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LncRNA GAPLINC promotes the growth and metastasis of glioblastoma by sponging miR-331-3p

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Abstract. – OBJECTIVE: Recent evidence shows that gastric adenocarcinoma predictive long intergenic noncoding RNA (GAPLINC) acts as a critical role in the proliferation and metastasis in several tumors. We aimed to explore the expression pattern, function, and potential mechanism of GAPLINC in glioblastoma multiforme (GBM).

PATIENTS AND METHODS: The expression levels of GAPLINC and its clinical significance were determined by analyzing TCGA datasets. RT-PCR was performed to detect the levels of GAPLINC and miR-331-3p. CCK-8, colony formation, EdU assays, wound healing assay and transwell invasion assay were performed to analyze the effect of GAPLINC on GBM behaviors. MiRNAs that may interact with GAPLINC were predicted using StarBase and RegRNA 2.0. A luciferase reporter assay was used to detect the targeting effect of GAPLINC on miR-331-3p.

RESULTS: We found that GAPLINC expression was significantly up-regulated in both GBM tissues and cell lines. The overexpression of GAPLINC was associated with shorter overall survival and disease-free survival. Functional assays indicated that GAPLINC silencing suppressed GBM cells proliferation, migration, and invasion, and promoted apoptosis. In the mechanism, we found that GAPLINC acted as a competing endogenous RNA to sponge miR-331-3p and knockdown of GAPLINC promoted the expression of miR-331-3p.

CONCLUSIONS: Our findings suggested that GAPLINC served as an oncogenic lncRNA in GBM through negative modulation of miR-331-3p, providing a novel treatment targeting for GBM.

Key Words:
LncRNA, GAPLINC, Glioblastoma multiforme, Metastasis, miR-331-3p, Prognosis.

Introduction

Human brain glioma represents the most common tumors in the central nervous system; it has very high mortality and morbidity rates not only in China but also in the worldwide[1,2]. Glioma is further classified as low-grade glioma (WHO grade I and II) and high-grade glioma (WHO grade III and IV) by the WHO according to their histopathology[3]. Glioblastoma, also known as glioblastoma multiforme (GBM), is the most aggressive glioma, accounting for 82% of cases of malignant glioma[4,5]. Despite multidisciplinary cooperation including surgical resection, target molecular therapy, and chemotherapy, the overall survival time of GBM patients has not improved markedly and the median survival time of patients with GBM is still only 15 months[6,7]. Up to date, the complex biology of GBM is still insufficiently understood. Therefore, understanding the molecular mechanisms involved in the progression of GBM is critical to identify novel prognostic biomarkers and improve therapeutic efficacy for GBM patients. Noncoding RNAs (ncRNAs), small (<200 kb) and long (lncRNAs) (>200 kb), have attained extensive attention recently because of their important biological roles in various diseases[8]. LncRNAs are non-protein-coding transcripts with a length greater than 200 nucleotides[9]. Several studies have demonstrated that lncRNAs could have critical roles in many biological processes, such as cellular development, growth, differentiation, and apoptosis[10,11]. The rapid development of RNA genomics has highlighted the important effects of lncRNAs in almost all human diseases, especially in tumors[12]. Notably, growing data have confirmed that lncRNAs can function as oncogenes, tumor suppressors, or even both, depending on the circumstance[13]. Although several lncRNAs have been identified to be important regulators in various tumors, such as lncRNA RGMB-AS1, lncRNA NNT-AS1, and lncRNA ATB[14-16], the number of lncRNAs is larger, and so whether other lncRNAs were also involved in the progression of

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GBM need further investigation. Gastric adenocarcinoma predictive long intergenic noncoding RNA (GAPLINC) is a 924-bp lncRNA which has been reported to be dysregulated in several tumors[17-19]. In addition, *in vitro* and *in vivo* assays have demonstrated that GAPLINC exerts oncogenic activity in a variety of tumors. However, limited knowledge is available concerning the roles of GAPLINC in the carcinogenesis of GBM, which needs to be well documented. In this study, we firstly reported that GAPLINC was highly expressed and acted as a tumor promoter in the progression of GBM, providing novel insight into the potential roles of GAPLINC in GBM.

