H19 functions as a competing endogenous RNA to regulate human epidermal growth factor receptor expression by sequestering let-7c in gastric cancer

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Abstract. Long non-coding RNA (lncRNA) H19 has been suggested to serve important roles in the progression of gastric cancer (GC); however, the mechanism involved is largely unknown. The present study aimed to investigate the mechanism underlying the effect of H19 on human epidermal growth factor receptor (HER2) expression. Let-7c belongs to the let-7 family, which has been reported to be downregulated in cancers and considered to serve as a tumor suppressor. Let-7c has been negatively associated with the expression of human epidermal growth factor receptor 2 (HER2). Reverse transcription-quantitative polymerase chain reaction was used to examine the expression levels of H19 and let-7c in GC tissues and cell lines. HER2 protein expression levels were examined using immunohistochemistry and western blot analyses. The effect of H19 on let-7c and HER2 expression was analyzed following transfection of small interfering RNA targeting H19 in GC cells. The results indicated that the expression levels of H19 lncRNA in GC tissue samples were significantly higher when compared with that in matched benign adjacent tissue samples (P<0.001). H19-silenced GC cells exhibited significantly increased let-7c expression and decreased HER2 protein expression levels. Assessment of tumor diameter and pathological tumor stage suggested that increased H19 expression was associated with a poorer prognosis in patients with GC. The results of the present study suggest that H19 may function as a competing endogenous RNA to regulate HER2 expression by sequestering let-7c in GC cells. The present study has aided the understanding of the mechanism of H19 lncRNA in GC, and has provided evidence for the application of lncRNA-based diagnostic and therapeutic strategies for GC.

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Introduction

Gastric cancer (GC) is one of the most common solid malignant tumors (1), and is typically associated with a poor prognosis due to the high frequency of metastases and relapse. In addition, the limitations of chemotherapy and surgery have contributed to the low survival rates of patients with GC. The five-year survival rate of patients with GC is 30-50% (2). Therefore, a comprehensive understanding of the mechanisms involved in the development and progression of GC is essential for improving the diagnosis, prevention and treatment of this disease.

Long non-coding RNA (lncRNA) is a type of non-protein encoding endogenous RNA that is ~200 nucleotides in length. This feature permits the formation of secondary structures. Some lncRNAs possess the same sequences corresponding to protein-coding genes (3). Previous studies have revealed that lncRNAs and mRNAs may compete for shared microRNA (miRNA) response elements (4-6). It has been reported that lncRNAs might function as competing endogenous RNAs (ceRNAs) to sequester miRNAs, thereby modulating the expression of miRNA target genes (4-7). The H19 lncRNA is a paternally imprinted gene located close to the telomeric region of chromosome 11p15.5 (8,9), which is a region that is frequently associated with tumor development (10,11). It has been demonstrated that H19 lncRNA binds and sequesters let-7, which inhibits its function (12). In addition, Gao *et al* (13) identified that let-7c is negatively associated with human epidermal growth factor receptor 2 (HER2) expression. The results of these studies suggest that a correlation and potential crosstalk between H19, let-7c and HER2 may exist. Therefore, the aim of the present study was to investigate this hypothesis.

Materials and methods

Tissue samples. A total of 24 GC and adjacent benign tissues (located 5 cm from the tumor margin) were collected during the surgery from patients admitted to the General Hospital of Daqing Oil Field (Daqing, China) between February 2016 and August 2016. None of the patients had received radiotherapy and chemotherapy before surgery. The differentiation and tumor, node and metastasis (TNM) stage of the tumor tissues were determined by histopathology. The sex ratio between female and

Table I. Correlation between H19 expression and clinicopathologic features of patients with gastric cancer.

