Decreased INPP5B Expression Predicts Poor Prognosis in Lung Adenocarcinoma

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Research Article

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Abstract

Background

Inositol Polyphosphate-5-Phosphatase B (INPP5B), ainositol 5-phosphatase, plays important roles in many biological processes through phosphorylating PI(4,5)P$_2$ and/or PI(3,4,5)P$_3$ at the 5-position. Nevertheless, little is known about its function and cellular pathways in tumors. This study aims to investigate the potential value of INPP5B as a diagnostic and prognostic biomarker for lung adenocarcinoma (LUAD), as well as its biological functions and molecular mechanisms in LUAD.

Methods

TCGA, GEO, CTPAC, and HPA datasets were used for differential expression analysis and pathological stratification comparison. The prognostic and diagnostic value of INPP5B was determined by Kaplan-Meier curves, univariate and multivariate Cox regression analysis, and receiver operating characteristics (ROC) curve analyses. The potential mechanism of INPP5B was explored through GO, KEGG, and GSEA enrichment analysis, as well as GeneMANIA and STRING protein-protein interaction (PPI) network. PicTar, PITA, and miRmap databases were used for exploring miRNA targeting INPP5B. In molecular biology experiments, immunohistochemical analyses and Western blot analyses were used to determine protein expression. CCK8 assays and colony formation assays were used for the measurement of cell proliferation. Cell cycle was assessed by PI staining with flow cytometry. Cell migration was performed by Transwell assays and wound healing assays.

Result

INPP5B was decreased in LUAD tissues compared with normal adjacent tissues. And low expression of INPP5B was associated with adverse-pathology features. In addition, INPP5B was found to be a significant independent prognostic and diagnostic factor for patients with LUAD. Hsa-miR-582-5p was predicted as a negative regulator of INPP5B mRNA expression. INPP5B was significantly correlated with the expression of PTEN and the activity of PI3K/Akt signaling pathways, as determined by enrichment analysis and PPI network. In vitro experiments partially confirmed the aforementioned findings. INPP5B overexpression inhibited LUAD cell proliferation and migration while downregulating the Akt pathway.

Conclusion

Our results demonstrated that INPP5B could inhibit the proliferation and metastasis of LUAD cells. It could serve as a novel diagnostic and prognostic biomarker for patients with LUAD.

Trial registration

LUAD tissues and corresponding para-cancerous tissues were collected from 10 different LUAD patients at Hangzhou First People's Hospital. The Ethics Committee of Hangzhou First People's Hospital have approved this study. (registration number: T-20210907-0031-01 registration date: 2021.09.13)
Introduction

Lung cancer is the leading cause of cancer-related death worldwide, with a disproportionately high incidence in Asia[1]. Among the major histological subtypes of lung cancer, lung adenocarcinoma (LUAD) is the most prevalent, accounting for more than 40% of lung cancer cases[2]. Although several effective treatment options, including surgery, chemotherapy, radiation therapy, and targeted molecular therapy, have been widely used for patients with LUAD, the prognosis is still far from satisfactory in the long run[2]. The progression of LUAD is influenced by many factors, such as genes, signaling pathways, and the tumor micro-environment, among others. These factors may influence the prognosis of LUAD vary from one to another[5, 6]. Therefore, exploring more and efficient biomarkers is crucial for better predicting the diagnosis and prognosis of LUAD.

Phosphoinositides (PIs) are recognized as important second messengers in eukaryotes that carry out an array of critical signal transductions and cellular functionalities[7, 8]. There is a total of 8 kind of PIs in mammalian cells that can undergo reversible conversions, two of which, PI(4,5)P$_2$ and PI(3,4,5)P$_3$, are associated with the activation of PI3K/Akt signaling pathway[7, 8]. PI3K mediates Akt activation and modulates its downstream cellular functions through phosphorylating PI(4,5)P$_2$ to PI(3,4,5)P$_3$ in many malignancies[10]. This process can be reversed by multiple phosphatase dephosphorylation at the 3-, 4-, and 5- positions[11]. For instance, PTEN, a well-characterized tumor suppressor gene, is a 3-phosphoinositide phosphatase that dephosphorylates PI(3,4,5)P$_3$ to PI(4,5)P$_2$ and thus acts as an Akt suppressor[12]. Similarly, 5-phosphatases can dephosphorylate PI(4,5)P$_2$ and/or PI(3,4,5)P$_3$ at the 5-position except for inositol polyphosphate 5-phosphatase A (INPP5A), suggesting they may have tumor suppressive properties similar to PTEN[12]. INPP5D was reported to negatively regulate PI3K-generated signals, and deletion of INPP5D might lead to the disease progression of spontaneous B cell lymphomas[12]. In addition, the down-regulation of INPP5E, INPP5J, and INPP5K was also observed in various cancers, such as gastric cancer, lung adenocarcinoma, and hepatocellular carcinoma[16-19].

