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Role of HOTAIR long noncoding RNA in metastatic progression of lung cancer

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Abstract. – OBJECTIVE: Lung cancer, the primary cause of cancer-related death worldwide, imperatively requires new therapeutic approaches that target the molecular regulatory machinery. Even though it has been reported that long noncoding RNAs are involved in different cancer types, limited data are available on the expression and function of long noncoding RNAs in lung cancer metastasis. The major objective of the current study was to profile expression and function of six long noncoding RNAs in five matched pairs of primary lung cancer and lymph node metastatic tissues.

MATERIALS AND METHODS: The study protocol was approved by the Institutional Review Board of the Chinese PLA General Hospital, China. All patients enrolled in the study provided signed informed consent.

RESULTS: Among the tested lncRNAs, HOTAIR and NEAT1 were most highly expressed in lymph node metastasis. However, only HOTAIR was subsequently found to be involved in lung cancer cell motility and invasion, as assessed by *in vitro* migration and invasion assay. Finally, our experiments suggest that HOTAIR promoted gelatinase activity in these cells.

CONCLUSIONS: Our data indicates that HOTAIR is overexpressed in metastatic lung cancer tissue, which is prospectively associated with the ability of HOTAIR to promote lung cancer cell motility and invasion.

Key Words:
HOTAIR, Long noncoding RNA, Lung cancer, Lymph node metastasis, Metastatic progression.

Introduction

According to recent estimates, lung cancer remains as the leading reason of cancer death worldwide in both men and women[1], with an estimated 1.4 million deaths per year[2]. Intriguingly, as with other solid carcinomas, most of lung cancer related deaths are due to secondary organ metastases and ensuing complications. Even though advances have been made in both diagnostics and treatment modalities lung cancer remains a serious disease because lung cancer is not usually diagnosed until advanced stage[3]. In addition, a significant number of lung cancer patients are found to be completely refractory to chemotherapy, whereas others would eventually develop acquired resistance even after combination therapy[4]. This highlights the imminent requirement for discovery of more molecular markers that can be potential and more efficacious therapeutic targets.

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[5-8]. Anomalous expression of lncRNAs have been reported in a wide variety of human diseases and cancers, inclusive of prostate[9-11] and breast[12,13]. MALAT-1 is the only lncRNA whose expression has been previously correlated to worse prognosis in a small cohort of lung cancer patients[14]. Cumulatively, these studies suggest that lncRNA might be involved in both the process of tumorigenesis as well as metastatic progression and, thus, merits detailed investigation. However, research on the expression and function of lncRNAs in lung cancer metastasis is still limited and deciphering the role of the same in lung cancer metastasis, if any, would be of significant clinical benefit.

Hence, the objective of the current study was to analyze the expression profiles of well documented metastasis-related lncRNAs[15] in 5 pairs of primary lung cancer and matched lymph node metastatic tissues and explore their role in promoting *in vitro* cell motility and invasion.

Aim

The major objective of the current study was to profile expression and function of six long noncoding RNAs in five matched pairs of primary lung cancer and lymph node metastatic tissues.

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Materials and Methods

Clinical Samples and Tissue Processing

The study protocol was approved by the Institutional Review Board of the Chinese PLA General Hospital, China. All patients enrolled in the study provided signed informed consent. Five pairs of primary lung cancer and matched lymph node metastatic tissues were obtained from the Respiratory Department at the Chinese PLA General Hospital, China. 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. Furthermore, tissue sections from each sample was reviewed and authenticated by a designated pathologist.

