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MiRNA-300 suppresses proliferation, migration and invasion of non-small cell lung cancer via targeting ETS1

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Abstract. – OBJECTIVE: To elucidate the biological function of microRNA-300 (miRNA-300) in influencing the progression of non-small cell lung cancer (NSCLC).

PATIENTS AND METHODS: The relative level of miRNA-300 in NSCLC tissues and cells was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The influences of miRNA-300 on the proliferative, migratory, and invasive potentials of A549 cells were assessed. The binding relationship between miRNA-300 and ETS1 was verified by the Dual-Luciferase Reporter Gene Assay. Finally, the regulatory effect of the interactive miRNA-300 and ETS1 on the progression of NSCLC was identified.

RESULTS: MiRNA-300 was downregulated in NSCLC tissues and cells. The overexpression of miRNA-300 suppressed A549 cells to proliferate, migrate, and invade. MiRNA-300 could bind to ETS1 3'UTR and suppress its level in A549 cells. Notably, the knockdown of miRNA-300 could reverse the regulatory role of ETS1 in the cellular behaviors of NSCLC.

CONCLUSIONS: MiRNA-300 suppresses NSCLC to proliferate, migrate, and invade by targeting ETS1.

Key Words:
MiRNA-300, ETS1, NSCLC.

Introduction

Lung carcinoma is a complex disease involving multiple genes and pathways. According to cell morphology and biological characteristics, lung carcinoma is pathologically classified into small cell lung cancer and non-small cell lung cancer (NSCLC). NSCLC is the major subtype of lung cancer, accounting for 85% of all lung carcinoma cases[1]. NSCLC is a solid tumor with high malignancy, rapid progression, and poor prognosis. Although the target therapy, the immune therapy, and other therapies for NSCLC treatment have been greatly advanced, the 5-year survival of NSCLC is lower than 15%[2,3]. Hence, it is urgent to uncover the pathogenesis of NSCLC, thus developing effective therapeutic targets to improve the clinical outcomes.

MicroRNAs (miRNAs) are non-coding, single-strand RNAs with 20-24 nt long that are extensively distributed in the animals and plants[4]. They are significant for the gene regulatory network. MiRNAs are capable of regulating gene expressions by binding to specific mRNAs to further suppress their translation or directly induce degradation[5]. Currently, miRNAs have been well investigated for their potentials in predicting the progression, metastasis, and prognosis of tumors, including NSCLC[6,7]. Li et al[8] demonstrated that miR-26b inhibited the metastasis of NSCLC by targeting and downregulating MIEN1 through the NF-κB/MMP-9/VEGF axis. Want et al[9] reported the role of miR-124 in inhibiting the proliferative ability and accelerating the ionizing radiation-induced apoptosis of NSCLC by downregulating STAT3. Therefore, it is of clinical significance to identify NSCLC-related miRNAs, thus developing novel therapeutic approaches for NSCLC.

MiRNA-300 is dysregulated in many types of tumors. In osteosarcoma[10,11] and gastric cancer[12], miRNA-300 is remarkably upregulated as an oncogene. On the contrary, miRNA-300 is lowly expressed in head and neck squamous cell carcinoma and hepatocellular cancer. By targeting ROS1, miRNA-300 inhibits cell proliferation and the metastasis of head and neck squamous cell car-

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cinoma[13]. MiRNA-300 suppresses hepatocellular cancer to invade and migrate by inhibiting EMT through the FAK/PI3K/AKT pathway[14]. He et al[15] showed that miRNA-300 regulates the insulin sensitivity by targeting p53 and apaf1 in NSCLC.

In this paper, the expression pattern and biological functions of miRNA-300 in NSCLC were mainly explored. By predicting and verifying the target gene of miRNA-300, the regulatory mechanism of miRNA-300 in NSCLC was further explored.

Patients and Methods

Sample Collection

NSCLC tissues and adjacent normal tissues were surgically resected from 20 NSCLC patients treated in the Zhongnan Hospital of Wuhan University from April 2016 to December 2018. They did not receive preoperative anti-tumor therapy and had no history of other malignancies. The clinical data of the enrolled NSCLC patients were collected. All subjects volunteered to participate in the study and signed a written informed consent. This study was approved by the Ethics Committee of Zhongnan Hospital of Wuhan University.

Cell Culture and Transfection

The lung carcinoma cell lines (A549, PC9, and H1299) and human bronchial epithelial cell line (HBE) were provided by Cell Bank (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA), 100 Ul/mL penicillin and 0.1 mg/mL streptomycin, at 37°C in a 5% CO_2 incubator.

