

HOTAIR Long Noncoding RNA Promotes Gastric Cancer Metastasis through Suppression of Poly r(C)-Binding Protein (PCBP) 1

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Abstract

The objective of this study was to evaluate the role of HOTAIR long noncoding RNA in gastric cancer metastasis. We analyzed HOTAIR expression levels by real-time reverse transcription PCR and Northern blot analysis in 100 gastric tissues (50 gastric cancer tissues and 50 adjacent normal mucosa), and in four gastric cancer cell lines. Transient RNAi-mediated knockdown and pcDNA-mediated overexpression of HOTAIR were performed. Stable shRNA-mediated knockdown and lentiviral-mediated overexpression of HOTAIR were to study the role of HOTAIR on *in vivo* tumorigenicity and metastatic burden in the context of xenograft assays. Proteomic profiling was performed to decipher differential protein expression in cells with different HOTAIR expression levels. One of the differentially regulated proteins, Poly r(C)-binding protein (PCBP) 1, was subsequently validated and its function evaluated through xenograft assays. Expression of

HOTAIR was significantly higher in cancerous tissues than in adjacent normal mucosa. HOTAIR expression levels dictated *in vitro* and *in vivo* tumorigenicity and metastatic potential in these cells. PCBP1 and HOTAIR have an inverse relationship, both at expression level and in function. A direct interaction between the two was confirmed through RNA immunoprecipitation coupled with quantitative real-time PCR. PCBP1 was confirmed to be an inhibitor of gastric cancer pathogenesis and as functionally opposite to HOTAIR long noncoding RNA. In conclusion, HOTAIR expression may serve as a potentially important disease biomarker for the identification of high-risk gastric cancer patients. Moreover, our findings provide mechanistic evidence for HOTAIR overexpression and PCBP1 downregulation and the ensuing malignant phenotype in both cultured and xenograft gastric cancer cells. *Mol Cancer Ther*; 14(5); 1162–70. ©2015 AACR.

Introduction

Gastric cancer, one of the most common causes of global mortality (1), is hardly benefited through surgical resection as it is mostly diagnosed at an advanced stage of the disease and is accompanied by malignant proliferation, extensive invasion, and lymphatic metastasis (2, 3). This highlights the imminent requirement for discovery of more molecular markers that can be potential and more efficacious therapeutic targets in patients with gastric cancer.

Noncoding RNAs (ncRNAs), small (<200 kb) and long (lncRNAs) (>200 kb), have been recently shown to be involved in both tumor-suppressive and oncogenic pathways (4–7). Anomalous expressions of lncRNAs have been reported in a wide variety of human diseases and cancers, including gastric (8, 9), prostate (10–12), and breast (13, 14) cancers.

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Both the aforementioned studies (8, 9) have suggested that the HOTAIR (*Hox transcript antisense intergenic RNA*), an approximately 2.2 kb long noncoding RNA transcribed from the *HOXC* locus, is upregulated in gastric cancer tissues and it might be involved in both the process of gastric cancer tumorigenesis as well as metastatic progression. In fact, it has been shown that oncogenic activity of HOTAIR in gastric cancer is dictated by its ability to function as a competing endogenous RNA (ceRNA) that can regulate the expression of HER2 through competition for miR-331-3p (9). However, the overall biologic role and the underlying molecular mechanism of HOTAIR in gastric carcinogenesis remain largely undefined.

Hence, the objective of the current study was to analyze the expression levels of HOTAIR lncRNAs in 50 matched pairs of gastric cancer and normal specimens and explore the underlying mechanisms of HOTAIR's role in gastric cancer pathogenesis.

Materials and Methods

Clinical samples, tissue processing, and ethical considerations

Fresh-frozen and paraffin-embedded gastric cancer tissues and corresponding adjacent nontumorous gastric samples were obtained from 50 Chinese patients at Renji Hospital of Shanghai Jiaotong University (Shanghai, P.R. China) between 2007 and 2010. All cases were included after review by pathologist and histologic confirmation as gastric cancer (stage II, III, and IV; 7th ed. AJCC) and only where complete clinical pathology information was available (Table 1). None of the 50 included patients underwent preoperative local or systemic treatment. The study

Table 1. Clinical correlation of HOTAIR expression with gastric cancer

Clinicopathologic features	n (= 50; %)	Relative HOTAIR expression	P
Gender			0.834
Male	27 (54)	11.97 (0.89–18.53)	
Female	23 (46)	12.39 (3.46–22.34)	
Tumor site			1.234
Distal third	8 (16)	8.9 (1.24–14.09)	
Middle third	12 (24)	7.34 (2.51–8.99)	
Proximal stomach	30 (60)	7.65 (1.39–14.23)	
Differentiation			0.038
Poor	33 (66)	16.3 (7.23–21.39)	
Moderate/high	17 (34)	7.96 (0.89–9.95)	
Metastatic disease			0.021
M0	31 (62)	8.37 (1.87–34.56)	
M1	19 (38)	30.87 (19.34–41.34)	
Lymph node metastasis			0.003
N0	9 (18)	1.09 (0.67–1.39)	
N1	9 (18)	2.98 (0.98–35.9)	
N2	14 (28)	13.38 (6.79–15.89)	
N3	18 (36)	31.59 (8.96–41.34)	

NOTE: Relative HOTAIR expression was calculated as the median of relative expression, with 25th to 75th percentile in parentheses. $P < 0.05$ was considered significant (Mann–Whitney U test between two groups and Kruskal–Wallis test for three groups).

protocol was approved by the Institutional Review Board of the Renji Hospital of Shanghai Jiaotong University. All patients enrolled in the study provided signed informed consent. Freshly harvested samples were immersed in RNAlater (Life Technologies) before snap freezing within 30 minutes after surgery. All tissue samples were stored in liquid nitrogen until further use.

