Mutant Isocitrate Dehydrogenase 1 Product, 2-Hydroxy Glutarate, Activates MutT Homolog 1 in Glioma Cells via Augmentation of Reactive Oxygen Species

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Research Article

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Abstract

MutT Homolog1 (MTH1) is an enzyme responsible for removing oxidized nucleotides from cells. Activation of MTH1 is reported in many cancer cells and is thought to be responsible for imparting resistance towards anticancer drugs. While there are several mechanisms for the activation of MTH1 in cancer cells, this study aimed to evaluate the role of mutant Isocitrate Dehydrogenase1 (mIDH1) - mediated reactive oxygen species (ROS) in the activation of MutT Homolog1 in glioma cells. MTH1 was found to be upregulated in both mIDH1 expressed and 2- HG treated cells. mIDH1 and its product, 2-HG, increased the ROS levels in the cultured glioblastoma cells. Further, increased expression and activity of MTH1 was observed in glioma tissues harboring mIDH1 compared to tissues with wild-type IDH1. Our study unveils a novel mechanism of activation of MTH1 in cells harboring mutant IDH1.

Introduction

Isocitrate dehydrogenase (IDH) enzymes are key players in various metabolic processes such as the Tri Carboxylic Acid (TCA) cycle, lipogenesis, glutamine metabolism and redox regulation (Han et al. 2020). These NAD(P)+-dependent enzymes catalyses the conversion of isocitrate to α-ketoglutarate (α-KG) via the oxidative decarboxylation and generates NAD(P)H (Shi et al. 2013). Mutations in IDH1 were first identified in human glioma tissue biopsies by whole genome sequencing, wherein it was observed that some mutations are commonly seen in most of the low-grade glioma (LGG) and secondary glioblastomas (GBM) (Parsons et al. 2008). The most important of these being a point mutation where the arginine gets replaced by histidine (R132H) at the 132nd position. IDH1 mutation status has now been included as an essential criteria for the glioma classification as per the WHO classification of the Central Nervous System Tumors of 2016 (Louis et al. 2016).

It has been earlier reported that IDH1 mutation generates increased ROS in cells via its enzymatic product, 2-Hydroxyglutarate (2-HG) (Garrett et al. 2018). ROS thus generated can interact with several biomolecules leading to activation of various signaling events leading to robust responses like antioxidant and DNA damage repair pathways. Among these, ROS mediated activation of various sanitation enzymes such as MutT Homolog1 (MTH1) has been reported (Qing et al. 2018). MTH1 belongs to a superfamily of enzymes called the nucleoside diphosphates linked to moiety-X (NUDIX) hydrolases and is potentially the only one enzyme involved in preventing mutations in DNA (Gad et al. 2014). In our previous study, we had reported that silencing of MTH1 can affect glioma cell migration, invasion and inhibits the regulators of angiogenesis (Bhavya et al. 2020b). In the present study, we hypothesized that mutant-IDH1 (mIDH1) and its product, 2-HG, could be responsible for the activation of MTH1, via the production of ROS. Two human glioblastoma cell lines (U87 and U251) were transfected with mIDH1 plasmid and were analyzed for MTH1 expression. mIDH1 expressed human glioblastoma cells showed an increased MTH1 expression. When 2-HG, a product of mutant IDH1 enzyme, was exogenously supplied to the cells, MTH1 expression was found to be significantly high. Our results also suggest that the 2-HG is a major contributor of increased ROS generation in mIDH1 cells. In order to verify the above findings originated from cell lines, we checked for MTH1 expression levels/activity in IDH1 wild

type gliomas and with IDH1 mutation. We found a positive correlation between MTH1 expression and IDH1 mutation in glioma patient biopsies. Consistent with this, 8-oxo-dG levels, which are indicative of the MTH1 activity were found to be high in mIDH1 harboring glioma biopsies than the wild-type IDH1 (wt-IDH1) patients. This study provides evidence of mIDH1/2-HG mediated ROS in activating MTH1 in both glioma cell lines and in mIDH1 gliomas.

Materials And Methods:

Collection of tissue biopsy

Human glioma tissue biopsies were collected from the Department of Neurosurgery, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum according to the protocols approved by the Institutional Ethical Committee (Institutional Ethics Committee Approval No.: SCT/IEC/932/AUGUST-2016). Informed written consents were obtained from the patients or relatives prior to tissue collection and further processing were cited elsewhere (Bhavya et al. 2020b).

