Activation of LncRNA HOTAIR by STAT3 Promotes Gefitinib Resistance and Tumourigenesis by Targeting MicroRNA-216a in NSCLC

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Primary research

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Abstract

Background:
Gefitinib resistance has become a major obstacle for cancer therapy of non-small cell lung cancer (NSCLC). Exosome-mediated transfer of long noncoding RNAs (lncRNAs) is associated with the drug-resistance in various tumors. However, the role of NSCLC-specific exosomal lncRNAs remains largely unknown. The aim of this study is to explore the role of exosomal Hox transcript antisense intergenic RNA (HOTAIR) on gefitinib resistance in NSCLC.

Methods:
We investigated the expression of lncRNAs in 5 paired gefitinib-sensitive and gefitinib-resistant tissues of NSCLC by microarray analysis. The qRT-PCR analysis was to investigate the expression pattern of HOTAIR in gefitinib-resistant NSCLC patient tissues and cell lines. Then, we investigated the effects of HOTAIR on gefitinib resistance in vitro and in vivo.

Results:
In this study, we found HOTAIR was evidently up-regulated in both tissues and serum exosome of gefitinib-resistant NSCLC patients. Moreover, by knocking down HOTAIR, we found that HOTAIR promoted the proliferation of NSCLC cells in vitro, as well as inhibited cell apoptosis and cell sensitivity to gefitinib. Extracellular HOTAIR could be incorporated into exosomes and transmitted to sensitive cells, thus disseminated gefitinib resistance. The expression level of HOTAIR from circulating exosomes is significantly higher in NSCLC patients with gefitinib resistance than those without gefitinib resistance. Mechanistically, bioinformatic analysis coupled with dual luciferase assay revealed that HOTAIR served as miR-216a sponge, and MAP1S was identified as a functional target of miR-216a.

Conclusions:
In conclusion, these data suggest that exosomal HOTAIR serves as an oncogenic role in gefitinib resistance of NSCLC cells CRC through activating miR-216a/MAP1S signaling pathway, providing a novel avenue for the treatment of NSCLC.

Introduction
As one of the frequent prevalent cancers, non-small cell lung cancer (NSCLC) has become the third most frequent cause of cancer-linked deaths globally \(^1\)\(^2\). Despite the notable clinical efficacy of EGFR tyrosine kinase inhibitor (TKI) gefitinib, the therapeutic efficacy is inevitably hampered by the development of
acquired resistance\textsuperscript{4–5}. Thus, further understanding the pathogenesis of NSCLC and to find new therapeutic strategies is quite urgent.

Recently, a kind of endogenous nanoparticle named exosomes, have been identified to play important roles on intercellular communication and are regarded as biomarkers for cancer diagnosis or drug carriers for cancer treatment\textsuperscript{6–7}. Moreover, IncRNAs can be encapsulated into exosomes and then transmitted to recipient cells to implement their biological functions\textsuperscript{8}. Previous studies have reported that exosomal IncRNA expression in NSCLC patients’ serum could be a biomarker or predictor for metastasis or prognosis. LncRNA plays a pivotal role in transcriptional regulation, epigenetic gene regulation, and tumors, especially in the regulation of glycolysis in cancers, including NSCLC\textsuperscript{9–11}. Although IncRNAs were regarded as merely transcriptional “noise” before, emerging evidence has shown that IncRNAs play a pivotal role in transcriptional regulation, epigenetic gene regulation, and tumors\textsuperscript{12}. A large number of researches have revealed that HOTAIR (Hox transcript antisense intergenic RNA), a 2,158 bp IncRNA located in the HOXC gene cluster (12q13.13), perform critical functions in various physiological or pathological processes\textsuperscript{13–15}. MiRNAs can pair with the mRNA bases of target genes to induce silencing complex (RISC) to degrade the mRNA or inhibit its translation, while circRNA can bind miRNAs, affect the binding of miRNA to target genes, indirectly up-regulate the target genes of miRNA, and thus regulate the occurrence and development of diseases, that is, act as a sponge for "adsorption" of miRNA. The circRNA-miRNA-mRNA axis has been shown to be a regulation of multiple tumor-related pathways, with an effect of inducing or inhibiting tumorigenesis. Previous studies have reported that exosomal IncRNA expression from chemo-resistant cells or patients’ serum may potentially influence the therapeutic response via transferring IncRNAs\textsuperscript{16–18}. However, few studies identified the functions of exosomes in gefitinib resistance of NSCLC. Therefore, our present study sought to find out a IncRNA which is critical for the gefitinib resistance and tumorigenesis of NSCLC.

In this study, bioinformatics analyses were applied to find out underlying gefitinib resistance-related IncRNAs in NSCLC. We found that exosomal HOTAIR is an important mediator of gefitinib resistance of NSCLC. Overexpression of HOTAIR triggers cell survival and gefitinib resistance. Knockdown of HOTAIR decreases cell survival and increases gefitinib sensitivity. We also revealed that HOTAIR upregulation was induced by STAT3. We identified miR-216a as an essential target for the HOTAIR pathway, which regulating expression of MAP1S. Taken together our study demonstrate the crucial role of HOTAIR in tumorigenesis and gefitinib resistance of NSCLC, and HOTAIR may be a prospective biomarker for antitumor therapy.

**Materials And Methods**

**Specimen collection**

The cancer tissues and the adjacent normal tissues were collected from 70 NSCLC patients at The Affiliated Tumor Hospital of Xinjiang Medical University. The tissues were stored in liquid nitrogen
immediately after resection. The study was proved by Ethics Committee of The Affiliated Tumor Hospital of Xinjiang Medical University. The written informed consent has been obtained from each patient.

