ORIGINAL ARTICLE

Glutamine synthetase mediates sorafenib sensitivity in β -catenin-active hepatocellular carcinoma cells

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The gene encoding β -catenin is frequently mutated in hepatocellular carcinoma cells. While the oncogenicity of β -catenin has been extensively studied, β -catenin's role in hepatocellular carcinoma tumor metabolism is currently less well understood. In this study, we found that β -catenin regulates the expression of glutamine synthetase and triggers a series of metabolic changes leading to induction of autophagy in hepatocellular carcinoma cells. β -Catenin-active Hep3B and HepG2 cells exhibited higher basal levels of autophagic activity than did β -catenin wild-type cells. We also found that autophagy in β -catenin-active cells was mediated by glutamine synthetase, as silencing of glutamine synthetase significantly reduced autophagic activity. We also showed that β -catenin-active hepatocellular carcinoma cells were more sensitive to sorafenib than were β -catenin wild-type cells. Our results demonstrated that glutamine synthetase-mediated autophagy explains the high sensitivity of β -catenin-active hepatocellular carcinoma cells to sorafenib. Our results highlight the importance of glutamine metabolism in the regulation of autophagy in hepatocellular carcinoma cells. More importantly, our study unravels the molecular mechanisms leading to sorafenib sensitivity in hepatocellular carcinoma.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the deadliest cancers in the world. HCC tumors are often advanced at diagnosis, and treatment options are limited. Therefore, overall mortality rates for HCC patients are higher than those for patients with many other cancers.^{1–3} At present, surgical resection is the preferred therapy for HCC. However, only about 15% of HCC patients are eligible for surgical resection, and up to 70% of patients who undergo resection experience disease recurrence within 5 years of surgery.⁴ Therefore, development of more individualized and effective systemic therapies is necessary for HCC patients who are not eligible for surgery and those with recurrence after surgery.⁵ Sorafenib, currently the only approved drug for first-line systemic treatment of advanced HCC, is an orally active multikinase inhibitor. While randomized phase III trials demonstrated that sorafenib has clinically significant anticancer activity,^{6,7} its survival benefits were only modest, suggesting that not all patients benefit from sorafenib treatment.

The liver's physiological homeostasis is maintained through high levels of metabolic activity in hepatocytes, which is

regulated through structural and functional units of the liver called hepatic lobules. The hepatocytes within a lobule express different transcriptional programs depending on which metabolic zone they are located in.⁸ Hepatocytes in the periportal metabolic zone express genes involved in gluconeogenesis and fatty acid metabolism, whereas hepatocytes in the perivenous zone express genes involved in detoxification. β -catenin is a master regulator of metabolic zonation in the liver, and its activity is limited in the perivenous zone, where it maintains the perivenous-specific transcriptome program.⁹ Glutamine synthetase (GS) is a metabolic enzyme that produces glutamine from glutamate and ammonia and is involved in detoxification of ammonia in the liver. Because GS is part of the perivenous transcriptome, its expression is restricted to hepatocytes in the perivenous zone and is tightly regulated by β -catenin.^{9–11} The gene encoding β -catenin, *CTNNB1*, is a frequently activated oncogene in HCC; its aberrant activation is largely attributable to somatic mutations, which occur in 20–30% of HCC patients.^{10–13} In the current study, we discovered unexpected roles of β -catenin and its downstream metabolic target gene that may be associated with treatment response in HCC patients.

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MATERIALS AND METHODS

Cell lines and cell culture

SK-Hep1, HepG2, Hep3B and Huh7 human HCC cells were obtained from the Health Science Research Resources Bank (Osaka, Japan), and FOCUS HCC cells were kindly provided by Dr Snorri S. Thorgeirsson at the National Cancer Institute (Bethesda, MD, USA). The cells were maintained in Dulbecco's modified Eagle's medium with high glucose and 4 mM glutamine (Invitrogen, Carlsbad, CA, USA) supplemented with heat-inactivated 10% fetal bovine serum and penicillin/streptomycin (Invitrogen).

Lentiviral GS overexpression vector and short hairpin RNAs

A GS-overexpressing lentiviral vector was constructed by cloning a full-length coding DNA fragment into the *NheI*/*NotI* sites of a pCDH-EF1-T2A-Puro vector (System Biosciences, Mountain View, CA, USA). Lentiviral-based short hairpin RNAs (shRNAs) targeting human GS (NM_002065.3-802s1c1, NM_002065.3-1450s1c1 and NM_002065.4-1890s21c1) and control shRNA targeting enhanced green fluorescent protein (GFP) (cat. no. SHC005V) were used for knockdown experiments.

