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# Prognostic Significance of BMI-1 But Not MEL-18 Expression in Pulmonary Squamous Cell Carcinoma

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**Abstract.** *Aim: We investigated the possibility of BMI-1 and MEL-18 to predict survival in patients with pulmonary squamous cell carcinoma. Materials and Methods: One hundred and ninety-nine patients underwent surgery in our Institute between 1995 and 2005. We used immunohistochemical (IHC) analysis to determine the expressions of BMI-1 and MEL-18 and compared them with clinicopathological factors and survival. Results: Forty-one of 199 cases (21%) were BMI-1-positive. No correlation was found between BMI-1 and MEL-18 expression by IHC and clinicopathological factors. Five-year overall survival in the BMI-1-positive group (66.8%), but not MEL-18, was significantly better than that in the negative group (45.5%, p=0.04). In multivariate analysis, positive BMI-1 was a better prognostic factor of overall survival (hazard ratio (HR)=0.561, 95% confidence interval (CI)=0.271-1.16, p=0.12). Conclusion: BMI-1 expression, but not MEL-18, is associated with a favorable prognosis and is a possible prognostic factor of pulmonary squamous cell carcinoma.*

Lung cancer is the major cause of malignancy-related death worldwide (1). Approximately, 85% of lung cancer is non-small cell lung cancer (NSCLC) and the most frequently diagnosed histologies are adenocarcinoma and squamous cell carcinoma. Although adenocarcinoma is becoming more well-characterized, driver mutations of epidermal growth factor receptor (EGFR) (2) or translocation of echinoderm microtubule-associated protein-like 4 (EML4)-anaplastic lymphoma kinase (ALK) (3) are known and characterized, yet squamous cell carcinoma, the second most frequent histology, is not well-studied. Although several molecularly targeted therapies have been developed, the benefits of these drugs may contribute to patients with adenocarcinoma but not squamous cell carcinoma. The mechanisms that underlie the carcinogenesis of squamous cell lung cancer are still poorly understood and should be clarified to develop new therapeutic approaches.

Polycomb group (PcG) proteins (discovered in *Drosophila* as epigenetic gene silencers) are conserved gene silencers playing a crucial role in the development of vertebrate organisms (4). These proteins regulate cell proliferation, senescence and tumorigenesis *via* well-known growth regulatory pathways. There is increasing evidence that PcG proteins play an important role in cancer development and recurrence (5). PcG proteins are subdivided into two multimeric protein complexes, that is, the polycomb repressive complex 1 (PRC1) and the polycomb repressive complex 2 (PRC2). The PRC1 complex includes B cell-specific Moloney murine leukemia virus integration site 1 (BMI-1), MEL-18, MPH1/RAE28, M33 and SCMH1 (4-6). BMI-1 is known as a key molecule in repressing $p16^{Ink4a}$ and $p19^{Arf}$, which are encoded by *INK4A*, for induction of cell growth arrest, senescence and apoptosis. Overexpression of BMI-1 has been found in several human malignancies, such as breast cancer, gastric cancer, colorectal cancer, nasopharyngeal carcinoma, melanoma, oral cancer and bladder cancer, as a poor prognostic factor (6-13).

In addition to BMI-1, mammalian cells express a BMI-1-related PcG protein, MEL-18, also known as polycomb group ring finger 2 (PCGF2) (14). BMI-1 and MEL-18, both belonging to the PRC1 complex, exhibit structurally high homology to each other. MEL-18, a BMI-1-related PcG protein, negatively regulates BMI-1 expression and its expression negatively correlates with BMI-1 in proliferating

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*Key Words:* Lung cancer, squamous cell carcinoma, BMI-1, MEL-18, prognosis.

Table I. *Patients' characteristics*.

