HOTAIR long noncoding RNA promotes gastric cancer metastasis through suppression of Poly r(C) Binding Protein (PCBP) 1

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Running title: HOTAIR and PCBP1 in gastric cancer

Key words: long noncoding RNA; HOTAIR; gastric cancer; Poly r(C) binding protein; PCBP1

Abbreviation list: ASF1A - Anti-silencing function protein 1 (histone chaperone); ceRNA – competing endogenous RNA; CEBPZ - CCAAT/enhancer-binding protein zeta; DTT – dithiothreitol; EDTA - Ethylenediaminetetraacetic acid; GPR1 - G protein-coupled receptor-1; HER2 - human epithelial growth factor receptor 2; HOTAIR - Hox transcript antisense intergenic RNA; HPLC – high performance liquid chromatography; INSIG2 - Insulin-induced gene 2; KCl – potassium chloride; lncRNA – long non coding RNA; MgCl₂ – magnesium chloride; MME - membrane metallo-endopeptidase; ncRNA – non coding RNA; MS – mass spectrometry; NaCl – sodium chloride; PBS – phosphate buffered saline; PCBP1 - Poly r(C) Binding Protein 1; qRT-PCR - quantitative reverse transcriptase polymerase chain reaction; RIP – RNA immunoprecipitation; RNF6 - ring finger protein (C3H2C3 type) 6; SD – standard deviation; STR – short tandem repeat; TBP – TATA Binding protein; TCEP - Tris 2-carboxyethyl phosphine; USP49 - ubiquitin specific peptidase 49; PCNP - PEST proteolytic signal containing nuclear protein.
Notes:


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**Conflict of interest:** The authors declare that they do not have any conflict of interest.
Abstract

The objective of this study was to evaluate role of HOTAIR long noncoding RNA in gastric cancer metastasis. We analyzed HOTAIR expression levels by real-time reverse transcription PCR and Northern blot 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 was performed. Stable shRNA-mediated knockdown and lentiviral-mediated overexpression of HOTAIR was 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 vivo tumorigenicity and metastatic potential in these cells. PCBP – 1 and HOTAIR has an inverse relationship, both at expression level and in function. 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 IncRNA. 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 over-expression and PCBP1 down-regulation and the ensuing malignant phenotype in both cultured and xenografts gastric cancer cells.
Introduction

Gastric cancer, one of the most common cause 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 gastric cancer patients.

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 expression of lncRNAs have been reported in a wide variety of human diseases and cancers, inclusive of gastric (8, 9), prostate (10-12) and breast (13, 14) cancer.

Both the aforementioned studies (8, 9) have suggested that the HOTAIR (Hox transcript antisense intergenic RNA), a ~2.2-kb long non-coding 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 human epithelial growth factor receptor 2 (HER2) through competition for miR-331-3p (9). However, the overall biological role and underlying molecular mechanism of HOTAIR in gastric carcinogenesis remains 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 non-tumorous gastric samples were obtained from 50 Chinese patients at Renji Hospital of Shanghai Jiaotong University between 2007 and 2010. All cases were included post review by pathologist and histological confirmation as gastric cancer (stage II, III, and IV; 7th Edition AJCC) and only where complete clinical pathology information was available (Table 1). None of the 50 included patients underwent pre-operative local or systemic treatment. The study 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, Carlsbad, CA) before snap freezing within 30 minutes post-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 Dulbecco’s modified Eagle’s media (DMEM) or RPMI 1640 medium supplemented with 10% fetal bovine...
serum (FBS) and 1% penicillin-streptomycin. Cell lines were characterized in the parent bank by the STR 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, and quantitative reverse transcriptase PCR (qRT-PCR)**

RNeasy kit (Qiagen, Grand Island, NY) was used to isolate total RNA from the stored tissue specimens or cell lines following the manufacturer’s instructions. Northern blot 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 manufacturer recommendations. Primers used for HOTAIR amplification were Forward - 5’-CAGTGGGGAACTCTGACTCG-3’ and Reverse – 5’-GTGCCTGGTGCTCTCTTACC-3’. All amplified amplicons were verified through agarose gel electrophoresis. The assays were performed in triplicates and done at least five different times and \(2^{-\Delta\Delta Ct}\) method was used to determine the relative IncRNA expression levels. *TBP* was used as an endogenous control for data normalization. Data is represented as post-normalization mean ± standard deviation (SD).

