Original Paper

Isocitrate Dehydrogenase 2 Suppresses the Invasion of Hepatocellular Carcinoma Cells via Matrix Metalloproteinase 9

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Key Words

Isocitrate dehydrogenase 2 (IDH2) • NF-κB • IκB • Matrix metalloproteinase 9 (MMP9) • Hepatocellular carcinoma (HCC) • Metastases

Abstract

Background/Aims: Isocitrate dehydrogenase 2 (IDH2) is a mitochondrial NADP-dependent isocitrate dehydrogenase, and has been found to be a tumor suppressor in several types of tumors. However, the roles of IDH2 in hepatocellular carcinoma (HCC) as well as underlying mechanisms remain unknown. **Methods:** The IDH2 and matrix metalloproteinase 9 (MMP9) levels in the specimens from 24 HCC patients were investigated by Western blot and ELISA, respectively. Their relationship was examined by correlation analyses. Patient survival with high IDH2 levels and low IDH2 levels was compared. IDH2 levels and MMP9 levels were modified in a human HCC cell line. The effects of IDH2 or MMP9 modulation on the expression of the other were analyzed. The effects of IDH2 on cell invasion were analyzed in a transwell cell invasion assay. The dependence of nuclear factor κB (NF-κB) signaling was examined using a specific inhibitor. **Results:** The IDH2 levels significantly decreased in HCC, and were lower in HCC with metastases, compared to those without metastases. IDH2 levels inversely correlated with MMP9 levels in HCC. HCC patients with Low IDH2 had lower 5-year survival. MMP9 levels did not regulate IDH2 levels, while IDH2 inhibited MMP9 levels in HCC cells, in a NF-κB signaling dependent manner, possibly through IκB, to suppress HCC cell invasion. **Conclusions:** Down regulation of IDH2 may promote HCC cell invasion via NF-κB-dependent increases in MMP9 activity. IDH2 may be a potential therapeutic target for HCC.

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Introduction

Hepatocellular carcinoma (HCC) is a prevalent primary malignant tumor, characterized by aggressive invasion, early metastasis and resistance to existing chemotherapeutics [1-4]. In spite of the continuous increases in 5-year survival for HCC due to advances in combined therapy with surgery, radiotherapy and chemotherapy, some patients still suffer from poor outcome due to malignant growth and metastases of HCC [1-4]. Thus, a further understanding of the molecular mechanisms underlying the progression and metastases of HCC would improve the current therapy [5-13].

NADP (+)-dependent isocitrate dehydrogenases (IDH) include IDH1 and IDH2, which catalyze oxidative decarboxylation and produces CO₂, NADPH and α -ketoglutarate from isocitrate in the mitochondria [14-19]. NADPH is a vital cofactor in fat and cholesterol biosynthesis and glutathione metabolism. Mitochondrial IDH plays an important role in cellular defense against oxidative damage by providing NADPH, which is required to regenerate the glutathione levels in the mitochondria [14-19]. Reactive oxygen species (ROS) are produced in mitochondria, and are involved in the pathogenesis of many human disorders [14-19]. Increased ROS levels promote cellular oxidative stress that contributes to various aspects of malignant tumors, including carcinogenesis, aberrant growth, angiogenesis and metastasis. Although IDH has been found to decrease in some types of cancer, resulting in defects in the mitochondrial redox balance and cellular oxidative damage through NADPH [20, 21], the role of IDH2 in HCC remains unknown.

Tumor cell growth and invasion are regulated by a network of factors, among which nuclear factor κ B (NF- κ B) is a critical one that regulates the expression of various genes involved in immunity, stress responses, inflammation and the inhibition of apoptosis, thus providing appropriate conditions for tumor cell progression [22-24]. NF- κ B is constitutively activated in some tumor cells, including HCC cells, and contributes to the maintenance of the highly proliferative malignant phenotype as well as cellular invasion [22-24]. NF- κ B regulates several metastasis-related matrix metalloproteinases (MMPs), such as MMP1, MMP3, and MMP9 [25-27]. MMP9 is an important invasion- or metastasis-related NF- κ B target gene that is directly associated with the metastatic processes in cancers [28-32].

