学位論文

Overexpression of Antiapoptotic MCL-1 Predicts Worse Overall Survival of Patients With Non-small Cell Lung Cancer.

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Overexpression of Antiapoptotic MCL-1 Predicts Worse Overall Survival of Patients With Non-small Cell Lung Cancer

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Abstract. *Background/Aim: Myeloid cell leukemia-1 (MCL-1) is a member of the B-cell lymphoma-2 (Bcl-2) family of proteins, which regulate the intrinsic (mitochondrial) apoptotic cascade. MCL-1 inhibits apoptosis, which may be associated with resistance to cancer therapy. Therefore, in this study, the clinical role of MCL-1 in non-small cell lung cancer (NSCLC) was explored. Patients and Methods: This retrospective study included 80 patients with stage 1-3A NSCLC, who underwent surgery without preoperative treatment between 2010 and 2011. MCL-1 expression and Ki-67 index were determined via immunohistochemical staining. Apoptotic index (AI) was determined via terminal deoxynucleotidyl transferase dUTP nick end labeling. Results: The receiver operating characteristic curve analysis (area under curve=0.6785) revealed that MCL-1 expression in 30.0% of the NSCLC tumor cells was a significant cut-off for predicting prognosis. Tumors were considered MCL-1-positive if staining was observed in >30% of the cells. Thirty-six tumors (45.0%) were MCL-1-positive. However, there were no significant differences between MCL-1 expression and clinical variables. AI was lower in MCL-1-positive (2.2±3.6%) than in MCL-1-negative (5.2±7.9%) tumors, although the difference was not significant (p=0.1080). The Ki-67 index was significantly higher in MCL-1-positive than in MCL-1-negative tumors (18.0% vs. 3.0%; p<0.001). Five-year survival rate was significantly worse in patients with MCL-1-positive tumors (68.3%) than in those with MCL-1-negative tumors (93.1%, p=0.0057). Univariate [hazard ratio (HR)=5.041, p=0.0013], and multivariate analyses revealed that MCL-1 expression was a significant prognostic factor (HR=3.983, p=0.0411). Conclusion: MCL-1 expression in NSCLC cells correlated inversely with AI and positively with Ki-67 index. MCL-1 may serve as a potential prognostic biomarker and a novel therapeutic target in NSCLC.*

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Key Words: Apoptosis, myeloid cell leukemia-1, non-small cell lung cancer, prognosis, survival.

Of the estimated 606,880 Americans who will die from cancer in 2019, corresponding to almost 1,700 deaths per day, one-quarter will be caused by lung cancer, which is the primary cause of cancer-related death worldwide (1). Approximately 85% of newly diagnosed lung cancers are non-small-cell lung cancers (NSCLC), which includes three cell types (adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma). Much progress has been made recently for lung cancer management, such as invasive techniques for diagnosis; advances in stereotactic ablative radiation therapy (SABR), targeted therapies, and immunotherapies (2-5). However, only 17.7% of the patients with lung cancer survive for ≥5 years after diagnosis because of the poor prognosis of NSCLC (6, 7).

Identification of activating mutations in the epidermal growth factor receptor-tyrosine kinase (*EGFR-TK*) and *ROS-1* genes as well as rearrangements in the anaplastic lymphoma kinase gene in NSCLC have enabled the identification of patients likely to achieve actual clinical benefits. More than 60% of the patients with EGFR mutation have been shown to respond to the *EGFR-TK* inhibitor and prolong their progression-free survival up to 10 months. Immune checkpoint inhibitors targeting programmed cell death protein 1 and programmed death-ligand 1 are a real advancement and have been approved for the treatment of certain subgroups of patients with advanced NSCLC (8). Landmark trials in patients with NSCLC have reported better treatment response and survival with checkpoint inhibitor therapy compared to standard-of-care first- and second-line chemotherapy, as well as a more durable response in a subgroup of patients. However, many patients do not respond to checkpoint inhibition (9). Chemotherapy and radiotherapy are still the primary treatment given to patients with NSCLC and the development of resistance is the primary reason for treatment failure (10).

