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LncRNA AWPPH accelerates the progression of non-small cell lung cancer by sponging miRNA-204 to upregulate CDK6

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Abstract. – OBJECTIVE: This study aims to uncover the function of long non-coding RNA (lncRNA) AWPPH in the progression of non-small cell lung cancer (NSCLC) and the potential mechanism.

PATIENTS AND METHODS: AWPPH and microRNA (miRNA-204) levels in NSCLC tissues and adjacent normal tissues were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Kaplan-Meier curves were introduced for assessing overall survival in NSCLC patients expressing high or low level of AWPPH. Potential correlation between expression levels of AWPPH and miRNA-204 in NSCLC tissues was analyzed by Spearman correlation test. Through Dual-Luciferase reporter gene assay, the interaction among AWPPH, miRNA-204, and CDK6 was identified. Potential impacts of AWPPH/miRNA-204/CDK6 regulatory loop on mediating proliferative, migratory, and invasive capacities of A549 cells were evaluated through cell counting kit-8 (CCK-8) and transwell assay.

RESULTS: Upregulated AWPPH and downregulated miRNA-204 were determined in NSCLC tissues. AWPPH level was negatively correlated to overall survival in NSCLC patients and miRNA-204 level in NSCLC tissues. Silence of AWPPH attenuated proliferative, migratory, and invasive capacities in A549 cells. MiRNA-204 was the downstream gene of AWPPH. Knockdown of miRNA-204 reversed the decreased viability, migratory, and invasive rates in A549 cells with AWPPH knockdown. In addition, CDK6 was the target gene of miRNA-204. Overexpression of miRNA-204 downregulated CDK6 level in A549 cells. The attenuated proliferative, migratory, and invasive capacities in A549 cells overexpressing miRNA-204 were reversed after CDK6 overexpression.

CONCLUSIONS: LncRNA AWPPH serves as the miRNA-204 sponge to upregulate CDK6 level, thus aggravating the progression of NSCLC.

Key Words:
NSCLC, AWPPH, MiRNA-204, CDK6, Metastasis, Proliferation.

Introduction

Non-small cell lung cancer (NSCLC) is a highly prevalent tumor, ranking the first in the incidence of malignancies[1,2]. Pathologically, NSCLC includes squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. NSCLC is characterized as slow growth and division of tumor cells, as well as late diffusion and metastasis[3]. About 75% NSCLC patients are initially diagnosed as middle or advanced stage, with a very low 5-year survival[4].

Long non-coding RNAs (lncRNAs) are non-coding RNAs with over 200 nucleotides long[5]. They are well explored in genetic researches owing to their crucial functions in life activities[6]. Several studies[7] have illustrated the involvement of lncRNAs in carcinogenesis, angiogenesis, and tumor metastasis. Oncogenic or tumor-suppressor lncRNAs are extensively involved in tumor metastasis and progression of NSCLC[8].

MicroRNAs (miRNAs) are endogenous, non-coding RNAs spanning 22 nucleotides, exerting post-transcriptional regulation in animals and plants[9]. A mature miRNA recognizes target mRNA through complementary base pairing, and further degrades mRNA or blocks mRNA translation according to the base pairing degree[10]. Through various mechanisms, miRNAs participate in tumor progression, virus defense, cell apoptosis,

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etc[11-13]. MiRNA-204 is a newly discovered miRNA involved in the occurrence and progression of tumors. In cervical cancer, miRNA-204 mediates proliferative and invasive abilities of tumor cells by targeting EphB2[14]. MiRNA-204 influence epithelial-mesenchymal transition (EMT) in gastric cancer through targeting Snail[15].

In this paper, we uncovered the biological role of lncRNA AWPPH in the progression of NSCLC. AWPPH sponged miRNA-204 as a ceRNA to upregulate CDK6, thus accelerating the malignant progression of NSCLC.

Patients and Methods

Sample Collection

NSCLC tissues and adjacent normal tissues were collected from NSCLC patients undergoing radical resection in Xiamen Fifth Hospital from October 2016 to December 2018. All tissues were pathologically confirmed. None of the patients had preoperative anti-tumor treatment. This investigation was approved by the Ethics Committee of Xiamen Fifth Hospital and the informed consent was signed from all the participants.

