Master of Science

miR-622 inhibits the tumorigenesis of glioblastoma through GLUT3 repression

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This certifies that the dissertation of Min Jung Kong is approved.

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Abstract

Histone deacetylases (HDAC) is modified histone-proteins. HDAC2 is a member of class Ⅰ HDAC and associated brain development. HDAC2 is highly expressed in various cancers and correlated with GBM progression. HDAC inhibitor is known to be effective in therapeutic cancer. GLUT3 is also highly expressed in GBM cells. Thus, we studied the tumorigenesis and apoptosis of GBM cells (U87MG and A172) by miR-622-regulated GLUT3 repression. miR-622 is highly correlated with apoptosis in GBM cells (U87MG and A172). MiR-622 significantly downregulated GLUT3 expression and induced apoptosis in GBM cells. Also, we showed that miR-622 suppressed the colony formation in GBM cells by colony formation assay. Additionally, we demonstrated that miR-622 induced apoptosis in GBM cells by the MTT and FACS analysis, and DNA damage by the TUNEL assay. The GBM tumor size decreased in orthotopic mouse model that was injected with miR-622. GLUT3 is essential for tumor metabolism and GLUT3 upregulation is involved in tumor progression and resistance to therapy. GLUT3-CDS was found to interact with miR-622. HDAC2 knockdown in GBM cells induces GBM cell death through miR-622 upregulation and GLUT3 downregulation. Thus, this study demonstrated that miR-622 expression in HDAC2-silenced GBM cells suppressed GLUT3 expression and induce GBM cell death.

Keyword

Glioblastoma (GBM), Histone deacetylase (HDAC2), GLUT3, miRNA, Apoptosis

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Introduction

GBM is the most common and aggressive malignant primary brain tumor in adult and involved in WHO grade IV glioma [1]. It is representing approximately 57% of all gliomas and 48% of all primary malignant central nervous system (CNS) tumors [2]. The survival is 15–23 months and 5-year survival is less than 6%, which is the lowest long-term survival rate among the malignant brain tumors [3]. Most chemotherapeutic agents currently used almost have no response and many fall into the range of10~20%, with delivery across the blood brain barrier (BBB). Also, chemoresistance contributing to the extremely poor out comes despite treatment [4]. The standard treatment for GBMs is surgical resection followed by chemoradiotherapy [5]. But GBM is known to contain a population of self-renewing cancer stem cells (CSCs) so the prognosis is poor [6]. GBM progression is related with epigenetic mechanisms and pathways [7].

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are two classes of enzymes that modulate histone acetylation [8]. HDAC enzymes eliminate the acetyls of the lysine and increase the electrostatic interaction between histones and DNA to generate a more compact chromatin structure that is associated with transcriptionally inactive states [9]. HDACs can be divided into three equally distinct groups: class 1, class 2, and a third class consisting of proteins related to the recently identified human HDAC11 gene. We term this novel group “class 4” to distinguish it from the unrelated “class 3” sirtuin deacetylases [10]. HDAC inhibitors interfere with HDAC activity and regulate biological events, such as cell cycle, differentiation and apoptosis in cancer cells [11]. The activity of class I HDACs in the cells is regulated through three main mechanisms: subcellular localization, association with other proteins into multi subunit complexes, and posttranslational modifications (PTMs) [12]. Histone acetylation plays key roles in modulating chromatin structure and function [13]. HDACs are increased in malignant cells and HDACs involved in acquisition of malignant

phenotypes in cancer genesis [14]. HDAC inhibitors cause changes in the acetylation status of chromatin and other non-histone proteins. Change of gene expression, induction of apoptosis, cell cycle arrest, and inhibition of angiogenesis and metastasis [15]. Various treatments are being studied using HDAC inhibitors.

Cellular uptake of glucose is a fundamental process for metabolism, growth and homeostasis1. The SLC2 family glucose transporters (GLUTs) catalyse facilitative diffusion of glucose and other monosaccharides across bio membranes [16]. Glucose enters eucaryotic cells via membrane associated carrier proteins, glucose transporter facilitators (GLUT) [17]. There are 14 mammalian facilitative glucose transporters and they are subdivided into three classes based on their protein sequence and structural similarity [18]. mor cells often increase their glucose consumption and lactate production even in the presence of physiological oxygen concentrations and functional mitochondria; this aerobic glycolysis is known as the Warburg effect. This metabolic shift toward aerobic glycolysis enables cancer cells to convert glucose more efficiently into macromolecules, which are needed for rapid cell growth [19]. The expression, regulation and activity of glucose transporters play an essential role in neuronal homeostasis, since glucose represents the primary energy source for the brain [20]. Consistent with the excessively high glucose consumption of tumor cells,1 aberrant GLUT family expression has been found in various cancers [21]. High expression of GLUT3 is associated with poor survival in most cancer types interrogated, including colorectal carcinoma, breast carcinoma, lung adenocarcinoma, squamous cell carcinoma, ovarian carcinoma, and glioblastoma [22]. The detection of immunoreactive GLUT3, in the high grade gliomas suggest the GLUT3 isoform may be the predominant glucose transporter in highly malignant glial cells of human brain [23].

MicroRNAs are small noncoding RNAs that regulate the expression of protein-coding genes. MicroRNA expression profiles also classify tumors by developmental lineage and differentiation state [24]. MiRNAs were found to play key roles in vital biological processes such as cell division and death cellular metabolism, intracellular signaling ,immunity and cell movement [25] And showed that the miR-15/16 cluster is frequently deleted in chronic lymphocytic leukemia (CLL) [26]. Some miRNAs act as oncogenes and act as tumor

suppressors [27]. We demonstrated that miR-622 performed tumor suppressor function by inhibiting GLUT3 and suppressing tumorigenesis of Glioblastoma.

Materials and Methods

1. Cell culture and Reagent

The human GBM Cell lines (A172, U87MG) were maintained in high glucose DMEM media supplemented with 10% FBS and 1% Antibiotics. To produce HDAC2 knockdown GBM stable cells, cell lines were infected with Lentivirus carrying pLKO/TetON shControl (DOX-inducible shcontrol) and HDAC2 shRNA (DOX-inducible shHDAC2) plasmids, and stable cells were selected by puromycin (5 μg/ml) and treated with doxycycline (1μg/ml) for HDAC2 knockdown.

2. Cell viability Assay

Cell viability was analyzed by the MTT assay. ALL cells were seeded in 96-well plates at a density of 5 × 10^3 cells. After incubation for 24 h, 20 μL of the MTT reagent (2 mg/mL) was added to each well and incubated for 90 min at 37 ℃ in a CO2 incubator. Cells were then changed with complete DMEM media. The absorption was measured at 570 nm with a micro plate reader. All MTT assay results were presented as the means ±SD of three independent experiments.

3. Western blotting

All cells were washed and harvest with cold PBS and scrapper. Cell extracts were prepared with lysis buffer [50mM Tris-HCl (pH7.5), 150mM NaCl, 1% NP-40, 10mM NaF, 10mM sodium pyrophosphate, and protease inhibitors], incubated for 30min on ice. After incubated on ice, Lysates were centrifuged at 13,000rpm for 20min at 4℃. Supernatants were collected and lysate protein concentrations were determined by using 660nm Protein Assay Reagent (Thermo,Rockford, USA). Equal protein of cell lysates were separated on 8% and 12% SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked by 5% skim milk (BD Biosciences, Franklin Lake, NJ, USA) with 1X PBST by incubating for 1h. The blocked membranes were incubated overnight at 4℃ with the primary antibody. After washing three times with 1X PBST, the membranes were incubated with secondary HRP-

conjugated antibody for 1h. The membranes were subjected to western blot analysis and visualized by developer with ECL solution.

4. RNA extraction and RT-PCR analysis

Total RNA was extracted with the RNA extraction kit (RNA EasySpin kit, Intron Biotechnology, Korea). Total RNA was reverse transcribed with random primer using Reverse Transcriptase (PrimeScriptTM Reverse Transcriptase, Takara) according to the protocols of the manufacturer. Real time PCR was performed using an iNtRON Thermo scientific PIKOREAL 96 Real time PCR instrument and using 2X PCR Master Mix (ElpisBIO EBT-1801). All samples were normalized to human GAPDH and expressed as fold changes. All reactions were done in triplicate. Primers for amplification of GLUT3 transcript were Fwd GCC GCT GCT ACT GGG TTT TAC and Rev AAT GAT GAT GGG CTG TCG GT. Primers for amplification of BAD were Fwd ACT GAG GTC CTG AGC CGA CA and Rev CGG CTC AAA CTC TGG GAT CTG. Primers for amplification of TP53 were Fwd GAA CAA GTT GGC CTG CAC TG and Rev GAA GTG GGC CCC TAC CTA GA. Primers for amplification of BAX transcript were Fwd GGT TTC ATC CAG GAT CGA GAC GG and Rev ACA AAG ATG GTC ACG GTC TGC C. Primers for amplification of GAPDH were Fwd TGA TGA CAT CAA GAA GGT GGT GAA G and Rev TCC TTG GAG GCC ATG TAG GCC AT. Primers for amplification of miR-622 were Fwd ACA GTC TGC TGA GGT TGG AGC. Primers for amplification of Poly adaptor reverse were GCG AGC ACA GAA TTA ATA CGA C. Primer for amplification of HDAC2 transcript were Fwd ATG GCG TAC AGT CAA GGA GG and Rev ACT GAA CCG CCA GTT GAG AG.