In the current study, we found that the IDH2 levels significantly decreased in HCC, and were lower in HCC with metastases, compared to those without metastases. IDH2 levels inversely correlated with MMP9 levels in HCC. HCC patients with Low IDH2 had lower 5-year survival. MMP9 levels did not regulate IDH2 levels, while IDH2 inhibited MMP9 levels in HCC cells, in a NF- κ B signaling dependent manner, possibly through κ B, to suppress HCC cell invasion.

Materials and Methods

Patient tissue specimens

A total of 24 resected specimens (paired with HCC tissue and adjacent non-tumor tissue (NT)) collected for this study. In all 24 samples, 12 were with distal metastases, while the other 12 were not. All specimens had been histologically and clinically diagnosed at the Jinan Central Hospital of Shandong University from 2008 to 2014. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

Cell lines

HepG2 is a human HCC cell line, which was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA) in a humidified chamber with 5% CO₂ at 37°C. MG132 (Sigma-Aldrich) is a specific NF- κ B signaling inhibitor, and was used at 5 μ mol/l.

Plasmid transfection

IDH2 and MMP9 constructs were generated by sub-cloning PCR-amplified full-length human IDH2 cDNA or MMP9 cDNA into pcDNA3.1EGFR. For depletion of IDH2 or MMP9, corresponding human short hairpin small interference RNA (shRNA) sequences were cloned into pcDNA3.1EGFR to generate the shRNA construct. Sequence: shIDH2: 5'-GTG GAC ATC CAG CTA AAG TAT-3'; shMMP9: 5'-ACC ACA ACA UCA CCU AUU GTT-3'. Transfection was performed with Lipofectamine 2000 reagent (Invitrogen), according to the instructions of the manufacturer. Transfected cells expressing IDH2, or shIDH2, or MMP9, or shMMP9, were selected by flow cytometry based on GFP. The cells that were transfected with plasmids carrying scrambled sequence were used as controls.

Western blot

The protein was extracted from the specimens from patients or from cultured cells, in RIPA lysis buffer (1% NP40, 0.1% SDS, 100 µg/mL phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, in PBS) on ice. The supernatants were collected after centrifugation at 12,000 × g at 4°C for 20 min. Protein concentration was determined using a BCA protein assay kit (Bio-rad, China), and whole lysates were mixed with 4 × SDS loading buffer (125 mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100mmol/l DTT, and 0.2% bromophenol blue) at a ratio of 1:3. Samples were heated at 100°C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies were rabbit anti-IDH2 (Millipore, Billerica, MA, USA), anti-p65, (Cell signaling, San Jose, CA, USA), anti-ikB (Cell signaling), anti-iNOS (Cell signaling), anti-MMP9 (Cell signaling) and anti-α-tubulin (Cell Signaling). Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). α-tubulin was used as a protein loading control. Blotting images were representative from 5 repeats. Densitometry of Western blots was quantified with NIH ImageJ software (Bethesda, MD, USA).

Quantitative RT-PCR

Total RNA was extracted from cultured cells with RNeasy kit (Qiagen, Hilden, Germany), respectively. Complementary DNA (cDNA) was randomly primed from 2µg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). RT-qPCR was subsequently performed in triplicate with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed using $2^{-\Delta\Delta Ct}$ method for quantification of the relative mRNA expression levels. Values of genes were first normalized against α-tubulin, and then compared to the experimental controls.

MMP9 ELISA and activity assay

The HCC cells were seeded in six-well plates and incubated at 37°C. After 24 h, the medium was removed. Then, the cells were washed and incubated in serum-free medium for 48 h. The MMP9 amount and activity in the media were detected using MMP9 ELISA kit (R&D Systems, Los Angeles, CA, USA) and Fluorokine E Human MMP9 Activity Assay kit (R&D Systems), respectively, according to the manufacturer's protocol.

