The Bcl-xL Inhibitor, ABT-737, Efficiently Induces Apoptosis and Suppresses Growth of Hepatoma Cells in Combination with Sorafenib

Hayato Hikita,[1]* Tetsuo Takehara,[1]* Satoshi Shimizu,[1] Takahiro Kodama,[1] Minoru Shigekawa,[1] Kyoko Iwase,[1] Atsushi Hosui,[1] Takuya Miyagi,[1] Tomohide Tatsumi,[1] Hisashi Ishida,[1] Wei Li,[1] Tatsuya Kanto,[1] Naoki Hiramatsu,[1] and Norio Hayashi[2]

Tumor cells are characterized by uncontrolled proliferation, often driven by activation of oncogenes, and apoptosis resistance. The oncogenic kinase inhibitor sorafenib can significantly prolong median survival of patients with advanced hepatocellular carcinoma (HCC), although the response is disease-stabilizing and cytostatic rather than one of tumor regression. Bcl-xL (B cell lymphoma extra large), an antiapoptotic member of the B cell lymphoma-2 (Bcl-2) family, is frequently overexpressed in HCC. Here, we present *in vivo* evidence that Bcl-xL overexpression is directly linked to the rapid growth of solid tumors. We also examined whether ABT-737, a small molecule that specifically inhibits Bcl-xL but not myeloid cell leukemia-1 (Mcl-1), could control HCC progression, especially when used with sorafenib. Administration of ABT-737, even at an *in vivo* effective dose, failed to suppress Huh7 xenograft tumors in mice. ABT-737 caused the levels of Mcl-1 expression to rapidly increase by protein stabilization. This appeared to be related to resistance to ABT-737, because decreasing Mcl-1 expression levels to the baseline by a small interfering RNA–mediated strategy made hepatoma cells sensitive to this agent. Importantly, administration of ABT-737 to Mcl-1 knockout mice induced severe liver apoptosis, suggesting that tumor-specific inhibition of Mcl-1 is required for therapeutic purposes. Sorafenib transcriptionally down-regulated Mcl-1 expression specifically in tumor cells and abolished Mcl-1 up-regulation induced by ABT-737. Sorafenib, not alone but in combination with ABT-737, efficiently induced apoptosis in hepatoma cells. This combination also led to stronger suppression of xenograft tumors than sorafenib alone. *Conclusion:* Bcl-xL inactivation by ABT-737 in combination with sorafenib was found to be safe and effective for anti-HCC therapy in preclinical models. Direct activation of the apoptosis machinery seems to unlock the antitumor potential of oncogenic kinase inhibitors and may produce durable clinical responses against HCC. (HEPATOLOGY 2010;52:1310-1321)

The B cell lymphoma-2 (Bcl-2) family proteins regulate the mitochondrial pathway of apoptosis, a major form of cell death.[1] They include five antiapoptotic proteins, Bcl-2, B cell lymphoma extra large (Bcl-xL), myeloid cell leukemia-1 (Mcl-1), Bcl-2–related protien A1 (Bfl-1), and Bcl-2-like 2 (Bcl-w), and two structurally related proapoptotic proteins, Bcl-2–antagonist/killer (Bak) and Bcl-2–

Abbreviations: ALT, alanine aminotransferase; Bad, Bcl-2–associated agonist of cell death; Bak, Bcl-2–antagonist/killer; Bax, Bcl-2–associated X protein; Bcl-2, B cell lymphoma-2; BH3, Bcl-2 homology domain-3; Bid, BH3-interacting domain death agonist; cDNA, complementary DNA; HA, hemagglutinin; HCC, hepatocellular carcinoma; Mcl-1, myeloid cell leukemia-1; mRNA, messenger RNA; RT-PCR, reverse-transcription polymerase chain reaction; siRNA, small interfering RNA; USP9X, ubiquitin-specific peptidase 9 X-linked; WST, water-soluble tetrazolium.

From the [1]Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan; and [2]Kansai-Rosai Hospital, Amagasaki, Hyogo, Japan.

Received May 26, 2010; accepted June 30, 2010.

**These authors contributed equally to this work This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to Tetsuo Takehara) and Grant-in-Aid for Research on Hepatitis and BSE from the Ministry of Health, Labour and Welfare of Japan.*

Address reprint requests to: Tetsuo Takehara, M.D., Ph.D., Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. E-mail: takehara@gh.med.osaka-u.ac.jp; fax: +81-6-6879-3629.

associated X protein (Bax), as well as several structurally diverse proapoptotic Bcl-2 homology domain-3 (BH3)-only proteins like Bcl-2–associated agonist of cell death (Bad), BH3-interacting domain death agonist (Bid), and Bcl-2-like 11 (Bim). Bak and Bax, effector molecules in this family, homo-oligomerize into proteolipid pores within the mitochondrial outer membrane, leading to release of cytochrome c followed by activation of downstream caspases, such as caspase-3/7, which dismantle a variety of cellular substrates, leading to cell death. Antiapoptotic Bcl-2 proteins function as regulators of apoptosis by directly or indirectly antagonizing Bak and Bax activity to maintain cellular integrity. BH3-only proteins, sensors of apoptosis, are activated by a variety of cellular stresses and either directly activate Bak and Bax or neutralize antiapoptotic Bcl-2 proteins, inducing cell death. Because tumor cells encounter a variety of cellular stresses, such as genotoxic and environmental factors, overexpression of antiapoptotic Bcl-2 family proteins is commonly observed and leads to survival of tumor cells.[2] We and others have reported that Bcl-xL is frequently overexpressed in human hepatocellular carcinomas (HCCs).[3–6] These reports point to the resistance of hepatoma cells to a wide variety of stress-inducing conditions. For example, Bcl-xL blocks p53-induced apoptosis in hepatoma cells, implying that Bcl-xL overexpression may be one of the mechanisms by which HCC survives under genotoxic conditions.[3] In addition, Bcl-xL overexpression was found to be associated with poor overall survival and disease-free survival after surgical resection for HCC.[7] These findings suggest that Bcl-xL may be a therapeutic target for HCC, although this possibility has not yet been addressed. Bcl-xL is also expressed in normal hepatocytes and plays a critical role in maintaining their integrity.[8] Thus, special caution is necessary when Bcl-xL inactivation is applied to therapeutic purposes.

Despite advances in understanding the mechanisms of cell death and the biology of Bcl-2 family proteins, therapeutic strategies for HCC targeting apoptotic molecules have been hampered due to a lack of specific inhibitors. ABT-737 is one of the first small-molecule inhibitors of Bcl-2 family proteins and opens the field for cancer treatment targeting the apoptosis machinery. ABT-737, designed as a Bad mimetic, binds and neutralizes Bcl-2, Bcl-xL, and Bcl-w, but not Mcl-1 or Bfl-1.[9-11] It has single-agent activity in a number of hematopoietic cancers and some solid tumors.[12,13] Its orally available derivative, ABT-263, is in early clinical trials against lymphoid malignancies, small-cell lung cancer, and chronic lymphocytic leukemia, with some promising results.[14] In this study, we investigated the impact of ABT-737 in treating human hepatoma in culture and using a xenograft model. We found that hepatoma cells are relatively resistant to ABT-737, presumably due to reciprocal up-regulation of Mcl-1 upon ABT-737 exposure. Although concomitant Mcl-1 inhibition appears to be effective for inducing apoptosis by ABT-737, it should be done in a tumor-specific manner, because administration of ABT-737 leads to liver deterioration in Mcl-1 knockout mice. Finally, sorafenib, an anti-HCC agent recently approved by the U.S. Food and Drug Administration, down-regulates Mcl-1 expression in a tumor-specific manner and induces apoptosis and tumor growth suppression in cooperation with ABT-737. Combination therapy with sorafenib and a Bcl-xL inhibitor seems to be an attractive strategy for controlling tumor progression in HCC.

Materials and Methods

Cell Lines and Reagents. Primary human hepatocytes were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured with the provided medium. Human hepatoma cell lines were cultured with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO). Cycloheximide was purchased from Nacalai Tesque (Kyoto, Japan), sorafenib tablets were purchased from Bayer HealthCare (Osaka, Japan), and ABT-737 was kindly provided by Abbott Laboratories (Abbott Park, IL). They were dissolved with dimethyl sulfoxide for *in vitro* use.

Hela Cells Expressed Bcl-xL with the Tet-on System. pcDNA3HABcl-xL, an expression vector coding human Bcl-xL tagged with hemagglutinin (HA), was provided by Dr. G. Nunez (University of Michigan Medical School, Ann Arbor, MI). The pcDNA4/TOHABcl-xL was constructed by inserting the complementary DNA (cDNA) for Bcl-xL gene with HA-tag from pcDNA3HABcl-xL into the EcoRI site of pcDNA4/TO (Invitrogen, Carlsbad, CA). TREx-Hela cells (Invitrogen) were transfected with pcDNA4/

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.23836
Potential conflict of interest: Nothing to report.
Additional Supporting Information may be found in the online version of this article.

TOHABcl-xL using Lipofectin (Invitrogen). The cells were cultured with DMEM containing 1.1 μg/mL zeocin, and zeocin-resistant clones were isolated. After examination of HA–Bcl-xL induction by doxycycline, two clones (Hela–Bcl-xL$^{Tet\text{-}on}$ clone A, clone B) were established and used for further experiments.

Mice. Conditional Bcl-xL knockout mice (*bcl-x$^{flox/flox}$ Alb-Cre* [albumin/cre recombinase]) and Mcl-1 knockout mice (*mcl-1$^{flox/flox}$ Alb-Cre*) were previously described.[15] Balb/c nude mice (CAnN.Cg-Foxn1^{nu}/CrlCrlj) were purchased from Charles River Laboratories (Yokohama, Japan). They were maintained in a specific pathogen–free facility and treated with humane care with approval from the Animal Care and Use Committee of Osaka University Medical School.

Apoptosis Assay. The *in vitro* apoptosis assay, measurement of caspase-3/7 activity, and the water-soluble tetrazolium salt (WST) assay, were described previously.[16] The *in vivo* apoptosis assay, measurement of serum alanine aminotransferase (ALT) level, and caspase-3/7 activity and histological analyses were also previously described.[15]

Western Blot Analysis. Whole-cell extracts from cultured cells or tissues were prepared and subjected to western blot. For immunodetection, the following antibodies were used: anti–Bcl-xL antibody and anti-human Mcl-1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA); anti-mouse Mcl-1 antibody from Rockland (Gilbertsville, PA); anti-Bid antibody, anti-Bax antibody, and anti-cleaved caspase-3 antibody from Cell Signaling Technology (Beverly, MA); anti-Bak antibody from Millipore (Billerica, MA); anti-Bim antibody from Assay Design (Ann Arbor, MI); anti-ubiquitin-specific peptidase 9, X-linked (USP9X) antibody from Abnova (Taipei, Taiwan); and anti–beta actin antibody from Sigma-Aldrich (St. Louis, MO) or Cell Signaling Technology.

Xenograft Tumor. To produce a xenograft tumor, 3 × 10^6 to 5 × 10^6 Hela–Bcl-xL$^{Tet\text{-}on}$ clone A or Huh7 cells were subcutaneously injected to Balb/c nude mice. For induction of HA–Bcl-xL, the mice that were injected with Hela–Bcl-xL$^{Tet\text{-}on}$ clone A cells were fed with water containing 100 μg/mL doxycycline. For anticancer therapy, ABT-737 was administered as described.[17] Sorafenib tablets were crushed and orally administered with water containing 12.5% Cremophor EL (Sigma-Aldrich) and 12.5% ethanol. We estimated the volume of the xenograft tumor using the following formula: tumor volume = $\pi/6$ × (major axis) × (minor axis)^2.

Small RNA Interference. Hepatoma cell lines were transfected with Stealth select RNAi (set of three oligonucleotides, Invitrogen) RNA interference (RNAi) directed against Mcl-1 or USP9X. A Stealth RNAi negative control kit (set of three oligonucleotides, Invitrogen) was used as a control for sequence-independent effects following Stealth RNAi delivery. The transfections were carried out using Lipofectamine RNAiMAX (Invitrogen) according to the reverse transfection protocol.

Real-Time Reverse-Transcription Polymerase Chain Reaction. Real-time reverse-transcription PCR (RT-PCR) was performed as previously described.[15] Mcl-1 messenger RNA (mRNA) expressions were measured using TaqMan Gene Expression Assays (Assay ID: Hs03043899_m1) and were corrected with the quantified expression level of beta actin mRNA measured using TaqMan Gene Expression Assays (Assay ID: Hs99999903_m1).

Statistical Analysis. Data are presented as mean ± standard deviation. Differences between two groups were determined using the Student *t* test for unpaired observations unless otherwise noted. Multiple comparisons were performed by analysis of variance followed by Scheffe post hoc correction. $P < 0.05$ was considered statistically significant.

Results

Bcl-xL Overexpression Is a Molecular Mechanism of Rapid* In Vivo *Tumor Growth. Research has shown that Bcl-xL overexpression confers resistance to apoptosis in a variety of tumor cells. To examine its impact on tumor growth *in vivo*, we generated the Hela–Bcl-xL$^{Tet\text{-}on}$ cell line which expresses the modified tetracycline repressor molecule (rtTA) and Bcl-xL under control of tetracycline-responsive cis-elements. We chose Hela cells as a model because they expressed a relatively small amount of Bcl-xL in comparison with human hepatoma cells including Huh7, Hep3B, and HepG2 (Fig. 1A). Tetracycline analogue doxycycline treatment efficiently induced Bcl-xL in Hela–Bcl-xL$^{Tet\text{-}on}$ cells as expected (Fig. 1B) and conferred resistance to apoptosis as evidenced by significantly lower levels of caspase-3/7 activity in culture (Fig. 1C), although it did not have a significant effect on cell growth assay (Fig. 1D). Next, we subcutaneously injected Hela–Bcl-xL$^{Tet\text{-}on}$ cells into nude mice. When subcutaneous tumors grew to approximately 1 cm, the mice were randomly assigned to two groups: a doxycycline-drinking group and a water-drinking group. Subcutaneous tumors grew rapidly in the doxycycline-drinking group compared with the water-drinking group (Fig. 1E). As expected, xenograft tumors displayed higher levels of Bcl-xL expression than those in the water drinking group (Fig.

