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Original Article

Up-regulation of lncRNA HULC predicts a poor prognosis and promotes growth and metastasis in non-small cell lung cancer

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Abstract: Introduction: Long non-coding RNAs (lncRNAs) play essential roles during tumorigenesis. Aberrant expression of lncRNA HULC has been declared in types of tumors. However, the function and underlying mechanism of HULC in non-small cell lung cancer (NSCLC) is still unclear. Methods: The relative expression level of lncRNA HULC was determined by quantitative real-time PCR (qRT-PCR) in a total of 58 NSCLC patients and 4 lung cancer cell lines. The prognostic significance was evaluated using Kaplan-Meier analyses. Biological function of HULC was explored by cell proliferation assay, migration assay and invasion assay. Furthermore, western blot was used to determine the activity of PI3K/Akt pathway in si-HULC transfected NSCLC cells. Results: Our findings showed that lncRNA HULC was significantly increased in NSCLC tissues and cell lines. Upregulation of HULC was significantly associated with poor tumor differentiation, advanced pathological stage and lymph-node metastasis. Kaplan-Meier analysis revealed that NSCLC patients with high HULC expression had shorter overall survival times. Additionally, the results of in vitro assays showed that suppression of HULC expression in NSCLC cells significantly reduced cell proliferation, migration and invasion ability. Moreover, we confirmed that the activity of PI3K/Akt pathway was suppressed in si-HULC transfected NSCLC cells. Conclusions: Our findings suggested that lncRNA HULC played a critical role in NSCLC tumorigenesis and could act as a novel prognostic biomarker and potential therapeutic target in the treatment of NSCLC.

Keywords: HULC, long non-coding RNA, NSCLC, PI3K/Akt, progression

Introduction

Lung cancer is the most common malignant disease and the leading cause of mortality in the world, and non-small cell lung cancer (NSCLC) accounts for 75-80% of lung cancer cases [1, 2]. Despite the recent advances in clinical and experimental oncology, the prognosis of lung cancer is still unfavorable, with a 5-year overall survival rate of approximately 11% [3]. Thus, a detailed understanding of the mechanisms underlying NSCLC development and progression are essential for improving the diagnosis, prevention and treatment of this disease.

Long non-coding RNA is an RNA molecule that is longer than 200 nucleotides and is not translated into a protein [4]. Although these long non-coding transcripts were once considered to be simply transcriptional "noise" or cloning artifacts [5], increasing evidence suggested that lncRNAs participate in a spectrum of biological function, including cell growth, differentiation, invasion and metastasis [6-8]. Dysregulation of lncRNA has been found in many types of cancer, including NSCLC. For example, Liu et al showed that lncRNA HOTAIR indicated a poor prognosis and promoted metastasis in NSCLC [9]. Xia et al found that downregulation of lncRNA MEG3 enhanced cisplatin resistance of lung cancer cells through activation of the Wnt/β-catenin signaling pathway [10]. Nie et al suggested that lncRNA UCA1 exerted oncogenic functions in NSCLC by targeting miR-193a-3p [11]. However, the roles of lncRNAs in NSCLC progression remain largely elusive.

In the present study, we focus on the lncRNA HULC (highly upregulated in liver cancer) which is located on chromosome 6p24.3. Transcription of HULC yields a ~500 nt long, spliced and polyadenylated ncRNA that localizes to the cytoplasm where it has been reported to associate with ribosomes [12]. Recent studies indicated that HULC was dysregulation in various kinds of tumors. For example, Uzan et al showed that elevated HULC expression was associated with poor clinical outcomes among the osteosarcoma patients, which suggested that HULC could be a potential prognostic biomarker in osteosarcoma [13]. Yang et al suggested that HULC promoted colorectal carcinoma progression through epigenetically repressing NKD2 expression [14]. Li et al HULC enhanced epithelial-mesenchymal transition to promote tumorigenesis and metastasis of hepatocellular carcinoma via the miR-200a-3p/ZEB1 signaling pathway [15]. These results suggested that HULC could play an essential role in tumorigenesis. However, the function and underlying mechanism of HULC in NSCLC are still unknown.

