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Cancer Research

Induction of DNA Damage-Inducible Gene GADD45β Contributes to Sorafenib-Induced Apoptosis in Hepatocellular Carcinoma Cells

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Abstract

Markers that could accurately predict responses to the general kinase inhibitor sorafenib are needed to better leverage its clinical applications. In this study, we examined a hypothesized role in the drug response for the growth arrest DNA damage-inducible gene 45β (GADD45β), which is commonly underexpressed in hepatocellular carcinoma (HCC) where sorafenib may offer an important new therapeutic option. The anticancer activity of sorafenib-induced GADD45β expression was tested in a panel of HCC cell lines and xenograft models. We found that GADD45β mRNA and protein expression were induced relatively more prominently in HCC cells that were biologically sensitive to sorafenib treatment. GADD45β induction was not found after treatment with either the mitogen-activated protein kinase–extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor U0126 or the Raf inhibitor ZM336372, suggesting that GADD45β induction by sorafenib was independent of Raf/MEK/ERK signaling activity. However, c-Jun NH_2-terminal kinase (JNK) kinase activation occurred preferentially in sorafenib-sensitive cells. Small interfering RNA–mediated knockdown of GADD45βor JNK kinase limited the proapoptotic effects of sorafenib in sorafenib-sensitive cells. We defined the −339/−267 region in the GADD45β promoter containing activator protein-1 and SP1-binding sites as a crucial region for GADD45β induction by sorafenib. Together, our findings suggest that GADD45β induction contributes to sorafenib-induced apoptosis in HCC cells, prompting further studies to validate its potential value in predicting sorafenib efficacy. *Cancer Res; 70(22); 9309–18. ©2010 AACR.*

Introduction

Sorafenib is the first molecular targeted agent that shows survival benefit for patients with advanced hepatocellular carcinoma (HCC; refs. 1, 2). Clinical trials of sorafenib in different stages of HCC and in combination with other treatment approaches are under way to find better treatment strategies for this difficult disease.

Although sorafenib represents a major breakthrough in the treatment of advanced HCC, several important issues need to be addressed to facilitate rational clinical trial design. First, the downstream mediators of the antitumor effects of sorafenib should be clarified. Sorafenib was developed in a program that was designed to find specific Raf kinase inhibitors (3). However, preclinical models indicate that the antitumor activity of sorafenib does not correlate completely with its inhibitory effects on Raf/mitogen-activated protein kinase (MAPK)–extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK activity (4). Both MEK/ERK-dependent and -independent mechanisms have been reported to account for the antitumor activity of sorafenib in various tumor models (5–8). Identification of signaling pathways that are critical for the antitumor efficacy of sorafenib will help the rational design of combination therapy for HCC (9).

Second, biomarkers that can predict clinical efficacy after sorafenib treatment should be developed. Previous studies on Raf/MEK/ERK signaling activity in HCC tumor cells yielded inconsistent results, and the predictive value of Raf/MEK/ERK signaling activity for the efficacy of sorafenib in HCC remains uncertain (10–12). In addition, the main effects of sorafenib in advanced HCC are seen in tumor stabilization and survival prolongation. It is difficult to measure efficacy in individual patients because the objective response rate of sorafenib is low (2–3%; refs. 1, 2). Surrogate biomarkers to predict the biological and clinical efficacy of sorafenib will help tailor treatment on an individual patient basis.

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Growth arrest DNA damage-inducible gene 45β (GADD45β) belongs to a family of small-molecule (~18 kDa) proteins that play important roles in cellular stress response, survival, senescence, and apoptosis regulation (13). GADD45 family proteins are frequently underexpressed in various types of cancers, including HCC (9, 14, 15). GADD45 family proteins are also important mediators of genotoxic stress-induced and transforming growth factor-β (TGF-β)–induced apoptosis (16–18). Recently, we found that GADD45β expression was induced in HCC cells after sorafenib treatment, and this induction was independent of MEK/ERK signaling activity (19). We hypothesized that GADD45β may be an important mediator of sorafenib-induced apoptosis in HCC cells. The present study aimed to explore the biological significance and possible mechanisms of sorafenib-induced GADD45β expression in HCC cells.

Materials and Methods

Cell culture

The HCC cell lines HepG2 and Hep3B were obtained from the American Type Culture Collection, and the Huh-7 cell line was from the Health Science Research Resources Bank. A sorafenib-resistant cell line, Huh-7R, was generated by continuous treatment of Huh-7 cells with sorafenib up to 10 μmol/L. Cells were cultured in DMEM containing 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), L-glutamine (2 mmol/L), and sodium pyruvate (1 mmol/L) at 37°C in a humidified incubator containing 5% CO_2. Primary human umbilical vascular endothelial cells (HUVEC) were cultured as described before (5).

Chemicals and other reagents

Sorafenib was provided by Bayer-Schering Pharma. The MEK inhibitor U0126, the Raf inhibitor ZM336372, and the c-Jun NH_2 terminal kinase (JNK) inhibitor SP600125 were purchased from Calbiochem, Merck KGaA, and AG Scientific, respectively. For *in vitro* experiments, the chemicals were dissolved in DMSO, and the final concentration of DMSO was kept below 0.1%. For *in vivo* experiments, sorafenib was dissolved in Cremophor EL/95% ethanol (50:50; Sigma-Aldrich). The antibodies used for Western blotting, immunohistochemical staining, and chromatin immunoprecipitation (ChIP) assays included GADD45β (AV48346 from Sigma-Aldrich and H-70 from Santa Cruz), ERK-2, phospho-ERK-1/2 (Santa Cruz Biotechnology), JNK, phospho-JNK (Thr^{183}/Tyr^{185}), c-Jun, phospho-c-Jun (Ser^{63}), phospho-c-Jun (Ser^{73}; Cell Signaling Technology), lamin B, α-tubulin (Calbiochem), and SP1 (Abcam).

Cell viability and apoptosis assays

Cell viability was assessed using an MTT assay as previously described (5). The IC_{50} values after drug treatment were calculated using CompuSyn software (ComboSyn) based on the changes of absorbance measured by spectrophometry (DTX 880; Beckman Coulter). The fraction of apoptotic cells after drug treatment was assessed by sub-G_1 fraction analysis and Annexin V analysis using flow cytometry (5). The potential synergistic or antagonistic antitumor effects between different drug treatments were measured by median dose-effect analysis using the combination index (CI)–isobologram method (20).