**Cell Lines**

The NSCLC cell lines (HCC827 and PC9) and human bronchial epithelial cells (16HBE) were obtained from the Cell Culture Center, Chinese Academy of Medical Sciences (Beijing, China). HCC827GR and PC9GR cells were generated by continually exposing to stepwise increased concentration of gefitinib over a period of 24 months. Cell lines were cultured in DMEM or RPMI1640 (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Hyclone), penicillin and streptomycin (Thermo Fisher Scientific) at 37 °C in 5% CO₂.

**lncRNA microarray**

The expression of lncRNAs was determined using Arraystar Human LncRNA Microarray v4.0 (Aksomics, China) which is designed for the global profiling of human LncRNAs and protein-coding transcripts. RNA quantity and quality were measured by NanoDrop ND-1000. RNA integrity was assessed by standard denaturing agarose gel electrophoresis or Agilent 2100 Bioanalyzer. Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology) with minor modifications. Finally, the hybridized arrays were washed, fixed, and scanned using the Agilent DNA Microarray Scanner (part number G2505C).

**RNA isolation, fractionation of nuclear-cytoplasm, RNase R and actinomycin D treatment and qRT-PCR assays**

The RNAiso Plus Reagent (Takara, Japan) was adapted to purify RNA from grown cells and tissues, per manufacturer’s method. The NE-PER Kit (Thermo Scientific, USA) was adapted for purification of nuclear, as well as cytoplasmic RNA fractions, per the method of the manufacturer. After that, the samples were inoculated with 4 U/μg of RNase R (Epicentre Biotechnologies, USA) at 37 °C for 10 minutes, 20 minutes, 30 minutes and 40 minutes, respectively. Besides, RNA from cells was inoculated with 100 ng/ml Actinomycin D (CST, Beverly, United States) for 12 hours and 24 hours, respectively. The PrimeScript RT Mix (Takara, China) was adapted to generate cDNA for mRNA and circRNA, per manufacturer’s manual. miRNA cDNA was generated with the miRNA 1st-Strand cDNA Synthesis Kit (Sangon Biotech, China). Afterwards, qPCR was run on the Quantstudio™ DX platform (Applied Biosystems, Singapore), with GAPDH along with small nuclear U6 serving as the normalization standards. Relative expression was determined via the $2^{-\Delta\Delta CT}$ approach.

**Isolation of exosomes and purification**

Exosomes were harvested from cells medium following ultra-spinning as standard protocols. Concisely, conditioned medium of cells was spun at 800g for 30 minutes, at 3,000g for 20 minutes, and at 12,000g for 30 minutes to remove cells and debris. Then the exosomes were purified via spinning 140,000g for
one hour. The exosomes were re-suspended in PBS, followed by repeated purification through ultracentrifugation as the last step. We re-suspended the exosomes in (a) 2% glutaraldehyde in 0.1 mol/L phosphate buffer for transmission electron microscopy; (b) RIPA buffer for western blot assays or precooled exosome re-suspension buffer for total exosome RNA extraction; and (c) FBS-free medium for nanoparticle tracking analysis (NTA) and cell treatment *in vitro* and *in vivo*.

**Western blotting**

The RIPA lysis buffer enriched with PMSF was adapted to isolate total proteins. After that, protein quantification was done using the Bradford approach. Next, equivalent protein amounts were fractionated using a 10% SDS-PAGE on an electrophoresis platform (Bio-Rad, America). The fractionated proteins were blotted onto PVDF membranes, followed by blocking for 20 minutes (in Quick Block: P0252; Beyotime Biotechnology, China). Afterwards, the membranes were inoculated overnight with primary antibodies at 4°C, with serving as the normalization standard. Subsequently, the membranes were rinsed and then inoculated for two hours with secondary antibody at room temperature. Then, enhanced chemiluminescence detection was adapted to visualize the blots.

**Plasmid construction and RNA transfection**

MiR-216a mimic, mimic nc, miR-216a inhibitor, inhibitor nc were purchased from GenePharma (Shanghai, China). siRNA was adapted to silence endogenous HOTAIR expression (si-HOTAIR#1, si-HOTAIR#2, and si-HOTAIR#3) and si-NC (negative control siRNA) were synthesized by RiboBio (Guangzhou, China). Mimic-, anti-miR-216a, and scrambled control were provided by GenePharma (Shanghai, China) and were propagated at a final level of 100 nM via transfection with Lipofectamine 2000 system (Invitrogen). In the overexpression of HOTAIR, the sequences of HOTAIR were propagated into vector pcDNA3.1 to create pcDNA3.1/HOTAIR and empty vector was regarded as negative control.

**CCK-8 assay**

We plated 10,000 cells/well in 96-well plates, followed by introduction of 10 μl of CCK-8 reagent (Dojindo Laboratories, Japan) to all the wells daily, as described by the manufacturer. Subsequently, we allowed the cells to grow for two hours at 37°C and then a microplate reader (BioTek, United States) was adapted to measure the OD values at 450nm.

**Flow cytometric analysis**

Cells were harvested and washed twice with phosphate-buffered saline (PBS). The cells were suspended in annexin V-binding buffer and the indicated amount of propidium iodide and annexin V-FITC (BD Pharmingen, San Diego, CA, USA). Flow cytometry was analyzed with a FACS Aria II instrument (BD Biosciences).

**Caspase-3/7 Assay**
Each cell line was plated in triplicate at a density of 5,000 cells per well (white, clear-bottomed 96-well plates) and was incubated at 37°C in a humidified chamber with 5% CO₂ overnight. The caspase-3/7 enzymatic activities of each cell line were measured using an Apo-ONE homogeneous caspase-3/7 assay (Promega, Madison, WI, USA) according to the manufacturer’s recommendations.

