

# Expression of CDCA7 Is a Prognostic Biomarker and Potential Therapeutic Target in Non-small Cell Lung Cancer

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# Abstract

## Background

Cell Division Cycle Associated 7 (CDCA7) was first identified as a direct target gene of c-Myc and dysregulated in various types of human cancer. However, it has limited implication in non-small Cell Lung Cancer (NSCLC). We aimed to explore the critical role of CDCA7 in NSCLC.

## Methods

In this study, we identified CDCA7 upregulation and association with the prognosis of NSCLC by integrating analysis of 3 Gene Expression Omnibus (GEO) databases. Real-time PCR and immunohistochemistry (IHC) were used to determine collected clinical NSCLC samples. Chi-square test was used to examine possible correlations between CDCA7 expression and clinicopathological factors. Univariate and multivariate Cox proportional hazards regression analysis were performed to determine whether CDCA7 is an independent risk factor for overall survival (OS). The effect of CDCA7 expression on proliferation, cell cycle and apoptosis ability of NSCLC cells was detected by cell counting kit-8 (CCK-8) and flow cytometry. CDCA7 stably knocking down cell line was established and Western blotting assay was applied to measure relevant protein expression. Xenograft models were used to examine the role of CDCA7 on tumorigenicity of NSCLC cells.

## Results

Analysis of clinical samples confirmed the CDCA7 high expression in tumor tissues compared with adjacent non-tumor tissues and predicted shorter OS time. COX proportional risk model analysis showed that the expression levels of CDCA7 was independent prognostic factors. We observed that CDCA7 silencing efficiently affect the proliferation, apoptosis and cycle distribution of NSCLC cells *in vitro*. Further results demonstrated that the expression of CDCA7 in A549/DDP cells was significantly higher than that in A549 cells, CDCA7 silencing efficiently down regulation cisplatin sensitivity in A549/DDP cells. Importantly, the depletion of CDCA7 strongly reduced the tumorigenicity of NSCLC cells *in vivo*. Furthermore, depletion of CDCA7 expression markedly affected the expression of cell division protein kinase 6 (CDK6) and caspase7 both *in vitro and in vivo*. In vitro study, we showed that CDCA7 silencing promotes A549 apoptosis via extracellular regulated protein kinases (ERK) pathway.

## Conclusion

Highly expressed CDCA7 plays a crucial role in the pathogenesis of NSCLC and might be a potential prognostic factor and therapeutic target in NSCLC.

## Background

Lung cancer is the most common cause of cancer-related deaths globally [1-2]. NSCLC accounts for about 80-85% of lung cancers. Surgery is the most effective treatment for early NSCLC, but more than

half of lung cancer patients are being diagnosed at a distant stage and the 5-year OS rate is only 18% [3]. Kinase inhibitors [4] and immunotherapies [5-6] has demonstrate great clinical efficacy in patient subgroups that exhibit specific molecular pattern if used at earlier stages of the disease. Therefore, there is an urgent need to identify novel biomarkers that could be used in early diagnosis and better treatment of NSCLC.

In 1997, Lewis et al [7] identified a novel c-Myc-responsive gene presently known as CDCA7, participates in c-Myc-mediated neoplastic transformation [8]. The fact that CDCA7 is highly expressed in the blast or terminal phases of chronic myeloid leukemia (CML), and *in vivo* tumorigenic studies in transgenic mice all supports the role for CDCA7 in tumorigenesis [9]. Cheng et al. found that high expression of CDCA7 might act as an oncogene possibly through the deregulation of cell proliferation and apoptosis in Esophageal Squamous Cell Carcinoma (ESCC) [10]. Overexpression of CDCA7 predicts poor prognosis and induces EZH2-mediated progression of triple-negative breast cancer [11]. Further study shown that CDCA7 is critical for invasion and migration of lymphoma cells [12]. However, it has limited implication in NSCLC, the mechanism of how CDCA7 is involved in tumorigenesis remains largely unknown.

Herein, we reported the high expression levels of CDCA7 in NSCLC compared to the adjacent tissues, and the high expression of CDCA7 was an independent risk factor for OS. We demonstrated that the expression of CDCA7 in A549/DDP cells was significantly higher than that in A549 cells, CDCA7 silencing efficiently down regulation cisplatin sensitivity in A549/DDP cells. Depletion of CDCA7 expression markedly reduced the proliferation the tumorigenicity of NSCLC cells via regulating the expression of CDK6 and caspase7 *in vitro* and *in vivo*. Our study uncovers a possible oncogenic role of CDCA7 in NSCLC progression, and may provide more effective therapeutic strategies against NSCLC.

## Materials And Methods

# Screening of novel biomarkers using GEO databases

3 GEO datasets were used to identify the mRNA profiles significantly upregulated in NSCLC using the online tool GEO2R, while  $P < 0.05$  and  $\text{Log}_2$  Fold Change  $> 2.5$  was considered the threshold to judge the upregulated mRNAs. GSE30219 [13] including 14 non-tumoral lung samples and 241 NSCLCs containing 85 ADCs (adenocarcinoma), 61 SCCs (squamous cell carcinoma), 39 BASs (basaloid tumor) and 56 LCNEs (large cell neuroendocrine tumor). GSE31210 [14] is a gene expression data for pathological stage I-II primary ADCs, which involved 20 non-tumor species and 226 ADCs. GSE19188 [15] is an expression data for early stage NSCLC with 91 NSCLCs (45 ADCs, 27 SCCs, 19 LCCs) and 65 adjacent normal lung tissue samples. The three datasets were processed on the Affymetrix Human Genome U133 Plus 2.0 Array (GPL570). The desired Affymetrix ID was valid: 224428\_s\_at (CDCA7).

The expression level of the mRNAs selected in The Cancer Genome Atlas (TCGA) database were analyzed using a free online tool, GEPIA (<http://gepia.cancer-pku.cn/>) [16]. We validated the expression level CDCA7 in TCGA NSCLC dataset, which include 483 lung adenocarcinoma (LUAD) tissues vs 347 normal lung tissues and 486 lung squamous cell carcinoma (LUSC) tissues vs 338 normal lung tissues.

### **The Kaplan–Meier plotter**

The prognostic significance of mRNA expression of CDCA7 in NSCLC was evaluated using the Kaplan–Meier plotter ([www.kmplot.com](http://www.kmplot.com)) [17]. The OS of 1,145 NSCLC were calculated according to the median expression of CDCA7 (high vs low expression) to obtain the Kaplan–Meier survival plots. Log-rank P-value and hazard ratio (HR) with 95% confidence intervals were calculated.

### **Sample Collection**

A total of 47 fresh specimens of tumors and matched surrounding non-tumor tissues were obtained from newly diagnosed NSCLC patients who received surgical resection in Department of Thoracic Surgery at Renmin Hospital of Wuhan University. Detailed information of samples is list in supplementary material (Table s1). Fresh samples were first incubated in TRIzol reagent (Invitrogen, Life Technologies) and then stored at  $-80^{\circ}\text{C}$  for further use. The collected tissue samples were used to extract RNA and detect the mRNA expression level of CDCA7. Aiming to further verify the protein expression level of CDCA7, 76 tumors and 74 matched surrounding non-tumor Paraffin-embedded tissues from NSCLC patients with follow up time was diagnosed according to surgical and pathological examination, which were based on the guidelines described by the 6th edition of AJCC/UICC (Table 2). This study was approved by Institutional Review Board of Department of Thoracic Surgery at Renmin Hospital of Wuhan University, and written informed consent was obtained from all participants.

### **Real-Time Quantitative PCR (RT-qPCR) Analysis**

Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) from the fresh tissues and cell lines according to the manufacturer's specifications. Total RNA (2  $\mu\text{g}$ ) was used for reverse transcription using the RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific). The expression of mRNA was examined by qRT-PCR with SYBR Premix Taq and Applied QuantStudio 6 Flex Real-Time PCR System.  $\beta$ -actin was used as the reference gene and the amplification results for qRT-PCR were calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method. The expression level of each gene was measured in triplicate. The primers used in this study are list in supplementary material (Table s2).

