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, $3 \times 10^6$ to $5 \times 10^6$ 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 = $\pi/6 \times$ (major axis) $\times$ (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.

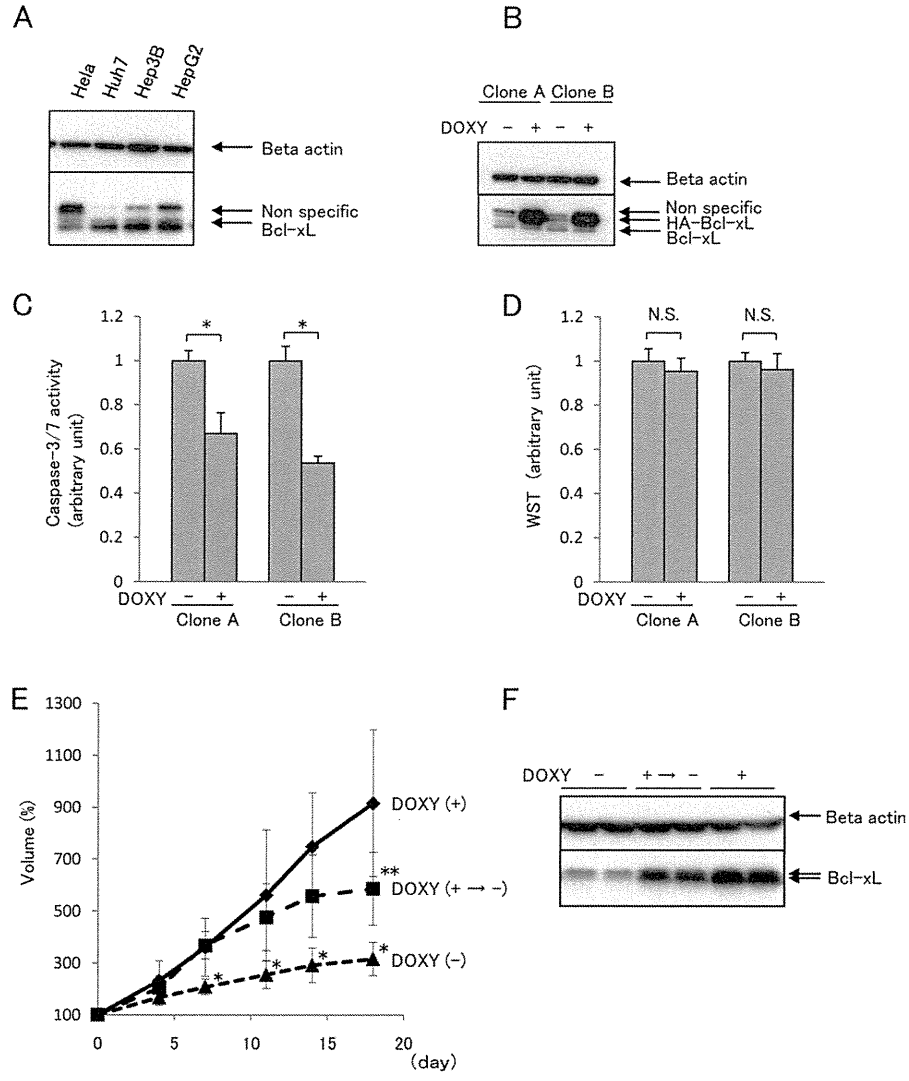


Fig. 1. Bcl-xL overexpression in vitro and in vivo by the Tet-on system. (A) Western blot analysis of Bcl-xL in human hepatoma cells and Hela cells. (B,C,D) Bcl-xL overexpression in vitro. Two independent clones of Hela-Bcl-xLTet-on cells were cultured with or without 1 $\mu$M doxycycline (DOXY) for 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

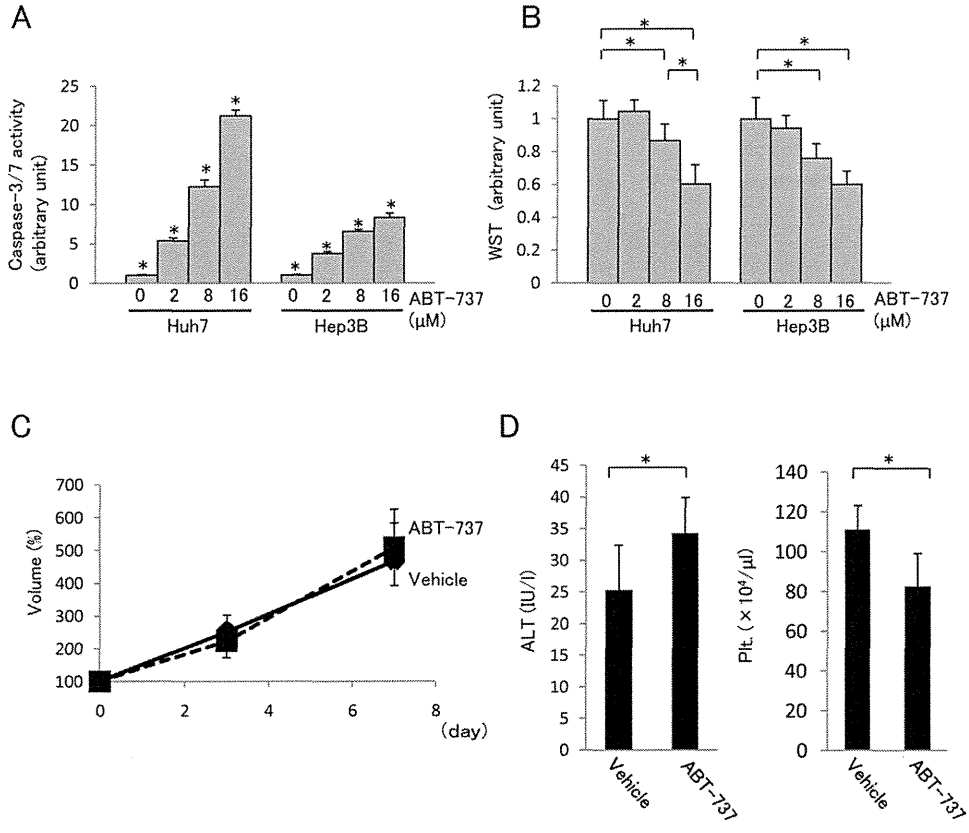


Fig. 2. Apoptosis and growth of hepatoma cells treated with ABT-737 *in vitro* and *in vivo*. (A,B) Huh7 and Hep3B cells were treated with indicated doses of ABT-737 for 24 hours (N = 4). (A) Caspase-3/7 activity of culture supernatant. $*P < 0.05$ versus all other groups. (B) Cell viability by the WST 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

