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Original Article

Glo1 genetic amplification as a potential therapeutic target in hepatocellular carcinoma

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Abstract: *Glyoxalase 1* (*Glo1*) gene aberrations is associated with tumorigenesis and progression in numerous cancers. In this study, we explored the role of *Glo1* genetic amplification and expression in Chinese patients with hepatocellular carcinoma (HCC), and *Glo1* genetic amplification as potential therapeutic target for HCC. We used fluorescence in situ hybridization (FISH) analysis and qRT-PCR to examine *Glo1* genetic aberrations and Glo1 mRNA expression in paired tumor samples obtained from HCC patients. *Glo1* genetic amplification was identified in a subset of HCC patient (6%, 3/50), and up-regulation of Glo1 expression was found in 48% (24/50) of tumor tissues compared with adjacent non-tumorous tissues. Statistic analysis showed that Glo1-upregulation significantly correlated with high serum level of alpha-fetoprotein (AFP). Interfering *Glo1* expression with shRNA knocking-down led to significant inhibition of cell growth and induced apoptosis in primarily cultured HCC cells carrying genetic amplified *Glo1* gene, while no inhibitory effects on cell proliferation were observed in HCC cells with normal copies of *Glo1* gene. *Glo1* knockdown also inhibited tumor growth and induced apoptosis in xenograft tumors established from primarily cultured HCC cells with *Glo1* gene amplification. In addition, *Glo1* knocking-down with shRNA interfering caused cellular accumulation of methylglyoxal, a Glo1 cytotoxic substrate. Our data suggested Glo1 pathway activation is required for cell proliferation and cell survival of HCC cells carrying *Glo1* genetic amplification. Intervention of Glo1 activation could be a potential therapeutic option for patients with HCC carrying *Glo1* gene amplification.

Keywords: Glyoxalase 1, hepatocellular carcinoma, genetic amplification, cell proliferation, apoptosis

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide [1-3]. HCC is also predominant in eastern Asia, which is associated with a variety of risk factors including prevalence of hepatitis B and C infections [4-6]. Although recent advances in diagnosis and clinical treatments, such as hepatic resection and liver transplantation, improve the outcomes for patients with early stage HCC, the overall survival rates for HCC with advanced disease remains disappointing [7-9]. Therefore, further investigations for pathophysiology of HCC and development of novel therapeutic strategies are clearly needed for the goal to improve outcomes for HCC patients.

Although HCC is a particularly heterogeneous disease and many pathogenic processes contribute to the tumorigenesis and cancer progression, the success for use of sorafenib, a multi-kinase inhibitor which has been proved to increase the survival rates of patient with unresectable HCC [10-12], suggested that targeting therapy may have important clinical impacts for HCC patients. In the past decades, a number of candidate oncogenes, including vascular endothelial growth factor receptor (VEGFR) [13], epidermal growth factor receptor (EGFR) [14, 15], fibroblast growth factor receptor (FGFR) [16, 17], insulin-like growth factor receptor (IGFR) signaling [18] and mammalian target of rapamycin (mTOR) [19, 20] pathway have been identified as potential therapeutic targets or pathways in cancer treatment. However, none of these targets/pathways was found to be crucial for HCC. Identification of novel molecular targets for HCC thus remains to be challenging.

Glyoxalase 1 (Glo1) is a ubiquitous cellular defense enzyme involved in the detoxification of methylglyoxal [21]. Insufficient metabolism of methylglyoxal causes accumulation of this reactive dicarbonyl metabolite, which reacts with nucleotides and proteins to form adducts in these molecular, and inhibit cell growth and induce cell death [22-24]. By virtue of catalyzing the removal of methylglyoxal, Glo1 was considered to promote cell growth. Increases of expression or activity of Glo1 was demonstrated in human tumors, including invasive ovarian, breast, lung, prostate, renal cancer, leukemia and melanoma [25-31]. In addition, Glo1 overexpression was also found to be associated with cancer cell survival and resistance to chemotherapeutic agents [23, 32]. Thus, Glo1 was proposed to be a potential therapeutic target for cancer treatment. In fact, cancer cell lines with Glo1 genetic amplification showed more sensitive to the growth inhibitory effect of bromobenzylglutathione cyclopentyl diester (BBGC), a potent Glo1 competitive inhibitor [25].

In this study, we examined the genetic amplification of Glo1 in a subset of Chinese patients with HCC. As an attempt, we also investigated the potential role of Glo1 in cancer cell proliferation and apoptotic response in primarily cultured cancer cells derived from patient's tumors and in nude mice xenografts with inoculation of primarily cultured HCC cells with genetically amplified Glo1 gene.

Materials and methods

Cell lines

HCC cell line HuH-7 was obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). HuH-7 was maintained in DMEM medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco BRL) respectively. The cell line was maintained in a humidified atmosphere containing 5% CO_2 at 37°C. The culture medium was renewed every 2 to 3 days.

Tissue samples

All tissue samples were collected from fresh biopsy or surgical specimens of HCC patients at Hangzhou first hospital, Zhejiang, China, including tumor tissues and adjacent non-tumorous tissues. Informed consent was obtained from all patients before operation and all the procedures were approved by the Medical Ethics Committee of Hangzhou first people's hospital. The adjacent tissues were obtained at lease 2 cm away from the tumor. All the tissues were immediately frozen in liquid N2 after surgery and then stored in -80°C. The tumor and adjacent non-tumorous tissues were assessed by pathologist review before following analysis.

Primary HCC cell culture from tumor tissues of HCC patients

Tumor tissues obtained from surgery were prepared for primary HCC cell culture. Informed consent was signed by corresponding patients according to institutionally-approved protocols before the procedure. Briefly, fresh tumor tissues were minced into small pieces, and were incubated in DMEM medium containing 1 mg/mL collagenase type IV (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 0.5 h. After filtrated with cell mesh, the cell suspension was washed in Hanks balanced salt solution, and then centrifuged (1,000 rpm for 5 min). The collected cells were cultured with MEDM/F12 medium (Gibco BRL) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 20% FBS. Fibroblasts were removed using cell scrapers during the cell culture. When tumor cells grow stably, they were then maintained in MEDM/F12 medium containing 10% FBS.