Cell culture

Human gastric cancer cell lines (SGC-7901, BGC-823, and AGS) and the immortalized human gastric epithelial mucosa cell line GES-1 were purchased from the Cell Bank of Shanghai Institute of Biochemistry & Cell Biology, Chinese Academy of Sciences (Shanghai, China; in January 2013) and maintained in humidified incubator at 37 °C in a CO₂ incubator in DMEM or RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin. Cell lines were characterized in the parent bank by the short tandem repeat method (in July 2012). All cell lines have been tested for mycoplasma contamination and verified to be mycoplasma free (last control August 2014).

RNA extraction, Northern blot analysis, and qRT-PCR

RNeasy kit (Qiagen) was used to isolate total RNA from the stored tissue specimens or cell lines following the manufacturer's instructions. Northern blot analysis using full-length HOTAIR was done as described before (15). For qRT-PCR, 1 µg total RNA was reverse transcribed using KAPA SYBR FAST One-Step qRT-PCR Kits as per the manufacturer's recommendations. Primers used for HOTAIR amplification were: forward 5'-CAGTGGG-GAACTCTGACTCG-3' and reverse 5'-GTGCCTGGTCTCTC-TTACC-3'. All amplified amplicons were verified through agarose gel electrophoresis. The assays were performed in triplicates and done at least five different times, and the 2^{-ΔΔC_t} method was used to determine the relative lncRNA expression levels. *TBP* was used as an endogenous control for data normalization. Data are represented as postnormalization mean ± SD.

Plasmid constructs, transfection, and transduction

Indicated cells (2×10^6 /well) were plated in 6-well plates overnight. Cells were then transfected with 30 nmol/L nontargeting siRNA control (siControl, Santa Cruz Biotechnology) or 30 nmol/L siRNA against HOTAIR (siHOTAIR I and siHOTAIR II; Sigma Aldrich) using Lipofectamine LTX transfection reagent (Carlsbad) according to the manufacturer's protocol. The transfectants were harvested after 24 hours for indicated experiments. For shRNA transfection, cells were transfected with HOTAIR (Santa Cruz Biotechnology) or *Renilla* Luciferase shRNAs using Lipofectamine LTX before being selected with puromycin for 3 weeks. The stable clones were pooled and used for further downstream experiments as indicated.

For transient overexpression constructs, PCBP1, HOTAIR, or *Renilla* cDNAs were cloned into the mammalian expression vector pcDNA3.1 (Life Technologies). For stable overexpression, HOTAIR, PCBP1, or *Renilla* cDNA was cloned into pLenti 6.3. Lentivirus was generated by transfecting the destination vector into HEK-293 cells using Lipofectamine 2000 (Life Technologies) according to manufacturer's recommendations. The viral titers were calculated based on the fact that an absorbance of 1 at 260 nm is equivalent to 1,012 plaque-forming units (pfu)/mL. All transduction was performed at multiplicity of infection 50. Cells were selected with 5 µg/mL blastocidin for 2 weeks to generate stable clones, which were pooled and used for further downstream experiments as indicated.

In vitro invasion assay

A modified *in vitro* Boyden chamber invasion assay with Matrigel-coated Transwell chambers (8 µm pore size) was performed. Indicated cells were resuspended at 5×10^4 cells in serum-free DMEM and were dispensed to Matrigel-coated inserts (Becton Dickinson) and placed in 24-well *trans*-plates with DMEM and 10% FBS (chemoattractant) to induce invasive cells to digest the coating and invade through the pores to the *trans* side. After 24 hours, the cells and Matrigel in the upper inserts were discarded, and the cells in the bottom *trans*-chambers were fixed with 3% glutaraldehyde and stained with crystal violet. Crystal violet-stained cells were counted in five randomly different fields. The experiments were performed in triplicate wells and each experiment was performed in triplicate.

In vitro migration assay

Indicated cells were deprived of serum overnight, treated with mitomycin-C, trypsinized and introduced into the upper chamber (1×10^5 /well) of the Transwell (8 µm pore size; BD Bioscience). The chemoattractant in the lower chamber was medium supplemented with 10% FBS. After 24 hours, the cells that migrated to the lower chambers were fixed with 3% glutaraldehyde, and stained with crystal violet. Crystal violet-stained cells were counted in five randomly different fields with an inverted fluorescence microscope. The experiments were performed in triplicate wells and each experiment was performed in triplicate.

Mass spectrometry and data analysis

pcDNA3 empty vector or pcDNA3-HOTAIR-treated (for 7 days) GES-1 cells in triplicate were lysed using NET buffer [50 mmol/L Tris-HCl, (pH 7.4), 150 mmol/L NaCl, 0.1% NP40, 1 mmol/L EDTA, 0.25% gelatin, 0.02% sodium azide, 1 mmol/L phenylmethylsulfonyl fluoride, and 1% aprotinin]. The lysates were centrifuged at $15,000 \times g$ for 30 minutes at 4°C. Collected

supernatant was dialyzed against PBS, and proteins were reduced with 5 mmol/L Tris 2-carboxyethyl phosphine (TCEP) and alkylated with 10 mmol/L iodoacetamide. Samples were digested with trypsin (Promega) in a 1:50 ratio for 12 hours at room temperature. Peptides were desalted on Ultra MicroTIP Columns (The Nest Group) and dried in a SpeedVac concentrator. Dried peptides were resolubilized in 20 μ L HPLC grade water containing 0.1% formic acid. Sample analysis was performed on a linear ion trap LTQ mass spectrometer (Thermo Electron) equipped with a nanoelectrospray ion source (Thermo Electron) coupled to an Agilent 1100 micro-HPLC system. Peptides were loaded with a cooled Agilent autosampler on a 2-cm long precolumn filled with C18 resin (Magic C18 AQ 5 μ m; Michrom Bioresources). A linear gradient of 80 minutes from 5% to 40% acetonitrile in H₂O with 0.1% formic acid was used to separate peptides on a 10-cm long fused silica emitter packed with C18 resin spraying directly into the mass spectrometer at a flow rate of 0.5 μ L/minute. The MS instrument was operated in positive ion mode. The data-dependent acquisition mode was set to acquire one MS scan followed by three collision-induced dissociation MS/MS scans. The MS full scans were recorded over a mass range of 400 to 1,600 m/z. Dynamic exclusion was enabled, the repeat count was set to 2, and the exclusion duration to 30 seconds. Further MS conditions were set as following: spray voltage 1.95 kV, transfer capillary temperature 230°C, normalized collision energy 35%, activation q 0.25, and activation time 30 ms.

