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UBE2S promotes the proliferation and survival of human lung adenocarcinoma cells

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Running title: UBE2S contributes to lung cancer cell growth

Abstract

Ubiquitin-conjugating enzyme E2S (UBE2S), a family of E2 protein in the ubiquitination process, is involved in the development of various cancers. However, its role in lung adenocarcinoma has not yet been well elucidated. In this report, we attempted to investigate the expression and function of UBE2S in lung adenocarcinoma. Up-regulation of UBE2S at mRNA and protein level was observed in human cancer tissues and lung adenocarcinoma cells. Higher UBE2S expression was correlated with poorer prognosis of lung adenocarcinoma patients. UBE2S expression was efficiently suppressed by lentivirus-mediated shRNA strategy in A549 cells and UBE2S silencing led to reduced cell proliferation, colony formation and enhanced apoptosis. Inverse results were observed in UBE2S over-expressed H1299 cells. Microarray analysis indicated that a large amount of genes were regulated by UBE2S and p53 signaling pathway might be critical to the role of UBE2S in cancer development. Taken together, UBE2S could be a potential target for lung adenocarcinoma.

Introduction

Lung cancer is the most common cause of cancer-related death in men and second most common cancer in women after breast cancer (1). The two main types of lung cancer are small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC). Lung adenocarcinoma is one of the most common subtypes of NSCLC, accounting for approximately 40% of all lung cancers (2, 3). In recent years, various therapeutic strategies were increasingly raised and utilized for lung adenocarcinoma, but its five-year overall survival (OS) is still very low (4). Therefore, there is still need to explore novel and effective therapeutic targets for lung adenocarcinoma.

Ubiquitination could affect proteins in many ways, mainly involved in activation, conjugation and ligation process, mediated by ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s), respectively. Ubiquitin-conjugating enzyme E2S (UBE2S) is a family of E2 protein in the ubiquitination process. Recently, it has been shown that UBE2S plays a vital role in regulating DNA damage-induced transcriptional silencing through catalyzing Lys11-linkage ubiquitination (5-8). Previous studies showed that aberrant expression of UBE2S was observed in various cancers including breast, esophageal, cervical and renal cancers (9-14). Furthermore, a previous study indicated that UBE2S played an important role in determining the malignancy properties of human colorectal cancers, and promoted its development (15). However, the critical function of UBE2S in lung adenocarcinoma remains largely unclear.

In this report, we investigated the expression and function of UBE2S in lung

adenocarcinoma. UBE2S was up-regulated in human lung cancer tissues and lung adenocarcinoma cells A549. Patients with higher expression of UBE2S had poorer prognosis compared with patients with lower UBE2S expression. UBE2S silencing suppressed the proliferation and colony formation and induced the apoptosis of lung cancer cells. Furthermore, microarray assay showed that numerous genes were regulated by UBE2S. p53 signaling pathway might be essential to the function of UBE2S in lung cancer. Thus, UBE2S might be a potential therapeutic target in lung adenocarcinoma.

Results

UBE2S expression in lung cancer tissues and cells

We firstly investigated the protein abundance of UBE2S in lung cancer samples using immuno-histochemical assay. The clinical and pathological characteristics of the patients were described in Table S1. Initially, UBE2S was moderately up-regulated in cancer tissues comparing with normal tissues (Figure 1A and Table S2). The expression of UBE2S was correlated with clinical T phase of lung cancer patients (Table S1) in our data. Then the mRNA expression of UBE2S in lung cancer and normal tissues was analyzed based on the TCGA database. The results showed that UBE2S mRNA level was increased in lung cancer tissues as compared with non-paired or paired normal tissues (Figure 1B and C). Interestingly, UBE2S mRNA expression was correlated with the clinical N migration and pathological stages of lung cancer patients in TCGA data (Table S3). Furthermore, UBE2S mRNA and protein expression was higher in lung cancer cells 95D, H1299, H1975 and lung adenocarcinoma cells A549 than in BEAS-2B normal cells (Figure 1D). Importantly, patients with higher UBE2S expression exhibited shorter overall survival than those with lower ones (Figure 1E). We predict that UBE2S is involved in lung cancer development.

UBE2S knockdown suppresses the proliferation and growth of A549 cells

To evaluate the effect of UBE2S on cell proliferation, lentivirus-mediated UBE2S knockdown and over-expression were performed in A549 cells and H1299 cells, respectively. qRT-PCR and Western blot results showed that UBE2S was efficiently

silenced in shUBE2S-1 and shUBE2S-2 A549 cells (Figure 2A and B) and it was over-expressed in H1299 cells (Figure 2C and D). Then the shCtrl and shUBE2S A549 cells were subjected to cell proliferation analysis. Cell growth screening assay showed that UBE2S knockdown inhibited the proliferation of A549 cells (Figure 2E and F). Consistently, MTT analysis also indicated that the viability of A549 cells was suppressed by shUBE2S-1 and shUBE2S-2 (Figure 2G). Furthermore, colony number was reduced in shUBE2S-1 and shUBE2S-2 A549 cells as compared with shCtrl cells (Figure 2I). By contrast, UBE2S over-expression increased the viability and colony formation capacity of H1299 cells (Figure 2H and J). Collectively, UBE2S promotes the proliferation and colony formation of A549 cells.

