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Original Article

Birth of MTH1 as a therapeutic target for glioblastoma: MTH1 is indispensable for gliomatumorigenesis

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Abstract: Malignant glioma is the most common primary tumor of the central nervous system. Chemotherapy and radiotherapy are the most common therapeutic approaches in glioma therapy. Both processes mainly kill cancer cells through generating high Reactive Oxygen Species (ROS) and lead to oxidative DNA damage. However, tumor resistance to ROS is always a challenge for cancer treatment. Human Mut T homolog 1 (MTH1, also known as NUDT1) is regarded as a protector of nucleotides against oxidization. Recent reports have verified that overexpression of MTH1 could remove oxidized dNTP pools. Here, we find that MTH1 is overexpressed both at mRNA and protein levels in GBM. MTH1 silencing inhibits colony formation; tumor spheres formation and xenograft tumor growth, and more importantly, the viability of glioma cells is significantly decreased in H_2O_2 after MTH1 was knocked down in glioma. PI staining show that H_2O_2 cause more glioma cell death after MTH1 silencing. So we speculate that overexpression of MTH1 is crucial for glioma survival, suppression of its expression can inhibit cancer cell survival *in vitro* and *in vivo*, MTH1 may be a potential target for human glioma therapy in future.

Keywords: GBM, MTH1, reactive oxygen species, viability, stemness

Introduction

Gliomas account for one of the most common central nervous system neoplasms and divided into four histopathologic grades based on the degree of malignancy [1, 2]. Malignant gliomas are among the most burdensome types of cancer, not only for their poor prognosis, but also because of the significant impacts on quality of life and cognitive function [3, 4]. Biologically, many reports have shown that oxidative stress is high in these cancer tissues [5]. A moderate level of reactive oxygen species (ROS) level may be needed for normal cell survival, but excessive amounts of ROS can cause oxidative damage to DNA [6-8]. A significant increase of 8-oxoguanine (8-oxoG) accumulated in the cytoplasm or mitochondria with a coincidentally elevated expression of human mutT homolog 1 was observed [9], so the possibility that human MTH1 (hMTH1) hydrolyzes oxidized DNA precursors other than 8-hydroxy-dGTP (8-OH-dGTP) was investigated very early [10]. Oxidative dNTPs, 8-oxoG, result in DNA damage and cell death [11, 12]. Recent discoveries have demonstrated that cancer cells require MTH1 activity to avoid incorporation of oxidized dNTPs which would lead to DNA damage and cell death [13, 14]. Several types of MTH1 inhibitors have been confirmed to be effective against cancer cells [15], for example small interfering RNA that inhibits MTH1 prevents removal of oxidized dNTP pools and causes cancer cell-selective DNA damage and cell death [16].

Chemotherapy and radiotherapy are essential treatment for many cancers, both processes mainly destroy cancer cells through directly damages DNA [17-20]. However, there always exists cancer cells could not be killed by these

therapies, resulting in the relapse of cancer [21, 22]. As we recommended previously, MTH1 plays a crucial role in tumor survival against the DNA lesions, so study of MTH1 may present a potential solution for cancer resistance to chemotherapy and radiotherapy. In this study, shRNAs were used to silence MTH1 in glioma cells. Proliferation assay, colony formation assay and tumor sphere formation assay were also performed for MTH1 knockdown glioma cell. Furthermore, xenograft growth was performed to verify the tumor suppressive effect of MTH1 knockdown *in vivo*. GBM cells viability to hydrogen peroxide (H_2O_2) was investigated after MTH1 silenced. Collectively, our study modified the expression status of MTH1 in glioma and provided evidence for its pivotal role for glioma survival. MTH1 may be a new and effective therapeutic target for malignant gliomas.

