

Long non-coding RNA HOTAIR mediates the switching of histone H3 lysine 27 acetylation to methylation to promote epithelial-to-mesenchymal transition in gastric cancer

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Abstract. HOX transcript antisense intergenic RNA (HOTAIR), a well-known long non-coding RNA, plays an important role in the regulation of epithelial-to-mesenchymal transition (EMT). In this study, we propose a novel mechanism through which HOTAIR promotes EMT by switching histone H3 lysine 27 acetylation to methylation at the E-cadherin promoter, which induces the transcriptional inhibition of E-cadherin. HOTAIR recruits polycomb repressive complex 2 (PRC2) to catalyze H3K27me₃; however, whether HOTAIR is associated with the acetylation of histone H3 lysine 27, a marker of transcriptional activation, and the mechanisms through which HOTAIR triggers the metastasis of gastric cancer (GC) by epigenetic regulation remain largely unknown. In this study, HOTAIR knockdown significantly reversed EMT by increasing the expression of E-cadherin in GC cells. Additionally, the loss of PRC2 activity induced by HOTAIR knockdown resulted in a global decrease in H3K27 methylation and an increase in H3K27 acetylation. Furthermore, HOTAIR recruits PRC2 (which consists of H3K27 methyltransferase EZH2, SUZ12 and EED), which may inhibit the reaction between the acetyltransferase CBP and H3K27 acetylation. On the whole, the findings of this study suggested that the HOTAIR-mediated acetylation to methylation switch was associated with the transcriptional inhibition of E-cadherin. HOTAIR can promote the development of GC through the epigenetic regulation of E-cadherin, switching the state of the E-cadherin promoter from the transcriptionally active to the transcriptionally repressive state.

Introduction

Epithelial-to-mesenchymal transition (EMT) is a multi-step process that converts epithelial cells into mesenchymal cells, which is mediated by series of transcription factors (TFs) (1). EMT has been linked to epithelial tumor characteristics, including the metastasis and invasiveness of gastric cancer (GC) cells (2-5). E-cadherin is an important EMT TF and epithelial factor that contributes to cell adhesions and cell junctions between adjacent cells (6). However, the molecular mechanisms of EMT require further exploration to reveal its association with the early metastasis of tumor cells.

Long non-coding RNA HOX transcript antisense intergenic RNA (HOTAIR) is a functional RNA that does not encode a protein, and was first identified by Rinn *et al* (7) in 2007. Long non-coding RNA HOTAIR is transcribed from one of the chromosomal loci, the HOXC locus, which contains the clustered HOX genes (8). HOTAIR is associated with different types of cancer, including breast cancer, gastrointestinal tumors, colorectal cancer and pancreatic cancer, and the overexpression of HOTAIR has also been shown to be associated with a poor prognosis and with increased metastasis (9-11). HOTAIR promotes EMT in various types of cancer through different mechanisms. It has previously been identified that HOTAIR regulates glioblastoma (GBM) invasion by increasing the expression of β -catenin (12). A previous study also demonstrated that HOTAIR serves as a modular scaffold for the polycomb repressive complex 2 (PRC2) complex, which consists of EZH2, SUZ12 and EED, and the LSD1/CoREST/REST complex, both of which are histone modification complexes (13). The methyltransferase EZH2 catalyzes H3K27 tri-methylation and induces the epigenetic silencing of gene expression (14). Based on this mechanism, HOTAIR epigenetically represses numerous factors, including microRNA (miRNA or miR)-200 (15) and miR-34a (16), which contribute to the GC cell-EMT process. Notably, long intergenic non-coding RNA HOTAIR is associated with EMT in multiple cancer types, including GC, through different mechanisms, but most importantly, through the epigenetic regulation of gene expression.

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Epigenetics refers to the regulation of genetic function and expression, which is mediated through non-DNA-encoded mechanisms (17). An important type of epigenetic modification is histone modification, which is a post-translational modification mediated by histone-modifying enzymes, including methylation and acetylation. Histone lysine methylation includes mono-, di- and tri-methylation at lysine residues. The methylation event is catalyzed by a group of lysine methyltransferases (KMTs). Notably, histone acetylation requires histone acetyltransferases (HATs) (18,19). Histone modifications regulate transcriptional activation or repression. For example, H3K27me3 is associated with gene repression, and H3K27ac is a hallmark of transcriptionally active genes (20,21). It is important to understand the association between histone modification and the mechanisms of tumorigenesis, which may provide new insight into effective treatment strategies and may improve the prognosis of epithelial tumors.

Recent studies have suggested that HOTAIR is involved in the development of GC and the overexpression of HOTAIR is positively associated with a poor clinical outcome (22-24). In this study, we explored the association between HOTAIR and EMT, particularly as regards the effects on E-cadherin expression. As HOTAIR targets PRC2 (EZH2) to catalyze H3K27me3, we examined whether HOTAIR influences the acetylation of H3K27 by indirectly interacting with HAT. We attempted to connect the HOTAIR-mediated antagonistic switch between histone H3K27 methylation and acetylation at the E-cadherin promoter with the expression of E-cadherin. Our study provides new insight into the mechanisms through which HOTAIR regulates EMT and promotes the carcinogenesis and progression of GC.

Materials and methods

Cell culture. The human gastric cancer cell lines, MGC-803 and SGC-7901 were purchased from China Academia Sinica (Shanghai, China). All cells were cultured in DMEM and RPMI-1640 medium supplemented with 10% FBS (HyClone, Logan, UT, USA) and were maintained in a humidified atmosphere with 5% CO₂ at 37°C.

Lentiviral infection and transient transfection. Lentiviruses containing the HOTAIR overexpression sequence (Lenti-HOTAIR), a HOTAIR inhibitor sequence (Lenti-HOTAIR si) and a negative control sequence (Lenti-NC and NC) were obtained from Shanghai GenePharma (Shanghai, China). For the transient transfection of cells, the tumor cells were transfected with negative control siRNA oligonucleotides against EZH2 and SUZ12 using X-treme GENE siRNA transfection reagent (Roche, Shanghai, China) according to the manufacturer's instructions. The siRNA sequences were as follows: si-EZH2, 5'-GUCGCA ACGGACCAGUAA-3'; and si-SUZ12, 5'-GCTTACGTTTAC TGGTTTCTT-3'. Subsequent experiments were then carried out 48 h following transfection.

Western blot analysis. RIPA lysis buffer was used to extract the total protein, and the Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Biotechnology, Shanghai, China) was used to extract the nuclear protein, according to the

manufacturer's instructions. Equal amounts of protein per lane (30 µg) were separated by 10 and 15% SDS-polyacrylamide gel and transferred onto PVDF membranes. The membranes were blocked in 5% skim milk at 37°C for 1 h and then incubated with a specific antibody at 4°C overnight. The antibodies used in this study were as follows: H3K4me3 (ab8580), H3K4ac (ab240195), H3K27me2 (ab24684), H3K27me3 (ab6002), HEK27ac (ab45173) and β-catenin (ab16051) antibodies (Abcam, Cambridge, UK; 1 µg/ml); EZH2 (5246), SUZ12 (3737), CBP (7389), E-cadherin (14472), N-cadherin (13116), snail (3879), slug (9585), twist (46702), H3K4me1 (5326), H3K4me2 (9725) and H3K27me1 (84932) (Cell Signaling Technology, Danvers, MA, USA; 1 µg/ml); H3 (AF0009; Beyotime Biotechnology; 0.2 µg/ml); GAPDH (TA-08; Zhongshan Bio Corporation, Beijing, China; 0.2 µg/ml). They were then incubated with their respective secondary antibodies (ZB-2305, ZB2306; Zhongshan Bio Corporation; 1:2,000 dilution). The membranes were stripped, and the bands were visualized using the SuperSignal protein detection kit (Pierce/Thermo Fisher Scientific, Waltham, MA, USA) following normalization with the density of GAPDH and H3. ImageJ 1.41 software (National Institutes of Health, Bethesda, MD, USA) was employed to quantify protein expression.

