

Long Noncoding RNA ANRIL Promotes Cervical Cancer Development by Acting as a Sponge of miR-186

Jun-Jun Zhang,* Dan-Dan Wang,* Chen-Xiang Du,† and Yan Wang‡

*Department of Obstetrics and Gynecology, The First Affiliated Hospital of Henan University of Science and Technology, Luoyang, Henan Province, P.R. China

†Department of Obstetrics, People's Hospital of Zhengzhou University, Zhengzhou, Henan Province, P.R. China

‡Department of Gynecology, The First Affiliated Hospital of Henan University of Science and Technology in The New Area, Luoyang, Henan Province, P.R. China

Cervical cancer is a common malignancy of the female reproductive system. Long noncoding RNAs (lncRNAs) have been reported to modulate tumor progression in multiple cancers. The lncRNA antisense noncoding RNA in the INK4 locus (ANRIL) has been identified as an oncogenic molecular target in several tumors; however, the function and underlying mechanism involved in cervical cancer oncogenesis are still unclear. In the present study, RT-PCR showed that ANRIL expression was significantly upregulated in cervical cancer tumors and cell lines. Nevertheless, ANRIL knockdown transfected with interference oligonucleotide inhibited the proliferation activity and invasive ability, and promoted apoptosis of cervical cancer cell lines. The bioinformatics prediction program and luciferase assay predicted and validated that miR-186 directly targeted ANRIL. The expression level of miR-186 was downregulated in cervical cancer tumors and cell lines and was negatively correlated to that of ANRIL. Moreover, rescue experiments showed that miR-186 inhibitor could reverse the suppression of ANRIL knockdown. In summary, our study demonstrated that the ANRIL/miR-186 axis might play a vital role in cervical cancer tumorigenesis.

Key words: Cervical cancer; ANRIL; miR-186; Long noncoding RNAs (lncRNAs)

INTRODUCTION

Cervical cancer is a common malignancy of the female reproductive system with thousands of incidences globally every year, accounting for nearly 10% of total tumor mortality¹. Accompanied by HPV infection, cervical cancer is the third most common cancer in women worldwide². Presently, the effective therapeutic method for cervical cancer mainly includes surgery excision, chemotherapy, and radiotherapy. To effectively cure and prevent cervical cancer, it is necessary to explore the internal complicated mechanisms of tumorigenesis.

Long noncoding RNAs (lncRNAs) are novel identified noncoding RNAs with more than 200 nucleotides in length, participating in physiological activity in nuclear or cytoplasmic compartments^{3,4}. lncRNA is a type of epigenetic modulation that refers to transcription and post-transcription regulation⁵. Accumulating evidence has revealed that numerous lncRNAs participate in tumor genesis and biological processes, such as proliferation, invasion, metastasis, and apoptosis⁶. The aberrant expression

levels of lncRNAs have the potential to act as biomarkers for tumor prediction. For example, lncRNA PVT1 is increased in cervical cancer patients' serum, acting as a novel biomarker for the detection of cervical cancer⁷. lncRNA antisense noncoding RNA in the INK4 locus (ANRIL) has been verified to be upregulated in tumor tissue and function as a tumor-promoting lncRNA in numerous malignancies, such as nasopharyngeal carcinoma, lung cancer, and multiple myeloma^{7,8}.

MicroRNAs (miRNAs) are a large group of noncoding RNAs found in most tissues, and they exert a considerable influence on physiological and pathological processes. For cervical cancer, a number of miRNAs have been verified to modulate tumorigenesis. For instance, miR-143 could inhibit the proliferative activity of HeLa cells through targeting the expression of K-ras gene *in vitro*⁹. In prostate cancer, miR-186 is downregulated in prostate cancer cells and tissues, inhibiting cell proliferation by targeting GOLPH3¹⁰. Hence, miRNAs play an important role in tumor biological regulation.

Address correspondence to Yan Wang, Department of Gynecology, The First Affiliated Hospital of Henan University of Science and Technology in The New Area, Xuefu Road, New District, Luoyang, 471000 Henan Province, P.R. China. E-mail: wangyan_jane@126.com

In the present study, we comprehensively investigated the role of ANRIL in cervical cancer and found that it was overexpressed in cervical cancer cell lines. Furthermore, bioinformatics analysis predicted the underlying miRNA “sponge” mechanism via targeting miR-186. Together, our results showed that ANRIL could be a promising molecular target for the development of diagnostic and therapeutic strategies in cervical cancer treatment.