Materials and methods

Cell culture and RNA interference

U87MG and U251MG glioma cell lines were used for the experiments. Cells were cultured in DMEM (Sigma) with 10% fetal bovine serum (FBS) from Gibco and 1% penicillin/streptomycin (Sigma), at 37°C and with 5% CO_2. Short hairpin RNA (shRNA) was used to silence MTH1. U251MG and U87MG were infected with lentivirus, followed by selection in puromycin (2 μg/mL) for 1 week. The target sequences are shown as following: shctrl, TTCTCCGAACGTGTCACGT; shMTH1-1, GAAATTCCACGGGTACTTCAA; shMTH1-2, CGACGACAGCTACTGGTTT.

Colony formation assay and tumor sphere formation assay

For clonogenicity, 1×10^3 cells/well were seeded in 24-well plate, the colonies were dyed by bromophenol blue after one week. For tumor sphere formation assay, cells (1×10^3/well) were seeded in the 24-well attachment plate in neurobasal medium supplemented with 20 ng/mL basic fibroblast growth factor (bFGF; Sigma), 20 mL/mL B27 supplement (Life Technologies), and 20 ng/mL EGF (Sigma). The medium was refreshed every 2 days. After 10 days, the tumor spheres diameters larger than 50 μm were counted and photographed using phase contrast microscope.

H_2O_2 toxicity assay

The glioblastoma cells were exposed to H_2O_2 to induce cell death mediated by oxidative damage. Cells were cultured in media containing different concentrations of H_2O_2 for indicated duration, and then cells were incubated in culture medium containing 10% WST-1 at 37°C for 30 min. Cell viability was calculated by measuring culture absorbance.

Western blotting assay

For Western blotting analysis, the total cell lysates were prepared in high KCl lysis buffer (10 mmol/L Tris-HCl, pH 8.0, 140 mmol/L NaCl, 300 mmol/L KCl, 1 mmol/L EDTA, 0.5% Triton X-100, and 0.5% sodium deoxycholate) with complete protease inhibitor cocktail (Roche). The protein concentration was determined using a BCA Protein Assay Kit (Pierce) and equal quantity of protein was loaded on SDS-PAGE gel and transferred to PVDF membrane (Roche Diagnostics GmbH Mannheim, Germany). Primary antibodies against β-actin (1:4000; Abmart) and MTH1 (1:1000; Santa Cruz) were used, respectively. Signals were developed with horseradish peroxidase-conjugated secondary antibodies.

Immunofluorescence staining assay

Total cellular MTH1 was stained by immunofluorescence using MTH1 polyclonal antibody. For detection of MTH1, cells were fixed in 4% paraformaldehyde, and MTH1 antibodies were diluted in blocking buffer and incubated at 4°C overnight.

Cell death assay

The glioma cells were exposed to H_2O_2 to induce cell death mediated by oxidative damage. 100 μM of H_2O_2 was added to cell cultures for the indicated time, and then cells were incubated in culture medium containing 5 mg/mL PI and 5 mg/mL Hoechst at 37°C for 30 min. Percentage of cell death was calculated by PI/Hoechst.

Animal studies

2×10^6 cells were suspended in 200 μL of DMEM and then implanted subcutaneously into both flanks of 5 male Balb/c nude mice

