Knockdown of long noncoding RNA GHET1 inhibits cell-cycle progression and invasion of gastric cancer cells

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Abstract. GHET1 is an oncogenic long noncoding RNA (lncRNA) that promotes the proliferation and invasion of many malignant cell types. However, the function and underlying mechanisms of lncRNA GHET1 in gastric cancer are not fully understood. In this study, the expression of GHET1 was investigated in gastric cancer and it was determined whether GHET1 may potentially be used as a biomarker for the disease. The gastric cancer cell lines MGC-803 and AGS were transfected with GHET1-directed small interfering RNA (siRNA) and the changes in phenotype and cell-cycle-related molecules were assessed. The downregulation of GHET1 induced G0/G1-phase arrest in gastric cancer cells and inhibited their proliferation, migration, and invasion. DNA synthesis and the expression of proliferating cell nuclear antigen (PCNA) decreased, which was consistent with the results of the CCK-8 assay. The levels of specific cell-cycle regulators were determined and the expression and activities of positive cell-cycle regulators (cyclin D, CDK4, CDK6, cyclin E, CDK2) were reduced, whereas those of a negative regulator (P21) were increased in GHET1-knockdown cells. Taken together, the present findings show that the downregulation of GHET1 not only inhibits the migration and invasion of gastric cancer cells, but also inhibits their proliferation, at least in part by upregulating P21 expression and downregulating cyclin and CDK expression to inhibit the G0/G1 to S phase transition. The present findings may provide a potential therapeutic target for gastric cancer.

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Introduction

Gastric cancer (GC) is an aggressive disease and a major health burden throughout the world, especially in China. It is currently the most common cancer in China, responsible for about 300,000 deaths per year (1,2). GC is commonly diagnosed at an advanced stage because there are no early noninvasive detection strategies, and it is therefore usually associated with a dismal outcome (3,4). The development of GC is a complex, multistep process involving multiple genetic and epigenetic changes to oncogenes, tumor suppressor genes, DNA repair genes, cell-cycle regulators, and signaling molecules (5-7). Therefore, the study of cell-cycle regulators is one of the most important approaches to understanding the molecular mechanisms involved in gastric carcinogenesis and to identifying the diagnostic markers for the early detection and targeted treatment of GC.

In the past decade, human genome sequencing and the GENCODE project have shown that <3% of the human genome encodes protein genes, whereas most of the remaining genome contains noncoding genes, yielding many noncoding transcripts, including microRNAs and long noncoding RNAs (lncRNAs) (8,9). lncRNAs are noncoding RNAs longer than 200 nucleotides. lncRNA expression is frequently dysregulated in human disease, and several specific lncRNAs are associated with cancer cell metastasis and a poor prognosis (10-12). Thus, lncRNAs have been identified as key players in cancer, and many studies have demonstrated that lncRNAs can function as tumor suppressors, oncogenes, or both, depending on the circumstances. Considerable evidence has recently demonstrated that lncRNAs are crucial regulators of the development and progression of GC.

Gastric carcinoma highly expressed transcript 1 (GHET1, AK123072) is a recently identified lncRNA (13). High GHET1 levels correlate with tumor size, tumor invasion, and poor survival in GC, and GHET1 promotes the proliferation of GC cells by increasing the stability and expression of c-MYC mRNA (14). The inhibition of GHET1 also reversed

the progression of the epithelial-mesenchymal transition in a bladder cancer cell line (13). However, the biological role and underlying mechanism of GHET1 in GC are not yet been fully understood. In this study, we investigated the clinical significance of GHET1 in GC patients. We also investigated the effect of GHET1 downregulation on the cell-cycle progression and metastasis of GC cells, and the underlying mechanisms.

