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Lentivirus-mediated silencing of HOTAIR lncRNA restores gefitinib sensitivity by activating Bax/Caspase-3 and suppressing TGF-α/EGFR signaling in lung adenocarcinoma

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Abstract. Secondary resistance is a major limitation in the efficacy of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor treatment of lung cancer. Previous studies have shown that expression of the long non-coding RNA HOX transcript antisense RNA (HOTAIR) is upregulated in lung cancer, which is correlated with metastasis and poor prognosis. However, the precise role of HOTAIR and its effects on gefitinib resistance in human lung adenocarcinoma are not known. To address this issue, in the present study we established a gefitinib-resistant (R)PC-9 human lung adenocarcinoma cell line and examined cell viability with the3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. We found that gefitinib concentrations <10 μM inhibited the viability of PC-9 but not RPC-9 cells in a dose-dependent manner. Lentivirus-mediated HOTAIR RNA interference induced cell apoptosis and S-phase arrest, as determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling and flow cytometry. Consistent with these observations, HOTAIR suppression was associated with tumor shrinkage and restoration of gefitinib sensitivity in RPC-9 xenograft mice. Immunohistochemical analyses and western blot revealed that HOTAIR silencing resulted in the upregulation of B cell lymphoma 2-associated X protein (Bax), Caspase-3 and transforming growth factor α (TGF-α) and downregulation of EGFR and B cell lymphoma 2 (Bcl-2) levels. These results indicate that HOTAIR normally prevents the activation of Bax/Caspase-3 while inducing TGF-α/EGFR signaling. Thus, targeting HOTAIR may be a novel therapeutic strategy for treating gefitinib-resistant lung adenocarcinoma.

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Introduction

Lung cancer is the malignancy with the highest morbidity and mortality among men and women worldwide (1,2). Non-small cell lung cancer (NSCLC) accounts for approximately 80-85% of cases, and can be classified into distinct histological subtypes (3), including adenocarcinoma and squamous cell carcinoma (3). Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKI) such as gefitinib are the first-line agent for treating advanced NSCLC (4). The majority of patients relapse within 6-12 months of treatment due to acquired resistance to EGFR-TKIs, with a 5-year survival rate of just 11% (5,6). Secondary resistance to EGFR-TKIs is a major factor limiting the success of lung cancer treatment. Therefore, developing novel strategies to resensitize lung tumors to these drugs is essential for improving the survival rate of patients.

Up to 90% of the genome is transcribed into non-coding RNA (ncRNA) (7,8), including long ncRNAs (lncRNA), which are over 200 nt long (9,10) and participate in a variety of biological processes (11,12). Although lncRNAs account for more than 68% of all ncRNAs, our knowledge of their functions is limited (13). Clarifying the roles of cancer-related lncRNAs can improve the survival rate of patients, especially those with tumor recurrence.

The lncRNA Homeobox (Hox) transcript antisense RNA (HOTAIR) is encoded by the antisense strand of the *HoxC* gene (14). HOTAIR recruits polycomb repressive complex 2 and the lysine-specific histone *demethylase*/repressor

element-1 silencing transcription factor (REST)/CoREST complex for trimethylation and dimethylation of histone H3 on lysines 27 and 4, respectively, leading to target gene silencing. Previous studies have demonstrated that HOTAIR is overexpressed in multiple types of cancer, including lung cancer, which is correlated with metastasis and poor prognosis (15-18). However, the mechanism by which HOTAIR mediates gefitinib resistance in human lung adenocarcinoma is not known.

To address this issue, we investigated the role of HOTAIR in a gefitinib-resistant lung adenocarcinoma PC-9 cell line (RPC-9) *in vitro* and *in vivo*. We found that HOTAIR silencing restored gefitinib sensitivity by activating B cell lymphoma 2-associated X protein (Bax)/Caspase-3 and suppressing transforming growth factor (TGF)-α/epidermal growth factor receptor (EGFR) signaling pathways, suggesting that it is a novel therapeutic target for lung cancer treatment.

Materials and methods

Cell lines and reagents. PC-9 human lung adenocarcinoma cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Roswell Part Memorial Institute (RPMI)-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; BI, Beit-Haemek, Israel), 2.05 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C and 5% CO_2/95% humidified air. Gefitinib was from Selleck Chemicals (Houston, Texas, USA). The gefitinib-resistant cell line RPC-9 was established by exposing PC-9 cells to increasing concentrations of gefitinib (0, 1.25, 2.5, 5, and 10 μM) for 1 month; cell viability was evaluated with the 5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-*tetrazolium* salt (MTS) assay.

Reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and 1 μg was reverse transcribed into cDNA with the PrimeScript RT Reagent kit with gDNA Eraser (RR047A; Takara Biotechnology Co., Ltd., Dalian, China). PCR was performed using SYBR Premix EX Taq II (RR820A; Takara Biotechnology Co., Ltd.) on an Mx3000P QPCR system (Agilent Technologies, Inc., Santa Clara, CA, USA) using the following forward and reverse primers synthesized by Sangon Biotech (Shanghai, China): HOTAIR, 5'-GGTAGAAAAAGCAACCACGAAGC-3' and 5'-ACATAAACCTCTGTCTGTGAGTGCC-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-TGCCTCCTGCACCACCAACT-3' and 5'-CCCGTTCAGCTCAGGGATGA-3'. The reaction conditions were as follows: 95˚C for 30 sec, and 40 cycles of 95˚C for 5 sec and 60˚C for 34 sec. Melting curve analysis was performed and relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH used as the reference gene. The experiment was performed using triplicate samples.

Lentivirus (LV) packaging and transduction. The LV vector GV113 (HU6-MCS-CMV-RFP) constructed by Shanghai Genechem Co., Ltd., (Shanghai, China) was used for stable knockdown of HOTAIR expression in RPC-9 cells. Short hairpin RNAs (shRNAs) used to target HOTAIR were: HOTAIR-sh1, 5'-AGAAATGCCACGGCCGCGTCC-3'; HOTAIR-sh2, 5'-ATGAGGAAAAGGGAAAATCTA-3'; and HOTAIR-sh3, 5'-CCAGTACCGACCTGGTAGAAA-3'. A negative control (NC) shRNA (5'-TTCTCCGAACGTGTCACGT-3') was used as a control. Cells were infected with LV in enhanced infection solution supplemented with polybrene according to the manufacturer's instructions and selected with puromycin (Sigma-Aldrich, St. Louis, MO, US) for 3 weeks to obtain stable cell lines.

