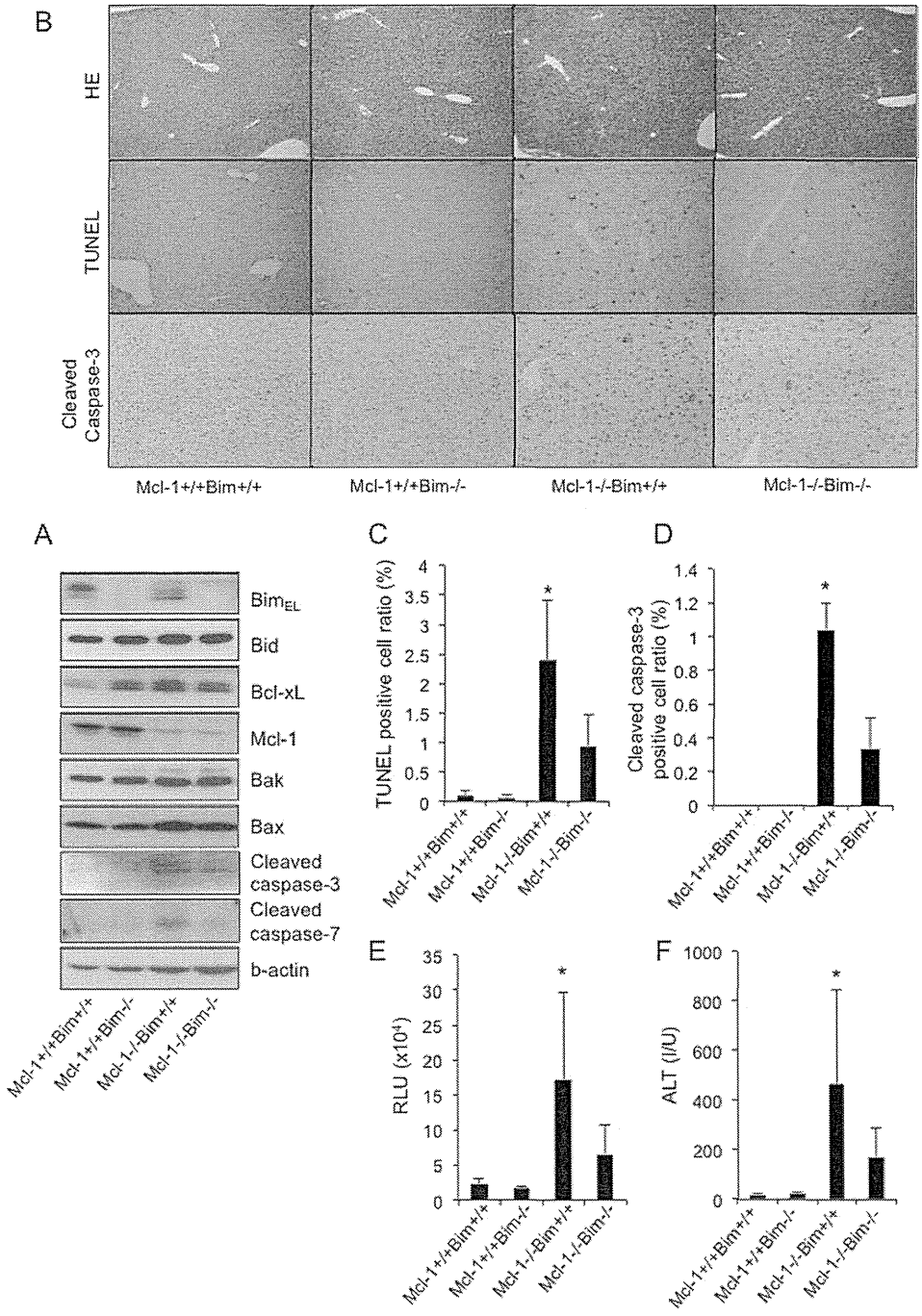


FIGURE 2. **The disruption of Bim alleviated spontaneous hepatocyte apoptosis in the absence of Mcl-1.** The offspring from the mating of *bim*$^{+-}$*mcl-1*$^{flox/flox}$*alb-cre* mice with *bim*$^{+/-}$*mcl-1*$^{flox/flox}$ mice were examined at 6 weeks of age. *Mcl-1*$^{+/+}$ and *Mcl-1*$^{-/-}$, *mcl-1*$^{flox/flox}$ and *mcl-1*$^{flox/flox}$*alb-cre*, respectively. *A*, Western blot analysis of whole liver lysates for the expression of Bim$_{EL}$, Bid, Bcl-xL, Mcl-1, Bak, Bax, cleaved caspase-3, cleaved caspse-7, and β-actin. *B*, representative images for liver histology stained with hematoxylin-eosin (*HE*), TUNEL, and cleaved caspase-3 (original magnification, ×100). *C*, TUNEL-positive cell ratio; $n = 3–6$ mice/group; *, $p < 0.05$ *versus* all. *D*, cleaved caspase-3-positive cell ratio; $n = 3$ mice/group; *, $p < 0.05$ *versus* all. *E*, serum caspase-3/7 activity; $n = 9–15$ mice/group; *, $p < 0.05$ *versus* all. *F*, serum ALT levels; $n = 9–15$ mice/group; *, $p < 0.05$ *versus* all. *Error bars*, S.D. *RLU*, relative light units; *I/U*, international units.

which are often dysregulated in malignant cells. ABT-737, which is a BH3 mimetic, could inhibit Bcl-xL, Bcl-2, and Bcl-w, and it has induced the regression of solid tumors (23). We previously reported that high dose ABT-737 administration caused hepatocyte apoptosis even in a normal liver, which was partly due to constitutive Bid-mediated BH3 stress (7). This finding led us to investigate the involvement of Bim and Bid in this ABT-737-mediated hepatotoxicity. Bim/Bid double knock-out mice (*bim*$^{-/-}$*bid*$^{-/-}$) were generated by mating Bim knock-out mice (*bim*$^{-/-}$) with Bid knock-out mice (*bid*$^{-/-}$), and the offspring were then treated with this drug. Western blot analysis confirmed the efficient deletion of Bim and Bid from the liver tissue of the double knock-out mice (Fig. 5*A*). Upon ABT-737 treatment, the Bim/Bid double knock-out mice showed complete prevention of ABT-737-induced hepatocyte apoptosis and hepatotoxicity (Fig. 5, *B–F*), in sharp con-

