

Original Article

LncRNA SNHG16 induced by TFAP2A modulates glycolysis and proliferation of endometrial carcinoma through miR-490-3p/HK2 axis

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Received August 4, 2019; Accepted October 26, 2019; Epub November 15, 2019; Published November 30, 2019

Abstract: Increasing evidence indicates the important roles of long noncoding RNA (lncRNA) in the endometrial carcinoma (ECa). Here, we identified the roles of SNHG16 in the ECa proliferation and glycolysis, and revealed the underlying mechanism. Results presented that SNHG16 expression was increased in the ECa tissue and cells, and the ectopic SNHG16 overexpression was closely correlated with the poor survival rate and recurrence free survival of ECa. As regarding the upstream, transcription factor TFAP2A bound with the promoter region of SNHG16 and activated its transcription. In functional experiments, SNHG16 knockdown suppressed the proliferation, glycolysis and tumor growth of ECa cells. In mechanical experiments, SNHG16 upregulated HK2, the target gene of miR-490-3p, by competitively sponging miR-490-3p and then promoted endometrial carcinoma proliferation and glycolysis. In conclusion, this finding illustrates the vital role of SNHG16 via the TFAP2A/SNHG16/miR-490-3p/HK2 axis in the ECa proliferation and glycolysis, providing an interesting insight for the ECa tumorigenesis.

Keywords: Endometrial carcinoma, SNHG16, glycolysis, miR-490-3p, HK2

Introduction

Endometrial carcinoma acts as the third leading cause of cancer-correlated deaths of gynaecological malignant tumor worldwide [1]. In the female reproductive system tumor, endometrial carcinoma could trigger high mortality rate for women [2]. The worse facts are that the incidence of endometrial carcinoma is still increasing. Therefore, this emergency requires the accurate diagnosis and effective treatment.

Long non-coding RNAs (lncRNAs) are a type of non-protein coding transcripts with longer than 200 nt in length [3, 4]. More and more evidences indicate that lncRNAs are involved in multiple physiopathologic functions. Including differentiation, metastasis and glycolysis [5-7]. For example, lncRNA GAS5 is under-expressed in the endometrial carcinoma tissue specimens, especially these samples with type 2 diabetes

mellitus, revealing the critical roles of GAS5/miR-222-3p/p27 axis in the endometrial carcinoma tumorigenesis [8]. Increased MIR22HG expression significantly inhibits the endometrial carcinoma cells' proliferation and induced the G0/G1 phase through regulating miR-141-3p/DAPK1 axis [9]. Overall, the roles of lncRNAs in the endometrial carcinoma are indeed essential.

The glycolysis has been reported to participate in the tumorigenesis of endometrial carcinoma [10, 11]. In this research, we found that SNHG16 was up-regulated in the endometrial carcinoma tissue and cells. SNHG16 targets miR-490-3p/HK2 axis to regulate the proliferation and glycolysis. SNHG16 upregulated the miR-490-3p target gene HK2 by competitively sponging miR-490-3p and then promoted endometrial carcinoma glycolysis and proliferation, acting as an element of the ceRNA network.

Materials and methods

Human tissue samples collection

The endometrial carcinoma tissue samples and non-tumor tissue were collected from patients at the Qilu Hospital of Shandong University. Tissue samples, including tumor tissue and non-tumor tissue, were immediately frozen in liquid nitrogen after surgical resection. Every patient had signed the informed consent before the surgery. This study had been approved by the Ethics Committee of hospital.

Cells and culture

Human endometrial carcinoma cell lines (HEC-1B, HEC-1A, RL95-2, AN3CA) and normal endometrial cell line (EMC) were provided by the China Center for Type Culture Collection (CC-TCC, China). Cells were cultured in RPMI-1640 (HyClone, Logan, UT, USA) medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/mL) in the environment of 5% CO₂ incubator at 37°C.

Transfection

The short hairpin RNA (shRNA) against human SNHG16, accompanied by miRNA mimics and inhibitors, were constructed by RiboBio (Guangzhou, China). The transactions were carried out using Lipofectamine 3000 Reagents (Life Technologies, Carlsbad, CA, USA) at the 70%-80% confluence. The sequences were presented in the [Table S1](#).