Transwell cell invasion assay

Cells (10^4) were plated into the top side of polycarbonate transwell filter coated with Matrigel in the upper chamber of the BioCoat™ Invasion Chambers (Becton-Dickinson Biosciences, Bedford, MA, USA) and incubated at 37°C for 22 hours. The cells inside the upper chamber with cotton swabs were then removed. Migratory and invasive cells on the lower membrane surface were fixed, stained with hematoxylin, and counted for 10 random 100X fields per well. Cell counts are expressed as the mean number of cells per field of view. Five independent experiments were performed and the data are presented as mean ± standard deviation (SD).

Statistical analysis

All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher's Exact Test for comparison of two groups (GraphPad Prism, GraphPad Software, Inc. La Jolla, CA,

USA). Bivariate correlations were calculated by Spearman's Rank Correlation Coefficients. Kaplan-Meier curves were used to analyze the patient survival by IDH2 levels. All values are depicted as mean $\pm$ standard deviation and are considered significant if $p < 0.05$.

Results

Decreased IDH2 and increased MMP9 are detected in HCC specimens

We examined the levels of IDH2 by Western blot, and the levels of MMP9 by ELISA on paired HCC tumor tissue (HCC) and the adjacent non-tumor normal tissues (NT) from the patients' specimens. Moreover, HCC was further separated into 2 groups, according to the presence of distal metastases (MS+) or not (MS-). We found that HCC contained significantly lower levels of IDH2 (Fig. 1A) and significantly higher levels of MMP9 (Fig. 1B), compared to NT. Moreover, the IDH2 levels in HCC with distal metastases were significantly lower than HCC without distal metastases (Fig. 1A), and the MMP9 levels in HCC with distal metastases were significantly higher than HCC without distal metastases (Fig. 1B).

Fig. 1. Correlated decreased IDH2 and increased MMP9 in HCC. (A-B) Western blot for IDH2 (A) and ELISA for MMP9 (B) was performed on paired HCC tumor tissue (HCC) with/without metastases (MS+; MS-) and the adjacent non-tumor normal tissues (NT) from the patients. (C) A correlation test between IDH2 and MMP9 levels was performed in 24 HCC patients. A strong inverse correlation was detected ($r = -0.79$, $p < 0.0001$, $N = 24$). (D) The 24 HCC patients were followed-up for 5 years. The median value of 24 cases was chosen as the cutoff point for separating IDH2-high cases ($n = 12$) from IDH2-low cases ($n = 12$). Kaplan-Meier curves were performed to evaluate the overall survival of the HCC patients, based on IDH2 levels. * $p < 0.05$. ** $p < 0.01$.

Fig. 2. MMP9 does not regulate IDH2 in HCC cells. We either overexpressed MMP9 by expression of MMP9 transgene (HepG2-MMP9), or inhibited MMP9 by expression of MMP9 shRNA (HepG2-shMMP9), through lipofectamine-mediated transfection of HepG2 cells. The HepG2 cells were also transfected with a plasmid carrying a scrambled sequence as a control (HepG2-scr). (A) The overexpression or inhibition of MMP9 in HepG2 cells was confirmed by RT-qPCR. (B-C) IDH2 levels in MMP9-modified HepG2 cells, by RT-qPCR (B), and by Western blot (C). * $p < 0.05$. NS: non-significant, $N = 5$.

IDH2 and MMP9 levels are inversely correlated in HCC

To figure out the relationship of IDH2 and MMP9 levels in HCC, we performed a correlation test in 24 HCC patients. A strong inverse correlation was detected (Fig. 1C, $r = -0.79$, $p < 0.0001$, $N = 24$), suggesting a possible regulatory relationship between IDH2 and MMP9. Next, we investigated whether the levels of IDH2 may correlate with overall survival of HCC patients. The 24 patients were followed-up for 5 years. The median value of all 24 cases was chosen as the cutoff point for separating IDH2-high cases ($n = 12$) from IDH2-low cases ($n = 12$). Kaplan-Meier curves were performed, showing that IDH2-low HCC patients had a worse 5-year survival, compared to IDH2-high HCC patients (Fig. 1D).

