Original Research

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miR-181a-3p inhibits malignant biological behavior of non-small cell lung cancer via down-regulating RAD21 expression

Qi Guo[1], Yan Yuan[2], Xi Zhang[3], Xuefen Lei[4], Qin Yuan[3], Heng Li[1, *]

Abstract

Lung cancer is one of the most common malignant tumors in the world, and it has become the first cause of death from malignant tumors in the Chinese urban population. Non-small cell lung cancer (NSCLC) is one of the main types, accounting for about 80% of all lung cancers. MicroRNA (miRNA) is a non-coding RNA with a length of about 22 nt, which can block the translation process of the protein by targeted binding with mRNA at the post-transcriptional level. Cumulative studies have shown that a variety of miRNAs play an important role in the occurrence and development of NSCLC. This study first found through UALCAN and Starbase databases that miR-181a-3p expression was abnormally low in NSCLC, which indicates that patient have a poor survival rate. Consistent results were also detected in the clinical samples we collected. In addition, we found that RAD21 was a downstream target of miR-181a-3p, and RAD21 was significantly highly expressed in NSCLC clinical samples. In NSCLC, the expression level of miR-181a-3p and RAD21 showed a negative correlation. Furthermore, we used CCK-8 kit, Annexin V-FITC/PI kit, Transwell and Wound-Healing assay to explore the effect of miR-181a-3p/RAD21 molecular axis on the malignant biological behavior of A549 cells. The results showed that knocking-down RAD21 significantly inhibited A549 cell proliferation, invasion and wound healing and induced its apoptosis, while knocking-down miR-181a-3p would rescue biological behaviors of A549 cells. In summary, we found for the first time that miR-181a-3p inhibits the proliferation, invasion and migration of NSCLC cells and induces their apoptosis by inhibiting the expression level of RAD21. It is suggesting that the miR-181a-3p/RAD21 molecular axis can be used as a target for the diagnosis and treatment of NSCLC.

Keywords: Non-small cell lung cancer; Malignant biological behavior; miR-181a-3p; RAD21

1. 2nd department of Thoracic surgery, The third affiliated hospital of Kunming medical university, No. 519 Kunzhou Road, Kunming, 650118, Yunnan, China

2. Critical care, The Second affiliated hospital of Kunming medical university, No. 374 Dianmian Road, 650106, Kunming, Yunnan, China

3. Kunming medical university, No. 1168 Chunrong West Road, 650504, Kunming, Yunnan, China

4. Oncology, The Second affiliated hospital of Kunming medical university, No. 374 Dianmian Road, 650106, Kunming, Yunnan, China

*Correspondence: romanticfat@126.com

Introduction

Lung cancer is the most common aggressive malignant tumor worldwide, accounting for 18.4% of the total deaths from cancer in men and women[1]. In China, lung cancer has the highest morbidity and mortality rate among all malignant tumors, and it is increasing year by year[2]. By 2015, it is estimated that there will be approximately 733,000 newly diagnosed lung cancer cases, and approximately 610,000 people will die of lung cancer[3]. NSCLC is the most common type of lung cancer, accounting for about 80% of all lung cancers. Since there are no obvious clinical symptoms in the early stage of NSCLC, most NSCLC patients are in the late stage with a poor prognosis after diagnosis[4]. More and more clinical studies have found that metastasis greatly hinders the treatment of NSCLC cancer[5]. Therefore, an in-depth understanding of its metastasis mechanism and possible targets is essential for effective treatment of non-small cell lung cancer.

miRNA is a small non-coding RNA molecule that is endogenously expressed, which can bind to incomplete sequence homology sites in the 3'untranslated region (3' UTR) of mRNA, thereby causing degradation of mRNA or inhibiting its protein translation[6, 7]. Accumulated studies have shown that miR-181a plays different roles in a variety of solid tumors including lung cancer, and plays an important regulatory role in key aspects of cell growth, development, angiogenesis, invasion, and metastasis[8, 9]. In lung cancer, miR-181a is usually down-regulated. In most cases, decreased expression of miR-181a can lead to poor prognosis of patients[10]. In addition, the targets of miR-181a are mostly key genes related to cell proliferation, evasion of growth inhibition or resistance to cell death, and replication and immortality[11, 12]. miR-181a-3p and miR-181a-3p are processed from the 3'end arm and 5'end arm of the hsa-miR-181 precursor, respectively. At present, more and more literature studies have shown that miR-181a-5p, as a tumor suppressor, can inhibit the proliferation, metastasis, drug resistance and immune escape of lung cancer by regulating downstream targets and other malignant biological behaviors[13, 14]. However, the regulatory mechanism of miR-181a-3p on the occurrence and development of NSCLC remains to be studied. A better understanding of the changes in miR-181a-3p expression in NSCLC may better improve the diagnosis and treatment of NSCLC.

