ACTIVATION OF MTH1 IN GLIOMA: THE DEFINING ROLE OF IDH AND ROS

BHAVYA BHARATHAN

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SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

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A THESIS PRESENTED

BY

BHAVYA BHARATHAN

TO

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM

Thiruvananthapuram

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE AWARD OF

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DECLARATION BY STUDENT

I, Ms. Bhavya Bharathan, hereby certify that I had personally carried out the work depicted in the thesis entitled "ACTIVATION OF MTH1 IN GLIOMA: THE DEFINING ROLE OF IDH AND ROS" under the direct supervision of Dr. G Srinivas, Scientist F, Department of Biochemistry, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, except where external help was sought and is acknowledged. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Date: 31/12/2020 Bhavya Bharathan

CERTIFICATE BY THE RESEARCH GUIDE

This is to certify that Ms. Bhavya Bharathan has fulfilled the requirements prescribed for the PhD degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The thesis entitled "ACTIVATION OF MTH1 IN GLIOMA: THE DEFINING ROLE OF IDH AND ROS" was carried out under my direct supervision. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Date: 31/12/2020 Dr. G. Srinivas (Guide)

The thesis entitled

ACTIVATION OF MTH1 IN GLIOMA: THE DEFINING ROLE OF IDH AND ROS

submitted by

BHAVYA BHARATHAN

for the Degree of

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List of Abbreviations

MTH1	MutT Homolog1
ROS	Reactive Oxygen species
IDH	Isocitrate Dehydrogenase
mIDH	Mutant Isocitrate Dehydrogenase
2-HG	2-Hydroxy glutarate
α-KG	Alpha Ketoglutarate
TMZ	Temozolomide
Hif1α	Hypoxia Inducible factor1 alpha
PHD	Prolyl Hydroxyl Domain
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
MMP	Matrix Metalloproteinase
8-oxodG	8-Oxo-7,8-dihydro-2'-deoxyguanosine
BER	Base Excision Repair
XRCC1	X-ray repair cross-complementing protein1
OGG1	8-oxoguanine glycosylase1
WHO	World Health Organization
CNS	Central Nervous System

GBM	Glioblastoma
RAC	Ras-related C3 botulinum toxin substrate 1
H_2O_2	Hydrogen peroxide
MnSOD/SOD2	Manganese superoxide dismutase
Gpx	Glutathione peroxidases
GSH	Reduced glutathione
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate

Synopsis

Gliomas are the tumors that affect the glial cells of the Central Nervous System (CNS) and are the frequently seen and most malignant brain tumor out of all the CNS tumors. The ability of gliomas to strongly migrate and their infiltrative nature make them more aggressive than other brain tumors and are characterized by prominent vascularization with poor prognosis. Gliomas are generally classified into low grade gliomas (LGG) which comprise grade I and II and the high-grade gliomas (HGG) consisting of grade III and IV, based on their aggressiveness and behavior. There are various risk factors for gliomas including the chromosomal or genetic aberrations, exposure to irradiation, head trauma and the like. Apart from all these, Reactive Oxygen Species (ROS) mediated signaling pathways have immense role in cell survival, proliferation, migration, adhesion and tumorigenesis in gliomas as in case of any other cancer. Out of several proteins involved in redox regulation, MutT Homolog 1 (MTH1) also called as NUDT1 is a Nudix hydrolase family enzyme that gets activated in the presence of increased ROS environment in cancer cells. In cancer cells, increased ROS generation leads to the formation of oxidized nucleotides in the cytoplasm. Such nucleotides may get incorporated into the DNA of cancer cells and can cause different types of damages to the DNA. When MTH1 gets activated, it prevents the incorporation of oxidized forms of nucleotides like 8-oxodGTP or 2-OH-dATP into the DNA by hydrolyzing them into their respective monophosphates, thereby preventing DNA damage and consequent death of cancer cells. However, activation of MTH1 during chemotherapy becomes counter productive as MTH1 can favor the cancer cell survival by removing the DNA damage-inducing effect of cancer therapies.

There are various studies that have targeted MTH1 in different cancers. Some of the commonly used MTH1 inhibitors include TH588, TH278 and (S)-crizotinib. MTH1 inhibition leads to the accumulation of 8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG). The increased levels of 8-oxo-dG indicate increased MTH1 activity. There are several reports of MTH1 inhibitors as promising cancer

treatment agents in various cancers like colorectal, neuroendocrine, pancreatic, and gastric, but not in normal cells. However, there are only a very few studies hitherto revealing the roles of MTH1 in gliomas.

Mutations in Isocitrate Dehydrogenase 1 (IDH1) are frequently observed in some of the tumors, especially in gliomas. IDH1 is an enzyme that catalyses isocitrate to alpha-ketoglutarate (α-KG) conversion, coupled to NADPH generation. The NADPH (approximately 60%) thus generated via IDH1 is the precursor for the synthesis of the anti-oxidant glutathione (GSH). IDH1 mutation is a heterozygous mutation and produces 2-hydroxyglutarate (2-HG) from the α-KG produced by the non-mutant isoforms and consumes NADPH in the process. This causes a decline in the NADPH level which affects the GSH level and in turn increases the ROS generation in the mutant IDH1 carrying patients. Thus, IDH1 mutation is a factor that causes a high ROS environment in glioma patients. This is a point mutation where the amino acid Arginine (R) at the codon number 132 is replaced by Histidine (H) or Serine (S) or Glycine (G) or Leucine (L) or Cysteine (C). The product 2-HG is an oncometabolite and it is also associated with various cellular and epigenetic signaling. Hence, this mutation is a gain-of-function mutation. The mutation is nevertheless a good prognostic factor during the time of treatment, since the survival period of the patients carrying IDH1 mutation is observed to be more than those with wild-type IDH1. This study primarily focuses on the relation between MTH1, ROS and IDH1 mutation, i.e., to check whether the ROS generated via mutant IDH1 is in anyway responsible for MTH1 activation. The scope of the study also lies on the role of MTH1 in glioma cell survival, cell migration, invasion and angiogenesis. The study also aims to check whether Hif1α modulates MTH1 expression in glioma.

Methods: Experiments were conducted in human glioma tissue biopsies and two human glioblastoma cell lines U87MG and U251MG. Tissue biopsies were obtained from the Department of Neurosurgery and grades of gliomas were

identified by Hematoxylin and Eosin staining by a pathologist. MTH1 expression in patient samples at the mRNA level was analyzed using RT-PCR and at the protein level was analyzed by Immunohistochemistry, ELISA and Western blotting. In order to check the MTH1 activity, 8-oxodG levels were measured using ELISA. Further experiments were done in cell lines using MTH1 siRNA (100 nM) or MTH1 inhibitor TH588 (10 μM). The cells were grown in low glucose (5.5 mM) DMEM. To study the role of MTH1 in preventing DNA damage, the γH2AX marker levels were checked by Immunofluorescence. Markers for Base Excision Repair, OGG1 and XRCC1 were analyzed by Western blotting. The cell death analysis after silencing MTH1 was done by Hoechst-PI double staining. Flow cytometry with Annexin-V/PI were done in order to identify the total number of early as well as late apoptotic cells after MTH1 knock-down. The apoptotic, invasion, migration and angiogenesis markers were checked after silencing MTH1 in the cells by means of Immunoblotting. The degree of glioma cell migration was checked by scratch wound healing assay. Flag-tagged mutant IDH1 overexpression plasmid was procured from Addgene and transfected into the cells in order to express the mutant IDH1. Further experiments were done in mutant IDH1 expressed U87 and U251 cells. The ROS level was measured by DCFHDA fluorimetric assay. The expression of tumorigenic and oxidative stress markers and MTH1 expression after treatment with 2-HG and in mutant IDH1 expressed cells were analyzed by Immunoblot. The role of mIDH1 in glioma cell migration was checked by scratch wound assay. Hif1α siRNA at a concentration of 100 nM and $CoCl_2$ at a concentration of 100 μM were used in order to check whether Hif1α modulates MTH1 levels in glioma. Pre-treatment with the ROS scavenger, N-Acetyl Cysteine (NAC), at a concentration of 1mM was used to quench the basal ROS levels in cell lines to filter out the effects of the treatments.

Major findings: MTH1 upregulation was observed in both LGG and HGG when compared to the non-tumor brain tissues at the mRNA as well as protein levels.

We also observed that the MTH1 activity (8-oxo-dG levels) was found to be high in glioma patient samples even though it is not statistically significant. Further experiments were done in cell lines after silencing or inhibiting MTH1. It was found that MTH1 is essential for preventing DNA damage and apoptosis in glioma. On silencing MTH1, an increase in γH2AX, a DNA damage marker was observed. Along with this the BER enzymes, OGG1 and XRCC1 were also upregulated indicating that MTH1 knock-down causes increased DNA damage in glioma. This was confirmed when shearing of DNA was observed on inhibiting MTH1 with TH588 in U87 cells. MTH1 silencing also results in the upregulation of the apoptotic marker cleaved PARP and the downregulation of anti-apoptotic marker Bcl2 in both the cell lines showing that MTH1 is an important factor for glioma cell survival. The Flow cytometry analysis with Annexin-V/PI staining shows an increased number of late apoptotic cells confirming the above result. It is found that there is a downregulation of the markers for invasion (MMP2, MMP9), migration (RAC1 and RhoA) and angiogenesis (VEGF) when MTH1 is silenced in U251 and U87 cells. We also observed a negative correlation of MTH1 expression with XRCC1 and cleaved PARP in patient samples, whereas a positive correlation with RAC1, MMP9 and VEGF levels. All these results suggest that MTH1 is upregulated in gliomas and has essential roles in glioma cell survival and physiology.

The role of mutant IDH1 and its product 2-HG in tumorigenesis was then analyzed. DCFHDA fluorimetric analysis showed that mutant IDH1 expressed as well as 2-HG treated cells shows increased ROS levels. It was found that in either of mutant IDH1 expressed cells or cells given 2-HG exogenously were showing upregulation of Hif1α and its downstream protein VEGF. It could be via 2-HG that the mutant IDH1 patients contributes to tumorigenesis. It is also found that mutant IDH1 is involved in glioma cell migration.

Interestingly, we observed an upregulation in MTH1 expression in those patients harboring IDH1 mutation, in comparison to the patients with wild-type IDH1, which shows a positive correlation of MTH1 expression with mutant IDH1. This was confirmed *in vitro* in both the cell lines. Mutant IDH1 expressing cells showed increased MTH1 expression and MTH1 was downregulated when mutant IDH1 was inhibited with mutant IDH1 inhibitor AGI-5198. Since, we have found a correlation between MTH1 expression and mutant IDH1, we also measured the 8-oxo-dG levels and found that those patients with mutant IDH1 were showing increased 8-oxo-dG levels indicating increased MTH1 expression and those patients with wild-type IDH1 were showing diminished levels of 8-oxo-dG. It could be via the ROS generated by mutant IDH1 that MTH1 is activated. This was proved by scavenging the basal ROS present in the cells and then treating the cells with 2-HG or transfecting with mutant IDH1. We found that MTH1 was upregulated even after the removal of the basal ROS.

It is already reported that Hif1α modulates MTH1 expression in colorectal cancer. We observed similar results with cell lines. When treated cells with the Hif1α inducer $CoCl_2$, MTH1 was found to be upregulated. When Hif1α was silenced, we found MTH1 downregulation in both the cell lines. This suggests that Hif1α modulates MTH1 expression in gliomas. We also found that the ROS level was diminished after silencing Hif1α. This could be the reason why MTH1 is downregulated when Hif1α was silenced, but the entailing molecular pathways are still unknown.

Significance of the study: This is the first study to investigate the activation of MTH1 via the activation of ROS consequent to IDH1 mutation. The roles of MTH1 in gliomas were observed after inhibiting or silencing MTH1 in glioma cell lines. It is also known that the activation of MTH1 adds to the chemoresistance shown by drugs like Temozolomide. Hence, MTH1 becomes a potential target for glioma therapy. The current study also shows that 2-HG could

I. Introduction

Glioma is the most malignant brain tumor which affects the glial cells of the central nervous system (CNS). It accounts for more than 60% of the primary brain tumor (Ostrom et al., 2017). The ability of this tumor to migrate strongly and their highly infiltrative nature make them aggressive than any other brain tumor and are well characterized by prominent vascularization with very poor prognosis (Monzo et al., 2016; Pudelko et al., 2017; Schneider et al., 2010). Patients with malignant glioma show poor prognosis. Major treatments for gliomas include surgery, radiation therapy and chemotherapy. Temozolomide (TMZ) is a commonly used chemotherapy agent for glioma treatment, which can exhibit therapeutic benefits in prolonging the survival of even the glioblastoma (GBM) patients (Chen et al., 2019). Resistance to treatment is the major reason for tumor recurrence in gliomas including GBM patients (Singer et al., 2015). Gliomas can be of various grades and types based on their cell of origin, behavior, malignancy and genetic abnormalities (Forst et al., 2014; Louis et al., 2016). Extensive research over past many years it is very understood that the signaling pathways mediated by Reactive Oxygen Species (ROS) have immense roles in cell proliferation, cell survival, cell adhesion, migration, and tumorigenesis in cancers (Kumari et al., 2018; Liou and Storz, 2010). In cancer, the high level of intrinsic ROS is related to various activities including oncogene stimulation, enhanced metabolism and so on. The production of free radicals in tumor cells especially hydrogen peroxide and ROS accumulation may result in the damage of DNA (Rinaldi et al., 2016). ROS generation can be through different ways: γ, X and UV radiation, biotransformation of dietary chemicals and during cellular metabolism. ROS are oxygen containing molecules which can be a radical or a non-radical (Tudek et al., 2010). Increased ROS in cancer leads to oxidative stress which causes the accumulation of oxidized bases like 8-oxoGuanine which in turn causes damage to DNA (Cadet and Wagner, 2013; Roszkowski et al., 2011).

I.1 Background studies

Exogenous as well as endogenous sources can cause oxidative damages to DNA including DNA strand break and chromosomal aberrations. Such damages to DNA can contribute to the pathology of cancer. Repair of such DNA lesions require effective

repairing system (Bohr, 2002; Martinet Wim et al., 2002). Base excision repair system (BER) is involved in the repair of DNA damage caused by oxidative stress. The enzymes involved in this system include 8- oxoguanine DNA glycosylase (OGG1), MutY homolog (MUTYH) and MutT Homolog 1 (MTH1) and it repairs the mutation involving 8-oxoguanine (Görgens et al., 2007). MUTYH excises misincorporated adenines opposite 8-oxo-dG base pairs during replication (Powell et al., 2005). OGG1 resides in the nucleus and mitochondria remove 8-oxodG that gets incorporated in the DNA (Van Houten et al., 2018), whereas MTH1 which exist among the nucleotide pool prevents the misincorporation of 8-OHdG and 2-OH-dA by hydrolyzing 8-oxo-dGTP or 2-OH-dATP to their respective monophosphates (Gad et al., 2014). The above enzyme system thus plays a crucial role in keeping the DNA without mutations or errors. However, altered expressions of these proteins have been reported in numerous cancers (Kumagae et al., 2018). MTH1 or NUDT1 comes under the Nudix hydrolase family of enzymes that becomes activated in the presence of a high ROS in cancer cells. By hydrolyzing the oxidized nucleotides in the nucleotide pool, the enzyme prevents DNA damage and consequent death of cancer cells (Gad et al., 2014). MTH1 favors the cancer cell survival and this was studied in various cancers. MTH1 resides in the cytoplasm where the main pool of free nucleotides exists, also present in the nucleus and mitochondria (Kang et al., 1995; Leon et al., 2016; Nakabeppu et al., 2006; Nakayama et al., 2011). In recent years, there has been increasing number of studies conducted targeting MTH1 in different cancers. Some of the common MTH1 inhibitors are (S)-crizotinib, TH588 and TH278 (Gad et al., 2014; Ikejiri et al., 2018; Pompsch et al., 2018; Prada et al., 2017; Waals et al., 2019; Zhou et al., 2019). Inhibition of MTH1 results in the accumulation of 8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) (Zhou et al., 2019). There are various reports of MTH1 inhibitors being used as a promising cancer treatment agents in tumors like colorectal cancer, neuroendocrine tumor and pancreatic cancer (McPherson et al., 2019; Pompsch et al., 2018; Prada et al., 2017), a recent one in gastric cancer cells where the inhibitor was showing increased cytotoxicity, but not in the normal cells (Zhou et al., 2019). However, only a few studies are demonstrating the functions of MTH1 in gliomas (Pudelko et al., 2017; Tu et al., 2016). A report in 2016 revealed that a malignancy-dependent MTH1 expression was found in gliomas (Tu et al., 2016). The observations

made by Pudelko *et al.*, claimed that GBM cultures with low and high aggressiveness, both have reduced viability on MTH1 inhibition (Pudelko et al., 2017).

Isocitrate Dehydrogenase (IDH) mutation is predominantly observed in gliomas after its discovery in 2008 (Parsons et al., 2008). Out of the different types of IDH mutation, IDH1 mutation is the most common mutation in glioma and is a heterozygous mutation where one allele is a mutant one and the other is the wild-type. The mutant allele of IDH converts alpha-ketoglutarate (α-KG) to 2-Hydroxyglutarate (2-HG) (Navis et al., 2013). This is a point mutation where at codon 132, arginine gets replaced by histidine (R132H). Due to the accumulation and secretion of the onco-metabolite 2-HG, the mutation is a gain-of-function mutation (Mondesir et al., 2016). It is a good diagnostic and prognostic biomarker in gliomas. Numerous studies conducted over the last decade on various aspects of IDH mutations has led to the accumulation of a wealth of data. IDH mutation is a factor that is responsible for the generation of elevated levels of reactive oxygen species (ROS) in glioma cells (Shi et al., 2014b; Shi et al., 2014a). Mutation in IDH1 leads to the reduction in NADPH levels which results in the decrease of GSH, thus generates high ROS in cells (Shi et al., 2014a). IDH in its mutated sate is found to induce Hypoxia-Inducible Factor 1 alpha (Hif1α) activation via 2-HG, leading to tumorigenesis (Zhao et al., 2009a). 2-HG further causes alterations in epigenetic signaling in gliomas. It induces DNA and histone hypermethylation thereby involved in blocking cell differentiation and promoting tumorigenesis in mutant IDH1 condition (Gagné et al., 2017). The role of 2-HG in TP53 inhibition, cellular signaling, metabolic flexibility, mitochondrial function and immunomodulation is also widely studied in gliomas (Choi and Curry, 2017; Han and Batchelor, 2017; Jiang et al., 2018; Waitkus et al., 2016).

I.2 Identification of the problem

In gliomas, ROS and ROS-mediated effects can lead to alterations in cell cycle phases and hence contributes to gliomagenesis. Gliomagenesis is a process that is characterized by various molecular mechanisms. The major reason for the difficulty in treating brain tumors is the existence of several interfering biological entities and mechanisms which include the presence of blood-brain barrier, failure of tumor response to therapies and resistance of tumor cells to drugs. IDH1 mutation is a factor that is known to create a

high ROS environment in cells. The primary scope of the current study is to present novel mechanisms of ROS action in activating an enzyme (MTH1) that is reported to have role in cancer cell survival and proliferation. Also, the less illuminated role of MTH1 and mIDH1 in gliomagenesis including cell migration and angiogenesis will be discussed. In the present study, we have evaluated the correlation of MTH1 with the factors controlling cell survival, migration, invasion and angiogenesis in glioma tissue biopsies. The role of MTH1 in preventing DNA damage, in apoptosis, migration and angiogenesis were analyzed using MTH1 siRNA in human glioma cell lines, U87 and U251. We also investigated a speculation that Hif1α may regulate MTH1 in glioma cells. The ultimate aim of this work is to investigate whether the ROS generated through mIDH1 is responsible for MTH1 activation in gliomas. This mechanism can propose the formulation of a novel therapeutic strategy that can improve the treatment response in glioma by modulating ROS and its detrimental effects in at least IDH1 mutant glioma.

I.3 Hypothesis

The presence of mutant IDH1 renders a high ROS environment increasing the propensity of nucleotides to oxidative damage which in turn might activate MTH1 in gliomas.

I.4. Broad Objectives and specific objectives

I.4.1 Broad Objective 1

To study the novel functions of MTH1 in glioma by silencing MTH1 gene expression in human glioblastoma cell lines, U87MG and U251MG.

Specific Objectives

I. Whether MTH1 is upregulated in gliomas.

II. To check whether MTH1 silencing/inhibition affects glioma cell physiology such as

(a) Survival

(b) Migration and Invasion

(c) Angiogenesis regulators

I.4.2 Broad Objective 2

To analyze whether MTH1 activity depends on the ROS generated by mutant IDH1 in gliomas.

Specific Objectives

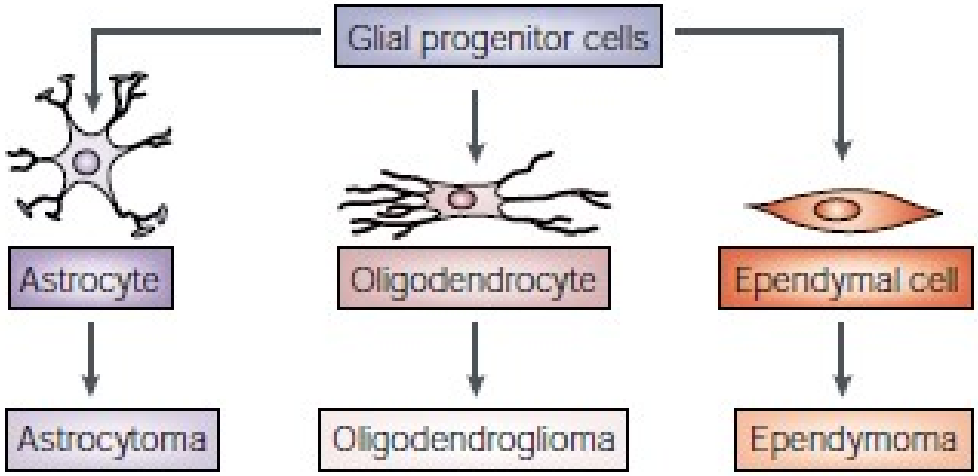
I. To study the role of mIDH1 and to know whether the ROS generated by mIDH1 can activate MTH1.

II. To verify whether Hif1α modulates MTH1 expression.

II. Review of Literature

II.1 Glioma- Major Etiologies

Gliomas are very diffusely infiltrative tumors which affect the glial cells of the brain (Mesfin and Al-Dhahir, 2020). Glioma is a type of primary brain tumors, and is classified according to their presumed cell of origin. These include astrocytomas, which arise from astrocytes, oligodendrogliomas arise from oligodendrocytes and ependymomas from ependymal cells (Agnihotri et al., 2013; Maher et al., 2001; Schwartzbaum et al., 2006). In 2016, gliomas were classified based on molecular and genetic markers (Louis et al., 2016). The treatment of brain tumors faces various challenges and one of the most notable one is the presence of the blood brain barrier (BBB). BBB is a highly selective semi-permeable barrier that separates blood from the brain. GBM is the frequently seen, aggressive and most malignant brain tumor. It accounts for 60% of brain tumors in adults. Over the last decade, the global incidence of GBM is less than 10 per 100,000 persons.



Rao, 2003

Fig.II.1 Glial cells and glioma

GBM patients have a poor prognosis and a median survival of ~10 months. There are three different modalities for glioma therapy; the initial approach is surgery, where complete resection is associated with longer progression-free survival (PFS) and overall survival (OS). Since resection is not a curative approach; patients usually undergo

radiotherapy and chemotherapy (Taylor et al., 2019). There are two major types of GBM based on the clinical characteristic; they are primary GBMs, which arises de novo without any evidence of precursor lesion, and secondary GBMs which grow out slowly from preexisting lower-grade gliomas. Recent findings in pediatric GBM have proposed a 3rd major category of GBM on the basis of the histone H3F3 gene mutation and this is different from primary and secondary GBM (Hanif et al., 2017).

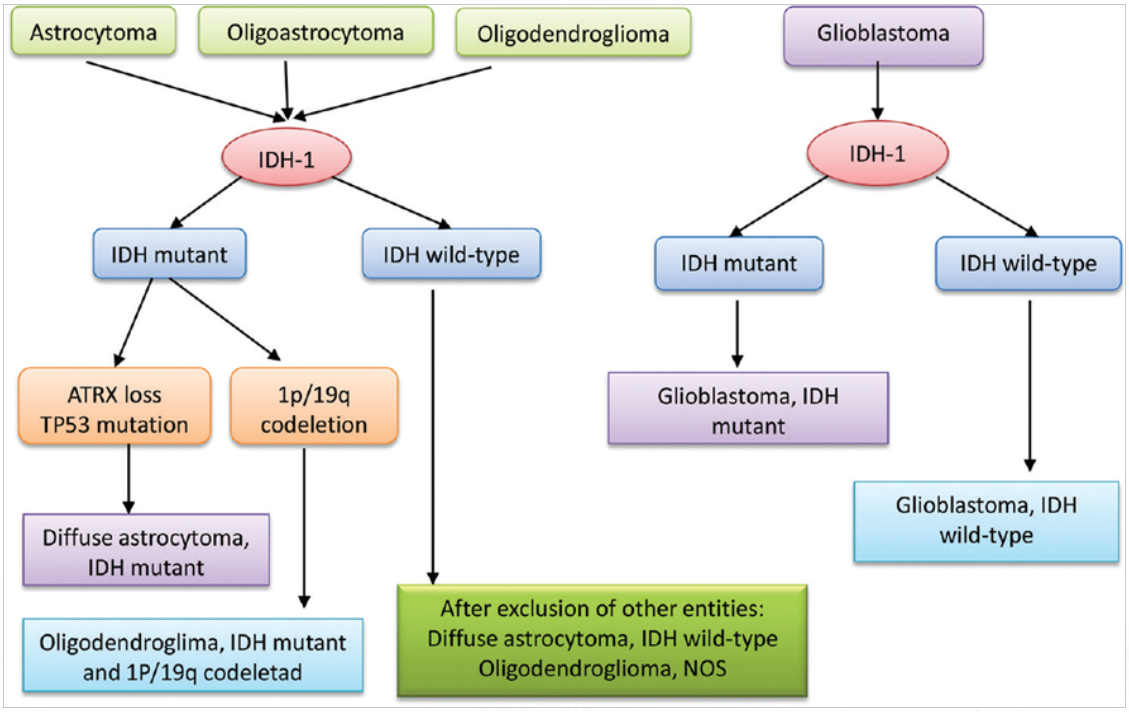
The commonly used chemotherapeutic agent for gliomas is Temozolomide (TMZ), the efficacy of which is limited by its acquired resistance (Chen et al., 2018) O6-methylguanine DNA methyltransferase (MGMT) inhibitor, Temozolomide (TMZ) was granted FDA approval for use in recurrent GBM in the year 1999. In 2005, it gained approval for using as a medication against primary GBM. Currently, TMZ is a standard of care chemotherapy for gliomas. Till date TMZ is the most effective alkylating agent for glioma. Along with drug resistance, tumor recurrence is yet another major challenge in glioma treatment. Use of angiogenesis (blood vessel formation) inhibitors like Bevacizumab (Avastin) was found to be effective in recurrent GBMs and the drug gained FDA approval in 2009 (Chowdhary et al., 2015). Several genetic and epigenetic alterations have been identified in the pathogenesis of gliomas in adults (Richterová and Kolarovszki, 2016). The genetic alterations include gene mutations, amplifications and deletions. The frequent genetic alterations seen in astrocytomas include mutations in isocitrate dehydrogenase genes 1 and 2 (IDH1/2), TP53 and TERT promoter (pTERT); EGFR amplification and pRB deletion (Bralten and French, 2011). But there are some genetic alterations that make primary glioblastomas distinct from secondary glioblastomas. Some of the prominent mutations seen in primary glioblastomas are EGFR amplification or mutation, PTEN deletion or mutation, CDKN2A-p16INK4a deletion. Frequently seen alterations in secondary glioblastomas are TP53 mutations, IDH1/2 mutations and (Platelet-derived growth factor receptor) PDGFR gene mutations (Crespo et al., 2015). IDH mutation was the frequently observed one and was reported in glioma in the year 2008 (Parsons et al. 2008) and later in some other tumors like Acute Myeloid Lymphoma and solid tumors like cholangiocarcinoma and chondrosarcoma (Dang et al., 2016). The current WHO classification of gliomas, especially glioblastomas is based on the status of IDH gene (wt/mutant) and aids in the defining an integrated basis for

assessment of genotypic and phenotypic parameters (Louis et al., 2016; Parsons et al., 2008). The IDH protein and its mutations which have significant prevalence in glioma are discussed in coming sections.

II.1.1 Glioma grading and WHO classification

The current international standard for the nomenclature and diagnosis of gliomas is WHO (World Health Organization) classification. It classifies gliomas into grade I to IV on the basis of level of malignancy that is determined by the histopathological criteria. Histological grading is a way of predicting the biological behavior of tumor. WHO grading helps in choosing the treatment mode, predict the therapeutic response and outcome of treatment. WHO grade I tumors are less malignant, hence is not life threatening. WHO grade II tumor patients usually survive for more than 5 years. Those patients affected with grade III tumors survive only for 2–3 years, at the same time the prognosis of patients with WHO grade IV tumors are even lesser than that (Louis et al., 2007). So, 2007 WHO classification was purely based on the histopathological nature of the tumor. Studies over a few decades have given us a clear idea regarding the genetic basis of tumorigenesis in glioma, raising the option of classifying the tumors based on genetic factors (Louis, 2012). Articles showing numerous genetic alterations in gliomas were published after the advent of the 2007 WHO classification of CNS, but at then it was not felt to include such an entity in the classification system. The 2007 WHO classification was revised and updated in 2016, incorporating certain genotypic parameters, one of them being the IDH mutations. Since discovery in 2008, many studies regarding IDH mutations were done world-wide in gliomas and other tumors. Due to their predominance in gliomas, it gained clinical importance and even became an essential criterion for classifying gliomas. Grade I gliomas relate to lesions that have low proliferative potential and can be cure by surgical procedure, whereas, grade II to IV gliomas are highly malignant and invasive. Glioblastoma multiforme is the most aggressive, invasive and undifferentiated type of tumor and has been designated Grade IV by WHO. As per the current nomenclature system, gliomas are named as (1) diffuse astrocytoma, IDH-mutant, (2) Oligodendroglioma, IDH-mutant, (3) Glioblastoma, IDH-wildtype (Louis et al., 2016). GBM is divided into three subgroups based on IDH1 and

IDH2 mutation status: IDH-mutant, IDH-wild-type and NOS (not otherwise specified) (Taylor et al., 2019).



