LncRNA-HEIH is a Novel Diagnostic and Predictive Biomarker in Gastric Cancer

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Background: Gastric cancer (GC) is associated with a high mortality rate. Long noncoding RNA (lncRNA)-high expression in hepatocellular carcinoma (HEIH) has recently gained interest as a marker for the detection of several cancer types. This study was designed to uncover the function of lncRNA-HEIH in GC.

Materials and Methods: Oncomine was used to analyze HEIH expression in cancerous and paired noncancerous tissues of GC patients. Subsequently, the expression levels of HEIH in GC cells was determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In addition, the effects of HEIH expression level on clinicopathological parameters and prognosis were further studied by statistical analysis and Kaplan-Meier survival curves. GC cell proliferation and the influence of HEIH on the sensitivity of cells to oxaliplatin following HEIH knockdown were assessed using sulforhodamine blue (SRB) assays in the MKN45 and AGS cell lines. In addition, the expression levels of p53 were detected by RT-qPCR following knockdown of HEIH.

Results: The lncRNA-HEIH was highly expressed in both GC tissues and GC cell lines. Patients with high HEIH expression were associated with medium-high differentiation ($p=0.0058$), distant metastasis (M, $p=0.0378$), lymph node metastasis (N, $p=0.0083$), and a deeper tumor invasion (T, $p=0.0204$). Elevated expression levels of HEIH in GC patients were associated with a worse prognosis compared to GC patients with low HEIH expression. This finding was supported by the parameters of overall survival ($p=3.3e-06$), first progression ($p=0.00028$), and postprogression ($p=1.5e-08$). Downregulation of HEIH expression inhibited cell proliferation, enhanced oxaliplatin sensitivity, and induced the expression of p53 in MKN45 and AGC cells.

Conclusion: These findings provide evidence that HEIH may be useful as a prognostic biomarker in GC. This lncRNA may also serve as a potential therapeutic target in GC patients.

Keywords: lncRNA-HEIH, gastric cancer, oncomine, proliferation, p53

Introduction

GASTRIC CANCER (GC) is the fifth most frequently diagnosed malignant tumor and exhibits the third highest cancer-related mortality rate worldwide (Smyth *et al.*, 2020). Approximately 80% of patients with GC in China are diagnosed at an advanced stage (Zong *et al.*, 2016). The disease is accompanied by regional lymph node invasion and distant metastasis (Rawla and Barsouk, 2019). Emerging evidence indicates that the 5-year overall survival (OS) rate for patients with metastatic cancer following diagnosis is only 5% (Thrift and El-Serag, 2020). Chemotherapy is recognized as the standard first-line treatment for patients with advanced GC, including fluoropyrimidine and oxaliplatin (Wang *et al.*, 2019). Despite improvements in chemotherapeutic options,

patients with GC often exhibit poor prognosis due to drug resistance and postoperative recurrence (Zhang *et al.*, 2016). Consequently, the exploration of novel biomarkers that can be used to assess the prognosis of GC is particularly significant for effective clinical decision making.

Long noncoding RNAs (lncRNAs) comprise a class of noncoding RNAs with ~200 nucleotides in length (Iyer *et al.*, 2015). lncRNAs are involved in cellular development and differentiation (Spizzo *et al.*, 2012; Fatica and Bozzoni, 2014). lncRNA high expressed in hepatocellular carcinoma (HEIH) is a member of the lncRNA family, which was initially reported and investigated in hepatocellular carcinoma (Yang *et al.*, 2011). Moreover, the tumor-promoting role of HEIH in multiple carcinoma types has been reported, including non-small cell lung cancer (Jia *et al.*, 2019), melanoma

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(Zhao *et al.*, 2017), and breast cancer (Li *et al.*, 2019). However, the association between lncRNA-HEIH and GC has not been previously studied.

Based on the results of previous studies, the expression levels of HEIH were investigated in GC tissues and cell lines and the clinical parameters and prognostic value of HEIH were analyzed in patients with GC. Cell viability and the function of HEIH in mediating oxaliplatin sensitivity in GC were explored by cell proliferation assays. The effect of HEIH on the expression of p53 was also investigated in GC samples. This study aimed to provide novel information on the vital role of HEIH in GC.

