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Original Article

SUMO-1 expression modulates non-small cell lung cancer progression

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Abstract: Previous studies indicate that the role of small ubiquitin-related modifier-1 (SUMO1) is responsible for multiple cancer progression including non-small-cell lung cancer (NSCLC). This study aims to explore the expression and significance of SUMO1 in NSCLC. 179 NSCLC patients with resected lung tissue were used for immunofluorescence to detect the expression level of SUMO1 in tumor cells. We also evaluated the association of SUMO1 expression with clinicopathological parameters, including the Ki-67 labeling index (LI). We used Kaplan-Meier survival analysis and Cox proportional hazards models to estimate the effect of SUMO1 expression on survival. We found that SUMO1 expression was significantly elevated in NSCLC tissues when compared with adjacent lung tissues ($p<0.01$). Moreover, SUMO1 expression was significantly associated with histological type ($p<0.01$), lymphatic metastasis ($P<0.001$), and pTNM stage ($p<0.05$). Kaplan-Meier survival analysis showed that overall survival of patients with high expression of SUMO1 was significantly shorter ($n = 163$, $p<0.05$). In conclusion, SUMO1 is closely related to the progression in NSCLC and could be served as a potential prognostic biomarker and therapeutic target for NSCLC.

Keywords: SUMO1, NSCLC, survival, oncogene

Introduction

Lung cancer is the leading cause of cancer-related death worldwide [1]. Most cases of lung cancer are non-small cell lung cancer (NSCLC), however, the long-term survival rate of NSCLC patients remains disappointing. A majority of NSCLC patients die from distant metastases and recurrent disease even after undergoing curative surgical resection [2-4]. There is an urgent need to identify new prognostic markers that can facilitate a better assessment of the survival probabilities and optimized therapies for individual patients.

Small ubiquitin-related modifier-1 (SUMO-1) is the best-characterized member of a rapidly growing family of ubiquitin-like proteins that are involved in post-translational modifications [5-7]. SUMO-1 is reversibly attached to other proteins in a ubiquitination-like manner. SUMO-1 congestion (sumoylation) affects the subcellular localization of substrates and stability as well as transcriptional activities [8-11]. Many transcriptional regulatory proteins have been identified as sumoylation substrates, and many more sumoylation substrates are expected to exist [12, 13]. A couple of oncoproteins, for example c-Myb [14], PML [15] and c-Jun [16], have been corroborated to be substrates of sumoylation. The significance of sumoylation in the function of the oncogenes varies among them. Given the substrates involved, protein sumoylation would be expected to play important role in the course of tumorigenesis and alter in different human cancer [17]. However, there is still no explicit evidence to support this theory.

To study the clinicopathologic features and prognostic implications of SUMO1 expression in patients with NSCLC, we investigated the expression of SUMO1 in NSCLC by immunohistochemical staining and analyzed the relationships between SUMO1 expression and clinicopathologic characteristics.

Table 1. Correlation between SUMO1 expression and clinicopathological features in 163 patients with NSCLC.

Features	All *n* = 163	SUMO1 expresion (IHC)		
		Overexpression (%)	Low expression (%)	*P*-value*
Sex				
Male	130	96	34	1.0
Female	33	25	8	
Age (years)				
<60	82	58	24	0.3712
≥60	81	63	18	
Smoking history				
Smoker	106	77	29	0.5773
Non-smoker	57	44	13	
Histological type				
Adenocarcinoma	46	35	11	0.9337
Squamous cell carcinoma	82	60	22	
Adenosquamous carcinoma	34	25	9	
Sarcomatoid carcinoma	1	1	0	
Differentiation				
Well + Moderately	110	74	36	0.0038
Poorly	53	47	6	
pT factor				
T_{1-2}	124	90	34	0.5292
T_{3-4}	39	31	8	
pN factor				
N_0	66	36	30	<0.0001
N_{1-2}	97	85	12	
pTNM stage				
I-II	87	58	29	0.0203
III-IV	76	63	13	
Ki-67 labeling index				
>33.5%	79	47	32	<0.0001
<33.5%	84	74	10	

