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ORIGINAL RESEARCH

Knockdown of Long Non-Coding RNA *HOTAIR* Suppresses Cisplatin Resistance, Cell Proliferation, Migration and Invasion of DDP-Resistant NSCLC Cells by Targeting *miR-149-5p/Doublecortin-Like Kinase 1* Axis

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Background: Long non-coding RNA (lncRNA) *HOTAIR* has been reported to be associated with cisplatin (DDP) resistance in different human cancers including non-small cell lung cancer (NSCLC). However, the mechanism of HOTAIR in cisplatin resistance of NSCLC remains largely undefined.

Materials and Methods: Expression of *HOTAIR*, *miR-149-5p* and *doublecortin-like kinase 1* (*DCLK1*) was detected using real-time quantitative PCR (RT-qPCR) and Western blotting. Cisplatin resistance was determined with cell counting kit (CCK)-8 assay and transwell assays in vitro, and xenograft tumor models in vivo. The target binding between *miR-149-5p* and either *HOTAIR* or *DCLK1* was predicted on Diana Tools website, and confirmed by dual-luciferase reporter assay and RNA immunoprecipitation.

Results: Expression of *HOTAIR* was upregulated in DDP-resistant NSCLC tumor tissues and cell lines (A549/DDP and H1299/DDP). Knockdown of *HOTAIR* decreased the acquired cisplatin resistance of A549/DDP and H1299/DDP cells, as evidenced by attenuated 50% inhibitory concentration (IC50) of DDP, cell proliferation, migration and invasion in vitro, as well as tumor growth inhibition in vivo. Mechanically, *HOTAIR* negatively regulated *miR-149-5p* expression via targeting, and DCLK1 was a downstream target for miR-149-5p. *DCLK1* was indirectly regulated by *HOTAIR* in DDP-resistant NSCLC cells as well. Functionally, *miR-149-5p* deletion could counteract the inhibitory effect of HOTAIR knock-down on cisplatin resistance; contrarily, restoring *miR-149-5p* exhibited the similar inhibition on cisplatin resistance in DDP-resistant cells in vitro, which was then abated by *DCLK1* upregulation.

Conclusion: Knockdown of *HOTAIR* enhances DDP-resistant NSCLC cells to overcome cisplatin resistance partially via regulating *miR-149-5p/DCLK1* axis.

Keywords: *HOTAIR*, NSCLC, cisplatin resistance

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Introduction

Lung cancer is the leading cause of cancer-related death among males, and non-small-cell lung cancer (NSCLC) accounts for approximately 85% in all primary lung cancers.[1] NSCLC is a type of heterogeneous tumor and classified into three different subtypes: squamous cell carcinoma, adenocarcinoma and large cell carcinoma.[2] In

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terms of the treatment of NSCLC, platinum-based chemotherapy following surgical resection has been a standard strategy.[3] Cisplatin (DDP), a platinum-containing compound, remains a reference standard for the first-line chemotherapy of multiple cancers including NSCLC. However, the clinical outcome remains disappointing in NSCLC patients, largely due to the acquired clinical resistance.[4] Therefore, reducing drug resistance may be a promising approach for the treatment of DDP-resistant NSCLC patients.

Recently, accumulating evidence has reported the link between the dysregulation of long non-coding RNAs (lncRNAs) and drug resistance in cancers.[5] LncRNAs are a type of transcripts with more than 200 nucleotides with little protein-coding capacity. Dysregulation of lncRNAs plays an essential role in the initiation and development of tumors.[6] *HOTAIR* is one of the key lncRNAs that is found to be upregulated in various human cancers. Acting as one oncogene, *HOTAIR* is closely related to the resistance of chemotherapy drugs.[7] In lung cancer, *HOTAIR* correlates with metastasis and poor prognosis in these patients, and acts as an oncogene in cell proliferation, metastasis and drug resistance.[8] Nevertheless, the complete biological roles of HOTAIR, especially on drug resistance, in NSCLC are undisclosed.

HOTAIR can potentially regulate lung cancer through multiple mechanisms such as crosstalk with microRNAs (miRNAs), which has become an emerging light-spot in the non-coding world.[9] MiRNAs are another group of noncoding transcripts with approximately 22 nucleotides. It has been well recognized about miRNAs as pivotal regulators of cisplatin resistance in lung cancers[10] including NSCLC.[11,12] *MiRNA (miR)-149-5p* was predicted as a novel potential target gene for *HOTAIR* according to DianaTools database in the present study. The linking between *miR-149-5p* and cisplatin resistance has already reported in several types of cancers[13,14] including NSCLC.[15]

Doublecortin-like kinase 1 (*DCLK1*) is often overexpressed in human cancers including NSCLC, and takes part in tumorigenesis, metastasis and drug resistance.[16–18] In this study, we detected the expression of *HOTAIR*, *miR-149-5p* and *DCLK1* in DDP-resistant and -sensitive NSCLC tissues and cells. Then, 50% inhibitory concentration (IC50), cell proliferation, migration and invasion in vitro and tumor growth in vivo were analyzed to determine the effect of *HOTAIR* dysregulation on cisplatin resistance. Furthermore, the relationship among *HOTAIR*, *miR-149-5p* and *DCLK1* in cisplatin resistance in NSCLC was confirmed.

Materials and Methods

Clinical Samples and Tissue Acquirement

Seventy patients with NSCLC were recruited from the Affiliated Tumor Hospital of Xinjiang Medical University, and the clinicopathological parameters of these NSCLC patients were presented in Table 1. All patients were received definitive chemotherapy with cisplatin after surgery. The NSCLC tumor tissues were acquired and directly preserved in liquid nitrogen during surgery. No enrolled patients in our study received anti-cancer therapy prior to surgery. According to the tracking survey, NSCLC tissue samples divided into two groups: DDP resistant (n=35) and DDP sensitive (n=35). DDP-resistant NSCLC was defined as tumor progression or recurrence within 6 months after the last DDP treatment, while those recurrence or progression more than 6 months were identified as DDP-sensitive NSCLC. This study was approved by the Ethics Committee

Table 1 Correlation Between *HOTAIR* Expression and Clinicopathological Parameters of Patients with NSCLC

Characteristics	Number	*HOTAIR* Expression		P
		High	Low	
		35	35	
Age (years)				0.810
<60	31	16	15	
≥60	39	19	20	
Gender				0.631
Male	38	20	18	
Female	32	15	17	
Tumor size (cm)				0.629
≤3	30	14	16	
>3	40	21	19	
TNM stage				0.017*
I–II	36	13	23	
III–IV	34	22	12	
Lymph node metastasis				0.016*
Negative	40	15	25	
Positive	30	20	10	
Chemotherapy response				0.031*
Sensitive	35	22	13	
Resistant	35	13	22	

Note: *$P < 0.05$, statistically significant.

of the Affiliated Tumor Hospital of Xinjiang Medical University and participators in the form of written informed consents.

Cells and Cell Culture

Human NSCLC cell lines A549 (CCL-185) and H1299 (CRL-5803) were obtained from American Type Culture Collection (Manassas, VA, USA). All cells were grown up in Roswell Park Memorial Institute 1640 medium (RPMI-1640; Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco) at 37°C in 5% CO_2.

