Downregulation of glucose-6-phosphate dehydrogenase by microRNA-1 inhibits the growth of pituitary tumor cells

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Abstract. Pituitary tumors are generally intracranial neoplasms with high incidence and mortality rates. The investigation of novel factors involved in the tumorigenesis of pituitary tumors and the characterization of the underlying molecular mechanisms is urgently required for the diagnosis and treatment of pituitary tumors. Accumulating evidence has indicated that microRNAs (miRs) serve important roles in the initiation and progression of cancer. The present study found that miR-1 was significantly downregulated in pituitary tumor tissues upon reverse transcription-quantitative polymerase chain reaction analysis. Decreased expression of miR-1 was associated with the progression and worse prognosis of patients with pituitary tumors. The MTT assay showed that overexpression of miR-1 significantly suppressed proliferation. Highly expressed miR-1 promoted the apoptosis of pituitary tumor cells upon fluorescence-activated cell sorting analysis. Further molecular study revealed that glucose-6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme of the pentose phosphate pathway (PPP), was one of the targets of miR-1. Western blot assays showed that overexpression of miR-1 significantly decreased the protein level of G6PD in pituitary tumor cells without changing the mRNA level of G6PD. Consequently, oxidative PPP flux analysis revealed that suppression of G6PD by miR-1 decreased the production of nicotinamide adenine dinucleotide phosphate and the glycolysis of pituitary cancer cells. Restoration of the expression of G6PD significantly reversed the inhibitory effect of miR-1 on the PPP and the growth of pituitary tumor cells. Collectively, the present results uncovered the critical involvement of miR-1 in pituitary tumors, indicating that miR-1 is a potential therapeutic target for the treatment of pituitary tumors.

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Introduction

Pituitary tumors are considered as one of the most common intracranial neoplasms of the central nervous system (1,2). Although the majority of pituitary adenomas are benign tumors, ~25% of the tumors invade areas of the intracranial region (3). The widely used surgical resection, chemotherapy and radiotherapy techniques are only partially effective or wholly ineffective for patients harboring invasive pituitary tumors, which leads to high mortality rates and a poor outcome (4). Therefore, it is important and urgent to identify novel targets and characterize the underlying molecular mechanisms to improve the treatment of pituitary tumors.

MicroRNAs (miRNAs/miRs) are characterized as a class of small non-coding RNAs with a length of ~22 nucleotides (5-7). miRNAs function as key regulators of gene expression via binding to the 3'-untranslated region (UTR) of the target mRNAs, and consequently inducing the degradation or translation inhibition of the mRNAs (5,7,8). Notably, emerging evidence has illustrated the critical roles of miRNAs in the pathogenesis of different cancer types (9-16). For example, miR-106b targeted the tumor suppressor phosphatase and tensin homolog and promoted the growth of pituitary cancer cells (17). Another study reported that miR-133 suppressed the migration and invasion of pituitary tumor cells by downregulating the expression of forkhead box C1 (14). Recently, a growing body of evidence has suggested that miRNAs regulate the progression of cancer by affecting the metabolism of cancer cells, particularly aerobic glycolysis (18-23).

It is well documented that the pentose phosphate pathway (PPP) serves an important role in glucose metabolism and biosynthesis (24). PPP generates nicotinamide adenine dinucleotide phosphate (NADPH) to facilitate the synthesis of lipid and ribose 5-phosphate for the biosynthesis of nucleotides, which maintains the rapid growth of cancer cells (25-27). The ribose-5-phosphate generated by the PPP is converted into the intermediates of the glycolytic pathways. As the hallmark of cancer, aerobic glycolysis triggers the conversion of glucose into lactate and generates adenosine triphosphate (ATP) in cancer cells (28-30). Glucose-6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme of the PPP, is essential for nucleotide precursor production and redox homeostasis maintenance (26,31). Increasing evidence has demonstrated that G6PD is highly expressed in human cancer and associated

with a worse patient prognosis (31-34). In previous studies, upregulated G6PD promoted reactive oxygen species (ROS) production by facilitating the NADPH-dependent activation of NADPH oxidase 4 (35-37). Inhibiting the activity of G6PD resulted in tumor suppressive functions. The negative regulation of G6PD by miRNAs has been demonstrated in recent studies (38,39); G6PD was modulated by miR-206 and shown to be critical for the differentiation of rhabdomyosarcoma (38). Additionally, it has been reported that miR-1 post-transcriptionally repressed the expression of G6PD, increased the ROS level and aggravated the cardiac oxidative stress (40). However, the regulatory association between G6PD and miR-1 in cancer remains unknown.

In order to investigate the involvement of miR-1 in pituitary tumor, the expression of miR-1 was detected in pituitary cancer tissues and cell lines in the present study. The effect of miR-1 on the growth of pituitary tumor cells was examined and the underlying molecular mechanism was further investigated.

