Long noncoding RNA-HOTAIR affects chemoresistance by regulating HOXA1 methylation in small cell lung cancer cells

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Homeobox (HOX) transcript antisense RNA (HOTAIR), a long intergenic noncoding RNA (lincRNA), has been reported to play an oncogenic role in various cancers including small cell lung cancer (SCLC). However, it is not known whether HOTAIR can modulate chemoresistance in SCLC. The aim of this study is to investigate the roles of HOTAIR in chemoresistance of SCLC and its possible molecular mechanism. Knockdown of HOTAIR was carried out in SCLC multidrug-resistant cell lines (H69AR and H446AR) and the parental cell lines (H69 and H446) to assess its influence on chemoresistance. The results showed that downregulation of HOTAIR increased cell sensitivity to anticancer drugs through increasing cell apoptosis and cell cycle arrest, and suppressed tumor growth *in vivo*. Moreover, HOXA1 methylation increased in the resistant cells using bisulfite sequencing PCR. Depletion of HOTAIR reduced HOXA1 methylation by decreasing DNMT1 and DNMT3b expression. The interaction between HOTAIR and HOXA1 was validated by RNA immunoprecipitation. Taken together, our study suggested that HOTAIR mediates chemoresistance of SCLC by regulating HOXA1 methylation and could be utilized as a potential target for new adjuvant therapies against chemoresistance.

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Lung cancer is one of the leading malignant tumors in the world. Small cell lung cancer (SCLC) accounts for ~15% of all lung cancers at present. First-line chemotherapy based on cisplatin is the current optimal treatment for SCLC.¹ SCLC is characterized by a propensity for early dissemination and rapid development of drug resistance during the course of disease, although patients have initial high response rates to chemotherapy. Several novel molecules are presently undergoing evaluation, and represent potential future additions to the treatment repertoire for SCLC.² However, the chemoresistance of SCLC remains an important issue worldwide.¹

Long noncoding RNAs (lncRNAs) have been demonstrated to regulate nearly every step in the life cycle of a gene over the past few years.³ Several lncRNAs may also be involved in oncogenesis and development of some kinds of cancers such as leukemia, lung cancer, and hepatocarcinoma.⁴ Recent studies showed that some lncRNAs such as BX648420, ENST00000366408, and AK126698 were correlated to resistance to cisplatin in NSCLC. AK126698 appeared to confer cisplatin resistance by targeting the Wnt pathway.⁵

LncRNA-HOTAIR, identified from a custom tiling array of the HOXC locus (12q13.13; Puglisi *et al*),⁶ can promote tumor progression such as colorectal cancer, pancreatic cancer, and NSCLC. Its molecular mechanism was reported to silence targeted genes such as *HOXD10* and *PGR* by inducing genome-wide retargeting of polycomb-repressive complex 2 (PRC2), which was composed of EZH2, SUZ12, and EED, leading to the methylation of H3K27.⁷ Homeobox (HOX) transcript antisense RNA (HOTAIR) has also been reported to affect cellular proliferation, invasiveness, and clinical relapse in SCLC.⁸ However, the involvement of lncRNA-HOTAIR in chemoresistance of SCLC remains unknown.

The aberrant DNA methylation has been well described as a novel marker for cancer diagnosis, prognosis, and prediction of response to chemotherapeutic drugs. In glioma, the hypermethylation at MGMT indicated resistance to a chemotherapeutic agent, whereas in breast cancer, the methylation of BRCA1 was indicative of successful chemotherapy.⁹ In NSCLC, SULF2 and IGFBP-3 have also been observed to affect drug sensitivity of NSCLC cells.^{10–12}

