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Original Article

Downregulation of microRNA-519 enhances development of lung cancer by mediating the E2F2/PI3K/AKT axis

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Abstract: MicroRNA-519 (miR-519) acts as an inhibitor in different kinds of tumors. The current study was set to probe the function of miR-519 in lung cancer and to explore the potential molecular mechanism. The expression difference of miRNAs between lung cancer and paracancerous tissues was analyzed by microarray. miR-519 expression was significantly diminished in lung cancer tissues and cells. After that, EdU staining, CCK-8 assay, Transwell assay, Hoechst 33258 staining and PI/Annexin-V staining revealed that overexpression of miR-519 in lung cancer cells inhibited their viability and promoted apoptosis. TragetScan and miRSearch were employed to predict the target mRNAs of miR-519, which were verified by a luciferase activity assay. miR-519 bound to the 3'untranslated region of E2F transcription factor 2 (E2F2) mRNA. Finally, the extent of PI3K/AKT signaling pathway phosphorylation was examined, which illustrated that upregulation of miR-519 repressed the phosphorylation of the PI3K/AKT pathway in SPC-A-1 and 95C cells. miR-519 reduces PI3K/AKT pathway activities by suppressing the transcription activity of E2F2, thereby potentially inhibiting the occurrence of lung cancer.

Keywords: Lung cancer, microRNA-519, E2F2, PI3K/AKT signaling pathway, proliferation, migration

Introduction

In 2012, around 1.8 million were diagnosed as lung cancer, and the mortality rates of lung cancer are largely similar to incidence rates as a consequence of low survival even in more developed countries [1]. Lung cancer survivors that smoke currently or had previous smoking history are exposed to higher risk of other smoking-associated cancers, particularly in the head, neck and urinary tract in addition to secondary lung cancers [2]. This lack of improvement in survivability may be caused by the high histological heterogeneity, the difficulties for diagnosis at early stage and the inaccessibility of rapid therapeutic effects [3]. Lung cancer can demonstrate as a persistent cough, chest pain, weight loss, difficulty in breathing, chronic obstructive pulmonary disease or pulmonary fibrosis [4].

MicroRNAs (miRNAs) are small non-coding RNAs which function principally by fine-tuning the expression patterns of genes post-transcriptionally, and various miRNAs are differentially expressed in tumors and play oncogene and/or tumor suppressor roles across different tumor types [5]. For instance, miR-192-5p was found to suppress bone metastasis of lung cancer by targeting Tripartite motif 44 [6]. Moreover, miR-519 was observed to robustly repress cell proliferation and tumor growth as well as encourage senescence [7]. Interestingly, E2F transcription factor 2 (E2F2) was reduced by miR-99a through directly interacting with their 3'untranslated region (3'UTR) in lung cancer [8], indicating a potential correlation between miRNAs and E2F2 in lung cancer cells. The prediction of TragetScan and miRSearch databases revealed binding sites between E2F2 and miR-519, which was further verified by a dual luciferase assay. E2F2 expression was promoted in breast cancer tissues versus normal breast tissues, and the overexpression closely correlated to advanced tumor stage [9]. Similarly, the expression of E2F2 was elevated

in lung adenocarcinoma and squamous cell lung carcinoma tissues relative to lung tissues, which was linked to advanced tumor stage and low relapse-free survival in patients with lung cancer [10]. In addition, E2F2 has the potency to regulate the induction of the phosphatidylinositol 3-kinase (PI3K)/AKT/NF-κB pathway in rheumatoid arthritis synovial fibroblasts [11]. PI3K/AKT pathway remains a renowned driver of lung cancer development for its stimulating role in metastasis and drug resistance, leaving it responsible for the high aggressiveness of lung cancer [12]. In this study, we hypothesized that miR-519 modulated the proliferation, invasion, and migration of lung cancer cells by binding to E2F2 and activating the PI3K/AKT signaling pathway. In order to authenticate the supposition, miR-519 expression in lung cancer tissues through microarray analysis as well as in cells was evaluated. By performing a series of *in vitro* experiments, we investigated the relevance of miR-519 in lung cancer.

