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Up-regulated circular RNA hsa_circ_0067934 contributes to glioblastoma progression through activating PI3K-AKT pathway

J. XIN[1], X.-Y. ZHANG[1], D.-K. SUN[1], L.-Q. TIAN[2], P. XU[1]

[1]Department of Neurosurgery, Linyi Center Hospital, Linyi, Shandong, China.
[2]Department of Neurosurgery, Linyi People's Hospital, Linyi, Shandong, China.

Jun Xin and Xi-Yun Zhang contributed equally to this work

Abstract. – OBJECTIVE: Circular RNAs (circRNAs) are involved in cancer progression and metastasis, although little is known about their role in glioblastoma (GBM). The aim of this study was to investigate the expression pattern and molecular mechanism of hsa_circ_0067934 in GBM.

PATIENTS AND METHODS: Hsa_circ_0067934 expression was detected in GBM tissues and cell lines by using RT-PCR. The prognostic value of the hsa_circ_0067934 expression in GBM patients was statistically analyzed. The effects of hsa_circ_0067934 expression on growth and apoptosis of GBM cells were analyzed by CCK-8, colony formation, and flow cytometry. Migration and invasion ability was evaluated by transwell and wound-healing assays. The protein expression of signaling pathways was determined by using Western blot.

RESULTS: The expression level of hsa_circ_0067934 was significantly increased in GBM tissues and cancer cells. Kaplan-Meier survival curves showed that patients with higher expression of hsa_circ_0067934 had a more favorable overall survival and disease-free survival. The functional investigations revealed that the knockdown of hsa_circ_0067934 suppressed GBM cell proliferation, metastasis and EMT, and promoted apoptosis. Moreover, the mechanistic analysis demonstrated that the down-regulation of hsa_circ_0067934 significantly suppressed the activation of the PI3K-AKT pathway.

CONCLUSIONS: Hsa_circ_0067934 is overexpressed and plays an oncogenic role in GBM by promoting cancer cell proliferation and metastasis via upregulation of PI3K-AKT pathway, which suggests that hsa_circ_0067934 is likely to serve as an efficient therapeutic approach in respect of GBM treatment.

Key Words
Circ_0067934, Proliferation, Metastasis, PI3K-AKT pathway, Glioblastoma.

Introduction

Glioma, characterized by high proliferation rate, increased angiogenesis, and invasive growth, is one of the common primary brain tumors in the central nervous system for both children and adults[1]. Each year, ~200,000 patients are diagnosed with glioma worldwide. Gliomas are divided into four grades (I–IV) by the WHO according to their histopathology[2]. Patients with the most malignant histopathologic subtype, the glioblastoma (GBM), display the poorest prognosis, with a median survival of only about 15 months[3,4]. Although substantial advances have been made in the therapy for gliomas, the long-term survivals of GBM patients have not been significantly improved[5,6]. The pathogenesis of GBM has been investigated extensively; however, the potential genetic factors involved in the progression of this malignancy remain largely unclear.

Cellular RNAs can be separated into two categories: coding and noncoding[7]. Non-coding RNAs have been reported to be involved in the regulation of the biological progression. In contrast to tRNA, miRNA and long non-coding RNA, circRNAs are less well characterized[8,9]. Circular RNAs (circRNAs) are a class of non-coding RNAs which are originally identified in plant viroid, virus, and archaea and are characterized by covalently closed continuous loops with neither 5' to 3' polarity nor a polyadenylated tail[10,11]. They were thought to be a result of splicing errors for several decades. Recent biological evidence shows that circRNAs can participate in the RNR-RNA regulation network or RNA-protein complex formation and thus they can act as potential regulators in the development and progression of several human diseases[12,13]. Importantly, increas-

Corresponding Author: Li-Qiang Tian, MD; e-mail: okppqq2234@163.com

ing studies have reported that circRNAs were abnormally expressed in various tumors and played a critical role in tumorigenesis[14,15]. However, the expression profiles and potential function of circRNAs in GBM remain largely unclear.

