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Original Article

Adsorption of miR-218 by lncRNA HOTAIR regulates PDE7A and affects glioma cell proliferation, invasion, and apoptosis

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Abstract: Objective: To evaluate the role of targeted adsorption of miR-218 by long-chain non-coding RNAHOTAIR to regulate PDE7A on glioma cell proliferation, invasion, and apoptosis. Methods: The expressions of lncRNA HOTAIR, miR-218, and PDE7A in glioma tissues and normal parcancer tissues, NHA and glioma cell lines were determined, and correlations among the three genes were analyzed. The subcellular localization of lncRNA HOTAIR was determined by fluorescent in situ hybridization. Dual-luciferase reporter assay was used to validate the targeted relationship between lncRNA HOTAIR/miR-218/PDE7A. Glioma cells were grouped to receive intervention of lncRNA HOTAIR or miR-218. MTT, transwell, and flow cytometry were performed to determine the proliferation, invasion, and apoptosis of cells. Results: Compared with the normal tissues and cells, the expression of lncRNA HOTAIR was increased while miR-218 was suppressed in glioma tissues samples and cells (all $P<0.05$). Inhibition of lncRNA HOTAIR expression, was able to induce apoptosis and suppress the proliferation and invasion of cells (all $P<0.05$). LncRNA HOTAIR is mainly localized in the cytoplasm, and is able to adsorb miR-218 as ceRNA. The effect of knockdown of HOTAIR on glioma cells could be partially rescued by miR-218 inhibitor. The expression of PDE7A was enhanced in glioma tissues and cells compared to normal tissues and cells (all $P<0.05$), which positively correlated with the expression of HOTAIR ($r=0.546$, $P<0.05$) and negatively correlated with the expression of miR-218 ($r=0.363$, $P<0.05$). The targeted relationship between miR-218 and PDE7A was validated: Overexpression of miR-218 was able to suppress the proliferation and invasion of glioma cells and restrain apoptosis compared to the miR-NC group (all $P<0.05$). The effect of miR-218 on glioma cells could be partially rescued by PDE7A. Conclusion: lncRNA HOTAIR can adsorb miR-218 to regulate expression of PDE7A and promote the malignant biologic behavior of glioma cells.

Keywords: lncRNA HOTAIR, miR-218, glioma, proliferation, invasion, apoptosis

Introduction

Gliomas, also known as neurogliocytoma, are common primary intracranial tumors that affect the central nervous system of the brain and spinal cord [1, 2]. They can be divided into two categories according to the degree of glioma malignancy: low-grade glioma (grade I-II) and high-grade glioma (grade III-IV), of which glioblastoma multiforme is the most malignant type [3-5]. Although modalities such as targeted therapy, surgery, radiotherapy and chemotherapy have greatly improved the prognosis, the overall prognosis remains poor [6-8]. The life expectancy of patients with low-grade glioma is about 5-10 years, while the that of patients with high-grade glioma is about 1 year. The tumor recurrence rate remains high [9, 10]. Therefore, it is necessary to explore new biomarkers and elucidate the molecular mechanisms of glioma progression for the diagnosis and treatment of glioma.

Long non-coding RNAs (LncRNAs) are more than 200 nucleotides in length and lack protein-coding functions [11, 12]. LncRNAs can interact with miRNAs as competing endogenous RNAs (ceRNAs) and adjust the expression of miRNA target genes, and recent research has found that lncRNAs play a role as carcinogenic factors or tumor-suppressor factors in the development of a variety of tumors. For example,

lncRNAZFPM2-AS1 is a miRNA sponge molecule to promote the invasion of liver cancer cells by regulating miR-13/GDF10 [13]. Li et al. found that lncRNA SNAI3-AS1 promoted PEG10-mediated proliferation and metastasis of liver cancer cells by inducing miR-27a-3p and miR-34a-5p [14]; lncRNA H1FX-AS1 axis regulated miR-324-3p/DACT1 as a tumor suppressor factor in cervical carcinoma. H1FX-AS1 overexpression was able to suppress the proliferation, migration, and invasion of cervical cancer cells and induce apoptosis [15]. As one of the long non-coding RNA molecules, the role of HOTAIR in glioma has been revealed; for example, Liu et al. found that HOTAIR as a competitive endogenous RNA promotes the progression of glioma through sponging miR-126-5p [16]. It has also been found that HOTAIR enhances angiogenesis by inducing the expression of VEGFA in glioma cells and transmitting it to endothelial cells through glioma cell-derived extracellular vesicles [17]. We further discuss the role of HOTAIR on glioma cell proliferation, invasion, and apoptosis and the regulatory relationship between HOTAIR and other factors.

The function of miR-218 in glioma has also been reported, and Luo et al. confirmed that miR-218 has low expression in glioma development [18]. Previous studies have also found that HOTAIR is able to regulate miR-218 involved in drug resistance in colorectal cancer cells [19]. But the ability of HOTAIR to regulate miR-218 and then play a role in glioma has not been fully elucidated.