Materials and Methods

Clinical samples

A total of 215 GC patient tissue specimens, including 125 samples from male subjects and 90 from female subjects with an age range from 37 to 86 years, were obtained from the Department of General Surgery, The First Affiliated Hospital of Soochow University (Suzhou, China). The clinical parameters were collected, including gender (male/female), age (<60/≥60 years old), differentiation (medium-high/low), pathological depth of invasion stage (T1+2/T3+4), pathological lymph node metastasis stage (–/+), distant metastasis stage (M₀/M₁). The inclusion criteria were as follows: (1) histopathological diagnosis of adenocarcinoma; (2) patients who had not received antitumor treatment before the study; and (3) computed tomography of the chest, abdomen, and pelvis did not show evidence of distant metastasis. The exclusion criteria were as follows: (1) patients who cannot tolerate surgical treatment and (2) patients who did not allow specimen provision. All the patients involved in this study signed informed consent agreements. The protocol of this study was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University (Suzhou, China; approval no. 2021015). None of the patients had received chemotherapy or radiotherapy before surgery.

Oncomine database analysis

The data were derived from the Oncomine database, an online cancer microarray/gene database for RNA and DNA sequences, and were used to analyze HEIH expression in GC and normal tissues by the *t*-test. The cut-off *p*-value and fold change were as follows: *p*-value ≤0.0001 and fold change: 2.

Cell culture

The normal gastric epithelial cell line GES1 and the human GC cell lines (MKN45, NCI-N87, AGS, and HGC-27) were provided by the Cell Bank of the Chinese Academy of Science (Shanghai, China). The cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere containing 5% CO₂.

Reverse transcription-quantitative polymerase chain reaction

Total RNA was extracted from GC cells using the TRIzol reagent (Invitrogen) according to the manufacturer's in-

structions. Subsequently, cDNA was amplified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using SYBR Premix Ex Taq kit (TaKaRa, Tokyo, Japan). The following thermal cycling conditions were used for the PCR: 95°C for 30 s; 45 cycles of 95°C for 5 s; and 60°C for 30 s. The relative mRNA expression of the target genes was calculated by the 2^{−ΔΔC_q} method. GAPDH was used as an internal control and all samples were run in triplicate. The primer sequences used were as follows: lncRNA-HEIH: forward CCTCTTGTGCCCCCTTTCTT and reverse ATGGCTTCTCGCATCCTAT; p53: forward TAACAGTTCTTGCATGGGCGGC and reverse AGGACAGGCACAAACACGCACC; and GAPDH: forward TTCCTGGGCATGAGTCCT and reverse TGATCTTCATTGTGCTGGGTG.

Kaplan-Meier plotter survival analysis

The prognostic value of HEIH mRNA expression in GC was evaluated by an online database, the Kaplan-Meier (KM) plotter. The parameters OS, first progression (FP), and postprogression survival (PPS) were assessed. The patient samples were divided into two groups (high vs. low expression) based on the median values of HEIH mRNA expression. The corresponding survival curve, hazard ratios (HR), 95% confidence intervals, and *p*-values were calculated and plotted in R. The log-rank test was also performed and a *p* < 0.05 was considered for significant differences.

Small interfering RNA transfection

MKN45 and AGS cells were seeded into six-well plates (Corning, Acton, MA) and left to grow to ~80% confluence. siHEIH-1 (siRNA-1, 5'-TGC GCC TTC CCT CTA ACC TTA ATT CAA GAG ATT AAG-3'), siHEIH-2 (siRNA-2, 5'-TGG CAA GAT GAA CGT CTG AAA TTT CAA GAG AAT TTC-3'), and negative control (NC, all Genescript, Shanghai, China) sequences were transfected into the cells. The small interfering RNA (siRNA) was transfected into MKN45 and AGS cells using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions.

Oxaliplatin treatment

Oxaliplatin (Selleck, Shanghai, China) was added at the indicated concentrations (12.5, 25, 50, 100, and 200 μM) into the culture medium of (siHEIH-) MKN45 and (siHEIH-) AGS cells. The cells were incubated with oxaliplatin for 48 h at 37°C in the presence of 5% CO₂.

