HOTAIR contributes to chemoresistance by activating NF-κB signaling in small-cell lung cancer

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Abstract: Our previous study showed that lncRNA HOTAIR affects the chemoresistance of SCLC by regulating HOXA1 methylation. However, the downstream regulatory mechanism remains unknown. The article aimed to further explore the potential downstream mechanism. In this study, we demonstrate that the knockdown of HOTAIR inhibits the NF-κB pathway in SCLC cells. The overexpression of HOXA1, the downstream gene of HOTAIR, also suppresses the NF-κB pathway, but the downregulation of HOXA1 shows the opposite results. Notably, the knockdown of HOXA1 in HOTAIR downregulated cells can rescue the inhibition of the NF-κB pathway mediated by HOTAIR downregulation. Meanwhile, we found that the NF-κB pathway is activated in multidrug-resistant SCLC cells (H69AR, H446AR) compared with the parental cells (H69, H446). The inhibition of the NF-κB pathway with celastrol increases cell sensitivity to anticancer drugs, cell apoptosis, and cell cycle arrest. Collectively, these results revealed that the NF-κB pathway may be involved in the chemoresistance of SCLC caused by HOTAIR methylating HOXA1.

Keywords: HOTAIR, HOXA1, SCLC, the NF-κB pathway

Introduction

Small-cell lung cancer (SCLC) is a neuroendocrine malignancy and accounts for 15% to 17% of all patients with lung cancer [1]. There were about 130,000 patients diagnosed with SCLC and 100,000 died of the disease in China in 2013 [2, 3]. In clinical practice, SCLC is generally divided into limited disease and extensive disease. Platinum plus etoposide is the first-line chemotherapy regimen for patients with SCLC. Although most people have high percentages of response to chemotherapy initially, this rapidly turns into drug resistance during treatment [4]. Therefore, the molecular mechanism involved in SCLC chemotherapy resistance needs to be further explored.

Long noncoding RNAs (IncRNAs), whose length are over 200 nucleotides, have been shown to regulate every step of cell growth and death [5]. Furthermore, many studies have confirmed that IncRNAs can regulate the occurrence, development and metastasis of tumors through classic signaling pathways [6-9]. Our previous research confirmed that HOTAIR induced HOXA1 methylation leads to the chemoresistance of SCLC [10], and the purpose of the present research is to explore whether classic signals are involved in the HOTAIR/HOXA1 axis or not. We reviewed the related literature and found that HOTAIR can regulate the NF-κB pathway in some cancers [11-13]. Meanwhile, the result of the enrichment analysis about differentially expressed proteins from H69AR and H69 revealed that the NF-κB pathway was activated in H69AR [14]. Therefore, we sought to explore whether the NF-κB pathway is involved in the process in which HOTAIR induced HOXA1 methylation contributes to the chemoresistance of SCLC.

In this research, we showed that HOTAIR induced HOXA1 methylation leads to the chemoresistance of SCLC via the NF-κB pathway. The inhibition of NF-κB signaling increases cell sensitivity to anticancer drugs, cell apoptosis, and cell-cycle arrest. The study adds to our understanding of the chemoresistance of SCLC.
Materials and methods

Cell culture

The human SCLC cell lines H69, H69AR and H446 were obtained from the American Type Culture Collection, and H446AR was established by exposing H446 cells to a high dose of Adriamycin for at least 6 months. All the cells were cultured in a 10-20% fetal calf serum (Gibco, USA) medium. However, H69AR and H446AR were alternately cultured with a medium containing 0.8 μM of ADM and a drug-free medium.

To inhibit the NF-κB pathway, the cells were treated with 5 μM celastrol (InvivoGen, USA) for 48 h.

Cell transfection

For downregulation, we transiently transfected cells with siRNAs (GenePharma, Shanghai) using Lipofectamine 2000 (Invitrogen) and OPTI-MEM (Invitrogen). The targeting sequences were as follows: siHOTAIR-1: 5'-cccauggacucauacatt-3' and 5'-uuguuuaugaguccauggtt-3'; siHOTAIR-2: 5'-gccuuuggaagcucuugatt-3' and 5'-uucaagagcuucaaaggctt-3'; siHOXA-1: 5'-acc-auaggauuacaacuutt-3' and 5'-aaaguuguaaucagtaatt-3'; siHOXA-2: 5'-uggaugaaagucaaaagaatt-3' and 5'-uucuuuugacuuucauccatt-3'.

For upregulation, the HOXA1 overexpression plasmid (HOXA1-eGFP) and the empty plasmid (eGFP) were purchased from Genepharma (Shanghai, China). The specific operational steps were the same as above.

Quantitative RT-PCR

TRIZOL reagent (TaKaRa) was used to obtain total RNA in the cell. The RNA concentrations were measured using NanoDrop 2000 (Thermo). And reverse transcription reactions were conducted using a PrimeScript RT Reagent Kit (TIANGEN Biotech, Beijing). Finally, qRT-PCR was run in an ABI Illumina instrument (San Diego, USA). The 2-ΔΔCT method was used to calculate the relative mRNA expression. The primer sequences designed by Shanghai Sangong Biotech were as follows: HOTAIR: F: 5'-ggtaggaagaagaacacagagac-3', R: 5'-acataaaccctctgctgcttgc-3'; HOXA1: F: 5'-aggtagaagtacgctactg-3', R: 5'-tgaggatcagcatctcacc-3'; GAPDH: F: 5'-gagtagggatttggtcatggt-3', R: 5'-cagtggtgagattttatt-3'.