In this study, we found through database and sample testing that miR-181a-3p is abnormally low in NSCLC. *In vitro*, we have found that miR-181a-3p can inhibit the proliferation, invasion and migration of NSCLC cell lines and induce their apoptosis by inhibiting the expression level of RAD21. It is suggested that the miR-181a-3p/RAD21 axis can be used as a target for the diagnosis and treatment of NSCLC.

Materials and Methods

2.1 Clinical sample and cells

Lung cancer tissue of grades II to IV, as well as adjacent normal tissue (>5 cm from tumor), were acquired from The third affiliated hospital of Kunming medical university in accordance with protocols approved by ethical committee of The third affiliated hospital of Kunming medical university. We collected 30 pairs in total. Written consent was acquired from each participant. BEAS2B, A549, HCC827, NCI-H23 and NCI-H1650 were acquired from American Type Culture Collection (ATCC; Rockville, MD) and maintained according to ATCC's recommendations.

2.2 Database analysis

In short, UALCAN database (http://ualcan.path.uab.edu/analysis.html)[15] analyzed the expression levels of miR-181a-3p and RAD21 in Lung adenocarcinoma (LUAD) and Lung squamous cell carcinoma (LUSC). The Starbase database (http://starbase.sysu.edu.cn/)[16] was used to analyze the correlation between miR-181a-3p and RAD21 with the survival of NSCLC patients, and the correlation between miR-181a-3p and RAD21 expression.

2.3 Cell culture and Transfection

Human normal lung epithelial cells BEAS2B and lung cancer cell lines (A549, HCC827, NCI-H23 and NCI-H1650) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), and 100 U/ml penicillin/streptomycin (Invitrogen Life Technologies) at 37 °C in a humidified 5% CO_2 incubator.

A549 cells at exponential stage were used for transfection. Before transfection, 1×10^6 cells were cultured in 6-well plates with 2 mL complete medium for 24 h until they were 90% confluent. sh-RAD21, hsa-miR-181a-3p mimics, has-miR-181a-3p inhibitor, and negative control (NC) were purchased from Shanghai GenePharma Inc. (Shanghai, China). Cells were grouped into NC, OV-miR-181a-3p, KD-miR-181a-3p, KD-RAD21 and Co-KD groups. The vectors and microRNAs were transfected, respectively, into A549 cells by Lipofectamine 3000 (Thermo Fisher, USA) reagents and cultured with Opti-MEM serum-free medium following the instructions.

2.4 RNA extraction and RT-qPCR

RNAs were extracted using the Trizol reagent (Invitrogen, Carlsbad, California), followed by removal of DNA with the TurboDNase kit (Ambion). Quantification of extracted RNA was performed using NanoDrop. Complementary DNA synthesis was performed using PrimeScript real-time (RT) reagent kit (Takara Bio, Japan) using 1000-ng of total RNA. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the SYBR Select Master Mix (Applied Biosystems, Waltham, Massachusetts) on an ABI 7900 system (Applied Biosystems). U6 was used as a control. The Ct value was calculated based on the ΔΔCt method. Fold change of gene expression was expressed as $2^{-\Delta\Delta Ct}$. The primers used in this study were as follows: miR-181a-3p sense: 5′-GAACATTCAACG CTGTCGGT-3′; miR-181a-3p antisense: 5′-GCTATAGGGTACAATCAACGGTC-3′; U6 sense: 5′-CTAGATAATGGTGCTGATAGATG GA-3′; U6 antisense: 5′-GGCACACCAG AAATCGAAGC-3′.

2.5 Western blotting assay

Protein was extracted from the indicated cells using RIPA lysis buffer, and a BCA Protein Assay Kit (Thermo Scientific, USA) was used to measure the protein concentration. In total, 60 μg of protein were separated on 10% SDS-PAGE gels by polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes. The membranes were blocked using 5% non-fat dry milk and incubated with primary mouse monoclonal anti-RAD21 (1:500, Abcam, Cambridge, UK) overnight at 4 °C. The membranes were washed with TBST and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. Enhanced chemiluminescence reagent was used to detect the signal on the membrane.

2.6 Luciferase reporter assay

T293 cells were transfected with 100 ng plasmids and 200 nmol/L miR-181a-3p mimics or their negative control. After 2 days, the cells were lysed with 80 μL 1×Passive Lysis Buffer

and tested through a dual luciferase assay (Promega). For RAD21 promoter analysis, the RAD21 promoter was amplified and cloned into a psiCHECK TM-2 vector (Promega). Luciferase activity was evaluated through the dual luciferase assay system (Promega).

