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ORIGINAL RESEARCH

MiR-490-3p Inhibits the Malignant Progression of Lung Adenocarcinoma

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Objective: To investigate the effects of miR-490-3p on the proliferation, migration, invasion and apoptosis of lung adenocarcinoma (LUAD) cells through the Wnt/β-catenin signaling pathway.

Methods: Differentially expressed miRNAs in LUAD tissues were analyzed by bioinformatics and the target miRNA went through GSEA enrichment analysis. qRT-PCR was used to detect the expression of miR-490-3p in human LUAD cells and normal bronchial cells. The constructed vectors were transfected into the LUAD cell lines using Lipofectamine 2000. Cell viability was detected by MTT, cell migration and invasion were detected by transwell assay, and cell apoptosis was detected by flow cytometry. Western blot was performed to detect the expression levels of the proteins related to the Wnt/β-catenin pathway and cell apoptosis. Xenograft tumor mouse models were used for in vivo validation.

Results: The results of qRT-PCR showed that miR-490-3p was relatively lowly expressed in LUAD cells, and the expression level was different in different LUAD cell lines. The results of MTT, transwell and flow cytometry exhibited that miR-490-3p could significantly inhibit the proliferation, migration, invasion and increase cell apoptosis rate of LUAD cells. Western blot results showed that miR-490-3p promoted the expression of Bax, Caspase-3 and E-cadherin as well as the phosphorylation of GSK-3β and inhibited the expression of Bcl-2, β-catenin and C-myc. Additionally, animal experiments were performed to prove that miR-490-3p suppressed LUAD malignant progression in vivo.

Conclusion: MiR-490-3p inhibited the proliferation, migration, invasion and promoted the apoptosis of LUAD cells by down-regulating the Wnt/β-catenin signaling pathway, suggesting that miR-490-3p may be an indicator for early diagnosis and prognosis of LUAD.

Keywords: miR-490-3p, lung adenocarcinoma cells, proliferation, migration, Wnt signaling pathway

Introduction

Lung cancer is one of the most common cancers with the highest morbidity and mortality.[1] There are two subtypes of lung cancer: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which account for 15% and 85% of all lung cancer cases, respectively. NSCLC can be further divided into lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LSCC), adenosquamous carcinoma (ASC) and lymphoepithelioma-like carcinoma (LELC). LUAD is the most common type of lung cancer, which accounts for about 40% of all lung cancer cases.[2] It arises from mutations of small airway epithelial type II alveolar cell, which secretes mucus and other substances.[3] Early lung cancer has no specific symptoms and manifestations, and is easy to be neglected due to similar symptoms

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as respiratory diseases, such as chest pain, cough, chest tightness, etc., resulting in a low rate of diagnosis in the early stage. Some patients have progressed to the middle and advanced stage, thus missing the best opportunity for treatment. At present, LUAD is mainly treated by surgery, supplemented by radiotherapy and chemotherapy, but LUAD is still one of the most aggressive and fatal cancers, with overall survival less than 5 years.[2] The purpose of this study was to explore the regulatory role of miR-490-3p in LUAD cells and to find its potential as a new therapeutic target for LUAD.

MicroRNAs (miRNAs) are a class of non-coding RNAs composed of 20–25 nucleotides, which can degrade mRNA or inhibit the translation process by binding to the 3′ untranslated regions (UTR) of mRNA, and then regulate the expression level of target genes.[4] Accumulating evidence shows that miRNAs are crucial in the occurrence and development of cancer, and the abnormal expression of various miRNAs is related to the inhibition of the malignant progression of LUAD.[5–7] Studies over the years have indicated that miR-490-3p is lowly expressed in a variety of cancers and can inhibit the malignant development of cancers. For example, the high expression of miR-490-3p can inhibit the proliferation, migration and invasion of prostate cancer cells.[8] MiR-490-3p inhibits the proliferation, delays cell cycle and promotes apoptosis through inhibiting autophagy of hepatocellular carcinoma cells.[9] However, the role and mechanism of miR-490-3p in LUAD cells have not been reported.

Wnt/β-catenin signaling pathway is considered to be one of the main signaling pathways involved in cell epithelial-mesenchymal transition (EMT), which can promote the occurrence and development of various malignant tumor diseases by regulating cell proliferation and apoptosis.[10] Glycogen synthase kinase-3β (GSK-3β) is an important negative regulator of the Wnt/β-catenin pathway, whose over-activation can lead to degradation of downstream β-catenin and promotion of cancer cell apoptosis.[11,12] The protein β-catenin is closely related to the differentiation of tumor cells. It abnormally aggregates in the cytoplasm of tumor cells or expresses in the nucleus of tumor cells while has low content in the normal cytoplasm. After the activation of Wnt signal, the accumulation of β-catenin in the nucleus increases, which activates the expression of its downstream target genes, thereby promoting the expression of C-myc and leading to the formation of tumors.[13]

The purpose of this study was to investigate the effect of miR-490-3p on the occurrence and development of LUAD and the inhibition to the Wnt/β-catenin signaling pathway, so as to explore the regulatory mechanism of miR-490-3p in LUAD cells and clarify its effect on LUAD malignant progression, ultimately providing a theoretical basis for miR-490-3p as a therapeutic target of LUAD.