RNA isolation and RT-qPCR. TRIzol reagent (Invitrogen/Thermo Fisher Scientific) was used to extract the RNA from the gastric cancer cells. Equal amounts of RNA were converted into cDNA using HOTAIR and CDH1 primers by using a PrimeScript RT Reagent kit (Takara Bio, Inc., Otsu, Japan). Quantitative PCR (qPCR) was performed using a Reverse Transcription System (Promega, Madison, WI, USA) on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) according to the instructions of the manufacturer. Each sample was analyzed in triplicate. The primers used were as follows: HOTAIR forward, 5'-AAATATGGCGGCGTCTACACGGA-3' and reverse, 5'-TCCAGAACCCTCTGACATTTGCCT-3'; CDH1 forward, 5'-ACGCCGAGAGCTACACGTTTC-3' and reverse, 5'-AGGCTGTCCTTTGTGCGACCG-3'; GAPDH forward, 5'-CCGGGAAACTGTGGCGTGATGG-3' and reverse, 5'-AGGTGGAGGAGTGGGTGTCGCTGTT-3'. Relative expression was calculated using the 2^{-ΔΔC_q} method (25). The PCR conditions were as follows: 35 cycles of 95°C for 30 sec, 56°C for 30 sec, 72°C for 90 sec, and a final extension at 72°C for 5 min.

Immunofluorescence. The MGC-803 cells were transfected with the Lenti-HOTAIR and Lenti-HOTAIR si, NC and Lenti-NC as a control to detect the amounts of H3K27me3/ac in the nuclei, and images were captured using a microscope (Olympus IX81; Olympus Corporation). The antibodies used were as follows: H3K27me3 (ab6002; 5 µg/ml), H3K27ac (ab45173; 5 µg/ml) (both from Abcam) and E-cadherin (14472; Cell Signaling Technology; 5 µg/ml). DAPI was used to dye the nuclei into blue.

Proliferation assay. The transfected and control cells were plated in 96-well plates at a cell density of 10⁴ cells/well with 6 replicate wells. The cells were incubated at 37°C for an additional 4 h by adding 10 µl of CCK-8 (5 g/l; Sigma, St. Louis, MO, USA) into each well. The optical density (OD) was measured at 450 nm at 0, 24, 48, 72, 96 and 120 h, and the

data were expressed as a percentage of the control, which are derived from triplicate samples.

Cell invasion analysis. Transwell invasion assays were performed using Matrigel-coated Transwell membranes (BD Biosciences, San Jose, CA, USA). The Matrigel solidified after a 30-min incubation at 37°C and post-transfection, the cells (10^5) in 100 μ l of serum-free RPMI-1640 medium were added to the upper compartment of the chamber. As a chemoattractant, 20% FBS in RPMI-1640 medium was added to the lower chambers of the wells. The cells were then incubated at 37°C in 5% CO₂ for 48 h, and the lower cells were stained with crystal violet (Zhongshan Bio Corporation) for 5 min at room temperature. The average number of cells per field was calculated by counting cells in 6 random fields per well under the microscope at x200 magnification (Olympus Corporation).

Chromatin immunoprecipitation (ChIP). To determine whether H3K27me3/ac binds to the E-cadherin promoter, ChIP was performed using the EZ-ChIP kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Cross-linked chromatin was extracted from the gastric cancer cells by adding ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0) and sonicated into 200-1,000 fragments. The chromatin was immunoprecipitated by the addition of anti-H3K27ac (ab45173, 1 μ g/ml) and anti-H3K27me3 (ab6002, 1 μ g/ml) antibodies, and a negative control IgG (ab171870, 1 μ g/ml) (both from Abcam), which was incubated overnight at 4°C with Protein G agarose. The Protein G agarose-antibody/chromatin complex was resuspended in wash buffer and centrifuged (70 x g, 3 min) to collect the protein/DNA complex, which were reversed to obtain free DNA. qPCR was conducted according to the method described above. Input sample represents the amount of chromatin used in the ChIP, the results of each antibody sample represent the percent of Input. The primers used were as follows: CDH1a forward, 5'-CCCTATGACTGCACCCCTTG-3' and reverse, 5'-GCAGATAACAGACCCCGCAT-3'; CDH1b forward, 5'-CAAATGGCCCTGGGAGGTTC-3' and reverse, 5'AGCCCTCTTGGCCCAATACA-3'.

The ENCODE Histone Modification Tracks embedded in the UCSC Genome Browser (University of California Santa Cruz, Santa Cruz, CA, USA) was used to find the promoter.

Animal experiments. A total of 12 BALB/c-A nude female mice at 6 weeks of age and weighing 8 g were purchased from the Animal Center of the Cancer Institute, Chinese Academy of Medical Science and bred at Tianjin Institute of Biological Engineering. The maintenance conditions for the mice were as follows: Temperature, 26-28°C; humidity, 40-60%; the food and water were bacteria-free without any special requirements. The mice were randomly assigned to 2 groups (6 mice a group), and were separately subcutaneously injected with 10^7 MGC-803 cells transfected with Lenti-NC and Lenti-HOTAIR si in 100 μ l of PBS. The tumor volumes and the weight of the mice were measured every 2 days, and the tumor weight was surveyed at the endpoint of the study. At 25 days post-tumor implantation, the xenograft samples were collected and subjected to immunohistochemistry (IHC). Animal experiments were approved by the Ethics Committee of Tianjin Medical University General Hospital.

IHC. For staining, 5- μ m-thick sections were cut and dried, deparaffinized, and immunostaining was performed on the sections using the avidin-biotin-complex method. Primary antibodies specific for H3K27me3 (ab6002), H3K27ac (ab45173) (Abcam; 1:100 dilution) and E-cadherin (14472) (Cell Signaling Technology; 1:50 dilution) were diluted in PBS with 0.1% Tween-20 and incubated overnight at 4°C. A secondary antibody (PV9003; Zhongshan Bio Corp.; 1:100 dilution) was added to the slides at 37°C for 1 h, followed by avidin-biotin complex solution for an additional 1 h. Protein expression was detected by coloration with diaminobenzidine (DAB) buffer for 5 min, and the sections were counterstained with hematoxylin for 5 min at room temperature. All images were captured using a microscope (Olympus Corporation).

Statistical analysis. All statistical analyses were performed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA) or GraphPad 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) software. Data are expressed as the means \pm standard deviation; the experiments were repeated 3 times. The statistical significance of differences between groups was determined using a Student's t-test, and one-way ANOVA was used to determine the differences among at least 2 groups. P-values <0.05 were considered to indicate statistically significant differences.

Results

HOTAIR knockdown inhibits the malignant properties of GC cells. Based previous results regarding HOTAIR (23), in this study, we examined the effect of HOTAIR on the malignant properties of GC cells. HOTAIR expression was significantly knocked down or overexpressed following lentiviral infection detected by qPCR (Fig. 1A). The results of Transwell assays revealed that the inhibition of HOTAIR decreased MGC-803 and SGC-7901 cell invasion (Fig. 1B). In addition, the CCK-8 assay was used to observe the proliferation of MGC-803 and SGC-7901 cells following HOTAIR knockdown *in vitro*. We estimated the proliferation rate of the GC cells at 0, 24, 48, 72, 96 and 120 h and compared this with that of the negative control. The results indicated that HOTAIR knockdown suppressed the growth of the GC cells (Fig. 1C). Taken together, these results indicate that HOTAIR is associated with the malignant properties of GC cells.

