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Effects of lncRNA-HEIH on proliferation, apoptosis and invasion of gastric cancer cells

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Abstract. – OBJECTIVE: The aim of this study was to explore the expression of long non-coding ribonucleic acid HEIH (lncRNA-HEIH) in gastric cancer (GC) tissues, and to investigate its effects on the proliferation, apoptosis and invasion of HGC-27 cells.

PATIENTS AND METHODS: A total of 80 tissue samples were collected from patients diagnosed with GC in Shenzhen People's Hospital. Meanwhile, para-carcinoma tissues were enrolled as normal controls (Control group). Total RNA was extracted from tissues, and the expression of lncRNA-HEIH was detected via quantitative reverse transcription-polymerase chain reaction (qRT-PCR). HGC-27 cells were cultured and transfected with small-interfering RNA-HEIH (si-HEIH group). At 48 h after transfection, cell proliferation, apoptosis and invasion were detected via methyl thiazolyl tetrazolium (MTT) assay, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and transwell assay, respectively.

RESULTS: (1) Compared with Control group, the expression of lncRNA-HEIH rose significantly in GC tissues ($p<0.01$). (2) The expression of lncRNA-HEIH in HGC-27 cells was significantly down-regulated in si-HEIH group compared with si-NC group ($p<0.01$). (3) Compared with those in si-NC group, the proliferation of HGC-27 cells was suppressed ($p<0.05$), while the apoptosis of HGC-27 cells was promoted ($p<0.01$) in si-HEIH group. (4) The invasion of HGC-27 cells was remarkably inhibited in Si-HEIH group than si-NC group ($p<0.05$).

CONCLUSIONS: LncRNA-HEIH is highly expressed in GC patients, which affects the proliferation, apoptosis and invasion of GC HGC-27 cells.

Key Words:
Gastric cancer (GC), LncRNA-HEIH, Proliferation, Apoptosis, Invasion.

Introduction

Gastric cancer (GC) is one of the most common causes of cancer-related death around the world. According to the latest statistics of the Cancer Control Program of the World Health Organization, there are more than 7 million cancer deaths every year globally, including 700,000 GC-related deaths[1]. Annually, about 934,000 cases are newly diagnosed with GC, of which about 43% (400,000) occur in China. Meanwhile, the morbidity and mortality rates of GC in China are twice higher than the world average[2,3]. Currently, great progress has been made in the targeted therapy of malignant tumors. However, the prognosis of GC is far from unsatisfactory, and the median survival time is 12 months[4]. Moreover, the early detection rate of GC is less than 10%, whose 5-year overall survival is lower than 24%. GC patients are usually diagnosed in the late stage or in the case of metastasis[5]. At present, the potential mechanism of the occurrence and development of GC remains unknown. Therefore, it is of great significance to screen specific and sensitive biomarkers and to enhance the research on the pathogenesis of GC for improving its diagnosis and treatment.

Long non-coding ribonucleic acids (lncRNAs) are a class of non-coding transcripts with more than 200 nucleotides in length. They have been confirmed with no or weak protein-coding potential[6]. LncRNAs play important roles in cell development, differentiation and other biological processes[7]. In terms of the mechanism, lncRNAs serve as ceRNAs to capture target miRNA response elements and affect their post-transcriptional regulation. LncRNAs are involved in tumor progression[8,9]. Of note, lncRNA DANCR is highly expressed in advanced colorectal cancer. Meanwhile, it promotes the proliferation and metastasis of colorectal cancer through miR-577[10]. LncRNA UCA1 facilitates chemical resistance of bladder cancer *via* regulating the Wnt signaling pathway[11]. In addition, linc00152 participates in apoptosis, cell cycle arrest, epithelial-mesenchymal transition, as well as the invasion and migration of GC cells[12].

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LncRNA-HEIH, a member of the lncRNA family, is located on chromosome 5. It is considered as a carcinogenic lncRNA able to promote tumor progression. LncRNA-HEIH was first identified and studied in hepatocellular carcinoma (HCC). It is highly expressed in HCC patients, serving as a key regulator of HCC progression[13]. The expression level of lncRNA-HEIH in hepatitis B virus-related HCC is closely related to the recurrence, which has been determined as an independent prognostic factor[13]. LncRNA-HEIH in serum and exosomes is a potential biomarker of hepatitis C virus-related HCC[14]. As a promoting factor[15], lncRNA-HEIH facilitates the proliferation and migration of melanoma cells[16]. The dysregulation of lncRNAs may lead to abnormal expression of target genes, thereby benefitting the occurrence and development of tumors including GC. However, the exact role of lncRNA-HEIH in the pathogenesis of GC remains unclear. In addition, its effects on the proliferation, apoptosis and migration of GC cells have rarely been elucidated.

