

Mutations in isocitrate dehydrogenase 2 accelerate glioma cell migration via matrix metalloproteinase-2 and 9

Yuejun Fu · Yali Zheng · Ke Li · Rui Huang ·
Shuhua Zheng · Na An · Aihua Liang

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Abstract The gene encoding isocitrate dehydrogenase (IDH) is somatically mutated predominantly in secondary glioblastoma multiforme. Glioma-specific mutations in IDH1 always produced a single amino acid substitution at R132, but mutations in IDH2 were exclusively at R172 which was the analogous site to R132 in IDH1. Mutations of IDH1 and IDH2 led to simultaneous loss and gain of activities in the production of α -ketoglutarate and 2-hydroxyglutarate, respectively. Matrix metalloproteinases (MMPs) are zinc-dependent endoproteinases involved in the degradation of the extracellular matrix. The exact role of IDH2 mutant on MMPs activity and cell migration has not been fully studied. Here, we show that in response to IDH2 mutations, low levels of α -ketoglutarate increased the stabilization of HIF-1 α which can contribute to tumor growth. Moreover, mutant IDH2-induced HIF-1 α improved the secretion levels of pro-MMP-2 and pro-MMP-9 as well as the conversion from pro-MMP-2 to its active form, giving C6 glioma

cells a higher migration potential. The HIF-1 α pathway is probably a critical pathway for release of MMPs in the glioma cancer harboring IDH mutant.

Keywords Glioma cancer · Isocitrate dehydrogenase-2 (IDH2) · Migration · Matrix metalloproteinase-2, 9

Introduction

Gliomas are the most common type of human brain tumors and contain specific histological subtypes. The major ones among these are astrocytomas, oligodendrogliomas and ependymomas (Fu et al. 2011b). WHO has classified gliomas into four clinical grades on the basis of histology and prognosis. The grade IV glioma, known as glioblastoma multiforme (GBM), is the most prevalent and malignant primary brain tumor in adults (Ji et al. 2011). Patients with it have a median survival of less than 1 year (Badiga et al. 2011). GBM can develop either de novo (primary GBM) or through progression from low grade tumors (secondary GBM) (Zhao et al. 2009).

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endoproteinases involved in the degradation of the extracellular matrix (ECM). They play roles in normal tissue remodeling during embryonic development, angiogenesis, ovulation, mammary, gland involution and wound healing (Ranogajec et al. 2011;

Y. Fu (✉) · Y. Zheng · R. Huang · S. Zheng ·
N. An · A. Liang
Key Laboratory of Chemical Biology and Molecular
Engineering of Ministry of Education,
Institute of Biotechnology, Shanxi University,
Taiyuan 030006, People's Republic of China
e-mail: yjfu@sxu.edu.cn

K. Li
Zhejiang Entry-Exit Inspection and Quarantine Bureau,
Hangzhou 310016, People's Republic of China

Castro et al. 2011). The expression level of MMPs correlates with the invasion of cancer cells because the degradation of the ECM is essential for tumor angiogenesis, invasion, and metastasis (Gladson 1999; Shao et al. 2011; Li et al. 2011; Lee et al. 2011). Amongst many MMPs that have been identified, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) have been hypothesized to be key enzymes as they degrade type IV collagen (the main component of ECM) (Chen et al. 2011; Ranogajec et al. 2011; Berg et al. 2011). Specifically, MMP-2 is up-regulated in gliomas and related cancers, but its expression level is trivial in normal brain tissue (Deshane et al. 2003).