5. Colony formation assay

GBM cells were seeded in 6-well plates at 1 × 10^3 cells/well and incubated with CO2 at 37 °C for 48 h. GBM cells transfected with miRNA were incubated for 14 days. Colonies were washed twice with PBS and fixed with distilled water containing 10% methanol and 2% paraformaldehyde. Colonies were stained with 0.5% crystal violet/20% methanol/PBS.

6. TUNEL assay

TUNEL assay (TUNEL Assay Kit – FITC, Abcam, Cambridge, UK; cat. #ab66108) was performed according to the manufacturer's protocol. In brief, 40,000 cells/cm2 were seeded, and 48 hours after transfection cells were trypsinized, washed, and fixed with 1% paraformaldehyde. Cells were then transferred to 70% ethanol, washed, and stained, as described in the manufacturer's protocol. Cells were analyzed with BD LSR Fortessa analyzer (BD Biosciences). For paraffin-embedded samples, the samples were deparaffinized, treated with 20 μg/mL proteinase K (Chemie Brunschwig; cat. #GEXPRK01-I5), and then stained, as described in the protocol.

7. Flow cytometric analysis

To assess the extent of apoptosis after DNA damage, cells were stained with both Annexin V-FITC and propidium iodide according to the manufacturer's protocol using the Dead Cell Apoptosis Kit with Annexin V Alexa Fluor® 488 & PI for Flow Cytometry (Invitrogen, V13241) for 15 min at room temperature. Cells were analyzed using a BD FACS Canto II cytometer (Becton Dickinson).

8. Mouse brain injection

The mouse was anesthetized by using the Avertin, the mouse skulls were fixed using a stereotactic device, and a hole was made in the skulls using a drill (SAESHIN, Strong207A).

Mouse was injected with 1 × 10^6 U87MG cells. After 1week, half of all was injected with miR-622.

9. Animal PET imaging and Analysis

PET-MRI fused imaging was performed using a nano ScanPET/ MRI system (1 T, Mediso, Hungary). Mice were kept warm, and 7.5 ± 1.0 MBq in 0.2 mL of FDG was administered intravenously via the tail vein to keep the mouse under anesthesia (1.5% isoflurane in 100% O2gas). MR brain imaging obtained T1 weighted with Gradient- echo (GRE) 3D sequence (TR = 25 ms, TEeff = 3, FOV = 50 mm, matrix = 128 × 128) and T2 weighted with Fast Spin Echo (FSE) 3D Sequence (TR = 2400 ms, TE eff = 110, FOV = 50 mm, matrix = 256 × 256)

images, which were acquired during the FDG uptake period. 20 min of static PET images were acquired in a 1–3 coincident in a single field of view with MRI range. Body temperature was maintained with heated air (37 °C) on the animal bed (Multicell, Mediso, Hungary). PET images were reconstructed by Tera-Tomo 3D, in full detector mode, with all the corrections on, high regularization and 8 iterations. Three-dimensional volume of interest (VOI) analysis of the reconstructed images was performed using the InterView Fusion software package (Mediso, Hungary) and applying standard uptake value (SUV) analysis. VOI were fixed with a diameter of 1.5 mm sphere and were drawn for the tumor and cerebellum site. The SUV of each VOI sites was calculated using the formula: SUVmean = tumor radioactivity in the tumor volume of interest with the unit of Bq/cc × body weight (g) divided by injected radioactivity.

10. Immunohistochemistry analysis

The tissues were sectioned 4 μm thick on paraffin-embedded slides. Tissue slides were incubated at 60°C for 1 h and then deparaffinized with xylenes and rehydrated with 100, 95, 90, 75, 50, and 0% ethanol. The primary antibody was incubated in the tissues overnight at 4°C. Antibodies used GLUT3 (Cell signaling, 1:1000). The IHC process was carried out using the PROCAM IHC kit (Abcam, MA USA). Digital images were obtained through (OLYMPUS-cellSens Standard). Quantitative analysis of the images was performed using Image J (NIH).

Results

1. Analysis of miRNAs and glucose transports in HDAC2 knockdown GBM cells

Previous studies have shown that overexpression of HDAC2 is key role to tumorigenesis. Silencing of HDAC2 promoted cell apoptosis and suppressed cell proliferation, migration, and invasion in GBM cells. We first investigated the expression of HDAC2 in human glioma patients via analysis of the TGCA public databases. HDAC2 was highly increased in GBM than Normal (Figure 1A). We also analyzed heatmap of miRNA expression in DOX-inducible shHDAC2 A172 cells w/wo doxycycline. These results showed that HDAC2 KD induced miRNA up-regulation. And the miR-622 expression level was most high among the other miRNA levels (Figure 1B). In addition, HDAC2 knockdown induced the glucose transporter protein level decreased. Glucose transporter 3 (GLUT3) was most downregulated among transcripts (Figure 1C). These finding suggest that HDAC2 knockdown regulates miRNA expression and glucose transport level.

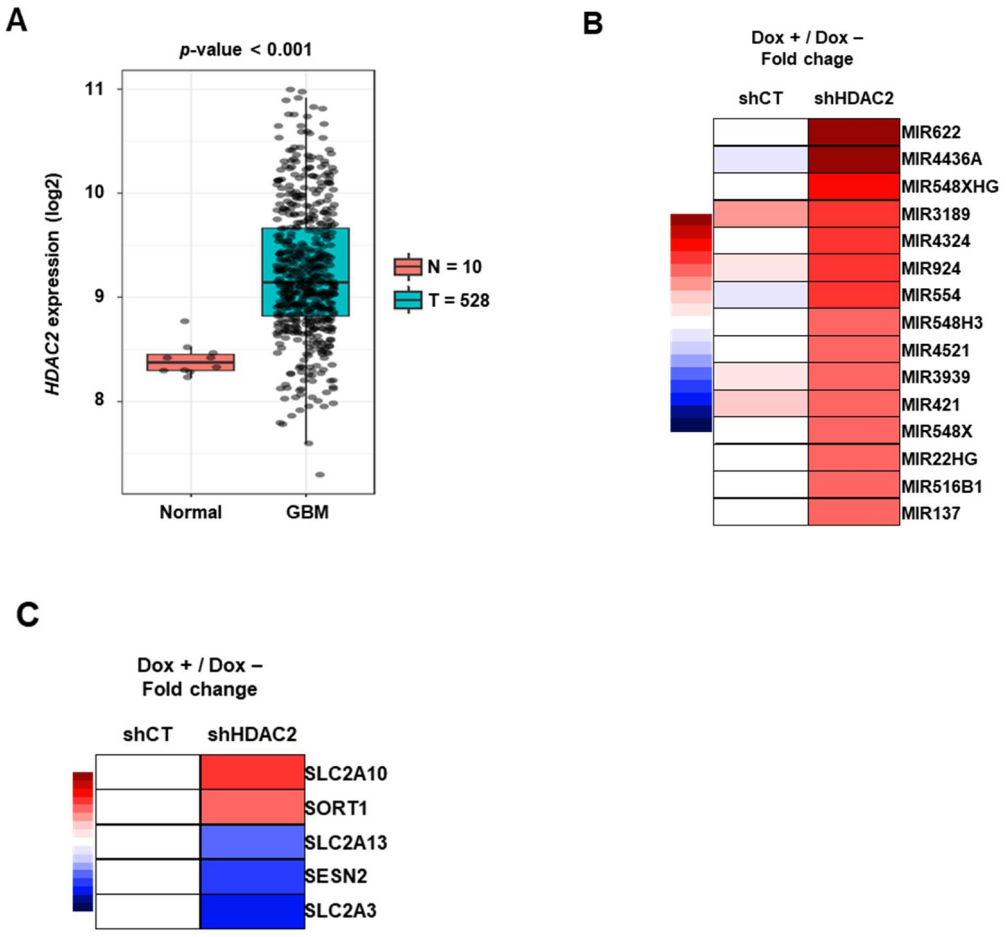

Figure 1. Bioinformation of GBM cells and analysis of mRNA sequence

(A) Expression of HDAC2 was detected high in GBM tissue more than in normal tissue.

