



Knockdown of growth differentiation factor-15 inhibited nonsmall cell lung cancer through inactivating PTEN/PI3K/AKT signaling pathway

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Abstract

Background Non-small cell lung cancer (NSCLC) is characterized by high morbidity and mortality in the world. Growth and differentiation factor 15 (GDF15) has been proved to play an important role in regulating tumor progression. However, the influence of GDF15 on NSCLC remains unclear.

Objective We aimed to investigate the regulatory role of GDF15 in NSCLC.

Methods The correlation between GDF15 expression and prognosis, stage of NSCLC was examined with bioinformatics method. The cell proliferation was detected with CCK8 and EdU staining. Wound healing, Transwell, flow cytometry assays were used to measure cell migration, invasion, and apoptosis, respectively.

Results High expression of GDF15 is correlated with worse survival and malignant progression of NSCLC. Knockdown of GDF15 restrained the proliferation, invasion, migration, but accelerated apoptosis of lung cancer cells through regulating PTEN/PI3K/AKT signaling pathway. sh-GDF15 suppressed epithelial mesenchymal transition (EMT) process and promoted the chemotherapy sensitivity of lung cancer cells.

Conclusion GDF15 plays an important role in NSCLC progression. GDF15 mediated PTEN/PI3K/AKT signaling pathway might be the potential therapeutic targets for the prevention and treatment of GDF15.

Keywords GDF15 · NSCLC · PTEN/PI3K/AKT · EMT

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Abbreviations

NSCLC	Non-small cell lung cancer
GDF15	Growth and differentiation factor 15
EMT	Epithelial mesenchymal transition
TGF-	Transforming growth factor-
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
LUAD	Lung adenocarcinoma
LUSC	Lung squamous cell carcinoma
MIC1	Macrophage inhibitory cytokine-1

Introduction

Lung cancer ranks the first in the number of morbidity and mortality in Chinese population (Italiano et al. 2022). The deaths caused by lung cancer account for about 1/5 of all cancer deaths. Its pathological type is mainly non-small cell lung cancer (NSCLC), with a high degree of malignancy, and bone metastasis often occurs in the late stage (Tsutani et al. 2021). The main treatments of lung cancer are surgery, chemotherapy and radiotherapy currently. Several reports

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indicated that targeted drugs may have the advantages of good curative effect and less side effects (Yamada et al. 2022). Therefore, it is necessary to find the exact therapeutic target gene.

Growth and differentiation factor 15 (GDF15) belongs to transforming growth factor- β (TGF- β) superfamily, and GDF15 participates in the regulation of many physiological activities (Lee et al. 2022). GDF15 has been proved to be closely related to the occurrence and development of a variety of cancers (Lodi et al. 2021). Studies have shown that GDF15 can regulate biological functions through the TGF- β /Smad pathway (Unsicker et al. 2013). GDF15 could activate Smad2 through binding TGF- β related receptor, and promote colon cancer metastasis (Okamoto et al. 2020). However, the regulatory role of GDF15 in the NSCLC remains unclear.

PI3K/AKT signaling pathway is an important tumor regulation signaling pathway, which involves apoptosis, proliferation, inflammatory response and glucose transport in the occurrence and development of tumors (Yang et al. 2017, 2020). It was found that PI3K/ATK pathway was activated in NSCLC, which promoted proliferation, migration, invasion and drug resistance (Yang et al. 2020). As a tumor suppressor, PTEN is involved in many processes of tumor formation and development (Luo et al. 2020). PTEN inactivation is usually related to the active state of PI3K pathway, and AKT phosphorylation level is linked with PTEN expression, which also indicates that AKT may be an important PTEN functional target (Cao et al. 2020). PTEN could dephosphorylate the inositol 3-phosphate product of PI3 kinase (PI3K), and thus negatively regulate the PI3K/Akt signaling pathway (He et al. 2019). However, if GDF15 could regulate NSCLC through PTEN/PI3K/AKT signaling pathway remains unknown.

In this study, bioinformatics method and measurement of GDF15 level in tumor tissues and cell lines were performed to analyze the correlation between GDF15 expression and prognosis, stage of NSCLC. We performed a series of experiments to validate the regulatory role of GDF15 in cell proliferation, invasion, migration, and apoptosis of lung cancer cells through PTEN/PI3K/AKT signaling pathway. This research provides more evidence for better understanding of GDF15 in NSCLC.

Materials and methods

Tissue samples collection

Tumor tissues and related adjacent tissues were collected from NSCLC patients who received surgery in the Second Affiliated Hospital of Air Force Medical University from June 2019 to March 2022. Written informed consent for publication was obtained from all participants. After samples collection, tissues were stored with liquid nitrogen before use. All experiment protocols have been approved by the ethics committee of the Second Affiliated Hospital of Air Force Medical University.

Cell culture

Human normal bronchial epithelial (HNBE) cells, some lung cancer cell lines (A549, H460, H1299, H1703, and H322) purchased from Chinese Academy of Cell Collection (Shanghai, China) were used in this research. RPMI-1640 medium (Invitrogen, US) containing 5% fetal bovine serum (FBS) was used for cell culture on the condition of 5% CO₂ at 37 °C.

Cell transfection

GDF15 siRNA (80 nM) and related control siRNA (80 nM) were purchased from Santa Cruz Biotechnology. Lipofectamine (Life Technologies, US) was used to for cell transfection. After 15 h incubation, the cells were applied for different experiments.