Materials and methods

Clinical samples

This study was conducted under the approval of the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University, and informed consent was also attained from each patient recruited. Sixty-seven human lung cancer and their paired adjacent paracancerous tissues were harvested from patients undergone surgery at the First Affiliated Hospital of Xinjiang Medical University between September 2012 and June 2013 with compete clinical data. The adjacent paracancerous tissues were harvested 5 cm from the tumor margin and confirmed by pathologists. Among the enrolled population, 37 were males and 30 were females, ranging from 49 to 79 years with an average age of 64.9 ± 7.3 years. The patients who received chemotherapy and radiotherapy and combined with other malignancies or chronic systemic diseases were excluded.

Microarray preparation

The cancer and adjacent tissues from 7 lung cancer patients were obtained, and the total RNA was extracted. The total RNA (0.5 μg) was applied to synthesize cDNA with a GeneChip 3'Invitro Transcription (IVT) Express Kit (902789, Thermo Fisher). The cDNA was then fragmented and hybridized to the human LncRNA expression array V3.0 (AS-LNC-H-V4.0, Arraystar Inc., Rockville, MD, USA). The images were produced from a confocal microarray scanner GeneChip.TM. Scanner 3000 7G system (000213, Thermo Fisher Scientific).

RNA extraction and quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

As per the protocol, the Total RNA was isolated using TRIzol (Invitrogen) from the samples. The primers for miR-519, U6, E2F2 and GAPDH were from Applied Biosystems (ABI, Foster City, CA, USA). qPCR was completed on an ABI7500 PCR detection system.

Cell culture

Lung cancer H1299, 95C, A549, SPC-A-1, L9981 cells and human bronchial epithelial cell (16HBEC) were from Cell Bank of Shanghai Institute of Cells, Chinese Academy of Science (Shanghai, China). Then the cells were seeded in Petri dishes at the density of 1×10^5 cells/cm^2 and grown in Roswell Park Memorial Institute-1640 medium, CM7-1 medium, F12K medium and LHC-9 medium containing 10% fetal bovine serum (all purchased from Gibco, Grand Island, NY, USA). After being cultured at a temperature of 37°C with 5% CO_2, the cells grew to 80%-90% confluence after a 48-h culture, trypsinized (Gibco) and passaged.

Cell viability assays

The viability of cells was determined by 5-ethynyl-2'-deoxyuridine (EdU) staining and a cell counting-kit 8 (CCK-8). The experimental steps of EdU staining were carried out according to the previous literature [13]. The experimental steps of detecting cell activity by CCK-8 kit were strictly carried out in accordance with the instructions.

Cell apoptosis detection

The apoptosis level of lung cancer cells was detected by Hoechst 33258 staining and flow cytometry after propidium iodide (PI)/Annexin-V labeling as previously reported [14].

Transwell assays for cell migration and invasion

The cell invasion and migration of was detected by Transwell assays as per previous literature [15].

Dual luciferase assay

The binding sequence of miR-519 to E2F2 3'UTR was predicted by the on-line prediction software StarBase, and E2F2-wild-type (WT) and 3'UTR binding sequence mutant (MT) were produced by Sangon (Shanghai, China) and inserted into pMIR-REPORT™ (Thermo Fisher Scientific) [16]. WT plasmid or MT plasmid was transfected into 293T cells with miR-519 mimic or miRNA negative control (NC) by a Lipofectamine 3000 transfection kit (Invitrogen) for 24 h. After being lysed, the Dual-Luciferase Reporter Assay System (Promega) was used to test the luciferase activity [17].

Western blot analysis

Western blot assays were carried out in strict accordance with the previous method [18]. In detail, the primary antibodies against $pPI3K^{Y607}$ (1:5000, ab182654), $pAKT^{T308}$ (1:5000, ab38449) and E2F2 (1:5000, ab138515) and the horseradish peroxidase-labelled secondary goat anti-rabbit antibody (1:15000, ab7090) were obtained from Abcam (Cambridge, UK).

Statistics

All sample data were analyzed by SPSS software (21.0, SPSS Inc., Chicago, IL, USA). Following the determination of the normality of the distribution by Kolmogorov-Smirnov test, data were represented as means ± standard deviation (SD). The *t*-test and one-way or two-way analysis of variance (ANOVA) were applied for comparison, followed by Tukey's post hoc test. Enumeration dates were examined using Fisher's exact test. $P < 0.05$ was considered significant.

