

Original Article

Long noncoding RNA GHET1 promotes the development of bladder cancer

Lin-Jin Li¹, Jian-Long Zhu¹, Wen-Shuo Bao¹, Da-Ke Chen¹, Wei-Wen Huang¹, Zhi-Liang Weng²

¹Department of Urology, The Third Clinical Institute Affiliated to Wenzhou Medical University (Wenzhou People's Hospital), Wenzhou, China; ²Department of Urology, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China

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Abstract: In spite of the advances in the diagnosis and treatment of bladder cancer, the prognosis of bladder cancer remains relatively poor. As a result, it is vital to identify novel diagnostic and prognostic marker of bladder cancer. A growing volume of literature has implicated the vital role of long noncoding RNA in the development of cancer. GHET1, a recently identified lncRNA, was initially characterized in gastric cancer. However, its role in bladder cancer remains largely unknown. In this study, we demonstrated that GHET1 was upregulated in bladder cancer tissues compared to adjacent normal tissues and its over-expression correlates with tumor size, advanced tumor and lymph node status, and poor survival. GHET1 knockdown suppressed the proliferation and invasion of bladder cancer cells *in vitro*. In the meantime, inhibition of GHET1 reversed the epithelial-mesenchymal-transition in bladder cancer cell line. Taken together, our study suggests that the potential use of GHET1 as a prognostic marker and therapeutic target of bladder cancer.

Keywords: Long noncoding RNA, bladder cancer, GHET1, prognosis, proliferation, epithelial-mesenchymal-transition

Introduction

Bladder cancer is the ninth most common cancer worldwide [1]. The majority of malignant bladder tumors are urothelial cell carcinomas evolved from urothelium. About 40% of the patients with bladder cancer experience multiple recurrences, which has a significant impact on the quality of life [2]. Bladder cancer is a heterogeneous disease which can be categorized into the low-grade papillary tumors and the high-grade invasive tumors. Invasive bladder tumor is more aggressive and patients with invasive disease have a much worse prognosis, with a 5-year survival rate around 50% [3]. Despite the recent advances in the early detection and more frequent surgical treatment, the mortality has not changed markedly [4]. Therefore, it is vital to clarify the molecular mechanism of the development of bladder cancer for the advances in novel diagnostic marker and therapeutic targets.

It is well known that protein-coding genes account for only about 2% of the human genome, whereas the vast majority of tran-

scripts are non-coding RNAs [5]. The ncRNAs include not only well-studied microRNAs and other noncoding transcripts less than 200 nucleotides (nt) but also a large class of long (> 200 nt) ncRNAs (lncRNAs), which have emerged as a new layer of cell biology [6, 7]. Although researches of miRNAs have dominated the field of RNA regulation in bladder cancer [8-10], accumulating evidence has indicated that long noncoding RNA may also play an important role in the development of cancer [11-13].

A recently identified long noncoding RNA, named gastric carcinoma high expressed transcript 1 (GHET1, AK123072), was upregulated in gastric cancer and the high expression level of this lncRNA correlates with tumor size, tumor invasion and poor survival [14]. Yang et al. [14] demonstrated that GHET1 promotes the proliferation via increasing c-Myc mRNA stability and expression. However, the role of GHET1 in bladder cancer is elusive.

In the present study, we explored GHET1 expression pattern in bladder cancer and examined its correlation with clinicopathological factors. In

addition, the prognostic value of GHET1 was evaluated. The oncogenic activity of GHET1 was investigated in bladder cell lines.

Materials and methods

Cell culture

Four human bladder cancer cell lines (RT4, RT112, 253J and T24), and a normal human uroepithelial cell line (CRL-9520) were obtained and maintained as recommended by American Type Culture Collection (ATCC, Manassas, VA). All cell lines have been passaged for fewer than 6 months.

Human tissue specimens

The study were undertaken with the understanding and written consent of each subject. The study methodologies conformed to the standards set by the declaration of Helsinki. This study was approved by the Human Ethics Committee of the Third Medical College of Wenzhou City affiliated to Wenzhou Medical University (Wenzhou, China). Eighty bladder cancer tissues and adjacent non-tumor bladder tissues were obtained from patients who underwent resection of the primary bladder cancer at the Third Medical College of Wenzhou City affiliated to Wenzhou Medical University Hospital between 2009 and 2011 and were diagnosed with bladder cancer based on histopathological evaluation. No anti-cancer treatments were given before biopsy collection. Complete clinicopathological data of the patients were available. No selection bias was introduced in bladder cancer samples collection for this study. The overall survival (OS) was defined as the interval between surgery and death or the last follow-up examination.