In this study, it was found that the expression of lncRNA-HEIH was significantly up-regulated in GC tissues. Such an up-regulation was correlated with the TNM stage of GC. The effect of lncRNA-HEIH on GC HGC-27 cells was further verified *via* cell biology experiments. The results showed that up-regulation of lncRNA-HEIH promoted GC cell proliferation and invasion, and inhibited GC cell apoptosis. This might eventually lead to malignant changes and promote deterioration of GC, as well as harm the prognosis of GC patients. All our findings demonstrated that lncRNA-HEIH was expected to be a potential target for early diagnosis of GC and provided a theoretical basis for its treatment.

Patients and Methods

Source of Cases

A total of 80 patients diagnosed with GC in the Digestive Surgery in Shenzhen People's Hospital from October 2016 to August 2018 were selected as research subjects, including 50 males and 30 females. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). The patient's information was shown in Table I. After resection, tissue samples were immediately placed into RNA fixative and stored at -80°C for use. All patients were diagnosed with GC by professional pathologists and clinicians. No patient underwent any preoperative chemotherapy or radiotherapy. All tissue samples were collected in accordance with the *Code of Ethics* of the World Medical Association. Informed consent was obtained from each patient before the study. The research program and implementation plan were approved by the Ethics Committee of Shenzhen People's Hospital.

Expression of LncRNA-HEIH In Tissues

A total of 20 mg of carcinoma tissues and para-carcinoma tissues were taken and cut into 1 mm^3 pieces. Total RNA in tissues and cells was extracted using TRIzol reagent (Invitrogen, Karlsruhe, Germany). The concentration and purity of RNA were determined using a NanoDrop-2000 spectrophotometer. Subsequently, extracted RNA was synthesized into complementary deoxyribose nucleic acid (cDNA) using the reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA), followed by qPCR using SYBR Mixture. The relative expression of lncRNA-HEIH was calculated using the $2^{-\Delta\Delta Ct}$ method, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference. Primer sequences of lncRNA-HEIH and GAPDH were as follows: LncRNA-HEIH: F: 5'-CCTCTTGTGCCCCTTTCTT-3', R: 5'-ATGGCTTCTCGCATCCTAT-3'; GAPDH: F: 5'-GAAGGTGAAGGTCGGAGTC-3', R: 5'-GAAGATGGTGATGGGATTTC-3'.

Cell Culture

Human normal gastric mucosal cell line (GES-1) and human GC cell line (HGC-27) were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences

Table I. Clinical characteristics of 80 GC patients.

	Proportion (%)
Age	
< 65	49 (61.25)
≥ 65	31 (38.75)
Gender	
Male	50 (62.5)
Female	30 (37.5)
Degree of differentiation	
Poor and moderate	38 (47.5)
High	42 (52.5)
TNM stage	
I/II	45 (56.25)
III/IV	35 (43.75)

(Shanghai, China). All cells were cultured in the Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin solution, and maintained in an incubator with 5% CO_2 at 37°C.

Cell Transfection

At 1 h before transfection, the old medium was replaced with FBS-free culture medium. HGC-27 cells in the logarithmic phase were taken, and transfected with transfection reagent, si-NC and si-HEIH according to the instructions of Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany). 6 h later, the medium was replaced with complete culture medium. Next, the cells were cultured in an incubator with 5% CO_2 at 37°C for 48 h. Finally, transfected cells were collected for subsequent assays.

Methyl Thiazolyl Tetrazolium (MTT) Assay

At 48 h and 72 h after transfection, the cells were inoculated into 96-well plates at a density of 3×10^3 cells/well. Next, MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well to make the final concentration of 0.5 mg/mL. After 4 h, dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added to dissolve formazan. Optical density at 570 nm (OD_{570}) was finally measured using an enzyme-linked immunosorbent assay reader (ELISA; Bio-Rad, Hercules, CA, USA).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Assay

Apoptotic cells were detected according to the manufacturer's instructions of TUNEL assay. In brief, transfected HGC-27 cells were placed on slides, and the percentage of apoptotic cells was evaluated using the *in situ* apoptosis detection kit (DeadEnd™ Colorimetric TUNEL System, Promega, Madison, WI, USA). After TUNEL labeling, the nucleus was labeled with 4',6-diamidino-2-phenylindole (DAPI). FITC-labeled TUNEL-positive cells were imaged under a fluorescence microscope at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Finally, the ratio of the number of TUNEL-positive cells to the total number of cells was calculated. Four fields of view were randomly selected in each section, and 100 cells were counted in each field.