UBE2S enhances the BrdU incorporation and suppresses the apoptosis of lung cancer cells

Next, BrdU incorporation assay was performed to verify the effect of UBE2S on cell proliferation. Consistent with the Figure 2, BrdU incorporation was reduced in shUBE2S-1 and shUBE2S-2 A549 cells, and it was enhanced in UBE2S over-expressed H1299 cells (Figure 3A and B). To determine the role of UBE2S on cell survival, shCtrl and shUBE2S A549 cells were subjected to apoptosis analysis through flow cytometry. We observed that the number of cell apoptosis was increased in shUBE2S A549 cells as compared to shCtrl cells (Figure 3C and D). Additionally, UBE2S silencing mediated by shUBE2S-1 and shUBE2S-2 resulted in increased caspase 3/7 activity and UBE2S ectopic expression led to decreased caspase 3/7 activity (Figure 3E and F). Western blot analysis showed that cleaved PARP1 was

up-regulated in shUBE2S-1 and shUBE2S-2 A549 cells and it was down-regulated in UBE2S over-expressed H1299 cells (Figure 3G and H). Taken together, UBE2S promoted cell proliferation and suppressed apoptosis in lung cancer cells.

Profiling the genes regulated by UBE2S

To explore the downstream genes and pathways regulated by UBE2S, we performed microarray analysis to identify differential gene expression in shCtrl and shUBE2S A549 cells. A total of 171 genes were up-regulated and 287 genes were down-regulated by UBE2S knockdown (Figure 4A). Pathway enrichment analysis showed that UBE2S-regulated genes were mainly involved in activation of P53, Integrin and ILK signaling pathway and suppression of Coagulation, ATM and Acute Phase Response signaling pathway (Figure 4B). We also performed correlation analysis of downstream targets regulated by UBE2S. As shown in Figure 4C, the green boxes represented down-regulated genes. Red ones represented up-regulated genes. Previous studies have shown that these dys-regulated genes were also regulated by NUPR1. Correlation analysis indicated that NUPR1 served as the mediator between the downstream targets and UBE2S (Figure 4C). This suggested that UBE2S knockdown might cause up-regulation of NUPR1, which was correlated with the dys-regulation of various genes. Therefore, NUPR1 maybe an important downstream target for UBE2S. Moreover, consistent with the microarray data, P53 and NUPR1 were up-regulated, and ILK was down-regulated by UBE2S knockdown in A549 cells (Figure 4D). Taken together, UBE2S regulation of these genes maybe important for its role in lung cancer.

Discussion

In the last decade, the treatment strategies, targeting specific molecular subsets of NSCLC, especially lung adenocarcinoma, have acquired remarkable success. Typically, small molecule tyrosine kinase inhibitors of epidermal growth factor (EGFR) and anaplastic lymphoma kinase (ALK) provided a comparable degree of clinical benefit to patients with mutations in EGFR and rearrangements involving ALK gene (16-22). However, the function of these targeted agents is still limited, partly due to primary or secondary resistance. These facts facilitated us to explore the molecular mechanisms underlying the progression of lung adenocarcinoma thus to identify potential targets for developing effective targeted treatment.

UBE2S, a family of E2 protein, mainly contributes to poly-ubiquitination or ubiquitin chain formation during ubiquitination process (23-25). It plays an essential role in timely degrading protein regulators of the cell cycle for the completion of cell division (26). Up-regulation of UBE2S was observed in various cancers. Previous studies have reported that UBE2S participates in the progression of human cancer. For example, UBE2S promotes colorectal cancer development and hepatocellular carcinoma cell proliferation and migration (15, 27). Reduction of UBE2S is also involved in dietary flavonoids, luteolin and quercetin inhibition of cervical cancer invasion (28). In this study, we found that UBE2S was up-regulated in lung cancer tissues and several cell lines, and UBE2S expression was inversely correlated with the prognosis of lung adenocarcinoma patients. This suggested that UBE2S might contribute to the development of lung adenocarcinoma. We subsequently knocked

down UBE2S using lentivirus strategy and found that UBE2S silencing inhibited the proliferation and colony formation of A549 cells and induced its apoptosis. These results suggested that UBE2S was a potential oncogene for lung adenocarcinoma.