In this study, we determined the expression lncRNA HULC in human NSCLC tissues and cell lines, and explored its correlation with clinicopathological features and patients' survival. Moreover, we determined the functional impact of HULC on NSCLC progression. Our findings provide novel insights into the role of HULC in the progression of NSCLC and identify a potential therapeutic target for the diagnosis and gene therapy.

Materials and methods

Tissue samples and patient data

Matched fresh NSCLC specimens and adjacent non-tumor tissues were collected from 58 patients who underwent surgery at Department of Thoracic Surgery, The Second Affiliated Hospital of Zhengzhou University between January 2009 and December 2010. All specimens were snap frozen in liquid nitrogen immediately following collection and stored at -80°C until use. None of the patients had received chemotherapy or radiotherapy prior to sampling. Overall survival was calculated from the date of initial surgical operation to death or last follow-up. The Research Ethics Committee of The Second Affiliated Hospital of Zhengzhou University approved this study and all patients provided written informed consent.

Cell culture and transfection

Four NSCLC cell lines (A549, SPC-A1, H23 and NCI-H520) and a normal human bronchial epithelial cell line (16HBE) were cultured in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C. Cells were used when they were in the logarithmic growth phase.

A549 and H23 cells were transfected with either 50 nM siRNA targeting lncRNA HULC (si-HULC) or scrambled negative controls (si-NC) (GenePharma) using the Lipofectamine 2000 reagent (Invitrogen) according to the instructions. The target sequences for HULC siRNAs were used in this study are as follow: si-HULC-1 (sense GGAGGUCGAUUCAUAUCAA and antisense UUGAUAUGAAUCGACCUCC) si-HULC-2 (sense GAAGUAAAGGCCGGAAUAU and antisense AUAUUCCGGCCUUUACUUC). After 48 h, the efficiency of HULC knockdown was confirmed by qRT-PCR.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from tissue samples or cells using TRIzol reagent (Invitrogen). Reverse transcription was carried out with 1 mg of total RNA. cDNA synthesis and qRT-PCR analyses were performed using the SYBR® Green master mix (TaKaRa). qRT-PCR was performed on the ABI Prism 7700 system (Applied Biosystems) and GAPDH was used for normalization. The PCR reaction conditions were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min. Relative HULC expression was calculated using the equation $2^{-\Delta\Delta Ct}$ method. The primers are as follows: HULC sense, 5'-ACCTCCAGAACTGTGATCCAAAATG-3' and reverse 5'-TCTTGCTTGATGCTTTGGTCTG-3'. GAPDH sense, 5'-CGCTCTCTGCTCCTCCTGTTC-3' and reverse 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western blot assay

The cells were lysed in SDS buffer. Extracts were sonicated and boiled for 5 min, and loaded and separated by SDS-PAGE (12%), and electro-transferred to PVDF. Residual binding sites on the membrane were blocked in 5% skim milk for 1 h at room temperature. The

