

Original Article

LncRNA ANRIL overexpression suppresses cell proliferation, migration and invasion but promotes apoptosis in glioma cells

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Abstract: Glioma has emerged as the most malignant form of brain tumors. Mounting evidence demonstrates that dysregulated long noncoding RNAs (lncRNAs) are involved in various cancers, including glioma. However, antisense non-coding RNA in the INK4 locus (ANRIL), a lncRNA involved in different types of tumors, was not fully elucidated for its roles in glioma. Relative ANRIL expression in glioma tissues and glioma cells was detected by quantitative real-time PCR (qRT-PCR). Overexpression of ANRIL was established by transfecting pcDNA3.1-ANRIL into U251 and A172 cells, and its effects on cell proliferation, migration, invasion, and apoptosis were examined using MTT assay, colony formation assay, Transwell migration and invasion assay, and flow cytometry, respectively. We found that ANRIL expression was downregulated in glioma tissues and glioma cells when compared with normal controls. Furthermore, ANRIL overexpression significantly inhibited glioma cell proliferation and invasion, but promoted cell apoptosis *in vitro*. Our findings demonstrate that ANRIL can exert a tumor suppressive role in glioma and may serve as a novel therapeutic target for glioma treatment.

Keywords: Glioma, long noncoding RNAs, ANRIL, malignancy, apoptosis

Introduction

Glioma is one of the most common and lethal types of adult primary brain tumors, accounting for more than 46% of all brain tumors. It can be divided into four histological grades (I to IV) based on the current WHO classification [1]. Although pilocytic astrocytoma (WHO I) is biologically benign and can be cured by surgical removal, malignant gliomas (WHO II-IV) exhibit poor clinical prognosis, and the overall 5-year survival rate of glioblastomas (GBMs, WHO IV) remains less than 5% [2]. Despite the progress in the treatment for glioma, the prognosis in malignant glioma patients remains unsatisfactory [3, 4]. Thus, it is urgent for us to discover the underlying molecular pathology and novel therapeutic targets of glioma, which can provide new ideas for more precise diagnosis and more effective treatment.

Long noncoding RNAs (lncRNAs), non-protein coding transcripts longer than 200 nucleotides, are involved in a wide range of biological pro-

cesses, including epigenetic regulation, transcription and post-transcriptional processing, dosage compensation, chromatin remodeling and nuclear organization [5, 6]. Moreover, current evidence demonstrates that dysregulated lncRNAs correlate with cancer progression and can be used to develop as attractive therapeutic targets in the fight against cancer [5-17].

Among them, the aberrant expression of antisense non-coding RNA in the INK4 locus (ANRIL) has captured substantial attention. ANRIL has been observed in a variety of tumors including bladder [18], colorectal [19], gastric [20], thyroid [21] cancer and nasopharyngeal carcinoma [22]. However, the potential role and underlying mechanism of ANRIL in glioma still remain unclear.

In this study, we explored the expression and biological functions of ANRIL in glioma. Our results showed that ANRIL expression was decreased in glioma tissues and glioma cells when compared with normal controls. In addi-

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tion, overexpression of ANRIL inhibited the proliferation, migration and invasion, but promoted apoptosis of glioma cells. Our results suggest that ANRIL has tumor-suppressive function in glioma and can facilitate the development of lncRNA-based diagnostics and therapeutics of glioma.

Materials and methods

Tissue collection

We collected 10 cases of glioma tissues with I-IV grades and 10 human occipital white matter specimens as non-tumor brain tissues. The diagnosis of glioma was determined using the WHO criteria. Glioma tissues were provided by Henan Provincial People's Hospital. This study was approved by the Research Ethics Committee of Henan Provincial People's Hospital. All patients provided written informed consent.

