INTRODUCTION

Lung cancer, one of the most prevalent malignancies worldwide, is becoming a leading contributor to tumor-associated death (1, 2). Non-small cell lung cancer (NSCLC) reportedly comprises 80% of all new lung cancer cases (3). Tobacco consumption has been regarded as one of the chief risk factors for lung cancer (4). Currently, substantial progress has been made in the clinical application of small molecule tyrosine kinase inhibitors (TKIs) and immunotherapy for treating NSCLC patients, but the overall cure and survival rates of these patients, especially in terms of metastatic disease, are very low (5). Existing clinical therapies for lung cancer mainly include radiotherapy, chemotherapy, surgical resection, and targeted drug therapy. All treatment options vary depending on the type of lung cancer and the phase of the tumor. Radiotherapy and chemotherapy are usually given for limited-stage lung cancer, but the chance of a cure becomes slim in patients with extensive metastatic lung cancer. Systemic chemotherapy indeed helps extend the survival period of patients (6). Unfortunately, many patients exhibit chemotherapy resistance, which is the most pivotal factor for chemotherapy failure (7, 8). Paclitaxel, cisplatin, and gemcitabine are chemotherapeutic drugs that are commonly used as frontline lung cancer chemotherapy agents and are capable of hindering tumor angiogenesis and cell proliferation and eliciting apoptosis (9-12). Thus, NSCLC cell resistance is a challenge in chemotherapy, and the key to enhancing the efficacy of cancer treatment lies in enhancing NSCLC cell chemotherapy sensitivity.

KINETOCHORE SCAFFOLD 1 DOWNREGULATION SUPPRESSED THE DEVELOPMENT OF NON-SMALL CELL LUNG CANCER BY INACTIVATING THE PHOSPHATIDYLINOSITOL 3 KINASE/PROTEIN KINASE B (AKT)/NUCLEAR FACTOR-KAPPA B PATHWAY

Kinetochore scaffold 1 (KNL1) is indispensable for generating motile micro-tubule attachments and isolating chromosomes. KNL1 is highly expressed in multiple middle-route tissues and promotes tumor development. However, how it functions in non-small cell lung cancer (NSCLC) is unclear. Real-time quantitative PCR (RT-qPCR) and Western blotting (WB) were used to determine KNL1 expression in NSCLC tissues and cells. The sh-KNL1 or oe-KNL1 was transfected into NSCLC cells. The colony formation assay, cell counting kit-8 (CCK-8) assay, and flow cytometry were used to evaluate cell proliferation and apoptosis. A transwell assay was used to monitor invasion and migration. The CCK-8 assay was used to measure NSCLC cell sensitivity to chemotherapy drugs. WB confirmed the protein levels of apoptosis-related proteins, cell cycle-associated proteins, and the phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT)/nuclear factor kappaB (NF-κB) pathway. A PI3K/AKT/NF-κB pathway inhibitor was used to intervene in NSCLC cell transfection along with oe-KNL1, thus revealing the function of the pathway in carcinogenicity mediated by KNL1. In result KNL1 expression was substantially increased in NSCLC tissues and cells. High-level KNL1 expression is related to the poor prognosis of NSCLC patients. KNL1 silencing bolstered promoted NSCLC cell apoptosis and inhibited proliferation, cell cycle progression, invasion, and EMT, whereas KNL1 silencing had the opposite effect. KNL1 knockdown increased NSCLC cell sensitivity to chemical drugs. KNL1 promoted PI3K/AKT/NF-κB pathway activation, while PI3K/AKT/NF-κB pathway inhibition weakened the procancer effect mediated by KNL1 overexpression but had little influence on KNL1 levels. We conclude that KNL1 activates the PI3K/AKT/NF-κB pathway to increase NSCLC progression and attenuate NSCLC sensitivity to chemotherapy drugs.

Key words: Kinetochore scaffold 1, non-small cell lung cancer, phosphatidylinositol 3 kinase, chemotherapy, nuclear factor kappaB, cell cycle, apoptosis, epithelial-mesenchymal transition
lung adenocarcinoma progression (15). Nonetheless, the influence of KNL1 on NSCLC development and its underlying mechanism have yet to be determined.

Phosphatidylinositol 3-kinase (PI3K)-protein kinase B (AKT), a significant signaling pathway in cells, is correlated with many cell functions and plays a critical role in multiple diseases, such as cancer (16). PI3K-AKT, one of the prevailing activated signaling pathways, mediates tumor cell proliferation, differentiation, migration, and invasion and is intricately associated with NSCLC occurrence and progression (17, 18, 19). NF-xB, a transcription factor and a significant downstream molecule of PI3K-AKT, is crucial for modulating immune stress, cell differentiation, and apoptosis (20). Studies have shown that inhibiting the PI3K-AKT/NF-xB signaling pathway can induce apoptosis in NSCLC (21). In addition, Akguns et al. (22) reported that NF-xB is a continuously active transcription factor in malignant lung cancer cells. MiR-548as-3p mediates the PI3K-AKT pathway and NF-xB-related genes, which enhance the invasiveness of NSCLC. However, whether KNL1 can regulate the PI3K-AKT-NF-xB pathway in the progression of NSCLC cells is still unclear.

