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LncRNA AWPPH promotes the invasion and migration of glioma cells through the upregulation of HIF1α

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Abstract. Long non-coding RNA (lncRNA) AWPPH has been revealed to serve pivotal roles in bladder cancer and liver cancer. The aim of the present study was to determine the involvement of lncRNA AWPPH in glioma. It was observed in the present study that the expression levels of AWPPH in plasma were significantly higher in patients with metastatic glioma compared with those in patients with non-metastatic glioma and healthy controls. In effect, overexpression of AWPPH allowed the differentiation of patients with metastatic glioma from patients with non-metastatic glioma and in healthy controls. Increased migration and invasion rates of glioma cells and upregulated hypoxia-inducible factor 1-α (HIF1α) expression were observed following AWPPH overexpression. HIF1α overexpression exhibited no significant effects on AWPPH expression but also promoted the migration and invasion of cancer cells. HIF1α small interfering RNA silencing attenuated the enhancing effects of AWPPH overexpression on the migration and invasion of glioma cells. From the results of the present study it was concluded that AWPPH may promote glioma metastasis by serving as an upstream activator of HIF1α.

Introduction

Although glioma is the most common type of primary brain malignancy in adults, it is a rare disease that only affects 6 cases out of 100,000 people worldwide (1). Despite the low incidence rate, this disease is considered a major cause of cancer-associated mortality worldwide, due to the unacceptably high mortality rate. Glioma causes seizures, headaches and progressive neurological disease, but also leads to changes in personality and behavior (2). Tumor metastasis is the major cause of failure in the treatment of glioma (3). The survival time of patients with metastatic glioma can be significantly prolonged with proper chemotherapy or radiation therapy (4). Therefore, proper treatment based on the existence of tumor metastasis is critical.

Hypoxia-inducible factor 1-α (HIF1α) is a transcriptional regulator that serves a central role in the regulation of cellular and developmental responses to hypoxia (5,6). It has been well established that genetic alternations or hypoxia in cancer cells can lead to the altered expression of HIF1α, and dysregulation of HIF1α further promotes the development of cancer (7). It has been revealed that inhibition of HIF1α signaling may serve as a promising target for cancer therapy (8). AWPPH is a recently identified lncRNA that, to date, has known functions only in bladder cancer (9) and hepatocellular carcinoma (10). Thus, the aim of the present study was to determine the involvement of lncRNA AWPPH in glioma. The present study revealed that AWPPH may contribute to the metastasis of glioma. The actions of AWPPH in the metastasis of glioma are likely to be achieved through the upregulation of HIF1α.

Materials and methods

Cell lines and human specimens. Hs 683 (ATCC® HTB-138™) and CCD-25Lu (ATCC® CCL-215™) human glioma cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells from the two cell lines were cultured in ATCC-formulated Eagle's minimum essential medium (catalog no. 30-2003) supplemented with 10% fetal bovine serum (FBS).

Plasma specimens were obtained from 66 patients (40 men and 24 women; age range, 54-72 years; mean age 64.4±5.1 years) with glioma and 42 healthy volunteers (28 men and 14 women; age range, 54-71 years; mean age, 64.2±5.0 years). The 66 patients with glioma were diagnosed and treated in the Chinese People's Liberation Army Rocket Force General Hospital (Beijing, China) between January 2014 and March 2018. Among those patients, 32 had non-metastatic glioma and 34 had metastatic glioma. Inclusion criteria were: i) Patients diagnosed by pathological examinations; ii) patients completely understood the experimental protocol and provided written informed consent. Exclusion criteria were: i) Patients who had other diseases present as well; ii) patients who were treated by any strategies

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Table I. Basic information for the three groups of participants.

Characteristics	Non-metastatic glioma	Metastatic glioma	Healthy control
Cases, n	32	34	42
Sex			
Male, n	15	19	20
Female, n	17	15	22
Lifestyle			
Smoking, n (%)	14 (43.8%)	16 (47.1%)	19 (45.2%)
Alcohol consumption, n (%)	17 (53.1%)	17 (50.0%)	19 (45.2%)

