Introduction

Lung cancer is the most common solid tumor and the leading cause of cancer-related mortality worldwide [1, 2]. Non-small cell lung cancer (NSCLC), including squamous cell carcinoma, adenocarcinoma, adenosquamous cell carcinoma, and large cell carcinoma, accounts for approximately 80% of all lung cancer cases [3]. Despite the recent advancements of the diagnosis and treatment of NSCLC, the prognosis of patients with lung cancer remains poor [4]. The main reason of lung cancer-related deaths is due to tumor metastasis [5]. However, the invasion and metastasis of NSCLC greatly limit treatment options, and no effective therapy for NSCLC patients with distant metastasis is currently available.

Therefore, understanding the mechanisms of NSCLC metastasis and identification of novel therapeutic targets are urgently needed.

Long noncoding RNAs (lncRNAs) are defined as a set of RNAs larger than 200 nt in length and without protein-coding potential. LncRNAs play pivotal roles in multiple biological processes, such as chromatin remodeling, transcriptional control, posttranscriptional regulation, and intercellular signaling [6, 7]. A growing number of studies have reported that the dysregulation of lncRNAs expression was involved in many cancers including lung cancer [8, 9]. Some lncRNAs have been demonstrated to play important roles in lung carcinogenesis and have emerged as biomarkers for tumor diagnosis and prediction of prognosis [10–12].

Keywords
Invasion, lncRNA, lncRNA-HIT, migration, non-small cell lung cancer, ZEB1

Abstract
Lung cancer is the most common solid tumor and the leading cause of cancer-related mortality worldwide. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancer cases. The main reason of lung cancer-related deaths is due to tumor metastasis. But, the mechanisms of NSCLC metastasis remains poorly understood. LncRNAs play pivotal roles in multiple biological processes. LncRNA-HIT (HOXA transcript induced by TGFβ) was recently identified. LncRNA-HIT promotes cell migration, invasion, tumor growth, and metastasis. However, the detailed role of lncRNA-HIT in NSCLC remains unknown. In this study, for the first time, we revealed a novel role of lncRNA-HIT in the migration and invasion of NSCLC cells. The expression of lncRNA-HIT was significantly upregulated in NSCLC tissues and cell lines, and the expression level of lncRNA-HIT correlates with advanced disease stage and predicts unfavorable prognosis of NSCLC patients. Functional assays demonstrated that lncRNA-HIT markedly increased the ability of NSCLC cells to migrate and invade. Furthermore, the molecular mechanism by which lncRNA-HIT affects NSCLC cells was associated with regulation of ZEB1 stability. LncRNA-HIT functions as a prometastasis oncogene by directly associating with ZEB1 to regulate NSCLC. The interaction of lncRNA-HIT and ZEB1 may be a potential target for NSCLC therapy.

Upregulation of LncRNA-HIT promotes migration and invasion of non-small cell lung cancer cells by association with ZEB1
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LncRNA-HIT (HOXA transcript induced by TGFβ) was recently identified [13]. LncRNA-HIT regulates TGF-β-induced epithelial-to-mesenchymal transition (EMT). Knockdown of lncRNA-HIT resulted in decreasing of cell migration, invasion, tumor growth, and metastasis [13]. However, the detailed role of lncRNA-HIT in NSCLC remains unknown. In this study, we elucidated the clinical significance of lncRNA-HIT on the prognosis and clinicopathological characteristics of NSCLC patients. We also focused on the underlying molecular mechanisms by which lncRNA-HIT promotes migration and invasion of NSCLC cells.

**Material and Methods**

**Cell culture and samples**

Five NSCLC cell lines (SK-MES-1, NCI-H1650, A549, NCI-H1975, 95D) and normal lung epithelial cells (NLEC) were purchased from ATCC. The cells were cultured in Dulbecco’s modified eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in 5% CO2. The tumor tissues and patient data were obtained from The Second Hospital of Jilin University. All of the patients were provided written informed consent.