Patients and methods

Patients and clinical samples. The clinical specimens were GC tissues and paired adjacent tissues (>5 cm from the tumor) from 42 GC patients with a diagnosis based on histopathological analysis at the Affiliated Hospital of Guizhou Medical University between 2012 and 2016. None of the patients received chemotherapy or radiotherapy before surgery. The disease of all the patients was staged based on the criteria of the WHO Classification of Tumors of the Digestive System, 2010 edition. The paired gastric cancer tissues and adjacent nontumor tissues were removed from 42 GC patients during surgery, and were sent to the Pathology Department for hematoxylin and eosin staining to confirm that they were gastric cancer tissues and adjacent nontumor tissues. All samples were immediately snap-frozen in liquid nitrogen and stored until analysis. The study was approved by the Ethics Committee of the Affiliated Hospital of Guizhou Medical University, and it was performed in compliance with the principles of the Declaration of Helsinki. All participating patients gave their informed consent.

Cell culture. All cell lines (AGS, BGC-823, HGC-27, SGC-7901, MGC-803, and GES-1) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). SGC-7901 and BGC-823 cells were cultured in RPMI-1640 medium (GE Healthcare Life Sciences, Logan, UT, USA). AGS, MGC-803, HGC-27, and the normal gastric epithelium cell line GES-1 were cultured in DMEM (GE Healthcare Life Sciences). All the human gastric cancer cell lines and GES-1 cells were supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated at 37˚C under 5% CO_2.

RNA extraction and RT-qPCR assays. RNA isolation and RT-qPCR were performed as described previously. Total RNA was extracted from the frozen tissues and cultured cells with TRIzol Reagent (Songon, Shanghai, China), according to the manufacturer's instructions. RNA was reverse transcribed to cDNA by using a PrimeScript RT kit (Takara, Dalian, China). qPCR analyses were performed with SYBR Premix Ex Taq (Takara) to quantify the expression of GHET1 in the GC tissues and cultured cells, with normalization to the gene encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following primers were used: GAPDH sense, 5'-GAGTCAACGGATTTGGTCGT-3' and antisense, 5'-GACAAGCTTCCCGTTCTCAG-3'; and GHET1 sense, 5'-GAACAAAGCAGGTAAACATTGG-3' and antisense, 5'-GCAAAGGCAGAGTGAAAGGT-3'. The cycling parameters were hot start at 95˚C for 30 sec, followed by 30 cycles of 95˚C for 30 sec and 60˚C for 30 sec. The relative fold change in mRNA was calculated with the $2^{-\Delta\Delta Ct}$ method.

Cell transfection. MGC-803 and AGS cells ($2x10^5$ cells/well) were seeded in six-well plates at 37˚C overnight until >50% confluence. MGC-803 and AGS cells in serum-free DMEM were transfected with si-NC sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3' or si-GHET1 sense, 5'-CGGCAGGCAUUAGAGAUGAACAGCA-3' and antisense, 5'-UGCUGUUCAUCUCUAAUGCCUGCCG-3' using the Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. After transfection for 6 h, the cells were incubated with medium containing 10% FBS for 42 h, and were then harvested for evaluation of the knockdown efficiency.

Cell proliferation assay. Cell viability was measured with the Cell Counting kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Transfected cells ($3x10^3$ cells/well) were incubated in 96-well plates and quantified every 24 h. Two h before the end of incubation, 10 μl of CCK-8 reagent was added to each well. The optical density at 450 nm (OD_{450}) of each well was determined with an enzyme immunoassay analyzer. Transfected MGC-803 and AGS cells were harvested after incubation for 48 h, stained with BD Cycle test™ Plus (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol, and subjected to a flow-cytometric analysis. The data were expressed as the percentage distribution of the cells in the G0/G1, S, and G2/M phases of the cell cycle.