### **Immunohistochemistry**

Paraffin-embedded tissue sections were dewaxed and rehydrated, and antigen retrieval was performed by microwaving in 10 mM sodium citrate buffer, pH 6.0, for 20 min. Sections were then incubated with 3% hydrogen peroxide for 30 min at room temperature to block the endogenous peroxidase, followed by blocking with 10% normal goat serum (AR1009; Boster, Pleasanton, CA, USA) for 0.5 h. Immunostaining

was performed by incubating the tissue slides with anti-CDCA7 antibody (1:100, HPA005565; Sigma, WGK), mouse anti-Caspase-7 antibody (1:100, ab69540, Abcam Inc., Cambridge, MA, U.K.), rabbit anti-Cdk6 antibody (1:200, ab151247, Abcam Inc., Cambridge, MA, U.K.) at 4°C overnight. Slides were then washed in phosphate-buffered saline with Tween 20 and incubated with secondary antibody (anti-rabbit and anti-mouse detection system, 1:200, GB23303; Wuhan Goodbio Technology, Wuhan, People's Republic of China) for 30 min at 37°C. Staining was visualized with 3,3-diaminobenzidine and counterstained with hematoxylin. The final staining score is the average of the percentage of stained area [scored as 0 (without staining), 1 (<25%), 2 (25-50%) and 3 (>50%)] and intensity [scored as 0 (without staining), 1 (light yellow), 2 (yellow) and 3 (brown yellow)] of stained cells. In this way, the patients were further divided into CDCA7-low (score  $\leq$ 3) and CDCA7-high (score >3) groups.

### **Cell culture and RNAi assay**

The human NSCLC cell lines (A549 and H661) and human bronchial epithelial cells BEAS-2B were purchased from the American Type Culture Collection (ATCC; Manassas, VA, US) and cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Gibco, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) at a 5% CO<sub>2</sub> air incubator at 37°C and passaged using 0.25% trypsin-EDTA (Gibco) when they reached confluence. After A549 cells were starved for 12 hours, DMSO was added to the Control Group and 1  $\mu$ M U0126 (Sigma, 19-147) was added to the experimental group for 24 hours.

Cells were transfected with small interfering RNA (siRNA) using Lipofectamine™ 3000 (Invitrogen, USA) according to the manufacturer's protocol with siRNA duplexes specific for human CDCA7 (Ribobio, Guangzhou, China) or negative control (NC) siRNA. The siRNA sequence targeting CDCA7 is as follows: CDCA7: 5'- GTGCATGCCTACTTGAAAA -3'.

Lentivirus-based puromycin-resistant shRNA was used for CDCA7 stable knockdown experiments. The targeting oligonucleotides were: 5'- GTGCATGCCTACTTGAAAA -3'. Forward / Reverse oligo sequences of CDCA7 shRNA were designed according to the website (<http://www.addgene.org/tools/protocols/plko/>) and synthesized by Tsingke Biological Technology. The target sequence was inserted into the AgeI/EcoRI sites in the pLKO.1-EGFP-Puro vector according to the manufacturer's recommendations. A scrambled shRNA was included as a negative control.

### **Western blot analysis**

Radio Immunoprecipitation Assay (RIPA) lysis buffer supplemented with 1% protease inhibitor Phenylmethanesulfonyl Fluoride (PMSF) was applied for total protein extraction of cells and Bicinchoninic Acid (BCA) kit (Thermo Scientific) was used to detect total protein concentration. Then the proteins were adjusted to suitable concentrations using deionized water, separated by SDS/PAGE, transferred on to PVDF membrane. The membranes were blocked with 5% non-fat milk and subsequently incubated with anti-human CDCA7 (1:200, ab69609, Abcam Inc., Cambridge, MA, U.K.), Caspase7 (1:1000, ab32522, Abcam Inc., Cambridge, MA, U.K.), CDK6 (1:1000, ab124821, Abcam Inc., Cambridge, MA, U.K.),

C-Caspase-3 (1:1000, ab32042, Abcam Inc., Cambridge, MA, U.K.), C-Caspase-7 (1:1000, ab256469, Abcam Inc., Cambridge, MA, U.K.), C-Caspase-9 (1:1000, ab2324, Abcam Inc., Cambridge, MA, U.K.), Bax (1:1000, ab32503, Abcam Inc., Cambridge, MA, U.K. ), Bcl-2 (1:1000, ab692, Abcam Inc., Cambridge, MA, U.K.), P-ERK (1:1000, 4370, CST), T-ERK (1:1000, 9102, CST), GAPDH (1:10000, 10494-1-AP, Proteintech). overnight at 4°C. After washing three times, the membranes were incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. The protein bands were visualized with Enhanced chemiluminescence (ECL) plus western blot analysis detection reagents (Thermo Scientific). GAPDH was set as the internal reference.

### **Cell proliferation assay**

Cells transfected in 6-well plates for 48 h were digested into single-cell suspension. Cells were then incubated in three 96-well plates at a density of 3,000/100 µl per well and each 96-well plate was equipped with five multiple wells for each group. To each well, 100 µl complete medium and 10 µl CCK-8 (Dojindo, Kumamoto, Japan) was added at indicated time points and incubated for another 3h. The absorbance of each well was obtained by PerkinElmer 2030 VICTOR X Multilabel Plate Reader (Perkin-Elmer, Waltham, MA, USA) at 450 nm.

### **Cell cycle assay**

NSCLC cells were transfected for 48 h and digested into single-cell suspension. After an overnight incubation with 70% ethanol, cells were washed with PBS three times for 5 min each. Subsequently, 100 µl RNA enzymes A was added to suspending cells and incubated for 30 min at 37 °C in a water bath. The cells were stained with 400 µl propidium iodide (PI) (KGA512, Jiangsu KeyGEN BioTECH Corp., Ltd, Jiangsu, China) for 30 min incubation on ice in the dark. The cells were then washed with ice cold PBS twice and PI intensity was detected by flow cytometry (Becton Dickinson, USA). The results were analyzed using ModFit software as directed by manufacturer's specifications.

### **Apoptosis analysis**

Cells were harvested and washed with ice-cold PBS twice. Then the cells were suspended in 100µL of Annexin V-FITC binding buffer, after which 5 µl Annexin-V-FITC (20 µg /ml) and 5 µl PI (50 µg /ml) was gently added into above system for 15 min incubation in the dark. At last, the cell apoptosis was measured using flow cytometry (Becton Dickinson, USA) within 30 min.

### **Immunofluorescence assay**

The cell density was adjusted to  $3 \times 10^6$ /ml and the cells were laid in the glass bottom dishes specially designed for laser confocal microscopy. Cells were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 10min; 3% BSA and sealed for 60 min at room temperature. After overnight incubation with anti-CDCA7 antibody (1:200, HPA005565; Sigma, WGK), the specimens were rinsed thoroughly and treated with anti-rabbit antibodies (1:200, BA1032, Boster), respectively.

Nuclei were stained using 0.3  $\mu$ M DAPI (C1002, Beyotime Biotechnology, China). The digital images were then captured with a cooled CCD camera and processed with Adobe Photoshop software.

### **Tumor xenografts in nude mice**

In this study, 6- to 8-week-old male nude mice were purchased from Center for Animal Experiment of Wuhan University, and maintained in Animal Biosafety Level- $\square$  Laboratory at Wuhan University. To initiate tumors,  $1 \times 10^6$  cells in 100  $\mu$ L of PBS were injected subcutaneously into left flank (negative control, NC) and right flank (CDCA7 knock down, CDCA7 KD) of nude mice (5 mice/group) respectively. The length (L) and width (W) of the tumors were measured using a digital caliper. Tumor proliferation was evaluated by their volume, which was calculated by the formula:  $V = 0.5 \times L \times W^2$ . At the end, the mice were euthanized and the tumors were removed and photographed. All experimental procedures were carried out in accordance with Guidelines for the management provided by Medical Laboratory Animal Center of Wuhan University.

### **Statistical analysis**

Statistical analysis was performed using SPSS ver. 18 (SPSS Inc., Chicago, IL, USA) GraphPad Prism V.5.00 software (GraphPad Software, San Diego CA, United States). Two-tailed t-test was used to compare the expression of CDCA7 in different groups and paired t-test was used for matched-samples. Data was expressed as mean  $\pm$  SD. Chi-square test was employed to examine possible correlations between CDCA7 expression and clinicopathological variables. Univariate and multivariate survival analyses were performed using Cox proportional hazards regression model. Factors with prognostic significance in the univariate analysis were included in the subsequent multivariate analysis. SPSS software was used to calculate and draw the receiver operating characteristic curve (ROC curve) and survival curve. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

## **Results**

### **High expression of CDCA7 is associated with the prognosis of NSCLC Patients**

Firstly, we selected three GEO datasets (GSE19188, GSE30219, and GSE31210), based on the pre-set screening threshold and taking intersection of these three data sets, 18 Affymetrix ID with 15 consistently significantly upregulated mRNAs were identified (Fig. 1A). We explored the prognostic value of the identified mRNAs using Kaplan–Meier plotter. Among the 15 identified mRNAs, the high expression of COL10A1 and CXCL13 predicted good OS in lung cancer while high expression of ANLN, CDCA7, COL11A1, DLGAP5, GJB2, GREM1, IGF2BP3, MELK, MMP12, RRM2, SPP1, TOP2A and UBE2T predicted poor OS in lung cancer. However, the mechanism of how CDCA7 is involved in tumorigenesis remains largely unknown. The information of all the upregulated mRNAs are summarized in Table 1.

