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Running Title: Role of SPINK1 in LAC progression.

Keywords: SPINK1, biomarker, cell growth, metastasis, lung adenocarcinoma.

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ABSTRACT

Serine protease inhibitor Kazal type 1 (SPINK1) plays a role in protecting the pancreas against premature activation of trypsinogen and is involved in cancer progression. SPINK1 promoted LAC cells growth, migration and invasion. Mechanistically, we found that SPINK1 promoted LAC cells migration and invasion via up-regulating matrix metalloproteinase 12 (MMP12). We observed that SPINK1 expression was only up-regulated in lung adenocarcinoma (LAC) tissues, and was an independent prognostic factor for poor survival. Our results indicate that SPINK1 might be a potential biomarker for LAC that promotes progression by MMP12.

INTRODUCTION

Lung cancer is one of the most common malignant tumours, which has become the leading cause of cancer deaths in both males and females worldwide (1). Non-small-cell lung cancer (NSCLC) accounts for about 80% of all diagnosed lung cancer, and lung adenocarcinoma (LAC) is the most common histologic subtype of NSCLC (2). Despite the advancement of targeted therapy with significant responses, LAC treatment remains a challenge because of acquired resistance and unknown driver factors (3). Therefore, there exists a need to identify new molecular regulators for LAC, providing insights into potential therapeutic targets.

The serine protease inhibitor Kazal type 1 (SPINK1) gene, also known as pancreatic secretory trypsin inhibitor (PSTI) or tumor associated trypsin inhibitor (TATI), encodes a 79 amino acid peptide, and mature SPINK1 consists of 56 amino acids (4,5). The physiological function of SPINK1 is to inhibit the activation of trypsin in pancreas, and prevents the "self digestion" of the organ (6). In the analysis of structure, SPINK1 shares 50% homology with epidermal growth factor (EGF) in the sequences of amino acid, and can bind with epidermal growth factor receptor (EGFR) (7). Therefore, it was conferred that SPINK1 has a role of a growth factor. Recent studies have proved that SPINK1 can promote tumor cell growth in prostate (8), colon (9, 10), pancreatic (11) and ovarian cancer (12), which indicated that the function of SPINK1 has a protumorigenic role through a mechanism distinct from its classical activity as a serine protease inhibitor. Previous studies suggested the potential role of SPINK1 in tumor development and progression, which might be a useful therapeutic target for cancer. However, its expression pattern and biological functions in LAC remain unclear.

In our study, SPINK1 promoted LAC cell growth, migration and invasion. Furthermore, we found that SPINK1 promoted LAC cells migration and invasion via up-regulating matrix metalloproteinase 12 (MMP12). Importantly, we showed that SPINK1 overexpression correlated with poor prognosis of LAC patients.

RESULTS

Down-regulation of SPINK1 attenuates LAC cells growth and metastasis.

To explore the differentially expressed gene (DEGs) in LAC tissues, microarray was performed in LAC patients (Supplementary Table S1). The heat map diagram showed the result of a two-way hierarchical clustering based on the 50 most up-regulated DEGs between LAC tissues and normal lung tissues (Supplementary Fig. S1A). The top 10 significantly up-regulated DEGs are listed in Supplementary Table S2 and ranked by the fold change compared to normal tissues. We found only SPINK1 mRNA was significantly up-regulated in LAC tissues (Supplementary Fig. S1B).

To explore SPINK1 role in LAC development, we first evaluated the SPINK1 expression levels in LAC cell lines and found that SPINK1 mRNA was higher in H1975 and A549 cells, and lower in H1299 and PC9 cells (Fig. 1A). SPINK1 siRNAs (siRNA 1, siRNA 2 and siRNA 3) were transfected into LAC cell lines (A549 and H1975), and it was confirmed that SPINK1 expressions were successfully down-regulated (Fig. 1B). As shown in Fig. 1C, SPINK1 inhibition significantly reduced the growth of A549 and H1975 cells. The EdU immunofluorescence staining showed that EdU -positive cells were reduced, in which SPINK1 expression was suppressed, both in A549 and H1975 cells (Fig. 1D). Further, we explored the effects of SPINK1 on the migration and invasion of LAC cells using the transwell system. Representative micrographs of transwell filters can be seen in Fig. 2E. The migrating and invasive potential of LAC cells were markedly weakened in SPINK1 siRNA (siRNA 1) group compared to the scramble group (Fig. 1E, $P < 0.01$). Additionally, the A549 xenografts displayed significant inhibition of tumor growth in the group of LV3-shSPINK1, compared to LV3-NC (Fig. 1F). Furthermore, a significant reduction in SPINK1 expression was observed in tumor tissues from LV3-shSPINK1 group, when compared to LV3-NC (Fig. 1G, Supplementary Fig. S2A). These results proved that SPINK1 inhibition suppressed the

development of lung adenocarcinoma.

SPINK1 up-regulation promotes LAC cell proliferation, migration, and invasion.

