Synaptotagmin 12 (SYT12) Gene Expression Promotes Cell Proliferation and Progression of Lung Adenocarcinoma and Involves the Phosphoinositide 3-Kinase (PI3K)/AKT/Mammalian Target of Rapamycin (mTOR) Pathway

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Background: This study aimed to use bioinformatics analysis to compare data from tissue microarrays from patients with lung adenocarcinoma (LUAD) and normal lung tissue, and human lung adenocarcinoma cells with normal lung epithelial cells *in vitro* to investigate the role of synaptotagmin 12 (SYT12) gene expression in LUAD.

Material/Methods: Human lung adenocarcinoma cell lines (A549, SPC-A-1, H1299, H1975, and PC9) and the normal HBE cell line were compared, and tumor xenografts were developed in mice. The Cancer Genome Atlas (TCGA) tissue microarray data were used to compare SYT12 expression and overall survival (OS). The *in vivo* and *in vitro* effects of down-regulation and upregulation of SYT12 were studied using short-interfering RNA (si-RNA) and overexpression plasmids, respectively. The Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) pathway analysis, quantitative reverse transcription-polymerase chain reaction (qRT-PCR), and Western blot investigated the molecular mechanisms of SYT12 expression in LUAD.

Results: SYT12 expression was increased in tissues from patients with LUAD from TCGA and was associated with advanced tumor stage and reduced prognosis. Knockdown of SYT12 suppressed the proliferation and migration of LUAD cells, and upregulation of SYT12 increased the proliferation and migration of LUAD cells *in vitro*. Phosphorylation of PIK3R3 activated the PI3K/AKT/mTOR pathway. In the mouse xenograft model, expression of SYT12 increased the volume and weight of the xenograft tumors.

Conclusions: Bioinformatics analysis, human LUAD cells, and mouse xenograft studies showed that SYT12 acted as a possible oncogene by phosphorylation of PIK3R3 to activate the PI3K/AKT/mTOR signaling pathway.

MeSH Keywords: Lung Neoplasms • Oncogene Protein v-akt • Oncogenes • Phosphatidylinositol 3-Kinases

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Background

Worldwide, lung cancer is the leading cause of cancer-related mortality [1]. In 85% of cases, the predominant histological subtype is non-small cell lung cancer (NSCLC), which mainly includes lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LSCC), and large cell carcinoma [2]. LUAD has recently become the most frequent subtype of lung cancer, with a reduced 5-year survival rate [3]. LUAD is often diagnosed at a late stage, and the mechanisms of tumor progression are complex [3]. Recent progress in the development of targeted therapy has resulted in new treatments for patients with molecular subgroups of LUAD. Also, in the last decade, there have been developments in the management of NSCLC, including early screening and targeted therapy for patients with advanced NSCLC [4,5]. In patients with LUAD, molecular studies have identified several therapeutic targets, which have resulted in the development of new targeted therapies [5]. Therefore, the identification of functional genes or biomarkers associated with the progression of LUAD may also lead to alternative therapeutic approaches.

SYT12 is a member of synaptotagmins family. Synaptotagmins are membrane proteins which compose of a short N-terminal non-cytoplasmic sequence, a transmembrane region, a linker sequence with variable length, and two C2-domains [6]. In 1996, Thompson initially reported that the expression of the SYT12 protein was induced by thyroid hormone and was homologous to SYT1 but without Ca^{2+}-binding sequences [7]. Recently, SYT12 was shown to be a prognostic biomarker for papillary thyroid cancer [8]. However, the underlying association SYT12 and human cancers have not been studied in detail. No previous studies have investigated the biological role of SYT12 in lung cancer.

The phosphoinositide 3-kinase (PI3K)/AKT/mTOR pathway plays a vital role in cellular processes and cancer progression by regulating cancer cell proliferation, metastasis, and apoptosis [9]. PIK3R3 is one of the regulatory subunits of Class IA PI3Ks which is involved in human cancer [10]. Therapeutic molecular targeting of PI3K, AKT, and mTOR have previously been used in patients with cancer [11,12].

Therefore, this study aimed to use bioinformatics analysis to compare data from tissue microarrays from patients with LUAD and normal lung tissue, and human LUAD cells with normal lung epithelial cells *in vitro* to investigate the role of synaptotagmin 12 (SYT12) gene expression. In this study, The Cancer Genome Atlas (TCGA) database was used to examine clinical samples to explore SYT12 expression in patients with LUAD.

Material and Methods

Data sources and bioinformatics analysis

The expression of SYT12 in tissues from patients with lung adenocarcinoma (LUAD) in The Cancer Genome Atlas (TCGA) database was analyzed using the online UALCAN cancer database (*http://ualcan.path.uab.edu*) [13]. The online Kaplan-Meier plotter (*http://kmplot.com/analysis/*) [14] was used to analyze the overall survival (OS) of patients with LUAD. The online cBioPortal database (*http://www.cbioportal.org/*) [15] was used to obtain SYT12 gene frequency changes in the genomic profiles of patients with LUAD and highly co-expressed genes with SYT12. The highly co-expressed genes were analyzed by Enrichr bioinformatics tools (*http://amp.pharm.mssm.edu/Enrichr/*) [16], and analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) pathways.

Tissue samples

A total of 50 paired samples were collected from patients with lung adenocarcinoma (LUAD) who underwent surgery in the Department of Cardiothoracic Surgery, Nanjing Jinling Hospital, from 2016 to 2018. Patients who received radiotherapy or chemotherapy before surgery or had a medical history of other primary tumors were excluded. The tissues were frozen in liquid nitrogen immediately after surgical resection and then stored at –80°C. Clinicopathological data were obtained from the patient medical records At least two experienced pathologists evaluated all tissues. Informed consent was obtained from all enrolled patients before surgery. This study was approved by the Ethics Committee of Jinling Hospital.

Cell culture

Human LUAD cell lines, including A549, SPC-A-1, H1299, H1975, and PC9, and the human normal human bronchial epithelial (HBE) cell line, were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (KeyGen Biotech Co. Ltd., Nanjing, China) with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin and streptomycin (KeyGen Biotech Co. Ltd., Nanjing, China) in a humidified incubator at 37°C containing 5% CO_2.

Cell transfection and reagents used

Small interfering RNA (siRNA) for SYT12 knockdown was designed and purchased from GenePharma (Shanghai, China). For SYT12 overexpression, a full-length human SYT12 cDNA was amplified by the polymerase chain reaction (PCR) and subcloned into the expression vector pEnter (Vigene Biosciences,

Table 1. The si-RNAs used in this study.

Name	Sequence (5'->3')
SYT12 siRNA-1 sense	GAGCUGACCUACAGCUCAAUC
SYT12 siRNA-1 antisense	UUGAGCUGUAGGUCAGCUCUG
SYT12 siRNA-2 sense	GAUUCUAGAACCAAAAUAAAG
SYT12 siRNA-2 antisense	UUAUUUUGGUUCUAGAAUCAU
Non-sense siRNA sense	UUCUCCGAACGUGUCACGUTT
Non-sense siRNA antisense	ACGUGACACGUUCGGAGAATT

Shandong, China). The recombinant vector was validated by Sanger sequencing. The empty vector pEnter (Vigene) was used as the negative control. Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) was used for transfection. The efficiency of transfection was verified by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot. The sequences of siRNAs are shown in Table 1.

RNA extraction and qRT-PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from cell lines or tissues according to the protocol. Prime Script RT reagent (Takara, Minato-ku, Tokyo, Japan) was used for reverse transcription with 1500 ng RNA in a total volume of 20 ul. The cDNA was amplified by qRT-PCR with SYBR Green Master Mix I (Takara, Minato-ku, Tokyo, Japan) on a QuantStudio™ 6 Flex Real-Time PCR System. The experiments were performed in triplicate. The comparative CT method ($\Delta\Delta$CT) was used to calculate the relative level of mRNA with β-actin as the internal reference. The primers we used are shown in Table 2.

Cell proliferation and colony formation assays

The cell counting kit-8 (CCK-8) assay was implemented to measure cell proliferation. After transfection, cells were cultured in 96-well plates (2,000/well) with 100 μl of medium. The cell proliferation was measured every 24 hours for five consecutive

days using the CCK-8 assay (KeyGen Biotech Co. Ltd., Nanjing, China), according to the manufacturer's protocol. The ELX-800 Universal Microplate Reader (BioTek, Winooski, VT, USA) was used to detect the absorbance at 450 nm.

For the colony formation assay, cells were seeded in the 6-well plate at a density of 200/well after transfection, then cultured in DMEM medium with 10% FBS for 10–15 days. The medium was replaced every 3–4 days. Then, 4% paraformaldehyde was used to fix the cells for 30 min, and 0.1% crystal violet was used to stain the cells. Visible stained colonies were photographed and counted.

Transwell migration and invasion assays

For migration assay, transfected cells (40,000/well) were placed into the upper chamber of transwell insert with an 8 μm pore size (MilliporeSigma, Canada) with 200 μl of serum-free DMEM medium, 800 μl of DMEM with 10% FBS was added to the lower chamber. After culture for 24 hours, cells that migrated to the lower membrane surface were fixed in 4% paraformaldehyde for 30 min, and then stained with 1% crystal violet for 15 min. A light microscope (Olympus, Tokyo, Japan) was used to photograph and calculated migrated cells.