Next, we validated the expression level of CDCA7 in TCGA NSCLC dataset (483 LUAD tissues with 347 normal lung tissues and 486 LUSC tissues with 338 normal lung tissues. Fig. 1B). Further, we explored

the expression of CDCA7 in different histological subtypes of NSCLC using published data from GEO datasets. CDCA7 was highly expressed in several histological subtypes including ADC, SCC, LCC, LCNE and BAS compared with nontumoral lung tissues (all  $P < 0.05$ ) in GSE31210 (Fig. 1C), GSE30219 (Fig. 1D), and GSE19188 (Fig. 1E). Furthermore, the expression of CDCA7 was significantly upregulated in stage IA NSCLC tissue (Fig. 1F and G).

We also explored the prognostic value of the expression of CDCA7 using Kaplan–Meier plotter. The desired Affymetrix ID was valid: 224428\_at (CDCA7), and patients were split into low- and high-expression groups by the median of CDCA7 expression values. A high expression of CDCA7 mRNA was related to significantly shorter OS for all lung cancer patients ( $n=1145$ , HR 1.48 [1.25–1.74],  $P=3.6e-06$ ) (Fig. 1H). In addition, among patients with the American Joint Committee on Cancer (AJCC) T1N0M0 stage ( $n=200$ ), high CDCA7 expression predicted shorter OS (HR 2.17 [1.40–3.36],  $P=0.00037$ ) (Fig. 1I).

### **CDCA7 is an independent prognostic factor for NSCLC**

GSE29013 with 55 NSCLC patients was applied to explore whether the higher CDCA7 expression could be an independent predictor of shorter OS in NSCLC patients by univariate and multivariate Cox proportional hazards regression analysis. Two variants, AJCC stage and CDCA7 expression were demonstrated to be significant in the univariate analysis, entered the multivariate Cox hazard model. Based on this analysis, higher stage and high CDCA7 mRNA expression were identified as independent prognostic factor of shorter OS ( $P=0.010$  and  $P=0.023$ , respectively) (Fig. 2A and 2B).

we did a ROC curve to check its prognostic ability. The area under the ROC curve (AUC) was 0.683 for OS (Fig. 2C).

### **CDCA7 up-regulated in NSCLC samples**

Based on the analysis of previously published public datasets, we found high CDCA7 expression in NSCLC tissues. Aiming to verify this expression trend, we checked the expression of CDCA7 gene on mRNA level in 47 pairs of NSCLC specimens (tumor and adjacent nontumor tissues), which consisted 20 SCCs and 27 ADCs. The clinical information of 47 NSCLC samples is listed in Table S1. The result show that CDCA7 mRNA level in NSCLC tissues was significantly higher than relative adjacent nontumor tissues ( $P<0.0001$ ) (Fig. 3A and B).

Furthermore, detected the protein expression levels of CDCA7 in 76 tumors and 74 matched surrounding non-tumor Paraffin-embedded tissues from ADC patients by immunohistochemistry (Fig. 3C). As expected, we found the stain score of tumors was significantly higher than adjacent nontumor tissues ( $P<0.0001$ ) (Fig. 3D), which shows the high expression of CDCA7 in lung cancer tissue compared to the adjacent normal tissue.

76 Paraffin-embedded ADC patients with median follow-up of 39 month was employed to calculate the survival curve for OS. As shown in Fig. 3E, the OS rate of patients with high CDCA7 expression was significantly lower than the survival rate of patients with low CDCA7 expression (log-Rank  $P=0.0366$ ).

## **The correlation of CDCA7 with NSCLC clinicopathological variables**

To explore the relationship between NSCLC and CDCA7, GSE29013 with 55 NSCLC patients and 76 Paraffin-embedded ADC tissues with corresponding clinical information collected from hospital were chosen to analyze the correlation of CDCA7 expression with NSCLC clinicopathological variables.

Notably, our analysis showed that high expression of CDCA7 portends high stage and grade of NSCLC (Table 2 and 3), which corroborated the essential role of CDCA7 in influencing the degree of tumor malignancy.

## **Knockdown of CDCA7 inhibits NSCLC cells proliferation and induces cell cycle arrest *in vitro***

We first detected the expression levels of CDCA7 in NSCLC cells and found the upregulation of CDCA7 in A549 and H661 cells compared to human bronchial epithelial cells BEAS-2B (Fig. 4A and 4B). Subsequent immunofluorescence analysis of A549 and H661 cells identified the subcellular localization of endogenous CDCA7 mainly in the nucleus and weakly in the cytoplasm (Fig. 4B).

To evaluate whether CDCA7 is biologically relevant to NSCLC cells malignant phenotypes, we first stably silenced CDCA7 in NSCLC cell lines A549 and H661 to perform loss of function experiments *in vitro* (Fig. 4C and 4D). Suppression of CDCA7 expression with siRNA significantly ( $P < 0.05$ ) inhibited cellular proliferation (Fig. 4E and 4F) compared with the control cells by arresting cell-cycle progression at the G1 phase compared with the control cells (Fig. 4G).

## **CDCA7 is unregulated in A549/DDP cell and knockdown of CDCA7 induce NSCLC cells apoptosis *in vitro***

To investigate the association between CDCA7 and cisplatin resistance in NSCLC, we investigated the expression levels of CDCA7 in A549 and A549/DDP cells by RT-qPCR and western blotting. As shown in Fig. 5A and 5B, the expression levels of CDCA7 in A549/DDP cells were significantly higher than that in A549 cells both on protein and mRNA level. We observed that the half-maximal inhibitory concentration (IC<sub>50</sub>) after 48 h was  $4.133 \pm 0.110$  and  $22.41 \pm 0.711$   $\mu\text{g/ml}$  for A549 and A549/DDP (Fig. 5C). To investigate whether CDCA7 mediates chemotherapy resistance in A549/DDP cells, we observed that CDCA7 down regulation using CDCA7 siRNA (Fig. 5D) had an effect on cisplatin sensitivity in A549/DDP cells, for which the IC<sub>50</sub> were  $23.11 \pm 0.411$  and  $15.33 \pm 0.372$   $\mu\text{g/ml}$  for A549/DDP siNC and siCDCA7, respectively ( $P = 0.0005$ ) (Fig. 5E). In addition, Annexin-V/PI staining showed that CDCA7 silencing triggered cell death in NSCLC cells (Fig. 5F and 5G). These results suggest that CDCA7 may be a potential therapeutic target for NSCLC.

## **Depletion of CDCA7 expression markedly affected the expression of CDK6 and caspase7**

We observed that CDCA7 silencing efficiently affect the proliferation, apoptosis and cycle distribution of NSCLC cells *in vitro*. To address the intrinsic mechanisms by which CDCA7 regulates NSCLC cell tumorigenesis. we performed qRT-PCR of cell cycle and apoptosis associated target genes, including CCNA1, CCNA2, CCNB1, CCND1, CCNE1, CDK1, CDK2, CDK4, CDK6, p18, p19, p21, p27 and CASP3,

CASP7, CASP9, CASP10, TNFRSF10C, IL1R1 in CDCA7 knockdown cells and cells transfected with siNC (used as controls). Indeed, we observed that CDK6 was significantly decreased 45% in A549 cells and 41% in H661 cells (Fig. 6B) and CASP7 was significantly increased in A549 and H661 cells upon CDCA7 depletion (Fig. 6C). WB analysis showed that by silencing CDCA7, the expression of CDK6 and caspase7 was significantly decreased and increased, respectively in NSCLC cells (Fig. 6D).

Moreover, CDCA7 expression was positively correlated with MKi-67 expression in three NSCLC datasets including Tumor Lung (NSCLC) - Chuang - 120 - MAS5.0 - u133p2 ( $r = 0.662$ ,  $P=1.81e-16$ ), Tumor Lung (NSCLC) - Muley - 100 - MAS5.0 - u133p2 ( $r=0.747$ ,  $P=4.30e-19$ ) and Tumor Lung (NSCLC) - spivack - 131 - rma\_sketch - hugene10t ( $r=0.626$ ,  $P=1.26e-15$ ) by using web R2: Genomics Analysis and Visualization Platform (Fig. 6A).

### **CDCA7 silencing promotes A549 apoptosis via ERK pathway**

We showed that silence CDCA7 increased the expression of Cleaved caspase-3, Cleaved caspase-7, Cleaved caspase-9 and Bax, and also attenuated levels of Bcl-2 and phosphor-ERK (Fig. 7A and 7B). In the presence of U0126, Cleaved caspase-3, Bax and Bcl-2 were not further regulated by shCDCA7 pretreatment (Fig. 7C and 7D), which suggested that the effects of shCDCA7 on the pro-apoptosis of A549 were exerted mainly by decreasing ERK activation induced.