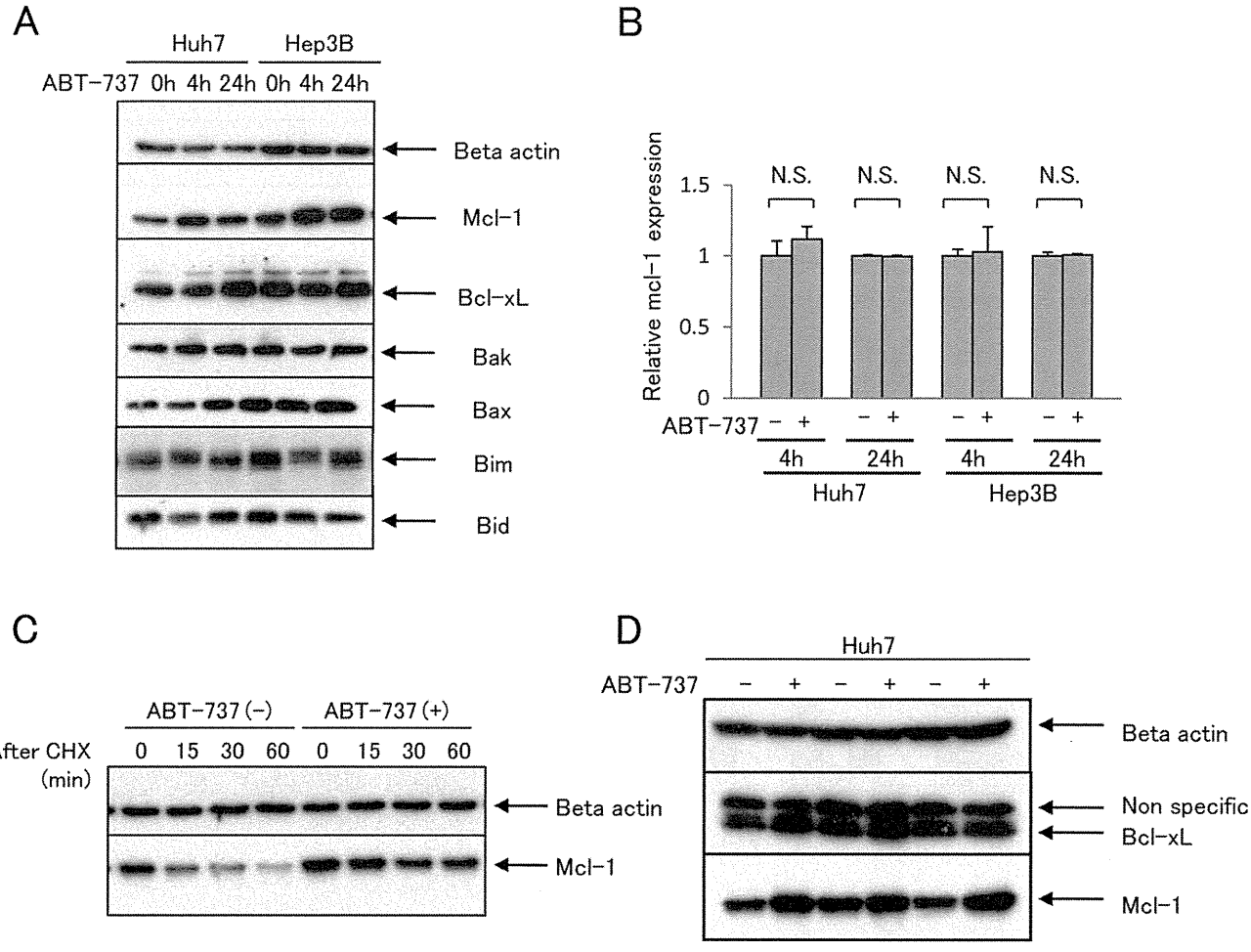


Fig. 3. Up-regulation of Mcl-1 in human hepatoma cells by ABT-737 in vitro and in vivo. (A,B) Huh7 and Hep3B cells were cultured with 4 $\mu$M ABT-737 for the indicated times. (A) Western blot analysis for the expression of Bcl-2 family proteins. (B) Real-time RT-PCR analysis for mcl-1 mRNA expression (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 $\mu$M 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

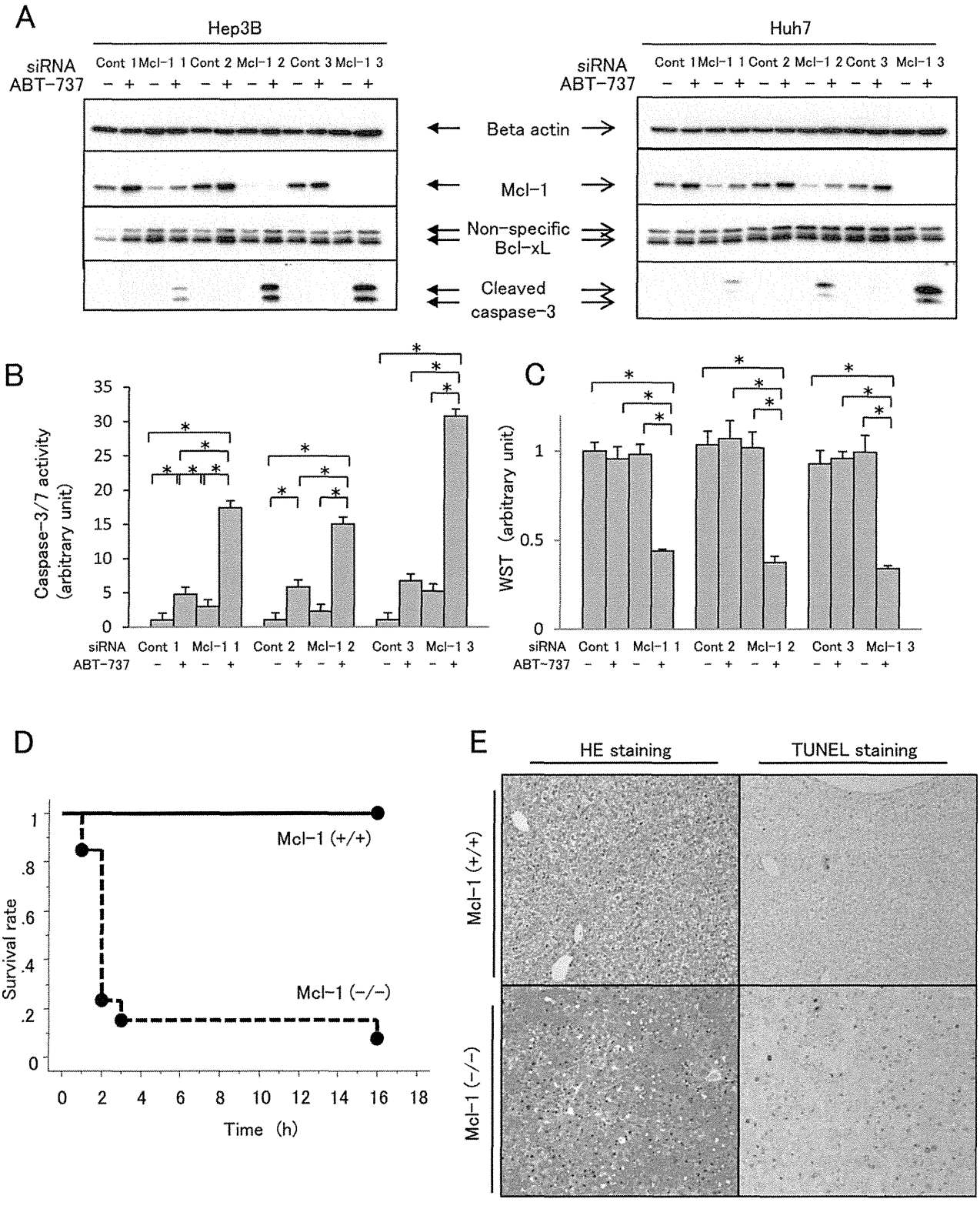


Fig. 4. Effects of ABT-737 under inhibition of Mcl-1 in vitro and in vivo. (A-C) Hep3B and Huh7 cells were transfected with Mcl-1 siRNAs (Mcl-1 1, Mcl-1 2, and Mcl-1 3) or control siRNAs (Cont 1, Cont 2, Cont 3). Forty-eight hours after transfection, they were treated with or without 4 $\mu$M ABT-737 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.