For the invasion assay, transfected cells (40,000/well) with 200 μl of serum-free DMEM medium were plated into the upper chamber coated by Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), 800 μl of DMEM with 10% FBS was added to the lower chamber. After culture for 72 hours, the cells that invaded the lower membrane surface were removed and analyzed in the migration assay. The experiments were performed in triplicate.

Cell cycle evaluation by flow cytometry

Transfected cells were collected and fixed in 4 ml of ice-cold 70% ethanol. After cell fixation at 4°C overnight in ethanol, the cells were centrifuged at 1,800×g for 5 min, then washed twice with ice-cold PBS, resuspended in 1 ml of propidium iodide (PI) staining solution (Beyotime, Shanghai, China) in the

Table 2. The primers used in this study.

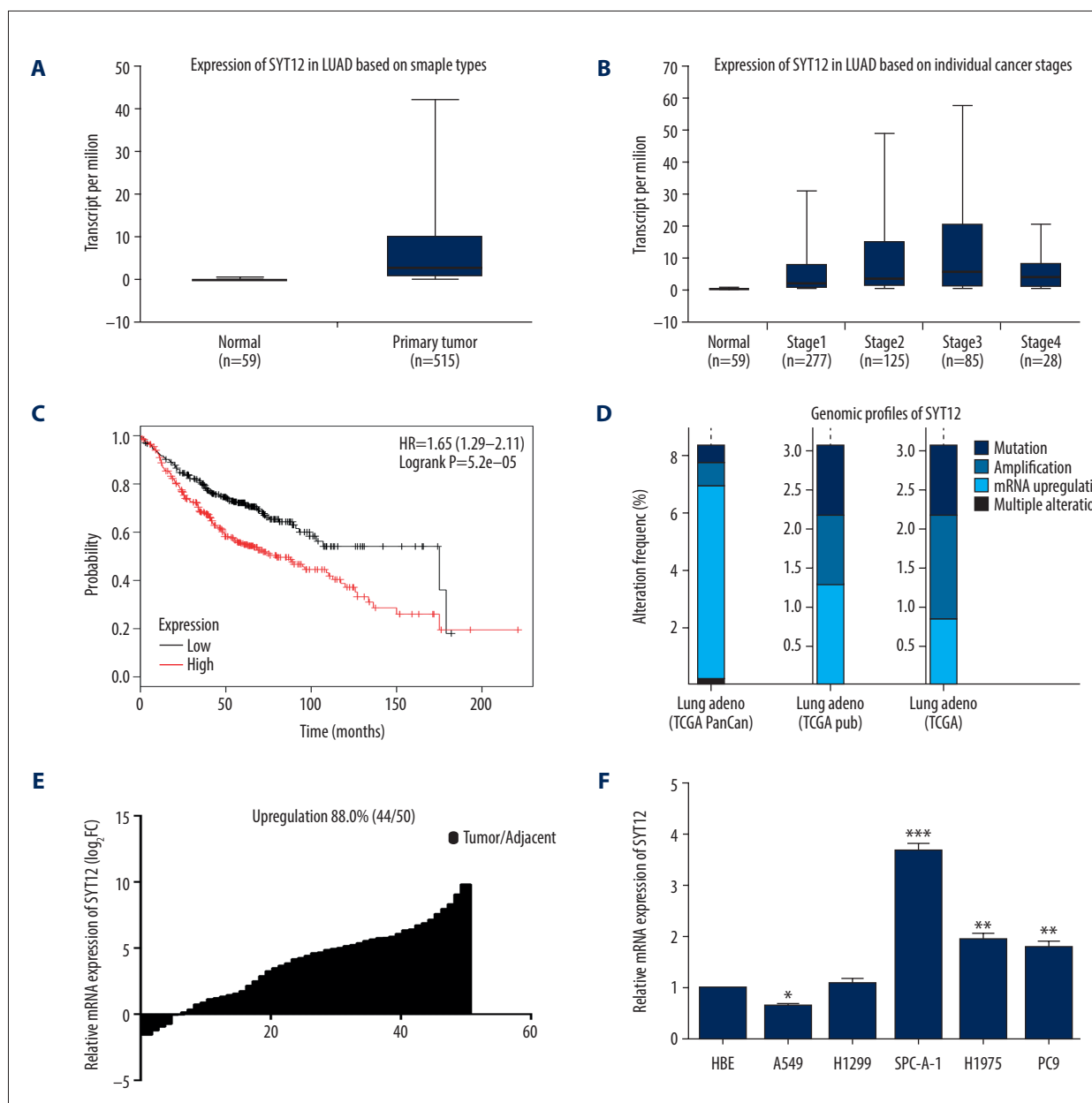| Name	Forward primer (5'->3')	Reverse primer (5'->3')
PIK3R3	CTTTGCGGAAGGGAGGCAATA	ACCACGGAATTAAATGTCAGAGG
PIK3R5	CCCCTGGTATGAGCGCAATG	CGGCAGTAGTAGAGTAGCATGT
AKT1	AGCGACGTGGCTATTGTGAAG	GCCATCATTCTTGAGGAGGAAGT
AKT2	AGGCACGGGCTAAAGTGAC	CTGTGTGAGCGACTTCATCCT
AKT3	TGTGGATTTACCTTATCCCCTCA	GTTTGGCTTTGGTCGTTCTGT
mTOR	ATGCTTGGAACCGGACCTG	TCTTGACTCATCTCTCGGAGTT
β-actin	GAAATCGTGCGTGACATTAA	AAGGAAGGCTGGAAGAGTG

dark at 37°C for 10 min. A FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used to analyze the distribution of cells in different phases of the cell cycle, including G0-G1, S, and G2-M phases. The experiments were performed in triplicate.
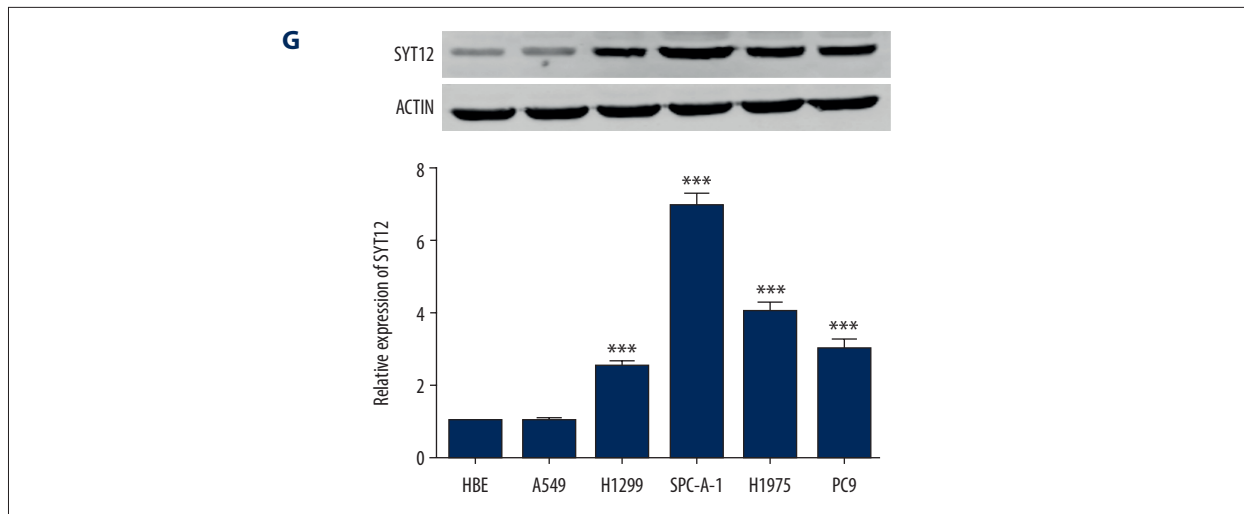
Protein extraction and Western blot

After transfection for 48 hours, cells were lysed in RIPA buffer (KeyGen Biotech Co. Ltd., Nanjing, China) containing protease inhibitors on ice. Then, 8% or 10% sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins according to the molecular weight. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes. TBS-T containing 5% bovine serum albumin (BSA) was used to block the PVDF membrane for 2 hours at room temperature.

