Basic Study

HOX transcript antisense intergenic RNA represses E-cadherin expression by binding to EZH2 in gastric cancer

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Abstract

AIM
To clarify the mechanisms of HOX transcript antisense intergenic RNA (HOTAIR) in gastric cancer (GC) migration and invasion.

METHODS
Quantitative real-time polymerase chain reaction (qPCR) was used to detect the expression level of HOTAIR in GC tissues. The correlation of its expression with clinicopathological features was analyzed. Area under receiver operating characteristic curve (AUCroc) was constructed to evaluate the diagnostic value of HOTAIR. Wound-healing assay and Transwell assay were performed to detect the biological effects of HOTAIR in GC cells. qPCR, western blot and
immunohistochemistry were used to evaluate the mRNA and protein expression of E-cadherin. RNA-binding protein immunoprecipitation was used for the analysis of EZH2 interactions with HOTAIR. Chromatin immunoprecipitation assay was performed to investigate direct interactions between EZH2 and E-cadherin.

RESULTS

The expression of HOTAIR was up-regulated in GC tumors compared with the para-tumorous tissues ($P < 0.001$). Its over-expression was correlated with tumor-node-metastasis (TNM) stage ($P = 0.024$), tumor invasion ($P = 0.018$), lymph node metastasis ($P = 0.023$), and poor prognosis ($P < 0.001$). Multivariate Cox regression analysis confirmed expression of HOTAIR as an independent predictor of overall survival ($P = 0.033$), together with TNM stage ($P = 0.002$) and lymph node metastasis ($P = 0.002$). The AUC was up to 0.709 (95%CI: 0.623-0.785, $P < 0.001$).

Knockdown of HOTAIR by siRNA in GC cells suppressed the migration and invasion of GC cells. Significantly negative correlation between HOTAIR and E-cadherin was found in GC tissues and cell lines, and HOTAIR contributed to the regulation of E-cadherin through binding to EZH2 with the E-cadherin promoter.

CONCLUSION

HOTAIR may play a pivotal role in tumor cell migration and invasion. It can be used as a potential diagnostic and prognostic biomarker for GC.

Key words: Long noncoding RNA; HOX transcript antisense intergenic RNA; Gastric cancer; Migration and invasion; E-cadherin

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Core tip: In this study, we found that HOX transcript antisense intergenic RNA (HOTAIR) expression was up-regulated in gastric cancer (GC) tissues. High expression of HOTAIR was associated with clinicopathological characteristics and poor prognosis in GC patients. Additional experiments revealed that HOTAIR knockdown significantly inhibited the invasion and migration of GC cells. We also tested whether HOTAIR recruited EZH2 to promote tumor cell migration and invasion by repressing E-cadherin in GC. The findings from our study will help clarify the role of HOTAIR in GC progression and its potential as a therapeutic target.

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INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies worldwide, having high rates of both incidence and mortality[1]. Even though remarkable progress has been made in surgical resection and chemotherapy, which are the main therapeutic approaches for GC, the prognosis continues to be dismal[2]. The main cause of treatment failure is cancer recurrence and metastasis[3,4].

Tumor metastasis is a complex process, in which cancer cells leave the primary tumor site and migrate to distant secondary organs. Metastatic cancer cells fractionally retain their epithelial properties and obtain mesenchymal characteristics that give them the ability to invade or distract[5]. This process is called epithelial-to-mesenchymal transition (EMT). EMT is characterized by E-cadherin expression loss and N-cadherin and vimentin up-regulation[6]. It is regulated by many signaling pathways, transcriptional factors and post-transcriptional factors[7]. Post-transcriptional regulatory networks include the miRNA and long noncoding (lnc)RNA families[8]. Emerging evidence shows that lncRNAs have an important role in regulating EMT and cancer metastasis[9]. Several important lncRNAs are reported to induce EMT, including highly upregulated in liver cancer (HULC)[10], metastasis-associated lung adenocarcinoma transcript (MALAT)-1[11], H19[12], and HOX transcript antisense intergenic RNA (HOTAIR)[13].

