

Effect of lncRNA ANRIL silencing on anoikis and cell cycle in human glioma via microRNA-203a

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Background: Glioma is a deadly nervous system tumor with a poor prognosis. Although there have been many efforts to overcome glioma, the molecular mechanism of its pathogenesis remains unclear.

Methods: We used human glioma U251 cells silenced for the oncogenic lncRNA ANRIL or overexpressing the anti-oncogene miR-203a to examine the role of lncRNA ANRIL silencing on anoikis and cell cycle arrest by flow cytometry. Meanwhile, the activity of caspase-3/8/9 was measured by fluorometric assay, the expression of tumor-related genes and activity of AKT signaling pathway was measured by Western blotting, real-time PCR, and dual luciferase reporter gene assay.

Results: lncRNA ANRIL was positively correlated with glioma grade and negatively correlated with miR-203a. lncRNA ANRIL silencing could induce anoikis and cell cycle arrest in G0/G1 phase, while regulating the activity of caspase-3/8/9 and the AKT signaling pathway, and the expression of tumor-related genes in the U251 cell line. miR-203a mimics could partially reverse these functions.

Conclusion: We consider that lncRNA ANRIL is a potential therapeutic and diagnostic target for glioma, and miR-203a plays an important role in the biological function of lncRNA ANRIL in glioma.

Keywords: glioma, long non-coding RNA, anoikis, cell cycle, lncRNA ANRIL, microRNA-203a

Introduction

Although there have been many efforts to overcome glioma, it remains a significant cause of cancer-related morbidity with poor prognosis in the People's Republic of China.¹ With the development of molecular biology technology, many studies have explored therapeutics and diagnosis of glioma using novel molecular biological targets.

lncRNAs are a type of non-coding RNA (ncRNA) more than 200 nucleotides in length.² Some reports have shown that lncRNAs can affect the expression of genes and play an important role in the tumorigenesis process.³

Some reports have shown that lncRNA ANRIL is associated with osteosarcoma and lung cancer;^{4,5} however, the role of lncRNA ANRIL in glioma has not been fully elucidated. An increased expression of lncRNA ANRIL in glioma correlating with grade and a negative correlation with the anti-oncogene miR-203a has been found;^{6,7} thus, we considered that there may be a potential relationship between lncRNA ANRIL, the glioma tumorigenesis process, and miR-203a expression. This study aimed to explore the effect of lncRNA ANRIL silencing on induced anoikis and cell cycle arrest in human glioma and the possible mechanism involved.

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Materials and methods

Tissue

Glioma samples of different grades (World Health Organization [WHO]-II, n=10; WHO-III, n=10; WHO-IV, n=10) were provided by the department of neurosurgery of Tianjin Huanhu Hospital from May 2015 to April 2017. The study was approved by the Ethics Committee of Tianjin Huanhu Hospital and all experiments were performed in accordance with approved guidelines and regulations of the Helsinki Declaration of 1975, as revised in 2008. Written informed consent was obtained from patients for the use of their tissue samples in this research.

Cell line and culture

Human glioma U251 cell line was provided by The Cell Bank of Type Culture Collection of Chinese Academy of Sciences and cultured in DMEM with 10% fetal bovine serum, 50 U/mL penicillin, and 50 µg/mL streptomycin at 37°C, 5% CO₂.

The siRNA pcDNA3.1-ANRIL (si-ANRIL) primer sequence was 5'-GGUCAUCUCAUUGCUCUAU-3', miR-203a mimic (si-miR-203a) was purchased from Thermo Fisher Scientific, Waltham, MA, USA, and empty vector, pcDNA3.1, was used as a negative control (NC) group. Plasmids harboring si-ANRIL, miR-203a, or NC were transfected into glioma U251 cells using Lipofectamine 2000 according to the manufacturer's instructions. The following experiments were performed 72 hours after transfection.

MTS assay

U251 cells (5,000/well) were seeded in a 96-well plate and cultured for 72 hours at 37°C, 5% CO₂. The medium was then refreshed, MTS was added, and it was cultured for 4 hours at 37°C, 5% CO₂. The OD of the culture was measured at 490 nm with a microplate reader (Thermo Fisher Scientific).