Transwell Invasion Assay

At 48 h after transfection, cell invasion was detected using transwell invasion assay. In the transwell chamber (24-well plates, pore size of 8 mm, Corning, Corning, NY, USA), 1×10^5 cells were inoculated into the upper chamber with serum-free medium. Meanwhile, complete medium containing 20% FBS was added to the lower chamber. After 48 h, the invading cells on the upper surface were wiped with a cotton swab, fixed with 4% paraformaldehyde and stained with 5% crystal violet. Five fields of view were randomly selected for each sample. The number of invading cells was finally observed and counted under a microscope.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Numerical variables were expressed as mean ± standard deviation. Independent-samples *t*-test was performed for the comparison between two groups. Kaplan-Meier method with the log-rank test was used for the survival analysis. $p<0.05$ was considered statistically significant.

Results

Expression of LncRNA-HEIH Was Up-Regulated In GC Tissues and Cells

QRT-PCR results showed that the expression of lncRNA-HEIH rose significantly in stage I/II GC patients (GC_I/II group) and stage III/IV GC patients (GC_III/IV group) compared with para-carcinoma tissues (Control group) ($p<0.05$). This indicated that lncRNA-HEIH expression was related to the stage of GC. Meanwhile, the expression of lncRNA-HEIH was significantly higher in GC_III/IV group than that in GC_I/II group ($p<0.01$) (Figure 1). According to the Kaplan-Meier analysis, the overall survival of GC patients with lower expression of lncRNA-HEIH was remarkably higher than that of those with higher expression ($p=0.012$) (Figure 2). Next, the expression of lncRNA-HEIH in HGC-27 cells and GES-1 cells was detected. The results showed that the expression of lncRNA-HEIH was significantly up-regulated in HGC-27 cells ($p<0.01$) (Figure 3).

Si-HEIH Transfection Reduced Expression of LncRNA-HEIH In HGC-27 Cells

To determine whether the expression of lncRNA-HEIH in HGC-27 cells was inhibited after

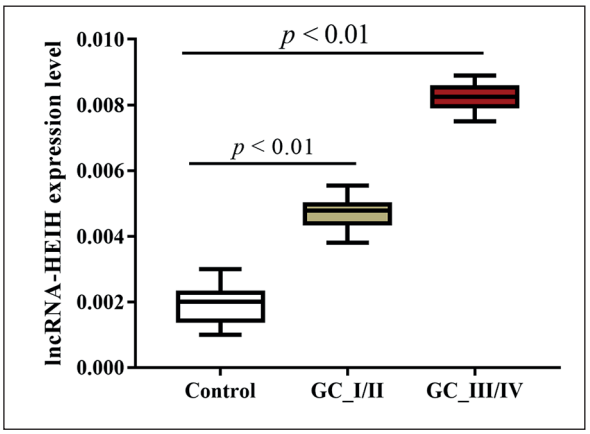

Figure 1. Increased expression of lncRNA-HEIH in GC tissues detected *via* qRT-PCR. Note: Compared with that in Control group, the expression of lncRNA-HEIH rises in GC tissues (**p<0.01).

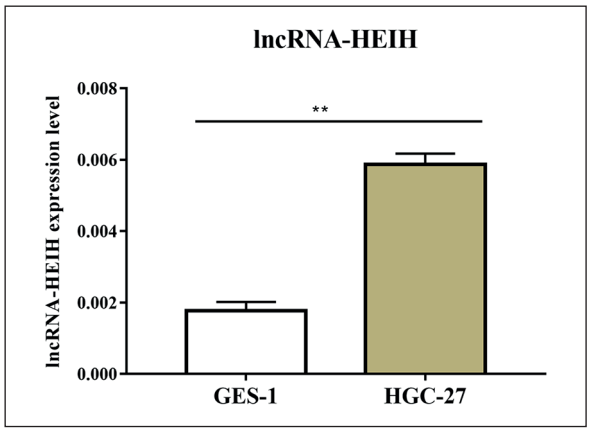

Figure 3. Increased expression of lncRNA-HEIH in HGC-27 cells detected *via* qRT-PCR. Note: Compared with that in GES-1 cells, the expression of lncRNA-HEIH rises in HGC-27 cells (**p<0.01).

siRNA transfection, qRT-PCR was performed. As shown in Figure 4, the expression of lncRNA-HEIH in HGC-27 cells was significantly inhibited in si-HEIH group compared with that in si-NC group (p<0.01).