CircRNA hsa_circ_0067934, generated from chromosomal region 3q26, has been confirmed to be frequently expressed in various cancer cells according to the circBase database[16]. Recently, up-regulation of hsa_circ_0067934 and its tumor-promotive roles have been reported in esophageal squamous cell carcinoma, hepatocellular carcinoma, and lung cancer[17-19]. However, whether hsa_circ_0067934 was abnormally expressed in GBM, and its potential function in the development of GBM have not been investigated.

Patients and Methods

Patients and Tissue Samples

A total of 157-paired human GBM samples and the corresponding normal brain tissue specimens were obtained between June 2010 and January 2013 following surgery at Linyi Center Hospital with the approval of the Ethics Committee. The patients did not receive any antitumor treatment before surgery. Tissue specimens collected during surgery were frozen using liquid nitrogen and sequentially stored until use. All GBM patients included in this study provided the written informed consents.

Cell Lines and Cell Transfection

The LN18, U251, LN229, T98G and A172 cells (GBM cell lines), and NHA cells (normal human astrocytes) maintained in RPMI-1640 medium containing 10% fetal bovine serum were all obtained from Wuhan Biofavor Biotechnology Co., Ltd. (Wuhan, Hubei, China). In addition, a cell transfection reagent which was purchased from SignaGen Biotechnology Co., Ltd. (Jinan, Shandong, China) was then utilized to transfect small interfering RNAs (siRNAs) specific targeting hsa_circ_0067934 (si-circRNA#1 and si-circRNA#2) into U251 and LN229 cells. The si-circRNA#1, si-circRNA#2 as well as the negative control siRNAs (si-NC) were all designed and synthesized by Genomeditech Biotechnology Co., Ltd. (Pudong, Shanghai, China).

Real-Time PCR Assay

TRIzol reagent (Haojia Biotechnology, Wuhan, Hubei, China) was applied to extract the total RNAs from GBM tissues or cells. Then, cDNA was synthesized by using a GeneCopoeia First-Strand cDNA Synthesis kit (FuLenGen, Guangzhou, Guangdong, China). Subsequently, the qRT-PCR analysis for hsa_circ_0067934 detection was carried out using an EvaGreen qRT-PCR assay kit (Chaoyang Biotechnology, Jinshan, Shanghai, China). GAPDH was used as internal, and the expression of hsa_circ_0067934 was calculated using $2^{-\Delta\Delta Ct}$ method. The primers for hsa_circ_0067934 and GAPDH were exhibited in Table I.

Western Blot Assay

A cell extraction kit (AmyJet Biotechnology, Wuhan, Hubei, China) was applied to lyse the U251 and LN299 cells after treatment with hsa_circ_0067934 siRNAs. The extracted proteins were loaded and then separated with 10% SDS separation gel as well as 5% concentrated gel, after being transferred into polyvinylidene difluoride membranes. The protein bands were detected by a National Diagnostics ProtoGlow ECL kit after the membranes were probed with primary antibodies and secondary antibodies sequentially. The primary antibodies against AKT, N-cadherin, PI3K, and GAPDH were purchased from Wuhan BOSTER Biotechnology Co., Ltd. (Wuhan, Hubei, China). The primary antibodies against p-PI3K (phosphorylated-PI3K), p-AKT (p-PI3K), and vimentin were obtained from Cell Signaling Technology Co., Ltd. (Beverly, MA, USA).

Cell Counting Kit-8 (CCK-8) and Colony Formation Assays

In brief, hsa_circ_0067934 siRNAs were separately transfected into U251 and LN299 cells, and the cells (1×10^4 cells per well) were subsequently

Table I. The primer sequences included in this study.

Names	Sequences (5′-3′)
Hsa-circ_0067934 (Forward)	TTGCTGATATTCAGGGACAC
Hsa-circ_0067934 (Reverse)	CCTGTTTGAGGATGACAACC
GAPDH (Forward)	GTCAACGGATTTGGTCTGTATT
GAPDH (Reverse)	AGTCTTCTGGGTGGCAGTGAT

planted into 96-well plates for attachment. Then, CCK-8 reagents (10 μl; YiSheng Biotechnology, Pudong, Shanghai, China) were added into the plates, after being incubated for 1 h. Finally, a microplate reader (SuPerMax 3001569AL; FSBT Co., Ltd., Minhang, Shanghai, China) was employed to measure the OD450 nm absorbance. For the colony formation assays, the cells treated with hsa_circ_0067934 siRNAs were planted into 6 well plates and cultured for 14 days. Then, crystal violet and a microscope (Yongke, Jinshan, Shanghai, China) were utilized to stain and observe the cell colonies, respectively.