HOTAIR is identified as an oncogene involved in many kinds of cancers, including breast cancer[14], esophageal squamous cell carcinoma[15], colorectal cancer[16], and GC[13]. HOTAIR acts mainly through the polycomb repressive complex (PRC2) (EZH2, RbAp46, RbAp48 and SUZ12)[17], which trimethylates histone H3 lysine-27 (H3K27) of the HOXD locus and represses HOXD gene expression[14]. EZH2 which is the core catalytic component of the PRC2, can affect cancer progression by altering H3K27 trimethylation and silencing transcription[17].

A previous study showed that silencing of HOTAIR inhibits GC cell migration, invasion and metastasis, and reverses the EMT in GC cells[13]. HOTAIR in combination with PRC2 could epigenetically inhibit miR34a, and control C-Met (HGF/C-Met/Snail pathway) and Snail, thus promoting the EMT process of GC cells and tumor metastasis[13]. However, the overall clinical role of HOTAIR in GC and the molecular mechanisms of HOTAIR involved in GC cell metastasis have not yet been fully investigated.

Up-regulation of HOTAIR expression in GC tissues was found in the present study. We also found that over-expression of HOTAIR was related to the clinicopathological characteristics of and poor prognosis in GC patients. Additional experiments revealed that HOTAIR knockdown significantly repressed migration and invasion. Finally, we tested whether HOTAIR recruited EZH2 to promote tumor cell migration and invasion by repressing E-cadherin in GC. These studies
will help clarify the role of HOTAIR in GC progression and its potential as a therapeutic target.

**MATERIALS AND METHODS**

**Tissue collection and ethics statement**
A cohort of 65 primary GC patients was enrolled in this study, each of who underwent surgery at the First Affiliated Hospital of Nanjing Medical University and Jining NO.1 People’s Hospital between 2008 and 2009. None received chemotherapy or radiotherapy prior to surgery. This study was approved by the University Ethics Committee. All patients participated after providing informed consent. Tumor stage was evaluated in accordance with the tumor-node-metastasis (TNM) classification system (UICC/AJCC 2002). Clinical characteristics are shown in Table 1. The relative expression was calculated using the equation: \( \Delta \text{Ct} = \text{Ct (target gene)} - \text{Ct (GAPDH)} \); Fold change = \( 2^{-\left(x \cdot \text{Ct (tumour)} - x \cdot \text{Ct (normal)}\right)} \) [18].

**Cell transfection**
HOTAIR small interfering (si)RNAs and negative control siRNA (si-NC) were purchased from Invitrogen. The siRNA sequences are listed in Supplementary Table 1. BGC-823 or SGC-7901 cells were grown in 6-well plates until confluent, then transfected with Lipofectamine 2000 (Invitrogen). At 48 h after transfection, cells were harvested for qPCR or western blot analysis.

**Wound-healing assay**
Cells were seeded in 6-well plates in normal cell growth medium and incubated to confluence. A 20-μL tip was used to make a straight scratch, simulating a wound. The medium was changed to medium containing 1% FBS. At different time points, images of the plates were acquired using a microscope.

**Cell migration and invasion assays**
For the migration assays, at 48 h after transfection, \( 5 \times 10^5 \) cells in serum-free medium were placed into the upper chamber of an insert (8-μm pore size; Millipore, Bedford, MA, United States). For the invasion assays, \( 10^5 \) cells in serum-free medium were placed into the upper chamber of an insert coated with Matrigel (Sigma-Aldrich, St. Louis, MO, United States). Medium containing 10% FBS was added to the lower chamber. After incubation for 24 h, the cells remaining on the upper membrane were removed with cotton wool. Cells that had migrated or invaded through the membrane were stained with methanol and 0.1% crystal violet, imaged and counted in five random fields per well using an IX71 inverted microscope (Olympus, Tokyo, Japan). Experiments were repeated independently three times.

**Western blot assay and antibodies**
Cell protein lysates were separated by 15% SDS-PAGE, transferred to 0.22-μm nitrocellulose membranes (Invitrogen, Shanghai, China), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen) in an incubator at 37 °C with 5% CO₂.

