

## SIRT6 Expression is Associated With Poor Prognosis and Chemosensitivity in Patients With Non-Small Cell Lung Cancer

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**Background:** Despite advances in the development of various therapeutic agents, non-small cell lung cancer (NSCLC) is associated with a poor prognosis. To improve the prognosis of patients with NSCLC, new therapeutic targets for overcoming drug resistance are required. The process of autophagy is required to support the tumorigenesis and drug resistance of cancer cells. We investigated the clinical significance of SIRT6, a member of the NAD<sup>+</sup>-dependent deacetylase family, which regulates a variety of cancer-related processes, including autophagy.

**Methods:** Immunohistochemistry analysis of SIRT6 expression and localization in 98 NSCLC clinical specimens and in vitro analysis using SIRT6-knockout lung carcinoma cell lines were performed.

**Results:** Patients with high cytoplasmic expression and low nuclear expression of SIRT6 (n = 33) had more aggressive cancer, shorter overall survival, and shorter recurrence-free survival than did patients with different SIRT6 expression profiles (P < 0.05). In vitro analysis revealed that SIRT6 knockdown lung adenocarcinoma cell line improved paclitaxel sensitivity (P < 0.05) and reduced the expression levels of both nuclear factor kappaB and autophagy marker Beclin1.

**Conclusion:** Our data demonstrated that SIRT6 expression in NSCLC could be a useful prognostic marker and that SIRT6 might represent a novel target gene for predicting sensitivity of chemotherapy in lung adenocarcinoma.

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**KEY WORDS:** sirtuin; sirtuin 6; autophagy; chemoresistance

### INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC) represents approximately 85% of all cases of lung cancer [1]. Targeted therapies such as epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) have become standard therapeutic agents, improving clinical outcomes and therapeutic options for patients with NSCLC [2]. However, the prognosis for patients with advanced NSCLC, which does not respond to conventional chemotherapies or EGFR-TKIs, remains very poor. The discovery of suitable biomarkers for predicting prognosis and chemosensitivity may be important for monitoring cancer recurrence and providing information on appropriate adjuvant or neoadjuvant chemotherapies. Therefore, further research is needed to identify new therapeutic targets and tools for overcoming NSCLC with drug resistance.

The seven members of the sirtuin family (SIRT1–7) belong to the family of NAD<sup>+</sup>-dependent deacetylases and are classified as class III histone deacetylases [3]. These enzymes are widely expressed in mammalian cells and modulate various biological processes, including cell survival, development, chromatin dynamics, DNA repair, metabolism, and cancer [4]. SIRT6 is predominantly localized in the nucleus of various cells and regulates transcription, genomic DNA stability and repair, metabolism, and aging through its histone deacetylation function [5]. Moreover, SIRT6 has been reported to regulate autophagy [6], an important process in cancer cell survival and chemoresistance, and has attracted attention for its function in overcoming drug resistance in hormone- and drug-refractory cancers [7]. SIRT6 has been shown to be downregulated in clinical samples from pancreatic cancer, colorectal cancer, head and neck squamous cell carcinoma, and hepatocellular carcinoma [8–10], and may function as a tumor suppressor. Other reports have shown that prostate

cancer and breast cancer patients with high SIRT6 expression have significantly poor prognoses and that prostate and breast cancer cells expressing SIRT6 show resistance to anticancer drugs [11,12]. However, few studies have investigated the relationship between SIRT6 expression levels and clinical outcomes in NSCLC.

The purpose of this study was to clarify the clinical significance of SIRT6, including its potential role in mediating anticancer drug sensitivity, in NSCLC. Therefore, we performed immunohistochemistry analysis of 98 clinical NSCLC samples to evaluate the relationship between SIRT6 expression and clinicopathological features, prognosis, and survival times after anticancer drug treatment. Furthermore, we explored the in vitro effects of siRNA-mediated SIRT6 knockdown on paclitaxel sensitivity and autophagy in human NSCLC cell lines.

