High expression of 14-3-3σ indicates poor prognosis and progression of lung adenocarcinoma

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Abstract. Lung adenocarcinoma (LUAD) is one of the leading causes of cancer-related death worldwide. 14-3-3σ is an intracellular phosphoserine-binding protein that has been proposed to be involved in tumorigenesis. However, the biofunctional role of 14-3-3σ and its clinicopathological/prognostic significance in LUAD have remained elusive. In the present study, western blot and immunohistochemical analyses of cancer tissues/cells and the corresponding normal controls were performed to verify that 14-3-3σ was upregulated in LUAD. Univariate and multivariate logistic regression analysis indicated that high expression of 14-3-3σ predicted poor overall survival and progression-free survival of patients with LUAD. Furthermore, *in vivo* and *in vitro* experiments demonstrated that overexpression of 14-3-3σ markedly promoted cell proliferation, colony formation, anchorage-independent growth and tumor growth, whereas 14-3-3σ depletion produced the opposite effects. Of note, 14-3-3σ was identified as an independent prognostic factor for patients with LUAD. Collectively, the present results revealed that high expression of 14-3-3σ may serve as an independent biomarker, contributing to poor prognosis and progression of LUAD.

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Introduction

Lung cancer, including non-small cell lung cancer (NSCLC) and SCLC, is one of the major causes of cancer-related death worldwide (1). NSCLC accounts for 80-85% of lung cancers and lung adenocarcinoma (LUAD) is the most prevalent sub-type of NSCLC (2). Difficulties in early diagnosis, high metastatic potential and the occurrence of treatment resistance in advanced disease are the reasons for the poor survival of patients with LUAD. Numerous targeted treatment drugs, such as gefitinib, erlotinib, and crizotinib, have been widely used in clinical treatment (3,4). Although molecularly targeted therapies have produced promising clinical outcomes, only a minority of patients with LUAD are ideal candidates for targeted therapies (5). Furthermore, curing patients with LUAD remains challenging due to target genetic resistance mutations or off-target mechanisms of resistance (6-8). Therefore, it is necessary to investigate more effective molecular pathological diagnosis and markers for predicting the prognosis of patients with LUAD.

The 14-3-3 protein family consists of at least seven isoforms, namely β, γ, ε, η, ξ, σ and τ/θ, which are present in mammalian cells (9). 14-3-3 proteins are involved in a variety of cell signal transduction processes, such as cell proliferation, cell cycle regulation, apoptosis and malignant transformation (10,11). In the 14-3-3 protein family, 14-3-3σ is most associated with tumor occurrence and development (12). 14-3-3σ was originally identified as a human mammary epithelium marker 1 and as a tumor suppressor gene (13,14), which was determined to be reduced or lost in numerous types of solid tumor (15). Loss or reduction of 14-3-3σ by CpG methylation or p53 mutation contributes to the progression of different types of carcinomas, including early stages of tumor development (16-21). This suggests that the function of 14-3-3σ may help prevent the malignant transformation of epithelial cells. However, a growing body of evidence suggested that 14-3-3σ does not act as a tumor suppressor in cancers. Enhanced 14-3-3σ has been observed in pancreatic cancer (22), gastric carcinomas (23) and prostate cancer (24), and which is positively associated with aggressive tumor behavior and poor prognosis (15,25,26). Therefore, the biological role of 14-3-3σ in tumorigenesis and progression of human cancers varies according to the specific tumor type.

In lung cancer, 14-3-3 protein was elevated in tumor tissues compared to normal tissues (9,27,28). Upregulation of 14-3-3σ was also observed in LUAD (27) and NSCLC tissues (29). Increased expression of 14-3-3 may be due to the decreased DNA methylation of 14-3-3σ (30) and has been associated with cisplatin resistance in NSCLC cells (29). These results suggest that 14-3-3σ may be a promising biomarker for the molecular pathology, diagnosis and prognosis of patients with LUAD. However, only a small number of studies have reported on this possibility (29,31). Furthermore, the biological function of 14-3-3σ in tumorigenesis and progression of LUAD has remained to be fully elucidated. In the present study, the prognostic significance of 14-3-3σ expression in LUAD was assessed by a bioinformatics analysis of the clinical features and survival information of patients from a public database and in clinical patients from our hospital. Furthermore, *in vitro* and *in vivo* experiments were performed to investigate the effect of 14-3-3σ expression on cell proliferation, colony formation and anchorage-independent growth, as well as tumor growth, of LUAD. Overall, 14-3-3σ was indicated to be involved in tumor progression and high expression of 14-3-3σ was associated with poor prognosis of patients with LUAD.

Materials and methods

Cell lines and culture. The human bronchial epithelial immortalized cell line Beas-2B was purchased from the American Type Culture Collection and cultured in Bronchial Epithelial Basal Medium (BEBM; Lonza) with 10% fetal bovine serum (FBS; Corning, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). The lung cancer cell lines NCI-H1299, NCI-H358, A-549 and NCI-H23 were purchased from Zhong Qiao Xin Zhou Biotechnology Co., Ltd. NCI-H1299, NCI-H358 and NCI-H23 were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1% penicillin/streptomycin; A-549 was grown in F-12K medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were maintained in a humidified incubator with 5% CO_2 at 37˚C.

Clinical specimens. To detect the protein level of 14-3-3σ in LUAD tissue, a total of four LUAD tissue samples and non-tumor adjacent tissues samples were derived from biopsy samples and pathologically confirmed as LUAD from December 2020 to August 2021. Specific information on the patients is provided in Table I. The present study was performed according to the Declaration of Helsinki and approved by Ethics Committee of the People's Hospital of Beilun District [Ningbo, China; no. 2021-23(YS)]. Each patient provided written informed consent prior to surgery.

To detect the relationship between 14-3-3σ and prognosis of patients with LUAD, a total of 106 paraffin-embedded LUAD specimens from the archives of the Department of Pathology at People's Hospital of Beilun (Ningbo, China) were included in the present study. All patients had undergone surgery from February 2002 to October 2003. All patients were pathologically confirmed as LUAD. Furthermore, they had no chemotherapy, radiation therapy or history of surgery. The patients, 66 males (62.3%) and 40 females (37.7%), ranged in age from 39 to 77 years (mean, 59 years). All patients received radical surgery for primary tumor and lymph nodes, and postoperative cisplatin-based adjuvant chemotherapy. The patients did not receive any radiotherapy or chemotherapy prior to surgery. The histological type and stage were determined according to the classification for NSCLC by the World Health Organization and the International Union against Cancer Tumor-Nodes-Metastasis staging system (32). All patients provided written informed consent for tissue use prior to surgery and this study was approved by the Institute Research Ethics Committee of People's Hospital of Beilun District (Ningbo, China). All patients had follow-up records for over 6 years. After the completion of therapy, patients were observed every 3 months for the first 3 years and every 6 months thereafter. Overall survival (OS) was defined as the time from diagnosis to the date of death, or at the latest date if patients were still alive. Progression-free survival (PFS) was determined from the first day of treatment to the earliest signs of disease progression as identified by CT or MRI, or death from any cause.