FISH assay

The Glo1 FISH probe was generated from BAC (RP11-992K23) from BAC DNA library (Invitrogen, Carlsbad, CA, USA) and directly labeled with spectrum Red (Abbott Molecular, Des Plaines, IL, USA). The Spectrum Green-labeled centromere of chromosome 6 (CEP6) probe (Abbott Molecular) was used as an internal control. For FISH staining, tissue sections (4 μm) from formalin-fixed and paraffin-embedded (FFPE) tissue samples were deparaffinized, and pretreated with SpotLight Tissue pretreatment Kit (00-8401, Invitrogen). Tissue samples and the Glo1/CEP6 probes were denatured at 80°C for 5 minutes, and then incubated together at 37°C for 48 hours. After hybridization, excess and unbound probes were removed by using incubation with posthybridization wash buffer (0.3% NP40/1 × saline-sodium citrate) at 75.5°C for 5 minutes followed by two washes with 2 × saline-sodium citrate at room tempera-

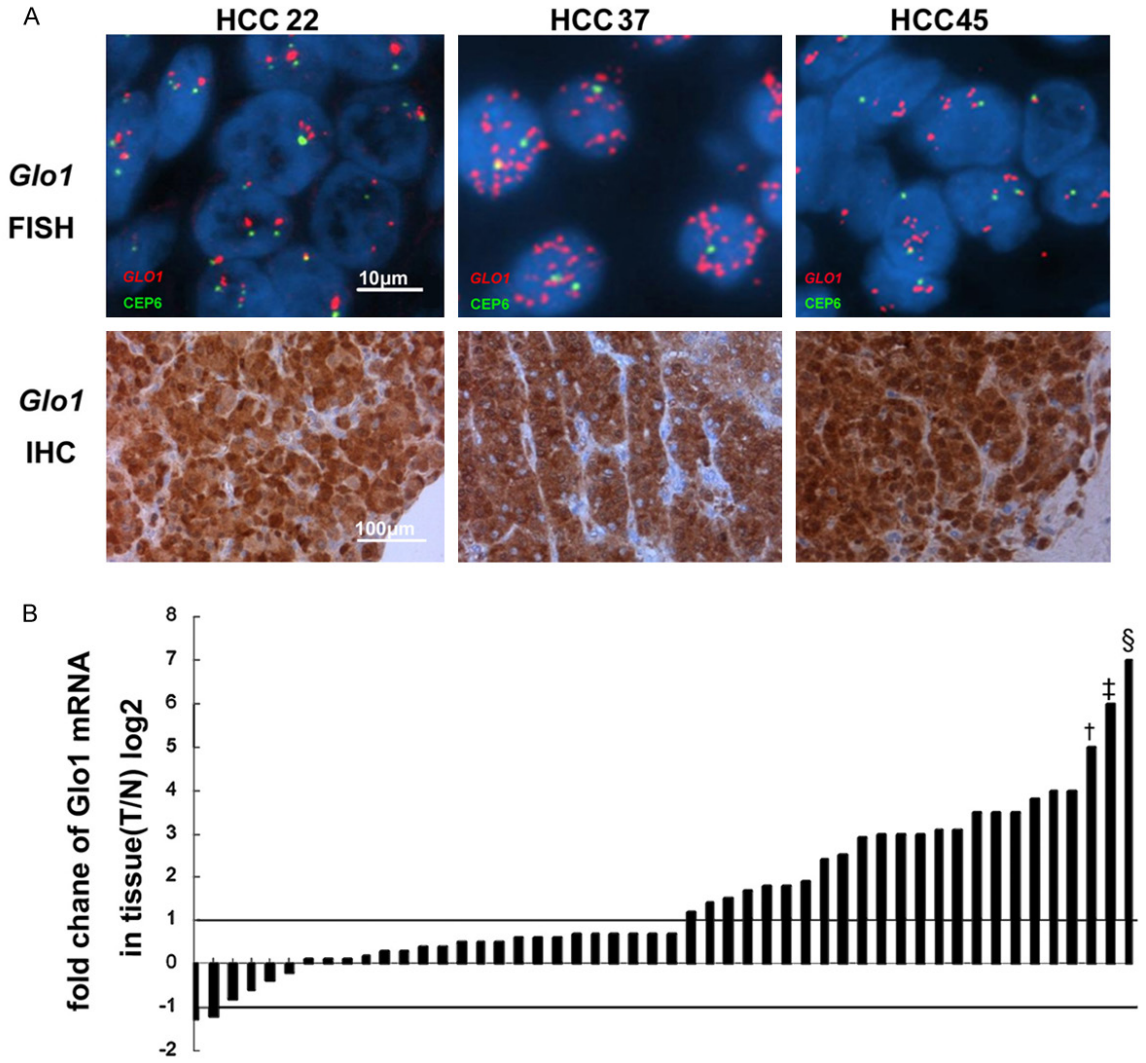


Figure 1. Glo1 gene amplification and mRNA up-regulation in HCC primary tumors. A. Representative images of Glo1 gene amplification from HCC tumor sample HCC22, HCC37 and HCC45. FISH analysis were performed in tissue sections using probes against Glo1 (Red) and CEP6 (green). Scale bars represent 10 μm for FISH images and 100 μm for IHC images. B. Glo1 mRNA expression levels were analyzed in paired tumor and adjacent non-tumorous tissues. Values were present as log2 transformed relative fold changes in mRNA expression level compared with the paired non-tumorous tissue. A twofold change threshold was set to identify obvious changes of gene expression. T: tumor tissue; N: non-tumorous tissue. †, ‡ and § represent HCC45, HCC22 and HCC37 respectively.

ture for 2 minutes, and nuclei were then counterstained with 4',6 diamidino-2-phenylindole (DAPI). Visualization of the fluorescent signals was conducted using a fluorescence microscope (Olympus, Tokyo, Japan) and image analysis was conducted with Cyto-Vision (Leica, Solms, Germany). The enumeration of the Glo1 gene and chromosome 6 was conducted in 50 tumor nuclei for each tissue section, which yielded a ratio of Glo1 to CEP6. Tumors with Glo1 to CEP6 ratio > 2 or presence of > 10% gene cluster were defined as amplified (AMP).