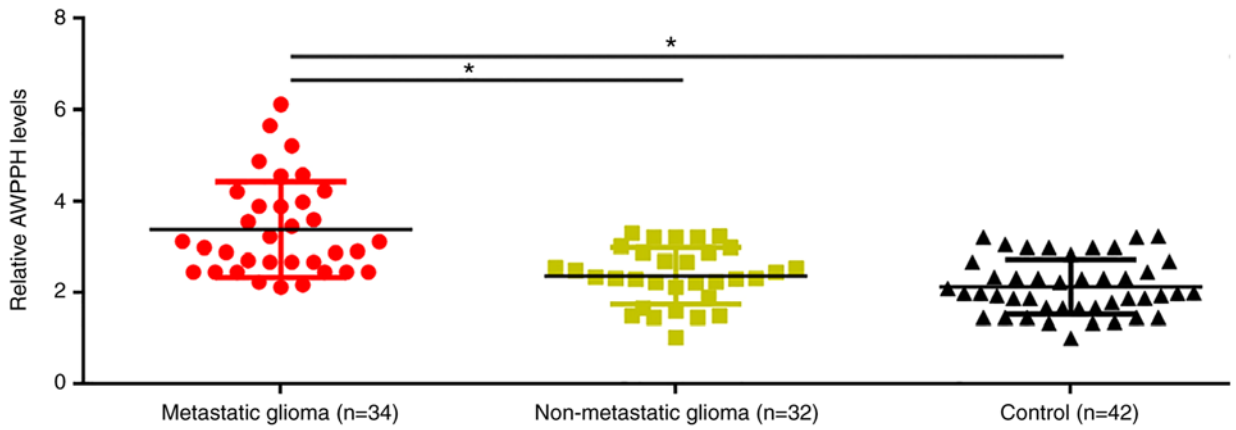

Figure 1. AWPPH expression is upregulated in metastatic glioma but not in non-metastatic glioma. *P<0.05.

within 3 months before admission. The 42 healthy volunteers received physical examinations in the Chinese People's Liberation Army Rocket Force General Hospital during the same period. No significant differences in age, sex or living habits were identified among the non-metastatic glioma, metastatic glioma and control groups. The basic information of the three patient groups is presented in Table I. The present study was approved by the Ethics Committee of the Chinese People's Liberation Army Rocket Force General Hospital. All patients completely understood the experiment protocol and provided written informed consent.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following total RNA extraction from plasma and Hs 683 and CCD-25Lu cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), reverse transcription was performed to synthesize cDNA. A SYBR® Green Quantitative RT-qPCR kit (Sigma-Aldrich; Merck KGaA) was used to prepare all PCR systems. The PCR conditions were: 57 sec at 95˚C, followed by 40 cycles of 16 sec at 95˚C and 38 sec at 57.5˚C. The primers used in the PCR were: Forward, 5'-CTGGATGGTCGCTGCTTTTTA-3' and reverse, 5'-AGG GGGATGAGTCGTGATTT-3' for human AWPPH; forward, 5'-TCCATTATGAGGCTGACCATC-3' and reverse, 5'-CCA TCCTCAGAAAGCACCATA-3' for human HIF1α; forward, 5'-GACCTCTATGCCAACACAGT-3' and reverse, 5'-AGT ACTTGCGCTCAGGAGGA-3' for β-actin. All data were normalized using the $2^{-\Delta\Delta Cq}$ method (11).

Vectors, small interfering (si)RNAs and cell transfection. AWPPH and HIF1α expression pIRSE2 vectors and empty pIRSE2 vectors were purchased from GeneCopoeia, Inc.. HIF1α siRNA (catalog no. AM16708) and Silencer® Negative Control #1 siRNA (catalog no. AM4611) were purchased from Thermo Fisher Scientific, Inc. Cell transfection was performed using Lipofectamine® 2000 Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) with vectors at a concentration of 10 nM and siRNAs at 50 nM. In this experiment, the control was the cells without transfection and the negative control was the cells transfected with empty vectors or Silencer® Negative Control #1 siRNA. Overexpression of AWPPH and HIF1α as well as HIF1α siRNA silencing was confirmed using RT-qPCR. An overexpression rate of 210-250% and a down-regulation rate of 30-50% was achieved prior to continuing experiments. Cells were transfected 24 h prior to subsequent experiments.