Construction of DDP-Resistant NSCLC Cells in vitro

A549 and H1299 cells were prepared to forcedly acquired DDP resistance. The cells were pre-treated with stepwise increasing concentrations of DDP (Sigma-Aldrich Co., St Louis, MO, USA). To maintain the resistance phenotype of DDP-resistant A549 and H1299 cells, 5 μM DDP was additionally added into the RPMI medium for long-time culture. After the determination for 50% inhibitory concentration (IC50), these cells were named as A549/DDP and H1299/DDP.

Cell Transfection

For overexpression, *miR-149-5p* mimic and miR-NC mimic were purchased from GenePharma (Shanghai, China); the sequence of *HOTAIR* and coding domain sequence of *DCLK1* were cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), respectively. For knockdown, *miR-149-5p* inhibitor (in-miR-149-5p), siRNA against *HOTAIR* (si-HOTAIR), and their controls were obtained from GenePharma. Cell transfection was carried out with Lipofectamine™ 2000 (Invitrogen) according to the manufacturers' instruction. After transfection for 24 h, A549/DDP and H1299/DDP cells were collected for further experiments. Sequences of siRNAs were as follows: si-HOTAIR: 5′- GAACGGGAGUACAGAGAGAUU-3′; si-NC: 5′-GAACGGAGCGAGCAGACCUUU-3′.

Cell Counting Kit (CCK)-8 Assay

For IC50 analysis, the parental cells and A549/DDP and H1299/DDP cells were seeded into 96-well plate (Corning, NY, USA) for overnight and then exposed to DDP (10, 20, 40, 60, 80, and 100 μM) for 48 h. 10 μL CCK-8 solution was added to each well and the cultures were incubated for another 4 h at 37°C. After Mixing on an orbital shaker for 5 min, optical density at 450 nm was recorded using a microplate reader. The experiments were conducted at least 3 times. The IC50 values were calculated by the relative dose–response survival curve. For cell viability assay, transfected A549/DDP and H1299/DDP cells were exposed to 60 μM DDP for 0, 24, 48 and 72 h. All the other operations were the same with IC50 analysis.

Real-Time Quantitative PCR (RT-qPCR)

Total RNA in tissues and cells was isolated with TRIzol (Invitrogen). The first-strand cDNA with SuperScript II reverse transcriptase (Invitrogen) with special stem-loop primer for miRNA and the quantitative PCR was performed with SYBR® Premix Ex Taq™ (Takara, Shiga, Japan) on Bio-Rad iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) and *U6 small nuclear RNA* (*U6*) were used as an internal control to *HOTAIR*, *DCLK1* and *miR-149-5p*. The expression was calculated according to the comparative threshold (Ct) method as normalized to that of U6 or GAPDH ($2^{-\Delta Ct}$) and the fold changes were calculated by the equation $2^{-\Delta\Delta Ct}$. The reactions were performed in triplicate for each sample and the primers involved were listed as follows: *HOTAIR*, 5′-TGCTACTTGTGTAGACCCAG-3′ (sense) and 5′-AGCAAAGGCTGGACCTTTGCT-3′ (anti-sense); *miR-149-5p*, 5′-TCGGCAGGUCUGGCUCCGU GUC-3′ (sense) and 5′-CCGAGGACGGGAGTG −3′ (anti-sense); *DCLK1*, 5′-GGAGTGGTGAAACGCCTGTAC-3′ (sense) and 5′-GGTTCCATTAACTGAGCTGG-3′ (anti-sense); *GAPDH*, 5′-CCCCTTCATTGACCTCAACTACAT -3′ (sense) and 5′-CGCTCCTGGAAGATGGTGA-3′ (anti-sense); *U6*, 5′-TTCACGAATTTGCGTGTCAT-3′ (sense) and 5′-CGCTCGGCAGCACATATAC-3′ (anti-sense).

Transwell Assay

The ability of cell migration and invasion was measured using Transwell chamber (8 μm pore size, Corning) with matrigel-free (for migration) or matrigel-coated (Bection Dickinson, Franklin Lakes, NJ, USA) (for invasion). Transfected A549/DDP and H1299/DDP cells (2×10^4 cells) in 200 μL serum-free medium were implanted in the upper chambers. The 500 μL medium containing 10% FBS was used as a chemo-attractant and loaded in the low chamber. Transwell system was stayed at 37°C for 24 h. After removing the cells on the top surface with a cotton swab, cells on the lower surface were stained with 0.1% crystal violet for 15 min at room temperature, followed with being photographed and counted under

a microscope in five predetermined fields (×200). Three independent experiments were carried out.

Western Blotting

Total protein from cultured A549/DDP and H1299/DDP cells was isolated in RIPA lysis buffer (Beyotime, Shanghai, China). According to Bradford protein assay reagent (Bio-Rad), equal amounts of protein (20 μg) from each sample were loaded for the standard procedures of Western blot assay. *β-actin* was used to normalize the *DCLK1* protein level. The primary antibodies including *DCLK1* (#62,257, 1:1000) and *β-actin* (#4967, 1:1000) were purchased from Cell Signaling Technology (CST; Danvers, MA, USA), and *Ki-67* (#ab197547, 1:500), cleaved *caspase-3* (#ab49822, 1:500) were from abcam (Cambridge, UK).

Luciferase Reporter Assay and RNA Immunoprecipitation (RIP)

According to in cilico data, there were potential complementary binding sites of miR-149-5p in human *HOTAIR* and 3′UTR of *DCLK1* (NM_004734.5). Then, the wild types of *HOTAIR* and 3′UTR of *DCLK1* (HOTAIR-WT and DCLK1 3′UTR-WT) were separately cloned into psi-CHECK-2 vector (Invitrogen) using PCR methods, as well as their mutants HOTAIR-MUT and DCLK1 3′UTR-MUT. A549/DDP and H1299/DDP cells were co-transfected with miR-149-5p/NC mimic and either HOTAIR-WT/MUT or DCLK1 3′UTR-WT/MUT. After 24 h incubation, cells were harvested to measure Firefly and Renilla luciferase activities using the dual-luciferase reporter assay system (Promega, Madison, WI, USA). The transfections were repeated at least three times.

The RIP assay was performed in A549/DDP and H1299/DDP cells after the transfection of miR-149-5p/NC mimic. Magna RIP™ RNA-binding protein immunoprecipitation kit (Millipore-Sigma, Billerica, MA, USA) was chosen to enrich HOTAIR from the samples bound to the *Ago2* or *IgG* antibody obeyed the standard instructions. The co-precipitated RNAs were detected by RT-qPCR.

Xenograft Mouse Model

Six-week athymic BALB/c mice were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. The animal experiments were approved by The Institutional Review Board of the Affiliated Tumor Hospital of Xinjiang Medical University and were untaken in accordance with National Institutions of Health Guide for Care and Use of Laboratory Animals. H1299/DDP cells were stably transfected lentiviral particles encoding shRNA against *HOTAIR* (sh-HOTAIR; Neuron Biotech, Shanghai, China) or the negative control (sh-NC) using Polybrene reagent (Sigma). Equal numbers (10^6) of transfected H1299/DDP cells/0.2 mL were subcutaneously injected in subcutaneous area of flanks (5 mice per group) for 35 days. One week later after transplantation, xenograft mice were subjected to intra-peritoneal injection of DDP at a dose of 3.0 mg/kg body weight or phosphate buffer solution (PBS; pH 7.4) every 7 days from the 7th day. Xenograft experiments were divided into three groups: sh-NC+PBS, sh-HOTAIR+PBS, sh-NC+DDP, and sh-HOTAIR+DDP. The tumors were measured with a caliper once 7 days, and the mice were practiced with euthanasia on day 35. The tumor volume was calculated using the formula: $0.5 \times l \times w^2$ (l is the length of tumor and w is the width of tumor). And the weight of tumors was evaluated with electronic balance. Immediately, the tumors were frozen in −80°C for further isolation of total RNA.