Scratch-healing assay. Each well of a six-well plate was seeded with $2x10^5$ cells and the transfected cells were cultured for 48 h to 100% confluence. The cell monolayers were scratched with the head of a 10 μl pipette tip, washed twice with phosphate-buffered saline (PBS), and then incubated for 0, 12, or 24 h in medium containing 1% FBS. The healing state of the scratched cells was observed under an inverted microscope, and the wound healing rate was calculated with the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell migration and invasion assay. Migration and invasion assays were performed in Transwell chambers. For the migration assay, $5x10^4$ transfected cells in serum-free medium were placed in the upper chamber (8 μm pore size; EMD Millipore, Billerica, MA, USA) and the lower chamber was filled with medium containing 10% FBS. The apparatus was incubated for 12 h. For the invasion assay, $5x10^4$ transfected cells in serum-free medium were placed into the upper chamber on an insert coated with Matrigel (Costar Corning Inc., Corning, NY, USA), and the lower chamber was filled with medium containing 20% FBS. The apparatus was incubated for 24 h. After incubation, the upper cells were removed with a cotton swab and the lower surface was fixed with 4% paraformaldehyde, stained with 1% crystal violet, and washed twice with PBS. The number of cells on the underside of the membrane was counted in five random fields under an inverted microscope and the average number was calculated.

Immunoblotting analysis. Transfected cells were harvested after incubation for 48 h in six-well plates and lysed with RIPA

Table I. Correlation between pathological parameters and the expression level of GHET1 in 42 gastric cancer patients.

Variables	n	Expression level of GHET1[a]		P-value[b]
		High	Low	
Total	42	21	21	
Sex				0.513
Male	28	15	13	
Female	14	6	8	
Age, years				0.757
≤60	23	12	11	
>60	19	9	10	
Tumor size, cm				0.346
≤5	25	14	11	
>5	17	7	10	
Differentiation				0.659
Well/moderate	6	4	2	
Poor	36	17	19	
Tumor status				0.028[a]
T1-T2	17	5	12	
T3-T4	25	16	9	
Lymph node invasion				0.408
N0	7	5	2	
N1-N3	35	16	19	
Distant metastasis				0.659
M0	36	17	19	
M1	6	4	2	

[a]The median ratio of the expression level of GHET1, [b]P<0.05 was considered to indicate a statistically significant difference. GHET1, gastric carcinoma highly expressed transcript 1.

Lysis Buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with Protease Inhibitor Cocktail (Roche Applied Science, Pleasanton, CA, USA). The total protein concentration was measured with a Bicinchoninic Acid Protein Assay kit (Solarbio, Beijing, China). The cell proteins were separated with sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE; Solarbio), transferred to polyvinylidene difluoride membranes (EMD Millipore), and incubated with antibodies, cyclin-dependent kinase 2 and 4 (CDK2, CDK4; Cell Signaling Technology Inc., Danvers, MA, USA), P21, cyclin E, cyclin D, CDK6 (Abcam, Cambridge, MA, USA), proliferating cell nuclear antigen (PCNA), and β-actin (Wuhan Sanying Biotechnology, Wuhan, China). The relative expression of the proteins was detected with Immobilon™ Western Chemiluminescent HPR Substrate (EMD Millipore) method. β-actin antibody was used as the control.

Statistical analysis. All data were presented as mean ± standard deviation from three independent experiments and the statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The expression level of GHET1 in gastric cancer tissues was compared with paired adjacent normal tissues utilizing the paired sample t-test, whereas the association between GHET1 expression and pathological parameters were evaluated using χ^2 test. The expression differences between cell lines, the expression changes after transfection, cell cycle, cell migration and invasion assays were analyzed using one-way ANOVA test followed by Tukey's post hoc test (Equal variances assumed) and Dunnett's C test (Equal variances not assumed). P<0.05 was considered to indicate a statistically significant difference.

Results

Elevated GHET1 expression in GC tissues correlated with pathological characteristics. We first examined the GHET1 expression in 42 paired gastric cancer tissues and adjacent nontumor tissues from GC patients using RT-qPCR. GHET1 expression was significantly upregulated in the GC tissues compared with the levels in the adjacent nontumor tissues (Fig. 1A; P<0.05). Table I shows that GHET1 expression also correlated with the pathological characteristics of the tumors, including the tumor invasion depth. We also quantified the expression of GHET1 in GC cell lines (AGS, SGC-7901, BGC-823, MGC-803, and HGC-27) and GES-1 cells. Compared with the GES-1 cells, GHET1 was more strongly expressed in the GC cell lines. As shown in Fig. 1B, MGC-803 and AGS cells were used in the subsequent experiments. These results suggest that GHET1 plays a vital role in the progression of GC.