Transwell migration and invasion assay. Following transfection, cell migration and invasion rates were measured using a Transwell migration and invasion assay. The assays were performed according to the same protocol except that the upper chamber was pre-coated with Matrigel (catalog no. 356234; EMD Millipore) prior to the invasion assay. Briefly, single cell suspensions were prepared ($5x10^4$ cells/ml of medium supplemented with 1% FBS). The upper chamber was filled with 0.1 ml single cell suspension and culture medium containing 20% FBS was added to the lower chamber. Cells were cultured

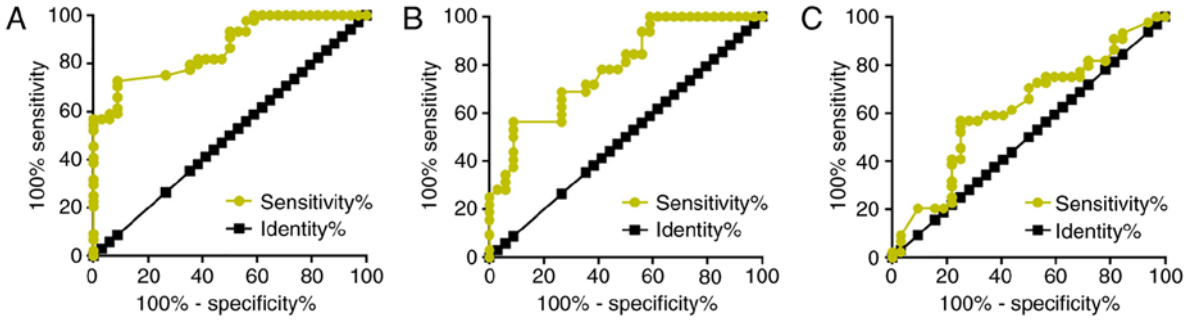

Figure 2. Identification of AWPPH distinguishes patients with metastatic glioma from patients with non-metastatic glioma and healthy controls. (A) ROC curve analysis of the diagnostic value of AWPPH for patients with metastatic glioma, with healthy controls as references. (B) ROC curve analysis of the diagnostic value of AWPPH for patients with metastatic glioma, with patients with non-metastatic glioma as references. (C) ROC curve analysis of the diagnostic value of AWPPH for patients with non-metastatic glioma, with healthy controls as references. ROC, receiver operating characteristic.

for 12 h, followed by membrane staining with 0.5% crystal violet (Sigma-Aldrich; Merck KGaG) for 25 min at room temperature. Invading and migrating cells were observed and counted using a light microscope (magnification, 40x).

Western blot analysis. Following total protein extraction using radioimmunoprecipitation assay solution (Thermo Fisher Scientific, Inc.) and protein quantification using BCA assay (Thermo Fisher Scientific, Inc.), SDS-PAGE (12% gel) was performed with 20 μg denatured protein in each well. Following gel transfer onto polyvinylidene difluoride membranes, blocking was performed with 5% skimmed milk in PBS for 1 h at room temperature. Subsequently, membranes were incubated with primary antibodies against HIF1α first (rabbit anti-human, 1:1,400; ab216842; Abcam, Cambridge, UK) and GAPDH (rabbit anti-human, 1:1,400; ab9485; Abcam) at 4˚C overnight, followed by incubation with horseradish peroxidase immunoglobulin secondary antibody (goat anti-rabbit, 1:1,300; MBS435036; MyBioSource) for 1 h at room temperature. Signals were developed using Pierce enhanced chemiluminescent Western Blot substrate (Thermo Fisher Scientific, Inc.). Signals were detected using a MYECL™ Imager (Thermo Fisher Scientific, Inc.) and normalized using ImageJ software (version 1.6; National Institutes of Health).

Statistical analysis. GraphPad Prism software (version 6; GraphPad Software, Inc., La Jolla, CA, USA) was used for the statistical analyses. All data were expressed as the mean ± standard deviation and compared using a one-way analysis of variance followed by a Tukey test. The diagnostic value of AWPPH for glioma was analyzed using the receiver operating characteristic (ROC) curve analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

AWPPH is upregulated in metastatic glioma but not in non-metastatic glioma. Plasma levels of AWPPH in patients with metastatic glioma, patients with non-metastatic glioma and healthy controls were determined using RT-qPCR. Compared with patients with non-metastatic glioma and healthy controls, significantly increased plasma levels of AWPPH were observed in patients with metastatic glioma ($P<0.05$; Fig. 1). However, no significant differences in plasma levels of AWPPH were