Cell viability assay. The CellTiter 96 Aqueous Cell Proliferation Assay (Promega, Madison, WI, USA) was used according to the manufacturer's protocol to evaluate the sensitivity of PC-9 and RPC-9 cells to gefitinib. Cells were seeded in a 96-well cell culture plate at a density of $1x10^4$ cells per well in 200 μl of medium for 24 h and allowed to adhere overnight. On the following day, cells were treated with different concentrations of gefitinib or with RPMI-1640 medium as a negative control for 24, 48, 72, or 96 h. A 20-μl volume of MTS reagent was added to each well, followed by incubation for an additional 4 h at 37˚C and 5% CO_2. The absorbance at 490 nm was measured on a Tecan Infinite M200 microplate reader (Tecan Group, Ltd., Mannedorf, Switzerland). The percentage of viable cells was calculated relative to untreated control cells.

Annexin V-allophycocyanin (APC)/7-aminoactinomycin D (7-AAD) apoptosis assay. Apoptosis was evaluated using the Annexin V-APC/7-AAD Apoptosis Detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer's protocol. Briefly, cells ($1x10^5$/well) were seeded in a 96-well cell culture plate in RPMI-1640 medium with 10% FBS and incubated overnight at 37˚C. They were then treated on the following day with 10 μM gefitinib or left untreated at 37˚C in 5% CO_2 and 95% humidified air for 48 h. Both adherent and suspended cells were harvested and washed twice with cold 1x phosphate-buffered saline (PBS), then resuspended in 500 μl binding buffer. Annexin V-APC (5 μl) and 7-AAD (5 μl) were added to 500 μl of the cell suspension, followed by incubation for 15 min in the dark. Samples were analyzed within 1 h on a Novocyte flow cytometer (ACEA Biosciences, San Diego, CA, USA). The experiment was performed using triplicate samples.

Cell cycle analysis. After treatment with 10 μM gefitinib or incubation without treatment for 48 h, cells ($2x10^6$) were collected with trypsin-EDTA, washed with 1x PBS, fixed with 4 ml chilled 70% ethanol, and store overnight at -20˚C. Fixed cells were washed with PBS, treated with 100 μl RNase A at 37˚C for 30 min, and stained with 400 μl propidium iodide (PI) at 4˚C for 30 min in the dark. Cell cycling was analyzed by flow cytometry.

Terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) assay. The TUNEL assay was carried out using an *in situ* colorimetric TUNEL Apoptosis Assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. Briefly, RPC-9 cells ($5x10^3$) were seeded on coverslips and grown

to 70-80% confluence, then treated with 10 μM gefitinib or left untreated at 37˚C for 48 h. The cells were fixed in 4% paraformaldehyde at 37˚C for 60 min and rinsed with PBS for 5 min. After incubation with 0.1% Triton X-100 in PBS for 2 min on ice followed by 0.3% H_2O_2 in methanol for 20 min and three rinses with PBS, the cells were incubated with TdT enzyme and biotin-dUTP for 60 min at 37˚C. The stop buffer was added for 10 min, and cells were treated with horseradish peroxidase (HRP)-streptavidin for 30 min, then stained with diaminobenzidine (DAB) and imaged under a light microscope. The percentage of TUNEL-positive cells was calculated in five random fields for each group.

Tumor xenograft model. Female and male BALB/c athymic nude mice (4-6 weeks old, weighing 15-20 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd., (Shanghai, China). Animal protocols were approved by the Ethics Committee of Zhejiang Provincial People's Hospital. Mice were subcutaneously injected in the left or right dorsal region with RPC-9 cells ($1x10^7$) infected with LV-HOTAIR-shRNA or LV-NC-shRNA resuspended in 100 μl PBS. After 1 week, when tumors were about 5 mm in diameter, gefitinib (20 mg/kg/day, n=4) or vehicle (0.05% Tween 80 as a control, n=5) was administered by oral gavage once daily. Tumor diameter was measured with digital calipers each week, and the tumor volume was calculated with the formula $V=\pi/6$ (length x width^2). After 28 days, mice were sacrificed and tumors were excised for the TUNEL assay and immunohistochemistry.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tumor tissue samples were sectioned at a thickness of 5 mm. The sections were mounted on Superfrost glass slides (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA), de-paraffinized, and rehydrated in a graded series of ethanol. Antigen retrieval was performed in 0.1 M trisodium citrate buffer at pH 6.0. To block endogenous peroxidase activity, sections were treated with 3% H_2O_2 for 5 min, then blocked with 10% normal goat serum (Abcam, Cambridge, MA, USA) before overnight incubation at 4˚C with primary antibody. After rinsing with PBS, sections were incubated with a biotin-labeled secondary antibody for 20 min followed by HRP-streptavidin for 20 min at room temperature. Following treatment with DAB substrate, sections were counterstained with hematoxylin, dehydrated, and mounted in Permount (Thermo Fisher Scientific, Inc.). As a negative control, immunohistochemistry was performed without primary antibodies.

Western blotting. Total protein was extracted from tissues with radioimmunoprecipitation buffer supplemented with 1% phenylmethylsulfonyl fluoride solution (Beyotime Institute of Biotechnology). Protein concentration was determined with a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology), and 20 μg of protein were separated by 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to 0.2-μm polyvinylidene difluoride membrane that was blocked with 5% non-fat milk in Tris-buffered saline with Tween-20 (TBST) for 2 h at room temperature. The membrane was then incubated overnight at 4˚C with primary antibodies against the following proteins: Bax (1:1,000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA), Caspase-3 (1:1,000 dilution; Cell Signaling Technology, Inc.), EGFR (1:1,000 dilution; Cell Signaling Technology, Inc.), TGF-α (1:1,000 dilution; Abcam, San Francisco, CA, USA), and Bcl-2 (1:1,000 dilution; Cell Signaling Technology, Inc.). After five washes with 1x TBST, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (Cell Signaling Technology, Inc.) for 1 h at room temperature. Protein bands were detected by enhanced chemiluminescence (GE Healthcare Life Sciences, Little Chalfont, UK). GAPDH served as a loading control.

Statistical analysis. Data are presented as mean ± SD from at least three independent triplicate experiments. Differences between groups were evaluated by one-way analysis of variance and the independent samples t-test using SPSS v.13.0 software (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

PC-9 and RPC-9 cells exhibit differential sensitivity to gefitinib. PC-9 and RPC-9 cells were treated with different concentrations of gefitinib for 24, 48, 72, or 96 h and cytotoxicity was evaluated with the MTS assay. At treated with gefitinib concentrations <10 μM for 24, 48, 72, or 96 h, the viability of PC-9 cells was decreased in a dose-dependent manner, whereas RPC-9 cells were unaffected (Fig. 1A).