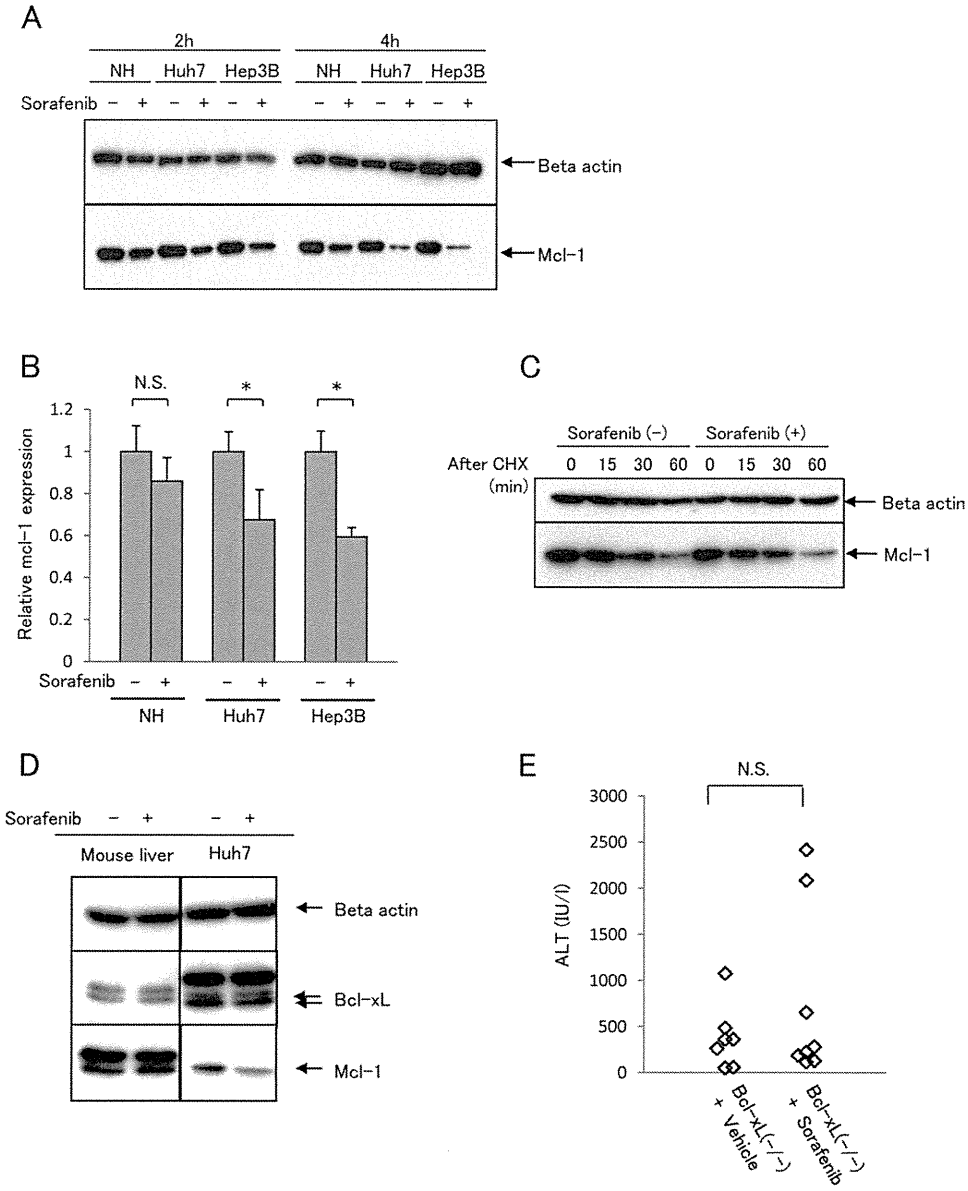


Fig. 5. Effects of sorafenib on the Mcl-1 expression in hepatoma cells and nontransformed cells in vitro and in vivo. (A,B) Normal hepatocytes (NH) and Huh7 and Hep3B cells were treated with 5 $\mu$M sorafenib. (A) Western blot analysis for the expression of Mcl-1 after treatment for 2 or 4 hours. (B) 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 $\mu$M 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

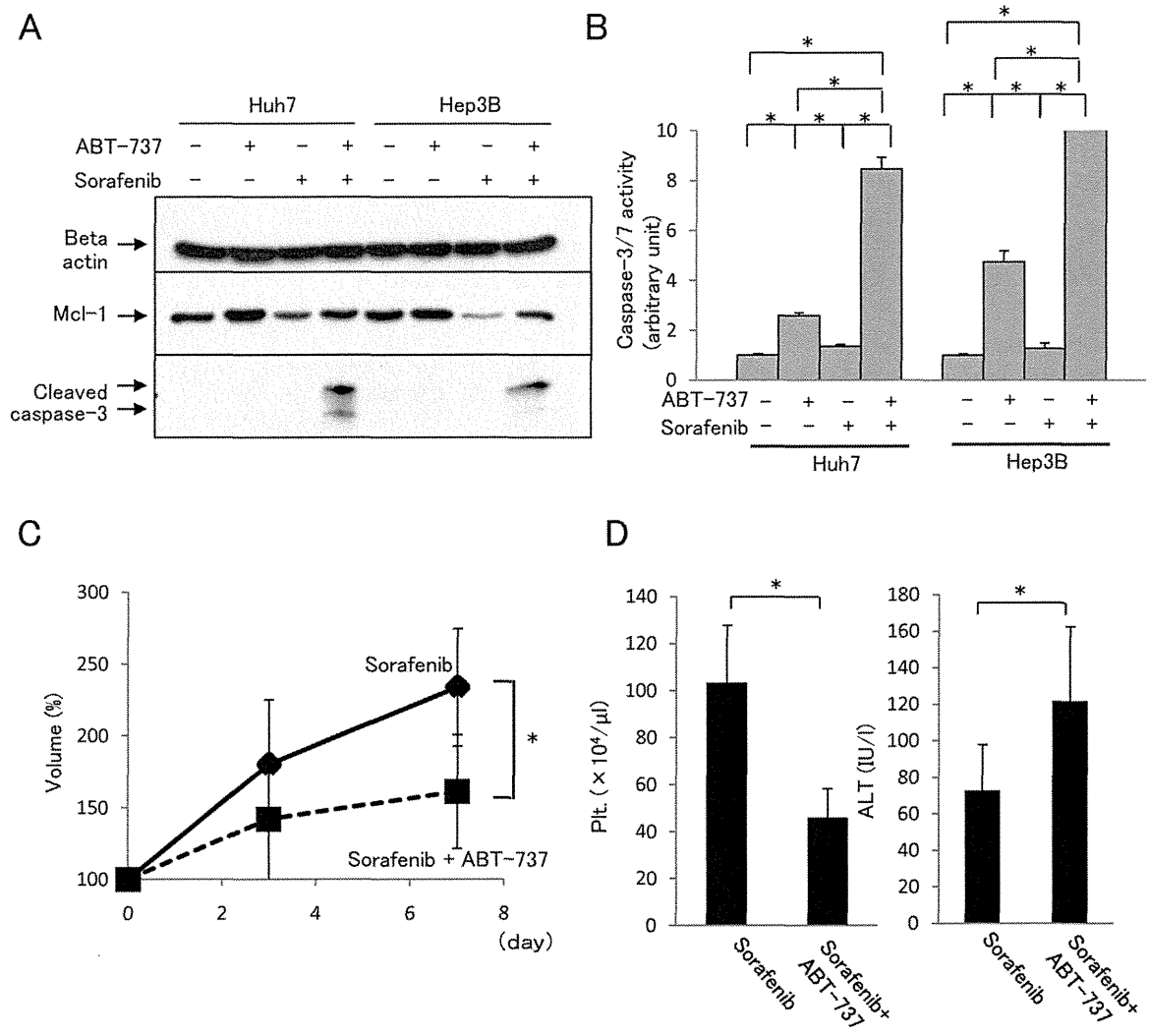


Fig. 6. Effects of ABT-737 with sorafenib treatment in vitro and in vivo. (A,B) Huh7 and Hep3B cells were treated with or without 4 $\mu$M ABT-737 together with or without 5 $\mu$M sorafenib. (A) Western blot analysis for the expression of Mcl-1 and cleaved caspase-3. (B) Caspase-3/7 activity of culture 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 $\mu$M) 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.

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Research Article

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# The *let-7* family of microRNAs inhibits Bcl-xL expression and potentiates sorafenib-induced apoptosis in human hepatocellular carcinoma

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**Background & Aims**: Bcl-xL, an anti-apoptotic member of the Bcl-2 family, is over-expressed in human hepatocellular carcinoma, conferring a survival advantage to tumour cells. The mechanisms underlying its dysregulation have not been clarified. In the present study, we explored the involvement of microRNAs that act as endogenous sequence-specific suppressors of gene expression.

**Methods**: The expression profiles of microRNAs in Huh7 hepatoma cells and primary human hepatocytes were compared by microarray analysis. The effect of *let-7* on Bcl-xL expression was examined by Western blot and a reporter assay. The involvement of *let-7* microRNAs in human tissues was analysed by western blot and reverse transcription-PCR.

**Results**: Microarray analysis, followed by *in silico* target prediction, identified *let-7* microRNAs as being downregulated in Huh7 hepatoma cells in comparison with primary human hepatocytes, as well as possessing a putative target site in the *bcl-xl* mRNA. Over-expression of *let-7c* or *let-7g* led to a clear decrease of Bcl-xL expression in Huh7 and HepG2 cell lines. Reporter assays revealed direct post-transcriptional regulation involving *let-7c* or *let-7g* and the 3′-untranslated region of *bcl-xl* mRNA. Human hepatocellular carcinoma tissues with low expression of *let-7c* displayed higher expression of Bcl-xL protein than those with high expression of *let-7c*, suggesting that low *let-7* microRNA expression contributes to Bcl-xL over-expression. Finally, expression of *let-7c* enhanced apoptosis of hepatoma cells upon exposure to sorafenib, which downregulates expression of another anti-apoptotic Bcl-2 protein, Mcl-1.

**Conclusions**: *let-7* microRNAs negatively regulate Bcl-xL expression in human hepatocellular carcinomas and induce apoptosis in cooperation with an anti-cancer drug targeting Mcl-1.



Keywords: Liver; Mcl-1; Bcl-2; Tumour; Epigenetic.

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*Abbreviations:* miRNA, microRNA; 3′UTR, 3′-untranslated region; mRNA, messenger RNA; HCC, hepatocellular carcinoma; CDK, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle medium; RT, reverse transcription; PCR, polymerase chain reaction; 7-AAD, 7-amino-actinomycin D; DMSO, dimethyl sulfoxide.

## Introduction

MicroRNAs (miRNAs), a novel class of non-coding, small RNAs, repress gene expression by binding to the 3′-untranslated region (3′UTR) of target messenger RNAs (mRNAs) [1]. More than 500 miRNAs have been identified in humans. Each miRNA is capable of modulating the expression of many mRNAs to which it binds by imperfect sequence complementarity, although only a limited number of targeted genes has been identified. Through its activity of gene silencing, miRNA functions in a variety of cellular processes, such as development, organ homeostasis, and cancer development and progression [2]. In the context of cancer development and progression, miRNAs targeting oncogenes function as tumour suppressors, whereas those targeting tumour suppressor genes serve as oncogenes [3]. Accumulating evidence has revealed the aberrant expression of miRNAs in human hepatocellular carcinoma (HCC) [4–6]. *miR-122a* has been reported to be downregulated in HCC, in turn, leading to upregulation of cyclin G1 [7]. On the other hand, recent reports have demonstrated that *miR-21* [8], *miR-221* [9], and *miR-224* [10] are upregulated in HCC, leading to downregulation of PTEN, CDK inhibitors, and API-5, respectively. Furthermore, the miRNA expression signature was reported to be related to the clinical outcome of patients with HCC [11,12]. Thus, miRNAs may play an important role in HCC development and progression by modulating a variety of gene expression and cellular processes.