Myeloid cell leukemia-1 (MCL-1) protein is a member of the B-cell lymphoma-2 (Bcl-2) protein family. It inhibits

apoptosis, and overexpression of MCL-1 has been associated with tumor progression and resistance to both traditional and targeted therapy, including Bcl-2 inhibitors such as ABT-263 (11). Expression of Bcl-2 family members has been reported in both NSCLC tissues and NSCLC cell lines (12, 13). The activities of Bcl-2 and Bcl-xL have been well-described; however, the role of MCL-1 has not been extensively investigated.

This study investigated the association between MCL-1 expression and prognosis in patients with NSCLC. To the best of our knowledge, this is the largest study to evaluate MCL-1 in patients treated with surgical resection only and the first to confirm the prognostic value of MCL-1 in patients with NSCLC.

Patients and Methods

Clinical characteristic of patients with NSCLC. Patients with NSCLC who underwent R0 resection without neoadjuvant chemotherapy at the Department of General Thoracic Surgery at Kagawa University between June 2010 and December 2011 were eligible. Eighty patients with stage 1-3A NSCLC, including 63 with adenocarcinoma, 14 with squamous cell carcinoma, 1 with adenosquamous cell carcinoma, and 2 with pleomorphic carcinoma were included (Table I). The mean patient age was 67 years (range=37-87). Tumor–node–metastasis staging was performed following the postoperative pathological international staging system criteria (14). The selected patient sample was not biased by differences in age, sex, or pathological stage. The clinical records and histopathological diagnosis were fully documented. Patient follow-up was continued to November 2017, while mean (SD) follow-up duration was 51.6 (14.6) months. The study was approved by the Certified Review Board of Kagawa University Hospital (#2019-090) and included an opt-out method.

Immunohistochemistry. MCL-1 expression in tumor tissue was assayed by immunohistochemistry. Tumor proliferation was assayed by the Ki-67 index. A monoclonal rabbit anti-MCL (1:100, ab32087; Abcam, Cambridge UK) and a monoclonal mouse anti-Ki-67 (1:40, MIB-1; Dako Cytomation, Glostrup, Denmark) were the primary antibodies. Isotype antibodies were used as negative controls. For staining, formalin-fixed paraffin-embedded tissue was cut into 4-μm serial sections and mounted on poly-L-lysine-coated slides (Muto Pure Chemicals Co. Ltd, Tokyo, Japan). The sections were deparaffinized and rehydrated. The slides were then heated in a microwave for 20 min in a 10 μmol/l citrate buffer solution (pH 6.0) and cooled to room temperature. After quenching endogenous peroxidase activity with 0.3% H_2O_2 in absolute methanol for 30 min, the sections were treated with 5% bovine serum albumin (Sigma-Aldrich, Tokyo, Japan) for 2 h at room temperature to block nonspecific staining. The sections were incubated overnight with the primary antibodies and were then incubated for 1 h with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA). The treated sections were incubated with the avidin–biotin–peroxidase complex (Vector Laboratories) for 1 h, and antibody binding was visualized with 3,3'-diamino-benzidine tetrahydrochloride. The sections were lightly counterstained with Mayer's hematoxylin. Sections of lung tumors known to express MCL-1 were used as positive controls.

Table I. *Distribution of MCL-1 status in 80 non-small cell lung cancer (NSCLC) patients according to clinical characteristics.*