Anoikis assay

U251 cells (3×10⁵/well) were seeded in a 6-well plate coated with Poly-HEMA (2-hydroxyethyl methacrylate) and cultured for 72 hours at 37°C, 5% CO₂. Cells were collected and stained with Annexin V-FITC at room temperature for 15 minutes in the dark. Fluorescence was then measured at 490 nm with a microplate reader (Thermo Fisher Scientific). PI3K inhibitor LY294002 (0.5 µM) was added for 72 hours to observe the effect of anoikis mediated by lncRNA ANRIL.

Caspase-3/8/9 activity assay

U251 cells (3×10⁵/well) were seeded in a 6-well plate coated with Poly-HEMA (2-hydroxyethyl methacrylate) and cultured

for 72 hours at 37°C, 5% CO₂. Cells were collected, and the activity of caspase-3/8/9 was measured by a Caspase 3, 8, 9 Multiplex Activity Assay Kit (Abcam, Cambridge, MA, USA) and fluorometric assay using a microplate reader (Thermo Fisher Scientific).

Cell cycle assay

U251 cells (3×10⁵/well) were seeded in a 6-well plate and cultured for 72 hours at 37°C, 5% CO₂. Cells were collected and fixed with 70% alcohol at 4°C overnight. The alcohol was removed, and cells were suspended in PBS; 200 µL propidium iodide (50 µg/mL) and 50 µL RNaseA (100 µg/mL) were then added to the cell suspension at room temperature for 30 minutes in the dark. Fluorescence was measured at 488 nm with a flow cytometer.

Real-time PCR assay

U251 cells (3×10⁵/well) were seeded in a 6-well plate and cultured for 72 hours at 37°C, 5% CO₂, and total RNA was extracted by using TRIzol (Invitrogen, Thermo Fisher Scientific). Expression of miR-203a was detected by using the miScript Reverse Transcription Kit (Qiagen NV, Venlo, the Netherlands), and expression of ANRIL and tumor-related genes was detected by using the miScript SYBR Green PCR kit (Qiagen NV) and an ABI 7500 PCR analyzer (Applied Biosystems, Thermo Fisher Scientific). The reaction conditions were initial denaturation at 93°C for 1 minute, followed by 40 cycles of denaturation at 93°C for 30 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 10 minutes, and a final extension at 72°C for 5 minutes. GAPDH was used for normalizing ANRIL and tumor-related gene expression, and U6 was used for normalizing miR-203a expression, all using the 2^{-ΔΔCq} method. Real-time PCR primers are given in Table 1.

Dual luciferase reporter gene assay

U251 cells (3×10⁵/well) were seeded in a 6-well plate and cultured for 72 hours at 37°C, 5% CO₂. The dual luciferase reporters *Twist1* and *c-jun* were purchased from YRGene (Changsha, People's Republic of China); Renilla Luciferase reporter for normalization was purchased from Promega Corporation (Fitchburg, WI, USA). The luciferase reporters were transfected in glioma U251 cells using Lipofectamine 2000 according to the manufacturer's instructions and cultured for 6 hours at 37°C, 5% CO₂; the medium was then refreshed, and the cells were cultured overnight at 37°C, 5% CO₂. Fluorescence intensity was measured by using a microplate reader (Thermo Scientific) via the Dual-Luciferase[®] Reporter Assay System (Promega Corporation).

Table 1 The real-time PCR primers

Gene	Primer sequences (5'-3')
lncRNA ANRIL	F: TGCTCTATCCGCAATCAGG R: GGGCCTCAGTGGCACATACC
miR-203a	F: GCGTGAAATGTTTAGGACCACT R: GCTGTCAACGATACGCTACG RT: GCTGTCAACGATACGCTACGTAACGGC ATGACAGTGTTTTTTTTTTTTTTTTTTTTTTTT
Bcl-2	F: ACCTACCCAGCCTCCGTTAT R: GAACTGGGGGAGGATTGTGG
p21	F: ATTCAGCATTGTGGGAGGAG R: TGGACTGTTTTCTCTCGGCT
CDK2	F: GGCCTAGCTTTCTGCCATTC R: CCCAGGAGGATTCAGGAGC
c-myc	F: CGAGGAGAATGTCAAGAGGCG R: CTGCTTGACGGACAGGATGT
U6	F: CTCGCTTCGGCAGCAC R: AACGCTTCACGAATTTGCGT
GAPDH	F: GTCTCCTCTGACTTCAACAGCG R: ACCACCTGTTGCTGTAGCCAA