Cell Culture

Human metastatic lung cancer cell line NCI-H2009 was obtained from the ATCC. NCI-H2009 cells were maintained at 37°C in a CO_2 incubator in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Quantitative Reverse Transcriptase PCR (qRT-PCR)

Total RNA was isolated from stored tissue specimens using the RNeasy kit (Qiagen, Grand Island, NY, USA) following the manufacturer's instructions. 1 µg total RNA was reverse transcribed using KAPA SYBR® FAST One-Step qRT-PCR Kits as per manufacturer recommendations. Real-time qPCR was performed on a Bio-Rad CFX-96 real-time PCR system (Bio-Rad, Hercules, CA, USA). Primers used for amplification are provided in Table I. All amplified amplicons were verified through agarose gel electrophoresis. The assays were performed in triplicates and done at least five different times. The $2^{-\Delta\Delta}$Ct method was used to calculate the relative expression of each lncRNA. GAPDH was used as an endogenous control for data normalization. Data is represented as post-normalization mean ± standard deviation (SD) of relative fold induction of lncRNA in metastatic over primary lung cancer tissue specimens.

siRNA Transfection

NCI-H2009 cells (3×10^4/well) were plated in 6-well plates overnight. Cells were then transfected with 30 nM non-targeting siRNA control (siControl) or 50 nM siRNA against HOTAIR I (siHOTAIR I and siHOTAIR II), or NEAT1 (siNEAT I and siNEAT II) (Sigma Aldrich, St Louis, MO, USA) for 24 hours using Lipofectamine LTX transfection reagent (Carlsbad, CA, USA) according to the manufacturer's protocol. Following the transfections, NCI-H2009 cells were then harvested for 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. NCI-H2009 cells, transfected with either siControl or siHOTAIR I, II and siNEAT I, II, were trypsinized, and 50,000 cells resuspended in serum free DMEM were added to rehydrated Matrigel-coated inserts (BioCoat Matrigel Invasion Chamber; Becton Dickinson, Franklin Lakes, NJ, USA) and placed in 24-well companion 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 cham-

Table I. Primers used to amplify the lncRNAs.

HOTAIR	Forward - 5'-CAGTGGGGAACTCTGACTCG-3' Reverse – 5'-GTGCCTGGTGCTCTCTTACC-3'
HULC	Forward – 5'-TCATGATGGAATTGGAGCCTT-3' Reverse – 5'-CTCTTCCTGGCTTGCAGATTG-3'
MEG3	Forward – 5'-GCCAAGCTTCTTGAAAGGCC-3' Reverse – 5'-TTCCACGGAGTAGAGCGAGTC-3'
NEAT1	Forward – 5'-TGGCTAGCTCAGGGCTTCAG-3' Reverse – 5'-TCTCCTTGCCAAGCTTCCTTC-3'
UCA1	Forward – 5'-CATGCTTGACACTTGGTGCC-3' Reverse – 5'-GGTCGCAGGTGGATCTCTTC-3'
MALAT-1	Forward – 5'-TAGGAAGACAGCAGCAGACAGG-3' Reverse – 5'-TTGCTCGCTTGCTCCTCAGT-3'

bers were removed, and the cells in the bottom trans chambers were fixed with 3% glutaraldehyde and stained with 4′,6-diamidino-2-phenylindole (DAPI) (1:5,000; Sigma-Aldrich) fluorescent staining. DAPI-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.

In vitro Migration Assay

NCI-H2009 cells, transfected with either siControl or siHOTAIR I, II and siNEAT I, II, 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, San Jose, CA, USA). The chemoattractant in the lower chamber was medium supplemented with 10% FBS. After overnight incubation, the migratory cells in the lower chambers were fixed with 3% glutaraldehyde, stained with 4′,6-diamidino-2-phenylindole (DAPI) (1:5,000; Sigma-Aldrich) fluorescent staining. DAPI-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.

In Situ Zymography

For the zymography assay, glass coverslips were cleaned before being coated with 0.2 mg/mL Oregon green 488-conjugated gelatin (Life Technologies, Carlsbad, CA, USA). They were subsequently cross-linked in 0.5% glutaraldehyde for 15 minutes at 4°C, before being incubated with 5mg/mL NaBH4 for 3 minutes. The coverslips were then sterilized using 70% ethanol for 15 minutes and incubated in serum-free media for a further 1 hour at 37°C. NCI-H2009 cells transfected with siRNA-control or siHOTAIR I were plated on gelatin-coated coverslips, incubated at 37°C for 24 hours before being imaged by fluorescent microscopy at an optimal magnification.