HOTAIR is more abundant in RPC-9 cells than in PC-9 cells. We measured and compared HOTAIR expression levels in RPC-9 and PC-9 cells by RT-qPCR. HOTAIR was more highly expressed in RPC-9 than in PC-9 cells, indicating that HOTAIR may be involved in mediating gefitinib resistance ($P<0.05$, Fig. 1B).

HOTAIR knockdown restores gefitinib sensitivity to RPC-9 cells. To investigate the role of HOTAIR in acquired gefitinib resistance, we silenced HOTAIR expression in RPC-9 cells with red fluorescent protein (RFP)-carrying LV-HOTAIR-shRNAs and visualized RFP expression by fluorescence microscopy 72 h post infection (Fig. 2A). The red fluorescent protein in Fig. 2A represents the infection efficiency of LV-NC-shRNA or LV-HOTAIR-shRNAs in RPC-9 cells. We found very high expression levels of RFP in the LV-NC-shRNA and LV-HOTAIR-shRNA groups. Therefore, PRC-9 cells were infected with LV-NC-shRNA or LV-HOTAIR-shRNAs with high infection efficiency. HOTAIR knockdown efficiency was confirmed by RT-qPCR (Fig. 2B). As shown in Fig. 2B, shRNA3 was one of the effective shRNAs in silencing HOTAIR. Therefore, we chose LV-HOTAIR-sh3 for further *in vitro* and *in vivo* experiments. In our preliminary experiments, LV-HOTAIR-sh1 and LV-HOTAIR-sh2 could not silence HOTAIR lncRNA in RPC-9 cells, restore gefitinib sensitivity to RPC-9 cells, or induce RPC-9 cell apoptosis and cell cycle arrest *in vitro*. Hence, LV-NC-shRNA resembled LV-HOTAIR-sh1 and LV-HOTAIR-sh2, and we chose only LV-NC-shRNA as the negative control. MTS is a classical method for testing the changes in cell viability after any cell treatment. Thus, we used this method to test the response effects of PC-9 or RPC-9 upon

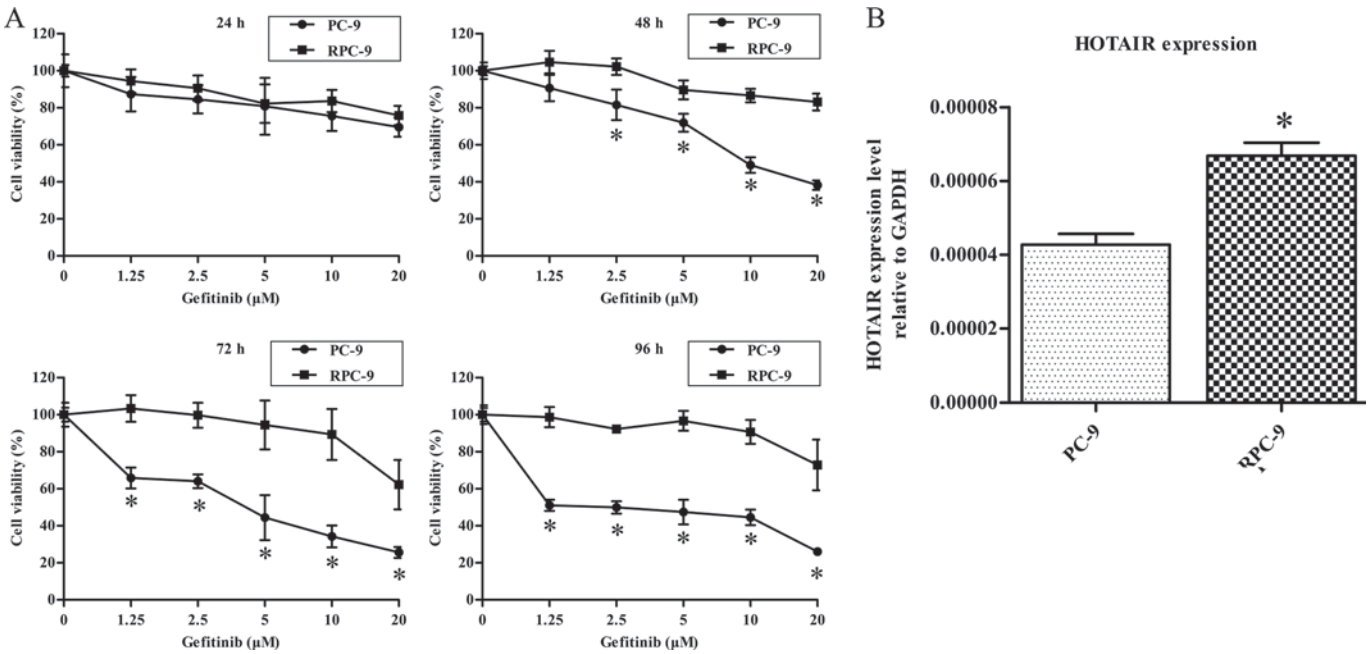

Figure 1. (A) PC-9 and RPC-9 cells exhibit differential sensitivity to gefitinib. Cells were treated with 0, 1.25, 2.5, 5, 10, or 20 μM gefitinib. After 24, 48, 72, or 96 h, cell viability was evaluated with the MTS assay. Data represent mean ± standard deviation (n=4). *P<0.05 vs. RPC-9 group. (B) HOTAIR expression level was higher in RPC-9 than in PC-9 cells. *P<0.05 vs. PC-9 group. HOTAIR, HOX transcript antisense RNA.

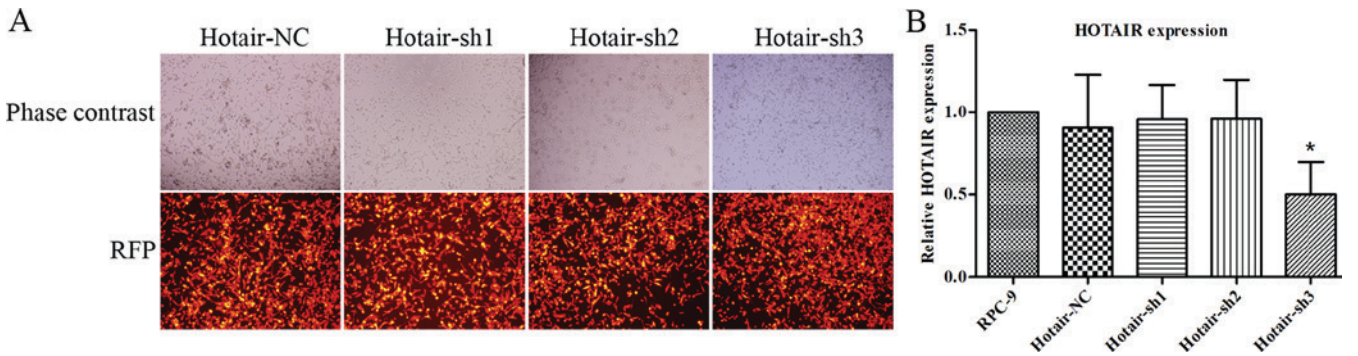

Figure 2. (A) RPC-9 cells were infected with LV-HOTAIR-shRNA or LV-NC-shRNA at a multiplicity of infection of 10. After 72 h, cells were examined by fluorescence and phase-contrast microscopy at 200x magnification. (B) HOTAIR expression in RPC-9 cells infected with LV-HOTAIR-shRNA or LV-NC-shRNA, as determined by RT-qPCR. LV-HOTAIR-sh3 inhibited HOTAIR expression in RPC-9 cells. *P<0.05 vs. the LV-NC-shRNA group. HOTAIR, HOX transcript antisense RNA; sh, short hairpin; NC, negative control; RFP, red fluorescent protein.

