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Long Noncoding RNA H19 Promotes Proliferation and Invasion in Human Glioma Cells by Downregulating miR-152

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miR-152 and lncRNA H19 have been frequently implicated in various cellular processes including cell proliferation, invasion, angiogenesis, and apoptosis. However, the interaction between miR-152 and H19 in glioma has never been reported. RT-qPCR was used to examine the expression of miR-152 and H19 in human glioma cell lines and normal human astrocytes (NHAs). The interaction between miR-152 and lncRNA H19 was assessed by dual-luciferase reporter assay. MTT assay and Transwell invasion assay were used to determine the proliferation and invasion of U251 and U87 cells. A xenograft tumor experiment was performed to confirm the role of H19 in vivo. The results showed that H19 expression was upregulated and miR-152 expression was downregulated in human glioma cell lines. H19 downregulation or miR-152 upregulation suppressed glioma cell proliferation and invasion in vitro. Moreover, H19 and miR-152 directly regulated each other. Furthermore, decreased miR-152 expression alleviated si-H19-induced inhibitory effects on proliferation and invasion in glioma cells. As expected, H19 silencing hindered glioma growth in vivo. Taken together, H19 promoted glioma cell proliferation and invasion by negatively regulating miR-152 expression, providing evidence for the potential application of H19 as a biomarker and therapy target for glioma.

Key words: H19; Glioma; Long noncoding RNAs (lncRNAs); MicroRNAs (miRNAs); Proliferation; Invasion

INTRODUCTION

Gliomas are the most prevailing and aggressive form of primary intracranial tumors in the central nervous system (CNS). Glioblastoma (GBM) is the main type of glioma with a high morbidity and mortality, which originates from poorly differentiated astrocytes with an average median survival time of approximately 10–14 months after diagnosis[1,2]. The therapeutic effect and prognosis with surgical resection treatment are always unfavorable due to the high brain invasiveness and rapid proliferation of glioma[3,4]. Despite the improvements in therapeutic protocol based on surgical resection combined with concurrent radiation therapy, chemotherapy, gene therapy, and other effective therapeutic strategies, the prognosis and survival rates of patients with glioma have not been significantly improved, especially for GBM patients[5,6]. Therefore, it is extremely useful to figure out the molecular mechanisms associated with glioma carcinogenesis, progression, and recurrence, and find novel and effective therapeutic approaches for glioma therapy.

Long noncoding RNAs (lncRNAs), a class of RNAs without protein-coding potential, consist of more than 200 nucleotides and are transcribed pervasively from the human genome, such as intergenic, intronic, and imprinted loci[7,8]. Many of these lncRNAs exert an important regulatory role in some crucial biological processes, such as gene expression, invasion, proliferation, and apoptosis of tumors, normal cellular function, and disease pathogenesis[9,10]. Besides, lncRNAs also have been reported to be involved in the mediation of cellular signaling, molecular guides at the chromatin sites, and scaffold modeling[11]. The transcriptions of most lncRNAs are with cell-type specificity, especially in CNS cells, where more than half of all lncRNAs are expressed[12,13].

MicroRNAs (miRNAs) are a series of single-stranded and small noncoding RNAs, which function as an important regulator at a posttranslational level by regulating their downstream target genes. A growing number of research studies suggest that miRNAs played critical roles in numerous biological processes, such as cell growth,

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differentiation, apoptosis, migration, and invasion. miRNAs, acting as oncogenes or tumor suppressors, were found to be abnormally expressed in diverse cancers by degrading or repressing translations of their mRNA targets[14]. The interplay of miRNAs and lncRNAs was reported to exert an important regulatory role in tumors[15,16]. For example, Wei et al. found that the lncRNA urothelial cancer-associated 1 (UCA1) was upregulated and UCA1-miR-507-FOXM1 was associated with cell growth, invasion, and cell cycle arrest in melanoma cells[17]. Wu et al. reported that cancer-related gastric adenocarcinoma-associated, positive CD44 regulator, long intergenic noncoding RNA (lncRNA-uc002kmd.1) was overexpressed and improved CD44-dependent cell proliferation by competing with miR-211-3p in colorectal cancer[18].

miR-152, a member of the miR-148/152 family, has been frequently implicated in various cellular processes, including cell growth, apoptosis, and invasion[19,20]. Several research studies showed that miR-152 expression was downregulated and had antitumor effects in many tumors including GBM[21,22]. H19 is an untranslated intergenic noncoding RNA that is located in chromosome 11 in humans and was reported to be upregulated in GBM cells and promoted cell growth and invasion[23]. Notably, a previous study demonstrated that H19 acted as an endogenous sponge by directly binding to miR-152 in breast cancer cells[24]. However, the interaction between miR-152 and H19 in glioma cells has never been reported. Therefore, the purpose of this study was to investigate the interaction and molecular mechanism between H19 and miR-152 in the glioma process.