The cells were cultured until they reached 60% of confluence and were subjected to transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 6 hours later, the complete medium was replaced. The transfected cells for 24-48 h were harvested for *in vitro* experiments.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The cells were lysed for harvesting the total RNA and subjected to reverse transcription for extracting the complementary deoxyribose nucleic acid (cDNA). The cDNA was used for conducting qRT-PCR using the SYBR® PrimeScript™ RT-PCR (TaKaRa, Otsu, Shiga, China) on the ABI 7300 PCR system (Applied Biosystems, Foster City, CA, USA). QRT-PCR was conducted for 5 min at 95°C, followed by 40 cycles for 30 s at 95°C, 30 s at 68°C, and 60 s at 72°C. The relative levels were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences were as follows: MiRNA-300: F: 5′-CGGAATTCGGCTATGGAAGGAATAGATGTGTG-3′, R: 5′-CGGGATCCGGCCAAGGTAGGCCCTTTTTG-3′; ETS1: F: 5'-AGCCGACTCTCACCATCATC-3', R: 5'-CAAGGCTTGGGACATCATTT-3'.

Cell Counting Kit-8 (CCK-8)

The cells were seeded in the 96-well plate with 1×10^4 cells per well and cultured overnight. Absorbance (A) at 450 nm was recorded at the appointed time points using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curves.

Transwell

The cell density was adjusted to 1×10^6 cells/mL. 200 μL of suspension was applied in the upper side, while 600 μL of medium containing 5% FBS was applied in the bottom of the transwell chamber (8 μm in pore size; Corning, Corning, NY, USA) pre-coated with 50 μg Matrigel gel. After 24 h of incubation, the cells penetrated to the bottom side were fixed in methanol for 15 min, stained with crystal violet for 20 min, and counted using a microscope. The number of migratory and invasive cells was counted in 5 randomly selected fields per sample (magnification 200×).

Dual-Luciferase Reporter Gene Assay

The wild-type and mutant-type Luciferase plasmids of ETS1 were constructed. The cells seeded in the 24-well plate with 2×10^5 cells per well were co-transfected with wild-type/mutant-type ETS1 and miRNA-300 mimic or NC. 48 hours later, the cells were lysed for determining the relative Luciferase activity (Promega, Madison, WI, USA).

Western Blot

The total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) and quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The protein sample was loaded for electrophoresis and transferred on polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). The membranes were blocked in 5% skim milk for 2 hours, and subjected to incubation with primary and second-

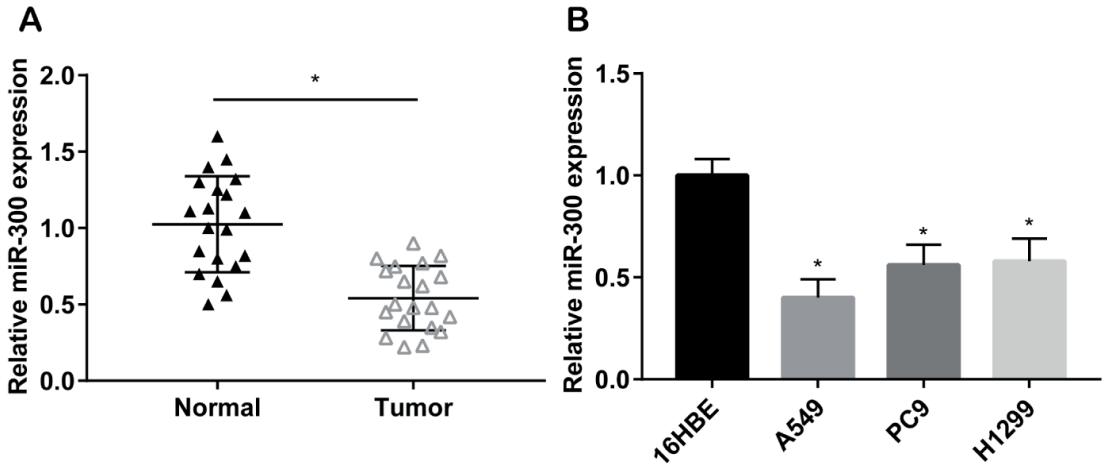

Figure 1. Downregulation of miR-300 in NSCLC. **A,** MiR-300 level in NSCLC tissues and adjacent normal tissues. **B,** MiR-300 level in 16HBE, A549, PC9, and H1299 cells.

ary antibodies. The bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software.