treatment with gefitinib or LV-HOTAIR-shRNAs. After incubation with different concentrations of gefitinib or RPMI-1640 medium as a negative control for 24, 48, 72, or 96 h, we found that gefitinib inhibited the proliferation of RPC-9 cells infected with LV-HOTAIR-sh3 as compared to LV-NC-shRNA at 48 h (gefitinib concentrations >2.5 μM), 72, and 96 h (Fig. 3), suggesting that HOTAIR is required for the proliferation of gefitinib-resistant RPC-9 cells.

HOTAIR knockdown induces RPC-9 cell apoptosis and cell cycle arrest. To confirm whether HOTAIR is required for RPC-9 cell survival, cells were treated with 10 μM gefitinib for 48 h after infection with LV-HOTAIR-sh3 or LV-NC-shRNA, and apoptosis was evaluated with the TUNEL assay. The bar plot in Fig. 4 shows the ratio of TUNEL-positive cells in various treatment groups. There were more TUNEL-positive cells in the LV-HOTAIR-sh3 + gefitinib group than in the LV-NC-shRNA + gefitinib group (31±1.41% vs. 6.5±0.71%; P<0.05) (Fig. 4), indicating that loss of HOTAIR increased apoptosis of RPC-9 cells. This was confirmed by annexin-V-APC and 7-AAD double staining followed by flow cytometry analysis; after treatment with 10 μM gefitinib for 48 h, LV-HOTAIR-sh3 infection increased the fraction of apoptotic RPC-9 cells as compared to infection with LV-NC-shRNA (32.17±1.61% vs. 12.94±0.65%; P<0.05) (Fig. 5A). Loss of HOTAIR inhibited RPC-9 cell proliferation by modulating cell cycling, as evidenced by the increased S-phase fraction in LV-HOTAIR-sh3 + gefitinib as compared to LV-NC-shRNA + gefitinib group (35.36±0.07% vs. 18.08±0.16%; P<0.05) detected by flow cytometry analysis (Fig. 5B). Moreover, HOTAIR silencing decreased the G1-phase (61.39±0.06% vs. 65.09±0.77%; P<0.05) and G2-phase (2.46±0.07% vs. 16.25±0.62%; P<0.05) fractions in the LV-HOTAIR-sh3 + gefitinib group as compared to the LV-NC-shRNA + gefitinib group. Similar results were found in the LV-HOTAIR-sh3 + vehicle and LV-NC-shRNA + vehicle

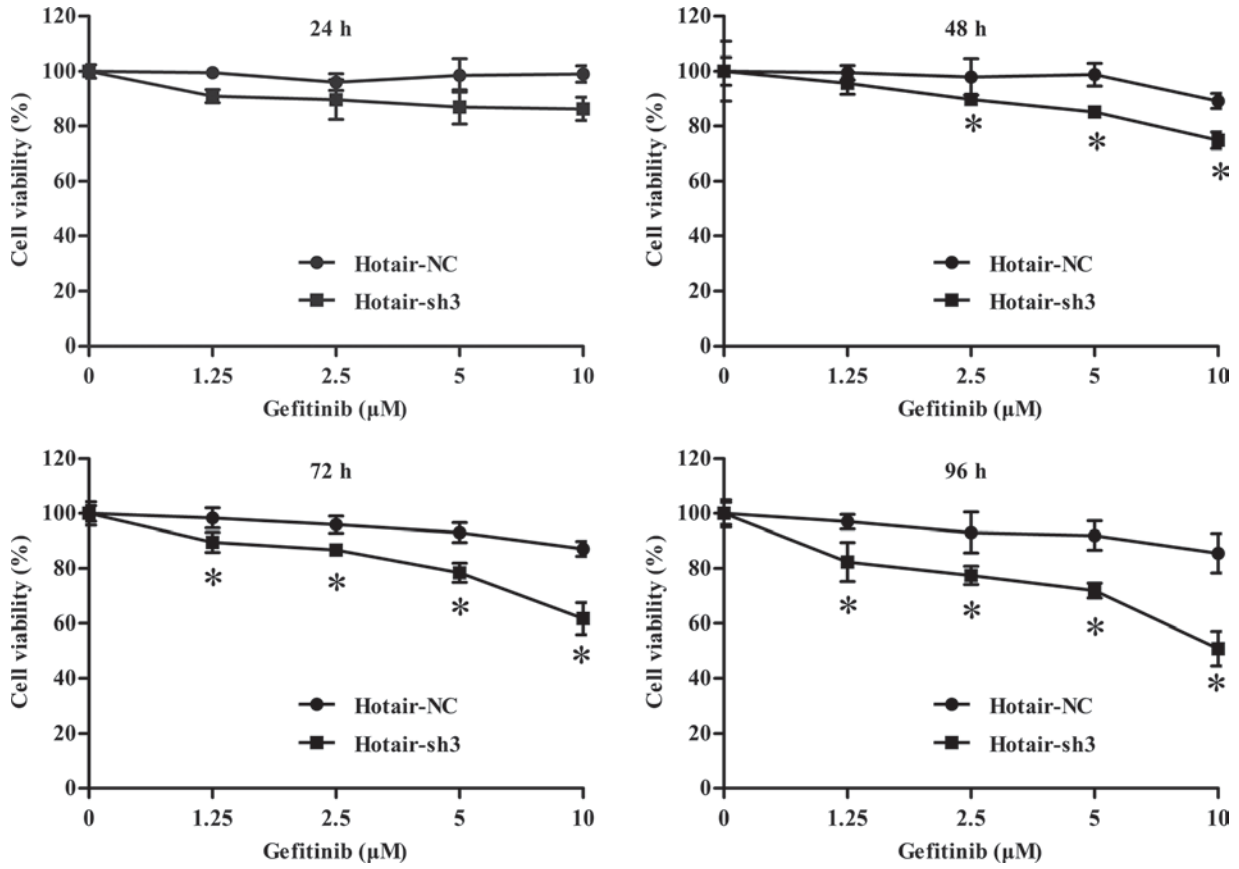

Figure 3. Viability of LV-infected RPC-9 cells treated with 0, 1.25, 2.5, 5, and 10 μM gefitinib for 24, 48, 72 and 96 h. Data represent mean ± standard deviation (n=3). *P<0.05 vs. LV-NC-shRNA group. LV, lentivirus; HOTAIR, HOX transcript antisense RNA; sh, short hairpin; NC, negative control.