Apoptosis resistance is an important characteristic of tumour cells, in addition to dysregulated proliferation and aberrant differentiation. Apoptosis is regulated by a fine balance of Bcl-2 family proteins, such as anti-apoptotic Bcl-xL and Mcl-1 and pro-apoptotic Bak and Bax. We previously demonstrated that Bcl-xL

is over-expressed in one-third of human HCC and confers resistance to hepatoma cells toward a variety of apoptotic insults generated by serum starvation and *p53* activation [13]. Patients with Bcl-xL-overexpressing HCC were shown to have significantly shorter disease-free survival after surgery [14]. Recently, it was proposed that autophagy defect is another mechanism of the malignant phenotype of Bcl-xL-overexpressing HCC through interaction between Bcl-xL and Beclin1 [15]. The underlying mechanisms of Bcl-xL over-expression in HCC are not clearly understood. Several reports show that transcription factors such as NF-κB [16] and STAT3 [17] could upregulate Bcl-xL expression in hepatoma cells. In addition, hepatitis C virus-related proteins, such as core [18] and NS5A [19], could upregulate Bcl-xL at a transcriptional level. However, we noticed that Bcl-xL-overexpressing hepatocarcinoma tissues do not always display upregulation of *bcl-xl* mRNA [13]. This observation led us to examine the possibility that post-transcriptional regulation by miRNAs may be involved in Bcl-xL expression in human HCC.

In the present study, we demonstrate that *let-7* family miRNAs, a prototype of human miRNAs [20], negatively regulate Bcl-xL expression in human HCC. *let-7* miRNAs are downregulated in human hepatoma cells and tissues in association with enhanced expression of Bcl-xL. Over-expression of *let-7* miRNAs in hepatoma cells downregulates Bcl-xL in a *bcl-xl* 3′UTR sequence-specific manner and enhances apoptosis induced by sorafenib, a recently approved anti-cancer drug for HCC [21]. The present study demonstrates for the first time that *let-7* miRNAs directly target Bcl-xL and induce apoptosis in cooperation with an anti-cancer drug targeting Mcl-1 in HCC.

## Materials and methods

*miRNA target predictions*

The algorithms miRanda (http://www.microrna.org/), Pictar (http://pictar.mdc-berlin.de/), and TargetScan (http://www.targetscan.org/) were used to predict miRNAs that could potentially bind to *bcl-xl* mRNA.

*Cell lines and tissues*

Primary human hepatocytes were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured with the provided medium. Human hepatoma cell lines, Huh7 and HepG2, were cultured with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (Sigma, St. Louis, MO). HCCs and adjacent non-tumour counterparts were obtained at the time of surgical resection. Written informed consent was obtained from each patient. All tissues were stored at −80 °C until the time of use.

*RNA extractions*

Total RNA including the miRNA fraction was isolated from cell lines and tissue samples using the miRNeasy Mini Kit (QIAGEN, Valencia, CA). After extraction, the quality of each RNA sample was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

*miRNA microarray analysis*

RNA labelling and hybridisation were performed using a human miRNA microarray kit and a miRNA complete labelling and hybridisation kit (Agilent Technologies). After washing with Gene Expression Wash Buffer, the slides were scanned with an Agilent Microarray Scanner and analysed by GeneSpring GX software.

*Western blot*

Cells or tissues were lysed and Western blotted as previously described [22]. For immunodetection, the following antibodies were used: anti-Bcl-xL polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Mcl-1 polyclonal antibody (Santa Cruz Biotechnology), anti-Bak polyclonal antibody (Millipore, Billerica, MA), anti-Bax polyclonal antibody (Cell Signaling Technology, Danvers, MA). Optical densities of bands in each blot were analysed using ImageJ 1.40 g (NIH, Bethesda, MD).

*Real time reverse transcription (RT)-PCR assays for mature miRNAs*

To quantify the expression of mature miRNA, we synthesised cDNA from 10 ng of RNA sample using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed with TaqMan MicroRNA Assays (Applied Biosystems) specific for *let-7c* (P/N 4373167) and *let-7g* (P/N 4395393). To normalise the expression levels of miRNAs, we used TaqMan MicroRNA Assays specific for RNU6B (P/N 4373381) as the endogenous control.

*Real time RT-PCR assays for bcl-xl mRNA*

We reverse-transcribed RNA with High Capacity RNA-to-cDNA Master Mix (Applied Biosystems), and *bcl-xl* mRNA expression was measured using TaqMan Gene Expression Assays (Applied Biosystems, Assay ID: Hs99999146_m1). We also quantified *β-actin* mRNA as an endogenous control (Assay ID: Hs99999903_m1).

*Transfections with miRNAs*

Huh7 and HepG2 cells were transfected with 50 nM Pre-miR miRNA precursor molecules (Ambion, Austin, TX) of either *let-7c* or *let-7g* using RNAiMAX (Invitrogen, Carlsbad, CA) in six-well plates according to the manufacturer's instructions. Pre-miR negative control (Ambion) was also used as a control.

*Luciferase assay*

To generate the pMIR-Bcl-xL-3′UTR construct that contains the putative binding site of *bcl-xl* 3′UTR downstream of the firefly luciferase gene, we synthesised oligonucleotides to mimic the target sequence and inserted them into the SpeI–HindIII site of pMIR-REPORT Luciferase vector (Ambion). We also generated a pMIR-Bcl-xL-3′UTR mutant that has a point mutation in the putative binding site, using the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

Each of these constructs was transfected into Huh7 cells together with 50 nM Pre-miR miRNA precursor molecules and pMIR-REPORT β-Gal vector (Ambion), which contains the β-galactosidase gene for normalisation of transfection efficiency. Transfection was performed using Lipofectamine 2000 (Invitrogen). We measured firefly luciferase activity 24 h after transfection using the Luciferase Assay System (Promega, Madison, WI) and normalised it to the β-galactosidase expression level.

*In vitro staurosporine or sorafenib treatment*

Huh7 cells were transfected with 50 nM Pre-miR miRNA precursor molecules as described above, and 48 h after transfection, the medium was changed to DMEM containing staurosporine (Calbiochem, Gibbstown, NJ) or sorafenib. Sorafenib was kindly provided by Bayer HealthCare Pharmaceuticals Inc. (Wayne, NJ). Cells were additionally cultured and assayed for apoptosis by monitoring the activity of caspase-3/7 using a luminescent substrate assay for caspase-3 and caspase-7 (Caspase-Glo assay, Promega, Madison, WI), or by flow cytometry using the Annexin V-PE Apoptosis Detection Kit I (BD Biosciences, San Jose, CA). We defined apoptotic cells as Annexin V-PE-positive and 7-amino-actinomycin D (7-AAD)-negative cells. Cell viability was determined by the WST assay using cell count reagent SF (Nacalai Tesque, Kyoto, Japan).

*Statistical analysis*

Data are presented as mean ± SD. Comparisons between two groups were performed by the unpaired *t*-test. Multiple comparisons were performed by ANOVA with the Scheffe post hoc test. $p < 0.05$ was considered statistically significant.

## Results

*let-7 miRNAs were downregulated in hepatoma cells with upregulated expression of Bcl-xL*

As observed in human HCC tissues, Bcl-xL was over-expressed, according to Western blot analysis, in Huh7 and HepG2 human hepatoma cell lines compared to normal hepatocytes (Fig. 1A). Previous research established that 30 and 32 kDa species are original and post-translationally modified Bcl-xL, respectively [23]. Mcl-1 was also over-expressed in human hepatoma cells, but the levels of expression of Bak and Bax did not differ between hepatoma cells and normal hepatocytes. We reasoned that miRNA regulating Bcl-xL expression would be downregulated in those hepatoma cell lines. To search for the candidate miRNA, microarray analysis was performed. More specifically, miRNA expression in Huh7 cells and normal hepatocytes was compared. When levels of expression less than 50% were considered significant, 26 miRNAs were identified as being downregulated in Huh7 cells: *let-7b*, *let-7g*, *let-7i*, *miR-127-3p*, *miR-214*, *miR-376a*, *miR-381*, *miR-409-3p*, *miR-376c*, *miR-493**, *miR-432*, *miR-487b*, *let-7d*, *let-7a*, *let-7f*, *let-7c*, *miR-200a*, *let-7e*, *miR-134*, *miR-503*, *miR-34a*, *miR-638*, *miR-150**, *miR-1225-5p*, *miR-21**, and *miR-223*. Among them, *in silico* analysis revealed that only the *let-7* family is capable of potentially targeting the 3′UTR of the *bcl-xl* mRNA. To confirm the results of the microarray analysis, quantitative real time RT-PCR was performed to evaluate the expression of *let-7c* and *let-7g* (Fig. 1B). After normalisation to endogenous RNU6B expression levels, the expression levels of both miRNAs were substantially lower in Huh7 cells than in normal hepatocytes. These results were consistent with the results of microarray analysis. Furthermore, the expression levels of both miRNAs were found to be downregulated in another human hepatoma cell line, HepG2, compared to normal hepatocytes.

