Original Article

Senescence marker protein 30 inhibits tumor growth by reducing HDAC4 expression in non-small cell lung cancer

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Background: Senescence marker protein 30 (*SMP30*), which plays a pivotal role as a suppressor protein in cell proliferation, among other regulatory actions, is a marker of aging that shows decreased expression during senescence. Decreased *SMP30* has been identified in several human cancers, but its expression and role in human non-small cell lung cancer (NSCLC) remain unclear.

Methods: Using tumor tissue and matched adjacent normal tissue from 341 patients with resected NSCLC, we assessed *SMP30* expression using immunohistochemical methods. The relationship between *SMP30* expression and clinicopathologic characteristics was investigated by Kaplan-Meier survival analysis and multivariate analysis. Cell viability assay, colony formation assay, EdU incorporation assay and *in vivo* tumor xenograft models were also performed to investigate NSCLC cell proliferation using A549 and H1299 cell lines. Recombinant lentivirus-meditated *in vivo* gene overexpression and Western blot were performed to clarify the underlying molecular mechanism of *SMP30* inhibiting NSCLC proliferation.

Results: *SMP30* expression was frequently downregulated in NSCLC tissue, as compared with adjacent non-tumor tissue. Kaplan-Meier survival analyses revealed NSCLC patients with low *SMP30* expression had a significantly worse overall survival (OS), with median OS of 18 *vs.* 67 months in high *SMP30* expression group. *SMP30* overexpression significantly inhibited A549 and H1299 cell proliferation both *in vitro* and in tumor xenografts and downregulated the expression of *c-Myc* and *CyclinD1* protein. Moreover, Western blot analyses confirmed that SMP30 overexpression significantly inhibited the histone deacetylase 4 (*HDAC4*) level in NSCLC cells, and *HDAC4* overexpression reversed *SMP30*-mediated NSCLC repression both *in vitro* and *in vivo*.

Conclusions: *SMP30* inhibited NSCLC proliferation by reducing *HDAC4* expression, and *SMP30* and

HDAC4 may serve as new prognostic biomarkers and future therapeutic targets for NSCLC.

Keywords: Senescence marker protein 30 (*SMP30*); proliferation; histone deacetylase 4 (*HDAC4*); non-small cell lung cancer (NSCLC)

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Introduction

Lung cancer remains the leading cause of cancer-related deaths worldwide, and non-small-cell lung cancer (NSCLC), mainly consisting of lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD), is the predominant type, accounting for approximately 85% (1). Despite great progress in therapeutic regimens, including surgical resection, chemotherapy, radiation therapy, immunotherapy, and targeted biological agents, the prognosis of NSCLC patients remains poor, with a 5-year survival rate of approximately 26% (2,3). Clearly, there is a desperate need of novel diagnostic and therapeutic targets that can promote better evaluation of prognosis and optimization of treatments for individual NSCLC patients. Recent studies reported that senescence marker protein 30 (*SMP30*), a calcium-binding protein, may function as a tumor suppressor against tumor development and progression (4,5).

SMP30, also known as regucalcin, shows decreased expression during senescence and is a marker of aging (6). *SMP30* was reported to be associated with aging and partial loss of hepatology function (7) and is involved in the regulation of intracellular Ca^{2+} levels, modulation of several cellular signaling pathways, and has antioxidant properties (8). Decreased levels of *SMP30* leads to increased generation of reactive oxygen species, regarded as the most influential factor causing senescence (9). *SMP30* has a protective action against oxidative damage through modulation of the activity of enzymes involved in generation of oxidative stress, as well as in antioxidant defense (9).

SMP30 plays a pivotal role as a suppressor protein in cell proliferation (4,5). It has been demonstrated that *SMP30* overexpression (OE) suppressed cell proliferation via decreasing DNA synthesis (10-12). Additionally, OE of *SMP30* upregulated p53 and p21, while downregulating the mRNA expression of *c-Myc* and H-ras, suggesting that *SMP30* inhibits cell proliferation by modulating the expression of pro-oncogenes and tumor suppressor genes (13,14). The expression of c-Jun and chk2 cell-cycle regulators was decreased in *SMP30*-transfected NRK52e cells (11). Interestingly, accumulating evidence demonstrates that suppression of *SMP30* expression is involved in carcinogenesis (4,15,16). Decreased *SMP30* expression has been found in several human cancers, such as pancreatic cancer, breast cancer, and liver cancer (5,17,18). Moreover, low *SMP30* level was also associated with poor prognosis in these cancers (5,16). However, *SMP30* expression and its role in NSCLC are still unknown.

HDAC4 is a member of the class IIa HDAC family and is associated with a wide range of cellular and epigenetic processes as a transcriptional corepressor, similar to other members of the family (*HDAC5*, *HDAC7*, *HDAC9*) (19). It was reported that the OE of *HDAC4* promotes proliferation, metastasis and invasion of gastric cancer (20), esophageal carcinoma (21) and glioma (22). Moreover, elevated *HDAC4* expression also correlated with poor prognosis (21,23,24). HDAC4 was also reported to promote progression of lung cancer by regulating epithelial mesenchymal transition, autophagy and apoptosis (25,26). Additionally, HDAC inhibitors were reported to exert growth arrest and apoptosis effect on tumor cells (27) and several HDAC inhibitors were used in NSCLC (28,29). However, the oncostatic actions of *HDAC4* and its detailed underlying molecular mechanisms in NSCLC remain largely unclear.

In the current study, we built the *SMP30* OE NSCLC cell lines to investigate the actions of *SMP30* on NSCLC proliferation both *in vitro* and *in vivo*. Western blot results revealed that *SMP30* OE dramatically affected *HDAC4* expression. Then we evaluated the role of *HDAC4* in *SMP30*-mediated tumor proliferation. Furthermore, the expression of *SMP30* in 341 paired human NSCLC tissues and its correlation with related clinicopathologic features and survival were analyzed. We also analyzed whether the expressions of *SMP30* could serve as predictive marker for prognosis of NSCLC patients. We present the following

article in accordance with the ARRIVE reporting checklist (available at https://dx.doi.org/10.21037/tlcr-21-982).

Methods

Database-mining

To evaluate the expression of *SMP30* in NSCLC tissue and paired normal tissue, data from Oncomine database (https://www.oncomine.org/, Human Genome U133 Plus 2.0 Array, Reporter ID: 210751_s_at) were analyzed. Correlation analysis of *SMP30* expression and clinicopathologic parameters was also performed. Moreover, we also analyzed the associations between *SMP30* expression and NSCLC patient prognosis using data from Kaplan-Meier plotter database (https://kmplot.com/, use earlier release of the database: all 2015 version, n=2,437) and The Human Protein Atlas database (the RNA-seq data based on The Cancer Genome Atlas, http://www.proteinatlas.org/).

NSCLC tissue samples and tissue microarray immunohistochemistry (IHC)

We included 341 patients who underwent NSCLC surgery at Tangdu Hospital between May 2009 and December 2013 in this retrospective study. Among them, 152 patients were of early clinical stage (stage I/II) and 189 patients were of late clinical stage (stage III/IV). None of the patients had received radiotherapy or chemotherapy before surgery, and the final follow-up was updated until death or December 2018, whichever came first. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of the Fourth Military Medical University (No. TDLL-202110-03) and informed consents were not required for this retrospective study. Using paraffin-embedded tissue microarray containing 341 pairs NSCLC and matched adjacent normal lung tissues, IHC staining was conducted using the primary antibodies of anti-*SMP30* (1:50, 17947-1-AP, Proteintech), and standard protocols were followed as previously described (30).

