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Long non-coding RNA HOTAIR regulates the development of non-small cell lung cancer through miR-217/DACH1 signaling pathway

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Abstract. – OBJECTIVE: Long non-coding RNA HOX transcript antisense RNA (HOTAIR) is reported to make chromatin state, cell growth and cancer metastasis. However, the role of HOTAIR in non-small cell lung cancer (NSCLC) remains unknown. The aim of this study was to explore the regulatory mechanism of HOTAIR in NSCLC in relation to miR-217/Dachshund homolog 1 (DACH1) signaling pathway.

MATERIALS AND METHODS: The expression levels of HOTAIR and miR-217 were measured by quantitative Polymerase Chain Reaction (qPCR) in NSCLC cell lines and human bronchial epithelial cell line HBE. The direct target of HOTAIR and miR-217 in NSCLC was confirmed by a Luciferase reporter assay. The expression of DACH1 protein was examined by Western blot (WB) assay. Cell migration and invasion were examined with transwell assays, and cell proliferation was measured by Cell Counting Kit-8 (CCK8) assay.

RESULTS: HOTAIR was up-regulated and miR-217 was down-regulated in NSCLC cell lines. Silencing of HOTAIR significantly repressed cell proliferation and inhibited cell migration and invasion in H1299 and A549 cells by facilitating miR-217 expression. Moreover, bioinformatics analysis and Luciferase reporter assay confirmed that DACH1 was a target of miR-217. Furthermore, the overexpression of miR-217 markedly repressed cell proliferation and inhibited cell migration and invasion in H1299 and A549 cells. DACH1 reverses the effects of miR-217 overexpression in NSCLC cells.

CONCLUSIONS: HOTAIR was up-regulated in NSCLC cell and regulates the proliferation, migration, invasion through the miR-217/DACH1 signaling pathway. It provides a novel potential treatment strategy for NSCLC.

Key Words:
HOTAIR, NSCLC, MiR-217, DACH1, Proliferation, Migration, Invasion.

Introduction

Non-small cell lung cancer (NSCLC) is a prevalent and high invasive malignant tumor[1,2]. NSCLC occurrence and development processes are all of a sudden, as well as other cancers[3]. There is a gradual development of this process, generally due to the disorder of cell growth regulation and the pattern change of cell growth, which lead to precancerous cell lesions and related gene changes. Researchers found that the main cause of this phenomenon depended on different epigenetic varies chromatin[4,5]. Changes of the chromatin modified products are likely to change the patterns of cell growth, leading the cells to lose their original character; thus, their presentation may possibly have harmful effects on a different feature of the body tissues[6]. Therefore, a better understanding of the mechanisms involved in the development of NSCLC and more effective therapeutic strategies are urgently needed.

It was shown that long non-coding RNAs (lncRNAs) have functions of regulating ontogenesis and act as tumor suppressor genes; then, lncRNAs have gradually become the focus of researches[7,8]. They are not only involved in cell cycle, differentiation, apoptosis, senescence, and intercellular or kernel plasma transportation and some other cell cytology process, but also have significant molecular biological functions such as epigenetic and transcriptional regulation, post-transcriptional regulation and translation regulation, formation of the nuclear body substructure and chromatin remodeling[9,10]. Long non-coding RNA HOX transcript antisense RNA (HOTAIR) has been found dysregulated in various cancers, including gastric cancer, breast cancer, hepatocellular carcinoma,

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and so on[11]. HOTAIR regulates autophagy in hepatic ischemia/reperfusion injury *via* the miR-20b-5p/ATG7 axis[12]. HOTAIR knockdown could enhance sensitivity to radiotherapy by reduction of autophagy and reversal of EMT through the suppression of the Wnt signaling pathway in human cervical cancer[13]. The overexpression of HOTAIR is a poor prognostic biomarker and promotes proliferation, migration and invasion in osteosarcoma[14]. However, little is known about the expression level and functional roles of HOTAIR in NSCLC.

This study aimed to investigate the mechanism of HOTAIR mediating miR-217/DACH1 signaling pathway in NSCLC cell growth, proliferation, invasion and migration, to have a better understanding of the molecular mechanisms of HOTAIR and provide a brand new perspective for NSCLC treatment.

Materials and Methods

Cell Culture

The NSCLC cell lines H23, H292, H1299, and A549 and human bronchial epithelial cell line (HBE) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) media (HyClone, South-Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, South-Logan, UT, USA), 100 U/ml of penicillin, and 50 μg/mL of streptomycin (Invitrogen, Carlsbad, CA, USA). All cells were maintained in a humidified incubator with 5% CO_2 at 37°C.