Materials and Methods

Bioinformatics Analysis

Gene expression profiles and clinical data of TCGA-LUAD were downloaded from the TCGA database. Differentially expressed miRNAs (DEmiRNAs) in LUAD tissues were analyzed using edgeR with $|logFC| > 2$ and $padj < 0.05$ as the threshold. Target miRNA was determined by survival analysis of DEmiRNAs based on the clinical information of samples. GSEA software was used to conduct single gene KEGG enrichment analysis on target miRNA to study its mechanism affecting LUAD.

Cell Culture

Normal bronchial cell line BEAS-2B (CBP60577) and human LUAD cell lines A549 (CBP60084), H1299 (CBP60053), H522 (CBP60140), PC9 (CBP60078) and H1703 (CBP60115) were purchased from Nanjing Cobioer Biotechnology Co., LTD. BEAS-2B cells were cultured in serum-free bronchial epithelial cell growth medium (BEGM), and LUAD cells were cultured in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS; Hyclon, USA). The A549 cell line was frozen in a liquid nitrogen tank with 90% complete medium+10% dimethylsulfoxide (DMSO; Solarbio, USA), and the other experimental cell lines were frozen in the liquid nitrogen tank with 90% FBS+10% DMSO freezing medium. The resuscitated cells were cultured in a humid incubator at 37°C with 5% CO_2. Cells were digested with 0.25% pancreatin-EDTA (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and passaged at 1:3 at 80%–90% in confluence. Logarithmic growth stage cells were collected for subsequent experiments.

qRT-PCR

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) according to the instructions.[14] cDNA was synthesized by PrimeScript RT Master Mix (Japan Takara Biotech). The reverse transcription system was as

follows (25 μL): 3 μL RNA, 1 μL 100 mmol/L primers and 13 μL dd H_2O were mixed and reacted at 72°C for 5 minutes. Then, 1.5 μL 10 mmol/L dNTP, 5 μL 5×MMLV buffer, 1 μL M-MLV RT and 0.5 μL RNasin were added and mixed. The reaction was conducted at 37°C for 1 hour to obtain cDNA.

With snRNA U6 used as internal reference, CFX96™ Real-time System (Bio-Rad Company, Hercules, CA, USA) was used for fluorescence quantitative reaction (the reaction conditions: 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute). $2^{-\Delta\Delta Ct}$ method was used for calculation. ΔCt and ΔΔCt were calculated with the following formulas: ΔCt = Ct miR-490-3p – Ct U6 and ΔΔCt = ΔCt case – ΔCt control.

The primer sequences for qPCR were as follows: miR-490-3p (forward): 5′-CGGCGGTCAACCTGGAGGACTCC-3′; miR-490-3p (reverse): 5′-CCAGTGCAGGGTCCGAGGTAT-3′; U6 (forward): 5′-CTCGCTTCGGCAGCACACATATA-3′; U6 (reverse): 5′- AAATATGGAACGCTTCACGA – 3′.

Vector Construction and Cell Transfection

At the logarithmic stage of cell growth, NC-mimic, miR-490-3p mimic, NC-inhibitor and miR-490-3p inhibitor (Guangzhou RiboBio, People's Republic of China) were transfected into LUAD cells by Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific), respectively. The transfection efficiency was detected by qRT-PCR after culture at 37°C for 24-48 hours.

The primer sequences of each gene were as follows: miR-490-3p mimic forward: 5′-CAACCUGGAGGACUCCAUGCCG-3′, miR-490-3p mimic reverse: 5′-AGACCGUCGAUUGGGCCAGUUG-3′; miR-490-3p inhibitor forward: 5′- UUUAGCUGGUACCGACUGUACG-3′, miR-490-3p inhibitor reverse: 5′- AUUCGGUAGUUUCAGGGCAUAG-3′; negative control forward: 5′-ACCUUCUCAGGCUUGACGUAGA-3′, negative control reverse: 5′- CCGUAUUAUCUGCCAAGUACGU-3′.

