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Original Article

MicroRNA-150 regulates glycolysis by targeting von Hippel-Lindau in glioma cells

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Abstract: Warburg effect, characterized by enhanced glycolysis and lactate production, even under aerobic conditions, is one of the hallmarks of cancer cells. However, the mechanism underlying this phenomenon remains poorly understood. Previous studies have shown that microRNA-150 (miR-150) is significantly up-regulated in various malignancies and represents a putative onco-miRNA in human cancers. In the present study, we aim to investigate the functional significance and molecular target of miR-150 in glioma. As a result, von Hippel-Lindau (VHL), which is a specific E3 ligase for hypoxia inducible factor 1 (HIF1α), was identified as a novel target of miR-150. Consistently, cells overexpressing miR-150 exhibited a metabolic shift, including enhanced glucose uptake and lactate production, which led to a rapid growth of glioma cells. Therefore, our results suggest that miR-150 modulates the Warburg effect in glioma via VHL/HIF1α and might provide a novel option for future treatments for glioma.

Keywords: VHL, HIF1α, MicroRNA-150, glioma, warburg effect

Introduction

In contract to normal somatic cells, most cancer cells preferentially rely on aerobic glycolysis. This metabolic phenotype is usually termed as the Warburg effect [1, 2]. Indeed, elevated expression and/or activity of several key glycolytic enzymes, including glucose transporter (GLUT) isoform 1 (GLUT1), hexokinase 2 (HK2) and lactate dehydrogenase A (LDHA) have been observed in many types of human cancers [3, 4]. These genes are transactivated by a transcription factor named hypoxia-inducible transcription factor 1α (HIF1α), a key mediator of cellular adaptation to oxygen stress [5]. HIF1α enhances messenger RNA expression of glycolytic genes through binding to HIF1α response elements (HRE) at their promoter regions [5]. Therefore, expression of HIF1α is tightly regulated at multiple levels, including von Hippel-Lindau (VHL)-mediated ubiquitination and degradation of HIF1α [6, 7]. Notably, loss of VHL function has been shown to regulate the metabolic reprogramming of cancer cells and promote tumor growth [8, 9].

MicroRNAs (miRNAs), a family of short, non-coding RNAs, negatively modulate gene expression by binding to the 3'-untranslated region (3'-UTR) of message RNA (mRNA), leading to mRNA degradation and/or translational suppression [10, 11]. One of miRNAs, miR-150, has been shown to play critical roles in the development of human malignancies, including breast, lung and gastric cancer [12-14]. In this study, we aim to explore the novel role of miR-150 in tumorigenesis and demonstrate that increased miR-150 expression is associated with rewiring of cancer cell metabolism by targeting VHL/HIF1α pathway.

Materials and methods

Clinical specimens and cell culture

30 pairs of glioma and para-carcinoma tissue samples were collected from patients who are diagnosed between May 2012 and July 2014. The design and implementation of this research was approved and documented by the clinical research ethical committee of the Third

Affiliated Hospital, Xinxiang Medical University. All cell lines were purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CAS, Shanghai, China). Cells were maintained at 37°C in an atmosphere of 5% CO_2 in DMEM medium (Gibco, Shanghai), supplemented with 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 mg/ml) (Gibco).

RNA extraction AND real-time quantitative PCR

miRNAs were extracted from tissues or cells using miRcute miRNA Kit (Tiangen, Shanghai, China) according to the manufacturer's instructions. Expression levels of miR-150 were measured using miScript SYBR Green PCR Kit (Qiagen). Quantitative real-time PCR was performed by using an Applied Biosystems 7300 Real-time PCR System. PCR conditions included an initial holding period at 95°C for 3 min, followed by a two-step PCR program consisting of 94°C for 5 s and 60°C for 35 s for 40 cycles. Expression of miR-150 was normalized to that of the U6 snRNA. The primers for miR-150 and U6 were purchased from Biosune Company (Shanghai, China).