A recent cancer genome-sequencing project revealed that the gene encoding isocitrate dehydrogenases 1 (IDH1) was somatically mutated predominantly in secondary GBM (Fu et al. 2010; Zhao et al. 2009). However, the frequency of IDH2 mutation in these gliomas was low. The human genome has five genes coding for three distinct catalytic isozymes of IDH: IDH1, IDH2 and IDH3. IDH enzymes catalyze the oxidative decarboxylation of isocitrate (ICT) into α -ketoglutarate (α -KG); IDH1 and IDH2 are NADP⁺-dependent and IDH3 is NAD⁺-dependent. Both IDH2 and IDH3 enzymes are localized in the mitochondria, whereas IDH1 is localized in the cytoplasm and peroxisomes (Fu et al. 2011b; Zhao et al. 2009). Intriguingly, glioma-specific mutations in IDH1 always produced a single amino acid substitution at R132, whereas mutations in IDH2 were exclusively at R172. IDH1 and IDH2 mutations led to simultaneous loss and gain of activities in the production of α -KG and 2-hydroxyglutarate (2-HG), respectively. In response to IDH1 mutations, low level of α -KG resulted in increasing the stabilization of HIF-1 α which could contribute to tumor growth (Zhao et al. 2009).

In our previous study, we reported that mutations of IDH1 and IDH2 were not detected in the rat C6 gliomas cell line model, suggesting that these mutations were not required for the development of glioblastoma induced by *N,N'*-nitroso-methylurea (Fu et al. 2011b). Moreover, we concluded that the low NADPH levels could sensitize tumors to chemotherapy and account for the prolonged survival of patients harboring the mutations. These data extrapolated potential importance of the in vitro rat C6 glioma cell model and showed that the IDH2^{R172G} mutation in glioma might give a benefit to traditional

chemotherapy of this cancer. Invasion of glioma cells into adjacent brain structures occurs through the activation of multigenic programs, including MMPs which degrade ECM to overcome the ECM barrier at the invasive fronts of tumors (Giese and Westphal 1996). Although IDH2 mutations in gliomas were low (Zhao et al. 2009), the function study on IDH2 mutations was needed for the diagnosis, prognosis, and treatment of patients with these tumors. Moreover, the exact role the mutant IDH2 plays on MMPs activity and cell migration has not been fully studied yet. In the present study, the effect of IDH2^{R172G} on rat C6 glioma cells migration is analyzed at the cellular level and the role of HIF-1 α pathway in MMPs release in the glioma cancer harboring IDH2 mutant is discussed.

Materials and methods

Materials

pEGFP-N1-IDH2 and pEGFP-N1-IDH2^{R172G} were constructed in our laboratory (Fu et al. 2011b). Rat C6 glioma cells were kindly provided by Prof. Wang (Institute of Clinical Medicine, Renmin Hospital, Yunyang Medical College, Shiyan, Hubei, China). Dulbecco's modified Eagle's medium (DMEM) was from Gibco BRL, calf serum was from Hangzhou Evergreen Corp. Lipofactamine 2000 was from Invitrogen Antibody of rabbit-anti-rattus HIF-1 α and fluorescent TRITC labeled goat-anti-rabbit IgG were purchased from Wuhan Boster Crop. All reagents used are of highest molecular grade.

Cell culture

Cell cultures were prepared and maintained according to standard cell culture procedures. The rat C6 glioma cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in DMEM supplemented with 10% (v/v) heat-inactivated calf serum.

Transfection of recombinant plasmids

C6 cells were cultured in the 6-, 96- and 24-well microtiter plates in immunofluorescence location assay, gelatin zymography assay and in vitro wound healing assay, respectively. For transient transfection,

cells were harvested by pancreatin digestion and seeded on microtiter plates. Transfection of plasmids (pEGFP-N1, pEGFP-N1-IDH2 and pEGFP-N1-IDH2^{R172G}) was performed with Lipofectamine 2000 according to manufacturer's instructions: for six-well microtiter plates, cells were incubated with 4 µg plasmid DNA and 10 µl Lipofectamine 2000 in Opti-MEM overnight before being analyzed. In the same way, for 96-well microtiter plates and 24-well microtiter plates, cells were incubated with 0.2 µg plasmid DNA and 0.5 µl Lipofectamine 2000 or 0.8 µg plasmid DNA and 2 µl Lipofectamine 2000, respectively.

