Prevalence of Enhancer of Zeste Homolog 2 in Patients with Resected Small Cell Lung Cancer

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Abstract. *Background/Aim:* Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase that is deeply involved in cancer pathogenesis. Although clinicopathological significance of EZH2 in non-small cell lung cancer has been gradually elucidated, such significance in small cell lung cancer (SCLC) has yet to be fully investigated. *Patients and Methods:* Forty patients with resected SCLC were analyzed for EZH2. EZH2 expression was evaluated using the Allred score (0-8) and was classified into negative (0-6) and positive (7 and 8). We evaluated the association between EZH2 and the clinicopathological characteristics and postoperative survivals. *Results:* Among 40 patients, 15 (37.5%) and 25 (62.5%) were classified as being negative and positive for EZH2, respectively. Fisher's exact test demonstrated no significant associations between the positivity for EZH2 and clinicopathological characteristics. No significant differences were observed in recurrence-free and overall survivals between EZH2-negative/low and EZH2-high patients. *Conclusion:* EZH2 was frequently observed in patients with resected SCLC, but no significant associations were found between its expression and the clinicopathological characteristics and postoperative survivals.

Small cell lung cancer (SCLC) is an extremely devastating neoplasm and its prognosis remains very poor. Although SCLC is known to respond well to radiotherapy and chemotherapy, relapse after such treatment is very frequently encountered (1). In addition to radiotherapy and

chemotherapy, clinical benefit of surgical resection for patients with SCLC has been also reported (2-5); however, most patients inevitably encounter recurrence and eventual death and therefore, novel effective treatments should be developed and applied for patients with SCLC. With regard to immunotherapy for SCLC, a recent phase 1/2 trial demonstrated the durable responses of nivolumab, a programmed death-1 antibody, alone and nivolumab combined with ipilimumab, a cytotoxic T-lymphocyte antigen-4 (CTLA-4) antibody in recurrent SCLC (6): objective response rates of nivolumab monotherapy and nivolumab plus ipilimumab were 10% and 19-33%, respectively. Despite the possible efficacy of immunotherapy for SCLC, primary and acquired resistances inevitably occur and further promising therapeutic targets should be therefore investigated in the treatment of SCLC.

Histone methyltransferases are deeply involved in the transcriptional regulation of various genes via the methylation of histone tails and their associations with human cancer have been gradually elucidated (7). Among the histone methyltransferases, enhancer of zeste homolog 2 (EZH2) has a catalytic SET domain as a conserved domain among species and methylated lysine residue 27 on histone H3 (H3K27), which leads to transcriptional repression. EZH2 was shown to be associated with murine embryonic development through the regulation of H3K27-methylation-related transcription. Regarding its involvement in human cancer, a line of reports has demonstrated that EZH2 was overexpressed in various types of human cancer, including lung cancer (8-11). Furthermore, somatic mutations within two residues in the catalytic SET domain of the *EZH2* gene (Y641 and A677) were identified to increase H3K27 tri-methylation and to alter the substrate specificity of the mutated *EZH2* in diffuse large B-cell lymphoma and follicular lymphoma (12). Such mutations were not found in our analysis focusing on patients with SCLC (13); however, EZH2 was reported to promote E2F-driven tumorigenesis of SCLC via the modulation of

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apoptosis and cell-cycle regulation (14). However, the clinicopathological significance of EZH2 protein expression in the resected SCLC remains to be clarified.

In the current study, the relationship between EZH2 expression and the clinicopathological characteristics of patients with resected SCLC and its prognostic influence on their postoperative survival were evaluated.

Patients and Methods

Study patients. Among 62 patients with SCLC who underwent surgery at the Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University between April 1974 and August 2015, 40 patients, whose resected specimens could be analyzed of EZH2, were included in this translational study. Pathological staging was performed using the 7th edition of the TNM Classification of Malignant Tumors. In addition to the pathological stage, the patients' age, gender, performance status, smoking history, pathological tumor and pathological lymph nodal factors, pleural invasion (pl), lymphatic invasion (ly), vascular invasion (v) and surgical procedure were examined. The present study was approved by our institutional review board (the Number of the Ethic Approval: 28-380).