INPP5B belongs to the 5-phosphatases type II family. It shares a conserved 5-phosphatase central domain with other members of the family, implying that it may perform similar functions and follow similar pathways[20]. INPP5B-deficient mice did not show the characteristics of Lowe syndrome, while double-knockout mice for INPP5F and INPP5B were embryonically lethal, indicating that they possessed functional redundancy and were capable of substituting for one another in a particular activity[21]. On the one hand, INPP5B can directly hydrolyze PI(3,4,5)P$_3$ in the early stages; on the other hand, it can dephosphorylate PI(4,5)P$_2$, further reducing the possibility of PI(3,4,5)P$_3$ formation[22-24]. Although this evidence indicates that INPP5B has the potential capacity to inhibit Akt phosphorylation and activation, relatively little is known about its function and molecular mechanisms in tumors.

Microarray and sequencing technology advancements have resulted in the establishment of multiple cancer gene expression databases and profiling, which have evolved into useful tools for analyzing tumor progression mechanisms. The purpose of this study is to determine the diagnostic and prognostic value of INPP5B in LUAD, as well as its biological functions and the role of the PI3K/Akt signaling pathway in
this situation through combining bioinformatics and experimentation. It may shed light on a previously unknown role for INPP5B in the development and progression of LUAD.

**Materials And Methods**

**Gene expression analysis**

We collected the mRNA expression of INPP5B in pan-cancer through the TNMplot online database (https://tnmplot.com/analysis/), which includes 56,938 unique multilevel quality controlled samples[25].

The UCSC Xena browser (https://xenabrowser.net/) was used to download Level 3 RNA-sequencing data from LUAD patients, which analyzed the difference in INPP5B expression between LUAD and normal lung tissues from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression Project (GTex).

GeneChip data of 49 paired samples were downloaded from GSE10072 in the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The differential expression of INPP5B mRNA in the paired samples from GeneChip data was analyzed.

The UALCAN portal (http://ualcan.path.uab.edu/analysis-prot.html) is a web-based tool for conducting protein expression analysis of the clinical proteomic tumor analysis consortium (CPTAC)[26].

The HPA database (https://www.proteinatlas.org/), a web-based resource for analyzing cancer Omics data, enabled us to conduct a search for INPP5B expression and distribution in LUAD patients and normal lung tissues[27].

**The correlation between INPP5Bexpression and clinicopathological features of LUAD patients**

The UCSC Xena browser (https://xenabrowser.net/) was used to download Level 3 RNA-sequencing data from LUAD patients, while keeping samples with clinical information recorded. An analysis of TCGA data was conducted to explore correlations between INPP5B mRNA expression and clinicopathological features in LUAD patients. The OCLR method developed by Malta et al. was used to compute stemness index[27].

**Survival prognosis analysis**

The R packages "survival" and "survminer" were used to conduct survival and cox analyses on TCGA data. On the basis of GeneChip data, the Kaplan-Meier plotter online database (http://kmplot.com/analysis/) was used to assess the prognostic potential of INPP5B in LUAD[29]. The examination probe ID of INPP5B was 220580_at. By selecting the "auto select best cut-off" option, all possible cut-off values were computed and the threshold with the best performance was used as the cut-off.
ROC curve analysis

By using the R package “pROC”, the diagnostic utility of INPP5B in LUAD patients was evaluated by receptor operating characteristic (ROC) curve analysis based on GTEx normal lung samples, TCGA LUAD samples, and GSE10072 datasets.

Biological functional analysis

The co-expressed genes of INPP5B were calculated by the R package “stat” based on the TCGA project’s expression datasets of the LUAD transcriptome. Using the R package “ClusterProfiler”, gene enrichment analysis of Gene Ontology (GO) and Kyoto Encyclopedia of Genes, Genome (KEGG) and Gene set enrichment analysis (GSEA) were performed on genes with Spearman rho greater than 0.30. Results were presented using the R package “ggplot2”.

Protein-protein interaction analysis

GeneMANIA (http://www.genemania.org)[29] and STRING database (https://string-db.org/)[31] were used to probe the INPP5B-involved protein-protein interaction (PPI) network. After combining the results of GeneMANIA and STRING, we selected overlapping genes as hub genes. Additionally, the expression of the genes coexpressed with INPP5B was analyzed in GEPIA browser (http://gepia.cancer-pku.cn/index.html)[32]. The statistical significance of correlations was assessed with Spearman’s rank correlation coefficient test.