Patients and Methods

Cell Lines and Cell Transfection

The normal human astrocytes (NHAs) and five GBM cell lines (T98G, U251, LN18, LN229, and A172) were obtained from SXBIO Co., Ltd. (Minhang, Shanghai, China). Roswell Park Memorial Institute-1640 (RPMI-1640) medium (containing 10% fetal bovine serum – FBS) was utilized as culture medium for these cells. A cell transfection reagent, NanoFect (Comiike, Nantong, Jiangsu, China) was used for cell transfection. The small interfering RNAs (siRNAs) targeting GAPLINC (si-GAPLINC#1, si-GAPLINC#2), negative control siRNA (si-Control), negative control miRNA mimic (Control) and miR-331-3p mimic were all purchased from Transheep Co., Ltd. (Suzhou, Jiangsu, China). In addition, Generay Biotechnology Co., Ltd. (Songjiang, Shanghai, China) constructed GAPLINC sequence into a pcDNA3.1 vector as a GAPLINC overexpressing plasmid, pcDNA3.1-GAPLINC.

Real-Time PCR Assay

Total RNA of GBM cells was isolated using an Eastep Total RNA extraction kit (Lab Biotech, Nankai, Tianjin, China). Then, we conducted qRT-PCR assays on a Roche LightCycler480 II instrument (Roche, Basel, Switzerland) using the quant one-step qRT-PCR kit (Kanglang, Minhang, Shanghai, China). GAPDH was used for mRNA or lncRNA normalization. Moreover, Mir-X miRNA qRT-PCR SYBR kit (ThinkFar Biotech, Wuhan, Hubei, China) was applied to detect miR-331-3p expression in U251 and LN299 cells. The expression of miR-331-3p was normalized to U6 snRNA. The primers of related miRNAs or mRNAs were all listed in Table I.

Cell Counting Kit-8 (CCK-8) Assay

Briefly, U251 and LN299 cells were transfected with GAPLINC siRNAs and sequentially placed into 96-well plates (2000 cells/well). Then, at the indicated time points (24 h, 48 h, 72 h, and 96 h), 10 μl CCK-8 reagents (Biolite Biotech, Binhai, Tianjin, China) were added into each well. Finally, we used a SM600 microreader (Yanhui Biotech, Jiading, Shanghai, China) to examine the absorbance (450 nm) values.

EdU Assay

The proliferation of U251 and LN299 cells transfected with GAPLINC siRNAs were also assessed by EdU (5-Ethynyl-2'-deoxyuridine) assays. Briefly, the treated cells were added with RPMI-1640 medium supplemented with EdU (10 μM) and sequentially cultured for 1-2 h. Afterwards, an EVOS fluorescence microscope (Thermo Fisher Scientific, Waltham, MA, USA) was applied to take photographs after the cells were fixed with 4% paraformaldehyde.

Colony Formation Assay

In short, GAPLINC siRNAs and control siRNAs were firstly transfected into U251 and LN299 cells. Then, the cells (500 cells) were added into each well of 6-well plates. After maintaining for two weeks at 37°C, we used 0.1% crystal violet (Chreagen Biotech, Fengtai, Beijing, China) to stain the cell colonies. Finally, an Olympus CKX31 microscope (Exsson, Daxing, Beijing, China) was utilized to take pictures.

Western Blot Assay

We employed a GeneLife total protein isolation kit (Mentougou, Beijing, China) to lyse the U251

Table I. Primer sets used in the present study.

Gene names	Forward (5′-3′)	Reverse (5′-3′)
GAPLINC	ACACACAGCAGCCTGGTTTC	ATGGCACAATCAGGGCTCTT
miR-331-3p	GCGCCCCTGGGCCTATC	CGATGACCTATGAATTGACA
U6	ACCCTGAGAAATACCCTCACAT	GACGACTGAGCCCCTGATG

and LN299 cells. Subsequently, an enhanced bicinchoninic acid (BCA) protein kit was applied to determine the protein concentration. Then, 20 μg of protein samples were loaded onto the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes, blocked with 5% non-fat milk, immunoblotted with primary antibodies, as well as corresponding secondary antibodies, and finally examined by a SuperBright ECL kit (SBS Genetech, Haidian, Beijing, China). The anti-vimentin antibody, anti-N-Cadherin antibody, and anti-GAPDH antibody were all obtained from Sino Biological Inc. (Yizhuang, Beijing, China).

RNA Immunoprecipitation (RIP) Assays

After U251 and LN299 cells were transfected with a pcDNA3.1-GAPLINC plasmid or miR-331-3p mimics, we performed RIP assays using a R&S RIP assay kit (R&S Biotech, Pudong, Shanghai, China). The cells were lysed by RIP lysis buffer. The magnetic beads which were connected to anti-Argonaute-2 (Ago2) antibodies (Cell Signaling Technology, Trask Lane, Danvers, MA, USA) were added into the cell lysates. Then, qRT-PCR assays were conducted to detect the co-precipitated RNAs.