Functional and mechanistic studies have identified various downstream targets of UBE2S in cancers. In hepatocellular carcinoma, UBE2S potentiates the ubiquitination of p53 and cancer cell proliferation and migration (27). p21, a downstream effecter of p53, is also regulated by UBE2S in oral squamous cell carcinoma (29). In addition, UBE2S promotes colorectal cancers through stabilizing β-Catenin via K11-linked polyubiquitination (15). Here, using microarray assay, we showed that UBE2S knockdown led to dys-regulation of various genes. Pathway enrichment suggested that p53 signaling pathway was negatively regulated by UBE2S. Additionally, UBE2S knockdown activated the integrin and ILK signaling and inhibited coagulation system. Still, further studies should be performed to illustrate the participation of these signaling pathways in lung cancer development.

In summary, our study showed that UBE2S was a potential oncogene in lung adenocarcinoma. UBE2S was up-regulated in human lung cancer tissues and cell lines. Knockdown of UBE2S significantly inhibited the proliferation and colony formation of lung adenocarcinoma cell A549, and induced its apoptosis. Microarray analysis revealed that UBE2S could regulated the expression of different genes, including p53 signaling pathway. Therefore, targeting UBE2S is a potential targeted strategy for lung adenocarcinoma.

Materials and Methods

Microarray of human lung adenocarcinoma and adjacent normal samples

The HLug-Ade060PG-01 microarrays of lung adenocarcinoma and adjacent normal samples were obtained from Outdo Biotech. This array contains a total of 30 samples with 5 stage I, 15 stage II, 10 stage III lung adenocarcinoma samples and 30 adjacent normal samples. A core represented a separate case and each sample was fixed in formalin. 5-μm-thick slices coated by paraffin were subjected to immunohistochemistry staining of UBE2S.

TCGA gene expression data

UBE2S transcriptome expression datasets and the clinical information were downloaded from the websites of The Cancer Genome Atlas (http://cancergenome.nih.gov). 522 tumor tissues, 379 normal tissues, and 57 pairs of cancer and adjacent normal tissues were used for analyzing the UBE2S expression in this study.

Cell culture

Lung epithelial cells BEAS-2B which are derived from bronchial epithelium of one normal subject were obtained from the American Type Culture Collection. Lung adenocarcinoma cells 95D, H1299, H1975 and A549 were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All the cells were cultured in RPMI1640 medium containing 10% fetal bovine serum and 1% antibiotics. These cells were maintained at 37 ℃ incubator containing 5% CO_2.

UBE2S knockdown assay

UBE2S was knocked down using pGCSIL-GFP lentivirus vectors in A549 cells. pGCSIL-GFP was co-transfected with pHelper1.0 and Helper2.0 into 293T cells using Lipofectamine TM 2000 (Invitrogen, Shanghai, China). Viral supernatants were collected 48 hours later and filtered through 0.45μm filters. Then, the supernatants were used to infect A549 cells. The targeted sequences were as follow: shCtrl, 5'-TTCTCCGAACGTGTCACGT-3'; shUBE2S-1, 5'-CATATGCTGGAGGTCTGTT-3'; shUBE2S-2, 5'-GGGCTCTCTTCCTCCTTCCAC-3'. qRT-PCR and Western blot were used to determine the infection efficacy.

UBE2S over-expression in H1299 cells

The cDNA sequence of UBE2S was inserted into the pCDH lentivirus vector. Ctrl or UBE2S pCDH vectors, accompained with the PSPAX2 and PDM2G packaging vectors, were transfected into 293T cells. 3 days later, the virus supernatants were collected, filtered through 0.45 μm filters and then subjected to infecting H1299 cells. UBE2S over-expression was detected by qRT-PCR and Western blot assays.

Western blot

A549 cells expressing shCtrl or shUBE2S lentivirus were cultured for 48 hours and used for protein isolation. In brief, the culture medium was removed and the cells were washed by PBS. Lysis buffer (100 mM Tris-HCl, pH = 7.4 l 0.15 M NaCl; 5 mM EDTA, pH = 8.0; 1% Triton X100; 5 mM DTT; 0.1 mM PMSF) was added into the 6 cm plates to extract total protein. BCA Protein Assay Kit (Pierce, Rockford, IL, USA) was used for protein quantification. A total of 30 μg protein lysis were loaded into

SDS-PAGE electrophoresis and subsequently transferred onto PVDF transmembrane. Membranes were blocked with 5% skim milk for 1 h at room temperature and then incubated with primary antibodies overnight at 4 °C. After washing the membranes with PBST, HRP conjugated secondary antibodies were added. ECL-Plus kit (Amersham Biosciences, Pollards Wood, UK) was used to detect the immunoactivity. Primary antibody against UBE2S (ab197945), p53 (ab1431) and NUPR1 (ab6028) were purchased from Abcam. Primary antibody against ILK (#3856) and Cleaved PARP1 (#5625) were purchased from Cell Signaling Technology. GAPDH primary antibody (SC-32233) and the secondary antibodies were obtain from Santa Cruz.