MATERIALS AND METHODS

Tissue Samples and Ethical Statement

A total of 60 pairs of primary cervical cancer tissues and the adjacent normal cervical epithelial tissues from cervical carcinoma patients were used. The patients had undergone surgery from March 2015 to October 2016 at the Department of Gynecology of the First Affiliated Hospital of Henan University of Science and Technology. None of the patients received radiotherapy or chemotherapy prior to hysterectomy. This study was approved by the research ethics review committees of the First Affiliated Hospital of Henan University of Science and Technology. All patients were informed of the aims of the study and signed informed consent.

Cervical Cancer Cell Lines and Culture

Cervical cancer cells (HeLa, CaSki, SiHa, HT-3, and C33A), human epidermal cells (HaCaT), and HEK293 cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cervical cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, L-glutamine (2 mM), 100 mg/ml penicillin, and 100 mg/ml streptomycin (Invitrogen). All cells were grown at 37°C in a cell incubator with a humidified atmosphere containing 5% CO₂.

siRNA and Plasmid Transfection

To determine the up- or downregulated expression of lncRNAs and miRNAs, oligonucleotide sequences were synthesized by GenePharma (Shanghai, P.R. China). For transfection, siRNA was transfected into cervical cancer cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The siRNA sequences were as follows: si-ANRIL-1, 5'-GUGGAG AUGAUCCAUUUCATTdTdT-3' (sense) and 5'-UGAA AUGGAUCAUCUCCACTTdTdT-3' (antisense); si-ANRIL-2, 5'-GUAUUUAUGACGGAUCCAdTdT-3' (sense) and 5'-UGUCUAAAUCCCAGGAACdTdT-3' (antisense); miR-186 inhibitor, 5'-CAGCGGAGA UCCAGCACUGdTdT-3'.

Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNAs were extracted from cervical cancer tissue and cells using RNAiso Plus reagent (Takara Biotechnology,

Dalian, P.R. China). Total RNAs were reversed using the SuperScript First-Strand Synthesis system (Invitrogen). The synthesized cRNA was used for RT-PCR with the RT-PCR System (StepOne™; Applied Biosystems, Darmstadt, Germany), and the conditions were performed according to the manufacturer's instructions. Value was normalized to the expression of U6 mRNA. The PCR primers are as follows: ANRIL, 5'-CTAAGGAGCAGAAGACA TC-3' (forward) and 5'-GTAGAATCTCTCAGACGGTT G-3' (reverse); miR-186, 5'-CTAGACGATTGAATAGA C-3' (forward) and 5'-GTCCGAGCTGGTCAGAATG-3' (reverse); U6, 5'-AGCCCCGCACTCAGAACATC-3' (forward) and 5'-GCCACCAAGACAATCATCC-3' (reverse). The relative gene expression results were quantified following normalization to U6 and expressed as fold change compared with internal standard with 2^{-ΔΔCT} method.

Luciferase Reporter Assay

The amplified full-length ANRIL 3'-UTR containing the miR-186 binding sites was cloned downstream of the firefly luciferase gene vector pGL3 (Invitrogen) to constitute the luciferase report vectors (pGL3-ANRIL-WT and pGL3-ANRIL-mutant). pGL3-ANRIL-WT and pGL3-ANRIL-mutant were then cotransfected into HEK293 cells with miR-186 mimics or negative control. *Renilla* luciferase plasmid pRL-TK (Promega, Madison, WI, USA) was used as control. Forty-eight hours later, luciferase activity was determined using the Dual-Luciferase Reporter Assay Kit (Promega). The relative firefly luciferase activity value was normalized to *Renilla* luciferase.

Colony Formation Assay

Resuspension fluid containing about 300 cells was added into 1640 medium with 10% FBS in six-well plates. After 14 days, cells were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet (Beyotime, Shanghai, P.R. China). After washing mildly with PBS, colonies containing >50 cells were manually counted.

CCK-8 Proliferation Assay

For cell proliferation assay, cervical cancer cells were seeded in a 96-well plate at a density of 1 × 10³ cells/well with serum-free 1640 medium. Cells were then incubated and assessed every 24 h at 450 nm using a spectrophotometer.

