Long non-coding RNA GACAT3 promotes liver cancer progression by regulating the proliferation, apoptosis and migration of tumor cells

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Abstract. Long non-coding RNA gastric cancer associated transcript 3 (GACAT3), is a newly identified non-coding RNA, which has been found to be involved in the tumorigenesis of gastric cancer. However, the biological function of GACAT3 in liver cancer remains unclear. The present study aimed to determine the expression level and function of GACAT3 in liver cancer. The authors cultured liver cancer cells *in vitro* and GACAT3 was silenced in the cells. Cell proliferation, apoptosis and migration were determined by MTT assay, flow cytometric analysis and transwell assay, respectively. It was demonstrated that GACAT3 was upregulated in liver cancer tissues. The inhibition of GACAT3 decreased the ability of hepatoma cells to proliferate and migrate, and increased apoptosis of the cells. These findings provide the first evidence, to the best of our knowledge, of the exact role of GACAT3 in liver cancer, suggesting GACAT3 as a potential target for liver cancer therapy in the future.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer worldwide and represents the third leading cause of cancer-related mortality (1). It is the fifth most common cancer in men and the seventh most common in women, with an increasing incidence, particularly in Western countries (1,2). Hepatitis B virus infection, liver cirrhosis and the occurrence of malignant liver neoplasia are considered the three gradations of HCC. This process involves a series of gene mutations in hepatocytes, and the accumulation of these mutations, which can lead to tumor cell proliferation and invasion/metastasis, is responsible for the formation of malignancy and metastases to the surrounding liver parenchyma/distant organs (3). Clinically, ablation, liver transplantation and surgical resection are used for the treatment of HCC and enable a high rate of complete excision of this disease (2). However, as HCC frequently relapses or is diagnosed at an advanced stage, these treatments may be ineffective. Furthermore, no adjuvant therapy has been demonstrated to improve recurrence-free survival rate following curative treatments (4).

In recent years, long non-coding RNAs (lncRNAs) have gained increasing attention in several fields in molecular and cellular medicine. As a product of transcription with little to no protein-coding functions, lncRNAs are involved in multiple biological functions, including intercellular signaling, protein localization or functions, post-transcriptional mRNA processing, control of gene transcription and regulation of gene expression (5-7). In addition to the investigation and recognition of lncRNAs, there is increasing evidence that they have significant roles as tumor oncogenes or/and suppressors (8-10). Furthermore, several lncRNAs are important in the progression and development of liver cancer (11,12).

The lncRNA gastric cancer associated transcript 3 (GACAT3), also known as AC130710), is located on human chromosome 2p24.3. It is upregulated in gastric cancer tissues and is significantly associated with distal metastasis, tumor-node metastasis stages and tumor size (13). In addition, GACAT3 promotes gastric cancer progression by negatively regulating the expression of microRNA (miR)-497, and the knockdown of GACAT3 significantly inhibits the invasion, migration, colony formation and proliferation of gastric cancer cells *in vitro* (14). However, the biological function of GACAT3 in HCC, including its role in proliferation, apoptosis and migration, remains to be elucidated. Therefore, the present study investigated the correlation between the expression levels of GACAT3 in HCC tissues and cell lines. Furthermore,

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the effects of decreasing the expression levels of GACAT3 on the proliferation, apoptosis and migration of liver cancer cells *in vitro* were examined.