Total RNA isolation and quantitative real-time PCR

shCtrl and shUBE2S A549 cells were cultured for 48 hours and washed by PBS. Trizol reagent (Invitrogen) was added into the wells and total RNA was isolated using RNeasy Mini kit (QIAGEN), according to the manufacturer's instructions. A total of 1 μg RNA was subjected to reverse-transcription using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO). TransStart Top Green qPCR SuperMix (TransGen Biotech) was used for the quantitative real-time PCR on an IQ-5 machine. The primer sequences are as follows: UBE2S forward, 5'-GTGCTCAAGAGGGACTGGACG-3', and reverse, 5'-GCAGACTCGGGGTTAGGGTG-3'; GAPDH forward, 5'-TGACTTCAACAGCGACACCCA-3', and reverse, 5'-CACCCTGTTGCTGTAGCCAAA-3'. GAPDH serves as internal control.

High-content screening assay

High-content screening (HCS) assay was used to detect the number of viable cells. This system is a computerized, automated fluorescence-imaging microscope which automatically detects stained cells and analyzes the intensity and distribution of fluorescence in each individual cell. Briefly, shCtrl and shUBE2S A549 cells were cultured in 96-well plates for 5 days. Cell numbers were scaned by fluorescence-imaging microscope of 20×objective using ArrayScan™ HCS software (Cellomics Inc). The intensity and distribution of the green fluorescence indicated the viable cells.

MTT assay

shCtrl and shUBE2S A549 cells were seeded in 96-well plates at a density of 3000 cells per well. 1, 2, 3, 4 and 5 days later, each well was washed by PBS and then 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) was added into the wells. 3 hours later, the MTT solution was removed and dimethyl sulfoxide (DMSO) was added. 10 minutes later, the cell viability was determined by detecting OD value at 490 nm on a micro-plate reader.

Colony formation assay

Equal number of shCtrl and shUBE2S A549 cells (800 cells per well) were seeded in six-well plates. After cultured for 12 days, cell colonies were fixed by methanol for half an hour and stained with Giemsa solution for 15 minutes. The colony number was analyzed by a fluorescence microscopy (Olympus).

BrdU incorporation assay

BrdU incorporation assay was performed to determine DNA synthesis in proliferating

cells using BrdU kit (Roche, No.11647229001), following the manufacturer's instructions.

Apoptosis analysis

Apoptosis of shCtrl and shUBE2S A549 cells detected by annexin V-APC kit (Ebioscience, USA), following the manufacturer's instructions. The cells were washed by PBS and were re-suspended by staining buffer. After adding 5μl annexin V-APC into the 100 μl cell suspension, the mixture was incubated for 15 min at room temperature. Then they were subjected to flow cytometry detection of cell apoptosis (FACSCalibur, Becton-Dickinson, USA).

Caspase 3/7 activity analysis

A total of 10000 cells were seeded into each well of the 96-well plates, and 100 μl Caspase-Glo reagent (Promega) was added into each well. Then the plates were rotated at 300-500 rpm for 30 min and incubated at room temperature for 90 min. The activity was detected by the microplate reader.

Gene expression profiling by microarray assay in A549 cells

RNA was extracted from shCtrl and shUBE2S A549 cells using Trizol reagents. Genes were expressed differentially when meet the criterion of fold change >2 and P value <0.05. Pathway enrichment or gene network analysis was performed using Ingenuity Pathway Analysis (IPA). Affymetrix human GeneChip primeview was used for gene expression analysis, according to the protocols as described previously (30).

Statistical analysis

GraphPad prism 6.0 was used to analyze the data as shown by mean ± SEM of at least

three independent repeats. All the results were based on three independent experiments. Difference between two groups was subjected to unpaired students' t tests. Difference among groups was analyzed by one-way ANOVA. P value less than 0.05 was considered statistical significance.

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None.

Conflicts of interest

The authors declare no conflict of interest.

285 **Figure Legends**

286 **Figure 1. UBE2S expression is increased in lung cancer tissues and cells and high**
287 **expression of UBE2S predicts poor outcome of lung cancer patients.**

288 (A) Immuno-histochemical analysis of UBE2S expression in lung cancer and normal
289 tissues.

290 (B) UBE2S mRNA expression in lung cancer (n=522) and normal tissues (n=379)
291 which is derived from the Cancer Genome Atlas (TCGA). $p<0.01$.

292 (C) UBE2S mRNA expression in lung cancer and adjacent normal tissues which is
293 derived from the Cancer Genome Atlas (TCGA). FC, Fold Change, n=57, $p<0.001$.

294 (D) UBE2S mRNA expression and protein level were determined respectively by
295 qRT-PCR and Western blot assays in BEAS-2B, 95D, H1299, H1975 and A549 cells.
296 **$p<0.01$, ***$p<0.001$.

297 (E) Lung cancer patients were divided into UBES2 high (Green line) and low (Blue
298 line) expression group. The overall survival of the patients was determined after
299 operation. $p<0.001$.