MATERIALS AND METHODS

Cell Lines and Culture

The human glioma cell lines A172, U251, U87, U373, and U563 were used in this study and were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, P.R. China) along with normal human astrocytes (NHAs) that were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). The cells above were cultivated in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Invitrogen, Grand Island, NY, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin supplement (Sigma-Aldrich, St. Louis, MO, USA) at 37°C with 5% CO_2 in a humid atmosphere.

Cell Transfection

miR-152 mimic and miRNA control (miR-control), anti-miR-152, and anti-miRNA control (anti-miR-control), siRNA against H19 (si-H19), and siRNA control (si-control), pcDNA-H19, pcDNA-vector, short hairpin RNA (shRNA) targeting H19 (sh-H19), and shRNA control (sh-con) were purchased from GenePharma (Shanghai, P.R. China). When the cells were grown to 70% confluency, 100 pmol/L of all molecular products was transiently transfected into U251 and U87 cells by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The sequences of si-H19 were as follows: sense, 5′-GCGGGUCUGUUUCUUUACUUU-3′; antisense, 5′-AGUAAAGAAACAGACCCGCUU-3′; the sequences of si-control were as follows: sense, 5′-GCGU UCUGGUCUUACU-3′; antisense, 5′-UCAGUGCAUG ACAGAACUUGG-3′; the sequences of miR-152 mimic were as follows: sense, 5′-UCAGUGCAUGACAGAAC UUGG-3′; antisense, 5′-AAGUUCUGUCAUGCACUG AUU-3′; the sequences of miR-152 control were as follows: sense, 5′-UCCUCCGAACGUGUCACGUTT-3′; antisense, 5′-ACGUGACACGUUCGGAGAATT-3′.

Quantitative Real-Time PCR (RT-qPCR)

Cells were collected and subjected to TRIzol reagent (Invitrogen) for total RNA extraction according to the standard protocol. MiRNA Extraction Kit (Tiangen, Beijing, P.R. China) was used to extract the miRNAs. For detection of miR-152 and H19 expression, the complementary DNA (cDNA) was synthesized by the miRNA-specific TaqMan miRNA Assay Kit (Applied Biosystems, Foster City, CA, USA) and the SuperScript III First-Strand Synthesis System (18080-051; Invitrogen), respectively. RT-qPCR was used to examine the expressions of miR-152 and H19 using SYBR Green PCR Master Mix Kit (Applied Biosystems) under the 7900 Real-Time PCR System (Applied Biosystems). The expressions of miR-152 and H19 were normalized to U6 snRNA. The primers of miR-152 were as follows: 5′-TCAGTGCATGACAGAACTTGG-3′ (forward) and 5′-GCGAGCACAGAATTAATACGAC-3′ (reverse); the primers of H19 were as follows: 5′-ATCGGTGCCTC AGCGTTCGG-3′ (forward) and 5′-CTGTCCTCGCCG TCACACCG-3′ (reverse); the primers of U6 were as follows: 5′-CTCGCTTCGGCAGCACA-3′ (forward) and 5′-AACGCTTCACGAATTTGCGT-3′ (reverse).

Cell Proliferation Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the cell proliferation. Briefly, 5×10^3 transfected U251 and U87 cells were reseeded into 96-well plates. Following incubation for 24, 48, and 72 h, 20 µl of MTT (5 mg/ml) was added and incubated for 4 h at 37°C. Then 200 µl of dimethyl sulfoxide (DMSO) was added to lyse formazan crystals at room temperature. After 10 min, the absorbance at 490 nm in each well was detected by a microtiter plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Transwell Invasion Assays

The invasion ability of transfected cells was performed using a Transwell chamber (Millipore, Billerica, MA, USA) inserted with 8-μm pore size polycarbonate membranes (Corning Incorporated, Corning, NY, USA). Transfected cells were trypsinized and washed once with DMEM. Then 5×10^4 cells in serum-free DMEM were seeded into the top of the invasion chambers, which were coated with 100 μg/well Matrigel (BD Biosciences, San Jose, CA, USA). DMEM supplemented with 10% FBS was added into the lower chamber. After 24 h of cell invasion at 37°C with 5% CO_2, the noninvading cells sticking on the top well were cleared using a cotton swab, and the invading cells that had migrated to the lower surface of the membrane were fixed with 20% precooled methanol for 5 min and stained with 0.5% crystal violet (Beyotime Institute of Biotechnology, Shanghai, P.R. China) for 30 min. The invading cell numbers were observed using an ECLIPSE TiS microscope (Nikon, Tokyo, Japan) and counted in five randomly selected fields.