Materials and methods

Cell culture and HCC samples. The QSG-7701 normal human liver cell line and the HepG2, HCCLM3, SK-Hep-1 and SMMC-7721 liver cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). The Huh7 HCC cell line was purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). All cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 100 U/ml penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a 5% CO_2 incubator. HCC and adjacent normal tissues were obtained with informed consent from 120 patients at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China) between January 2016 and December 2017. The adjacent noncancerous liver tissues were obtained from >5 cm away from the tumor sites, and verified simultaneously by two pathologists separately. All specimens were placed in liquid nitrogen immediately following resection. Patient charts were obtained regarding age, sex, tumor size, α-fetoprotein levels, hepatitis B surface antigen positivity, tumor differentiation and Tumor, Node, Metastasis stage (American Joint Committee on Cancer; Table I) (15). The study protocol was approved by the Ethics Committee of the Yinzhou People's Hospital Affiliated to Ningbo University School of Medicine (Ningbo, China).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA from the HCC tissues and the liver cancer cell lines was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The total RNA was eluted with RNase-free water and the concentration was determined by ultraviolet spectrophotometry. RNA was reverse transcribed to cDNA and RT-qPCR was performed using Synergy Brands (SYBR)-green PCR Master mix (Takara Biotechnology Co., Ltd., Dalian, China) in a Fast Real-time PCR 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling steps were as follows: 5 min at 95˚C, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 30 sec. The specific primers for GACAT3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used were from Invitrogen; Thermo Fisher Scientific, Inc., and as described previously (16). The primer sequence details were as follows: GACAT3 (forward, 5'-CTTCCGGAGCAGGTC TGAGT-3' and reverse, 5'-CTTTCCCTGCAGAGACCAG T-3'), GAPDH (forward, 5'-GTCAACGGATTTGGTCTGTAT T-3' and reverse, 5'-AGTCTTCTGGGTGGCAGTGAT-3'). Relative quantification was analyzed via the comparative threshold cycle ($2^{-\Delta\Delta Cq}$) method (17) and the results were internally normalized to the expression of GAPDH.

Gene silencing of GACAT3 in liver cancer cell lines. To downregulate the expression of GACAT3, a small interfering RNA (siRNA) sequence targeting GACAT3 (5'-AUCAGG GCUUGUGGA AUGGGA AG-3'; GeneChem Co., Ltd., Shanghai, China) was inserted into the lentivirus vector pLL3.7 (Sigma-Aldrich; Merck KGaA) containing green fluorescent protein, and designated as si-GACAT3. A hairpin siRNA with no human sequence homology was used as the negative control (si-NC; 5'-UCACA ACCUCCUAGA A AG AGUAGA-3'; Shanghai GeneChem Co., Ltd.). All constructs were verified by sequencing. Recombinant lentivirus was established from human 293T cells (American Type Culture Collection) by co-transfection of pdelta-8.91 and pVSVG, together with si-GACAT3 or si-NC using polybrene (1:1,000; Sigma-Aldrich; Merck KGaA) and FBS-free MEM. After 12 h, the FBS-free MEM was changed with DMEM containing 10% FBS. After 48 h the cells were observed under fluorescence microscopy (magnification x40), to identify GFP, then DMEM containing 10% FBS and 0.2% puromycin was added to select the transfected cells for 7 days. The cells were cultured in DMEM containing 10% FBS. The knockdown efficiency of si-GACAT3 was determined by RT-qPCR.

Determination of cell viability with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The liver cancer cells were seeded into 96-well plates and treatments were performed following adherence. Following incubation periods of 24, 48, 72, or 96 h, 20 μl of a 5 mg/ml MTT solution was added to each well and the plate was incubated at 37˚C for 4 h. Thereafter, the medium was aspirated and the wells washed with phosphate-buffered saline. Following drying for ~2 h, 200 μl of dimethyl sulfoxide was added to each well. The formazan crystals were dissolved by placing the microtiter plate on a shaker, and the absorbance was determined spectrophotometrically at 570 nm using a reference wavelength of 630 nm on an ELX800 UV universal microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Determination of apoptosis by flow cytometric analysis. The HepG2 and HCCLM3 cells transfected with si-GACAT3 or si-NC for 48 h were subjected to analysis of apoptosis using Annexin V/propidium iodide (PI), as described previously (18). Liver cell apoptosis was examined using an Annexin V-FITC apoptosis detection kit (Sigma-Aldrich; Merck KGaA) according to the manufacturer's recommendations. Annexin V-positive cells were considered early apoptotic cells, and PI-positive cells were considered late apoptotic/necrotic cells. All liver cell nuclei were counterstained with Hoechst 33342 dye.

Western blot analyses. Following transfection of the HepG2 and HCCLM3 cells with si-GACAT3 or si-NC for 48 h, total protein was extracted with radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Jiangsu, China), followed by quantification using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal quantities of protein (20 g) from each sample were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 8-12% gels and transferred onto a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). Membranes were blocked using 5% skimmed milk for 2 h at room temperature, washed with TBST, and incubated primary antibodies

Table I. Clinicopathological characteristics of patients.