We constructed overexpression and knockdown models of KNL1 in NCI-H1650 and NCI-H1299 cells (both are non-small cell lung cancer cell lines) to investigate the impact of KNL1 on the biological development and chemosensitivity of NSCLC cells. We discovered that KNL1 could serve as an oncogene to promote the malignant biological behaviors of NSCLC cells and attenuate the sensitivity of NCI-H1650 and NCI-H1299 cells to paclitaxel, cisplatin, and gemcitabine. The carcinogenic function of KNL1 might be correlated with PI3K-AKT-NF-xB pathway activation. These discoveries provide us with new insights into targeted molecular therapies for NSCLC patients.

MATERIAL AND METHODS

Clinical specimen collection

Cancerous and normal paracancerous tissues were harvested from 40 NSCLC patients at the Thoracic Surgery Department of The First Affiliated Hospital of Bengbu Medical College from December 2019 to December 2020. The inclusion criteria for patients were as follows: 1) pathologically diagnosed with NSCLC; 2) not receiving neoadjuvant chemotherapy or radiotherapy prior to surgery; and 3) not receiving immunosuppressive therapy, biotherapy, or targeted therapy. The exclusion criteria for patients were as follows: 1) had small cell lung cancer and 2) quit their own initiative.

The research was approved by the Ethics Committee of The First Affiliated Hospital of Bengbu Medical College, and the participants provided informed consent.

Bioinformatics analysis

LinkedOmics (https://www.linkedomics.org/) is an open database that includes multi-omics data from all 32 TCGA cancer types (23). We used this database for analyzing KNL1-associated genes in LUAD. Step 1, TCGA LUAD cohort is selected. Step 2, chose data type “RNAseq” on the “HiSeq RNA” platform. Step 3, input “CASC5”. Step 4, select data type “RNAseq” on the “HiSeq RNA” platform. Step 5, input “CASC5”. Step 6, select “Pearson correlation test” and click “submit query”.

Cell culture

NSCLC cell lines (NCI-H1650, NCI-H1299), obtained from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), were grown in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA) medium supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin (Invitrogen, Carshad, CA, USA) supplemented with 5% CO₂ at 37°C. The medium was renewed every three days. The cells in the logarithmic growth phase were passaged after being trypsinized with 0.25% trypsin (Thermo Fisher HyClone, Logan, UT, USA) and harvested for the following experiments.

Cell transfection

NCI-H1650 and NCI-H1299 cells in the logarithmic growth stage were inoculated into 6-well plates at 5×10⁵ cells/well and transfected when they achieved stable growth. KNL1 overexpression plasmids (oe-KNL1), blank control vectors (Vector, Beijing, China), KNL1 low expression plasmids (sh-KNL1), and negative control plasmids (sh-NC) were transfected into NCI-H1650 and NCI-H1299 cells according to the instructions of the FuGENE® HD Transfection Reagent (Roche, Shanghai, China). The cells in each group were incubated in an incubator with 5% CO₂ at 37°C. The transfection reagent was removed 48 hours later and the cells were incubated in a new fresh medium. 5 days after transfection, KNL1 level was determined by Western blot to confirm the transfection efficiency (14).

Cell treatment

When the NCI-H1650 and NCI-H1299 cells entered the logarithmic growth stage, medium supplemented with 5 µM paclitaxel/cisplatin/gemcitabine was added for 24 hours of treatment. Then, the medium was replaced with normal complete medium for trypsinization and passage. When the cells reached 80% confluence, 0.5 µM was used as the starting concentration. Forty eight hours later, the cells were cultured in normal medium. The concentrations of the drugs were gradually increased after the cells reached normal and steady growth. These procedures were repeated until stable growth and passage could be achieved via cells treated with 5 µM paclitaxel/cisplatin/gemcitabine (12).

Cell counting kit-8 assay

CCK-8 assay was used to test cell viability. NCI-H1650 and NCI-H1299 cells in the logarithmic growth phase were trypsinized and then inoculated into 96-well plates, after which the cell density was adjusted to 2×10⁴ cells/well. 100 µL of the cells treated with 5 µM paclitaxel/cisplatin/gemcitabine was added for 24 hours of treatment. Then, the medium was replaced with normal complete medium for trypsinization and passage. When the cells reached 80% confluence, 0.5 µM was used as the starting concentration. Forty eight hours later, the cells were cultured in normal medium. The concentrations of the drugs were gradually increased after the cells reached normal and steady growth. These procedures were repeated until stable growth and passage could be achieved via cells treated with 5 µM paclitaxel/cisplatin/gemcitabine (12).