The IHC staining score was based on two criteria: (I) percentage of positive cells (0, ≤5%; 1, 6–25%; 2, 26–50%; 3, 51–75%; and 4, >75%); (II) staining intensity (0, negative; 1, yellow; 2, brown; and 3, tan). The two scores were multiplied to produce the total score and the median score was used to divide the NSCLC samples into those with low and high *SMP30* expression.

Cell culture and lentivirus (LV) infection

Human NSCLC A549 (ATCC Cat# CRM-CCL-185, RRID: CVCL_0023) and H1299 (ATCC Cat# CRL-5803, RRID: CVCL_0060) cell lines were purchased from the American Type Culture Collection (ATCC, VA, USA) in January 2018, and the A549 and H1299 cells were authenticated by the ATCC upon purchase (5 months prior to experimental research) using morphology, karyotyping, and PCR based approaches to profile the cytochrome C oxidase I gene (COI analysis) and short tandem repeat to confirm the identity of human cell lines and to rule out both intra- and interspecies contamination (see ATCC website https://www.atcc.org/CellAuthenticationMatters.aspx). Cells were cultured in DMEM with high glucose (Gibco, NY, USA), supplemented with 10% fetal bovine serum (Gibco), penicillin-streptomycin solution (100 units/mL; Solarbio, Beijing, China). *SMP30*, *HDAC4* and paired empty vector LVs were purchased from Genechem (Shanghai, China). LV infection of A549 and H1299 cells was performed according to the Genechem protocols. Next, *SMP30* and *HDAC4* OE cells or negative controls (NCs) were selected with puromycin.

Cell viability assay

The A549 and H1299 cell viability assay used the CCK-8 kit according to the manufacturer's instructions (7Sea, Shanghai, China). Optical density values were measured at 450 nm through a microplate reader (SpectraMax 190, Molecular Device, USA).

Colony formation assay

A total of 500 A549 and H1299 cells were seeded and cultured in 6-well plates for 12–14 days, after which the plates were photographed and colonies containing >50 cells were counted after staining with 0.1% crystal violet.

5-Ethynyl-2'-deoxyuridine (EdU) incorporation assay

Cell proliferation was assessed by EdU incorporation assay. A BeyoClick EdU Cell proliferation kit with Alexa Fluor 594 (Beyotime, Shanghai, China) was used according to the manufacturer's instructions. Cells were imaged by Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan).

EdU-positive cells were manually counted and expressed as the percentage of cells calculated from nuclear labeling with Hoechst 33342.

In vivo tumor xenograft models

Animal experiments were performed under a project license (No. IACUC-20210609) granted by the Animal Ethics Committee of the Fourth Military Medical University, in compliance with the institutional guidelines for the care and use of animals. A protocol was prepared before the study without registration. For each part, 5 athymic nude mice (male, 4–6 weeks, 18–20 g) were obtained from the university's Laboratory Animal Center. They were housed singly under the conditions of 12 h light/12 h dark cycle, 65–75 °F (18–23 ℃) and 40–60% humidity with adequate food and water. The left and right flanks of 5 nude mice (male, 4–6 weeks, 18–20 g) were subcutaneously inoculated with LV-control and LV-*SMP30* cells separately while another 5 mice received LV-*SMP30* + LV-*HDAC4* and LV-*SMP30* + LV-control cells, respectively. Different groups of 5×10^6 H1299 cells were separately inoculated subcutaneously into the right or left flank of 6-week-old male nude mice for *in vivo* tumor xenograft assay. The tumor size and body weight were assessed every 3 days (the longer diameter of the tumor should not be >20 mm). Twenty-one to Twenty-eight days after injection, the animals were anesthetized and the tumors were excised for additional analysis before the humane death of the animal. After photographing and weighing the tumor, part of each sample was fixed in formalin, and another part was prepared for further analysis.

Western blot

Western blot was performed as previously described (31). Antibodies against *SMP30* (1:1,000, 17947-1-AP, Proteintech), against *HDAC4* (1:1,000, Proteintech Cat# 17449-1-AP, RRID: AB_2118864), anti-*CyclinD1* (1:1,000, 60186-1-Ap, Proteintech), anti-*c-Myc* (1:1,000, #5605, CST) anti-*β-actin* (1:5,000, ab6276, Abcam), and anti-*tubulin* (1:1,000, #2148, CST) were used. The 1:5,000 dilution of horseradish-peroxidase-linked anti-mouse or rabbit IgG was used as the secondary antibody (Zhongshan Company, Beijing, China).

Cell treatment

The pan-HDAC inhibitor vorinostat (SAHA), and the specific class IIa inhibitor TMP269, were obtained from MedChemExpress and prior to the experiment were prepared in DMSO and diluted in culture media immediately. One day after seeding onto culture dish, normal cells and *HDAC4* overexpressing cells were divided into four groups: (I) control group; (II) 0.625 μM SAHA/20 μM TMP269 group; (III) 1.25 μM SAHA/40 μM TMP269 group; (IV) 2.5 μM SAHA/60 μM TMP269 group. After the above treatments for 48 h, cells were analyzed.

Statistical analysis

Data analyses were carried out with SPSS 23.0 (SPSS Inc., IL, USA) software. The relationships between *SMP30* expression and the clinicopathologic parameters of the NSCLC patients were evaluated by χ^2 test or Fisher's exact test. Survival analyses were examined by Kaplan-Meier method, then compared with the log-rank test. Univariate and multivariate survival analyses were conducted using Cox hazards regression models. Results were compared between groups by Student's *t*-test. Data are presented as the mean ± standard deviation (SD). $P<0.05$ was considered as statistically significant.

Results

Expression of SMP30 in NSCLC patients

To investigate the expression of *SMP30* in NSCLC, we initially conducted database-mining to compare the gene expression profiles of *SMP30* between normal lung and cancer tissues. Oncomine database (https://www.oncomine.org/) analysis revealed that the *SMP30* mRNA level was significantly lower in various cancer types compared with normal tissues, but especially in lung cancer (Figure S1A). Results showed that *SMP30* mRNA levels in NSCLC, including large-cell lung carcinoma, LUAD and LUSC, were significantly decreased compared with normal lung tissue samples (Figure S1B). To further validate the *SMP30* expression in NSCLC tissues, we performed IHC analysis to detect the *SMP30* level in a tissue microarray containing 341 paired tumor-normal lung tissues (*Figure 1A*) and it was clearly lower in NSCLC than in adjacent noncancerous lung samples (*Figure 1B*).

In order to characterize the roles of *SMP30* in NSCLC, we analyzed whether its expression was associated with clinicopathologic variables (*Table 1*). Of the 341 samples, 133 had a score of 0 while two samples had a score of 12.