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The HOX family, including 39 HOX genes, is divided into four groups (A–D) in tightly linked clusters on different chromosomes, and has the equivalent position in each cluster. HOXA1, one of the HOX family members, has been reported to be involved in many important biological processes including cell growth, apoptosis, transformation, and survival.¹³ Our previous study showed that HOXA1, targeted by miR-100, played a role in SCLC cell survival and chemoresistance.¹⁴ HOXA1 was found to stimulate STAT3 and STAT5B in oncogenic transformation of the immortalized mammary epithelial cells,¹⁵ and regulated the p44/42 MAP kinase pathway in mammary cancer cells.¹⁶ The overexpression of HOXA1 was associated with poor outcome in patients with HCC, whereas the downregulation of HOXA1 can inhibit cell growth, anchorage-independent growth, migration, and invasion of HepG2 cells.¹⁷

In the present study, we first detected the level of lncRNA-HOTAIR in SCLC multidrug resistance cells (H69AR and H446AR) and found the overexpression of HOTAIR in H69AR and H446AR cells. Next, we investigated the biological functions of lncRNA-HOTAIR in SCLC, *in vitro* and *in vivo*. Knockdown of HOTAIR increased cell sensitivity to anticancer drugs, cell apoptosis, and inhibited tumor growth. Moreover, we found that HOTAIR modulated HOXA1 methylation, through decreasing DNMT1 and DNMT3b expression. These findings revealed a novel mechanism by which HOTAIR mediated chemoresistance of SCLC.

MATERIALS AND METHODS

Cell Culture

The human small cell lung cancer cell lines NCI-H446 and NCI-H69 and the drug-resistant subline H69AR were purchased from American Type Culture Collection (ATCC, USA), and the drug-resistant subline H446AR was constructed by exposing at intervals and repeatedly to high dose of adriamycin (ADM).¹⁸ All cell lines were maintained in RPMI-1640 medium with 10% fetal calf serum in an incubator at 37 °C with 5% CO₂. H69AR and H446AR were alternately fed with drug-free medium and medium containing 0.8 μM of ADM. The resistant cell lines were tested regularly for maintaining resistance to the selected drugs. Growth and morphology of all cell lines were monitored on a weekly basis.

Small Interference RNA (siRNA), Short Hairpin RNA (shRNA), and Transfection

The lncRNA-HOTAIR siRNA and HOXA1 siRNA were purchased from Sigma Aldrich (Shanghai, China), and the oligonucleotides encoding siRNA were as follows: siHOTAIR-1115: 5'-CCCAUGGACUCAAAACAATT-3' and 5'-UUGUUUAUGAGUCCAUGGGTT-3'; siHOTAIR-1560: 5'-GC-CUUUGGAAGCUCUUGAATT-3' and 5'-UUCAAGAGCUUCCAAAGGCTT-3'. Lipofectamine 2000 was purchased from Invitrogen (Shanghai, China) and siRNA transfection was done according to the protocol supplied by Invitrogen.

To stably silence endogenous HOTAIR, two shRNA oligonucleotides were purchased from Sigma Aldrich (Shanghai, China), and the oligonucleotides were as follows: shHOTAIR-I: 5'-CACCGCCTTTGCTTCGTGCTGATTCCGAAGAATCAGCACGAAGCAAAGGC-3' and 5'-AAAAGCCTTTGCTTCGTGCTGATTCTTCGGAATCAGCACACGAAGCAAAGGC-3';¹¹ shHOTAIR-II: 5'-GATCCCGAACGGGAGTACAGAGAGATTCAAGAGATCTCTCTGTACTCCCGTTCTTTTTTGGAAA-3' and 5'-CGCGTTTCCAAAAAAGAACGGGAGTACAGAGAGATCTCTTGAATCTCTCTGTACTCCCGTTCCGG-3';¹² they were cloned into the pGCSi-neo-GFP vector to generate pGCSi-neo-GFP-HOTAIR-RNAi(s). SCLC cells were transfected with shRNA via Lipofectamine 2000 (Invitrogen). Cells were sorted at 24 h after transfection by GFP expression on a FACSaria cell sorter machine that produced a population that was 80% GFP positive. At 48 h, cells were exposed to G418 (400 ng/ml, Sigma-Aldrich, St Louis, MO, USA) selection for 1 month. G418-resistant cells were ring-cloned and expanded to establish stable shHOTAIR and shNC transfectants.

