miR-148b-3p inhibits malignant biological behaviors of human glioma cells induced by high HOTAIR expression

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Abstract. Increasing evidence suggests that long non coding (lnc)RNA and microRNA (miRNA/miR) both regulate the expression of key genes in tumorigenesis and have considerable theranostic potential. Rapid advances in bioinformatics indicate that miRNA may potentially interact with lncRNA to modulate their regulatory roles. miR-148b-3p has been reported to have a vital role in regulating tumor progression. However, the expression pattern of miR-148b-3p in glioma remains largely unknown, and interactions between miR-148b-3p and lncRNA has yet to be identified. The aim of the present study was to insight into the regulatory role of miR-148b-3p in glioma. Using online software, the HOTAIR gene was identified as a possible lncRNA target of miR-148b-3p in the present study. siRNA was used to suppress the expression of HOTAIR and reverse transcription-quantitative polymerase chain reaction was used to detect the expression of miR-148b-3p. The results confirmed that HOTAIR mRNA expression was inversely correlated with miR-148b-3p expression in A172 glioma cells. Furthermore, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to detect the viability of cells, flow cytometry was performed to test cell cycle and a matrigel invasion assay was performed to test cell invasion. The results showed that HOTAIR promotes factors associated with malignancy, including cell proliferation, cell cycle progression and invasion, whereas miR-148b-3p suppresses malignancy. Bioinformatics and luciferase reporter assays showed that miR-148b-3p modulates HOTAIR expression by directly targeting the HOTAIR gene sequence. In summary, the results indicated that miR-148b-3p inhibits malignant biological behaviors of glioma cells by directly targeting HOTAIR. The current data provide important evidence for understanding the key roles of the lncRNA miRNA functional network in glioma.

Introduction

Advances in contemporary molecular biology have revealed that a large number of RNAs that have no protein-coding ability [non-coding (nc)RNAs] exist in various organs and tissues (1-4). Many of these ncRNAs are key factors in gene regulation and are important for normal cellular function as well as disease pathogenesis (5). Among the different classes of ncRNAs, long ncRNAs (IncRNAs), which are most commonly defined as non-protein-coding RNA molecules of >200 nucleotides, have recently received increasing attention. IncRNAs can be transcribed from intergenic, intronic and imprinted loci, or from overlapping or antisense loci adjacent to protein-coding genes. These diverse transcription patterns of IncRNAs have significant implications for their various functions, including imprinting, DNA methylation and X-chromosome dosage compensation, as well as transcriptional, post-transcriptional and epigenetic regulation (6-11). The majority of IncRNAs are transcribed in a developmentally-regulated and cell type-specific manner (12,13), particularly in the central nervous system (CNS), where over half of all IncRNAs are expressed (13-15). Therefore, IncRNAs are now considered to be highly important for mediating CNS form and function, and alteration of their expression may cause certain CNS pathologies, such as Alzheimer's disease, multiple sclerosis, Down's syndrome, schizophrenia and brain tumors (16).

Gliomas are the most common and aggressive type of primary adult brain tumor, with an prevalence of 3-6 per 100,000 population in China (17). Their highly malignant and invasive nature gives rise to a median survival time of <15 months for patients with glioblastoma (GBM) undergoing conventional treatment. The pathogenesis of glioma is complex and involves the aberrant activation of proto-oncogenes and the inactivation of tumor suppressor genes (18-20). Growing evidence in the literature has revealed that aberrant expression of IncRNAs in glioma influence cell proliferation, apoptosis and invasion through interactions with different molecules and diverse signaling pathways (21-23), thereby acting as critical...
components in the progression of gliomas. For example, H19, a well-characterized lncRNA in glioma, drives tumor transformation and contributes to malignant glioma phenotypes through binding with transcription factor c-Myc (22,24). CRNDE, the most upregulated lncRNA in GBM (16), regulates gene expression through histone methylation/demethylation epigenetic changes; these histone modifications are induced via interaction with chromatin modifying complexes CoREST and polycomb repressive complex 2 (PRC2) (25). In addition, MEG3, a tumor suppressor lncRNA (23,26), appears to have an anti-proliferative function in glioma through the suppression of MDM2 and subsequent activation of the p53 signaling pathway (27). HOTAIR, which also participates in epigenetic regulation through PRC2, was demonstrated to be closely associated with tumor staging, poor prognosis and the molecular subtype of glioma (17), with knockdown of HOTAIR in glioma cells exerting a tumor suppressive function accompanied by significant downregulation of cell-cycle related genes (28,29).