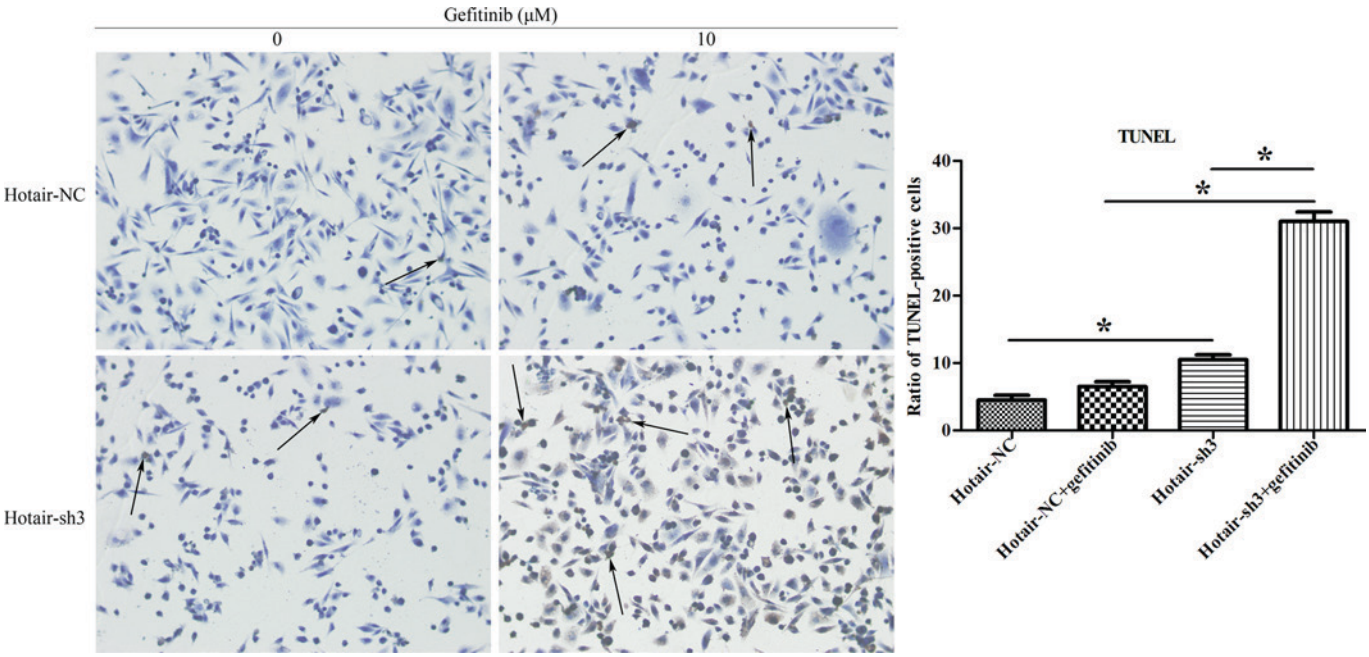

Figure 4. RPC-9 cells with or without 10 μM gefitinib treatment for 48 h following infection with LV-HOTAIR-sh3 or LV-NC-shRNA, as determined by the TUNEL assay. There were more TUNEL-positive cells in the LV-HOTAIR-sh3 + gefitinib than in the LV-NC-shRNA + gefitinib group. Arrows indicate TUNEL-positive cells. *P<0.05. HOTAIR, HOX transcript antisense RNA; sh, short hairpin; NC, negative control.

groups (Fig. 5B). Hence, it seems that the cell cycle changes occurred mainly because of HOTAIR silencing, and additional treatment of gefitinib seemed to have no effect. The data of one representative experiment are shown in the quadrants of Fig. 5.