*let-7c and let-7g downregulate Bcl-xL expression by directly targeting the 3′UTR of bcl-xl mRNA*

To examine whether *let-7* miRNAs are capable of suppressing translation of Bcl-xL, hepatoma cell lines were transfected with *let-7c* or *let-7g* or the negative control. Three days after transfection, Huh7 cells showed a decrease in Bcl-xL protein levels in both the *let-7c*-transfected group and the *let-7g*-transfected group in comparison with the negative control group (Fig. 2A). The transfection of *let-7c* and *let-7g* showed suppression of Bcl-xL protein levels in HepG2 cells as well (Fig. 2B). It did not affect expression of Bak and Bax, but increased Mcl-1 expression, which may be a secondary phenomenon of suppression of Bcl-xL. Normal hepatocytes were also transfected with *let-7c* or *let-7g* (Suppl. Fig. 1). The transfection led to a decrease in Bcl-xL expression in normal hepatocytes, but the decline was lesser than that observed in hepatoma cells. This finding may be explained by the observation that endogenous expression of *let-7c* and *let-7g* was extremely high in normal hepatocytes.

To examine whether the downregulation of Bcl-xL by *let-7c* or *let-7g* is caused by direct binding to a putative targeting site in the *bcl-xl* mRNA, we constructed the luciferase reporter plasmid pMIR-Bcl-xL-3′UTR containing the putative *let-7* binding site of *bcl-xl* 3′UTR downstream of the luciferase open reading frame (Fig 3A). The pMIR-Bcl-xL-3′UTR construct was cotransfected with the control pMIR-REPORT β-gal vector into Huh7 cells together with *let-7c* or *let-7g* or the negative control. When *let-7c* or *let-7g* Pre-miR was cotransfected with pMIR-Bcl-xL-3′UTR, the expression of firefly luciferase was significantly reduced compared to the negative control cotransfected group. There was no difference in firefly luciferase expression levels when pMIR-REPORT, which does not contain the putative *let-7* binding site, was cotransfected with *let-7c*, *let-7g* or the negative control (Fig. 3B). We also generated a pMIR-Bcl-xL-3′UTR mutant with a single base mutation in the seed region of the putative binding sequence to investigate whether the downregulation of firefly luciferase can be attributed to the insert (Fig. 3A). A single base mutation prevented the downregulation of firefly luciferase

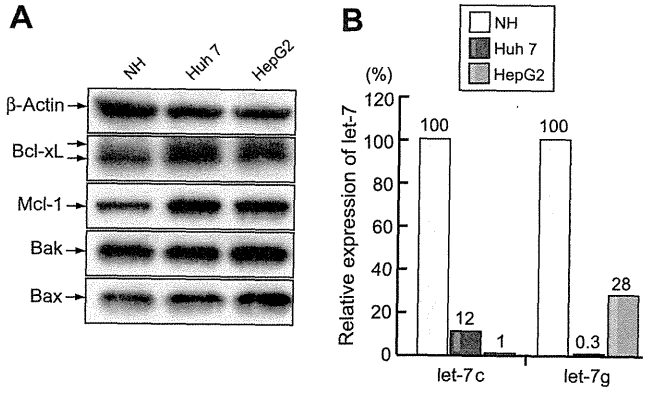


**Fig. 1. Expression of Bcl-xL and *let-7* miRNAs in cultured human hepatocytes and hepatoma cells.** Human hepatoma cell lines, Huh7 and HepG2, and normal hepatocytes (NH) were cultured and then lysed. (A) Western blot analysis for Bcl-2 family proteins. Bcl-xL migrates as a doublet band (see text). (B) Real time RT-PCR analysis for *let-7c* and *let-7g* expression. After normalisation to endogenous RNU6B expression, the expression of each miRNA in hepatoma cells was expressed in comparison to the levels observed in normal hepatocytes.

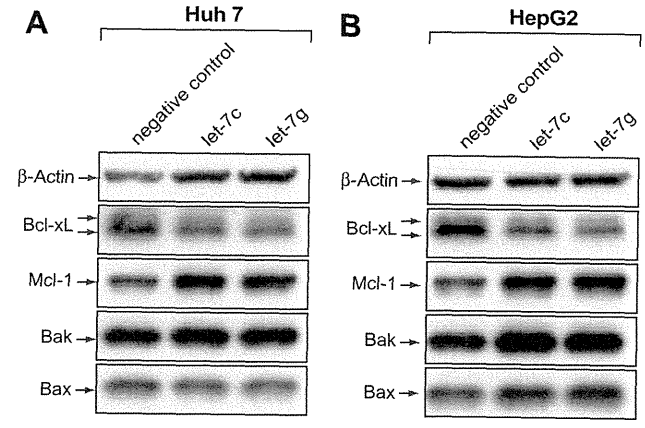


**Fig. 2. Over-expression of *let-7* miRNAs downregulates Bcl-xL expression in hepatoma cells.** Hepatoma cell lines Huh7 (A) and HepG2 (B) were transfected with *let-7c*, *let-7g*, or negative control miRNA at 50 nM and cultured for 3 days. Expression levels of Bcl-2 family proteins were determined by Western blot analysis.

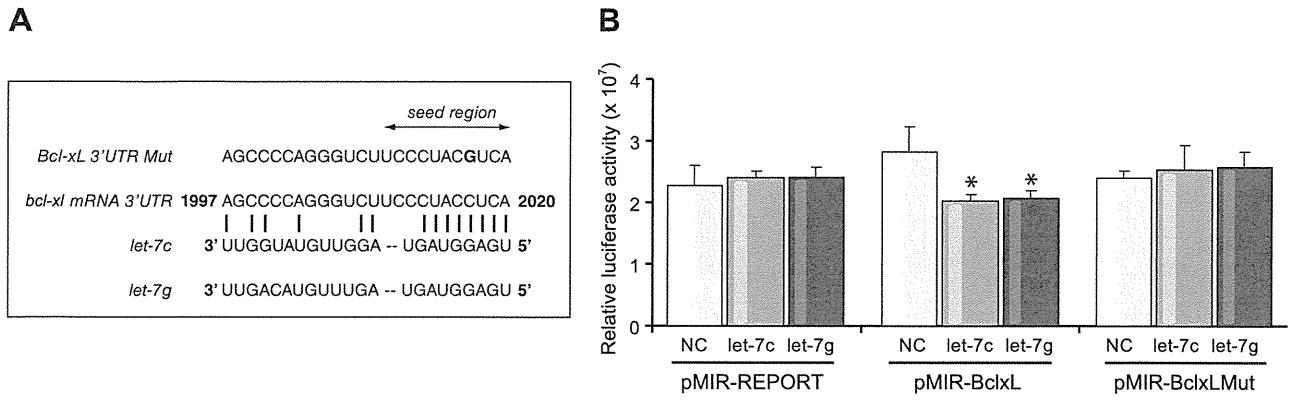


**Fig. 3. Sequence-specific suppression of *bcl-xl* gene expression by *let-7c* or *let-7g* miRNAs.** (A) The putative target site of *bcl-xl* mRNA 3'UTR determined by computational predictions. The target sequence was cloned into pMIR-REPORT vector (pMIR-Bcl-xL-3'UTR). pMIR-Bcl-xL-3'UTR mutant was also generated with a single mutation (indicated by a bold character) in the target site. (B) Each of these constructs was transfected into Huh7 cells together with *let-7c*, *let-7g* or negative control miRNA (NC). At 24 h after transfection, the activity of firefly luciferase was measured and normalised to β-galactosidase expression levels ($n = 3$). *$p < 0.05$.

induced by *let-7c* or *let-7g*, which strongly suggests a direct inhibitory effect of *let-7* on Bcl-xL expression (Fig. 3B).

