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Original Article

Long non-coding RNA SNHG7 promotes proliferation and self-renewal of glioblastoma cells

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Abstract: Glioblastoma multiform (GBM) is the most lethal brain tumor due to the aggressive proliferation and resistance to radio- and chemotherapy. In the present study, we demonstrated that long non-coding RNA SNHG7 was a new target for GBM cells proliferation and self-renewal which could contributed to the tumor progression. SNHG7 was significantly up-regulated in GBM tissues and cell lines and correlated with high stage and metastasis status. Moreover, high SNHG7 suggested poor prognosis in our observation. *In vitro* studies further revealed that SNHG7 promoted cell proliferation and inhibited apoptosis. Also of note was the fact that SNHG7 induced tumor cells self-renewal by regulating stem cell associated transcriptional factors such as Nanog and Oct4 as well as Wnt/beta-catenin signaling. In addition, we noticed that miR-34a-5p might be involved in SNHG7 regulated cell stemness as we found that the expression of miR-34a-5p was higher in non GBM tissues compared to GBM and was negatively correlated with SNHG7 expression. Collectively, our results showed a critical role of SNHG7 in GBM for modulating tumor cells proliferation and self-renew ability.

Keywords: Long non-coding RNA, SNHG7, glioblastoma, prognosis, self-renew

Introduction

Glioblastoma multiform (GBM) is the most common malignancy of brain tumor, which is highly aggressive and resistant to therapeutics. The prognosis of GBM is extremely poor with median survival of less than 15 months [1, 2]. The recurrence and chemotherapy resistance are the major factors for the unfavorable prognosis [3]. As the increasing understanding of histopathology of GBM, it is well recognized that multiple genetic and molecular aberrant expression are involved in tumor development and progression [4]. The tumor stem cell-like cells is implied in a variety of studies which are proved to be responsible for these criterion [5, 6]. Therefore, it is urgently needed to identify new targets for the treatment of GBM and reveal the mechanisms regulating GBM stem cells.

Long non-coding RNAs (lncRNAs) are a type of conserved non coding RNAs which are larger than 200 nucleotides in length. It has been considered that lncRNAs regulated diverse biological processes, such as cell growth, migration and metabolism, by affecting transcriptional activation, protein stability and microRNAs expression [7]. Therefore, identification of cancer-associated lncRNAs and the mechanisms underlying the gene regulation are important and urgently needed in cancer biology. SNHG7 (small nucleolar RNA host gene 7) is one of the recognized lncRNAs, which is located at chromosome 9q34.3, with a length of 2176 bp [8]. Recently, SNHG7 has been demonstrated to regulated by insulin-like growth factor (IGF1) and was necessary for proliferation [9]. Meanwhile, SNHG7 was also identified to compose the lncRNA complex in eIF4E protein with SNHG4, SNHG12 and some other lncRNAs, causing dysregulation of eIF4E in B-cell lymphoma [10]. However, little is known about the impacts of SNHG7 on GBM initiation and progression.

In this study, we revealed that lncRNA-SNHG7 was significantly up-regulated in GBM tissues and correlated with bad prognosis. In vitro functional analysis indicated that SNHG7 promoted GBM cells proliferation and stemness property

and inhibited apoptosis. Mechanically, SNHG7 could induce stem-associated transcriptional factors expression such as Nanog and Oct4. Also, the beta-catenin was elevated. Furthermore, we found that expression of SNHG7 and miR-34a-5p were inversely correlated in glioblastoma tissues.

Materials and methods

Ethics and human tissue samples

The study was supported and approved by the Ethics Committee of Dalian Municipal Central Hospital and written informed consent was obtained from all patients. 46 cases of glioblastoma tissue samples and normal brain tissues were obtained from the Department of Neurosurgery from 2008 to 2011. Tissues samples were collected at surgery and immediately frozen in liquid nitrogen and stored at -80°C.

