

# Upregulation of the Kank1 gene inhibits human lung cancer progression *in vitro* and *in vivo*

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**Abstract.** Kank1, an important member of the Kank gene family, plays an important role in the development of many malignant tumors. As a brand new tumor-suppressor gene, the expression of Kank1 gene has been revealed to be downregulated or absent in many malignant tumors. However, there is no study concerning the specific role of Kank1 in the development and progression of lung cancer. In the present study, we found that the Kank1 gene was significantly downregulated in human lung cancer and lung cancer cells. When the Kank1 gene was upregulated, we found that the proliferation of lung cancer cells was significantly inhibited, the cells were arrested in the G0/G1 phase, the apoptosis of cells was observed, the expression of the caspase family of genes was altered with mainly the activation of caspase-3 and -9, and Bcl-2/Bax ratio was significantly imbalanced. In addition, we also found that upregulation of the Kank1 gene resulted in the inhibition of tumor cell invasion and metastasis. Concurrently, *in vivo* nude mice experiments also confirmed that upregulation of Kank1 expression led to reduced tumor formation in nude mice and significantly reduced tumor volume. These results revealed that upregulation of the Kank1 gene inhibited the progression of lung cancer both *in vitro* and *in vivo*, and its mechanism was closely related to cell apoptosis as well as tumor invasion and metastasis.

## Introduction

Lung cancer is the leading cause of cancer-related deaths, particularly in underdeveloped countries since it produces the highest percentage of deaths than any other cancer (1). The tendency of metastasis is very high in the late stage of lung cancer, and the clinical treatment of lung cancer is mainly

radiotherapy and chemotherapy. However, the results are often unsatisfactory due to the short survival time of patients and the tendency of recurrence which is high. In recent years, there has been more and more research conducted on molecular targeted therapy of lung cancer, and it is increasingly important to explore the molecular mechanisms of lung cancer development (2,3).

The specific mechanism of tumor genesis and development is complex and changeable. The deletion or mutation of a tumor-suppressor gene and the expression of a large number of oncogenes have important impacts on tumorigenesis. In 2002, Japanese scholars cloned a candidate tumor-suppressor gene, the Kank1 gene in renal cell carcinoma using heterozygous deletion analysis (4). The Kank family is mainly composed of 4 genes, Kank1-Kank4, of which Kank1 plays a certain role in the inhibition of tumorigenesis and development (5). The present study revealed that Kank1 exhibited a low expression in various tumor tissues including kidney (6), liver (7,8) and lung cancer (9,10). Kank1 is mainly distributed in normal tissues, including renal tubular epithelial cells and the glandular cells of other digestive organs. Its main function is the polymerization with actin of the cytoplasm adjusting the cytoskeleton, and driving cell motility (11). In addition, it can form a complex with  $\beta$ -catenin shuttling it in the nucleus to regulate  $\beta$ -catenin subcellular distribution. Studies have revealed that downregulation of the Kank1 gene participates in the genesis and development of bone tumors by activating the JAK2/Stat tumor-promoting signaling pathway (12).

In the present study, we found that the expression of the Kank1 gene was low in lung cancer tissues and lung cancer cells. Following upregulation of the Kank1 gene, tumor cell proliferation was significantly inhibited, and apoptosis occurred. Concurrently, the expression of Bax decreased and the expression of Bcl-2 increased, while the expression of the caspase family was also altered, mainly with the activation of caspase-3 and -9. In addition, upregulation of the Kank1 gene also reduced the invasion and metastasis of tumor cells. In an *in vivo* experiment, we found that the implantation of upregulated Kank1 lung cancer cells into nude mice resulted in lower tumorigenicity and the significant decrease of tumor volume in nude mice. In summary, we concluded that upregulation of the Kank1 gene inhibited the progression of lung cancer both *in vitro* and *in vivo*, and its mechanism was closely related to cell apoptosis and tumor invasion and metastasis.

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## Materials and methods

**Cell lines and culture.** Human lung cancer cell lines NCI-H292, A549 and PGCL3 cells, and normal human lung cell lines MRC-5 and WI-38 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The aforementioned 5 cell lines were all cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) in an incubator at 37°C, with 5% CO<sub>2</sub> and saturated humidity. RPMI-1640 medium and FBS were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

**Antibodies and reagents.** Kank1 (dilution 1:2,000; cat. no. AM8596b) was purchased from Abgent, Inc. (San Diego, CA, USA). Bax (dilution 1:1,000; cat. no. 2772T), Bcl-2 (dilution 1:1,000; cat. no. 2872T), caspase-3 (dilution 1:1,000; cat. no. 9662S), caspase-9 (dilution 1:1,000; cat. no. 9502T), MMP-7 (dilution 1:1,000; cat. no. 71031S) and MMP-9 (dilution 1:1,000; cat. no. 3852S) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). siRNA and plasmids were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). An RT-PCR kit was obtained from Takara Bio, Inc. (Otsu, Japan). PI was purchased from Sigma-Aldrich (St. Louis, MO, USA). An Annexin V-FITC apoptosis assay kit was purchased from BD Biosciences (San Jose, CA, USA).