Variable	Number
Sex	
Male	86
Female	34
Age (years)	
≤50	48
>50	72
Tumor size (cm)	
≤3	36
>3	84
α-fetoprotein (ng/ml)	
≤400	92
>400	28
Hepatitis B surface antigen	
Positive	70
Negative	50
Liver cirrhosis	
Yes	91
No	29
Barcelona clinic Liver Cancer	
0-B	104
C	16
Tumor, node, metastasis stage	
I-II	113
III-IV	7

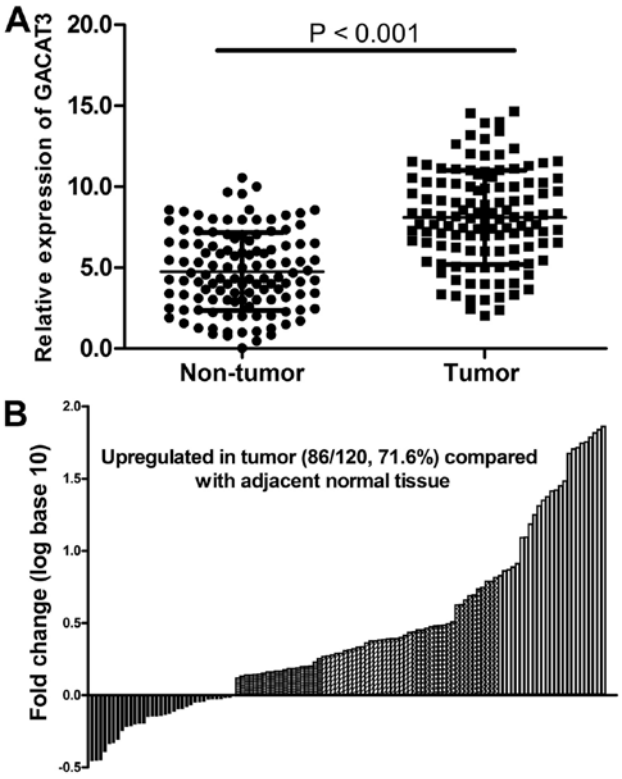

Figure 1. mRNA expression levels of GACAT3 in tissues of patients with HCC. (A) Relative mRNA expression of GACAT3 in tumor and adjacent normal tissues from 120 patients with HCC, measured by reverse transcription-quantitative polymerase chain reaction analysis. (B) Fold change in GACAT3 mRNA was upregulated in 71.6% of tumor tissues of 120 patients with HCC compared with adjacent tissues. Data are expressed as the mean ± SEM. GACAT3, gastric cancer associated transcript 3; HCC, hepatocellular cancer.

against Bax (cat. no. sc-20067; diluted 1:1,000), Bcl-2 (cat. no. sc-509; diluted 1:500), E-cadherin (cat. no. sc-71008; diluted 1:1,000), N-cadherin (cat. no. sc-7939; diluted 1:1,000; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA), TGF-β1 (cat. no. ab155264; diluted 1:500; Abcam), β-catenin (cat. no. 9582; diluted 1:500), β-actin (cat. no. 4970; diluted 1:2,000) and GAPDH (cat. no. 8884; diluted 1:1,000; all Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4˚C. The membranes were washed with TBST three times, incubated with horseradish peroxidase-conjugated goat anti-rabbit (cat. no. sc-2004; diluted 1:1,000; Santa Cruz Biotechnology, Inc.) and goat anti-mouse (cat. no. ab97040; 1:5,000; Abcam) secondary antibodies for 2 h at room temperature and again washed with TBST. The protein bands were detected using a chemiluminescent detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). GAPDH or β-actin was used as the loading control.

Transwell migration assay. The migration ability of liver cancer cells was assessed using a Transwell assay with 8 μm porous membrane inserts (Corning, Inc., Corning, NY, USA). The siRNA-transfected liver cancer cells were treated with a trypsin/EDTA solution, and then washed with PBS and suspended in serum-free medium. The cells ($5x10^4$) in 200 μl serum-free medium were inoculated into the upper chamber and 500 μl of medium containing 20% fetal bovine serum were added into the lower chamber. Following incubation at 37˚C for 48 h, cells that migrated to the bottom surface of the Transwell chamber were fixed with 100% methanol at room temperature for 10 min. The cells were then stained with 0.5% crystal violet for 10 min and washed three times with phosphate-buffered saline. The cells on the top surface of the Transwell chamber were removed with a cotton swab. The migration capacity was quantified by counting the number of migratory cells in five fields of each Transwell using an inverted microscope (x200 magnification; Olympus Corporation, Tokyo, Japan). Each experiment was repeated three times.

