

Original Article

LncRNA linc01116 promotes glioma cell migration and invasion by modulation of radixin targeted by miR-31

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Abstract: Background: Long non-coding RNA (lncRNA) linc01116 was found to be abnormally expressed in many malignant tumor tissues and involved in cancer progression, but its expression and role in glioma tissue is still unclear. This study was designed to investigate the expression of linc01116 in glioma tissues and the role of linc01116 in glioma cell migration and invasion. Methods: Linc01116 and miR-31 expression was measured in 135 cases of human glioma tissues and normal brain tissues using Real-time quantitative PCR (RT-qPCR). The function of linc01116 in glioma cells was determined by Transwell invasion assays and nude mice metastasis assay. Luciferase reporter system was used to confirm the connection between linc01116 and miR-31, or miR-31 and radixin. Results: Linc01116 is highly expressed in glioma tissue and cells, along with low expression of miR-31, and there was a negative correlation between the expression of linc01116 and miR-31 in glioma tissue. In addition, the expression of linc01116 in glioma patients with metastasis was significantly higher than that in patients without metastasis, while miR-31 was significantly lower. In vitro and in vivo studies shown that linc01116 promoted invasion and migration of glioma cells. The luciferase gene reporter system had confirmed that linc01116 targeted miR-31 and miR-31 targeted radixin in U251 cells. Moreover, radixin was downregulated and decreased E-cadherin protein expression, but increased MMP-9 and vimentin protein expression in U251 cells. Conclusion: LncRNA linc01116 is highly expressed in glioma tissues, and it promotes glioma cell migration and invasion by modulation of radixin targeted by miR-31.

Keywords: Linc01116, miR-31, radixin, glioma

Introduction

Glioma is a primary brain tumor caused by carcinogenesis of brain and spinal glial cells, and is one of the most common intracranial tumors. About 45% of the patients with intracranial tumors have gliomas, and the annual incidence rate is about 3-8 people per 100,000 population [1, 2]. Although many previous studies have partially revealed the mechanism of glioma disease development, and clinical progress has been made in the diagnosis and treatment of glioma [3], the mortality rate and postoperative survival rate of glioma patients have not significantly improved.

Long non-coding RNA (lncRNA) is a type of RNA that is over 200 bp in length, has little or no open reading frame, and cannot encode, or rarely encodes a protein [4, 5]. With the devel-

opment of gene sequencing, gene chips, and genomics, more lncRNAs have been found to be involved in the development of tumors [6]. At the same time, lncRNA has become a new hot spot in tumor research due to its remarkable tumor tissue specificity and its potential for application in tumor diagnosis and prognosis [7, 8]. LncRNA linc01116 is a newly discovered lncRNA that has been shown to be up-regulated in human prostate cancer cell lines but can be reduced by treatment with sulphoraphane [9]. Yuan et al. founded that lncRNA linc01116 was highly expressed in non-small cell lung cancer tissues, and inhibiting the expression of linc01116 in NSCLC cells in vitro could target FAT4 by inhibiting activation of the Hippo signaling pathway, thereby inhibiting cell proliferation, migration, invasion, and promotion of apoptosis in NSCLC [10].

We first detected the expression of linc01116 and miR-31 in normal human glioma tissue and human glioma cell lines by RT-qPCR. After obtaining the results of up-regulation of linc01116 in human glioma tissue, we conducted this study. In this paper, we present a novel molecular mechanism by which linc01116 promoted the proliferation and metastasis of human glioma cells, and show that lncRNA linc01116 could promote glioma cell migration and invasion by modulation of radixin targeted by miR-31.

Materials and methods

Tissue and consent

All glioma tissues were collected from 135 patients who had undergone curative resections from glioma at Beijing Anzhen Hospital, Capital Medical University. There were 79 male patients and 56 female patients; the ages of the 135 cases glioma patients were from 12 years to 71 years, and 49 was their median age. According to the WHO criteria, these comprised 62 stage I and II tumors, and 73 stage III and IV tumors. All subjects (or their guardians) included in this study consented the research protocol and signed an informed consent form. The ethics committee of Beijing Anzhen Hospital, Capital Medical University approved this research protocol.