Wang et al, 2018

Fig.II.2 WHO 2016 Classification of gliomas

II.1.2 Common genetic alterations in glioma

There are numerous genetic alterations that have been associated with the overall survival of LGG. Some of them are co-deletions of chromosomes 1p and 19q, mutations in the Isocitrate dehydrogenase (IDH) genes, of the O6-methylguanine-DNA methyltransferase (MGMT) gene methylation and mutation or deletion in p53 in some gliomas as early step in the development of gliomas (Claus et al., 2015). The Cancer Genome Atlas (TCGA) have validated gene-expression-based classification profiles for grade IV glioma (GBM) which includes Platelet-derived growth factor receptor factor A (PDGFRA) alterations, IDH1 and TP53 mutations, EGFR amplification and cyclin-dependent kinase inhibitor

2A (CDKN2A) alterations, Neurofibromin 1 (NF1) and alterations in hepatocyte growth factor (HGF) receptor (McLendon et al., 2008; Vitucci et al., 2011). Major hallmarks of primary GBM include mutation and amplification of epidermal growth factor receptor (EGFR) gene, mouse double minute 2 (MDM2) over expression, loss of heterozygosity (LOH) of chromosome 10q and deletion of p16 of phosphatase and tensin homolog (PTEN) and mutation of telomerase reverse transcriptase (TERT) promoter. Over expression of PDGFA/PDGFRA and retinoblastoma (RB), LOH of 19q and IDH1/2 mutations, TP53 and (Alpha thalassemia/mental retardation syndrome X-linked) ATRX mutations are the major alterations of secondary GBMs (Agnihotri et al., 2013; Ohgaki and Kleihues, 2007). The frequently observed genetic alterations in all types of gliomas are listed in the table below **(Table II.1).**

Gene Symbol	Gene Name	Type of Mutation
EGFR	Epidermal growth factor receptor	Point mutation, amplification and deletion
IDH1/2	Isocitrate dehydrogenase1/2	Missense mutation
1p19q	Short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q)	Co-deletion
MGMT	O-6-methylguanine-DNA methyltransferase	Promoter methylation
ATRX	Alpha thalassemia/mental retardation syndrome X-linked	Mutation
BRAF	Encodes proto-oncogene B-Raf	Fusion and mutation
TERT	Telomerase reverse transcriptase	Mutation
Histone H3K27M	Histone H3Lysine27Methionine mutation	Trimethylation
PTEN	Phosphatase and tensin homolog	Mutation/deletion
NF1	Neurofibromin 1	Mutation/deletion
RB1	Retinoblastoma 1	Mutation/deletion
TP53	Tumor protein p53	Mutation
PIK3CA	Phosphoinositide-3-kinase catalytic alpha	Missense mutation
PIK3R1	Phosphoinositide-3-kinase	Somatic mutation

	regulatory 1	
PTPRD	Protein tyrosine phosphatase receptor type D	Deletion and mutation

Table II.1 Various genetic alterations seen in gliomas

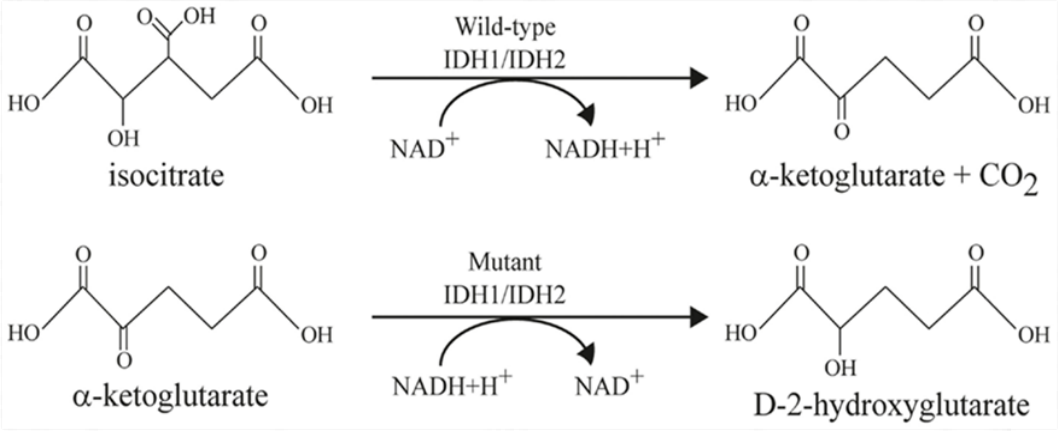
II.1.3 Isocitrate Dehydrogenase

Isocitrate dehydrogenase (IDH) is a small molecule protein that is widely distributed in the heart muscle, skeletal muscle, liver (LaPorte and Koshland, 1983) and also in brain (Jennings et al., 1994). It is involved in various cellular processes, including oxidative phosphorylation, lipogenesis, glutamine metabolism, glucose sensing, and regulation of redox status in cells (Reitman and Yan, 2010; Ständer et al., 2004). *IDH* is an $NAD(P)^+$dependent enzyme which catalyzes the formation of α-KG via an oxidative decarboxylation step involving reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H) generation (Shi et al., 2014a). Of the 3 isoforms of IDH, IDH1 resides in the cytosol and in peroxisomes, whereas IDH2 and IDH3 in the mitochondria (Krell et al., 2011). IDH1/2 (NADP-dependent) are homodimeric enzymes, whereas IDH3 (NAD-dependent) is a heterodimer, which is not related to the other two isoforms structurally (Mellai et al., 2013). IDH plays an essential role in the regulation of Kreb's cycle and is also involved in cell's energy metabolism in almost all tissues (Huang et al., 2019).

II.1.4 IDH mutations

IDH mutation is commonly seen in acute myelogenous leukemia (AML)and in gliomas (DiNardo et al., 2015). *IDH* mutations were first identified in gliomas by Parsons *et al.,* when they sequenced human glioma tissue samples in 2008. The mutation was observed in all but one of the secondary GBM, although none of the primary exhibited *IDH* mutation (Parsons et al., 2008). In gliomas, IDH1 mutation is the frequently observed mutation. It is a point mutation, which occurs either at the first or second base of the codon 132 of the *IDH1* gene, where histidine is formed instead of arginine (R132H). Several other types of mutations also occur in IDH1 at the same codon where arginine is replaced by glutamine, cysteine, serine or lysine (Watanabe et al., 2009). Some of the commonly seen IDH2 mutations in gliomas are R172K, R172M, R172W and R172G. Most of the IDH1 and IDH2 mutations in gliomas are frequently observed in AML and

some cartilaginous tumors (Yang et al., 2012). *IDH3* mutations are not found in glioblastomas at an appreciable rate (Huang et al., 2019). Secondary glioblastomas which develop on a background of low grade astrocytoma possess *IDH1* mutation (Ohgaki and Kleihues, 2013). The *IDH1* mutation is a heterozygous mutation where one allele is a mutant one and the other is the wild-type. The mutant allele of *IDH* further converts α-KG formed by wild-type IDH to 2-Hydroxy Glutarate (2-HG) (Navis et al., 2013). Normally, wild-type *IDH1* produces α-KG in the cytoplasm, which enters the mitochondria where NAD(P)H is generated, which in turn helps in the formation of glutathione (GSH), a major antioxidant. GSH is responsible for preventing the damage caused by oxidative stress and also has anti-apoptotic functions (Circu and Aw, 2012).



Voelxen, 2016

Fig.II.3 Enzymatic reactions catalyzed by wild-type and mutant IDHs

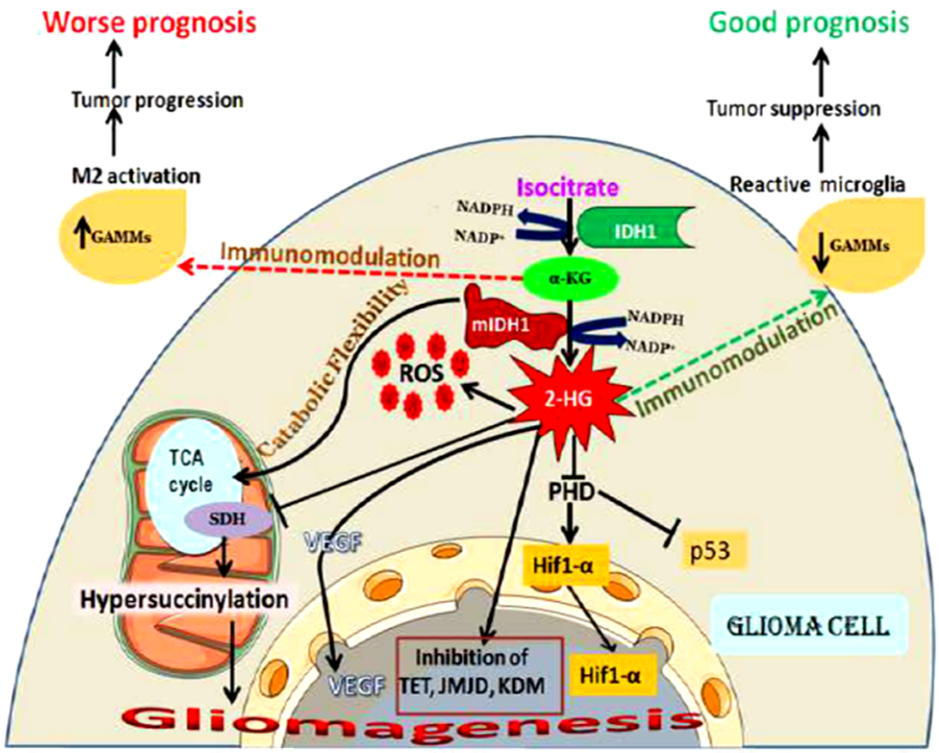
Homozygous *IDH1* mutation was also found recently in a few number of patients, but the prognostic significance and effects of the 2-HG produced by the same is not fully understood (Singh et al., 2017). It remains to be understood whether the quantity of 2-HG produced be directly linked to the heterozygous or homozygous nature of the mutation. Also mIDH1 harboring tumor cells are metabolically different from the tumor cells with wild type IDH1 (Garrett et al., 2018).

II.2 2-HG "an oncometabolite"

Once mutation occurs in the *IDH1* gene, the production of NADPH that helps in the formation of GSH gets reduced. Moreover, NADPH will be consumed by the mutant form of *IDH1(mIDH1*) to produce 2-HG which leads to the decreased production of GSH, thereby increasing the cellular ROS level (Shi et al., 2014a). This decline in the level of NADPH will cause a reduction in the GSH (an antioxidant) level which elevates the ROS generation in the glioma cells. 2-HG inhibits various α-KG dependent dioxygenases like TET (Ten Eleven Translocase) family of DNA hydroxylases, lysine histone demethylases (KDMs), prolyl hydroxylase domain (PHD) and JMJDs (Jumonji C-domain-containing enzymes) and thus is involved in tumorigenesis. In case of wild-type IDH1 cells, PHD acts on its substrate Hif1α and promotes Hif1α degradation because Hif1α accumulation results in tumorigenesis. 2-HG inhibits Hif1α degradation and p53 downregulation by inhibiting PHD, promoting gliomagenesis. 2-HG is also involved in the hypersuccinylation of TCA enzymes in the mitochondria via inhibition of Succinate dehydrogenase (SDH), which leads to apoptotic resistance in tumor cells and thus results in tumorigenecity. 2-HG favors VEGF (Vascular Endothelial Growth factor) upregulation leading to angiogenesis. mIDH1 cells have higher proportion of pro-inflammatory GAMMs (Glioma Associated Microglia and Macrophages), although fewer in total. wt-IDH1 and mIDH1 shows distinct immunomodulatory functions which may render opposite prognostic values (Bhavya et al., 2020).

2-HG can be present either in the S-form (L-2-HG) or in the R-form (D-2-HG). Out of the two, D-2-HG is the one that accumulates in IDH1-mutant glioma cells(Dang et al., 2009). Patients with D-2-HG have increased risk of brain tumors. Normally, D-2-HG is converted by the enzyme D-2HGDH (D-2HG Dehydrogenase) to α-KG and thereby decreasing its level in the cell. The increased L-2HG level is seen in a disease condition called 2-Hydroxyaciduria (a rare neurological disorder) (Madala et al., 2018). L-2-HG patients do not have brain tumors, because they all die in infancy. This is in contrast to D-2-HG which accumulates in gliomas with IDH mutation. It is the presence of 2-HG that helps in identifying the IDH mutation in glioma patients (Kranendijk et al., 2012). 2-HG is also associated with various other processes of the cell including the epigenetic

regulation and cellular signaling and repression of the tumor-associated immune system in glioma (Gagné et al., 2017).



Bhavya et al., 2020

Fig.II.4 Gliomagenesis, prognosis and immunomodulation rendered by IDH1 status

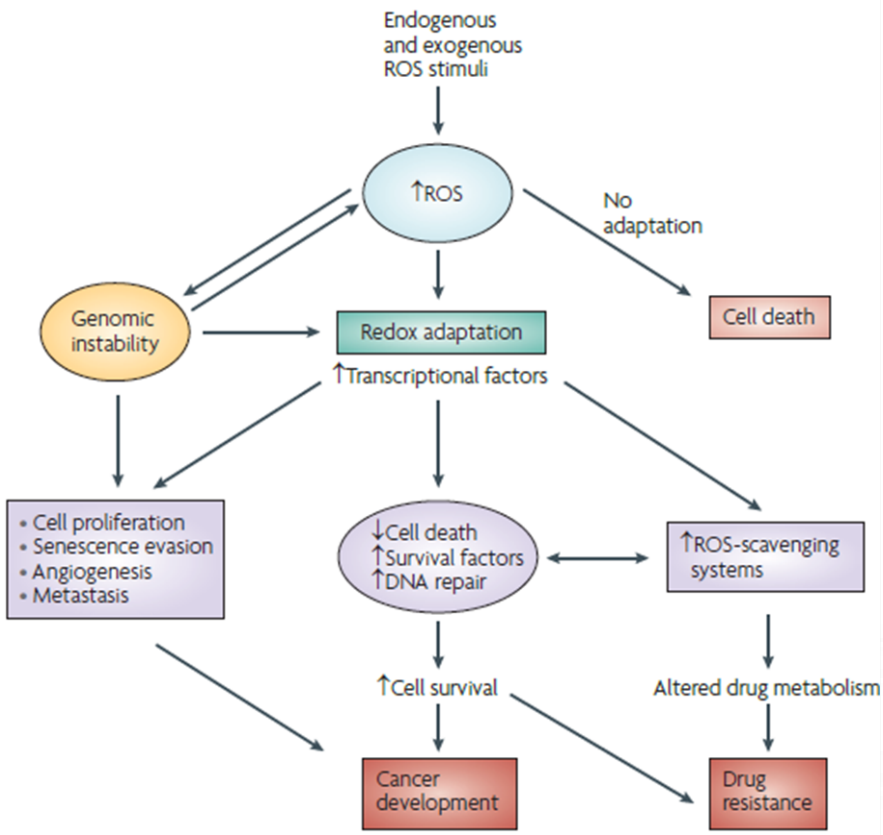
Dang *et al.* first depicted that 2-HG is synthesized by the mutant form of IDH1 and it has properties that enable us to refer to it as an 'oncometabolite'. 2-HG is a metabolite that is present in micromolar levels even in normal cells. Elevated level of 2-HG in blood, other body fluids like plasma and urine, or tissues is an indication of metabolic disorders. 2-HG in the mitochondria is produced by the enzyme, hydroxyacid-oxoacid-transhydrogenase (HOT) and in the cytosol by phosphoglycerate dehydrogenase from a common substrate, α-KG. 2-HG dehydrogenase catalyzes the conversion of 2-HG into α-KG, and any impairment in this enzyme leads to the accumulation of 2-HG (Struys, 2006).

In a recent report, it shows that 2-HG inhibits the pathways involved in complement activation, with a reduction in proliferation of tumor infiltrating and activated T cells, phagocytosis and cytokine secretion in human glioma tissue samples. This suppression of

host immune system by 2-HG in mIDH1 tumors helps in the escape of tumors from the action of immune system against tumor cells. Thus this could be one of the answers for the frequently asked question, how 2-HG favors tumorigenesis (Zhang et al., 2018). It remains to be understood whether the quantity of 2-HG produced be directly linked to the heterozygous or homozygous nature of the mutation (Garrett et al., 2018). Homozygous IDH1 mutation was also found recently in a few number of patients, but the prognostic significance and effects of the 2-HG produced by the same is not fully understood (Singh et al., 2017). Also mIDH1 harboring tumor cells are metabolically different from the tumor cells with wild type IDH1 (Garrett et al., 2018).

II.3 Reactive Oxygen Species

Reactive oxygen species (ROS) are chemically reactive oxygen contacting radicals as well as non-radicals (Halliwell, 2006). They comprise a group of highly reactive molecules that are the key regulators of various signaling pathways. Moderate levels of ROS are essential for several cellular functions, including gene expression, for example; H_2O_2 has an important role as a second messenger in some of the pathways (Perillo et al., 2020). ROS includes the production of superoxide ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O2$), peroxyl radical ($LOO^{\bullet}$), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), ozone (O_3), and some others by various pathways (Li et al., 2016). Numerous articles have been in published since 1945 relating ROS to various physiological processes and pathological conditions including diabetes, cancer, artherosclerosis, ageing, cardiovascular diseases, and so on (Alfadda and Sallam, 2012). ROS are mainly produced in mitochondria through the electron transport chain, in peroxisomes and also in the endoplasmic reticulum (Bedard and Krause, 2007). ROS levels can be elevated in tumor cells due to various reasons including high metabolic rate, mutations, hypoxia, and so on. But excess ROS are reduced by increased antioxidant mechanism present in the cells. Increase in ROS levels contribute to several pathologic conditions like tumor progression and survival (Perillo et al., 2020; Reczek and Chandel, 2017).



Trachootham et al., 2009

Fig.II.5 Redox adaptation in cancer development and drug resistance

ROS levels have been found to be elevated in most of the cancers. The major functions played by ROS in cancers include cell survival and apoptosis, cell morphology, cell cycle progression, cell proliferation, energy metabolism, cell-cell adhesion, angiogenesis, metastasis, invasion and tumor stemness maintenance (Liou and Storz, 2010; Shwetha et al., 2016). Any imbalance in level of ROS can result in oxidative stress and further production of free radicals leads to the damage of nucleic acids, proteins, lipids, cell membranes and mitochondrial malfunctioning (Chio and Tuveson, 2017; Latunde-Dada, 2017). Hence, the tight regulation of ROS in cells is very essential (Perillo et al., 2020). Most of the anticancer drugs kill their target cells through the production of elevated amounts of ROS inside the cells. The effect of ROS on different cells may vary according to the metabolic rate of the cell (Singer et al., 2015). In healthy cells, the high ROS levels

are balanced via various detoxification processes including the antioxidant enzymes (Aggarwal et al., 2019).

II.4 Oxidative stress and its markers

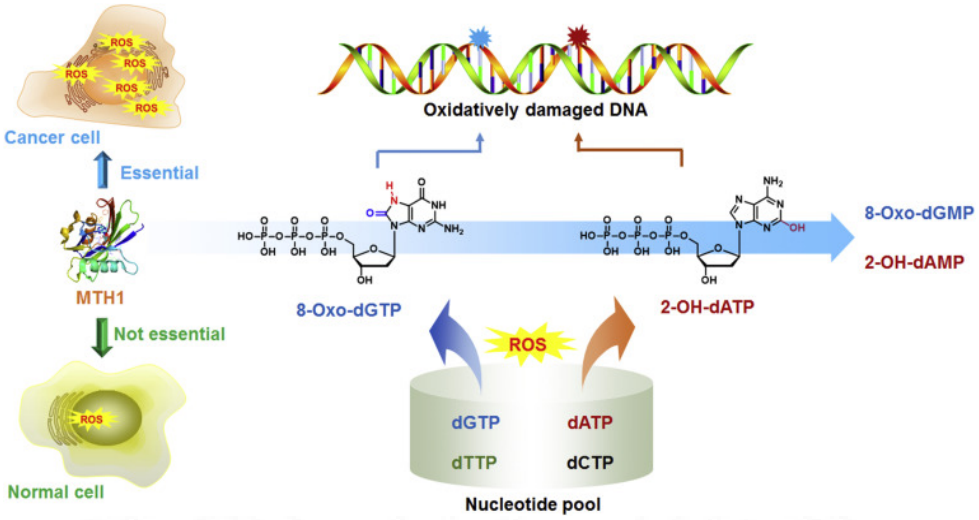
Oxidative stress is well defined as any imbalance in the anti-oxidant system and the ROS production in the cells (Betteridge, 2000). This in turn causes oxidation of cellular components, activation of signal transduction pathways in cytoplasm and nucleus, alteration of gene expression, protein expression and modulation of activities of nucleic acid polymerases (Tudek et al., 2010). Some of the major DNA alterations that can occur due to oxidative stress include strand break, base substitution, DNA-protein cross linkages and all these can contribute to carcinogenesis (Rinaldi et al., 2016). Oxidative stress can lead to the formation of lesions to the DNA of the cell (Bedi et al., 2017; Mencalha et al., 2014; Pourahmad et al., 2016). Being the base with low redox potential, guanine is highly sensitive to oxidation (Aguiar et al., 2013). Out of the various oxidative lesions, 8-hydroxy-2-deoxyguanosine (8-OHdG), 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanine (8-oxoGua) are some of the reliable biomarkers of oxidative stress and ROS-induced carcinogenesis, further genomic instability and cell death (Oberley, 2002; Roszkowski et al., 2011; Wu et al., 2004).

As discussed above tight regulation of ROS is an essential process for cells. Therefore, in order to scavenge the extra ROS present in cells, eukaryotic cells possess a complex anti-oxidant system (Perillo et al., 2020). Antioxidants have the capacity to neutralize and scavenge free radicals/ROS in cells (Hawk et al., 2016). They are of two major types: enzymatic and non-enzymatic antioxidants. Glutathione peroxidases (Gpx), catalase and superoxide dismutases (SODs) are the important enzymatic antioxidants (Asadi-Samani et al., 2019). Gpx, another important anti-oxidant enzyme resides in cytoplasm, and also in the mitochondria of brain since brain mitochondria is the major site of hydrogen peroxide (H_2O_2) production, where it converts H_2O_2 to water (Huang and Philbert, 1995; Li et al., 2000). Among the various types of Gpxs, Gpx4 plays an essential role in detoxification of oxidative damage caused by hydroperoxides to membrane lipids. There are different types of Gpxs; Gpx4 reduces complex lipids from phospholipids, cholesterol and cholesteryl esters. It also plays an essential role in apoptosis (Ran et al., 2007).

SODs are located mainly in the cytoplasm and mitochondria (Perillo et al., 2020). They helps in eliminating the superoxide radicals present in the cell (Hileman et al., 2001). Manganese superoxide dismutase (SOD2 or MnSOD), is the mitochondrial SOD that regulate the oxidative stress and energy metabolism in the mitochondria. Apart from this, SOD2 also have crucial role in the development of cerebral cortex, and its expression is usually significantly high in brain cancer than the normal brain. It is a key factor in the oxidative defense especially in GBMs (Chien et al., 2009).

II.5 MutT Homolog 1

ROS-mediated DNA damage is a major hallmark of most of the cancers (Gad et al., 2014; Kumari et al., 2018). Such high ROS in cells results in the accumulation of oxidized nucleotides. Further, the incorporation of such oxidized nucleotides into the cancer cell DNA may cause damage to DNA, leading to cell death (Abbas et al., 2018). There are altogether 24 genes coming under the Nudix hydrolase family in humans, out of which two of the genes encode the MTH1 and MTH2 enzymes which are capable of hydrolyzing 8-oxo-dGTP. MTH1 vis-à-vis MTH2 has higher preference to 8-oxo-dGTP and MTH2 activity does not have much role in sanitation of oxidized nucleotides (Abbas et al., 2018; Maki and Sekiguchi, 1992). The enzyme MTH1 prevents the incorporation of oxidized forms of nucleotides like 8-oxodGTP or 2-OH-dATP into the DNA or RNA by hydrolyzing them into their respective monophosphates and pyrophosphate, thus preventing DNA damage and further cell death (Gad et al., 2014). The 8-oxo-dG level reflects the activity of MTH1 although it does not account for the actual incorporation of 8-oxo-dGTP to the DNA. Since, MTH1 is the Nudix enzyme that has highest affinity for the oxidized nucleotide substrate 8-oxo-dGTP (McLennan, 2006), the absence of MTH1 could lead to increased oxidative DNA damage in cells (Gad et al., 2014; Kumari et al., 2018). Even though this mechanism provides a mode of safeguarding DNA from various mutations it can also favor the cancer cell survival by removing the DNA damaging effect of cancer therapies. While some of the previous reports showed that MTH1 mainly resides in the cytoplasm where the main pool of free nucleotides exists, at the same time there are some other studies which showed that MTH1 is mainly located in nucleus and mitochondria (Kang et al., 1995; Leon et al., 2016; Nakabeppu et al., 2006; Nakayama et al., 2011).



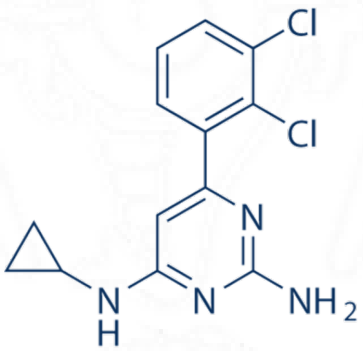
Yin and Chen, 2020

Fig.II.6 MTH1 as a therapeutic target for cancer eradication

In the year 2014, a "nature" article by Gad *et al.;* have stated that MTH1 might be indispensable for sanitizing the free oxidized nucleotide pool, thus prevents DNA damage in cancer cells. They have also shown *in vivo* that the silencing of MTH1 resulted in DNA damage and apoptosis in cancer cells, but is non-essential for normal cells (Gad et al., 2014). In a different study by Huber *et al.;* have identified that (S)-crizotinib is effective as an MTH1 activity inhibitor *in vivo*. They also shown that (S)-crizotinib treatment induced the oxidized nucleotide accumulation in DNA and significantly impaired xenograft tumor growth (Huber et al., 2014). Both the above mentioned studies showed have that MTH1 was overexpressed in almost all human cancers. A novel role for MTH1 was discovered by Gad et al in 2019. They found that MTH1 have a mitotic role where it binds tubulin which is required for microtubule polymerization, for spindle assembly and mitosis progression. Potent MTH1 inhibitors like TH588 and TH278 have the ability to break the MTH1-tubulin interaction, leading to mitotic arrest (Gad et al., 2019).

There are various studies that targeted MTH1 in different cancers. MTH1 inhibition leads to the accumulation of 8-oxo-dG (Zhou et al., 2019). Some of the commonly used MTH1 inhibitors are (S)-crizotinib, TH588 and TH278 (Gad et al., 2014; Ikejiri et al., 2018;

Pompsch et al., 2018; Prada et al., 2017; Waals et al., 2019; Zhou et al., 2019). At the same time, a report in 2019 says that the 8-oxodGTPase activity of MTH1 can be replaced by an alternative mechanism like MTH2 in tumor cells. Also they have suggested that the use of MTH1 inhibitors in a combinatorial treatment regimen can enhance their therapeutic index instead using as a single agent (Samaranayake et al., 2020).



Gad et al., 2014

Fig.II.7 Structure of MTH1 inhibitor TH588

There are some reports which shows that brain is more vulnerable to oxidative DNA damage and the reason behind this is the high endogenous metabolic activity in brain (Canugovi et al., 2013; Lovell and Markesbery, 2007). There are only a limited studies regarding MTH1 in glioma (Iida et al., 2001; Pudelko et al., 2017; Tu et al., 2016; Versano et al., 2018). 8-oxo-dG accumulation was found to be high in high grade gliomas (HGG). MTH1 is an enzyme that can prevent the DNA damage caused by oxidized nucleotides (Iida et al., 2001). Recently, inhibition of MTH1 along with inhibitors of histone deacetylases (HDAC) and PARP enhance the response of GBM to chemotherapy and radiotherapy were reported (Versano et al., 2018). Pudelko *et al.*, in 2017 claimed that GBM cultures with both low and high aggressiveness showed reduced cell viability after inhibition of MTH1 (Pudelko et al., 2017).

II.6 8-oxodeoxyguanosine

Elevated levels of 8-oxodG were found to be accumulated in various cancers including lung cancer, basal cell carcinoma, bladder cancer, colorectal cancer, lymphoblastic

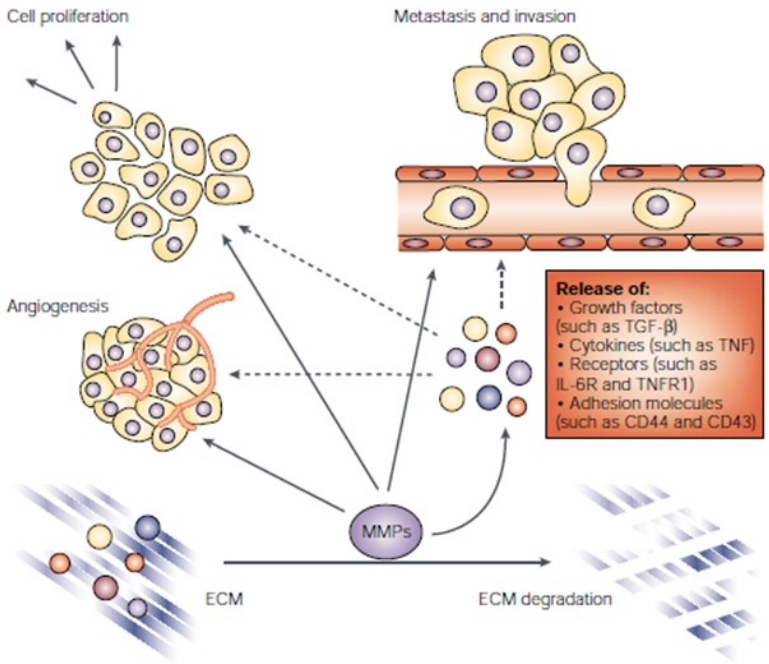
leukaemia, high grade cervical dysplasia, prostate cancer, gastric intestinal metaplasia, renal cell carcinoma and gastric adenocarcinoma when compared with their healthy subjects (Sova et al., 2010). Another study shows that increased 8-oxodG levels were also observed in serum samples of patients with breast cancer (Cho et al., 2009). From these it is clear that 8-oxodG levels would be a marker of increased oxidative stress in cancers and hence induces DNA damage. As mentioned earlier, since MTH1 have important roles in DNA damage prevention in cancer, its inhibition causes increased DNA damage. ROS attack can lead to DNA oxidation which can generate more than 20 oxidized base adducts (Lovell and Markesbery, 2007). 8-oxoG being the major oxidized base present in nucleic acids (both DNA and RNA) and nucleotide pools is found to be accumulated in most of the diseases including cancer (Sheng et al., 2012). The basal level of metabolic activity is always high in brain cells and hence use various oxidative damage repair mechanisms to remove oxidative damage from DNA and from free nucleotide pools (Canugovi et al., 2013). 8-oxoG has the ability to pair with adenine as well as cytosine, hence causes G to T transversion mutations (Ohno et al., 2014). 8-oxoG levels can be kept lowered by two main enzymes: one is MTH1 and the other is 8-oxoguanine glycosylase1 (OGG1). OGG1 excises the 8-oxoG opposite cytosine in the DNA to initiate a base excision repair (BER) process. Just like MTH1, OGG1 too resides in nuclei, cytoplasm and mitochondria (Leon et al., 2016; Wang et al., 2018). In glioma treatment, a majority of the TMZ induced lesions like N7-methylguanine and N3-methyladenine are BER substrates which will be readily repaired by the BER enzymes. Hence, blocking the BER pathway can make the TMZ therapy more effective and also BER pathway has emerged as another attractive target for reversing TMZ resistance in gliomas (Goellner et al., 2011). Another important protein X-ray repair cross-complementing protein 1 (XRCC1) participates in single strand break repair in DNA along with many other BER enzymes (London, 2015). XRCC1 is better defined as a non-enzymatic scaffold protein that is involved in BER by interacting with other BER proteins and has no enzymatic activity (Wallace et al., 2012). Polymerase-β, poly (ADP-ribose) polymerase [(PARP)] is the enzyme that recruits XRCC1 to the site of DNA lesion (Torgovnick and Schumacher, 2015). XRCC1 in combination with Ligase III helps in the ligation of DNA ends (Marsden et al., 2017).

II.7 Cancer metastasis

Cancer metastasis is the hallmark of cancer malignancy. It is a leading cause of deaths among cancer patients. Metastasis is a process that includes mainly migration and invasion of cancer cells. Both these processes require the involvement of various cellular mechanisms led by cytoskeleton dynamics and molecular alterations including cell adhesion. Cell migration is a process that includes formation of cytoplasmic protrusions, attachment, traction, production of ROS, mutations of genes involved in DNA damage repair and so on (Tahtamouni et al., 2019). Cell invasion is a key factor for cancer progression and metastasis to other tissues and organs. A moving cell possess a "leading edge" or "leading front" which uses proteases and integrins (Krakhmal et al., 2015). As it is well known that there are different modalities for GBM treatment, none of those therapies have been successful in curing GBM. Two of the reasons for this are: the (1) presence of blood–brain barrier (BBB) and (2) the invasive nature of brain tumor cells (Cuddapah et al., 2014). In case of GBM, metastasis occurs mainly through cerebrospinal fluid, but GBM metastasis outside CNS happens rarely (Liu et al., 2018). GBM have become the most aggressive tumor because of the tumor-propagating property of human glioma stem cells. Due the high rate of migration, the cells migrate into the surrounding brain parenchyma (Giese et al., 1996; Zepecki et al., 2019). Cilengitide is a drug for GBM that targets glioma cell migration and invasion (Zepecki et al., 2019). In order to migrate, the cell changes its shape and stiffness according to the surrounding ECM. For moving, the cells become polarized, develop membrane protrusions like extensions at the leading edge of pseudopodia, lamellipodia and filopodia (Demuth and Berens, 2004).