Variables	N	MCL-1 status		*p*-Value
		Positive N (%)	Negative N (%)	
Total	80	36 (45.0)	44 (55.0)	
Age* (years)		67.69±9.26	67.20±9.72	0.819
Gender				
Male	54	26 (72.2)	28 (63.6)	0.477
Female	26	10 (27.8)	16 (36.4)	
Smoking history				
Current	15	8 (22.2)	7 (15.9)	0.288
Ex	40	20 (55.6)	20 (45.5)	
Never	25	8 (22.2)	17 (38.6)	
Histology				
Ad	53	28 (77.8)	35 (79.5)	0.486
Sq	14	6 (16.7)	8 (18.2)	
Other	3	2 (5.6)	1 (2.3)	
Pathological T				
T1a	36	14 (38.9)	22 (55.0)	0.793
T1b	23	12 (33.3)	11 (25.0)	
T2a	21	11 (30.6)	10 (22.7)	
T2b	2	1 (2.8)	1 (2.3)	
Pathological N				
N0	72	30 (83.3)	42 (95.5)	0.225
N1	5	4 (11.1)	1 (2.3)	
N2	3	2 (5.6)	1 (2.3)	
Pathological stage				
IA	54	22 (61.1)	32 (72.7)	0.451
IB	16	7 (19.4)	9 (20.5)	
IIA	7	5 (13.9)	2 (4.5)	
IIIA	3	2 (5.6)	1 (2.3)	

Ad, Adenocarcinoma; Sq, squamous cell carcinoma. *Data presented as mean±SD.

The sections were evaluated by two independent, blinded investigators (T. N. and D. L.). Discrepancies were jointly re-evaluated until a consensus was reached. MCL-1 expression was reported as the percentage of carcinoma cells exhibiting positive staining. The percentage of Ki-67-positive carcinoma cells in each tumor specimen reported the Ki-67 index. Apoptosis was assayed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) as described by Sgonc and Wick (15). TUNEL staining was performed using the *In Situ* Apoptosis Detection kit (MK500; Takara Bio, Inc., Otsu, Shiga, Japan) following the manufacturer's instructions. A total of 10,000 tumor cells (1,000 cells/field in 10 different fields) were evaluated at a high magnification (×400). The apoptotic index (AI) was the number of apoptotic cells/1000 tumor cells.

Statistical analysis. Easy R (EZR), which is based on R, was used for statistical analysis. R commander was used to perform the analysis (16). Data were expressed as means±SD. MCL-1 expression by 30.0% of tumor cells was found by receiver operating characteristic (ROC) curve analysis to be a significant cut-off for predicting prognosis. The significance of associations of MCL-1 status and patient characteristics was determined by the chi-square