Transwell Invasion Assay

For the invasion assay, a 24-well BD BioCoat Matrigel Invasion Chamber (pore size: 8 mm; BD Biosciences, Bedford, MA, USA) was used according to the manufacturer's protocol. In brief, cervical cancer cells were seeded into the Matrigel-coated upper chamber (BD Transduction Lab, San Jose, CA, USA) at a density of 1 × 10⁴ cells/well. Then 200 ml of serum-free medium and 600 ml of medium containing 20% FBS were respectively added into the

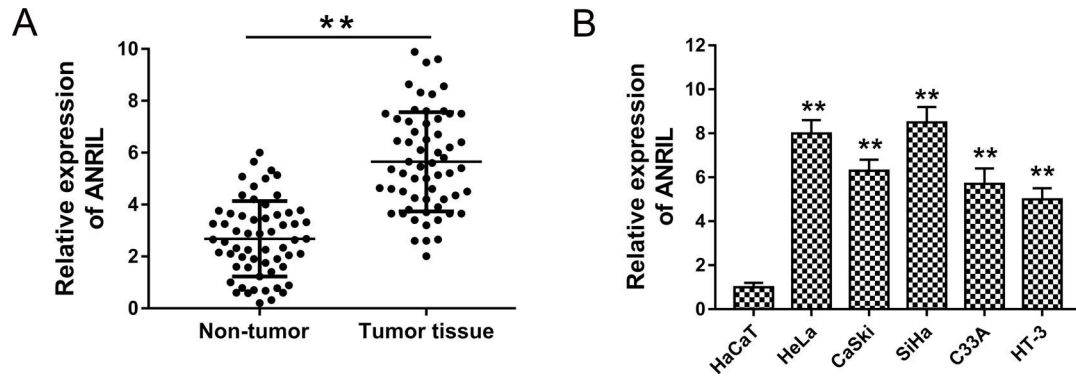


Figure 1. Antisense noncoding RNA in the INK4 locus (ANRIL) expression was significantly upregulated in cervical cancer tissues and cell lines. (A) Expression of ANRIL in cervical cancer tissues detected by real-time polymerase chain reaction (RT-PCR) compared to adjacent nontumor tissues. (B) The affirmative detection for ANRIL in cervical cancer cell lines (HeLa, CaSki, SiHa, HT-3, and C33A) compared to human epidermal cells (HaCaT). Data are presented as the mean \pm SEM. $**p < 0.01$ compared to HaCaT cells or the noncancerous group.

upper and lower chambers. After incubation for 48 h, the nonmigrating cells on the upper surface of the membrane were wiped with a cotton swab. The cells on the lower surface were stained with the Quik Stain Kit (Triangle Biomedical Sciences, Inc., Durham, NC, USA). The number was quantified by counting at 200 \times magnification. The experiment was repeated three times.

Apoptosis Assay

The apoptotic cells were tested by the Annexin-V-FITC Apoptosis Detection Kit (Invitrogen). In brief, cervical cancer cells were digested by trypsin and washed with cold PBS. The cells were resuspended in binding buffer with annexin V and PI added for incubation at