Western blotting

U251 cells (3×10^5 /well) were seeded in a 6-well plate and cultured for 72 hours at 37°C, 5% CO₂. Total protein was collected, and the concentration was measured by BCA assay. Fifty nanograms total protein was separated by 10% SDS-PAGE, transferred to a PVDF membrane, and blocked with 5% milk at room temperature for 1 hour. After washing with PBST, primary antibody against p21 (1:1,500), Bcl-2 (1:1,500), CDK2 (1:1,500), c-myc (1:1,500), p-AKT (1:1,500), or β -actin (1:4,000) was added and incubated overnight at 4°C. After washing again with PBST, horseradish peroxidase conjugated IgG (1:3,000) was added and incubated for 1 hour at 37°C. An ECL kit (Merck Millipore, Billerica, MA, USA) was used for visualization with β -actin as the internal reference.

Statistical analysis

SPSS 11.0 software was used for statistical analysis. Data are expressed as mean \pm SD. Differences between groups were evaluated by one-way analysis of variance. $P < 0.05$ was considered significantly different.

Results

Expression of lncRNA ANRIL and miR-203a in glioma tissues

We analyzed the expression of lncRNA ANRIL and miR-203a in glioma tissues and found that there was an increased expression of lncRNA ANRIL in glioma samples with increasing tumor grade (WHO-II, n=10; WHO-III, n=10; WHO-IV, n=10) (Figure 1A). The trend in expression of miR-203a was

in contrast to that of lncRNA ANRIL (Figure 1B). Further, there was a negative correlation between lncRNA ANRIL and miR-203a in glioma tissues (Figure 1C).

Effect of lncRNA ANRIL silencing on proliferation of human glioma U251 cells

lncRNA ANRIL silencing could suppress the expression of lncRNA ANRIL and improve the expression of miR-203a; meanwhile, miR-203a mimics suppressed the expression of miR-203a and could not affect the expression of lncRNA ANRIL in U251 cells (Figure 2A and B), indicating that miR-203a may play a role in the function of lncRNA ANRIL. We explored the effect of lncRNA ANRIL silencing on the proliferation of human glioma by MTS assay and found that lncRNA ANRIL silencing could suppress proliferation of U251 cells and that miR-203a mimics could partially reverse this function (Figure 2C).

Effect of lncRNA ANRIL silencing on anoikis and the cell cycle in human glioma U251 cells

We explored the effect of lncRNA ANRIL silencing on anoikis and the cell cycle in human glioma, and found that lncRNA ANRIL silencing could induce anoikis and cell cycle arrest in G₀/G₁ phase in U251 cells and that miR-203a mimics could partially reverse this function. We found that the anoikis induced by lncRNA ANRIL was partially suppressed after PI3K inhibitor LY294002 (0.5 μ M) was added, indicating the effect of AKT on anoikis mediated by lncRNA ANRIL (Figure 3).

Effect of lncRNA ANRIL silencing on the activity of caspase and expression of tumor-related genes in human glioma U251 cells

We explored the effect of lncRNA ANRIL silencing on the activity of caspase and the expression of tumor-related genes in human glioma and found that lncRNA ANRIL silencing could improve the activity of caspase-3/8/9 (Figure 4A). Further, lncRNA ANRIL silencing could improve the expression of p21 while suppressing the expression of Bcl-2, CDK2, c-myc, and p-AKT in U251 cells (Figure 4B and C). At the same time, lncRNA ANRIL silencing could suppress the transcriptional activity of *Twist1* and *c-jun* in U251 cells (Figure 4D); miR-203a mimics could partially reverse this suppression.