We identified PDE7A through the targeted relationship prediction website. PDE7A is a downstream target gene of miR-218 and it is a member of the PDE superfamily. It has been confirmed that PDE7A expression is up-regulated in endometrial cancer, and silencing of PDE7A significantly inhibits the migration and invasion of cancer cells [20]. While there have been no relevant reports on PDE7A in gliomas, we speculated that PDE7A may be involved in the development of glioma.

Thus, the purpose of this study was to investigate the effects of HOTAIR-targeted regulation of the miR-218/PDE7A axis in glioma.

Materials and methods

Tissue specimen collection

In this study, we collected tumor tissues from 43 glioma patients who underwent surgery at The Second People's Hospital of Guiyang (Jinyang Hospital) as well as normal brain tissues from patients who were treated for traumatic brain injury in our hospital. The ages of glioma patients ranged from 33 to 62 years old and included 27 males and 16 females.

Inclusion criteria: Patients confirmed to have glioma by histopathological examination; patients were newly diagnosed glioma cases; patients haven't received any treatment. Exclusion Criteria: Patients who had other clinical diseases; those with recurrent cases of glioma.

All patients signed an informed consent form, and the trial was approved by the Ethics Committee of The Second People's Hospital of Guiyang (Jinyang Hospital). All the tissue samples were confirmed by histopathologic examination, and all tissue samples after resection were stored at -80°C.

Cell culture

Human normal astrocyte cell line NHA was purchased from ATCC (USA) and glioma cell lines A172, LN229, SHG44, U87 and U251 were cultured in RPMI 1640 medium (Beijing Solaibao Technology Co., Ltd., China) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5% CO_2. The expression differences of HOTAIR and miR-218 in NHA and glioma cell lines were observed by qRT-PCR and the glioma cell lines with the most significant differences were selected for subsequent experiments.

Cell grouping and transfection

After screening glioma cell lines, the cell lines used for subsequent experiments were divided into the following groups: control group (glioma cells without treatment), NC group (cells transfected with HOTAIR negative control sequence), sh-HOTAIR group (cells transfected with HOTAIR shRNA), miR-NC group (cells transfected with miR negative control sequence), miR-218 mimic group (cells transfected

Table 1. Primer sequences

Name	Sequence
HOTAIR	Forward: 5'-TAGGCAAATGTCAGAGGGTT-3'
	Reverse: 5'-ACACAAGTAGCAGGGAAAGG-3'
PDE7A	Forward: 5'-GGAAATAGTCTAGTAAGCTTAACC-3'
	Reverse: 5'-GGCAGATGTGAGAATAAGCCTG-3'
GAPDH	Forward: 5'-CGAGATCCCTCCAAAATCAA-3'
	Reverse: 5'-TTCACCCATGACGAACAT-3'
MiR-218	Forward: 5'-TCTTCCTGGATGTTGTTCCCGA-3'
	Reverse: 5'-GCCTACTTCAGCTAGTCAC-3'
U6	Forward: 5'-CTCGCTTCGGCACA-3'
	Reverse: 5'-AACGCTTCACGAATTTGCGT-3'

with miR-218 inhibitor), miR-218 mimic group (cells transfected with miR-218 inhibitor), sh-HOTAIR + miR-NC group (cells transfected with HOTAIR shRNA and miR negative control sequence), sh-HOTAIR + miR-218 mimic group (cells transfected with HOTAIR shRNA and miR-218 inhibitor), and miR-218 + PDE7A (cells transfected with miR-218 mimic + PDE7A inhibitor). Both HOTAIR and PDE7A were transfected using vector vectors (Hunan Fenghui Biotechnology Co., Ltd., China). MiRNA and negative control sequences were devised and synthesized by Shanghai Jima Pharmaceutical Technology Co., Ltd. (China). Transfection steps were performed according to the Lipofectamine-TM 3000 kit (Thermo Fisher, USA) instructions.

qRT-PCR

Total RNA was extracted from tissue samples and cells by TRIzol reagent (Beijing Solaibao Technology Co., Ltd., China). The concentration and purity of RNA were determined by UV spectrophotometer (Shanghai Meipida Instrument Co., Ltd., China), and cDNA was synthesized by reverse transcription of RNA according to PrimeScript™ RT-PCR kit (Thermo Fisher, USA). The expression of HOTAIR, miR-218, and PDE7A was determined by PCR according to the instructions of SYBR Premix Ex Taq kit (Beijing Zhijie Fangyuan Technology Co., Ltd., China). U6 was used as an internal control for miR-218, and GAPDH was used as housekeeping gene for the remaining genes. All primers in this study were designed and synthesized by BGI GENE (China). Correlation factor expression levels were calculated with the $2^{-\Delta\Delta Ct}$. The primer sequences are shown in **Table 1**.

Fluorescence in situ hybridization

The subcellular localization of HOTAIR was detected with FISH kit (Guangzhou Ruibo Biotechnology Co., Ltd., China). The cell suspension was placed on the bottom of a 24-well plate, 5×10^3/well. After culture for 24 h, the supernatant was removed, the cells were cleaned with 1× PBS (Shanghai Kanglang Biotechnology Co., Ltd., China), and after fixation with 4% paraformaldehyde (Shanghai Yibo Biotechnology Co., Ltd., China), 0.5% TritonX-100 (Shanghai Yuanmu Biotechnology Co., Ltd., China) in PBS was added. The prehybridization solution was blocked at 37°C, the HOTAIR probe was hybridized overnight at 37°C, and the cells were cleaned with hybridization wash solution at 42°C in the dark. The hybridization area of the section was stained with DAPI, and the cells were fixed on the slide with mounting medium in the dark. Experimental specimens were viewed under a confocal laser scanning microscope.