Cell culture

Normal human astrocytes (NHA) and human glioblastoma cell lines (U87, U251, SNB19 and A172) were maintained in DMEM (Gibco) medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Plasmid construction and cell transfection

To establish SNHG7 expressing cells, we amplified full-length SNHG7 fragment by PCR from cells with the primers showed in Table S1. The PCR product was subcloned into the expression vector pCDNA3.1 (Invitrogen, USA). Three individual siRNAs and negative control were purchased from Genepharma (Shanghai, China). The cells were seeded into 6-well plates and transfection was performed using Lipofectamine 2000 according to the manufacturer's instruction.

Cell proliferation assay

Cell proliferation assay was performed using Cell Counting Kit-8 (CCK-8, Dojindo) according to the manufacturer's recommendation. Briefly, 100 μl of cell suspension from each group was seeded in the 96-well plate at concentration of 3000 cells per well. After incubation at indicated time point, 10 μl of CCK-8 was added to each well and the absorbance at 450 nm was measured using microplate spectrophotometer. The experiments were repeated at least three times.

Colony formation assay

Cells were plated into 6-well plates at concentration of 2000 cells per well and incubated for 15 days to form cell colonies. Then cells were washed with PBS, fixed with 4% paraformaldehyde and stained with crystal violet. The number of colonies was imaged and measured.

Apoptosis assay

Each group of cells was harvested and double staining with FITC-Annexin V and 7-AAD. Then cells were analyzed by flow cytometry (BD Calibur, BD Biosciences) using CellQuest software. The rate of apoptotic cells were measured and compared to the control group. All samples were performed in triplicate.

Spheroid formation assay

To quantify growth of spheroids formation under non-adherent conditions, single cell suspensions (2×10^4 per well) were seeded in 6-well ultra-low attachment plates. The medium used in this assay was DMEM/F12 supplemented with B27 (Gibco), 10 μg/ml basic fibroblast growth factor and 20 μg/ml epidermal growth factor. Cells were incubated for 10 days and the number and size of spheroids were quantified [11].

Western blot

Cells were lysed using RIPA buffer (Pierce) containing protease inhibitor and phosphatase inhibitor cocktail (Roche). The protein concentration was measured using BCA method (Pierce). 70 μg of total protein were loaded and electrophoresed in 10% SDS-PAGE and transferred onto PVDF membranes (Millipore). The membranes were blocked with 5% BSA and then incubated with primary antibody over night. Then membranes were washed and incubated with HRP-labeled secondary antibody. The proteins were detected using ECL method. The primary antibodies used were as followed: anti-Nanog, anti-Oct4, anti-AKT, anti-phosphorylated AKT, anti-β-cetanin, anti-GAPDH was used as internal control.