Cell Apoptosis Analysis

In brief, U251 and LN299 cells transfected with GAPLINC siRNAs or negative control siRNAs were resuspended in binding buffer supplemented with Annexin V/PI in an apoptosis detection kit (Servicebio, Wuhan, Hubei, China). Afterwards, a MoFloAstrios EQ flow cytometer (Beckman-Coulter Inc., Fullerton, CA, USA) was utilized to analyze the apoptotic cell rates.

Dual-Luciferase Reporter Assay

The wild-type GAPLINC (GAPLINC wt) and mutant GAPLINC (GAPLINC mut) luciferase reporter plasmids were constructed by Transduction Bio Co., Ltd. (Wuhan, Hubei, China). After U251 and LN299 cells were co-transfected with GAPLINC wt plasmids or GAPLINC mut plasmids, as well as corresponding miRNA mimics, the luciferase activities in U251 and LN299 cells were evaluated by a Dual-Luciferase Reporter kit (Shrbio, Nanjing, Jiangsu, China).

Wound Healing Assay

In short, the GAPLINC siRNAs or control siRNAs were firstly transfected into U251 and LN299 cells. Then, 70 μl of the treated cells (5×10^5 cells/ml) were added into an Ibidi 3.5 cm μ-dish (Biocytocare Biotech, Guangzhou, Guangdong, China). After the inserts of the μ-dish were removed, an Olympus CKX31 microscope (Exsson, Daxing, Beijing, China) was utilized to take pictures of the wounded areas at 0 h and 24 h.

Transwell Invasion Assay

The transwell inserts were firstly treated with Matrigel (70 μl). Then, U251 and LN299 cells transfected with GAPLINC siRNAs or control siRNAs were collected and resuspended in serum-free RPMI-1640 medium. Subsequently, the U251 or LN299 cell suspensions was added into the upper chamber of the inserts, and the complete medium (containing 15% FBS) were added into the lower chamber of the transwell. Finally, an Olympus CKX31 microscope (Exsson, Daxing, Beijing, China) was applied to take images of the invaded cells after the cells were stained with 0.1% crystal violet.

Statistical Analysis

Statistical analyses were performed using Prism 6 (GraphPad, La Jolla, CA, USA) and the SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). The comparison between groups was performed using the unpaired *t*-test. The comparison between the three groups was done using the One-way ANOVA test followed by the Least Significant Difference (LDS). Statistics with $p<0.05$ were considered as statistically significant.

Results

GAPLINC Was Highly Expressed in GBM and Associated with Prognosis of GBM Patients

In this study, we first analyzed RNA sequencing data of GBM and normal brain tissues downloaded from TCGA datasets. As shown in Figure 1A and 1B, abnormally expressed lncRNAs were shown by Heat map and Volcano Plot. In addition, we observed that GAPLINC was significantly upregulated in GBM tissues compared to normal brain tissues (Figure 1C). Then, we used online tools (GEPIA), which collected the data of TCGA datasets to explore the clinical significance of GAPLINC in GBM patients. As shown in Figure 1D and 1E, we found that high GAPLINC expression was significantly associated with poor overall survival of

GBM patients. On the other hand, in order to confirm the above results, we performed RT-PCR to detect the expression of GAPLINC in GBM cells, finding that GAPLINC expression in five GBM cell lines was remarkable upregulated compared to NHA (Figure 1F). Overall, our results firstly reported that GAPLINC was overexpressed in GBM and was associated with poor prognosis in patients with GBM.

