USP52 inhibits cell proliferation by stabilizing PTEN protein in non-small cell lung cancer

**Running title: USP52 Inhibits Proliferation of NSCLC.**

Maoshu Zhu¹ ² #, Hui Zhang¹ ² #, Fuhua Lu¹ ² #, Zhaowei Wang¹ ², Yulong Wu¹ ², Huoshu Chen¹ ², Xin Fan³, Zhijiang Yin¹ ² *, Fulong Liang¹ ² *

1. The Fifth Hospital of Xiamen, Xiamen, 361101, China.

2. Xiang’an Branch, The First Affiliated Hospital of Xiamen University

3. Oncology Department, Xiamen Haicang Hospital, Xiamen 361026, China

*These authors contributed equally to this work and should be considered co-first authors

**Correspondence:** Zhijiang Yin, The Fifth Hospital of Xiamen, Xiamen, 361101, China. Email: Yzj701023@163.com.

Fulong Liang, The Fifth Hospital of Xiamen, Xiamen, 361101, China. Email: fulong_liang1964@126.com.

Acknowledgements

This research was supported by Scientific research personnel training project of Fujian (grant no.2019-ZQNB-34), Science and technology project of Fujian (grant no.2019D028), Science and technology project of Xiamen (grant no. 3502Z20184042, 3502Z20209226), The Science and Technology Program of Haicang District of Xiamen city (Grant No. 350205Z20174006).

**Data Availability Statement:** all supporting data are included within the main article

**Conflict of interest:** The authors declare no conflicts of interest.

**Author Contributions:** Conception and Design: Maoshu Zhu, Hui Zhang, Zhijiang Yin, Fulong Liang; Development of Methodology: Maoshu Zhu, Hui Zhang, Fuhua Lu, Zhaowei Wang; Acquisition of Data and Material: Yulong Wu, Huoshu Chen; Analysis and Interpretation of Data: Maoshu Zhu, Hui Zhang, Zhaowei Wang; Writing, Fulong Liang; Review, and/or Revision of the Manuscript: Maoshu Zhu, Hui Zhang, Fuhua Lu, Xin Fan, Zhijiang Yin, Fulong Liang.
Abstract
Non-small cell lung cancer (NSCLC) is the most common subtype of lung cancer. Ubiquitination is closely related to the development of lung cancer. However, the biological importance of newly discovered ubiquitin specific peptidase 52 (USP52) in NSCLC remained unclear. Here, our findings identify USP52 as a novel tumor suppressor of NSCLC, the low expression of USP52 predicts a poor prognosis for NSCLC patients. This study demonstrates that USP52 inhibits cancer cell proliferation through downregulation of cyclin D1 as well as AKT/mTOR signaling pathway inhibition. Meanwhile, USP25 also suppresses NSCLC progression via enhancing PTEN stability in cancer cells, which further indicates the significance/importance of USP52 in NSCLC suppression.

Key Words: USP52; NSCLC; PTEN; Proliferation; Stabilization
Introduction

Lung cancer is a leading cause of cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC) is the most frequent subtype of lung cancer. In recent years, great progress has been made in the research of lung cancer. The understanding of genetic alterations that drive NSCLC is evolving. Nonetheless, the prognosis of NSCLC remains poor owning to the lack of effective treatment.

Ubiquitin proteases are a large family of cysteine proteases that specialize in cleavage of ubiquitin conjugates. The ubiquitin system regulates essential cellular processes in eukaryotes. Ubiquitin binds to substrate proteins in a monomeric or chain formation/structure, and the ubiquitin modified topological structure regulates the interactions between substrate and specific proteins. Therefore, ubiquitination directs the fate of many substrates, including proteasome degradation. The deubiquitinases drive the process of ubiquitin cleavage from substrates. This process controls the stability of most cellular proteins, and its abnormal regulation leads to the occurrence of various human diseases, including cancer. Deubiquitinase inhibition can induce the degradation of selected proteins, which potentially includes some otherwise undruggable targets. For instance, the inhibition of ubiquitin-specific protease 7 (USP7) results in the degradation of the oncogenic E3 ligase MDM2 and the reactivation of the tumor suppressor p53 in various cancers.