Lentiviral transduction

To produce lentiviral particles, the GS overexpression vector or shRNA expression vectors were co-transfected with the lentiviral packaging plasmids pLP1, pLP2 and pLP/VSVG (Invitrogen) into 293FT lentivirus packaging cells. Lipofectamine 2000 (Invitrogen) was used as the transfection reagent. At 48–72 h after transfection, the virus-containing cell culture medium was harvested and frozen in aliquots. A moderate multiplicity of infection was used for transduction of cells to minimize negative effects on cell proliferation. All experiments were performed 2–4 days after infection

Figure 1 Sensitivity to sorafenib is associated with glutamine synthetase (GS) expression levels in hepatocellular carcinoma (HCC) cells. **(a)** Representative western blots showing expression of GS in five HCC cell lines. **(b)** Results of cell viability assays performed 48 h after treatment with the indicated doses of sorafenib. IC₅₀ values were compared using a Student *t* test. Data shown are means $\pm$ s.e.'s. of the mean from three independent experiments. **P* < 0.01.

Antibodies

Antibodies against phospho-p70 S6 kinase (Thr389), p70 S6 kinase, phospho-S6 (Ser235/236), S6, LC3B and α -tubulin were purchased from Cell Signaling Technology (Danvers, MA, USA; cat. nos. 9205, 9202, 4858, 2217, 2775 and 3873). Antibodies against GS were obtained from EMD Millipore (Danvers, MA, USA; cat. no. MAB302). Antibodies against β -actin were purchased from Sigma-Aldrich (St. Louis, MO, USA; cat. no. A5441). Horseradish peroxidase-conjugated anti-mouse immunoglobulin G and horseradish peroxidase-conjugated anti-rabbit immunoglobulin G were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA; cat. nos. sc-2748 and sc-2749).

MTT cell viability assay. To assess cell viability. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich) was performed as described previously^{5,6} using a VMax kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 570 nm. Cells were fixed in 10% formaldehyde and stained with 10% crystal violet (Sigma-Aldrich) for 10 min at ambient temperature.

Flow cytometry

Samples were collected at 48 h after sorafenib treatment and fixed in 70% ethanol overnight. To measure the proportion of apoptotic cells, we used an Annexin V apoptosis detection kit (Santa Cruz Biotechnology) according to the manufacturer's protocol. Cells were stained with Annexin V-fluorescein isothiocyanate and DAPI after sorafenib treatment. Cells were evaluated for apoptosis via flow cytometry using an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA).

Autophagic puncta analysis

SK-Hep1 cells were stably transfected with pEGFP-LC3 and pDsRed2-C1 or pDsRed2-GS constructs and plated on coverslips. The plated cells were exposed to sorafenib for 24 h and then fixed with 4% paraformaldehyde in phosphate-buffered saline. Autophagic puncta were counted using an ECLIPSE TE2000-E fluorescence microscope (Nikon, Melville, NY, USA).

RESULTS

β -catenin-active HCC cells are sensitive to sorafenib

Because expression of GS is tightly regulated by β -catenin in perivenous hepatocytes and reflects β -catenin activity,^{9–11} we determined the activation status of β -catenin by assessing GS expression in five HCC cell lines. HepG2 cells were used as positive controls for assessments of β -catenin activation because β -catenin in HepG2 cells is known to be constitutively activated by deletion mutations in negative regulatory sites.¹³ As expected, GS was highly expressed in HepG2 cells (Figure 1a). GS was also highly expressed in Hep3B cells, suggesting that β -catenin is highly active in this cell line. This observation is in good agreement with a previous study demonstrating that β -catenin is highly active in Hep3B and that the tumorigenicity of xenografted Hep3B cells depends on β -catenin expression.¹⁴ In contrast, expression of GS was absent in FOCUS, SK-Hep1 and Huh7 cells, indicating that β -catenin is not active in these cell lines.