Mouse Studies

All animal experiments with cell line xenografts were performed in accordance with the guidelines approved by the Animal Ethics Boards of Xinjiang Medical University. Five-week-old female nude mice (Shanghai SLAC Laboratory Animal Co., Ltd) were raised in specific pathogen-free animal facilities with humane care. A total of 5 × 10⁶ cells stably transfected with sh-HOTAIR or sh-NC in 100 μL PBS mixed with 100 μL Matrigel (BD Biosciences, CA, USA) were subcutaneously injected into both flanks of nude mice. Tumor volume was measured in two dimensions every 3 days, starting at after 9 days of implantation, and was used to calculate the volume as V = ab²/2. At 29 days after tumor implantation, tumors were excised and fixed in 4% neutral PFA solution for morphological and IHC analyses.

Immunohistochemistry

Paraffin sections were dewaxed to water. Rinse with distilled water and soak in PBS for 5 minutes. The sections were incubated in 3%H₂O₂ at room temperature for 10 minutes to eliminate endogenous peroxidase activity and then washed with PBS for 3 times. Thereafter, sections were blocked in 5% normal goat serum and incubate at room temperature for 10 minutes. Then, the sections were incubated with the primary antibodies (ki67, 1:800, Proteintech, China;) overnight at 4°C. Subsequently, HRP conjugated second antibodies was added and incubate for 1 h at room temperature. A DAB kit (Beyotime, Shanghai, China) was used to visualize the positive staining. Finally, the slides were observed and imaged under a microscopy (Olympus, Japan).

RNA fluorescence in situ hybridization (RNA-FISH) assay

FISH Tag™ RNA Multicolor Kit (Invitrogen, USA) was used to detect lncRNA HOTAIR. In brief, tissues were fixed in formaldehyde, permeabilized by Triton X-100, and then hybridization was performed using labeled probes in a moist chamber at 42°C overnight in the darkroom. Cells and tissues were added with 2 × 10 μl antifade reagent containing DAPI under coverslips. The fluorescence images were observed using the LSM 5 Pascal Laser Scanning Microscope (Zeiss, Germany). If necessary, the protein immunofluorescence assays were performed after the FISH assays completed.

Chromatin immunoprecipitation (ChIP) assays

The EZ-Magna ChIP kit (Millipore, Billerica, USA) was used to conduct ChIP assays. Briefly, cells were treated with formaldehyde and incubated for 10 min to generate DNA–protein cross-links. qPCR was used to analyze the precipitated DNA fragments, which generated from ranging 200 to 300 bp using
sonication. ChIP assays employed Antibodies including anti-STAT3 (Cell Signaling Technology, USA) and IgG for each immunoprecipitation.

**Luciferase reporter assay**

HOTAIR sequences containing the mutant (MUT) or wild-type (WT miR-216a-docking site was amplified using Genepharma and propagated (separately) via transfection into the psi-CHECK2 luciferase enzyme reporter vector (Promega Corporation, United States). The prepared luciferase enzyme reporter vectors are termed as HOTAIR-WT and HOTAIR-MUT, respectively. The Lipofectamine® 2000 system was adapted to co-insert HEK-293 T cells with either the miRNA-216a mimic or miR-NC and either HOTAIR-WT or HOTAIR-MUT for the reporter assay. Luciferase enzyme activities were explored in the Dual-Luciferase enzyme Reporter Assay (Promega Corporation) at 48 hours after insertion. The Renilla luciferase enzyme activity acted as the standard for firefly luciferase enzyme activity.

**RNA immunoprecipitation assay (RIP)**

Cells were washed in pre-cold PBS and lysed in RIP buffer at °C for half an hour and treated with magnetic beads conjugated to antibodies against Ago2 (Millipore). Immunoprecipitated RNA was extracted for quantitative real-time PCR. For MS2-RIP assay, cells treated with pMS2-GFP were co-transfected with pcDNA3.1-MS2 and pcDNA3.1-HOTAIR-MS2 for 48 hours. Thereafter, cells were incubated with GFP antibody (Roche Diagnostics GmbH, Mannheim, Germany) and the Magna RIP RNA-Binding Protein Immunoprecipitation Kit in accordance with the guidebook for user. After collecting and lysing, cell suspension was cultured with magnetic beads. The antibody was added and incubated all night. After RNA purification, the isolated RNA was detected by quantitative real-time PCR to quantify the presence of the binding targets.

**Statistical analysis**

All data were presented at least three independent experiments and were shown as means ± standard deviation (SD) via GraphPad Prism 7.0 software (Graph Pad Software, La Jolla, CA, USA). The student's t-test or Mann-Whitney U test was used to compare the difference between the two groups when appropriate. And paired samples were analyzed using the paired t-test. χ2 analysis or Fisher exact probability analysis was used to analyze the clinical characteristics of pancreatic cancer when appropriate. Multiple comparisons were performed using the one-way ANOVA test. Spearman's correlation coefficient was used to analyze the correlation between the expressions of two genes via R software (version 3.6.3). Kaplan-Meier analysis and a log-rank test were used to compare the different survival rates. Cox regression models were performed to assess survival differences and hazard ratios (HR). All statistical tests were two-sided.

**Results**

**Upregulation of HOTAIR is associated with gefitinib resistance in NSCLC**
We screened 5 pairs of patients with different gefitinib-resistant-related lncRNAs in gefitinib-resistant NSCLC tissues and gefitinib-sensitive NSCLC tissues by IncRNA microarray analysis to identify gefitinib-resistant NSCLC-specific exosomal IncRNAs. After the combined analysis, a total of 110 significantly up-regulated and 128 significantly down-regulated IncRNAs were found in gefitinib-resistant NSCLC tissues compared with gefitinib-sensitive NSCLC tissues (fold change $\geq 2$ and $P \leq 0.05$). The heatmap showed the different expression of most 5 up- and 5 down-regulated IncRNAs and we preferentially selected top 10 elevated IncRNAs for further verification by qRT-PCR (Fig. 1A). The qRT-PCR showed that both the top 5 up-regulated IncRNAs were up-regulated in gefitinib resistant NSCLC tissues, which was consistent with the microarray data (Fig. 1B). Furthermore, we found that inhibition of HOTAIR in HCC827GR and PC9GR cell lines could reverse gefitinib resistance, while other 4 IncRNAs showed little effect (Fig. 1C).

