

MicroRNA-146b-5p/EPHA7 axis regulates cell invasion, metastasis, proliferation, and temozolomide-induced chemoresistance via regulation of IRAK4/TRAF6/NF- κ B signaling pathway in aggressive pituitary adenoma

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Summary. Background. Aggressive pituitary adenoma (APA) is a huge challenge for neurosurgeons. Temozolomide (TMZ) is conventionally used in chemotherapy against APA, but acquired resistance developed during long-term therapy limits its benefits. MiRNA-146b-5p has been confirmed to inhibit tumor metastasis. This study aimed to explore the underlying biological functions of miRNA-146b-5p in APA.

Methods. Sixty confirmed APA tissues and corresponding adjacent normal tissues were collected. We established a TMZ-resistant cell line (GH3/TMZ) by exposing GH3 cells to gradually increasing doses of TMZ for 5 months. Cell Counting Kit-8 assay, flow cytometric analysis, RNA pull-down assay, 5-ethynyl-20-deoxyuridine assay, dual-luciferase reporter gene assay, wound healing assay, and invasion assay were used to explore the malignant biological characteristics of cells. Immunohistochemistry (IHC), western blotting analysis, and real-time quantitative PCR (qRT-PCR) were used to analyze the expression level of related proteins and nucleic acids.

Results. The expression of miRNA-146b-5p was down-regulated not only in APA tissues but also in PA cell lines compared with the matched adjacent non-tumor tissues or normal human astrocyte (NHA) cells. Low expression of miRNA-146b-5p was notably associated with poorer disease-free survival rate ($P=0.032$), overall survival rate ($P=0.039$), larger tumor size ($P=0.028$), poorer Knosp grade ($P=0.020$), and poorer Hardy grade ($P=0.006$) in APA patients. MiRNA-146b-5p negatively regulated cell proliferation, invasion, migration, and induced apoptosis in GH3 cells.

Overexpression of miRNA-146b-5p suppressed IRAK4 and TRAF6 protein expression and negatively regulated NF- κ B phosphorylation. The restoration of EPHA7 expression in GH3 cells notably reversed the inhibitory effects of miRNA-146b-5p. MiRNA-146b-5p expression was significantly down-regulated and EPHA7 gene expression was significantly up-regulated in GH3/TMZ cells, compared to the parental cell line. Similarly, EPHA7 was up-regulated, while the miRNA-146b-5p level was down-regulated in chemoresistance tissues more than in chemosensitive tissues. The autophagic activity was decreased markedly with increasing miRNA-146b-5p expression, while it was enhanced after Lv-EPHA7 treatment in GH3/TMZ cells.

Conclusions. MiRNA-146b-5p can inhibit EPHA7 expression, suppress the IRAK4/TRAF6/NF- κ B signaling pathway, and weaken PA cell invasion, metastasis, proliferation, and TMZ-induced chemoresistance *in vitro*.

Key words: Expression, Ephrin receptor A7, Apoptosis, Autophagy, Prognosis

Introduction

Aggressive pituitary adenoma (APA), which exhibits aggressive behaviors and resistance to conventional treatments, is a huge challenge for neurosurgeons (Lizzul et al., 2020). Patients with APA are often diagnosed in the advanced stage, so the prognosis is very poor. APA can

Abbreviations. APA, aggressive pituitary adenoma; CCK-8, Cell Counting Kit-8; EPHA7, ephrin receptor A7; IC50, half maximal inhibitory concentration; IL-1R, interleukin 1 receptor; IHC, immunohistochemistry; miRNA, microRNA; NHA, normal human astrocyte; PA, pituitary adenoma; qRT-PCR, RNA extraction and real-time PCR analyses; TLR, Toll-like receptor; TMZ, Temozolomide

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compress the peripheral cranial nerves, invade the adjacent dura mater, and surround the internal carotid artery and other important brain tissues (Ceccato et al., 2018). Surgical treatment is the main clinical method for APA. However, the postoperative recurrence rate is as high as 30% (Ceccato et al., 2018). It is worth noting that chemotherapy resistance reduces the sensitivity of APA to chemotherapy drugs and limits the clinical application of chemotherapy. The current effectiveness of various chemotherapy regimens is only about 30%, and multi-drug resistance is a common problem (Kim et al., 2021). Temozolomide (TMZ) is the cornerstone drug for the treatment of APA patients and can significantly inhibit cancer progression (Syro et al., 2018; Burman et al., 2020; Lizzul et al., 2020). However, the key mechanism of TMZ-induced chemoresistance in APA remains unclear. Therefore, exploring the internal essence of the occurrence, development, and metastasis of APA and looking for new prognostic markers and prevention targets have important clinical significance for improving the cure rate of APA.

MicroRNAs (miRNAs) are highly conserved small noncoding RNA with lengths of approximately 17-25 nucleotides (Chen et al., 2020). Recently, miRNA-424-3p was found to be involved in multiple steps that control the occurrence and development of APA (Chen et al., 2020). MiRNA-146b-5p has been confirmed to show antitumor effects in multiple types of carcinoma (Li et al., 2019; Al-Abdallah et al., 2020). However, the underlying mechanisms and significance of miRNA-146b-5p in APA remain unclear.

Epfrin receptor A7 (EPHA7) mainly mediates the inflammatory response to cell injury as an adaptor protein binding to the Toll-like receptor (TLR) and interleukin 1 receptor (IL-1R) (Dong et al., 2013). Previous studies have shown that the abnormal expression of EPHA7 is closely related to tumor development and drug resistance (Manso et al., 2016). EPHA7 is abnormally highly expressed in many kinds of malignant tumor cells and tissues, such as lung cancer (Liu et al., 2020), and colorectal cancer (Di et al., 2019). However, there is still no information about the role of EPHA7 in the occurrence and development of APA.

In this study, we first identified that miRNA-146b-5p is down-regulated in APA tissues and cells. The dysregulation of miRNA-146b-5p was markedly correlated with poor prognosis in APA patients. Furthermore, we demonstrated that miRNA-146b-5p inhibited EPHA7 expression, suppressed the IRAK4/TRAF6/NF- κ B signaling pathway, and weakened PA cell invasion, metastasis, proliferation, and TMZ-induced chemoresistance *in vitro*.

Materials and methods

The definition of APA

Pituitary adenoma (PA) is a type of tumor originating from the anterior pituitary gland, which

accounts for about 10-15% of intracranial tumors. Some of them are APA, which accounts for about 25-55%. APA shows strong invasiveness, it can invade surrounding tissue structures, including sphenoid sinus, ethmoid sinus, cavernous sinus and even the third ventricle.

Surgical specimens and clinicopathological data

From January 2013 to January 2018, primary tumor tissues with pathologically confirmed APA (n=60) and their matching adjacent non-tumor tissue samples were obtained at the Beijing Tiantan Hospital, Capital Medical University. Among them, 28 cases were non-functional APA and 32 cases were functional APA. Functional APA patients included 11 cases of prolactin-secreting APA, 15 cases of GH-secreting APA, and 6 cases of ACTH-secreting APA. These clinical characteristics of APA patients are described in Table 1. After surgery, APA patients were followed up for 30 months. Patients' disease-free survival rates were determined by the operation date and the date of recurrent disease diagnosis or the end of follow-up. Survival rates were determined from the operation date to the date of death or the end of follow-up. All tissues were acquired with written informed consent. Our research plans were approved by the Clinical Research Ethics Committee of the Capital Medical University,

Table 1. Correlation of miRNA-146b-5p expression with clinicopathological characteristics in aggressive pituitary adenoma.

Variables	miRNA-146b-5p expression			P value
	Cases (n=60)	Low (n=30)	High (n=30)	
Age (years)				0.432
≤35	25	11	14	
>35	35	19	16	
Gender				0.371
Male	45	21	24	
Female	15	9	6	
Classification				0.392
Non-functional	28	12	16	
Prolactin-secreting	11	6	5	
GH-secreting	15	10	5	
ACTH-secreting	6	2	4	
Tumor size (cm)				0.028*
≤2	40	16	24	
>2	20	14	6	
Knosp grade				0.020*
Grade III	16	4	12	
Grade IV	44	26	18	
Hardy grade				0.006*
Grade III	20	5	15	
Grade IV	40	25	15	
Chemosensitivity				0.002*
Chemosensitive	32	10	22	
Chemoresistant	28	20	8	

* Statistically significant.

MiRNA-146b-5p/EPHA7 regulates aggressive pituitary adenoma

Beijing, China (NO.: KL2013014D), according to the Declaration of Helsinki (as revised in 2013).