Flow cytometry

Propidium iodide (PI) and fluorescein isothiocyanate (FITC) doublestaining was performed to analyze cell apoptosis. After transfection, cells were suspended with binding buffer. After incubation with PI and Annexin V-FITC (G003-1-3, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) for 20 min in the dark. Cytomics™ FC500 Flow Cytometer (Beckman, USA) was used for cell apoptosis measurement.

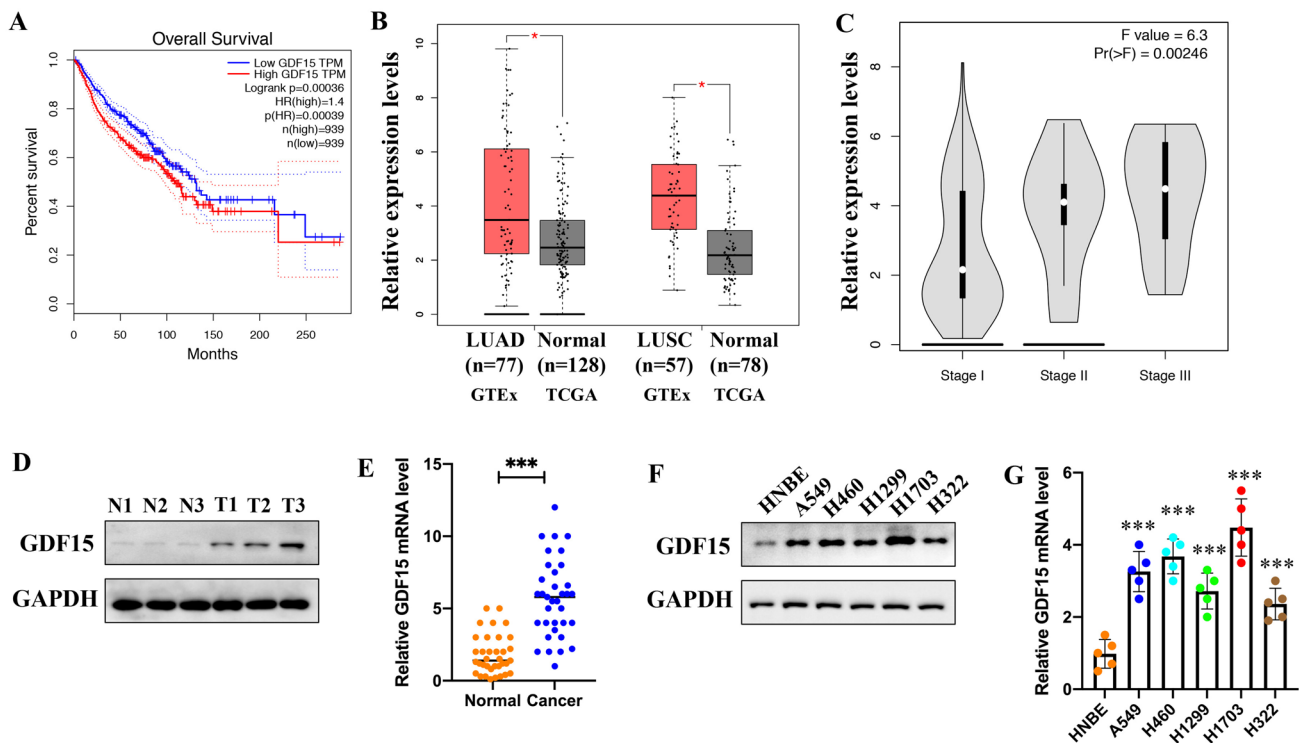


Fig. 1 High expression of GDF15 is correlated with worse survival and malignant progression of NSCLC. **A** Higher expression of GDF15 indicated lower survival. **B** Higher expression of GDF15 was observed in LUAD and LUSC tissues compared with control. **C** Overexpression of GDF15 is correlated with advanced tumor. **D** Significant higher protein expression of GDF15 was observed in tumor tissues compared with control. **E** Significant higher mRNA expres-

sion of GDF15 was observed in tumor tissues compared with control. **F** The protein expression of GDF15 was highly expressed in H322, H1703, H1299, H460, and A549 cell lines compared with control. **G** The mRNA expression of GDF15 was highly expressed in H322, H1703, H1299, H460, and A549 cell lines compared with control. *** $P < 0.001$ compared with group normal or HNBE.

Bioinformatics analysis

The relationship between GDF15 expression analysis and prognosis, survival, and tumor stage was performed with TCGA, GTEX, and GEPIA databases as described previously (Xu et al. 2022).

RNA extraction and qRT-PCR

RNA extraction was performed with TRIzol reagent (Invitrogen, US), and RNeasy Mini Kit (QIAGEN) was used for purification. ProtoScript First Strand cDNA Synthesis Kit was used for cDNA generation, and qRT-PCR analysis was conducted with a ABI7500 (Applied Biosystems, US). The primers were listed as follows: GDF15: F: 5'-tccgga-tactcagccagaa-3', R: 5'-tctgcaaggctgagctgac-3'; PTEN: F: 5'-GGGTCTGAGTCGCCTGTCA-3', R: 5'-CCGTGTTGG AGGCAGTAGAAG-3'; AKT F: 5'-ACTCATTCCAGA CCCACGAC-3', R: 5'-AGCCCGAAGTCCGTTATCTT-3'; PI3K F: 5'-CTCTCCTGTGCTGGCTACTGT-3', R: 5'-GCT

CTCGGTTGATTCCAAACT-3'; GAPDH: F: 5'-TGTGGG CATCAATGGATTGG-3', R: 5'-ACACCATGTATTCCG GGTCAT-3'.