Drug sensitivity examination

The tumor cells (NCI-H1650 and NCI-H1299) in the logarithmic growth phase were collected using 0.25% trypsin for 1-2 hours, after which the absorbance of each well was measured at 450 nm (24).
BrdU experiment

NCI-H1650 and NCI-H1299 cells were inoculated into 24-well plates (5×10^5 cells/well) for 24 hours of culture. Next, BrdU (20 μM) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well, and the cells were incubated for 4 hours. The cells were fixed with paraformaldehyde (4%) for 30 min, washed with PBS three times, and permeabilized with 0.1% Triton X-100 in PBS. After that, the cells were blocked with 5% FBS solution and incubated with an anti-BrdU antibody (ab220074, 1:100) for 18 hours at 4°C. The nuclei were counterstained with DAPI (Beyotime, Shanghai, China). The fluorescence images were observed under a fluorescence microscope (BX53, Olympus, Tokyo, Japan). Three replicate wells were established for each group. The experiment was performed in duplicate. The mean value was taken (25).

Flow cytometry

Cells were flushed in PBS twice and resuspended in 150 μL of binding buffer. After that, 10 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) staining solution were added to the buffer. The cells were gently shaken in a centrifuge tube to mix, after which the mixture was incubated in the dark for 15 minutes at 4°C. An Annexin V-FITC/PI Apoptosis Detection Kit (Yeasen Biotech Co., Ltd., Wuhan, China) was used to examine apoptosis in each group, as instructed, via flow cytometry (24).

Transwell assay

For the cell migration test, the cells in the logarithmic growth phase were added to the upper Transwell compartments of each group at a density of 2×10^4 cells/well, and the lower chamber was given 600 μL of culture solution containing 20% FBS. The Transwell was maintained at 37°C. After 12 hours, the cells in the upper compartments were removed, while the residual cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, dried, photographed, and counted. For the cell invasion test, upper Transwell chambers were coated with Matrigel prior to the addition of cells, and the other steps were identical to those used in the migration experiment. An inverted microscope was used to capture images and observe cell migration and invasion in each group (15).

Gene expression analysis

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA of KNL1 from NCI-H1650 and NCI-H1299 cells as well as from the corresponding drug-resistant strains (NCI-H1650R and NCI-H1299R). The PrimeScript™ RT Reagent Kit (Invitrogen, Shanghai, China) was used to reverse transcribe mRNA into cDNA. Real-time fluorescence quantitative PCR (RT-qPCR) was subsequently performed with SYBR Green PCR reagent and an ABI 7500 FAST Real-Time PCR system. The 2^(-∆∆Ct) approach was used to assess the relative expression of KNL1 (after normalization to GAPDH as the internal parameters) (11).

The sequences of primers used are detailed below:

- KNL1: forward (5’-3’) GCTCCTCCTTCCTCCTCCTTTT; reverse (5’-3’) CCAAGGAGTCTGCAATGGTG
- GAPDH: forward (5’-3’) TTGGTTGACGACAGGGAACCTT; reverse (5’-3’) CCAAGGAGTAAGACCCCTGG

Western blot

Precooled PBS was used to flush 2×10^6 cells from each group three times, after which the cells were lysed using RIPA lysis buffer. As the supernatant was harvested, the BCA method was used to determine the protein concentration. After 20 μg of total protein was subjected to polyacrylamide gel electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk powder at room temperature for 2 hours and washed with TBST three times. Then, the sections were incubated with primary antibodies (1:1000) against KNL1 (ab70537), Bax (ab32503), Bcl-2 (ab218123), Bad (ab32445), E-cadherin (ab40772), vimentin (ab92547), Snail (ab85936), PI3K (ab278245), PI3K (ab32089), p-AKT (ab8805), AKT (ab131168), p-NF-κB p65 (ab28849), NF-κB p65 (ab7549), CDK2 (ab32147), CDK4 (ab108357), cyclin D (ab16663), cyclin E (ab33911), anti-p21 (109199), and β-actin (ab115777) overnight at 4°C.

These antibodies were all obtained from Abcam (Waltham, MA, USA). After the membranes were washed with TBST, they were incubated with horseradish peroxidase (HRP)-labelled anti-rabbit secondary antibody (concentration: 1:300) for an additional hour. The membranes were then rinsed with TBST

Fig. 1. KNL1 expression is increased in NSCLC cell line tissues. (A): KNL1 expression in NSCLC tissues was investigated via a database (https://www.proteinatlas.org/). (B-C): RT-qPCR and WB confirmed KNL1 expression in NSCLC tissues and normal paracancerous tissues. ***p<0.001. N=20.
three additional times. Finally, WB reagent (Invitrogen, Shanghai, China) was used for colour development and imaging, and ImageJ was used to analyse the grey value of each protein (11).

Nude mouse xenograft experiments

The animal experiments were reviewed, approved and carried out under the guidance of the Animal Care and Committee. C57BL/6 mice (male, 8 weeks old) were acquired from Shanghai Slac Laboratory Animal Co., Ltd. (Shanghai, China) and housed in a specific pathogen-free animal room for at least one week of domestication. The room temperature was maintained at 20–22ºC, the relative humidity was 40–60%, and the light/dark cycle was 12 hours. Control NSCLC cells or NSCLC cells stably transfected with the corresponding plasmids (blank group, vector group, KNL1 group) were subcutaneously injected into nude mice (1×10⁷ cells per mouse) to induce tumor formation. The mice were monitored every day for 35 days to observe tumor growth. Tumor length (L) and width (W) were measured. The longest diameter of the tumor (a) and the shortest diameter perpendicular to it (b) were assessed, and the tumor volume was calculated according to the formula $V = 0.5 \times a \times b^2$. Changes in tumor volume were observed. At the end of the experiments, the tumors from each mouse were weighed (24).