**P*-value of χ^2-test are shown.

Material and methods

Subjects

Study population and tissue samples collection: Paraffin-embedded tissue specimens from 163 patients with confirmed NSCLC were obtained from the thoracic oncology tissue repository in the Department of Thoracic Surgery of Tangdu Hospital, between March 2006 to September 2009. Patients who received preoperative chemotherapy, radiotherapy or *EGFR*-targeted therapy were excluded from this study. Detailed information was obtained from the medical records of the enrolled patients in a computerized registry database including age, gender, smoking history, clinical manifestation, tumor status, histological differentiation, nodal status and follow-up information. Follow-up lasted through December 1, 2013, with a median follow-up period of 52 months for living patients (range, 45-80 months). The day of surgery was considered as the starting day for estimating postoperative survival time. Histological classification of tumors was reviewed by pathologists and based on the World Health Organization criteria. All tumors were staged according to the pathological tumor/node/metastasis (pTNM) classification (7th edition) of the International Union against Cancer [18]. The study protocol was approved by the Regional Ethics Committee for Clinical Research of the Fourth Military Medical University. Informed consent was taken from all subjects.

Immunohistochemistry

Paraffin-embedded tissue blocks were cut into 5-μm serial slides. The slides were then dewaxed in xylene and rehydrated through a graded series of ethanol solution. Endogenous peroxidase activity was neutralized by immersing the slides in a solution of 3% hydrogen peroxide in methanol for 15 min at room temperature. Combined sodium citrate (pH 6.0) and incubation in a pressure cooker (3 min, 125°C) was used for antigen retrieval. To reduce nonspecific binding, slides were blocked with goat serum for 30 min. Then, the slides were incubated in a humidified chamber at 4°C overnight with primary anti-SUMO1 (diluted 1:400, Abcam, USA) or anti-Ki-67 (diluted 1:50,

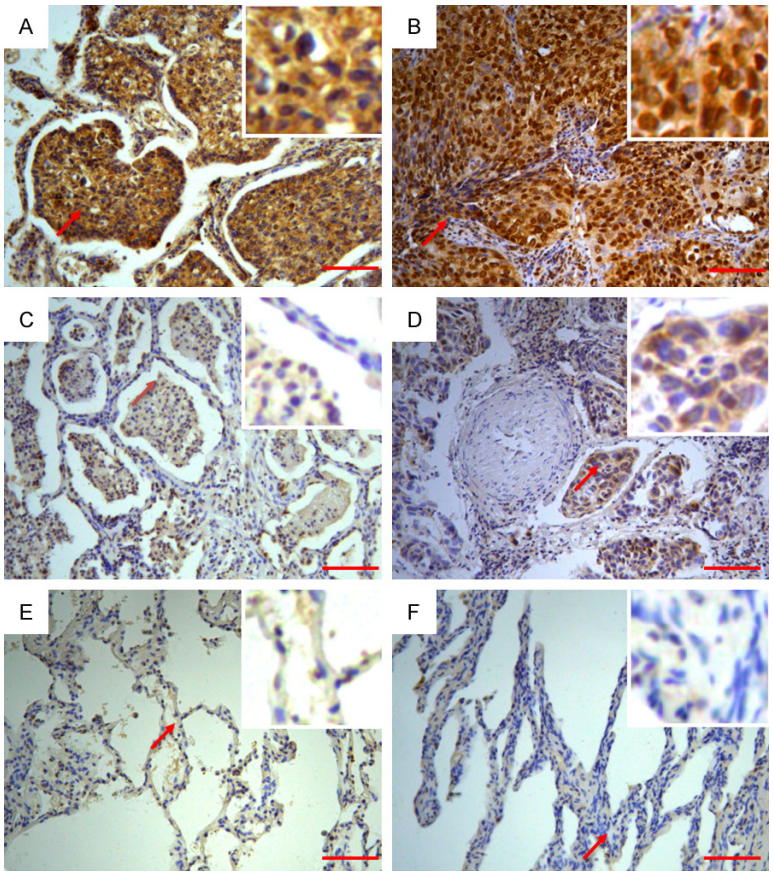

Figure 1. Expression of SUMO1 in NSCLC. A. Optical overexpression SUMO1 staining in ADC. B. Overexpression SUMO1 staining in SCC. C. Low expression SUMO1 staining in ADC. D. Low expression SUMO1 staining in SCC. E. Low expression SUMO1 staining in corresponding adjacent non-cancerous tissue. F. Absence of SUMO1 in corresponding adjacent non-cancerous tissue staining in corresponding adjacent non-cancerous tissue. Scale bar = 100 μm.