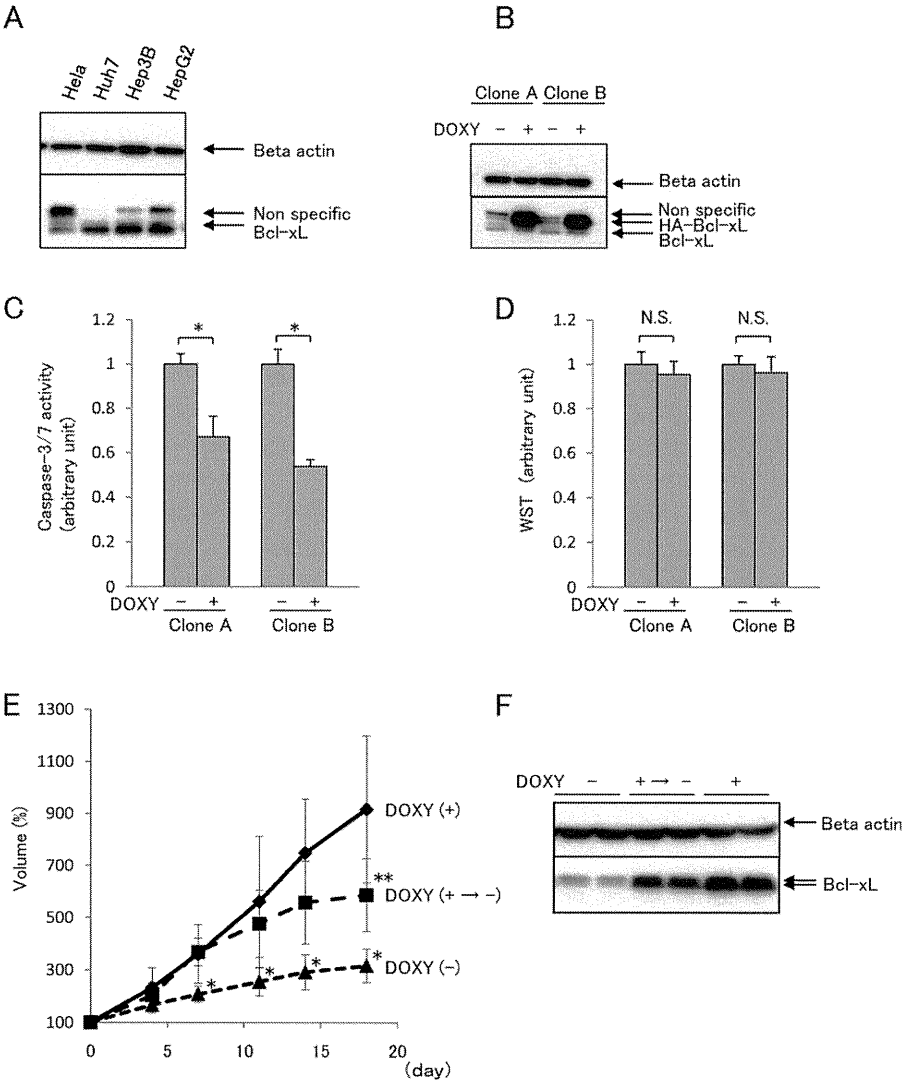

Fig. 1. Bcl-xL overexpression in vitro and in vivo by the Tet-on system. (A) Western blot analysis of Bcl-xL in human hepatoma cells and Hela cells. (B,C,D) Bcl-xL overexpression in vitro. Two independent clones of Hela-Bcl-xLTet-on cells were cultured with or without 1 μM doxycycline (DOXY) for 24 hours. (B) Western blot analysis of Bcl-xL. (C,D) Caspase-3/7 activity in culture supernatant and cell viability by the WST assay (N = 4). *P < 0.05. N.S., not significant. (E,F) Bcl-xL overexpression in vivo. Nude mice carrying xenograft tumors of Hela-Bcl-xL$^{Tet\text{-}on}$ clone A were randomly assigned to water given with or without 100 mg/mL doxycycline for 7 days. After 7 days, the mice of the doxycycline-drinking group were randomly assigned to two groups: one in which doxycycline drinking was continued and the other in which water was given instead (N = 5 or 6 per group). (E) The percentage of xenograft tumor volume. (F) Western blot analysis of xenograft tumor for the expression of Bcl-xL. *P < 0.05 versus the other two groups. **P < 0.05 versus the DOXY (+) group.

1F). In addition, switching the mice to water drinking at 7 days after doxycycline drinking decreased Bcl-xL expression and retarded tumor growth compared with continuing doxycycline drinking (DOXY + → − versus DOXY +, respectively; Fig. 1F). These results indicate that Bcl-xL overexpression was directly linked to rapid growth of tumors *in vivo* and suggest that Bcl-xL may be a therapeutic target for inhibiting tumor progression, especially for Bcl-xL–overexpressing tumors.

Bcl-xL Inhibitor ABT-737 Dose-Dependently Induces Apoptosis of Hepatoma Cells but Fails to Suppress Tumor Growth in a Xenograft Model. To examine the impact of pharmaceutical inactivation of Bcl-xL overexpressed in hepatoma cells, Huh7 and Hep3B hepatoma cells were cultured with escalating doses of ABT-737. ABT-737 dose-dependently activated caspase-3/7 in hepatoma cells and suppressed tumor growth at high dosages (Fig. 2A,B). To examine the *in vivo* effect of ABT-737, nude mice were subcutaneously injected with Huh7 cells to generate xenograft tumors and were randomly assigned into two groups when the diameter of the subcutaneous tumors reached approximately 1 cm: ABT-737 injection group and vehicle injection group. Administration of ABT-737 at 50 mg/kg body weight/day for 7 days failed to suppress tumor growth (Fig. 2C). In contrast, mild ALT elevation and thrombocytopenia were observed in ABT-737–injected mice (Fig. 2D). Previous research has demonstrated that both are observed in mice after ABT-737 administration,[17,18] confirming that the dose injected in the present experiment is sufficient for inducing a biological effect of ABT-737 *in vivo*.

ABT-737 Posttranscriptionally Increases Expression of Mcl-1. To examine the mechanisms underlying relative resistance of hepatoma cells to ABT-737, we examined the expression profile of the Bcl-2 family

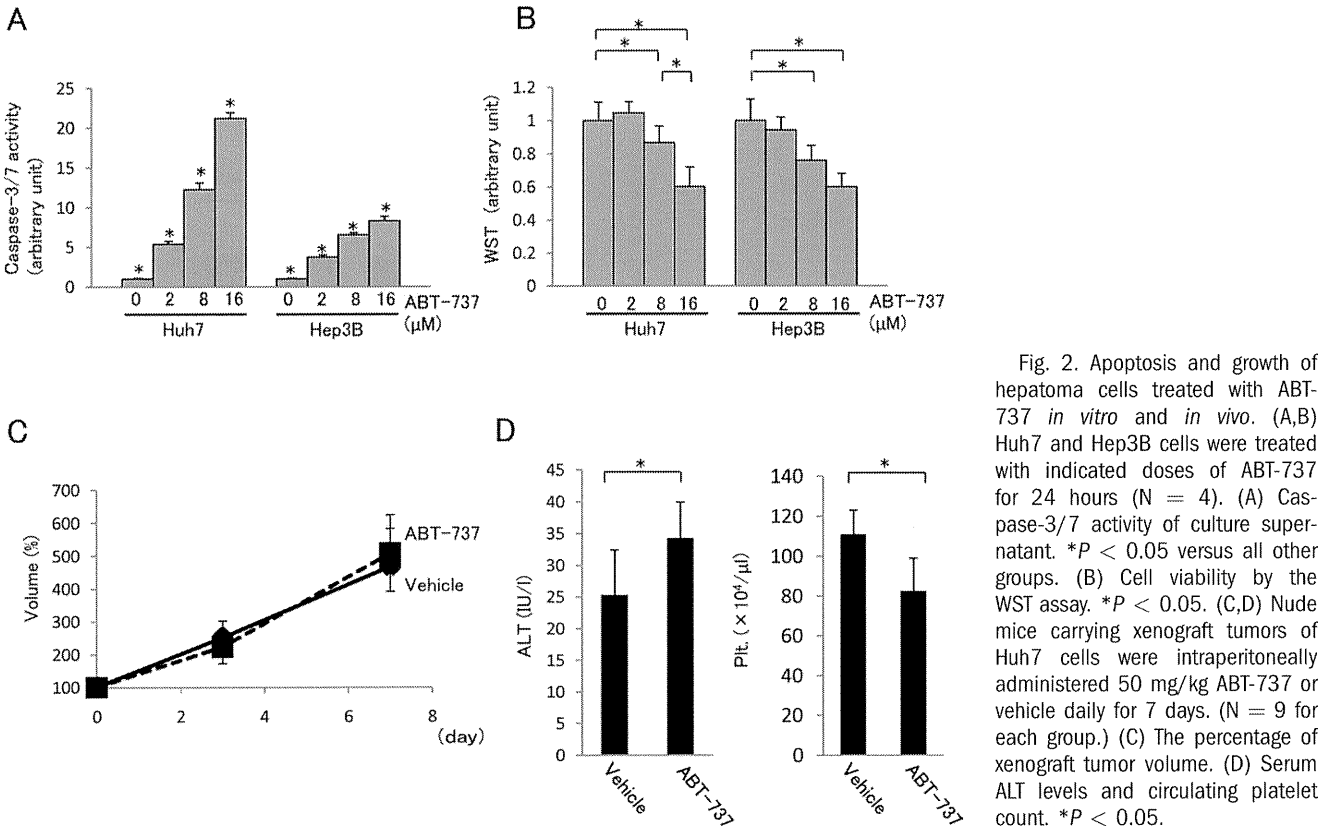

Fig. 2. Apoptosis and growth of hepatoma cells treated with ABT-737 *in vitro* and *in vivo*. (A,B) Huh7 and Hep3B cells were treated with indicated doses of ABT-737 for 24 hours (N = 4). (A) Caspase-3/7 activity of culture supernatant. $*P < 0.05$ versus all other groups. (B) Cell viability by the WST assay. $*P < 0.05$. (C,D) Nude mice carrying xenograft tumors of Huh7 cells were intraperitoneally administered 50 mg/kg ABT-737 or vehicle daily for 7 days. (N = 9 for each group.) (C) The percentage of xenograft tumor volume. (D) Serum ALT levels and circulating platelet count. $*P < 0.05$.

proteins. Administration of ABT-737 did not affect expression of proapoptotic multidomain members Bak and Bax or BH3-only proteins Bid and Bim in cultured hepatoma cell lines Huh7 and Hep3B (Fig. 3A). Although the slower migrating species of Bim at 4 hours was increased, this change disappeared at 24 hours. In agreement with previous research,[19,20] Mcl-1 was constitutively expressed in hepatoma cells. Of importance is the finding that the levels of Mcl-1 expression were rapidly increased as early as 4 hours after ABT-737 exposure. Expression of mcl-1 mRNA did not differ between ABT-737–treated cells and vehicle-treated cells (Fig. 3B), suggesting the involvement of a posttranscriptional mechanism. Because Mcl-1 is a rapid-turnover protein, the levels of Mcl-1 may be regulated by protein degradation.[21] To examine this, we treated hepatoma cells with cycloheximide, a well-established protein synthesis inhibitor, in the presence or absence of ABT-737. Cycloheximide-induced rapid decline in Mcl-1 expression was substantially blocked in the presence of ABT-737, suggesting that ABT-737 significantly delays degradation and prolongs the stability of Mcl-1 (Fig. 3C). Recently, it was reported that the deubiquitinase USP9X is involved in stabilization of Mcl-1.[22] In this study, western blot analysis revealed that the levels of USP9X expression were not changed in Huh7 and Hep3B with ABT-737 (Supporting Fig. 1A). Furthermore, USP9X down-regulation by small interfering RNA (siRNA) could not block the Mcl-1 up-regulation induced by ABT-737 (Supporting Fig. 1B).These results suggest that USP9X was not involved in Mcl-1 up-regulation induced by ABT-737. Of importance is the finding that Mcl-1 expression was also up-regulated after administration of ABT-737 in our xenograft model (Fig. 3D). Because Mcl-1 is not a target of ABT-737, relative resistance to ABT-737 of hepatoma cells may be due, at least in part, to posttranscriptional induction of Mcl-1.