Drug-Resistance Assay

In vitro drug cytotoxicity was measured by Cell Counting Kit-8 (CCK-8) assays. The cells incubated without drugs (i.e., control wells) were set at 100% survival and were used to calculate the concentration of each cytostatic drug lethal to 50% of the cells (IC₅₀). The ranges of drug concentrations were based on earlier studies and aimed at obtaining an IC₅₀ value for all cases. A total of three anticancer drugs (Cisplatin (CDDP; Shandong, China), Adriamycin (ADM; Jiangsu, China), and Etoposide (VP-16; Jiangsu, China)) were obtained from commercial sources and were dissolved according to the manufacturer's instructions and tested in eight concentrations. Cells were seeded into 96-well plates (4000 cells/well) and then treated for 24 h in 100 μl of medium with anticancer drugs. CCK-8 reagent (Dojindo, Kumamoto, Japan) was then added and the cells were incubated at 37 °C for 4 h before reading the absorbency using a microplate reader (μQuant, Bio-Tek Instruments) at 450 nm. The assay was conducted in five replicate wells for each sample and three parallel experiments were performed.

Flow Cytometry

For cell cycle analysis, cells were seeded in six-well plates (1 × 10⁶ cells /well) and then treated for 24 h in 2 ml of medium with anticancer drugs or mock as described above. The cells were washed twice in cold PBS buffer and fixed with 70% ethanol. After centrifugation at 2000 g for 5 min, the cells were then treated with 0.1 mg/ml RNase A (Boehringer Mannheim) and stained with 50 mg/ml propidium iodide (Sigma Chemical). All samples were prepared following the manufacturer's instructions and analyzed by flow cytometry (FACSCalibur Becton Dickinson) using CellQuest software (BD Bioscience). For apoptosis assays, cells were seeded in six-well plates (1 × 10⁶ cells/well) and then treated for 24 h in 2 ml of medium with anticancer drugs or mock as described

above, and were washed twice in cold PBS buffer and fixed with 70% ethanol. Apoptosis was detected by Annexin V-fluorescein isothiocyanate (FITC).

In Vivo Tumor Xenograft Model

Male BALB/c nude mice aged 4–5 weeks were purchased from the Laboratory Animal Centre of Zhongshan University (Guangzhou). All procedures were performed according to the internationally accepted ethical guidelines. The BALB/c nude mice were randomly assigned to groups ($n = 5/\text{group}$). For the tumorigenicity studies, cells were harvested, washed with PBS, and resuspended in normal culture medium at a concentration of 1×10^7 cells per 0.2 ml. Each subculturing situs was injected with viable 1×10^7 cells per 0.2 ml in the back leg to establish the tumor model, respectively. At 1 month after injection, all the mice were killed. The tumors were excised. Tumor volumes were calculated using the ellipsoid volume formula ($0.5 \times l \times w^2$, where l is length and w is width).

Bisulfite Sequencing PCR (BSP)

DNA from SCLC cell lines were isolated, bisulfate modified, and then used for BS analysis as previously described.¹³ Primer sequences for PCR were as follows: BSP-HOXA1-F: 5'-GT TTAGTTTTAGAATAGAGGAGGTGGTT-3'; BSP-HOXA1-R: 5'-AAAATCAATAAACCAA AACTC-3'. The region of HOXA1 promoter is shown in Supplementary Figures S1 and S7.

Bioinformatics-Based Tool

We used catRAPID algorithm to compute the interaction propensity of HOXA1 protein with lncRNA-HOTAIR. CatRAPID predicts protein–RNA associations by estimating the interaction propensity between amino acids and nucleotides using secondary structure information, hydrogen bonding, and Van der Waals forces. The approach was previously applied to predict associations between different types of proteins and RNA molecules. Although each protein binds to distinct types of RNA structures, we observe that the contribution of hairpin loops accounts for 57% of the overall interaction propensity.

