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Knockdown of Long Noncoding RNA GHET1 Inhibits Cell Proliferation and Invasion of Colorectal Cancer

Jianyu Zhou,* Xiaorong Li,* Meirong Wu,† Changwei Lin,* Yihang Guo,* and Buning Tian*

*Department of General Surgery, The Third Xiangya Hospital of Central South University, Changsha, Hunan, China
†Operation Center, The Third Xiangya Hospital of Central South University, Changsha, Hunan, China

Emerging evidence has identified the vital role of long noncoding RNAs (lncRNAs) in the development of colorectal cancer. In this study, we aimed to investigate the role of lncRNA gastric carcinoma highly expressed transcript 1 (GHET1) in colorectal cancer. We analyzed the expression of GHET1 in colorectal cancer (CRC) tissues by using ISH. We found that GHET1 expression was significantly increased in the CRC samples compared with adjacent tissues. Furthermore, the cancer tissues had higher GHET1 mRNA levels than their matched adjacent tissues. GHET1 expression was also significantly increased in the CRC cell lines compared with human normal colon epithelial cells. Downregulation of GHET1 mediated by shRNA suppressed the proliferation, cell cycle arrest, migration, and invasion of colorectal cancer cells in vitro. In addition, inhibition of GHET1 reversed the epithelial–mesenchymal transition in colorectal cancer cell lines. Taken together, our results suggest the potential use of GHET1 as a therapeutic target of colorectal cancer.

Key words: Long noncoding RNAs (lncRNAs); Colorectal cancer (CRC); Gastric carcinoma highly expressed transcript 1 (GHET1); Invasion; Proliferation; Epithelial–mesenchymal transition (EMT)

INTRODUCTION

The number of deaths caused by colorectal cancer (CRC) is ranked third in the world (1). Cancer cell invasion and metastasis are the main reasons for high mortality (approximately 30%) of CRC patients (2). Epithelial–mesenchymal transition (EMT) is able to promote cancer cell invasion and metastasis (3). EMT is a process for epithelial cells transforming into mesenchymal cells under specific pathological conditions. When epithelial cells undergo EMT, they lose typical intercellular junction structure, undergo cytoskeleton remodeling, and change shape to fibrocyte form, subsequently resulting in apoptosis resistance and enhanced motor ability. Thus, inhibition of EMT would be effective for suppression of cancer cell metastasis (4). Recent studies reveal that not only the coding RNA but also noncoding RNA can regulate EMT (5,6).

Noncoding RNAs include microRNAs and long noncoding RNAs (lncRNAs). Although microRNAs have been well studied, lncRNAs are newly discovered RNAs that make up 80% of noncoding RNAs and are needed to be deeply illustrated (7). lncRNAs have been linked to every stage of cell life, including cell proliferation, differentiation, apoptosis, and motility (8). lncRNAs have been identified as oncogene or tumor suppressors or a predictor of prognosis (9,10). For example, stable expression of lncRNA H19 significantly promotes EMT progression and accelerates colorectal tumor growth in vivo and in vitro (11), and the expression of lncRNA CCAT1 in colorectal tumor tissue was significantly higher than that in normal paracarcinoma tissue and was significantly correlated with local infiltration depth, tumor staging, and vascular invasion (12).

Gastric carcinoma highly expressed transcript 1 (GHET1, AK123072) is a recently identified long noncoding RNA. High GHET1 levels are correlated with tumor size, tumor invasion, and poor survival in gastric cancer, and GHET1 promotes gastric carcinoma cell proliferation via increasing c-Myc mRNA stability and expression (13). In addition, inhibition of GHET1 is able to reverse the EMT progression in a bladder cancer cell line (14). However, its role in CRC remains unclear.

In the present study, we explored the GHET1 expression pattern in CRC tissues and cell lines. In addition, we also evaluated the oncogenic role of GHET1 in CRC cells.