To further explore the effect of SPINK1 on the LAC development, LAC cells (PC9, H1299) that had very low SPINK1 expression levels were infected with LV5-SPINK1 or LV5-NC. Our results showed that SPINK1 expression was significantly increased in the group of LV5-SPINK1, compared with the group of LV5-NC (Fig. 2A). Results also showed that the growth rate of LV5-SPINK1-infected cells was significantly higher than that of the control group (Fig. 2B). Furthermore, we determined that the EdU-positive cells were increased in both PC9 and H1299 cells, in which SPINK1 expression was overexpressed (Fig. 2C). We also examined the effect of SPINK1 on the migrated and invading abilities of LAC cells, and it was found that SPINK1 overexpression promoted the migration and invasion of PC9 and H1299 by the transwell assay (Fig. 2D). To further elucidate the role of SPINK1 overexpression in tumor growth ability in vivo, we established mouse xenograft models with stable SPINK1 overexpression PC9 cells or the control by subcutaneous injection. SPINK1 overexpression significantly promoted tumor formation and tumor growth (Fig. 2E and 2F). SPINK1 expression was up-regulated in tumor tissues from the group of LV5-SPINK1, when compared with the LV5-NC (Fig. 2G, Supplementary Fig. S2B). These results demonstrated that SPINK1 played a pro-tumor role by promoting cell proliferation and invasion.

SPINK1 facilitates migration and invasion in LAC cells via up-regulating MMP-12.

Matrix metalloproteinases (MPPs) play important roles in tumor invasion and metastasis. Thus, we investigated whether SPINK1 was involved in regulating MMPs expressions in LAC cells. As shown in Fig. 3A, MMP12 mRNA expression was down-regulated while others were not significantly down-regulated in A549 and H1975 cells. We also confirmed that MMP12 protein was decreased in SPINK1 knockdown cells (Fig. 3B). Compared to the control, MMP12 protein levels were up-regulated in SPINK1-overexpressing

PC9 and H1299 cells (Fig. 3C). Next, we examined the MMP12 expression of tumor tissues in xenograft models with LAC cells, which SPINK1 expressions were knock-down or over-expressed. As shown in Fig. 3D, MMP12 mRNA was down-regulated in the group of SPINK1 knockdown and up-regulated in the group of SPINK1 overexpression. Furthermore, the level of SPINK1 mRNA expression was significantly correlated with that of MMP12 mRNA in LAC tissues ($R = 0.709$, $P < 0.001$) (Fig. 3E). To further elucidate whether the effects of SPINK1 on LAC cells migration and invasive depend on MMP12, MMP12 was knocked down by siRNA (Fig. 3F) and results showed that the enhanced migratory and invasion ability of SPINK1 overexpressing LAC cells were dramatically reversed (Fig. 3G). Moreover, when recombinant human MMP12 was added back to SPINK1-knockdown A549 and H1975 cells, the numbers of migrating and invasive cells were rescued (Fig. 3H). Collectively, SPINK1 promoted the migration and invasion of lung adenocarcinoma by up-regulating MMP12 expression.

Increased SPINK1 expression in LAC tissues significantly correlates with poor prognosis.

Since SPINK1 can promote LAC cell growth and metastasis, we next explored the correlation between SPINK1 expression and prognosis in LAC patients. First, we detected SPINK1 expression in tissues with LACs, lung squamous carcinoma (Sq) or nonmalignant lung nodules (NLNs) and our results showed that SPINK1 protein was only up-regulated in LAC tissues (Fig. 4A, Supplementary Fig. S3 and Table S3). Second, we explored the correlation between SPINK1 expression and clinicopathological features and results indicated that SPINK1 expression was significantly positively associated with male gender, carcinoembryonic antigen (CEA) level, tumor size, invasive histology (Fig. 4B), lymph node metastasis and advanced stage

(Supplementary Table S4). More importantly, higher expression of SPINK1 in LAC tissues was negatively correlated with progression-free survival (PFS) and overall survival (OS) in the cohort of patients (Fig. 4C). Furthermore, a Cox proportional hazards model showed that SPINK1 remained significantly associated with PFS and OS. Supplementary Table S5 showed the results of the univariate and multivariate analyses. Taken together, our results suggested that SPINK1 might act as a novel prognostic biomarker for LAC.

DISCUSSION

Several studies reported that SPINK1 was shown to stimulate growth of several cancers, including pancreatic cancer (13), prostate cancer (14), ovarian cancer (12), colorectal cancer (15), suggesting that SPINK1 may significantly promote cancer cell growth and progression. In our study, we found that knockdown of SPINK1 decreased the proliferation ability of LAC cells, whereas overexpression of SPINK1 had the opposite effect. Our findings can be explained by the fact that SPINK1 structure is similar with EGF (7) and can stimulate cell proliferation by EGFR/MAPK signaling pathway (16). The results of our current study are consistent with that of the previous studies and indicate that SPINK1 plays an important role in the promoting the growth of LAC cells, which could offer a new target for pharmacological intervention in LAC patients with SPINK1-expressing tumors.

Tumor metastasis, occupying more than 90% of all deaths in tumors, is considered to be the leading cause of mortality in cancer patients, but the underlying mechanisms remain unclear. SPINK1 has been reported to be related to tumor metastasis (17, 18). Our data showed that SPINK1 expression levels in IAC tissues were higher than that of MIA or AIS, and increased expression of SPINK1 was closely correlated with lymph node metastases, strongly suggesting that SPINK1 may be involved in the LAC metastatic processes. In this study, knockdown of SPINK1 can inhibit the migration and invasion of LAC cells, whereas SPINK1 overexpression can promote this effect. The results can be explained by the findi