Cell lines and culture

Four glioma cell lines (U87, U251, A172, D54) and normal human astrocytes (NHA) were obtained from the American Type Culture Collection. The five cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were maintained in humidified air at 37°C with 5% CO₂.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from tissues and cells by using Trizol reagent (Invitrogen) and was then reverse transcribed into cDNAs using the Reverse Transcription Kit (Takara). The cDNA template was synthesized through qRT-PCR using SYBR Premix Dimmer Eraser kit (TaKaRa) by the ABI7900 system (Applied Biosystem). The relative expression of ANRIL was calculated by the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as the internal control. The primer sequences were as follows: GAPDH forward, 5'-GTCAACG-GATTTGGTCTGTATT-3' and reverse, 5'-AGTCTT-CTGGGTGGCAGTGAT-3'. ANRIL forward, 5'-TGC-TCTATCCGCCA-ATCAGG-3', and reverse, 5'-GG-GCCTCAGTGGCACAACC-3'.

Plasmid construction and transfection

ANRIL cDNA of full length was subcloned into the pcDNA3.1 expression vector (Invitrogen).

Cells were transfected with pcDNA3.1-ANRIL or empty pcDNA3.1 vector (pcDNA3.1-NC) as a negative control using Lipofectamine 2000 (Invitrogen). After 24 h of transfection, the transfected cells were subjected to selection in medium containing G418 (400 µg/mL, Invitrogen). ANRIL expression in the transfected cells was detected by qRT-PCR.

Cell proliferation assays

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate cell proliferation. Briefly, cells were harvested for 48 h after transfecting with pcDNA3.1-ANRIL or the empty vector. Subsequently, cells were seeded at a density of 1000-1500 cells/well into 96-well plates for 24 h of incubation. Afterward, 20 µL MTT (Sigma, 5 mg/mL) was added into each well. After 4 h of incubation, the medium was discarded and 150 µL DMSO (Sigma) was added for 10 min of incubation. The absorbance at 490 nm was measured by an enzyme-labeled analyzer. Cellular viability was normalized to control well.

Cell colony formation assay

After transfection, cells were harvested for 48 h and were placed in 6-well plates at density of 500 cells/well. After 10 days of incubation, the colonies were stained with 0.05% crystal violet. The number of colonies containing at least 50 cells was counted. The colony forming rate was calculated by dividing the number of colonies with the number of inoculated cells.

Cell migration and invasion assay

For the cell migration assay, approximately 1×10^5 transfected cells were resuspended in serum-free medium, after which, cells were seeded in the upper chamber of Transwell inserts (8.0 µm, BD Biosciences). Full-serum medium containing DMEM with 10% FBS was added into the lower chamber wells. After 24 h of incubation, non-migratory cells on the upper chambers were scraped off with a cotton swab. Cells cling to the bottom side were fixed with methanol, stained with 0.1% crystal violet and counted in five random fields under a microscope. Cell numbers were averaged and converted to percent migration. The same procedures were followed for the invasion assay, except that the upper chamber was pre-coated

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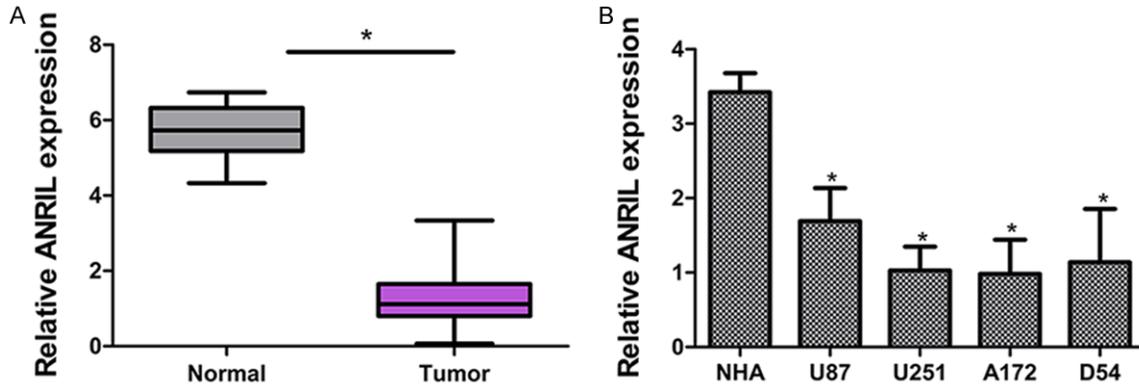


Figure 1. LncRNA ANRIL expression was downregulated in glioma tissues and cells. A. Relative expression of ANRIL in glioma tissues from diverse stages compared with adjacent normal brain tissues. B. ANRIL levels in normal human astrocytes (NHA) and glioma cells (U87, U251, A172, D54). ANRIL expression was determined by qRT-PCR and normalized to GAPDH expression. Results are expressed as mean \pm SD for three independent experiments. * $P < 0.05$ vs. Normal or NHA group.

with a Matrigel (BD Biosciences, San Jose, CA, USA).