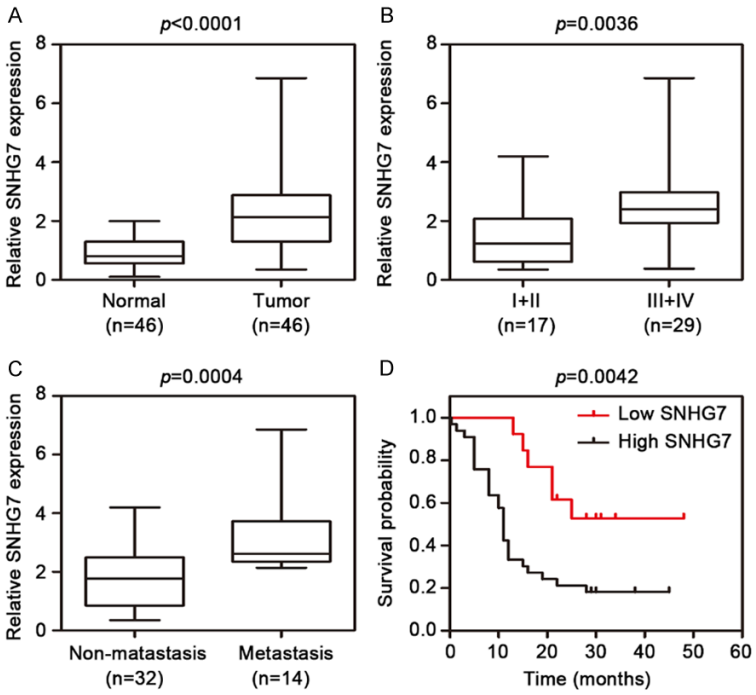

Figure 1. lncRNA SNHG7 is significantly higher in GBM tissues and correlated with poor prognosis. A: Relative expressions of SNHG7 in 46 paired samples of GBM tissues (T) and non-tumor tissues (N) were measured by q RT-PCR. B: The expression of SNHG7 was measured according to the patients with early stages (I and II) group or advanced stages (III and IV) group, respectively. C: The expression of SNHG7 was measured according to patients with metastasis or non-metastasis, respectively. D: Kaplan-Meier curves for the survival rate of 46 patients according to SNHG7 expression.

Quantitative real-time PCR

Total RNA was extracted from tissues or cells using Trizol reagent (Invitrogen, USA) according to the manufacturer's instruction. The first strand cDNA was synthesized using PrimeScript RT regent Kit. The expression of SNHG7 was determined by SYBR Green method. Results were normalized to GAPDH expression. The primers used were as shown in Table S1. The expression level of miR-34a-5p was determined by TaqMan miRNA assays (Applied Biosystems, USA) according to the provided protocol, and U6 small nuclear RNA was used to normalize the expression. The experiments were performed on ABI7500 real-time system (Applied Biosystems, USA).

Statistical analysis

Statistical analyses were performed by SPSS 21.0 (IBM) and GraphPad Prism 5. The comparisons of the expression of SNHG7 and miR-34a-5p in GBM samples and normal tissues were performed using Wilcoxon signed rank test. Kaplan-Meier analysis was used to test survival between two groups. The correlation between SNHG7 and miR-34a-5p expression was analyzed using Spearman's rank test. The results of *in vitro* experimental assay were presented as the mean ± SEM of three independent experiments. Differences between groups were tested using Student's t test or one-way ANOVA. A *p*-value of less than 0.05 was considered statistically significant.

Results

SNHG7 is up-regulated in glioblastoma and associated with poor prognosis

The expression level of SNHG7 was detected in 46 GBM samples and normal tissues by qRT-PCR. SNHG7 was significantly up-regulated in tumor tissues compared with corresponding normal tissues (**Figure 1A**). Furthermore, correlation of SNHG7 expression with clinical pathological features of GBM patients, indicating a significant association between SNHG7 high expression and advanced clinical stages and metastasis (**Figure 1B** and **1C**). More importantly, Kaplan-Meier survival analysis with log-rank test was performed to evaluate the correlation between SNHG7 expression and prognosis. We found that high SNHG7 expression had significantly shorter survival times (**Figure 1D**). Taken together, these results suggested that up-regulated SNGH7 might play important roles in GBM.

SNHG7 promotes cell proliferation and inhibit apoptosis

We next examined the expression of SNHG7 in human GBM cell lines. Of these, U87 and U251 expressed higher levels of SNHG7 compared with normal human astrocytes, while A172 cells expressed relatively low levels (**Fig-**

