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LncRNA H19 is overexpressed in glioma tissue, is negatively associated with patient survival, and promotes tumor growth through its derivative miR-675

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Abstract. – OBJECTIVE: Glioma is one of the most common and invasive tumors of the central nervous system. Long non-coding (lnc) RNAs are involved in many cancers, but their function and mechanism in glioma remain largely unknown. We wished to delineate the role of lncRNA H19 and its derivative miR-675 in this tumor.

PATIENTS AND METHODS: Using qPCR, we compared expression of lncRNA H19 in 35 specimens of glioma vs control tissue, and in two glioma cell lines U251 and U87 vs Normal Human Astrocyte (NHA) cells. Cell proliferation was evaluated after shRNA silencing of lncRNA H19 in glioma cell lines. The role of miR-675 was tested using antagomir and the mimic.

RESULTS: LncRNA H19 was overexpressed in glioma tissue and cell lines. In tissue, higher expression levels were observed in more advanced stages of the tumor. Furthermore, lncRNA H19 was negatively associated with patient survival time. In cell culture experiments, silencing of lncRNA H19 diminished proliferation of glioma cell lines. These effects of lncRNA H19 appeared to be intermediated by miR-675. The latter was overexpressed in glioma tissue and was negatively associated with patient survival. Supporting the involvement of miR-675, its antagomir decreased proliferation of glioma cell lines, whereas its mimic increased proliferation of NHA cells.

CONCLUSIONS: LncRNA H19 is overexpressed in glioma tissue, and is positively associated with the tumor grade and negatively associated with patient survival. In cell culture studies, lncRNA H19 promotes glioma cell proliferation. These tumor-promoting effects of lncRNA H19 appear to be mediated by miR-675.

Key Words:
lncRNA H19, Glioma, miR-675, Proliferation.

Introduction

Glioma is one of the most common and invasive tumors of the central nervous system. Glioma accounts for about 80% of primary malignant brain tumors[1]. These tumors are divided into four histological subtypes: astrocytoma, oligodendroglioma, ependymoma and mixed tumors[2-4]. Treatment options include surgical resection, radiotherapy, and chemotherapy[5], but the overall efficacy is still unsatisfactory. The average survival time of patients with glioma is only 4.5 months without treatment, increasing to 15 months upon temozolomide treatment[6,7]. Insufficient treatment efficacy prompts studies to discover new therapeutic targets in glioma.

Long non-coding RNAs (lncRNAs) are RNAs with the length of more than 200 nucleotides. LncRNAs regulate gene expression at transcriptional, post-transcriptional, and epigenetic levels[8]. Recent studies demonstrated regulatory roles of lncRNAs in different tumors. This was shown with hepatocellular carcinoma (lncRNA ATB[9]) gastric cancer (lncRNA GAPLINC[10]), and glioma (SPRY4-IT1[11]). Furthermore, lncRNAs may regulate cancer malignancy by interacting with signaling proteins, including the regulators of DNA demethylation TCF21 and GADD45A (lncRNA TARID[12]). As studies indicate the involvement of lncRNAs in the regulation of cancer malignancy, there is interest in evaluating lncRNAs as potential targets for cancer treatment. The involvement of lncRNAs in glioma remains largely unknown to date.

In the current study, we quantified expression of lncRNA H19 in 35 specimens of glioma or control tissues and analyzed the relationship between

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expression of this lncRNA and patient survival. In supporting cell studies, we demonstrated that lncRNA H19 promotes proliferation of glioma cells via its derivative miR-675. These observations indicate that lncRNA H19 may be the therapeutic target in glioma.

Patients and Methods

Specimens

Thirty-five specimens of glioma and adjacent normal brain tissue were obtained from patients of the Department of Neurosurgery, Institute of Surgery Research, Daping Hospital, Third Military Medical University (Daping, China). Patient enrollment took place from June 2012 to January 2013. The study protocol was approved by the Institutional Review Board of Daping Hospital, Third Military Medical University, and informed consents were obtained from all patients.