Clinical pathologic features	Number of patients (%)	Relative expression of H19 (95% CI)	P-value
Sex			0.660
Male	16 (66.7)	3.21 (0.75-4.53)	
Female	8 (33.3)	4.01 (1.34 -4.85)	
Tumor size (cm)			0.038
>5	14 (58.3)	3.20 (1.34-4.85)	
<5	10 (41.7)	1.90 (0.75-3.12)	
Differentiation			0.019
Poor	13 (54.2)	3.60 (1.25-4.36)	
High/moderate	11 (45.8)	1.10 (0.75-3.14)	
Lymph node metastasis			0.015
N0	8 (33.3)	1.14 (0.75-1.42)	
N1-3	16 (66.7)	3.44 (1.15-4.85)	
Metastatic disease stage			0.383
M0	7 (29.2)	2.35 (1.53-3.64)	
M1	17 (70.8)	3.82 (0.75-4.85)	

95% CI, 95% confidence interval.

male was 1:2. The average age of the patients was 63 ± 2.6. The clinicopathological features of all patients are listed in Table I. The present study was approved by the Ethics Committee of the General Hospital of Daqing Oil Field, and written informed consent was obtained from all patients. Tumor diameters were measured with a Vernier caliper and were stored in liquid nitrogen.

Cell culture. Human GC cell lines, BGC823 and SGC7901, and the normal gastric epithelial GES-1 cell line, were purchased from the China Academy of Chinese Medical Sciences (Beijing, China). Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Sanofi Genzyme, Cambridge MA, USA) at 37°C in an atmosphere containing 5% CO_2.

Immunohistochemistry (IHC). Tissues were fixed in 4% paraformaldehyde and subjected to standard (4 mm) paraffin sectioning. For immunohistological staining, the sections were treated with hydrogen peroxide to black the endogenous peroxidase activity, followed by heating to 96°C for 10 min for antigen retrieval. Following blocking with 1% goat serum (G9023; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at room temperature for 1 h, the sections were then incubated with anti-HER2 primary antibody (1:800, cat. no. 4290; Cell Signaling Technology, Inc., Danvers, MA, USA) for 2 h at room temperature. The anti-PCNA antibody (1:50, BZ00678; Bioworld Technology Inc., St. Louis Park, MN, USA) was also used to stain the nucleus. Immunoperoxidase staining was performed with the 3,3' diaminobenzidine chromogen (K3647, Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 5 min at room temperature. The images were captured by light microscopy (Leica Microsystems Ltd., Milton Keynes, UK)

under high magnification (x200). IHC-staining was confirmed independently by three pathologists. The IHC scoring system was used to determine the scores of HER2 expression (14-19). Scores ≥2+ were defined as HER2-positive, and IHC scores of 0 and 1+ were defined as HER2-negative, indicating tumor differentiation stage.

Transfection of GC cells. All plasmid vectors for transfection were extracted from DH5α competent cells (Thermo Fisher Scientific, Inc.) using a DNA Midiprep kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Three individual H19 small interfering (si)RNAs (si-H19) and a scrambled negative control siRNA (si-NC) were purchased from Invitrogen; Thermo Fisher Scientific, Inc. Target sequences for H19 siRNAs were listed as H19-siRNA1, 5'-UAAGUCAUUUGCACUGGUUdTdT-3'; H19-siRNA2, 5'-GCAGGACAUGACAUGGUCCdTdT-3'; and H19-siRNA3 5'-CCAACAUCAAAGACACCAUdTdT-3'. BGC-823 and SGC7901 cells were transfected with si-NC and the three siRNAs of H19 using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

RT-qPCR. Total RNA was extracted from frozen GC tissue samples or BGC823 and SGC7901 cells using TRIzol reagent (Thermo Fisher Scientific, Inc.), and the reverse transcription reactions were performed using the SuperScript™ IV First-Strand Synthesis System (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. H19 and let-7c expression levels were quantified relative to GAPDH expression.

The forward and reverse primers were as follows: GAPDH, forward, 5'-CATGAGAAGTATGACAACAGC CT-3' and reverse, 5'-AGTCCTTCCACGATACCAAAGT-3';
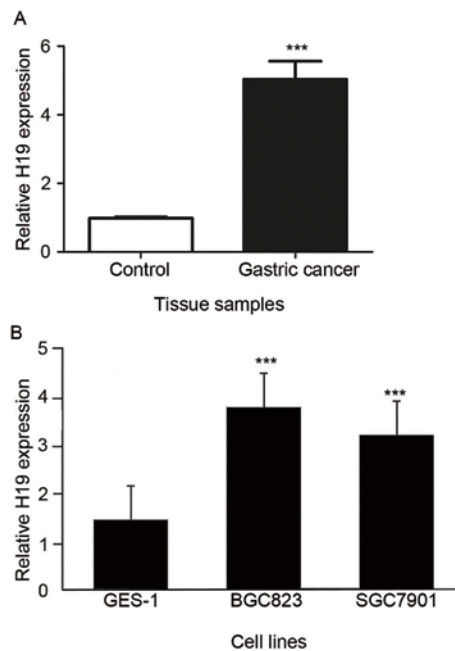
Figure 1. H19 is highly expressed in gastric cancer tissues and cell lines. Reverse transcription-quantitative polymerase chain reaction analysis was performed to detect the expression of H19 within (A) gastric cancer and normal adjacent tissue samples. ***P<0.001. (B) Expression levels of H19 within gastric cancer cell lines, BGC823 and SGC7901, and control GES-1 cells were detected. ***P<0.001 vs. GES-1.