Cell culture, transfection, and transduction

Our study used several PA cell lines, of which AtT-20 (ATCC Cat# CCL-89, RRID: CVCL_2300), HP75 (ATCC Cat# CRL-2506, RRID: CVCL_3516), GH3 (BCRJ Cat# 0090, RRID: CVCL_0273), GT1-1 (RRID: CVCL_6236) were purchased from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Normal human astrocyte (NHA, ATCC Cat# CRL-2541, RRID: CVCL_6379) cell line was purchased from ATCC (Manassas, VA, USA). All cells were cultured in DMEM (Thermo Fisher, Carlsbad, CA, USA) according to the manufacturer's recommendations. miRNA-146b-5p mimics (miR115129137648-1-5), NC mimics (miR321117137437-1-5), and miRNA-146b-5p inhibitor (miR2164753092366-1-5) were obtained from RiboBio (Guangzhou, China). miRNA-146b-5p inhibitor was dissolved in DMSO, cells treated with vehicle (Veh) without miRNA-146b-5p inhibitor served as the control. The transfection was conducted using Lipofectamine 3000[®] Transfection Reagent (Invitrogen, USA) according to the manufacture's instructions. EPHA7 lentiviral overexpression vectors (VB010000-9343tfj) and control lentiviral vectors (VB010000-9298rtf) were purchased from VectorBuilder (Guangzhou, China). All these vectors were packaged with lentivirus. Viral supernatants were used to transduce cells. Stable transductions were selected by growth in media containing Ampicillin.

Establishment of TMZ-resistant cell line

TMZ-resistant cell line (GH3/TMZ) was established by exposing GH3 cells to gradually increasing the dose of TMZ (0.1–2.0 µg/mL) for 5 months, and the cell viability was tested.

Cell counting Kit-8 (CCK-8) assay

Two groups of GH3 cells were digested with digestive enzymes and replaced in 96-well plates. An enzyme-linked immunosorbent detector (Dojindo Molecular Technologies, Kumamoto, Japan) was applied to estimate the absorbance at 450 nm to generate a growth curve.

Flow cytometric analysis of apoptosis

GH3 cells were harvested after 48 h, and apoptotic rates were detected according to the Annexin-V/FITC Kit (BD Biosciences, San Jose, CA, USA).

Transwell chamber assay

GH3 cells were trypsinized and resuspended in a serum-free medium. Approximately 40,000 cells were

added to the upper layer, and 500 µl of DMEM medium was supplied to the lower layer. After washing 3 times with PBS, it was stained with 0.1% methyl violet for 25 min. After washing, 5 fields under the microscope were randomly selected to count the stained cells.

Wound healing assay

The transfected GH3 cells or NHA cells were evenly spread on a 6-well plate. After the cells adhered to 90% confluence, the cells were uniformly scratched with a 200 µl pipette tip. After 24 h, they were observed again, and the migration areas were measured in 5 random fields.

Database analysis

We used the online target gene prediction databases TargetScan 7.2 (http://www.targetscan.org/vert_72/) and MiRanda (<http://www.microrna.org/microrna/home.do>) to jointly predict the miRNA-146b-5p target genes, and then the two results were intersected. Besides, target gene databases with experimental verification support, miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw>) and miRwalk (<http://129.206.7.150/>), were jointly used to detect possible targets of miRNA-146b-5p. The results were combined as the final miRNA-146b-5p target gene prediction result.

Dual-luciferase reporter gene assay

A luciferase reporter assay with the EPHA7 3'-UTR was used to verify that EPHA7 is the direct target of miRNA-146b-5p. The negative control vector or miRNA-146b-5p mimics were cotransfected with the constructed WT-EPHA7-3'-UTR (wild-type seed sequence: CUACCCU) or MT-EPHA7-3'-UTR (mutant seed sequence: CUGCCGU) psiCHECK2 reporter vector (Promega, Madison, WI, USA) into GH3 cells using Lipofectamine 2000.

RNA pull-down assay

The RNA sequences used for RNA pull-down assay were transcribed from their corresponding plasmids *in vitro*, and biotin-labeled using the Biotin RNA Labeling Mix (Roche, 11685597910). Total RNAs from GH3 cell extracts were mixed with biotin-labeled miRNA-146b-5p, incubated with Dynabeads M-280 Streptavidin (Invitrogen, 11205D) at 4°C for 3h. Finally, the expression of EPHA7 contained in the pulled-down complexes was tested by qRT-PCR. In this study, Biotin-labeled non-sense RNA with a similar length to miRNA-146b-5p (NS) and miRNA-146b-5p without biotin label (NC) served as the control.

Western blotting

Total protein was extracted separately from PA cells.

A separation gel and concentrated gel were prepared, and the proteins were separated by electrophoresis and then transferred to membranes. The membranes were blocked with 5% skim milk and incubated with antibodies against EPHA7 (1:1,000; Acris Antibodies GmbH Cat# AP06735PU-N, RRID: AB_1615652), IRAK4 (1:1000; GenWay Biotech Inc. Cat# GWB-B3A5CF, RRID: AB_10123603), TRAF6 (1:1000; LSBio (LifeSpan) Cat# LS-C92947-100, RRID: AB_1936834), NF- κ B (1:1000; Cell Signaling Technology Cat# 3034, RRID: AB_330561), phospho-NF- κ B (1:1000; Cell Signaling Technology Cat# 13346, RRID: AB_2798185), GAPDH (1:2,000; ABFrontier Cat# LF-MA0038, RRID: AB_1616728) and washed after incubation. Finally, the secondary antibody was incubated for 2 hours, and the gray band of the protein band was detected by chemiluminescence.

RNA extraction and real-time PCR analyses (qRT-PCR)

The primer sequences used for qRT-PCR were as follows: 5'-AATCGAGGCTGCCAAGCGCAC-3' (forward) and 5'-GCCGAGCTTTGACAGGCACAGA-3' (reverse) for miRNA-146b-5p; 5'-GGCTGCTCTCAACATGCGA-3' (forward) and 5'-CTGTGTCCG CACGTTCAAGA-3' (reverse) for EPHA7; and 5'-CTCGCTTCGGCAGCACA-3' (forward) and 5'-AACGCTTCACGAATTTGCGT-3' (reverse) for U6. EPHA7 data were normalized to GAPDH, which was detected using the following primers: 5'-GCACCACCAACTGCTTAGCA-3' (forward) and 5'-TCTTCTGGGTGGCAGTGATG-3' (reverse), while U6 was used as an endogenous control for miRNA-146b-5p. The amplification efficiencies of EPHA7, miRNA-146b-5p, GAPDH, and U6 were 97.6%, 95.4%, 101.7%, and 99.3%, respectively. The relative level was calculated by $2^{-\Delta CT}$ method.

Immunohistochemistry (IHC) staining of EPHA7

EPHA7 (1:300; Acris Antibodies GmbH Cat# AP06735PU-N, RRID: AB_1615652) was used as the primary antibody to incubate the slides at 4°C overnight and then incubated with a secondary antibody. Immunohistochemical staining was observed by two pathologists who did not know the patient's clinical data.

Cell viability assay

Culture medium was replaced with media containing different concentrations of TMZ (0, 0.5, 0.75, 1, and 2 μ g/ml) for 48h. Cell viability was assessed using a CCK-8 assay. The equation of half maximal inhibitory concentration (IC50) of TMZ was [viability% = 100/(1 + 10^{[TMZ concentration]log IC50})].

5-ethynyl-20-deoxyuridine assay

GH3 and NHA cells were incubated with 5-ethynyl-

20-deoxyuridine (EdU, Shanghai Huicheng Biological Technology Co., Ltd., Shanghai, China) for 5 hours. After three washes with phosphate buffer saline, cells were treated with a 1xApollo[®] reaction cocktail (100 μ l/well) for 30 min. After that, each well was stained with Hoechst 33342 (5 μ g/ml, 100 μ l/well) for 30 min.

Transmission electron microscopy

Cells were fixed with 2.5% glutaraldehyde in phosphate buffer. The specimens were post-fixed in 1% Osmium Tetroxide with 0.1% potassium ferricyanide, dehydrated through a graded series of ethanol (30-90%). Images were taken on a HITACHI HT7700 transmission electron microscopy system (HITACHI, Japan) at 80 kV.

Statistical analysis

Data expressed as the mean $\pm$ SEM were analyzed by SPSS 22.0 software (IBM SPSS Statistics, RRID: SCR_019096). Experiments were performed at least 3 times. We used the chi-square test to explore the correlation between the two variables. For continuous variable analysis, independent t-tests and variance A-tests were used. The overall survival and disease-free survival rates of the patients were measured by the Kaplan-Meier method, and the survival rate between the groups was compared by a log-rank test. Correlation curves were constructed, and differences between groups were calculated using Spearman rank correlation analysis. P values less than 0.05 were considered to indicate a statistically significant difference.