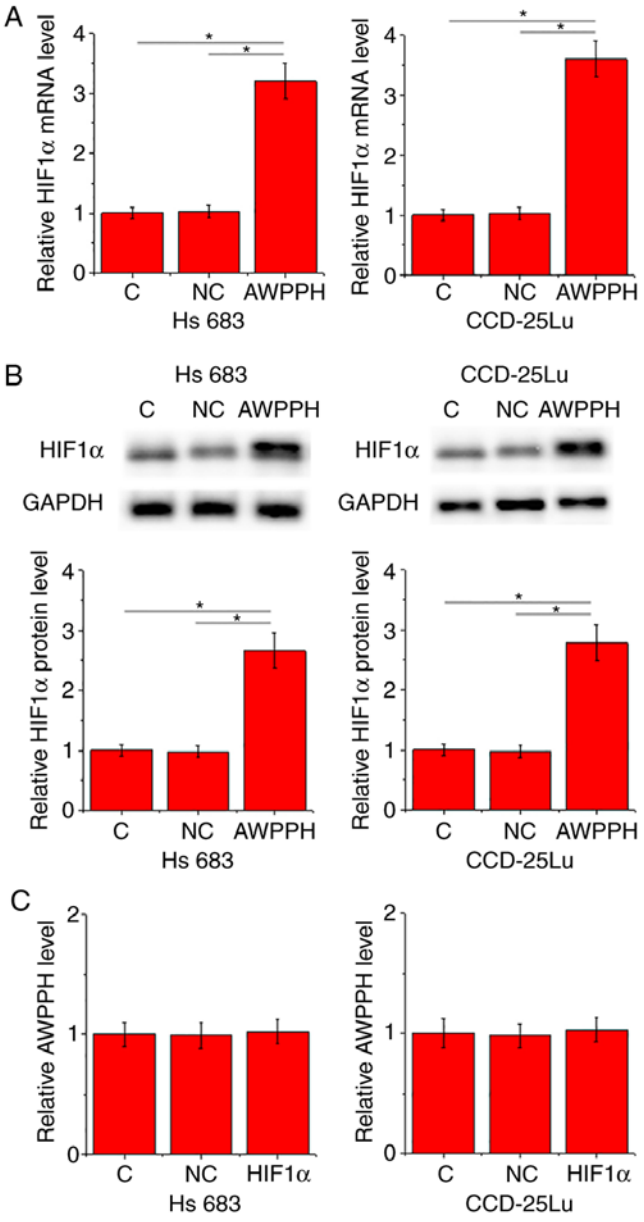

Figure 3. AWPPH overexpression leads to upregulated HIF1α expression in glioma cells. (A) AWPPH led to upregulated HIF1α expression in glioma cells at the mRNA level. (B) AWPPH led to upregulated HIF1α in glioma cells at the protein level. (C) HIF1α overexpression failed to significantly affect AWPPH expression. *$P<0.05$. HIF1α, hypoxia-inducible factor 1-α; C, control; NC, negative control.