Several studies have suggested that the interaction between lncRNAs and microRNAs (miRNA/miR)s may have a regulatory role in cancer (30-33). For example, MEG3 is methylation-dependent tissue-specific lncRNA that is regulated by miR-29a and has been reported to contribute to hepatocellular carcinoma growth (34). Furthermore, UCA1, an oncogene lncRNA in breast cancer, modulates breast cancer cell growth and apoptosis by decreasing tumor suppressive miR-143 (35), and miR-148b-3p, a member of the miR-148 family, was underexpressed (reduced compared with normal cells) in several cancer cell lines (36-38). In a study by Zhang et al., miR-148b-3p was found to induce cell apoptosis by activating caspase-3 and caspase-9, inducing S phase arrest by regulating cyclin D1 and p21, and inhibiting cell invasion (39). Although a number of mRNA targets of miR-148b-3p have been identified, interaction between miR-148b-3p and an lncRNA has not been reported to date. Using the online software program starBase v2.0, the present study identified HOTAIR as a potential lncRNA target of miR-148b-3p. Subsequently, the current study showed that mutated HOTAIR promotes the aggressiveness of A172 glioma cells, and its was determined that miR-148b-3p binds HOTAIR in a sequence-specific manner. Furthermore, miR-148b-3p reduced proliferation, cell cycle progression and invasion of A172 cells through the suppression of HOTAIR expression. Thus, the current data, at least in part, contributes insight into the development of glioma.

Materials and methods

Human tissue samples and cell lines. mRNA and miRNA expression microarray data from 180 samples were downloaded from the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/; accession no. GSE4290). The data were compiled from 23 non-tumor, 26 astrocytoma (7 grade II, 19 grade III), 50 oligodendrogliaoma (38 grade II, 12 grade III) and 81 GBM samples. The tumor sample expression profile, including HOTAIR expression data, was also downloaded. HA1800 human astrocytes and A172 glioma cells were purchased from the Cell Resource Center of Shanghai Institute of Life Sciences (Shanghai, China). The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C in 5% CO₂.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cells using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Briefly, 1 ml TRIzol per 5x10⁶ cells was added to cells, prior to adding 0.2 ml of chloroform per 1 ml TRIzol. The mixture was mixed vigorously by hand and allowed to stand for 2-3 min at room temperature. The mixture was then centrifuged at 10,000 x g for 10 min at 4°C. The upper clear phase was transferred to a fresh tube and 0.5 ml isopropanol per 1 ml of the clear phase was added, which was mixed vigorously by rapid shaking and left to stand for 10 min. The precipitated RNA was collected by centrifugation at 10,000 x g for 10 min at 4°C and then carefully decanting/pipetting the supernatant. The RNA precipitate was washed once with 70% ethanol, dissolved in 25 µl RNase free water, and then stored at -80°C. The concentration of the Recombinant DNase I RNase-free (Takara Biotechnology Co., Ltd., Dalian, China) used to treat the RNA sample was 5 units/µl. cDNA was synthesized using the HiFi-MMLV cDNA kit (Beijing ComWin Biotech Co., Ltd., Beijing, China) and qPCR was conducted using the UltraSYBR Mixture (Beijing ComWin Biotech Co., Ltd.). Briefly, 5 µg purified RNA sample was mixed with Primer Mix, dNTP Mix, DTT, RT-buffer, HiFi-MMLV and RNase-free water using a pulled pipette (total volume, 20 µl). All qRT-PCR reactions were run in a StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The mixture was then incubated at 42°C for 30-50 min and then 55°C for 5 min. The RT products were quickly centrifuged and stored at -20°C. No cDNA was used as a negative control. To amplify hsa-miR-148b-3p cDNA, specific RT primers were used based on its sequence, and the U6 RT primer for was the same as the U6 reverse PCR primer. The hsa-miR-148b-3p RT primer was 5'-GGTTGGCTCTGTGGGCAGGTCGGTCTACAGGAGCTAAGCCATCCACAAAAG-3'. PCR primers were as follows: Forward, 5'-GGCACCACACCTTCTCAAT-3' and reverse, 5'-GGCTGTGTCCAGAGCTTGAACC-3' for the β-actin gene; forward, 5'-CAGTGGAGAATCTGACGTTCCGAGGT-3' for hsa-miR-148b-3p; and forward, 5'-CCTGCTACAGCAGTCCCAAGT-3' for U6. All primers were synthesized by GenScript (Nanjing, China). The relative fold change in mRNA expression level was calculated using the 2⁻ΔΔCq method (40).
antisense, 5’-ACGUGACACGUUCGAGAATT-3’ for si-NC (22). Cells were grown to until they reached 10^5 in number in 6-well plates prior to transfection. One day before transfection, the control group cells were plated at the same density as the si-HOTAIR and si-NC groups. The cells were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s instructions.