Address correspondence to Buning Tian, Department of General Surgery, The Third Xiangya Hospital of Central South University, Tongzipo Road No. 138, Changsha, Hunan 410013, China. Tel: +86-0731-88953543; Fax: +86-0731-88953543; E-mail: skypeblue1977@163.com

MATERIALS AND METHODS

Tissue Samples

A total of 20 colorectal carcinoma tissues and the paired adjacent tumor tissues were collected from The Third Xiangya Hospital of Central South University. Informed consents have been signed by all subjects. All samples were collected and identified by histopathological evaluation and stored at −80°C until used.

Cell Culture

The human CRC cell lines, Lovo, HCT-116, and Caco-2, and the human colonic epithelial cells, HCoEpiC, were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All the cells were cultured in RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA), supplemented with 10 % (v/v) fetal bovine serum (FBS; Invitrogen Life Technologies) at 37°C in a humidified 5% CO_2 incubator.

Cell Treatment

The RNA interference sequence (5′-CGGCAGGCATTA GAGATGAACAGCA-3′) was inserted into pRNAT-U6.1/ Neo plasmid to generate the pRNAT-U6.1/Neo-GHET1 plasmids (GHET1-shRNA). The empty pRNAT-U6.1/Neo plasmid was used as a negative control. To knock down GHET1 expression, Lovo and HCT-116 cells were transfected with empty vector or GHET1-shRNA for 48 h. The expression of GHET1 was detected by real-time PCR, and the transfected cells were used for further analysis.

In Situ Hybridization

The ISH probe used for detecting GHET1-labeled digoxin was designed and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The probe sequence was designed as GCAAAAAGACAGUAGGGAUGTGUC UGGAGCAACAAGGAUCGGGCUGUUCCUGGG GCAGCC. Slices were processed using Enhanced Sensitive ISH Detection Kit I (POD) (cat: MK1030; Boster, Wuhan, China) according to the manufacturer's protocol. The slides were visualized with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (BCIP/ NBT) (Solarbio, Beijing, China) for 5 min and counterstained with nuclear fast red for 60 s. The slides were mounted and dried. The positive cells were stained in purple red. Slides were photographed with an Olympus BX51 microscope (Olympus, Japan).

Real-Time Quantitative PCR (qPCR) Analysis

TRIzol reagent (Invitrogen, USA) was used to extract total RNA from the indicated cells and tissues according to the manufacturer's instructions. One-Step Real-Time RT-PCR Master Mixes (Thermo Fisher Scientific, Grand Island, NY, USA) was used for real-time PCR to detect the expression of GHET1. The primers of GHET1 and β-actin are as follows: GHET1 sense CCCC ACAAATGAAGACACT and antisense TTCCCAACA CCCTATAAGAT; β-actin sense AGGGGCCGGACTCG TCATACT and antisense GGCGGCACCACCATGTAC CCT. GHET1 expression was normalized by β-actin. All the qPCR data were processed using $2^{-\Delta\Delta CT}$ method.

CCK-8 Cell Proliferation Assay

Cell growth was measured by CCK-8 assay. One thousand Lovo or HCT-116 cells transfected with empty vector or GHET1-shRNA were seeded in each 96-well plate for 12 h and further incubated for 0, 24, 48, and 72 h, respectively. One hour before the ending of incubation, 10-μl CCK-8 reagents (Dojindo, Japan) were added to each well. Optical density (OD) 570 nm value in each well was determined by an enzyme immunoassay analyzer.

Flow Cytometric Analysis of the Cell Cycle

Cells from each group were trypsinized and washed with cold PBS. The cells were incubated in 75% ethanol at 4°C overnight. After washing with PBS once, cells were treated with RNAase (Takara, Japan) for 15 min at 37°C and then stained by BD Cycletest™ Plus (BD Biosciences) according to the manufacturer's instructions. The cell cycle was analyzed by flow cytometry (Beckman Coulter, USA). The experiments were independently performed in triplicate.

Scratch Assay

Cells in each group were collected and resuspended in RPMI-1640 medium. Each well of a six-well plate was seeded with 5×10^5 cells and cultured for 24 h to 100% confluence. The cells were scratched with the head of a 200-μl tip and washed with serum-free medium. These cells were further cultured for 24 h in serum-free medium. After that, serum-free medium were replaced with RPMI-1640 medium containing 3% FBS and continued to culture for 72 h; these cells in each group were then photographed for analysis.