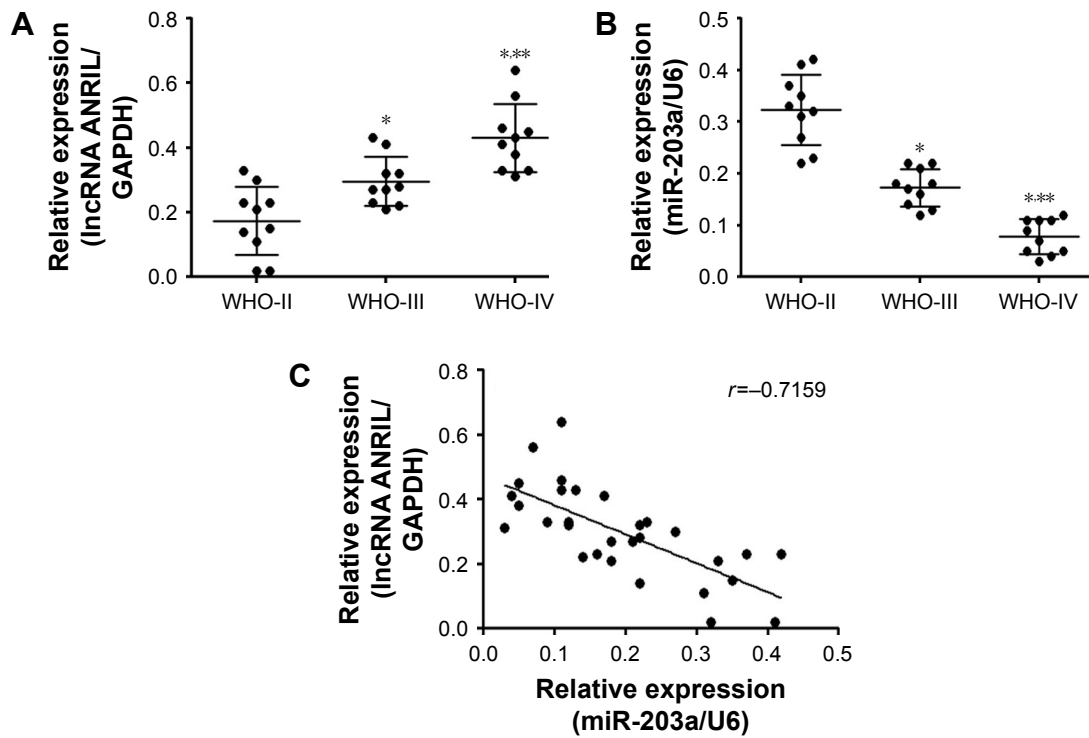


Figure 1 Expression of lncRNA ANRIL and miR-203a in glioma tissues. **Notes:** (A) Increased expression of lncRNA ANRIL in glioma tissues correlating with tumor grade. (B) Decreased expression of miR-203a in glioma tissues correlating with tumor grade. (C) Negative correlation between lncRNA ANRIL and miR-203a in glioma tissues. *Compared with negative control group, $P < 0.05$; **compared with si-ANRIL group, $P < 0.05$.

