Effects of miR-221 on the apoptosis of non-small cell lung cancer cells by IncRNA HOTAIR

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Abstract. OBJECTIVE: The aim of this study was to investigate the effects of miR-221 on the proliferation of non-small cell lung cancer (NS-CLC) cells through long non-coding RNA (lncRNA) HOX transcript antisense RNA (HO-TAIR), and to explore the possible underlying mechanism.

PATIENTS AND METHODS: Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was applied to detect the expression level of HOTAIR in 38 NSCLC patients. The correlations of HOTAIR expression with clinic-pathological features, as well as the correlation between HOTAIR expression and miR-221 expression was analyzed by RT-PCR. Furthermore, NSCLC cell lines were cultured in vitro, and the expressions of HOTAIR and miR-221 in NSCLC cells were also detected. A549 cells were transfected with miR-221 mimics, miR-221 inhibitors, HOTAIR-small interfering RNAs (siRNAs) and plasmid cytomegalovirus deoxyribonucleic acid (pcDNA)3.1-HOTAIR. The interaction between miR-221 and HOTAIR in transfected cells was analyzed via RT-PCR and Northern blotting. Ultimately, flow cytometry was adopted to analyze the effects of miR-221 in the apoptosis of NSCLC cells through HOTAIR.

RESULTS: The expression of HOTAIR in tissues of clinical patients only exhibited a correlation with the stage of cancer. The expressions of HOTAIR in patients with stage I and II were remarkably lower than those with stage III and IV. Additionally, the expression of HOTAIR was negatively correlated with the expression of miR-221 ($r$=-0.7651, $p<0.0001$). Further studies revealed that there was a negatively regulatory interaction between miR-221 expression and HOTAIR expression. Apoptosis assay results manifested that miR-221 significantly promoted the apoptosis of NSCLC cells by negatively regulating HOTAIR expression.

CONCLUSIONS: MiR-221 promotes the apoptosis of NSCLC cells through negative regulation of IncRNA HOTAIR, which can be used in the treatment of NSCLC.

Key Words: MiR-221, HOTAIR, Non-small cell lung cancer (NS-CLC), Apoptosis.

Introduction

According to the analysis of whole-genome sequencing and high-throughput transcriptome in recent years, a large proportion of human genomes can be transcribed into numerous short or long non-coding ribonucleic acids (IncRNAs)1,2. Micro RNAs (miRNA) have attracted attention among different types of ncRNAs. It has been shown that silencing after the transcription of specific target messenger RNAs (mRNAs) exerts various important effects on cancer3. MiRNA-221 (miR-221), as one of the most abundant miRNA species in human brain4, plays a vital role in cell survival5,6, apoptosis7-10 and neurogenesis11. Recent studies have indicated that the expression of miR-221 in melanoma is closely related to pigmentation and invasion ability12. Besides, two independent miRNA studies in patients with peritoneal dialysis have verified that reduced level of miR-221 in serum can be regarded as a biomarker for the diagnosis and evaluation of disease staging13.

Nevertheless, a newly discovered type of IncRNAs is one of the emerging fields of ncRNA research. The involvement of IncRNAs in a wide range of biological processes has been extensively studied14. Multiple evidence has verified that IncRNA disorders are related to diversified human diseases, especially cancer. Several studies have manifested that many IncRNAs may be correlated with chromatin-modified complexes. Therefore, this can affect epigenetic information and confer multiple properties that are needed for tumor progression and metastatic phenotype15,16. HOX transcript antisense RNA (HOTAIR) is one of the few well-recorded IncRNAs with a length of 2158 bp, which exerts a crucial role in trans-silencing17. It has been confirmed that HOTAIR is a negative prognostic indicator for the survival
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rate of patients with breast cancer, colon cancer, liver cancer and pancreatic cancer. Meanwhile, HOTAIR expression is significantly increased in patients with breast cancer and colon cancer, which is related to increased metastasis. Furthermore, knockdown of HOTAIR suppresses cell invasion and proliferation, changes cell cycle progression and induces cell apoptosis, indicating that HOTAIR directly regulates the progression of cancer.

However, the effects of miRNA and HOTAIR on the development or metastasis of non-small cell lung cancer (NSCLC) remain unclear. In this study, therefore, we investigated the effects of miR-221 and IncRNA HOTAIR on the apoptosis of NSCLC.