**Cell transfection.** Human lung cancer cells in the logarithmic growth phase were collected and cultured in 6-well plates at a density of 6x10<sup>5</sup> cells/ml. Negative oligonucleotides were used as a control. The medium was replaced with serum-free and antibiotic-based medium, according to the procedure on the transfection of reagents. Following the transfection of siRNA, the culture plates were placed in a CO<sub>2</sub> incubator for 4-6 h, the medium was then replaced with the serum-containing medium and cells continued to culture. The transfected cells were screened after transfection with plasmids. After 3 weeks of selection, monoclonal antibodies (KANK1 antibody; dilution 1:2,000; cat. no. AM8596b) were purchased from (Abgent, Inc.) and chosen to expand the culture to establish an overexpressed Kank1 human lung cancer cell line. Subsequently, the cells underwent RT-PCR detection.

**RNA extraction and RT-PCR detection.** Human lung cancer cells were divided into untransfected, blank plasmid pCMV-vec and the experimental group pCMV-Kank1. Total RNA was collected and extracted from each group of cells to assess purity and integrity before reverse transcription. Following calculation of the RNA concentration, the RT-PCR reaction was performed using an RT-PCR kit (Takara Bio), and the procedure was performed according to the manufacturer's instructions. Kank1 and  $\beta$ -actin gene primers were designed and synthesized by Invitrogen; Thermo Fisher Scientific, Inc. The primers were as follows: Kank1 gene upstream primer, 5'-CTTGACACAGTATTTTCACGCTTTTG-3' and downstream primer, 5'-AAGTAAATGTGACACGGTAAAAGG-3';  $\beta$ -actin gene upstream primer, 5'-CTGGGACGACATGGAGAAA-3' and downstream primer, 5'-AAGGAAGCGTGAAGAGTGC-3'. The PCR reaction system was 25  $\mu$ l and the reaction conditions were as follows: 94°C for 2 min,

denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec, for a total of 31 cycles. The resulting PCR product was electrophoresed on a 1.5% agarose gel and scanned using a Gel Doc™ XR+ Gel Documentation System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Cell viability detection.** Trypsin was used for the digestion and collection of the untransfected group, the blank plasmid pCMV-vec group and the experimental pCMV-Kank1 cell group, and then the cells were inoculated in 96-well plates, with each set consisting of 5 complex wells. The transfected group was replaced with complete culture solution after 24 h, and after 48 h, 20  $\mu$ l of MTT solution (0.5 mg/ml)/well was added and incubated at 37°C for 4 h. The supernatant was removed and 150  $\mu$ l of dimethyl sulphoxide (DMSO) was added to each well and shaken for 10 min until the purple crystals were completely dissolved. A microplate reader was used to detect the optical density of each well at 490 nm, and the average value of each group was calculated. The cell growth inhibition rate (%) = (1 - average A value of the treated group/average A value of the blank group) x 100%. The experiment was repeated 3 times.

**Caspase activity assay.** Caspase-3 and -9 activities were determined using the Caspase activity assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions after transfected human lung cancer cells, untransfected human lung cancer cells and blank plasmid transfected cells were collected. The assessment was performed with a fluorescence spectrophotometer at an excitation wavelength of 400 nm and an onset wavelength of 505 nm.

**Flow cytometric analysis detects apoptosis and the cell cycle.** The transfected human lung cancer cells and untransfected human lung cancer cells as well as the blank plasmid transfected cells were digested with 0.25% trypsin-EDTA and collected, then suspended in phosphate-buffered saline (PBS), and single cell suspensions were prepared. Flow cytometry (BD Biosciences) was used to detect apoptosis following the Annexin V-FITC apoptosis assay kit manufacturer's instructions. In addition, the cells were fixed with 70% ethanol overnight at 4°C. The cells were then washed with PBS 2 times, add 1 ml of propidium iodide (PI) comprehensive dye solution (containing RNase 10  $\mu$ g, Triton X-100 5  $\mu$ l), was added to stain the cells in the dark at 4°C for 30 min in order to assess the cell cycle. The stained cells were analyzed using a FACSCalibur flow cytometer (Becton-Dickinson; BD Biosciences).