Western blots

Proteins were extracted using modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4; 120 mM NaCl; 1% Nonidet P-40; 0.25% deoxycholate; 0.1% sodium dodecyl sulfate) supplemented with protease inhibitor cocktail (Beyotime, Shanghai, China). Samples were then normalized for protein concentration using a BCA protein assay kit (Pierce, Rockford, IL). Protein extracts were equally loaded onto 10% SDS polyacrylamide gels, electrophoresed, and transferred to nitro cellulose membranes (Amersham Bioscience). After blocking with 5% non-fat milk in PBS, the membranes were probed with antibodies against VHL (sc-135657, mouse monoclonal antibody) (Santa Cruz Company, California, USA), HIF1α (sc-13515, mouse monoclonal antibody) (Santa Cruz Company) and -actin (sc-47778, mouse monoclonal antibody) (Santa Cruz Company), followed by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Company). The signals were detected by chemiluminescent substrate kit (Millipore Company, Bedford, MA, USA).

Transient transfection

For transient transfection, miR-150 mimics (Genepharm, Shanghai, China), miR-150 inhibitors (Genepharm) and negative controls were used and transfected into glioma cells using the Lipofectamine 2000 Transfection Regent (Invitrogen, USA) according to the manufacturer's instructions.

Glucose uptake analysis

To determine the rate of glucose uptake, cells were washed with phosphate buffered saline (PBS) and then incubated for 3 hr in DMEM containing 1-mCi/mL 2-deoxy-2[1, 2-^{3}H] glucose (PerkinElmer Life Sciences, Waltham, MA). The cells were washed with ice-cold PBS and solubilized in 1% of SDS. A scintillation counter (Beckman LS 6500, CA) was used to determine the radioactivity of each aliquot. Each assay was performed in triplicate. All values were normalized to total protein levels (BCA protein assay kit, Thermo Scientific, Waltham, USA).

Lactate production measurements

Lactate production was quantified using the lactate assay kit (BioVision, Mountain View, USA) following the manufacturer's protocol. ATP levels were measured using a CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega, Madison, WI, USA). All values were normalized to total protein levels.

Luciferase reporter assays

The 3'-untranslated region of human VHL gene was amplified by RT-PCR using cDNA from U251 cells. The mutant construct was also generated by replacing the 3'-UTR with custom made synthetic whole 3'-UTR DNAs with mismatched seed region mutations. Renilla and firefly luciferase activities were measured by the Dual-Luciferase Reporter system (Promega, Madison, USA). All the transient transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions.

Cell viability and proliferation analysis

The cell viability was determined by assaying the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-di-phenyltetrazolium bromide (MTT) to forma-