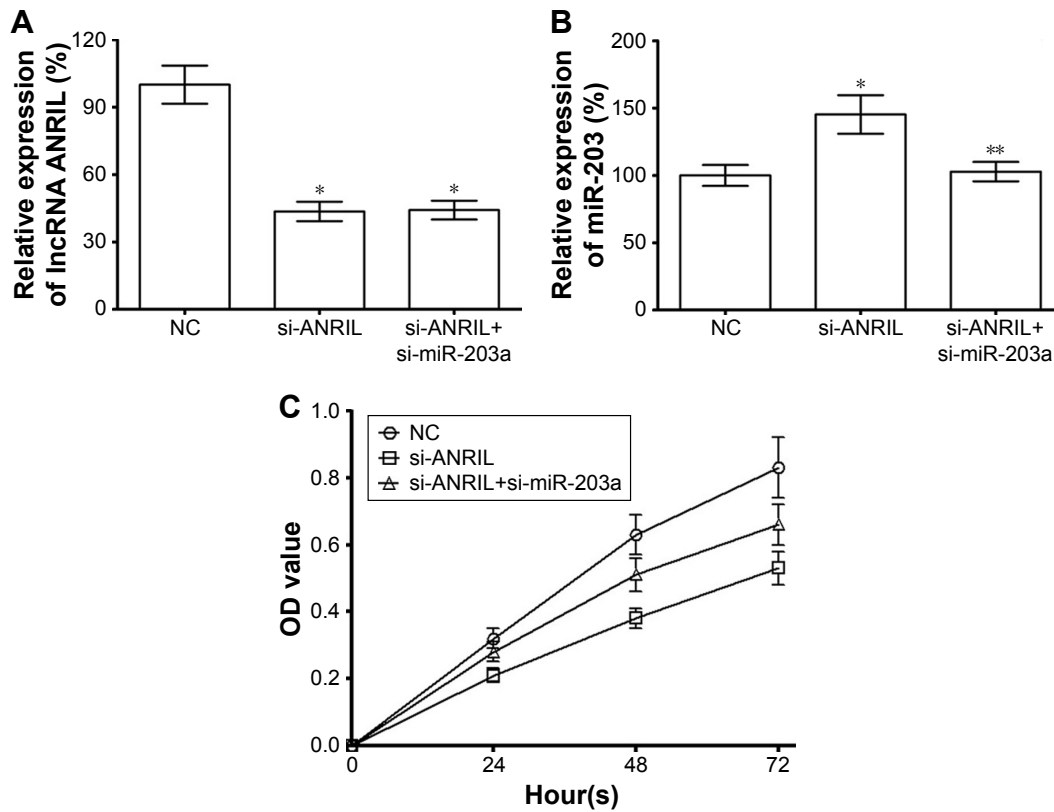


Figure 2 Effect of lncRNA ANRIL silencing on the proliferation of U251 cells. **Notes:** (A) Decreased expression of lncRNA ANRIL in lncRNA ANRIL silenced U251 cells; miR-203a mimics could not reverse the decreased expression. (B) Increased expression of miR-203 in lncRNA ANRIL silenced U251 cells; miR-203a mimics could reverse this increased expression. (C) Decreased proliferation of lncRNA ANRIL silenced U251 cells; miR-203a mimics could partially reverse this decrease. *Compared with negative control (NC) group, $P < 0.05$; **, compared with si-ANRIL group, $P < 0.05$.

