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From Cell Biology to Tissue Engineering

Long non-coding RNA SNHG7 promotes malignant melanoma progression through negative modulation of miR-9

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Summary. Long non-coding small nucleolar RNA host gene 7 (lncRNA SNHG7) was verified to act as an onco-gene in human cancers. Nevertheless, the role of SNHG7 in malignant melanoma remains elusive. The present study showed an increase of SNHG7 expression in malignant melanoma tissues and cell lines. Besides, SNHG7 knockdown inhibited proliferation and migration in malignant melanoma cells. Bioinformatics analysis demonstrated that SNHG7 functions as a molecular sponge for miR-9 in biological behavior of melanoma cells. And miR-9 could inhibit the expression of PI3KR3 by binding with the 3' -UTR. Furthermore, PI3KR3, pAKT, cyclin D1 and Girdin expression was down-regulated after SNHG7 knockdown by siRNA. In addition, SNHG7 knockdown decreased xenograft growth in vivo. Taken together, this research demonstrated that SNHG7 was an oncogene in malignant melanoma, providing a novel insight for the pathogenesis and new potential therapeutic target for malignant melanoma.

Key words: Malignant melanoma, SNHG7, miR-9, PI3K/AKT signaling pathway

Introduction

Malignant melanoma (MM), a kind of malignant tumor, is the most common skin cancer besides skin squamous cell carcinoma and basal cell carcinoma (Bai et al., 2017). The incidence of MM is estimated to increase by 3–7% per year among different populations with approximately 50,000 deaths worldwide per year (Miller and Mihm, 2006; Tuong et al., 2012). Despite great advances in treatment technology, the prognosis of MM remains poor due to therapy failure (Eggermont et al., 2016).

Long non-coding RNAs (lncRNAs), greater than 200 nucleotides in length and without protein-coding potential, have been verified to regulate diverse cellular processes such as cell cycle, autophagy and apoptosis (Wu et al., 2014; Shang et al., 2016). In addition, lncRNAs might interact with miRNAs (Juan et al., 2013), functioning as a competing endogenous RNA (ceRNA) (Su et al., 2013). Long non-coding small nucleolar RNA host gene 7 (lncRNA SNHG7) is located on chromosome 9q34.3 with a length of 984bp (Wang et al., 2017a). Previous studies have confirmed that SNHG7 acted as an onco-gene in human cancers, such as gastric cancer (Wang et al., 2017b), lung cancer (She et al., 2016) and esophageal cancer (Xu et al., 2018). Nevertheless, the role of SNHG7 in MM remains elusive.

MicroRNAs are a class of highly conservative non-coding small RNAs (around 19-23 nucleotides in length) that regulate gene expression of target mRNAs, which play an important role in maintaining homeostasis (Valencia-Sanchez et al., 2006). Moreover, aberrant upregulation or downregulation of miRs has been observed in MM, some of which have been confirmed to play key roles in the development and progression of this disease (Liu et al., 2014; Zhou et al., 2014). Recently, several studies revealed that miR-9 was involved in the pathogenesis of MM including progression, invasion and metastasis (Liu et al., 2012a,b). However, whether SNHG7 could interact with miR-9 to regulate the development of MM remains unclear.

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Hence, the purpose of this study was to investigate the level of SNHG7 expression in MM tissues and cell lines, and explore its underlying mechanism.

Materials and methods

Patients and tissue specimens

We collected a cohort of 80 pairs of primary malignant melanoma tissues and adjacent normal tissues from patients who were admitted to the Department of Plastic and Burn Surgery, Tianjin First Center Hospital from January 2012 to December 2015. None of the patients had previously received other treatment (radiotherapy and chemotherapy). The characteristics of the patients included in this study are shown in Table 1. This study was approved by the Ethics Committee of Tianjin First Center Hospital, and all patients and their families had signed the written informed consent. The specimens were stored in liquid nitrogen immediately after surgery for further analysis.

Cell culture

The human melanoma cell lines (A375, B16, MNT-1, WM451) and the normal epidermal melanocytes HEM-l (Institute of Biochemistry and Cell Biology, SIBS, CAS, Beijing, China) were cultured in Dulbecco' s Modified Eagle' s Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), 100 U/ml penicillin G and 100 μg/mL streptomycin (both from Sigma). Cell culture was maintained in a humidified thermostat incubator with 5% CO_2 at 37°C.

Cell transfection

Si- SNHG7, miR-9 mimic, miR-9 inhibitor and their parental negative controls (NCs) were synthesized from GenePharma (Shanghai, China). A375 and WM451 cells were seeded in six-well plates at 60% confluence. After 48 hours, cells were transfected with oligonucleotide or plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer' s instructions.

Cell proliferation

Cells (10,000cells/well) were plated into a 96-well plate, and cultured at 37°C in an atmosphere containing 5% CO_2 for 0, 24, 48, 72 and 96 h and stained with 5 mg/l MTT for 4 h. Supernatant was discarded and 200 μl of dimethylsulfoxide (DMSO) was added to dissolve precipitates. Absorbance was detected by determining the optical density at 490 nm using an enzyme immunoassay analyzer.