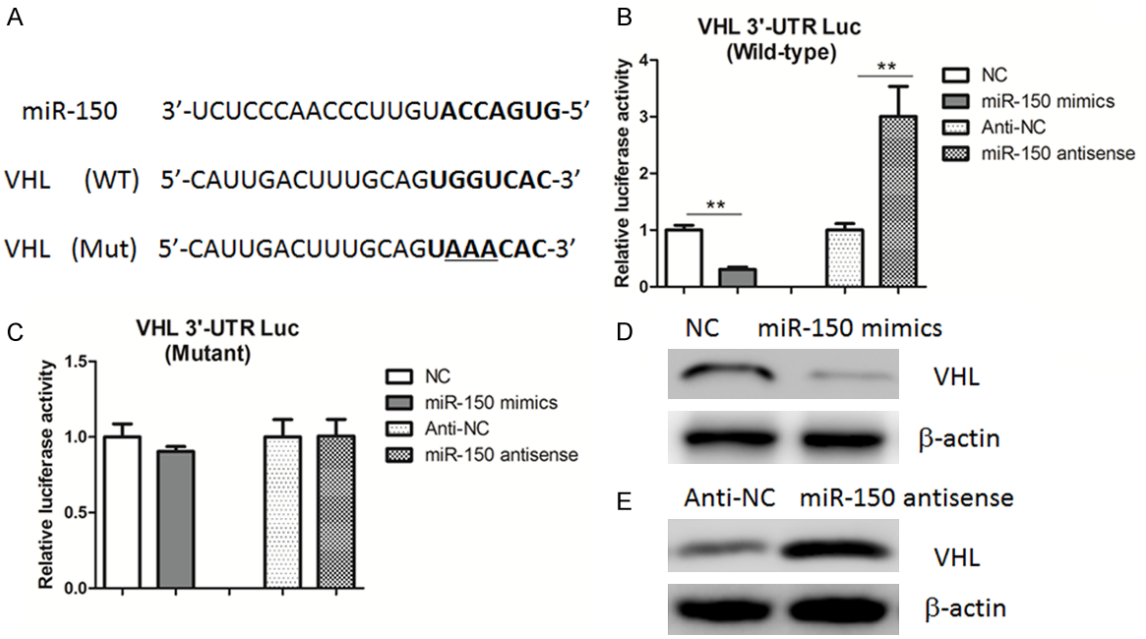

Figure 1. miR-150 inhibits VHL expression. (A) The wild-type (WT) and mutant (Mut) 3'-UTR of VHL. Computer prediction of miR-150 binding sites were shown in bold. (B, C) Luciferase reporter assays in U251 cells. Cells were transfected with wild-type (B) or mutant (C) 3'-UTR-reporter constructs together with miR-150 mimics, antisense or negative control (NC). (D, E) Representative protein levels of VHL in U251 cells were determined by western blots. Cells were transfected with miR-150 mimics (D), antisense (E) or negative control (NC) for 48 hr. Comparisons between groups were analyzed by the Student t-test. $^{**}P<0.01$ between two groups.

zan. For BrdU analysis, a cell proliferation enzyme-linked immunosorbent assay (BrdU kit; Beyotime) was used to analyze the incorporation of BrdU during DNA synthesis following the manufacturer's protocols.

Mouse experiments

Male BALB/c nude mice aged 5 weeks were purchased from Shanghai Laboratory Animal Company (SLAC, Shanghai). 5.0×10^6 U251 cells stably expressing miR-150 mimics or negative control were injected subcutaneously to the skin under the front legs of the nude mice. The mice were observed over 16 days for tumor formation. Mice were then sacrificed and the wet weights of each tumor were determined.

Statistical analysis

The data shown represent the mean ± standard error (SE) values of at least three independent experiments. Comparisons between groups were analyzed by the Student t-test. A value of $P<0.05$ was considered statistically significant.

Results

miR-150 inhibits VHL expression in glioma cells

To identify novel mRNA(s) that are targeted by miR-150, a stringent bioinformatics approach (TargetScan software: www.targetscan.org) was used. Among several putative genes, the gene encoding von Hippel-Lindau (VHL) that harbored a miR-150 binding site (**Figure 1A**) was selected for further studies, since it has been shown to interact with HIF1α and mark it for subsequent proteasome degradation [6, 7]. Therefore, the VHL 3'-untranslated region (3'-UTR) was cloned into a luciferase reporter construct and co-transfected into U251 cells with miR-150 mimics, antisense or negative control (NC). As a result, the luciferase activity was reduced by overexpression of miR-150 mimics and increased by inhibition of miR-150, when the reporter construct contained the wild-type 3'-UTR (**Figure 1B**). In contrast, mutation of the miR-150 binding motif abrogated the luciferase expression (**Figure 1C**). Besides, endogenous VHL protein expression was suppressed