Quantitative real-time PCR

Total RNA from tissues or cells was extracted using Trizol reagent (Invitrogen, CA). RNA was reverse transcribed to cDNA by a Reverse Transcription Kit (Takara, Dalian, China). The cDNA template was amplified by real-time RT-PCR using the SYBR® Premix Dimmer Eraser kit (TaKaRa, Dalian, China). Real-time PCR was performed using a standard SYBR Green PCR kit (Toyobo, Osaka, Japan) protocol on Applied Biosystems 7300 Real Time PCR system (Applied Biosystems, Foster City, CA) according to the instructions. Glyceraldehyde-

3-phosphate dehydrogenase (GAPDH) was measured as an internal control. The $2^{-\Delta\Delta Ct}$ method was employed to determine the relative quantitation of gene expression levels. The primer sequences were as follows: GAPDH: 5'-GTCAACGGATTTGGTCTGTATT-3' (forward), 5'-AGTCTTCTGGGTGGCAGTGAT-3' (reverse); β -actin: 5'-GAAATCGTGCCTGACATTAA-3' (forward), 5'-AAGGAAGGCTGGAAGAGTG-3' (reverse); GHET1: 5'-CCCCACAAATGAAGACT-3' (forward), 5'-TTCCCAACACCCTATAAGAT-3' (reverse). Each sample was analyzed in triplicate. qRT-PCR results were analyzed and expressed relative to CT (threshold cycle) values, and then converted to fold changes.

Small interfering RNA (siRNA)

siRNA specifically targeting GHET1 was synthesized by Invitrogen. The GHET1 specific siRNA sequences were as follows: GHET1 siRNA (siGHET-1): CGGCAGGCATTAGAGATGAACAGCA. Allstars Negative Control siRNA were purchased from Qiagen, Hilden, Germany. Cells were grown on six-well plates to 70% confluency and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were harvested for qRT-PCR or western blot analyses.

Cell proliferation assay

Cell proliferation assays were performed using the CCK-8 assay kits (Roche) following the manufacturer's instructions. EdU immunofluorescence staining was performed using an EdU kit (Roche).

Flow-cytometric analysis

Cells transiently transfected with si-NC or si-GHET1 were harvested 48 h after transfection. Cells were treated with PI (50 mg/L) for 30 min at 4°C in the dark. The cell-cycle profiles were assayed at 488 nm on an EPICS 752 flow cytometer (Coulter, Hialeah, FL) equipped with MPLUS software (Phoenix 140 Flow Systems, San Diego, CA). Data were expressed as percentage distribution of cells in G0/G1, S and G2/M phases of the cell cycle.

Cell migration and invasion assay

Cell transfected with si-NC or si-GHET1 were harvested 48 h after transfection. For migra-

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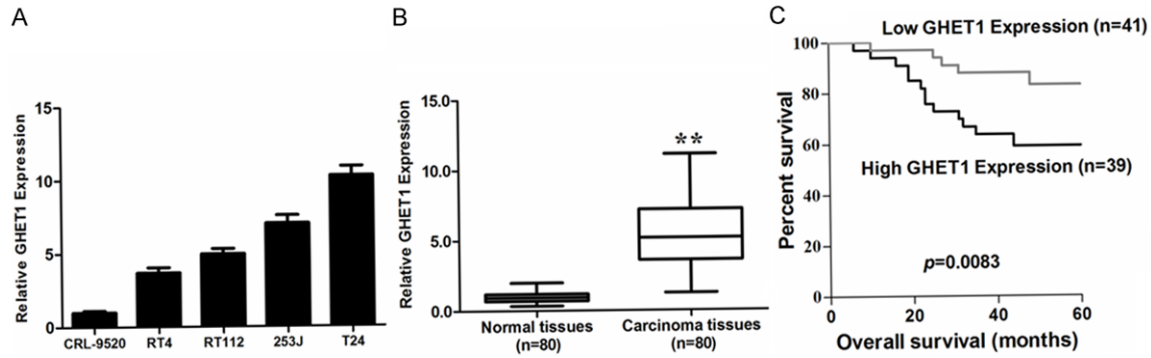


Figure 1. GHET1 expression in bladder cancer cell lines, cancer tissues and its clinical significance. A: qRT-PCR analysis of GHET1 expression levels in bladder cancer cell lines (RT4, RT112, 253J and T24) compared with the normal human uroepithelial cell line (CRL-9520). B: Difference in expression levels of GHET1 between bladder cancer tissues and matched non-tumor bladder tissues. The expression of GHET1 was normalized to GAPDH. The statistical differences between samples were analyzed with paired samples t-test ($n = 80$, $P < 0.0001$). C: Patients with high levels of GHET1 expression showed reduced overall survival times compared with patients with low levels of GHET1 expression ($P = 0.0083$, log-rank test). * $P < 0.05$; ** $P < 0.01$.