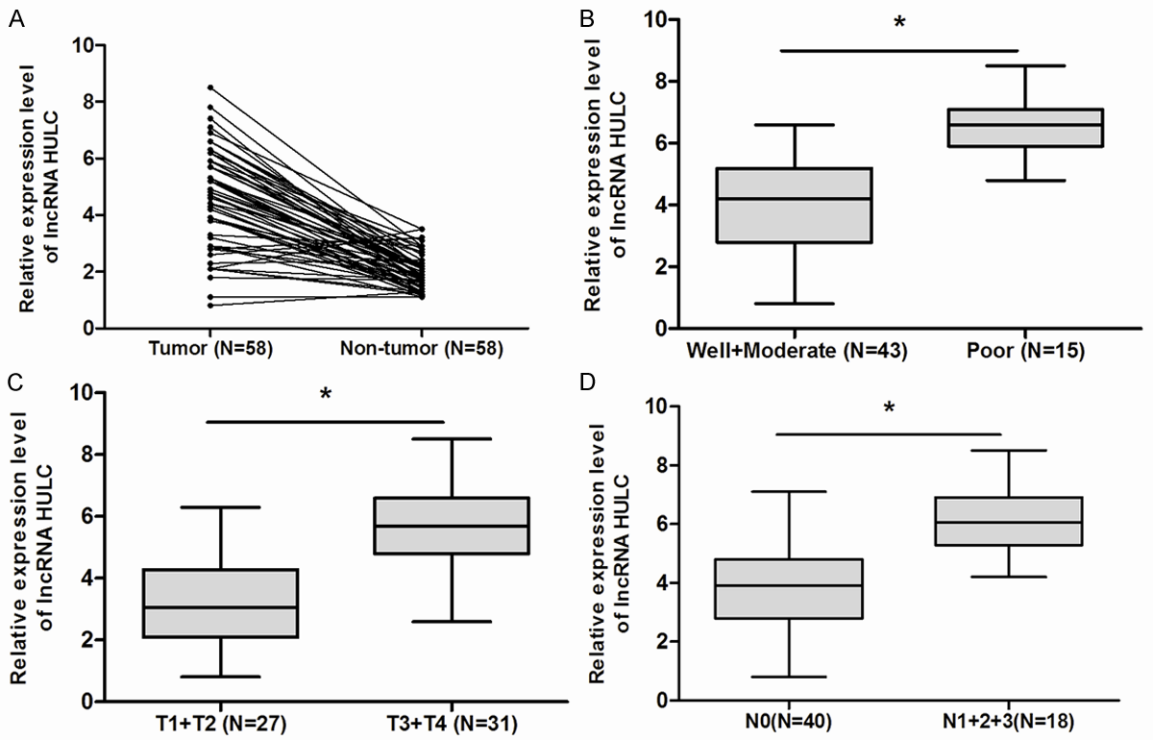

Figure 1. HULC expression in NSCLC tissues and its clinical significance. A: Relative expression of HULC in NSCLC tissues and adjacent non-tumor tissues analyzed by qRT-PCR. B: HULC expression was higher in NSCLC patients with poor tumor differentiation than those patients with well or moderate differentiation. C: HULC expression was higher in NSCLC patients in T3+T4 than those patients in T1+T2. D: HULC expression was higher in NSCLC patients with lymph-node metastasis (N1+2+3) than those patients with no lymph node-metastasis (N0). *P<0.05.

blots were incubated with primary antibodies overnight at 4°C and then with appropriate secondary antibody for 1 h at room temperature. After washing, the membranes were developed with an ECL plus western blotting detection system (Amersham).

Cell proliferation assay

Cell proliferation was measured using Cell Counting Kit-8 (CCK-8) assay kit purchased from Beyotime (Shanghai, China) according to the manufacturer's instructions. 3000 cells were seeded in a 96-well plate after transfection with siRNA for 48 h. A 10 μl CCK-8 was added to each well and incubated at 37°C for 3 h. The proliferation of cells was assessed at 24, 48, and 72 h. Absorbance values at 450 nm were detected by the microplate reader. Assays were performed in triplicate.

In vitro cell invasion migration assays

For cell migration and invasion assays, 24-well Transwell chambers with 8 μm pore size polycarbonate membrane were used (Corning). Cells were planted on the top side of the membrane pre-coated with Matrigel (BD) (without Matrigel for cell migration assay) and incubated for 24 h. Cells inside the upper chamber were obliterated with cottons swabs, while cells on the lower membrane surface were fixed and then stained with 0.5% crystal violet solution. Five fields were counted randomly in each well.

Statistical analysis

Statistical analysis was processed using SPSS 17.0 software. Experimental data were expressed as the mean ± SD. All experimental assays were performed in triplicate. The differences between groups were analyzed by the Student's t-test, ANOVA, or χ^2 test. P<0.05 was considered to be statistically significant.