RNA isolation and quantitative RT-PCR

RNA was isolated using TRIzol (Life Technologies, Invitrogen, CA, USA) based on the manufacturer's protocol. The isolated RNA was purified by RNeasy mini kit (QIAGEN, Hilden, Germany). The cDNA reverse transcription reaction was synthesized using the SuperScript First-Strand Synthesis system (Invitrogen, US) using random primers. Quantitative RT-PCR was carried out using SYBR Green PCR Master Mix (Life Technologies) on 7500 Fast Real-time PCR System (Applied Biosystems). See [Table S1](#) for the quantitative PCR primers.

Western blot

Tissue and cellular proteins were extracted using cell lysis RIPA buffer (Beyotime Institute

of Biotechnology) on ice for 30 min as previously described [12]. The cells lysate by RIPA lysis buffer was quantitatively analyzed by the bicinchoninic acid (BCA) kit (Thermo Scientific, Waltham, MA, USA). Protein constituents were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Roche). Antibodies (Abcam, anti-HK2, 1:1000) and mouse anti-GAPDH were incubated with the membranes overnight at 4°C. The reactivity was detected with an enhanced chemiluminescence system using Odyssey infrared imaging system (LI-COR, Inc., Lincoln, NE, USA). Quantitative analysis of the band intensity was carried out using the ImageJ software.

Colony formation assay

Colony formation assay was performed for the cell proliferation evaluation. In brief, HEC-1A and AN3CA cells were transfected with oligonucleotides and then seeded in the 96-well plates. 10 µL CCK-8 reagent (Dojindo, Japan) was treated with the cells at 24, 48, 72, and 96 hours for 1 hour. The absorbance of each group was measured at the wavelength of 450 nm under microplate reader.

Glucose consumption and lactate production

The glucose consumption was measured using a glucose detection mix detected at 460 nm wavelength (Biosino Bio-Technology). The lactate production was measured using lactate assay kit (Sigma, St-Louis, MO, USA) as described by the manufacturer protocol [13].

Subcellular fractionation assays

The subcellular fractionation assay was performed as previously recorded using a PARIS kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Dual-luciferase reporter assay

The SNHG16 promoter sequences and 3'-UTR were amplified from genomic DNA using primer and sub-cloned into the pGL3-Basic vectors (Promega) for the luciferase reporter vectors. For the mutation type of binding sites were constructed by re-placed the binding sites. Luciferase activity was measured normalized by firefly/Renilla ratio as previously described [14].

Chromatin immunoprecipitation (ChIP)

ChIP-PCR was performed using Chroma Flash One-Step Magnetic ChIP Kit (#P-2026-48, Epigentek, NY, USA) according to the manufacturer's instructions with minor modifications as previously described [14]. Briefly, the protein-DNA complexes were de-crosslinked to be DNA fragments with average length of 200-1000 bp. The fragments were incubated with ChIP-grade anti-TFAP2A and control nonimmune IgG antibody. Finally, the precipitated DNA was Quantified using PCR by EpiQuik™ Quantitative PCR Kit (#P-1029, Epigentek).

Tumor xenograft implantation

Male nude BALB/c mice (five week old) were provided by the Slac Laboratory Animal Center (Shanghai, China). For the injection, the stably transfected HEC-1A cells were constructed by lentiviral transfection and then implanted into the flank of mice (1×10^6 cells in 100 μ l). All these processes were performed in accordance with the protocol approved by the Administrative Panel on Laboratory Animal Care. Tumors volume was measured every three days according to the formula: volume (mm^3) = length \times width²/2.

Statistical analysis

All the data were calculated by SPSS 22.0 statistical software and presented as means \pm SD. The differences were analyzed using Student's t-test or one-way ANOVA. Overall survival and relapse-free survival (RFS) were calculated using the Kaplan-Meier analysis. The interaction within two index was calculated by Pearson's correlation analysis. $P < 0.05$ and $P < 0.01$ were considered as statistical significance.

Results

LncRNA SNHG16 indicated the poor clinical prognosis of endometrial carcinoma individuals

The abundance of lncRNA SNHG16 in the endometrial carcinoma (ECa) tissue samples were measured using the RT-PCR, illustrating the ectopic overexpression of SNHG16 (**Figure 1A**). In the GEPIA database, the SNHG16 expression was identified to be up-regulated or increased

as compared to the controls (**Figure 1B**). The overall survival rate of endometrial carcinoma patients was also estimate by the Kaplan-Meier plotter (TCGA data, <http://kmplot.com/analysis/>), suggesting the lower survival rate of endometrial carcinoma patients who were marked with higher SNHG16 level (**Figure 1C**). Recurrence free survival (RFS) was computed using the Kaplan-Meier plotter, suggesting the lower survival level of endometrial carcinoma patients with higher SNHG16 level (**Figure 1D**). Thus, the overexpression of lncRNA SNHG16 indicated the poor clinical prognosis of endometrial carcinoma individuals.