Cell Transfection

Short hairpin RNA (shRNA) sequences target HOTAIR (sh- HOTAIR) or negative control (sh-NC) were designed by GenePharma (Shanghai, China). After annealing, double strands of shRNA were inserted into a lentiviral pU6-Luc-Puro vector (GenePharma, Shanghai, China). To overexpress DACH1, the full-length sequences of DACH1 were amplified by Polymerase Chain Reaction (PCR) and then subcloned into a pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). pcDNA3.1 vectors containing full-length HOTAIR, mutant HOTAIR or DACH1 were obtained from RiboBio (Guangzhou, China) and transfected into cells using Lipofectamine 2000 (Life Technologies, Gaithersburg, MD, USA). The miR-217 mimics, inhibitor and miRNA control (miR-NC) were synthesized by GenePharma (Shanghai, China) and transfected into cells using Lipofectamine 2000 (Life Technologies, Gaithersburg, MD, USA).

Real Time-Quantitative Polymerase Chain Reaction assays

Total RNA was obtained from NSCLC cells by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized from 200 ng of extracted total RNA using the First Strand cDNA Synthesis Kit (TaKaRa, Otsu, Shiga, Japan). For the measurement of the miRNA expression, miRNA cDNA was synthesized using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed with SYBR Green (TaKaRa, Otsu, Shiga, Japan), and the data collection was performed on the Applied Biosystems 7500 system (Foster City, CA, USA). The primers were synthesized by GeneScript (Nanjing, China). The relative expression level of indicated genes was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or U6, and the expression fold changes were calculated using the $2^{-\Delta\Delta Ct}$ methods.

Cell Counting Kit-8 Assays

Cell proliferation capacity of treated H1299 and A549 cells was detected using Cell Counting Kit-8 (CCK-8, CK04, Dojindo Molecular Technologies, Kumamoto, Japan) referring to the manufacturer's instructions. Briefly, H1299 and A549 cells after transfection were seeded in 96-well plates in growth medium at approximately 5×10^3 cells/well. Then, 10 μL of CCK-8 solution was added into each well of 96-well plates for another 3 h of incubation at the indicated time points (0, 24, 48, and 72 h) after transfection. At last, the absorbance was measured by a microplate reader (9200, Bio-Rad Laboratories, Hercules, CA, USA) at the wavelength of 450 nm.

Transwell Assay

For cell migration assay, transwell chamber (BD Biosciences, Franklin Lakes, NJ, USA) with 8 mm pore size polycarbonate inserts was used to detect the capability of cell migration. Briefly, H1299 and A549 cells (3×10^4) in 200 μl of serum-free Roswell Park Memorial Institute-1640 (RPMI-1640) were inoculated into the upper chambers, while the medium with 10% fetal bovine serum (FBS) was added to the lower

chambers. After 48 h of incubation at 37°C, the cells on the upper side of the membranes were removed using a cotton swab. The cells that adhered to the lower surface were photographed and counted after fixing with 100% pre-cold methanol and straining using 0.1% crystal violet solution. For cell invasion assay, the same experimental procedures were performed except that the transwell chambers (Corning, Tewksbury, MA, USA) were coated with 20 μl of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA).

Western Blot Assays

Total protein was obtained from H1299 and A549 cells using radioimmunoprecipitation assay lysis buffer (RIPA; Sigma-Aldrich, St. Louis, MO, USA) and the protein concentrations were determined by a BCA protein assay kit (Pierce, Waltham, MA, USA). Equal amounts of protein were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk for 1 h at room temperature, the membranes were incubated with primary antibody against DACH1 and β-actin (Abcam, Cambridge, MA, USA) overnight at 4°C. Then, horseradish peroxidase (HRP) conjugated goat anti-rabbit and sheep anti-mouse IgG (Cell Signaling Technology, Danvers, MA, USA) were used as secondary antibodies, and the immunoblots were visualized by using electrochemiluminescence (ECL) Reagents (Pierce, Waltham, MA, USA) followed by densitometry using the Image J software (NIH, Bethesda, MD, USA).

Luciferase Reporter Assays

The wild-type HOTAIR, DACH1 and a mutant HOTAIR, DACH1 sequences (mutant in miR-217 binding site) were cloned into a pmirGLO plasmid. The pmirGLO-HOTAIR, DACH1 or pmirGLO- HOTAIR, DACH1-mut was co-transfected with miR-217 mimics or miRNA negative control (miR-NC) by Lipofectamine 2000 (Life Technologies, Gaithersburg, MD, USA). Next, the constructed Luciferase reporters were respectively co-transfected with miR-NC or miR-217 into HEK 293 cells. At 48 h after transfection, Luciferase activity was determined by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The Luciferase activity was normalized to Renilla Luciferase activity.

