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RESEARCH PAPER

SNHG15 promotes gallbladder cancer progression by enhancing the autophagy of tumor cell under nutrition stress

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ABSTRACT

Gallbladder cancer (GBC) is a major malignant carcinoma of the biliary tract with extremely poor prognosis. Currently, there is no useful therapy strategies for GBC treatment, indicating the unmet mechanism researches for GBC. In this study, our data showed that SNHG15 expression significantly up-regulated and its high expression associated with poor overall survival of patients suffer from GBC. Functional experiments showed that SNHG15 depletion delayed the proliferation and enhanced the apoptosis of GBC tumor cells under the nutrition stress condition, which further confirmed in the subcutaneous xenograft model and liver metastasis model. Mechanistically, SNHG15 could interact with AMPK and facilitate the phosphorylation of AMPK to Tuberous sclerosis complex TSC2, resulting in mTOR suppression and autophagy enhancement, and finally, conferring the GBC cell sustain proliferation under nutrition stress. Taken together, our findings revealed that SNHG15 promotes GBC tumor progression by enhancing the autophagy under poor nutrition tumor microenvironment, which could be a promising targets for GBC.

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Gallbladder cancer; SNHG15; autophagy; TSC2

Introduction

Gallbladder cancer (GBC), the major malignant carcinoma of the biliary tract, ranks as the sixth most common form of digestive-tract neoplasm [1]. Compared to the other biliary-tract carcinomas, GBC is usually diagnosed late with unsatisfactory treatment, resulting in most of patients having no opportunity to receive surgical treatment [2]. GBC is with an extremely poor prognosis with 5-year survival rate varying from 0% to 12% in most reported series and the median survive time is less than 12 months [3,4]. Thus, it is urgent to understand the mechanisms that facilitate the progression of this unusual cancer, screen potential early diagnostic biomarkers and therapeutic targets for gallbladder cancer.

Long non-coding RNAs (lncRNAs) belong to ncRNA with a length no less than 200 nucleotides and without protein code sequencing, which are initially considered as transcriptional noises [5–7]. Currently, more and more evidences have shown that lncRNAs play important roles in diverse normal and pathology by both transcriptional and post transcriptional ways [8–10]. lncRNA usually exerts its functions by competing with mRNAs that prevent the miRNA binding to the mRNA [11,12]. In cancer, lncRNAs were reported to promote cancer cells growth and chemotherapy resistance [13,14]. lncRNAs could be detected in multiple body fluids, providing the chance that lncRNAs act as potential biomarkers for diagnosis and prognosis and as promising therapeutics in different cancers [15,16]. In the prostate cancer, lncRNA PCA3 was reported as a potential diagnostic indicator, which is associated with the development of prostate cancer. Although extensive evidences had reported that lncRNA is involved in the cancer progression, only a less extent mechanism had been referred to lncRNAs in the pathological regulation of GBC.

SNHG15, also called small nucleolar RNA host gene 15 [17], is located on chromosome 7p13 with a length of 783 bp nucleotide [18]. Recently, researches gradually reveal the roles of SNHG15 in the pathology of tumor development. SNHG15 expression was reportedly upregulated in multiple cancer tissues

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such as colorectal cancer, gastric cancer, breast cancer, hepatocellular cancer, lung cancer, osteosarcoma, ovarian cancer, pancreatic cancer as well as renal cell carcinoma, and down regulated in thyroid cancer [19].

In current study, our results showed that lncRNA SNHG15 expression is dramatically enhanced in gallbladder cancer compared to the non-tumor gallbladder tissues. Functional experiments showed that SNHG15 depletion significantly suppressed the tumor cell growth and enhanced the apoptosis of cancer cells under nutrition stress. Furthermore, we demonstrate that lncRNA-SNHG15 could interact with AMPK, enhancing the phosphorylation of TSC2, leading to the increased autophagy in gallbladder cancer to facility tumor cell survival and growth under poor nutrition tumor microenvironment. Taken together, our research found that lncRNA-SNHG15 promotes the growth of GBC tumor cells by enhancing the autophagy under stress, which could be a potential therapy targets for GBC.

Material and methods

Cell culture

The gallbladder cancer cell lines, including GBC-SD, SGC-996, NOZ and EH-GB-1, were kept in the Department of Biliary-Pancreatic Surgery, Ren Ji Hospital. These cells were cultured in standard condition for passaging: DMEM with 10% fetal bovine serum (Gibco), 100 μg/ml streptomycin and 100 U/ml penicillin (Gibco). For nutrition stress, the GBC cells were cultured with DMEM supplied with 1% FBS. The cell lines were tested with mycoplasma free and the passages of cell lines between 15–25.

qRT-PCR

The total RNA extraction in this study was performed according to the manufacturer's suggestion (#12183018APureLink™ RNA). The GBC tumor cell lines were harvested and lysed by the lysis buffer, following RNA binding and washing, and finally eluting the RNA into new RNAase free tubes. The extracted RNA was reversely transcript into cDNA by taking use of RT Kit, following PCR process to detect the expression of SNHG15. The ΔCT method was applied to compare the genes and lncRNA expression in different groups.

Cell transfection

The cell transfections were performed by using lipofectamine™ 3000 Reagent. Briefly, the GBC cells were seeded at conference 50–70%, and then the plasmids and lipofectamine™ 3000 mixed with opti-MEM, which was further mixed, following 15 min incubation. After that, the DNA-lipid complex was gradually added into the cells drop by drop. The medium was replaced after 6 h of transfection. The cells was harvested and analyzed after 48 h of transfection.