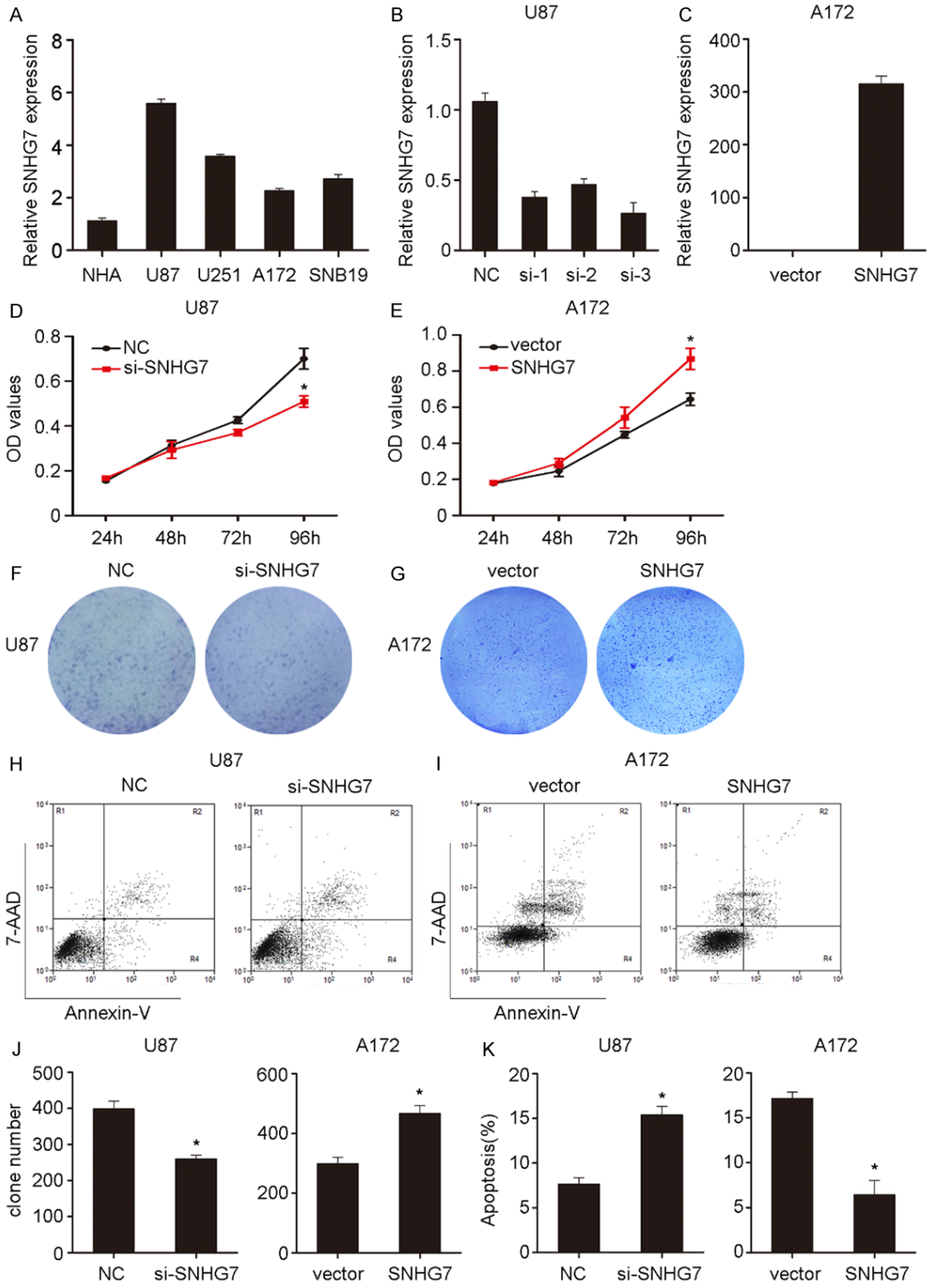

Figure 2. SNHG7 promotes GBM cells proliferation and inhibits apoptosis. (A) qRT-PCR analysis of SNHG7 expression levels in GBM cell lines. qRT-PCR analysis of knockdown of SNHG7 in U87 cells (B) and overexpression of SNHG7 in A172 cells (C). Cell proliferation assays for knockdown of SNHG7 (D) and overexpression of SNHG7 (E). Colony formation assays for knockdown (F) and overexpression of SNHG7 (G). Apoptosis assays for knockdown (H) and overexpression of SNHG7 (I). (J) Statistics of colony formation assays. (K) Statistics of apoptosis assays. The results were expressed as the mean ± SEM of triplicated independent experiments (*P<0.05).