Materials and methods

Pituitary tumor tissues. Snap-frozen pituitary tumor tissues were obtained from 50 pituitary tumor patients (age range, 45-68 years; mean age, 58.4 years; female:male ratio, 1:1.32) between October 2013 and August 2015. Patients who had received chemotherapy or radiotherapy were excluded. The pituitary samples from patients without a diagnosis of pituitary cancer were included as the non-tumor control group to compare the expression of miR-1 in cancer tissues (n=50; mean age, 55.8 years; age range, 41-69 years; female:male ratio, 1:1.27). These tissues were used for analyzing the expression of miR-1 in cancer, and for evaluating the association between the expression of miR-1 and the metastasis and tumor size of the patients. Another 120 patients, who were used for analyzing the association between the expression of miR-1 and the 5-year overall survival rate, were enrolled between April 2008 and July 2011 (n=120; mean age, 60.2 years; female:male ratio, 1.18).

All the tissue samples were obtained from the People's Hospital of Yichang City Center affiliated to China Three Gorges University (Yichang, Hubei, China). Patients were not subjected to radiotherapy or chemotherapy prior to the surgical resection. All tissues were stored in liquid nitrogen until use. Written informed consent was provided by all participants and the study was approved by the Ethical Committee of China Three Gorges University. The clinicopathological parameters, including age, gender, differentiation, tumor size, lymph node metastasis and clinical stage, of all 170 patients are summarized in Table I.

Cell culture and transfection. The human pituitary adenoma HP75 cell line and the rat pituitary gland neoplasm MMQ cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 12.5% horse serum and 2.5% fetal bovine serum (all Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), with 5 U/ml penicillin and 5 g/ml streptomycin (Beyotime Institute of Biotechnology, Shanghai, China) in an atmosphere with 5% CO_2 at 37˚C. For the cell transfection, MMQ and HP75 cells were seeded in 6-well plates and when the cell confluence reached 70-80%, miR-1 mimics (5'-UGGAAUGUAAAGAACU-3') or negative control miRNA (5'-UUUGUACUACACAAAAGUACUG-3') were transfected into the cells at the final concentration of 20 nM for 48 h with Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols.

Plasmid construction. The full length of G6PD was amplified by PCR (as described below) and inserted into the pcDNA3.0-Flag vector. To investigate the effect of G6PD on the growth of pituitary cancer cells, Flag-G6PD was transfected into the cells with Lipofectamine 2000.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for gene expression. Total RNA was extracted from pituitary tissues and cell lines with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The purity and quality of RNA were determined with the NanoDrop-2000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). cDNA was obtained with the Omniscript Reverse Transcription kit (Qiagen GmbH, Hilden, Germany) by reverse transcription with 0.5 μg RNA. Using the cDNA as template, the relative expression of miR-1 was determined with SYBR green mix (Tiangen Biotech Co., Ltd., Beijing, China) on the ABI7500 quantitative PCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a 10-μl reaction system. The primers of miR-1 and U6 were designed as below: miR-1 forward, 5'-CAGTGCGTGTCGTGGAGT-3' and reverse, 5'-GGCCTG GATGTAAAGAAGT-3'; and U6 forward, 5'-GCTTCGGCA GCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACG AATTTGCGTGTCAT-3'. The reaction conditions were set as: 95˚C for 5 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Data were analyzed with the $2^{-\Delta\Delta Cq}$ method (41). The level of miR-1 was normalized using U6 RNA as the internal reference.

Luciferase reporter assay. The wild-type or mutant 3'-UTR sequences of G6PD containing the putative binding sites of miR-1 were cloned into the pmirGLO reporter vector (Promega Corporation, Madison, WI, USA), respectively. MMQ and HP75 cells were co-transfected with this luciferase reporter vector and the indicated miRNA using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Subsequent to transfection for 48 h, the cells were harvested and the luciferase activity was determined with the Dual-luciferase Reporter assay system (cat. no. E1910; Promega Corporation). *Renilla* luciferase activity was measured as the method of endogenous normalization.

Western blot analysis. MMQ and HP75 cells were transfected with miR-1 mimics or control miRNA using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Subsequent to transfection for 48 h, the cells were collected and lyzed with the NP-40 lysis buffer (Beyotime Institute of Biotechnology). The protein concentration was determined with the bicinchoninic acid assay (BCA) kit (Beyotime Institute of

Table I. Clinical parameters of the pituitary tumor patients.

Clinical characteristics	n
Age, years	
≤60	63
>60	107
Sex	
Male	77
Female	93
Differentiation	
Poor	81
High/moderate	89
Tumor size, cm	
<4	75
≥4	95
Lymph node metastasis	
Present	74
Absent	96
Clinical stage	
I-II	82
III-IV	88

Biotechnology). Protein from each sample (20 μg) was loaded and separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membranes. The membranes were blocked with 5% skimmed milk in PBS at room temperature for 1 h and then incubated with anti-G6PD antibody (1:3,000 dilution; cat. no. PA5-18734; Thermo Fisher Scientific, Inc.) at room temperature for 2 h. The membranes were washed twice with Tris-buffered saline plus Tween-20 and incubated with goat anti-mouse horseradish-peroxidase-conjugated secondary antibody (1:5,000 dilution; cat. no. AS003; ABclonal Biotech Co., Ltd., Woburn, MA, USA) for 1 h at room temperature. The expression of β-actin (1:3,000; cat. no. AA128; Beyotime Institute of Biotechnology) was detected as the loading control. The protein bands were visualized with electrochemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.) using the typhoon scanner (GE Healthcare, Chicago, IL, USA).