**HOTAIR was highly expressed in gefitinib-resistant NSCLC tissues and cells**

Moreover, the expression level of HOTAIR was examined by quantitative real-time PCR analysis in NSCLC samples and paired adjacent normal tissues (ANT) collected from NSCLC patients. Among all differentially expressed IncRNAs between tumor and normal tissues, HOTAIR was the most upregulated in tumor samples (Fig. 1D), and its expression was highest in gefitinib-resistant NSCLC tissues (Fig. 1E). Furthermore, the ROC curve and AUC (area under the curve) was generated to evaluate the diagnostic accuracy of HOTAIR and the AUC value of HOTAIR was 0.8510 (95% CI= 0.782–0.921, $P < 0.0001$; Fig. 1F). These results suggested that HOTAIR may be a potential noninvasive biomarker for the diagnosis of NSCLC.

Subsequently, we found that higher level of HOTAIR was determined in patients with advanced tumor stage ($P = 0.0004$) and large tumor size ($P = 0.007$) (Table 1). To analyze the prognostic potential of HOTAIR in NSCLC patients, we plotted Kaplan-Meier surviving curves. According to the data shown in Figure 1G, high level of HOTAIR was closely associated with the low overall survival rate of NSCLC patients, indicating the potential prognostic role of HOTAIR in NSCLC patients ($P=0.016$). Then, experiments were performed at the cellular level. HOTAIR expression was distinctively higher in gefitinib-resistant HCC827GR and PC9GR cells, and obviously lower in human bronchial epithelial cells 16HBE (Fig. 1H; $P < 0.01$), indicated that HOTAIR might be a participant in tumorigenesis of NSCLC.

**HOTAIR can be transferred from resistant cells to parental cells via exosomes**

It was shown that IncRNA can be transferred from cells to cells via exosomes. Thus, we hypothesized whether HOTAIR could be transferred from gefitinib-resistant cells to gefitinib-sensitive cells via exosomes. We measured the abundance change of extracellular HOTAIR by treatment with RNase or RNase + Triton X100, and we found that HOTAIR expression in culture medium was little affected through the treatment of RNase alone but notably reduced when simultaneously treated with RNase and Triton X100, indicating that extracellular HOTAIR was secreted by packaging into exosomes and protected by membrane (Fig. 2A). Then, we purified exosomes from culture medium (CM), which was identified through electron microscopy (TEM), nanoparticle tracking analysis (NTA) and Western blot analysis. It
was revealed that the separated extracellular vesicles (EVs) were indeed exosomes and the exosomes isolated from gefitinib-resistant cells had similar typical goblet morphology, size and number. Exosomes extracted from condition media of gefitinib-resistant and control cells were identified as membrane-encapsulated particles with a range of 50 to 100 nm in size by transmission electron microscopy (Fig. 2B-C). The NTA results demonstrated that isolated exosomes showed a similar size distribution, and the peak size range was 80-135 nm. Western blot analysis confirmed the presence of three well-known exosomal markers, CD63, TSG101, and Hsp 70 (Fig. 2D). Subsequently, we explored whether HOTAIR was incorporated into exosomes. ExoQuick purification kit (System Biosciences) was used to isolate exosomes from culture medium of gefitinib-resistant cells, and the exosomes were then subjected to qRT-PCR. As anticipated, the abundance of HOTAIR was prominently increased in secreted exosomes collected from culture medium of gefitinib-resistant cells compared with that in culture medium from parental cells (Fig. 2E). We analyzed the expression of HOTAIR in 70 NSCLC patients by qRT-PCR. We observed that exosomal HOTAIR expression level was significantly upregulated in NSCLC patients compared with healthy controls. Furthermore, HOTAIR was more highly expressed in gefitinib-resistant group than gefitinib-sensitive group (Fig. 2F; P<0.01).

Then, we examined whether these exosomes could deliver HOTAIR to recipient cells. After coculture of the parental cells with the purified exosomes for 12 h, the expression of HOTAIR was upregulated in the parental cells (Fig. 2G). We further investigated whether the HOTAIR transferred via exosomes conferred a gefitinib resistant phenotype of the parental cells. Parental cells were stably transfected with sh-HOTAIR after coculture with the exosomes isolated from gefitinib-resistant cells. It was found that co-culture of parental cells with exosomes increased the IC50 and reduced gefitinib-induced apoptosis, but inhibition of HOTAIR reversed these effects induced by exosomes (Fig. 2H-I). Collectively, these results suggest that HOTAIR is contained in cancer-secreted exosomes and exosomes from gefitinib-resistant cells could confer the resistance to gefitinib by delivering HOTAIR.

**HOTAIR expression modulates gefitinib sensitivity in NSCLC cells**

To further validate the expression level of HOTAIR on gefitinib resistance, we overexpressed HOTAIR in HCC827 and PC9 cells but silenced it in HCC827GR and PC9GR cells. As a result, the knockdown of HOTAIR rendered HCC827GR and PC9GR cells more sensitive to gefitinib compared with control group, as demonstrated by the decreased IC50 value of gefitinib (Fig. 3A-B). Moreover, silencing of HOTAIR significantly inhibited the percent of HCC827GR and PC9GR cells in G2 phase and promoted the gefitinib-induced cell apoptosis of HCC827GR and PC9GR cells after exposure to gefitinib (1 μM) (Fig. 3C-E, Fig. S1A and C). However, the opposite phenomenon was observed after overexpression of HOTAIR (Fig. 3F-G; P<0.01). Overexpression of HOTAIR significantly decreased the percent of cells in G0/G1 phase and inhibited the cell apoptosis of HCC827 and PC9 cells in the presence of gefitinib (1 μM) (Fig. 3H-J; Fig. S1B and D; P<0.01).