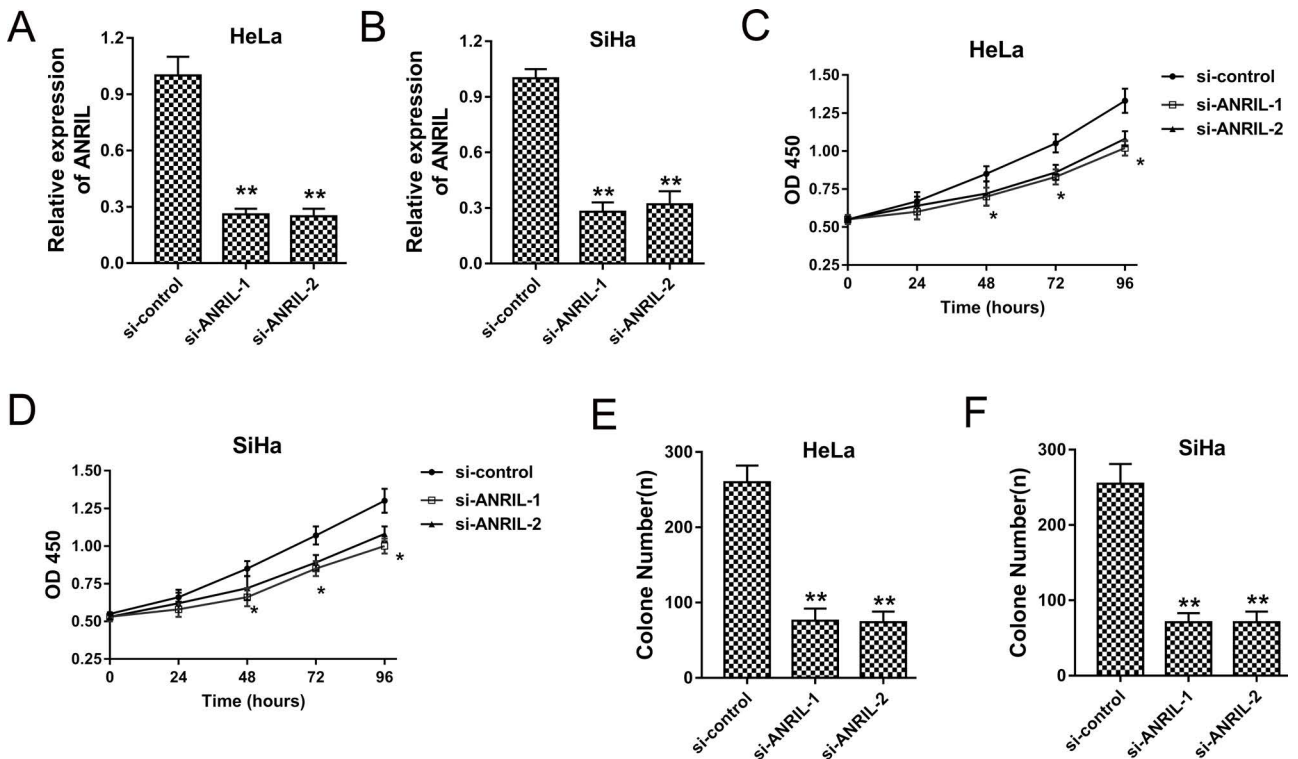


Figure 2. ANRIL knockdown suppressed cervical cancer proliferation. (A, B) Expression of ANRIL in HeLa and SiHa cells transfected with si-ANRIL-1 and si-ANRIL-2. (C, D) CCK-8 assay showed the proliferative activity of HeLa and SiHa cells. (E, F) Colony formation assay showed the clone number of HeLa and SiHa cells. Data are presented as the mean \pm SEM. $*p < 0.05$, $**p < 0.01$ compared to the si-control group.

room temperature for 20 min in the dark. Apoptotic cells were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

Data are presented as mean \pm SEM. Statistical analysis was performed using SPSS (version 18.0; Chicago, IL, USA) and GraphPad 5 (La Jolla, CA, USA) and by Student's *t*-test and one-way ANOVA. A value of $p < 0.05$ was considered to be significant.

RESULTS

ANRIL Expression Was Significantly Upregulated in Cervical Cancer Tissues and Cell Lines

To assess the expression of ANRIL, we performed RT-PCR to detect the levels in cervical cancer tissue and cell lines. The results showed that ANRIL expression was significantly upregulated in cervical cancer tissue compared to adjacent nontumor tissue (Fig. 1A). Because of the overexpression of ANRIL in cervical cancer tumors, in order to confirm the upregulated expression, we also detected the expression of ANRIL in cervical cancer cell lines. As expected, ANRIL expression was similarly upregulated in cervical cancer cell lines (Fig. 1B). Hence, the results strongly revealed that ANRIL was

upregulated in both cervical cancer tumors and cell lines, suggesting the probable oncogenic role in cervical cancer tumorigenesis.

ANRIL Knockdown Suppressed Cervical Cancer Proliferation

Because ANRIL was upregulated in cervical cancer tissues and cells, our team established ANRIL knockdown induced by interference sequence to decrease the expression of ANRIL. Cervical cancer cells HeLa and SiHa were transfected with si-ANRIL, and the ANRIL expression was significantly decreased, indicating effective silencing (Fig. 2A and B). The CCK-8 assay showed that ANRIL knockdown induced by si-ANRIL significantly inhibited the proliferation vitality (Fig. 2C and D). Moreover, the colony formation assay demonstrated that ANRIL knockdown decreased the colony number of HeLa and SiHa cells (Fig. 2E and F). The results indicated that ANRIL knockdown could significantly suppress cervical cancer proliferation.

