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ORIGINAL ARTICLE

Long-Non Coding RNA SNHG16 Supports Colon Cancer Cell Growth by Modulating miR-302a-3p/AKT Axis

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Abstract

Small nucleolar RNA host gene 16 (SNHG16) is reported to be involved in the tumorigenesis of various kinds of tumors. SNHG16 expression was reported to be upregulated in colon cancer, however, the underlying mechanism of how SNHG16 affects the colon cancer development remains poorly elucidated. In our study, with the aim to identify the role of SNHG16 on colon cell proliferation, SNHG16 was overexpressed or knocked down in vitro, respectively. SNHG16 overexpression accelerated colon cancer cell growth, while cell growth ability was impaired in SNHG16 silencing cells. Furthermore, the starBase database predicted that miR-302a-3p was the target gene of SNHG16, which was supported by dual luciferase assay. The effect of promoting cell proliferation ability induced by SNHG16 overexpression could be partly reversed by co-transfection of miR-302a-3p mimic. Application of the miRanda database indicated that AKT may be modulated by SNHG16, further evidenced by western blot and quantitative PCR assays. AKT overexpression could partly reverse the attenuated colon cancer cell growth caused by miR-302a-3p mimic transfection. Meanwhile, the combination of miR-302a-3p inhibitor and shAKT achieved the parallel result. In conclusion, our study revealed the SNHG16/miR-302a-3p/AKT axis might play a crucial role in colon cancer cell proliferation, thus participating in the process of colon cancer development.

Keywords SNHG16 · miR-302a-3p · AKT · Colon cancer · Cell growth

Introduction

Colon cancer is among the most common malignancies worldwide, ranking the third leading cause of cancer-related deaths [1]. The occurrence of colon cancer is a complex and long-term process, in which genetic factors, chronic inflammation stimuli and immune dysfunction are involved [2, 3]. High incidence and mortality make colon cancer arouse high attention. At present, clinical treatments available for patients diagnosed with colon cancer mainly conclude surgery, radiotherapy and chemotherapy [4]. Early diagnosis and appropriate treatment can effectively reduce the mortality rate of colon cancer, however, the incidence is still high and the five-year survival rate is far from satisfactory. Therefore, in-depth study of the specific mechanism to promote the development of colon cancer, looking for specific target genes for the proliferation process of colon cancer cells, provide useful evidence for the development of new immunotherapy targets, in order to effectively extend the survival rate of patients.

Long non-coding RNAs (lncRNAs) are a set of transcripts including more than 200 nucleotides in length [5]. The absence of open reading frame makes lncRNAs functionally have no potential to encode proteins, but they could modulate gene expression at pre-transcriptional, post-transcriptional and epigenetic levels [6, 7]. Emerging evidence has uncovered that multiple lncRNAs are closely related to various biological behaviors of tumors such as proliferation, apoptosis and metabolism, as well as migration and metastasis [8–10]. For instance, NKILA, which is associated with the poor prognosis of breast cancer patients, could serve as a class of NF-κB modulators to suppress breast cancer metastasis [11]. MITA1, upregulated in hepatocellular carcinoma, contributed

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to metastasis through promoting epithelial-mesenchymal transition [12].

Small nucleolar RNA host gene 16 (SNHG16) was first identified as an oncogene in neuroblastoma [13]. Recent studies have confirmed that SNHG16 are related to the occurrence and development of various tumors [14–16]. Zhao, Wenke et al. found that SNHG16 affected the tumor biological behaviors of human hemangioma endothelial cell, such as proliferation, migration, and invasion [17]. By competitively binding miR-98 with E2F5, SNHG16 facilitated the migration capacity of breast cancer cell [18]. Compared to paracancerous normal tissue, SNHG16 expression levels were evidently downregulated in HCC tissues. Further investigation showed that during the development of hepatocellular carcinoma, SNHG16 exerted a crucial effect on HCC via functionally sponging hsa-miR-93 [19]. In addition, several reports have examined the role of SNHG16 in colon cancer [20]. The researchers found that SNHG16 was significantly up-regulated in colorectal cancer and SNHG16 might participate in the tumorigenesis of colon cancer by affecting the lipid metabolism [21]. However, there is still much work need to be done to elucidate the specific mechanisms of how SNHG16 take part in the progression of colon cancer.