Western blot analysis

Whole-cell lysates of HCC cells after drug treatment were prepared and quantified as previously described (5). Nuclear and cytoplasmic fractions were extracted with a CMN Compartment Protein Extraction kit (BioChain). SDS-PAGE and Western blot analysis were performed to measure protein expression. Signals were visualized using a UVP Imaging System (UVP) or with X-ray film.

Quantitative reverse transcription-PCR

RNA was extracted using Trizol reagent (Invitrogen). cDNAs were synthesized from total RNA (1 μg) using a high-capacity cDNA archive kit (Applied Biosystems) and quantified using the TaqMan-Universal or SYBR Green PCR Master Mix (Applied Biosystems) on an ABI PRISM 7900 sequence detection system (Applied Biosystems). The primers for the GADD45β and c-Jun genes were purchased from Applied Biosystems (ABI TaqMan assay ID: Hs00169587_ml and Hs00277190_s1). Primers for the hypoxanthine phosphoribosyltransferase (sense 5-TGACACTGGCAAAACAATGCA-3 and antisense 5-GGTCCTTTTCACCAGCAAGCT-3) gene or α-fetoprotein (AFP; ABI TaqMan Hs00173490_m1) gene were used as endogenous controls. Conditions for PCR were as follows: 50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles of 95°C for 15 seconds (denaturation) and 60°C for 1 minute (annealing/extension). The relative mRNA amount of the target/control genes was calculated using the ΔC_t (threshold cycle) method: relative expression = $2^{-\Delta Ct}$, where $\Delta C_t = C_t$ (target gene) − C_t (control gene).

Small interfering RNA knockdown of GADD45β expression

The GADD45β and scrambled nonspecific (negative control) small interfering RNAs (siRNA) were purchased from Ambion. The sequences for GADD45β siRNA are as follows: si-GADD45β-a, sense 5′-GCAUACGAGAGACUUGGUUtt-3′ and antisense 5′-AACCAAGUCUCUCGUAUGCag-3′; si-GADD45β-b, sense 5′-GAAUUAUAGAGACAAUCUAtt-3′ and antisense 5′-UAGAUUGUCUCUAUAAUUCgc-3′. Cells were transfected with GADD45β or scrambled siRNA using the siPORT NeoFx siRNA transfection reagent (Ambion), treated with the drugs at the indicated concentrations for 48 hours, and collected for subsequent Western blot or quantitative reverse transcription-PCR (qRT-PCR) analysis.

Overexpression of GADD45β in HCC cells

HCC cells were transiently transfected with the pCMV6-AC-GFP-GADD45β vector (RG213354; Origene Technologies) or empty vector (pCMV6-AC-GFP; Origene Technologies). Twenty-four hours after transfection, cells were treated with sorafenib (10 μmol/L) or control. Effects of GADD45β overexpression on the sensitivity of HCC cells to sorafenib were measured by flow cytometry.

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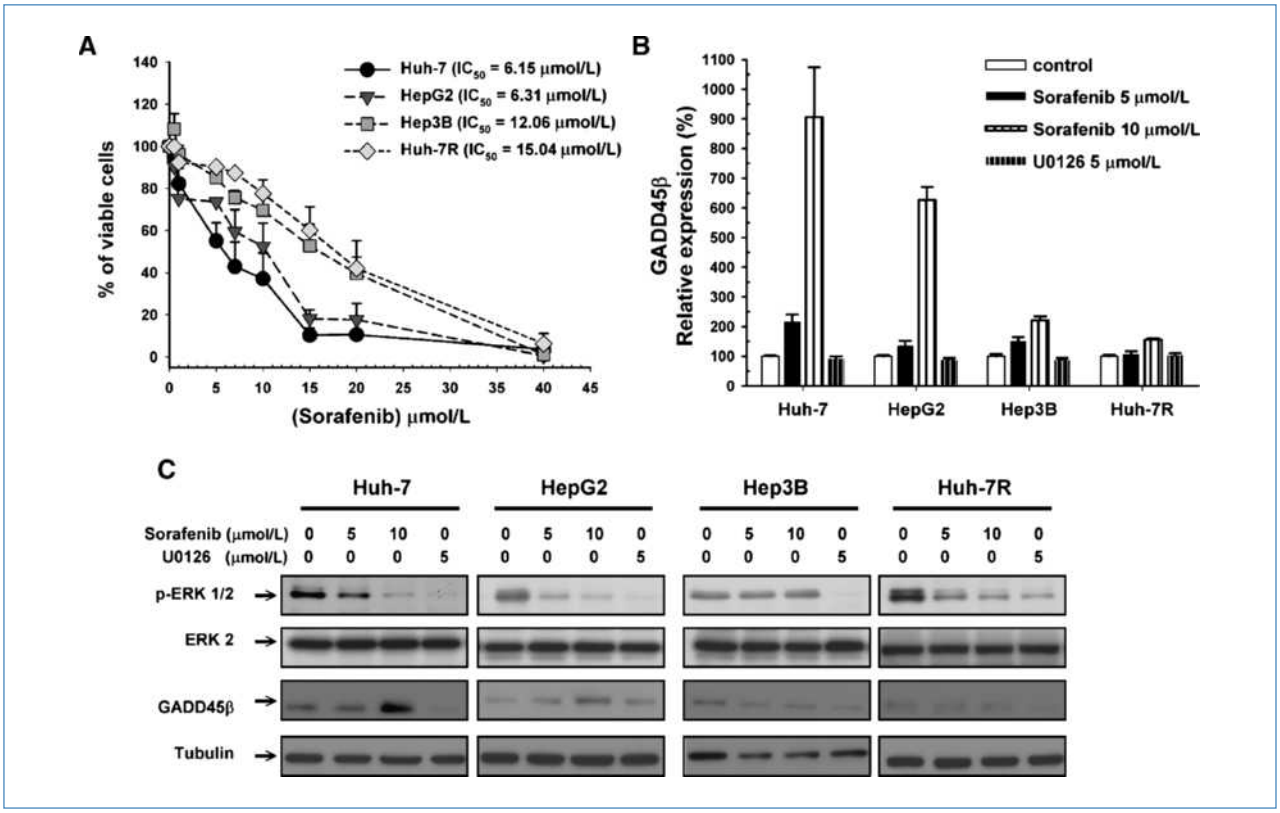

Figure 1. Sorafenib induces GADD45β expression in HCC cells independent of MEK/ERK inhibition. A, IC_{50} of HCC cell lines after sorafenib treatment. HCC cells were treated with sorafenib at the indicated concentrations in 96-well plates for 72 h, and cell viability was assessed by MTT assay. Points, mean (n = 3); bars, SD. B, GADD45β mRNA induction after sorafenib or U0126 treatment. HCC cells were treated for 24 h, and GADD45β mRNA levels were assessed by real-time qRT-PCR. C, GADD45β, total-ERK, and phospho-ERK protein levels in HCC cells after sorafenib or U0126 treatment. Whole-cell lysates after drug treatment were examined by Western blotting.