Mcl-1 Knockdown Sensitizes Hepatoma Cells to ABT-737. To examine the impact of Mcl-1 induction in hepatoma cell resistance to ABT-737, we silenced Mcl-1 expression through use of three different siRNAs. Western blot analysis revealed that Mcl-1 siRNA2 and siRNA3 completely knocked down Mcl-1 expression in Hep3B cells, whereas Mcl-1 siRNA1 did so only partially (Fig. 4A). Mcl-1 knockdown or a medium dose of ABT-737 (4 μM) modestly activated caspase-3/7 in Hep3B cells, whereas both substantially activated caspase-3/7 (Fig. 4B). In addition, Mcl-1 knockdown or ABT-737 alone failed to suppress the growth of tumor cells but caused significant suppression when used together (Fig. 4C). Caspase-3 activation was also confirmed by western blots (Fig. 4A). It should be noted that Mcl-1 siRNA1 reduced Mcl-1

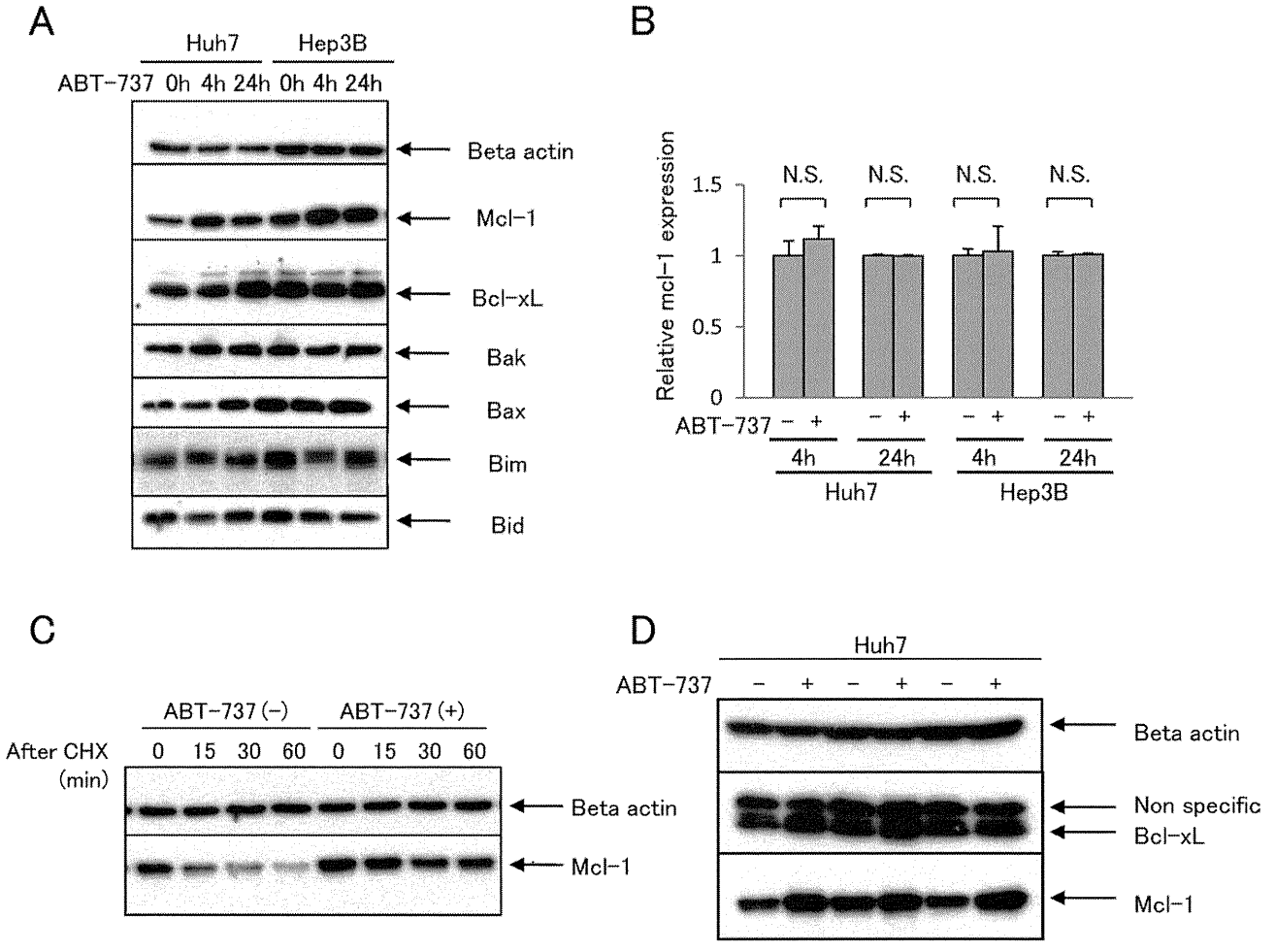

Fig. 3. Up-regulation of Mcl-1 in human hepatoma cells by ABT-737 in vitro and in vivo. (A,B) Huh7 and Hep3B cells were cultured with 4 μM ABT-737 for the indicated times. (A) Western blot analysis for the expression of Bcl-2 family proteins. (B) Real-time RT-PCR analysis for mcl-1 mRNA expression (N = 6). The levels were normalized to each group without ABT-737. N.S., not significant. (C) Huh7 cells were cultured with or without 4 μM ABT-737 for 4 hours and then further treated with 1 mM cycloheximide (CHX) for the indicated times. Western blot analysis for Mcl-1 expression. (D) Nude mice carrying xenograft tumors of Huh7 cells were intraperitoneally administered 50 mg/kg ABT-737 or vehicle daily for 7 days. Western blot analysis of xenograft tumor after 7-day treatment for the expression of Bcl-2 family proteins.

expression in ABT-737-treated cells to levels similar to those of untreated cells (Fig. 4A). Even in this case, mcl-1 knockdown enhanced caspase activation and growth suppression of Hep3B cells induced by ABT-737. Similar data were obtained with another hepatoma cell line, Huh7 (Fig. 4A and Supporting Fig. 2). These results indicate that Mcl-1 up-regulation induced by ABT-737 is involved in the resistance of hepatoma cells to ABT-737 and suggest that combination therapy with ABT-737 and Mcl-1 inhibitor may be predictably effective *in vivo*.

We previously reported that, similar to Bcl-xL, Mcl-1 plays an important role in apoptosis resistance of normal hepatocytes. In addition, knockdown of both Mcl-1 and Bcl-xL led to impaired liver development during embryogenesis.[15] Thus, the concern arises that simultaneous inactivation of both Bcl-xL and Mcl-1 may cause severe liver injury in adults. To examine this possibility, we injected ABT-737 into hepatocyte-specific Mcl-1 knockout mice or wild-type littermates. ABT-737 injection into wild-type mice led to mild liver apoptosis, which is consistent with our previous finding,[17] whereas injection into Mcl-1 knockout mice induced massive liver apoptosis leading to severe liver injury, and most animals died within 1 day (Fig. 4D,E). This result indicates that inactivation of both Bcl-xL and Mcl-1 may be hazardous and that Mcl-1 inactivation should be done in a tumor-specific manner.

Sorafenib Down-Regulates Mcl-1 Expression in Hepatoma Cells Much More Strongly than in Normal Liver Cells. Previous research has shown that sorafenib down-regulates Mcl-1 expression in hepatoma cells in a mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK)-independent manner.[16,23] In the present study, to examine whether Mcl-1

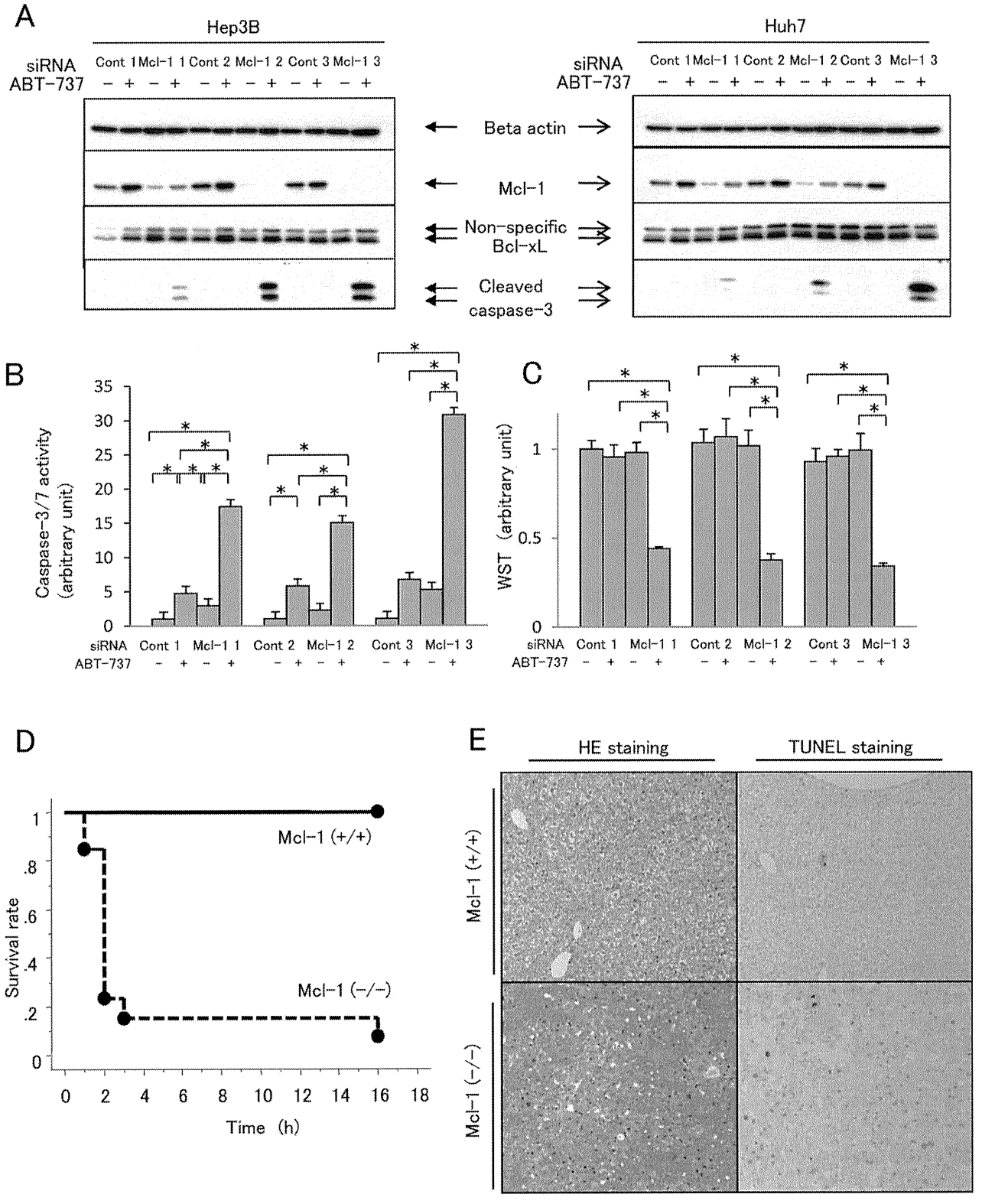

Fig. 4. Effects of ABT-737 under inhibition of Mcl-1 in vitro and in vivo. (A-C) Hep3B and Huh7 cells were transfected with Mcl-1 siRNAs (Mcl-1 1, Mcl-1 2, and Mcl-1 3) or control siRNAs (Cont 1, Cont 2, Cont 3). Forty-eight hours after transfection, they were treated with or without 4 μM ABT-737 for 24 hours (N = 4). (A) Western blot analysis for the expression of Mcl-1, Bcl-xL, and cleaved caspase-3. (B) Caspase-3/7 activities of supernatant in Hep3B culture dishes. *P < 0.05. (C) Cell viability of Hep3B cells by the WST assay. *P < 0.05. (D,E) Wild-type mice (Mcl-1 +/+) and hepatocyte-specific Mcl-1 knockout mice (Mcl-1 −/−) were intraperitoneally administered 50 mg/kg of ABT-737. (D) Survival curve of the mice (N = 13 or 15). (E) Hematoxylin and eosin (HE) and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining of the liver sections 16 hours after administration of ABT-737 with wild-type mice and immediately after death with Mcl-1 knockout mice. Representative photographs are shown.

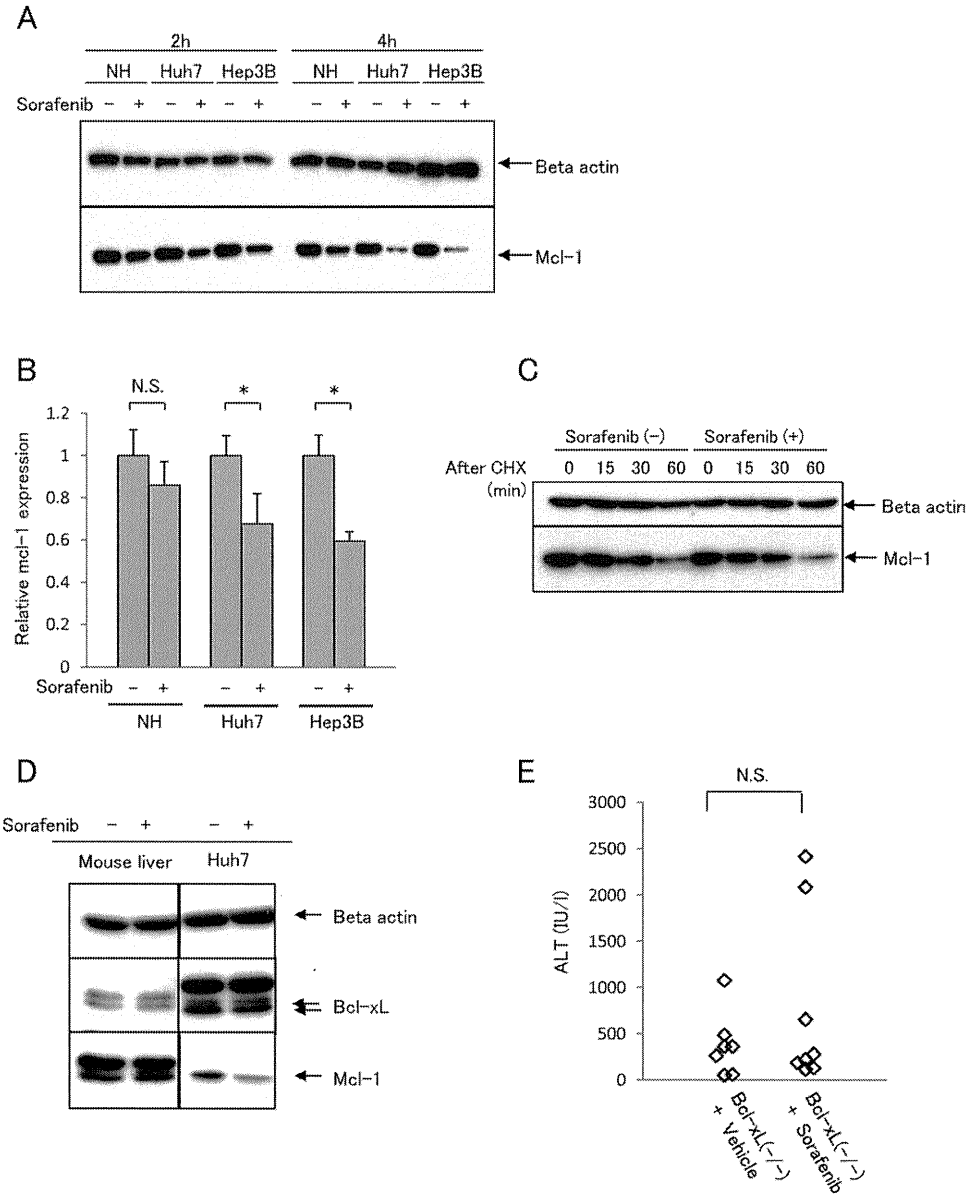

Fig. 5. Effects of sorafenib on the Mcl-1 expression in hepatoma cells and nontransformed cells in vitro and in vivo. (A,B) Normal hepatocytes (NH) and Huh7 and Hep3B cells were treated with 5 μM sorafenib. (A) Western blot analysis for the expression of Mcl-1 after treatment for 2 or 4 hours. (B) Real-time RT-PCR analysis for mcl-1 expression after treatment for 4 hours (N = 6). The levels were normalized to each group without sorafenib. N.S., not significant. *P < 0.05. (C) Huh7 cells were cultured with 1 mM cycloheximide (CHX) with or without 5 μM sorafenib for the indicated times. Western blot analysis for Mcl-1 expression. (D) Nude mice carrying xenograft tumors of Huh7 cells were orally administered 30 mg/kg sorafenib or vehicle daily for 3 days. Western blot analysis for the expression of Bcl-xL and Mcl-1 in the xenograft tumor of Huh7 cells and the liver of mice. (E) Hepatocyte-specific Bcl-xL knockout mice (Bcl-xL −/−) were orally administered daily 30 mg/kg sorafenib or vehicle daily for 3 days. The serum ALT levels are shown (N = 7 or 8). The difference between two groups was determined using Mann-Whitney's U test. N.S., not significant.