The membranes were incubated with primary antibodies overnight at 4°C. Primary antibodies were to SYT12 (1: 1,000) (Cat. No. TA342629; Origene, Rockville, MD, USA), PIK3R3 (1: 1,000) (Cat. No. 11889; Cell Signaling Technology, Danvers, MA, USA), phospho-PIK3R3 (1: 1,000) (Cat. No. 4228; Cell Signaling Technology, Danvers, MA, USA), PIK3R5 (1: 1,000) (Cat. No. A505893; Origene, Rockville, MD, USA), pan AKT (1: 1,000) (Cat. No. 44691; Cell Signaling Technology, Danvers, MA, USA), phospho-Akt (Ser473) (1: 1,000) (Cat. No. 4015; Cell Signaling

A Expression of SYT12 in LUAD based on smaple types

B Expression of SYT12 in LUAD based on individual cancer stages

C HR=1.65 (1.29–2.11) Logrank P=5.2e−05

D Genomic profiles of SYT12

E Upregulation 88.0% (44/50)

F

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Figure 1. SYT12 was overexpressed in lung adenocarcinoma (LUAD) and was associated with poor prognosis. (**A**) The results of the UALCAN cancer database analysis of LUAD (n=515) and normal lung (n=59) tissues in The Cancer Genome Atlas (TCGA) database. The mRNA level of SYT12 in normal lung samples: maximum: 0.468 transcripts per million (TPM); upper quartile: 0.189 TPM; median: 0.085 TPM; lower quartile: 0.056 TPM; minimum: 0.010 TPM. The mRNA level of SYT12 in LUAD samples: maximum: 42.277 TPM; upper quartile: 10.221 TPM; median: 2.827 TPM; lower quartile: 0.895 TPM; minimum: 0.025 TPM. ($p < 0.001$). (**B**) The median mRNA level of SYT12 in tumor tissue samples from patients with different stages of LUAD are 1.894, 3.327, 5.430, and 3.591 TPM for stages 1-4, respectively. (**C**) The online Kaplan-Meier plotter was used to analyze the overall survival (OS) of 673 patients with LUAD. The patients were divided according to the median expression of SYT12. Patients (n=337) with low expression of SYT12 had a significantly higher OS compared with patients (n=336) with high SYT12 expression. (Log-rank, $p < 0.001$). (**D**) Analysis of the online cBioPortal database showed that the frequency of SYT12 gene changes, including mutation, amplification, and mRNA upregulation, was approximately 3–8% in the genomic profiles of patients with LUAD, and the frequency of SYT12 gene changes was 8.35% in the TCGA database (PanCancer Atlas) of 566 LUAD samples, 3.04% in the TCGA database (TCGA, Provisional) of 586 LUAD samples, and 3.04% in the TCGA database (TCGA, Nature 2014) of 230 LUAD samples. (**E**) SYT12 was upregulated in 44 out of 50 tissues from patients with LUAD compared with paired adjacent normal lung tissues collected from our institute. (**F**) The mRNA expression levels of SYT12 in LUAD cell lines (SPC-A-1, H1975, PC9) were significantly higher than the normal human bronchial epithelial (HBE) cell line. (**G**) The protein expression of levels of SYT12 in LUAD cell lines (H1299, SPC-A-1, H1975, PC9) were significantly higher than in the HBE cell line. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Technology, Danvers, MA, USA), phospho-Akt (Thr308) (1: 1,000) (Cat. No. 4056; Cell Signaling Technology, Danvers, MA, USA), mTOR (1: 1,000) (Cat. No. 2983; Cell Signaling Technology, Danvers, MA, USA), phospho-mTOR (Ser2448) (1: 1,000) (Cat. No. 5536; Cell Signaling Technology, Danvers, MA, USA), and beta-actin (1: 1,000) (Cat. No. 2947; Cell Signaling Technology, Danvers, MA, USA). The membranes were washed three times in TBST. Horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit or anti-mouse) were incubated on the membranes for 2 hours. The enhanced chemiluminescence (ECL) method (Thermo Fisher Scientific, Waltham, MA, USA) was used to visualize the blots. β-actin was used as the control.

Immunohistochemistry (IHC) of tissues from patients with LUAD

A tissue microarray (TMA) containing 90 pairs of human LUAD and normal adjacent lung tissues (Cat. No. HLug-Ade180Sur-01) (Outdo Biotech Co., Shanghai, China) was used to detect the SYT12 expression by IHC. The TMA was incubated with the primary antibody to SYT12 (1: 100) (Cat. No. 820401) (Biolegend San Diego, CA, USA) according to the manufacturer's instructions. Stained tissues were evaluated and scored independently by two or more experienced pathologists basing on the staining intensity and proportion of positive cells. The intensity score included four grades: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. The percentage score was divided into five levels (0, 25, 50, 75, 100), according to the percentage of positive cells. The final IHC score (a minimum of 0 and a maximum of 300) was the product of the score of the intensity and percentage of stained cells. Clinicopathologic data included the TNM stage and the overall survival (OS).

LUAD tumor xenografts in nude mice

The animal study was approved by the Animal Care Committee of Nanjing Medical University. Thirty nude mice were purchased from the accredited Animal Facility of Nanjing Medical University. The nude mice were divided equally into six groups. After transfection, 2×10^6 LUAD cells in 100 μl of sterile PBS

Table 3. Correlation between SYT12 expression and clinicopathological characteristics.

Characteristics	Low level of SYT12 expression (n=25)	High level of SYT12 expression (n=25)	P-value
Age (years)			0.571
≤65	12	14	
>65	13	11	
Gender			0.765
Male	17	16	
Female	8	9	
Smoker			0.571
Yes	14	12	
No	11	13	
Primary tumor			**0.037**
T1–T2	20	13	
T3–T4	5	12	
Lymph node			0.083
N0	18	12	
N1–NX	7	13	
Metastasis			1.000
M0	23	24	
M1–MX	2	1	
TNM stage			**0.031**
I–II	21	14	
III–IV	4	11	

were injected subcutaneously into each mouse. The tumors were harvested six weeks after injection. The weight and volume of each tumor were measured according to the following equation: length×width^2/2.

Statistical analysis

Data were presented as the mean±standard deviation (SD). Statistical differences between groups were compared using the chi-squared (χ^2) test, Student's t-test, and one-way analysis of variance (ANOVA) using SPSS version 24.0 (IBM, Chicago, IL, USA). GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA, USA) was used for the graphical presentation of data. A P-value <0.05 was considered to be statistically significant.

Results

SYT12 expression was increased in tissues from patients with lung adenocarcinoma (LUAD) and LUAD cell lines and was significantly associated with advanced tumor stage

The Cancer Genome Atlas (TCGA) tissue microarray data were analyzed using the online UALCAN cancer database (*http://ualcan.path.uab.edu*). SYT12 mRNA levels were significantly greater in tissues from patients with LUAD compared with normal lung tissues (Figure 1A), and there was an increasing expression of SYT12 from stage I to stage III LUAD (Figure 1B). The online Kaplan-Meier plotter was used to analyze the overall survival (OS) of 673 patients with LUAD. By ranking the SYT12 expression from high to low, 673 patients were divided into two. Patients with low expression of SYT12 (n=337) had significantly improved OS compared with patients with high expression of SYT12 (n=336) (Figure 1C). Analysis of the online cBioPortal database showed

that the frequency of SYT12 gene changes, including mutation, amplification, and mRNA upregulation, was approximately 3–8% in the genomic profiles of patients with LUAD. The frequency of SYT12 gene changes was 8.35% in the TCGA database (PanCancer Atlas) of 566 LUAD samples, 3.04% in the TCGA database (TCGA, Provisional) of 586 LUAD samples, 3.04% in the TCGA database (TCGA, Nature 2014) of 230 LUAD samples (Figure 1D). In 50 paired LUAD and normal lung tissues from Jinling Hospital, the mRNA level of SYT12 was upregulated in 88.0% (44/50) of tissues from patients with LUAD (Figure 1E). By ranking SYT12 level in the 50 paired tissues from high to low, half of the patients were in the high SYT12 expression group (n=25), and half of the patients were in the low SYT12 expression group (n=25). Analysis using the chi-squared (χ^2) test showed that SYT12 expression was significantly associated with T stage and TNM stage (Table 3). In the LUAD cell lines, SPC-A-1, H1975, and PC9, the mRNA level of SYT12 was also significantly increased. In the LUAD cell lines, H1299, SPC-A-1, H1975, and PC9, the protein level of SYT12 was significantly increased when compared with the normal HBE cells (Figures 1F, 1G).