Real-time quantitative PCR

Trizol was used to extract the total RNA of the tissue or cells. The extracted RNA was reverse transcribed into cDNA by using PrimeScriptTM RT Master Mix reverse transcription kit (RR036B, Takara, Beijing, China). 20 µl Real-time fluorescence quantitative PCR (RT-qPCR) system was prepared according to the SYBR Green qPCR Master Mix kit instructions (638320, Takara, Beijing, China) and amplified using ABI 7500 fluorescence quantitative PCR instrument (Applied Biosystems, Maryland, USA). PCR primers: linc01116-F: 5'-CTTCTTCCCTCCAAGTGA-T-3', linc01116-R: 5'-TTAGCAAGTCAGCAAGTCC-T-3'; miR-31-F: 5'-CAGGCAAGATGCTGGCATAGC-3'; miR-31-R: 5'-TGGTGTCGTGGAGTCG-3'; U6-F, 5'-CTCGCTTCGGCAGCACA-3', U6-R, 5'-AACGCTTACGAATTTGCGT-3'; GAPDH-F, 5'-CTCGCC-TAGAGTGAGCTCC-3', GAPDH-R, 5'-AACTGCTG-CGTTGACGGGTATG-3'.

Western blot

Tissue or cell lysates were separated by SDS-page and then transferred to PVDF membrane. Primary antibody: anti-radixin (ab52495, 1:2000, Cambs, UK), anti-MMP-9 (ab73734, 1:1000, Cambs, UK), or anti-Vimentin (ab8978, 1:500, Cambs, UK), or anti-E-Cadherin (ab760-55, 1:500, Cambs, UK), or anti-VEGFA (ab1316, 1:2000, Cambs, UK), or anti-GAPDH (ab9484, 1:3000). Second antibody: goat anti-rabbit (ab150077, 1:1000, abcam, Cambs, UK), or goat anti-rat (ab150117, 1:1000, abcam, Cambs, UK). Primary antibody was incubated overnight at 4°C and second antibody was incubated for 1 hour at room temperature.

Transwell invasion assay

Corning BioCoatTM Matrigel transwell invasion chamber (Corning, USA) was used to evaluate the ability to migrate (Upper chamber without glue, 3422) and invade (Upper chamber with glue, 354480) in SHG-44 and U251, and their specific experimental steps are the same.

Nude mouse metastasis assay

4-week-old nude mice (male/female = 1:1) that were adaptive for feeding (room temperature of 20-24°C, half day and night, air humidity of 60%) were selected. $1 \times 10^6/0.1$ ml logarithmic phase of Vector SHG-44 or up-linc01116 SHG-44 cells were injected through the tail vein. After 8 weeks, mice were sacrificed, and the fresh lung samples were harvested and fixed with 10% formalin for histopathology analysis. Tissues were paraffin-embedded and sectioned at a thickness of 5 µm. The sections were stained with H&E and then examined under the microscope to count the number of tumor nodules.

Cell and cell transfection

HA cells (C0402, Shanghai Honsun Biological Technology Co., Ltd., Shanghai, China), SHG-44 cells (YB-ATCC-4187, Shanghai Yu Bo Biological Technology Co., Ltd., Shanghai, China), U87 (HTB-14, ATCC, VA, USA), U118 MG (HTB-15, ATCC, VA, USA) and U251 (ml028960, Shanghai Enzyme-linked Biotechnology Co., Ltd. Shanghai, China) was cultured with DMEM medium to which was added 10% of fetal bovine serum