Glioma cells have the ability to migrate through extracellular matrix by degrading proteins present in the matrix and thus create a path for cell invasion. There are various matrix-metalloproteinases (MMPs) involved in this process in most of the cancer cells including glioma. Hence, most of the pathways that promote glioma invasion upregulate numerous MMPs (Nishida et al., 2006). MMPs being the key players in invasion facilitate tumor cell extravasation to promote invasion (Stamenkovic, 2000). Generally, in cancers MMP upregulation and activation is a sign of tumor progression and high level MMP expresssion correlates with poor survival (Falk et al., 2018). According to their substrate specificity, MMPs are classified into four groups: colleganase, gelatinase,

stromelysins and a fourth one containing various heretogenous groups (Klein and Bischoff, 2011; Sternlicht and Werb, 2001). MMP-2 and MMP-9 comes under the gelatinase family of MMPs, hence the major substrates are gelatin and collagen Type IV. Increased MMP-2 and MMP-9 expression is correlated with poor survival outcome in various cancers (Li et al., 2017; Mendes et al., 2005; Salem et al., 2016).



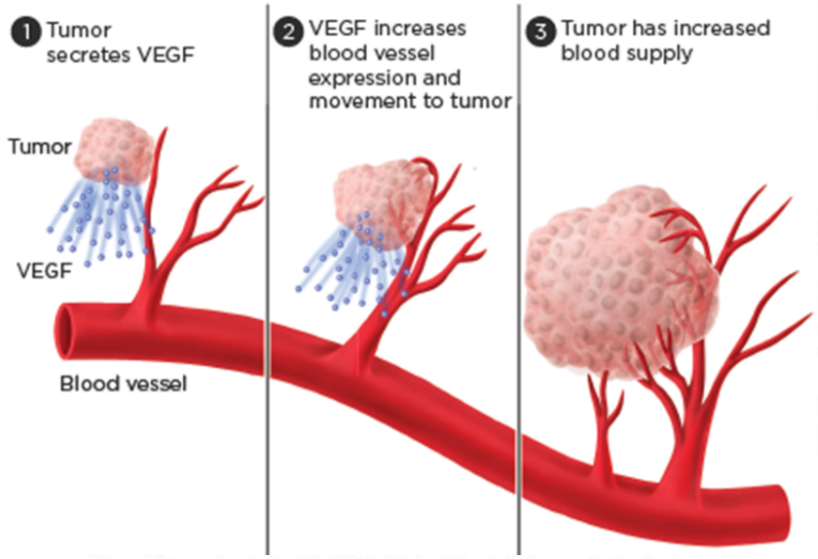
Rao, 2003

Fig.II.8 MMPs promote cancer cell growth

Rho/Rac proteins and Cdc42 are some of the major protein involved in the cell migration process. Rac1 (Ras-related C3 botulinum toxin substrate 1) and Rho A are GTP binding proteins which are crucial for cell migration. Rho, Rac and other Ras-related GTP-binding proteins control the organization of the actin cytoskeleton during the process of migration (Etienne-Manneville and Hall, 2002; Tapon and Hall, 1997). Rac subfamily of proteins includes Rac1, Rac2 and Rac3. Rho molecules regulate the stress fibers and focal adhesion formation, whereas Rac control membrane ruffling and Cdc42 regulates the filopodium (Parri and Chiarugi, 2010).

II.8 Angiogenesis in cancer

Angiogenesis is a process that is regulated by the equilibrium between activators and inhibitors. Angiogenesis gets stimulated when tumor tissues require nutrients and oxygen. The upregulation of angiogenic activators and at the same time the downregulation of angiogenic inhibitors are required for the angiogenesis of the tumor. Some of the common activators are Vascular endothelial growth factor (VEGF), angiogenin, transforming growth factor alpha (TGF-α) and beta (TGF-β), basic fibroblast growth factor (bFGF), tumor necrosis factor alpha (TNF-α). VEGF and its receptors (VEGFR) plays central role in the process of neoplastic vascularization. VEGF is a powerful angiogenic agent in cancer tissues and it also helps in neovascularization (Nishida et al., 2006). VEGF was reported to be upregulated in gliomas than the non-tumor brain tissues (WANG et al., 2016). An increased VEGF levels in glioblastomas show poor prognosis (Xu et al., 2013). VEGF inhibition affects tumor growth, causes DNA damage and also reduces cell survival in glioma (Michaelsen et al., 2018). VEGFR-2 (Flk1 in mice) is a major signal transducer for blood vessel formation or angiogenesis (Shibuya, 2011) and it is reported that Flk1 activation is essential for angiogenic responses and glioma growth (Hardee and Zagzag, 2012; Reynolds et al., 2004).



Oliver and Waxman, 2019

Fig.II.9 VEGF: A key mediator of angiogenesis

II.9 Hypoxia Inducible factor 1 alpha

Hif1α is a one of the major mediator of tumorigenic responses under hypoxic condition. Hif1α modulates MTH1 expression in colorectal cancer was reported earlier (Qiu et al., 2015). Hif1α being a transcriptional factor is found to be elevated in various cancers and this indicates a poor prognosis (Iommarini et al., 2017; Jun et al., 2017).

Hypoxia or low oxygen concentrations initiates a sequence of adaptive responses through the upregulation of various genes involved in angiogenesis, glycolysis and many other responses which are mediated by transcription factor called Hif1 (Jung et al., 2008). It is a dimeric protein complex that plays a central role in the body's response to hypoxia. It is a transcription factor for a number of target genes. It is necessary for immunological responses and is a vital physiological regulator of vascularization (in tumors), homeostasis and anaerobic metabolism (Ziello et al., 2007). Hif1 is a heterodimeric transcription factor which consists of Hif1α and Hif1β, out of which β-subunit is a constitutively expressed and α-subunit is regulated by oxygen. Both Hif1α and Hif1αβ contains basic helix-loop-helix motifs that binds with DNA and can cause subunit dimerization (Kietzmann and Görlach, 2005; Zhao et al., 2014; Ziello et al., 2007). In normoxic state, Hif1α is degraded by an enzyme called PHD which utilize oxygen as a substrate. ROS is found to play a critical regulator of Hif1α in non-hypoxic conditions. Moreover, ROS were also reported to induce Hif1α. But the mechanism by which ROS regulates Hif is yet to be elucidated. Both Hif1α as well as ROS plays a central role in cancer development, the link between Hif1α and ROS has to be investigated (Jung et al., 2008; Kietzmann and Görlach, 2005). The regulation of Hif1α involves multiple mechanisms and pathways. Among these includes the regulatory role of ROS on Hif1α (Movafagh et al., 2015). Active Hif1α can reprogramme the glucose metabolism from oxidative phosphorylation to glycolysis, which results in a reduction in cytotoxic ROS levels, which then contribute to formation and survival of metastatic cancer cells (Zhao et al., 2014). Hypoxia is one of the main factors in the pathology of diseases like aging, diabetes, stroke and ischemia along with cancer. Hif1α targets expressed in normoxic and hypoxic areas may provide a novel target to eradicate solid tumors (Cimmino et al., 2019). It is reported that in ischemic patients, Hif1α cause angiogenesis, hence it can promote the blood vessel proliferation required for oxygenation. In case of cancer, the

survival and proliferation of cancerous cells is mainly due to its angiogenic property, so targeting Hif1α can prevent the spread of cancer by interacting with other transcription factors and enzymes (Ziello et al., 2007). For instance, Hif1α regulates the transcription of vascular endothelial growth factor (VEGF) which is the major regulator involved in angiogenesis, which promotes the migration of endothelial cell towards the hypoxic environment (Carmeliet et al., 1998; Genbacev et al., 1997). Such endothelial cells later helps in the formation of new blood vessels and supply oxygenated blood to other areas (Ziello et al., 2007).

II. Materials and Methods

III.1 Reagents, antibodies and drugs

TH588, an MTH1 inhibitor was kindly gifted by Prof. Thomas Helleday, (Karolinska Institutet, Sweden). Acrylamide-Bis-acrylamide solution, West Femto and Pico Enhanced Chemiluminescent reagents, RIPA buffer, BCA protein assay kit, Protease inhibitor and Phosphatase Inhibitor cocktail were from Pierce Biotechnology (MA, USA). OptiBlot ECL was from Abcam (Cambridge, UK) and ClarityMax ECL Kit was from Bio-Rad Laboratories (CA, USA). TaqMan probes and TaqMan gene expression assay master mix were purchased from Applied Biosystems (Thermo). The Relia Prep RNA Tissue Miniprep was fromPromega (WI, USA). Antibodies against Vinculin, α-Tubulin, cleaved PARP, γH2AX, RAC1, RhoA, Horse Radish Peroxidase (HRP)-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Cell Signaling Technology (MA, USA). Anti MTH1 antibody was procured from Novus Biologicals (CO, USA). Anti β-actin and mutant IDH1 R132H antibodies and RNA Later were from Sigma. Syringe filters, and filter membranes (for media filtration) and PVDF membrane were from Millipore (MA, USA). Antibodies against FLAG, VEGF, VEGFR-2, MMP2, MMP9, MnSOD, and GPx4 were from Abcam (Cambridge, UK) and XRCC1, OGG1 from ImmunoTag (MO, USA). Hif1α antibody was purchased from Santa Cruz Biotechnology (TX, USA).

Transfection reagent from Polyplus Transfection SA (Illkirch-Graffenstaden, France) and MTH1 and Hif1α siRNAs were purchased from Eurogentec (Seraing, Belgium). Consumables/plasticwares were purchased from Axygen Scientific (CA, USA) and Tarsons (Kolkata, India). Culturewares was procured from Becton Dickinson (NJ, USA) and Nunc (Thermofisher Scientific, MA, USA).

III.2 Instruments

ELISA plate reader (Bio-Tek Instruments, VT, USA), PCR Thermal Cycler (Bio-Rad Laboratories, CA, USA), Semi Dry Blot Apparatus and Wet-transfer Apparatus, Powerpac universal, Mini-PROTEAN Tetra Cell Electrophoresis Unit (Bio-Rad Laboratories, CA, USA), Water bath (Julabo GmbH, Seelbach, Germany), UV-visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan), pH meter (Eutech, Singapore), Weighing balance (Sartorius, Gottingen, Germany), Magnetic Stirrer (Schott, Mainz, Germany), Micro-Pipettes (Eppendorf, Hamburg, Germany), -80^{o}C Freezer (New

Brunswick Scientific, NJ, USA), -20^{o}C Freezer (Vest frost, Falkevej, Denmark), Centrifuge (REMI, Mumbai, India), Cooling centrifuge (Eppendorf, Hamburg, Germany), Laminar Air Flow Hood (MicroFilt, Pune, India), CO_2 incubator (Sanyo, Osaka, Japan), Phase Contrast Microscope with fluorescence filters (Olympus Life Science, Tokyo, Japan), BD FACS Jazz Cell Sorter (Becton and Dickinson, NJ, USA), 7500 Real-Time PCR System (Applied Biosystems, CA, USA), Gel Documentation System (Bio-Rad Laboratories, CA, USA).

III.3 Collection of tissue biopsy:

Experiments were conducted in human glioma tissue biopsies (SCT/IEC/932/AUGUST-2016) and temporal lobe epilepsy tissue biopsies (SCT/IEC/1254/DECEMBER-2018), which serve as the control, collected from the Department of Neurosurgery, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, after obtaining the Institute Ethical Committee approval. Informed written consents were obtained from the patients or relatives prior to tissue collection. The samples were collected in 1X PBS for DNA and protein studies (immunoblot), in formalin for IHC studies and in RNA Later for analyzing the mRNA level expression of MTH1 and the tissues (for protein and DNA studies) immediately after surgical resection were collected and stored at -80˚C, for IHC will be kept at room temperature (27˚C) and for RNA, will be stored for one day at 4˚C and then at -80˚C.

III.4 Cell culture and maintenance

III.4.A Procedure

U87MG and U251MG glioma cell lines procured from National Centre for Cell Science (NCCS, Pune, India) were used for the experiments. Both the cells were cultured in DMEM (low glucose) (Sigma-Aldrich, Gillingham, UK) with 10% fetal bovine serum (FBS) (PAN-Biotech GmbH, Aidenbach, Germany) and antibiotic-antimycotic cocktail solution (PAN-Biotech GmbH, Aidenbach, Germany) at 37°C and with 5% CO2. The cells were removed from the culture flask/dish using Trypsin-Phosphate-Versene-Glucose (TPVG). The cells were then centrifuged at 2500 rpm for 5 min and the supernatant was removed. For freezing the cells, the cells were resuspended in 1 ml of the

freezing medium (50% FBS and 5% DMSO in the culture medium) and stored at -80 °C freezer.

III.4.B. Reagents Used

III.4.B.1.DMEM preparation (1 litre) (pH 7.4)

2g $NaHCO_3$ (Sodium bicarbonate), Low Glucose DMEM powder (16g), 10 mL Antibiotic-antimycotic cocktail (100x) mixed in sterile distilled water, filtered and then stored in autoclaved bottles.

III.4.B.2. Phosphate-buffered saline (PBS) (pH 7.4)

Potassium chloride- 2.7 mM, Sodium chloride- 137 mM, potassium dihydrogen phosphate- 1.76 mM and disodium hydrogen phosphate- 10.14 mM in sterile deionized water.

III.4.B.3. TPVG solution (pH 7.4)

0.1% trypsin, 0.2% EDTA and 0.05% glucose dissolved in 1X PBS.

III.4.C. Other chemicals used

III.4.C.1. DCFDA stock (10 mM)

5 mg in 1.026 mL DMSO

III.4.C.2 TH588 HCl (10 mM)

3mg were dissolved in 150 µL DMSO and 10 µM were used for experiments.

III.4.C.3 N-Acetyl Cysteine (1 M)

163mg in 1mL deionized sterile water

III.4.C.4 Hydrogen Peroxide (H_2O_2)

1M H_2O_2 was prepared from 8.82M (30% w/v) H_2O_2 by adding 1/133ml of H_2O_2 in 8.867ml 1X PBS. 100 µM was used for the experiment.

III.4.C.5 2-Hydroxy glutarate (50mM)

10mg were dissolved in 1.04 mL DMSO and 30 mM were used for experiment.

III.4.C.6 Mutant IDH1 inhibitor- AGI-5198 (10mM)

5mg in 1.081mL 1X PBS and 2 µM were used for experiment.

III.4.C.7 Cobalt chloride (100nM)

13mg were dissolved in 1mL PBS. 50µM and 100 µM were used for U251 and u87 cells respectively.

III.5 Cell viability assays

III. 5.A Hoechst –PI staining procedure

Cell death analysis was performed by Hoechst/ PI double-staining method. Hoechst 33342 stain ($C_{27}H_{37}Cl_3N_6O_4$) is a cell-permeable fluorescent dye, that can enter both live and dead cells, and it stains the nuclei of any cell. Hoechst 33342 (Sigma-Aldrich, MO, USA) dissolved in 1X PBS at a concentration of 1mg/ml. The experiment was performed in 24-well plate at a seeding density of $5x10^4$ cells per well, in order to check the number of non-viable cells due after inhibiting MTH1. Hoechst was used at a final concentration of 1µg/ml for each well. The cells were then incubated at room temperature for 15-20 min to allow the cells to uptake the Hoechst stain. The cells were then washed with HBSS and then incubated with Propidium Iodide (PI) (Propidium Iodide, Sigma-Aldrich, MO, USA), another fluorescent dye, which specifically stains the dead cells (with damaged cell membrane). The dye was used at a concentration of 1mg/ml and incubated at room temperature for 5 min. The imaging of each well was done using fluorescence microscope to determine the total number of dead cells, which fluoresces red. The percentage of cell death was calculated using the formula; $[(PI/H)_{Test} / (PI/H)_{Control}]x100$, where PI= total number of PI stained nuclei and H= total number of Hoechst stained nuclei.

III. 5.B Reagents Used

III.5.B.2 Hoechst 33342 stock (10 mM)

2 mg in 1 mL DMSO

III.5.B.3 Propidium iodide stock (10 mg/mL)

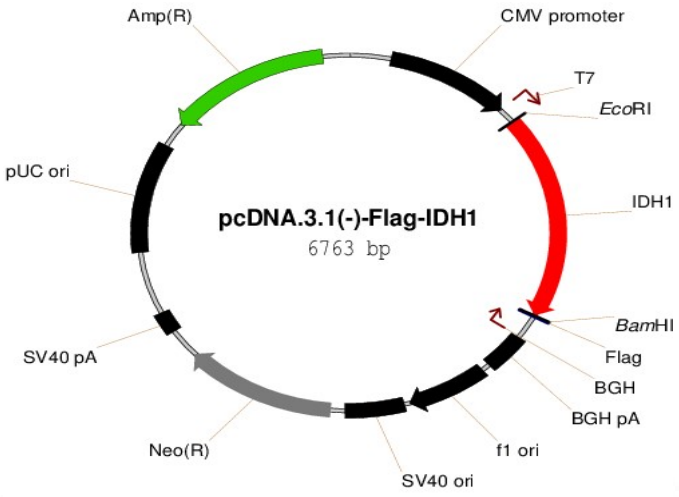
5 mg in 500 µL DMSO

III.6 RNA Interference

MTH1 siRNA and Hif1α siRNA (Eurogentech, Seraing, Belgium) were used to silence MTH1 and Hif1α gene expression respectively. The cells were transfected using Jetprime from Polyplus-transfection kit (Illkirch, France) containing jetPRIME® buffer and jetPRIME® reagent. U87 and U251 were cells cultured in 60 mm dishes and grown till attaining 60-80% confluence. For 100 nM concentration, 440 pmoles of MTH1 siRNA/ Hif1α siRNA (10 µL) was mixed with 400 µL of jetPRIME® buffer and mixed well by vortexing. To this, 5 µL jetPRIME® reagent was added and the mixture was vortexed for 2 sec, spun down and then incubated for 10 min at room temperature. The transfection mix was then added to 1600 µL of serum-free culture medium. After addition of the medium containing the transfection mix into each culture dishes, the dishes were then incubated in CO_2 incubator at 37°C. The media was changed to 5% DMEM after 4 h to avoid the toxicity of transfection reagent. The transfection was analyzed after 48 h of incubation at 37°C. The target sequences are shown as following: Negative control siRNA (Catalogue Number: SR-CL000-05), MTH1 siRNA: 5'-UUCCACGGGUACUUCAAGUdTdT-3'; 5'-ACUUGAAGUACCCGUGGAAdTdT-3' and Hif1α siRNA: 5'-CAUGAAAGCACAGAUGAAUdTdT-3'; 5'-AUUCAUCUGUGCUUUCAUGdTdT-3'.

III.7 Plasmid DNA isolation and transfection

pcDNA3-Flag-IDH1-R132H was a plasmid deposited in the repository by Yue Xiong (Addgene plasmid # 62907 ; http://n2t.net/addgene:62907 ; RRID:Addgene_62907) (Zhao et al., 2009b).



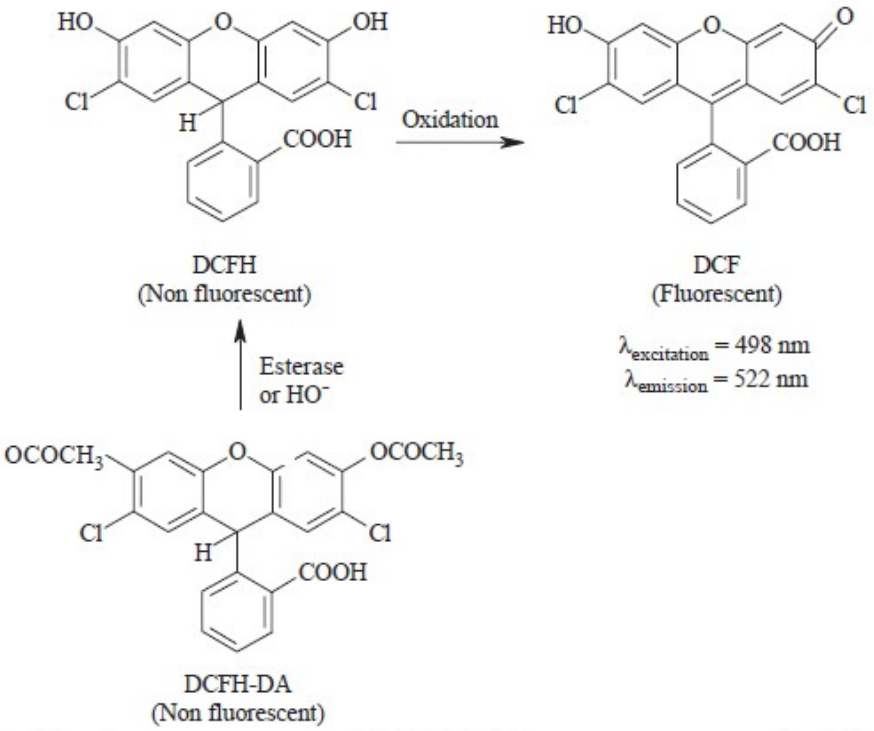
Source: Addgene

Fig.III.1 Plasmid carrying Flag-tagged R132H

The desired insert was confirmed by restriction digestion at either EcoRI or BamHI site and electrophoresed on 1% agarose gel. The plasmid containing *E. coli* was grown in an ampicillin coated LB agar. After 24 h of incubation at 37˚C, single colony was selected and inoculated into LB broth for another 24 h at 37˚C. The broth becomes turbid indicating the growth of bacteria. The plasmid DNA was isolated from the bacteria culture using SmartPure Plasmid DNA Isolation Kit (Eurogentech, Seraing, Belgium) as per the kit protocol. The DNA after estimation was used for transfecting the glioma cell lines. The transfection was performed using Jetprime transfection kit.

III.8 Intracellular ROS measurement by H_2DCFDA staining

Intracellular ROS levels in cultured cells after various treatments were measured using Dichlorodihydrofluorescein diacetate (DCFH-DA) (Gomes et al., 2005). The non-fluorescent dye will be hydrolyzed by cellular esterases to release the diacetate groups on it. Further the ROS present in the milieu of the dye oxidizes it to the fluorescent form, Dichlorofluorescein.



Gomes et al., 2005

Fig.III.2 Mechanism of DCFH-DA de-esterification and oxidation

The steps for DCFH-DA treatment were standardized at various points including the concentration and incubation time of DCFHDA. For fluorimetry, cells were cultured in a 96-well black plate at a seeding density of $1x10^4$ per well with low glucose DMEM medium. After various treatments, the wells were then washed twice with HBSS and then incubated with 10 µM DCFH-DA at 37°C in the dark for half an hour. Cells treated with H_2O_2 were used as a positive control. The wells were washed twice with HBSS to remove excess dye, and the DCF fluorescence developed in proportion to the amount of intracellular ROS was measured using Fluorimeter (BioTek instruments, Winooski, VT, USA) at 530 nm (excitation 488 nm), with Gen5 software. The relative fluorescence in the treated group compared to the control was calculated from the obtained fluorescence intensity values.

III.9 Immunofluorescence staining

The cells were grown over sterile cover slips in 12 well culture plates. After various treatments, the cells were pre-fixed by adding 4% formaldehyde to the medium (1:2 v/v)

and incubating at 37˚C for 2 min. Cells were washed with 1X PBS (pH 7.6) once, followed by fixation adding 2mL of 4% formaldehyde at 37˚C for 15 min. The cells were then washed thrice with 1X PBS for 5 min. each, and then permeabilized using 0.1% Triton-X 100 for 10 min. at 4˚C. The cells were again washed using PBS for 5 minutes (three times). Blocking buffer (3% BSA) was added into each dish (for 30 min.) in order to block the non-specific sites. Immediately after blocking, the respective primary antibody (prepared in 1.5% BSA with 0.05% Triton-X) was added and the dishes were incubated over-night at 4˚C. The cover slips with cells were washed on the next day for 5 min thrice with wash buffer (1% BSA with 0.05% Triton-X). Fluorescence tagged secondary antibody was then added and incubated for 1 hour at room temperature. The cover slips were washed thrice with PBS (5 min.). The cells were then observed under fluorescence microscope after adding a drop of DAPI fluoroshield (Sigma-Aldrich, Gillingham, UK) onto the cover slips.

III.10 Flow cytometry

To determine apoptosis, cells after treatment were trypsinized and washed twice with cold PBS and resuspended the cells at a concentration of $1x10^6$ cells/mL in 500µL of 1X binding buffer. 5µLAnnexin V-FITC (Miltenyi Biotech, Bergisch-Gladbach, Germany) were added to each of the tubes (treated as well as control) and incubated at room temperature in dark for 15 min. 5µL of PI (100µg/mL) was added and the samples were taken for Flow Cytometry (BD FACS Jazz, Franklin Lakes, NJ, US) analysis to detect the total number of apoptotic cells. Cells stained with both Annexin V-FITC and PI was used as a compensation control and the reading was done at 488nm and 530nm.

III.11 Scratch Wound Assay

The cultures cells after becoming confluent were subjected to a wound by scratching the wells of culture plates with a 10 µL pipette tip. After the 6-10 h of incubation after various treatments, the cells were washed and stained with crystal violet and checked to determine the degree of cell migration towards the scratch. The images were captured using ProgRes Capture Pro V2.8.0 Software (Suboj et al., 2012).

III.12 Agarose gel electrophoresis

In apoptotic cells, DNA fragmentation turns to be evident in electrophoresis analysis. This is a simple and quick method to qualitatively measure the DNA damage due to any treatment in cells. DNA from cells after silencing of MTH1 was isolated according to the kit protocol using DNA isolation kit (Smartpure Extract DNA kit, Eurogentech, Seraing, Belgium). The isolated DNA was mixed with 5µl of 6X DNA loading dye and was then loaded into 1% agarose gel prepared in 1X TBE containing 1µg/ml EtBr and electrophoresed for 20 min at 35V. The bands were then visualized by ultraviolet transilluminator. A typical ladder pattern of fragmented DNA was observed in the apoptotic cells.

III.13 Haematoxylin and Eosin staining

The formalin-fixed paraffin-embedded sections were deparaffinized in the oven at 70°C for 30 min and in xylene for 10 min. For gradual rehydration, the slides were incubated in anhydrous alcohol for 2-3 min and then in diluted alcohol (70% and 50%). The slides were then rinsed with tap water followed by distilled water. The slides were kept in haematoxylin (Harris stain) stain for 15 min, followed by two quick rinsing in acid-alcohol mixture (10% acetic acid in 95% alcohol) to remove excess stain. Haematoxylin stains the nuclear chromatin and possibly other acidic cellular elements. The slides were washed in tap water for 10 min and then dipped in lithium solution (saturated lithium carbonate) for 2 min. the slides were then incubated with eosin for 2 min and then 10 sec in 95% alcohol. Eosin is a cytoplasmic counter stain. This was followed by xylene wash for a few seconds and then with tap water. The slides were wiped and observed under the microscope.

III.14 Immunohistochemical analysis

Immunohistochemistry was performed using the standard protocol used in the Department of Pathology. Briefly, 4 µm formalin-fixed paraffin-embedded sections were deparaffinized for 30 min in the oven at 70°C and in xylene for 10 min, followed by dehydration in graded isopropanol. The antigen retrieval was performed using Tris-EDTA buffer (PathnSitu Biotechnologies, India) in microwave oven at 100°C for 20 min. The endogenous peroxidase activity was blocked using 3% hydrogen peroxide followed

by washing with immuno wash buffer (PathnSitu Biotechnologies) for 5 min twice and non-specific background staining was blocked using protein block (Agilent Dako, CA, USA). The slides were incubated with MTH1 primary antibody at 1:100 dilutions for 1 h and washed with immuno wash buffer for 5 min twice. The sections were visualized using horseradish peroxidase labelled anti-Rabbit secondary antibody system (Dako REAL EnVision, Agilent Dako) for 30 min, washed with immuno wash buffer for 5 min twice followed by incubation with DAB chromogen system (Dako REAL EnVision) for 10 min. The sections were finally washed with distilled water, counterstained with haematoxylin, dried and mounted using DPX (Dibutylphthalate Polystyrene Xylene). The MTH1 immunostained sections were assigned scores by two pathologists based on the percentage of tumor cells stained and intensity of staining modified from Yu CJ, et al (Cj et al., 2011). Percentage of tumor cells staining: <25% = 1, 26-50% = 2, 51-75% = 3 and >75% = 4; intensity of staining: weak = 1, moderate = 2 and strong = 3. The total score was the sum of the two scores. A table representing the genetic classification of a subset of glioma patient samples used for the study is given **(Table. III.1).**

III.15 Protein expression analysis

III.15.A Protein isolation from cells

The culture plates having either U87MG or U251MG were decanted off the media and washed thrice thoroughly with ice-cold PBS to remove all traces of media and other chemicals. After decanting off the PBS, the cells were incubated for 5 min in ice-cold Radio Immunoprecipitation Assay (RIPA) buffer containing protease/phosphatase inhibitor cocktail. The cells were scrapped using a cell scrapper and the lysate was then collected in a microcentrifuge tube. The lysates were then incubated 30 min in ice with vortexing at regular intervals of 5 min. The cell lysates were centrifuged at 16,500 g for 15 min at 4°C and the supernatants were stored at -80°C.

III.15.B Protein isolation from tissues

The glioma as well as non-tumor tissues after weighing were pulverized using RIPA buffer with protease/phosphatase inhibitor cocktail. The supernatant was collected and stored as said above.

III.15.C Protein quantification

The isolated proteins were quantified using Bicinchoninic acid assay method (Pierce Biotechnology, Massachusetts, USA). For microplate assay, the cell lysates diluted 10-times and BSA protein standards containing a range of 1.25 to 15 µg protein were added to the wells. A 50:1 mix of Pierce BCA Reagent 'A' and BCA Reagent 'B' was prepared and 200 µL was added to each well. The plate was incubated for half an hour in dark at 50°C and absorbance was measured at 562 nm after the plate cooled to room temperature. A standard curve of absorbance vs. micrograms protein of standard was plotted to get a linear equation using which the concentration of protein in µg/µL in the lysates were determined.

III.15.D Electrophoresis and Blotting

The protein lysates (30-60 µg) were mixed with 6x Laemmli buffer containing 2-mercaptoethanol (2-ME), heat denatured for 5 min at 95°C and resolved on 5-12% polyacrylamide gels using Tris-Glycine-SDS buffer. The gels after the Sodium Dodecyl Sulphate- Poly Acrylamide Gel Electrophoresis (SDS-PAGE) at 100V were equilibrated with transfer buffer and excess SDS was removed. The resolved proteins were transferred to a PVDF membrane (pre-wetted with 100% methanol) using a Trans Semi Dry Blot apparatus (Bio-Rad Laboratories, CA, USA) at 10V for 30-40 min. The membrane was blocked for 1 h at room temperature using either 5% skimmed milk (for non-phosphoprotein detection) or 1% Bovine Serum Albumin (BSA) solutions in TBST. The membrane was then probed with antibodies (prepared in 3% BSA-TBST) specific to target proteins at 4°C overnight. Secondary antibodies conjugated to Horse Radish Peroxidase (HRP); anti rabbit IgG; 1:5000-1:8000, anti mouse IgG; 1:10000-1:20000 were used to probe the primary antibodies by incubation for 1 h at room temperature.

III.15.E Chemiluminescent detection

Protein bands were visualized using Enhanced Chemiluminescence detection. Equal volumes of luminol and peroxide solutions were mixed and added on to the membranes. Light emitting bands were captured on an X-ray film and developed and then documented in Gel Doc™ XR Imaging System (Bio-Rad Laboratories, CA, USA) and quantified using Quantity One 1 D Analysis Software.