Statistical Analysis

Statistical analysis was performed using the SPSS 20.0 software (SPSS IBM, Armonk, NY, USA). Every experiment was conducted three times and data were presented as the mean ± standard deviation (SD). The significance of differences was determined using Student's *t*-test for two groups and Tukey's Post-Hoc test was used to validate the ANOVA for comparing measurement data between the groups. $p < 0.05$ was considered statistically significant.

Results

HOTAIR Downregulation Repressed NSCLC Cell Proliferation, Migration and Invasion

We investigated the expression levels of HOTAIR in NSCLC cells. HOTAIR expression was upregulated in NSCLC cells (H23, H292, H1299, and A549) compared with human bronchial epithelial cell line (HBE; Figure 1A). A549 cells exhibited the highest level of HOTAIR, followed by H1299 cells. Sh-HOTAIR was used to decrease the expression of HOTAIR in H1299 and A549 cells and the transfection efficiency was tested using qRT-PCR (Figure 1B). The expression levels of HOTAIR were suppressed after using sh-HOTAIR, compared with the negative control group (Figure 1B). Then, the effects of HOTAIR on proliferation, migration and invasion of NSCLC cells were detected by CCK-8, transwell migration and invasion assay. The results showed that the down-regulation of NSCLC by sh-HOTAIR significantly suppressed proliferation (Figure 1C and 1D), invasion (Figure 1E) and migration (Figure 1F) in H1299 and A549 cells.

HOTAIR Suppressed MiR-217 Expression by Direct Interaction

To further investigate the effects of HOTAIR in NSCLC progression, the potentially targeted miRNAs of HOTAIR were forecasted by bioinformatics analysis software, including Starbase and TargetScan. As a result, miR-217 was obtained as the candidate miRNA (Figure 2A). Dual-Luciferase reporter assay proved that the overexpression of miR-217 markedly attenuated the Luciferase activity of HOTAIR-WT reporter but not that of HOTAIR-MUT reporter, compared with miR-NC (Figure 2B). To further verify the influence of HOTAIR on miR-217 expression, H1299 and A549 cells were transfected with sh-HOTAIR. H1299