**Viral infections**

LncRNA-HIT cDNA was cloned into the pLVX lentiviral vector (Addgene, Cambridge, MA). Virus was made using Turbofect transfection (Thermo, Boston, MA) into 293T cells. Virus was filtered and then infected into cells with polybrene for 24 h. Cells were selected with 3 μg/mL puromycin (Invitrogen, Carlsbad, CA). The pLKO.1 shRNA lentiviral system was used to knockdown genes of interest. pLKO.1, pLKO.1-shHIT-1, and pLKO.1-shHIT-2 were purchased from Genechem Company, Shanghai, China. Target sequences for the target genes were as follows: shHIT-1: GTCTACGACATCTCCTACTCTAG, shHIT-2: CTCGAAAGGTCTGTGACCTAA. Puromycin at 3 μg/mL was used to select stable cells.

**Western blot**

Western blotting was performed as described previously [14]. Primary antibodies 1:1000 anti-ZEB1 (Abcam), 1:1000 anti-E-cadherin (CST), 1:1000 anti-N-cadherin (CST), 1:1000 anti-α-tubulin (CST), and 1:1000 anti-GAPDH (Santa Cruz) were used. Secondary detection antibodies were anti-mouse IgG-HRP conjugate (Jackson) used at 1:10000 or anti-rabbit IgG-HRP conjugate (Jackson). Blots were developed with ECL substrate (Millipore Boston, MA) and analyzed on an imager (GE Healthcare, London, UK).

**Migration and invasion assays**

A total of 5 × 10^4 cells were seeded in a serum-free DMEM in the upper chamber of a 24-well transwell migration or invasion insert (BD biosciences, Franklin lakes, NJ). The lower chamber was filled with medium containing 20% FBS. After 24 h of culture at 37°C, cells in the upper chamber were removed, and the cells that had traversed the membrane were fixed in 4% paraformaldehyde, and then stained by crystal violet.

**RNA isolation and quantitative real-time PCR (qRT-PCR)**

Total RNAs of cells or tissue samples were isolated using TRIzol (Invitrogen) according to the manufacturers’ instructions. First-strand cDNA was generated using the Reverse Transcriptase (Transgene, Beijing, China). qRT-PCR was performed in the ABI 7500 Real-Time PCR System using SYBR Green Mixture (Takara, Dalian, China). Data were normalized to GAPDH or to control samples. Primers sequences for the target genes were as follows: lncRNA-HIT-F: 5′-TGAAA_GGGAGAAGAAGGAAAGG-3′, lncRNA-HIT-R: 5′-GACAGTCTAGGCATTGCTGAT-3′, ZEB1-F: 5′-CCCAAGACAGCAGTAAAT-3′, ZEB1-R: 5′-GATGGTGTACTACTTCTGGAACC-3′.

**RNA immunoprecipitation assay**

RNA immunoprecipitation assay (RIP) was performed as described previously [15]. RIP products were analyzed by qRT-PCR. A total quantity of 5 μg Snail1 (Abcam), Snail2 (Abcam), ZEB1 (Abcam), ZEB2 (Abcam), Twist1 (Abcam), or Twist2 (Abcam) antibodies were used for RIP reaction.

**Chromatin immunoprecipitation assay (ChIP)**

ChIP assays were performed as described previously [16]. The primers for the CDH1 promoter were as follows: 5′-ACTCCAGGCTAGAGGTCCAC-3′ (sense) and 5′-CC GCAAGCTCACAGCTTGGAC-3′ (antisense).

**RNA pull-down assay**

RNA pull-down and deletion mapping were performed as described previously [15]. Briefly, biotin-labeled lncRNA-HIT were in vitro transcribed with the Biotin RNA Labeling Mix (Roche, Cambridge, UK) and T7 RNA polymerase (Roche), treated with RNase-free DNase I (Roche), and purified with the RNeasy Mini Kit (Qiagen, Dusseldorf, Germany). Cell nuclear proteins were extracted using the Cytoplasmic and Nuclear Protein Extraction Kit (Tiangen, Beijing China). Cell
nuclear extract was then mixed with biotin-labeled lncRNA-HIT. Washed streptavidin agarose beads (Invitrogen) were added to each binding reaction and further incubated at room temperature. Beads were washed briefly five times and boiled in sodium dodecyl sulfate buffer, and the retrieved protein was detected by the standard western blotting.

Statistics

All experiments were repeated at least three times. Results are expressed as mean ± SD as indicated. A two-tailed Student’s t test was used for intergroup comparisons. P < 0.05 was considered statistically significant.