suppression of sorafenib is tumor-specific, nontransformed human hepatocytes and hepatoma cell lines were treated with sorafenib. Sorafenib down-regulated Mcl-1 expression in all hepatoma cell lines tested, but had a lesser effect on nontransformed human hepatocytes (Fig. 5A). Sorafenib down-regulated mcl-1 mRNA expression in Huh7 and Hep3B hepatoma cells but not in nontransformed hepatocytes (Fig. 5B). To examine the posttranscriptional effect of sorafenib for Mcl-1 expression, we treated Huh7 cells with cycloheximide in the presence or absence of sorafenib. Cycloheximide-induced decline in Mcl-1 expression was not accelerated by sorafenib (Fig. 5C). This result indicated that, in contrast to the case of ABT-737, sorafenib does not affect the degradation process of Mcl-1.

We also examined Mcl-1 expression in the liver as well as xenograft tumors. Administration of sorafenib significantly suppressed Mcl-1 expression in Huh7 xenograft tumors but not in the liver (Fig. 5D). To examine the safety of sorafenib in the absence of Bcl-xL *in vivo*, we administered sorafenib to hepatocyte-specific Bcl-xL knockout mice. These mice had elevated levels of serum ALT at baseline, as we reported previously,[8] but displayed neither further ALT elevation nor lethal liver failure upon sorafenib administration (Fig. 5E). Taken together, these results indicate that sorafenib did not affect Mcl-1 expression in the liver and therefore did not cause further liver injury even if Bcl-xL was inactivated.

ABT-737 Induced Apoptosis of Hepatoma Cells and Suppressed Growth of Xenograft Tumor with Sorafenib Coadministration. To examine the impact of coadministration of sorafenib and ABT-737 on

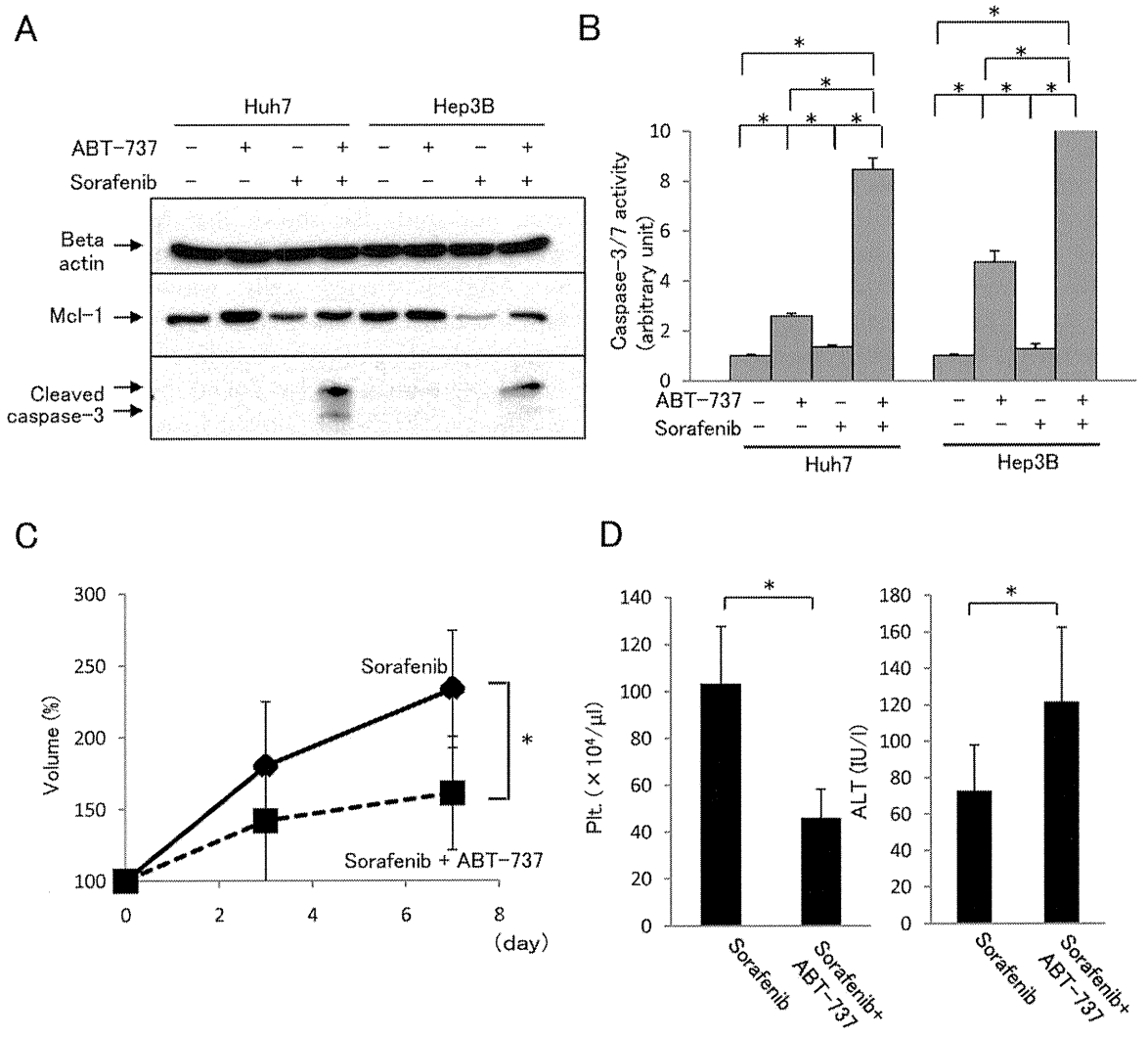

Fig. 6. Effects of ABT-737 with sorafenib treatment in vitro and in vivo. (A,B) Huh7 and Hep3B cells were treated with or without 4 μM ABT-737 together with or without 5 μM sorafenib. (A) Western blot analysis for the expression of Mcl-1 and cleaved caspase-3. (B) Caspase-3/7 activity of culture supernatants. *$P < 0.05$. (C,D) Nude mice carrying xenograft tumors of Huh7 cells were intraperitoneally administered daily 50 mg/kg ABT-737 or vehicle with daily oral administration of 30 mg/kg sorafenib for 7 days ($N = 8$ or 11). (C) The percentage of xenograft tumor volume. (D) Circulating platelet (Plt.) count and serum ALT levels. *$P < 0.05$.

inducing apoptosis, we treated Huh7 and Hep3B hepatoma cells with ABT-737 and/or sorafenib. Although ABT-737 up-regulated Mcl-1 expression in Huh7 and Hep3B cells, sorafenib abolished the Mcl-1 up-regulation induced by ABT-737; the levels of Mcl-1 expression of cells treated with both were similar to those of nontreated cells (Fig. 6A). Sorafenib failed to activate caspase-3/7 in hepatoma cells by itself, but efficiently activated it in the presence of ABT-737 (Fig. 6B). It was also confirmed by efficient cleavage of caspase-3 on western blot analysis (Fig. 6A).

To examine whether ABT-737 has an antitumor effect in the presence of sorafenib, we administered ABT-737 and sorafenib together to nude mice bearing Huh7 xenograft tumors. Although even sorafenib alone significantly suppressed tumor growth compared with the vehicle alone (Supporting Fig. 3), coadministration of ABT-737 and sorafenib led to significant further suppression of tumor growth compared to administration of sorafenib alone (Fig. 6C). Similar to administration of ABT-737 as a single agent, coadministration of sorafenib and ABT-737 also induced mild thrombocytopenia and ALT elevation (Fig. 6D). However, coadministration did not induce further morbidity or mortality in mice, suggesting that this regimen is safe and effective for controlling HCC progression.

Discussion

Tumor cells have two characteristic features: uncontrolled proliferation and apoptosis resistance. Uncontrolled proliferation, driven by activation of a variety

of oncogenes, is directly linked to tumor growth. Apoptosis resistance is believed to be required for the oncogene-induced aberrant proliferation, because without it, tumor cells tend to undergo apoptosis.[24] However, the direct link between apoptosis resistance and growth of solid tumors *in vivo* has not been well studied. Clarifying this point is very important, especially because a very recent study by Weber et al.[25] produced the contradictory finding that aged hepatocyte-specific Mcl-1 knockout mice develop HCC-like lesions, suggesting a link between hepatocarcinogenesis and increased proliferation resulting from increased apoptosis. In the present study, we used conditional expression of Bcl-xL in tumor cells to show that Bcl-xL overexpression, which is frequently found in human HCC, can be directly linked to tumor growth *in vivo*, although it did not promote significant cell growth *in vitro*. Our results suggest that tumor cells encounter a variety of cellular stresses and require antiapoptosis to survive *in vivo* rather than *in vitro*. Thus, we consider antiapoptosis to be an important mechanism for progression of a solid tumor, even if it may not be the case for tumor development as suggested by Weber et al.[25] Our finding also provides proof of the concept that Bcl-xL may be a target for therapy against HCC progression.

In the present study, we showed that, unlike hematopoietic malignancy, hepatoma cells are relatively resistant to ABT-737. Although ABT-737 dose-dependently induced apoptosis in hepatoma cells, a relatively high dose of ABT-737 (more than 8 μM) was required to suppress tumor growth *in vitro*. Importantly, administration of an *in vivo* effective dose of ABT-737 (50 mg/kg) failed to suppress xenograft tumors. We found increased expression of Mcl-1 in cultured hepatoma cells as well as xenograft tumors upon ABT-737 treatment. This may be part of the mechanism of their relative resistance to ABT-737 because hepatoma cells were highly sensitive to this agent if Mcl-1 expression levels were kept constant by an siRNA strategy. Previous articles have reported that Mcl-1 knockdown makes some tumor cells sensitive to ABT-737.[26,27] The present study showed that ABT-737 up-regulation of Mcl-1 rather than Mcl-1 expression itself may be a mechanism of tumor cell resistance to this agent.

A recent study demonstrated that long-term exposure to ABT-737 made initially sensitive lymphoma cell lines resistant to this agent via up-regulation of Mcl-1.[28] In this study, Mcl-1 up-regulation in the ABT-737–resistant lymphoma cells were reported to be mediated by transcriptional up-regulation. In the present study, hepatoma cells showed immediate, posttranscriptional up-regulation of Mcl-1. This rapid response may contribute to the difficulty of treating hepatoma cells with ABT-737 compared with lymphoma cells in which ABT-737 is reported to be effective not only *in vitro*[29] but also *in vivo*.[30] The mechanism by which hepatoma cells posttranscriptionally up-regulate Mcl-1 upon ABT-737 exposure is not clear at present. However, our study has shown that Mcl-1 up-regulation was mediated by delayed degradation of Mcl-1 protein in ABT-737–treated cells without involving the USP9X deubiquitinase. ABT-737 is a Bad mimetic small molecule and preferentially binds with the BH3-binding groove of Bcl-xL. This binding may release endogenous BH3-only proteins such as Bim and Bid and presumably Bak and Bax from Bcl-xL and these unleashed Bcl-2 proteins may then bind Mcl-1. The interaction between Mcl-1 and the unleashed Bcl-2 proteins may cause increased Mcl-1 stability. Because Bak/Bax and Bid/Bim function as effectors and activators for the mitochondrial pathway of apoptosis, respectively, their binding with Mcl-1 may also cause apoptosis resistance to ABT-737.

Not only efficacy but also safety is an important point when considering a therapeutic strategy for cancer. Tumor cells sometimes share similar mechanisms for survival with normal cells. Indeed, HCCs overexpress Bcl-xL, but this molecule also plays an important role in maintaining the integrity of normal hepatocytes.[8] In the present study, we administered ABT-737 to Mcl-1 knockout mice and demonstrated that inactivation of both Bcl-xL and Mcl-1 could induce lethal hepatitis. We previously reported that Bcl-xL and Mcl-1 are required for liver development during embryogenesis,[15] and the present study also revealed the critical importance of both molecules in the adult liver. Recently, the possibility of combination therapy for down-regulation of Bcl-xL and Mcl-1 has been reported *in vitro*.[26,27,31] The present study, for the first time, focused on the *in vivo* safety of this strategy.

Regarding safety concerns about the inactivation of both Mcl-1 and Bcl-xL, sorafenib is an attractive agent because as we have revealed in this study, it down-regulates Mcl-1 expression in a relatively specific manner in tumor cells. Experiments with sorafenib administration into Bcl-xL knockout mice confirmed the safety of coadministration of sorafenib and ABT-737. The underlying mechanisms by which sorafenib down-regulates Mcl-1 in a tumor-specific manner are not clear. Some reports have shown that the down-regulation of Mcl-1 by sorafenib is independent of MEK/ERK,[16,23,32] but is dependent on Raf, AKT (protein kinase B), and Tyr705 phosphorylation of signal transducer and activator of transcription 3 (STAT3).[33,34]

Together with the report that activation of Ras/Raf and STAT3 pathways was found in HCC,[35] these pathways in tumor cells may be more activated than in healthy cells and result in the specificity of Mcl-1 down-regulation in tumor cells by sorafenib. Further experiments are needed to clarify this point.