Statistical Analysis

Statistics were analyzed by SPSS 21.0 (SPSS Inc, Chicago, IL, USA) and presented as the mean ± standard deviation. Unpaired Student's *t*-test method was utilized for comparison between two groups, while one-way analysis of variance was used for data comparison in multiple groups. $P < 0.05$ was considered as statistically significant.

Results

HOTAIR Was Upregulated in DDP-Resistant NSCLC Tissues and Cells

To investigate the association of *HOTAIR* expression with NSCLC chemoresistance against DDP, RT-qPCR was carried out to confirm its expression in patients with NSCLC. As shown in Figure 1A, HOTAIR levels were significantly higher in DDP-resistant tumors (n=35) than DDP-sensitive tumors (n=35). In clinic, high *HOTAIR* level was significantly associated with TNM stage, lymph node metastasis and DDP response (Table 1). The DDP-resistant NSCLC cells (A549/DDP and H1299/DDP) in vitro were developed based on their parental cell lines (A549 and H1299). CCK-8 assay further confirmed this acquired cisplatin resistance, as depicted by the increased IC50 values of DDP in A549/DDP and H1299/DDP cells than that in parental cells (Figure 1B and C). To further testify whether

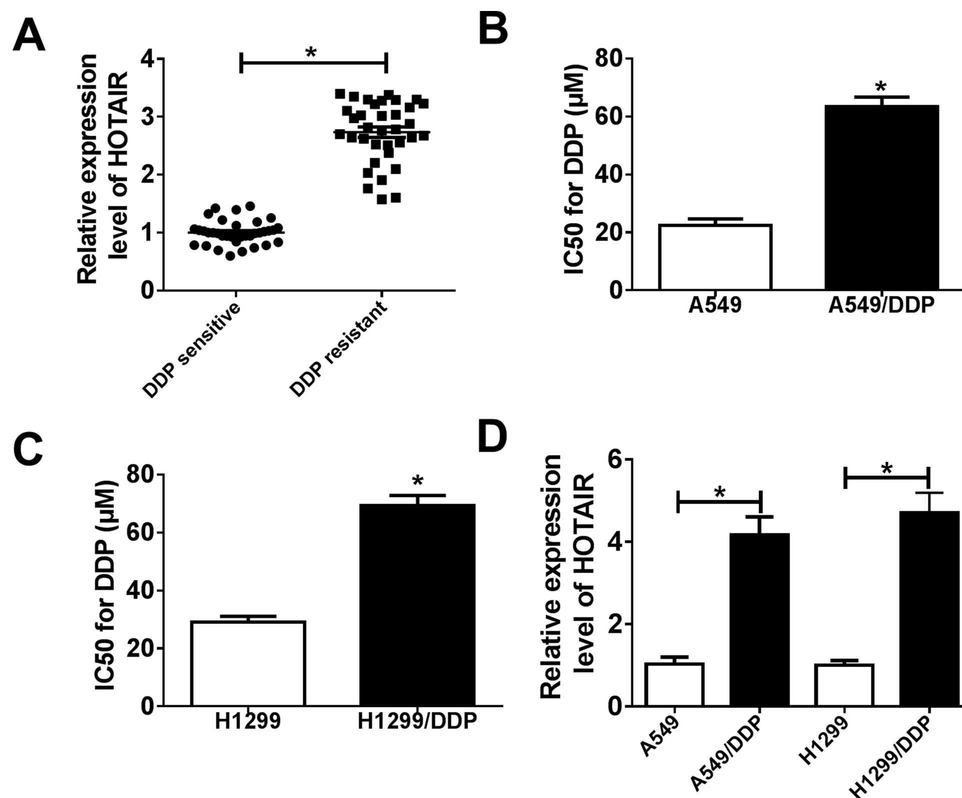

Figure 1 Expression of lncRNA *HOTAIR* (*HOTAIR*) in cisplatin (DDP)-resistant non-small cell lung cancer (NSCLC) tissues and cells. (**A**) Real-time quantitative PCR (RT-qPCR) assay showed the expression levels of *HOTAIR* in DDP-resistant and -sensitive NSCLC tissues. (**B, C**) The 50% inhibitory concentration (IC50) values of DDP were determined by Cell Counting Kit (CCK)-8 assay in A549/DDP and H1299/DDP cells with their parental cells. The cells were exposed to different concentrations (10, 20, 40, 60, 80, and 100 μM) of DDP for 48 h. (**D**) RT-qPCR assay showed *HOTAIR* levels in A549/DDP and H1299/DDP cells with their parental cells. *$P < 0.05$.

HOTAIR plays a critical role in the acquired DDP resistance of NSCLC cells, the expression of *HOTAIR* was also detected utilizing RT-qPCR. Expectedly, *HOTAIR* was upregulated in A549/DDP and H1299/DDP cells (Figure 1D). These data showed an increase of HOTAIR in DDP-resistant NSCLC tissues and cells.