To detect the role of SNHG16 in colon cancer progression, firstly, we utilized two methods of interference and overexpression to demonstrate the effect of SNHG16 on the proliferation of colon cancer cell HCT116. SNHG16 overexpression promoted the proliferation of HCT116, while which was impaired in SNHG16 silencing cells. To identify the mechanism of how SNHG16 affect HCT116 cell growth ability, we searched the targeted genes of SNHG16 via the starBase database. Dual luciferase assay confirmed that miR-302a-3p could be sponged by SNHG16. The effect of promoting cell proliferation ability induced by SNHG16 overexpression could be partly reversed by co-transfection of miR-302a-3p mimic, which was dependent on AKT. At last, the pro-proliferative effect of SNHG16 was further verified in a *KRAS*-wild type colon cancer cell line CaCO-2.

Material and Methods

Reagents

The antibody AKT (11E7), GAPDH (D16H11) and HRP-conjugated polyclonal goat anti-rabbit (#7074) were from Cell Signaling (Danvers, MA, USA). The antibody PE anti-AKT (Ser473, M89–61), PE mouse IgG1 (Isotype, MOPC-21) Fixation buffer (554655) and Perm buffer III (558050) were bought from BD Biosciences (San Diego, CA, USA). Cell Counting Kit-8 (CCK8) was purchased from Transgen Biotech (Beijing, China).

Cells and Cell Culture

The human colon cancer cell lines HCT116 and CaCO-2 were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in 1640 culture medium containing 10% fetal bovine serum (FBS) at 37 °C with 5% CO2. To up-regulate or knockdown SNHG16 in vitro, HCT116 or CaCO-2 cells were transfected with oe-Ctrl or oe-SNHG16 and Sh Scr or Sh SNHG16. To up-regulate or knockdown miR-302a-3p in vitro, HCT116 or CaCO-2 cells were transfected with Ctrl inhi or miR-302a mimic and sh Scr or sh SNHG16. To up-regulate or knockdown AKT in vitro, HCT116 or CaCO-2 cells were transfected with oe Ctrl or oe AKT and sh Scr or sh AKT.

Cell Proliferation Assay

Different indicated groups of HCT116 or CaCO-2 cells (2×10^5) were cultured for 48 h, and 20 μl of CCK8 was added to each well and cells were incubated for an additional 4 h. The absorbance of each well was read at 450 nm. Cell proliferation was calculated by dividing the ODs of the treated cells with the ODs of the control cells.

Quantitative Real-Time PCR

Total RNA was extracted with the TRIzol reagent and cDNA was synthesized using a PrimeScript RT reagent kit (Takara Bio, Inc. Otsu, Shiga, Japan). The following PCR conditions were used: 1 cycle at 95 °C for 30 s and then 40 cycles of 5 s at 95 °C and 34 s at 60 °C. Real-time PCR was performed on an Applied Biosystems 7500 real-time PCR system (Foster City, CA, USA). The results were normalized against β-actin RNA. The sequences of PCR primers used were as follows: sense, 5'-CACCATTGGCAATGAGCGGTTC-3′ and anti-sense, 5'-AGGTCTTTGCGGATGTCCACGT-3′ for *β-actin*; sense, 5′-TGGACTACCTGCACTCGGAGAA-3′ and anti-sense, 5′-GTGCCGCAAAAGGTCTTCATGG-3′ for *Akt*. sense, 5′-CAGAATGCCATGGTTTCCCC-3′ and anti-sense, 5′-TGGCAAGAGACTTCCTGAGG-3′ for *Snhg16*.

Western Blot Analysis

A total of 30 μg crude proteins extracted from cell lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked with 5% BSA in Tris-buffered saline plus

0.05% Tween-20 and then incubated with corresponding primary antibodies at 4 °C overnight. After washing with Tris-buffered saline plus 0.05% Tween-20, the membranes were incubated with corresponding HRP-conjugated secondary antibodies. Proteins were visualized using SuperSignal West Femto Maximum (Thermo, IL, USA).

Luciferase Assay

The luciferase reporter constructs including SNHG16 WT and SNHG16 MUT, together with the pRL-TK Vector (Promega, Madison, WI), were co-transfected into cells with ctrl mimic or miR-302a-3p mimic by lipofectamine 2000 (Life Technologies, Grand Island, NY). 48 h after transfection, firefly and renilla luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's protocol. Firefly luciferase activity was normalized to renilla luciferase activity.