**Plasmid constructs, transfection, and transduction**

Indicated cells (2 × 10⁶/well) were plated in 6-well plates overnight. Cells were then transfected with 30 nM non-targeting siRNA control (siControl, Santa Cruz Biotechnology, Shanghai, China) or 30 nM siRNA against HOTAIR (siHOTAIR I and siHOTAIR II) (Sigma Aldrich) using Lipofectamine LTX transfection reagent (Carlsbad, CA, USA) 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, Shanghai) 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, Beijing, China). For stable overexpression, HOTAIR, PCBP1 or Renilla cDNA was cloned into pLenti 6.3. Lentivirus were generated by transfecting the destination vector into HEK-293 cells using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) 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 1012 plaque-forming units (pfu)/ml. All transduction was performed at multiplicity of infection (MOI) 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 x 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 5 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 × 10⁵/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, stained with crystal violet. Crystal violet-stained cells were counted in 5 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 × g for 30 minutes at 4°C. Collected supernatant was dialyzed against PBS, proteins were reduced with 5 mM Tris 2-carboxyethyl phosphine (TCEP) and alkylated with 10 mM iodoacetamide. Samples were digested with trypsin (Promega) in a 1:50 ratio for 12 h at room temperature. Peptides were desalted on Ultra MicroTIP Columns (The Nest Group, Southborough, MA, USA) and dried in a SpeedVac concentrator. Dried peptides were resolubilized in 20 µl HPLC grade water containing 0.1% formic acid. Sample analysis was performed on a linear ion trap LTQ mass spectrometer (Thermo Electron, San Jose, CA) 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 pre-column filled with C18 resin (Magic C18 AQ 5 µm;
Michrom Bioresources, Auburn, CA, USA). A linear gradient of 80 minutes from 5% to 40% acetonitrile in H$_2$O 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 µl/min. 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 400-1600 m/z. Dynamic exclusion was enabled, the repeat count was set to 2 and the exclusion duration to 30s. 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 was normalized to TBP expression levels. Log (2) 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 × 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 × 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, Richmond, CA). Ten micrograms of total cell lysates were 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 (1:2000 dilution in 10% milk/PBS-T (Abcam, Cambridge, MA)). Post-exposure blots were stripped and probed with anti-TBP antibody (1:5000 dilution; Abcam (Cambridge, MA)). The detection was done using horseradish peroxidase–labeled secondary antibodies and enhanced chemiluminescence detection reagent.

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

10 × 10⁶ GES-1, AGS, SGC-7901, and BGC-823 cells were lysed for 15 min on ice in lysis buffer containing 100 mM KCl, 5 mM MgCl₂, 10 mM HEPES, pH 7.0, 0.5% Nonidet P-40 detergent supplemented with fresh 1 mM dithiothreitol (DTT), 1000 units/ml RNAsin (Promega,
Madison, WI, USA), and Mini protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Post-nuclear cytosolic fraction was collected by spinning the cells at 15,000 × g for 15 min at 4 °C and 1% (v/v) was removed for input sample. Four mg of lysates were subjected to immunoprecipitation using 10 µg of PCBP1 antibody or 10 µg of mouse IgG using Pierce Crosslink IP Kit (Thermo Scientific, Rockford, USA). Immunoprecipitated complex was digested with 30 µg of proteinase K to release the ribonucleoprotein complex. TRIzol LS reagent (Life Technologies, Carlsbad, CA) was subsequently used to extract RNA from the immunoprecipitates and the input samples following manufacturer’s recommendations.

The RNAs isolated above were used for first stand cDNA using SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, CA) 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, Wilmington, MA) 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, 10^6 indicated cells were injected subcutaneously into eight-week-old male nude mice (Beijing HFK Bio-Technology Co. Ltd., China). Tumor diameters were measured on alternative days, and volumes calculated using
the estimation: width² × length × 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
Two-tailed Student’s t test was used to calculate statistical significance between two comparator groups. The differences of lncRNA expression between paired tissue sample was evaluated with Wilcoxon matched pairs signed ranks test. A P value < 0.05 was considered as 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. 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 to the cohort with relatively lower HOTAIR expression (P<0.001) (Fig. 1B), cumulatively reinforcing previous findings (8, 9) that HOTAIR expression might be a useful as a diagnostic and prognostic marker for gastric cancer.

**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). Since 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 post-transfection. Whereas, 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 to 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 to 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. 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 pro-tumorigenic and pro-metastatic in gastric cancer.

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

In order to determine the underlying mechanism of HOTAIR in gastric cancer pathogenesis we sought to perform proteomic analyses in the GES-1 cells ectopically overexpression 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 post-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), Anti-silencing function protein 1 (histone chaperone) (ASF1A)) showed 3 fold downregulation in HOTAIR overexpressing GES-1 cells compared to parental cells (Figure 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 to parental cells (Figure 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 cases where higher HOTAIR expression were noted (Fig. 1A, 2A). Cumulatively, our data suggest that PCBP1 is robustly downregulated when the relative expression of HOTAIR is enhanced.

To confirm direct interaction between HOTAIR IncRNA 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 4 cell lines are 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 to 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 non-injected liver lobes (Fig. 5C). Cumulatively, and in comparison to results depicted in Fig. 3, our results suggested that whereas HOTAIR expression is both pro-tumorigenic and pro-metastatic in gastric cancer, PCBP1 expression seems to have a diametrically opposite effect in being anti-tumorigenic and anti-metastatic.

**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 is not merely a secondary readout.
for cellular transformation. In fact, IncRNAs 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 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 TNM staging in gastric cancer (23).