Transwell Assay

The indicated cells were starved for 24 h and then resuspended in serum-free medium and added to the upper chamber. The lower chamber was filled with medium containing 10% FBS. Following 48 h of culture, cells attached to the bottom were fixed and stained with crystal violet for 45 min and dried in air. The OD at 570 nm of crystal violet dissolved by 10% acetic acid was detected by an enzyme immunoassay analyzer (Synergy™ Mx; BioTek, Winooski, VT, USA).

Western Blot

RIPA lysis buffer (Auragene, Changsha, China) was used to extract protein from indicated cells. Bradford Protein Assay Kit (Beyotime, Shanghai, China) was used to measure the protein concentration. A total of 50 μg of protein was separated on 10% SDS-PAGE gels and blotted

onto 0.22-μm nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk for 2 h and incubated with primary antibodies (rabbit polyclonal anti-E-cadherin, cat No. ab15148, 1:1,000 dilution), rabbit polyclonal anti-fibronectin (cat No. ab2413, 1:1,000 dilution), mouse monoclonal anti-vimentin (cat No. ab8978, 1:500 dilution) from Abcam (Cambridge, UK), and mouse monoclonal anti-β-actin (cat No. 60008-1-Ig, 1:5,000 dilution) from Proteintech (Wuhan, China) overnight at 4°C. The membranes were washed with Tris-buffered saline containing 0.1% Tween 20 (TBST), and then incubated with appropriate horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit, 1:2,000; goat anti-mouse, 1:5,000; Auragene) for 1 h at 37°C. Enhanced chemiluminescence reagent (Merck Millipore, Germany) was used to detect the signal on the membrane. The data were analyzed via densitometry using Image-Pro plus software 6.0 (Media Cybernetics, Rockville, MD, USA) and normalized to the expression of the internal control (β-actin).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA), and the data are presented as the mean ± standard deviation (SD). An unpaired two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) with Bonferroni posttest was used to analyze the data depending on conditions. A value of $p < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

GHET1 Is Significantly Upregulated in CRC Cancer Tissues and Cell Lines

To investigate the role of GHET1 in CRC, we first analyzed the expression of GHET1 in CRC tissues by using ISH. We found that GHET1 expression was significantly increased in the CRC samples compared with the adjacent tissues (Fig. 1A). Furthermore, we analyzed GHET1 expression in the cancer tissues and their matched adjacent tissues by using real-time qPCR. All of the cancer tissues had higher GHET1 levels than their matched adjacent tissues (Fig. 1B). In addition, similar results were observed in the CRC cell lines (Fig. 1C). These results indicate that GHET1 may act as an oncogene contributed to tumor development.