Statistical Analysis

Statistical significance between two comparator groups was ascertained by two-tailed Student's *t* test. For paired lung cancer tissues, the differences of lncRNA expression were evaluated with Wilcox matched pairs signed ranks test. A p value < 0.05 was considered as statistically significant.

Results

LncRNA Expression in Primary Lung Cancer and Matched Lymph Node Metastatic Tissues

Expression profiles of six lncRNAs (HOTAIR, HULC, MEG3, NEAT1, UCA1, and MALAT-1) previously shown to involved in cancer metastasis[15] were examined by qRT-PCR in 5 pairs of primary lung cancer and matched lymph node metastatic tissues. Among the six, two (HOTAIR and NEAT1) showed significant differential expression in primary lung cancer versus matched metastatic tissues (Figure 1) ($p < 0.01$ for HOTAIR and $p < 0.05$ for NEAT1), suggestive of the fact that HOTAIR and NEAT1 expression is correlated to the progression and metastasis of lung cancer.

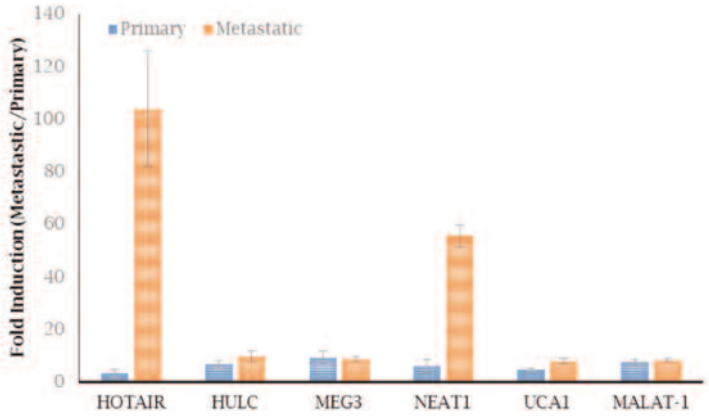

Figure 1. HOTAIR and NEAT1 is up regulated in lymph node metastasis of lung cancer. Expression profiles of lncRNAs in primary lung cancer and matched lymph node metastatic tissues. Quantitative RT-PCR was performed on six lncRNAs in five pairs of primary lung cancer and their matched metastatic tissue samples.

Knockdown of HOTAIR, but not NEAT1, suppressed metastatic lung cancer NCI-H2009 cell motility and invasion

Since cell migration and invasion are essential prerequisites for cancer metastasis, we next determined if increased expression of HOTAIR and NEAT1 lncRNA observed in metastatic lymph node tissue specimens is correlated to increased motility and invasive potential. HOTAIR and NEAT 1 expression in NCI-H2009 was knocked down by siRNAs and confirmed by real-time qPCR (Figure 2A and data not shown). Whereas, siControl-transfected NCI-H2009 cells showed robust *in vitro* migration, knocking down HOTAIR with either of the two siRNAs significantly inhibited *in vitro* migration (Figure 2B, C) ($p < 0.001$ for each HOTAIR siRNA). However, knockdown of NEAT1 did not have any effect on *in vitro* migration capacity of NCI-H2009 cells (Figure 2B, C). This suggested that HOTAIR can promote lung cancer cell motility. Furthermore, knockdown of HOTAIR, but not NEAT1, significantly ($p < 0.001$ for each HOTAIR siRNA) inhibited invasiveness of NCI-H2009 cells (Figure 3). Cumulatively, these indicate that knockdown of HOTAIR, but not NEAT1, inhibited *in vitro* properties associated with metastasis inclusive of motility and invasion.