Dual-Luciferase Reporter Assay

The putative sequence of an H19 fragment containing the predicted miR-152 binding sites was cloned into *Xho*I and *Kpn*I sites of a dual-luciferase reporter vector pGL3 (Promega, Madison, WI, USA), and the sequence of the putative binding site was replaced as indicated. The constructed luciferase reporters were called pGL3-WT-H19 (wild type) and pGL3-MUT-H19 (mutated), respectively. For the dual-luciferase reporter assay, U251 and U87 cells (1×10^5 cells/well) were seeded into 24-well plates and cotransfected with 50 pmol/L of miR-152 mimic or miR-control, and 50 ng of reporter vectors WT-H19 or MUT-H19 by Lipofectamine 2000 (Invitrogen). After 48 h of transfection, the luciferase activity was detected using a dual-luciferase reporter assay system (Promega).

Xenograft Tumor Experiment

All animal experiments were approved by the Institutional Animal Care and Use Committee of the l01st Hospital of PLA (Wuxi Taihu Hospital, Wuxi, P.R. China). Four-week-old female BALB/c nude mice were obtained from the Chinese Academy of Sciences (Shanghai, P.R. China). U251 cells (3×10^6) stably transfected with sh-H19 or sh-con were subcutaneously inoculated into the right side of the groin of nude mice in a volume of 0.1 ml of PBS. Seven days later, the volume of tumor xenografts was measured every 6 days using a caliper and calculated using the following formula: volume = (length × width^2)/2. After 25 days of injection, the tumor-bearing mice were sacrificed, and visible tumors were excised and weighed.

Statistical Analysis

Data from each group were expressed as the mean ± SD from three independent experiments. Statistical differences were performed by Student's *t*-test or ANOVA in GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA). Values of $p<0.05$ were considered to be statistically significant.

RESULTS

H19 Was Upregulated and miR-152 Was Downregulated in Glioma Cell Lines

The expressions of miR-152 and H19 in NHAs and glioma cell lines were examined by RT-qPCR. As shown in Figure 1A and B, we found that H19 was significantly upregulated while miR-152 was abnormally downregulated in glioma cell lines A172, U251, U87, U373, and U563 in comparison with that in NHAs, particularly in U251 and U87 cell lines.

Downregulation of H19 Suppressed Proliferation and Invasion in Glioma Cell Lines

To observe the effect of H19 on proliferation and invasion in glioma cells, si-H19 and si-control were transfected into the U251 and U87 cell lines. RT-qPCR results showed a significant decrease in H19 in the si-H19 group compared with the si-control group for U251 and U87 cells (Fig. 2A and B). MTT assay results showed that

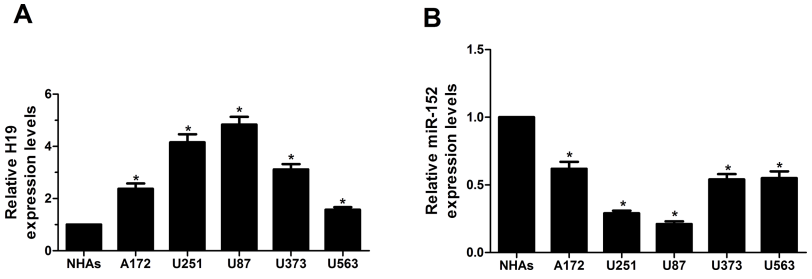

Figure 1. H19 was upregulated and microRNA-152 (miR-152) was downregulated in glioma cell lines in comparison with that in normal human astrocytes (NHAs). (A) H19 expression in glioma cell lines (A172, U251, U87, U373, and U563) and NHAs was assessed by quantitative real-time PCR (RT-qPCR). (B) miR-152 expression in glioma cell lines (A172, U251, U87, U373, and U563) and NHAs was determined by RT-qPCR. *$p<0.05$, $n=3$.

the proliferation rates of U251 and U87 cells were strikingly decreased in the si-H19 group compared with that in the si-control group at 48 and 72 h (Fig. 2C and D), revealing that H19 downregulation inhibited the proliferation of U251 and U87 cells. Additionally, Transwell invasion assays indicated that the invasion cell numbers in the si-H19 group were notably decreased compared with that in the si-control group (Fig. 2E and F), suggesting that H19 downregulation inhibited the invasion ability of U251 and U87 cells.