### **CDCA7 silencing reduces the tumorigenicity of NSCLC cells *in vivo***

To verify the function of CDCA7 *in vivo*, we carried out subcutaneous tumorigenesis experiment in nude mice. We found that tumors in CDCA7 knock down group (Fig. 8A and 8B) grew more slowly compared to the negative control (Fig. 8C-E). The subsequent tissue sections showed reduced CDK6 expression and increased caspase7 in CDCA7 knock down group (Fig. 8F and 8G). These data were consistent with our results *in vitro*. These results suggest that downregulation of CDCA7 may inhibits the tumorigenicity of NSCLC cells both *in vitro* and *in vivo*.

## **Discussion**

The majority of lung cancer patients are diagnosed in advanced or metastatic stages, which are largely considered unresectable [18]. The early diagnosis of NSCLC is possible by the screening the signature gene [19]. Our study found that the expression level of CDCA7 was significantly increased in the early stage of NSCLC (AJCC T1N0M0), Thus CDCA7 could be used as a sensitive screening indicator for early-stage NSCLC. Although the TNM staging system has been widely used to evaluate the prognosis of lung cancer, However, 40% of lung cancer patients at early TNM stage suffer from relapse after receiving potentially curative treatment [20]. Therefore, additional molecular markers are urgently needed to recognize those early-stage lung cancer patients receiving surgical management who are at high risk to recurrence. Studies have shown that the high expression of other members of the CDCA7 family, such as CDCA2 [21], CDCA3 [22], CDCA5 [23], and CDCA8 [24] in lung cancer tissues is closely related to poor prognosis. We found that among AJCC T1N0M0 stage patients, those with high expression of CDCA7

showed significant propensities for poor OS compared with CDCA7-low-expression cohorts (HR 2.17[1.4-3.36], P=00037). Therefore, CDCA7 might be an additional molecular marker in combination with TNM staging system to predict OS for early-stage lung cancer patients.

Cell division is necessary to produce the large number of cells required for living. However, cell division can lead to a variety of cancer-promoting errors and promote tumorigenesis [25]. The genes of the CDCA family are characterized by their association with known cell cycle related genes including CDC2, CDC7, CDC23, cyclin [26]. Nuf2/CDCA1 knockdown induced cell cycle arrest via the suppression of Cyclin B1, Cdc2 and Cdc25A in pancreatic cancer [27] and hepatocellular carcinomas [28] cell lines. Depletion of CDCA2 suppressed the proliferation of lung adenocarcinoma cells by G1 phase arrest via downregulating cyclin E1 [29]. Inhibition of CDCA3 markedly impairs the proliferation by inducing cell cycle arrest in the G2 phase of the cell cycle in NSCLC cell lines [30]. CDCA family contains regulatory factors that play an important role in cell division via regulation the expression of cell cycles-related genes. However, the molecular mechanism of CDCA7 implication in NSCLC tumorigenesis remains elusive. In 2002, Whitfield et al. [31] speculated that CDCA7 was involved in cell cycle progression. Recently, Hongying Wang et al showed that silencing CDCA7 inhibited cell proliferation in LUAD through G1 phase arrest [32]. In this study, we further concluded that interfering CDCA7 could inhibit proliferation and blocks cells at G0/G1 phase of NSCLC cells by regulating the expression of CDK6 both in vitro and in vivo. The mRNA levels of CCNE1 did not show any significant change in si-cdca7-transfected A549 and H661 cells, which was different with Hongying Wang et al [32]. Abemaciclib represents the first selective inhibitor of CDK4 and CDK6 has been evaluated the safety and antitumor activity in a multicenter phase I clinical trial for NSCLC, breast cancer and other solid tumors [33]. Abemaciclib in combination with ionizing radiation enhances NSCLC radiosensitivity in preclinical models, potentially providing a novel biomarker-driven combination therapeutic strategy for patients with NSCLC [34], suggesting that CDK6 was an anticancer drug target.

CDK4/CDK6 interact with D-type cyclins to phosphorylate the retinoblastoma (RB) tumor suppressor protein and pRB releases the inhibition of E2F1 transcription factor which regulates the synthesis of s-phase related proteins and promotes the cell progresses from G1 phase to S phase [35]. Our results showed that interference with CDCA7 expression resulted in cell arrest in G1 phase and decreased CDK6 expression. We speculate that CDCA7/CDK6/RB/E2F1/CDCA7 may act as a circulating regulatory axis, because it has been reported that the promoter region of CDCA7 has binding sites for E2F1, and the expression of CDCA7 is regulated by E2F1 [36].

Our result showed that CDCA7 knockdown promoted cell apoptosis and significantly induced the expression of Cleaved caspase-3, Cleaved caspase-7, Cleaved caspase-9 and Bax, and also attenuated levels of Bcl-2 in NSCLC cells. Previous study has reported that the activation of an effector caspase, caspase-3 or -7, is performed by an initiator caspase, caspase-9, through an internal cleavage to separate the large and small subunits [37]. The alteration of Cleaved caspase-3 and Cleaved caspase-7 may be triggered by Cleaved caspase-9. In vitro study, we showed that silence CDCA7 led to the decrease of phosphor-ERK, an essential downstream component of MAPK pathway regulating cell proliferation, which

has been reported that lead the alteration of Bax and Bcl-2 expression [38]. Collectively, we supposed that ERK might be responsible for CDCA7 silence-mediated apoptosis. To further confirm silence CDCA7 exerted pro-apoptosis action in ERK dependent manner, U0126, ERK inhibitor was used in our vitro study. In the presence of U0126, cell apoptosis protein levels were not further inhibited by shCDCA7 pretreatment, which suggested that the effects of shCDCA7 on the pro-apoptosis of A549 were exerted mainly by decreasing ERK activation induced. Furthermore, it has been reported that the strategy of suppressing ERK activation may be a therapy for patients with NSCLC [39]. Together, these data indicated that shCDCA7 treatment was capable of promoting A549 apoptosis via ERK pathway.

Our study found that CDCA7 expression level was significantly upregulated in A549/DDP cells than that of wild-type A549 cells. Interference with CDCA7 expression resulted in decreased cisplatin sensitivity in A549/DDP cells, which suggesting that CDCA7 may be a potential therapeutic target for NSCLC. Xie C et al showed that c-Myc expression in A549/DDP cells were higher than that in A549 cells [40]. Osthus RC et al have reported that the expressions of MYC and CDCA7 are concordant in colorectal carcinomas, whereas only CDCA7 is overexpressed and MYC and CDCA7 are not concordant in lung cancers [9]. The relationship between CDCA7 and cisplatin resistance in lung cancer may be an issue that needs further study.

## Conclusions

Highly expressed CDCA7 predicts poor prognosis in NSCLC and CDCA7 silencing inhibits proliferation, promotes apoptosis and blocks cells at G0/G1 phase of NSCLC cells by regulating the expression of CDK6 and caspase7 both *invivo and vitro*. CDCA7 is unregulated in A549/DDP cell and CDCA7 silencing efficiently down regulation cisplatin sensitivity in A549/DDP cells. Our study uncovers a possible oncogenic role of CDCA7 in NSCLC progression, and may provide more effective therapeutic strategies against NSCLC.

## Abbreviations

CDCA7: Cell Division Cycle Associated 7

NSCLC: Non-Small Cell Lung Cancer

GEO: Gene Expression Omnibus

IHC: immunohistochemistry

CCK-8: Cell counting kit-8

OS: overall survival

CDK6: cell division protein kinase 6

ERK: extracellular regulated protein kinases

ADC: adenocarcinoma

SCC: squamous cell carcinoma

LCC: large-cell carcinoma

LCNE: large-cell neuroendocrine tumor

BAS: basaloid

PI: Propidium Iodide

DAPI: 4', 6-diamidino-2-phenylindole

IC50: half-maximal inhibitory concentration

AJCC: American Joint Committee on Cancer

TCGA: The Cancer Genome Atlas

## **Declarations**

## **Author Contributions**

Qiuping Zhang and Songping Xie conceived and designed the study. Wen Yuan, Tian Xie and Jingyuan Li conducted the experiments. Hengya Song and Muhammad Jamal were involved in statistical analysis. Wanli Jang and Jie Huang collected clinical sample and interpreted the experimental results. Wenhui Zeng and Wen Yuan wrote and revised the manuscript. All authors have read and approved the final manuscript.

## **Acknowledgements**

Not applicable.

## **Competing interests**

The authors declare that they have no competing interests.

## **Availability of data and materials**

The GEO datasets are available on National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD, USA) (<http://www.ncbi.nlm.nih.gov/geo/>). The online database Kaplan-Meier

Plotter can be accessed on (<http://kmplot.com/analysis/>).