MTT

The transfected cells were inoculated into 96-well plates with 2×10^4 cells per well. At 24, 48, 72 and 96 hours, 20 μL MTT solution was added to the medium and cells were incubated for 4 hours. After discarding the medium, 200 μL DMSO was added to the cells and the formazan crystal was dissolved for 15 minutes. The optical density (OD) value at 490 nm was detected by the enzyme-linked immunometric meter (Thermo Fisher Scientific).

Transwell

Single-cell suspension was prepared using serum-free medium for each group 48 hours after transfection, and 5×10^3 LUAD cells were inoculated in the upper transwell chamber (Thermo Fisher Scientific). Next, 750 uL RPMI-1640 medium containing 10% FBS was added to the lower chamber and the cells were incubated for 6 hours. The cells were washed with PBS once and fixed with 4% paraformaldehyde for 20 minutes. After being stained with 0.1% crystal violet, the cells were observed under a microscope. The number of migrated cells in five random independent fields was calculated.

The transwell invasion experiment was conducted by the following procedures. Matrigel was added to the upper chamber and after Matrigel solidification, cells were incubated for 4 hours. The rest of the operations were the same as the transwell migration experiment.

Flow Cytometry (FCM)

The LUAD cells were seeded into 6-well plates and cultured in a saturated humidity incubator containing 5% CO_2 at 37°C for 48 hours. Trypsin was used to digest the cells, and the serum-containing medium was used to terminate digestion and resuspend cells. The cells were centrifuged at 4°C for 10 minutes at 1000 r/min, and the supernatant was discarded. Then, 1 mL PBS was used to resuspend cells and cells were centrifuged for 10 minutes to discard the supernatant. After the cells were washed twice, the apoptosis rate of H1299 cells was tested using Annexin V-FITC/PI (Merck Sigma-Aldrich, Germany) by FCM (Thermo Fisher Scientific).

Western Blot

The total proteins were extracted and the protein concentration was detected using a protein extraction kit and the BCA kit (Thermo Fisher Scientific), respectively. Proteins were insolated on SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Thermo Fisher Scientific). The membrane was cultured with rabbit polyclonal antibody Bax (1:1000), Bcl-2 (1:1000), Caspase-3 (1:500), E-cadherin (1:1000), β-catenin (1:5000), p-GSK-3β (1:1000), GSK-3β (1:5000), C-myc (1:2000) and β-actin (1:1000) antibodies at 4°C overnight. The next day, the residual primary antibodies were washed with PBST,

and the goat anti-rabbit IgG H&L (HRP) antibody (1:5000) was added for incubation at room temperature for 2 hours. The enhanced ECL kit (Thermo Fisher Scientific, USA) was used to measure the OD value, and the protein level was quantified by Image-Pro Plus software. Relative quantification of target protein was carried out with the β-actin as the internal reference. All antibodies were purchased from Abcam (Shanghai) Trading Co., Lld.

Animal Experiments

To validate the effect of miR-490-3p on LUAD tumor in vivo, a total of 20 BALB/c male nude mice (4–6 weeks) were ordered from Shanghai SLAC Laboratory Animal (Shanghai, People's Republic of China), and then were divided into four groups at random with mice mice per group. LUAD cell lines, which were incubated with agomir NC/miR-490-3p agomir or antagomir NC/miR-490-3p antagomir (Guangzhou Ribobio, People's Republic of China) for 3 days, were subcutaneously injected into nude mice for tumor formation. Tumor volume was measured once a week and tumor growth curve was correspondingly plotted with a formula set as: Volume = Length × Width^2 × 0.5. Five weeks later, the mice were executed and tumors were isolated for weighing.

Statistical Analysis

All experiments were repeated three times. Data analysis was conducted using Graphpad Prism 5 statistical software. *t*-test was used for analyzing comparison between two groups, and one-way analysis of variance was used for comparison among multiple groups. $P<0.05$ indicated a significant difference, and $P<0.01$ indicated a highly significant difference.

Results

MiR-490-3p is Down-Regulated in LUAD Cells

A total of 46 normal tissue samples and 521 tumor tissue samples were screened from the TCGA database. EdgeR analysis results indicated that miR-490-3p was significantly down-regulated in LUAD tissues (Figure 1A). Survival analysis results showed that the expression of miR-490-3p significantly affected the prognosis of patients, and the survival time of patients with high expression was significantly longer than that of patients with low expression (Figure 1B). In order to confirm the bioinformatics results, we used qRT-PCR to detect the expression of miR-490-3p in the cells, and found that miR-490-3p was significantly lower expressed in the LUAD cells than that in the normal bronchial lung cells (BEAS-2B), with the lowest expression in H1299 cell line (Figure 1C). Therefore, H1299 cell line was selected for subsequent experiments. These results fully demonstrated that miR-490-3p was down-regulated in LUAD and may be closely related to the prognosis of LUAD.