Cell Culture and Transfection

Human normal bronchial epithelial cells HBE and lung cancer cells A549, NCI-H1650, and HCC827 were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 0.1 mg/mL streptomycin, in a 37°C, 5% CO_2 incubator. The fresh medium was regularly replaced.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Extraction of total RNA in cells was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNAs were subjected to reverse transcription. The extracted complementary deoxyribose nucleic acid (cDNA) was applied for PCR using SYBR Green method (TaKaRa, Otsu, Shiga, Japan) at 94°C for 5 min, and 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s.

Dual-Luciferase Reporter Gene Assay

Wild-type and mutant-type vectors were constructed by cloning of binding sites in the promoter regions of two genes into psi-CHECK2 vectors. Cells were co-transfected with wild-type/mutant-type vectors and miRNA-204 mimic/control for 48 h. Afterwards, cells were lysed for determining Luciferase activity (Promega, Madison, WI, USA).

Plasmids Construction and Transfection

After constructing the pcDNA3.0-CDK6 vector based on amplification with specific primers, CDK6 cDNA was cloned into the mammalian expression vector pcDNA3.0 (Invitrogen, Carlsbad, CA, USA). The pcDNA3.0-CDK6 vector was transfected into A549 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were collected after 48 h of transfection.

Cell Counting Kit-8 (CCK-8)

Cells were seeded in the 96-well plate with 2×10^3 cells per well and cultured overnight. Absorbance (A) at 450 nm was recorded at the appointed time points using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curves.

Migration Assay

3×10^4 cells suspended in 500 μL of medium were inoculated on the bottom of transwell chamber placed in a 24-well plate. 100 μL of serum-free medium was applied on the bottom. After 12-h incubation, cells were fixed, dyed with 3 mL of 0.1% crystal violet and washed with phosphate-buffered saline (PBS) twice. Penetrating cells were captured in 3 randomly selected fields per sample (magnification 40×).

Invasion Assay

Fibronectin (FN) was diluted to a final concentration of 100 μg/mL. Matrigel was diluted at 1:9 with serum-free medium. The bottom of each chamber was coated with 50 μL of FN and placed in a clean bench for 2 h to air dry. The inside of the chamber was coated with 100 μL of Matrigel and placed in an incubator overnight. Cell density was adjusted to 1×10^6 cells/mL. 100 μL of suspension was applied on the upper layer of the transwell chamber, while 600 μL of medium containing 10% FBS was added to the bottom. After 24-h incubation, cells were fixed, dyed with trypan blue, washed 3 times with PBS, and captured in 3 randomly selected fields per sample (magnification 40×).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA) was

used for data analyses. Data were expressed as mean ± standard deviation. Intergroup differences were analyzed by the *t*-test. Spearman correlation test was conducted to analyze the relationship between expression levels of AWPPH and miRNA-204 in NSCLC tissues. $p<0.05$ was considered as statistically significant.

Results

Upregulated AWPPH Predicts Poor Prognosis in NSCLC Patients

First, we detected AWPPH and miRNA-204 levels in NSCLC tissues and adjacent normal ones. AWPPH was upregulated, while miRNA-204 was downregulated in NSCLC tissues (Figure 1A, 1C). By analyzing the follow-up data of enrolled NSCLC patients, worse survival was observed in NSCLC patients expressing high level of AWPPH (Figure 1B). Moreover, a negative relationship was identified between levels of AWPPH and miRNA-204 in NSCLC tissues (Figure 1D). It is indicated that AWPPH may be an oncogene involved in the progression of NSCLC.

Silence of AWPPH Suppresses NSCLC to Proliferate, Migrate, and Invade

Compared with normal bronchial epithelial cells, AWPPH was identically upregulated in lung cancer cells (Figure 2A). A549 cells expressed the highest level of AWPPH among the three tested cell lines and were used for the following experiments. Transfection of si-AWPPH greatly downregulated AWPPH level in A549 cells, showing a satisfactory transfection efficacy (Figure 2B). CCK-8 assay showed the reduced viability in A549 cells transfected with si-AWPPH (Figure 2C). In addition, invasive and migratory capacities were attenuated after knockdown of AWPPH in A549 cells (Figure 2D).