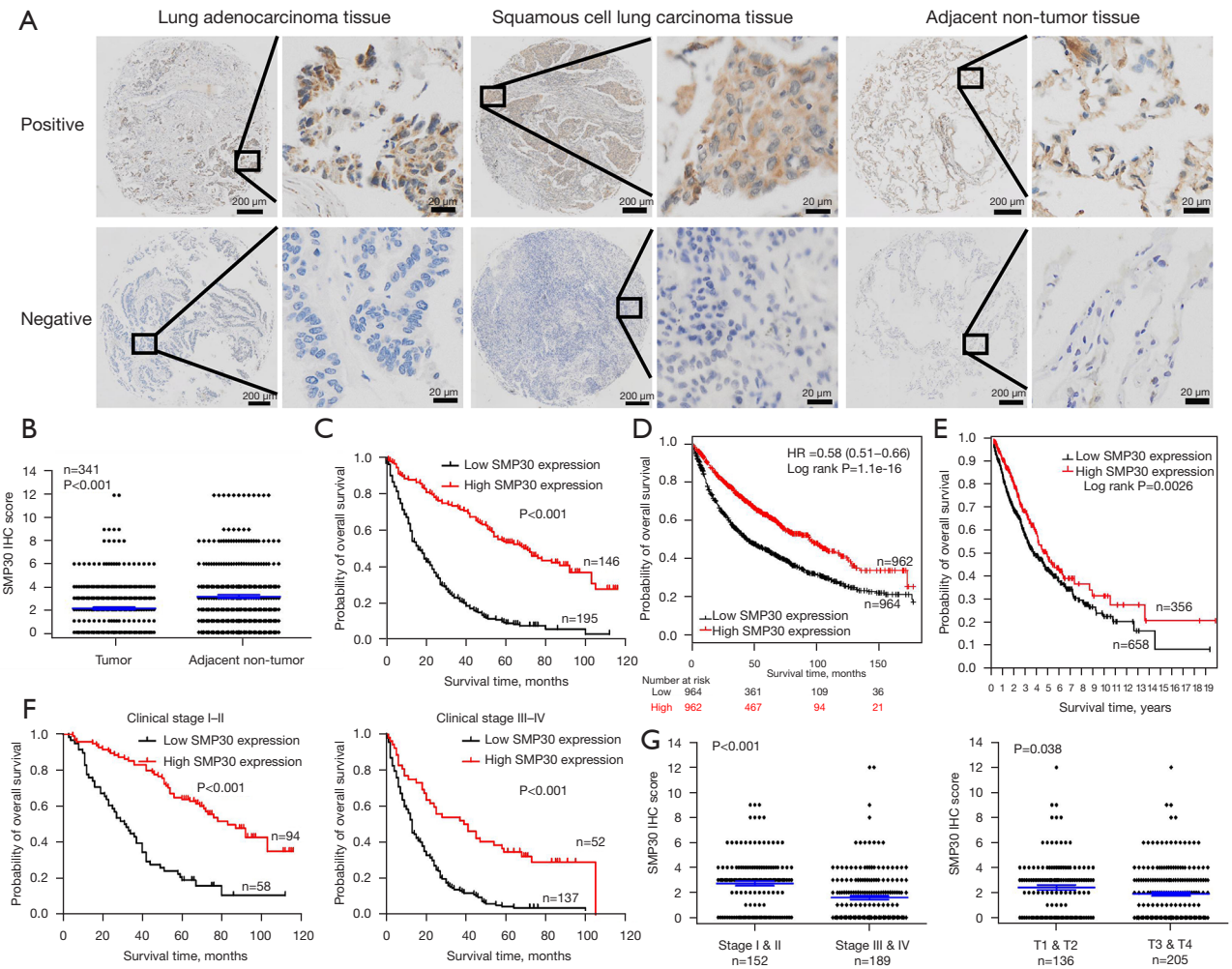

Figure 1 Correlation of low *SMP30* level with worse overall survival in NSCLC patients. (A) Representative immunohistochemical (IHC) images for *SMP30* expression in NSCLC (lung squamous cell carcinoma and lung adenocarcinoma) and adjacent noncancerous tissues. Scale bar, 200 and 20 μm (inset) respectively. (B) Statistical analysis of *SMP30* expression in tumor tissue and adjacent noncancerous tissue of 341 NSCLC patients through IHC staining. (C-F) Kaplan-Meier survival analyses of high/low *SMP30* expression based on tissue microarray IHC results for 341 NSCLC patients (C), 1,926 NSCLC patients in the Kaplan-Meier plotter database (D), 1,014 NSCLC patients from The Human Protein Atlas (original RNA-seq data from TCGA) (E), 152 NSCLC patients in the early clinical stages, and 189 NSCLC patients in the late clinical stages (F). (G) Statistical analysis of *SMP30* expression in different clinical stages and T classifications of 341 NSCLC patients through IHC staining. *SMP30*, senescence marker protein 30; NSCLC, non-small cell lung cancer; TCGA, The Cancer Genome Atlas.

The median score for the entire cohort was 2. On univariate analysis, *SMP30* expression was significantly associated with tumor size, tumor invasion, lymphatic metastasis, distant metastasis, cancer differentiation and clinical stage respectively (*Table 1*). These results indicated that abnormal *SMP30* expression was involved in NSCLC progression.

Correlation of SMP30 expression and NSCLC prognosis and survival

We analyzed the association between *SMP30* expression and NSCLC patient prognosis using Kaplan-Meier analysis and log-rank test for significance estimates. The Kaplan-Meier survival curves showed that NSCLC patients with

Table 1 Association of *SMP30* expression with clinicopathologic parameters of patients with NSCLC

Parameter	N	*SMP30* expression		
		Low	High	P value
Age, years				0.911
<60	153	88	65	
≥60	188	107	81	
Sex				0.503
Male	272	158	114	
Female	69	37	32	
Tumor location				0.803
Left lung	142	89	53	
Right lung	199	106	93	
Tumor size, cm				0.003
<5	124	58	66	
≥5	217	137	80	
T classification				0.001
T1	6	3	3	
T2	130	62	68	
T3	127	71	56	
T4	78	59	19	
Lymph node metastases				<0.001
N0	156	72	84	
N1–N3	185	123	62	
Metastasis				0.022
No	330	185	145	
Yes	11	10	1	
Differentiation				<0.001
Well and moderate	235	108	127	
Poorly and not	106	87	19	
Clinical stage				<0.001
I	48	12	36	
II	104	46	58	
III	178	127	51	
IV	11	10	1	

SMP30, senescence marker protein 30; NSCLC, non-small cell lung cancer.

low *SMP30* expression were associated with worse overall survival (OS) (log-rank P<0.001, *Figure 1C*). Consistently, the Kaplan-Meier plotter database (https://kmplot.com/) survival analysis based on 1,926 NSCLC cases suggested that a low *SMP30* level was significantly correlated with poor prognosis [hazard ratio (HR) =0.58, log-rank P<0.001, *Figure 1D*]. Furthermore, we analyzed the survival of 1,014 NSCLC cases through The Human Protein Atlas database (the RNA-seq data based on The Cancer Genome Atlas, https://www.proteinatlas.org/), and verified that low *SMP30* level was related to poor prognosis (*Figure 1E*). Interestingly, we found that NSCLC patients with low *SMP30* expression had worse OS when the patients were analyzed based on stage at diagnosis. This was evident in both early stage (stage I/II) and more advanced stage (stage III/IV) (P<0.001, *Figure 1F*). Our results also indicated that NSCLC patients with larger tumors (T3/T4) and high clinical stage (stage III/IV) had lower expression of *SMP30* than those with smaller tumor invasion (T1/T2) and low clinical stage (stage I/II) (P<0.001, *Figure 1G*).

To further assess whether *SMP30* expression represents a prognostic factor for patients with NSCLC, we performed Cox proportional hazard regression analysis of the patients' OS. Univariate analysis showed that low *SMP30* expression negatively correlated with NSCLC survival (*Table 2*). In the multivariate survival analysis, when other risk factors such as tumor invasion, lymphatic invasion, distant metastasis, cancer differentiation, and clinical stage were taken in consideration, the *SMP30* expression was still associated with a lower risk of death 0.383 (95% CI: 0.282–0.518, P<0.001).