Results

MiRNA-146b-5p is down-regulated in APA tissues and cells.

In this study, we first measured the expression of miRNA-146b-5p in 60 pairs of APA tissues and matching adjacent non-tumor tissues by real-time PCR. Notable down-regulation of miRNA-146b-5p was observed in APA tissues compared with the matched adjacent non-tumor tissues (Fig. 1A). To explore whether miRNA-146b-5p is dysregulated in PA cells, the expression of miRNA-146b-5p in PA cell lines (AtT-20, HP75, GH3, GT1-1) and normal NHA cells was quantified. We confirmed that the expression of miRNA-146b-5p was also reduced in PA cell lines compared with NHA cells (Fig. 1B). Therefore, we demonstrated the frequent down-regulation of miRNA-146b-5p in APA.

Down-regulation of miRNA-146b-5p is related to poor prognosis.

To reveal the clinic-pathological characteristics of miRNA-146b-5p in APA, we divided APA patients into two groups according to the median expression of miRNA-146b-5p: the miRNA-146b-5p low expression

MiRNA-146b-5p/EPHA7 regulates aggressive pituitary adenoma

group (n=30) and the miRNA-146b-5p high expression group (n=30). The results suggested that low expression of miRNA-146b-5p was notably related to APA invasive

pathological features, including larger tumor size, poorer Knosp grade, poorer Hardy grade, and TMZ resistance (Table 1). Because poorer cellular differentiation and

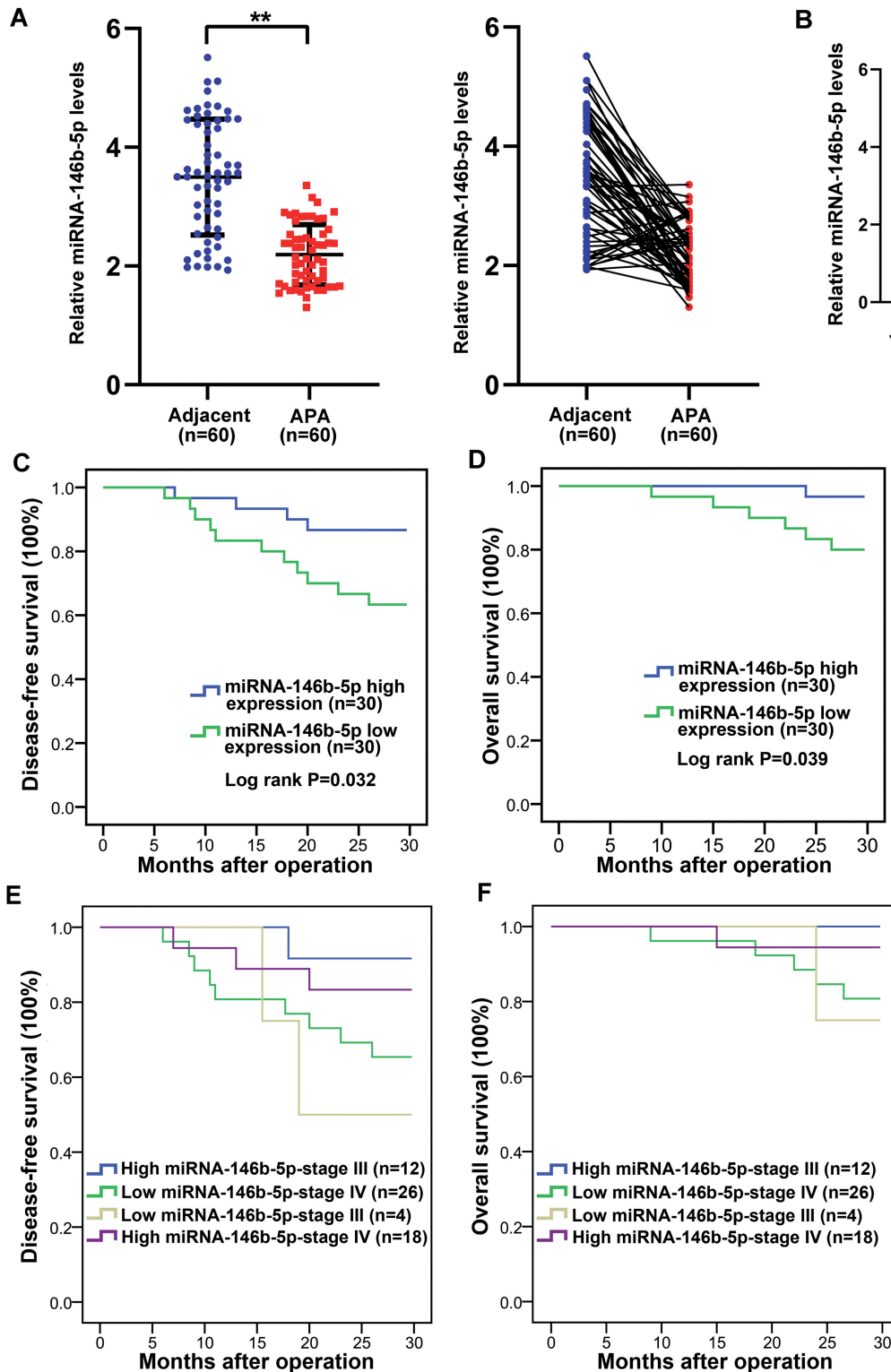


Fig. 1. MiRNA-146b-5p was down-regulated in APA and was related to poor prognosis. **A.** Expressions of miRNA-146b-5p were measured by real-time PCR analyses in APA tissues and their matching adjacent non-tumor tissues. The test was repeated four times for each sample. **: compared with adjacent non-tumor tissues group, $P < 0.001$. **B.** The expression of miRNA-146b-5p in PA cell lines (AtT-20, HP75, GH3, and GT1-1) and a normal cell line (NHA). The test was repeated four times for each sample. Data are presented as the mean $\pm$ standard deviation. *: compared with NHA, $P < 0.05$. **: compared with NHA, $P < 0.001$. **C, D.** The disease-free survival rate and overall survival rate were calculated by the Kaplan-Meier method. **E, F.** When considering the tumor staging of APA patients, the disease-free survival rate and overall survival rate were calculated by the Kaplan-Meier method.

MiRNA-146b-5p/EPHA7 regulates aggressive pituitary adenoma

advanced tumor stage are related to APA metastasis, the down-regulation of miRNA-146b-5p may participate in the metastatic growth of APA. Besides, to reveal the relationship between miRNA-146b-5p down-regulation and patient prognosis, we also investigated the correlation between low miRNA-146b-5p expression and patient survival. After 30 months of follow-up, we found that the 30-month disease-free survival rates (Log-rank $P=0.032$, Fig. 1C) and 30-month overall survival rates (Log-rank $P=0.039$, Fig. 1D) of patients in the low miRNA-146b-5p expression group were notably poorer than those in the high expression group. When considering the tumor staging of APA patients, we found that compared with patients with high miRNA-146b-5p expression, 30-month disease-free survival rates and 30-month survival rates of patients with stage III (Log-rank

$P=0.024$, Fig. 1E; Log-rank $P=0.033$, Fig. 1F) or IV (Log-rank $P=0.038$, Fig. 1E; Log-rank $P=0.045$, Fig. 1F) in the low miRNA-146b-5p expression groups were significantly reduced. All the above data revealed that the loss of miRNA-146b-5p expression may promote the metastasis of APA and is correlated with the poor prognosis of APA.

MiRNA-146b-5p negatively regulates PA cell proliferation, invasion, migration, and induces apoptosis.