Cell apoptosis assay

Cell apoptosis assay was conducted using an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) cell apoptosis detection kit following the instructions ((BD Biosciences). Apoptotic cells were quantified with a flow cytometry system (FACScan®, BD Biosciences) equipped with CellQuest software (BD Biosciences).

Statistical analysis

SPSS 18.0 software was used to perform all statistical analyses. The data are expressed as the mean \pm SD from three independent experiments. The unpaired Student's *t*-test was used to analyze differences between the two groups. One-way ANOVA was used to analyze differences among multiple groups. $P < 0.05$ was considered statistically significant.

Results

LncRNA ANRIL was downregulated expression in glioma tissues and cell lines

To explore the potential roles of LncRNA ANRIL in glioma, we performed qRT-PCR assay to detect the expression of ANRIL in 10 non-neoplastic brain tissues and in 10 human glioma tissues specimens with diverse stages. Data revealed that relative ANRIL expression was lower

in glioma tissues than that in adjacent non-tumor tissues (**Figure 1A**). Then, we evaluated the levels of ANRIL in primary normal human astrocytes (NHA) and the glioma cell lines including U87, U251, A172 and D54. ANRIL expression of each glioma cell line was compared to the average expression of NHA. Our results showed that ANRIL expression was significantly decreased in glioma cells compared with NHA. In addition, U251 and A172 expressed lower levels of ANRIL than that of D54 and U87 (**Figure 1B**). Taken together, these findings suggested that downregulation of ANRIL was correlated with gliomas pathogenesis.

Overexpression of LncRNA ANRIL inhibited proliferation, migration and invasion of glioma cells in vitro

To determine the effect of ANRIL overexpression on glioma cell growth, U251 and A172 cells which exhibited the lowest ANRIL expression were selected for further gain-of-function experiments. We overexpressed ANRIL expression by transfecting cells with pcDNA3.1-ANRIL or empty vector alone. The qRT-PCR analysis showed that levels of ANRIL expression were effectively elevated after transfection with pcDNA3.1-ANRIL (**Figures 2A** and **3A**). Subsequently, the effect of ANRIL overexpression on glioma cell proliferation was investigated *in vitro* using MTT assay and colony formation assay. We found that ANRIL overexpression notably decreased the cell viability of both U251 and A172 cells compared with the control