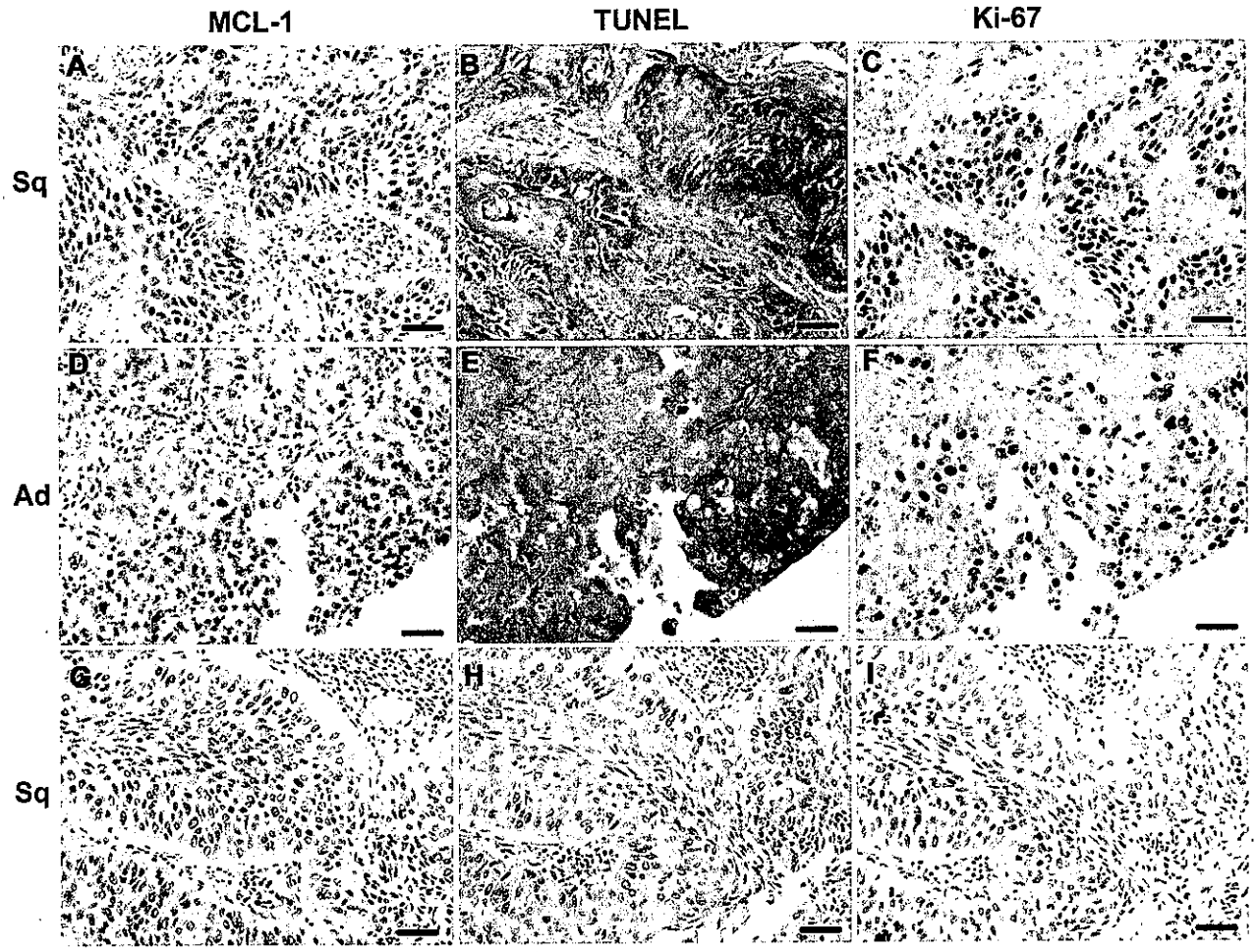

Figure 1. *Representative immunohistochemical images show myeloid cell leukemia-1 (MCL-1) positive staining in squamous cell carcinoma (A), adenocarcinoma (D) and MC-1-negative squamous cell carcinoma (G). Apoptotic index (TUNEL) and Ki-67 staining of serial sections from the same tumor were listed in corresponding rows. Scale bar=50 μm.*

or the *t*-test. Overall survival, defined as the time from surgical resection to the date of death from any cause, was estimated by the Kaplan–Meier method and differences were compared using Mantel's log-rank test. Univariate and multivariate analyses were performed using Cox regression models to determine the significance of the association between patient variables and survival. The *p*-values were two-tailed, and $p<0.05$ was considered statistically significant.

Results

MCL-1 expression in NSCLC. MCL-1 expression in tumor tissue primarily appeared as nuclear membrane and nuclear staining, and rarely was seen in the cytoplasm. MCL-1 staining in normal alveolar epithelium was weak. Only cells with nuclear membrane/nuclear staining were included in the percentage of positively stained cells. The percentage of MCL-1 expression in the tumor cells varied significantly (25.0±20.9%; median=26.8%). ROC curve analysis of MCL-1 expression established 30.0% (sensitivity=0.786; specificity=0.621; accuracy=0.679) a cut-off for predicting prognosis. Tissue specimens with staining of >30% of the tumor cells were considered MCL-1-positive. Thirty-six of the 80 patients (45.0%) had MCL-1-positive tumors. Twenty-eight of the 63 adenocarcinomas (44.4%) were positive for MCL-1, while 6 of the 14 squamous cell carcinomas (42.8%) were MCL-1-positive. The difference in MCL-1 expression was not significant between these two histological types of NSCLC. The MCL-1 status did not differ significantly with patient age, sex, smoking history, tumor status, nodal status, or pathological stage (Table I).