Patients and Methods

Main Reagents and Equipment

One-step Reverse Transcription-Polymerase Chain Reaction (RT-PCR) kits were purchased from Beijing CW Biotech Co., Ltd. (Beijing, China); SYBR® Premix Ex Taq™ GC (Perfect Real Time) kits were obtained from Hunan Oracle Biomedical Co., Ltd. (Changsha, Hunan, China); HOTAIR small interfering RNAs (siRNAs) were purchased from Beijing OriGene Biotech Co., Ltd. (Beijing, China); Primer-Script™ one-step RT-PCR kits and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, USA); GeneScreen Plus membranes were purchased from PerkinElmer; UltraHyb-Oligo buffer was obtained from Ambion (Austin, TX, USA); Typhoon 9410 variable imager was purchased from GE (Little Chalfont, Buckinghamshire, UK); CELL Quest software was obtained from BD Biosciences (Franklin Lakes, NJ, USA).

Cell Lines and Patients

NSCLC cell lines (A549, H322 and H1299) and normal lung cell line (BEAS-2B) were purchased from Shanghai BioLeaf Co., Ltd. (Shanghai, China). All cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 37°C, 5% CO2 incubator. All specimens of NSCLC tissues and adjacent normal tissues were collected 38 NSCLC patients who received treatment in the Affiliated Jiangyin Hospital of Southeast University Medical College. Clinicopathological features of 38 patients, including age, gender, cancer stage and vascular infiltration, were collected and statistically analyzed. Obtained tissue samples were quickly frozen at -80°C for storage. This study was approved by the Ethics Committee of Affiliated Jiangyin Hospital of Southeast University Medical College. The signed written informed consents were obtained from all participants before the study.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Detection

Total RNA was extracted in accordance with the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into complementary deoxyribonucleic acid (cDNA). The expression levels of HOTAIR gene, miR-221 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were detected via Real Time-Polymerase Chain Reaction (RT-PCR) amplification using the SYBR® Premix Dimmer Eraser kit. Then the mRNA expression levels were normalized to GAPDH. Real-time PCR amplification was carried out using the ABI7500 system. The fold changes in the relative expression of mRNA were calculated by the 2-ΔΔCt method. Primers used in this study were as follows: HOTAIR, F: 5’-CCAG-GAGCCCTCCTTACTC-3’, R: 5’-ATCTAGGG-GTGTCGAAAGGA-3’; miR-221, F: 5’-AGGGGTTGTAACATCCTCGA-3’, R: 5’-TATTGG-CGGTCGAGGATGCG-3’; U6: F: 5’-GGTAGCGTGTCAT-3’, R: 5’-GCTTCGGCAGCACATATAACTAAAAT-3’. Construction of Plasmid

Cytomegalovirus DNA (pcDNA 3.1-HOTAIR)

PCR amplification was conducted with human genomic DNAs as the template by referring to the primers of Ma. PCR products were cloned into the mammalian expression vector pcDNA3.1, and the pcDNA3.1-HOTAIR plasmid was constructed.

Cell Transfection

After the cells grew into a monolayer on a 6-well plate, cell transfection was performed according to the instructions of Liposome 2000. 48 h after transfection, the cells were harvested for quantitative RT-PCR (qRT-PCR) or Northern blotting analysis.
**Northern Blotting Analysis**

Total RNA of extracted samples was separated by 15% denaturing polyacrylamide gel, transferred onto a GeneScreen Plus membrane and hybridized with UltraHyb-Oligo buffer. The end of T4 kinase was labeled with a mature miR-221 complementary oligonucleotide (5'-AGCUA-CAUUUGUCUGCGGGUUUC-3'), which was applied as a probe for hybridization. After hybridization overnight, the membrane was exposed to a storage screen for 8 h, followed by imaging using the Typhoon 9410 variable imager. 18S ribosomal RNA (rRNA) and cDNA were used as a negative control.

**Flow Cytometry**

Cell apoptosis was analyzed by flow cytometry in strict accordance with the Annexin V-enhanced green fluorescent protein (EGFP)/propidium iodide (PI) Apoptosis Detection Kit. Cells grown into monolayer were washed with phosphate-buffered saline (PBS), fixed in pre-cooled 75% ethanol and treated with Annexin V-EGFP/PI Apoptosis Detection reagents for 30 min at 4°C in the dark. Finally, cell apoptosis was detected via BioRad (Hercules, CA, USA) flow cytometer and analyzed using the CELL Quest software.