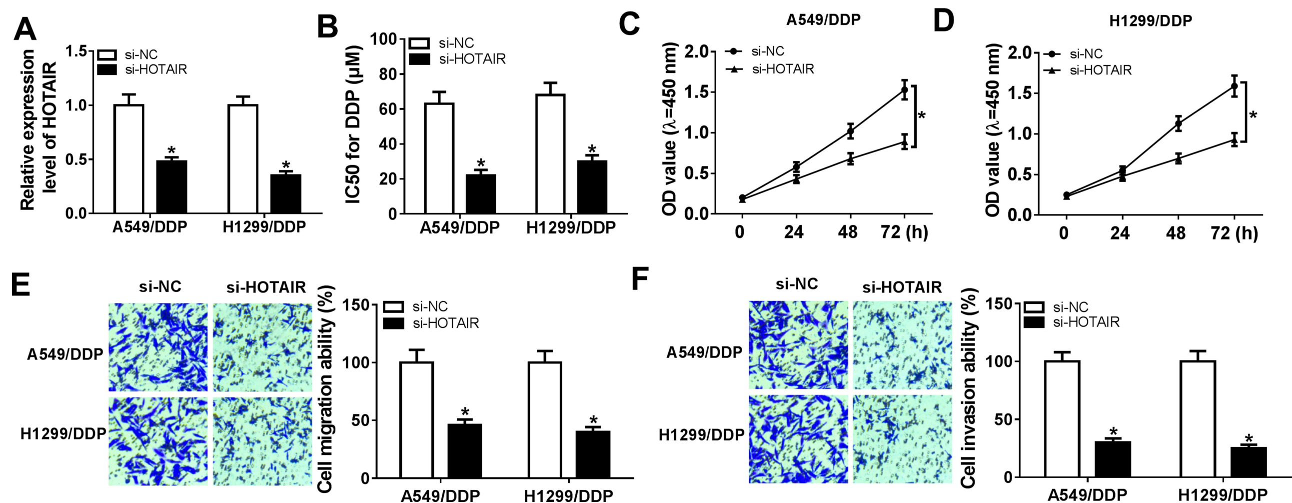

Figure 2 Effects of *HOTAIR* knockdown on cisplatin resistance in NSCLC cells. A549/DDP and H1299/DDP cells were transfected with siRNA against *HOTAIR* (si-HOTAIR) or its negative control (si-NC). (**A**) RT-qPCR assay showed *HOTAIR* expression levels after transfection for 24 h. (**B**) IC50 values of DDP were determined by CCK-8 assay after treated with different concentrations (10, 20, 40, 60, 80, and 100 μM) of DDP for 48 h. (**C, D**) CCK-8 assay detected cell viability after transfection for 0, 24, 48 and 72 h. (**E, F**) Transwell assays measured cell migration and invasion after transfection for 24 h. The cell migration/invasion ability was calculated as % of total cells. *$P < 0.05$.

HOTAIR Knockdown Inhibited Cisplatin Resistance in DDP-Resistant NSCLC Cells in vitro

The role of *HOTAIR* in cisplatin resistance was determined in loss-of-function experiments. The siRNA against *HOTAIR* was used in A549/DDP and H1299/DDP, and RT-qPCR confirmed the transfection efficiency (Figure 2A). Secondly, drug resistance was assessed by IC50 value, cell proliferation, migration and invasion. The CCK-8 results showed that si-HOTAIR1 could significantly decrease the IC50 of DDP in A549/DDP and H1299/DDP cells (Figure 2B), as well as cell proliferation (Figure 2C and D); the ability of cell migration and invasion analyzed by transwell assays was lowered by si-HOTAIR transfection for 24 h (Figure 2E and F). Thus, knockdown of *HOTAIR* could reverse the high IC50 of DDP, cell proliferation, migration and invasion in A549/DDP and H1299/DDP cells. On the contrary, overexpression of *HOTAIR* via pcDNA vector transfection led to increased malignant behaviors of the parental cells (A549 and H1299), as evidenced by increased IC50 value of DDP, cell proliferation, migration and invasion (Supplementary Figure 1A–F).

miR-149-5p Sponged by *HOTAIR* Was Downregulated in DDP-Resistant NSCLC Tissues and Cells

A possible target miRNAs of HOTAIR were retrieved on DianaTools website, and *miR-149-5p* was further identified to be highly expressed in *HOTAIR*-silenced A549/DDP and H1299/DDP cells (Figure 3A). The sequences of the putative binding site of miR-149-5p in HOTAIR-WT were mutated as the complementary sequences (Figure 3B). Next, a dual-luciferase reporter assay was performed to show that *miR-149-5p* mimic significantly diminished luciferase activity of HOTAIR-WT in both A549/DDP and H1299/DDP cells (Figure 3C and D); whereas there was little influence on the luciferase activity of HOTAIR-MUT whenever transfected with *miR-149-5p* mimic or miR-NC mimic. In addition, RIP assay was carried out to further verify this target binding. As a result, *HOTAIR* was abundantly enriched by *Ago2* in A549/DDP and H1299/DDP cells with *miR-149-5p* overexpression (Figure 3E). These results indicated that *miR-149-5p* was sponged by *HOTAIR* via targeting. Moreover, expression of *miR-149-5p* was lower in DDP-resistant NSCLC tissues and cells (Figure 3F and G); its expression was downregulated in A549/DDP and H1299/DDP cells transfected with pcDNA-HOTAIR and upregulated with si-HOTAIR transfection (Figure 3H).

Reduced *miR-149-5p* Counteracted the Inhibitory Effect of *HOTAIR* Knockdown on Cisplatin Resistance

Then, a series of rescue experiments were performed to testify the occurrence of *HOTAIR*/*miR-149-5p* axis. A549/DDP and H1299/DDP cells were transfected with si-HOTAIR or si-NC and co-transfected with si-HOTAIR and in-miR-149-5p or in-miR-NC. As depicted in Figure 4A, si-HOTAIR transfection led to increased expression of *miR-149-5p*, whereas this upregulation was then impaired by in-miR-149-5p transfection. CCK-8 assay demonstrated that si-HOTAIR-mediated the decrease of IC50 of DDP (Figure 4B) and cell proliferation (Figure 4C and D in A549/DDP and H1299/DDP cells) were apparently improved by introducing in-miR-149-5p. Consistently, si-HOTAIR-induced inhibition on cell migration and invasion was attenuated after in-miR-149-5p transfection (Figure 4E and F). These outcomes showed that blocking *miR-149-5p* could counteract the inhibitory effect of *HOTAIR* knockdown on acquired cisplatin resistance in A549/DDP and H1299/DDP cells, suggesting that *HOTAIR* modulated cisplatin resistance in DDP-resistant NSCLC cells via at least partially targeting *miR-149-5p*.

DCLK1 Was Positively Regulated by *HOTAIR via miR-149-5p*

We suspected *DCLK1* as one target gene of *miR-149-5p* according to DianaTools website, and the putative binding sites between *DCLK1* and *miR-149-5p* were presented (Figure 5A). Dual-luciferase reporter assay further identified this potential target binding, as evidenced by the decline of luciferase activity in A549/DDP and H1299/DDP cells co-transfected with DCLK1 3′UTR-WT and miR-149-5p mimic (Figure 5B and C). Expression of *DCLK1* was measured by RT-qPCR and *DCLK1* mRNA levels were higher in DDP-resistant NSCLC tissues and cells (Figure 5D and E); moreover, its protein level analyzed by Western blotting was downregulated in A549/DDP and H1299/DDP cells transfected with miR-149-5p mimic and upregulated with in-miR-149-5p transfection or pcDNA-HOTAIR transfection (Figure 5F and G); meanwhile, *HOTAIR* overexpression-mediated upregulation of *DCLK1* was further attenuated with the presence of miR-149-5p mimic. These results indicated that *HOTAIR*