The acquired raw files were converted to mzXML files using ReAdW with default settings and searched against the mouse IPI database version 3.26 with the Sequest search algorithm. The Sequest search parameters contained the static modification of cysteine +57.02 Da, at least one tryptic terminus, and one missed cleavage was allowed. The data were further processed using the Trans-Proteomic Pipeline TPP, including PeptideProphet and ProteinProphet, to estimate the false-discovery rate in the datasets. A protein probability of 0.5 was set as a cutoff corresponding to a false-discovery rate of approximately 5%. The protein list was annotated for secreted proteins using the algorithm SignalP and further manually curated for secreted proteins using UniProt database and literature search. Functional annotation was assigned using the PANTHER Classification system.

Relative enrichment or depletion in the GES-1 cells ectopically overexpressing HOTAIR was determined relative to mock-treated GES-1 cells and data were normalized to TBP expression levels. Log₂ fold change (increase or decrease) above 3 was considered significant.

Immunoblot analysis

Indicated cells were washed twice in PBS (pH 7.4), scraped into 15-mL conical tubes, and centrifuged at 1,000 \times g at 4°C for 5 minutes. Cell extracts were prepared by lysis in NET buffer [50 mmol/L Tris-HCl, (pH 7.4), 150 mmol/L NaCl, 0.1% NP40, 1 mmol/L EDTA, 0.25% gelatin, 0.02% sodium azide, 1 mmol/L phenylmethylsulfonyl fluoride, and 1% aprotinin]. The lysates were centrifuged at 15,000 \times g for 30 minutes at 4°C. The protein concentration of the supernatants was determined according to the method of Bradford using a Bio-Rad protein assay kit (Bio-Rad Laboratories). Ten micrograms of total cell lysates was resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in PBS/Tween 20 (0.05%), followed by incubation with an anti-PCBP1 antibody (1:2,000 dilution in 10% milk/PBS-T; Abcam). Postexposure

blots were stripped and probed with anti-TBP antibody (1:5,000 dilution; Abcam). The detection was done using horseradish peroxidase-labeled secondary antibodies and enhanced chemiluminescence detection reagent.

RNA immunoprecipitation (RIP), qRT-PCR, and data analyses

A total of 10 \times 10⁶ GES-1, AGS, SGC-7901, and BGC-823 cells were lysed for 15 minutes on ice in lysis buffer containing 100 mmol/L KCl, 5 mmol/L MgCl₂, 10 mmol/L HEPES, pH 7.0, 0.5% Nonidet P-40 detergent supplemented with fresh 1 mmol/L dithiothreitol (DTT), 1,000 U/mL RNAsin (Promega), and Mini protease inhibitor cocktail (Roche Diagnostics). Postnuclear cytosolic fraction was collected by spinning the cells at 15,000 \times g for 15 minutes at 4°C and 1% (v/v) was removed for input sample. Lysates (4 mg) were subjected to immunoprecipitation using 10 μ g of PCBP1 antibody or 10 μ g of mouse IgG using the Pierce Crosslink IP Kit (Thermo Scientific). An immunoprecipitated complex was digested with 30 μ g of proteinase K to release the ribonucleoprotein complex. TRIzol LS reagent (Life Technologies) was subsequently used to extract RNA from the immunoprecipitates and the input samples following the manufacturer's recommendations.

The RNAs isolated above were used for first-stand cDNA using SuperScript III Reverse Transcriptase (Life Technologies) and random hexamers. Control reactions were set up without any reverse transcriptase. The cDNAs were subsequently used to template qRT-PCR reactions using KAPA SYBR FAST Universal 2X qPCR Master Mix (KAPA BIOSYSTEMS) and HOTAIR and TBP primers.

In vivo tumor growth assay

Experiments were approved by the Institutional Animal Care and Use Committee of Renji Hospital of Shanghai Jiaotong University and were performed in accordance to the "Guidelines for the Welfare of Animals in Experimental Neoplasia" published by The United Kingdom Coordinating Committee on Cancer Research. For xenograft assays, 10⁶ indicated cells were injected subcutaneously into 8-week-old male nude mice (Beijing HFK Bio-Technology Co. Ltd.). Tumor diameters were measured on alternative days, and volumes calculated using the estimation: width² \times length \times 0.5. Animals were sacrificed on day 56 and tumor weights were measured off excised tumors.

To measure metastatic potential, 10⁶ indicated cells were injected into the right lower lobe of the liver in mice following instructions described before (14). Animals were sacrificed on day 56 and all tumor nodules were counted. For each set of animal experiments, 5 mice were used per experimental group.

Statistical analysis

A two-tailed Student *t* test was used to calculate statistical significance between two comparator groups. The differences of lncRNA expression between paired tissue sample were evaluated with the Wilcoxon matched pairs signed ranks test. A *P* value of <0.05 was considered statistically significant.

Results

HOTAIR lncRNA expression is upregulated in gastric cancer tissues and correlated with poorer survival

The level of HOTAIR expression was determined in 50 paired gastric cancer samples and matched adjacent, histologically normal tissues by qRT-PCR, and normalized to TBP expression.