AWPPH Regulates NSCLC Through Sponging MiRNA-204

The presence of binding sites between AWPPH and miRNA-204 was predicted by TargetScan (Figure 3A). Declined Luciferase activity was seen in A549 cells co-transfected with miRNA-204 mimic and AWPPH WT, verifying the binding between AWPPH and miRNA-204 (Fig-

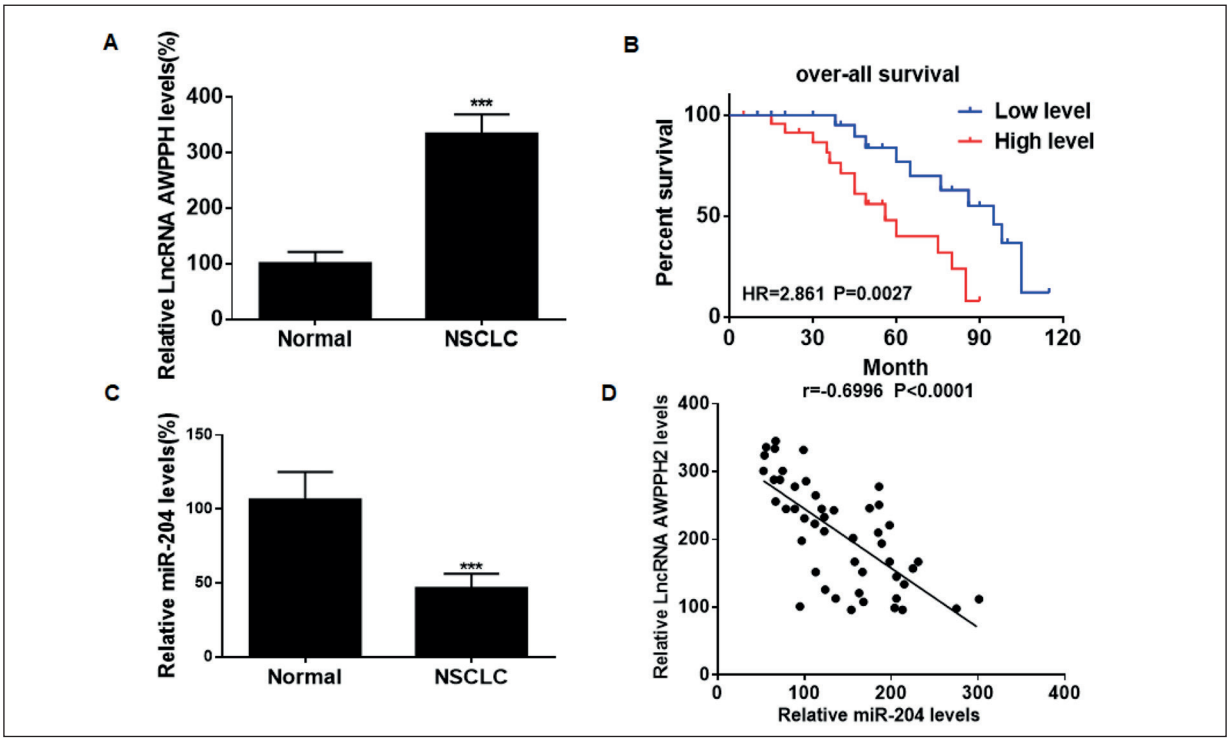

Figure 1. Upregulated AWPPH predicts poor prognosis in NSCLC patients. **A,** AWPPH levels in NSCLC tissues and adjacent normal tissues. **B,** Overall survival in NSCLC patients with low or high level of AWPPH. **C,** MiRNA-204 levels in NSCLC tissues and adjacent normal tissues. **D,** Negative relationship between levels of AWPPH and miRNA-204.