HCC with activated β -catenin has a distinct clinical course; studies showed that HCC patients with activated β -catenin

have better prognoses than do those with wild-type β -catenin.^{15,16} Therefore, we determined whether β -catenin-active HCC cells and HCC cells with wild-type β -catenin responded differently to sorafenib.^{6,7} To measure the half maximal inhibitory concentration (IC_{50}) of sorafenib in each HCC cell line, we treated five HCC cell lines with various concentrations of sorafenib for 48 h and counted the viable cells. Interestingly, the two HCC cell lines with activated β -catenin, HepG2 and Hep3B, were more sensitive to sorafenib than were those with inactive β -catenin activity (Figure 1b). The IC_{50} values of sorafenib in β -catenin-active HepG2 and Hep3B cells were two to three times lower than in cell lines with inactive β -catenin ($P<0.001$ for all comparisons), suggesting a link between β -catenin activation and sensitivity to sorafenib.

Sensitivity to sorafenib in β -catenin-active HCC cells is mediated by GS

Because the most distinctive liver-specific function of β -catenin is control of glutamine metabolism through expression of GS,¹⁷ we next tested whether GS plays a role in sorafenib sensitivity in β -catenin-active HCC cells. First, we assessed sorafenib sensitivity after silencing GS expression with lentiviral shRNAs in GS-high HCC cell lines (HepG2 and Hep3B). When GS was depleted by transduction with two GS-specific shRNAs, HepG2 cells develop resistant to sorafenib (Figure 2a). The IC_{50} value of sorafenib increased from 5.4 μ M in parental HepG2 cells to 9.1 μ M and 9.9 μ M (both $P<0.001$) in HepG2 cells with silenced GS expression ($n=3$). Similarly, depletion of GS via transduction with two shRNAs made Hep3B cells resistant to

sorafenib ($P<0.001$; Figure 2b), strongly suggesting that GS regulates the sensitivity of HCC cells to sorafenib.

We next overexpressed GS in GS-low SK-Hep1 cells by transducing them with a lentivirus harboring an exogenous GS gene. We examined the viability of the transduced cells after 48 h of sorafenib treatment. GS overexpression sensitized SK-Hep1 cells to sorafenib (Figure 3a). The IC_{50} value decreased from 11.9 μ M in parental SK-Hep1 cells to 4.7 μ M in GS-overexpressing cells ($n=3$; $P<0.001$). GS overexpression also significantly sensitized FOCUS cells to sorafenib (Figure 3b; $P<0.001$), confirming that the sensitivity of β -catenin-active HCC cells to sorafenib is mediated by GS.

Finally, we examined apoptosis rates in GS-overexpressing SK-Hep1 cells using flow cytometry. Sorafenib treatment induced apoptosis at a significantly higher rate in GS-overexpressing SK-Hep1 cells than in low-GS parental SK-Hep1 cells ($P<0.001$, Figure 3c and d). Because exogenous overexpression of GS had little effect on the apoptosis rate in untreated SK-Hep1 cells, it is unlikely that the observed increase in sensitivity to sorafenib could be attributed to GS-mediated toxicity in HCC cells. Therefore, these results suggested that sensitivity to sorafenib strongly depends on GS expression levels in HCC cells.

GS induces autophagy

A previous study showed that increased intracellular production of glutamine was correlated with induction of autophagy,¹⁸ suggesting that β -catenin-active HCC cells should exhibit high levels of autophagy. Autophagic activity is also known to be high in the perivenous zone, where β -catenin is highly active.¹⁹ Thus, we next tested whether β -catenin induces autophagy in HCC cells.

We estimated basal autophagy levels by measuring the conversion of microtubule-associated light chain 3 (LC3-I) to its lipidated form (LC3-II), which is a key step in autophagosome formation and a biomarker of autophagy initiation.²⁰ As expected, basal levels of autophagic activity as represented by the LC3-I/LC3-II ratio were substantially higher in β -catenin-active Hep3B cells than in the other cell lines (Figure 4a). The LC3-II level in Hep3B cells was several times higher than that in SK-Hep1 and FOCUS cells. Because GS is a major metabolic target of β -catenin in HCC cells, we next determined whether the high levels of GS expression in Hep3B cells accounted for their high basal level of autophagic activity. We assessed autophagic activity by measuring the LC3-II/LC3-I ratio in GS-silenced Hep3B cells and GS-overexpressing SK-Hep1 cells. Depletion of GS in Hep3B cells by transduction with two independent shRNAs reduced LC3-II formation (Figure 4b). Conversely, exogenous GS expression increased autophagic activity in SK-Hep1 cells (Figure 4c). To verify autophagy induction, we generated a transient construct that expressed GFP/LC3 and transfected it into SK-Hep1 cells. Fluorescence microscopy showed that GFP-LC3 was expressed diffusely in the cytoplasm of mock-transfected SK-Hep1 cells. However,