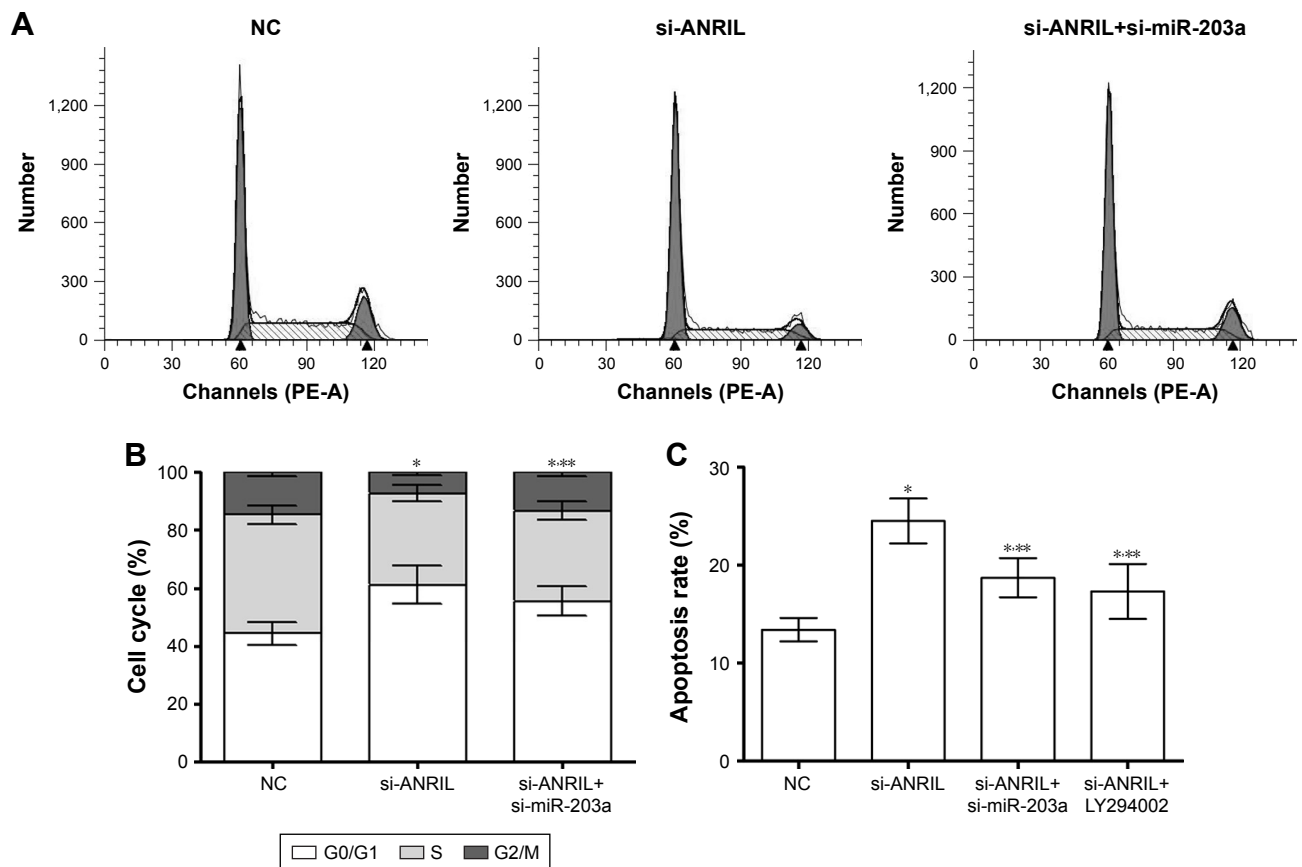


Figure 3 Effect of lncRNA ANRIL silencing on anoikis and cell cycle in U251 cells.

Notes: (A) G0/G1 phase arrest in lncRNA ANRIL silenced U251 cells; miR-203a mimics could partially reverse this arrest. (B) Cell cycle in lncRNA ANRIL silenced U251 cells. (C) Increased anoikis in lncRNA ANRIL silenced U251 cells; miR-203a mimics could partially reverse this increase. *Compared with negative control (NC), $P < 0.05$; **compared with si-ANRIL group, $P < 0.05$.

Discussion

There are a large number of ncRNAs in the human genome.⁸ Although ncRNAs do not encode proteins, they can regulate the expression of miRNAs and their biological function. lncRNAs are a type of ncRNA and play an important role in the tumorigenesis process.⁹ lncRNA ANRIL is overexpressed in osteosarcoma and lung cancer; however, the role and biological mechanism of lncRNA ANRIL in glioma have not been fully understood. We found that there was an increased expression of lncRNA ANRIL in glioma tissue samples, positively correlated with grade. Meanwhile, the expression of lncRNA ANRIL was negatively correlated with the anti-oncogene miR-203a. Further, we also silenced lncRNA ANRIL in glioma U251 cells and observed that lncRNA ANRIL silencing could induce anoikis and cell cycle arrest in G0/G1 phase in U251 cells.

Bcl-2 is an anti-apoptosis gene that can suppress the activity of caspase.¹⁰ We found that lncRNA ANRIL silencing could suppress the expression of *Bcl-2* in U251 cells; meanwhile, the activity of caspase-3/8/9 was up-regulated. *CDK2* is a cell cycle related gene,¹¹ and we found that

lncRNA ANRIL silencing could suppress the expression of *CDK2* in U251 cells. *p21* is a tumor suppressor gene that can induce apoptosis by regulating *Bcl-2*,¹² and induce cell cycle arrest by regulating *CDK2* in tumors.¹³ *c-myc* is also an oncogene that can negatively regulate the expression of *p21*.¹⁴ We found that lncRNA ANRIL silencing could suppress the expression of *c-myc* and improve the expression of *p21*.

AKT is an important signaling molecule in the tumorigenesis process and shows increased expression in tumors.¹⁵ *Twist1* and *c-jun* are transcription factors that are downstream signaling molecules of the AKT pathway and are up-regulated in tumors.^{16,17} We found that lncRNA ANRIL silencing could suppress the phosphorylation of AKT and the transcriptional activity of *Twist1* and *c-jun*, indicating lncRNA ANRIL could regulate the tumorigenesis process by regulating the phosphorylation of AKT and the transcriptional activity of *Twist1* and *c-jun* in U251 cells. We also found that the anoikis induced by lncRNA ANRIL was partially suppressed by LY294002, a PI3K inhibitor, certifying the role of AKT in anoikis mediated by lncRNA ANRIL.