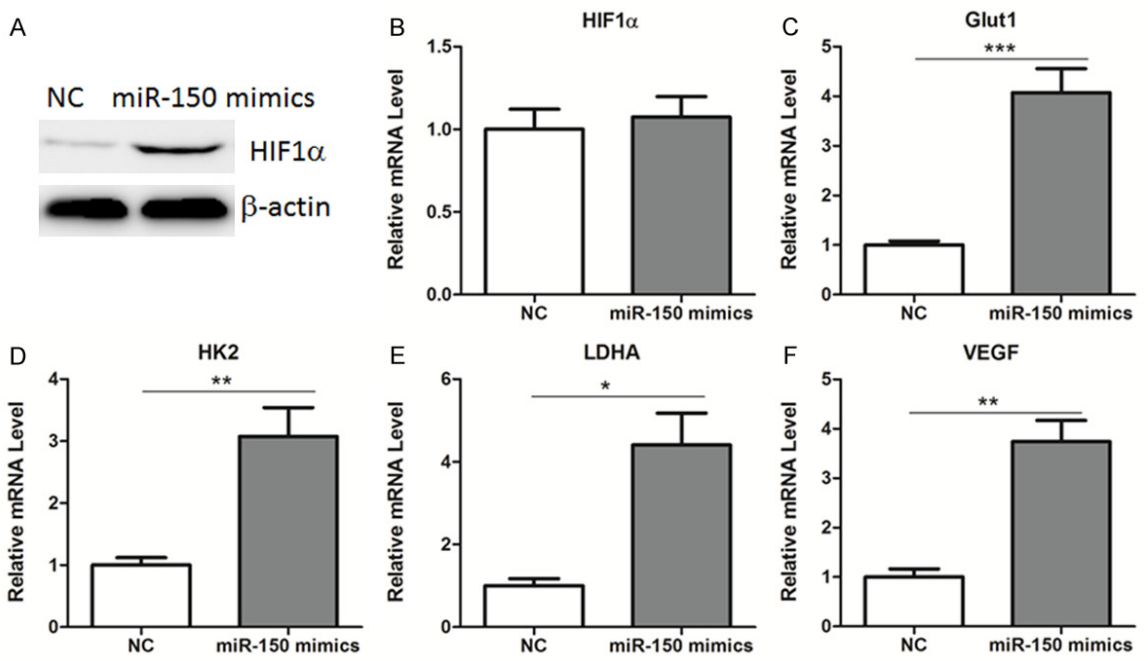

Figure 2. miR-150 mimics up-regulates HIF1α and its target genes. (A) Representative protein levels of HIF1α in U251 cells were determined by western blots. Cells were transfected with miR-150 mimics or negative control (NC) for 48 hr. (B-F) Relative mRNA levels of HIF1α (B), Glut1 (C), HK2 (D), LDHA (E) and VEGF (F) in U251 cells were determined by quantitative real-time PCR. Cells were transfected with miR-150 mimics or negative control (NC) for 48 hr. Comparisons between groups were analyzed by the Student t-test. *P<0.05, **P<0.01, ***P<0.001 between two groups.

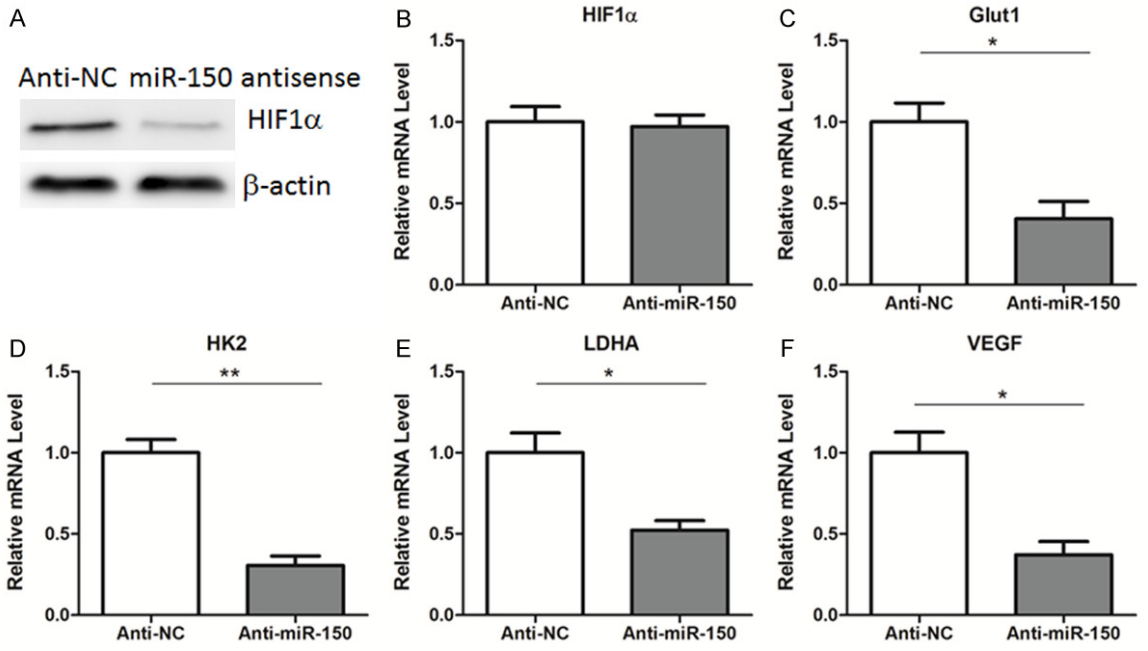

Figure 3. miR-150 antisense down-regulates HIF1α and its target genes. (A) Representative protein levels of HIF1α in U251 cells were determined by western blots. Cells were transfected with miR-150 antisense or negative control (NC) for 48 hr and placed under hypoxic conditions (1% O_2) for 6 hr. (B-F) Relative mRNA levels of HIF1α (B), Glut1 (C), HK2 (D), LDHA (E) and VEGF (F) in U251 cells were determined by quantitative real-time PCR. Cells were transfected with miR-150 mimics or negative control (NC) for 48 hr. Comparisons between groups were analyzed by the Student t-test. *P<0.05, **P<0.01 between two groups.