Cell culture, Plasmid DNA isolation and transfection

U87MG and U251MG human glioblastoma cell lines used for the experiments were procured from National Centre for Cell Science (NCCS, Pune, India). Cells were grown in DMEM (low glucose) (Sigma-Aldrich, Gillingham, UK) at 37°C with 5% CO2. pcDNA3-Flag-IDH1-R132H was a kind gift from Yue Xiong (Addgene plasmid # 62907; http://n2t.net/addgene:62907; RRID:Addgene_62907) (Zhao et al. 2009). Pure colonies were selected from ampicillin containing LB agar plates and then grown in LB broth at 37°C. The plasmid DNA was then isolated using SmartPure Plasmid DNA Isolation Kit (Eurogentech, Seraing, Belgium). The DNA (10 µg/mL) mixed with Transfection reagent and its buffer was used for transfecting the glioma cell lines. The transfection was performed using Jetprime transfection kit (Polyplus-transfection SA, Illkirch-Graffenstaden, France). The concentrations of 2-HG, NAC and mutant IDH1 inhibitor used were reported earlier in various studies. 30 mM of 2-HG, 2 µM of mutant IDH1 inhibitor- AGI-5198 and 1M N-Acetyl Cysteine (Sigma-Aldrich, Gillingham, UK) were used for the experiments.

Intracellular ROS measurement

The intracellular ROS levels were measured in cultured cells after various treatments using Dichlorodihydrofluorescein diacetate (DCF-DA). Cells were cultured in a 96-well black plate at a seeding density of $1x10^4$ per well. The wells were washed with HBSS after treatment and incubated with 10 µM DCFH-DA at 37°C in the dark for 1 h. Cells treated with H_2O_2 were used as a positive control. The wells were washed 2 times with HBSS to remove excess dye, and the DCF fluorescence developed was measured using Fluorimeter (BioTek instruments, Winooski, USA) at 530 nm (excitation 488 nm), with Gen5 software. The relative fluorescence of the treated groups to the control was calculated using the fluorescence intensities from triplicates.

Immunoblot

Glioblastoma cells and glioma tissues were processed for protein expression analysis and probed for desired proteins along with loading controls as described elsewhere (Bhavya et al. 2020b). The primary antibodies used were: MTH1 (1:500; Novus Biologicals, Centennial, Colorado, United States), vinculin, β-actin (1:1000; Sigma-Aldrich, Gillingham, UK), OGG1 and mIDH1 (1:1000, ImmunoTag, St. Louis, MO, USA), GPx4 and MnSOD (1:1000; Abcam, Cambridge, United Kingdom).

Enzyme Immuno Assays

Universal 8-oxo-dG ELISA kit (ImmunoTag, St. Louis, MO, USA) was used in order to measure the levels of 8-oxo-dG which is an indicator of MTH1 activity. The cell/tissue lysates were prepared as per the kit protocol and added in triplicates into the 96-well plate coated with antibodies against 8-oxo-dG. Briefly, the biotinylated antibodies were added which was followed by Streptavidin-HRP to label them. Then the substrate (provided with the kit) for HRP was added for color development and the reaction was terminated after 10 min. The absorbance was then measured at 450 nm using ELISA plate reader (Bio-Tek Instruments, VT, USA) and the 8-oxo-dG concentrations were extrapolated from their respective standard curves.

Immunofluorescence staining

OGG1 expression was analyzed in cells using immunocytochemistry. Briefly, the cells were fixed, permeabilized and incubated with blocking buffer. The cells were then incubated with anti-OGG1 antibody (1:200) for overnight. After washing, the cells incubated with secondary antibody (anti-rabbit; 1:10000-Cell Signaling Technology, Danvers, MA, USA) and then observed under fluorescence microscope (Olympus Life Science, Tokyo, Japan) once stained with DAPI (Sigma-Aldrich, Gillingham, UK). The images captured were analyzed using ImageJ software v1.49 and the Mean Fluorescence Intensities (MFI) normalized to the cell count was estimated.