Immunofluorescence location assay for HIF-1 α

The effect of EGFP, EGFP-IDH2 and EGFP-IDH2^{R172G} on the stabilization of HIF-1 α was analyzed by immunofluorescence location assay. C6 cells transfected with recombinant plasmids were fixed with 4% (v/v) paraformaldehyde in PBS, washed with PBS, then permeabilized with 0.3% Triton X-100 for 20 min. Cells were then washed gently with PBS and blocked with BSA in PBS for 1 h. Rabbit-anti-rattus HIF-1 α (1:100) antibody was added and incubated at 4°C overnight. The antibody of fluorescent TRITC labeled goat-anti-rabbit IgG (1:64) was then added for 1 h at room temperature. Coverslips are then washed with PBS, mounted onto slides and observed with a laser scanning confocal microscope.

Gelatin zymography assay

Gelatinolytic activity of MMP-2 and MMP-9 was analyzed by gelatin zymography assay. C6 cells transfected with recombinant plasmids were incubated for 24 h. Culture media were then changed into serum-free cell culture media. To understand the effect of HIF-1 α on MMPs activity in the glioma cells harboring IDH2 mutant, CoCl₂ was used for its induction as mentioned earlier (Zhao et al. 2009). As a control sample, CoCl₂ was added into the serum-free cell culture media at 200 µM. Serum-free media conditioned for 24 h were subjected to 10% (v/v) SDS-PAGE containing 0.1% gelatin. The gel was incubated twice (for 30 min at room temperature) in 2.5% Triton X-100. The gel was then incubated in activation buffer (5 mM CaCl₂, 1 µM ZnCl₂, and 0.005% Brij, 50 mM Tris/HCl, pH 8.0) at 37°C overnight, stained with

Coomassie Brilliant Blue R-250 and briefly destained in 10% (v/v) acetic acid and 40% (v/v) methanol (Fu et al. 2011a). Gelatinolytic activity of MMPs is detected as transparent bands on the blue background.

In vitro wound healing assay

The effect of EGFP, EGFP-IDH2 and EGFP-IDH2^{R172G} on cell migration was examined using in vitro wound healing assay. After transfection with recombinant plasmids for 24 h, a 'scratch' was introduced by scraping the C6 cells monolayer with a p200 pipette tip and then washed gently three times with cell culture media to remove cell debris. As a control group, CoCl₂ was added into the serum-free cell culture media before the cells were scratched. The cells were then cultured by serum-free culture media. Percent migration rate (MR%) was calculated as: $MR\% = (1 - T/C) \times 100\%$, where T and C represent, respectively, the scrape distance at the indicated treatment times and at the previous treatment time.

Results and discussion

IDH2^{R172G} increases the stabilization of HIF-1 α

Compared to the EGFP and IDH2 treatment groups, the amounts of fluorescence of IDH2^{R172G} and HIF-1 α in IDH2^{R172G} treatment group were significantly elevated (Fig. 1). This demonstrates that: (1) mutant IDH2 can elevate the stabilization of HIF-1 α , and (2) improve the growth rate of C6 cells. The link between IDH2^{R172G}, HIF-1 α and tumor growth was confirmed: mutant IDH2 improved tumor growth by elevating the stabilization of HIF-1 α . This link highlights an emerging theme in which mutationally-altered metabolic enzymes are thought to contribute to tumor growth by stimulating HIF-1 α pathway.

IDH2^{R172G} improves the secretion levels of pro-MMP-2 and 9

Matrix metalloproteinases production in response to induced HIF-1 α has not been elucidated in the glioma cells harboring IDH2 mutant. CoCl₂ treatment was used as a control group since previous results showed that CoCl₂ at 200 µM induced HIF-1 α in U-87MG cells (Zhao et al. 2009). As shown in Fig. 2a, transparent