Transcription factor TFAP2A promoted the transcriptional level of lncRNA SNHG16

In the clinical endometrial carcinoma individuals' samples, transcription factor TFAP2A was found to be up-regulated (**Figure 2A**). The transcriptional targeting motifs of TFAP2A were predicted using the JASPAR database (<http://jaspar.genereg.net/>) (**Figure 2B**). There were two potential binding region of TFAP2A in the SNHG16 promoter. The chromatin immunoprecipitation (ChIP) followed by PCR showed that the TFAP2A antibody could specifically bind with the site 1 sequences and precipitate it (**Figure 2C**). The wild type sequences of site 1 and corresponding mutant sequences were constructed for the luciferase reporter assay (**Figure 2D**). Luciferase reporter assay showed that the wild type of promoter region site 1 sequences could bind with the TFAP2A (**Figure 2E**). The TFAP2A overexpression vectors using pcDNA3.1 plasmids was constructed, suggesting the increased level of SNHG16 by the TFAP2A overexpression (**Figure 2F**). These data showed that transcription factor TFAP2A promoted the transcriptional level of lncRNA SNHG16.

Knockdown of SNHG16 repressed the proliferation and glycolysis of endometrial carcinoma cells

The level of SNHG16 was found to be up-regulated in the endometrial carcinoma cells (HEC-1B, HEC-1A, RL95-2, AN3CA) using the RT-PCR (**Figure 3A**). The silencing short hairpin RNAs (shRNAs) were transfected into the HEC-1A and AN3CA cells to silence the SNHG16 level (**Figure 3B**). Colony formation assay illustrated that the