Sanger Sequencing

Genomic DNA from FFPE sections was isolated using HiPura™ Mammalian Genomic DNA Purification Kit (HiMedia). The target region (Exon 4 of IDH1) was amplified using the following forward and reverse primer set: IDH1f 5’ CGGTCTTCAGAGAAGCCATT 3’ and IDH1r 5’ GCAAAATCACATTATTGCCAAC 3’. After purification of the PCR product, cycle sequencing was carried out using the ABI Big Dye Terminator v.3.1 kit (Thermo Scientific, San Diego, CA, USA). PCR products were resolved by electrophoresis on an 8 capillary ABI 3500 model sequencer (Thermo Fisher Scientific, Waltham, USA). In this case cycle sequencing was performed separately in both the forward and reverse directions. The sequences obtained were analyzed using BioEdit tool (Tom Hall, Ibis biosciences, Carlsbad, CA) and the corresponding genotypes were recorded.

Statistical analysis

All data are represented as mean ± SEM. The statistical analyses were done using GraphPad Prism 5 software. For comparison between two groups, unpaired t-test was used for cell line data and Mann Whitney test for patient data. In case of multiple group comparisons, for parametric analysis one-way

ANOVA followed by Tukey's or Dunnett's multiple comparisons test was performed. For non-parametric analysis, Kruskal-Wallis with Dunn's post-hoc test was used. Pearson's correlation coefficient was used for testing the correlation of MTH1 expression with mIDH1. 'p' values of 0.05 or less were considered statistically significant.

Results

Augmented expression of MTH1 in mIDH1 expressed and 2-HG treated glioma cells

In order to test whether mIDH1 has any effect on the expression of MTH1, plasmid carrying the mutant IDH1 (R132H) was expressed in U87MG and U251MG human glioblastoma cell lines. Western blot analysis of the protein isolated from U87MG cells showed that MTH1 was significantly upregulated ($p<0.0001$, 2.79±1.22) in mIDH1 expressed cells compared to the empty vector transfected wtIDH1 cells. On inhibiting mIDH1 using a specific inhibitor (AGI-5198), MTH1 protein levels was significantly reduced ($p<0.0001$, 1.37±0.07) (Fig. 1A). Similar results were obtained with the U251 cells as well (Fig. 1B). Subsequently, U87MG cells treated with 2-HG showed significant upregulation of MTH1 ($p=0.0308$, 1.65±0.20) compared to the untreated control (Fig. 1C). When checked for the expression of OGG1, a base excision repair enzyme was significantly upregulated in mIDH1 expressed and 2-HG treated U251 cells, but upon inhibiting mIDH1 with its specific inhibitor, reduced its expression (Fig. 1D).

mIDH1/2-HG contributes to high ROS levels in glioma cells

The DCF-DA assay was performed to check whether ROS is produced due to mIDH1 expression or its product, 2-HG in U87 glioblastoma cells. Cells transfected with plasmid carrying mIDH1 or treated with 2-HG were subjected to fluorometric analysis for the detection of ROS. Measurements of the DCF fluorescence revealed a significant increase in the ROS levels in cells expressing mIDH1. mIDH1 expressed cells were showing 8.51-fold fluorescence intensity ($p<0.0001$, 422.7 ± 7.45) than cells harboring empty vector (49.67 ± 1.67) (Fig. 2A). Also, in 2-HG treated cells, the ROS fluorescence levels were increased to 7.0-fold ($p<0.0001$, 443 ± 13.43) when compared to untreated control (63.33 ± 3.18). (Fig. 2B). Meanwhile, in mIDH1 expressed cells treated with a specific mIDH1 inhibitor, the fold DCF fluorescence decreased to 3.40 ($p<0.0001$, 112 ± 7.94) vs. mIDH1 expressed cells (Fig. 2A).

N-Acetyl Cysteine (NAC), a ROS scavenger, pretreatment given in both mIDH1 expressed cells and 2-HG treated cells could not inhibit the formation of ROS in those conditions [6.06-fold ($p<0.0001$, 384 ± 19.92)] and [7.67-fold ($p<0.0001$, 381 ± 16.56)] respectively compared to their control groups (Fig. 2A, 2B). H_2O_2 was used as a positive control in these experiments. The levels of two anti-oxidant enzymes, GPx4 and MnSOD, was determined upon treatment of U87MG cells with 2-HG by Western blot. The anti-oxidant enzymes GPx4 ($p=0.0136$, 1.26 ± 0.06) and MnSOD ($p=0.0005$, 1.83 ± 0.08) were increased in the presence of 2-HG (Fig. 2C and 2D).