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## MATERIALS AND METHODS

### Clinical Samples and Cell Lines

We analyzed tumor specimens from 98 patients with lung cancer who underwent surgery for excision of a primary tumor between January 1999 and February 2006 in the Department of General Surgical Science of Gunma University School of Medicine. The patients included 65 men and 33 women with a median age of 65 years (range, 32–84 years) at surgery. Sixty-five patients were former/current smokers, with a median Brinkman index (BI; number of cigarettes per day times years) of 1,051, and 33 patients had no history of smoking. Eighty-one patients had adenocarcinomas, and 17 had squamous cell carcinomas. Fifty-seven patients had stage I lung cancer, 9 patients had stage II lung cancer, 29 patients had stage III lung cancer, and 3 patients had stage IV lung cancer at the time of surgery. Forty-eight patients received chemotherapy after operation, and eleven patients received radiation after operation. All patients provided written informed consent.

The human lung adenocarcinoma cell lines A549, H1975, and H2009 and the lung squamous cell carcinoma cell lines EBC-1 and RERF-LC-AI were all maintained in RPMI1640 medium containing 10% fetal bovine serum (Equitech-Bio, Inc., Kerrville, TX) and 100 U/ml penicillin and streptomycin sulfate (Life Technologies, Carlsbad, CA). Cells were cultured in a humidified incubator with 5% CO<sub>2</sub> at 37°C. All cell lines were obtained from the American Type Culture Collection (Manassas, VA) or Riken BioResource Center (Japan). It was previously validated that these cells were not cross-contaminated with other cell lines by STR-PCR in each cell bank.

### Immunohistochemistry

The resected surgical specimens were fixed with 10% formaldehyde, embedded in paraffin blocks, cut into 4- $\mu$ m-thick sections, and mounted on glass slides. Staining was performed using standard methods, and detection was facilitated by formation of the streptavidin-biotin peroxidase complex (S-ABC). All sections were incubated at 60°C for 60 min, deparaffinized in xylene, rehydrated, and then incubated with fresh 0.3% hydrogen peroxide in 100% methanol for 30 min at room temperature to block endogenous peroxidase activity. After rehydration through a graded ethanol series, antigen retrieval was carried out in 10 mM citrate buffer (pH 6.4) at 98–100°C for 20 min. The sections were then passively cooled to 30°C. After rinsing the sections in 0.1 M phosphate-buffered saline (PBS, pH 7.4), nonspecific binding sites were blocked by incubating the sections with 10% normal goat serum for 30 min. The sections were then incubated overnight at 4°C and at room temperature for 30 min with mouse monoclonal anti-SIRT6 antibodies (Abnova, Taiwan) at a dilution of 1:200 in PBS containing 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO). The sections were washed in PBS, incubated with biotinylated anti-mouse IgG, A, M solution (Nichirei Co., Tokyo, Japan) for 30 min at room temperature, and finally incubated in S-ABC solution (Nichirei Co.) for 30 min. The chromogen, 3,3'-diaminobenzidine tetrahydrochloride, was applied as a 0.02% solution containing 0.005% hydrogen peroxide in a 50 mM ammonium acetate-citrate acid buffer (pH 6.0). The sections were lightly counterstained in Mayer's hematoxylin and mounted on glass slides.

The level of SIRT6 immunoreactivity was defined as follows: (i) low expression = no staining, weak staining or strong complete cytoplasm/nuclear staining in <20% of tumor cells; (ii) high expression = strong complete cytoplasm/nuclear staining in  $\geq$ 20% of tumor cells. The expression levels were evaluated by two independent investigators who reached a consensus for all samples.

