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The lncRNA H19 interacts with miR-140 to modulate glioma growth by targeting iASPP

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ABSTRACT

H19, one of the first found cancer-associated long non-coding RNAs (lncRNAs), is involved in the development and progression of many types of tumors. An aberrant expression of H19 was observed in hepatocellular carcinoma, cervical cancer, breast cancer, ovarian cancer, and colorectal cancer. However, the exact effects and molecular mechanisms of H19 in glioma progression are still unknown up to now. In this study, we investigated the role of H19 in human glioma cell lines and clinical tumor samples in order to determine the function of this molecule. In our research, lncRNA-H19 was specifically upregulated in glioma cell lines and promoted glioma cell growth through targeting miR-140. Knockdown of H19 inhibited the proliferation and invasion of human glioma cell and suppressed its metastasis in vitro and in vivo. In addition, miR-140 dependent inhibitor of apoptosis-stimulating protein of p53 (iASPP) regulation was required in H19 induced glioma cell growth. These findings indicated that H19 might regulate the tumor growth and metastasis via miR-140 dependent iASPP regulation. Taken together, our data indicated that H19 might be an oncogenic lncRNA that promoted proliferation and metastasis of glioma and could be regarded as a therapeutic target in human glioma.

1. Introduction

Glioma is a highly malignant phenotype characterized by rapid progression, early metastasis, and a limited response to radiotherapy and chemotherapy. In the past 10 years, despite of FDA approved therapeutic regimens and great improvements in medical care, we observed no momentous effect on glioma patient survival [1]. Therefore, to identify biomarkers which will promote early diagnosis and allow personalized treatment strategies for patients at high risk of glioma has become an urgent need [2].

MicroRNA (miRNAs) are especially important in almost all tumor development studies because they can be targets of genomic lesions, controlled by classic tumor signals, and they themselves present as a class of oncogenes or tumor suppressors [3,4]. In recent, a close association between miRNAs and glioma tumorigenesis has been suggested [5,6]. MiR-140 has been noted because it is involved in the development and progression of so many types of cancers, including breast cancer [7], osteosarcoma [8] and colon cancer [9]. Yuan et al. reported that miR-140 is significantly downregulated in non-small cell lung cancer (NSCLC) tissues and cell lines; and that miR-140 suppresses tumor growth and metastasis of non-small cell glioma by targeting insulin-like growth factor 1 receptor [10]. These findings suggest that miR-140 may play a tumor-suppressor role in these cancers; however, as far as we know, its roles and the potential mechanisms in glioma remain unclear.

Human genome sequence data indicates that more than 90% of the DNA sequences actively transcribed but only 2% of it encodes protein, thus the majority of transcripts are referred to as non-coding RNAs (ncRNAs) [11,12]. Small non-coding RNAs such as microRNAs have been studied extensively and their roles in gene regulation and cell function have been elucidated innumerous cancers [12]. Recent studies have shown that lncRNAs play important roles in both normal development and diseases including cancer [13]. LncRNAs have emerged as new players in

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cancer research and several studies have shown that some lncRNAs function as oncogenes, tumor suppressor genes or both, depending on the circumstance [14].

The mechanisms by which lncRNAs exert their effect varies under different conditions, however, emerging evidences have revealed that the interaction between lncRNAs and microRNAs plays a major role [15,16]. Han et al. reported that lncRNA H19 indicates a poor prognosis of colorectal cancer and promotes tumor growth by recruiting and binding to eIF4A3 [17]. Increased level of H19 long non-coding RNA promotes invasion, angiogenesis, and stemness of glioblastoma cells [18].

In this study, we report an interaction between H19 and miR-140 which regulates glioma cell growth through directly targeting iASPP. Our findings provide a novel understanding of the role of H19 and miR-140 in glioma metastasis and the mechanism involved.

2. Materials and methods

2.1. Tissue samples, cell lines and cell transfection

We collected 28 paired primary glioma tissues and the matched adjacent normal tissues. We obtained all samples from patients who underwent surgical resection at Xiangya Hospital of Central South University (Changsha, China). The tissues were snap-frozen in liquid nitrogen, and then stored at −80 °C. This project was approved by the Ethic Committee of Xiangya Hospital of Central South University.