III.15.F Reagents Used

III.15.F.1 Acrylamide 40%

Acrylamide- 38.67 % (w/v) and N, N'-methylene bisacrylamide- 1.33% (w/v) in 100 ml in deionized water.

III.15.F.2 Blocking solution

Skimmilk- 5% (w/v) in 1X TBST (For detecting Non-phosphoproteins)

BSA- 1% (w/v) in 1X TBST

III.15.F.3 10 X TGS buffer (Running Buffer, pH 8.3)

Trizma base – 25 mM, Glycine –192 mM, SDS –1% in deionized water.

III.15.F.4 Ponceau S stain

1% Ponceau in 5% glacial acetic acid.

III.15.F.5 8 X Resolving gel buffer (pH - 8.8)

SDS - 0.2%, Tris – 3 M in deionized water.

III.15.F.6 RIPA (Radio ImmunoPrecipitationAssay) BufferpH – 8.0

25 mMTrisHCl (p^H-7.6), Sodium chloride – 150 mM, Nonidet-P-40 (NP-40) – 1.0%, Sodium deoxycholate – 0.5%, SDS – 0.1% in deionized water.

Protease and Phosphatase Inhibitors (Cocktails) added as required

III.15.F.7 SDS gel loading buffer (6X) pH – 6.8

SDS – 12%, 2- mercaptoethanol – 12.5% (Add fresh), Glycerol – 60%, Bromophenol blue – 0.012%, Tris-, HCl - 0.375 M (p^H-6.8) in deionized water.

III.15.F.8 4 X Stacking gel buffer (pH – 6.8)

SDS – 0.1%, Trizma base 0.5 M in deionized water.

III.15.F.9 10 X Towbin's buffer (Transfer buffer, pH 8.3)

Trizma base – 25 mM, Glycine –192 mM, 20% methanol in deionized water.

III.15.F.10 Tris-buffered saline (10 X, pH 7.6)

Tris base- 24.2 g, sodium chloride- 80 g in 1L deionized water.

III.15.F.11Tris-buffered saline with Tween-20 (TBST) [1 X]

1X TBS containing 0.5% Tween-20.

III.15.F.12 To prepare 10% resolving gel (~10 mL)

40% acrylamide: bis-acrylamide (29:1)	- 2.5 mL
8X resolving gel buffer	- 1.25 mL
TEMED	- 10 µL
20% APS	- 20 µL
Deionized water	- 6 mL

III.15.F.13. To prepare 5% stacking gel (~5 ml)

40% Acrylamide- bis acrylamide gel (29:1)	- 0.625 ml
Stacking gel buffer	- 1.25 ml
TEMED	- 5 µl
20% APS	- 10 µl
Deionized water	- 3.125 ml

III.16 Gene Expression Assays

III.16.A Reagents for Agarose Gel electrophoresis

III.16.A.1 TBE Buffer (5X)

1.1M Tris, 900 mM Borate & 25 mM EDTA, pH 8.3.

III.16.A.2 1% agarose gel

Low EEO Agarose (1%) molten in 1X TBE and casted in a gel cast

III.16.A.3 Ethidium bromide (EtBr)

EtBr dissolved at 3% concentration in distilled water.

III.16.B RNA extraction and cDNA synthesis

The glioma/non-tumor tissue biopsies collected in RNA Later was washed with PBS. RNA was isolated with the Relia Prep RNA Tissue Miniprep, Promega. Briefly, the tissues after adding lysis buffer were homogenized using tissue homogeniser **(Potter S, Sartorius, Göttingen**, Germany). The lysate was centrifuged at 16000 rcf and the supernatant was added with one volume of 70% ethanol and mixed well. The mixture was transferred in to spin cartridges, centrifuged and the flow-through was discarded. The columns were then washed with wash buffer I and wash buffer II. The RNA bound to the spin columns were eluted in 40 µL RNase free water. The concentration and impurity of the extracted RNA was checked using BioPhotometer Plus (Eppendorf AG, Hamburg, Germany) by measuring A260, A260/A230 and A260/A280 absorbance. Intactness of RNA was ascertained by 1% agarose gel electrophoresis by observation of intact 28S and 18S rRNA bands. The RNA samples were aliquoted and stored at -80^{o}C till use. Repeated freeze-thaw was avoided to minimize RNA degradation. Using 1 µg RNA for each sample, first strand cDNA was synthesized in a Thermocycler- iCycler (Bio-Rad, Hercules, CA, USA), as per manufacturer's protocol, using the Reverse Transcriptase Core Kit (Eurogentec, Seraing, Belgium). Briefly, the reaction mix was prepared as per Table III.1. keeping all reagents on ice.

Component	Volume (µL)	Final concentration
10x reaction buffer	1	1x
25 mM $MgCl_2$	2	5 mM (or as required)
2.5 mM dNTP	2	500 µM each dNTP
Random nonamer	0.5	2.5 µM
RNAse Inhibitor	0.2	0.4 U/µL
EuroScript RT	0.25	1.25 U/µL
RNase free water	3.05	-
Template	1	1µg - 2µg Total RNA
Total Mix	**10 µL**	

Table. III.2. Reaction mix for reverse transcription PCR

Equal quantities of the reaction mix were taken in PCR tubes and the template RNA were added for samples while adding water instead of template in negative control. The steps for amplification were set as below in the thermal cycler.

Initial step	10 min at 25°C
Reverse Transcriptase step	30 min at 48°C
Inactivation of the RT enzyme	5 min at 95°C

III.16.C Quantitative PCR for MTH1

The cDNA was used for qPCR analysis of MTH1 mRNA expression using Applied Biosystems TaqMan Gene Expression Assays. The assay primers/probes used were: MTH1 (Hs00159343_m1) with FAM-MGB reporter-quencher labels and α-tubulin (Hs03045184_g1) with VIC-MGB reporter-quencher labels and Taqman master mix (Applied Biosystems, CA, USA) were used to amplify the target genes in glioma tissues of different grades along with non-template controls (NTC). The pre-optimized protocol for qPCR using TaqMan forward and reverse probes with similar T_m and the TaqMan Gene Expression Master Mix was performed in the thermal cycler. The temperature and time of PCR run was as follows: 2 min hold at 50°C followed by 10 min hold at 95°C and then 35 repetitions of Denaturation: 15 sec at 95°C and Annealing/Extension: 1 min at 60°C. The Ct values obtained for each reaction were used to calculate the ΔCt values by taking the difference between the Ct values of MTH1 and the Ct values of β-actin as a reference gene. The average ΔCt of target gene MTH1 vs. reference gene α-tubulin obtained for each treatment group were subtracted by the corresponding ΔCt of the control to obtain the ΔΔCt values and then fold changes calculated as $2^{-\Delta\Delta Ct}$.

- $\Delta Ct = Ct_{target} - Ct_{reference\ gene}$
- $\Delta\Delta Ct = (Avg\ \Delta Ct)_{treatment} - (Avg\ \Delta Ct)_{control}$
- Relative fold of target mRNA levels of treated with respect to control = $2^{-\Delta\Delta Ct}$

III.17 Enzyme Linked Immuno Sorbent Assays

Universal 8-oxo-dG ELISA kit (ImmunoTag, MO, USA) was used to measure the levels of 8-oxo-dG, an indicator of MTH1 activity while NUDT1 ELISA kit (ImmunoTag, MO, USA) was used to measure MTH1 protein levels. The cell/tissue lysates were freshly

prepared and added into the 96-well plate coated with antibodies against 8-oxo-dG and MTH1 respectively. Remaining procedures were performed as per the kit protocol.

Briefly, the biotinylated antibodies against the antigens were added followed by Streptavidin-HRP to label them. Then the substrate solution for HRP was added for color development and the reaction terminated after 10 min. The absorbances were measured at 450 nm spectrophotometrically and the 8-oxo-dG and MTH1 concentrations were extrapolated from their respective standard curves.

III.18 Statistical Analysis

All data are represented as mean±SEM. The statistical analysis was done using GraphPad Prism 5 software. For multiple group comparison, one-way ANOVA with either Dunnett's or Tukey's post-hoc tests were performed or non-parametric Kruskal- Wallis analysis with Dunn's post hoc tests were done. Pearson's correlation coefficient is used for testing the correlation of MTH1 expression with XRCC1, Cleaved-PARP, MMP-9, RAC and VEGF. P values of 0.05 or less were considered statistically significant.

Sl. No	Diagnosis	WHO grade	Immunohistochemistry							
			R132H-mutant IDH1	ATRX (nuclear expression)	p53	Ki-67	MTH1			
							Score for % of tumor cells staining	Score for intensity of tumor cells staining	Total score	Pattern of staining
1	Glioblastoma, IDH-wildtype	IV	-	intact	-	25%	2	1	3	cytoplasmic
2	Glioblastoma, NOS	IV	-	intact	+	30%	3	1	4	cytoplasmic
3	Anaplastic astrocytoma, IDH-mutant	III	+	loss	+	12%	4	2	6	cytoplasmic
4	Anaplastic oligodendroglioma, NOS	III	+	intact	-	15%	2	1	3	cytoplasmic
5	Glioblastoma, NOS	IV	-	loss	+	35%	4	3	7	cytoplasmic & nuclear
6	Anaplastic oligodendroglioma, NOS	III	+	intact	-	15%	3	2	5	cytoplasmic
7	Anaplastic oligodendroglioma, NOS	III	+	intact	-	10%	2	1	3	cytoplasmic
8	Anaplastic oligodendroglioma, NOS	III	+	intact	-	10%	2	1	3	cytoplasmic
9	Glioblastoma, NOS	IV	-	loss	+	20%	3	2	5	cytoplasmic
10	Anaplastic astrocytoma, IDH-mutant	III	+	loss	+	12%	0	0	0	-
11	Anaplastic oligodendroglioma, NOS	III	+	intact	-	13%	3	2	5	cytoplasmic
12	Anaplastic astrocytoma, NOS	III	-	loss	+	18%	4	3	7	cytoplasmic
13	Pilocytic astrocytoma	I	ND	ND	ND	2%	1	2	3	nuclear
14	Oligodendroglioma, NOS	II	+	intact	-	6%	4	2	6	cytoplasmic
15	Diffuse astrocytoma, IDH-mutant	II	+	loss	+	5%	3	1	4	cytoplasmic
16	Oligodendroglioma, NOS	II	+	intact	-	4%	1	1	2	cytoplasmic
17	Diffuse astrocytoma, IDH-mutant	II	+	loss	NA	4%	3	1	4	cytoplasmic & nuclear
18	Diffuse astrocytoma, IDH-mutant	II	+	NA	+	4%	4	2	6	cytoplasmic

Table. III.1. Histopathological diagnoses and immunohistochemical features of glioma patient biopsies

(NOS: Not otherwise specified, ND: not done, NA: not available)

IV. Results

IV.1 Haematoxylin and Eosin staining for determining glioma grades

We have performed Haematoxylin and Eosin (H & E) staining in order to determine grades and types of tumor biopsies collected from the glioma patients. This was done in Department of Pathology, Sree Chitra Tirunal Institute for Medical Sciences and Technology. After confirming the grades, the tissues were selected for various experiments.

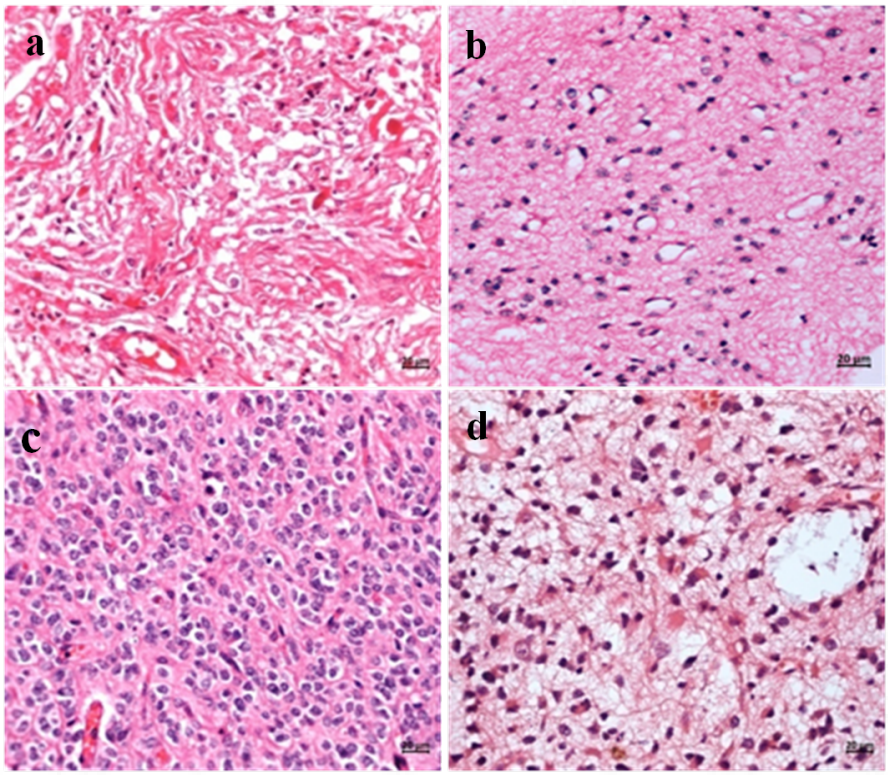


Fig.IV.1 H & E staining showing different types of glioma

(a) *Pilocytic astrocytoma, Grade I,* ***(b)*** *Diffuse astrocytoma, grade II,* ***(c)*** *Oligodendroglioma, grade III,* ***(d)*** *GBM, Grade IV. All tissues were selected for experiment after determining grades and types of tumor*

IV.2 Elevated expression of MTH1 in glioma patient samples

MTH1 expression was reported to be upregulated in glioblastomas when compared to the normal brain tissues (Tu et al., 2016). In the present study, we have checked the MTH1 expression both at mRNA as wells as protein levels. It was found that the glioma patients

show an upregulation of MTH1 expression at mRNA using RT-PCR level when compared to the non-tumor patients. We found a significant upregulation of MTH1 in LGG, but not in HGG even though a trend towards increase is observed **(Fig.IV.2)**. MTH1 expression at protein levels in tissues were first analyzed by IHC. We observed a grade independent expression of MTH1 in glioma patient samples. At the same time, our results indicate that MTH expression is upregulated in gliomas in general and is not dependent on the grades of glial tumors. IHC results from the current study show moderate and strong MTH1 expression in various grades of tumors **(Fig.IV.3)**. In order to confirm the above data, we have performed western blotting with more number of samples (n=50). Upon that, it is found that both HGG as well as LGG show significant increase in MTH1 expression when compared to the non-tumor tissues **(Fig.IV.4)**.Our ELISA data generated using MTH1 ELISA kit is also in concordance with the above observations, suggesting unequivocally the high MTH1 expression in gliomas than the non-tumor patient samples **(Fig.IV.5)**.

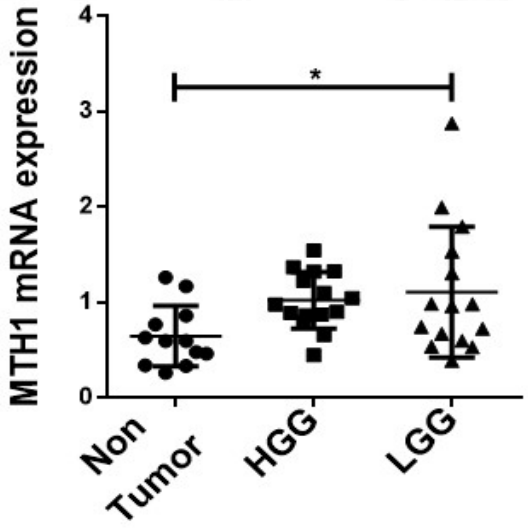


Fig.IV.2 Elevated MTH1 expression at mRNA level in glioma

*Dot plot representation of qPCR data showing relatively high RNA expression of MTH1 in glioma tissue biopsies (LGG, n=15; HGG, n=15) when compared to the non-tumor brain tissues (n=12). MTH1 expression is significantly upregulated in LGG. α-tubulin was used as the internal control. Kruskal-Wallis multiple comparison analysis with Dunn's post-hoc tests was used to compare the three data sets. *p<0.05.*

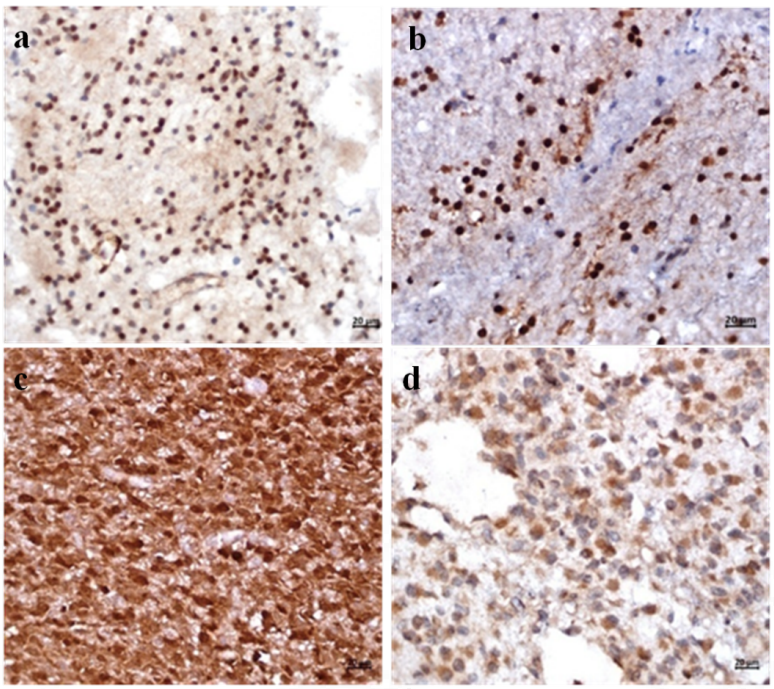


Fig.IV.3 MTH1 expression in diffuse gliomas of various WHO grades

(a) Pilocytic astrocytoma, grade I with moderate intensity nuclear MTH1 expression in microcystic areas. (b) Diffuse astrocytoma, grade II with strong nuclear MTH1 expression. (c) Anaplastic oligodendroglioma, NOS, grade III with strong cytoplasmic MTH1 expression. (d) Glioblastoma, grade IV with moderate cytoplasmic MTH1 expression. [(i)-(iv)-upper lane represents Haematoxylin and Eosin; (i)-(iv)-lower lane: MTH1 immunostaining. All images: original magnification 400X, Scale bar 20 μm]; n=18.

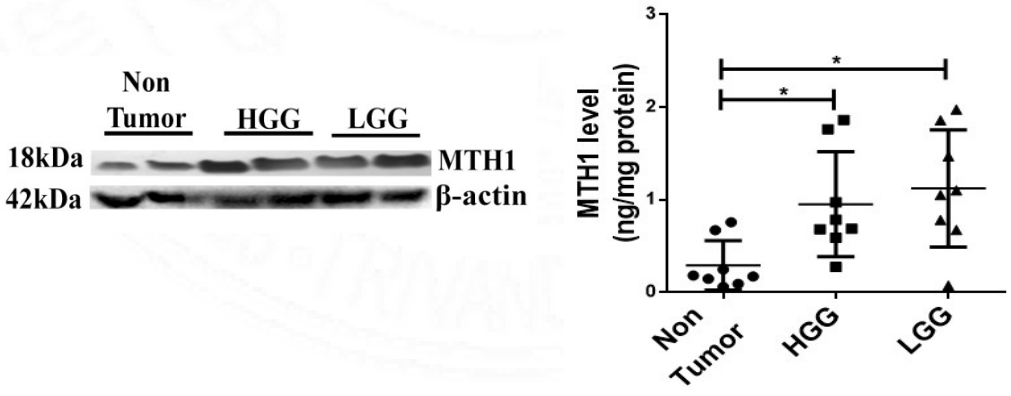


Fig.IV.4 Increased MTH1 expression at protein levels in gliomas

*Western blot data showing high level of MTH1 expression at the protein level in glioma tissues (LGG, n=25; HGG, n=25) when compared to the non-tumor tissues (n=12). β-actin serves as the loading control. Statistical analysis was done using Kruskal-Wallis multiple comparison analysis followed by Dunn's post-hoc tests. ***p<0.001.*

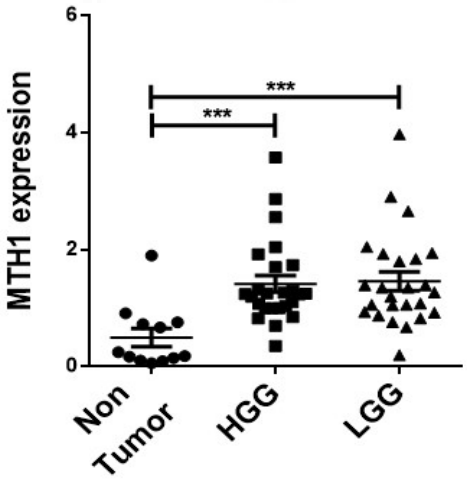


Fig.IV.5 Increased MTH1 expression at protein levels in gliomas

*Graphical representation of MTH1 Elisa data in glioma tissues (LGG-n=12, HGG-n=12) and non-tumor brain tissues (n=12) confirms the same result. Statistical analysis was done by Tukey's multiple comparison tests, following one-way ANOVA. *p<0.05.*

IV.3 Increased MTH1 activity in patient samples

MTH1 is the enzyme that hydrolyses 8-oxo-dGTP into 8-oxo-dGMP (8-oxo-dG). MTH1 gets activated in the presence of high ROS environment in cancer cells. The levels of 8-oxo-dG indicate the activity of MTH1. Hence, we measured the 8-oxo-dG levels in glioma patient samples as well as in non-tumor tissues by ELISA. It is found that glioma patients show a high 8-oxo-dG levels than the non-tumor samples though it is not statistically significant(p=0.6161)**(Fig.IV.6)**.This indicates that along with the high MTH1 expression pattern in glioma samples, there is a similar trend in the activity level too.

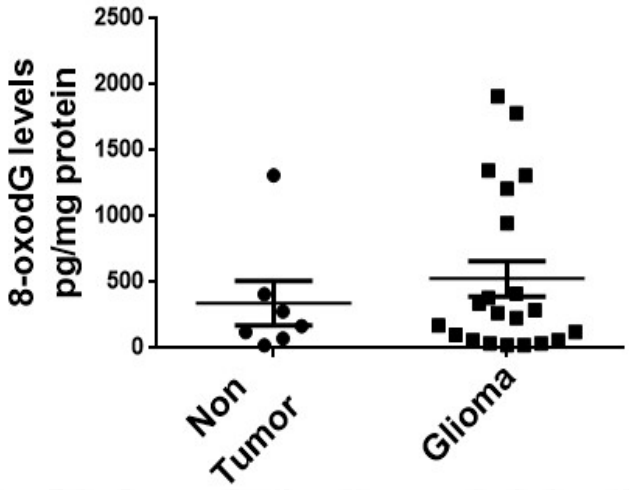


Fig.IV.6 MTH1 activity in patient samples

Dot plot representation of 8-oxodG levels in patient samples. Statistical analysis was performed by Mann Whitney test. The result is not statistically significant.

IV.4 MTH1 upregulation in the presence of high ROS

As discussed above, MTH1 gets activated in the presence of elevated ROS. Hence, we used H_2O_2, a ROS inducer at a concentration of 100 µM for both the cell lines, U87 and U251. We have also used an N-Acetyl Cysteine (1 mM) pre-treated group as a negative control. NAC is a ROS scavenger and the concentration of same was selected from previous reports. After protein extraction from the treated cells, immunoblotting for MTH1 was done. It is found that MTH1 gets upregulated in the H_2O_2 treated group, whereas in the NAC pre-treated group, MTH1 is found to be downregulated. Thus, for further experiments, we have used H_2O_2 as a positive control **(Fig.IV.7)**.

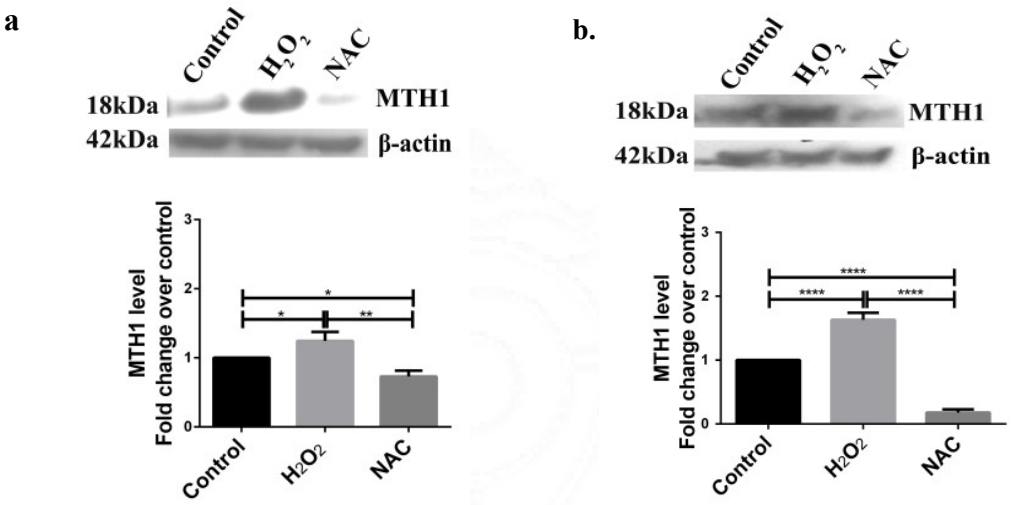


Fig.IV.7 H_2O_2 increases MTH1 upregulation

Western blot analysis showing increased MTH1 expression in the presence of high ROS environment (H_2O_2 treated group) and diminished MTH1 expression in the NAC (N-acetyl cysteine- ROS scavenger) treated group in U87 ***(a)*** *and U251* ***(b)*** *cells. β-actin is taken as the loading control. *p<0.05, **p<0.01, ****p<0.0001, (n=3).*

IV.5 MTH1 inhibition by TH558

TH588 is the MTH1 activity inhibitor that binds to the active site of the enzyme and blocks its action. As explained above, the inhibitor concentration was standardized and was used for various experiments. Since it is already proved that MTH1 gets upregulated in the presence of H_2O_2, we have used the inhibitor in the presence of basal ROS (without H_2O_2) as well as high ROS (with H_2O_2). The following changes were observed when MTH1 was inhibited using TH588:

IV.5.1 Effect of TH588 on MTH1 activity in glioma cells

Glioma cells, both U251 and U87 cells were exposed to TH588 at a final concentration of 10 μM for 24 h in the presence of basal as well as high ROS level. The following groups were taken for the experiment: Control, TH588, H_2O_2, TH588+H_2O_2. A diminished 8-oxo-dG level was observed when MTH1 was inhibited in the presence of basal ROS level in both the cell lines. However, on H_2O_2 treatment, the 8-oxo-dG levels rose in the both

the cells whether MTH1 was inhibited or not indicating the inability of the inhibitor to reduce MTH1 activity under high ROS environment **(Fig.IV.8)**.

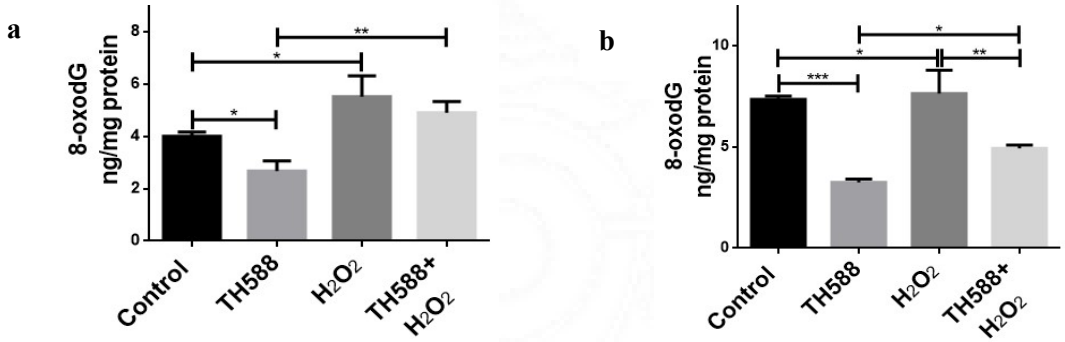


Fig.IV.8 Diminished 8-oxo-dG levels when MTH1 was inhibited

Histograms showing 8-oxodG levels in ***(a)*** *U87 and* ***(b)*** *U251 cells after inhibiting MTH1. Diminshed 8-oxodG levels were observed in TH588, MTH1 inhibitor (10 μM) treated cells. Statistical analysis was done using Tukey's multiple comparisons test. *p<0.05, **p<0.01.*

IV.5.2 TH588 causes DNA damage in U87 cells

In U87 cells, after MTH1 was inhibited with the drug TH588, the genomic DNA was isolated and electrophoresed in 1% agarose gel. We observed a shearing of DNA in the groups where MTH1 was inhibited **(Fig.IV.9)**. This indicates that MTH1 is required for preventing DNA damage in glioma cells both at basal as well as high ROS level.

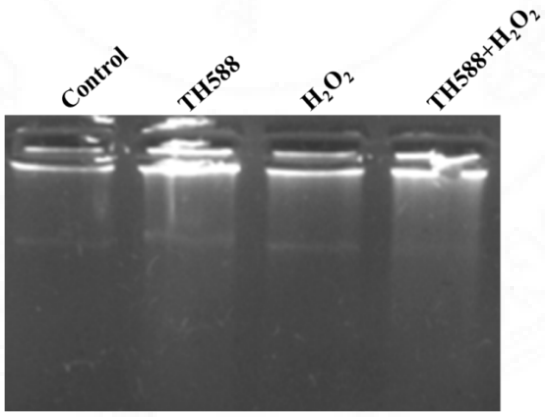
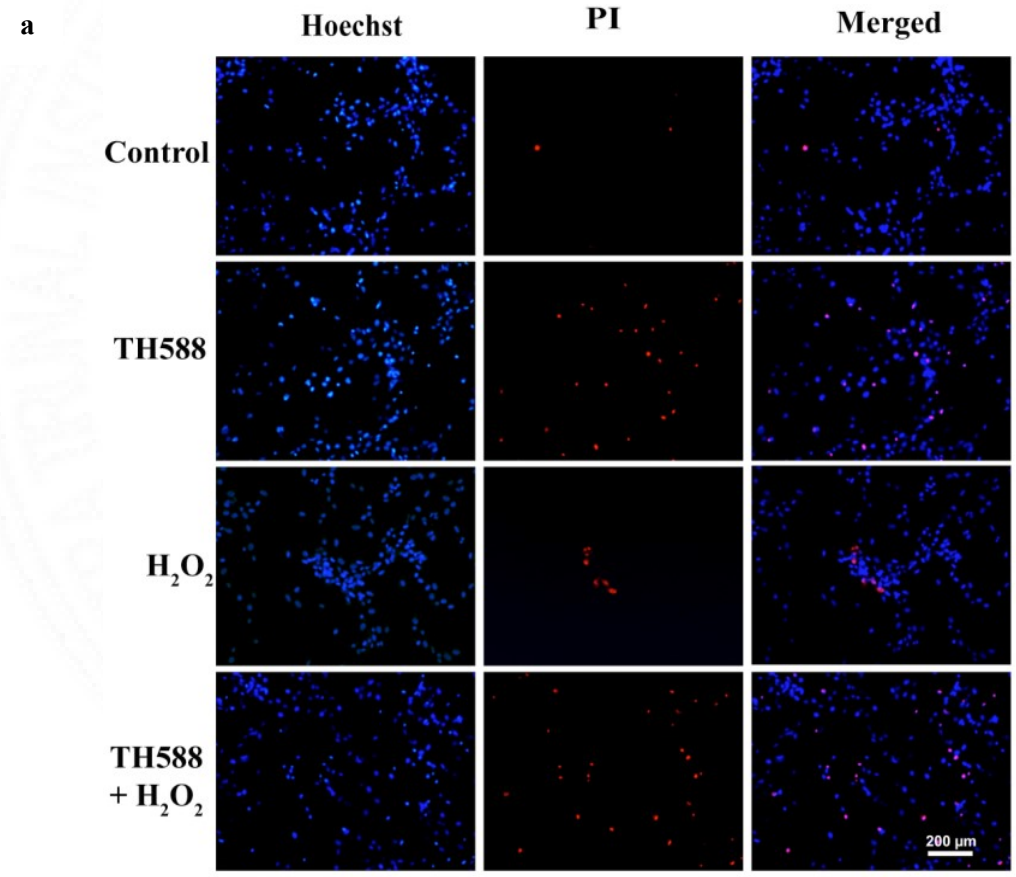


Fig.IV.9 MTH1 inhibition causes DNA fragmentation

Agarose gel showing sheared bands in TH588 treated U87 cells indicate increased DNA fragmentation when MTH1 was inhibited. DNA isolated from control, TH588, H_2O_2 and TH588+ H_2O_2 treated cells were isolated and loaded into 1% agarose gel and electrophoresed to check the intensity of fragmented DNA. (n=4).