U87 MG cells expressing mIDH1 were pre-treated with NAC and then probed for MTH1 protein levels. In another group, NAC pretreated U87MG cells were exposed to 2-HG for 48 h. Western blot analysis of proteins isolated from these NAC pre-treated cells showed elevated MTH1 expression both after mIDH1 transfection (p=0.0158, 1.67 ± 0.21) and 2-HG treatment (p=0.0073, 1.79 ± 0.05) (Fig. 2E). A significant increase in 8-oxodG levels was also observed in the NAC pre-treated cells expressing mIDH1 (p=0.0218, 5.84 ± 0.38) as well as treated with 2-HG (p=0.0115, 6.23 ± 0.63) (Fig. 2F).

Increased MTH1 expression and activity in mIDH1 harboring glioma tissues

The DNA sequencing data of a subset of the patient samples (n=21) denoting the IDH1 status shows that five of them had R132H mutation (Fig. 3A). Out of the 57 glioma patient samples, MTH1 expression was found to be elevated in patients harboring mIDH1 when compared to patients with wild-type IDH1. The Western blot analyses of glioma tissues showed that MTH1 expression was significantly upregulated (p=0.0249) in patients harboring IDH1 mutation (n=27, 1.51±0.15) when compared to patients carrying wild-type IDH1 (n=30, 1.04±0.13) (Fig. 3B and 3C). In patients with mIDH1, there is a moderate positive correlation with MTH1 expression (n=27; r= 0.4354, p= 0.0232) (Fig. 3D).

Next, 8-oxo-dG levels, which are indicative of activity of MTH1 enzyme was measured in glioma tissue biopsy extracts by immunoassay. We found increased 8-oxo-dG levels in those patients harboring mIDH1 (n= 15; 855.3 ± 242.6 ng/mg) compared to patients with wtIDH1 (n=9; 332.6 ± 114.1 ng/mg) (p=0.0148) (Fig. 3E), which is in concordance with the correlation found between MTH1 and mIDH1 expression. This indicates that concurrent with the high MTH1 expression pattern observed in mIDH1 glioma samples; there is a relative increase in MTH1 activity too.

Discussion

Oxidative damage of biomolecules like proteins, DNA and lipids due to elevated ROS levels promote pro-tumorigenic signaling, cancer cell proliferation, cell survival, cancer metastasis, apoptosis, adaptation to hypoxia and so on (Behrend et al. 2003; Reczek and Chandel 2017). There are numerous studies going on world-wide pertaining to IDH mutations in the pathobiology of cancers, especially gliomas. R132H substitution, the frequently observed IDH1 mutation in gliomas is caused by the G◊A transition at nucleotide position 395 of codon 132 (Yusoff et al. 2016). Cancer-associated IDH mutations leads to the formation of an oncometabolite, 2-HG (Bhavya et al. 2020a) associated increased ROS environment in cells and the subsequent oxidative stress is a major hallmark of cancers with IDH mutations (Shi et al. 2015). 2-HG being the structural analogue of α-ketoglutarate, competitively inhibits various α-KG dependent enzymes, α-keto acid transaminase, inhibition of DNA break repair and methylation of histones like H3K4, H3K9 and H3K27 (Reiter-Brennan et al. 2018) (Xu et al. 2011) (Molenaar et al. 2018) (Miyata et al. 2019).

Since increased ROS is associated with greater propensity for oxidant mediated DNA damage in cells, we hypothesized that mIDH1/2-HG may induce the expression of MTH1, an enzyme responsible for

sanitizing oxidant nucleotide pool in cells. Our results were in line with our hypothesis and 2-HG treated and mIDH1 expressed U87MG as well as U251 cells showed high level of MTH1 expression. The increased MTH1 expression in mIDH1 expressed cells was significantly reduced upon treatment with a specific mIDH1 inhibitor AGI-5198, which establishes the role of mIDH1/2-HG in regulating MTH1 levels in cells.

