Original Article

Upregulation of Neural Precursor Cell Expressed Developmentally Downregulated 4-1 is Associated with Poor Prognosis and Chemoresistance in Lung Adenocarcinoma

Ying-Hua Song[1], Cai-Qing Zhang[1], Fang-Fang Chen[1], Xiao-Yan Lin[2]

[1]Department of Respiratory Medicine, Shandong Qianfoshan Hospital, Shandong University, Jinan, Shandong 250014, China
[2]Department of Pathology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250021, China

Abstract

Background: The E3 ubiquitin ligase neural precursor cell expressed developmentally downregulated 4-1 (NEDD4-1) negatively regulates phosphatase and tensin homolog deleted on chromosome 10 (PTEN) protein levels through polyubiquitination and proteolysis, but its significance in lung cancer is still unclear. This study investigated the expression and the role of NEDD4-1 in tumor development and chemosensitivity of lung adenocarcinoma (ADC).

Methods: We retrospectively investigated the expression and significance of NEDD4-1, PTEN, and p-Akt proteins in 135 paired ADC and adjacent noncancerous tissue specimens using immunohistochemistry. Furthermore, we evaluated the relationship between NEDD4-1 expression and clinicopathologic characteristics and prognosis. The effects of small interfering RNA against NEDD4-1 on proliferation and chemosensitivity were examined in A549 cells *in vitro* using 3- (4,5-dimethylthiazol-2-yl) -5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)- 2H-tetrazolium method. The ability of migration and invasion of A549 cells was tested by transwell assay. Moreover, reverse-transcription quantitative polymerase chain reaction and Western blotting analyses were used to determine the expression of NEDD4-1, PTEN, phosphoinositide 3-kinase (PI3K)/Akt activity, and its downstream target proteins.

Results: NEDD4-1 protein was significantly upregulated in lung ADC tissues, whereas it was weak or negative in normal lung epithelial cells. The expression of NEDD4-1 in ADC (78.5%, 106/135) was significantly much higher than that in adjacent normal lung tissue (13.3%, 29/135, $P < 0.01$), and it was associated with lymph node metastasis, tumor-node-metastasis (TNM) stage, and chemotherapy resistance. PTEN expression was downregulated in lung ADC (60.7% vs. 100.0% in noncancerous specimens, $P = 0.007$), and was negatively correlated with lymph node metastasis, histological variants, clinical stage, chemoresistance. In addition, expression of p-Akt in ADC tissues (71.1% 96/135) was much higher than that in adjacent lung epithelial cells (6.7%, 9/135, $P < 0.01$). Kaplan-Meier and multivariate analysis demonstrated that expressions of NEDD4-1 and PTEN were both independent risk factors for survival in patients with lung ADC. NEDD4-1 knockdown *in vivo* decreased proliferation, migration, and invasion and improved chemosensitivity to cisplatin and paclitaxel in A549 cells. NEDD4-1 knockdown also significantly enhanced PTEN expression and inhibited p-Akt activity and downstream target proteins.

Conclusions: NEDD4-1 upregulation may contribute to the progression of lung ADC. NEDD4-1 may regulate the proliferation, invasion, migration, and chemoresistance of lung ADC cells through the PI3K/Akt pathway, suggesting that it may be regarded as a therapeutic target for the treatment of lung ADC.

Key words: Chemoresistance; Lung Adenocarcinoma; NEDD4-1; PTEN 10; Prognosis

Introduction

Lung cancer is the leading cause of malignancy mortality all over the world, and its incidence has been increasing rapidly in recent years.[1] Non-small cell lung cancer (NSCLC) accounts for 85% of all the pulmonary malignancies, and lung adenocarcinoma (ADC) accounts for 60% of NSCLC, which means ADC is the most common histologic type.[2]

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.4103/0366-6999.221262

Address for correspondence: Dr. Xiao-Yan Lin, Department of Pathology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250021, China
E-Mail: linxiaoyanyan@163.com

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

© 2017 Chinese Medical Journal ¦ Produced by Wolters Kluwer - Medknow

Received: 26-07-2017 **Edited by:** Yi Cui
How to cite this article: Song YH, Zhang CQ, Chen FF, Lin XY. Upregulation of Neural Precursor Cell Expressed Developmentally Downregulated 4-1 is Associated with Poor Prognosis and Chemoresistance in Lung Adenocarcinoma. Chin Med J 2018;131:16-24.

Available treatment strategies for ADC include conventional surgery, radiotherapy, immunotherapy, chemotherapy, and combinations of these therapies.[3] Recently, targeted therapies such as epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) have been identified as effective therapeutic strategies capable of improving clinical outcomes in patients with ADC. However, the prognosis of lung ADC is still poor, even in patients with Stage I tumor whose 5-year survival rate is also less than 30%. Especially in patients with advanced ADC, which is often resistant to conventional chemotherapies or TKIs.[4] Because most of the patients are diagnosed in the advanced stage, chemoresistance becomes the most obstacle to clinical therapy to lung ADC patients. Thus, it is essential to discover new biomarkers for predicting prognosis and chemosensitivity to determine new therapeutic targets for the treatment and drug-resistant reversal of lung ADC.

At present, loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) protein and activation of phosphoinositide 3-kinase (PI3K)/Akt signaling pathway are important mechanisms for promoting tumor cell malignant transformation and tolerance to chemotherapy in lung ADC. The PI3K/Akt pathway is one of the most important intracellular signaling pathways, which can be activated by various growth factors, and phospho-Akt (p-Akt) phosphorylation can activate many downstream target genes such as BAD, mTOR, NF-kB, GSK3 beta, and MDM2 so as to promote cell survival, proliferation, and malignant transformation. Tumor suppressor gene PTEN can dephosphorylate the second messenger phosphatidylinositol-3-phosphate (PIP3) to PIP2, antagonizing the activity of PI3K/Akt signal pathway. Loss of PTEN and activation of Akt have been certificated in many human malignant tumors. Several studies have indicated that loss rate of PTEN is about 30–50%, which is the most common early molecular events of lung ADC. Despite gene mutation and deletion, the post-translational modification is another important inactivation mechanism of PTEN. Ubiquitin modification process is the main post-translational regulation way of PTEN degradation in proteasome system, which maybe an important mechanism of loss expression of PTEN.

The E3 ubiquitin ligase neural precursor cell expressed developmentally down-regulated 4-1 (NEDD4-1) negatively regulates PTEN protein stability through polyubiquitination.[5] This regulation may activate the PI3K/Akt pathway, which functions in the regulation of proliferation and survival of cells. Negative regulation of PTEN by NEDD4-1-mediated polyubiquitination is reportedly involved in biologic and pathologic processes including axon branching, T-cell activation, keloid formation, and insulin-mediated glucose metabolism.[6] Retrospective clinical studies have found that NEDD4-1 overexpression plays a proto-oncogenic role in cancer development in human malignant tumors such as bladder, breast, colon, and prostate cancers.[7,8] Several studies have also indicated that loss of PTEN confers a chemoresistance and multidrug resistance (MDR) phenotype *in vitro*,[9] suggesting that NEDD4-1 may be a mechanism of chemoresistance in lung cancer. However, few studies have investigated the expression and clinical significance of NEDD4-1 in lung ADC.

