Phenotypic Changes of LncRNA Hotair in Non-Small-Cell Lung Cancer and Its Clinical Application

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Received 1 September 2021; Revised 12 October 2021; Accepted 18 October 2021; Published 5 November 2021

1. Introduction

Non-small-cell lung cancer (NSCLC) is one of the most common malignant tumors of the respiratory system. The main causes of death are smoking, low air quality, gene mutation, and other adverse factors, and its new cases in the world are increasing year by year, especially in China [1, 2]. Although some progress has been made in clinical and experimental research, the long-term prognosis of NSCLC patients is still very poor due to late discovery of the disease, high recurrence rate, and lack of an effective treatment intervention for advanced tumors. Previous studies have revealed many dysregulated genes and signaling pathways associated with NSCLC, but the highly complex molecular mechanism of its carcinogenesis and progression is still unclear [3]. Therefore, it is urgent to find reliable biomarkers for early diagnosis, effective treatment, and prognosis evaluation of NSCLC.

Long non-coding RNA (lncRNA) is a subcategory of the nontranslated RNA sequence defined by more than 200 base pairs of any length [4, 5]. Studies have found the physical location and protein coding of most lncRNAs. The genes are very close, and they can be further classified according to the order of coding and the positional relationship. They are divided into intergenic lncRNA, antisense lncRNA, and other subtypes [6, 7]. The change of lncRNA content in plasma, serum, or urine has become an emerging technology for noninvasive diagnostic applications [8, 9]. For example, plasma lncRNA-Pou3f3 can be used as a potential biomarker for the diagnosis of rectal cancer, especially early tumor screening [10]. LncRNA Loc389332 was found to be significantly increased in the plasma and urine of patients with renal clear cell carcinoma, which can be used to
distinguish cancer patients from the health examination group
[11]. LncRNA Hotair is composed of 2158 nucleotides, located
between Hoxc11 and Hoxc12 on chromosome 12q13.135 [12].
It can bind to PRC2, resulting in histone methylation and
transcriptional silencing [13]. Studies have shown that, in oral
squamous cell carcinoma, LncRNA Hotair overexpression
enhances the metastatic potential and epithelial mesenchymal
transformation characteristics of cancer cells. It is also found
that LncRNA Hotair expression is positively correlated with
mesenchymal markers and negatively correlated with epithelial
markers in clinical samples [14, 15]. In addition, LncRNA
Hotair can realize its function by reprogramming the chro-
mosome state to control the expression of multiple genes
represented by Hoxc10; Hoxc10 has been proved to be a gene
markers in clinical samples [14, 15]. In addition, LncRNA
Hotair overexpression enhances the metastatic potential and epithelial
between Hoxc11 and Hoxc12 on chromosome 12q13.135 [12].

Based on the conclusions of previous studies, this paper
further studied the relationship between the expression level and
pathological parameters of LncRNA Hotair in clinical samples
of NSCLC and the related mechanism of exogenous LncRNA
Hotair on cell proliferation, migration, and invasion, so as to
fully grasp the role of LncRNA Hotair in NSCLC. It provides
valuable clinical auxiliary reference for the treatment of NSCLC.

2. Materials and Methods

2.1. Patient Samples and Materials. During the research
process, 62 patients with NSCLC in the hospital from January
2018 to December 2020 were selected from cancer tissues and
adjacent paracancerous tissue specimens. In the course of the
study, all patients had not received chemotherapy or targeted
therapy before surgery and signed an agreement to participate
in the experiment with the patients. This study was approved by
the ethics committee of Shanghai General Hospital, Shanghai
Jiao Tong University, School of Medicine. Clinical data in-
cluding age, sex, smoking history, stage, and lymph node
metastasis of these patients were collected. All specimens were
placed in liquid nitrogen for cryopreservation after sampling.
The lung cancer cells used can be divided into an adenocar-
cinoma cell line (A549, SPC-A1), squamous cell carcinoma cell
line (SK-MES-1), and human normal bronchial epithelial cell
line, and they were purchased from the Institute of Bio-
chemistry and Cell Biology, Chinese Academy of Sciences,
Shanghai, China. RPMI 1640 medium, DMEM medium, 10%
fetal bovine serum, penicillin, and streptomycin were added
to the culture medium. (“The absorbance was measured at 450nm using
a microplate spectrophotometer.”