Sorafenib belongs to a recently approved new class of targeted therapeutics that inhibit the oncogenic kinase pathway for HCC. It has been found to significantly prolong survival of patients with advanced HCC, although its effect appeared to be one of maintaining a stable disease state rather than inducing tumor regression.[36,37] It is speculated that sorafenib produces anticancer effects through a variety of ways such as suppression of angiogenesis and cell cycle arrest of tumor cells. Although it down-regulates Mcl-1,[16,23,32-34] its effect on apoptosis has not been clearly understood. In the present study, we found that sorafenib could not efficiently induce apoptosis in hepatoma cells by itself. This might explain why this agent elicits predominantly disease-stabilizing, cytostatic responses rather than tumor regression. Adding ABT-737 efficiently induced apoptosis of hepatoma cells, clearly indicating that the target of ABT-737, presumably Bcl-xL, blocks the apoptosis-inducing potency of sorafenib. Furthermore, coadministration of ABT-737 and sorafenib led to stronger suppression of xenograft tumor growth than did administration of sorafenib alone. These results suggest that combining sorafenib with ABT-737 may be an attractive strategy for producing durable clinical responses to combat HCC.

In conclusion, we have demonstrated that the inhibition of Bcl-xL by ABT-737 under sorafenib administration was safe and effective for anti-HCC therapy in preclinical models. ABT-737, a direct activator of apoptosis machinery, may unlock the potent antitumor potential of oncogenic kinase inhibitors such as sorafenib.

Acknowledgment: We sincerely thank Abbott Laboratories for providing ABT-737, Dr. You-Wen He (Department of Immunology, Duke University Medical Center, Durham, NC) for providing the *mcl-1* floxed mice and Dr. Lothar Hennighausen (Laboratory of Genetics and Physiology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD) for providing the *bcl-x* floxed mice.

References

1. Tsujimoto Y. Cell death regulation by the Bcl-2 protein family in the mitochondria. J Cell Physiol 2003;195:158-167.
2. Yip KW, Reed JC. Bcl-2 family proteins and cancer. Oncogene 2008; 27:6398-6406.
3. Takehara T, Liu X, Fujimoto J, Friedman SL, Takahashi H. Expression and role of Bcl-xL in human hepatocellular carcinomas. HEPATOLOGY 2001;34:55-61.
4. Watanabe J, Kushihata F, Honda K, Mominoki K, Matsuda S, Kobayashi N. Bcl-xL overexpression in human hepatocellular carcinoma. Int J Oncol 2002;21:515-519.
5. Takehara T, Takahashi H. Suppression of Bcl-xL deamidation in human hepatocellular carcinomas. Cancer Res 2003;63:3054-3057.
6. Ding ZB, Shi YH, Zhou J, Qiu SJ, Xu Y, Dai Z, et al. Association of autophagy defect with a malignant phenotype and poor prognosis of hepatocellular carcinoma. Cancer Res 2008;68:9167-9175.
7. Watanabe J, Kushihata F, Honda K, Sugita A, Tateishi N, Mominoki K, et al. Prognostic significance of Bcl-xL in human hepatocellular carcinoma. Surgery 2004;135:604-612.
8. Takehara T, Tatsumi T, Suzuki T, Rucker EB 3rd, Hennighausen L, Jinushi M, et al. Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. Gastroenterology 2004;127:1189-1197.
9. Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature 2005;435:677-681.
10. Vogler M, Dinsdale D, Dyer MJ, Cohen GM. Bcl-2 inhibitors: small molecules with a big impact on cancer therapy. Cell Death Differ 2009;16:360-367.
11. Mott JL, Gores GJ. Piercing the armor of hepatobiliary cancer: Bcl-2 homology domain 3 (BH3) mimetics and cell death. HEPATOLOGY 2007;46:906-911.
12. Del Gaizo Moore V, Schlis KD, Sallan SE, Armstrong SA, Letai A. BCL-2 dependence and ABT-737 sensitivity in acute lymphoblastic leukemia. Blood 2008;111:2300-2309.
13. Hann CL, Daniel VC, Sugar EA, Dobromilskaya I, Murphy SC, Cope L, et al. Therapeutic efficacy of ABT-737, a selective inhibitor of BCL-2, in small cell lung cancer. Cancer Res 2008;68:2321-2328.
14. Tse C, Shoemaker AR, Adickes J, Anderson MG, Chen J, Jin S, et al. ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. Cancer Res 2008;68:3421-3428.
15. Hikita H, Takehara T, Shimizu S, Kodama T, Li W, Miyagi T, et al. Mcl-1 and Bcl-xL cooperatively maintain integrity of hepatocytes in developing and adult murine liver. HEPATOLOGY 2009;50:1217-1226.
16. Shimizu S, Takehara T, Hikita H, Kodama T, Miyagi T, Hosui A, et al. The let-7 family of microRNAs negatively regulates Bcl-xL expression and potentiates sorafenib-induced apoptosis in human hepatocellular carcinoma. J Hepatol 2010;52:698-704.
17. Hikita H, Takehara T, Kodama T, Shimizu S, Hosui A, Miyagi T, et al. BH3-only protein bid participates in the Bcl-2 network in healthy liver cells. HEPATOLOGY 2009;50:1972-1980.
18. Mason KD, Carpinelli MR, Fletcher JI, Collinge JE, Hilton AA, Ellis S, et al. Programmed anuclear cell death delimits platelet life span. Cell 2007;128:1173-1186.
19. Sieghart W, Losert D, Strommer S, Cejka D, Schmid K, Rasoul-Rockenschaub S, et al. Mcl-1 overexpression in hepatocellular carcinoma: a potential target for antisense therapy. J Hepatol 2006;44:151-157.
20. Fleischer B, Schulze-Bergkamen H, Schuchmann M, Weber A, Biesterfeld S, Müller M, et al. Mcl-1 is an anti-apoptotic factor for human hepatocellular carcinoma. Int J Oncol 2006;28:25-32.
21. Zhong Q, Gao W, Du F, Wang X. Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. Cell 2005;121:1085-1095.
22. Schwickart M, Huang X, Lill JR, Liu J, Ferrando R, French DM, et al. Deubiquitinase USP9X stabilizes MCL1 and promotes tumour cell survival. Nature 2010;463:103-107.
23. Liu L, Cao Y, Chen C, Zhang X, McNabola A, Wilkie D, et al. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. Cancer Res 2006;66:11851-11858.
24. Cotter TG. Apoptosis and cancer: the genesis of a research field. Nat Rev Cancer 2009;9:501-507.

25. Weber A, Boger R, Vick B, Urbanik T, Haybaeck J, Zoller S, et al. Hepatocyte-specific deletion of the antiapoptotic protein myeloid cell leukemia-1 triggers proliferation and hepatocarcinogenesis in mice. HEPATOLOGY 2010;51:1226-1236.
26. van Delft MF, Wei AH, Mason KD, Vandenberg CJ, Chen L, Czabotar PE, et al. The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. Cancer Cell 2006;10:389-399.
27. Keuling AM, Felton KE, Parker AA, Akbari M, Andrew SE, Tron VA. RNA silencing of Mcl-1 enhances ABT-737-mediated apoptosis in melanoma: role for a caspase-8-dependent pathway. PLoS One 2009;4: e6651.
28. Yecies D, Carlson NE, Deng J, Letai A. Acquired resistance to ABT-737 in lymphoma cells that up-regulate MCL-1 and BFL-1. Blood 2010;115:3304-3313.
29. Vogler M, Dinsdale D, Sun XM, Young KW, Butterworth M, Nicotera P, et al. A novel paradigm for rapid ABT-737-induced apoptosis involving outer mitochondrial membrane rupture in primary leukemia and lymphoma cells. Cell Death Differ 2008;15:820-830.
30. Mason KD, Vandenberg CJ, Scott CL, Wei AH, Cory S, Huang DC, et al. In vivo efficacy of the Bcl-2 antagonist ABT-737 against aggressive Myc-driven lymphomas. Proc Natl Acad Sci U S A 2008;105: 17961-17966.
31. Lin X, Morgan-Lappe S, Huang X, Li L, Zakula DM, Vernetti LA, et al. 'Seed' analysis of off-target siRNAs reveals an essential role of Mcl-1 in resistance to the small-molecule Bcl-2/Bcl-XL inhibitor ABT-737. Oncogene 2007;26:3972-3979.
32. Yu C, Bruzek LM, Meng XW, Gores GJ, Carter CA, Kaufmann SH, et al. The role of Mcl-1 down-regulation in the proapoptotic activity of the multikinase inhibitor BAY 43-9006. Oncogene 2005;24: 6861-6869.
33. Ulivi P, Arienti C, Amadori D, Fabbri F, Carloni S, Tesei A, et al. Role of RAF/MEK/ERK pathway, p-STAT-3 and Mcl-1 in sorafenib activity in human pancreatic cancer cell lines. J Cell Physiol 2009;220: 214-221.
34. Blechacz BR, Smoot RL, Bronk SF, Werneburg NW, Sirica AE, Gores GJ. Sorafenib inhibits signal transducer and activator of transcription-3 signaling in cholangiocarcinoma cells by activating the phosphatase shatterproof 2. HEPATOLOGY 2009;50:1861-1870.
35. Calvisi DF, Ladu S, Gorden A, Farina M, Conner EA, Lee JS, et al. Ubiquitous activation of Ras and Jak/Stat pathways in human HCC. Gastroenterology 2006;130:1117-1128.
36. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008; 359:378-390.
37. Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, et al. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol 2009;10:25-34.

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i44.4890

World J Gastroenterol 2011 November 28; 17(44): 4890-4898
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Serum manganese superoxide dismutase and thioredoxin are potential prognostic markers for hepatitis C virus-related hepatocellular carcinoma

Tsutomu Tamai, Hirofumi Uto, Yoichiro Takami, Kouhei Oda, Akiko Saishoji, Masashi Hashiguchi, Kotaro Kumagai, Takeshi Kure, Seiichi Mawatari, Akihiro Moriuchi, Makoto Oketani, Akio Ido, Hirohito Tsubouchi

Tsutomu Tamai, Hirofumi Uto, Yoichiro Takami, Kouhei Oda, Akiko Saishoji, Masashi Hashiguchi, Kotaro Kumagai, Takeshi Kure, Seiichi Mawatari, Akihiro Moriuchi, Makoto Oketani, Akio Ido, Hirohito Tsubouchi, Department of Digestive and Lifestyle Related Diseases, Human and Environmental Sciences, Health Research, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan
Author contributions: Tamai T and Takami Y performed the majority of experiments; Uto H and Tsubouchi H were involved in editing the manuscript; Uto H, Oda K, Saishoji A, Hashiguchi M, Kumagai K, Kure T, Mawatari S, Moriuchi A, Oketani M and Ido A coordinated the collection of and provided all the human material for this work; Tamai T and Uto H designed the study and wrote the manuscript.
Supported by (in part) Grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Ministry of Health, Labour and Welfare of Japan
Correspondence to: Hirofumi Uto, MD, PhD, Department of Digestive and Lifestyle Related Diseases, Human and Environmental Sciences, Health Research, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan. hirouto@m2.kufm.kagoshima-u.ac.jp
Telephone: +81-99-2755326 Fax: +81-99-2643504
Received: April 26, 2011 Revised: August 17, 2011
Accepted: October 14, 2011
Published online: November 28, 2011

Abstract

AIM: To evaluate the clinical significance of oxidative stress markers in patients with hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC).

METHODS: Sixty-four consecutive patients who were admitted to Kagoshima University Medical and Dental Hospital were enrolled in this retrospective study. All patients had chronic liver disease (CLD) due to infection with HCV. Thirty patients with HCV-related HCC, 34 with HCV-related CLD without HCC (non-HCC), and 20 healthy volunteers (HVs) were enrolled. Possible associations between serum manganese superoxide dismutase (MnSOD) and thioredoxin (TRX) levels and clinical parameters or patient prognosis were analyzed over a mean follow-up period of 31.7 mo.

RESULTS: The serum MnSOD levels were significantly higher in patients with HCV-related HCC than in patients without HCC ($P = 0.03$) or HVs ($P < 0.001$). Similarly, serum TRX levels were also significantly higher in patients with HCV-related HCC than in patients without HCC ($P = 0.04$) or HVs ($P < 0.01$). However, serum levels of MnSOD and TRX were not correlated in patients with HCC. Among patients with HCC, the overall survival rate (OSR) was lower in patients with MnSOD levels $\geqslant$ 110 ng/mL than in patients with levels < 110 ng/mL ($P = 0.01$), and the OSR tended to be lower in patients with TRX levels < 80 ng/mL ($P = 0.05$). In addition, patient prognosis with HCC was poorest with serum MnSOD levels $\geqslant$ 110 ng/mL and serum TRX levels < 80 ng/mL. Furthermore, a multivariate analysis using a Cox proportional hazard model and serum levels of five factors (MnSOD, prothrombin time, serum albumin, serum α-fetoprotein (AFP), and serum des-γ-carboxy prothrombin) revealed that MnSOD levels $\geqslant$ 110 ng/mL (risk ratio: 4.12, 95% confidential interval: 1.22-13.88, $P = 0.02$) and AFP levels $\geqslant$ 40 ng/mL (risk ratio: 6.75; 95% confidential interval: 1.70-26.85, $P < 0.01$) were independent risk factors associated with a poor patient prognosis.

CONCLUSION: Serum MnSOD and TRX levels are potential clinical biomarkers that predict patient prognosis in HCV-related HCC.