Results

Expression profile of miR-519 in lung cancer tissues and cells

We first selected 30 differentially expressed miRNAs from 7 lung cancer patients by microarray analysis to plot the heat map (**Figure 1A**). We noted that miR-519 was markedly reduced in tumor tissues relative to the adjacent tissues. Moreover, it has been previously reported that miR-519 inhibited malignant biological behavior in nasopharyngeal carcinoma cells [19]. Therefore, we measured the miR-519 expression in cancer and paracancerous tissues of 67 lung cancer patients by RT-qPCR. miR-519 was monitored to be significantly reduced in lung cancer tissues (**Figure 1B**). Subsequently, we examined the miR-519 expression in lung cancer cells and immortalized human lung epithelial cells, and miR-519 was found to be significantly diminished in lung cancer cells (**Figure 1C**). In order to verify the functional relevance of miR-519 on malignant biological behavior of lung cancer cells, we overexpressed miR-519 in 95C cells and SPC-A-1 cells and carried out RT-qPCR to verify the success of delivery (**Figure 1D**).

Overexpression of miR-519 inhibits cell malignant biological behavior in lung cancer

The lung cancer cell viability and proliferation were investigated by CCK-8 assay and EdU staining, which implied that overexpression of miR-519 exerted an anti-proliferative role in lung cancer cells (**Figure 2A**, **2B**). Then, PI/Annexin V staining and Hoechst 33258 staining were used to detect apoptotic cells (**Figure 2C**, **2D**). We found that miR-519 promoted apoptosis of 95C cells and SPC-A-1 cells. Furthermore, Transwell assays discovered that miR-519 mimic markedly repressed the invaded and migrated 95C and SPC-A-1 cell number (**Figure 2E**, **2F**). Therefore, miR-519 may act as a suppressor by lowering lung cancer cell proliferation, migration, and invasion as well as facilitating apoptosis.

E2F2 is a putative target of miR-519 in lung cancer cells

In order to determine the downstream mechanism of miR-519, we predicted the possible target mRNAs of miR-519 by TargetScan and miRSearch. We found that miR-519 had the potency to target E2F2, and we proved that miR-519 shared a binding site with the E2F2 3'UTR (**Figure 3A**). As a family of transcription factors, the E2F family is involved in the initiation and development of several cancers [9]. High expression of E2F2 in patients with lung cancer was analyzed by GEPIA in The Cancer Genome Atlas (TCGA) database (**Figure 3B**). Then, the expression of E2F2 in 95C cells and SPC-A-1 cells was assessed by RT-qPCR and western blot. miR-519 mimic was found to repress the E2F2 expression in lung cancer cells (**Figure 3C**, **3D**).