GHET1 downregulation inhibited GC cell proliferation and induced cell-cycle arrest. The significantly increased expression of GHET1 in GC tissues prompted us to investigate its biological role in GC cells. To determine the effect of GHET1 on GC cell proliferation and cell-cycle progression, small interfering RNA (siRNA) was used to knockdown its expression. As showed in Fig. 2A, siRNA significantly reduced GHET1 in both MGC-803 and AGS cells. CCK-8 assays also showed that knockdown of GHET1 impaired MGC-803 and AGS cell proliferation (Fig. 2B). PCNA was expressed in both MGC-803 and AGS cells transfected with si-GHET1, but its expression was reduced, which is consistent with the role of PCNA in cell proliferation (Fig. 2C and D). To determine whether the effect of GHET1 on GC cell growth was attributable to cell-cycle arrest, cell-cycle progression was analyzed with flow cytometry. MGC-803 and AGS cells transfected with si-GHET1 showed obvious cell-cycle arrest at the G1/G0 phase (Fig. 3A and B). The levels of several cell-cycle regulators were determined. The level of P21 was increased, but the levels of cyclin D, CDK4, CDK6, cyclin E, and CDK2 were reduced in the GHET1-knockdown cells (Fig. 3C and D).

Knockdown of GHET1 inhibited GC cell migration and invasion. We also investigated the effect of GHET1 on GC cell migration and invasion. As shown in Fig. 2B, a scratch-healing assay demonstrated that the migratory ability of MGC-803 and AGS cells transfected with si-GHET1 was inhibited compared with that of si-NC-transfected cells and the control (Fig. 4A and B). A Transwell migration assay

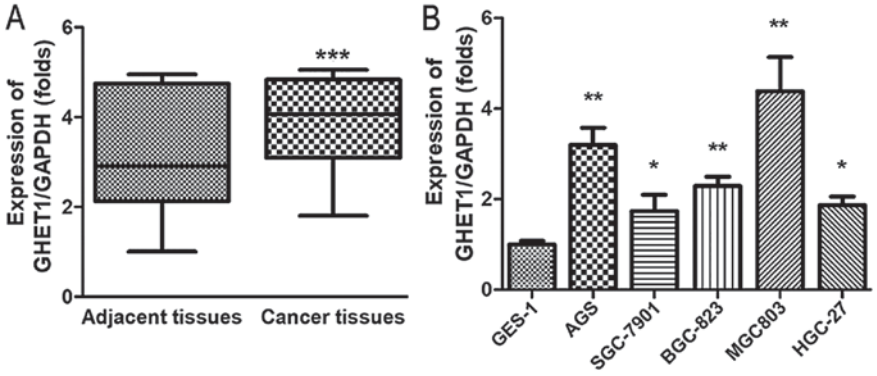

Figure 1. Expression of GHET1 is significantly upregulated in gastric cancer tissues and cell lines. (A) Differences in GHET1 expression between gastric cancer tissues and paired adjacent normal tissues. The results are displayed on a log scale and accord with normal distribution at the 95% confidence level. The expression of GHET1 was normalized to GADPH. The statistical differences between samples were analyzed with paired samples t-test (n=42, ***P<0.001 vs. paired adjacent normal tissues). (B) Relative expression of GHET1 in different gastric cancer cell lines. GHET1 expression was significantly higher in GC cell lines than in GES-1 cells. (*P<0.05, **P<0.01 vs. GES-1 cells). GHET1, gastric carcinoma highly expressed transcript 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