MiR-490-3p Inhibits the Proliferation and Promotes Apoptosis of LUAD Cells

Transfection efficiency was detected by qRT-PCR after transfection (Figure 2A). Compared with the control group, the expression of miR-490-3p was increased significantly in the miR-490-3p mimic group ($P<0.05$), but decreased significantly in the miR-490-3p inhibitor group ($P<0.05$). In order to detect the effect of miR-490-3p on H1299 cell vitality, MTT assay was conducted and the results demonstrated that compared with the control group, overexpression of miR-490-3p could remarkably inhibit the proliferation of LUAD cells in a time-

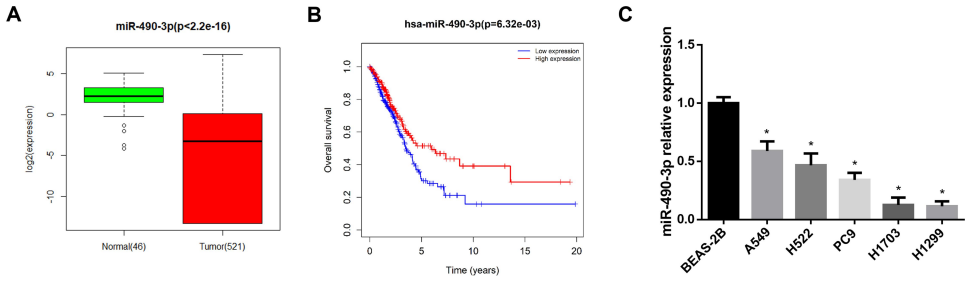

Figure 1 Low expression of miR-490-3p in LUAD cells. (**A**) The boxplots of miR-490-3p expression in the normal group and tumor group from the TCGA-LUAD dataset; (**B**) The survival curves of the prognosis affected by the expression level of miR-490-3p, which was divided into a high-expression group (red) and a low-expression group (blue) by the median value of the miR-490-3p expression level; (**C**) The expression of miR-490-3p in bronchial cell line and five LUAD cell lines; n=3, * indicates significant difference, $P<0.05$.