Cell Apoptosis Analysis

The cell apoptosis was evaluated by flow cytometry using an apoptosis detection kit (See-Bio, Pudong, Shanghai, China). Briefly, the hsa_circ_0067934 siRNAs or control siRNAs transfected U251, and LN299 cells were collected by trypsinization, resuspended in binding buffer, and FITC-annexin V, as well as PI, were then added into the treated U251 and LN299 cells. After incubating for 15 min in the dark, the treated U251 and LN299 cells were collected by centrifuging and analyzed by a NovoCyte flow cytometer system (ACEA Bioscience Inc., Hangzhou, Zhejiang, China).

Wound Healing Assays

In short, the hsa_circ_0067934 siRNAs or control siRNAs treated U251, and LN299 cells were grown to 100% confluence in 12-well plates, and wounded areas were then created by applying a pipette tip (200 μl) to vertically scratch the cells. After rinsing the cells with PBS for three times, the distance of blank areas at 0 h and 24 h were imaged through a microscope (Yongke, Jinshan, Shanghai, China).

Transwell Invasion Assay

For the cell invasion assays, the hsa_circ_0067934 siRNAs or control siRNAs transfected U251 and LN299 cells (2.5×10^4 /well) were maintained in 250 μl serum-free RPMI-1640 medium in the upper chambers (pre-coated with Matrigel) of the Costar transwell inserts (XinFan Biotechnology, Minhang, Shanghai, China). In addition, the lower chambers were loaded with 750 μl complement RPMI-1640 medium (with 15% FBS). Twenty-four hours later, the methanol was applied to fix the invaded U251 and LN299 cells on the lower surfaces of the transwell inserts, after being stained with crystal violet. After rinsing with phosphate-buffer saline three times, the invasive cells were observed and imaged by a microscope (Yongke, Jinshan, Shanghai, China).

Statistical Analysis

The statistical analysis in this study was achieved by the software SPSS version 19.0 (IBM Corp. IBM SPSS Statistics for Windows, Armonk, NY, USA). The comparisons between quantitative variables were assessed by using (the use of) the Student's *t*-test or one-way ANOVA. Multiple comparisons between groups were performed using the Student-Newman-Keuls post-hoc test. For overall survival (OS) and disease-free survival (DFS) analysis, the Kaplan-Meier method (with the log-rank test) was employed. A *p*-value of $p<0.05$ was taken to be statistically significant.

Results

Up-Regulation of Hsa_circ_0067934 Predicted Poor Prognosis in GBM

Firstly, in order to explore whether hsa_circ_0067934 was abnormally expressed in GBM, hsa_circ_0067934 expression was detected by using quantitative real time-polymerase chain reaction (qRT-PCR) and normalized to GAPDH. As shown in Figure 1A, we found that hsa_circ_0067934 was significantly overexpressed in 157 pairs of GBM tissues compared with the adjacent noncancerous tissues ($p<0.01$). Furthermore, the up-regulation of hsa_circ_0067934 has been observed in GBM cell lines compared to NHAs (Figure 1B). Subsequently, we studied the significance of hsa_circ_0067934 in 157 GBM patients, and the results of the Kaplan-Meier method showed that the patients with higher hsa_circ_0067934 expression had both poorer overall survival ($p=0.021$, Figure 1C) and disease-free survival ($p<0.011$, Figure 1D). Overall, our results indicated that hsa_circ_0067934 was highly expressed in GBM and could predict poor prognosis.