**Assessment of cell invasion and migration abilities.** Each group of cells was cultured in BD 6-well Transwell culture plates (BD Biosciences). The cells were scratched using a standard 200  $\mu$ l pipette tip, and were removed by washing with serum-free medium. Serial images were obtained at different time-points using a phase contrast microscope. The cell invasion assay was performed using Matrigel-coated Transwell chambers. The 8- $\mu$ m filter in the upper chamber was covered with Matrigel diluted at 1:6, and each well was seeded with ~2x10<sup>6</sup> cells. After 36 h of culturing, the cells that reached the bottom of the culture well through the membrane were fixed with formalin and stained with crystal violet. Cells

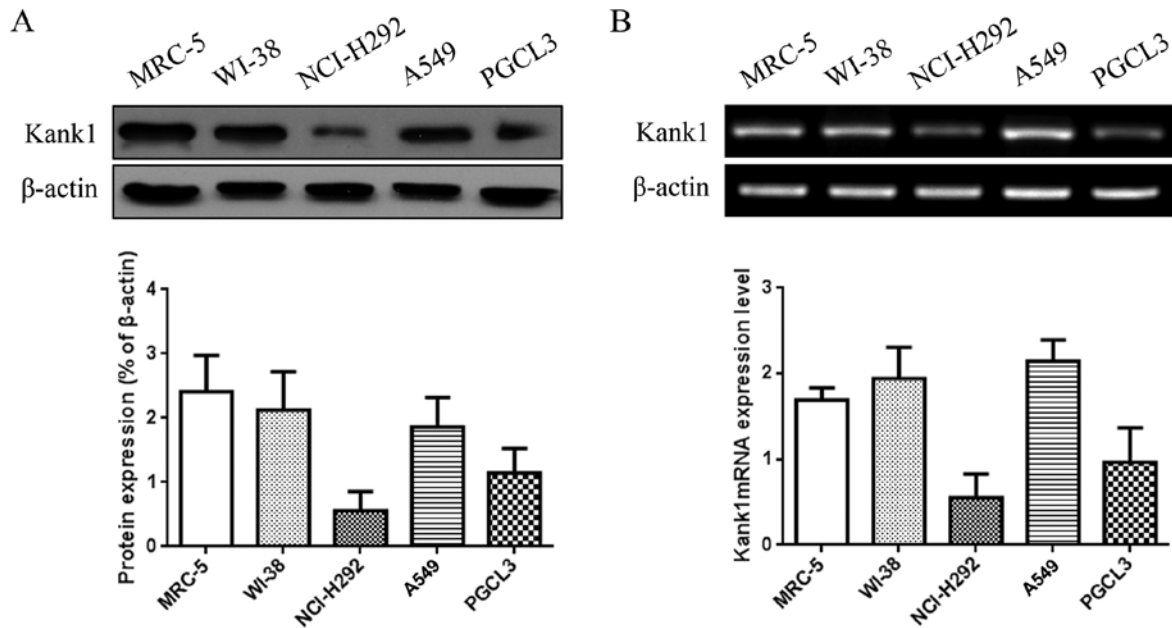


Figure 1. Kank1 gene and protein expression in lung cancer cells. (A) The expression level of the Kank1 protein in lung cancer cells. (B) The expression level of Kank1 mRNA in lung cancer cells. Each independent experimental sample was repeated 3 times.

were randomly counted in 10 fields of view (magnification,  $\times 200$ )/well (Olympus Corp., Tokyo, Japan).

**Western blot analysis.** Human lung cancer cells and their stably transfected cells were harvested and washed twice with PBS and 1 ml of protein lysis buffer (Sigma-Aldrich) containing 10  $\mu$ l of protease inhibitor was added for cell lysis. The protein concentration was determined using bicinchoninic acid assay (BCA) method. The same amount of protein was added to each well for loading. The gel was separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The protein was then transferred to a polyvinylidene fluoride (PVDF) membrane using a semi-dry method and blocked with 5% non-fat dry milk overnight at 4°C. Subsequently, it was diluted with Tris-buffered saline containing Tween-20 (TBST) solution and a primary antibody was added at 37°C for 1 h. The primary antibody dilution ratio (phospho-PI3K antibody, dilution 1:500; cat. no. 4228S; phospho-Akt antibody, dilution 1:1,000; cat. no. AP3434a; and MMP-7 antibody, dilution 1:800; 71031S; all were purchased from Cell Signaling Technology, Inc.). The membrane was washed 3 times with TBST. The secondary antibody anti-rabbit IgG, HRP-linked antibody (cat. no. 7074P2; Cell Signaling Technology) was added for incubation at 37°C for 1 h, and the membrane was then washed with TBST solution 4 times by shaking. An ECL luminescence reagent was added, X-ray exposure imaging was performed, the bands were scanned, and the grayscale analysis was performed.  $\beta$ -actin was used as a reference standard.

**Nude mice inoculation.** The animal experiment program was approved by the Medical Ethics Committee of Jiangsu College of Nursing. Twenty nude male mice, 6-8 weeks old and weighing ~20 g, were purchased from the Animal Experiment Center of Nanjing University. Housing conditions were controlled

for temperature (18-22°C) and relative humidity (50-60%) on a light/dark cycle 10 to 14 h. Access to food and water was *ad libitum*. Nude mice were randomly divided into 2 groups and each group consisted of 10 mice. The constructed PGCL3 cell line and the blank plasmid pCMV-vec cell line with stably expressed Kank1 gene transfection were implanted into the subcutaneous right forelimbs of the 2 groups of nude mice, respectively, to observe the effect of Kank1 on the tumorigenicity of the transplanted tumors of the nude mice. The growth of the tumor was observed and assessed at 7, 14, 21 and 28 days. After 4 weeks, the nude mice were sacrificed by cervical spine dislocation, and the subcutaneously transplanted tumor tissue was excised under aseptic conditions for index analysis.