GAPLINC Affected the Proliferation and Apoptosis of GBM Cells

Given that GAPLINC was highly expressed in GBM, we next aimed to explore whether GAPLINC played critical roles in modulating the biological functions of U251 and LN299 cells. Hence, we synthesized siRNAs specific against GAPLINC (si-GAPLINC#1 and si-GAPLINC#2) and transfected them into U251 and LN299 cells. The qRT-PCR assays suggested that the silencing efficiency was higher in the GAPLINC siRNAs transfected U251 and LN299 cells (Figure 2A). Subsequently, we conducted CCK-8 assays to assess the influence of GAPLINC on cell growth. As the data presented in Figure 2B, transfection of GAPLINC siRNAs remarkably reduced the proliferative rates of both U251 and LN299 cells. Consistent with the results of CCK-8 assays, the data of EdU assays also confirmed that knockdown of GAPLINC significantly suppressed the proliferation of U251 and LN299 cells (Figure 2C and D). Similarly, the silence of GAPLINC markedly impaired the colony formation capabilities of U251 and LN299 cells (Figure 2E). Thereafter, we investigated the apoptosis of U251 and LN299 cells after they were transfected with GAPLINC siRNAs using flow cytometry. The data revealed that the suppression of the expression of GAPLINC notably promoted the apoptotic rates of U251 and LN299 cells (Figure 2F). Therefore, our results demonstrated that GAPLINC could modulate the development of GBM.

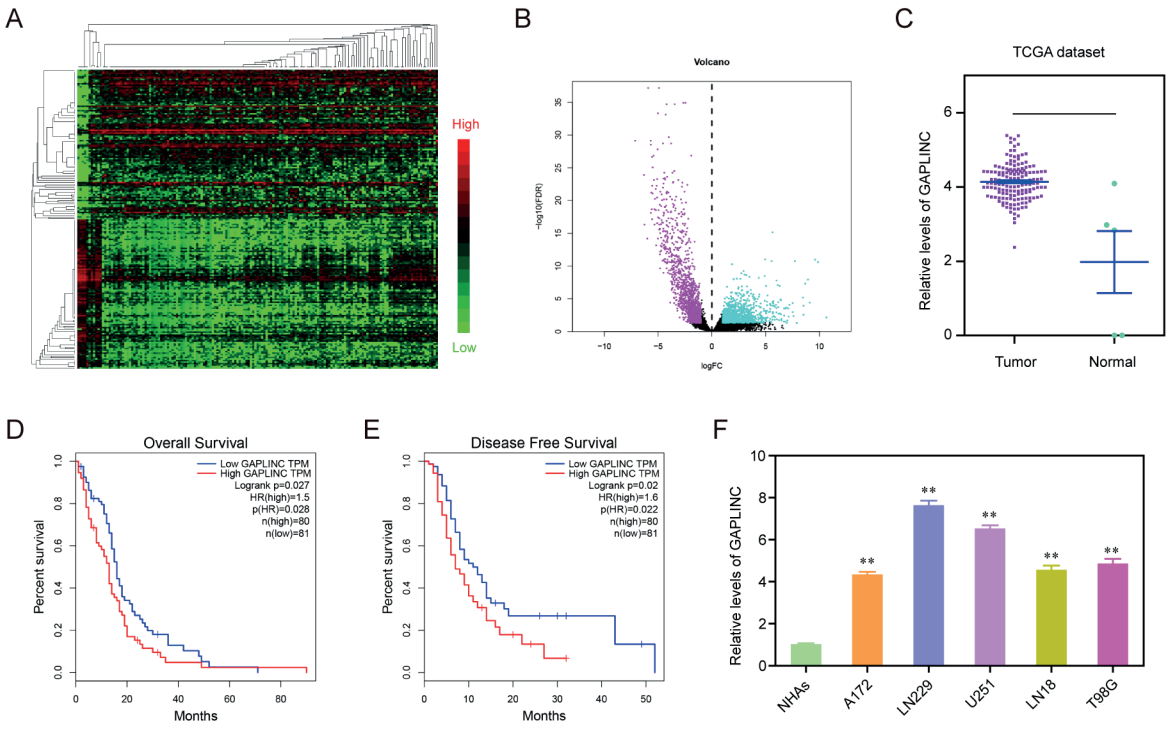

Figure 1. Bioinformatics analysis of differentially expressed lncRNAs in GBM. ***A,*** Heatmap of abnormally expressed lncRNAs in GBM tissues compared to normal brain tissues according to TCGA datasets. ***B,*** Volcano plot of the aberrantly expressed lncRNAs between GBM and normal brain tissues. ***C,*** The expression of GAPLINC in GBM tissues and normal brain tissues by analyzing TCGA datasets. ***D,*** Patients with high GAPLINC expression had a shorter overall survival than those with low expression by analyzing TCGA datasets. ***E,*** Patients with high GAPLINC expression had a shorter disease-free survival than those with low expression by analyzing TCGA datasets. ***F,*** The expression of GAPLINC in NHAs and GBM cell lines (A172, LN229, U251, LN8, and T98G) were detected by RT-PCR. *$p < 0.05$, **$p < 0.01$.