Since we observed that the down-regulation of miRNA-146b-5p was closely correlated with the metastasis and poor prognosis of APA, and that tumor cell proliferation, apoptosis, and invasion play important roles in tumor growth and metastasis, we speculated that

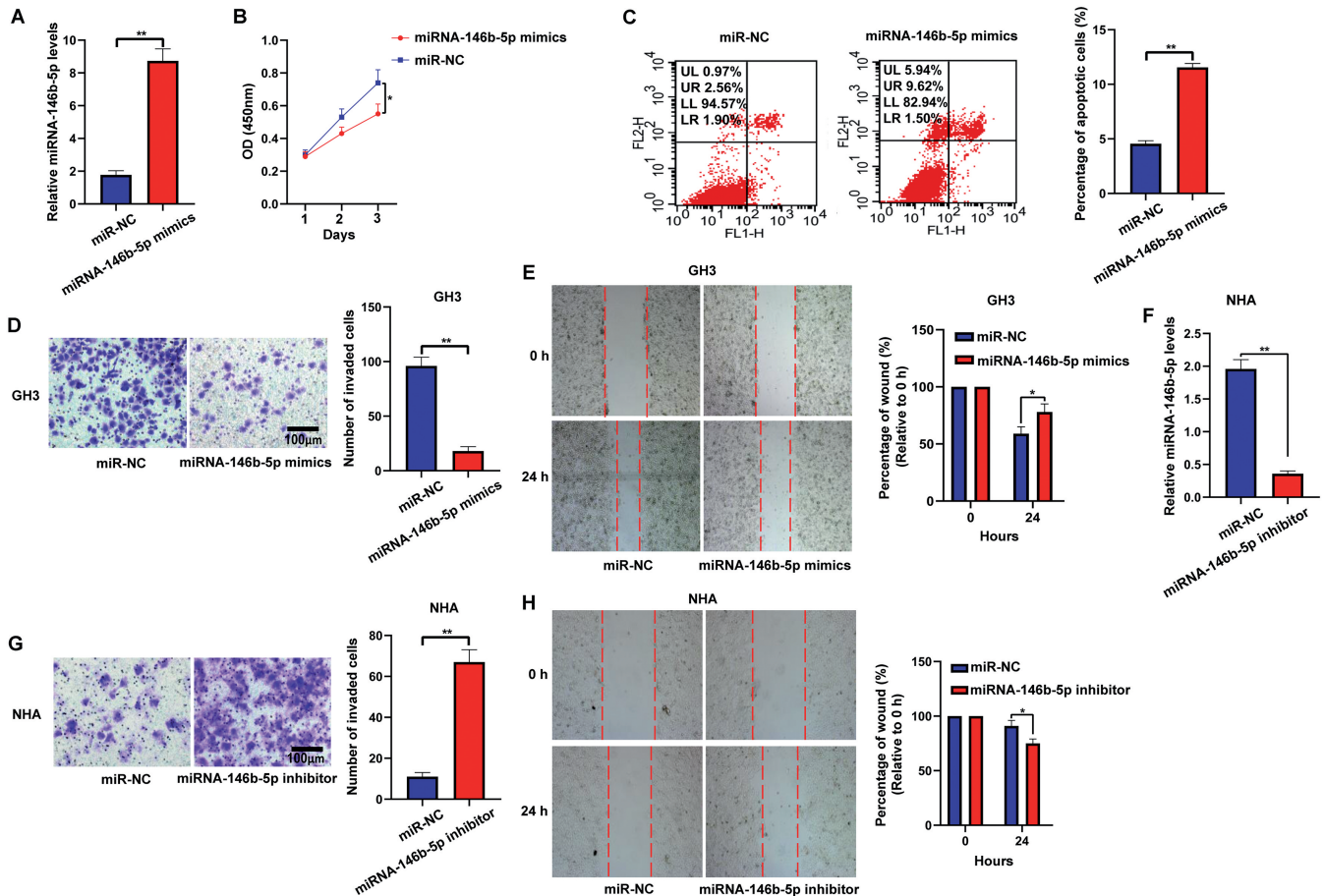


Fig. 2. MiRNA-146b-5p inhibited proliferation, migration, invasion, and induced apoptosis in PA cell line. **A.** MiRNA-146b-5p mimics and negative control were efficiently transfected into GH3 cells. The test was repeated four times for each sample. **B.** Cell proliferation rates were measured in GH3 cells of two groups through CCK-8 assay after 24 h, 48 h, and 72 h. The experiment was performed three times. **C.** Flow cytometric analysis was used to identify apoptotic rates of GH3 cells. The experiment was performed three times. **D.** Invasive abilities of GH3 cells after transfection were identified by the Transwell assay. The experiment was performed three times. **E.** Migration rates of GH3 cells were determined by the wound healing assay. The experiment was performed three times. **F.** The expression of miRNA-146b-5p was measured in NHA cells transfected with miRNA-146b-5p inhibitor and negative control. The test was repeated four times for each sample. **G.** Invasive abilities of NHA cells after transfection were confirmed by the Transwell assay. The experiment was performed three times. **H.** Migration rates of NHA cells were confirmed by the wound healing assay. The experiment was performed three times. Data are presented as the mean $\pm$ standard deviation. *: compared with miR-NC, $P<0.05$. **: compared with miR-NC, $P<0.001$. D, G, $\times 200$.

the restoration of miRNA-146b-5p may inhibit PA cell proliferation and invasion. Because of the most marked down-regulation of miRNA-146b-5p in GH3 cells (Fig. 1B), we first established GH3 cells that could stably express miRNA-146b-5p. Compared with the negative control group, miRNA-146b-5p expression was confirmed to notably increase in the miRNA-146b-5p mimic transfection group (Fig. 2A). Through the CCK-8 assay, we observed that miRNA-146b-5p re-expression notably inhibited GH3 cell proliferation *in vitro* (Fig. 2B). We then used flow cytometry to reveal the effect of miRNA-146b-5p re-expression on apoptotic rates. The data indicated that the apoptotic rate of GH3 cells also increased markedly with increasing miRNA-146b-5p expression, which demonstrated that miRNA-146b-5p induced cell apoptosis in APA (Fig. 2C). In the transwell cell invasion assay, overexpression of miRNA-146b-5p reduced the cells which invaded the lower chamber (Fig. 2D). Similarly, in the miRNA-146b-5p high expression

group, the ability of cells to migrate was notably impeded compared with that in the low expression group (Fig. 2E). To further detect the effects of miRNA-146b-5p on cell migration and invasive ability, NHA cells expressing relatively high endogenous miRNA-146b-5p were transfected with miRNA-146b-5p inhibitors. The down-regulation of miRNA-146b-5p was verified by qRT-PCR (Fig. 2F). Interestingly, inhibition of miRNA-146b-5p markedly promoted cell invasion (Fig. 2G) and migration in NHA cells (Fig. 2H). Our earlier-described results demonstrated that miRNA-146b-5p suppressed APA metastasis by negatively regulating cell proliferation, migration, invasion, and inducing cell apoptosis.

EPHA7 is a direct target of miRNA-146b-5p in PA cells

Previous research results revealed that miRNA-146b-5p functions as a tumor suppressor gene in APA. Accordingly, we further sought to identify the