2.7 CCK-8 assay

In total, 2000 cells were seeded into each well of a 96-well plate for 12 h and further incubated for 24, 48, or 72 h. One hour before the end of incubation, 10 µl of CCK-8 reagent (Solarbio, Beijing, China) was added to each well. The OD 450 nm in each well was determined using an enzyme immunoassay analyzer.

2.8 Annexin V-FITC/IP assay

Cells from each group were trypsinized, washed with cold phosphate buffer saline (PBS), and stained with Annexin-V FITC and PI according to the manufacturer's instructions. The apoptosis rate was analyzed by flow cytometry (BD Biosciences). The experiments were independently performed in triplicate.

2.9 Transwell assay

For the invasion assay, matrigel chambers (BD Biosciences, San Jose, CA, USA) were carried out conforming to manufacturer's instructions. Briefly, transfected A549 cells (200 µL, 5000 cells per well) were collected, resuspended in medium without serum, and then shifted to the hydrated matrigel chambers (50 µL). The bottom chambers were incubated overnight in 500 µL DMEM culture medium with 10% FBS. The cells on the upper surface were scraped, whereas the invasive cells on the lower surface were fixed and colored with 0.1% crystal violet for half an hour.

2.10 Wound-Healing assay

First, A549 cells were seeded in 6-well plates. When the cells reached confluence, the surface of the plates was lightly scratched using a sterile micropipette tip. The floating cells then was added with DMEM medium with 10% FBS. An inverted optical microscope (×200) (Nikon, Japan) was used to monitor the closure of the wound at 0 and 24 h. Each group repeated the process thrice.

2.11 Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.2 software (GraphPad Software, Inc., La Jolla, CA, USA), and data are presented as the mean ± standard deviation. An unpaired two-tailed Student's t-test or one-way ANOVA analysis of variance with Bonferroni's post-test were used to analyze the data as appropriate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

3.1 Clinical features of miR-181a-3p

We first analyzed the expression level of miR-181a-3p in NSCLC and its relationship with survival through the database. UALCAN database analysis found that miR-181a-3p was highly expressed in LUAD, but the opposite was true in LUSC (Fig. 1A-B). In addition, an analysis of the Starbase database found that high expression of miR-181a-3p predicts a better survival rate for patients (Fig. 1C). Furthermore, we tested the collected clinical samples. The results showed that miR-181a-3p was significantly lower expressed in NSCLC compared to paired adjacent tissues (Fig. 1D). Cell-level detection revealed that the expression level of miR-181a-3p in NSCLC cell lines was significantly lower than that of human normal lung epithelial cells BEAS2B, and the expression level of miR-181a-3p in A549 cells was the lowest (Fig. 1E). The abnormally low expression of miR-181a-3p may be related to the occurrence and