5-Ethynyl-2'-Deoxyuridine (EdU) Assay

The cells were inoculated into 96-well plates with 1×10^5 cells per well, and labeled with 100 μL of EdU reagent (50 μM) per well for 2 h. After washing with Phosphate-Buffered Saline (PBS), the cells were fixed in 50 μL of fixation buffer, decolored with 2 mg/mL glycine and permeated with 100 μL of penetrant. After washing with PBS once, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI) in the dark for 30 min. The EdU-positive cells, DAPI-labeled cells, and their merged images were determined under a fluorescent microscope.

Statistical Analysis

The GraphPad Prism 7.0 (La Jolla, CA, USA) was used for data analyses. The data were expressed as mean ± standard deviation. The intergroup differences were analyzed by the *t*-test. $p<0.05$ was considered as statistically significant.

Results

Downregulation of MiRNA-300 in NSCLC

To determine the expression pattern of miRNA-300 in NSCLC, its level was firstly examined in NSCLC tissues and adjacent normal ones. QRT-PCR data showed a lower abundance of miRNA-300 in NSCLC tissues relative to normal ones (Figure 1A). Furthermore, miRNA-300 was downregulated in lung carcinoma cells A549, PC9, and H1299 cells than that of human bronchial epithelial cells 16HBE (Figure 1B). In the three selected tumor cell lines, A549 cells expressed the lowest level of miRNA-300, and were used for the following experiments. The above data suggested that miRNA-300 was downregulated in NSCLC and may be involved in the progression of NSCLC.

MiRNA-300 Suppressed NSCLC to Proliferate, Migrate, and Invade

To uncover the biological role of miRNA-300 in NSCLC, miRNA-300 mimic was first constructed. The transfection of miRNA-300 mimic markedly upregulated the miRNA-300 level in A549 cells (Figure 2A). The CCK-8 assay revealed lower viability in A549 cells transfected with miRNA-300 mimic relative to those of the controls (Figure 2B). Besides, the EdU-positive ratio decreased after transfection of miRNA-300 mimic in A549 cells, indicating the attenuated proliferative potential (Figure 2C). Furthermore, the transwell assay uncovered the inhibited migratory and invasive capacities in A549 cells overexpressing miRNA-300 (Figure 2D). Hence, miRNA-300 was believed to be a tumor-suppressor gene in NSCLC to suppress the proliferation and metastasis of the tumor cells.

ETS1 Was the Target Gene of MiRNA-300

The potential binding sites in the promoter region of miRNA-300 and ETS1 were predicted on the TargetScan (Figure 3A). Furthermore, the Luciferase activity decline was observed after the