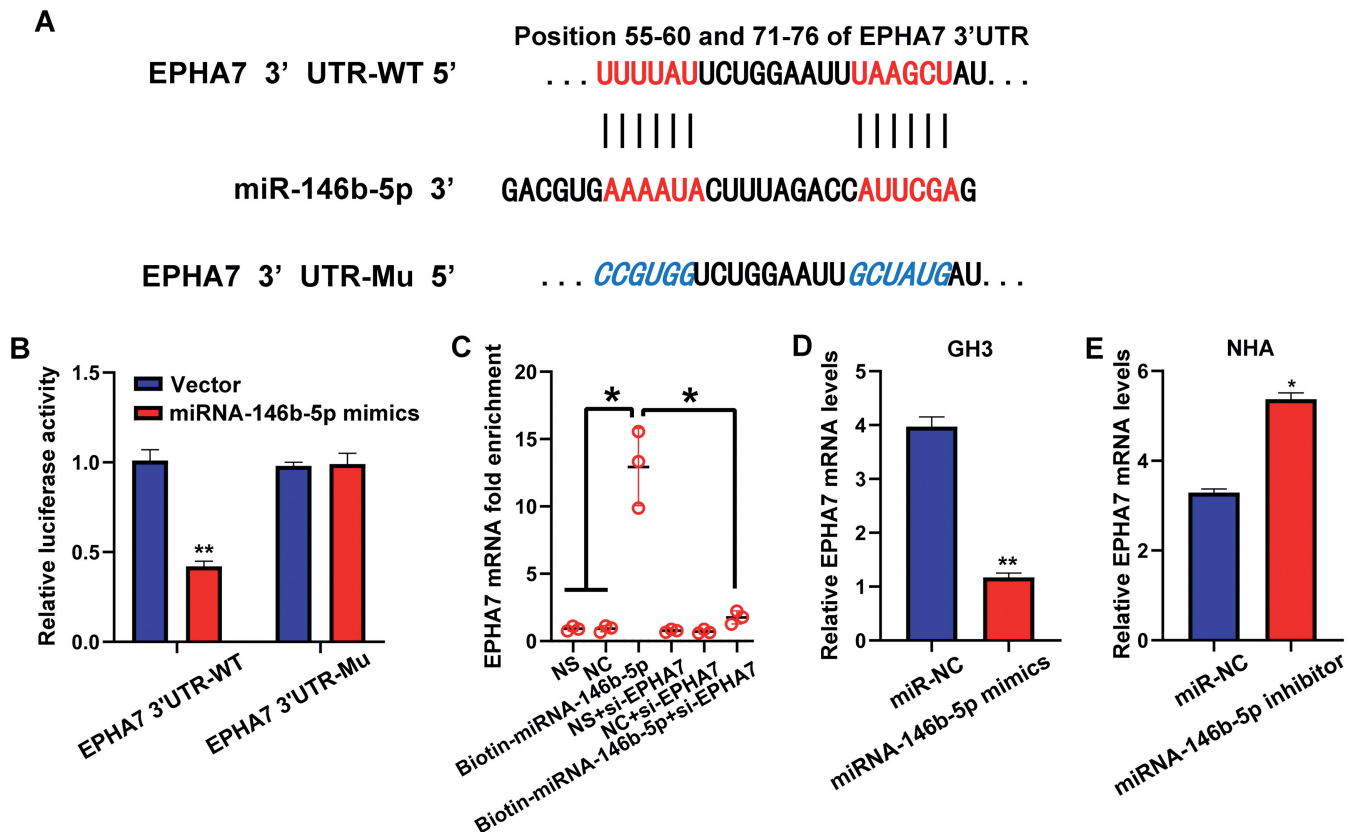


Fig. 3. EPHA7 was proven to be a novel target of miRNA-146b-5p in GH3 cells. **A.** EPHA7 has a predicted consequential pairing with miRNA-146b-5p at bases 55-60 and 71-76. psiCHECK2 dual-luciferase reporter vectors containing WT or MT EPHA7 3'-UTR were constructed. **B.** Renilla luciferase activity was standardized to firefly luciferase activity in GH3 cells with WT-EPHA7 or MT-EPHA7. The experiment was performed three times. **C.** RNA pull-down assay was used, and we found that EPHA7 could specifically interact with miRNA-146b-5p. The experiment was performed three times. NS was the biotin-labeled non-sense RNA with a similar length to miRNA-146b-5p, NC was the miRNA-146b-5p without biotin label. One-way ANOVA was used. *: compared with biotin-miRNA-146b-5p group, $P < 0.05$. **D.** After transfection with miRNA-146b-5p mimics, the EPHA7 mRNA level of GH3 cells was decreased significantly. The test was repeated four times for each sample. **E.** qRT-PCR was performed to identify the expression level of EPHA7 mRNA in NHA cells. The test was repeated four times for each sample. *: compared with miR-NC, $P < 0.05$. **: compared with miR-NC, $P < 0.001$. Data are presented as the mean $\pm$ standard deviation.

underlying targets of miRNA-146b-5p that are related to tumor metastasis. We explored miRNA-146b-5p downstream gene targets through four different miRNA targeting prediction tools: TargetScan, MiRanda, miRTarBase, and miRwalk. A potential candidate target gene, EPHA7, was then selected, which has a predicted consequential pairing with miRNA-146b-5p at bases 55-60 and 71-76 (Fig. 3A). To further explore whether the anti-metastatic properties of miRNA-146b-5p in APA were related to EPHA7, we first performed a dual-luciferase reporter gene assay in GH3 cells. The luciferase activity of GH3 cells with wild-type EPHA7 after transfection with miRNA-146b-5p mimics was markedly reduced compared with that of the mimic NC-

transfected cells. However, in GH3 cells with mutated EPHA7, the decrease in luciferase activity was significantly reversed (Fig. 3B). To verify the interaction between miRNA-146b-5p and EPHA7, we performed an RNA pull-down assay. As shown in Fig. 3C, the EPHA7 was able to specifically interact with miRNA-146b-5p. We further verified whether miRNA-146b-5p could regulate EPHA7 expression at the cellular level. After transfection with miRNA-146b-5p mimics, the EPHA7 level of GH3 cells was decreased by approximately 75% (Fig. 3D). The inhibition of miRNA-146b-5p in normal NHA cells markedly increased EPHA7 expression (Fig. 3E). Accordingly, EPHA7 was proven to be a potential target of miRNA-146b-5p in PA cells.

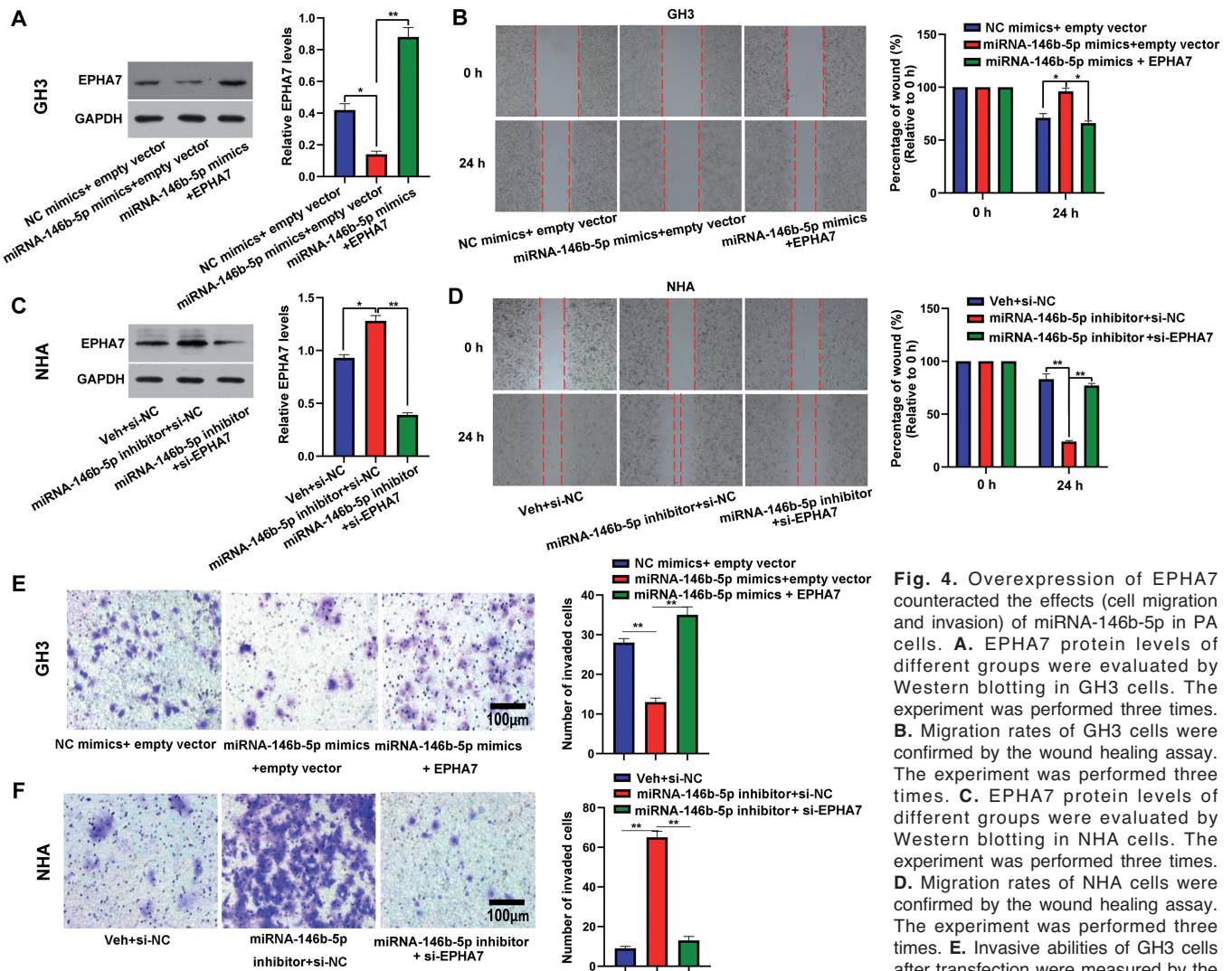


Fig. 4. Overexpression of EPHA7 counteracted the effects (cell migration and invasion) of miRNA-146b-5p in PA cells. **A.** EPHA7 protein levels of different groups were evaluated by Western blotting in GH3 cells. The experiment was performed three times. **B.** Migration rates of GH3 cells were confirmed by the wound healing assay. The experiment was performed three times. **C.** EPHA7 protein levels of different groups were evaluated by Western blotting in NHA cells. The experiment was performed three times. **D.** Migration rates of NHA cells were confirmed by the wound healing assay. The experiment was performed three times. **E.** Invasive abilities of GH3 cells after transfection were measured by the Transwell assay. The experiment was performed three times. **F.** Invasive abilities of NHA cells after transfection were measured by the Transwell assay. The experiment was performed three times. *: compared with miRNA-146b-5p mimics+empty vector group/miRNA-146b-5p inhibitor+si-NC group, $P < 0.05$. **: compared with miRNA-146b-5p mimics+empty vector group/miRNA-146b-5p inhibitor+si-NC group, $P < 0.001$. Data are presented as the mean $\pm$ standard deviation. E, F, $\times 200$.