The specimens were collected and kept in liquid nitrogen after surgery. Clinical data were procured, and patients were followed for 3 years. According to the World Health Organization (WHO) classification, glioma specimens were classified into two groups: low-grade glioma (LGG, WHO I-II, 15 specimens), and high-grade glioma (HGG, WHO III-IV, 20 specimens).

Cell Culture

Human glioma cell lines U251 and U87 were obtained from CAMS (Chinese Academy of Medical Sciences, Beijing, China). Normal Human Astrocyte (NHA) cells were obtained from American Type Culture Collection (Manassas, VA, USA); these cells were used as control cells in our studies. Both glioma and NHA cells were cultured in Dulbecco's modified Eagle medium (DMEM)/high glucose (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA).

qPCR

Total RNA from glioma and normal brain tissues were extracted using RNAiso Plus kit (TaKaRa, Otsu, Japan), after the specimens were ground to powder. Total RNA from cells was also extracted with RNAiso Plus kit. RNA concentration of both tissue specimens and cells was measured, and cDNA was synthesized using 1 μg of RNA (PrimeScript RT reagent kit with gDNA Eraser; TaKaRa). Expression of lncRNA H19 was quantified by qPCR, using SYBR Premix Ex Taq (TaKaRa). Expression of GAPDH was used as the reference gene. The primers were listed as follows: GAPDH primers: sense: TGTGGGCATCAATGGATTTGG; anti-sense: ACACCATGTATTCCGGGTCAAT; H19 primers: sense: GGCTCTGGAAGCTAGAGGAA; anti-sense: CTGGGATGATGTGGTGGC.

shRNA Silencing

The short-hairpin (sh) RNA directed against lncRNA H19 was constructed in pGPU6/GFP/Neo vector (shH19): CUUUCUGUCACAUUGACCACACCUG or UCUGAUUGCAGCAUCUUCUUGAUUC. We further used antagomir against miR-675 (GenePharma, Shanghai, China) with the following sequence: TGAGCGGTGAGGGCATACAG[13]. The sequence of microRNA-675 mimic was as follows: CTGTATGCCCTCACCGCTCA[13]. Transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Cell Proliferation (CCK8) Assay

Cells were seeded in 96-well plates at the density of 1500 cells per well, with five replicate wells per each outcome. After respective treatments, the wells received 100 μl of culture medium and 10 μl of the reaction mixture from the CCK8 kit (Dojundo, Kumamoto-ken, Japan). The plate was incubated for 2 hours at 37º C. Then, cell proliferation was estimated by measuring absorbance at 450 nm.

Statistical Analysis

Each experiment was repeated at least three times, and data were presented as means ± standard deviation (SD). Descriptive and comparative analyses were done using GraphPad Prism software (GraphPad, La Jolla, CA, USA). The Student's t-test or one-way ANOVA test was used to compare outcomes. Survival analysis was performed by using the log-rank test. The $p < 0.05$ was considered to be significant.

Results

lncRNA H19 is Overexpressed in Glioma Tissue and is Negatively Associated with Patient Survival Time

We first quantified lncRNA H19 expression in 35 specimens of glioma tissue and adjacent (control) tissue by qPCR. LncRNA H19 was overexpressed in glioma tissue (Figure 1A). Furthermore, the level of lncRNA H19 expression