Table 1. Correlation between GHET1 expression and clinicopathologic characteristics

Clinicopathologic characteristics	Number of patients (%)	Relative expression of GHET1 ^a	P value ^b
Gender			
Male	43 (53.75%)	5.33	0.66
Female	37 (46.25%)	5.56	
Age			
< 60	32 (40.00%)	5.01	0.17
≥ 60	48 (60.00%)	5.76	
Number of tumors			
single	66 (82.50%)	4.97	0.09
multiple	14 (17.50%)	5.98	
Tumor status			
Ta-T1	65 (81.25%)	4.17	0.005
≥ T2	15 (18.75%)	8.12	
Grade			
G1/2	31 (38.75%)	3.45	0.003
G3	49 (61.25%)	7.91	

^aMedian of relative expression; ^b $P < 0.05$ was considered significant (Mann-Whitney U test).

tion assay, 1×10^5 cells were plated on the non-coated membrane in the top chamber (24-well insert; 8-mm pore size, Corning Costar Corp). Cells were plated in medium without serum. Medium supplemented with 10% FBS was used as a chemo-attractant in the lower chamber. For invasion assay, the upper chamber was pre-coated with Matrigel (Sigma, USA). After 24 h of incubation, cells that did not migrate or invade through the pores were removed using a cotton swab, whereas the

cells on the lower surface were fixed with methanol and stained with 0.05% crystal violet. Finally, cells were counted under a microscope and the relative number was calculated. Experiments were independently repeated in triplicate.

Immunofluorescence analysis

Cells transfected with si-NC or si-GHET1 were cultured and fixed on 12×12 mm glass slides. For membrane staining (E-cadherin), cells were fixed with cold 100% methanol for 12 minutes. For intracellular staining (vimentin, fibronectin), the cells were fixed with 4% (wt/vol) paraformaldehyde in PBS and permeabilized by incubation with 0.5% Triton X-100 in PBS for 2 minutes. The cells were incubated with 3% bovine serum albumin in PBS for 30 minutes at room temperature. After washing with PBS, the cells were incubated with specific primary antibody at 4°C overnight. The cells were then washed and incubated with Alexa Fluor 633-conjugated goat anti-rabbit IgG for 1 hour. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Sections were visualized by fluorescence microscopy.

Western blot analysis

Cells were lysed using RIPA buffer (Pierce) containing protease inhibitors cocktail (Roche).

Total cell lysates were prepared in a 1 × sodium dodecyl sulfate buffer. Identical quantities of proteins (30-50 µg) were separated by 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto PVDF Immobilon-P membranes. The membrane was blocked with 5% skim milk in TBST. After an incubation with antibodies specific for ZEB1 (Sigma-Aldrich), Twist (Sigma-Aldrich), E-cadherin (Abcam), fibronectin (Abcam), Snail (Abcam), vimentin (Cell Signaling Technology), or β-actin (Sigma-Aldrich) with gentle shaking at 4°C overnight, the blots were incubated with Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody. Antibody-bound proteins were detected by BeyoECL Plus kit. β-actin was used as a loading control for Western blots.

Statistical analysis

All data were presented as mean ± standard error from three independent experiments. All statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, USA). The gene expression level of GHET1 in tumors was compared with adjacent normal tissues utilizing the paired sample *t*-test, whereas the association between GHET1 expression and clinical characteristics was evaluated using the Mann-Whitney test. Survival curves were plotted with the Kaplan-Meier method, and the long-rank comparison was carried out to assess differences between stratified survival groups using the median value as the cutoff. The expression differences between cell lines, the expression changes after transfection, cell cycle, cell migration and invasion assays were analyzed using independent samples *t*-test. All tests performed are two sided. Differences were considered statistically significant at $P < 0.05$.

Results

Upregulation of GHET1 in bladder cancer cell lines

To determine the expression profile of GHET1 in bladder cancer, we at the first place measured the expression level of GHET1 in bladder cancer cell lines. When normalized to human uroepithelial cell line (CRL-9520), the expression level of GHET1 was upregulated in bladder cancer cell lines (RT4, RT112, 253J and T24) (**Figure 1A**).