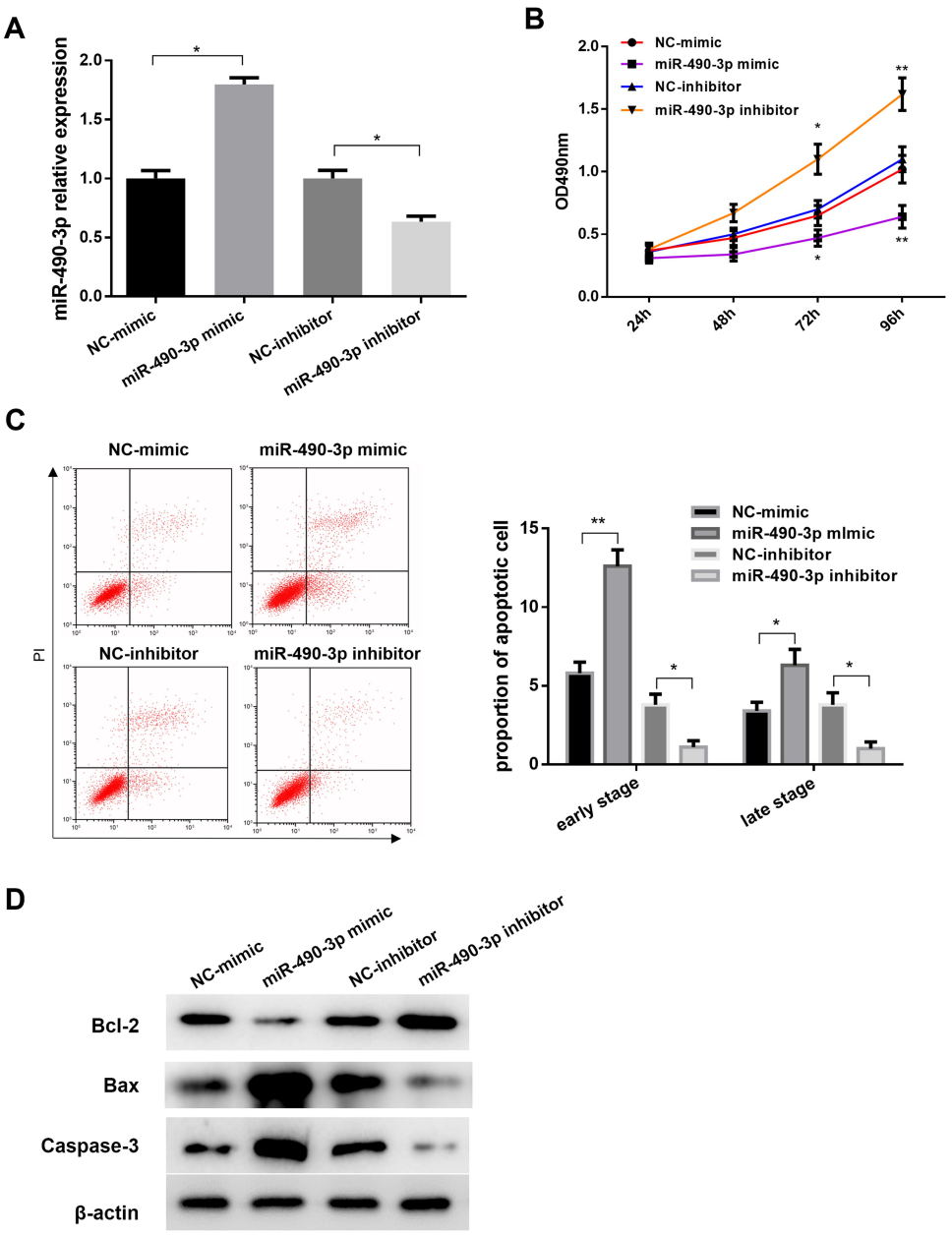

Figure 2 MiR-490-3p inhibits the proliferation and promotes apoptosis of LUAD cells. (**A**) After miR-490-3p mimic/inhibitor was transfected into H1299 cells, the expression level of miR-490-3p was detected by qRT-PCR in each treatment group; (**B**) Cell viability was tested by MTT assay at 24, 48, 72 and 96 hours; (**C**) Cell apoptosis rate was detected by FCM. The lower limit on the left was normal cell group, the lower limit on the right was early apoptotic cell group, the upper limit on the left was autophagic death and non-specific death cell group, and the upper limit on the right was the late apoptotic cell group and the necrotic cell group; (**D**) The expression levels of Bcl-2, Bax and Caspase-3 were detected by Western blot; n=3, * indicates significant difference in comparison with corresponding NC, *P*<0.05; ** indicates highly significant difference in comparison with corresponding NC, P<0.01.

dependent manner, while silencing miR-490-3p caused a significant increase in cell vitality, suggesting that miR-490-3p overexpression effectively inhibited the proliferation of LUAD cells (Figure 2B).

After transfection for 48 hours, the apoptosis level and expression of apoptosis-related proteins in each group were detected. FCM observed that the early apoptotic ratios of miR-490-3p mimic group and miR-490-3p inhibitor group were 12.6% and 1.1%, respectively, and the late apoptotic ratios were 6.3% and 1.0%, respectively, both of which were greatly different from those of the corresponding control group, indicating that miR-490-3p could significantly promote the apoptosis of H1299 cells (Figure 2C). Meanwhile, the effects of miR-490-3p on anti-apoptosis protein Bcl-2 and pro-apoptosis proteins Bax and Caspase-3 were detected by Western blot. The results exhibited that overexpression of

miR-490-3p significantly reduced Bcl-2 expression and increased Bax and Caspase-3 expression, while silencing miR-490-3p caused opposite results, which verified that miR-490-3p promoted the apoptosis of LUAD cells at the molecular level (Figure 2D).

MiR-490-3p Inhibits the Migration and Invasion of LUAD Cells

In order to observe the effect of miR-490-3p on the migration and invasion of LUAD cells, transwell assay was performed to detect the migration and invasion abilities of cells, respectively. The results of the migration experiment showed that, compared with the control group, overexpression of miR-490-3p led to an extremely obvious decrease in migration ability while silencing miR-490-3p significantly improved the migration ability (Figure 3A, $P<0.05$). The same trend could be observed in the results of the invasion experiment (Figure 3B, $P<0.05$). E-cadherin is one of the main adhesion molecules of epithelial cells. We detected the expression of E-cadherin, a marker protein of EMT, by Western blot. The results suggested that overexpression of miR-490-3p significantly increased the expression of E-cadherin, but silencing miR-490-3p significantly inhibited the expression of E-cadherin (Figure 3C).