H19 forward, 5'-GGGTCTGTTTCTTTACTTCCTCCAC-3' and reverse, 5'-GATGTTGGGCTGATGAGGTCTGG-3'; let-7c forward, 5'-UGAGGUAGUAGGUUGUAUGGUU-3' and reverse, 5'-UGAGGUAGUAGGUUGUAUGGUU-3'. qPCR was performed using the SYBR-Green PCR kit with the ABI Prism 7900 HT Sequence Detection System (both from Applied Biosystems; Thermo Fisher Scientific, Inc.) The PCR reaction conditions were: Forty-two cycles at 95°C for 30 sec, 1 cycle at 60°C for 30 sec and 1 cycle at 72°C for 30 sec. The relative expression levels were determined with the $2^{-\Delta\Delta Cq}$ method (20).

Agarose gel. The amplified cDNA was separated by 2% agarose gel electrophoresis. The bands were visualized with the ethidium bromide staining [Tiangen Biotech (Beijing) Co., Ltd., Beijing, China]. The DNA fragments were visualized with a long wave UV light monitor at 254 nm.

Western blot analysis. Total protein was extracted from the tissues and GC cells with the Protein Extraction kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). The protein concentration was determined with the bicinchoninic protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Cell protein lysates (20 µg/lane) were separated by 10% SDS-PAGE, transferred to 0.22 µm nitrocellulose membranes (Sigma-Aldrich; Merck KGaA). The membrane was firstly blocked with 5% non-fat milk at room temperature for 1 h. The membrane was then incubated with primary antibodies, including anti-GAPDH (1:2,000, ab37168; Abcam, Cambridge, MA, USA) and anti-HER2 (1:1,000, cat. no. 4290; Cell Signaling Technology, Inc., Danvers, MA, USA) for 2 h at room temperature. GAPDH served as the control. Subsequently, the

Table II. Concordance of HER2 status with H19 expression levels in gastric cancer and adjacent normal gastric tissue samples.

A, Gastric cancer

HER2 score	H19 high	H19 low
0/1+	4	6
2+/3+	12	2
Percentage of HER2 positive (%)	50.0 (12/24)	8.3 (2/24)

B, Adjacent benign tissues

HER2 score	H19 high	H19 low
0/1+	14	7
2+/3+	2	1
Percentage of HER2 positive (%)	8.3 (2/24)	4.2 (1/24)

HER2, human epidermal growth factor receptor 2.

membrane was incubated with horseradish peroxidase-linked goat anti-rabbit or anti-mouse immunoglobulin G (1:5,000, sc-2007 or sc-2005, respectively; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The bands were visualized with the Pierce™ ECL Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.). Autoradiograms were quantified by densitometry analysis using Quantity One software (version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA). GAPDH was used as the control of protein expression level.

Statistical analysis. The results are presented as the mean ± standard error of the mean of five independent experiments. Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The Student's t-test was used to analyze differences between groups. The association between H19 expression and pathological characteristics were analyzed by one-way analysis of variance followed by a Tukey's test and binary logistic regression analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

H19 is highly expressed in GC tissues and cell lines. RT-qPCR analysis indicated that H19 expression was significantly higher in 24 GC tissues when compared the normal adjacent control tissue samples (5.23±0.34, P<0.001; Fig. 1A). In addition, the expression levels of H19 in GC cell lines, BGC823 and SGC7901 (3.68±0.23 and 3.14±0.24, respectively), were significantly increased when compared with normal control GES-1 cells (P<0.001; Fig. 1B).