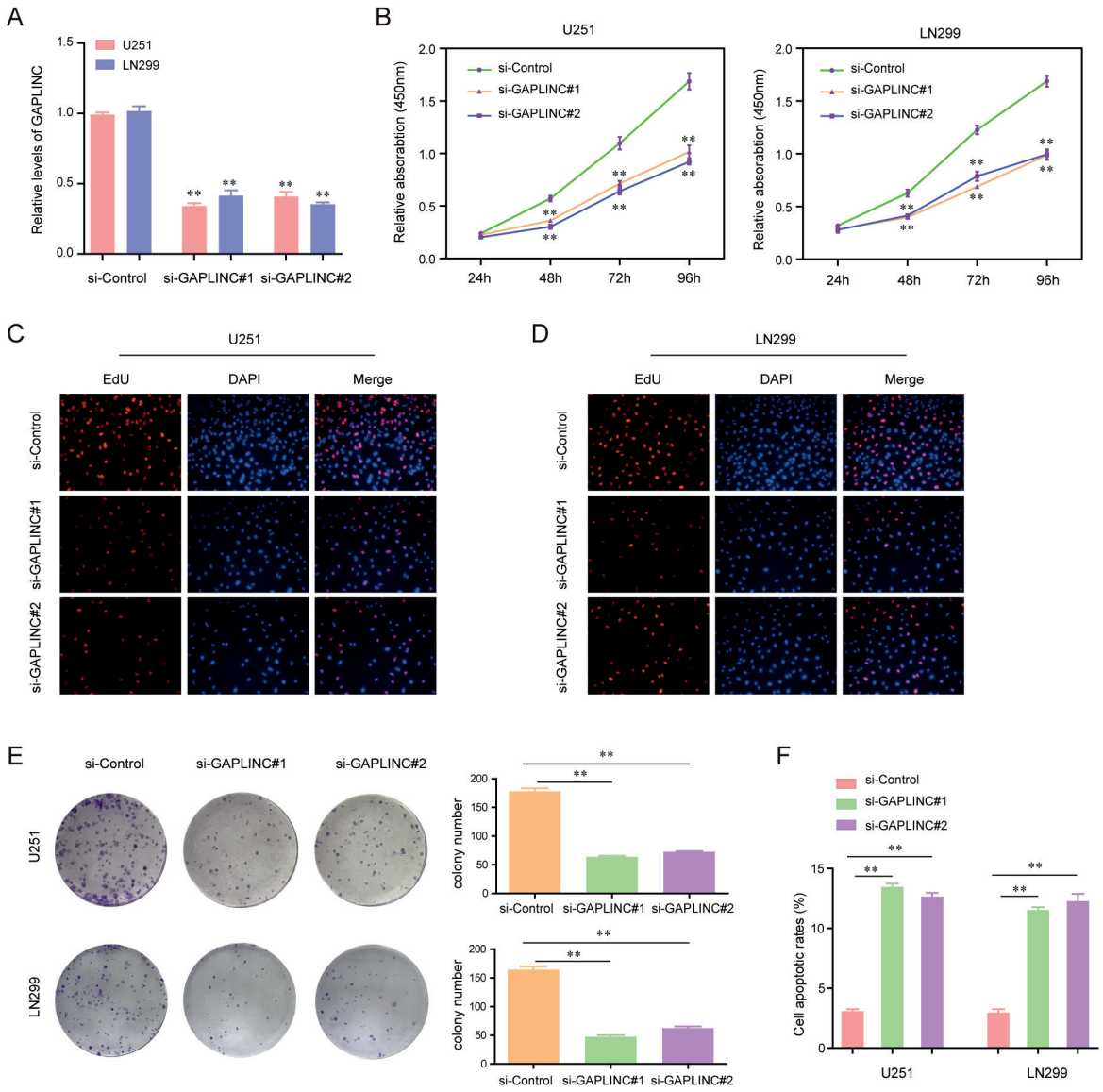

Figure 2. The effects of GAPLINC on cell proliferation and apoptosis. ***A,*** The relative expression levels of GAPLINC in U251 and LN299 cells after transfection with GAPLINC siRNAs (si-GAPLINC#1 and si-GAPLINC#2) or negative control siRNAs (si-Control). ***B,*** The proliferation of U251 and LN299 cells evaluated by CCK-8 assays. ***C*** and ***D,*** EdU assays determined the proliferation of U251 and LN299 cells. The proliferative cells were labeled with red fluorescence, and the blue fluorescence was nuclear stained by DAPI. ***E,*** The colony formation assays detected the colony formation abilities of U251 and LN299 cells. ***F,*** Knockdown of GAPLINC induced the cell apoptosis. *$p < 0.05$, **$p < 0.01$.