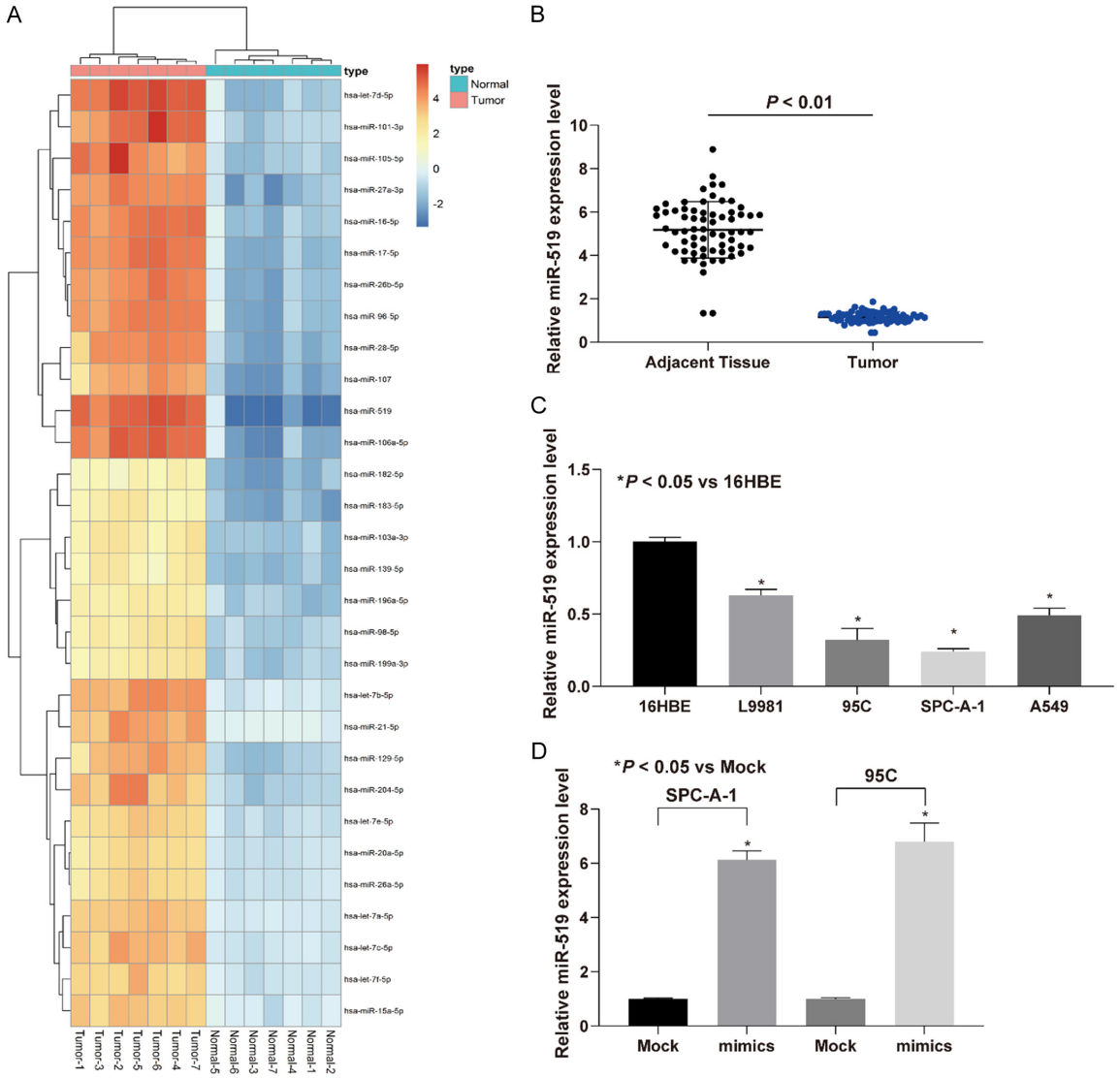


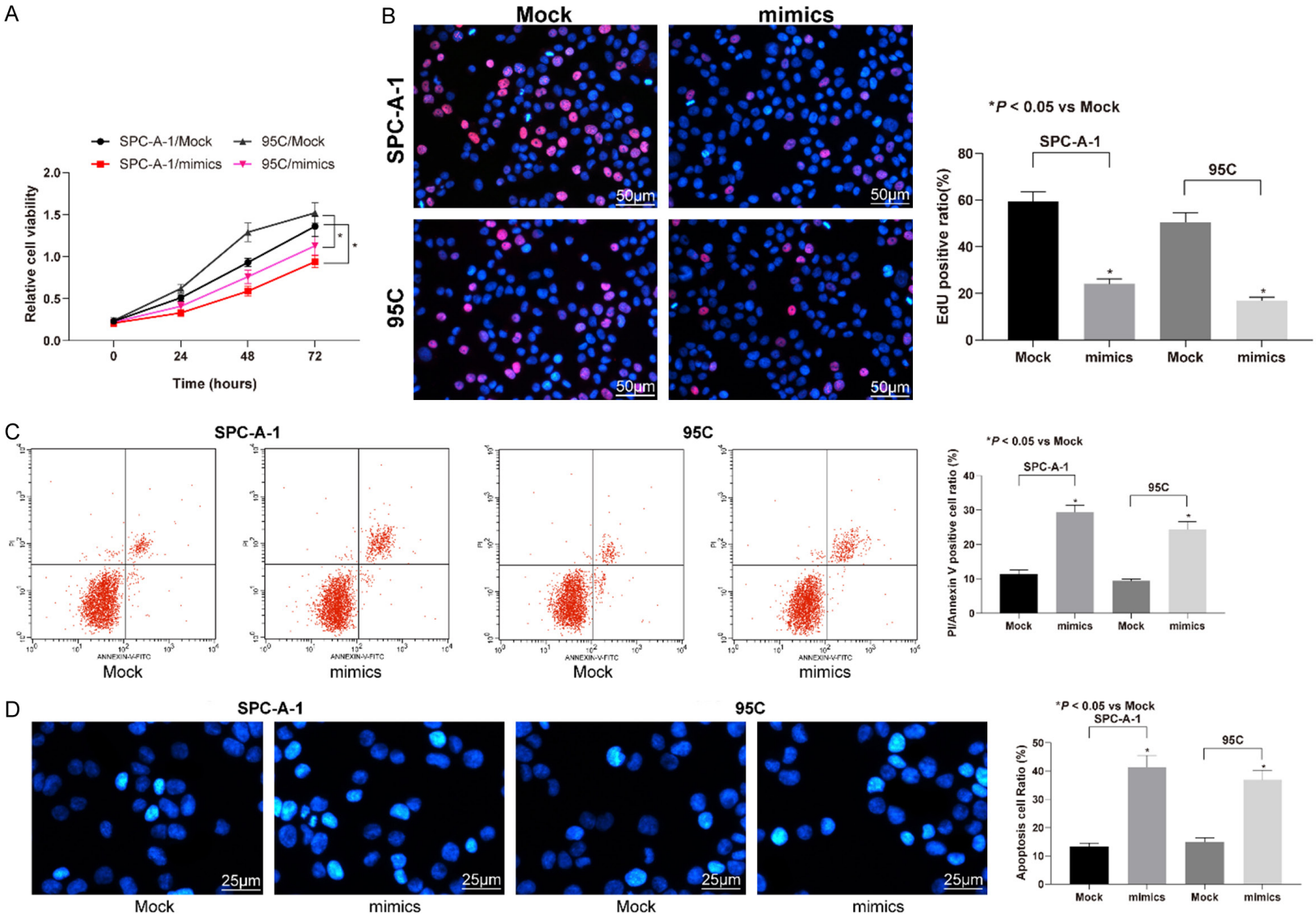
Figure 1. miR-519 is downregulated in lung cancer tissues. A. Dysregulated miRNAs determined using miRCURY LNA™ Universal RT microRNA PCR Human panel. B. miR-519 expression of tumor tissue and adjacent tissue in 67 lung cancer patients measured by RT-qPCR. C. miR-519 expression of lung cancer cell lines and normal lung epithelial cells measured by RT-qPCR. D. miR-519 expression in SPC-A-1 and 95C cells transfected with miR-519 mimic and mimic control. Data are expressed as mean ± SD. One-way ANOVA and Tukey's multiple comparisons test were used to determine statistical significance. *P < 0.05 compared with Mock (mimic control).

E2F2 activates the PI3K/AKT pathway

Finally, the extents of PI3K and AKT phosphorylation in 95C and SPC-A-1 cells were detected, and were decreased tremendously after overexpression of miR-519. After further overexpression of E2F2, the phosphorylation was partially restored (**Figure 4**). These data strongly suggest that the miR-519/E2F2/PI3K/AKT axis was involved in the modulation of cell proliferation, migration, and invasion in lung cancer.

Discussion

In lung cancer, chemotherapy is the mainstream therapy at present, and progress in improving survival has been halted, while novel drugs including gefitinib and erlotinib will fail sooner or later due to the development of chemoresistance. New treatment strategies are urgently needed consequently to enhance the prognosis of lung cancer patients [20]. In this report, miR-519 impaired lung cancer cell pro-


A
SPC-A-1/Mock
95C/Mock
SPC-A-1/mimics
95C/mimics
Relative cell viability
Time (hours)
B
Mock
mimics
SPC-A-1
95C
50μm
*P < 0.05 vs Mock
EdU positive ratio(%)
C
SPC-A-1
95C
ANNEXIN-V-FITC
PI
Mock
mimics
PI/Annexin V positive cell ratio (%)
D
25μm
Apoptosis cell Ratio (%)