**Statistical analysis.** All experimental data were analyzed using SPSS 18.0 statistical software (SPSS, Inc., Chicago, IL, USA). A t-test and analysis of variance were used by ANOVA followed by Fishers' least significant difference.  $P < 0.05$  was considered to indicate a statistically significant result.

## Results

**The Kank1 gene is downregulated in human lung cancer cells.** We detected the expression of Kank1 protein and mRNA in human lung cancer cell lines NCI-H292, A549 and PGCL3 and human normal lung cell lines MRC-5 and WI-38 by western blotting and RT-PCR. The results revealed that Kank1 protein and mRNA expression levels were significantly decreased in NCI-H292 and PGCL3 cells compared to the normal human lung cell lines (Fig. 1).

**Upregulation of the Kank1 gene inhibits human lung cancer cell growth.** To further confirm the effect of the Kank1 gene on the growth of human lung cancer cells, we successfully constructed and transfected Kank1 plasmid to upregulate the

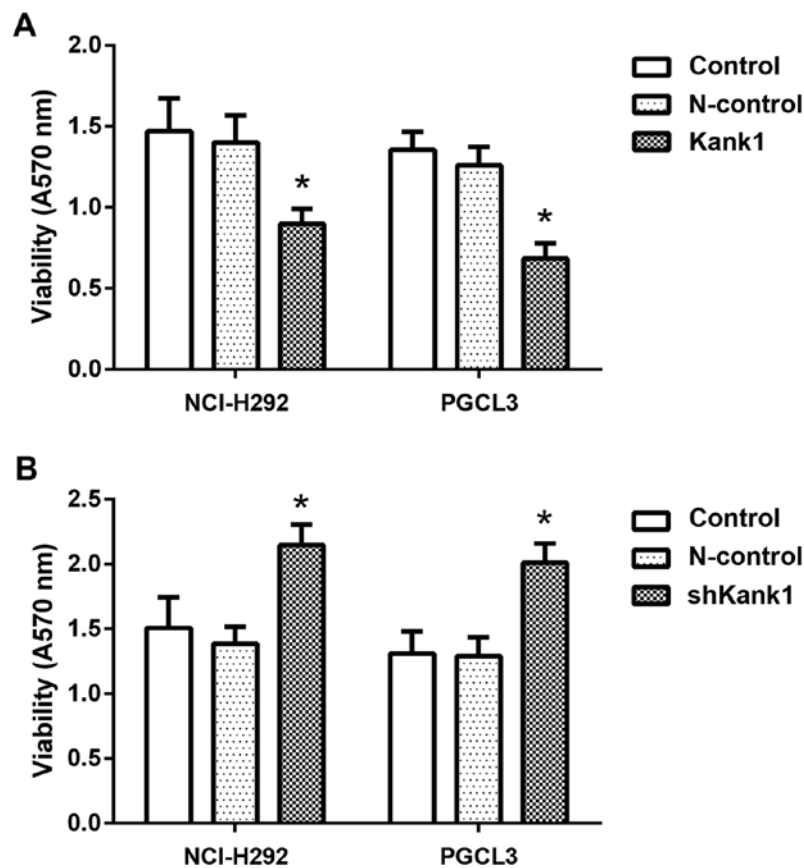


Figure 2. Impact of the Kank1 gene on human lung cancer cell growth. (A) Effect of the upregulation of the Kank1 gene on the proliferation of NCI-H292 and PGCL3 cells. (B) Effect of the downregulation of the Kank1 gene on the proliferation of NCI-H292 and PGCL3 cells. A significant decrease was observed compared with the control group and the transfected negative control oligonucleotide group. \* $P < 0.05$ . Data from 3 independent experiments.

expression level of the Kank1 gene in human lung cancer cells. Concurrently, we downregulated the expression of the Kank1 gene in human lung cancer cells by shRNA and used MTT for analysis. We found that the proliferation of NCI-H292 and PGCL3 cells was significantly decreased after upregulation of Kank1 gene expression compared with the control group and negative control oligonucleotide-transfection group. In contrast, the proliferation of NCI-H292 and PGCL3 cells was increased upon downregulation of Kank1 gene expression. Therefore, it was demonstrated that the Kank1 gene had the ability to regulate the proliferation of human lung cancer cells (Fig. 2).