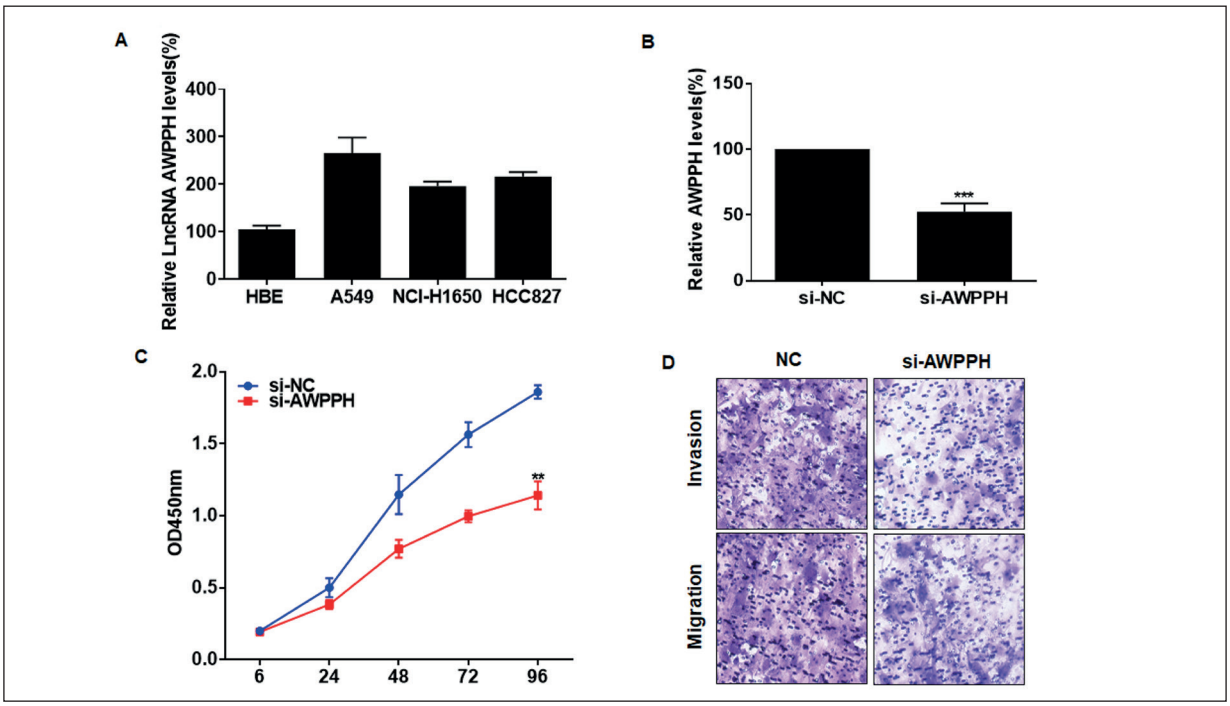

Figure 2. Silence of AWPPH suppresses NSCLC to proliferate, migrate, and invade. **A,** AWPPH levels in human normal bronchial epithelial cells HBE and lung cancer cells A549, NCI-H1650, and HCC827. **B,** AWPPH level in A549 cells transfected with si-NC or si-AWPPH. **C,** Viability at 6, 24, 48, 72, and 96 h in A549 cells transfected with si-NC or si-AWPPH. **D,** Invasion and migration in A549 cells transfected with si-NC or si-AWPPH (magnification: 200×).