(B) RNA-seq of compared pLKO/TetON HDAC2 shRNA infected A172 cell with DOX and without DOX. HDAC2 knockdown regulated miRNA gene.

(C) RNA-seq of compared pLKO/TetON HDAC2 shRNA infected A172 cell with DOX and without DOX. HDAC2 knockdown regulated GLUT family gene.

2. miR-622 promotes apoptosis by regulating GLUT3 expression

In order to identify the effect of miR-622 in GBM cells (U87MG and A172), we transfected miR-Control and miR-622 to GBM cells. We found that the transfected GBM cells (U87MG and A172) with 300pmol for 24h hours affected cell death. First, we detected miR-622 expression level by analyzed qRT-PCR, A172 increased 20 times and U87MG increaed 5 times than control GBM cells (U87MG and A172) (Figure 2A). We confirned the miR-622 was expressed. And as demonstrated in figure 2B, we analyzed protein levels by western blotting. We detected transfected with miR-622 increased PARP cleavge, Apaf1, Cleavged Caspase3 and BAX. However the protein level of GLUT3 was decreased. Also we tested the transcription level of pro-apoptosis target genes, BAD, TP53 and BAX. We carried out the qRT-PCR analysis in GBM cells (U87MG and A172) transfected with miR-Control and miR-622. The tanscription levels of BAD, TP53 and BAX were enhanced by expressing miR-622 in GBM cells (U87MG and A172) (Figure 3B-3D). And the transcription level of GLUT3 is decreased (Figure 3A). Thus these reults show that expression of miR-622 down regulated GLUT3 and induced apoptosis.

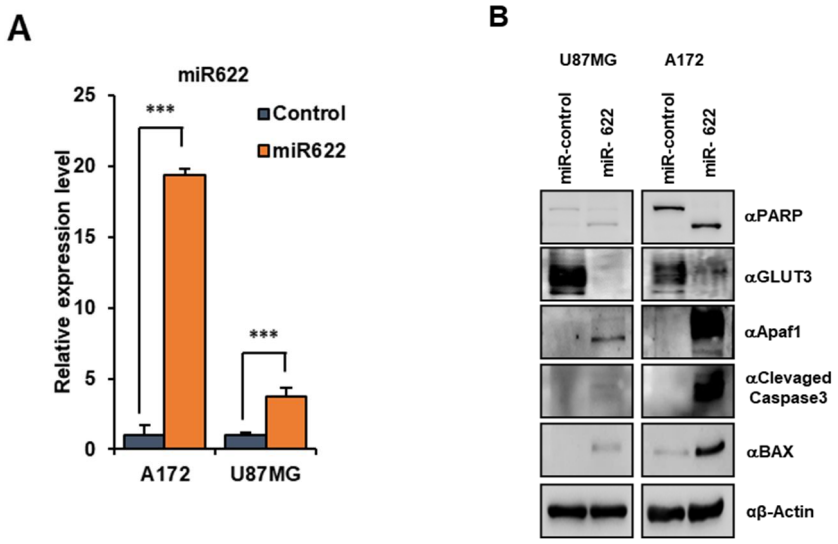

Figure2. miR-622 promotes apoptosis by regulating GLUT3 expression in GBM cells

(A) GBM cells (U87MG and A172) were transfected with miR-control, miR-622 for 24h and mRNA expression level was analyzed by qRT-PCR. All data are expressed as the mean ± SD for triplicates.

(B) Western blotting of PARP, GLUT3, Apaf1, Clevaged Caspase3, Bax, and β-Actin in GBM cells (U87MG and A172)

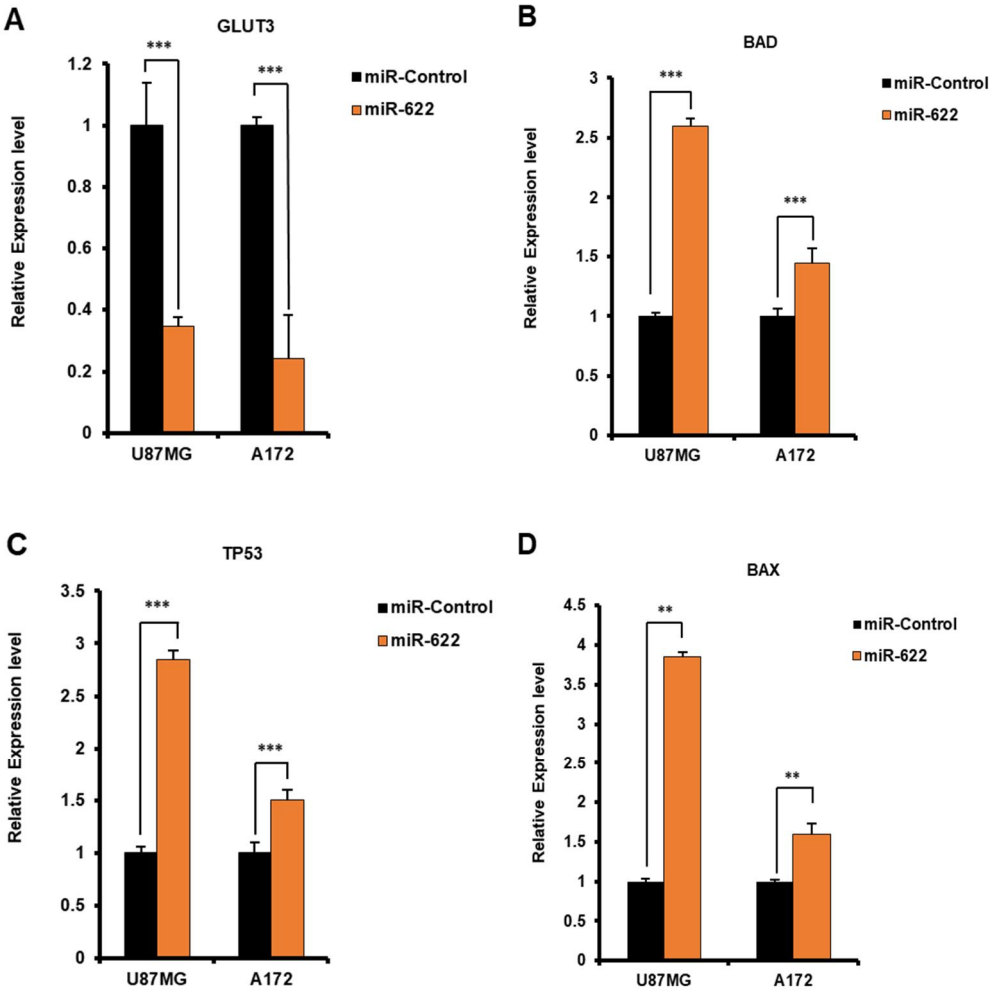

Figure 3. The transcription level of GLUT3, BAD, TP53 and BAX in GBM cells

(A) GBM cells (U87MG and A172) transfected with miR-Control and miR-622 for 24h. GLUT3 mRNAs expression was analyzed by perforemd RT-PCR

(B) GBM cells (U87MG and A172) transfected with miR-Control and miR-622 for 24h. BAD mRNAs expression was analyzed by perforemd RT-PCR

(C) GBM cells (U87MG and A172) transfected with miR-Control and miR-622 for 24h. TP53 mRNAs expression was analyzed by perforemd RT-PCR

(D) GBM cells (U87MG and A172) transfected with miR-Control and miR-622 for 24h. BAX mRNAs expression was analyzed by performed RT-PCR.

All data are expressed as the mean ± SD for triplicates.

3. miR-622 suppressed the colony formation of GBM cells

Cancer progression is related to colonization. Thus we perforemd colony formation assay to confrimed that miR-622 involved in colony formation. First we subcultured GBM cells (U87MG and A172) in 1 × 10^3 on 6-well plates. After 48h, GBM cells (U87MG and A172) were transfected with miR-Control and miR-622 for 300pmol. And we confirmed colony formation continuously for 14 days. After 14 days we counted colony unit and detected that the colony formation was increased in GBM cells (U87MG and A172) transfected with miR-Control but miR-622 was not compared with miR-Control. GBM cells (U87MG and A172) transfected with miR-622 significantly decreased the colony formation (Figure 3A-3B). These data demonstrated that miR-622 reduced colony forming unit. And we expected that miR-622 have an affect to formation of cancer.