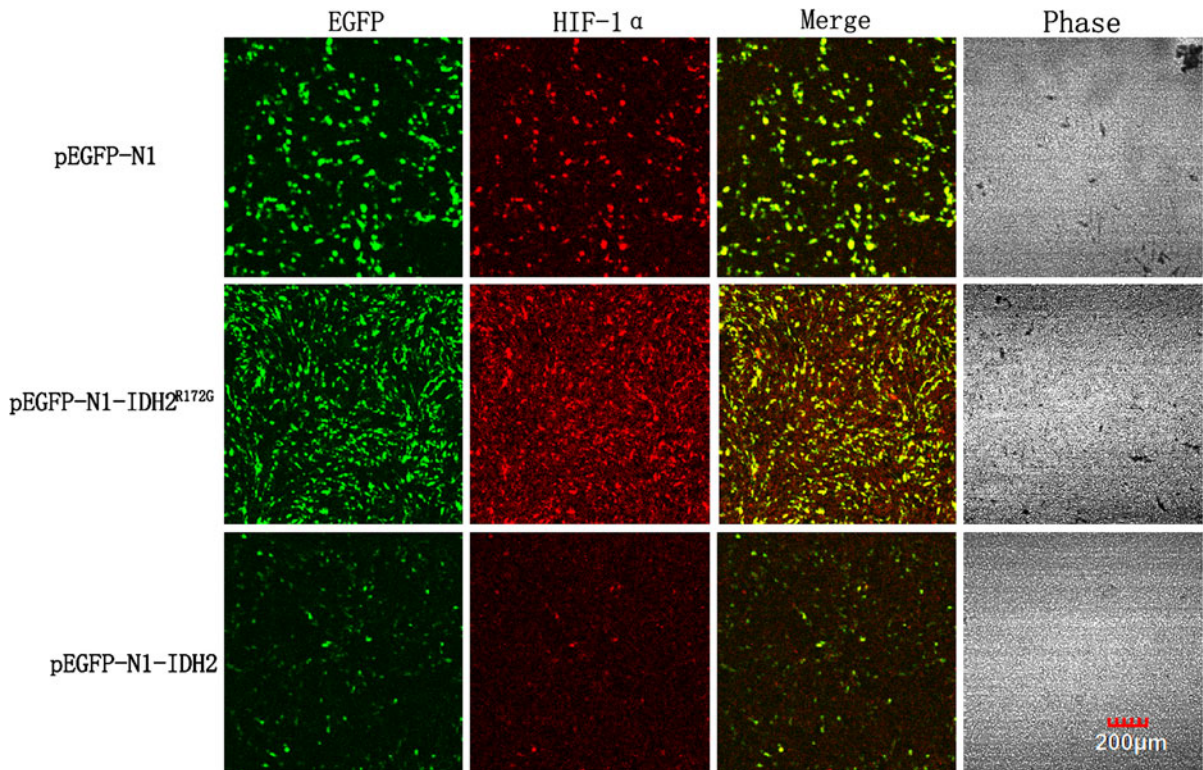


Fig. 1 The role of IDH2^{R172G} on the stabilization of HIF-1 α : C6 cells are transfected with pEGFP-N1, pEGFP-N1-IDH2 and pEGFP-N1-IDH2^{R172G}, respectively. Photographs of transfected C6 glioma cells were obtained with the laser scanning confocal microscope at 24 h post-transfection. EGFP was excited at 488 nm with an argon laser and fluorescence was detected at 500–540 nm, and TRITC was excited at

550–596 nm and fluorescence was detected at 570–620 nm. The differential interference contrast confocal imagings were also present to record the growth status of cells. The amount of HIF-1 α was elevated prominently in IDH2^{R172G} treatment group which demonstrates IDH2^{R172G} can improve the stabilization of HIF-1 α . Scale bar 200 μ m

bands of pro-MMP-9 (~90 kDa), pro-MMP-2 (~70 kDa) and MMP-2 (~60 kDa) were detected in the gelatin zymography assay. CoCl₂-induced HIF-1 α increased the protein levels of pro-MMP-2, 9 in the conditioned media of cells. The activity of pro-MMP-2, 9 was elevated in the CoCl₂ and IDH2^{R172G} treatment groups compared to that in the IDH2 and EGFP treatment groups which demonstrates that mutant IDH2 can also elevate the secretion levels of MMP-2 and MMP-9 by stimulating HIF-1 α pathway according to the results obtained in above section. Moreover, IDH2^{R172G} induced HIF-1 α also increased the conversion from pro-MMP-2 to its active form (Fig. 2b).