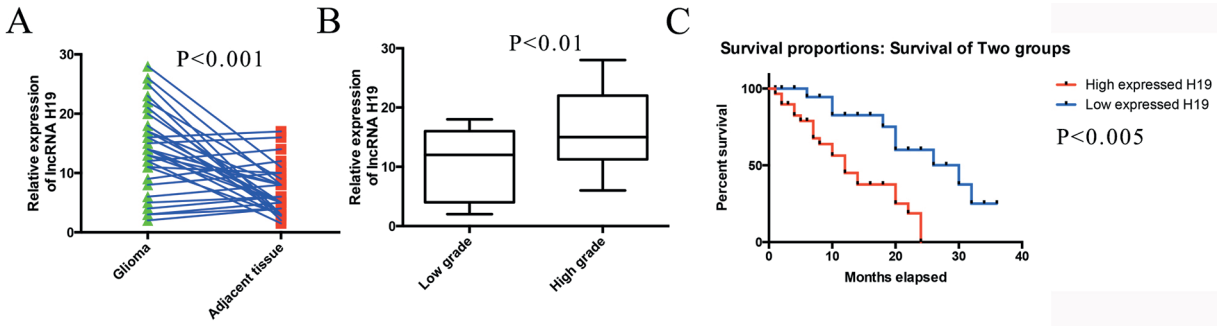

Figure 1. LncRNA H19 is overexpressed in glioma tissue and is negatively associated with patient survival time. ***(A)*** Expression level of lncRNA H19 in 35 glioma and normal tissue specimens. ***(B)*** Expression level of lncRNA H19 in specimens of different tumor grades. ***(C)*** Association between patient survival time (log-rank test) and expression of lncRNA H19.

correlated with the tumor grade. In particular, specimens of grade III-IV glioma showed markedly higher levels of lncRNA H19 expression than specimens of grade I-II glioma (Figure 1B). Following this, we analyzed the association between lncRNA H19 expression and patient survival. Higher levels of lncRNA H19 expression were negatively associated with the overall survival, which was calculated using the log-rank test (Figure 1C).

LncRNA H19 is highly expressed in glioma cell lines and determines their proliferation

To further define biological role of lncRNA H19 in glioma cells, we quantified the expression of lncRNA H19 in U251 and U87 glioma cells and compared this expression to that in control NHA cells. lncRNA H19 was overexpressed in both glioma cell lines (Figure 2A). Afterwards we depleted lncRNA H19 by shRNA in these cell lines (Figure 2B). Suppression of lncRNA H19 expres-