Figure 2 Depletion of glutamine synthetase (GS) expression decreased sorafenib sensitivity in hepatocellular carcinoma (HCC) cells. Results of cell viability assays performed 48 h after sorafenib treatment in HepG2 (a) and Hep3B (b) cells transduced with GS-silencing shRNAs (shGS1, shGS2) or a control shRNA against green fluorescent protein (shGFP). IC_{50} values were compared using a Student *t* test. Data shown are means $\pm$ s.e.s. of the mean from three independent experiments. * $P<0.001$.

Figure 3 Glutamine synthetase (GS) overexpression significantly increased sorafenib sensitivity in hepatocellular carcinoma (HCC) cells. (a, b) Results of cell viability assays performed 48 h after sorafenib treatment in GS-overexpressing (GS_{OE}) or mock-transfected (Mock) SK-Hep1 and FOCUS cells. IC₅₀ values were compared using a Student *t* test. Data shown are means ± s.e.s. of the mean from three independent experiments. **P* < 0.001. (c) Results of flow cytometry apoptosis assays using Annexin V-FITC and DAPI staining in SK-Hep1 cells with (GS_{OE}) or without (MOCK) overexpression of GS after treatment with the indicated concentrations of sorafenib or dimethyl sulfoxide (DMSO) control. (d) Quantified flow cytometry data from three independent experiments. Data are presented as means ± s.e.s. of the mean. *P* values were determined by Student *t* test.

GFP-LC3 formed autophagic puncta in GS-expressing SK-Hep1 cells (Figure 4d), indicating that the increased formation of autophagosomes was mediated by GS. These results suggest that GS expression induces autophagy in HCC cells.

Sorafenib sensitivity is mediated by autophagy

The results of previous studies indicate that sorafenib can induce autophagy in HCC cells and that excessive autophagy can induce cell death.^{21,22} Thus, we hypothesized that the increase in sorafenib sensitivity observed in GS-expressing HCC cells is largely attributable to excessive autophagy that is jointly mediated by GS and sorafenib. Consistent with the results of previous reports,^{21,22} we found that sorafenib treatment increased autophagic activity in Hep3B cells and that GS depletion via knockdown using shRNAs substantially reduced sorafenib-induced autophagy (Figure 5a). These

findings strongly suggest that the reduction in autophagic activity caused by GS inactivation explains resistance to sorafenib in HCC cells. To validate this conclusion, we overexpressed GS in SK-Hep1 cells and measured LC3-II using western blot analysis. Consistently, expression of the autophagy marker LC3-II was substantially increased by exogenous overexpression of GS (Figure 5b), and this increase in autophagic activity coincided with increased sorafenib sensitivity in SK-Hep1 cells (Figure 3a). These findings suggest that the high sensitivity of β-catenin-active HCC cells to sorafenib is mediated by their high basal level of GS-induced autophagy. In agreement with the results of the western blot analysis, analysis of autophagic puncta demonstrated that exogenous GS overexpression increased GFP-LC3 punctate formation in sorafenib-treated SK-Hep1 cells (Figure 5c). These results further validated our conclusion that the increased formation of autophagosomes was indeed mediated by GS. Taken

Figure 4 Glutamine synthetase (GS) activates autophagy in hepatocellular carcinoma (HCC) cells. **(a)** Western blots showing that high-GS Hep3B cells had higher LC3-II/LC3-I ratios than did low-GS FOCUS and SK-Hep1 cells. Expression of GS and autophagy markers LC3-I and LC3-II in HCC cells was assessed using western blotting. The LC3-II/LC3-I ratios and LC3-II levels were analyzed by quantitative densitometry ($n=3$ independent experiments). **(b)** Western blots showing that GS knockdown decreased autophagic activity in Hep3B cells. Autophagy was measured by western blotting for LC3-I and LC3-II in Hep3B cells after GS expression was depleted by transduction with shRNAs shGS1, shGS2 or shGFP. The LC3-II/LC3-I ratios and LC3-II levels were analyzed as in **a**. **(c)** Western blots showing increased autophagic activity in GS-overexpressing (GS_{OE}) SK-Hep1 cells. The LC3-II/LC3-I ratios were measured as in **a**. **(d)** Representative cell images showing autophagic puncta as speckles of LC3-GFP in GS-overexpressing SK-Hep1 cells. SK-Hep1 cells were stably transfected with pEGFP-LC3 (LC3-GFP), pEGFP-LC3 and pDsRed-C1 (RFP), or pEGFP-LC3 and pDrRed-GS (GFP-LC3) and analyzed using fluorescence microscopy. Scale bars, 20 μ m.