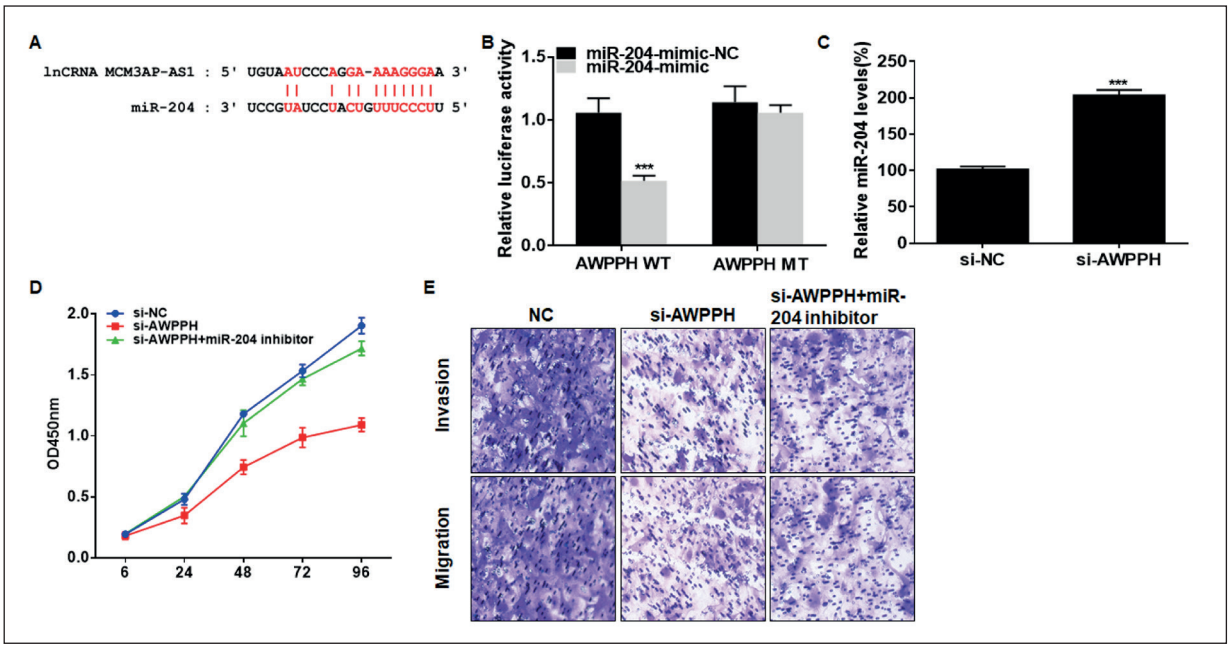

Figure 3. AWPPH regulates NSCLC through sponging miRNA-204. **A,** Binding sites between AWPPH and miRNA-204. **B,** Luciferase activity in A549 cells co-transfected with miRNA-204 mimic/NC and AWPPH WT/AWPPH MT. **C,** MiRNA-204 level in A549 cells transfected with si-NC or si-AWPPH. **D,** Viability at 6, 24, 48, 72 and 96 h in A549 cells transfected with si-NC, si-AWPPH or si-AWPPH + miRNA-204 inhibitor. **E,** Invasion and migration in A549 cells transfected with si-NC, si-AWPPH or si-AWPPH + miRNA-204 inhibitor (magnification: 200×).

ure 3B). MiRNA-204 level was remarkably up-regulated after transfection of si-AWPPH in A549 cells (Figure 3C). Interestingly, the attenuated viability, invasive and migratory capacities in A549 cells transfected with si-AWPPH were partially reversed after co-transfection of miRNA-204 inhibitor.

MiRNA-204 Regulates NSCLC Through Targeting CDK6

Similarly, CDK6 was predicted to be the downstream gene binding to miRNA-204 and further verified through Dual-Luciferase reporter gene assay (Figure 4A, 4B). CDK6 level was negatively regulated by miRNA-204 in A549 cells (Figure 4C). Overexpression of miRNA-204 suppressed A549 cells to proliferate, migrate, and invade, while these inhibitory effects were blocked by co-overexpression of CDK6 (Figure 4D). Collectively, AWPPH stimulated the progression of NSCLC through sponging miRNA-204 to upregulate CDK6.

Discussion

Lung cancer is a common solid tumor and the major reason for tumor death[16,17]. NSCLC is the most prevalent subtype of lung cancer, accounting for 80% of all lung cancer cases[18]. The 5-year survival of NSCLC is extremely low even though great strides have made on NSCLC treatment[19]. Distant metastasis is responsible for the high rate of tumor mortality, which is also the therapeutic difficulty[20]. It is necessary to clarify the molecular mechanism underlying metastasis and progression of NSCLC.

Noncoding RNAs are a type of functional RNAs that could not be translated into proteins, including miRNAs, circRNAs, and lncRNAs[21,22]. Increasing evidence has proved the vital functions of lncRNAs in epigenetics[23]. Dysregulated lncRNAs exert carcinogenic or tumor-suppressor effect on NSCLC, and they are utilized as novel hallmarks[8,24].

LncRNA AWPPH is highly expressed in hepatocellular cancer[25], bladder cancer[26], and

