Identification of candidate responders for anti-PD-L1/PD-1 immunotherapy, Rova-T therapy, or EZH2 inhibitory therapy in small-cell lung cancer

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Abstract. A useful candidate for small-cell lung cancer (SCLC) therapy is immune checkpoint blockade therapy targeting programmed death-1 (PD-1) and its ligand, PD-L1. Furthermore, rovalpituzumab tesirine (Rova-T), a delta-like protein 3 (DLL3)-targeted antibody-drug conjugate, and enhancer of zeste homologue 2 (EZH2) inhibitor are expected to be the first targeted therapy for SCLC. The aim of the present study was to evaluate PD-L1, DLL3 and EZH2 expression in SCLCs to find a candidate responder to those therapies. Immunohistochemical (IHC) staining for PD-L1, DLL3 and EZH2 was performed in 20 patients with SCLC and the clinicopathological characteristics and IHC staining intensity were compared. It was demonstrated that 1/20 patients (5.0%) exhibited positive PD-L1 expression in the metastatic lesions, as well as in the primary lung tumor. DLL3 was highly expressed in 14/20 patients (70%) and EZH2 was positive in 17/20 patients (85%). None of these cases exhibited any correlation with age, sex, smoking, stage or treatment, whereas IHC staining was able to identify candidate responders to anti-PD-L1/PD-1 immunotherapy, Rova-T therapy, or EZH2 inhibitor therapy.

Introduction

Small-cell lung cancer (SCLC) is an aggressive type of lung cancer exhibiting rapid growth and widespread metastases, with a poor prognosis (1). Unlike therapy for lung adenocarcinoma (LADC), the treatment for SCLC has not significantly advanced over the last three decades (2-4). To understand the genomic landscape and identify candidate therapeutic targets in SCLC, large-scale genomic analyses were performed, which revealed that mutations in *TP53* and *RB1*, and amplifications of the *MYC* family members *SOX2* and *SRSF1*, have been recurrently identified (5-8). Although it was reported that somatic genomic rearrangements of *TP73* contribute to SCLC tumorigenesis (5), druggable gene aberrations are rarely identified. As there is no standard targeted therapy for SCLC, platinum-based doublet chemotherapy is recommended as first-line treatment for advanced SCLC; however, its effectiveness is limited (2). Therefore, there is a need for development of further treatment options for patients with SCLC.

The blockade of immune checkpoints in cancer immunotherapy has exhibited durable positive efficacy in non-small-cell lung cancer (NSCLC), particularly LADC (9-11). Recent clinical trials have further investigated the efficacy of monotherapy with nivolumab (12) and of combination therapy with platinum-based doublet regimens (13). Due to these positive treatment effects on NSCLC, the blockade of immune checkpoints is also expected to be a useful therapy for SCLC. Although efforts have been made to develop a biomarker to identify patients who may benefit from immunotherapy (14), such a biomarker has yet to be determined. Previous reports demonstrated that the programmed death-ligand 1 (PD-L1) protein in tumor cells is a potential predictive biomarker of response to anti-PD-1/PD-L1 immunotherapy (10,15,16). Furthermore, PD-L1 expression in tumors was reported to be associated with improved efficacy of pembrolizumab (15) and with significantly longer progression-free and overall survival (17). We recently revealed the mechanism through which high expression of PD-L1 is caused by focal amplification of *CD274*, encoding the PD-L1 protein in SCLC (18). Although only a subset of SCLC tumors highly express PD-L1, such SCLC tumors may be particularly susceptible to immune checkpoint blockade therapy. Therefore, the aim of the present

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study was to investigate PD-L1 expression in an independent cohort of SCLCs, in order to identify a candidate responder to PD-L1 blockade therapy.