Cell proliferation analysis. The growth of the cells was evaluated using the CellTiter 96 AQueous One Solution Cell Proliferation assay kit (Promega Corporation) according to the manufacturer's protocols. Briefly, the cells transfected with miR-1 mimics or control miRNA were re-seeded into a 96-well plate at a density of 1,000 cells/well. Subsequent to being cultured for 24 h, 20 μl MTT was added once into the DMEM at the time point of 1, 2, 3, 4 or 5 days and incubated at 37˚C for 3 h. The absorbance (formazan was dissolved in DMSO) of each well at 480 nm was measured with a microplate reader. The experiment was performed in triplicate.

Cell apoptosis. The cell apoptosis percentage of the pituitary tumor cells was determined with the Annexin V-Fluorescein Isothiocyanate Apoptosis Detection kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Briefly, cells were transfected with miR-1 mimics or control miRNA. Subsequent to transfection for 48 h, the cells were stained with Annexin V-FITC and propidium iodide working solution for 15 min in the dark at room temperature. The cell apoptosis rate was analyzed using flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA).

Colony formation assay. Pituitary tumor cells transfected with the indicated miRNA were seeded in the 6-well plate at 1,000 cells/well. Subsequent to being cultured for 2 weeks, the cells were washed twice with PBS and fixed with methanol for 15 min at room temperature. The cell colonies were stained with 1% crystal violet for 10 min at room temperature. The colonies were counted by light microscopy.

Prediction of the targets of miR-1. The downstream targets of miR-1 were found using the TargetScan database (http://www.targetscan.org/mamm_31/), with the species set as human and the conserved microRNA family as miR-1/206 HMRDC. Upon submitting the search request, the putative downstream targets of miR-1 were summarized in a table, which included the gene name and the binding sites in the 3'-UTR.

Oxidative PPP flux analysis. Pituitary tumor cells were transfected with miR-1 or control miRNA using Lipofectamine 2000 (Invitrogen: Thermo Fisher Scientific, Inc.). When the cell confluence reached 60-70%, the cells were washed with fresh DMEM without glucose and incubated with medium containing 10 mM [2-^{13}C] glucose for 10 h. The medium from the experimental and control groups was analyzed in a 20-nm nuclear magnetic resonance tube with a 9.4T spectrometer at 100.66 MHz. Incorporation of ^{13}C in the carbon 2 and 3 of the lactate represented the glucose metabolism from glycolysis and the PPP, respectively. The oxidative PPP flux was determined by the ratio of ^{13}C in carbon 2 (glycolysis) and carbon 3 (oxidative PPP flux) of lactate, as well as the rate of glucose uptake. The number of cells that were transfected with miR-1 or control miRNA was compared to eliminate the influence caused by the variation of cell amounts.

NADPH level determination. Pituitary tumor cells were transfected with miR-1 mimics or control miRNA. Subsequent to transfection for 48 h, the cells were lysed with buffer containing 0.1 M Tris-HCl (pH 8.0), 0.05% Triton X-100 and 0.01 M EDTA. The lysates were sonicated and centrifuged at 3,000 x g for 10 min at 4˚C. The level of NADPH was detected for the absorbance at the wavelength of 341 nm by spectrometry. The protein concentration was determined using the BCA kit (Beyotime Institute of Biotechnology) to normalize the influence of the cell number.

Statistical analysis. The data from three independent experiments are presented as the mean ± standard error. The statistical analysis was performed using the SPSS software (13.0, IBM Corp., Armonk, NY, USA). The difference between two groups was analyzed with Student's t-test. The differences between more than two groups were detected by one-way analysis of variance followed by Dunnett's post hoc test.

Kaplan-Meier survival analysis was performed to compare the overall survival rates of patients with pituitary tumors grouped by high and low miR-1 expression levels (the mean value of miR-1expression in pituitary cancer tissues was considered as the cut-off). The log-rank test was used to analyze the statistical significance. Spearman's rank correlation test was performed to analyze the correlation between the expression of miR-1 and G6PD in the pituitary tumor tissues. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-1 is downregulated in pituitary tumor tissues and associated with a poor prognosis in patients with pituitary cancer. To understand the function of miR-1 in pituitary tumors, the expression of miR-1 in pituitary tumor tissues and adjacent normal pituitary tissues was evaluated by RT-qPCR analysis. As shown in Fig. 1A, the expression of miR-1 was significantly decreased in pituitary tumor tissues compared with that in the control group. The decreased expression of miR-1 in pituitary tumor tissues motivated investigation of the association between the level of miR-1 and the clinicopathological factors of patients with pituitary cancer. The analysis data showed that among the 50 patients, the expression of miR-1 was significantly lower in the patients with lymph metastasis compared with that in the patients without metastasis (Fig. 1B). However, there was no significant difference in the expression of miR-1 with regard to the size of the tumors (Fig. 1C). Based on these data, Kaplan-Meier survival analysis was performed to investigate the association between the expression of miR-1 and the 5-year overall survival of patients was analyzed in another 120 pituitary tumor patients. As shown in Fig. 1D, low expression of miR-1 was significantly associated with a worse prognosis in the patients. These results suggested that decreased abundance of miR-1 was a possible biomarker for predicting the prognosis of patients with pituitary cancer.