Cell proliferation assay

The cell growth was performed by employing a cell proliferation analyzing kit (CCK-8 Kit, Dojindo Laboratory). First, the cells were seeded into 96-well plates at 5000 per well, and the regent of CCK-8 diluted by culture medium (1:10) was added into the well, which further incubated at 37°C for 1 h. The OD450nm was measured, and the growth curve was drawn by the normalized values of OD450 at day 0, day 1, day 2, day 3 and day 4.

In situ hybridization

In this study, the in situ hybridization assays were conducted to measure the SNHG15 expression and localization in the GBC tumor tissues and cells. The procedure was performed according to previous report [20]. First of all, the tumor or cell section was pre-hybridized with buffer for 4 h, which was further incubated with digoxin-labeled probe overnight. Then, the slides were washed three times with TBST and blocked with 3% BSA; after that, the biotin conjugated antibody was applied for 2 h at room temperature, following with three times of washing, and then incubated with SABC-HRP. The images were captured by using microscope (Leica).

Immunohistochemistry

The immunohistochemistry analysis was performed according standard procedure. First, the tumor sections were deparaffinized, following rehydration. Then, the boiled water was used for antigen retrieval. Next, 5%BSA was used for slides blocking. After that, the primary antibodies were incubated with these slides overnight at 4°C. Then, the slides were

incubated with HRP linked secondary antibody. DAB system was used for signal detection. The positive staining was determined by two different pathologists with blindness.

RNA pull-down

The GBC cells were harvested and lysed with IP buffer. Next, the cytoplasmic extract proteins were incubated with biotin-labeled SNHG15 or control at room temperature for 2 h, in RIP buffer for 1 h, and incubated with streptavidin agarose beads for 1 h. Then the agarose beads were collected. The perception was harvested and analyzed by mass spectrum.

RNA immunoprecipitation

For RNA immunoprecipitation, the experiments were conducted according to the manufacturer's guidelines. The AMPK antibody was first incubated with dynabeads (10006D, INVITORGEN) at room temperature for 10 min. After that, the antibody beads were incubated with GBC cell lysate at room temperature for 2 h. After washing with PBST twice, the precipitations were collected, following the total extraction, further reverse transcription RNA to cDNA, and finally the qRT-PCR was conducted for RNA detection.

Animal study

In this study, the mice model experiments were already approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiaotong University (NO.202101471). For the subcutaneous xenograft model, 1×10^6 GBC cells were injected into the subcutaneous blank of 6–8 week age nude mice, $n = 5$ of each group of mice. The tumor volumes were monitored every 5 days by measuring the width and length of tumor by using calipers (V = [(length ×width ^{2})/2]. The liver metastasis model was generated by intrasplenic injection, $n = 5$ of each group of mice. Briefly, the 1×10^6 GBC tumor cells were injected to the spleen. The livers were resected and fixed for immunohistochemistry analysis.

Statistical analysis

The statistical analyses in this study were performed by using GraphPad-Prism 8. All data are presented as means ± SD. In this study, the experiments were conducted for three times. The student *t*-test was used for two group comparisons, and one-way ANOVA was conducted for multiple groups comparisons. *P* values less than 0.05 were considered to be statistically significant. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Results

SNHG15 expression increased in GBC tumor and associated with poor prognosis

To reveal the function of lncRNA in the GBC, the different expressed lncRNAs among GBC and matched adjacent non-tumor tissues were analyzed by using microarray analysis [21]. The results showed that SNHG15 is one of most significantly upregulated lncRNA. Thus, we aimed to determine the roles of SNHG15 in the GBC progression. To further confirm the microarray data, we detected the SNHG15 expression in 40 cases of GBC cohort from Renji Hospital (referred as Renji cohort) (Figure 1a). The real-time PCR results demonstrated that the expression of SNHG15 greatly increased in the cancer tissues compared to the matched adjacent non-tumor tissues (Figure 1a). Furthermore, to figure out whether SNHG15 involves in the development of GBC, the association between SHNG15 expression and the overall survival time was applied. The Renji cohort was equally divided into SNHG15 high and low group according to the SNHG15 expression of clinical specimens. The log-rank statistical results showed that patients with high SNHG15 expression presented shorter overall survival time (Figure 1b). Next, we determined the association between SNHG15 and Tumor Node Metastasis classification (TNM) tumor stage, histology differentiation and Lymph node Metastasis. Consistently, high SNHG15 expression group presents high TNM stage and more lymph node metastasis and poor differentiation status. To further validate the upregulation, Fluorescent in Situ Hybridization (FISH) was

performed in the GBC tumor and adjacent non-tumor sections; the data indicated that the SNHG15 expression indeed increased in tumor tissues. Taken together, these results suggested that SNHG15 plays an important role in the progression of GBC.