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**Table 1** Relationships between the expression of HOTAIR and clinicopathological characteristics in 65 patients with gastric cancer

| Characteristic               | Expression of HOTAIR | \( P \) value |
|------------------------------|----------------------|---------------|
| Sex             | Low \( n = 32 \) | High \( n = 33 \) |
| Female          | 7                    | 11            | 0.408 |
| Male            | 25                   | 22            |
| Age in yr       | \( \leq 60 \)         | 17            | 21 |
| \( > 60 \)      | 15                   | 12            | 0.455 |
| Histological grade |                     |               | 1.000 |
| Well/moderate   | 14                   | 15            |
| Other           | 18                   | 18            |
| Tumor invasion depth, T     |                     |               | 0.018 |
| Tis, T1, T2     | 15                   | 6             |
| T3 or above     | 17                   | 27            |
| Lymph node metastasis, N    |                     |               | 0.023 |
| N0              | 12                   | 4             |
| N1 or above     | 20                   | 29            |
| TNM stage       |                      |               | 0.024 |
| I/II            | 23                   | 14            |
| III/IV          | 9                    | 19            |
| Tumor location   |                      |               | 0.102 |
| Antrum          | 8                    | 15            |
| Cardia          | 10                   | 9             |
| Angulus         | 13                   | 5             |
| Body            | 1                    | 3             |
| Full stomach    | 0                    | 1             |
| Laure’s classification |                 |               | 0.373 |
| Intestinal      | 17                   | 15            |
| Diffuse         | 12                   | 17            |
| Mixed           | 3                    | 1             |

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Expression of HOTAIR (in Invitrogen, Shanghai, China), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen) in an incubator at 37 °C with 5% CO₂.

**RNA extraction and quantitative polymerase chain reaction (qPCR)**
Total RNA was extracted from the frozen tissues using TRIzol reagent (Invitrogen). RNA concentrations were estimated by spectrophotometer absorbance readings of 260 nm. One microgram of total RNA was used for reverse transcription to cDNA with a Reverse Transcription Kit (TaKaRa, Shiga, Japan). An ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, United States) was used to quantify HOTAIR. Ten microliters of SYBR Premix ExTag (TaKaRa) was mixed. Primer sequences are listed in Supplementary Table 1. The relative expression was calculated using the equation: \( \Delta \text{Ct} = \text{Ct (target gene)} - \text{Ct (GAPDH)} \); Fold change = \( 2^{-\left(x \cdot \text{Ct (tumour)} - x \cdot \text{Ct (normal)}\right)} \) [18].

**Cell lines and culture conditions**
Human GC cell lines SGC-7901 and BGC-823 were obtained from the Chinese Academy of Sciences Committee on Type Culture Collection Cell Bank. Cells were cultured in RPMI 1640 or Dulbecco’s modified Eagle’s medium (Gibco-BRL, Shanghai, China) supplemented with 10% fetal bovine serum (FBS)
expression. Receiver operating characteristic (ROC) correlation between HOTAIR and E-cadherin mRNA performed to adjust for other covariates. Spearman survival (OS) time. Multivariate Cox regression was performed to calculate the hazard ratio (HR) and the 95% confidence interval (CI) to evaluate the extent of immunoreactivity ranged from 0 to 3, moderate staining; and 3, strong staining. The scores achieved by multiplying the scores for intensity and field of view (0, < 25%; 1, 25%-50%; 2, 50%-75%; 3, 75%-100%). A final score ranging from 0 to 9 was calculated as a percentage relative to the input DNA by the equation of $2^{(\text{Input Ct} - \text{Target Ct})} \times 0.1 \times 100$. The wound-healing assay showed that HOTAIR knockdown cells were significantly slower than control cells that showed positive staining in each microscopic view of GC tissues. A two-tailed $t$-test were used. The results revealed that patients in the high-expression group had a shorter OS (median OS: 42.5 mo) than those in the low-expression group (median OS: 25.9 mo) than those in the low-expression group ($P < 0.001$; Figure 1A). The sensitivity was 64.62%, the specificity was 75.38%, and Youden's index was 0.4.