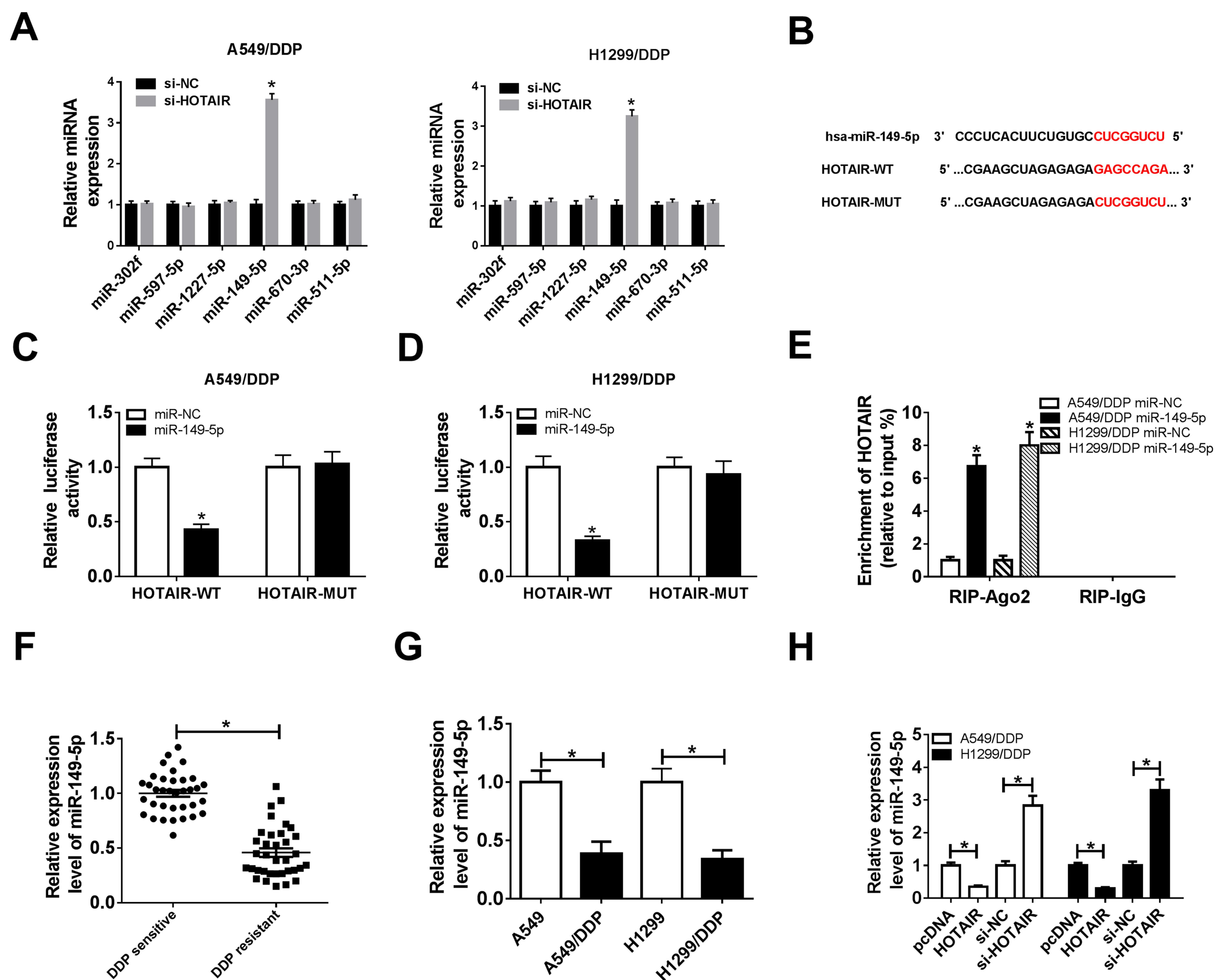

Figure 3 Identification of the negative regulatory relationship between *HOTAIR* and *miR-149-5p*. (**A**) RT-qPCR assay measured expression levels of miRNAs in A549/DDP and H1299/DDP cells transfected with si-*HOTAIR* or si-NC. (**B**) The predicted *hsa-miR-149-5p* binding sites in *HOTAIR* according to DianaTools. The corresponding sequence in the mutated version was shown as well. (**C, D**) Luciferase activity of *HOTAIR* wild type (HOTAIR-WT) and mutant (HOTAIR-MUT) was examined by dual-luciferase reporter assay in A549/DDP and H1299/DDP cells when transfected with *miR-149-5p* mimic (miR-149-5p) or miR-NC mimic (miR-NC). (**E**) Expression of *HOTAIR* was detected with RNA immunoprecipitation (RIP) assay in A549/DDP and H1299/DDP cells transfected with miR-149-5p/NC. The enrichment of *HOTAIR* level was showed as % of input. (**F, G**) RT-qPCR assay showed the expression levels of *miR-149-5p* in DDP-resistant NSCLC tissues and cells (A549/DDP and H1299/DDP), compared to DDP-sensitive NSCLC tissues and parental cells. (**H**) RT-qPCR assay measured *miR-149-5p* expression levels in A549/DDP and H1299/DDP cells transfected with pcDNA-HOTAIR (HOTAIR), si-HOTAIR or their controls. *$P < 0.05$.

sponging *miR-149-5p* indirectly regulated *DCLK1* expression in DDP-resistant NSCLC.

Elevated *DCLK1* Abated the Suppressive Effect of *miR-149-5p* Overexpression on Cisplatin Resistance in DDP-Resistant NSCLC Cells in vitro

The role of *miR-149-5p* in cisplatin resistance in NSCLC cells was researched, as well as the presence of *miR-149-5p/DCLK1* axis. A549/DDP and H1299/DDP cells were transfected with miR-149-5p/NC mimic and co-transfected with miR-149-5p mimic and pcDNA-DCLK1 or pcDNA empty vector. As depicted in Figure 6A and B, IC50 of DDP was decreased with miR-149-5p mimic transfection accompanied with downregulated *DCLK1* protein. Cell proliferation of A549/DDP and H1299/DDP cells was diminished by miR-149-5p mimic transfection (Figure 6C and D), as well as the ability of cell migration and invasion (Figure 6E and F). More importantly, the inhibition of *miR-149-5p* overexpression on *DCLK1* expression, IC50 of DDP, cell proliferation, migration and invasion was overall significantly abated in A549/DDP and H1299/DDP cells co-transfected with pcDNA-DCLK1 (Figure 6A–F). These data showed a suppressive role of *miR-149-5p*