Analysis of Glo1 mRNA expression

RNAs were extracted from tissues and cell lines using TRIzol. 1-2 μg RNA was reverse transcribed to cDNA using High Capacity RNA-to-cDNA master mix (Applied Biosystems, Foster City, CA, USA). Real-time PCR analyses were performed using the Glo1 TaqMan assay Hs00198702_m1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) TaqMan assay Hs99999905_m1 http://www.lifetechnologies.com/order/genome-database/AssayDetails?r

Table 1. Correlation analyses of Glo1 mRNA up-regulation and clinicopathologic parameters

		Glo1 mRNA expression (T versus N)		
Parameters	Median (range)	≥ 2 folds N (%)	< 2 folds N (%)	*P* value
Gender				
Male		22 (53.7)	19 (46.3)	0.2694
Female		3 (33.3)	6 (66.7)	
Age(year)				
≤ 58.0	58.0 (32-83)	13 (46.4)	15 (53.6)	0.8018
> 58.0		11 (50.0)	11 (50.0)	
AFP (ng/ml)				
≤ 45	45 (2-144630.00)	7 (28.0)	18 (72.0)	0.0046
> 45		17 (68.0)	8 (32.0)	
TNM				
1, 2		11 (37.9)	18 (62.1)	0.0940
3, 4		13 (61.9)	8 (28.1)	
HBV				
Positive		20 (46.5)	23 (53.5)	0.6016
Negative		4 (57.1)	3 (42.9)	

NOTE: Patient clinicopathologic information was obtained from 50 Chinese HCC tumor specimens and the correlations with Glo1 mRNA expression analyzed. Up-regulation was defined as log2 value > 1 whereas non up-regulation defined as log2 value < 1. Significance was determined using the χ^2 likelihood ratio test.

ev=V60&impInfo=true&assayid=Hs9999990-5_m1&assaytype=ge (Invitrogen, Carlsbad, CA, USA) on the ABI 7900HT (Applied Biosystems). qRT-PCR results for Glo1 gene expression were normalized using GAPDH as internal control. Relative Glo1 expression levels normalized to GAPDH were determined with the formula $2^{-\Delta Ct}$, in which $\Delta Ct = Ct_{Glo1}-Ct_{GAPDH}$. To calculate the fold changes of Glo1 expression in tumor tissues compared to adjacent non-tumorous tissues (T/N), the $2^{-\Delta\Delta Ct}$ method was used, in which $\Delta\Delta Ct = \Delta Ct_{tumor}-\Delta Ct_{non\text{-}tumorous}$, and log2 values were calculated. A twofold change and above in gene expression was considered to be up-regulated.

Microsatellite analysis

DNA was extracted from blood and tissue samples (Puregene DNA extraction kit, QIAGEN, Valencia, CA), and was applied to microsatellite characterization by evaluating nine short tandem repeat (STR) loci and one amelogenin for gender identification (Cell ID™ System, Promega, Foster City, CA), which provide a genetic profile with a random match probability of 1 in 2.92 × 109 population. The GeneMapper 4.0 software (Applied Biosystems) was used to analyze the alleles.

Western blot analysis

20 μg of cell lysates in RIPA buffer was used for western blot analysis with anti-Glo1 (ab96032, 1:1500, Abcam, Cambridge, UK), anti-cleaved-Caspase 3 (CC3) (9661, 1:1000, Cell Signaling Technology, Danvers, MA, USA) and anti-GAPDH (5174, 1:5000, Cell Signaling Technology) antibodies.

Immunohistochemistry

Antigen retrieval was conducted on FFPE tissues for 5 minutes with retrieval buffer (S1699, DAKO, Glostrup, Denmark) followed by washing in running water for 5 minutes. Tissue samples were then rinsed in TBS containing 1% Tween (TBST) and incubated with endogenous peroxidase blocker on a LabVision autostainer for 10 minutes. Slides were washed twice in TBST and incubated with primary antibodies (Glo1, ab96032, 1:100, Abcam; CC3, 9661, 1:100, Cell Signaling Technology) for 60 minutes at room temperature and then washed twice in TBST. DAKO EnVision™ + System-HRP was used as second antibody for vitalization and staining was detected using diaminobenzidine (K3468, DAKO). For Ki67 immunohistochemical analysis, sections were incubated with biotinylated primary antibody (M7240, 1:100, DAKO) for 1 minute at room temperature and then washed twice with TBST. Following 15 minutes streptavidin-peroxidase treatment and washing in TBST, slides were counterstained with DAPI and visualized by chemiluminescence as described earlier. Quantification of positive signals was conducted using the Ariol system (Genetix, San Jose, CA, USA).