MiRNA-146b-5p/EPHA7 regulates aggressive pituitary adenoma

Overexpression of EPHA7 counteracts the effects of miRNA-146b-5p in PA cells.

The above findings indicated that miRNA-146b-5p inhibits metastasis by negatively regulating PA cell migration and invasion and that EPHA7 is directly targeted by miRNA-146b-5p. Therefore, we suspected that EPHA7 may participate in miRNA-146b-5p-mediated anti-metastatic properties. To verify that

miRNA-146b-5p directly targets EPHA7 to perform its function, miRNA-146b-5p-transfected GH3 cells were infected with EPHA7 lentiviral activation particles or control lentiviral activation particles. EPHA7 protein levels were significantly increased by transfection of EPHA7 lentiviral activation particles (Fig. 4A), and mRNA levels exhibited the same trend with qRT-PCR detection (Fig. 4A). In wound healing assays, the recovery of EPHA7 expression partially promoted the

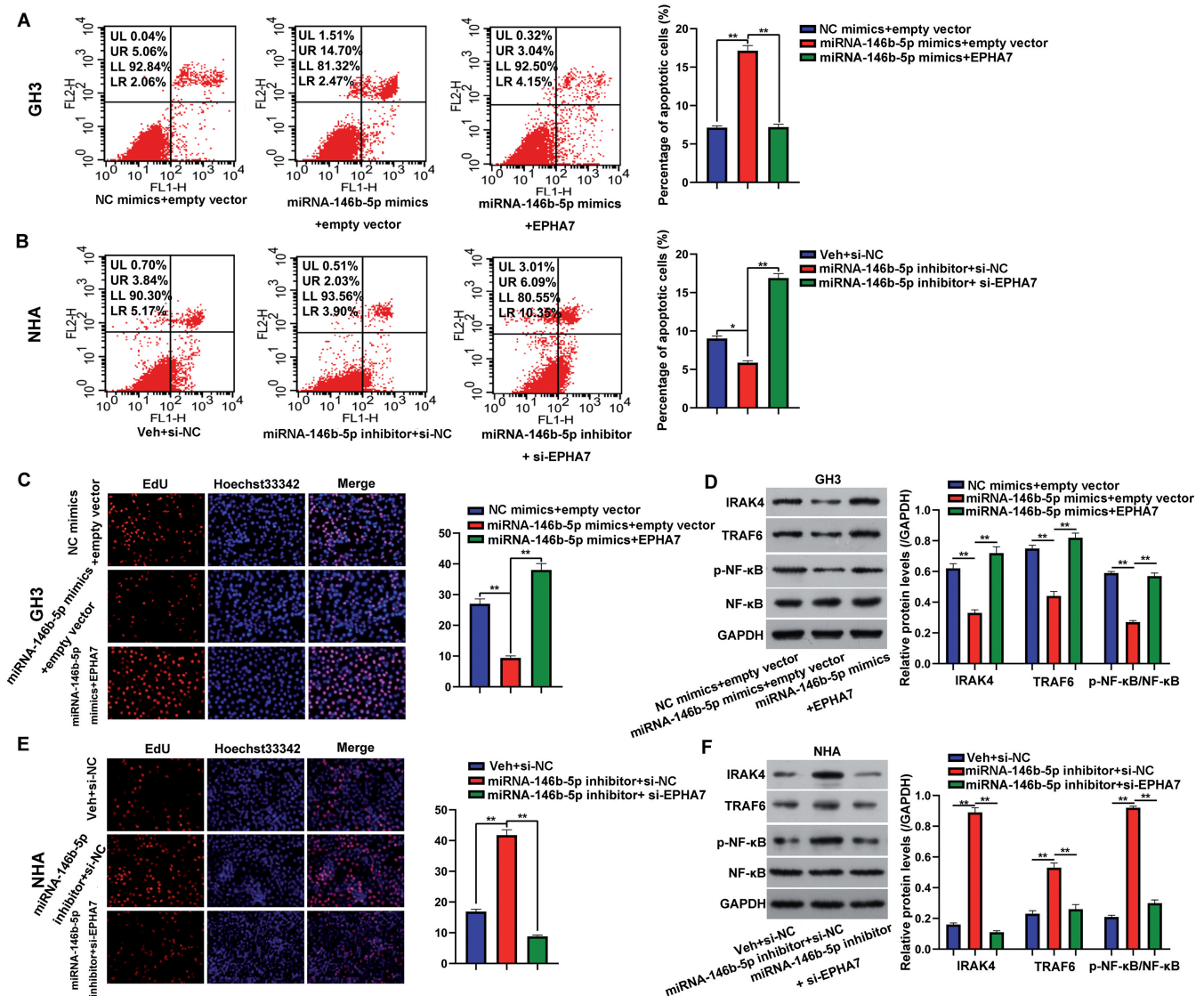


Fig. 5. Overexpression of EPHA7 counteracted the effects (cell growth) of miRNA-146b-5p in PA cells. **A.** Flow cytometric analysis was used to identify apoptotic rates of GH3 cells. The experiment was performed three times. **B.** Flow cytometric analysis was used to identify apoptotic rates of NHA cells. The experiment was performed three times. **C.** MiRNA-146b-5p overexpression prominently inhibited the number of EdU-positive cells in GH3 cells. The experiment was performed three times. **D.** Western blotting analysis of the protein expression of IRAK4, TRAF6, NF- κ B, and p-NF- κ B in GH3 cells. The experiment was performed three times. **E.** MiRNA-146b-5p knockdown prominently enhanced the number of EdU-positive cells in NHA cells. The experiment was performed three times. **F.** Western blotting analysis of the protein expression of IRAK4, TRAF6, NF- κ B, and p-NF- κ B in NHA cells. The experiment was performed three times. *: compared with miRNA-146b-5p mimics+empty vector group/miRNA-146b-5p inhibitor+si-NC group, $P < 0.05$. **: compared with miRNA-146b-5p mimics+empty vector group/miRNA-146b-5p inhibitor+si-NC group, $P < 0.001$. Data are presented as the mean $\pm$ standard deviation.

migration of GH3 cells overexpressing miRNA-146b-5p (Fig. 4B). In transwell assays, increased expression of EPHA7 enhanced the invasion ability of miRNA-146b-5p-overexpressing GH3 cells, which was suppressed by miRNA-146b-5p (Fig. 4E). Accordingly, the findings showed that miRNA-146b-5p inhibited migration and invasion by targeting EPHA7, and the underlying mechanism was further studied. The inhibition of miRNA-146b-5p in normal NHA cells markedly increased the expression of EPHA7 (Fig. 4C) and enhanced the migration ability (Fig. 4D) and invasion ability (Fig. 4F) of NHA cells. However, these effects were reversed after inhibiting the expression of EPHA7 in NHA cells (Fig. 4C,D,F). The above results suggest that miRNA-146b-5p can interfere with the invasion activity of PA cells through EPHA7.

Then we further studied the effects of miRNA-146b-

5p/EPHA7 on PA cell growth *in vitro*. We found that miRNA-146b-5p overexpression/knockdown prominently enhanced/inhibited cell apoptosis (Fig. 5A,B) and inhibited/enhanced the number of EdU-positive cells (Fig. 5C,E) in GH3 and NHA cells, respectively.

The IRAK4/TRAF6/NF- κ B signaling pathway, which has been reported to be an important pathway for regulating the differentiation, survival, and movement of normal and cancer cells, was closely related to the physiological role of EPHA7 (Dries et al. 2011). Therefore, we further investigated the influences of miRNA-146b-5p and EPHA7 on the expression changes of IRAK4, TRAF6, NF- κ B, and p-NF- κ B. Western blotting analysis revealed that overexpression of miRNA-146b-5p suppressed IRAK4 and TRAF6 protein expression and negatively regulated NF- κ B