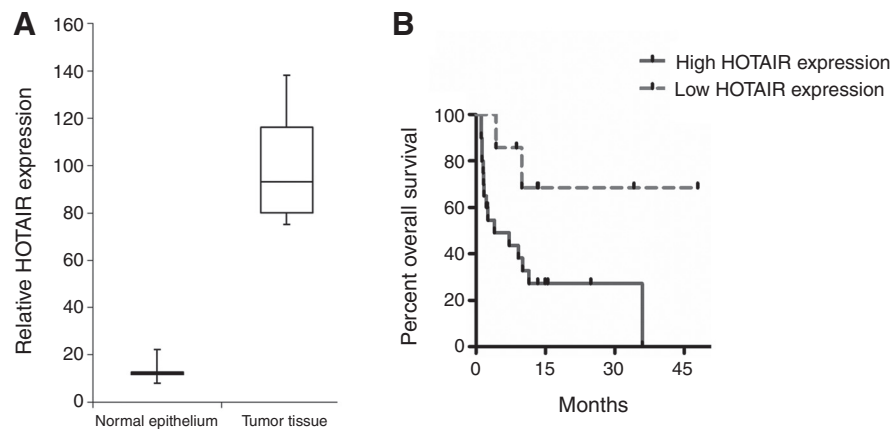


Figure 1. HOTAIR lncRNA is upregulated in gastric cancer and correlates with a worse survival rate among gastric cancer patients. A, expression profiles of HOTAIR lncRNA in metastatic gastric cancer ($n = 50$) and matched normal gastric epithelium tissues ($n = 50$). HOTAIR expression was examined by qRT-PCR and normalized to *TBP* expression. Data are presented as normalized fold-change in tumor tissues relative to normal epithelium. B, Kaplan-Meier overall survival curves based on differential HOTAIR lncRNA expression level. The overall survival of the High-HOTAIR group ($n = 35$; HOTAIR expression ratio \geq median ratio) was significantly higher than that of the Low-HOTAIR group ($n = 15$; HOTAIR expression ratio \leq median ratio; $P = 0.0039$, log-rank test).

HOTAIR expression was significantly upregulated in cancerous tissues (mean ratio of 93.14-fold, $P < 0.01$) compared with normal counterparts (Fig. 1A). HOTAIR expression was not associated with gender ($P = 0.834$) and tumor site (distal third, middle third, or proximal stomach; $P = 1.234$; Table 1). However, HOTAIR expression was significantly associated with tumor cell differentiation ($P = 0.038$), distant metastasis ($P = 0.021$), and

lymph node metastasis ($P = 0.003$; Table 1). Furthermore, the patient cohort with relatively higher HOTAIR expression had a significantly less overall survival compared with the cohort with relatively lower HOTAIR expression ($P < 0.001$; Fig. 1B), cumulatively reinforcing previous findings (8, 9) that HOTAIR expression might be useful as a diagnostic and prognostic marker for gastric cancer.

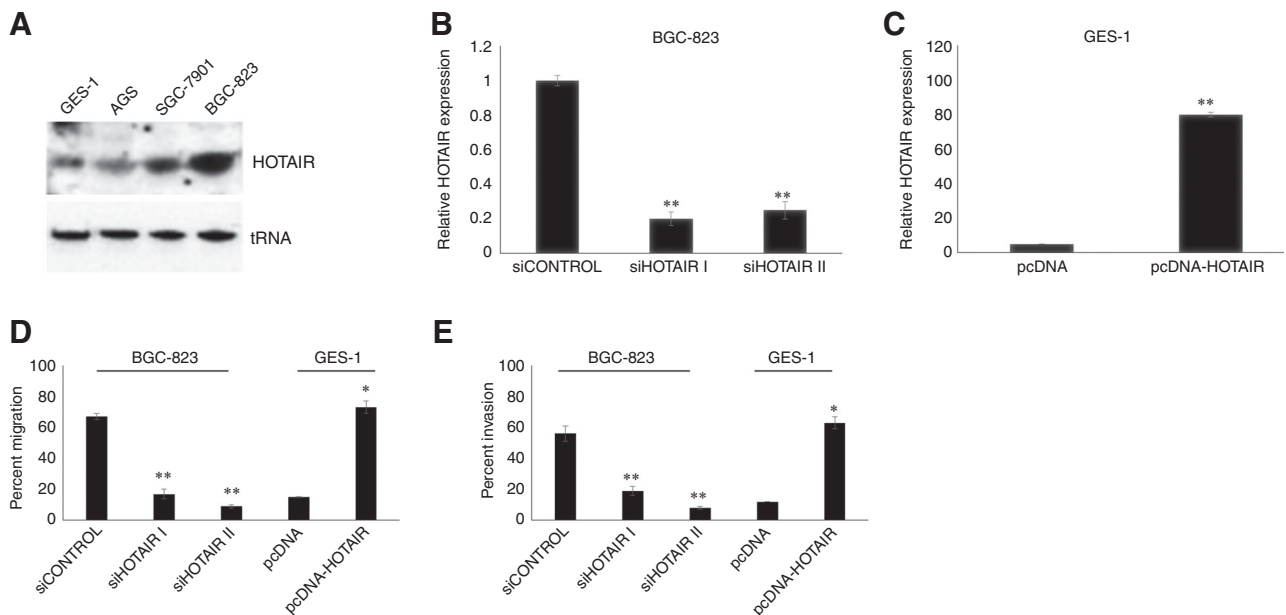


Figure 2. The level of HOTAIR lncRNA in gastric cancer cell lines and its effect on *in vitro* invasion and migration. A, Northern blot analysis was performed to examine HOTAIR lncRNA levels in gastric cancer cell lines AGS, SGC-7901, and BGC-823, and the immortalized human gastric epithelial mucosa cell line GES-1. The blot was stripped and reprobed with tRNA probe for loading control. B and C, qRT-PCR analyses of HOTAIR expression level following transient transfection of BGC-823 cells with siRNAs targeting HOTAIR or a scrambled control (B) and transient transfection of GES-1 cells with pcDNA/HOTAIR or the empty control vector (C). D and E, quantification (percent migration and invasion, respectively) of *in vitro* migration (D) and invasion (E) assays in BGC-823 cells transfected with two different siRNAs against HOTAIR (HOTAIR I and II) or a scrambled control (CONTROL) and GES-1 cells transfected with HOTAIR cDNA (pcDNA-HOTAIR) or the empty vector control (pcDNA). *, $P < 0.05$; **, $P < 0.01$.