Mutation analysis

The TCGA mutation and expression data were obtained from cBioportal (http://www.cbiointerportal.org)[33]. The OncoPrinter (v1.0.1) tools of cBioPortal were used to produce the mutation graphs.

Targeted miRNA Prediction

PicTar, PITA, and miRmap databases were used for exploring miRNA targeting INPP5B[34]. After combining the results of the above databases, we selected the overlapping one and predicted its binding site with INPP5B. In addition, we analyzed the correlation between the miRNA and INPP5B expression in the TCGA database and analyzed the expression of the miRNA in LUAD patients and the impact on patient survival.

Patients and clinical samples
LUAD tissues and corresponding para-cancerous tissues were collected from 10 different LUAD patients at Hangzhou First People's Hospital. The Ethics Committee of Hangzhou First People's Hospital have approved this study. After formalin fixation, all samples were embedded in paraffin.

**Immunohistochemistry (IHC) analysis**

Paraffin-embedded pathology specimens were analyzed by immunohistochemistry following the standard procedure with anti-human INPP5B (proteintech, 15141-1-AP, 1:200), followed by HRP conjugated secondary antibody. The intensity of staining was defined by H-score: $H$-score = (weak intensity% × 1) + (moderate intensity% × 2) + (strong intensity% × 3).

**Cell culture**

Human lung carcinoma cells (A549, H358, H838, PC9) were cultured in 90% RPMI-1640 medium (HyClone, USA) with 10% with 10% (v/v) fetal bovine serum (HyClone, USA). Human lung bronchus epithelial cells (BEAS-2B) were cultured in medium containing 90% DMEM (HyClone, USA) and 10% fetal bovine serum. The cells were cultured at 37°C in a humidified atmosphere composed of 95% air and 5% CO$_2$. All cell lines were obtained from the Shanghai institute of biochemistry and cell biology (Shanghai, China), and were validated using short tandem repeat DNA profiling and mycoplasma testing.

**Western blot analysis**

Western blot analysis was performed in accordance with earlier published methods[35]. Briefly, protein was extracted from the cells, and concentration was measured with the Bicinchoninic Acid (BCA) protein assay kit (Beyotime). SDS-PAGE was used to separate samples, and they were electrophoretically transferred to PVDF membranes (BioRad, Hercules, CA, USA). After blocking with 5% BSA in TBST, membranes were incubated overnight at 4°C with primary antibodies, followed by 1 hour at room temperature with secondary antibodies. Prior to chemiluminescence visualization of samples, membranes were washed with TBST.

The primary antibodies were as follows: INPP5B (proteintech, 15141-1-AP, 1:1000), p-Akt$^{(Ser473)}$ (CST, 4060S, 1:1000), Wee1 (CST, 13084S, 1:1000), CDK1 (CST, 9116S, 1:1000), Cyclin B1 (CST, 12231S, 1:1000), p-S6$^{(Ser240/244)}$ (CST, 5364S, 1:1000), β-Actin (Santa Cruz, sc-47778, 1:1000)

**Transfection**

The full-length human INPP5B gene was cloned using BamHI–EcoRI restriction sites into the eukaryotic HA-tag fusion expression vector pcDNA3.1(-)-HA-tag. Following that, the recombinant plasmids were extracted from Escherichia coli DH-5α. Transient transfection was performed according to the
manufacturer's instructions using jetPRIME (Polyplus, NY, USA). The empty vector was used as a negative control. The transfection efficiency was tested by Western blotting.

**CCK8 assay**

Cell viability was determined using the Cell Counting Kit-8 (CCK8) assay (MedChem Express, USA). Following transfection, cells were seeded at a density of 500 cells per well in 96-well plates; absorbance values were determined 0 to 96 hours after transfection. 100 µL RPMI 1640 medium solution containing 10 µL of CCK8 was added to each well of the 96-well plates and incubated for an additional 2 hours. The absorbance at 450 nm was then determined using a microplate reader (Bio-Rad, USA). Each experiment had three duplicate wells and was performed three times.

**Colony formation assay**

Following the transfection, cells were counted and cultured in six-well plates with culture media at a density of 500 cells per well. Every three days, the medium of cultivation was changed. Colonies were counted only if they contained at least 50 cells. The cells were stained with 0.5% crystal violet for 30 minutes at room temperature. Each experiment was carried out three times in all.