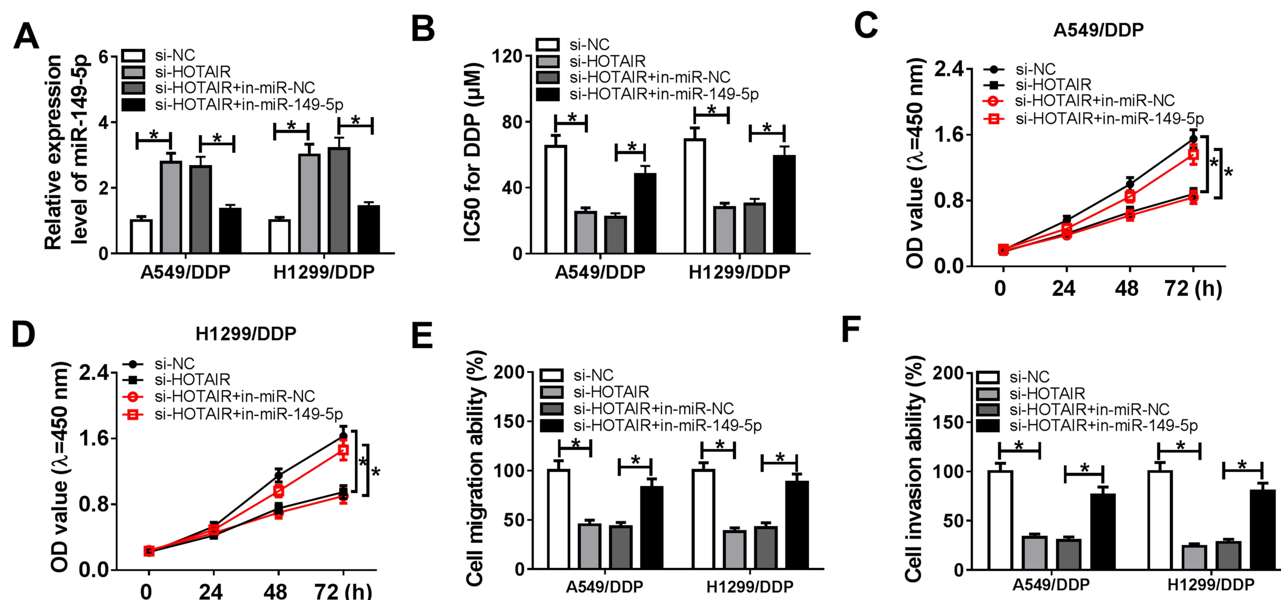

Figure 4 Influence of *miR-149-5p* reduction on the inhibitory effect of *HOTAIR* knockdown on cisplatin resistance. A549/DDP and H1299/DDP cells were transfected with si-HOTAIR or si-NC, and co-transfected with si-HOTAIR and miR-149-5p/NC inhibitor (in-miR-149-5p/NC). (**A**) Expression levels of *miR-149-5p* were detected by RT-qPCR after transfection. CCK-8 assay detected (**B**) IC50 values of DDP after transfection for 48 h and (**C, D**) cell viability after transfection for 0, 24, 48 and 72 h. (**E, F**) Transwell assays measured cell migration and invasion after transfection for 24 h. $^{*}P < 0.05$.

overexpression in cisplatin resistance in DDP-resistant NSCLC cells in vitro partially through downregulating *DCLK1*.

Knockdown of *HOTAIR* Inhibited Tumor Growth and Cisplatin Resistance of DDP-Resistant NSCLC Cells in vivo

To confirm the effects of *HOTAIR* on cisplatin resistance of NSCLC cells in vivo, H1299/DDP cells stably infected with sh-HOTAIR or sh-NC was subcutaneously injected into BALB/c nude mice (n=5), followed with DDP (3.0 mg/kg body weight) administration or PBS treatment. As shown in Figure 7A and B, xenograft tumor was generated after implantation for 7 days; sh-HOTAIR extremely decreased tumor volume and tumor weight in both groups treated with DDP or PBS. RT-qPCR analysis clarified that sh-HOTAIR transfection resulted in lower *HOTAIR* and *DCLK1* expression, and higher *miR-149-5p* expression in xenograft tumor tissues, accompanied with downregulated *Ki-67* and upregulated cleaved *caspase-3* (Figure 7C and D). Collectively, these results implicated that *HOTAIR* knockdown could suppress cisplatin resistance of H1299/DDP cells in vivo partially through upregulating *miR-149-5p* and downregulating *DCLK1*.

Discussion

Previous studies have shown dysregulation of lncRNAs participate in tumor progression and chemoresistance.[19] *HOTAIR* contributes to cisplatin resistance of NSCLC through several mechanisms such as downregulating *p21*,[20] upregulating *Kruppel-like factor 4*,[21] targeting *miR-326/specificity protein 1* axis,[22] and activating *Wnt* signaling pathway.[23] In the present study, we observed the upregulation of *HOTAIR* in DDP-resistant NSCLC tissues and investigated the promoting effect of *HOTAIR* on acquired cisplatin resistance in NSCLC cells through targeting *miR-149-5p/DCLK1* axis.

HOTAIR takes part in different drug resistance of NSCLC cells. For instance, silencing of *HOTAIR* decreased crizotinib resistance by suppressing the phosphorylation of *ULK1* in inactivating autophagy.[24] Liu et al[25] indicated that elevated *HOTAIR* upregulated expression of *KLF4*, tumor stem cell-related biomarkers, which might lead to cisplatin resistance in A549/DDP cells. Liu et al[26] reported that the *HOTAIR* downregulation could restore gefitinib sensitivity through activating *Bax/Caspase3* pathway and suppressing *TGFα/EGFR* signaling. *HOTAIR* targeting miRNAs has been a well-documented mechanism in regulating cisplatin resistance of human cancers. For example, *HOTAIR* targeted *miRNA-126*[27] or *miRNA-34a*[28] to promote cisplatin resistance in gastric cancer cells. It was depicted that *miRNA-326* targeting *SP1* was declared to reverse chemoresistance