Overexpression of miR-1 suppresses the proliferation and induces the apoptosis of pituitary tumor cells. Given the decreased expression of miR-1 in pituitary tumors, the influence of miR-1 on the growth of pituitary tumor cells was then investigated. MMQ and HP75 cells were transfected with miR-1 mimics or control miRNA. The expression of miR-1 was confirmed by RT-qPCR analysis, as presented in Fig. 2A. An MTT assay was performed to detect the proliferation rate of pituitary tumor cells expressing miR-1 mimics or control miRNA. The results showed that upregulation of miR-1 significantly decreased the proliferation of the MMQ and HP75 cells (Fig. 2B and C). To further characterize the inhibitory effect of miR-1 on pituitary tumor cells, an *in vitro* colony formation assay was performed with MMQ and HP75 cells harboring overexpressed miR-1 or control miRNA. The data indicated that in the MMQ and HP75 cells, highly expressed miR-1 significantly suppressed the colony formation of the pituitary tumor cells (Fig. 2D). The cell apoptosis of MMQ and HP75 cells with overexpressed miR-1 was also evaluated. As illustrated in Fig. 2E, a significantly increased cell apoptosis rate was observed in the pituitary tumor cells with highly expressed miR-1. These results demonstrated that overexpression of miR-1 inhibited the growth of pituitary tumor cells.

G6PD is a downstream target of miR-1 in pituitary tumor cells. To understand the functional mechanism of miR-1 in pituitary cancer, the downstream targets of miR-1 were predicted by the TargetScan database, and ~440 possible conserved targets of miR-1 were found. These targets mainly function in regulating the proliferation and differentiation of cells. G6PD ranked top among all the predicted target candidates of miR-1. The putative binding sites of miR-1 at the 3'-UTR of G6PD are shown in Fig. 3A. To test this observation, a luciferase reporter assay was performed by co-transfecting miR-1 mimics with the wild-type or mutant 3'-UTR of G6PD into the pituitary tumor cells. The data showed that overexpression of miR-1 significantly decreased the luciferase activity of the wild-type but not the mutant 3'-UTR of G6PD in the MMQ and HP75 cells (Fig. 3B and C). To further confirm this result, the protein level of G6PD in pituitary tumor cells expressing miR-1 mimics or control miRNA was detected by western blotting with anti-G6PD antibody. A decreased protein level of G6PD was observed with the high expression of miR-1 in the MMQ and HP75 cells (Fig. 3D). At the same time, the mRNA level of G6PD was also evaluated with overexpressed miR-1. However, the results showed that no significance was obtained for the mRNA abundance of G6PD in the presence of miR-1 compared with that of the control cells (Fig. 3E). These results demonstrated that G6PD was a target of miR-1 and negatively regulated the protein expression of G6PD in pituitary tumor cells.

miR-1 targets G6PD to suppress the NADPH production and glycolysis of pituitary cancer cells. It has been well documented that the PPP generates NADPH and ribose-5-phosphate to facilitate the biosynthesis of nucleotides and glycolysis, respectively, which maintains the rapid growth of cancer cells (42). G6PD has been demonstrated as the first and rate-limiting enzyme of the PPP (42). Due to the negative regulation of miR-1 on G6PD, an assessment of whether overexpression of miR-1 modulated the PPP in pituitary tumor cells was performed. The oxidative PPP flux (flux through the oxidative branch of PPP and glycolysis), glucose consumption and lactate production of pituitary tumor cells transfected with overexpressed miR-1 were detected. As shown in Fig. 4A, the ectopic expression of miR-1 resulted in a significant decrease in the oxidative PPP flux. Since PPP serves important roles in the production of cellular NADPH, the influence of miR-1 on the generation of NADPH was also measured. The data showed that overexpression of miR-1 suppressed the level of NADPH compared with that of the control group (Fig. 4B). The glucose uptake and lactate production were also significantly decreased in the MMQ and HP75 cells expressing miR-1 mimics (Fig. 4C and D). These results indicated that miR-1 suppressed the PPP and glucose metabolism of the pituitary tumor cells.

Overexpression of G6PD reverses the inhibitory effect of miR-1 on the growth of pituitary tumor cells. To further characterize the association between miR-1 and G6PD, the expression of G6PD in paired pituitary tumor tissues and adjacent normal tissues was detected by RT-qPCR. The data showed that the mRNA level of G6PD was significantly increased in the pituitary tumor tissues compared with that in