ng that SPINK1 can promote the process of epithelial-mesenchymal transition (EMT) (19). EMT is an important biological process for malignant tumor cells to acquire migration and invasion. Furthermore, we explored the underlying mechanisms responsible for SPINK1 promoting-metastasis in LAC. MMPs are known to degrade almost all protein components in extracellular matrix and play a key role in the invasion and metastasis of tumors. Many studies have confirmed that MAPK signaling pathway can regulate the expression of MMPs, thereby affecting the migration and invasion of tumors (20, 21). Pornchai et al. found that ligands of EGFR could regulate MMP9 in different degrees in head and neck cancer (22), suggesting that SPINK1, as an EGFR ligand, could regulate the expression of MMPs. Therefore, we detected the expressions of MMPs family members in SPINK1 modifying LAC cells and found that only MMP12 expression was dysregulated, which has been also proved in vivo experiments. Moreover, it was confirmed that SPINK1 expression was positively correlated with MMP12 expression in LAC clinical samples. All of these data suggested that MMP12 might be an important mediator in SPINK1-regulated the invasiveness and metastasis of LAC cells. Subsequently, we used MMP12 siRNA and rhMMP12 to further confirm MMP12 mediated the metastatic potential of SPINK1 in LAC cells. Previous studies have demonstrated that MMP12 was involved in the invasion of LAC cells (23), which further supports that SPINK1 enhanced the metastasis of LAC cells via MMP12. Taken together, our findings shed new light on the role of SPINK1 related with metastasis of LAC.

SPINK1 was overexpressed and correlated with prognosis in a variety of tumors (12, 24-26). In our study, it was confirmed that SPINK1 was expressed only in LAC tissues but not in tissues of Sq and NLNs. In the physical condition, SPINK1 is secreted from pancreatic acinar cells. Lung adenocarcinoma is named for its structure with glandular cavity, which is similar with the structure of pancreas. This may be the reason SPINK1 is expressed only in

adenocarcinoma. Furthermore, our study showed that higher SPINK1 in tumor tissues was correlated with shorter PFS and OS in LAC patients. This can be explained by our finding that SPINK1 can promote LAC cells growth. Thus, we present very important clinical evidence, suggesting that SPINK1 might serve as a novel prognostic biomarker for LAC and is also involved in LAC progression.

In summary, we initially demonstrated the effect of SPINK1-promoting growth and the underlying molecular mechanisms that SPINK1 promotes metastasis of lung adenocarcinoma by MMP12. Importantly, our results suggested that SPINK1 might be a potential biomarker for lung adenocarcinoma. Our findings provide new insight into the lung adenocarcinoma pathogenesis mediated by SPINK1 and a novel candidate target for the effective therapy of lung adenocarcinoma.

MATERIALS AND METHODS

Patients and tumors

For mRNA array analysis, tumor and paired adjacent non-tumor tissues from 6 LAC patients were used (Supplementary Figure S4). For qRT-PCR analysis, we used fresh tissues of 62 primary LACs. For immunohistochemical analysis, primary tumor and paired adjacent nontumor tissues were collected from 382 LACs, 45 Sq, and 51 NLNs, which underwent surgical resection between January 2010 and June 2013 at the Department of Cardiothoracic Surgery of Zhoushan hospital. No patients had received chemotherapy or radiation before resection. Informed consent was obtained from all patients before the study was initiated with approval of the Zhoushan Hospital Ethics Committee in accordance with the Declaration of Helsinki.

Gene expression analysis by microarray

To accurately acquire tumor cells and normal cells from lung tissues, samples were

microdissected using a laser capture microdissection system (Applied Biosystems® ArcturusXT™, ABI). mRNA profiling was performed by using Illumina Technologies “Human Genome U133 Plus 2.0 Array” according to the manufacturer’s protocol. GenomeStudio 1.0 was used to perform average normalization of the results from mRNA microarray. Microarray data were deposited in a Gene Expression Omnibus (GEO) database (accession no. GSE118370).

RNA extraction and quantitative real-time PCR (qRT-PCR)

Isolation of total RNA and synthesis of cDNA were made according to the manufacture’s protocol. qRT-PCR was performed with SYBR Green Realtime PCR Master Mix (Applied Biosystems), and was run on ABI 7500 Real-time PCR system (Thermo, Waltham, MA) . The target gene Ct values were normalized to GAPDH using the $2^{-\Delta\Delta Ct}$ method. Gene-specific primers were listed in Supplementary [Table S6](#).

Cell lines, siRNA, Recombinant protein and Plasmids

The human LAC cell lines NCI-H1975, A549, NCI-H1299 and PC9 were purchased from the Shanghai Cell Collection. The siRNAs were purchased from Life Technologies Corporation (Thermo Fisher Scientific). The SPINK1 siRNAs were used: siRNA1 (HSS144065), siRNA 2 (HSS144065), siRNA 3 (HSS186064) and Silencer™ Negative Control #1 siRNA (AM 4611). The ID of MMP12 siRNA (AM16708) was 104022 and the control catalog was AM 4613. Recombinant human MMP12 (rhMMP12, 917-MPB) and the control IgG (cIgG, 1-001-A) were purchased from R&D Systems. Human SPINK1 expression plasmids pcDNA3.0-SPINK1 (pcSPINK1) and the control plasmid pcDNA3.0 were purchased from GenePharma (Shanghai, China).