ANRIL Knockdown Suppressed Cervical Cancer Invasion and Promoted Apoptosis

It has been indicated that ANRIL knockdown suppressed cervical cancer proliferation; nevertheless, the role of ANRIL in cervical cancer cell invasion and

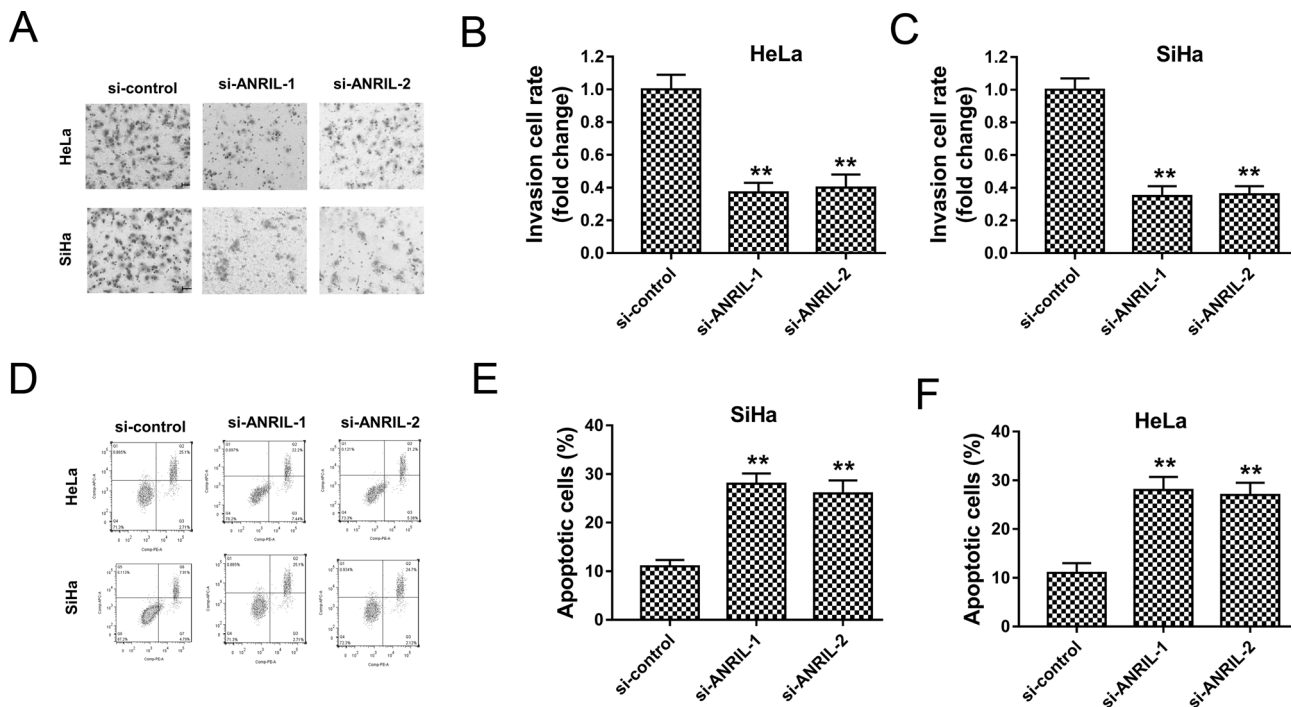


Figure 3. ANRIL knockdown suppressed cervical cancer invasion and promoted apoptosis. (A) Representative images of the Transwell assay showed the invasion of HeLa and SiHa cells. (B, C) Quantitative counting of invasive number for HeLa and SiHa cells. (D) Representative images of flow cytometry show the apoptotic cells. (E, F) Apoptotic cell rate of HeLa and SiHa cells. Data are presented as the mean \pm SEM. ** $p < 0.01$ compared to si-control group.

apoptosis was undefined. Subsequently, our team investigated the role of ANRIL knockdown on cervical cancer cell invasion and apoptosis. The Transwell assay showed that ANRIL knockdown induced by interference oligonucleotide significantly inhibited the invasive ability of HeLa and SiHa cells (Fig. 3A–C). In addition, flow cytometry revealed that ANRIL knockdown enhanced the apoptotic cell rates in HeLa and SiHa cells (Fig. 3D–F). Overall, the results indicated that ANRIL knockdown suppressed the invasive ability and promoted the apoptosis of HeLa and SiHa cells.