IV.5.3 MTH1 inhibition leads to reduced viability in glioma cells

In order to determine whether MTH1 has a role in glioma cell survival, we have performed Hoechst-PI double staining for cells (both U251 and U87) after inhibiting MTH1. There was no significant loss of viability in the control as well as the H_2O_2 alone groups. But in those groups where MTH1 was inhibited, we observed a significant increase in cell death **(Fig.IV.10)**. Based on these observations, it is clear that MTH1 is essential for preventing DNA damage and essential for glioma cell survival.



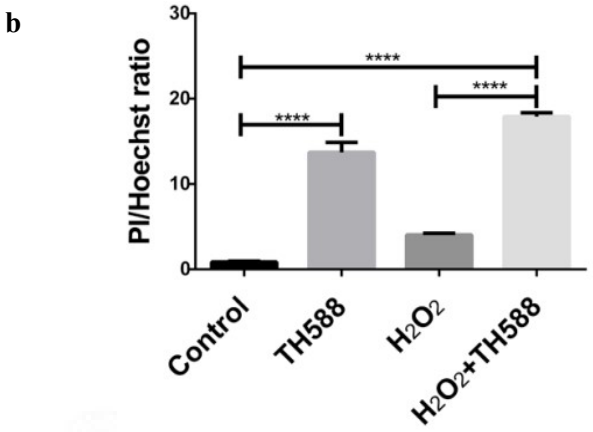
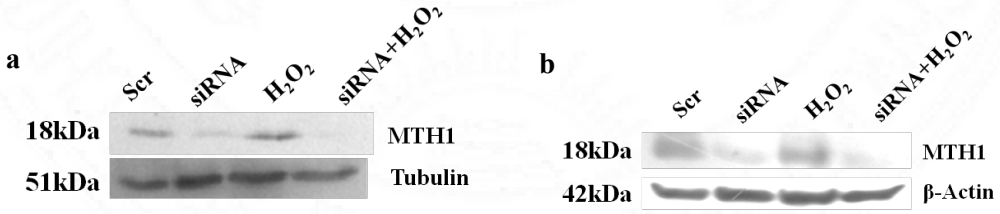


Fig.IV.10 MTH1 silencing affects glioma cell viability

Representative images of percentage PI stained U87 cells after inhibiting MTH1 ***(a)*** *and its corresponding histograms* ***(b).*** *First panel towards down represents Hoechst staining (total nuclei), PI stained cells indicating total number of dead cells (second panel) and third panel is the representation of merged images of Hoechst and PI stained cells. Statistical analysis was done using Tukey's multiple comparisons tests. *p<0.05, (n=4).*

IV.6 Effects of MTH1 silencing in glioma cells

As discussed earlier since MTH1 is found to be upregulated in the presence of high ROS environment; we have silenced MTH1 in the presence of basal and high ROS levels. MTH1 siRNA was used at a concentration of 100 nM with 48 h incubation for both the cell lines**(Fig.IV.11)**. The role of MTH1 in preventing DNA damage, its part in glioma cell survival, cell migration, invasion and angiogenesis were also studied.



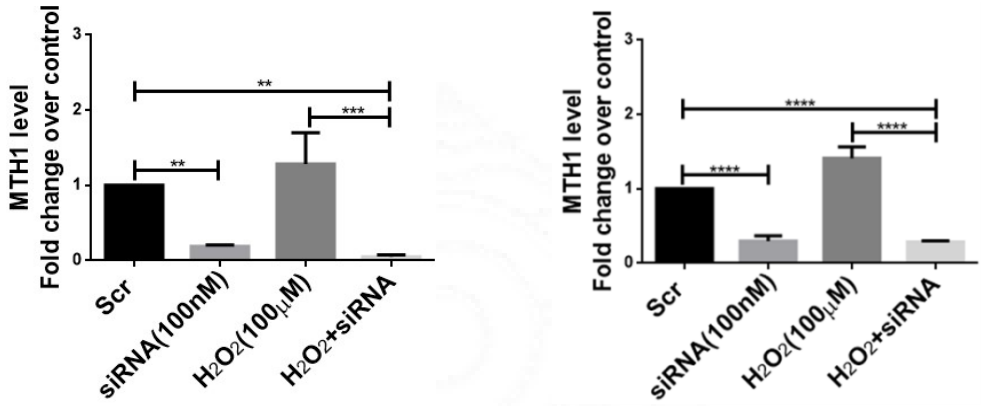


Fig.IV.11 Transfection of glioma cell lines with MTH1 siRNA

*U87**(a)** and U251 **(b)**cells were transfected with MTH1 specific siRNA (100nM) alone and in combination with H_2O_2 (100μM). α-tubulin is taken as the loading control for MTH1 in U87 cells and β-actin is taken as the loading control for U251. **p<0.01, ***p<0.001, ****p<0.0001, (n=3).*

IV.6.1 The level of DNA damage repair enzymes on silencing MTH1

In order to determine the various roles of MTH1 in glioma, MTH1 was silenced in glioma cell lines. After silencing MTH1 in U87 cells, we checked the expression level of a marker of DNA double-strand break, phosphorylated H2A histone family member X (γH2AX) by means of immunofluorescence labeling. We found an increased expression of γH2AX in the MTH1 silenced cells in the presence of basal ROS as well as high ROS levels when compared to the control cells. Also, among the H_2O_2 treated groups an increased expression of γH2AX was observed in the MTH1-silenced group in comparison with scrambled siRNA treated group **(Fig.IV.12)**. MTH1 is reported to have essential roles in preventing DNA damage in cancer, hence, MTH1 inhibition in glioma cells is expected to have increased DNA damage. It is already reported that 8-oxo-7,8-dihydroguanine (8-oxoG), a major base lesion occurs due to DNA oxidation is repaired by 8-oxoguanine glycosylase1 (OGG1)-mediated base excision repair (BER) pathway (Wang et al., 2018). Another important BER enzyme is X-ray repair cross-complementing protein 1 (XRCC1), which participates in single strand DNA break repair along with other enzymes (London, 2015). MTH1 silencing in the presence of high and

basal ROS resulted in increased expressions of XRCC1 and OGG1 in U251 as well as U87 cells **(Fig.IV.13 and Fig.IV.14)**.

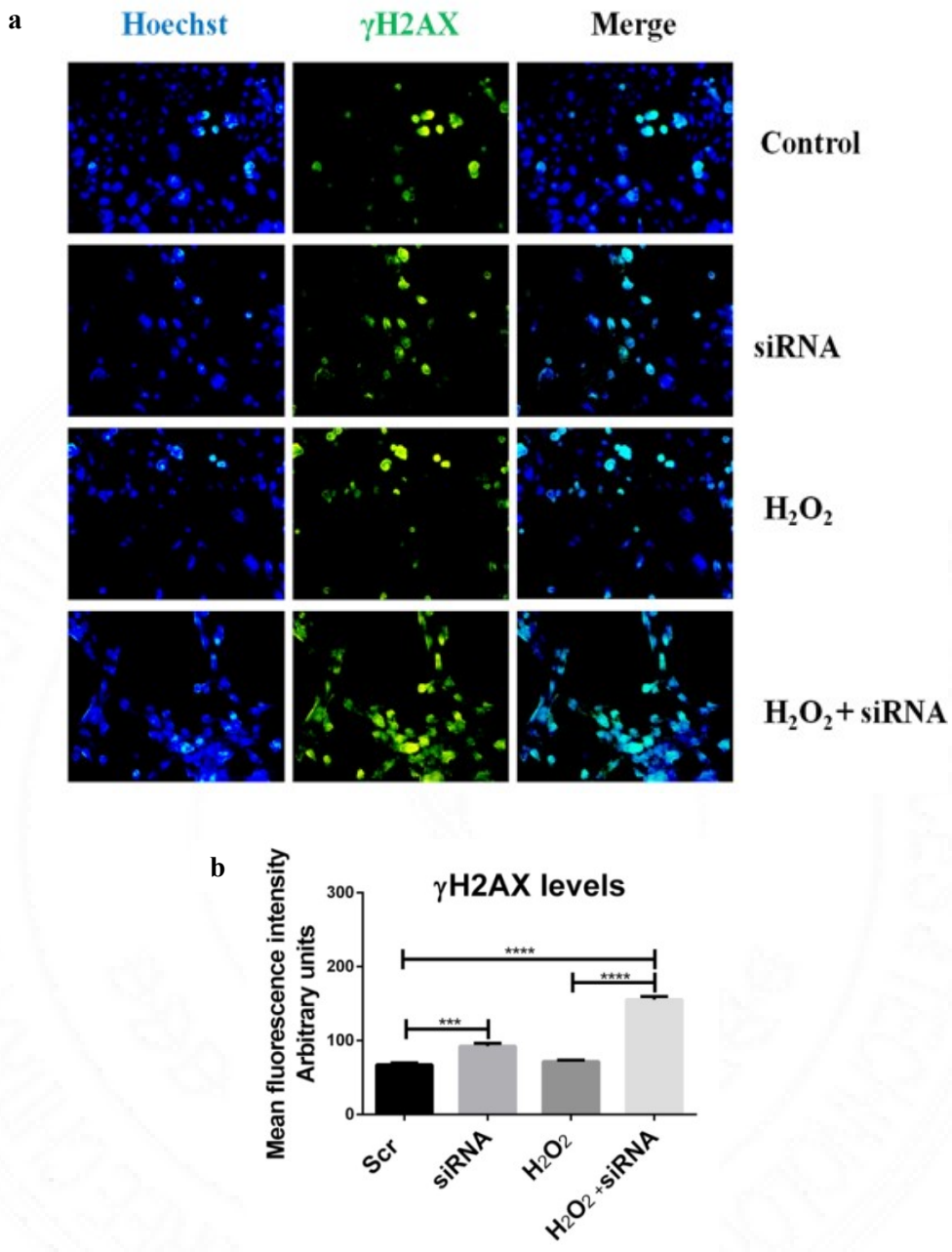


Fig.IV.12 Increased γH2AX levels showing increased DNA damage

Mean Fluoresence Intensity (MFI) calculated for γH2AX localization. ***(a)****Representative images at 100X magnification on fluorescent microscope are given for control as well as*

siRNA groups. MTH1 silenced cells showed high γH2AX expression in U87 cells (second and fourth panel) when compared to the control group (top panel) and H_2O_2 group (third panel). ***(b)*** *Graphical representation of MFI (Mean ± SEM), showing highest intensity for MTH1 silenced group in the presence of H_2O_2, ***p<0.001, ****p<0.0001.*

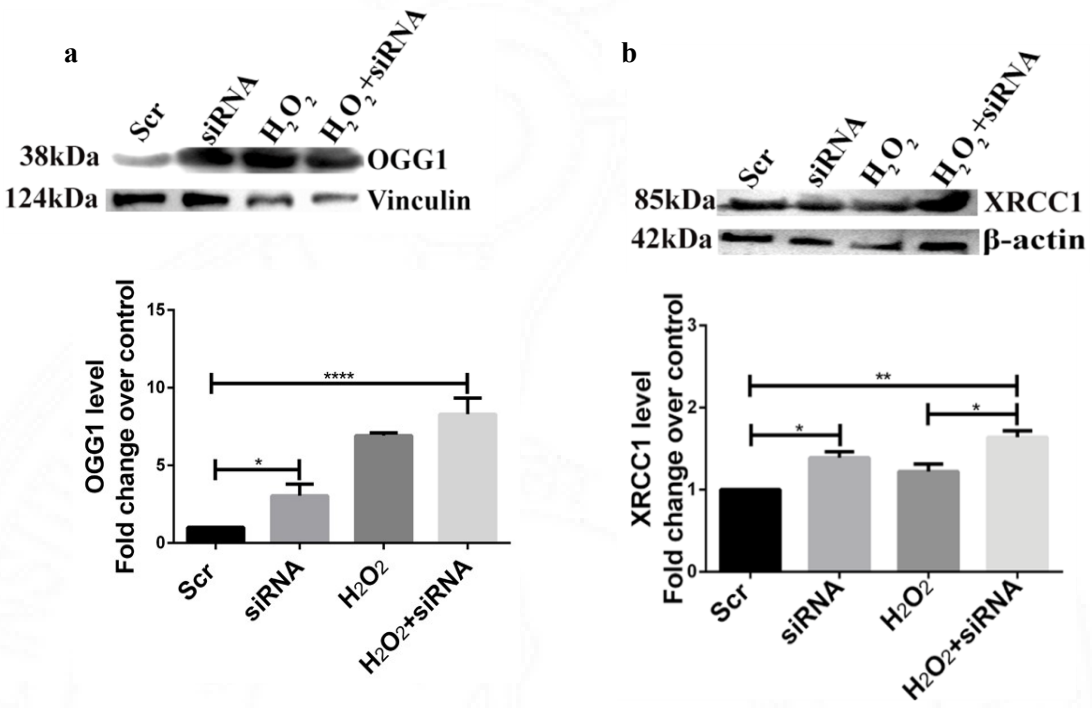
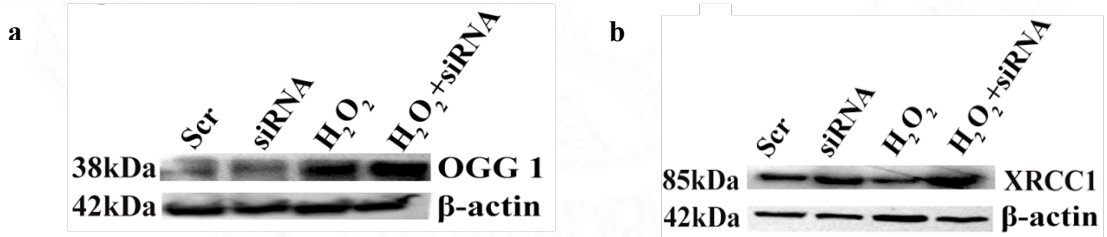


Fig.IV.13 Increased levels of base excision repair enzymes in U251 cells

(a)*Western blot analysis showing increased expression of OGG1 and* ***(b)****increased expression of XRCC1 in MTH1 silenced U251 cells. *p<0.05, **p<0.01, ****p<0.0001, (n=3).*



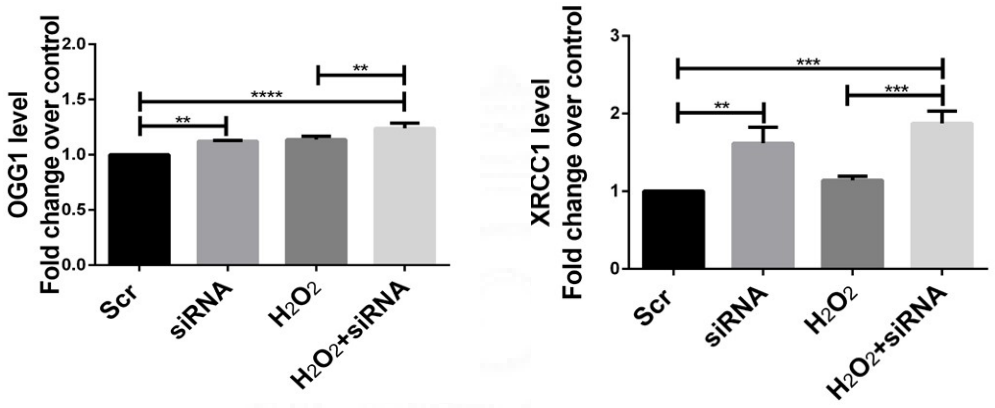


Fig.IV.14 Increased BER enzyme level in MTH1 knock-down U87 cells

(a) *Western blot analysis showing increased expression of OGG1 in MTH1 silenced U87 cells.* ***(b)*** *Western blot analysis showing increased expression of XRCC1 in MTH1 silenced U87 cells. Statistical analysis was done using Tukey's multiple comparison tests. **p<0.01, ***p<0.001, ****p<0.0001, (n=3).*

IV.6.2 MTH1 knockdown resulted in apoptosis in glioma cells

In order to check the role of MTH1 in glioma cell survival, MTH1-silenced cells were analyzed for cell death markers. Hence, the fraction of early and late apoptotic cells was measured in MTH1 silenced cells by Annexin V-FITC/PI flow cytometric analysis. From the flow cytometric analysis, it was observed that there was an increased fraction of early and late apoptotic cells in the MTH1 silenced cells in the presence of high ROS [H_2O_2(+)] environment in both the cell lines. In U87 cells, the percent count of early apoptotic cells increased to 2.39 fold ($p<0.0001$) while that of late apoptotic cells increased to 12.14 fold ($p<0.0001$) of scrambled-siRNA control **(Fig.IV.15)**. In case of U251 cells, the percent count of early apoptotic cells increased 18.037 fold ($p<0.0001$) while that of late apoptotic cells increased to 16.283 fold ($p<0.0001$) of scrambled-siRNA control. Even at basal ROS level [H_2O_2(-)], there was a significant increase in early as well as late apoptotic cells in MTH1-knockdown groups of U87 and U251 cells. **(Fig.IV.16).** Western blotting analysis for cleaved PARP (poly ADP ribose polymerase); an apoptotic marker, also confirmed the induction of apoptosis in MTH1-knockdown cells on H_2O_2 exposure. We found an upregulation in cleaved PARP in both the cell lines.

In case of MTH1 silenced cells under basal ROS levels, U251 cells showed significant increase in PARP cleavage, but in U87 cells, it was unaltered **(Fig.IV.17).**

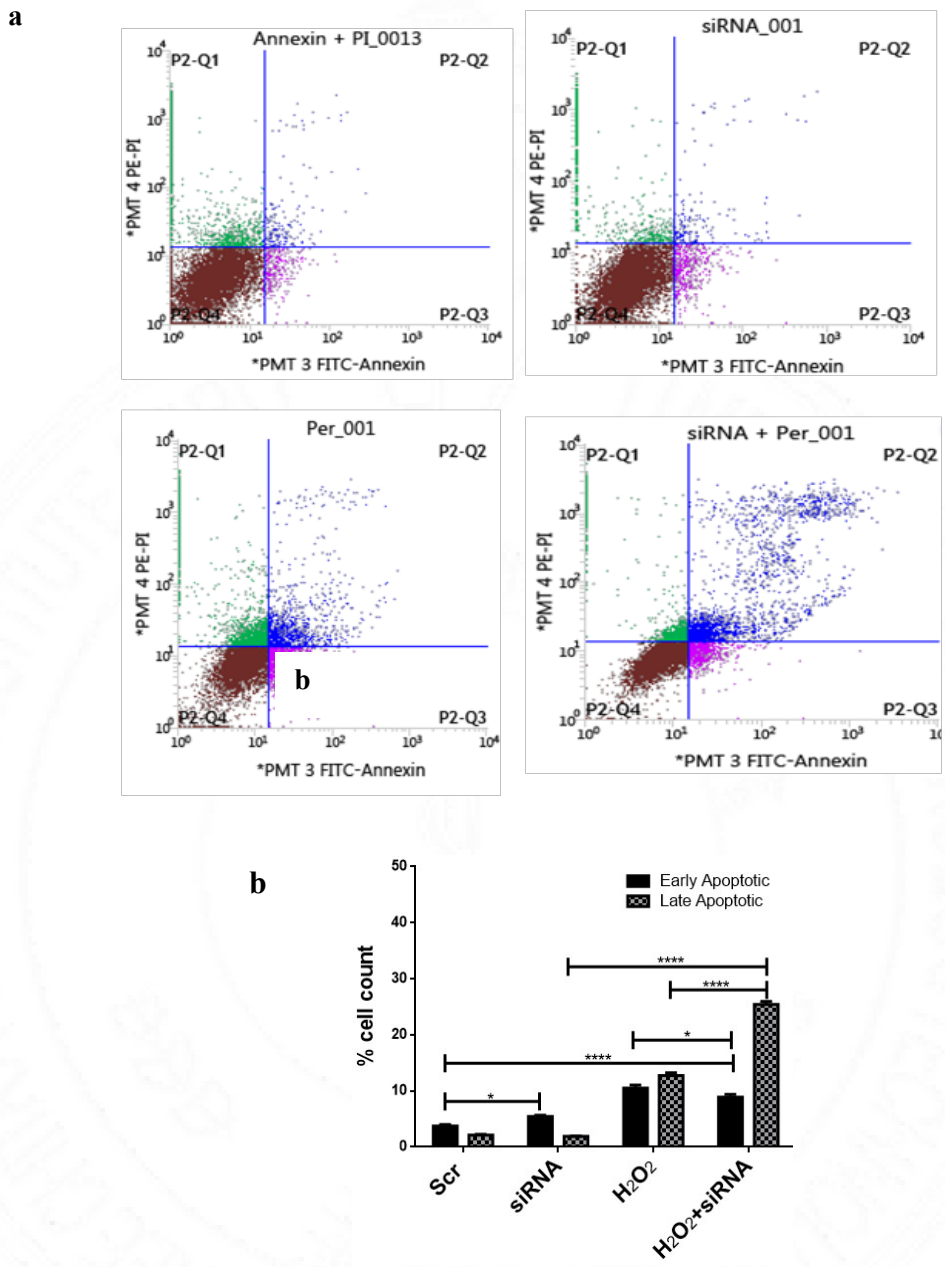


Fig.IV.15 Increased apoptosis in MTH1 knock-down U87 cella

(a)*FACS-flow cytometry analysis with Annexin V-FITC/PI illustrates increased number of late apoptotic cells in the MTH1 silenced groups in U87 cells.* ***(b)*** *Representative*

*histograms of apoptotic cell counts after silencing MTH1 in U87 cells respectively. Statistical analysis was done using Tukey's multiple comparisons tests. *p<0.05, ****p<0.0001, (n=2).*

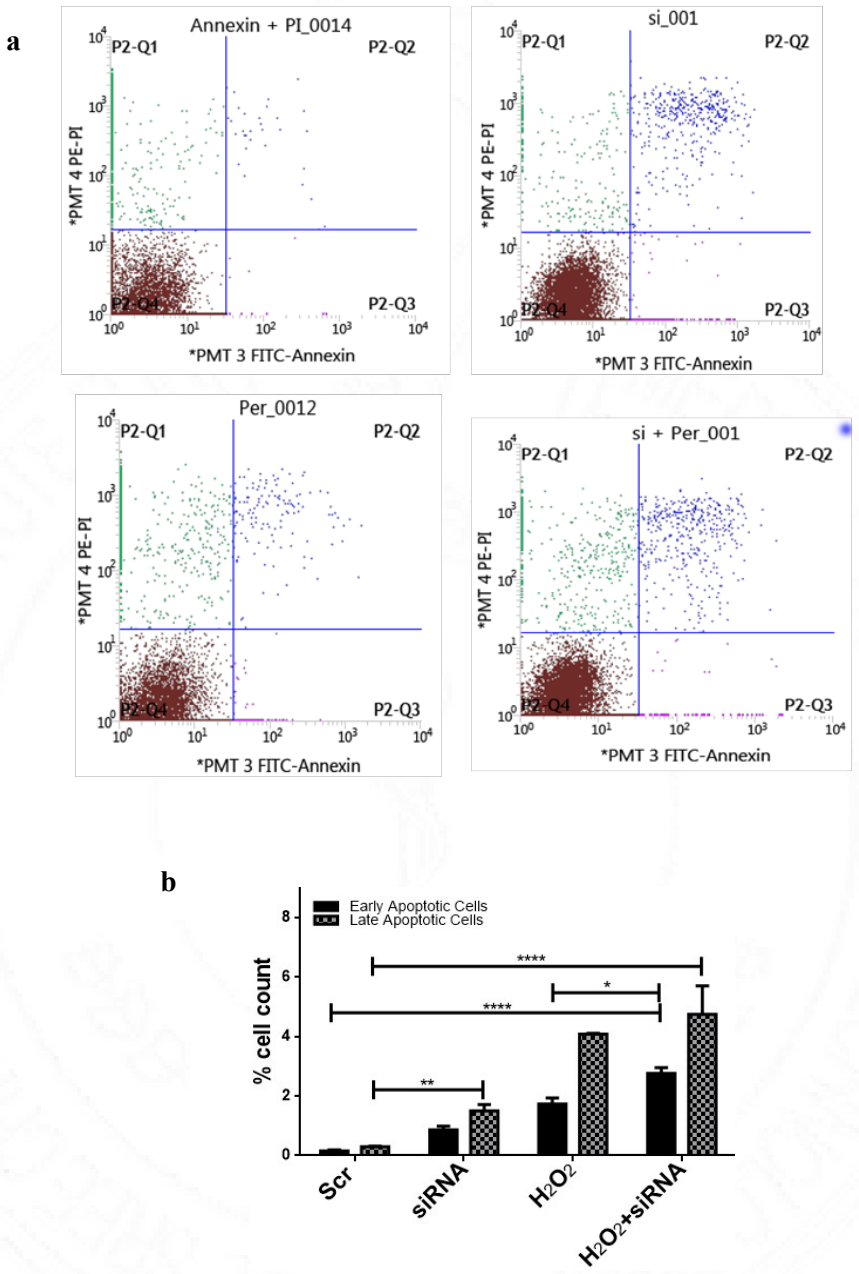


Fig.IV.16 Increased apoptosis in MTH1 knock-down U251 cella

(a)*FACS-flow cytometry analysis with Annexin V-FITC illustrates increased number of late apoptotic cells in the MTH1 silenced groups in U251 cells.* ***(b)*** *Representative histograms of apoptotic cell count after silencing MTH1 in U251 cells respectively.*

*Statistical analysis was done using Tukey's multiple comparison tests. *p<0.05, **p<0.01, ****p<0.0001, (n=2).*

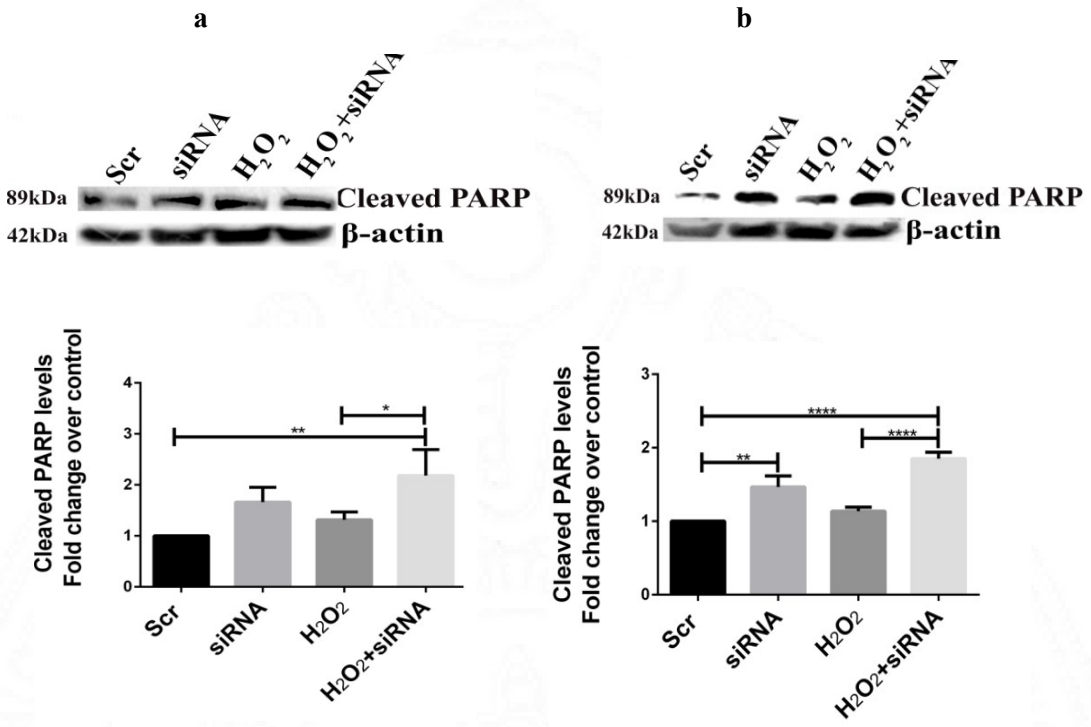


Fig.IV.17 Increased apoptosis when MTH1 is knockdown

*Western blot analysis showing the downregulation of apoptotic marker Cleaved PARP in the MTH1-knockdown cells in U87 and U251 cells respectively indicating the cell survival is affected in the absence of MTH1. Statistical analysis was done using Tukey's multiple comparisons test. *p<0.05, **p<0.01, ****p<0.0001, (n=3).*

IV.6.3 MTH1 knockdown curbed glioma cell migration

To check the role of MTH1 in cell migration, we have performed migration assays with cultured cells. The scratch wound cell migration assay indicated that MTH1 silencing lead to less migration of both U87 and U251 cells towards the wounded area when compared to the control and H_2O_2 groups **(Fig.IV.18).**

a

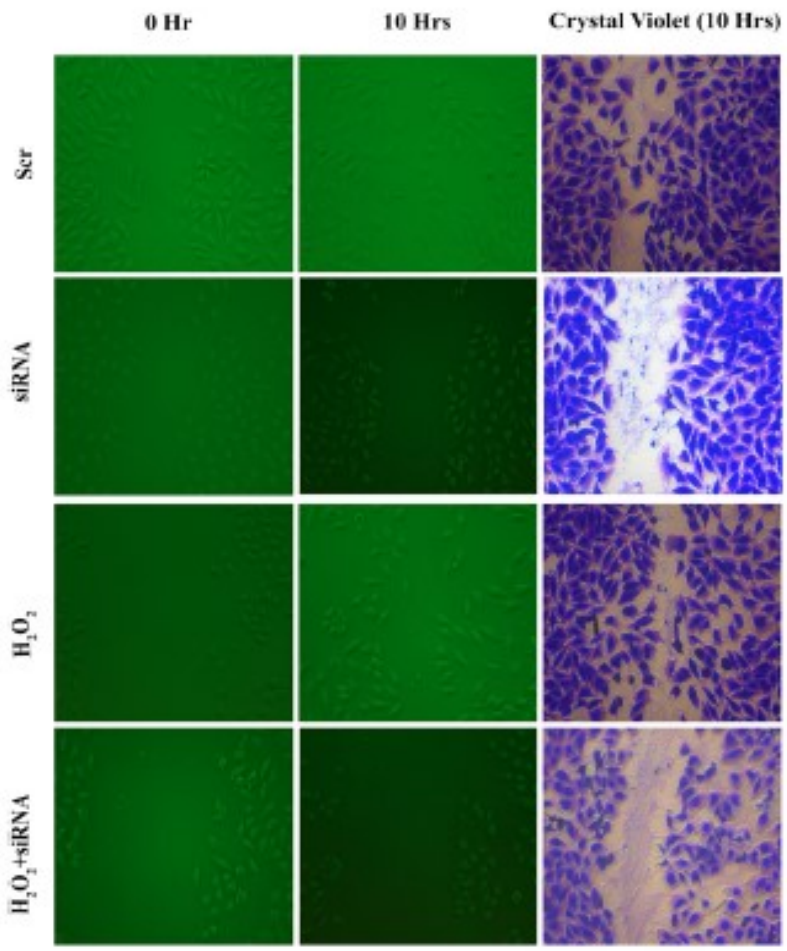


Fig.IV.18a MTH1 silencing affects U251 cell migration

Scratch wound healing assay showing reduced migration in U251 cells on MTH1 silencing. The dotted lines represent the point where scratch was given. After 10 h the cells migrated beyond the dotted lines in control and H_2O_2, but not in MTH1 silenced cells indicating that the cell migration capacity in glioma cells are affected due to MTH1 knockdown; (n=3).