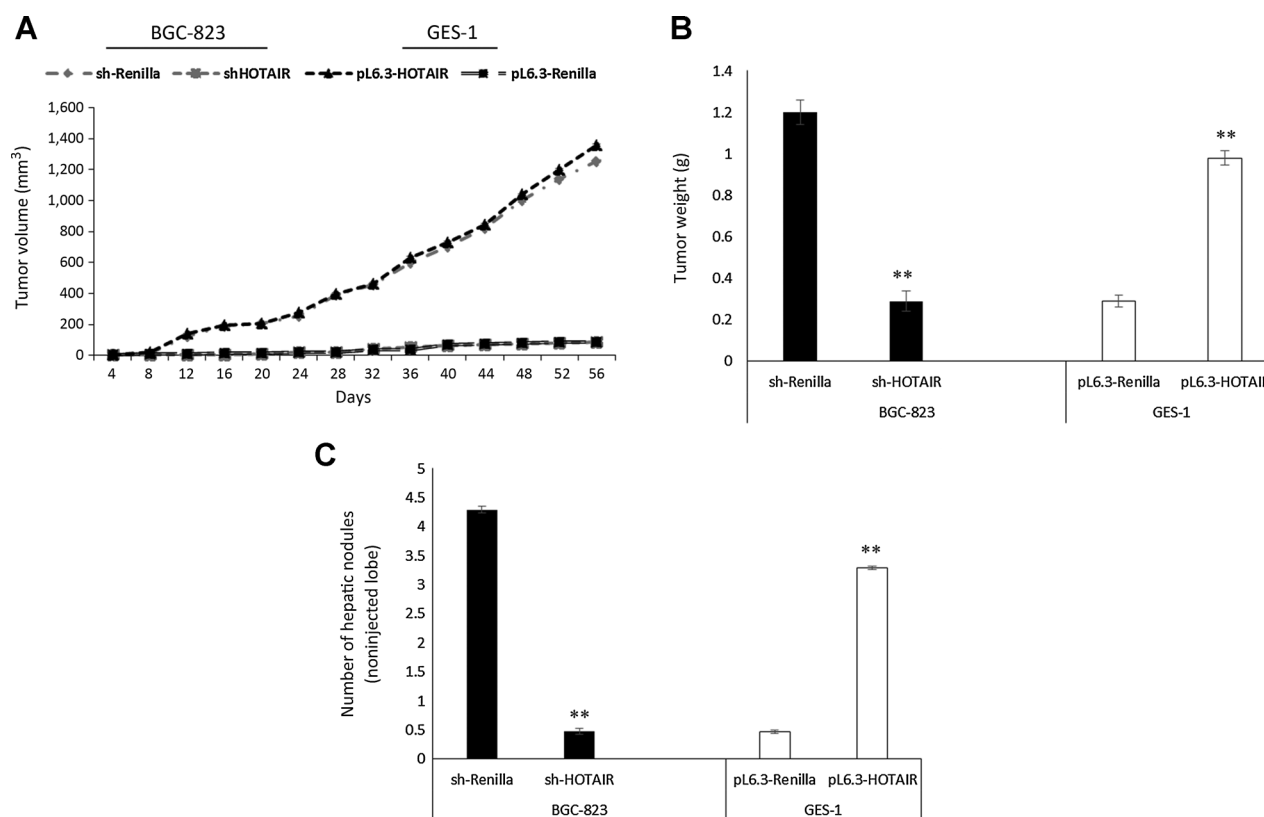


Figure 3.

Stable HOTAIR lncRNA downregulation in BGC-823 cells or upregulation in GES-1 cells attenuates and induces, respectively, subcutaneous tumor growth and metastatic hepatic burden *in vivo*. A, the effects of HOTAIR knockdown or overexpression on tumor growth were investigated in a subcutaneous tumor model. HOTAIR-shRNA-transduced BGC823 cells showed a decelerated *in vivo* growth rate, as compared with *Renilla*-shRNA-transduced cells (**, $P < 0.01$ for both at day 56). pLenti 6.3-HOTAIR GES-1 cells showed an accelerated *in vivo* growth rate, as compared with pLenti 6.3 *Renilla*-transduced cells (**, $P < 0.01$ for both at day 56). B, changes in tumor growth rate were also reflected by final tumor weights at day 56 for both BGC823-shRNA-HOTAIR and GES1-pL6.3-HOTAIR-transduced cells (**, $P < 0.01$). Bars, mean \pm SEM. C, whereas animals in the BGC823-HOTAIR-shRNA group, compared with the BGC823-*Renilla*-shRNA, had a markedly attenuated hepatic tumor burden, as determined by measuring the occurrence of hepatic nodules in liver lobes other than the injected lobe, those in the GES1-pL6.3-HOTAIR group had a marked induction of hepatic tumor burden compared to the GES1-pL6.3-*Renilla* (**, $P < 0.01$).