**HOTAIR knockdown enhanced the anti-tumor effect of gefitinib in NSCLC in vivo**
We further examined the possibility of HOTAIR being involved in gefitinib resistance of NSCLC in vivo. Inhibition of HOTAIR plus gefitinib decreased tumor volumes and weights compared with control group (Fig. 4A-B). In consistent with this, inhibition of HOTAIR plus gefitinib decreased the positive rate of Ki67 of the nude mice models (Fig. 4 C-D). These findings suggested that HOTAIR could modulate gefitinib sensitivity in vivo.

**HOTAIR may act as a ceRNA of miR-216a in NSCLC cells**

The primary sub-localization of HOTAIR was probed to be cytoplasmic using LncLocator prediction (http://www.csbio.sjtu.edu.cn/bioinf/IncLocator/) (Fig. 5A). In order to verify this hypothesis, then we examined the subcellular localization of HOTAIR abundance and found that HOTAIR is abundant and stable in the cytoplasm of HCC827GR and PC9GR cells using a nuclear and cytoplasmic protein extraction assay (Fig. 5B). Experiment result from FISH assay indicated that abundance of HOTAIR was in cytoplasmic of PC9GR cells (Fig. 5C). Five potential miRNAs for HOTAIR were predicted by DIANA and starbase (Fig. 5D). Subsequently, MS2-RIP assay demonstrated the binding of these five potential miRNAs (miR-20a-5p, miR-122, miR-216a, miR-331-3p and miR-138) to HOTAIR. As a result, miR-216a showed the strongest affinity to HOTAIR-MS2 beads (Fig. 5E). Then, we examined the expression level of miR-216a in cells with high or low HOTAIR level. The results showed that miR-216a was efficiently upregulated by silenced HOTAIR but was downregulated by overexpressed HOTAIR (Fig. 5F). The predicted binding sequence between HOTAIR and miR-216a was shown (Fig. 5G). The dual luciferase reporter assay was preformed to confirm the direct interaction between HOTAIR and miR-216a,. As presented in Fig. 5H, miR-216a mimics efficiently decreased the luciferase activity of reporter containing wild type HOTAIR (HOTAIR-WT) vector in HCC827GR and PC9GR cells, but it had little effect on the luciferase activity of mutant type HOTAIR (HOTAIR-MUT) vector. All these experimental results indicated that HOTAIR might act as a ceRNA to regulate miR-216a.

**HOTAIR knockdown inhibited gefitinib resistance by upregulating miR-216a in NSCLC cells**

Next, we measured the levels of miR-216a expression in a panel of NSCLC cell lines. The expression of miR-216a was obviously decreased in HCC827GR and PC9GR cells (Fig. 6A). Also, miR-216a was expressed at low level in gefitinib-resistant patients in contrast with that in gefitinib-sensitive patients (Fig. 6B), indicating that miR-216a was involved in gefitinib resistance in NSCLC. To gain insight into whether HOTAIR affected gefitinib resistance of NSCLC cells via modulation of miR-216a, we further performed rescue assays to confirm how miR-216a modulated gefitinib resistance. To change the expression level of miR-216a in NSCLC cells, miR-216a inhibitor and miR-216a mimics were separately transfected into NSCLC cells (Fig. 6C). CCK-8 assay suggested that the HOTAIR propelled gefitinib-resistant NSCLC cells viability promotion was ameliorated via miR-216a inhibition in the presence of gefitinib (1 μM) (Fig, 6D-E). Simultaneously, flow cytometry analysis disclosed that deficiency of HOTAIR expedited gefitinib-induced apoptosis, while silence of miR-216a effectively attenuated the promoting effect of HOTAIR knockdown on gefitinib-induced apoptosis (Fig. 6F and G). Caspase-3 activity of HOTAIR-silencing gefitinib-resistant NSCLC cells was increased and later partially inhibited
following the silence of miR-216a in the presence of gefitinib (1 μM) (Fig. 6H and I). In summary, deletion of miR-216a partly abolished the promotion effect of HOTAIR down-regulation on gefitinib sensitivity in gefitinib-resistant NSCLC cells.

**HOTAIR positively regulated MAP1S expression by interacting with miR-216a in gefitinib-resistant NSCLC cells**

Subsequently, four bioinformatics tools (miRWalk, miRanda, RNAhybrid, and Targetscan) unveiled four shared target genes (Bax, FoxO1, SMAD7 and MAP1S) of miR-216a (Fig. 7A). To further verify the downstream targets of HOTAIR, mRNA levels of four candidate target genes were detected after silencing HOTAIR, and we found only MAP1S was downregulated (Fig. 7B). To verify whether MAP1S was the direct target of miR-216a, we first performed the miRNA biotin pull-down assay. We found that miR-216a could significantly enrich the 3’UTR of MAP1S mRNA in HCC827GR and PC9GR cells (Fig. 7C). The binding sequence of miR-216a to MAP1S 3’UTR was predicted and obtained (Fig. 7D). Then, we examined the luciferase activity of reporter containing wild type MAP1S (MAP1S-WT) or mutant type MAP1S (MAP1S-MUT) in HCC827GR and PC9GR cells transfected with miR-216a mimics. The results suggested that the luciferase activity of MAP1S-WT vector but not MAP1S-MUT vector was decreased by miR-216a mimics (Fig. 7E).