Delta-like protein 3 (DLL3) and enhancer of zeste homologue 2 (EZH2) expression was also investigated in SCLC tumors. DLL3 is highly expressed in the majority of SCLCs and inhibits the Notch receptor pathway, promoting SCLC tumorigenesis (19). A recent clinical trial demonstrated that rovalpituzumab tesirine (Rova-T), a DLL3-targeted antibody-drug conjugate, exhibited marked antitumor activity and durability in recurrent or refractory SCLC (20). EZH2 is also highly expressed in SCLC, and its inhibition by EZH2 inhibitor enhances the effectiveness of current standard chemotherapy (21). Higher expression of DLL3 and EZH2 in SCLC was associated with a higher rate of response to Rova-T and EZH2 inhibitor, respectively; thus, DLL3 and EZH2 expression may be a candidate predictive biomarker for SCLC treatment.

Patients and methods

Patients. The present study included 20 primary and two metastatic tumors obtained from 20 SCLC patients at surgery or autopsy performed between 1991 and 2013 at Akita University (Akita, Japan). We retrospectively collected information regarding age, sex, ethnicity, pathological TNM stage (22,23), and smoking status. The present study was approved by the Ethics Committee of Akita University (reference nos. 1241 and 1246) and written informed consent was obtained from all patients.

Immunohistochemical (IHC) staining and evaluation. IHC staining for PD-L1 was performed as previously described (18). In addition, IHC staining for DLL3 and EZH2 were performed. Briefly, IHC staining was performed on 4-μm paraffin-embedded histological sections that had been fixed in 10% buffered formalin using a polymer peroxidase method (EnVision+/HRP; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). Following deparaffinization with xylene and rehydration using a descending alcohol series, the tissue sections were treated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature to block endogenous peroxidase activity. Following rinsing in PBS, the sections were incubated with rabbit monoclonal anti-PD-L1 (1:400; cat. no. 13684, E1L3N; Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal anti-DLL3 (1:100; ab103102, Abcam, Cambridge, MA, USA), and rabbit monoclonal anti-EZH2 (1:100; cat. no. 12408, D2C9, Cell Signaling Technology) at 4°C overnight. An additional wash in PBS was followed by treatment with a ready-to-use peroxidase-labeled polymer conjugated to goat anti-rabbit immunoglobulins (catalog no. SM801; EnVision+ kit; Dako; Agilent Technologies, Inc.) as the secondary antibody for 30 min at room temperature. The staining was visualized with diaminobenzidine, followed by counterstaining with hematoxylin.

IHC staining for PD-L1, DLL3 and EZH2 was evaluated by two independent observers (M.S. and A.G.), including an expert pathologist (A.G.). For the evaluation of PD-L1 and EZH2, the H-score method was used (18). Briefly, staining percentages (0-100%) and the intensity (0, negative; 1, very weak; 2, moderate; and 3, strong expression) in tumor cells were evaluated, and immunostained slides were scored ranging

Table I. Patient characteristics of this study cohort (n=20).

Characteristics	No. (%)
Age, years	
Mean (range)	68.0 (45-82)
Sex	
Male	20 (100)
Female	0 (0)
Ethnicity (%)	
Asian	20 (100)
Caucasian	0 (0)
African american	0 (0)
Smoking status	
Never smoker	1 (5)
Current or former smoker (pack years)	19 (95)
<20	17 (85)
≥20	0 (0)
Unknown	2 (10)
TNM stage	
I	6 (30)
II	1 (5)
III	8 (40)
IV	5 (25)

from 0 to 300 by multiplying the percentage of tumor area. For the evaluation of PD-L1, the score was divided into two intensity levels (positive, ≥3; and negative, 0-2) due to antibody specificity (18). For the evaluation of EZH2, the score was divided into two intensity levels (positive, >100; and negative, ≤100), according to the previous study (24).

For the evaluation of DLL3, cytoplasmic or membranous staining at any intensity in the tumor cells was scored. The staining percentage (0-100%) was then evaluated and divided into two intensity levels (high, ≥50%; and low, 0-49%), according to the previous study (20).