Statistical analysis. Statistical analysis was performed using GraphPad Prism5 software (GraphPad Software, Inc., La Jolla, CA, USA). The results are presented as the mean ± standard error of the mean. Statistical analyses were performed with Student's t-test or one-way ANOVA followed by Tukey's post hoc test. For all experiments, $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of GACAT3 is increased aberrantly in HCC tissues. The mRNA expression level of GACAT3 was measured in tissues from 120 patients with HCC. As indicated in Fig. 1A and B, the mRNA expression of GACAT3 was significantly

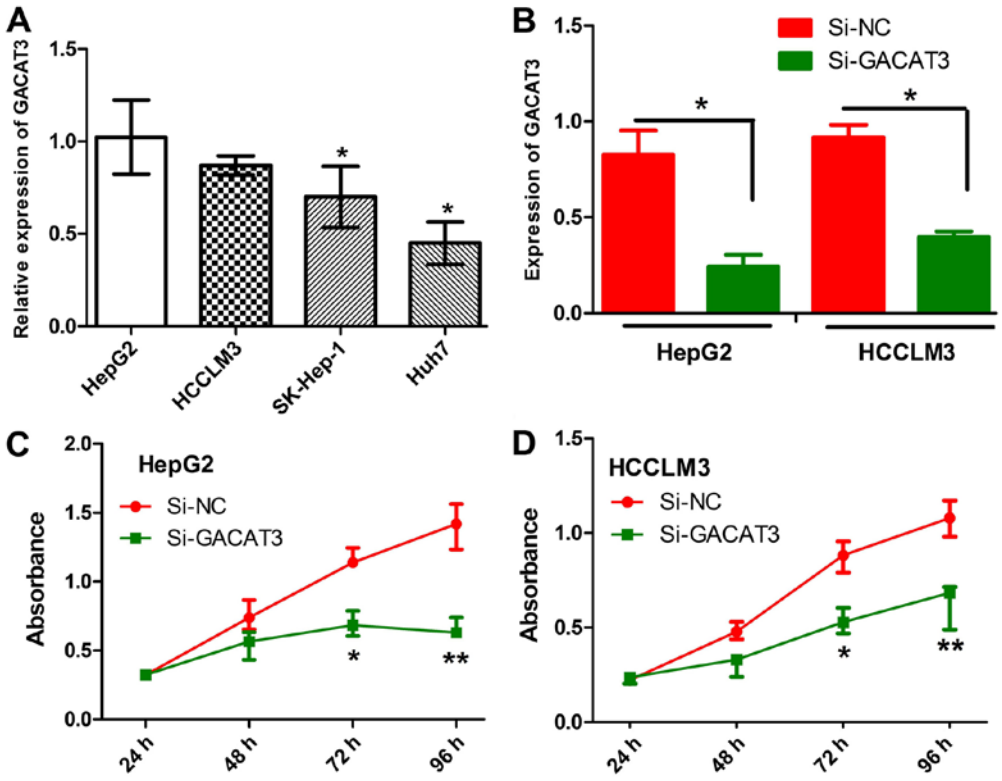

Figure 2. Effects of GACAT3 knockdown on liver cancer cell lines. (A) mRNA expression levels of GACAT3 in HepG2, HCCLM3, SK-Hep-1, SMMC-7721, Huh7 and QSG-7701 cells were detected by the RT-qPCR analysis. (B) Relative mRNA expression of GACAT3 in HepG2 and HCCLM3 cells transfected with si-GACAT3 mRNA or si-NC was detected by RT-qPCR analysis. Viability of (C) HepG2 and (D) HCCLM3 cells transfected with si-GACAT3 or si-NC was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. All data are expressed as the mean ± SEM (*P<0.05, **P<0.01). GACAT3, gastric cancer associated transcript 3; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; si, small interfering RNA; NC, negative control.