HOTAIR negatively regulates E-cadherin expression. Since the malignant properties of cancer cells are associated with EMT (2), we examined the effects of HOTAIR on EMT processes in GC cells. Previous research revealed that HOTAIR is involved in GC cell ECM function (16). Consequently, we detected the of mRNA and protein expression levels of EMT-related TFs following HOTAIR knockdown or overexpression. The expression levels of the epithelial marker, E-cadherin (also known as CDH1), in cells in which HOTAIR was knocked down were increased compared with the control cells. By contrast, the expression levels of mesenchymal markers, including N-cadherin, Snail, Slug, Twist and β -catenin, were decreased in the GC cells in which HOTAIR was knocked down. However, in the HOTAIR-overexpressing cells, the expression levels of EMT markers exhibited the opposite effects (Fig. 2A and B). We then performed immunofluorescence staining to evaluate E-cadherin-related morphological changes induced by

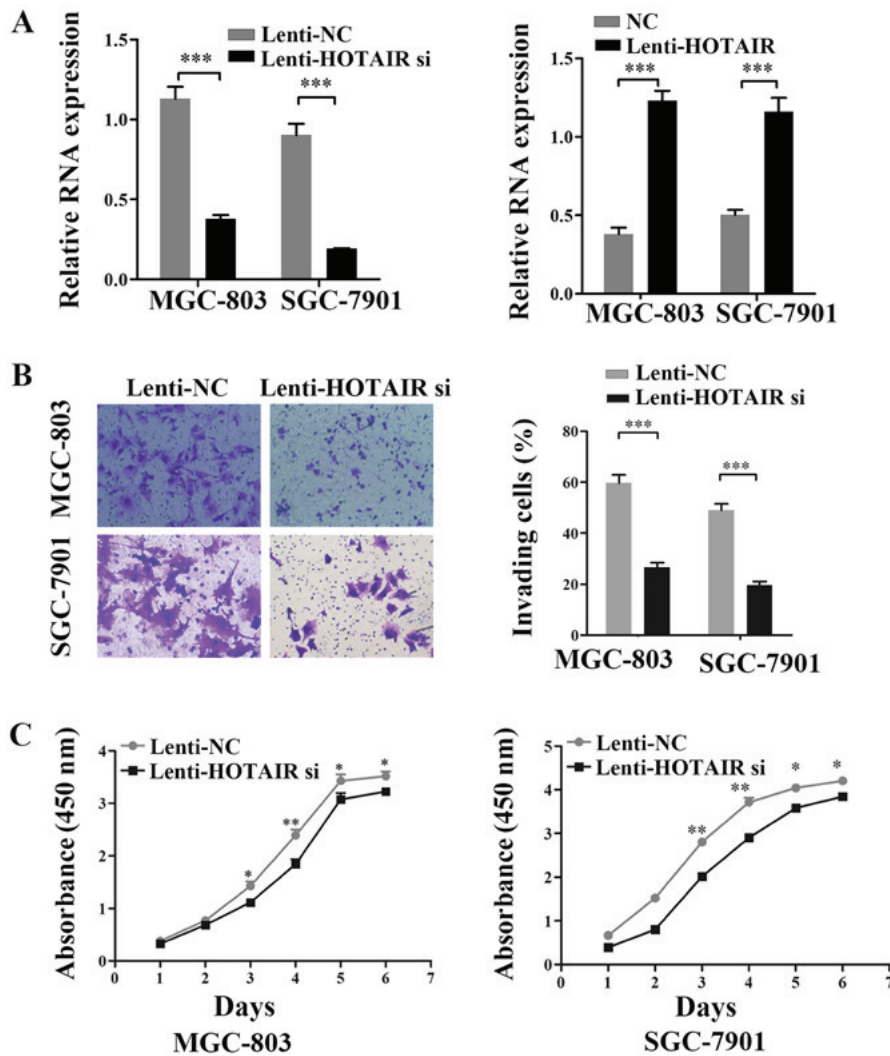


Figure 1. HOTAIR knockdown inhibits the malignant properties of gastric cancer cells. (A) RT-qPCR was used to detect HOTAIR expression in SGC-7901 and MGC-803 cells infected with Lenti-HOTAIR si or Lenti-HOTAIR (** $P < 0.001$). (B) Transwell assays indicated that the knockdown of HOTAIR by infection with Lenti-HOTAIR si inhibited the invasion of SGC-7901 and MGC-803 cells (magnification, $\times 200$, *** $P < 0.001$). (C) Silencing of HOTAIR suppressed the proliferation of SGC-7901 and MGC-803 cells as determined by CCK-8 assay (* $P < 0.05$ and ** $P < 0.01$ vs. control).

HOTAIR knockdown or overexpression. As shown in Fig. 2C, the Lenti-HOTAIR si-infected cells exhibited an epithelial morphology, which was characterized by the increased expression of E-cadherin. By contrast, the cells infected with Lenti-HOTAIR exhibited a mesenchymal morphology, as indicated by the decreased expression of E-cadherin in the cytoplasm. The E-cadherin protein is a hallmark of EMT and is crucial for maintaining an epithelial phenotype (26). These results suggested that HOTAIR may promote GC cell metastasis by negatively regulating E-cadherin expression at the mRNA and protein level.

HOTAIR regulates histone methylation and the acetylation of H3K4 and H3K27. Previous research has indicated that long intergenic non-coding RNA HOTAIR serves as a scaffold for linking a histone methylase and a demethylase, which separately affects histone H3 lysine 27 methylation and lysine 4 demethylation (12). It has also been reported that the loss of H3K27me3 results in increased levels of H3K27ac, which are specifically dependent on PRC2 activity (27). It is believed that the methylation of H3K27 mediates transcriptional repression.

However, the methylation and acetylation of H3K4, and the acetylation of H3K27 are regarded as transcriptionally active markers (28). Therefore, in this study, we performed western blot analysis to explore the molecular mechanisms through which HOTAIR regulates transcription in SGC-7901 and MGC-803 GC cells. The expression levels of H3K4me1/2/3 and H3K27ac were increased, whereas those of H3K4ac and H3K27me1/2/3 were decreased following infection with Lenti-HOTAIR si. By contrast, infection with Lenti-HOTAIR increased the H3K4ac and H3K27me1/2/3 expression levels, and the decreased H3K4me1/2/3 and H3K27ac expression levels (Fig. 3A). The results of western blot analysis indicated that HOTAIR regulated the methylation and acetylation of H3K4/27 and mediated the transcriptional repression or activation. Furthermore, we analyzed the gray value of H3K27me3/ac. The results indicated that HOTAIR may regulate histone H3K27 methylation and acetylation using an antagonistic approach (Fig. 3B).