In this study, we assessed the clinical significance of NEDD4-1 and its role in chemoresistance in lung ADC. We performed immunohistochemical analysis of 135 samples obtained from a consecutive series of patients with lung ADC to evaluate the relationship between NEDD4-1 expression and clinicopathologic features, chemosensitivity, and prognosis. Furthermore, we explored the *in vitro* effects of small-interfering RNA (siRNA)-mediated NEDD4-1 knockdown on cell proliferation, migration and invasion, drug sensitivity, and PI3K/Akt pathway in the human A549 cell line.

Methods

Ethical approval

This study was approved by the Research Ethics Committee of Shandong Medical University. Informed consent was obtained from all participants of this study.

Clinical specimens

We examined 135 paired paraffin-embedded specimens and adjacent tissues obtained from a series of patients who underwent complete pulmonary lobectomy at the Department of Thoracic Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, China, between January 2008 and December 2009. Major patient demographic and clinical characteristics, including tumor size, histologic type and lymph node metastasis, are summarized in Table 1. Tumors were staged according to the seventh edition of the American Joint Committee on Cancer tumor-node-metastasis (TNM) staging system.[10] A pathological diagnosis and histological type was confirmed according to the fourth edition of the WHO classification of tumors of the lung, pleura, thymus, and heart by two separate pathologists.[11] No patients received neoadjuvant chemotherapy or radiochemotherapy before operation. All patients received platinum-based chemotherapy. Curative efficacy was evaluated according to the WHO curative effect evaluation standards for solid tumors after two cycles of treatment. Chemotherapeutic effects were divided into stable disease and progressive disease (PD). All patients in this study had complete follow-up data for >5 years.

Immunohistochemistry and evaluation

Immunohistochemical staining was performed using an EnVision™ Detection Kit (Dako, Santa Clara, CA, USA). Briefly, tissue blocks were cut into sections 4 μm thick, deparaffinized, and rehydrated. The tissue sections were placed in boiling sodium citrate buffer (10 mmol/L sodium citrate, 0.05% Tween-20, pH 6.0) for 15 min for antigen retrieval. Next, the slides were blocked with

Table 1: Correlation between NEDD4-1 and PTEN expression and clinicopathologic factors in 135 consecutive patients with lung adenocarcinoma (*N*=135), *n* (%)

Factors	*n*	NEDD4-1		*P*	PTEN		*P*	P-Akt		*P*
		High	Low		High	Low		High	Low	
Sex										
Male	56	42 (75.0)	14 (25.0)	0.402	31 (55.4)	25 (44.6)	0.281	41 (73.2)	15 (26.8)	0.542
Female	79	64 (81.0)	15 (19.0)		51 (64.6)	28 (35.4)		54 (68.4)	25 (31.6)	
Age										
≥50 years	83	67 (80.7)	16 (19.3)	0.431	50 (60.2)	33 (39.8)	0.881	62 (74.7)	21 (25.3)	0.245
<50 years	52	39 (75.0)	13 (25.0)		32 (57.7)	20 (42.3)		34 (65.4)	18 (34.6)	
Smoking history										
Smoker	45	32 (71.1)	13 (28.9)	0.138	23 (51.1)	22 (48.9)	0.105	35 (77.8)	10 (22.2)	0.227
Nonsmoker	90	74 (82.2)	16 (17.8)		59 (65.6)	31 (34.4)		61 (67.8)	29 (32.2)	
Tumor size										
≤3 cm	92	68 (73.9)	24 (26.1)	0.057	60 (65.2)	32 (34.8)	0.119	63 (68.5)	29 (31.5)	0.324
>3 cm	43	38 (88.4)	5 (11.6)		22 (51.2)	21 (48.8)		33 (76.7)	10 (23.3)	
Lymph node status										
N0	98	72 (73.5)	26 (26.5)	0.020	65 (66.3)	33 (33.6)	0.030	67 (68.4)	31 (31.6)	0.252
N1–N2	37	34 (91.9)	3 (8.1)		17 (45.9)	20 (54.0)		29 (78.4)	8 (21.6)	
Histology type										
Lepidic	35	24 (62.9)	11 (37.1)	0.209	28 (80.0)	7 (20.0)	0.010	19 (54.3)	16 (45.7)	0.061
Acinar	46	35 (76.1)	11 (23.9)		31 (67.4)	15 (32.6)		37 (80.4)	9 (19.6)	
Papillary	23	20 (87.0)	3 (13.0)		13 (56.5)	10 (43.5)		18 (78.3)	5 (21.7)	
Solid	31	27 (93.5)	4 (6.5)		10 (32.3)	21 (67.7)		22 (71.0)	9 (29.0)	
Pleural invasion										
No	78	58 (74.4)	20 (25.6)	0.169	50 (69.2)	28 (30.8)	0.349	52 (66.7)	26 (33.3)	0.182
Yes	57	48 (84.2)	9 (15.8)		32 (49.1)	25 (50.9)		44 (77.2)	13 (22.8)	
Clinical stage										
II/III	62	58 (93.5)	4 (6.5)	0.001	31 (50.0)	31 (50.0)	0.019	55 (88.7)	7 (11.3)	0.001
I	73	48 (65.8)	25 (34.2)		51 (69.9)	22 (30.1)		41 (56.2)	32 (43.8)	
Chemotherapy										
Stable disease	93	66 (71.0)	27 (29.0)	0.001	66 (71.0)	27 (29.0)	0.001	61 (65.6)	32 (34.4)	0.035
Progressive disease	42	40 (95.2)	2 (4.8)		16 (38.1)	26 (61.9)		35 (83.3)	7 (16.7)	
EGFR status										
Mutation/deletion	68	55 (80.9)	13 (19.1)	0.500	35 (54.4)	33 (45.6)	0.026	46 (67.6)	22 (32.4)	0.371
Wild-type	67	51 (76.1)	16 (23.9)		47 (67.2)	20 (32.8)		50 (74.6)	17 (25.4)	
ALK										
Positive	8	6 (75.0)	2 (25.0)	0.803	3 (37.5)	5 (62.5)	0.165	5 (62.5)	3 (37.5)	0.579
Negative	127	100 (78.7)	27 (21.3)		79 (62.2)	48 (37.8)		91 (71.7)	36 (28.3)	

ALK: Anaplastic lymphoma kinase; EGFR: Epithelial growth factor receptor; NEDD4-1: Neural precursor cell expressed, developmentally down-regulated 4; PTEN: Phosphatase and tensin homolog deleted on chromosome 10; p-Akt: Phosphorylated-Akt.