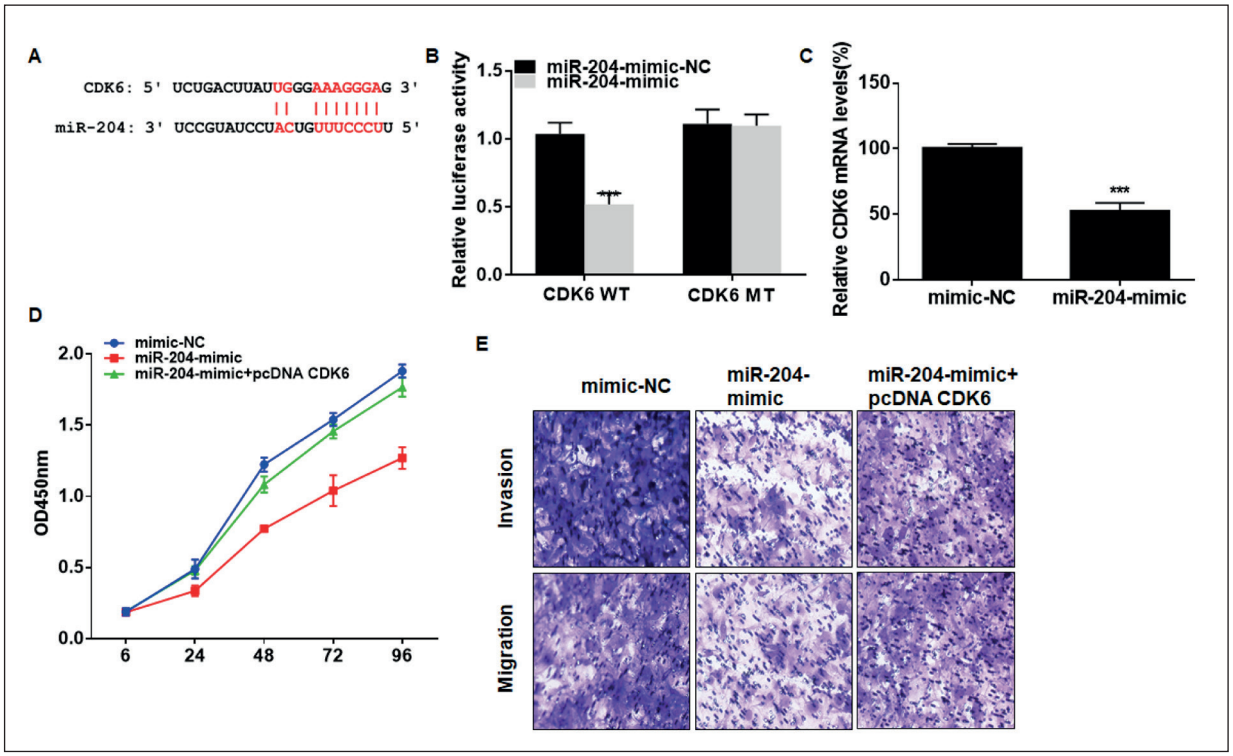

Figure 4. MiRNA-204 regulates NSCLC through targeting CDK6. **A,** Binding sites between CDK6 and miRNA-204. **B,** Luciferase activity in A549 cells co-transfected with miRNA-204 mimic/NC and CDK6 WT/CDK6 MT. **C,** CDK6 level in A549 cells transfected with NC or miRNA-204 mimic. **D,** Viability at 6, 24, 48, 72, and 96 h in A549 cells transfected with NC, miRNA-204 mimic or miRNA-204 mimic + pcDNA CDK6. **E,** Invasion and migration in A549 cells transfected with NC, miRNA-204 mimic or miRNA-204 mimic + pcDNA CDK6 (magnification: 200×).

osteosarcoma[27], which stimulates the malignant progression and distant metastasis of tumors. In ovarian cancer, AWPPH accelerates tumor cells to migrate and invade by activating the Wnt/β-catenin pathway[28]. By activating the Wnt pathway, AWPPH/miR-93-3p/FZD7 regulatory loop aggravates the progression of osteosarcoma[27]. In NSCLC, AWPPH is capable of promoting proliferative ability and inhibiting apoptosis of tumor cells[29]. Postoperative recurrence of NSCLC is found to be influenced by AWPPH by targeting TGF-β1[30]. Our findings showed upregulation of AWPPH in NSCLC patients. High level of AWPPH predicted worse survival in NSCLC patients. Subsequently, *in vitro* experiments confirmed that AWPPH promoted lung cancer cells to proliferate, migrate, and invade. MiRNA-204 was verified to be the target of AWPPH and it was downregulated in NSCLC tissues. Importantly, miRNA-204 was responsible for the regulatory effects of AWPPH on cellular behaviors of lung cancer cells.

It is well known that tumorigenesis is a multistep complex process[31]. Genetic and epigenetic changes trigger carcinogenic transformation from normal cells to tumor cells[32,33]. CDK6 is not only a cyclin-dependent kinase, but also a transcription regulator[34]. CDK6 could be regulated by multiple miRNAs[35]. It is reported that CDK6 is extensively involved in the metastasis and progression of tumors[36], and lncRNA NEAT1 aggravates laryngeal squamous cell carcinoma by mediating miR-107/CDK6 axis[37]. MiR-892b influences proliferative, migratory, and invasive capacities of bladder cancer *via* targeting the p19ARF/Cyclin D1/CDK6 and Sp-1/MMP-9 axis[38]. In this paper, CDK6 was certified to be the downstream gene of miRNA-204. Overexpression of miRNA-204 downregulated CDK6 level in A549 cells. Notably, overexpression of CDK6 reversed the regulatory effect of miRNA-204 on A549 cells. Therefore, AWPPH/miRNA-204/CDK6 regulatory loop was identified to aggravate the malignant progression of NSCLC.

Conclusions

In summary, lncRNA AWPPH is upregulated in NSCLC. Serving as a ceRNA, AWPPH sponges miRNA-204 to upregulate CDK6 level, thus aggravating the progression of NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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