IDH2^{R172G} accelerates C6 glioma cells migration

Matrix metalloproteinases-2 and MMP-9 have been hypothesized to be key enzymes responsible for the

degradation of type IV collagen, the main component of ECM. Presumably, these two enzymes may be critical for C6 glioma cells migration or metastasis. As shown in Fig. 3, compared to that of the EGFP and IDH2 treatment groups, migration rates of C6 glioma cells were promoted by approx. 47% in the presence of both IDH2^{R172G} and CoCl₂ at 16 h. These results show that: (1) IDH2^{R172G} displays a higher activity in promoting cell migration than IDH2 does, and (2) MMP-2 and MMP-9 are critical for C6 cell metastasis and angiogenesis at least in this aspect.

Our recent studies have shown the relations of mutated IDH2, NADPH and chemotherapy sensitivity in C6 gliomas (Fu et al. 2011b): (1) IDH2^{R172G} can sensitize glioma sensitivity to chemotherapy through NADPH levels; (2) IDH2^{R172G} can give a benefit to traditional chemotherapy of glioma. In the present study, the relationships of mutated IDH2, HIF-1 α ,

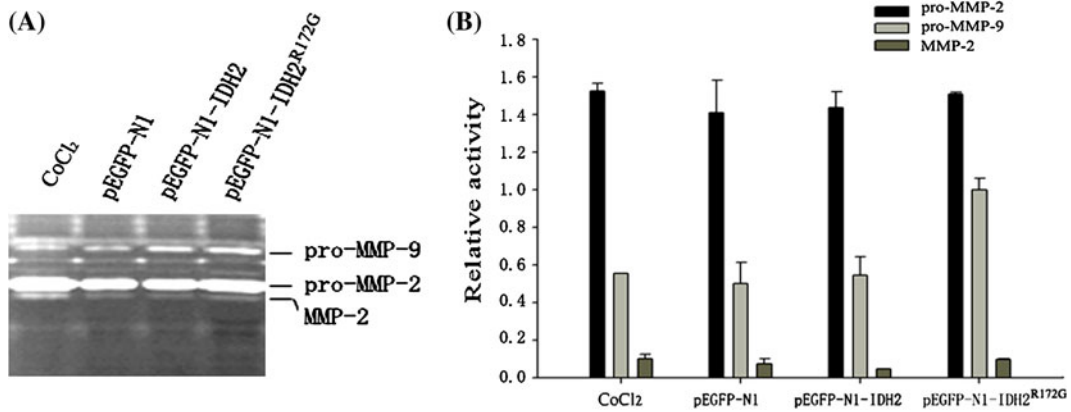


Fig. 2 Effect of IDH2^{R172G} on MMP-2,9 activity in C6 glioma cells. **a** Gelatin zymographic analysis of the conditioned media collected from cells treated with CoCl₂, EGFP, EGFP-IDH2, and EGFP-IDH2^{R172G}. Gelatinolytic activity of pro-MMP-9 (~90 kDa), pro-MMP-2 (~70 kDa), and active-MMP-2 (~60 kDa) was detected as three transparent bands on the blue

background. The gel shown is representative of four treatment groups. **b** Relative activity analysis of pro-MMP-9, pro-MMP-2 and active-MMP-2. Densitometry analysis of MMPs bands in gel was performed using Gene Tools 4.01.02 software (Synoptics Ltd) and relative activity was calculated using Sigma Plot 10.0 (Jandel Corp.). Error bars represent the standard deviation