SYT12 expression increased LUAD cell proliferation *in vitro*

To investigate the biological function of SYT12 in LUAD cells, siRNAs and overexpression plasmid of SYT12 (oe-SYT12) were transfected into SPC-A-1 and A549 cell lines, respectively. The transfection efficiency was verified (Figure 2A–2D). Both siRNAs reduced the mRNA level of SYT12 effectively, and siRNA-1 was selected in the following experiments using si-SYT12. The cell counting kit-8 (CCK-8) assays confirmed that the down-regulation of SYT12 significantly reduced SPC-A-1 cell proliferation (Figure 2E). However, overexpression of SYT12 significantly increased A549 cell proliferation (Figure 2F). Knockdown of SYT12 reduced the number of SPC-A-1 cells in the S phase of the cell cycle and increased the number of SPC-A-1 cells in the G1 phase and G2 phase. Overexpression of SYT12 upregulated A549 cells in the S phase of the cell cycle and reduced the number of A549 cells in the G2 phase (Figure 2G–2J). These results showed that SYT12 had an oncogenic role by increasing cell proliferation and resulting in changes in the cell cycle of LUAD cells.
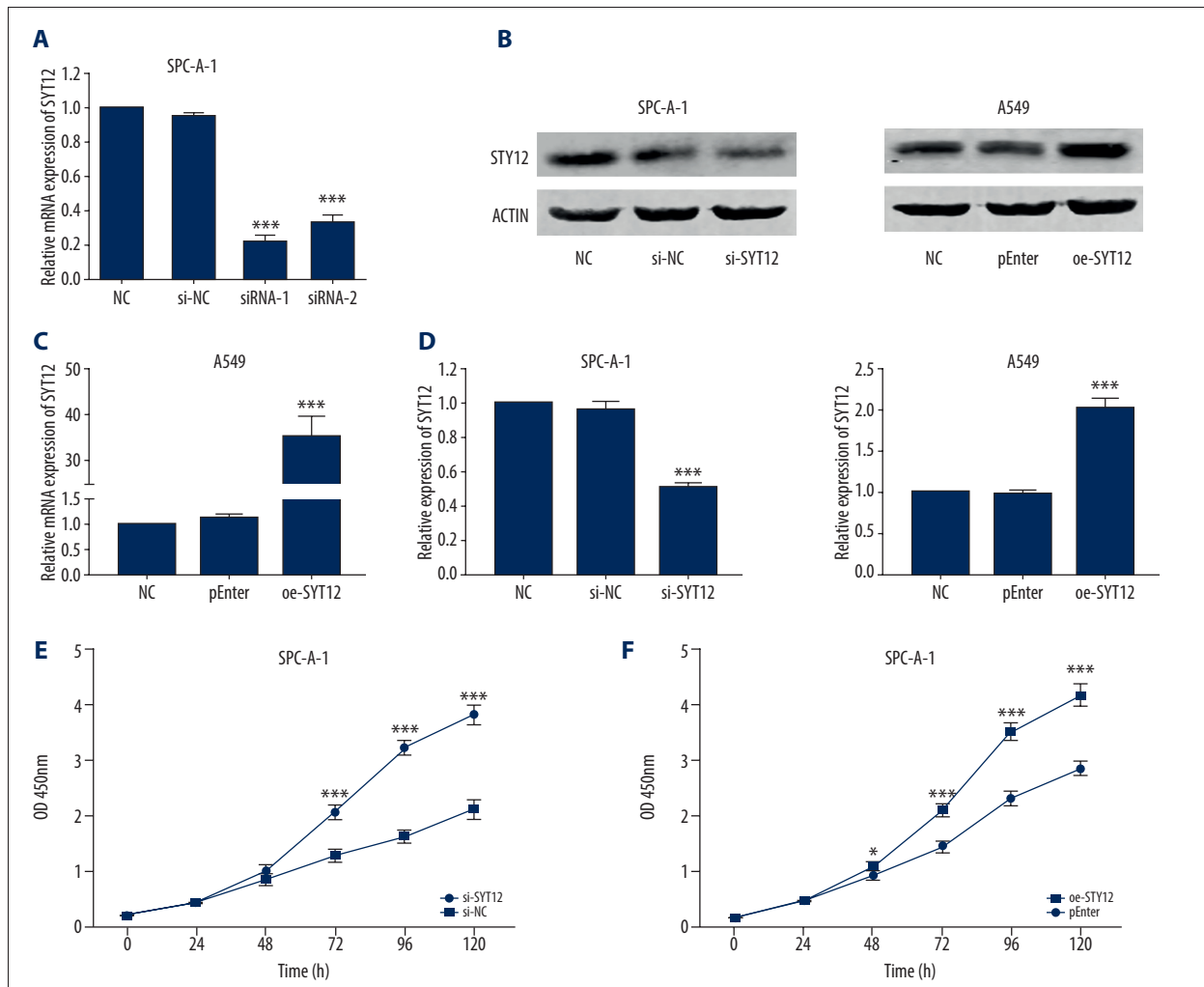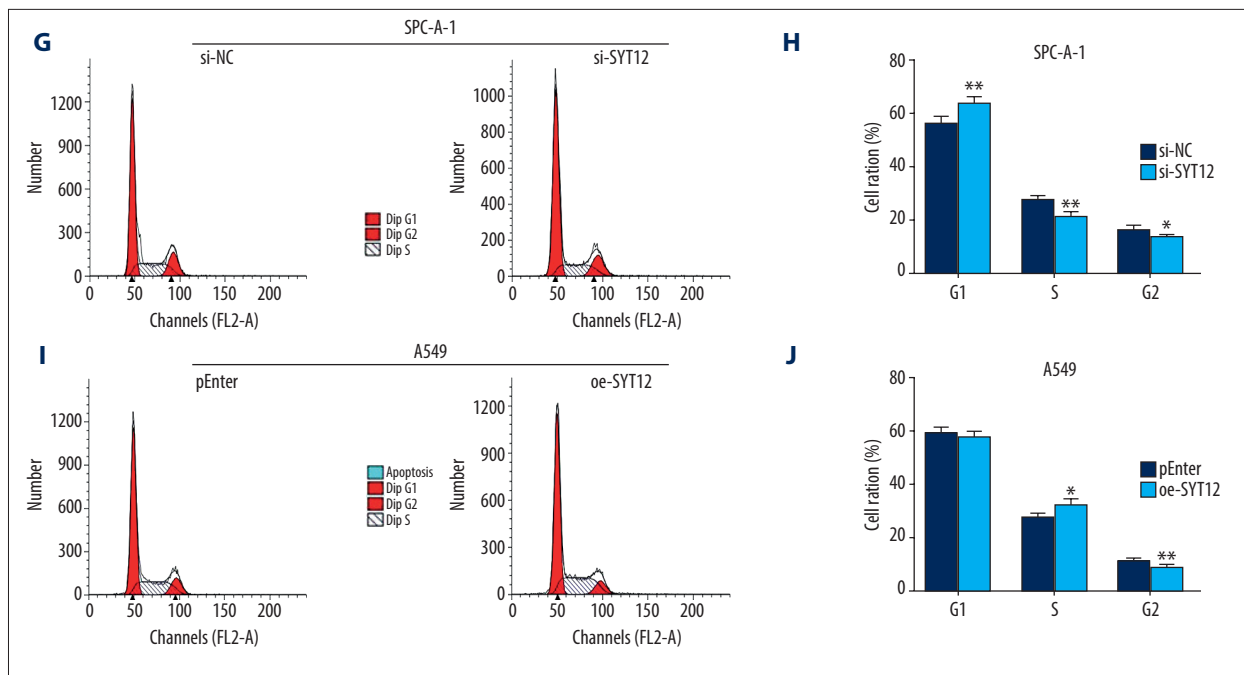
Figure 2. Knockdown of SYT12 suppressed the proliferation of lung adenocarcinoma (LUAD) cells, and overexpression of SYT12 promoted the proliferation of LUAD cells *in vitro*. (**A**) The two specific SYT12-siRNA (siRNA-1, siRNA-2) both effectively down-regulated the mRNA level of SYT12 in SPC-A-1 cells. (**B**) The pENTER vector containing the total coding sequence of SYT12 (oe-SYT12) was significantly increased the mRNA level of SYT12 in A549 cells. (**C, D**) SYT12 siRNA-1 and the oe-SYT12 vector effectively inhibited and increased the protein level of SYT12 in LUAD cells, respectively. (**E, F**) The cell counting kit-8 (CCK-8) assay showed that knockdown of SYT12 significantly inhibited the growth of SPC-A-1 cells, while overexpression of SYT12 promoted the growth of A549 cells. (**G–J**) Down-regulation of SYT12 decreased cells in the S phase of the cell cycle and induced G1/S cell cycle arrest. Upregulation of SYT12 increased the percentage of cells in the S phase. (* P<0.05, ** P<0.01, *** P<0.001).

Knockdown of SYT12 expression reduced cell migration and colony formation of LUAD cell lines

As shown in Figure 3A and 3B, knockdown of SYT12 significantly reduced SPC-A-1 cell migration, and overexpression of SYT12 increased A549 cell migration. Inhibition of SYT12 expression also suppressed the cell invasion of SPC-A-1 cells, and overexpression of SYT12 increased A549 cell invasion (Figure 3C, 3D). Colony formation assays showed that the colonies formed by SPC-A-1 cells were significantly reduced by knockdown of SYT12. However, the overexpression of SYT12 increased the colony numbers of A549 cells (Figure 3E, 3F). These findings supported that SYT12 expression promoted the malignant phenotype of LUAD cells *in vitro*.
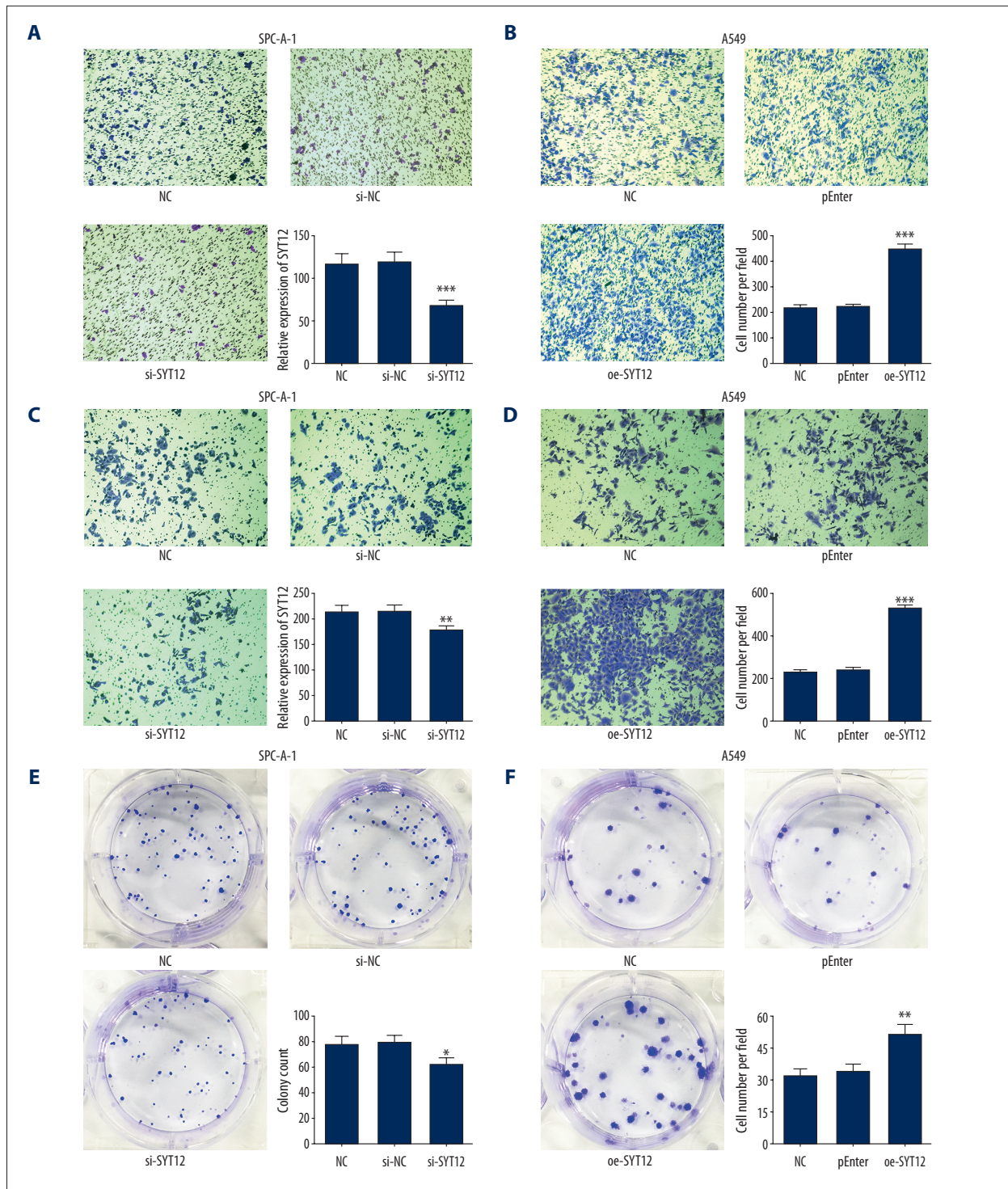
SYT12 enhanced the malignant phenotype of LUAD by regulating the phosphorylation of PIK3R3, AKT, and mTOR