Results

PD-L1 expression in SCLC. We previously reported that a subset (4/210, 1.9%) of SCLC cases exhibited high expression of PD-L1 caused by high-level amplification of *CD274* (18). As PD-L1-positive cases are rarely identified in SCLC, we further expanded the investigation to include 20 Japanese patients with SCLC (Table I). We performed IHC analyses of PD-L1 protein expression with the same anti-PD-L1 antibody used in our previous study (18), and identified one case (1/20, 5.0%) with positive PD-L1 expression (Fig. 1). This patient (AK014) had liver and lymph node metastases, and the metastatic tumors were positive for PD-L1 expression.

DLL3 and EZH2 expression in SCLC. IHC staining was next performed for DLL3 and EZH2 to identify candidate responders for Rova-T and EZH2 inhibitor therapy (Figs. 1 and 2). DLL3 expression of ≥1% was observed in 18/20 cases (90%) and of ≥50% in 14/20 cases (70%). The staining intensity of DLL3 was
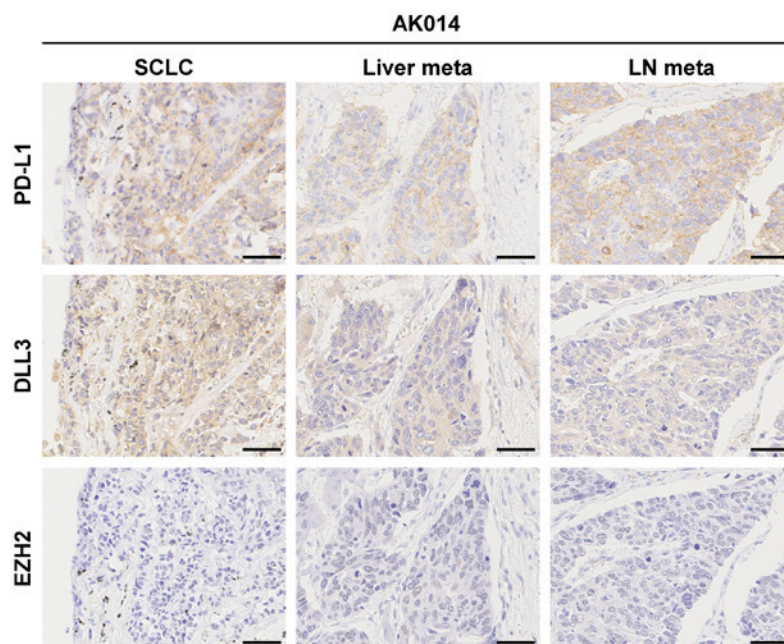
Figure 1. Immunohistochemical staining of PD-L1, DLL3, and EZH2 in SCLC. Positive expression for PD-L1, high expression for DLL3, and negative expression for EZH2 in SCLC and metastatic liver tumor and lymph node in a patient (AK014). Scale bars, 50 μm. SCLC, small-cell lung cancer; PD-L1, programmed death-ligand 1; DLL3, delta-like protein 3; EZH2, enhancer of zeste homologue 2; meta, metastasis.
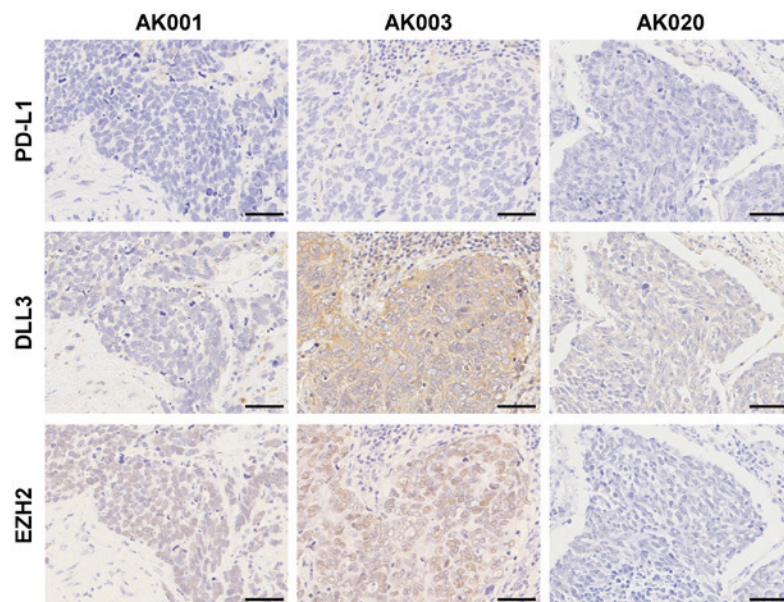