Thermo, USA) antibodies. A two-step polymer-HRP method (Dako, Carpinteria, CA) was used for detection. No staining was observed for negative controls, which included incubation of lung tissue with a non-immune primary antibody.

Evaluation of immunohistochemical staining

Five random fields from each slide were viewed under a light microscope (Leica DM4000B, Germany) at ×400 magnification. The expression of SUMO1 was scored by multiplication of the percentage of positive tumor cells and the staining intensity. Initially, the percentage of positive cells was scored as 0 (0%), 1 (1-10%), 2 (11-50%) and 3 (51-100%). Thereafter, intensity of staining was scored as follows: 0 (negative), 1 (weakly positive), 2 (moderately positive) and 3 (strongly positive). By ROC analyses, the case with a final scores ≥5 was classified as overexpression (Sensitivity 81.9, Specificity 95.1), the case with a final scores between 3-5 was classified as low expression. For Ki-67, the expression of Ki-67 was assessed based on the labeling index (LI) determined by counting 500-1000 tumor cells randomly selected in a high-power field. The median value of positive tumor cells was 33.5% in the current series, therefore, we defined tumors with ≥33.5% of Ki-67 as high Ki-67. All slides were assessed by 3 independent investigators who were blinded to the clinical features and outcomes. The final immunohistochemical staining score reported is the average of the scores from the three investigators.

Statistical analysis

Associations between SUMO1 expression and clinicopathological parameters were evaluated using the Fisher's exact test. Survival was examined using the Kaplan-Meier method, and the significance of the difference was evaluated using the log-rank test. Correlation analyses of the survival time and various clinicopathological variables were performed by univariate and multivariate analyses using the Cox regression model. $P<0.05$ was considered to be statistically significant. All analyses were performed with Prism 5.01 software (GraphPad Software, Inc.) and SPSS 18 (Inc., Chicago IL, USA).

Results

General characteristics of the subjects

The clinicopathologic characteristics of the patients are summarized in **Table 1**. There were 33 female and 130 male patients with a median age of 59 years (range, 30-81 years). The

patients were diagnosed with squamous cell carcinoma (SCC; n = 82, 50.3%), adenocarcinoma (ADC; n = 46, 28.2%), adenosquamous carcinoma (ASC; n = 34, 20.9%) and sareomatodes carcinoma (SC; n = 1, 0.61%). Histopathologic diagnosis included: 29 well differentiation (17.8%), 81 moderately differentiation (49.7%), and 53 poorly differentiation tumors (33.1%). Postoperative staging evaluation demonstrated stage I disease in 24 patients, stage II disease in 64 patients, stage III disease in 71 patients, and stage IV disease in 4 patients.

Expression pattern of SUMO1 in NSCLC and correlation with clinicopathological characteristics

Overall, 74.23% (121/163) tumor sections were classified as SUMO1 overexpression (**Figure 1A**, **1B**), the rest of 42 (25.774%) tumor sections were classified as low expression (**Figure 1C**, **1D**), while 5.52% (9/163) homologous adjacent non-cancerous tissue sections were classified as SUMO1 low expression (**Figure 1E**) and the rest of adjacent non-cancerous tissues were classified as negative (**Figure 1F**). Positive staining was mainly located in the cytoplasm. In order to evaluate the role of SUMO1 in NSCLC, we analyzed whether SUMO1 expression was associated with any of the clinicopathological features (**Table 1**). Statistical results showed that SUMO1 overexpression was significantly associated with differentiation (P = 0.0038), pN factor (P< 0.0001), pTNM stage (P = 0.0203) and Ki-67 labeling index (P<0.0001). No significant relationship was noted between SUMO1 expression and sex (P = 1.0), age (P = 0.3712), smoking history (P = 0.5773), histological type (P = 0.9337), and pT factor (P = 0.5292).