*Downregulation of let-7c miRNA in human HCC tissues overexpressing Bcl-xL but not bcl-xl mRNA*

To investigate the relationship between *let-7* expression levels and Bcl-xL protein levels in human HCC samples, we used 22 pairs of surgically resected human HCC tissue samples and adjacent non-tumour tissue samples with highly preserved RNA. Compared to the non-tumour counterparts, *bcl-xl* mRNA was found to be over-expressed in HCC tissue samples in only two cases; Bcl-xL was also over-expressed at the protein level in these cases. To assess the significance of *let-7* in post-transcriptional regulation of Bcl-xL *in vivo*, we selected 20 pairs of HCC tissue samples that did not over-express *bcl-xl* mRNA. When these samples were divided into two groups according to relative *let-7c* expression levels, the relative expression of Bcl-xL protein was significantly higher in the *let-7c* low expression group than in the *let-7c* high expression group (Fig. 4). By contrast, there was no significant difference in *bcl-xl* mRNA expression between the two groups. We also examined the relationship between relative *let-7g* expression and Bcl-xL expression. The *let-7g* low expression group tended to over-express Bcl-xL protein compared to the *let-7g* high expression group, although the difference did not reach statistical significance (data not shown). These results are consistent with the hypothesis that *let-7* miRNAs negatively regulate Bcl-xL expression independent of transcriptional regulation.

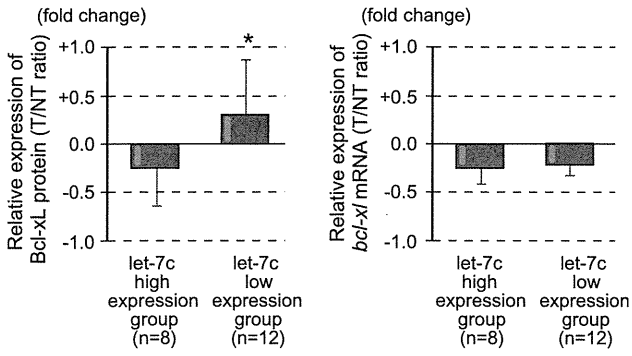


**Fig. 4. Expression of Bcl-xL, *bcl-xl* mRNA and *let-7* miRNAs in human HCC tissue.** Relationship between *let-7* and Bcl-xL expression in human HCC tissue samples. HCC tissue samples that did not show transcriptional upregulation of *bcl-xl* mRNA were divided into two groups according to relative *let-7c* expression levels with the threshold set at a 0.4-fold change in the tumour to non-tumour (T/NT) ratio. Relative expression of Bcl-xL protein and *bcl-xl* mRNA was calculated as the optical densities of the Bcl-xL blots normalised with the β-actin blots and those of real time RT-PCR assays, respectively, and are shown as the ratio of expression in the tumour to non-tumour expression in $\log_{10}$ scale. *$p < 0.05$.

*let-7c miRNA sensitises human Huh7 cells to sorafenib, which downregulates Mcl-1 expression*

To investigate the effect of *let-7* in the resistance of hepatoma cells to apoptosis, we transfected Huh7 hepatoma cells with *let-7c* miRNAs and then subjected them to apoptosis analysis and a cell viability assay. There was no significant difference in caspase-3/7 activation or cell viability between *let-7c* miRNA-transfected Huh7 cells and control miRNA-transfected Huh7 cells (represented by the DMSO-treated group of Fig. 5A and B). These results are in agreement with our previous finding that anti-sense oligonucleotide-mediated knockdown of Bcl-xL sensitised hepatoma cells to apoptotic stimuli, such as serum starvation and *p53* activation, but did not induce apoptosis by itself [13]. Next, we exposed miRNA-transfected Huh7 cells to staurosporine, which is a well-established apoptosis inducer. Staurosporine treatment induced apoptosis, as determined by caspase-3/7 activation and decreased the viability of Huh7 cells by itself, but *let-7c* miRNA-transfected Huh7 cells were more susceptible to staurosporine treatment than control miRNA-transfected cells. *let-7c* miRNA-transfected Huh7 cells showed a significant decrease in cell viability, even upon exposure to low-dose of staurosporine at which control miRNA-transfected Huh7 did not show a significant difference in cell viability (Fig. 5B). In addition, the activation of caspase-3/7 was more intense in *let-7c* miRNA-transfected Huh7 cells than in control miRNA-transfected Huh7 cells (Fig. 5A). Thus, suppression of *let-7* expression leading to over-expression of Bcl-xL, may be a mechanism by which hepatoma cells resist apoptotic stimuli. While normal hepatocytes were more sensitive to staurosporine than hepatoma cells, transfection of *let-7* miRNA did not affect sensitivity to staurosporine

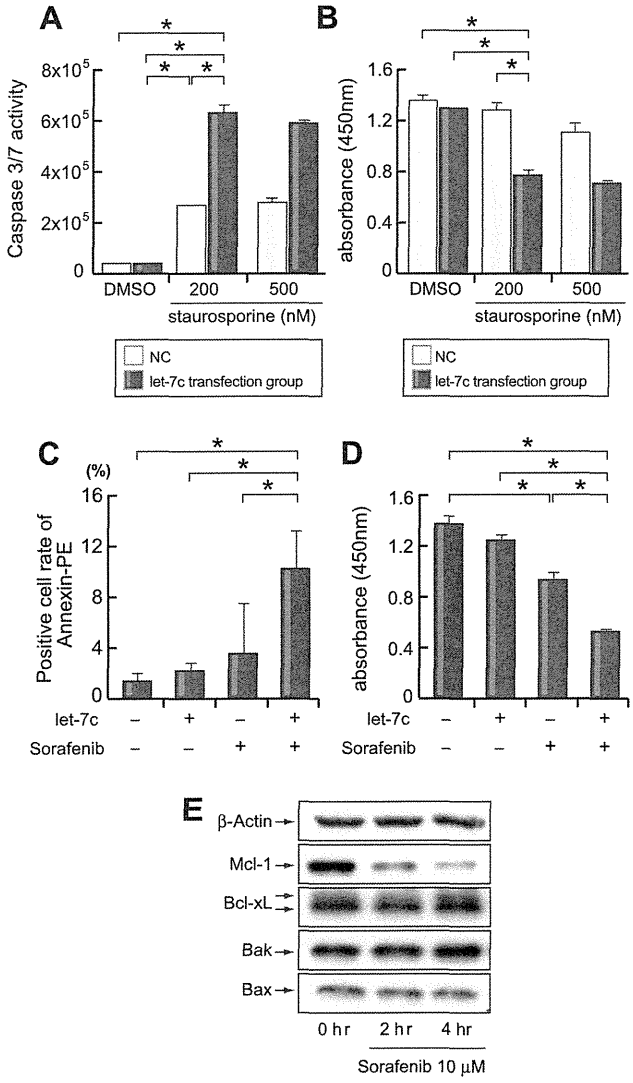


**Fig. 5. Introduction of *let-7* miRNAs sensitises hepatoma cells to apoptotic stimuli.** (A and B) Response to staurosporine treatment. Huh7 cells were transfected with *let-7c* (grey bars) or control miRNA (white bars) for 48 h and then further treated with staurosporine or DMSO alone for 12 h. The activities of caspase-3 and -7 were determined by luminescent substrate assays for caspase-3 and -7 ($n = 4$) (A). Cell viability was determined by the WST assay ($n = 4$) (B). *$p < 0.05$. (C and D) Response to sorafenib treatment. Huh7 cells were transfected with *let-7c* or control miRNA for 48 h and then further treated with sorafenib (5 μM) or DMSO alone for 48 h (C) or 72 h (D) 7-AAD negative cells were gated and the positive cell rate for annexin V-PE was determined ($n = 4$) (C). Cell viability was determined by the WST assay ($n = 4$) (D). *$p < 0.05$. E. Western blot analysis for Bcl-2 family proteins in lysates of Huh7 cells treated with sorafenib.

in normal hepatocytes (Suppl. Fig. 2), which is in agreement with the modest decline of Bcl-xL expression described earlier.

To examine the impact of *let-7* family miRNAs as a therapeutic tool, we investigated the effect of *let-7* miRNAs on apoptosis resistance to sorafenib, a recently approved anti-cancer drug for HCC. It has been reported that sorafenib was capable of downregulating Mcl-1 expression in tumour cells [24], and HCC has been reported to over-express Mcl-1, which is another anti-apoptotic Bcl-2 protein capable of conferring resistance to apoptosis [24–27]. In agreement with these findings, sorafenib treatment clearly downregulated Mcl-1 expression in hepatoma cells, but did not affect Bcl-xL expression (Fig. 5E). In contrast, sorafenib treatment did not affect Mcl-1 expression in normal hepatocytes (Suppl. Fig. 3). We hypothesised that *let-7* miRNA targeting Bcl-xL may induce apoptosis of hepatoma cells in cooperation with sorafenib. Apoptosis determined by Annexin V staining did not increase in *let-7c* miRNA-treated Huh7 cells compared to control miRNA-treated cells (represented by the DMSO-treated group in Fig. 5C). Sorafenib treatment of Huh7 cells led to a slight increase in the annexin V-positive cell rate, although the difference did not reach statistical significance levels under our experimental conditions (Fig. 5C). Of importance is the finding that sorafenib-induced apoptosis was markedly enhanced in *let-7c* miRNA-transfected cells. In addition, sorafenib treatment significantly reduced the viability of Huh7 cells and this decrease was markedly enhanced in cells transfected with *let-7c* miRNA (Fig. 5D). This finding implies that *let-7* miRNA transfection potentiates sorafenib-induced apoptosis and toxicity in hepatoma cells.