Overexpression of miR-152 Suppressed Proliferation and Invasion in Glioma Cell Lines

To figure out the effects of miR-152 on proliferation and invasion in glioma cells, miR-152 mimic and miR-control were transfected into U251 and U87 cells. The RT-qPCR results showed an evident increase in miR-152 expression in the miR-152 mimic group compared with the miRNA-control group for U251 and U87 cells (Fig. 3A and B). MTT assays revealed that the proliferation rates of U251 and U87 cells were prominently lowered in the miR-152 mimic group compared with that in the miRNA-control group (Fig. 3C and D), indicating that miR-152 overexpression suppressed the proliferation of U251 and U87 cells. In addition, Transwell invasion assays clearly displayed that there was a significant decrease in the number of invasion cells in the miR-152 mimic group compared with that in the miR-control group (Fig. 3E and F), demonstrating that miR-152 overexpression inhibited the invasion capability of both U251 and U87 cells.

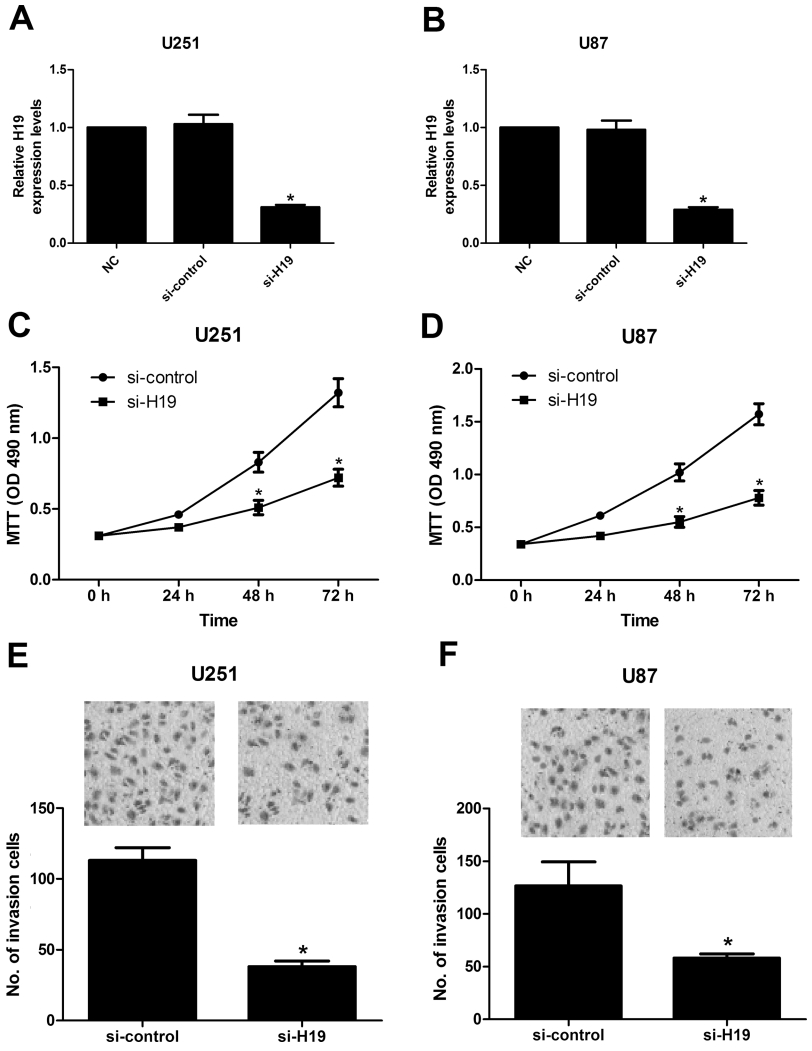

Figure 2. Downregulation of H19 suppressed cell proliferation and invasion in glioma cell lines. H19 expression in U251 (A) and U87 (B) cells transfected with siRNA against H19 (si-H19) or siRNA control (si-control) was determined by RT-qPCR. MTT assays were performed to assess the proliferation of U251 (C) and U87 (D) cells transfected with si-H19 or si-control. Transwell invasion assays were used to evaluate the invasive ability of U251 (E) and U87 (F) cells transfected with si-H19 or si-control. $*p<0.05$, $n=3$.

