European Review for Medical and Pharmacological Sciences 2018; 22: 8722-8730

LncRNA HULC promotes non-small cell lung cancer cell proliferation and inhibits the apoptosis by up-regulating sphingosine kinase 1 (SPHK1) and its downstream PI3K/Akt pathway

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Abstract. – OBJECTIVE: LncRNA HULC has been proved to have important functions in the pathogenesis of several types of cancers. While its involvement in non-small cell lung cancer (NSCLC), which is one of the most common malignancies, still hasn't been reported to date. Therefore, we aimed to investigate the role of HULC in NSCLC and to explore the possible mechanisms.

PATIENTS AND METHODS: Tumor tissues and adjacent healthy tissues were collected from NSCLC patients, and blood samples were collected from both NSCLC patients and healthy controls. Expression of HULU in those tissues was detected by qRT-PCR. All patients were followed up for 5 years. Diagnostic and prognostic values of serum HULU for NSCLC were investigated by ROC curve analysis and survival curve analysis, respectively. HULC overexpression NSCLC cell lines were established and its effects on cell proliferation as well as apoptosis were investigated by CCK-8 assay and MTT assay, respectively. Effects of HULC overexpression on sphingosine kinase 1 (SPHK1) and its downstream PI3K/Akt pathway were investigated by Western blot. Results: HULC expression level was increased in tumor tissues compared with adjacent healthy tissues in most patients. Serum level of HULC was higher in cancer patients than that in healthy control. Serum level of HULC was increased with the increased stage of primary tumor (T stage). Serum HULC can be used to accurately predict NSCLC and its prognosis. HULC overexpression promoted tumor cell proliferation, but inhibited cell apoptosis. HULC overexpression also increased expression level of SPHK1 and phosphorylation level of Akt in NSCLC cell, but showed on significant effects on Akt expression. Treatment with SPHK1 inhibitor and Akt reduced the effects of HULC overexpression on proliferation and apoptosis of NSCLC cells. But the treatment showed no significant effects on HULC expression. SPHK1 inhibitor treatment inhibited phosphorylation of Akt, while Akt inhibitor treatment showed no significant effects on SPHK1 expression.

CONCLUSIONS: LncRNA HULC overexpression can promote NSCLC cell proliferation and inhibit cell apoptosis by up-regulating sphingosine kinase 1 (SPHK1) and further induce the activation of its downstream PI3K/Akt pathway.

Key Words:
Non-small cell lung cancer, lncRNA HULC, PI3K/Akt pathway, Sphingosine kinase 1.

Introduction

Lung cancer is a type of malignancy that develops from lung tissues, and it is considered to be one of the major causes for cancer-related death[1]. In the United States, lung cancer affects about 234,030 new cases and causes about 154,050 deaths every year[2]. World widely, more than 1.2 million people died of this disease every year[3]. Even worse, incidence of lung cancer is predicted to be continually increased due to the worsening global environment[4]. Non-small cell lung cancer (NSCLC) is a major type of lung cancer and it accounts for more than 85 % of the cases[5]. At present, surgical resection is the only radical treatment for lung cancer, while most patients with

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lung cancer are diagnosed at advanced stages, which is not appropriate for surgical operation[6]. Therefore, the early diagnosis and treatment are pivotal to improve treatment outcomes of NSCLC. It has been well established that[7] noncoding RNAs play pivotal roles in nearly all-critical normal physiological and pathological processes in the human body. As a subgroup of noncoding RNAs, long non-coding RNA (lncRNA) is composed of more than 200 nucleotides and it participates in the onset and development of a variety of human diseases including different types of malignancies[8-10]. LncRNA HULC has been proved to play an oncogenic role in several types of cancers such as prostate cancer[11], while its involvement in NSCLC remains unclear.