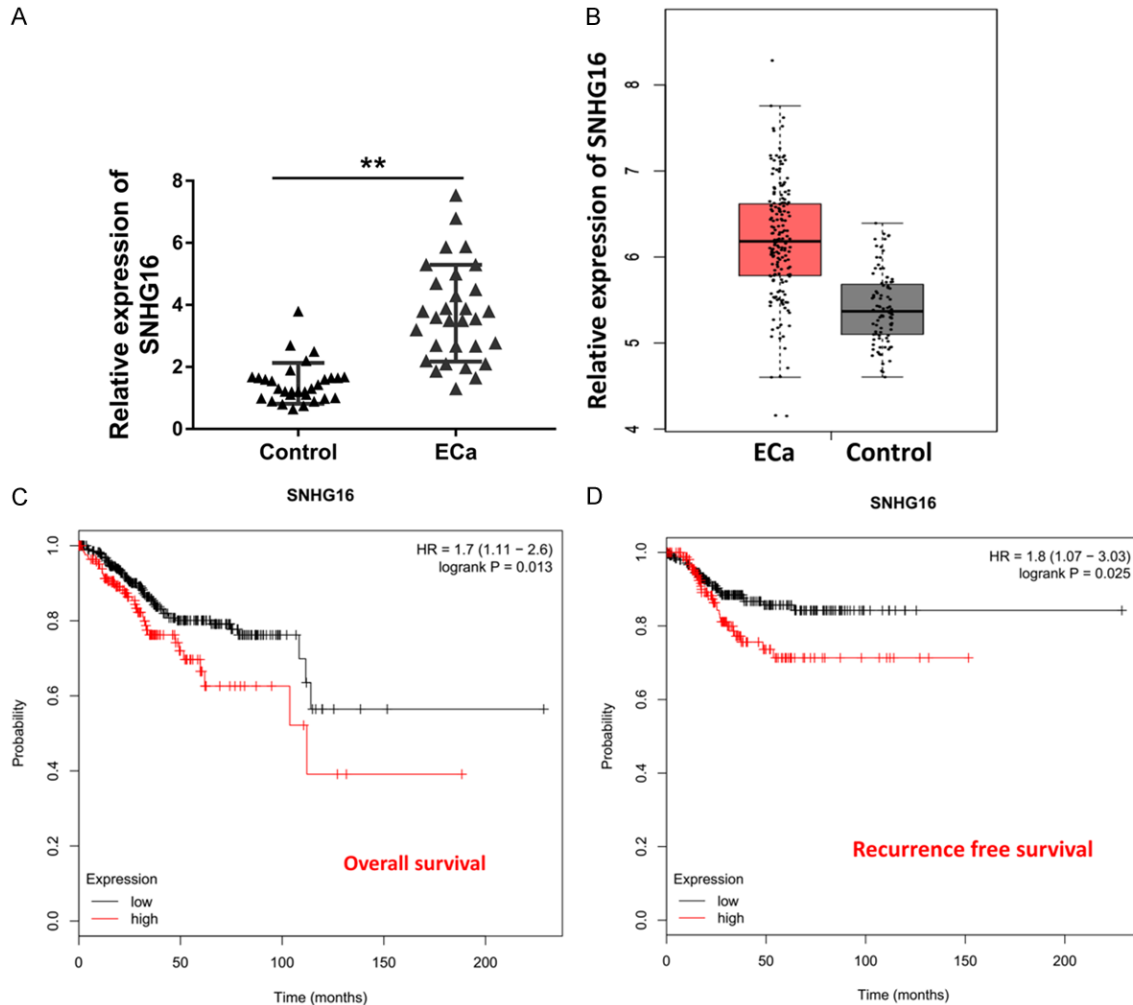


Figure 1. LncRNA SNHG16 indicated the poor clinical prognosis of endometrial carcinoma individuals. A. The abundance of lncRNA SNHG16 in the endometrial carcinoma (ECa) tissue samples were measured using the RT-PCR. B. The SNHG16 expression in the GEPIA database. C. The overall survival rate of endometrial carcinoma patients was also estimate by the Kaplan-Meier plotter (TCGA data <http://kmplot.com/analysis/>). D. Recurrence free survival (RFS) was computed using the Kaplan-Meier plotter. **Shows the significant difference P < 0.01.

SNHG16 silencing suppressed the proliferation of endometrial carcinoma cells (Figure 3C). The SNHG16 silencing could also suppress the glucose uptake, lactate production and ATP level compared to the blank control (Figure 3D-F). In vivo xenotransplantation assay in mice showed that the SNHG16 silencing inhibited the tumor growth of HEC-1A injection (Figure 3G). Overall, knockdown of SNHG16 repressed the proliferation and glycolysis of endometrial carcinoma cells.

SNHG16 targeted the miR-490-3p/HK2 axis

The subcellular location of SNHG16 was found to be enriched in the cytoplasm, suggesting the

potential post-transcriptional regulation (Figure 4A). The bioinformatics tools (StarBase, <http://starbase.sysu.edu.cn/>) indicated the potential binding sites within SNHG16 and miR-490-3p in the post-transcriptional regulation (Figure 4B). Therefore, we constructed the luciferase reporter assay to identify the binding within them, and results suggested that miR-490-3p could target the SNHG16 3'-UTR sequences with complementary binding (Figure 4C). In the endometrial carcinoma cells (HEC-1A, AN3CA), we found that the SNHG16 knockdown transfection by the sh-SNHG16 could up-regulate the miR-490-3p level (Figure 4D). The StarBase bioinformatics tool inspired the binding within

