LncRNA HOTAIRM1 Inhibits the Proliferation and Invasion of Lung Adenocarcinoma Cells via the miR-498/WWOX Axis

Background: Lung adenocarcinoma (ADC) is a major form of lung cancer, which is a main cause of global cancer-related death in male and female patients. LncRNAs are implicated in tumor development. However, the functions and mechanisms of the LncRNA HOTAIRM1 in ADC are not known.

Materials and Methods: Here, the downregulated HOTAIRM1 in ADC was selected by TCGA analysis. Subsequently, qRT-PCR, CCK-8, EdU, cell apoptosis, cell cycle and cell invasion assays were utilized for evaluating the roles of HOTAIRM1 in ADC. Finally, we explored the mechanism of HOTAIRM1 in ADC.

Results: HOTAIRM1 expression was considerably decreased in ADC tissues. The knockdown of HOTAIRM1 promoted the cell cycle, growth, and invasion of ADC. Moreover, HOTAIRM1 competitively bound miR-498 to regulate the expression of WWOX.

Conclusion: HOTAIRM1 suppressed the proliferation and invasion of ADC cells via the modulation of miR-498/WWOX axis. This finding suggested that it might be clinically valuable as a biomarker for ADC. Furthermore, the findings suggest LncRNA HOTAIRM1 as a candidate therapeutic target in ADC.

Keywords: HOTAIRM1, lung adenocarcinoma, miR-498, WWOX, cell proliferation

Introduction
Lung cancer, one of the most commonly diagnosed malignant tumors, is a major cause of cancer-related deaths around the world. Lung cancer can be categorized into five histological types: adenocarcinoma, squamous cell carcinoma, large cell carcinoma, small cell carcinoma, and bronchoalveolar carcinoma. Among them, lung adenocarcinoma (ADC) is the most prevalent and has high metastatic and invasion likelihood. The 5-year overall survival rate of patients with lung cancer is about 18.1%; this rate is dependent on tumor histology, cancer stage, general health status of the host and other factors. Currently, no biomarker is available for the early diagnosis and prognosis of ADC. Therefore, it is important to find new targets and biomarkers for its early diagnosis.

Long noncoding RNAs (LncRNAs) are a new class of host factors that has been attracting many attentions in recent years. As the most common subtype of noncoding RNAs, LncRNAs have over 200 nucleotides. In addition, they also participate in many physiopathologic processes, including epigenetic gene expression regulation, genomic imprinting, chromatin organization, immunoregulation, cell development and differentiation, oncogenesis and viral pathogenesis.
instance, LncRNA ODIR1 suppresses osteogenic differentiation of hUC-MSCs via the FBXO25/H2BK120ub/H3K4me3/OSX axis. LncRNAs participate in the development of colorectal cancer and are a new target biomolecule. LncRNA LCAT1 sponges miR-4715-5p in lung cancer as a ceRNA to regulate RAC1 functions.

HOXA transcript antisense RNA, myeloid-specific 1 (HOTAIRM1), an LncRNA between the HOXA1 and HOXA2 loci, was first determined as a myeloid-specific regulator of HOXA gene family, which modulates the transcription of target genes by chromosome remodeling during myeloid cell maturation and differentiation. HOTAIRM1 serves as a tumor inhibitor in head and neck, colorectal, and hepatocellular carcinoma; however, its roles in ADC are unclear. WW domain-containing oxidoreductase which is known as WWOX, FOR and WOX1 initially considered as a tumor suppressor protein.

We identified that HOTAIRM1 expression is decreased in ADC tissues and that this downregulation enhances the growth and invasion of ADC cells. Moreover, HOTAIRM1 may exert its actions via sponging of miR-498. Overall, our results suggest that HOTAIRM1 might have a crucial role in ADC pathogenesis.

Materials and Methods

Sample Collection from ADC Patients and Culture of ADC Cell Lines

Forty-six pairs of tissue samples were collected from ADC patients of the First Affiliated Hospital of Xi’an Jiaotong University. Ethical approval for this study was obtained from the First Affiliated Hospital of Xi’an Jiaotong University’s Ethics Committee, and written informed consents were obtained from all participants. All population-related studies were performed per the World Medical Association Declaration of Helsinki.