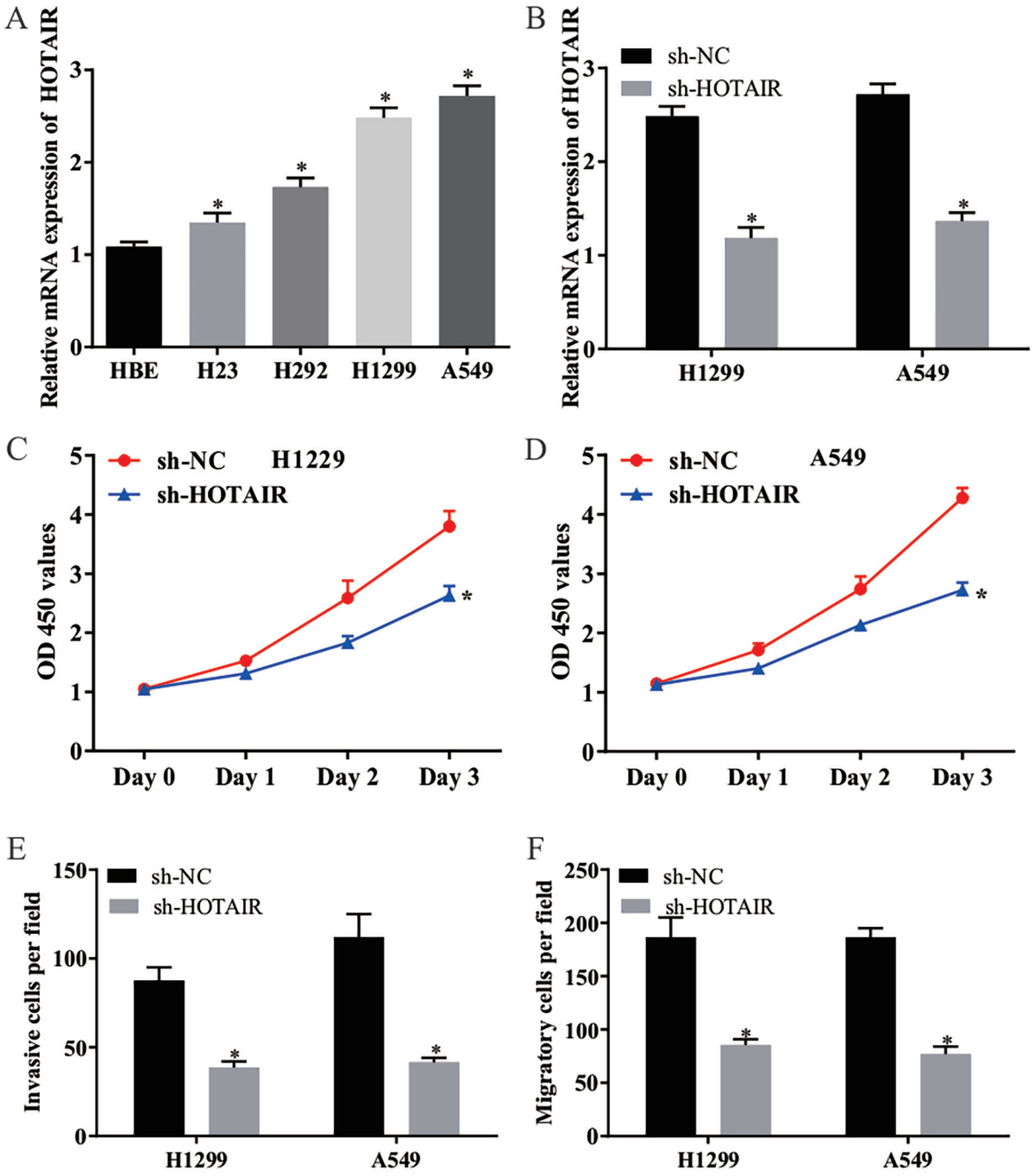

Figure 1. HOTAIR is up-regulated in non-small cell lung cancer (NSCLC) cell lines and HOTAIR down-regulation repressed NSCLC cell proliferation, migration and invasion. ***A,*** The expression levels of HOTAIR were measured by qRT-PCR in NSCLC cell lines and human bronchial epithelial cell line HBE. ***B,*** Sh-HOTAIR could decrease the levels of HOTAIR in H1299 and A549 cells. ***C-D,*** The cell proliferation of H1299 and A549 cells after transfection with sh-HOTAIR was measured using CCK-8 assay. ***E,*** The cell invasion of H1299 and A549 cells after transfection with sh-HOTAIR was measured using transwell assay. ***F,*** The cell migration of H1299 and A549 cells after transfection with sh-HOTAIR was measured using transwell assay. The data are expressed as mean ± SD. *$p<0.05$.

and A549 cells transfected with sh-HOTAIR presented remarkably increased miR-217 expression (Figure 2C). Meanwhile, miR-217 expression was also downregulated in NSCLC cells (H23, H292, H1299, and A549) compared with human bronchial epithelial cell line (HBE) (Figure 2D).

MiR-217 Reverses the Effects of Silencing of HOTAIR in NSCLC Cells

To investigate whether the effects of HOTAIR in NSCLC progression were mediated by miR-217, the miR-217 inhibitor was introduced into sh-HOTAIR-transfected H1299 and A549 cells. The results stated that sh-HOTAIR-induced decrease on proliferation (Figure 3A and 3B), invasion (Figure 3C) and migration (Figure 3D) were remarkably attenuated by knockdown of miR-217. These data indicated that HOTAIR exerted its oncogenic effect partially by suppressing miR-217 expression.

MiR-217 Suppressed DACH1 Expression by Direct Interaction

By using TargetScan software, we identified the complementary sequences between miR-217 and DACH1-3'UTR (Figure 4A). Subsequent Dual-Luciferase reporter assay showed that miR-217 decreased the Luciferase activity of DACH1-WT reporter rather than miR-NC, while little change was observed in the Luciferase activity of DACH1-MUT reporter between miR-NC and miR-217 groups (Figure 4B). Moreover, ectopic expression of miR-217 dramatically suppressed DACH1 expression at protein level in H1299 and A549 cells (Figure 4C). Additionally, HOTAIR knockdown resulted in a prominent decline of DACH1 protein expression in H1299 and A549 cells (Figure 4D). In all, DACH1 was a direct target of miR-217 in NSCLC cells.

