
RESEARCH ARTICLE

IDH1 Overexpression Induced Chemotherapy Resistance and IDH1 Mutation Enhanced Chemotherapy Sensitivity in Glioma Cells in Vitro and in Vivo

Ju-Bo Wang¹, Dan-Feng Dong², Mao-De Wang¹*, Ke Gao¹

Abstract

Isocitrate dehydrogenase (IDH) is of great importance in cell metabolism and energy conversion. IDH mutation in glioma cells is reported to be associated with an increased overall survival. However, effects biological behavior of therapy of gliomas are unclear. Here, we investigated the influence of wild-type and mutated IDH genes on glioma cell biological behavior and response to chemotherapy. Relevant mechanisms were further explored. We designed our study on the background of the IDHR132H mutation. Stable cell lines were constructed by transfection. The CCK-8 method was used to assess cell proliferation, flow cytometry for the cell cycle and cell apoptosis, and the transwell method for cell invasion. Nude mouse models were employed to determine tumorigenesis and sensitivity to chemotherapy. Western blotting was used to detect relevant protein expression levels. We found that overexpression of wild IDH1 gene did not cause changes in the cell cycle, apoptosis and invasion ability. However, it resulted in chemotherapy resistance to a high dose of temozolomide (TMZ) in vivo and in vitro. The IDH1 mutation caused cell cycle arrest in G1 stage and a reduction of proliferation and invasion ability, while raising sensitivity to chemotherapy. This may provide an explanation for the better prognosis of IDH1 mutated glioma patients and the relative worse prognosis of their wild-type IDH1 counterparts. We also expect IDH1 mutations may be optimized as new targets to improve the prognosis of glioma patients.

Keywords: Isocitrate dehydrogenase 1 - cell cycle - proliferation - invasion - apoptosis - glioma - chemotherapy

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Introduction

Gliomas are the most frequent and most devastating brain malignancies. It makes up 45% of primary brain tumors in Chinese (Yu et al., 2008). Glioblastomas (GBMs) are by far the most aggressive and infiltrating type, accounting for more than half of all gliomas (CBTRUS, 2010). Despite of aggressive methods of surgery, radiotherapy and chemotherapy, GBMs are invariably associated with unavoidable tumor recurrence and overall poor prognosis. The overall survival of patients with GBMs is only 12-15 months (Stupp et al., 2005; Wen et al., 2008; Buonerba et al., 2011; Sherman et al., 2011; Babu et al., 2012). Prognosis is even poorer in elderly GBM patients. Patients usually face different prognosis, even in the same pathological grade of glioma. Parsons et al. reported that IDH1 mutation occurred in a wide frame of glioma patients [Parsons et al., 2008]. IDH1 mutation was reported to be associated with an increased overall survival (Parsons et al., 2008; Nobusawa et al., 2009; Yan et al., 2009) More effective treatment is expected to be explored by the finding of IDH1 mutation (Turcan et al., 2012; BaldehPersad et.al., 2013). TMZ is now the most common anti-tumor drug used in glioma patients and often works as the basic chemotherapy drug in scientific researches on glioma (Li et al., 2013). We hope to find any biological or chemotherapy sensitivity differences caused by IDH1 mutation and also want to reveal the reason for the different prognosis between the glioma subtypes. This may provide clinicians with a more comprehensive understanding of the IDH1 gene and the IDH1 mutation event and provide us a reason for using IDH1 mutation as a chemotherapy sensitization target or an anti-tumor target by IDH1 interference.

Materials and Methods

Cells and reagents

U87 cell line was kindly donated to us by Doctor Liang of our department. Dulbecco's Modified Eagles Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco. Human malignant glioma cell line U87 and the following stable cell lines were maintained in DMEM medium of high glucose (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 2 mM glutamine (Sigma, USA) and antibiotics (penicillin, 100 U/mL and streptomycin, 100 μg/mL; China), in 5% CO₂ atmosphere at 37°C. Cells were collected by trypsin method. Nude

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mice were bought from the Chinese Academy of Sciences Animal Center. TMZ was donated to us by the Beijing Tian-shili company. Anti-CDC2, Anti-caspase-3, Anti-caspase-9, Anti-Bax, Anti-Bcl-2 monoclonal antibodies were purchased from Signalway. Anti-MMP-2 and Anti-MMP-9 monoclonal antibody were purchased from Bioworld. Horseradish peroxidase-conjugated 2nd antibodies were purchased from Zhong-shan Jinqiao (Beijing, China). Lipofectamine 2000 reagent was purchased from Invitrogen (Carlshad, CA). All the chemicals not included above were from Sigma.