Results

Expression of HULC is upregulated and correlation with clinicopathological features in NSCLC

The expression level of lncRNA HULC was examined by qRT-PCR in NSCLC tissues and adja-

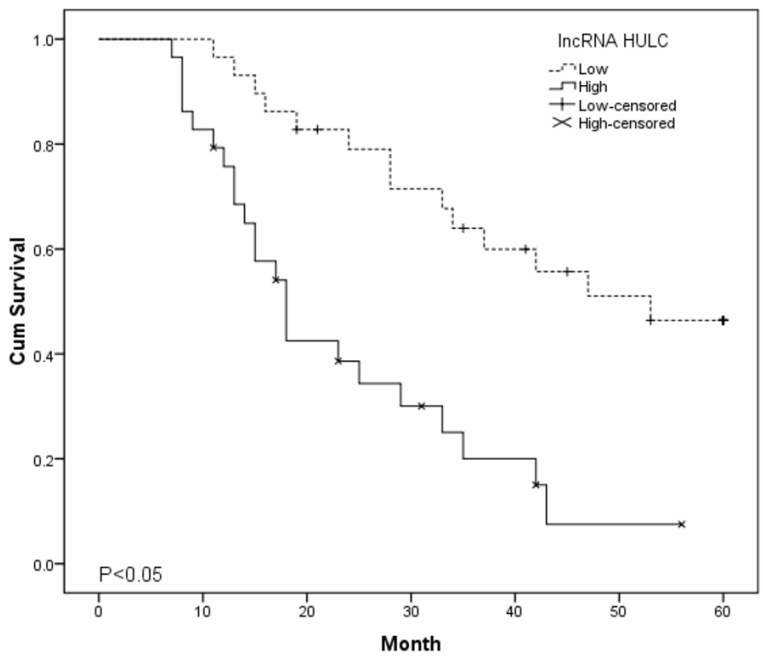

Figure 2. Kaplan-Meier curves of overall survival of patients with NSCLC stratified by HULC expression levels. Patients with high HULC levels had a shorter overall survival than patients with low HULC levels.

cent non-tumor tissues from 58 NSCLC patients. Our data revealed that the expression level of HULC was upregulated in NSCLC tissues compared to ADJACENT non-tumor tissues (P<0.05, **Figure 1A**). Moreover, we also explored the correlation of HULC expression with the clinicopathological features. We found that HULC upregulation was correlated with poor tumor differentiation, advanced pathological stage and lymph-node metastasis (P<0.05, **Figure 1B-D**). Those findings suggested that upregulated expression of HULC play critical roles in the progression and development of NSCLC.

Upregulation of HULC predicts poor prognosis of NSCLC

The Kaplan-Meier survival analysis and log-rank test was performed to detect the association between lncRNA HULC and prognosis of NSCLC patients. According to the median ratio of relative HULC expression in tumor tissues, NSCLC tissue samples were divided into the low expression group (n=29) and the high expression group (n=29). Kaplan-Meier analysis showed that patients with high HULC expression had a poorer overall survival than patients with low HULC expression (P<0.05, **Figure 2**), indicating that HULC could acted as a predictor for the overall survival of NSCLC patients.

Inhibition of HULC suppressed NSCLC cell proliferation

To understand the function of HULC in NSCLC progression, we determined HULC expression in four NSCLC cell lines via qRT-PCR. Our results showed that the expression of lncRNA HULC was upregulated in all four NSCLC cell lines (A549, SPC-A1, H23 and NCI-H520) compared to a normal human bronchial epithelial cell line (16HBE) (P<0.05, **Figure 3A**). To determine the effect of HULC expression on proliferation of NSCLC cells, si-HULC was transiently transfected into A549 and H23 cells. We found that si-HULC efficiently knocked-down HULC expression in NSCLC cells (P<0.05, **Figure 3B**). CCK8 assay showed that A549 and H23 cells transfected with si-HULC was observed to grow more slowly than those cells transfected with scrambled negative controls (si-NC) (P<0.05, **Figure 3D**). Thus, the results of our study indicated that reduce HULC expression could inhibit in vitro proliferation of NSCLC cells.