Immunohistochemistry (IHC)

After fixation with formaldehyde (10%), the tumor tissues were embedded in paraffin and cut into 4 µm thick sections. After the sections were incubated with the primary antibody against Ki67 (ab15580; 1:200; Abcam, Cambridge, UK), they

Fig. 2. KNL1 expression is associated the prognosis of NSCLC patients. (A): The GEPIA database (http://gepia.cancer-pku.cn/) corroborated the profile of KNL1 in LUAD and LUSC. (B): The GEPIA database was used to evaluate the prognostic survival period of LUAD patients.

Fig. 3. KNL1 overexpression facilitates NSCLC cell proliferation and invasion. (A): KNL1-overexpressing cell model was established in the NSCLC cell line NCI-H1650. (A-B): RT-qPCR and WB were used to determine KNL1 expression. (C-D): CCK-8 and BrdU assays were used to evaluate NCI-H1650 cell viability. (E): Transwell assays were used to measure NCI-H1650 cell invasion and migration. **P<0.01, ***P<0.001 (vs. the vec group). N=3.
were further probed with a secondary antibody (ab171870, 1:2000; Abcam, Cambridge, UK). Following staining with diaminoaniline (DAB; Sangon Biotech, Shanghai, China) and counterstaining with hematoxylin (Sangon Biotech, Shanghai, China), the sections were observed using an Olympus microscope (24).

Statistical analysis

All the statistical analyses were conducted using SPSS 22.0 statistical software (SPSS Inc., Chicago, IL, USA), and all the data are presented as the means ± standard deviations. The χ2 analysis method was used for comparisons of measurement data.
or percentages (%). One-way ANOVA was applied to compare multiple groups, while a t-test was utilized for comparisons between two groups. P<0.05 was regarded as statistically significant.

RESULTS

**KNL1 expression was elevated in NSCLC cell lines**

To determine whether KNL1 functions in NSCLC development, we consulted a database (https://www.proteinatlas.org/) and discovered that the KNL1 level was notably greater in NSCLC tissues than in normal paracancerous tissues (Fig. 1A). RT-qPCR (P<0.05; Fig. 1B) and WB (Fig. 1C) revealed that KNL1 expression was markedly greater in NSCLC tissues than in normal paracancerous tissues. In addition, we evaluated the expression features of KNL1 in lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) through the GEPIA database (http://gepia.cancer-pku.cn/). KNL1 indeed presented high expression in LUAD and LUSC (Fig. 2A). Moreover, the GEPIA database also suggested that LUAD patients with high KNL1 expression had a shorter overall survival (OS) period and a lower prognostic survival rate (Fig. 2B). All these findings demonstrated that KNL1, which is associated with the malignant phenotype of NSCLC cells, has a carcinogenic function.

**KNL1 overexpression facilitates NSCLC cell proliferation and invasion and hampers apoptosis**

To investigate the influence of KNL1 on NSCLC progression, we established a KNL1-overexpressing cell line, the NCI-H1650 cell line (P<0.05; Fig. 3A and 3B). CCK-8 and BrdU assays revealed that KNL1 overexpression substantially increased cell viability (P<0.05; Fig. 3C and 3D). Transwell assays revealed that KNL1 overexpression enhanced NSCLC cell invasion and migration (P<0.05; Fig. 3E). As shown by flow cytometry, apoptosis was lower in the oe-KNL1 group than in the vector group (P<0.05, Fig. 4A). WB analysis revealed that KNL1 overexpression increased the expression of the antiapoptotic protein Bcl-2 and decreased the expression of the proapoptotic proteins Bax and Bad (P<0.05; Fig. 4B). KNL1 overexpression evidently decreased the expression of E-cadherin, an epithelial marker, and increased the protein expression of vimentin and Snail, interstitial markers (P<0.05; Fig. 4C). To better understand the impact of KNL1 on the NSCLC cell cycle, we performed a WB to evaluate the profiles of cell cycle-related proteins. The oe-KNL1 group exhibited a distinct downregulation of p21 and a marked upregulation of CDK2, CDK4, cyclin D and cyclin E in contrast with the control group (P<0.05; Fig. 4D). These findings indicated that KNL1 overexpression enhances the malignant biological behaviors of NSCLC cells.