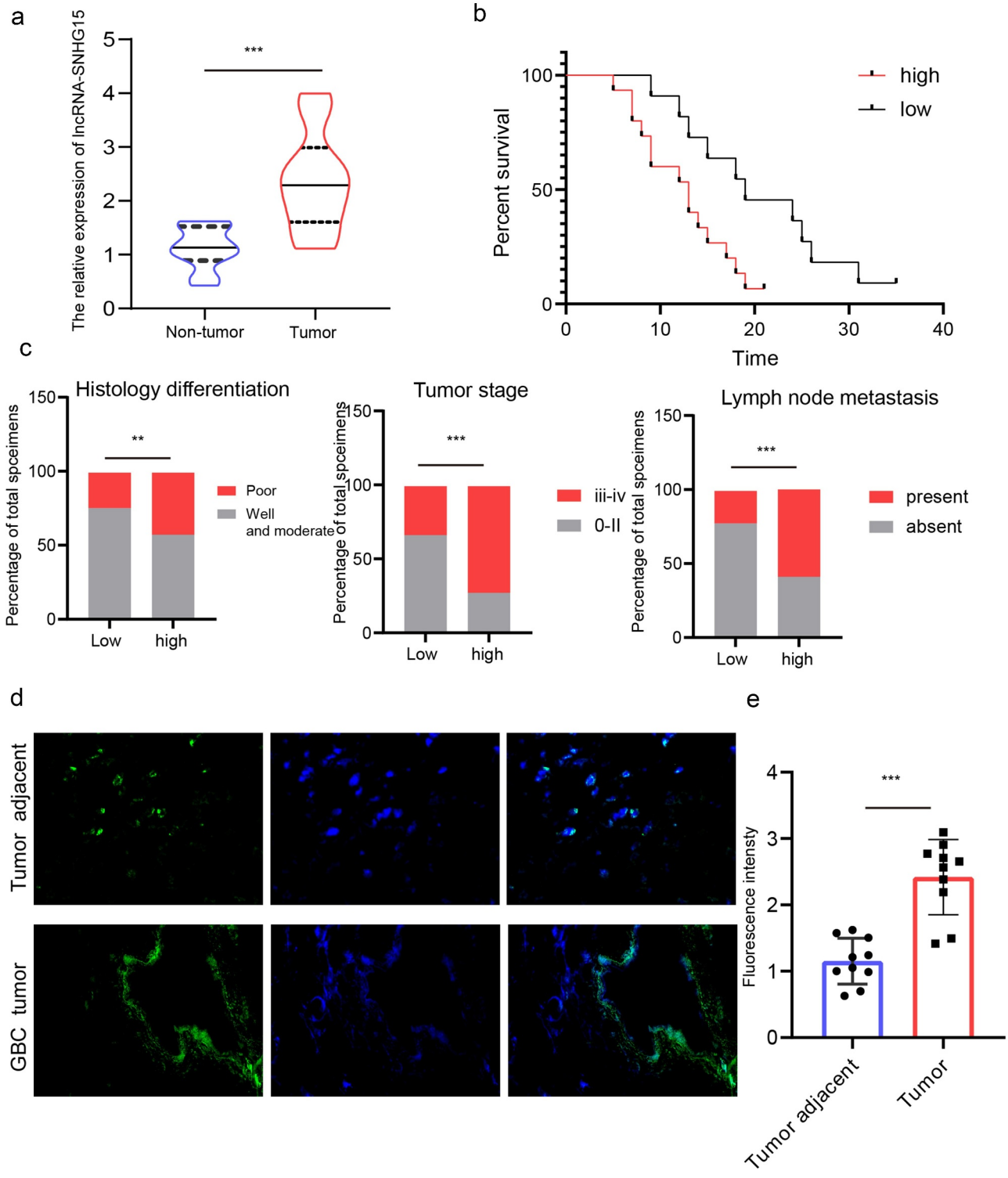

Figure 1. SNHG15 expression increased in GBC tumor and its expression is associated with poor prognosis.

(a) The QPCR of SNHG15 expression of GBC tumor tissues and adjacent tissues. (b) Kaplan – Meier analysis of SNHG15 high and low expression to overall survival of Renji cohort. (c) The association of SNHG15 expression and TNM tumor stage, histology differentiation and Lymph Node Metastasis. (d) The FISH results of GBC tumors and corresponding adjacent normal tissues Scale bar, 50 μm. (E) The statistical results of FISH. $^{**}p < 0.01$, $^{***}p < 0.001$.

SNHG15 depletion suppressed the proliferation and enhanced the apoptosis of GBC cells

Next, we aimed to determine the function of SNHG15 in the GBC cancer cells. The SNHG15 expression of four GBC cancer cell lines, EH-GB1, SGC-996, GBC-SD and QNZ, was measured by RT-PCR (Figure 2a). The results revealed that QNZ and SGC-996 exhibited higher SNHG15 expression, which was chosen to knockdown SNHG15. The stable SNHG15 knockdown cell lines QNZ and SGC-996 were generated by infecting with sh SNHG15 packaged lenti-virus. Further, RT-PCR was performed to validate the knockdown efficiency (Figure 2b). The results showed that more than 85% SNHG15 expression was suppressed by the sh SNHG15. Next, the cell proliferation of negative control (referred as shNC) and sh SNHG15 was measured by CCK 8 kit. The cell growth curve suggested that SNHG15 deletion slightly suppressed the QNZ and SGC-996 cell proliferation under nutritious culture condition (medium supplied with 10% FBS) (Figure 2c). Considering the poor nutrition microenvironment of GBC, we further assay the cell growth of shNC and sh SNHG15 GBC cell under nutrition stress condition (medium supplied with 1% FBS). Interestingly, the proliferation of shSNHG15 cells dramatically reduced compared to the negative control group shNC cells (Figure 2d). To further confirm, we next measured the effects of SNHG15 overexpression on the growth of GBC cells. SNHG15 was ectopic, expressed in EH-GB1 and GBC-SD, which presents relatively low SNHG15 expression. Consistently, SNHG15 overexpression dramatically increased the GBC cell growth under nutrition stress (Figure 2e). Given that nutrition stress induces cell death, we next detected the GBC cell apoptosis upon SNHG15 depletion and over expression by Caspase 3/7 Activity Apoptosis Assay Kit. Fluorescence measurement results showed that SNHG15 knockdown and overexpression only slightly influenced the apoptosis of GBC cell under full nutrition culture condition (Figure 2f,g). Therefore, we further detected the apoptosis under nutrition stress condition. In line with this, we found the apoptosis index of GBC sharply increased upon SNHG15 knockdown (Figure 2h). On the contrary, SNHG15 overexpression significantly reduced the apoptosis of GBC cells (Figure 2i). In summary, these data indicated that SNHG15 promotes the growth of GBC cells under nutrition stress by enhancing the proliferation and suppressing the apoptosis.