Hsa_circ_0067934 Affected Cellular Proliferation and Apoptosis of GBM Cells

To investigate whether the knockdown of hsa_circ_0067934 could influence the cellular growth of GBM cells *in vitro*, we performed loss-of-function studies using the siRNAs specific targeting hsa_circ_0067934 siRNAs (si-circRNA#1 and si-circRNA#2). Thereafter, qRT-PCR assays were applied to detect the knockdown efficien-

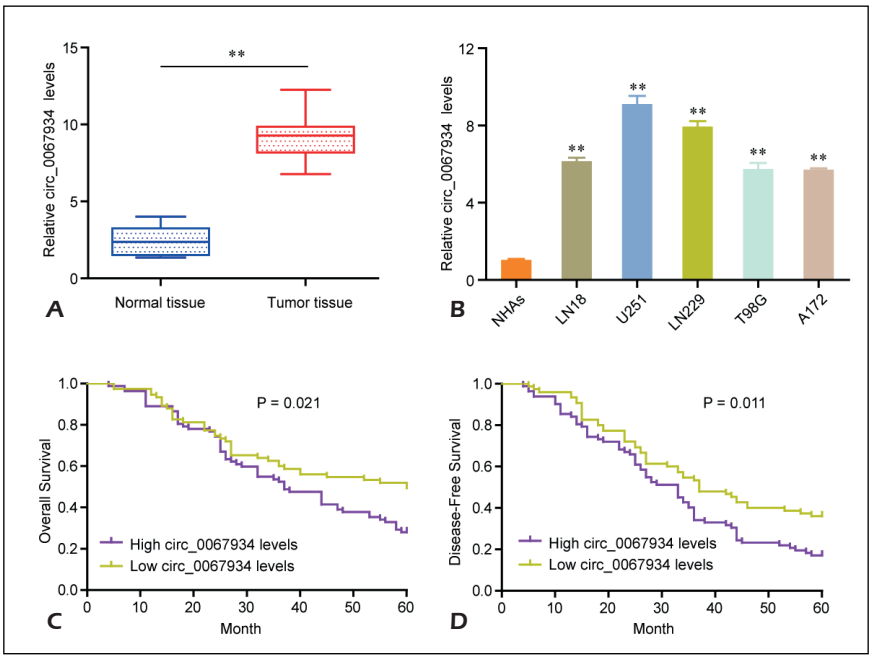

Figure 1. Hsa_circ_0067934 was up-regulated in GBM tissues and cells. **A**, Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of hsa_circ_0067934 expression in glioma tissues and normal brain tissues. **B**, qRT-PCR analysis of hsa_circ_0067934 expression in NHAs and five GBM cell lines (LN18, U251, LN229, T98G and A172). Transcript levels were normalized by GAPDH expression. **C**, Kaplan-Meier overall survival curves according to hsa_circ_0067934 expression level. **D**, Kaplan-Meier disease-free survival curves according to hsa_circ_0067934 expression level. *p<0.05, **p<0.01.

cy of these siRNAs, and the results revealed that the transfection of hsa_circ_0067934 siRNAs significantly reduced the expression of hsa_circ_0067934 in both U251 and LN299 cells (Figure 2A). In addition, with the down-regulation of hsa_circ_0067934, the proliferative rates of U251 and LN299 cells at 48 h, 72 h, and 96 h were remarkably decreased when they were examined by CCK-8 assays (Figure 2B). Similarly, according to the results of the colony formation assays, the transfection of hsa_circ_0067934 siRNAs dramatically impaired the clonogenic capacity of U251 and LN299 cells (Figure 2C). To elucidate the effects of hsa_circ_0067934 on cell apoptosis, GBM cells after hsa_circ_0067934 siRNAs treatment were next analyzed using flow cytometry. The data suggested that the silencing of the expression of hsa_circ_0067934 resulted in a notably increasing of apoptotic U251 and LN299 cells (Figure 2D). Hence, these data indicated that hsa_circ_0067934 played essential roles in regulating the proliferation and apoptosis of GBM cells.