## Consent for publication

## Not applicable.

### Ethics approval and consent to participate

This study was approved by Institutional Review Board of Department of Thoracic Surgery at Renmin Hospital of Wuhan University, and written informed consent was obtained from all participants. The animal experimental procedures were carried out in accordance with Guidelines for the management provided by Medical Laboratory Animal Center of Wuhan University.

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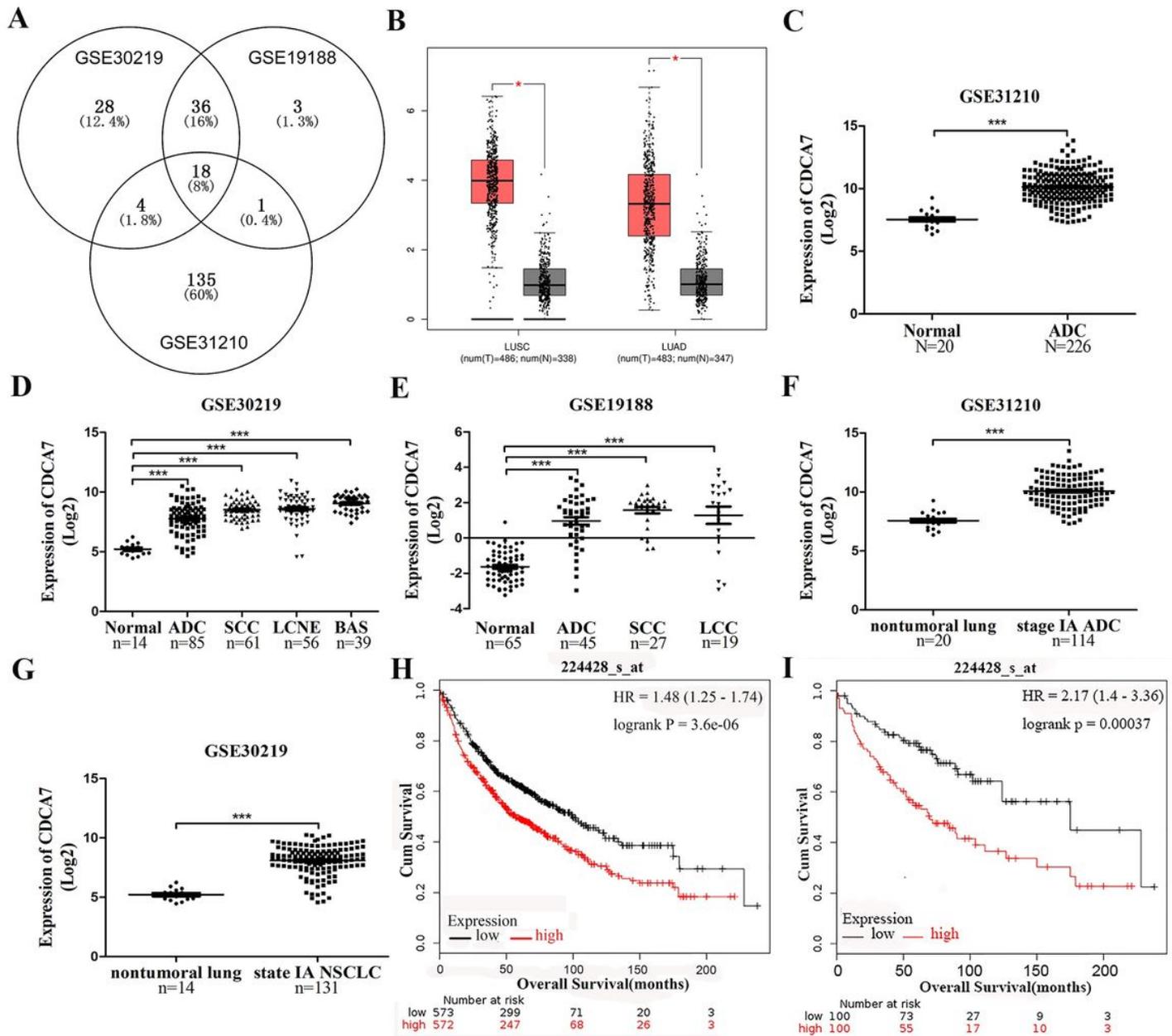
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## Tables

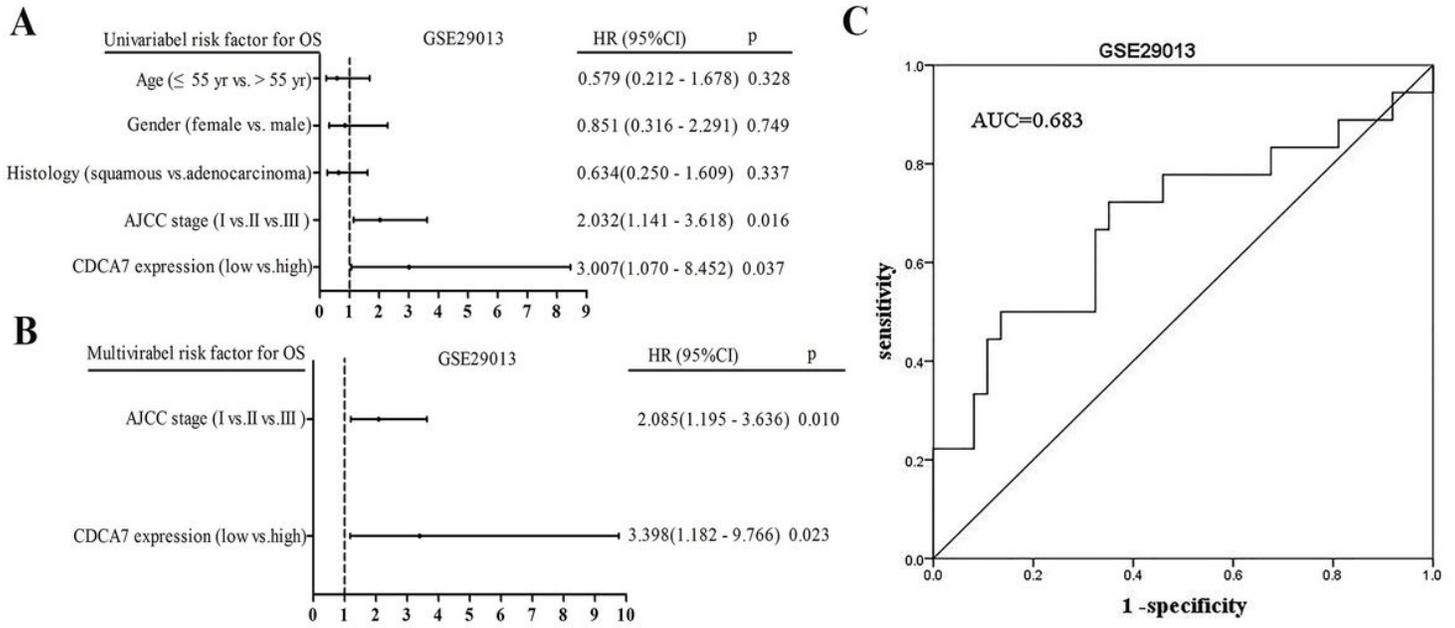
Due to technical limitations, table 1-3 is only available as a download in the Supplemental Files section.