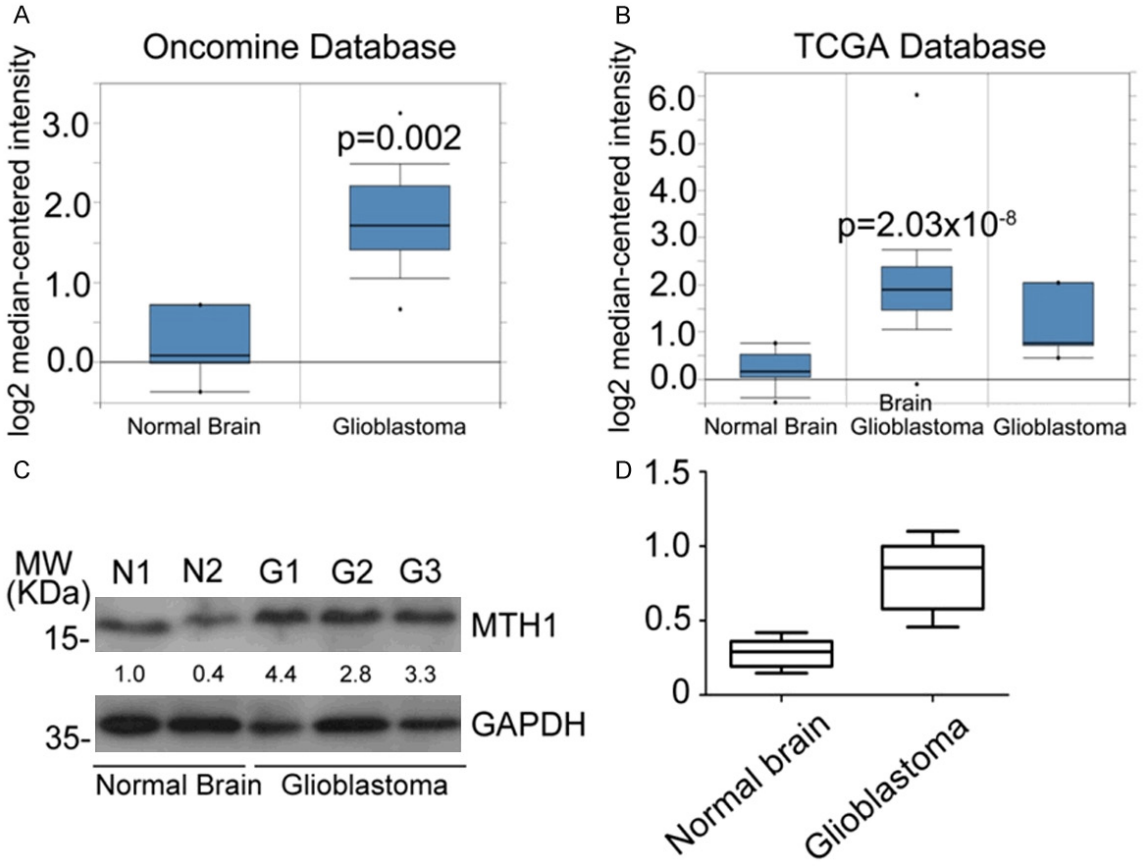

Figure 1. Bioinformatics analysis of MTH1 expression in GBM tissues. (A) Oncomine microarray data analysis for MTH1 expression in glioblastoma versus normal brain was shown. The student test was conducted using the Oncomine software (http://www.oncomine.org); (B) The Cancer Genome Atlas (TCGA) data for MTH1 expression in glioblastoma versus normal brain; (C) Western blotting analyzing of MTH1 expression in GBM tissues and normal brain; (D) Statistic analysis of MTH1 expression level in GBM tissues and normal brain.

aged 4 weeks. Tumor growth was monitored every 2 days for 30 days. Tumor volume was determined by measuring the length (a) and width (b). The tumor volume (V) was calculated according to the formula $v = ab^2/2$. All mice experiments were carried out in accordance with institutional guidelines and government regulations.

Hematoxylin and eosin staining

For hematoxylin and eosin (H&E) staining, we disposed the tumor into frozen section and stained them with H&E. Slides were photographed using an optical microscope (Leica).

Statistical analysis

Quantifications were performed from at least three independent experiments, and data were presented as the mean ± SEM in the graphs. Student's t test was used to compare two sets of data, and a value of $P < 0.05$ was considered statistically significant.

Results

MTH1 expresses at high levels in human glioblastomas

To examine the roles of MTH1 in GBM, we investigated the expression of MTH1 using Oncomine database (http://www.oncomine.org) and TCGA database (http://tcga-data.nci.nih.gov). Analysis from oncomine database based on a set of data including 557 GBM samples showed that MTH1 mRNA levels were increased in brain cancer tissue compared with normal tissue (**Figure 1A**). TCGA data set including 542 GBM samples was also analyzed for MTH1 RNA levels, and the results showed sig-