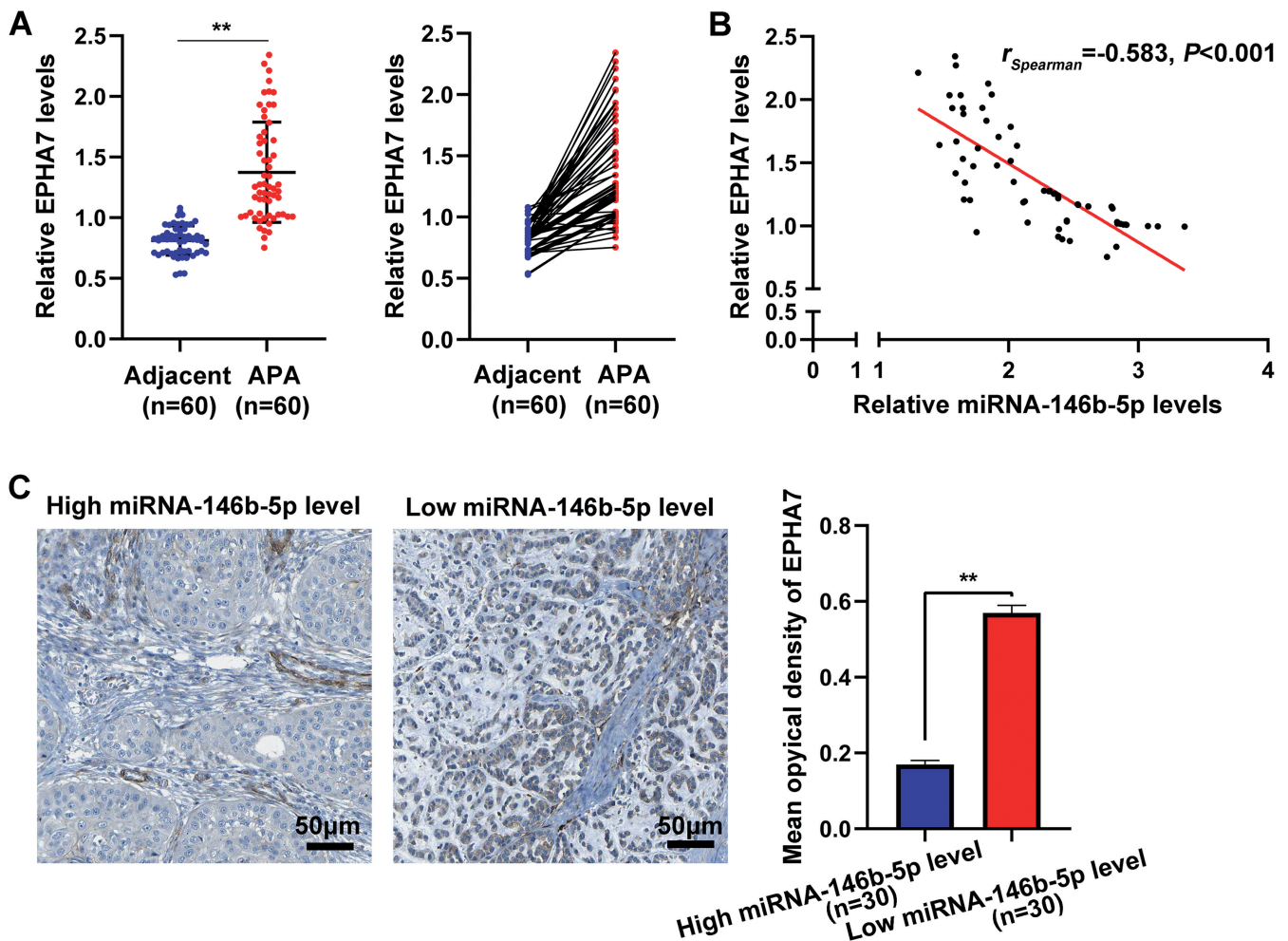


Fig. 6. EPHA7 was significantly upregulated in APA tissues and negatively correlated with miRNA-146b-5p in APA. **A.** Real-time PCR was performed to identify the mRNA levels of EPHA7 in APA tissues and their matching adjacent non-tumor tissues. The test was repeated four times for each sample. **: compared with adjacent non-tumor tissues group, $P < 0.001$. **B.** In APA tissues, the levels of EPHA7 mRNA were negatively correlated with miRNA-146b-5p. **C.** Representative pictures of EPHA7 immunohistochemistry in APA tissues with different miRNA-146b-5p levels. The test was repeated four times for each sample. **: compared with the high miRNA-146b-5p level group, $P < 0.001$.

MiRNA-146b-5p/EPHA7 regulates aggressive pituitary adenoma

phosphorylation. The restoration of EPHA7 expression in GH3 cells notably reversed the inhibitory effects of miRNA-146b-5p (Fig. 5D). All these results suggested that miRNA-146b-5p inhibited invasion and metastasis by directly targeting EPHA7, possibly through the IRAK4/TRAF6/NF- κ B signaling pathway. The inhibition of miRNA-146b-5p in normal NHA cells markedly increased IRAK4 and TRAF6 protein expression and NF- κ B phosphorylation levels (Fig. 5F).

EPHA7 is significantly overexpressed in APA and negatively correlated with the expression of miRNA-146b-5p.

Due to the metastatic effect of EPHA7 in many

tumors (Manso et al. 2016; Di et al. 2019; Liu et al. 2020), we first determined the expression of EPHA7 in APA tissues. APA tissues showed significantly higher expression of EPHA7 than adjacent non-tumor tissue samples (Fig. 6A). Interestingly, we also found a negative correlation between miRNA-146b-5p and EPHA7 expression ($r=-0.583$, $P<0.001$, Fig. 6B). We further quantized the expression levels of EPHA7 in APA tissues with different miRNA-146b-5p levels by immunohistochemistry (Fig. 6C). Tumors with lower levels of miRNA-146b-5p ($n=30$) tended to overexpress EPHA7, and conversely, tumors that overexpressed miRNA-146b-5p ($n=30$) showed a lower level of EPHA7. Collectively, these data suggested that dysregulation of miRNA-146b-5p was related to the

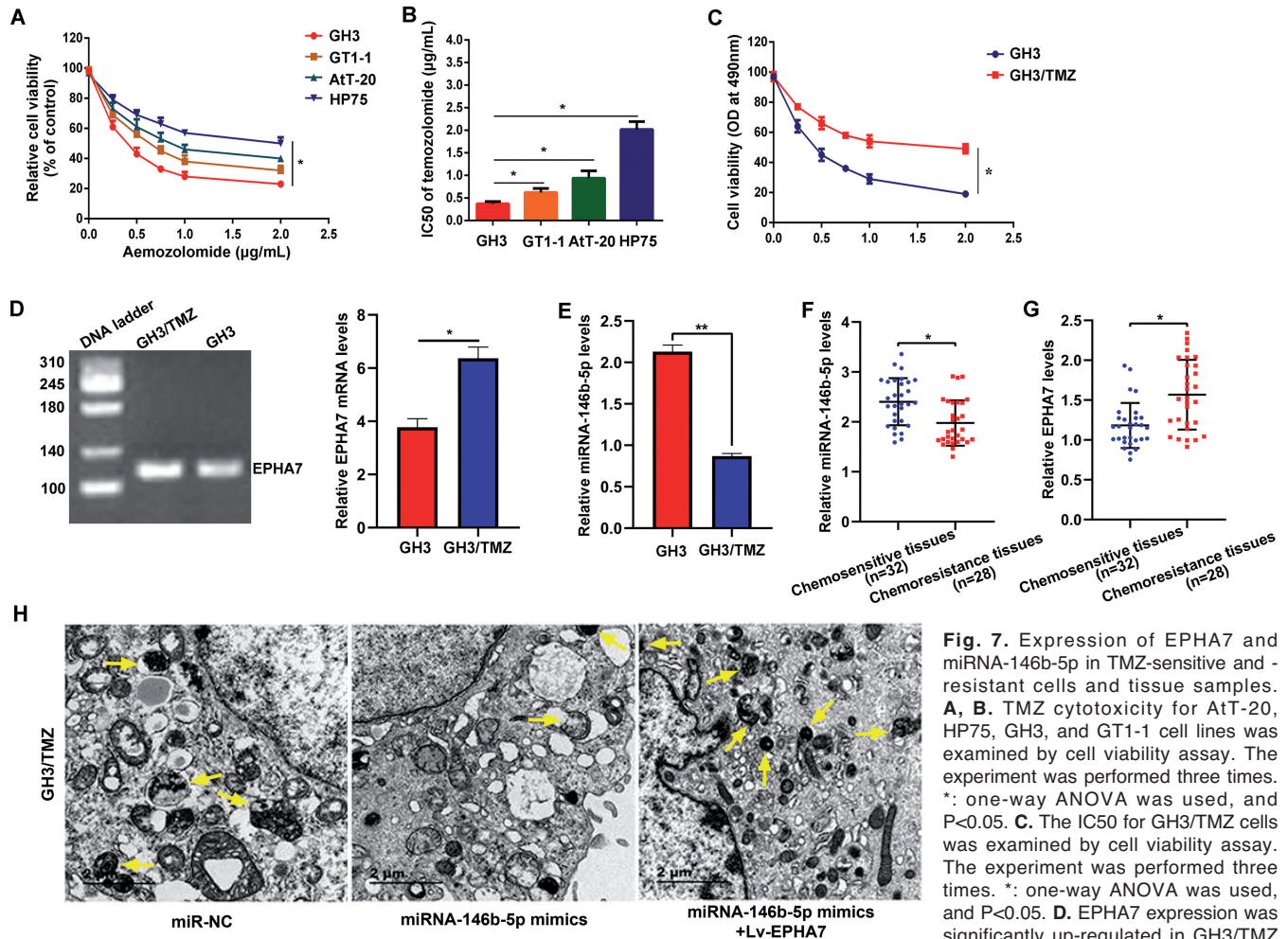


Fig. 7. Expression of EPHA7 and miRNA-146b-5p in TMZ-sensitive and -resistant cells and tissue samples. **A, B.** TMZ cytotoxicity for AtT-20, HP75, GH3, and GT1-1 cell lines was examined by cell viability assay. The experiment was performed three times. *: one-way ANOVA was used, and $P<0.05$. **C.** The IC50 for GH3/TMZ cells was examined by cell viability assay. The experiment was performed three times. *: one-way ANOVA was used, and $P<0.05$. **D.** EPHA7 expression was significantly up-regulated in GH3/TMZ