Dual-luciferase reporter assay

The relationships between HOTAIR and miR-218, or miR-218 and PDE7A were calculated by the targeted relationship prediction website. Afterwards, wild-type and mutant luciferase reporter plasmids for HOTAIR, and wild-type, and mutant luciferase reporter plasmids for PDE7A were constructed, and all plasmids were co-transfected with miR-218 mimic or mimic NC into HEK-293T cells (purchased from ATCC, USA). The luciferase activity of the cells was detected by the dual-luciferase reporter assay kit (Beijing Solaibao Technology Co., Ltd., China) after 48 hours.

Western blot

RIPA lysis solution (Beijing Solaibao Technology Co., Ltd., China) precooled on ice was added to the cells to lyse the cells, and the supernatant was collected after centrifugation. We quantified the protein using a BCA protein quantification kit (Beijing Solaibao Technology Co., Ltd., China), and after adjusting the protein concentration, the protein was placed in boiling water for boiling denaturation. Afterwards, proteins were separated by 12% SDS-PAGE and transferred to PVDF membranes. After blocking by TBST (Beijing Solaibao Technology Co., Ltd., China) containing 5% non-fat dry milk 1 h, the

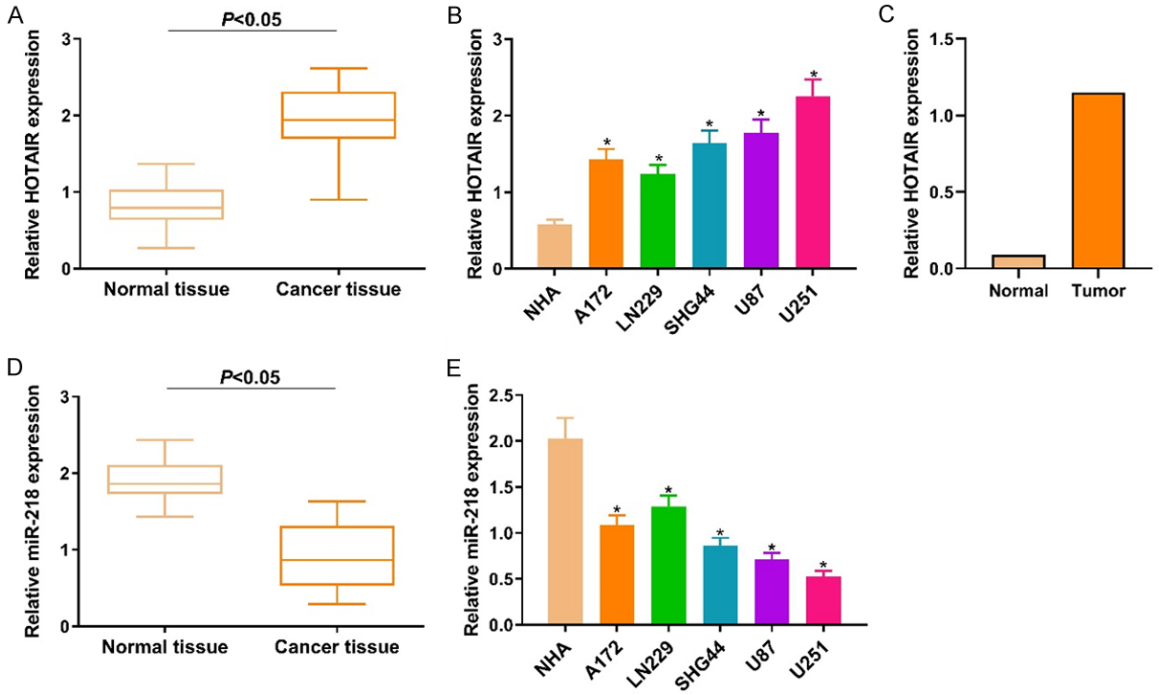


Figure 1. Expression of HOTAIR and miR-218 in glioma tissues and cells. A. Expression of HOTAIR in tissues; B. Expression of HOTAIR in cell lines; C. High expression of HOTAIR in glioma was shown in the lncRNA SNP database; D. Expression of miR-218 in tissues; E. Expression of miR-218 in cell lines. Compared with NHA, *P<0.05.

membranes were incubated with primary antibody PDE7A (1:1000, ab249158, Abcam, UK) overnight at 4°C, and then incubated with secondary antibody (1:1500, ab150077, Abcam, UK) for 1 h at room temperature. Finally, after rinsing the membrane three times, it was developed with an ECL kit (Beijing Solaibao Technology Co., Ltd., China) in the dark. Image software was used for quantitative analysis of proteins, and GAPDH (1:1000, ab8245, Abcam, UK) was used as housekeeping gene.