**Statistical Analysis**

Statistical Product and Service Solutions 17.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (La Jolla, CA, USA) were used for all statistical analysis. Paired sample t-test was used to compare the expression levels of HOTAIR in tumor tissues and adjacent normal tissues. Differences in the expression levels of HOTAIR in cell lines, expression changes after transfection and the apoptosis difference among different groups were analyzed by independent samples t-test. All data were expressed as mean ± standard deviation. p<0.05 was considered statistically significant.

**Results**

**HOTAIR Expression in NSCLC Tissues and its Correlation with Clinic-Pathological Features**

We first detected the expression level of HOTAIR in the tissues of 38 NACLC patients. Meanwhile, the correlation between HOTAIR expression and clinic-pathological features were analyzed. Results (Table I) manifested that the expression of HOTAIR only exhibited a certain correlation with the stage of NSCLC patients. Furthermore, the expression levels of HOTAIR in patients with stage I and II were markedly lower than those with stage III and IV.

**Correlation between HOTAIR Expression and MiR-221 Expression in NSCLC Tissues**

The expressions of HOTAIR and miR-221 in 38 pairs of NSCLC tissues and adjacent normal tissues were detected. Meanwhile, the correlation between HOTAIR expression and miR-221 expression in 38 NSCLC patients was analyzed. The results revealed the expression level of HOTAIR in NSCLC tissues was significantly higher

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<th>Clinical feature</th>
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Table I. Correlations of the HOTAIR expression with clinicopathological features.
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than that of adjacent normal tissues (Figure 1A) \((p<0.01)\). However, the expression level of miR-221 in NSCLC tissues was significantly lower than that of adjacent normal tissues (Figure 1B) \((p<0.01)\). Besides, linear correlation analysis showed that HOTAIR expression was significantly negatively correlated with miR-221 expression in NSCLC tissues (Figure 1C) \((r=-0.7651, p<0.0001)\).

Expressions of HOTAIR and MiR-221 in NSCLC Cell Lines

To further verify the expression levels of HOTAIR and miR-221, three NSCLC cell lines (A549, H322 and H1299) and one normal lung cell line (BEAS-2B) were cultured. Subsequently, the expression levels of HOTAIR and miR-221 were detected. RT-PCR results illustrated that the expression levels of HOTAIR in the three NSCLC cell lines were significantly higher than those of the normal lung cell line (Figure 2A) \((p<0.01)\). However, the expression levels of miR-221 in NSCLC cell lines were remarkably lower than those of the normal lung cell line (Figure 2B) \((p<0.01)\).

Regulatory Effect of MiR-221 on HOTAIR

A549 cells were then selected as the research object. The cells were transfected with miR-221 mimics and inhibitors, and the expression of HOTAIR was detected via RT-PCR. It was found that compared with the mimics transfection control group, the expression of HOTAIR was significantly decreased in the miR-221 mimics transfection group (Figure 3A) \((p<0.01)\). On the contrary, after transfecting with miR-221 inhibitors in A549 cells, the expression of HOTAIR was significantly increased (Figure 3B) \((p<0.01)\).

Negative Regulation of HOTAIR on MiR-221

A549 cells were transfected with siRNA and pcDNA3.1-HOTAIR plasmids, respectively. The regulatory effect of HOTAIR on miR-221 was investigated. We detected the expression of miR-221 via RT-PCR and Northern blotting. The targeting of si-HOTAIR and pcDNA3.1-HOTAIR was first verified. The results revealed that si-HOTAIR markedly reduced the expression of HOTAIR (Figure 4A). However, pcDNA3.1-HO-
TAIR significantly up-regulated the expression of HOTAIR (Figure 4B). Moreover, si-HOTAIR significantly increased the expression of miR-221 (Figure 4C) \( (p<0.01) \), whereas pcDNA3.1-HOTAIR markedly reduced the expression of miR-221 in A549 cells (Figure 4D) \( (p<0.01) \).