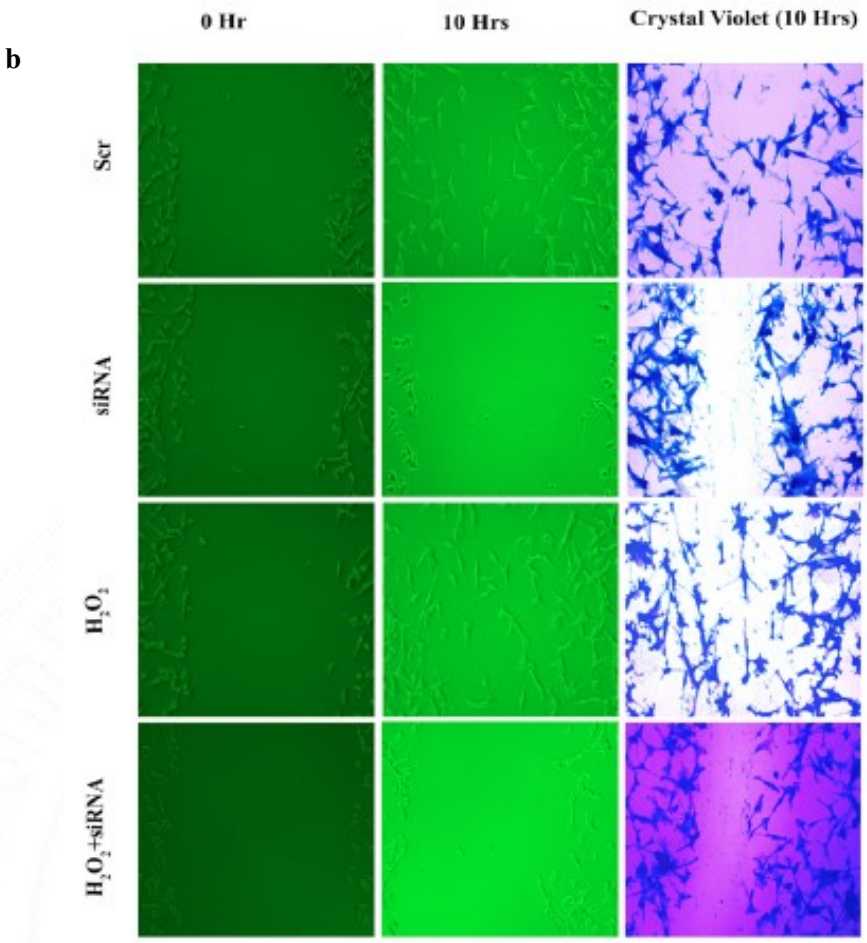


Fig.IV.18b MTH1 silencing affects U87 cell migration

Scratch wound healing assay showing reduced migration in U87 cells on MTH1 silencing. The dotted lines represent the point where scratch was given. After 10 h the cells migrated beyond the dotted lines in control and H_2O_2, but not in MTH1 silenced cells indicating that the cell migration capacity in glioma cells are affected due to MTH1 knockdown; (n=3).

Therefore, the prominent markers involved in cell migration were evaluated using Western blot. Rac1 (Ras-related C3 botulinum toxin substrate 1) and Rho A are GTP binding proteins that exert an important regulatory role in cell migration. We found a downregulation of Rac1 in the MTH1 silenced U87 cells after H_2O_2 treatment. RhoA

expression decreased in MTH1 silenced groups in U251 cells in the presence of high ROS. Even at basal ROS levels, MTH1 silencing caused a downregulation of Rac1 in U87 and RhoA expression in U251 cells. These results suggest that MTH1 play key roles in glioma cell migration **(Fig.IV.19).**

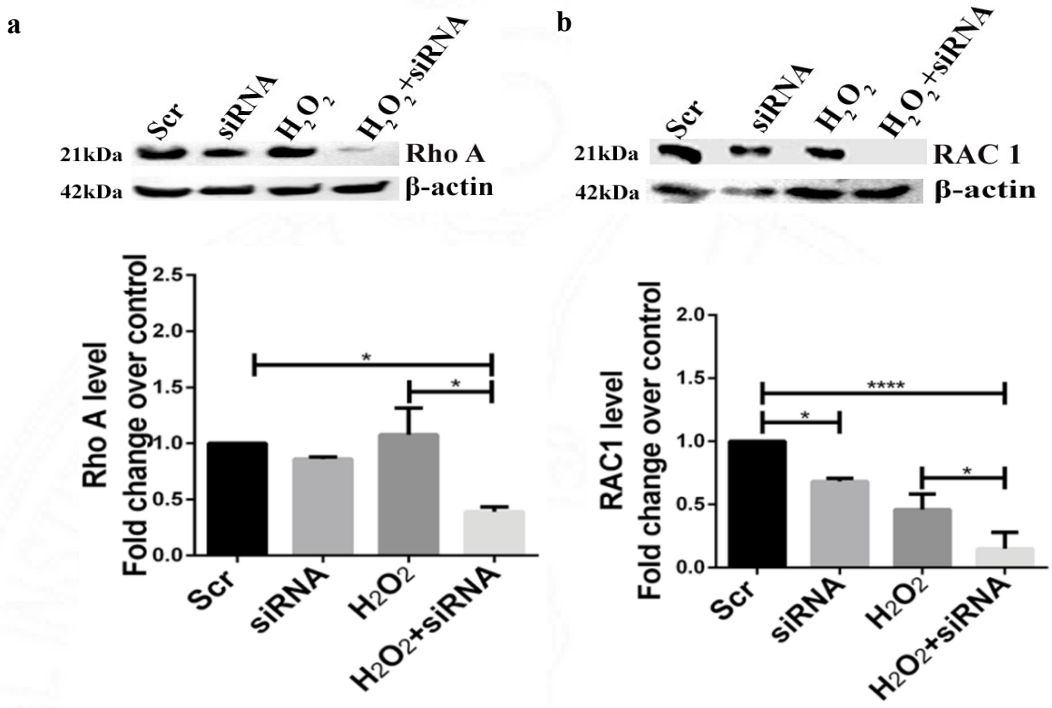


Fig.IV.19 MTH1 affects glioma cell migration

*MTH1 knockdown showing downregulation of migration markers (RhoA and RAC1) indicating that MTH1 is involved in glioma cell migration in U251 and U87 cells respectively. Statistical analysis was done using Tukey's multiple comparison test. *p<0.05, ****p<0.0001, (n=3).*

IV.6.4 MTH1 knockdown perturbed the invasion signals in glioma cells

To examine the effect of MTH1 silencing on cell invasion, the expression levels of MMP2 and MMP9 (Matrix metalloproteinase 2 and 9- proteins involved in cell invasion) were checked. We found a downregulation of MMP2 in MTH1 silenced U87 cells in the presence of high as well as basal ROS environment **(Fig.IV.20b).** Similarly, MMP9 showed a significant decrease in the expression levels in MTH1 silenced U251 glioma cells in the presence of high ROS as well as basal ROS **(Fig.IV.20a).**

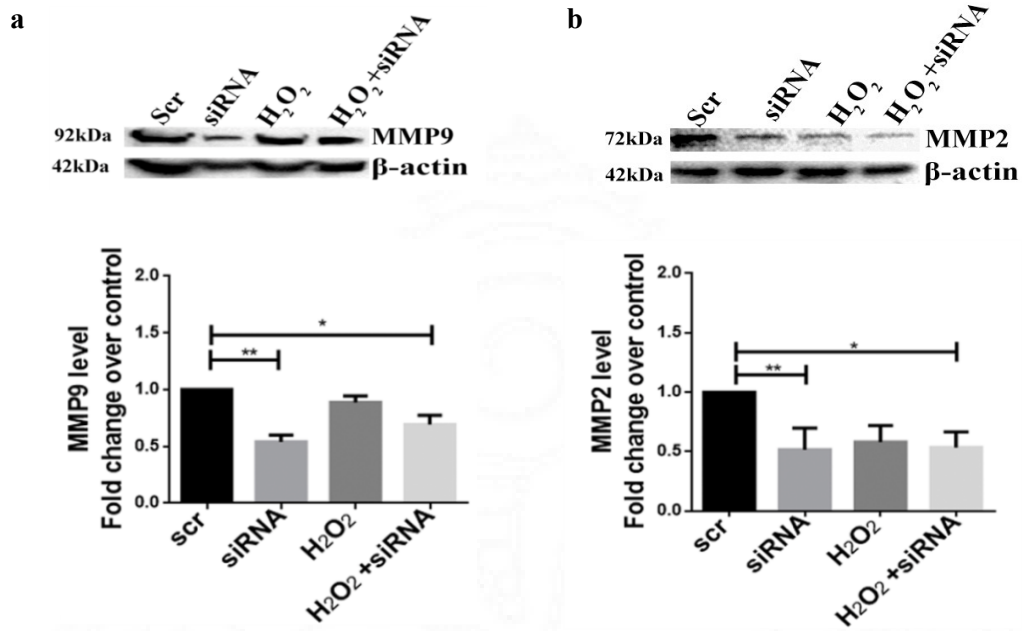


Fig.IV.20 MTH1 silencing affects cell invasion in glioma cells

*MTH1 silenced cells showing downregulation of invasion markers (MMP9 in U251 and MMP2 in U87 cells). Statistical analysis was done using Tukey's multiple comparisons test. *p<0.05, **p<0.01 (n=3).*

IV.6.5 MTH1 knockdown can affect angiogenesis by downregulating VEGF and its receptor

Vascular endothelial growth factor (VEGF) expression is already reported to be upregulated in glioblastomas when compared to the non-tumor brain tissues. An increased VEGF levels in glioblastomas correspond to poor prognosis. Targeting VEGF is also reported to affect tumor growth *in vivo.* VEGF inhibition causes DNA damage and reduces glioma cell survival. VEGFR-2 (Flk1 in mice) is a major signal transducer for blood vessel formation or angiogenesis (Shibuya, 2011) and it is reported that Flk1 activation is essential for angiogenic responses and glioma growth (Hardee and Zagzag, 2012; Reynolds et al., 2004). To check whether MTH1 have any role in glioma angiogenesis, VEGF and VEGFR-2 expression were checked after silencing MTH1. MTH1 silencing in U251 cell lines showed a downregulation of VEGF to 0.29±0.01 ($p<0.0001$) and 0.78±0.04 ($p=0.0016$) in the presence of high ROS as well as basal ROS

respectively. Also, the VEGF levels significantly declined to 0.32 fold in H_2O_2 treated MTH1 silenced cells compared to H_2O_2 alone treatment group respectively **(Fig.IV.21a).** When the expression of VEGFR-2 was checked on silencing MTH1 in U251, similar result was observed **(Fig.IV.21b).** MTH1 silencing in U251 cell lines showed a downregulation of VEGFR-2 to 0.15±0.005 ($p<0.0001$) and 0.21±0.04 ($p<0.0001$) in the presence of high ROS as well as basal ROS respectively. This suggests that MTH1 is involved in glioma cell angiogenesis. The mechanism of which is yet to be elucidated.

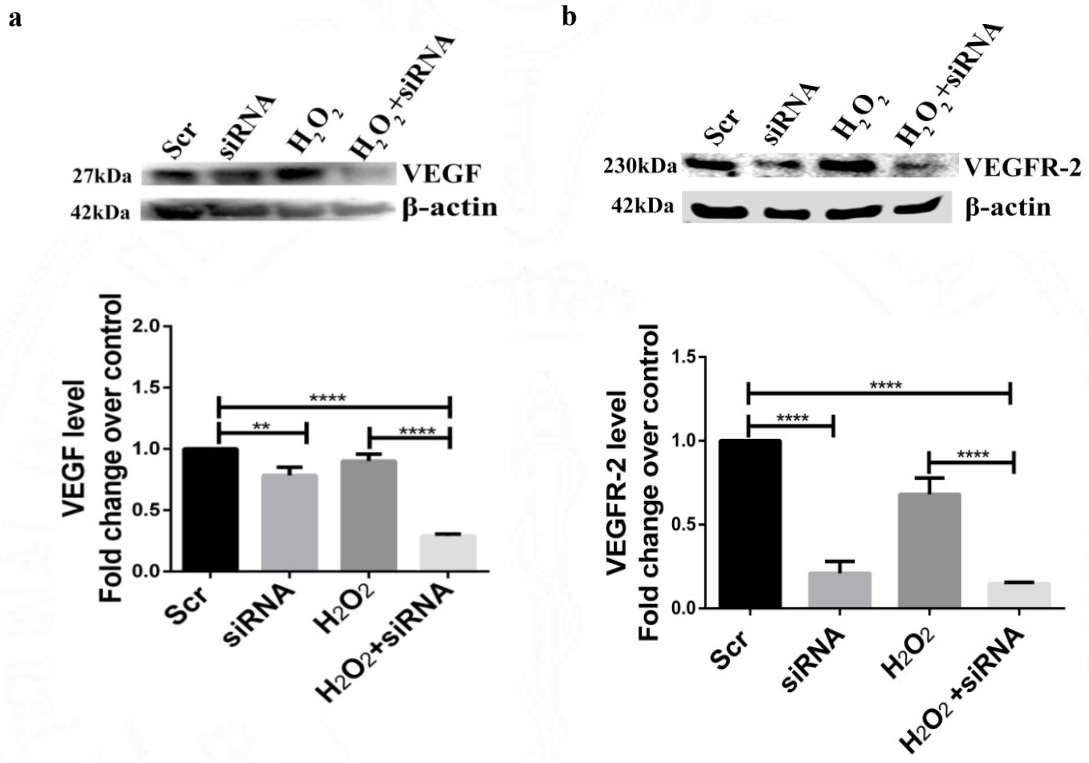


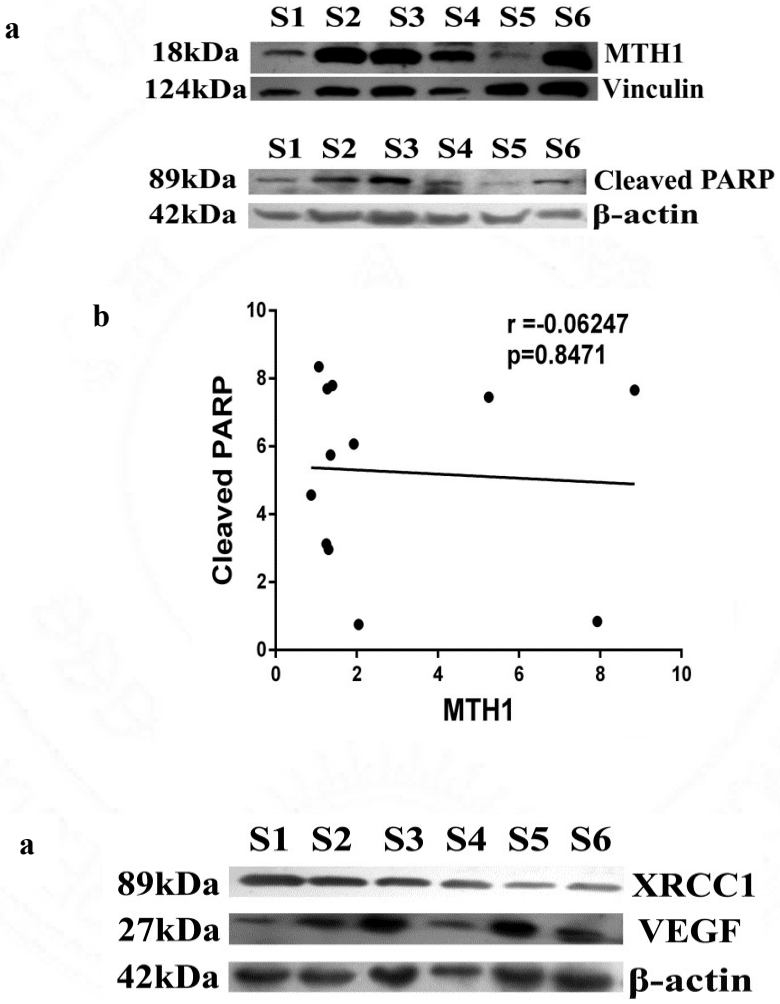
Fig.IV.21MTH1 silencing downregulates angiogenesis markers expression in glioma

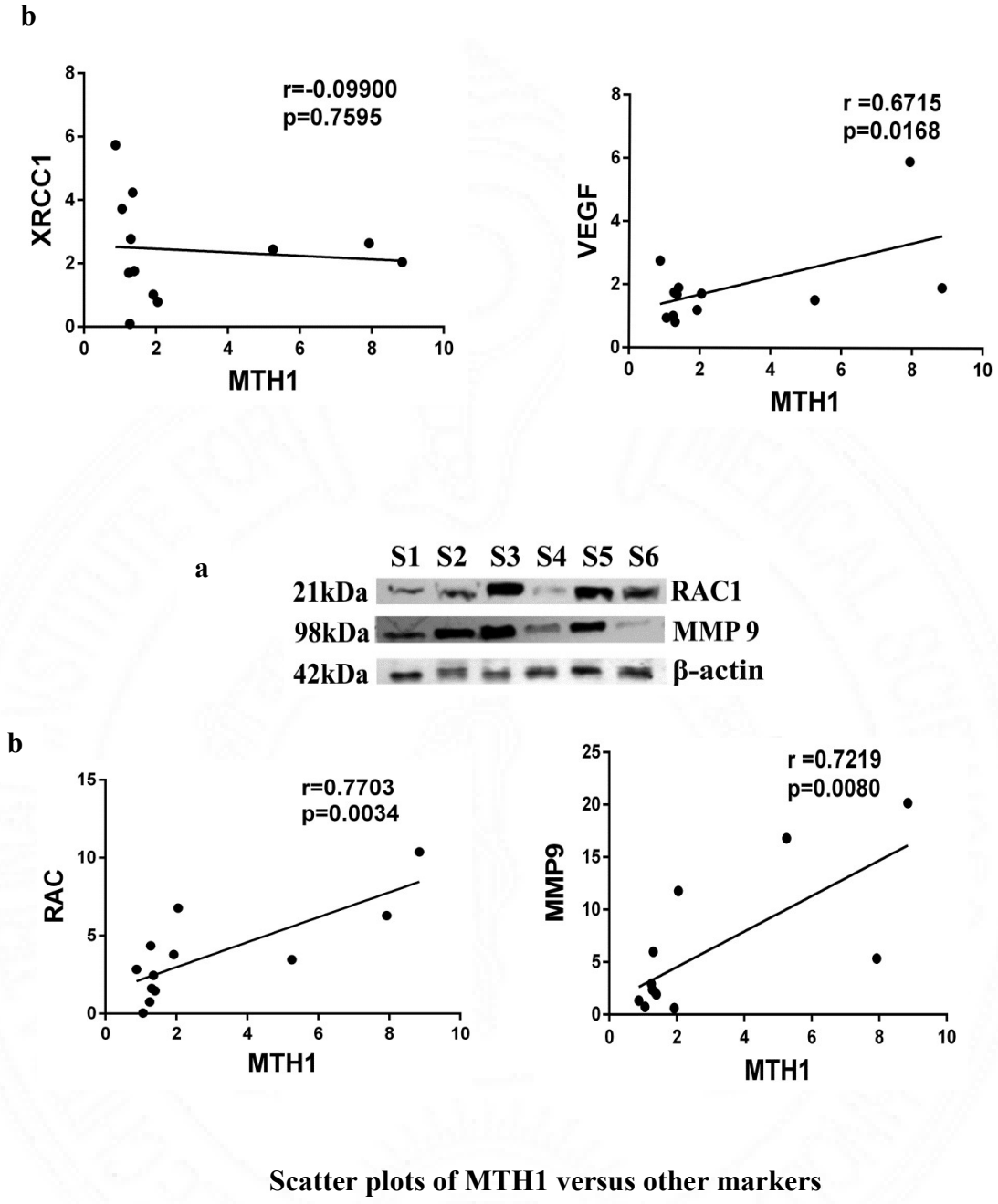
*Western blot analysis showing downregulation of VEGF and VEGFR-2 in the MTH1 silenced U251 cells indicating that MTH1 have important role in angiogenesis. Statistical analysis was done using Tukey's multiple comparisons test. **p<0.01, ***p<0.001, ****p<0.0001, (n=3).*

IV.7 Correlation of MTH1 with other markers in patient samples

Twelve glioma patient samples (6 LGG and 6 HGG) were selected to study the correlation of their MTH1 expression with the markers of DNA damage, apoptosis,

migration, invasion and angiogenesis. Cell lysate samples from LGG and HGG biopsies were selected based on their differential MTH1 expression to check the following markers: XRCC1, Cleaved-PARP, RAC1, MMP9 and VEGF. We found weak negative correlations for XRCC1 and cleaved PARP with MTH1, albeit statistically non-significant. This suggests that there is a propensity of increased DNA damage and consequent cell death in those samples showing reduced MTH1 expression. Moreover, statistically significant positive correlations were observed for MMP9, RAC1 and VEGF with MTH1 expression. This supports our *in vitro* data that MTH1 silencing downregulates the invasion, migration and angiogenesis markers in glioma **(Fig.IV.22).**





Scatter plots of MTH1 versus other markers

Fig.IV.22 Correlation of MTH1 with other markers in glioma patient samples

Spearman's rank correlation coefficients showing correlation of MTH1 with other markers: ***a.*** *Representative blots for MTH1, XRCC1, Cleaved PARP, RAC1, MMP9 and VEGF.* ***b.*** *Histograms representing the correlation of MTH1 with each marker in glioma tissue samples: XRCC1 (r= -0.09900, p= 0.7595, ns), Cleaved PARP (r= -0.06241, p=*

0.8471, ns) MMP9 (r= -0.7219, p= 0.0080), RAC1 (r= 0.7703, p= 0.0034) and VEGF expression (r= 0.6715; p= 0.0168), (n=12), ns=non-significant.

IV.8 Correlation of MTH1 with the IDH1 status in glioma patient samples

To check whether glioma patient samples show any correlation between MTH1 expression and the status of IDH1, we have performed western blotting. Those patients who are positive for IDH1 mutation were showing increased MTH1 expression. It is found that most of the patients carrying wild-type IDH1 show reduced MTH1 expression **(Fig.IV.23).** This indicates that MTH1 expression has positive correlation with IDH1 status.

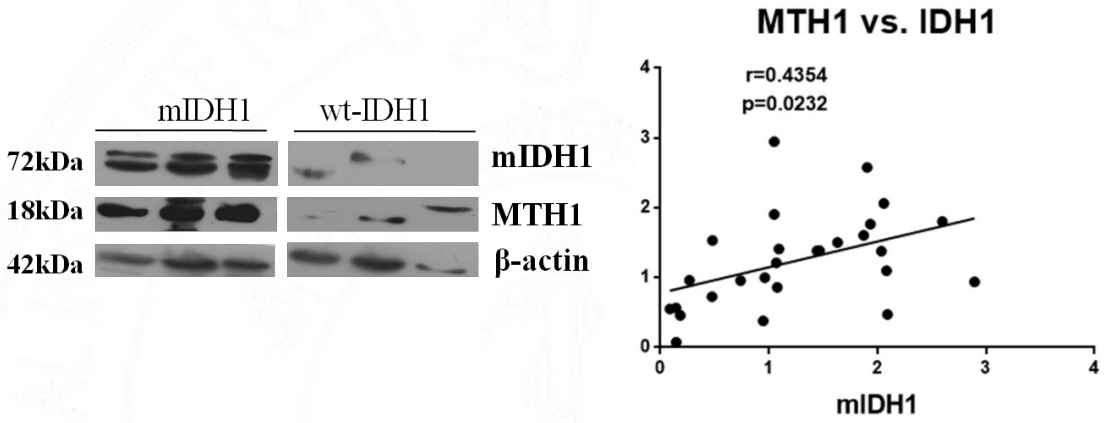


Fig.IV.23 Correlation of MTH1 expression with IDH1 mutation

(a) Representative blots and ***(b)*** *graphs showing upregulation of MTH1 in mIDH1 harboring glioma patient samples and diminished MTH1 expression in most of the patients with wild-type IDH1.Pearson's correlation coefficients was used for analysis, r=0.4354, p=0.0232; n=27.*

IV.9 Increased MTH1 activity in patients with IDH1 mutation

Since it was found that MTH1 expression is high in patients harboring IDH1 mutation, we have checked the levels of 8-oxo-dG in the same set of patients. Interestingly, it was observed that most of the mutant IDH1 harboring patients had significantly high 8-oxo-dG levels, showing increased MTH1 activity **(Fig.IV.24).**

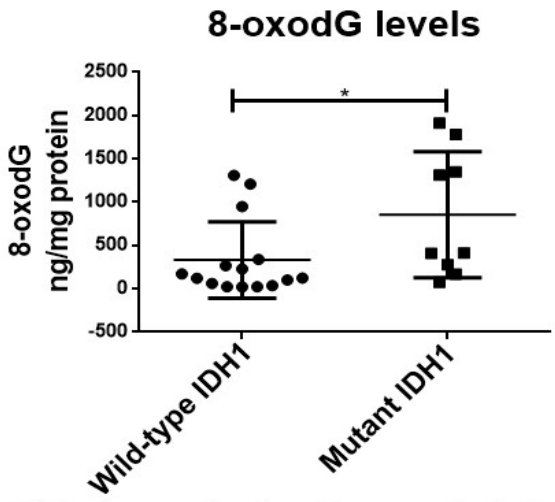


Fig.IV.24 Correlation of MTH1 activity with IDH1 mutation

Dot plot representation of 8-oxodG levels in glioma patient samples. Statistical analysis was performed by Mann Whitney test. 8-oxo-dG levels indicating increased MTH1 activity in glioma patients with IDH1 mutation; n= 24.

IV.10 Transfection of plasmid DNA

In order to study the role of mIDH1 on glioma cells, R132H overexpression plasmid was procured from Addgene. The insert (R132H) was confirmed by restriction enzyme digestion either at EcoRI site or BamHI site**(Fig.IV.25a).**Over expression of IDH1 R132H in U87 and U251 cells was done by transfecting cells with IDH1 R132H plasmid. The transfection was done and tested using anti-Flag antibody by western blotting**(Fig.IV.25b).**DCFDHDA, scratch wound assay and western blot were performed to study the effects of IDH1 R132H mutation on ROS production, glioma cell migration and to correlate MTH1 with mIDH1 respectively.

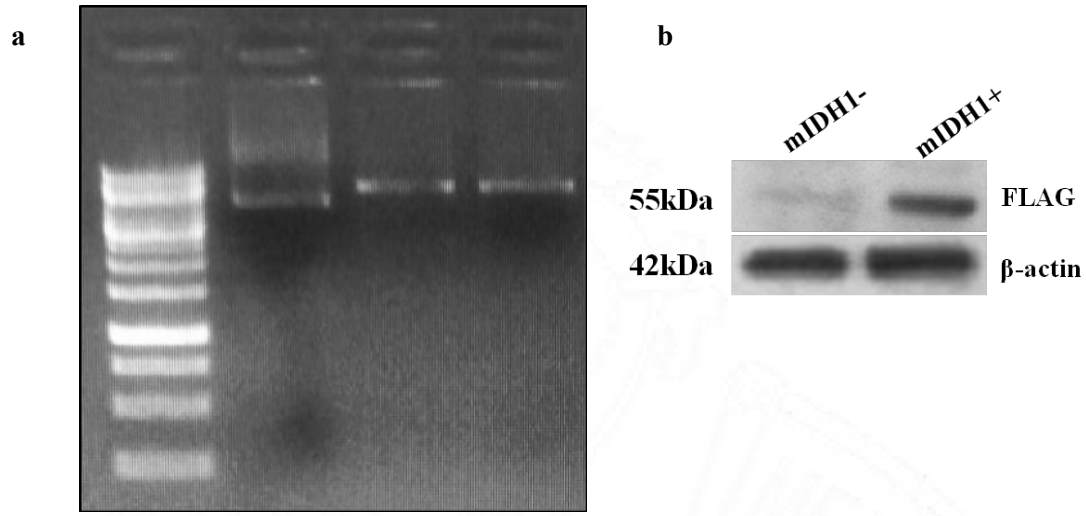


Fig.IV.25 Plasmid carrying Flag-tagged R132H

(a)*Flag-tagged R132H IDH1 mutant plasmid with EcoR1 and BamHI restriction sites flanking on both sides.* ***(b)*** *Representative immunoblot showing Flag expression indicating successful transfection of mIDH1 plasmid in U87 cells.*

IV.10.1 MTH1 upregulated in mIDH1 expressed and 2-HG treated glioma cells

In order to confirm the correlation of MTH1 with IDH1 mutation in glioma patient samples, mIDH1 plasmid was transfected into both U87 and U251 cells. It is found that mIDH1 expressed cells showed an upregulation of MTH1 expression, whereas downregulation of MTH1 was observed when mIDH1 was inhibited when we have performed western blotting **(Fig.IV.26).** To check whether 2-HG, the mIDH1 product have any link with MTH1, we have treated U87 cells with 2-HG and found that MTH1 was markedly upregulated in cells exposed to 2-HG **(Fig.IV.27).**.This shows that mIDH1 may have direct or indirect relation with MTH1, but the actual mechanism behind the same is currently unknown.