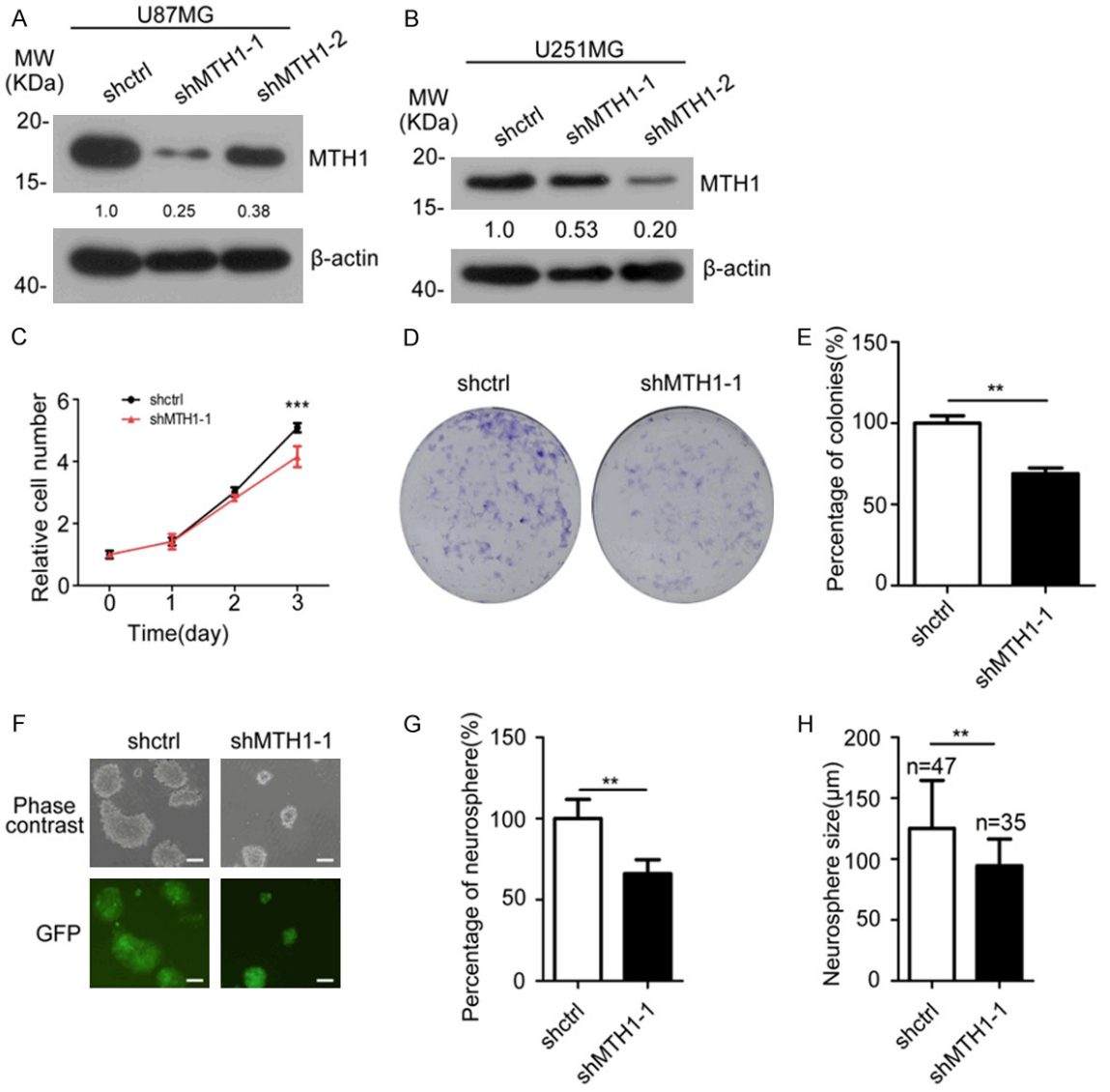

Figure 2. MTH1 is high expressed in GBM tissues. (A) Western blotting analysis of the expression levels of MTH1 in U87MG, take β-actin as loading control; (B) Western blotting analysis of the expression levels of MTH1 in U251MG, take β-actin as loading control; (C) Growth curves after cell transfections were assessed by WST-1 assay; (D) Representative images of colonies formed by MTH1-knockdown and negative control U87MG; (E) Statistics of the colony number in **Figure 2C**; (F) The morphology of tumor spheres formed by the cancer stem cells from MTH1-knockdown U87MG and control cells; (G) Number of glioma tumor spheres in negative control and MTH1-knockdown U87MG; (H) Diameter of glioma tumor spheres negative control and MTH1-knockdown U87MG.