Quantitative RT-PCR

Our observations were made in an ABI Prizm 7300 Sequence Detection System using SYBR Green (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Primers for quantitative RT-PCR were designed based on sequences from the GenBank as follows:

HOTAIR: F: 5'-GGTAGAAAAAGCAACCACGAAGC-3', R: 5'-ACATAAACCTCTGTCTGTGAGTGCC-3'; HOXA1: F:

5'-AGTTGGAGAGTACGGCTACCTG-3', R: 5'-TGCAGGGA TGCAGCGATCTCCAC-3'; GAPDH: F: 5'-GAGTCAACGGAT TTGGTCGT-3', R: 5'-CATGGGTGGAATCATATTGGA-3'. The relative mRNA expression level was calculated by using the comparative expression level $2^{-\Delta\Delta CT}$ method.

Western Blotting Analysis

Protein extract (30 μg) was separated in a 15% SDS–polyacrylamide gel and electrophoretically transferred onto a PDVF membrane (Millipore, Etten-Leur, The Netherlands). Membranes were blocked overnight with 5% non-fat dried milk and incubated for 2 h with an anti-HOXA1 antibody (Sigma, USA) or anti-GAPDH antibody (Sigma, USA). After washing with TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween-20), the membranes were incubated for 1 h with horseradish peroxidase-linked goat-anti-rabbit antibody (KPL, Gaithersburg, MD, USA). The membranes were washed again with TBST, and the proteins were visualized using ECL chemiluminescence.

RNA Immunoprecipitation

We performed RNA immunoprecipitation (RIP) method using Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer's instructions. The HOXA1 antibody (Santa Cruz, USA) was used for RIP. The coprecipitated RNA was detected by reverse transcription-PCR and quantitative PCR. The primer sequences were listed as above. Total RNA (input controls) and isotype controls were assayed simultaneously to demonstrate that the detected signals were the result of RNA specifically binding to HOXA1.

Enzyme-Linked Immunosorbent Assay (ELISA)

For DNA methyltransferase (DNMT) measurement, 100 μl standard or samples were added to each well of 96-well plates. After incubation for 2 h at 37 °C, reagents were added to the wells according to the manufacturer's instructions of ELISA kit (GENMED DNMT total activity kit, Genmed Scientifics, USA). A microplate reader (μQuant , Bio-Tek Instruments) was used to read the absorbency at 450 nm. The duplicate readings were averaged. Software curve expert 1.30 was used to construct a standard curve and calculate the concentration of each sample.

Statistical Analysis

The data are presented as means $\pm$ s.d. Data of related assay were assessed by one-way ANOVA. SPSS 16.0 software (SPSS, Chicago, IL, USA) was used for all statistical analyses, with $P < 0.05$ considered statistically significant.

Figure 1 lncRNA-HOTAIR is required for refractoriness to treatment with anticancer drugs. (a) RT-PCR analysis of lncRNA-HOTAIR expression in resistant cells (H69AR and H446AR) and their parental cells (H69 and H446). (b) Downregulation of lncRNA-HOTAIR by siRNA was validated by RT-PCR. (c) IC_{50} was measured by CCK-8 assay when lncHOTAIR-downregulated cells were exposed to CDDP, ADM, and VP-16. (d and e) Cell early apoptosis and cell cycle progression were determined by flow cytometric analysis in lncHOTAIR-downregulated cells after drug exposure. Results are mean $\pm$ s.d. * $P < 0.05$, ** $P < 0.01$.