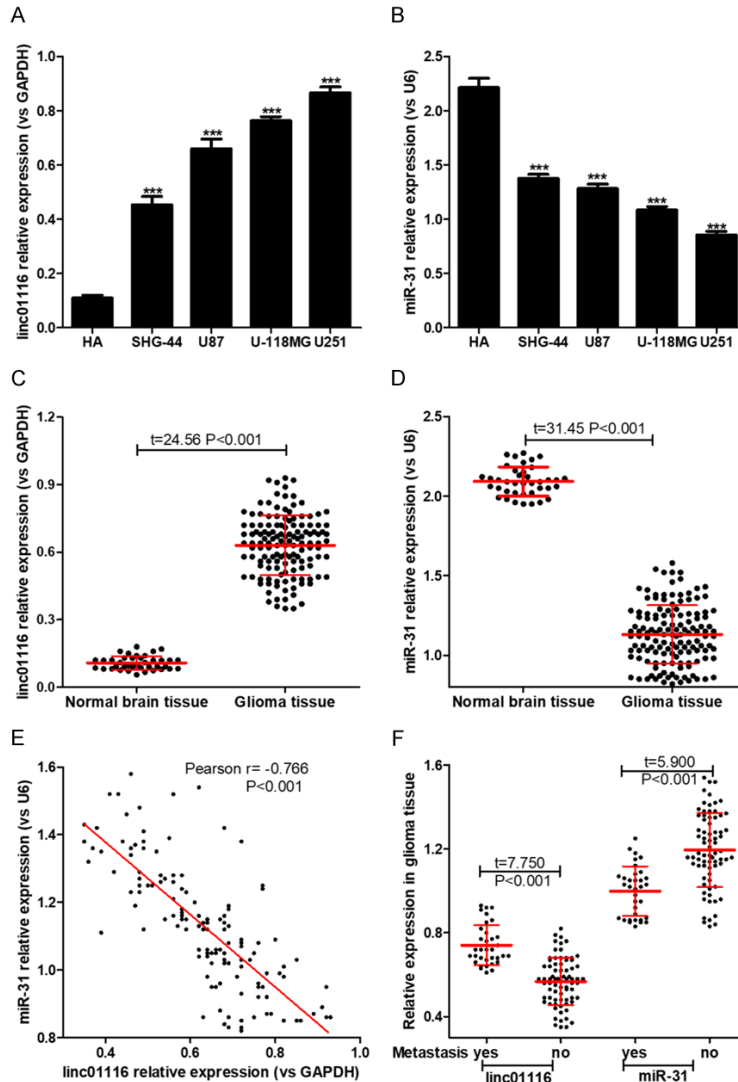


Figure 1. Linc01116 and miR-31 expression in glioma cells and tissue. A and C. The expression of linc01116 in glioma cells and tissue was higher than that in normal astroglia and normal brain tissue; B and D. The expression of miR-31 in glioma cells and tissue was less than that in normal astroglia and normal brain tissue; E. linc01116 and miR-31 were negatively correlated in glioma tissue; F. Expression of linc01116 and miR-31 in metastatic or non-metastatic glioma tissue.

and 1% penicillin-streptomycin. si-linc01116 (F: 5'-CCCAUUCUUGUUGUCACUTT-3', R: 5'-AGUGACAACAAGAAUGGGTT-3'), si-radixin (F: 5'-AUGAUUUCUUUUUUUUGAGAC-3', R: 5'-CUCAAUAAUAAGAUUACAUUA-3'), si-NC (F: 5'-ACCGCAUAGUGUAAACUUUATT-3', R: 5'-GAAAGUUAGACUAGCGGCTT-3') were designed and synthesized by Sheng Gong Bioengineering Co., Ltd. (Shanghai, China). si-RNA, si-NC, microRNA-mimic and microRNA-NC were directly transferred into cells by Lipofectamine™ 2000 trans-

fection reagent (11668019, Invitrogen, CA, USA). Wild type or mutated 3'-UTRs were first connected to pISCHECK2 (Promega, WI, USA) and then were transfected into cells as si-RNA.