The ADC cell lines H1650 and PC-9 were procured from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in a humid 5% CO₂ atmosphere at 37°C in RPMI-1640 medium containing 10% fetal bovine serum (Gibco™, Thermo Fisher Scientific, CA).

Real-Time PCR Assay, Cell Transfection, Lentivirus Production and Transduction

RNAs were detached from cells with the Trizol reagent (Invitrogen, Life Technologies). GoScript™ Reverse Transcription System (Promega, Madison, WI, USA) was utilized for converting total RNAs to cDNAs, while GoTaq® qRT-PCR Master mixes (Promega, Madison, WI, USA) and C1000 Touch™ Real-Time Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) were used for all the qRT-PCR reactions. The following primers were used in this study: GAPDH Forward: 5′-AATGGG CAGCCGTAGGAAA-3′; Reverse: 5′-TGAAGGGGTCT AT TGATGGA-3′, HOTAIRM1 Forward: 5′- GCGGTT CTCTTGCTCAA-3′; Reverse: 5′- CCCGGTCG CAAAGCATTAG-3′, miR-498 Forward: 5′- ACACTCC AGCTGGCCUUUUUGCGGGGACCG-3′; Reverse: 5′- CTCAAACTGTGTTCGAGTGGACATCGTT GAGAAAGTTGC-3′, WWOX Forward: 5′- CTCCCA GGGATGTTTGTGC-3′; Reverse: 5′- CACGCCAAC CAGGTTTTTAC-3′. All experiments were carried out in triplicate. 2⁻ΔΔCT method was applied for measuring the expression of target genes.

Ruibo (Guangzhou, China) was used for synthesizing miRNA-NC, miR-498, anti-miR-NC and anti-miR-498 for transfection. The plasmid WWOX was commercially acquired from Santa Cruz. Subsequently, oligonucleotide transfection was conducted with Lipofectamine® 2000 reagent (Invitrogen, Thermo Fisher Scientific) following its protocol.

HOTAIRM1-specific oligonucleotides encoding short hairpin RNAs (shRNAs) were subcloned into GV102 (Genechem, Shanghai, China). Neomycin or puromycin (Invitrogen) was added for the screening of stable cell lines. Sh-NC was applied as the control.

Cell Counting Kit-8 (CCK8) Assay and EdU Assay

After seeding preconditioned cells into a 96-well plate, cells were incubated for 2 h with CCK-8 reagent (DingGuo Bio) at 37°C. We measured the absorbance with a microplate reader (BioTek) at 450 nm.

During EdU assay, Cell-Light EdU DNA Cell Proliferation Kit (RiboBio, Guangzhou, China) was used. Cells were incubated using EdU for 2 h, fixed in 4% paraformaldehyde, dyed using Apollo Dye Solution and mounted using Hoechst 33342. The mounted samples were then photographed and EdU positive cells were counted.

Transwell Assay, Cell Cycle and Apoptosis Assay

Transwell® chambers were coated with Matrigel™ (BD biosciences) for 30 min at 37 °C. Cells treated with
mitomycin were seeded into the upper chambers and 500 μL complete medium was added to the lower chambers. After 48 h, the cells on the lower chambers were washed with PBS, fixed with 4% paraformaldehyde, and stained with crystal violet solution. Images were captured under a microscope. Each group was analyzed in triplicate.

After isolation by trypsinization, cells were rinsed twice using precooled PBS and then fixed in 70% ethanol at −20°C overnight. Subsequently, fixed cells suspended in 50 μg/mL of propidium iodide (KeyGen BioTECH) and 100 μg/mL of RNaseA (KeyGen BioTECH), cells were incubated for 40 min at room temperature and in the absence of light. Flow cytometry was used for determining the cycle of filtered cells.

In the apoptosis assay, cells were washed using PBS and stained with Annexin V-FITC Apoptosis Detection Kit (Affymetrix eBioscience) according to manufacturer instructions. Flow cytometer (BD Biosciences, BD FACSTM Flow Cytometer) was used for cell analysis.