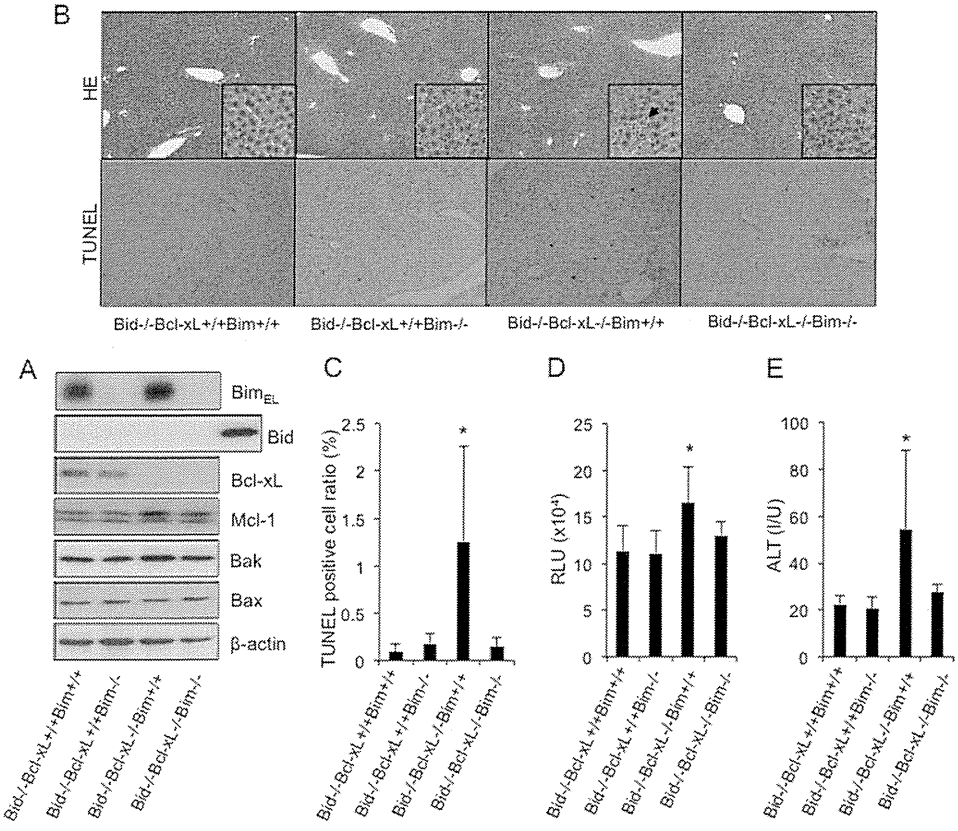


FIGURE 3. **The disruption of Bim and Bid prevented spontaneous hepatocyte apoptosis in the absence of Bcl-xL.** The offspring from the mating of *bim*$^{+/-}$*bid*$^{-/-}$*bcl-x*$^{flox/flox}$*alb-cre* mice with *bim*$^{+/-}$*bid*$^{-/-}$*bcl-x*$^{flox/flox}$ mice were examined at 6 weeks of age. *Bcl-xL*$^{+/+}$ and *Bcl-xL*$^{-/-}$, *bcl-x*$^{flox/flox}$ and *bcl-x*$^{flox/flox}$*alb-cre*, respectively. *A*, Western blot analysis of whole liver lysates for the expression of Bim$_{EL}$, Bid, Bcl-xL, Mcl-1, Bak, Bax, and β-actin. *B*, representative images of liver histology stained with hematoxylin-eosin (*HE*) and TUNEL (original magnifications, ×100 (*large panels*) and ×400 (*insets*)). *Black arrows* indicate apoptotic bodies. *C*, TUNEL-positive cell ratio; more than 5 mice/group; *, $p < 0.05$ *versus* all. *D*, serum caspase-3/7 activity; more than 6 mice/group; *, $p < 0.05$ *versus* all. *E*, serum ALT levels; more than 6 mice/group; *, $p < 0.05$ *versus* all. *Error bars*, S.D. *RLU*, relative light units; *I/U*, international units.

trast to their Bid-knock-out littermates, which still showed moderate hepatocyte apoptosis (Fig. 5, *C–E*) and increased serum ALT levels (Fig. 5*F*). These findings suggested that Bim- and Bid-mediated constant BH3 stress evoked hepatotoxicity by promoting the intrinsic pathway of apoptosis with the use of the inhibitors of the Bcl-2 family.

## DISCUSSION

At least eight BH3-only proteins are known, and five have been reported to exist in hepatocytes: Bid, Bim, Noxa, Puma, and Bad (22). We also confirmed these five proteins in the liver tissue of our mice (Fig. 1*A*), and we detected at least the mRNA expression of three other genes (supplemental Fig. 1). These proteins are considered to function as pro-apoptotic sensors upon activation by a variety of apoptotic stimuli, thereby promoting an intrinsic pathway of apoptosis in a manner that is dependent on the presence of Bak and Bax. In previous studies, bile acids or death receptor stimuli activated Bid and induced liver injury, which was alleviated by Bid disruption (12, 22). Bim activation was involved in hepatocyte lipoapoptosis, which is a critical feature of non-alcoholic steatohepatitis, and in reactive oxygen species-induced hepatocyte apoptosis (10, 11, 14). Additionally, a recent *in vivo* study revealed that the activation of Bid and Bim played a central pro-apoptotic role in fatal TNF-α-induced hepatitis (24). Taken together, these findings indicated the importance of these two BH3-only proteins in the pathogenesis of various liver diseases (12, 24, 25). Conversely, the systemic knock-out of Bid or Bim in mice did not result in any liver abnormalities under normal conditions; therefore, there has not been much interest in studying their physiological involvement in the healthy liver (12, 26). However, our present study showed that spontaneous hepatocyte apoptosis in the absence of Bcl-xL was alleviated by the deletion of either Bim or Bid, and it was diminished by the deletion of both. These results indicated that these BH3-only proteins are functionally active even in the healthy liver, but they are fully restrained by the anti-apoptotic Bcl-2 family proteins in the physiological state.