Western blotting analysis

A RIPA lysate (Biyuntian, China) was used to obtain the total protein in the cells. Cell lysates with the same content were separated using 10% SDS-polyacrylamide gel electrophoresis and then transferred to a PVDF membrane (Millipore). The membranes were blocked with 5% skim milk for 1 h and then incubated with an anti-BCL-XL antibody (Santa Cruz, USA), anti-HOXA1 (Sigma, USA), and an anti-GAPDH antibody (Sigma, USA) overnight at 4°C. After they were washed three times with 1 × TBST, the membranes were incubated for 1 h with an HRP-linked anti-rabbit secondary antibody (EarthOx, San Francisco). Finally, the proteins were visualized using chemiluminescence (ECL).

Luciferase reporter assays

The cells were transfected with a pNF-κB luciferase reporter plasmid (NF-κB-dependent firefly luciferase reporter) and a pRL-CMV vector (a kind gift from Metastasis Research Laboratory, Peter MacCallum Cancer Centre, Melbourne, Australia) using Lipofectamine 2000 (Invitrogen) and OPTI-MEM (Invitrogen). After 48 h transfection, the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Drug-resistance assay (CCK8)

Drug sensitivity was detected using a CCK-8 assay. Transfected cells were incubated with different concentrations of chemotherapy drugs (Adriamycin (ADM; Jiangsu), cisplatin (CDDP; Shandong), and etoposide (VP16; Jiangsu). After 24 h, 10 μl CCK-8 reagent (Dojindo, Japan) was added into cells and incubated for 3 h. Then, the OD value was measured at 450 nm. According to the OD value measured, the IC50 of each drug was calculated. Each group had five vice holes, and three parallel experiments were performed.

Flow cytometry

The cells were treated with anticancer drugs for 24-48 h and then collected for further experiments. For cell cycle assays, the cells were fixed with 70% ethanol and stained with
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50 mg/ml propidium iodide (Sigma, USA). For the apoptosis assays, Annexin V450/eff660 APC (eBioscience, USA) was used according the manufacturer’s protocol. All samples were analyzed using a BD FACS-Verse flow cytometer.

Statistical analysis

The data were represented as the mean ± SD. An independent-samples t test or a one-way ANOVA were used to analyze the differences between groups. SPSS (Chicago, USA) was used for the statistical analysis, and \( P < 0.05 \) was considered statistically significant.

Results

**HOTAIR regulates the NF-κB pathway in SCLC cells**

Our team has confirmed that HOTAIR affects the chemoresistance of SCLC by regulating HOXA1 methylation. To understand the role of HOTAIR in the NF-κB pathway, we transfected HOTAIR siRNAs into SCLC cells. And the qRT-PCR results showed that the expression of HOTAIR was downregulated in the SCLC cells [10]. We found that the downregulation of HOTAIR in chemoresistant SCLC cells significantly decreased the NF-κB induced luciferase activity (Figure 1A). Meanwhile, western blot showed that the expression of BCL-XL (a classical NF-κB target gene) was downregulated in HOTAIR-silenced SCLC cells (Figure 1B). These results indicate that HOTAIR may regulate the NF-κB pathway in SCLC cells.

**HOXA1 inhibits the NF-κB pathway in SCLC cells**

After revealing the relationship between HOTAIR and the NF-κB pathway, we sought to explore whether HOTAIR downregulation inhibits the NF-κB pathway by increasing HOXA1 expression in SCLC. To further verify the negative regulatory effect of HOXA1 on the NF-κB pathway, we constructed HOXA1 downregulated and overexpressed SCLC cells (Figure 2A). We found that NF-κB induced luciferase activity and the expression of BCL-XL was markedly enhanced in HOXA1 downregulated SCLC cells. On the other hand, the luciferase activity and the expression of BCL-XL were downregulated in SCLC cells with HOXA1 overexpression (Figure 2B). Meanwhile, the knockdown of HOXA1 by siRNA in HOTAIR downregulated cells could rescue the effects of HOTAIR’s silencing of luciferase activity (Figure 2C). A previous study confirmed that DNA methylation can reduce the expression of HOXA1 in SCLC [10]. Therefore, we treated SCLC cells with the DNA methyltransferase inhibitor 5-Aza-2-deoxycytidine (5-Aza-CdR) and found that the NF-κB pathway was inhibited in the treatment group compared to the control group (Figure 2D). Our results suggest that HOTAIR affects the NF-κB pathway partly by regulating HOXA1 in SCLC cells.