Cell viability assay. The effect of HOTAIR downregulation on the viability of A172 cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were trypsinized and seeded at a density of 1x10^4 cells/well in 96-well plates immediately after siRNA transfection. Then, 10 µl MTT solution (5 mg/ml) was added and the plates were incubated for an additional 4 h at 37°C. Following removal of the medium, formazan crystals were dissolved in 150 µl dimethyl sulfoxide. The absorbance of the MTT formazan was measured at 550 nm using a SpectraMax M3 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Experiments were repeated three times using 6 wells for each treatment to ensure the reproducibility of results.

Flow cytometry analysis (FCM). Cells were separately harvested 0, 24, 48 and 72 h after siRNA transfection, fixed in 70% ethanol, and stained with propidium iodide (1 mg/ml; Takara Biotechnology Co., Ltd., Nantong, China) containing RNase A (1 mg/ml; Promega, Madison, WI, USA), downstream of the luciferase gene, to generate the recombinant vectors pmirGLO-wild-type (WT) and pmirGLO-mutant (MUT). For the pmirGLO-MUT construct, 12 mismatches were introduced into the HOTAIR fragment containing mutated binding sites was synthesized. The constructs were validated by Sanger sequencing by Sangon Biotech Co., Ltd. (Shanghai, China).

Luciferase reporter assay. For the luciferase reporter assay, A172 cells were co-transfected with miRNA (miR-148b-3p mimics or miR-148-3p mimic negative control; Biomics Biotechnologies Co., Ltd., Nantong, China) and reporter vectors (pmirGLO-WT or pmirGLO-MUT) using Lipofectamine 2000. Luciferase activity was assayed 48 h after transfection using a Dual-Luciferase Reporter Assay system (Beyotime Institute of Biotechnology, Haimen, China). The values were normalized to those obtained for miRNA negative control transfection. All transfection experiments were performed in triplicate.

Statistical analysis. All statistical analyses were performed using SPSS software version 18.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as means ± standard deviation of experiments performed in triplicate. HOTAIR expression in normal tissues and tumor tissues was compared using analysis of variance (ANOVA) followed by Student-Newman-Keuls analysis. GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA) was used for the graphing. Statistical analysis on other experiments was performed using one-way ANOVA and the Student-Newman-Keuls test for multiple comparisons.

Figure 1. HOTAIR expression in glioma tissues and cells. (A) Expression levels of HOTAIR in glioma tissues from an NCBI GEO dataset, as analyzed by whole genome gene expression profiling. (B) mRNA expression level of HOTAIR in HA1800 and A172 cell lines, as determined by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean ± standard deviation of three independent experiments. NBT, normal brain tissue.
and unpaired Student's t-tests for comparisons between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

HOTAIR expression in glioma tissues and cells. First, the expression pattern of HOTAIR was analyzed through whole genome gene expression profiling of 157 glioma and 23 normal tissue samples from NCBI GEO data (accession no. GSE4290). As shown in Fig. 1A, HOTAIR expression was significantly higher in carcinoma tissues compared with normal tissues (P=0.010), and grade IV and III tumor tissues both demonstrated a significant increase in HOTAIR transcription levels compared with grade II tumor tissues (P=0.047). However, no significant difference in HOTAIR expression was observed between grade IV and III samples. Next, HOTAIR expression was detected in HA1800 human astrocytes and A172 glioma cells by RT-qPCR. The HOTAIR mRNA level was significantly increased in A172 cells compared with HA1800 cells.
These findings indicate that HOTAIR may have an important role in glioma progression.