Colony formation assay

Cells were seeded on a 6-well plate (1000/well), and, 3 days later, medium was changed. After 14 days, colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Beyotime, Shanghai, China) followed by cell count and photographing.

Cell migration assay

Cells at density of 1×10^5 per well were seeded into the upper chamber filled with medium containing 1% FBS. The lower chamber was filled with culture medium with 10% FBS medium. After 24 h of incubation, cotton swabs were used to remove the cells inside the upper chamber, while the cells on the other side of the membrane were fixed and stained with 0.1% crystal violet and counted under a microscope (five fields per well).

Real time quantitative PCR analysis

The total RNA was extracted from tissues and cells using Trizol reagent (Invitrogen, Shanghai, China) and then 1 mg of total RNAwas reverse-transcribed in a volume of 20 ml using random and oligo(dT) primers under standard conditions, according to the instructions of the PrimeScript RT Kit (TaKaRa, Dalian, China). Gene expression was evaluated using SYBR Green qPCR master mix (Roche, USA). GAPDH or U6 was used as an internal control for mRNA or microRNA, respectively. The primers were as follows: circMTO1 forward 5' - GTGACTTCGCCTGTGATGGA-3' and reverse 5' - GGC CTCTATCTGTACCTTTATTCC-3' ; miR-9 forward 5' --GCCCGCTCTTTGGTTAT CTAG-3' and reverse 5' --CCAGTGCAGGGTC CGAGGT-3' ; U6 forward 5' -GCTCGCTTCGG CAGCACA-3' and reverse 5' -GAGGTATTCG

Table1. The Correlation between lncRNA SNHG7 expression and clinical pathological characteristic of malignant melanoma (n=80).

Variables	Clinical parameters	SNHG7 expression		P value
		High (n=46)	Low (n=34)	
Gender	Male	23	18	0.824
	Female	23	16	
Age (years)	< 65	20	17	0.652
	≥65	26	17	
Thickness (mm)	≤2	19	18	0.367
	≥2	27	16	
Differentiation	Well/Moderate	12	19	0.010*
	Poor	34	15	
TNM stage	I/II	16	20	0.042*
	III/IV	30	14	
Lymphatic metastasis	Yes	28	12	0.041*
	No	18	22	
Distant metastasis	Yes	27	11	0.025*
	No	19	23	

*P<0.05.

CACCAGAG GA-3' ; and GAPDH forward 5' -TGTGGGCATCAATGGATTTGG-3' and reverse 5' -ACACCATGTATTCCGGGTCAAT-3' .

Luciferase reporter assay

The 3' -UTR fragment of SNHG7 and TGFBR2 containing the putative binding sequences of miR-9 were cloned into the psiCHECK-2 vector (Promega Corp., Madison, WI, USA). After culturing overnight, cells were co-transfected with the wild-type (wt-SNHG7, wt-TGFBR2-3' UTR)/mutated (mut- SNHG7, mut-TGFBR2-3' UTR) SNHG7 or TGFBR2 reporter plasmid and miR-9 mimics or miR-9 inhibitor. The activities of Renilla luciferase and Firefly luciferase were examined using the luciferase reporter assay system (Promega Corp.), according to the manufacturer' s instructions. The Renilla luciferase activity was normalized to Firefly luciferase activity.

Western blotting analysis

Protein was separated using 12% SDS-PAGE, transferred to a polyvinylidene fluoride membrane (PVDF; Thermo Fisher Scientific, Inc.), and blocked in 5% non-fat dried milk in phosphate buffered saline (Thermo Fisher Scientific, Inc.) at 4°C overnight. After electrophoresis, the protein samples were incubated with the primary antibody purchased from Abcam Campany (anti-PI3KR3, anti-pAkt, anti-Cyclin D1 and anti-Girdin 1:1000 dilution). Membranes were washed and incubated with secondary antibodies conjugated by horseradish peroxidase (HRP), the protein bands were visualized using chemiluminescence dissolvent (Thermo, USA).

Tumor xenograft model in nude mice

$5x10^5$ A375 cells transfected with si-NC or si-SNHG7 were subcutaneously injected into the backs of BALB/c nude mice (Beijing Hua-Fu-Kang Biosciences, n=6 per group). After the first injection, tumor volume of all mice was calculated using a digital caliper every 5 days according the following equation: Volume= 0.5×length×width×width. Twenty-five days after injection, the mice were sacrificed by euthanasia and the tumor weights were measured.

Statistical analysis

All data were expressed as mean ± SD. All calculations were carried out using SPSS 17.0 software (IBM Software, Chicago, IL, USA) and GraphPad (vision 6.0, USA). Kaplan-Meier method and log rank test were utilized to analyze the overall survival rate of patients. A value of $P<0.05$ was considered statistically significant.