Patients and Methods

Patients

A total of 102 patients (61 males and 51 females) with NSCLC were enrolled in Peking Union Medical College Hospital (PUMCH) from March 2010 to March 2012. Age of the patients ranged from 21 to 76 years, with an average age of 48±7.7 years. All patients were diagnosed by pathological and imaging tests. Patients with other lung diseases, other malignancies and mental disorders were excluded. Primary tumors were classified into different stages using the following standards: greatest dimension <= 3 cm, superficial spreading of tumor in the central airways, tumors were surrounded by visceral or lung pleura, T1; 3 cm < greatest dimension <= 7 cm, or tumor invades visceral pleura, or tumor invades the main bronchus >= 2 cm distal to the carina, or invasion is associated with atelectasis/obstructive pneumonitis extending to hilar region but not involving the entire lung, T2; greatest dimension > 3 cm, or tumor invade chest wall (including superior sulcus tumors), diaphragm, phrenic nerve, mediastinal pleura, or parietal pericardium; or tumor invade the main bronchus < 2 cm distal to the carina but without involvement of the carina, T3; tumor invades mediastinum, great vessels, heart, trachea, esophagus, vertebral body, recurrent laryngeal nerve, or carina; or separate tumor nodule(s) in a different ipsilateral lobe, T4. There were 18 cases of T1, 22 cases of T2, 34 cases of T3 and 28 cases of T4. Tumor tissues and adjacent healthy tissues were collected during surgical operations and were confirmed pathologically. A total of 32 with normal physical conditions were also enrolled at the same time to serve as controls. No significant differences in age and gender were found between patients and control group. The Ethics Committee of PUMCH approved this study, and all participants signed informed consent.

Cell Lines and Cell Culture

Human NSCLC cell lines NCI-H23 and NCI-H522 were obtained from ATCC (Manassas, VA, USA). Cells were cultured under the conditions recommended by ATCC (Manassas, VA, USA). Cells were collected during logarithmic growth phase for subsequent experiments.

Construction of HULC Overexpression Cell Lines

HULC expression vector was constructed by inserting full length HULC cDNA (V0728, GeneCopoeia, Rockville, MD, USA) into pIRSE2-EGFP vector (Clontech, Palo Alto, CA, USA). Cells of two cell lines were cultured in Eagle's Minimum Essential Medium (ATCC, Manassas, VA, USA) containing 10 % fetal bovine serum (FBS was not added in case of chemical treatment) overnight to reach 80-90% confluence. Empty vector without HULC cDNA was used as a negative control. Lipofectamine 2000 reagent (Cat. No. 11668-019; Invitrogen, Carlsbad, CA, USA; Thermo Fisher Scientific, Waltham, MA, USA) was used to transfect 10 nM vector into $4x10^5$ cells. After that, cells were cultured with (Roswell Park Memorial Institute-1640) RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences, South Logan, UT, USA) for 48 h at 37°C before the subsequent experiments.

Preparation of Serum Samples

Fasting blood (about 20 ml) was extracted from patients and healthy controls in the morning of the day just after admission. Blood was kept at room temperature for 3 h and centrifuged at 1875 rpm for 15 min to separate serum. Serum samples were stored at -80ºC before use.

Cell Proliferation Assay

Cells were collected during logarithmic growth phase to prepare cell suspension. Next, 100 μl of cell suspension were added into each well of 96-well plate, and 10 uL of CCK-8 solution were added 24, 48, 72 and 96 h later. OD values at 450 nm were measured using Fisherbrand™ accuSkan™ GO UV/Vis Microplate Spectrophotometer (Fisher Scientific, Waltham, MA, USA) after another 4 hours' cell culture.

MTT Assay

Cell suspension was diluted using medium containing 10 mM tetraethylammonium (TEA, to induce cell apoptosis) to make a final cell density of $4x10^4$ cells /ml. Next, 100 µl of cell suspension containing $4x10^3$ cells were added into each well of 96-well plate. 10 µl of MTT were added into each well at 6 h after the beginning of cell culture. Cells were cultured for another 4 h, and absorbance at 570 nm was measured using Fisherbrand™ accuSkan™ GO UV/Vis Microplate Spectrophotometer (Fisher Scientific, Waltham, MA, USA). Cell apoptosis was presented as the proportion to that of control group.