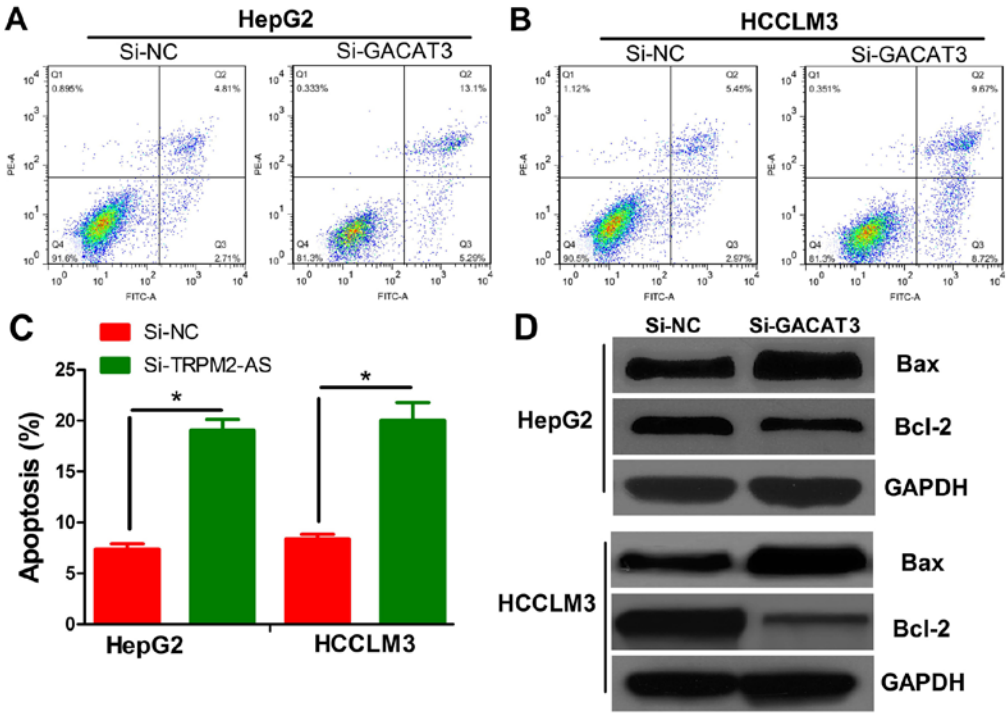

Figure 3. Effects of GACAT3 knockdown on the apoptosis and apoptosis-related proteins of HepG2 and HCCLM3 cells. Extent of apoptosis in (A) HepG2 and (B) HCCLM3 cells transfected with si-GACAT3 or si-NC was determined by flow cytometric analysis. (C) Histogram showing the extent of apoptosis from (A) and (B). Data are expressed as the mean ± SEM (*P<0.05). (D) Expression levels of Bax and Bcl-2 were measured by western blot analysis following GACAT3 knockdown with siRNA in HepG2 and HCCLM3 cells. GAPDH was used as the internal control. GACAT3, gastric cancer associated transcript 3; si/siRNA, small interfering RNA; NC, negative control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