**KNL1 inhibition attenuated NSCLC cell proliferation and invasion and promoted their apoptosis**

To determine the influence of KNL1 on NSCLC development, we established a KNL1-knockdown cell model in the NSCLC cell line NCI-H1299 (P<0.05; Fig. 5A and 5B). CCK-8 and BrdU assays revealed that KNL1 knockdown substantially decreased cell viability (P<0.05; Fig. 5C and 5D). Transwell assays revealed that KNL1 knockdown considerably decreased NSCLC cell invasion and migration (P<0.05; Fig. 5E). As revealed by flow cytometry, apoptosis was strongly increased in the sh-KNL1 group compared with the control group (P<0.05; Fig. 6A). WB confirmed that KNL1 knockdown decreased Bcl-2 expression and increased Bax and Bad expression (P<0.05; Fig. 6B). Additionally, KNL1 knockdown

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**Fig. 6. KNL1 downregulation enhances NSCLC cell apoptosis. (A): Flow cytometry was used to assess apoptosis. (B): WB analysis of Bcl-2, Bax, and Bad expression. (C-D): WB analysis of the expression of E-cadherin, vimentin, Snail, p21, CDK2, CDK4, cyclin D, and cyclin E. **P<0.01, ***P<0.001 (vs. the sh-NC group). N=3.
elevated E-cadherin expression and inhibited vimentin and Snail expression (P<0.05; Fig. 6C). To further investigate the impact of KNL1 on the NSCLC cell cycle, we conducted WB to evaluate the profiles of cell cycle-related proteins. In contrast to those in the control group, the p21 levels in the sh-KNL1 group were elevated, and the CDK2, CDK4, cyclin D and cyclin E levels were notably downregulated (P<0.05; Fig. 6D). These findings indicated that KNL1 inhibition weakened the malignant biological behaviors of NSCLC cells.

The influence of KNL1 on NSCLC cell sensitivity to chemotherapy

To determine the influence of KNL1 on the sensitivity of NSCLC cells (NCI-H1650 and NCI-H1299) to chemotherapy, we employed a CCK8 assay to examine the impact of KNL1 overexpression and low KNL1 expression on the sensitivity of these cells to the chemotherapeutic drugs, including cisplatin, paclitaxel, and gemcitabine. As a result, cell viability was vastly greater in the oe-KNL1 group than in the vec group. In contrast, cell viability was distinctively lower in the sh-KNL1 group than in the sh-NC group (P<0.05; Fig. 7A-7C). These data showed that KNL1 overexpression strengthened the resistance of NCI-H1650 and NCI-H1299 cells to cisplatin, paclitaxel, and gemcitabine, whereas lower KNL1 expression enhanced the chemosensitivity of these cells to these drugs.

KNL1 activated the PI3K/AKT/NF-κB axis

To investigate the underlying mechanism of KNL1, we analysed the co-expressed genes of KNL1 (CASC5) in LUAD using the LinkedOmics database. PIK3CA was shown to be positively related to KNL1 in LUAD (Fig. 8A and 8B). To confirm the influence of KNL1 on the PI3K/AKT/NF-κB pathway, we determined the protein expression of KNL1 in the KNL1-overexpressing cell model through WB. PI3K/AKT/NF-κB phosphorylation was dramatically upregulated in the oe-KNL1 group compared with the vector group (P<0.05; Fig. 9A). Compared to that in the sh-NC group, the phosphorylation of the pathway was distinctly suppressed in the sh-KNL1 group (P<0.05; Fig. 9B). These findings indicated that KNL1 activated the PI3K/AKT/NF-κB pathway.

Fig. 7. The impact of KNL1 on NSCLC cell chemotherapy sensitivity. (A-E): CCK8 assays were used to determine the influence of KNL1 overexpression or knock-down on the chemosensitivity of NCI-H1650 and NCI-H1299 cells to cisplatin, paclitaxel, and gemcitabine. *P<0.05, **P<0.01 (vs. the vec group). #P<0.05, ## P<0.01 (vs. the sh-NC group). N=3.
Fig. 8A. KNL1 has a positive relationship with PI3K pathway in NSCLC. (A): The coexpressed genes of KNL1 (CASC5) in LUAD (LindOmnics). The top 50 genes coexpressed with KNL1 in LUAD patients are shown as heatmaps. (B): PIK3CA has a positive relationship with KNL1 in LUAD.
KNL1 overexpression clearly facilitates NSCLC cell malignant behaviors of NSCLC cells. Thus, KNL1 is believed to be a potential therapeutic target for NSCLC.