## Figures



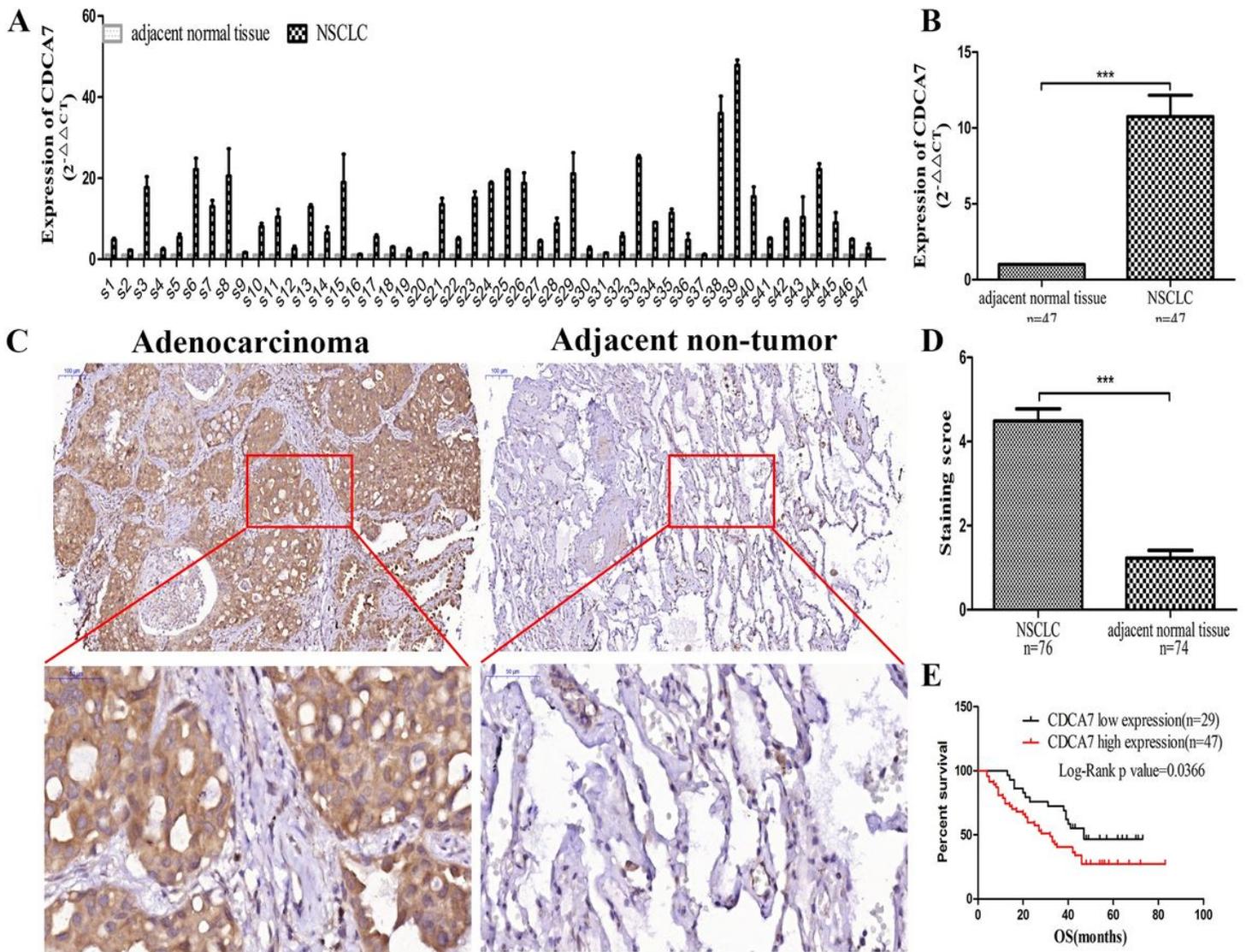
**Figure 1**

After an integrated analysis of GEO datasets, CDCA7 was selected to further explore its potential prognostic ability. (A) Venn diagram of the integrated analysis of GSE19188, GSE30219, and GSE31210 (B) CDCA7 was significantly upregulated in NSCLC tissues than normal tissues in the TCGA database. (C, D and E) in different histological subtypes of NSCLC tissues than normal tissues. (F and G) in stage IA NSCLC tissue. (H and I) Kaplan–Meier survival curves indicating OS based on the expression of CDCA7.



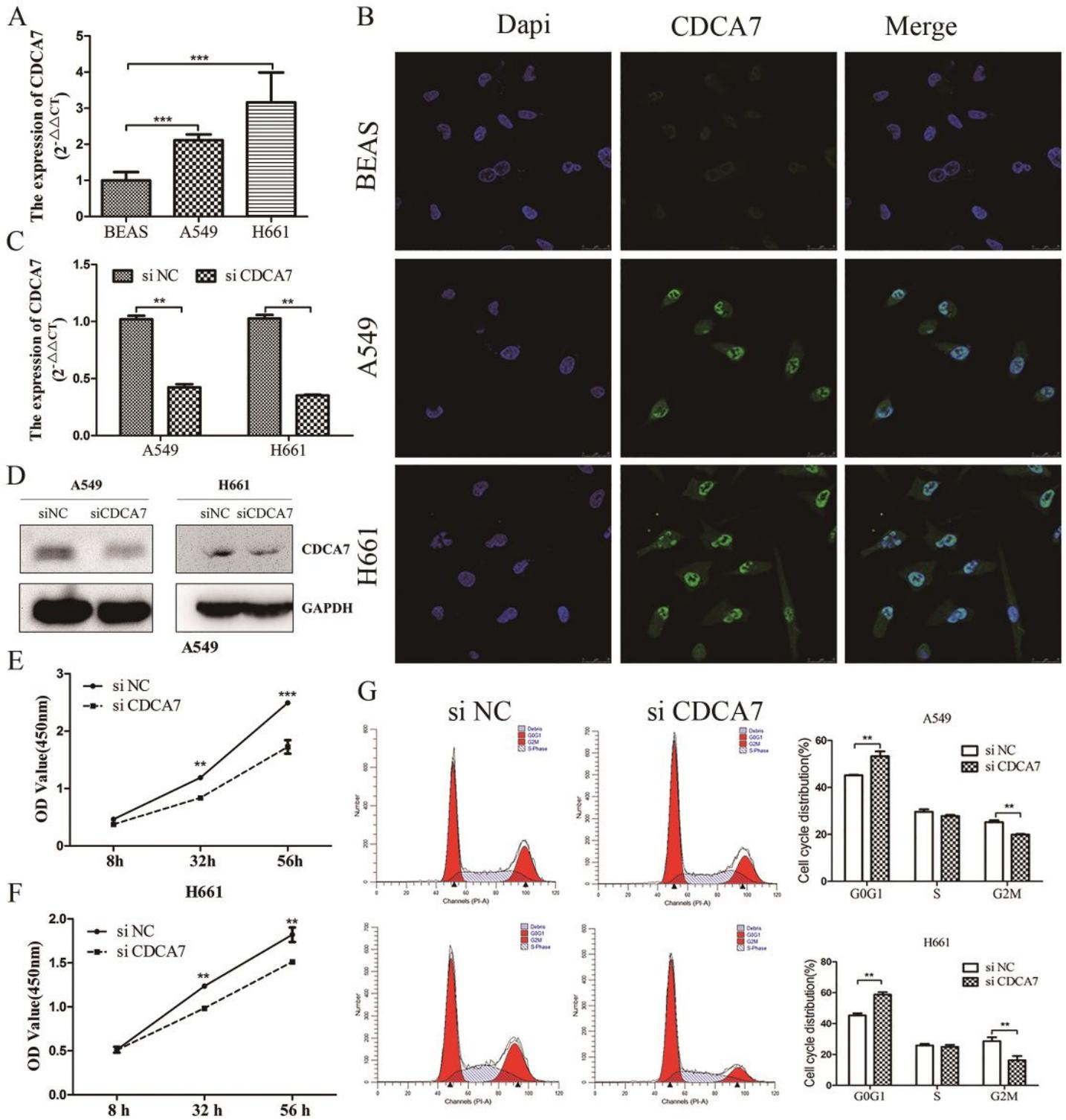
**Figure 2**

CDCA7 is an independent prognostic factor for NSCLC. (A) Univariate analysis of factors affecting the OS of NSCLC patients (B) Cox proportional hazards regression analysis of factors affecting the OS of NSCLC patients (C) ROC curve for NSCLC patients.