### Knockdown of SIRT6 Expression by RNA Interference

SIRT6-specific small interfering RNA (siRNA) #1 (5'-GGGAC-AAACUGGCAGAGC AGCUCUGCCAGUUUGUCC-3'), siRNA #2

(5'-GGAAGAAUGGCCAAGUGACACUUGGCACAUUCUUC-3'), and negative control siRNA (siBonac Negative Control 1; scrambled siRNA) were purchased from Bonac and Hokkaido System Science (Japan), respectively. RNA interference assays for the target cell lines EBC-1 and A549 were performed using an in vitro electroporation protocol. In brief, the cells were suspended in Opti-MEM 1 (Life Technologies) without serum at a density of  $1 \times 10^6$  cells/ml. siRNA was added to the cell suspension at a concentration of 1.5  $\mu$ M. Next, 100  $\mu$ l of the cell suspension was transferred to a 2-mm gap cuvette electrode and then subjected to electroporation using an electroporator (CUY21EDIT II; BEX Co., Ltd., Tokyo, Japan). The conditions for electroporation were as follows: one pulse of 125 V with a 10-ms duration and 40-ms interval; followed by five pulses of 10 V with a 50-ms duration and 50-ms interval at 940  $\mu$ F capacity; and five pulses of 10 V with a 50-ms duration and 50-ms interval at 940  $\mu$ F capacity with reversed polarity.

### Proliferation and Drug Sensitivity Assay

Proliferation and drug sensitivity assays were performed using cells that had been transfected with siRNA targeting SIRT6 transcripts. For proliferation assays, EBC-1 cells were plated at approximately 1,500 cells/well and A549 cells were plated at 3,000 cells/well in 96-well plates with 100  $\mu$ l of medium. The water-soluble tetrazolium (WST)-8 assay (Dojindo Laboratories, Tokyo, Japan) was used to quantify cell viability. Ten microliters of cell-counting solution was added to each well, and the plates were incubated at 37°C for 2 hr. The cell proliferation rate was then determined by measuring the absorbance of the medium at 450 nm with the reference wavelength set at 650 nm. The absorbance values were read using a microtiter plate reader (Molecular Devices, Sunnyvale, CA). A549 and EBC-1 cells were plated at approximately 3,000 cells/well in 96-well plates with 100  $\mu$ l of medium in each well. Various concentrations of paclitaxel (Sawai Seiyaku, Osaka, Japan) were added (EBC-1 cells: 0, 5, 10, 20, 40, or 60 nM; A549 cells: 0, 25, 50, 100, 200, or 400 nM). After incubation for 48 hr at 37°C, cell viability was quantified by WST-8 assay using the same method as described for the proliferation assay above. Each experiment was carried out with 10 replicate wells for each concentration and experiments were repeated twice.

### Protein Extraction and Western Blot Analysis

Western blot analysis was used to confirm the expression of target proteins using anti-SIRT6 (Cell Signaling Technology, Danvers, MA), anti-NF $\kappa$ B (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bcl-1 (Novus Biologicals, Littleton, CO), anti-BCL2 (Cell Signaling Technology), and anti- $\beta$ -actin (Sigma, St. Louis, MO) antibodies in each lung cancer cell line. Four hundred micrograms of total protein was extracted with PROPREP protein extraction solution (iNtRON Biotechnology, Inc., Korea). Total protein was then electrophoresed on NuPAGE 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and then transferred to nitrocellulose membranes using a wet transfer protocol. The membranes were blocked with 5% skim milk, and the proteins were detected using anti-SIRT6 rabbit monoclonal antibodies (1:1,000), anti-NF $\kappa$ B rabbit polyclonal antibodies (1:200), anti-Bcl-1 rabbit polyclonal antibodies (1:1,000), and anti-BCL2 rabbit polyclonal antibodies (1:1,000). Anti- $\beta$ -actin mouse monoclonal antibodies (clone AC-74; 1:2,000) served as a control. Bands were detected, and band intensities were calculated using ECL Prime Western blotting detection reagent (GE Healthcare, Wauwatosa, WI) and an Image Quant LAS 4000 (GE Healthcare Life Sciences, UK).