Relationship between SUMO1 expression and survival in NSCLC patients

To investigate the relationship between SUMO1 expression level and the clinical outcome of NSCLC patients, we compared the correlation between patient survival and SUMO1 expression status. Patients with overexpression SUMO1 had an obviously worse prognosis than those with low level of SUMO1 expression (P<0.0001, **Figure 2A**). NSCLC patients with SUMO1-overexpression (n = 121) had a 19-month median survival time and 23.17-month mean survival time (95% CI = 19.91-26.42 months). The mean survival time of patients with low level of SUMO1 expression (n = 42) was 46.76 months (95% CI = 42.41-51.11 months), whereas the median survival time had not reached. Compare with poorly differentiated patients (n = 53, median survival time = 19 months), patients with well or moderately differentiated tumors (n = 110, median survival time = 50) had a longer survival time (P< 0.001). The median survival time of SCC (Squamous cell carcinoma) patients with SUMO1 overexpression (n = 60) was 12 months (95% CI = 5-19 months), while the median survival time of those with SUMO1 low-expression SCC (n = 22) was 51 months (95% CI was not reached). SCC patients with SUMO1 overexpression had a worse prognosis than those with SUMO1 low expression (P = 0.0056, **Figure 2B**). For the ADC (Adenocarcinoma) patients, the median and mean survival time of patients with SUMO1-overexpression (n = 35) was 25 months, whereas the mean survival time of those with SUMO1 low expression ADC (n = 11) had not yet been reached and the mean survival time was 45.45 months. ADC patients with SUMO1 overexpression expression had a worse prognosis than those with SUMO1 low expression (P = 0.0301, **Figure 2C**). For the ASC + SC (Adenosquamous carcinoma and Sarcomatoid carcinoma) patients, the median and mean survival time of patients with SUMO1-overexpression (n = 26) was 15 months, whereas the mean survival time of those with SUMO1 low expression (n = 9) had not yet been reached and the mean survival time was 54.11 months. ASC + SC patients with SUMO1 overexpression expression had a dramatically worse prognosis than those with SUMO1 low expression (P = 0.0004, **Figure 2D**). The survival of patients with NSCLC was related with the grade of tumor differentiation and pTNM stage. Patients with pTNM I/II tumors (n = 87) had a median survival time of 49 months. The median survival time of NSCLC patients with TNM III/IV tumors (n = 76) was 20 months (P = 0.0019, Figure S1).

We divided the enrolled NSCLC patients into pTNM I/II and pTNM III/IV groups to investigate the relationship between SUMO1 expression and the clinical outcome. Among the I/II-stage group, the median survival time of patients with SUMO1-overexpression (n = 58) was 24 months, whereas the mean survival time of those with SUMO1 low expression (n = 29) was