nificant differences between GBM tissues and normal tissues (**Figure 1B**). MTH1 level was also higher in GBMs compared to other less malignant tumors (for example astrocytoma, oligodendroglioma, mixed glioma), suggesting a correlation between MTH1 level and tumor grade (Supplemental Figure 1). Western blotting analyzing also verified the high expression of MTH1 in GBM tissues (**Figure 1C** and **1D**). These results certified that there was a high level of MTH1 in human GBM.

MTH1 silencing could suppress proliferation, colony formation and tumor sphere formation GBM

To detect the effect of MTH1 on glioma cell proliferation, MTH1 was silenced using two short hairpin RNAs (shRNAs) in U251MG (**Figure 2A**) and U87MG (**Figure 2B**), respectively. We used WST-1 cell viability assay to examine the cell proliferation. As shown in **Figure 2C**, the rate of cell proliferation was decreased after MTH1

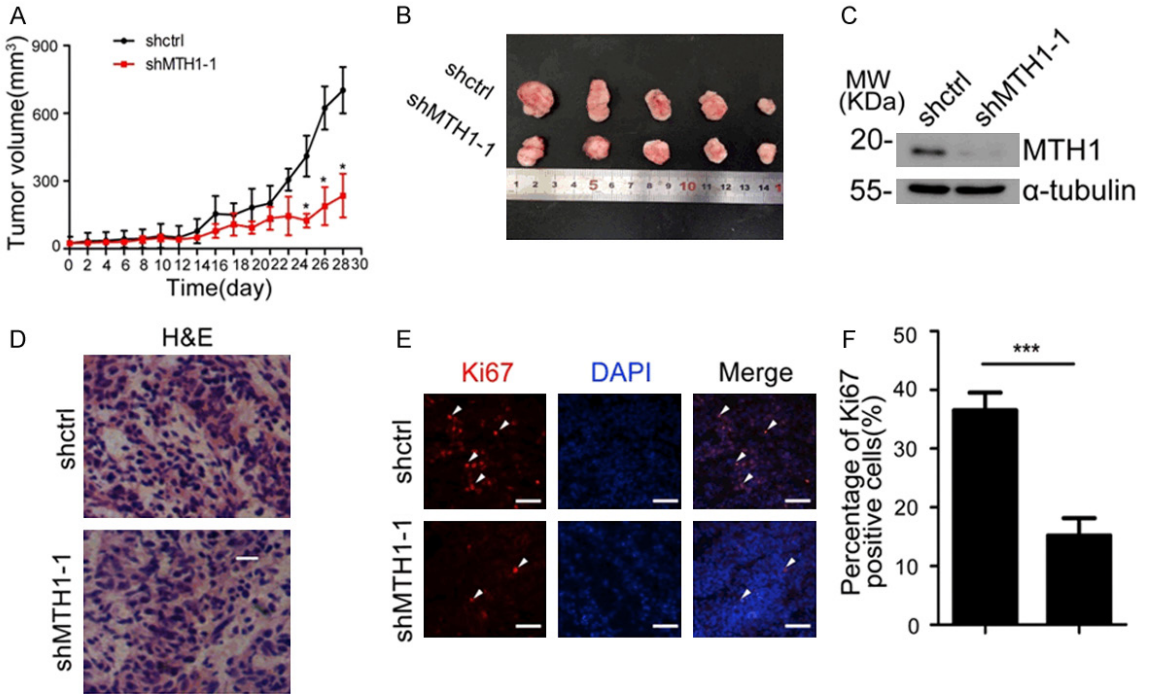

Figure 3. MTH1 silencing inhibited xenograft tumor growths in nude mice. (A) Determination of the tumor growth. Tumor volume was calculated every two days after injection; (B) Image for tumor growth was shown. Nude mice were subcutaneously injected with 2.0 × 10^6 MTH1 knockdown-control stable transfected U87MG cells; (C) Western blots analysis of MTH1 expression in nude mice tumor; (D) H&E staining of xenograft tumor tissues. Magnification, 40 ×; (E) Immunofluorescence images showing expression levels of Ki67 (red) in negative control tumors and MTH1-konckdown tumors. DAPI (blue) was used to stain nuclei; (F) Ratio of ki67 positive cells in control tumors and MTH1-konckdown tumors. Scale bar, 100 μm. Error bars represent SEM. *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