Si-HEIH Transfection Suppressed HGC-27 Cell Proliferation

To detect the changes in HGC-27 cell proliferation after si-HEIH transfection, MTT assay was conducted. The results manifested that OD_{570} significantly declined in si-HEIH group at 48 h and 72 h after transfection when compared with si-NC group (p<0.01) (Figure 5). This indicated that lncRNA-HEIH promoted the proliferation of HGC-27 cells.

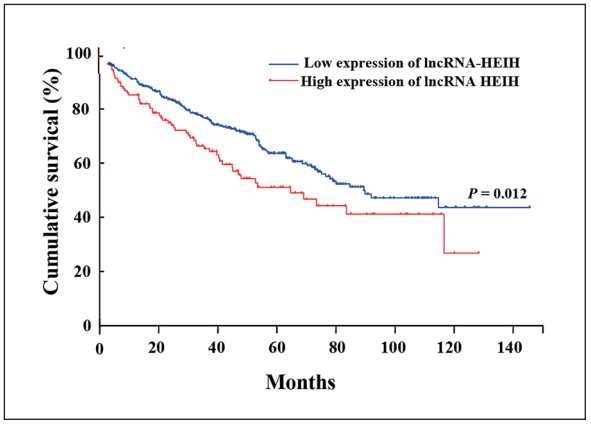

Figure 2. Kaplan-Meier survival analysis based on lncRNA-HEIH expression in GC patients.

LncRNA-HEIH Silencing Facilitated HGC-27 Cell Apoptosis

To detect the changes in HGC-27 cell apoptosis after si-HEIH transfection, TUNEL assay was performed. The results showed that the number of apoptotic HGC-27 cells in si-HEIH group was evidently larger than that in si-NC group (p<0.01) (Figure 6), suggesting that lncRNA-HEIH silencing facilitated HGC-27 cell apoptosis.

LncRNA-HEIH Silencing Inhibited HGC-27 Cell Invasion

Transwell assay was performed to further explore whether si-HEIH transfection induced changes in cell invasion. The results demonstrated that the number of cells decreased significant-

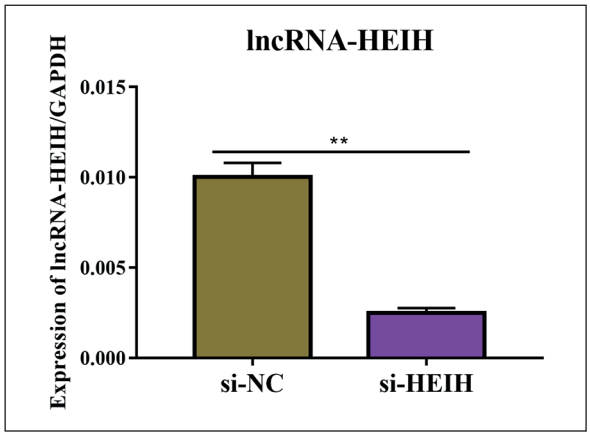

Figure 4. Si-HEIH transfection inhibits lncRNA-HEIH expression. Note: The expression of lncRNA-HEIH significantly declines in si-HEIH group compared with that in si-NC group (**p<0.01).

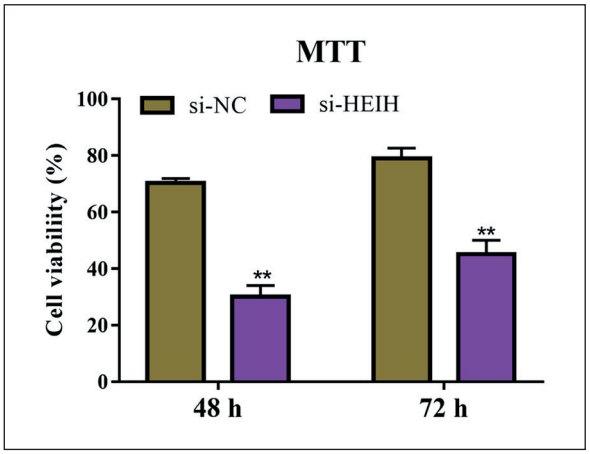

Figure 5. Si-HEIH transfection suppresses HGC-27 cell proliferation. Note: The OD_{570} significantly declines in si-HEIH group at 48 h and 72 h compared with that in si-NC group (**p<0.01).

ly in si-HEIH group compared with si-NC group (p<0.01) (Figure 7). It could be concluded that lncRNA-HEIH silencing inhibited HGC-27 cell invasion.