**The NF-κB pathway affects the chemoresistance of SCLC**

The above experiments have demonstrated that the NF-κB pathway can be regulated by the HOTAIR/HOXA1 axis in SCLC cells. In addition, we found that the NF-κB pathway is activated in chemoresistant SCLC cells (H69AR, H446AR) than in parental cells (H69, H446) (Figure 3A). In order to assess whether the NF-κB pathway was involved in the chemoresistance of SCLC, we treated cells with NF-κB inhibitors (celast-
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Interaction in HOTAIR and NF-κB signaling in small-cell lung cancer. Dual luciferase reporter assays and western blot analysis showed that both the NF-κB induced luciferase activity and the expression of BCL-XL were decreased after treatment with celastrol in chemoresistant SCLC cells (Figure 3B). CCK8 assays were conducted to evaluate the chemosensitivity of the SCLC cells to various drugs (ADM, CDDP, and VP-16). The results showed that the IC50 values were significantly decreased after treatment with celastrol in the H69AR and H446AR cells (Figure 3C). These results suggest that the NF-κB pathway may be involved in the multidrug resistance of SCLC.

**Inhibiting the NF-κB pathway increases cell sensitivity to chemotherapy drugs by enhancing cell apoptosis and cell cycle arrest**

We further evaluated the effect of celastrol on cell apoptosis and the cell cycle upon exposure of the cells to chemotherapy drugs. Flow cytometry results demonstrated that cell apoptosis increased and the cell cycle was arrested in H69AR and H446AR cells treated with celastrol after anticancer drug exposure (Figure 4A and 4B). These observations showed that the inhibition of the

Figure 2. HOXA1 inhibits the NF-κB pathway in SCLC cells. A. Inhibition or overexpression of HOXA1 by transfection of HOXA1 siRNA or plasmid in SCLC cells. B. NF-κB induced luciferase activity and BCL-XL expression were measured by dual luciferase reporter assays and western blot analysis in HOXA1 downregulated and overexpressed cells. C. After the cells were co-transfected with HOXA1 siRNA and HOTAIR siRNA, NF-κB-induced luciferase activity was examined using dual luciferase reporter assays. D. After SCLC cells are treated with 5-Aza-CdR, the NF-κB induced luciferase activity was measured by dual luciferase reporter assays. *P < 0.05, **P < 0.01, ***P < 0.001.
Discussion

Patients with SCLC usually have a poor prognosis, which is closely related to the occurrence of chemoresistance. But the precise mechanism of the chemoresistance remains unknown. Many recent studies have reported that lncRNAs are involved in tumor chemotherapy resistance. Zhang et al. demonstrated that lncRNA foxc2-as1 promotes Adriamycin resistance in osteosarcoma [15]. Xiong et al. showed that lncRNA HULC attenuates the chemosensitivity of HCC cells by increasing autophagy [16], and so on.

Our laboratory also confirmed that lncRNA HOTAIR affects the chemoresistance of SCLC by regulating HOXA1 methylation [10]. However, the downstream regulatory mechanism is still unclear. Through a literature review, we found that HOTAIR can regulate the NF-κB pathway in many cancers [11-13], and an enrichment analysis of mass spectrometry resulting from H69AR and H69 also revealed that the NF-κB pathway was activated in H69AR [14]. Thus, we hypothesized that the HOTAIR/HOXA1 axis modulated the chemoresistance of SCLC by the NF-κB pathway. To confirm this hypothesis, we changed the expressions of HOTAIR and HOXA1 in SCLC cells and found that HOTAIR downregulation and HOXA1 overexpression inhibited the NF-κB pathway. In contrast, the downregulation of HOXA1 produced the opposite results. To further confirm the results, we conducted a rescue experiment and found that HOXA1 downregulation could rescue the effects of HOTAIR silencing on the NF-κB pathway. As we previously reported, HOXA1 methylation induced by HOTAIR contributes to the chemoresistance of SCLC. We found that NF-κB-induced luciferase activity decreased in cells treated with 5-Aza-CdR compared with the control group. Therefore, our studies showed that the HOTAIR/HOXA1 axis can regulate the NF-κB pathway in SCLC cells.

The NF-κB pathway has been reported to be closely related to the occurrence and development of many tumors [17-19]. In addition, some studies have shown a relationship between the NF-κB pathway and treatment resistance in cancers. For example, Jacamo et al. demonstrated that reciprocal leukemia-stroma VCAM-1/VLA-4 mediates chemoresistance by activating NF-κB signaling [20]. And Zhang et al. showed that icariin increases the radiosensitivity of colorectal cancer by inhibiting NF-κB activity [21]. In our study, we showed that the NF-κB pathway was activated in multidrug-resistant SCLC, which is involved in the chemoresistance of SCLC.
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Aa

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Annexin V V450-A

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resistant cell lines compared with the parental cell lines. Moreover, we found that the inhibition of the NF-κB pathway would increase cell sensitivity to ADM, CDDP, and VP-16 by enhancing cell apoptosis and cell cycle arrest. These results suggested that inhibiting the NF-κB pathway improved the drug sensitivity of SCLC in vitro.

In conclusion, our findings reveal that HOXA1 methylation induced by HOTAIR contributes to the chemoresistance of SCLC by regulating the NF-κB pathway. NF-κB inhibitors may be used in chemotherapy resistant patients with increased HOTAIR expression.

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Disclosure of conflict of interest

None.

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