**Figure 3**

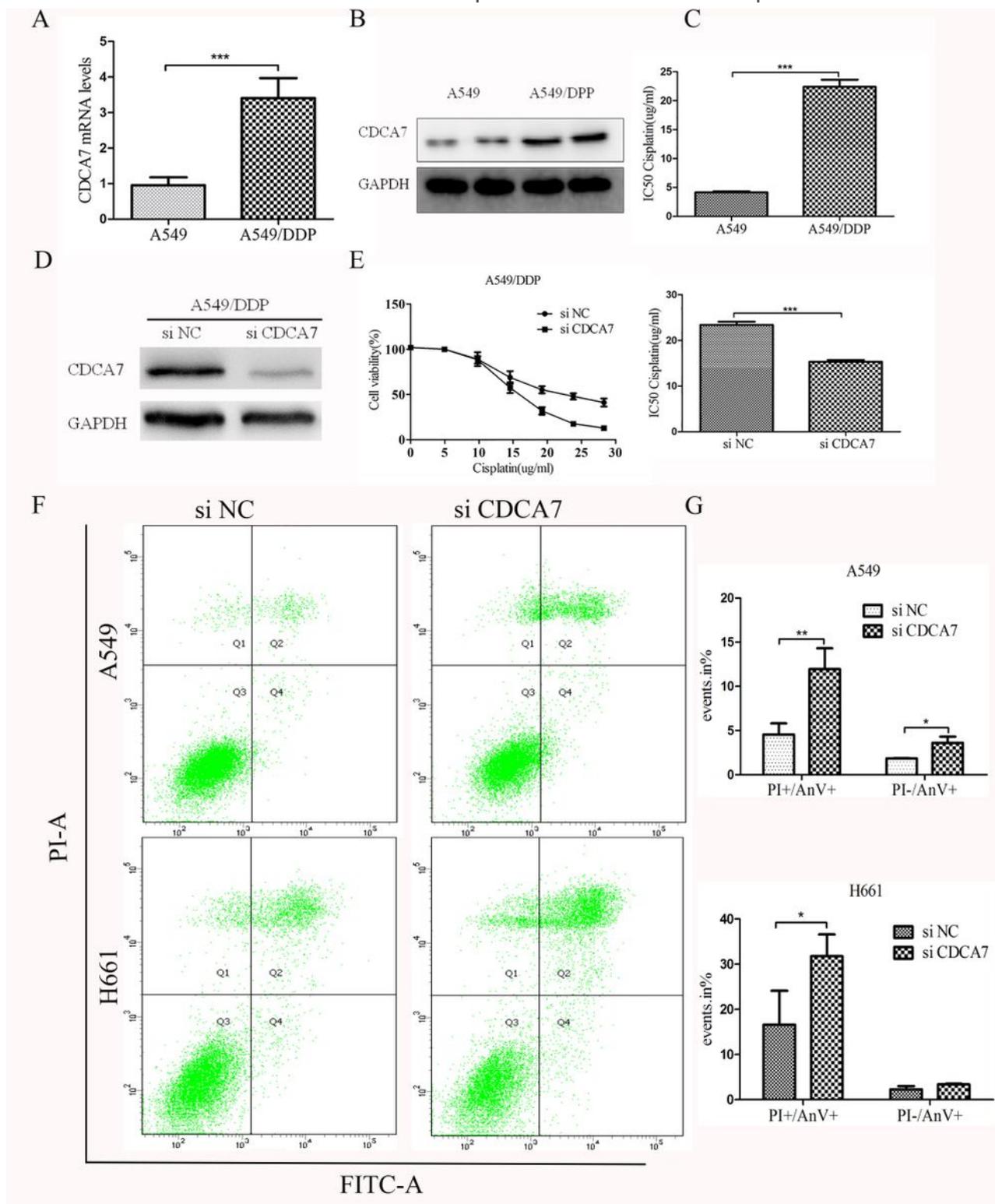
The expression of CDCA7 in NSCLC samples. (A and B) real-time PCR analysis of CDCA7 expression in 47 lung cancer specimens and adjacent nontumor tissues. (C) Representative images of NSCLC patients by IHC. (D) Stain score of NSCLC and adjacent nontumor tissues. (E) Survival curve of OS for 76 ADC patients based on the expression of CDCA7.  $\beta$ -actin was used as the internal control for real-time PCR. All the experiments were done in triplicate.



**Figure 4**

CDCA7 Silencing inhibits the proliferation and induces cell cycle arrest of NSCLC cells. (A) Real-time PCR analysis detecting the CDCA7 expression in cultured NSCLC cell lines. (B) Immunofluorescence analysis detecting the CDCA7 expression and subcellular endogenous localization in NSCLC cells. Cells were stained with a rabbit polyclonal anti-CDCA7 antibody (green) and DAPI (blue). Real-time PCR (C) and western blotting (D) analysis of the interference efficiency of CDCA7 siRNA in A549 and H661 cells. (E

and F) The proliferation rate of the indicated cells, as determined by the CCK-8 assay. (G) Representative FACS analysis showing the number of cells in the G0/G1 phase is significantly increased in siCDCA7-transfected A549 and H661 cells. All the experiments were done in triplicate.



**Figure 5**

CDCA7 overexpression in cisplatin-resistant A549 cells and knockdown of CDCA7 induce NSCLC cells apoptosis. (A) Real-time PCR and (B) Western blot analysis detecting the CDCA7 expression in A549 and

A549/DDP cells. (C) Chemosensitivity assay was conducted at 48h after cisplatin treatment for A549 and A549/DDP cells. (D) Western blot analysis detecting the CDCA7 expression in A549/DDP cells with CDCA7 siRNA transfection. (E) Cisplatin IC50 detection after CDCA7 siRNA transfection in A549/DDP cells. (F and G) Flow cytometry assay for the ratio of apoptotic cells in A549 and H661 cells when treated with/without CDCA7 siRNA. All the experiments were done in triplicate.

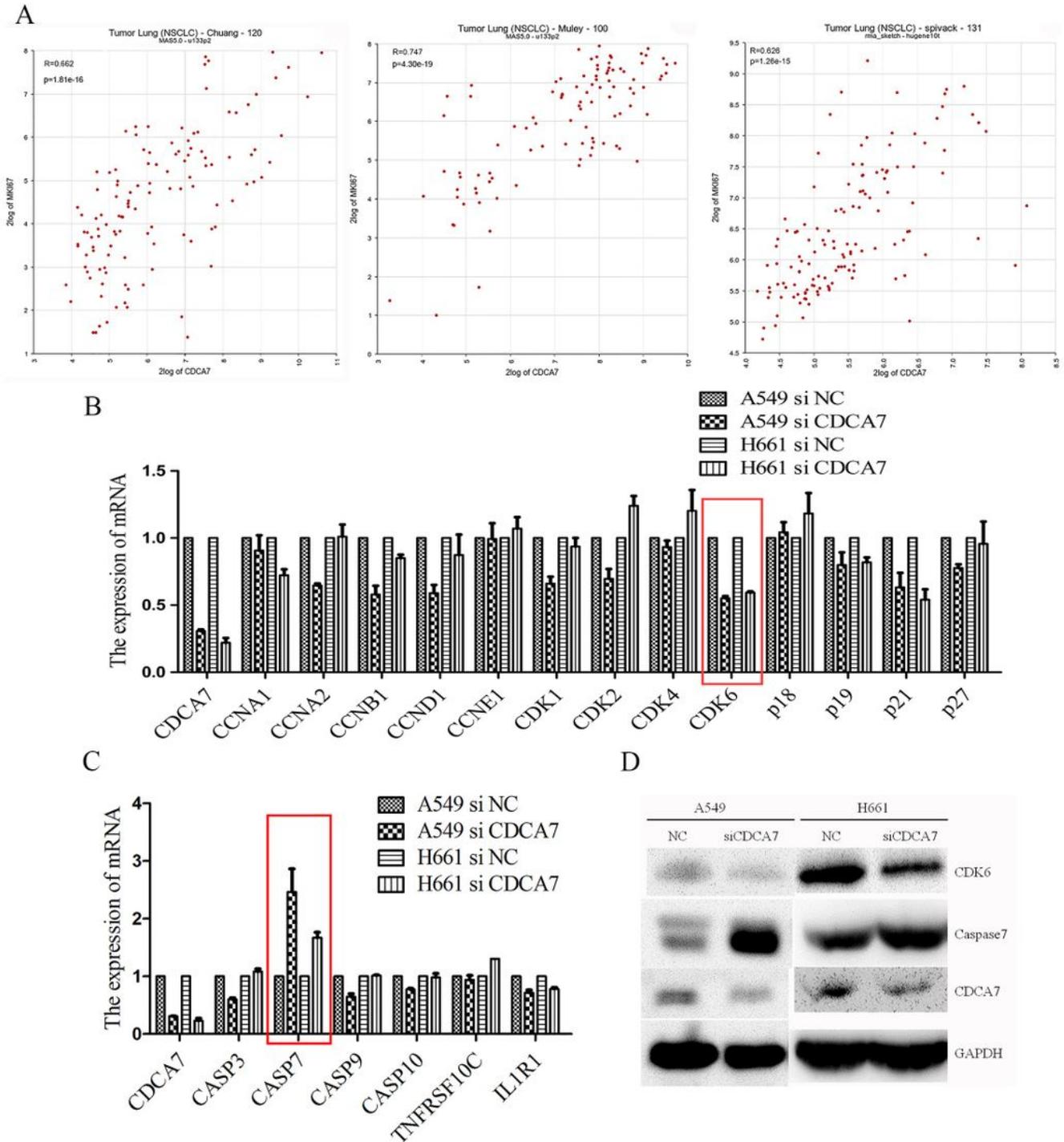
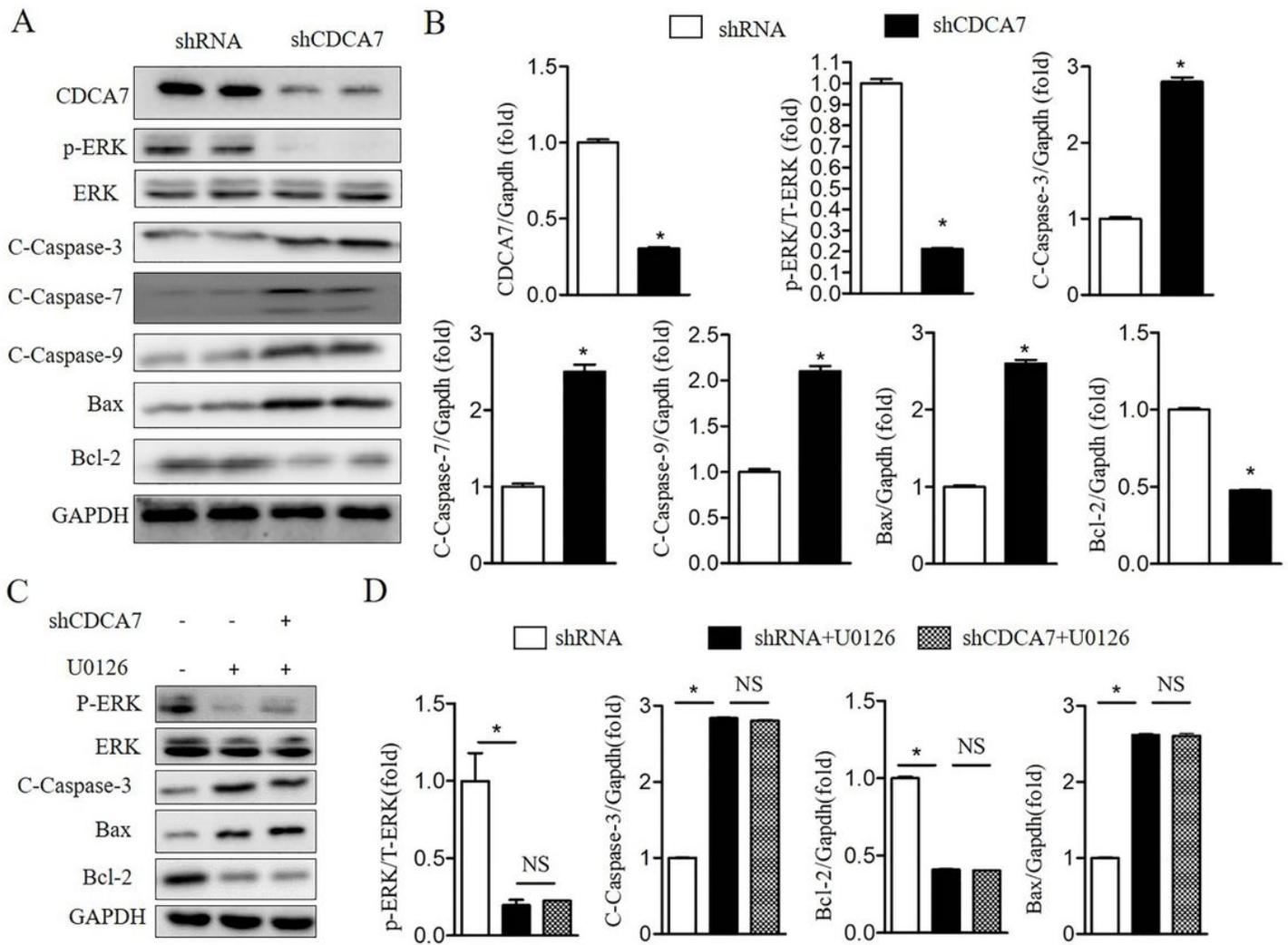