Flow Cytometry

To detect p-AKT (S473) in the indicated HCT116 cells, cells were collected and washed twice with PBS and stained with fluorescently-labeled Abs against p-AKT according to the manufacturer's instructions from BD Biosciences. Flow cytometry was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Statistical Analysis

Data are presented as the mean ± SD. Comparisons between two groups were performed using two-tailed Student's *t* test, and paired Student's *t* test was used to analyze paired data. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

SHNG16 Promoted the Growth of Colon Cancer Cell

In order to explore the biological function of SHNG16 in colon cancer cell proliferation, SHNG16 plasmid was used to increase the level of SHNG16 in HCT116 cells while the specific siRNA for SHNG16 was used to decrease SHNG16 expression in HCT116 cells. Satisfactory transfection efficiency was obtained at 48 h post-transfection (Fig. 1a, b). Then, we used the CCK8 assay and cell counting to ascertain the role of SHNG16 in HCT116 cells growth. As showed in Fig. 1c, compared with oe-Ctrl HCT116 cells, oe-SHNG16 HCT116 cells dramatically promoted the proliferation of HCT116 cells. At the same time, down-regulation of SHNG16 suppressed HCT116 cells growth (Fig. 1c). Besides, the result of cell counting was consistent with that achieved in CCK8 assay (Fig. 1d). Taken together, these results revealed that SHNG16 engaged in the development of colon cancer cell tumorigenesis.

SNHG16 Affected the Proliferation of HCT116 Cells Via miR-302a-3p

LncRNAs including SNHG16 has been identified as ceRNAs for certain miRNAs [13, 18]. To study whether SNHG16 exerting its function by acting as a ceRNA in colon cancer cells, we utilized StarBase database to predict which miRNAs might participate in binding SNHG16. Surprisingly, results from StarBase analysis disclosed that miR-302a-3p could form complementary base pairing with SNHG16 (Fig. 2a). To further confirm the interaction between SNHG16 and miR-302a-3p, dual- luciferase reporter assay was performed. As displayed in Fig. 2b, we found that only the luciferase activity of the wild-type SNHG16 could be largely reduced by miR-302a-3p mimics but not that of mutant report vector. Next, we also detected whether SNHG16 influenced colon cancer cell growth through miR-302a-3p. Ctrl mimic or miR-302a-3p mimic were transfected into oe Ctrl HCT116 cells or oe SNHG16 HCT116 cells. CCK8 assay indicated that over-expression of miR-302a-3p mimic mediated inhibiting proliferation of HCT116 cells could be completely reversed by oe SNHG16 (Fig. 2c). In addition, knock down of miR-302a-3p mediated promoting HCT116 cells growth could be also completely abolished by sh SNHG16 (Fig. 2d). Therefore, we could conclude that miR-302a-3p was a target of SNHG16 and SNHG16 influenced the proliferation of HCT116 cells via targeting miR-302a-3p.

SNHG16 Regulated AKT by Targeting miR-302a-3p in Colon Cancer Cells

It has been reported that AKT was relatively related with tumor progression [22–24]. To investigate how miR-302a-3p exerting its role in the development of colon cancer cell, miRanda database analysis was used and we found that AKT might be a target of miR-302a-3p (Fig. 3a). Besides, we analyzed the mRNA and protein level of AKT when miR-302a-3p was up-regulation or down-regulation in HCT116 cells. We observed that both AKT mRNA and protein level were decreased when HCT116 cells were transfected with miR-302a-3p mimic (Fig. 3b), while AKT expression was increased in HCT116 cells with miR-302a-3p inhi (Fig. 3c), indicating that AKT was negatively relative with miR-302a-3p in HCT116 cells. Because total AKT