## Discussion

Anti-apoptotic members of the Bcl-2 family, which consists of five members, Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and Bfl-1, are critically involved in the mitochondrial pathway of apoptosis [28]. Cancer cells frequently over-express one or more members of this family to acquire a survival advantage [29]. These proteins are over-expressed in a variety of ways, including genetic translocation, particularly in the case of Bcl-2, and transcriptional regulation. Unlike the case of the *bcl-2* gene, mutations or amplification of the *bcl-x* gene have not been demonstrated in tumour cells. With regard to miRNA regulation, previous research clearly demonstrated that Bcl-2 is a direct target of *miR-15* and *miR-16*. The expression levels of *miR-15* and *miR-16* inversely correlate with Bcl-2 expression in chronic lymphocytic leukaemia [30]. More recently, Mcl-1 was reported to be suppressed by *miR-29* [31]. Our present study is the first demonstration of miRNA-mediated regulation of Bcl-xL expression. Since Bcl-xL is over-expressed not only in HCC but also in other tumours, the present findings may shed light on the mechanisms of Bcl-xL over-expression in other malignancies.

While more than 500 human miRNAs have been identified, *let-7* is a prototype of human miRNA and was first identified in 2001 [32]. *let-7* miRNAs are downregulated in several malignancies. A highly characterised example is non-small cell lung cancer in which downregulation of *let-7* miRNAs is well correlated with poor prognosis in patients [33]. In HCC, some reports showed downregulation of *let-7*, while others did not [7]. In the present study, *let-7c* miRNA was under-expressed at less than 40% of the normal level in approximately half of the HCC tissues. Further study is needed to determine the clinical significance of *let-7* miRNA in HCC. Several target genes have been identified for *let-7* miRNA, including Ras [34], Myc [35], HMGA2 [36], CDC25A, and CDK6 [37]. The major function of this miRNA is to promote cell proliferation. Since these proteins could act as oncogenes in tumour cells, *let-7* miRNA is believed to serve as a tumour suppressor [38]. In the present study, we have demonstrated that *bcl-xl* is a direct target for *let-7* miRNA, implying that this well-known tumour suppressor miRNA directly regulates apoptosis, another important process in malignancy.

Sorafenib is a recent FDA-approved anti-cancer drug for HCC [21]. It functions as a multi-kinase inhibitor and can induce

apoptosis at least in part by downregulating Mcl-1 in tumour cells [24]. Like Bcl-xL, several reports have identified Mcl-1 as being over-expressed in some HCCs [25–27]. Since Bcl-xL and Mcl-1 share a similar structure and functions, we reasoned that downregulation of both proteins would efficiently kill hepatoma cells. To verify this hypothesis, we treated hepatoma cells with sorafenib and *let-7* miRNA. As expected, sorafenib treatment downregulated Mcl-1 expression as early as 2 h post-treatment; however, it did not efficiently induce apoptosis. Transfection of *let-7* miRNA itself was not capable of inducing apoptosis of hepatoma cells despite a clear reduction in Bcl-xL expression. Importantly, *let-7* miRNA substantially increased sensitivity to sorafenib. Since both *let-7* miRNA and sorafenib may have pleiotropic effects on gene expression and cellular processes, downregulation of Bcl-xL and Mcl-1 may not be a single mechanism for killing hepatoma cells. However, our study revealed that Bcl-xL-targeting miRNA, *let-7*, controls resistance of hepatoma cells to this novel class of anti-HCC drug.

In conclusion, we have demonstrated that *let-7* miRNA negatively regulates Bcl-xL expression in HCCs. Reconstitution of *let-7* miRNA may reduce apoptosis resistance to anti-cancer drugs targeting Mcl-1 in HCC. Further study is needed to examine the significance of *let-7* miRNA expression for predicting responses to sorafenib therapy in patients with HCC.

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### Disclosures

All authors have nothing to disclose.

### Conflicts of interest

All authors have no conflicts of interest.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2009.12.024.

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# Difference in serum complement component C4a levels between hepatitis C virus carriers with persistently normal alanine aminotransferase levels or chronic hepatitis C

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**Abstract.** Certain hepatitis C virus (HCV) carriers exhibit persistently normal alanine aminotransferase (ALT) levels (PNALT) (≤30 IU/l) accompanied by normal platelet counts (≥15x10$^4$/$\mu$l); these individuals show milder disease activity and slower progression to cirrhosis. This study aimed to elucidate the characteristics of HCV carriers with PNALT using serum proteomics. The first group of subjects, who underwent clinical evaluation in the hospital, consisted of 19 HCV carriers with PNALT (PNALT-1) and 20 chronic hepatitis C (CHC-1) patients. The second group of subjects was part of a cohort study on the natural history of liver disease, and included 37 PNALT (PNALT-2) and 30 CHC (CHC-2) patients. Affinity bead-purified serum protein was subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis. Serum proteomics showed that 6 protein peaks with mass-to-charge ratios ranging from 1,000 to 3,000 differed significantly between the PNALT-1 and CHC-1 groups. Among these peaks, a 1738-m/z peak protein was identified as a fragment of complement component 4 (C4) and correlated significantly with serum C4a concentrations as determined by enzyme immunoassay. Serum C4a levels were also significantly higher in the PNALT-2 group compared to the CHC-2 group and healthy volunteers. Furthermore, in the PNALT-2 group, serum C4a levels negatively correlated with transaminase levels, but not with other biochemical tests, HCV core antigen levels, peripheral blood cell counts or serum hepatic fibrosis markers. This study indicates that host factors such as C4a not only differ between HCV carriers with PNALT and CHC, but that proteomic approaches could also contribute to the elucidation of factors in PNALT as more differences are discovered.

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*Key words:* hepatitis C virus, persistent normal alanine aminotransferase, complement component C4a, C4 fragment, serum proteomics

## Introduction

Hepatitis C virus (HCV) infection, one of the main causes of chronic hepatitis, is estimated to affect 170 million people worldwide (1). The natural history of HCV infection is characterized by acute and eventually chronic infection, and may progress from a long-lasting asymptomatic condition to decompensated liver cirrhosis or hepatocellular carcinoma (HCC) (2). However, the long-term impact of HCV infection is highly variable; some patients with persistent HCV infection exhibit persistently normal alanine aminotransferase (ALT) levels (PNALT), which are associated with milder disease activity and slower progression to cirrhosis (3). In addition, the differences between PNALT patients and those with chronic hepatitis C (CHC) who exhibit elevated ALT levels have not yet been fully elucidated (4).

Persico *et al* reported that the grade of disease activity does not increase over a period of years, and that progression to cirrhosis is slow or absent in patients with HCV-related chronic hepatitis associated with PNALT (5). We have previously reported that the ALT level is a predictor of HCV-associated HCC incidence in a community-based population in Japan (6). In addition, a number of studies have shown that interferon (IFN)-based therapy reduces HCC in patients with CHC,

even in those in whom HCV RNA remains detectable (7,8). Continuous normalization of aminotransferase and α-feto protein (AFP) for more than 1 year during IFN therapy is associated with a reduced risk of HCC development following the termination of the IFN therapy (9).

A recent application of proteomic technology has identified a spectral pattern from the serum of patients with liver disease (10-12), and proteomic techniques will be able to identify serum biomarkers that are present in the serum of patients with PNALT. Furthermore, a biomarker or biomarker panel may also help to elucidate a possible mechanism for chronic hepatitis from PNALT and could perhaps lead to the development of more effective treatments for chronic hepatitis. However, proteomic approaches focused on PNALT have not been previously explored.

In this study, we verified differentially expressed protein in serum samples and showed that the level of the complement component 4a (C4a) in serum was higher in HCV carriers with PNALT compared to CHC patients or healthy volunteers. The present study reveals that C4a increases with HCV infection, but decreases with disease progression. Identification of these and other proteins will help clarify the underlying mechanisms and contribute to improved clinical outcomes for HCV carriers.

## Patients and methods

*Study population.* Anti-HCV seropositive subjects with detectable HCV core antigen (HCVcAg) or HCV RNA were considered to be persistently infected with HCV and were classified as HCV carriers. ALT levels >30 IU/l and platelet counts $<15x10^4/\mu l$ were considered to be abnormal. HCV carriers exhibiting persistently normal ALT levels accompanied by normal platelet counts during the observation period were defined as the PNALT group in this study (13). Subjects who underwent oral or intravenous administration of medical herbs or other palliative therapies were not excluded from this study, but those who had received IFN therapy were excluded. All subjects were negative for hepatitis B virus surface antigen (HBsAg).