Generation of stable cells using lentiviral infection

To generate stable SPINK1 knockdown cells, A549 and H1975 cells were infected with LV3-

pGLV-SPINK1 shRNA lentivirus (LV3-shSPINK1, GenePharma, Shanghai, China). To generate stable SPINK1 overexpression cells, PC9 and H1299 cells were infected with LV5-EF1a-SPINK1 lentivirus (LV5-SPINK1, GenePharma, Shanghai, China). Cells infected with vector-scramble sequence were used as controls (LV3-NC, LV5-NC). Single-cell clones were isolated by 5 μ g/ml Puromycin for 48h followed by 1 μ g/ml puromycin treatment. SPINK1 expressions were confirmed by qRT-PCR (Supplementary Figure S5).

Enzyme-linked Immunosorbent Assay (ELISA)

The concentration of SPINK1 in the cell culture supernatant was determined using the ELISA kit from Abnova (KA4275, Taiwan). The assays were performed according to the manufactures's instructions. All samples were examined in duplicate.

Cell Counting Kit-8 analysis

LAC cells were seeded in 96-well cell culture plates. Cell viability was measured using Cell Counting Kit-8 (CCK-8, C008-3, 7 sea biotech, Shanghai, China).

EdU immunofluorescence staining

Immunofluorescence staining was performed according to standard protocols using Click-iT EdU Imaging Kits (C10338, Invitrogen).

In vitro migration and invasion assays

Cell migration assays were performed using 6.5-mm transwell chambers (8 μ m pore size, Corning, USA). Cell invasion assays were performed using the Cell Invasion Assay Kit (ECM550, Millipore, USA) according to the manufactures's protocol.

In vivo tumor homograft assay

Male BALB/c nude mice (6-8 weeks of age, 16-18g) were purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). All mice were housed in the animal facilities under the specific pathogen-free (SPF) conditions. The welfare of experimental mice followed Guide for the Care and Use of Laboratory Animals.

All mice were randomly divided into four groups, and a xenograft model was established by subcutaneous injection of 0.2 ml (5×10^6) A549 cells stable knockdown of SPINK1, PC9 cells stably overexpressing SPINK1 and their controls. Tumor size was monitored 2 times weekly for 20-30 days. Tumor volumes (V) were calculated by the equation ($V = \text{length} \times \text{width} \times \text{width}/2$). Mice were killed and the tumors were isolated and weighed. Animal experiments were repeated two times and at least six mice were included in each cohort.

Western blot analysis

Cells were lysed and proteins were detected as previous description. Immunoblotting was carried out with the anti-MMP12 (sc133151, Santa Cruz Biotechnology, USA) and the anti- β -actin (TA-09, Zhongshanjinqiao Biotechnology Co., Beijing, China).

Immunohistochemical (IHC) staining

IHC staining was performed according to standard protocols using the following antibody: anti-SPINK1 (ab183034, Abcam, UK). Positive staining areas in the entire tissue section were graded as follows: 0, for 5%; 1, for 5-25%; 2, for 26-50% 3, 51-100%. Tumors scored as 0 or 1 were considered “negative”, and those scored as 2 or 3 were classified as “positive”.

Statistical analysis

GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used for data analysis.

P-values of < 0.05 were considered as statistically significant.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

FIGURE LEGENDS

Fig. 1 SPINK1 inhibition suppresses the growth, migration and invasion of LAC cells. (A) SPINK1 mRNA levels were detected in LAC cell lines by qRT-PCR normalizing to GAPDH. (B) Detection of the interference effect of SPINK1 siRNAs in LAC cell lines by qRT-PCR. (C) At 0 h and 72 h, cell growth analysis of A549 and H1975 cells transfected with SPINK1 siRNAs or scramble by CCK-8 assay. (D) At 72 h, cell proliferation was assayed by EdU. Scale bars, 100 μ M. (E) The migrating and invasion ability were assessed by the Transwell assay. Scale bars, 100 μ M. (F) SPINK1- knockdown (LV3-shSPINK1) A549 cells (5×10^6) or the control (LV3-NC) in 200 μ l PBS were subcutaneously injected into the left axillae of the BALB/c nude mice. The tumor size was monitored 2 times weekly. Tumor volume was calculated using the formula: $V = \text{length} \times \text{width} \times \text{width}/2$. Subsequently, growth curve was drawn. After 26 days, the mice were killed and the tumors were isolated and weighed. (G) The SPINK1 expression in xenograft tissues was measured by IHC (200 \times). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 2 SPINK1 overexpression promotes the growth, migration and invasion of LAC cells. (A) SPINK1 protein level was detected by ELISA in cultures of stable SPINK1-overexpressing PC9 and H1299 cells. (B) The cell growth was monitored by CCK-8 assay for 7 days. (C) Cell proliferation was assayed by EdU staining. Scale bars, 100 μ M. (D) The migrating and invasion ability were assessed by the Transwell assay. Scale bars, 100 μ M. (E) PC9-LV5-control or PC9-LV5-SPINK1 cells (5×10^6) in 200 μ l PBS were subcutaneously injected into the left axillae of the BALB/c nude mice. The tumor size was monitored 2 times weekly. Tumor volume was calculated using the above formula and growth curve was drawn. (F) After 26 days, the mice were killed and the tumors were isolated, imaged and weighed. (G) The SPINK1 expression in xenograft tissues was measured by