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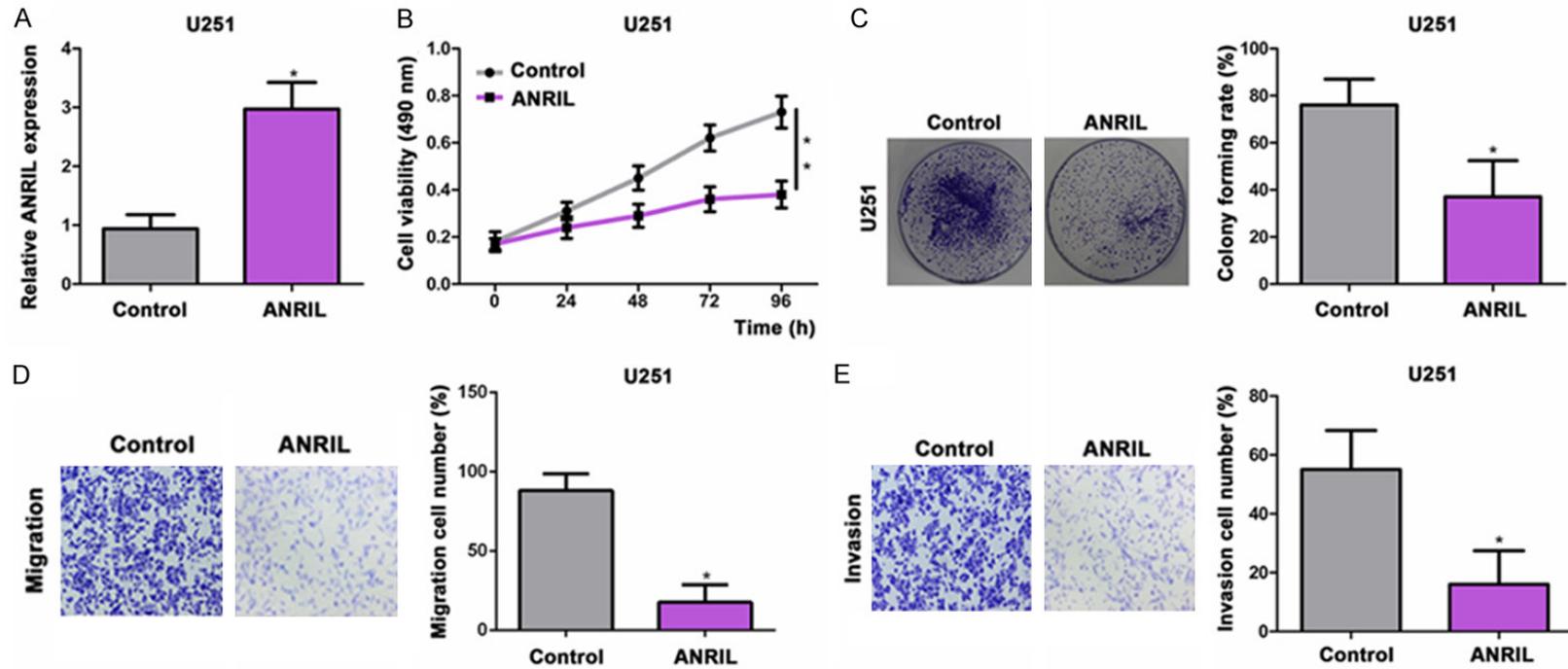


Figure 2. Upregulation of lncRNA ANRIL significantly inhibited the proliferation, migration and invasion of U251 cells. A. Relative ANRIL expression in U251 cells transfected with pcDNA3.1-NC or pcDNA3.1-ANRIL was assessed by qRT-PCR. B. The cell viability of U251 cells transfected with pcDNA3.1-NC or pcDNA3.1-ANRIL was determined by MTT assay. C. Colony formation assay was performed to investigate the colony formation of pcDNA3.1-NC or pcDNA3.1-ANRIL transfected U251 cells. D. Transwell migration assay was conducted to investigate the migration of U251 cells transfected with pcDNA3.1-NC or pcDNA3.1-ANRIL. E. Transwell invasion assay was used to determine the invasion of U251 cells transfected with pcDNA3.1-NC or pcDNA3.1-ANRIL. Results are expressed as mean \pm SD for three independent experiments. ** $P < 0.01$, * $P < 0.05$ vs. Control group (pcDNA3.1-NC).