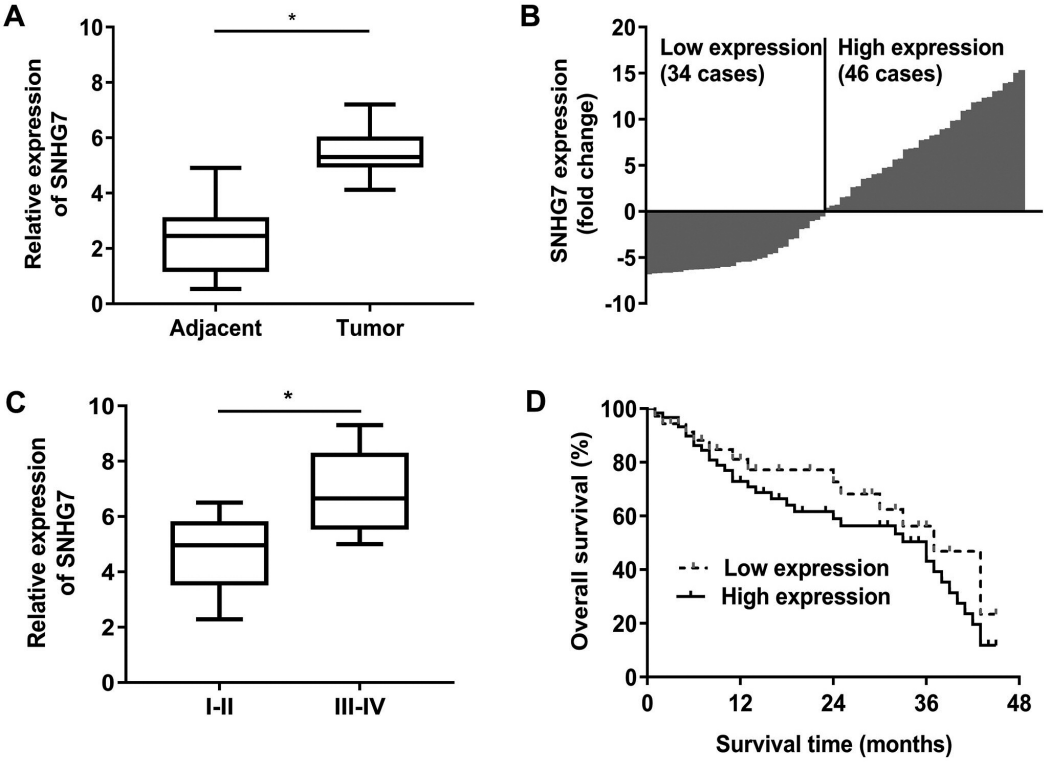

Fig. 1. SNHG7 was increased in MM tissues and correlated with poor prognosis of patients with MM. **A.** Expression of SNHG7 in MM tissues and adjacent normal tissues samples. **B.** The fold changes of SNHG7 expression level (malignant melanoma tissues samples) in each patient were measured by real-time PCR. **C.** SNHG7 expression was significantly higher in patients with advanced clinical stage (III-IV phase) thanthose with early clinical stage (I-II phase). **D.** Kaplan-Meier curves of overall survivals demonstrated that patients with high SNHG7 levels had poorer overall survival than that with low SNHG7 levels. *P<0.05 compared to control group.

Results

SNHG7 was increased in MM tissues and correlated with poor prognosis of patients with MM

We initially measured the level of SNHG7 in MM tissues and adjacent normal tissues using qRT-PCR (Fig. 1A). The fold changes of SNHG7 level in each patient with MM are indicated in Figure 1B. Furthermore, the results revealed that high SNHG7 was associated with differentiation, advanced TNM stage, lymphatic and distant metastases (Fig. 1C, Table 1). However, no correlation was found between SNHG7 elevation and patient gender, age, and thickness (Table 1). Moreover, Kaplan-Meier curves of overall survivals showed that patients with high SNHG7 levels had poor overall survival (Fig. 1D). These data indicated that SNHG7 was up-regulated in MM tissue and was positively associated with poor prognosis.

SNHG7 knockdown inhibited proliferation and migration in MM cells in vitro

We further detected SNHG7 expression in 4 MM cell lines and the normal cell line (Fig. 2A). Subsequently, we stably established SNHG7 silencing in A375 and WM451 cell lines in vitro (Fig. 2B). The MTT assay showed SNHG7 knockdown markedly repressed the proliferative ability of A375 and WM451 cells (Fig. 2C). SNHG7 knockdown could suppress colony formation of A375 and WM451 cells lines significantly (Fig. 2D). Besides, Besides, SNHG7 knockdown inhibited cell migration of A375 and WM451 cells lines obviously (Fig. 2E).