HOTAIR is associated with an antagonistic switch between histone H3K27 methylation and acetylation. The above-mentioned findings demonstrated that H3K27 methylation and acetylation

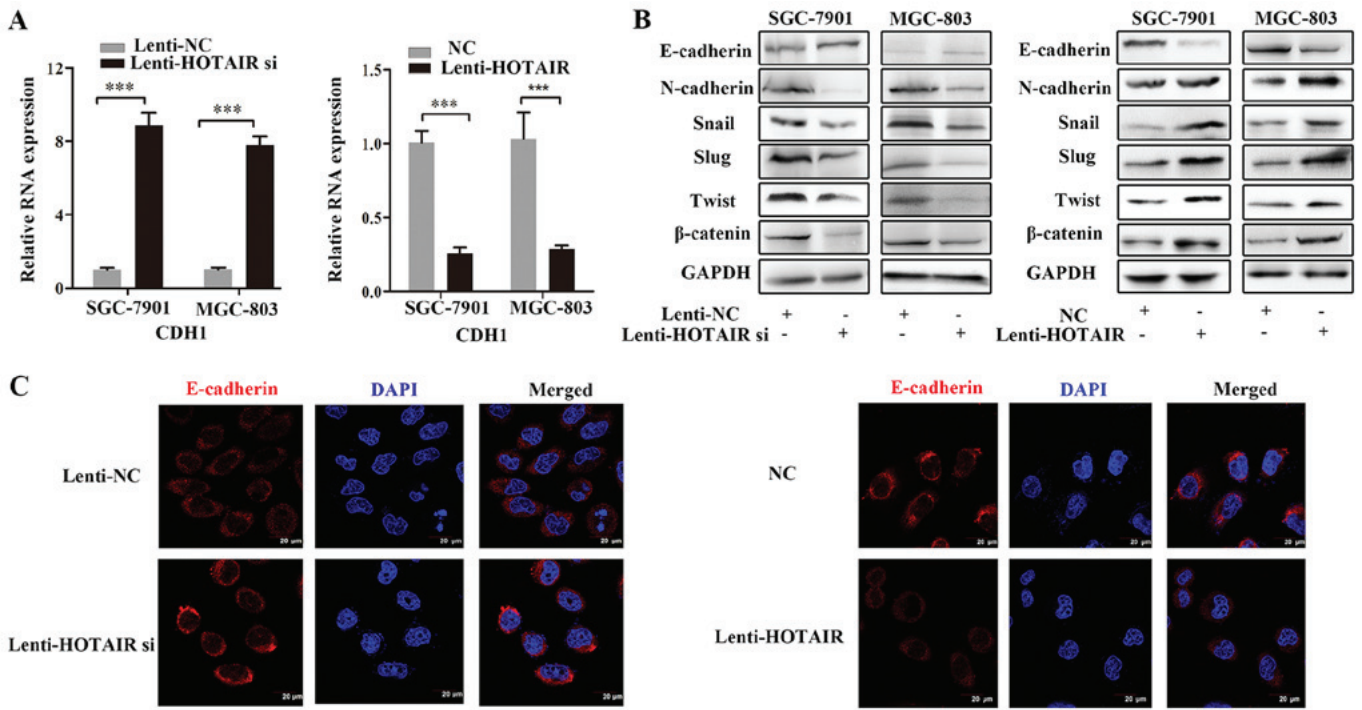


Figure 2. HOTAIR negatively regulates E-cadherin expression. (A) RT-qPCR was used to detect CDH1 expression in SGC-7901 and MGC-803 cells infected with Lenti-HOTAIR si or Lenti-HOTAIR (***) $P < 0.001$). (B) Western blot analysis was performed to examine the expression of E-cadherin, N-cadherin, snail, slug, twist and β -catenin in the SGC-7901 and MGC-803 cells. All experiments were performed in triplicate with 3 technical replicates. GAPDH was used as a control. (C) Immunofluorescence staining revealed the levels of cytoplasmic E-cadherin following infection with Lenti-HOTAIR si or Lenti-HOTAIR in MGC-803 gastric cancer cells (magnification, $\times 1,000$).

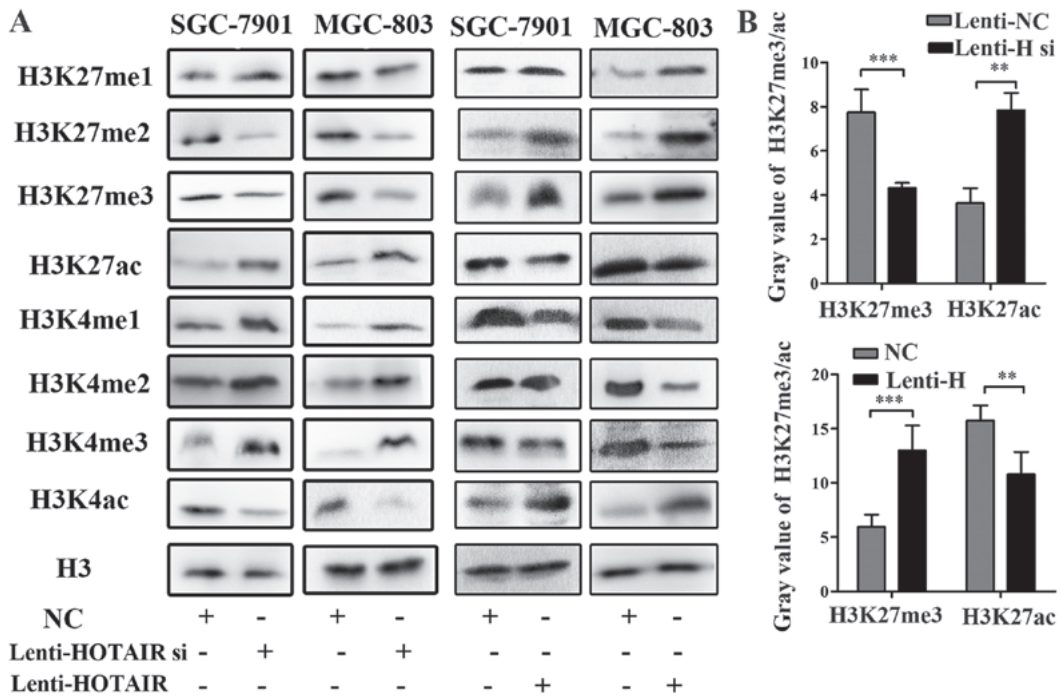


Figure 3. HOTAIR regulates the histone methylation and acetylation of H3K4 and H3K27. Western blot analysis was used to examine the expression levels of H3K4me1/me2/me3/ac and H3K27me1/me2/me3/ac. Histone H3 was used as the nuclear extraction loading control. (A) Infection with Lenti-HOTAIR si or Lenti-HOTAIR regulated the expression of histone H3K4/K27 modification. (B) The gray value of H3K27me3/ac following infection of the gastric cancer cells with Lenti-HOTAIR si or Lenti-HOTAIR (** $P < 0.01$ and *** $P < 0.001$).

were regulated by HOTAIR in opposing ways. Subsequently, we aimed to identify the histone-modifying enzymes that may be involved in HOTAIR-associated H3K27 methylation and

acetylation. It is known that HOTAIR targets PRC2, which consists of H3K27 methyltransferase EZH2, SUZ12 and EED (29). In this study, the GC cell lines, SGC-7901 and MGC-803, were infected