Plasmids, siRNA and transfection. Plasmids of HA-14-3-3σ were obtained from Professor Jie Xu from The Second Affiliated Hospital of the Third Military Medical University (Army Medical University, Chongqing, China). The targeting plasmids were delivered into NCI-H1299 LUAD cells using the Lipofectamine® 2000 transfection reagent (Cat No: 11668019; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. siRNA designed to target 14-3-3σ (si-14-3-3σ) was purchased from Sangon Biotech Co., Ltd., including si-14-3-3σ-1# (5'-GGAUCCCACUCUUCUUGC A-3') and si-14-3-3σ-2# (5'-GACCAUGUUUCCUCUCAA U-3'). The sequence for the scrambled control siRNA was 5'-AUUGUAUGCGAUCGCAGACUU-3'. For transient transfection, control and si-14-3-3σ siRNA (#1 and #2) were mixed with Lipofectamine® 2000 and then added to the cell culture medium of A-549 LUAD cells according to the manufacturer's protocol. Finally, western blot analysis was used to assess the transfection efficiency.

ATP-lite cell proliferation assay. Cells were seeded into 96-well plates in triplicate at an initial density of $0.2x10^4$ cells/well. Cells were processed using the ATP-lite assay (cat. no. 6016739; Perkin-Elmer, Inc.) to assess cell proliferation at various time-points (24, 48, 72, 96 and 120 h) according to the manufacturer's protocol (33). Luminescence was read using a LD 400 plate reader (Beckman Coulter, Inc.). Each experiment was performed in triplicate.

Colony-formation assay. After transduction with targeting plasmids or small interfering (si)RNA, the stably transfected A-549 or NCI-H1299 cells were seeded into 6-cm dishes in triplicate at a density of $0.5x10^3$ cells/well and incubated at 37˚C for 14 days. The colonies were fixed with 4% paraformaldehyde, stained with crystal violet and then counted (33).

Soft agar assay. A standard soft agar assay was done as detailed previously (34). In brief, $5x10^3$ of parental cells or their transfectants (14-3-3σ-overexpressing or 14-3-3σ silenced, along with the vector controls), were suspended in

Table I. Clinicopathological data of the patients whose samples were used for western blot analysis.

ID	Sex	Age, years	Pathologic type	TNM stage
3397749	Male	51	Infiltrating adenocarcinoma of the left upper lung	T1cN0M0
2296185	Female	41	Infiltrating adenocarcinoma of the left upper lung	T1bN0M0
2295463	Male	43	Infiltrating adenocarcinoma of the left upper lung	T1bN0M0
2287639	Male	41	Infiltrating adenocarcinoma of the right upper lung	T1cN0M0

0.33% agar (cat. no. A5431; MilliporeSigma) containing 10% FCS in 6-cm dishes. After culture at 37˚C for 14 days, the cells were stained with *p*-iodonitrotetrazolium (cat. no. V900870; 1 mg/ml; MilliporeSigma) overnight at 37˚C and the colonies with cell numbers of more than eight were counted in five randomly selected areas from each dish.

Western blot analysis. Total protein was extracted from cells using RIPA lysis buffer (50 mM Tris-pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS; cat. no. P0013B; Beyotime Institute of Biotechnology) supplemented with phosphatase (cat. no. B15001; Bimake) and protease inhibitors (cat. no. B14001; Bimake). BCA assay kit (cat. no. P0011; Beyotime Institute of Biotechnology) was used to determine the protein concentration and 40 μg protein was loaded onto the gel per lane. The equal amounts of total protein were separated by SDS-PAGE (8-15%) and then transferred onto nitrocellulose (NC) membranes (EMD Millipore). The NC membranes were blocked with 5% nonfat milk (cat. no. P0216; Beyotime Institute of Biotechnology) for 1 h at room temperature, followed by incubation with primary antibodies overnight at 4˚C. The following primary antibodies were used: 14-3-3σ (cat. no. sc-100638; 1:1,000 dilution; Santa Cruz Biotechnology, Inc.), poly (ADP-ribose) polymerase (PARP; cat. no. 13371-1-AP; 1:1,000 dilution; ProteinTech Group, Inc.), Caspase-3 (cat. no. 66470-2-Ig; 1:1,000 dilution; ProteinTech Group, Inc.), GAPDH (cat. no. 60004-1-Ig; 1:1,000 dilution; ProteinTech Group, Inc.) and hemagglutinin (HA)-tag (cat. no. 51064-2-AP; 1:1,000 dilution; ProteinTech Group, Inc.). The NC membranes were then incubated with the following corresponding secondary antibodies: Goat anti-rabbit IgG (cat. no. SA00001-2; 1:2,000 dilution; ProteinTech Group, Inc.); goat anti-mouse IgG (cat. no. SA00001-1; 1:2,000 dilution; ProteinTech Group, Inc.); goat anti-rat IgG (cat. no. SA00001-15; 1:2,000 dilution; ProteinTech Group, Inc.) for 1 h at room temperature. ECL reagent (cat. no. 32106; Thermo Fisher Scientific, Inc.) was used to detect the signals.

Immunohistochemical (IHC) analysis and scoring. IHC analysis was used to study 14-3-3σ protein expression in human LUAD tissues and normal adjacent tissues as controls. The slides were dewaxed in xylene, rehydrated with an alcohol gradient, immersed in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity and subjected to antigen retrieval by pressure cooking in Tris/EDTA (pH=8.0) for 10 min. The slides were then incubated with 14-3-3σ primary antibody overnight at 4˚C. After incubation with the secondary antibody for 30 min at 37˚C, the specimens were stained with a DAB staining kit (cat. no. ab64238; Abcam). Finally, the sections were counterstained with hematoxylin, dehydrated and fixed. The brown granules in the cytoplasm were considered as positive staining for 14-3-3σ. For the evaluation of nuclear and cytoplasmic staining, the staining intensity was scored as follows (35): Negative (score 0); weak (score 1); moderate (score 2) and strong (score 3). The degree of staining was divided into four categories according to the percentage of stained cells in the field: Negative (score 0%), 0-25% (score 1), 26-75% (score 2), 56-100% (score 3) and 76-100% (score 4). The results reported as the expression score were the product of the above two scores. Protein expression was then evaluated by jointly assessing the intensity and extent of staining. IHC staining was assessed and scored by two independent researchers (JL and CDZ), who were blinded to the clinicopathological data.