SNHG7 sponged miR-9 in melanoma cells

An increasing number of studies have demonstrated that lncRNAs might function as a ceRNA or a molecular

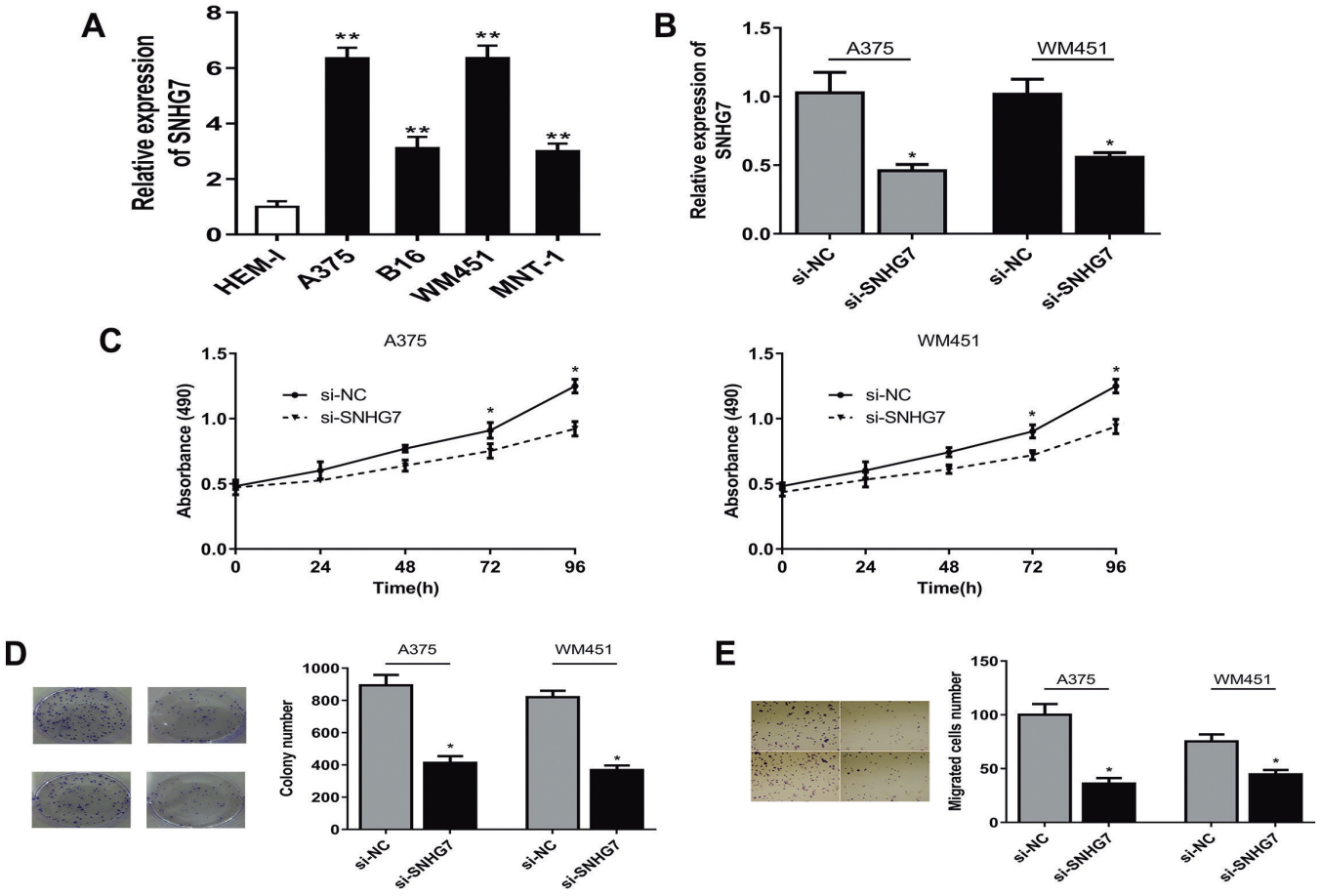

Fig. 2. SNHG7 knockdown inhibited proliferation and migration in MM cells in vitro. **A.** SNHG7 expression level was significantly up-regulated in 4 MM cell lines compared to the normal cell line. **B.** SNHG7 silencing was established via siRNA transfection in A375 and WM451 cell lines. **C.** the MTT assay showed SNHG7 knockdown significantly repressed the proliferative ability of A375 and WM451 cells. **D.** Colony formation assay revealed that decreased SNHG7 could induce two cell lines formed significantly fewer colonies than control cells. **E.** Cell migration assay showed SNHG7 silencing inhibited the migration ability of MM cells. *P<0.05, **P<0.01 compared to control group.

sponge in modulating miRNA (Mercer et al., 2009). Using bioinformatics analysis, we observed the 3' UTR of SNHG7 was highly conserved to bind with miR-9. The binding site is presented in Fig. 3A. MiR-9 level was significantly decreasetd in MM tissues compared to adjacent normal tissues (Fig. 3B). Luciferase reporter assay confirmed the binding with decreasing fluorescence within miR-9 mimic and SNHG7 wild type (Fig. 3C). MTT assay revealed that miR-9 inhibitor was able to rescue the suppression by si-SNHG7 in the proliferation of cells (Fig. 3D).