Low SMP30 level and poor prognosis in both LUAD and LUSC patients

We further studied the prognostic role of *SMP30* expression in LUAD and LUSC patients. The Kaplan-Meier plotter database survival analysis including 719 LUAD cases indicated that low *SMP30* expression in LUAD patients was significantly associated with poor prognosis (HR =0.44, log-rank P<0.001, *Figure 2A*). Analysis of SMP30 expression in 131 LUAD patients in our cohort, verified that low *SMP30* level significantly correlated with worse OS, with median OS in low *SMP30* expression group and high *SMP30* expression group was 22 and 47 months respectively (log-rank P<0.001, *Figure 2B*). Moreover, we found the prognosis of LUAD patients with low SMP30 expression was worse in both early clinical stage (stage I/II) (median OS of

Table 2 Univariate and multivariate analyses of the correlation between clinicopathological variables for survival of patients with non-small cell lung cancer

Variable	Category	Univariate analysis			Multivariate analysis		
		HR	95% CI	P value	HR	95% CI	P value
Age	<60/≥60 years	1.171	0.919–1.491	0.201	–	–	–
Sex	Male/female	1.067	0.789–1.442	0.675	–	–	–
Tumor location	Left lung/right lung	0.879	0.689–1.121	0.299	–	–	–
T classification	I–II/III–IV	2.002	1.552–2.584	<0.001	1.393	1.005–1.930	0.046
Lymphatic invasion	Yes/no	1.740	1.359–2.229	<0.001	0.991	0.714–1.377	0.959
Distant metastasis	Yes/no	2.425	1.323–4.445	0.004	1.171	0.537–2.553	0.692
Differentiation	Well and moderate/poorly and not	5.150	3.913–6.777	<0.001	3.272	2.452–4.365	<0.001
Clinical stage	I/II/III/IV	1.549	1.332–1.800	<0.001	1.378	1.018–1.867	0.038
SMP30 expression	Low/high	0.263	0.201–0346	<0.001	0.383	0.282–0.518	<0.001

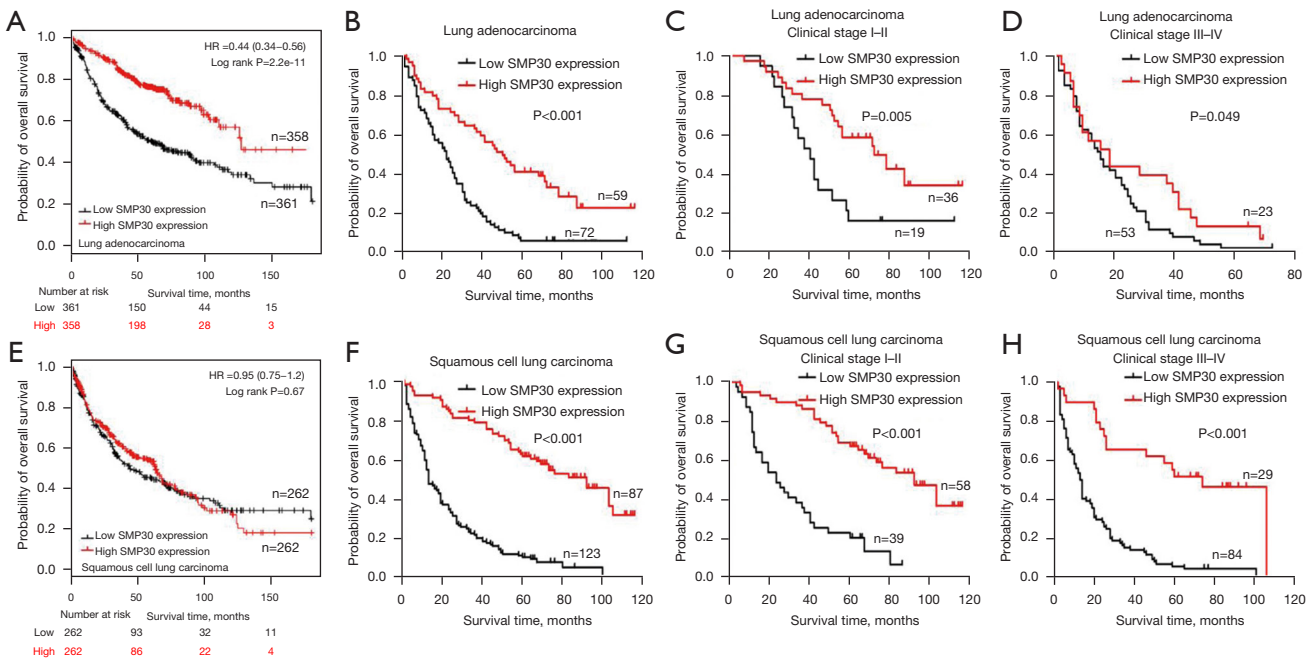

Figure 2 Correlation of low *SMP30* level with worse overall survival in both LUAD and LUSC patients. (A,E) Kaplan-Meier survival analyses of high/low *SMP30* expression in 719 LUAD patients and 524 LUSC patients, respectively, based on the Kaplan-Meier plotter database. (B-D,F-H) Kaplan-Meier survival analyses of high/low *SMP30* expression in 131 LUAD patients and 210 LUSC patients based on the tissue microarray immunohistochemical results. *SMP30*, senescence marker protein 30; HR, hazard ratio; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.

31 *vs.* 103 months) and late clinical stage (stage III/IV) (median OS of 13 *vs.* 39 months) subgroups (log-rank P<0.05, *Figure 2C*,*2D*).

Interestingly, the Kaplan-Meier plotter database showed no significance between *SMP30* expression and LUSC patients' OS (HR =0.95, log-rank P=0.67) (*Figure 2E*), but our Kaplan-Meier survival analysis based on 210 LUSC patients showed that low *SMP30* level was associated with poor prognosis (median OS of 13 *vs.* 103 months) (log-rank P<0.001, *Figure 2F*). Furthermore, we found LUSC patients with low *SMP30* expression had significantly worse OS in both the early and late clinical stage subgroups (log-rank P<0.001, *Figure 2G*,*2H*).

Effect of SMP30 OE on proliferation of NSCLC A549 and H1299 cells

To verify the potential antitumor actions of *SMP30* in NSCLC, we first examined its expression in multiple NSCLC cell lines (*Figure 3A*) and established stable *SMP30* overexpressing A549 and H1299 cell lines through use of the *SMP30* LV. *SMP30* expression was confirmed by Western blot (*Figure 3B*). Subsequently, we performed cell viability and colony formation assays to evaluate the role of *SMP30* in A549 and H1299 cell proliferation. Compared with the NC group, we found *SMP30* OE significantly decreased the A549 and H1299 cell viability determined by CCK-8 analysis (P<0.05, *Figure 3C*). This anti-proliferative effect was further validated by the colony formation assay, in which *SMP30* OE significantly decreased the colony formation ability in A549 and H1299 cells (P<0.05, *Figure 3D*). We also used the EdU incorporation assay to analyze the role of *SMP30* on proliferation, and we found *SMP30* OE remarkably decreased the EdU-positive cells compared with the NC group (P<0.05, *Figure3E*). To further validate the effect of *SMP30* OE on inhibiting NSCLC cell proliferation, we established H1299 cell xenograft in athymic nude mice and measured their tumor volumes. We found that all nude mice developed subcutaneous tumors, but the tumor volume in *SMP30* OE group was clearly reduced compared with NC group (*Figure 3F*). Aberrant activation or expression of *c-Myc* contributes to tumor progression. Western blot analyses showed that *SMP30* OE inhibited the expression of *c-Myc* both *in vitro* and *in vivo* (*Figure 3B*,*3G*). We further examined cell-cycle proteins with Western blot analysis and found that the level of *CyclinD1* was dramatically decreased in the *SMP30* overexpressing groups both *in vivo* and *in vitro* (*Figure 3B*,*3G*). However, other cell cycle-related proteins did not significantly change in these cell lines (Figure S2A). Taken together, the results revealed that *SMP30* had an antiproliferative action on NSCLC cells.