The first group of subjects, who were undergoing hospital-based clinical evaluation, consisted of 39 HCV carriers. Of these, 19 with PNALT (PNALT-1 group) and 20 with CHC and abnormal ALT levels (CHC-1 group) were enrolled. HCV carriers with PNALT (PNALT-1 group) were defined as those who had normal serum ALT levels (≤30 U/l) over a 12-month period and on at least 3 different occasions, and platelet counts of $\geq 15x10^4/\mu l$. Blood samples from the PNALT-1 and CHC-1 groups were obtained during the last observation period.

The second group of subjects was part of a larger cohort being followed-up as part of a study on the natural history of liver disease; data on these individuals were acquired from 1994 through 2005 (14). An analysis was conducted of HCV carriers who had undergone at least 3 independent ALT measurements obtained during annual general health examinations or liver disease screenings. In total, 37 HCV carriers with persistently normal ALT levels and platelet counts $\geq 15x10^4 \mu l$ (PNALT-2 group) and 30 HCV carriers with persistently abnormal ALT levels and platelet counts $<15x10^4/\mu l$ (CHC-2) were investigated. Blood samples from PNALT-2 or CHC-2 subjects were obtained during the last observation period in this study from 2002 to 2005. Serum samples were also obtained from healthy volunteers without HCV infection (n=12).

After the blood samples were collected, serum was stored at -80°C. Written informed consent was obtained from each subject and the study protocol was approved by the Ethics Committee of Kagoshima University Hospital; the Faculty of Medicine, University of Miyazaki and Kyoto Prefectural Medical School.

*Serum pre-treatment with ClinProt magnetic beads.* Serum samples (5 μl) were purified and concentrated using magnetic bead-based weak cation exchange chromatography resins (WCX) (Bluker Daltonics, Bremen, Germany). 2-Cyano-4-hydroxycinnamic acid (CHCA) matrix solution (Bluker Daltonics) was diluted to 0.3 g/l in an ethanol:acetone (2:1) solution. Purified serum samples and diluted CHCA solutions were mixed (1:9), and 1 μl of the solution was applied onto a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) AnchorChip.

*Mass spectrometry (MS) and peptide identification.* The AnchorChip target plate was placed in an AutoFlex II TOF/TOF mass spectrometer (Bluker Daltonics). Spectra were acquired in the positive linear mode in a molecular mass range from 1,000 to 3,000 Da. The MALDI-TOF MS spectrum was subjected to a Mascot database search (Matrix Science, Boston, MA, USA) using the SwissProt database.

*Serum markers.* The presence of serum anti-HCV antibody (Ab) was determined using a commercially available third-generation enzyme-linked immunosorbent assay (ELISA). Serum levels of HCVcAg were determined by a chemiluminescence enzyme immunoassay (HCV core protein; SRL, Tokyo, Japan), with a detection threshold of 20 fmol/l. The serologically defined HCV genotype (HCV serotype) was tested with a serological genotyping assay kit (Immunocheck F-HCV Grouping, International Reagents Co., Tokyo, Japan). In some patients, the HCV genotype was examined (HCV Core Genotype, SRL, Tokyo, Japan). HCV genotype 1b was included with serotype I, and genotypes 2a and 2b with serotype II. No other HCV genotype was detected in this study population.

The serum concentration of C4a was determined using a C4a ELISA kit (Human C4a ELISA kit, BD Biosciences, San Diego, CA, USA).

*Statistical analysis.* The results are presented as the means ± standard deviation (SD). All spectra in MALDI-TOF MS were analyzed using Bluker Daltonics FlexAnalysis 2.2 software and ClinProTools 2.0 software. Statistical analysis of other clinical data was performed using StatView 4.5 software (Abacus Concepts, Berkeley, CA, USA) or SPSS software (SPSS Inc., Chicago, IL, USA). Differences were evaluated by the Mann-Whitney U test, the Fisher's exact test or the Chi-square test as appropriate. Any probability value $<0.05$ was considered to indicate a statistically significant difference.

Table I. Patient characteristics in the hospital-based group.

| Characteristics | PNALT-1 (n=19) | CHC-1 (n=20) | P-value[a] |
|---|---|---|---|
| Age | 55.2±15.1 | 52.3±11.7 | 0.17 |
| Gender (male/female) | 3/16 | 7/13 | 0.27 |
| HCV core antigen (fmol/l) | 1163±803 | 1072±669 | 0.72 |
| HCV serotype (I/II/UD) | 9/5/5 | 11/5/4 | 0.87 |
| Platelet count (x$10^4$/$\mu$l) | 21.3±5.5 | 14.8±4.0 | <0.001 |
| AST (IU/l) | 25.4±3.9 | 73.6±37.5 | <0.001 |
| ALT (IU/l) | 23.5±5.1 | 90.9±54.8 | <0.001 |
| γ-GTP (IU/l) | 18.6±8.5 | 89.4±77.9 | <0.001 |
| Total cholesterol (mg/dl) | 200.0±24.4 | 180.1±27.4 | 0.03 |
| Albumin (g/dl) | 4.5±0.2 | 4.5±0.4 | 0.76 |

PNALT, persistently normal ALT; CHC, chronic hepatitis C; n, number of patients; HCV, hepatitis C virus; UD, undetermined; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyltranspeptidase. Data are presented as the means ± standard deviation or number. [a]Differences between mean values were evaluated using either the Fisher's exact test, the Chi-square test or the Mann-Whitney U test, as appropriate.

Table II. Protein peaks expressed differentially between HCV carriers with persistent normal ALT levels (PNALT) and chronic hepatitis C (CHC) patients with abnormal ALT levels.

| | Peak intensity | | |
|---|---|---|---|
| m/z | PNALT-1 (n=19) | CHC-1 (n=20) | P-value[a] |
| 1738 | 109.4±67.1 | 83.9±54.0 | <0.01 |
| 1896 | 105.0±64.8 | 111.3±63.3 | <0.05 |
| 1943 | 191.3±149.5 | 139.6±73.6 | <0.01 |
| 2858 | 104.0±34.3 | 85.3±25.6 | <0.001 |
| 2928 | 31.8±9.7 | 64.0±28.9 | <0.001 |
| 2947 | 59.3±34.9 | 80.7±36.2 | <0.001 |

Data are presented as the means ± standard deviation. [a]Differences between mean values were evaluated using the Mann-Whitney U test ALT, alanine aminotransferase.

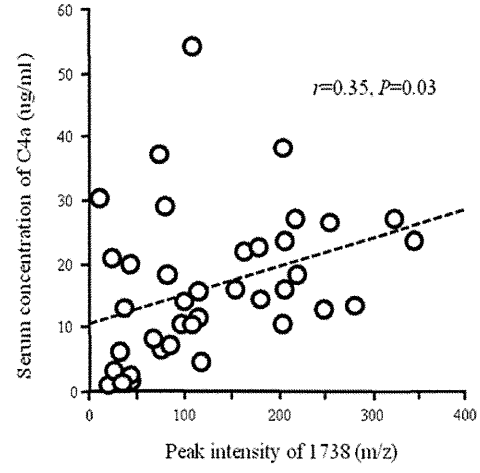


Figure 1. Association between the peak intensity of 1738 m/z (C4 fragment) and the serum level of C4a as determined by enzyme-linked immunosorbent assay. The peak intensity of 1738 m/z correlated with serum C4a levels (r=0.35, P=0.03). C4a, complement component 4a.

## Results

*Profiling sera from patients with PNALT and chronic hepatitis C using MALDI-TOF MS analysis.* In the first hospital-based group, serum levels of ALT, aspartate aminotransferase (AST), and γ-glutamyltranspeptidase (γ-GTP) were lower and platelet counts and total cholesterol were higher in PNALT-1 patients than in CHC-1 patients (Table I). In this group, the sera of patients were analyzed to identify protein peaks that differed most between patient subsets. Serum proteomics revealed that 6 serum protein peaks with mass-to-charge ratios ranging from 1,000 to 3,000 differed significantly between PNALT-1 and CHC-1 groups (Table II). In these protein peaks, a 1738-m/z peak protein was identified as a fragment of C4, with the sequence NGFKSHALQLNNRQI.

*Correlation between the protein peak of 1738 m/z and serum levels of C4a determined by ELISA.* Although the identified C4 fragment is part of C4c, serum concentrations of C4c could not be determined by commercially available methods, such as ELISA. By contrast, the recalibrated peak intensity of this fragment significantly correlated with the serum level of C4a, which could be determined with a commercially available assay kit (Fig. 1). In addition, serum concentrations of C4a were significantly higher in PNALT-1 subjects [means ± SD ($\mu$g/ml), 20.6±11.9] compared with those in CHC-1 subjects (12.2±10.2) (P=0.01).

*Serum levels of C4a determined by ELISA in the second group.* In the cohort-based population, age, the prevalence of females and of serotype II, platelet counts, and serum albumin