Associations among MCL-1 expression, AI, and Ki-67 index. AI of the tumor samples from the 80 patients varied considerably. The mean of all the samples was 3.8±6.1%,

and although it was lower in MCL-1-positive (2.2±3.6%) than in MCL-1-negative (5.2±7.9%) tumors, the difference was not significant (p=0.108, Figure 3A). In serial sections, fewer apoptotic cells were observed in the MCL-1-positive than in the MCL-1 negative tumors (Figure 1B, E). The overall mean Ki-67 index in the 80 patients was 13.7±14.7%; it was significantly higher in MCL-1-positive than in MCL-1-negative tumors (20.3±16.1% *vs.* 8.41±11.2%, p<0.001; Figure 3B). In serial sections, more Ki-76-positive cells were observed in MCL-1-positive than in MCL-1-negative tumors (Figure 1C, F).

Association between survival and MCL-1 status. The overall 5-year survival rate of the 80 patients with NSCLC was 82%. Five-year survival was significantly worse in patients with MCL-1-positive than in those with MCL-1-negative tumors (68.3% *vs.* 93.1%, p=0.00572; Figure 4A). Overall survival was significantly worse in patients with MCL-1-positive than in those with MCL-1-negative adenocarcinomas (p=0.0071, Figure 4B). It was also worse in patients with MCL-1-positive than in those with MCL-1-negative squamous cell carcinomas; however, the difference was not significant (Figure 4C).

As shown in Table II, univariate Cox regression analysis showed that MCl-1 expression status (HR=5.041; p=0.0013), tumor status (HR=12.310; p=0.0001), and nodal status (HR=6.263; p=0.0011) were all significant prognostic factors associated with survival in these patients with NSCLC. Multivariate analysis also demonstrated that MCL-1 expression, tumor, and nodal status were independently associated with survival (Table II).

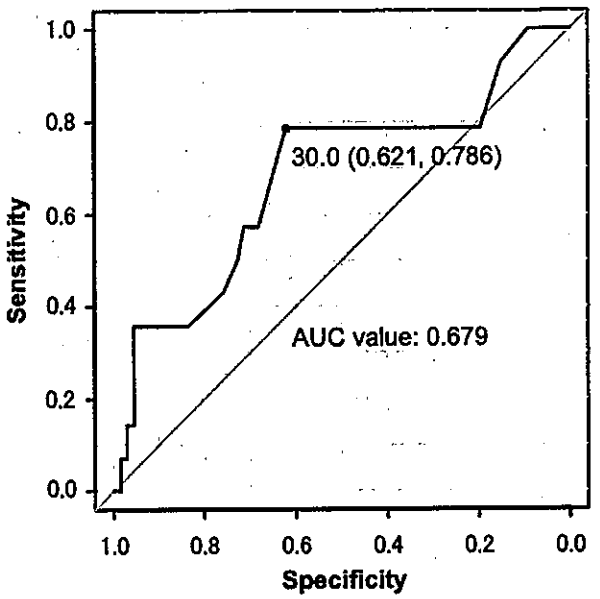

Figure 2. *Receiver operating characteristics (ROC) curve for predicting overall survival based on the percentage of positively stained MCL-1 cells in 80 non-small cell lung cancer (NSCLC) patients. The area under the curve (AUC) value of percentage of MCL-1 for prognosis was 0.6785 (95% CI=0.506-0.8512; p<0.0010).*

Discussion

MCL-1 is an immediate-early gene induced in the ML-1 human myeloid leukemia cell line by tetradecanoylphorbol acetate (17). MCL-1 is a member of the Bcl-2 family of proteins, and it has antiapoptotic activity, while other family members are proapoptotic or BH3-only proteins (18). MCL-1 protein protects cells from apoptosis and is overexpressed in a number of human cancer cell lines, and tumors, including hematological malignancies and solid tumors, including NSCLC, breast cancer, gastric cancer, prostate cancer, and pancreatic cancer (19-24). MCL-1 expression in NSCLC has been investigated in various cell lines, but in few clinical samples or tissue microarrays (12, 13, 25). To the best of our knowledge, this is the largest clinical study of MCL-1 expression and its association with prognosis in patients with NSCLC.