Key words: Oxidative stress; Manganese superoxide dismutase; Thioredoxin; Hepatitis C virus; Hepatocellular carcinoma

Peer reviewers: Assy Nimer, MD, Assistant Professor, Liver Unit, Ziv Medical Centre, BOX 1008, Safed 13100, Israel; Andre Castro Lyra, MD, Associate Professor, Federal University of Bahia, Head, Gastro-Hepatology Unit, Hospital Sao Rafael, Monte Tabor Foundation, Salvador, Bahia 40296 720, Brazil

Tamai T, Uto H, Takami Y, Oda K, Saishoji A, Hashiguchi M, Kumagai K, Kure T, Mawatari S, Moriuchi A, Oketani M, Ido A, Tsubouchi H. Serum manganese superoxide dismutase and thioredoxin are potential prognostic markers for hepatitis C virus-related hepatocellular carcinoma. *World J Gastroenterol* 2011; 17(44): 4890-4898 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i44/4890.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i44.4890

INTRODUCTION

As a significant cause of global cancer morbidity and mortality, hepatocellular carcinoma (HCC) is the fifth- and seventh-most frequently diagnosed cancer worldwide in men and women, respectively, and is the second- and sixth-most frequent cause of cancer deaths in men and women, respectively[1]. HCC is most frequently caused by persistent infection with hepatitis C or B virus. Early HCC diagnosis and better treatments have helped to improve the prognosis for patients with HCC. Also, interferon (IFN)-based treatments not only eliminate hepatitis C virus (HCV) infection, but also prevent HCC in patients with chronic hepatitis C (CHC)[2]. However, IFN-based therapies do not always effectively eliminate HCV infection or prevent HCC. Thus, biomarkers that are indicative of HCC pathological condition would have many clinical benefits, including aiding in the selection of the most appropriate treatment for a patient's disease.

Oxidative stress results from an imbalance in the production of reactive oxygen species (ROS) and the antioxidative defenses that maintain a cellular redox state. ROS include superoxide anions, hydrogen peroxide, hydroxyl radicals and nitric oxide, all of which are indispensable elements in many biochemical processes[3]. ROS are mainly derived from Kupffer and inflammatory cells in the liver[4], and upon exposure to other cells are thought to induce apoptosis, necrosis, inflammation, immune responses, fibrosis and tissue regeneration[5]. In liver disease, there is an overproduction of ROS from endogenous sources such as the mitochondria, peroxisomes, and activated inflammatory cells. In particular, ROS of mitochondrial origin were recently reported to be elevated in patients with alcoholic liver disease, non-alcoholic steatohepatitis (NASH)[6,7] and HCV-related chronic liver disease (CLD)[8]. Conversely, cells are protected from oxidative stress by intracellular antioxidants such as glutathione (GSH) and thioredoxin (TRX) and by various antioxidant enzymes such as superoxide dismutase (SOD), GSH peroxidase, catalase, and heme oxygenase-1[9-11]. Collectively, the relative expression levels of these molecules may serve as biomarkers for various liver diseases, including HCV-related HCC.

Manganese SOD (MnSOD) is an antioxidant enzyme that catalyzes the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2. We have previously demonstrated that MnSOD expression was induced in primary cultured hepatocytes that were loaded with hydrogen peroxide *in vitro* and that serum MnSOD levels can be used to distinguish between NASH and simple steatosis in patients with nonalcoholic fatty liver disease[7]. However, the clinical significance of serum MnSOD levels in HCV-related CLD has not been fully investigated.

TRX was originally discovered in *Escherichia coli* as a proton donor for ribonucleotide reductase[12]. Subsequently, the human *TRX* gene was cloned as an adult T-cell leukemia-derived factor and was originally described as an interleukin-2 receptor inducer present in the cell culture supernatant of human T-lymphotropic virus type-1 -transformed cells[13]. TRX expression is induced by various oxidative stressors in patients with acquired immunodeficiency syndrome[14], Sjögren's syndrome[15], rheumatoid arthritis[16], and malignant neoplasms[17,18]. Previous studies have reported that serum TRX is an oxidative stress marker and that serum TRX levels increase in patients with HCV-related CLD during liver fibrosis progression[19]. In addition, serum TRX levels are reported to be elevated in patients with NASH compared to patients with simple steatosis[20]. However, the clinical significance of elevated TRX levels among patients infected with HCV in relation to HCC diagnosis and prognosis has not been elucidated.

In this study, we aimed to clarify the clinical significance of serum levels of MnSOD and TRX in patients with HCV-related CLD, and in particular among patients with HCC.

MATERIALS AND METHODS

Patients

Sixty-four consecutive patients who were admitted to Kagoshima University Medical and Dental Hospital between December 2006 and November 2008 were enrolled in this retrospective study. All patients had CLD due to an HCV infection and were diagnosed with HCC (30 patients; HCC group) or without HCC (34 patients; non-HCC group). Twenty healthy volunteers (HVs) were also enrolled in this study.

In this study, HCC was diagnosed based on findings from abdominal ultrasound, abdominal computed tomography, and serum levels of α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP, also known as PIVKA-Ⅱ). Patients were excluded from this study if they were positive for hepatitis B surface antigen; other types of hepatitis, including autoimmune hepatitis and alcoholic liver disease; or other malignancies.

The study endpoint was patient death, the available follow-up date, or December 31, 2010. Patient follow-up

periods ranged from 5.1 to 44.6 mo, with a mean observation time of 31.7 mo. Informed consent was obtained from all study patients and healthy controls. This study was approved by the ethical committees of Kagoshima University Graduate School of Medical and Dental Sciences and Kagoshima University Medical and Dental Hospital.

Laboratory markers

The clinical laboratory parameters assessed included platelet count (Plt), prothrombin time (PT), albumin (Alb), total bilirubin (T-Bil), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), AFP and DCP. The serologically defined HCV genotype (HCV serotype) was determined using a serological genotyping assay kit (Immunocheck F-HCV Grouping; International Reagents Co., Tokyo, Japan). If the HCV serotype could not be determined, the HCV genotype was evaluated using the HCV Core Genotype assay (SRL, Tokyo, Japan). HCV genotype 1b was included with serotype Ⅰ, while genotypes 2a and 2b were included with serotype Ⅱ. No other HCV genotype was detected in this study population. HCV RNA titers were quantified using either quantitative RT-PCR (Amplicor monitor version 2, Roche, Tokyo, Japan) or the Cobas TaqMan PCR assay (Roche, Tokyo, Japan). Patients were categorized as having a high viral load if their values were 100 KIU/mL or greater based on quantitative RT-PCR analysis, or 5 log IU/mL or more based on the Cobas TaqMan PCR assay.

Evaluation of clinical stage

Hepatic function was assessed in the HCC group using Child-Pugh staging based on both clinical (ascites and encephalopathy) and laboratory (Alb, T-Bil, and PT) parameters. HCC clinical stage was assessed based on a patient's Cancer of the Liver Italian Program (CLIP) score, which was calculated by adding points for the following four variables: Child-Pugh stage, tumor morphology, AFP value, and portal venous invasion[21,22]. The Japan Integrated Staging (JIS) system[23,24], developed by the Liver Cancer Study Group of Japan and based on a combination of Child-Pugh stage and HCC TNM classification, was used to clinically stage HCC.

Serum MnSOD and TRX levels

Serum was obtained from peripheral blood samples by centrifugation at 4000 *g* for 5 min at room temperature. Serum samples were frozen at -80 ℃ until further use. Serum MnSOD or TRX levels were measured using the Human Superoxide Dismutase 2 (AbFRONTIER, Seoul, Korea) and human thioredoxin (Redox Bio Science, Kyoto, Japan) ELISAs, respectively.

Statistical analysis

Results are expressed as the mean and standard deviation. *P* values less than 0.05 were regarded as statistically significant. Statistical analyses were performed using the Fischer's exact test or the Mann-Whitney *U* test, as appropriate. The area under the curve (AUC) was calculated for the receiver operating characteristic (ROC) curve in order to measure the overall accuracy of the test. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of diagnostic test were additionally determined according to the protocol described previously[25]. Differences among the three groups were evaluated using the Kruskal-Wallis test followed by Dunn's multiple comparison tests. Correlation coefficients were calculated using Spearman's rank correlation analysis. The Kaplan-Meier method was used to estimate death for each parameter that had been identified at enrollment, and the death distribution curves were compared using the log-rank test. Univariate and multivariate analyses of patient outcome risk ratios were performed using Cox's proportional hazards regression analyses. All statistical analyses were conducted using PASW Statistics v. 18 (SPSS Inc., Chicago, IL).

Table 1 Patient clinical characteristics

Characteristics	Non-HCC group (*n* = 34)	HCC group (*n* = 30)	*P* value[1]
Age (yr)	62.3 ± 11.0	72.2 ± 7.5	< 0.001
Sex (male/female)	10/24	21/9	< 0.01
Plt (× 10^4/μL)	17.0 ± 5.5	10.3 ± 5.2	< 0.001
PT (%)	99.7 ± 13.3	77.6 ± 11.8	< 0.001
Alb (g/dL)	4.3 ± 0.4	3.6 ± 0.6	< 0.001
T-Bil (mg/dL)	0.8 ± 0.3	1.5 ± 0.8	< 0.001
ALT (IU/L)	44.8 ± 30.2	52.0 ± 28.2	0.12
γ-GTP (IU/L)	31.3 ± 16.1	56.2 ± 44.3	< 0.01
AFP (ng/mL)	7.2 ± 22.8	85.9 ± 197.6	< 0.001
DCP (mAU/mL)	22.8 ± 14.7	485.5 ± 1982.6	0.001
HCV serotype group (1/2)	18/10 (*n* = 28)	21/3 (*n* = 24)	0.06
HCV RNA level (high/low)	28/5 (*n* = 33)	21/4 (*n* = 25)	0.99

Data are shown as the mean ± SD. *n*: Number of patients or the number of samples analyzed. [1]Differences between mean values were evaluated using either the Fischer's exact test or the Mann-Whitney *U* test, as appropriate. Plt: Platelet count; PT: Prothrombin time; Alb: Albumin; T-Bil: Total bilirubin; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; AFP: alpha-fetoprotein; DCP: des-γ-carboxy prothrombin; HCV: Hepatitis C virus; RNA: Ribonucleic acid.

RESULTS

Patient characteristics and classification according to the presence of hepatocellular carcinoma

Table 1 summarizes the baseline clinical characteristics of the 64 patients who were classified based on the presence or absence of HCC. Age, sex, and clinical laboratory parameters, including Plt, PT, Alb, T-Bil, γ-GTP, AFP and DCP, were significantly different between these two groups.

Serum MnSOD and TRX levels in hepatocellular carcinoma patients

Serum MnSOD levels were significantly higher in patients with HCC compared to patients without HCC ($P = 0.03$) and HVs ($P < 0.001$) (Figure 1A). The serum TRX levels were also significantly higher in the HCC group compared to the non-HCC group ($P = 0.04$) and HV group ($P < 0.01$) (Figure 1B). However, there was no correlation between these two markers in the HCC group ($P = 0.28$, $r = 0.20$) (Figure 1C).

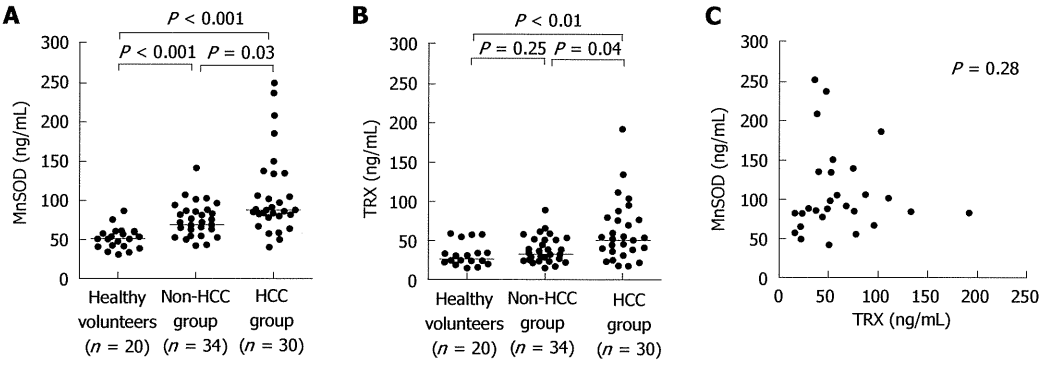

Figure 1 Serum levels of manganese superoxide dismutase and thioredoxin in the hepatocellular carcinoma, non-hepatocellular carcinoma and healthy volunteer groups. A: Serum manganese superoxide dismutase (MnSOD) levels were significantly higher in the hepatocellular carcinoma (HCC) group than in either the non-HCC group ($P = 0.03$) or the healthy volunteers (HV) group ($P < 0.001$); B: Serum thioredoxin (TRX) levels were also significantly higher in the HCC group than in either the non-HCC group ($P = 0.04$) or the HV group ($P < 0.01$); C: No significant correlation was detected between serum MnSOD and TRX levels in the HCC group.

Table 2 Sensitivity, specificity, positive predictive value, negative predictive value and accuracy of manganese superoxide dismutase and α-fetoprotein serum levels for diagnosis of hepatocellular carcinoma in all patients (%)

Factors	Sensitivity	Specificity	PPV	NPV	Accuracy
MnSOD (≥ 110 ng/mL)	26.7	97.1	88.9	60.0	64.1
AFP (≥ 40 ng/mL)	33.3	97.1	90.9	62.3	67.2
Combination[1]	46.7	94.1	87.5	66.7	71.9

[1]MnSOD ≥ 110 ng/mL and/or AFP ≥ 40 ng/mL. PPV: Positive predictive value; NPV: Negative predictive value; MnSOD: Manganese superoxide dismutase; AFP: α-fetoprotein.

Table 3 Correlation between serum manganese superoxide dismutase or thioredoxin levels and laboratory data in the hepatocellular carcinoma group

Factors	HCC group (n = 30)			
	Serum MnSOD levels		Serum TRX levels	
	Correlation coefficient	P value	Correlation coefficient	P value
Age (yr)	-0.97	0.61	0.11	0.55
Plt (× 10^4/μL)	0.03	0.89	0.66	< 0.001
PT (%)	-0.36	0.05	0.12	0.53
Alb (g/dL)	-0.63	< 0.001	0.19	0.33
T-Bil (mg/dL)	0.25	0.18	0.05	0.79
ALT (IU/L)	0.12	0.52	0.15	0.42
γ-GTP (IU/L)	0.30	0.11	0.28	0.13
AFP (ng/mL)	0.38	0.04	0.11	0.57
DCP (mAU/mL)	0.57	0.001	0.12	0.52

P values were assessed by Spearman's rank correlation analysis. MnSOD: Manganese superoxide dismutase; TRX: Thioredoxin; HCC: Hepatocellular carcinoma; Plt: Platelet count; PT: Prothrombin time; Alb: Albumin; T-Bil: Total bilirubin; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; AFP: α-fetoprotein; DCP: des-γ-carboxy prothrombin.