Statistical analysis

Data was presented in (mean \pm standard deviation) and analyzed by SPSS 20.0. Student's t-test or chi-square test was used to compare differences between two groups. The correlation between two groups was analyzed by Pearson. Cox regression model was used to test univariate and multivariate analysis for survival times of glioma patients. Survival curves of glioma patients based on linc01116 or miR-31 protein expression were drawn by Kaplan-Meier method. Log-rank test was used to compare differences of survival curves. $P < 0.05$ indicated significant difference. $P < 0.05$ indicated significant difference.

Results

Linc01116 and miR-31 expression in glioma tissues

RT-qPCR was used to measure the expression of linc01116 and miR-31 in normal astroglia (HA cell), glioma cells (SHG-44, U87, U-118MG and U251 cells), glioma tissues ($n = 135$) and normal brain tissues ($n = 39$), and we found that the expression of linc01116 in glioma cells and tissues was significantly higher than that in normal astroglia and normal brain tissues (Figure 1A and 1C), and miR-31 was just the opposite (Figure 1B and 1D). Pearson was used to analyze the correlation between linc01116 and miR-31 expression in glioma tissue. As shown in Figure 1E, there was a negative correlation between the expression of linc01116 and miR-

Table 1. Univariate and multivariate Cox regression analysis for prognosis of glioma patients

Clinical parameters	Univariate analysis			Multivariate analysis		
	OR	95% CI	P	OR	95% CI	P
Age	3.209	1.254-10.957	0.025	2.895	1.271-10.324	0.040
Gender	1.682	0.845-3.997	0.072			0.458
Tumor diameter	3.628	1.342-5.324	0.049			0.162
WHO grade	4.032	1.605-11.271	0.003	2.692	1.004-10.628	0.047
KPS	3.164	0.986-4.238	0.062			0.084
linc01116 expression	6.259	3.200-9.397	< 0.001	5.982	3.251-14.127	< 0.001
miR-31 expression	4.138	2.514-9.029	< 0.001	4.257	2.132-8.627	0.002

Note: OR = odds ratio, 95% CI = 95% confidence interval; SPSS statistical analysis software was used for multivariate Cox regression analysis. When *P* was higher than 0.05, the results do not show the OR and 95% CI.

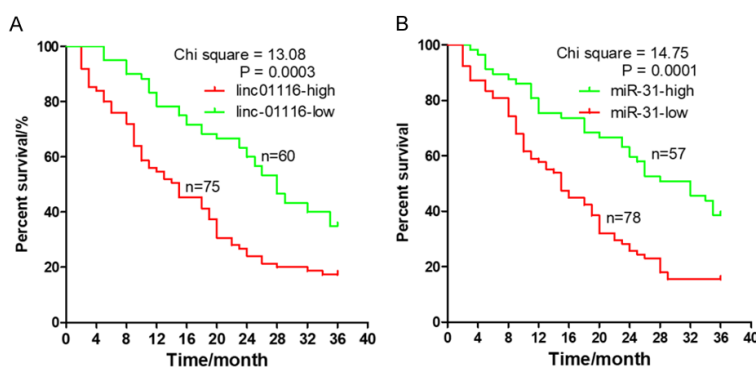


Figure 2. Survival curves of glioma patients with different linc01116 or miR-31 expression. A and B. The 3-year overall survival of glioma patients with high linc01116 or low miR-31 expression was significantly lower than that with low linc01116 or high miR-31 expression.

31 in glioma tissue. In addition, there were 34 cases of glioma patients who were confirmed to have cancer cell metastasis, 74 cases without metastasis, and 27 cases were not clear. The expression of linc01116 in glioma patients with metastasis was significantly higher than that in patients without metastasis, and miR-31 was significantly lower (**Figure 1F**). These results indicated that abnormal expression of linc01116 and miR-31 in glioma tissues was associated with metastasis of glioma cells.