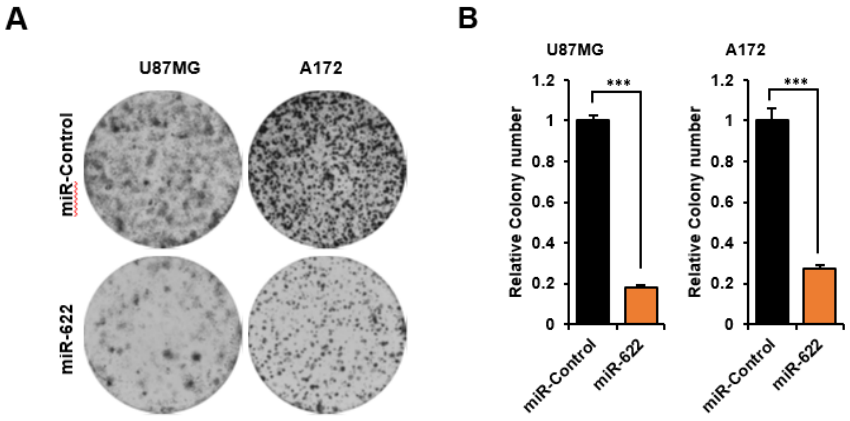

Figure 4. miR-622 suppressed the colony formation of GBM cells

(A) GBM cells (U87MG and A172) were subcultured in 1 × 10^3 media (10% FBS, 1% Antibiotics) on 6 well plates. 48 hours later miR-Control and miR-622 transfected in GBM cells (U87MG and A172). The plate shown in was stained with crystal violet to count colonies after transfection for 14 days.

(B) The quantification results are presented on the right. All data are expressed as the mean ± SD for triplicates.

4. miR-622 induced apoptotic cell death in GBM cells

To assess the miR-622 reduces cell viability in GBM cells, we transfected miR-control and miR-622 in GBM cells (U87MG and A172). We performed MTT assays to confirmed cell viability. We found that expression of miR-622 decreased the cell viability in GBM cells (U87MG and A172) compared to transfected with miR-control (Figure 4A). So, we demonstrated that miR-622 is related with GBM cells viability. Furthermore, we detected apoptotic cell death by FACS analysis. FACS data showed the increase ratio of Q2+Q4 apoptosis cell by expression of miR-622 (Figure 4B). These data demonstrated that miR-622 induced cell apoptosis.

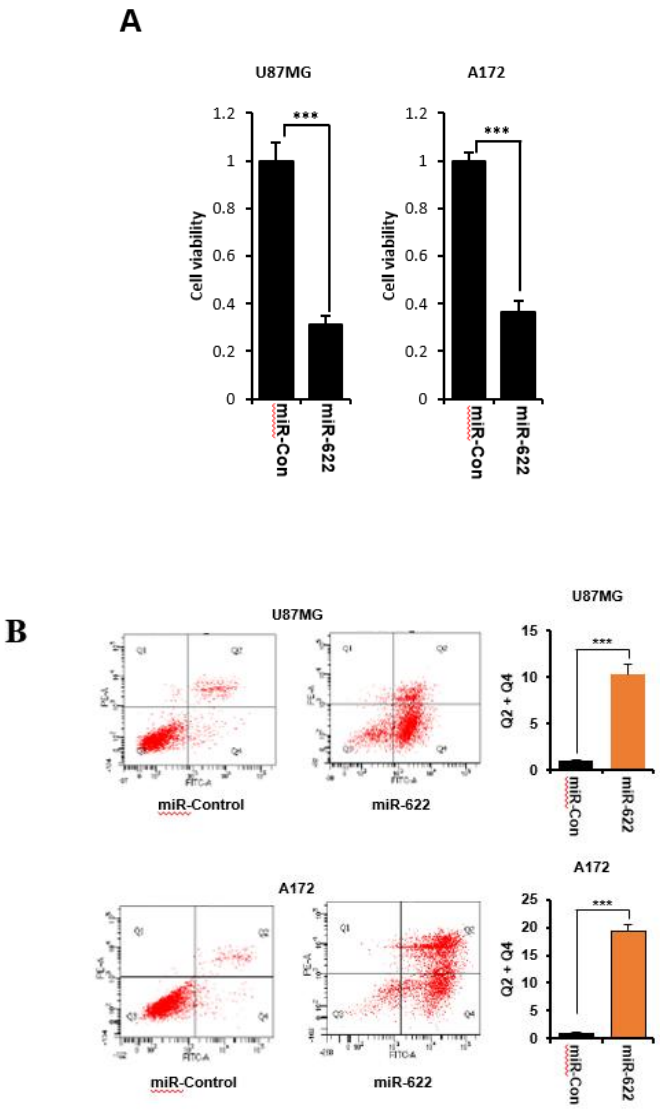

Figure5. miR-622 decreased the cell viability of GBM cells

(A) GBM cells (U87MG and A172) were transfected with miR-control and miR-622 and the cell viability was detected by MTT assays.

(B) FACS analysis of Annexin V/PI staining of apoptotic GBM cells (U87MG and A172) expressing miR-control and miR-622. Graphic representation of apoptosis rate of each group.

All data are expressed as the mean ± SD for triplicates.

5. miR-622 promotes DNA damage in GBM cells

Apoptosis is a common feature of many cancers. One of the characteristics of apoptosis is DNA fragment by nucleus. DNA is fragmented by damage and generates DNA double-strand breaks (DSBs). The DSBs was detected by dUTP staining. Thus, we analyzed TUNEL assay to confirm DNA apoptotic level by miR-622. We transfected 300pmol of miR-control and miR-622 in GBM cells (U87MG and A172) for 24h. We collect single cell and staining with staining solution. The result showed that dUTP was significantly increased GBM cells (U87MG and A172) transfected with miR-622. But we couldn't confirm the expression of dUTP staining in miR-Control (Figure 6A). This result demonstrated that miR-622 involved in DNA damage in GBM cells (U87MG and A172).

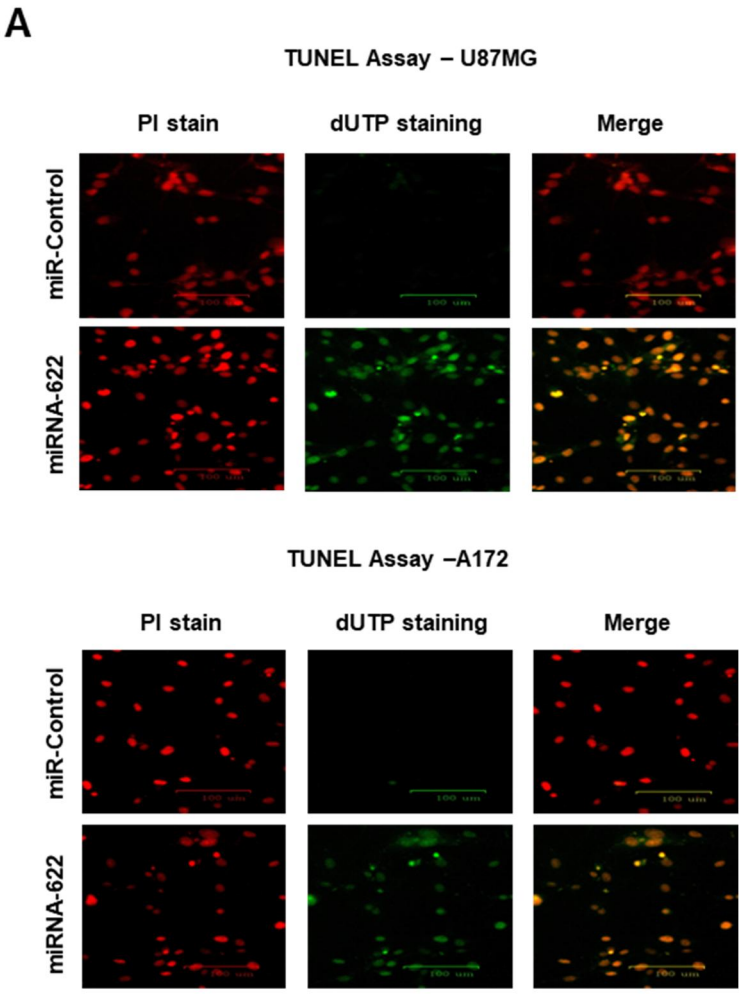

Figure 6. miR-622 increased DNA damage apoptosis of GBM cells

(A) Detection of DNA fragment and analyzed by TUNESL assay in GBM cells (U87MG and A172) transfected with miR-control and miR-622. TUNEL-positive cells were stained with green and nuclei were counterstained with PI (red).