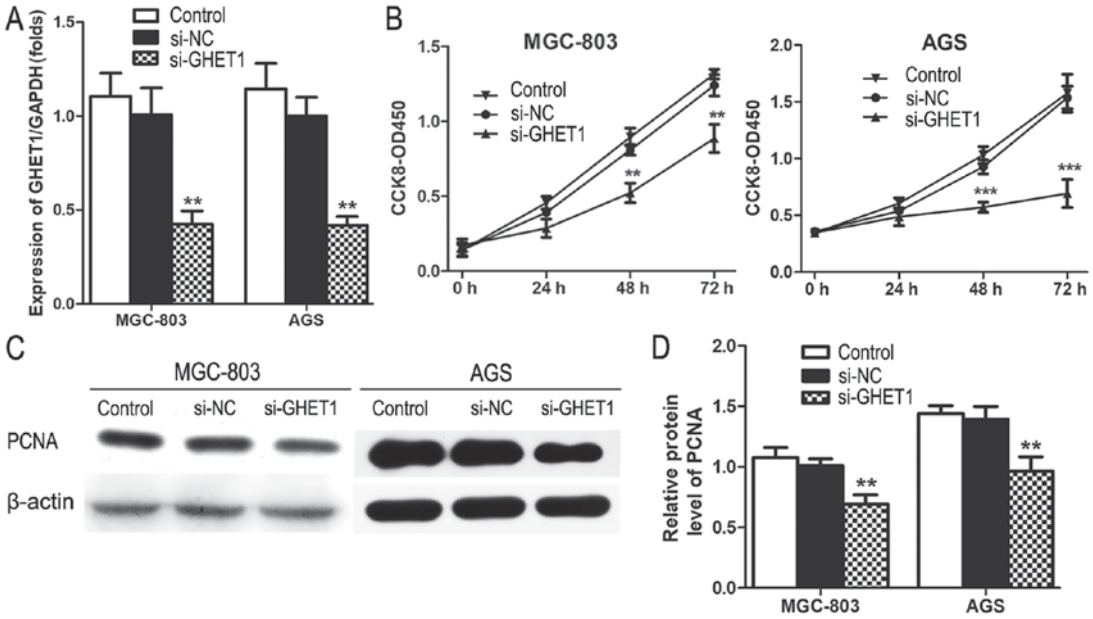

Figure 2. Effect of GHET1 on gastric cancer cell proliferation. (A) RT-qPCR analysis of GHET1 expression in MGC-803 and AGS cells transfected with GHET1 siRNA or NC siRNA, and in the control group. (B) Growth curves for MGC-803 and AGS cells after transfection with GHET1 siRNA were determined with CCK-8 assays. (C and D) PCNA protein levels were detected in MGC-803 and AGS cells after transfection with si-GHET1 or si-NC, and in the control group with an immunoblotting analysis. (*P<0.05, **P<0.01, ***P<0.001 vs. si-NC). GHET1, gastric carcinoma highly expressed transcript 1.

showed that the inhibition of GHET-1 dramatically suppressed AGS and MGC-803 cell migration compared with that of the negative control. Consistent with this result, the knockdown of GHET1 caused a significant decline in the invasion capacity of GC cells (Fig. 4C and D). These findings suggest that the expression of GHET1 is closely associated with the migration and invasion in GC cell lines.

Discussion

Gastric cancer is one of the most frequent cancers of the digestive system and has a high mortality rate worldwide. Accumulating evidence has recently demonstrated that lncRNAs are critical players in the tumorigenesis and progression of GC (15). And can be used as a diagnostic marker of GC (16).

This study suggests that lncRNA GHET1 is strongly expressed in clinical GC tissue specimens. An analysis of different clinical pathological characteristics showed that high lncRNA GHET1 expression is closely associated with GC tumor invasion and TNM stage. This conclusion is consistent with the report of Yang *et al* (14). Tumor cells often invade the surrounding tissues through blood and lymphatic vessels, and form distant secondary tumors, which are critical in cancer prognosis (17). In this study, GHET1 expression correlated with the pathological characteristics of 42 GC patients and also correlated positively with tumor invasion. The downregulation of GHET1 also inhibited the migration and invasion of MGC-803 and AGS cells in a Transwell assay and scratch-healing assay. These data suggest that the knockdown of GHET1 inhibits cell metastasis in GC.