Western blotting

The cells were lysed with RIPA buffer, and protein concentration was detected with BCA method (Nanjing Jiancheng, China). Each protein sample (20 μ g) was loaded on 10% SDS-PAGE. The protein samples were transferred to a nitrocellulose membrane. 5% fat-free milk was used to block non-specific protein at 25 $^{\circ}$ C for 1 h. The membrane was incubated with related primary antibodies overnight at 4 $^{\circ}$ C. After washing with PBS for 3 times, the membrane was incubated with second primary 25 $^{\circ}$ C for 2 h. Chemiluminescence (Thermo, US) was used to visualize target proteins, and ImageJ was used to analyze protein bands. The antibodies were listed as follows: rabbit monoclonal to GDF15 (ab206414, abcam, UK), rabbit monoclonal to PTEN (ab267787), rabbit monoclonal to p-PI3K

Table 1 Relationship between GDF15 expression and characteristics in NSCLC patients

Features	Number of patients	High expression of GDF15	Low expression of GDF15	p value
All cases	266	130	136	
Gender				0.136
Male	125	70	55	
Female	141	75	66	
Age				0.461
< 60	129	62	67	
≥ 60	137	71	66	
Smoking status				0.003*
Non-smoker	84	34	50	
Smoker	182	101	81	
Diameter (cm)				0.125
< 3	176	95	81	
≥ 3	90	52	38	
T stage				0.025*
T1, T2	208	108	100	
T3, T4	58	38	20	
N stage				0.041*
N0	188	91	97	
N1–N3	78	45	33	
M stage				0.311
M0	169	80	89	
M1	97	45	52	

Results were presented as mean ± SD

* < 0.05

(ab278545), rabbit monoclonal PI3K (ab140307), rabbit polyclonal to p-AKT (ab38449), rabbit monoclonal to AKT (ab179463), rabbit polyclonal to GAPDH (ab9485), rabbit monoclonal to N-Cadherin (ab76011), rabbit monoclonal to Vimentin (ab92547), rabbit monoclonal to E-Cadherin (ab40772), rabbit monoclonal to cleavage caspase-3 (ab32042), rabbit monoclonal to cleavage PARP (ab32064), rabbit monoclonal to H2AX (ab229914), goat Anti-Rabbit IgG (ab6721).

EdU staining

EdU reagent (Beyotime, Shanghai, China) was used to incubate transfected cells for 4 h. Then, cells were stained with DAPI (Beyotime, Shanghai, China). Finally, a fluorescence microscope (Olympus, Japan) was used to capture fluorescence images.

CCK8 assay

After transfection, cells (3000 cells/well) were seeded into a 96-well plate. The CCK8 reagent (Beyotime, Shanghai, China) was added at 0, 24, 48, and 72 h, respectively. After 1 h incubation, the cell proliferation was measured at 450 nm.

10 wound healing assay

Cells were seeded into a 6-well plate. After reaching 70% confluence, a 200 µL pipette tip was used to scratch wound. PBS was used to remove floating cells. The cells were photographed at 0 and 24 h with an inversion microscopy. Related migrated distance was calculated with ImageJ software.

Transwell assay

After transfection, cells (2×10^5) were seeded into the upper chamber of a Transwell chamber (BD Biosciences, US) with Matrigel (BD Biosciences). 1 mL medium containing 10% FBS was added to lower chamber. After 24 h, methanol was used for fixation and 0.3% crystal violet was used for staining. Invasive cells were photographed with an inversion microscopy.

Statistical analysis

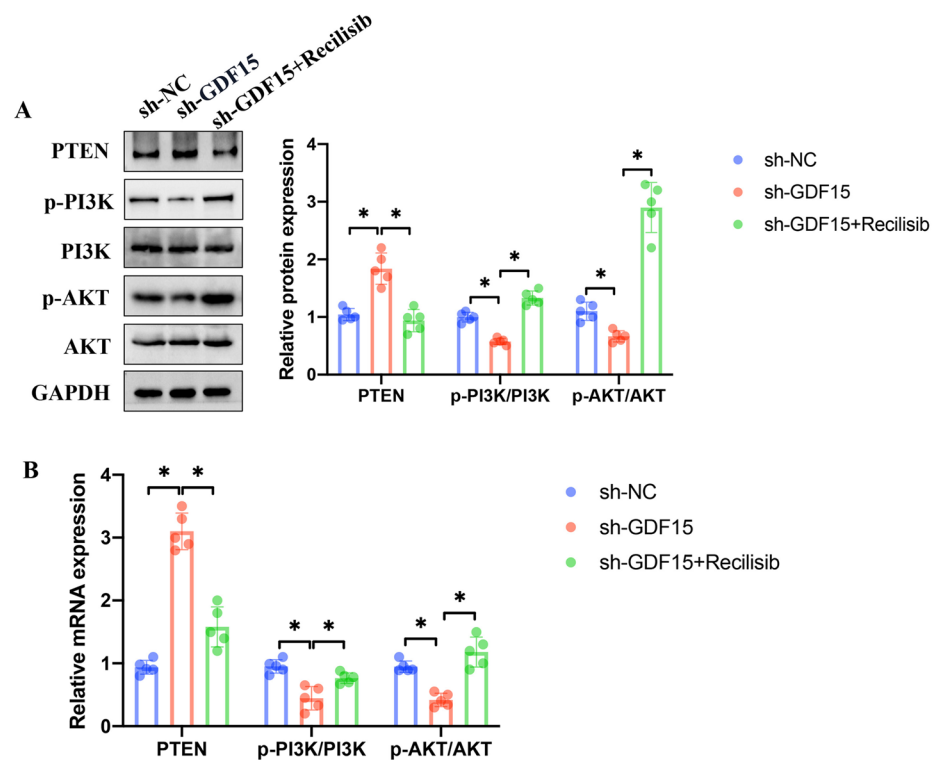
SPSS 22.0 software and GraphPad Prism 9.0 were applied for data analysis. Student's t-test or One-way ANOVA or were performed for the statistical analysis. $p < 0.05$ was considered statistically different.