The USP52, also called as poly(A) specific ribonuclease subunit PAN2, was reported as a key component of P-bodies required to prevent HIF1α mRNA degradation. It was also being reported that USP52 play an important role in inhibiting antiviral immunity. Additionally, USP52 could promotes histone chaperone ASF1A stabilization. However, the antitumor function of USP52 has not been reported.

Rapid proliferation is a malignant phenotype of NSCLC. The proliferation of cancer cells is regulated by a variety of proteins and genes. Long noncoding RNA PVT1 promotes NSCLC cell proliferation through epigenetically regulating LATS2 expression. IL-6 induces neuroendocrine dedifferentiation and cell proliferation in NSCLC cells. Prognostic significance of CCND1 (cyclin D1) as a cell cycle promotor can be overexpressed in primary resected non-small-cell lung cancer. The expression level of cyclin-dependent kinase (CDK2) can also be elevated in NSCLC for the enhancement of cancer cell proliferation.
It is well known that PTEN (phosphatase and tensin homolog) is an important tumor suppressor in NSCLC. The PTEN/PI3K/AKT pathway regulates multiple cellular functions, including cell growth, proliferation, survival, motility, differentiation, invasion and intracellular trafficking. Alterations in this pathway, particularly PTEN inactivation, have been associated with resistance to the therapy of EGFR-tyrosine kinase inhibitor and results in lower survival in NSCLC patients.

PTEN, as another commonly mutated, deleted, and epigenetically silenced tumor suppressor in human lung cancers, is altered in 15% of human lung small cell cancers (SCCs). PTEN negatively regulates the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which is also altered in more than half of human NSCLCs. Here, we used human NSCLC and cell lines to research the expression of USP52, and the regulatory relationship between USP52 and PTEN. We discovered that USP52 plays an important role in NSCLC suppression by inhibiting cancer cell proliferation via PTEN stabilization, which further indicated that USP52 plays an important role in NSCLC suppression. Thus, the low expression of USP52 predicts a poor prognosis for NSCLC patients.

Materials and Methods

Survival analysis

USP52 gene expression profile (USP52 data) for lung cancer patients was obtained from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/). Clinical data such as gender, age, histological type, survival and outcome were also downloaded from TCGA data portal.

Molecular biology

HA-tagged USP52 constructs were made using the pCDH-GFP-Puro vector (https://www.addgene.org/167463/). Sequences encoding the HA epitope were added by PCR through replacement of the first Met-encoding codon in the respective cDNA clones.

To produce USP52-overexpressing cell lines, H292 and H460 cells were infected with lentivirus particles expressing HA-USP52 plasmids in the presence of 10 µg/ml polybrene. Cells infected with retroviruses expressing pCDH-GFP-Puro plasmids were used as controls.

Cell Culture and Treatments
Human bronchial epithelial cells (HBE), NSCLC cell lines H292, H460 and LTEP-A-2 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in a medium containing 10% fetal bovine serum (FBS; HyClone Laboratories Inc., Logan, UT, USA) and 1% penicillin- streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C in the presence of 5% CO2. Media containing cells were passaged every 2-3 days. Four independent series of treatments were conducted to obtain the three technical repeats that were used for all studies.

**Study of human primary lung cancer specimens**

This study was approved by the Medical Ethics Committee of The Fifth Hospital of Xiamen and Patients provided written informed consent in accordance with the legal and institutional ethical guidelines defined by the hospital (Ethical approval number: 2019-XMSDWYYLL-051). We collected 18 lung cancer specimens with tumor-adjacent lung tissues from patients who had undergone resection at the Fifth Hospital of Xiamen and stored the tissues at the tissue bank of the Fifth Hospital of Xiamen. The patients were diagnosed with NSCLC at the Fifth Hospital of Xiamen, and diagnoses were made according to the World Health Organization criteria.

**RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-qPCR)**

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA was synthesized using Invitrogen SuperScript III Reverse Transcriptase kit according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). Besides, SYBR Green-Based RT-qPCR was performed using Mx3000P qPCR System (Stratagene California, San Diego, CA, USA) according to the manufacturer’s instructions. Three independent amplifications were performed for each sample in each technique. In addition, GAPDH was taken as an internal reference into consideration. The primer sequences used for RT-qPCR are shown in Table 1.