GC tissues that express high levels of H19 expression are HER2-positive. Immunostaining analysis of the 24 GC
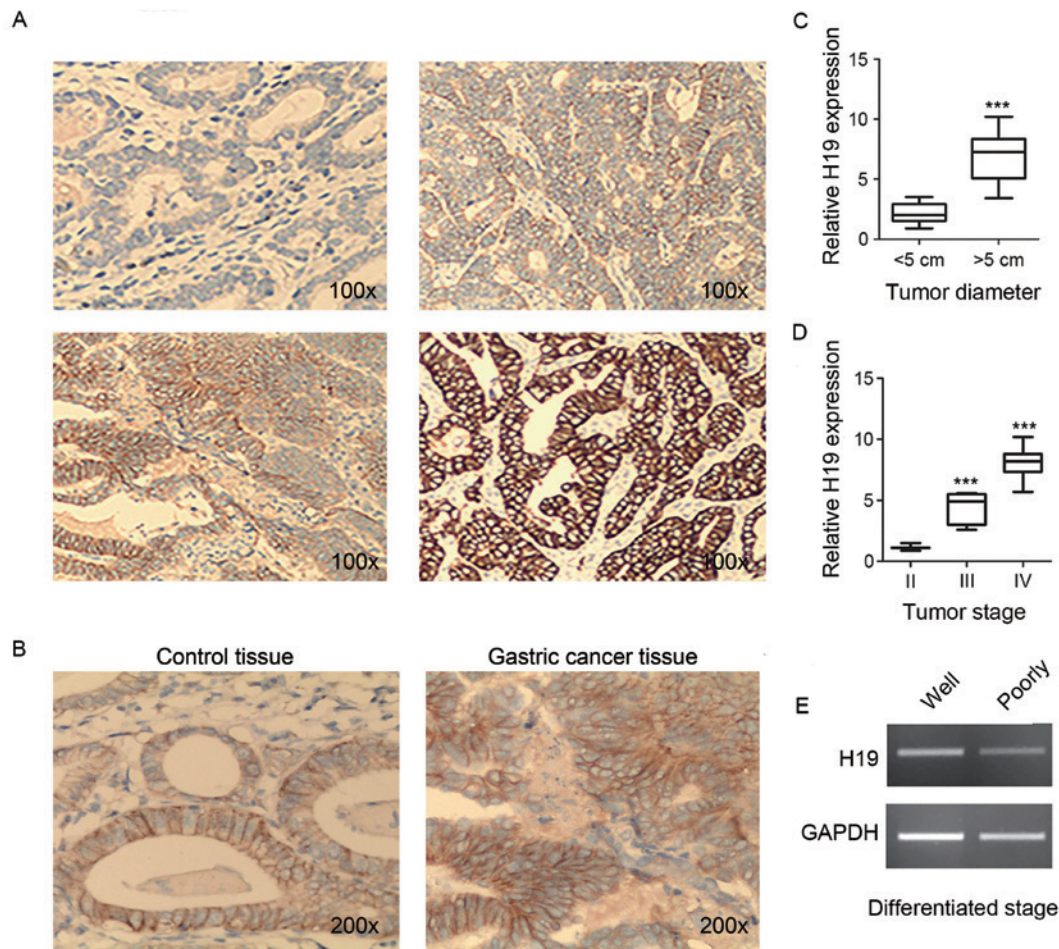
Figure 2. Gastric cancer tissues expressing high levels of H19 are HER2-positive. (A) Standard scores of HER2 in representative immunostaining images. Top left panel=0; top right panel=1; bottom left panel=2; bottom right panel=3. Magnification, x100. (B) Standard HER2 score of gastric cancer tissue was markedly higher (2) comapared with in the control, 0. Magnification, x200 (C) H19 expression levels were significantly higher within the group with tumor diameters >5 cm compared with in tumors <5 cm. ***P<0.001. (D) Advanced tumor, node and metastasis stage groups exhibit a greater average expression levels of H19. ***P<0.001. (E) H19 expression levels were higher in poorly differentiated tissues compared with that of well-differentiated tissues. HER2, human epidermal growth factor receptor 2.