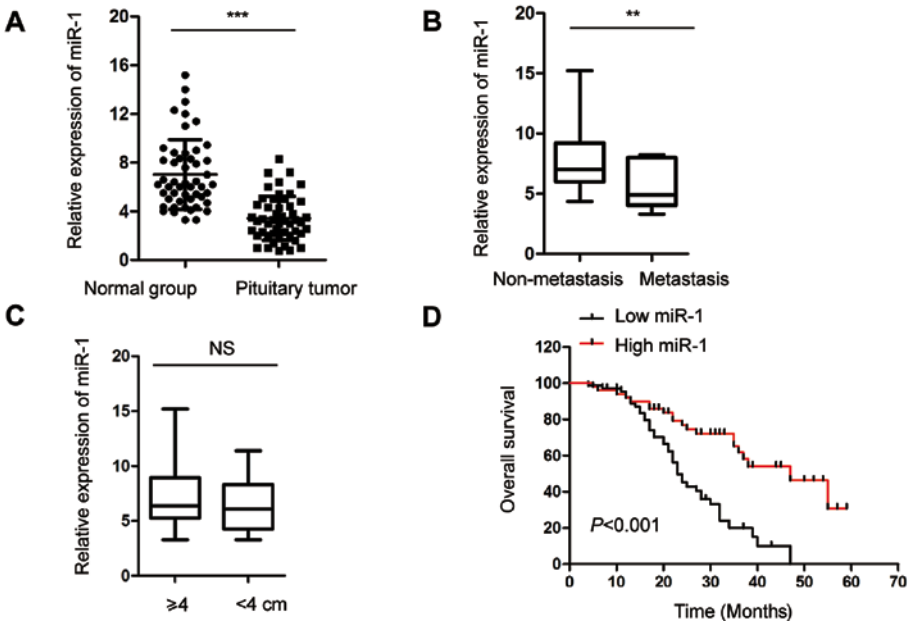

Figure 1. miR-1 is downregulated in pituitary tumors and associated with a worse prognosis. (A) Relative expression of miR-1 in pituitary tumor tissues and non-tumor pituitary issues (n=50) (***P<0.001 vs. control). (B) Expression level of miR-1 in pituitary tumor tissues with (n=20) or without (n=30) metastasis (**P<0.01 vs. control). (C) Expression of miR-1 in different tumor sizes. (D) Kaplan-Meier curves of the overall survival of the patients with pituitary tumors with low or high miR-1 expression (n=120). miR, microRNA; NS, no significance.

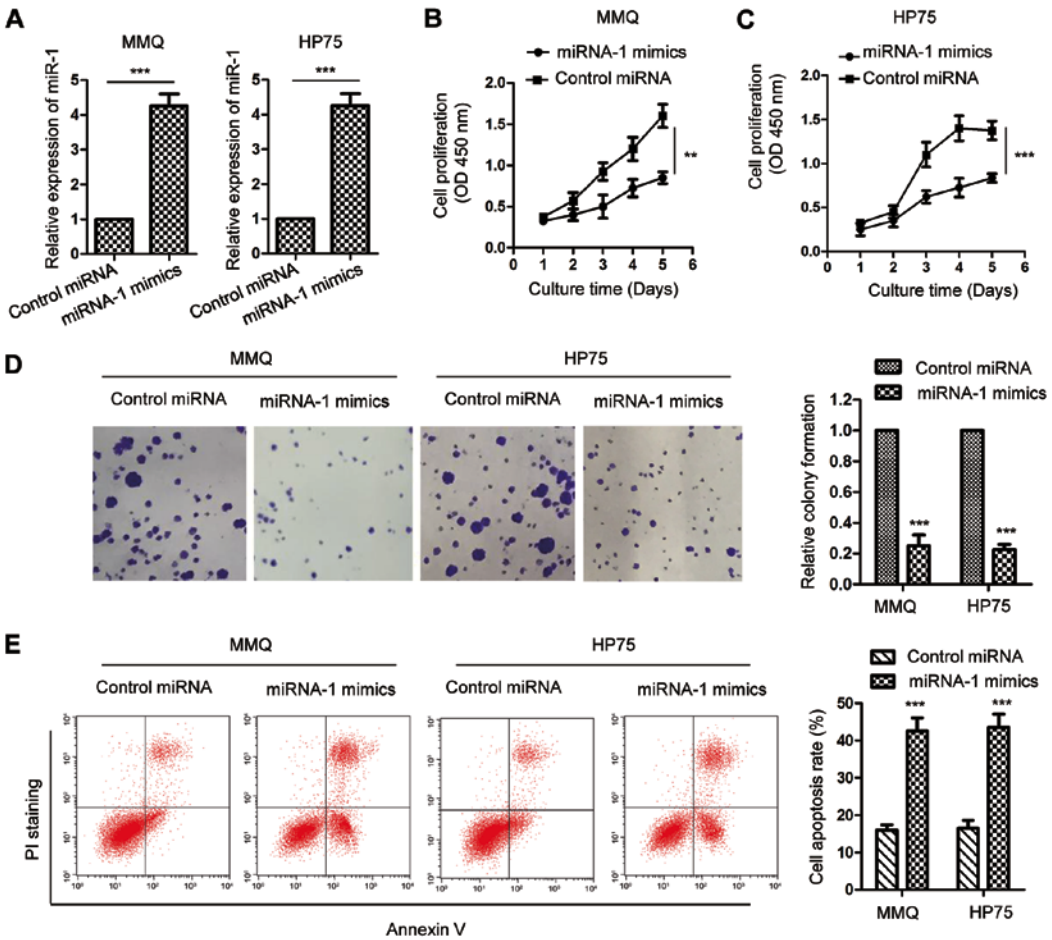

Figure 2. Overexpression of miR-1 inhibits the growth of pituitary tumor cells. (A) Pituitary tumor MMQ and HP75 cell lines were transfected with miR-1 mimics or control miRNA. The expression of miR-1 was confirmed by reverse transcription-quantitative polymerase chain reaction (***P<0.001 vs. control). An MTT assay was performed to evaluate the proliferation of (B) MMQ and (C) HP75 cells transfected with miR-1 or control miRNA (**P<0.01 and ***P<0.001 vs. control). (D) *In vitro* colony formation of pituitary tumor cells following transfection with miR-1 or control miRNA (***P<0.001 vs. control). (E) The cell apoptosis rate of MMQ and HP75 cells overexpressing miR-1 or control miRNA using FACS analysis (***P<0.001 vs. control). miR/miRNA, microRNA; PI, propidium iodide.