RESULTS

HOTAIR Knockdown Increased Drug Sensitivity *In Vitro*

We tested lncRNA-HOTAIR expression by RT-PCR in H69/H69AR and H446/H446AR cells and found that the expression was elevated by 2.8–3.6-fold in the resistant cells compared with the parental cells (Figure 1a). To confirm the role of lncRNA-HOTAIR in multidrug resistance of SCLC, we performed CCK-8 assay and flow cytometry to test the effect of the lncRNA-HOTAIR on cell sensitivity to anticancer drugs, cell apoptosis, and cell cycle progression in H69, H69AR, H446, and H446AR cells. We repressed the lncRNA-HOTAIR expression by siRNA and showed the expression was downregulated by 35–97-fold (Figure 1b). CCK-8 assays showed that IC₅₀ values of CDDP, ADM, and VP-16 decreased significantly after transfection with siHOTAIR (Figure 1c).

CDDP and VP-16 should induce DNA damage, and result in S-phase arrest. To investigate the possible mechanisms of HOTAIR in chemoresistance, we evaluated the impact of HOTAIR on apoptosis and cell cycle control when cells were exposed to chemotherapeutic drugs. Flow cytometry results showed that the apoptosis rates increased significantly in the lncRNA-HOTAIR-downregulated cells after drug exposure (Figure 1d), the S phase was prolonged, and the cell cycle progression was delayed after drug exposure (Figure 1e; cell apoptosis rates and cell cycle progression upon CDDP and VP-16 exposure are given in Supplementary Figures S2 and S3). These observations suggested that knockdown of

lncRNA-HOTAIR increased drug sensitivity, enhanced cell apoptosis, and decelerated cell cycle progression.

HOTAIR Knockdown Suppressed Tumor Growth *In Vivo*

To examine the effect of lncRNA-HOTAIR on tumor growth, we subcutaneously injected SCLC cells transfected with shRNAs specific against lncRNA-HOTAIR or negative control (NC) into nude mice. Mice were killed after 4 weeks. The tumors of shHOTAIR-treated cells grew significantly slowly compared with those of shNC-treated cells (Figure 2a and c). Growth curves showed the tumor volumes of shHOTAIR-treated cells decreased significantly (Figure 2b and d).

Methylation Status of HOXA1 Was Associated with Chemoresistance of SCLC

DNA hypermethylation has been found to be one of the mechanisms in activating genes required for drug cytotoxicity in the resistant phenotype development.⁹ In this study, we found that the methylation levels of HOXA1 were higher in resistant cells than in parental cells. Meanwhile, the methylation levels of HOXA1 decreased after treatment with DNA methyltransferase inhibitor 5-Aza-2-deoxycytidine (5-Aza-CdR) (Figure 3a). Subsequently, the IC₅₀ values of H69AR and H446AR cells decreased in response to CDDP, ADM, and VP-16 after treatment by 5-Aza-CdR (Figure 3b). Meanwhile, chemosensitivity was measured by CCK-8 assays when resistant cell lines (H69AR and H446AR) were treated with siHOXA1 and 5-Aza-CdR. The results showed that there is no significant difference between

Figure 2 lncRNA-HOTAIR suppressed tumor growth in nude mice subcutaneously inoculated with shHOTAIR-treated cells compared with shNC-treated cells. (a) H69 and H69AR cells; (c) H446 and H446AR cells. (b and d) The curves of tumor growth. The tumor volume was calculated using the ellipsoid volume formula ($0.5 \times l \times w^2$, where l is length and w is width) every week. $**P < 0.01$.

Figure 3 Methylation status of HOXA1 was associated with multidrug resistance. (a) Analysis of the HOXA1 CpG islands in H69/H69AR/5-Aza-CdR-treated H69AR cells (top set) and H446/H446AR/5-Aza-CdR-treated H446AR cells (bottom set) by bisulfite sequencing PCR. (b) IC₅₀ of H69AR and H446AR cells to anticancer drugs after demethylation with 5-Aza-CdR. Results are mean $\pm$ s.d. ** $P < 0.01$. (c and d) HOXA1 expression was measured by RT-PCR and western blot after treatment with 5-Aza-CdR.

control groups and siHOXA1-5-Aza-CdR groups in H446AR cells upon CDDP and ADM treatment (Supplementary Figures S5 and S6). The mRNA and protein expressions of HOXA1 in H69AR and H446AR with treatment of 5-Aza-CdR were upregulated by 4-15-fold and 2-3-fold, respectively (Figure 3c and d). The results suggested that aberrant methylation of HOXA1 may be involved in multidrug resistance of SCLC.