Figure 2. Immunohistochemical staining of PD-L1, DLL3, and EZH2 in SCLC. Negative expression for PD-L1, low expression of DLL3, and positive expression for EZH2 in a patient (AK001). Negative expression for PD-L1, high expression of DLL3, and positive expression for EZH2 in a patient (AK003). Negative expression for PD-L1, low expression of DLL3, and negative expression for EZH2 in a patient (AK020). Scale bars, 50 μm. SCLC, small-cell lung cancer; PD-L1, programmed death-ligand 1; DLL3, delta-like protein 3; EZH2, enhancer of zeste homologue 2.

consistent with a previous report from the USA (Table II) (20). EZH2 was positively expressed in 17/20 patients (85%) in our cohort. A case with positive PD-L1 expression exhibited high DLL3 and negative EZH2 expression in a metastatic liver tumor and a lymph node metastasis, as well as in a primary SCLC tumor (Fig. 1).

Therapeutic possibilities for SCLC. The clinicopathological characteristics, treatment history and results of IHC staining for PD-L1, DLL3 and EZH2 in the 20 cases are summarized in Table III. The cases with high DLL3 or positive EZH2 expression did not have any specific characteristics associated with age, sex, smoking, stage or treatment (Table III). Among the 20 cases, 1 exhibited positive PD-L1 expression, 14 exhibited high DLL3 expression, and 17 exhibited positive EZH2 expression. This result suggested that PD-L1 blockade therapy, Rova-T therapy, or EZH2 inhibitor therapy, may be used in 19/20 cases (95%).

Table II. Comparison of delta-like protein 3 expression in small-cell lung cancer.

Positively stained tumor cells (%)	Present study, n/total (%) (n=20)	Rudin *et al* (20), n/total (%) (n=48)
≥1	18/20 (90)	42/48 (88)
≥50	14/20 (70)	32/48 (67)

Table III. Clinicopathological characteristics and IHC staining results of SCLC.

Patient ID	Age, years	Sex	Smoking (pack years)	pStage	1st treatment	2nd treatment	IHC staining		
							PD-L1	DLL3	EZH2
AK001	66	Male	46	I	Surgery		Negative	Low	Positive
AK002	80	Male	65	III	Surgery		Negative	High	Positive
AK003	82	Male	58	I	Surgery		Negative	High	Positive
AK004	79	Male	40	I	Surgery		Negative	High	Positive
AK005	79	Male	87	I	Surgery		Negative	Low	Positive
AK006	45	Male	25	II	Chemotherapy	Surgery	Negative	High	Positive
AK007	77	Male	N/A	III	Surgery		Negative	Low	Positive
AK008	60	Male	40	I	Surgery		Negative	High	Positive
AK009	77	Male	53	I	Surgery		Negative	High	Positive
AK010	71	Male	25	III	Surgery		Negative	Low	Positive
AK011	74	Male	120	IV	Chemotherapy/radiation		Negative	High	Positive
AK012	59	Male	50	IV	Chemotherapy		Negative	High	Positive
AK013	63	Male	75	III	Chemotherapy		Negative	High	Positive
AK014	68	Male	0	IV	Radiation		Positive	High	Negative
AK015	74	Male	100	III	Chemotherapy		Negative	High	Positive
AK016	68	Male	23	IV	Chemotherapy/radiation		Negative	High	Positive
AK017	64	Male	50	III	Chemotherapy/radiation		Negative	High	Positive
AK018	47	Male	60	III	Chemotherapy/radiation		Negative	Low	Positive
AK019	59	Male	40	IV	Chemotherapy/radiation		Negative	High	Negative
AK020	68	Male	N/A	III	Chemotherapy		Negative	Low	Negative