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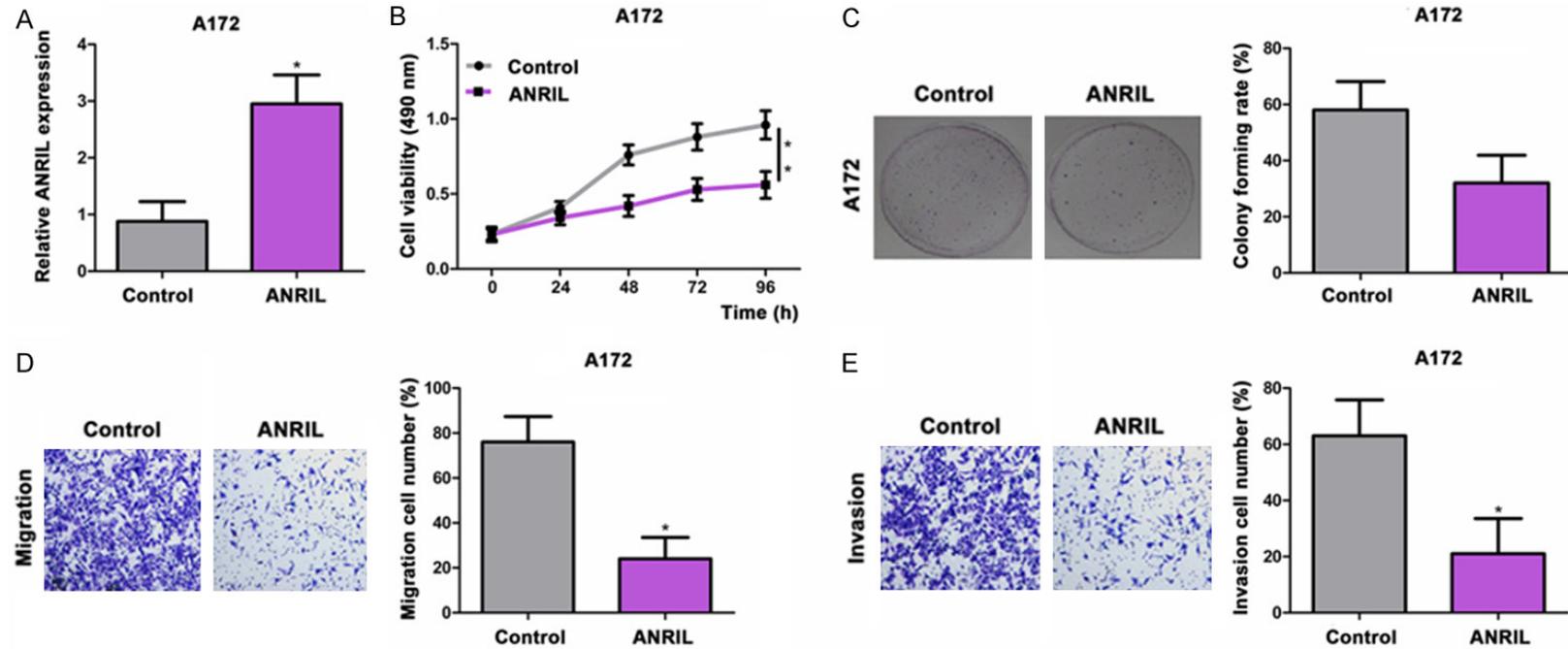


Figure 3. Upregulation of lncRNA ANRIL inhibited the proliferation, migration and invasion of A172 cells in vitro. A. Relative ANRIL expression in A172 cells transfected with pcDNA3.1-NC or pcDNA3.1-ANRIL was assessed by qRT-PCR. B. The cell viability of A172 cells transfected with pcDNA3.1-NC or pcDNA3.1-ANRIL was determined by MTT assay. C. Colony formation assay was performed to investigate the colony formation of pcDNA3.1-NC or pcDNA3.1-ANRIL transfected A172 cells. D. Transwell migration assay was conducted to investigate the migration of A172 cells transfected with pcDNA3.1-NC or pcDNA3.1-ANRIL. E. Transwell invasion assay was used to determine the invasion of A172 cells transfected with pcDNA3.1-NC or pcDNA3.1-ANRIL. Results are expressed as mean \pm SD for three independent experiments. ** $P < 0.01$, * $P < 0.05$ vs. Control group (pcDNA3.1-NC).

group (**Figures 2B and 3B**). Moreover, upregulated expression of ANRIL resulted in a significant decline in colony formation of both U251 and A172 cells compared with the control cells (**Figures 2C and 3C**). These results suggested that overexpression of ANRIL inhibited proliferation of U251 and A172 cells. Next, Transwell migration and invasion assays were performed to analyze the impact of ANRIL overexpression on glioma cell metastasis. Transwell migration assay indicated that the migration ability of U251 and A172 cells transfected with pcDNA3.1-ANRIL was obviously downregulated in comparison with that pcDNA3.1-NC (**Figures 2D and 3D**). Similarly, Transwell invasion assay revealed that upregulation of ANRIL reduced the number of invasive U251 and A172 cells (**Figures 2E and 3E**). Overall, these results demonstrated that overexpression of ANRIL could exert a significant inhibitory effect on proliferation, migration and invasion of glioma cells *in vitro*.