Construction of vectors and stable cell lines

Vectors carrying wild IDH1 gene (wIDH1) or mutated IDH1 gene (mIDH1) were constructed by DNA recombination technology. The stable cell lines on the base of U87 cell line carrying wIDH1, mIDH1 or the empty p-EGFP-C1 vectors were separately constructed by transfection method and were finally selected by G418 selection method.

Cell cycle analysis

Cells were collected by trypsin method, washed with PBS, fixed in 70% ethanol and kept at −20°C for 24 h. They were then washed in cold PBS and resuspended in 50 μg/mL propidium iodide and RNase-DNase free (10 μg/mL). The cell suspension was incubated for 30 min at room temperature and cell cycle distribution was determined by flow cytometry with Cell-Quest software analysis.

Cell proliferation and viability assay

To detect the proliferation ability of the three stable cell lines, trypsin digestion and cell counting method was used. Briefly, cells of the same number were cultured at routine condition for 48 h, and were then collected by trypsin method and counted on a cell counting plate. The proliferation rate was calculated by ratio of the final cell number with the initial cell number.

The detection of the proliferation inhibition rate was based on the CCK-8 method. Cells were seeded in a 96-well plate at a density of 2×10⁴ cells/mL (100 μl/well). They were co-cultured with different doses of TMZ and were allowed to grow for 48 h and the zero dose cells were allowed to grow for 48 h as a corresponding control group. At the end of the experiment, the media was added with PBS, fixed in 70% ethanol and kept at −20°C for 24 h. After 36 h, the cells that had migrated through the matrigel and the 8 μm pore size membranes were fixed, stained and counted under a light microscope. The wells without TMZ were used as control groups. The wells with TMZ were worked as treated groups respectively. Cell numbers in each perspective of the control and the treated groups were calculated under the inverted microscope. Values for invasion inhibition rate were obtained by (1-numberTMZ/numbercontrol) ×100% (mean ± SD).

Western blot analysis

Cells were lysed in ice-cold RIPA lysis buffer. The protein concentration was estimated using the Bio-Rad protein assay according to the manufacturer’s protocol. Cell lysates were separated by SDS-PAGE and transferred onto PVDF membranes. Blots were blocked in TBS buffer containing 5% non-fat dried milk for 1 h at room temperature. The membranes were then incubated overnight at 4°C with the 1st antibody. Membranes were then washed and incubated with horseradish peroxidase conjugated 2nd antibody for 1 h at room temperature before washes. The membranes were then adequately washed with tris-buffered saline containing Tween20 after each treatment with antibody. Detection of antibody binding was performed by enhanced chemiluminescence according to the manufacturer’s instructions. Data collection and processing was performed using a luminescent image analyzer. The same blot was stripped and reprobed with anti-β-actin or anti-GAPDH for use as an internal control.

Construction of nude mice models and followed procedure

30 male mice were randomly divided into 3 groups and were separately injected with mIDH1 glioma cells, wIDH1 glioma cells and empty control vector glioma cells (5×10⁶ cells for each) under the back skin of the nude mice. The tumor models were allowed to grow for 21 d. The tumor sizes were measured as wide (a) and length (b). The tumor size were calculated by the function V (mm³) = π (a²b)/6. A second 30 male nude mice were treated by cell injection separately and were treated with TMZ therapy by gastric gavage for 5 d according to the surface areas of the nude mice. The surface area is calculated according to the weight by the function S (m²) = 0.066 × (KG)²/3. The Tumor formation rates were finally calculated.

A third 30 male nude mice were treated with cell injection separately and feeding for 14 d. The nude mice were then treated with TMZ by gastric gavage for 5 d. The followed observation period lasted for 21 d. The final tumor sizes were calculated and the total chemotherapy efficient rates were calculated. The tumor models were used for western blot analysis.