300 **Figure 2. UBE2S knockdown suppresses the proliferation of A549 cells.**

301 (A and B) shCtrl, shUBE2S-1 and shUBE2S-2 A549 cells were subjected to qRT-PCR
302 (A) and Western blot (B) analysis of UBE2S. GAPDH serve as internal control.
303 **$p<0.01$.

304 (C and D) Control (Ctrl) and UBE2S over-expressed (UBE2S-OE) H1299 cells were
305 subjected to qRT-PCR (C) and Western blot (D) analysis of UBE2S. GAPDH serve as
306 internal control. **$p<0.01$.

(E and F) (E) Representative images of high-content screening (HCS) of shCtrl (top) and shUBE2S-1 (bottom) A549 cells from day 1 to day 5. HCS detected the numbers of viable cells. (F) Relative quantification of the HCS results (Cell count). **p<0.01, ***p<0.001.

(G) The viability of shCtrl, shUBE2S-1 and shUBE2S-2 A549 cells was determined by MTT assay. *p<0.05, **p<0.01.

(H) The viability of Control (Ctrl) and UBE2S over-expressed (UBE2S-OE) H1299 cells was determined by MTT assay. *p<0.05, **p<0.01.

(I) shCtrl, shUBE2S-1 and shUBE2S-2 A549 cells were subjected to colony formation assay. **p<0.01.

(J) Control (Ctrl) and UBE2S over-expressed (UBE2S-OE) H1299 cells were subjected to colony formation assay. *p<0.05.

Figure 3. UBE2S induces the BrdU incorporation and suppresses the apoptosis of lung cancer cells.

(A) The BrdU incorporation of shCtrl, shUBE2S-1 and shUBE2S-2 A549 cells. **p<0.01.

(B) The BrdU incorporation of Control (Ctrl) and UBE2S over-expressed (UBE2S-OE) H1299 cells. **p<0.01.

(C and D) The apoptosis of shCtrl and shUBE2S-1 A549 cells was determined by Flow cytometry. (C) Representative images of the flow cytometry results, and (D) quantification of apoptosis. **p<0.01.

(E) The caspase 3/7 activity of shCtrl, shUBE2S-1 and shUBE2S-2 A549 cells. **p<0.01.

(F) The caspase 3/7 activity of Ctrl and UBE2S-OE H1299 cells. *p<0.05.

(G) Western blot analysis of cleaved-PURP1 in shCtrl, shUBE2S-1 and shUBE2S-2 A549 cells.

(H) Western blot analysis of cleaved-PURP1 in Ctrl and UBE2S-OE H1299 cells.

Figure 4. Dys-regulated genes in A549 cells after UBE2S knockdown.

(A) A total of 458 genes were regulated (171 genes up-regulated and 287 genes down-regulated) by UBE2S knockdown in A549 cells (P<0.05, fold change >1.5).

(B) Pathway enrichment was analyzed by IPA software. Orange, activated pathway. Blue, suppressed pathway.

(C) Correlation map of the up-regulated NUPR1 and its potential downstream targets. Red and orange box, up-regulated gene. Green box, down-regulated gene. Blue arrow, consistent repression. Orange arrow, consistent activation. Yellow arrow, inconsistent correlation.

(D) Western blot analysis of P53, NUPR1 and ILK in shCtrl and shUBE2S A549 cells.