To investigate the potential mechanism of SYT12 affecting malignant phenotype of LUAD cells, the online cBioPortal database was used to analyze three TCGA databases of LUAD, to identify three highly co-expressed genes with SYT12 (Supplementary Table 1). There were 337 genes that were from the TCGA LUAD database (Provisional, 586 samples), 459 genes from the TCGA LUAD database (PanCancer Atlas, 566 samples), and 274 genes from the TCGA LUAD database (Nature 2014, 230 samples). These highly co-expressed genes were submitted for analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) pathways, respectively. The bar graphs of the KEGG pathway of the three groups are shown in Figure 4A–4C. The bar graphs of the GO pathway are shown in Figure 4D–4F. The clustergrams of the KEGG and the GO pathway analysis are shown in Figure 4G and Figure 4H, respectively. However, no significant oncogenic pathways, such as cell cycle, apoptosis, and cell migration, were observed in the pathway analysis. PIK3R3 appeared in 4/6 clustergrams and PIK3R5 in 5/6 clustergrams. PIK3R3 and PIK3R5 are two subunits of PI3Ks, and the PI3K/AKT/mTOR pathway affects several aspects of cancer progression, indicating that SYT12 promoted the malignant phenotype of LUAD cells by PIK3R3 or PIK3R5.

Also, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot were performed to detect the mRNA and protein level of PIK3R3, PIK3R5, AKT, and mTOR, respectively. As shown in Figure 4I and 4J, the mRNA levels of PIK3R3, PIK3R5, AKT1, AKT2, AKT3, and mTOR were

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Figure 3. Knockdown of SYT12 reduced cell migration and colony formation of lung adenocarcinoma (LUAD) cells and overexpression of SYT12 reversed these effects *in vitro*. (**A, B**) Transwell assays showed that knockdown of SYT12 inhibited SPC-A-1 cells from migrating through the membrane of the transwell insert, while upregulation of SYT12 promoted the migration of A549 cells. (**C, D**) Matrigel assays showed that down-regulation of SYT12 effectively suppressed SPC-A-1 cell invasion ability, while overexpression of SYT12 promoted the invasion of A549 cells. (**E, F**) Colony formation assays showed that knockdown of SYT12 decreased colony numbers, while overexpression of SYT12 increased colony numbers. (* P<0.05, ** P<0.01, *** P<0.001).

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not significantly affected by SYT12 expression. The results of Western blot analysis showed no significant differences in total protein levels of these molecules. However, the phosphorylation levels of PIK3R3, AKT, and mTOR were significantly altered by SYT12. These results indicated that SYT12 could activate the PI3K/AKT/mTOR pathway at the level of post-translational protein modification by promoting phosphorylation of PIK3R3, AKT (Ser473 and Thr308), and mTOR (Ser2448).