tissue samples and paired normal controls indicated that 50.0% of GC tissue samples that highly expressed H19 were HER2-positive, and lower H19 expression samples were observed to exhibit a lower HER2-positive rate (8.3%; Table II). As presented in Fig. 2A, the standard score of HER2: Top left panel, 0; top right panel, 1; bottom left panel, 2; and bottom right panel, 3. The standard score indicated that the score of HER2 was significantly higher in gastric tissue compared with in the control. As presented in Fig. 2B, the score of HER2 was nearly 0 within the control and nearly 2 with in the gastric cancer tissue. In addition, H19 expression levels were significantly higher in the group with tumor diameters >5 cm compared with in the group with diameters <5 cm (Fig. 2C). The more advance the TNM stage, the grater the average expression levels of H19 (Fig. 2D). Furthermore, H19 expression levels were higher in poorly differentiated tissues compared with in well-differentiated tissues (Fig. 2E).

H19 silencing increases let-7c expression in GC cells. To assess the effect of H19 silencing on the expression levels of let-7a, let-7b and let-7c miRNAs in GC, BGC-823 cells were transfected with H19 siRNA sequences, and RT-qPCR was used to analyze the expression of let-7 miRNAs. BGC-823 cells transfected with H19 siRNA expressed significantly lower levels of H19 expression when compared with scrambled controls (P<0.01; Fig. 3A and B). In addition, the expression of let-7a/b/c with or without si-H19 transfection was detected; the expression of let-7c was significantly increased in the H19 siRNA-transfected BGC-823 cells compared with in si-NC transfected cells (P<0.01; Fig. 3C and D). As presented in Fig. 3C, let-7a expression levels were and let-7b exhibited high expression levels within BGC-823 cell expressing si-NC. The results of the present study revealed that the expression of let-7a/b may not significantly change with or without H19 siRNA transfection.

H19 silencing decreases HER2 expression in GC cells. The expression levels of HER2 in BGC823 and SGC7901 cells transfected with H19 siRNA were assessed using western blot analysis. As demonstrated in Fig. 4, H19-silenced BGC823 and SGC7901 cells expressed markedly reduced levels of HER2 protein expression when compared with their respective scrambled controls.
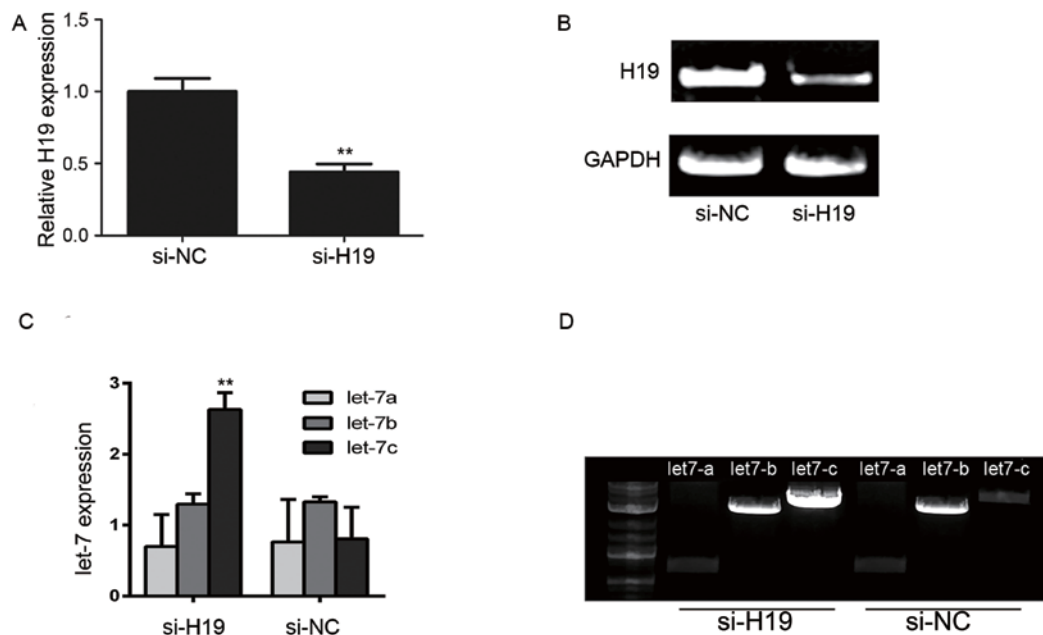
Figure 3. H19 silencing increases let-7c expression levels in BGC823 cells. (A and B) BGC823 cells transfected with si-H19 expressed significantly decreased levels of H19 compared with the si-NC controls (**P<0.01 vs. si-NC). (C and D) The expression of let-7a/b/c with si-H19 or si-NC transfection. Data are presented as the mean ± standard deviation (**P<0.01 vs. let 7c in si-NC transfected cells). si-H19, small interfering RNA targeting H19; si-NC, scrambled negative control small interfering RNA.
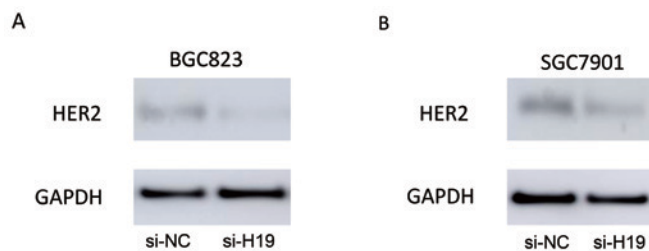