Effects of Hsa_circ_0067934 on the Migration and Invasion of GBM Cells

To explore the effects of hsa_circ_0067934 on the GBM cell migration and invasion, we firstly performed wound healing assays, and the results suggested that the inhibition of hsa_circ_0067934 led to a significant decrease in the migratory ability of U251 and LN299 cells because the wounded areas of the hsa_circ_0067934 siRNAs transfected cells were remarkably wider than the cells transfected with negative control siRNAs (Figure 3A and B). We next conducted transwell invasion assays, and the results showed that the invasion of U251 and LN299 cells was markedly reduced by about 70% following the down-regulation of hsa_circ_0067934 expression (Figure 3C). To explore the potential mechanisms by which hsa_circ_0067934 regulated the migration and invasion of GBM cells, we next utilized Western blot to detect the protein expression of epithelial-mesenchymal transition makers, N-cadherin, and vimentin. According to the results, after U251 and LN299 cells were transfected with hsa_circ_0067934 siRNAs, the protein levels of N-cadherin and vimentin were remarkably decreased (Figure 3D). Therefore, our data indicated that the silencing of hsa_circ_0067934 impeded the metastatic potentials of GBM cells via modulating epithelial-mesenchymal transition.

Silence of Hsa_circ_0067934 Impeded the Activation of PI3K-AKT Signaling in GBM Cells

To investigate the detailed molecular mechanisms by which hsa_circ_0067934 contributed to the regulatory effects of GBM development and progression, we focused on a well-studied signaling pathway, the PI3K-AKT signaling, because the molecules involved in this signaling accounted