IHC (200×). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 3 SPINK1 enhances the migration and invasion of LAC cells by up-regulating MMP12. (A) MMPs expressions were analyzed by qRT-PCR in A549 cells and H1975 cells transfected with Scramble or SPINK1 siRNAs. (B) MMP12 protein expressions were analyzed by western blot in A549 cells and H1975 cells transfected with Scramble or SPINK1 siRNA 1. (C) MMP12 protein levels were measured by western blot in PC9 cells and H1299 cells transfected with control or SPINK1. (D) MMP12 mRNA expression in xenograft tissues was measured by qPCR. (E) The correlation between SPINK1 expression and MMP12 expression in LAC tissues was analyzed with Pearson correlation. (F) The interference effect of MMP12 siRNA was verified by western blot in PC9 and H1299 cells stably overexpressing SPINK1. And the number of migrating and invasive cells was counted (G). (H) Recombinant human MMP12 (rhMMP12) or control IgG (cIgG) at a concentration of 50 ng/ml was added back into SPINK1 knockdown A549 and H1299 cells. And the number of migrating and invasive cells was counted. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 4 Enhanced SPINK1 expression in LAC predicts poor prognosis. (A) Representative images of IHC staining of SPINK1 protein in NLNs, Sq, LAC and matched nor-tumor tissues (×200). (B) SPINK1 expressions in the subtypes of LAC. Representative images of IHC staining of SPINK1 protein in AIS, MIA, IAC and nor-tumor tissues (×200). (C) The rate of the PFS and OS of LAC patients with higher SPINK1 expression was significantly lower than that of the patients with lower SPINK1 expression. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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Fig. 1

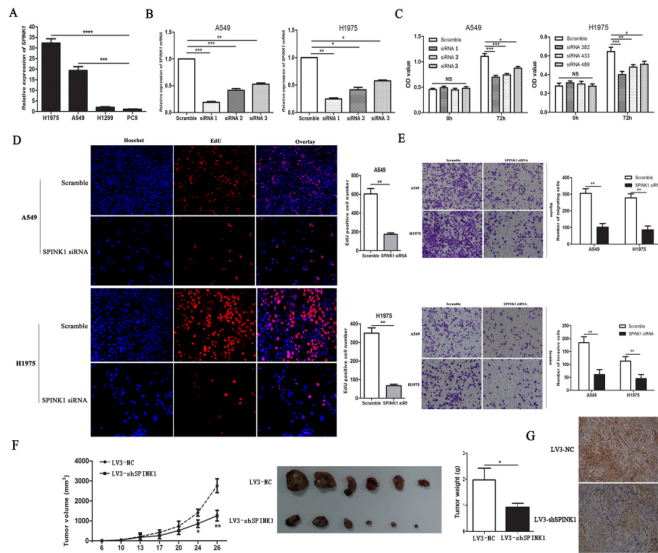


Fig. 1.

Fig.2

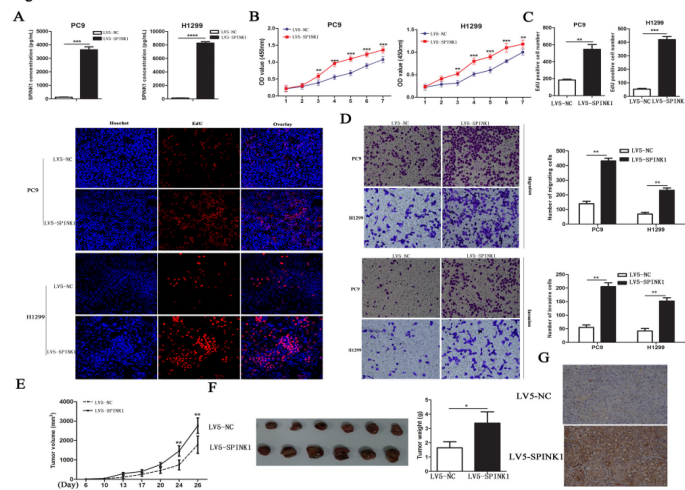


Fig. 2.

Fig. 3

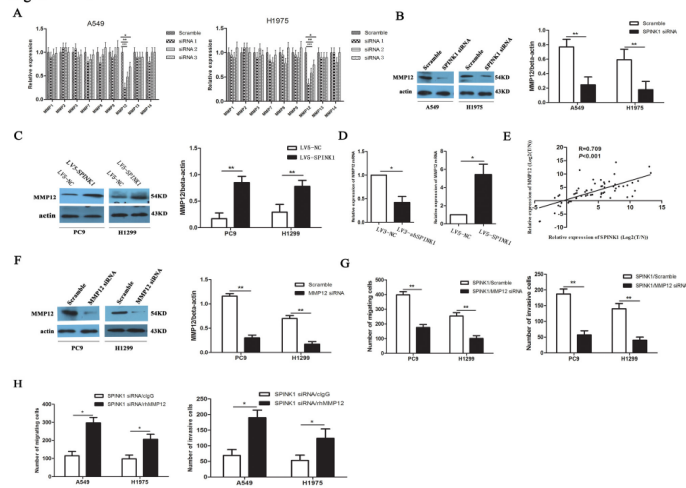


Fig. 3.

Fig.4

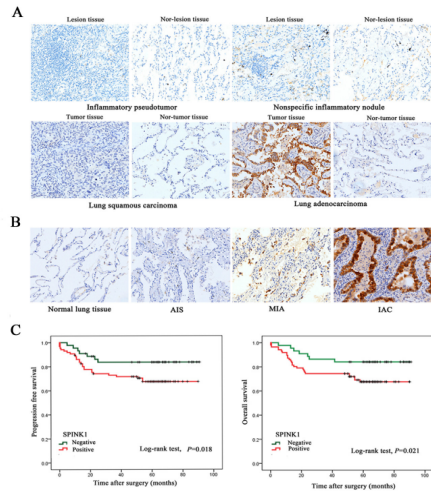


Fig. 4.