Effect of linc01116 and miR-31 expression on prognosis

According to the expression level of linc01116 or miR-31 in glioma tissues, 135 glioma patients were divided into two groups. The linc01116 low expression group was defined as having expression level of linc01116 < the median value of 135 glioma patients. The same division into groups was done for miR-31. 135

glioma patients were followed up at least once in four months or when patients came to hospital for review. The factors influencing the survival times of glioma patients were analyzed by COX regression model. The results showed (**Table 1**) that linc01116 expression level (OR = 5.982, 95% CI = 3.251-14.127) and miR-31 protein expression level (OR = 4.257, 95% CI = 2.132-8.627) were independent risk factors influencing glioma prognosis. Postoperative survivals in glioma patients with low expres-

sion of linc01116 were significantly higher than in patients with high expression of linc01116 (*P* = 0.0003) (**Figure 2A**), and postoperative survivals in glioma patients with low expression of miR-31 were significantly lower than patients with low expression of miR-31 (*P* = 0.0001) (**Figure 2B**).

Linc01116 promoted cell migration and invasion in glioma cell lines

Transwell invasion assay revealed that the ability of cell motility and invasion increased in up-linc01116 SHG-44 cells as compared with those with vector SHG-44 cells (both *P* < 0.001, **Figure 3A** and **3C**), whereas the ability of cell motility and invasion was decreased in si-control U251 cells as compared with those with si-linc01116 U251 cells (both *P* < 0.001, **Figure 3B** and **3D**). To evaluate the in vivo effects of linc01116 on tumor metastasis, two groups of eight mice each were injected intravenously in