**Cell proliferation analysis**

According to the kit manual instructions, cell proliferation assay was performed using the CCK-8. The transfected cells (5x10^3) were seeded in 96-well plates with 90 µl medium containing 10% FBS in each well. After different treatments, 10 ul CCK-8 solution was added to each well with 100 ul culture medium following 2 h incubation at 37 °C, the number of cells in each well was calculated by measuring the absorbance at 450 nm using Microplate Reader.
Western blot analysis

Protein extracts were prepared by lysis in a buffer containing 50 mM Tris-HCl (pH 7.4), 0.1% Triton X-100, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1 mM Na3VO4, and protease inhibitors (Roche, Basel, Switzerland). Proteins were separated by SDS–PAGE, and then transferred onto polyvinylidene difluoride (PVDF) membranes. Blots were probed with the following antibodies: anti-USP52 mouse monoclonal (Santa Cruz Biotechnology, Inc., Dallas, TX, USA, 1:1000), anti-GAPDH mouse monoclonal (Invitrogen, Carlsbad, CA, USA, 1:3000), anti-cyclinD1 mouse monoclonal (Invitrogen, Carlsbad, CA, USA, 1:1000), anti-CDK2 mouse monoclonal (Invitrogen, Carlsbad, CA, USA, 1:1000), anti-p53 mouse monoclonal (Invitrogen, Carlsbad, CA, USA, 1:2000), anti-p-AKT mouse monoclonal (Invitrogen, Carlsbad, CA, USA, 1:1000), anti-AKT mouse monoclonal (Invitrogen, Carlsbad, CA, USA, 1:1000), anti-p-mTOR mouse monoclonal (Invitrogen, Carlsbad, CA, USA, 1:1000), anti-mTOR mouse monoclonal (Invitrogen, Carlsbad, CA, USA, 1:1000), and mouse monoclonal anti-PTEN antibody (Cell Signaling Technology, Inc., Danvers, MA, USA, 1:1000).

Immunohistochemical analysis

All specimens were formalin-fixed and paraffin-embedded. Immunohistochemistry (IHC) staining were performed. In briefly, Tissue sections were dehydrated in gradient concentration of ethanol solution and fixed with 4% paraformaldehyde at room temperature for 30 min, and then paraffin-embedded. Consecutive 4-µm sections were used for analysis. Antigen retrieval was performed by microwaving sections in citrate buffer (pH 6.0). Subsequently, slides were incubated overnight with USP52 antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA, 1:100) at 4˚C. Subsequently, slides were block with goat serum (cat. no. AR0009; Wuhan Boster Biological Technology, Ltd; 1:20) at room temperature for 1 h and then incubated with goat anti-rabbit second antibody (cat. no. ab6721; Abcam; 1:2,000) at RT for 1 h. Streptococcal avidin-biotinylated peroxidase system (Thermo Fisher Scientific, Inc.) was used to develop the color according to the manufacturer’s instructions. Tissue sections were visualized using an Olympus microscope IX50 at 20x magnification and the images were analyzed using Image J 1.49 version software (National Institutes of Health). The American Joint Committee on Cancer (AJCC) Staging Manual system was used to assess tumor grades.

Protein Degradation Experiment

H292 cell lines were secondly transfected with control or USP52-HA, respectively, and then, were
treated with cycloheximide (CHX) or MG-132 for indicated time periods. After 48 h, cells were harvested, and the expression of PTEN was detected by Western blot analysis and quantification analysis of gray scanning.

Statistical analysis

All data were presented as mean ± standard deviation (SD). These data were evaluated by one-way analysis of variance (ANOVA) using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). P < 0.05 was considered statistically significant.

Results

1. Low USP52 predicts a poor prognosis for NSCLC patients.

Deubiquitination plays a decisive role in lung cancer, such as USP7(5). USP52 acts as a deubiquitinase and promotes histone chaperone ASF1A stabilization(8). However, the function of USP52 in NSCLC has not been reported. In order to explore it, we screened publicly available datasets to determine the prognostic correlation between the USP52 level and survival of NSCLC patients. Kaplan-Meier survival analysis revealed that the survival time for patients with low USP52 expression was obviously shorter than those with high USP52 expression (p = 0.00041) (Fig.1A).