Knockdown of HOTAIR Suppressed Degradation of Matrix in Situ

Given that tumor metastasis needs both specialized cell migration and the ability to degrade basement membrane by secreted or membrane-bound proteases[16], we next determined if matrix metalloproteinases (MMPs) are involved in HOTAIR-potentiated invasion by assessing gelatinase activity that is readout for MMP-2 and MMP-9 activities. The ability of NCI-H2009 cells to degrade matrix *in situ* was markedly suppressed by siRNA-HOTAIR I, as indicated by decreased matrix degradation (Figure 4, *bottom panels*). This indicated that HOTAIR promoted gelatinase activity, at least in this lung cancer cells tested.

Discussion

Previous studies have indicated that lncRNAs are key players in gene regulatory processes and can influence both normal and transformed cellular functionality[17,18]. 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

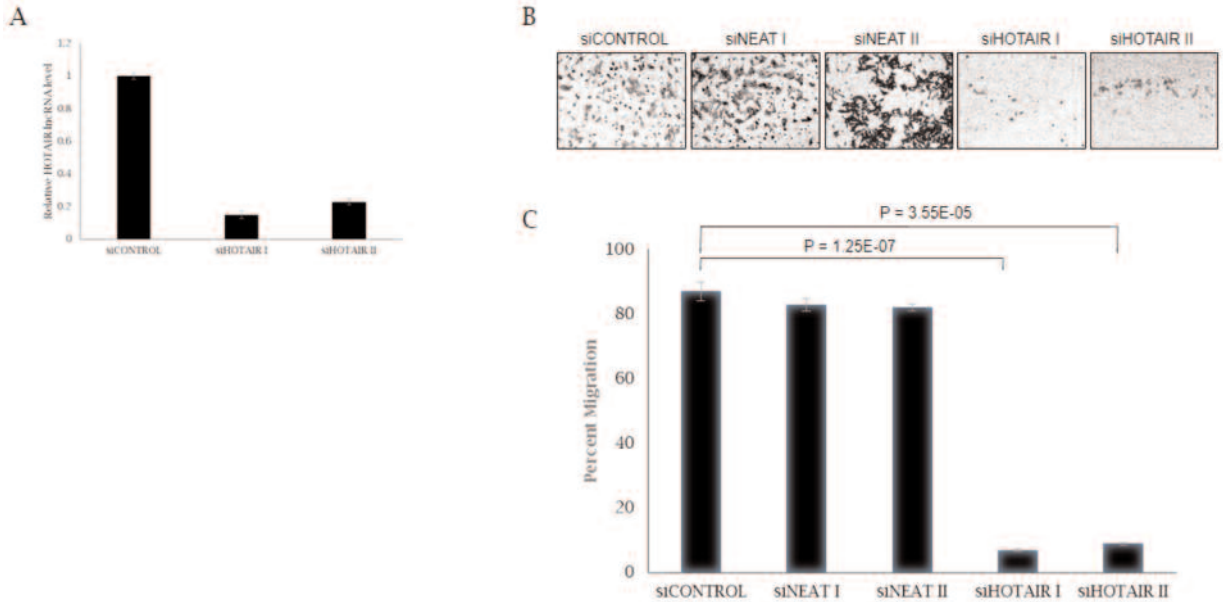

Figure 2. Knockdown of HOTAIR, but not NEAT1, decreases metastatic lung cancer cell line, NCI-H2009, motility. ***(A)*** qRT-PCR analysis was performed to examine HOTAIR RNA levels in NCI-H2009 cells transfected with siControl, siHOTAIR I, or siHOTAIR II. ***(B)*** Representative images and ***(C)*** quantification (percent migration) of *in vitro* migration assays in NCI-H2009 cells transfected with siControl, siHOTAIR I, siHOTAIR II, siNEAT1, or siNEAT II.