### Statistical Analysis

Differences between two groups were estimated with Student's *t*-tests,  $\chi^2$  analysis, and analysis of variance (ANOVA). Survival curves

were generated according to the Kaplan–Meier method. The differences between survival curves were examined by using the log-rank test. In addition, univariate and multivariate survival analyses were performed by using the Cox proportional hazards model. A result was considered statistically significant when the relevant  $P$ -value was less than 0.05. All statistical analyses were performed with JMP 5.0 software (SAS Institute, Inc., Cary, NC).

## RESULTS

### SIRT6 Immunohistochemical Staining in Clinical NSCLC Samples

First, we investigated SIRT6 expression in 98 NSCLC samples by immunohistochemistry. Representative immunohistochemical images of different expression levels of SIRT6 are shown in Figure 1. Our data demonstrated that SIRT6 was expressed at higher levels in cancer tissues than in the corresponding noncancerous tissues. Additionally, SIRT6 expression was predominantly observed in the cytoplasm. Fifty-five (56.1%) NSCLC specimens were assigned to the cytoplasmic high-SIRT6 expression group, and 43 (43.8%) were assigned to the cytoplasmic low-SIRT6 expression group.

### Association Between SIRT6 Expression and Clinicopathological Factors of NSCLC

Next, we evaluated SIRT6 expression in the cytoplasm and nucleus of cells within the set of NSCLC tissues and analyzed the correlation between SIRT6 cellular localization and clinicopathological factors and prognosis. The associations between cytoplasmic SIRT6 expression in the NSCLC specimens and ten clinicopathological characteristics (i.e., gender, age, smoking status, histological type, T factor, lymph node metastasis, pathological stage, chemotherapy after operation, radiation after operation, and recurrence) are shown in Table I. There was a significant correlation between cytoplasmic SIRT6 expression and histological type ( $P = 0.0018$ ). However, no significant differences were observed for other factors.

Next, we examined the cytoplasmic and nuclear expression of SIRT6 simultaneously, and classified each specimen into one of two groups: specimens with cytoplasmic high-SIRT6 expression with nuclear low-SIRT6 expression (the SIRT6 cH/nL group) and specimens with other expression profiles. The relationships between cytoplasmic/nuclear expression of SIRT6 in the NSCLC specimens and the ten clinicopathological characteristics are shown in Table II. There were significant correlations between cytoplasmic/nuclear expression of SIRT6 and histological type ( $P = 0.018$ ) and pathological staging ( $P = 0.01$ ).

### Prognostic Significance of SIRT6 Expression in Patients With NSCLC

The overall survival rates of patients with tumors that were assigned to the cytoplasmic high-SIRT6 expression group were significantly lower than those of patients with tumors that were assigned to the cytoplasmic low-SIRT6 expression group ( $P = 0.0223$ ; Fig. 2A). In contrast, patients in the nuclear high-SIRT6 expression group tended to have better prognoses ( $P = 0.0924$ ; Fig. 2B), but no significant difference was observed. The SIRT6 cH/nL group had significantly shorter overall survival times ( $P < 0.01$ ) and shorter recurrence-free survival times ( $P = 0.01$ ) than did patients with other expression profiles (Fig. 2C, D). Moreover, we analyzed overall survival rate according to pathological stage or histological type. In stage III, patients exhibiting the SIRT6 cH/nL expression profile had poorer prognoses than did other patients with  $P < 0.01$ , Supplementary Figure S1C. Patients with lung adenocarcinoma exhibiting the SIRT6 cH/nL expression profile had poorer prognoses than did other patients with adenocarcinoma ( $P < 0.01$ ; Supplementary Figure S2). The evaluation of SIRT6 cH/nL was an independent prognostic factor for poor survival in multivariate analysis (Supplementary Table S1).