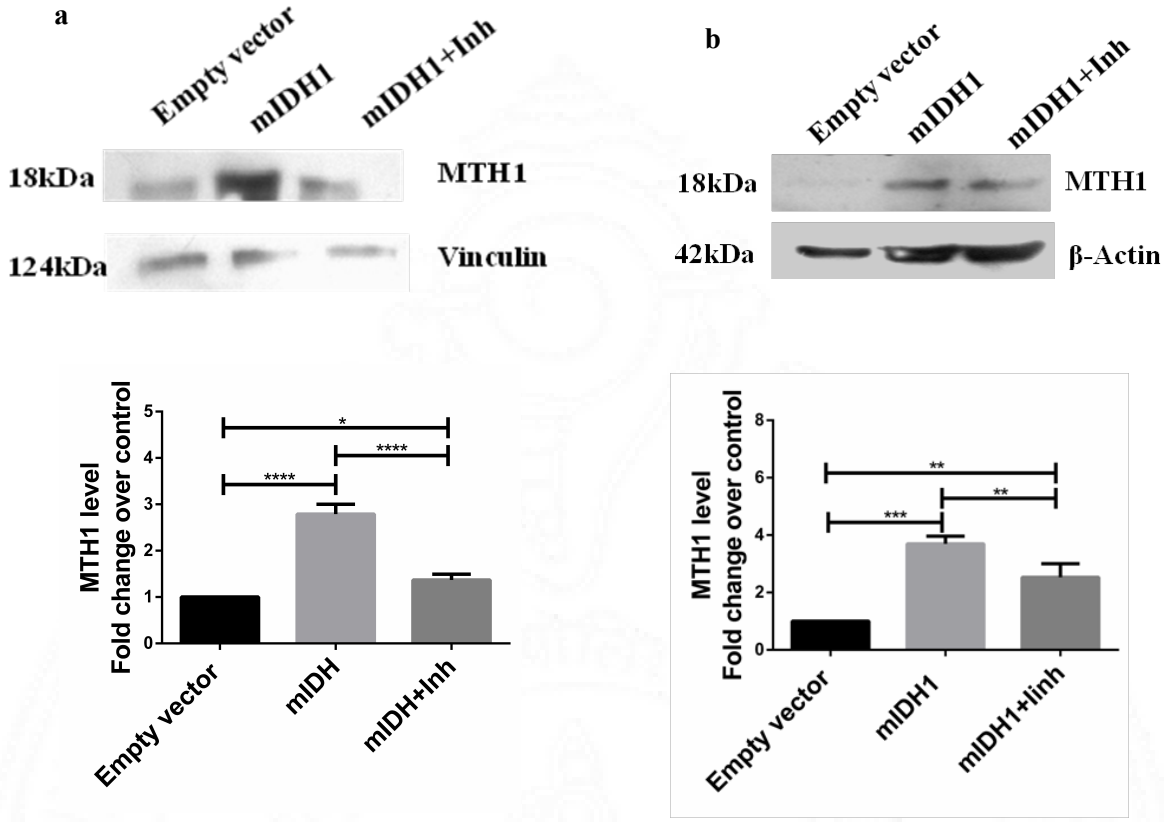


Fig.IV.26 mIDH1 expressed glioma cells upregulates MTH1

Immunoblot and corresponding histograms representing upregulation of MTH1 in mIDH1 expressed ***(a)*** *U87 and* ***(b)*** *U251 cells.MTH1 was downregulated when mIDH1 was inhibited. *p<0.05,**p<0.01, ***p<0.001, ****p<0.0001, (n=3).*

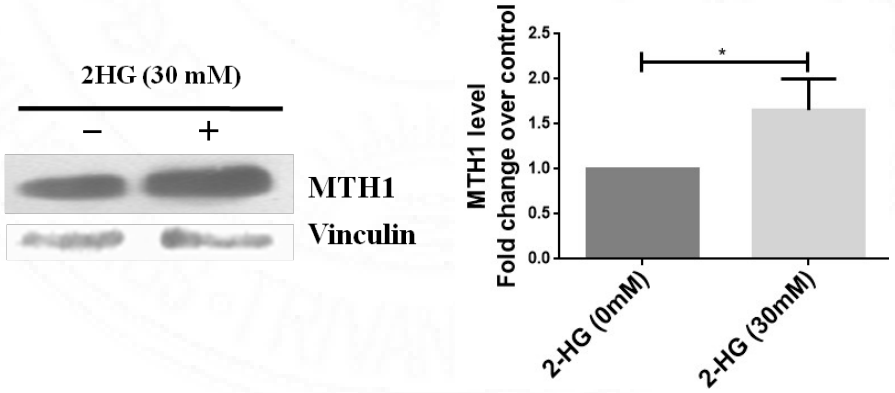


Fig.IV.27 2-HG upregulates MTH1 expression in U87

*Immunoblot and corresponding histograms representing upregulation of MTH1 in 2-HG treated (30mM)U87 cells.*p<0.05, (n=3).*

IV.10.2 mIDH1 or 2-HG modulates MTH1 expression/activity via ROS

In order to check whether mIDH1/2-HG directly modulates MTH1 expression/activity, the cells were pre-treated with 1 mM NAC. After scavenging the basal ROS, the cells were transfected with mIDH1 DNA or exposed to 2-HG treatment. The proteins isolated were taken for western blotting. In order to check the MTH1 activity, 8-oxodG levels were measured using ELISA after the treatment. It is found that MTH1 expression was upregulated even after scavenging the basal ROS in the cells indicating that the ROS via mIDH1 or 2-HG may contribute to MTH1 expression **(Fig.IV.28a).** The increased 8-oxodG levels indicate MTH1 activity via the ROS generated by mIDH1 or 2-HG **(Fig.IV.28b).**

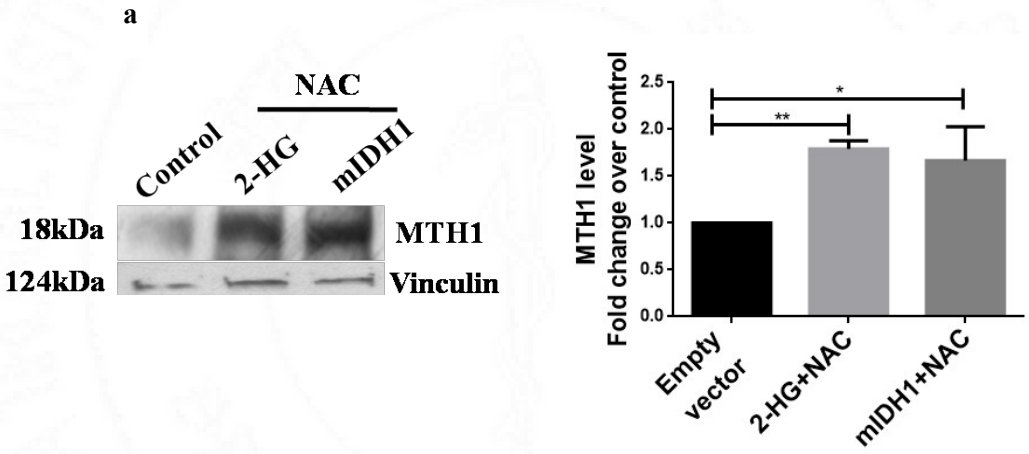


Fig.IV.28a mIDH1 and 2-HG upregulates MTH1 via ROS

*Western blot showing upregulation of MTH1 in mIDH1 expressed and 2-HG treated U87 cells were pre-treated with 1mM NAC. **p<0.01, (n=3).*

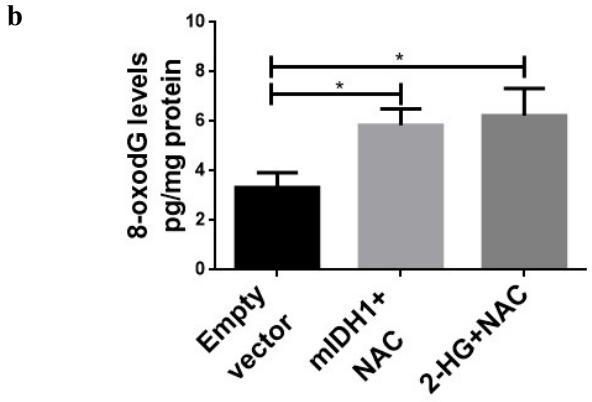


Fig.IV.28b Increased 8-oxodG levels when basal ROS was depleted in mIDH1 and 2-HG treated U87 cells

*8-oxodG levels showing MTH1 activity in mIDH1 expressed and 2-HG treated U87 cells which were pre-treated with 1mM NAC. *p<0.05, (n=3).*

IV.10.3 mIDH1 and 2-HG contributes to increased ROS levels in glioma cells

After expressing mIDH1 in the glioma cells, we have performed DCFHDA assay in order to measure the total intracellular ROS production. In this experiment, U87 cells were transfected with mIDH1 or treated with 2-HG. In one of the groups, mutant IDH1 inhibitor AGI-5198 was used at a concentration of 2 μM for 24 hours. The mIDH1 inhibitor blocks the production of 2-HG from mIDH1. In yet another experiment, 2-HG, the mIDH1 product was exogenously supplied to the cells at a concentration of 30mM for 24 h. Cells exposed to 100 μM H_2O_2 was used as a positive control. In one of the groups, cells were pre-treated with the ROS scavenger, NAC before mIDH1 transfection or 2-HG treatment. The cells were then exposed to the fluorescent probe DCFHDA for fluorimetric analysis. It was observed that both the mIDH1 expressed as well as 2-HG treated cells showed increased ROS generation. This result suggests that 2-HG could also be a factor that contributes to the increased ROS production in mIDH1 harboring glioma patients. It was found that ROS generation was diminished when mIDH1 was inhibited when compared to the empty vector control. Interestingly, we also observed that on depleting the basal ROS and further transfecting with mIDH1 or treating with 2-HG

increases the total intracellular ROS which indicates that mIDH1/2-HG contributes to the ROS generation in the mIDH1 harboring cells **(Fig.IV.29).**

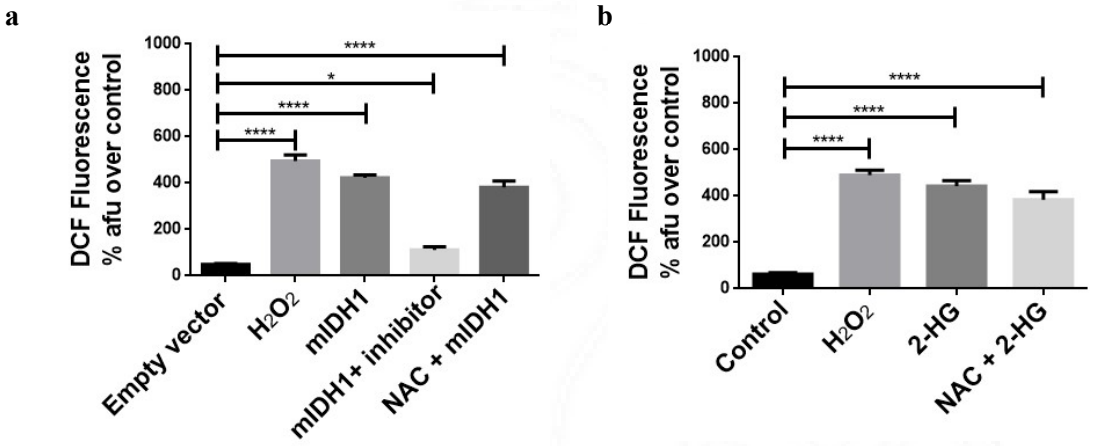
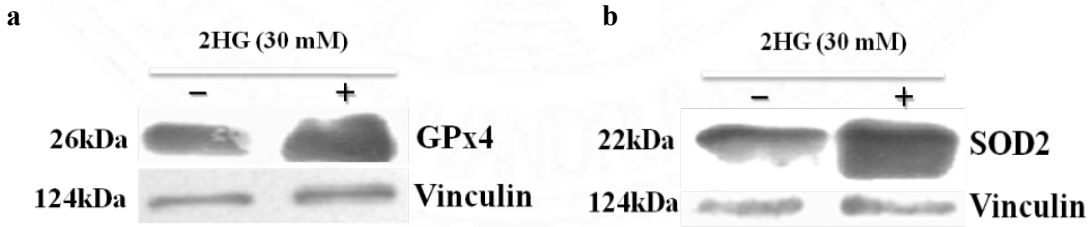


Fig.IV.29 mIDH1 and 2-HG produces high ROS

DCFHDA fluorimetric analysis showing high ROS generated by mIDH1 expressed and 2-HG treated U87 cells ***(a)*** *mIDH1 groups* ***(b)*** *2-HG groups. *p<0.05, ****p<0.0001, (n=3).*

IV.10.4 2-HG treatment elevated the expression of oxidative stress markers

After treating the U87 cells with 2-HG, we have checked the expression levels of two major oxidative stress markers, GPx4 and MnSOD. It is found that both the markers showed elevated expression when treated with 2-HG **(Fig.IV.30).** This confirms the above result suggesting that the ROS generated via 2-HG causes an increased oxidative stress in the cell.



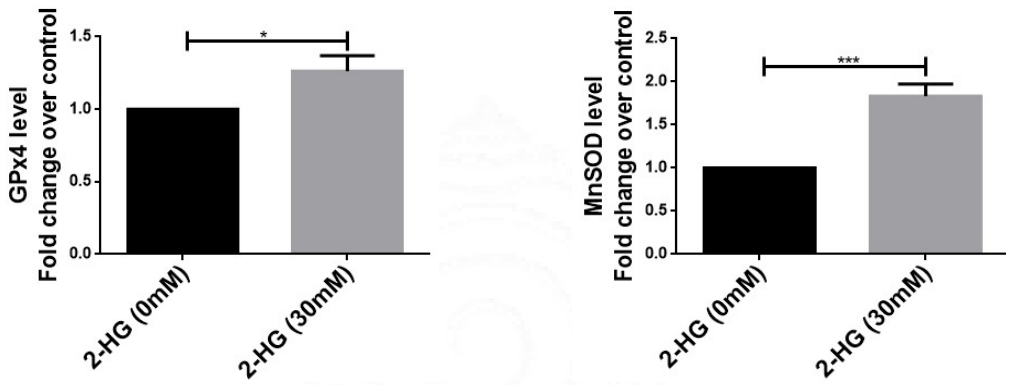
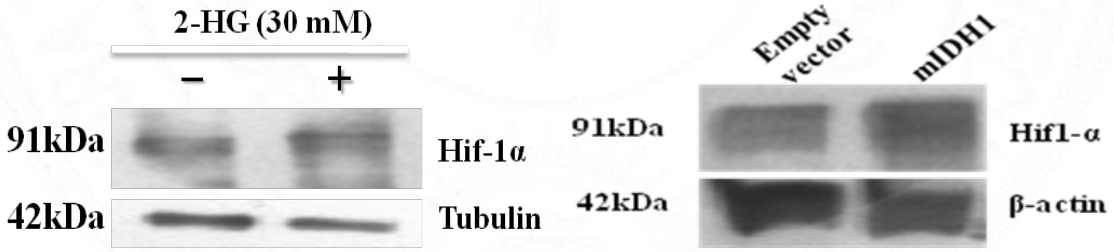


Fig.IV.30 2-HG increases oxidative stress in U87 cells

*Western blot analysis showing **(a)** the increased GPx4 and **(b)** MnSOD levels when U87 cells were treated with 30mM 2-HG for 24 h. *p<0.05, ***p<0.001, (n=3).*

IV.10.5 mIDH1 and 2-HG contributes to gliomagenesis

In order to check whether mIDH1 and 2-HG has any role in gliomagenesis as reported earlier, we have probed for Hif1α and it downstream VEGF. Our western blot data shows that both mIDH1 expressed as well as 2-HG treated cells show a significantly high expression of the transcription factor, Hif1α **(Fig.IV.31)** as well as VEGF levels **(Fig.IV.32).** This shows that mIDH1 though is a good prognostic factor for glioma patients, at the same time is responsible for the tumorigenic and angiogenic properties shown by the cells.



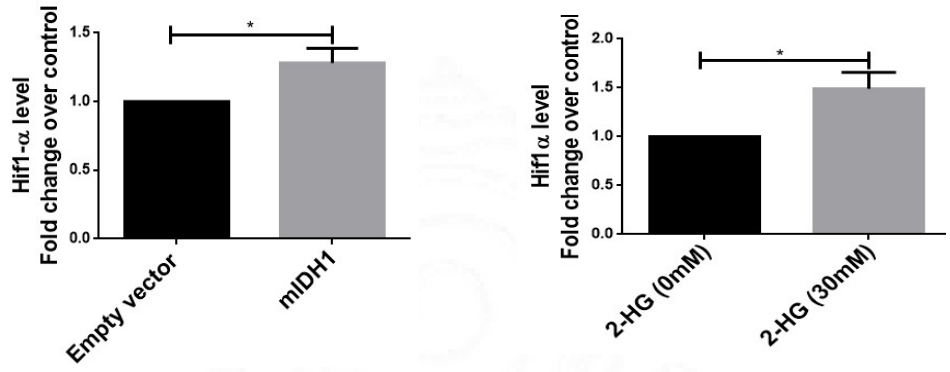


Fig.IV.31 Hif1α upregulation in mIDH1 expressed and 2-HG treated cells

*Western blot data showing increased Hif1α expression in mIDH1 expressed and 2-HG treated U87 cells. Statistical analysis was performed by t-test. *p<0.05, (n=3).*

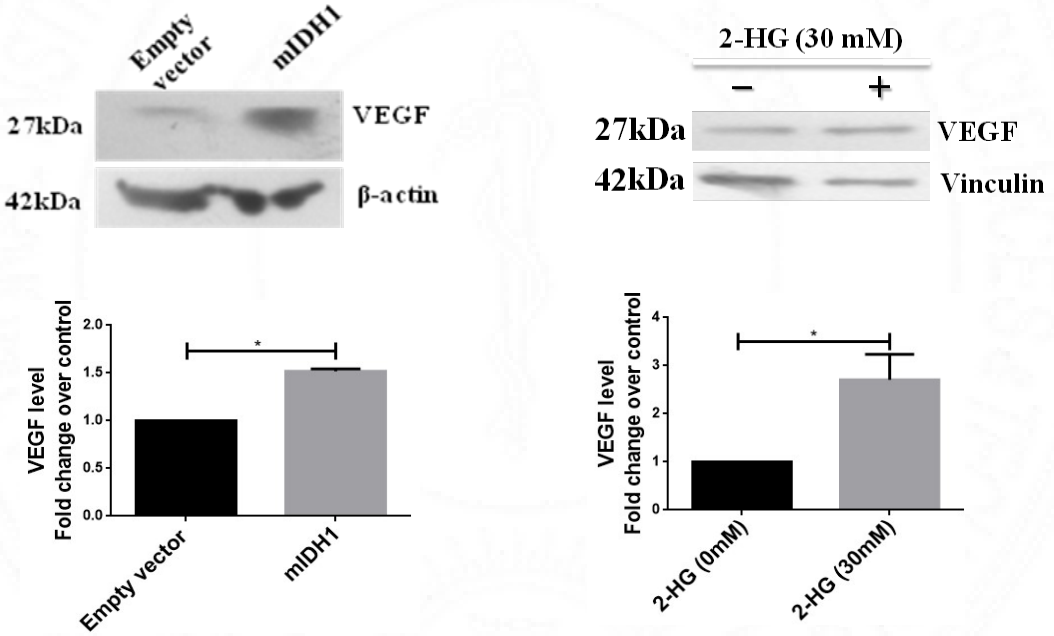


Fig.IV.32 VEGF upregulation in mIDH1 expressed and 2-HG treated cells

*Western blot data showing increased VEGF expression in mIDH1 expressed and 2-HG treated U87 cells. Statistical analysis was performed by t-test. *p<0.05, (n=3).*

IV.10.6 mIDH1 contributes to glioma cell migration

In order to check the role of mIDH1 on glioma cell migration, we have used the following groups in U87 cells: Empty vector, mIDH1 expressed cells, mIDH1 inhibited cells, It is found that the migration efficiency of mIDH1 expressed cells increased when compared to the parental control, whereas the rate of migration was gradually sluggish when mIDH1 was inhibited **(Fig.IV.33).**

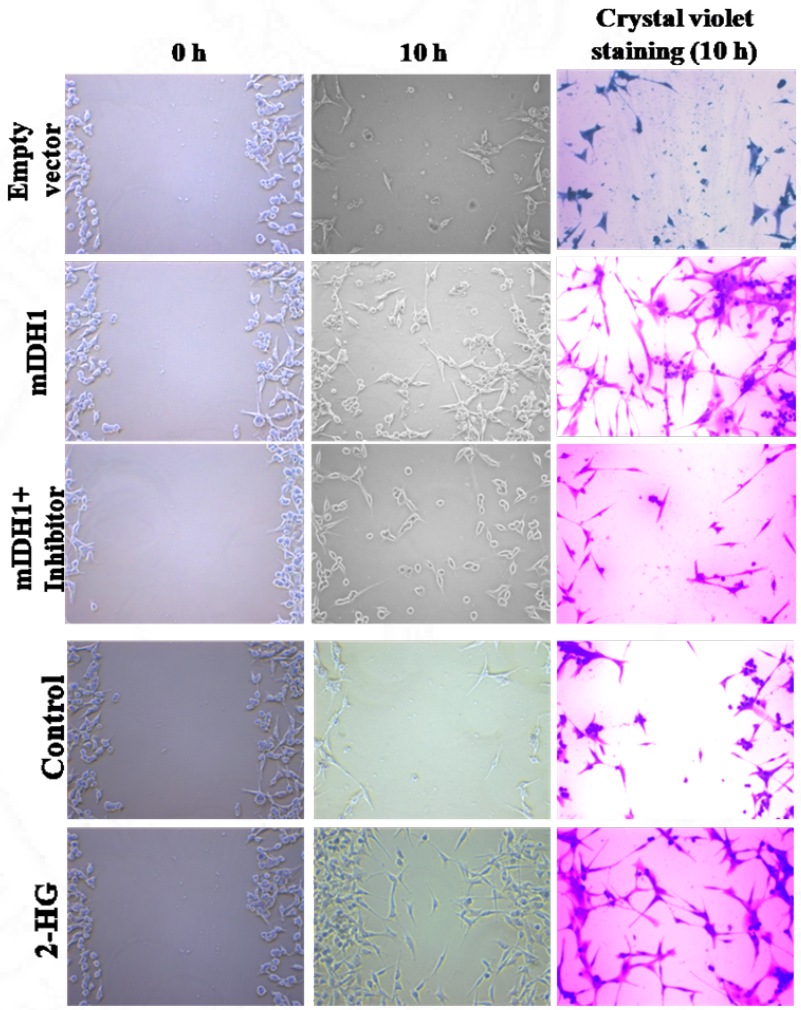


Fig.IV.33 mIDH1 increases glioma cell migration

Scratch wound healing assay showing increased cell migration in mIDH1 expressed and 2-HG treated U87 cells and less migration in mIDH1 inhibited cells (n=4).

IV.11 Hif1α modulates MTH1 expression in glioma

Increased MTH1 expression was found to be correlated with Hif1α expression in glioma patient samples **(Fig.IV.34).** Hif1α is reported to be elevated in several cancers and portend a poor prognosis and an increased malignancy (Iommarini et al., 2017; Jun et al., 2017) Hence, the cells were treated with 100 μM $CoCl_2$,an inducer of Hif1α for 24 h for U87 cells and 50 μM $CoCl_2$ for 16 h for U251 cells. Very interestingly, we found that MTH1 is upregulated along with the elevation of Hif1α **(Fig.IV.35 and Fig.IV.36).** Thus, the expression of Hif1α was silenced in glioma cell lines using Hif1α siRNA and checked whether MTH1 expression is altered. MTH1 was found to be downregulated when Hif1α was silenced in both U87 and U251 cells **(Fig.IV.37 and Fig.IV.38).** This indicates that Hif1α may modulate MTH1 expression in glioma. We also observed that there is a no change in the ROS level when Hif1α was silenced **(Fig.IV.39)**. Hence, in order to check whether there is any direct interaction between Hif1α and MTH1, a co-immunoprecipitation assay was performed. On pulling down MTH1 in the presence of basal as well as high ROS, we couldn't observe any bands for Hif1α **(Fig.IV.40)**.

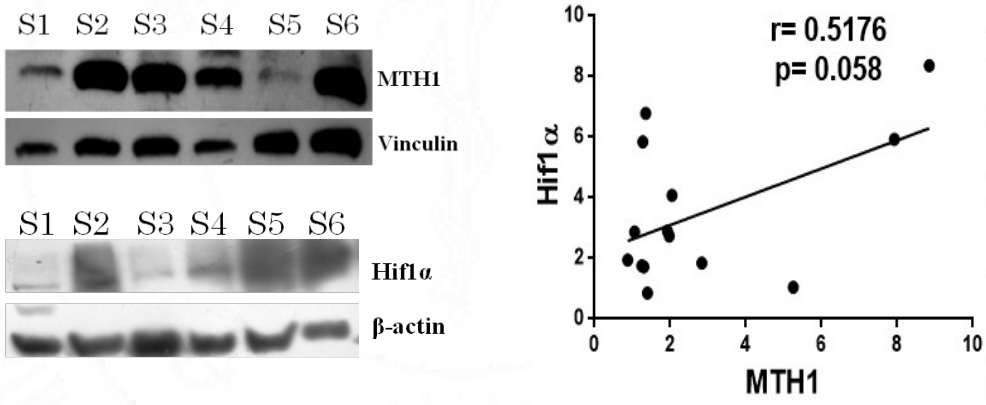


Fig.IV.34 Correlation of MTH1 with Hif1α expression

Spearman's correlation coefficient shows trend towards increase in the expression of Hif1α with MTH1 expression in glioma patient samples. r=0.5176, p=0.058; n=14.

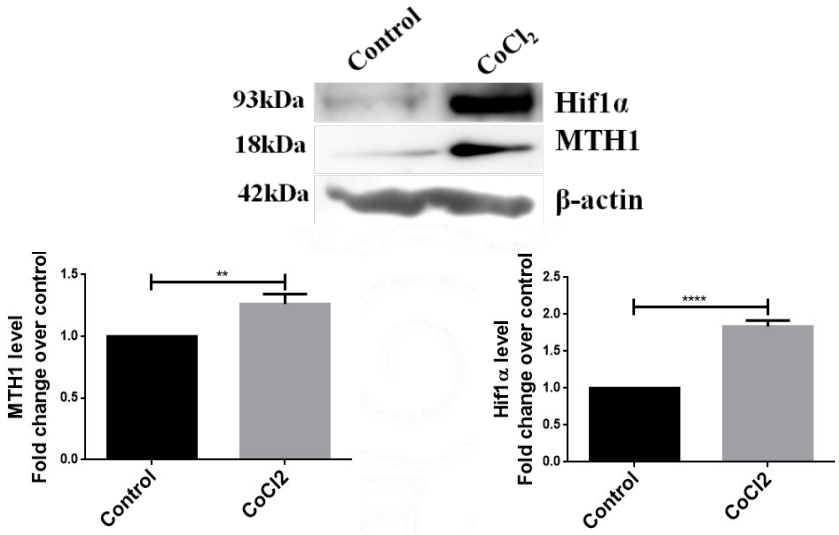


Fig.IV.35 CoCl2 induces MTH1 expression in U87 cells

*Western blot analysis showing upregulation of Hif1α on treatment with 100 μM $CoCl_2$ for 24 h. Further probing for MTH1 shows an upregulation of MTH1 in U87 cells. **p<0.01, ****p<0.0001, (n=3).*

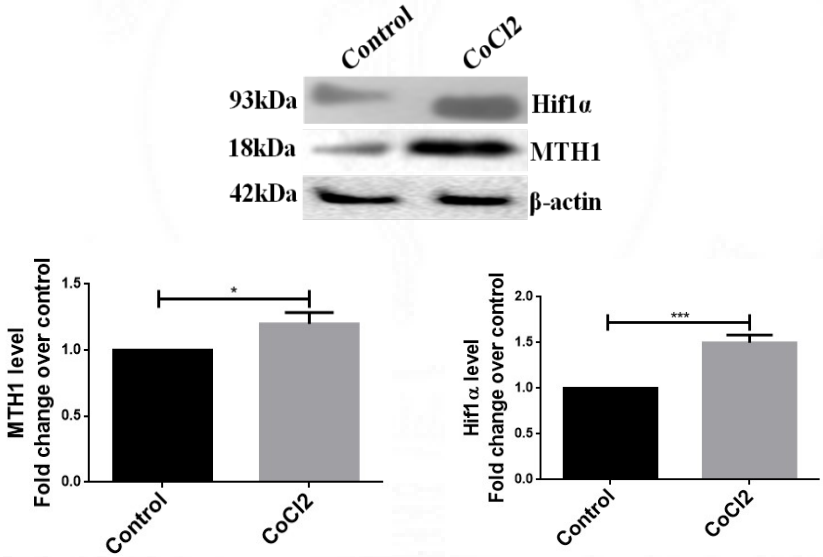


Fig.IV.36 CoCl2 induces MTH1 expression in U251 cells

*Western blot analysis showing upregulation of Hif1α on treatment with 50 μM $CoCl_2$ for 16h. Further probing for MTH1 shows an upregulation of MTH1 in U251 cells. *p<0.05, ***p<0.001, (n=3).*

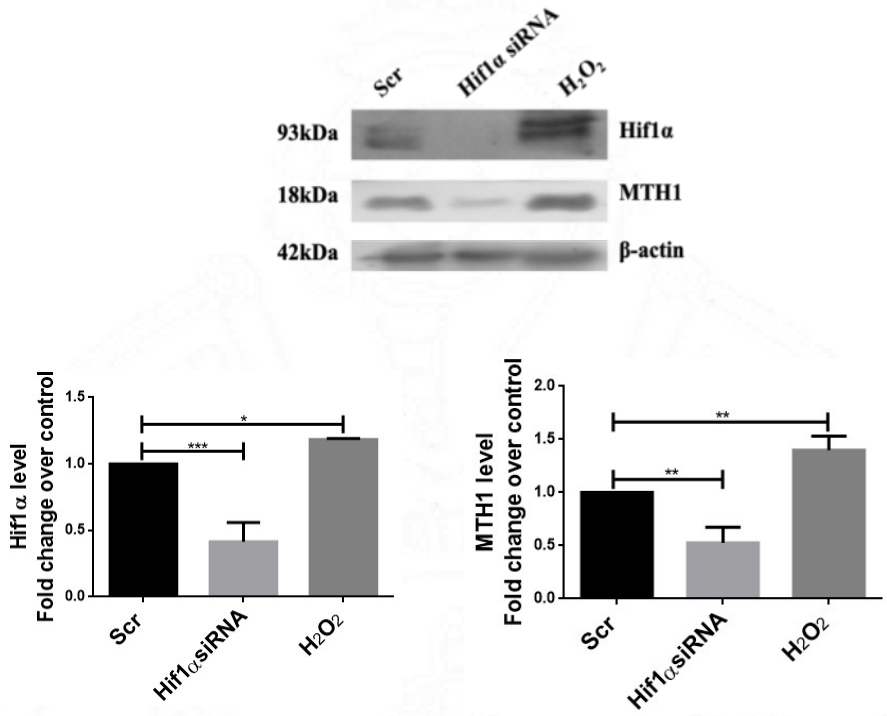
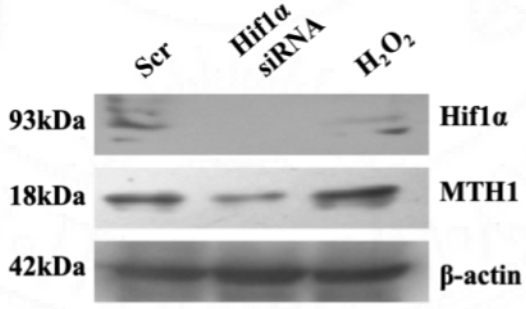


Fig.IV.37 Hif1α silencing downregulates MTH1 expression in U87 cells

*Western blot analysis showing silencing of Hif1α on treatment with 100 nMHif1α siRNA for 48 h in U87 cells. Further probing for MTH1 shows a downregulation of MTH1. *p<0.05, **p<0.01, ***p<0.001, (n=3).*



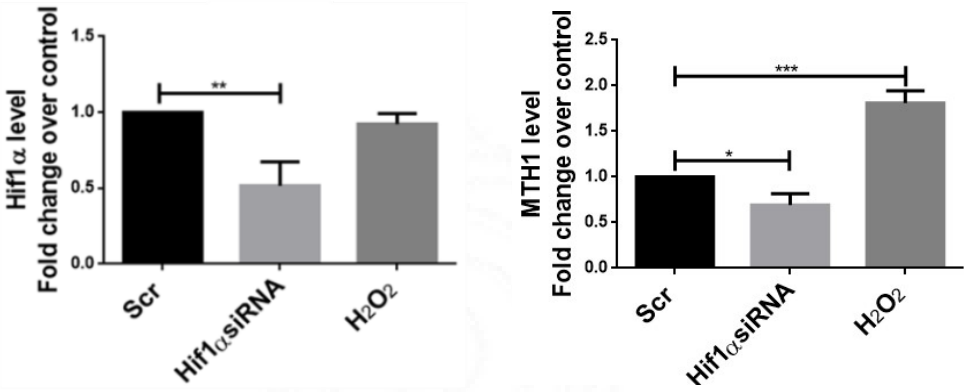


Fig.IV.38 Hif1α silencing downregulates MTH1 expression in U251 cells

*Western blot analysis showing silencing of Hif1α on treatment with 100 nM Hif1α siRNA for 48 h in U251 cells. Further probing for MTH1 shows a downregulation of MTH1. *p<0.05, **p<0.01, ***p<0.001, (n=3).*

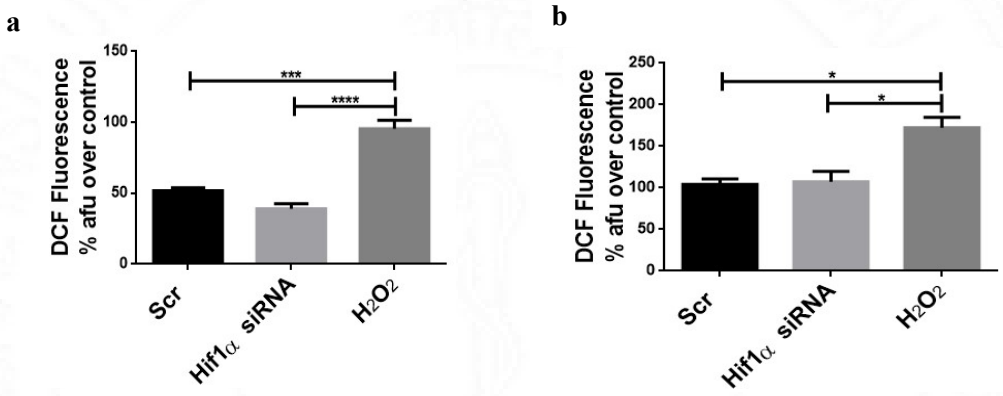


Fig.IV.39 Hif1α silencing reduces ROS level in glioma cells

DCFHDA showing ROS levels when Hif1α was silenced in both the cells, ***(a)*** *U87 and* ***(b)*** *U251. *p<0.05, ***p<0.001, ****p<0.0001, (n=3).*

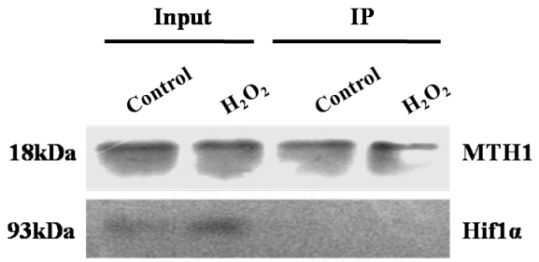
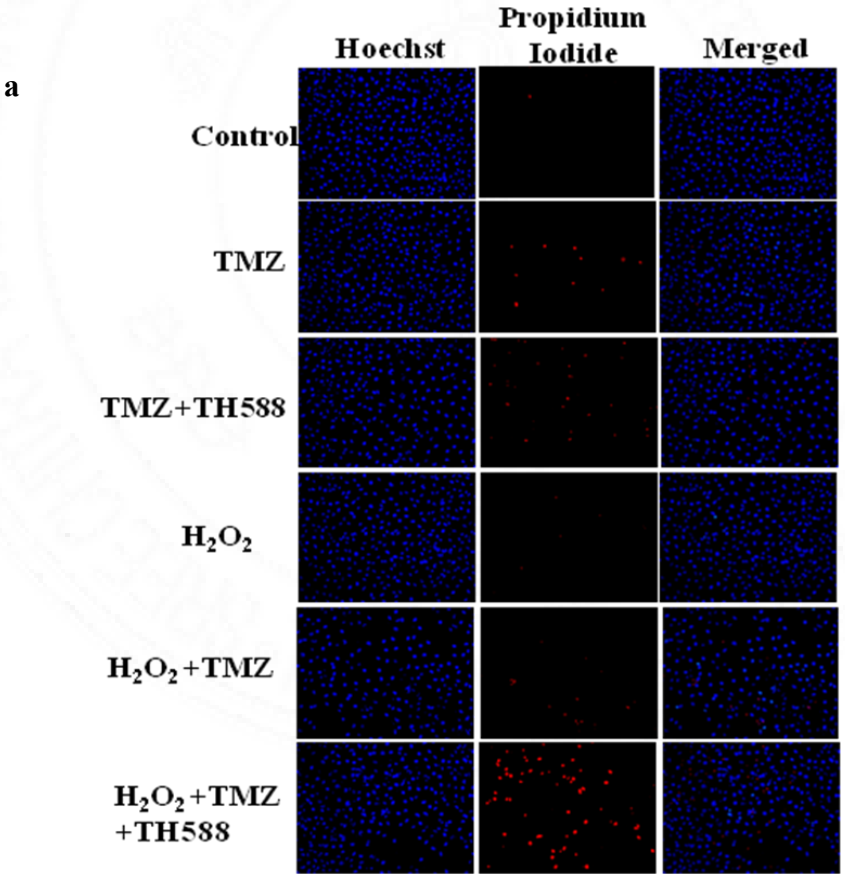


Fig.IV.40 No direct interaction between MTH1 and Hif1α proteins

Representative Western blot image of MTH1 and Hif1α in U251 cells after immunoprecipitation of MTH1 in the presence of basal as well as high ROS levels. (n=3).