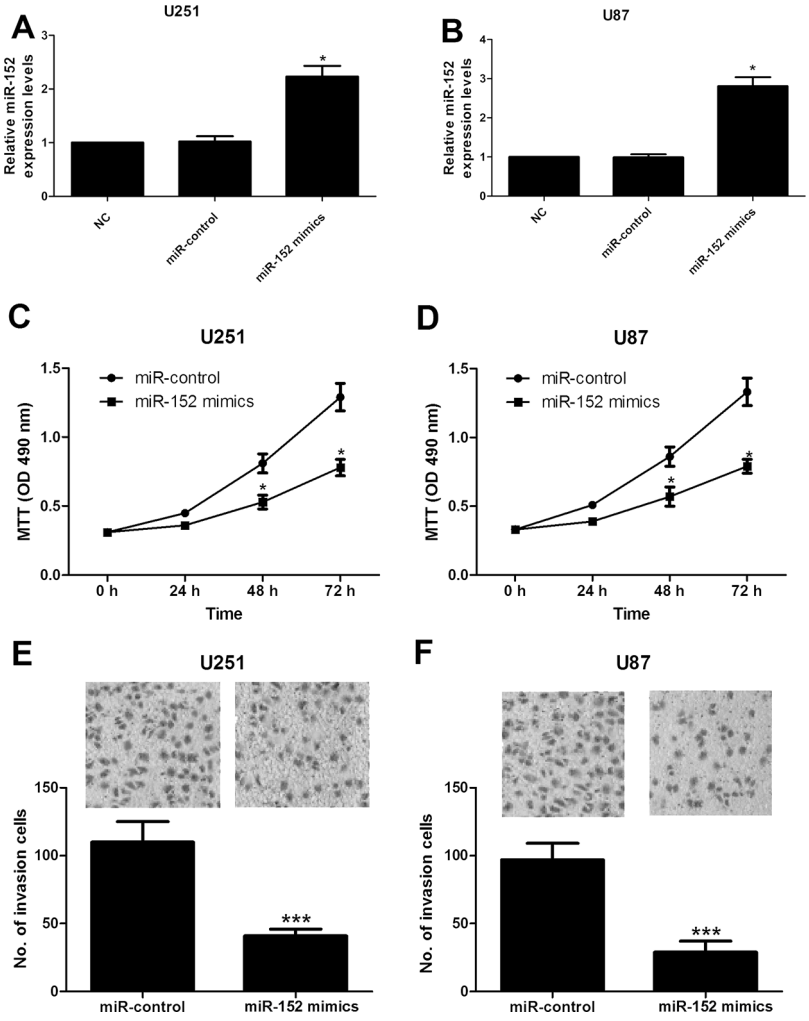

Figure 3. Overexpression of miR-152 suppressed cell proliferation and invasion in glioma cell lines. Expression of miR-152 in U251 (A) and U87 (B) cells transfected with miR-152 mimic or miR-control was quantified by RT-qPCR. MTT assays were applied to observe the proliferation of U251 (C) and U87 (D) cells transfected with miR-152 mimic or miR-control. Transwell invasion assays were performed to assess the invasive ability of U251 (E) and U87 (F) cells transfected with miR-152 mimic or miR-control. *$p<0.05$, ***$p<0.001$, $n=3$.

The Expression of H19 Was Inversely Associated With miR-152 Expression in Glioma Cell Lines

Bioinformatic databases (miRCode and DIANA-LncBase) were used to predict potential miRNA candidates of H19. The predicted sites of miR-152 binding to the H19 sequence are illustrated in Figure 4A. Luciferase activity assay was used to figure out the relationships between miR-152 and H19. We constructed a luciferase reporter that contained wild-type (WT) or mutated (MUT) miR-152 binding sites within H19. The results indicated that miR-152 overexpression apparently inhibited the WT-H19 luciferase activity in U251 and U87 cells, but did not affect MUT-H19 luciferase activity, suggesting the direct binding between H19 and miR-152 (Fig. 4B and C). To further verify the reciprocal regulatory effect of H19 and miR-152, RT-qPCR was performed to determine the expression of H19 in U251 and U87 cells transfected with miR-152 mimic, anti-miR-152, and miR-control, respectively. We found that the expression of H19 was conspicuously decreased in the miR-152 mimic group and drastically increased in the anti-miR-152 group compared with the miR-control group (Fig. 4D and E). Meanwhile, the expression of miR-152 in U251 and U87 cells transfected with si-H19, si-control, pcDNA-H19, or pcDNA-vector was also examined by RT-qPCR. The results showed that downregulation of H19 significantly increased the expression of miR-152 in U251 and U87 cells compared with the si-control group, and overexpression of H19 by pcDNA-H19 dramatically decreased the expression of miR-152 compared with the pcDNA-vector group (Fig. 4F and G). These findings demonstrated that miR-152 was inversely correlated with H19 expression in both U251 and U87 cells.