### Regulation of Autophagy by SIRT6 was Associated With Paclitaxel Sensitivity in the Lung Adenocarcinoma Cell Line A549

Western blot analysis was used to determine the expression of SIRT6, NF $\kappa$ B, Beclin1 (an autophagy marker), and BCL2 (an anti-apoptotic

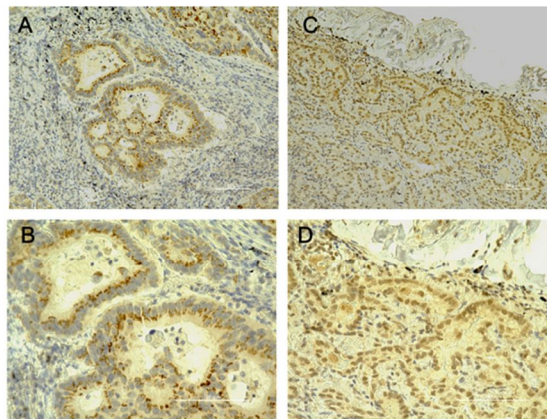


Fig. 1. Immunohistochemical staining of SIRT6 in primary NSCLC. A: High cytoplasmic expression and low nuclear expression of SIRT6 in a representative NSCLC sample (200 $\times$  magnification). B: High cytoplasmic expression and low nuclear expression of SIRT6 in a representative NSCLC sample (400 $\times$  magnification). C: Low cytoplasmic expression and high nuclear expression of SIRT6 in a representative NSCLC sample (200 $\times$  magnification). D: Low cytoplasmic expression and high nuclear expression of SIRT6 in a representative NSCLC sample (400 $\times$  magnification).

TABLE I. Relationships Between Clinicopathological Factors and SIRT6 Expression in the Cytoplasm

Factors	Cytoplasm SIRT6		P-value
	Low expression n = 43	High expression n = 55	
Age at operation	65.5 + 1.61	66.0 + 1.42	0.83
Gender			
Male	25	40	0.13
Female	18	15	
Smoking status			
Current, former	25	40	0.13
Never	18	15	
Histological type			
Adeno	41	40	0.0018*
Squamous	2	15	
T factor			
T1	17	25	0.14
T2	23	21	
T3	0	3	
T4	3	6	
Lymph node metastasis			
Absent	30	30	0.14
Present	13	25	
Pathological stage			
1	29	28	0.55
2	3	6	
3	9	20	
4	2	1	
Chemotherapy after operation			
Absent	23	22	0.35
Present	18	30	
Unknown	2	3	
Radiation after operation			
Absent	37	45	0.6
Present	4	7	
Unknown	2	3	
Recurrence			
Absent	29	30	0.19
Present	14	25	

\*P &lt; 0.05.

protein) in six human lung carcinoma cell lines, that is, A549, H1975, H2009, EBC-1, and RERF-LC-AI. Of the three adenocarcinoma cell lines, SIRT6 was highly expressed only in A549 cells. In contrast, both of the two squamous cell carcinoma cell lines showed high SIRT6 expression levels (Fig. 3A). Moreover, A549 cells exhibited accumulation of both NFκB and Beclin 1. However, the expression levels of these two proteins were low in lung squamous cell carcinoma cell lines (Fig. 3A).

To investigate the function of SIRT6 in NSCLC cells, A549 and EBC-1 cells were transfected with *SIRT6* siRNA. Reduced expression of SIRT6 protein was confirmed in both A549 and EBC-1 cells (Fig. 3B). The expression levels of NFκB and Beclin1 in A549 cells were decreased in cells transfected with *SIRT6* siRNA compared to control cells, whereas no remarkable changes were observed in EBC-1 cells. BCL2 expression levels were not altered in A549 or EBC-1 cells (Fig. 3B). These data confirmed that *SIRT6* siRNA was effective at knocking down SIRT6 expression.

Next, we examined the effects of SIRT6 knockdown on cell proliferation and paclitaxel sensitivity in lung cancer cells. Interestingly, knockdown of SIRT6 did not affect the proliferation of A549 or EBC-1 cells (Supplementary Figure S3). However, in A549 cells, knockdown of SIRT6 improved cell sensitivity to paclitaxel (Fig. 3C, D). These data confirmed the role of SIRT6 in mediating drug sensitivity in patients with lung cancer.