Role of HDAC4 in SMP30-mediated NSCLC inhibition

To further explore the underlying mechanism of *SMP30* OE on inhibiting NSCLC proliferation, we measured the level of HDAC family members in A549 and H1299 cells among the NC and *SMP30* overexpressing groups. Our results showed that *HDAC4* was downregulated after *SMP30* OE both *in vivo* and *in vitro* (*Figure 4A*,*4B*) while other members showed no significant changes (*Figure 4A*,*4B*, Figure S2B). To further explore the role of *HDAC4* in NSCLC, we measured its levels in several NSCLC cell lines (*Figure 4C*) and then established stable *HDAC4* overexpressing A549 and H1299 cell lines using the *HDAC4* LV, and *HDAC4* expression was confirmed by Western blot (*Figure 4D*). Colony formation assay was performed to show that *HDAC4* OE significantly increased colony formation ability of both cell lines (*Figure 4E*). These results were also confirmed by the EdU incorporation assay (*Figure 4F*) and furthermore, Western blot results showed that the levels of *c-Myc* and *CyclinD1* were elevated after *HDAC4* OE in both the A549 and H1299 cell lines (*Figure 4D*).

Involvement of HDAC4 in SMP30-mediated NSCLC suppression

To further confirm the involvement of *HDAC4* in *SMP30*-mediated NSCLC repression, we upregulated *HDAC4* by transfecting *HDAC4* LV into SMP30 overexpressing H1299 cells. The OE of *HDAC4* was verified by Western blot and had no effect on the levels of *SMP30* protein (*Figure 5A*). *HDAC4* OE markedly reversed *SMP30* OE-induced *c-Myc* and *CyclinD1* inhibition in NSCLC cells (*Figure 5A*). Moreover, we found *HDAC4* OE partially reversed the repressed proliferative ability of H1299 cells overexpressing *SMP30* (*Figure 5B*). These results were confirmed by the EdU incorporation assay (*Figure 5C*). Furthermore, the *in vivo* study showed that *HDAC4* OE significantly increased the mean volume of tumors in the H1299 *SMP30* overexpressed xenograft group (*Figure 5D*). Western blot analyses of subcutaneous tumors indicated that the OE of *HDAC4* reversed the *c-Myc* and *CyclinD1* inhibition both *in vivo* and *in vitro* (*Figure 5A*,*5E*). Taken together, these results suggested that *SMP30*-mediated NSCLC inhibition

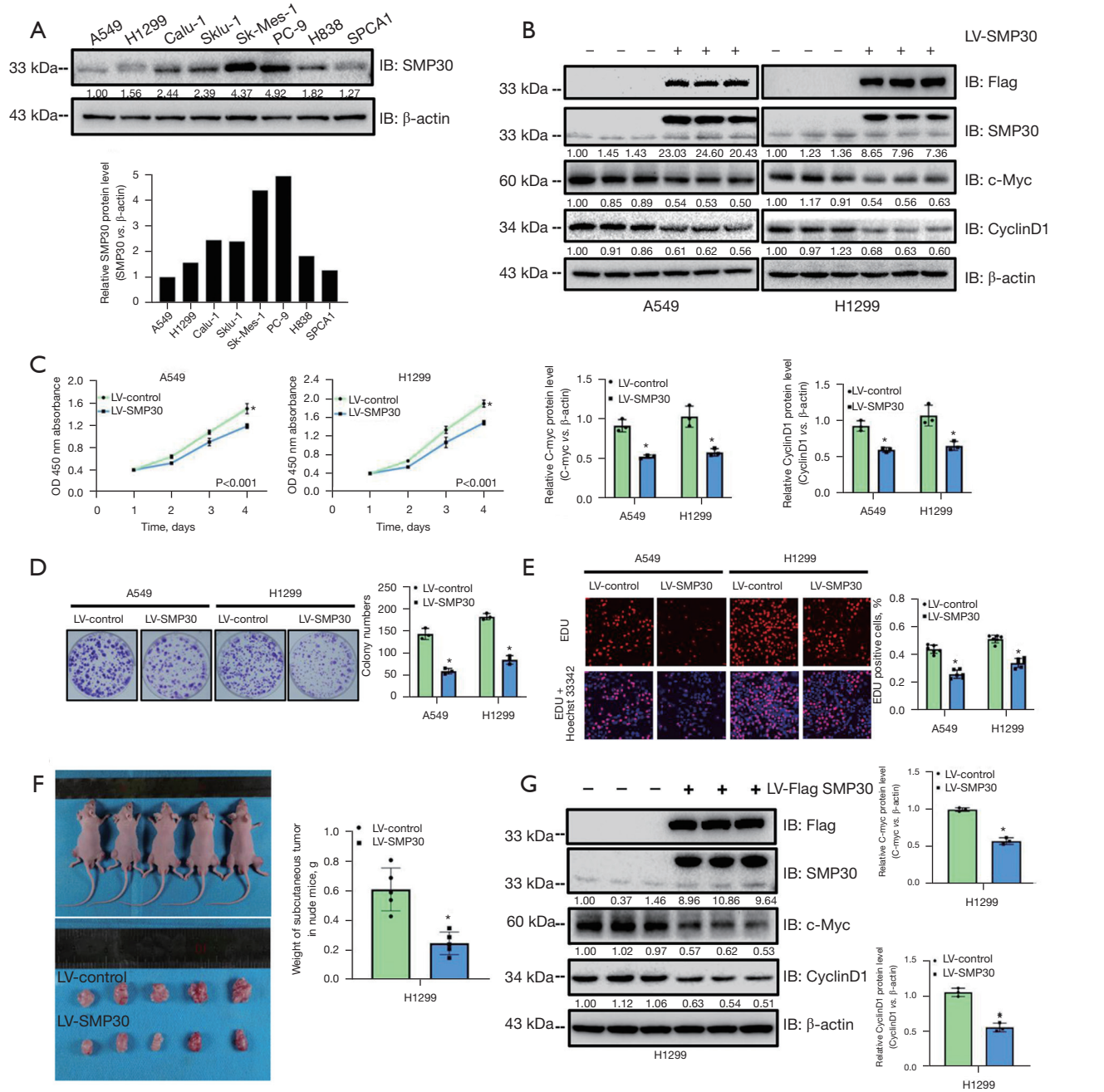

Figure 3 Effect of *SMP30* overexpression on proliferative ability of A549 and H1299 cells, and tumor growth in H1299 cell tumor xenografts. (A) Relative protein levels of *SMP30* in cell lines as determined by Western blot. *β-actin* was used as a loading control. (B) Representative Western blot results of Flag, *SMP30*, *c-Myc* and *CyclinD1* in LV-*SMP30* A549 and LV-*SMP30* H1299 cells. (C) Growth curves of the A549 and H1299 cells. Cell viability was detected by CCK-8 assay and expressed as optical density values. (D) Representative images and results of the colony formation assay. Colonies were visualized by crystal violet staining. (E) Representative images (×20) and statistical analysis of EdU incorporation assay. The results are presented as the ratio between the number of EdU-positive cells (red fluorescence) and the total number of Hoechst 33342-stained cells (blue fluorescence). (F) Representative results of tumor weight changes after subcutaneous injection of H1299 cells. Photographs show tumor xenograft morphologies in the LV-control and LV-*SMP30* H1299 groups. (G) Representative Western blot results of Flag, *SMP30*, *c-Myc* and *CyclinD1* in H1299 xenograft tumor tissues. *β-actin* was used as a Western blot loading control. The values below the Western blot band represent the relative gray values. All the data are shown as the mean ± SD. Student's *t*-test, *, P<0.05 *vs.* LV-control group. LV, lentivirus; *SMP30*, senescence marker protein 30.