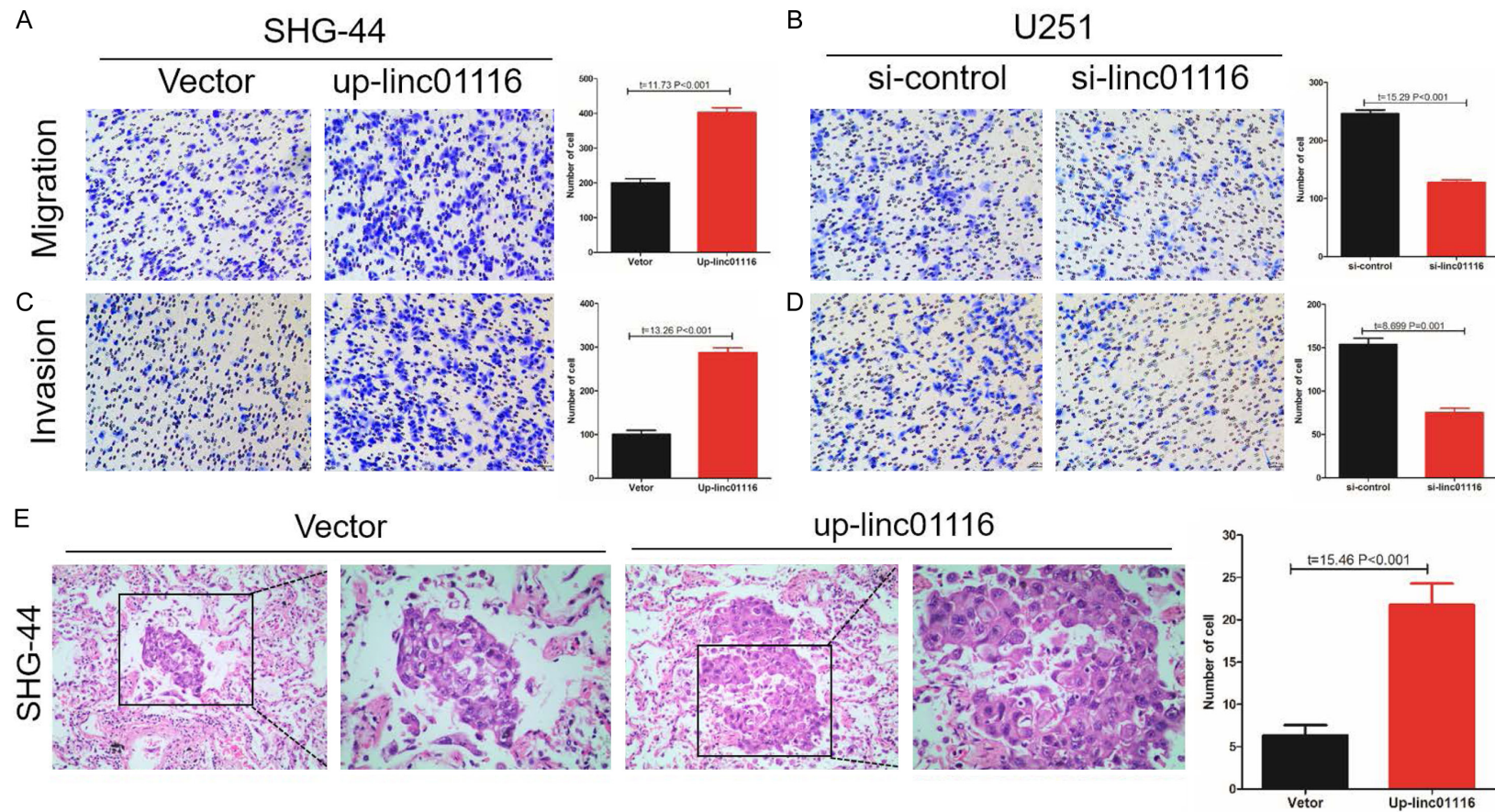


Figure 3. Linc01116 promoted cell migration and invasion in glioma cell lines. A and C. Linc01116 was upregulated in SHG-44 cells and promoted cell migration and invasion; B and D. Linc01116 was downregulated in U251 cells and inhibited cell migration and invasion; E. Lung morphology and H&E staining of nude mice inoculated with SHG-44 up-linc01116 or control cells via tail vein, and the number of lung metastatic foci in each group were also calculated. Each experiment repeated 3 times independently.

