LncRNA HOTAIR Increases Inflammatory Responses by Activating NF- κ B Pathway in LPS-Induced Acute Lung Injury

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Abstract

Background Nuclear factor kappa-B (NF- κ B) activation increased the expression of cytokines and further lead to lung injury was considered the main mechanism of acute lung injury (ALI). Here, we focus on exploring the potential regulatory mechanism between long noncoding RNA (LncRNA) HOX transcript antisense RNA (HOTAIR) and NF- κ B on LPS-induced ALI.

Methods A549 cells were then divided into 4 groups: HOTAIR group, NC group, si-HOTAIR group and si-NC group. These 4 groups were then treated with 1 μ g/mL lipopolysaccharides (LPS) or without LPS at 37°C for 24 h. The expression level of cytokines (tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6) and LncRNA HOTAIR were evaluated by quantitative Real Time Polymerase Chain Reaction (qRT-PCR) and Enzyme-linked immunosorbent assay (ELISA). Western Blot analysis was adopted for evaluating the level of p-I κ B α /I κ B α and p-p65/p65. Nuclear translocation of p65 was observed by immunofluorescence staining.

Results qRT-PCR and ELISA assay showed that the expression of cytokines (IL-1 β , IL-6 and TNF- α) and inflammatory gene HOTAIR was remarkably increased with LPS treatment ($p < 0.01$). Over-expression of HOTAIR significantly increased the expression of cytokines (including IL-1 β , IL-6 and TNF- α) and NF- κ B pathway associated proteins (including p-I κ B α /I κ B α and p-p65/p65), while knockdown of HOTAIR had the opposite effect ($p < 0.01$). The immunofluorescence assay showed that the level of p65 in the nucleus was significantly higher in the HOTAIR group and significantly lowers in the si-HOTAIR group ($p < 0.01$).

Conclusion HOTAIR may play a pro-inflammatory response through NF- κ B pathway in LPS-induced ALI, which may provide a perspective for further understanding the pathogenic mechanism of ALI.

1. Background

ALI is a morbid pulmonary inflammatory disease which may lead to acute respiratory failure diseases [1]. Bacterial infection was considered the main cause of ALI, in which LPS on bacteria could activate NF- κ B signaling pathway through TLR4 signals and further lead to the enhancement of cytokines expression and further lead to lung injury [2] [3] [4]. Thus, restrain inflammatory reaction via TLR4 signaling was suggested a possible mechanism of ALI treatment [5].

LncRNAs were able to regulate varied genes expressed in cells and tissues [6]. It is reported that multiple lncRNAs have contribute to the progress of ALI, for example, high levels of the lncRNAs nuclear paraspeckle assembly transcript 1 (NEAT1) aggravated the progression of ALI via HMGB1/RAGE signaling, increased metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was positively correlated with increased severity and unfavorable prognoses via TSC2-mTOR and NF- κ B signaling pathways[7][8][9][10]. Long noncoding RNA taurine up-regulated gene 1 (TUG1) targeted to miR-34b-5p and activated GRB2 associated binding protein 1 (GAB1), which showed a reducing pulmonary injury in ALI mice [11]. However, specific prognostic and therapeutic target of lncRNAs in ALI remain uncertain.

LncRNA HOTAIR have impacts on multiple cancer diseases [12]. It was regarded as a potential prognostic and therapeutic target in lung cancer [13]. Until recently, the role of HOTAIR in LPS-induced ALI remains unknown. Here, we presented a research focus on exploring the potential regulatory mechanism between HOTAIR and NF- κ B signaling pathway on LPS-induced ALI.

2. Methods

2.1 Cell culture and LPS treatment

EML4-ALK Fusion-A549 Isogenic Cell Line Human (ATCC® CCL-185IG™) was purchased from American Type Culture Collection (ATCC) (ATCC, Manassas, VA, USA). Cells were cultured in complete medium of DMEM with 10% FBS and 1% penicillin streptomycin at 37°C under 5% CO₂. LPS induced ALI cell model was induced by incubated with 1 μ g/mL LPS (*Escherichia coli* 055:B5, Sigma Aldrich, Saint Louis, Missouri, USA) at 37 °C for 24 h as described in our previous report [14].