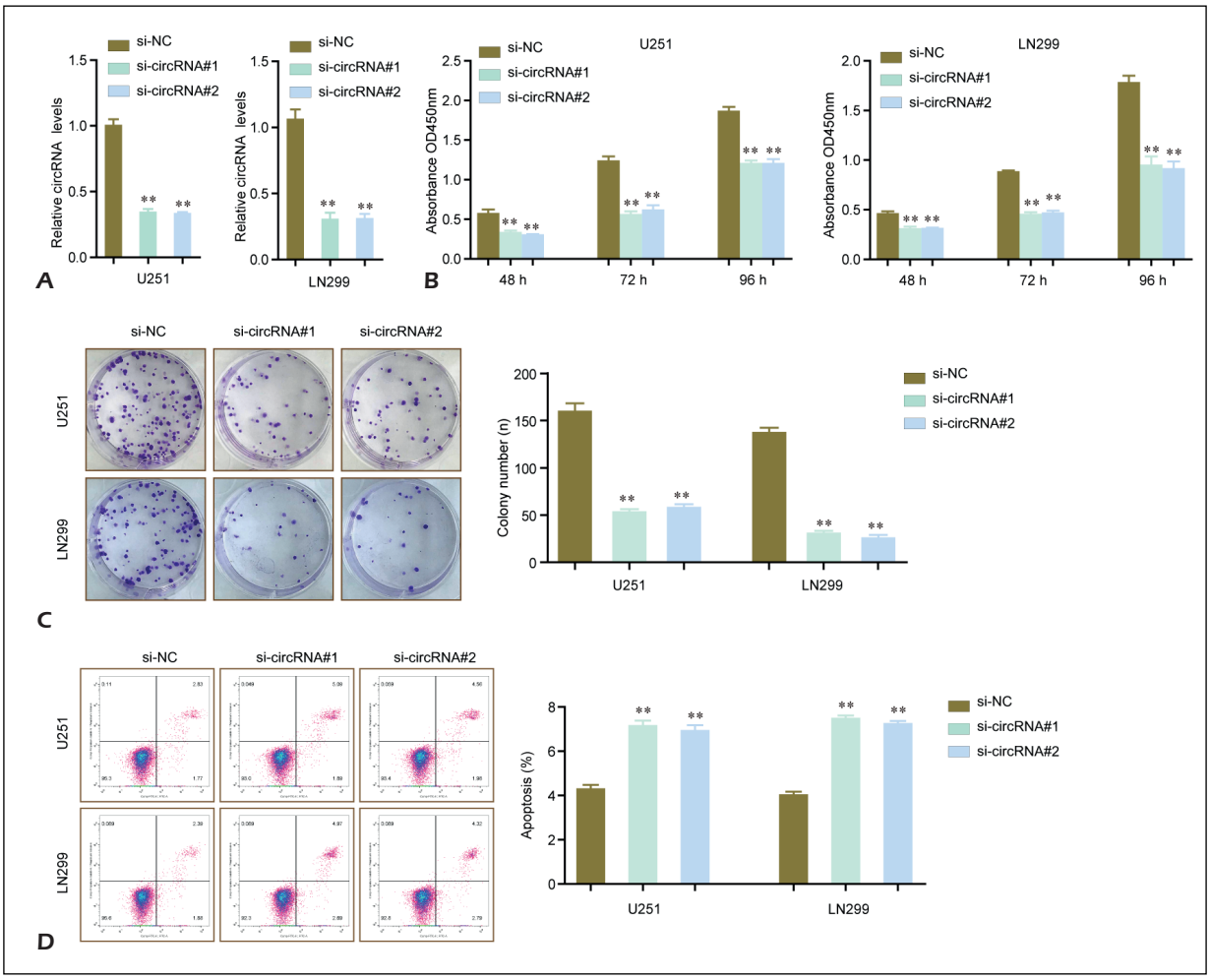

Figure 2. Knockdown of hsa_circ_0067934 suppressed the proliferation and induced the apoptosis of U251 and LN299 cells. **A**, The synthesized siRNAs (si-circRNA#1 and si-circRNA#2) could efficiently inhibit the expression levels of hsa_circ_0067934 in U251 and LN299 cells. **B**, CCK-8 assay detected the cell proliferation after hsa_circ_0067934 siRNAs transfection at 48 h, 72 h and 96 h. **C**, Colony formation assay evaluated the clonogenic ability of U251 and LN299 cells after treatment with hsa_circ_0067934 siRNAs (Magnification: 10 ×). **D**, Flow cytometry examined the apoptotic rates of U251 and LN299 cells after transfecting with hsa_circ_0067934 siRNAs. **p*<0.05, ***p*<0.01.

for a plethora of functional regulation in multiple types of cancer including GBM. Hence, we firstly transfected hsa_circ_0067934 siRNAs into U251 and LN299 cells and subsequently conducted Western blot assays to evaluate the changes of the protein levels. As expected, the U251 and LN299 cells after treatment with hsa_circ_0067934 siRNAs were dramatically decreased the protein levels of p-PI3K and p-AKT, while the protein levels of PI3K and AKT were not changed, indicating that the silencing of hsa_circ_0067934 inhibited the activation of PI3K-AKT signaling (Figure 4A and B). Taken together, our data indicated that hsa_circ_0067934 functioned as an oncogenic promoter via activating PI3K-AKT signaling.

Discussion

GBM is ranked as one of the most common cancers in China, and its incidence increases annually and will continue to rise in the next few years[20]. Up to date, the prognosis of GBM patients remains poor. The identification of sensitive biomarkers for glioma early diagnosis and prognosis is urgently needed[21]. However, the molecular biological mechanism of GBM is complicated, and it is not yet fully understood, which makes it hard to screen critical biomarkers. Recently, growing evidence reveals that circRNAs play an important role in the progression of GBM and frequent dysregulations of circRNAs are observed in can-