Real-Time Quantitative PCR

Total RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Before the addition of TRIzol reagent, tumor tissues and adjacent healthy tissues were ground in liquid nitrogen. NanoDrop™ 2000 Spectrophotometers (Thermo Fisher Scientific, Waltham, MA, USA) was used to test RNA quality, and RNA samples with an A260/A280 ratio between 1.8 and 2.0 were subjected to reverse transcription to synthesize cDNA. PCR reaction system was prepared using cDNA and SYBR® Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, Waltham, MA, USA). The following primers were used in PCR reactions: 5'-ACCTCCAGAACTGTGATCCAAAATG-3' (forward) and 5'-TCTTGCTTGATGCTTTGGTCTG-3' (reverse) for HULC; 5'-GACCTCTATGCCAACACAGT-3' (forward) and 5'-AGTACTTGCGCTCAGGAGGA-3' (reverse) for β-actin. PCR reaction conditions were: 95°C for 50 s, followed by 40 cycles of 95°C for 15 s and 60°C for 35 s. Ct values were processed using $2^{-\Delta\Delta CT}$ method, and relative expression level HULC was normalized to endogenous control β-actin.

WesternB

Total protein was extracted from *in vitro* cultured cells using RIPA solution (Thermo Fisher Scientific, Waltham, MA, USA). Protein samples were quantified by BCA method, followed by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis using 20 µg protein from each sample. After transmembrane, polyvinylidene difluoride (PVDF) membranes were blocked with 5 % skimmed milk for 1 h at room temperature. Membranes were then incubated with primary antibodies including rabbit anti-p-Akt (1:2000, ab38449, Abcam, Cambridge, MA, USA), anti-SPHK1(1: 2000, 46719, Abcam, Cambridge, MA, USA), and anti-GAPDH primary antibody (1: 1000, ab8245, Abcam, Cambridge, MA, USA) overnight at 4°C. After that, membranes were washed with PBS there times, 10 min each tine, and further incubated with anti-rabbit IgG-HRP secondary antibody (1:1000, MBS435036, MyBioSource, San Diego, CA, USA) at room temperature for 4 h. Membranes were incubated with ECL (Sigma-Aldrich, St. Louis, MO, USA) solution and signals were detected by MYECL™ Imager (Thermo Fisher Scientific, Waltham, MA, USA). Relative expression level of each protein was normalized to endogenous control GAPDH using image J software.

Statistical Analysis

SPSS19.0 (IBM, Armonk, NY, USA) was used to analyze the data. Chi-square test was used to analyze count data. Measurement data were expressed as ($\bar{x}\pm s$), and comparisons between two groups were performed by *t*-test. Comparisons among multiple groups were performed by analysis of variance and LSD test. $p < 0.05$ indicated a difference with statistically significance.

Results

Expression of lncRNA HULC in Tumor Tissues and Adjacent Healthy Tissues of Patients With NSCLC

The expression of HULC in tumor tissues and adjacent healthy tissues of 102 patients with NSCLC was detected by qRT-PCR. As shown in Figure 1, expression of HULC was significantly higher in tumor tissues than that in adjacent healthy tissues in 84 out of 102 patients ($p < 0.05$). Expression of HULC was significantly lower in tumor tissues compared with adjacent healthy tissues in only 5 patients ($p < 0.05$). No significant difference in expression level of HULC was found between tumor tissues and adjacent healthy tissues in 13 cases ($p > 0.05$). Those results suggest that upregulation of HULC is very likely to be involved in the pathogenesis of NSCLC.

Expression of HULC in Serum of Healthy Controls and Patients With Different Stages of NSCLC

Expression of HULC in serum of NSCLC patients with different stages of primary tumor as well as healthy controls was detected by qRT-