**Apoptosis of NSCLC Cells Promoted by Negative Regulation of MiR-221 on HOTAIR**

Compared with the control group, the apoptosis rate of cells after si-HOTAIR transfection was significantly increased [si-HOTAIR group: (6.41±0.32) % and control group: (2.24%±0.21) %]. The apoptosis rate in the miR-221 inhibitor group [(1.44%±0.15) %] was notably lower than that of the control group (Figure 5A) \( (p<0.05) \), indicating that suppressing miR-221 expression could reduce the apoptosis of NSCLC cells. In addition, the apoptosis rate in the si-HOTAIR + miR-221 inhibitor group was (2.32%±0.19) %, which was remarkably lower than that of the si-HOTAIR group (Figure 5B) \( (p<0.05) \). MiR-221 inhibitors not only decreased the expression of miR-221 but also promoted the expression of HOTAIR and reduced cell apoptosis. Conversely, miR-221 promoted the apoptosis of NSCLC cells through negative regulation on HOTAIR expression.

**Figure 3.** A. The expression of HOTAIR was significantly decreased after transfection with miR-221 mimics. B. MiR-221 inhibitor significantly promoted the expression of HOTAIR.

**Figure 4.** A, Transfection of si-HOTAIR dramatically reduced the expression of HOTAIR. B, Transfection of pcDNA3.1-HOTAIR evidently promoted the expression of HOTAIR. C, Transfection of si-HOTAIR significantly promoted the expression of miR-221. D, Transfection of pcDNA3.1-HOTAIR markedly reduced the expression of miR-221.
Discussion

NSCLC is one of the most common and lethal diseases in human beings, which also accounts for a very high proportion of cancer deaths globally\(^2\). In the early stage, studies have revealed that the poor prognosis of NSCLC is closely correlated with the occurrence and metastasis of the tumor\(^2,3,4\). However, these processes are still unknown. Hence, it is of great significance to investigate the mechanisms of NSCLC occurrence, development and migration for improving the diagnosis, prevention and treatment of NSCLC. In recent years, increasing evidence has confirmed that lncRNA plays important roles in the occurrence and metastasis of NSCLC. Meanwhile, lncRNA can be used as a prognostic marker for the metastasis and survival of patients with NSCLC\(^5\).

HOTAIR, a HOXC cluster-derived lncRNA, binds to the transcriptional co-suppressor polycomb repressive complex 2 (PRC2) and recruits PRC2 to silence its target gene\(^6\). Since its elevated expression, HOTAIR has been regarded as an oncogene in several cancers. Previous studies have found that HOTAIR mediates the invasion and metastasis of breast cancer cells\(^7,8\). However, the regulatory mechanism of HOTAIR expression in NSCLC remains unclear, which was studied in this work.

According to some studies\(^9,10\), HOTAIR exerts its effects by regulating a train of target genes. Tang et al.\(^11\) have demonstrated that HOTAIR is involved in the invasion and metastasis of various cancers, including liver cancer. Hence, we first analyzed the expression and clinic-pathological features of HOTAIR in NSCLC tissue samples. The results revealed that HOTAIR expression only had a certain correlation with the cancer stage. Meanwhile, the expressions of HOTAIR in patients with stage I and II were notably lower than those with stage III and IV. Secondly, the correlation between miR-221 expression and HOTAIR expression in NSCLC tissues was analyzed. Our findings revealed that there was a significantly negative relationship between the expression of miR-221 and the expression of HOTAIR. To further validate their intrinsic association and effects on the apoptosis of NSCLC cells, NSCLC cell lines were cultured in vitro. The expressions of HOTAIR and miR-221 in NSCLC cell lines were detected, and the results were consistent with those detected in tissues. Furthermore, miR-221 mimics, miR-221 inhibitors, HOTAIR-siRNAs and pcDNA3.1-HOTAIR were transfected into A549 cells. RT-PCR and Northern blotting were adopted to analyze the interaction between miR-221 and HOTAIR. It was discovered that there was a negatively regulatory interaction between miR-221 expression and HOTAIR expression. Finally, whether miR-221 triggered the apoptosis of NSCLC cells by inhibiting HOTAIR expression was analyzed by flow cytometry. Results manifested that miR-221 negatively regulated the expression of HOTAIR, thereby stimulating the apoptosis of NSCLC cells.

![Figure 5. A. The apoptosis rate in the miR-221 inhibitor group was notably lower than that of the control group. B. MiR-221 inhibitors significantly reduced cell apoptosis.](image-url)
Conclusions

We demonstrated that miR-221 promoted the apoptosis of NSCLC cells by negative regulation of IncRNA HOTAIR. Moreover, it can be used as a target molecule for the treatment of NSCLC.

Conflict of Interests
The authors declare that they have no conflict of interest.

References
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