ure 2A). To evaluate the role of SNHG7 in glioblastoma, we modulated SNHG7 expression. As shown in **Figure 2B** and **2C**, we knocked down the expression of SNHG7 in U87 cells while overexpressed in A172 cells. Then we assessed the effect of knockdown or ectopic expression of SNHG7 on cell proliferation and apoptosis. CCK-8 results revealed that cell growth was significantly repressed when knocking down of SNGH7 (**Figure 2D**). Similarly, colony formation assays revealed that inhibition of SNGH7 reduced the clone numbers (**Figure 2F** and **2J**). On the contrary, overexpression of SNHG7 in A172 cells promoted cell growth and colony formation ability (**Figure 2E** and **2G**). We also tested the effect of SNHG7 on apoptosis. Overexpressed SNHG7 inhibited cell apoptosis while knockdown induced it (**Figure 2H**, **2I** and **2K**). Taken together, these results indicated that SNHG7 promoted cell growth and inhibit apoptosis.

SNHG7 induces stem-cell like properties of glioblastoma

Emerging evidence suggests that tumor stem cells is responsible for the initiation and drug resistance of GBM [12, 13]. Therefore, we explored if SNHG7 had a direct function in facilitating GBM cells stemness properties. We used non-adherent culturing and detected the tumor spheroids formation. We noticed that knockdown of SNGH7 significantly reduced the number and volume of U87 spheres compared with control cells (**Figure 3A**). However, overexpression of SNHG7 in A172 increased the spheroid formation ability (**Figure 3B**). Furthermore, the self-renewal capacity of stem-like cells in the A172 cells was measured by a limiting dilution assay. The results indicated that less required cells was needed to generate tumor sphere (**Figure 3C**). We also detected stem cell markers, such as CD133, EpCAM, Oct4 and Nanog by qRT-PCR (**Figure 3D**) and found that these markers were up-regulated, suggesting that overexpression of SNGH7 increased GBM cells stemness property [14].

SNHG7 regulates the expression of Nanog and Oct4 in glioblastoma cells

To explore the molecular mechanisms by which SNHG7 contributes to the stem-cell like properties of GBM cells, we detected potential targets involved in tumor stem cell formation. In this study, western blot assay were employed to detect the expression of stemness transcriptional activators (Nanog and Oct4) and important signaling (AKT and β-catenin) between SNHG7 overexpression and knockdown cells with control cells, respectively (**Figure 4A**). These results revealed Nanog and Oct4 were significantly up-regulated with SNHG7 overexpression. Furthermore, β-catenin was elevated between SNHG7 overexpression cells and control cells, however, there was no significantly changes of AKT and phosphorylated AKT. On the contrary, knocking down of SNHG7 had the opposite effects (**Figure 4B**). In addition, previous study has reported that miR-34a-5p could inhibit tumor stem-cell like property by repressing key molecules such as CD44 and Oct4 [15, 16]. We investigated the expression of miR-34a-5p in GBM tissues. As shown in **Figure 4C**, miR-34a-5p was significantly higher in normal tissues compared to tumor tissues. More importantly, we found that the expression of SNHG7 and miR-34a-5p were inversely correlated in 46 clinical samples (**Figure 4D**). We also explored the TCGA (The Cancer Genome Atlas) database containing 151 samples of GBM [17, 18]. The results indicated validated our observation (**Figure 4E**). Taken together, we suggested that SNHG7 could influence the stem-cell like property by altering stemness-associated transcriptional factors which might be partially by regulating miR-34a-5p.

Discussion

GBM is characterized by high malignancy and ranks among the most lethal cancers. Poor prognosis of GBM is due to the metastasis and resistance of chemotherapy. Over the last decade, accumulating studies have demonstrated that lncRNAs played important roles in key molecular and cellular signaling governing GBM initiation and progression [19, 20]. However, the mechanisms of lncRNAs in the carcinogenesis of GBM are far more needed to be elucidated.