6. miR-622 directly regulates GLUT3 expression

Previous western blot data showed that miR-622 regulates GLUT3 expression and induced apoptosis in GBM cells (U87MG, A172) (Figure 2B). Furthermore, to investigate miR-622 directly regulated GLUT3, we performed luciferase reporter assays. First, we found that miR-622 contained a complementary sequence to the GLUT3-CDS, which might suppress GLUT3 expression. (Figure 7A). And miR-622 and GLUT3-CDS primrGLO-WT plasmid or GLUT3-CDS primrGLO-MT plasmid were co-transfected in 293T and GBM cells (U87MG, A172). We confirmed 293T was transfected with miR-622 by performed qRT-PCR. Luciferase activity expression level decreased in 293T, GBM cells (U87MG, A172) transfected with miR-622 and GLUT3-CDS primrGLO-WT. These data showed that miR-622 bind to GLUT3-WT sequence and regulation GLUT3 expression. However, luciferase activity upon pmirGLO-GLUT3-MT did not regulate luciferase activity in 293T and GBM cells (U87MG, A172) because miR-622 didn't bind to GLUT3-MT sequence. (Figure 7B). These results indicated that miR-622 suppressed GLUT3 expression by binding GLUT3-CDS and miR-622 directly regulated GLUT3.

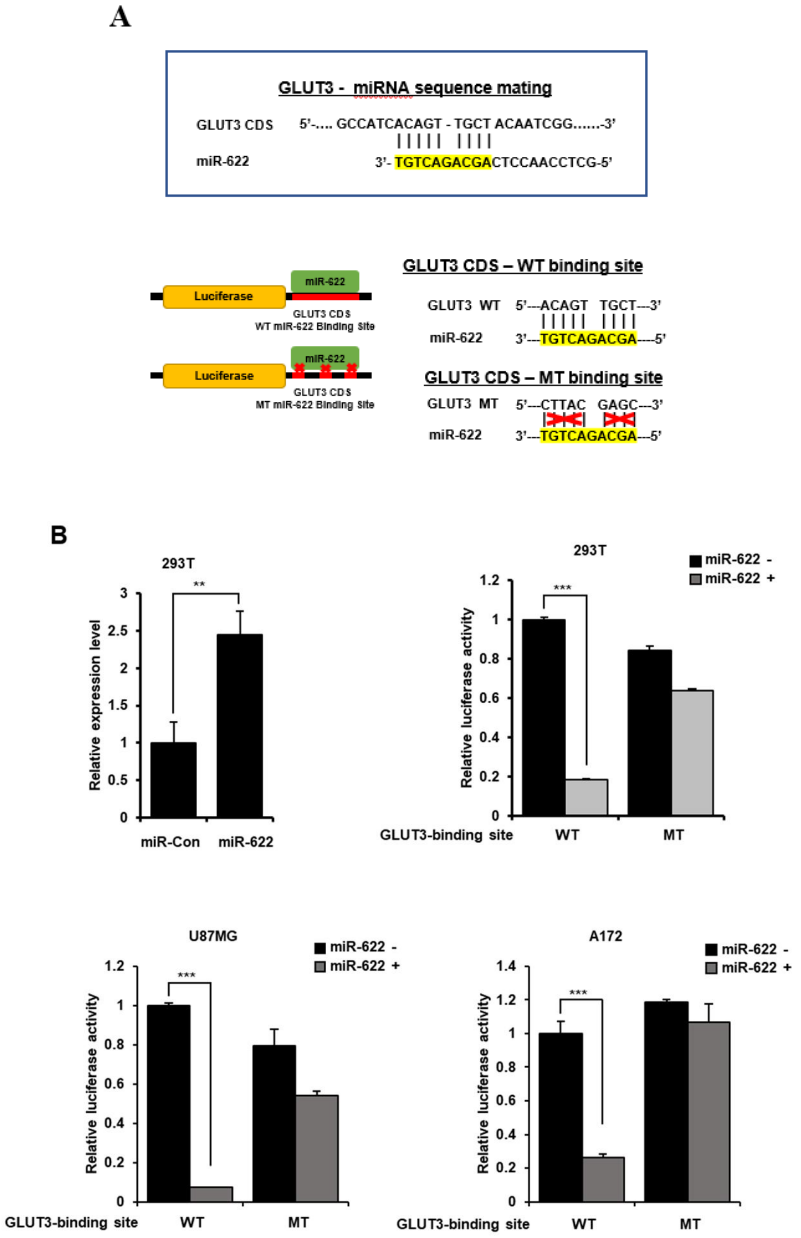

Figure7. GLUT3 expression is regulated by miR-622

(A) Binding site sequence GLUT3 and miR-622. 293T cell were transiently transfected with reporter construct primrGLO-GLUT3WT-Luc or primrGLO-GLUT3MT-Lu.

(B) 293T and GBM cells (U87MG and A172) were transfected with miR-control and miR-622 were harvested and protein extracted, and the dual luciferase activity was measured. All data are expressed as the mean ± SD for triplicates.

7. HDAC2 knockdown regulates miR-622-mediated GLUT3 expression

HDAC2 is known as promotes GBM cells proliferation and affect to GBM cells death. And silence of HDAC2 suppress growth of GBM cells. Previously, we studied that HDAC2 is overexpression in GBM than normal (Figure 1A). Also, we confirmed that HDAC2 knockdown induced downregulated GLUT3 (Figure 1C). We analyzed miR-622 directly regulated GLUT3 (Figure 2B, Figure 7). Moreover, to demonstrate that mir-622 mediated GLUT3 expression is regulated by HDAC2, we performed next study. Firstly, we transfected miR-622 in DOX inducible shHDAC2 GBM cells (U87MG, A172) w/wo doxycycline. We detected miR-622 expression level by analyzed qRT-PCR. The results showed that miR-622 expression increased in DOX-inducible shHDAC2 GBM cells. These data demonstrated that silencing of HDAC2 promoted miR-622 expression (Figure 8A). Next, we perforemd the luciferase reporter assay. We transfected pmirGLO-GLUT3WT and pmirGLO-GLUT3MT containing the miR-622 binding site. We sought the lucuferase activity of pmirGLO-GLUT3WT in HDAC2 knockdown GBM cells was decreased. However there is almost no activity change in transfected pmirGLO-GLUT3MT (Figure 8B). We validated HDAC2 knockdown significantly decreased the luciferase activity and induced GBM cell death via inhibition of miR-622-mediated GLUT3 expression.

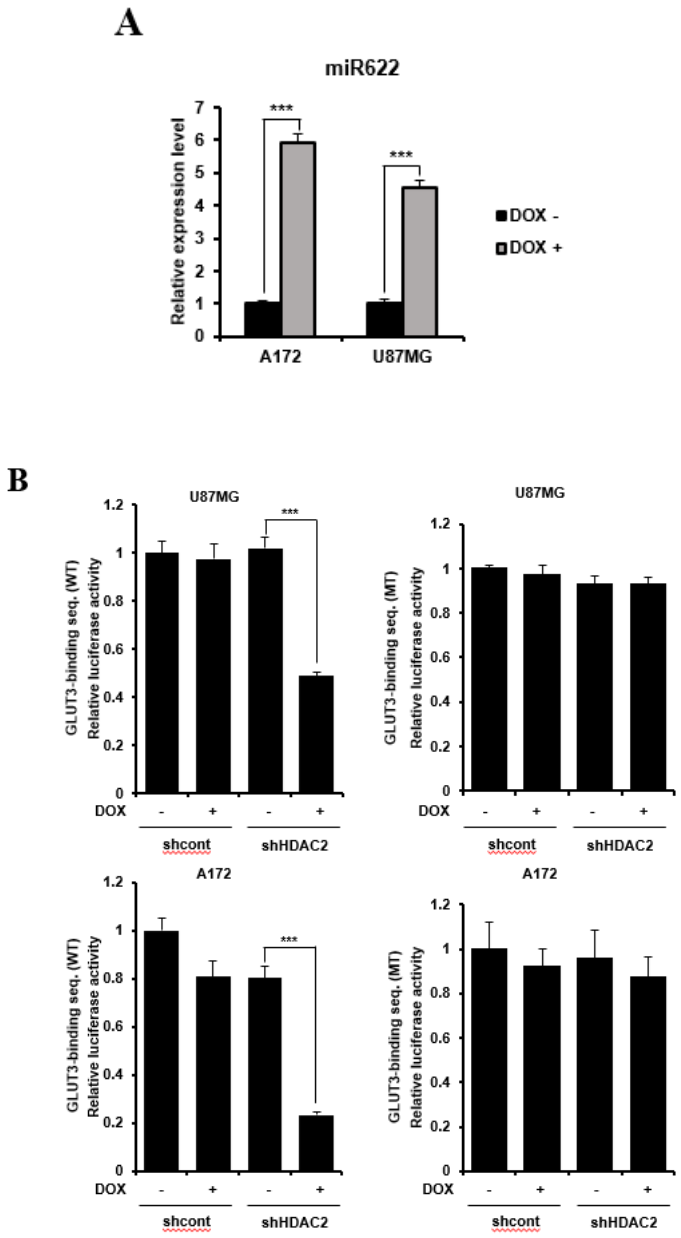

Figure8. HDAC2 knockdown inhibited miR-622-mediated GLUT3 transcription activity

(A) GBM cells (U87MG and A172) were transfected with DOX-inducible shHDAC2 GBM cells w/wo doxycycline.