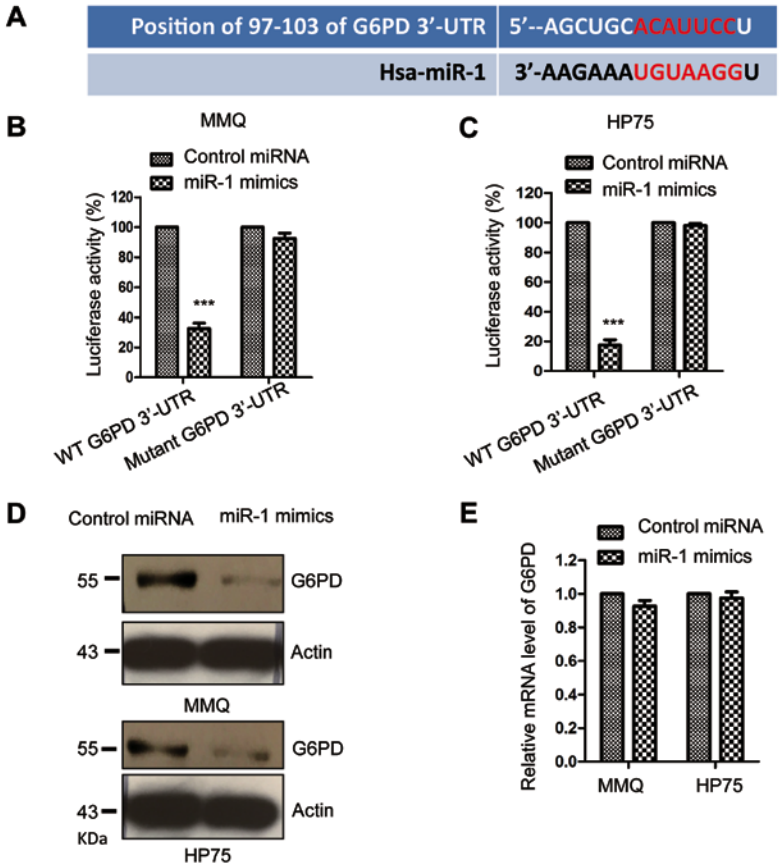

Figure 3. G6PD is a target of miR-1 in pituitary tumor cells. (A) The predicted binding site of miR-1 at the 3'-UTR of G6PD. (B) MMQ and (C) HP75 cells were transfected with miR-1 mimics or control miRNA in the presence of wild-type or mutant luciferase reporter vector of the 3'-UTR of G6PD (***P<0.001 vs. control). The (D) protein and (E) mRNA levels of G6PD in MMQ and HP75 cells transfected with miR-1 mimics or control miRNA were determined by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. G6PD, glucose-6-phosphate dehydrogenase; miR/miRNA, microRNA; UTR, untranslated region; WT, wild-type.

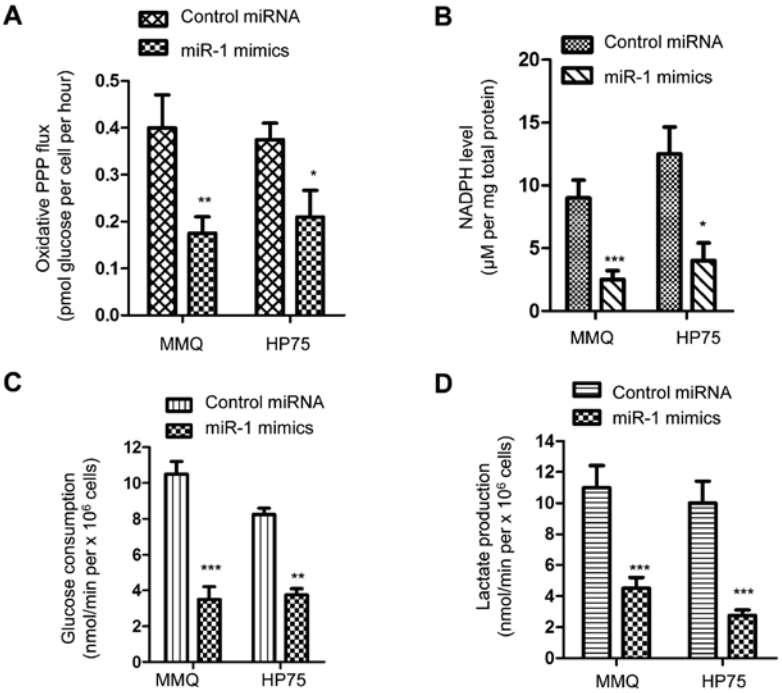

Figure 4. miR-1 suppresses the NADPH production and glycolysis of pituitary tumor cells. (A) MMQ and HP75 cells were transfected with miR-1 mimics or control miRNA. The oxidative PPP flux was measured based on the rate of glucose consumption and the ratio of ^{13}C incorporated into carbon 2 and carbon 3 of the lactate (*P<0.05, **P<0.01). (B) The NADPH level of pituitary tumor cells expressing miR-1 or control miRNA was determined (*P<0.05, ***P<0.001). The (C) glucose consumption (**P<0.01, ***P<0.001), and (D) lactate production of MMQ and HP75 cells expressing miR-1 mimics or control miRNA were compared (***P<0.001). NADPH, nicotinamide adenine dinucleotide phosphate; miR/miRNA, microRNA; PPP, pentose phosphate pathway.