Luciferase reporter constructs for the GADD45β promoter

Proximal promoter fragments of GADD45β, spanning −736 to +3, were cloned upstream of the luciferase gene in the pLuc-MCS-base luciferase expression plasmid (Stratagene). Eleven different GADD45β promoter deletion fragments were generated by PCR using the sense and antisense primers listed in Supplementary Table S1. A *Hind*III site was incorporated into the sense primers, and a *Bgl*II site was incorporated into the antisense primers. The TFSEARCH program (version 1.3; http://www.cbrc.jp.reserach/db/TFSEARCH.html) was used to identify possible binding sites for transcription factors in the GADD45β promoter.

Site-directed mutagenesis was performed to obtain sequences that were mutated at either the activator protein-1 (AP-1m) or SP1 (SP1m) binding sites or mutated at both sites (AP-1m +SP1m) using a one-side splicing by overlap extension method (21). The primers used for site-directed mutagenesis are listed in Supplementary Table S1. Conditions for PCR were as follows: one cycle at 95°C for 15 minutes followed by 34 cycles at 95°C for 40 seconds, 60°C for 40 seconds, 72°C for 2 minutes, and a final cycle at 72°C for 10 minutes. Huh-7 genomic DNA was used as the PCR template. PCR products were digested with *Hind*III and *Bgl*II and purified by phenol-chloroform extraction and ethanol precipitation. Fragments were then cloned into the corresponding sites of the pLuc-MCS basic plasmid and confirmed by DNA sequencing.

Luciferase activity assay

HCC cells were transfected with individual GADD45β reporter constructs using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were treated with 10 μmol/L sorafenib and incubated for an additional 8 hours. Cell lysates were prepared to determine GADD45β promoter activity using a Luciferase Reporter Gene Assay kit (Packard) according to the manufacturer's instructions.

ChIP assay

Huh-7 cells (~5×10^6) with or without sorafenib treatment were used for ChIP using an EZ-ChIP assay kit (Millipore). PCR amplification was performed using primers spanning the c-Jun and SP1 site on GADD45β promoter from nucleotides −341 to −122 (forward 5′-TAGCTGCACTCGCCCTTGT-3′ and reverse 5′-GGGAAGCAGCGAAATCCTTC-3′).

Tumor xenograft experiments

The protocol for the *in vivo* studies was approved by the Institutional Animal Care and Use Committee of College of

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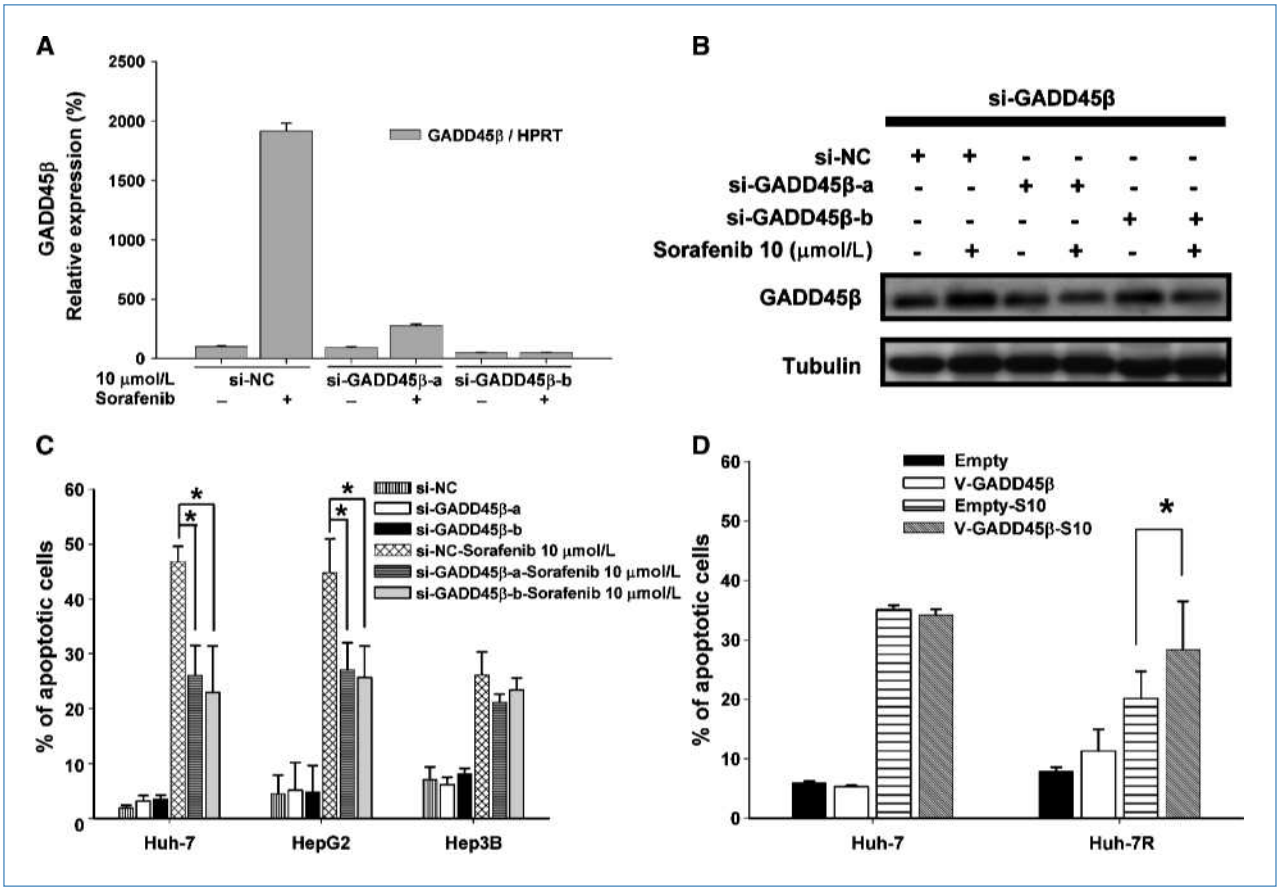