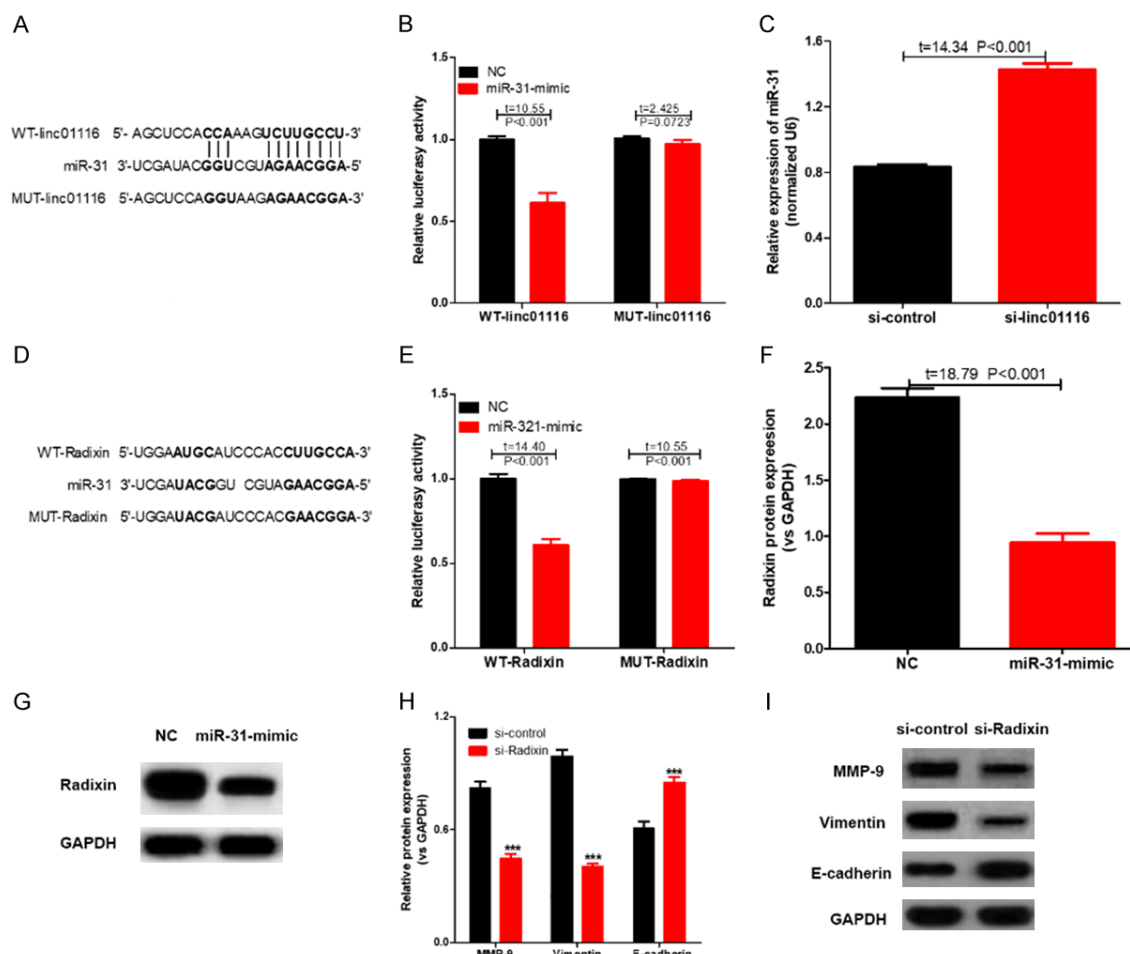


Figure 4. Linc01116 targets miR-31 and miR-31 targets Radixin. A. WT-linc01116 3'UTR and MUT-linc01116 3'UTR luciferase reporter vector were constructed; B and E. NC/miR-31-mimic were transfected into U251, and luciferase activity was detected; C. Linc01116 was downregulated and it increased the expression of miR-31 in U251 cells; D. WT-Radixin 3'UTR and MUT-Radixin 3'UTR luciferase reporter vectors were constructed; F and G. miR-31 was up-regulated and it decreased the expression of radixin protein in U251 cells; H and I. Radixin was downregulated and it decreased E-cadherin protein expression, and increased MMP-9 and vimentin protein expression in U251 cells; *** is $P < 0.001$ vs si-control group. Each experiment repeated 3 times independently.

the tail vein with Vector SHG-44 cells or up-linc01116 SHG-44 cells, respectively. After 8 weeks, the mice were killed and the metastatic nodules at the lung surfaces were counted. There were larger numbers of metastatic nodules at the surface of the lungs of mice injected with the up-linc01116 SHG-44 cells than those with the Vector SHG-44 cells ($P < 0.001$, **Figure 3E**). HE staining confirmed that the nodules on the surfaces of mice lungs were metastatic tumors (**Figure 3E**).

Linc01116 regulated glioma cell EMT via miR-31/radixin

We found that there are mutual binding sequences between linc01116 and miR-31 by

analyzing the sequences of them (**Figure 4A**), and the luciferase reporter system confirmed that they could bind in U251 cells (**Figure 4B**), and down-regulation of linc01116 could reduce the expression of miR-31 in U251 cells (**Figure 4C**). That meant that linc01116 targeted miR-31 in glioma cells. Previous studies [11] had confirmed that human miR-31 targeted radixin and inhibited migration and invasion of glioma cells, and we also found that miR-31-mimic could decrease the expression of radixin protein (**Figure 4D-G**). In addition, radixin was downregulated by transferring in si-radixin, and this decreased E-cadherin protein expression, and increased MMP-9 and vimentin protein expression in U251 cells. The above results indicated that down-regulation of linc01116

inhibited glioma cell EMT by modulation of radixin targeted by miR-31.