We then examined the mRNA expression of USP52 in ten pairs of fresh primary NSCLC tissues compared with individual normal para-cancerous tissues. The result showed that, the mRNA expression levels of USP52 were dramatically reduced in NSCLC tissues compared to normal tissues (Fig.1B). Consistently, IHC staining assays further confirmed that, the expression of USP52 protein was also dramatically reduced in the NSCLC tissues compared with normal tissues (Fig.1C). Moreover, the expression of USP52 were also detected in human NSCLC cell lines (H292, NCI-H460 and LTEP-A-2). Consistent with the human specimen, the result show that USP52 were also significantly low expression compared with the human bronchial epithelial cells (HBE) (Fig.1D).

Collectively, it manifested that low level of USP52 expression was associated with poor prognosis for NSCLC patients.

2. Overexpression of USP52 inhibits cell growth in NSCLC cells.
The above data revealed that the USP52 is lowly expressed in NSCLC, and we speculated that USP52 is a tumor suppressor gene in NSCLC. In order to study whether USP52 inhibits the proliferation of NSCLC tumor cells, we took the following research.

Firstly, we established H292 and H460 cell lines with overexpression of USP52. Overexpression of USP52 was successfully verified in protein expression (Fig.2A). Then the CCK-8 analysis was used to analyze the cell proliferation at 0, 1, 3 and 5 days after USP52 overexpressed. The OD value measured at OD450 nm showed that the proliferation of the H292 and H460 cells were significantly inhibited compared to the control group after USP52 overexpression (P < 0.05) (Fig.2B). It is proving that USP52 suppresses NSCLC by inhibiting cell proliferation.

Further, we wanted to demonstrate the mechanism of how USP52 inhibits the proliferation of NSCLC. It was well known that Cyclin D1 regulates cell proliferation(18). Cyclin D1 is believed to promote NSCLC cell proliferation and functions as regulators for Cyclin-dependent Kinases (CDK)(19). The cell cycle is a tightly regulated process controlled by cyclin-dependent kinase (CDK)-cyclin complex(20). CDK2 was also known as promote cell proliferation(21). In this study, the expression of Cyclin D1 and CDK2 were detected in H292 and H460 cells by overexpression USP52. The result shows that the expression of Cyclin D1 and CDK2 were decreased after USP52 overexpression. Conversely, the expression of tumor suppressor p53 was increased (Fig. 2C, D).

Together, these results indicated that the overexpression of USP52 could inhibit cell proliferation of NSCLC, and it was declared that USP52 suppresses NSCLC through the mechanism of inhibiting cell cycle regulatory proteins of Cyclin D1 and CDK2, as well as promotes p53 expression.

3. **USP52 inhibits AKT signaling in NSCLC cells.**

Same as cyclin D1 and CDK2, it has been reported that AKT/mTOR pathway also play crucial role in promote cell growth(22). In order to further understand whether USP52 decreased cell growth by regulating AKT/mTOR signaling, H292 and H460 cells were transfected with dose-dependent USP52 plasmid. In response to USP52 overexpression, the level of phospho-AKT (p-AKT) and phospho-mTOR (p-mTOR) was markedly reduced (Fig. 3A, B, C and D), which verifies the activation status of AKT/mTOR pathway. These results indicated that USP52
suppresses NSCLC via inhibiting the activation of AKT/mTOR pathway.

4. **USP52 and PTEN were decreased in NSCLC tissues.**

PTEN inhibits the PI3K/AKT pathway by catalyzing the dephosphorylation of PIP3, while the loss of PTEN induces the activation of PI3K/AKT cascade, thereby stimulating cell growth and survival(23). To confirm the correlation between PTEN and USP52 in lung cancer, firstly, we have quantified the expression of PTEN and USP52 in lung cancer tissues. Western blot results further revealed that PTEN and USP52 expression were also notably decreased compared with normal para-cancerous tissues (Fig. 4A and B). Further experiments found that the expression levels of PTEN and USP52 in tumor tissues have a certain correlation (R^2=0.6211, Fig. 4C and D).

We hypothesized that the decrease of PTEN was on account of the low expression of USP52. That is, USP52 inhibited the tumorigenesis of NSCLC by upregulating the tumor suppressor of PTEN.