**Cell cycle assay**

For cell cycle analysis, cells were collected and then digested using trypsin with 0.25% EDTA. After centrifugation to remove supernatant, the cells were washed three times with PBS, followed by the addition of 70% chilled ethanol and overnight storage at 4 °C; the cells were then washed again, centrifuged after resuspension, and cycle detection reagent (550825, BD, USA) was added for 30 minutes. Following incubation, samples were examined by flow cytometrically (BD Biosciences) and the results were processed using ModFit LT 2.0. The experiment was repeated three times independently.

**Wound healing assay**

A micropipette tip was used to scrape the cell monolayer at 100% confluence 24 hours after transfection. Photographs were obtained at 0h and 24h post-scratch with an inverted microscope. The scratch area was calculated to obtain the migration percentage. The experiment was repeated independently three times.

**Transwell migration assay**
After 24 hours of transfection, serum-free medium was used to prepare cell suspensions. The upper chamber of a 24-well plate was supplemented with 200 μL of 1640 medium containing $5 \times 10^4$ cells, and the lower chamber was supplemented with 800 μL of 1640 medium containing 20% FBS. After 24 hours of incubation at 37°C, cells were washed twice with PBS solution, fixed for 20 minutes at room temperature with methanol, stained with 0.5% crystal violet solution, and observed under an optical microscope. Each experiment was conducted a minimum of three times.

**Statistical Analysis**

The Wilcoxon signed-rank test was used to determine the expression of INPP5B. The associations between INPP5B expression and the clinicopathological parameters of the LUAD patients were evaluated using the Wilcoxon signed-rank test or the Kruskal-Wallis test. Paired t-test was used to compare two paired groups, while a two-sample t-test was used for non-paired data. The R programming language (version 3.6.3), GraphPad 8.0, ImageJ 1.48, and Adobe Illustrator CS6 were used to conduct all data analyses (*p* < 0.05, **p** < 0.01, and ***p*** < 0.001)

**Results**

**INPP5B is down-regulated in LUAD**

Currently, the relationship between INPP5B and oncogenesis is poorly understood. To address this question, we conducted a comprehensive analysis of 22 different types of tumors from the TCGA. As a result, the expression of INPP5B was decreased in 17 different types of solid tumors (Fig.1a). We further assessed the expression of INPP5B in the LUAD samples of TCGA compared with the normal controls of TCGA and GTEx. We found that INPP5B mRNA expression level had significant reductions in LUAD tissues (Fig.1b). This result was validated in paired samples of GSE10072(Fig.1c). The CPTAC database analysis revealed that the INPP5B protein expression was significantly reduced in LUAD tissues compared to normal lung tissues (Fig.1d). Likewise, IHC on tissue microarray from the HPA database indicated that LUAD tissues expressed significant lower levels of INPP5B than normal lung tissues (Fig.1e). Our experimental data was consistent with the results of bioinformatics analysis. The expression of INPP5B was significantly down-regulated in LUAD tissues compared with the paired para-cancerous tissues, as determined by IHC (Fig.1f). Western blot analysis confirmed that INPP5B protein levels had dramatically decreased in LUAD cells (Fig.1g). In addition, the potential for INPP5B to serve as a diagnostic biomarker for patients with LUAD was investigated using Receiver operating characteristic (ROC) curve analysis. ROC curve analysis based on TCGA database showed that INPP5B appeared to be a promising diagnostic biomarker for patients with the area under curve (AUC) equal to 0.899 (Fig.1h). ROC curve analysis in GSE10072 data revealed the similar result with the AUC value equal to 0.779 (Additional file 1: Fig. S1a). Taken together, these findings indicated that INPP5B exhibited higher expression in normal lung tissues comparing with LUAD tissues at both transcriptional and proteomic levels. INPP5B had the potential to be a novel diagnostic biomarker for patients with LUAD.
Low expression of INPP5B is associated with adverse-pathology features

Given the differences in expression of INPP5B between LUAD tissues and adjacent normal tissue, we sought to determine whether the expression of INPP5B was correlated with clinical-pathological parameters of patients with LUAD. As shown in Fig.2a,b,c, low INPP5B mRNA level is related to patients’ pathologic stage (a), tumor stage (b), and lymph node metastasis status(c). Namely, INPP5B expression was down-regulated in advanced stages LUAD compared with early stages LUAD. Meanwhile, LUAD tissues with lower INPP5B expression exhibited higher cancer stemness(Fig.2d). And the expression of INPP5B in the proximal proliferative (PP) and proximal inflammatory (PI) subtypes were lower than in the terminal respiratory unit (TRU) subtype (Fig.2e). Furthermore, The metastatic tumor expressed lower levels of INPP5B rather than the non-metastatic primary tumor or normal lung tissues (Fig.2f). These results suggested low INPP5B levels might be an unfavorable prognostic factor for patients with LUAD.