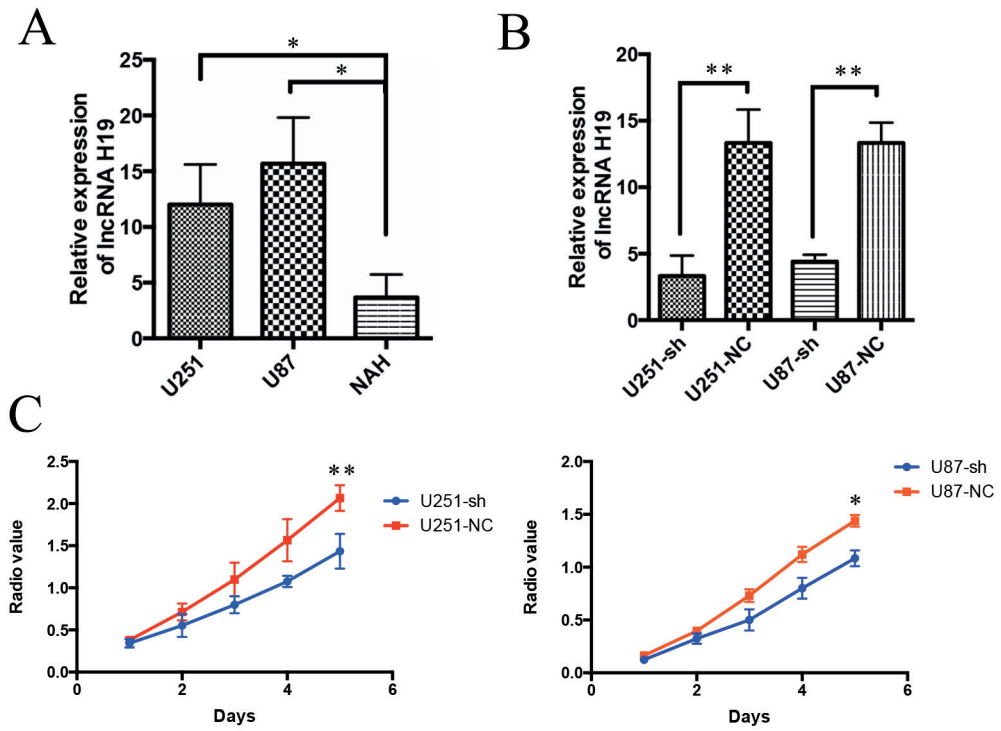

Figure 2. LncRNA H19 is overexpressed in glioma cell lines and determines their proliferation. ***(A)*** Expression level of lncRNA H19 in U251 and U87 glioma cell lines, and NHA cells. ***(B)*** Transfection of shRNA against lncRNA H19 significantly diminishes expression of lncRNA H19 in U251 and U87 glioma cell lines. ***(C)*** Proliferation of U251 and U87 glioma cell lines (respectively, left and right plots) after transfection of shRNA against lncRNA H19. $^{*}p < 0.05$; $^{**}p < 0.01$.

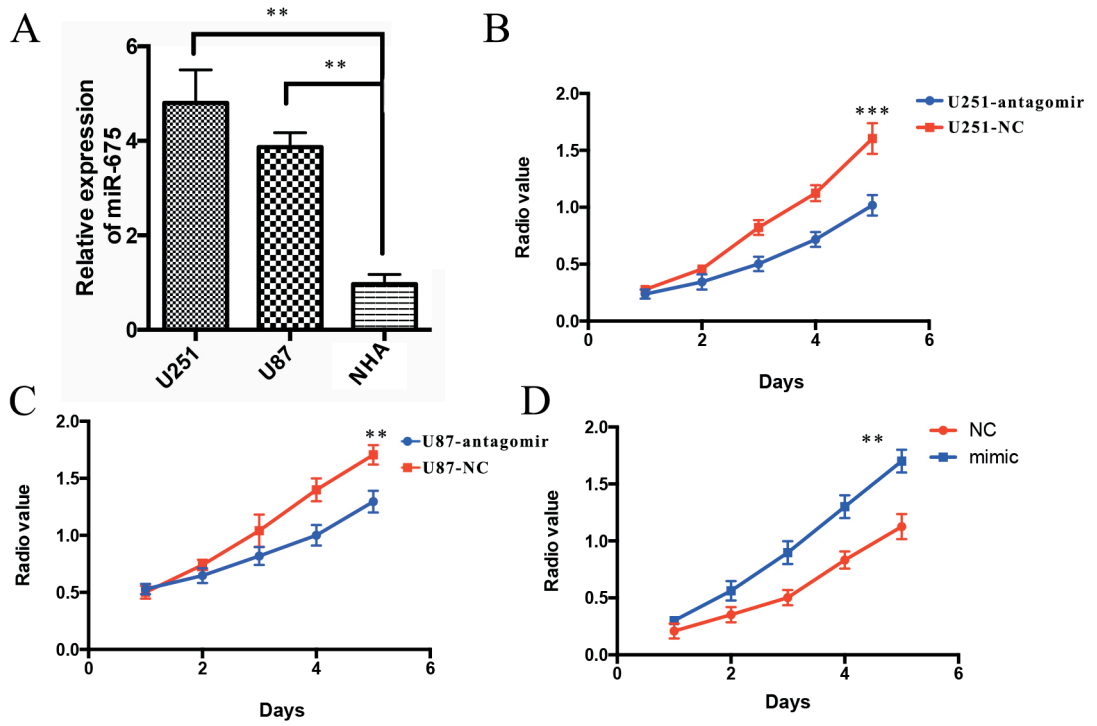

Figure 3. miR-675 is overexpressed in glioma cell lines and determines their proliferation. ***(A)*** Expression of miR-675 in U251 and U87 glioma cell lines, and NHA cell lines. ***(B*** and ***C)*** Respectively, proliferation of U251 and U87 glioma cell lines after transfection of antagomir of miR-675. (***D)*** Proliferation of NHA cells after transfection of the mimic of miR-675. $^{**}p < 0.01$; $^{***}p < 0.001$.

sion in U251 and U87 glioma cell lines led to a marked decline in their proliferation (Figure 2C). These observations are consistent with clinical findings (Table I).