GHET1 is upregulated in bladder cancer tissues and is associated with poor prognosis

The expression level of GHET1 in bladder cancer tissues and pair-matched adjacent normal bladder tissues from 80 bladder cancer patients was examined by quantitative real-time PCR. As illustrated in **Figure 1B**, GHET1 was significantly upregulated in bladder cancer tissues compared to adjacent normal tissues ($P < 0.0001$ by Wilcoxon signed-rank test). Then we analyzed the correlation between the expression level of GHET1 and the clinicopathological characteristics of the 80 bladder cancer patients. Correlation regression analysis showed that increased GHET1 expression correlated with advanced tumor status ($P = 0.005$) and grade ($P = 0.003$). However, the GHET1 expression level was not associated with other parameters such as age and gender (**Table 1**).

We further determined whether the expression level of GHET1 correlated with the clinical outcome of bladder cancer patients. Kaplan-Meier survival analysis and log-rank tests using patient postoperative survival were conducted to further evaluate the correlation between GHET1 and prognosis of patients with bladder cancer. According to the median ratio of relative GHET1 expression (5.44) in tumor tissues, the 80 bladder cancer patients were classified into two groups: High-GHET1 group ($n = 39$): GHET1 expression ratio \geq median ratio; and Low-GHET1 group ($n = 41$): GHET1 expression ratio \leq median ratio. Kaplan-Meier survival analysis showed that high GHET1 expression in bladder carcinoma tissues is significantly associated with worse overall survival ($P = 0.0083$, log-rank test) (**Figure 1C**). These results suggest that GHET1 may play an important role in the progression of bladder cancer.

Knockdown of GHET1 suppressed the proliferation of bladder cancer cells

Yang et al. [14] demonstrated the GHET1 promoted the proliferation of gastric cancer cells. We would like to explore the effect of GHET1 on bladder cancer cells. The expression level of GHET1 was significantly downregulated in bladder cancer cell line T24 by si-GHET1 (**Figure 2A**).

CCK-8 assays indicated the inhibition of GHET1 suppressed the T24 cell proliferation (**Figure**