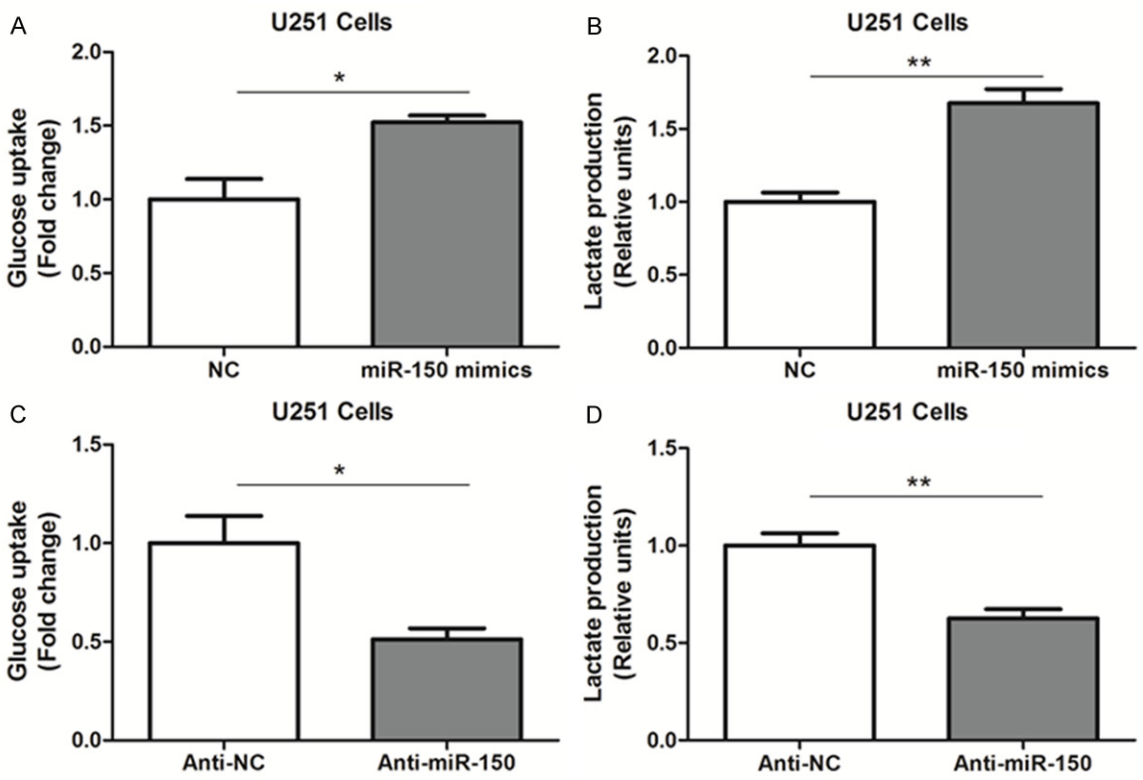

Figure 4. miR-150 regulates glucose metabolism in glioma cells. (A-D) Glucose uptake (A, C) and lactate production (B, D) in U251 cells. Cells were transfected with miR-150 mimics (A, B) or antisense (C, D) for 36 hr. The data were normalized by total protein concentration. Comparisons between groups were analyzed by the Student t-test. *P<0.05, **P<0.01 between two groups.

by miR-150 mimics and up-regulated by miR-150 antisense (**Figure 1D**, **1E**).

miR-150 promotes HIF1α expression in glioma cells

Next, expression level of HIF1α in U251 cells was determined. As expected, HIF1α protein level was increased, even in normoxic conditions, when basal level of HIF1α was low (**Figure 2A**). However, its mRNA level was not affected (**Figure 2B**), indicating that miR-150 regulated HIF1α at post-transcription level. Expression levels glycoytic enzymes, including Glut1, HK2 and LDHA, were increased by miR-150 overexpression (**Figure 2C-E**). Besides, vascular endothelial growth factor (VEGF), a potent angiogenic factor, was also up-regulated (**Figure 2F**). On the other hand, expression levels of target genes of HIF1α were inhibited by miR-150 antisense under hypoxic conditions (**Figure 3A-F**). Together, these results indicate that VHL is a direct target of miR-150 in glioma cells.