Results

High expression of GDF15 is correlated with worse survival and malignant progression of NSCLC

Based on the GEPIA database analysis, we found that higher expression of GDF15 suggested poor prognosis and lower survival (Fig. 1A). Lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) are the most common types of non-small cell lung cancer. Significant higher expression of GDF15 was observed in LUAD and LUSC tissues compared with normal tissues based on the databases of TCGA and GTEx (Fig. 1B). In addition, overexpression of GDF15 is correlated with advanced tumor (Fig. 1C). The protein and mRNA expression levels of GDF15 in the tumor tissues and adjacent

Fig. 2 The inhibition of PTEN/PI3K/AKT pathway by sh-GDF15 was reversed by Recilisib. **A** The protein expression of PTEN/PI3K/AKT signaling pathway was evaluated with western blotting. **B** The mRNA expression of PTEN/PI3K/AKT signaling pathway was evaluated with qRT-PCR. * indicates $P < 0.05$ between two groups



tissues from NSCLC patients in our hospital were analyzed. The expression of GDF15 was markedly increased in the tumor tissues compared to group normal (Fig. 1D, E). Meanwhile, five types of lung cancer cell lines were used to evaluate the expression of GDF15. The protein and mRNA levels of GDF15 were highly expressed in H322, H1703, H1299, H460, and A549 cell lines compared with in HNBE cell line (Fig. 1F, G). H1299 and A549 cell lines are commonly used to study NSCLC, and they were applied in this research for in vitro study. The relationship between GDF15 expression and characteristics in NSCLC patients was listed in the Table 1. Statistical differences were observed in terms of smoking status, tumor stage/grade, or metastasis condition.

The inhibition of PTEN/PI3K/AKT pathway by sh-GDF15 was reversed by Recilisib

PTEN/PI3K/AKT signaling pathway is closely linked with the progression of several types of tumors, and we investigated the potential influence of sh-GDF15 on expression of PTEN/PI3K/AKT. The levels of PTEN was promoted, but p-PI3K/PI3K and p-AKT/AKT were restrained by sh-GDF15 (Fig. 2A, B). However, Recilisib, the activator of PTEN/PI3K/AKT signaling pathway, changed the effects

of sh-GDF15 on PTEN/PI3K/AKT signaling pathway significantly (Fig. 2A, B).

Knockdown of GDF15 restrained the proliferation, invasion, migration, but accelerated apoptosis of lung cancer cells

EdU staining (Fig. 3A, B) and CCK8 (Fig. 3C) methods were used to detect the proliferation ability of tumor cells. Knockdown of GDF15 greatly inhibited the proliferation of H1299 and A549 cells, but the inhibition effects of sh-GDF15 were reversed by Recilisib (Fig. 3A–C). Similarly, the suppression of cell migration and invasion after transfecting sh-GDF15 in both H1299 and A549 cell lines was also reversed by Recilisib (Fig. 4A, B). Significant increase of cell apoptosis caused by sh-GDF15 was markedly restrained by Recilisib (Fig. 5A). The cell percentage in the S stage was greatly elevated in the group sh-GDF15, and the cell percentage in the G2 stage was decreased (Fig. 5B). The data of cell cycle suggest that sh-GDF15 arrested tumor cells in the S stage, and prevented cells from entering G2 stage. In addition, the expression of apoptosis specific markers including cleavage caspase-3 and PARP was measured. We found that the significant increase of cleavage caspase-3 and PARP caused by sh-GDF15 was greatly decreased by