Statistical analysis

The relative variation rate was statistically analyzed
Table 1. Cell Cycle Detection in Glioma Cells of the wIDH1, mIDH1 and Control Groups

<table>
<thead>
<tr>
<th>Stage</th>
<th>wIDH1</th>
<th>Control</th>
<th>mIDH1</th>
</tr>
</thead>
<tbody>
<tr>
<td>G&lt;sub&gt;0&lt;/sub&gt;</td>
<td>(48.57 ± 4.15)%</td>
<td>(50.17 ± 4.48)%</td>
<td>(65.67 ± 4.65)%</td>
</tr>
<tr>
<td>S</td>
<td>(28.68±3.51)%</td>
<td>(31.04±3.75)%</td>
<td>(26.75±3.48)%</td>
</tr>
<tr>
<td>G&lt;sub&gt;0&lt;/sub&gt;/M</td>
<td>(22.74±3.35)%</td>
<td>(18.81±2.95)%</td>
<td>(7.57% ± 1.97)%</td>
</tr>
</tbody>
</table>

Figure 1. Comparison of Glioma Cells in Vitro (A) and in Vivo (B). A: The mIDH1 glioma cells had a higher proliferation rate than the glioma cells of the wIDH1 group and the control group (P<0.05), there was no significant difference between the wIDH1 group and the control group of glioma cells. B: The tumor volume of the mIDH1 group was smaller than that of the wIDH1 group and the control group (P<0.05), there was no significant difference between the wIDH1 group and the control group of glioma volume by SPSS 16.0 software. Data was presented as (mean ± SD). Comparison between groups was valued by variance (One-Way ANOVA), SNK-q test method was used to describe the difference between each group in multiple groups. P < 0.05 was considered statistically different.

Results

Cell cycle detection by flow cytometry

To detect the cell cycle of the stable cell lines, flow cytometry analysis was used. We found that mIDH1 arrested cell cycle in G<sub>0</sub> stage and reduced the G<sub>0</sub>/M population, while over expression of wIDH1 affected little to the cell cycle compare with the control group. As shown in Table 1, mIDH1 group got a higher G<sub>0</sub> stage (65.67% ± 4.65%) under routine condition. It tended to be arrested in the G<sub>0</sub> stage and reduced G<sub>0</sub>/M stage proportion (7.57% ± 1.97%) compared with the wild IDH1 and the control groups (P<0.05). But the wIDH1 group (48.57% ± 4.15%) showed fuzzy difference with the control group (50.17% ± 4.48%) in G<sub>0</sub> stage (P<0.05). When treated with TMZ of different dose, the cell cycle of the stable cell lines changed little which was not list in the table. It was because TMZ was not a cell cycle depended anti-tumor drug.

mIDH1 delayed cell proliferation.

Cells that cultured for 48 h at routine condition were counted. The proliferation rates were shown in Figure 1A. Over expression of wIDH1 didn’t raise cell proliferation compared with the control group, while the ectopic expression of mIDH1 did inhibit cell proliferation. The mIDH1 group got the lowest proliferation rate (150±4.3%) and was significant different with the other two groups (P<0.05). The wIDH1 group (189±2.4%) showed little difference with the control group (185±2.9%) (P<0.05). In vivo study showed that all injection accepted mice finally formed glioma models. Beyond of these, the size of each glioma mass was measured at the end of the 21st day. The size differences of the three groups were also analyzed (Figure 1B). We found the proliferation result was similar with that in vitro. The mIDH1 tumors grew much slower than the wild IDH1 group and the control group. The glioma size analysis showed that the mIDH1 over expression group display lowest tumor volume in size compared with the rest two groups (P<0.05). The wIDH1 over expression group showed no significant difference with the control group (P<0.05).

wIDH1 displayed different proliferation inhibition response by TMZ with mIDH1

Cell proliferation inhibition rates among the three groups were affected by TMZ, and the difference among the three groups was little at a dose of no more than 75 μg/mL Figure 2A. But the result changed when cells were treated with TMZ of a high dose of 100 μg/mL. The wIDH1 group showed the lowest inhibition rate (P<0.05) and the mIDH1 group showed the highest inhibition rate in vitro (P<0.05).