| Characteristics | All patients | BMI-1-positive | BMI-1-negative | *p*-Value | MEL-18-positive | MEL-18-negative | *p*-Value |
|---|---|---|---|---|---|---|---|
| Gender | | | | | | | |
| Male | 181 | 40 | 141 | 0.13 | 44 | 137 | 0.57 |
| Female | 18 | 1 | 17 | | 3 | 15 | |
| Age (Years) | | | | | | | |
| <65 | 48 | 7 | 41 | 0.31 | 12 | 36 | 0.79 |
| ≥65 | 151 | 34 | 117 | | 35 | 116 | |
| Smoking index | | | | | | | |
| <400 | 38 | 9 | 29 | 0.48 | 9 | 29 | 0.83 |
| ≥400 | 129 | 23 | 106 | | 29 | 100 | |
| Unknown | 32 | 9 | 23 | | 9 | 23 | |
| pT | | | | | | | |
| 1 | 45 | 10 | 35 | 0.49 | 12 | 33 | 0.39 |
| 2 | 88 | 21 | 67 | | 22 | 66 | |
| 3 | 38 | 7 | 31 | | 5 | 33 | |
| 4 | 28 | 3 | 25 | | 8 | 20 | |
| pN | | | | | | | |
| 0 | 128 | 24 | 104 | 0.7 | 31 | 97 | 0.98 |
| 1 | 23 | 5 | 18 | | 5 | 18 | |
| 2 | 40 | 11 | 29 | | 9 | 31 | |
| 3 | 5 | 1 | 4 | | 1 | 4 | |
| Unknown | 3 | 0 | 3 | | 1 | 2 | |
| Stage | | | | | | | |
| 1a | 36 | 7 | 29 | 0.79 | 9 | 27 | 0.81 |
| 1b | 45 | 10 | 35 | | 12 | 33 | |
| 2a | 30 | 7 | 23 | | 5 | 25 | |
| 2b | 25 | 3 | 22 | | 4 | 21 | |
| 3a | 48 | 12 | 36 | | 13 | 35 | |
| 3b | 15 | 2 | 13 | | 4 | 11 | |
| Adjuvant therapy | | | | | | | |
| (–) | 178 | 34 | 144 | 0.15 | 41 | 137 | 0.59 |
| (+) | 21 | 7 | 14 | | 6 | 15 | |
| Total | 199 | 41 | 158 | | 47 | 152 | |

and senescing human fibroblasts (15). Overexpression of MEL-18 leads to repression of BMI-1 and reduction of the transformed phenotype in malignant breast cancer cells. Furthermore, the repression of BMI-1 is accompanied by the reduction of Akt/protein kinase B (PKB) activity in breast cancer cells. MEL-18 and BMI-1 may regulate the Akt pathway in breast cancer cells, while MEL-18 functions as a tumor suppressor by repressing the expression of BMI-1 and, consequently, down-regulating Akt activity (15, 16).

In this study, we evaluated the association of BMI-1 and MEL-18 expression with clinicopathological factors and prognostic possibility in patients with stage I to III squamous cell lung carcinoma.

## Materials and Methods

*Patients and samples*. Clinicopathological characteristics of 199 patients with lung squamous cell carcinoma were investigated. Patients diagnosed as stage I to III primary lung squamous cell carcinoma had been surgically resected at the Department of General Thoracic, Breast and Pediatric Surgery, Fukuoka University School of Medicine and Hospital, Fukuoka, Japan, from January 1, 1995 to December 31, 2005. Our Institutional ethical committee approved the retrospective study and waived the need for patient consent.

We reviewed each patient's medical records for clinical information, including follow-up status and outcome information. Clinicopathological parameters were evaluated, including age, gender, smoking index (the number of cigarettes smoked per day multiplied by the number of years of smoking), tumor size, lymph node (LN) metastasis and adjuvant therapy. The pathological stage was determined according to the tumor/node/metastasis (TNM) classification of malignant tumors (Union for International Cancer Control (UICC)).

*Immunohistochemical analysis*. BMI-1 and MEL-18 were detected in paraffin sections of lung squamous carcinoma tissue. All slides were interpreted by two independent observers in a blinded fashion. For evaluation reliability, two independent assessors estimated the staining positivity of two serial sections. Four micrometer sections were prepared for tissue slides. Antigen retrieval was performed at 121˚C for 15 min in an autoclave with citrate buffer (pH 9.0) after

Table II. *Cox regression models for overall survival.*

| | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| Characteristics | HR | 95%CI | *p*-Value | HR | 95%CI | *p*-Value |
| Age 70< *vs*. <70 | 0.91 | 0.61-1.36 | 0.64 | 1.07 | 0.63-1.79 | 0.81 |
| Smoking index 400> *vs*. 400< | 2.66 | 1.08-6.56 | 0.034 | 5.49 | 1.33-22.7 | 0.02 |
| T factor T1 *vs*. T2-4 | 1.32 | 0.82-2.12 | 0.26 | 1.84 | 1.03-3.3 | 0.039 |
| Node status N0 *vs*. N1-2 | 2.02 | 1.34-3.1 | 0.001 | 1.8 | 1.01-3.19 | 0.045 |
| Adjuvant With *vs*. without | 1.19 | 0.64-2.24 | 0.58 | 0.98 | 0.46-2.12 | 0.97 |
| BMI-1- positive *vs*. negative | 0.55 | 0.31-0.97 | 0.04 | 0.56 | 0.27-1.16 | 0.12 |
| MEL-18- positive *vs*. negative | 0.87 | 0.53-1.43 | 0.59 | 0.85 | 0.45-1.6 | 0.85 |

HR, Hazard ratio; CI, confidence interval.