MiR-490-3p Inhibits the Expression of Wnt Signaling Pathway-Related Proteins

Single gene KEGG pathway enrichment analysis was conducted on miR-490-3p using GSEA software based on the miRNA expression data. It was observed that miR-490-3p was significantly enriched in the Wnt signaling pathway

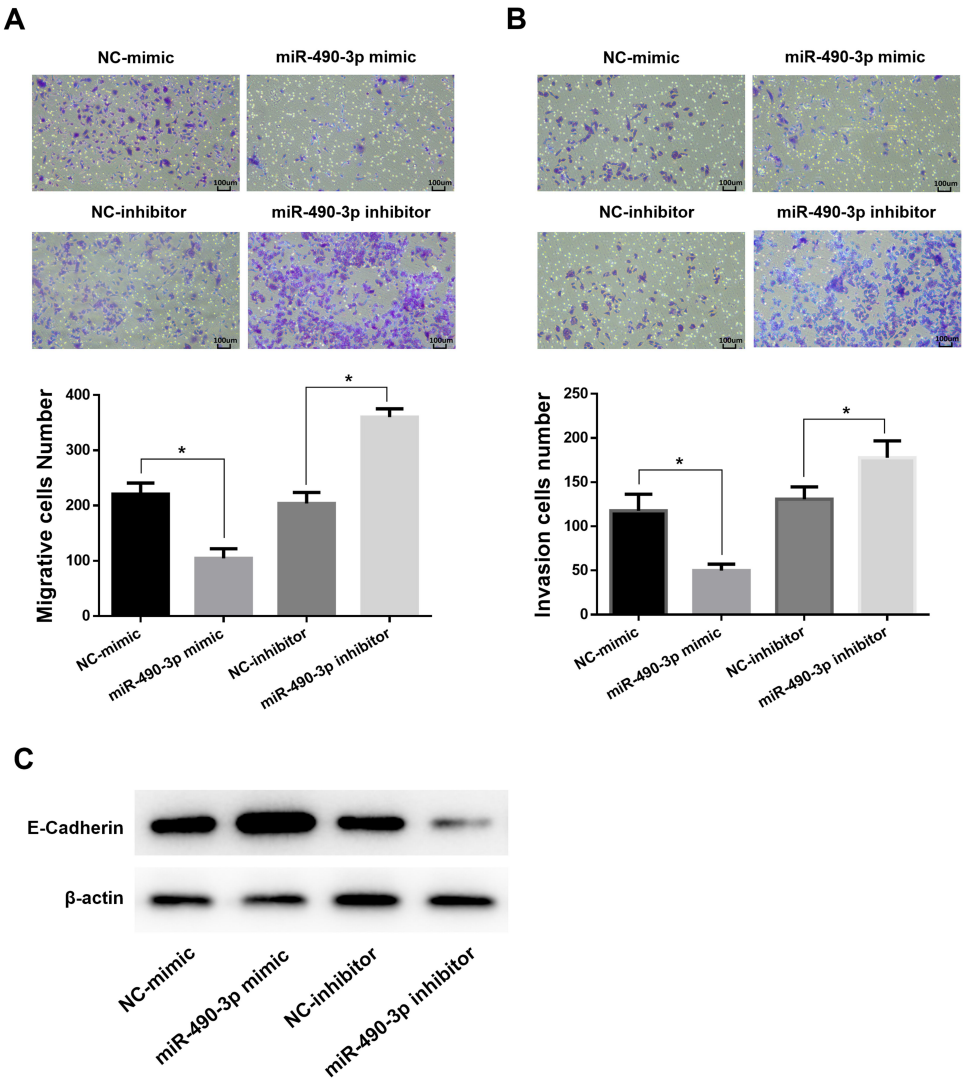

Figure 3 MiR-490-3p inhibits the migration and invasion of LUAD cells. (**A**) Cell migration, (**B**) invasion and (**C**) the effects of miR-490-3p on E-cadherin expression in each group were determined by transwell assay and Western blot; n =3, * indicates significant difference, $P<0.05$.

(Figure 4A). To verify the above result and explore the effects of up-regulation of miR-490-3p on the expression levels of Wnt signaling pathway-related proteins, we detected the expression of three important Wnt/β-catenin signaling pathway-related proteins. Results showed that compared with the control group, overexpression of miR-490-3p dramatically reduced the expression of β-catenin and C-myc, while increasing the expression of phosphorylated negative regulatory factor GSK-3β greatly. Silencing miR-490-3p had the opposite effects on these proteins (Figure 4B), which indicated that miR-490-3p activated GSK-3β phosphorylation,[15] and hindered signal transduction of the Wnt/β-catenin pathway, thus leading to degradation of pathway-related proteins.