To further confirm the effects of HOTAIR on MAP1S expression, PC9GR cells were transfected with the HOTAIR shRNA, and the MAP1S protein levels were detected using western blotting. The results showed that knockdown of HOTAIR expression significantly reduced the MAP1S protein levels in PC9GR cells (Fig. 7F). Moreover, inhibition of HOTAIR mediated decrease of MAP1S protein expression was significantly recuperated following miR-216a inhibitors (Fig. 7F). To determine the expression levels of MAP1S in NSCLC, we analysed the MAP1S expression in NSCLC tissues by qRT-PCT and IHC. The expression of MAP1S mRNA was obviously increased in gefitinib resistant NSCLC patient tissues than that in gefitinib sensitive NSCLC patient tissues (P<0.01; Fig. 7G). The results of IHC showed that MAP1S expression in gefitinib resistant NSCLC patient tissues was significantly upregulated compare with that in gefitinib sensitive NSCLC patient tissues (P<0.01; Fig. 7H). All of these data made us draw a conclusion that HOTAIR positively regulated MAP1S expression by interacting with miR-216a in gefitinib-resistant NSCLC cells.

**STAT3 activated HOTAIR expression in the NSCLC cells**

Accumulating evidences have been reported that several epigenetic regulators and key transcription factors (TFs) contributed to IncRNAs dysregulation in human cancers. To explore the mechanism of HOTAIR upregulation in NSCLC, we applied online bioinformatical software programs JASPAR (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl) to analyze promoter region of HOTAIR and we found that there are four STAT3 binding sites with scores >10 at the regions E1 (~1330 to −1320, TCTCCTCCTCT), E2 (~766 to −756, GCTCCGCCCAG), E3 (~44 to −34, CCCCACCCCG) and E4 (~19 to −9, TCCCCTCCTCT) in the HOTAIR promoter regions (Fig. 8A).
Firstly, we designed three paired primers and ChIP assay was performed to detect which region in the HOTAIR promoter mediated STAT3-binding to the HOTAIR promoter. The ChIP data showed that STAT3 could bind to E2 sites (Fig. 8B). To further verify this result, we cloned the full promoter region of HOTAIR and E2 deleted promoter region into pGL3-basic reporter. The results showed that the deletion of E2 sites significantly impaired the effect of STAT3 on HOTAIR transcription activation (Fig. 8C), revealing that STAT3 could bind to the promoter of HOTAIR to regulate HOTAIR transcription. Moreover, we explored whether the overexpression of HOTAIR is mediated by STAT3. We silenced endogenous STAT3 expression in NSCLC cells by transfecting with siRNAs (siSTAT3–1, siSTAT3-2) targeting the STAT3 gene. HOTAIR levels were markedly decreased in NSCLC cells transfected with siRNAs (Fig. 8D). Furthermore, we detected the expression of STAT3 mRNA in NSCLC tissues and found that STAT3 mRNA was markedly upregulated in NSCLC tissues (Fig. 8E). These results indicated that HOTAIR upregulation in NSCLC is mediated by STAT3.

Discussion

Recently, the non-coding RNAs (ncRNAs) including miRNAs and IncRNAs have received growing attention in human tumors particularly in NSCLC\(^{16-17}\). A few studies have evaluated the involvement of ncRNAs in the drug resistance of various cancers, and the underlying mechanisms remain to be elucidated \(^{18}\). Previous studies of the competing endogenous RNA (ceRNA) focused on the network regulation between ncRNAs \(^{19-21}\). However, there is a lack of studies on the mutual regulation of gene expression between ncRNAs. In this study, we identified that drug resistance-related IncRNA HOTAIR, as an independent prognostic marker for progression and good outcome of NSCLC, was underexpressed in NSCLC tissues. Mechanistically, HOTAIR regulated the levels of MAP1S by regulating the HOTAIR-miR-216a pathway. Moreover, STAT3 regulates HOTAIR transcription, indicating that STAT3/HOTAIR/miR-216a/MAP1S pathway play a novel therapeutic strategy against gefitinib resistance.

LncRNAs were recognized as important contributors in facilitating the development of drug resistance of NSCLC \(^{22}\). Liu et al. found that knockdown of HOTAIR increased susceptibility to apoptosis and restored gefitinib sensitivity in resistant NSCLC cells \(^{23}\). What was consistent with these previous investigations, IncRNAs microarray could provide valuable information for screening functional IncRNAs for further study candidate circRNAs. Here, we found HOTAIR via IncRNA microarray assessment that was dramatically upregulated in gefitinib resistant cells and tissues of individuals with NSCLC. It is very important to us that the IncRNA identified in our study will successfully achieve clinical transformation and application. As a tumor suppressor, HOTAIR is expressed at a higher level in NSCLC. It possesses the potential to be a prognostic biomarker or a therapeutic target for NSCLC. Our findings demonstrated that knockdown of HOTAIR promoted gefitinib-induced apoptosis but inhibited the proliferation of NSCLC cells \textit{in vitro}. Moreover, the results of xenografts in nude mice demonstrated that HOTAIR conferred gefitinib resistance \textit{in vivo}. 
Recent studies have documented that cancer cells-derived exosomes engage in initiation and progression of various cancer processes, including drug-resistance. Those vesicles could influence surrounding cells by delivering alternative signals and thus gives advantages to cancer cells to survive chemotherapy. Exosomes could secrete lncRNAs from cancer cells into body fluids, suggesting that exosomal lncRNAs can be potential biomarkers. Here, we performed TEM to reveal the shapes and size of exosomes and used exosome markers to verify. Furthermore, we also demonstrated that the parental cells treated with exosomes containing HOTAIR could induce an elevated gefitinib resistance. Therefore, these findings indicated that exosomal HOTAIR promoted the development of gefitinib resistance in NSCLC cells.