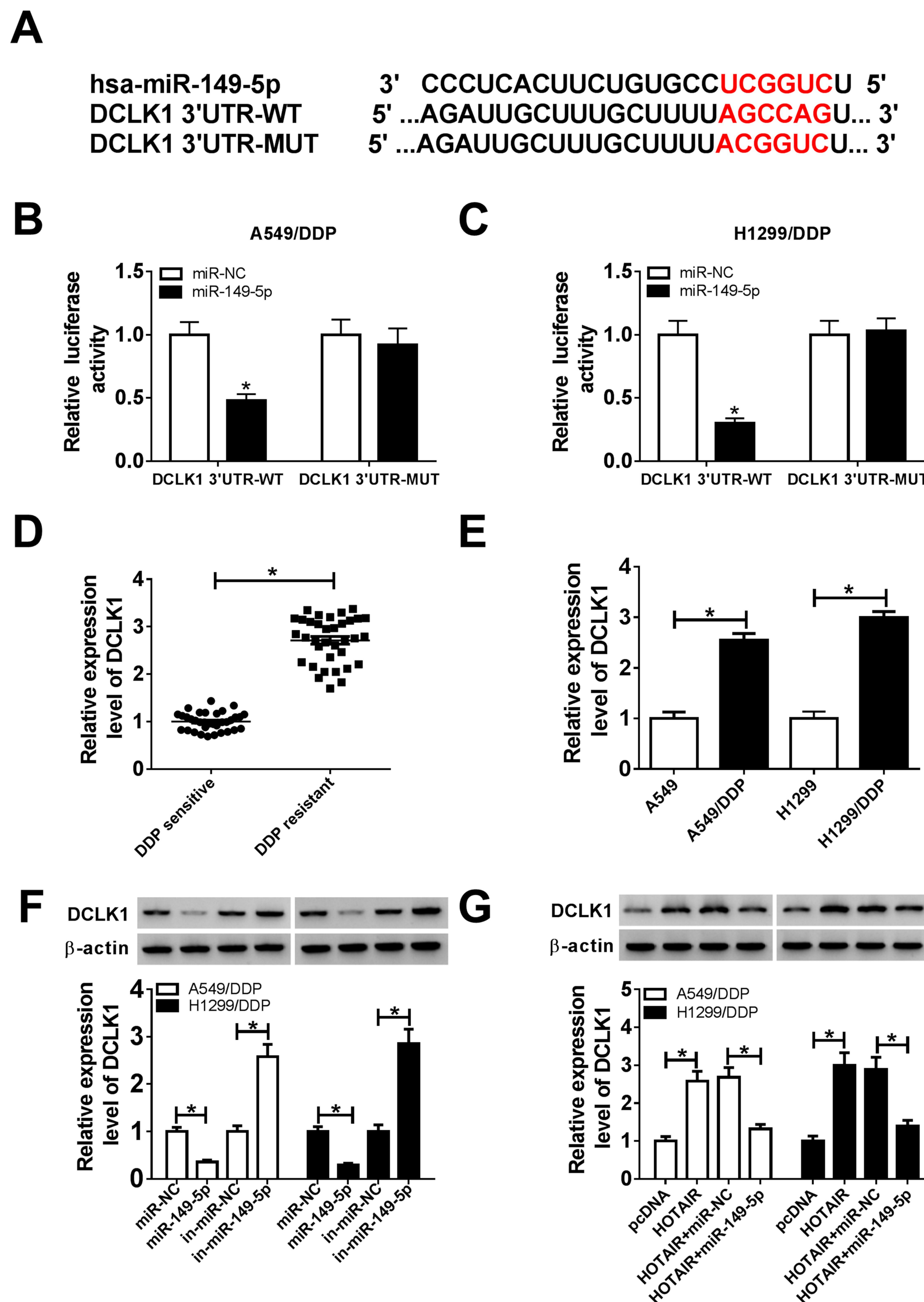

Figure 5 Verification of the target relationship between *miR-149-5p* and *doublecortin-like kinase 1* (*DCLK1*). (**A**) The predicted *hsa-miR-149-5p* binding sites in *DCLK1* 3′ untranslated regions (3′UTR) according to DianaTools. The corresponding sequence in the mutated version was shown as well. (**B, C**) Luciferase activity of *DCLK1* 3′UTR wild type (DCLK1 3′UTR-WT) or mutant (DCLK1 3′UTR-MUT) in A549/DDP and H1299/DDP cells transfected with miR-149-5p or miR-NC. (**D, E**) RT-qPCR assay showed the expression levels of *DCLK1* mRNA in DDP-resistant NSCLC tissues and cells (A549/DDP and H1299/DDP), compared to DDP-sensitive NSCLC tissues and parental cells. (**F, G**) Western blotting measured *DCLK1* protein expression levels in A549/DDP and H1299/DDP cells when transfected with miR-149-5p, in-miR-149-5p, pcDNA-HOTAIR, si-HOTAIR, or their controls. β-actin was detected as the internal reference. *$P < 0.05$.

of A549/DDP cells both in vitro and in vivo, which was mediated by *HOTAIR* repression.[22] Here, we noticed a decrease in cell viability, migration and invasion, as well as attenuated IC50 values and tumor growth in A549/DDP and H1299/DDP cells after HOTAIR was knocked down. Besides, overexpressing *HOTAIR* could induce A549 and H1299 cells to acquire cisplatin resistance as well. These outcomes suggested a promoting role of *HOTAIR* in cisplatin resistance in NSCLC. Besides, the anti-growth and anti-metastasis properties of *HOTAIR* deficiency in A549 and H1299 cells both in vitro and in vivo as well.[24,29,30] Notably, luciferase reporter assay and RIP assay testified

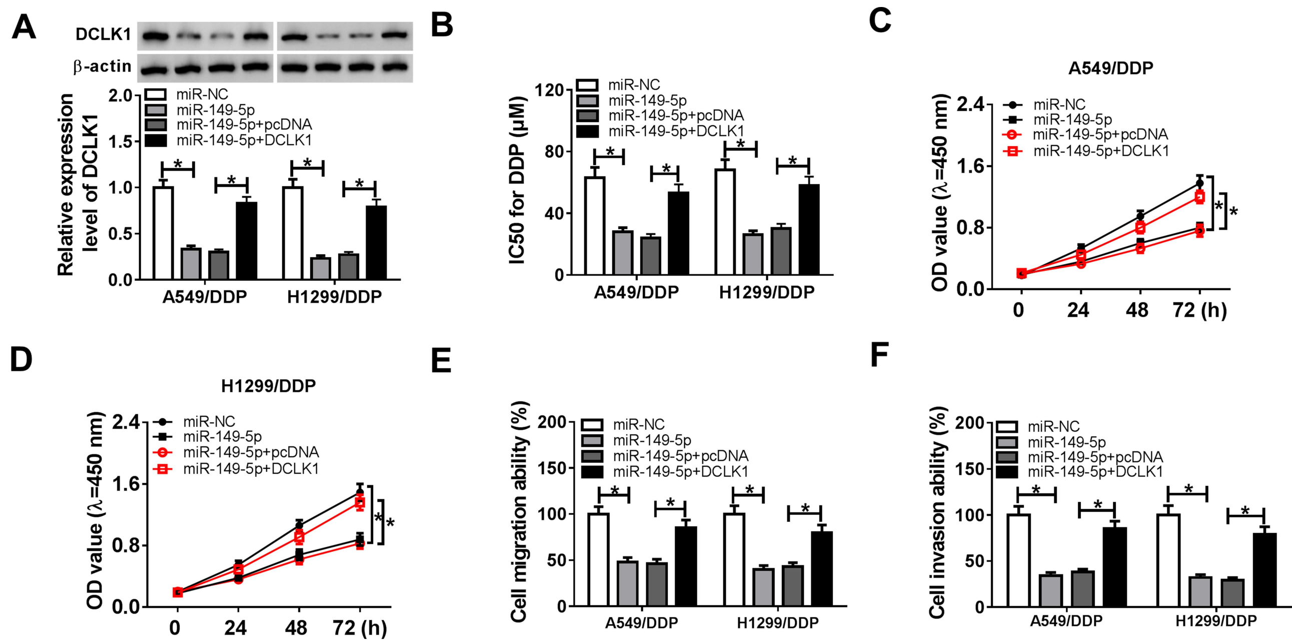

Figure 6 Influence of *DCLK1* elevation on the role of *miR-149-5p* in cisplatin resistance. A549/DDP and H1299/DDP cells were transfected with miR-149-5p or miR-NC, and co-transfected with miR-149-5p and pcDNA-DCLK1 (DCLK1) or pcDNA. (**A**) Expression levels of *DCLK1* were detected by Western blotting after transfection. CCK-8 assay detected (**B**) IC50 values of DDP after transfection for 48 h and (**C, D**) cell viability after transfection for 0, 24, 48 and 72 h. (**E, F**) Transwell assays measured cell migration and invasion after transfection for 24 h. *$P < 0.05$.