Inhibition of HULC suppressed NCSLC cell migration and invasion

To investigate the function of lncRNA HULC on the migration and invasion of NSCLC, Transwell assays were performed to determine the migratory and invasive abilities of cells after transfection of si-HULC. Transwell migration assay demonstrated that decreased expression of HULC suppressed A549 and H23 cell migration ability compared to si-NC group (P<0.05, **Figure 4A**). In addition, Transwell invasion assay revealed that the inhibition of HULC reduced the invasive ability of A549 and H23 cells compare with si-NC group (P<0.05, **Figure 4B**). These results suggested that decreased expression of HULC could inhibit NSCLC cell migration and invasion capacity in vitro.

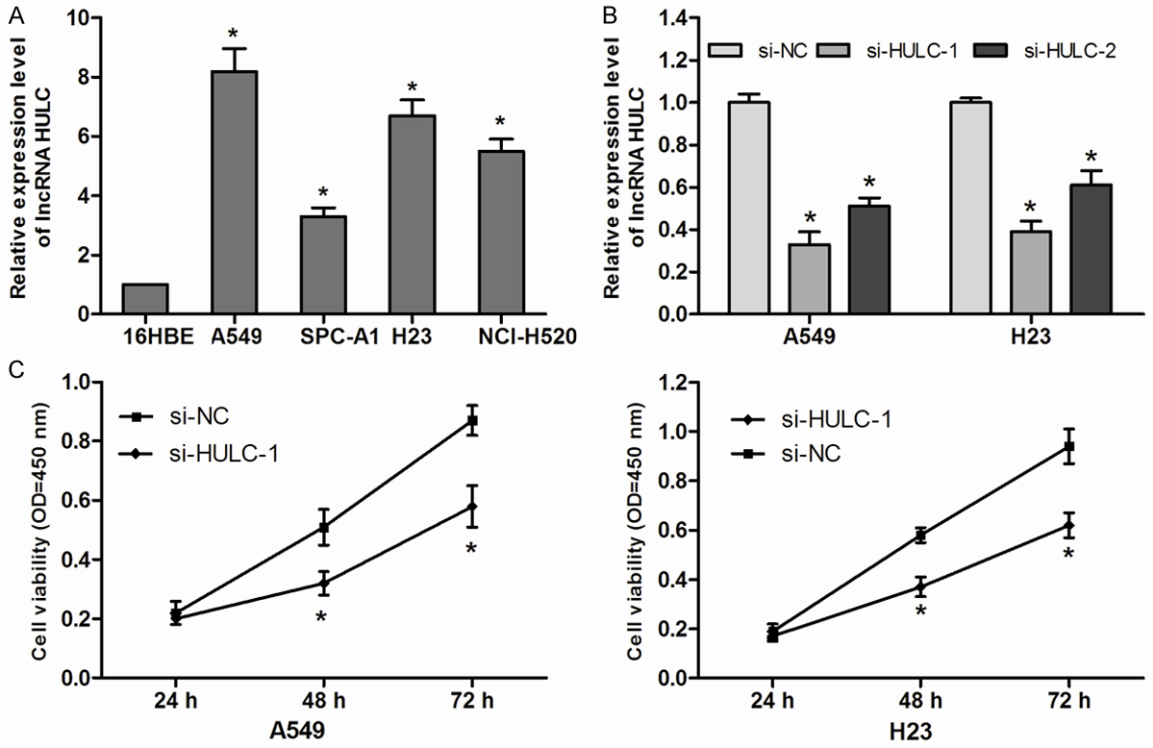

Figure 3. Effects of HULC on the proliferation of NSCLC cells in vitro. A: Expression of HULC in NSCLC cell lines (A549, SPC-A1, H23 and NCI-H520) and a normal human bronchial epithelial cell line (16HBE) was determined by qRT-PCR. B: qRT-PCR analysis of HULC expression following treatment with siRNAs targeting HULC in A549 and H23 cells. C: Inhibition HULC levels in A549 and H23 cells significantly reduced their proliferation ability as determined by CCK-8 assay. *P<0.05.