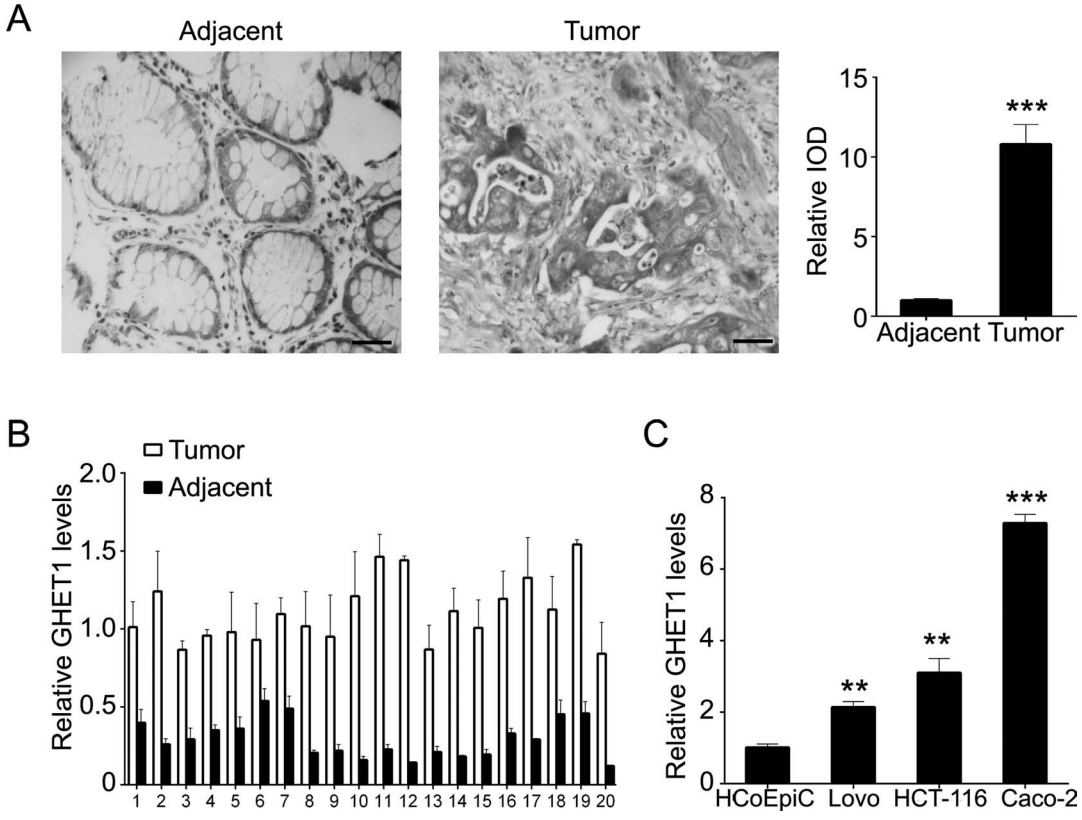

Figure 1. Expression of GHET1 in CRC cancer tissues and CRC cell lines. (A) Representative images of ISH for GHET1 in CRC adjacent tissues and tumor tissues (left), quantification of integral optical intensity (IOD) of staining (right). Scale bars: 100 μm. (B) Real-time PCR analysis for GHET1 in 20 paired CRC adjacent and tumor tissues. (C) Real-time PCR analysis for GHET1 in CRC cell lines. Data are presented as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$.