MiR-9 was directly targeted by PI3KR3

PI3KR3 was predicted to be a target gene of miR-9 according to bioinformatics analysis. 3' UTR of PI3KR3 was highly conserved to bind with miR-9 in Fig. 4A. PI3KR3 level was much lower in MM tissues than adjacent normal tissues (Fig. 4B). Luciferase reporter assay showed that transfection of miR-9 could significantly restrict the relative luciferase activity in cells (Fig. 4C). Furthermore, increased miR-9 level remarkably downregulated PI3KR3 expression in A375 and WM451 cells at the mRNA and protein levels (Fig. 4D). In short, our study found that miR-9 inhibited the expression of PI3KR3 by binding with the 3' -UTR.

SNHG7 silencing repressed the PI3K/AKT signaling pathway

The differences were identified in the expression of PI3KR3 between the two groups after SNHG7

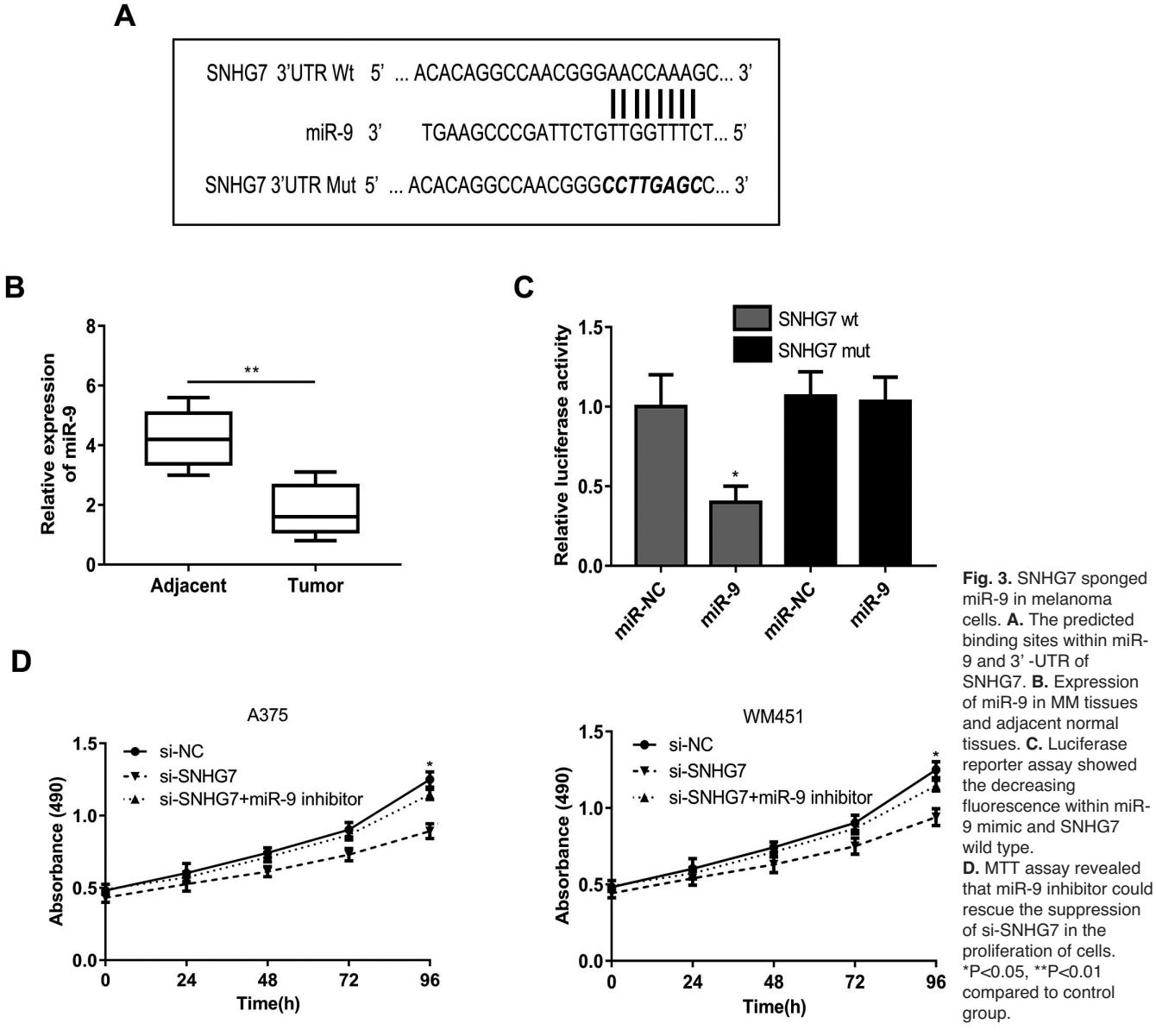

Fig. 3. SNHG7 sponged miR-9 in melanoma cells. **A.** The predicted binding sites within miR-9 and 3' -UTR of SNHG7. **B.** Expression of miR-9 in MM tissues and adjacent normal tissues. **C.** Luciferase reporter assay showed the decreasing fluorescence within miR-9 mimic and SNHG7 wild type. **D.** MTT assay revealed that miR-9 inhibitor could rescue the suppression of si-SNHG7 in the proliferation of cells. *P<0.05, **P<0.01 compared to control group.