**SPINK1 promotes cell growth and metastasis in lung adenocarcinoma
and acts as a novel prognostic biomarker**

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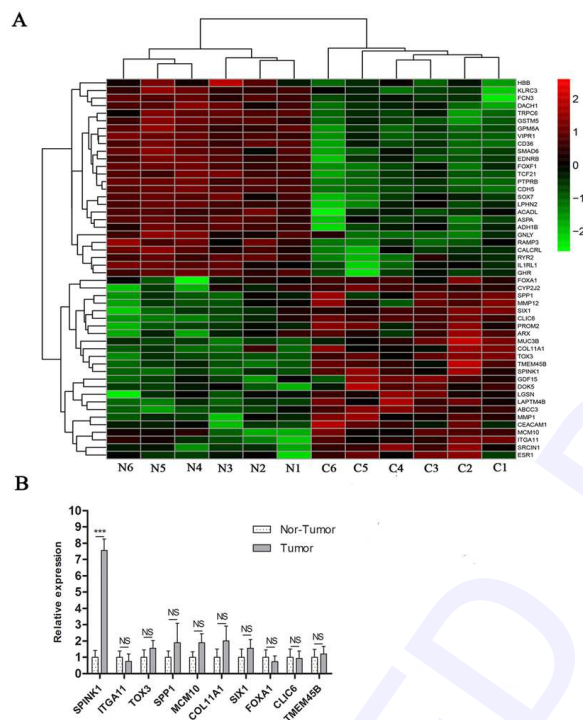
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Supplementary Information

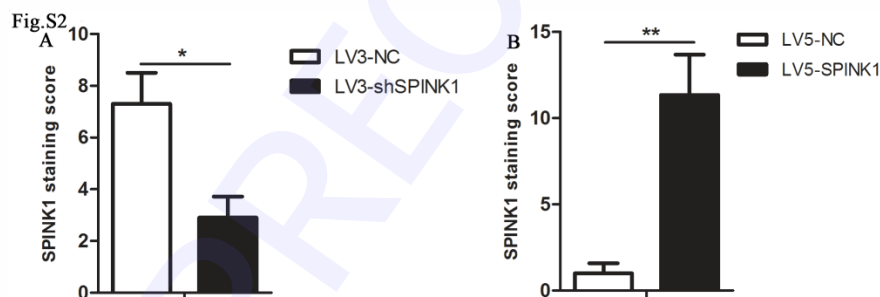
Supplementary Figure 1 Screening of up-regulated genes in LAC tissues. (A)

Microarray results of the up-regulated genes in LAC. We assayed 6 paired LAC vs. normal tissues. C and N stand for cancer and adjacent normal tissues, respectively. (B) Validation of microarray results by qRT-PCR. We selected 10 up-regulated genes in LAC and performed qPCR in 62 cases for the validation of relative mRNA expression in reference to GAPDH.

Fig. S1

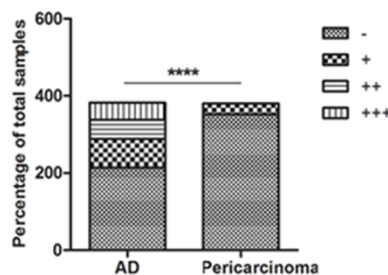


Supplementary Figure 2 The expression rate and staining intensity of SPINK1 in xenograft tumour tissues according to the German semiquantitative scoring system (* $P < 0.05$, ** $P < 0.01$).

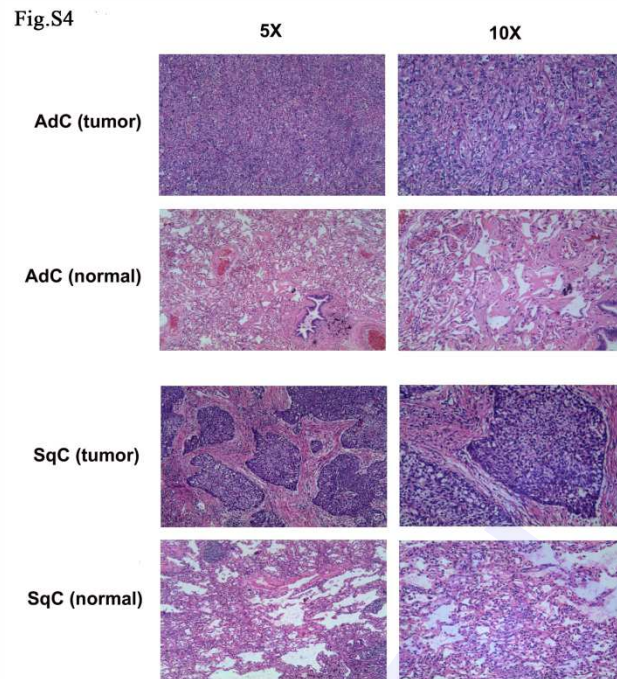


Supplementary Figure 3 Staining intensity of SPINK1 in lung adenocarcinoma (AD) tissues was significantly higher than that of the pericarcinoma tissues.