2.2. Cell Culture. In the experiment, DMEM medium,
RPMI1640 medium, fetal bovine serum (10%), and cell
culture medium were used for sample cell culture, and 10%
fetal bovine serum, penicillin, and streptomycin were added
to the culture medium. The cells of the small cell lung cancer
tissue sample were cultured at 37 degrees Celsius and 5%
of cells in each group were observed under a fluorescence microscope.

2.8. Apoptosis Was Detected by TUNEL and Merge Staining. SPC-A1 cells in the logarithmic growth stage were treated with $1 \times 10^5/L$ and inoculated into 96-well plates, $100 \mu L$ per well, cultured in a $37^\circ C$ incubator for 24 hours, and the culture was terminated after the cells adhered to the wall and continued to be cultured at $37^\circ C$ for 48 hours, 4% paraformaldehyde was fixed for 30 min, and then cleaned twice with PBS, equilibrium buffer was added and incubated at room temperature for 5 min, finally $50 \mu L$ reaction buffer was added, incubated in the dark for 60 min, and centrifuged with a centrifuge, the supernatant was discarded and $5 \times$ Wash with $10-3 \mathrm{mg} / \mathrm{L} BSA$ was added, and the morphological changes of cells were observed under a fluorescence microscope and photos were taken.

2.9. Western Blot. The total protein of cells in each group after 48 hours of transfection was extracted and quantified according to each $1 \mu L$ sample added $0.25 \mu L$. The ratio of sample buffer was mixed, boiling water heating denatured 5 min, separated by SDS-PAGE gel electrophoresis, and migrated to an nitrocellulose membrane, and 5g defatted milk powder and $100 \mathrm{~mL}$ TBST SDS-PAGE gel electrophoresis, and migrated to an nitrocellulose membrane, and 5g defatted milk powder and $100 \mathrm{~mL}$ TBST were added and placed in an oscillating incubator at $26^\circ C$ closed for 2 h. The primary antibodies used in this study include Ki-67, proliferating cell nuclear antigen (PCNA), Bax, Bcl-2, matrix metalloproteinase-3 (MMP-3), vascular endothelial growth factor (VEGF), epithelial cadherin (E-cadherin), vimentin, and GAPDH. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody for 1 h at room temperature. Then, the antibody-bound proteins were detected using the ECL system.

2.10. Statistical Analysis. After completing the experiment, based on the quantitative detection and recorded experimental data, all statistical analyses were performed using SPSS software package version 22.0 (SPSS, Chicago, Illinois, USA). All graphics were drawn by Graphpad prism 9.0 software (GraphPad software, La Jola, California). A paired-sample $t$-test was used to compare the difference of Hotair expression in paired tissues of NSCLC patients. Other experimental data were compared by an independent $t$-test; * represents $<0.05$, ** represents $<0.01$, and *** represents $<0.001$.

3. Results

3.1. Higher Expression of LncRNA Hotair in NSCLC Tissue. In order to investigate the expression of LncRNA Hotair in NSCLC patients, we first performed qRT-PCR to determine the expression level of LncRNA Hotair in NSCLC and adjacent tissues. In the independent study group of 62 original NSCLC patients, the expression level of LncRNA Hotair was $(25.30 \pm 2.23)$ in NSCLC tissues and $(3.52 \pm 0.29)$ in adjacent tissues (Figure 1). The expression level of LncRNA Hotair was significantly higher than that of adjacent tissues ($P<0.0001$).

3.2. Correlation between Clinicopathological Parameters and Relative Expression of LncRNA Hotair. In order to further study the role of LncRNA Hotair in the occurrence and development of NSCLC, this study statistically analyzed the relationship between serum LncRNA Hotair level and clinicopathological parameters in patients with NSCLC. The results showed that there was no significant correlation between serum LncRNA Hotair level and gender, age, and smoking ($P>0.05$). The serum LncRNA Hotair level of lung cancer patients with tumor size $>3 \mathrm{~cm}$ was significantly higher than that of patients with tumor size $\leq 3 \mathrm{~cm}$, the serum LncRNA Hotair level of patients with stage III + IV NSCLC was significantly higher than that of patients with stage I + II, and the serum LncRNA Hotair level of NSCLC patients with lymphatic metastasis was significantly higher than that of patients without lymphatic metastasis ($P<0.001$). The results are shown in Table 1.