Western Blot Detection
RIPA lysis buffer (Thermo Fisher Scientific) was used for protein extraction, while BCA Protein assay kit (Beyotime, Nantong, China) was used for the assay of protein concentrations. After protein separation through electrophoresis, proteins were transferred onto PVDF membranes and blocked using 5% defatted milk. Primary antibodies were incubated on polyvinylidene fluoride (PVDF) membranes overnight at 4°C, and horseradish peroxidase (HRP)-conjugated secondary antibodies (Beyotime, Nantong, China) were applied for 1 h at room temperature to allow the blocking of proteins. BioSpectrum 600 Imaging System

**Figure 1**
HOTAIRM1 was decreased in ADC and related to unfavorable overall survival. (A and B) Expression profile of HOTAIRM1 in TCGA ADC samples. (C) HOTAIRM1 expression was remarkably reduced in ADC tissues in comparison with no-tumor lung tissues. (D) High expression of HOTAIRM1 was related to better overall survival, consistent with the survival results of (E) TCGA data analysis. Data were shown as the mean ± SEM of three experiments. ***P<0.001.
(UVP, CA, USA) was used for imaging. The primary antibody WWOX was obtained from Abcam (1:1000; Lot No. ab238144) and GAPDH (1:500; Lot No. ab9484).

**RNA-Binding Protein Immunoprecipitation (RIP) Assay**

EZ-Magna RIP Kit from Millipore (Billerica, MA, USA) was applied for RIP assay. Cell lysis was conducted using RIP lysis buffer containing RNase and proteinase inhibitor (Millipore). Subsequently, RIP lysates were treated using RIP buffer containing magnetic beads post-conjugation with nonspecific mouse IgG antibody or human anti-Ago2 antibody (Millipore). After digesting proteinase K into the immunoprecipitate, qRT-PCR and gel-staining analyses were conducted for detecting HOTAIRM1 enrichment. The concentrations of RNAs were detected using NanoDrop spectrophotometer, with purified RNAs used for qRT-PCR analysis.

**Luciferase Reporter Assay**

3’-UTR of WWOX and HOTAIRM1 was amplified and cloned into the downstream the firefly luciferase gene in pGL3 vector (Promega) respectively. This vector was called wild-type (WT) 3’-UTR accordingly. Quick change site-directed mutagenesis kit (Stratagene, Cedar Creek, USA) was used for the mutation of miR-498 binding sites in WWOX and HOTAIRM1 3’-UTR. Thus, this vector was called mutant (MUT) 3’-UTR. ADC cells were transfected with mut-3’-UTR or wt-3’-UTR and miR-NC or miR-498. Dual Luciferase reporter assay system (Promega) was applied for 48 h post transfection. All groups were separately determined in triplicate.

**Immunofluorescence Staining**

After fixation and sealing, tissues were incubated in the antibody of WWOX (Invitrogen, Carlsbad, California, USA) at 4°C for 24 h. After washing, cells were incubated in FITC-labeled IgG (H+L) (Beyotime, Nantong, China) for 60 min. DAPI was used for nuclei staining. The protocol followed was described in detail in our previous study.

**Mouse Model**

A total of 6 female BALB/c-nu/nu mice (age, 5 weeks; weight, 16–22 g) were purchased from the Model Animal Research Center of Nanjing University. The mice were housed in a sterile room under a 12-h light/dark cycle at ~23°C and 50% humidity, with ad libitum access to food and water. 2×10⁶ H1650 (sh-NC or sh-HOTAIRM1) were loaded into the flanks of BALB/c-nu/nu mice by subcutaneous inoculation. The mice were euthanized after harboring tumors for four weeks. Animals were maintained and tested according to the US National Institute of Health Guidelines for Use of Experimental Animals. Ethical approval was obtained from the Ethnic Committee for Experimental Animals in Xi’an Jiaotong University. Animal experiments took place in SPF Animal Laboratory at Xi’an Jiaotong University.