wIDH1 over expression displayed chemotherapy resistance and mIDH1 enhanced chemotherapy sensitivity in vitro

When cells were cultured for 48 h with different dose of TMZ, Annexin V/RFP staining method was applied for flow cytometry apoptosis analysis. We confirmed that there was no significant apoptosis difference at routine culture condition among the three cell lines. Even at low doses of TMZ, there was still no significant difference among the three groups. But the result was different when cells were cultured with TMZ higher than 75 μg/mL. As is shown in Figure 2B, when treated with 75 μg/
Table 2. Invasion Ability Detection among the Three Groups by Transwell Method

<table>
<thead>
<tr>
<th>Groups</th>
<th>Invasion cells</th>
<th>Inhibition rate of invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>mIDH1</td>
<td>28.3 ± 4.4</td>
<td>84.50%</td>
</tr>
<tr>
<td>Control</td>
<td>54.2 ± 6.6</td>
<td>71.60%</td>
</tr>
<tr>
<td>wIDH1</td>
<td>56.4 ± 7.6</td>
<td>64.90%</td>
</tr>
</tbody>
</table>

mL of TMZ, mIDH1 group displayed a higher apoptosis rate (22.4% ±2.7%) than the other two groups (P<0.01). The wIDH1 group (18.0% ±2.2%) showed no difference with the control group (17.9% ±2.3%). When cells were treated with TMZ of 100 μg/mL, the mutated IDH1 group got the highest apoptosis rate (38.5% ±3.1%) among the three groups and was significant different with the rest two groups (P<0.05). The wIDH1 group had the lowest apoptosis rate (24.8% ±2.4%). It was different with the control group (28.8% ±2.5%) (P<0.05). Over expression of wIDH1 seemed to play a protective role in chemotherapy procedure on glioma cells, while mIDH1 enhanced this procedure oppositely.

In vivo study showed that when the nude mice were treated with injection of glioma cell cells and TMZ gastric gavage at the same time, the wIDH1 over expression group had lowest tumor formation inhibition rate (40%), while the mIDH1 over expression group had the highest tumor formation inhibition rate (80%) and the control group was 50%. Even in the tumor formatted mice, the sizes were also of different. The tumor sizes of the mIDH1 mice were much smaller than that of the wIDH1 group. In the TMZ therapy groups, the total effective rate in mutated IDH1 over expression group was 70%, the total effective rate of the wIDH1 over expression group was 20%. The result in the control group was 40%. These results indicated that over expression of wIDH1 might provide chemotherapy resistance in vivo while mIDH1 might raise chemotherapy sensitivity.

Over expression of wIDH1 increased invasion while mIDH1 enhanced the reduction of invasion by TMZ.

Transwell assay was used to detect the effects on invasion ability by the aimed genes under serum chemotaxis. As shown in Table 2, the mIDH1 group got the least invasion cells for each microscopic field, about (28.3 ± 4.4) cells per microscopic view, obviously lower than the other two groups (P<0.01). The wIDH1 group’s was about (56.4 ± 7.6) cells per microscopic view and displayed few difference (P>0.05) with the control group’s (54.2 ± 6.6). That meant mIDH1 reduced cell invasion. When treated with TMZ (100 μg/mL), the invasion cell number was (4.4 ± 2.4) for each view, the invasion cells were reduced by 84.5%. The number of the invasion cells in the wIDH1 group was (19.8 ± 4.2) and was reduced by 64.9%. The result in the control group was (15.4 ± 3.2) with a reduction of 71.6%. The inhibition rates of invasion in the three groups were significantly different with each other (P<0.05).

Changes of molecules involved in cell cycle, invasion and apoptosis

To investigate the potential mechanisms involved in cell cycle, cell invasion and cell apoptosis induced by mIDH1, wIDH1 and TMZ, western blot was applied to analyze protein changes of the related molecules.

As shown in Figure 3A, cell lysates of normal condition were supplied for western blot analysis. The expression level of CDC2 in mIDH1 glioma cells was lower than the other two group by gray scanning analysis (P<0.05). There was no significant difference between the wIDH1 glioma cell group and the control glioma cell group.