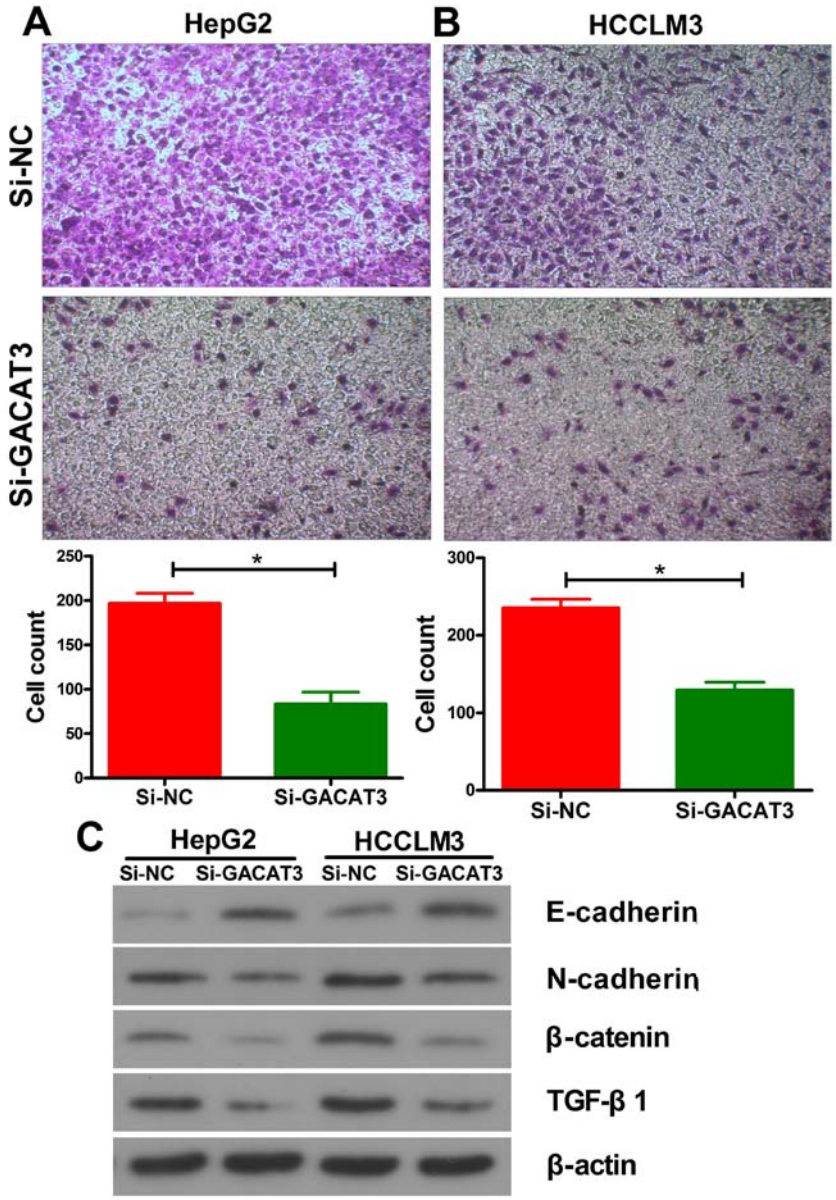

Figure 4. Effect of GACAT3 on cell migration in liver cancer cell lines. Cell migration was determined using a Transwell assay following GACAT3 knockdown with siRNA in (A) HepG2 and (B) HCCLM3 cells (magnification, x40). Data are expressed as the mean ± SEM (*P<0.05). (C) Protein levels of E-cadherin, N-cadherin, β-catenin and TGF-β1 were detected following the downregulation of GACAT3 in HepG2 and HCCLM3 cells. GACAT3, gastric cancer associated transcript 3; si/siRNA, small interfering RNA; NC, negative control.

upregulated in HCC tissues compared with that in adjacent normal tissues (P<0.001). These results indicate that GACAT3 may serve a vital role in the development of HCC.

mRNA expression of GACAT3 in liver cancer cell lines and the effect of GACAT3 knockdown on the viability in HepG2 and HCCLM3 cells. To assess the biological function of GACAT3 in HCC, the mRNA expression level of GACAT3 was examined in the QSG-7701 normal human liver cell line and the HepG2, HCCLM3, SK-Hep-1, SMMC-7721 and Huh7 liver cancer cell lines. The results from RT-qPCR analysis showed that the mRNA levels of GACAT3 were significantly upregulated in the liver cancer cell lines compared with that in the QSG-7701 cells (Fig. 2A).

HepG2 and HCCLM3 cells, with the highest mRNA levels of GACAT3, were selected for functional investigation. GACAT3 was silenced in these cells by transfecting them with the si-GACAT3 lentivirus. An MTT assay was used to determine their viability. The efficiencies of GACAT3 knockdown were verified by the RT-qPCR assay. The results showed that the mRNA expression of GACAT3 was significantly decreased in the HepG2 and HCCLM3 cells transfected with the si-GACAT3 lentivirus, compared with that in the control cells (Fig. 2B). The MTT assay indicated that the knockdown of GACAT3 significantly decreased the viability of HepG2 and HCCLM3 cells (Fig. 2C and D, P<0.05 and P<0.01). These results demonstrated that the expression of GACAT3 was high in liver cancer cell lines and its knockdown significantly inhibited liver cancer cell viability.