SYT12 promoted tumor progression *in vivo* in the mouse xenograft model of LUAD
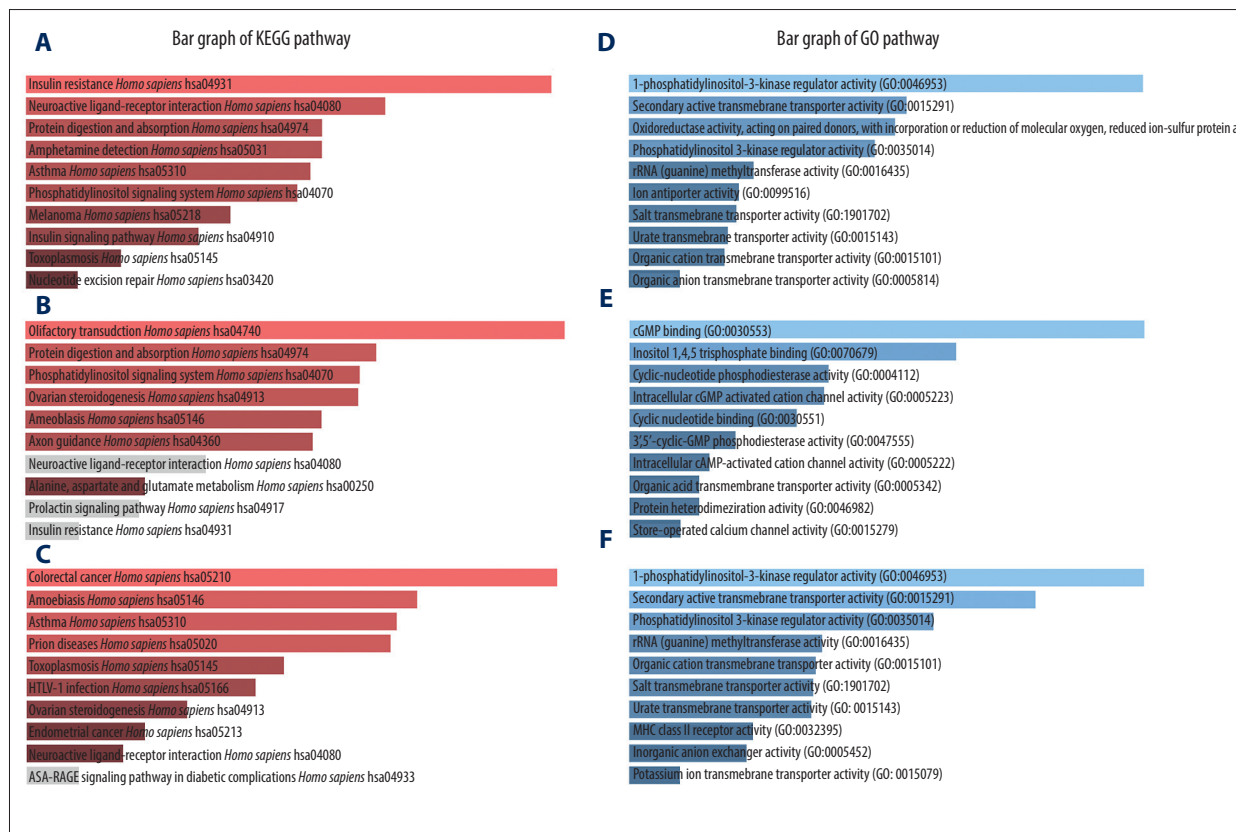
The mouse xenograft model was established in nude mice to evaluate the oncogenic role of SYT12 *in vivo*. Transfected LUAD cells were injected subcutaneously to nude mice. Thirty nude mice (5 per group) all developed subcutaneous tumors. The LUAD xenograft tumors were collected six weeks after injection (Figure 5A). Knockdown of SYT12 suppressed xenograft tumor growth (volume and weight) compared with the control groups, while overexpression of SYT12 significantly promoted xenograft tumor growth (Figures 5B–5E).
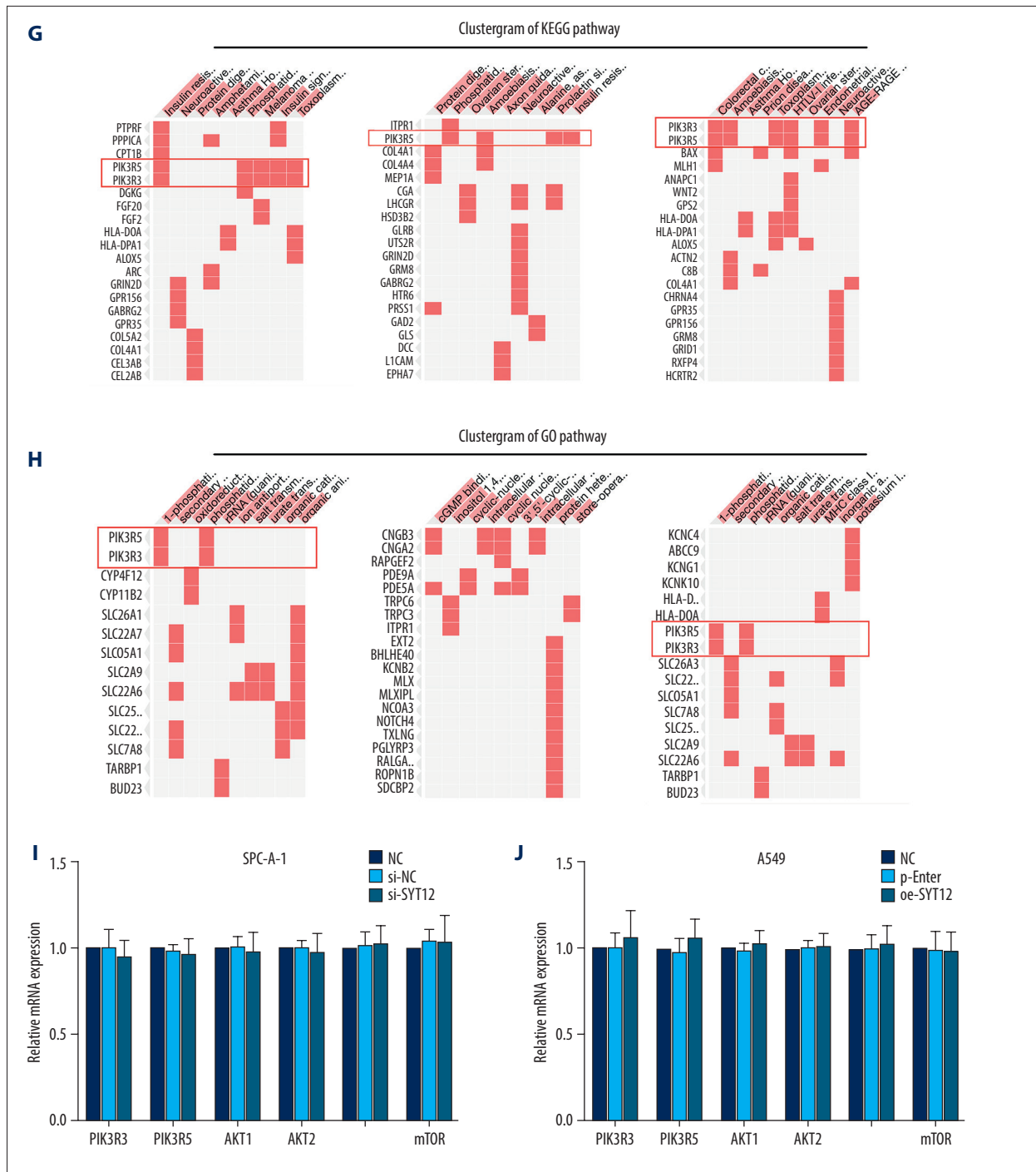
The LUAD tissue microarrays (TMAs) were examined histologically and by immunohistochemistry (IHC), which showed that SYT12 expression in tissues from patients with LUAD was significantly increased when compared with paired adjacent
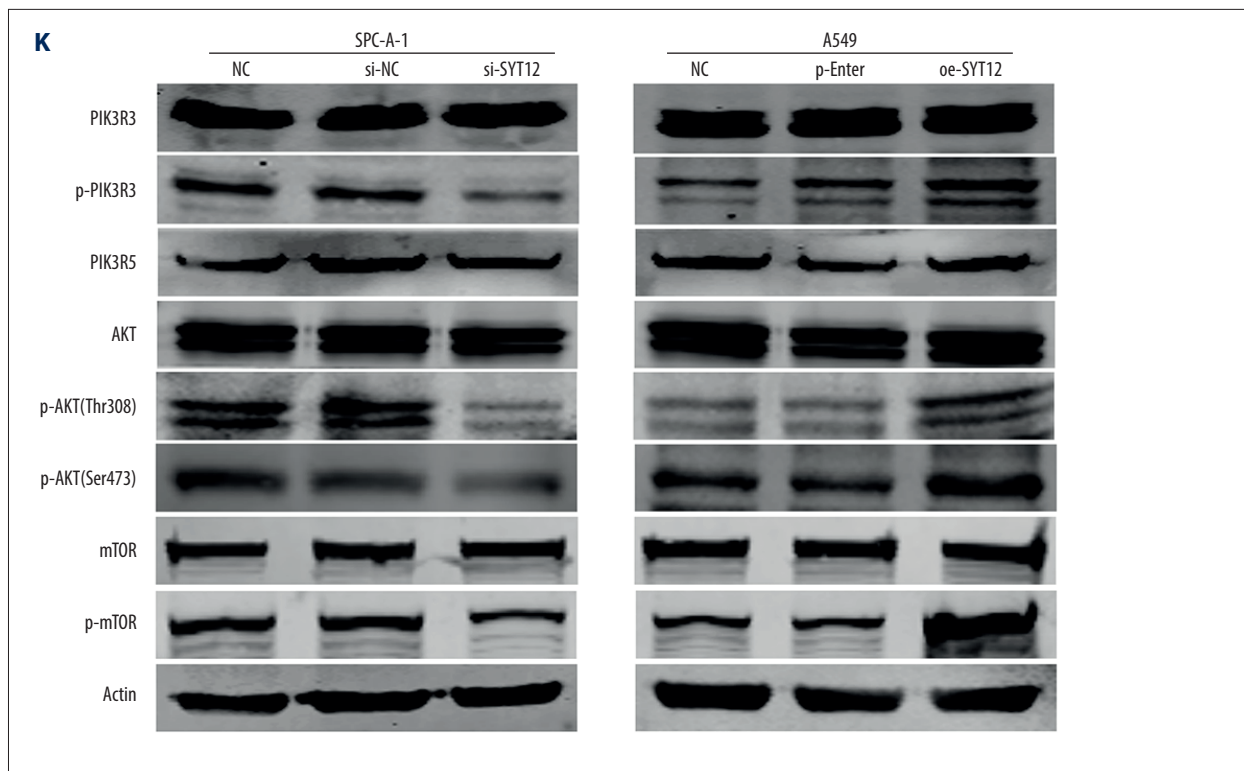
normal tissues (Figure 5F, 5G). The SYT12 level was higher in patients with advanced T stage (Figure 5H), and lymph node metastasis (Figure 5I). Patients with LUAD who were in the upper quartile of SYT12 immunostaining scores had a significantly reduced prognosis compared with patients with lower quartile scores (Figure 5J). These results showed that increased expression levels of SYT12 were significantly associated with tumor stage and prognosis in patients with LUAD.

Discussion

Non-small cell lung cancer (NSCLC) includes lung adenocarcinoma (LUAD), which has a poor prognosis due to late diagnosis and the complex molecular mechanisms involved in its development and progression [3–5]. Multiple molecular genomic changes result in cancer occurrence and progression [17], and for LUAD, targeted molecular therapies have been developed based on molecular changes in the EGFR, ALK, and RET genes [5]. However, drug resistance during treatment has become the main obstacle to targeted molecular therapy [18]. Although several molecular genomic changes in patients with LUAD have been detected using genome sequencing, the specific roles of these changes remain unclear [19]. Therefore, there remains a need to identify novel oncogenes and the molecular

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Clustergram of KEGG pathway

Clustergram of GO pathway

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Figure 4. SYT12 had an oncogenic role in lung adenocarcinoma (LUAD) by regulating the phosphorylation of PIK3R3, AKT, and mTOR. (**A–C**) The bar graphs of The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway for SYT12. (**D–F**) The bar graphs of the Gene Ontology (GO) pathway for SYT12. (**G, H**) The clustergram of the KEGG pathways of Figure 4A–4C and the GO pathways of Figure 4D–4F show that PIK3R3 and PIK3R5 participated in most of the predicted pathways. (**I, J**) The results of quantitative reverse transcription-polymerase chain reaction (qRT-PCR) showed that SYT12 did not affect the mRNA level of PIK3R3, PIK3R5, AKT1, AKT2, AKT3, and mTOR. (**K**) Western blot showed that knockdown of SYT12 reduced the level of phosphorylation of PIK3R3, AKT (Ser473 and Thr308), and mTOR. Overexpression of SYT12 had the opposite effect.

mechanisms involved in the progression of LUAD that may provide potential targets for the treatment of LUAD.