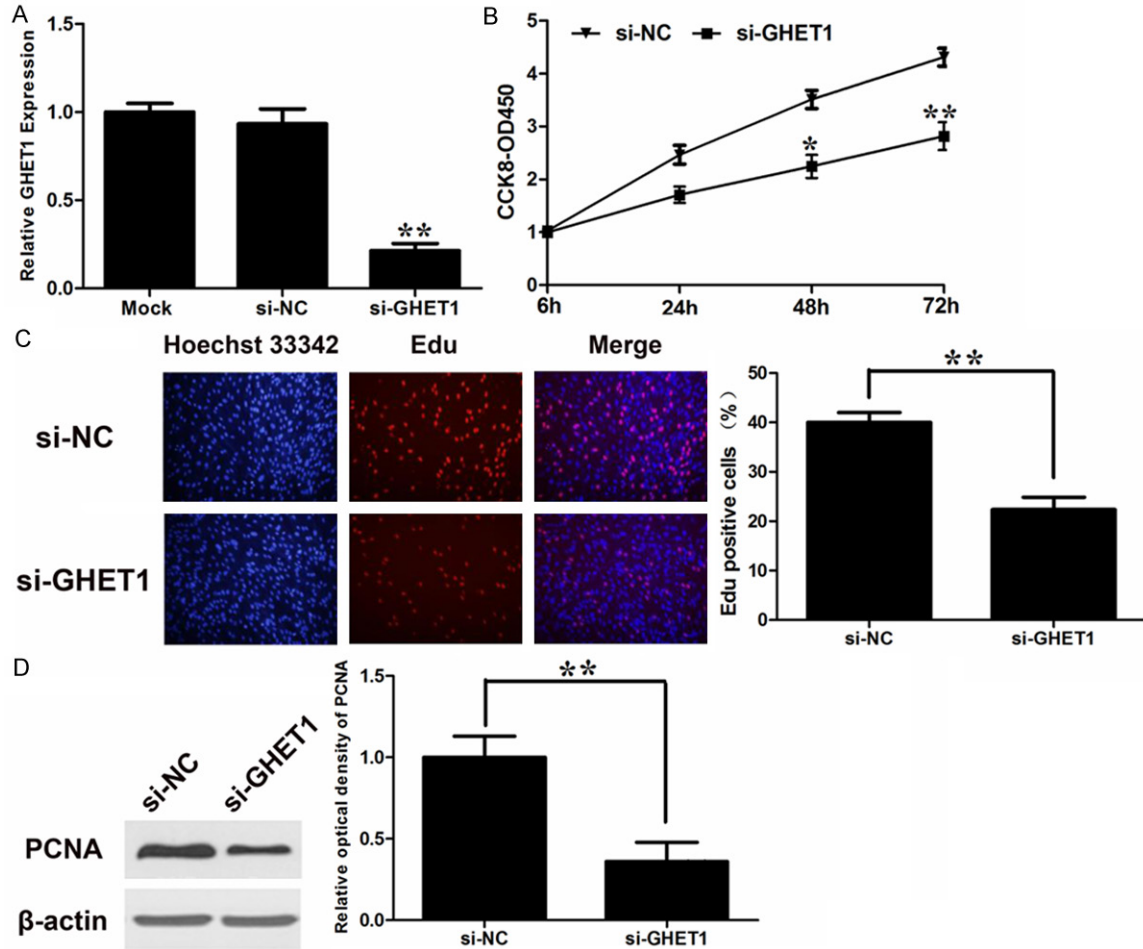


Figure 2. Effect of GHET1 on cell proliferation. (A) qRT-PCR analysis of GHET1 expression following treatment of T24 cells with specific siRNA targeting GHET1. (B) GHET1 silencing inhibited DNA replication in bladder cancer cells compared to controls as determined by the EdU incorporation assay, Original magnifications: $\times 200$. EdU-positive cell counts were analyzed by using software of the light microscope (C) T24 cells were transfected with si-GHET1 or si-NC. CCK8 assays were performed to determine the proliferation of T24 cells. Data represent the mean \pm S.D. from three independent experiments. (D) Changes in the proliferation marker, PCNA, were shown by western blotting analysis and normalized to β -actin after si-GHET1 transfection. Relative protein expression was identified (n = 3). Data represent the mean \pm S.D. from three independent experiments. * $P < 0.05$; ** $P < 0.01$.

2B). Furthermore, the percentage of EdU-positive cells was reduced with GHET1 knock-down (Figure 2C). Proliferating cell nuclear antigen (PCNA) is deemed as one of the most important index to estimate cell proliferation. Consistent the attenuated proliferation, T24 cells had markedly decreased expression of PCNA with GHET1 knockdown (Figure 2D). These data suggest that GHET1 may take a part in the regulation of cell proliferation. To explore the underlying mechanism of proliferation-promoting effect of GHET1 on bladder cancer cell, we studied the effect of GHET1 knock-down on cell cycle. As demonstrated Figure 3A, knockdown of GHET1 resulted in a blockade of

T24 cells in G0/G1 phase compared to the control cells. It hints that knockdown of GHET1 may inhibit the proliferation of bladder cancers via inducing G0/G1 arrest. To verify our hypothesis, we examined the protein levels of G0/G1 arrest markers, p16 and p21 after GHET1 knockdown. The western blot analysis showed that p16 and p21 proteins were increased with GHET1 knockdown (Figure 3B).

GHET1 promoted the invasion of bladder cancer cells

As correlation regression analysis showed that increased GHET1 expression correlated with