In vitro and in vivo shRNA knockdown of Glo1 expression

The constructs containing Glo1-specific short hairpin RNA (shRNA) sequences under the con-

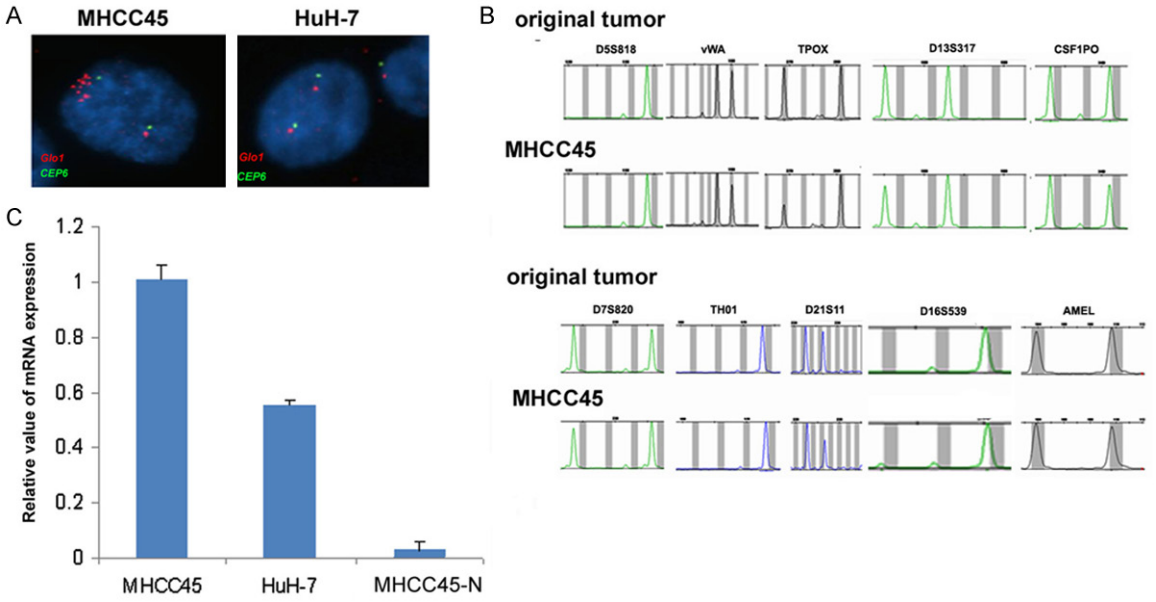


Figure 2. Characterization of HCC tumor cells. A. Representative images for FISH analysis staining with probes of Glo1 (red) CEP6 (green) in MHCC45 and HuH-7 cells. B. Microsatellite analysis showing all 10 loci of MHCC45 completely matched with those in original tumor. C. Expression level of Glo1 mRNA in MHCC45, HuH-7 and MHCC45 paired adjacent non-tumorous tissue. mRNA level of Glo1 was determined by qRT-PCR as described in Materials and Methods. mRNA level of HuH-7 and MHCC45 was normalized to that of MHCC45. MHCC45-N: MHCC45 original paired adjacent non-tumorous tissue.

trol of the tetracycline promoter was used for shRNA knocking-down experiments. The shRNA oligos (TRCN0000118628, TRCN0000118630 and TRCN0000118631 designated as sh-Glo1-A, sh-Glo1-B, and sh-Glo1-C respectively) targeting Glo1 were purchased from Sigma-Aldrich, USA. Briefly, primarily cultured HCC cells with genetic Glo1 amplification (MHCC45) and ATCC-established HuH-7 cells were transfected with the Glo1 shRNA constructs, or a construct containing enhanced GFP (EGFP)-specific shRNA sequence as a negative control. Engineered cells were selected with puromycin (P9620, Sigma-Aldrich). In these engineered cells, the expression of shRNA was induced by doxycycline (D9891, Sigma-Aldrich).

For *in vitro* study, MHCC45 and HuH-7 cells were treated with or without doxycycline at 1 mg/mL for 3 days, and cell proliferations were measured using tetrazolium-based CellTiter 96 AQueous One Solution Cell Proliferation assay (Promega, Fitchburg, WI, USA) as per manufacture's instruction. Expressions of Glo1 and CC3 in treated cells were determined by western blot analysis. Cells were also plated at 500 cells/well in six-well plates for colony formation assay. In this experiment, cells were treated with doxycycline or left untreated as control, and were then cultured for an additional 10-12 days. Colonies with more than 50 cells were counted, and the effect on survival colony was analyzed using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA).

For *in vivo* study, stably transfected MHCC45 cells were suspended in 200 μL of 50% Matrigel and inoculated subcutaneously into the right flank of balb/c nude mice (Charles River, Beijing, China). Mice bearing tumors with volumes approximating 200 mm^3 were randomized into 2 groups, with 10 mice per group. Mice were then either treated with 2 mg/mL of doxycycline (dissolved in 5% sucrose solution and administered via the drinking water) or sucrose solution as control for two weeks. Tumor size and mice body weight were measured twice a week. Tumor volumes were calculated by measuring 2 perpendicular diameters with calipers [formula: $V = (length \times width^2)/2$]. Percentage tumor growth inhibition [% TGI = 1-[change of tumor volume in treatment group/change of tumor volume in control group) × 100] was used for the evaluation of antitumor efficacy. Tumor tissues from two sacrificed mice in both treatment group and correspond-