ANRIL Was Directly Targeted by miR-186

Numerous studies have revealed that lncRNAs played an important role in pathological progression through binding miRNAs to silence expression, also known as miRNA “sponge.” Hence, to uncover the underlying carcinogenic mechanism of ANRIL, the potential miRNAs were predicted using a bioinformatics program (TargetScan, starBase). The results revealed that miR-186 matched with ANRIL at 3′-UTR containing

complementary binding sites (Fig. 4A). The luciferase reporter assay showed the decreased fluorescence between ANRIL wild type and miR-186 (Fig. 4B). The expression of miR-186 detected by RT-PCR showed that miR-186 expression was significantly downregulated in cervical cancer tissues compared to adjacent noncancerous tissues (Fig. 4C). miR-186 expression was downregulated in cervical cancer cell lines (HeLa, CaSki, SiHa, HT-3, and C33A) compared to human epidermal cells (HaCaT) (Fig. 4D). The results revealed that miR-186 directly targeted ANRIL and was downregulated in cervical cancer tissue and cell lines.

miR-186 Rescued the Role of ANRIL on Cervical Cancer Tumorigenesis

The present study revealed the complementary binding within miR-186 and ANRIL; thus, their interactive regulation on cervical cancer tumorigenesis attracted our interest. To identify the interaction, we performed rescue experiments in HeLa cells transfected with si-ANRIL and/or miR-186 inhibitor. As shown in Figure 5A, HeLa

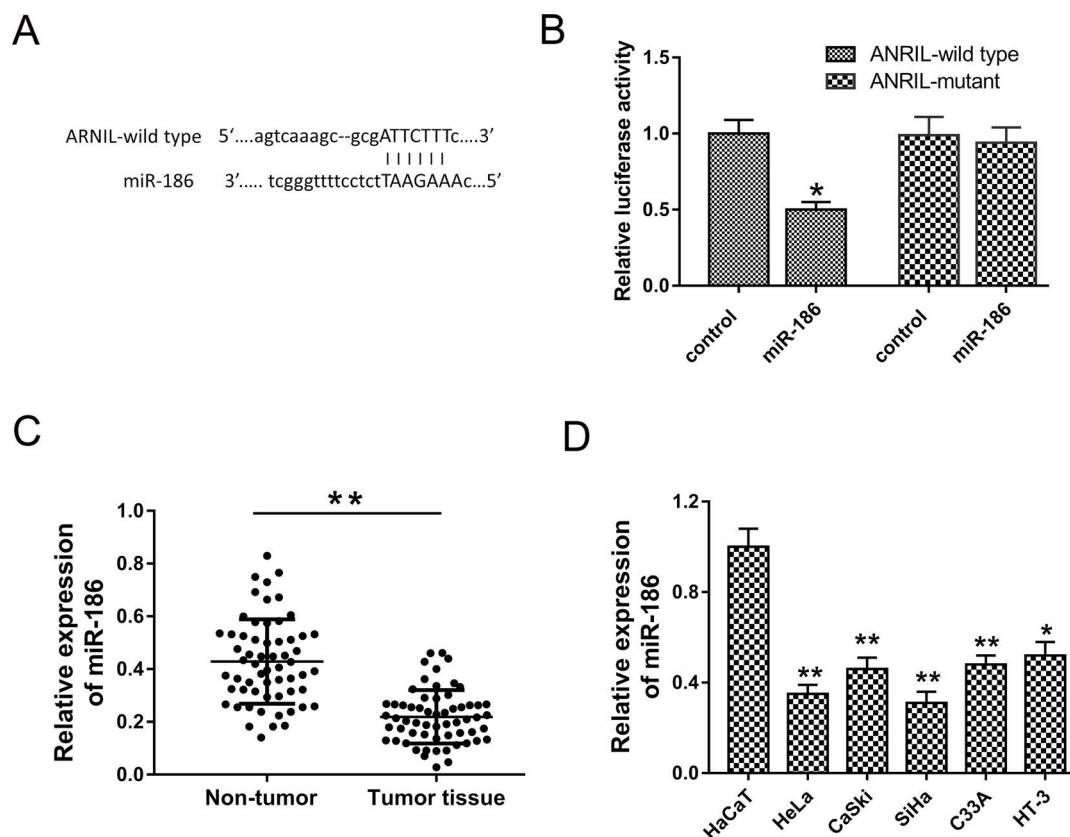


Figure 4. ANRIL was directly targeted by miR-186. (A) Complementary binding sites predicted using bioinformatics program (TargetScan, starBase). (B) Luciferase reporter assay verified the fluorescence between ANRIL wild type/mutant and miR-186 mimics/control. (C) miR-186 expression was downregulated in cervical cancer tissues compared to adjacent noncancerous tissues. (D) miR-186 expression was downregulated in cervical cancer cell lines (HeLa, CaSki, SiHa, HT-3, and C33A) compared to human epidermal cells (HaCaT). Data are presented as the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ compared to the si-control group.