The lncRNA H19 Derived miR-675 Promotes Proliferation of Glioma Cells

Recent studies demonstrated that miR-675, encoded by the exon 1 of lncRNA H19, is an important mediator of the effects of this lncRNA in different types of cancer. We, therefore, wanted to verify whether the effects of lncRNA H19 observed in the aforementioned experiments were mediated by miR-675. We first studied the expression of miR-675 in U251 and U87 glioma cell lines. As expected, miR-675 was overexpressed in both cell lines (Figure 3A). To further verify the role of this miRNA, we suppressed its expression by transfecting U251 and U87 cell lines with the respective antagomir. As expected, the proliferation of both glioma cell lines was markedly diminished when expression of miR-675 was suppressed (respectively, Figures 3B and 3C). To further confirm the role of miR-675, we transfected NHA cells with the mimic of miR-675. This dramatically increased proliferation of control cells (Figure 3D).

miR-675 is overexpressed in glioma tissue and is negatively associated with patient survival time

Following our findings in glioma cell lines, we retested 35 specimens of glioma and control tissue to quantify expression of miR-675. This assessment demonstrated overexpression of miR-675 in glioma tissue (Figure 4A). Similar to lncRNA H19, we observed a negative association between the expression level of miR-675 and patient survival (Figure 4B). Also, expression of miR-675 was closely associated with the tumor size in these glioma specimens (Table I).

Discussion

Our studies demonstrated that lncRNA H19 is overexpressed in glioma tissue, with higher expression levels observed in more advanced stages of the tumor. Furthermore, lncRNA H19 is negatively associated with the patient survival time. In cell culture experiments, silencing of lncRNA H19 diminished proliferation of glioma cells. We also provide

Table I. Clinical characteristics of patients with glioma stratified by lncRNA H19 and miR-675 expression levels.

		H19			miR-675		
Characteristics	**No.**	**High expressione**	**Low xpression**	***p***	**High expression**	**Low expression**	***p***
Age (years)				0.387			0.219
<50	21	13	8		15	6	
>50	14	9	5		6	8	
Gender				0.465			0.332
Male	18	12	6		13	5	
Female	17	10	7		8	9	
Family history of cancer				0.238			0.285
Yes	13	5	8		6	7	
No	22	17	5		15	7	
WHO grade				0.0035			0.0021
I-II	15	9	6		5	10	
III-IV	20	17	3		16	4	
Tumor size (cm)				0.0002			0.0003
<5 cm	11	4	7		2	9	
>5 cm	24	18	6		19	5	

evidence that the aforementioned effects of lncRNA H19 are mediated by miR-675. Similarly to lncRNA H19, miR-675 is overexpressed in glioma tissue and is negatively associated with patient survival. Furthermore, negating the effects of miR-675 decreased proliferation of glioma cell lines akin to lncRNA H19 silencing. Finally, increasing the levels of miR-675 enhances proliferation of normal cells.