Selection of cutoff score for 14-3-3σ expression. A receiver operating characteristic (ROC) curve analysis was performed for the selection of the cutoff value of the 14-3-3σ IHC score for OS and PFS, as described previously (36). In brief, the sensitivity and specificity of the outcome being studied for each score were plotted to generate an ROC curve. The score localized closest to the point of maximum sensitivity and specificity, the point on the curve (0.0, 1.0), was selected as the cutoff score leading to the highest number of tumors correctly classified as with or without the respective outcome. To facilitate the ROC curve analysis, the patients' outcome characteristics were dichotomized by survival [death vs. other outcome (censored, alive or death from other causes)].

Animal experiment. All experimental procedures using mice were performed in accordance with protocols approved by the Laboratory Animal Welfare and Ethics Committee of The Third People's Hospital of Yunnan Province (Kunming, China). A total of eight BALB/C nude mice (nu/nu; male; aged, 4-6 weeks; body weight, 18-20 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and each experimental group consisted of four mice. In brief, $5x10^5$ NCI-H1299 cells stably transfected with Vector or HA-14-3-3σ were mixed 1:1 with Matrigel® in a total volume of 0.2 ml and injected subcutaneously into the right flank of each mouse (37). Tumor size and mouse body weight were measured twice a week with a vernier caliper and precise analytical balance, respectively. On day 28 after tumor cell injection, mice were euthanized by carbon dioxide asphyxiation (30% vol/min) when certain tumors reached the size limits set by the guidelines 'Tumor Induction in Mice and Rats-UCSF Animal Care and Use' (38). The largest sizes of

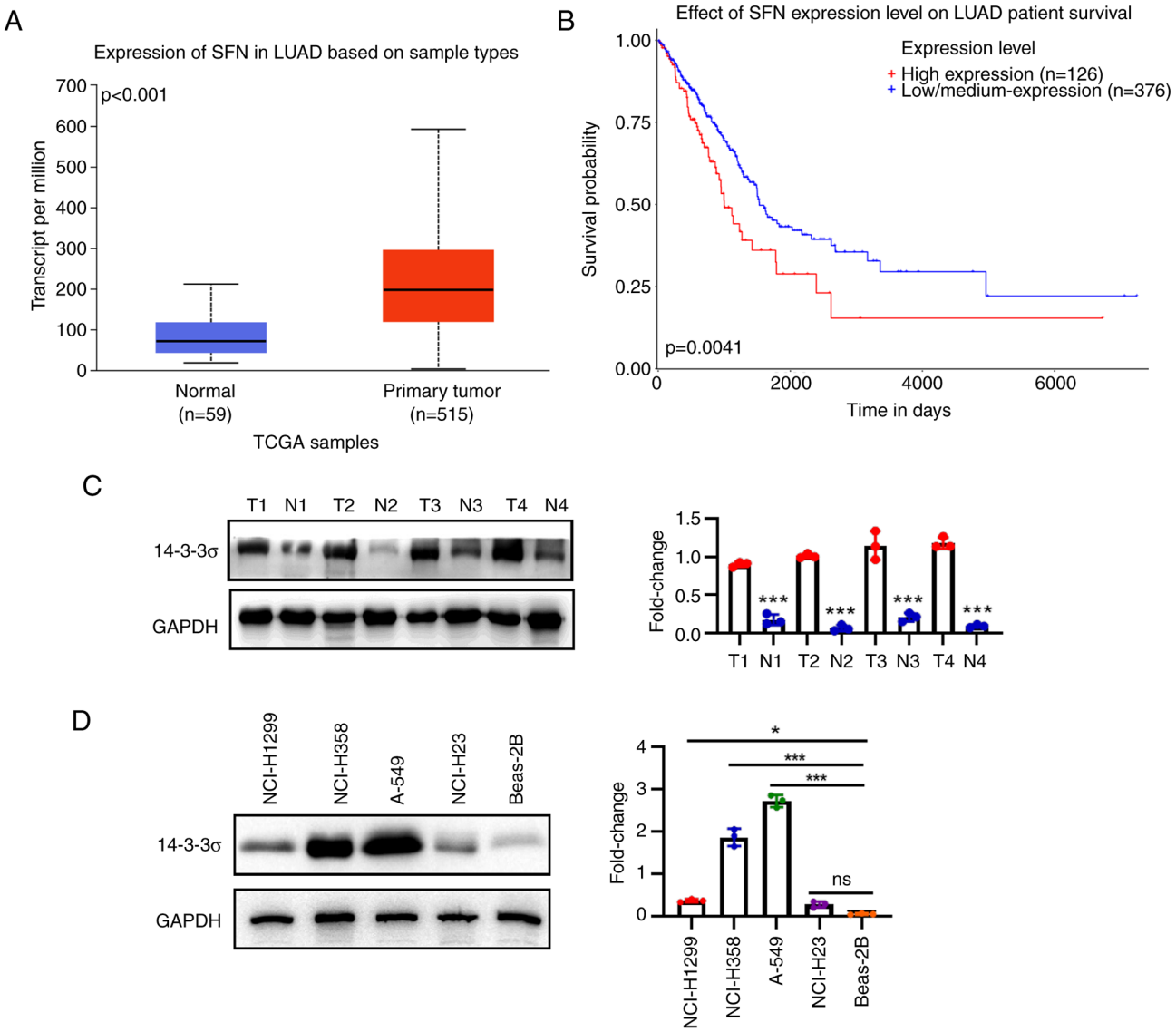

Figure 1. 14-3-3σ is upregulated in LUAD and predicts poor prognosis. (A) The mRNA level of 14-3-3σ has a higher expression value in LUAD. Bar graph displaying expression values of the 14-3-3σ in the UALCAN (http://ualcan.path.uab.edu.) with 95% confidence interval. (B) mRNA levels of 14-3-3σ in LUAD and association with patient survival: Kaplan-Meier survival analysis indicated that patients with higher expression of 14-3-3σ had unfavorable overall survival (log-rank test, P=0.0041). (C) Western blot analysis of 14-3-3σ protein expression in LUAD tissues (T) and normal adjacent tissues (N). (D) Basal levels of 14-3-3σ in human LUAD cell lines and Beas-2B cells. The protein levels were normalized to GAPDH. ns, not significant; *P<0.05, ***P<0.001; n=3 independent experiments; two-tailed paired Student's t-test or one-way ANOVA with Tukey's test. LUAD, lung adenocarcinoma; UALCAN, University of Alabama Cancer Database; N, normal tumor-adjacent tissue; T, tumor tissue.

tumor in the study were no bigger than 16 mm. At 10 min after the animals were euthanized by carbon dioxide asphyxiation, the absence of vital signs was checked to confirm that the animals had died. In this study, the nude mice did not suddenly lose 20-25% weight (cachexia); therefore, no other humane endpoint was used in this experiment except for tumor size limitation. Tumors were excised, weighed and frozen or fixed in 4% paraformaldehyde and paraffin-embedded for IHC analysis. The mean tumor volume (TV) was calculated according to the following equation: TV=(L x W^2)/2, where L is the length and W is the width of the tumor.