MTT

About 3×10^4 cells/well were seeded into a 96-well plate. After culture for 24 h, 48 h and 72 h, 10 μL of 0.5% MTT solution (Shanghai Biyuntian Biotechnology Co., Ltd., China) was added into each well. The absorbance value at 490 nm of each well was detected by a microplate reader (Thermo Fisher, USA) after continuous culture for 2 h.

Transwell

The upper chamber of the transwell was coated with matrigel (Beijing Solaibao Technology Co., Ltd., China). 1×10^5/cells were suspended in 200 μL serum-free DMEM medium (Beijing Solaibao Technology Co., Ltd., China). 500 μL DMEM medium containing 10% fetal bovine serum (Beijing Solaibao Technology Co., Ltd., China) was added to the lower chamber of the transwell, and the invasive cells were wiped off with a cotton swab after 48 hours of incubation at room temperature. Successfully invaded cells were fixed with 4% paraformaldehyde (Shanghai Biyuntian Biotechnology Co., Ltd., China) and stained with 0.5% crystal violet (Shanghai Biyuntian Biotechnology Co., Ltd., China). Cells were counted under an inverted microscope (OLYMPUS, Japan).

Flow cytometry

The cells were re-suspended at 1×10^6/mL, and Annexin V-FITC and PI staining solutions were prepared according to Annexin V-FITC Apoptosis Detection Kit (Shanghai Biyuntian Biotechnology Co., Ltd., China). Then, 1 mL cells suspension was added with 10 μL Annexin V-FITC and PI staining solutions and incubated for 20 min in the dark. Cell apoptosis rate of each group was detected by flow cytometry (Thermo Fisher, USA).

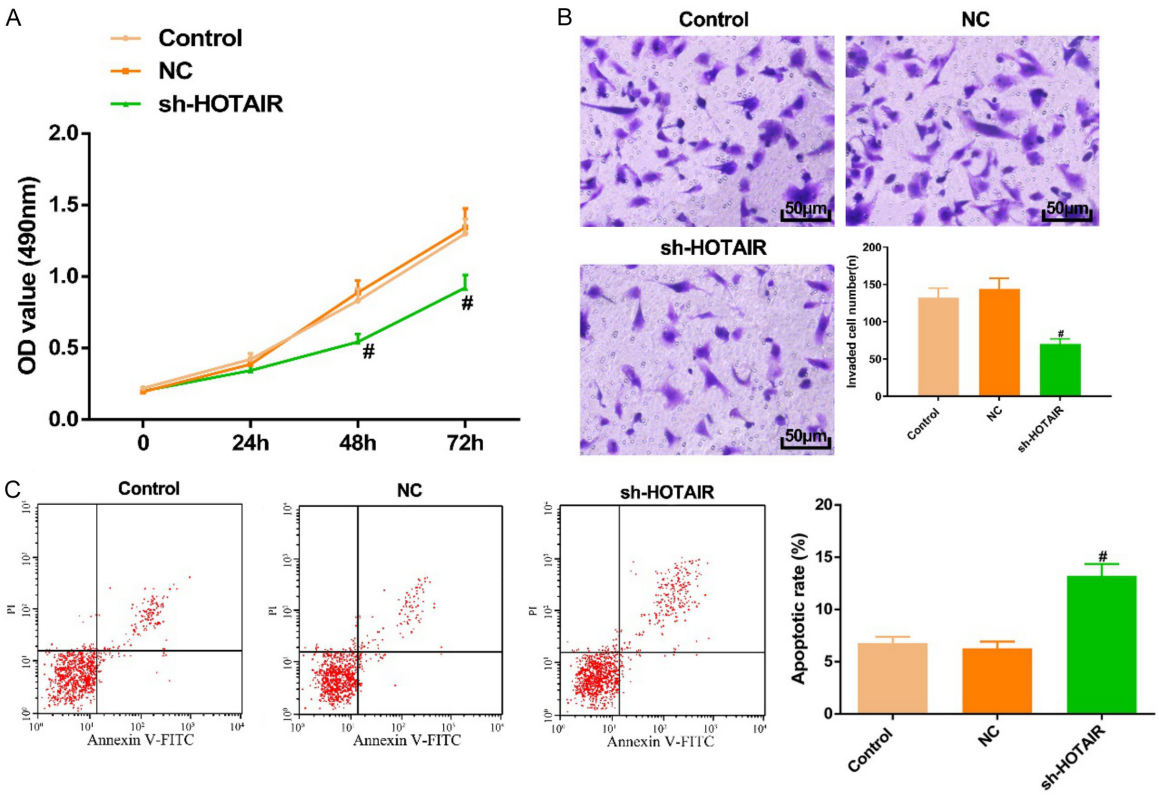


Figure 2. The role of HOTAIR knockdown in U251. A. Cell proliferation; B. Cell invasion (200×); C. Apoptosis detection. Compared with the control group, #P<0.05.

Statistical analysis

SPSS 21.0 software was used for data analysis, and measurement data were expressed in the form of mean ± standard deviation. Comparisons between two groups were handled using the t-test, and one-way analysis of variance was employed for comparisons between multiple groups, followed by Bonferroni post-hoc test. P<0.05 was deemed significant.