MCL-1 has a short half-life and is a highly regulated protein; its expression is induced by survival and differentiation signals. It is downregulated in many cell systems during apoptosis and appears to be responsive to rapidly changing conditions (26). Regulation of MCL-1 expression can be either transcriptional, post-transcriptional, or translational. MCL-1 gene amplification is frequently found in human cancers, and several key oncogenic pathways lead to increased MCL-1 protein expression (27-29).

In this cohort of patients with NSCLC, MCL-1 expression was tumor-specific. Of the 80 patients, 36 (45%) were considered MCL-1-positive following ROC curve analysis (Table I). A previous study reported 58% MCL-1 positivity in a group of 49 tumor specimens and did not find an association between MCL-1 expression and prognosis (13). We did not find significant associations between MCL-1 status and patient age, sex, smoking history, tumor status, nodal status, or pathological stage (Table I). However, MCL-1 expression had a tendency, though not significant, toward an inverse correlation with tumor apoptosis. The reason of not reaching significance may be the low accuracy of AI, which was evaluated as the number of apoptotic cells/1000 tumor cells. In the serial sections, there were clearly fewer apoptotic cells in the MCL-1-positive than in the MCL-1-negative tumors (Figure 1). The Ki-67 index was significantly higher in the MCL-1-positive than in MCL-1-negative tumors. This result is consistent with the findings in gastric cancer reported by Lee *et al.* (22). The Bcl-2 family is usually considered a group of oncogenes that promote oncogenesis by maintaining viability through the inhibition of apoptosis and not by the promotion of cell proliferation (30). Our findings suggest that MCL-1 plays a

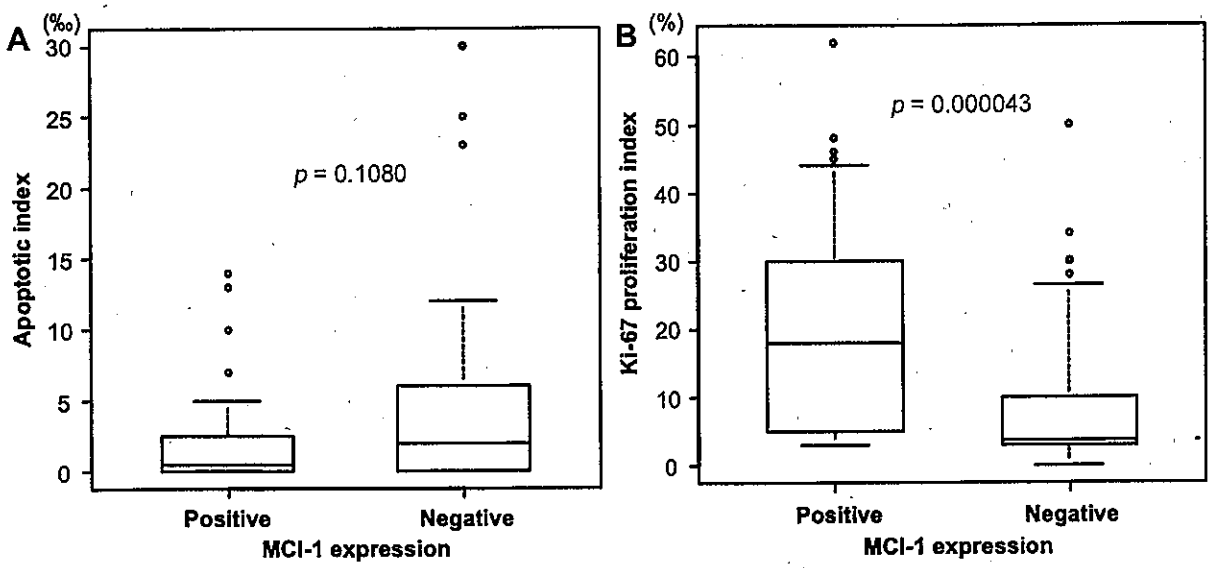

Figure 3. *Correlation between MCL-1 expression and biological properties in 80 non-small cell lung cancer (NSCLC) tumors. AI was lower in MCL-1-positive compared with MCL-1-negative tumors, but the difference did not reach significance (A). The Ki-67 index was significantly higher in the MCL-1-positive than in MCL-1-negative tumors (B).*