Diagonal value of using HOTAIR as a marker

An ROC curve was generated by comparing HOTAIR expression in GC tissues to expression in matched adjacent non-tumor tissues. With a cut-off value of 9.31, the area under the ROC curve (AUCROC) reached 0.709 (95%CI: 0.623-0.785, $P < 0.001$; Figure 1B). The sensitivity was 64.62%, the specificity was 75.38%, and Youden's index was 0.4. Patients with GC were divided into two groups based on the cut-off ratio of HOTAIR expression (2.35-fold) in tumor tissues: high-expression group ($n = 33$) and low-expression group ($n = 32$) (Figure 1C). Table 1 showed that HOTAIR over-expression was significantly correlated with tumor invasion ($P = 0.018$), lymph node metastasis ($P = 0.023$) and higher TNM stage ($P = 0.024$). However, there was no correlation between HOTAIR expression and age, sex and histological grade ($P > 0.05$).

Up-regulation of HOTAIR associated with poor survival of GC

To assess the impact of HOTAIR expression on OS of GC patients, Kaplan-Meier analysis and log rank test were used. The results revealed that patients in the high-expression group had a shorter OS (median OS: 25.9 mo) than those in the low-expression group (median OS: 42.5 mo, $P < 0.001$; Figure 1D). Univariate analyses of clinical variables considered as potential predictors of survival are shown in Table 2. Further analysis in a multivariate Cox proportional hazards model showed that HOTAIR expression ($P = 0.033$), along with TNM stage ($P = 0.002$) and lymph node metastasis ($P = 0.002$), were strongly associated with OS. These results revealed that HOTAIR expression was an independent prognostic indicator of OS.

 Knockdown of HOTAIR suppressed migration and invasion of GC cells

The wound-healing assay showed that HOTAIR knockdown cells were significantly slower than control cells that showed positive staining in each microscopic view of GC tissues. A two-tailed $t$-test were used. The results revealed that patients in the high-expression group had a shorter OS (median OS: 42.5 mo) than those in the low-expression group (median OS: 25.9 mo, $P < 0.001$; Figure 1A). The sensitivity was 64.62%, the specificity was 75.38%, and Youden's index was 0.4. Patients with GC were divided into two groups based on the cut-off ratio of HOTAIR expression (2.35-fold) in tumor tissues: high-expression group ($n = 33$) and low-expression group ($n = 32$) (Figure 1C). Table 1 showed that HOTAIR over-expression was significantly correlated with tumor invasion ($P = 0.018$), lymph node metastasis ($P = 0.023$) and higher TNM stage ($P = 0.024$). However, there was no correlation between HOTAIR expression and age, sex and histological grade ($P > 0.05$).

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 Knockdown of HOTAIR suppressed migration and invasion of GC cells

The wound-healing assay showed that HOTAIR knockdown cells were significantly slower than control
Figure 1  HOTAIR up-regulation correlates with poor survival in patients with GC. A: HOTAIR expression was significantly up-regulated in tumor tissues compared with their corresponding para-carcinoma tissues (shown as -ΔCt); B: ROC curves for observing the diagnostic value of HOTAIR; C: HOTAIR expression was classified into two groups according to HOTAIR expression levels (median split); D: Kaplan-Meier analysis of OS was analyzed according to HOTAIR expression levels; E: Different factors (including HOTAIR, tumor invasion depth, lymph node metastasis, TNM stage, histological grade, gender, tumor location, Lauren’s classification and age) were analyzed for their association with patient survival using Cox regression model. The hazard ratio and 95%CI are plotted for each factor.

CI: Confidence interval; GC: Gastric cancer; HOTAIR: HOX transcript antisense intergenic RNA; OS: Overall survival; ROC: Receiver operating characteristic; TNM: Tumor-node-metastasis.
confirmed that knockdown of HOTAIR inhibited the migration and invasion of GC cells.

HOTAIR was inversely correlated with E-cadherin in GC tissue and cell lines
The HOTAIR and E-cadherin expression levels in GC tissues were detected by qPCR and immunohistochemistry. E-cadherin expression was significantly down-regulated in tumor tissues ($P = 0.001$), as compared with the para-tumor tissues (Figure 3A and B). E-cadherin mRNA levels were negatively associated with HOTAIR expression in GC tissues ($r^2 = -0.298, P = 0.016$) (Figure 3C). HOTAIR silencing strikingly enhanced the expression of E-cadherin at transcript (Figure 3D and E) and protein (Figure 3F and G) levels in BGC-823 and SGC-7901 cells. These results demonstrated that HOTAIR promoted EMT in GC tissues and cell lines, and that HOTAIR over-expression was significantly associated with migration and invasion.