INPP5B expression associated with survival prognosis of LUAD patients

To determine the effect of the INPP5B expression on survival in patients with LUAD, we analyzed the prognostic value of INPP5B expression using TCGA data and GEO data. As demonstrated by the KM curve, there was a strong correlation between INPP5B expression and LUAD patients’ survival, such that low INPP5B expression resulted in poor overall survival (OS) (Fig.3a) and progression-free interval (PFI) (Fig.3b). In terms of treatment-related prognosis, LUAD patients with higher expression of INPP5B had a higher clinical benefit rate(Fig.3c). Besides, LUAD patients with higher INPP5B expression had a higher overall survival rate after surgery (Fig.3d), chemotherapy (Fig.3e) or radiotherapy (Fig.3f). The Univariate Cox analysis revealed that pathologic stage, T stage, M stage, and N stage were all risk factors, whereas INPP5B was a protective factor(HR=0.657; 95%CI: 0.490-0.881; p=0.005) (Fig.3g). Multivariate Cox analysis showed that INPP5B might be an independent factor improving the prognosis of patients with LUAD(HR=0.688; 95%CI: 0.478-0.972; p=0.034) (Fig.3h). Therefore, INPP5B expression was an independent predictor of survival in patients with LUAD.

Enrichment analysis explored INPP5B-related signaling pathways in LUAD

To investigate the potential biological functions and molecular mechanisms of INPP5B in regulating LUAD, enrichment analysis was performed. The GSEA analysis revealed that the terms "phosphoinositol signaling system" and "cell adhesion molecules CAMs" were differentially enriched in LUAD samples expressing high levels of INPP5B. The terms "cell cycle" and "oxidative phosphorylation," on the other hand, had a strong negative correlation with INPP5B expression (Fig.4a). In line with GSEA results, the
outcomes of GO and KEGG analyses revealed that the co-expressed genes of INPP5B were mainly associated with “positive regulation of cell adhesion”, “cell-cell junction”(Fig.4c), and “PI3K/Akt signaling pathway”(Fig.4b). All of these terms indicated that INPP5B exerted a significant effect on tumor proliferation and metastasis, which were consistent with the results in Fig.2. To elucidate this finding further, we used GEPIA database to analyze the association between INPP5B expression and various cell cycle-related genes. The results showed that INPP5B was significantly positively correlated with the expression of Wee1, while negatively correlated with CyclinB1, CDK1, and PCNA(Fig.4d). Combining the results mentioned above, we speculated that INPP5B might play a role in PI3K/Akt signaling pathway. Our experimental results verified this conjecture. Western blot analysis showed that the overexpression of INPP5B inhibited the activation of the Akt signaling pathway, which in turn increased Wee1 protein expression, whereas reduced CDK1 and Cyclin B1 protein levels(Fig.4e). Altogether, these data indicated that INPP5B might modulate the Akt signaling pathways to influence cell proliferation and migration, thereby limiting the occurrence and progression of LUAD.

**PPI network analysis constructed interaction networks for INPP5B**

In order to further investigate the molecular mechanism of INPP5B, we constructed INPP5B-involved PPI networks using GeneMANIA and STRING databases, respectively(Fig.5a, b). By taking the intersection of results from GeneMANIA (a) and STRING (b), the two most relevant genes were identified as PTEN. We then examined the expression correlations between INPP5B and PTEN using TCGA database. The results revealed that INPP5B expression was statistically positively associated with PTEN ($R=0.306, p<0.001$)(Fig.5c). The results of the TCGA and GTEx datasets showed lower expression of PTEN in LUAD tissues than that in normal tissues(Fig.5d). And Kaplan-Meier plotter overall survival (OS) analysis revealed that lower PTEN expression was associated with worse OS in LUAD patients(Fig.5e). Based on the above-mentioned results, there might be functional or structural interactions between PTEN and INPP5B.