HOTAIR Associated with HOXA1

We performed bioinformatics-based tool (catRAPID algorithm) to predict the interaction between lncRNA-HOTAIR and HOXA1, and found the highest interaction score (normalized) was 3.68 (location: lncRNA-HOTAIR region: 349–444, HOXA1 region: 226–277) (Figure 4a and Supplementary Table S1). RIP method was further carried out to show a significant enrichment of lncRNA-HOTAIR with the HOXA1 antibody as compared with the nonspecific IgG control antibody (Figure 4b and Supplementary Figure S4). Next, we observed the upregulation of the HOXA1 mRNA expression by 12.7–886.3-fold and protein expression by 12.5–24.9-fold in the lncHOTAIR-downregulated cells (Figure 4c and d).

lncRNA-HOTAIR Epigenetically Silenced HOXA1 through DNMT1 and DNMT3b

We found the methylation level of HOXA1 decreased consistently with lncRNA-HOTAIR downregulation (Figure 5a).

To better characterize the way by which lncRNA-HOTAIR regulated the HOXA1 methylation, we performed ELISA assay to measure the expression of DNMTs (DNMT1, DNMT3a, and DNMT3b). The results revealed that both DNMT1 and DNMT3b expression decreased significantly in H69AR and H446AR cells after transfection with siHOTAIR, but no significant difference was found in the DNMT3a expression (Figure 5b). Therefore, these findings suggested that loss of lncRNA-HOTAIR demethylated HOXA1 by decreasing expression of DNMT1 and DNMT3b.

DISCUSSION

Recently, forced expression of lncRNA-HOTAIR has emerged as a powerful predictor of eventual metastasis and chemoresistance in several kinds of tumors such as colorectal cancer, sarcoma, and lung adenocarcinoma.^{7,19–22} However, the role of lncRNA-HOTAIR in the chemoresistance of SCLC remains unknown. In this study, we initially identified lncRNA-HOTAIR as a regulator in chemoresistance of SCLC by qRT-PCR in H69/H69AR and H446/H446AR cells. We further demonstrated that downregulation of lncRNA-HOTAIR led to increased cell sensitivity to CDDP, ADM, and VP-16, enhanced cell apoptosis and cell cycle arrest, and reduced tumor growth *in vivo*. To our knowledge, this is the first report to investigate the function of lncRNA-HOTAIR in mediating chemoresistance of SCLC.

Some previous studies have shown a relationship between gene silencing by targeted DNA methylation and chemotherapy response to SCLC. For example, DNA methylation of caspase-8 in SCLC cells resulted in resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).²³ We found that downregulation of HOXA1 gene affects SCLC cell survival and chemoresistance before.¹⁶ In our study, BSP method showed hypermethylation status of HOXA1 CpG islands in the resistant cells as compared with the parental cells. The methylation level of HOXA1 became lower and the expression level was improved remarkably when H69AR and H446AR cells were treated with 5-Aza-CdR. Then, the IC₅₀ values of H69AR and H446AR cells became lower in response to CDDP, ADM, and VP-16 after treatment with 5-Aza-CdR. To further investigate whether aberrant methylation of HOXA1 is necessary in multidrug resistance, we knocked down HOXA1 in the context of 5-aza-CdR treatment, and the results showed that there is no significant difference between control groups and siHOXA1-5-Aza-CdR groups in H446AR cells upon CDDP and ADM treatment. In addition to caspase-8 reported by Kaminsky et al,²³ our results suggested DNA methylation of HOXA1 was closely associated with chemoresistance of SCLC.