SNHG7 is a member of recently identified lncRNAs and mentioned in a variety of cancers including gastric cancer, lymphoma and ovarian cancer. However, little is known about the expression and function of SNGH7 in GBM. In this study, we revealed that SNHG7 was upregulated in GBM samples compared to normal

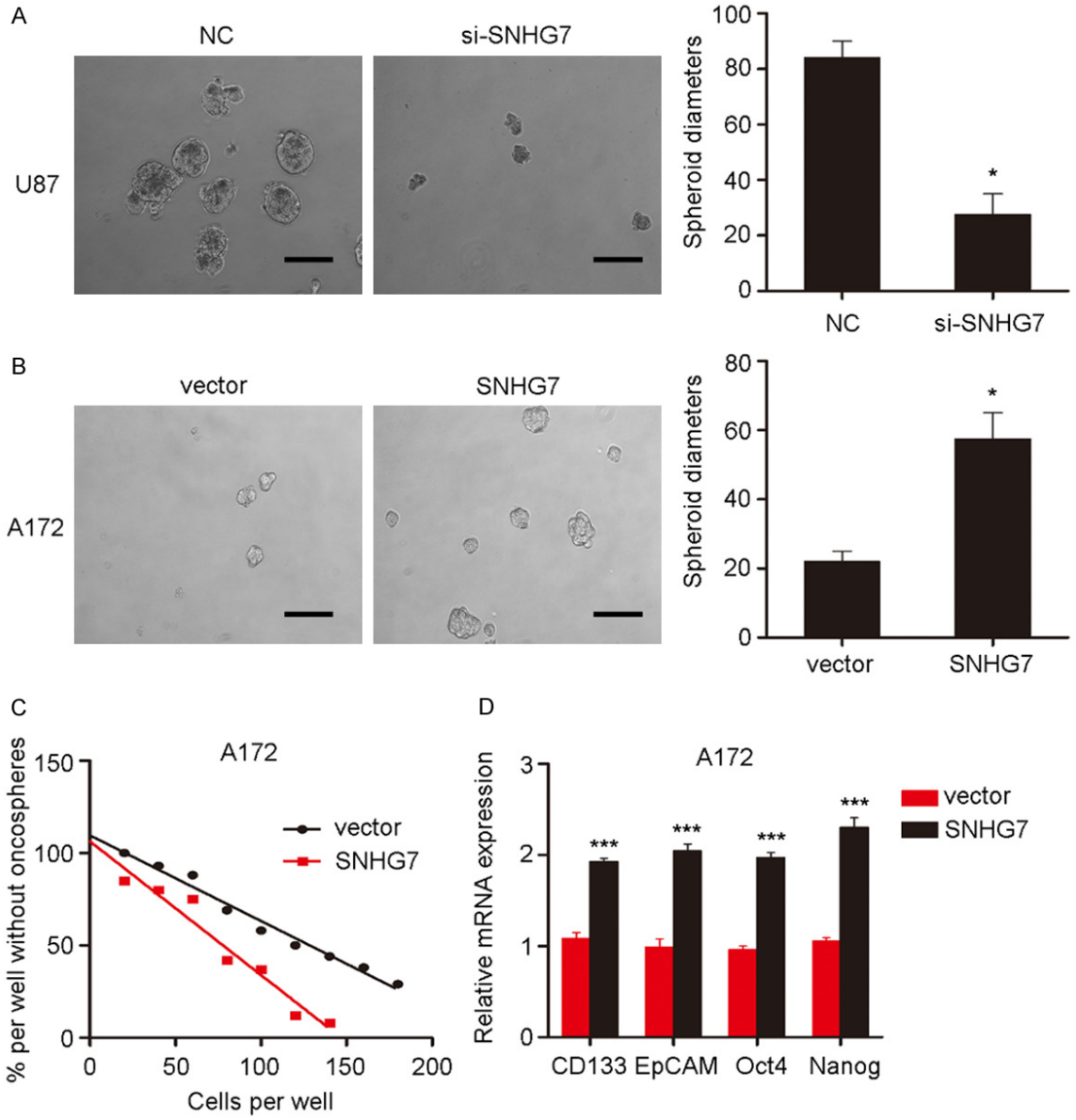

Figure 3. SNHG7 induces tumor cell stemness ability. Spheroid formation assays for knockdown (A) and overexpression (B) of SNHG7 (Bar, 100 μm). (C) Limiting dilution assay of overexpressing SNHG7 in A172 cells. (D) qRT-PCR analysis of cells overexpressing SNHG7. The results were expressed as the mean ± SEM of triplicated independent experiments (*P<0.05, ***P<0.001).

tissues. According to GSE51575, Fei et al indicated that SNHG7 expression in gastric cancer tissues was more than 2.5 fold changes compared with corresponding non cancer tissues [21]. Also, it was reported that SNHG7 has copy number amplification in BRCA1-associated ovarian cancer [22]. All these results supported us to investigate the effect of SNHG7 in GBM. We demonstrated that SNHG7 promoted cell proliferation and induced stem-cell like property. This study revealed a mechanism for the SNGH7 in GBM cells.

Our results showed a significantly increase in the self-renewing capacity, expression of stem-cell like markers and the volume of A172 tumor spheroid with ectopic expression of SNGH7. We also observed that modulating SNHG7 expression in U87 and A172 cells could affect the expression of Nanog, Oct4 and β-catenin (**Figure 4A**). However, there was no significantly changes in the level of Akt activation. Meanwhile, our results suggested that SNHG7 and miR-34a-5p were inversely correlated in GBM samples, which implying the role of SNHG7 reg-