Figure 2. GADD45β knockdown partially abrogates the proapoptotic effects of sorafenib. A and B, efficacy of GADD45β knockdown was measured by quantitative RT-PCR (A) and Western blotting (B). Huh-7 cells were transfected with siRNA directed against GADD45β (si-GADD45β-a and si-GADD45β-b) or a negative control (NC) siRNA and treated with sorafenib (10 μmol/L; S10) for 48 h. C, effects of GADD45β knockdown on sorafenib-induced apoptosis were assessed by Annexin V analysis. After drug treatment, both floating and adherent HCC cells were collected for analysis by flow cytometry. Proportions of apoptotic cells were indicated by the percentage of Annexin V (+) cells. Each value is the mean ± SD of three independent experiments. *, $P < 0.05$ compared with S10-si-NC. D, effects of GADD45β overexpression on the sensitivity of HCC cells to sorafenib-induced apoptosis. Huh-7 and Huh-7R cells were transfected with GADD45β or empty vectors. Cells were treated 24 h after transfection with sorafenib (10 μmol/L; S10) or control for an additional 48 h. Both floating and adherent cells were collected for flow cytometry. Proportions of apoptotic cells were indicated by the percentages of cells in the sub-G_1 fraction. Columns, mean of three independent experiments; bars, SD. *, $P < 0.05$ compared with empty-S10.

Medicine, National Taiwan University. All the animal studies were performed according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by National Academy of Sciences and published by NIH. Male BALB/c athymic (nu+/nu+) mice were inoculated s.c. with Huh-7 cells or Huh-7R cells (~1×10^6). When the tumor volume reached ~100 mm^3 [volume (mm^3) = $(width)^2 \times length \times 0.5$], the mice were randomized to two treatment groups ($n \geq 3$ in each group): (*a*) vehicle control and (*b*) sorafenib 15 mg/kg/d. Drug treatment was given daily by gavage. Tumor volume and body weight were recorded every 4 days. Fresh-frozen tumor samples after drug treatment were collected to measure the levels of pertinent mRNA and proteins by real-time PCR and Western blot, respectively. To avoid the contamination of GADD45β mRNA measurement by adjacent nontumor tissue, 6-μm frozen sections of the tumor tissues were stained with hematoxylin, and then the tumor cells were collected by laser capture microdissection using the PALM MicroLaser System (MicroLaser Technologies AG). Total RNA from the dissected specimens was extracted using the RNeasy Micro kit (Qiagen), and cDNA synthesis and real-time PCR were then performed according to the protocols described above. Formalin-fixed, paraffin-embedded tumor samples after drug treatment were collected for immunohistochemical analysis of pertinent protein expression and tumor angiogenesis, as described before (5). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed to measure the extent of tumor cell apoptosis (5).

Statistical analysis

All data were representative of at least three independent experiments. Quantitative data are expressed as mean ± SD. Comparisons were analyzed using the Student's *t* test and ANOVA. Significance was defined as $P < 0.05$.

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Results

Sorafenib induces GADD45β expression in HCC cells independent of MEK/ERK inhibition

The IC_{50} values of HCC cells after sorafenib treatment are shown in Fig. 1A. Hep3B and Huh-7R cells were categorized as sorafenib-resistant because their IC_{50} values (12–15 μmol/L) exceeded the steady-state concentration of sorafenib that can be achieved in patients after the recommended dosage (400 mg twice daily; ref. 22). Sorafenib upregulated GADD45β expression, at both the mRNA and protein levels, in sorafenib-sensitive, but not in sorafenib-resistant, HCC cells (Fig. 1B and C). Treatment of HCC cells with the MEK inhibitor U0126 did not upregulate GADD45β expression, suggesting that GADD45β upregulation by sorafenib treatment was independent of MEK/ERK signaling activity (Fig. 1B and C). In addition, treatment of HCC cells with the Raf inhibitor ZM336372, alone or in combination with sorafenib, did not change the effects of sorafenib on GADD45β expression (Supplementary Fig. S1), suggesting that GADD45β induction by sorafenib was independent of Raf signaling activity.

Because vascular endothelial growth factor receptor (VEGFR) is an important target of sorafenib, we also explore the effects of VEGFR inhibition on GADD45β expression in Huh-7 cells and HUVEC. Two multikinase inhibitors, sunitinib (from Pfizer) and AEE788 (from Novartis), which also target VEGFR, were used as comparison. As shown in Supplementary Fig. S2, sorafenib can induce GADD45β mRNA expression in both Huh-7 cells and, to a lesser extent, HUVEC. Sunitinib and AEE788 had negligible effects on GADD45β induction in either Huh-7 cells or HUVEC. The effects of these drugs on GADD45β induction do not correlate with their effects on VEGFR phosphorylation. Therefore, our data do not support a direct relationship between inhibition of VEGFR activity and induction of GADD45β.