Augmented ROS levels seen in both mIDH1 expressed and 2-HG treated cells are in concordance with an earlier report (Gilbert et al. 2014). This shift in the redox status of the cells is likely to trigger the escalation of the antioxidant enzymes, GPx4 and SOD2, as seen from our results. In order to check, whether obliteration of basal ROS would significantly affect the prooxidant influences imparted by mIDH1/2-HG, cells were pretreated with NAC and probed for ROS levels. Intriguingly, NAC pretreatment had little or no influence on the ROS inducing capabilities of mIDH1/2-HG. When probed for MTH1 expression under NAC pretreatment in mIDH1/2-HG treated cells, there was nearly 2-fold increase in protein levels and in the MTH1 enzyme activity, determined by the 8-oxodG levels in cells. These results indicate the augmented MTH1 activity in the treated cells, even after scavenging the basal ROS that clearly suggests ROS generated via mIDH1/2-HG is responsible for the activation MTH1 in glioma cells.

Interestingly, in glioma patient biopsies with IDH1 mutation, we observed a significantly higher MTH1 expression compared to the wtIDH1 samples. Concomitant increase in the activity (as assessed by the increased 8-oxo-dG levels) supported the protein expression results in glioma tissues.

The highlight of the current study is that, we found a link between mIDH1 and MTH1 activation, mediated by ROS. This forms the basis for the increased MTH1 expression seen in mIDH1 glioma tissues and is the first study linking IDH1 mutation, ROS and activation of MTH1 in cells. As it was found that mIDH1/2-HG causes increased DNA damage (more OGG1 expression) in U251 cells, this might yet be another factor that played a role in the upregulation of MTH1 in gliomas. Further experiments are required in order to decipher the mechanistic molecules involved in relation to MTH1 and mIDH1.

Abbreviations

MTH1	MutT Homolog1
ROS	Reactive Oxygen species
mIDH1	mutant Isocitrate Dehydrogenase
wt-IDH1	wild type-Isocitrate Dehydrogenase
2-HG	2-Hydroxyglutarate
8-oxodG	8-Oxo-7,8-dihydro-2’-deoxyguanosine

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

Conceptualization and methodology**:** BB, CRA, MU, KS, AND, SG; Validation: BB, CRA, MU, HVE, KS, GRE, KK, AND, SG; Formal analysis: BB, CRA, MU, HVE, KS, GRE, KK, AND, SG; Resources: BB, CRA, HVE, KS, GRE, KK; Writing- Original draft and visualization: BB, CRA, MU, SG; Writing- Review and editing: BB, CRA, MU, SG; Supervision: MU, SG; Project administration: SG; Funding acquisition: SG.

Compliance with Ethical Standards

Research Involving Human Participants and/or Animals

1. Ethical Approval

All procedures performed with human subjects in the above study were in accordance with the ethical standards of the Institutional Ethics Committee of Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum and with the 1964 Helsinki declaration and its later amendments or comparable standards. This article does not contain any studies with animals performed by any of the authors.

2. Consent to participate

Informed consent was obtained from all individual participants included in the study.