knockdown could increase miR-186 expression, which was suppressed by miR-186 inhibitor. The CCK-8 assay and colony formation assay showed that miR-186 inhibitor reversed the suppression of ANRIL knockdown on proliferation vitality (Fig. 5B and C). The Transwell assay showed that miR-186 inhibitor significantly reversed the inhibition of ANRIL knockdown on the invasive ability of HeLa cells (Fig. 5D). Flow cytometry revealed that miR-186 inhibitor significantly decreased the apoptotic cell rate compared to the ANRIL knockdown group (Fig. 5E). Comprehensively, the results indicated that miR-186 inhibitor rescued the role of ANRIL knockdown on cervical cancer tumorigenesis.

DISCUSSION

The pathological development of cervical cancers has been identified as a complicated pathogenetic process, involving a series of dysregulated tumor suppressor genes and oncogenes¹¹. The function of lncRNAs has received increased attention in multiple tumors due to their widely acting range, referring to transcription and posttranscription regulation¹².

Presently, a number of lncRNAs have been discovered and identified, involving tumors, cardiovascular system disease, and nervous system disease¹³. The lncRNA ANRIL (CDKN2B antisense RNA 1) was initially identified from familial melanoma patients, encoding 3834 nt RNA and containing 19 exons at the antisense orientation of the INK4B-ARF-INK4A gene cluster. ANRIL has been identified as an oncogenic lncRNA in several tumors, such as nasopharyngeal carcinoma¹⁴, thyroid cancer¹⁵, and osteosarcoma¹⁶. In the present study, we detected the expression of lncRNA ANRIL in cervical cancer tissues and cell lines and found that ANRIL was upregulated compared to nontumor tissues and normal cells. In addition, ANRIL knockdown silenced by si-ANRIL could significantly suppress the proliferation and invasive ability and enhance the apoptosis of cervical cancer cells, indicating the oncogenic role on cervical cancer genesis.

Emerging evidence has demonstrated that lncRNAs participate in tumorigenesis by regulating transcription and posttranscription¹⁷. For instance, ANRIL is increased in thyroid cancer cells and decreases p15INK4B expression and promotes the invasion through the TGF- β /Smad