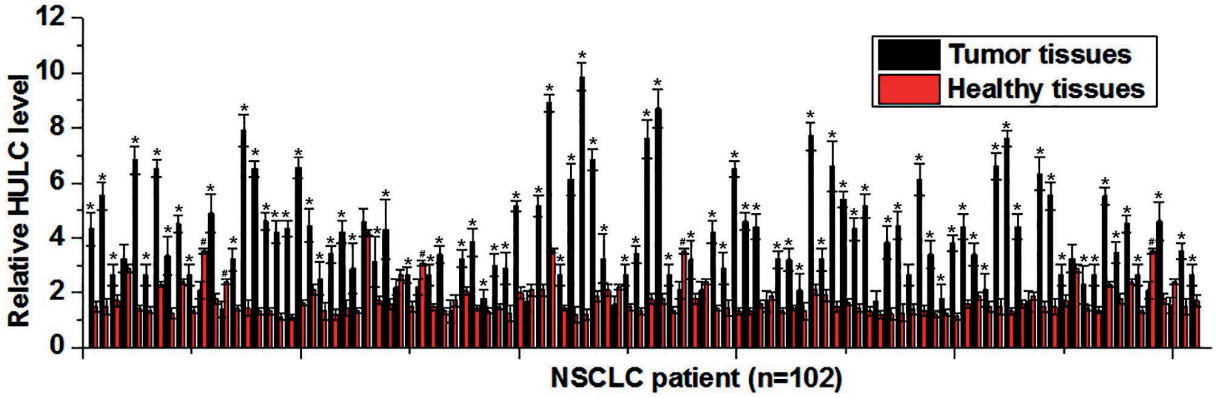

Figure 1. Expression of lncRNA HULC in tumor tissues and adjacent healthy tissues of 102 patients with NSCLC. Notes: *compared with adjacent healthy tissue, $p < 0.05$; #compared with tumor tissue, $p < 0.05$

PCR. As shown in Figure 2, serum level of HULC in was upregulated in patients with different stages of NSCLC compared with healthy controls ($p < 0.05$). In addition, expression level of HULC significantly increased with the increased primary tumor stage (T1-T4, $p < 0.05$). Those results suggest that upregulation of HULC expression is very likely to be involved in the progression of NSCLC.

Diagnostic and Prognostic Value of Serum HULC for NSCLC

ROC curve analysis showed that the area under the curve (AUC) of serum HULC in the detection of NSCLC was 0.9392 with 95 interval of 0.9020 to 0.9763 ($p < 0.0001$, Figure 3a). According to the median serum level of HUCL, patients were divided into high HULC expression group and low HULC expression group. All patients were followed-up for 5 years or until their death. Survival curves of those two groups of patients were plotted using Kaplan-Meier method and compared by log rank *t*-test. As shown in Figure 3b, the overall survival rate of patients with high expression level of HULC was significantly lower than that of patients with low expression level of HULC. Those data suggest that serum HULC may serve as a biomarker for the diagnosis and prognosis of NSCLC.

LncRNA HULC Upregulate SPHK1 Expression and Further Activate its Downstream PI3K/Akt Pathway

As shown in Figure 4a, HULC overexpression significantly increased the expression level of both SPHK1 and phosphorylation level of Akt in both NSCLC cell lines ($p < 0.05$). SPHK 1 Inhibitor 5C (5 μM, CAS 120005-55-2, Santa Cruz Biotechnology, Santa Cruz, CA, USA) is a selective inhibitor of SPHK1 and shows no significant effects on SPHK1. Treatment with 5C showed no significant effects on HULC expression but significantly promoted the phosphorylation of Akt ($p < 0.05$, Figure 4b). LY-294,002 hydrochloride (1 μM Sigma-Aldrich, St. Louis, MO, USA) is an inhibitor of PI3K/Akt pathway. Treatment with LY-294 showed no significant effects on HULC expression and phosphorylation of Akt (Figure 4c). These results indicate that HULC may serve as a positive upstream regulator of SPHK1, which can activate downstream PI3K/Akt pathway in NSCLC cells.

Effects on HULC Overexpression, SPHK1 Inhibitor and PI3K/Akt Inhibitor on Proliferation and Apoptosis of NSCLC Cells

HULC overexpression cell lines were confirmed by measuring the expression level of HULC by qRT-PCR (data not shown). As shown

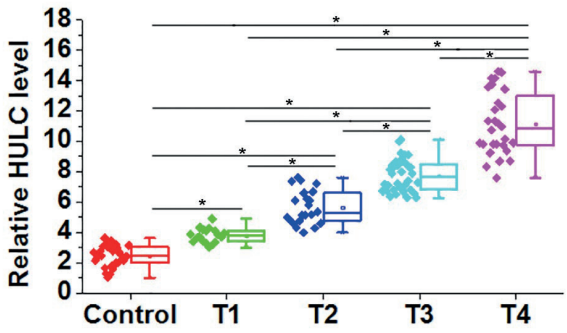

Figure 2. Expression of HULC in serum of healthy controls and patients with different stages of NSCLC. Notes: *, $p < 0.05$.