To confirm these results, we analyzed the correlation between prognosis and SIRT6 expression profiles in patients who had received chemotherapy. We found that SIRT6 cH/nL patients who had been

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TABLE II. Relationships Between Clinicopathological Factors and SIRT6 Expression in the Cytoplasm/Nucleus

Factors	Cytoplasm/Nuclear SIRT6		P-value
	High/Low expression n = 33	Others n = 65	
Age at operation	66.1 + 1.83	65.6 + 1.30	0.8
Gender			
Male	25	40	0.15
Female	8	25	
Smoking status			
Current, former	26	39	0.05*
Never	7	26	
Histological type			
Adeno	23	58	0.018*
Squamous	10	7	
T factor			
T1	15	27	0.18
T2	11	33	
T3	2	1	
T4	5	4	
Lymph node metastasis			
Absent	16	43	0.07
Present	17	21	
Pathological stage			
1	15	42	0.01*
2	2	7	
3	16	13	
4	0	3	
Chemotherapy after operation			
Absent	13	32	0.56
Present	18	30	
Unknown	2	3	
Radiation after operation			
Absent	26	56	0.51
Present	5	6	
Unknown	2	3	
Recurrence			
Absent	15	24	0.41
Present	18	41	

\*P &lt; 0.05.

given chemotherapeutic agents in the adjuvant and/or recurrent settings tended to show poorer prognoses than did patients with other SIRT6 expression profiles ( $P = 0.05$ ; Fig. 4). The evaluation of SIRT6 cH/nL was an independent prognostic factor for poor survival in multivariate analysis (Supplementary Table S2).

## DISCUSSION

In this study, we showed that the high expression of SIRT6 in the cytoplasm in primary cancer tissues from patients with NSCLC was associated with poor prognosis. Moreover, patients with high cytoplasmic expression and low nuclear expression of SIRT6 exhibited poorer prognosis than did patients with other SIRT6 expression profiles. Moreover, in our in vitro analysis of the effects of SIRT6 knockdown, we found that paclitaxel sensitivity was improved in A549 cells, but not EBC-1 cells, transfected with *SIRT6* siRNA.

SIRT6 has been reported to be predominantly localized in the nucleus [5]. However, in the present study, we found that SIRT6 was mainly present in the cytoplasm in NSCLC. Histone deacetylation activity by nuclear SIRT6 inhibits the expression of Hif1α and NFκB, both of which are associated with chemotherapy resistance [13,14]. On the other hand, cytoplasmic SIRT6 coexists with spindle fibers in the S phase of the cell cycle; [15] therefore, cytoplasmic SIRT6 may reflect the cell cycle regulatory function of SIRT6. From these observations, we hypothesize that intracellular localization of SIRT6, as measured by immunohistochemical staining, could be a useful marker of chemosensitivity and cell cycle progression in patients with NSCLC.