In vivo Experiments Validate That MiR-490-3p Suppresses the Malignant Progression of LUAD

To further elucidate the tumor suppressive role of miR-490-3p in vivo, xenograft tumor mouse models were established. According to the experimental results it was found that the tumor weight and volume of the mice injected with miR-490-3p agomir were both significantly decreased compared to those of the mice with agomir NC, while those of the mice with miR-490-3p antagomir were reversely increased relative to the control values (Figure 5A and B). Additionally, the proteins related to cell apoptosis, EMT and the Wnt/β-catenin pathway were measured using Western blot, the results of which indicated that transfection of miR-490-3p agomir promoted the expression of Bax, Caspase-3, E-cadherin and induced the phosphorylation of GSK-3β, yet the expression of Bcl-2, β-catenin and C-myc was suppressed. Adversely, transfection of miR-490-3p posed opposite effects (Figure 5C). Overall, all the above findings identified the in vivo inhibitory role of miR-490-3p in LUAD malignant progression.

Discussion

LUAD is a common clinical NSCLC, and mostly originates from bronchial mucosal epithelial cells, with an increasing incidence in recent years. Unlike other types of lung cancer, LUAD often occurs in non-smokers or women at a young age.[16] Surgery and chemotherapy are still the main clinical treatment methods and chemotherapy is mainly used for advanced inoperable patients, but the drug resistance during chemotherapy and the high recurrence rate after intervention are the primary causes of death in LUAD patients.[17] Therefore, in-depth study of the molecular mechanism of the occurrence and development of LUAD is of great significance to the discovery of diagnostic indicators and new effective treatment methods for LUAD.

MiRNAs are involved in the occurrence and development of various tumors by regulating the expression levels of tumor promoter or tumor suppressor in the post-transcriptional level. MiR-490-3p is located on chromosome 7q33 and is composed of 22 nucleotides.[18] Multiple studies have confirmed that silencing miR-490-3p affects the development of a variety of cancers, which has an anticancer effect, and its expression is negatively correlated with the incidence of disease and drug resistance.[18,19] These studies all indicate that the abnormal low expression of miR-490-3p is closely related to the malignant progression and poor prognosis of cancers. However, the role and mechanism of miR-490-3p in the malignant progression of LUAD remain unclear. Here, by activating or silencing miR-490-3p in LUAD cells, it was found to inhibit the proliferation,

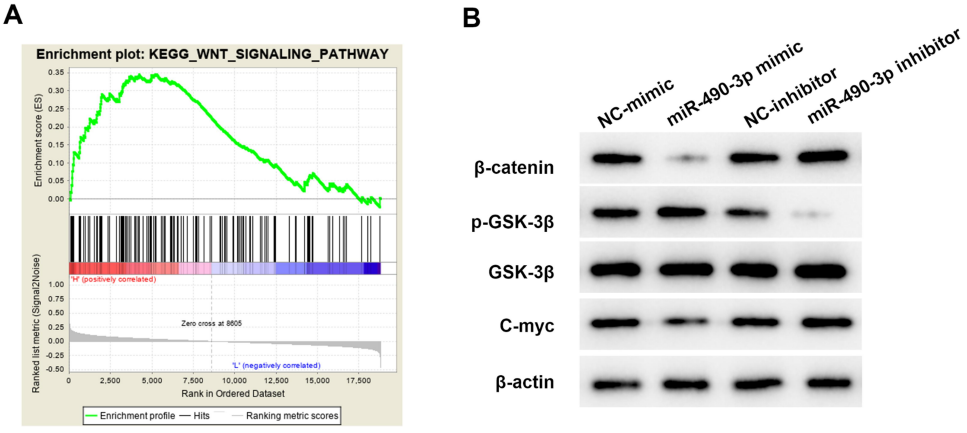

Figure 4 MiR-490-3p inhibits the expression of Wnt pathway-related proteins. (**A**) GSEA pathway enrichment analysis results of miR-490-3p; (**B**) Western blot was used to detect the effects of miR-490-3p on the expression levels of β-catenin, GSK-3β and C-myc; n=3.