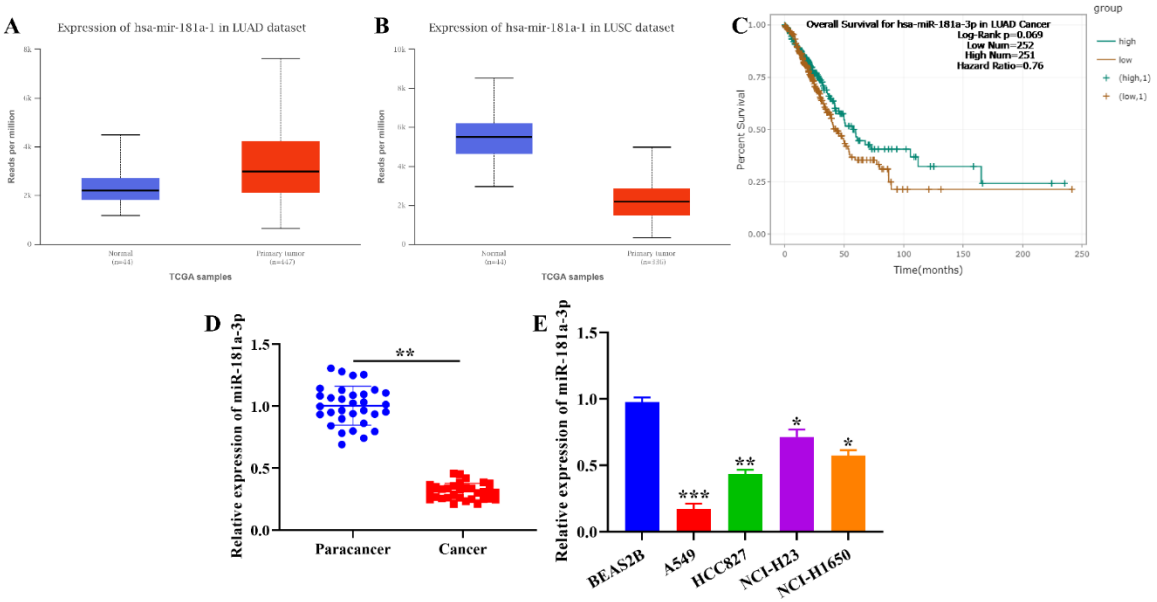

Figure 1 Clinical features of miR-181a-3p. UALCAN database analyzed the expression differences of miR-181a-3p in (a) LUAD and (b) LUSC clinical samples. (c) Starbase database analyzed the correlation between the expression level of miR-181a-3p and the survival rate of NSCLC patients. RT-qPCR was used to detect the expression level of miR-181a-3p in (d) NSCLC tissues and (e) cell lines. In all cases, Values are mean ± SD (*P<0.05, **P<0.01, ***P <0.001).

development of NSCLC. We chose A549 cells for follow-up experiments in vitro.

3.2 miR-181a-3p inhibits the malignant biological behavior of A549 cells

We further explored the effects of miR-181a-3p on the proliferation, apoptosis, invasion and apoptosis of A549 cells by exogenously regulating the expression level of miR-181a-3p. RT-qPCR results showed that transfection of miR-181a-3p mimic or miR-181a-3p inhibitor into A549 cells significantly up-regulated or down-regulated the expression level of miR-181a-3p (Fig. 2A). Subsequently, we tested the proliferation of A549 cells with the CCK-8 kit. The results showed that compared with the control group, overexpression of miR-181a-3p could significantly down-regulate the proliferation of A549 cells, while knockdown of miR-181a-3p was the opposite (Fig. 2B). In addition, the results of the Annexin V-FITC/PI kit showed that the apoptosis level of A549 cells in the OV-miR-181a-3p group was significantly higher than that of the control group, and the KD-miR-181a-3p group was lower than the control group (Fig. 2C). Furthermore, we tested the effects of miR-181a-3p on the invasion and migration of A549 cells through Transwell and Wound-Healing assay, respectively. Transwell results showed that overexpression of miR-181a-3p significantly inhibited the invasion number of A549 cells in the Transwell chamber (Fig. 2D). The Wound-Healing assay also showed that, after 24 h, the wound healing rate of the OV-miR-181a-3p group was significantly lower than that of the NC group, while the KD-miR-181a-3p was the opposite (Fig. 2E). The above results suggest that miR-181a-3p can be used as a tumor suppressor to inhibit the proliferation, invasion and migration of A549 cells and induce their apoptosis.