IHC staining of mouse tumors was performed as described above. In brief, after deparaffinization, rehydration, antigen retrieval and blocking, the tissue slides were incubated overnight at 4˚C with the indicated antibodies. The following primary antibodies were used: Ki-67 (1:1,000 dilution; cat. no. 9449; Cell Signaling Technology, Inc.), p21 (1:200 dilution; cat. no. 10355-1-AP; ProteinTech Group, Inc.), p27 (1:200 dilution; cat. no. 25614-1-AP; ProteinTech Group, Inc.) and cleaved-Caspase-3 (Asp175) (1:200 dilution; cat. no. 9664; Cell Signaling Technology, Inc.).

Statistical analysis. All data were expressed as the mean ± standard deviation and statistical charts were prepared using GraphPad Prism 7.0 software (GraphPad Software, Inc.) and SPSS 20.0 (IBM Corporation). Transcripts per million (TPM) values were used and survival analysis was applied for assessing differential expression of 14-3-3σ mRNA levels in UALCAN. For survival analysis, the χ^2 test or Fisher's exact test was used to assess the association

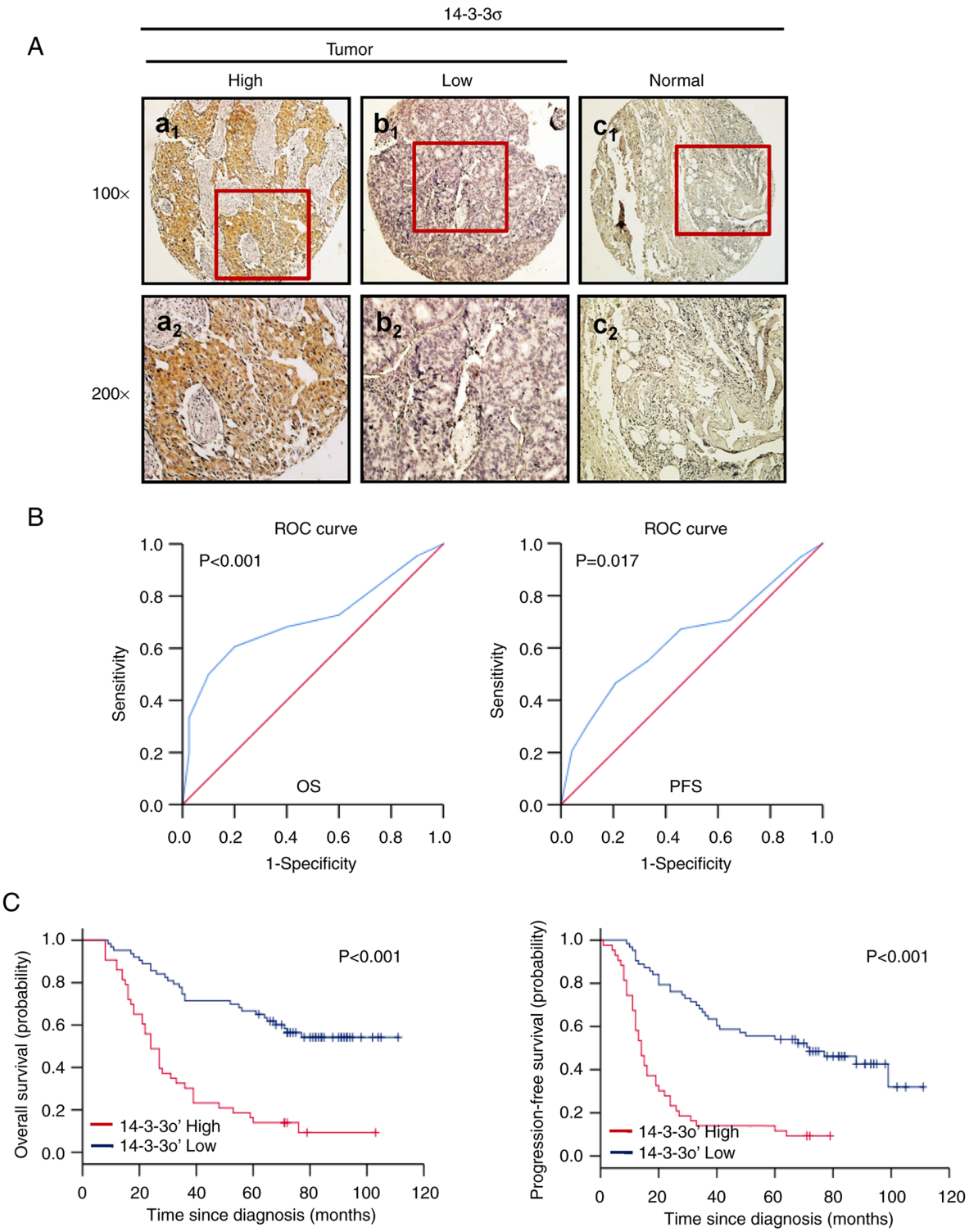

Figure 2. Protein levels of 14-3-3σ are significantly upregulated in LUAD and predict poor patient survival. (A) Representative IHC staining of 14-3-3σ in LUAD and normal adjacent tissues: (a_1) 14-3-3σ was strongly expressed in the cytoplasm of LUAD tissue; (b_1) 14-3-3σ was weakly expressed in the cytoplasm of LUAD tissue; (c_1) 14-3-3σ was negatively expressed in paired normal adjacent tissues from the same case (x100). (a_2, b_2 and c_2) provide magnified windows (x200) from a_1, b_1 and c_1, respectively. The images of the IHC staining are representative of all samples. (B) ROC curve analyses of the predictive value of 14-3-3σ in patients with LUAD. OS (left) or PFS (right) in the overall patients. For each IHC score, the sensitivity and specificity for the outcome being studied were plotted, thus generating an ROC curve. The optimal cutoff score for 14-3-3σ for OS and PFS was 4.1 and 3.8, respectively. (C) Kaplan-Meier survival analysis of the impact of 14-3-3σ expression on survival of patients with LUAD. High expression of 14-3-3σ was significantly associated with poor OS (left) and PFS (right) in the dataset. LUAD, lung adenocarcinoma; OS, overall survival; PFS, progression-free survival; ROC, receiver operating characteristic; IHC, immunohistochemistry.