The findings from the present bioinformatics and *in vitro* cell study showed that expression of the synaptotagmin 12 (SYT12) gene promoted the progression of LUAD through the PI3K/AKT/mTOR pathway. SYT12 was first described as being homologous to SYT1, which is a thyroid hormone-induced protein [7]. The synaptotagmins, including SYT1, were identified in 1990 [20], and have been established as the primary Ca²⁺ sensors for vesicular exocytosis and endocytosis [6]. Mammalian cells contain 17 genes that encode synaptotagmins, and all 17 synaptotagmins have similar molecular structures, but only a minority have been studied in detail [6]. Few previous studies have reported the association between synaptotagmin family members and human cancer. Recently, Kanda et al. identified SYT7 to be a novel molecule that promoted hepatic metastasis of gastric cancer [21]. Increased expression of SYT13 is significantly associated with an increased risk of peritoneal recurrence in patients with gastric cancer, and down-regulation of SYT13 inhibited migration and invasion of gastric cancer

cells *in vitro* [22]. The findings from the present study support these previous studies, which showed that SYT12 expression significantly promoted the migration and invasion of LUAD cells (Figure 3A–3D).
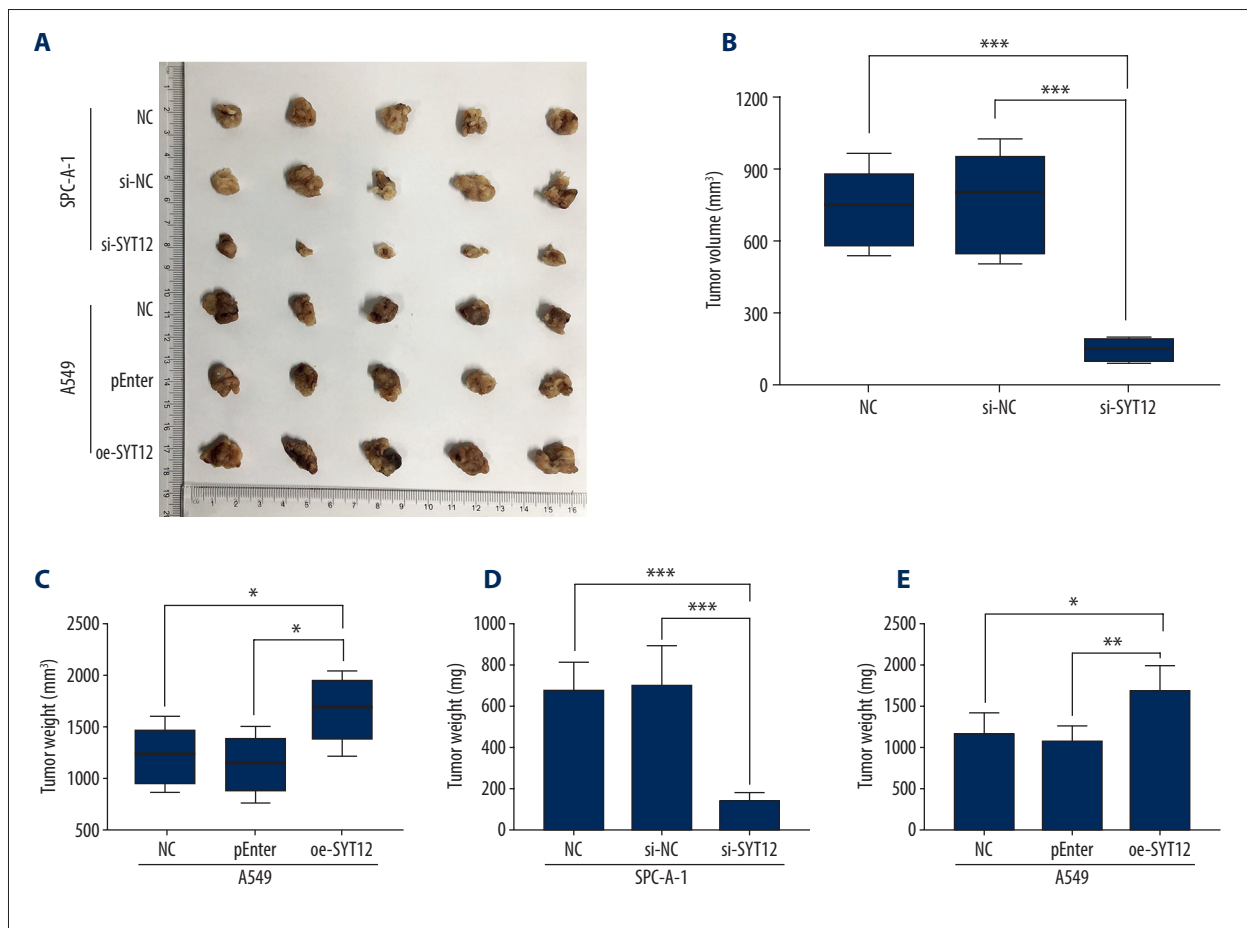
The SYT12 gene is located on the human chromosome at 11q13.2 and encodes a protein that is similar to other synaptotagmins. SYT12 is expressed at relatively low levels in several tissues, including normal lung, but shows increased expression in tissues from patients with LUAD (Figure 1A). Jonklaas et al. [8] reported that SYT12 could be a prognostic marker for papillary thyroid cancer. To our knowledge, the oncological roles of SYT12 in LUAD have not been previously studied. In the present study, SYT12 expression was significantly increased in The Cancer Genome Atlas (TCGA) tissue microarray data and 50 paired LUAD tissue samples. In support of the findings from the TCGA database, the SYT12 was upregulated 88.0% (44/50) of patients with LUAD (Figure 1E) and was significantly associated with advanced tumor stage. Several common human LUAD cell lines were used to detect SYT12 expression levels, and the findings showed that SYT12 was

highly expressed in LUAD cell lines when compared with the normal lung cell line, HBE. The SPC-A-1 LUAD cell line showed the highest expression of SYT12, and the A549 LUAD cell line had the lowest SYT12 expression (Figure 1F, 1G). Therefore, the SPC-A-1 cell line was selected for the gene expression studies on the knockdown of SYT12, while A549 cell lines were selected to study the effects of increased expression of SYT12. *In vitro* studies showed that SYT12 expression levels affected cell proliferation, the cell cycle, cell migration, and cell invasion to promote the malignant phenotype of LUAD cells. The Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) pathway enrichment analysis identified the possible molecular mechanisms involved in the promotion of the progression of LUAD by SYT12 gene expression, which indicated that two subunits of PI3Ks, PIK3R3, and PIK3R5, were involved in most predicted pathways.
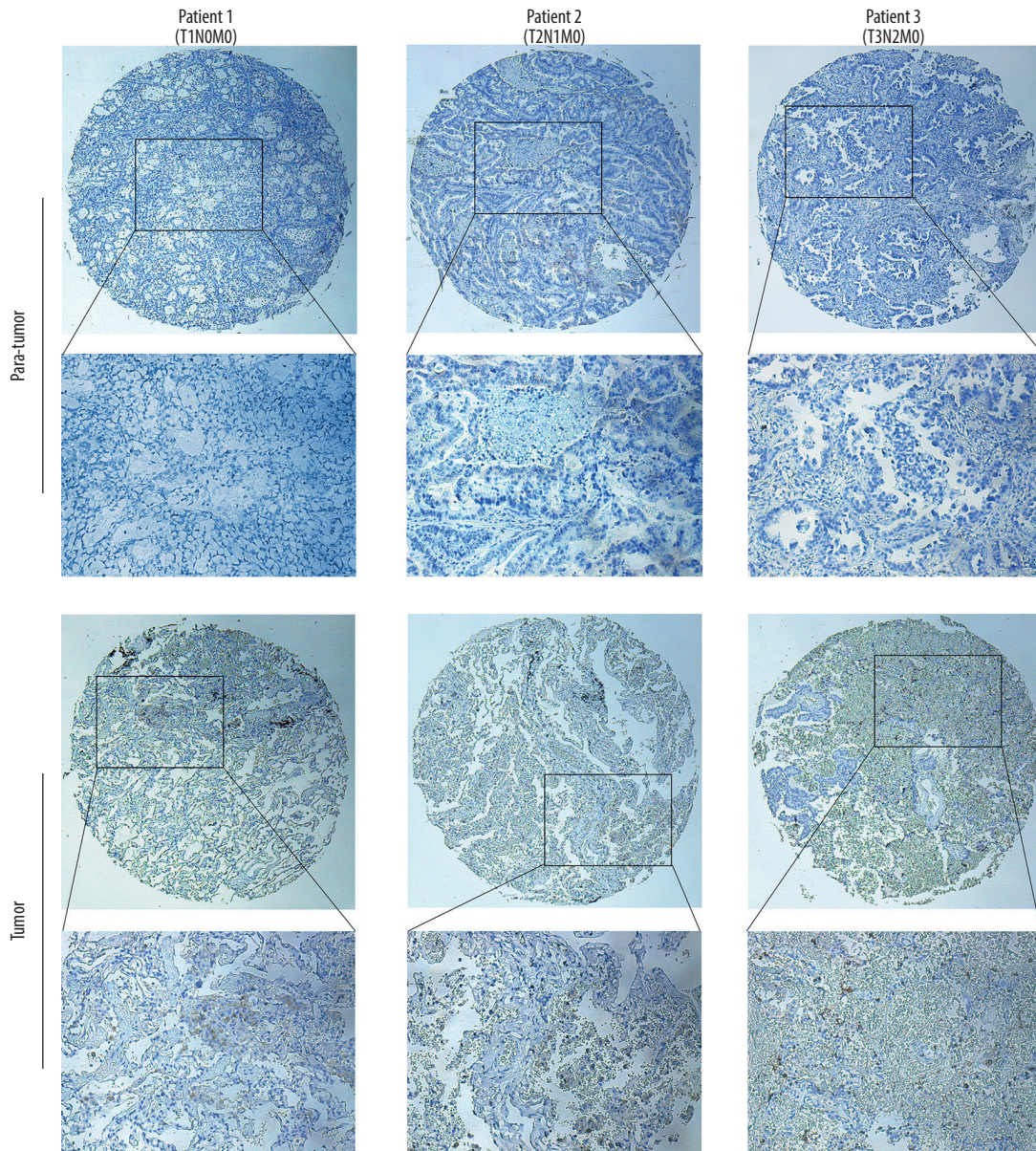
PI3Ks are a group of plasma membrane-associated lipid kinases that include three subtypes, class I, II, and III, based on their structure and function [10]. Class I PI3Ks are composed of a catalytic and a regulatory subunit, and according to the difference in the regulatory subunits, class I PI3Ks are grouped into class I_A PI3Ks and class I_B PI3Ks [23]. The regulatory subunit of class I_A PI3Ks, also known as p85, is composed of p85α, p85β, and p55, which are encoded by PIK3R1, PIK3R2, and PIK3R3, respectively [10]. The p85 subunit integrates various signaling pathways for transmembrane and intracellular molecules, including protein kinase C (PKC), hormone receptors, tyrosine kinase-linked receptors, and mutated RAS, to activate the catalytic subunit, p110 [12]. PIK3R3 encoded p55 as a part of p85, plays a critical role by the combination of p110 activation with several signaling pathways [24].
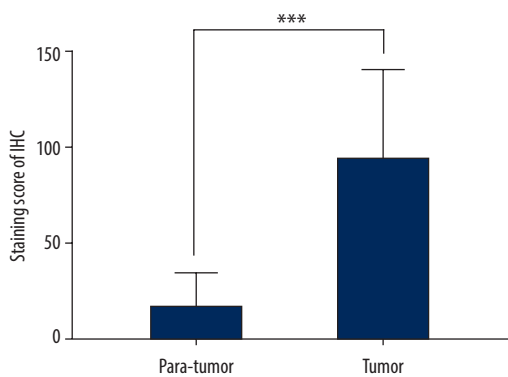
Previous studies have shown that PIK3R3 facilitates the proliferation, metastasis, and invasion of cancer cells [25–27]. The findings from the present study supported the findings from previous studies and showed that by activating PIK3R3, SYT12 enhanced proliferation, migration, and invasion of LUAD cells. However, protein p101 encoded by PIK3R5 is one of the regulatory subunits of class I_B PI3Ks. Currently, class I_A PI3Ks have been associated with human cancer through multiple mechanisms [28,29], while the relationship between class I_B PI3Ks and human cancer has rarely been studied [30]. Therefore, although the GO and KEGG analysis in this study indicated that PIK3R3 and PIK3R5 were involved in most of the predicted pathways, it was assumed that PIK3R3 had a main role in the effects of
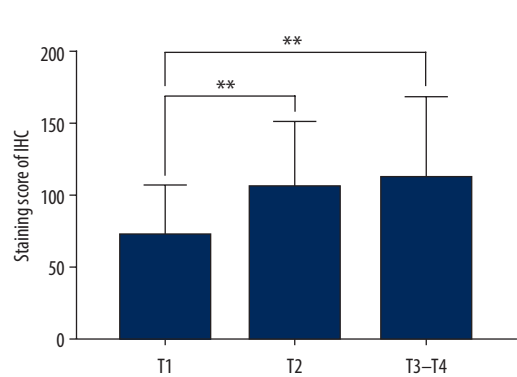
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[Chemical Abstracts/CAS]