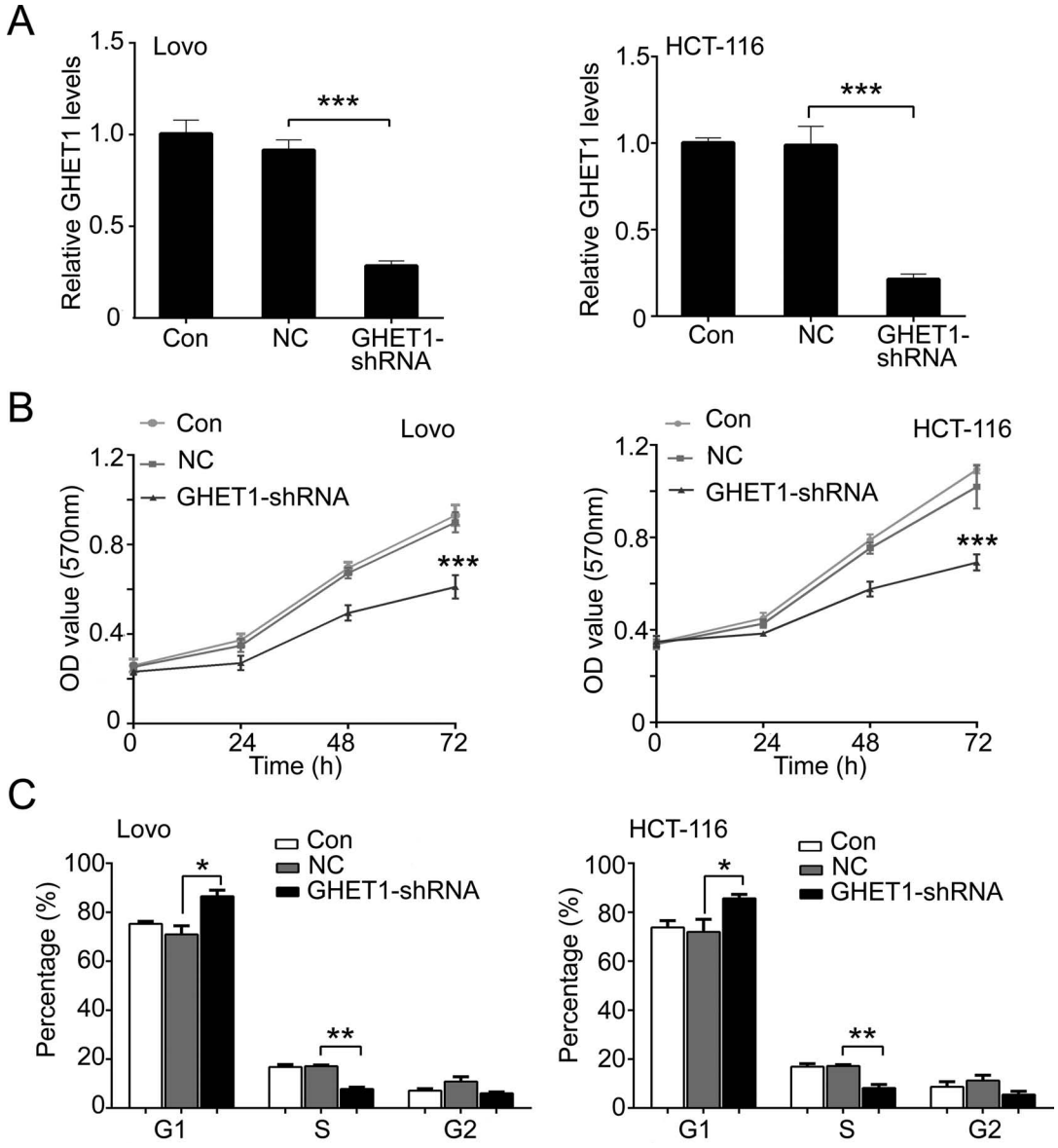

Figure 2. Knockdown of GHET1 inhibits cell proliferation and arrests cell cycle at S phase in Lovo and HCT-116 cells. (A) Real-time PCR analysis for GHET1 in Lovo and HCT-116 cells transfected with negative control sequence (NC) or GHET1-shRNA. (B) MTT assay in Lovo and HCT-116 cells transfected with negative control sequence (NC) or GHET1-shRNA. (C) Flow cytometric analysis for cell cycle in Lovo and HCT-116 cells transfected with negative control sequence (NC) or GHET1-shRNA. Data are presented as mean ± SD. Experiments were independently repeated three times. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Knockdown of GHET1 Reduces the Ability of Cell Proliferation and Invasion in Lovo and HCT-116 Cells

In order to investigate the role of GHET1 in CRC cells, we transfected the Lovo and HCT-116 cells with GHET1-shRNA or empty vector plasmid (Fig. 2A). CCK-8 was used to detect the Lovo and HCT-116 cell proliferation. We found that inhibition of proliferation was induced by downregulation of GHET1 (Fig. 2B). The results of flow cytometric analysis revealed that knockdown of GHET1 induced cell cycle arrest with a significant decrease in S phase in Lovo and HCT-116 cells (Fig. 2C). Scratch assay showed that the migration ability was significantly decreased in GHET1 group compared with negative control group (Fig. 3A). Invasive ability was also significantly reduced in Lovo and HCT-116 cells transfected with GHET1-shRNA compared with negative control (Fig. 3B).