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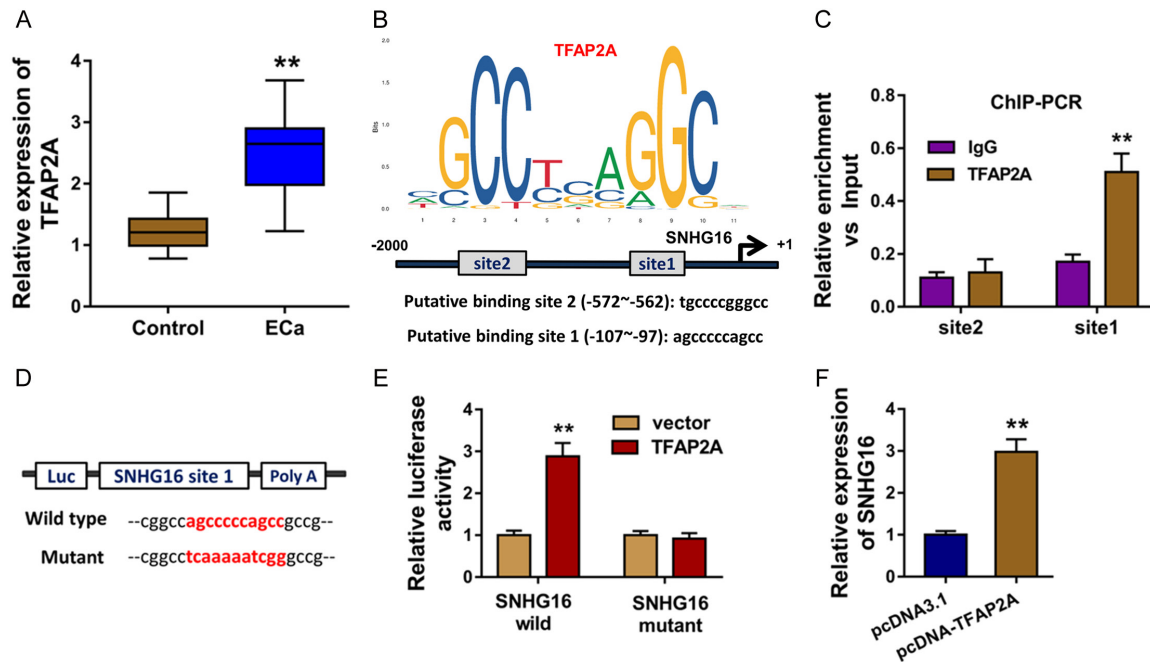


Figure 2. Transcription factor TFAP2A promoted the transcriptional level of lncRNA SNHG16. A. RT-PCR showed the up-regulated level of TFAP2A in the clinical endometrial carcinoma individuals' samples. B. Schematic diagram presented the transcriptional targeting motifs of TFAP2A predicted by JASPAR database (<http://jaspar.genereg.net/>). C. Chromatin immunoprecipitation (ChIP) followed by PCR showed the abundance of precipitated DNA fragment by TFAP2A antibody. D. The wild type sequences of site 1 and corresponding mutant sequences were constructed for the luciferase reporter assay. E. Luciferase reporter assay showed the binding efficiency of wild type of promoter region site 1 sequences with the TFAP2A. F. RT-PCR showed the level of SNHG16 induced by TFAP2A overexpression vectors using pcDNA3.1 plasmids. **Shows the significant difference $P < 0.01$.

miR-490-3p and HK2 mRNA 3'-UTR (**Figure 4E**). It has been reported that HK2 functions as the critical regulator in the cancer glycolysis [15]. Luciferase reporter assay using the wild type and mutant vectors to confirm that the miR-490-3p mimics could effectively integrate with the HK2 mRNA wild vector (**Figure 4F**). Western blot clued that the transfection by the sh-SNHG16 could decrease the HK2 protein level, while the co-transfection of sh-SNHG16 and miR-490-3p inhibitor (miR-490-3p inhib) could recover the HK2 protein level (**Figure 4G**). The GEPIA database inspired that the remarkably overexpression of HK2 in the endometrial carcinoma tissues (**Figure 4H**). In the enrolled endometrial carcinoma samples, Pearson's correlation analysis indicated that miR-490-3p was negative correlated with SNHG16 (**Figure 4I**). In conclusion, SNHG16 targeted the miR-490-3p/HK2 axis in the endometrial carcinoma.

Discussion

The roles of long noncoding RNA (lncRNA) have been regarded as the important element in the

human cancer tumorigenesis [16]. More and more evidence support that the lncRNA participate in the endometrial carcinoma oncogenesis, involving the proliferation, migration, metastasis and glycolysis [17-19].

In this research, we found that the lncRNA SNHG16 was observably up-regulated in the endometrial carcinoma tissue and cells. Besides, the up-regulated expression of SNHG16 in the endometrial carcinoma samples indicated the poor clinical prognosis, hinting the oncogenic element of SNHG16. Moreover, the transcription factor TFAP2A could trigger the transcriptional level of SNHG16 in the endometrial carcinoma. In other type of human cancer, SNHG16 could regulate the tumorigenesis. For example, SNHG16 was induced by the upstream transcription factor 1 (USF1) in glioma tissues and cells, demonstrating the USF1/SNHG16/miR-212-3p/ALDH1A1 pathway [12]. In osteosarcoma tissues and cell lines, ectopic overexpression of SNHG16 indicated poor prognosis and lower overall survival rate of osteosarcoma patients and the knockdown of SNHG16