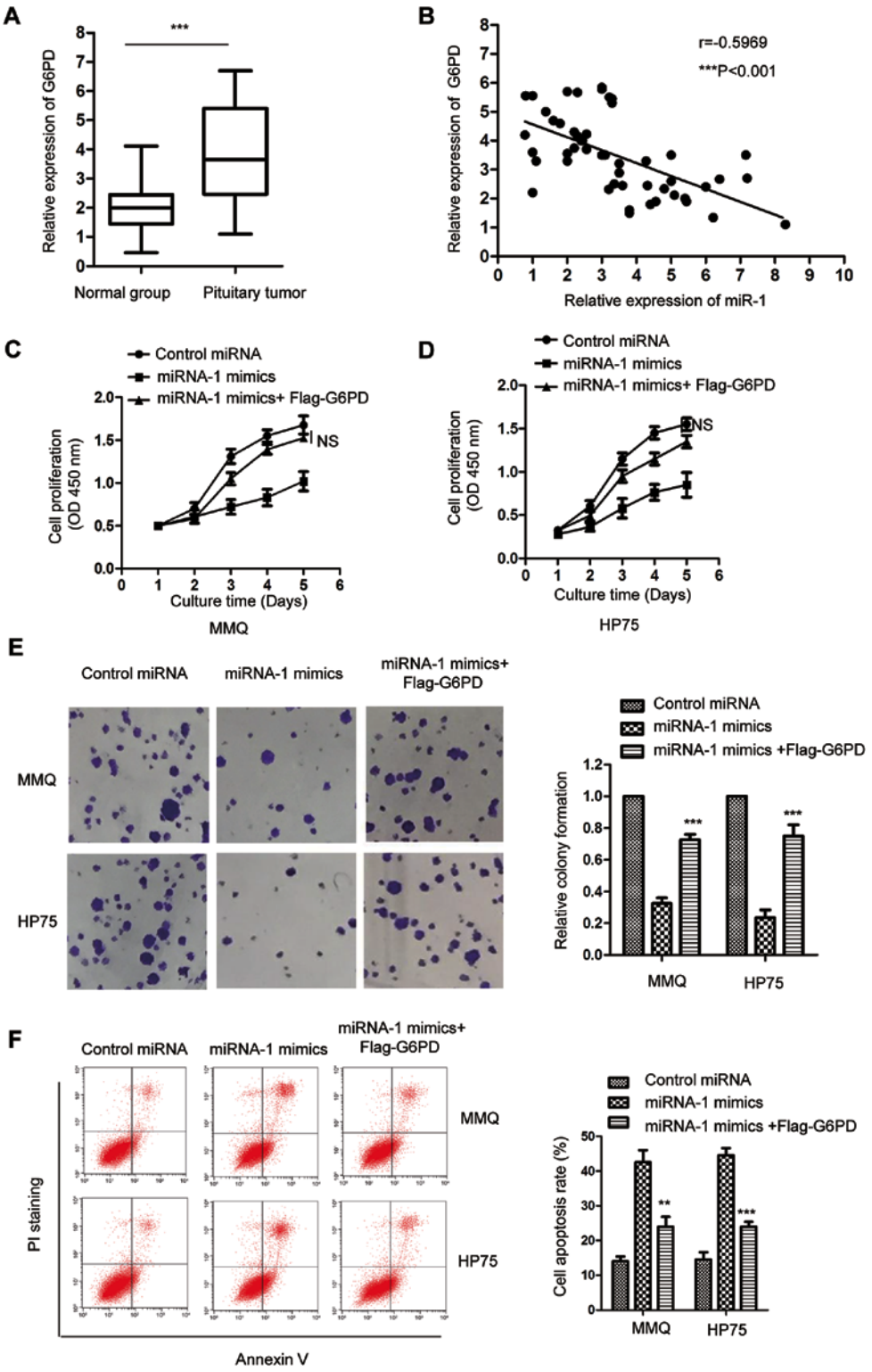

Figure 5. Overexpression of G6PD reverses the inhibitory effect of miR-1 on the growth of pituitary tumor cells. (A) Reverse transcription-quantitative polymerase chain reaction analysis was performed to detect the mRNA level of G6PD in the paired pituitary tumor tissues and corresponding normal tissues. (B) Spearman's correlation analysis of the correlation between the expression of miR-1 and G6PD in pituitary tumor tissues. (C) MMQ and (D) HP75 cells were transfected with miR-1 or control miRNA with or without Flag-G6PD. The cell proliferation was determined by the MTT assay. (E) The colony formation of pituitary tumor cells that transfected with miR-1, control miRNA or the combination of miR-1 with Flag-G6PD was compared (***P<0.001 vs. control). (F) MMQ and HP75 cells expressing miR-1 mimics were transfected with Flag-G6PD, and the cell apoptosis rate was determined by the FACS analysis (**P<0.01 and ***P<0.001 vs. control). NS, no significance; G6PD, glucose-6-phosphate dehydrogenase; miR/miRNA, microRNA; PI, propidium iodide; OD, optical density.