Effect of manipulation of HOTAIR expression level in gastric cancer cell lines

We first evaluated relative expression of HOTAIR through Northern blot analysis in the immortalized human gastric epithelial mucosa cell line GES-1 and human gastric cancer cell lines, SGC-7901, BGC-823, and AGS (Fig. 2A). Along expected lines, GES-1 had the minimum HOTAIR expression. Of the gastric cancer cell lines tested, BGC-823 had the highest HOTAIR expression (Fig. 2A). Because cell migration and invasion are essential prerequisites for cancer metastasis, we next evaluated the effect of differential HOTAIR expression on *in vitro* migration and invasion. To manipulate HOTAIR levels in BGC-823 cells, two separate HOTAIR-specific siRNAs or a scrambled control were transiently transfected. Conversely, a pcDNA-HOTAIR vector was transiently transfected to ectopically overexpress HOTAIR in the GES-1 cell line. Successful RNAi-mediated knockdown and ectopic expression of HOTAIR in BGC-823 (Fig. 2B) and GES-1 (Fig. 2C) cells, respectively, were confirmed by qRT-PCR on harvested RNA 48 hours after transfection. Although siControl-transfected BGC-823 cells showed robust *in vitro* migration, knocking down HOTAIR with either of the two siRNAs significantly inhibited *in vitro* migration (Fig. 2D; $P < 0.01$ for each HOTAIR siRNA).

Conversely, ectopic expression of HOTAIR in the GES-1 cells induced significantly more *in vitro* migration compared with the empty vector-transfected cells (Fig. 2D; $P < 0.01$). Similar pattern was observed in *in vitro* invasion assays (Fig. 2E), suggesting that HOTAIR can promote both *in vitro* gastric cancer cell motility and invasion.

Impact of differential HOTAIR expression on *in vivo* tumor growth and metastasis

The effects of HOTAIR expression on tumor growth *in vivo* were determined in a subcutaneous xenograft assay using the BGC-823 and GES-1 cells, the former either stably expressing a *Renilla* or HOTAIR shRNA, and the later either stably overexpressing *Renilla* or HOTAIR cDNA. Our results showed that shRNA-mediated silencing of HOTAIR expression lead to a significant attenuation of tumor growth rate, as compared with *Renilla*-shRNA-transfected control in BGC-823 cells (Fig. 3A; $P < 0.01$) and tumor burden (Fig. 3B; $P < 0.01$). Conversely, forced expression of HOTAIR in the GES-1 cells induced rapid tumor growth rate (Fig. 3A; $P < 0.01$) and tumor burden (Fig. 3B; $P < 0.01$). Finally, we investigated the role of HOTAIR expression on tumor metastasis by using a hepatic tumor growth model.

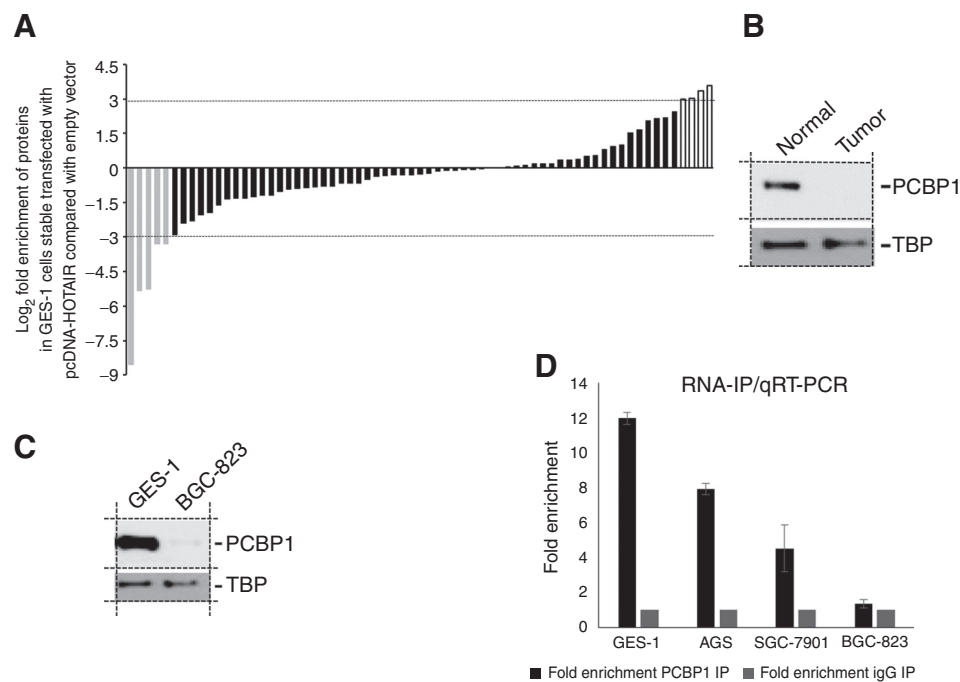


Figure 4.

HOTAIR induces potent downregulation of PCBP1 protein expression in GES1 cells. A, observed fold changes of differentially expressed proteins up- or downregulated following transient overexpression of HOTAIR in GES1 cells in comparison with GES1 cells transiently transfected with the empty vector. The data represent experiments done in triplicate and postnormalization to TBP expression. Grey and white bars represent, respectively, downregulated and upregulated showing at least 3-fold change. B and C, immunoblot analysis of PCBP1 (top) protein levels in normal and gastric cancer tumor epithelium (B) and gastric cancer cell line BGC823 and the immortalized human gastric epithelial mucosa cell line GES1 (C). The blots were stripped and reprobed for TBP (bottom) as a loading control. D, RIP experiment on indicated samples using anti-PCBP1 or control IgG antibodies. Immunoprecipitated material was analyzed by real-time RT-PCR, normalized versus the relative input and plotted as fold enrichment versus the IgG. RT-minus control experiments showed the absence of DNA contamination (data not shown).

HOTAIR-shRNA-transfected stable BGC-823 cells significantly inhibited, and HOTAIR-overexpressing stable GES-1 cells significantly induced, formation of hepatic tumor nodules in non-injected liver lobes (Fig. 3C). Cumulatively our results suggested that HOTAIR expression is both protumorigenic and prometastatic in gastric cancer.

PCBP1 expression is inversely correlated to HOTAIR expression in gastric cancer cells

To determine the underlying mechanism of HOTAIR in gastric cancer pathogenesis, we sought to perform proteomic analyses in the GES-1 cells ectopically overexpressing HOTAIR. The choice of GES-1 cells over BGC-823, which has higher HOTAIR expression, was based on the fact that our goal was to determine what changes occur when HOTAIR expression starts going up as is observed during gastric cancer pathogenesis as opposed to what happens after knockdown of HOTAIR expression.