between 14-3-3σ protein expression and clinicopathological variables. The multivariate Cox proportional hazards model was used to estimate the hazard ratios and 95% confidence intervals for patient outcomes. The associations between 14-3-3σ protein expression and OS or PFS were determined by Kaplan-Meier analysis. The log-rank test was performed

to assess differences in survival probability between patient subsets. Differences between two groups were analyzed using an unpaired Student's t-test, while the expression of 14-3-3σ in tumor tissues and matched normal tissues was compared using a paired Student's t-test. Comparisons of multiple groups were performed using one-way ANOVA followed by Dunnett's or Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Integrated analysis indicates that 14-3-3σ is an oncogene in LUAD. It was previously reported that 14-3-3σ expression is present in LUAD (27), but the biofunction role of 14-3-3σ and its clinicopathological/prognostic significance in LUAD have remained elusive. In the present study, 14-3-3σ mRNA expression was analyzed in LUAD using the data from UALCAN (http://ualcan.path.uab.edu.). Significant differences were identified between LUAD tissues and their normal counterparts (Fig. 1A). For the dataset from UALCAN, Kaplan-Meier survival analysis also indicated that patients with higher 14-3-3σ mRNA expression had poor overall survival (Fig. 1B). Next, the protein expression of 14-3-3σ was examined in LUAD and adjacent normal tissues by western blot analysis (Fig. 1C), and it was observed that 14-3-3σ was upregulated in LUAD tissues, whereas its expression was low in the normal adjacent tissue. Consistent with this result, the expression of 14-3-3σ in four established LUAD cell lines (A-549, NCI-H1299, NCI-H358 and NCI-H23) and a human bronchial epithelial cell line (Beas-2B) was analyzed by western blot analysis, indicating that 14-3-3σ was highly expressed in LUAD cell lines (Fig. 1D). Taken together, 14-3-3σ may function as an oncogene for LUAD.

14-3-3σ expression and survival: Univariate analysis. Consistent with the western blot results, IHC analysis revealed that 14-3-3σ was present in the cytoplasm and highly expressed in LUAD tissues (Fig. 2Aa_1 and a_2), while it was weakly expressed in normal paired lung tissues (Fig. 2Ac_1 and c_2). Next, ROC curve analysis was employed to determine a cutoff score for 14-3-3σ expression to predict survival (Fig. 2B). The cutoff score for 14-3-3σ to predict OS and PFS in patients with LUAD (n=106) was 3.8 (P<0.001) and 4.1 (P=0.017), respectively. Therefore, a 14-3-3σ expression score of 4.0 (>4.0 vs. ≤4.0) was selected as the unified cutoff point for survival analysis in patients with LUAD. The ROC-derived 14-3-3σ cutoff score for patients with LUAD was 4 and the cohort (n=106) was divided into a high (52/106, 49.1%) and a low (54/106, 50.9%) expression group. Kaplan-Meier analysis further indicated that high expression of 14-3-3σ predicted an inferior OS and PFS in patients with LUAD (P<0.001, Fig. 2C).

14-3-3σ expression in LUAD tissues and clinical features of patients. The association between 14-3-3σ expression and the clinical characteristics of patients with LUAD was then examined. As presented in Table II, high expression of 14-3-3σ in LUAD tissues was significantly positively associated with the tumor stage (P=0.001). In addition, high expression of 14-3-3σ was associated with tumor size (P=0.036) and recurrence (P<0.001, Table II). However, no association between 14-3-3σ and other patient characteristics was observed, including patient age, sex or node stage.

Table II. Association of 14-3-3σ expression with characteristics of patients with lung adenocarcinoma.

		14-3-3σ		
Variable	Total n	High	Low	P-value
Age, years				0.078
≥59.00	56	32	24	
<59.00	50	20	30	
Sex				0.803
Male	66	33	33	
Female	40	19	21	
Tumor size (mm)				0.036
<20	7	5	6	
20-50	53	18	31	
>50	46	29	17	
Tumor stage				0.001
T_1+T_2	52	17	35	
T_3+T_4	54	35	19	
Node stage				0.227
N_0+N_1	45	19	26	
N_2+N_3	61	33	28	
Recurrence				<0.001
Positive	61	46	15	
Negative	45	6	39	

Multivariate Cox regression analysis. To avoid the influence of various factors in the univariate analysis, the expression of 14-3-3σ as well as other parameters were examined in a multivariate Cox regression analysis (Table III). Among the patients with LUAD, 14-3-3σ was indeed determined to be a significant independent prognostic factor for OS (hazard ratio, 4.878; 95% CI, 2.895-8.219; P<0.001; Table III) and PFS (hazard ratio, 3.041; 95% CI, 1.878-4.923; P<0.001; Table III). Furthermore, the node stage was also identified as an independent prognostic parameter for OS (hazard ratio, 2.396; 95% CI, 1.115-5.148; P=0.025; Table III) and PFS (hazard ratio, 2.471; 95% CI, 1.168-5.228; P=0.018; Table III) in the patients with LUAD. However, other important prognostic factors, including age, sex, smoking, tumor stage and metastasis, were no significant independent prognostic factors for LUAD according to this analysis, implying that a larger cohort may be required in future studies.

14-3-3σ overexpression promotes growth of LUAD cells in vitro. The above results suggested that high expression of 14-3-3σ was associated with progression and poor prognosis of LUAD. Thus, the biological function of 14-3-3σ in LUAD was further investigated *in vitro*. First, 14-3-3σ plasmid was transfected into NCI-H1299 LUAD cells (Fig. 3A), as the native cell line expressed 14-3-3σ at a low level (Fig. 1D), and it was indicated that overexpression of 14-3-3σ promoted cell

Table III. Results of multivariate Cox proportional hazards analysis in lung adenocarcinoma.

	For death			For survival with relapse		
Variable	Hazard ratio	95% confidence interval	P-value	Hazard ratio	95% confidence interval	P-value
Age (≥59.00 vs. <59 years)	1.390	(0.888-2.176)	0.150	1.168	(0.749-1.822)	0.492
Sex (male vs. female)	0.624	(0.301-1.292)	0.204	0.869	(0.407-1.858)	0.718
Smoking (yes vs. no)	0.939	(0.468-1.883)	0.859	1.052	(0.510-2.168)	0.892
Tumor size, mm						
<20	1.024	(0.357-2.937)	0.965	0.781	(0.275-2.218)	0.642
20-50	1.287	(0.758-2.185)	0.351	1.153	(0.679-1.958)	0.598
>50	1 (Reference)	1		1 (Reference)	1	
Tumor stage ($T_4 + T_3$ vs. $T_2 + T_1$)	1.130	(0.667-1.915)	0.649	1.307	(0.777-2.201)	0.313
Nodal stage ($N_3 + N_2$ vs. $N_1 + N_0$)	2.396	(1.115-5.148)	0.025	2.471	(1.168-5.228)	0.018
14-3-3σ (high vs. low)	4.878	(2.895-8.219)	<0.001	3.041	(1.878-4.923)	<0.001

proliferation and survival, as measured by ATP-lite (Fig. 3B) and colony formation assays (Fig. 3C), respectively. Furthermore, the anchorage-independent growth of NCI-H1299 cells, measured by the soft agar assay, was increased upon 14-3-3σ overexpression (Fig. 3D). Thus, 14-3-3σ appeared to have growth-promoting activity in LUAD cells.