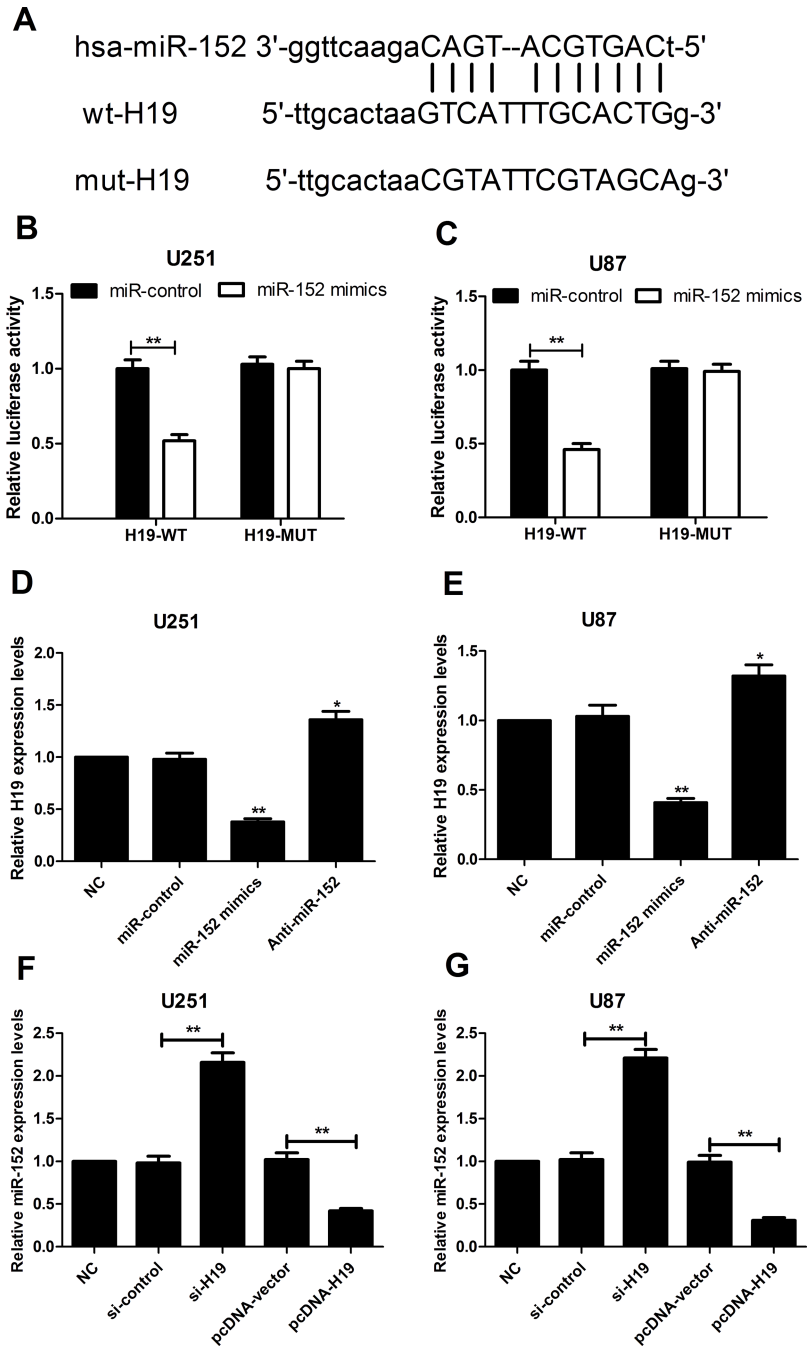

Figure 4. The expression of H19 was inversely associated with miR-152 expression. (A) The potential binding sites for miR-152 on H19. (B, C) Luciferase activity was detected after U251 and U87 cells were cotransfected with miR-152 mimic or miR-control and WT-H19 (wild type) or MUT-H19 (mutated) reporter. (D, E) The expression of H19 in U251 and U87 cells transfected with miR-control, miR-152 mimics, or anti-miR-152 was quantified by RT-qPCR. (F, G) The expression of miR-152 in U251 and U87 cells transfected with si-control or si-H19, and either with pcDNA-vector or pcDNA-H19 was examined by RT-qPCR. $^{**}p<0.01$, $^{*}p<0.05$, $n=3$.

H19 Promoted Proliferation and Invasion by Downregulating miR-152 in Glioma Cell Lines

To investigate the potential interaction of H19 and miR-152 on cell proliferation and invasion, U251 and U87 cells were transfected with si-H19 or in combination with anti-miR-152. MTT assay indicated that the proliferation ability was evidently inhibited due to H19 downregulation in U251 and U87 cells, while anti-miR-152 prominently alleviated the inhibition effect on cell proliferation induced by H19 downregulation (Fig. 5A and B). Transwell invasion assays suggested that downregulation

of H19 distinctly suppressed cell invasion, while anti-miR-152 abated si-H19-elicited blockade on invasion capability (Fig. 5C and D). All the results revealed that H19 facilitated proliferation and invasion by downregulating miR-152 in glioma cells.