Immunohistochemical analyses. Immunohistochemistry was performed as described previously (15, 16). 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% H₂O₂ in methanol, the sections were pretreated with Target Retrieval Solution (Dako, Glostrup, Denmark) in a decloaking chamber at 110°C for 15 min and then incubated with monoclonal antibodies at 4°C overnight. Immune complexes were detected with a DAKO EnVision Detection System (Dako). The sections were finally reacted in 3,3'-diaminobenzidine, counterstained with hematoxylin, and mounted. The primary antibody for EZH2 was an anti-human EZH2 mouse monoclonal antibody (clone 6A10, dilution 1:100; Leica Biosystems, Newcastle, UK). Carcinoma cells showing nuclear staining for EZH2 were considered to be positive cells. Allred scores were applied to evaluate EZH2 expression (17). The primary antibody for programmed death-ligand 1 (PD-L1) was an anti-human PD-L1 rabbit monoclonal antibody (clone SP142, dilution 1:100; Spring Bioscience, Ventana, Tucson, AZ, USA). Carcinoma cells showing membranous staining for PD-L1 were evaluated as positive cells. The proportion of PD-L1-positive cells was estimated as the percentage of total carcinoma cells. The cut-off values were set at 5% as described previously (18). Human tonsils and placentas were used as positive controls for EZH2 and PD-L1, respectively. The evaluations were performed independently by three investigators (G.T., K.T. and Y.Y.). If the independent assessments did not agree, the slides were reviewed together to achieve consensus. The consensus judgments were adopted as the final results.

Spread through air spaces (STAS). STAS was defined as tumor cells within air spaces in the lung parenchyma beyond the edge of the main tumor (19, 20). In addition, STAS was also classified into the following three categories: "no STAS" for cases without definite STAS, "low STAS" for cases with 1 to 4 single cells or clusters of STAS, and "high STAS" for cases with five or more single cells or clusters of STAS (21). All tumors were evaluated at a magnification of ×200 using an optical microscope (BX40; Olympus, Tokyo, Japan).

Table I. Patient characteristics.

Factors	N
Age (years)	
<70	21
≥70	19
Gender	
Female	6
Male	34
Smoking history*	
Never-smoker	4
Smoker	34
TT1	16
≥T2	24
N	
Negative	23
Positive	17
Pathological stage	
I	20
≥II	20
pl*	
Negative	22
Positive	15
ly*	
Negative	28
Positive	11
v*	
Negative	18
Positive	21
Surgical procedure*	
≥Lobectomy	29
Sublobar resection	6
Adjuvant chemotherapy*	
None	13
Administered	10
PD-L1	
Negative	34
Positive	6
STAS*	
Negative	5
Positive	25

*Cases whose data are available. pl: Pleural invasion; ly: lymphatic invasion; v: vascular invasion; PD-L1: programmed death-ligand 1; STAS: spread through air spaces.

Statistical analyses. The associations between EZH2 and patient characteristics were analysed using Fisher's exact test. The overall survival (OS) was defined as the time from the initial surgery until death from any cause, while the recurrence-free survival (RFS) was defined as the time from the initial surgery until recurrence. The Kaplan-Meier method was used to estimate the survival probabilities, and the curves of the two or three groups were statistically compared using the log-rank test. All of the statistical analyses were conducted using the JMP version 12 software program (SAS Institute, Cary, NC, USA). *p*-Values of <0.05 were considered to indicate statistically significant differences.

Figure 1. Histogram showing the Allred scores of enhancer of zeste homolog 2 in 40 patients with resected small cell lung cancer.

Results

Clinicopathological characteristics of patients. Table I shows the characteristics of the patients included in this translational study. The number of patients <70 and ≥70 was 21 (52.5%) and 19 (47.5%), respectively. The number of female and male patients was six (15%) and 34 (85%), respectively. Thirty-four patients (85%) had a history of smoking. Sixteen (40%) and 24 (60%) patients had T1 and ≥T2 tumor, respectively, and 17 (43%) patients were positive for lymph node metastasis. Twenty (50%) and 20 (50%) patients were diagnosed with pathological stage I and II or III, respectively. Pathological examinations revealed pl, ly, and v in 15 (41%), 11 (28%), and 21 (54%) patients, respectively. Twenty-nine (83%) patients underwent surgical resection of more than one lobe, while six (17%) received sublobar resection. Ten (43%) patients received adjuvant chemotherapy. PD-L1 and STAS were positive in six (15%) and twenty-five (83%) patients, respectively.

Prevalence of EZH2 expression and its association with the clinicopathological characteristics. Among 40 patients, one (2.5%), zero (0%), two (5%), one (2.5%), one (2.5%), one (2.5%), nine (22.5%), 10 (25.0%) and 15 (37.5%) showed Allred score of 0, 1, 2, 3, 4, 5, 6, 7 and 8, respectively (Figure 1). Mean Allred score for EZH2 was 6.5 and therefore, cut-off value was set as Allred score of 7. Therefore, EZH2 positivity and negativity were found in 25 (62.5%) and 15 (37.5%) patients, respectively. According to the Fisher's exact test, positivity for EZH2 was not significantly associated with any clinicopathological features (Table II).