The PI3K inhibitor LY294002 was further administered to suppress PI3K expression, thus investigating the impact of PI3K inhibition on KNL1-mediated NSCLC development. CCK-8 and BrdU assays revealed that, in contrast with those in the vector group, NSCLC cell proliferation in the KNL1 group substantially increased. However, compared to those in the KNL1 group, the KNL1 + LY294002 group exhibited a considerable reduction in NCI-H1650 cell proliferation (P<0.05; Fig. 10A and 10B). Transwell assays revealed that invasion and migration were strongly enhanced in the KNL1 group compared with the vector group, whereas PI3K inhibition significantly decreased invasion and migration (P<0.05; Fig. 10C). As reflected by flow cytometry, cell apoptosis was lower in the KNL1 group than in the vector group, whereas apoptosis was strongly augmented in the KNL1 + LY294002 group compared with that in the KNL1 group (P<0.05; Fig. 11A). WB revealed that Bax and Bad expression was decreased and that Bcl-2 expression was increased in the KNL1 group compared with the vector group. However, PI3K inhibition reversed these changes (P<0.05; Fig. 11B). In contrast to those in the vector group, E-cadherin expression was evidently lower, and vimentin and Snail expression was conspicuously greater in the KNL1 group. PI3K inhibition had the opposite effect (P<0.05; Fig. 11C). Moreover, in comparison with those in the vector group, there were notable increases in the protein profiles of CDK2, CDK4, cyclin D, and cyclin E and a distinct reduction in p21 in the KNL1 group. In contrast to those in the oe-KNL1 group, the oe-KNL1 + LY294002 group exhibited a decrease in the protein expression of p21, cdk2, cyclin D3, and cyclin E2 and an increase in the expression of cyclin B1 and cyclin A (P<0.05; Fig. 11D). As revealed by WB, the protein expression of the PI3K/AKT/NF-κB pathway was dramatically increased in the KNL1 group compared with the vector group, whereas PI3K inhibition led to a marked decrease in the expression of the pathway in NCI-H1650 cells (P<0.05; Fig. 11E). These findings indicate that KNL1 overexpression clearly facilitates NSCLC cell malignant development and that PI3K inhibition vigorously attenuates the carcinogenic effects mediated by KNL1.

KNL1 overexpression stimulated the growth of NSCLC tumors in vivo

We further studied how KNL1 regulates NSCLC tumor growth and invasion in vivo by using a nude mouse xenograft model. We injected nude mice with blank-transfected, vector-transfected or KNL1-transfected NCI-H1650 cells. As shown in Fig. 12A and 12B, the tumors in mice injected with KNL1-transfected NSCLC cells grew rapidly, and the tumor volume progressively increased. However, the tumor volume in the vector groups was significantly smaller than that in the KNL1 group (P<0.05; Fig. 12A and 12B). In parallel, the tumor weight in the vector group was significantly greater than that in the vector group (P<0.05; Fig. 12C). As indicated by the RT-qPCR results, overexpressing KNL1 led to the upregulation of KNL1 (P<0.05; Fig. 12D). IHC analysis revealed that overexpressing KNL1 increased the expression of Ki67 (a proliferation marker) in excised tumor masses (P<0.05; Fig. 13A). The protein expression of E-cadherin, vimentin and Snail was further checked by WB, which demonstrated that the expression of the epithelial marker E-cadherin decreased, while the expression of the mesenchymal markers vimentin and Snail was notably augmented in the KNL1 group (compared with the vector group) (P<0.05; Fig. 13B). Therefore, we concluded that overexpression of KNL1 facilitates NSCLC growth and metastasis in vivo.

DISCUSSION

NSCLC is the most common histological type of lung cancer, and 60% of patients exhibit distant metastases at diagnosis. By the time patients reach advanced stages, their 5-year survival rate is less than 5%. Thus, early diagnosis and treatment are instrumental in attenuating NSCLC invasion (26, 27). On the other hand, overcoming the chemoresistance of lung cancer also has great significance in the clinical treatment of this disease. A recent study has revealed that sulforaphane can overcome T790M mutation-mediated gefitinib resistance in lung cancer (28). Identification of novel regulators involved in NSCLC development is highly important for better targeted treatment (29). Here, we found that KNL1 has upregulation in NSCLC. Its down-regulation resulted in repressed malignant behaviors of NSCLC cells. Thus, KNL1 is believed to be a potential therapeutic target for NSCLC.

KNL1 is located at human chromosome 15.15.1 and spans approximately 70 kbp with 27 exons. It was first characterized as a short cDNA fragment that was found to be overexpressed in growth-suppressed melanoma cells via human chromosome 6 transfer mediated by microcells (30). KNL1 is also highly expressed in multiple cultured cancer cell lines, primary tumors, and normal testes (31-33). Recently, studies have found that KNL1 level is associated with the prognosis of cancer patients. For instance, KNL1 exhibits a high level of expression in prostate adenocarcinoma (PRAD). Elevated KNL1 level has a significant relationship with the poor prognosis of PRAD patients. Moreover, KNL1 expression is correlated with various tumor-infiltrating immune cells, particularly Treg and Th2 cells. KNL1 could serve as an independent prognostic indicator in PRAD and is connected to immune infiltration (34). Moreover, KNL1 is a potential prognostic and diagnostic biomarker in patients with uterine corpus endometrial carcinoma (UCEC) (35) and osteosarcoma (36). Presently, our study also found that a higher KNL1 level predicts poorer overall survival and...
disease-free survival of LUAD, suggesting that KNL1 might be a diagnostic biomarker of lung cancer patients.