Following mass spectrometry, data analysis and normalization to TBP expression levels and setting cutoff value to at least 3-fold enrichment/depletion, 5 proteins [Poly r(C)-binding protein-1 (PCBP1), G protein-coupled receptor-1 (GPR1), membrane metallo-endopeptidase (MME), CCAAT/enhancer-binding protein zeta (CEBPZ), antisilencing function protein 1 (histone chaperone; ASF1A)] showed 3-fold downregulation in HOTAIR-overexpressing GES-1 cells compared with parental cells (Fig. 4A; Table 2). Conversely, four proteins [insulin-induced gene

2 (INSIG2), ubiquitin-specific peptidase 49 (USP49), PEST proteolytic signal containing nuclear protein (PCNP), and ring finger protein (C3H2C3 type) 6 (RNF6)] showed 3-fold upregulation in HOTAIR-overexpressing GES-1 cells compared with parental cells (Fig. 4A; Table 2). PCBP1 showed the maximal downregulation (8.54019 ± 1.3457 -fold) following HOTAIR overexpression and hence we decided to validate and further evaluate its role in gastric cancer pathogenesis and progression.

We subsequently validated the steady state expression level of PCBP1 protein in normal and gastric cancer tumor tissue from patients and observed that PCBP1 expression was almost absent in tumor tissue sample (Fig. 4B) and BGC-823 cells (Fig. 4C), both

Table 2. Proteins showing the maximum differential expression between gastric cancer and matched normal epithelium

Protein name	Fold enrichment	Log ₂
PCBP1	0.002686249	-8.54019
GPR1	0.024563913	-5.34732
MME	0.026034958	-5.26341
CEBPZ	0.101168032	-3.30517
ASF1A	0.100708805	-3.31174
INSIG2	8.01793942	3.003232
USP49	8.056980057	3.010239
RNF6	10.26361656	3.359467
PCNP	12.03440121	3.589092

NOTE: Cutoff was set as log₂ 3-fold upregulation or downregulation.

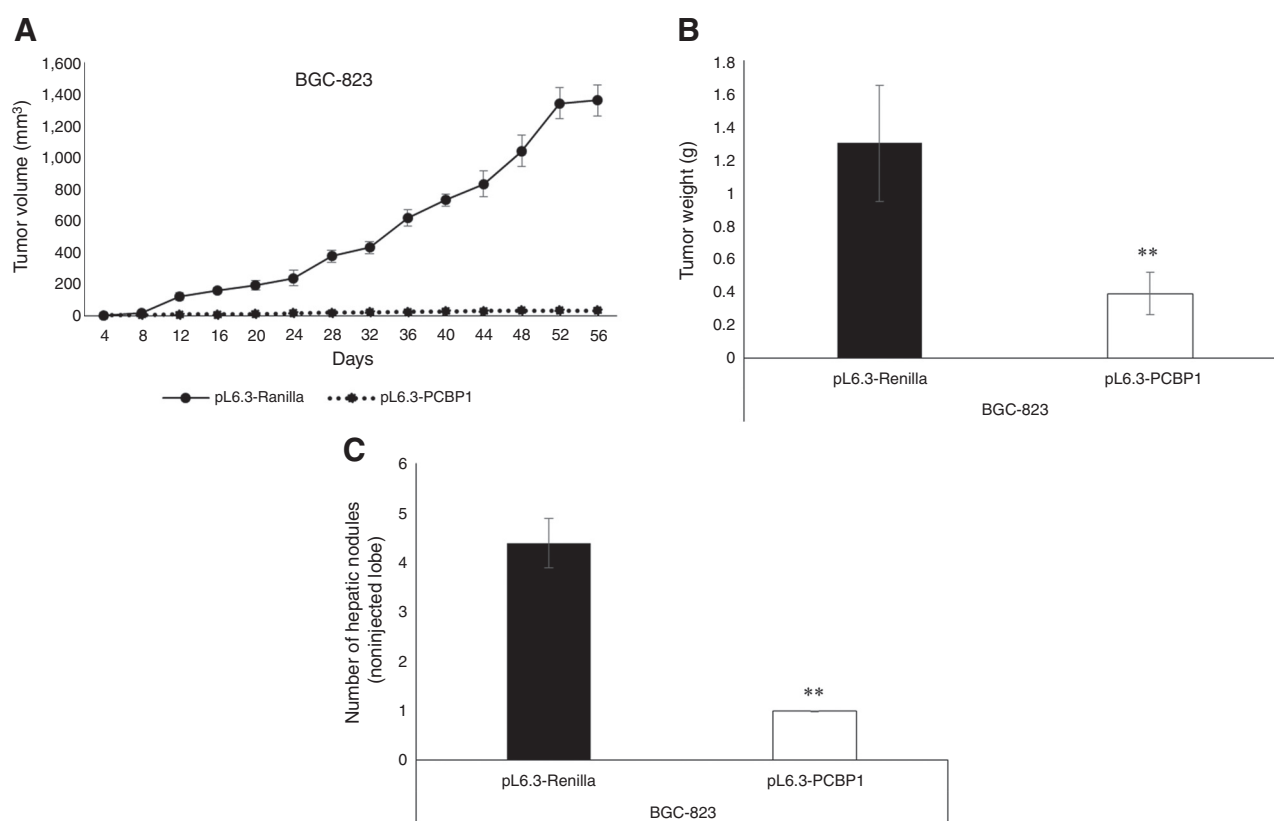


Figure 5. Ectopic expression of *PCBP1* in BGC-823 cells can reverse subcutaneous tumor growth and metastatic hepatic burden observed in parental cells *in vivo*. A, the effects of *PCBP1* overexpression on tumor growth were investigated in a subcutaneous tumor model. pLenti 6.3-*PCBP1* BGC-823 cells showed an attenuation *in vivo* growth rate, as compared with pLenti 6.3-*Renilla*-transduced cells (**, $P < 0.01$ for both at day 56). B, changes in tumor growth rate were also reflected by final tumor weights at day 56 for both BGC823-pL6.3-*PCBP1* and BGC823-pL6.3-*Renilla*-transduced cells (**, $P < 0.01$). Bars, mean \pm SEM. C, animals in the BGC823-pL6.3-*PCBP1* group had a markedly attenuated hepatic tumor burden compared with the BGC823-pL6.3-*Renilla* group as determined by measuring the occurrence of hepatic nodules in liver lobes other than the injected lobe (**, $P < 0.01$).