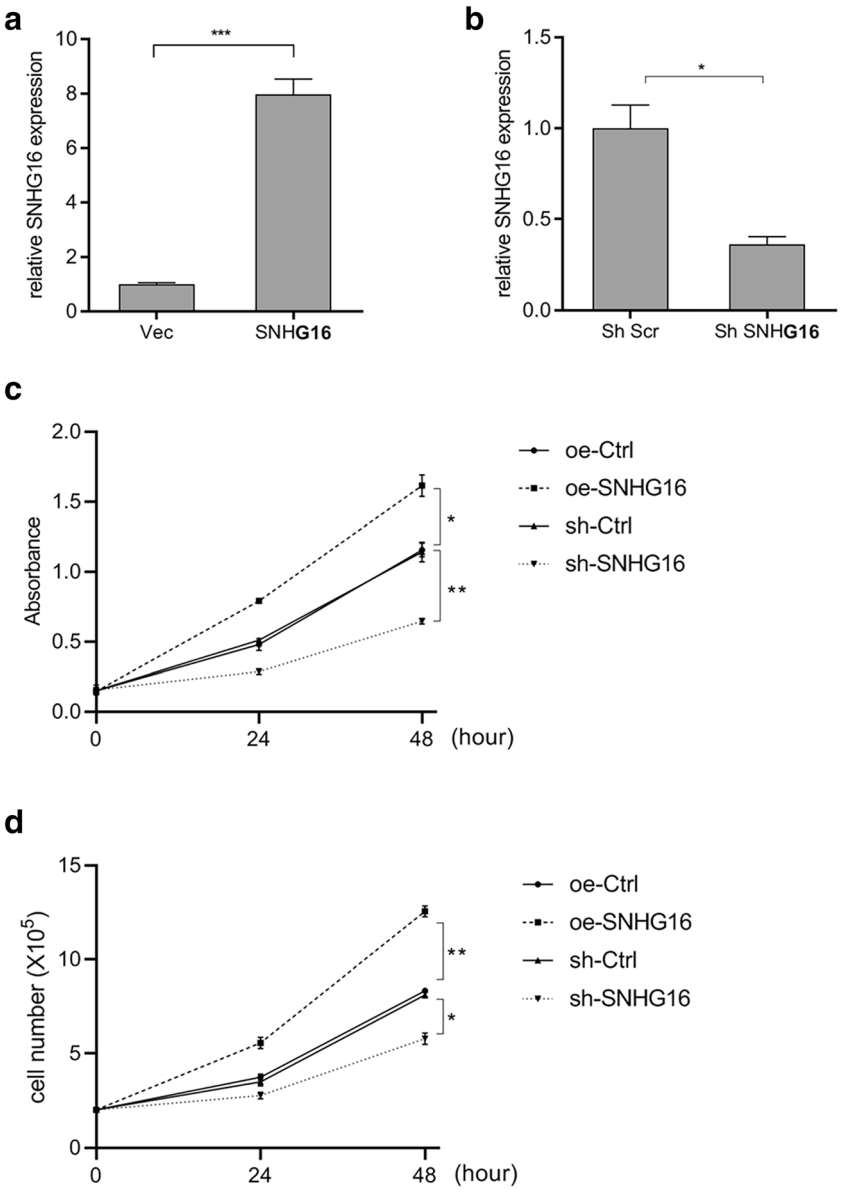

Fig. 1 SNHG16 influenced the proliferation of HCT116 cells. **a** HCT116 cells were transfected with oe-Ctrl or oe-SNHG16 and transfection efficiency was examined by q-PCR. **b** HCT116 cells were transfected with Sh Scr or Sh-SNHG16 and transfection efficiency was examined by q-PCR. **c**, **d** CCK8 (**c**) and cell counting assays (**d**) were conducted to investigate the proliferative ability of oe-Ctrl HCT116 cells or oe-SNHG16 HCT 116 cells and Sh Scr or Sh-SNHG16 HCT116 cells. Error bars represented the mean ± SD of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

protein expression was decreased after miR-302a-3p of HCT116 cells was over-expression, we adopted flow cytometry analysis to identify the phosphorylation of AKT (S473) in oe Ctrl and oe SNHG16 HCT116 cells. As was shown in Fig. 3d, SNHG16 did not influence the the phosphorylation of AKT (S473). But the q-PCR assay presented that the AKT mRNA level was up-regulation in oe SNHG16 HCT116 cells while it was down-regulation in sh SNHG16 HCT116 cells (Fig. 3e), which demonstrated that AKT was positively relative with SNHG16 in colon cancer cells. Collectively, the above results illustrated that SNHG16 negatively regulated miR-302a-3p by negatively targeting AKT, resulting SNHG16 positively regulated AKT in colon cancer cells.