Silencing HOTAIR inhibits the malignant properties of A172 cells. To further investigate the oncogenic role of HOTAIR in glioma pathogenesis, A172 cell lines were transfected with si-HOTAIR. Successful transfection was confirmed by RT-qPCR (Fig. 2A). Cells were separately collected at 0, 24, 48 and 72 h post-transfection, and cell growth ability was determined using the MTT assay. Silencing of HOTAIR expression significantly reduced growth rates compared with the si-NC and untransfected cell groups at 48 (P = 0.012) and 72 h (P < 0.001) post-transfection (Fig. 2B). Additionally, FCM was performed following transfection to assess cell cycle distribution. The results showed that the cell population in the G1 phase was increased but the S phase population was decreased after HOTAIR gene silencing compared with the results observed for the si-NC cells (Fig. 2C), further indicating that knockdown of HOTAIR expression may suppress cancer cell proliferation. In addition, the effect of HOTAIR knockdown on A172 cell invasion was investigated using a Matrigel invasion assay. The in vitro Matrigel invasion assay revealed that the invasiveness of A172 cells transfected with si-HOTAIR was significantly suppressed compared with the control and si-NC cells (P = 0.007; Fig. 2D).

Figure 3. Reciprocal negative regulation of miR-148b-3p and HOTAIR. (A) miR-148b-3p expression in HA1800 and A172 cell lines. (B and C) Effect of miR-148b-3p on HOTAIR expression. A172 cells were transfected with si-NC, (B) miR-148b-3p mimic or (C) miR-148b-3p inhibitor. (D) Upregulation of miR-148b-3p induced by ectopic expression of HOTAIR. A172 cells were transfected with si-NC or si-HOTAIR. At 24, 48 and 72 h post-transfection, mRNA expression was analyzed by reverse transcription-quantitative polymerase chain reaction. (E) miR-148b-3p binding sequence in HOTAIR. The red letters in the HOTAIR sequence show mutations. (F) A172 cells were co-transfected with the pmirGLO luciferase construct (pmirGLO-mutant or pmirGLO-wild-type) and miR-148b-3p mimic. After 24 h of incubation, cells were harvested and assayed using a luciferase reporter assay system. *P < 0.05, **P < 0.01 vs. si-NC. Data are presented as the mean ± standard deviation of three independent experiments. miR, microRNA. si-NC; siRNA-negative control; si-HOTAIR, siRNA HOTAIR.

Reciprocal negative regulation of miR-148b-3p and HOTAIR. RT-qPCR revealed that the expression level of miR-148b-3p was significantly lower in A172 cells compared with HA1800 cells (P < 0.001; Fig. 3A). To determine whether miR-148b-3p is able to suppress HOTAIR expression, miR-148b-3p mimic or inhibitor were transfected into A172 cells. As shown in Fig. 3B, the miR-148b-3p mimic reduced HOTAIR expression in a dose-dependent manner, decreasing it by ~62% 72 h after transfection (P < 0.001). By contrast, the miR-148b-3p inhibitor increased the level of HOTAIR in a dose-dependent manner (Fig. 3C). Furthermore, si-HOTAIR transfection significantly increased the expression of miR-148b-3p in A172 cells at all time points (P < 0.001; Fig. 3D), indicating that there is a strong inverse correlation between HOTAIR and miR-148b-3p expression levels.