Discussion

In this study, we found that linc01116 was up-regulated in glioma tissues and cells. There were many lncRNAs found to be involved in gliomas, such as MEG3 [12], HOTAIR [13], and CASC2 [14]. Although no previous studies had reported the expression of linc01116 in gliomas, there were many studies had found that linc01116 was abnormally expressed in many malignant tumor tissues and was involved in cancer progression [9]. Moreover, Hu et al. [15] found that linc01116 was highly expressed in breast cancer, and promoted the proliferation of breast cancer cells by modulation of ESR1 targeted by miR-145; Fang et al. found that [16] linc01116 promotes the progression of epithelial ovarian cancer regulating cell apoptosis. To investigate the effect of linc01116 expression on glioma patients, we found that the expression of linc01116 in patients with metastasis was significantly higher than that in patients without metastasis, and the glioma patients with low linc01116 had longer survival time. These results indicate that linc01116, which is abnormally expressed in glioma tissues, is involved in the metastasis of glioma cells.

Because lncRNA is a non-coding RNA, it can only exert biological functions by regulating the expression of other genes. Previous research had shown there were many mechanisms regulating the expression of genes by lncRNA [17, 18], and the interaction mechanism between lncRNAs and miRNAs was one of the important ways that lncRNAs regulated gene expression [19]. On one hand, lncRNA can not only act as a decoy of miRNA to inhibit the binding of miRNA and target gene mRNA, and exhibit the function of endogenous miRNA sponges to inhibit miRNA expression, but also indirectly inhibits the negative control of miRNA on target gene mRNA by competing with miRNA to bind to the 3'-UTR of the target gene mRNA. On the other hand, miRNAs can target a large number of protein-coding genes and can also target lncRNAs. In this study, we also found that miR-31 was down-regulated in glioma tissue and cells, and had a negative correlation with linc01116 in glioma tissues.

miR-31 is located on human chromosome 9p21.3. Many previous studies have found that miRNA-31 expression was dysregulated in lung cancer [20], tongue cancer [21], and colorectal cancer [22], and played a role of tumor suppressor gene in the majority of malignant tumors [23, 24]. In gliomas, miR-31 was found to be abnormally expressed in glioma tissues and was involved in advanced tumor progression and unfavorable prognosis in patients with gliomas [25, 26]. In addition, Hua et al. [11] found that human miR-31 targeted radixin and inhibited migration and invasion of glioma cells. In this study, we found that the expression of miR-31 was not only negatively correlated with the expression of linc01116 in human glioma tissues, but also interacted with linc01116 in human glioma cells. According to previous research, we know that miR-31 functions as a tumor suppressor in liver cancer, so linc01116 may function as a tumor-promoting factor, which was confirmed by the migration and invasion of human glioma cells in vivo and in vitro.

MicroRNA is also a non-coding RNAs that can only exert their biological functions through the regulation of target gene expression. As noted above, we found that miR-31 could target the inhibition of radixin protein expression. Radixin is a cytoskeletal protein that in humans is encoded by the RDX gene, and may be important in linking actin to the plasma membrane. Much previous research had confirmed that radixin functioned as an oncogene to promote the metastasis of cancer cells in gastric cancer [27, 28], breast cancer [29], and colon cancer [30].

Furthermore, we also found radixin knockdown could significantly inhibit the expression of MMP-9, and vimentin proteins, and increase the expression of E-cadherin protein in hepatoma cells. As we all know, MMP-9, E-cadherin and vimentin are biomarkers of epithelial-mesenchymal transition (EMT). EMT is a biological process by which epithelial cells are transformed into a mesenchymal phenotypic cell by a specific procedure. The main features of EMT are the reduction of the expression of cell adhesion molecules (such as E-cadherin) and the conversion of cytokeratin in the cytoskeleton to vimentin. Vimentin mainly has cytoskeletal and morphological characteristics of mesenchymal cells [31]. For cancer cells, EMT is an important

biologic process for the ability of epithelial cell-derived malignant tumor cells to gain migration and invasion.

All in all, this study showed that lncRNA linc01116 is highly expressed in glioma tissues, and it promotes glioma cell migration and invasion by modulation of radixin targeted by miR-31.

Disclosure of conflict of interest

None.

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