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Figures

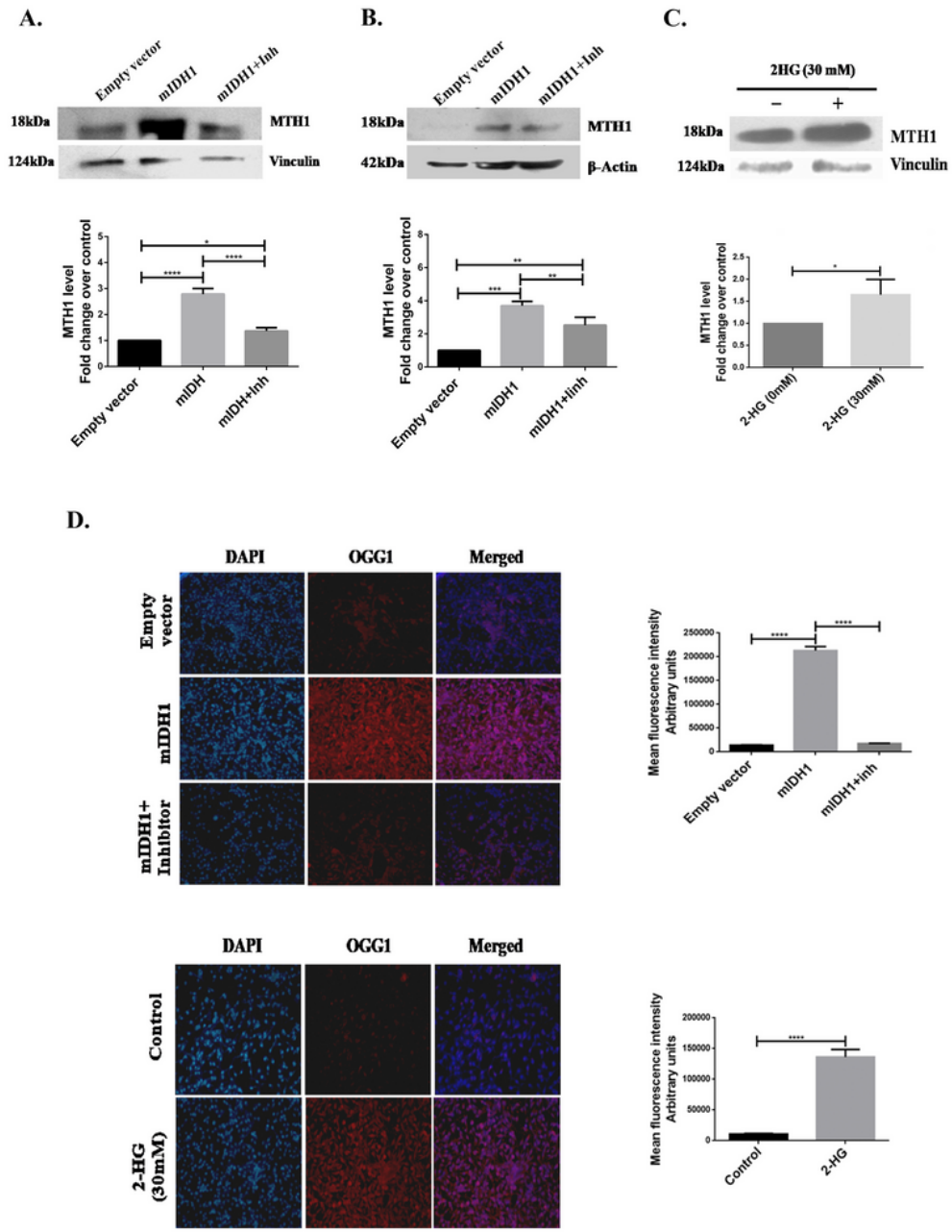

Figure 1

Augmented expression of MTH1 in mIDH1 expressed and 2-HG treated glioma cells

A. Western blot analysis showing the expression of MTH1 in mIDH1 expressing and mIDH1 inhibited U87 cells. The mIDH1 inhibitor AGI-5198 was used. Vinculin is used as the internal control. Statistical analysis was done using Tukey's multiple comparison tests. *p<0.05, ****p<0.0001, (n=4).

B. Western blot analysis for checking MTH1 expression in mIDH1 expressed cells and in the mIDH1 inhibited U251 cells. β-actin is taken as the loading control. **p<0.01, ***p<0.001, (n=3).

C. Western blot analysis showing expression of MTH1 in 2-HG treated U87 cells. Vinculin is used as the internal control. Statistical analysis was done using Student's t-test. *p<0.05, (n=3).

D. Representative images of Immunofluorescence staining of OGG1 expression in mIDH1 expressed and 2-HG treated U251 cells; ****p<0.0001, (n=3).