MMP9 does not regulate IDH2 in HCC cells

In order to examine the relationship between IDH2 and MMP9, we used HepG2, which is the most widely used human HCC cell line, in our study. We either overexpressed MMP9 by expression of MMP9 transgene (HepG2-MMP9), or inhibited MMP9 by expression of MMP9 shRNA (HepG2-shMMP9), through lipofectamine-mediated transfection of HepG2 cells. The HepG2 cells were also transfected with a plasmid carrying a scrambled sequence as a control (HepG2-scr). The overexpression or inhibition of MMP9 in HepG2 cells was confirmed by RT-qPCR (Fig. 2A). We found that the alteration of MMP9 levels in HepG2 cells did not alter IDH2

Fig. 3. IDH2 inhibits MMP9 in HCC cells. We either overexpressed IDH2 by expression of IDH2 transgene (HepG2-IDH2), or inhibited IDH2 by expression of IDH2 shRNA (HepG2-shIDH2), through lipofectamine-mediated transfection of HepG2 cells. The HepG2 cells were also transfected with a plasmid carrying a scrambled sequence as a control (HepG2-scr). (A) The overexpression or inhibition of IDH2 in HepG2 cells was confirmed by RT-qPCR. (B-C) MMP9 levels in IDH2-modified HepG2 cells, by RT-qPCR (B), and by ELISA (C). (D) MMP9 activity in IDH2-modified HepG2 cells, by MMP9 activity assay. (E-F) Transwell cell invasion assay, shown by quantification (E), and by representative images (F). * $p < 0.05$, $N=5$.

levels, by RT-qPCR (Fig. 2B), and by Western blot (Fig. 2C). These data suggest that MMP9 does not regulate IDH2 in HCC cells.

IDH2 inhibits MMP9 in HCC cells

Next, we either overexpressed IDH2 by expression of IDH2 transgene (HepG2-IDH2), or inhibited IDH2 by expression of IDH2 shRNA (HepG2-shIDH2), through lipofectamine-mediated transfection of HepG2 cells. The HepG2 cells were also transfected with a plasmid carrying a scrambled sequence as a control (HepG2-scr). The overexpression or inhibition of IDH2 in HepG2 cells was confirmed by RT-qPCR (Fig. 3A). We found that overexpression of IDH2 significantly decreased MMP9 levels in HepG2 cells, while inhibition of IDH2 significantly increased MMP9 levels in HepG2 cells, by RT-qPCR (Fig. 3B), and by ELISA (Fig. 3C). Moreover, the IDH2-regulated changes in MMP9 resulted in changes in MMP9 activity (Fig. 3D). Thus, these data suggest that IDH2 inhibits MMP9 in HCC cells. Furthermore, the IDH2-regulated alterations in MMP9 levels resulted in changes in cell invasion in a transwell cell invasion assay, shown by quantification (Fig. 3E), and by representative images (Fig. 3F). Hence, IDH2 regulates HCC cell invasion through MMP9.

IDH2 inhibits MMP9 in HCC cells through NF- κ B pathway

Then we examined the underlying mechanisms. First, modification of IDH2 levels in IDH2-modified HCC cells was confirmed (Fig. 4A-B). We found that increases in IDH2 in HCC cells decreased p65, a major NF- κ B player. On the other hand, decreases in IDH2 in HCC

Fig. 4. IDH2 inhibits MMP9 in HCC cells through NF- κ B pathway. In order to see whether NF- κ B signaling is required for the regulation of MMP9 by IDH2, we gave MG132, a NF- κ B signaling inhibitor, to IDH2-depleted HepG2 cells. Western blot was performed and shown by representative blots (A), and by quantification for IDH2 (B), p65 (C), MMP9 (D), iNOS (E) and κ B (F). * $p < 0.05$. NS: non-significant, N = 5.