miR-150 modulates glucose metabolism in glioma cells

Given that miR-150 was able to regulate glycolytic enzymes, we sought to test whether dysregulation of miR-150 could induce a metabolic shift in glioma cells. Indeed, overexpression of miR-150 enhanced, while inhibition of miR-150 reduced glucose uptake and lactate production in U251 cells (**Figure 4A-D**).

miR-150 promotes glioma cell proliferation in vitro

Next, we determined the biological functions of miR-150 in glioma cells. MTT and BrdU incorporation assays showed that cell viability and proliferation abilities were enhanced or inhibited by miR-150 mimics or antisense, respectively (**Figure 5A-D**). Moreover, the cells expressing miR-150 mimics were growing faster than the control and the cells expressing miR-150 antisense (**Figure 5E**).

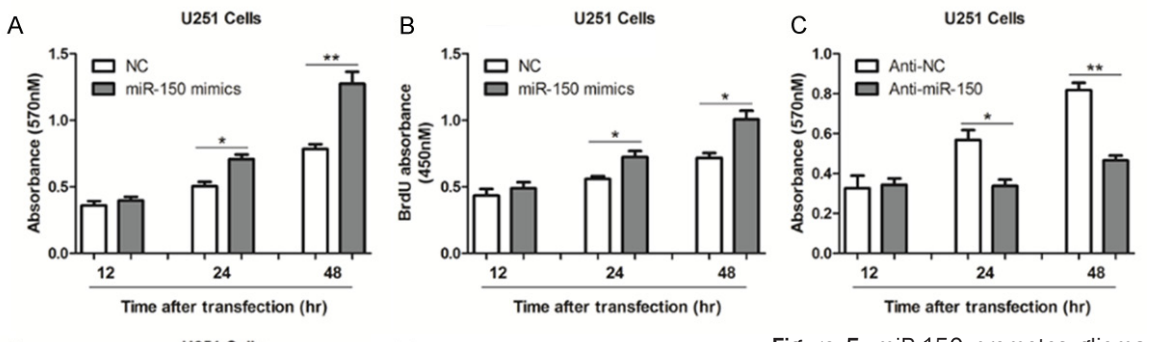

Figure 5. miR-150 promotes glioma cell proliferation. (A-D) The cell viability (A, C) and proliferative potential (B, D) was analyzed in U251 cells. Cells were transfected with miR-150 mimics (A, B), antisense (C, D) or negative control (NC). (E) Cell number was determined in U251 cells with miR-150 overexpression or inhibition. Comparisons between groups were analyzed by the Student t-test. *P<0.05, **P<0.01 between two groups.