To verify whether miR-148b-3p regulates HOTAIR as a direct target, a predicted binding site for miR-148b-3p was identified using the online software program starBase v2.0 (42) (Fig. 3E) an wild-type or mutant miR-148b-3p target binding sequences were cloned into the pmirGLO luciferase vector. Following co-transfection with the miR-148b-3p mimic in A172 cells, a Dual-Luciferase Assay was performed determine the luciferase activity. The data shows that cells co-transfected with the constructs containing the pmirGLO-WT and miR-148b-3p mimic had significantly lower luciferase activity compared with that of those transfected with pmirGLO-MUT and miR-148b-3p mimic (P = 0.007; Fig. 3F). All the results indicate that miR-148b-3p suppresses HOTAIR by binding to HOTAIR in a sequence-specific manner.

miR-148b-3p inhibits HOTAIR function. To determine whether the aggressiveness of A172 cells could be restored by miR-148b-3p, an MTT assay, FCM and a Transwell invasion assay were performed following transfection with the miR-148b-3p mimic.
Transient transfection with the miR-148b-3p mimic decreased the proliferation of A172 cells by ~15% at 72 h compared with the mimic negative control group (P=0.002; Fig. 4A). The miR-148b-3p mimic also increased the cell population in the G1 phase and decreased the S-phase population (Fig. 4B), demonstrating that miR-148b-3p blocks the cell cycle of A172 cells. Furthermore, the Transwell invasion assay results clearly revealed that the miR-148b-3p mimic decreased cell invasion to ~70% compared with the untreated and miR-148b-3p mimic negative controls (P=0.002; Fig. 4C).

**Discussion**

Years of research have revealed the essential role of non-coding lncRNAs and miRNAs in gene regulation, and their involvement in diverse biological processes, such as development and disease. Recently, rapid advances in bioinformatic analysis have expanded understanding of the transcriptome to a genome-wide level, and the interaction between lncRNA and miRNA has been shown to provide an additional element of control in gene regulation (43-45). In support an interaction...
between miRNA and IncRNA, the present study demonstrated the reciprocal repression of miRNA-148b and HOTAIR. The addition of a miR-148b-3p mimic mimicked the expression levels of HOTAIR, while miR-148b-3p inhibitor increased the levels of HOTAIR. In addition, knockdown of HOTAIR induced the upregulation of miR-148b-3p.

HOTAIR is a 2,158-bp IncRNA that is located within the HOXC cluster of chromosome 12, and is flanked by HOXC11 and HOXC12. HOTAIR activity is partially regulated by its interaction with PRC2, which is comprised of EZH2 (a histone H3K27 methylase), SUZ12 and EED. Since Gupta et al (41) first observed that loss of HOTAIR can inhibit the invasiveness of cancer cells, increasing evidence for a significant role of HOTAIR in carcinogenesis has been documented for several types of cancer, including glioma. The current study confirmed that HOTAIR is highly expressed in glioma tissues and the A172 glioma cell line. Furthermore, knockdown of HOTAIR decreased growth rates, blocked the cell cycle and suppressed the invasion of glioma cells, as well as increasing the expression of miR-148b-3p.

Previous studies have shown that miR-148b-3p is dysregulated in numerous types of cancer (46-48). For example, in colorectal cancer, it is downregulated and acts as a tumor suppressor by targeting the CCKBR gene (38). miR-148b-3p is also downregulated in pancreatic cancer and suppresses cell growth by targeting the AMPKα1 gene (49). However, it is upregulated in ovarian cancer and may be involved in the early stages of ovarian carcinogenesis (50). These results reflect the controversial roles of miR-148b-3p in different types of cancer. To the best of our knowledge, the present study is the first to demonstrate that miR-148b-3p is downregulated in glioma cells. Additionally, an association between miR-148b-3p and IncRNA expression in cancer has not been reported until now. In the current study, luciferase assays indicated that miR-148b-3p reduces HOTAIR expression through the putative miR-148b-3p binding site in HOTAIR. To investigate whether miR-148b-3p has a metastasis-suppressing function in glioma cells, a miR-148b-3p mimic was transfected into A172 cells, resulting in reduced expression levels of HOTAIR. The data also showed that miR-148b-3p inhibits cell proliferation, blocks the cell cycle and reduces cell invasion.

In conclusion, the results of the current study indicate that miR-148b-3p has a tumor-suppressive role by downregulating HOTAIR in glioma, providing evidence for the importance of the interaction between IncRNA and miRNA in gliomagenesis. These results will aid in providing novel considerations for the molecule-targeted therapies of glioblastoma. Additional investigations are underway to further investigate other molecules involved in the HOTAIR-miR-148b-3p interaction.

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