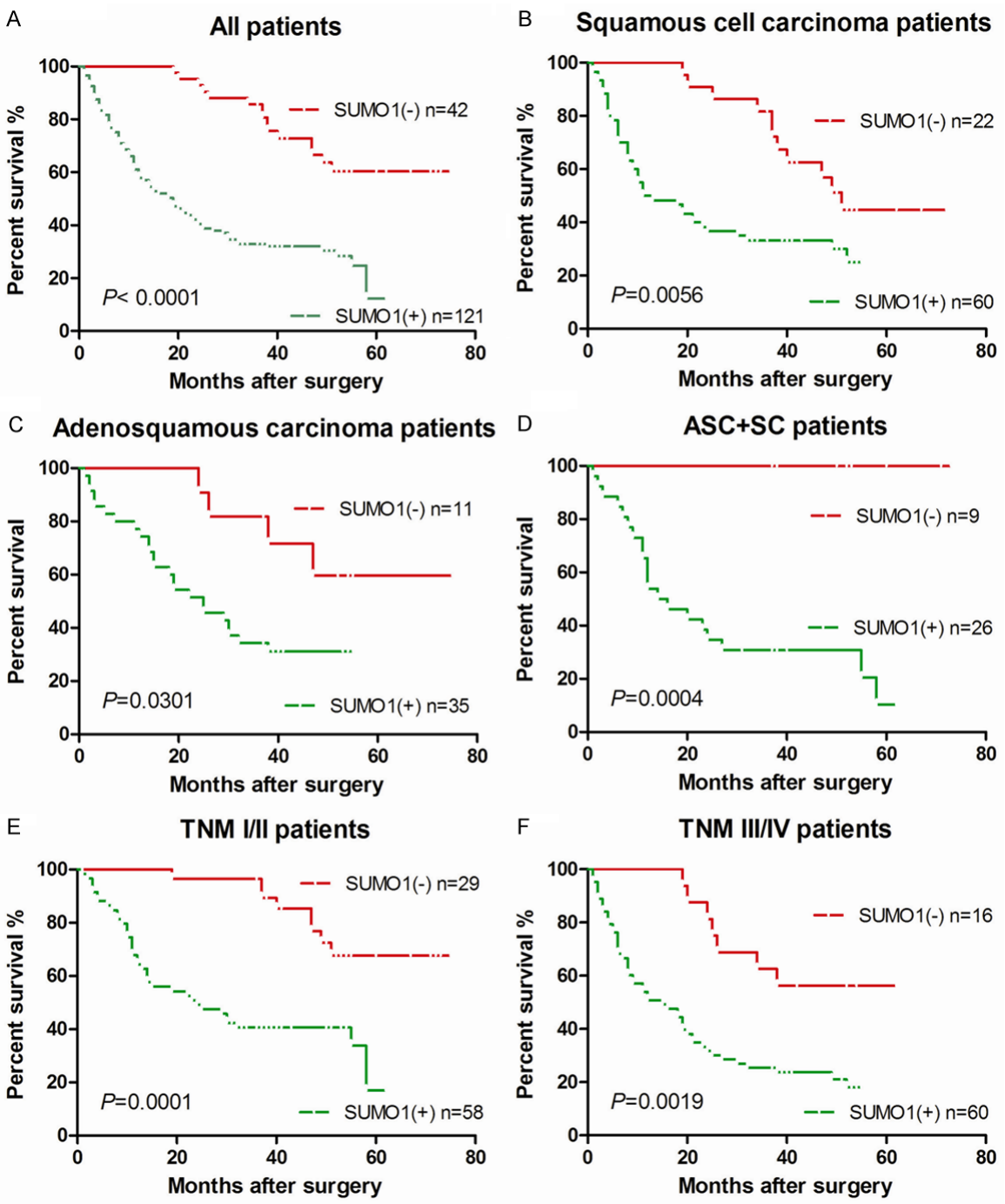

Figure 2. Survival analysis for NSCLC patients. A. Comparison of the overall survival (OS) between SUMO1 low expression and SUMO1 overexpression NSCLC patients. B. Comparison of the OS between SUMO1 low expression and SUMO1 overexpression SCC patients. C. Comparison of the OS between SUMO1 low expression and SUMO1 overexpression ADC patients. D. Comparison of the OS between SUMO1 low expression and SUMO1 overexpression ASC + SC patients. E. Comparison of the OS between SUMO1 low expression and SUMO1 overexpression pTNM I/II patients. F. Comparison of the OS between SUMO1 low expression and SUMO1 overexpression pTNM III/IV patients. The *P*-value was determined using the log-rank (Mental-Cox) Test.

52.17 months and the median survival time had not been reached. There were significant differences in the survival rate with SUMO1 expression level (P = 0.0001, **Figure 2E**). Among the III/IV-stage group, the median survival time of SUMO1 overexpression patients (n =

Table 2. Cox proportional hazards model analysis of variables affecting survival in NSCLC patients

Variables	Categories	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*P*-valve	HR (95% CI)	*P*-valve
Age (years)	≥60/<60	0.857 (0.49-1.36)	0.255		
Sex	Male/Female	0.921 (0.79-1.87)	0.573		
Smoking history	Smoking/Non-smoking	0.855 (0.63-1.67)	0.499		
Histological type	ADC/SCC/ASC + SC	1.341 (0.89-2.02)	0.250		
Grade of differentiation	Poor/Well + Moderate	2.438 (1.65-3.82)	<0.001	1.912 (1.23-2.86)	0.005
pTNM stage	I/II; III/IV	2.287 (1.69-3.04)	<0.001	2.057 (1.58-2.77)	<0.001
SUMO1 expression	Overexpression/Low expression	2.450 (1.78-3.12)	0.0016	1.731 (1.02-2.94)	0.0059

60) was 15 months, whereas the median survival time of SUMO1 low expression patients (n = 16) had not yet been reached and the mean survival time was 38.5 months. There also existed momentous difference in the survival rate with SUMO1 expression in this group (P = 0.0019, **Figure 2F**). To further assess whether SUMO1 expression represents a prognostic criterion in patients with NSCLC, we carried out regression analysis using the Cox's proportional hazards model. The covariate parameters included several clinicopathological factors in addition to SUMO1 were shown in **Table 2**. In univariate analysis, SUMO1 expression, grade of differentiation and pTNM stage showed a significantly higher hazard ratio for a poor prognosis. Moreover, multivariate analysis was performed using the significant factors observed in univariate analysis. The results showed that, along with the TNM stage and grade of differentiation, SUMO1 expression level was an autonomous prognostic factor (P = 0.0059, **Table 2**). These results adequately pointed out that the SUMO1 expression in NSCLC patients was firmly related to a poor prognosis.