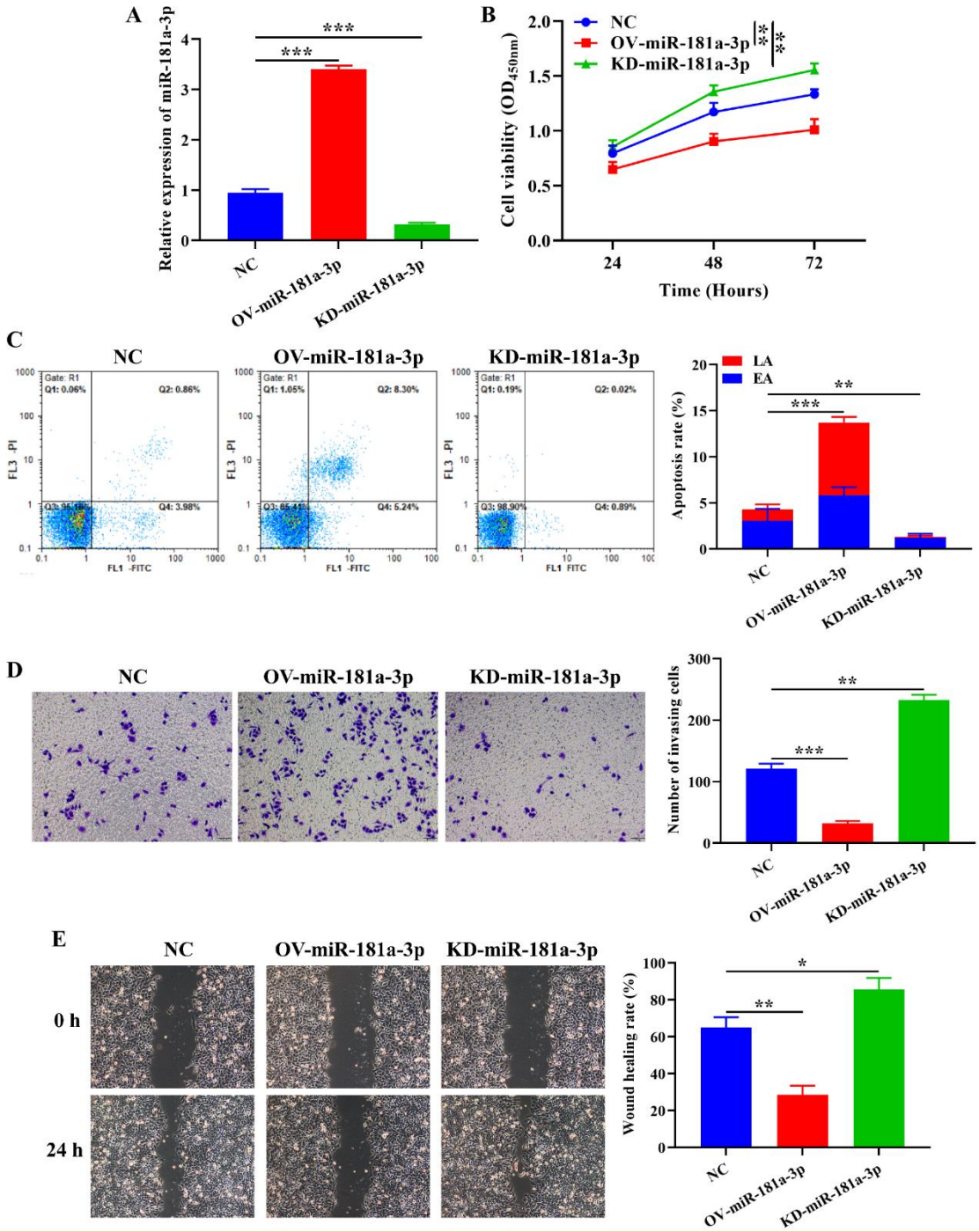

Figure 2 miR-181a-3p inhibits the malignant biological behavior of A549 cells. After transfecting miR-181a-3p mimic or miR-181a-3p inhibitor into A549 cells, (a) RT-qPCR was performed to detect miR-181a-3p in A549 cells. (b) A549 cell proliferation was detected by CCK-8 kit. (c) Annexin V-FITC/PI exhibited A549 cell apoptosis. (d) Transwell was used to indicate A549 cell invasion. (e) Wound-Healing Assay showed wound width of A549 cells in 0 h and 24h. In all cases, Values are mean ± SD (n=3 for each group; *P<0.05, **P<0.01, ***P <0.001).

3.3 RAD21 is the target gene of miR-181a-3p

Further, we explored the downstream pathways of miR-181a-3p. First, we predicted the downstream targets of miR-181a-3p through the Starbase database. As shown in Fig. 3A, miR-181a-3p and RAD21 had potential target binding sequences. We further verified it

through the dual luciferase reporter gene experiment. The results showed that compared with the NC group, miR-181a-3p in the miR-181a-3p group could significantly inhibit the luciferase activity of the RAD21 wild-type vector, but had no significant effect on the luciferase activity of the mutant RAD21 vector (Fig.3B). Furthermore, Western blotting results showed that overexpression or knockdown of miR-181a-3p significantly inhibited or promoted the expression level of RAD21 in A549 cells (Fig. 3C). In addition, an analysis of the UALCAN database found that the high expression of RAD21 in NSCLC predicts poor patient survival (Fig. 3D). Analysis of the Starbase database found that RAD21 was significantly highly expressed in both LUSC and LUAD (Fig. 3E). Similarly, our clinical samples also show that RAD21 was significantly highly expressed (Fig. 3F). We also found that the expression of miR-181a-3p and RAD21 was negatively correlated in LUAD and LUSC (Fig. 3G). Consistent conclusions were also obtained in the clinical samples we collected (Fig. 3H). It indicated that miR-181a-3p may regulate the malignant biological behavior of NSCLC cells through RAD21.