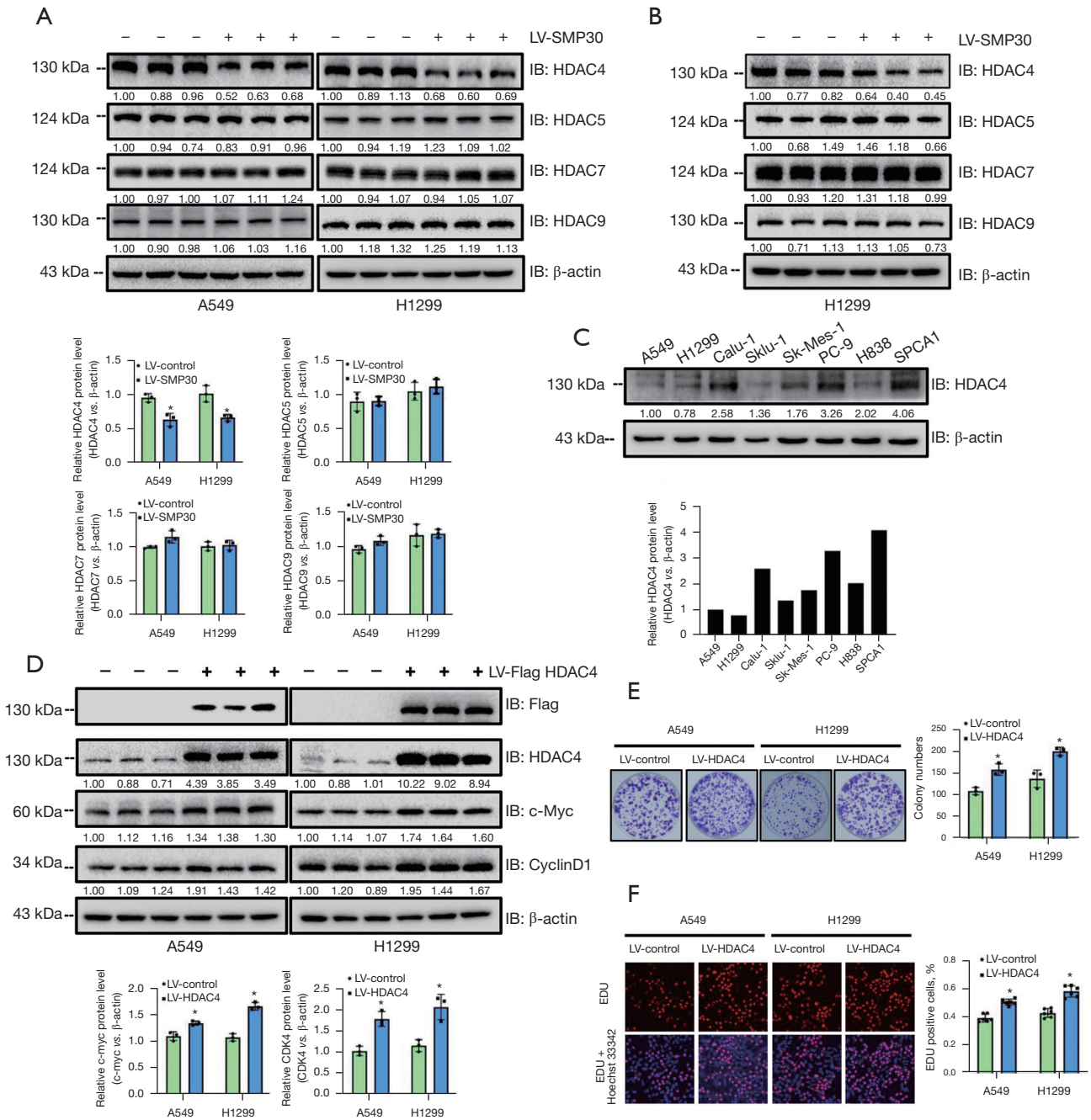

Figure 4 Effect of *HDAC4* level of *SMP30* overexpression both *in vivo* and *in vitro*, and effect of *HDAC4* overexpression on NSCLC cell proliferation. (A) Representative Western blot results of *HDAC4*, *HDAC5*, *HDAC7* and *HDAC9* in LV-*SMP30* A549 and LV-*SMP30* H1299 cells. (B) Representative Western blot results of *HDAC4*, *HDAC5*, *HDAC7* and *HDAC9* in H1299 xenograft tumor tissues. (C) Relative protein levels of HDAC4 in cell lines as determined by Western blot. (D) Representative Western blot results of Flag, *HDAC4*, *c-Myc* and *CyclinD1* in LV-*HDAC4* A549 and LV-*HDAC4* H1299 cells. (E) Representative images and results of colony formation assay. Colonies were visualized by crystal violet staining. (F) Representative images (×20) and statistical analysis of EdU incorporation assay. The results are presented as the ratio between the number of EdU-positive cells (red fluorescence) and the total number of Hoechst 33342-stained cells (blue fluorescence). *β-actin* was used as a Western blot loading control. The values below the Western blot band represent the relative gray values. All the data are shown as the mean ± SD. Student's *t*-test, *, P<0.05 *vs.* LV-control group. HDAC histone deacetylase; LV, lentivirus; *SMP30*, senescence marker protein 30; SD, standard deviation; NSCLC, non-small cell lung cancer.