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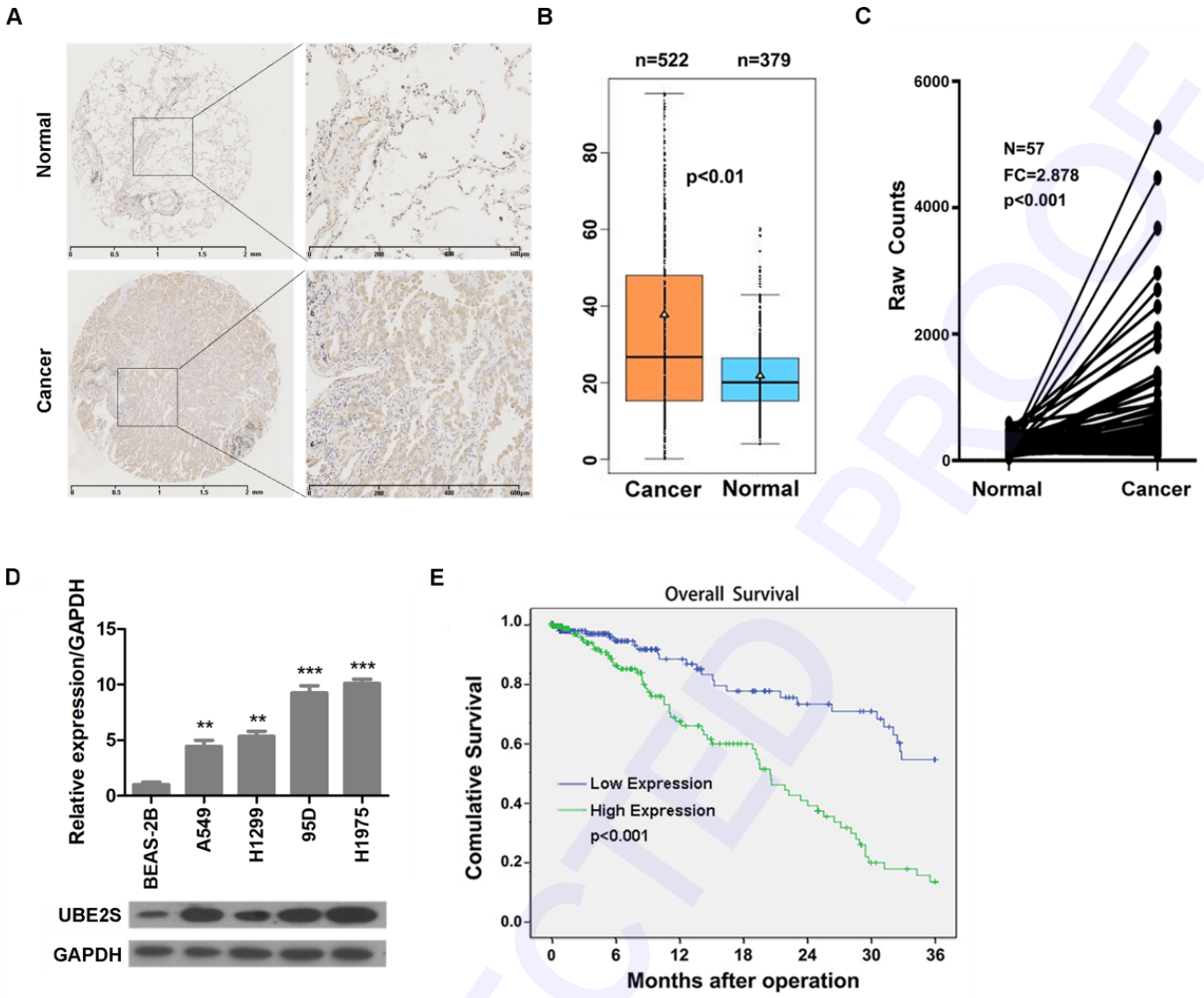