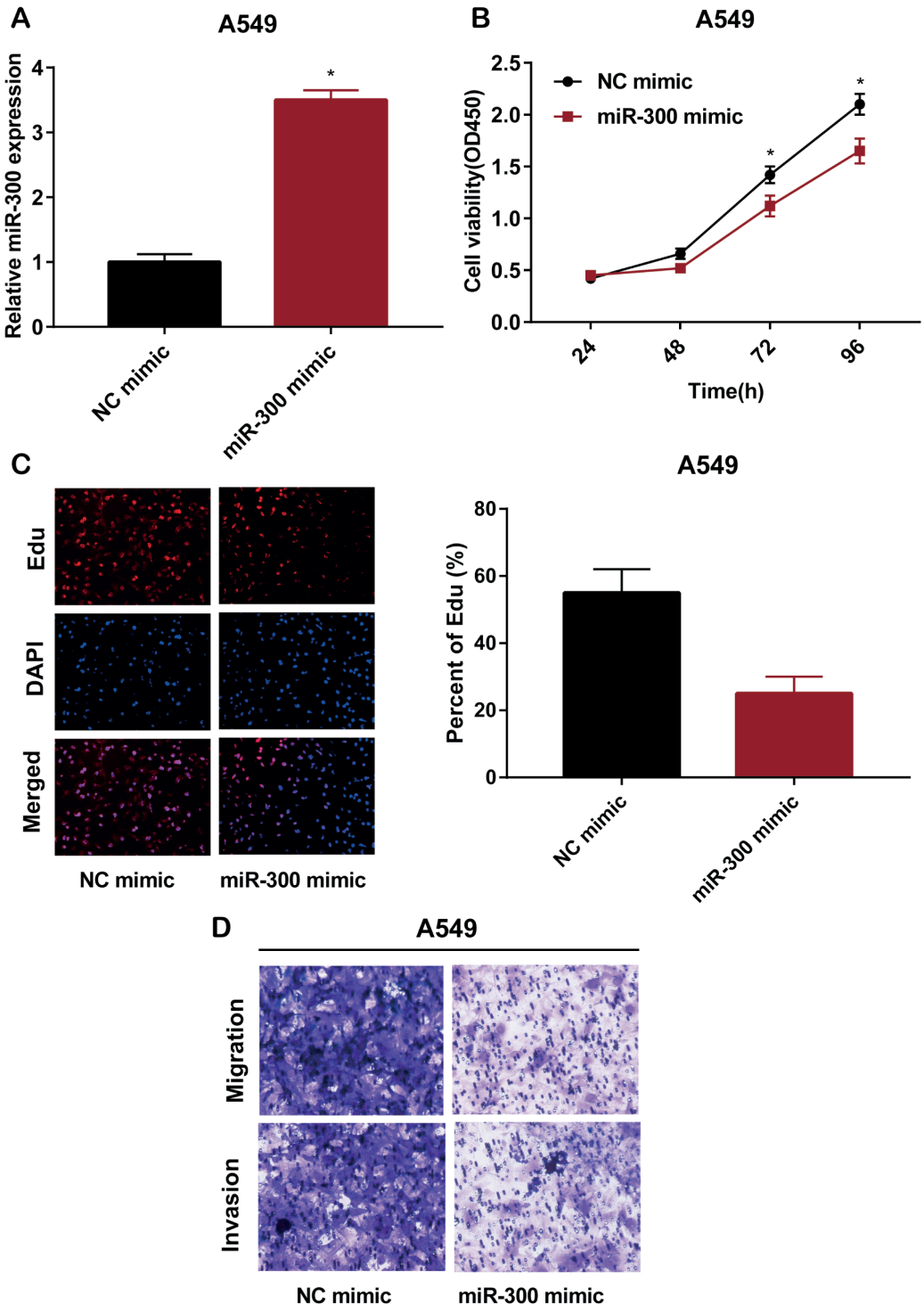

Figure 2. MiR-300 suppressed NSCLC to proliferate, migrate, and invade. **A,** The transfection efficacy of miR-300 mimic in A549 cells. **B,** Viability in A549 cells transfected with miR-300 mimic or NC mimic at 24, 48, 72, and 96 h. **C,** EdU-positive cells, DAPI-labeled cells, and their merged images of A549 cells transfected with miR-300 mimic or NC mimic (magnification: 40×). **D,** Migration and invasion of A549 cells transfected with miR-300 mimic or NC mimic (magnification: 40×).