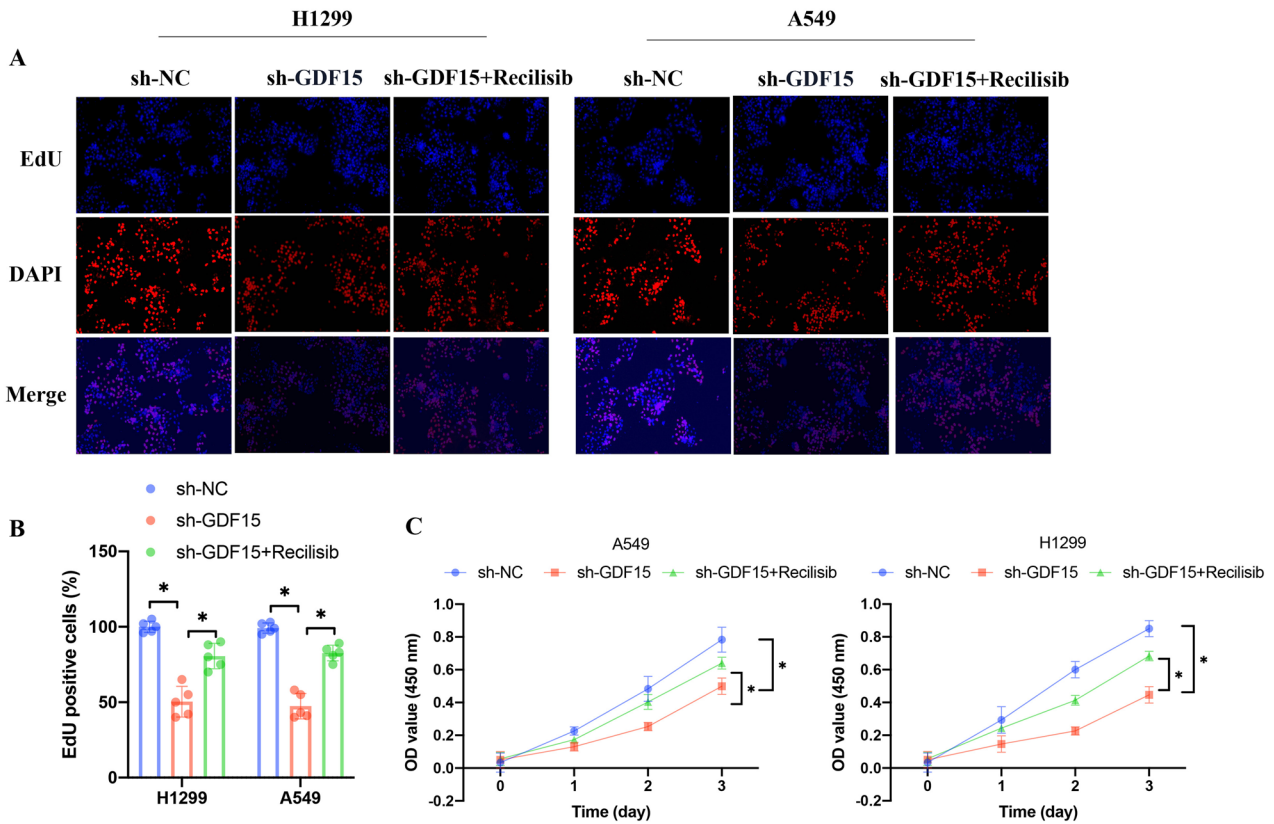


Fig. 3 Knockdown of GDF15 restrained the proliferation ability of lung cancer cells. **A** EdU staining was used to detect the proliferation ability of tumor cells. **B** The EdU staining results were analyzed. **C**

CCK8 method was used to detect the proliferation ability of tumor cells. * indicates $P < 0.05$ between two groups

Recilisib (Fig. 5C). Actually, we previously measured the expression levels of cleavage of caspase-3, PARP and H2AX in sh-NC treated cells and untreated cells. No significant difference was observed, indicating that the cells used in this study were apoptotic to some degree. H2AX is a marker of DNA damage, and its level was remarkably elevated by sh-GDF15, but simultaneous treatment with Recilisib greatly suppressed the expression of H2AX.

Influence of sh-GDF15 on chemotherapy sensitivity of lung cancer cells

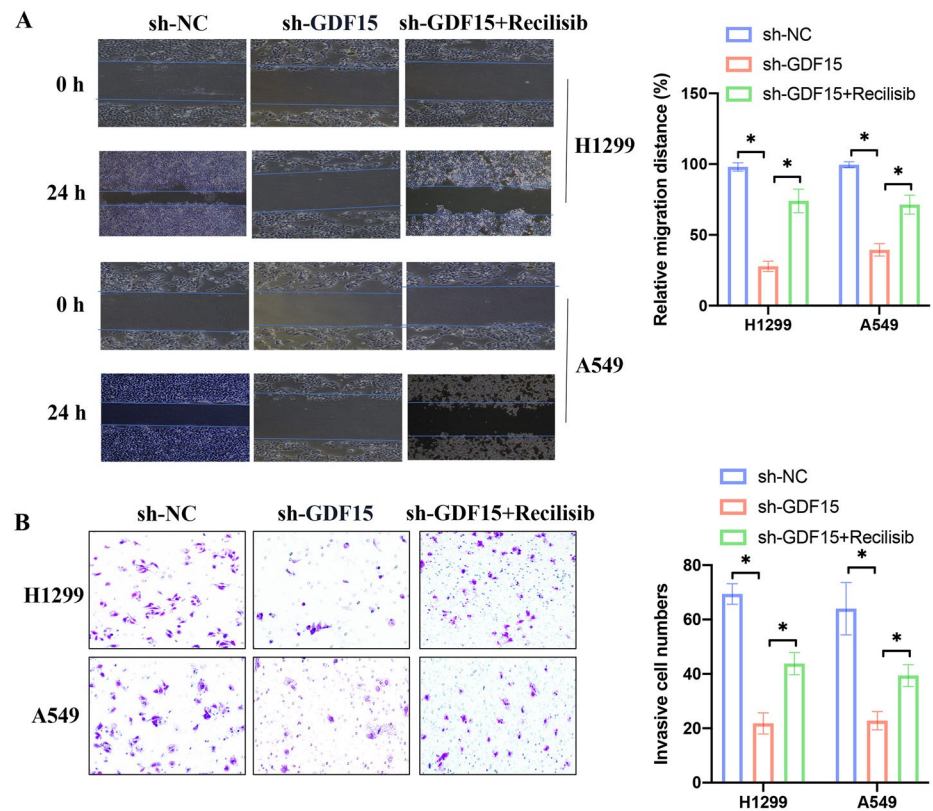
Docetaxel and doxorubicin, two common chemotherapy drugs in the clinic, were used to investigate the influence of sh-GDF15 on chemotherapy sensitivity of lung cancer cells. After transfection with sh-GDF15, the cell proliferation was remarkably restrained (Fig. 6 A-B), but simultaneous

treatment with Recilisib increased cell proliferation ability (Fig. 6 A-B). Therefore, sh-GDF15 could increase the chemotherapy sensitivity of H1299 and A549 cell lines to both docetaxel and doxorubicin.