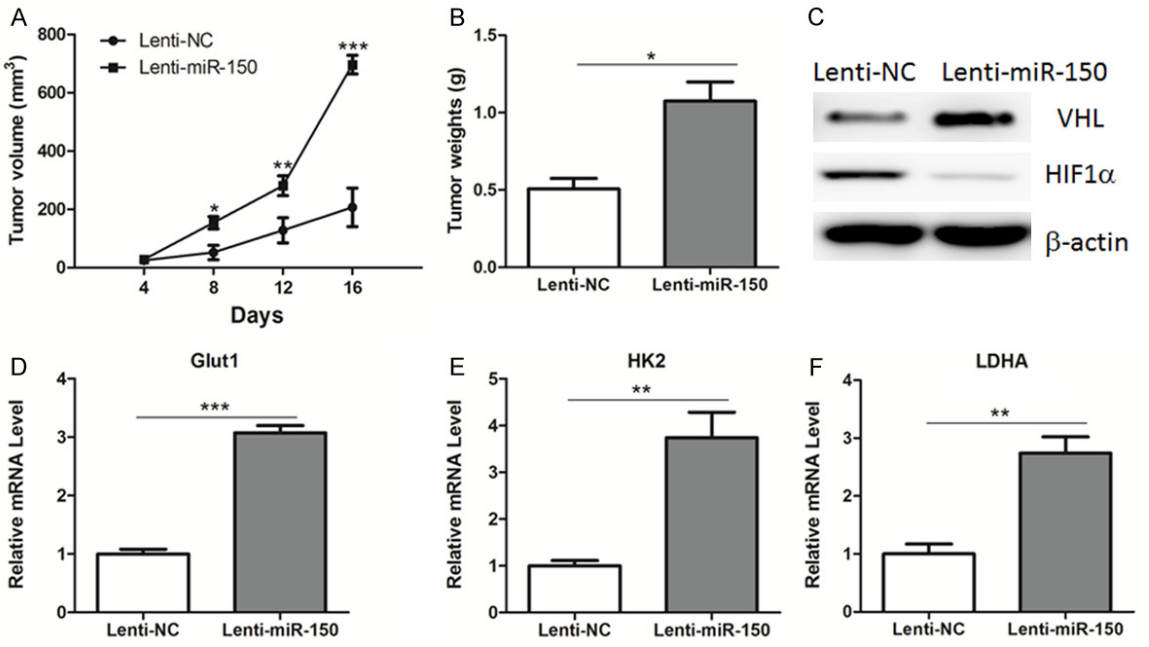

Figure 6. miR-150 facilitates tumor growth *in vivo*. (A, B) U251 cells stably transfected with lent-virus containing miR-150 mimics or negative control (NC) were injected into nude mice (n=8 for each group) and followed up for tumorigenesis. Growth curve of tumor volumes (A) and tumor weights (B) were taken 16 days after injection. (C) Representative protein levels of VHL and HIF1α in tumors were determined by western blots. (D-F) Relative mRNA levels of Glut1 (D), HK2 (E) and LDHA (F) in tumors were determined by quantitative real-time PCR. Comparisons between groups were analyzed by the Student t-test. **P<0.01, ***P<0.001 between two groups.

miR-150 facilitates tumor growth in vivo

To further evaluate the potential effect of miR-150 on glioma cell growth *in vivo*, U251 cells with or without miR-150 over-expression were subcutaneously inoculated into the skin under the front legs of the nude mice. The mean volume and wet weight of the tumors of the miR-