Cell proliferation assay

Cell proliferation was determined by the sulforhodamine blue (SRB) assay (Orellana and Kasinski, 2016). The cells were seeded into a 96-well plate (Corning) for the indicated time points (1, 2, and 3 days) with 1 × 10⁴ cells (100 μL per well). The cells were fixed with 50 μL 30% (v/v) trichloroacetic acid (TCA; Sinopharm Reagent, Shanghai, China) for 60 min at 4°C and washed five times using distilled water. Following washing and fixation, the cells were stained with 100 μL 0.4% (w/v) sulforhodamine B (SRB, prepared in 1% acetic acid) (Sigma-Aldrich, St. Louis, MO) for 60 min at room temperature. The wells were rinsed with 1% acetate acid solution and air dried. The stain was solubilized

FIG. 1. (A) The lncRNA-HEIH expression levels were assessed in 25 tumor and adjacent nontumor tissues from Oncomine ($p < 0.0001$). (B) The lncRNA-HEIH mRNA expression levels were evaluated in GC and normal gastric epithelial cell lines by RT-qPCR. ** $p < 0.01$ and * $p < 0.05$ versus GES1. GC, gastric cancer; lncRNA-HEIH, long noncoding RNA high expressed in hepatocellular carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction. Color images are available online.

in 100 μ L 10 mM Tris (pH 10.5). The absorbance was recorded with a spectrometer at 490 nm (Infinite M200, Tecan, Austria).

Statistical analysis

The GraphPad Prism 7.0 software was used for statistical analysis. The data were processed by *t*-test and presented as mean $\pm$ standard deviation. The association between HEIH expression and clinicopathological parameters was measured by the χ^2 test. The differences were considered significant at $p < 0.05$.

Results

High expression of HEIH in GC tissues and cell lines

The mRNA expression levels of HEIH in GC and normal GC tissues were determined in a cohort of 25 patients from the Oncomine database. The results revealed that HEIH mRNA levels were significantly higher in GC tissues than those in normal tissues ($p < 0.0001$; Fig. 1A).

In addition, higher expression levels of HEIH were observed in the human GC cell lines (MKN45, NCI-N87, AGS, and HGC-27) compared with those noted in the normal gastric epithelial cell line GES1, as determined by RT-qPCR ($p < 0.05$, Fig. 1B). Finally, the MKN45 and AGS cell lines were selected for subsequent studies.

High HEIH expression levels are associated with more aggressive clinicopathological characteristics in GC patients

A total of 215 GC patients were selected, which included 125 male patients and 90 female patients. The association between HEIH expression levels and GC patient clinicopathological parameters is shown in Table 1. A significant difference was noted between the expression levels of HEIH and the T, N, and M stage. High expression levels of HEIH were significantly associated with medium-high differentiation ($p = 0.0058$),

M stage ($p = 0.0378$), N stage ($p = 0.0083$), and T stage ($p = 0.0204$). The parameters gender and age were not associated with high HEIH expression levels ($p < 0.05$).

High HEIH expression predicts poor survival in GC patients

Patients with GC and elevated HEIH mRNA expression levels exhibited decreased OS (HR = 1.86; $p = 3.3 \times 10^{-6}$;

TABLE 1. THE ASSOCIATION BETWEEN LONG NONCODING RNA HIGH EXPRESSED IN HEPATOCELLULAR CARCINOMA EXPRESSION AND CLINICOPATHOLOGICAL CHARACTERISTICS OF PATIENTS WITH GASTRIC CANCER ($n = 215$)

Variable	Patients (n)	HEIH levels		p
		Low (n = 64)	High (n = 151)	
Gender				
Male	125	33	92	0.2031
Female	90	31	59	
Age				
<60	121	36	85	0.9955
≥ 60	94	28	66	
Differentiation				
Medium-high	100	39	61	0.0058
Low	115	25	90	
M				
M0	165	55	110	0.0378
M1	50	9	41	
N				
–	82	33	49	0.0083
+	133	31	102	
T				
T1 + 2	95	36	59	0.0204
T3 + 4	120	28	92	

M₀, no distant metastasis; M₁, distant metastasis; N (–), no lymph node metastasis; N (+), lymph node metastasis; T, depth of invasion. HEIH, high expressed in hepatocellular carcinoma.

FIG. 2. The prognostic values of lncRNA-HEIH in GC. (A) High mRNA levels of HEIH were associated with lower OS in all GC patients (female or male). (B) High mRNA levels of HEIH were associated with lower OS in stage III and IV, but not in stage II GC patients. (C) High mRNA levels of HEIH were associated with OS in GC patients who received single treatment with surgery, 5FU-based adjuvant therapy and other adjuvant therapy. OS, overall survival. Color images are available online.