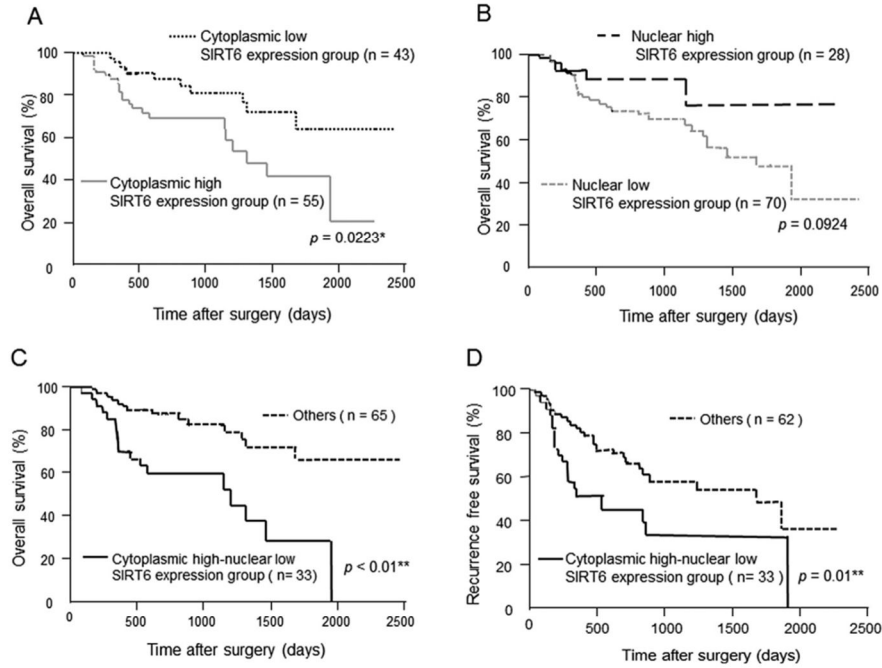


Fig. 2. Kaplan-Meier curves for patients with NSCLC according to the level of SIRT6 expression. A: Overall survival curves for patients with NSCLC (n = 98) based on the level of cytoplasm SIRT6 expression ( $P = 0.0223$ ). B: Overall survival curves for patients with NSCLC based on the level of SIRT6 expression in the nucleus ( $P = 0.0924$ ). C: Overall survival curves for patients with NSCLC based on the level of SIRT6 expression in the cytoplasm and nucleus ( $P < 0.01$ ). D: Recurrence-free survival curves for patients with NSCLC based on the level of SIRT6 expression in the cytoplasm and nucleus ( $P = 0.01$ ).

In this study, we examined SIRT6 expression in three lung adenocarcinoma cell lines; of these cell lines, only A549 cells expressed SIRT6. However, SIRT6 expression was high in both EBC-1 and RERF-LC-A1 cells, which are derived from squamous cell carcinoma. Similarly, in NSCLC clinical specimens, high expression of cytoplasmic SIRT6 was frequently observed in squamous cell carcinoma samples. The high rate of genomic loss of 19p13.3, the location of the *SIRT6* gene, has been reported in patients with lung adenocarcinoma [16]. Moreover, array CGH data from patients with NSCLC showed higher rates of 19p13.3 gains in patients with squamous cell carcinoma than in patients with adenocarcinoma [17]. According to these reports, one of the reasons that patients with adenocarcinoma and squamous cell carcinoma exhibit different levels of SIRT6 expression may be due to such genomic alterations.

Previous studies have reported that SIRT6 regulates cellular sensitivity to chemotherapy by controlling BCL2 expression in prostate cancer cells [11]. However, we could not validate the association between SIRT6 and BCL2 expression in our *in vitro* SIRT6 suppression analysis. Therefore, we focused on analyzing the relationship between SIRT6 and autophagy, a key mechanism mediating chemotherapy resistance [7]. SIRT6 suppression has also been shown to inhibit the induction of autophagy via regulation of

mTOR signaling [6]. Furthermore, induction of autophagy has been reported to be necessary for activation of NFκB signaling [18], which is known to be associated with cancer progression and chemoresistance. Indeed, in our study, A549 lung adenocarcinoma cells expressing Beclin1 and NFκB showed improved paclitaxel sensitivity in SIRT6-knockdown cells. However, this change was not observed in EBC-1 cells, a lung squamous cell carcinoma cell line, because of the absence of NFκB and the autophagy maker Beclin1. Therefore, we hypothesized that the regulation of autophagy by SIRT6 may enhance paclitaxel sensitivity only in A549 cells, which may utilize autophagy for cell survival and chemoresistance. Thus, targeting SIRT6 may be useful for the treatment of lung adenocarcinoma patients with paclitaxel-resistant tumors via induction of autophagy and activation of NFκB signaling. Further studies are needed to determine the clinical relevance of SIRT6 in lung squamous cell carcinoma.