**Hsa-miR-582-5p upregulation might be responsible for the loss of INPP5B in LUAD patients**

There are multiple causes for the loss of tumor suppressor genes, the most common of which include genetic mutations and epigenetic alterations[35]. By searching TCGA and cbioportal databases, we found that the expression of INPP5B was not related to typical driver-gene mutations of LUAD, such as EGFR mutation (Fig.6a), KRAS mutation (Fig.6b), and ALK mutation (Fig.6c). And INPP5B exhibited a low mutation rate in LUAD tissues (2.5%, 14/566) (Fig.6d). In addition, we found that the expression of INPP5B did not correlate with methylation levels (Fig.6e, f). MicroRNAs can be combined with the 3’ untranslated region (UTR) of the target gene to down-regulate the expression level of their target gene. We identified miRNAs that target INPP5B by taking the intersection of results from PicTar, PITA, and miRmap databases (Fig.6g). We got hsa-miR-582-5p and predicted its binding site to INPP5B (Fig.6h). The
correlation analysis results revealed that the expression of INPP5B and hsa-miR-582-5p was negatively correlated (R=-0.172, p=9.44e-05) (Fig.6i). The result from TCGA indicated that the expression level of hsa-miR-582-5p was increased in LUAD patients (Fig.6j). And higher hsa-miR-582-5p expression was associated with worse OS (Fig.6k). Together, these results implied that increased hsa-miR-582-5p might account for the loss of INPP5B rather than genomic alterations or DNA methylation in LUAD patients.

**INPP5B inhibited proliferation and migration of LUAD cells *in vitro***

Given that the results of bioinformatics analysis suggested that INPP5B was associated with proliferation and metastasis of LUAD cells, we further verified its biological functions by in vitro experiments. CCK8 assay revealed that the overexpression of INPP5B noticeably inhibited the proliferation of LUAD cells begins at 48-72h after transfection (p<0.01)(Fig.7a). Colony formation assay revealed a marked reduction of colonies formed by INPP5B overexpressing cells (p<0.001) (Fig.7b). Flow cytometry analysis demonstrated that overexpression of INPP5B caused 28.46±1.51% or 30.16±2.53% cell arrested in the G2/M phase in A549 and H358 cells respectively (Fig.7c). Additionally, INPP5B overexpression significantly impaired both the vertical and horizontal migration abilities of LUAD cells after 24 hours observation (p<0.01) (Fig.7d, e). As a result of our findings, INPP5B could inhibit the proliferation and migration abilities of LUAD cells *in vitro*.

**Discussion**

Despite great strides have been made in the understanding of fundamental molecular mechanisms of INPP5B, little is known about its function and cellular pathways in tumors[23]. Here, we demonstrated a causal relationship between decreased INPP5B expression and the development of lung adenocarcinoma (LUAD) by using combinatorial tools consisting of both bioinformatics analysis and experimental studies. In the first place, we discovered that INPP5B expression was decreased in a variety of solid tumors, especially LUAD. And the low expression of INPP5B is associated with adverse-pathology features and poor prognosis in LUAD. These results were consistent with a recent study in which a high mutation rate of INPP5B was observed in the genome-wide mapping of melanocytic neoplasms[37]. Furthermore, changes in the expression of INPP5B have also been found in the metabolomic data of hepatocellular carcinoma[38]. These findings suggested that loss of function or lower functioning of INPP5B might be related to the initiation and progression of tumors.

In view of the differential expression of INPP5B between LUAD and adjacent normal tissue, INPP5B might serve as a potential diagnostic and prognostic indicator in LUAD. This was partially supported by a recent study that was published when we were preparing the current manuscript. Han et al.[39] developed a four-gene signature including INPP5B which could effectively predict recurrence of early lung cancer patients and associated survival chances following surgery. However, their study was based on a model of multi-gene signatures. And it remained unknown whether INPP5B could be used as an independent diagnostic
or prognostic biomarker of LUAD. Besides, their study made it a priority to focus on the post-operative survival impact of the model, rather than its role in the development of LUAD. Therefore, the biological function of INPP5B in LUAD even in tumorshave not been reported so far. Our study further supplemented the biological function of INPP5B in LUAD. We found that low INPP5B expression was associated with advanced pathological stage and malignant progression in patients with LUAD. Interestingly, the differential expression of INPP5B in TMN staging and proliferative subtypes of LUAD, to some extent, reflected that INPP5B might affect tumor proliferation and metastasis. These part of results were consistent with enrichment analysis data which showed that INPP5B was associated with terms of “molecular adhesion” and “cell cycle functions”. We further validated this hypothesis in vitro.

Overexpression of INPP5B significantly inhibited the proliferation and migration of LUAD cells, blocking the cells in G2/M phase. Certainly, further work in vivo was necessary to confirm the anti-proliferation and anti-metastasis functions of INPP5B in LUAD.