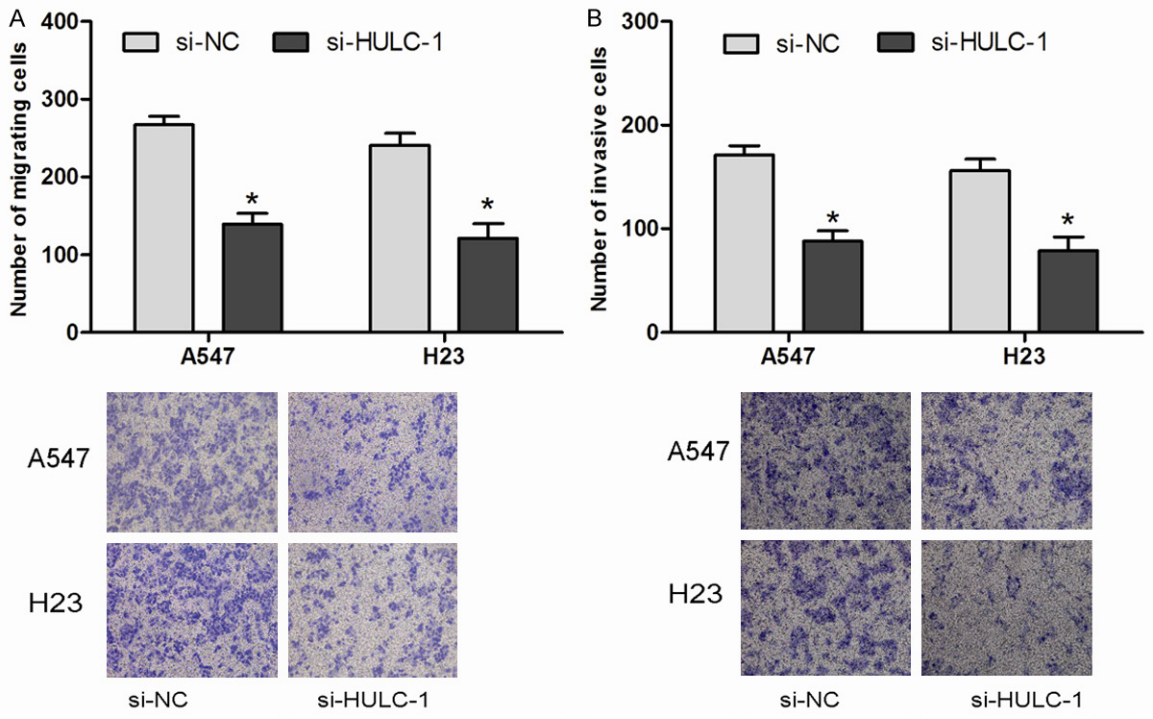

Figure 4. Effects of HULC expression on metastasis of NSCLC cells in vitro. A: Inhibition HULC levels in A549 and H23 cells significantly reduced their migration ability as determined by migration assay. B: Inhibition HULC levels in A549 and H23 cells significantly reduced their invasion ability as determined by invasion assay. *P<0.05.

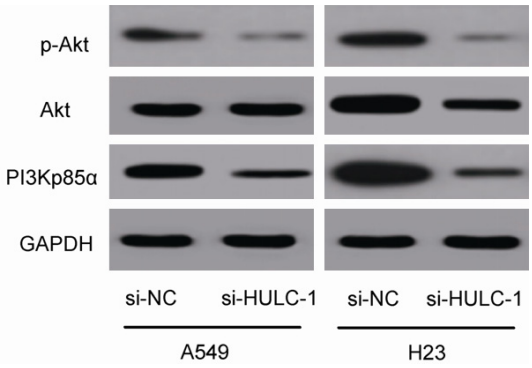

Figure 5. The effects of HULC knockdown on PI3K/Akt pathway. Western blot showed that inhibition of HULC could reduce the expression of phosphorylated PI3Kp85α and p-Akt in NSCLC cells.