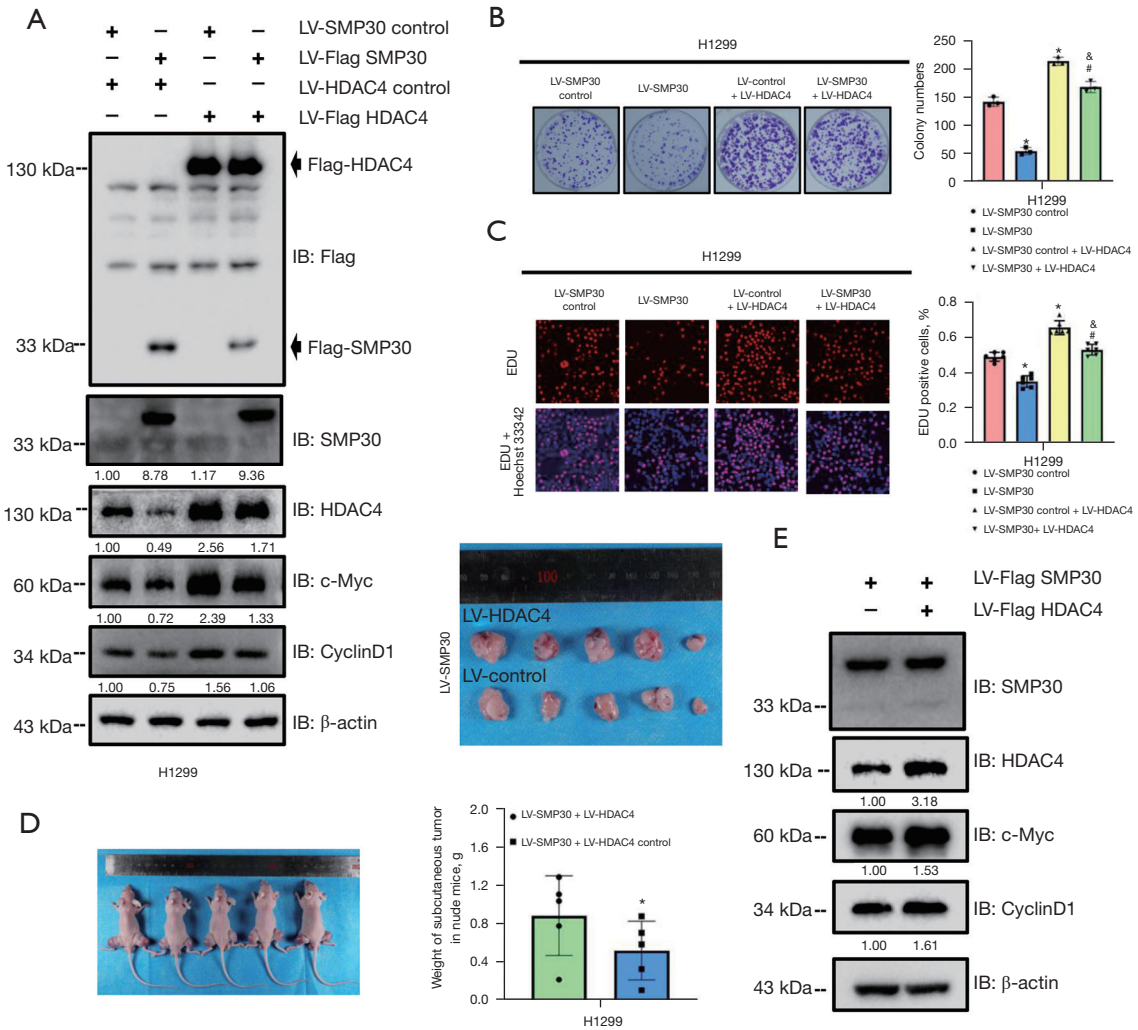

Figure 5 Essential requirement for *HDAC4* in *SMP30*-mediated NSCLC cell proliferation. (A) Representative Western blot results of Flag, *SMP30*, *HDAC4* and *c-Myc* and *CyclinD1*. (B) Representative images and results of colony formation assay. Colonies were visualized by crystal violet staining. (C) Representative images (×20) and statistical analysis of EdU incorporation assay. The results are presented as the ratio between the number of EdU-positive cells (red fluorescence) and the total number of Hoechst 33342-stained cells (blue fluorescence). (D) Representative results for tumor weight changes after subcutaneous injection of H1299 cells. Photographs show tumor xenograft morphologies in the LV-*SMP30* + LV-*HDAC4* and LV-*SMP30* + LV-control H1299 groups. (E) Representative Western blot results of *SMP30*, *HDAC4*, *c-Myc* and *CyclinD1* in H1299 xenograft tumor tissues. *β-actin* was used as a Western blot loading control. The values below the Western blot band represent the relative gray values. All the data are shown as the mean ± SD. Student's *t*-test, *, P<0.05 *vs.* the LV-SMP30 control group; #, P<0.05 *vs.* the LV-SMP30 group; &, P<0.05 *vs.* the LV-SMP30 control + LV-HDAC4 group. HDAC, histone deacetylase; LV, lentivirus; *SMP30*, senescence marker protein 30; SD, standard deviation; NSCLC, non-small cell lung cancer.

can be reversed by *HDAC4* upregulation.

Effect of pharmacological inhibition of HDAC4 on proliferative abilities of H1299 cells

To further confirm the role of *HDAC4* in NSCLC, normal cells and *HDAC4* overexpressing cells were incubated with the pan-inhibitor vorinostat (SAHA) and the specific class IIA inhibitor TMP269 respectively. Western blot results showed that, with increasing concentrations of SAHA and TMP269, the level of *HDAC4* decreased (*Figure 6A*,*6B*). Consistently, the levels of *c-Myc* and *CyclinD1* were also

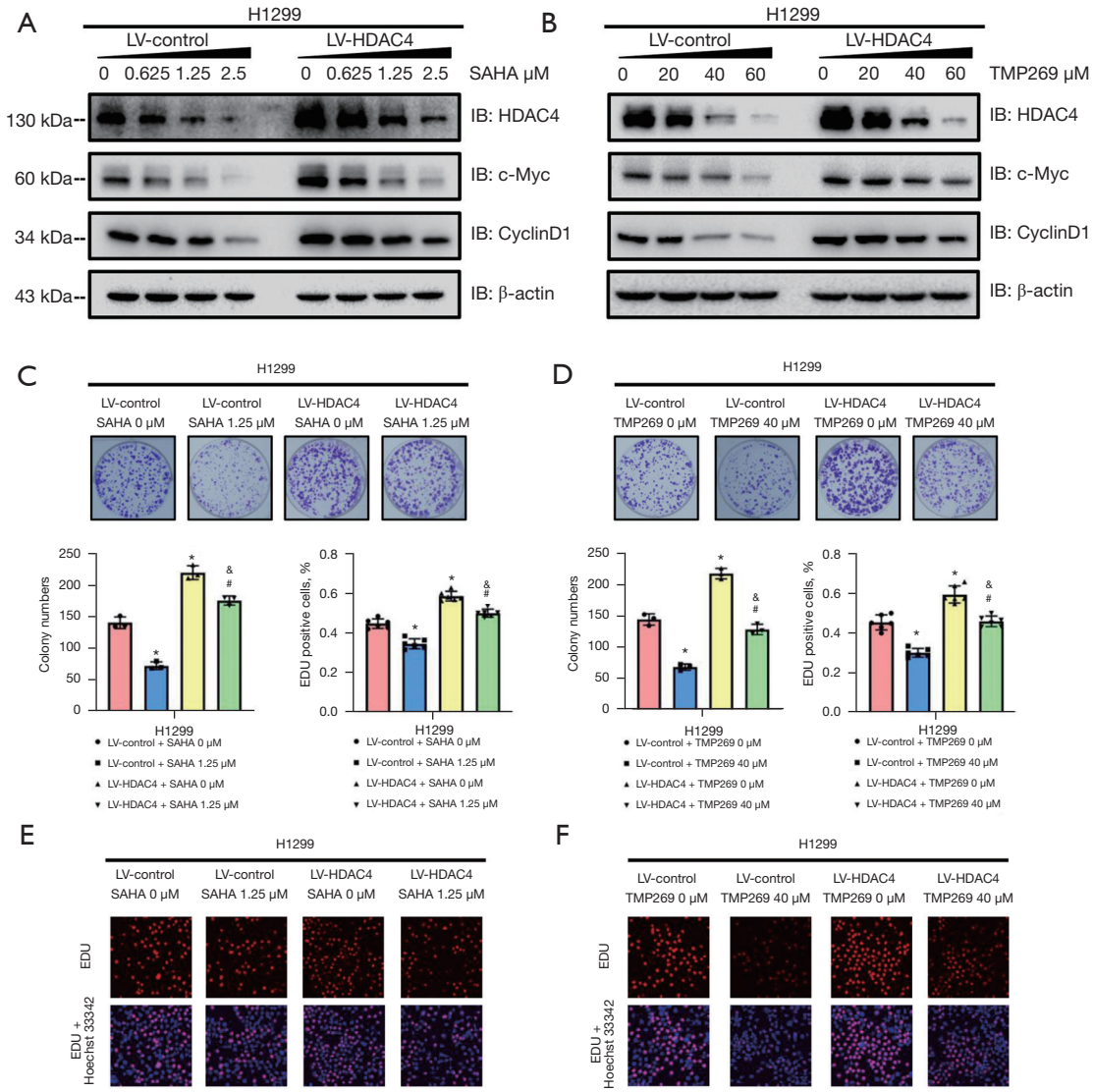

Figure 6 Effect of pharmacologic inhibition of *HDAC4* on proliferative abilities of H1299 cells. LV-control and LV-*HDAC4* H1299 cells were pretreated with SAHA or TMP269 for 48 h. (A,B) Representative Western blot results of *HDAC4*, *c-Myc*, and *CyclinD1*. (C,D) Representative images and results of colony formation assay. Colonies were visualized by crystal violet staining. (E,F) Representative images (×20) and statistical analysis of EdU incorporation assay. The results are presented as the ratio between the number of EdU-positive cells (red fluorescence) and the total number of Hoechst 33342-stained cells (blue fluorescence). *, P<0.05 *vs.* the LV-control +SAHA/TMP269 0 μM group; #, P<0.05 *vs.* the LV-control + SAHA1.25 μM/TMP269 40 μM group; &, P<0.05 *vs.* the LV-HDAC4 + SAHA/TMP269 0 μM group. HDAC, histone deacetylase; LV, lentivirus; *SMP30*, senescence marker protein 30.