2.2 Cell transfection

pcDNA 3.1-HOTAIR (HOTAIR group), pcDNA 3.1 empty vector (NC group), siRNAs specifically targeting HOTAIR (si-HOTAIR) and siRNA negative control (si-NC) were purchased from Sigma. MH-S and MLE-12 cells were seeded into 96-well plates and cultured for overnight at 37 °C. All of these oligonucleotides were transfected into A549 cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) incubated for 48 h according to the manufacturer's protocols.

2.3 qRT-PCR analysis

Total RNAs were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNAs were used for synthesizing cDNA with a miRcute miRNA first-strand cDNA synthesis kit (TIANGEN Biotech, Beijing, China) and eliminating gDNA with PrimeScript RT Reagent Kit with gDNA Eraser (TIANGEN Biotech, Beijing, China). IL-1 β , IL-6, TNF- α and LncRNA HOTAIR were evaluated using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with custom designed primers (Table 1). The relative expression was normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) qRT-PCR was performed on a 7500 Real-Time PCR system (Applied Biosystems, USA) and conducted under the following conditions: 95 °C for 5 min, 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The experiment was performed in triplicate. The relative gene expression was calculated d using the $2^{-\Delta\Delta CT}$ method and normalized to the expression of internal control.

Table 1
primers

	Forward	Reverse
HOTAIR	CAGTGGGGAACTCTGACTCG	GTGCCTGGTGCTCTCTTACC
IL-1 β	CTTGGTGATGTCTGGTCCAT'	CCTTGTACAAAGGACATGGAG
IL-6	CCAGAGCTGTGCAGATGAGT	CTGCAGCTTCGTCAGCAGGC
TNF- α	CCCACCTTTGCCCGGGGTTC'	CCTCCCGGGCGTCAGCACTA
GAPDH	GCTCATTTGCAGGGGGGAG	GTTGGTGGTGCAGGAGGCA-

2.4 ELISA analysis

Proteins of A549 cells was isolated with lysis buffer (Elabscience, Wuhan, China) and the concentration was confirmed with Pierce™ BCA protein assay kit (Elabscience, Wuhan, China). The level of IL-1 β , IL-6 and TNF- α were detected using ELISA kits (Beyotime Biotechnology, Shanghai, China) on a multiskan FC spectrum system (Thermofish, USA) and measured at an absorbance at 450 nm.

2.5 Western Blot Assay

Total cellular proteins were isolated with pre-cold RIPA buffer (Beyotime Institute of Biotechnology, Nantong, China) with protease inhibitor. The proteins were separated by SDS-PAGE electrophoresis and transferred into polyvinylidene fluoride (PVDF) membranes and fixed in PBS with 0.1% Tween-20 (PBST) containing 5% non-fat milk for 2 h at room temperature. After the block, the membrane was incubated with primary antibodies (anti-p-IkB α monoclonal antibody (1:1,000; abcam, No. ab133462), anti-IkB α monoclonal antibody (1:1,000; abcam, No. ab32518), p-p65 NF- κ B monoclonal antibody (1:10,000; abcam, No. ab76302), anti-p65 NF- κ B monoclonal antibody (1:10,000; abcam, No. ab732536), and anti-human GAPDH polyclonal antibody (1:10,000; abcam, No. ab181602,) at 4°C overnight and then incubated with the horseradish peroxidase (HRP) labeled secondary antibody (1:10,000) at 25°C for 2 h. GAPDH was used as the loading control. The protein bands were detected using the enhanced chemiluminescence reagent (Thermo Scientific, USA).

2.6 Immunofluorescence analysis

A549 cells were seeded on a microscope slide and fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature. Then we blocked the cells with 1 × PBS containing 5% goat normal serum and 0.3% Triton-x100 for 1 h. The cells were then incubated with rabbit anti-p65 NF- κ B primary antibodies (1:200, 710048, Ebioscience, US) at 4 °C overnight and Anti-Goat secondary antibody (Ebioscience, US) for 1 h at room temperature. Finally, the cells were stained with DAPI for 10 min and mounted with fluorescent mounting solution. Images were taken by fluorescence microscope with excitation wavelength of 360 nm (Nikon ECLIPSE TE2000-U).