5. **USP52 stabilizes PTEN protein in NSCLC cells.**

It has been reported that CK1α could suppresses lung tumor growth by stabilizing PTEN(24). In order to confirm the regulatory relationship between USP52 and PTEN, we analyzed the expression of PTEN in USP52 overexpressed H292 cells. Western blot and qPCR have been used to test the protein and mRNA, respectively. The Western blot results exhibited that the expression of PTEN protein in USP52 overexpressed H292 cell lines was significantly higher than that in control group in a dose-dependent manner (Fig.5A, B). Whereas the expression levels of PTEN mRNA in USP52 overexpressed H292 cell lines has no significant difference compare with the control group (Fig.5C). It is suggested that USP52 may regulate the expression of PTEN through a posttranscriptional mechanism.

Furthermore, we determined to explore whether USP52 affects PTEN protein stability. Here, we used the protein synthesis inhibitor cycloheximide (CHX) to inhibit the transport step in protein synthesis (the movement of mRNA and two tRNA molecules on the ribosome) and block the extension of peptides during translation, resulting in cell growth arrest even death. Indeed,
PTEN was remarkably attenuated and degraded slower in H292 cells after USP52 overexpression (Fig. 5 D and E). When the proteasome pathway is inhibited by MG-132, the ability of USP52 to regulate PTEN is inhibited, which further proves that USP52 affects the protein level of PTEN through the deubiquitination pathway (Fig. 5 F and G). In general, the above results imply that the USP52 inhibit NSCLC cell lines proliferation through the mechanism of strongly promotes the protein stability of antioncogene PTEN.

Discussion

Ubiquitination regulation is widely involved in the regulation of various biological process including autophagy(25), cell-cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, and endocytosis(26). Abnormalities in ubiquitin-mediated processes have been shown to cause pathological conditions including malignant transformation(26). Although USP52 exhibits no deubiquitylating activity on account of the lack of an active-site cysteine residue(27), it was required for HIF1A mRNA stability (6). Recently, it has been reported that USP52 could promotes histone chaperone ASF1A stabilization(8). In this study, we have found that USP52 was expressed at a low level in NSCLC specimen in NSCLC specimen, and the survival time for patients with low USP52 expression was obviously shorter than those with high USP52 expression. This indicated that USP52 play an important role in NSCLC suppression.

Tumor-related cell cycle defects are usually mediated by alterations in the activity of cyclin-dependent kinase (CDK). Dysregulated CDKs induce unscheduled proliferation as well as genomic and chromosomal instability(28). Emerging evidence suggests that tumor cells may also require specific interphase CDKs for proliferation(28). The overexpression of cyclin D1, a cell cycle-regulating protein, is associated with poor histological differentiation in NSCLC (11, 29). In this study, we have found that USP52 overexpression in NSCLC cell lines could inhibit cyclin D1 and CDK2 expression, which suggested that USP52 suppresses NSCLC through the mechanism of inhibits the expression of cell cycle regulatory proteins of cyclin D1 and CDK2.

The gene encoding the components of the PI3K-AKT-mTOR signaling axis is often mutated in cancer, but mTOR, which encodes mTOR kinase, is rarely mutated. The researchers used publicly
available tumor genome sequencing data identifying 33 mTOR mutations that confer pathway hyperactivation(30). In this research we have identified that USP52 overexpression inhibits the activation of AKT/mTOR pathway, which suggested that USP52 also through inhibits AKT/mTOR pathway to suppresses NSCLC. Whereas the mechanism remains to be further explored.

The anticancer activity of PTEN mainly depends on its lipid phosphatase activity, which is in contrary to the activation of PI3K/AKT. As a typical tumor suppressor gene that inhibits the cascade of PI3K/AKT/mTOR pro-growth signals, PTEN dysfunction leads to the miss adjustment of this pathway and other pathways, leading to excessive growth(23). PTEN regulates many cellular processes, including proliferation, survival, energy metabolism, cell structure, and movement. More than a decade of research has expanded our knowledge of how PTEN is controlled at the transcriptional level and regulates its enzyme activity, protein stability, and cell location through extensive post-transcriptional modifications(31). In this study we found that the overexpression of USP52 could upregulates PTEN. In addition, USP52 strongly stabilizes PTEN protein in NSCLC cells.

In conclusion, this study demonstrated that USP52 is essential for inhibiting the NSCLC cells proliferation. The low expression of USP52 predicts a poor prognosis for NSCLC patients. And indicated that USP52 suppresses the NSCLC cells proliferation through the mechanism of inhibits the cell cycle regulator of cyclin D1, CDK2 and AKT/mTOR pathway. Furthermore, USP52 suppresses the NSCLC cells proliferation through the mechanism of promotes the protein stability of PTEN via the deubiquitination manner.