14-3-3σ silencing inhibits LUAD cell growth by inducing apoptosis. To further validate 14-3-3σ as an oncogene for LUAD, siRNA-based knockdown experiments were performed in A-549 LUAD cells, as the native cell line has high expression of 14-3-3σ (Fig. 1D). The results indicated that 14-3-3σ depletion suppressed cell proliferation and the colony-forming ability of the cells (Fig. 4A-C). The nature of survival inhibition upon 14-3-3σ depletion was demonstrated to be induction of apoptosis, as evidenced by an increase in PARP cleavage and Caspase-3 cleavage (Fig. 4D). Furthermore, the anchorage-independent growth of A-549 LUAD cells was inhibited by up to 60% on 14-3-3σ depletion (Fig. 4E). Thus, growth suppression upon 14-3-3σ depletion may be mediated via apoptosis induction.

Overexpression of 14-3-3σ promotes LUAD tumor growth in vivo. Next, the above *in vitro* findings were validated in a xenograft model. NCI-H1299 cells with stable overexpression of 14-3-3σ were inoculated into the right flank of nude mice and, as expected, overexpression of 14-3-3σ profoundly promoted tumor growth *in vivo*, increasing the tumor size and weight compared to the vector control (Fig. 5A-C). Likewise, IHC staining of tumor tissues also revealed that overexpression of 14-3-3σ markedly promoted tumor growth (increase of Ki-67 and decrease of p21 and p27) and inhibited apoptosis (decrease of cleaved-Caspase-3) (Fig. 5D). Collectively, the results of the *in vitro* cell experiments and *in vivo* xenograft models consistently demonstrated that overexpression of 14-3-3σ significantly promoted the growth of LUAD. At the same time, clinical data analysis also confirmed that 14-3-3σ high expression was positively correlated with poor prognosis of patients with LUAD.

Discussion

LUAD is one of the most common causes of global cancer-related mortalities worldwide (39). Although previous studies have indicated that numerous abnormally expressed genes in LUAD may help classify prognostic risks (40-42), there is still an urgent requirement for novel molecular markers to identify tumor progression and predict prognosis. The 14-3-3 family of proteins have received considerable attention in the past few years due to their involvement in cancers by regulating a variety of cellular processes (10,11). 14-3-3σ was originally identified as a tumor suppressor gene (13-15), which was upregulated by p53 upon DNA damage, and reported to sequester the essential mitosis initiation complex cdc2-cyclin B1 into the nucleus, thus preventing the initiation of mitosis (42-45). As a result, 14-3-3σ induces G2 arrest, allowing DNA damage repair to take place (18-20). However, there is increasing evidence that 14-3-3σ is an oncogene in cancers (22-24). Although 14-3-3σ expression was also observed in LUAD (27), its significance in the prognosis and function of patients with LUAD has remained largely elusive.

In the present study, certain findings regarding the important role of 14-3-3σ in LUAD were presented and supporting evidence for the following was provided: i) 14-3-3σ is strongly expressed in both LUAD cells and tissues, Similar to the findings in previous studies (27); ii) overexpression of 14-3-3σ significantly promotes cell growth and survival in both *in vitro* and *in vivo* LUAD cancer models, whereas 14-3-3σ depletion produces opposite effects; iii) 14-3-3σ depletion also induces cell apoptosis, as evidenced by the increase of PARP cleavage and Caspase-3 cleavage; iv) high expression of 14-3-3σ positively correlates with tumor size, tumor stage and recurrence, worse OS and PFS in LUAD; v) multivariate analyses further