F

Patient 1 (T1N0M0)   Patient 2 (T2N1M0)   Patient 3 (T3N2M0)

Para-tumor

Tumor

G

Staining score of IHC

***

Para-tumor   Tumor

H

Staining score of IHC

**   **

T1   T2   T3–T4
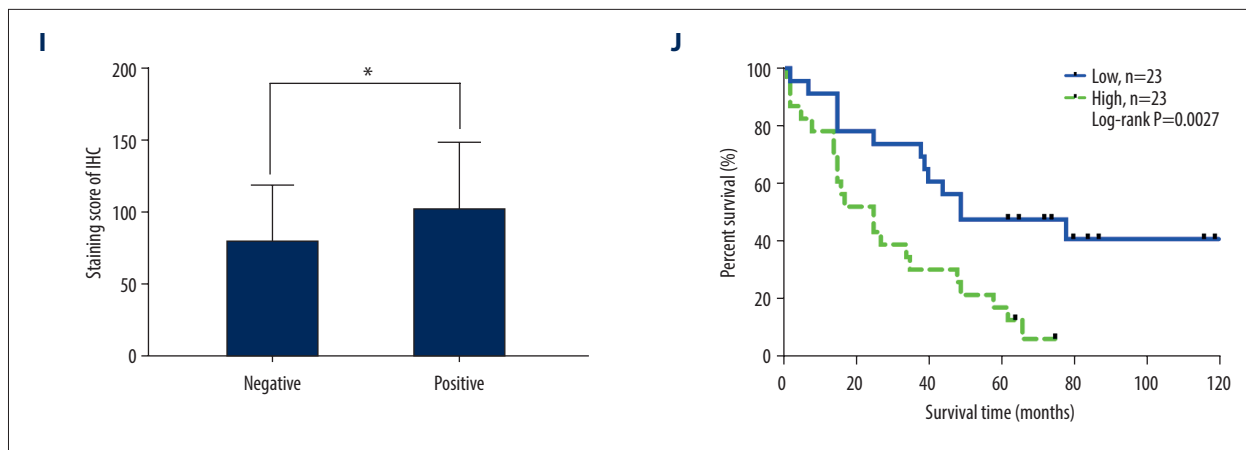
Figure 5. SYT12 promoted the progression of lung adenocarcinoma (LUAD) *in vivo*. (**A**) Harvested xenograft tumors are shown. (**B–E**) The volume and weight of xenograft tumors of each group were measured. The results show that SYT12 promoted the proliferation of LUAD *in vivo*. (**F**) Representative immunohistochemistry (IHC) staining photomicrographs of the tissue microarrays (TMAs) are shown. (**G**) The SYT12 staining score was upregulated in tissues from patients with LUAD compared with paired adjacent normal lung tissue. (**H, I**) The SYT12 staining score was significantly higher in patients with LUAD and advanced T stage and positive lymph node metastasis. (**J**) Patients with upper quartile scores of SYT12 expression had significantly reduced prognosis compared with those with lower quartile scores. (* P<0.05, ** P<0.01, *** P<0.001).

SYT12 expression in promoting the progression of LUAD. In the present study, the phosphorylation level of PIK3R3 instead of PIK3R5 was studied (Figure 4K). A further reason for the lack of detection of the phosphorylation level of PIK3R5 was that a primary antibody was unavailable. However, the total protein level of PIK3R5 was measured, which was not significantly affected by SYT12 expression (Figure 4K).

AKT can be activated by phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3), also known as PIP$_3$, which is generated by class I PI3K phosphorylation of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) [31]. PI3K recruits AKT to the plasma membrane, and AKT is activated by phosphorylation of the conserved Thr308 and Ser473 molecules. Thr308 is phosphorylated by PDK1, while Ser473 is phosphorylated by mTOR complex 2 (mTORC2) [32]. In this study, down-regulation or upregulation of SYT12 affected the phosphorylation level of AKT on both the conserved sites, Thr308 and Ser473 (Figure 4K). Aberrant PI3K/Akt signaling has previously been reported as a significant activator of the mTOR pathway, which results in both cancer progression and resistance to treatment [33]. Also, mTOR is a serine-threonine protein kinase composed of two functionally different complexes, mTOR complex 1 (mTORC1) and mTORC2 [34]. Class I PI3K-AKT signaling can induce the elimination of the upstream inactivator of mTORC1, allowing GTP to stimulate mTORC1 activity. Also, mTORC2 activation leads to the phosphorylation of Akt at Ser473, which promotes AKT to phosphorylate Thr308 by PDK1 further, resulting in total activation of AKT [35]. The findings from the present study were consistent with the findings from previous studies that overexpression of SYT12 activated PIK3R3 resulting in an increased phosphorylation level of AKT and mTOR (Figure 4K).

To the best of our knowledge, this study was the first to demonstrate that SYT12 promoted LUAD progression and to investigate the possible mechanism. However, this study had several limitations. This study did not investigate the molecular mechanisms for the effects of SYT12 on the phosphorylation of PIK3R3. Although PIK3R5 belongs to class I$_B$ PI3K, which is not as important as class I$_A$ PI3K in cancer progression, it might be better important to identify the phosphorylation of PIK3R5 in LUAD. Also, this study did not analyze the correlation between SYT12 expression and prognosis of the patients from our institution.

Conclusions

This study aimed to use bioinformatics analysis to compare data from tissue microarrays from patients with lung adenocarcinoma (LUAD) and normal lung tissue, and human lung adenocarcinoma cells with normal lung epithelial cells *in vitro* to investigate the role of synaptotagmin 12 (SYT12) gene expression in LUAD. The findings showed that mRNA expression of SYT12 in tissues from patients with LUAD was significantly increased when compared with normal lung tissues and correlated with advanced tumor stage and reduced overall survival (OS). SYT12 had an approximately 3–8% rate of change in the genomic profiles of patients with LUAD. Bioinformatics analysis and molecular studies showed that SYT12 had a possible oncogenic role in the progression of LUAD and was associated with the phosphorylation of phosphoinositide-3-kinase regulatory subunit 3 (PIK3R3), AKT, and mammalian target of rapamycin (mTOR).

Acknowledgments

Supplemenatry Data

Supplemenatry Table 1.

Supplementary/raw data available from the corresponding author on request.

Conflict of interest

None.

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