Influence of sh-GDF15 on epithelial mesenchymal transition (EMT) process in lung cancer cells

The protein expression levels of key EMT process related proteins including N-cadherin, Vimentin, and E-cadherin were measured. sh-GDF15 greatly inhibited the levels of N-cadherin and Vimentin, but promoted the expression of E-cadherin in both H1299 and A549 cell lines (Fig. 7A, B). In addition, Recilisib treatment reversed the influence of sh-GDF15 on EMT process related proteins expression (Fig. 7A, B).

Fig. 4 Knockdown of GDF15 restrained the migration and invasion ability of lung cancer cells. **A** Wound healing assay was used to investigate the influence of sh-GDF15 on cell migration. **B** Transwell assay was used to investigate the influence of sh-GDF15 on cell invasion. * indicates $P < 0.05$ between two groups



Discussion

The incidence rate of lung cancer is the highest among all tumors, and its annual death toll ranks the first, and this figure is still increasing year by year (Tan et al. 2022). Lung cancer has become a serious threat to human health, in which NSCLC accounts for more than 80% of lung cancer (Provencio et al. 2021). Bone tissue is one of the most common metastatic sites in patients with lung cancer. The rate of bone metastasis in patients with lung cancer is about 35% (Kang et al. 2021). It is necessary to unfold novel target gene for precise treatment of lung cancer.

GDF15 is also known as macrophage inhibitory cytokine-1 (MIC1). The role of GDF15 in the occurrence and development of cancer is complex and contradictory (Husaini et al. 2020; Rybicki et al. 2021). Some experimental evidence shown that GDF15 had tumor suppressive activity, while other data suggested that it had carcinogenic activity (Myojin et al. 2021). Whether GDF15 promotes or inhibits tumor growth seems to depend on the type and stage of the tumors (Gao et al. 2021; Li et al. 2020). Previous studies have shown that GDF15 plays different or even

opposite roles in different tumors and different stages of tumors, which is similar to the controversial role of its family members (Zhang et al. 2021).

Clinical data indicated that high levels of GDF15 in serum predicted higher incidence of colonic adenomatous polyps, faster tumor progression and higher risk of recurrence (Danta et al. 2017). GDF15 is also called macrophage inhibitory factor because of its inhibitory effect in the process of inflammation (Guo et al. 2021). It was reported that GDF15 could inhibit the inflammatory cytokines of lipopolysaccharide-treated macrophages, indicating that GDF15 could play an anti-tumor role by reducing inflammation (Lewandowska et al. 2021). In this study, we proved that knockdown of GDF15 presented anti-tumor effect through modulating PTEN/PI3K/AKT signaling pathway by inhibiting cell proliferation, invasion, and migration of NSCLC cell lines.

PI3K/AKT signaling pathway has been proved to be closely related with the regulation of many kinds of disease process, and the relationship between PI3K/AKT and GDF15 was reported. GDF15 secreted by esophageal squamous cell carcinoma cells promoted tumor progression by