Fig. 1. Figure 1

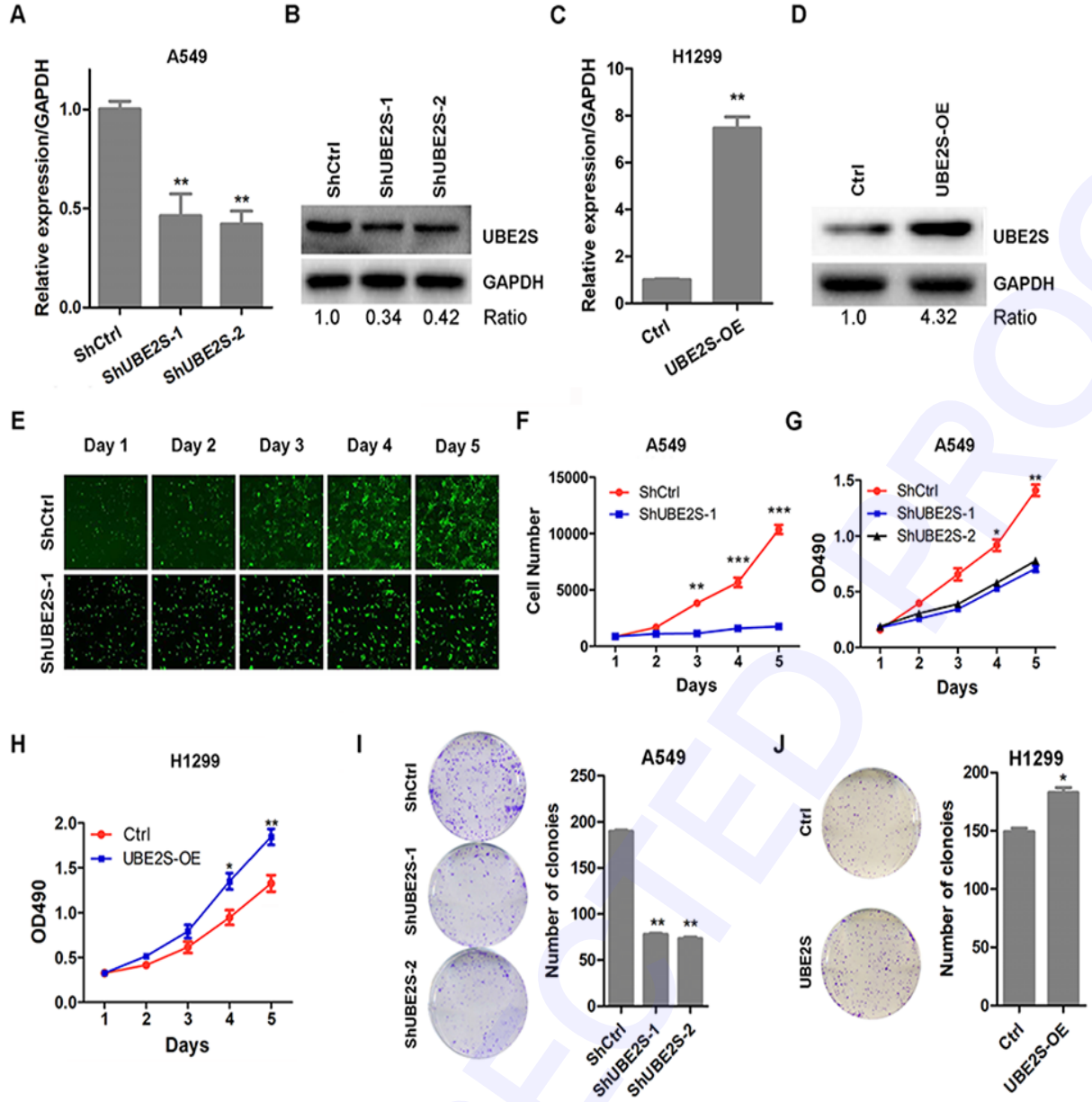

Fig. 2. Figure 2

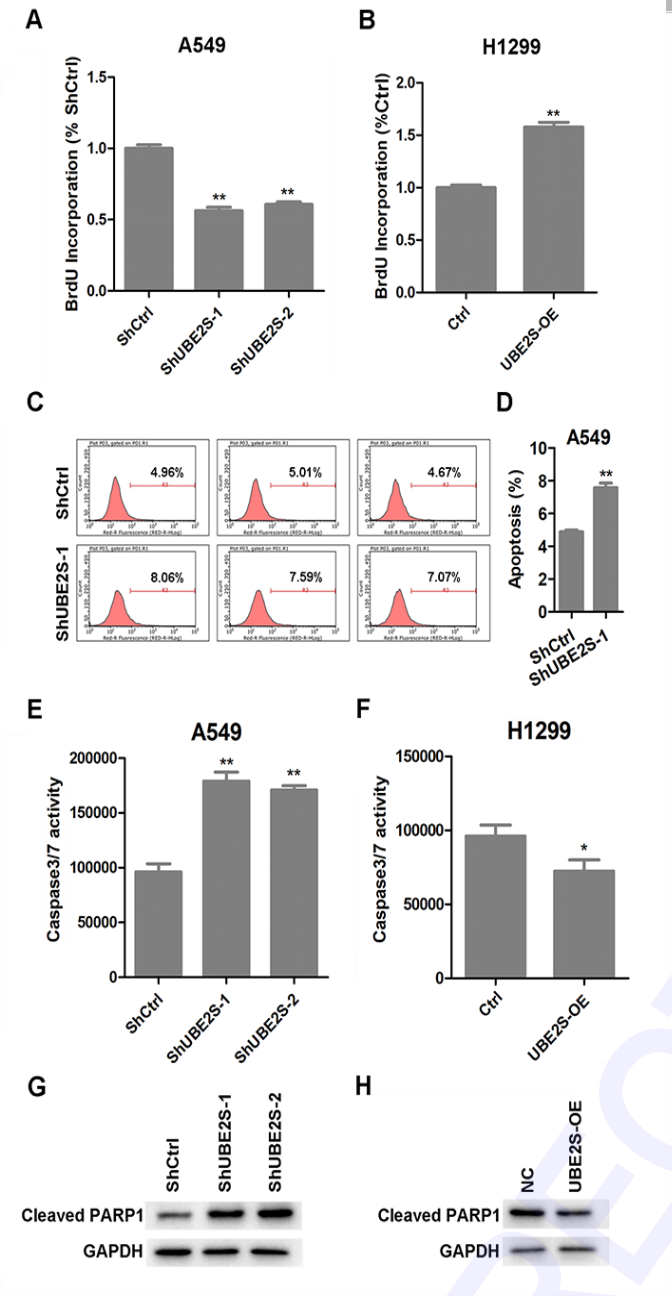

Fig. 3. Figure 3

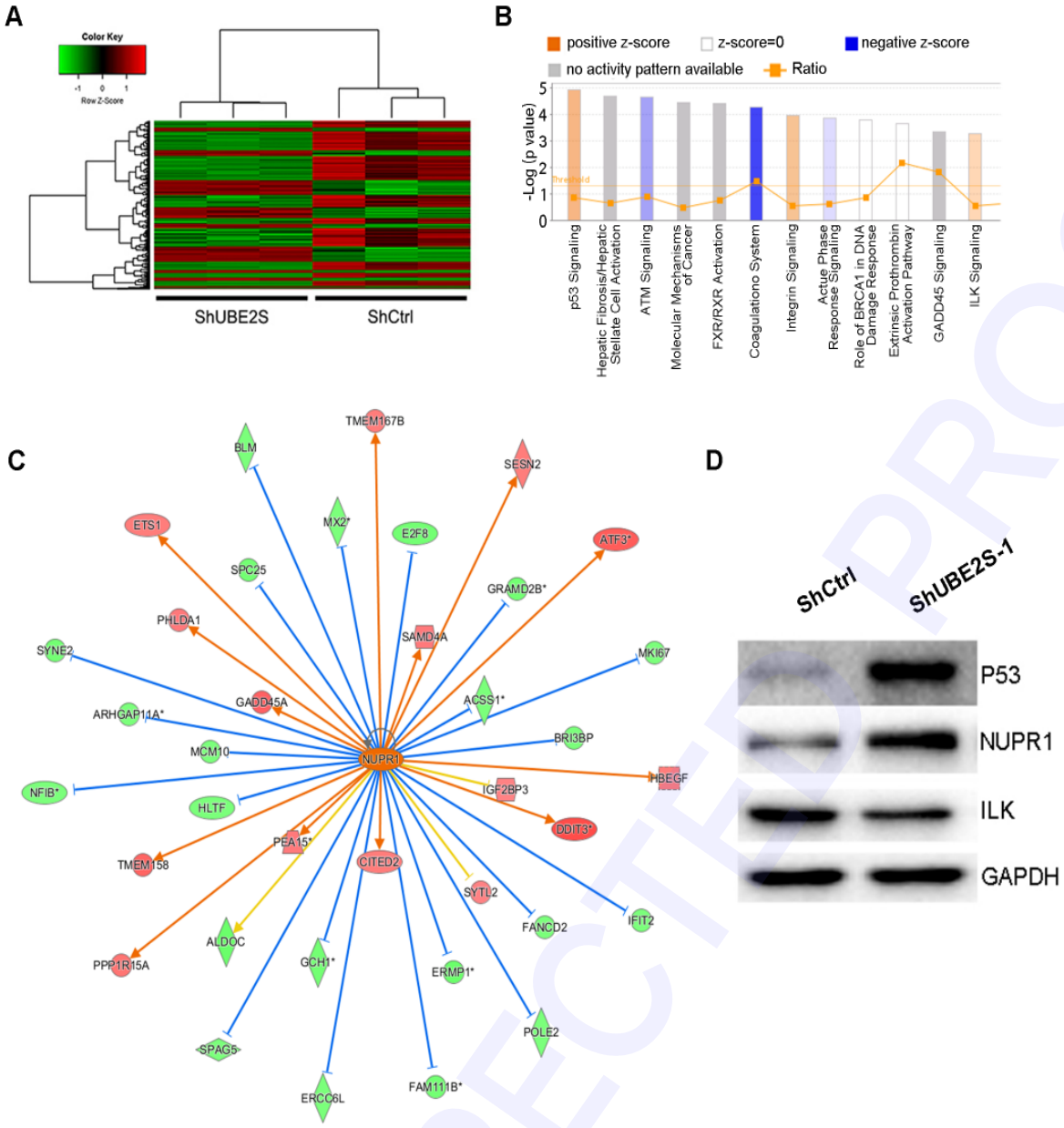

Fig. 4. Figure 4

Table S1 Relationship between UBE2S protein level and clinical pathological parameters by IHC staining

		Expression of UBE2S		Total	P value
		Low	High		
Age					
	≤61	10	4	14	.942
	>61	11	4	15	
Total		21	8	29	
Gender					
	Male	8	3	11	.982
	Female	14	5	19	
Total		22	8	30	
Tumor Size					
	<3cm	5	0	5	.368
	≥3cm	17	8	25	
Total		22	8	30	
T Phase					
	T1	14	1	15	.035
	T2/3	8	7	15	
Total		22	8	30	
N Migration					
	N0	4	0	4	.418
	Nx	15	8	23	
Total		19	8	27	
Stage					
	I	3	0	3	.565
	II	12	5	17	
	III	7	3	10	
Total		22	8	30	

Table S2 The protein level of UBE2S in human lung cancer and normal tissues

	n	UBE2S-negative	UBE2S-positive	χ^2	*p*
Normal	30	30 (100.0%)	0 (0%)	9.231	0.002
Lung cancer	30	22 (73.3%)	8 (26.7%)		

Table S3 Relationship between UBE2S mRNA expression and clinical pathological parameters by TCGA

		Expression of UBE2S		Total	P value
		Low	High		
T Phase					
	T1	94	71	165	.058
	T2	123	144	267	
	T3	20	25	45	
	T4	10	8	18	
Total		247	248	495	
N Migration					
	N0	170	151	321	.021
	N1/2/3	70	97	167	
Total		240	248	488	
M Metastasis					
	M0	166	167	333	.555
	M1	10	13	23	
Total		176	180	356	
Pathological Stage					
	Stage I	151	121	272	.007
	Stage II	54	65	119	
	Stage III	33	50	83	
	Stage IV	11	13	24	
Total		249	249	498	