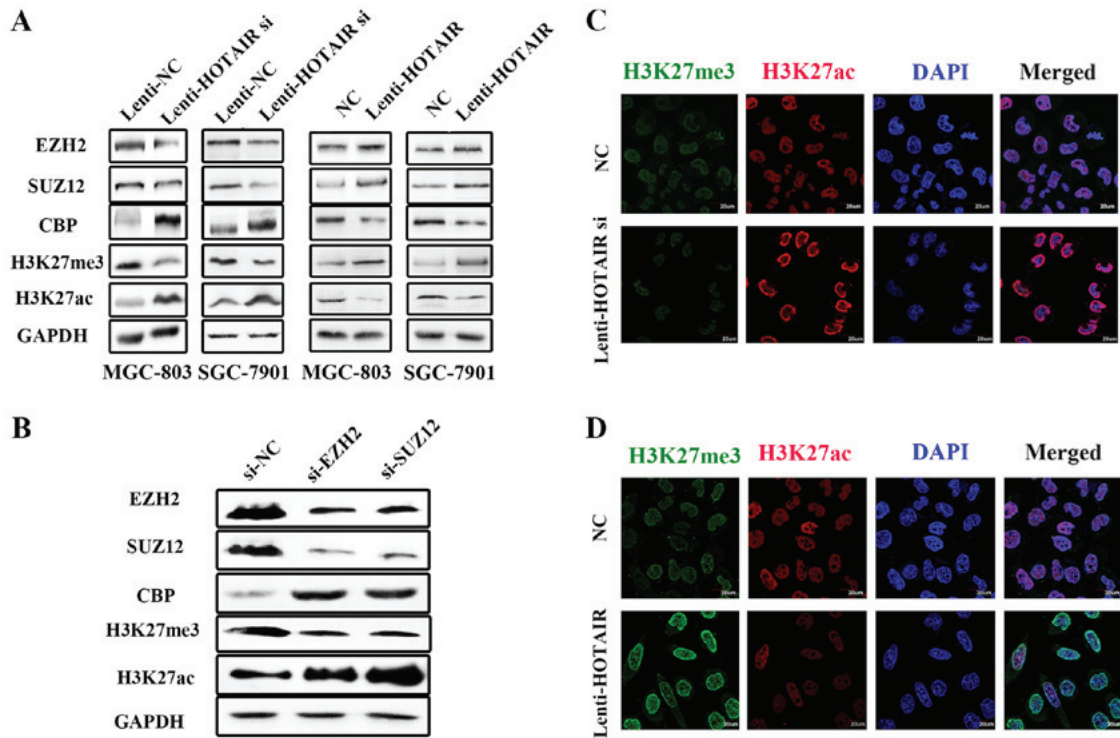


Figure 4. HOTAIR is associated with an antagonistic switch between histone H3K27 methylation and acetylation. (A and B) Western blot analysis was used to examine the expression levels of EZH2, SUZ12, CBP, H3K27me3 and H3K27ac in the SGC-7901 and MGC-803 cells infected with Lenti-HOTAIR si, Lenti-HOTAIR, si-EZH2 and si-SUZ12. GAPDH was used as a loading control. (C and D) Immunofluorescence staining revealed the expression of H3K27me3 and H3K27ac located in the nuclei of gastric cancer cells infected with Lenti-HOTAIR si or Lenti- (magnification, x1,000).

with Lenti-HOTAIR si or Lenti-HOTAIR. Western blot analysis was then used to examine the expression levels of EZH2 and SUZ12 and the results revealed that these were not markedly altered by HOTAIR knockdown or overexpression. However, the expression levels of acetyltransferase CBP increased with Lenti-HOTAIR si infection and decreased with the overexpression of HOTAIR. Additionally, the expression levels of H3K27me3 were decreased, whereas the expression levels of H3K27ac were increased by Lenti-HOTAIR si infection. The opposite effects were observed as regards the expression levels of these factors with HOTAIR overexpression (Fig. 4A). To further explore the association between the PRC2 complex and CBP, the expression levels of EZH2, SUZ12, CBP, H3K27me3 and H3K27ac were detected following the inhibition of EZH2 and SUZ12 expression. The results were similar to those observed with Lenti-HOTAIR si infection (Fig. 4B). Following this, immunofluorescence assays were performed to further verify the HOTAIR-mediated antagonistic switch between histone H3K27 methylation and acetylation in GC cells (Fig. 4C and D). All the results mentioned above indicated that the antagonistic switch of methylation and acetylation of H3K27 were specifically dependent on PRC2 activity, which was induced by HOTAIR. Conversely, acetyltransferase CBP was considered to play an important role in histone H3K27ac without the existence of the PRC2 complex.

Epigenetic regulation of E-cadherin expression by the methylation and acetylation of H3K27. To examine the underlying mechanisms through which E-cadherin transcription is suppressed by HOTAIR-mediated H3K27 methylation and acetylation, UCSC Genome Browser was used to identify H3K27me3 and H3K27ac enrichment peaks in the E-cadherin

promoter region (Fig. 5A). ChIP was performed to obtain specific DNA, including the DNA of the CDH1 promoter binding to H3K27me3 and H3K27ac using agarose gel electrophoresis (Fig. 5B). The results suggested that H3K27me3 and H3K27ac were enriched in the promoter region of the CDH1 gene. In addition, qPCR analysis revealed that HOTAIR knockdown decreased the binding of H3K27me3 to the E-cadherin promoter in GC cells (Fig. 5C); however, HOTAIR knockdown induced the recruitment of H3K27ac to the E-cadherin binding site at the promoter of E-cadherin (Fig. 5D). Therefore, the knockdown of HOTAIR appears to relieve the repressive histone marker, H3K27me3, and induces a switch to the active marker, acetylated H3K27, at the promoter of E-cadherin. Taken together, these data provide evidence of a HOTAIR-mediated competition between H3K27me3 and H3K27ac in regulating CDH1 expression.

HOTAIR knockdown inhibits GC growth in vivo. To further verify the role of HOTAIR in GC cells and to determine the therapeutic potential of HOTAIR knockdown, we established a subcutaneous tumor model of GC using MGC-803 GC cells. Nude mice were subcutaneously injected with MGC-80 cells that were infected with Lenti-NC or Lenti-HOTAIR si. From 10 days post-injection, the weight of the mice and tumor volume were measured every 2 days for a total of 25 days. The tumor weight was measured after the mice were sacrificed (Fig. 6A and B). Compared with the nude mice injected with Lenti-NC-infected MGC-803 cells, the mice bearing tumors derived from MGC-803 cells in which HOTAIR was knocked down exhibited suppressed GC tumor growth. In addition, IHC was performed to evaluate the pathological changes in each of the GC orthotopic tumors (Fig. 6C). Compared with the Lenti-NC tumors, treatment