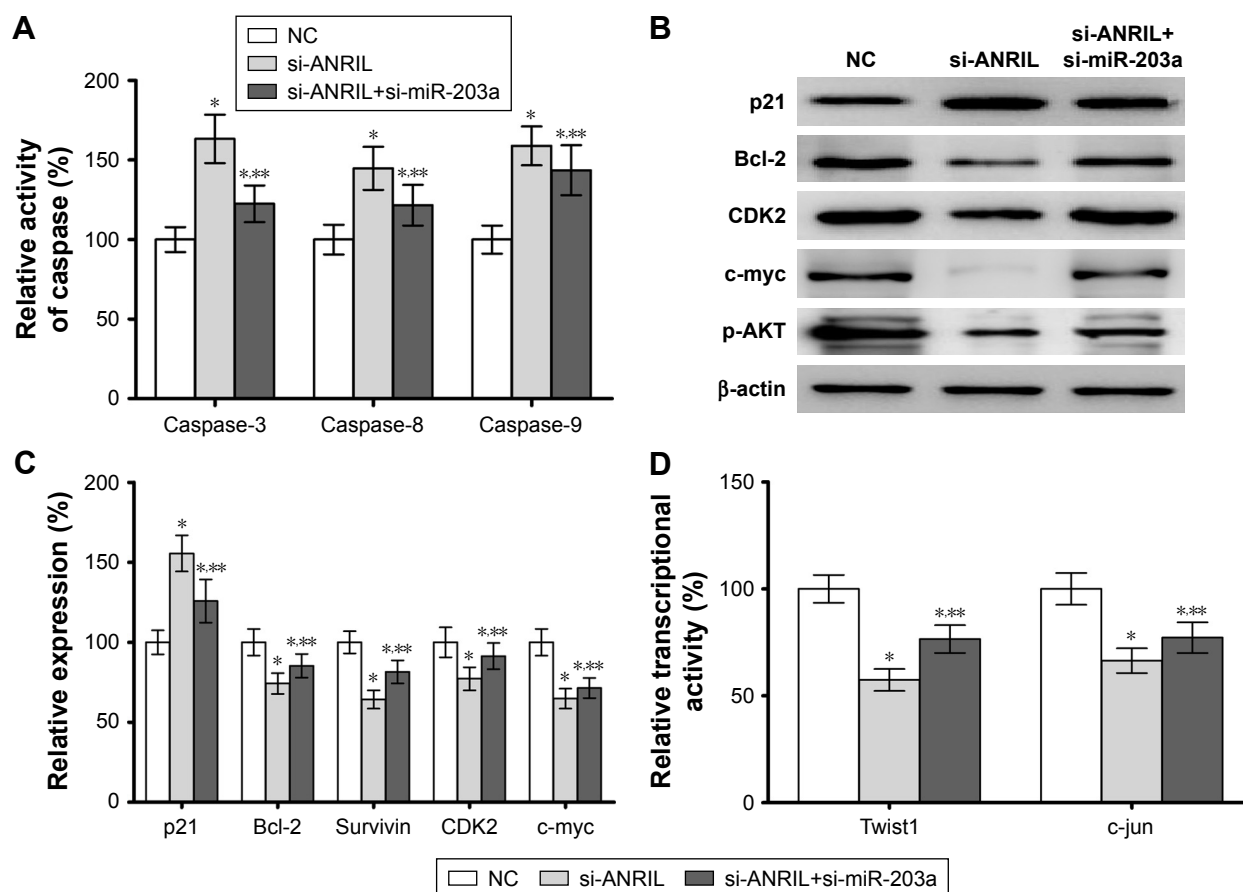


Figure 4 Effect of lncRNA ANRIL silencing on the activity of caspase and expression of tumor-related genes in U251 cells.

Notes: (A) Increased activity of caspase-3/8/9 in lncRNA ANRIL silenced U251 cells; miR-203a mimics could partially reverse this increase. (B) Increased expression of p21 protein and decreased expression of Bcl-2, CDK2, c-myc, and p-AKT proteins in lncRNA ANRIL silenced U251 cells; miR-203a mimics could partially reverse this effect. (C) Increased expression of p21 mRNA and decreased expression of Bcl-2, CDK2, and c-myc mRNA in lncRNA ANRIL silenced U251 cells; miR-203a mimics could partially reverse this function. (D) Decreased transcriptional activity of Twist1 and c-jun in lncRNA ANRIL silenced U251 cells; miR-203a mimics could partially reverse this decrease. *Compared with negative control (NC) group, $P < 0.05$; **compared with si-ANRIL group, $P < 0.05$.

miR-203a is an anti-oncogene that is overexpressed in tumors, and we also found decreased expression in glioma tissues. We found that lncRNA ANRIL silencing could improve the expression of miR-203a, and miR-203a mimics could partially reverse this effect. The result showed that lncRNA ANRIL affected the oncogenic function via miR-203a.

Disclosure

The authors report no conflicts of interest in this work.

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