*Upregulation of the Kank1 gene induces apoptosis in human lung cancer cells.* To investigate whether the Kank1 gene is associated with apoptosis in human lung cancer cells, we upregulated the expression of the Kank1 gene and detected the apoptosis rate by Annexin V-FITC/PI double-labeling method. We found that the apoptosis rate of NCI-H292 and PGCL3 cells was significantly increased after upregulation of Kank1 gene expression compared with the N-control group (Fig. 3A). Concurrently, the activity of caspase-3 and -9 increased after upregulation of Kank1 gene expression. However, the activity of caspase-8 was not significantly altered (Fig. 3B). In addition, Western blotting was used to detect the expression of Bcl-2, Bax and CytC in the cytoplasm, and we found that the levels of Bcl-2 and CytC in the cytoplasm were significantly

increased, while the expression level of the Bax protein was decreased (Fig. 3C and D). These results indicated that upregulation of the Kank1 gene led to the mitochondrial apoptosis of human lung cancer cells.

*Upregulation of the Kank1 gene induces cell cycle arrest in human lung cancer cells.* To further investigate the molecular mechanism by which the upregulated Kank1 gene inhibits the proliferation of human lung cancer cells, we used flow cytometry to analyze the changes of the cell cycle in lung cancer. It was revealed that the ratio of the G0/G1 phase of NCI-H292 and PGCL3 cells after upregulation of the Kank1 gene was significantly higher than that of the negative control oligonucleotide group (Fig. 4A). Concurrently, the expression levels of CDK4 and cyclin D1 were significantly decreased, and p21 was significantly increased (Fig. 4B). This demonstrated that upregulation of the Kank1 gene inhibited human lung cancer cell proliferation by arresting the cell cycle at the G0/G1 phase.

*Upregulation of the Kank1 gene inhibits invasion and metastasis of human lung cancer cells.* We found significant decreases in both the invasion and metastasis of human lung cancer cells with the upregulation of the Kank1 gene as compared with the negative control oligonucleotide group (Fig. 5A and B). The expression of EMT-related proteins E-cadherin, N-cadherin and fibronectin in human lung cancer cells upon upregulation of the Kank1 gene was revealed to be significantly altered as

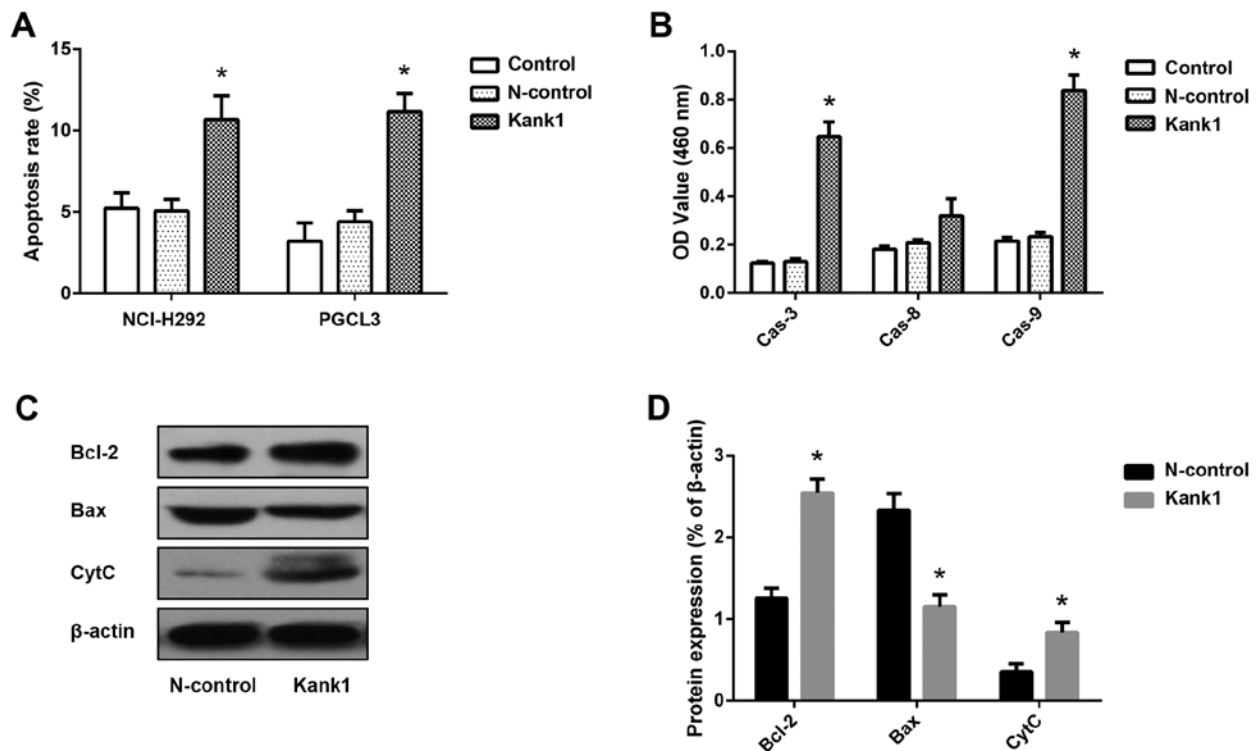


Figure 3. Upregulation of the Kank1 gene leads to apoptosis of human lung cancer cells. (A) Annexin V-FITC/PI double-labeling flow cytometric analysis of the apoptosis rate. (B) Viability assay of caspase-3, caspase-8 and caspase-9. (C) Western blot analysis of Bcl-2, Bax and CytC protein expression levels in the cytoplasm. (D) The results of (C) were analyzed with Gel-Pro Analyzer 4.0 software. \*P<0.05 as compared to the N-control group, and the data were from 3 independent experiments.