**Statistical Analysis**

Fisher’s exact test was applied for identifying any difference between categorical variables. For normally distributed data with equal variance, the difference was evaluated by two-tailed Student’s t-test (two group comparisons) or one-way ANOVA followed by the Bonferroni post hoc test (multigroup comparisons). For non-normally distributed data or data with unequal variances, the difference was evaluated by a nonparametric Mann–Whitney U-test (two group comparisons) or the Kruskal–Wallis test followed by the Bonferroni post hoc test (multigroup comparisons).

**Table 1** The Correlation of HOTAIRM1 Expression with Clinical Parameters in Patients with Lung Adenocarcinoma

| Clinicopathological Features | Number of Cases | HOTAIRM1 Expression | p value |
|------------------------------|-----------------|----------------------|---------|
|                              |                 | Low (n=23)           | High (n=23) |        |
| Gender                       |                 |                      |         |
| Male                         | 25              | 10                   | 15      | 0.2362 |
| Female                       | 21              | 13                   | 8       |        |
| Age                          |                 |                      |         |
| ≤60                          | 24              | 13                   | 11      | 0.7683 |
| ≥60                          | 22              | 10                   | 12      |        |
| Tumor size                   |                 |                      |         |
| ≤5                           | 24              | 8                    | 16      | 0.0377*|
| >5                           | 22              | 15                   | 7       |        |
| TNM stages                   |                 |                      |         |
| I/II                         | 28              | 9                    | 19      | 0.0058*|
| III/IV                       | 18              | 14                   | 4       |        |
| Lymph node metastasis        |                 |                      |         |
| Absent                       | 24              | 7                    | 17      | 0.0072*|
| Present                      | 22              | 16                   | 6       |        |

**Note:** *p* < 0.05.
Data are presented as the mean ± SD. \( P<0.05 \) was considered to indicate a statistically significant difference.

**Results**

**HOTAIRM1 Expression Was Decreased in ADC Tissues**

The analysis was conducted via the TCGA database and the downregulated HOTAIRM1 in ADC was selected as the topic of interest (Figure 1A and B). qRT-PCR assay was conducted for determining HOTAIRM1 expression in ADC tissues and corresponding normal tissues, which uncovered that HOTAIRM1 expression was remarkably reduced in ADC tissues compared with those in normal lung tissues (Figure 1C).

**Low Expression of HOTAIRM1 Was Associated with Unfavorable Prognosis of ADC Patients**

The association between HOTAIRM1 expression and patients’ clinicopathological features are shown in Table 1. Forty-six ADC patients were stratified equally into low expression group and high expression group with median HOTAIRM1 expression as the cutoff. No apparent association existed between HOTAIRM1 expression and gender.

![Figure 2](image_url)

**Figure 2** HOTAIRM1 down-regulation promoted the proliferation and cell cycle of ADC cells. (A) HOTAIRM1 expression in H1650 and PC-9 cells post transduction with shRNA vector (sh-NC) or HOTAIRM1 shRNA vector (sh-HOTAIRM1) control. (B and C) HOTAIRM1 down-regulation notably increased cell proliferation in CCK-8 and EdU assays. (D) sh-HOTAIRM1 cells presented with significantly high percentage in S phase. Data were shown as the mean ± SEM of three experiments. *\( P<0.05 \), **\( P<0.01 \), ***\( P<0.001 \).
(P=0.2362) or age (P = 0.7683). However, an overt association was observed between the knockdown of HOTAIRM1 expression and clinical stage (P=0.0058), and lymph node metastasis (P=0.0072) and tumor size (P=0.0377). Additionally, the knockdown of HOTAIRM1 was linked to worse overall survival of ADC patients, as indicated by Kaplan-Meier analysis (Figure 1D). This result was consistent with the TCGA database analysis (Figure 1E).

HOTAIRM1 Down-Regulation Promoted ADC in vitro
We established H1650 and PC-9 cells, which proved to be stable for transfection with sh-HOTAIRM1 (Figure 2A). HOTAIRM1 downregulation notably enhanced the cell proliferation of cells as shown observed in the CCK-8 assay (Figure 2B). During the EdU assay, cells incorporating EdU were considerably higher in the sh-HOTAIRM1 group than in the sh-NC group (Figure 2C). Additionally, based on the flow cytometry analysis, less cells in G1 phase and more cells in S phase were observed in sh-HOTAIRM1 cells (Figure 2D). Cell apoptosis was analyzed to identify whether LncRNA HOTAIRM1 had any effect on the apoptosis, which suggested that downregulated HOTAIRM1 reduced cell apoptosis in ADC cells (Figure 3A). Following that, Transwell Invasion Assay showed that inhibition of HOTAIRM1 increased ADC cell invasion (Figure 3B).