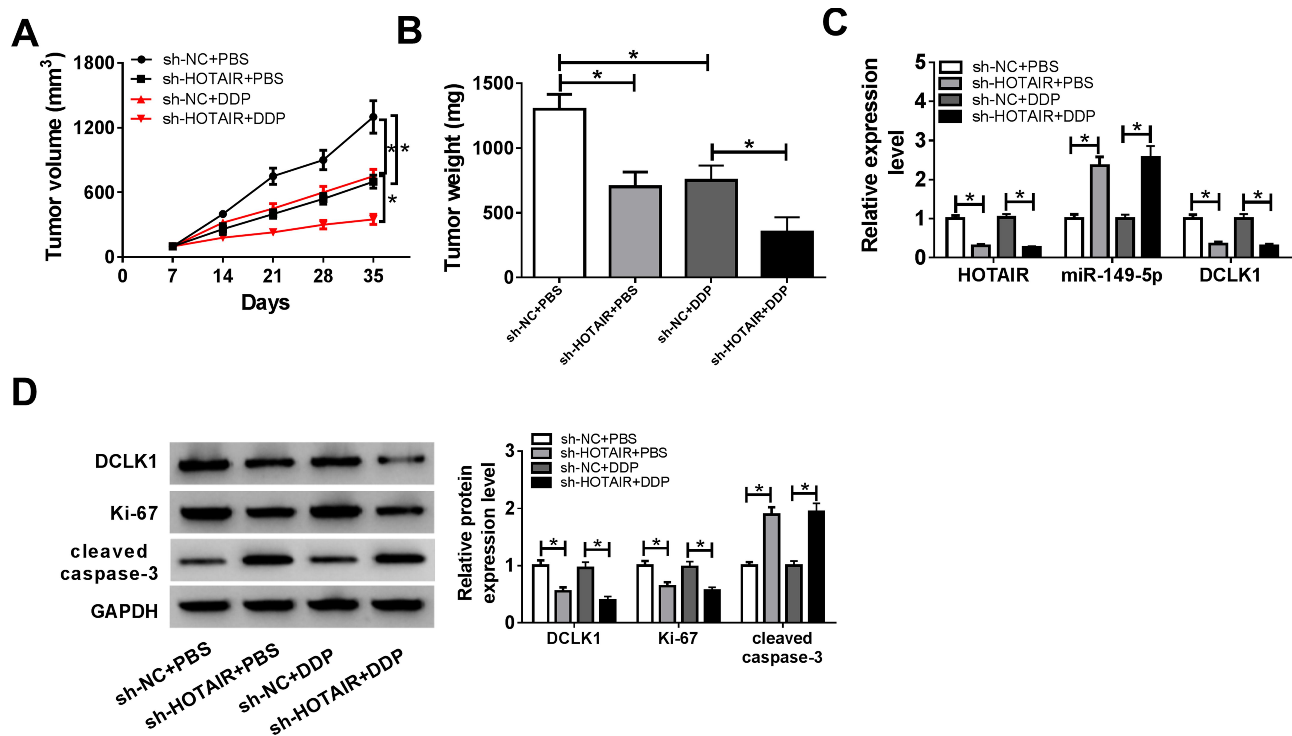

Figure 7 Knockdown of *HOTAIR* inhibited tumor growth of H1299/DDP cells in vivo. H1299/DDP cells were lentiviral infected with short hairpin RNA against HOTAIR (sh-HOTAIR) or its negative control sh-NC prior to injection into BALB/c nude mice (n=5). Xenograft tumors were exposed to DDP (3.0 mg/kg body weight) or phosphate buffer solution (PBS; pH 7.4) every 7 days from 7th day after transplantation. (**A**) The volumes were calculated every week and the growth curve was drawn. (**B**) Tumor weight was recorded on day 35 after transplantation. (**C**) Expression of *HOTAIR*, *miR-149-5p* and *DCLK1* was confirmed in xenograft tumors using RT-qPCR. (**D**) Western blotting evaluated protein levels of *DCLK1*, *Ki-67* and cleaved *caspase-3*. *$P < 0.05$.

miR-149-5p was a novel target for *HOTAIR*. Thus, our results implied that inhibiting *HOTAIR/miR-149-5p* axis contributed to reverse chemoresistance in DDP-resistant NSCLC cells.

In NSCLC, six miRNAs including *miR-149-5p* and nine target genes were validated to distinguish squamous cell carcinoma and adenocarcinoma,[31] which are two different subtypes of NSCLC. According to the miRNA expression profile, expression of *miR-149-5p* was observed to be down-regulated in NSCLC tissues and cells than normal controls.[32] Functionally, Zhao et al[33] demonstrated that *miR-149-5p* inhibited tumor growth, epithelial–mesenchymal transition (EMT) phenotype, invasion and metastasis in NSCLC by negatively modulating *Forkhead box M1/cyclin D1/MMP2* axis. In terms of the relationship between *miR-149-5p* and chemoresistance, *miR-149-5p* downregulation could effectively attenuate gefitinib resistance, as described by decreased cell viability and colony formation ability, and increased apoptosis rate and caspase 3 expression.[34] *MiR-149-5p* was also declared to be linked to cisplatin-vinorelbine response and progression-free survival in NSCLC patients.[35] However, the part of *miR-149-5p* in chemoresistance of many chemotherapy drugs including cisplatin in NSCLC remains largely unknown. Herein, we wondered whether *miR-149-5p* was complicated in cisplatin resistance in DDP-resistant NSCLC cells. Expression level of *miR-149-5p* was lower in drug-resistant NSCLC tissues and cells, which is in consistent with the previous study.[32,33,36] Its forced high expression could decrease IC50 of DDP, cell proliferation, migration and invasion of A549/DDP and H1299/DDP cells. Our data implied that *miR-149-5p* upregulation could descend the acquired DDP resistance in NSCLC cells in vitro, which supports the announcement of *miR-149-5p* role in cisplatin resistance in ovarian cancer,[13] esophageal cancer[14] and gastric cancer.[37] Furthermore, we testified that the suppressive effect of *miR-149-5p* in cisplatin resistance in NSCLC cells probably via directly interacting with upstream HOTAIR and downstream *DCLK1*. This study established a new evidence of *HOTAIR/miR-149-5p* axis in functioning in cisplatin resistance.

Next, we searched for the potential gene effectors involved in its functions in NSCLC. In plenty of potential gene effectors, *DCLK1* was further confirmed due to many reasons. For example, Powrozek et al[38] firstly appointed out that it could be detected of *DCLK1* gene promoter methylation in the plasma of lung cancers including NSCLC and SCLC, and this phenomenon was associated with lower overall survival. Later, expression of *DCLK1* in pathological stage I NSCLC tumors was investigated and suggested *DCLK1* as a new target in clinic.[39] Functionally, *DCLK1* was confirmed to take part in cell proliferation, migration and invasion of human lung squamous cell carcinoma cells in vitro through serving as target gene for *miR-448*.[40] Besides, cisplatin resistance and *PI3K/AKT/mTOR* signaling pathway were also altered by *miR-539/DCLK1* axis.[18] That is why we selected *DCLK1* as a potential downstream target of *HOTAIR/miR-149-5p* axis in regulating the acquired cisplatin-resistant NSCLC cells. Here, we observed the upregulation of *DCLK1* in DDP-resistant patients with NSCLC and in A549/DDP and H1299/DDP cells. Upregulation of oncogene *DCLK1* could contribute to cisplatin resistance of A549/DDP and H1299/DDP cells with *miR-149-5p* overexpression through increase IC50, cell proliferation, migration and invasion. Taken together, *DCLK1* may be a key target of adjuvant chemotherapy to reverse DDP resistance in patients with NSCLC.

In this study, we provided a novel, promising mechanism underlying *HOTAIR* at least through activating *miR-149-5p/DCLK1* axis. However, the influence of *HOTAIR/miR-149-5p/DCLK1* on other cell processes including apoptosis and EMT remains to be further uncovered, as well as the involved signaling pathways such as *ERK1/2 MAPK* signaling pathway.[41]

Conclusion

In conclusion, *HOTAIR* is significantly upregulated in human DDP-resistant NSCLC tissues and cells. Knockdown of *HOTAIR* partially reverses the acquired cisplatin resistance in DDP-resistant NSCLC cells both in vitro and in vivo through *miR-149-5p/DCLK1* axis. This work suggests a novel *HOTAIR/miR-149-5p/DCLK1* pathway in the occurrence, development and treatment of cisplatin resistance in NSCLC.

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Disclosure

The authors report no conflicts of interest for this work.

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