knockdown compared with the control group at 72 hours after lentivirus infection (P < 0.001 for each), indicating that MTH1 silencing could inhibit the proliferation of glioma cells. A colony formation assay data showed the colony number decreased by 30% after MTH1 was knocked down (**Figure 2D** and **2E**), indicating that MTH1 knockdown could also inhibit colony formation of glioma cells. Simultaneously, we found a significant decrease in the number of tumor spheres compared with controls in MTH1-knockdown U87MG (**Figure 2F** and **2G**). The volume of tumor spheres decreased by nearly 40% in MTH1 silencing U87MG (**Figure 2H**). This suggests that MTH1 may be related with the regulation of glioma stemness.

MTH1 knockdown suppresses glioma xenograft tumor growth in nude mice

The effect of MTH1 overexpression on tumor growth *in vivo* was assessed to analyze the role of MTH1 in glioma carcinogenesis. U87MG-MTH1 knockdown cells and their respective control cells were implanted into the right and left flanks (2.0 × 10^6 cells per flank) of nude mice by subcutaneous injection. At 30 days postinjection, the mean volumes of xenograft tumors generated from U87MG-MTH1 knockdown cells were significantly smaller than those originating from control-U87MG cells (**Figure 3A-C**). H&E staining showed MTH1 knockdown had no observably influence on tumor tissue density of xenografts (**Figure 3D**). Immunohistochemical staining of Ki67 showed a decreased percent of Ki67 positive cells in MTH1 knockdown tumors compared with control the control group (**Figure 3E** and **3F**). Thus, MTH1 knockdown significantly inhibited the proliferation of glioma cells both *in vitro* and *in vivo*.

Suppression of MTH1 expression reduces cell viability in high ROS

It is well known that MTH1 could remove oxidized dNTPs and protect cell from oxidative damage. Therefore, we used WST-1 cell viability