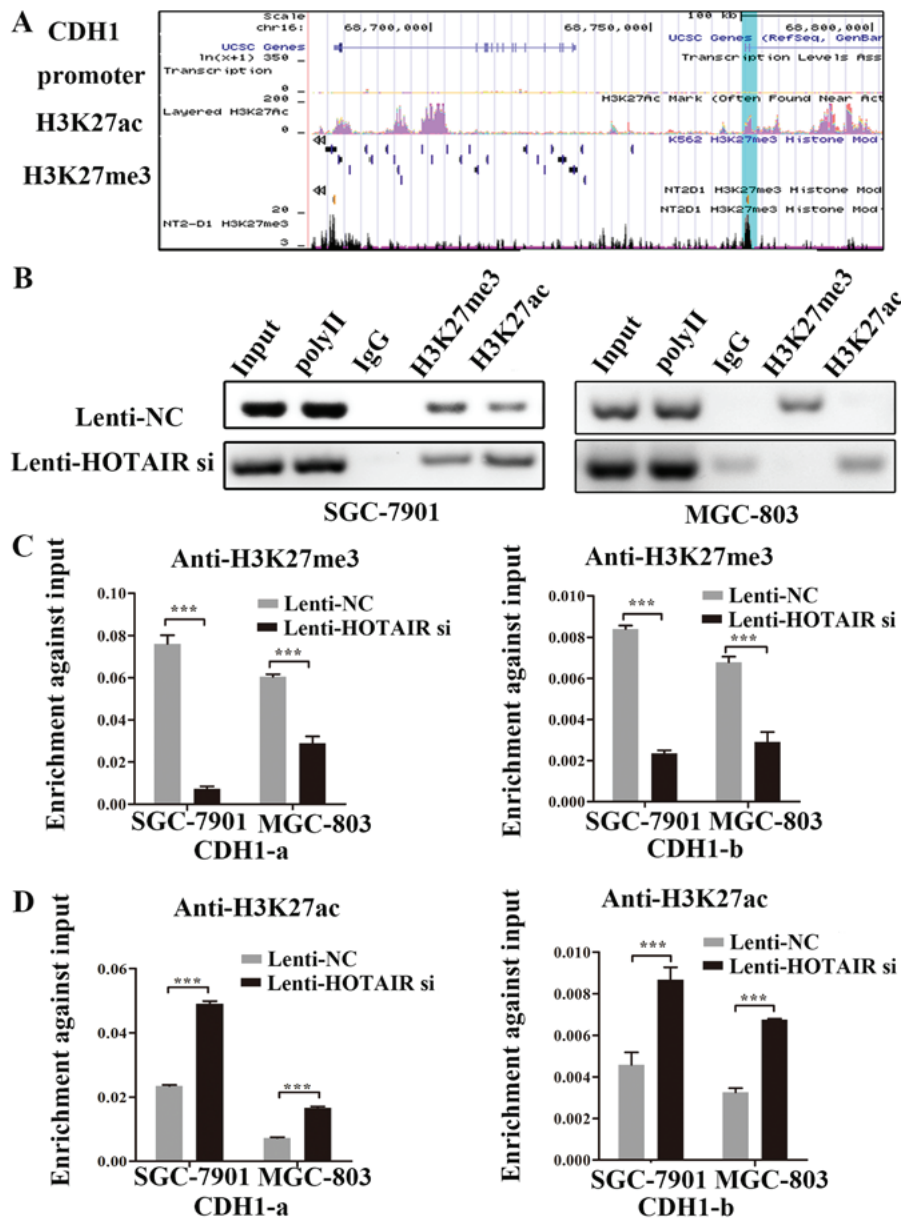


Figure 5. Epigenetic regulation of E-cadherin expression by the methylation and acetylation of H3K27. (A) Genome browser analysis of H3K27me3 and H3K27ac enrichment peaks in the E-cadherin promoter region. (B) ChIP analysis of SGC-7901 and MGC-803 cells infected with Lenti-HOTAIR si was conducted on the E-cadherin promoter. (C) ChIP assay demonstrated that the level of H3K27me3 in the E-cadherin promoter was decreased following infection with Lenti-HOTAIR si ($^{***}P<0.001$). (D) ChIP demonstrated that the level of H3K27ac in the E-cadherin promoter was increased following infection with Lenti-HOTAIR si ($^{***}P<0.001$). The H3K27me3 or H3K27ac binding to the E-cadherin promoters was assessed by ChIP assays followed by qPCR as described in the Materials and methods.

with Lenti-HOTAIR si significantly elevated E-cadherin expression. Furthermore, the methylation of H3K27 was decreased and the acetylation of H3K27 was increased following infection with Lenti-HOTAIR si, which was in line with our results *in vitro*.

Taken together, the findings of this study indicate that HOTAIR promotes the EMT of gastric cancer by inhibiting the expression of E-cadherin through an antagonistic switch of histone H3K27 acetylation to methylation at the E-cadherin promoter (Fig. 7).

Discussion

Since the introduction of HOTAIR by Rinn *et al* (7) in 2007, numerous studies have been conducted regarding HOTAIR

and the associated mechanisms of carcinogenesis and tumor progression. It has been reported that overexpression of long non-coding RNA HOTAIR is associated with various types of cancer (30-33), which suggests that HOTAIR functions as an oncogene in a variety of human cancer types. Previous studies have revealed that, compared with adjacent non-tumor tissues, HOTAIR is upregulated in GC tumor tissues and is also associated with diffuse type GC, venous invasion and other clinicopathological factors, including TNM stage and lymph node metastasis (11,34,35). In this study, as shown in Fig. 1, we performed Transwell assays to detect the invasion and metastasis of GC cells. Cell proliferation was also evaluated by CCK-8 assay. Notably, the results *in vivo* had the same trend as those *in vitro*. All the results indicated that the

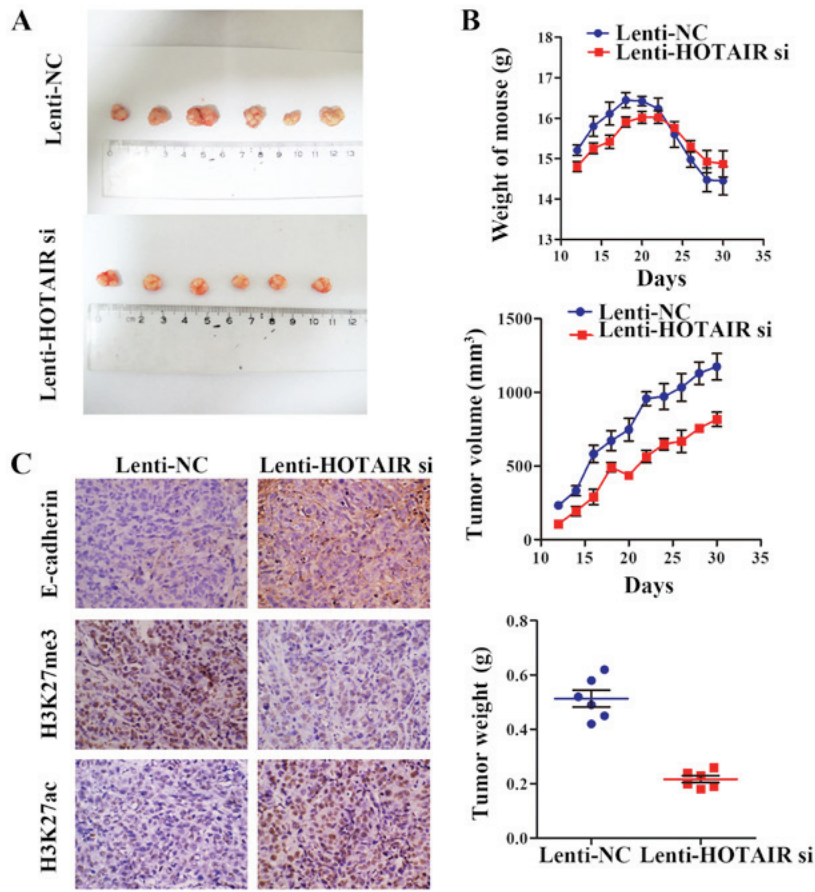


Figure 6. HOTAIR knockdown inhibits gastric cancer growth *in vivo*. The 2 mouse groups: the MGC-803/Lenti-NC and MGC-803 Lenti-HOTAIR si groups. (A) Images of and tumor tissue specimens *in vivo*. (B) The mouse body weights, and tumor volume and weight were monitored in the MGC-803 Lenti-NC and Lenti-HOTAIR si orthotopic gastric cancer models. (C) Representative images of immunohistochemical staining of E-cadherin, H3K27me3 and H3K27ac in tissues from mice with orthotopic tumors derived from MGC-803 cells infected with Lenti-NC or Lenti-HOTAIR si (magnification, x200).