REFERENCES

1. Gester F, Paulus A, Sibille AL, Duysinx B, Louis R. [PROGNOSTIC FACTORS IN NON SMALL CELL LUNG CANCER]. Revue medicale de Liege. 2016;71(1):34-9.
2. Burki TK. Treatment options not taken for non-small-cell lung cancer. The Lancet Oncology. 2017;18(3):e135.
3. Wilson SM, Bhattacharyya B, Rachel RA, Coppola V, Tessarollo L, Householder DB, et al. Synaptic defects in ataxia mice result from a mutation in Usp14, encoding a ubiquitin-specific protease. Nat Genet. 2002;32(3):420-5.
4. Katgeya L, Di Lello P, Rougé L, Pastor R, Clark KR, Drummond J, et al. USP7 small-molecule inhibitors interfere with ubiquitin binding. Nature. 2017;550(7677):534-8.
5. Turnbull AP, Ioannidis S, Krajewski WW, Pinto-Fernandez A, Heride C, Martin ACL, et al.
Molecular basis of USP7 inhibition by selective small-molecule inhibitors. Nature. 2017;550(7677):481-6.

6. Bett JS, Ibrahim AF, Garg AK, Kelly V, Pedrioli P, Rocha S, et al. The P-body component USP52/PAN2 is a novel regulator of HIF1A mRNA stability. The Biochemical journal. 2013;451(2):185-94.

7. Liu Q, Wu Y, Qin Y, Hu J, Xie W, Qin FX, et al. Broad and diverse mechanisms used by deubiquitinase family members in regulating the type I interferon signaling pathway during antiviral responses. Science advances. 2018;4(5):eaar2824.

8. Yang S, Liu L, Cao C, Song N, Wang Y, Ma S, et al. USP52 acts as a deubiquitinase and promotes histone chaperone ASF1A stabilization. Nature communications. 2018;9(1):1285.

9. Wan L, Sun M, Liu GJ, Wei CC, Zhang EB, Kong R, et al. Long Noncoding RNA PVT1 Promotes Non-Small Cell Lung Cancer Cell Proliferation through Epigenetically Regulating LATS2 Expression. Molecular cancer therapeutics. 2016;15(5):1082-94.

10. Chang KT, Tsai CM, Chiou YC, Chiu CH, Jeng KS, Huang CY. IL-6 induces neuroendocrine dedifferentiation and cell proliferation in non-small cell lung cancer cells. American journal of physiology Lung cellular and molecular physiology. 2005;289(3):L446-53.

11. Betticher DC, Heighway J, Hasleton PS, Altermatt HJ, Ryder WD, Cerny T, et al. Prognostic significance of CCND1 (cyclin D1) overexpression in primary resected non-small-cell lung cancer. British journal of cancer. 1996;73(3):294-300.

12. Wang X, Y, LU, Feng W, Chen Q, Guo H, Sun X, et al. A two kinase-gene signature model using CDK2 and PAK4 expression predicts poor outcome in non-small cell lung cancers. 2016.

13. Xu L, Wang C, Wen Z, Yao X, Liu Z, Li Q, et al. Selective up-regulation of CDK2 is critical for TLR9 signaling stimulated proliferation of human lung cancer cell. Immunology letters. 2010;127(2):93-9.

14. Song L, Li D, Gu Y, Wen ZM, Jie J, Zhao D, et al. MicroRNA-126 Targeting PIK3R2 Inhibits NSCLC A549 Cell Proliferation, Migration, and Invasion by Regulation of PTEN/PI3K/AKT Pathway. Clinical lung cancer. 2016;17(5):e65-e75.

15. Pérez-Ramírez C, Cañas-Garre M, Molina M, Faus-Dáder MJ, Calleja-Hernández M. PTEN and PI3K/AKT in non-small-cell lung cancer. Pharmacogenomics. 2015;16(16):1843-62.