Recently, accumulating data have showed that STAT3 was a key transcription factor in various cancers. We determined that STAT3 could interact with the promoter of HOTAIR, indicated that STAT3 activated HOTAIR translational expression to modulate HOTAIR in NSCLC. Previous study showed that lncRNA play a role in the miRNA sponge to remove the suppressive effect of miRNA on its target genes. LncRNAs typically participate in posttranscriptional regulation by interacting with microRNAs. We found that HOTAIR promoted drug resistance through antagonizing miR-216a. The localization of lncRNAs is important for their function and HOTAIR mainly locates in the cytoplasm. More importantly, the loss function assay indicated that knockdown HOTAIR increased the expression level of miR-216a in NSCLC cells. In general, miRNAs regulate cell proliferation, apoptosis and differentiation. Furthermore, downregulation of miR-216a dramatically rescued HOTAIR expression that was downregulated by HOTAIR silencing, as well as the cell proliferation and chemoresistance abilities of NSCLC cells. Besides, public bioinformatics websites have reported that miR-216a can directly target MAP1S. Our data showed that miR-216a bound to the MAP1S mRNA 3'-untranslated region (3'UTR) and affected gefitinib sensitivity of NSCLC cells, which indicated that miR-216a/MAP1S axis was one of the important mechanisms in HOTAIR-promoted drug resistance of NSCLC.

**Conclusions**

In conclusion, we found that HOTAIR directly regulated miR-216a expression to promote gefitinib resistance in NSCLC, and demonstrated that knockdown of HOTAIR could increase the gefitinib sensitivity of NSCLC cells. Thus, combining gefitinib with targeting HOTAIR may be a promising strategy to enhance gefitinib sensitivity in NSCLC.

**Declarations**

**Ethics approval and consent to participate**

Written informed consents were obtained from all participants and this study was permitted by the Ethics Committee of The Affiliated Tumor Hospital of Xinjiang Medical University.

**Consent for publication**

All authors have read and approved the final manuscript.
Competing interests

There are no conflicts to declare.

Authors' contributions

HZ mainly did the experiment and wrote the paper, XW and XZ helped do the cell experiment and review the manuscript, SL, GG, and CL helped do analysis and interpretation of data. CL mainly constructed the idea of this article and provided administrative, technical and material support. All authors read and approved the final manuscript.

Research involving patients

All procedures performed in studies involving humans were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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### Tables

**Table 1 Correlation between HOTAIR expression and clinicopathologic characteristics of NSCLC patients.**

|               | Overall (n=70) | HOTAIR Low (n=35) | HOTAIR High (n=35) | P  |
|---------------|----------------|-------------------|-------------------|----|
| **Gender**    |                |                   |                   |    |
| Male          | 42             | 22                | 20                | 0.807 |
| **Age, y**    |                |                   |                   |    |
| ≥ 60          | 52             | 24                | 28                | 0.412 |
| **Smoking status** |            |                   |                   |    |
| yes           | 40             | 18                | 22                | 0.469 |
| **Tumor size** |                |                   |                   |    |
| ≥ 4           | 30             | 9                 | 21                | 0.007 |
| **TNM stage** |                |                   |                   |    |
| I-II          | 45             | 30                | 15                | 0.0004 |

### Figures
Figure 1

Upregulation of HOTAIR is associated with gefitinib resistance in NSCLC. A. The heat map showed the top 10 dysregulated lncRNAs between gefitinib-resistant NSCLC tissues vs. gefitinib-sensitive NSCLC tissues; B. The qRT-PCR results indicated HOTAIR showed highest fold-change in the gefitinib resistant NSCLC tissues than in the gefitinib sensitive NSCLC tissues; C. Inhibition of HOTAIR reversed gefitinib resistance in both HCC827GR and PC9GR cells; D. The level of HOTAIR was significantly increased in NSCLC tissues compared with paired adjacent normal tissues; E. The level of HOTAIR was significantly increased in gefitinib-resistant NSCLC tissues compared to gefitinib-sensitive NSCLC tissues; F. Evaluation the diagnostic performance of HOTAIR for NSCLC diagnosis; G. Kaplan-Meier curve revealed that high expression of HOTAIR was relative to a poor overall survival in NSCLC patients; H. HOTAIR expression was distinctively higher in gefitinib-resistant NSCLC cell lines HCC827GR and PC9GR. All tests were at least performed three times. Data were expressed as mean ± SD. ***P < 0.001; **P < 0.01;
Exosome-Mediated Transfer of HOTAIR Induced Gefitinib Resistance in NSCLC Cells

A. The expression level of HOTAIR was detected by qRT-PCR following treatment with RNase or RNase + Triton X100; 
B. TEM scanning showed the exosomes' images released by HCC827GR cells; 
C. TEM scanning showed the exosomes' images released by PC9GR cells; 
D. Exosomal protein markers (CD63, TSG101, hsp70) were determined using the Western blot analysis; 
E. The qRT-PCR analysis was used to examine exosomal HOTAIR expression in gefitinib-resistant and -sensitive NSCLC cells; 
F. Serum exosome HOTAIR level was more highly expressed in gefitinib-resistant group than gefitinib-sensitive NSCLC patients; 
G. qRT-PCR revealed an increased expression of HOTAIR in HCC827 and PC9 cells incubated with exosomes (extracted from HCC827GR and PC9GR cells); 
H. Exosomes promoted gefitinib resistant for HCC827 cells; 
I. Exosomes promoted gefitinib resistant for PC9 cells. Data were expressed as mean ± SD. **P < 0.01;

Figure 2
HOTAIR enhances gefitinib chemosensitivity of NSCLC in vitro. A. Downregulation of HOTAIR rendered HCC827GR cells more sensitive to gefitinib compared with control group; B. Downregulation of HOTAIR rendered PC9GR cells more sensitive to gefitinib compared with control group; C. Downregulation of HOTAIR significantly increased the percent of HCC827GR cells in G0/G1 phase; D. Downregulation of HOTAIR significantly increased the percent of PC9GR cells in G0/G1 phase; E. Downregulation of HOTAIR significantly promoted the cell apoptosis of HCC827GR and PC9GR cells in the presence of gefitinib (20 μM); F. Overexpression of HOTAIR rendered HCC827 cells more resistant to gefitinib compared with control group; G. Overexpression of HOTAIR rendered PC9 cells more resistant to gefitinib compared with control group; H. Overexpression of HOTAIR significantly increased the percent of HCC827 cells in G0/G1 phase; I. Overexpression of HOTAIR significantly increased the percent of PC9 cells in G0/G1 phase; J. Overexpression of HOTAIR significantly inhibited the cell apoptosis of HCC827 and PC9 cells in the presence of gefitinib (20 μM). All tests were at least performed three times. Data were expressed as mean ± SD. **P < 0.01;
Figure 4