3.3. Expression Level of Exogenous LncRNA Hotair in NSCLC Cells and Changes in Silencing and Overexpression Levels. The expression content of LncRNA Hotair in NSCLC tissues was higher than that in adjacent tissues. It can be seen from the table that the relative expression level of SPC-A1 and SK-MES-1 cell lines is higher than that of normal cell lines, and the relative expression level of A549 cell line is lower than that of normal cell lines, as shown in Figure 2(a). In order to further verify the correlation between the expression level of LncRNA Hotair, the expression level of exogenous LncRNA Hotair in NSCLC cells was observed by transfection and overexpression, and an A549 cell line with the lowest expression and an SPC-A1 cell line with the highest expression were selected for verification. Also, from the histogram of the relative expression level, the relative expression level showed an obvious difference, which was more obvious in the Hotair group. Based on the abovementioned analysis, it can be clearly seen that, after 2 days of transfection with siRNA, the expression level of LncRNA Hotair in SK-MES-1 and SPC-A1 cells was lower than before transfection ($P<0.05$). RNA interference technology can inhibit the proliferation of NSCLC cells by hindering the expression level of LncRNA Hotair. Transfection of pcDNA3.1-Hotair promoted the expression of Hotair and inhibited the proliferation of NSCLC cells. After 2 days of transfection with pcDNA3.1-Hotair, the expression level of LncRNA Hotair in SK-MES-1 and SPC-A1 cells was higher than before transfection (Figures 2(b) and 2(c)).

3.4. Downregulation of LncRNA Hotair Suppressed Cell Proliferation and Invasion and Promoted Apoptosis of NSCLC Cells. In order to study the role of LncRNA Hotair in NSCLC, siRNA was used to specifically downregulate the expression of LncRNA Hotair. Because the expression of LncRNA Hotair in SPC-A1 and SK-MES-1 cell lines was higher, we chose the SPC-A1 cell line for the next experiment. We found that compared with NC and anti-NC groups, LncRNA Hotair downregulated inhibited cell
proliferation and promoted apoptosis, and DAPI staining showed that the tumor cells in the Si-Hotair group and Si-Hotair + anti-NC group showed shrinkage, dense cytoplasm, edge collection of nuclear chromatin, nuclear lysis, and formation of apoptotic bodies containing nuclear fragments and organelles. The downregulation of LncRNA Hotair suppressed cell proliferation (Figure 3(a)). TUNEL and Merge staining showed that the proportion of green fluorescence positive cells in the Si-Hotair group and Si-Hotair + anti-NC group increased gradually, indicating that the proportion of apoptosis increased gradually (Figures 3(b) and 3(c)). Si-Hotair also inhibited the invasive ability of SPC-A1 cells (Figures 3(d) and 3(e)). Si-Hotair decreased the expression of proliferation-related proteins Ki-67 and proliferating cell nuclear antigen (PCNA), E-cadherin and vimentin, anti-apoptotic protein Bcl-2, and invasion-related protein matrix metalloproteinase-3 (MMP-3) and vascular endothelial growth factor (VEGF), while the expression of proapoptotic protein Bax increased significantly (Figures 4(a)–4(c)). In addition, the expression of epithelial marker E-cadherin was upregulated, while the expression of mesenchymal marker vimentin was downregulated. Si-Hotair could inhibit epithelial mesenchymal like phenotypic transformation (EMT) in NSCLC (Figure 4(d)). In conclusion, our results showed that the downregulation of LncRNA Hotair inhibited cell proliferation and invasion and promoted the apoptosis of NSCLC cells.

**Figure 1:** LncRNA Hotair expression was significantly increased in human NSCLC tissues compared to normal lung tissues.

**Table 1:** Relationship between clinicopathological parameters and serum Hotair level in NSCLC.

| Clinical pathological parameters | Cases (n) | Relative expression of Hotair (mean ± SD) | t  | P   |
|---------------------------------|----------|------------------------------------------|----|-----|
| Age (yr)                        |          |                                          |    |     |
| >65                             | 21       | 2.87 ± 0.74                              | 1.901 | 0.061 |
| ≤65                             | 41       | 3.27 ± 0.79                              |     |     |
| Gender                          |          |                                          |    |     |
| Male                            | 28       | 2.81 ± 0.72                              | 0.528 | 0.600 |
| Female                          | 34       | 2.91 ± 0.70                              |     |     |
| Smoking                         |          |                                          |    |     |
| Yes                             | 29       | 3.17 ± 0.84                              | 0.194 | 0.847 |
| No                              | 33       | 3.21 ± 0.65                              |     |     |
| Tumor size                      |          |                                          |    |     |
| >3 cm                           | 37       | 2.98 ± 0.82                              | 4.685 | <0.001 |
| ≤3 cm                           | 25       | 2.20 ± 0.11                              |     |     |
| TNM stage                       |          |                                          |    |     |
| I + II                          | 33       | 2.86 ± 0.64                              | 4.280 | <0.001 |
| III + IV                        | 29       | 2.31 ± 0.25                              |     |     |
| Lymph node metastasis           |          |                                          |    |     |
| Negative                        | 30       | 3.32 ± 0.47                              | 7.308 | <0.001 |
| Positive                        | 32       | 2.60 ± 0.29                              |     |     |
Figure 2: Expression level of exogenous LncRNA Hotair in NSCLC cells and changes in silencing and overexpression levels. (a) Relative expression values of LncRNA Hotair in different cell lines; (b) expression of LncRNA Hotair after transfection; and (c) expression of pcDNA3.1-Hotair after transfection.