GADD45β expression contributes to sorafenib-induced apoptosis in HCC cells

Knockdown of GADD45β expression by siRNA diminished the expression of GADD45β induced by sorafenib (Fig. 2A and B). GADD45β knockdown partially abrogated the apoptosis induced by sorafenib in sorafenib-sensitive HCC cells (Huh-7 and HepG2) but not in sorafenib-resistant Hep3B

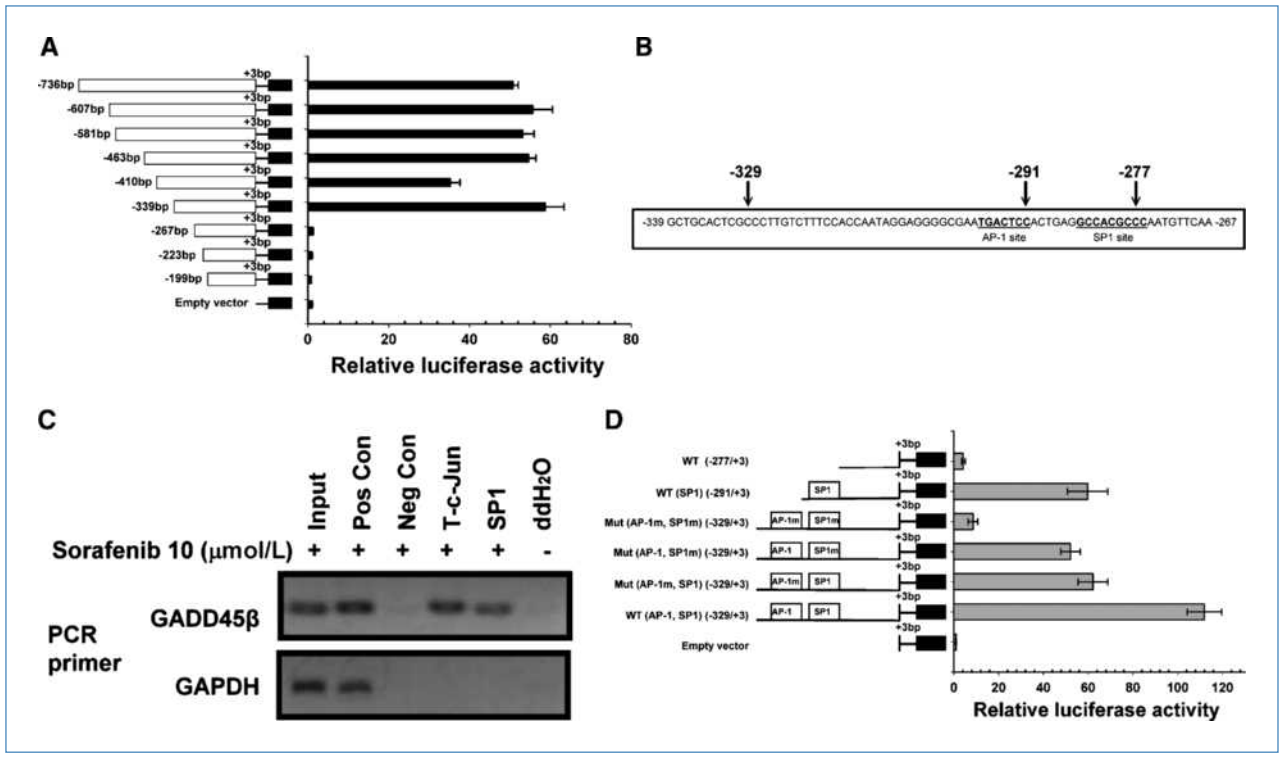

Figure 3. AP-1 and SP1 regulate GADD45β expression induced by sorafenib. A, localization of the transcriptional regulatory region of the GADD45β promoter by 5′ deletion analysis. The 5′ deletion constructs of the GADD45β promoter (shown on the left) were transfected into Huh-7 cells, and the relative luciferase activity of each promoter fragment after sorafenib treatment is shown on the right. B, sequence of the –339/–267 region of the human GADD45β promoter. The AP-1 (–298/–292) and SP1 (–285/–277) binding sites, identified by the TFSEARCH program, are underlined. C, chromatin immunoprecipitation assay of c-Jun and SP1 association with the GADD45β promoter-enhancer in Huh-7 cells. Negative control (Neg Con) was chromatin immunoprecipitated with a normal mouse IgG. Positive control (Pos Con) was chromatin immunoprecipitated with anti-RNA polymerase II antibody. Input was 0.1% of the sonicated chromatin before immunoprecipitation. D, identification of functional sites within the human GADD45β promoter region. The 5′ deletion constructs of the GADD45β promoter with mutations in the AP-1 or SP1 binding sites (shown on the left) were transfected into Huh-7 cells, and the relative luciferase activity of each promoter fragment after sorafenib treatment is shown on the right.

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cells (Fig. 2C; Supplementary Fig. S3). Transfection of GADD45β into the sorafenib-resistant Huh-7R cells increased apoptosis induced by sorafenib but had no effects on the parental Huh-7 cells (Fig. 2D).

AP-1 and SP1 regulate GADD45β expression induced by sorafenib

To explore the regulatory mechanisms of GADD45β transcription, a series of luciferase reporter plasmids with deletions in the 5′-flanking region of the GADD45β promoter were generated (Fig. 3A). The results of luciferase assays indicated that the region −339/−267 in the 5′-flanking region of the GADD45β promoter was crucial for GADD45β induction by sorafenib (Fig. 3A). Potential binding sites for AP-1 (−298/−292) and SP1 (−285/−277) were identified using the TFSEARCH program (Fig. 3B). Binding of c-Jun, the main factor of the AP-1 transcription factor complex, and SP1 to the GADD45β promoter was confirmed by ChIP (Fig. 3C). To confirm the biological role of these potential binding sites, the pLuc-MCS-GADD45β (−339/+3) construct was used to generate mutations in the AP-1 and SP1 binding sites. Results of luciferase activity assays indicated that mutations in both the AP-1 (AP-1m) and the SP1 (SP1m) sites completely abolish the luciferase activity of the promoter constructs after sorafenib treatment, whereas mutation in either site alone partially decreased the luciferase activity.

Inhibition of JNK signaling partially abrogates GADD45β expression and apoptosis induced by sorafenib

The effects of sorafenib treatment on JNK signaling activity are shown in Fig. 4. Sorafenib, but not the MEK inhibitor U0126, increased phosphorylation of JNK in sorafenib-sensitive, but not in sorafenib-resistant, HCC cells. Both total and phospho-c-Jun levels were also increased more prominently in sorafenib-sensitive HCC cells. On the other hand, the level of SP1 did not change significantly after sorafenib treatment (Fig. 4A). Activation of JNK signaling by sorafenib increased the cytosolic and nuclear levels of c-Jun and GADD45β (Fig. 4B), which may then have regulated downstream effectors of apoptosis. The specific JNK inhibitor SP600125 was used to examine the effects of JNK signaling on GADD45β expression. SP600125 inhibited c-Jun and GADD45β mRNA and protein expression induced by sorafenib in Huh-7 cells (Fig. 5A and B). Although SP600125 could also induce apoptosis in Huh-7 cells (Fig. 5C), its effects antagonized the proapoptotic effects of sorafenib, as shown by median dose-effect analysis (Fig. 5D). The above results indicated that sorafenib induced GADD45β expression by activating the JNK signaling pathway.