GHET1 Regulates EMT-Related Gene Expression

To further investigate the underlying mechanism of GHET1 in CRC cancer cell invasion, we analyzed

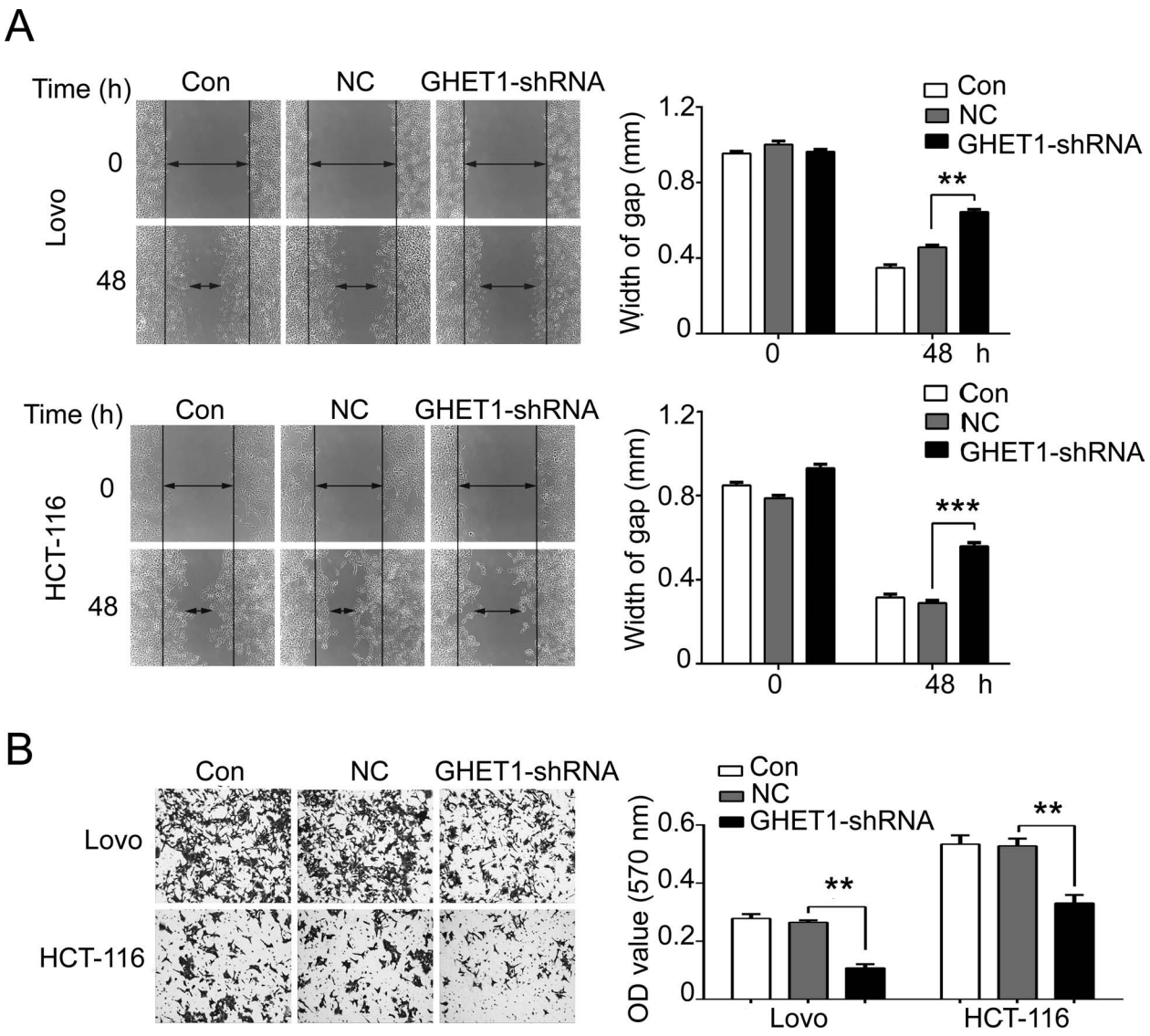

Figure 3. Knockdown of GHET1 suppresses the ability of cell migration and invasion. (A) Scratch assay in Lovo and HCT-116 cells transfected with negative control sequence (NC) or GHET1-shRNA and quantification of the gap. (B) Transwell assay in Lovo and HCT-116 cells transfected with negative control sequence (NC) or GHET1-shRNA and quantification of the optical density (OD) of staining. Data are presented as mean ± SD. Experiments were independently repeated three times. $^{**}p<0.01$, $^{***}p<0.001$.

whether GHET1 regulated EMT signaling. We found that knockdown of GHET1 dramatically increased E-cadherin expression, while reducing fibronectin and vimentin levels (Fig. 4).