3% H_2O_2-methanol followed by 10% normal goat serum for 10 min. After washing with phosphate-buffered saline (PBS), the tissues were incubated with the following primary antibodies overnight at 4°C: anti-NEDD4-1 (1:300; Upstate Biotechnology, NY, USA), anti-PTEN/MMAC1 (1:200; Maxim Biotechnology, Fuzhou, China), and anti-phosphorylated (p)-Akt (1:400; Santa Cruz Biotechnology, Dallas, USA). Then, the slides were incubated for 30 min with a horseradish peroxidase (HRP)-labeled polymer conjugated to a secondary antibody. In addition, nuclei were counterstained with hematoxylin and stained with 3,3'-diaminobenzidine (DAB). Negative controls were prepared by substituting PBS for the primary antibody. Positive slides provided by the companies were used as positive controls. Cytoplasmic staining was scored as NEDD4-1-positive or p-Akt staining, and nuclear staining was scored as PTEN-positive staining. We evaluated the staining results using a dual semi-quantitative scale that combined staining intensity and the percentage of positive cells. Staining intensity was scored as follows: 0, negative; 1, weak; 2, moderate; or 3, strong. The percentage of positive cells was scored as follows: 0, negative or ≤5%; 1, 6–25%; 2, 26–50%; 3, 51–75%; or 4, >75%. The final staining score was calculated by adding the scores for the percentage of positive cells and staining intensity and ranged from 0 to 7. A total score <4 indicated low expression and a score ≥4 indicated high expression. Two pathologists assessed the sections, and we used the average of their scores in the final analyses.

Cell lines and small-interfering RNA transient transfection

The human lung ADC A549 cell line was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin and streptomycin sulfate (Life Technologies, Carlsbad, CA, USA). The cells were cultured in a humidified incubator with 5% CO_2 at 37°C. A siRNA against NEDD4-1 (siNEDD4-1) and a negative control (NC) siRNA were designed and purchased from RiboBio Co., Ltd. (Guangzhou, China). The sequences of the siRNAs targeting human NEDD4-1 at nucleotide 556 were as follows: sense, *5'-CACC*GCAGAACAGGC TGAGGAATTA*TTCAAGAGA*TAATTCCTCAGCCTGT TCTGC*TTTTG-3'*; antisense, *5'-GATCCAAAAAA*G C A G A A C A G G C T G A G G A A T T A *T C T C T T G* AATAATTCCTCAGCCTG*TTCTGC-3'*. The 1 × 10^5 A549 cells were seeded in 12-well plates and incubated for 12 h before transfection. Then, the A549 cells were transfected with 100 nmol/L of siNEDD4-1 or the NC siRNA in 4 μl of Lipofectamine 3000 Transfection Reagent (Invitrogen Inc., USA). The cells were harvested after 48 h and subjected to next experiment.

Reverse-transcription quantitative polymerase chain reaction

Total RNA was obtained from the cells using an RNA Isolation Kit (Takara Bio Inc., Otsu, Japan). According to the manufacturer's protocol, RNA samples were reverse-transcribed to cDNA with random primers in a 20 μl final reaction volume. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to detect *NEDD4-1* mRNA levels. RT-qPCR was performed using SYBR® Green Real-Time PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan) in a final reaction volume of 25 μl using an iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primers used were as follows: *NEDD4-1* forward, 5'-GGTGGAGGTGTTCGGGCT-3', reverse: 5'-GCAAGGCCTATTCCGGCTA-3'. The amplification data were calculated using the ΔΔCq method, which was also used to calculate the relative mRNA expression. The relative target gene expression was calculated using the formula for $2^{-\Delta\Delta Cq}$, where ΔΔCq = target Cq − control Cq, and ΔΔCq = ΔCq target − ΔCq calibrator. The PCR products (10 μl) were separated by electrophoresis on a 2% agarose gel containing ethidium bromide and visualized using ultraviolet (UV) light to identify the specificity.

Western blotting analysis

Frozen tissue (100 mg) was collected from each sample and homogenized for 10 min in tissue protein lysis buffer containing 20 mmol/L Tris-HCl, 1 mmol/L ethylenediaminetetraacetic acid, 50 mmol/L NaCl, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, 1% Triton X-100, 1 mmol/L phenylmethanesulfonyl fluoride, and phosphatase inhibitor using a homogenizer, chilled on ice for 30 min, and centrifuged at 10,000 g for 5 min at 4°C. The resulting supernatant was collected and stored at −70°C. Proteins (60 μg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Following transfer to polyvinylidene fluoride blotting membranes, the blots were incubated in blocking buffer for 2 h at room temperature, followed by incubation with the anti-NEDD4-1 (Upstate Biotechnology, Inc.), anti-PTEN/MMAC1 (Maxim Biotechnology), and anti-p-Akt (Santa Cruz Biotechnology, Inc.,) primary antibodies. The blots were then incubated with an HRP-conjugated secondary antibody. The blots were treated with enhanced chemiluminescence reagents and exposed to film. Images were obtained using a transmission scanner, with β-actin protein as the internal control, and relative quantitative analysis was conducted based on the photodensity ratio of protein:β-actin. All experiments were performed in triplicate and one representative experiment is shown.

Cell viability and chemosensitivity assay

We performed 3-(4,5-dimethylthiazol-2-yl) -5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) -2H-tetrazolium (MTS) assays to detect the proliferation rate and chemosensitivity to paclitaxel and cisplatin of A549 cells. Briefly, the cells were seeded in 96-well culture plates at a density of 5 × 10^3 cells/well and cultured for 24, 48, 72, or 96 h. Cisplatin (5, 10, 25, 50, or 100 μmol/L) or paclitaxel (25, 50, 100, 200, or 400 nmol/L) in medium was then added to the cells for another 24 h. After washing with PBS, 80 μl of serum-free medium and 20 μl of MTS solution were added to each well, and the cell plates were incubated at 37°C for 1 h. A microplate reader (Bio-Rad Laboratories, Inc.,) was used to measure the absorbance at 490 nm. The survival rates of the cells were calculated as follows: cell viability = (A490 [anticancer drug +]/A 490 [anticancer drug−]) × 100%. Growth inhibition (%) = 100 − cell survival rate (%). A dose-response curve was plotted, and the half-maximal inhibitory concentrations (IC_{50}) were calculated from at least three independent experiments.

Invasion and migration assay

Cancer cell invasion/migration assays were performed using a 24-well transwell insert with 8 μm pores (Corning Inc., Corning, NY, USA) with/without Matrigel® matrix (BD Biosciences, San Jose, CA, USA). Forty-eight hours after transfection with siNEDD4-1 or the NC siRNA, 1 × 10^4 cells were resuspended in 200 μl of serum-free medium and seeded into the upper chambers. The lower chambers were filled with 600 μl of RPMI 1640 medium with 10% FBS. After incubation at 37°C for 24 h, cells on the upper surface were gently removed by wiping using a cotton-tipped swab, whereas migrated or invaded cells were attached to the bottom of the membrane. The cells that invaded the membrane to the bottom chamber were fixed in 4% paraformaldehyde for 10 min and then stained with 1% crystal violet for 15 min at room temperature. The number of invading or migrating cells was determined by counting five random fields for each insert and calculated as the mean number of cells per field.