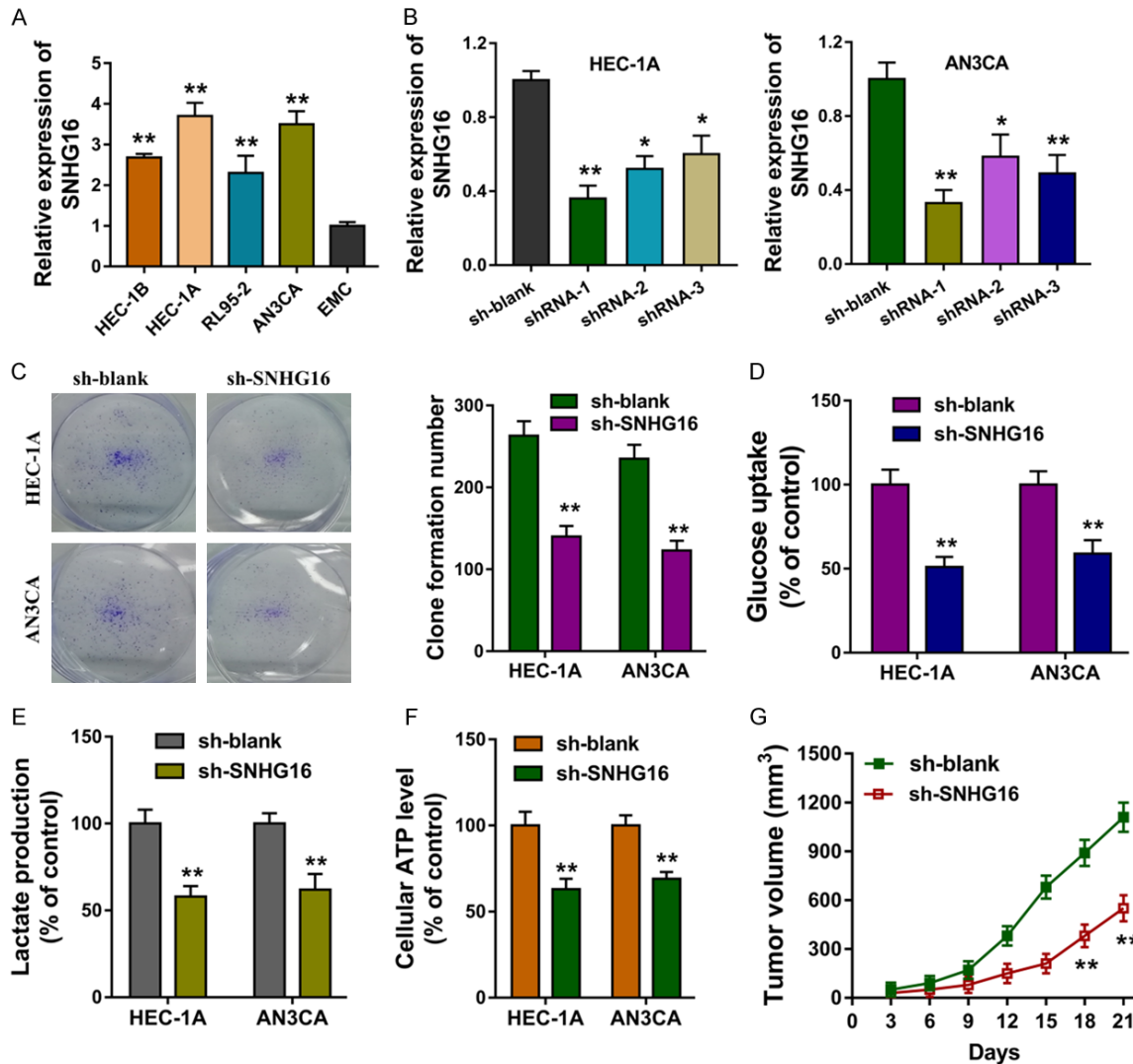


Figure 3. Knockdown of SNHG16 repressed the proliferation and glycolysis of endometrial carcinoma cells. A. RT-PCR showed the level of SNHG16 in the endometrial carcinoma cells (HEC-1B, HEC-1A, RL95-2, AN3CA). B. The silencing short hairpin RNAs (shRNAs) were transfected into the HEC-1A and AN3CA cells to silence the SNHG16 level. C. Colony formation assay illustrated the proliferation of endometrial carcinoma cells. D-F. The glucose uptake, lactate production and ATP level was measured in SNHG16 silencing and blank control. G. In vivo xenotransplantation assay presented the tumor growth of HEC-1A cells. **Shows the significant difference $P < 0.01$.

inhibited the cellular proliferation, migration, invasion, cell cycle and promoted apoptosis in osteosarcoma [20].