Downregulation of H19 Hindered Glioma Growth In Vivo

We further explored the functional role of H19 in glioma in vivo. U251 cells stably transfected with sh-H19 or sh-con were subcutaneously inoculated into the right side of the groin of nude mice. At the injection site, all mice developed xenograft tumors. As shown in Figure 6A, H19 silencing led to a marked suppression on tumor growth compared with the sh-con group. Moreover, the tumor size and weight in the sh-H19 group were both distinctly reduced relative to the sh-con group (Fig. 6B). Hence, we concluded that downregulation of H19 inhibited glioma tumor growth in vivo.

DISCUSSION

A growing number of research studies have proven that miRNA deregulation was associated with carcinogenesis and invasion of various malignancies, including glioma[25]. Therefore, miRNAs are frequently regarded as effective molecular biomarkers for glioma development, prognosis, and diagnosis[26]. For example, Guo et al. reported that miR-451 inhibited cell proliferation and invasion of glioma cells by regulating glucose transporter 1 (GLUT1), which suppressed glucose metabolism to reduce the energy supply[27]. Song et al. found that miR-92b could regulate glioma cell proliferation, migration, invasion, and apoptosis by targeting the PTEN/Akt signaling pathway[28]. Li et al. showed that miR-661 acted as a tumor suppressor and repressed glioma proliferation, migration, and invasion by modulating hTERT[29]. Cheng et al. found that miR-184 was downregulated in glioma tissues and cell lines and played a tumor-suppressive role in glioma by regulating

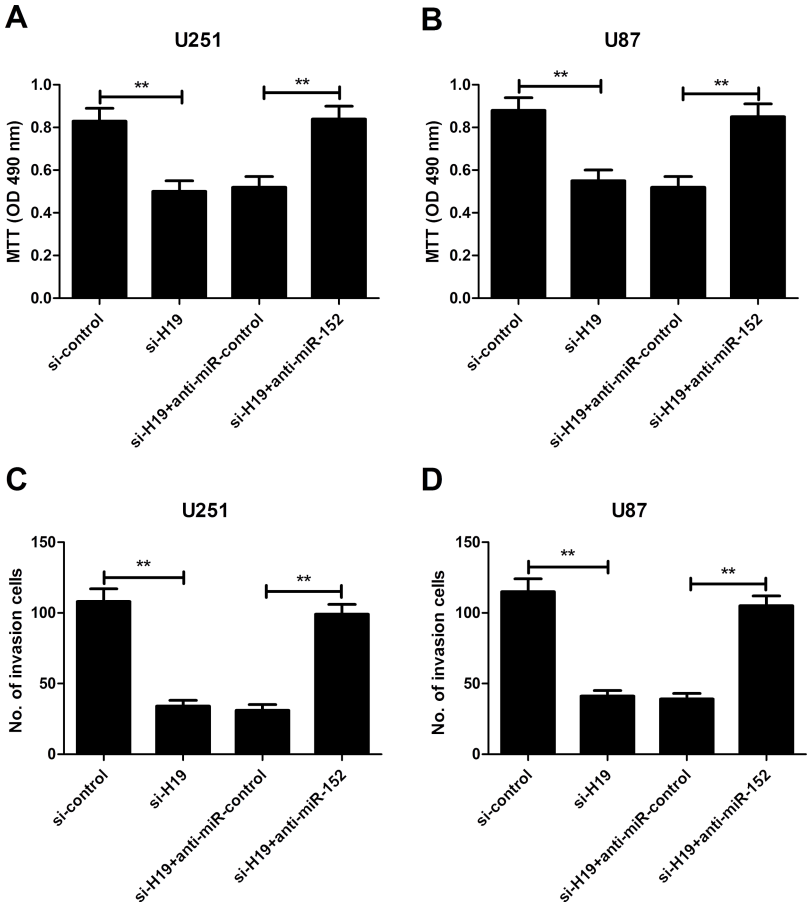

Figure 5. H19 promoted cell proliferation and invasion by downregulating miR-152. (A, B) Cell proliferation was tested by MTT assay after U251 and U87 cells were transfected with si-control, si-H19, si-H19 + anti-miR-control, or si-H19 + anti-miR-152. (C, D) Cell invasion was examined by Transwell invasion assay after U251 and U87 cells were transfected with si-control, si-H19, si-H19 + anti-miR-control, or si-H19 + anti-miR-152. $^{**}p < 0.01$, $n = 3$.