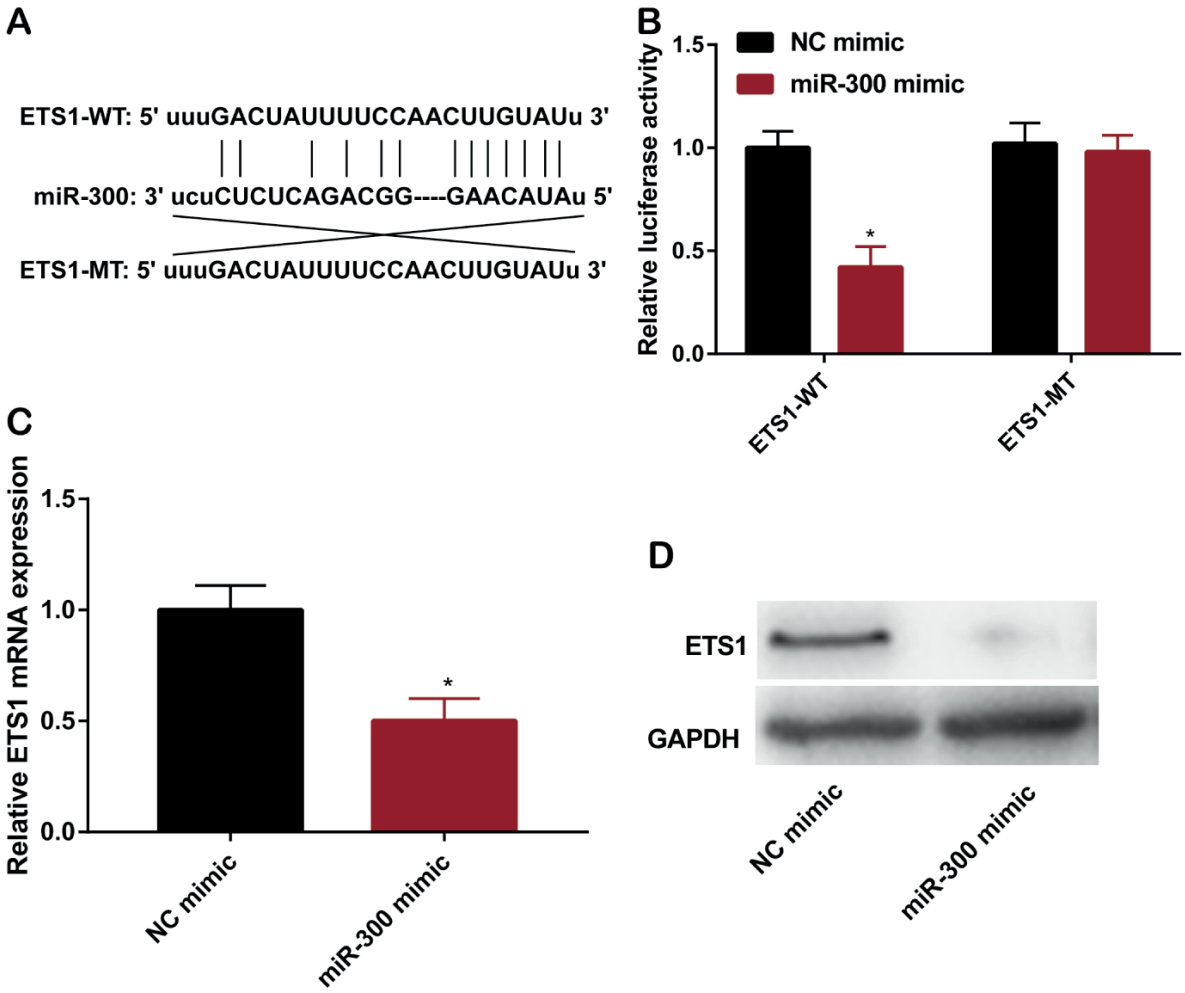

Figure 3. ETS1 was the direct target of miR-300. **A,** Binding sites in the promoter region of miR-300 and ETS1 predicted on TargetScan. **B,** Luciferase activity in A549 cells co-transfected with ETS1-WT/ETS1-MUT and miR-300 mimic/NC mimic, respectively. **C,** The mRNA level of ETS1 in A549 cells transfected with miR-300 mimic or NC mimic. **D,** The protein level of ETS1 in A549 cells transfected with miR-300 mimic or NC mimic.

co-transfection of miRNA-300 mimic and ETS1-WT, by verifying the binding between miRNA-300 and ETS1 (Figure 3B). Both mRNA and the protein levels of ETS1 were downregulated in A549 cells transfected with miRNA-300 mimic (Figures 3C, 3D). As a result, ETS1 was the direct target of miRNA-300, and the ETS1 level was negatively regulated by it.

MiRNA-300 Regulated the Progression of NSCLC by Targeting ETS1

Concerning the target gene of miRNA-300, we next focused on the interaction between miRNA-300 and ETS1 in the progression of NSCLC. The transfection of si-ETS1 markedly downregulated ETS1 level in A549 cells, confirming its transfection efficacy. The downregulated level of ETS1 in A549 cells transfected with si-ETS1 was partially reversed by the co-transfection of miRNA-300 inhibitor (Figure 4A). Transfection of si-ETS1 reduced the viability and EdU-positive ratio in A549 cells, and was further reversed by the knockdown of miRNA-300 (Figures 4B, 4C). Moreover, the attenuated migratory and invasive capacities of A549 cells with ETS1 knockdown were partially reversed after the knockdown of miRNA-300 (Figure 4D). Collectively, miRNA-300 attenuated the proliferative, migratory, and invasive potentials of NSCLC by targeting and downregulating ETS1, thus exerting a tumor-suppressor effect.