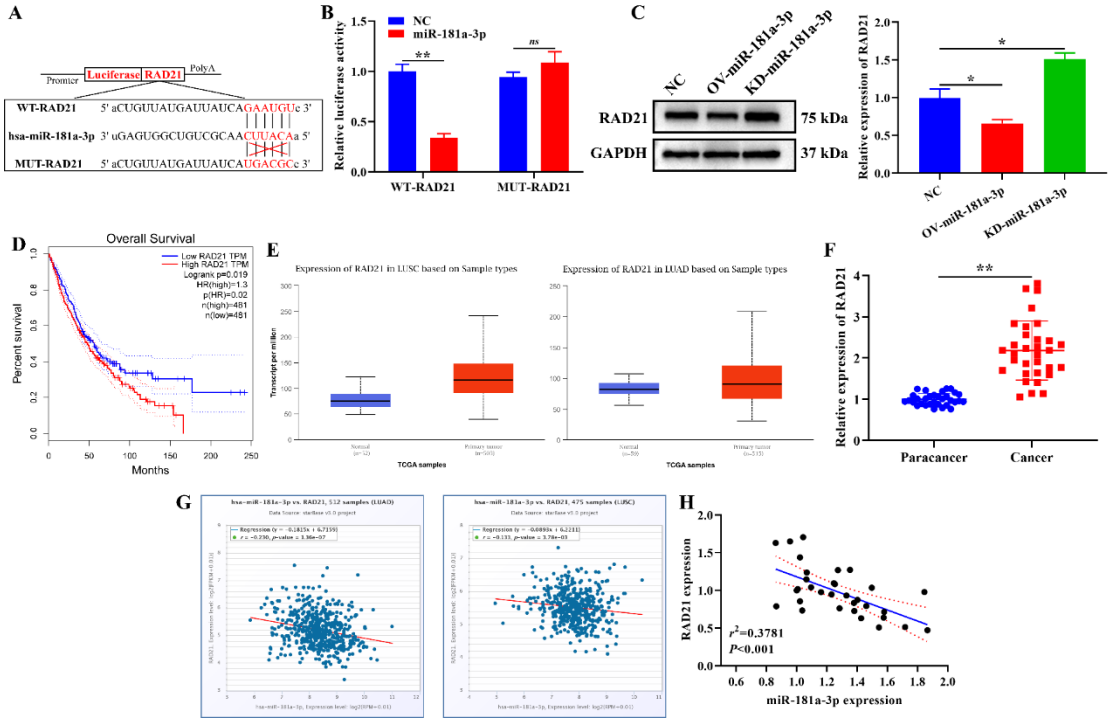

Figure 3 RAD21 is the target gene of miR-181a-3p. (a) The binding region of RAD21 and miR-181a-3p predicted by the Starbase database. (b) Dual-luciferase reporter gene verified the targeting relationship between RAD21 and miR-181a-3p. (c) Western blotting detected expression level of RAD21 in A549 cells after exogenously regulated miR-181a-3p expression. (d) Sarbase database was used to predict the correlation between RAD21 expression and survival rate of NSCLC patients. (e) The UALCAN database analyzes RAD21 expression in LUSC and LUAD clinical samples. (f) RT-qPCR was used to detect RAD21 expression in clinical samples of NSCLC. (g) Starbase database analyzed the correlation between miR-181a-3p and RAD21 expression in clinical samples of NSCLC. (h) Graphpad prism 8.0 analyzes the correlation between miR-181a-3p and RAD21 expression in the NSCLC samples we collected. In all cases, Values are mean ± SD (n=3 for each group; *P<0.05, **P<0.01, ***P <0.001).

3.4 miR-181a-3p inhibits the malignant biological behavior of A549 cells through RAD21

We first transfected A549 cells with sh-RAD21 and sh-RAD21+miR-181a-3p inhibitor (co-transfected with sh-RAD21 and miR-181a-3p inhibitor). Western blotting results showed that after sh-RAD21 was transfected, the expression level of RAD21 in A549 cells was significantly down-regulated (Fig. 4A). However, after transfection of sh-RAD21 and miR-181a-3p inhibitor at the same time, the expression level of RAD21 in A549 cells was not significantly different from that in the NC group (Fig. 4A). CCK-8 results showed that knocking down RAD21 significantly inhibited the proliferation of A549 cells, while knocking down miR-181a-3p at the same time could rescued the proliferation level of A549 cells (Fig. 4B). As expected, knocking down RAD21 significantly promoted the apoptosis of A549 cells, and further knocking down the expression level of miR-181a-3p could restore the apoptotic level of A549 cells (Fig. 4C). Transwell results showed that compared with the NC group, the invasion number of A549 cells in the KD-RAD21 group was significantly increased, while the Co-KD did not change significantly (Fig. 4D). The results of the Wound-Healing assay were consistent with the results of Transwell (Fig. 4E). It suggested that miR-181a-3p inhibits the proliferation, invasion, and migration of A549 cells and induces apoptosis by inhibiting the expression level of RAD21.