2.6 Statistical analysis

Continuous variables are presented as mean $\pm$ standard deviation (SD). Difference between control and LPS-induced group was performed through student's t test and the differences between multiple groups were analyzed using a one-way analysis of variance (ANOVA) followed by a post-hoc LSD test. Statistical analyses were performed with the statistical package for the social sciences (SPSS) version 22.0 software (IBM Inc., USA) and P-values < 0.05 were considered statistically significant.

3. Results

LPS induced increasing IL-1 β , IL-6, TNF- α and lncRNA HOTAIR

A LPS-induced ALI cell model was established and the expression of cytokines (IL-1 β , IL-6, TNF- α) and inflammatory gene HOTAIR were detected by both qRT-PCR and ELISA, which have been described as our previous report [14]. The results showed that the expression of IL-1 β , IL-6 and TNF- α (Fig. 1a & Fig. 1b) and HOTAIR (Fig. 1c) was remarkably increased with LPS treatment compared with the control group.

lncRNA HOTAIR correlated with inflammation response induced by LPS

To explore whether effect of lncRNA HOTAIR knockdown in LPS-induced ALI, A549 cells were transfected with NC-pcDNA 3.1, HOTAIR-pcDNA 3.1, si-NC and si-HOTAIR for 48 h and treated with or without LPS for 24 h. As shown as Fig. 2a, the expression of HOTAIR in the HOTAIR-pcDNA 3.1 group was more 20 times higher than other groups without LPS. Compared with the si-HOTAIR groups, the expression of HOTAIR in the si-NC group was more 3 times higher without LPS. Moreover, the expression of HOTAIR in the HOTAIR-pcDNA 3.1 group was also more 20 times higher than other groups and the expression of HOTAIR in the si-NC group was more 5 times higher with LPS (Fig. 2b).

To explore the effect of over-expression and knockdown of HOTAIR on LPS-induced inflammatory response in ALI, A549 cells were transfected with NC-pcDNA 3.1, HOTAIR-pcDNA 3.1, si-NC and si-HOTAIR for 48 h and treated with LPS for 24 h. As shown as Fig. 3a, the results showed that the expression of IL-1 β , IL-6 and TNF- α was remarkably increased in the HOTAIR-pcDNA 3.1 group compared with the other groups. ELISA results demonstrated that the expression of IL-1 β , IL-6 and TNF- α was significantly decreased in the si-HOTAIR group compared with the other groups, (Fig. 3b).

The role of HOTAIR in modulating NF- κ B pathway activation

To investigate over-expression and knockdown of HOTAIR on the activation of NF- κ B pathway, A549 cells were transfected with NC-pcDNA 3.1, HOTAIR-pcDNA 3.1, si-NC and si-HOTAIR and treated with LPS. Our result showed that the levels of p-I κ B α /I κ B α and p-p65/p65 were increased in the HOTAIR-pcDNA 3.1 group and decreased in the si-HOTAIR group (Fig. 4).

Knockdown HOTAIR inhibit nuclear translocation of p65 induced by LPS

The nuclear translocation of p65 NF- κ B induced by LPS in A549 cells was then detected by immunofluorescence assay. Our result showed that p65 NF- κ B protein level in the nucleus was

significantly higher in the HOTAIR-pcDNA 3.1 group than the other groups. P65 NF- κ B protein levels in the nucleus was the lowest in si-HOTAIR groups compared with the other groups (Fig. 5).

4. Discussion

Our study revealed that the expression level of cytokines (IL-1 β , IL-6, TNF- α) and inflammatory gene (HOTAIR) were increased in LPS-induced ALI model. Knockdown of HOTAIR can inhibit the secretion of IL-1 β , IL-6, and TNF- α via inhibiting p65 transfer from cytoplasm to nucleus.