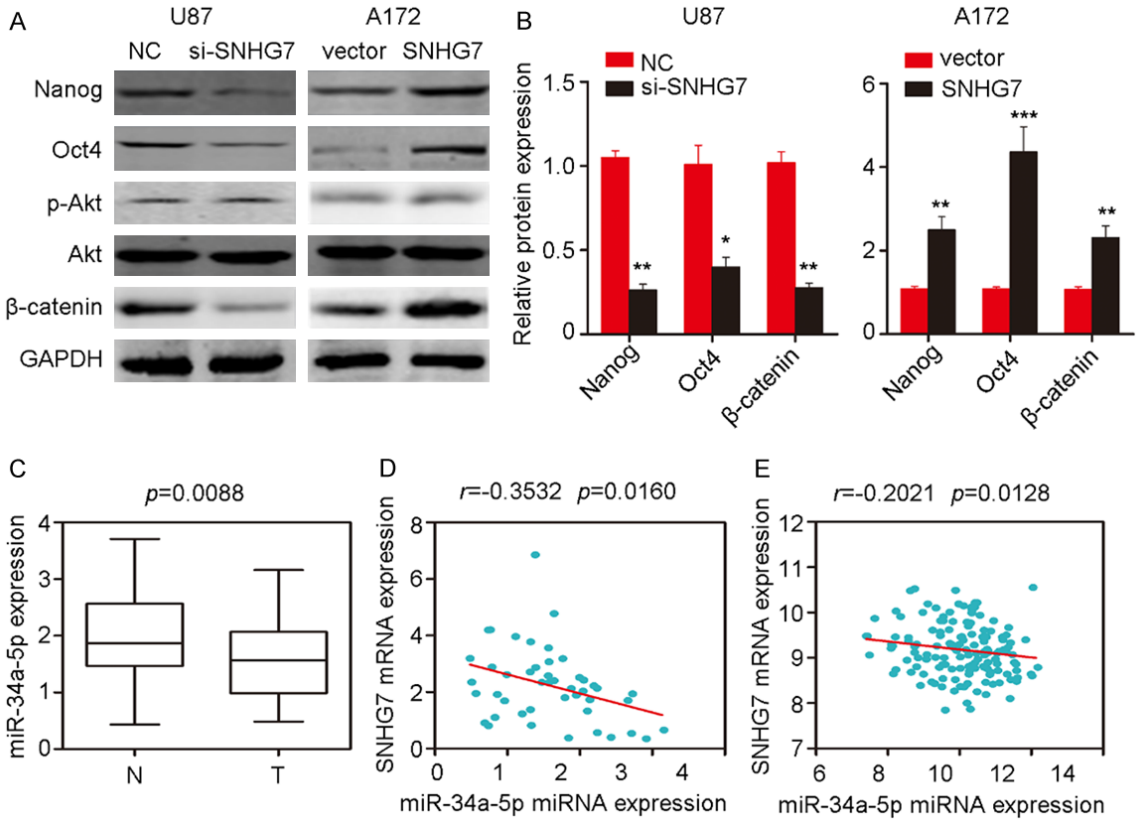

Figure 4. SNHG7 regulates beta-catenin expression and negatively correlated with miR-34a-5p expression. A: Western blot analysis of transcriptional factors and signaling activators when SNHG7 knockdown or overexpression. B: Statistics of western blot results. C: The expression of miR-34a-5p in 46 cases of GBM tissues samples. D: The correlation between miR-34a-5p and SNHG7 was examined in 46 cases of GBM samples. E: The correlation between miR-34a-5p and SNHG7 was examined in TCGA database. The results were expressed as the mean ± SEM of triplicated independent experiments (*P<0.05, **P<0.01, ***P<0.001).

ulating GBM cells stemness. MiR-34a-5p was reported to be the tumor suppressor gene in prostate cancer, colorectal cancer and endometrial cancer [15, 23, 24]. Mechanically, miR-34a-5p could target a numerous genes which involved in tumor initiation and progression, such as Oct4, p53, KLF4 [25, 26]. miR-34a also mediated Axin2 expression and involved in p53 regulated nuclear GSK-3 levels [27]. So these observations were consistent with our hypothesis, that SNHG7 could regulate tumor cell stemness by modulating miR-34a-5p expression. Further studies would proceed to investigate the regulation pattern of SNHG7 to miR-34a-5p.