Discussion

In the past two decades, important progress has been made in the treatment of NSCLC, which has improved our understanding of disease biology and tumor progression mechanisms, and advanced early detection and multimodal therapy. However, the overall cure rate and survival rate of NSCLC are still very low, especially in metastatic disease. In our research, we found for the first time that miR-181a-3p/RAD21 can be used as a promising diagnosis and treatment for NSCLC. The abnormally low expression of miR-181a-3p in NSCLC tissues is related to the poor survival rate of NSCLC patients. Further research found that RAD21 is the downstream target of miR-181a-3p in NSCLC, and the expression of the two is negatively correlated in NSCLC. In addition, we also found that miR-181a-3p inhibits the proliferation, invasion and migration of NSCLC cells and induces their apoptosis by inhibiting the expression level of RAD21.

miRNA inhibits gene expression by binding to the complementary sequence in the 3'untranslated region (3' UTR) of the target mRNA, thereby making it targeted for degradation and preventing its translation[17]. At present, more than 1,000 individual miRNA genes have been identified. A single miRNA can target hundreds or thousands of different mRNAs, and a single mRNA can be inhibited by a variety of different miRNAs[18]. Therefore, the biogenesis pathway of miRNA plays an important role in gene expression. In the past decade, miRNAs have been shown to play a vital role in cancer. The most typical is that let-7 family miRNAs target important oncogenes, such as MYC, RAS family members (HRAS, KRAS and NRAS) and high mobility HMGA2 to inhibit tumor growth[19, 20]. Therefore, cancer-related changes in