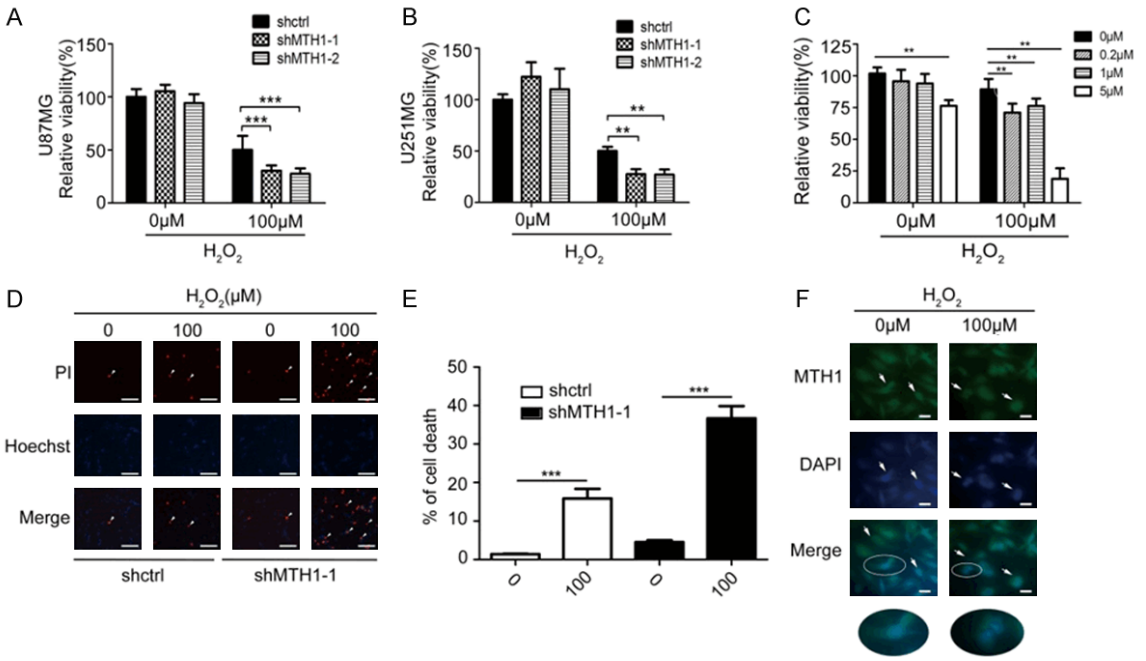

Figure 4. MTH1 knockdown reduces high ROS resistance of glioma cell. (A) U87MG was exposed with 100 μM H_2O_2 for 12 h, then cell viability were assessed by a WST-1 assay; (B) U251MG was exposed with 100 μM H_2O_2 for 12 h, then cell viability was assessed by a WST-1 assay; (C) U251 GBM cells were exposed with 0 μM and 100 μM (S)-crizotinib for 12 h, then cell viability was detected by WST-1. Scale bar, 100 μm; (D, E) U87MG cells were treated with 0 or 100 μM H_2O_2 for 12 h, cell death rate was determined by propidium iodide (PI)/Hoechst; (F) Total cellular immunofluorescence staining of MTH1 (green) in U251 after exposed with 100 μM H_2O_2 for 8 h. DAPI (blue) was used to stain nuclei. Error bars represent SEM. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

assays to examine whether MTH1 silencing affected glioma viability in high ROS created by H_2O_2. Glioma cell line U87MG and U251-MG were exposed in 100 μM H_2O_2 for 12 hours and cell viability was then detected by WST-1. Results showed that both MTH1-knockdown U87MG and U251MG cells exhibited a lower viability in contrast to the control cells (**Figure 4A** and **4B**).

A MTH1 inhibitor (S)-crizotinib has been developed and proved to selectively inhibit activity of MTH1 [9]. U87MG cells were treated with different concentrations of (S)-crizotinib and cell viability was also examined to exam the effect of this inhibitor on glioma ROS resistance. Result showed that (S)-crizotinib led MTH1 knockdown cells to a significant decrease of cell viability in H_2O_2 (**Figure 4C**). This illustrated that MTH1 shRNA possessed the same ability with MTH1 inhibitor. Immunochemical staining showed that MTH1 translocated from cytoplasm to nucleus after H_2O_2 treatment and the cell nucleus shrank but was not broken (**Figure 4F**). Propidium iodide (PI)/Hoechst staining showed that a significant more PI stained cells were detected in MTH1-knockdown U87MG than control cells (**Figure 4D** and **4E**). This suggested that the ROS resistance of glioma cell was declined after MTH1 down-regulated and glioma emerge more apoptosis in high ROS environment.

Discussion

Chemotherapy and radiotherapy are essential treatments for many cancers, while radiotherapy destroys cancer cells by ionizing radiation that directly damages cancer cells DNA. Most of the chemotherapeutic agents generate high ROS, oxidative stress from high ROS leads to oxidative damage in various molecules include of DNA in cancer cell [23-25]. However, both processes mainly induce apoptosis to kill cancer cells. There constantly exist some cancer cells that could not be killed by chemotherapy or radiotherapy, one reason is the existence of molecular function of DNA damage resistance. It has long been recognized that one of the most important mutagenic and cytotoxic