Figure 3: Continued.
4. Discussion

Lung cancer is the leading cause of cancer-related death, and NSCLC accounts for 85% of all lung cancer patients. In recent years, with the development of molecular biology of lung cancer, many new drugs have appeared, but the 5-year survival rate of patients with advanced lung cancer is still only 15% [17]. In recent years, it has been found that the abnormal expression of lncRNA is closely related to human diseases and is involved in the process of tumor occurrence, growth, invasion, metastasis, recurrence, and drug resistance [18–20].

LncRNA Hotair is the transcriptional antisense RNA of HOX. It is the first LncRNA recognized to have transcriptional regulation and related to malignant tumors. Studies have shown that LncRNA Hotair mainly interacts with Polycomb repressive complex 2 (PRC2) to regulate chromosome rearrangement and promote tumorigenesis[21]. Duan, et al. Found that LncRNA Hotair was highly expressed in liver cancer tissues and promoted
hepatocarcinogenesis by inhibiting the expression of tumor suppressor genes; conversely, downregulating LncRNA Hotair expression can inhibit the growth and migration of hepatoma cells [22]. It was found that the overexpression of Hotair in gastric cancer can promote lymph node metastasis, EMT progression, and vascular invasion of gastric cancer [23]. The overexpression of LncRNA Hotair is the main marker of poor prognosis in patients with gastric cancer. In human glioma, LncRNA Hotair can affect the progression of glioma by affecting the expression of inflammatory factors and VEGF [24]. Therefore, LncRNA Hotair explored the biological behavior and possible mechanism of NSCLC cells. The results showed that LncRNA Hotair was abnormally highly expressed in NSCLC cell lines; transfection knockdown of LncRNA Hotair expression significantly inhibited the proliferation, migration, and invasion of NSCLC cells. It is suggested that LncRNA Hotair has the role of oncogene and can promote the proliferation, migration, and invasion of cancer cells.

From the experimental results, it can be seen that the relative expression level of LncRNA Hotair in SPC-A1 and SK-MES-1 cells is higher than that in normal bronchial epithelial cells (P < 0.05); the relative expression level in A549 cells was lower than in normal bronchial epithelial cells (P < 0.05). After 2 days of transfection with siRNA, the expression level of LncRNA Hotair in A549 and SPC-A1 cells was lower than before transfection (P < 0.05). DAPI/TUNEL/Merge experiment showed that Si-Hotair could gradually increase the proportion of apoptosis. At the same time, through cell proliferation comparison experiments, it was found that, after 2 days of transfection with pcDNA3.1-Hotair, the expression level of LncRNA Hotair in A549 cells was higher than before transfection (P < 0.05). Transfection of pcDNA3.1-Hotair to promote the expression of Hotair can inhibit the proliferation of NSCLC cells. On this basis, we conducted a series of studies on the phenotypic changes of NSCLC regulated by LncRNA Hotair. Compared with the Si-NC group and NC group, the number of invasion and migration of SPC-A1 cells in Si-Hotair group decreased significantly, and Si-Hotair decreased the expression of proliferation-related proteins (Ki-67 and PCNA), anti-apoptotic protein Bcl-2, and invasion-related proteins (MMP-3 and VEGF), while the expression of proapoptotic protein Bax increased significantly. We found that the expression of E-cadherin was upregulated and vimentin was downregulated and then speculated that Si-Hotair inhibited the development of EMT. Therefore, a series of experimental results show that Si-Hotair can inhibit tumor occurrence and metastasis by regulating the content expression in NSCLC cells. In conclusion, the experimental results show that LncRNA Hotair has a certain impact on the proliferation, migration, and apoptosis of NSCLC tissues and cells, which has a very important reference value for the treatment and early detection of NSCLC.

Data Availability

The simulation experiment data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

Authors’ Contributions

Haihua Huang and Jin Wang contributed equally to this work.

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