Figure 4. H19 silencing decreases HER2 expression levels in gastric cancer cells. Protein expression levels of HER2 in (A) BGC823 and (B) SGC7901 cells transfected with si-H19 or si-NC, as determined by western blot analysis. GAPDH was used as a loading control. HER2, human epidermal growth factor receptor 2; si-H19, small interfering RNA targeting H19; si-NC, scrambled negative control small interfering RNA.

Discussion

lncRNAs are RNA transcripts consisting of ~200 nucleotides with no protein-encoding functions (21). An increasing number of studies have suggested that the molecular mechanisms of carcinogenesis are relevant not only to protein-encoding genes, but also to non-coding RNAs (22-27). Previous findings have indicated that functional alterations of specific lncRNAs promote tumor formation, progression and metastasis in various human malignancies (28).

The H19 lncRNA was first identified to be expressed in developing embryos and adult muscles (29). H19 binds to and sequesters let-7 miRNA family members, thus inhibiting their function (30). The let-7-binding sites on H19 have been demonstrated to sequester let-7 miRNAs in a variety of cell types and among various species.

The majority of studies have indicated that decreased miRNA expression is associated with cancer progression (31-35). These studies suggested the tumor suppressor or oncogenic function of miRNAs in tumors. A previous study revealed that the let-7 family may serve estrogen-dependent and estrogen-independent roles in estrogen receptor-positive cancer types (36).

The present study examined the expression of HER2 in GC tissues and cell lines. The results indicated a positive correlation between H19 and HER2 expression in GC tissue samples. The results of the current study suggest that high levels of H19 expression in GC tumors may be associated with increased tumor size and a more advanced tumor stage compared with tumors expressing lower levels of H19. Similarly, previous studies have reported that GC patients with increased H19 or HER2 expression demonstrated a poorer outcome and response to endocrine therapy (37-39). Notably, Peng et al (39) identified that HER2 expression may correlate with the expression of Lin28 and its homolog Lin28b. These proteins bind to the stem-loop of let-7 miRNA precursors to directly inhibit the Drosha- and Dicer-mediated processing of their primary-miRNA precursors into mature let-7 miRNAs (40). Furthermore, Lin28 expression has been demonstrated to regulate the expression of let-7 miRNA family members in tumors and cell lines (41). Lin28 is transcriptionally regulated by Myc, which is an estrogen receptor-regulated gene that is associated with H19 expression (42). In addition, Lin28 is targeted by let-7, which suggests that H19, Lin28, let-7 and Myc may function as part of a regulatory loop. These findings suggest a novel endogenous gene target as a therapeutic strategy for GC.

In conclusion, the results of the present study demonstrated that H19 may function as a ceRNA to regulate HER2 expression by sequestering let-7c in GC cells. In addition, high expression levels of H19 may be associated with poorer prognosis for patients with GC. Further studies may be encouraged to investigate the outcome of GC patients with high or low

expression abundance of H19 and determine the correlation between the expression of H19 and the overall survival of GC patients.

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