Statistical analysis

All statistical analyses were performed using SPSS Statistics 19.0 (IBM Corp., Armonk, NY, USA) and Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). The Student's *t*-test was used to analyze differences between the groups. The Chi-squared and Fisher's exact tests were used to analyze the relationship between NEDD4-1 or PTEN expression and clinicopathologic parameters. The Kaplan-Meier method and log-rank test were used to plot survival curves and compute differences between curves. A Cox proportional-hazards model was constructed for the univariate and multivariate analyses of the prognostic value of NEDD4-1 expression. Data represent the means ± standard deviation (SD) of three independent experiments. Statistical significance was defined as $P < 0.05$.

Results

Neural precursor cell expressed, developmentally downregulated 4 is overexpressed in lung adenocarcinoma tissue

We summarized the pathologic characteristics of lung ADC tumors in Table 1. Positive immunohistochemical staining signal for NEDD4-1 was yellow and brown granules in the cytoplasm. It was strongly and diffuse positive in lung ADC tissues, but this staining was weak or negative in normal lung epithelium cells [Figure 1]. The expression of NEDD4-1 in ADC (78.5%, 106/135) was significantly much higher than that in adjacent normal lung tissue (13.3%, 29/135, $\chi^2 = 30.38$, $P < 0.01$). Positive immunocytochemical staining for PTEN was evident as yellow and brown granules in the nucleus. PTEN was expressed in 60.7% (82/135) tumor specimens and 100.0% (135/135) noncancerous specimens ($P = 0.007$). The expression of p-Akt in ADC was 71.1% (96/135), which was much higher than that in adjacent lung epithelial cells (6.7%, 9/135, $P < 0.01$). Tumor tissue overexpressing NEDD4-1 always exhibited the loss of expression of PTEN and upregulation of p-Akt. There was a significant negative correlation between NEDD4-1 and PTEN expression ($r = -0.632$, $P = 0.010$) and positive correlation between NEDD4-1 and p-Akt ($r = 0.679$, $P = 0.050$; Figure 2).

Relationship between neural precursor cell expressed, developmentally downregulated 4 expression and clinicopathologic characteristics of lung adenocarcinoma

The relationship between NEDD4-1 protein expression and patients' clinicopathologic features was shown in Table 1. NEDD4-1 expression was correlated with lymph node metastasis, clinical stage, and chemosensitivity ($P = 0.020$, 0.001, and 0.001, respectively), but was not correlated with other clinicopathologic features. The frequencies of lymph node metastasis and II/III TNM stage were significantly higher in the high-NEDD4-1 expression group than the low-NEDD4-1 expression group (lymph node metastasis: 32.1% vs. 10.3%, $P = 0.020$; II/III TNM stage: 54.7% vs. 13.8%, $P = 0.001$). Of the 135 patients, 66 exhibited a stable disease, but 42 exhibited PD after chemotherapy. The frequency of PD after conventional chemotherapy was significantly higher in the high-NEDD4-1 group than the low-NEDD4-1 group (37.7% vs. 6.9%; $P = 0.001$).

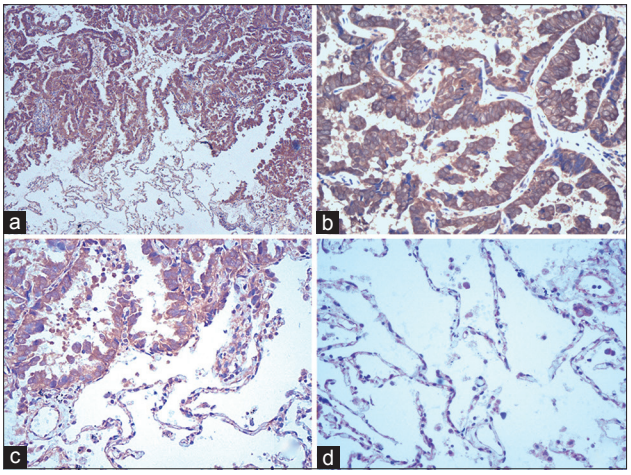

Figure 1: Expression of NEDD4-1 protein in lung ADC cells. (a) NEDD4-1 expression in ADC and adjacent normal lung tissues (immunohistochemistry; original magnification ×100). (b) Strong NEDD4-1 expression in ADC tissue (immunohistochemistry, original magnification ×400). (c) NEDD4-1 expression in ADC and adjacent normal lung tissues (immunohistochemistry; original magnification ×200). (d) Weak NEDD4-1 expression in adjacent normal lung tissue (immunohistochemistry; original magnification ×400). NEDD4-1: Neural precursor cell expressed, developmentally downregulated 4; ADC: Adenocarcinoma.

PTEN expression was negatively correlated with lymph node metastasis, histological type, clinical stage, and chemosensitivity ($P = 0.030$, 0.010, 0.019, and 0.001, respectively) and was also correlated with EGFR status ($P = 0.026$), but had no relationship with other clinicopathologic parameters. PTEN expression in the EGFR mutation group was lower than that in the wild-type EGFR group (67.2% vs. 54.4%; $P = 0.026$). P-Akt was correlated with clinical stage and chemotherapy response ($P = 0.010$ and 0.035, respectively) but without association with other characteristics.

Neural precursor cell expressed, developmentally downregulated 4 overexpression is predictive of poor prognosis

According to the last follow-up data obtained from all patients, 25 (18.5%) had disease-free survival, 26 (19.3%) survived with tumors, and 84 (62.2%) died of tumor recurrence or distant metastasis. The median overall survival was 35.0 months, and the mean overall survival was 37.8 ± 1.4 months (95% *CI*: 35.1–40.7 months). Survival curves analysis using Kaplan-Meier method and log-rank test showed that the overall 5-year survival of patients with high-NEDD4-1 expression was much worse than that of patients with low-NEDD4-1 expression (34.6 ± 1.6 months vs. 47.1 ± 2.8

months, $P = 0.001$). In contrast, patients with high PTEN expression showed a longer survival time, compared with patients with low PTEN expression (42.6 ± 1.7 months vs. 29.9 ± 2.0 months, $P = 0.001$). In a multivariate Cox regression analysis, TNM stage ($P = 0.023$, risk ratio [*RR*] =1.385, 95% *CI:* 0.521–2.621), chemotherapeutic efficacy ($P = 0.043$, $RR = 1.280$, 95% *CI:* 0.709–2.044), NEDD4-1 ($P = 0.023$, $RR = 2.002$, 95% *CI:* 1.002–4.896), and PTEN ($P = 0.031$, $RR = 1.899$, 95% *CI:* 0.877–3.926) showed a significant association with overall survival [Figure 3a and 3b].