GAPLINC Modulated the Migration and Invasion of GBM Cells

We also employed wound healing and transwell invasion assays to evaluate the effects of GAPLINC on the metastatic potentials of GBM cells. The wound-healing assays demonstrated that transfection of GAPLINC siRNAs significantly reduced the migration of U251 and LN299 cells (Figure 3A and B). Also, the data of transwell invasion assays certified that inhibiting the expression of GAPLINC led to a remarkable decline of the invasive cell number of U251 and LN299 cells (Figure 3C). We next aimed to uncover the molecular mechanisms of GAPLINC regulating the migration and invasion of GBM cells. The results of Western blot assays validated that transfection of GAPLINC siRNAs resulted in a remarkable reduction of protein levels of two epithelial-mesenchymal transition related molecules, N-cadherin and vimentin (Figure 3D). Overall,

our results provided evidence that silencing the expression of GAPLINC inhibited the metastatic potentials of GBM cells.

GAPLINC Acted as a ceRNA Sponge of MiR-331-3p in GBM Cells

We next aimed to discover the potential molecular mechanisms by which GAPLINC affected the cell growth and metastasis of GBM cells. At present, a plethora of studies have demonstrated that lncRNAs modulated the development and progression of multiple cancers via competitive "sponge" miRNAs. Therefore, we next searched the "miRDB" (http://www.mirdb.org/) database and found that miR-331-3p might be a potential target of GAPLINC (Figure 4A). Subsequently, we performed luciferase reporter assays to validate miR-331-3p was directly interacted with GAPLINC. The data confirmed that co-transfection of miR-331-3p mimic and GAPLINC wildtype (GAPLINC wt) plasmid remarkably reduced the luciferase activities of U251 and LN299 cells, while there were no changes of luciferase activities in the miR-331-3p mimic as well as GAPLINC mutant (GAPLINC mut) plasmid transfected cells (Figure 4B). Furthermore, RIP assays demonstrated that GAPLINC as well as miR-331-3p were significantly enriched in Ago2-containing beads, while there were no enrichments of GAPLINC and miR-331-3p in the input group (Figure 4C). Besides, overexpression of GAPLINC notably reduced the miR-331-3p le-