Fig. 2A), FP (HR=1.71, $p=0.00028$; Fig. 3A), and PPS (HR=2.17, $p=1.5e-08$; Fig. 4A). In addition, the prognosis of patients with high and low HEIH expression levels and different gender and stage (II, III, IV) was assessed according to the treatment selection, which included surgical treatment, 5FU-based adjuvant, and other adjuvant therapies. The statistical analysis of the variables demonstrated the following results: female subjects (OS, HR=2.77, $p=0.00014$; FP, HR=2.57, $p=3e-06$; and PPS, HR=3.1, $p=5.7e-05$), male subjects (OS, HR=1.75, $p=0.00024$; FP, HR=1.53,

$p=0.00052$; and PPS, HR=2.12, $p=6e-06$), stage II (PPS, HR=2.9, $p=0.00098$), stage III (OS, HR=2.3, $p=0.00072$; FP, HR=2.14, $p=0.0012$; and PPS, HR=2.36, $p=8.1e-05$), stage IV (OS, HR=1.53, $p=0.047$, and PPS, HR=1.89, $p=0.0074$), patients receiving surgical treatment alone (OS, HR=2.01, $p=0.00022$; FP, HR=1.79, $p=0.0016$; and PPS, HR=2.09, $p=2.3e-06$), 5FU-based adjuvant (OS, HR=0.3, $p=0.018$), and other adjuvant (OS, HR=3.46, $p=0.00032$; FP, HR=3.52, $p=0.00081$; and PPS, HR=3.02, $p=0.013$). Moreover, the data indicated that high HEIH

FIG. 3. Association between lncRNA-HEIH expression and FP of patients with GC. (A) High mRNA levels of HEIH were associated with lower FP in all GC patients (female or male). (B) High mRNA levels of HEIH were associated with shorter FP in stage III, but not in stage II and IV GC patients. (C) High mRNA levels of HEIH were associated with FP in GC patients who received single treatment with surgery and those who received additional adjuvant therapy. This effect was not noted in GC patients with 5FU-based adjuvant therapy. FP, first progression. Color images are available online.

expression was significantly associated with worse OS, FP, and PPS compared with that noted in patients with low HEIH expression. Taken together, these results indicated that GC patients with high HEIH expression levels exhibited lower OS, FP, and PPS, and that HEIH may be a poor prognostic factor associated with the survival of GC patients.

Knockdown of HEIH inhibits cell proliferation of GC

SRB assay indicated that the proliferative activity of MKN45 and AGS cells in both siRNA-1 and siRNA-2 groups

was significantly reduced compared with that of the NC group ($p < 0.05$, Fig. 5A), which indicated that the interference of HEIH expression inhibited cell proliferation of GC cells.

Knockdown of HEIH enhances oxaliplatin sensitivity of GC cells

In both MKN45 and AGS cells treated with increasing concentrations of oxaliplatin, lncRNA-HEIH interference significantly reduced cell viability compared with that of the

FIG. 4. Association between lncRNA-HEIH expression and PPS of patients with GC. (A) High mRNA levels of HEIH were associated with reduced PPS in all GC patients (female or male). (B) High mRNA levels of HEIH were associated with lower PPS in stage II, III, and IV GC patients. (C) High mRNA levels of HEIH were associated with PPS in GC patients who received single treatment with surgery and GC patients who received additional adjuvant therapy. This finding was not noted in GC patients who received 5FU-based adjuvant therapy. PPS, postprogression survival. Color images are available online.

siRNA groups ($p < 0.05$; Fig. 5B). These results indicated that interference of HEIH expression could enhance oxaliplatin sensitivity in GC cells.

Knockdown of HEIH upregulates the expression of p53 in GC

Following lncRNA-HEIH interference, the p53 expression levels of MKN45 and AGS cells were detected by RT-qPCR. The relative expression of p53 was significantly upregulated

in the siRNA-HEIH group of GC cells compared with that of the NC groups (Fig. 5C, $p < 0.05$). HEIH inhibited the expression of p53 in MKN45 and AGS cells, suggesting that it may be a downstream molecule of HEIH.