While it is well established that inositol phosphatases fulfil their functions by dephosphorylating phosphoinositides which are required as a second messenger in the PI3K/Akt pathway, the role of inositol 5-phosphatases in regulating Akt is controversial[11]. On the one hand, 5-phosphatases can inhibit the activation of Akt by dephosphorylating PI(4,5)P$_2$ and/or PI(3,4,5)P$_3$ at the 5-position. On the other hand, dephosphorylation of PI(3,4,5)P$_3$ to PI(3,4)P$_2$ enhances Akt activation because PI(3,4)P$_2$ is considered to be another potent Akt agonist[9, 40, 41]. It is therefore hard to predict how inositol 5-phosphatases affect Akt in advance. Oomset al observed that Loss of INPP5J in breast cancer promotes Akt activation, leading to the promotion of tumor growth[42]. In contrast, Bohdanowicz M et al. found that APPL1 recruited INPP5F and INPP5B, effectively terminating PI(3,4,5)P$_3$ synthesis via substrate depletion and direct dephosphorylation, thereby limiting the duration of Akt activation[43]. Our findings were consistent with the latter. GSEA enrichment analysis showed that INPP5B was significantly enriched in PI3K/Akt pathway. Western blot analysis revealed that INPP5B increased the expression of Wee1 by dephosphorylating Akt, inhibiting the formation of CyclinB1-CDK1 complexes, and thereby arresting the cells in the G2/M phase. Furthermore, PPI analyses indicated that INPP5B and PTEN might interact. PTEN is known to be a tumor suppressor by inhibiting the activation of PI3/Akt pathway[44]. Although our experimental results indicated that overexpression of INPP5B had no effect on PTEN expression(Additional file 1: Fig. S1b), we hypothesized that the interaction between INPP5B and PTEN may be functionally or structurally complementary, rather than acting directly upstream or downstream of PTEN. Other researchers also shared similar interpretations. Hellsten et al.[45] observed that the dephosphorylation function of INPP4B could act as a "backup" mechanism when PTEN is deficient. It will be of great interest to explore whether INPP5B can also exert its dephosphorylation effect through interacting with PTEN. The investigation now in progress in our laboratory may clarify this question.

**Conclusions**

This study has integrated public sequencing data in a systematic manner to guide this research of INPP5B in LUAD. Our present study suggested that the expression of INPP5B was decreased in LUAD,
which was associated with tumor progression and poor prognosis in patients with LUAD. Additionally, INPP5B might act on downstream target genes to inhibit the proliferation and metastasis of LUAD cells by dephosphorylating Akt via interacting with PTEN. Finally, we demonstrated that increased hsa-miR-582-5p might account for the loss of INPP5B rather than genomic alterations or DNA methylation in LUAD patients.

This study aims to investigate the potential value of INPP5B as a diagnostic and prognostic biomarker for LUAD. In conclusion, INPP5B, as a tumor suppressor gene, has the potential to be used as a novel biomarker in LUAD patients, which may shed new lights on LUAD prevention and treatment in the future.

Declarations

- **Ethics approval and consent to participate**

The Ethics Committee of Hangzhou First People's Hospital have approved this study. (registration number:T-20210907-0031-01, registration date:2021.09.13)

- **Consent for publication**

Not applicable.

- **Availability of data and materials**

The datasets generated and/or analyzed during the current study are available in the [TCGA] repository [https://tcgadata.nci.nih.gov/tcga/] [34] and [GEO] repository [http://www.ncbi.nlm.nih.gov/geo] [35]. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

- **Competing interests**

The authors declare that they have no competing interests.

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- **Authors' contributions**
JD made substantial contributions to conception and design, acquisition of data, and analysis and interpretation of data. XL performed the experiments and wrote the main manuscript text. QL, LW and XC was involved in performing the experiments. BZ, YL and JH revised the article critically for important intellectual content; NL had given final approval of the version to be published. All authors read and approved the final manuscript.

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**Figures**

**Figure 1**

INPP5B expression in LUAD tissues at the mRNA and protein level. a Different expression of INPP5B in pan-cancer by RNASeq obtained from the TNMplot. Siginificant differences by Mann-Whitney U test marked with red*; black indicated no difference. b Expression of INPP5B in LUAD tissues (TCGA, n=374) and healthy lung tissues (TCGA and GTEx, n=515) by RNASeq. c Paired expression data for LUAD and adjacent normal tissue by RNASeq in GSE10072 dataset. (n=58). d The expression level of INPP5B total protein between normal tissue and primary tissue of LUAD based on the CPTAC dataset. (n=111) e Immunohistochemistry tissue microarray images of proteins INPP5B detected in the HPA database. f Immunohistochemistry images of proteins INPP5B for LUAD tissue samples and tumor adjacent tissue (TAT). g Western blot analysis of INPP5B expression in BEAS-2B cells and four LUAD cell lines. *p =0.05, **p =0.01, ***p =0.001. h ROC (Receiver operator characteristic curve) analysis of INPP5B in patients with LUAD based on TCGA and GTEx data. AUC area under the curve.