(B) Luciferase reporter assays using pmirGLO-GLUT3-Luc plasmid transiently transfected into DOX-inducible shHDAC2 GBM cells.

All data are expressed as the mean ± SD for triplicates.

8. Downregulated GLUT3 promotes GBM cell death by expressing HDAC2 knockdown and miR-622

GBM is contained high expression of HDAC2 and HDAC2 knockdown promoted miR-622 expression. To analyzed both HDAC2 knockdown and miR-622 promote GBM cells apoptosis we transfected doxycycline 1ul/ml for 24h in GBM cells (U87MG and A172) to induce HDAC2 knockdown. After 24h we transfected miR-Control and miR-622 300pmol for 24h. First, we confirm that HDAC2 expression level is decreased in shHDAC2 expression GBM cells (U87MG and A172) with doxycycline. And when HDAC2 knockdown in GBM cells (U87MG and A172) showed increased PARP cleavge and apoptosis marker such as Apaf1, Cleavged Caspase3 and BAX. However, the GLUT3 expression level is decrease. Likewise, GBM cells (U87MG and A172) transfected with miR-622 increased apoptosis marker and decreased GLUT3. Also, HDAC2 knockdown GBM cells (U87MG and A172) transfected with miR-622 showed more increased level of apoptosis marker. So, we demonstrated that silenced of HDAC2 and induced of miR-622 promotes apoptosis by downregulated GLUT3 (Figure 9A). And we confirmed miR-622 mRNA expression levels were increased in HDAC2 knockdown GBM (U87MG and A172) cells by treated DOX. Moreover, we conducted RT-PCR to confirm the mRNA expression level of HDAC2, GLUT3 and BAX. When the miR-622 mRNA level was increased and HDAC2 mRNA level is decreased the GLUT3 mRNA level was downregulated. And it significantly induced cell apoptosis by analyzed BAX mRNA expression level (Figure 9C-9E). We expected more effective way for death of GBM cells by regulated HDAC2 through miR-622-mediated GLUT3.

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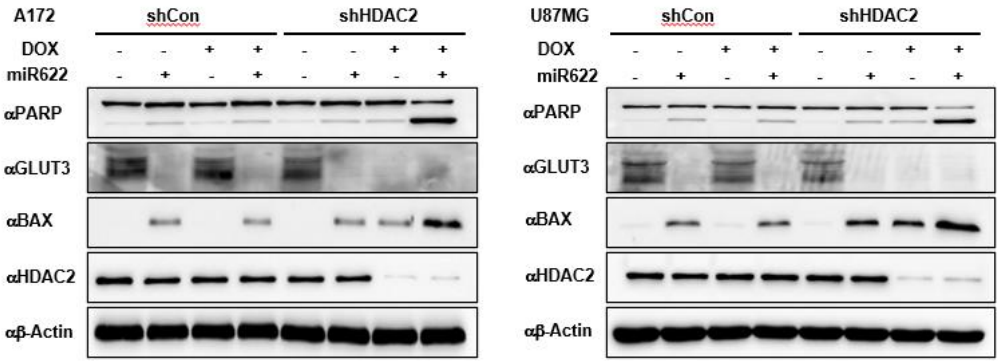
A172
shCon
shHDAC2
DOX
miR622
αPARP
αGLUT3
αBAX
αHDAC2
αβ-Actin
U87MG
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shHDAC2
DOX
miR622
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αHDAC2
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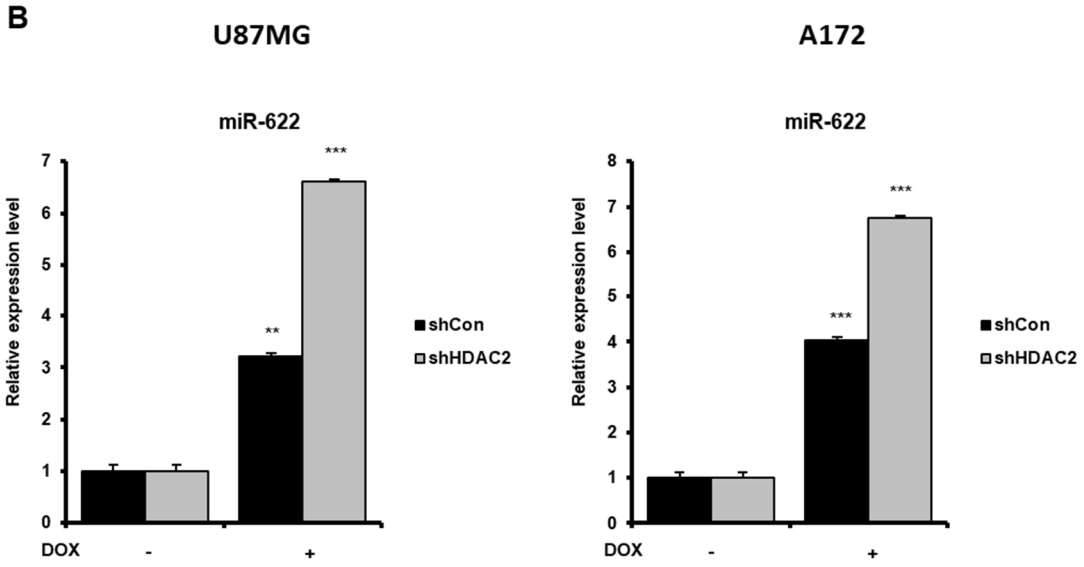
U87MG
miR-622
Relative expression level
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A172
miR-622
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shCon
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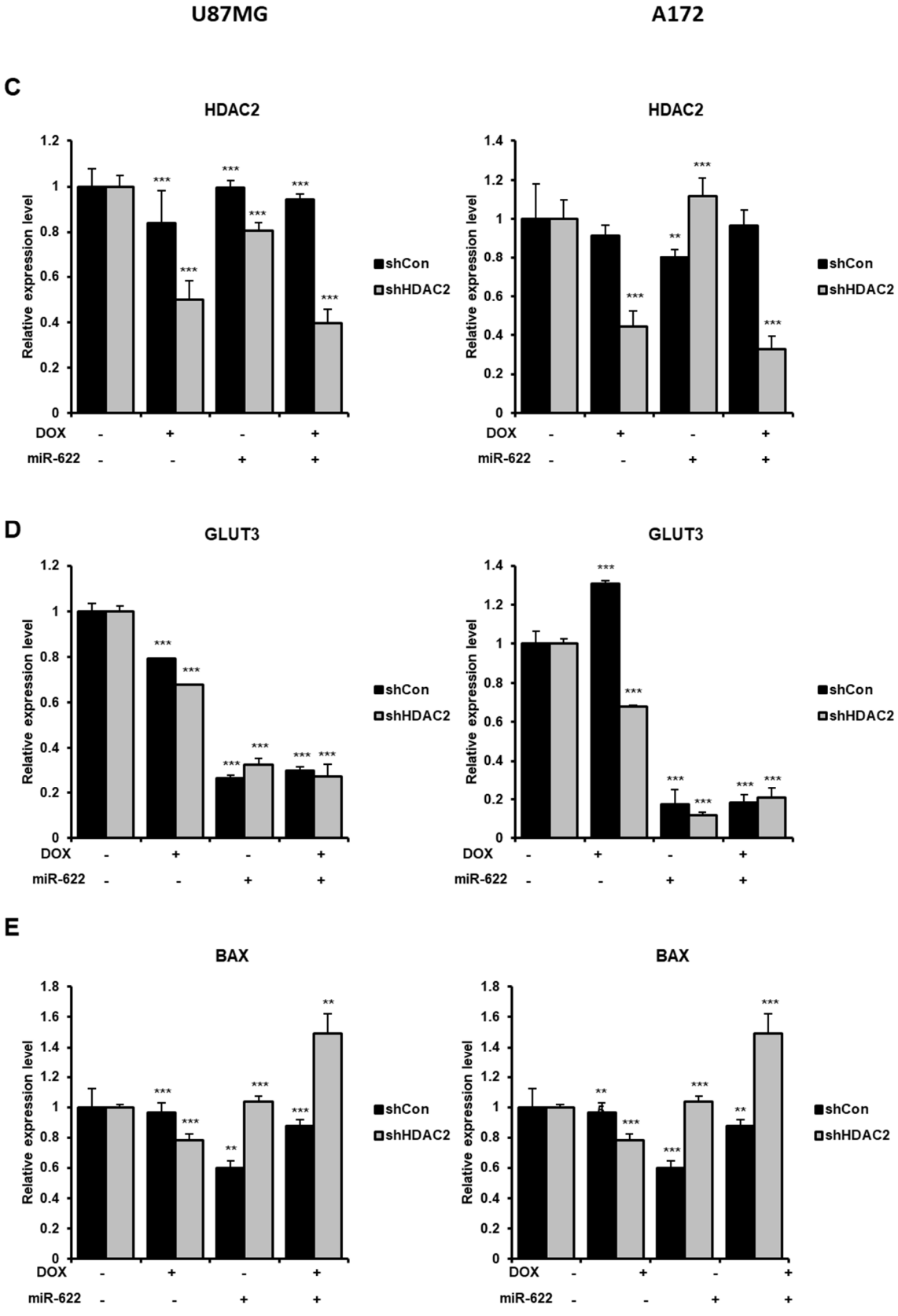