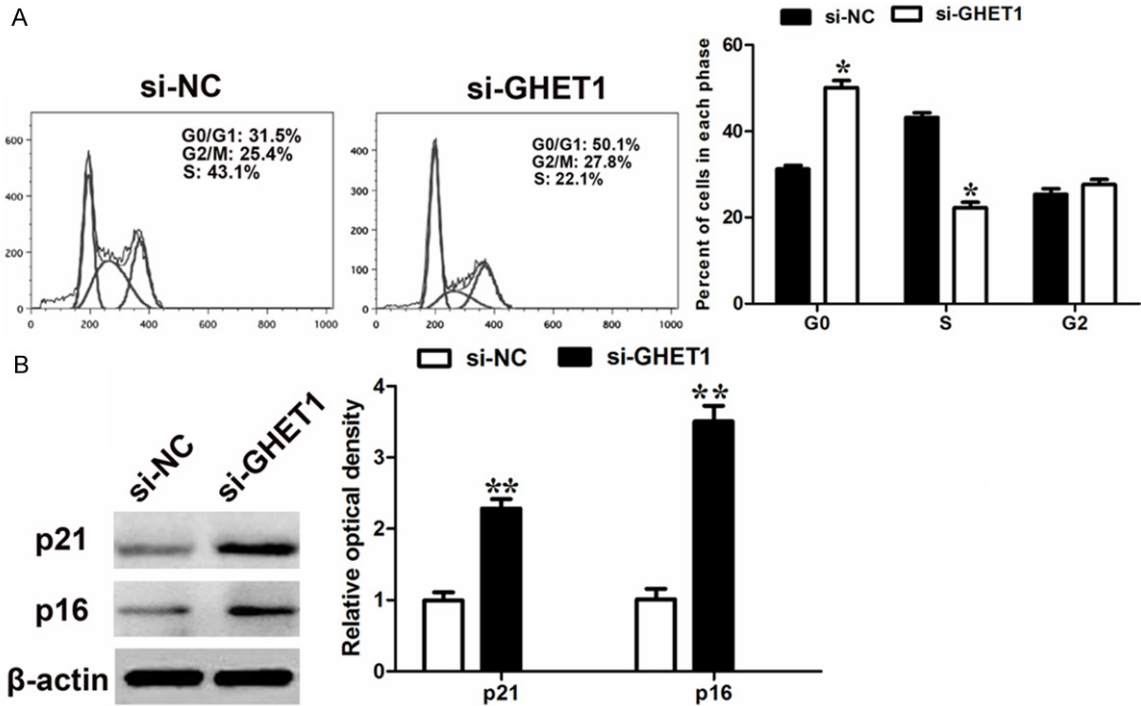


Figure 3. Knockdown of GHET1 may inhibit the proliferation of bladder cancers via inducing G0/G1 arrest. A: Cell cycle analysis determined the relative cell numbers in each cell-cycle phase after propidium iodide staining of GHET1-downregulated T24 cells. Numbers inside bars represent percentages of cells in each phase. B: T24 cells, treated as described in **Figure 2C, 2D** were collected for western blotting analysis of the G0/G1 arrest markers, p16 and p21. Relative protein expression was identified (n = 3). Data represent the mean ± S.D. from three independent experiments. * $P < 0.05$; ** $P < 0.01$.

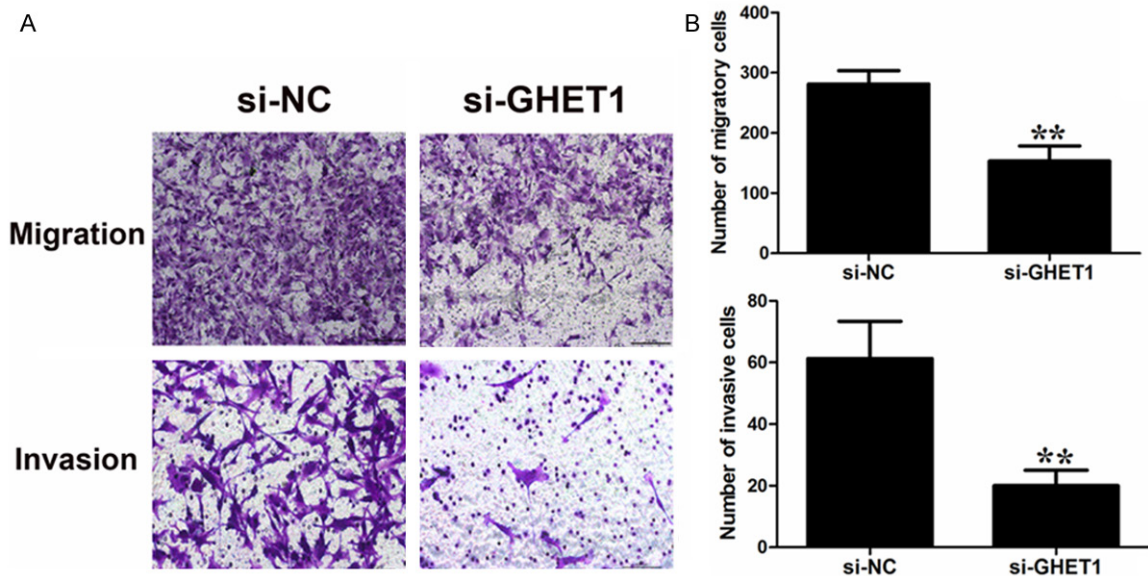


Figure 4. Effect of GHET1 on cell migration and invasion. A, B: T24 cells were transfected with GHET1 siRNA or si-NC. Transwell assays were performed to investigate the migratory and invasive ability of bladder cancer cells. Data represent the mean ± S.D. from three independent experiments. * $P < 0.05$; ** $P < 0.01$.

and tumor status ($P = 0.005$), we would like to explore whether GHET1 has a role in facilitating

bladder cancer migration and invasion. A significant decrease in cancer cell migration and