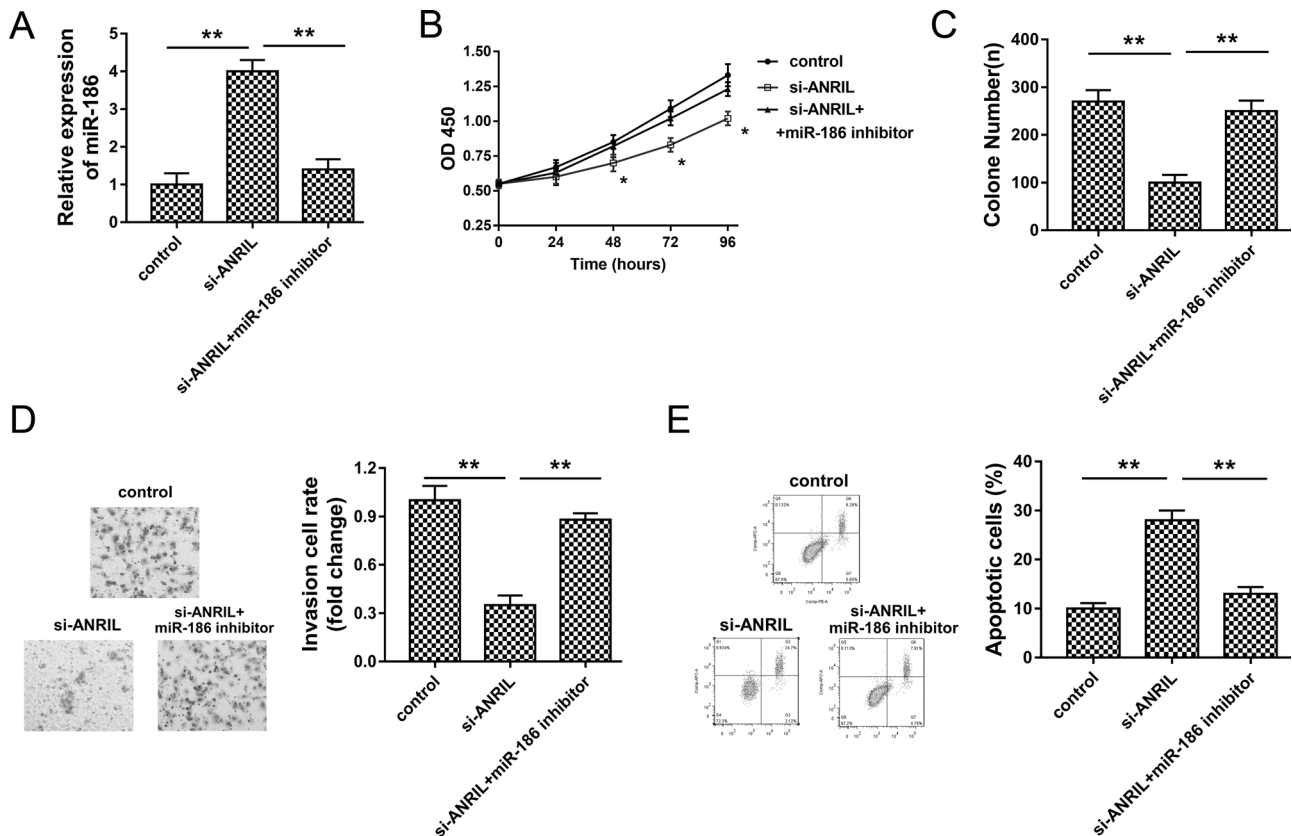


Figure 5. miR-186 inhibitor rescued the role of ANRIL knockdown on cervical cancer tumorigenesis. (A) miR-186 expression in HeLa cells transfected with si-ANRIL and miR-186 inhibitor. (B) CCK-8 assay shows the proliferation activity. (C) Colony formation assay shows the number of HeLa cells. (D) Invasion ability detected by the Transwell assay. (E) Apoptotic cell rate detected by flow cytometry. Data are presented as the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ compared to the si-control group.

signaling pathway¹⁵. The lncRNA RP1-193H18.2 is increased in HepG2 cells induced by cisplatin and downregulated in a number of target proteins, including CDKN1A, TP53I3, and PPM1D¹². The transcription level of lncRNA LINC00261 was significantly decreased in choriocarcinoma tissues, and LINC00261 overexpression suppressed cell proliferation and arrested cell cycle at the G₀/G₁ phase¹⁸. Therefore, lncRNAs could regulate multiple types of carcinogenesis and exert a vital role in the whole physiological process.

It is well known that lncRNAs function as an miRNA “sponge” to indirectly modulate the functional target mRNAs to regulate physiological activity¹⁹. lncRNAs and miRNAs share complementary pairing sequence at 3'-UTR, allowing molecular-level binding and counteraction. For instance, lncRNA TUG1 promotes gallbladder carcinoma cell proliferation, metastasis, and EMT progression by functioning as an miR-300 sponge to abrogate its endogenous effect on tumorigenesis²⁰. In the present study, we predicted target miRNAs for ANRIL using a bioinformatics program, demonstrating the targeting of miR-186 with seven complementary binding sites. The prediction was confirmed by luciferase reporter assay. Rescue experiments showed that miR-186 could reverse the function of ANRIL in cervical cancer cells, indicating the physiological antagonism within ANRIL and miR-186.

The competing endogenous RNA (ceRNA) mechanism, also well known as a “sponge,” is regarded as the major regulating type modulating physiological and pathological activities²¹. For example, TUG1 is downregulated in breast cancer tissues and cell lines and negatively regulates the expression of miR-9 and positively regulates the expression of MTHFD2 in MCF-7 cells, revealing the TUG1/miR-9/MTHFD2 axis regulating pathway²². ANRIL is highly expressed in nasopharyngeal carcinoma, and let-7a was downregulated by ANRIL in tumor tissues and cells²³.

In summary, the present study reveals the ANRIL/miR-186 regulating pathway in cervical cancer in vitro, demonstrating a vital role in carcinogenesis progression. Hence, the miRNA “sponge” acted by lncRNAs might be a promising therapeutic target for cervical cancer.

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