In summary, we showed that SNHG7 and its association with GBM clinical pathological features and prognosis. The upregulation of SNHG7 in GBM suggested that its oncogenic roles. Further experiments demonstrated that SNHG7 promoted cell proliferation and self-renewing capacity which might be related with miR-34a-5p expression. Further studies are required to determine the detail mechanisms underlying SNGH7 modulating miR-34a-5p and a clinical treatment strategy for GBM patients.

Disclosure of conflict of interest

None.

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Table S1. Primer sequences

Primer name	Sequence (5' to 3')
Primers for qRT-PCR	
homo-GAPDH-F	ACAACTTTGGTATCGTGGAAGG
homo-GAPDH-R	GCCATCACGCCACAGTTTC
homo-SNHG7-F	TTGCTGGCGTCTCGGTTAAT
homo-SNHG7-R	GGAAGTCCATCACAGGCGAA
homo-Nanog-F	TTTGTGGGCCTGAAGAAAACT
homo-Nanog-R	AGGGCTGTCCTGAATAAGCAG
homo-CD133-F	AGTCGGAAACTGGCAGATAGC
homo-CD133-R	GGTAGTGTTGTACTGGGCCAAT
homo-Oct4-F	GGGAGATTGATAACTGGTGTGTT
homo-Oct4-R	GTGTATATCCCAGGGTGATCCTC
homo-EpCAM-F	TGATCCTGACTGCGATGAGAG
homo-EpCAM-R	CTTGTCTGTTCTTCTGACCCC
Primers for clone	
SNHG7-sense	GCCAGTTCTCGAGCGCCTCAC
SNHG7-antisense	GTCCAGGCTGGAGTGCAGTGGTG