SNHG15 promotes the tumor cell proliferation by enhancing the autophagy

Next, we aimed to dissect the mechanism that SNHG15 facilitates GBC tumor cell growth under nutrition stress. Considering that cell enhanced the autophagy to overcome the nutrition stress, we measured the autophagy of GBC cell under SNHG15 depletion. We first generated an autophagy luciferase report system by stably expressing LC3-luc in the GBC cancer cells. The luciferase reporter showed that the level of autophagy in SNHG15 depletion cells significantly reduced compared to that in the control group cells (Figure 3a). To further validate, the effects of SNHG15 overexpression to the autophagy in GBC cells were also measured. As expectedectopic SNHG15 expression greatly enhanced the luciferase expression (Figure 3b). To further confirm the results of luciferase report system, we further detected the LC3 expression by immunofluorescence. Consistent with previous results, SNHG15 suppression dramatically decreased the LC3 fluorescence (Figure 3c). Conversely, SNHG15 suppression greatly enhanced the autophagy level (Figure 3d). Next, to revel whether the growth protective effect of SNHG15 depends on the autophagy in GBC cells, we first over expressed SNHG15 and then treated the GBC cells with autophagy inhibitor 3 MA. The results showed that SNHG15 could enhance the growth of GBC cells under nutrition stress condition, but not under the treatment of 3 MA (Figure 3e), which indicated that the growth promote effects depend on the autophagy of GBC cells.

SNHG15 interacted with AMPK to regulate the autophagy of GBC cells

Next, we aim to reveal the mechanism of how SNHG15 enhances the autophagy in GBC cell. Considering that the location of SNHG15 is