DACH1 Reverses the Effects of MiR-217 Overexpression in NSCLC Cells

To further explore whether the effects of miR-217 in NSCLC progression were mediated by DACH1, miR-217 mimics and pcDNA-DACH1 were co-transfected into H1299 and A549 cells. The results showed that miR-217 overexpression led to a significant decrease in proliferation (Figure 5A and 5B), invasion (Figure 5C) and migration (Figure 5D) of H1299 and A549 cells, while these effects were substantially abated following increased DACH1 expression. All these data suggested that miR-217 blocked NSCLC cells proliferation, migration and invasion *via* down-regulating DACH1. HOTAIR regulates the proliferation, migration and invasion through the miR-217/DACH1 signaling pathway.

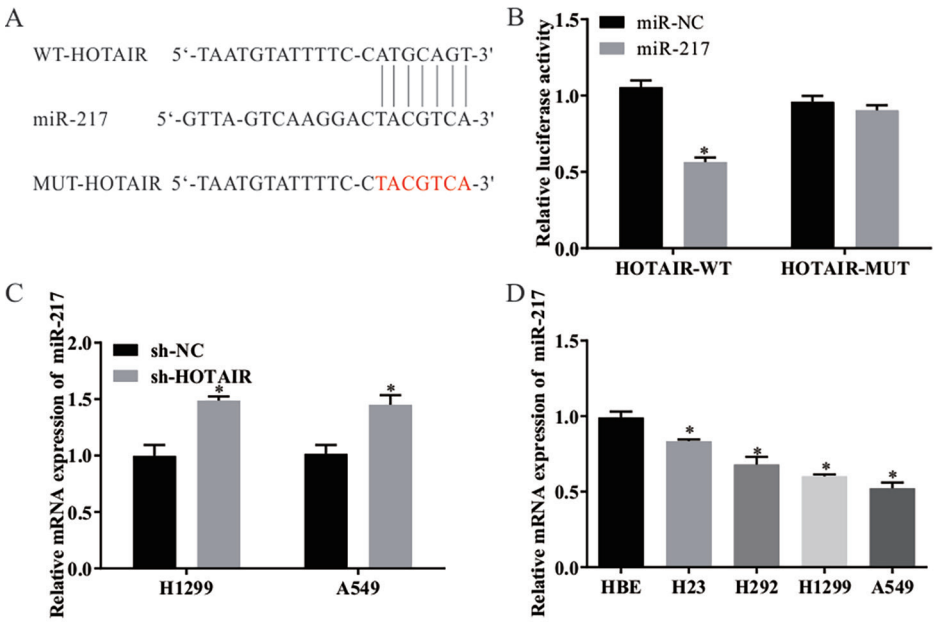

Figure 2. HOTAIR suppressed miR-217 expression by direct interaction. ***A,*** The predicted miR-217 binging sites in HOTAIR mRNA 3'-UTR. ***B,*** Effects of miR-217 on the Luciferase activity of HOTAIR -WT and HOTAIR -MUT reporter were detected by Luciferase assays in HEK 293 cells. ***C,*** The expression levels of miR-217 were determined by qPCR in H1299 and A549 cells after transfection with sh-HOTAIR. ***D,*** The expression levels of miR-217 were measured by qRT-PCR in NSCLC cell lines and human bronchial epithelial cell line HBE. The data are expressed as mean ± SD. **p* <0.05.