IV.12 Combinatorial effects of TH588 and TMZ

When U251 cells were treated with TH588 and TMZ, interesting results were observed. In the presence of basal as well as high ROS, cells treated with TMZ and TH588 were showing increased OGG1 expression and further increased cell death than the TMZ alone group **(Fig.IV.41).**



b

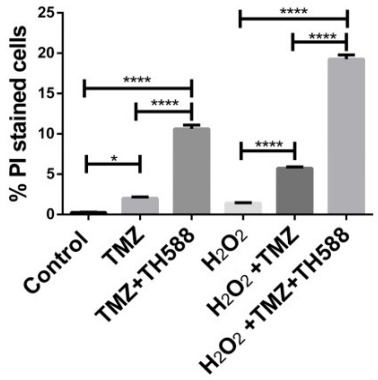
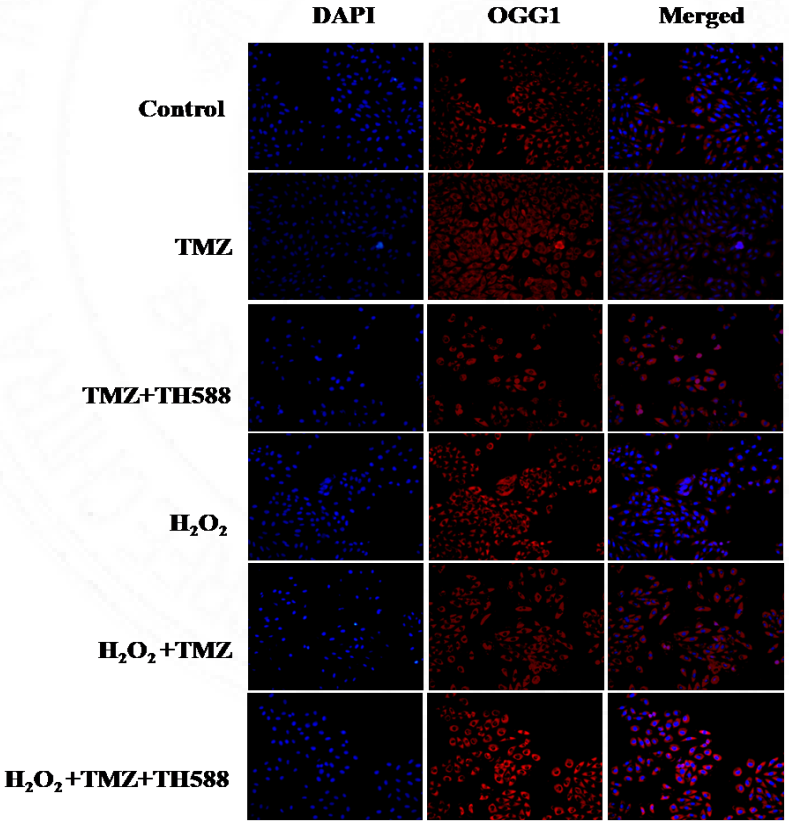


Fig.IV.41 Increased cell death in TMZ+TH588 groups

Representative images of percentage PI stained U251 cells after inhibiting MTH1 along with TMZ treatment ***(a)*** *and its corresponding histograms* ***(b).*** *First panel towards down represents Hoechst staining (total nuclei), PI stained cells indicating total number of dead cells (second panel) and third panel is the representation of merged images of Hoechst and PI stained cells. Statistical analysis was done using Tukey's multiple comparisons tests. *p<0.05, ****p<0.0001 (n=3).*

a



b

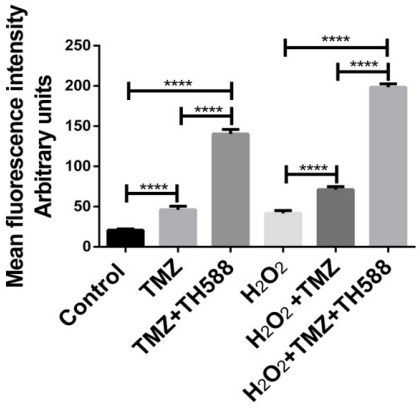


Fig.IV.42 Increased DNA damage in TMZ+TH588 groups

Mean Fluoresence Intensity (MFI) calculated for OGG1 expression. ***(a)*** *Representative images at 100X magnification on fluorescent microscope are given for control as well as treated groups. TMZ+TH588 groups showed high OGG1 expression in U251 cells when compared to the control group as well as the TMZ alone group* ***(b)*** *Graphical representation of MFI (Mean ± SEM), showing highest intensity for TMZ+TH588 group in the presence and absence of H_2O_2, ****$p<0.0001$ $(n=3)$.*

IV.13 Key findings

The key findings from this study are:

- MTH1 is upregulated in glioma patient samples of all grades.
- The expression and activity of MTH1 is independent of the grades of glioma.
- MTH1 is activated in response to H_2O_2 and its inhibition with pharmacological inhibitor TH588 enhances the DNA damage in human glioblastomas cell lines (U87MG and U251MG).
- Silencing of MTH1 affected glioma cell survival, migration and angiogenesis.
- Silencing of MTH1 downregulated the invasion markers in glioma.
- Hif1α modulates MTH1 expression in glioma cells.
- The ROS generated by mIDH1 is also contributed by 2-HG.
- mIDH1/2-HG is involved in gliomagenesis by upregulating Hif1α and VEGF levels.

- mIDH1 enhances the migration ability of glioma cells.
- MTH1 expression and activity is upregulated in most of the glioma patients harboring mIDH1.
- Increased MTH1 expression was seen in mIDH1 expressed and 2-HG treated cell lines.
- ROS generated via mIDH1/2-HG contributes to the activation of MTH1.

V. Discussion

ROS is one of the major hallmarks of most cancers (Gad et al., 2014; Kumari et al., 2018). High levels of ROS in cells will result in the accumulation of oxidized nucleotides. The ensuing incorporation of such oxidized nucleotides into cancer cell DNA lead to DNA damage and consequent cell death (Abbas et al., 2018). The accumulation of 8-oxodG which is a major oxidative DNA damage product was reported to be high in gliomas. The activity of MTH1 is essential in such conditions, since the enzyme hydrolyzes the 8-oxodGTP to 8-oxodGMP and thus prevent the DNA damage caused by the oxidized nucleotides (Iida et al., 2001). Tu et al. in 2016 showed that a malignancy-dependent MTH1 expression is in gliomas as they had found a high MTH1 expression in glioblastoma when compared to other less malignant gliomas like mixed gliomas and astrocytoma (Tu et al., 2016). Tu *et al.* found novel functions of MTH1 in glioma cell proliferation, tumor sphere formation and colony formation in GBM. They also showed that the MTH1 mRNA as well as protein levels were upregulated in GBM patient samples when compared with the normal brain tissues when they analyzed 557 GBM samples. They found that when compared to all the other less malignant gliomas, GBMs show higher MTH1 expression indicating a positive correlation between MTH1 expression and tumor grade. In contrast to the findings of aforementioned report, the current study shows that MTH1 expression is irrespective of glioma malignancy atleast in the Asian Indian population. However, MTH1 expression is markedly high in different grades of gliomas than the non-tumor brain tissues. This is in concordance with the observations made by Pudelko *et al.*, which claimed that GBM cultures with both low as well as high aggressiveness have reduced viability on inhibition of MTH1 (Pudelko et al., 2017). The upregulation of MTH1 in glioma tissue biopsies prompted us to explore the other major functions of MTH1 in glioma, for which we have carried out further experiments *in vitro* in human glioblastoma cell lines, U87MG and U251MG. Most of the glioma patient samples harboring IDH1 mutation showed elevated expression and activity of MTH1. IDH mutation is a factor that is responsible for the generation of elevated levels of ROS in glioma cells (Shi et al., 2015). This resulted in the birth of our hypothesis that whether the IDH1 mutation has any role in the activation of MTH1 via the ROS generated by the mutant form. With the clues developed from the patient samples, we also verified the above hypothesis in cell lines too.

Although the available literature on the generation of ROS by the mIDH1 product 2-HG is few, we were able to observe an increase in ROS levels when 2-HG was given to U87 as well as U251 cells. Even though the role of 2-HG in ROS production is not studied widely, our observations indicate that 2-HG is also contributing to the ROS production in mIDH1 cells, apart from the reduction in the levels of anti-oxidant GSH which were reported in numerous studies (Mellai et al., 2013; Shi et al., 2013; Wahl et al., 2017). Further, the level of oxidative stress markers Gpx4 and SOD2 were too found to be high in 2-HG treated U87 cells supporting the above observation. We also observed that MTH1 gets activated in the presence of H_2O_2 (high ROS) which actually makes a strong association between MTH1 and mIDH1 in gliomas. Interestingly, majority of the glioma patients carrying mIDH1 were showing significant upregulation of MTH1. This is a novel finding, where a positive correlation between MTH1 and mIDH1 was observed in glioma, which was not studied in any of the cancers before. Accordingly, mIDH1 expressed U87 and U251 cells were also showing elevated MTH1 expression and on inhibiting mIDH1, we observed downregulation of MTH1. This confirms our observation in patient samples. Similar to the above observation seen in mIDH1 expressed cells, it is also demonstrated that the MTH1 expression was markedly high on treatment of U87 cells with 2-HG. Hence, to conclude, it could be via the 2-HG that mIDH1 showing this significant upregulation of MTH1 in gliomas. To prove our hypothesis whether the ROS generated by mIDH1 have a role in MTH1 activity, after scavenging the basal ROS present in U87 cells, still a significant upregulation was found in mIDH1 expressed and 2-HG treated cells. This shows that the ROS generated by mIDH1 have a defining role in the activation of MTH1. More studies have to be done in order to know whether mIDH1 patients showing MTH1 upregulation via any other mechanism.

There are only a few studies relating MTH1 and glioma (Iida et al., 2001; Pudelko et al., 2017; Tu et al., 2016; Versano et al., 2018). Although the reliance of glioma cells on the deeds of MTH1 for tumorigenesis is *fait accompli* (Tu et al., 2016; Versano et al., 2018), the concept that MTH1-mediated removal of oxidized nucleotides is essential for glioma cell survival faces disputes by performing some gene silencing and proteomic studies (Kawamura et al., 2016; Prada et al., 2017; Zhou et al., 2019). Nevertheless, targeting MTH1 and using it as a therapeutic target in combination with other DNA damage

inducing drugs or along with radiotherapy holds the attention of scientific community to a certain extent due to its elevated expression in some difficult to cure tumors. There was a report in 2014 that an upregulation in BER enzyme OGG1 was observed when MTH1 was silenced (Ke et al., 2014). Similarly, we observed MTH1 silencing showed an upregulation of BER proteins (XRCC1 and OGG1) and this indicates DNA damage in both the cell lines. Enhanced BER protein expression can be one of the reasons of TMZ resistance in gliomas (Lee, 2016), because majority (almost 80%) of the TMZ lesions are the substrates of BER proteins and those lesions can be easily repaired. Hence, the combination of TMZ with BER inhibitors could be a promising therapeutic strategy against gliomas (Goellner et al., 2011; Zhang et al., 2012). Silencing of MTH1 in U251 cells showed an increased expression of phospho-γH2AX. Phosphorylation of this histone protein helps in the recruitment of DNA damage repair proteins. This was further augmented when the silencing was accompanied by ROS exposure. Thus, this shows an increased DNA damage in the absence of MTH1 in glioma cells. This suggests that the inactivation of MTH1 can lead to cell death in glioma. This also suggests that MTH1 is essential for stress management of glioma under high redox pressure as previously proposed (Bräutigam et al., 2016; Pudelko et al., 2017). Interestingly, while investigating the association between mIDH1 and MTH1, we came across a report showing that mutant IDH1 in gliomas impairs DNA repair mechanism and hence causes increased DNA damage (Lu et al., 2017). This impairment of DNA damage repair by mIDH1 might activate MTH1.

Nevertheless, some recent studies reported that MTH1 inhibition solely cannot induce DNA damage due to the insufficient incorporation of the oxidized nucleotides (Zhou et al., 2019) and assistance from DNA damage inducing drugs and/or irradiation therapies might be required for efficient induction of cancer cell death (Prada et al., 2017). This prerequisite for combinatorial therapeutic approaches while entailing MTH1 inhibitors in cancer curing regimen is supposedly due to the DNA repair mechanisms active during replication averting the redundant oxidized nucleotides from being incorporated to the DNA. One such approach being sought after is the inhibition of OGG1, the major BER protein repairing 8-oxoG, in combination with MTH1 inhibitors (Michel et al., 2019). Recently, MTH1 inhibition along with histone deacetylase (HDAC) and PARP

inhibitions were found to enhance the GBM's response to treatment with drugs and radiation (Versano et al., 2018).

In order to examine whether the MTH1 silencing-induced DNA damage response were wielding any apoptotic signals, cell death analyses were performed. MTH1 silencing resulted in an upregulation of an apoptotic marker; Cleaved PARP on silencing MTH1 indicates an increased apoptosis in MTH1-knockdown cells. PARP is a cell survival factor which is essential for recognizing DNA damage and helps recruit DNA damage repair proteins to the site of damage. In the presence of PARP inhibitor, recruitment of BER proteins is prevented which leads to DNA damage and hence kills cancer cells (Chen, 2011; Horton et al., 2014; Lawrence et al., 2015). Since PARP has an important role in BER, PARP inhibitor along with TMZ may improve the response of glioma to TMZ. In case of mutant IDH1, it promotes apoptosis were previously reported in numerous studies (Li et al., 2015; Yao et al., 2018; Zhang et al., 2019). Apart from this, a very interesting fact is that IDH1 mutant tumors are more vulnerable to PARP inhibition because of the homologous recombination (HR) defect seen in mIDH1 tumors caused by 2-HG (Wang et al., 2020). HR is a mechanism of double strand DNA break repairs; the defect in this alone with the single strand break repair mediator PARP inhibition makes the mIDH1 tumors more sensitive to treatment (Sulkowski et al., 2017). The extent of PARP cleavage found in MTH1 silenced cells is in concordance with the results of cell death analyses on MTH1 inhibited cells. Further, the downregulation of invasion and migration markers in MTH1 silenced cells points to the vital role of MTH1 during tumor progression and metastasis in glioma cells. Thus we further analyzed the levels of some of the aforesaid markers in human brain tumor biopsies vis-à-vis MTH1 expression. Correlation analyses of Cleaved-PARP, XRCC1, MMP9 and RAC1 protein levels with respect to the MTH1 expression in glioma tissues substantiated the above data obtained from MTH1 silencing experiments. Proteins essential for migration and invasion, RAC1 and MMP9 were showing a positive correlation with MTH1 in patient samples. Our observation regarding the role of mIDH1 in glioma cell migration goes in line with the earlier report that mIDH1 carrying U87 cells have higher ability of cell migration than the wild-type U87 cells (WANG et al., 2014). It is also understood from the current study that both mIDH1 and MTH1 favors glioma cell migration.

MTH1 has a higher preference to 8-oxo-dGTP when compared to MTH2 which does not involve much in sanitation of oxidized forms of nucleotides (Carter et al., 2015). The 8-oxo-dGTP in the cells gets converted into 8-oxo-dG primarily through the action of MTH1. The 8-oxo-dG levels indirectly reflect the MTH1 activity although it does not account for the actual incorporation of 8-oxo-dGTP to the DNA. MTH1 has high affinity for substrate 8-oxo-dGTP (McLennan, 2006), hence its absence could result in DNA damage. Our results indicate that the MTH1 activity is significant in glioma cells in the presence of both high as wells as basal ROS level, but high level of oxidative stress turn them addictive to MTH1. Diminished 8-oxo-dG levels were found in the TH588 treated U87 cells which implies that effective inhibition of MTH1 activity caused by the inhibitor may increase the propensity of the oxidized nucleotides being incorporated into the DNA. So, it was considered that MTH1 inhibition under high ROS environment (H_2O_2 treatment) may lead to excess accumulation of oxidized dNTPs (especially 8-oxo-dGTP) and comparatively lesser amount of the neutralized product 8-oxo-dG to be formed *versus* the H_2O_2 alone treated cells. Nevertheless, TH588 treated cells when exposed to H_2O_2 produced 8-oxo-dG to levels comparable with the H_2O_2 alone group indicating an inefficient inhibition of MTH1. The inability of TH588 to reduce 8-oxo-dG levels in H_2O_2 treated cells may be due to its competitive mode of inhibition, where the inhibitor gets displaced from the active site of the MTH1 in the presence of high concentrations of oxidized dNTPs. There could be other enzymes that have the same property of MTH1 which can act on 8-oxo-dGTP and produce 8-oxo-dG in the presence of high ROS environment. This may be the reason why the addition of MTH1 inhibitor in the presence of peroxide failed to reduce 8-oxo-dG production. At the same time, samples from patients with IDH1 mutation were also showing an increased 8-oxodG level indicating relatively higher MTH1 activity than wildtype samples.

Another aspect yet to be uncovered is the molecular signals in response to high concentrations of oxidized nucleotides that upregulate MTH1. Hif1α is a known mediator of tumorigenic responses mainly under hypoxic condition, though non-canonical Hif1α activation pathways do exist under normoxia. A study showed that Hif1α modulates MTH1 expression in colorectal cancer (Qiu et al., 2015). Consistent with this, we have also observed the similar result in glioma. In addition, our patient data shows that Hif1α

has correlation with MTH1 expression. When Hif1α was silenced in glioma cell lines, we found an inhibition in the MTH1 expression showing that Hif1α regulates MTH1 expression. Also on inducing Hif1α with $CoCl_2$, MTH1 was found to be upregulated in glioma cells as reported previously (Cheng et al., 2017; Okail, 2010). The actual mechanism by which mIDH1 is involved in gliomagenesis is yet to be elucidated. But there are growing evidence that mIDH1 upregulates Hif1α (Huang et al., 2019; Zhao et al., 2009b) and further, Hif1α activates several other genes that are involved in multiple cellular processes, including cell survival, cell proliferation and angiogenesis, mainly via VEGF (Wang et al., 2014). Similar to this, the present study demonstrated the upregulation of Hif1α and VEGF in mIDH1 expressed U87 cells. Importantly, we also found similar upregulation in Hif1α and VEGF markers in 2-HG treated U87 cells indicating that it could be the mIDH1 product 2-HG that is responsible for the tumorigenic properties shown by mIDH1. Furthermore, the downregulation of VEGF and its receptor in MTH1 silenced cells points to the key role of MTH1 during angiogenesis in glioma cells. Further analysis of VEGF levels in human glioma biopsies goes in line with the data obtained from *in vitro* silencing experiments. Further studies are required to find the molecular mechanism by which Hif1α modulates MTH1 and how MTH1 silencing affects VEGF expression.

No promising and long lasting therapy targeting against the most malignant grade IV gliomas without causing any harm to the neighboring normal cells of the brain have been yet developed. mIDH1 is a positive prognostic factor for glioma patients, but at the same time, its product 2-HG contributes to tumorigenesis. It is hitherto unclear as to how and what factors contribute to gliomagenesis in IDH1 mutant tumor. Why a mutation that phenotypically favors tumorigenesis, has positive effect on the survival of glioma patients than the wild-type IDH patients still intrigues the scientific community (Bhavya et al., 2020).

On a concluding note, our results show that MTH1 silencing affects the cell migration, downregulates the expression of invasion as well as angiogenesis markers. As per our results it is verified that the MTH1 expression is not dependent on the glioma malignancy or grade. In addition, we have observed that MTH1 may modulate Hif1α expression in

gliomas even though the signaling pathways involved are not known. Hif1α accumulation can lead to the increase or decrease in the ROS levels in cells. We observed that the ROS levels are diminished on silencing Hif1α. For the activation of MTH1, a high ROS environment is necessary. This could be a reason that MTH1 gets inhibited on silencing Hif1α. More experiments are required in order to understand the relation between Hif1α and ROS in glioma cells. This can help develop a therapeutic strategy in the treatment of gliomas. Also this study offers a novel prospective on the defining role of mIDH1 and the ROS generated by mIDH1 on the activation of MTH1.

Significance of the study

This study expands our understanding of the function of MTH1 in glioma pathobiology and also provides a hint about the novel mechanism of MTH1 modulation by Hif1α in glioma. By establishing that mIDH1 activates MTH1 via ROS, the current study provides a fresh prototype to use a combinatorial therapeutic module incorporating inhibitors of MTH1 and mIDH1 against glioma.

Limitations and future directions of the study

Although the current study succeeded in establishing that the ROS generated via mutant IDH1 activates MTH1, the findings are from an *in vitro* cell culture models. Future investigations must be performed in an *in vivo* model of glioma and to look into the effect of tumor microenvironment on MTH1 activation in mIDH1 carrying glioma patients. In addition, *in vivo* studies can test out the systemic effects of a combinatorial treatment with MTH1 and mIDH1 inhibitors with proper controls. The experiments performed in glioblastoma cell lines were not compared with the normal human astrocytes.

VI. Summary and Conclusion

MutT Homolog 1 is upregulated in glioma patient samples than the non-tumor brain tissues. The expression and activity of MTH1 is independent of the glioma grades and malignancy. It was found to be involved in glioma cell survival, migration and angiogenesis. Also the markers of invasion were found to be downregulated when MTH1 was silenced in cell lines. HIf1α was found to be modulating MTH1 expression in cell lines. The role of mutant IDH1 in glioma cell migration and tumorigenesis were investigated and found that mIDH1 favors cell migration in glioma. 2-Hydroxyglutarate, the product of mIDH1 was found to be contributing to the ROS produced by mIDH1 in glioma cells. MTH1 was found to be upregulated in mIDH1 expressed and 2-HG treated cells. mIDH1/2-HG also have role in gliomagenesis through upregulating Hif1α and VEGF levels when compared to the wild-type IDH1 cells. mIDH1 harboring glioma patient samples were showing increased MTH1 expression than the patients with wild-type IDH1. The 8-oxo-dG levels were found to be significantly high in patients with mIDH1 than those with wild-type IDH1 indicating MTH1 activity. Increased MTH1 expression was seen in mIDH1 expressed and 2-HG treated cell lines. It could be via the ROS generated by mIDH1/2-HG that MTH1 gets it activated in mIDH1 harboring gliomas. In conclusion, this study displays the role of MTH1 and also shows that Hif1α regulates MTH1 in glioma. Notably, the novel finding of the contribution of ROS generated by mIDH1 to MTH1 activation proposes a prototype therapeutic regimen for glioma.

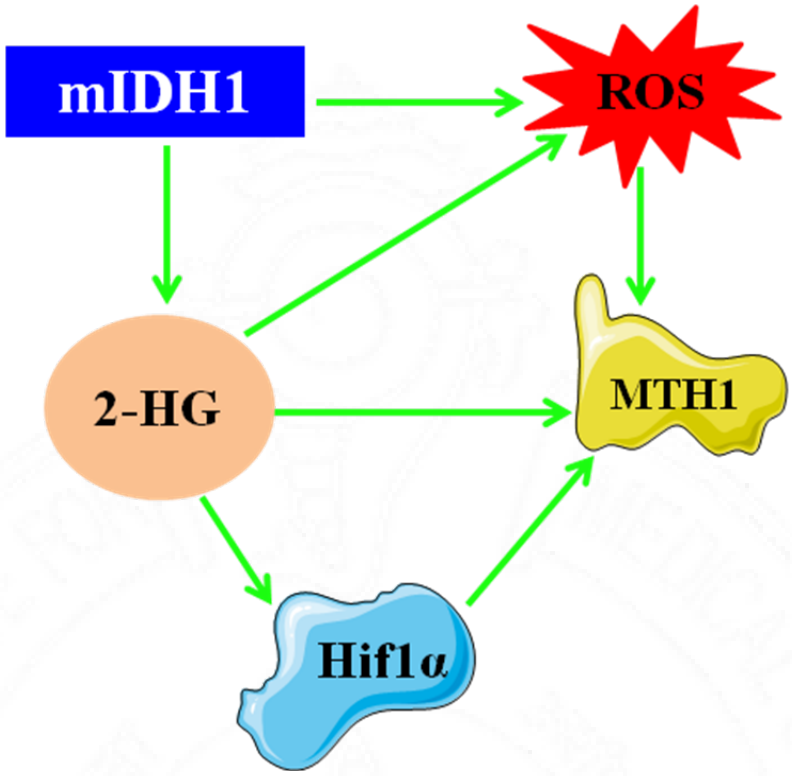


Fig.VI.1 Summary of the major observations in the study

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VIII. List of Publications and Abstracts

1) ***Bhavya Bharathan****, HV Easwer, GC Vilanilam, CR Anand, K Sreelakshmi, Madhusoodanan Urulangodi, P Rajalakshmi, Issac Neena, CJ Padmakrishnan, Girish R Menon, K Krishnakumar, AN Deepti, Srinivas Gopala;* MutT Homolog1 has multifaceted role in glioma and is under the apparent orchestration by Hypoxia Inducible Factor1α; **Life Sciences (2020);** https://doi.org/10.1016/j.lfs.2020.118673.

2) *CR Anand,* ***Bhavya Bharathan****, K Jayakumar, VS Harikrishnan, Srinivas Gopala;* Inorganic nitrite alters mitochondrial dynamics without overt changes in cell death and mitochondrial respiration in cardiomyoblasts under hyperglycemia; ***Toxicology in vitro*** **(2020)**; https://doi.org/10.1016/j.tiv.2020.105048.

3) *C Jayakrishnan P, H Venkat E, M Ramachandran G, K Kesavapisharady K, N Nair S,* ***Bharathan B****, Radhakrishnan N, Gopala S;* In vitro neurosphere formation correlates with poor survival in glioma; **IUBMB Life (2019);** 71(2):244-253.

4) ***Bharathan Bhavya,*** *C.R. Anand, U.K. Madhusoodanan, P. Rajalakshmi, K. Krishnakumar, H.V. Easwer, A.N. Deepti, Srinivas Gopala;* To be Wild or Mutant: Role of Isocitrate Dehydrogenase 1 (IDH1) and 2 Hydroxy Glutarate (2 HG) in Gliomagenesis and Treatment Outcome in Glioma; **Cellular and Molecular Neurobiology (2020);** 40(1):53-6.

Poster Presentations

1) MTH1 Expression in Different Grades of Glioma: Action of MTH1 inhibitor TH588; Bhavya Bharathan, Easwer HV, Sandhyamani S, Srinivas Gopala at "Recent Biochemical Approaches in Therapeutics-IV" on January 24, 2018, University of Kerala, Trivandrum (International Conference).

2) Activation of Mut T Homologue 1 in Glioma: Is there a Link with IDH Mutation?; Bhavya Bharathan, Easwer HV, Sandhyamani S, Srinivas Gopala at 37th Annual

Conference of Indian Association for Cancer Research on February 24, 2018, Bose Institute, Kolkata (International Conference).

Oral Presentation

3) Activation of MutT Homologue 1 in Glioma: The Defining Role of Isocitrate Dehydrogenase and Reactive Oxygen Species; Bhavya Bharathan, Easwer HV, Srinivas Gopala at "39th Annual Conference of Indian Association for Cancer Research" on February 5, 2020, Kovalam, Trivandrum (International Conference).

IX. Annexure

Document Information

Analyzed document	Activation of MTH1 in Glioma The Defining Role of IDH and ROS Bhavya Bharathan.pdf (D75544659)
Submitted	6/24/2020 8:05:00 AM
Submitted by	Harikrishnan VS
Submitter email	harikrishnan@sctimst.ac.in
Similarity	0%
Analysis address	harikrishnan.sctims@analysis.urkund.com

Sources included in the report

W	URL: http://n2t.net/addgene:62907 Fetched: 6/24/2020 8:07:00 AM	1
W	URL: https://www.spandidos-publications.com/10.3892/or.2013.2669?text=fulltext Fetched: 6/24/2020 8:07:00 AM	1