HOTAIR knockdown enhanced the anti-tumor effect of gefitinib in NSCLC in vivo. A. Volume of tumors that developed in xenografts from different groups; B. Weights of tumors that developed in xenografts from different groups; C. The immunohistochemistry assay showed that the tumors treated with sh-HOTAIR plus gefitinib displayed an increased proliferation percentage of Ki-67 positive tumor cells compared with the control group; D. The percentage of Ki67 positive cells in xenografts from different groups. All tests were at least performed three times. Data were expressed as mean ± SD. **P < 0.01;
HOTAIR may act as a ceRNA of miR-216a in NSCLC cells. A. LncLocator found that HOTAIR is predominantly presented in the cytoplasm; B. qRT-PCR analysis of HOTAIR expression in cytoplasm or nucleus after separation of cytoplasm or nucleus of HCC827GR and PC9GR cells; C. HOTAIR was observed to be enriched in PC9GR cytoplasm by fluorescence in situ hybridization (FISH) assay; D. Five potential miRNAs for HOTAIR were predicted by DIANA and starbase; E. MS2-RIP assay indicated that
miR-216a showed the strongest affinity to HOTAIR-MS2 beads; F. The results showed that miR-216a was efficiently upregulated by silenced HOTAIR but was downregulated by overexpressed HOTAIR; G. The predicted binding sequence between HOTAIR and miR-216a; H. The luciferase reporter systems showed that miR-1299 mimic considerably reduced the luciferase activity of the WT-HOTAIR luciferase reporter vector compared with negative control, while miR-216a mimic did not pose any impact on the luciferase activity of MUT-HOTAIR-transfected HCC827GR and PC9GR cells; All tests were at least performed three times. Data were expressed as mean ± SD. **P < 0.01.

**Figure 6**

HOTAIR knockdown inhibited gefitinib resistance by upregulating miR-216a in NSCLC cells A. The expression of miR-216a was obviously decreased in HCC827GR and PC9GR cells; B. miR-216a was expressed at low level in gefitinib-resistant patients in contrast with that in gefitinib-sensitive patients; C. miR-216a inhibitor and miR-216a mimics were separately transfected into NSCLC cells; D. CCK-8 assay
suggested that the HOTAIR propelled HCC827GR cells viability promotion was ameliorated via miR-216a inhibition in the presence of gefitinib (20 μM); E. CCK-8 assay suggested that the HOTAIR propelled PC9GR cells viability promotion was ameliorated via miR-216a inhibition in the presence of gefitinib (20 μM); F. Flow cytometry analysis disclosed that silence of miR-216a effectively attenuated the promoting effect of HOTAIR knockdown on gefitinib-induced apoptosis in HCC827GR cells; G. Flow cytometry analysis disclosed that silence of miR-216a effectively attenuated the promoting effect of HOTAIR knockdown on gefitinib-induced apoptosis in PC9GR cells; H. Caspase-3 activity of HOTAIR-silencing HCC827GR cells was increased and later partially inhibited following the silence of miR-216a in the presence of gefitinib (20 μM); I. Caspase-3 activity of HOTAIR-silencing PC9GR cells was increased and later partially inhibited following the silence of miR-216a in the presence of gefitinib (20 μM); All tests were at least performed three times. Data were expressed as mean ± SD. **P < 0.01.
HOTAIR positively regulated MAP1S expression by interacting with miR-216a in gefitinib-resistant NSCLC cells. A. Venn diagram showing 4 genes that are putative miR-216a targets computationally predicted by four algorithms (miRanda, RNAhybrid, miRWalk and TargetScan); B. mRNA levels of 4 candidate target genes were detected after silencing HOTAIR; C. miR-216a could significantly enrich the 3'UTR of MAP1S mRNA; D. The binding sequence between miR-216a and MAP1S; E. Luciferase reporter assay demonstrated miR-216a mimics significantly decreased the luciferase activity of MAP1S-wt in HCC827GR and PC9GR cells; F. Inhibition of HOTAIR mediated decrease of MAP1S protein expression was significantly recuperated following miR-216a inhibitors in PC9GR cells; G. The level of MAP1S was significantly upregulated in NSCLC tissue compared with normal tissue from the TCGA database; H. Kaplan–Meier survival analysis from TCGA NSCLC datasets suggested that high MAP1S expression in NSCLC tissues is not significantly associated with overall survival; I. Kaplan–Meier survival analysis from TCGA NSCLC datasets suggested that high MAP1S expression in NSCLC tissues is not significantly associated with disease free survival. All tests were at least performed three times. Data were expressed as mean ± SD. **P < 0.01.
Figure 8

The transcription factor STAT3 is involved in HOTAIR upregulation. A. The predicted positions of putative STAT3 binding motif in ~2500 bp human HOTAIR promoter. B. Quantitative ChIP assays were performed to show direct binding of STAT3 to endogenous HOTAIR promoter regions. C. A luciferase reporter assay was used by cotransfecting the full HOTAIR promoter (HOTAIR-pGL3-F) or deleted HOTAIR promoter fragment E2 (HOTAIR-pGL3-D) with STAT3 expression plasmid or blank vector in 293T cells. Luciferase activities were expressed as relative to that of the pGL3 vector. D. qPCR analysis of HOTAIR expression levels following the treatment of siSTAT3-1, siSTAT3-2 in NSCLC cells. E. STAT3 mRNA was markedly upregulated in NSCLC tissues. Data were shown as mean ± SD of three independent experiments. **P < 0.01, ***P < 0.001