Results

lncRNA-HIT is upregulated in NSCLC tissues and cell lines

We first examined the expression level of lncRNA-HIT in NSCLC tissues and cell lines using qRT-PCR. The results showed that the expression of lncRNA-HIT of five NSCLC cell lines, namely SK-MES-1, NCI-H1650, A549, NCI-H1975, and 95D, was significantly higher than normal lung epithelial cells (NLEC) (Fig. 1A). In parallel, lncRNA-HIT expression was markedly increased in NSCLC tumor tissues (T) compared to matched adjacent nontumor tissues (NT) from 60 NSCLC patients. (P < 0.001) (Fig. 1B). Taken together, these data suggest that lncRNA-HIT expression is upregulated in NSCLC.

High-level expression of lncRNA-HIT correlates with advanced disease stage and predicts unfavorable prognosis

To investigate the clinical significance of lncRNA-HIT in NSCLC, the correlation between lncRNA-HIT expression and clinicopathological features were analyzed (Table 1). LncRNA-HIT expression significantly correlated with the clinical staging (P = 0.02) and distant metastasis (P = 0.002). Furthermore, Kaplan–Meier analysis showed that NSCLC patients with high-level lncRNA-HIT expression group had a shorter median survival time than those in the low-level group (Fig. 1C). These results suggested that lncRNA-HIT expression may be a novel valuable marker for the prognosis of NSCLC patients.
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Table 1. The relationship between IncRNA-HIT expression and the clinicopathological characteristics of Non-Small Cell Lung Cancer (NSCLC) patients.

| Features                  | IncRNA-HIT       |  |  |  |
|---------------------------|------------------|---|---|---|
|                           | Low | High |  |  |  |
| Age                       |     |      |  |  |  |
| ≤60                       | 23  | 21   | 0.559 |  |
| >60                       | 7   | 9    |      |  |
| Gender                    |     |      |  |  |  |
| Male                      | 24  | 23   | 0.754 |  |
| Female                    | 6   | 7    |      |  |
| Histology                 |     |      |  |  |  |
| Squamous cell carcinoma   | 10  | 9    | 0.898 |  |
| Adenocarcinoma            | 13  | 12   |      |  |
| Adenosquamous carcinoma   | 2   | 3    |      |  |
| Bronchioalveolar carcinoma| 5   | 6    |      |  |
| Clinical stage            |     |      |  |  |  |
| I, II                     | 20  | 11   | 0.02 |  |
| III, IV                   | 10  | 19   |      |  |
| Distant metastasis        |     |      |  |  |  |
| No                        | 21  | 9    | 0.002 |  |
| Yes                       | 9   | 21   |      |  |
| Drug/radiation therapy    |     |      |  |  |  |
| No                        | 9   | 10   | 0.781 |  |
| Yes                       | 21  | 20   |      |  |

P-values were derived with a two-sided Pearson chi-square test.

Silence of IncRNA-HIT inhibits the migration and invasion of NSCLC cells

Based on the above results, we hypothesized a relationship between IncRNA-HIT and NSCLC cell migration and invasion. To determine the role of IncRNA-HIT in NSCLC cell migration and invasion, we silenced IncRNA-HIT expression using lentivirus-mediated shRNAs in A549 and SK-MES-1 cells (Fig. 2A). Notably, IncRNA-HIT depletion significantly reduced the expression of mesenchymal markers, N-cadherin and vimentin, and increased the expression of epithelial markers, E-cadherin and ZO-1 (Fig. 2B). The migration assay showed that IncRNA-HIT knockdown inhibited the migratory capabilities of both A549 and SK-MES-1 cells compared to their control cells (Fig. 2C). Moreover, IncRNA-HIT-silenced NSCLC cells showed much weaker abilities to invade through Matrigel than did control cells (Fig. 2D).

Overexpression of IncRNA-HIT promotes the migration and invasion of NSCLC cells

In contrast, gain-of-function of IncRNA-HIT with lentivirus-mediated overexpression was performed to evaluate whether ectopic IncRNA-HIT expression promotes the invasiveness of NSCLC cells (Fig. 3A). Overexpression of IncRNA-HIT significantly inhibited the expression of epithelial markers, E-cadherin and ZO-1, and increased the expression of mesenchymal markers, N-cadherin and vimentin (Fig. 3B). In addition, overexpression of IncRNA-HIT obviously promoted the migratory and invasive abilities of both A549 and SK-MES-1 cells (Fig. 3C and 3D). Taken together, these data demonstrated that IncRNA-HIT promotes EMT and remarkably induces the invasive phenotype of NSCLC cell lines.