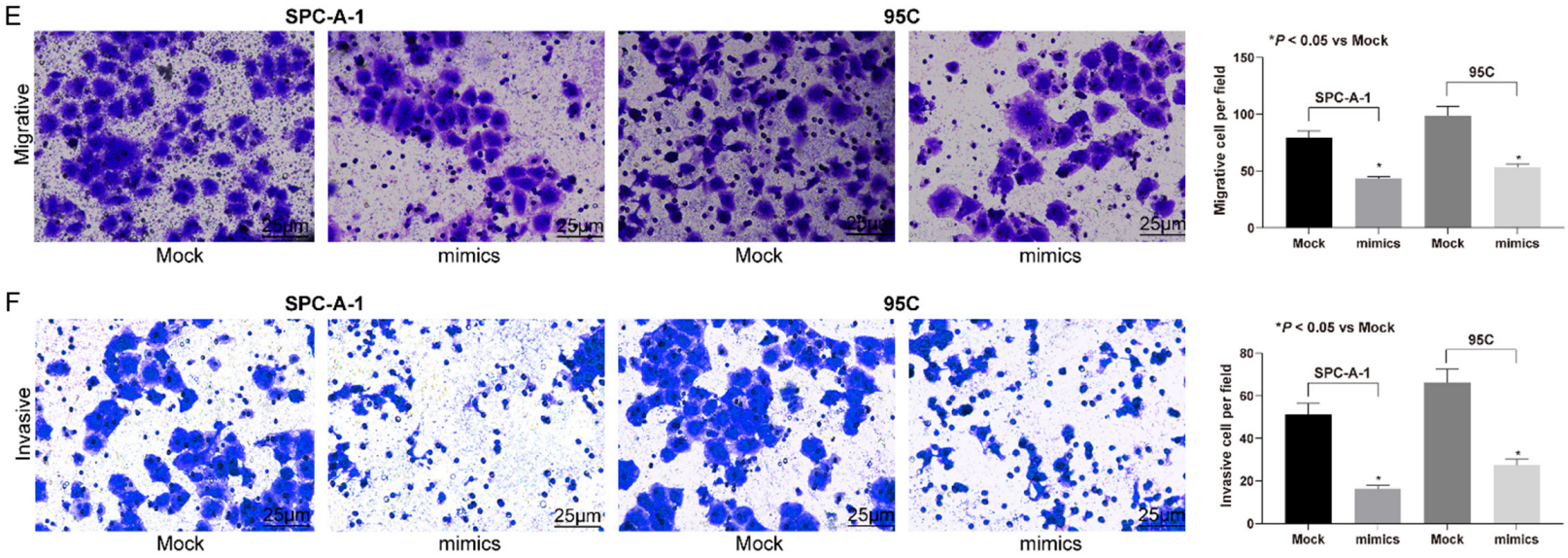


Figure 2. miR-519 overexpression suppresses the proliferation, migration, and invasion, while inducing apoptosis of lung cancer cells. A. Cell viability determined by CCK-8 assay. B. Cell viability determined by EdU staining. C. Apoptosis index was determined by PI/Annexin-V staining and then determined by flow cytometry. D. Hoechst 33258 staining of cells and apoptosis rate. E. Cell migration determined by Transwell assays. F. Cell invasion determined by Transwell assays. Data are expressed as mean ± SD. One-way ANOVA and Tukey's multiple comparisons test were used to determine statistical significance. *$P < 0.05$ compared with Mock (mimic control).