Table III. *Cox regression models for disease-free survival.*

| | Multivariate analysis | | |
|---|---|---|---|
| Characteristics | HR | 95%CI | *p*-Value |
| Age 70< *vs*. <70 | 0.71 | 0.38-1.3 | 0.27 |
| Smoking index 400> *vs*. 400< | 0.97 | 0.4-2.33 | 0.94 |
| T factor T1 *vs*. T2-4 | 0.48 | 0.27-0.87 | 0.015 |
| Node status N0 *vs*. N1-2 | 2.11 | 1.16-3.84 | 0.014 |
| BMI-1- positive *vs*. negative | 0.97 | 0.5-1.88 | 0.93 |
| MEL-18- positive *vs*. negative | 1.27 | 0.66-2.47 | 0.47 |

HR, Hazard ratio; CI, confidence interval.

deparaffinization. DAKO blocking solution (DAKO, Glostrup, Denmark) was used to block non-specific binding. For BMI-1, staining with anti-BMI-1 clone F6 (Millipore, MA, USA) with diluents, 1:300, was performed overnight at 4˚C. After reaction with 3% hydrogen peroxide for 15 minutes at room temperature, RealTM Envision HRP Rabbit/Mouse antibody (DAKO) for BMI-1 was applied and incubated for 30 min at room temperature. For MEL-18, staining with MEL-18 (H-115) (Santa Cruz Biotechnology, CA, USA) with diluents, 1:100, was performed overnight at 4˚C. After reaction with 3% hydrogen peroxide for 15 min at room temperature, Real™ Envision HRP Rabbit/Mouse antibody (DAKO) for MEL-18 was applied and incubated for 30 min at room temperature.

Negative controls were incubated without the primary antibody. IHC staining was evaluated as previously described (17).

*Statistical analysis*. All statistical analyses were performed using SPSS 14.0 (SPSS Japan Inc., Tokyo, Japan). The different variables of the tumors and normal tissues were analyzed with chi-square tests or Fisher's exact tests. Disease-free and overall survival were analyzed using the Kaplan-Meier method and evaluated by the log-rank test. Significant differences were accepted at $p<0.05$.

## Results

*Relationship between clinicopathological characteristics, BMI-1 and MEL-18 expression*. The correlation between the clinicopathological characteristics of pulmonary squamous cell carcinoma and BMI-1 and MEL-18 expression was investigated. A total of 41 of the 199 cases (21%) were BMI-1-positive. As shown in Table I, no significant correlations between BMI-1 expression and clinicopathological factors were found. The expression pattern of BMI-1 is shown in Figure 1. BMI-1-positive cases showed strong granular staining in the nuclei of pulmonary squamous cell carcinoma from the resected specimens, although normal bronchial cells did not show any positive staining of BMI-1. MEL-18 was also stained in the nuclei of pulmonary squamous cell carcinoma specimens (positive: 47 of 199, 23.6%; Figure 2). However, normal bronchial epithelium was not stained. MEL-18 was related with neither clinicopathological factors nor BMI-1 expression.

*Prognostic values of BMI-1 and MEL-18*. The possibility of using these proteins to predict the survival of pulmonary squamous cell carcinoma was then examined. Figure 3 shows disease-free, disease-specific and overall survival according to the stratification of BMI-1 expression. The median follow-up period was 63 months. Five-year disease-free survival in the BMI-1-positive group was better than in the negative group (78.4% and 63.8%, respectively, $p=0.045$; Figure 3A). However, five-year disease-specific survival showed a trend of better survival in the BMI-1-positive group (positive 71.2%, negative 57.4%, $p=0.08$; Figure 3B). Five-year overall survival, including other causes of death, showed significant difference between the two groups (positive 66.8%, negative 45.5%, $p=0.04$; Figure 3C). On the other hand, MEL-18 showed prognostic significance in neither disease-free, nor disease-specific, nor overall survival ($p=0.15$, $p=0.54$ and $p=0.59$, respectively; Figures 4A-C). We found that smoking index, nodal involvement and BMI-1 expression showed prognostic significance by univariate analysis in overall survival (Table II); however, only the smoking index, T factor and nodal involvement showed significant prognostic value by multivariate Cox regression analysis. BMI-1 expression showed a potential for prognostic impact by multivariate analysis (Table II). Multivariate analysis in disease-free survival showed that T factor and