cells increased p65 (Fig. 4A, C). In order to see whether NF- κ B signaling is required for the regulation of MMP9 by IDH2, we gave MG132, a NF- κ B signaling inhibitor, to IDH2-depleted HepG2 cells. We found that inhibition of NF- κ B signaling did not alter IDH2 levels, but abolished the activation of p65, and abolished the activation of MMP9 (Fig. 4A, B, D). Since IDH2 has been implicated in the control of oxidative stress and one of the main mechanisms of NF- κ B activation is via ROS, we thus examined iNOS levels after the overexpression or depletion of IDH2, and found that iNOS levels were not changed by IDH2 modification (Fig. 1A, E). Therefore, these data do not support that oxidative stress may collaborate with IDH2 to regulate MMP9 and NF- κ B activation in HCC cells. Since the proteasome inhibitor MG132 blocks activation of NF- κ B by preventing degradation of κ B, we thus examined the expression of κ B in both IDH2-overexpressed or IDH2-depleted HepG2 cells. Our data showed that increases in IDH2 in HCC cells increased κ B, and decreases in IDH2 in HCC cells decreased κ B (Fig. 4A, C). Thus, IDH2 inhibits MMP9 in HCC cells in a NF- κ B signaling dependent manner, possibly through κ B, to suppress HCC cell invasion (Fig. 5).

Fig. 5. A Schematic model. IDH2 inhibits MMP9 in HCC cells in a NF- κ B signaling dependent manner, possibly through $\text{I}\kappa\text{B}$, to suppress HCC cell invasion.

Discussion

HCC is one of the most prevalent human cancers. The alteration of IDH2 levels has been identified in several types of carcinoma. However, the expression and significance of IDH2 in HCC is still unknown. Moreover, the molecular mechanisms underlying the IDH2-regulated cancer cell metastases are so far poorly understood.

Here, we first confirmed that the level of IDH2 was significantly decreased in HCC tissues, compared with the adjacent normal tissues from human patients. Moreover, from examination of key MMPs, we detected significant upregulation of MMP9 in HCC. MMPs have been well-known to control cell metastasis. Since we want to know whether IDH2 plays a role in control of HCC metastasis, we examined the correlation between MMP9 and IDH2 in HCC. A strong negative correlation prompted us to test a causal link.

We thus modified IDH2 levels in a HCC cell line, and found that it directly affected the levels of MMP9. Moreover, by inhibiting NF- κ B pathway, we completely inhibited the effect of IDH2 on MMP9. Nuclear factor- κ B (NF- κ B) has been well recognized as a pivotal regulator of cancer initiation, progression and invasion, whereas recent developments have shown a broad involvement of NF- κ B in other aspects of cancer pathology. NF- κ B is a group of dimeric transcription factors comprised of the Rel family of proteins that include RelA (p65), c-Rel, RelB, NF- κ B1 (p50), and NF- κ B2 (p52) [33-35]. The most abundant form in activated cells is the RelA/NF- κ B1 (p65/p50) heterodimer, referred to as 'classic' NF- κ B [33-35]. NF- κ B resides in the cytoplasm in a latent form, and translocate to the nucleus to function upon stimulation [33-35]. The cytoplasmic retention of NF- κ B results from its interaction with inhibitory proteins known as $\text{I}\kappa\text{B}$ [33-35]. Insufficient $\text{I}\kappa\text{B}$ allows the detachment of NF- κ B from $\text{I}\kappa\text{B}$, and its subsequent entering the nucleus to initiate gene transcription [33-35]. Indeed, by examination of the possible involved pathways, the effects of NF- κ B pathway in the regulation of MMP9 by IDH2 were most pronounced, compared to JNK, ERK/MARK, or PI3k signaling pathway. Thus, we conclude that IDH2 may inhibit MMP9 activation in HCC cells through inhibiting NF- κ B pathway, possibly through increasing $\text{I}\kappa\text{B}$ activities.

The limitation of this study rests on that it is a retrospective study including a relative small number of patients' specimen. In addition, the lower IDH2 levels in HCC specimens do not necessarily indicate a mutation in this gene. Furthermore, gain-of-function and loss-of-function experiments using MMP9 or IDH2 were only performed in one cell line. Further studies are needed to confirm the precise molecular regulation of IDH2 and NF- κ B and their interaction to elucidate the role of IDH2 in cell growth and its effects on cell invasion. In summary, our study highlights IDH2 as a novel therapeutic target for HCC treatment.

Disclosure Statement

The authors have declared that no competing interests exist.

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