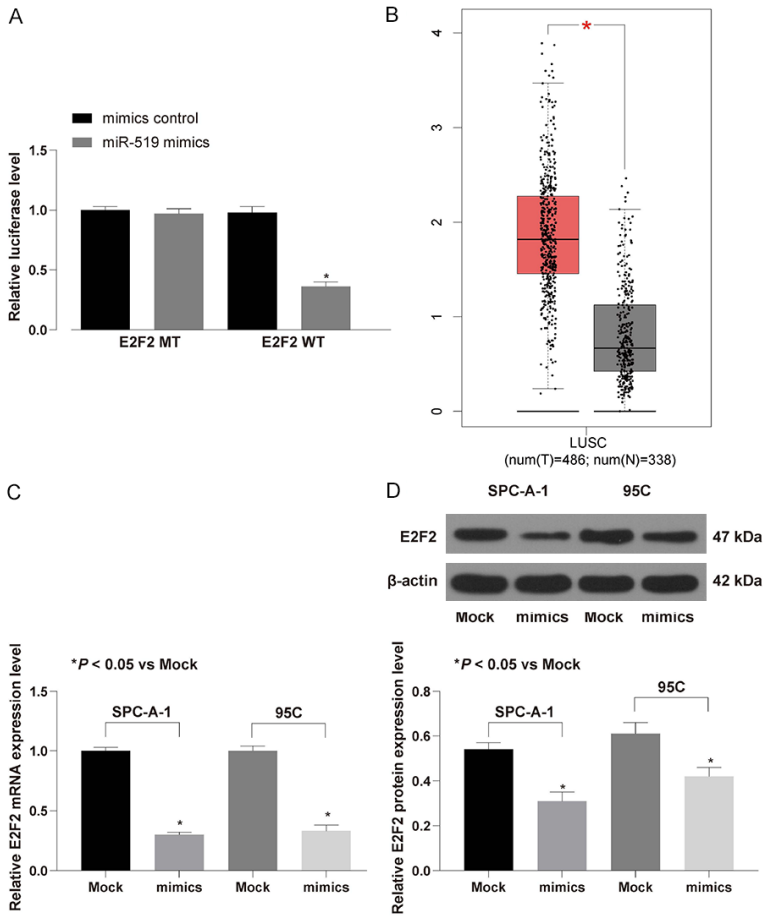


Figure 3. E2F2 is a direct functional target of miR-519 in lung cancer cells. A. The combination between miR-519 and E2F2 3'UTR sequence is verified by a dual luciferase assay. B. E2F2 expression in the TCGA dataset by GEPIA. C. E2F2 mRNA expression determined by RT-qPCR. D. E2F2 protein expression determined by western blot analysis. Data are expressed as mean ± SD. One-way ANOVA and Tukey's multiple comparisons test were used to determine statistical significance. *P < 0.05 compared with Mock (mimic control).

liferation, migration, and invasion, while it facilitated apoptosis. Mechanism analysis proposed E2F2 as a target gene of miR-519, and that miR-519 directly downregulated its expression to prevent the PI3K/AKT pathway from induction. In order to confirm miR-519 blocked the PI3K/AKT pathway in an E2F2-dependent manner, we assessed the effect of miR-519 and E2F2 on the extents of PI3K and AKT phosphorylation by western blot assays. It was suggested that miR-519 inhibited the phosphorylation of PI3K and AKT, while E2F2 overexpression rescued the repressed PI3K/AKT signaling pathway.

In this study, miR-519 was expressed at a low level in lung cancer tissues relative to the corresponding paracancerous tissues revealed by microarray analysis and in lung cancer cells. The downregulation of miR-519 was proven in 90 patients with esophageal squamous cell carcinoma and linked to the radiosensitivity of cancer cells [21]. In addition, Abdelmohsen *et al.* examined the miR-519 expression in cancer tissues and adjacent healthy tissues extracted from organs such as ovary, lung, and kidney and revealed markedly reduced miR-519 expression in the three sample collections [22]. miR-519 restoration repressed cell viability, migratory, and invasive capabilities, while accelerating apoptosis in lung cancer. Similarly, miR-519 was underexpressed in nasopharyngeal carcinoma cells; its upregulated inhibited cell proliferation and arrested the cell cycle at G0/G1 stage [19]. In lung adenocarcinoma cell line, miR-519b-3p inhibitor was monitored to recover the cell proliferation, migration, and invasion that was hindered by LINC01419 knockdown [23]).

Identification of miR-519 targets is critical for the comprehension of its underlying mechanism in the carcinogenesis of lung cancer. Bioinformatics analysis confirmed E2F2 as a target of miR-519 in lung cancer cells. Moreover, promoted E2F2 was identified in non-small cell lung cancer (NSCLC) tissues and cells, which was conversely related with the miR-936 expression in the same condition [24]. E2F2 has been established as an oncogene in osteosarcoma [25], ovarian cancer [26], and glioma [27]. Also, E2F2 was upregulated and accountable for the circPVT1-mediated carcinogenesis of NSCLC [28]. Furthermore, E2F2 was found to be indispensable for SIRT1 to suppress apoptosis in melanoma cells [29]. The Akt/GSK3β signaling pathway contributed to the oncogenic role of