Results

HOTAIR is up-regulated and miR-218 is down-regulated in glioma tissues and cells

As shown in **Figure 1**, the expression of HOTAIR was significantly increased in glioma tissues and various glioma cell lines compared with normal paracancer tissues and normal astrocyte cell lines NHA (all P<0.05; **Figure 1A**, **1B**), which is consistent with the high expression of HOTAIR in glioma (GBM) shown in the lncRNA SNP database (**Figure 1C**). In addition, compared with paracancer tissues and normal astrocyte cell line NHA, miR-218 expression was significantly reduced in glioma tissues and cells (all P<0.05) and its expression changed most significantly in U251 cells (**Figure 1D**, **1E**). Thus, U251 was selected for a series of subsequent experiments.

Inhibition of HOTAIR can induce apoptosis and suppress the proliferation and invasion of cells

The expression of HOTAIR was inhibited in cell line U251. Compared with the NC group, the proliferation and invasion of sh-HOTAIR group cells were weakened, and the apoptosis rate showed an increasing trend (all P<0.05). However, there was no significant difference between the control group and NC group (all P> 0.05) (**Figure 2**).

Targeted adsorption of miR-218 by HOTAIR as ceRNA

The results of FISH showed that HOTAIR was expressed in the cytoplasm and nucleus in U251 cells, but mainly localized in the cytoplasm, where it could function as ceRNA. The bioinformatics prediction website revealed that there was a binding site between HOTAIR and miR-218, and dual-luciferase reporter assay sh-

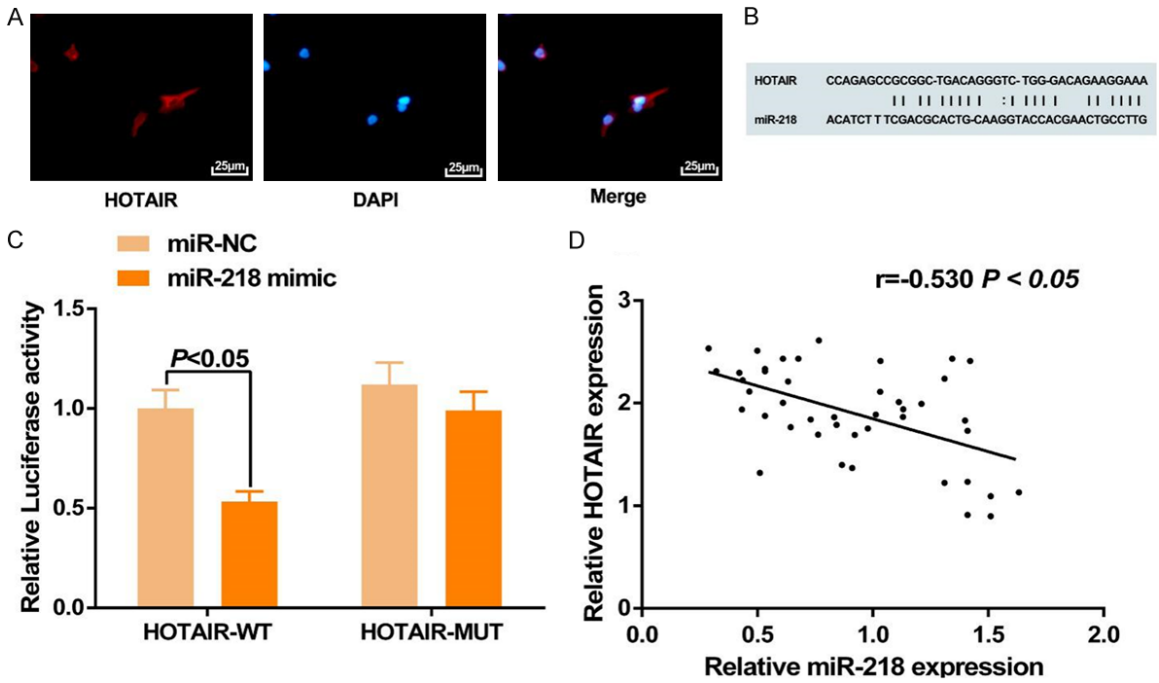


Figure 3. HOTAIR acts as a ceRNA for miR-218. A. FISH assay results (400×); B. The bioinformatics prediction website results of HOTAIR and miR-218; C. Dual-luciferase reporter assay results of HOTAIR and miR-218; D. Correlation analysis of HOTAIR and miR-218.

owed that miR-218 was able to suppress the luciferase activity of HOTAIR wild-type luciferase reporter plasmid (P<0.05) and did not have an actual effect on HOTAIR mutant luciferase reporter plasmid (P>0.05). Correlation analysis indicated a negative correlation between HOTAIR and miR-218 (P<0.05) (**Figure 3**).

Knockdown of the effect of HOTAIR could be partially rescued by miR-218 inhibitor

The expression of HOTAIR and miR-218 were regulated in cell line U251, and the results showed that, silencing HOTAIR was able to induce apoptosis and suppress the proliferation and invasion of the cells when compared with the NC group (all P<0.05). However, there was no significant difference in cell apoptosis, proliferation and invasion between sh-HOTAIR group and sh-HOTAIR + inhibitor NC group (P> 0.05). The effects of sh-HOTAIR on apoptosis, proliferation and invasion in U251 cells could be partially reversed by miR-218 inhibitor, (**Figure 4**).