Discussion

In this study, lncRNA-HEIH was found highly expressed in GC patients, whose increased expression was related to the TNM stage. The higher the TNM stage of GC indicated significantly higher lncRNA-HEIH expression. Therefore, lncRNA-HEIH can serve as a marker for early diagnosis of GC malignancy. Previous studies have pointed out that lncRNA-HEIH is highly expressed in GC. However, its mechanism of action remains unclear. Our results showed that lncRNA-HEIH promoted proliferation and invasion, and inhibited apoptosis of GC cells.

LncRNAs play important roles in cell proliferation, differentiation, migration, apoptosis and immune response through affecting the expressions of DNA, RNA and protein[17-19]. It has been reported that many lncRNAs are closely related to the occurrence and development of GC[20-22]. According to recent reports, the abnormal expression of some lncRNAs, such as SAMMSON, HOTAIR and GAPLINC, may be diagnostic biomarkers and therapeutic targets for melanoma, pancreatic cancer and GC[23-25]. All these findings demonstrate that lncRNAs can be used as markers or targets in GC patients. Therefore, thorough understanding of the functions and mechanisms of related lncRNAs in GC is of great importance for clarifying its pathogenesis. LncRNA-HEIH, with increased expression in non-small cell lung cancer tissues and cells, is mostly applied in tumor treatment in clinic. Current studies have found that it positively regulates cell viability. Overexpression of lncRNA-HEIH promotes cell migration and invasion[26]. Cui et al[27] have demonstrated that lncRNA-HEIH is remarkably up-regulated in human colorectal cancer, acting as a predictive index of overall survival. Zhang et al[28] have also reported that lncRNA-HEIH is significantly up-regulated in liver cancer cell lines. Meanwhile, lncRNA-HEIH up-regulation promotes the invasion of MHCC97L and HepG2 cells, similar to the findings in this study. The above research results reveal that the dysregulation of lncRNA-HEIH exhibits great application potential in the early detection and diagnosis of malignant tumors. However, little is known about the expression, pathophysiological effect and clinical significance of lncRNA-HEIH in GC. Due to the reason that the early diagnosis and treatment of GC has been a global problem so far, there is an urgent need to better understand the ex-

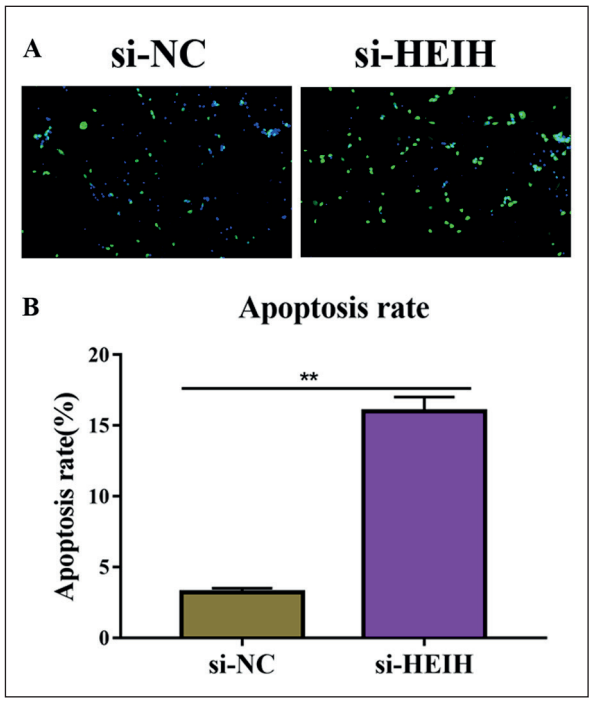

Figure 6. LncRNA-HEIH silencing facilitates HGC-27 cell apoptosis. **A,** Micrographs. (magnification: 200×) **B,** The number of apoptotic cells is evidently larger in si-HEIH group than that in si-NC group (**p<0.01).