Neural precursor cell expressed, developmentally downregulated 4 downregulation increases A549 cell proliferation and migration/invasion

To evaluate the effects of NEDD4-1 on A549 cells, we inhibited endogenous NEDD4-1 expression by transfecting

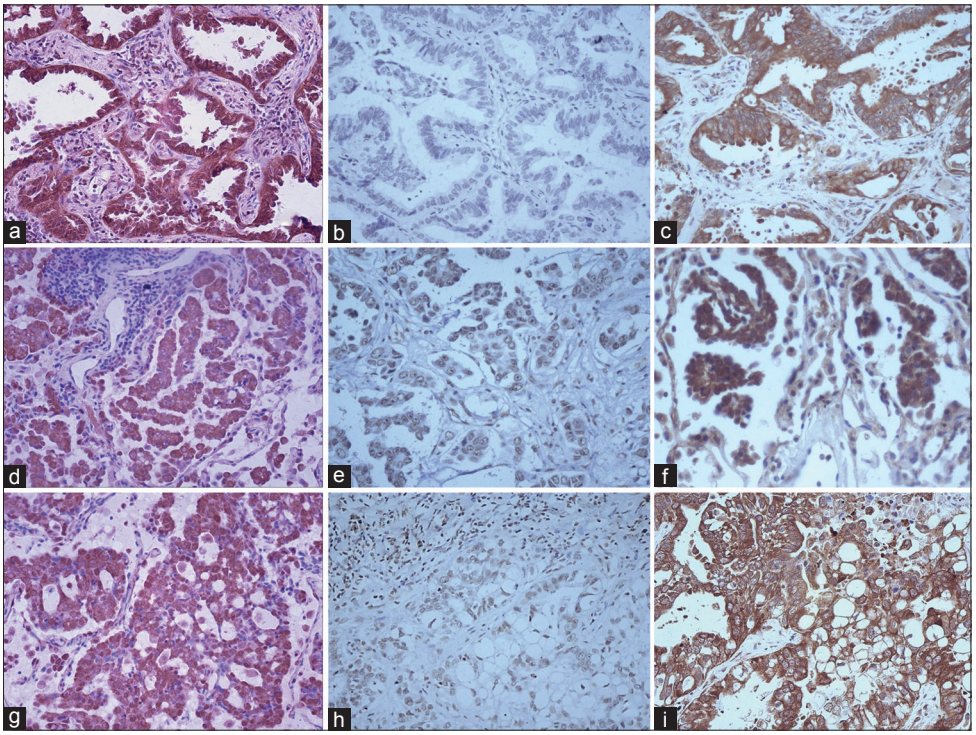

Figure 2: Expression of NEDD4-1 and PTEN protein and p-Akt in lung ADC tissue (immunohistochemistry; original magnification, ×400). (a) NEDD4-1 is strongly positive in acinar ADC. (b) PTEN is weakly positive in acinar ADC. (c) P-Akt is strongly positive in acinar ADC. (d) NEDD4-1 is strongly overexpressed in papillary ADC tumor tissue. (e) PTEN is moderately positive in papillary ADC tumor tissue. (f) P-Akt is strongly overexpressed in papillary ADC tumor tissue. (g) NEDD4-1 is strongly overexpressed in solid ADC tumors. (h) PTEN is weakly positive in solid ADC tumors. (i) P-Akt is strongly overexpressed in solid ADC tumors. NEDD4-1: Neural precursor cell expressed, developmentally downregulated 4; PTEN: Phosphatase and tensin homolog deleted on chromosome 10; p-Akt: Phosphorylated-Akt; ADC: Adenocarcinoma.

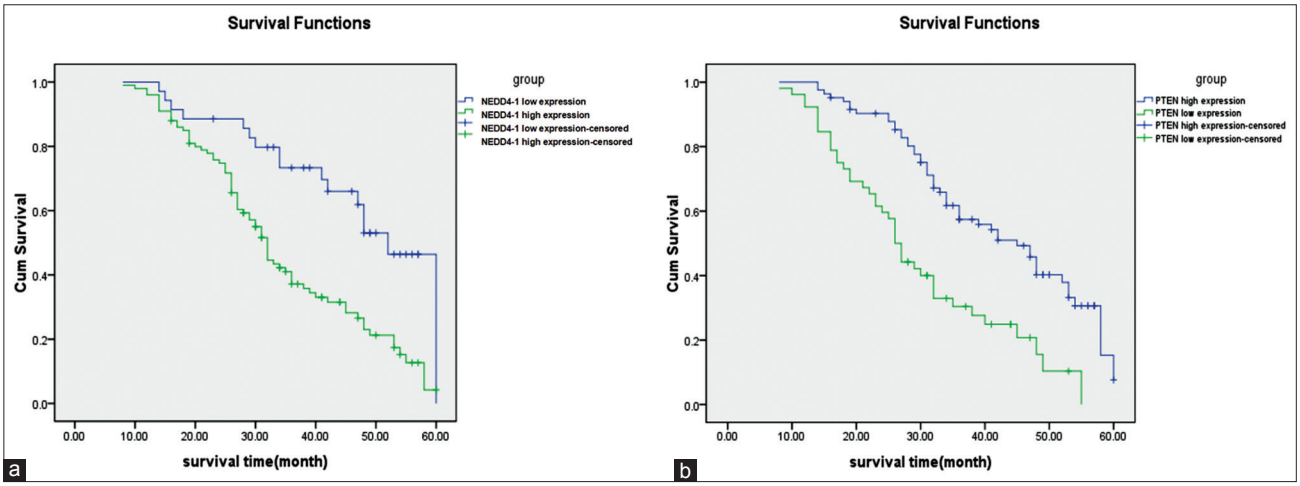

Figure 3: Kaplan-Meier curves with log-rank test for patients with low versus high-NEDD4-1 and PTEN expression in ADC tumors. (a) OS curves of patients with lung ADC according to NEDD4-1 immunostaining. (b) OS curves of patients with lung ADC according to PTEN immunostaining. *P* values were obtained using the log-rank test ($P < 0.05$). NEDD4-1: Neural precursor cell expressed, developmentally downregulated 4; PTEN: Phosphatase and tensin homolog deleted on chromosome 10; ADC: Adenocarcinoma; OS: Overall survival.

siNEDD4-1. As shown in Figure 4, after siNEDD4-1 transfection, *NEDD4-1* mRNA and protein expression were effectively reduced [Figure 4a and 4e]. Moreover, MTS assay demonstrated that NEDD4-1 knockdown suppressed cell proliferation compared with the NC siRNA in A549 cells in a concentration- and time-dependent manner [Figure 4b, $P < 0.05$]. A plot of cell growth showed cell viability was significantly decreased by 43.0 ± 5.0% in A549 cells transfected with siNEDD4-1 at 48 h [Figure 4b, all $P < 0.01$]. A549 cells were transfected with siNEDD4-1 to investigate its role in cell migration and invasion using transwell assay. After NEDD4-1 knockdown, A549 cells displayed significantly decreased migration and invasion compared with that of cells transfected with the NC siRNA [Figure 4c and 4d; $P < 0.05$].