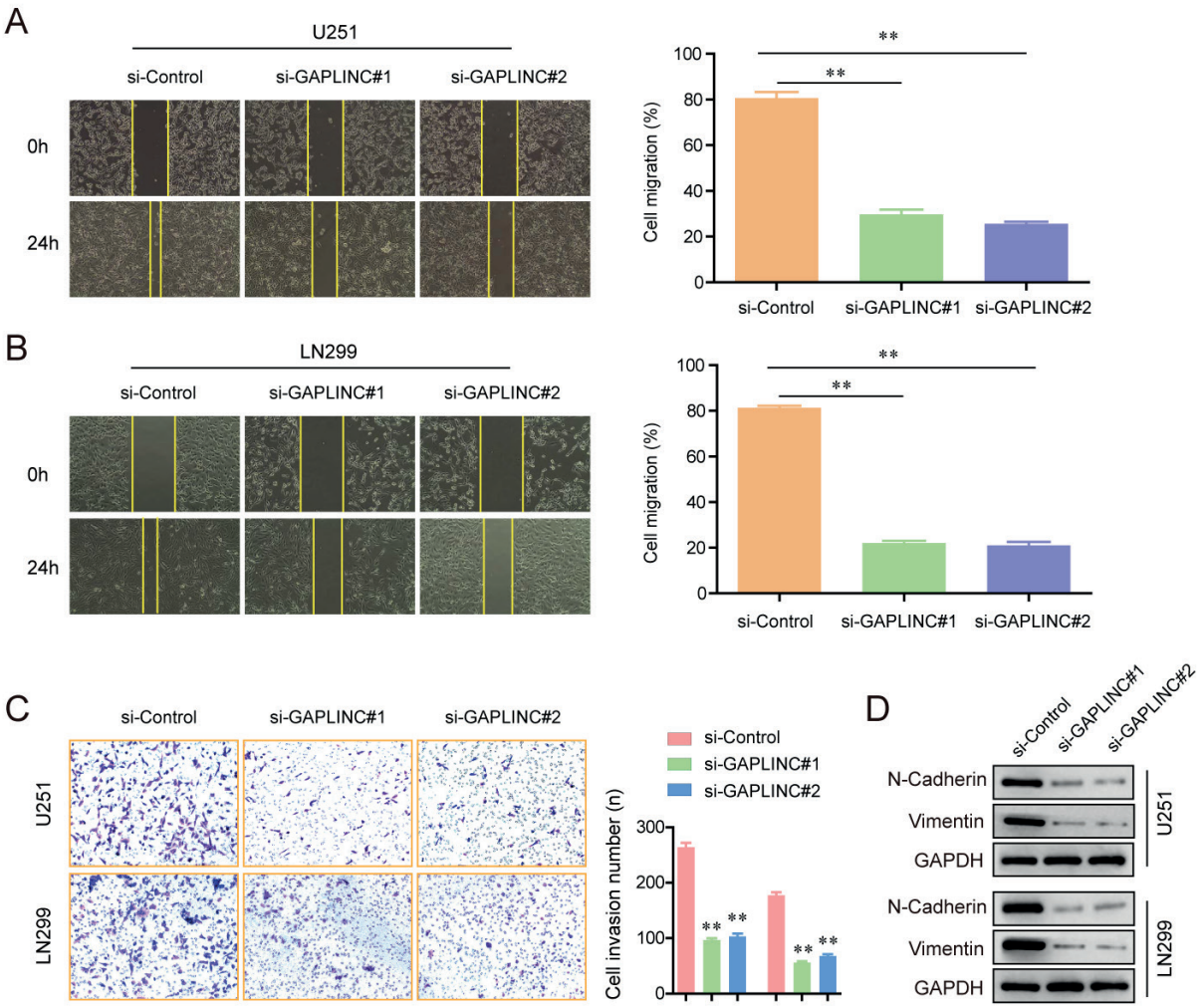

Figure 3. The influence of GAPLINC on the invasion and migration of U251 and LN299 cells. **A** and **B,** Transfection of GAPLINC siRNAs reduced the migration of U251 and LN299 cells. **C,** Silence of GAPLINC reduced the invasive cell number of U251 and LN299 cells. **D,** The protein levels of N-cadherin and vimentin detected by Western blot assays. *$p < 0.05$, **$p < 0.01$.

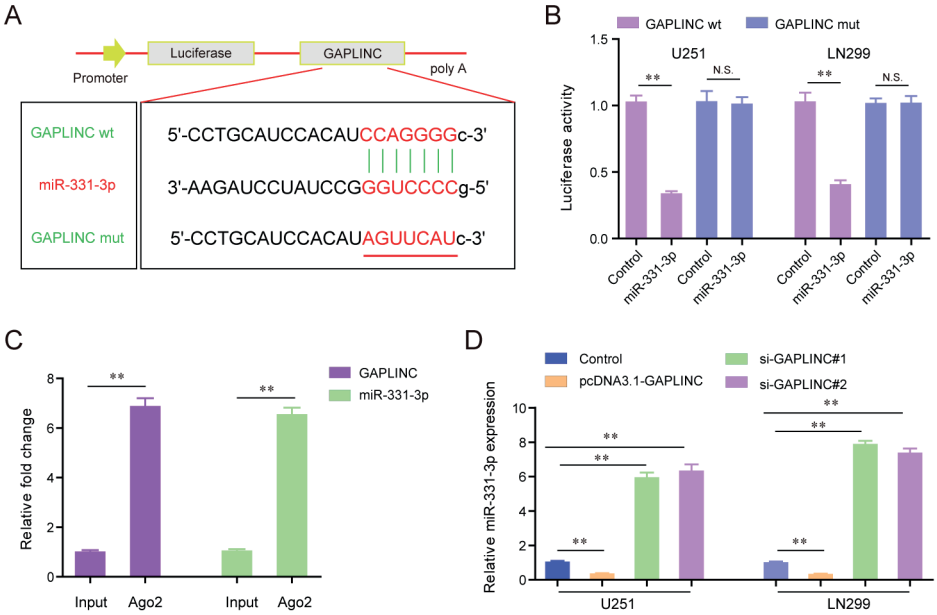

Figure 4. MiR-331-3p was a direct target of GAPLINC. ***A,*** "miRDB" predicted the binding site of miR-331-3p in the sequence of GAPLINC. ***B,*** The relative luciferase activities of U251 and LN299 cells examined by dual luciferase reporter assays. ***C,*** RIP assays evaluated the enrichment of GAPLINC and miR-331-3p. ***D,*** The relative expression of miR-331-3p in U251 and LN299 cells. **p* < 0.05, ***p* < 0.01.

vels, whereas increased miR-331-3p levels were observed in the GAPLINC siRNAs transfected U251 and LN299 cells (Figure 4D). Taken together, our data proved that miR-331-3p was the target of GAPLINC in GBM cells.