cells, compared to GH3 cells. The experiment was performed three times. **E.** MiRNA-146b-5p expression was significantly down-regulated in GH3/TMZ cells, compared to GH3 cells. The test was repeated four times for each sample. **: compared with GH3 cells, $P<0.001$. **F.** MiRNA-146b-5p level was down-regulated in chemoresistance tissues more than in chemosensitive tissues. The test was repeated four times for each sample. *: compared with chemosensitive tissues, $P<0.05$. **G.** EPHA7 was up-regulated in chemoresistance tissues more than in chemosensitive tissues. The test was repeated four times for each sample. *: compared with chemosensitive tissues, $P<0.05$. **H.** Morphology observation by transmission electron microscopy demonstrated that the autophagic activity was decreased markedly with increasing miRNA-146b-5p expression. After Lv-EPHA7 treatment, the autophagic activity was enhanced in GH3/TMZ cells. The experiment was performed three times.

overexpression of EPHA7 in APA.

Expression of EPHA7 and miRNA-146b-5p in TMZ-sensitive and -resistant cells and tissue samples

TMZ cytotoxicity for AtT-20, HP75, GH3, and GT1-1 cell lines was examined. GH3 cells possessed the most sensitivity to TMZ accompanied by the lowest IC50 (Fig. 7A,B). Furthermore, we characterized TMZ resistance in GH3/TMZ cells. After exposure to a gradually increasing dose of TMZ (0.1–2.0 µg/mL) for 2 days, the IC50 value was 1.93 ± 0.27 µg/mL for GH3/TMZ cells, showing a 4.7-fold increase as compared to the parental cell line (GH3 cells: 0.41 ± 0.11 µg/mL, Fig. 7C). Furthermore, we found that miRNA-146b-5p expression was significantly down-regulated and EPHA7 gene expression was significantly up-regulated in GH3/TMZ cells, compared to the parental cell line (Fig. 7D,E). Similarly, EPHA7 was up-regulated, while the miRNA-146b-5p level was down-regulated in chemoresistance tissues more than in chemosensitive tissues (Fig. 7F,G). Therefore, we speculate that the abnormal expressions of miRNA-146b-5p and EPHA7 may be involved in the development of TMZ resistance in APA. *In vitro*, morphology observation by transmission electron microscopy demonstrated that the autophagic activity was decreased markedly with increasing miRNA-146b-5p expression (Fig. 7H), while after Lv-EPHA7 treatment, the autophagic activity was enhanced in GH3/TMZ cells (Fig. 7H).

Discussion

PA is the third most common tumor in intracranial tumors, most of which are benign monoclonal tumors and require surgical treatment (Carrillo-Najar et al., 2021). Because of its invasion and surrounding important neurovascular and dural structures, the postoperative recurrence rate is as high as 50% (Ceccato et al., 2018). At present, the ideal treatment goal for pituitary tumors is not only to achieve an imaging cure, but also to achieve an endocrinology cure or biological cure (Lizzul et al., 2020). Therefore, in addition to surgical resection, it is necessary to actively study the pathogenesis of APA from the molecular level, which is of great significance to biological and gene therapy. There have been reports on changes in cell cycle-related proteins, loss of balance between cell proliferation and apoptosis, mutations of oncogenes and tumor suppressor genes (Cheng et al., 2020; García-Guzmán et al., 2020). Besides, TMZ is the cornerstone drug for the treatment of APA patients. TMZ mainly inhibits the synthesis of nucleic acid by intercalating DNA, which has strong cytotoxicity and can kill tumor cells of various growth cycles (Lizzul et al., 2020). But so far, there are still no definite aggressive molecular markers to predict and evaluate the aggressiveness and chemotherapeutic drug resistance behavior of pituitary tumors.

MiRNA-146b-5p plays a role in the diagnosis, prognosis, anti-metastasis, and treatment of various cancers via targeting different genes in various complex ways (Li et al., 2019; Al-Abdallah et al., 2020). MiRNA-146b-5p is negatively correlated to lymphatic metastasis and distant metastasis rates of papillary thyroid carcinoma, and it can attenuate proliferative and migratory potentials of papillary thyroid carcinoma cells by targeting the natural killer group 2 member D (NKG2D) (Al-Abdallah et al., 2020). However, whether miRNA-146b-5p is involved in the resistance of APA to TMZ has not been reported.

More and more reports indicate that EPHA7 combined with NF- κ B promotes tumor development (Włodarczyk et al., 2006). NF- κ B is one of the important signaling molecules downstream of EPHA7 (Dries et al., 2011). Notably, we confirmed that EPHA7 was not only increased in many PA cell lines but also significantly higher in APA tissues than in adjacent non-tumor tissue samples. EPHA7 was then predicted to be a downstream gene target of miRNA-146b-5p by four different miRNA targeting prediction tools, and the targeting relationship was confirmed through the dual-luciferase reporter gene assay and RNA pull-down assay. Interestingly, the present data revealed that miRNA-146b-5p was negatively correlated with EPHA7 in APA tissues. Tumors with lower levels of miRNA-146b-5p tended to overexpress EPHA7, and conversely, tumors that overexpressed miRNA-146b-5p showed a lower level of EPHA7. Furthermore, restoration of EPHA7 rescued miRNA-146b-5p-inhibited tumor migration and invasion. All the findings showed that miRNA-146b-5p inhibits tumor metastasis by targeting EPHA7 *in vitro*.

Previous studies have shown that EPHA7 is required for IRAK4/TRAF6/NF- κ B signaling, which plays a pivotal role in the phenotypic changes, invasion, and metastasis of malignant tumor cells by promoting metastasis and local adhesion dynamics (Włodarczyk et al., 2006). Overexpression of EPHA7 is involved in NF- κ B phosphorylation activation, inducing cell proliferation, anti-apoptosis, and cell metastasis (Dries et al., 2011). With increased p-NF- κ B expression levels, cell migration and invasion capabilities are enhanced (Dries et al., 2011). Therefore, we further detected the influences of miRNA-146b-5p and EPHA7 on the expression changes of IRAK4, TRAF6, NF- κ B, and p-NF- κ B. We confirmed that upregulation of miRNA-146b-5p inhibited IRAK4 and TRAF6 protein expression and negatively regulated the phosphorylation of NF- κ B. The restoration of EPHA7 in GH3 cells notably reversed the inhibitory effects of miRNA-146b-5p. Therefore, we postulated that miRNA-146b-5p inhibits invasion and metastasis in APA by directly targeting EPHA7, possibly through IRAK4/TRAF6 signaling pathways. Further research is needed to adequately illuminate miRNA-146b-5p/EPHA7-related signaling pathway and their functions in APA.

Furthermore, we identified which pathway was involved in the regulation of autophagy by miRNA-

MiRNA-146b-5p/EPHA7 regulates aggressive pituitary adenoma

146b-5p. We found that miRNA-146b-5p treatment inhibited the autophagic activity in GH3/TMZ cells. After Lv-EPHA7 treatment, the autophagic activity was enhanced in GH3/TMZ cells. Thus, we speculated that miRNA-146b-5p may regulate autophagy at least partly through the miRNA-146b-5p/EPHA7 axis. Our findings suggested that miRNA-146b-5p enhanced the efficacy of TMZ in APA *in vitro*. However, our research still has the following main shortcoming, that is, we did not conduct further analysis in mice. Therefore, we still need to further verify our results *in vivo*. In summary, we confirmed the down-regulation of miRNA-146b-5p in APA, which is related to poor prognosis. MiRNA-146b-5p can inhibit EPHA7 expression, suppress the IRAK4/TRAF6/NF- κ B signaling pathway, and weaken PA cell invasion, metastasis, proliferation, and TMZ-induced chemoresistance *in vitro*.

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Conflict of interest statement. none declared.

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