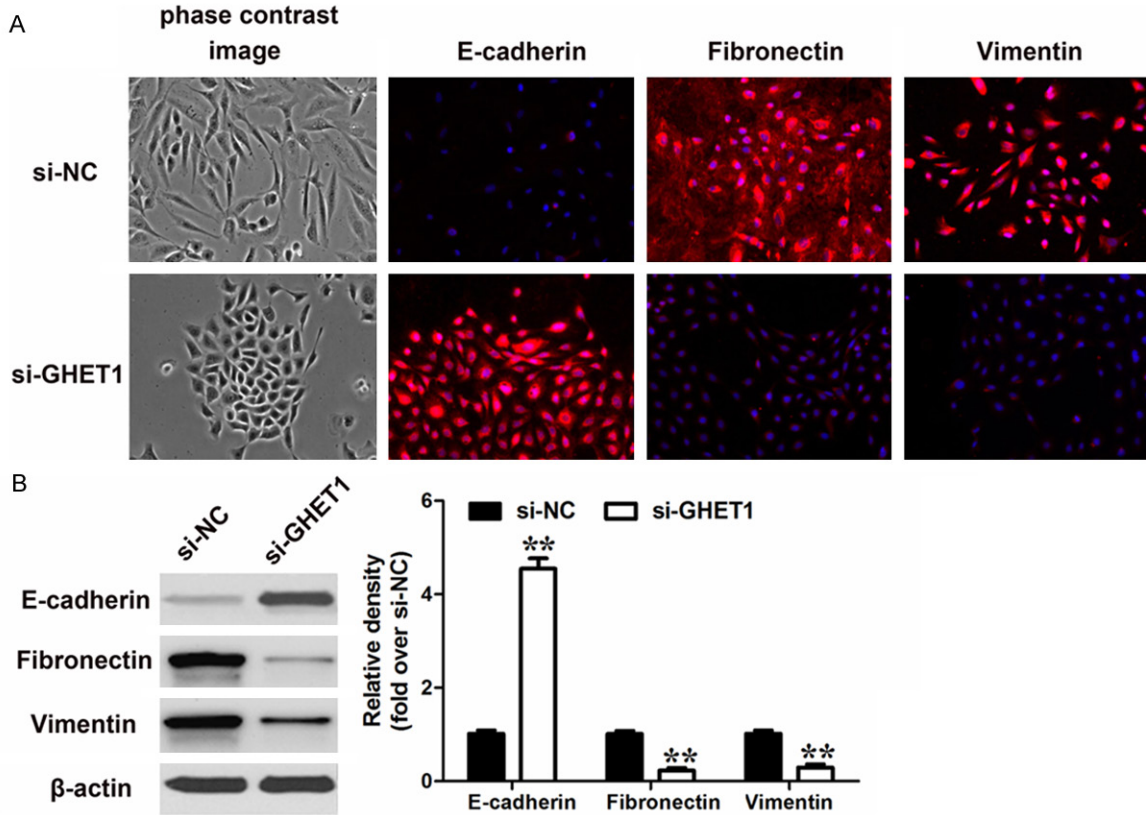


Figure 5. Effect of GHET1 on the EMT process. A: Phase-contrast images (up) and immunofluorescence images (down) of T24 cells stained using antibodies against E-cadherin, Fibronectin or Vimentin after transfected with si-GHET1. B: Western-blot analysis of phenotypic markers after GHET1 knockdown in T24 cells. Relative protein expression was identified (n = 3) and normalized to β -actin. Data represent the mean \pm S.D.

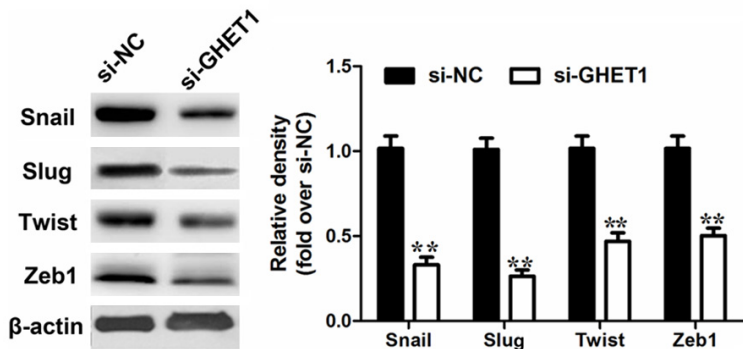


Figure 6. Effect of GHET1 on the EMT-related TFs. Western-blot analysis of EMT-related TFs after GHET1 knockdown in T24 cells. Relative protein expression was identified (n = 3) and normalized to β -actin. Data represent the mean \pm S.D.

invasion was observed in T24 cells with GHET1 knockdown (Figure 4).