IncRNA-HIT associates with ZEB1

Suppression of E-cadherin, induced by Snail, ZEB, or Twist family, is considered not only a hallmark of EMT but also a key driver of EMT and metastasis [17]. We suspected that whether IncRNA-HIT regulates E-cadherin expression through association with these EMT-related transcription repressors. We detected the association between IncRNA-HIT and these repressors by performing RIP assays. The results showed that IncRNA-HIT was significantly enriched by ZEB1 antibody than the non-specific IgG control antibody, Snail1, Snail2, ZEB2, Twist1, or Twist2 antibody (Fig. 4A). To validate the association and determine the specific binding region between IncRNA-HIT and ZEB1, we performed RNA pull-down assay and deletion-mapping experiments. We found a 423-nt region at the 5' end of IncRNA-HIT required for the association with ZEB1 (Fig. 4B). Taken together, we demonstrated a specific association between IncRNA-HIT and ZEB1.

Next, we determined the function of IncRNA-HIT-ZEB1 association. Silence of IncRNA-HIT significantly suppressed the protein level of ZEB1, but had no effect on the ZEB1 mRNA level (Fig. 4C and D). In contrast, overexpression of IncRNA-HIT increased the protein level, but not mRNA level, of ZEB1 (Fig. 4E and F). These results strongly indicated that the association of IncRNA-HIT and ZEB1 may influence the stability of ZEB1 protein. To further confirm the IncRNA-HIT-mediated ZEB1 regulation, we treated control and IncRNA-HIT knockdown A549 cells with cycloheximide (CHX). We found that the half-life of ZEB1 was much shorter in IncRNA-HIT knockdown A549 cells than that in control cells (Fig. 4G). When MG132, an inhibitor of proteasome degradation was used, the ZEB1 protein level in IncRNA-HIT knockdown A549 cells was markedly upregulated and reached a level that was comparable to that in control cells (Fig. 4H). Taken together, these data suggested that IncRNA-HIT is important for the stability of ZEB1 protein.

Next, we examined whether IncRNA-HIT affected ZEB1 occupancy of the promoter region in CDH1. The effect of IncRNA-HIT on the ZEB1 occupancy of CDH1 promoter was evaluated using a ChIP assay followed by qPCR. We found that overexpression of IncRNA-HIT
significantly increased the occupancy of ZEB1 on the promoter region of CDH1, while silence of lncRNA-HIT reduced the binding of ZEB1 on the CDH1 promoter (Fig. 4I and J).

**LncRNA-HIT promotes migration and invasion via regulation of ZEB1 expression**

Finally, we determined whether lncRNA-HIT promotes migration and invasion via regulation of ZEB1 expression. We found that overexpression of ZEB1 (Fig. 5A) significantly rescued the cell migration and invasion phenotypes induced by lncRNA-HIT knockdown (Fig. 5B and C). Together, these results demonstrate that lncRNA-HIT exerts its function at least in part through regulating ZEB1 expression.

**Discussion**

In this study, for the first time, we revealed a novel role of lncRNA-HIT in the migration and invasion of NSCLC cells. The expression of lncRNA-HIT was significantly upregulated in NSCLC tissues and cell lines, and the expression level of lncRNA-HIT correlates with advanced
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disease stage and predicts unfavorable prognosis of NSCLC patients. Silence of IncRNA-HIT effectively decreased the migration and invasion of NSCLC cells. In contrast, the overexpression of IncRNA-HIT markedly increased the ability of NSCLC cells to migrate and invade. The molecular mechanism by which IncRNA-HIT affects NSCLC cells was associated with regulation of ZEB1 stability. IncRNA-HIT may function as a prometastasis oncogene by directly associating with ZEB1 to regulate NSCLC.