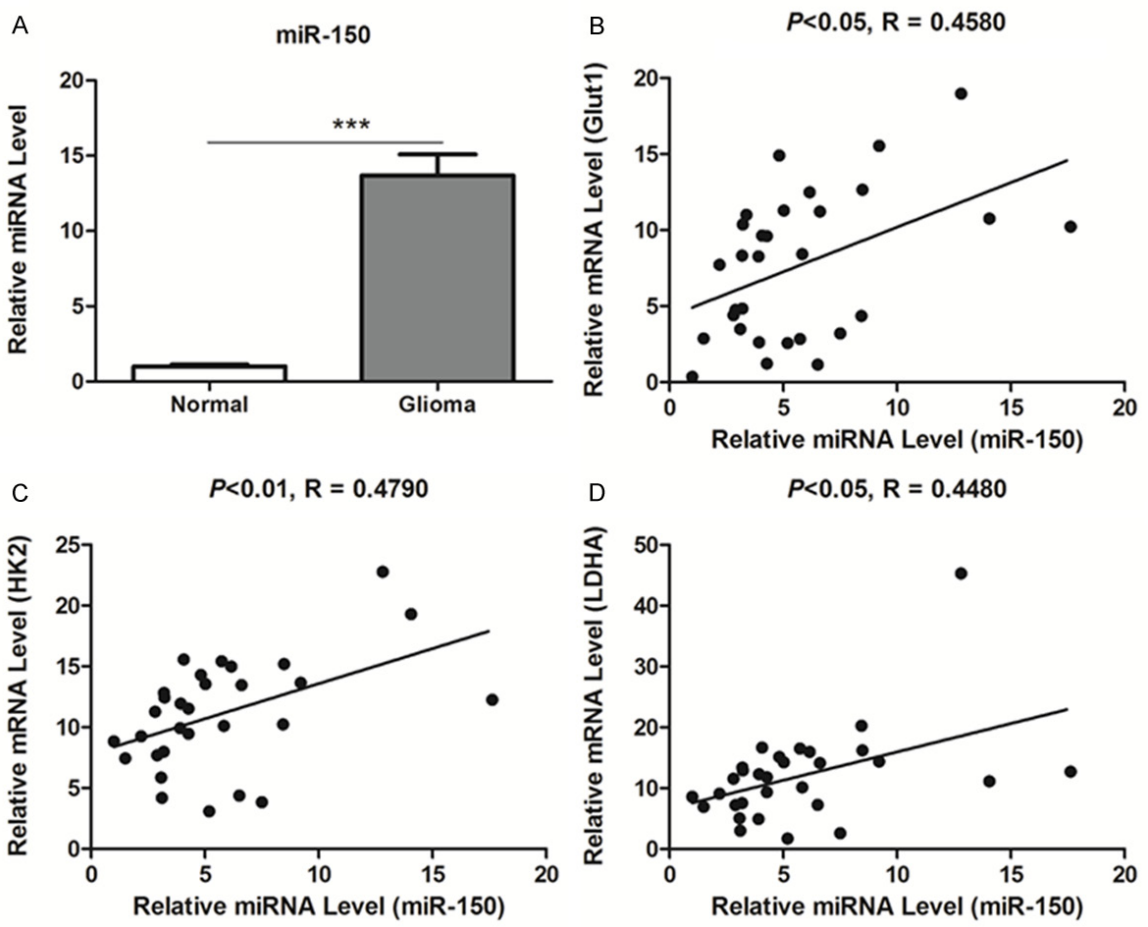

Figure 7. Expression levels of miR-150 in glioma tissues. (A) miR-150 expression was determined by quantitative real-time PCR in 30 paired of human glioma tissues and adjacent noncancerous tissues (Normal). (B-D) The correlation between miR-150 and Glut1 (B), HK2 (C) and LDHA (D) expression levels in glioma tissues (n=30). Comparisons between groups were analyzed by the Student t-test. ***P<0.001 between two groups.

150 over-expressing group were significantly higher than that of the tumors of the control group (**Figure 6A**, **6B**). Consistently, protein levels of VHL were also down-regulated in tumors overexpressing miR-150 mimics (**Figure 6C**), while expression of HIF1α and its target genes were increased (**Figure 6D**, **6E**).

Up-regulation of miR-150 in glioma tissues

Given that miR-150 could promote glioma cell survival and growth, its expression levels were analyzed by quantitative real-time PCR in human glioma tissues and pair-matched adjacent normal tissues. We found that miR-150 was significantly up-regulated in cancer tissues (**Figure 7A**). Moreover, the expression of miR-150 was correlated with levels of Glut1, HK2 and LDHA (**Figure 7B-D**), further suggesting that miR-150 might be a key regulator of glycolysis in glioma.

Discussion

Large-scale screens of miRNA-mRNA interactions have unveiled that one miRNA has multiple mRNA targets [15]. Indeed, miR-150 was shown to regulate expression of several target genes in normal and malignant tissues, such as MUC4, ZEB1, P53 and c-Myb [16-19]. Therefore, miR-150 could function as an oncogene or tumor suppressor gene in different solid tumors [20], which might rely on its regulation of target genes.

In the present study, we investigated a novel role of miR-150 in the regulation of glucose metabolism. We found that miR-150 inhibited

VHL expression in glioma cells by binding to its 3'-UTR region. As a result, endogenous expression of VHL was suppressed by miR-150 mimics and up-regulated by miR-150 antisense. Therefore, by down-regulation of VHL and up-regulation of HIF1α, miR-150 promoted glycolysis in glioma cells and facilitated xenograft tumor formation in mouse models. To our knowledge, this is the first report that this miRNA also plays an important role in regulating cancer cell energy metabolism. However, further studies are still needed to test whether this phenomenon occurs in other types of human cancers. Moreover, the molecular determinants for the aberrant expression of miR-150 in tumorigenesis remain largely unknown. A recent study showed that KLF family DNA-binding sites are necessary for miR-150 promoter activity and KLF2 or KLF4 overexpression induces miR-150 expression [21]. Interestingly, several members in the KLF family have been reported to function in the pathogenesis of human cancers in multiple ways [22, 23]. Therefore, we speculate that dysregulation of certain KLF proteins might be responsible for the up-regulation of miR-150 in glioma tissues.

Recent studies demonstrated that dysregulation of miRNAs could alter expression and/or activity of key glycolytic enzymes in tumor cells [24, 25], including glioma. For instance, miR-495 mediates metabolic shift in glioma cells via directly suppressing Glut1 [26]. miR-29b regulates the Warburg effect in ovarian cancer by targeting AKT2 and AKT3 [27]. Besides, miR-199a-5p is negatively associated with malignancies and regulates glycolysis by targeting hexokinase 2 in (HCC) is a leading cause of cancer-related [28], suggesting that these gene regulators might be promising therapeutic targets for cancer treatment.

Taken together, our results demonstrate that miR-150 could modulate the Warburg effect in glioma via VHL/HIF1α and might provide a novel option for cancer therapy.

Disclosure of conflict of interest

None.

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