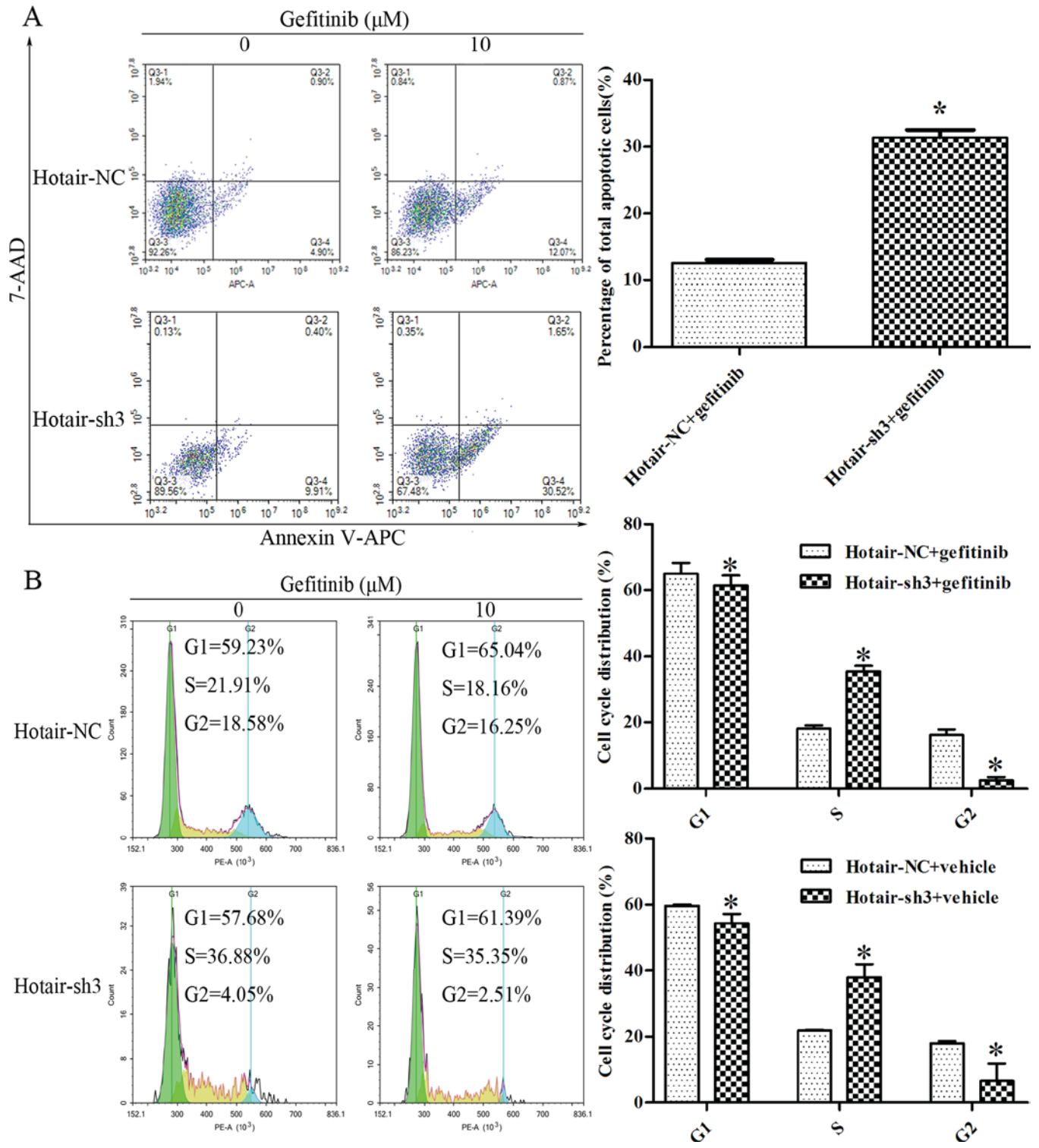

Figure 5. HOTAIR knockdown induces apoptosis and S-phase arrest in RPC-9 cells. (A) Apoptosis of RPC-9 cells was assessed by flow cytometry. HOTAIR silencing increased the fraction of apoptotic RPC-9 cells treated with gefitinib. (B) Cell cycling was analyzed by flow cytometry. HOTAIR silencing increased and decreased the percentage of RPC-9 cells in S and G1/G2 phases, respectively. *P<0.05 vs. LV-NC-shRNA + gefitinib group. HOTAIR, HOX transcript antisense RNA; sh, short hairpin; NC, negative control; APC, allophycocyanin.

Thus, HOTAIR knockdown inhibits RPC-9 cell proliferation by inducing cell cycle arrest and apoptosis.

HOTAIR knockdown suppresses the tumorigenicity of RPC-9 cells in vivo. To assess the antitumor effects of HOTAIR silencing *in vivo*, RPC-9 cells infected with LV-HOTAIR-sh3 or LV-NC-shRNA were subcutaneously injected into nude mice and growth of the resultant tumors was compared. After 21 days of gefitinib administration, the growth of tumors with HOTAIR knockdown was slower as compared to those infected with the negative control (P<0.05; Fig. 6A, B). Moreover, the growth of tumors in the LV-HOTAIR-sh3 + gefitinib group was slower than that in the LV-HOTAIR-sh3 + vehicle group, indicating that