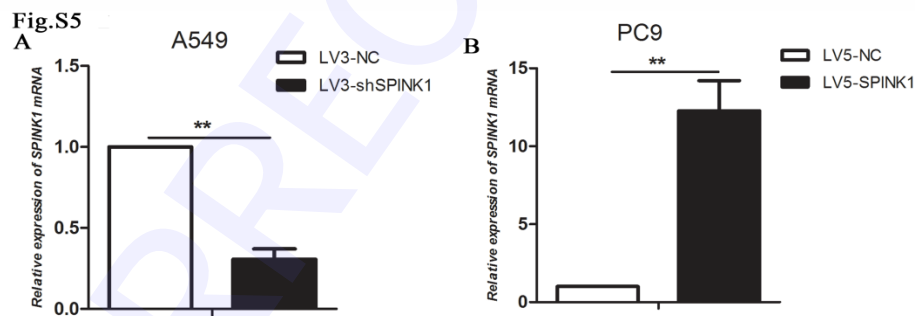
Fig. S3



Supplementary Figure 4 Histological images of lung adenocarcinoma tissues and the adjacent normal lung tissues (hematoxylin-eosin staining).



Supplementary Figure 5 Validation of stable SPINK1 knockdown or overexpression cells by qRT-PCR (** $P < 0.01$).



Supplementary Table 1 Information for patients that were used in performing microarray, qRT-PCR and IHC

Clinical Characteristics	No. (microarray)	No. (qPCR of mRNA)	No. (IHC for Nor)	No. (IHC for Sq)
Gender				
Male	3	27	26	42
Female	3	35	25	3
Age (years)				
<60	3	40	26	16
≥60	3	22	25	29
Smoking history				
No	3	50	35	6
Yes	3	12	16	39
CEA (IU/mL)				
<5.0	3	56	51	30
≥5.0	3	6	0	15
Size (cm)				
≤1.0	0	21	27	0
> 1.0-≤2.0	3	25	9	5
> 2.0-≤3.0	3	13	3	7
> 3.0	0	3	12	33
Pathological diagnosis				
AIS	0	8	NA	NA
MIA	0	10	NA	NA
IAC	6	42	NA	NA
Pleural invasion				
No	6	11	NA	35
Yes	0	51	NA	10
Lymph node metastasis				
No	5	54	NA	31
Yes	1	8	NA	14
TNM stage				
0	0	6	NA	NA
I	4	25	NA	23
II	2	27	NA	14
III	0	4	NA	8
Tumor stage				
Tis	0	6	NA	NA
T1	4	23	NA	14
T2	2	33	NA	25
T3	0	0	NA	6

Abbreviation: qPCR = Real-time Polymerase Chain Reaction; IHC = Immunohistochemistry; Nor = Normalinant nodules; Sq = Squamous carcinoma; CEA =Carcino-embryonic antigen; AIS =Adenocarcinoma in situ; MIA = Minimally invasive adenocarcinoma; IAC = Invasive adenocarcinoma; NA = Not applicable. A "T" score is based upon the size and/or extent of invasion.

The "N" score indicates the extent of lymph node involvement. The "M" score indicates whether distant metastases are present. We made this classification based on the 8th edition of UICC.

Supplementary Table 2 The top ten most significantly up-regulated DEGs.

Gene Symbol	Description	Fold Change	P-value
SPINK1	Serine Peptidase Inhibitor, Kazal Type 1	45.682	0.029
ITGA11	Integrin Subunit Alpha 11	22.488	0.014
TOX3	TOX High Mobility Group Box Family Member 3	15.609	0.000
SPP1	Secreted Phosphoprotein 1	14.952	0.008
MCM10	Minichromosome Maintenance 10	11.282	0.003
COL11A1	Collagen Type XI Alpha 1	9.1511	0.012
SIX1	SIX Homeobox 1	8.909	0.003
FOXA1	Forkhead Box A1	8.562	0.019
CLIC6	Chloride Intracellular Channel 6	6.25	0.003
TMEM45B	Transmembrane Protein 45B	5.856	0.003

Abbreviation: DEGs = Differentially expressed genes

Supplementary Table 3 Comparison of SPINK1 expression in lung cancer and nonmalignant lesions

Variables	Total	Negative	Positive
Non	51	51 (100%)	0
Sq	45	45 (100%)	0
Ad	382	225 (58.90%)	157 (41.10%)

Abbreviation: Ad = Lung adenocarcinoma; Non = Nonmalignant nodules; Sq = Squamous carcinoma.

Supplementary Table 4 Correlation between clinicopathological features and SPINK1 expression

Clinical Characteristics	Cases	SPINK1		<i>P</i> -value
		Negative	Positive	
Gender				0.024 ^a
Male	147	76	71	
Female	235	149	86	
Age (years)				0.081
<60	201	110	91	
≥60	181	115	66	
Smoking history				0.484
No	275	165	110	
Yes	107	60	47	
CEA (IU/mL)				0.001 ^b
<5.0	310	195	115	
≥5.0	72	30	42	
Tumor size (cm)				0.000 ^b
≤1.0	143	127	16	
> 1.0-≤2.0	124	65	59	
> 2.0-≤3.0	68	23	45	
> 3.0	47	10	37	
Histological type				0.000 ^b
AIS	103	96	7	
MIA	79	63	16	
IAC	200	66	134	
Pleural invasion				0.015 ^a
No	316	195	121	
Yes	66	30	36	
Lymph node metastasis				0.003 ^b
No	324	201	123	
Yes	58	24	34	
TNM stage				0.001 ^b
0	94	90	4	
I	213	115	98	
II	37	12	25	
III	38	8	30	
Tumor stage				0.001 ^b
Tis	95	91	4	
T1	201	114	87	
T2+T3	86	20	66	

Abbreviation: CEA = Carcino-embryonic antigen; AIS = Adenocarcinoma in situ; MIA = Minimally invasive adenocarcinoma; IAC = Invasive adenocarcinoma. aP < 0.05, bP < 0.01.