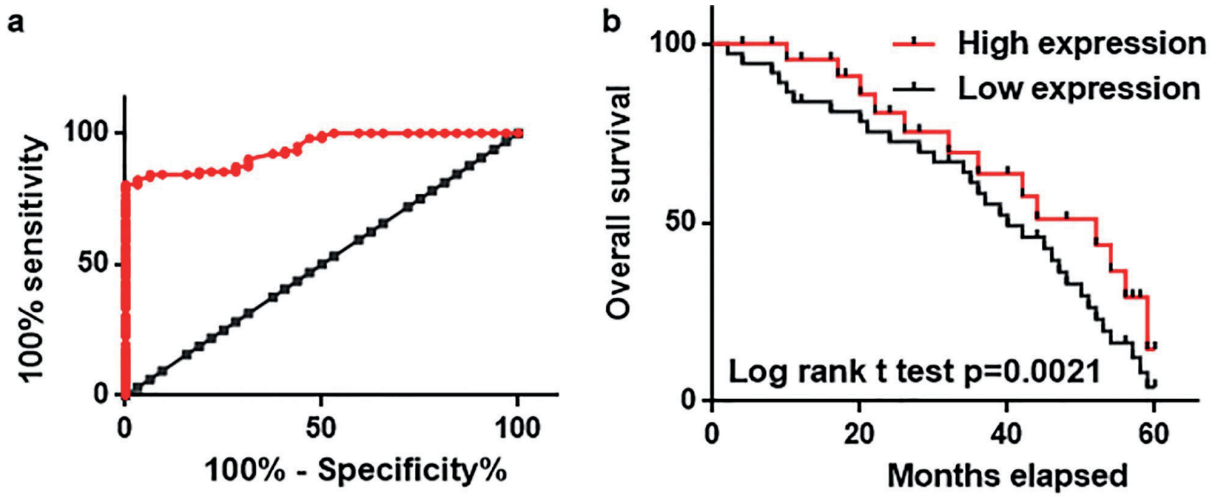

Figure 3. Diagnostic and prognostic value of serum HULC level for NSCLC. a Diagnostic values of serum HULC for NSCLC analyzed by ROC curve analysis; b Comparison of survival curves of patients with high and low serum level of HULC.

in Figure 4a, HULC overexpression promoted tumor cell proliferation, while treatments with SPHK1 inhibitor or PI3K/Akt inhibitor significantly reduced the enhancing effects of HULC overexpression on NSCLC cell proliferation ($p<0.05$). In addition, HULC overexpression also inhibited tumor cell apoptosis, while treatment with SPHK1 inhibitor or PI3K/Akt inhibitor significantly reduced the inhibitory effect of HULC overexpression on NSCLC cell apoptosis ($p<0.05$, Figure 4b). These data suggest that lncRNA HULC can promote NSCLC cell proliferation and inhibit cell apoptosis by upregulating SPHK1 expression, further activating its downstream PI3K/Akt pathway.