effects of ROS is the xidation of guanine residues in DNA to 8-oxoguanine. Because 8-oxoguanine in DNA readily base pairs with adenine and that could initiate transversion mutagenesis [26-28]. The mutT gene in E. coli, which preferentially cleaves the oxidized nucleotide 8-oxo-dGTP to the corresponding deoxyribonucleoside monophosphate 8-oxo-dGMP plus pyrophosphate, has been proved to be related with counteracting ROS-stimulated mutagenesis. This damage might actually occur at the nucleotide level originated from the demonstration that free intracellular nucleotides are orders of magnitude more reactive with modifying reagents than the corresponding nucleotides in DNA [29, 30]. A human homologue MTH1 (also known as NUDT1) has similar activity, and two other human nucleotidases [31, 32], MTH2 (also known as NUDT15) and MTH3 (also known as NUDT18), have related substrate specificities, including the ability to cleave oxidized ribonucleotides and nucleoside diphosphates [33, 34].

MTH1 has been identified as a potential anticancer target by different researchers. Gad et al. observed that overexpression of MTH1 prevents DNA damage and genome instability induced by HRAS [15]. Meanwhile, Huber et al. used a compound which identified MTH1 as the target known to suppress the growth of KRAS-transformed fibroblasts [16]. A MTH1 inhibitor (S)-crizotinib has been developed by these authors, and it has been approved for the treatment of some lung carcinomas. These studies acknowledged the anti-tumor characteristics of MTH1 inhibition, and suggested an appropriate tumor therapeutic approach using MTH1 inhibitors.

In this study, we verified that the MTH1 was highly expressed in malignant gliomas both at RNA level and protein level, and it was mainly located in the nucleus in glioma cells. MTH1 could translocate to the cytoplasm when glioma cells are exposed to H_2O_2, and more importantly, the viability of glioma cells was significantly decreased in H_2O_2 after MTH1 was knocked down by shRNA in both U87MG and U251MG. PI staining showed that H_2O_2 caused more cell death after MTH1 was knocked down, this suggests that MTH1 was crucial for glioma survival in high ROS environment.

The results acknowledge that MTH1 is indispensable in tumor survival on account of its function to eliminate DNA damage. This means that MTH1 may be a potential solution to cancer resistance for chemotherapy and radiotherapy. MTH1 may be a potential therapeutic strategy for glioma treatment in the future.

Acknowledgements

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Disclosure of conflict of interest

None.

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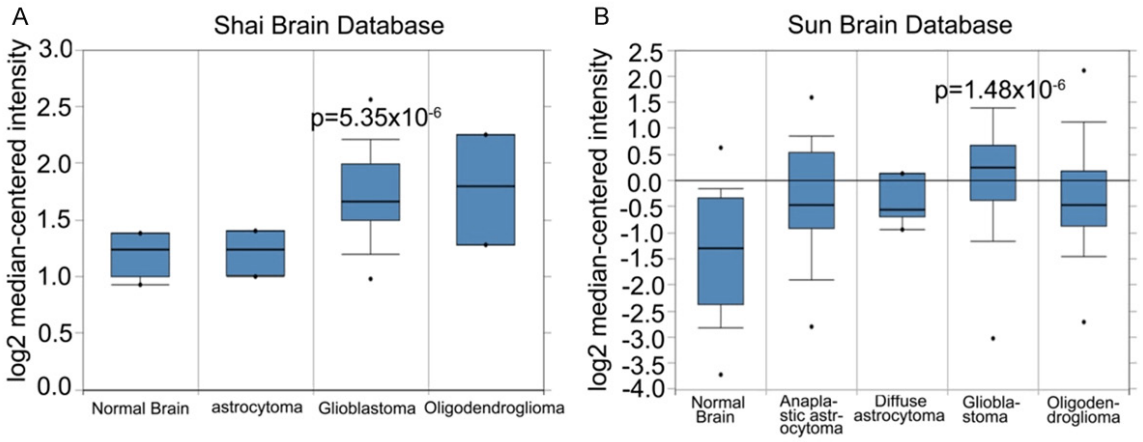

Supplemental Figure 1. MTH1 expression level in less malignant tumors. A. MTH1 expression in normal Shai brain database, data from oncomine; B. MTH1 expression in normal Sun brain database, data from oncomine.