Overexpression of lncRNA ANRIL promoted apoptosis of glioma cells in vitro

Next, flow cytometry assay was performed to examine the impact of ANRIL on apoptosis of glioma cells, data showed that upregulation of ANRIL increased the total number of apoptotic cells and apoptosis rates in both U251 and A172 cells compared to the control group (**Figure 4**), indicating that overexpression of ANRIL could obviously induce apoptosis.

Discussion

Currently, clinical guidelines for treatment of lower-grade gliomas are less well standardized [23]. Standard treatment for glioblastoma (WHO IV) is surgical resection followed by concomitant radiotherapy and chemotherapy with temozolomide [4]. Despite progress in conventional diagnosis and treatment, the clinical outcome of glioma patients remains far from satisfactory. Metastasis and spread of gliomas constitute the major causes of death in glioma patients [24]. Thus, identification of novel therapeutic targets or methods to effectively suppress the growth and metastasis of gliomas is imperative. Recently, therapeutic strategies have focused on lncRNAs expressed aberrantly in gliomas.

Mounting evidence implied that lncRNAs could drive multiple crucial cancer phenotypes [11].

For instance, Zhang *et al.* stated that lncRNA SPRY4-IT1 (SPRY4 intronic transcript 1) was highly expressed in clear cell renal cell carcinoma tissues and predicted poor prognosis [25]. Sun *et al.* reported that upregulation of lncRNA GAS5 (growth arrest-specific 5) inhibited the proliferation but induced the apoptosis of gastric cancer cells *in vitro* [26]. In glioma, lncRNAs also appeared to exert key roles in tumorigenesis [27, 28]. Differential expression of lncRNAs between glioma and normal tissues has been described in genome-wide association studies. For example, both SPRY4-IT1 and HOTAIR (Hox transcript antisense intergenic RNA) were upregulated in glioma and associated with poor clinical outcome [29, 30]. Additionally, overexpression of HOXA11-AS (the antisense transcript of the HOXA11 gene) promoted glioma cell proliferation *in vitro* [31]. Similarly, upregulation of H19 promoted invasion and angiogenesis of glioblastoma cells [32]. Knockdown of NEAT1 (nuclear enriched abundant transcript 1) reduced glioma proliferation, invasion, and migration [33]. Contrary to the above oncogenic lncRNAs, growing evidence reported a few lncRNAs downregulated in glioma tissues which could act as tumor suppressors. For instance, overexpression of lncRNA CASC2 (cancer susceptibility candidate 2) suppressed glioma cell proliferation, migration, and invasion but promoted cell apoptosis [34]. Overexpression of lncRNA MDC1-AS (mediator of DNA damage checkpoint protein 1 antisense RNA) inhibited glioma cell proliferation [35]. lncRNA TUG1 (taurine upregulated gene 1) has been described to promote glioma cell apoptosis [36]. However, ANRIL, a lncRNA observed to be upregulated in many tumors including lung cancer [37], hepatocellular carcinoma [38], gastric cancer [39], bladder cancer [18], ovarian cancer [40], and nasopharyngeal carcinoma [22], remains still under investigation for its expression pattern and functional role in glioma.