LncRNA has been described to mediate maintenance of DNA methylation and transcriptionally silence protein-coding genes.²⁴ Therefore, we sought to investigate whether lncRNA-HOTAIR was involved in regulating HOXA1 methylation. Using bioinformatics-based tools (catRAPID algo-

rithm), we found there was a possible interaction between lncRNA-HOTAIR and HOXA1. Next, we observed that the methylation level of HOXA1 was obviously consistent with lncRNA-HOTAIR downregulation. RIP method was subsequently carried out to validate the interaction between lncRNA-HOTAIR and HOXA1. These results support that lncRNA-HOTAIR can affect the methylation level of HOXA1.

DNA methylation is catalyzed via DNMTs: DNMT1, DNMT3a, and DNMT3b. DNMT1 is the most abundant DNA methyltransferase in mammalian cells and codes for maintenance methylation and *de novo* methylation. DNMT3a and DNMT3b are responsible for catalyzing *de novo* methylation during early embryogenesis, thereby establishing a somatic methylation pattern.²⁵ To better understand the way by which lncRNA-HOTAIR regulated the HOXA1 methylation level, we measured DNMT expression including DNMT1, DNMT3a, and DNMT3b in H69AR and H446AR cells by ELISA assay. The results showed that the expression of DNMT1 and DNMT3b decreased significantly after the downregulation of lncRNA-HOTAIR, but no significant difference was observed in DNMT3a expression. Previous studies have shown that genetic disruption of both DNMT1 and DNMT3b nearly eliminated methyltransferase activity, and reduced genomic DNA methylation by >95% in colorectal cancer cells.²⁶ Inhibiting DNMTs by 5-Aza-CdR treatment led to significant inhibition of expression of DNMT1 and DNMT3b but not DNMT3a and enhanced expression of PTEN and p21 analyzed in glioma.²⁷ All these

Figure 4 HOXA1 expression was regulated by lncRNA-HOTAIR. (a) Bioinformatics prediction of the interaction between lncRNA-HOTAIR and HOXA1. (b) RIP enrichment was determined as RNA associated with HOXA1 antibody relative to IgG negative control. (c and d) The mRNA and protein expression of HOXA1 after cells were transfected with siHOTAIR. * $P < 0.05$, ** $P < 0.01$.

Figure 5 lncRNA-HOTAIR epigenetically silenced HOXA1 through DNMT1 and DNMT3b. (a) HOXA1 methylation status of H69/H69AR/siHOTAIR-treated H69AR cells (top set) and H446/H446AR/siHOTAIR-treated H446AR cells (bottom set). (b) Quantification of DNMTs after transfection with siHOTAIR in both resistant cells by ELISA. Results are mean $\pm$ s.d. ** $P < 0.01$.

Figure 6 Flow diagram of the lncRNA-HOTAIR on SCLC chemoresistance by regulating HOXA1 methylation.

results including ours suggested that DNMT1 and DNMT3b cooperatively maintained DNA methylation and gene silencing in oncogenesis and chemoresistance of tumors.

In summary, our results demonstrated that lncRNA-HOTAIR was overexpressed in the resistant cells. Down-regulation of lncRNA-HOTAIR led to increased cell sensitivity to anticancer drugs, enhanced cell apoptosis and cell cycle arrest, and reduced tumor growth *in vivo*. DNA methylation of HOXA1 was required for the chemoresistance of SCLC. lncRNA-HOTAIR epigenetically silenced HOXA1 through DNMT1 and DNMT3b. Our findings revealed that lncRNA-HOTAIR can modulate chemoresistance of SCLC by regulating HOXA1 methylation (Figure 6). lncRNA-HOTAIR might be a predictor for chemotherapy response and a therapeutic target against chemoresistance of SCLC.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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