HOTAIRM1 Functioned as a ceRNA in ADC by Sponging miR-498
miRNAs capable of complementary base paring with HOTAIRM1 were searched by using online software program starbase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php). miR-498 was selected from miRNAs that promoted the cell proliferation and invasion in many cancers.20,21 Figure 4A depicted the binding site of miR-498 on HOTAIRM1. A mutated miR-498 binding site of HOTAIRM1 (HOTAIRM1-MUT) and predicted miR-498 binding site of HOTAIRM1 (HOTAIRM1-WT) were cloned into reporter plasmids. Luciferase activity was notably reduced by co-transfecting miR-498 and HOTAIRM1-WT. On the other hand, luciferase activity remained unchanged after co-transfection with miR-498 and HOTAIRM1-MUT (Figure 4A). miRNA is distributed in the cytoplasm, which is a component of the RNA-induced silencing complex (RISC) containing Ago2. Ago2 is
required for miRNA-mediated gene silencing. In this study, we analyzed whether HOTAIRM1 and miR-498 contained the same RISC and affected RIP assay similarly in H1650 and PC-9 cells. It was observed that HOTAIRM1 and miR-498 were enhanced in Ago2 immunoprecipitate compared to IgG immunoprecipitate controls (Figure 4B). Moreover, miR-498 expression was higher in ADC tissues in comparison with adjacent normal lung tissues (Figure 4C). To further verify the correlation between HOTAIRM1 and miR-498, miR-498 expression in ADC cells was changed by transfecting with miR-498 overexpressed plasmids and anti-miR-498 (Figure 4D). Whether the levels of miR-498 was affected, HOTAIRM1 expression levels was determined. As displayed in Figure 4E, lower HOTAIRM1 expression was observed in miR-498 treated cells, as opposed to HOTAIRM1 expression in anti-miR-498 treated cells. Then, we determined whether miR-498 expression was regulated by HOTAIRM1. Results showed that sh-HOTAIRM1 could significantly increase the level of miR-498 in H1650 and PC-9 cells (Figure 4F). In summary, our

![Figure 4](https://example.com/figure4.png)

**Figure 4** Reciprocal repression between LncRNA HOTAIRM1 and miR-498. (A) Binding sites of miR-498 on HOTAIRM1 and dual-luciferase reporter assay for the validation of the interaction between HOTAIRM1 and miR-498. (B) RIP assay was performed to reveal the combination between HOTAIRM1 and miR-498. (C) Up-regulated miR-498 was seen in ADC tissues by qRT-PCR assay. (D) miR-498 expression was reduced by anti-miR-496 and increased by miR-496 mimics. (E) miR-498 negatively regulated HOTAIRM1 expression. (F) miR-498 expression was increased by sh-HOTAIRM1. Data were shown as the mean ± SEM of three experiments. **P<0.01, ***P<0.001.
findings include that miR-498 directly bound to HOTAIRM1 had negative regulatory effects on HOTAIRM1 expression.

**WWOX Was the Direct Target of miR-498**

MicroRNAs exerted their biological functions mainly by means of regulating their downstream targets. WWOX was selected from the predicted target genes of miR-498 for further studies. Dual-luciferase reporter assay revealed that miR-498 mimics efficiently decreased the luciferase activity of WWOX-WT instead of that of WWOX-MUT (Figure 5A). Furthermore, WWOX expression was also observed in ADC tissues. Western blot assay uncovered that WWOX expression was reduced in ADC tissues compared with those in adjacent normal lung tissues (Figure 5B). Furthermore, miR-498 repressed WWOX expression in H1650 and PC-9 cells at both RNA and protein levels (Figure 5C and D). On the other hand, WWOX level was decreased by sh-HOTAIRM1 and recovered by anti-miR-498 treatment in sh-HOTAIRM1 cells (Figure 5E and F). In all, our data uncovered that the WWOX was a downstream target of miR-498 and under the regulation of HOTAIRM1.