Many large-scale sequencing studies have demonstrated that >9000 genomic loci express IncRNAs [18, 19]. However, the vast majority of these IncRNAs remain functionally uncharacterized in NSCLC. Increasing evidence suggests that IncRNAs play important roles in oncogenesis and metastasis in NSCLC. For example, IncRNA AGAP2-AS1 promotes NSCLC growth through interacting with EZH2 and LSD1 and repressing LAT52 and KLF2 expression [8]. LncRNA TATDN1 promotes NSCLC

Figure 3. Overexpression of IncRNA-HIT promotes the migration and invasion of NSCLC cells. (A) The relative expression of IncRNA-HIT in control and IncRNA-HIT overexpressed cells. (B) The EMT markers detected by western blot in control and IncRNA-HIT overexpressed cells. (C) Overexpression of IncRNA-HIT promoted migration in NSCLC cells. (D) Overexpression of IncRNA-HIT promoted invasion in NSCLC cells. Data are shown as mean ± SD. *P < 0.05. EMT, epithelial-to-mesenchymal transition.

Figure 4. IncRNA-HIT associates with ZEB1. (A) RIP assays for IncRNA-HIT were performed using indicated antibodies. (B) Deletion mapping of ZEB1-binding domain in IncRNA-HIT (Up). Western blot for ZEB1 in samples pulled down by different IncRNA-HIT fragments (Down). Antisense IncRNA-HIT was used as a negative control. (C) The relative ZEB1 mRNA expression in control and IncRNA-HIT knockdown cells. (D) The ZEB1 protein level in control and IncRNA-HIT knockdown cells. (E) The relative ZEB1 mRNA expression in control and IncRNA-HIT overexpressed cells. (F) The ZEB1 protein level in control and IncRNA-HIT overexpressed cells. (G) The stability of ZEB1 protein over time was measured by western blot relative to time 0 after blocking new protein synthesis with 100 μg/ml CHX in control and IncRNA-HIT knockdown A549 cells. (H) ZEB1 protein expression in control and IncRNA-HIT knockdown A549 cells treated with vehicle control (DMSO) or 10 μmol/L MG132 for 12 h. (I) The occupancy of ZEB1 in the promoter of CDH1 was measured by ZEB1 ChIP assay followed by qRT-PCR in control and IncRNA-HIT knockdown A549 cells. (J) The occupancy of ZEB1 in the promoter of CDH1 was measured by ZEB1 ChIP assay followed by qRT-PCR in control and IncRNA-HIT overexpressed A549 cells. Data are shown as mean ± SD. *P < 0.05. RIP, RNA immunoprecipitation assay.
metastasis through suppression of E-cadherin [20]. These and other examples suggest that lncRNAs are critical to many oncogenic processes. IncRNA-HIT, an oncogene, has been documented to promote tumor metastasis in breast cancer [13]. However, the role of IncRNA-HIT in NSCLC remains unclear. We first analyzed the expression of
lncRNA-HIT on the NSCLC tissues cell lines. LncRNA-HIT was significantly upregulated, correlates with advanced disease stage, and predicts unfavorable prognosis, indicating that lncRNA-HIT may play an important role in NSCLC invasion and metastasis.

Functional assays demonstrated an important role of lncRNA-HIT in NSCLC progression, including migration and invasion. Mechanistic study revealed that lncRNA-HIT exerts prometastasis function at least in part through association with ZEB1. ZEB factors contain multiple domains to interact with other transcription factors, which is essential for regulation of EMT. For example, ZEB proteins can recruit histone deacetylases, methyltransferases, and polycomb group proteins [21–23]. ZEB1 also suppressed transcription through recruitment of the SWI/SNF chromatin remodeling ATPase BRG1 [24]. Recent studies have found a set of lncRNA that can regulate ZEB1 expression through sponging miRNAs, such as lncRNA-ATB and lncRNA-NEAT1 [25, 26]. In this study, for the first time, we revealed a novel posttranslational manner, in which lncRNA regulates ZEB1. We found that lncRNA-HIT could evaluate the stability of ZEB1 and then increase the ZEB1 occupancy of the promoter region in CDH1. The interaction of lncRNA-HIT and ZEB1 may be a potential target for NSCLC therapy.

Overall, our study is the first to show that lncRNA-HIT plays an important role in metastasis in NSCLC. LncRNA-HIT promotes cell migration and invasion at least in part through interaction with ZEB1 regulating its expression. However, it should be pointed out that our in vitro observations may not be completely applied to in vivo situations in the absence of in vivo studies. The possible role of lncRNA-HIT in NSCLC required further studies using animal models of tumors.

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Conflict of Interest

None declared.

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