16. Salmena L, Carracedo A, Pandolfi PP. Tenets of PTEN tumor suppression. Cell. 2008;133(3):403-14.

17. Hammerman PS LM, Voet D, Jing R, Cibulskis K, Sivachenko A, Stojanov P, McKenna A, Lander ES, Gabriel S, Getz G, Sougnez C, Imielinski M, Helman E, Hernandez B, Pho NH, Meyerson M, Chu A, Chun HJ, Mungall AJ, Pleasance E, Robertson A, Sipahimalani P, Stoll D, Balasundaram M, Birol I, Butterfield YS, Chuah E, Coope RJ, Corbett R, Dhallan N, Guin R, He A, Hirst C, Hirst M, Holt RA, Lee D, Li HI, Mayo M, Moore RA. Comprehensive genomic characterization of squamous cell lung cancers. Nature. 2012;489(7417):519-25.

18. Mohamed TMA, Ang YS, Radzinsky E, Zhou P, Huang Y, Elfenbein A, et al. Regulation of Cell Cycle to Stimulate Adult Cardiomyocyte Proliferation and Cardiac Regeneration. Cell. 2018;173(1):104-16.e12.

19. Liu B, Li X, Sun F, Tong X, Bai Y, Jin K, et al. HP-CagA+ Regulates the Expression of CDK4/CyclinD1 via reg3 to Change Cell Cycle and Promote Cell Proliferation. International journal of molecular sciences. 2019;21(1).

20. Malumbres M. Cyclin-dependent kinases. Genome biology. 2014;15(6):122.
21. Jablonska B, Aguirre A, Vandenbosch R, Belachew S, Berthet C, Kaldis P, et al. Cdk2 is critical for proliferation and self-renewal of neural progenitor cells in the adult subventricular zone. The Journal of cell biology. 2007;179(6):1231-45.

22. Di Leo A, Johnston S, Lee KS, Ciruelos E, Lønning PE, Janni W, et al. Buparlisib plus fulvestrant in postmenopausal women with hormone-receptor-positive, HER2-negative, advanced breast cancer progressing on or after mTOR inhibition (BELLE-3): a randomised, double-blind, placebo-controlled, phase 3 trial. The Lancet Oncology. 2018;19(1):87-100.

23. Yehia L, Keel E, Eng C. The Clinical Spectrum of PTEN Mutations. Annual review of medicine. 2020;71:103-16.

24. Cai J, Li R, Xu X, Zhang L, Lian R, Fang L, et al. CK1α suppresses lung tumour growth by stabilizing PTEN and inducing autophagy. Nature cell biology. 2018;20(4):465-78.

25. Grumati P, Dikic I. Ubiquitin signaling and autophagy. The Journal of biological chemistry. 2018;293(15):5404-13.

26. Hershko A, Ciechanover A. The ubiquitin system. Annual review of biochemistry. 1998;67:425-79.

27. Quesada V, Diaz-Perales A, Gutierrez-Fernandez A, Garabaya C, Cal S, Lopez-Otin C. Cloning and enzymatic analysis of 22 novel human ubiquitin-specific proteases. Biochemical and biophysical research communications. 2004;314(1):54-62.

28. Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. Nature reviews Cancer. 2009;9(3):153-66.

29. Jin M, Inoue S, Umemura T, Moriya J, Arakawa M, Nagashima K, et al. Cyclin D1, p16 and retinoblastoma gene product expression as a predictor for prognosis in non-small cell lung cancer at stages I and II. 2001;34(2):207-18.

30. Grabiner BC, Nardi V, Birsoy K, Possemato R, Shen K, Sinha S, et al. A diverse array of cancer-associated MTOR mutations are hyperactivating and can predict rapamycin sensitivity. Cancer discovery. 2014;4(5):554-63.

31. Worby CA, Dixon JE. PTEN. Annual review of biochemistry. 2014;83:641-69.
| Name | accession numbers | Forward sequence | Reverse sequence | amplicon size |
|------|------------------|------------------|------------------|---------------|
| USP52 | NM_001394701     | GTGGGTGTACCTGTTTCCGT | GGATGAGTAGCGCTCCAAGG | 117 |
| GAPDH | NM_001256799     | AAGCCTGCCCCGTTGACTAAC | GTAAAAAGCAGCCCTGTTGAC | 174 |
| PTEN | NM_000314        | TCCCAGACATGACAGCCATC | TGTTTGAATCCAAAAACCTTACT | 190 |

Table 1. Primer sequences
**Figure legends**

**Figure 1 Low expression of USP52 predicts a poor prognosis for NSCLC patients.**

A. The overall survival periods of lung cancer patients with low or high expression of USP52 were estimated by Kaplan-Meier plotter (http://kmplot.com). B. Ten pairs of fresh primary NSCLC tumor tissues (T) and individual normal para-cancerous tissues (N) were prepared for qRT-PCR against USP52. C. Representative images of the Immuno-histochemistry (IHC) staining for USP52 in NSCLC tissues and normal para-cancerous tissues. D. Three NSCLC cell lines and one human bronchial epithelial cell were prepared for qRT-PCR against USP52. *P < 0.05, **P < 0.01.