Discussion

HULC is generally considered to be an oncogenic long non-coding RNA with significantly upregulated expression in the onset, development and progression of different types of malignant tumor[11,12]. In the study of prostate cancer, Yu et al[12] reported that HULC expression level was significantly higher in prostate cancer tissues and cell lines than that in adjacent non-tumor tissues and normal prostate cell lines, and upregulation of HULC expression was found to be closely correlated with epithelial-mesenchymal transition in prostate cancer. Wang et al[13] showed that higher expression level of HULC was detected in patients with liver cancer compared with normal healthy controls, and the expression level of HULC was proved to be positively correlated with tumor progression. In our study, expression of HULC was significantly higher in tumor tissues than that in adjacent healthy tissues in 84 out of 102 patients while, lower expression level of HULC in tumor tissues than in adjacent healthy tissues was only observed in 5 patients. In addition, serum level of HULC was upregulated in patients with different stages of NSCLC compared with healthy controls, and expression level of HULC was further significantly increased with the increased primary tumor stage. Data demonstrate that upregulation of HULC expression is very likely to be involved in the development and progression of NSCLC. Most NSCLC lost the best timing of surgical resections by the time of diagnosis due to the lacking of classic symptoms during the early stages of disease[6]. Therefore, development of early diagnosis and treatment program is urgently demanded to improve the treatment outcomes of NSCLC. The development of human disease is usually accompanied with the changes of substance in blood, and the detection of certain substances in blood may provide references for the diagnosis and prognosis of the diseases[14]. In our study, ROC curve analysis showed that serum levels of HULC could be applied to effectively and accurately distinguish NSCLC patients from normal healthy controls. In addition, 5 years' follow-up showed that overall survival rate of patients with high expression level of HULC was significantly lower than that of patients with low expression level of HULC. Data suggest that serum HULC