Figure 5 Glutamine synthetase (GS) expression augments autophagy induced by sorafenib treatment. **(a)** Expression of autophagy markers LC3-I and LC3-II induced by sorafenib treatment was assessed using western blotting. Autophagic activity was decreased by GS knockdown with shGS2 RNA in Hep3B cells. shGFP was used as a control. Cells were treated with sorafenib at the indicated concentrations for 48 h. The LC3-II/LC3-I ratios and LC3-II levels were measured by quantitative densitometry ($n=3$ independent experiments). **(b)** Western blots showing that autophagic activity induced by sorafenib treatment was increased by GS overexpression in SK-Hep1 cells. The LC3-II/LC3-I ratios were measured as in **a** after induction of GS overexpression. **(c)** Representative fluorescence microscopy images showing autophagic puncta as speckles of LC3-GFP after sorafenib treatment in GS-overexpressing SK-Hep1 cells. SK-Hep1 cells were stably transfected with pEGFP-LC3 (LC3-GFP), pEGFP-LC3 and pDsRed-C1 (RFP), or pEGFP-LC3 and pDrRed-GS (GFP-LC3) and treated with 2.5 μ M sorafenib for 24 h. Scale bars, 20 μ m.

together, these results strongly support the notion that sorafenib sensitivity in β -catenin-active HCC cells is mediated by GS, which triggers a high basal level of autophagy.

DISCUSSION

In the current study, we identified a novel GS-mediated mechanism by which β -catenin induces autophagy. This

finding is similar to recent findings that the oncogenes *MYC* and *RAS* can stimulate autophagy.^{23,24,25} Because autophagy is activated to relieve cells in stress conditions, such as those under metabolic or environmental stress, autophagy generally promotes cell survival. In fact, autophagy induction by oncogenic *RAS* is necessary for cancer cell survival and oncogenic transformation.²⁶ Our data strongly suggest that β -catenin activation relieves stress in HCC cells and promotes cell survival through activation of a liver-specific transcriptome involving GS.

Although sorafenib is the only approved systemic drug for the treatment of patients with HCC, its efficacy is limited, as evidenced by the only 2- to 3-month increase in survival duration observed in clinical studies.^{6,7} Only a small subset of patients experiences a response to treatment. Sorafenib is well known to exert its antitumor effects by inhibiting several kinases. It also induces autophagy as other chemotherapy agents.^{21,22} While autophagy is best known as a pro-survival mechanism, recent studies have shown that prolonged autophagy impedes cell recovery and survival, triggering cell death.^{27,28} HCC cells with high levels of GS expression may be particularly sensitive to sorafenib because they already have high basal levels of autophagy. Our data clearly demonstrated that β -catenin-activated HCC cells were more sensitive to sorafenib. We further demonstrated that sensitivity to sorafenib was mediated by high GS expression, which is a direct downstream target of β -catenin in HCC cells.

Taken together, our data suggest that HCC with activated β -catenin is not only a genetically distinct subtype of HCC but also a clinically distinct subtype of this disease. Understanding how a tumor's genetic subtype may affect its sensitivity to standard treatment may be useful for guiding treatment decisions. For instance, patients with HCC with activated β -catenin mutations may be more likely to benefit from sorafenib treatment.

In summary, we uncovered a connection between a liver-specific transcriptome regulated by β -catenin and the sensitivity of HCC cells to sorafenib, the standard treatment for HCC. Our results highlight the importance of glutamine metabolism in the regulation of autophagy in HCC cells. When activated by oncogenic mutations that lead to hepatotumorigenesis, β -catenin also activates a liver-specific transcriptome program that produces GS overexpression in HCC cells. More importantly, high GS expression in β -catenin-active HCC increases autophagy by modulating metabolic flux, which contributes to increased sorafenib sensitivity. Collectively, our results suggest that β -catenin-regulated metabolic activity may dictate clinical outcomes for patients with HCC.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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