cases where higher HOTAIR expression were noted (Figs. 1A and 2A). Cumulatively, our data suggest that *PCBP1* is robustly downregulated when the relative expression of HOTAIR is enhanced.

To confirm direct interaction between HOTAIR lncRNA and *PCBP1*, we performed RNA-immunoprecipitation (RIP) with an antibody against *PCBP1* before performing qRT-PCR with the immunoprecipitated mRNA. As shown in Fig. 4D, we saw an inverse relationship between HOTAIR expression level (Fig. 2A) and interaction with *PCBP1* in the gastric cancer cells lines tested. One possible and perhaps obvious explanation of the differential interaction of HOTAIR and *PCBP1* observed in the four cell lines is the differential *PCBP1* expression levels in these cells. However, this still proves direct interaction between HOTAIR lncRNA and *PCBP1*.

Modulating *PCBP1* expression is enough to mimic effect of HOTAIR expression on *in vivo* tumor growth and metastasis

The effects of *PCBP1* expression on tumor growth *in vivo* were determined in a subcutaneous xenograft assay using the BGC-823 cells stably overexpressing *Renilla* or HOTAIR cDNA. Our results showed that ectopic expression of *PCBP1* in the BGC-823 cells attenuated rapid tumor growth rate (Fig. 5A; $P < 0.01$) and

significantly decreased the tumor burden (Fig. 5B; $P < 0.01$), compared with the BGC-823/*Renilla* xenografts. We next investigated the role of *PCBP1* expression on tumor metastasis by using a hepatic tumor growth model. *PCBP1*-overexpressing stable BGC-823 cells significantly inhibited formation of hepatic tumor nodules in noninjected liver lobes (Fig. 5C). Cumulatively, and in comparison with results depicted in Fig. 3, our results suggested that although HOTAIR expression is both protumorigenic and prometastatic in gastric cancer, *PCBP1* expression seems to have a diametrically opposite effect in being antitumorigenic and antimetastatic.

Discussion

Previous studies have indicated that lncRNAs are key players in gene regulatory processes and can influence both normal and transformed cellular functionality (16, 17). lncRNAs do not code for proteins; however, they have been reported to control transcription, indicative that differences observed in lncRNAs between normal and transformed cells are not merely a secondary readout for cellular transformation. In fact, lncRNAs are reportedly associated with metastatic cancer progression (18–21). Our findings are corroborated by two recent reports

(22, 23). RNAi-mediated knockdown of HOTAIR was shown to induce gene expression changes characteristics of inhibition epithelial-to-mesenchymal transition (EMT) and also functionally inhibited *in vitro* migration and invasion (22). In a study conducted in Iran, it was shown that HOTAIR expression correlated with perineural invasion, distant metastasis, and tumor-node-metastasis staging in gastric cancer (23). However, little information about the underlying mechanism of lncRNAs in gastric cancer pathogenesis is available. In that perspective, our current profiling of HOTAIR and subsequent elucidation of PCBP1 is of paramount significance in advancing the knowledge base.

HOTAIR is a member of the human *HOX* loci-associated 231 ncRNAs (14) and it is evident that nuclear HOTAIR can target polycomb repressive complex 2, altering H3K27 methylation and gene expression patterns across the genome (14, 24). Recent work reported a scaffold function for HOTAIR in the cytoplasm as an inducer of ubiquitin-mediated proteolysis (25).

Our proteomic profiling, RIP/qRT-PCR and subsequent validation indicate that PCBP1 and HOTAIR have inverse roles in gastric cancer pathogenesis and metastatic progression. Several studies corroborate our current finding that PCBP1 can function as a robust tumorigenic and metastatic inhibitor. In one of those, a transcript-selective translational regulatory pathway was described in which a ribonucleoprotein (mRNP) complex, consisting of PCBP1, silences translation of mRNAs that are involved in mediating EMT and metastatic progression (26). It was shown that TGF- β activates a kinase cascade terminating in the phosphorylation of PCBP1 by isoform-specific stimulation of protein kinase B β /Akt2, inducing the release of the mRNP complex from the 3'-UTR element, in turn resulting in the reversal of translational silencing and increased expression of transcripts that mediates EMT (26–28). In the second study, PCBP1 was shown to downregulate production of the prometastatic PRL-3 phosphatase (29). Thus it might be possible that downregulation of PCBP1 dictates mesenchymal cell formation in a context-dependent fashion, as observed by us in the current study and the others (26–29).

It would be interesting to identify what potentially causes downregulation of PCBP1 in gastric cancer cells overexpressing

HOTAIR. Ongoing endeavors in the laboratory are focusing on identifying the exact mechanism of PCBP1 downregulation by PCBP1. Our preliminary results suggest that it is not at the level of *PCBP1* transcription and is perhaps being mediated by a secondary mechanism (data not shown). Another interesting objective would be to determine whether a similar mechanism is operative in the sustained enrichment of HOTAIR expression or PCBP1 suppression in other tumor types.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H. Cao, J. Xu

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z.-Z. Zhang, J. Xu, E.-H. Zhao, H. Cao

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.-Y. Shen, J. Xu, E.-H. Zhao, M. Wang, C.-J. Wang, H. Cao

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.-J. Wang

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