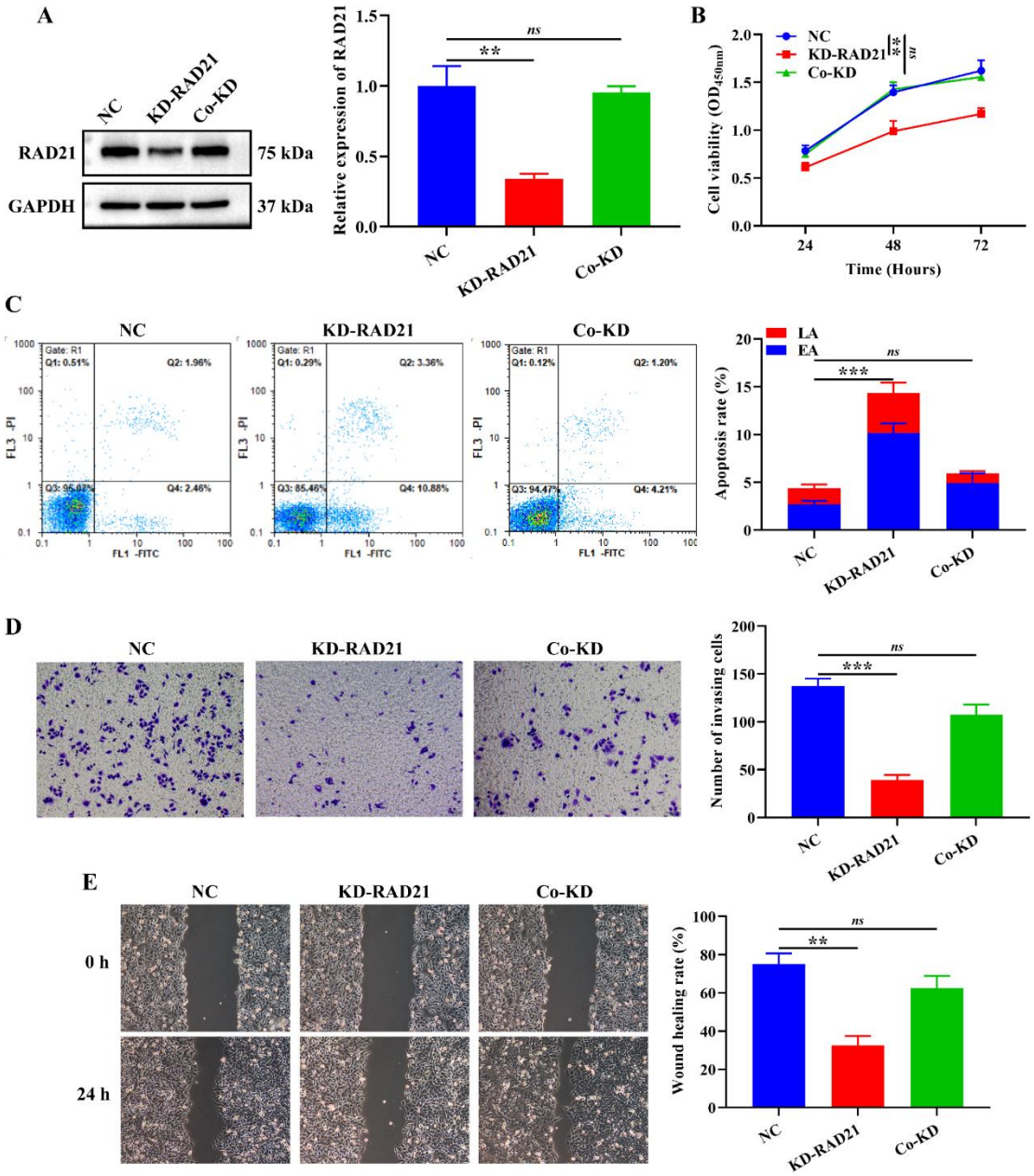

Figure 4 miR-181a-3p inhibits the malignant biological behavior of A549 cells through RAD21. (a) Western blotting was performed to detect RAD21 in A549 cells. (b) CCK-8 kit, (c) Annexin V-FITC/PI kit (d) Transwell assay and (e) Wound-Healing assay was performed to display proliferation, apoptosis, invasion and migration, respectively. In all cases, Values are mean ± SD (n=3 for each group; $^{**}P<0.01$, $^{***}P<0.001$, *ns* means no significant difference).

miRNA expression patterns are becoming promising diagnostic markers, usually associated with disease progression and patient survival[21]. miR-181a-3p is a miRNA processed from the 3'end arm of the hsa-miR-181 precursor. At present, the role of miR-181a in malignant tumors has been thoroughly studied. By down-regulating GRP78, miR-181a leads to a lower cell proliferation rate and oxaliplatin resistance in cervical cancer[22]. miR-181a inhibits NSCLC cell proliferation, colony formation and cell invasion by down-regulating CDK1[23]. miR-181a-5p inhibits cell proliferation and migration by targeting

Kras in non-small cell lung cancer A549 cells[24]. In contrast, Hu et al. showed that miR-181a reduces the radiosensitivity of NSCLC cells by inhibiting PTEN[25]. Similarly, Ping et al. showed that miR-181a promotes the resistance of non-small cell lung cancer cells to gefitinib by targeting GAS7[26]. In this study, we confirmed that miR-181a-3p acts as a tumor suppressor in NSCLC and can be used as a prognostic indicator for patients. In addition, we also found that the regulation of NSCLC by miR-181a-3p is achieved through RAD21.

RAD21 (RAD21 Cohesin Complex Component) is an essential gene in organisms, encoding DNA double-strand break repair protein, which is evolutionarily conserved in all eukaryotes. RAD21 protein is a structural component of a highly conserved adhesive protein complex composed of RAD21, SMC1a, SMC3 and SCC3 proteins, and is involved in the adhesion of sister chromatids[27]. This function is essential for correct chromosome separation, DNA repair after replication, and prevention of inappropriate recombination between repeated regions. During the interphase, RAD21 also controls gene expression by binding to many sites in the genome[28]. In addition to playing a role in the normal cell cycle and repair of DNA double-strand breaks, RAD21 is also involved in the apoptosis pathway[29]. In recent years, the accumulated literature shows that RAD21 plays an important role in the occurrence and development of a variety of cancers. The high expression of RAD21 indicates that the survival rate of NSCLC patients is poor, and RAD21 may become a new prognostic biomarker and therapeutic target for NSCLC patients[30]. RAD21 overexpression is a prognostic and predictive marker, which can aggravate the poor prognosis of KRAS mutant colorectal cancer[31]. Enhanced RAD21 expression confers poor prognosis in BRCA2 and BRCAX, but not BRCA1 familial breast cancers[32]。It is suggested that abnormally high expression of RAD21 can be used as a predictive and therapeutic target for a variety of malignant tumors. RAD21 inhibits the transcription of the tumor suppressor gene MIR4697HG and leads to the occurrence of glioma[33]. miR-320b inhibits the expression of RAD21 and enhances the radiosensitivity of liver cancer to ionizing radiation therapy through DNA damage repair signal transduction[34]. Inhibition of RAD21 induces the senescence of MDA-MB-231 human breast cancer cells by down-regulating c-Myc to activate the RB1 pathway[35]. However, the specific mechanism of RAD21 in NSCLC remains to be elucidated. In this study, we found that RAD21 is abnormally highly expressed in NSCLC, and this result is consistent with Zhu et al. [30]. In addition, we have found that the expression of RAD21 and miR-181a-3p is negatively correlated in NSCLC, and miR-181a-3p can target RAD21 to negatively regulate the expression level of RAD21, thereby inhibiting the malignant biological behavior of NSCLC. In conclusion, this study suggests that miR-181a-3p/RAD21 can be used as a therapeutic target for NSCLC.

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