Abnormally expressed lncRNAs have been shown in many tumors, including breast, lung, colorectal, and gastric cancer. There are still substantial knowledge gaps on the clinical relevance of lncRNAs. Some abnormally expressed lncRNAs have been reported in glioma. Similar to our observations, other studies reported that abnormally expressed lncRNA could be used as a biomarker to predict patient survival. This was shown with regard to lncRNA SPRY4-IT1 and MALAT1[14]. It was further shown that suppression of lncRNA MALAT1 induces apoptosis in glioma cells[15] and leads to the reduction of SOX2, the biomarker for stemness (that is, the ability to self-renew)[16]. Similarly, the studies with cell lines demonstrated the association of lncRNA with glioma. For instance, it was shown[17] that suppression of lncRNA H19 in U251 and U87 glioma cell lines decreased expression of the stemness markers CD133, NANOG, Oct4 and Sox2, suggesting that lncRNA H19 is important for maintaining

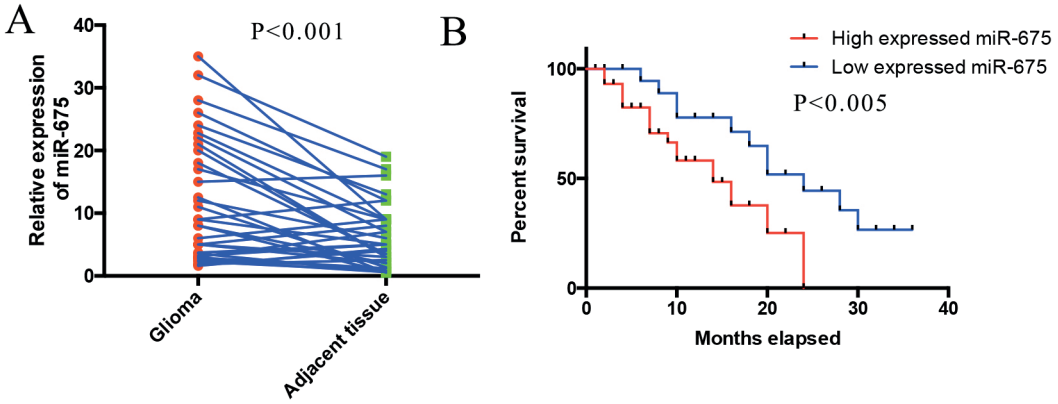

Figure 4. miR-675 is overexpressed in glioma tissue and is negatively associated with patient survival time. ***(A)*** Expression level of miR-675 in 35 specimens of glioma and normal tissue. ***(B)*** Association of patient survival time (log-rank test) and expression of miR-675.

the stemness of glioma cells. Other researchers[18] demonstrated that lncRNA H19 is involved in chemoresistance, indicating that this lncRNA may be a therapeutic target in temozolomide-resistant glioma.

Similar to us, other studies[17,19] implicated lncRNA in cancer proliferation. In addition, some researchers demonstrated positive and negative interactions of lncRNA H19 with miRNAs. Thus, lncRNA H19 was shown to compete with miR-141 to promote proliferation of gastric cancer cells[20]. In other cancers, lncRNA H19 was found to exert its effects by competitively binding to miR-17-5p[21]. Similarly, lncRNA H19 was found to suppress miR-630 in nasopharyngeal carcinoma[22]. In our studies, lncRNA H19 was associated with miR-675. Specifically, the latter was the mediator of pro-cancer effects of lncRNA H19. While in previous studies lncRNA H19 simply interacted with miRNAs, the relationship with miR-675 is because of this miR is the derivative of lncRNA H19.

Involvement of miR-675 in cancer-promoting effects of lncRNA H19 was confirmed by other studies as well. For instance, this was demonstrated with regard to bladder cancer[23], breast cancer[24], and lung cancer[25].

Our study had some limitations. While we were able to demonstrate that lncRNA H19, via miR-675, increases proliferation of glioma cells, the exact mechanism of this cancer-promoting effect remains unclear. The clinical relevance of this lncRNA will need to be further confirmed by subsequent studies.

Conclusions

lncRNA H19 is overexpressed in glioma tissue, and is positively associated with the tumor grade and negatively associated with patient survival. In cell culture studies, lncRNA H19 promoted glioma cell proliferation. These tumor-promoting effects of lncRNA H19 were found to be mediated by miR-675.

Conflicts of interest

The authors declare no conflicts of interest.

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