U87MG
A172
C
HDAC2
HDAC2
Relative expression level
shCon
shHDAC2
DOX
miR-622
D
GLUT3
GLUT3
E
BAX
BAX

Figure9. HDAC2 knockdown and miR-622 promotes cell apoptosis

(A) Western blotting of PARP, GLUT3, BAX, HDAC2, β-Actin in stable GBM cells (U87MG and A172) by treated DOX that were transfected with miR-622.

(B) Stable GBM cells (U87MG and A172) by treated DOX. miR-622 mRNAs expression was analyzed by perforemd RT-PCR.

(C) Stable GBM cells (U87MG and A172) by treated DOX that were transfected with miR-622. HDAC2 mRNAs expression was analyzed by perforemd RT-PCR.

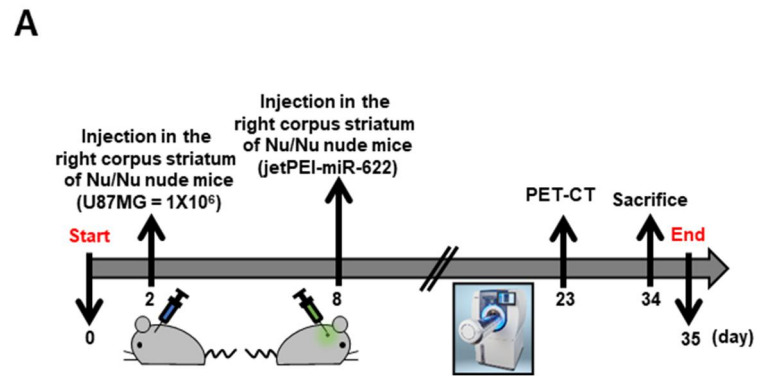
(D) Stable GBM cells (U87MG and A172) by treated DOX that were transfected with miR-622. GLUT3 mRNAs expression was analyzed by perforemd RT-PCR.

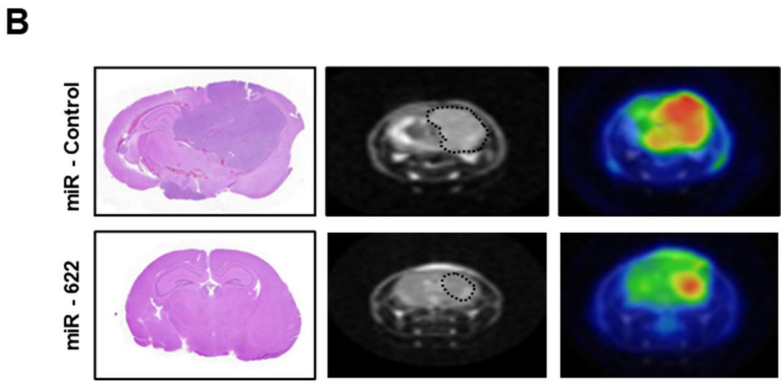
(E) Stable GBM cells (U87MG and A172) by treated DOX that were transfected with miR-622. BAX mRNAs expression was analyzed by perforemd RT-PCR.

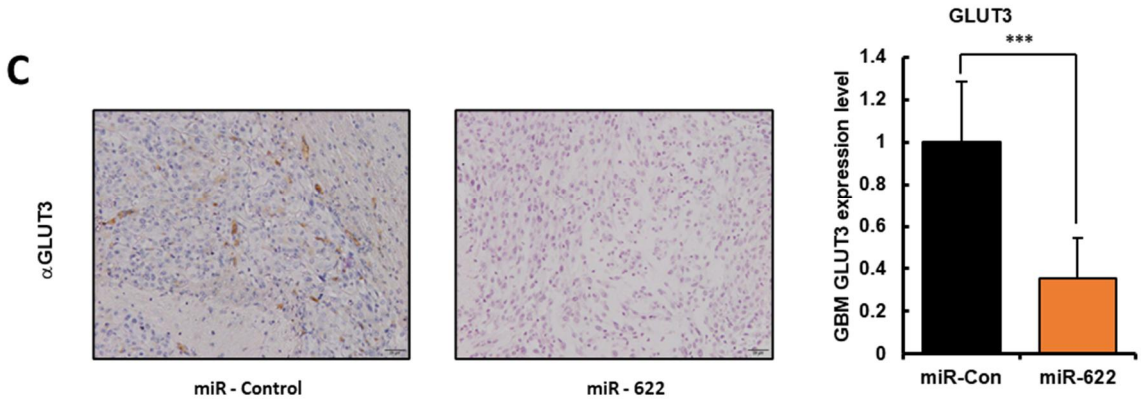
All data are expressed as the mean ± SD for triplicates.

9. miR-622 suppresses GBM tumorigenesis in orthotopic mouse model

To demonstrate miR-622 suppresses tumorigenesis in vivo we injected 1 × 10^6 U87MG cells into the brain of immune-deficient BALB/C nu/nu mice, after 1week JetPEI-miR-622 was directly injected in same sites (Figure 10A). We sectioned the brain of mice and stained with H&E. Also, we detected MRI images and PET-CT images to measure the in vivo efficacy of miR-622. We confirmed that SUV ratio of the red image in the mice brain highly increased in miR-control treated mice but not in miR-622 treated mice. And the tumor growth in U87MG injected mice by miR-622 treatment was inhibited than miR-control treatment by H&E analysis. These results indicated that miR-622 effectively inhibited GBM tumorigenesis (Figure 10B). Previous study, we have identified that miR-622 regulated GLUT3 protein and mRNA expression level. We also confirmed that GLUT3 expression level was significantly decreased in U87MG injected mice brain tissues treatment with miR-622 using IHC analysis (Figure 10C). Moreover, we measured the mouse weight continuous for 35 days. We observed the bodyweight of U87MG-injected mice by miR-Control and miR-622 treatment and were steadily maintained by miR-622 treatment. However, miR-622 untreated mice rapidly decreased (Figure 10D). So, we predicted that miR-622 related with cancer prognosis and survival.


A
Injection in the right corpus striatum of Nu/Nu nude mice (U87MG = 1X10⁶)
Injection in the right corpus striatum of Nu/Nu nude mice (jetPEI-miR-622)
PET-CT
Sacrifice
Start
End
0
2
8
23
34
35 (day)


B
miR - Control
miR - 622


C
αGLUT3
miR - Control
miR - 622
GLUT3
***
GBM GLUT3 expression level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
miR-Con
miR-622

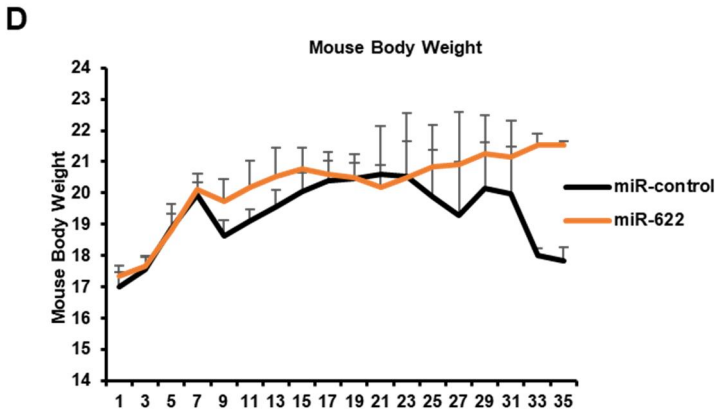


Figure 10. GBM Tumor growth suppressed by miR-622 expression with in vivo orthotopic mouse model

(A) Schematic outline of in vivo experimental procedure.