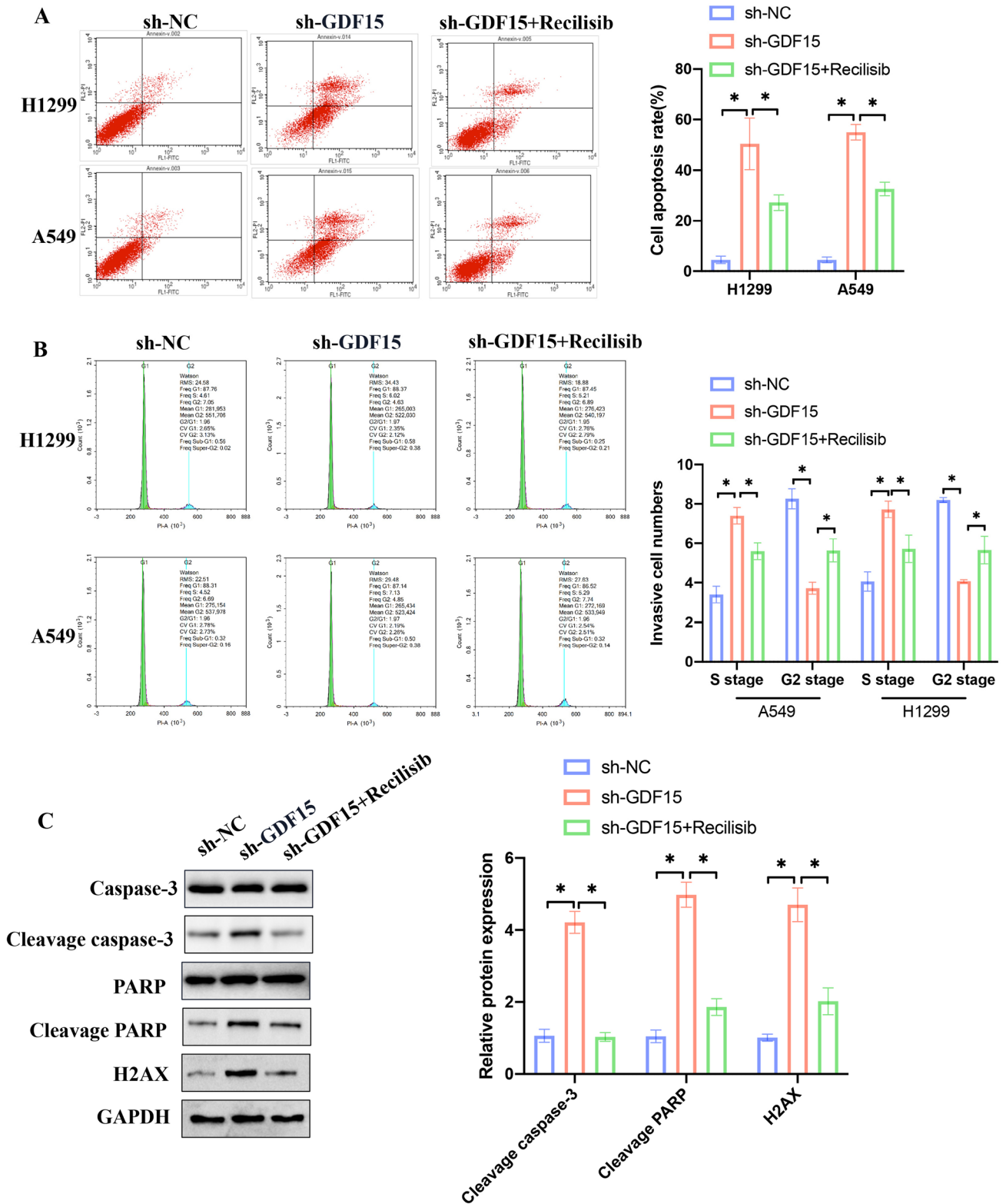


Fig. 5 Knockdown of GDF15 promoted apoptosis of lung cancer cells. **A** The cell apoptosis was analyzed with flow cytometry. **B** The cell cycle was analyzed with flow cytometry. **C** The protein expres-

sion levels of apoptosis related genes and DNA damage marker were measured. *P<0.05 between two groups

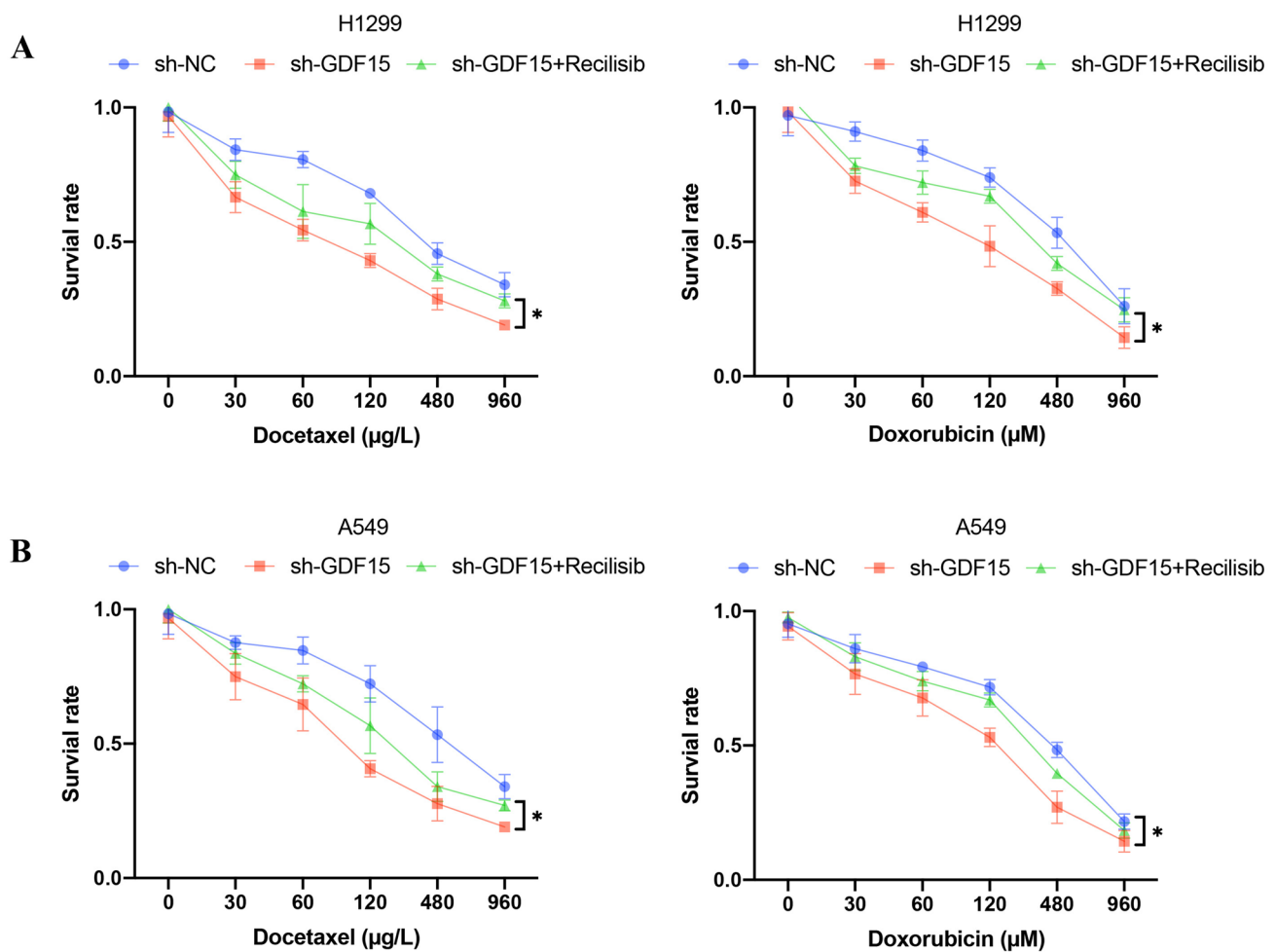


Fig. 6 Influence of sh-GDF15 on chemotherapy sensitivity of lung cancer cells. **A** sh-GDF15 increased the chemotherapy sensitivity of H1299 cells to both docetaxel and doxorubicin. **B** sh-GDF15