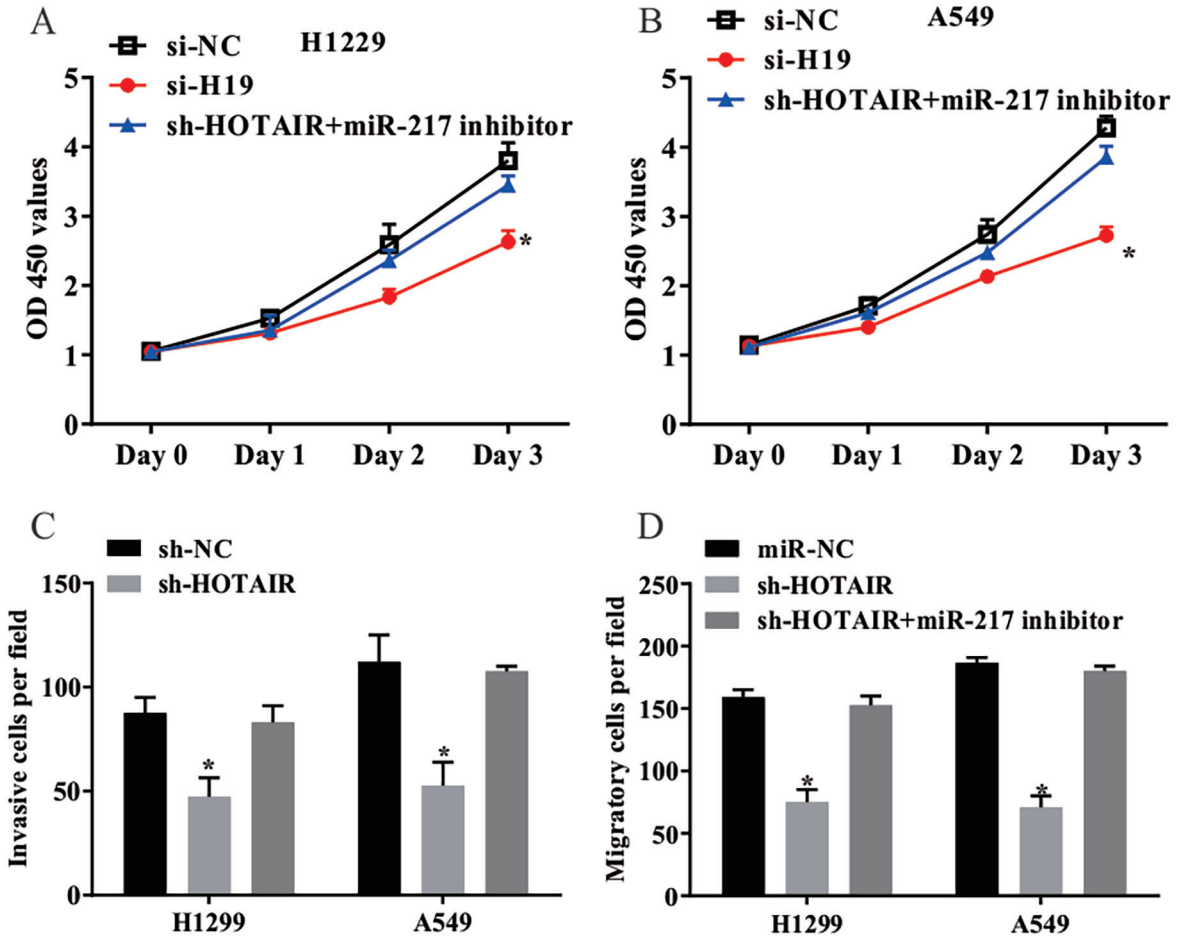

Figure 3. miR-217 reverses the effects of silencing of HOTAIR in NSCLC cells. ***A-B,*** The cell proliferation of H1299 and A549 cells after transfection with sh-NC or sh-HOTAIR along with miR-217 inhibitor was measured using CCK-8 assay. ***C,*** The cell invasion of H1299 and A549 cells after transfection with sh-NC or sh-HOTAIR along with miR-217 inhibitor was measured using transwell assay. ***D,*** The cell migration of H1299 and A549 cells after transfection with sh-NC or sh-HOTAIR along with miR-217 inhibitor was measured using transwell assay. The data are expressed as mean ± SD. **p* <0.05.

Discussion

LncRNAs are pervasively transcribed in the genome, but the mechanism of its potential involvement in human disease is not well understood[15]. Recent studies[16,17] of dosage compensation, imprinting and homeotic gene expression suggest that individual LncRNAs can function as the interface between DNA and specific chromatin remodeling enzyme. HOTAIR is highly expressed in invasive NSCLC[18] and its expression level in primary NSCLC is a powerful predictor of prognosis[19]. The enforced expression of HOTAIR in NSCLC cells can alter histone H3 lysine 27 methylation, and increase NSCLC invasiveness and metastasis[20]. Conversely, loss of HOTAIR can inhibit NSCLC metastasis[21]. In our work, we found that HOTAIR was significantly overexpressed in NSCLC cell lines. More importantly, the knockdown of HOTAIR markedly repressed cell proliferation, migration and invasion. Our findings are similar to previous studies in which NSCLC contributed to the oncogenesis of various tumors. These findings suggested that HOTAIR may function as an oncogene in NSCLC; the up-regulation of HOTAIR contributes to NSCLC development and progression.