(B) Tumorigenesis in orthotopic mouse model transfected with miR-622.

(C) IHC Staining level of GLUT3 in mouse GBM tissues transfected with miR-622 (Left). Quantitative analysis of the images was performed using Image J (Right).

(D) Mouse body weight of in vivo mouse GBM models by miR-622 transfection.

Discussion

Glioblastoma, the most common primary brain tumor in adults, is usually rapidly fatal. Many multimodal treatments are known but prognosis poor, less than 5% of patients surviving at 5 years following initial diagnosis [28]. Genetics, epigenetics, and many other factors affects GBM oncogenesis [29].

HDAC2 is one of the HDAC family and HDAC members are highly expressed in various types of cancers. Their function is related to regulate oncogenic and malignant disease [29]. HDAC2 is also overexpression in GBM. Recently HDAC2 inhibitors were reported to be used for GBM treatment. HDAC2 knockdown inhibited cell growth and regulate cancer metabolism [30]. We confirmed that HDAC2 is overexpression in tumor than normal by analyzed bioinformation. Also, when HDAC2 is silenced miR-622 expression was increased so we expected that miR-622 can suppress tumorigenesis.

MicroRNAs are known to function as tumor inhibitor. Also, deregulation of miRNA expression led to a variety of disorders, including human cancer [31]. MicroRNAs induced cell apoptosis, reduced cell growth and survival. To demonstrate miR-622 efficacy we transfected miR-Control and miR-622 in GBM cells (U87MG and A172). We detected cell apoptosis markers PARP, Apaf1 and BAX. The protein expression levels were increased in overexpression miR-622 GBM cells (U87MG and A172) more than transfected with miR-Control in GBM cells (U87MG and A172). And we can confirm that the GLUT3 expression level was decreased by miR-622. We predicted miR-622 regulates GLUT3 downregulation. And we performed colony formation assay to demonstrate whether miR-622 is related to colony formation.

Cancer is related with tumorigenesis, and it is involved in cell migration and metastasis. Metastasis has a lot of influence on the prognosis of patients [32]. And it is involved in cancer cells colonization. We detected colony formation was reduced in GBM cells (U87MG and A172) transfected with miR-622. These results showed that miR-622 suppressed cancer cell colonization. Moreover, we detected cell viability and apoptosis by MTT assay and FACS analysis. The cell viability was decreased in overexpression miR-622 compared to transfected

miR-Control. The FACS analysis also showed that miR-622 induces apoptosis in GBM cells (U87MG and A172).

Cellular responses of DNA damage was reported to related with cancer development and chemotherapy [33]. We detected the DNA damage by TUNEL assay. The GBM cells (U87MG and A172) transfected with miR-622 was detected DNA damage by dUTP staining. We investigated miR-622 regulated GLUT3 expression by Western blotting. Cancer cells showed increased glucose uptake and it mediated by glucose transporter. GLUT3 is known as overexpression in many human cancers [34].

We further demonstrated that miR-622 directly involved in regulation of GLUT3 by luciferase reporter assays. The expression level of luciferase activity was decreased in GBM cells transfected with miR-622 and GLUT3-CDS primrGLO-WT. But there is no change with expression level in GBM cells transfected with miR-622 and GLUT3-CDS primrGLO-MT. These result revealed that miR-622 contained a complementary sequence to the GLUT3-CDS, can suppress GLUT3 expression. miR-622 expression level is correlated with HDAC2 knockdown. Silence of HDAC2 promoted overexpression of miR-622. To demonstrate this database, we transfected GBM cells with transfected doxycycline and induced HDAC2 knockdown GBM cells (U87MG and A172) and analyzed miR-622 mRNA expression level by qRT-PCR. And luciferase reporter assays showed that primrGLO-GLUT-WT in DOX-inducible shHDAC2 GBM cells decreased the activity level of luciferase. GBM cells transfected with GLUT3-CDS primrGLO-WT luciferase activity was decreased. We sought GLUT3 regulation mediated miR-622 is regulated by HDAC2.

Several studies showed that HDAC2 knockdown regulated the glycolysis. To analyze HDAC2 knockdown regulate GLUT3 expression level and cell apoptosis, we performed western blotting and RT-pcr. GBM cells (U87MG and A172) expressing shControl and shHDAC2 transfected with miR-622 showed the protein level of GLUT3 was downregulated and cell apoptosis level was increased. Moreover, expressing shHDAC2 GBM cells (U87MG and A172) showed apoptosis marker expression was more upregulated than shControl when transfected with miR-622. These results suggested that HDAC2 knockdown and overexpression of miR-622 regulated GLUT3 and induced cell apoptosis.

Next, we conducted mouse brain injection to demonstrate that miR-622 suppressed tumorigenesis in vivo. The H&E staining, MRI images and PET images showed that the tumor size was decreased in U87MG injected with miR-622 mouse. And we confirmed that GLUT3 expression level was decreased in U87MG injected mice brain tissues upon miR-622 treatment.

In conclusion, we demonstrated that miR-622 induced cell apoptosis in GBM (U87MG and A172) cells by repressing GLUT3 activation. Also, we anticipated that HDAC2 depletion could be a new effective biomarker with miR-622 for therapy of GBM by regulating glucose metabolism activation.

Conclusion

Glioblastoma (GBM) is the most common and aggressive primary brain tumor. HDAC is known to be overexpressed in various cancer and regulates expression of several other genes related to angiogenesis, differentiation, proliferation, and apoptosis. HDAC inhibitors act as tumor suppressor genes. Thus, we confirmed that HDAC2 is overexpression in tumor. When HDAC2 is silenced, miR-622 upregulated. To study the effects of miR-622 in GBM cells, we transfected miR-622 in GBM cells and demonstrated miR-622 induced cell apoptosis and regulated GLUT3 expression level. HDAC2 knockdown also induced GBM cells apoptosis. The increased of miR-622 inhibited GLUT3 expression by attachment of GLUT3-CDS binding site. MiR-622 was showed that the tumor size is decreased in U87MG injected the mouse in vivo model. This result showed that miR-622 is effective to suppress GBM tumorigenesis. In conclusion, our results suggested the novel therapy of GBM by regulating glucose metabolism through the effect of miR-622 and HDAC2 inhibition to suppress tumorigenesis.

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Abstract (In Korean)

GLUT3를 표적으로 하는 miR-622를 이용한 glioblastoma 종양화 억제 기전 연구

교모세포종은 가장 흔하고 공격적인 악성 뇌종양으로 사망률이 높으며 예후 또한 좋지 않다. 히스톤 탈아세틸라제 2 는 다양한 암에서 과발현되며 GBM 진행과 상관관계가 있다. 또한 GLUT3는 GBM에서 상향조절 되어있다. 우리는 GLUT3 를 조절함으로써 GBM 세포에서 마이크로 RNA-622 의 역할을 연구했다. 마이크로RNA-622는 GBM의 세포 사멸과 밀접한 관계가 있었다. 마이크로RNA-622는 GLUT3 단백질 수준을 상당히 하향 조절하고 U87MG 및 A172 세포에서 세포 사멸을 유도하였다. 또한, 콜로니 형성 분석을 통해 GLUT3 조절에 의해 마이크로 RNA-622 가 과발현된 GBM 세포에서 콜로니 형성이 감소되었음을 보여주었다. GBM 세포 생존 수준은 마이크로R-622에 의해 감소된다. 또한, FACS 분석 결과는 마이크로 RNA-622 가 세포 사멸을 유도함을 보여주었다. 그리고 TUNEL assay 를 통해 마이크로 RNA-622 가 DNA 손상을 유도함을 확인하였다. 히스톤 디아세틸라제 2 의 결실은 GLUT3 를 하향 조절한다. GLUT3-CDS 는 마이크로 RNA-622 와 상호 작용하는 것으로 밝혀졌다. GBM 세포의 히스톤 디아세틸라제 2의 결실은 마이크로RNA-622로 세포 사멸을 유도하고 GLUT3은 감소한다. 마우스 모델에서도 마이크로RNA-622가 GBM 종양 형성을 억제한다는 것을 보여주었다. 이러한 결과에서 우리는 히스톤 디아세틸라제 2 의 결실이 마이크로 RNA-622 를 통해 GLUT3 의 하향 조절을 유도하여 종양 억제 기능 활성화에 관여하다는 것을 확인하였다.

중심단어 : 교모세포종, 히스톤 디아세틸라제 2, 마이크로RNA-622, 세포자살, 포도당 전달체 3

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