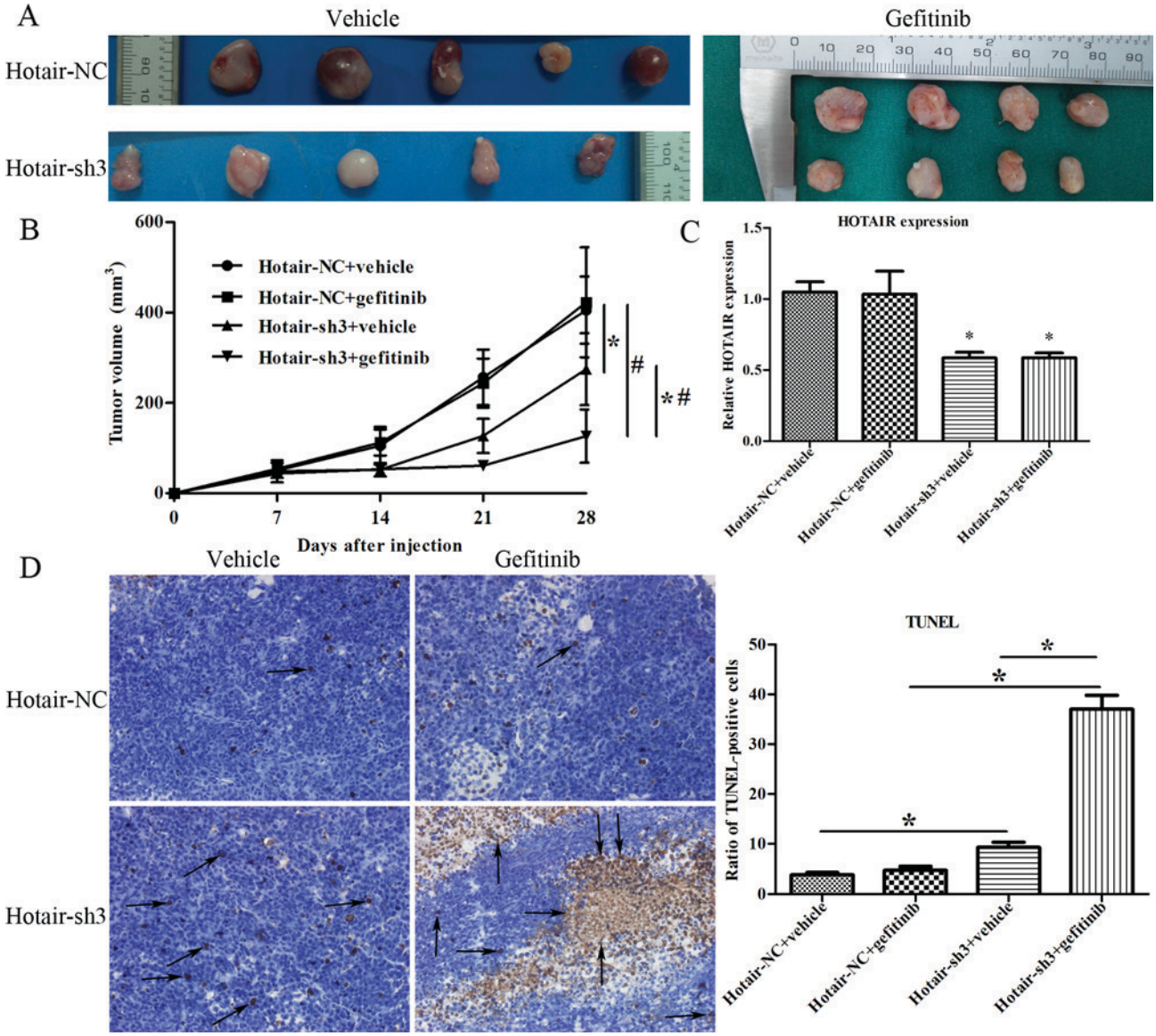

Figure 6. HOTAIR silencing suppresses tumorigenicity in RPC-9 cells *in vivo*. (A) Representative images of tumors originating from RPC-9 cells infected with LV-HOTAIR-sh3 or LV-NC-shRNA after treatment with gefitinib or vehicle for 28 days. (B) Tumor growth curves. The mean volumes of tumors derived from RPC-9 cells infected with LV-HOTAIR-sh3 and treated with gefitinib were smaller than those originating from cells infected with LV-NC-shRNA and treated with gefitinib. *P<0.05 vs. LV-NC-shRNA + vehicle group; #P<0.05 vs. LV-NC-shRNA + gefitinib group; *#P<0.05 vs. LV-HOTAIR-sh3 + vehicle group. (C) HOTAIR expression was downregulated in tumors derived from RPC-9 cells infected with LV-HOTAIR-sh3. *P<0.05 vs. LV-NC-shRNA group. (D) More TUNEL-positive cells were observed in tumors originating from RPC-9 cells infected with LV-HOTAIR-sh3 and treated with gefitinib as compared to LV-NC-shRNA and treated with gefitinib. Arrows indicate TUNEL-positive cells. *P<0.05. HOTAIR, HOX transcript antisense RNA; sh, short hairpin; NC, negative control; APC, allophycocyanin.

HOTAIR silencing could effectively restore the sensitivity of RPC-9 cells to gefitinib (P<0.05; Fig. 6A, B). There was no significant difference in the growth of tumors between the LV-NC-shRNA + vehicle and LV-NC-shRNA + gefitinib groups (P>0.05). HOTAIR is a lncRNA, which does not encode a protein; thus, RT-qPCR was implemented to confirm HOTAIR expression in LV-HOTAIR-sh3 tumors (19). RT-qPCR confirmed that HOTAIR expression was similarly downregulated in LV-HOTAIR-sh3 + vehicle and LV-HOTAIR-sh3 + gefitinib tumors as compared to LV-NC-shRNA + vehicle tumors (Fig. 6C). The bar plot in Fig. 6D shows the ratio of TUNEL-positive cells in various treatment groups. The ratio of TUNEL-positive cells in LV-HOTAIR-sh3 + vehicle tumors was significantly higher than that in LV-NC-shRNA + vehicle tumors (9.25±1.06% vs. 3.85±0.49%; P=0.023 <0.05). This is in accordance with the tumor growth data in Fig. 6B, as well as the upregulation of Caspase-3 in Fig. 7. Moreover, tumors derived from RPC-9 cells infected with LV-HOTAIR-sh3 showed higher ratio of TUNEL-positive cells than those originating from LV-NC-shRNA-infected cells following gefitinib treatment (P<0.05, Fig. 6D). The ratio of TUNEL-positive cells in the LV-HOTAIR-sh3 + gefitinib group was higher than that in the LV-HOTAIR-sh3 + vehicle group, indicating that HOTAIR silencing could effectively restore the sensitivity of RPC-9 cells to gefitinib (P<0.05; Fig. 6D). These results indicate that HOTAIR knockdown restores gefitinib sensitivity *in vivo*.