However, little information regarding the underlying mechanism of IncRNAs 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 HOTAI 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 has 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 Poly(C) binding protein (PCBP) 1, silences translation of mRNAs that are involved in mediating epithelial to mesenchymal transition (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 Bβ/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 pro-metastatic 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 lab 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 if a
similar mechanism is operative in the sustained enrichment of HOTAIR expression or PCBP1
suppression in other tumor types.
References


Table 1. Clinical correlation of HOTAIR expression with gastric cancer. Relative HOTAIR expression was calculated as the median of relative expression, with 25th-75th percentile in parentheses. P<0.05 was considered significant (Mann–Whitney U test between 2 groups and Kruskall-Wallis test for 3 groups).

<table>
<thead>
<tr>
<th>Clinicopathologic features</th>
<th>n (=50) (%)</th>
<th>Relative HOTAIR expression</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td>0.834</td>
</tr>
<tr>
<td>Male</td>
<td>27 (54)</td>
<td>11.97 (0.89-18.53)</td>
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<tr>
<td>Female</td>
<td>23 (46)</td>
<td>12.39 (3.46-22.34)</td>
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<tr>
<td><strong>Tumor Site</strong></td>
<td></td>
<td></td>
<td>1.234</td>
</tr>
<tr>
<td>Distal third</td>
<td>8 (16)</td>
<td>8.9 (1.24-14.09)</td>
<td></td>
</tr>
<tr>
<td>Middle third</td>
<td>12 (24)</td>
<td>7.34 (2.51-8.99)</td>
<td></td>
</tr>
<tr>
<td>Proximal stomach</td>
<td>30 (60)</td>
<td>7.65 (1.39-14.23)</td>
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<tr>
<td><strong>Differentiation</strong></td>
<td></td>
<td></td>
<td>0.038</td>
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<tr>
<td>Poor</td>
<td>33 (66)</td>
<td>16.3 (7.23-21.39)</td>
<td></td>
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<tr>
<td>Moderate/High</td>
<td>17 (34)</td>
<td>7.96 (0.89-9.95)</td>
<td></td>
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<tr>
<td><strong>Metastatic Disease</strong></td>
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<td>0.021</td>
</tr>
<tr>
<td>M0</td>
<td>31 (62)</td>
<td>8.37 (1.87-34.56)</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>19 (38)</td>
<td>30.87 (19.34-41.34)</td>
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<td><strong>Lymph Node Metastasis</strong></td>
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<tr>
<td>N0</td>
<td>9 (18)</td>
<td>1.09 (0.67-1.39)</td>
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<tr>
<td>N1</td>
<td>9 (18)</td>
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<td>N3</td>
<td>18 (36)</td>
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Table 2. Proteins showing the maximum differential expression between gastric cancer and matched normal epithelium. Cutoff was set as Log (2) 3 fold up- or down-regulation.

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<th>Protein Name</th>
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Figure legends

**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 is 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 ≥ median ratio) was significantly higher than that of Low-HOTAIR group (n=15; HOTAIR expression ratio ≤ median ratio; P = 0.0039, log-rank test).

**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, 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, 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, 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).
Figure 3. Stable HOTAIR lncRNA down-regulation 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 to Renilla-shRNA transduced cells (***P <0.01 for both at day 56). pLenti 6.3-HOTAIR GES-1 cells showed an accelerate in vivo growth rate, as compared to 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 ± SEM. (C) Whereas animals in the BGC823-HOTAIR-shRNA group, compared to 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).

Figure 4. HOTAIR induces potent downregulation of PCBP1 protein expression in GES1 cells. (A) Observed fold changes of differentially expressed proteins up- or down-regulated following transient overexpression of HOTAIR in GES1 cells in comparison to GES1 cells transiently transfected with the empty vector. The data represents experiment done in triplicate and post-normalization to TBP expression. Grey and white bars represent, respectively, downregulated and upregulated showing at least 3 folds change. (B, C) Immunoblot analysis of PCBP1 (upper panels) 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 re-probed for TBP (bottom panels) as a loading control. (D) RNA-immunoprecipitation (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).

Figure 5. Ectopic expression of PCBP1 in BGC-823 cells can revert to 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 to 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 ± SEM. (C) Animals in the BGC823-pL6.3-PCBP1 group had a markedly attenuated hepatic tumor burden compared to 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).
Figure 2

(A) Western blot analysis of HOTAIR expression in GES-1, AGS, SGC-7901, and BGC-823 cell lines. 

(B) Hotair expression in BGC-823 cells transfected with siCONTROL, siHOTAIR I, and siHOTAIR II. 

(C) Hotair expression in GES-1 cells transfected with pcDNA, pcDNA-HOTAIR. 

(D) Migration assay results showing percent migration for BGC-823 and GES-1 cells treated with siCONTROL, siHOTAIR I, siHOTAIR II, pcDNA, and pcDNA-HOTAIR. 

(E) Invasion assay results showing percent invasion for BGC-823 and GES-1 cells treated with siCONTROL, siHOTAIR I, siHOTAIR II, pcDNA, and pcDNA-HOTAIR.
Molecular Cancer Therapeutics

HOTAIR long noncoding RNA promotes gastric cancer metastasis through suppression of Poly r(C) Binding Protein (PCBP) 1

Zi-Zhen Zhang, Zhi-Yong Shen, Yan-Ying Shen, et al.

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