We purchased human glioma cell lines, including U373, A172, U251, T98G and U87MG cells from the American Type Culture Collection (Manassas, VA, USA). They were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, CA, USA) at 37 °C in a humidified atmosphere with 5% CO_2. The expression of miR-140 was achieved by transfection of miR-140 mimics or miR-140 inhibitor (Genepharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen). A si-iASPP vector was used to achieve knockdown of iASPP (GeneCopoecia, Guangzhou, China). Cells were plated in 6-well plates or 96-well plates, transfected, incubated for 24 h or 48 h and used for further assays or RNA/protein extraction.

2.2. RNA extraction and SYBR green quantitative PCR analysis

We extracted total RNA from cells using Trizol reagent (Invitrogen, CA, USA) and detected mature miR-140 expressions in cells using a Hairpin-it TM miRNAs qPCR kit (Genepharma, Shanghai, China). We used expression of RNU6B as an endogenous control. Fbxw8 expression was measured by SYBR green qPCR assay (Takara, Dalian, China). Data were processed using $2^{-\Delta\Delta CT}$ method.

2.3. MTT assay

A modified MTT assay was used to evaluate cell viability. After seeding 2×10^3 transfected cells/well into 96-well culture plates we assessed the viability of U251 and U87MG cells at five time points (on day 1, 2, 3, 4 and 5). In brief, quantification of mitochondrial dehydrogenase activity was achieved through the enzymatic conversion of MTT [3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, MO, USA] to a colored formazan product. MTT (10 μl, 10 mg/ml) was added to the cells, incubated for 4 h, and we terminated the reaction by removal of the supernatant and addition of 100 μl DMSO to dissolve the formazan product. After 0.5 h, the optical density (OD) of each well was measured at 570 nm using a plate reader (ELx808 Bio-Tek Instruments, City, ST, USA).

2.4. Cell cycle analysis

U87MG and U251 were processed after 48 h of transfection with Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Thermo Fisher Scientific Inc.), according to the manufacturer's protocol. Cells were then stained with propidium iodide and cell cycle analysis was performed on a Navios flow cytometer (Beckman Coulter) and analysed using Kaluza software (v1.2, Beckman Coulter).

2.5. Western blot analysis

The expression of iASPP, ΔNp63, MMP2 and MMP9 in glioma cells was detected by performing immunoblotting. We lysed cultured or transfected cells in RIPA buffer with 1% PMSF and loaded protein onto a SDS-PAGE minigel and transferred them onto PVDF membrane. After probed with 1:1000 diluted rabbit polyclonal iASPP, ΔNp63, MMP2 and MMP9 antibody (Abcam, MA, USA) at 4 °C overnight, the blots were subsequently incubated with HRP-conjugated secondary antibody (1:5000). ECL Substrates was used to visualize signals (Millipore, MA, USA). β-actin was used as an endogenous protein for normalization.

2.6. Luciferase reporter assay

U251 cells were seeded into a 24-well plate. After cultured overnight, cells were co-transfected with the wild-type and mutated iASPP 3′UTR reporter plasmid, and pRL-TK plasmids, or transfected with miR-140 mimics and miR-140 inhibitor. Luciferase assays were performed 48 h after transfection using the Dual Luciferase Reporter Assay System (Promega, WI, USA).

2.7. Statistical analysis

Data were exhibited as mean ± SD of three independent experiments and processed using SPSS 17.0 statistical software (SPSS, Chicago, IL, USA). By using Wilcoxon's paired test we compared the expression of miR-140 in glioma tissues and the paired adjacent normal tissues. The differences between groups in the migration and invasion assays were evaluated using the one-way ANOVA. P values of <0.05 were considered statistically significant.

3. Results

3.1. H19 is frequently upregulated in glioma tissues and cell lines

SYBR green quantitative PCR analysis was performed to quantify the expression of H19 in glioma tissues and cell lines. In a large panel of 28 cases of primary glioma tissues and the adjacent normal tissues, the expression of H19 was significantly increased in glioma tissues, compared with the paired adjacent normal tissues (Fig. 1A). Besides, the expression of H19 were at a significantly higher level in the five human glioma cell lines compared to normal human astrocyte (NHA) (Control) (Fig. 1B). Among the five cell lines, H19 expressed at higher levels in U87MG and U251 cell lines.

3.2. H19 knockdown inhibited glioma cell growth and induces growth arrest

To validate experimentally the effect of H19 expression on glioma cell growth, H19 expression was knocked down by si-H19 transfection (Fig. 2A). Then the cell growth and cell cycle of U87MG and U251 cells were monitored. Cell growth of both U87MG and U251 was reduced by H19 knockdown (Fig. 2B and C). For a better understanding of the nature of the observed impact on proliferation, the effect of H19 knockdown on cell cycle was