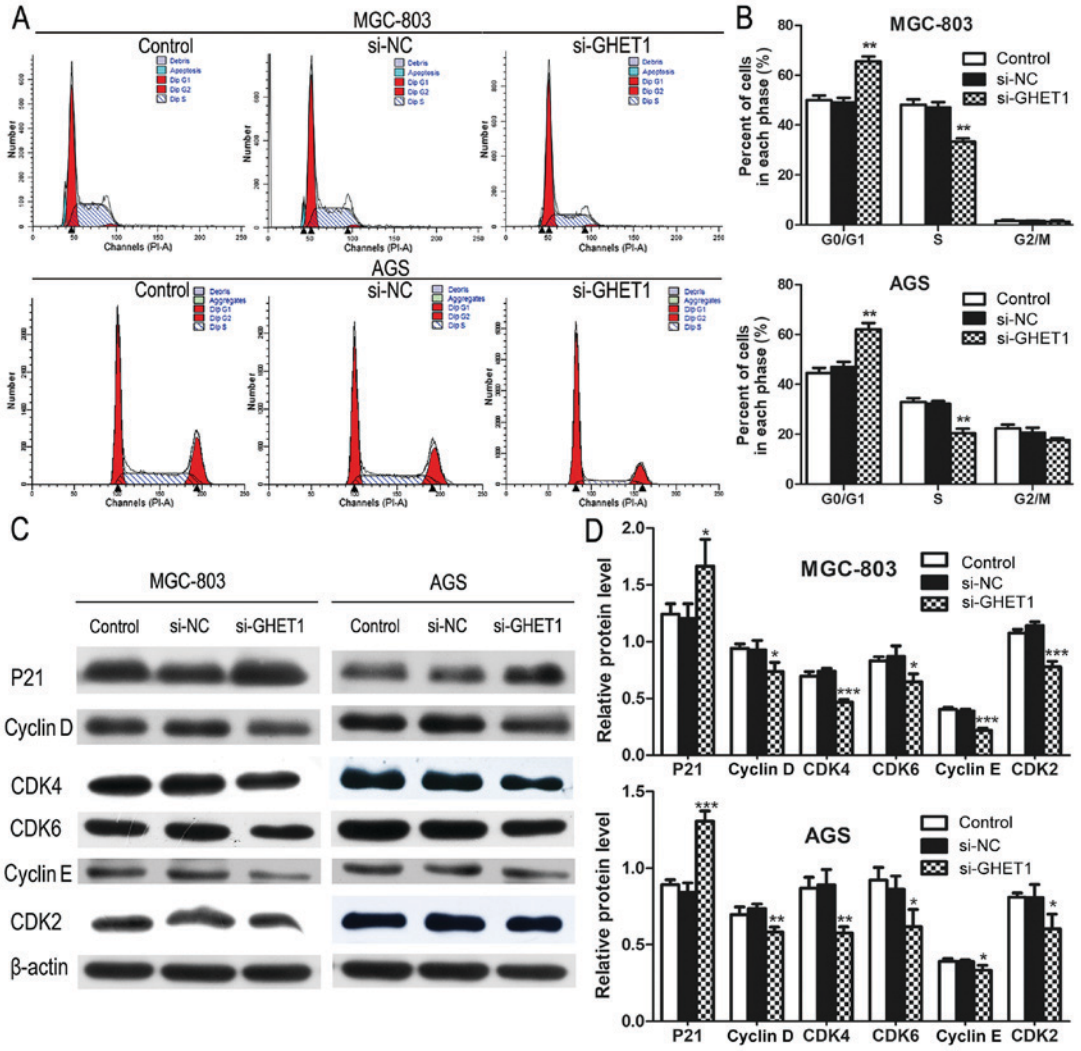

Figure 3. Effect of GHET1 on gastric cancer cell cell-cycle progression. (A and B) Cell-cycle progression in MGC-803 and AGS cells was evaluated with flow cytometry 48 h after transfection with si-GHET1 or si-NC and in the control group. (C and D) P21, cyclin D, CDK4, CDK6, cyclin E, and CDK6 protein levels were determined in MGC-803 and AGS cells after transfection with si-GHET1 or si-NC and in the control group with an immunoblotting analysis. (*P<0.05, **P<0.01, ***P<0.001 vs. si-NC).