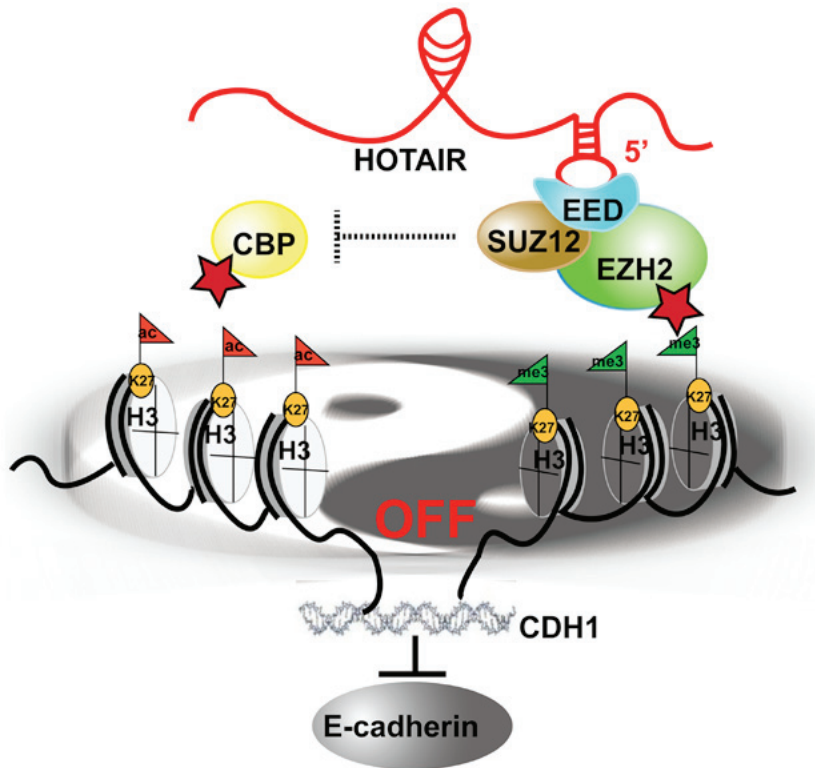


Figure 7. HOTAIR promotes epithelial-mesenchymal transition (EMT) of gastric cancer by inhibiting the expression of E-cadherin through an antagonistic switch of histone H3K27 acetylation to methylation at the E-cadherin promoter.

overexpression of HOTAIR promoted the development of GC. These findings suggest that HOTAIR plays an important role in the aggressiveness and metastasis of GC, and may serve as a novel biomarker for the metastasis or prognosis of GC.

HOTAIR serves as a modular scaffold of histone modification complexes, with the 5' domain binding the PRC2 and the 3' domain binding the LSD1/CoREST/REST complex (12). The methyltransferase EZH2 is a catalytic subunit of PRC2 that can epigenetically silence downstream gene transcription by the addition of three methyl groups to lysine 27 of histone 3 (36). However, the demethylase LSD1 performs the enzymatic demethylation of H3K4me2. Despite the influence of the HOTAIR-mediated methylation of histone 3, lysine 27 and lysine 4, H3K4/27 acetylation is significantly altered in an opposite manner compared with methylation. In this study, the results of western blot analysis in Fig. 3 demonstrated that the knockdown of HOTAIR decreased H3K27 methylation and increased H3K27 acetylation, whereas H3K4 methylation was increased and the acetylation of H3K4 was decreased. Therefore, we attempted to explore the underlying mechanisms of HOTAIR and histone acetylation. Previous research has indicated that histone H3 lysine 27 methylation and acetylation act as an antagonistic switch in regulating gene expression. In addition, the loss of SUZ12, a subunit of PRC2, results in a significant increase of global H3K27ac, which requires the activity of acetyltransferase CBP (37). Accordingly, in this study, we performed western blot analysis to examine the levels of EZH2, SUZ12, CBP, H3K27me3 and H3K27ac following infection with Lenti-HOTAIR si, Lenti-HOTAIR, si-EZH2 and si-SUZ12. The results in shown in Fig. 4 indicated a potential mechanism with regard to the switch between H3K27 methylation and acetylation. HOTAIR induced the methylation of H3K27 by recruiting EZH2, and the acetylation of H3K27 was decreased in the presence of SUZ12, which may inhibit the binding of CBP and H3K27ac. Thus, HOTAIR mediates a switch between the acetylation and tri-methylation of H3K27, which are associated with the transcriptional activation and repression of target genes, respectively.

EMT is associated with multiple cancer cells (38). During the metastasis and invasion of GC cells, the cells lose the function of epithelial factors, such as E-cadherin, and increase the expression of mesenchymal markers, including Twist, Snail, Slug, N-cadherin and β -catenin (39). E-cadherin is considered an important epithelial marker and its loss of function may contribute to cancer progression by increasing proliferation, invasion and/or metastasis. Multiple studies have reported that HOTAIR is negatively associated with the expression of E-cadherin in cancer cells (30,31,40). Therefore, we performed experiments to detect the levels of CDH1 and E-cadherin in GC cells. The results indicated that E-cadherin may be the pivotal marker that promotes HOTAIR-mediated GC cell migration and metastasis. Recent evidence has linked EMT with epigenetic modifications (41-43), particularly histone modifications. It has been proposed that the E-cadherin promoter, EZH2, and H3K27me3 have binding sites that can result in the silencing of E-cadherin. As a result, we performed ChIP assay in the present study. The results indicated that HOTAIR increased the binding of H3K27me3 instead of H3K27ac at the E-cadherin promoter, which causes a switch from the transcriptional activation to the transcriptional repression of E-cadherin.

In this study, we validated that HOTAIR promoted EMT by epigenetically silencing E-cadherin expression in GC.

Long non-coding RNA HOTAIR and histone modification are critical factors during carcinogenesis and tumor progression. In addition to the previously mentioned mechanisms of HOTAIR and EMT, we propose a new regulation of EMT by HOTAIR through an competitive switch of histone H3K27 acetylation and methylation at the E-cadherin promoter, which differs from the traditional model of HOTAIR concerning PRC2 and H3K27me3. This finding provides new insight into HOTAIR and epigenetic modification. However, the exact mechanism of PRC2, CBP and H3K27ac requires further exploration. Additionally, the association between HOTAIR and other EMT-TFs, particularly mesothelial factors, is not explained by the HOTAIR-mediated switch of histone acetylation and methylation and therefore requires further exploration.

In conclusion, the findings of the present study indicate that HOTAIR promotes EMT in GC by switching between the acetylation and the tri-methylation of H3K27, which is associated with the change of transcriptional activation to inhibition of E-cadherin. Taken together, these findings indicate a novel mechanism involving HOTAIR, histone modification and EMT (Fig. 7).

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Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Authors' contributions