PDE7A is a target of miR-218 and is regulated through HOTAIR

The bioinformatics prediction website showed that there might be a binding site between miR-218 and PDE7A, and the dual luciferase reporter system assay confirmed that PDE7A was a target of miR-218. In addition, we found that knockdown of HOTAIR was able to significantly inhibit PDE7A protein expression in cells (P< 0.05), while this phenomenon was reversed after transfection with miR-218 inhibitor. Correlation analysis showed that HOTAIR was positively correlated with PDE7A, and miR-218 was negatively correlated with PDE7A (all P< 0.05) (**Figure 5**).

The effect of miR-218 on cells could be partially rescued by PDE7A overexpression

By regulating the expression of miR-218 and PDE7A in U251 cells, the proliferation activity and invasion ability of the miR-218 mimic group were lowered, and the rate of apoptosis was elevated compared with the miR-NC group (P<0.05). The effect of miR-218 overexpression on glioma cells could be partially rescued by PDE7A overexpression. There was no significant difference in the indexes between the control group and miR-NC group (all P>0.05). The experimental results indicated that the effect of overexpression of miR-218 could be reversed by up-regulation of PDE7A (**Figure 6**).

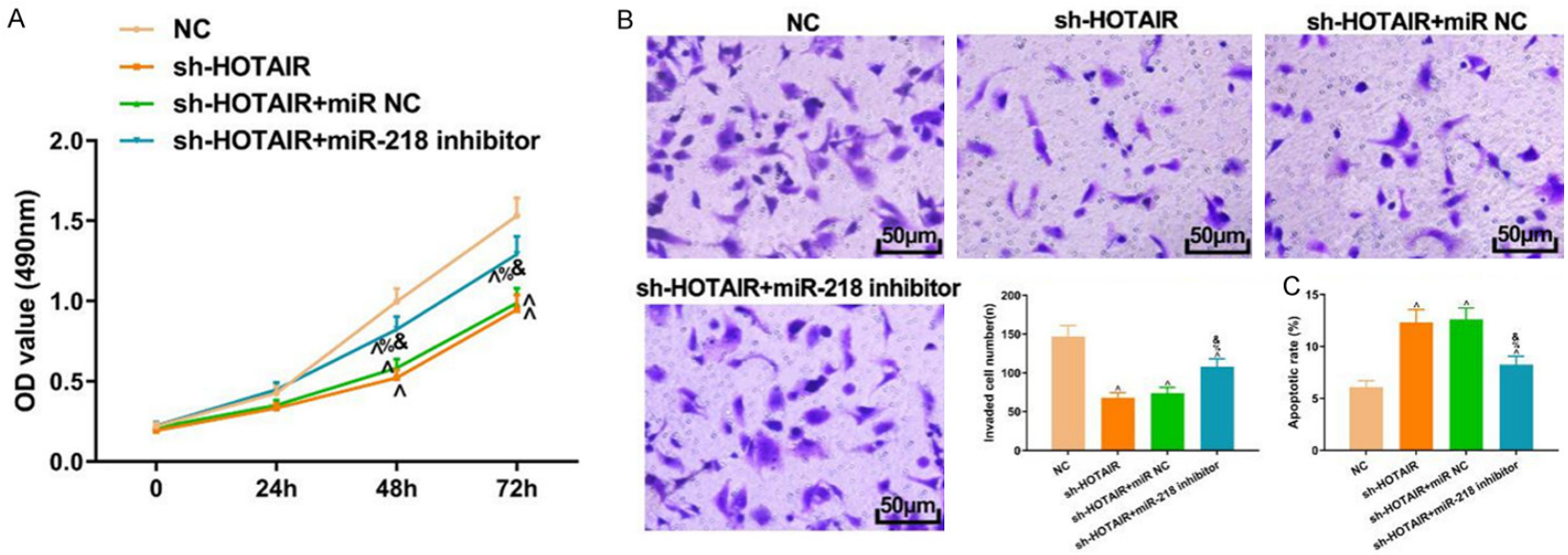
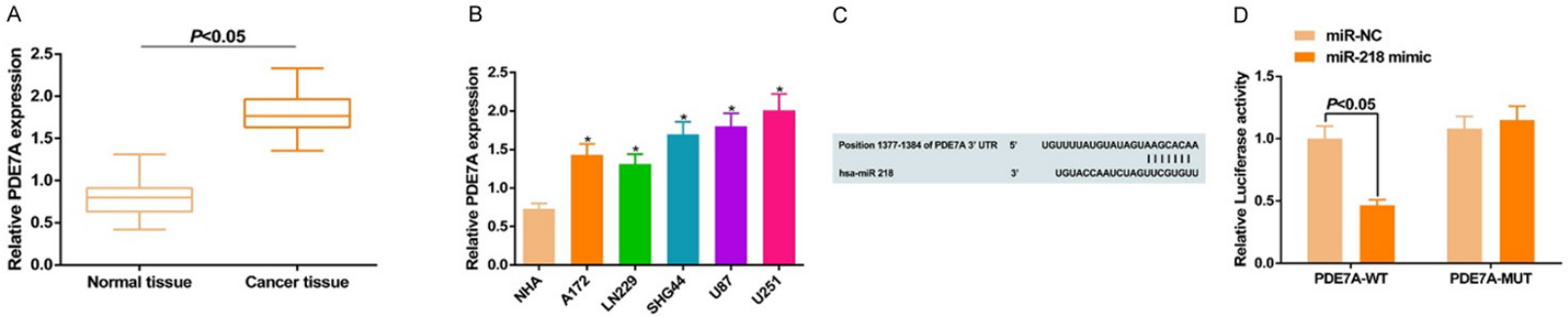