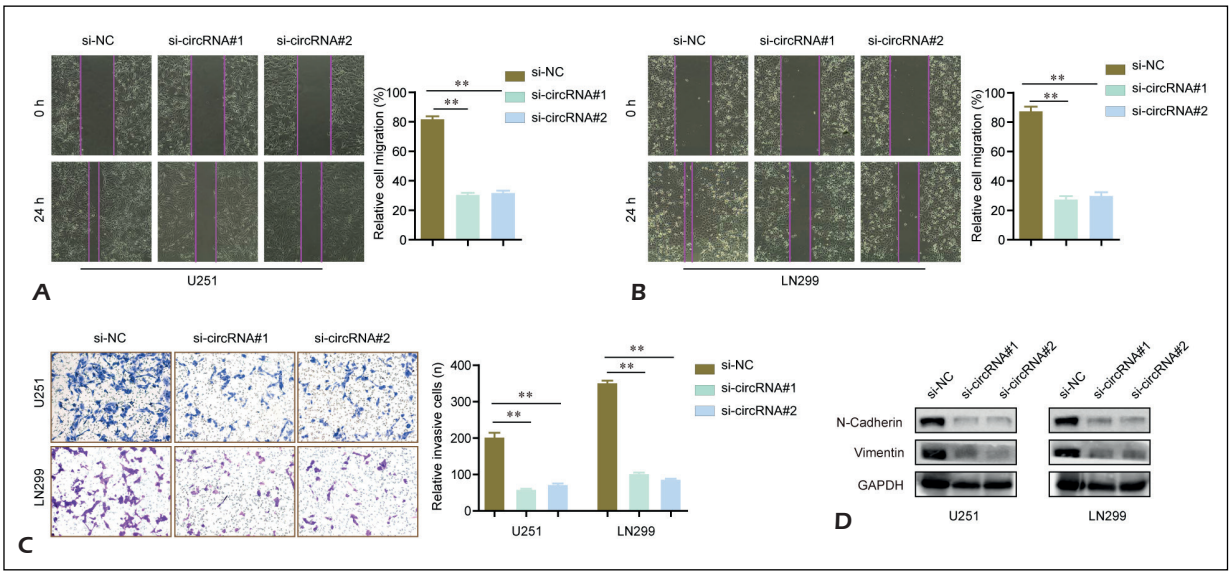

Figure 3. Silencing of hsa_circ_0067934 suppressed the invasion and migration of U251 and LN299 cells. **A**, and **B**, Inhibition of hsa_circ_0067934 reduced the migration of U251 and LN299 cells (Magnification: 10 ×). **C**, Transfection of hsa_circ_0067934 siRNAs reduced the invaded cell number of U251 and LN299 cells (Magnification: 40 ×). **D**, The protein levels of N-cadherin and vimentin were decreased in U251 and LN299 cells after they were transfected with hsa_circ_0067934 siRNAs. *p<0.05, **p<0.01.

cer tissues, suggesting that circRNAs could become a prognostic marker and a potential therapeutic target for GBM[22,23]. In addition, several circRNAs have been identified and elucidated in recent years. However, there are a large number of circRNAs which remain to be studied. Hsa_circ_0067934 was a newly identified circRNA which has been found to be up-regulated in esophageal squamous cell carcinoma and hepatocellular carcinoma[17,18]. However, its expression and clinical significance in GBM have not been investigated. In this study, we firstly detected the expression pattern of hsa_circ_0067934 in GBM patients by performing RT-PCR, finding that hsa_circ_0067934 expression was significantly increased in GBM tissues compared to matched normal tissues, in line with the trend of hsa_circ_0067934 expression in other two tumors. Furthermore, we explored the prognostic value of hsa_circ_0067934 in GBM patients,