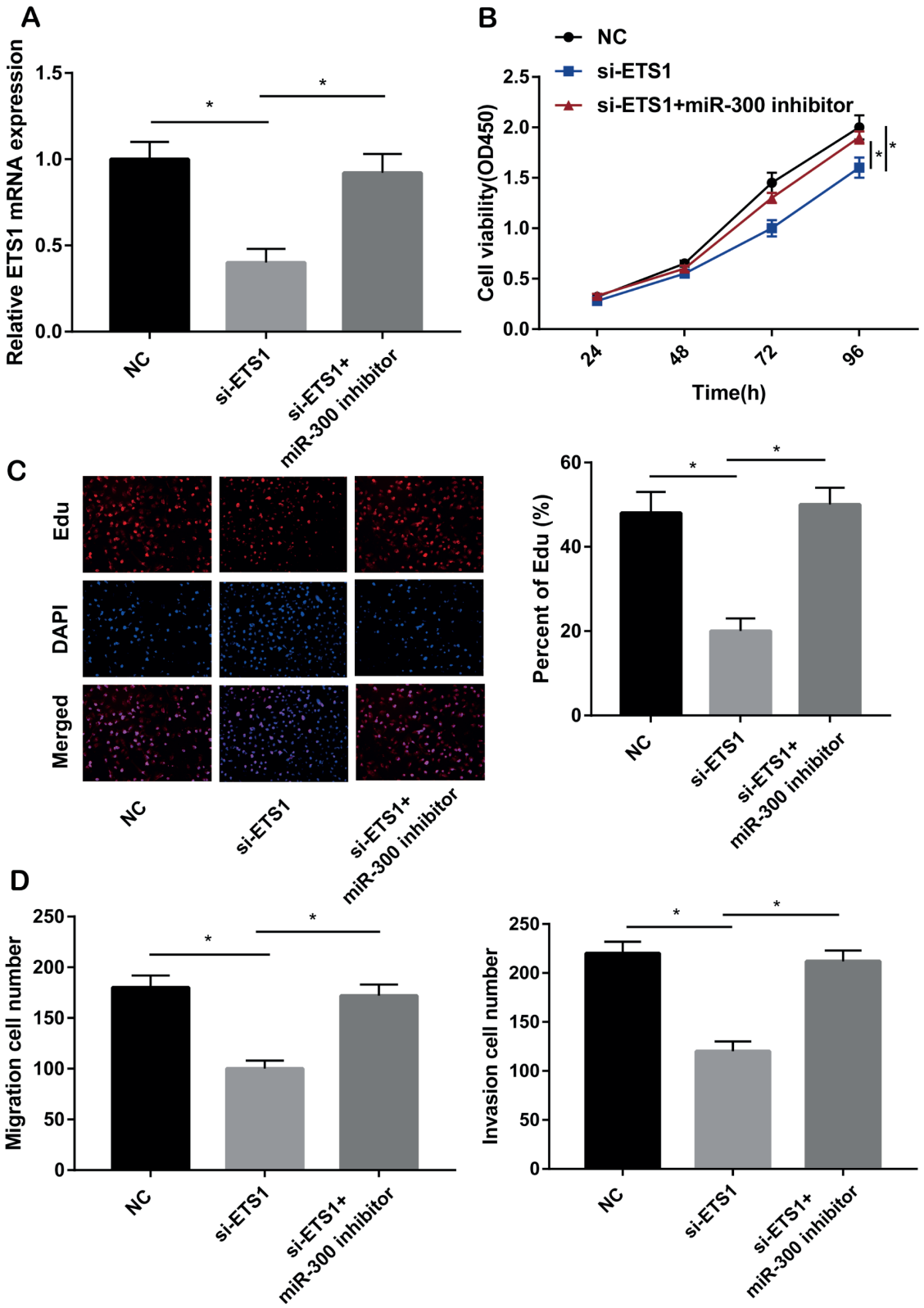

Figure 4. MiR-300 regulated the progression of NSCLC by targeting ETS1. A549 cells were transfected with NC, si-ETS1, or si-ETS1+miR-300. **A,** Relative level of ETS1. **B,** Viability at 24, 48, 72, and 96 h. ***C,*** EdU-positive cells, DAPI-labeled cells, and their merged images (magnification: 40×). **D,** Migratory and invasive cell numbers.

Discussion

The deterioration of NSCLC is a multi-step process, involving various pathological changes and gene expression changes[16,17]. With the rapid progression of molecular biology and therapeutic technology, plenty of genes have been discovered to be related to the occurrence and progression of NSCLC[18-20]. These certain genes may be utilized for developing diagnostic, therapeutic, and prognostic markers of NSCLC. In this paper, miRNA-300 was downregulated in NSCLC tissues and cells. *In vitro* experiments showed that the overexpression of miRNA-300 could attenuate viability, migratory, and invasive potentials of A549 cells.