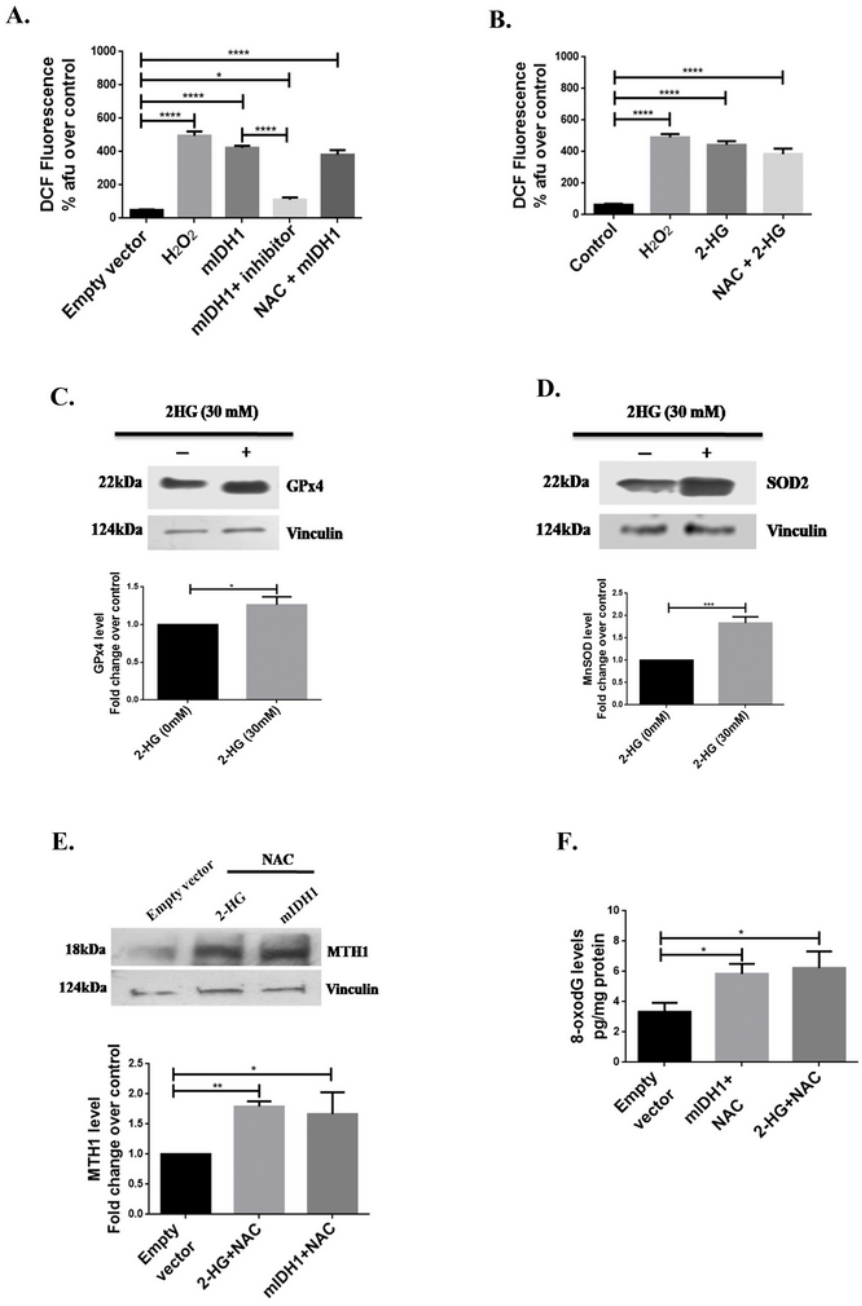

Figure 2

mIDH1/2-HG contributes to high ROS levels in glioma cells

A. Histograms representing ROS levels measured by DCFHDA fluorimetric analysis on transfecting U87 cells with mIDH1-R132H plasmid. Statistical analysis was done using Tukey's multiple comparison tests. *p<0.05, ****p<0.0001 (n=3).

B. Histograms representing ROS levels measured by DCFHDA fluorimetric analysis on treating U87 cells with 2-HG. Statistical analysis was done using Tukey's multiple comparison tests. ****p<0.0001 (n=3).

C. Representative immunoblots and histogram showing increased expression of GPx4 in U87 cells treated with 30 mM 2-HG. Vinculin is used as the internal control. Statistical analysis was done using Student's t-test. *p<0.05 (n=3).

D. Immunoblot analysis showing increased expression of MnSOD in U87 cells treated with 30 mM 2-HG. Vinculin is used as the internal control. Statistical analysis was done using Student's t-test. ***p<0.001 (n=3).

E. Representative immunoblots showing MTH1 expression in U87 cells pre-treated with NAC, a ROS scavenger and subsequently either transfected with mIDH1 or treated with 2-HG. Vinculin is used as the internal control. Statistical analysis was done using Tukey's multiple comparison tests. *p<0.05, **p<0.01, (n=3).

F. Histogram representation of 8-oxodG levels in U87 cells with the above treatments. Statistical analysis was performed by Tukey's multiple comparison tests. *p<0.05, (n=3).