knockdown by siRNA (Fig. 5A). Meanwhile, si-SNHG7 obviously repressed downstream molecules cyclin D1 and Girdin expression that regulate cell proliferation and migration (Fig. 5B).

SNHG7 knockdown decreased xenograft growth in vivo

To further determine the biological significance of SNHG7 on tumor growth in vivo, we injected A375 cells transfected with si-NC or si-SNHG7 into nude mice. The average tumor volume, tumor size and weight in si-SNHG7 group were significantly lower than that in the si-NC group (Fig. 6A-C). These data indicated that SNHG7 silencing suppressed tumor growth *in vivo*.

Discussion

Among human malignancies, melanoma has a very poor prognosis due to its early metastasis with no obvious inchoate symptom, so the stage of disease is often diagnosed later and cannot be controlled in time. This is the main reason for the high mortality of melanoma (Rigel et al., 2010). Therefore, further study of the progression of metastatic melanoma and the innovation of new, targeted therapies are desperately needed.

Non-coding RNAs (ncRNAs) have been found to be closely associated with melanoma tumorigenesis (Hombach and Kretz, 2013). As a highly heterogeneous group of noncoding RNAs, lncRNA was believed to be the "noise" in transcription of human genome without any biological effect in human originally (Tang et al., 2017). However, more and more research has shown that lncRNAs, though without any protein-encoding potential, exhibit critical effect in diverse cellular processes such as cell cycle, autophagy and apoptosis (Wu et al., 2014; Shang et al., 2016). As one of the recognized lncRNAs, SNHG7 promotes proliferation, migration, and invasion and inhibits apoptosis in many cancers, although, the clinical significance and biological mechanisms of SNHG7 in the progression of MM remain largely to be elucidated. The results revealed that SNHG7 expression was increased in MM tissue and cells and the high SNHG7 level was associated with poor prognosis. To further explore the function of SNHG7 in the development of MM, we established SNHG7 silencing in cell lines and observed SNHG7 knockdown could suppress proliferation and migration in MM cells.

Increasing evidence has confirmed that lncRNAs could function as ceRNAs to regulate miRNA pathways (Tay et al., 2014). For instance, long non-coding RNA-SNHG7 acts as a target of miR-34a to regulate PI3K/Akt/mTOR pathway in colorectal cancer progression (Li et al., 2018), and long noncoding RNA