MicroRNAs (miRNAs), a class of endogenously small non-coding RNAs with about 22 nt, can regulate the translation and stability of messenger RNAs (mRNAs) at the post-transcriptional level[22]. Emerging evidence shows that miRNAs

can act as oncogenes or tumor suppressors in human cancers by modulating the processes associated with tumorigenesis, such as inflammation, cell cycle, stress response, differentiation and invasion[23,24]. MiR-217 has been elucidated as an anti-cancer gene in multiple cancer. Wang et al[14] reported HOTAIR promotes osteosarcoma cell growth, migration and invasion, and induce cell apoptosis by sponging miR-217. Hong et al[25] reported that HOTAIR promotes renal cell carcinoma proliferation, migration, and EMT process *via* the miR-217/HIF-1α/AXL signaling. By using bioinformatics analysis software, we identified some candidate miRNAs that might be bind with PVT1, including miR-16-5p. Besides bioinformatics analysis, Luciferase reporter assay defined that miR-217 is the target of HOTAIR. Moreover, miR-217 inhibitor could partly reverse the regulatory effects of downregulation of HOTAIR. These findings imply that miR-217 may function as a tumor inhibitor.

Dachshund homolog 1 (DACH1) is known as cell fate determination factor in tumorigenesis, particularly those of the breast, prostate, ovarian, brain and lung[26]. The role of DACH1 in the inhibition of oncogene induced cellular migration and metastasis is well established in NSCLC cells[27]. Then, we used web-based tools to find that DACH1-3'URT possessed possible binding sites of miR-217. Moreover, Luciferase reporter and Western blot assays validated that DACH1 was a direct target of miR-217. Furthermore, ectopic expression of miR-217 dramatically suppressed DACH1 expression at the protein level in NSCLC cells. Additionally, HOTAIR knockdown resulted in a prominent decline of DACH1 protein expression in NSCLC cells. Meanwhile, miR-217 overexpression led to a significant decrease in proliferation, invasion (Figure 5C) and migration of NSCLC cells, while these effects were substantially abated following increased DACH1 expression. All these data suggested that miR-217 blocked NSCLC cells proliferation, migration and invasion *via* down-regulating DACH1.

Conclusions

We revealed that the HOTAIR level was upregulated in NSCLC cell lines, and HOTAIR knockdown suppressed proliferation, migration

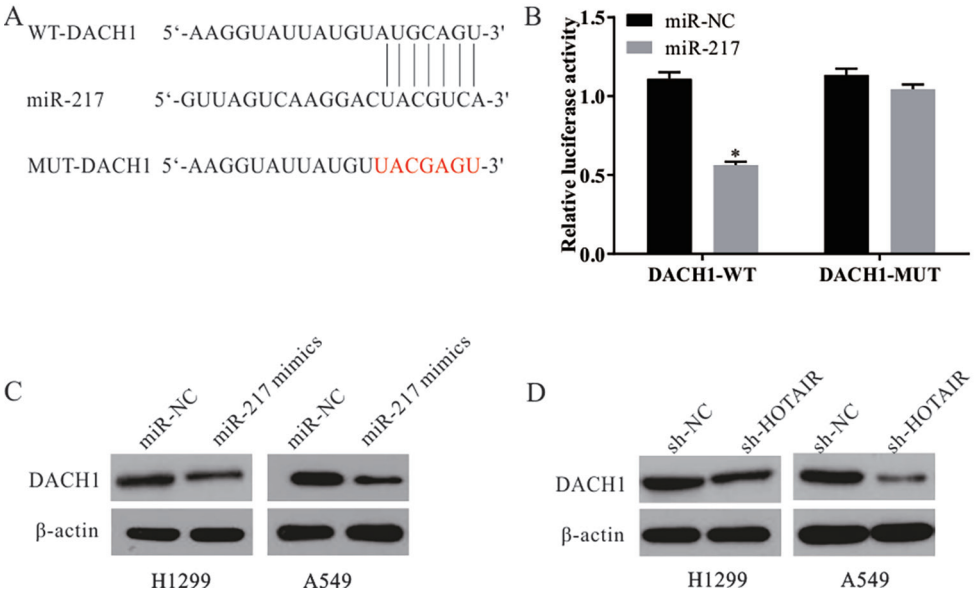

Figure 4. miR-217 suppressed DACH1 expression by direct interaction. ***A,*** The predicted miR-217 binging sites in DACH1 mRNA 3'-UTR. ***B,*** Effects of miR-217 on the Luciferase activity of DACH1-WT and DACH1-MUT reporter were detected by Luciferase assays in HEK 293 cells. ***C,*** Western blot assay of DACH1 protein expression in H1299 and A549 cells after transfection with miR-NC or miR-217 mimics. ***D,*** Western blot assay of DACH1 protein expression in H1299 and A549 cells after transfection with sh-NC or sh-HOTAIR. The data are expressed as mean ± SD. **p* <0.05.