**Figure 2**

The correlation of INPP5B mRNA expression with risk factors and the clinicopathological characteristics of LUAD patients in TCGA database. a Pathologic stage(Stage I&Stage II=417;Stage III&Stage IV=110) b T
stage(T1&T2=464;T3&T4=68)  c N stage (N0&N1=443,N2&N3=76) d Cancer cell stemness( High=127;Low=126) e LUAD gene expression subtypes included terminal respiratory unit (TRU), proximal proliferative (PP), and proximal Inflammatory (PI). The data were collected from TCGA Portal (http://www.tcgaportal.org/TCGA/Lung_TCGA_LUAD/index.html). f Compare Tumor, Normal and Metastasis. The data were collected from Tnmplot (https://tnmplot.com/analysis/).*p≤0.05, **p≤0.01, ***p≤0.001.

Figure 3

Prognostic role of INPP5B in lung adenocarcinoma. a,b KM survival curves for overall survival (a) and progression free interval (b) in LUAD patients from the Kaplan-Meier plotter online database or TCGA database. Grouping by “auto select best cutoff”. c Primary therapy outcome included complete response (CR), partial response (PR), stable disease (SD), progressive disease(PD). Clinical benefit rate (CBR) = CR + PR + SD (PR&CR&SD=375,PD=71). d-f Overall survival of patients with lung adenocarcinoma after successful surgery (d), Chemotherapy (e) and Radiotherapy (f), respectively. The data were collected from the Kaplan-Meier plotter online database. Grouping by "auto select best cutoff". g,h Univariate analysis (g) and Multivariate analysis (h) of TCGA database. HR>1 is poorly prognostic; HR<1 indicates improved OS and any characteristic crossing the line at 1 is not significant.

Figure 4

Pathway enrichment analysis of INPP5B. a Enrichment plots from GSEA. NES, normalized enrichment score of GSEA. p<0.05 and FDR<0.25 were considered statistically significant for GSEA. b KEGG signaling pathway enrichment analysis. c Gene ontology analysis included biological process (BP), molecular function (MF), and cellular component (CC). d The expression relationship between INPP5B expression and cell cycle-related genes. e Western blot analysis validation of Akt signaling pathway in LUAD cell lines. Experiments were repeated three times, and data from a representative experiment are shown.

Figure 5

INPP5B-involved protein-protein interaction network. a-c The INPP5B-involved protein-protein interaction network constructed by STRING database (a) and GeneMANIA database (b), respectively. The intersections of above datasets identified as key genes (c). d The expression relationship between INPP5B and PTEN in lung adenocarcinoma from TCGA database. e Expression of PTEN in LUAD tissues (TCGA) and healthy
lungs (TCGA and GTEx) by RNASeq. e Survival analysis of PTEN in LUAD determined by Kaplan-Meier plotter database.

Figure 6

Reasons for the decreased expression of INPP5B in LUAD patients. a Correlation of EGFR mutations and INPP5B expression in LUAD tissues. b Correlation of KRAS mutations and INPP5B expression in LUAD tissues. c Correlation of ALK mutations and INPP5B expression in LUAD tissues. d Mutation rate of INPP5B in patients with LUAD from the TCGA dataset, n=566 e Correlation of INPP5B expression and INPP5B methylation level in LUAD. f Survival analysis of INPP5B methylation level in LUAD patients. g The intersection results of PicTar, PITA, and miRmap databases for microRNA targeting INPP5B prediction. h Possible binding sites between hsa-miR-582-5p and INPP5B. i Correlation between INPP5B expression and hsa-miR-582-5p expression in LUAD tissues. j Expression of hsa-miR-582-5p in LUAD patients. k Survival analysis of hsa-miR-582-5p in LUAD patients.

Figure 7

Validation of overexpressed INPP5B as a tumor suppressor in vitro. a The CCK-8 assay showed the overexpression of INPP5B inhibited proliferation of lung adenocarcinoma cells. b The colony formation assay showed INPP5B overexpression suppressed the proliferation abilities of lung adenocarcinoma cells. c Cell cycle and statistical analysis showed significant G2 arrest after INPP5B overexpression. d Results of wound healing assay showed that INPP5B overexpression inhibited the migration ability of lung adenocarcinoma cells. Scale bar: 50μm. e Results of transwell assay evaluating showed significant decrease in the migration ability of lung adenocarcinoma cells after INPP5B overexpression. Scale bar: 50μm. *p < 0.05, **p < 0.01, ***p < 0.001.

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