Accumulated studies have revealed that KNL1 participates in the malignant development of tumors by mediating cell proliferation and apoptosis (14, 37). The tumor suppressor gene P53 has been found to perform crucial functions in regulating cell cycle, DNA repair following damage, and triggering cell apoptosis. In glioma, KNL1 upregulation is significantly

Fig. 9. KNL1 blocks PI3K/AKT/NF-κB axis activation. (A, B): WB was used to determine the PI3K/AKT/NF-κB protein expression in KNL1-overexpressing and KNL1-low cells. *P<0.05, **P<0.01, ***P<0.001. N=3.
associated with poorer prognosis of the patients. Meanwhile, the survival analysis showed that KNL1 level and P53 gene level are closely related (38). Urata et al. (39) found that knocking down KNL1 promoted apoptotic cell death and inhibited cell growth of tumor cells under wild-type p53 and mutant-type p53 status. Here, we discovered that KNL1 overexpression enhanced NSCLC cell proliferation and inhibited apoptosis by mediating apoptosis-related and cell cycle-related proteins. By contrast, KNL1 knockdown led to the opposite results. These findings reveal that KNL1 may be a target of NSCLC treatment.

Metastasis is the major cause of mortality of multiple cancers, including NSCLC (40). KNL1 has a potential role in mediating the metastasis of cancers. For example, He et al. (34) found that KNL1 is not only associated with a poor prognosis of uterine corpus endometrial carcinoma patients, and KNL1 knockdown suppressed the invasion and metastasis of UCEC cells. Notably, Wang et al. suggested that LINCO2418 promoted KNL1 expression in lung adenocarcinoma (LAD)(15). Since LINCO2418 can also enhance the migration of LAD cells, it’s believed that KNL1 has a role in mediating the metastasis of NSCLC cells (15). Here, we found that KNL1 overexpression significantly promoted the migration and invasion of lung cancer cells, and downregulated the expression of the epithelial marker E-cadherin, promoted the protein expression of the interstitial markers vimentin and Snail, and promoted the epithelial-mesenchymal transition (EMT) process in NSCLC. These data suggested that KNL1 is potentially involved in the metastasis of NSCLC. EMT is an evolutionarily conserved developmental program that has been implicated in carcinogenesis and confers metastatic properties upon cancer cells by enhancing mobility, invasion, and resistance to apoptotic stimuli (41). The key proteins regulating EMT include E-cadherin, vimentin, ZEB1, slug, and Snail (42). During the malignant progression of tumors, EMT enables tumor cells to infiltrate and metastasize to distant sites (43, 44). For example, a study revealed that KNL1 could facilitate the resistance of NCI-H1650 and NCI-H1299 cells to cisplatin, paclitaxel, and gemcitabine, while low KNL1 expression considerably augmented the chemotherapeutic sensitivity of NSCLC cells.

PI3K/AKT signaling is known as a typical signaling pathway in tumor cells, and its aberrant activation is associated with tumor cell growth, proliferation, and differentiation (46, 50). As reported, PI3K/AKT is highly expressed in prostate cancer, breast cancer, ovarian cancer, and lung cancer (30, 51, 52). The pathway also plugs into the drug resistance mechanisms of tumor cells. For instance, miR-1269b targets PENT, initiates PI3K/AKT signaling (53), boosts NSCLC cell resistance to cisplatin, enhances cell proliferation and tumor growth ex vivo, and impedes apoptosis (54). NF-κB, a downstream molecule of PI3K/AKT, also participates in the regulation of NSCLC. Baicalein hampers PI3K/AKT/NF-κB pathway activation, induces EMT, suppresses anti-apoptotic protein expression in A549 cells that are resistant to cisplatin, and enhances A549 cell chemosensitivity to cisplatin (55). The PI3K/AKT/NF-κB signaling pathway has been shown to exert pro-cancer and drug resistance functions in the context of NSCLC. Here, we revealed that KNL1 activated the PI3K/AKT/NF-κB pathway in tumor cells, and its aberrant activation is associated with poorer prognosis of the patients. Meanwhile, the survival analysis showed that KNL1 level and P53 gene level are closely related (38). Urata et al. (39) found that knocking down KNL1 promoted apoptotic cell death and inhibited cell growth of tumor cells under wild-type p53 and mutant-type p53 status. Here, we discovered that KNL1 overexpression enhanced NSCLC cell proliferation and inhibited apoptosis by mediating apoptosis-related and cell cycle-related proteins. By contrast, KNL1 knockdown led to the opposite results. These findings reveal that KNL1 may be a target of NSCLC treatment.

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Primary or secondary drug resistance is a leading contributor to failed targeted treatment for solid tumors such as NSCLC. Tumor cells resist targeted drugs via a multitude of mechanisms, hence weakening therapeutic efficacy (46, 47). Cisplatin, paclitaxel, and gemcitabine, all of which are chemical drugs often used for NSCLC and other solid tumors, trigger apoptosis by combining with DNA or through other methods. They have been demonstrated to efficaciously improve the survival rate and quality of life of patients with NSCLC (48). Dysregulation of miRNAs, proteins, and diverse transcription factors contributes to NSCLC cell resistance to drugs. For instance, miR-539 overexpression attenuates DCLK1 expression, represses PI3K/AKT/mTOR pathway activation, and strengthens NSCLC cell chemosensitivity to cisplatin (49). Here, we revealed that KNL1 could facilitate the resistance of NCI-H1650 and NCI-H1299 cells to cisplatin, paclitaxel, and gemcitabine, while low KNL1 expression considerably augmented the chemotherapeutic sensitivity of NSCLC cells.