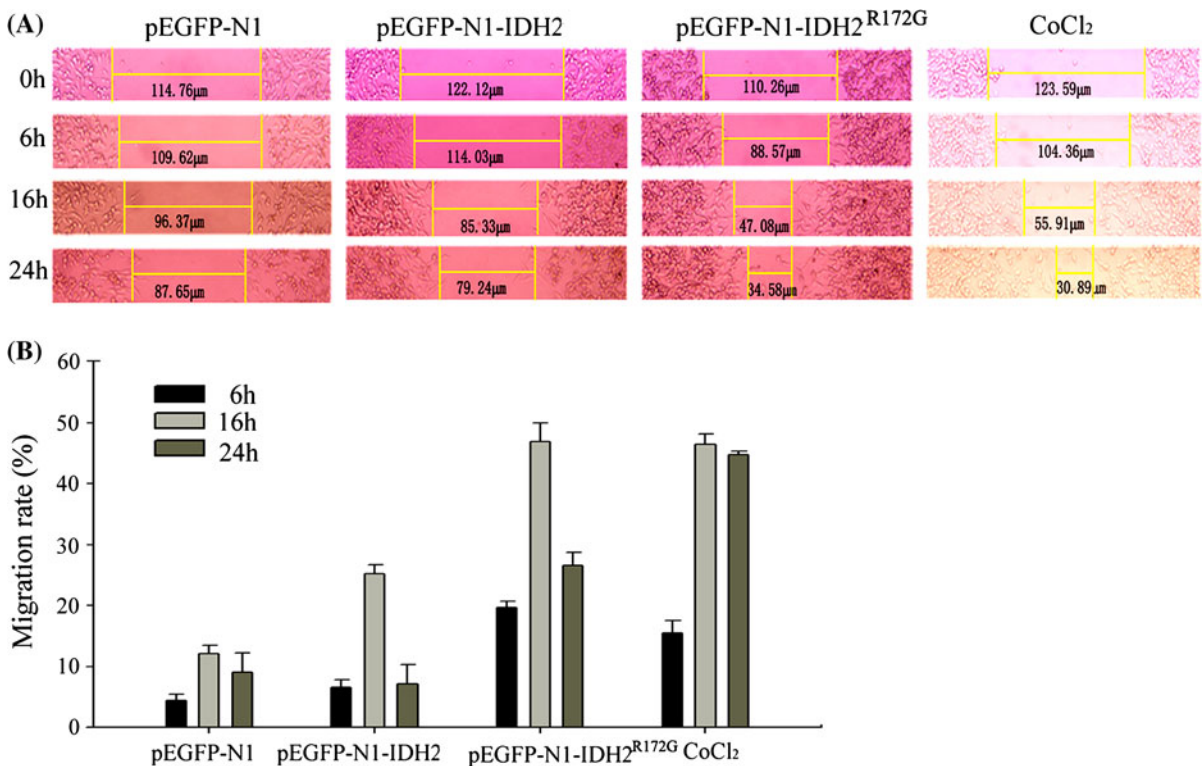


Fig. 3 IDH2^{R172G} increases C6 cell migration rate. **a** Migration of C6 cells was routinely monitored after confluent monolayer of C6 cells were gently scratch with a plastic pipette tip. Migration distance of C6 cells was detected at 6, 16, and 24 h after transfection with pEGFP-N1, pEGFP-N1-IDH2, pEGFP-

N1-IDH2^{R172G}, and treatment with CoCl₂, respectively. **b** Migration rate analysis showed that IDH2^{R172G} and CoCl₂ display a higher activity in promoting cell migration than IDH2 and EGFP do. Error bars represent the standard deviation

MMPs and glioma migration were disclosed: (1) HIF-1 α pathway is critical in MMPs release in glioma cancer harboring IDH2 mutant; (2) Mutant IDH2-induced HIF-1 α can improve the secretion levels of MMP-2 and 9; 3) Mutant IDH2-induced HIF-1 α provides glioma cells with a higher migration potential via up-regulation of MMP-2 and 9. This study may therefore serve as an important complement to existing research on this topic.

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