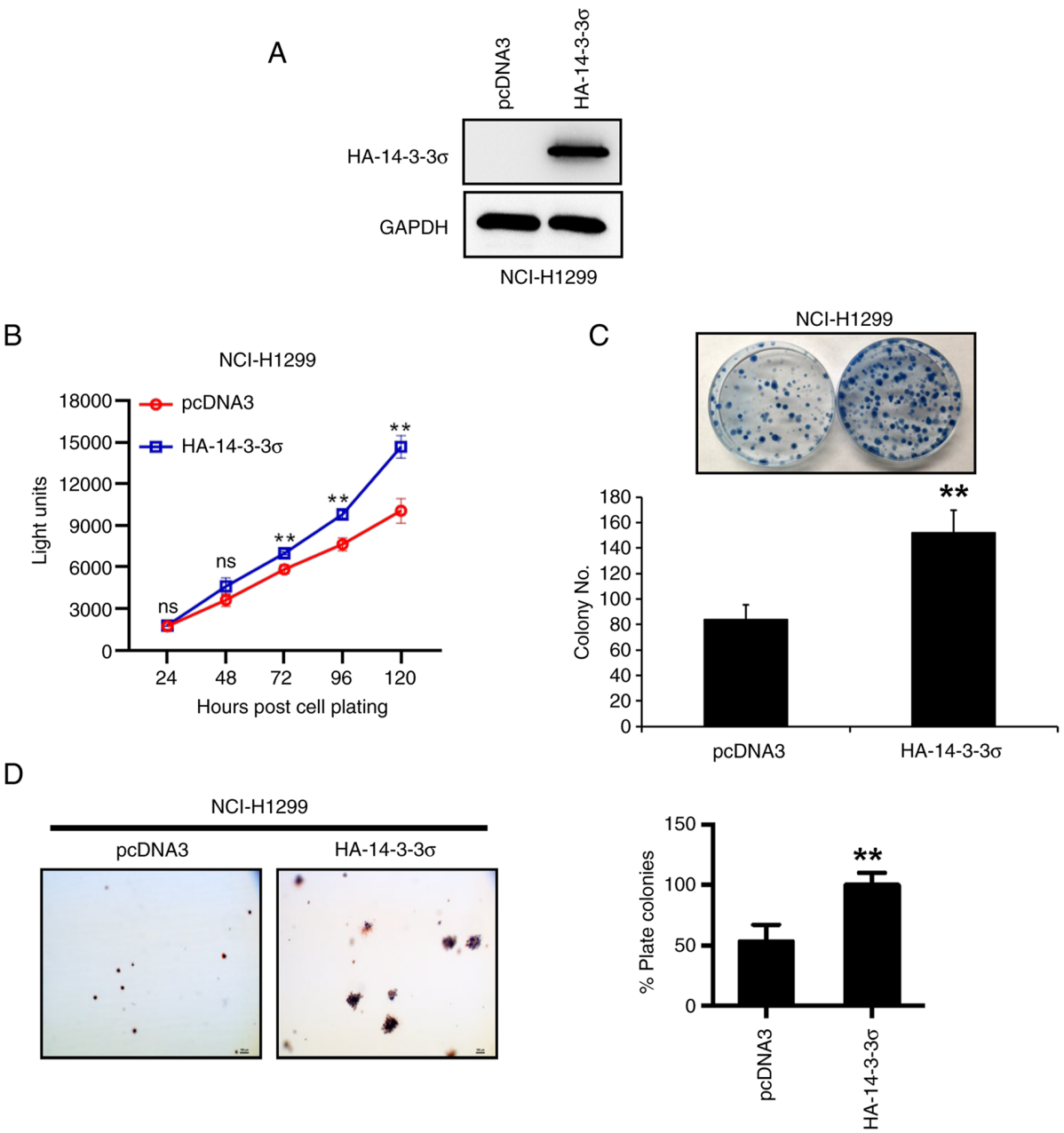

Figure 3. Overexpression of 14-3-3σ promotes the growth of LUAD cells. (A) 14-3-3σ was overexpressed in NCI-H1299 LUAD cells by targeted plasmids. (B) Overexpression 14-3-3σ significantly stimulated cell proliferation in NCI-H1299 cells. Cell viability was detected with an ATP-lite assay. (C) Overexpression 14-3-3σ significantly promoted cell colony formation by NCI-H1299 cells. (D) The anchorage-independent growth of NCI-H1299 cells in a soft agar assay was increased upon 14-3-3σ overexpression (scale bar, 100 μm). ns, no significance; **P<0.01; n=3 independent experiments; Student's two-tailed t-test, paired or unpaired. LUAD, lung adenocarcinoma.

revealed that 14-3-3σ is an independent prognostic biomarker for patients with LUAD. Taken together, the findings of the present study provided evidence that overexpression of 14-3-3σ may contribute to an increased degree of malignancy and unfavorable prognostic phenotype in LUAD.

Data reported by certain studies are completely contradictory regarding the prognostic impact of 14-3-3σ in different human cancers. Loss of expression of 14-3-3σ has been documented to be associated with poor prognosis in patients with breast cancer (46,47), endometrial (48) and ovarian cancers (49). However, consistent with findings in pancreatic cancer (50) and colorectal cancer (51), the results of the present study suggested that high expression of 14-3-3σ was positively associated with poor survival. The underlying mechanism(s) by which 14-3-3σ affected cancer prognosis may depend on the intrinsic properties of the tumor type. More recently, studies have indicated that overexpression of 14-3-3σ is associated with tumor progression in pancreatic cancer (26,50), which may further explain the present findings that higher 14-3-3σ expression was predominantly detected in more advanced tumor stages.

In conclusion, in the present study, 14-3-3σ was identified as an independent prognostic biomarker for OS and PFS in LUAD. The results suggested that 14-3-3σ has clinical value in predicting the prognosis of LUAD and identifying patients with LUAD at high risk of progression and recurrence.