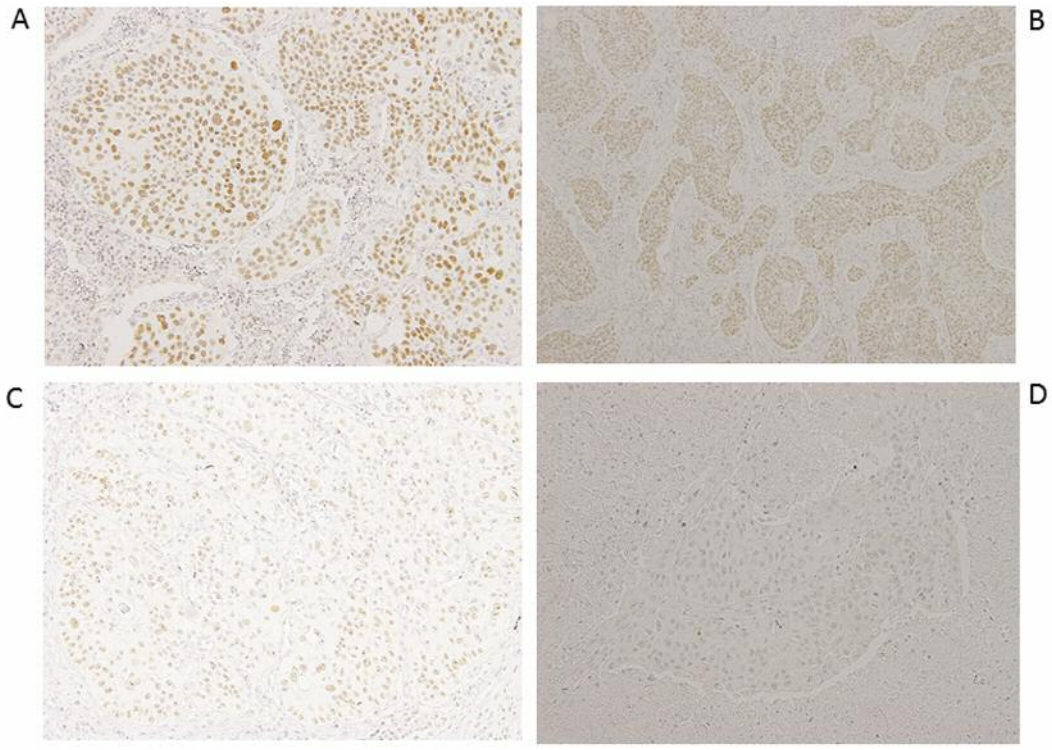
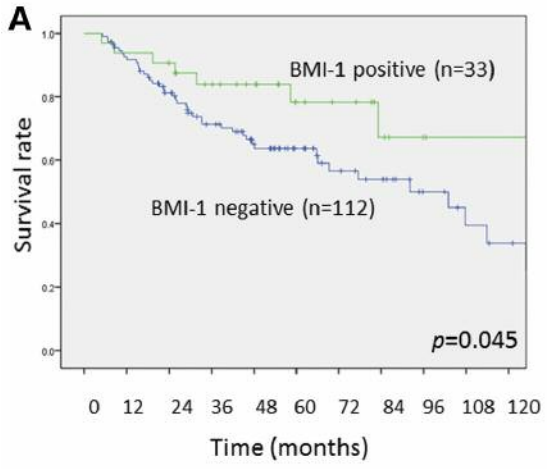
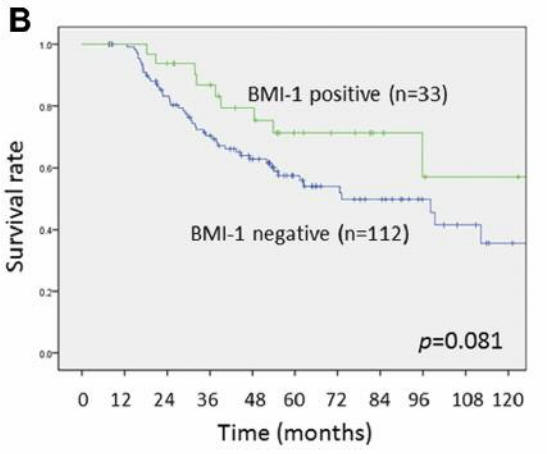



