated with anti-PD-1/PD-L1 agents should be examined in prospective or retrospective studies.

The results of this study help clarify the frequency of PD-L1 protein expression in surgically resected SCLC specimens. These findings may be a useful reference for identifying patients with PD-L1-expressing SCLC who might benefit from immunotherapy.

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## Conflicts of Interest

The Authors declare no conflicts of interest in association with this study.

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