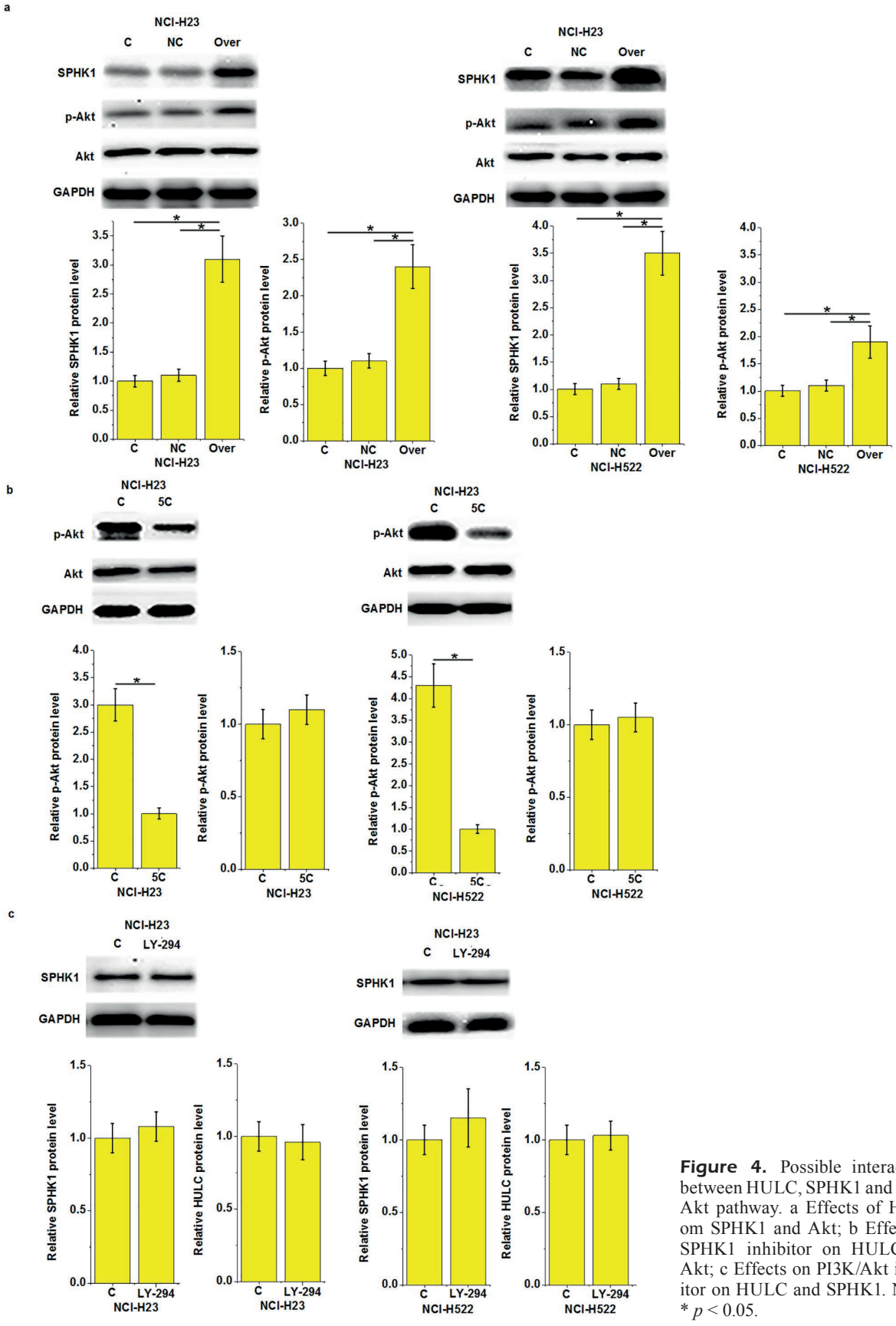

Figure 4. Possible interactions between HULC, SPHK1 and PI3K/Akt pathway. a Effects of HULC om SPHK1 and Akt; b Effects of SPHK1 inhibitor on HULC and Akt; c Effects on PI3K/Akt inhibitor on HULC and SPHK1. Notes: * $p < 0.05$.