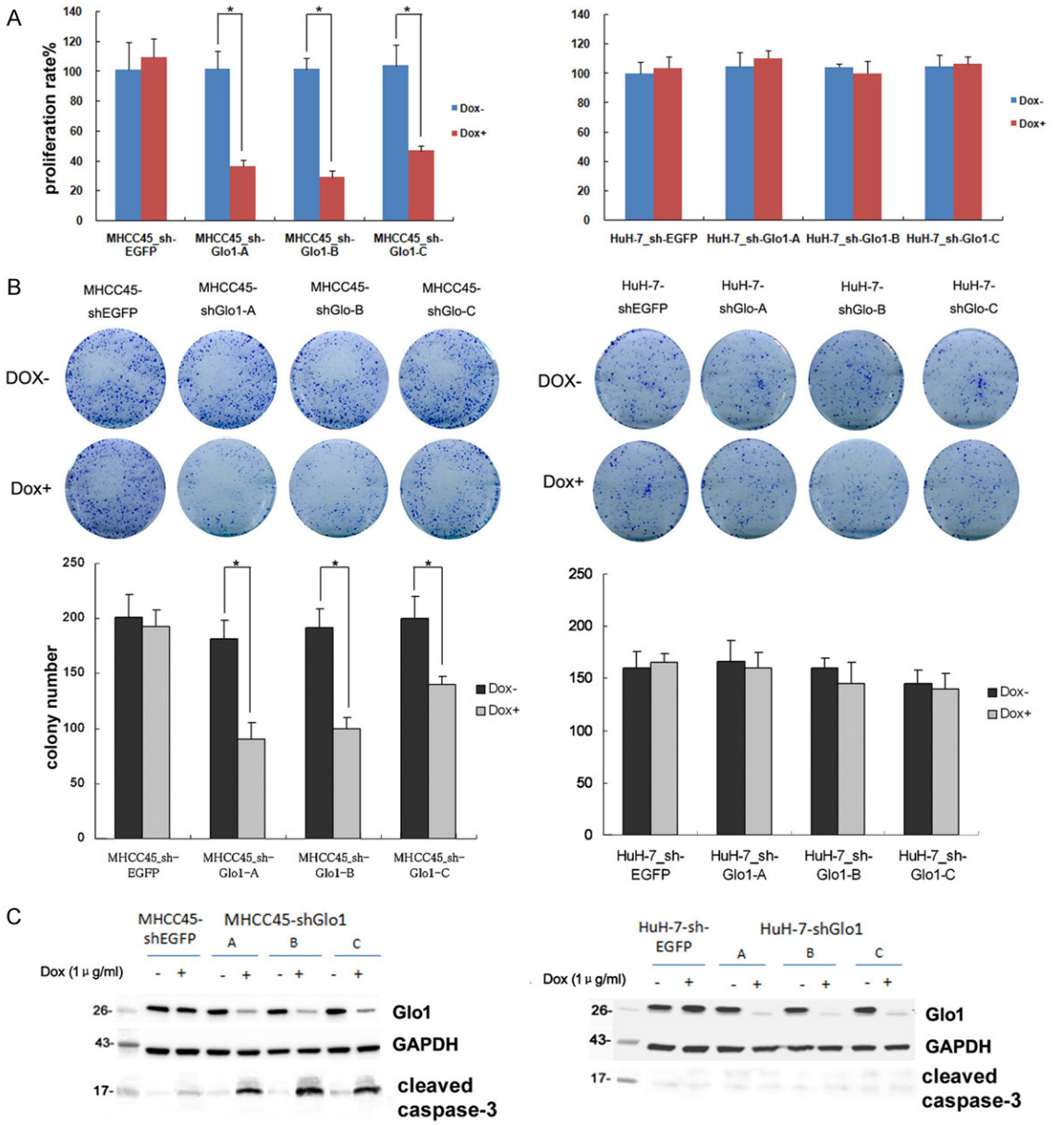
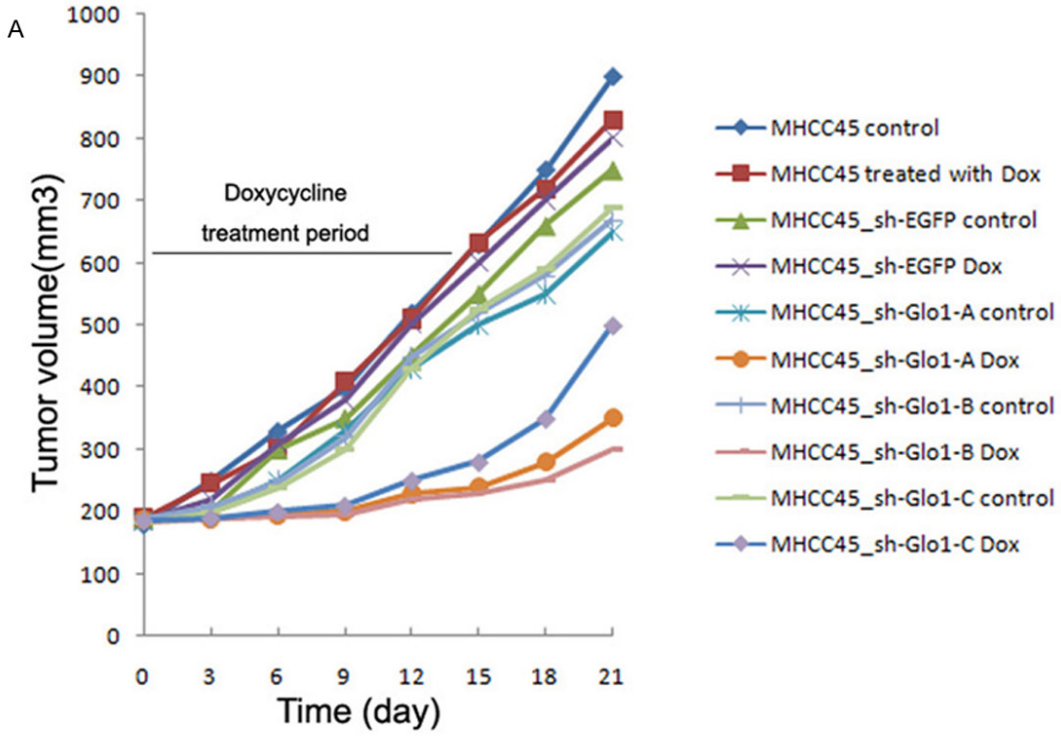


Figure 3. Effect of Glo1 knockdown by shRNA on cell growth in MHCC45 cells. A. Effect of Glo1 knockdown on cell proliferation. MHCC45 cells engineered with inducible Glo1 shRNA were treated with doxycycline for 72 hours, or left untreated as control. Cell proliferation was assessed with MTS proliferation assay. Data represent the mean ± SD, **P* < 0.05. B. Effect of Glo1 knockdown on colony formation. Colony formation assay was performed in MHCC45 and HuH-7 cells with engineered expressing Glo1 shRNAs and vector control (EGFP). Cells were seeded at 500 cells/well in 6-well plate, and treated with/without doxycycline. Surviving colonies with more than 50 cells were counted. Data showed colony number of each groups. Data represent the mean ± SD, *indicates *P* < 0.05. C. Effects of Glo1 knockdown on protein levels of Glo1 and Cleaved caspase 3 protein. Cells were treated with doxycycline or left untreated. Cell lysates were collected 72 hours post treatment and analyzed by western blot.

ing control group were analyzed by IHC staining.