The glycolysis is regarded as the main way of energy suppletion in the tumor. Moreover, the enhancement of glycolysis is closely correlated with the tumour malignant degree. The activity of the key glycolytic enzyme is the vital initiating factor for the tumor progression. The increasing of tumor cellular glycolysis is caused by the enhanced glycolytic enzyme activity. HK2 is a key glycolytic enzyme in the multiple human

cancers, including breast cancer, non-small cell lung cancer cells and multiple myeloma [21, 22]. In this research, we found that HK2 acted as the target of SNHG16, through the miR-490-3p and competing endogenous RNA (ceRNA) regulation.

In the functional assay, we found that the knockdown of SNHG16 could decrease the glucose uptake, lactate production and ATP level in the endometrial carcinoma cells. This finding inspire us that the SNHG16 might regulate the metabolic level of endometrial carcinoma cells.

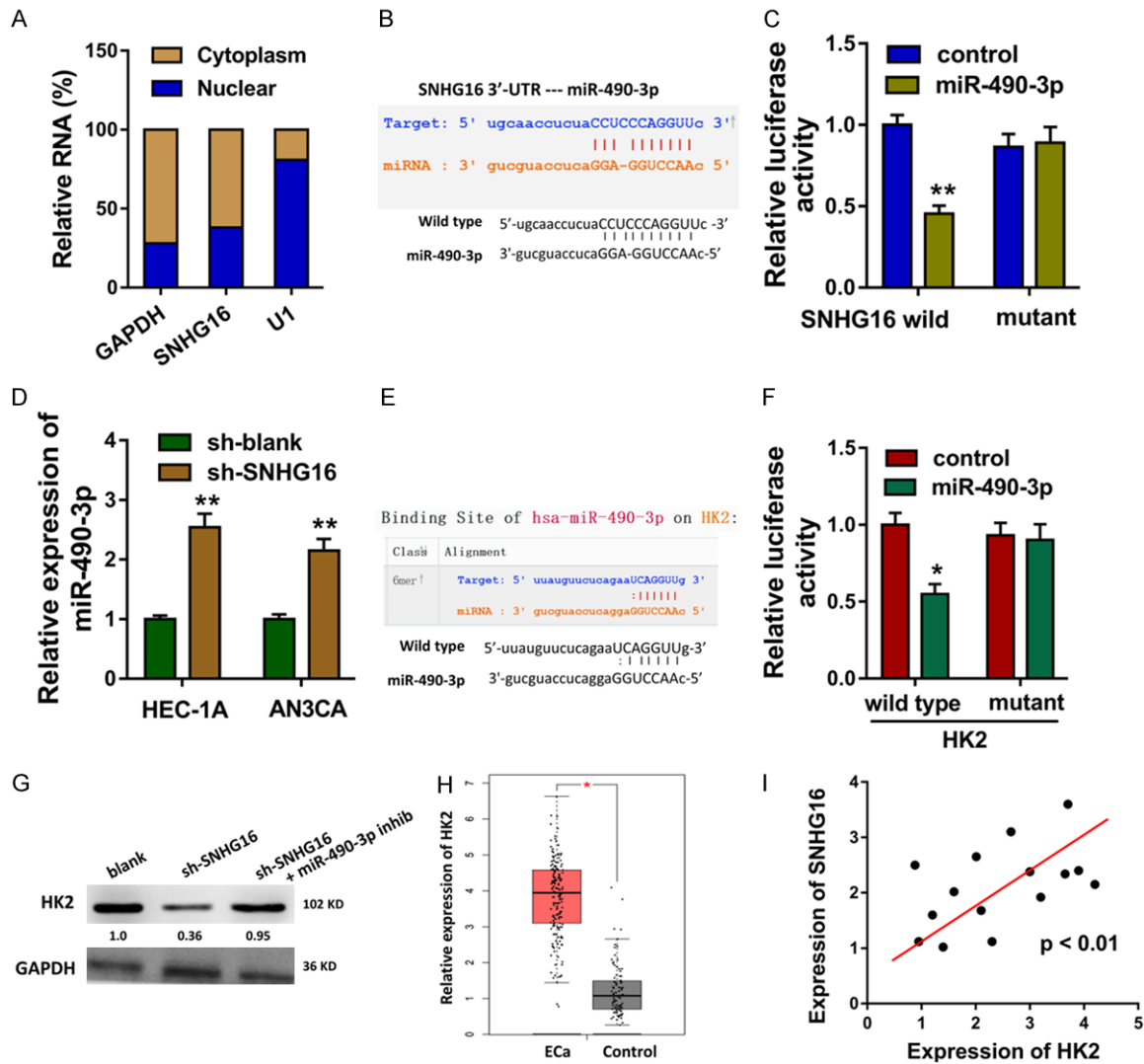


Figure 4. SNHG16 targeted the miR-490-3p/HK2 axis. A. The subcellular location analysis showed that SNHG16 was enriched in the cytoplasm. B. The bioinformatics tool (StarBase, <http://starbase.sysu.edu.cn/>) indicated the potential binding sites within SNHG16 and miR-490-3p. C. Luciferase reporter vectors were constructed to identify the binding within miR-490-3p and SNHG16. D. RT-PCR found that miR-490-3p level was up-regulated after SNHG16 knockdown transfection. E. The StarBase bioinformatics tool inspired the binding within miR-490-3p and HK2 mRNA 3'-UTR. F. Luciferase reporter assay using the wild type and mutant vectors was performed to identify the interaction within miR-490-3p and HK2 mRNA. G. Western blot clued the HK2 protein level in HEC-1A cell transfected by the sh-SNHG16 or miR-490-3p inhibitor (miR-490-3p inhib). H. The GEPIA database inspired the overexpression of HK2 in the endometrial carcinoma tissues. I. Pearson's correlation analysis indicated that miR-490-3p was negative correlated with SNHG16. **Shows the significant difference P < 0.01.