To explore the biological functions of GHET1, a loss-of-function approach was used in MGC-803 and AGS cells. Knockdown of GHET1 significantly inhibited cell proliferation and the level of PCNA protein. PCNA is a good indicator of cellular proliferation, which is closely related to DNA synthesis (18-20). This result suggests that the upregulation of GHET1 is associated with cell proliferation.

Regulation of the cell cycle is important in cell proliferation, and the loss of cell-cycle control is associated with carcinogenesis (21). We performed a cell-cycle analysis to investigate the mechanism through which GHET1 promotes the proliferation of GC cells. The data suggested that the knockdown of GHET1 inhibited cell proliferation by inducing G0/G1 arrest. In mammalian cells, the G1-S transition is controlled by cyclins, CDKs, and CDK inhibitors (CKIs) (22). Cyclin D interacts and forms complexes with CDK4 and CDK6 to regulate G1 phase, whereas cyclin E forms a complex with CDK2 to regulate the G1-S transition in the cell cycle (23). CDKIs such as $P21^{WAF1}$ are important CKI family members and are frequently dysregulated in cancer (24). $P21^{WAF1}$ arrests cell-cycle progression from G0/G1 phase to S-phase and inhibits the kinase activities of cyclin-CDK complexes by binding to CDKs, preventing their association with cyclins (25,26). Previous study proved that knockdown of GHET1 could suppress the expression of cyclin D in AGS cells (27). Our western blotting assay further showed that the downregulation of GHET1 negatively regulated the expression of the proteins involved in cyclin-CDK complexes and promoted the expression of CKIs. The expression of cyclin D, cyclin E, CDK2, CDK4, and CDK6 was decreased and that of P21 was elevated. Here, we provide the first evidence that GHET1 promotes cell proliferation by downregulating P21 expression and increasing the cyclin-CDK complexes in GC cells, accelerating the progression of GC. Previous studies have shown that GHET1 promotes the stability and expression of c-MYC by interacting with insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1) to promote the proliferation of GC cells (14). The MYC family is one of the proteins upregulated in many human cancers (28,29). It regulates the expression of lncRNAs, and some of these transcripts participate in the transcriptional functions mediated by MYC (30-33). MYC also both activates and