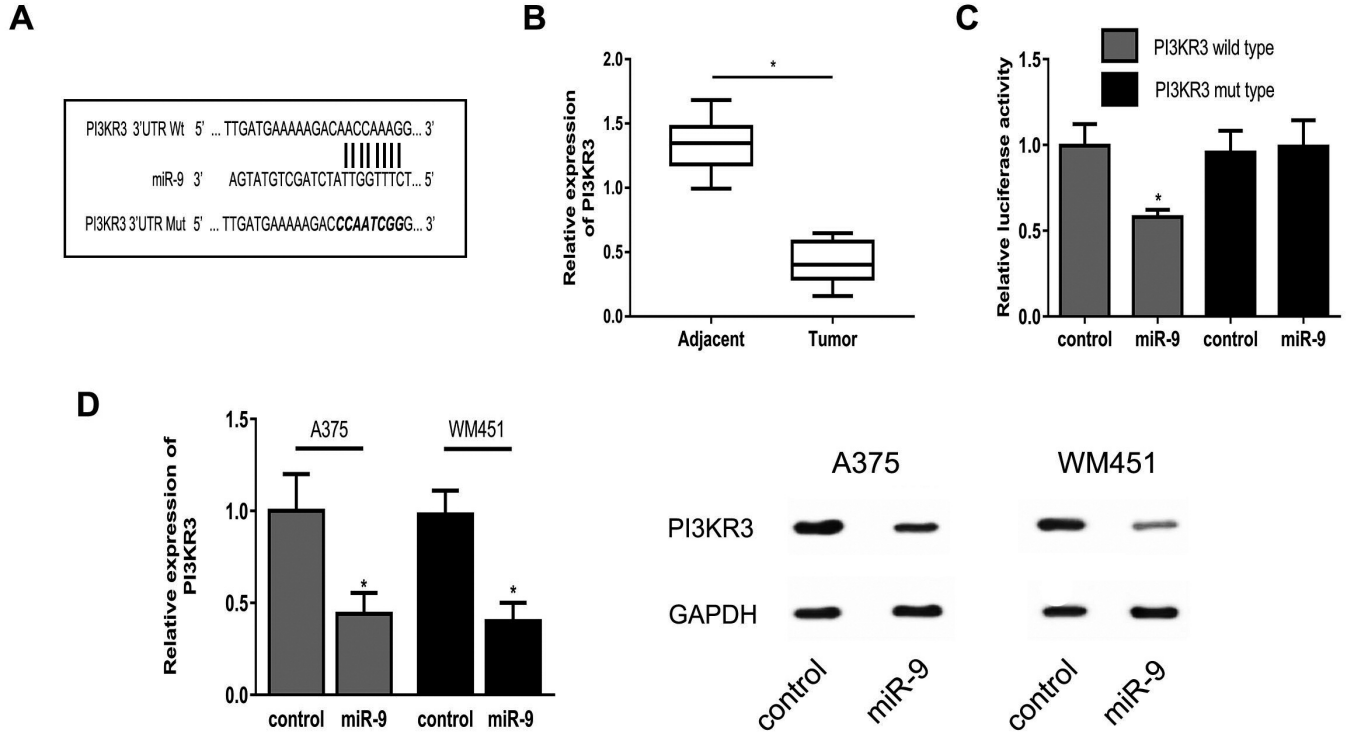

Fig. 4. MiR-9 was directly targeted by PI3KR3. **A.** The predicted binding sites within miR-9 and 3' -UTR of PI3KR3. **B.** Expression of PI3KR3 in MM tissues and adjacent normal tissues. **C.** Luciferase reporter assay showed the decreasing fluorescence within miR-9 and PI3KR3 wild type. **D.** PI3KR3 expression in A375 and WM451 cells transfected with miR-9 mimic at the mRNA and protein levels. *P<0.05 compared to control group.