MiRNAs are able to stimulate mRNA degradation by binding to them, thus mediating the gene expressions at post-transcriptional level[21-23]. On the other hand, miRNAs could regulate the gene expressions by inhibiting the initiation of mRNA translation[24]. Jin et al[25] demonstrated that the interferon regulatory factor 2 (IRF2) is the target gene of miR-1290 and is negatively regulated by the latter. Our results verified that miRNA-300 could bind to ETS1 3'UTR, suggesting the direct binding between miRNA-300 and ETS1. Moreover, both mRNA and the protein levels of ETS1 were negatively regulated by miRNA-300.

ETS1 is extensively expressed in embryos and adult tissues, which is important in lymphoid development, T cell activation, and angiogenesis. ETS1 is also able to influence cellular behaviors[26]. ETS1 has been identified to be highly expressed in multiple types of tumors[27-29]. Cao et al[30] illustrated that miR-512-5p affects proliferative, migratory, invasive, apoptotic, and differentiation capacities of NSCLC by targeting ETS1. Here, the silence of ETS1 attenuated the viability, migration, and invasiveness of A549 cells. More importantly, the knockdown of miRNA-300 reversed the regulatory effect of ETS1 on the cellular behaviors of A549 cells. It is concluded that miRNA-300 influenced the malignant progression of NSCLC *via* targeting ETS1.

Conclusions

The above findings discovered that miRNA-300 is downregulated in NSCLC tissues and cells. It inhibits NSCLC to proliferate, migrate, and invade by downregulating ETS1, thus suppressing the malignant progression of NSCLC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

References

1) Zheng X, Hu Y, Yao C. The paradoxical role of tumor-infiltrating immune cells in lung cancer. Intractable Rare Dis Res 2017; 6: 234-241.
2) He M, Zhu J, Yu N, Kong H, Zeng X, Xie W, Xu H. The superior antitumor effect of self-assembled paclitaxel nanofilaments for lung cancer cells. Curr Drug Deliv 2019; 16: 171-178.
3) Shi X, Liu Z, Liu Z, Feng X, Hua F, Hu X, Wang B, Lu K, Nie F. Long noncoding RNA PCAT6 functions as an oncogene by binding to EZH2 and suppressing LATS2 in non-small-cell lung cancer. EBioMedicine 2018; 37: 177-187.
4) Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. Trends Mol Med 2014; 20: 460-469.
5) Zong L, Zhu Y, Liang R, Zhao HB. Gap junction mediated miRNA intercellular transfer and gene regulation: a novel mechanism for intercellular genetic communication. Sci Rep 2016; 6: 19884.
6) Yu T, Liu L, Li J, Yan M, Lin H, Liu Y, Chu D, Tu H, Gu A, Yao M. MiRNA-10a is upregulated in NSCLC and may promote cancer by targeting PTEN. Oncotarget 2015; 6: 30239-30250.
7) Del VV, Grasso M, Barbareschi M, Denti MA. MicroRNAs as lung cancer biomarkers. World J Clin Oncol 2014; 5: 604-620.
8) Li D, Wei Y, Wang D, Gao H, Liu K. MicroRNA-26b suppresses the metastasis of non-small cell lung cancer by targeting MIEN1 via NF-κB/MMP-9/VEGF pathways. Biochem Biophys Res Commun 2016; 472: 465-470.
9) Wang M, Meng B, Liu Y, Yu J, Chen Q, Liu Y. MiR-124 inhibits growth and enhances radiation-induced apoptosis in non-small cell lung cancer by inhibiting STAT3. Cell Physiol Biochem 2017; 44: 2017-2028.
10) Karbasy SH, Taheriazam A, Mirghasemi A, Sedaghati F, Shakeri M, Yahaghi E, Bahador R. RETRACTED ARTICLE: upregulation of miR-300 and downregulation of miR-125b act as potential predictor biomarkers in progression, metastasis, and poor prognosis of osteosarcoma. Tumour Biol 2015. [Epub ahead of print].
11) Xue Z, Zhao J, Niu L, An G, Guo Y, Ni L. Up-regulation of miR-300 promotes proliferation and invasion of osteosarcoma by targeting BRD7. PLoS One 2015; 10: e0127682.
12) Shen Z, Li C, Zhang K, Yu W, Xiao H, Li B, Liu T. The up-regulation of miR-300 in gastric cancer and its effects on cells malignancy. Int J Clin Exp Med 2015; 8: 6773-6783.
13) Ge W, Han C, Wang J, Zhang Y. MiR-300 suppresses laryngeal squamous cell carcinoma proliferation and metastasis by targeting ROS1. Am J Transl Res 2016; 8: 3903-3911.