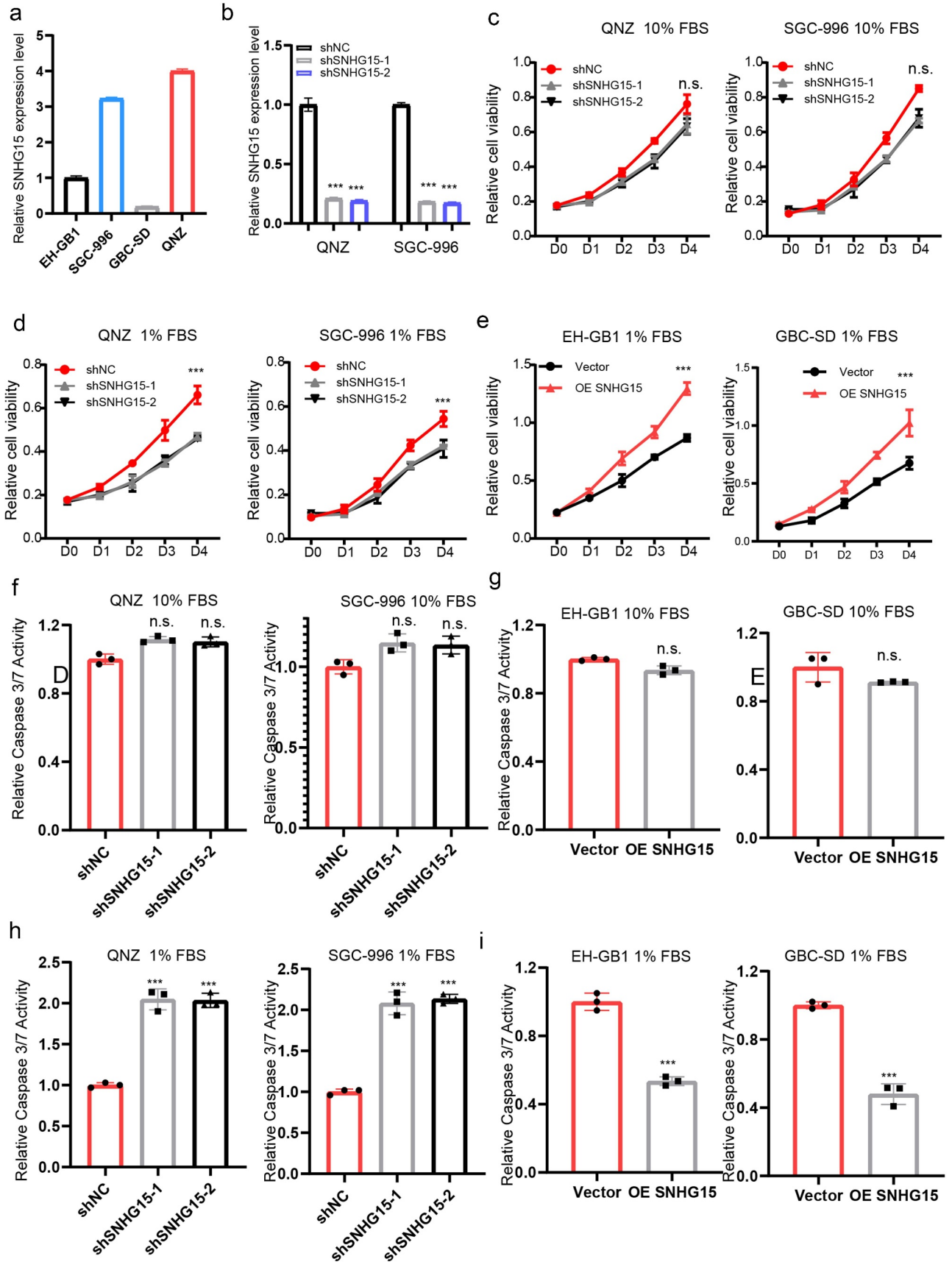

Figure 2. SNHG15 depletion suppresses the growth of GBC cells.

(a) SNHG15 expression in GBC cancer cell lines. (b) The SNHG15 silencing efficiency in GBC cells. (c–e) The cell proliferation assay of GBC cell lines that knockdown or over express SNHG15 under normal or nutrition stress condition. (f–g) Represented results of the Caspase 3/7 activity in SNHG15 knockdown cell lines or overexpression under normal culture condition. (h–i) Represented results of the Caspase 3/7 activity in SNHG15 knockdown cell lines or overexpression under nutrition stress. $^{**}p < 0.01$, $^{***}p < 0.001$.

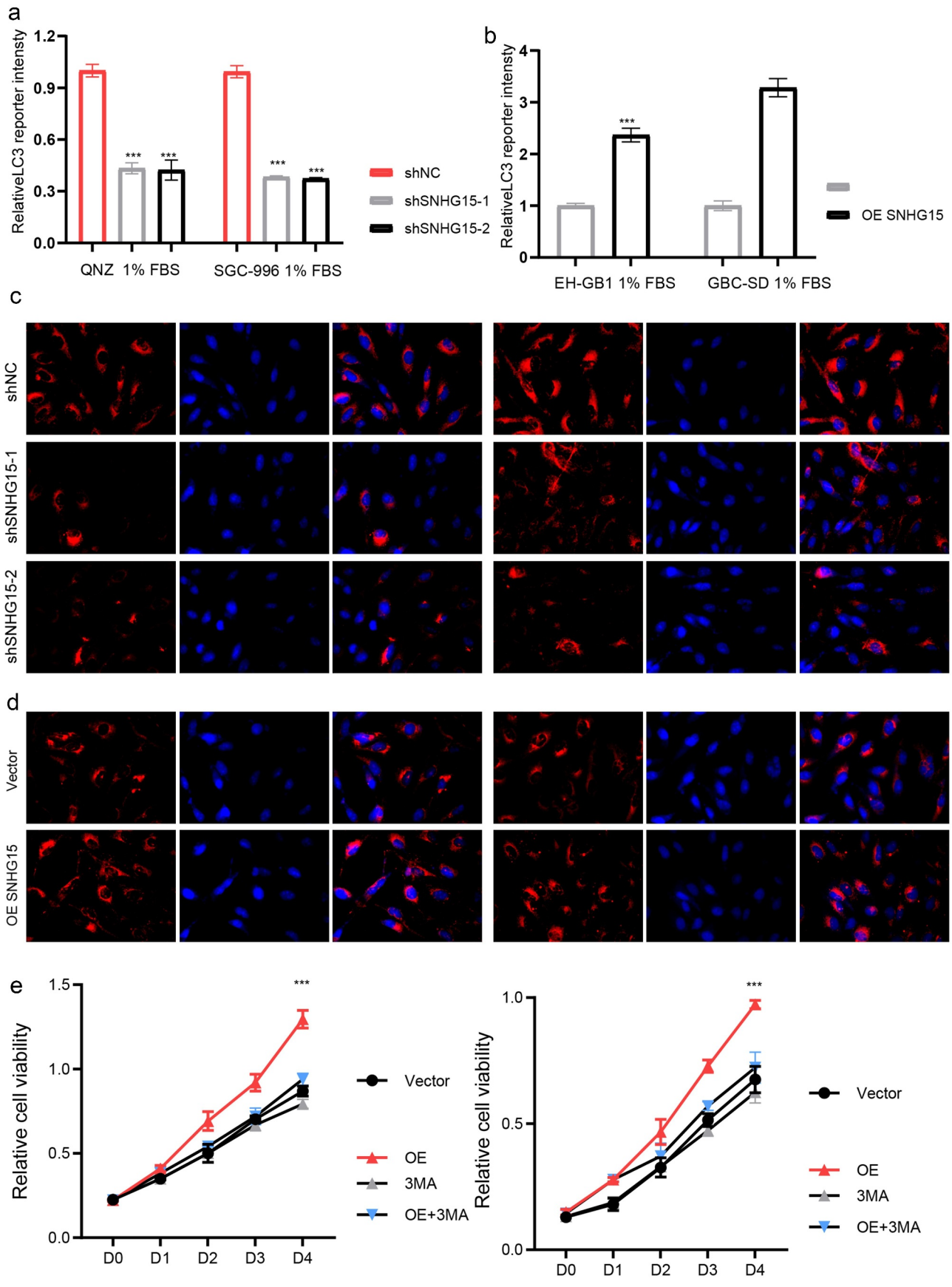

Figure 3. SNHG15 enhances the autophagy of GBC cells.