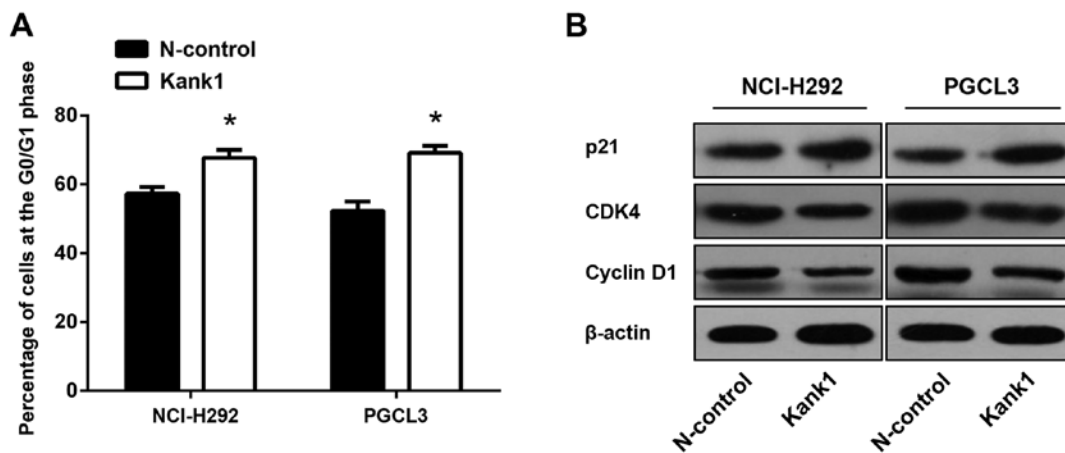


Figure 4. Upregulation of the Kank1 gene blocks human lung cancer cells in the G0/G1 phase. (A) Cell cycle changes of NCI-H292 and PGCL3 cells after Kank1 gene upregulation were analyzed by flow cytometry. (B) Western blot analysis of CDK4, cyclin D1 and p21 protein expression levels. \*P<0.05, each independent experimental sample was repeated 3 times.

determined by western blotting (Fig. 5C and D). Our results revealed that upregulation of the Kank1 gene inhibited the invasion and metastasis of human lung cancer cells.

*Upregulation of the Kank1 gene inhibits the growth of xenografts in nude mice.* To further determine whether Kank1 was associated with tumor growth, we implanted lung cancer cells with upregulated Kank1-expression into nude mice. After 4 weeks, we found that upregulation of the Kank1 gene significantly reduced the rate of tumor

formation in nude mice and significantly reduced the volume of the tumor xenografts (Fig. 6A and B). We found that upon surgical resection of the tumor, the tumor weight in the Kank1-upregulated group was significantly smaller than that in the control group (Fig. 6C). At each time-point, the volume of the tumors in the nude mice with the upregulated Kank1 gene was significantly smaller than that in the control group (Fig. 6D). The *in vivo* experiments revealed that upregulation of the Kank1 gene significantly inhibited the progression of lung cancer *in vivo*.