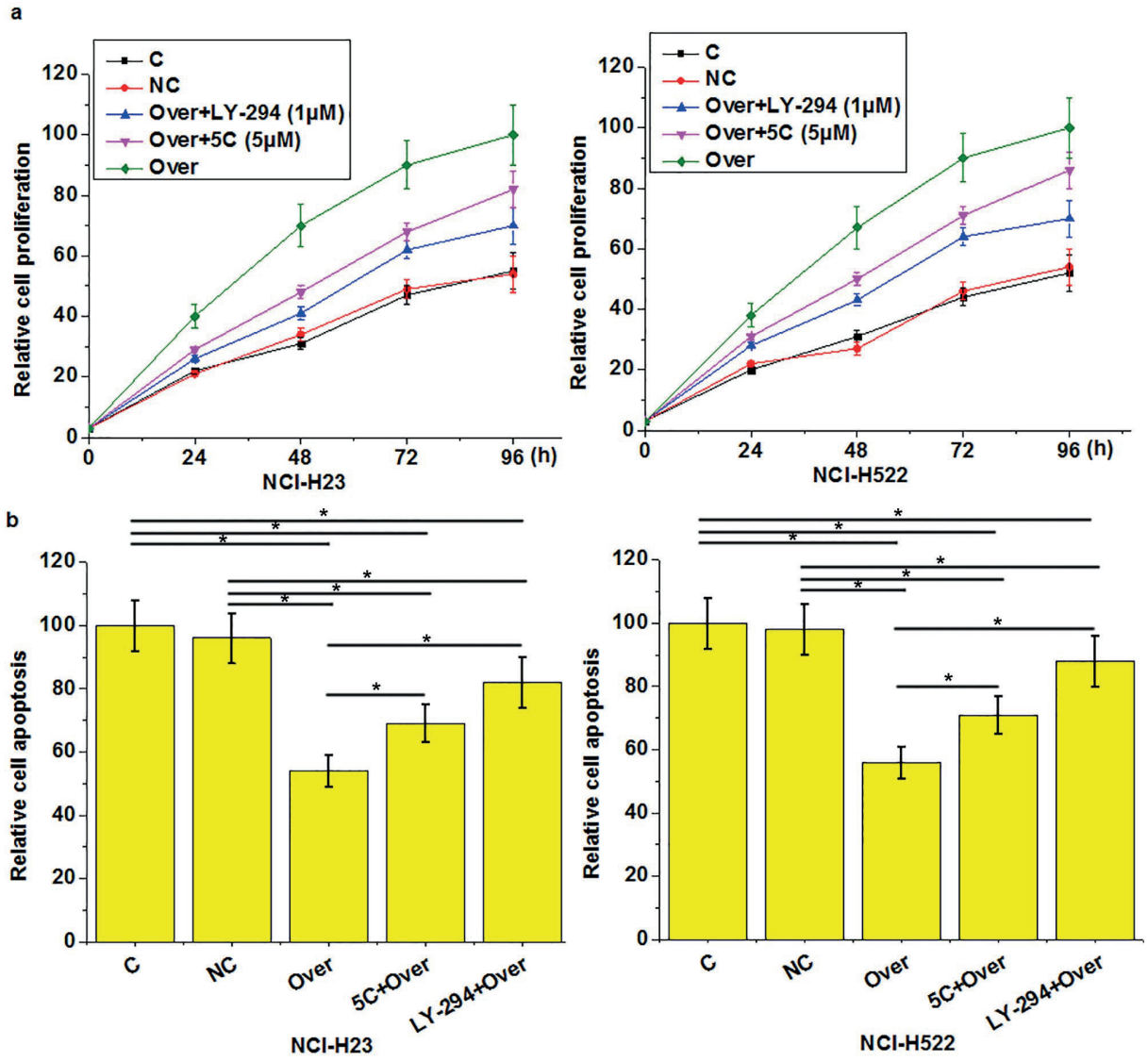

Figure 5. Effects on HULC overexpression, SPHK1 inhibitor and PI3K/Akt inhibitor on proliferation and apoptosis NSCLC cells. a Cell proliferation of different cell lines with different treatment; b Cell apoptosis of different cell lines with different treatment. Notes: *, $p < 0.05$.

may serve as a biomarker for the diagnosis and prognosis of NSCLC. However, it has been reported that HULC has diagnostic and prognostic values for multiple malignancies, which may affect its specificity. Therefore, combination of multiple biomarkers is needed to improve the diagnosis and prognosis of NSCLC. HULC is an oncogenic long non-coding RNA that not only promotes tumor cell proliferation but also inhibits tumor cell apoptosis in different malignancies such as bladder cancer[16], while inhibition of HULC expression promotes tumor cell apoptosis and inhibits proliferation of the tumor cell[17]. In our study, HULC overexpression significantly increased the proliferation rate and reduced the apoptosis rate of two NSCLC cell lines. As a sphingosine kinase, SPHK1 is usually overexpressed in tumor tissues to promote tumor development[18]. Activation of PI3K/AKT signaling is also critical to the growth and metastasis of different malignancies[19]. HULC has been proved to participate in liver cancer by up-regulating SPHK1[20], and SPHK1 activates PI3K/AKT signaling in breast cancer[21]. In our study, HULC overexpression significantly increased both the expression level of SPHK1 and phosphorylation

level of Akt. Treatment with SPHK1 inhibitor 5C showed no significant effects on HULC expression but significantly promoted the phosphorylation of Akt, while treatment with PI3K/Akt inhibitor LY-294,002 hydrochloride showed no significant effects on HULC expression and phosphorylation of Akt. Besides that, treatment with SPHK1 inhibitor or PI3K/Akt inhibitor significantly reduced the effects of HULC overexpression on NSCLC cell proliferation and apoptosis. Those data suggest that lncRNA HULC can promote NSCLC cell proliferation and inhibit cell apoptosis by upregulating SPHK1 expression and further activating its downstream PI3K/Akt pathway.

Conclusions

We found that HULC expression was upregulated in NSCLC tissues and serum of patients with NSCLC. Serum level of HULC was increased with the increased stage of primary tumor. Serum HULC can be used to accurately predict NSCLC and its prognosis. HULC overexpression promoted tumor cell proliferation, but inhibited cell apoptosis. HULC overexpression also increased expression level of SPHK1 and phosphorylation level of Akt in NSCLC cell, but show on significant effects on Akt expression. Treatment with SPHK1 inhibitor and Akt reduced the effects of HULC overexpression on proliferation and apoptosis of NSCLC cells, but showed no significant effects on HULC expression. SPHK1 inhibitor treatment inhibited phosphorylation of Akt, while Akt inhibitor treatment showed no significant effects on SPHK1 expression. Therefore we conclude than LncRNA HULC overexpression can promote NSCLC cell proliferation and inhibit cell apoptosis by up-regulating SPHK1 and further induce the activation of its downstream PI3K/Akt pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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