Figure 1. *Representative BMI-1 protein expression in pulmonary squamous cell carcinoma by immunohistochemistry (IHC). Nuclei of cancer cells were stained strongly in 3+ cases (200×, A), 2+ (B) and 1+ (C). Negative staining of pulmonary squamous cell carcinoma (D).*


Figure 2. *Representative MEL-18 protein expression in pulmonary squamous cell carcinoma by immunohistochemistry (IHC). Nuclei of cancer cells were stained strongly in 3+ cases (200×, A), 2+ (B) and 1+ (C). Negative staining of pulmonary squamous cell carcinoma (D).*



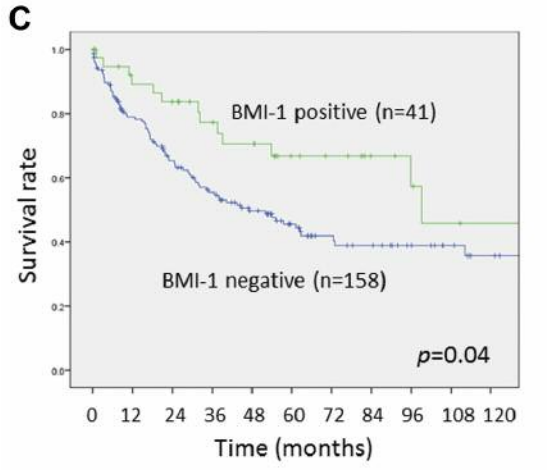
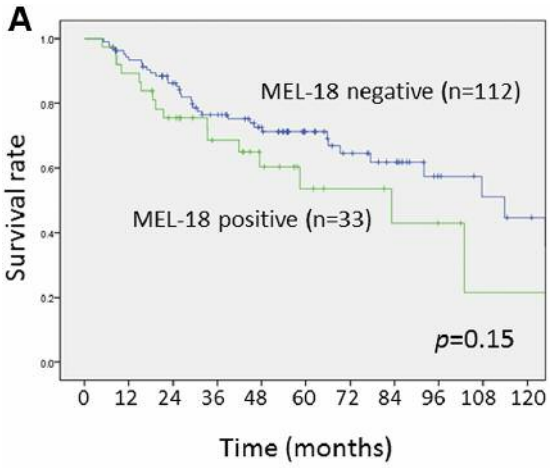
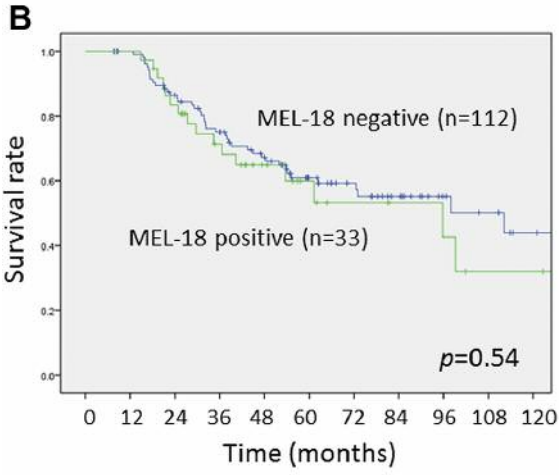




Figure 3. *Disease-free survival (A), disease-specific survival (B) and overall survival (C) according to BMI-1 protein expression. Five-year disease-free survival was 78.4% in the BMI-1-positive group and 63.8% in negative group (p=0.045). Five-year disease-specific survival was 71.2% in the BMI-1-positive group and 57.4% in the negative group (p=0.08). Five-year overall survival was 66.8% in BMI-1-positive group and 45.5% in thenegative group (p=0.04).*



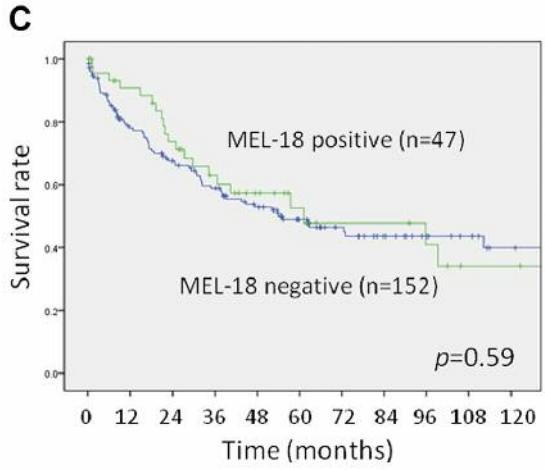




Figure 4. *Disease-free survival (A), disease-specific survival (B), and overall survival (C) according to MEL-18 protein expression. Five-year disease-free survival was 53.6% in the MEL-18-positive group and 71.2% in the negative group (p=0.15). Five-year disease-specific survival was 59.9% in the MEL-18-positive group and 60.9% in negative group (p=0.54). Five-year overall survival was 54.2% in the MEL-18-positive group and 47.9% in the negative group (p=0.59).*

nodal status were significant prognostic factors; BMI-1 and MEL-18 were not (Table III).