**Figure 2 Overexpression of USP52 inhibits cell growth in NSCLC cells.**

A. H292 and H460 cells were stably infected with lentiviral LV-NC or LV-USP52, followed by Western blot against USP52. GAPDH was used as a loading control. B. H292 and H460 cells stably infected with LV-NC or LV-USP52 were cultured for indicated time, followed by CCK-8 assay at day 0, 1, 3, or 5. C. H292 and H460 cells were stably infected with 1 LV-NC or LV-USP52, followed by Western blot against Cyclin D1, CDK2, p53, and GAPDH. D. The relative abundance of Cyclin D1, CDK2 AND p53 in H292 and H460 from (C) were quantified by gray scanning after LV-USP52 plasmids transformed at 0, 1, 2, 4 μg. These experiments were repeated for three times. One-way ANOVA, *P< 0.05, **P<0.01. NS, not significant.

**Figure 3 USP52 inhibits AKT signaling in NSCLC cells.**

A. H292 cells were transfected with empty vector (EV) or USP52-HA plasmids for 48 h, followed by Western blot against p-AKT, AKT, p-mTOR, mTOR and HA. GAPDH was used as a loading control. B. The relative abundance of p-AKT and p-mTOR in H292 from (A) were quantified by gray scanning after USP52-HA plasmids transformed at 0, 1, 2, 4 μg. C. H460 cells were transfected with empty vector (EV) or USP52-HA plasmids for 48 h, followed by Western blot against p-AKT, AKT, p-mTOR, mTOR and HA. GAPDH was used as a loading control. D. The relative abundance of p-AKT and p-mTOR in H460 from (C) were quantified by gray scanning after USP52-HA plasmids transformed at 0, 1, 2, 4 μg. These experiments were repeated for three times. One-way ANOVA, *P< 0.05, **P<0.01. NS, not significant.

**Figure 4 USP52 and PTEN were decreased in NSCLC tissues.**
A. Two representative pairs of fresh primary NSCLC tumor tissues (T) and individual normal para-cancerous tissues (N) were prepared for Western blot against USP52 and PTEN.  

B. The relative abundance of USP52 and PTEN in N and T from (A) were quantified by gray scanning as 2# and 5# patients, respectively. These experiments were repeated for three times. One-way ANOVA test, *P< 0.05, **P<0.01. NS, not significant.  

C. Eight representative pairs of fresh primary NSCLC tumor tissues were prepared for Western blot against USP52, PTEN and GAPDH.  

D. Use linear function to analyze the correlation between USP52 and PTEN expression, and fit linear function Y=1.0034x-0.182, R²=0.6211.  

Figure 5 USP52 stabilizes PTEN protein in NSCLC cells.  

A. Control or USP52-HA plasmids were transfected into H292 cells for 48 h, followed by Western blot against PTEN and HA.  

B. The relative abundance of PTEN in H292 from (A) were quantified by gray scanning after USP52-HA transformed at 0,1,2,4 μg. These experiments were repeated for four times. One-way ANOVA, *P< 0.05, **P<0.01. NS, not significant.  

C. qRT-PCR against PTEN after USP52-HA transformed at 0,1,2,4 μg.  

D. H292 cells were transfected with USP52-HA or empty vector (Control) for 24 h, followed by CHX chase assay at indicated time. Then, Western blot was performed to assess the expression levels of PTEN and USP52-HA.  

E. Statistically analysis of (D).  

F. H292 cells were transfected with USP52-HA plasmid (control) and treated with MG-132 respectively, and then Western blot was performed on PTEN, USP52 and GADPH, where - means untreated (control).  

G. The relative abundance of PTEN, USP52 and GADPH in H292 from (F) was analyzed by quantitative statistical analysis, **P<0.01. NS, not significant.