Table II. *Cox hazard regression analysis for predicting the survival of 80 non-small cell lung cancer (NSCLC) patients.*

	Univariate			Multivariate		
Variables	HR	95%CI	*p*-Value	HR	95%CI	*p*-Value
Gender						
Male/Female	6.936	0.9072-53.030	0.0620			
Age (years)						
<65/≥65	0.585	0.183-1.865	0.3644			
Smoking status						
Former & present/never	6.620	0.866-50.620	0.0686			
Histology						
Non-Ad/Ad	3.445	1.194-9.944	0.0222			
Tumor status						
T2/T1	12.310	3.413-44.430	<0.0001	10.340	2.754-38.800	0.0005
Nodal status						
N1-2/N0	6.263	2.088-18.790	0.0011	2.366	0.739-7.580	0.1471
MCL-1 status						
Positive/negative	5.041	1.405-18.080	0.0013	3.983	1.058-15.000	0.0411

HR, Hazard ratio; CI, confidence interval; MCL-1, myeloid cell leukemia-1.

role in regulating cell proliferation in NSCLC tumors. Further studies are warranted to verify these results.

Overall survival was significantly shorter in patients with MCL-1-positive than in those with MCL-1-negative tumors (Figure 4A). It was also significantly shorter in patients with MCL-1-positive than in those with MCL-1-negative adenocarcinomas (Figure 4B). MCL-1 status did not influence survival in patients with squamous cell carcinoma (Figure 4C); however the small number of patients with squamous cell carcinoma may have influenced the results.

Univariate and multivariate Cox regression analyses revealed that MCL-1 expression predicted survival in patients with NSCLC (Table II). To the best of our knowledge, this study is the first to identify a correlation between MCL-1