The expression levels of MMP-2 and MMP-9 were lowest in the mIDH1 glioma group (P<0.05), while the wIDH1 group showed little difference with the control group. The expression levels of MMP-2 and MMP-9 in glioma tissue were also detected. We could find from Figure5 that the expression levels of MMP-2 and MMP-9 in mIDH1 glioma tissue were lowest (P<0.05). The tissue of the control group seemed to have higher expression levels of MMP-2 and MMP-9 than the wIDH1 group glioma tissue.

As shown in Figure 3B, when glioma cells were treated with 100 μg/mL of TMZ, the wIDH1 over expression glioma cells showed lowest inhibition rate in MMP-2 and MMP-9 expression levels. It was significant different with the other two group by the ratio of MMP-2 or MMP-9 with β-actin according the gray scale scanning results (P<0.05). The mIDH1 glioma cell group showed highest inhibition rate in MMP-2 and MMP-9 (P<0.05). Since there was no
significant difference in apoptosis among the three glioma cell groups under routine condition, the TMZ treated cells but the cells under routine culture condition were provided for the detection of apoptosis related molecules by western blot analysis (Figure 3B). wIDH1 glioma cell group induced highest Bcl-2 expression level and lowest Bax and Caspase-3 expression levels (P<0.05) and the mIDH1 gliom cells induced lowest Bcl-2 expression level and highest Bax and Caspase-3 levels (P<0.05) when treated with TMZ of 100 μg/mL.

Tumor models treated with TMZ therapy (chemotherapy effective models) were also provided for western blot analysis (Figure 3C). The study in vivo showed the MMP-2 and MMP-9 expression levels in the wIDH1 glioma tissue were highest among the three groups. They were significantly higher than the control group and the mIDH1 group. The MMP-2 and MMP-9 expression levels were still lowest in the mIDH1 group. The differences became more significantly by the gray scale scanning result compared with the result in cell lysates. The mIDH1 group still had the highest expression levels in Bax, Caspase-3 and the lowest expression level in Bcl-2 (P<0.05). The wIDH1 group had the lowest expression levels in Bax, Caspase-3 and the highest expression level in Bcl-2 (P<0.05).

To summarize, mIDH1 induced cell cycle arrest in G1 stage. It also induced much lower invasion ability by down regulating the MMP-2 and the MMP-9 levels in vivo and in vitro. mIDH1 didn’t increase cell apoptosis rate and wIDH1 over expression didn’t reduce apoptosis rate either at routine condition, but wIDH1 over expression lead to chemotherapy resistance in TMZ induced cytotoxic effect and invasion inhibition. The ectopic over expression of wIDH1 displayed the chemotherapy resistance characteristic or at least induced up regulation of the Bcl-2 expression level and the down regulation of the Bax and Caspase-3 levels. It meant that wIDH1 might play a supplementary role in chemotherapy resistance. mIDH1 enhanced chemotherapy sensitivity on glioma cells by down regulating the Bcl-2 level and up regulating the Bax, Caspase-3 levels in vivo and in vitro on the contrary which meant that mIDH1 might work as a chemotherapy enhanced target.

Discussion

AIDH plays an important role in cell substance and energy metabolism. The IDH gene encodes three IDH enzymes. Among which, IDH1 is present in the cytoplasm and in peroxisomes. It contributes to the NADPH pool which is of great important in keeping the oxidation-reduction balance. mIDH1 fails to finish the conversion of isocitrate to a-ketoglutarate (a-KG) and causes 2-hydroxyglutarate accumulation. It consumes rather than produces NADPH (Dang et al., 2009). It may thus fail to maintain the balance of oxidation-reduction. It may raise the risk of tumorigenesis that is to say when NADPH is consumed or exhausted, the cellular oxidative metabolism disorder appears, leading to tumor development. That may be the reason why glioblastoma occurred frequently in elder people. But it is does not meet the relative better prognosis compared with the wild IDH1 glioma carriers. The existence of IDH1 mutation in glioma patients means that IDH1 mutation is not a lethal factor. It just changes the cell metabolic pathway.