More and more evidence showed that non-coding RNA (ncRNA) is an important part of various cellular and physiological signaling processes including ALI [7][15][16][17]. HOTAIR is an antisense transcript that play a role in suppression [18][19][20]. HOTAIR was reported to participate in various cancer and other diseases like acute myocardial infarction, and also involved in immune and inflammatory responses [20][21][22][23][24]. Monira et al suggests that HOTAIR was a key factor in NF- κ B activation of macrophages, regulated the activation of NF- κ B and its target genes expression of IL-6 and iNOS by promoting the degradation of I κ B α [24]. Human immune response is complex biochemical processes, modulated by various proteins and lipids [25]. Among them, Toll-like receptors play an important role in the innate response to bacterial infections and to the immune response to pathogens [26][27]. TLR4 activation triggers the activation of NF- κ B, inducing increased expression of downstream signaling cascades including cytokines, chemokines, and pro-inflammatory genes. A variety of extracellular stimulation signals including Inflammatory factors, LPS, mitogens of T cells and B cells, viral double-stranded RNA and various physical and chemical pressures was able to trigger the activation of NF- κ B signaling pathway [28][29][30][31]. LPS triggers the body's inflammatory response by activating the classic NF- κ B pathway, thereby promoting the expression of cytokines such as IL-1, IL-6 and TNF- α and these cytokines can also induce NF- κ B activation [32]. In this study, we explored the potential role of HOTAIR in the regulation of cytokines and immune response in A549 cells. Our results showed that LPS-induced ALI increased the expression of various cytokines including IL-1 β , IL-6 and TNF- α , and the expression of inflammatory gene HOTAIR was also up-regulated. This result suggests that there may be a correlation between the expression of HOTAIR and cytokines in LPS-induced ALI.

We knockdown HOTAIR and detecting the expression of I κ B protein and NF- κ B member p65 for further exploring whether HOTAIR increase the expression of related inflammatory factors by activating NF- κ B in LPS-induced ALI. Our results suggest that knockdown HOTAIR significantly decreased the level of p-I κ B α and p-p65. As we know that in the classical pathway of NF- κ B, the activation of IKK kinase complex can phosphorylates I κ B α , and further triggering its polyubiquitination and proteasome degradation, leading to the release and activation of NF- κ B dimer, which accompanied by the activation of p65 [32][33][34]. Ozes et al found that HOTAIR regulated NF- κ B by decreased I κ B α transcription during DNA damage response) in ovarian cancer [35]. Park et al have reported that the activity of the NF- κ B pathway can be influenced by the expression of lncRNA HOTAIR [36]. Therefore, the decreased level of p-I κ B α and p-p65 indicates the effective inhibition of NF- κ B pathway by HOTAIR knockdown in our study. In addition, our immunofluorescence results indicate that the nuclear translocation of p65 in LPS-induced ALI was

inhibited after HOTAIR knockdown. Studies have shown that the inhibition of p65 nuclear translocation is also observed after LPS stimulation of macrophages [24]. Thus, our result indicates that HOTAIR may be involved in the degradation of I κ B α and activation of NF- κ B in LPS-induced ALI.

5. Conclusion

In summary, based on our biochemical studies in A549 cells, it is proved that HOTAIR triggered NF- κ B activation and promote pro-inflammatory response in LPS-induced ALI, which may provide a perspective for further understanding the pathogenic mechanism of ALI.

6. Abbreviations

Long noncoding RNA	LncRNA
Non-coding RNA	ncRNA
HOX transcript antisense RNA	HOTAIR
Acute lung injury	ALI
Lipopolysaccharides	LPS
Activation of nuclear factor kappa-B	NF- κ B
Inhibitor of NF- κ B	I κ B
I κ B kinase	IKK
Tumornecrosis factor	TNF
Interleukin	IL
Inducible nitric oxide synthases	iNOS
Toll-like receptor 4	TLR4
Nuclear paraspeckle assembly transcript 1	NEAT1
Metastasis-associated lung adenocarcinoma transcript 1	MALAT1
Taurine Up-regulated Gene 1	TUG1
GRB2 associated binding protein 1	GAB1
High Mobility Group Protein B1/Receptor for Advanced Glycation Endproducts	HMGB1/RAGE
4',6-Diamidino-2-Phenylindole, Dihydrochloride	DAPI
Enzyme-linked immunosorbent assay	ELISA
quantitative Real Time Polymerase Chain Reaction	qRT-PCR
Paraformaldehyde	PFA
Polyvinylidene fluoride	PVDF
PBS with 0.1% Tween-20	PBST
Horseradish eroxidase	HRP
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
One-way analysis of variance	ANOVA
Statistical package for the social sciences	SPSS
Standard deviation	SD