14) Wang R, Yu Z, Chen F, Xu H, Shen S, Chen W, Chen L, Su Q, Zhang L, Bi J, Zeng W, Li W, Huang X, Wang Q. MiR-300 regulates the epithelial-mesenchymal transition and invasion of hepatocellular carcinoma by targeting the FAK/PI3K/AKT signaling pathway. Biomed Pharmacother 2018; 103: 1632-1642.

15) He J, Feng X, Hua J, Wei L, Lu Z, Wei W, Cai H, Wang B, Shi W, Ding N, Li H, Zhang Y, Wang J. MiR-300 regulates cellular radiosensitivity through targeting p53 and apaf1 in human lung cancer cells. Cell Cycle 2017; 16: 1943-1953.

16) Benderli Cihan Y. ALK inhibitors and cranial radiotherapy in brain metastasis from NSCLC. J BUON 2018; 23: 1558.

17) Yusen W, Xia W, Shengjun Y, Shaohui Z, Hongzhen Z. The expression and significance of tumor associated macrophages and CXCR4 in non-small cell lung cancer. J BUON 2018; 23: 398-402.

18) Tiseo M, Gelsomino F, Boggiani D, Bortesi B, Bartolotti M, Bozzetti C, Sammarelli G, Thai E, Ardizzoni A. EGFR and EML4-ALK gene mutations in NSCLC: a case report of erlotinib-resistant patient with both concomitant mutations. Lung Cancer 2011; 71: 241-243.

19) Ulivi P, Delmonte A, Chiadini E, Calistri D, Papi M, Mariotti M, Verlicchi A, Ragazzini A, Capelli L, Gamboni A, Puccetti M, Dubini A, Burgio MA, Casanova C, Crino L, Amadori D, Dazzi C. Gene mutation analysis in EGFR wild type NSCLC responsive to erlotinib: are there features to guide patient selection? Int J Mol Sci 2015; 16: 747-757.

20) Chen S, Li P, Yang R, Cheng R, Zhang F, Wang Y, Chen X, Sun Q, Zang W, Du Y, Zhao G, Zhang G. MicroRNA-30b inhibits cell invasion and migration through targeting collagen triple helix repeat containing 1 in non-small cell lung cancer. Cancer Cell Int 2015; 15: 85.

21) Zheng HE, Wang G, Song J, Liu Y, Li YM, Du WP. MicroRNA-495 inhibits the progression of non-small-cell lung cancer by targeting TCF4 and inactivating Wnt/β-catenin pathway. Eur Rev Med Pharmacol Sci 2018; 22: 7750-7759.

22) Liu Z, Jiang L, Zhang G, Li S, Jiang X. MiR-24 promotes migration and invasion of non-small cell lung cancer by targeting ZNF367. J BUON 2018; 23: 1413-1419.

23) Qi YJ, Zha WJ, Zhang W. MicroRNA-217 alleviates development of non-small cell lung cancer by inhibiting AKT3 via PI3K pathway. Eur Rev Med Pharmacol Sci 2018; 22: 5972-5979.

24) Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, Basyuk E, Bertrand E, Filipowicz W. Inhibition of translational initiation by Let-7 microRNA in human cells. Science 2005; 309: 1573-1576.

25) Jin JJ, Liu YH, Si JM, Ni R, Wang J. Overexpression of miR-1290 contributes to cell proliferation and invasion of non small cell lung cancer by targeting interferon regulatory factor 2. Int J Biochem Cell Biol 2018; 95: 113-120.

26) Dittmer J. The role of the transcription factor Ets1 in carcinoma. Semin Cancer Biol 2015; 35: 20-38.

27) Takai N, Miyazaki T, Fujisawa K, Nasu K, Miyakawa I. Expression of c-Ets1 is associated with malignant potential in endometrial carcinoma. Cancer 2000; 89: 2059-2067.

28) Nakayama T, Ito M, Ohtsuru A, Naito S, Sekine I. Expression of the Ets-1 proto-oncogene in human colorectal carcinoma. Mod Pathol 2001; 14: 415-422.

29) Yamaguchi E, Nakayama T, Nanashima A, Matsumoto K, Yasutake T, Sekine I, Nagayasu T. Ets-1 proto-oncogene as a potential predictor for poor prognosis of lung adenocarcinoma. Tohoku J Exp Med 2007; 213: 41-50.

30) Cao B, Tan S, Tang H, Chen Y, Shu P. MiR5125p suppresses proliferation, migration and invasion, and induces apoptosis in Nonsmall Cell Lung Cancer cells by targeting ETS1. Mol Med Rep 2019; 19: 3604-3614.