The epithelial-mesenchymal-transition (EMT) process has been known to take a part in the

cancer invasion, metastasis, expansion of the population of cancer stem cells and therapeutic resistance [15]. We then investigated whether knockdown of GHET1 would have an effect on the EMT in bladder cancer cells. We observed that with GHET1 knockdown, the resultant cells acquired a round, epithelial morphology (Figure 5A). With the immunofluorescence analysis, T24 cells exhibited a significant decreased expression of vimentin and fibronectin (mesenchymal marker) and an

increase in E-cadherin protein level (epithelial marker) after GHET1 knockdown (Figure 5A). Moreover, western blot analysis confirmed the results from the immunofluorescence analysis (Figure 5B).

A number of transcription factors (TFs) are known to be involved in the EMT process; we assessed the effect of GHET1 silencing on the expression of the following TFs which are known to promote EMT: Snail, Slug, ZEB1 and Twist. We found that T24 cells expressed lower levels of Snail, Slug, Twist and ZEB1 in response to GHET1 silencing (Figure 6).

Discussion

Bladder cancer is one of most common carcinomas worldwide. With the advances in the high-throughput gene sequencing analysis, our understanding of bladder cancer pathogenesis has improved through the identification of activating mutations in and amplifications of oncogenes, including MDM2 [16], PIK3CA [17], CDKN2A/B [17], KRAS [18], and inactivating mutations in tumor suppressive genes, such as CDKN1A [16] and p53 [16]. In the past decade, a growing volume of literature has identified a large number of miRNAs that contribute to the progression of bladder cancer [8-10]. However, the mechanism of bladder cancer progression, including the factors that promote cancer cell invasion, proliferation, apoptosis-resistance and chemo-therapy resistance remain largely unknown. Although accumulating evidence has indicated the role of lncRNAs in cancer, only a relatively small proportion of lncRNAs have been characterized. Our study demonstrates that, GHET1, an lncRNA, is clinically and functionally relevant to the development of bladder cancer.

GHET1 was initially characterized in gastric cancer and found to promote gastric cancer cell proliferation via increasing c-Myc mRNA stability and expression [14]. Inspired by the observation that GHET1 is associated with the development of gastric cancer, we would like to explore its role in bladder cancer progression.

In the present study, we discovered that GHET1 was upregulated in bladder cancer tissues compared to adjacent normal tissues. What's more, increased GHET1 expression correlated with tumor size and tumor status.

A great reduction in cancer cell proliferation and invasion with GHET1 knockdown was observed. To investigate the underlying mechanism through which GHET1 promoted the proliferation of gastric cancer cells, we performed

cell cycle analysis. GHET1 induced a significant G0/G1 arrest. Furthermore, we found that GHET1 silencing markedly increased the protein levels of G0/G1 arrest markers, p16, p21 and p27. The data suggest that knockdown of GHET1 might inhibit cell proliferation through inducing G0/G1 arrest.

The epithelial-mesenchymal-transition (EMT) process occurs during embryonic development and tumorigenesis [19]. More and more studies have confirmed the vital role of EMT in cancer invasion and metastasis, a process in which cells loses an epithelial phenotype and other components of cell junctions and acquire a mesenchymal phenotype [19, 20]. Essential hallmarks of EMT include loss of the E-cadherin expression and increased expression of vimentin and fibronectin [19]. Previous studies suggest that the EMT process is regulated by a set of transcription factors including Snail, Slug, ZEB1 and Twist [20]. Recent studies have indicated that long noncoding RNAs can also play a role in the regulation of EMT. For example, HOTAIR promoted EMT by increasing the expression levels of Slug, Snail and Twist [21]. MALAT-1 promoted EMT by activating Wnt signaling [22]. Similar regulations have been reported for of BRAF activated non-coding RNA [23] and lncRNA-activated by TGF- β (lncRNA-ATB) [24]. Thus, we examined the expression levels of hallmarks of EMT with GHET1 knockdown. Knockdown of GHET1 downregulated vimentin and fibronectin expression while induced an upregulation of E-cadherin expression, restoring T24 cells to more of an epithelial phenotype. Furthermore, a reduction in the expression of a series of transcription factors that regulate the EMT was observed with GHET1 knockdown. Our results suggest that the inhibitory effect on cell invasion of GHET1 silencing were associated with EMT.

In general, we demonstrated that the expression of GHET1 was significantly upregulated in bladder cancer tissues compared to adjacent normal tissues. We also showed that GHET1 promoted the proliferation and invasion of bladder cancer cells. Our study also deepen the understanding of the molecular mechanism through which GHET1 contribute to the tumor progression and may lead to the development of a novel diagnostic marker and therapeutic strategy for bladder cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhi-Liang Weng, Department of Urology, The First Affiliated Hospital, Wenzhou Medical University, 2 Fuxue Xiang, Wenzhou 325000, People's Republic of China. Fax: +86-0577-55578033; E-mail: 2834589118@qq.com

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