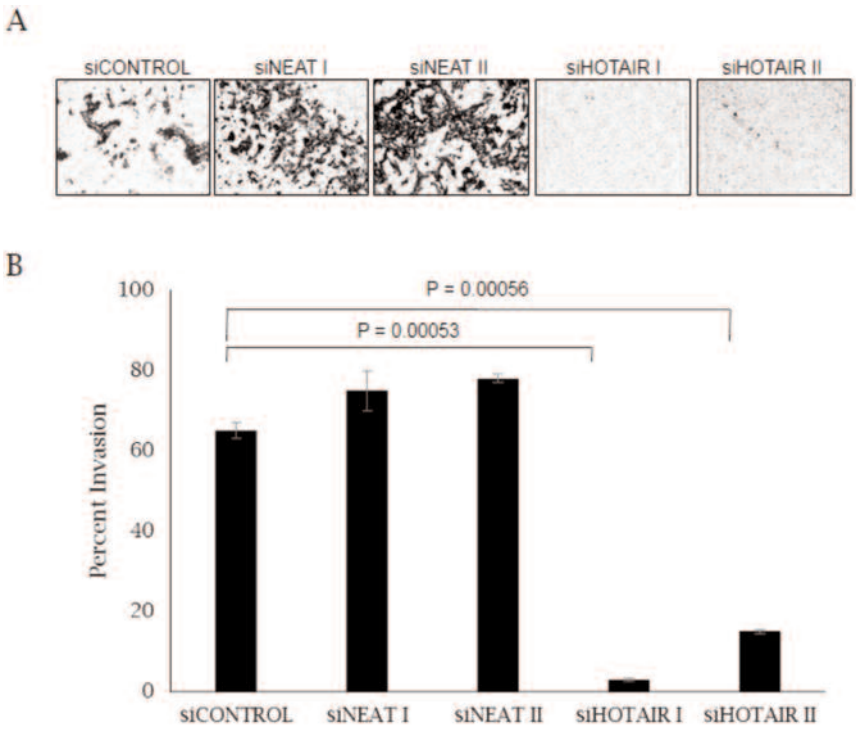

Figure 3. Knockdown of HOTAIR inhibits the invasion of metastatic lung cancer cell line, NCI-H2009. Matrigel-based invasion assay was performed using modified Boyden chambers with 10% FBS as a chemoattractant. Representative images are presented ***(A)*** and quantified (percent invasion) ***(B).***