Measurement of methylglyoxal (MG) level

The level of MG was measured with an enzyme-based immunoassay (OxiSelect™ Methylglyoxal ELISA Kit, Cell BioLabs Inc., San Diego, CA, USA). To measure the level of MG accumulation after Glo1 knockdown, the cell clones of MHCC45_sh-EGFP, MHCC45_sh-Glo1-A, MHCC45_sh-Glo1-B, and MHCC45_sh-Glo1-C were treated with or without 1 mg/mL of doxycycline for 3 days, and cells were then lysed for MG


A
Tumor volume(mm3)
Time (day)
Doxycycline
treatment period
MHCC45 control
MHCC45 treated with Dox
MHCC45_sh-EGFP control
MHCC45_sh-EGFP Dox
MHCC45_sh-Glo1-A control
MHCC45_sh-Glo1-A Dox
MHCC45_sh-Glo1-B control
MHCC45_sh-Glo1-B Dox
MHCC45_sh-Glo1-C control
MHCC45_sh-Glo1-C Dox

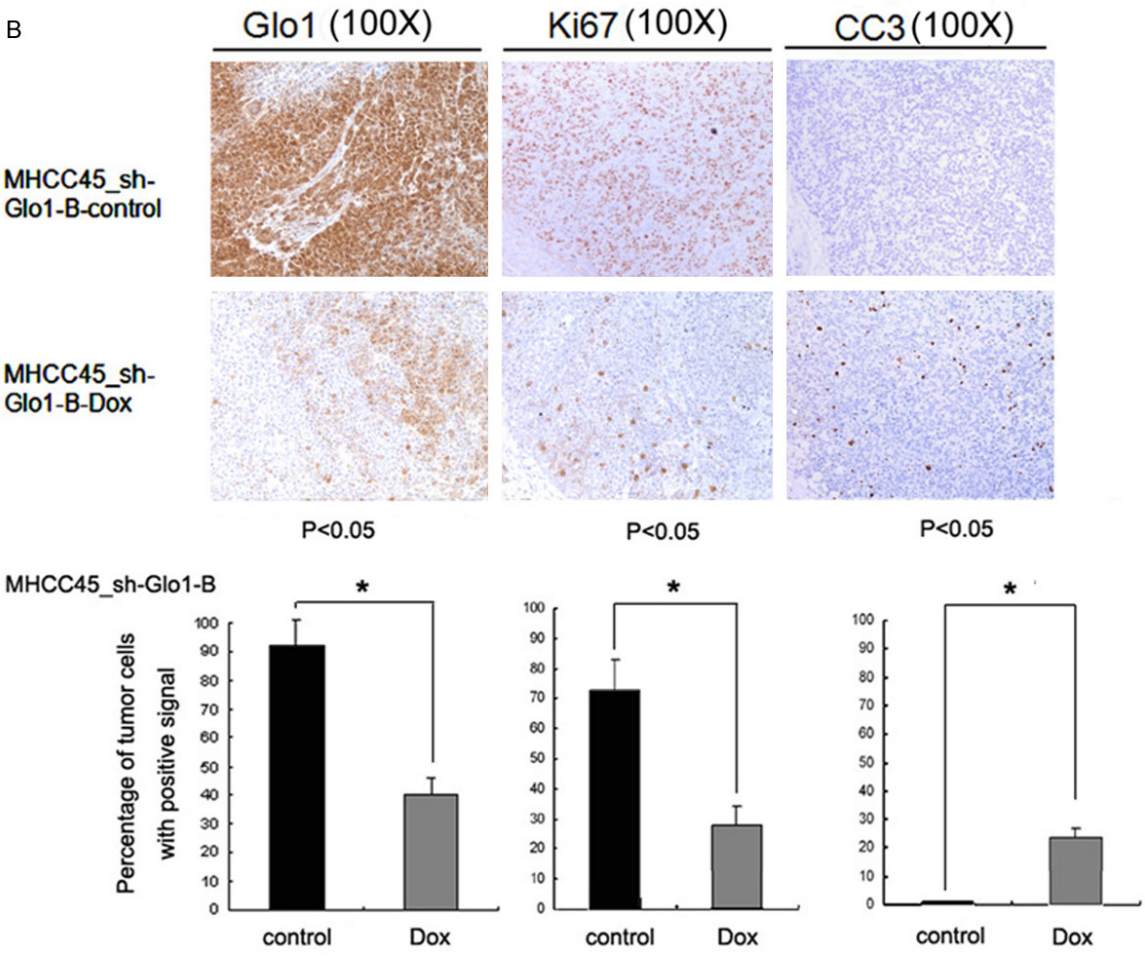

B
Glo1 (100X)
Ki67 (100X)
CC3 (100X)
MHCC45_sh-Glo1-B-control
MHCC45_sh-Glo1-B-Dox
P<0.05
P<0.05
P<0.05
MHCC45_sh-Glo1-B
Percentage of tumor cells with positive signal
control
Dox
control
Dox
control
Dox

Figure 4. Inhibitory effect of Glo1 knockdown on growth of MHCC45 xenografts in vivo. A. MHCC45 xenograft developed from parental control cells, or cells carrying inducible engineered expression of tet-controlled Glo1 shRNA (MHCC45-sh-Glo1-A, MHCC45-sh-Glo1-B, MHCC45-sh-Glo1-C), or EGFP control (MHCC45-sh-EGFP) were randomized into indicated groups and treated with/without doxycycline for 14 days. Tumor volume was measured as described in Materials and Methods. B. Immunohistochemical analysis. IHC were performed using anti-Glo1, anti Ki67 and anti CC3 in tumor samples excised from xenograft of MHCC045-sh-Glo-B treated with or without doxycycline. Glo1, Ki67 and CC3 analysis was conducted after 14-day treatment with/without doxycycline. Quantification of positive signals was conducted using the Ariol system automatically. *indicates $P < 0.05$.