MiR-302a-3p Suppressed the Growth of Colon Cancer Cells Via AKT Signaling

In order to elucidate the actual effects of miR-302a-3p/STAT3 axis on HCT116 cells proliferation ability, function assay was utilized. Firstly, HCT116 cells were transfected with Ctrl mimic or miR-302a-3p mimic. At the same time, we also treated HCT116 cells with oe Ctrl or oe AKT. Consistent with the above results, the results of CCK8 assay suggested that decreased colon cancer cell proliferation caused by miR-302a-3p was rescued by miR-302a-3p mimic and oe AKT (Fig. 4a). In addition, HCT116 cells transfected with both miR-302a inhi and sh AKT abolished

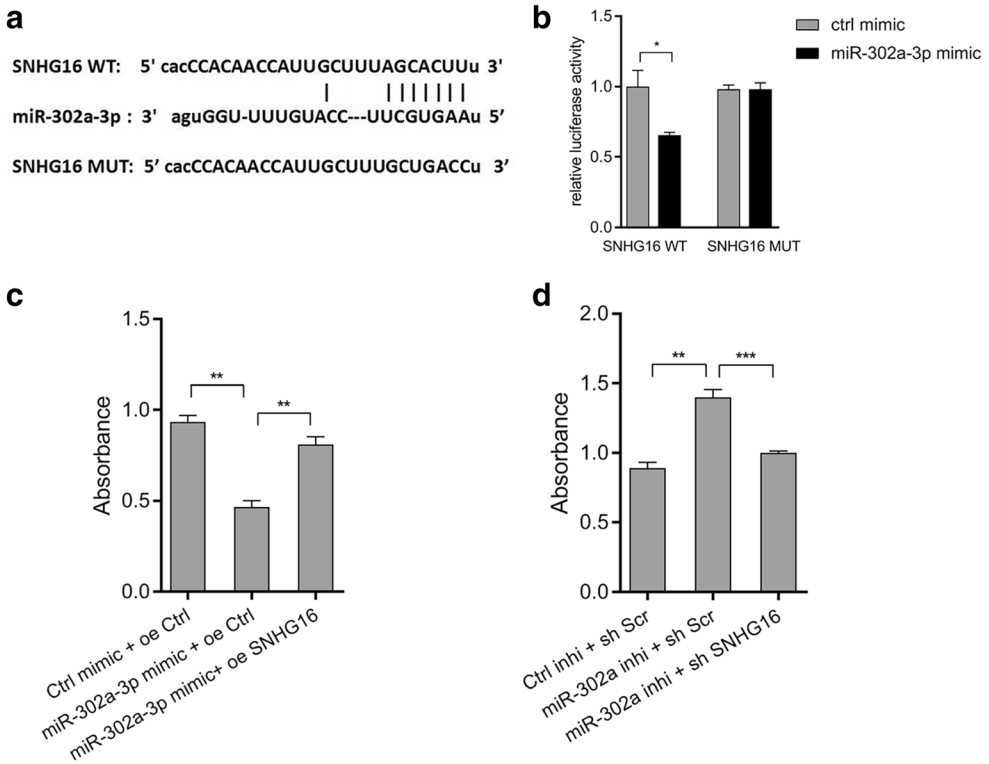

Fig. 2 SNHG16 influenced the proliferation of HCT116 cells by targeting miR-302a-3p. **a** MiR-302a-3p was predicted as a target of SNHG16 (StarBase). **b** Dual luciferase activity was monitored after plasmids with SNHG16 WT or SNHG MUT was transfected into HCT116 cells with Ctrl-mimic or miR-302a-3p mimic, respectively. **c** Cell proliferation was determined by CCK8 assay using HCT116 cells after transfection of Ctrl-mimic or miR-302a-3p mimic and oe Ctrl or oe SNHG16. **d** Cell proliferation was determined by CCK8 assay using HCT116 cells after transfection of Ctrl inhi or miR-302a-3p inhi and sh Scr or sh SNHG16. Error bars represented the mean ± SD of at least three independent experiments. Error bars represented the mean ± SD of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

cell proliferation caused by miR-302a inhi (Fig. 4b). Therefore, we documented that miR-302a-3p suppressed the growth of colon cancer cells via AKT signaling.