HOTAIR recruited EZH2 to inhibit the expression of E-cadherin
HOTAIR plays its role mainly in the epigenetic regulation of gene expression mainly by binding to PRC2 complex. We firstly used RIP assay to investigate whether HOTAIR could bind to EZH2, which is the core catalytic component of the PRC2. The results showed that endogenous HOTAIR was abundant in the anti-EZH2 RIP fraction (Figure 4A), validating that HOTAIR can bind to EZH2 in GC cells. Then we conducted ChIP assay to determine whether HOTAIR regulates E-cadherin via recruiting EZH2 in GC cell lines. ChIP assay demonstrated that knockdown of HOTAIR significantly reduced the binding of EZH2 and H3K27me3 with the E-cadherin promoter in GC cells (Figure 4B). The rescue experiments showed that up-regulation of HOTAIR increased the binding of EZH2 and H3K27me3 with the E-cadherin promoter in GC cells (Figure 4C). These results indicated that HOTAIR inhibits E-cadherin expression partly through combining with EZH2.

**DISCUSSION**
LncRNAs are non-protein-coding transcripts > 200 nucleotides. Dysregulation of lncRNAs has been found in GC cells and their aberrant expression is correlated with tumorigenesis, metastasis, prognosis or diagnosis[20,21].

We found that HOTAIR was markedly up-regulated in GC tissues, which was correlated with the invasion of primary tumor, lymph node metastasis, and TNM stage. The ROC curve used for distinguishing between GC tissues and normal tissues showed that HOTAIR is a promising cancer biomarker. Univariate and multivariate Cox regression analyses demonstrated that HOTAIR is a valuable prognostic factor independent of major clinicopathological features. We also found that decreased HOTAIR expression inhibited migration and invasion of tumor cells.

To clarify the molecular mechanism of HOTAIR action contributing to GC metastasis, we also investigated potential target genes involved in cell migration and invasion. Previous studies have revealed that the expression of epithelial markers (such as E-cadherin) are increased following HOTAIR knockdown in GC cell lines[13,22,23]. Further mechanistic studies have shown that by recruiting and binding to PRC2, HOTAIR could epigenetically silence miR34a expression to promote GC cell EMT and metastasis[12]. However, there are still some issues that need to be clarified. For one thing, E-cadherin is a target gene of EZH2. Because EZH2 can mediate transcriptional silencing of E-cadherin through trimethylation of H3 lysine 27[24,25], and HOTAIR binds to EZH2[14]. Furthermore, a significantly negative correlation was found between HOTAIR and E-cadherin in GC tissues in the present study, and knockdown of EZH2 also up-regulated E-cadherin expression in GC cells as shown by another study[26]. So, why does HOTAIR not regulate expression of E-cadherin directly by binding to EZH2? We speculate that HOTAIR promotes tumor cell migration and invasion by repressing E-cadherin via EZH2 directly. To confirm this, we performed ChIP analysis in HOTAIR-silenced GC cell lines. Not unexpectedly, HOTAIR contributed to the regulation of E-cadherin via recruiting EZH2 and H3K27me3 to the E-cadherin promoter. Our findings provide additional insight into the mechanisms by which HOTAIR promotes GC migration and invasion.