DISCUSSION

CRC is one of the most common carcinomas worldwide. To our knowledge, many mutations in oncogenes and tumor-suppressive genes have been identified, such as MDM2 and p53 (15). Recently, emerging evidence demonstrates that a large number of noncoding RNAs, including miRNAs and lncRNAs, also contribute to the progression of CRC (16,17). However, the mechanisms of CRC cell invasion, proliferation, and apoptosis resistance remain largely unknown. lncRNAs have been involved in several types of cancer (18,19). lncRNA GHET1 was initially characterized in gastric cancer and found to promote gastric cancer cell proliferation via increasing c-Myc mRNA stability and expression (13). In addition, GHET1 was also clinically and functionally relevant to the development of bladder cancer, which increased GHET1 expression correlated with tumor size and tumor status (14).

In this study, we found that GHET1 expression was significantly increased in CRC tissues compared with the matched adjacent tissues. This increased expression was also observed in CRC cell lines. We knocked

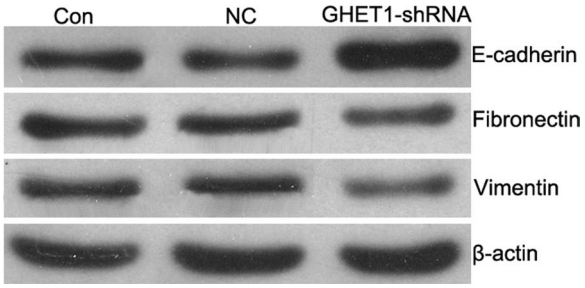

Figure 4. GHET1 regulates EMT-related genes. Western blot analysis for E-cadherin, fibronetin, and vementin in HCT-116 cells transfected with negative control sequence (NC) or GHET1-shRNA.

down GHET1 expression in CRC cells by using shRNA transfection and found a great reduction in cancer cell proliferation and invasion with GHET1 knockdown. To investigate the underlying mechanism through which GHET1 promoted the proliferation of gastric cancer cells, we performed cell cycle analysis. GHET1 induced a higher percentage of G_1 phase and a lower percentage of S phase, indicating a significant G_0/G_1 arrest. The data suggest that knockdown of GHET1 might inhibit cell proliferation through inducing G_0/G_1 arrest.

The EMT process occurs during embryonic development and tumorigenesis (20). The EMT process plays a vital role in cancer invasion and metastasis (3). Essential hallmarks of EMT include loss of the E-cadherin expression and increased expression of vimentin and fibronectin (21). Previous studies showed that the transcription factors, such as ZEB1 and Twist, were able to regulate the EMT process (22,23). Recent studies also demonstrate that long noncoding RNAs can play a role in the regulation of EMT (24,25). For example, SPRY4-IT1 promoted EMT in glioma cells (26). lncTCF7 promoted hepatocellular carcinoma aggressiveness through EMT (27). lncRNA Hh functioned as an important regulator endowing Twist-induced EMT cells to gain the CSC-like stemness properties in breast cancer (28). Thus, we examined the expression levels of hallmarks of EMT in colorectal cancer cells with GHET1 knockdown. We found that knockdown of GHET1 induced an upregulation of E-cadherin expression, whereas it downregulated vimentin and fibronectin expression, restoring CRC cells to more of an epithelial phenotype. Our results suggest that the inhibitory effect on cell invasion of GHET1 silencing was associated with the EMT process.

In summary, we demonstrated that the expression of GHET1 was significantly upregulated in CRC tissues compared with the matched adjacent normal tissues. We also showed that GHET1 promoted the proliferation and invasion of CRC cells. Our study also indicated that the molecular mechanism by which GHET1 contributed to the tumor progression might be via the EMT process and may lead to the development of a novel diagnostic marker and therapeutic strategy for CRC.

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