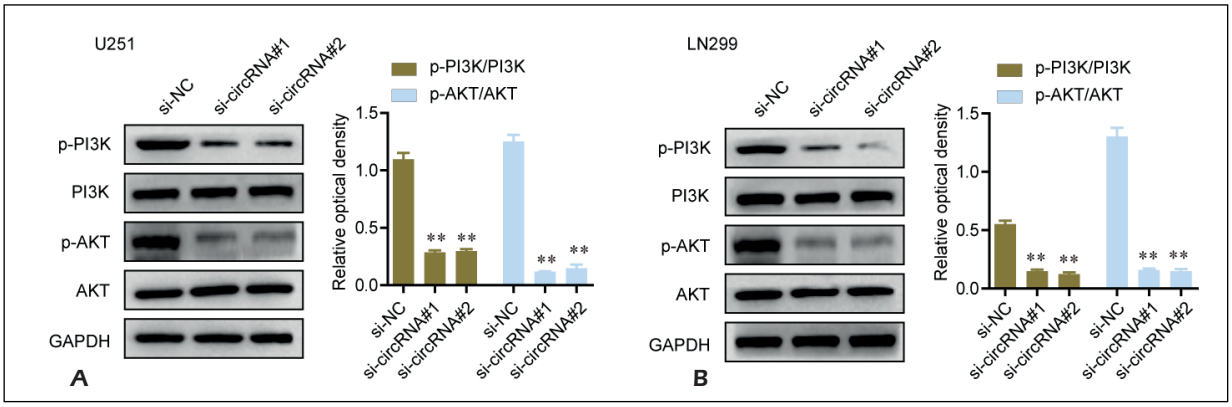

Figure 4. The activity of PI3K/AKT signaling in U251 and LN299 cells was inhibited by repressing hsa_circ_0067934 expression. **A**, The protein levels of p-PI3K, PI3K, p-AKT and AKT in U251 cells were assessed using Western blot assays. The protein levels of p-PI3K and p-AKT, but not PI3K and AKT were changed in U251 cells after they were transfected with hsa_circ_0067934 siRNAs. **B**, Western blot assays detected the protein levels of p-PI3K, PI3K, p-AKT and AKT inLN299 cells. *p<0.05, **p<0.01.

and the results indicated that patients with higher hsa_circ_0067934 expression exhibited a shorter overall survival and disease-free survival, suggesting that hsa_circ_0067934 expression may be used as a novel prognostic biomarker for GBM patients. However, the population involved in the study is relatively small. In addition, due to limitations in time and funds, the COX regression analysis was not been performed in this study. Therefore, further studies should be conducted to make up these limitations. Recently, the function of hsa_circ_0067934 in tumors has been reported. For instance, Xia et al[17] reported that the knockdown of hsa_circ_0067934 significantly suppressed esophageal squamous cell carcinoma cells proliferation and invasion, suggesting its tumor-promotive roles in the progression of esophageal squamous cell carcinoma. Then, Zhu et al[18] found that hsa_circ_0067934 expression acted as a tumor promoter in hepatocellular carcinoma because its forced expression could promote cells proliferation and metastasis through regulation of miR-1324/FZD5/Wnt/β-catenin axis. In this study, we also explored the potential function of hsa_circ_0067934 in the progression of GBM, finding that the down-regulation of hsa_circ_0067934 significantly suppressed GBM cells proliferation and promoted apoptosis. More importantly, we performed Wound healing assays and transwell invasion assay to study the effects of hsa_circ_0067934 on the ability of migration and invasion of GBM cells, finding that the knockdown of hsa_circ_0067934 significantly suppressed the migration and invasion of GBM cells. Subsequently, we explored the potential mechanism by detecting the expression of EMT-related proteins using Western blot, finding that the knockdown of hsa_circ_0067934 displayed a negative role in EMT progression. Thus, our results revealed that hsa_circ_0067934 functioned as a tumor promoter in GBM, which was consistent with the oncogenic roles of hsa_circ_0067934 in other tumors previously reported. The phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathways are crucial to various biological progressions, such as cell proliferation, angiogenesis, metabolism, differentiation, and survival[24,25]. The evidence identifies that the activation of PI3K-AKT signaling is one of the most frequent events in cancer[26,27]. In addition, the upstream regulation of PI3K-AKT signaling was involved in very complicated molecular mechanism[28]. Interestingly, several studies reported that circRNAs displayed functional effects by modulating PI3K-AKT signaling. For instance, circular RNA ciRS-7 was found to promote gastric cancer cells proliferation by PTEN/PI3K/AKT signaling pathway[29]. However, the association between hsa_circ_0067934 and PI3K-AKT signaling in GBM remains largely unclear. In this study, to elucidate the possible mechanism by which hsa_circ_0067934 regulates GBM cell proliferation and metastasis, we performed Western blot to explore the possible roles of hsa_circ_0067934 on PI3K-AKT signaling; our results indicated that knockdown of hsa_circ_0067934 suppressed the activity of PI3K-AKT signaling, suggesting that hsa_circ_0067934 exhibited its oncogenic roles in GBM through activating PI3K-AKT signaling.

Conclusions

We firstly demonstrated that hsa_circ_0067934 was highly expressed in GBM and associated with shorter overall survival and disease-free survival. We also showed that hsa_circ_0067934 plays a role in regulating PI3K-AKT signaling and affecting GBM cells proliferation and metastasis; other potential mechanisms still need further investigations. Our study revealed that hsa_circ_0067934 may be a therapeutic target for the treatment of GBM.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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