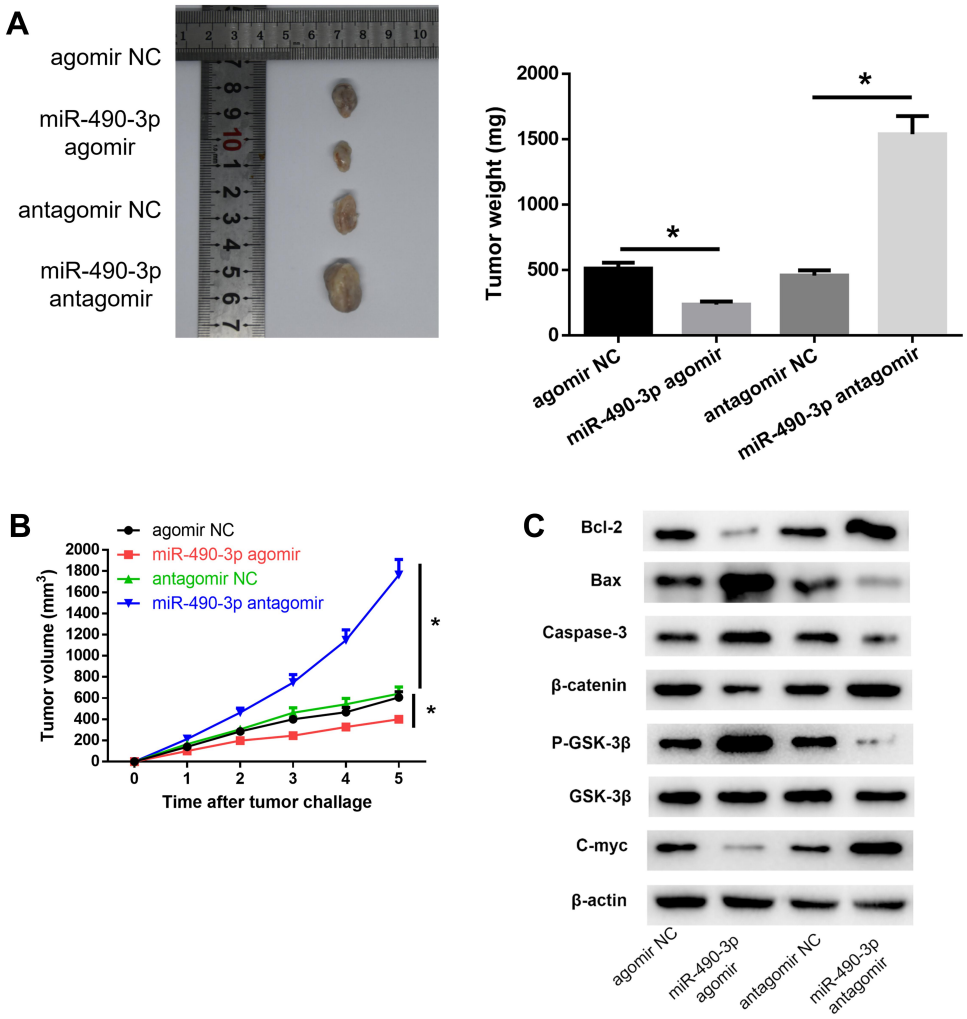

Figure 5 MiR-490-3p suppresses the malignant progression of LUAD in vivo. (**A**) Tumor pictures and tumor weight in each transfection group (agomir NC, miR-490-3p agomir, antagomir NC, miR-490-3p antagomir); (**B**) Tumor growth curve in each transfection group (means ± standard deviation, n=5/group); (**C**) Western blot was applied to test the expression of the proteins related to cell apoptosis, EMT and the Wnt/β-catenin pathway. * indicates statistically significant difference, $P<0.05$.

migration and invasion of LUAD cells in vitro. Besides, in vivo animal experiments further proved that miR-490-3p was able to suppress the malignant progression of LUAD. Bax and Bcl-2 belong to the Bcl-2 gene family, and Bcl-2 is an inhibitor of apoptosis while Bax can antagonize the inhibitory effect of Bcl-2 and promote apoptosis. Caspase-3 is the most important terminal shear enzyme in cell apoptosis. MiR-490-3p was proved to increase the expression of pro-apoptotic protein and improve the apoptosis rate of LUAD cells by FCM and Western blot.

Single gene KEGG pathway enrichment analysis found that miR-490-3p was likely to function on the Wnt/β-catenin signaling pathway. The Wnt/β-catenin signaling pathway is tightly regulated at the cellular level and is dysregulated in LUAD.[20] Herein, the high expression of phosphorylated GSK-3β and the low expression of β-catenin and C-myc indicated that the malignant progression of LUAD cells was associated with the inhibitory effect of miR-490-3p on the Wnt/β-catenin signaling pathway. However, the specific molecular mechanism is still unclear and will be explored in future studies.

In summary, miR-490-3p is important in inhibiting the proliferation, migration and invasion of LUAD cells, and is a vital tumor suppressor miRNA. It has also been observed that the miR-490-3p regulates the occurrence and development of LUAD cells through the Wnt/β-catenin signaling pathway. MiR-490-3p may be a diagnostic and prognostic indicator of LUAD, and may become a new target for the treatment of LUAD after further study on its targeting effect.

Data Sharing Statement

The data and materials in the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Author Contributions

All authors made a significant contribution to the work reported, whether that was in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas. All authors had agreed on the journal to which the article will be submitted, gave final approval of the version to be published. All authors reviewed and agreed on all versions of the article before submission, during revision, the final version accepted for publication, and any significant changes introduced at the proofing stage. All authors agreed to take responsibility and be accountable for the contents of the article.

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Disclosure

The authors report no conflicts of interest in this work.

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