Diagnostic value of serum MnSOD and TRX levels for patients with hepatocellular carcinoma and hepatitis C virus infection

Serum AFP and DCP concentrations are established diagnostic markers for HCC. To evaluate the utility of MnSOD and TRX for the diagnosis of HCC, we measured AFP and DCP expression in addition to MnSOD and TRX expression. In an AUC-ROC analysis, AFP was the strongest diagnostic marker for HCC (AUC-ROC, 0.90). AUC-ROCs for MnSOD, TRX and DCP were 0.73, 0.77 and 0.77, respectively. Additional analyses showed that the accuracy of AFP (≥ 40 ng/mL) for diagnosis of HCC was higher than that of MnSOD (≥ 110 ng/mL) (Table 2), while the combination of AFP and MnSOD was a more accurate marker of HCC than either marker alone.

Association of serum MnSOD or TRX levels with laboratory data in the HCC group

Serum MnSOD levels for the 30 patients in the HCC group were positively correlated with serum AFP and DCP levels and were negatively correlated with serum Alb levels (Table 3). Serum MnSOD levels were also significantly higher in patients with two or more HCC tumors than in patients with a single HCC tumor [average ± SD (ng/mL), 125.4 ± 50.9 *vs* 87.4 ± 48.8, $P = 0.008$], although HCC tumor size was not associated with serum MnSOD levels. In addition, HCC patient serum MnSOD levels increased in parallel with the Child-Pugh stage, CLIP score and JIS score (Figure 2A-C). In contrast, serum TRX levels were only associated with platelet counts (Table 3). Serum TRX levels were not associated with HCC tumor number or size. Furthermore, there were no significant correlations between serum TRX levels for various scores (Figure 2D-F).

Overall survival rate based on serum MnSOD or TRX levels in the HCC group

In the HCC group, the overall patient survival rate was significantly lower ($P = 0.01$) in patients with MnSOD levels ≥ 110 ng/mL compared to patients with levels < 110 ng/mL (Figure 3A). In addition, the overall survival rate tended to be lower ($P = 0.05$) in patients with TRX levels < 80 ng/mL compared to those with levels ≥ 80 ng/mL (Figure 3B). Furthermore, among all HCC groups, patients who had both serum MnSOD levels ≥ 110

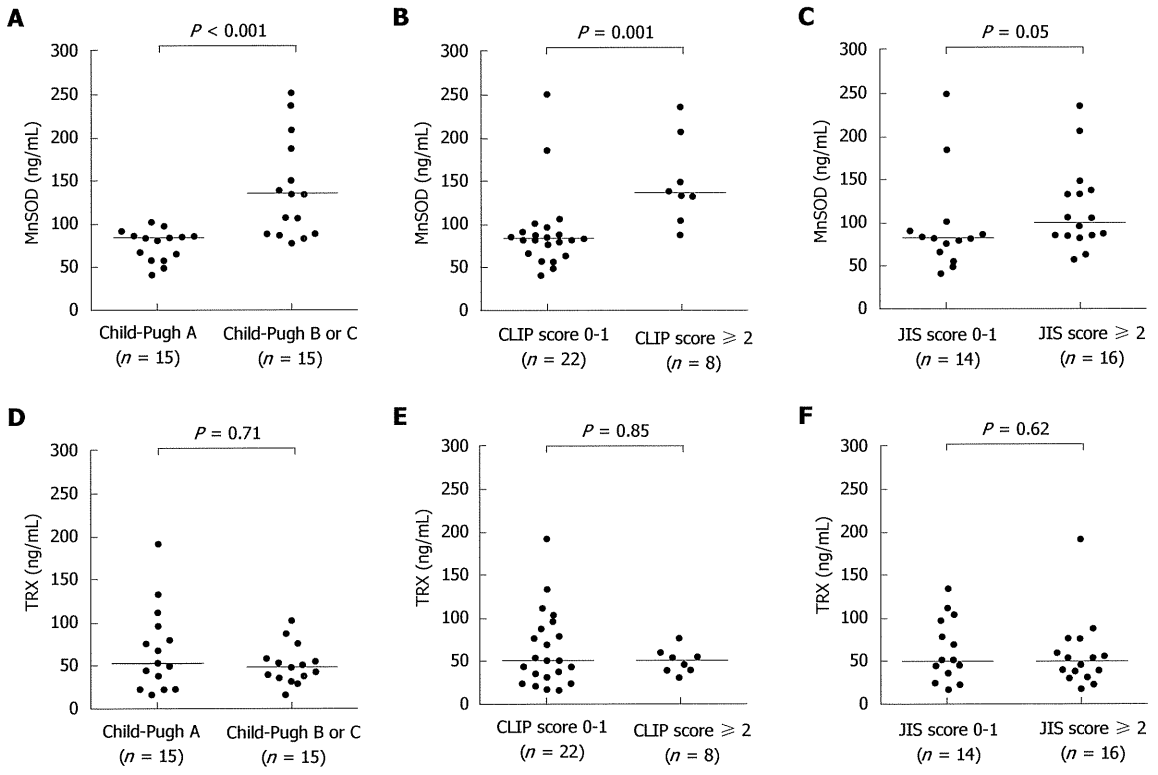

Figure 2 Clinical significance of serum manganese superoxide dismutase and thioredoxin levels in hepatocellular carcinoma. In the hepatocellular carcinoma (HCC) group, differences in serum manganese superoxide dismutase (MnSOD) and thioredoxin (TRX) levels were evaluated based on Child-Pugh stage, cancer of the liver italian program (CLIP) score and Japan integrated staging (JIS) score. A: Serum MnSOD levels were significantly higher in patients with Child-Pugh B or C compared to those with Child-Pugh A ($P < 0.001$); B: Serum MnSOD levels in patients with a CLIP score of 2 or greater were significantly higher compared to levels in patients with a CLIP score of 0 or 1 ($P = 0.001$); C: In addition, serum MnSOD levels tended to be higher in patients with a JIS score of 2 or greater compared to patients with a JIS score of 0 or 1 ($P = 0.05$); D-F: In contrast, serum TRX levels were not significantly different based on Child-Pugh stage, CLIP score or JIS score.

ng/mL and TRX levels < 80 ng/mL had a significantly poorer prognosis. Conversely, patients with a serum TRX level ≥ 80 ng/mL had a favorable prognosis, regardless of their serum MnSOD level (Figure 3C).

In addition to serum MnSOD and TRX levels, other possible prognostic factors were also investigated in the HCC group. A univariate analysis (log-rank test) revealed that the survival rate was significantly different between patients with high and low levels of MnSOD, PT, Alb, AFP and DCP, but not other factors such as TRX (Table 4). A multivariate analysis using a Cox proportional hazard model and five markers (MnSOD, PT, Alb, AFP and DCP) selected based on the results of the univariate analysis revealed that MnSOD levels ≥ 110 ng/mL and AFP levels ≥ 40 ng/mL were independent risk factors that were associated with a poor patient prognosis (Table 5). In addition, similar results were obtained from a similar multivariate analysis using the same five factors and TRX, supporting the finding that TRX is not an independent risk factor associated with HCC prognosis. Furthermore, patient Child-Pugh stage, CLIP score and JIS score, which were calculated based on several factors including clinical symptoms and laboratory data, were also prognostic factors for patients with HCC (Table 4). A multivariate analysis using the three markers of MnSOD, Child-Pugh stage and CLIP score indicated that Child-Pugh stage was also a significant prognostic factor (risk ratio: 6.19, 95% confidential interval: 1.33-28.95, $P = 0.02$).

DISCUSSION

HCV infection is the most important known contributor to the etiology of HCC. An increasing incidence of HCC has been largely attributed to a rise in HCV infections in the general population during the last 50 to 60 years[26]. During HCV infection, ROS production increases and persists throughout the infection. In addition, ROS are thought to play a major role in the pathogenesis of chronic inflammatory changes in the liver, leading to increased hepatic fibrosis and decreased hepatic function. In this study, we have shown that both serum MnSOD and TRX levels are elevated in patients with HCV-related HCC, with no correlation between these two markers. In addition, serum MnSOD and TRX levels were a useful predictor of overall patient survival. Serum MnSOD and TRX levels are reported to be biomarkers of oxidative stress in several diseases, including liver disease[7,14,17,19,27-29]. There were a small number of enrolled patients in this study and other contributors to liver diseases such as chronic hepatitis B infection should be further evaluated. However, our

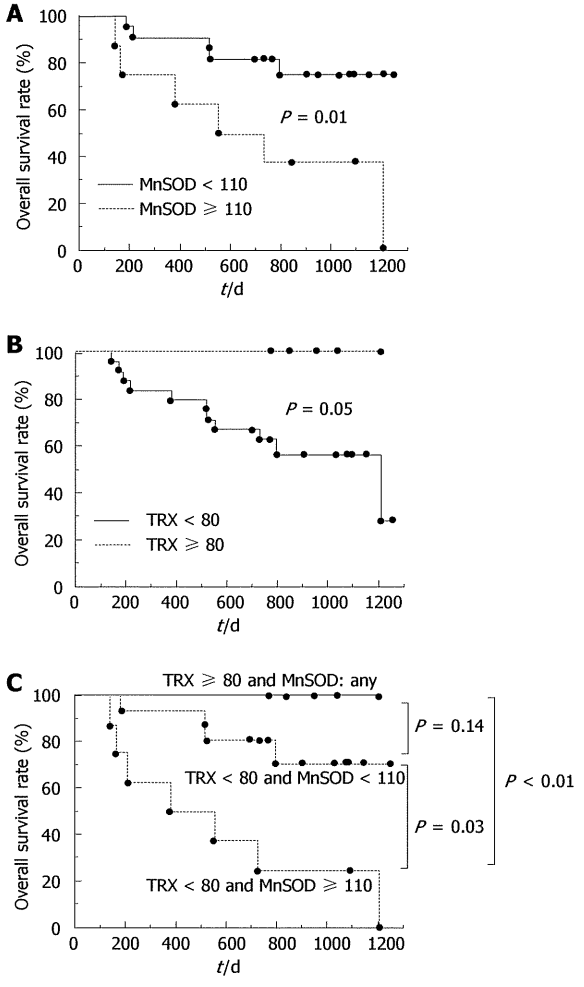

Figure 3 Overall hepatocellular carcinoma patient survival based on serum levels of manganese superoxide dismutase or thioredoxin. Overall survival was plotted using the Kaplan-Meier method after separation into two or three groups defined as follows: A: Manganese superoxide dismutase (MnSOD) < 110 ng/mL or ≥ 110 ng/mL; B: Thioredoxin (TRX) < 80 ng/mL or ≥ 80 ng/mL; TRX ≥ 80 ng/mL, TRX < 80 ng/mL; C: MnSOD < 110 ng/mL, or TRX < 80 ng/mL and MnSOD ≥ 110 ng/mL. The overall survival rate was lower in patients with MnSOD levels ≥ 110 ng/mL (P = 0.01) (A). Also, cumulative patient survival rate tended to be lower in patients with TRX levels < 80 ng/mL (P = 0.05) (B). Among these groups, patients with serum TRX levels < 80 ng/mL and serum MnSOD levels ≥ 110 ng/mL had the poorest prognosis (C).

Table 4 Univariate analysis of prognostic factors in the hepatocellular carcinoma group

Factors	Category	Number	*P* value[1]
Single marker			
MnSOD (ng/mL)	< 110/≥ 110	22/8	0.01
TRX (ng/mL)	< 80/≥ 80	24/6	0.05
Age (yr)	< 70/≥ 70	12/18	0.23
Plt (× 10^4/μL)	< 10/≥ 10	19/11	0.38
PT (%)	< 80/≥ 80	15/15	0.02
Alb (g/dL)	< 3.5/≥ 3.5	15/15	0.02
T-Bil (mg/dL)	< 1.5/≥ 1.5	18/12	0.34
ALT (IU/L)	< 40/≥ 40	11/19	0.58
γ-GTP (IU/L)	< 50/≥ 50	17/13	0.98
AFP (ng/mL)	< 40/≥ 40	20/10	< 0.01
DCP (mAU/mL)	< 40/≥ 40	16/14	0.02
Staging system			
Child-Pugh stage	A/≥ B	16/14	< 0.01
CLIP score	0-1/≥ 2	22/8	0.01
JIS score	0-1/≥ 2	14/16	0.41

[1]*P* values were assessed using the log-rank test. MnSOD: Manganese superoxide dismutase; TRX: Thioredoxin; Plt: Platelet count; PT: Prothrombin time; Alb: Albumin; T-Bil: Total bilirubin; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; AFP: Alpha-fetoprotein; DCP: Serum des-γ-carboxy prothrombin; CLIP: Cancer of the Liver Italian Program; JIS: Japan Integrated Staging.

Table 5 Multivariate analysis of prognostic factors in the hepatocellular carcinoma group

Factors	Risk ratio	95% CI	*P* value
MnSOD (≥ 110 ng/mL)	4.12	1.22-13.88	0.02
AFP(≥ 40 ng/mL)	6.75	1.70-26.85	< 0.01

95% CI: 95% confidence interval; MnSOD: Manganese superoxide dismutase; AFP: α-fetoprotein.

study has clearly demonstrated the clinical significance of these markers in patients with HCV-related HCC.