Supplementary Table 5 Univariate and multivariate Cox regression analyses for progression free survival (PFS) and overall survival (OS) of patients (n=130)

Variables	PFS		OS	
	HR (95%CI)	P value	HR (95%CI)	P value
Univariate analysis				
Sex (Male vs.Female)	0.887 (0.591-1.330)	0.561	0.914 (0.610-1.369)	0.663
Age (years) (<60 vs. ≥60)	1.151 (0.764-1.734)	0.502	1.185 (0.787-1.785)	0.417
Smoking history (No vs.Yes)	0.991 (0.656-1.496)	0.964	1.052 (0.697-1.587)	0.81
CEA (IU/mL) (<5.0 vs. ≥5.0)	1.522 (0.989-2.342)	0.056	1.675 (1.084-2.588)	0.02 ^a
Tumor size (cm) (≤2.0 vs. > 2.0)	2.574 (1.646-4.026)	<0.001 ^b	1.780 (1.182-2.678)	0.006 ^b
Pleural invasion (No vs. Yes)	1.462 (0.931-2.221)	0.426	1.200 (0.765-1.833)	0.426
Lymph node metastasis (No vs. Yes)	3.581 (1.646-8.620)	0.012 ^a	1.033 (0.612-1.744)	0.903
TNM stage (0/ I vs. II/III)	1.830 (1.362-2.458)	0.002 ^b	1.709 (0.832-1.399)	0.568
Tumor stage (Tis/T1 vs.T2/T3)	1.472 (0.976-2.221)	0.005 ^b	1.139 (0.752-1.724)	0.539
SPINK1 expression (negative vs. positive)	1.840 (1.189-2.848)	0.006 ^b	2.304 (1.321-3.130)	0.001 ^b
Multivariate analysis				
Sex (Male vs.Female)	NA		NA	
Age (years) (<60 vs. ≥60)	NA		NA	
Smoking history (No vs.Yes)	NA		NA	
CEA (IU/mL) (<5.0 vs. ≥5.0)	1.004 (0.575-1.695)	0.063	1.404 (0.892-2.212)	0.143
Tumor size (cm) (≤2.0 vs. > 2.0)	2.398 (1.373-4.188)	0.002 ^b	1.403 (0.902-2.181)	0.037 ^a
Pleural invasion (No vs. Yes)	NA		NA	
Lymph node metastasis (No vs. Yes)	2.838 (1.038-7.765)	0.042 ^a	NA	
TNM stage (0/ I vs. II/III)	1.174 (1.082-2.020)	0.039 ^a	NA	
Tumor stage (Tis/T1 vs.T2/T3)	NA		NA	
SPINK1 expression (negative vs. positive)	1.223 (1.142-2.009)	0.022 ^a	1.751 (1.110-2.762)	0.016 ^a

Abbreviation: CEA = Carcino-embryonic antigen; PFS = Progression-free survival; OS = Overall survival; HR = Hazards ratio; CI = Confidence interval. ^a $P < 0.05$, ^b $P < 0.01$.

Supplementary Table 6. Primer list for qRT-PCR

ID	Sequence(5'...3')
SPINK1 forward primer	ATATGACCCTGTCTGTGGGAC
SPINK1 reverse primer	CAGCAAGGCCAGATTTTGA
ITGA11 forward primer	ACCCAATCTGCACACTCCAG
ITGA11 reverse primer	TGGGTTCAATTCTTCGGGAGC
TOX3 forward primer	AGTGGCATAGGAGGGAAAAGC
TOX3 reverse primer	GACACTTGAGAGGACCGTTTGA
SPP1 forward primer	TGCTTCTTTCTCAGTTTATTGGTTG
SPP1 reverse primer	AGGGAGTTTCCATGAAGCCAC
MCM10 forward primer	CACCAGGTTGGTGTCTGAGC
MCM10 reverse primer	GCTCACCCTCAGCCTTTACA
COL11A1 forward primer	GCACAGACGGAGATAAATGGC
COL11A1 reverse primer	ATACCAGGCTCAACCACTGC
SIX1 forward primer	AAACCAACAGCGATCTCAAGC
SIX1 reverse primer	AACAGGCGTATCAGTTGCCC
FOXA1 forward primer	AAGTTACAAGGACCCCAACCC
FOXA1 reverse primer	GAAGCAGAGTTCTTGAGGGCA
CLIC6 forward primer	AGTGCTGGTGGTATCGTGTG
CLIC6 reverse primer	ACTCCCTCCCTAACTGGACC
TMEM45B forward primer	GGCACAGGTGTCCTGATGG
TMEM45B reverse primer	GCGGGTACTTCACTGACCAA
MMP12 forward primer	AGTTACCTTCAAAGGCCAAGAGA
MMP12 reverse primer	AGTCCAAGGATGTTAGGAAGCA
GAPDH forward primer	ACTAGGCGCTCACTGTTCTC
GAPDH reverse primer	CGACCAAATCCGTTGACTCC