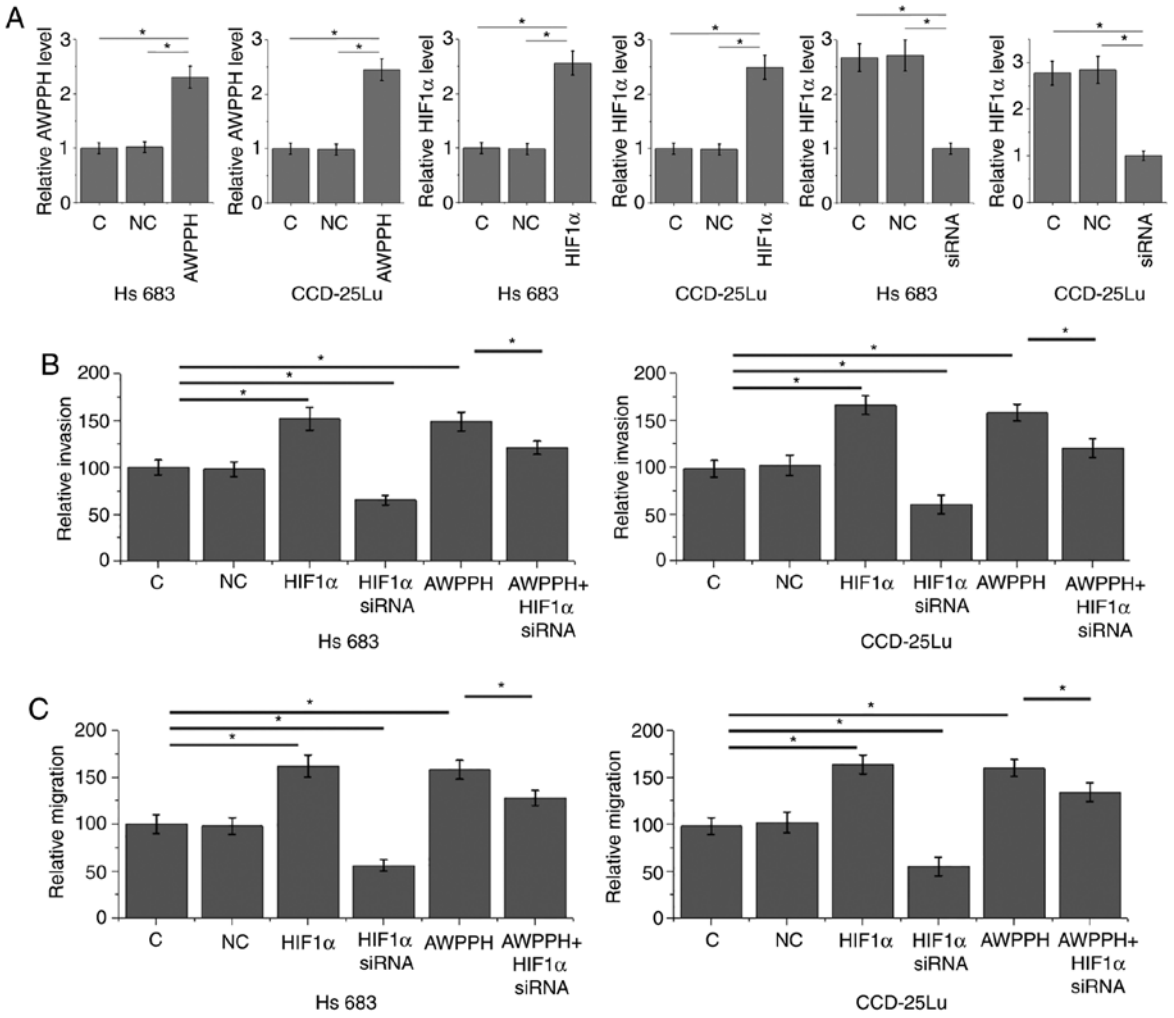

Figure 4. AWPPH promotes glioma cell migration and invasion possibly by upregulating HIF1α. (A) Overexpression of AWPPH and HIF1α and silencing of HIF1α siRNA was achieved following transfection. (B) AWPPH and HIF1α expression significantly promoted the invasion of cells of the Hs 683 and CCD-25Lu human glioma cell lines, and HIF1α siRNA silencing inhibited the invasion of the two cell lines. (C) AWPPH and HIF1α expression significantly promoted the migration of cells of the Hs 683 and CCD-25Lu human glioma cell lines, and HIF1α siRNA silencing inhibited the migration of the two cell lines. *P<0.05. HIF1α, hypoxia-inducible factor 1-α; siRNA, small interfering RNA.

identified between patients with non-metastatic glioma and healthy controls (Fig. 1).

Overexpression of AWPPH distinguishes patients with metastatic glioma from patients with non-metastatic glioma and healthy controls. The diagnostic value of AWPPH for glioma was analyzed by ROC curve analysis. For metastatic glioma with healthy controls as references, the area under the curve (AUC) was 0.8640 [standard error, 0.03950; 95% confidence interval (CI), 0.7865-0.9414; P<0.0001; Fig. 2A]. For metastatic glioma with patients with non-metastatic glioma as references, the AUC was 0.7881 (standard error, 0.05451; 95% CI, 0.6813-0.8950; P<0.0001; Fig. 2B). For non-metastatic glioma with healthy controls as references, the AUC was 0.6186 (standard error, 0.06578; 95% CI, 0.4896-0.7476; P=0.07899).

AWPPH overexpression leads to upregulated HIF1α expression in glioma cells. In the present study the interactions between AWPPH and HIF1α were investigated in cells of the two human glioma cell lines Hs 683 and CCD-25Lu. Compared with control cells (C) and negative control cells (NC), overexpression of AWPPH led to significantly promoted expression of HIF1α in the two cell lines at the mRNA (P<0.05; Fig. 3A) and protein (P<0.05; Fig. 3B) levels. By contrast, compared with C and NC groups, there were no significant changes in the expression level of AWPPH revealed in cells with HIF1α overexpression (Fig. 3C).