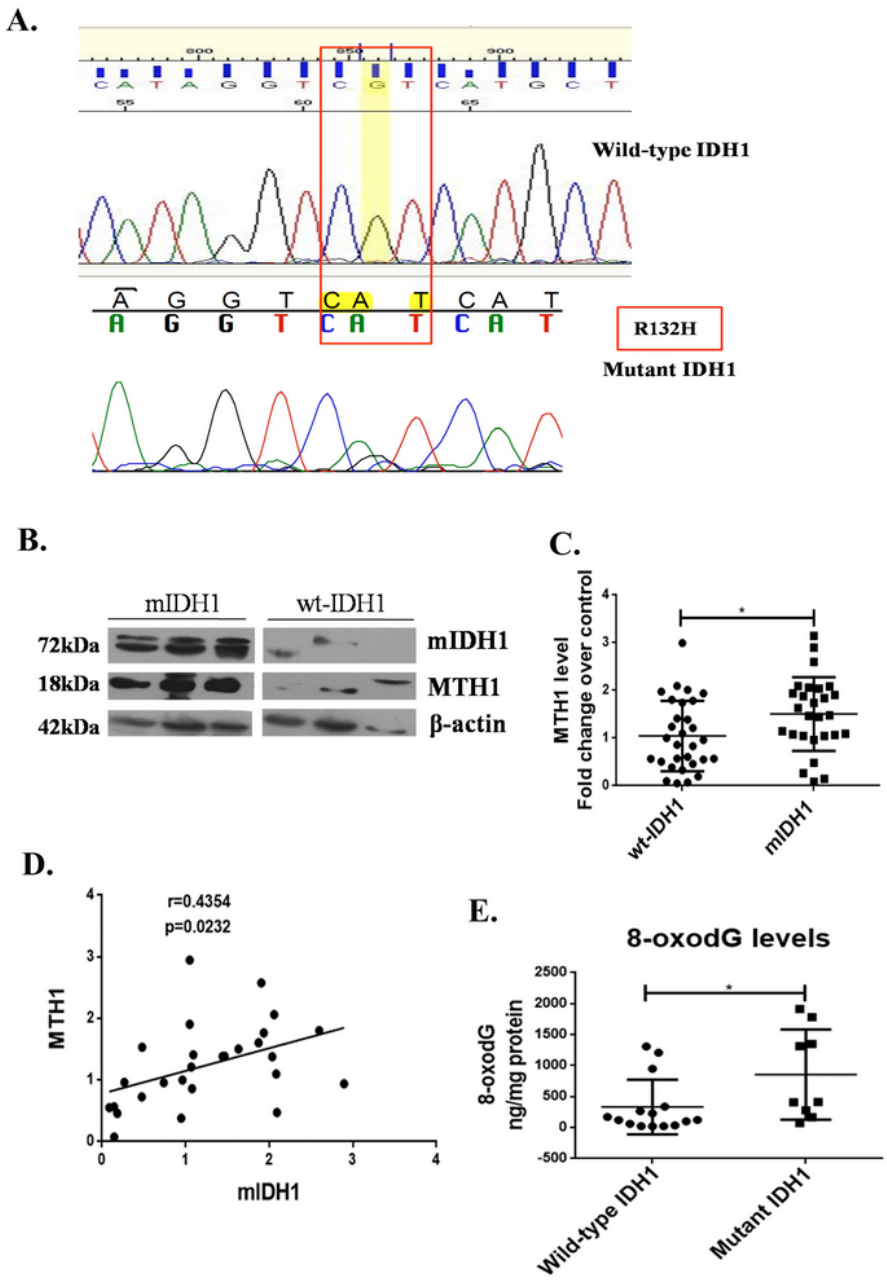

Figure 3

Increased MTH1 expression and activity in mIDH1 harboring glioma tissues

A. Sequencing data showing IDH1 R132H mutation in glioma patient samples (n=21).

B. Representative immunoblots showing MTH1 expression in glioma tissues (wt-IDH1; n=30, mIDH1; n=27). β-actin was used as the internal control.

C. Dot plot representation of MTH1 expression in glioma tissues with and without IDH1 mutation. Statistical analysis was done by Mann-Whitney test. *p<0.05.

D. Scatter plot showing correlation of MTH1 with mIDH1 in glioma patient samples (r=0.4354, p= 0.0232). Pearson's correlation analysis is used; n=27.

E. Dot plot representation of 8-oxodG levels in glioma patient samples with mIDH1 compared with patient samples without IDH1 mutation. Statistical analysis was performed by Mann Whitney test. *p<0.05.