(a) Represented results of the LC3 reporter activity in SNHG15 knockdown cell lines under nutrition stress condition. (b) Represented results of the LC3 reporter activity in SNHG15 overexpression cell lines under nutrition stress condition. (c–d) LC3 immunofluorescence staining results of GBC cells under nutrition stress condition. (e) The cell proliferation assay of GBC cell lines that over express SNHG15 under 3 MA treatment. $^{**}p < 0.01$, $^{***}p < 0.001$.

majorly in the cytosol of GBC cells, we thus performed a RNA pulldown by using a biotin labeled probe of SNHG15. The precipitation was applied for mass spectrometry analysis, which, in order to identify proteins, interacted with SNHG15 (Figure 4a). After analyzing the mass spectrometry results, we found that Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK), which is one of the most famous autophagy regulator, is the candidate protein that interacted with SNHG15. To further validate this, RNA pull down was further performed, following immunoblotting with AMPK antibody. Consistently, the immunoblotting results demonstrated that SNHG15 could interact with AMPK. However, the antisense of SNHG15 could not interact with AMPK in the GBC cells (Figure 4b). To further confirm this, RNA Binding Protein Immunoprecipitation (RIP) was conducted in QNZ cell lines. The gel electrophoresis results showed that the SNHG15 could be amplified from the precipitation of anti-AMPK antibody but not the IgG precipitation (Figure 4c). Moreover, the RIP-PCR results also confirmed that AMPK could interact with SNHG15 (Figure 4d). Next, to map the specific sequence that mediated the interaction of SNHG15 and AMPK, differenced SNHG15 truncated mutation was generated and applied for RNA pull down. The immunoblots results showed that Nucleotides 200 to 400 of SNHG15 disrupted the interaction of SNHG15 and AMPK, indicating that this region response the interaction between SNHG15 and AMPK (Figure 4e). Given that AMPK can phosphorylate mTOR to enhance the autophagy in the tumor cell, we proposed that SNHG15 may facilitate the phosphorylation of mTOR. Proceeding researches reported that AMPK regulates mTOR by phosphate TSC2. Thus, we detected the phosphation TSC2 level

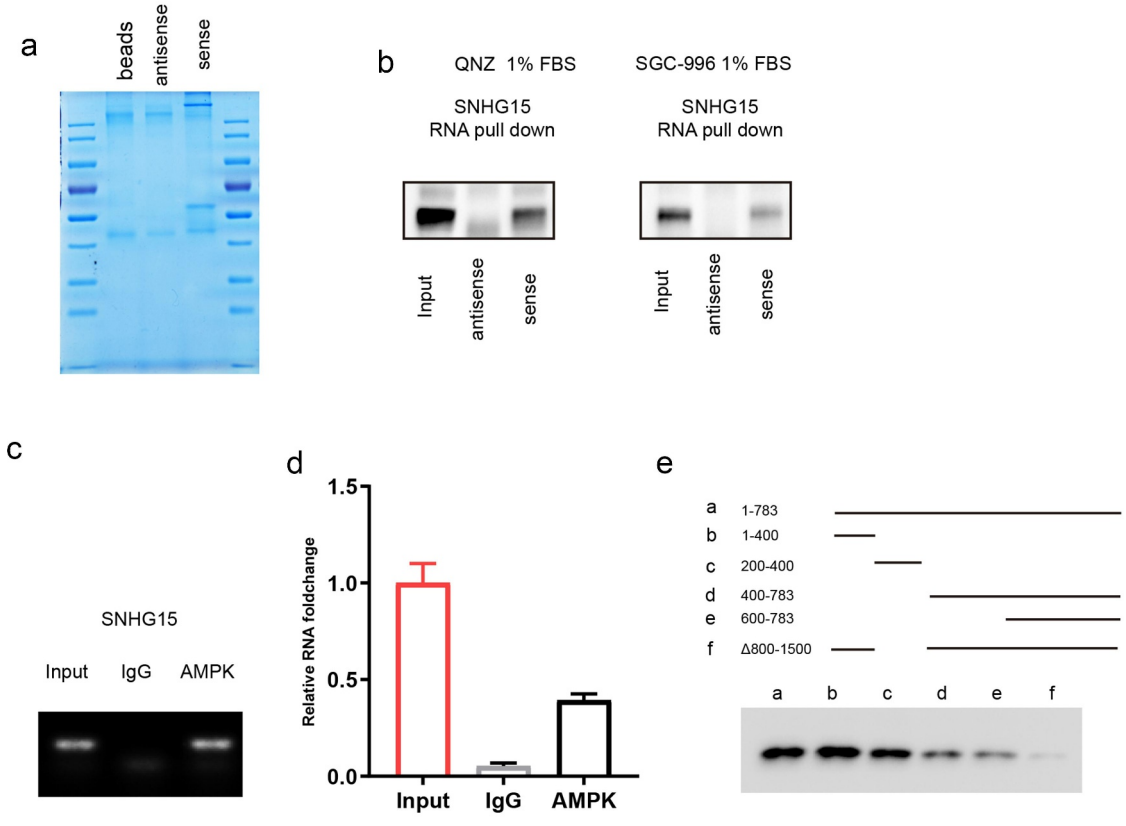

Figure 4. SNHG15 interacts with AMPK.