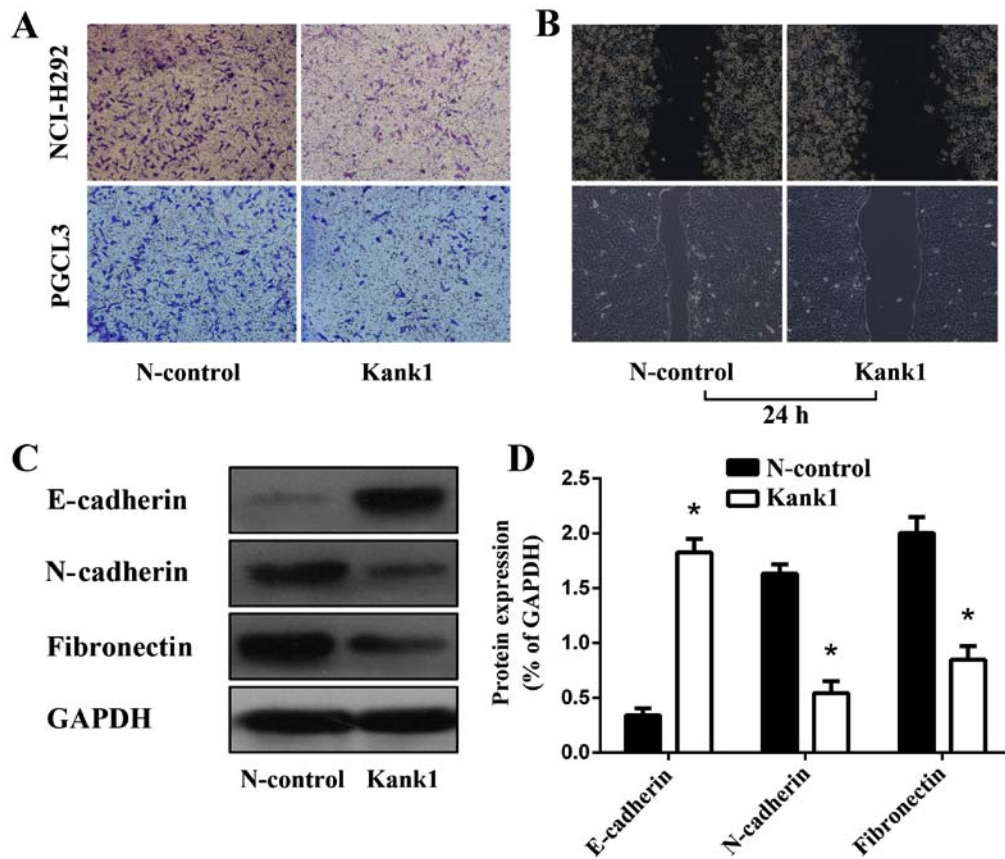


Figure 5. Upregulation of the Kank1 gene inhibits the invasion and metastasis of human lung cancer cells. (A and B) Invasion and migration assays to detect the invasion and metastasis of human lung cancer cells with upregulated Kank1 gene expression. (C) Western blot analysis of E-cadherin, N-cadherin and fibronectin protein expression levels. (D) The western blot results were further analyzed using Gel-Pro Analyzer 4.0 software. \*P<0.05, each independent experimental sample was repeated 3 times.

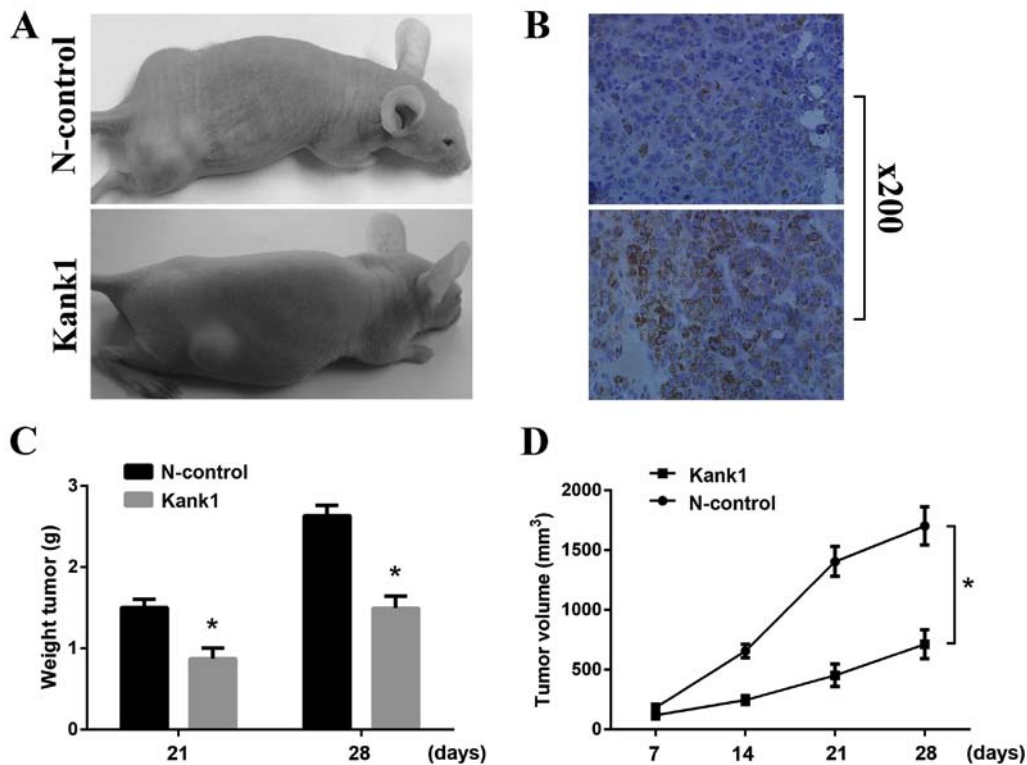


Figure 6. Upregulation of the Kank1 gene inhibits lung cancer growth in nude mice. (A) The tumor on the 28th day after inoculation. (B) Immunohistochemical staining of the tumor. (C) The weight of the 2 groups of tumors 21 and 28 days after inoculation. (D) The tumor growth volume was assessed at different time-points after inoculation. Compared with the control group, \*P<0.05.