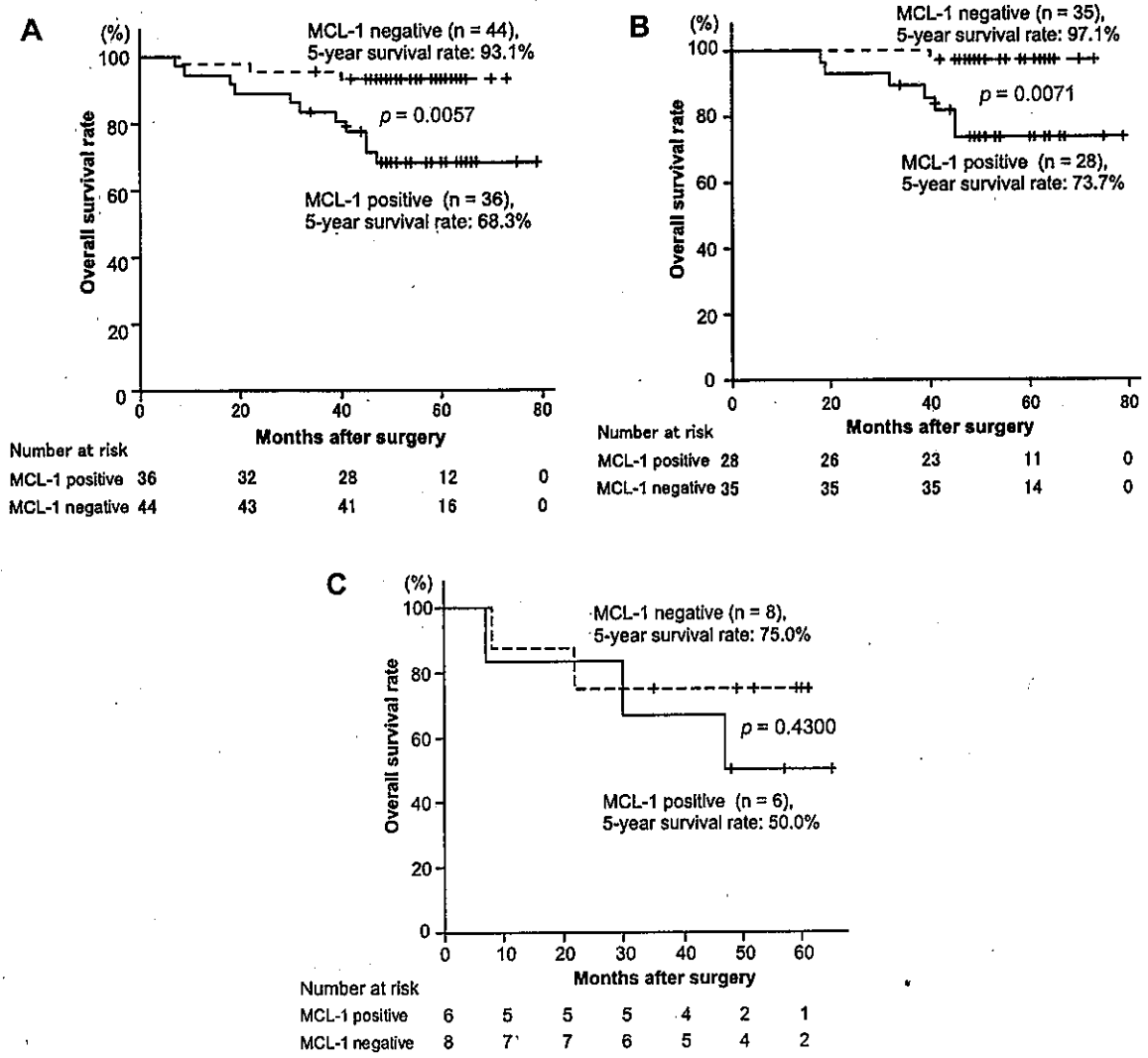

Figure 4. *Overall survival of 80 non-small cell lung cancer (NSCLC) patients in relation to MCL-1 status: (A) in total of 80 NSCLC patients; (B) in 63 patients with adenocarcinomas; (C) in 14 patients with squamous cell carcinomas.*

expression and clinical outcomes in patients with NSCLC and to find that MCL-1 expression is independently associated with prognosis. In the same context, previous studies have shown that MCL-1, which is highly amplified in cancer, is essential for cell survival in normal and malignant tissues. Moreover, its anti-apoptotic activity has been was associated with chemoresistance in different tumors (31, 32). Specific inhibitors of MCL-1 have been discovered, making it a potential therapeutic target (33, 34).

The study limitations include the small number of patients. In our institution, patients with advanced lung cancer, such as stage cN2, are often administered preoperative induction chemotherapy. The number of advanced cases in which surgery is performed without preoperative therapy is decreasing. Consequently, few pN2 patients were eligible for inclusion in this study. In addition, as the incidence of squamous carcinoma is decreasing, few patients were available.

In conclusion, MCL-1 expression correlated inversely with AI and positively with Ki-67 index in NSCLC. Moreover, the present results suggested that MCL-1 may serve as a potential prognostic biomarker for and a novel therapeutic target in NSCLC.

Availability of Data

The datasets used/or analyzed in this study are available in the corresponding author on reasonable request.

Conflicts of Interest

None declared.

Authors' Contributions

TN and DL designed the study; TN and NN performed research; TG, NN and TN provided sample collection and contribute to data interpretation; TN and DL wrote the manuscript, and TN, NN, TG and HY revised the manuscript and participated in interpreting the data.

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