In vivo evidence of GADD45β induction as a predictive biomarker of the antitumor efficacy of sorafenib

To compare the effects of sorafenib in sensitive and resistant HCC cells *in vivo*, Huh-7 and Huh-7R cells were used to develop xenograft models. Treatment with sorafenib (15 mg/kg/d) inhibited tumor growth significantly in Huh-7 xenograft ($P < 0.0001$, compared with vehicle treatment) but not in Huh-7R xenograft ($P = 0.10$; Fig. 6A). Induction of JNK signaling activity (Fig. 6B) and GADD45β mRNA and protein expression (Fig. 6C) was evident in Huh-7, but not in Huh-7R, xenografts. The above data suggested that the induction of JNK signaling and GADD45β expression after sorafenib treatment may help predict the therapeutic efficacy of sorafenib in HCC. Induction of tumor cell apoptosis (measured by TUNEL assay) and inhibition of tumor angiogenesis is prominent in Huh-7 model, but negligible in Huh-7R model (Fig. 6D).

Discussion

In this study, we showed that sorafenib can induce GADD45β expression in HCC cells, and failure of GADD45β induction may confer resistance to sorafenib-induced

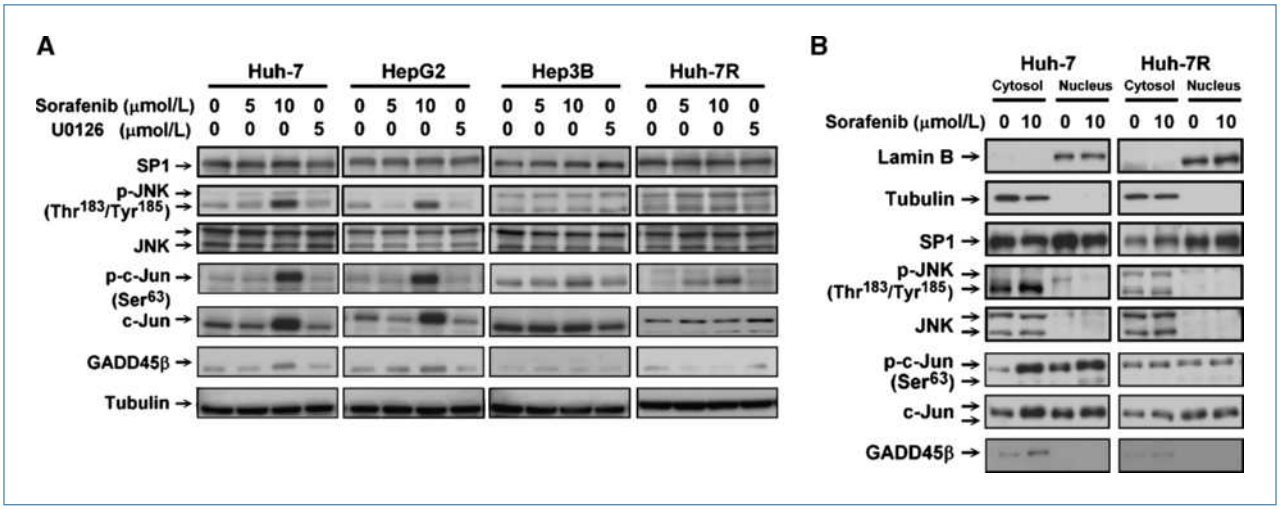

Figure 4. Sorafenib increases the phosphorylation of JNK/c-Jun in HCC cells. A, HCC cells were treated with sorafenib or U0126 at the indicated concentrations for 24 h. Whole-cell lysates were subjected to Western blotting. B, Huh-7 and Huh-7R cells were treated with sorafenib (10 μmol/L) for 24 h. Cytoplasmic and nuclear fractions were subjected to Western blotting. Lamin B and α-tubulin were used as loading controls.

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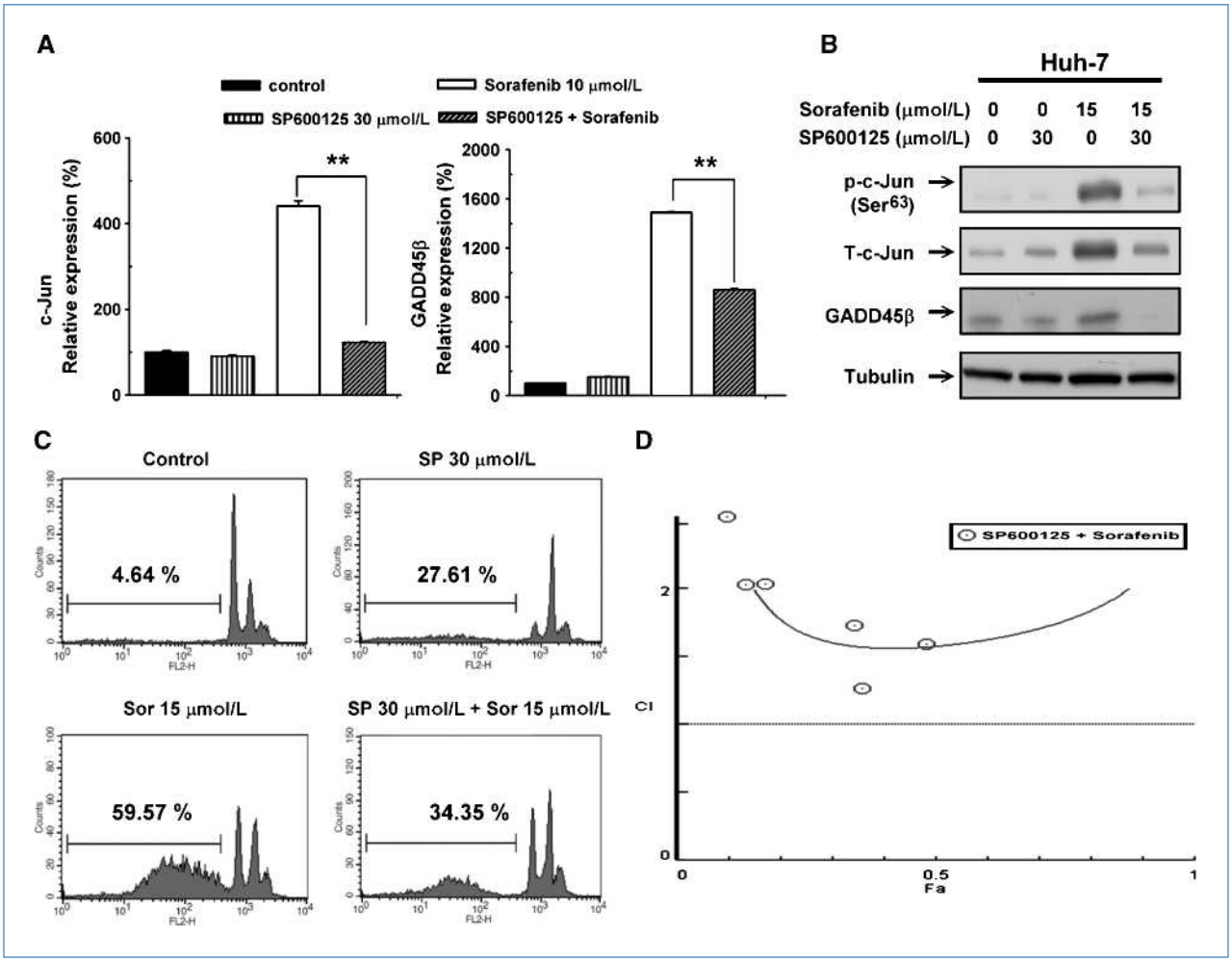