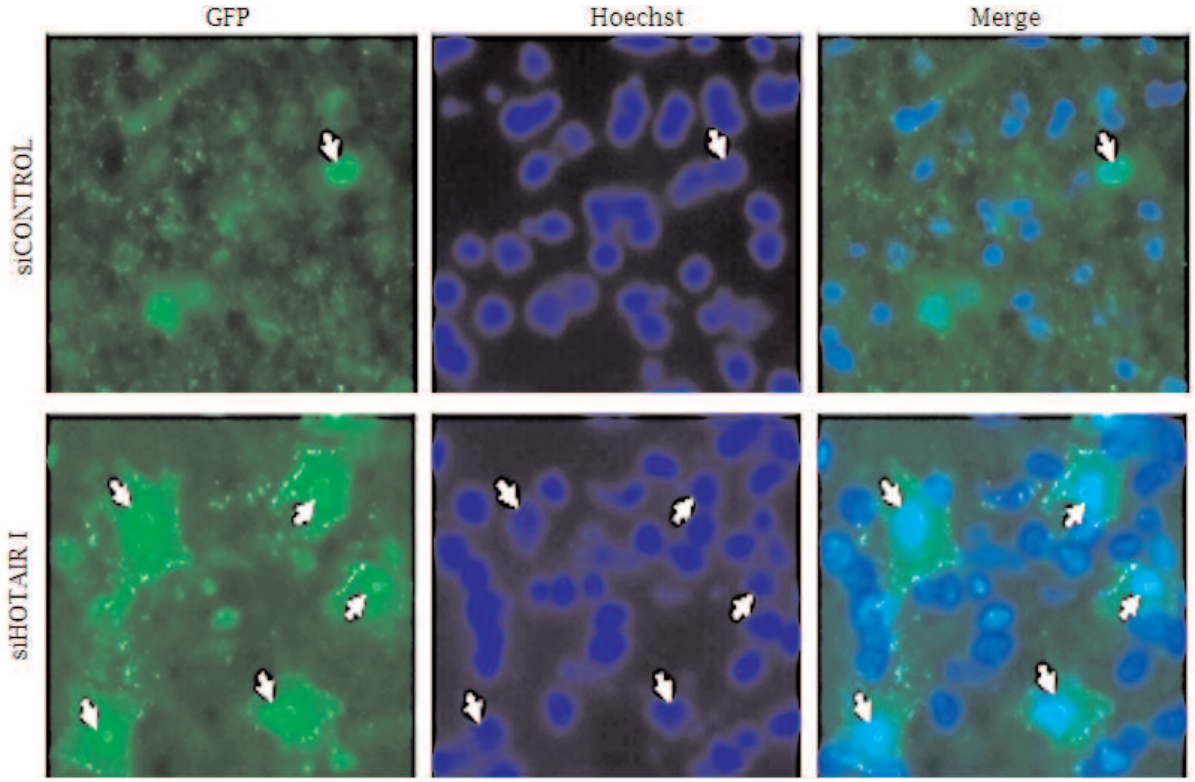

Figure 4. Knockdown of HOTAIR suppressed degradation of matrix *in situ* as assesses by zymography assay. NCI-H2009 cells were transfected with siControl or siHOTAIR (I) for 24 hours. *In situ* zymography was performed with Oregon green 488-conjugated gelatin where the cells were plated on gelatin matrix and incubated for 24 hours. Gelatin degradation was visualized by fluorescence microscopy. Images were obtained at 40X magnification.

for cellular transformation. In fact, lncRNAs are reportedly associated with metastatic cancer progression[19]. However, little information regarding the expression profiles of lncRNAs in lung cancer is available. In that perspective our current profiling of six promiscuous lncRNAs is of paramount significance in advancing the knowledge base.

We observed that HOTAIR and NEAT1 were the only consistently overexpressed lncRNAs among the 6 lncRNAs in lung cancer metastasis compared to the matched primary tumors. Four other lncRNAs were differentially expressed in primary lung cancer versus lymph node metastasis, even though not significant, and thus merits further investigation in an expanded series of samples. However, it was HOTAIR, and not NEAT1 that was probably responsible for acquired *in vitro* motility and invasive potential in the metastatic lung cancer cell line, NCI-H2009.

HOTAIR is one of the 231 ncRNAs associated with human HOX loci[13] and was shown to facilitate breast cancer metastasis[13]. Other cancer types where a similar role of HOTAIR has been suggested are hepatocellular carcinoma[20,21] and pancreatic cancer[22]. In fact, HOTAIR expression was also shown to correlate with lymph node metastasis in hepatocellular carcinoma[21]. Furthermore, siRNA-mediated knockdown of HOTAIR in HCC cells was accompanied by a down regulation of MMP-9, suggesting that MMP-9 may be involved in HOTAIR-mediated regulation of HCC progression[21]. In fact, when we assessed gelatinase activity too, knockdown of HOTAIR suppressed degradation of matrix, supporting the hypothesis that HOTAIR promotes the activity of MMP-9 and/or MMP-2. However, it is imperative that this hypothesis is further tested through direct assays.

Conclusions

Further research on lncRNA expression profiles is needed to define the impact of lncRNAs on the progress of lung cancer and expand on the current findings. It will be interesting to see if HOTAIR expression also correlates to lung cancer metastasis to other organs, which in turn will shed light if there is cancer type specific or organ type specific lncRNA expression and requirement, respectively. It is imperative to have further mechanistic investigation, which was beyond the scope of the current study, into the regulation of metastasis by HOTAIR. However, our experimental results do support a pro-metastatic activity for HOTAIR in lung cancer and perhaps qualifies it as a promising target for lung cancer metastasis diagnosis and therapeutic strategy design.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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