| Table 2 Univariate and multivariate analysis of clinical pathological characteristics and overall survival of 65 patients with gastric cancer |
| --- |
| Factor | Univariate analysis | Multivariate analysis |
| | HR | 95%CI | $P$ value | HR | 95%CI | $P$ value |
| HOTAIR expression | 2.943 | 1.580-5.483 | 0.001 | 1.998 | 1.058-3.772 | 0.033 |
| Tumor invasion depth, T1, T2/above | 2.651 | 1.316-5.341 | 0.006 | 1.676 | 0.691-4.067 | 0.254 |
| Histology grade, well, moderate/others | 3.470 | 1.459-8.250 | 0.005 | 4.324 | 1.701-10.993 | 0.002 |
| Histology grade, well, moderate/others | 4.494 | 2.351-8.592 | < 0.001 | 3.598 | 1.624-7.975 | 0.002 |
| Age, < 60/> 60 | 0.967 | 0.535-1.750 | 0.913 | 0.396-1.344 | 0.312 |
| Sex, male/female | 0.822 | 0.428-1.581 | 0.557 | 0.482-1.021 | 0.059 |
| Tumor location | 0.839 | 0.623-1.131 | 0.250 | 0.913-3.772 | 0.106 |
| Lauren’s classification | 1.256 | 0.757-2.084 | 0.378 | 1.580-5.483 | 0.001 |

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Role of HOTAIR in gastric tumorigenesis

We found that HOTAIR was markedly up-regulated in GC tissues, which was correlated with the invasion of primary tumor, lymph node metastasis, and TNM stage. The ROC curve used for distinguishing between GC tissues and normal tissues showed that HOTAIR is a promising cancer biomarker. Univariate and multivariate Cox regression analyses demonstrated that HOTAIR is a valuable prognostic factor independent of major clinicopathological features. We also found that decreased HOTAIR expression inhibited migration and invasion of tumor cells.

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In conclusion, we demonstrated that HOTAIR was significantly up-regulated in GC tissues, and its overexpression was correlated with tumor progression and poor prognosis. We can distinguish between...
Figure 3  HOTAIR negatively correlates to E-cadherin expression in GC tissues and cell lines. A: E-cadherin immunostaining in para-carcinoma tissues (denoted as ‘P’) and GC tissues (denoted as ‘T’); B: Representative E-cadherin protein levels and scores in GC tissues and para-carcinoma tissues was analyzed by immunohistochemistry (n = 30); C: Negative correlation between E-cadherin mRNA levels and HOTAIR levels in 65 GC samples; D, E: Relative expression of HOTAIR and E-cadherin in BGC-823 and SGC-7901 cells treated with siRNA for 48 h; F, G: Western blot analysis of E-cadherin after HOTAIR-siRNA (Si1 and Si2) treatment for 48 h in BGC-823 and SGC-7901 cells. *P < 0.05, **P < 0.01. GC: Gastric cancer; HOTAIR: HOX transcript antisense intergenic RNA; si: Small interfering.
cancerous and non-cancerous lesions based on the expression of HOTAIR. HOTAIR may regulate the invasive ability of GC cells, partially via EMT regulation. Our findings have provided new insight into the GC pathogenesis, which benefits diagnosis and therapy in cancer.

**COMMENTS**

**Background**

Gastric cancer (GC) is one of the most aggressive malignancies with high morbidity and mortality worldwide. Treatment options are limited due to the lack of knowledge of the molecular and genetic bases of gastric carcinogenesis. A deeper understanding of the molecular mechanisms of GC will shed light on its
pathogenesis, and identification of new biomarkers for diagnosis and prognosis may improve individualized treatment strategies in the future.

Research frontiers
Emerging evidence suggest that long non-coding (lnc)RNA may play a role in gastric carcinogenesis. HOX transcript antisense intergenic RNA (HOTAIR) is one of the well-documented lncRNAs, which is aberrantly expressed in several tumors and plays a crucial role in cancer development. However, the overall clinical role of HOTAIR in GC and the molecular mechanisms of HOTAIR involved in GC cell metastasis has not yet been well investigated.

Innovations and breakthrough
The authors demonstrated that HOTAIR promotes tumor cell migration and invasion by repressing E-cadherin via EZH2 directly. Their findings have provided additional insight into the mechanisms of gastric carcinogenesis.

Applications
The results from this study provide novel clues for further investigation of HOTAIR as a potential biomarker and therapeutic target for GC.

Terminology
RNA-binding protein immunoprecipitation assay was used for the analysis of EZH2 interactions with HOTAIR. ChIP assay was performed to investigate direct interactions between EZH2 and E-cadherin.

Peer-review
The study aimed to investigate the clinical significance and the mechanism behind lncRNA HOTAIR in GC. The overall study is solid and well designed. The results are consistent with the proposed hypothesis.

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