decreased and the average expressions of *HDAC4*, *c-Myc* and *CyclinD1* in the *HDAC4* overexpressing groups were significantly higher than in the normal cell groups (*Figure 6A*,*6B*). Moreover, the colony formation assay showed that as the level of HDAC4 decreased, the cells' proliferative ability was inhibited (*Figure 6C*,*6D*). These results were confirmed by the EdU incorporation assay (*Figure 6E*,*6F*).

Discussion

SMP30, a multifunctional protein, plays a pivotal role in cell homeostasis (4), through maintaining calcium homeostasis,

inhibiting various signaling pathways involving various protein kinases and protein phosphatases, suppressing nuclear DNA and RNA synthesis, and preventing cell proliferation and apoptosis (16,32). Furthermore, downregulation of *SMP30* during aging might contribute to the deterioration of cellular functions and interactivity. It is well known that cancer is more common with aging (33). Previous studies suggest that *SMP30* is downregulated and exerts anticancer actions in human tumor tissues (15), but its oncostatic effects and detailed molecular mechanisms in NSCLC remain unclear. Consistent with previous research, our databases-mining results indicated that *SMP30* mRNA levels were lower in NSCLC compared with normal lung tissues. Furthermore, that outcome was confirmed by our IHC analysis results based on tissue microarray containing 341 paired NSCLC tumor and normal samples. *SMP30* expression negatively correlated with tumor size, tumor invasion, lymphatic metastasis, distant metastasis, cancer differentiation and clinical stage.

To the best of our knowledge, this study is the first to show a connection between *SMP30* negativity and poor prognosis, and to indicate that *SMP30* may be an independent prognostic factor for NSCLC patients. NSCLC patients, both LUAD and LUSC with low *SMP30* expression had a significantly shorter OS time than those with high expression. Furthermore, we also found that NSCLC patients with low *SMP30* expression had poor prognosis regardless of stage at diagnosis. Interestingly, the Kaplan-Meier plotter database showed that low *SMP30* expression was a predictor of poor prognosis in LUAD but not in LUSC patients, but our analysis demonstrated that low *SMP30* level was associated with the poor prognosis of both LUAD and LUSC patients. We supposed that the differences may be due to that the Kaplan-Meier plotter database was based on mRNA and the expression of *SMP30* in our study was based on IHC, which may be more reliable.

Several studies report that *SMP30* OE inhibited cell proliferation in pancreatic cancer (34), colorectal carcinoma (17), liver cancer (18), and breast cancer (5) *in vitro*. Those findings support the idea that *SMP30* may play an important inhibitory role in human cancer cells and that downregulation of *SMP30* expression may lead to carcinogenesis. We found *SMP30* OE significantly inhibited the proliferative ability (cell viability and colony formation assays) of A549 and H1299 cells. Additionally, the suppression of tumor growth by *SMP30* was further confirmed in a subcutaneous xenograft tumor nude mice model. Western blot analysis suggested that *SMP30* OE downregulated the expression of *HDAC4* both *in vitro* and *in vivo*.

HDAC4 is a member of the class IIa HDAC family and controls a complex cellular signaling cascade (19). It has diverse roles in cellular regulation, mainly including promoting chondrocyte differentiation (35), preventing neuronal death (36), promoting myocyte differentiation (37) and promoting the proliferation of satellite cells (38). Recently, numerous studies reported that OE of *HDAC4* promoted cancer proliferation, invasion and metastasis via various signaling pathways, such as inhibition of *p21* and/or *p27* (20,22,24,39) or elevation of proliferating cell nuclear antigen (*PCNA*) (40). These results indicate that *HDAC4* may act as a putative tumor promoter. In our study, the Western blot results showed that *HDAC4* levels were significantly suppressed in *SMP30* overexpressing A549 and H1299 cell lines. Previous studies showed that pharmacological inhibition of HDAC suppressed the levels of *c-Myc* and *CyclinD1* (41,42). Similarly, in our study, *HDAC4* OE in the A549 and H1299 cell lines increased the levels of *c-Myc* and *CyclinD1*, and pharmacologic inhibition of *HDAC4* decreased both. Intriguingly, *HDAC4* OE dramatically reversed the inhibition of *SMP30* OE on cell proliferation both *in vivo* and *in vitro* but had no effect on the level of *SMP30* expression.

Transcription factor *c-Myc*, product of gene *MYC*, correlates with tumor aggression and poor clinical outcome in a variety of tumors (43). *CyclinD1*, encoded by *CCND1* gene in humans, is an important regulator of the cell-cycle G1/S transition (44). *c-Myc* and *CyclinD1*, which were often dysregulated and constitutively activated in human NSCLC, play important roles in many cancer types, suggesting that they may be key targets for treatment (45,46). Previous studies report that *c-Myc* increases the levels of *CyclinD1*, and further, activates *CDK4* and *CDK6*, promoting G1-phase cell-cycle progression (47). In this study, *SMP30* OE significantly inhibited the levels of *c-Myc* and *CyclinD1* both *in vivo* and *in vitro*, and this was dramatically reversed by *HDAC4* OE. These results indicated that *SMP30* suppresses NSCLC proliferation via the *HDAC4/c-Myc*, *CyclinD1* pathways.

In conclusion, we demonstrated for the first time that *SMP30* functions as a novel tumor suppressor factor in NSCLC. The level of *SMP30* expression was significantly decreased in NSCLC tumors and a low level strongly correlated with worse OS of patients. Our findings indicated that *SMP30* inhibited the development and progression of

NSCLC through inhibiting cell proliferation via inhibition of *HDAC4* expression. Therefore, targeting *SMP30* may be a novel therapeutic strategy for NSCLC.

There are some limitations to this study that should be noted. Firstly, SMP30 was reported to be decreased during senescence (6) while SMP30 expression showed no significant changes with aging in our cohort, which may be due to our small size of data and deserve more efforts. Secondly, in the two parts: involvement of *HDAC4* in *SMP30*-mediated NSCLC suppression and effect of pharmacological inhibition of *HDAC4*, we used the H1299 cell line nor the combination of A549 and H1299 cell lines, which may affect the reliability of our study and will be our subsequent work.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://dx.doi.org/10.21037/tlcr-21-982

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/tlcr-21-982). TH reports that he has received personal fees from Chugai Pharmaceutical outside the submitted work. XY serves as an unpaid editorial board member of *Translational Lung Cancer Research* from July 2021 to June 2023. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of the Fourth Military Medical University (No. TDLL-202110-03) and informed consents were not requested for this retrospective study. Animal experiments were performed under a project license (No. IACUC-20210609) granted by the Animal Ethics Committee of the Fourth Military Medical University, in compliance with the institutional guidelines for the care and use of animals.

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