SNHG16 Promotes the Growth of *KRAS*-Wild Type Colon Cancer Cells

Because HCT116 cells carry a $KRAS^{G13D}$ mutation, we further used a *KRAS*- and *BRAF*- double wild type colon cancer cell line CaCO-2 to investigate if the pro-proliferative ability of SNHG16 is a general effect [25]. Therefore, SNHG16 was overexpressed or silenced in CaCO-2 cells (Fig. 5a). The results showed that SNHG16 overexpression significantly enhanced the proliferation of CaCO-2 cells, whereas SNHG16 inhibition decreased cell proliferation (Fig. 5b). These results were further supported by cell counting assay (Fig. 5c). Moreover, although miR-302a-3p overexpression suppressed CaCO-2 proliferation, this effect was abrogated by the concomitant overexpression of SNHG16. Conversely, SNHG16 silencing inhibited the pro-proliferative function of miR-302a-3p inhibitor in CaCO-2 cells (Fig. 5d, e). To investigate if miR-302a-3p modulates CaCO-2 growth by targeting AKT, we co-transfected miR-302a-3p mimic and AKT-expressing plasmid in CaCO-2 cells. AKT overexpression rescued the impaired proliferation of miR-302a-3p mimic-transfected cells. On the other hand, AKT inhibition abrogated the enhanced growth of miR-302a-3p inhibitor-transfected cells (Fig. 5f, g). Taken together, the above results indicate that SNHG16 promotes the growth of colon cancer cells by antagonizing miR-302a-3p-mediated downregulation of AKT signaling.

Discussion

With the improvement of people's living standards and changes in dietary habits, the incidence of colon cancer has continued to grow in recent years [4, 26]. The common clinical treatment is radical resection. However, it is reported that after radical tumor resection, the patient's five-year survival rate is only 50–60% [27]. Postoperative tumor recurrence and metastasis are frequent causes of death in

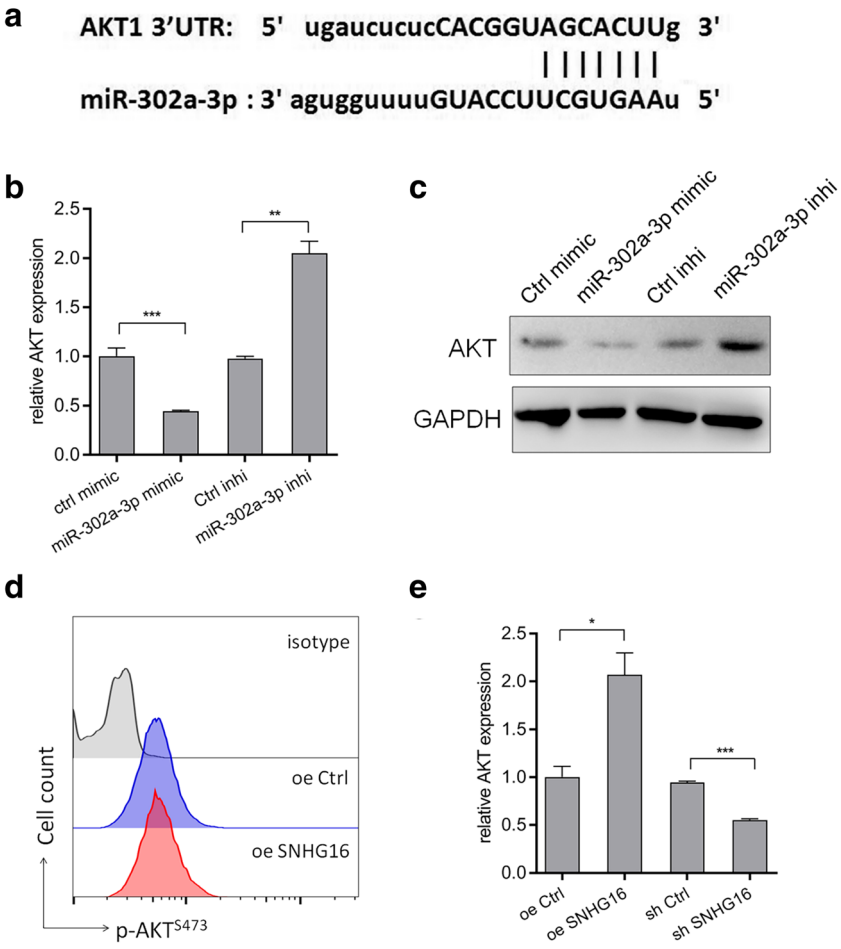

Fig. 3 SNHG16 regulated AKT by targeting miR-302a-3p in colon cancer cells. **a** AKT was predicted as a target of miR-302a-3p (miRanda). **b**, **c** The mRNA (**b**) or protein (**c**) expression of AKT was detected by q-PCR using HCT116 cells after transfection of Ctrl mimic or miR-302a-3p mimic and Ctrl inhi or miR-302a-3p inhi. **d** The p-AKT (S473) was measured by flow cytometry in HCT116 cells transfected wit oe Ctrl or SNHG16. **e** The mRNA expression of AKT was detected by q-PCR using HCT116 cells after transfection of oe Ctrl or oe SNHG16 and sh Ctrl or sh SNHG16. Error bars represented the mean ± SD of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

patients. Hence, it is imperative to further elucidate the potential molecular mechanisms of colon cancer, for the purpose of searching out prognostic biomarkers and newly therapies.