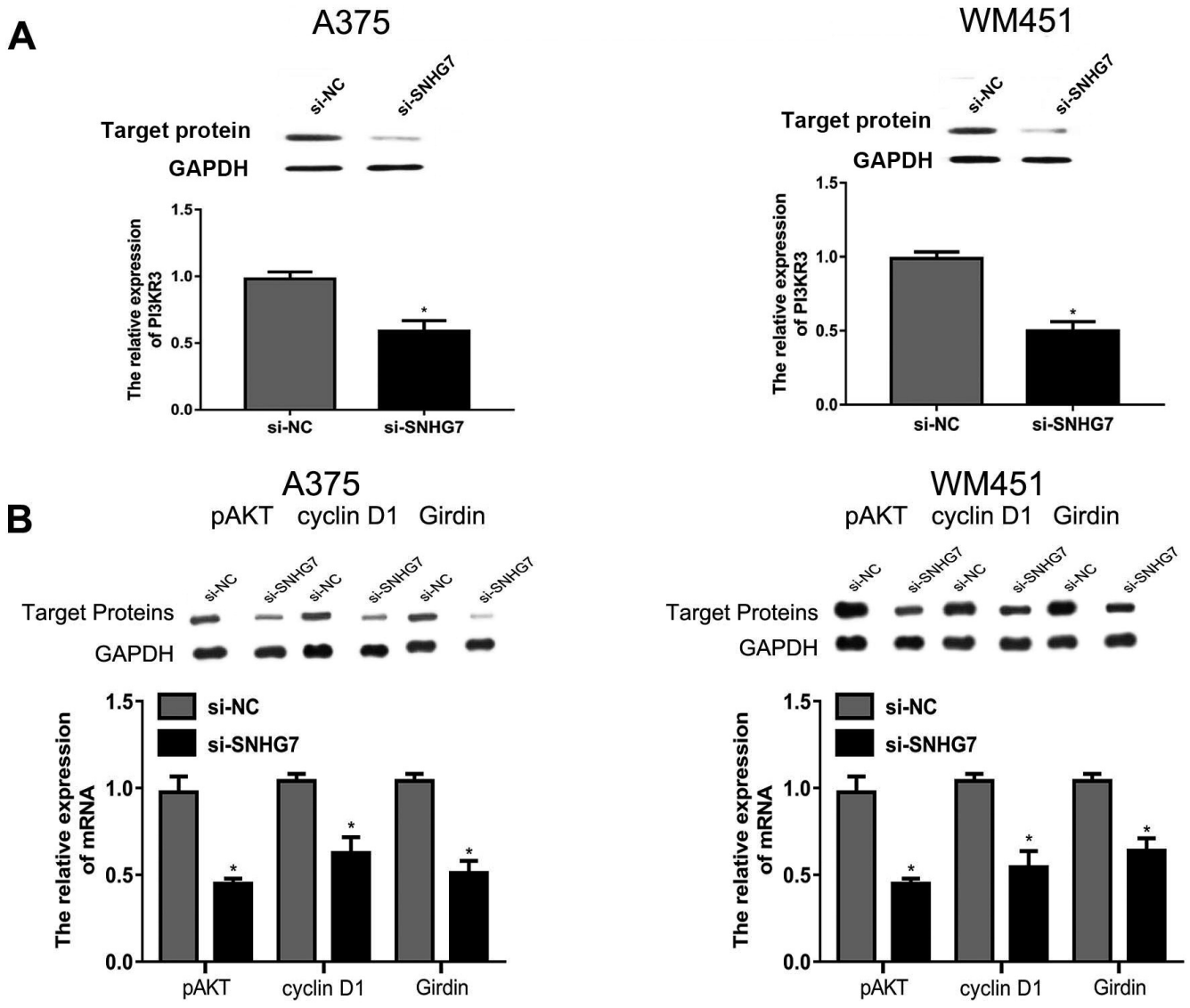

Fig. 5. SNHG7 silencing repressed the PI3K/AKT signaling pathway. **A.** Expressions of PI3KR3 in A375 and WM451 cells 48h after transfection was detected by qRT-PCR and western blot. **B.** Expressions of pAKT, cyclin D1 and Girdin in A375 and WM451 cells 48h after transfection was detected by qRT-PCR and western blot. *P<0.05 compared to control group.

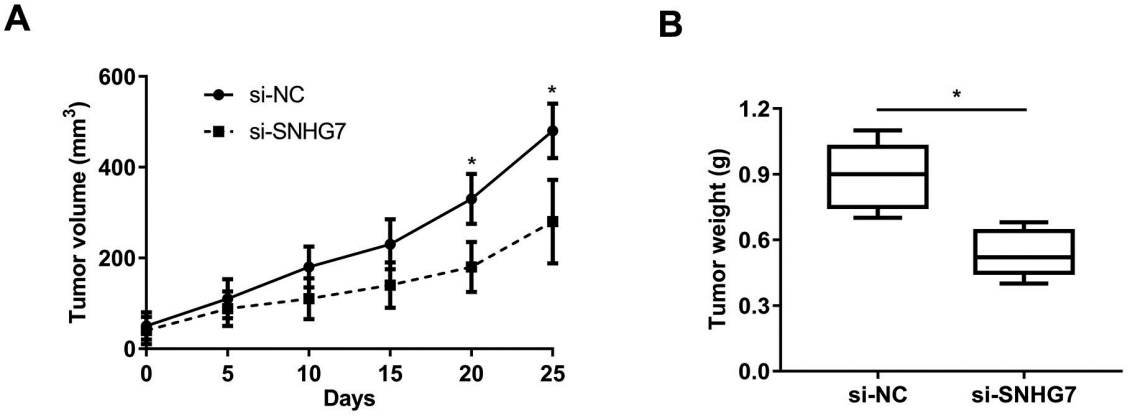

Fig. 6. SNHG7 knockdown decreased xenograft growth in vivo. **A.** The tumor volume of subcutaneous implantation models of colon cancer. **B.** Tumor weight of si-NC group and si-SNHG7 group. Data are presented as the mean ± SD. *P<0.05 compared to control group.

SNHG7 promotes the progression and growth of glioblastoma via inhibition of miR-5095 (Xu et al., 2016). Bioinformatics analysis demonstrated that SNHG7 functions as a molecular sponge for miR-9 in biological behavior of melanoma cells. Meanwhile, miR-9 levels were significantly decreased in MM tissues. Some studies have reported miR-9 might inhibit the progression of malignant melanoma by affecting target proteins (Bu et al., 2017). Our study identified PI3KR3 as a functional target gene of miR-9 with 3' -UTR.

Phosphatidylinositol 3' -kinases (PI3K) is a heterodimer that consists of a p110 catalytic subunit and a p85 regulatory subunit. Although much research has identified the role of catalytic subunits in a wide range of cellular processes associated with cancer development and progression (Samuels et al., 2004), current understanding of the function of PI3K regulatory subunits such as PI3KR3 is not very clear.

Previous studies showed increased PI3KR3 expression in glioblastoma, ovarian cancer and gastric cancer, suggesting an oncogenic role of PI3KR3 in these cancers (Soroceanu et al., 2007; Zhang et al., 2007; Zhou et al., 2012). Likewise, we found PI3KR3 level was much higher in MM tissues and cell lines. Then, we focused on the downstream regulators of PI3K signaling. The results showed decreased pAkt level in MM cells after SNHG7 konckdown. In addition, we observed SNHG7 knockdown suppressed significantly cyclin D1 and Girdin expression after siRNA, implying si-SNHG7 could inhibit MM cell proliferation and migration by repressing the PI3K/AKT signaling pathway. Taken together, these data suggested SNHG7 could promote the progression of malignant melanoma by activating PI3K/AKT signaling pathway.

Conclusion

The present study demonstrated that SNHG7 was an oncogene in malignant melanoma, providing a novel insight for the pathogenesis and new potential therapeutic possibility for malignant melanoma.

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Conflict of interest. The authors declare no conflicts of interest.

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