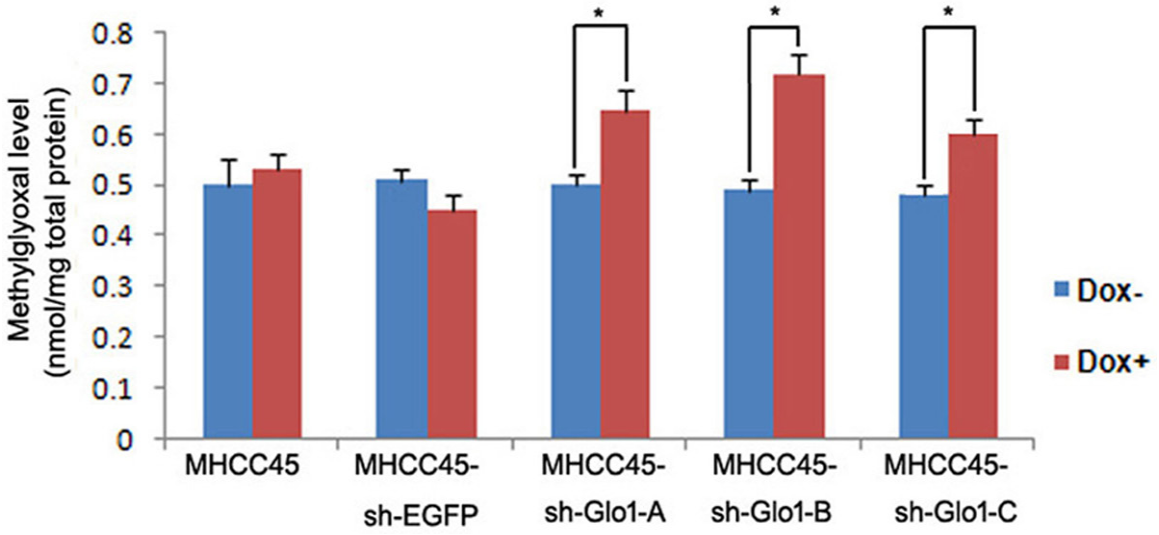


Figure 5. The MG level increased after Glo1 knockdown in MHCC045 cells. MG levels in MHCC045 cells with inducible engineered expression of Glo1 shRNAs (sh-Glo1-A, sh-Glo1-B and sh-Glo1-C) and vector control (EGFP) were measured 72-hour post treatment of doxycycline. Data represent the mean ± SD, *indicates $P < 0.05$.

measurement as per manufacture's instruction.

Statistical analysis

Statistical analyses for Glo1 mRNA up-regulation and its correlations with clinicopathologic parameter were performed using χ^2 likelihood ratio test. The experimental data in this study were present as the mean ± SD. Statistical significance was evaluated using a one-tailed two-sample *t* test.

Results

Genetic amplification and overexpression of Glo1 in tumor tissues of HCC patients

We first examined Glo1 genetic amplification and up-regulation of Glo1 expression in HCC tumor cells. For this, fifty paired fresh tumor tissues and adjacent non-tumorous tissues were collected from patients after surgery in Hangzhou first people's hospital. The media age of these patients were 58.0 years (range, 32-83) and majority were male (n = 41; 82%). Of them, 86% of patients (n = 43) had history of HBV infection, 2% of patients (n = 1) had history of HCV infection. With FISH analysis performed in tissue sections, we identified Glo1 gene amplification (defined as ratio of Glo1/CEP6 probes > 2 or cluster signals > 10%) in 3 (6%) of fifty tumor samples (**Figure 1A**), whereas paired adjacent non-tumorous tissues only showed normal copy number of Glo1 gene in nuclear.

qRT-PCR analysis showed that Glo1 expression were significantly up-regulated in 24 tumor tissues (48%) when compared to their adjacent non-tumorous tissue controls (log2 transformed fold change > 1). However, as shown in **Figure 1B**, we also noticed that Glo1 were lower expressed in tumor tissues from two patients (log2 < -1). Statistical analysis showed a clear association of Glo1-upregulation with elevated serum level (> 45 ng/ml) of alpha-fetoprotein (AFP) ($P = 0.0046$, χ^2 likelihood ratio test). No statistic significances were found with Glo1 up-regulation and patient gender, age, tumor stage or HBV infection (**Table 1**). However, we noticed

that the incidence of HCC expressing high level of Glo1 were 1.2 folds higher than that of HCC with non up-regulated level of Glo1 expression in HCC with TNM stage of 3/4, indicating a potential association of Glo1 expression with HCC progress and TNM staging (**Table 1**). Of note, we also found that all three tumor tissues with genetic Glo1 amplification had up-regulated expression of Glo1 mRNA.

shRNA knocking-down of Glo1 expression inhibits cell growth in primarily culture HCC cells with multiple copies of Glo1 gene

To test the potential role of Glo1 expression on HCC cell growth, we performed primary HCC cell cultures from surgically resected tumor tissues from HCC patients. Of these primarily cultured HCC cells, we found that MHCC45 HCC cells had multiple copies of Glo1 gene in genome (**Figure 2A**). MHCC45 cells grew as adherent monolayer, and were maintained in regular cell culture medium for more than 12 months with > 50 passages before it was used for shRNA experiments and for establishment of tumor xenograft. Microsatellite analysis showed genetic background of MHCC45 cells matched that of the original tumor that it was derived from (**Figure 2B**). MHCC45 cells also expressed high-level of Glo1 mRNA when compared to its original paired adjacent non-tumorous tissue. In shRNA experiments, we also included ATCC-established HuH-7 cells as control which carried normal copy of Glo1 gene and moderate level of Glo1 mRNA expression (**Figure 2C**).