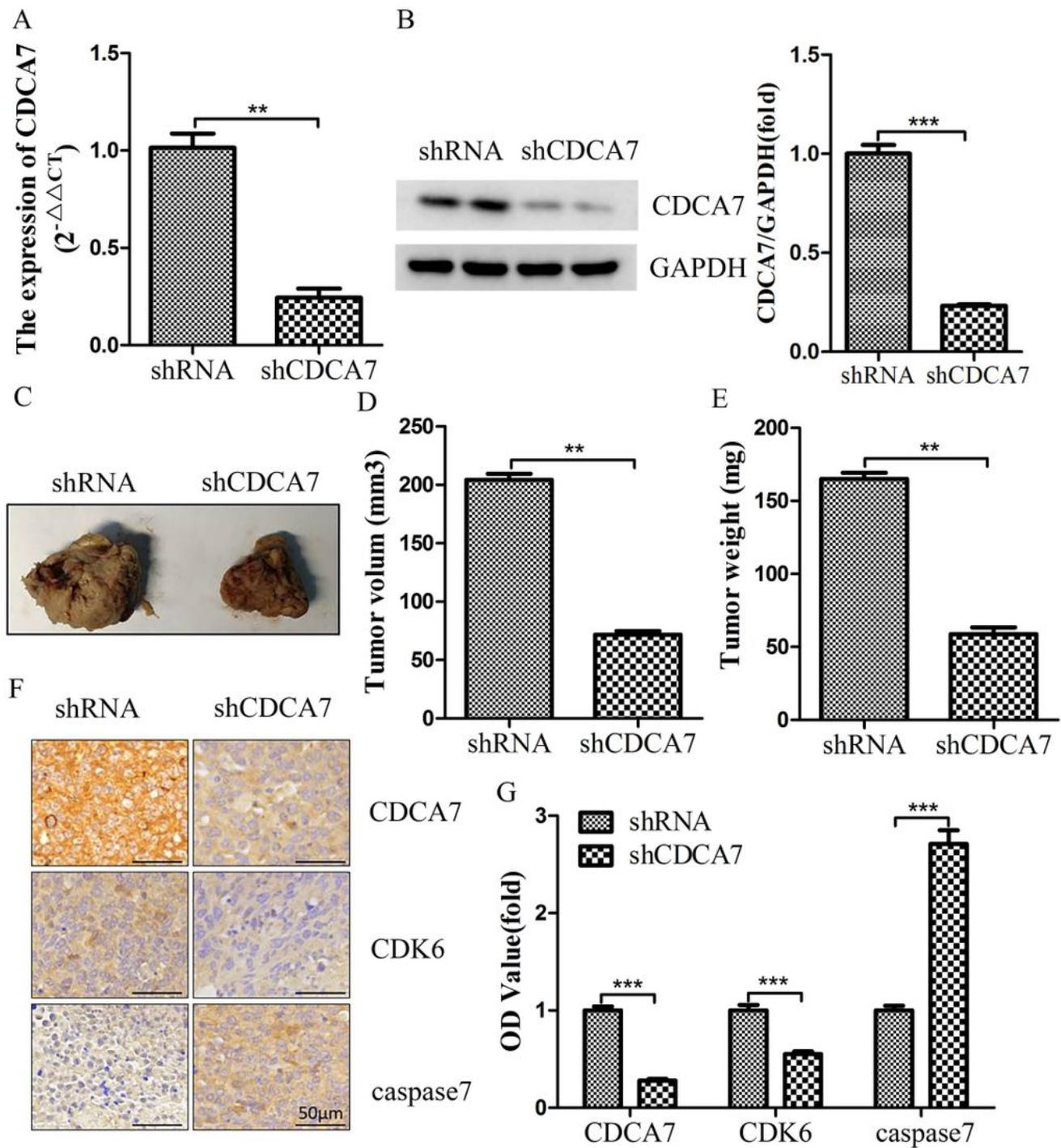
Figure 6

Depletion of CDCA7 expression markedly affected the expression of CDK6 and caspase7. (A) CDCA7 was found to be positively correlated with Ki-67 in three NSCLC datasets. (B) qRT-PCR showing the decrease CDK6 mRNA levels after siRNA-CDCA7 transfection in both A549 and H661 cells. (C) qRT-PCR and analysis showed that CASP7 significantly increased after siRNA-CDCA7 transfection in both A549 and H661 cells. (D) Western blot analysis showing the dysregulated expressions of CDK6 and caspase7 after siRNA-CDCA7 transfection in both A549 and H661 cells. All the experiments were done in triplicate.



**Figure 7**

CDCA7 silencing promotes A549 apoptosis via ERK pathway. (A and B) Western blot analysis detecting the Cleaved caspase-3, Cleaved caspase-7, Cleaved caspase-9, Bax and Bcl-2 after shCDCA7 transfection in A549 cells. (C and D) Western blot analysis detecting the Cleaved caspase-3, Bax and Bcl-2 after shCDCA7 transfection and treatment of U0126.



**Figure 8**

Knockdown of CDCA7 inhibits tumor growth in vivo. (A) Real-time PCR and (B) Western blot analysis detecting the CDCA7 expression in A549 cells. (C) Nodules harvested from shRNA and shCDCA7 group of xenograft model in nude mice. (D and E) Tumor nodules derived from shCDCA7-transfected A549 cells are significantly smaller than those in shRNA group. (E and F) IHC showing that CDK6 staining as well as CDCA7 was weaker and caspase7 was stronger in shCDCA7 group.

## Supplementary Files

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