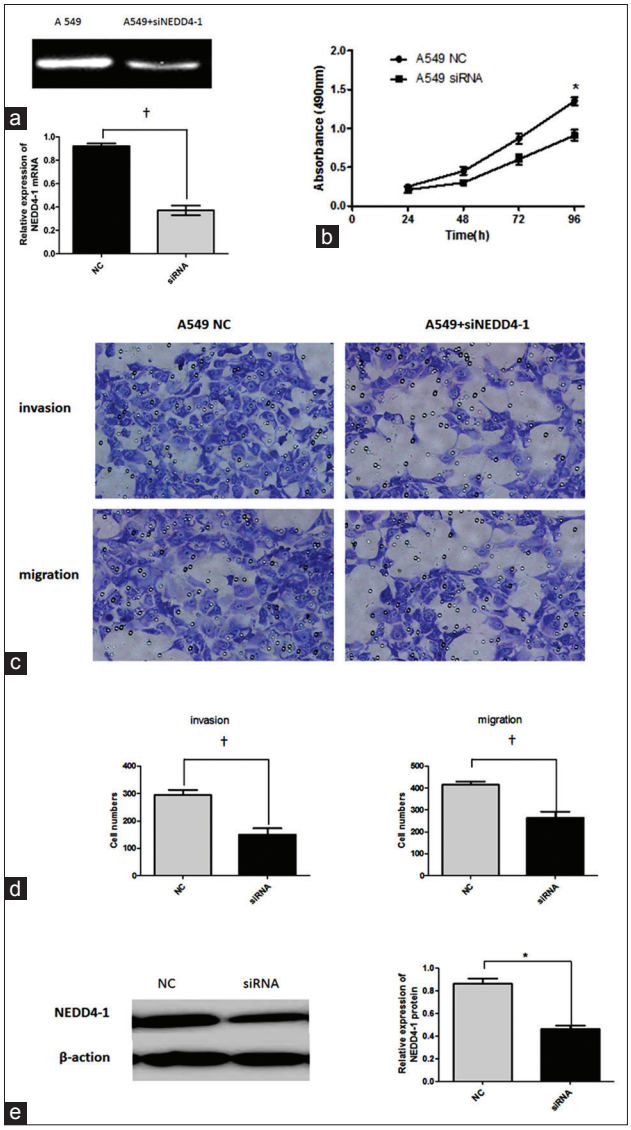

Figure 4: Knockdown of NEDD4-1 suppresses cell proliferation. (a) siNEDD4-1 transfection significantly reduced *NEDD4-1* mRNA expression in A549 cells. (b) siNEDD4-1 transfection significantly suppressed the proliferation rate of A549 cells. The invasion (c) and migration (d) abilities of A549 cells transfected with siNEDD4-1 were decreased in comparison with those of the negative control. (e) Western blotting showed that siNEDD4-1 transfection decreased expression of NEDD4-1. *$P < 0.05$, †$P < 0.01$. NC: Negative control; NEDD4-1: Neural precursor cell expressed, developmentally down-regulated 4; siNEDD4-1: Small-interfering RNA for NEDD4-1.

Neural precursor cell expressed, developmentally downregulated 4 knockdown increases A549 cell chemosensitivity by suppressing phosphoinositide 3-kinase/Akt signaling

In this study, NEDD4-1 knockdown improved the sensitivity to paclitaxel and cisplatin in a concentration-dependent manner in A549 cells. The IC_{50} values of cisplatin and paclitaxel in A549 cells were 21.3 ± 2.7 μmol/L and 484.2 ± 22.5 nmol/L, respectively. However, after siNEDD4-1 transfection, the IC_{50} values of cisplatin and paclitaxel were reduced to 8.5 ± 1.9 μmol/L and 239.6 ± 26.4 nmol/L, respectively ($P = 0.002$ and 0.001). Western blotting showed that, following inhibition of NEDD4-1 by siRNA transfection, PTEN protein was enhanced in A549 cells ($P < 0.05$; Figure 5a and 5b). To verify the relationship between NEDD4-1 expression and PI3K/Akt signaling, we monitored the expression of key biomarkers of PI3K/Akt activity after NEDD4-1 was silenced. Western blotting analysis revealed that NEDD4-1 knockdown significantly decreased the level of p-Akt ($P < 0.05$; Figure 5c), but the total Akt was not decreased. In addition, we also detect the expression changes of several downstream proteins of PI3K/Akt pathway. Specifically, NEDD4-1 knockdown significantly decreased the level of NF-kB, p-mTOR, and increased the level of BAD.

Discussion

E3 ubiquitin ligase NEDD4-1 plays a proto-oncogenic role in tumors through regulating the tumor suppressor PTEN expression by ubiquitination and causing its proteasomal degradation and/or nuclear translocation.[5,12] Recent studies have found that monoubiquitination of PTEN by NEDD4-1 promotes nuclear accumulation, and polyubiquitination of PTEN inducing its degradation in the cytoplasm, but the concrete mechanism is still unclear. Upregulation expression of NEDD4-1 is a characteristic finding in malignant tumors including breast, colon, bladder, and gastric cancer by immunohistochemical staining. However, limited data are available on NEDD4-1 expression in lung cancer specimens.[13] In this study, we evaluated the expression and clinical significance of NEDD4-1 in lung ADC, as well as PTEN and p-Akt using immunohistochemistry in a cohort of 135 clinical ADC samples. We also examined the relationships between expressions of NEDD4-1, PTEN, and p-Akt and patients' clinicopathologic features and prognoses.

In our study, NEDD4-1 overexpression was evident in ADC specimens compared with normal adjacent tissues. Using immunohistochemistry, several researchers have assessed that NEDD4-1 was upregulated in malignant tumors than in normal tissues including breast, colon, and bladder cancers.[7,8,14] In contrast, in our study,