Subsequent mechanical exploration suggests that SNHG16 sponged the miR-490-3p, thereby promoted the HK2 expression. This results concluded the TFAP2A/SNHG16/miR-490-3p/HK2 axis in the endometrial carcinoma cells.

Compared with normal cells, tumor cells still utilizes the glycolysis as their primary means of energy productivity, even there is adequate oxygen [23]. This physiological phenomenon is

named as the 'Warburg effect' [24]. In the endometrial carcinoma, we found that SNHG16 could trigger the aerobic glycolysis via the ceRNA regulation.

In conclusion, this research discovered the potential regulation of SNHG16 in the endometrial carcinoma oncogenesis. SNHG16, induced by the transcription factor TFAP2A, could target the miR-490-3p/HK2 axis to regulate the prolifer-

eration and glycolysis of endometrial carcinoma cells. This finding might bring an interesting insight for the endometrial carcinoma.

Acknowledgements

This study was supported by Youth foundation of Shandong Academy of Medical Sciences (2015-73, 2015-74).

Disclosure of conflict of interest

None.

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Table S1. Primers sequences for qRT-PCR and sequences of shRNA

| | Sequences 5'-3' |
|----------------------|--|
| SNHG16 | forward, 5'-TCAGGGTAAGTTAACAAGGTCT-3' reverse, 5'-GATCGACAATCGAACCCCGTTA-3' |
| miR-490-3p | forward, 5'-GAGCACAATTCTGTTTGGTTGC-3' reverse, 5'-CTCCTTCTTCGCTTTTTTGTAT-3' |
| TFAP2A | forward, 5'-AGGTCAATCTCCCTACACGAG-3' reverse, 5'-GGAGTAAGGATCTTGCGACTGG-3' |
| GAPDH | forward, 5'-CTAAGGCCAACCGTGAAAAG-3' reverse, 5'-ACCAGAGGCATACAGGGACA-3' |
| sh-SNHG16-1 | 5'-GCCTAACCGTTTATACTAC-3' |
| sh-SNHG16-2 | 5'-GCTGATCACCTACTCTAAT-3' |
| sh-SNHG16-3 | 5'-GGAACCTAGGTTACTACCC-3' |
| miR-490-3p inhibitor | 5'-ACGCAAGGAUCACGAUGUUAC-3' |