Discussion

GBM widely invades the surrounding brain but rarely metastasizes to other organs and are among the most resistant to radiation and cytotoxic chemotherapy, causing a very poor prognosis for GBM patients[20]. On the other hand, diagnostic tests for GBM are currently limited to MRI and CT, which have limited roles when GBM patients are at early stages[21]. Thus, investigating the pathogenesis of GBM is necessary to identify novel biomarkers and develop new potential therapeutic targets. Recently, several lncRNAs have been found to be involved in the regulation of tumor progression and be key regulators of the protein signalling pathways underlying carcinogenesis[22]. For instance, Lu et al[23] reported that lncRNA SNHG16 was highly expressed and associated with poor prognosis in glioma patients. In their lost-function assay, it was found that lncRNA SNHG16 promoted glioma cells proliferation by sponging MiR-4518. Gao et al[24] reported that knockdown of HSP90AA1-IT1 significantly suppressed cells proliferation, migration, and invasion by targeting miR-88. These findings highlighted the important roles of lncRNA in GBM and encouraged us to identify more lncRNAs. There is emerging evidence that GAPLINC might be playing an important role in several tumors. For instance, Yang et al[25] reported that GAPLINC expression was up-regulated in colorectal cancer and may be associated with advanced clinical stages and shorter overall survival of colorectal cancer patients. *In vitro and in vivo* experiments indicated that knockdown of GAPLINC suppressed cells proliferation and invasion in colorectal cancer via targeting SNAI2 through binding with PSF. Diao et al[19] showed that GAPLINC was highly expressed in gastric cancer and its down-regulation suppressed gastric cancer cell proliferation by sponging miR-378 to modulate MAPK1 expression. The oncogenic roles and the potential of GAPLINC as novel biomarkers were also reported in several other tumors, such as bladder cancer[26], osteosarcoma[27], and cholangiocarcinoma[18]. However, whether GAPLINC had similar roles in GBM remains unknown. In this study, we showed that GAPLINC was significantly up-regulated in both GBM tissues and cell lines, and increased GAPLINC predicted poor prognosis for GBM patients. A functional investigation indi-

cated that knockdown of GAPLINC significantly suppressed GBM proliferation and metastasis, and promoted apoptosis. Our findings demonstrated that GAPLINC served as a tumor promoter in GBM, which was consistent with the oncogenic roles of GAPLINC in other tumors. Acting as a noncoding RNA, lncRNAs lack of protein-coding ability. Growing evidence indicated that lncRNA could serve as competing endogenous RNAs (ceRNAs) to absorb miRNA via sequence complementarity and modulating the expression of miRNAs to affect the biological tumor-related genes[28,29]. Previously, several lncRNAs have been reported to sponge miRNA in almost all types of tumors. Also, GAPLINC was confirmed to function as ceRNAs to be involved in the regulation of gastric cancer and colorectal cancer[19,25]. However, it could be interesting to investigate whether GAPLINC promotes gastric cancer behaviors. In this study, using bioinformatics prediction program, we predicted the downstream target for GAPLINC, finding that miR-331-3p directly targeted 3'-UTR of GAPLINC. Generally, miR-331-3p is downregulated in a variety of tumors, such as cervical cancer, prostate cancer, and GBM[30-32]. A functional investigation indicated that miR-331-3p displayed its oncogenic roles by modulating NRP2 and ERBB-2[33,34]. Then, a luciferase activity assay and RT-PCR confirmed the direct binding relationship between GAPLINC and miR-331-3p. Overall, GAPLINC plays an oncogenic role in the tumorigenesis of GBM by binding miR-331-3p.

Conclusions

We showed that GAPLINC was increased in GBM and associated poor prognosis of GBM patients. Furthermore, GAPLINC promoted GBM progression by binding miR-331-3p. Therefore, these findings contribute to a better understanding of the potential mechanism of the progression of GBM, which could be a novel diagnostic and therapeutic target for GBM.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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