Figure 5. Inhibition of JNK signaling partially abrogates GADD45β expression and apoptosis induced by sorafenib. A and B, the mRNA and protein levels of c-Jun and GADD45β in Huh-7 cells after addition of sorafenib or the JNK inhibitor SP600125 were assessed by qRT-PCR and Western blotting, respectively. **, $P < 0.01$ compared with treatment with sorafenib alone. C, apoptosis of Huh-7 cells after treatment with sorafenib or SP600125 was assessed by flow cytometry. Proportions of apoptotic cells were indicated by the percentages of cells in the sub-G_1 fraction. D, median dose-effect analysis of the antagonistic effects between sorafenib and SP600125. Huh-7 cells were treated with sorafenib and SP600125 at a fixed ratio (2:1) for 72 h, and apoptosis was measured by flow cytometry (sub-G_1 fraction analysis). The CI was calculated using the CI-isobologram method by Chou and Talalay (20). CI = 1, additive effect; CI < 1, synergistic effect; CI > 1, antagonistic effect.

apoptosis. We also showed that GADD45β induction is mediated by the cellular JNK/c-Jun signaling pathway but not by Raf/MEK/ERK signaling. Our data broaden the understanding of the antitumor mechanism of sorafenib in HCC and suggest the possibility of using GADD45β as a predictive biomarker for sorafenib sensitivity in HCC.

Extensive studies have been performed to verify the role of the Raf/MEK/ERK signaling pathways in the antitumor mechanisms of sorafenib, but cumulative evidence indicates that mediators independent of MEK/ERK signaling also play important roles in sorafenib-induced apoptosis in cancer cells (10, 23). Examples of these kinds of mediators include myeloid cell leukemia-1 (Mcl-1), the translation initiation factor eIF4E, and apoptosis-inducing factor (7, 8). The fact that sorafenib can induce GADD45β expression, whereas the MEK inhibitor U0126 and the Raf inhibitor ZM 336372 cannot, indicated that GADD45β may be another factor that mediates sorafenib-induced apoptosis through MEK/ERK-independent mechanisms. The correlation between GADD45β induction and the sensitivity of HCC cells to sorafenib treatment shown in this study suggests that GADD45β induction may be related to the resistance mechanisms of HCC cells to sorafenib. Further studies are needed to explore the relationship between GADD45β and other MEK/ERK-dependent or independent mediators in predicting the treatment efficacy of sorafenib. Validation of their predicting values in human HCC tumor tissue is also necessary.

The effects of GADD45β induction on cell survival depends on the types of stimuli, the cell types tested, and the interaction between GADD45β and the different cellular MAPK pathways. GADD45β mediates TGF-β–induced apoptosis in