## Discussion

It is well known that the complicated and changeable pathogenesis of malignant tumors is closely related to the deletion or mutation of tumor-suppressor genes and the large number of oncogenes as well as some signaling pathways. This topic explores the occurrence and development of lung cancer from the point of view of tumor suppressor gene deletion or mutation. The Kank1 gene, identified as a candidate tumor suppressor gene in renal cell carcinoma in 2002 by Japanese scientists using loss of heterozygosity analysis and other methods, belongs to the Kank family and is one of its important members (13). Kank1 is mainly distributed in the cytoplasm, and related to cell motility. It affects the occurrence and development of tumors in a variety of diseases to some extent, as it often forms a compound with  $\beta$ -catenin and shuttles in the nucleoplasm, regulating the distribution of  $\beta$ -catenin in the nucleus and enhancing  $\beta$ -catenin transcription in the occurrence and development of malignant tumors and invasion and metastasis (14).

As a candidate tumor-suppressor gene, the Kank1 gene is expressed at a low level in many malignant tumors, such as kidney (15) and liver cancer (8). However, no research on lung cancer has been reported yet. The incidence of lung cancer and mortality is ranked first among malignant tumors, however the pathogenesis is still not clear. In the present study, we found that the Kank1 gene and protein expression in human lung cancer cells was significantly lower than in normal cells. Therefore, we concluded that the Kank1 gene was closely related to the development and progression of lung cancer, and the Kank1 gene may be a potential therapeutic target for lung cancer. To further explain the association between the Kank1 gene and the development and progression of lung cancer, we upregulated the expression of the Kank1 gene in human lung cancer cells and observed the biological changes of human lung cancer cells with overexpressed Kank1 through a series of basic experiments. We determined that upregulation of Kank1 gene expression significantly inhibited human lung cancer cell proliferation with cells arrested in the G0/G1 phase, and cell apoptosis was promoted. Therefore, we surmised that the low expression of Kank1 in lung cancer plays a certain role in promoting the proliferation of tumor cells.

The pathways of apoptosis are complex, and the signaling pathways that regulate apoptosis are diverse. Studies have shown that the death receptor pathway of the mitochondrial pathway is a classic pathway of apoptosis in many tumor cells (16). Bcl-2 and Bax play a key role in the mitochondrial apoptotic pathway (17). In many tumors, including lung cancer, the expression of Bcl-2 is increased in tumors and Bax is decreased (18). To date, the understanding of how the Kank1 gene regulates the apoptosis mechanism of human lung cancer cells is still very limited. Therefore, following upregulation of the Kank1 gene in human lung cancer cells, we found through western blotting and RT-PCR that Bax and Bcl-2 translocated, Bax expression decreased, and Bax translocated from the cytoplasm to the mitochondrial membrane to alter the permeability of the mitochondrial membrane, promote the release of cytochrome *c* from the mitochondria into the cytoplasm, and then start the cascade

of apoptosis to eventually lead to cell apoptosis. Therefore, we hypothesized that the Kank1 gene promoted apoptosis in human lung cancer cells by regulating the anti-apoptotic proteins (Bcl-2 and Bax) in the Bcl-2 family and also confirmed that the Kank1 gene and mitochondrial membrane permeability finally resulted in tumor cell apoptosis. It is well-known that caspase family activation and cascade amplification are the necessary conditions for apoptosis in cells, both from the external and internal pathways (19). Following upregulation of the Kank1 gene in human lung cancer cells, we found that procaspase-9 and procaspase-3 expression levels were significantly decreased. In summary, we found that the Kank1 gene, by regulating the mitochondrial potential change, activated the mitochondrial membrane to release caspase to activate caspase and then promote human lung cancer cell apoptosis.

Tumor invasion and metastasis are important causes of cancer death. Therefore, elucidating the molecular mechanism of tumor metastasis has important clinical value for the treatment of lung cancer. In this study, we found that upregulation of the Kank1 gene significantly inhibited the invasion and metastasis of human lung cancer cells. In the human lung cancer cells with upregulated Kank1, the expression levels of EMT-related proteins E-cadherin, N-cadherin and fibronectin were significantly altered, the tumorigenicity in nude mice decreased and the volume of xenograft tumors decreased significantly. The aforementioned results revealed that the Kank1 gene affected the invasion and metastasis of human lung cancer cells.

In conclusion, we determined in the present study that Kank1 gene expression was decreased in lung cancer. After upregulation of the Kank1 gene, the cells were arrested in the G0/G1 phase and the proliferation of lung cancer cells was markedly inhibited. Concurrently, the mitochondrial pathway regulated Bcl-2/Bax and induced the apoptosis in human lung cancer cells. The Kank1 gene in the future may become a potential therapeutic target for the clinical treatment of lung cancer and provide a theoretical basis.

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## Funding

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## Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

## Authors' contributions

YG and MZ conceived and designed the study. YG performed the experiments. MZ wrote the paper. YG and MZ reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

### Ethics approval and consent to participate

The animal experiment program was approved by the Medical Ethics Committee of Jiangsu College of Nursing.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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