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Epidermal growth factor receptor (EGFR) is a growth factor receptor that induces cell differentiation and proliferation after

![Fig. 10. PI3K inhibition weakens KNL1-mediated NSCLC cell proliferation and migration.](image-url)

The PI3K inhibitor LY294002 was administered to suppress PI3K expression. (A-B): CCK-8 and BrdU assays were used to evaluate NCI-H1650 cell proliferation. (C): Transwell assays were used to assess NCI-H1650 cell invasion and migration. **P<0.01 (vs. the vec group). *P<0.05, **P<0.01 (vs. the KNL1 group). N=3.
Fig. 11. PI3K inhibition affects KNL1-mediated NSCLC cell apoptosis and EMT. The PI3K inhibitor LY294002 was administered to suppress PI3K expression. (A): Flow cytometry was used to detect apoptosis. (B): WB confirmed the expression of Bcl-2, Bax, and Bad. (B-D): WB analysis of the expression of E-cadherin, vimentin, Snail, p21, CDK2, CDK4, cyclin D, and cyclin E. (E): WB analysis of the protein profiles of KNL1 and the PI3K/AKT/NF-κB signaling pathway in NCI-H1650 cells. **P<0.01, ***P<0.001 (vs. the vec group). #P<0.05, ## P<0.01 (vs. the KNL1 group). N=3.
activation by binding to one of its ligands. The receptor is located on the cell surface where ligand binding activates tyrosine kinases in the receptor intracellular region. This tyrosine kinase phosphorylates many intracellular substrates. The activation of substrates leads to cell growth, DNA synthesis and oncogene expression. Oncogenes are usually amplified and/or mutated in cancer cells. Therefore, EGFR is considered to be closely related to the development of cancer (56). Research has shown that EGFR mutations, such as inframe deletion of amino acids within exon 19 and the L858R substitution mutation, activate EGFR, leading to prolicence and antiapoptotic signaling (57). It has been reported that more than 60% of NSCLC patients express EGFR, and EGFR has become an important therapeutic target for the treatment of these tumors (58, 59). For example, studies have shown that the activation of EGFR expression promotes the EMT phenotype in lung cancer cell lines. As an EMT marker, vimentin can predict the development of brain metastasis (BM) in patients with EGFR-mutant NSCLC (60). In addition, studies have reported that the PI3K/AKT pathway is the key downstream signaling pathway of EGFR, and abnormal activation of the PI3K/AKT signaling pathway is one of the most relevant mechanisms of acquired targeted treatment resistance in patients with EGFR-mutant NSCLC. Liu et al. showed that in patients with EGFR-mutant NSCLC, exosomes can transmit mutation-induced resistance through activation of the PI3K/AKT pathway, thus hindering the sensitivity of tumors to chemotherapy drugs (61, 62). Therefore, we speculate that KNL1 may treat EGFR-mutated NSCLC by regulating these pathways. On this basis, we will further carry out follow-up experiments in EGFR-mutated cells.

Fig. 12. KNL1 overexpression promoted the growth and metastasis of NSCLC tumors in vivo. (A): Representative images of tumors from the individual groups. (B): Quantification of the tumor volume in the individual groups at the indicated time points. (C): Quantification of the tumor weights from the individual groups on day 35. (D): RT-qPCR was used to measure the mRNA level of KNL1. NS P>0.05, *P<0.05, **P<0.01, ***P<0.001 (vs. the vector group). N=5.

Fig. 13. KNL1 overexpression promoted the proliferation and EMT of NSCLC tumors in vivo. (A): IHC was used to detect the expression of Ki67 in tumor tissues. (B): WB was used to measure the expression of E-cadherin, vimentin, and Snail. ***P<0.001 (vs. the vector group). N=5.
Nonetheless, our experiments had limitations as follows: 1) more clinical samples, especially from multiple centers, should be collected to confirm the diagnostic value of KNL1 in NSCLC; 2) the regulatory role of KNL1 in NSCLC should be confirmed in more lung cancer cells both in vitro and in vivo; 3) the mechanism of KNL1 in promoting the PI3K-AKT-NF-κB pathway and EMT should be further investigated. Besides, the upstream mechanism of KNL1 has not been investigated. Clarifying these mechanisms might help the targeted therapy of NSCLC. In the future, we will further analyze the diagnostic value of KNL1 in NSCLC and explore the regulatory role of KNL1 in the immune microenvironment of NSCLC. We hope future studies will help the precise diagnosis and treatment of NSCLC.

To summarize, our work has demonstrated that KNL1 has a distinct pro-cancer function in NSCLC. KNL1 functions as an oncogene in the malignant development of NSCLC by promoting the PI3K/AKT-NF-κB pathway. KNL1 is a potential tumor marker of NSCLC and targeting KNL1 might be a novel strategy for NSCLC treatment.

Authors’ contributions: Ligao Wu conceived and designed the experiments; Ligao Wu and Guanghui Zhang performed the experiments; Qing Zhu, Yuanli Huang - statistical analysis; Ligao Wu, Guanghui Zhang, Qing Zhu, and Yuanli Huang wrote the paper.

All authors read and approved the final manuscript.

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