Discussion

Protein sumoylation can be expected to be important in the course of tumorigenesis [11, 19-21]. However, there is no evidence to support this notion in lung cancer. In this study, we performed immunohistological analysis to detect the expression of SUMO-1 in 163 patients with NSCLC. The result showed that 74.23% (121/163) of tumor sections were overexpression for SUMO-1 expression, while none of corresponding adjacent non-cancerous tissue was overexpression. SUMO1 was highly expression in NSCLC tissues and almost undetectable in normal lung tissue. This indicated that SUMO-1 may be a new biomarker or diagnostic tool for lung cancer. We also compared the correlation between patient survival and SUMO1 expression status and found that SUMO1 over expression in NSCLC patients was notably associated with some clinical characteristic, such as pN factor and pTNM stage. These finding indicated that SUMO1 may play an important role in the metastasis of NSCLC.

To the best of our knowledge, this is the first time to indicate an association between SUMO1-positivity in NSCLC patients and poor prognosis and to suggest that SUMO1 overexpression may be an independent prognostic factor of NSCLC patients. Furthermore, the prognostic significance of SUMO1 overexpression was apparent in all kinds of NSCLC patients. We also investigated whether SUMO1 overexpression was associated with prognosis in early stage NSCLC patients. The results showed apparent prognostic significance of SUMO1 overexpression in early stage patients and in late stage patients. This result indicated that SUMO1 overexpression may also take part in the development of NSCLC. We hypothesize that SUMO1 overexpression may be related to cancer cell proliferation and metastasis.

Some shortcomings present existed in this study. Subject to the limitations of sample size and follow-up, the median survival time in many groups was absent. To respond the patient's survival, we listed the mean survival time, which was failed to accurately respond the patient's survival as the median survival time. In addition, this study did not investigate the relationship between the expression level of SUMO1 gene and the pathogenesis of lung cancer, which was very significant for a biomarker validation. To remedy these limitations, further multicenter clinical studies will be carried out

and an expanding sample size will enrich the means of detection.

Taken together with the results in this study, we indicate that SUMO1 may be a potential drug therapy target for NSCLC patients. However, the mechanism underlying the promotion of NSCLC by SUMO1 needs to be further examined.

Conclusions

We showed that a high proportion of NSCLCs express SUMO1 and that SUMO1 overexpression is significantly associated with grade of tumor differentiation, pTNM stage, and lymphatic metastasis. Moreover, our study is the first to indicate a role for SUMO1 as an independent factor predictive of poor prognosis in NSCLCs.

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Disclosure of conflict of interest

None.

Abbreviations

NSCLC, Non-small cell lung cancer; SUMO1, small ubiquitin-related modifier-1; SCC, Squamous cell carcinoma; ADC, Adenocarcinoma; SC, sareomatodes carcinoma; ASC, adenosquamous carcinoma; CI, Confidence interval; HR, Hazard Ratio.

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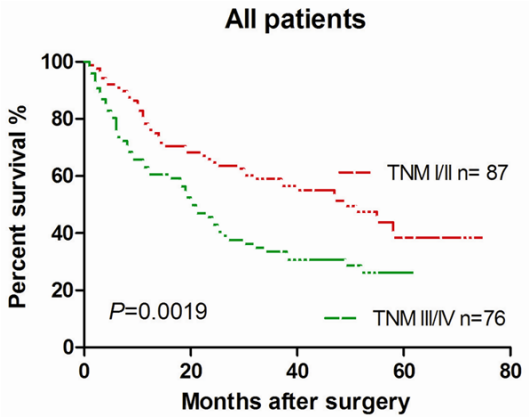

Figure S1. Survival analysis of pTNM I/II and pTNM III/IV patients. Comparison of the OS between pTNM I/II and pTNM III/IV patients.