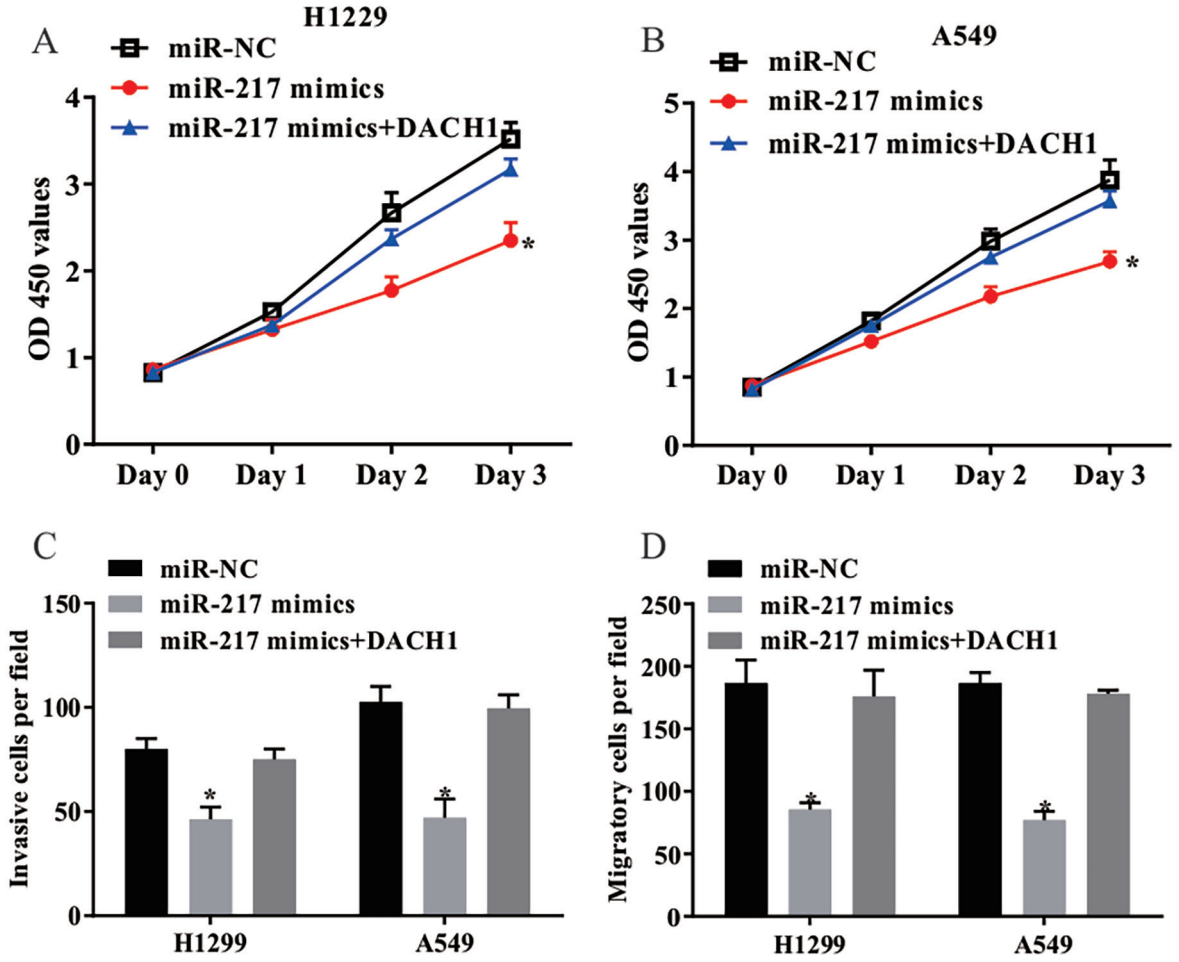

Figure 5. DACH1 reverses the effects of miR-217 overexpression in NSCLC cells. ***A-B,*** The cell proliferation of H1299 and A549 cells after transfection with miR-NC or miR-217 mimics along with DACH1 was measured using CCK-8 assay. ***C,*** The cell invasion of H1299 and A549 cells after transfection with miR-NC or miR-217 mimics along with DACH1 was measured using transwell assay. ***D,*** The cell migration of H1299 and A549 cells after transfection with miR-NC or miR-217 mimics along with DACH1 was measured using transwell assay. The data are expressed as mean ± SD. **p* <0.05.

and invasion through the 217/DACH1 signaling pathway in NSCLC cell line. Our study contributes to a better understanding of the molecular mechanisms involved in NSCLC progression and provides a novel potential treatment strategy for NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgments

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