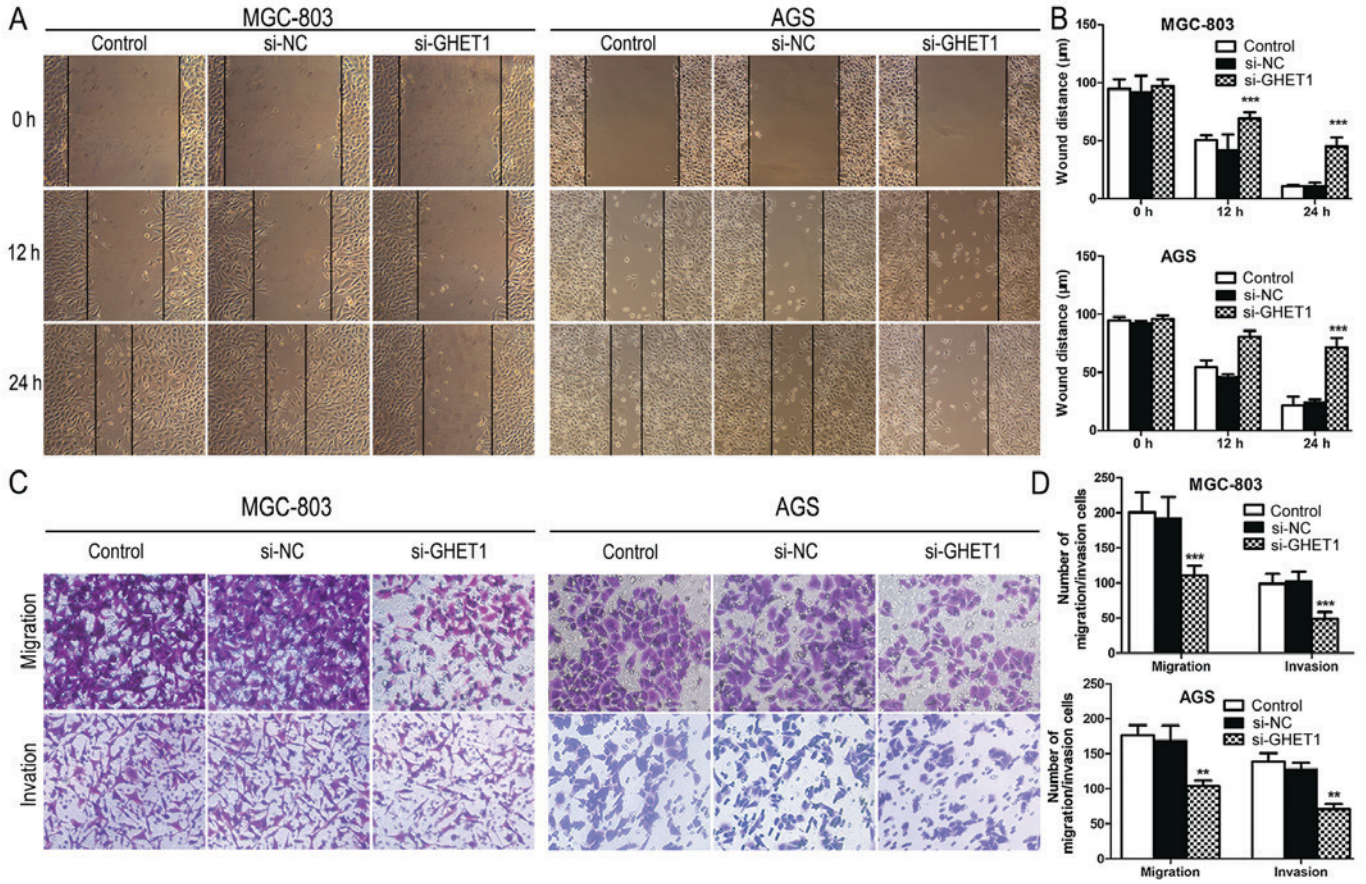

Figure 4. Knockdown of GHET1 inhibited the migration and invasion of MGC-803 and AGS cells. (A and B) Knockdown of GHET1 inhibited cell migration, as detected in scratch-healing assays of MGC-803 and AGS cells. (C and D) Knockdown of GHET1 inhibited cell invasion and migration in MGC-803 and AGS cells, as detected with Transwell assays. (**P<0.01, ***P<0.001 vs. si-NC). GHET1, gastric carcinoma highly expressed transcript 1.

represses the expression of cyclin and CDK genes (34). Taken together, these findings clearly show that aggressive GC cells are characterized by higher GHET1 expression, which in turn increases the expression of cell-cycle-related proteins, accelerating the progression of GC.

In general, we suggest that knocking down the expression of lncRNA GHET1 inhibits cell-cycle progression and metastasis in GC. The expression of GHET1 is significantly upregulated in GC tissues compared with that in adjacent normal tissues. The downregulation of GHET1 inhibits cell proliferation by reducing the expression of cyclins and CDKs and upregulatig their inhibitors, arresting the cell cycle at the G1-S phase transition. We have also shown that the knockdown of GHET1 inhibits the migration and invasion of GC cells. Our study, comprising patients from several minor ethnical populations in Guizhou, China, strengthens the overarching conclusion that GHET1 may have diagnostic potential in gastric cancer diagnosis.

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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

YX and YW performed the functional *in vitro* assays. JL, JZ and YB performed qPCR and western blot assays. YX, SW and YW analyzed the data and wrote manuscript. ZY collected and analyzed clinical samples. HH and DJL designed the present study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by Ethics Committee of the Affiliated Hospital of Guiyang Medical College. All the study participants gave their written informed consent to participation in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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