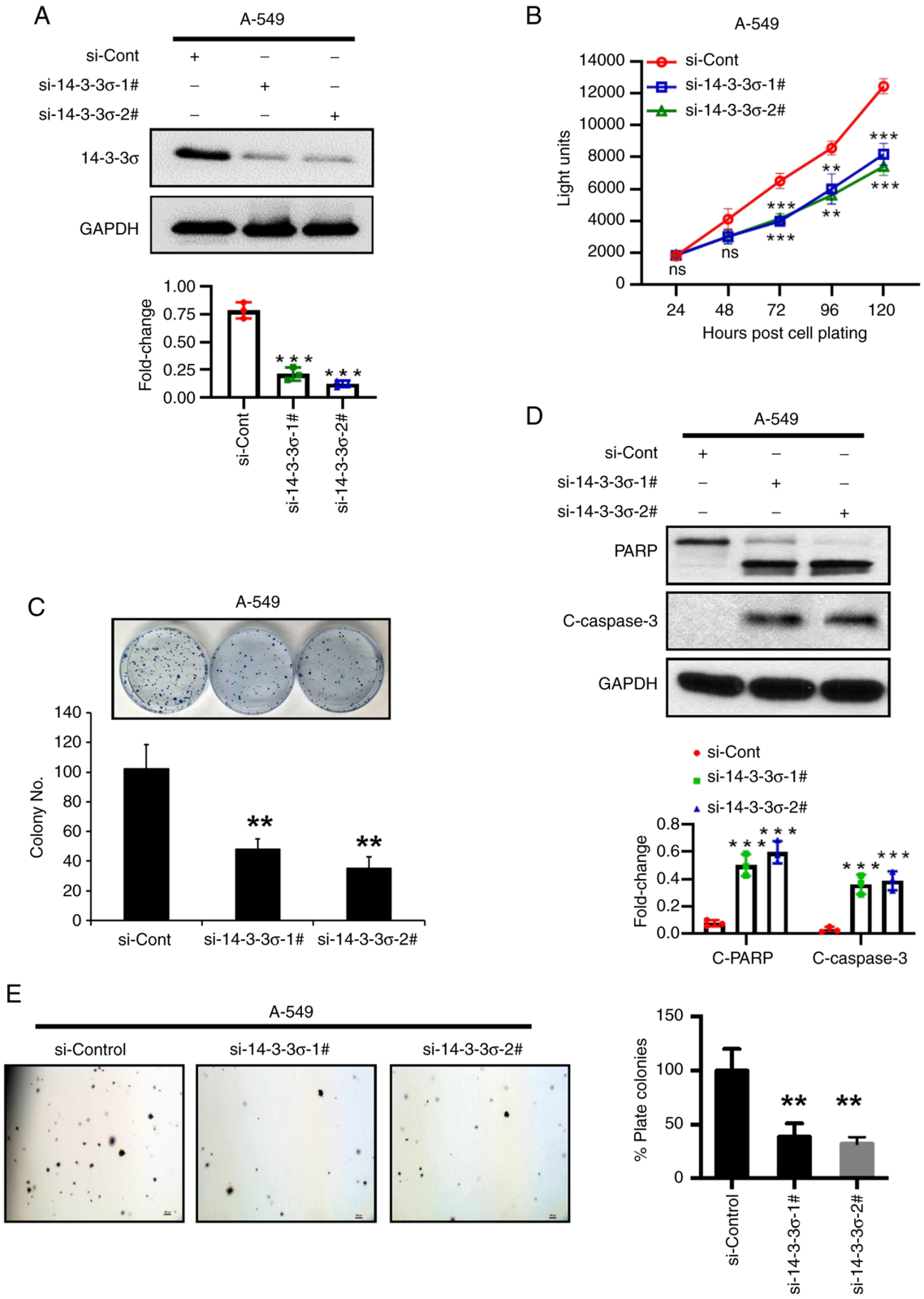

Figure 4. 14-3-3σ silencing inhibits LUAD cell growth by inducing apoptosis. (A) 14-3-3σ was knockdown in A-549 LUAD cells by targeted siRNA. (B) 14-3-3σ silencing significantly suppressed cell proliferation in A-549 cells. Cell viability was detected with an ATP-lite assay. (C) 14-3-3σ silencing significantly suppressed colony formation by A-549 cells. (D) 14-3-3σ depletion induced apoptosis by increasing PARP and C-Caspase-3. A-549 LUAD cells were transfected siRNAs targeting 14-3-3σ, followed by western blot analysis. (E) The anchorage-independent growth of A-549 cells sd measured by a soft agar assay was reduced upon 14-3-3σ silencing (scale bar, 100 μm). ns, no significance; **P<0.01, ***P<0.001; n=3 independent experiments; one-way ANOVA with Dunnett's test. LUAD, lung adenocarcinoma; siRNA, small interfering RNA; si-Cont, control siRNA; C-PARP, cleaved poly (ADP-ribose) polymerase.

Although the present study reported the effects of 14-3-3σ in LUAD, the regulatory mechanisms remain to be elucidated. In the future, further research will be performed to investigate how 14-3-3σ regulates cellular processes in LUAD.

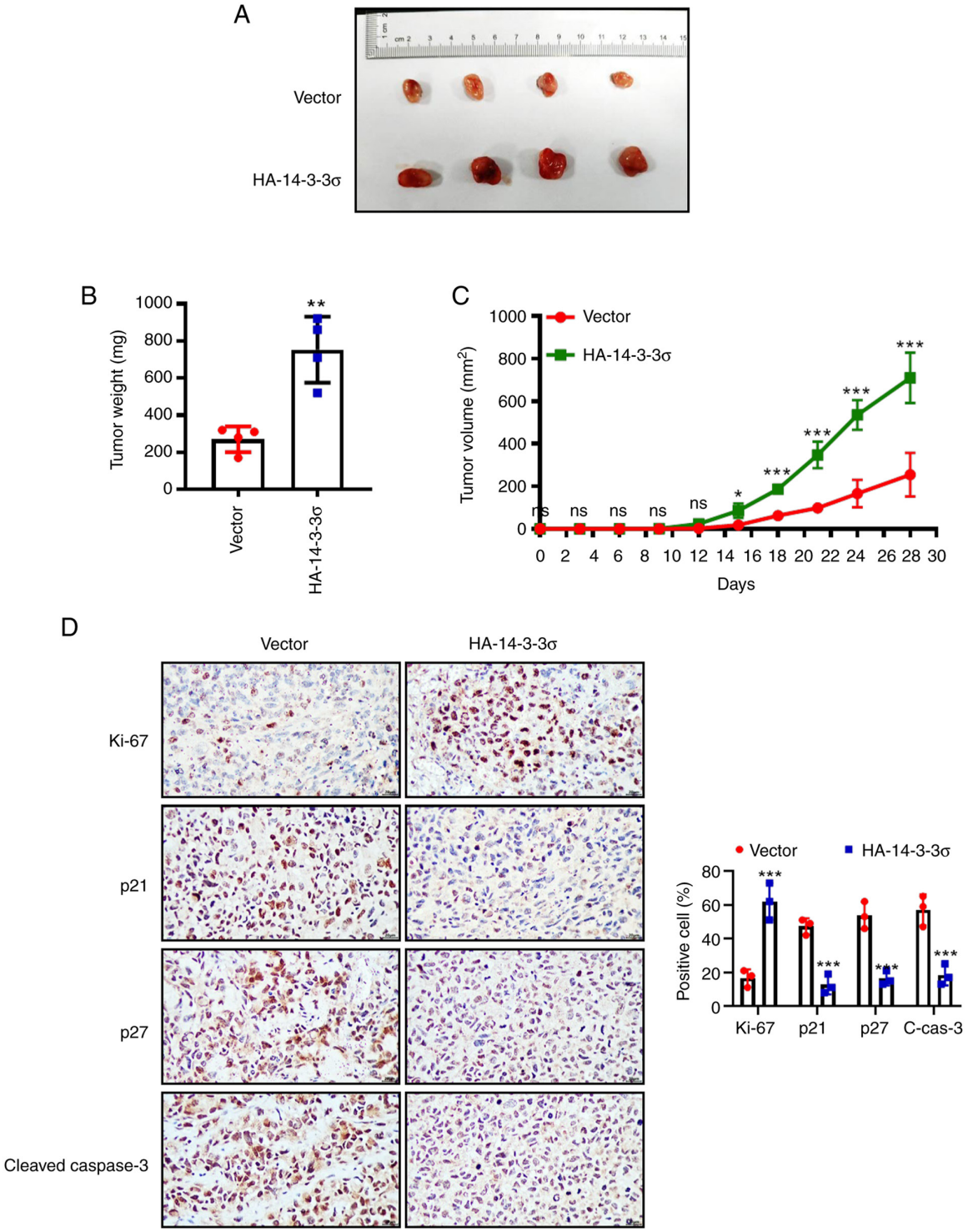

Figure 5. Overexpression of 14-3-3σ promotes LUAD tumor growth *in vivo*. (A-C) Overexpression of 14-3-3σ promoted tumor growth *in vivo* xenograft LUAD models. NCI-H1299 cells stably expressing HA-14-3-3σ or transfected with vector were subcutaneously inoculated in the right flank of nude mice. After the indicated times, the tumors were harvested, (A) images were captured and (B) their weight was determined. (C) The tumor growth was measured and plotted. (D) Immunohistochemical staining of xenograft tumor tissues. Tumor tissues from two groups of nude mice were fixed, sectioned and stained with indicated antibodies (scale bars, 100 μm). Values are expressed as the mean ± standard error of the mean (n=4). ns, no significance; *P<0.05; **P<0.01; ***P<0.001; paired or unpaired two-tailed Student's t-test. LUAD, lung adenocarcinoma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author's contributions

HFL and YBC were involved in the conception and design of the research. HFL edited and revised the manuscript. JFF, JL and CDZ performed the experiments, interpreted the results of the experiments and prepared the figures. YBC and JG drafted the manuscript. JG was also responsible for the collation of clinical data. HFL and YBC confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocol for the study involving human participants was reviewed and approved by the Ethics Committee of the People's Hospital of Beilun District of China [Ningbo, China; no. 2021-23(YS)]. The patients/participants provided their written informed consent to participate in this study. The animal experiment was approved by the Institutional Animal Care and Use Committee of the People's Hospital of Beilun District of China (Ningbo, China; no. NBU20220098).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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