It has been reported that SNHG16 participated in the tumorigenesis of colon cancer by affecting the lipid metabolism [21]. An improved understanding of the role of SNHG16 in the progression of colon cancer will open new avenues for

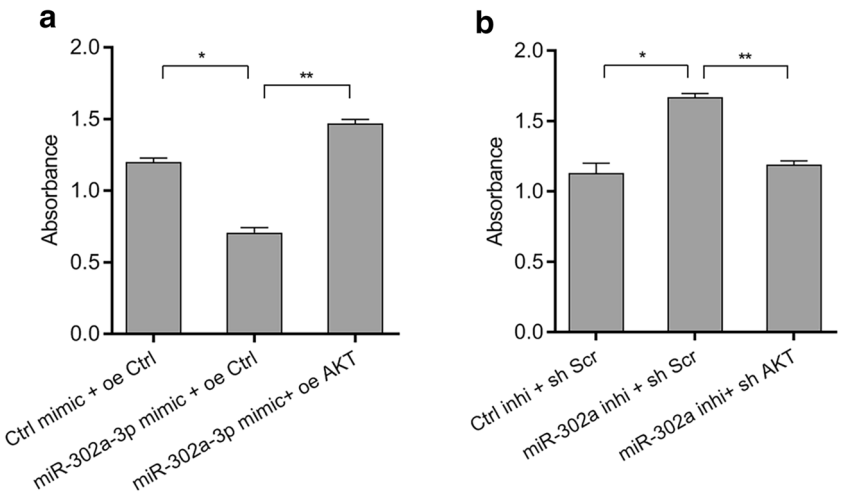

Fig. 4 MiR-302a-3p suppressed the growth of colon cancer cells via AKT signaling. **a**, **b** The proliferation ability of indicated HCT116 cells was detected by CCK8 assay. Error bars represented the mean ± SD of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$