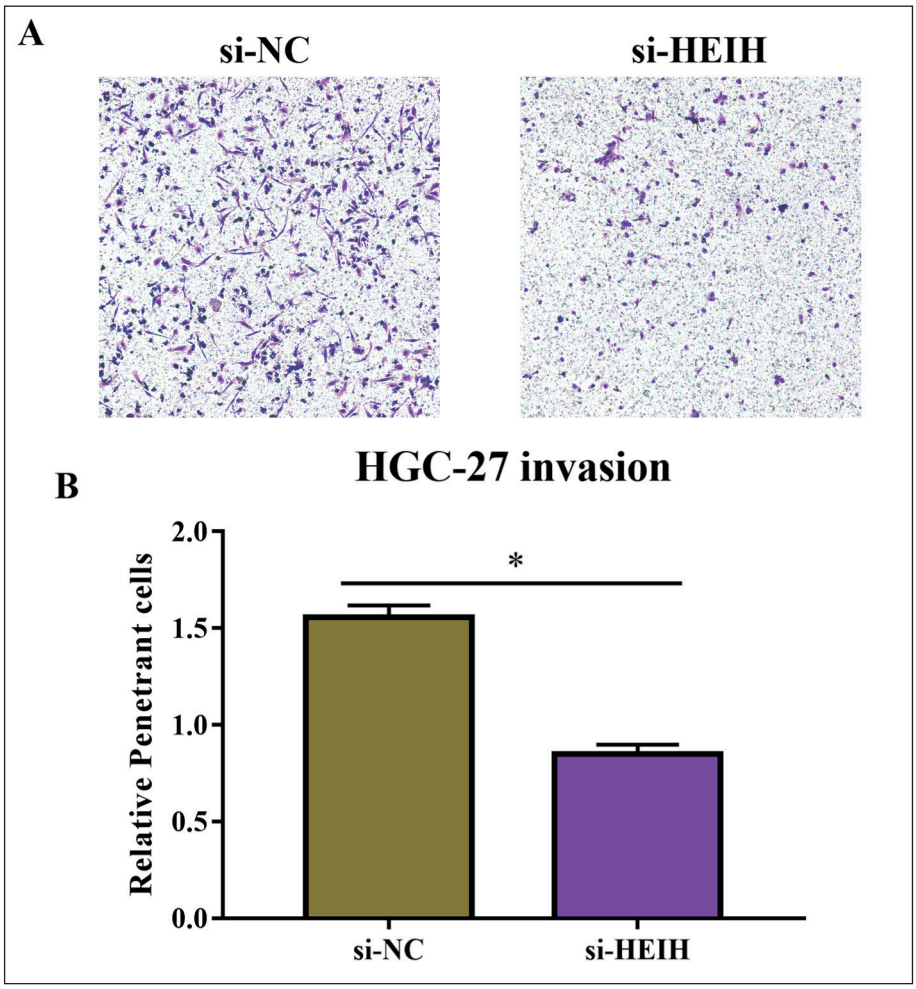

Figure 7. Effect of lncRNA-HEIH silencing on invasion ability of HGC-27 cells. **A,** Micrographs (magnification: 200×). **B,** Column diagram of HGC-27 invasion assay results. Note: The number of cells decreases significantly in si-HEIH group compared with that in si-NC group (*p<0.05).

pression pattern of lncRNAs in GC. Therefore, the expression pattern of lncRNA-HEIH in GC tissues and cells, and its effects on the proliferation, apoptosis and invasion of GC cells were explored in this study. Our findings might help to better understand the clinical significance of lncRNA-HEIH for GC patients.

There are still some limitations in this study. First, more GC tissue samples are needed to further detect the expression of lncRNA-HEIH. Second, lncRNA-HEIH can play an important role *via* regulating more miRNAs or target genes. Therefore, two or more GC cell lines should be used, so as to understand the mechanism of lncRNA-HEIH in GC more deeply. To sum up, lncRNA-HEIH is significantly up-regulated in human GC samples. Up-regulated expression of lncRNA-HEIH is related to the TNM stage. The higher malignancy of GC corresponds to the higher expression of lncRNA-HEIH. These findings indicate that lncRNA-HEIH can serve as an index for GC malignancy to a certain extent. In addition, overexpression of lncRNA-HEIH promotes the proliferation and invasion, and inhibits the apoptosis of GC cells, thereby further aggravating GC. Therefore, inhibiting the expression of lncRNA-HEIH in GC patients by some means may suppress the proliferation and spread of cancer cells to a certain extent.

Conclusions

LncRNA-HEIH is highly expressed in GC patients, which affects the proliferation, apoptosis and invasion of GC HGC-27 cells. The novelty

of this study was that our findings may provide a certain reference for the prevention and treatment of GC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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