Discussion

GC has become a major threat to human health due to its late detection and poor prognosis (Van Cutsem *et al.*, 2016). lncRNAs, which have limited protein-coding ability,

FIG. 5. (A) SRB assay demonstrating that knock-down of lncRNA-HEIH inhibits cell proliferation ability of MKN45 and AGS cells. (B) The results from the SRB assay indicated that siRNA-1 or siRNA-2 GC cells exhibited increased sensitivity to treatment following an increase in the concentrations of oxaliplatin in MKN45 and AGS cells. (C) P53 expression was up-regulated following knock-down of lncRNA-HEIH in both MKN45 and AGS cells. * $p < 0.05$ versus NC. SRB, sulforhodamine blue; siRNA-1, siRNA-HEIH-1; siRNA-2, siRNA-HEIH-2; NC, negative control; siRNA, small interfering RNA. Color images are available online.

play critical roles in cancer progression and metastasis (Mirhosseini *et al.*, 2019). lncRNA-based clinical tools have been rapidly developed, including diagnostic and prognostic biomarkers, as well as therapeutic targets (Mao *et al.*, 2018; Yan *et al.*, 2019; Zhang *et al.*, 2019). Although the role of HEIH in diverse cancer types has been previously reported, the expression of this lncRNA and its clinical significance in GC remain unclear.

To the best of our knowledge, this study provides the first preliminary evidence to support the association between HEIH expression and GC. HEIH was highly expressed in human GC tissues and cell lines. A significant association between HEIH expression levels and specific clinical parameters was reported, including medium-high differentiation, distant metastasis, lymph node metastasis, and a larger tumor size. These results suggested the potential role of lncRNA-HEIH as a molecular biomarker of GC. Subsequently, the prognostic value of HEIH in GC was assessed. This

study further assessed whether high HEIH expression could predict poor prognosis of GC patients by KM survival analysis. The results suggested that HEIH expression may aid the prediction of the postoperative tumor progression and were consistent with those of Cui *et al.* (2018). Therefore, lncRNAs may be used as markers for cancer diagnosis and prognosis.

A previous study has indicated that lncRNA-HEIH is involved in the modulation of tumor progression (Wan *et al.*, 2020). To explore the role of HEIH in GC, its expression was knocked down by siRNAs in GC cells. SRB analysis indicated that HEIH knockdown significantly inhibited the viability of MKN45 and AGS cells. Several studies have previously demonstrated that lncRNAs are involved in drug resistance of GC (Yang *et al.*, 2015; Ayers and Vandesompele, 2017; Heery *et al.*, 2017). Guo *et al.* (2019) reported that downregulation of lncRNA-HEIH reversed paclitaxel resistance of endometrial cancer cells by activation

of the MAPK signaling pathway. Based on these conclusions, the effect of HEIH expression was explored on oxaliplatin sensitivity in GC cells by the SRB assay. The data demonstrated that HEIH weakened chemotherapeutic sensitivity to oxaliplatin in GC cells. However, the precise molecular mechanism of this process requires further investigation.

P53 is widely accepted as one of the most important tumor suppressor proteins (Kruiswijk *et al.*, 2015). Mutation and/or deletion of the p53 gene plays a prominent role across numerous malignancies, including GC (Lin *et al.*, 2019; Blanchet *et al.*, 2021). Increasing evidence has shown that lncRNAs are associated with the p53 pathway in a number of *in vitro* and *in vivo* models (Chen *et al.*, 2020; Zhao *et al.*, 2020). It was also shown that knockdown of lncRNA-HEIH downregulated the expression levels of p53, which suggested a possible lncRNA-mediated transcriptional regulation of p53.

The limitation of this study was the lack of *in vivo* experiments. Animal experiments may aid the clarification of the exact function and regulatory mechanisms of HEIH in GC.

Conclusion

In conclusion, this study examined the role of lncRNA-HEIH by validating its oncogenic and prognostic potential in GC. High HEIH expression was associated with poor prognosis in GC patients. Moreover, knockdown of HEIH exhibited antitumor properties by inhibition of cell viability, promotion of sensitivity to oxaliplatin, and induction of p53 expression in GC. These findings suggest the potential application of HEIH as a biomarker for the prognosis of GC. However, additional studies are required to verify the findings of this study.

Authors' Contributions

X.C. and X.S. conceived and designed the experiments. X.L., X.C., and X.S. performed all the experiments. W.Y. and L.X. wrote and revised the article. All authors read and approved the final version of the article.

Author Disclosure Statement

No competing financial interests exist.

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