(a) RNA pulldown assay with SNHG15, followed by coomassie staining. (b) SNHG15 pulldown assay immunoblots with AMPK antibody in GBC cell lines QNZ and SGC-996. (c) The agarose gel electrophoresis of SNHG15. (d) Q-PCR results of SNHG15 in the precipitation of AMPK antibody derived from GBC tumor cells. (e) RNA pulldown assay for full-length or truncated SNHG15, followed by immunoblots with AMPK in GBC cells. $^{**}p < 0.01$, $^{***}p < 0.001$.

upon SNHG15 silencing. As expected, p-TSC2 expression was significantly reduced in shSNHG15 cells compared to the control cell. Taken together, these data indicated that SNHG15 interacted with AMPK to enhance the phosphorylation of AMPK to TSC, leading to autophagy enhancement in the GBC cells.

Targeting SNHG15 delayed the development of GBC in vivo

Further, we aimed to assess whether targeting SNHG15 impedes the progression of GBC in mouse model. First of all, subcutaneous xenograft model was generated by injecting shNC and shSNHG15 cells in the nude mice, respectively. After 25 days of cell implantation, the mice were sacrificed and tumors resected from mice. The results showed that the tumor developed from shSNHG15 GBC cell was much smaller than that in shNC GBC cell (Figure 5a). Next, the tumor growth curve was drawn by measuring the tumor volume every 5 days. Tumor growth curve statistical analysis suggested that the tumor grow much more rapidly than that in the control group mice (Figure 5b). In line with this, subcutaneous xenograft tumor weight measurement also confirmed that SNHG15 silencing dramatically delayed the progress of GBC tumor (Figure 5c). Furthermore, the tumor sections were applied for immunohistochemistry (IHC) staining. The shSNHG15 group tumor section presents weaker PCNA, p-AMPK, p-TSC2 than that in the control group mice (Figure 5d). To further determine the potential of targeting in vivo, liver metastasis model of GBC was established by intrasplenic injection of GBC tumor cell. The results demonstrated that the burden of shSNHG15 group mice significantly reduced (Figure 5e). Furthermore, the livers were applied for H&E staining, and the metastasis area analysis demonstrated that SNHG15 depletion decreased the liver metastasis area from 70% to 45% (Figure 5f,g). In line with this, the liver sections in the shNC group exhibited stronger PCNA, p-AMPK, p-TSC2 staining than that in the SNHG15 knockdown group (figure 7h). Collectively, these data suggested that targeting SNHG15 could impede the tumor growth and attenuate liver metastasis of GBC in vivo.

Discussion

Accumulated evidences reported that lncRNAs play important roles in physiology and pathology, rather than in transcriptional "noise" [22]. Currently, lncRNA is majorly reported as a sponge of microRNA to exert its function [23,24]. In this study, we found that SNHG15, a lncRNA localized in the cytosol of GBC cell expression, was significantly increased in the GBC tumor tissues when compared to the matched non-tumor tissue. Moreover, SNHG15 expression was positively associated with the overall survival time of GBC patients, indicating that SNHG15 could be a potential biomarker and target of GBC.

Here, by analyzing datasets and FISH, our results showed that the expression of SNHG15 significantly enhanced in GBC tumor tissues when compared to the adjacent tissues. In line with previous studies, SNHG15 positively associated with clinicopathologic features, including in tumor volume, distant metastasis, tumor stage and overall survival. Even though SNHG15 is widely reported in cancer-promoted role, the function of SNHG15 is diverse in different cancers. SNHG15 reported to enhance the EMT process to facilitate the migration and invasion in breast cancer and renal cell carcinoma [25]. In lung cancer, SNHG15 was reported to suppress the CDK14 activity to promote the growth of lung cell [26]. In thyroid cancer, SNHG15 was found to inhibit hippo pathway to prevent the proliferation of thyroid tumor cells [27]. In this study, we found that SNHG15 could enhance the apoptosis of GBC cell under nutrition stress condition, which help the tumor cell for survival in the poor nutrition tumor microenvironment. Our study provides new insight to the function of SNHG15 in the tumor development.

The precise role of AMP-activated kinase (AMPK) in cancer is controversial, acting as both a "conditional" tumor suppressor and "contextual" oncogene. The mechanism for AMPK tumor suppressor activity includes: (1) AMPK could exert as a metabolism suppressor. AMPK could decrease the expression of hypoxia-