We stably transfected these cells with inducible Glo1 shRNA, and treated engineered cells with doxycycline to induce Glo1 shRNA expression. We found that shRNA knocking-down of Glo1 expression significantly reduced the proliferation and inhibited the clonogenic survival of MHCC45 cells *in vitro*. Glo1 knockdown also increased CC3 level, an apoptotic marker, in MHCC45 when compared to corresponding control cells. However, no similar effects were observed in Huh-7 cells that has normal genetic copy number of Glo1 gene and moderate expression level of Glo1 (**Figure 3**).

Knockdown of Glo1 expression inhibits tumor growth in xenografts in vivo

To investigate the role of Glo1 activity on tumor growth, we also established a xenograft with MHCC45 cells expressing tet-controlled Glo1 shRNA. Our data showed that treatment with doxycycline led to obvious tumor growth inhibition by 32.3%, 75.4% and 68.2% in xenograft groups of Glo1-sh-RNA-A, B, C when compared to corresponding control groups ($P < 0.05$) (**Figure 4A**). As expected, doxycycline treatment did not cause growth inhibition in MHCC45 xenograft control that had no Glo1 shRNA. In tumors excised from Glo1-shRNA-B xenografts, we also noticed significant down-regulation of Glo1 expression in tumor cells from mouse treated with doxycycline (56% reductions in Glo1 staining, $P < 0.05$), but not in doxycycline-untreated control cells. Doxycycline treatment also led to increased staining of cleaved caspase 3 (CC3) (25% of cells IHC-positive in Dox group, $P < 0.05$), and decreased staining of Ki67 (72% of cells IHC-negative in Dox group, $P < 0.05$) (**Figure 4B**). These results suggested that knockdown of Glo1 expression inhibits *in vivo* tumor growth and increases apoptosis in MHCC45 cells. Taken together, our data demonstrated that Glo1 plays important role in cell proliferation and apoptosis in HCC cells with Glo1 gene amplification.

Intracellular methylglyoxal level increases in HCC primary cells after Glo1 knockdown

To test whether the inhibitory effect of Glo1 knockdown on cell proliferation is caused by toxic methylglyoxal accumulation, we examined the methylglyoxal levels in MHCC45 cells. As expected, we found that methylglyoxal levels significantly accumulated in cells with treatment of doxycycline for 72 hours, when compared to control cells that were not treated with doxycycline (**Figure 5**).

Discussion

Glo1 and glyoxal 2 (Glo2) comprise the glyoxalase system which catalyses the conversion of methylglyoxal to D-lactate via the intermediate S-D-lactoylglutathione. Previous studies demonstrated that Glo1 expression was up-regulated in some of human tumors, and Glo1 activity was involved in the regulation of tumor cell survival [25-31]. Studies also revealed that tumors with high expression of Glo1 were sensitive to Glo1 inhibitors. In addition, a link between high expression of Glo1 in tumors and multiple drug resistance (MDR) in cancer chemotherapy was also discovered [25, 32, 33].

Recently, genetic aberration of Glo1 gene has been reported in different types of cancers. Amplification of Glo1 gene was found to be a frequent genetic event in breast cancer, sarcoma and non small cell lung cancer [25]. However, whether Glo1 gene is also genetically amplified and the potential therapeutic effects for targeting Glo1 in HCC remain unclear. In this study, we examined Glo1 gene amplification and expression in HCC tumor tissues. Our results showed that Glo1 gene amplification is also a genetic event in Chinese HCC patients with incidence rate of 6% (3/50) in cohorts. When compared to paired non-tumorous tissues, 48% (24/50) of HCC tumor expresses higher level of Glo1, which is consistent with the result from a recent study showing that Glo1 was up-regulated in HCC [34]. Interestingly, we noticed that all three primary tumor samples that have genetic Glo1 amplifications express high levels of Glo1 mRNA, indicating that genetic amplification of Glo1 gene may correlate with up-regulation of Glo1 expression in some human tumors. In addition, we found that Glo1 high expression is clearly associated with up-regulated serum level of AFP, and may also correlated to higher HCC TNM staging, suggesting that Glo1 may play essential roles for HCC tumor cell proliferation and progress.

With shRNA interfering of Glo1 gene, we found that down-regulation of Glo1 expression could significantly inhibit both tumor cell proliferation or tumor growth in HCC cells carrying genetic amplification and high expression of Glo1 gene. The inhibitory effects of Glo1 shRNA were further demonstrated to be associated with accumulated methylglyoxal and increased cellular apoptosis. We also noticed that shRNA interfering of Glo1 expression in HCC HuH-7 cells with normal genetic copy and moderate expression level had no obvious effects on cell proliferation and apoptosis. In addition, Sakamoto et al reported that NCI-H23 cells that have low gene copy number of Glo1 but express high level of Glo1 mRNA were not sensitive to Glo1 inhibition [33]. Thus, these results suggest a potential role of targeting Glo1 amplification in clinical HCC treatment.

Recent studies suggested a vital role of Glo1 against cellular damage by removal of methylglyoxal (MG), an essential component in glycolysis and is known to be an inducer of apoptosis in tumor cells [35, 36]. However, few data collected in HCC. HCC, one typical solid tumor with the elevated glucose metabolism, is demonstrated to have high glycolytic rates and be accompanied by increased MG formation. Therefore, accumulation of cytotoxic MG may cause cell growth inhibition through apoptosis in HCC cells. Indeed, our data showed higher MG accumulation and increased apoptosis induction in HCC cells with Glo1 knockdown interfering.

In conclusion, our data demonstrated that Glo1 gene amplification is also a genetic event in Chinese HCC. Tumor cells with genetic amplified Glo1 gene express higher level of Glo1, and are more sensitive to the cell killing effects of targeted down-regulation of Glo1 expression. These data strongly support the potential of Glo1 as therapeutic target and Glo1 inhibitors as therapeutic agents HCC patient with genetic Glo1 amplification. However, because of limited case number and incidence percentage of tumor with genetic Glo1 amplification included in this cohort study, the potential for future clinical development of a therapeutic strategy targeting Glo1 gene requires further investigation.

Disclosure of conflict of interest

None.

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