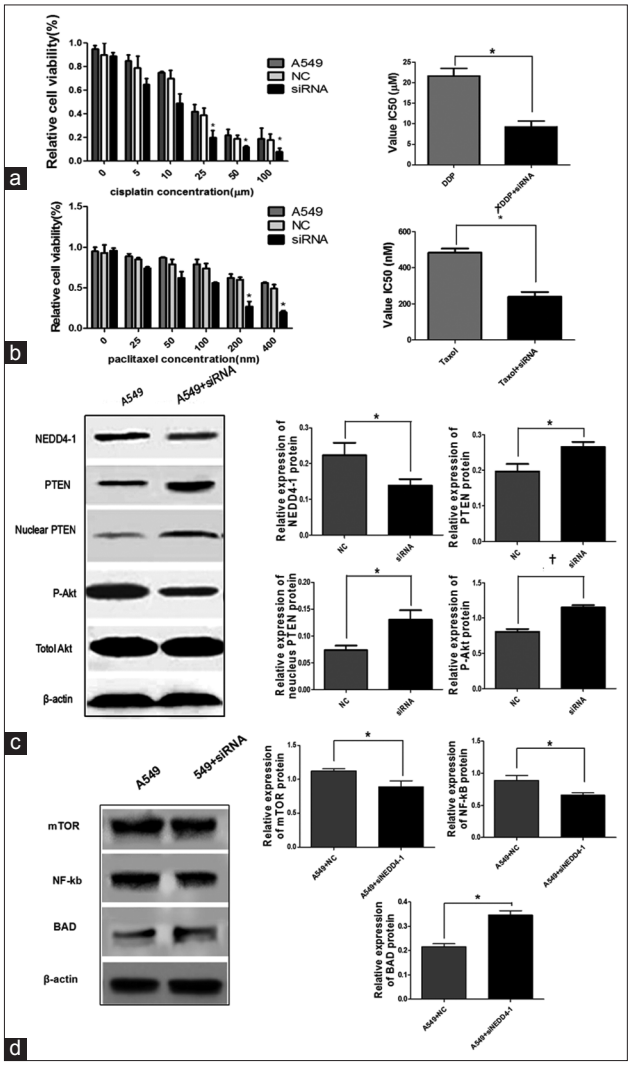

Figure 5: Knockdown of NEDD4-1 improved cell sensitivity to paclitaxel and cisplatin in a concentration-dependent manner. The half-maximal inhibitory concentrations in A549 cells of cisplatin (a) and paclitaxel (b) were reduced from 21.3 ± 2.7 μmol/L and 500 ± 20.0 nmol/L, to 9.5 ± 1.8 μmol/L and 220.5 ± 19.1 nmol/L, respectively (P = 0.002 and 0.001, respectively). (c) siNEDD4-1 reduced the expression of phosphorylated-Akt, mTOR, nuclear factor-κB and enhanced the expression of PTEN and BAD. *P < 0.05, †P < 0.01. NC: Negative control; NEDD4-1: Neural precursor cell expressed, developmentally downregulated 4; siNEDD4-1: Small-interfering RNA for NEDD4-1.

PTEN staining was absent in 63 (39.3%) patients, but all the adjacent lung tissues showed PTEN expression, which is consistent with the previous findings.[15,16] We also investigated the relationship between the clinicopathologic features of patients with ADC and NEDD4-1 expression, and found that high-NEDD4-1 expression was significantly associated with lymph node metastasis, TNM Stage II/III, and chemoresistance but was not correlated with other clinicopathologic features. The correlation between tumor stage/lymph node metastasis and NEDD4-1 expression suggests that its overexpression may occur in tumors with a more malignant biologic behavior and maybe predictor of poor prognosis. These results are consistent with those studies on breast and bladder tumors.[8] Furthermore, the frequency of PD after conventional chemotherapy was significantly higher in the high-NEDD4-1 group than in the low-NEDD4-1 group, which suggests that NEDD4-1 upregulation is closely related to chemoresistance.

As shown in this study, loss of PTEN expression and overexpression of p-Akt were both positively correlated with lymph node metastasis, clinical stage, and chemosensitivity; in addition, PTEN also related to EGFR status and histological variants. Shin *et al*.[17] examined PTEN expression and p-Akt in lung ADC and found that both of them were significantly associated with tumor stage, metastasis, and prognosis, which is consistent with our results. Other studies have shown that aberrations in downstream pathways (Akt mutations and loss of PTEN) are one mechanism of resistance to EGFR-TKIs.[18] Kaplan-Meier and Cox regression analyses showed that NEDD4-1 and PTEN expression was associated with poor prognosis in ADC and both were independent prognostic factors. Many studies have confirmed that NEDD4-1 is an independent risk factor for poor prognosis in human cancers including gastric and breast cancers.[8,14] Our results suggest that there was a significant negative correlation between NEDD4-1 and PTEN and positive correlation between NEDD4-1 and p-Akt. Therefore, NEDD4-1 maybe closely related to tumor cell behavior and prognosis in patients with lung ADC through downregulating PTEN expression and activating PI3K/Akt signaling. However, some studies report contradictory results. In contrast, study of Amodio *et al*.[7] did not show that the expression of NEDD4-1 and PTEN was associated with clinicopathological factors, and no correlation was found between tumor size, lymph node involvement, and staging. This discrepancy may be attributable to differences in the study populations, the region, and time of follow-up.

To evaluate NEDD4-1 role in lung ADC cells, we identified that silencing of NEDD4-1 by siRNA in A549 cells *in vitro*. MTS assay demonstrated that NEDD4-1 knockdown suppressed cell viability, and transwell assay displayed decreased migration and invasion compared with the NC siRNA in A549 cells. However, NEDD4-1 and its potential mechanisms of action in A549 cells are poorly understood. A recent study reported that the biologic functions of NEDD4-1 in inducing invasion, migration, and metastasis of cancer cells are achieved by its inhibition of GTP-Rap2a[19] and active ras genes.[20]

Recently, much attention has been focused on the involvement of PTEN–PI3K/Akt signaling in chemoresistance and MDR. Numerous studies have shown that loss of PTEN and upregulation of the p-Akt is considered related to MDR in some cancer cell lines.[22] Once overactivated, Akt phosphorylates multiple downstream effectors such as members of the caspase family, cell cycle proteins, and nuclear factor-κB, which collectively contribute to promoting cell proliferation, malignancy, invasion, and metastasis.[22] In a study involving 618 patients with

advanced ADC treated with platinum-based agents, PTEN polymorphisms were shown to contribute to survival and chemotherapy efficacy.[23] Despite being a regulator of PTEN and an oncogene, the role of NEDD4-1 in the chemosensitivity of lung ADC cells has not been described. NEDD4-1 was shown to induce chemosensitization through ubiquitination of SAG/RBX2.[24,25] In this study, Western blotting analysis revealed that NEDD4-1 knockdown significantly increased PTEN expression and decreased the level of p-Akt and improved sensitivity to paclitaxel and cisplatin in a concentration-dependent manner. Furthermore, NEDD4-1 knockdown significantly decreased the level of NF-kB and mTOR and increased the level of BAD, which are the downstream of PI3K/Akt pathway. The genes are key molecular of cell growth, invasion, and apoptosis and may contribute and explain the effects of NEDD4-1 in lung cancer cells. These findings suggest that NEDD4-1 may function in the chemoresistance of A549 cells through regulating the PTEN–PI3K/Akt pathway and downstream target proteins, but the mechanism needs further confirmation with more experiments. Inhibition of NEDD4-1 may provide a new molecular target for the treatment of lung ADC.

In conclusion, our results demonstrate that NEDD4-1 expression is overexpression in lung ADC and also associated with chemosensitivity and prognosis. NEDD4-1 plays a key role in disease progression that promotes cell proliferation, migration, and invasion through the inhibition of PTEN expression. NEDD4-1 may be regarded as a potential prognostic biomarker and therapeutic target for the treatment of lung ADC. The mechanism of NEDD4-1 in tumorigenesis and tumor progression warrants further investigation in more detailed studies.

Financial support and sponsorship

This work was supported by a grant from the National Natural Science Foundation of China (No. 81402185).