**HOTAIRM1 Down-Regulation Increased Tumor Growth in vivo**

In vivo experiment was utilized for verifying the roles of HOTAIRM1 in tumorigenesis. In Figure 6A, the down-regulation of HOTAIRM1 promoted tumor growth. Xenograft tumors from sh-HOTAIRM1 group had higher volume and weight of xenograft tumors than those from the sh-NC group (Figure 6B and C). HOTAIRM1 expression was then determined in excised tumor tissues, which
revealed the downregulation of HOTAIRM1 expression in the sh-HOTAIRM1 group relative to that of the sh-NC group (Figure 6D). Tumor section staining was performed for WWOX expression, which was decreased in sh-HOTAIRM1 group (Figure 6E). In summary, these findings supported the growth inhibition effect of HOTAIRM1 in vivo.

HOTAIRM1 Regulated ADC Cell Proliferation, Invasion, Apoptosis and Cycle Through miR-498/WWOX Axis

In order to verify the roles of HOTAIRM1/miR-498/WWOX axis in lung adenocarcinoma, we performed a “rescue experiment” in H1650 and PC-9 cells. H1650 (sh-HOTAIRM1) and PC-9 (sh-HOTAIRM1) cells which were stable in transfection with sh-HOTAIRM1 were stratified into three groups for transfection with NC, anti-miR-498, and WWOX overexpressed plasmids. CCK-8 and EdU experiments indicated that the proliferation of anti-miR-498 group and WWOX group was notably decreased (Figure 7A and B). Additionally, in both anti-miR-498 and WWOX groups, the invasion of H1650 (sh-HOTAIRM1) and PC-9 (sh-HOTAIRM1) cells was inhibited (Figure 7C) and the G1 arrest and apoptosis of ADC cells were promoted (Figure 7D and E).

Discussion

ADC, as the most prevalent non-small cell lung cancer, causes over 500,000 deaths per year around the world.24 At present, there is an urgent demand for effective new therapies for ADC; however, efforts should first be made to deepen the understanding of underlying mechanisms of the development and progression of ADC. Increase evidence shows that LncRNAs may modulate multiple tumorigenesis processes, especially ADC.25-27 In this study, through TCGA data analysis of ADC related LncRNAs, downregulated HOTAIRM1 was selected as the topic of interest and was subsequently verified in human tissues.

During functional analysis, HOTAIRM1 inhibition promoted the proliferation and invasion of H1650 and PC-9 cells. Furthermore, downregulated HOTAIRM1 contributed to the development from the G1 phase into the S phase of H1650 and PC-9 cells and prevented them from apoptosis. In addition, the reduction of HOTAIRM1 expression resulted in worse overall survival of ADC patients. These data identified HOTAIRM1 as a suppressor gene in ADC. Moreover, HOTAIRM1 may be a candidate prognostic marker for ADC.
Human WWOX gene is distributed on chromosomal site FRA16D or 16q23. FRA16D, which is a focus of genomic instability and is predisposed to breakage and germline and somatic copy number variation. Thus, WWOX is a commonly deleted target in various cancers, such as breast cancer, non-small cell lung cancer, bladder cancer, prostate cancer. In the present study, WWOX was reduced in ADC tissues. In addition, stable downregulation of HOTAIRM1 expression significantly promoted the proliferative and invasive capabilities of ADC cells, but overexpression WWOX reversed these influences.

**Conclusion**

In conclusion, HOTAIRM1 is downregulated in ADC and this downregulation enhances the proliferation and invasion of ADC cells via the miR-498/WWOX axis (Supplementary Figure 1).

**Abbreviations**

ADC, lung adenocarcinoma; LncRNAs, long noncoding RNAs; HOTAIRM1, HOXA transcript antisense RNA, myeloid-specific 1; CCK8, Cell counting kit-8; RIP, RNA-binding protein immunoprecipitation.
Data Sharing Statement
The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

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Disclosure
The authors report no conflicts of interest in this work.

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