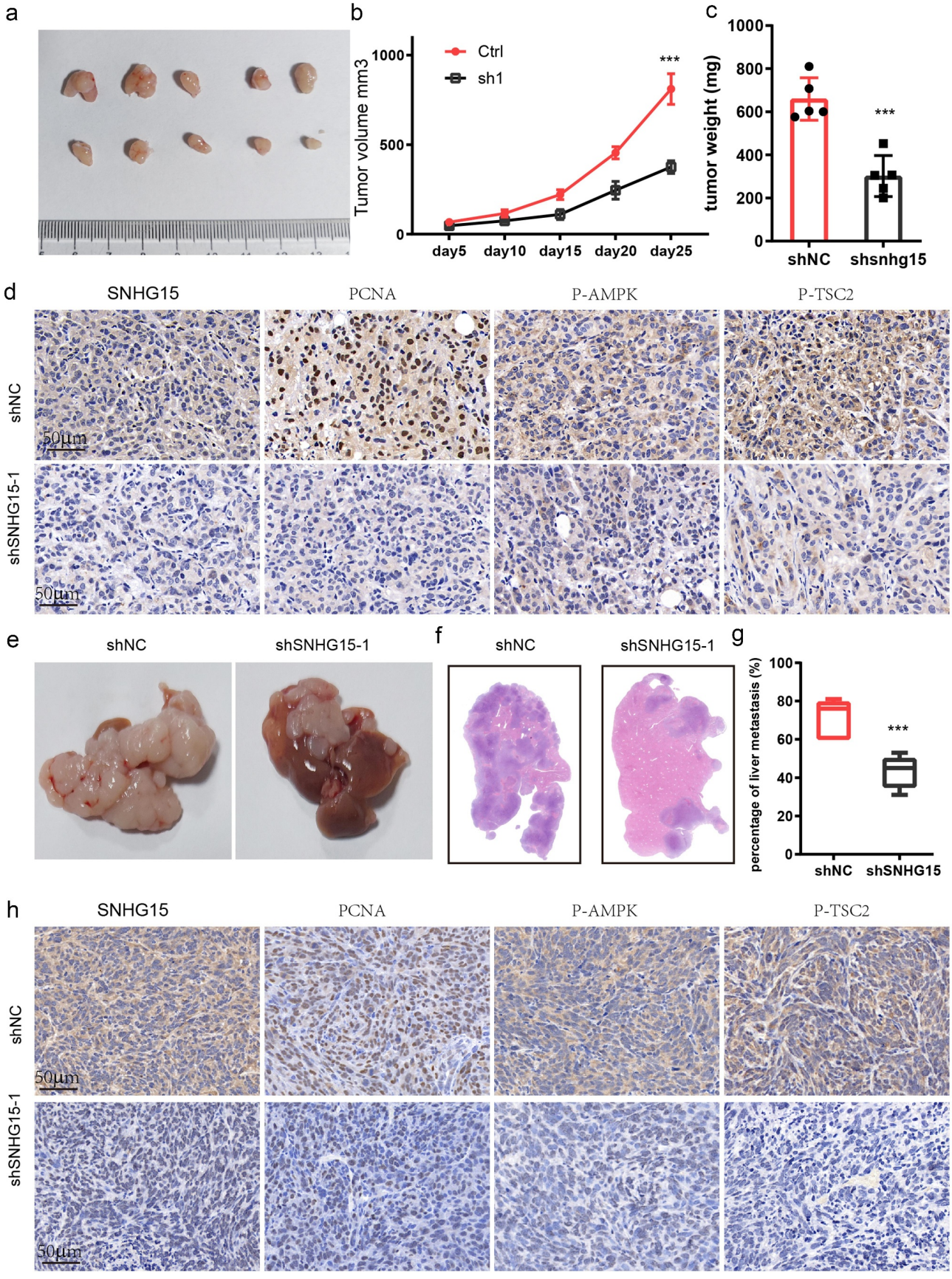

Figure 5. SNHG15 depletion delayed the growth of GBC *in vivo*.

(a) Subcutaneous xenografts transplanted with SNHG15 NC or knockdown cells ($n = 5$). Scale bar, 1 cm. (b) Tumor growth curve of subcutaneous xenografts. (c) Tumor weight of subcutaneous xenografts. (d) Representative IHC staining results of SNHG15, PCNA, P-AMPK and P-TSC2 in subcutaneous xenografts. (e) Liver metastasis results of mice transplanted with SNHG15 NC or knockdown cells. (f) h&e staining of liver resected from shNC and shSNHG15 group mice. (g) statistical results of liver metastasis. (h) Representative IHC staining results of SNHG15, PCNA, P-AMPK and P-TSC2 in liver metastasis.

inducible factor 1-alpha (HIF1a) and AMPK has also been shown to inhibit unchecked mTORC1 activity and de novo lipogenesis, required both during G1–S and G2–M phases [28]. (2) AMPK-dependent phosphorylation of the oncogene BRAF at Ser729. This phosphorylation prevents BRAF interaction with the scaffolding protein kinase suppressor of Ras 1 (KSR1), leading to the suppression of the oncogenic MEK–ERK signaling and consequent impairment of cell proliferation and cell-cycle progression [29]. In addition, AMPK/mTOR pathway mediated the extrinsic apoptosis by trifolirhizin in CRC [30]. On the other side, the AMPK pathway supporting cancer progression has been described. These include: (1) AMPK could promote FA oxidation (FAO) to generate ATP, supporting the growth of tumor cells [31]. (2) AMPK could phosphorylate the core histone H2B, leading to gene transcription enhancement [32]. (3) AMPK increase intracellular NADPH levels through the activation of FAO to neutralize cytotoxic ROS [33]. (4) When in nutrient-replete conditions, the AMPK energy-sensing pathway and the PI3K/Akt cascade converge on mTOR with opposing regulatory effects; under glucose depletion, both AMPK and Akt are activated and coordinately support cell survival [34]. Consistently, our data showed that SNHG15 facilitated the phosphorylation of AMPK to TSC2 to resist the nutrient depletion stress.

In this study, we found SNHG15 significantly increased in GBC tumor and promote GBC cell proliferation and metastasis. Mechanistically, SNHG15 can interact with AMPK, resulting in the phosphorylation enhancement of TSC2 and GBC cell proliferation. In summary, our data suggested that SNHG15-AMPK-TSC2 axis exerts crucial role in GBC progression.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contribution

H.M., L.H.Y, X.X conducted experiments and collected the data; H.M., L.H.Y, X.X performed experiments and provided intellectual discussion. H.M., Y.G., F.L. designed the experiments. H.M., Y.B. L. wrote the manuscript. All authors critically revised and approved the manuscript.

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