Effects of GACAT3 knockdown on apoptosis and apoptosis-related proteins of HepG2 and HCCLM3 cells. To

examine whether GACAT3 knockdown is involved in the apoptosis of liver cancer cell lines, the HepG2 and HCCLM3 cells were transfected with si-GACAT3 or si-NC lentivirus. These cells were then stained with Annexin-V/PI, followed by the detection of apoptosis using flow cytometry. The results showed that the knockdown of GACAT3 in both cell lines significantly promoted apoptosis (Fig. 3A-C $P<0.05$). Two apoptosis-related proteins (Bax and Bcl-2) were measured by western blot analysis to confirm the effects of GACAT3 on apoptosis. Bax was upregulated and Bcl-2 was downregulated in the HepG2 and HCCLM3 cells transfected with si-GACAT3 lentivirus, compared with levels in the siNC-transfected cells (Fig. 3D).

Effects of GACAT3 on cell migration in liver cancer cell lines. To evaluate the effect of GACAT3 on the development of HCC, the present study observed its effect on the migration in HepG2 and HCCLM3 cells using a Transwell assay. The results showed that GACAT3 knockdown significantly suppressed the migration ability of the HepG2 (Fig. 4A) and HCCLM3 (Fig. 4B) cells. These results suggested that the inhibition of GACAT3 decreased the migration ability of HCC cells. In order to analyze the mechanism of inhibition, the expression of EMT-related proteins were detected. As is shown in Fig. 4C, the expression levels of N-cadherin, β-catenin and TGF-β1 were decreased and that of E-cadherin was increased following the inhibition of GACAT3 in both liver cancer cell lines.

Discussion

lncRNAs perform crucial and complicated roles in cancer progression and carcinogenesis at the chromatin organizational, translational and post-translational levels (19). Accumulated evidence has shown that numerous lncRNAs are closely associated with the progression and development of HCC. For example, lncRNA UCA1 downregulated miR-216 and activated the fibroblast growth factor-1/extracellular signal-regulated kinase pathway, inhibiting the proliferation of HCC cells *in vitro* (20). lncRNA linc-USP16 regulated the phosphatase of tensin homolog and negatively regulated the invasion/migration of HCC cells (21). *In vitro*, the upregulation of the lncRNA HULC inhibited the expression of p18 and promoted HCC cell proliferation (22). However, numerous novel lncRNAs remain unknown.

Increasing evidence indicates that lncRNA GACAT3 serves complex roles in various aspects of cancer progression and carcinogenesis. Zhong *et al* (23) found that GACAT3 predicted poor prognosis and promoted cell proliferation in breast cancer through the regulation of miR-497/CCND2. Zhou *et al* (24) reported that GACAT3 promoted colorectal cancer cell proliferation, invasion and migration through miR-149. Yang *et al* (25) demonstrated that a high expression of GACAT3 inhibited the invasion and metastasis of non-small cell lung cancer, enhancing the effect of radiotherapy. In addition, GACAT3 promotes gastric cancer progression and cell proliferation by negatively regulating the expression of miR-497 and the interleukin-6/STAT3 signaling pathway, respectively (14,13). However, there has been no investigation on the effects of GACAT3 in HCC.

In the present study, it was demonstrated that the expression of GACAT3 was elevated in HCC tissues and cell lines. The results indicated that GACAT3 may be an important factor that can affect the pathological characteristics of HCC. Furthermore, experiments were performed at the tissue and cell level to confirm the biological function and mechanism of proliferation and metastasis of GACAT3 in HCC *in vitro*. The results revealed that the knockdown of GACAT3 in liver cancer cells significantly inhibited cell proliferation and migration by promoting apoptosis and influencing EMT. These results indicate that GACAT3 may be an oncogene in liver cancer. Therefore, GACAT3 may be a therapeutic target for the treatment of liver cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MC and DL were involved in the collection of HCC tissues. CJ and LC were mainly involved in cell culture and western blot analyses. LD was involved in designing the experiment, processing the main content of this experiment, and drafting the manuscript and revising it critically for important intellectual content.KZ was involved in the conception and design of the present study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Human HCC tissues were obtained with written and signed informed consent under a general waiver for the appropriate secondary use of human material and were obtained from Yinzhou People's Hospital. The study protocol was approved by the Ethics Committee of Yinzhou People's Hospital Affiliated to Ningbo University School of Medicine (Ningbo, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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