Serum MnSOD and TRX levels should both reflect hepatic oxidative stress. The results of the current study showed that both of these markers were increased in the HCC group relative to levels in the non-HCC group and the HV group (Figure 1A and B). However, there was no correlation between these two markers in the HCC group (Figure 1C). MnSOD is primarily localized to the mitochondrial matrix[3] and abnormal mitochondrial morphologies are frequently observed in CHC[8]. Therefore, MnSOD may be an indicator of mitochondrial disorders that are induced by oxidative stress. On the other hand, there are two TRX proteins, cytoplasmic TRX1 and mitochondrial TRX2[30]. TRX1 negatively regulates the apoptosis signal-regulating kinase 1 (ASK1)-c-Jun N-terminal kinase/P38 apoptotic pathway by binding to and inhibiting the kinase activity of ASK1, which plays an important role in ROS-induced cellular responses[31]. TRX2 is an essential regulator of mitochondrial ROS levels that has been associated with mitochondrial outer membrane permeability[32]. In the present study, we examined the serum levels of TRX1, but not TRX2, using a sandwich ELISA. Thus, the MnSOD and TRX proteins that were examined in this study have different origins in the mitochondria and cytoplasm, respectively, which could contribute to the lack of correlation between these two markers.

Several studies have shown that the HCV core protein directly inhibits the electron transport system and modulates apoptosis, transcription, and cell signaling[33]. Abdalla *et al*[34] reported that expression of not only the HCV core protein but also the HCV NS proteins increases ROS and further showed that the presence of these proteins can increase endogenous expression levels of antioxidant enzymes and prooxidants such as MnSOD. Several reports have shown that serum MnSOD levels in patients with HCV-related CLD[35-37] are associated with

various clinical findings, such as fibrosis and hepatic oxidative stress. However, the significance of serum MnSOD levels has not been fully examined in patients with HCC. We previously reported that serum MnSOD levels may be correlated with fibrosis in patients with NAFLD[7]. In addition, serum MnSOD levels decreased in patients with CHC after administration of an interferon-based treatment (data not shown). These results indicate that serum MnSOD levels are likely associated with hepatic fibrosis or oxidative stress in patients with CHC. In the present study, however, MnSOD levels were not associated with platelet counts, which is a simple predictor of hepatic fibrosis in this patient population[38]. Thus, advanced hepatic fibrosis or oxidative stress may be one reason why serum MnSOD levels have diagnostic and prognostic utility with HCC, but other mechanisms should also be considered.

The present study revealed that serum MnSOD levels were significantly higher in the HCC group than in the non-HCC group (Figure 1A). In the HCC group, serum MnSOD levels were negatively correlated with serum Alb and tended to negatively correlate with PT (Table 3); these results showed an association between MnSOD and Child-Pugh stage (Figure 2A). It is known that in humans, MnSOD activity is comparatively higher in the liver compared to other tissues[39]. In addition, although a previous immunohistochemical study showed that MnSOD expression was higher in both cancerous and non-cancerous liver tissues from patients with HCC, this positive immunoreactivity was strongly observed in non-cancerous liver tissues, especially in normal hepatocytes surrounding HCC, regenerative small hepatocytes in the tumor boundary, and mononuclear inflammatory cells in necroinflammatory lesions[40]. Furthermore, ROS are overproduced by Kupffer cells and inflammatory cells in liver disease[5,41]. In the present study, serum MnSOD levels were also positively correlated with the serum tumor markers AFP and DCP (Table 3) and with Child-Pugh stage and CLIP score (Figure 2). These results indicate that increased MnSOD expression reflects hepatocyte oxidative stress and correlates with decreased hepatic function, increased hepatic fibrosis and ROS production by inflammatory cells in liver cirrhosis. These features comprise the main background characteristics leading to HCC and may be associated with the indirect effects of liver cancer progression. These associations may also explain why serum MnSOD levels predicted the overall survival of patients with HCC.

It was previously reported that serum levels of TRX, which is a stress-induced protein, increase relative to the degree of hepatic fibrosis, and that high serum concentrations of TRX may indicate advanced hepatic fibrosis[19,20]. In contrast, it has also been reported that a higher degree of hepatic fibrosis is associated with lower platelet counts[38]. Therefore, the present study may present a conflict, since results indicated that serum TRX level was positively correlated with platelet count. A previous report showed that the survival rate following LPS plus GalN-induced hepatitis was much higher in transgenic mice overexpressing TRX than in wild-type mice, and that thioacetamide-induced hepatic fibrosis was suppressed in TRX transgenic mice compared to wild-type mice[42]. Although it is still unclear why TRX and platelet counts are positively correlated, we speculate that elevated serum TRX in patients with HCC and advanced hepatic fibrosis potentially improves overall survival by suppressing oxidative stress[43]. In addition, patients with HCC, low levels of TRX, and high levels of MnSOD, which may be indicative of excessive oxidative stress without TRX attenuation, have the poorest prognosis. This result supports the hypotheses presented above. In order to better assess these findings, future studies are needed that incorporate sequential observations of serum TRX and MnSOD levels over time in patients with chronic hepatitis, cirrhosis and HCC.

Serum MnSOD and TRX may be useful biomarkers for HCC diagnosis (Figure 1). AFP is also a diagnostic marker for HCC, and the present results indicate that AFP can be used to distinguish between patients with and without HCC (Table 2). However, AFP is not a sufficiently sensitive marker for identification of the majority of patients with small HCCs[44,45], and AFP testing is not currently included in the recommendations for HCC surveillance in the updated HCC guidelines published by the American Association for the Study of Liver Disease[46]. Therefore, clinicians and clinical researchers should consider using MnSOD and TRX as diagnostic biomarkers for early HCC or as additional markers in a HCC surveillance program using ultrasonography or AFP. In addition, it is highly important to know whether these markers decrease in response to HCC therapy and reductions in tumor burden. These markers also may have utility in patients on a transplant waiting list who are treated with neo-adjuvant therapy for tumor downstaging.

Our study demonstrated that elevated serum AFP level is indicative of a poor prognosis for patients with HCC (Table 4), as was previously reported[47]. The CLIP score, which is calculated based on four factors such as the AFP value, was also useful to predict the prognosis of HCC patients in this study as well as in a previous report[48]. Other markers such as the protein survivin have been reported as poor prognostic factors for HCC[49]. Similarly, MnSOD was an independent predictive factor for overall survival in the HCC group (Figure 3A, Table 5). Although TRX was not an independent predictor of overall survival in patients with HCC (Table 4), we speculate that a combination assay using both MnSOD and TRX could be used to predict overall patient survival. It will be important to conduct further prospective evaluations of each individual marker as well as a combination of these markers using a large number of patients.

In conclusion, serum MnSOD and TRX levels increased as HCV-related chronic liver disease progressed, especially among patients with HCC. Although there was no correlation between serum levels of MnSOD and TRX, higher serum MnSOD levels and lower TRX levels in patients with HCC trended towards an indication of poor

patient prognosis. These results suggest that serum Mn-SOD and TRX levels are not only a potential biomarker for HCV-related progressed liver disease, but may also serve as prognostic markers in HCC.

ACKNOWLEDGMENTS

We thank Ms. Yuko Nakamura for technical assistance.

COMMENTS

Background

During hepatitis C virus (HCV) infection, production of reactive oxygen species (ROS) is persistently increased throughout HCV infection. ROS are thought to play an important role in the pathogenesis of chronic inflammatory changes in the liver, which may lead to the development of hepatic fibrosis, decreased hepatic function or hepatocellular carcinoma (HCC). However, there is little information currently available regarding serum oxidative stress markers in patients with HCV-related HCC.

Research frontiers

Cells are protected from oxidative stress by antioxidant enzymes such as superoxide dismutase (SOD) and by intracellular antioxidants such as thioredoxin (TRX). Serum manganese SOD (MnSOD) and TRX are thought to be biomarkers for various liver diseases, including HCV-related liver disease, but these possibilities have not been fully investigated. In this study, the authors demonstrated the clinical significance of serum levels of MnSOD and TRX in patients with HCV-related HCC.

Innovations and breakthroughs

Although there was no correlation between serum levels of MnSOD and TRX, serum levels of both markers increased as HCV-related chronic liver disease progressed, and in particular among patients with HCC. In addition, higher serum MnSOD levels and lower TRX levels tended to indicate a poor prognosis among patients with HCC.

Applications

Serum MnSOD and TRX levels are not only potential biomarkers for progression of HCV-related liver disease, but they may also serve as prognostic markers for patients with HCC. Therefore, clinicians should consider using serum levels of MnSOD and TRX as diagnostic biomarkers for early HCC or as additional markers in HCC surveillance programs. In addition, it will be important to know whether these markers change after therapy for liver disease, including HCC.

Peer review

Oxidative stress is closely associated with carcinogenesis. If oxidative stress markers could be useful in predicting clinical outcome in chronic hepatitis C and HCV-related HCC, they would provide us with a practical and informative tool. However, there are some limitations of this investigation, including a relatively small number of patients studied. Thus, the overall assessment is "good".

REFERENCES

1 **Jemal A**, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2001; **61**: 69-90
2 Zhang CH, Xu GL, Jia WD, Li JS, Ma JL, Ge YS. Effects of interferon treatment on development and progression of hepatocellular carcinoma in patients with chronic virus infection: A meta-analysis of randomized controlled trials. *Int J Cancer* 2010; Epub ahead of print
3 **Matés JM**, Pérez-Gómez C, Núñez de Castro I. Antioxidant enzymes and human diseases. *Clin Biochem* 1999; **32**: 595-603
4 **Jaeschke H**, Farhood A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. *Am J Physiol* 1991; **260**: G355-G362
5 **Loguercio C**, Federico A. Oxidative stress in viral and alcoholic hepatitis. *Free Radic Biol Med* 2003; **34**: 1-10
6 **Niemelä O**, Parkkila S, Juvonen RO, Viitala K, Gelboin HV, Pasanen M. Cytochromes P450 2A6, 2E1, and 3A and production of protein-aldehyde adducts in the liver of patients with alcoholic and non-alcoholic liver diseases. *J Hepatol* 2000; **33**: 893-901
7 **Takami Y**, Uto H, Tamai T, Sato Y, Ishida Y, Morinaga H, Sakakibara Y, Moriuchi A, Oketani M, Ido A, Nakajima T, Okanoue T, Tsubouchi H. Identification of a novel biomarker for oxidative stress induced by hydrogen peroxide in primary human hepatocytes using the 2-nitrobenzenesulfenyl chloride isotope labeling method. *Hepatol Res* 2010; **40**: 438-445
8 **Mottola G**, Cardinali G, Ceccacci A, Trozzi C, Bartholomew L, Torrisi MR, Pedrazzini E, Bonatti S, Migliaccio G. Hepatitis C virus nonstructural proteins are localized in a modified endoplasmic reticulum of cells expressing viral subgenomic replicons. *Virology* 2002; **293**: 31-43
9 **Tsan MF**. Superoxide dismutase and pulmonary oxygen toxicity: lessons from transgenic and knockout mice (Review). *Int J Mol Med* 2001; **7**: 13-19
10 **Immenschuh S**, Ramadori G. Gene regulation of heme oxygenase-1 as a therapeutic target. *Biochem Pharmacol* 2000; **60**: 1121-1128
11 **Guo X**, Shin VY, Cho CH. Modulation of heme oxygenase in tissue injury and its implication in protection against gastrointestinal diseases. *Life Sci* 2001; **69**: 3113-3119
12 **Ludwig J**, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980; **55**: 434-438
13 **Teshigawara K**, Maeda M, Nishino K, Nikaido T, Uchiyama T, Tsudo M, Wano Y, Yodoi J. Adult T leukemia cells produce a lymphokine that augments interleukin 2 receptor expression. *J Mol Cell Immunol* 1985; **2**: 17-26
14 **Nakamura H**, De Rosa SC, Yodoi J, Holmgren A, Ghezzi P, Herzenberg LA, Herzenberg LA. Chronic elevation of plasma thioredoxin: inhibition of chemotaxis and curtailment of life expectancy in AIDS. *Proc Natl Acad Sci USA* 2001; **98**: 2688-2693
15 **Kurimoto C**, Kawano S, Tsuji G, Hatachi S, Jikimoto T, Sugiyama D, Kasagi S, Komori T, Nakamura H, Yodoi J, Kumagai S. Thioredoxin may exert a protective effect against tissue damage caused by oxidative stress in salivary glands of patients with Sjögren's syndrome. *J Rheumatol* 2007; **34**: 2035-2043
16 **Lemarechal H**, Allanore Y, Chenevier-Gobeaux C, Ekindjian OG, Kahan A, Borderie D. High redox thioredoxin but low thioredoxin reductase activities in the serum of patients with rheumatoid arthritis. *Clin Chim Acta* 2006; **367**: 156-161
17 **Deng ZH**, Cao HQ, Hu YB, Wen JF, Zhou JH. TRX is upregulated by fibroblast growth factor-2 in lung carcinoma. *APMIS* 2011; **119**: 57-65
18 **Cha MK**, Suh KH, Kim IH. Overexpression of peroxiredoxin I and thioredoxin1 in human breast carcinoma. *J Exp Clin Cancer Res* 2009; **28**: 93
19 **Sumida Y**, Nakashima T, Yoh T, Nakajima Y, Ishikawa H, Mitsuyoshi H, Sakamoto Y, Okanoue T, Kashima K, Nakamura H, Yodoi J. Serum thioredoxin levels as an indicator of oxidative stress in patients with hepatitis C virus infection. *J Hepatol* 2000; **33**: 616-622
20 **Sumida Y**, Nakashima T, Yoh T, Furutani M, Hirohama A, Kakisaka Y, Nakajima Y, Ishikawa H, Mitsuyoshi H, Okanoue T, Kashima K, Nakamura H, Yodoi J. Serum thioredoxin levels as a predictor of steatohepatitis in patients with nonalcoholic fatty liver disease. *J Hepatol* 2003; **38**: 32-38
21 A new prognostic system for hepatocellular carcinoma: a retrospective study of 435 patients: the Cancer of the Liver Italian Program (CLIP) investigators. *Hepatology* 1998; **28**: 751-755
22 Prospective validation of the CLIP score: a new prognostic system for patients with cirrhosis and hepatocellular carcinoma. The Cancer of the Liver Italian Program (CLIP) Investigators. *Hepatology* 2000; **31**: 840-845
23 The general rules for the clinical and pathological study of primary liver cancer. Liver Cancer Study Group of Japan. *Jpn J Surg* 1989; **19**: 98-129