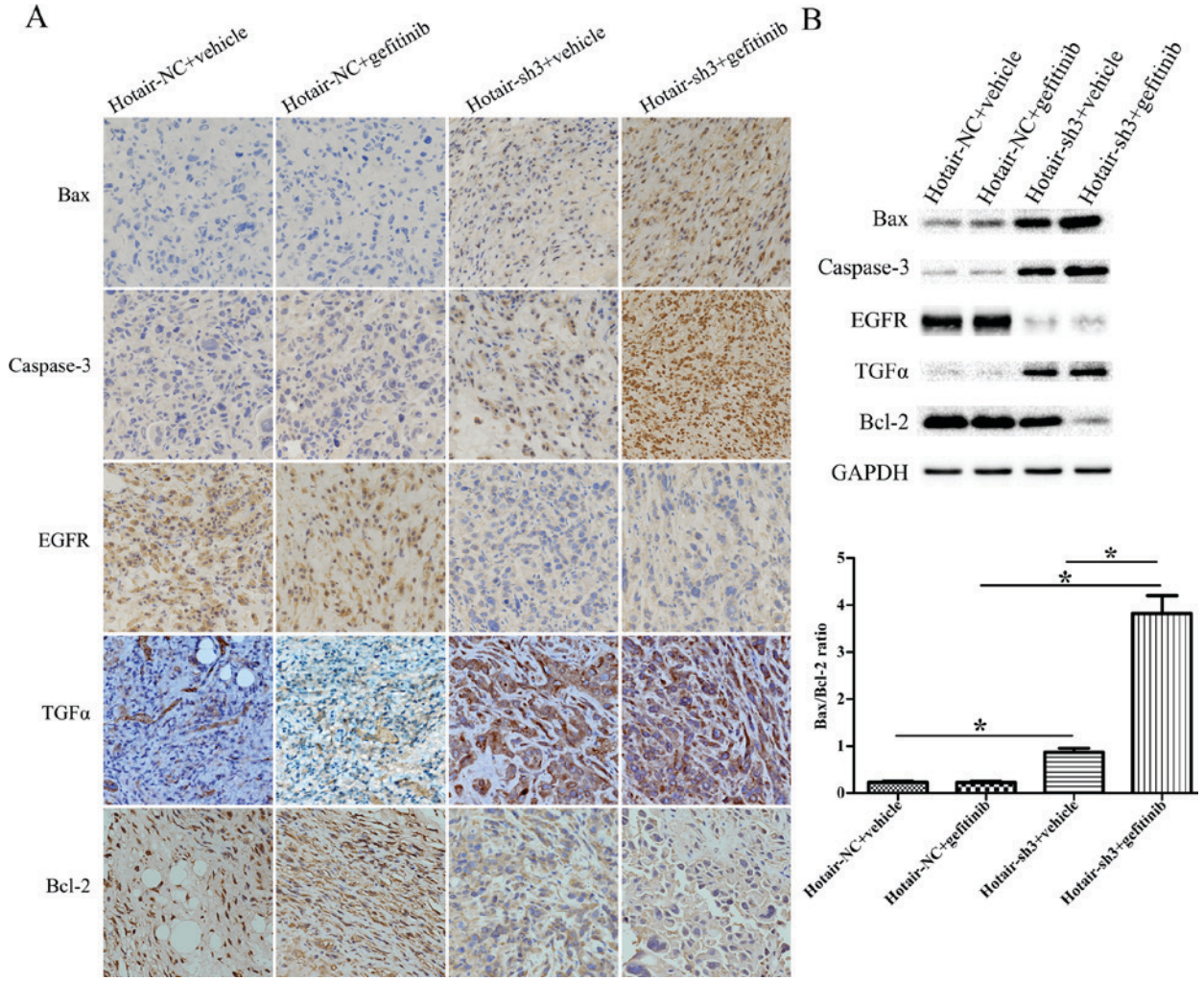

Figure 7. (A) Immunohistochemical analysis of Bax, Caspase-3, EGFR, TGF-α, and Bcl-2 expression in xenograft tumors originating from RPC-9 cells infected with LV-HOTAIR-sh3 or LV-NC-shRNA (400x magnification). (B) Western blot analysis of Bax, Caspase-3, EGFR, TGF-α, and Bcl-2 expression in xenograft tumors originating from RPC-9 cells infected with LV-HOTAIR-sh3 or LV-NC-shRNA. *P<0.05. HOTAIR, HOX transcript antisense RNA; sh, short hairpin; NC, negative control; APC, allophycocyanin; Bax, B-cell lymphoma 2-associated X protein; TGF-α, Transforming growth factor-α; EGFR, epithelial growth factor receptor.

HOTAIR knockdown restores gefitinib sensitivity by activating Bax/Caspase-3 and suppressing TGF-α/EGFR signaling. To clarify the mechanism by which HOTAIR silencing restores gefitinib sensitivity to RPC-9 cells, we examined the expression of genes related to apoptosis (Bax, Caspase-3, Bcl-2) and EGFR signaling (TGF-α, EGFR) in xenograft tumors by immunohistochemistry and western blotting. The Bax/Bcl-2 ratio and Caspase-3 level were upregulated in LV-HOTAIR-sh3 + gefitinib tumors compared to LV-NC-shRNA + gefitinib tumors. Moreover, the Bax/Bcl-2 ratio and Caspase-3 level were upregulated in LV-HOTAIR-sh3 + gefitinib tumors compared to LV-HOTAIR-sh3 + vehicle tumors. EGFR levels were downregulated in tumors originating from RPC-9 cells infected with LV-HOTAIR-sh3 as compared to LV-NC-shRNA; the opposite trend was observed for TGF-α expression. There was no significant difference in the Bax/Bcl-2 ratio or levels of Caspase-3, EGFR, and TGF-α between the LV-NC-shRNA + vehicle and LV-NC-shRNA + gefitinib groups (P>0.05) (Fig. 7A, B). Thus, gefitinib resistance in RPC-9 cells can be overcome by HOTAIR knockdown, which induces apoptosis via activation of Bax/Caspase-3 and blocks cell proliferation via modulation of TGF-α/EGFR signaling.

Discussion

Various mechanisms of EGFR-TKI resistance have been reported in NSCLC, including EGFR T790M mutation (20,21), MET amplification (22), human epidermal growth factor *receptor* 2 amplification (23), hepatocyte growth factor overexpression (24), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha mutation (25), and histologic transformation to small-cell lung cancer (26), among others. About 50-60% of cases of acquired resistance are attributed to T790M mutation (27). Second-generation EGFR-TKIs such as afatinib that target T790M-induced resistance lack clinical efficacy due to on-target toxicity and side effects (28). Third-generation TKIs including AZD9291 (osimertinib), CO-1686 (rociletinib), and HM61713 (olmutinib) that target T790M while having no effect on wild-type EGFR are currently in clinical trials. However, it is possible that resistance to these drugs will eventually emerge. Therefore, novel therapeutic strategies that reverse acquired resistance are needed.

HOTAIR is associated with chemoresistance in lung, breast, and ovarian cancers (29-31). For example, inhibiting HOTAIR reverses the resistance of lung adenocarcinoma to cisplatin via downregulation of p21 expression (29). In the present study, we found that HOTAIR knockdown blocked the proliferation of RPC-9 cells and restored their sensitivity to gefitinib *in vitro* and *in vivo*. These effects were accompanied by increases in cell apoptosis and cell cycle arrest. In a xenograft model, loss of HOTAIR resulted in tumor shrinkage and restored gefitinib sensitivity. Bax is a pro-apoptotic signaling molecule (32), whereas Caspase-3 is an effector in the terminal stages of apoptosis (33). In serous ovarian cancer, HOTAIR knockdown was found to induce Caspase-3 expression (34); this is consistent with our observation that Bax and Caspase-3 expression was upregulated by silencing HOTAIR. Zou *et al* demonstrated that the knockdown of Bcl-2 using siRNAs increases the sensitivity to gefitinib in a gefitinib-resistant H1975 lung cancer cell line (35). In our present study, we found that Bcl-2 was downregulated by silencing HOTAIR (Fig. 7), which is in accordance with the study of Zou *et al*.

Aberrant EGFR expression and signaling contribute to the malignant transformation of various human cancers, including lung cancer (36). HOTAIR knockdown was found to suppress EGFR expression by inhibiting of miR-545 levels in colorectal cancer (37). Ishikawa and Masago *et al* reported that TGF-α is a serum biomarker for gefitinib resistance in patients with advanced NSCLC (38,39). TGF-α is a ligand of EGFR, and the function of EGFR somehow depends on the ligand quantity around cells (40). In the present study, HOTAIR silencing suppressed the expression of EGFR and induced that of its ligand TGF-α in the cytoplasm. This leads us to believe that HOTAIR silencing inhibits the release of TGF-α into the extracellular environment, which is in accordance with the study of Ishikawa and Masago *et al*. HOTAIR lncRNA might be restoring gefitinib sensitivity by suppressing TGF-α/EGFR signaling. The underlying molecular mechanism needs to be explored further.

Thus, inhibiting HOTAIR can reverse acquired resistance to gefitinib by activating Bax/Caspase-3-mediated apoptosis and suppressing TGF-α/EGFR signaling.

Although Shien *et al* have demonstrated that the knockdown of EGFR using siRNAs suppressed RPC-9 cell proliferation (41), the role HOTAIR in gefitinib resistance in human lung adenocarcinoma is unknown. The original aim of our study was to investigate the role of HOTAIR in lung adenocarcinoma, but during our experiment, we found that HOTAIR silencing could effectively restore the sensitivity of RPC-9 against gefitinib. Therefore, this is a novel study about the relationship of HOTAIR lncRNA and gefitinib sensitivity in lung adenocarcinoma.

In conclusion, these results demonstrate for the first time that HOTAIR knockdown can reverse acquired resistance to gefitinib in human lung adenocarcinoma. Based on these findings, we propose that HOTAIR is a novel therapeutic target for NSCLC cases exhibiting gefitinib resistance.

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