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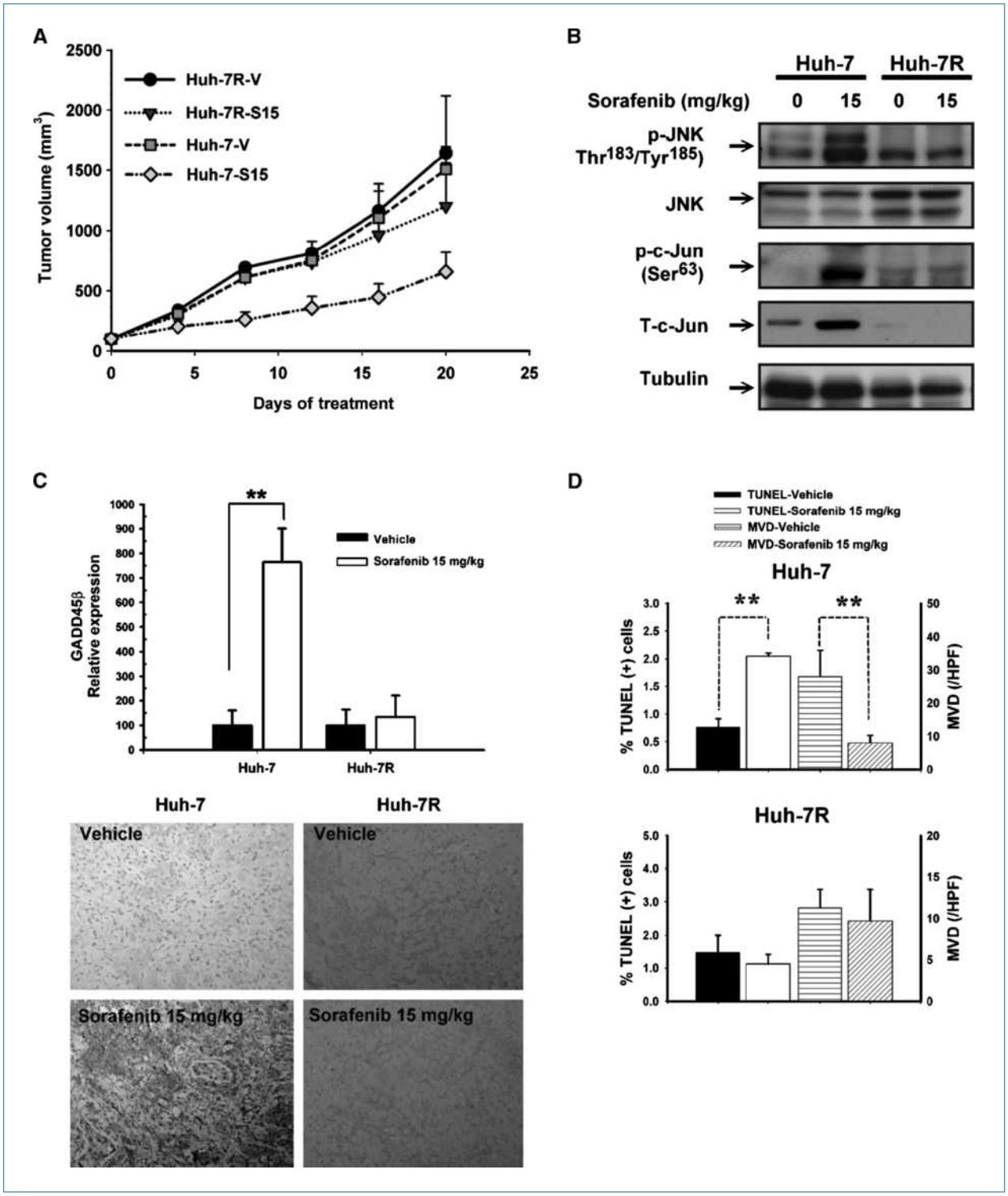

Figure 6. *In vivo* evidence of GADD45β induction as a predictive biomarker of the antitumor efficacy of sorafenib. Huh-7 or Huh-7R cells were injected s.c into male BALB/c athymic nude mice. Mice were treated daily by gavage as indicated (V, vehicle; S15, sorafenib 15 mg/kg/d). A, difference in tumor growth. B, difference in JNK and c-Jun phosphorylation measured by Western blotting. C, difference in GADD45β mRNA and protein expression measured by real-time qRT-PCR and immunohistochemical staining, respectively. GADD45β mRNA was expressed relative to endogenous AFP expression. D, difference in tumor cell apoptosis and tumor angiogenesis measured by TUNEL assay and microvessel density (MVD). Quantification of apoptosis and MVD was done by manual counting of TUNEL (+) cells and CD31 (+) microvessels, respectively, under high-power field (HPF, 200×). The numbers were the average of counting 4 HPF in each sample. **, $P < 0.01$, compared with the control (vehicle-treated) group.

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pancreatic cancer cell lines and in murine hepatocytes by activating the p38 MAPK pathway (18, 24). On the other hand, GADD45β antagonizes tumor necrosis factor-α–induced apoptosis in murine embryonal fibroblasts and UV- or chemotherapy-induced apoptosis in hematopoietic cells by inhibiting the JNK signaling pathway (25–27). The latter seemed contradictory to our findings. Although we found that JNK activation by sorafenib can induce GADD45β expression and apoptosis, other investigators showed that GADD45β can inhibit JNK signaling activity by interacting with the upstream MAPK kinase-4 and MAPK kinase-7 and thus prevent apoptosis (25, 28). These findings suggest that GADD45β and the JNK signaling pathway may regulate each other in a feedback loop to control the cellular response to environmental stress.

GADD45β and the JNK signaling pathway may play contradictory roles in hepatocyte survival and liver carcinogenesis. GADD45β is induced early in the liver regeneration process after partial hepatectomy and promotes hepatocyte survival by inhibiting MKK-7 and JNK signaling (29, 30). However, the findings that GADD45β expression is frequently suppressed in HCC tumor cells suggest that GADD45β may play growth inhibitory roles during the carcinogenesis process (15). Similarly, JNK signaling may act either as a proapoptotic or a prosurvival factor in different context. In liver regeneration models, JNK signaling is proapoptotic, and inhibition of JNK signaling via the interaction between GADD45β and upstream MKK-7 can promote hepatocyte survival (28). In the diethylnitrosamine-induced HCC model, on the other hand, JNK signaling activity can increase HCC cell proliferation by inducing c-Myc expression and inhibiting the expression of the cell cycle inhibitor p21 (31). The effects of molecular targeted therapy on JNK signaling activity in cancer cells have not yet been clarified, and further investigation is warranted to explore new therapeutic strategies.

In addition to the JNK signaling pathway, GADD45β expression is also regulated by other mechanisms, including the NF-κB and the Smad signaling pathways (24, 25). Besides, GADD45β expression may be affected by the methylation status of its promoter region (32, 33). It has been suggested that GADD45 proteins, as cellular sensors to environmental stress, may coordinate the cellular stress response, depending on the types and intensity of stress, the types and levels of interacting proteins, and the specific cell types (34, 35). Other GADD family proteins, including GADD45α and GADD45γ, have been shown to inhibit cell growth and induce apoptosis in various cancer models (36–38). The expression levels and functional significance of other GADD45 family proteins in HCC should also be explored for a better understanding of the regulatory roles of GADD45 family proteins in HCC cell growth and apoptosis.

In conclusion, GADD45β induction contributes to sorafenib-induced apoptosis in HCC cells. Future studies are needed to validate its value in predicting sorafenib efficacy.

Disclosure of Potential Conflicts of Interest

Dr. Ann-Lii Cheng is a consultant for and a member of the speaker's bureau of Bayer-Schering Pharma. Dr. Chiun Hsu is a member of the speaker's bureau of Bayer-Schering Pharma. Other authors have nothing relevant to this manuscript to disclose.

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Induction of DNA Damage-Inducible Gene GADD45β Contributes to Sorafenib-Induced Apoptosis in Hepatocellular Carcinoma Cells

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