Survival analyses according to the EZH2 expression. RFS and OS after surgical resection were not significantly different between EZH2-negative and EZH2-high patients ($p=0.68$ and $p=0.80$, respectively; Figure 2A and 2B). The five-year RFS in EZH2-negative and EZH2-positive patients

Table II. Association between EZH2 and the clinicopathological factors in patients with resected small cell lung cancer.

Factors	n	EZH2 Negative/Positive	p-Value
Age (years)			
<70	21	10/11	0.20
≥70	19	5/14	
Gender			
Female	6	2/4	1.00
Male	34	13/21	
Smoking history*			
Never-smoker	4	1/3	1.00
Smoker	34	12/22	
T			
T1	16	5/11	0.74
≥T2	24	10/14	
N			
Negative	23	9/14	1.00
Positive	17	6/11	
Pathological stage			
I	20	8/12	1.00
≥II	20	7/13	
pl*			
Negative	22	9/13	0.74
Positive	15	5/10	
ly*			
Negative	28	12/16	0.48
Positive	11	3/8	
v*			
Negative	18	10/8	0.06
Positive	21	5/16	
Surgical procedure*			
≥Lobectomy	29	14/15	0.06
Sublobar resection	6	0/6	
Adjuvant chemotherapy*			
None	13	1/12	0.28
Administered	10	3/7	
PD-L1			
Negative	34	14/20	0.38
Positive	6	1/5	
STAS*			
Negative	5	1/4	1.00
Positive	25	8/17	

*Cases whose data are available. EZH2: Enhancer of zeste homolog 2; pl: pleural invasion; ly: lymphatic invasion; v: vascular invasion; PD-L1: programmed death-ligand 1; STAS: spread through air spaces.

were 32.0% and 23.8%, respectively. The five-year OS in EZH2-negative and EZH2-positive patients were 48.1% and 50.6%, respectively.

Discussion

In the current study, EZH2 positivity was identified in 25 (62.5%) among 40 patients with SCLC. As we reported previously, the frequency of EZH2 expression in the resected lung adenocarcinoma was 34.5%, which was lower than that

Figure 2. The (A) recurrence-free and (B) overall survival according to enhancer of zeste homolog 2 expression in 40 patients with resected small cell lung cancer.

in the resected SCLC (16). In contrast, the frequency of EZH2 expression in the resected squamous cell carcinoma was as high as 88.9% (16). However, when setting the cut-off of EZH2 expression at 3 (16), EZH2 positivity was found in 37 (92.5%) among 40 SCLC patients. These discrepant frequencies suggested that EZH2 expression might vary between the different histological types. Furthermore, although the EZH2 expression was significantly associated with the clinicopathologically invasive features in lung adenocarcinoma (16), the present study demonstrated no significant associations between EZH2 positivity and clinicopathological features. Furthermore, RFS and OS were not significantly different between EZH2-negative and EZH2-positive patients, while the presence of EZH2 was reported to negatively affect postoperative survivals in lung adenocarcinoma (8, 16). Several reasons can be considered for these discrepancies: the difference of EZH2 roles in each histological type and the limited sample size of the present.

High expression of EZH2 in patients with SCLC suggested that such patients might be targetable by EZH2 inhibitors. In non-Hodgkin lymphoma, an EZH2 inhibitor, EPZ6438, is under investigation by a phase I/II study (22). Although EZH2 has not yet been examined for its clinical targetability by EZH2 inhibitors, preclinical data showed that growth of SCLC could be suppressed by the knockdown of EZH2 (23). Furthermore, a report by Gardner *et al.* demonstrated that EZH2 drove acquired resistance to chemotherapeutic agents in SCLC and combination of an EZH2 inhibitor, EPZ011989, with standard cytotoxic chemotherapy suppressed the emergence of acquired resistance (24). Recently, EZH2 was reported to control the mechanisms of adaptive resistance to tumor immunotherapy in human skin cutaneous melanoma (25). The inactivation of EZH2 reversed resistance to CTLA-4 or Interleukin-2 (IL-2) immunotherapy and synergized with

anti-CTLA-4 and IL-2 immunotherapy, resulting in the suppression of melanoma proliferation. Thus, EZH2 has attracted much attention in the treatment of lung cancer, including both non-small cell lung cancer and SCLC and the above-mentioned preclinical evidence should be clinically investigated in the future studies.

The current study is associated with several limitations, including that it examined a small cohort of patients with resected SCLC. In addition to the further analysis in a larger cohort of patients with resected SCLC, future studies are warranted to elucidate the significance of EZH2 expression in patients with advanced SCLC.

In conclusion, the present study revealed that EZH2 was frequently observed in the resected SCLC and no impacts on postoperative survivals were identified.

Conflicts of Interest

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

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