In the present study, we found that lncRNA ANRIL was significantly downregulated in human glioma tissues compared with the normal brain tissues. Our findings were supported by bi-hierarchical clustering of all 90 investigated lncRNAs in 30 glioma tissue specimens (WHO II-IV) [41]. Moreover, lower expression of ANRIL was also found in glioma cells compared with normal human astrocytes. To further explore the underlying mechanism of ANRIL in

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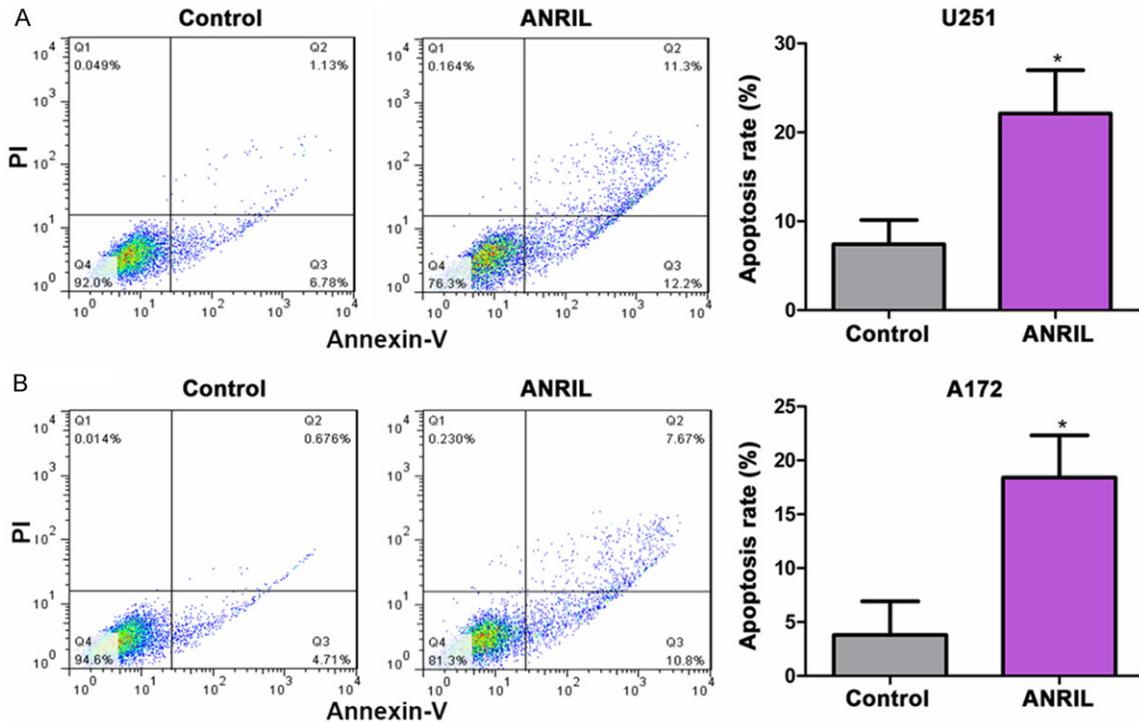


Figure 4. Upregulation of lncRNA ANRIL significantly promoted glioma cell apoptosis *in vitro*. A. Flow cytometry assay was performed to analyze the cell apoptosis of U251 cells transfected with pcDNA3.1-NC or pcDNA3.1-ANRIL. B. Cell apoptosis in A172 cells transfected with pcDNA3.1-NC or pcDNA3.1-ANRIL was detected by flow cytometry assay. Results are expressed as mean \pm SD for three independent experiments. * $P < 0.05$ vs. Control group (pcDNA3.1-NC).

glioma, we evaluated the effects of ANRIL overexpression on the malignancy of glioma cells. Our results showed that overexpression of ANRIL significantly repressed the proliferation, migration and invasion but promoted the apoptosis of glioma cells *in vitro*, which is similar to the expression patterns and tumor suppressive functions possessed by the above-mentioned CASC2, MDC1-AS as well as TUG1. Although the effects of ANRIL on the cell malignancy and apoptosis has been elucidated for the first time with our efforts, more studies are needed to explore the regulatory network of ANRIL in glioma and examine the association between the downregulated expression of ANRIL and clinicopathological characteristics in patients with glioma.

Conclusion

In summary, ANRIL was downregulated in glioma tissues and cells. Overexpression of ANRIL could significantly repress the proliferation, migration and invasion but promote the apoptosis of glioma cells. These findings suggest

that ANRIL may serve as a tumor suppressor in glioma and a novel therapeutic target for future therapy for glioma.

Disclosure of conflict of interest

None.

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