Conflicts of interest

There are no conflicts of interest.

References

1. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin 2014;64:9-29. doi: 10.3322/caac.21208.
2. Breathnach OS, Freidlin B, Conley B, Green MR, Johnson DH, Gandara DR, *et al.* Twenty-two years of phase III trials for patients with advanced non-small-cell lung cancer: Sobering results. J Clin Oncol 2001;19:1734-42. doi: 10.1200/jco.2001.19.6.1734.
3. Reck M, Heigener DF, Mok T, Soria JC, Rabe KF. Management of non-small-cell lung cancer: Recent developments. Lancet 2013;382:709-19. doi: 10.1016/S0140-6736(13)61502-0.
4. Fennell DA, Summers Y, Cadranel J, Benepal T, Christoph DC, Lal R, *et al.* Cisplatin in the modern era: The backbone of first-line chemotherapy for non-small cell lung cancer. Cancer Treat REV 2016;44:42-50. doi:10.1016/j.ctrv.2016.01.003.
5. Wang X, Trotman LC, Koppie T, Alimonti A, Chen Z, Gao Z, *et al.* NEDD4-1 is a proto-oncogenic ubiquitin ligase for PTEN. Cell 2007;128:129-39. doi: 10.1016/j.cell.2006.11.039.
6. Shi Y, Wang J, Chandarlapaty S, Cross J, Thompson C, Rosen N, *et al.* PTEN is a protein tyrosine phosphatase for IRS1. Nat Struct Mol Biol 2014;21:522-7. doi: 10.1038/nsmb.2828.
7. Chen Y, van de Vijver MJ, Hibshoosh H, Parsons R, Saal LH. PTEN and NEDD4 in human breast carcinoma. Pathol Oncol Res 2016;22:41-7. doi: 10.1007/s12253-015-9971-2.
8. Sun A, Yu G, Dou X, Yan X, Yang W, Lin Q, *et al.* Nedd4-1 is an exceptional prognostic biomarker for gastric cardia adenocarcinoma and functionally associated with metastasis. Mol Cancer 2014;13:248. doi: 10.1186/1476-4598-13-248.
9. McCubrey JA, Steelman LS, Abrams SL, Lee JT, Chang F, Bertrand FE, *et al.* Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. Adv Enzyme Regul 2006;46:249-79. doi: 10.1016/j.bbamcr.2006.10.001.
10. Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A, editors. AJCC Cancer Staging Manual. 7th ed. New York: Springer; 2010. p. 237-46.
11. Travis WD, Brambills E, Burke AP, Marx A, Nicholson AG. World Health Organization Classification of Tumours of the Lung, Pleura, Thymus and Heart. 4th ed. Lyon: IARC Press; 2015.
12. Trotman LC, Wang X, Alimonti A, Chen Z, Teruya-Feldstein J, Yang H, *et al.* Ubiquitination regulates PTEN nuclear import and tumor suppression. Cell 2007;128:141-56. doi: 10.1016/j.cell.2006.11.040.
13. Amodio N, Scrima M, Palaia L, Salman AN, Quintiero A, Franco R, *et al.* Oncogenic role of the E3 ubiquitin ligase NEDD4-1, a PTEN negative regulator, in non-small-cell lung carcinomas. Am J Pathol 2010;177:2622-34. doi: 10.2353/ajpath.2010.091075.
14. Yang Z, Yuan XG, Chen J, Lu NH. Is NEDD4-1 a negative regulator of phosphatase and tensin homolog in gastric carcinogenesis? World J Gastroenterol 2012;18:6345-8. doi: 10.3748/wjg.v18.i43.6345.
15. Guyard A, Danel C, Théou-Anton N, Debray MP, Gibault L, Mordant P, *et al.* Morphologic and molecular study of lung cancers associated with idiopathic pulmonary fibrosis and other pulmonary fibroses. Respir Res 2017;18:120. doi: 10.1186/s12931-017-0605-y.
16. Yanagawa N, Leduc C, Kohler D, Saieg MA, John T, Sykes J, *et al.* Loss of phosphatase and tensin homolog protein expression is an independent poor prognostic marker in lung adenocarcinoma. J Thorac Oncol 2012;7:1513-21. doi: 10.1097/JTO.0b013e3182641d4f.
17. Shin E, Choi CM, Kim HR, Jang SJ, Park YS. Immunohistochemical characterization of the mTOR pathway in stage-I non-small-cell lung carcinoma. Lung Cancer 2015;89:13-8. doi: 10.1016/j.lungcan.2015.04.003.
18. Sun H, Ma H, Wang J, Xia L, Zhu G, Wang Z, *et al.* Phosphatase and tensin homolog deleted on chromosome 10 degradation induced by NEDD4 promotes acquired erlotinib resistance in non-small-cell lung cancer. Tumour Biol 2017;39:1010428317709639. doi: 10.1177/1010428317709639.
19. Wang L, Zhu B, Wang S, Wu Y, Zhan W, Xie S, *et al.* Regulation of glioma migration and invasion via modification of Rap2a activity by the ubiquitin ligase Nedd4-1. Oncol Rep 2017;37:2565-74. doi: 10.3892/or.2017.5572.
20. Zhang H, Nie W, Zhang X, Zhang G, Li Z, Wu H, *et al.* NEDD4-1 regulates migration and invasion of glioma cells through CNrasGEF ubiquitination *in vitro*. PLoS One 2013;8:e82789. doi: 10.1371/journal.pone.0082789.
21. Li X, Zhang R, Liu Z, Li S, Xu H. The genetic variants in the PTEN/PI3K/AKT pathway predict susceptibility and CE(A)F chemotherapy response to breast cancer and clinical outcomes. Oncotarget 2017;8:20252-65. doi: 10.18632/oncotarget.15690.
22. Lim HJ, Crowe P, Yang JL. Current clinical regulation of PI3K/PTEN/Akt/mTOR signalling in treatent of human cancer. J Cancer Res Clin Oncol 2015;141:671-89. doi:10.1007/s00432-014-1803-3.
23. Yang Y, Xu W, Liu D, Ding X, Su B, Sun Y, *et al.* PTEN polymorphisms contribute to clinical outcomes of advanced lung adenocarcinoma patients treated with platinum-based chemotherapy. Tumour Biol 2016;37:7785-96. doi: 10.1007/s13277-015-4651-0.
24. Pérez-Ramírez C, Cañadas-Garre M, Molina MÁ, Robles AI, Faus-Dáder MJ, Calleja-Hernández MÁ, *et al.* Contribution of genetic factors to platinum-based chemotherapy sensitivity and prognosis of non-small cell lung cancer. Mutat Res 2017;771:32-58. doi: 10.1016/j.mrrev.2016.11.003.
25. Zhou W, Xu J, Zhao Y, Sun Y. SAG/RBX2 is a novel substrate of NEDD4-1 E3 ubiquitin ligase and mediates NEDD4-1 induced chemosensitization. Oncotarget 2014;5:6746-55. doi: 10.18632/oncotarget.2246.