AWPPH promotes glioma cell migration and invasion possibly by upregulating HIFα. The aforementioned data indicated the involvement of AWPPH in glioma metastasis. AWPPH and HIF1α overexpression, as well as HIF1α siRNA silencing, were achieved following transfection (P<0.05; Fig. 4A). Compared with the C and NC groups, AWPPH and HIF1α overexpression significantly promoted the invasion (Fig. 4B) and migration (Fig. 4C) of cells of the two human glioma cells lines Hs 683 and CCD-25Lu (P<0.05). In addition, compared with glioma cells that only presented with AWPPH overexpression, cells transfected with both AWPPH expression vector and HIF1α siRNA demonstrated significantly decreased migration and invasion rates, but they remained lower compared with those of the C and NC groups.

Discussion

AWPPH is a characterized oncogenic lncRNA in bladder cancer (9) and hepatocellular carcinoma (10), although its involvement in other diseases is currently unknown. The present study revealed that AWPPH may contribute to the metastasis of glioma. The actions of AWPPH in the metastasis of glioma are likely to be achieved through the upregulation of HIF1α.

The development of glioma led to altered signaling transduction of multiple molecular pathways in the human body (12). The dysregulation of lncRNAs may serve as a mediator between signaling pathways to participate in the different aspects of the development and progression of glioma (13). Certain lncRNAs, such as lncRNA taurine upregulated gene 1 (14), have lower expression levels in glioma tumor tissues compared with in healthy tissues, and overexpression of those lncRNAs inhibits tumor progression, indicating the role they serve as a tumor suppressor in glioma. In contrast, certain lncRNAs, such as lncRNA activated by transforming growth factor β, serve an oncogenic role in glioma and demonstrate an upregulated expression pattern (15). However, all those lncRNAs appear to be involved in the growth and metastasis of glioma, and lack the potential to predict a certain stage of glioma, such as tumor metastasis. In bladder cancer, upregulation of AWPPH was observed prior to the occurrence of tumor metastasis (stage Ta-T1) (9). The study on hepatocellular carcinoma did not distinguish between the expression of AWPPH prior to and following tumor metastasis (10). In contrast with the aforementioned studies, the present study revealed upregulated expression of AWPPH only in metastatic glioma, not in non-metastasis glioma. Therefore, AWPPH may participate only in the metastasis of glioma. The present study provided novel insights into the pathogenesis of glioma, and suggests that tumor growth and metastasis may require the involvement of different cellular factors. Therefore, inhibition of tumor growth and metastasis in clinical trials should have different targets.

Proper treatment strategies designed based on accurate clinical stages are critical for the treatment of cancer. Even though the survival of patients with metastatic brain tumors is generally poor, proper treatment strategies can still significantly prolong survival time (16,17). In the present study, it was demonstrated that the overexpression of AWPPH can allow the differentiation of patients with metastatic glioma from patients with non-metastatic glioma and healthy controls. However, the present study failed to demonstrate the differentiation of patients with non-metastatic glioma from healthy controls. Therefore, upregulation of plasma AWPPH may serve as a biomarker for the metastasis of glioma. It has been well established that HIF1α may participate in cancer biology through interactions with different lncRNAs (18,19). Activation of HIF1α promotes cancer metastasis, and inhibition of HIF1α has been proven to be a promising target for the treatment of cancer (20,21). The results of the present study indicated that AWPPH may be an upstream activator of HIF1α in the regulation of migration and invasion of glioma cells under non-hypoxic condition; however, only the sequential signaling of AWPPH-HIF1α in glioma was reported. Whether this regulation is direct or indirect is still unknown.

The present study did not include *in vivo* studies; therefore, future studies should attempt to establish animal models for glioma to further confirm the conclusions of the present study. In addition, future studies should try to use double fluorescence reporter enzyme systems to verify whether lncRNA AWPPH is a direct regulator of HIF1α mRNA.

In conclusion, AWPPH may specifically participate in the metastasis of glioma through the upregulation of HIF1α.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TZ and BZ designed the experiments. TZ and FW performed all of the experiments. YL and LY collected and analyzed data. TZ and BZ drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Chinese People's Liberation Army Rocket Force General Hospital. All patients completely understood the experiment protocol and provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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