Figure 4. The effect of knockdown HOTAIR on U251 cells could be partially rescued by miR-218 inhibitor. A. Cell proliferation; B. Cell invasion (200×); C. Apoptosis detection. Compared with the NC group, ^P<0.05; compared with the sh-HOTAIR group, %P<0.05; compared with the sh-HOTAIR + miR NC group, &P<0.05.



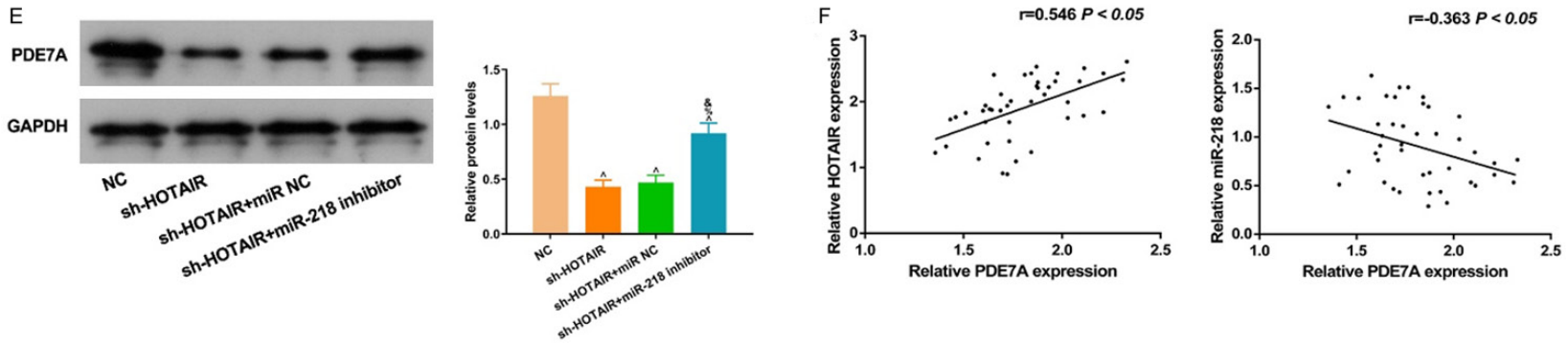


Figure 5. HOTAIR regulates PDE7A expression in U251 cells by regulating miR-218. A. mRNA expression of PDE7A in tissues; B. mRNA expression of PDE7A in cells; C. The result of a bioinformatics prediction website regarding miR-218 and PDE7A; D. The results of a dual-luciferase reporter assay of miR-218 and PDE7A; E. Cellular protein expression level; F. Correlation analysis. Compared with NHA, $^{*}P<0.05$, compared with NC group, $^{\wedge}P<0.05$; compared with sh-HOTAIR group, $^{\%}P<0.05$; compared with sh-HOTAIR + miR NC group, $^{\&}P<0.05$.