IHC, immunohistochemical; SCLC, small-cell lung cancer; PD-L1, programmed death-ligand 1; DLL3, delta-like protein 3; EZH2, enhancer of zeste homologue 2.

Discussion

In the present study, it was reconfirmed that tumor expression of PD-L1 is rarely found to be upregulated at the protein level in SCLC. IHC analyses of PD-L1 protein expression was performed, employing the PD-L1 antibody E1L3 N, which was used in our previous study (18). The specificity of this antibody for PD-L1 was considered as high, since one case with high-level focal *CD274* amplification and high transcript levels only exhibited positive PD-L1 protein expression in our previous study (18). Therefore, we concluded that focal *CD274* amplification is associated with high PD-L1 antigen expression (18). The present study evaluated PD-L1 expression in cases with SCLC; however, the amplification of *CD274* was not investigated due to the lack of frozen tissue samples.

Anti-PD-1/PD-L1 immunotherapy, alone or in combination with other treatment modalities, exhibits a significant efficacy for patients with various malignant tumors, including NSCLC.

Predictive biomarkers, enabling the selection of patients who will benefit the most from PD-1/PD-L1-targeted therapy and the prevention of adverse events, are required to further increase positive outcomes. Although several studies have attempted to develop a predictive biomarker using IHC, including staining for PD-L1, there is currently no reliable predictive biomarker of this immunotherapy due to cancer immune complexity. In addition, PD-L1 antibodies used in IHC staining differ among various studies and different cut-off values of PD-L1 positivity are used, making it difficult to compare results across studies. Indeed, different companion antibodies of PD-L1 made by various pharmaceutical manufacturers have been used in clinical trials (25). Although PD-L1 expression was evaluated by IHC staining of SCLC cells in the present study, it has not been established whether PD-L1 expression is correlated with clinical response and outcome.

DLL3 expression was also evaluated in SCLC tumors. DLL3 is a novel druggable target of Rova-T. Rova-T is a

first-in-class antibody-drug conjugate directed against DLL3, and early-phase clinical trials of Rova-T assessed DLL3 expression by IHC staining. While objective responses were recorded in patients with high DLL3 expression, those with low DLL3 expression had no recorded objective responses (20). Therefore, DLL3 expression may be a therapeutic biomarker, as well as a therapeutic target. In fact, it was reported that DLL3 was expressed on the surface of tumor cells in ~85% of SCLC patients (19,20). Consistently, DLL3 was highly expressed in our Japanese SCLC patients, and may be expected to be a candidate responder to Rova-T therapy. In addition to DLL3, EZH2 expression was evaluated in SCLC tumors. EZH2 is also expected to be a candidate therapeutic target for SCLC. However, no clinical trials targeting EZH2 in SCLC patients are currently underway.

The early success of Rova-T is accentuating the significance of targeted therapy for improving the prognosis of patients with SCLC. Thus, EZH2 inhibition, as well as Rova-T therapy, may be an option for patients with recurrent SCLC. In cases where first-line treatment results in failure, monitoring the status of PD-L1, DLL3, or EZH2 in recurrent or metastatic tumors may serve as the next regimen.

In conclusion, PD-L1, DLL3 and EZH2 expression was evaluated in additional SCLC patients following our previous study, to investigate the adoption of precision medicine. In addition to anti-PD-1/PD-L1 immunotherapy, Rova-T therapy or other DLL3- and EZH2-targeted drugs may be expected to be proven useful for the treatment of SCLC patients.

Acknowledgements

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