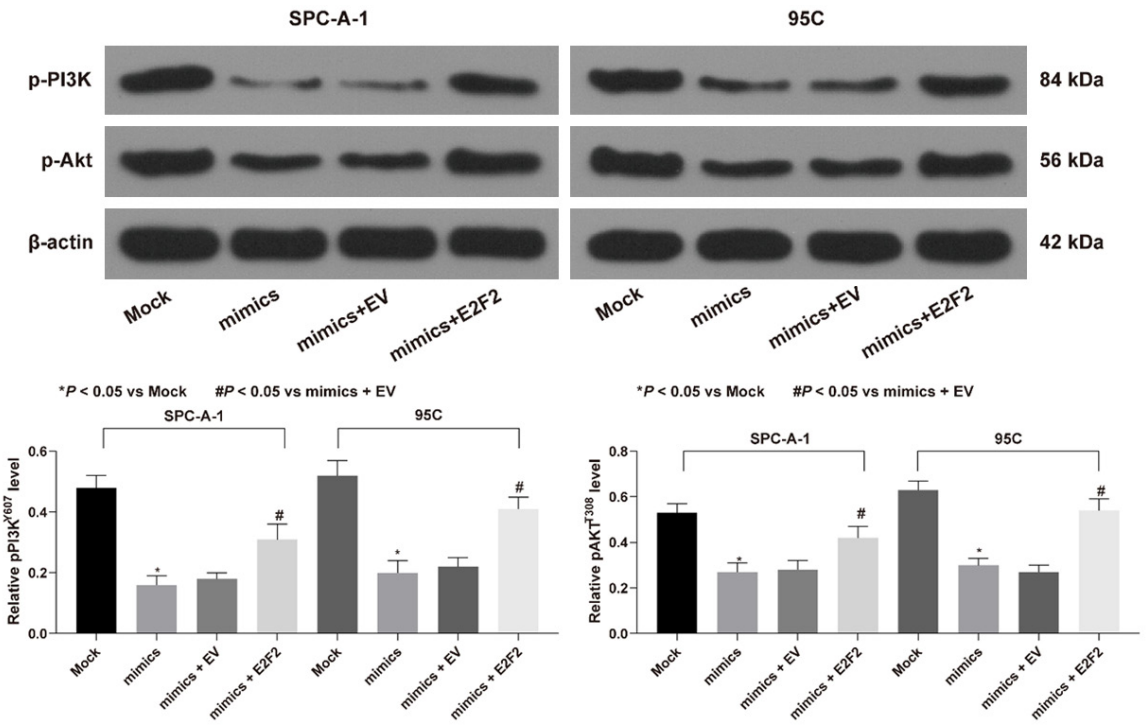


Figure 4. The extents of PI3K and AKT phosphorylation are diminished by miR-519, while they are potentiated by E2F2. Data are expressed as mean ± SD. One-way ANOVA and Tukey's multiple comparisons test were used to determine statistical significance. *$P < 0.05$ compared with Mock (mimic control).

Orai1 in colorectal cancer cells, and Orai1 was a target gene of miR-519 [30]. More specifically, enforced miR-519 diminished the extents of AKT and mTOR phosphorylation, thus blocking the PI3K/AKT/mTOR signaling after irradiation in esophageal squamous cell carcinoma [21]. The correlation between E2F2 and the PI3K/AKT signaling pathway has been rarely studied so far. Our western blot assays revealed that miR-519 lowered the levels of AKT and mTOR phosphorylation, while restoration of E2F2 partially recovered the phosphorylation, thereby activating the PI3K/AKT signaling pathway in lung cancer. The PI3K/AKT pathway was activated in many patients with lung cancer and epitomizes a target for therapy [31]. AKT, a serine/threonine kinase, functions vitally in numerous cellular processes, such as proliferation and apoptosis, and drugs targeting PI3K/AKT pathway have the potency to repress cell proliferation and to induce cell death in cancer cells [32]. Nevertheless, due to the funding and time limits, the specific role of the PI3K/AKT signaling in lung cancer cells was not investigated in the current study, which will be the focus of our further experiments.

Conclusions

We demonstrated a significant role of miR-519 in lung cancer cells. In detail, miR-519 curtailed the proliferation, migration, and invasion of lung cancer cells through regulating the E2F2-depedent PI3K/AKT signaling pathway. The discoveries proposed that the miR-519/E2F2/PI3K/AKT network might function as a possible target for a lung cancer therapeutic strategy.

Acknowledgements

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Disclosure of conflict of interest

None.

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