7. Declarations

Ethics approval and consent to participate:

Not applicable.

Consent for publication:

Not applicable.

Availability of data and material:

The datasets supporting the conclusions of this article are included within the article.

Competing interests:

None.

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Authors` contributions:

XMH, ZXM and SQW contributed to the literature search and study design, ZXM contributed to the analysis of data, XMH contributed to the manuscript preparation, HH, KWW, NNX, WHG and WNT collecting data, YJL and SQW contributed to the review of manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

LPS promotes IL-1β, IL-6, TNF-α and lncRNA HOTAIR expression. (a) Increased expression of IL-1β, IL-6 and TNF-α in LPS-induced ALI detected by qRT-PCR. (b) Increased expression of IL-1β, IL-6 and TNF-α in LPS-induced ALI detected by ELISA. (c) Increased expression of lncRNA HOTAIR in LPS-induced ALI detected by qRT-PCR. Each experiment was repeated three times. ***P<0.001.

Figure 2

Overexpression and knockdown of lncRNA HOTAIR with/without LPS stimulation. (a) RT-qPCR analysis of HOTAIR expression in A549 cells after transfected with NC-pcDNA 3.1, HOTAIR-pcDNA 3.1, si-NC and si-HOTAIR for 48h and treated without LPS for 24h. (b) RT-qPCR analysis of HOTAIR expression in A549 cells after transfected with NC-pcDNA 3.1, HOTAIR-pcDNA 3.1, si-NC and si-HOTAIR for 48h and treated with LPS for 24h. *** $P < 0.001$.

Figure 3

Overexpression and knockdown of HOTAIR on LPS-induced inflammatory response. (a) RT-qPCR analysis of HOTAIR expression in A549 cells after transfected with NC-pcDNA 3.1, HOTAIR-pcDNA 3.1, si-NC and si-HOTAIR for 48h and treated with LPS for 24h. (b) ELISA assay of IL-1 β , IL-6 and TNF- α level in the culture supernatant in A549 cells, which were transfected with NC-pcDNA 3.1, HOTAIR-pcDNA 3.1, si-NC and si-HOTAIR for 48h and treated with LPS for 24h. ***P<0.001. GAPDH

Figure 4

Overexpression and knockdown of HOTAIR on NF- κ B pathway by treated with LPS. A549 cells after transfected with NC-pcDNA 3.1, HOTAIR-pcDNA 3.1, si-NC and si-HOTAIR for 48h and treated with LPS for 24h. (a) Increased levels of p-I κ B α , I κ B α , p-p65 NF- κ B and p65 NF- κ B in HOTAIR-pcDNA 3.1 group and significantly decreased level in si-HOTAIR group by western blot. (b) The relative expression levels of p-I κ B α /I κ B α and p-p65 NF- κ B and p-p65 NF- κ B/NF- κ B were increased in HOTAIR-pcDNA 3.1 group and significantly decreased level in si-HOTAIR group. ***P<0.001.

Figure 5

Knockdown of HOTAIR inhibited LPS-induced translocation of p65 NF- κ B protein from cytoplasm to nucleus. A549 cells after transfected with NC-pcDNA 3.1, HOTAIR-pcDNA 3.1, si-NC and si-HOTAIR for 48h and treated with LPS for 24h. P65 NF- κ B protein level in the nucleus was significantly higher in the HOTAIR-pcDNA 3.1 group than the other groups. Images were taken by fluorescence microscope (Magnification, 400 $\times$).

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