In this study, we found that mIDH1 displayed different in biological behaviors and chemotherapy sensitivity. We demonstrated here that mIDH1 caused proliferation, invasion progression inhibition and chemotherapy sensitivity. It enhanced the inhibition of cell proliferation, cell invasion and cell apoptosis caused by TMZ. wIDH1 over expression didn’t promote proliferation and invasion progression, but it displayed chemotherapy resistance characteristic in TMZ induced invasion and apoptosis inhibition. Due to these differences, glioma may be divided into two subtypes which existed obvious different prognosis (Nobusawa et al., 2009; Parsons et al., 2009; Lewandowska et al., 2013). It is important to know these differences and explore the mechanisms of the relative better prognosis induced by mIDH1.

The prognosis of tumor patients depends on two factors. They are the tumor cells themselves and the treatment done to it. The former is a multiple factors involved event including cell cycle, cell invasion ability, cell apoptosis et al. The latter includes surgery, chemotherapy and radiotherapy which are now the standard treatment on newly diagnostic glioma patients (Combs et al., 2005). Cell cycle control is realized by checkpoint controlment via cell cycle regulatory proteins such as cyclins and cdkks. It is an important means of inhibiting cancer cell growth and division (Chen et al., 2002). CDC2 is known as cell cycle engineer and has been reported to be associated with cell cycle, cell proliferation and the outcome of patients (Jansen et al., 2012; Que et al., 2013).

In cell cycle detection, we found that mIDH1 arrest cells in G1 stage of cell cycle, and we confirmed that came from the CDC2 down regulation. mIDH1 leads to cell metabolic changes that can’t provide more energy for phosphorylation and thus delays the progression from G1 to S stage. Down regulation of CDC2 level may further lead to H1 histone and nuclear lamins phosphorylation deficiency and hinder the mitotic spindle formation and finally cause cell cycle arrest in G1 stage. The block of cell cycle further inhibits the cell proliferation. What’s more, the inhibition effect is further enhanced by TMZ. It means that mIDH1 cells are easier to be hurt by TMZ induced cytotoxic effect. But the over expression of wIDH1 caused little change in cell cycle.

Invasion ability is another reason for shortening survival. Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes. Much evidence has linked MMPs to aggressive, malignant behavior, and poor outcome in various cancer cells, including prostate cancer, breast cancer, lung cancer et al. They remodel the extracellular matrix and increase the invasive potential of tumor cells. The expression level is associated with tumor invasion ability and survival (Quaranta et al., 2007; Chen et al., 2012; Irena et al., 2012; Langers et al., 2012; Dai et al., 2013). Our study showed mIDH1 reduced the invasion ability of glioma cells and was further reduced by TMZ. It highlights the negative regulation of MMP-2 and MMP-9. This had been confirmed by vivo and vitro study and this
is also consistent with its’ better prognosis. The invasion ability of the wIDH1 group was affect least when treated with TMZ. In other words, wIDH1 over expression displays a characteristic of TMZ resistance in the invasion experiment.

Cell apoptosis is a programmed cell death process. It may be started by some endogenous or exogenous factors which is known as the death signal-induced death receptor mediated pathway and the mitochondrion-dependent pathway. Apoptosis is important in maintaining the homeostasis of cell numbers. Caspase-cascade is controlled by some molecules and is crucial in cell apoptosis. The ratio of Bax/Bcl-2 usually determined the cell apoptosis (Timme et al., 2013; Yang et al., 2013) and activated caspase-cascade or not. In our present study, mIDH1 and wIDH1 over expression didn’t cause apoptosis changes under routine condition. wIDH1 over expression inhibited TMZ induced apoptosis, at least in part by decreasing Bax/Bcl-2 ratio and Caspase-3 activity. It highlighted the possibility of IDH1 over expression in resistance to TMZ chemotheraphy in glioma. mIDH1 ectopic expression enhanced cell apoptosis by increasing Bax/Bcl-2 ratio and Caspase-3 activity.

According to the above results, we conclude that mIDH1 causes biological changes in glioma cells which reduced the cell malignant behavior. mIDH1 also enhances chemotheraphy sensitivity of glioma cells in vivo and in vitro while the wIDH1 displays a chemotherapy resistance characteristic. These findings well explain why there are different prognoses between glioma subtypes and provide reason for mIDH1 as an enhanced chemotheraphy sensitivity target.

Acknowledgements

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