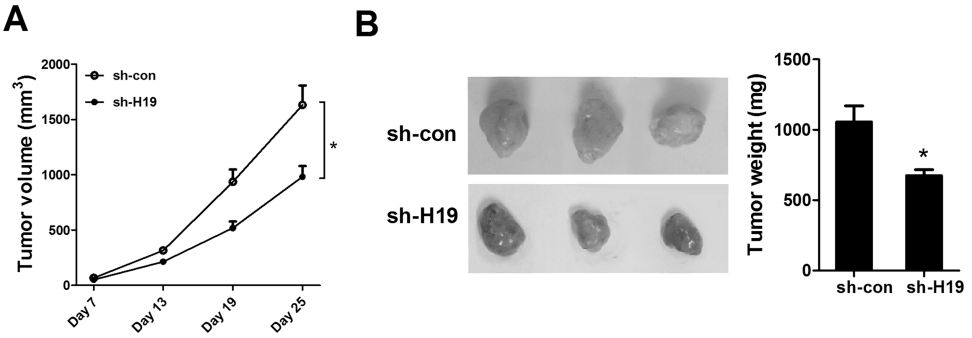

Figure 6. Downregulation of H19 inhibited glioma growth in vivo. U251 cells stably transfected with short hairpin RNA (shRNA) targeting H19 (sh-H19) or shRNA control (sh-con) were subcutaneously inoculated into the right side of the groin of nude mice. (A) Seven days later, the volume of the tumor xenografts was measured every 6 days using a caliper. (B) After 25 days of injection, the tumor-bearing mice were sacrificed, and visible tumors were excised and weighted. $*p<0.05$, $n=3$.

TNF-α-induced protein 2 (TNFAIP2)[30]. In this study, we found that expression of miR-152 was lower in glioma cell lines than that in NHAs. Besides, miR-152 overexpression inhibited cell proliferation and invasion in vitro, indicating that miR-152 may be a potential therapy target for glioma. It was reported that miR-152 exerted an important role in tumor suppression in other malignant tumors. For example, Dang et al. discovered that increased miR-152 expression resulted in an obvious suppression on cell growth, motility, and apoptosis by binding to TNF receptor superfamily member 6B (TNFRS6B) signaling in hepatocellular carcinoma[31]. Tsuruta et al. stated that miR-152, which was silenced by DNA hypermethylation, functioned as a tumor suppressor in endometrial cancer[32].

lncRNAs played a vital regulatory role in cell proliferation and invasion of diverse malignancies, making lncRNAs an attractive therapeutic target for cancers[33]. Accumulating evidence highlighted that aberrant expression of H19 was closely related to embryogenesis, fetal growth, and tumorigenesis[34]. For example, Li et al. manifested that upregulation of H19 facilitated tumorigenesis and metastasis of gastric cancer[35]. Han et al. delineated that H19 served as a prognostic biomarker and growth regulator, and promoted tumor growth by targeting eukaryotic translation initiation factor 4A3 (eIF4A3)[36]. In addition, Jiang et al. found that H19 was overexpressed in GBM tissues and contributed to the invasion, angiogenesis, and stemness of GBM cells[37]. Moreover, Li et al. clarified that suppressing H19 inhibited tumorigenicity and stemness in glioma cells[38]. Consistent with these previous studies, the results of our study demonstrated that H19 expression was upregulated in glioma cell lines. Moreover, H19 silencing suppressed proliferation and invasion in glioma cell lines in vitro and impeded glioma growth in vivo.

Recently, a growing number of research studies have suggested that the interaction between lncRNA H19 and miRNAs played an effective role in gene regulation and was involved in various biological processes, including cancer development. Liu et al. hinted that H19-derived miR-675 enhanced proliferation and invasion of gastric cancer cells by regulating tumor suppressor runt domain transcription factor 1 (RUNX1)[39]. Vennin et al. reported that H19-derived miR-675 promoted tumorigenesis of breast cancer by targeting Casitas B-lineage lymphoma proto-oncogene (c-Cbl) and Cbl-b[40]. Zhou et al. highlighted that H19 expression was inversely related to miR-141 expression, and miR-141 and H19 competed with each other in regulating cell proliferation and migration in gastric cancer[41]. In this study, luciferase activity assay indicated that H19 and miR-152 directly regulated each other. Besides, the expression of miR-152 was inversely associated with H19 expression. Furthermore, the results of the MTT assay and Transwell invasion assay further revealed that H19 promoted cell proliferation and invasion by downregulating miR-152 expression. Importantly, miR-152 has been shown to regulate and be regulated by X-inactive specific transcript (XIST) in glioma[42,43]. Therefore, the reciprocal effects of H19 and miR-152 might also involve other effector genes (miRNAs, lncRNAs, or coding genes). Further studies are needed to explore the possible target genes and signaling pathways of miR-152 involved in glioma progression.

Taken together, we found that H19 expression was increased and miR-152 expression was decreased in glioma cell lines. Moreover, downregulation of H19 suppressed cell proliferation and invasion by negatively regulating miR-152 expression. This study provided important clues to understand the interaction between lncRNAs and miRNAs in glioma, illuminating a potential therapeutic target for glioma in the future.

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