To target SIRT6 in NSCLC patients, using the small interfering RNA or microRNA of SIRT6 for recovery of chemo resistance in SIRT6 high expressing patients. Actually, small RNAs, including microRNAs, have attracted attention as potential new tools for cancer therapeutic strategies [19,20]. Moreover, it is required that evaluation of cross activation of preexisting SIRT inhibitors for SIRT6, and screening of new candidate drugs as the inhibitor of SIRT6.

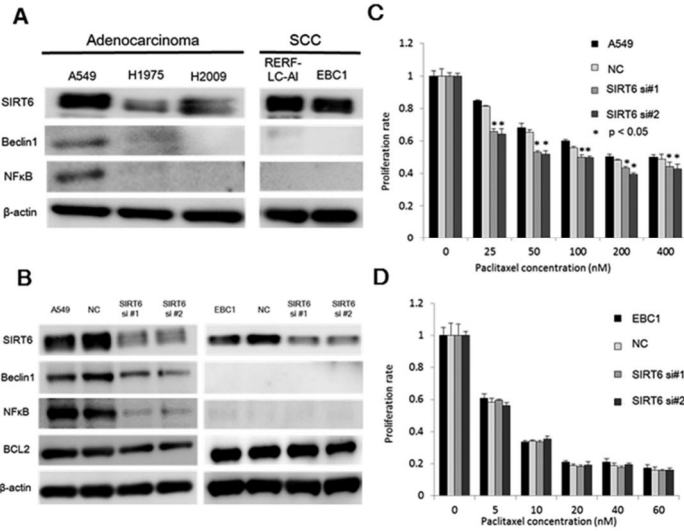


Fig. 3. Functional analysis of human NSCLC cell lines transfected with SIRT6 siRNA. A: Protein expression levels of SIRT6, the autophagy marker Beclin1, NFκB, and β-actin were examined in the adenocarcinoma cell lines A549, H1975, and H2009 in the squamous cell carcinoma cell lines RERF-LC-AI and EBC1 were measured by Western blotting. β-Actin was used as the loading control. B: Protein expression levels of SIRT6, Beclin1, NFκB, and BCL2 in A549 and EBC1 cells transfected with SIRT6 siRNA were measured by Western blotting. β-Actin was used as the loading control. N-C: negative control. C: A WST assay was performed to evaluate the paclitaxel sensitivity of A549 cells transfected with SIRT6 siRNA. N-C: negative control (\**P* < 0.05). D: A WST assay was performed to evaluate the paclitaxel sensitivity of EBC1 cells transfected with SIRT6 siRNA. N-C: negative control.

In conclusion, the predominantly cytoplasmic localization of SIRT6 expression was correlated with poor prognosis and reduced chemosensitivity in patients with NSCLC. SIRT6 expression and localization could be a useful prognostic marker for patients with

NSCLC. In our in vitro SIRT6 siRNA analysis, we found that SIRT6 could regulate paclitaxel sensitivity via induction of autophagy. Therefore, during the development of new molecular cancer therapies, SIRT6 may be a promising candidate for targeting chemoresistance in lung adenocarcinoma.

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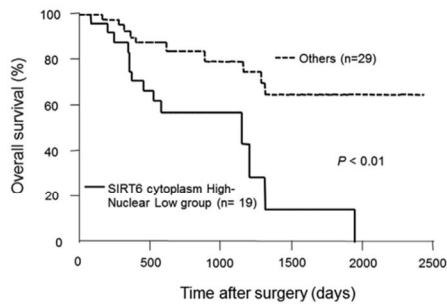


Fig. 4. Kaplan-Meier curves according to the level and localization of SIRT6 expression in patients with NSCLC treated with postoperative chemotherapy. Patients received chemotherapy in the adjuvant and recurrent settings. Curves were constructed to show differences between patients with high cytoplasmic and low nuclear expression of SIRT6 versus patients with other SIRT6 expression profiles (n = 19 vs. n = 29, respectively; *P* < 0.01).

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