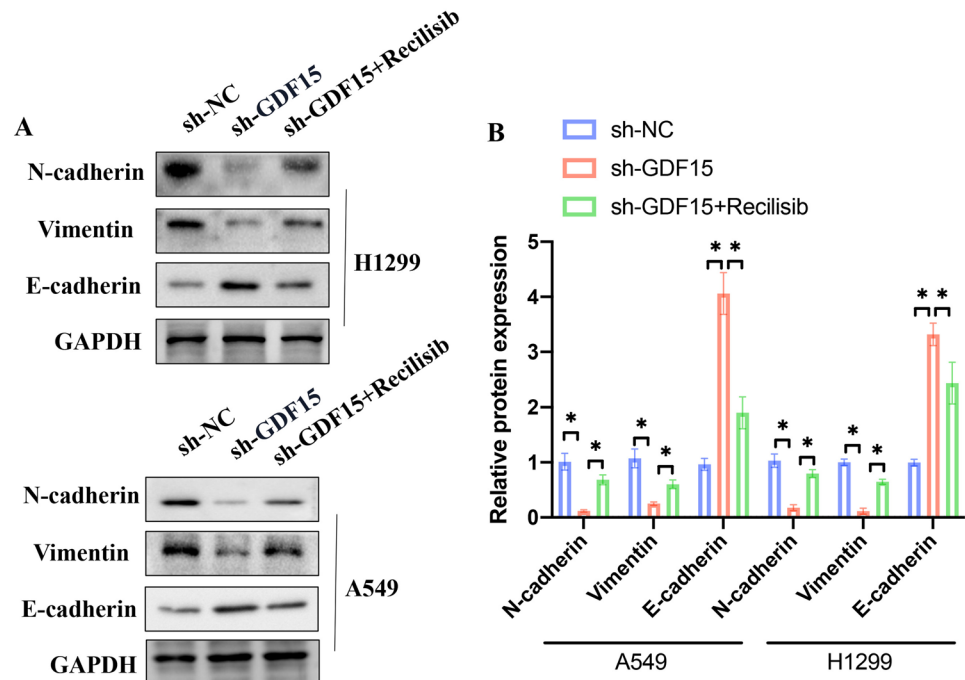
increased the chemotherapy sensitivity of A549 cells to both docetaxel and doxorubicin. * indicates $P < 0.05$ between two groups

activating AKT (Urakawa et al. 2015). On the other hand, GDF15 promoted the proliferation, migration, invasion and clone formation of oral squamous cell carcinoma cells by inhibiting AKT phosphorylation (Yang et al. 2014). Meanwhile, blocking PI3K can significantly inhibit the myocardial hypertrophy induced by GDF15 (Heger et al. 2010). Therefore, in different cells, GDF15 has both positive and negative regulation on its downstream signal pathway.

EMT plays an important role in the process of tumor cell invasion and metastasis. Epithelial cells obtain the phenotype of stromal cells, the cell adhesion ability is decreased

or disappeared, and the migration ability is increased (Chen et al. 2018). During EMT process, the expression of E-cadherin was decreased, while the expression of vimentin and N-cadherin was increased (Wang et al. 2019). β -Catenin is transferred to the nucleus to promote abnormal high expression of related genes leading to the enhancement of migration and invasion ability (Mulholland et al. 2012). In this study, we found that sh-GDF15 significantly suppressed the EMT process, and it might be the potential mechanism how GDF15 affect NSCLC.

Fig. 7 Influence of sh-GDF15 on EMT process in lung cancer cells. **A** The protein expression of EMT related genes was evaluated with western blotting. **B** The protein expression of EMT related genes was analyzed. * $P < 0.05$ between two groups



Conclusion

Significant higher expression of GDF15 was observed in the lung cancer tissues and cell lines compared to related controls. Overexpression of GDF15 is correlated with poor prognosis. Knockdown of GDF15 restrained the proliferation, invasion, migration, but accelerated apoptosis of lung cancer cells through regulating PTEN/PI3K/AKT signaling pathway. sh-GDF15 increased the chemotherapy sensitivity of lung cancer cells, and inhibited EMT process.

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Author contributions YL and XJ conceived and designed the experiments; JL, XJ, XC, JW, JD, HZ and YL performed the experiments, analyzed data. YL and XJ wrote the paper. All authors read and approved the final manuscript.

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Data availability The data support this study could be requested from corresponding author.

Declarations

Conflict of interest The authors have declared that no competing interests exist.

Ethical approval and consent to participate Written informed consent for publication was obtained from all participants. All experiment protocols have been approved by the ethics committee of the Second Affiliated Hospital of Air Force Medical University.

Consent for publication The authors agree for publication of this manuscript.

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