## Discussion

BMI-1 is one of the proteins that is a member of the polycomb group family and has a potentially critical role for tumor progression (5). BMI-1 is known as an oncogene that has a critical role associated with *C-MYC* in B-cell lymphoma cells (15). Most studies have shown that overexpression of BMI-1 is associated with poor prognosis in many human cancers, including cervical, breast, lung, esophageal, stomach and colon (6-9, 16, 18, 19). However, the opposite result, that BMI-1 may be a favorable prognostic factor in breast cancer, was also reported (20). It has been shown that BMI-1 is significantly correlated with negative axillary node metastasis and estrogen receptor (ER) status; furthermore, prognosis in BMI-1-positive cases is significantly more favorable than in negative cases. The suggested reason was that comparable methods of IHC for expression levels of BMI-1 were different in the evaluation process, including distribution and intensity of positive cells between the two studies. Saeki *et al*. reported that *BMI-1* mRNA expression was inversely correlated with nodal involvement and staging in breast cancer. These results suggested that BMI-1 may cause carcinogenesis in early events, but not late stage progression, but this remains controversial. Another study of BMI-1 expression in lung cancer showed significant correlation with early and decline in late stages of disease, which is negatively correlated with nodal involvement (18). They reported that silencing of BMI-1 expression induced invasiveness and metastasis in human lung cancer cell lines *in vitro*. These results support our results that the staging of lung squamous cell cancer was not correlated with BMI-1 expression. It has also been reported that BMI-1 repression induces epithelial-mesenchymal transition and progression of lung cancer *in vitro*. In our study, overall survival in lung squamous cell carcinoma was inversely correlated with BMI-1 expression, and these results were supported by a previous report (18). In another report, *BMI-1* mRNA expression in early stage breast cancer was higher than in late stage and was inversely related with lymph node metastasis (21). Taken together, BMI-1 expression may play a crucial role in carcinogenesis but not progression in lung cancer.

In this study, we focused on the expression of BMI-1 in squamous cell carcinoma but not adenocarcinoma. The prognostic value of BMI-1 was evaluated and positive BMI-1 expression was a favorable prognostic factor in squamous cell lung cancer. Multivariate analysis showed that smoking index, T factor and nodal status were of significant value for overall survival but the value of BMI-1 was weak. These well-known confounding factors affected prognosis in our study; however, BMI-1 may be a weak independent prognostic factor. Furthermore, disease-free survival was associated with BMI-1 expression but weakly related with disease-specific survival. The reason why this discrepancy between overall and disease-specific survival existed remains unclear. A possible reason is that other causes of death, such as pneumonia because of a high prevalence of smoking, affected the analysis. Most studies about BMI-1 in lung cancer were estimated in adenocarcinoma or non-small cell carcinoma (22-24). The function of BMI-1 in pulmonary squamous cell carcinoma may be different from in adenocarcinoma of the lung. Although there are several driver mutations, such as *EGFR* of lung adenocarcinoma, squamous cell carcinoma of the lung is neither well-known nor characterized. BMI-1 may be one of the critical functions for carcinogenesis and should be studied in a larger population.

MEL-18, which was inversely expressed with BMI-1, was evaluated in our study. However, no correlation with any clinicopathological factors was shown and it did not have any prognostic value in this study. MEL-18 is a regulator of cell proliferation and quittance by BMI-1 suppression acting as a tumor suppressor gene or oncogene (15). The reason why our study showed no correlation between the two proteins is unknown.

The limitations of this study are that it involves retrospective analysis and is a single-Institution study. Another limitation is the relatively small number of patients used to observe differences in survival between the two groups. Longer follow-up and an increased number of patients in both groups may lead to further confirmation of the results.

In conclusion, BMI-1 protein was expressed in pulmonary squamous cell carcinoma and may be a possible prognostic marker. Further studies should be undertaken to clarify this hypothesis.

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