YS and RW performed the molecular studies. LWL and XL performed the animal experiments. YFW and QXW provided experimental technical support and performed the statistical analysis. QYZ designed the study and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal experiments were approved by the Ethics Committee of Tianjin Medical University General Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- De Craene B and Berx G: Regulatory networks defining EMT during cancer initiation and progression. *Nat Rev Cancer* 13: 97-110, 2013.
- Thiery JP, Acloque H, Huang RY and Nieto MA: Epithelial-mesenchymal transitions in development and disease. *Cell* 139: 871-890, 2009.
- Huang L, Wu RL and Xu AM: Epithelial-mesenchymal transition in gastric cancer. *Am J Transl Res* 7: 2141-2158, 2015.
- Yan Y, Zhang J, Li JH, Liu X, Wang JZ, Qu HY, Wang JS and Duan XY: High tumor-associated macrophages infiltration is associated with poor prognosis and may contribute to the phenomenon of epithelial-mesenchymal transition in gastric cancer. *Onco Targets Ther* 9: 3975-3983, 2016.
- Peng Z, Wang CX, Fang EH, Wang GB and Tong Q: Role of epithelial-mesenchymal transition in gastric cancer initiation and progression. *World J Gastroenterol* 20: 5403-5410, 2014.
- Bedi U, Mishra VK, Wasilewski D, Scheel C and Johnsen SA: Epigenetic plasticity: A central regulator of epithelial-to-mesenchymal transition in cancer. *Oncotarget* 5: 2016-2029, 2014.
- Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Bruggmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E, *et al*: Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* 129: 1311-1323, 2007.
- Marchese FP and Huarte M: Long non-coding RNAs and chromatin modifiers: Their place in the epigenetic code. *Epigenetics* 9: 21-26, 2014.
- Kogo R, Shimamura T, Mimori K, Kawahara K, Imoto S, Sudo T, Tanaka F, Shibata K, Suzuki A, Komune S, *et al*: Long noncoding RNA HOTAIR regulates polycomb-dependent chromatin modification and is associated with poor prognosis in colorectal cancers. *Cancer Res* 71: 6320-6326, 2011.
- Amicone L, Citarella F and Cicchini C: Epigenetic regulation in hepatocellular carcinoma requires long noncoding RNAs. *Biomed Res Int* 2015: 1-10, 2015.
- Hajjari M and Salavaty A: HOTAIR: An oncogenic long non-coding RNA in different cancers. *Cancer Biol Med* 12: 1-9, 2015.
- Zhou X, Ren Y, Zhang J, Zhang C, Zhang K, Han L, Kong L, Wei J, Chen L, Yang J, *et al*: HOTAIR is a therapeutic target in glioblastoma. *Oncotarget* 6: 8353-8365, 2015.
- Tsai MC, Manor O, Wan Y, Mosammamaparast N, Wang JK, Lan F, Shi Y, Segal E and Chang HY: Long noncoding RNA as modular scaffold of histone modification complexes. *Science* 329: 689-693, 2010.
- Baylin SB and Ohm JE: Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? *Nat Rev Cancer* 6: 107-116, 2006.
- Ning X, Shi Z, Liu X, Zhang A, Han L, Jiang K, Kang C and Zhang Q: DNMT1 and EZH2 mediated methylation silences the microRNA-200b/a/429 gene and promotes tumor progression. *Cancer Lett* 359: 198-205, 2015.
- Liu YW, Sun M, Xia R, Zhang EB, Liu XH, Zhang ZH, Xu TP, De W, Liu BR and Wang ZX: LincHOTAIR epigenetically silences miR34a by binding to PRC2 to promote the epithelial-to-mesenchymal transition in human gastric cancer. *Cell Death Dis* 6: e1802-e1811, 2015.
- Margueron R and Reinberg D: Chromatin structure and the inheritance of epigenetic information. *Nat Rev Genet* 11: 285-296, 2010.
- Pradeepa MM, Grimes GR, Kumar Y, Olley G, Taylor GC, Schneider R and Bickmore WA: Histone H3 globular domain acetylation identifies a new class of enhancers. *Nat Genet* 48: 681-686, 2016.
- Greer EL and Shi Y: Histone methylation: A dynamic mark in health, disease and inheritance. *Nat Rev Genet* 13: 343-357, 2012.
- Li KK, Luo C, Wang D, Jiang H and Zheng YG: Chemical and biochemical approaches in the study of histone methylation and demethylation. *Med Res Rev* 32: 815-867, 2012.
- Kim YW and Kim A: Characterization of histone H3K27 modifications in the β -globin locus. *Biochem Biophys Res Commun* 405: 210-215, 2011.
- Xu ZY, Yu QM, Du YA, Yang LT, Dong RZ, Huang L, Yu PF and Cheng XD: Knockdown of long non-coding RNA HOTAIR suppresses tumor invasion and reverses epithelial-mesenchymal transition in gastric cancer. *Int J Biol Sci* 9: 587-597, 2013.
- Hajjari M, Behmanesh M, Sadeghizadeh M and Zeinoddini M: Up-regulation of HOTAIR long non-coding RNA in human gastric adenocarcinoma tissues. *Med Oncol* 30: 670-673, 2013.
- Fang XY, Pan HF, Leng RX and Ye DQ: Long noncoding RNAs: Novel insights into gastric cancer. *Cancer Lett* 356: 357-366, 2015.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) methods. *Methods* 25: 402-408, 2001.
- Perl AK, Wilgenbus P and Dahl U: Acausal role for E-cadherin in the transition from adenoma to carcinoma. *Nature* 392: 190-193, 1998.
- Pasini D, Malatesta M, Jung HR, Walfridsson J, Willer A, Olsson L, Skotte J, Wutz A, Porse B, Jensen ON, *et al*: Characterization of an antagonistic switch between histone H3 lysine 27 methylation and acetylation in the transcriptional regulation of Polycomb group target genes. *Nucleic Acids Res* 38: 4958-4969, 2010.
- Latham JA and Dent SY: Cross-regulation of histone modifications. *Nat Struct Mol Biol* 14: 1017-1024, 2007.
- Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, Hung T, Argani P, Rinn JL, *et al*: Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. *Nature* 464: 1071-1076, 2010.
- Battistelli C, Cicchini C, Santangelo L, Tramontano A, Grassi L, Gonzalez FJ, de Nonno V, Grassi G, Amicone L and Tripodi M: The Snail repressor recruits EZH2 to specific genomic sites through the enrollment of the lncRNA HOTAIR in epithelial-to-mesenchymal transition. *Oncogene* 10: 1-14, 2016.
- Liu L, Xu Z, Zhong L, Wang H, Jiang S, Long Q, Xu J and Guo J: Enhancer of zeste homolog 2 (EZH2) promotes tumour cell migration and invasion via epigenetic repression of E-cadherin in renal cell carcinoma. *BJU Int* 117: 351-362, 2016.
- Wu Y, Zhang L, Zhang L, Wang Y, Li H, Ren X, Wei F, Yu W, Liu T, Wang X, *et al*: Long non-coding RNA HOTAIR promotes tumor cell invasion and metastasis by recruiting EZH2 and repressing E-cadherin in oral squamous cell carcinoma. *Int J Oncol* 46: 2586-2594, 2015.
- Zhang JX, Han L, Bao ZS, Wang YY, Chen LY, Yan W, Yu SZ, Pu PY, Liu N, You YP, *et al*: Chinese Glioma Cooperative Group: HOTAIR, a cell cycle-associated long noncoding RNA and a strong predictor of survival, is preferentially expressed in classical and mesenchymal glioma. *Neuro Oncol* 15: 1595-1603, 2013.
- Pan W, Liu L, Wei J, Ge Y, Zhang J, Chen H, Zhou L, Yuan Q, Zhou C and Yang M: A functional lncRNA HOTAIR genetic variant contributes to gastric cancer susceptibility. *Mol Carcinog* 55: 90-96, 2016.
- Zhao W, Dong S, Duan B, Chen P, Shi L, Gao H and Qi H: HOTAIR is a predictive and prognostic biomarker for patients with advanced gastric adenocarcinoma receiving fluorouracil and platinum combination chemotherapy. *Am J Transl Res* 7: 1295-1302, 2015.
- Chase A and Cross NC: Aberrations of EZH2 in cancer. *Clin Cancer Res* 17: 2613-2618, 2011.
- Tie F, Banerjee R, Stratton CA, Prasad-Sinha J, Stepanik V, Zlobin A, Diaz MO, Scacheri PC and Harte PJ: CBP-mediated acetylation of histone H3 lysine 27 antagonizes *Drosophila* Polycomb silencing. *Development* 136: 3131-3141, 2009.
- Christiansen JJ and Rajasekaran AK: Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. *Cancer Res* 66: 8319-8326, 2006.
- Yang J and Weinberg RA: Epithelial-mesenchymal transition: At the crossroads of development and tumor metastasis. *Dev Cell* 14: 818-829, 2008.
- Zha L, Cao Q, Cui X, Li F, Liang H, Xue B and Shi H: Epigenetic regulation of E-cadherin expression by the histone demethylase UTX in colon cancer cells. *Med Oncol* 33: 21-31, 2016.
- Wang Y and Shang Y: Epigenetic control of epithelial-to-mesenchymal transition and cancer metastasis. *Exp Cell Res* 319: 160-169, 2013.
- Wang R, Chen X, Xu T, Xia R, Han L, Chen W, De W and Shu Y: MiR-326 regulates cell proliferation and migration in lung cancer by targeting phox2a and is regulated by HOTAIR. *Am J Cancer Res* 6: 173-186, 2016.
- Wolff EM, Liang G and Jones PA: Mechanisms of Disease: Genetic and epigenetic alterations that drive bladder cancer. *Nat Clin Pract Urol* 2: 502-510, 2005.