the corresponding normal tissues (Fig. 5A). Spearman's rank correlation test indicated that the expression of miR-1 and G6PD in pituitary tumor tissues was significantly inversely correlated (Fig. 5B). To confirm that the inhibitory effect of miR-1 on the growth of pituitary tumor cells was through the regulation of G6PD, MMQ and HP75 cells expressing miR-1

mimics were transfected to ectopically express G6PD. As shown in Fig. 5C and D, the MTT assay showed that highly expressed miR-1 decreased the cell proliferation, while restoring the expression of G6PD significantly inhibited the suppressive function of miR-1 on the growth of pituitary tumor cells. To further confirm this observation, the colony formation of pituitary tumor cells that were transfected with miR-1 or control miRNA with G6PD was performed. The data showed that in comparison with the cells expressing miR-1, the overexpression of G6PD significantly promoted the colony formation of the MMQ and HP75 cells (Fig. 5E). The fluorescence-activated cell sorting (FACS) analysis showed that overexpression of G6PD attenuated the cell apoptosis of pituitary tumor cells that were induced with the transfection of miR-1 (Fig. 5F). These results suggested that G6PD serves important roles for mediating the inhibitory effect of miR-1 on the growth of pituitary tumor cells.

Discussion

Abnormal expression of miRNAs has been involved in the initiation and progression of human cancer (10,11). The tumor suppressive function of miR-1 in cancer has been revealed in recent years (39,43-45). Downregulation of miR-1 was shown to enhance the tumorigenesis and invasiveness of oral squamous cell carcinoma (46). In ovarian cancer, miR-1 inhibited cell proliferation and migration through regulation of the c-Met pathway (47). The present study revealed that miR-1 was downregulated in pituitary tumor tissues and was associated with the poor prognosis of patients. Overexpression of miR-1 suppressed the proliferation of pituitary tumor cells, which was consistent with the previously described tumor suppressive roles of miR-1 in other types of cancer.

It is well known that miRNAs exert their functions by negatively regulating the expression of downstream target genes. In gastric cancer, miR-1 was found to suppress the expression of vascular endothelial growth factor A and endothelin-1, which consequently inhibited the tube formation of endothelial cells (48). Additionally, miR-1 suppressed the growth of esophageal carcinoma cells and enhanced the sensitivity of cells to anticancer drugs (49). In the present study, the bioinformatics and luciferase reporter assay revealed that miR-1 bound the 3'-UTR of G6PD. Overexpression of miR-1 decreased the protein level of G6PD but exhibited no significant effect on the mRNA level of G6PD. The binding of miR-1 with the 3'-UTR of G6PD may affect the structure of G6PD mRNA and block the translation of this mRNA into G6PD protein, which finally results in the decreased protein abundance of G6PD in miR-1-overexpressing pituitary tumor cells. Decreased expression of miR-1 was associated with the metastasis of pituitary tumor patients in this study. Notably, previous publications reported that the overexpression of G6PD was associated with the high risk of recurrent metastasis in primary breast carcinoma and esophageal squamous cell carcinoma (50,51). Elevated G6PD level promoted the migration and invasion of hepatocellular carcinoma cells by inducing the epithelial-mesenchymal transition (52). All these results suggested the critical involvement of G6PD in the metastasis of cancer. Thus, the potential function of G6PD in regulating the metastasis of pituitary tumor cells deserves further investigation.

As the hallmark of cancer, cancer cells specifically reprogram the metabolism from oxidative phosphorylation to glycolysis, which accelerates the generation of ATP and intermediate materials for the biosynthesis of molecules in cells (28-30). Notably, the PPP, as the major source of NADPH, provides the reducing power for the biosynthesis of cancer cells (25,27). As the rate-limiting factor of PPP, the expression of G6PD was previously found to be highly upregulated in several cancer types (31). Transcriptional activation of G6PD by Tap73, the homolog of p53, enhanced the PPP and facilitated the growth of cancer cells (53,54). Due to its oncogenic function, decreasing the level of G6PD is a novel strategy to suppress tumorigenesis. In the present study, the downregulation of G6PD by miR-1 inhibited the generation of NADPH, and decreased the glucose consumption and lactate production. These results suggested that miR-1 was a novel regulator of the PPP in pituitary tumor cells.

In conclusion, this study found that miR-1 was downregulated in pituitary tumor cells and tissues. Overexpression of miR-1 suppressed cell growth by targeting G6PD to inhibit the metabolism of cancer cells. The downregulation of miR-1 was significantly associated with a worse prognosis in patients with pituitary tumors. These results suggested that miR-1 and G6PD may be potential targets for the therapy of human pituitary cancer.

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Availability of data and materials

All the data and materials in this study are available from the corresponding author upon request.

Authors' contributions

CH designed the study and performed the majority of the experiments. JuY and JD collected the clinical samples and detected the expression of miR-1 in the tissues. SL and HW performed the western blotting. YX and FZ performed the FACS analysis. YJ and LT performed the statistical analysis. JiY designed the study, wrote the manuscript, revised it critically for important intellectual content and gave the final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethical Committee of China Three Gorges University. Written informed consent was provided by all participants.

Patient consent for publication

Consent for publication was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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