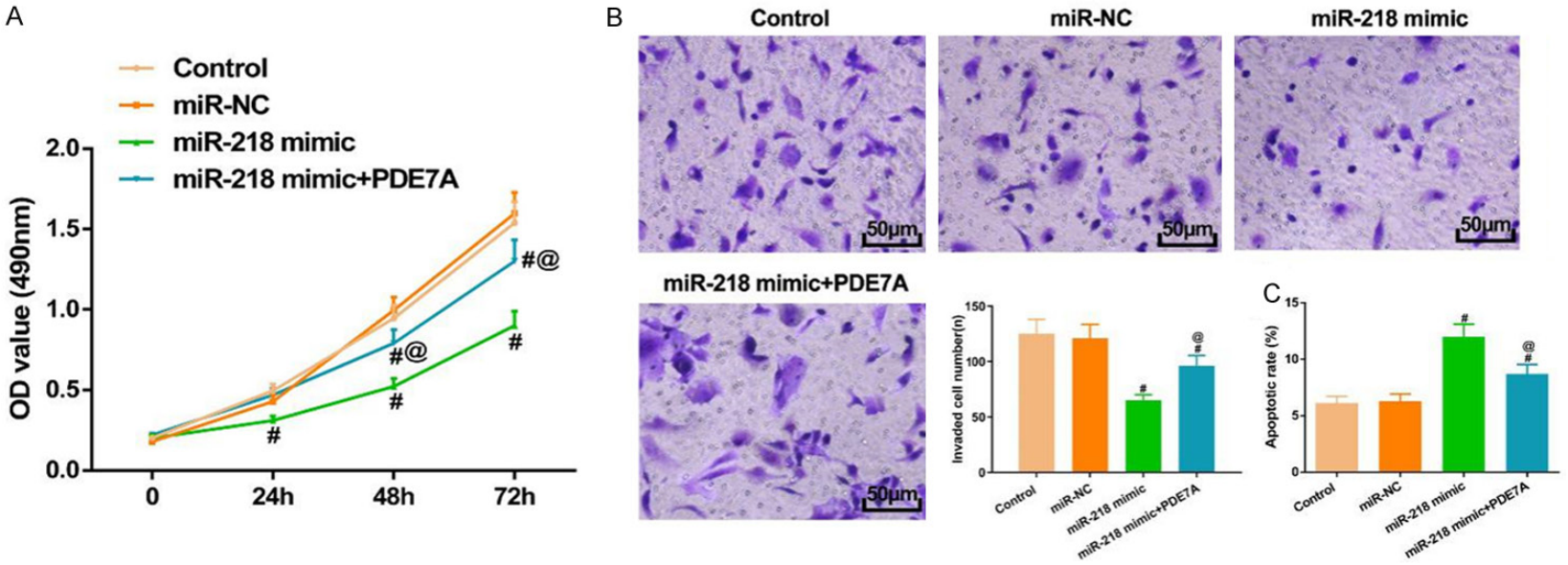


Figure 6. The effect of miR-218 on U251 cells could be rescued by PDE7A overexpression. A. Cell proliferation; B. Cell invasion (200×); C. Apoptosis detection. Compared with the control group, $^{\#}P<0.05$; compared with miR-218 mimic, $^{@}P<0.05$.

Discussion

Glioma has a high recurrence rate and mortality, with median survival often less than 14 months as gliomas are characterized by high migration and invasion [21-23]. Recently it has been found many lncRNAs are dysregulated in glioma and can be used as effective therapeutic targets for identifying glioma patients, and it has been reported that lncRNA TTN-AS1 upregulates RUNX1 through sponging miR-27b-3p to promote glioma progression [24]. Related studies have also confirmed that lncRNA SNHG11 promotes glioma cell proliferation, invasion, and migration through targeted miR-154-5p [25]. Therefore, it is of great significance to investigate the function of lncRNAs in gliomas.

Recently, it has been found that HOTAIR is dysregulated in ovarian cancer, renal cancer, breast cancer and colorectal cancer, and it plays an important role in the proliferation, migration, invasion, autophagy, and resistance of cancer cells. For example, knocking down its expression can inhibit the malignant biologic behavior of the above cancer cells [26-29]. The role of HOTAIR in glioma has been revealed to some extent, which was consistent with previous studies [16, 17]. We found that silencing HOTAIR was able to effectively inhibit the proliferation and invasion of glioma cells and promote apoptosis.

LncRNAs can function by sponges adsorbing downstream MiRNAs and regulating their expression. To further validate the role of HOTAIR, we found miR-218 was a potential target of HOTAIR by bioinformatics prediction web site. As one of the microRNA molecules, miR-218 plays a role as a tumor suppressor in prostate adenocarcinoma, non-small cell lung cancer, triple-negative breast cancer, and hepatic carcinoma [30-33]. It has been reported that miR-218 shows low expression in glioma [18]. This study confirmed that HOTAIR could function as a ceRNA of miR-218 by nucleocytoplasmic separation assay, dual-luciferase reporter, and correlation analysis. In addition, the role of miR-218 as a tumor suppressor in glioma was further confirmed. Up-regulation of miR-218 could induce apoptosis of glioma cells and enhance cell proliferation and invasion; however, down-regulation of miR-218 has the opposite experimental changes. Inhibition of miR-218 expression was able to partially reverse the effects of silencing HOTAIR on glioma cell proliferation, invasion, and apoptosis.

miRNAs act by regulating downstream target gene levels. We found PDE7A was a downstream target gene of miR-218 through the targeted relationship prediction website. PDE7A was previously found to play a role as a cancer-promoting factor in intestinal and endometrial cancer [34, 35]. In addition, some studies have found PDE7A gene polymorphism is closely related to genetic variation and cognitive function in patients with brain tumors [36]. However, there was no study on its involvement in glioma cell proliferation, invasion, and apoptosis. This study confirmed that PDE7A is a target of miR-218 by dual-luciferase reporter assay and correlation analysis, and western blot results showed inhibition of HOTAIR was able to effectively inhibit PDE7A expression in glioma cells. Inhibition of miR-218 could rescue this phenomenon, suggesting that HOTAIR is able to upregulate PDE7A by downregulating the expression of miR-218. Although overexpression of miR-218 can effectively inhibit the proliferation and invasion of glioma cells and promote apoptosis, this effect can be partially reversed by up-regulation of PDE7A. These experimental results showed that PDE7A was negatively regulated by miR-218 and positively regulated by HOTAIR. In this study, we confirmed the role of PDE7A in the malignant biologic behavior of glioma cells and explored its upstream regulation mechanism on the basis of previous studies.

In summary, HOTAIR knockdown could act as a ceRNA for miR-218 to regulate the expression of PDE7A, thereby blocking glioma progression. Therefore, the HOTAIR/miR-218/PDE7A axis may be a target for the treatment of glioma.

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Disclosure of conflict of interest

None.

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