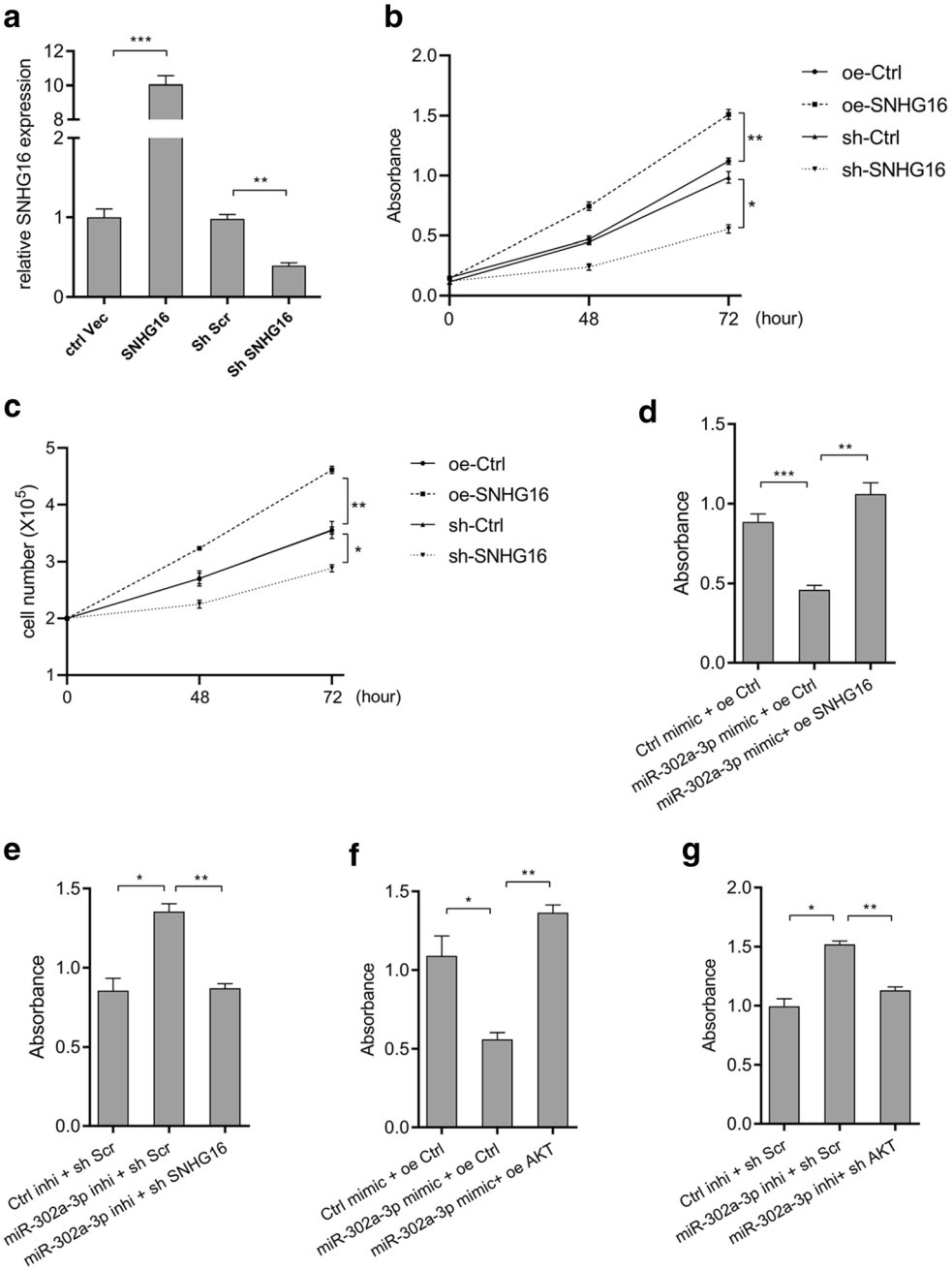

Fig. 5 The effect of SNHG16 on the growth of *KRAS*-wild type colon cancer cell CaCO-2. **a** CaCO-2 cells were transfected with oe-Ctrl, oe-SNHG16, Sh Scr or Sh-SNHG16, the expression of SNHG16 was examined by q-PCR. **b**, **c** CCK8 (**b**) and cell counting assays (**c**) were conducted to investigate the role of SNHG16 on the proliferation of CaCO-2 cells. **d**, **e** Cell proliferation was determined by CCK8 assay using CaCO-2 cells transfected with Ctrl-mimic or miR-302a-3p mimic and oe Ctrl or oe SNHG16 (**d**), or CaCO-2 cells transfected with Ctrl inhi or miR-302a-3p inhi and sh Scr or sh SNHG16 (**e**). **f**, **g** Cell proliferation was determined by CCK8 assay in CaCO-2 cells transfected with Ctrl-mimic or miR-302a-3p mimic and oe Ctrl or oe AKT (**f**), or CaCO-2 cells transfected with Ctrl inhi or miR-302a-3p inhi and sh Scr or sh AKT (**g**). Error bars represented the mean ± SD of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

searching out therapeutic approaches for the clinical treatment of colon cancer patients.

In our study, we applied the CCK8 assay and cell number counting to identify the effect of SNHG16 on the proliferation ability of colon cancer cell HCT116. Silencing SNHG16 impaired cell growth, which could be reversed under the condition of SNHG16 overexpression. Mechanistically, predicted by StarBase databases, we supposed miR-302a-3p was a target of SNHG16, which was confirmed by both dual luciferase reporter and cell proliferation assays. Besides, miRanda database predicted that Akt was a target of miR-302a-3p. It has been well known that AKT has a profound impact on colon tumor progression [28, 29]. Functionally, SNHG16 mimic transfection could effectively enhance the expression level of AKT, while overexpression could partly reverse the attenuated colon cancer cell growth caused by miR-302a-3p

mimic transfection. The summarized results indicated that SNHG16 modulated colon cancer cell proliferation through targeting miR-302a-3p/AKT axis.

To our best knowledge, it is the first time we uncovered that the SNHG16/miR-302a-3p/AKT axis might be critical to the cell proliferation to further affect the development of colon cancer. In addition, the effect of SNHG16 on other tumor characteristics of colon cancer cells is worth to be further explored, for the aim to fully enrich our findings about the precise role of SNHG16 in colon tumor progression.

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Compliance with Ethical Standards

Conflict of Interest There is no conflict of Interest.

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