Inhibition of HULC suppressed PI3K/Akt pathway in NSCLC

In order to define the possible mechanism modulated by lncRNA HULC in NSCLC, PI3K/Akt pathway which was aberrantly activated in tumors and contributed to cell proliferation and metastasis was chosen [16]. Western blot showed that reduced expression of HULC decreased the expression level of PI3Kp85α and p-Akt in NSCLC cells (**Figure 5**). These data suggested that PI3K/Akt pathway might participate in the HULC induced proliferation and metastasis of NSCLC cells.

Discussion

The role of lncRNAs in development and progression of NSCLC remains ambiguous and discovery of new specific therapeutic targets may provide effective management of disease [17]. Dysreulation of lncRNAs have been previously suggested in many types of cancer. For example, Li et al demonstrated that overexpression of lncRNA H19 enhanced carcinogenesis and metastasis of gastric cancer [18]. Shi et al showed that lncRNA ATB promotes trastuzumab resistance and invasion-metastasis cascade in breast cancer [19]. Guo et al reported that lncRNA MEG3 inhibited cell proliferation of endometrial carcinoma by repressing Notch signaling [20]. However, the role of lncRNAs in the carcinogenesis of human cancer is far from being fully elucidated.

In the present study, we first detected the expression levels of HULC in NSCLC, our data showed that HULC was significantly upregulated in NSCLC tissues compared to adjacent non-tumor tissues. By clinicopathologic features analysis, we found that HULC upregulation was associated with poor tumor differentiation, advanced pathological stage and lymph-node metastasis. Then, we explore the correlation between HULC expression and NSCLC prognosis, Kaplan-Meier analysis revealed that the overall survival of the high HULC level group was poor than the low HULC level group, suggesting that lncRNA HULC plays an important role in NSCLC progression.

To explore the roles of HULC in NSCLC progression, two cell lines (A549 and H23) with higher HULC expression were chosen. Then, we transfected si-HULC and scrambled negative controls (si-NC) into the two NSCLC cell lines. Function assay showed that downregulated expression of HULC suppressed NSCLC cell proliferation, migration and invasion ability. Thus, the current study suggested that HULC was involved in functionally important elements in the development and progression of NSCLC.

The PI3K-Akt signaling pathway regulates many normal cellular processes including cell proliferation, survival, growth, and motility-processes that are critical for tumorigenesis [21]. Aberrant activation of the PI3K/Akt pathway has been widely implicated in many cancers, including NSCLC. For example, Chen et al showed that transforming growth factor-β1 induced epithelial-to-mesenchymal transition in human lung cancer cells via PI3K/Akt and MEK/Erk1/2 signaling pathways [22]. Xu et al found that miR-7 regulated TLR9 signaling-enhanced growth and metastatic potential of human lung cancer cells by altering the PI3K/Akt pathway [23]. Pan et al suggested that lncRNA BC087858 induced non-T790M mutation acquired resistance to EGFR-TKIs by activating PI3K/AKT and MEK/ERK pathways and EMT in non-small-cell lung cancer [24]. Thus, in the present study, we investigated that whether knockdown HULC could affect the activity of PI3K/Akt pathway in NSCLC cells. Western blot showed that reduced expression of HULC significantly inhibit the expression level of PI3Kp85α and p-Akt. Thus, those findings suggested that HULC could regulate tumor proliferation and metastasis by regulate the PI3K/Akt pathway.

In conclusion, lncRNA HULC was increased NSCLC, the expression of HULC was associated with advanced clinical features and poor prognosis of NSCLC patients. Knockdown of HULC suppressed NSCLC cell proliferation, migration and invasion in vitro. Furthermore, inhibition of HULC led to the inactivation of the PI3K/Akt pathway. Therefore, HULC could act as a potential prognosis biomarker and therapeutic target in the NSCLC treatment.

Disclosure of conflict of interest

None.

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