What type of stimuli constitutively activate these BH3-only proteins remains unknown. The liver is a specific organ that can be continuously exposed to a variety of stimuli, such as bile acids and enteric endotoxin, as well as interactions with immune cells. These stimuli might cause constitutive BH3-only stress through the activation of death receptors, such as Fas, tumor necrosis factor (TNF), and TNF-related apoptosis-inducing ligand (TRAIL) receptors. To explore the involvement of Fas signaling in generating this BH3-only stress, we studied the effect of *fas* inhibition in the hepatocyte apoptosis induced by the genetic disruption of Bcl-xL or ABT-737 administration. siRNA-mediated *in vivo* knockdown of *fas* did not alleviate their hepatocyte apoptosis (supplemental Fig. 2, *B* and *D*), suggesting that Fas signaling may not be the origin of this BH3-only

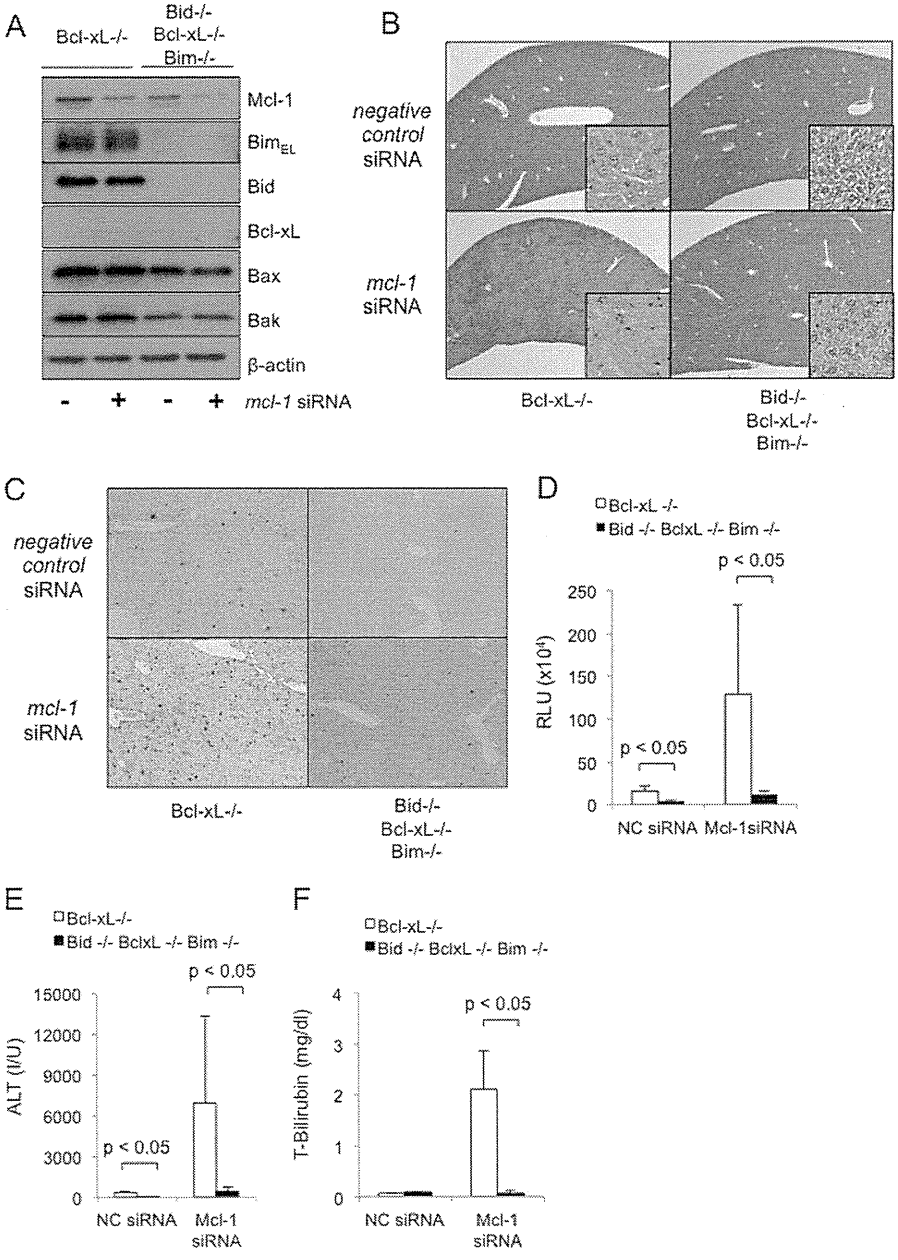


FIGURE 4. **Bim and Bid are essential regulators involved in the intrinsic pathway of apoptosis in hepatocytes in the absence of anti-apoptotic Bcl-2 family proteins.** *bcl-x$^{flox/flox}$alb-cre* mice and *bim$^{-/-}$bid$^{-/-}$bcl-x$^{flox/flox}$alb-cre* mice were injected with *mcl-1* or with negative control siRNA via the tail vein and were sacrificed 24 h (*A* and *C–F*) or 48 h (*B*) later. *Bcl-xL$^{+/+}$* and *Bcl-xL$^{-/-}$*, *bcl-x$^{flox/flox}$* and *bcl-x$^{flox/flox}$alb-cre*, respectively. *NC*, negative control. *A*, Western blot analysis of whole liver lysates for the expression of Bim$_{EL}$, Bid, Bcl-xL, Mcl-1, Bak, Bax, and β-actin. *B*, representative images of liver histology stained with hematoxylin-eosin (original magnifications, ×100 (*large panels*) and ×400 (*insets*)). *C*, representative images of liver histology stained with TUNEL (original magnification, ×100). *D*, serum caspase-3/7 activity; $n = 3–4$ mice/group. *E*, serum ALT levels; $n = 4$ mice/group; data are presented as means ± S.E. (*error bars*). *F*, serum T-bilirubin levels; $n = 4$ mice/group. *RLU*, relative light units; *I/U*, international units.

stress. However, it should be noted here that siRNA administration only decreased *fas* mRNA levels to around half (supplemental Fig. 2, *A* and *C*). Therefore, genetic study is still necessary to clarify its involvement. In order to examine the involvement of T and B cells, which comprise about 50% of intrahepatic resident immune cells (27), in producing the BH3-only stress in the healthy liver, we crossed hepatocyte-specific Mcl-1 knock-out mice with homozygous SCID mutant mice, which are characterized by an absence of functional T cells and B cells (28). The spontaneous hepatocyte apoptosis of the Mcl-1 knock-out mice was unchanged even in the homozygous SCID mutant background, monitored by serum ALT levels and serum caspase-3/7 activity (supplemental Fig. 3, *A–D*). These data indicate that these immune cells are not the major source of the BH3-only stress in the liver under physiological conditions. Therefore, further study is required to identify the main source of constitutive BH3-only stress in the healthy liver. We previously reported that Mcl-1 and Bcl-xL individually worked as apoptotic antagonists in differentiated hepatocytes (13). However, the hepatocyte-specific deletion of both led to early postnatal death due to the failure of hepatocyte development in the fetal liver (13), thus hampering the clarification of their

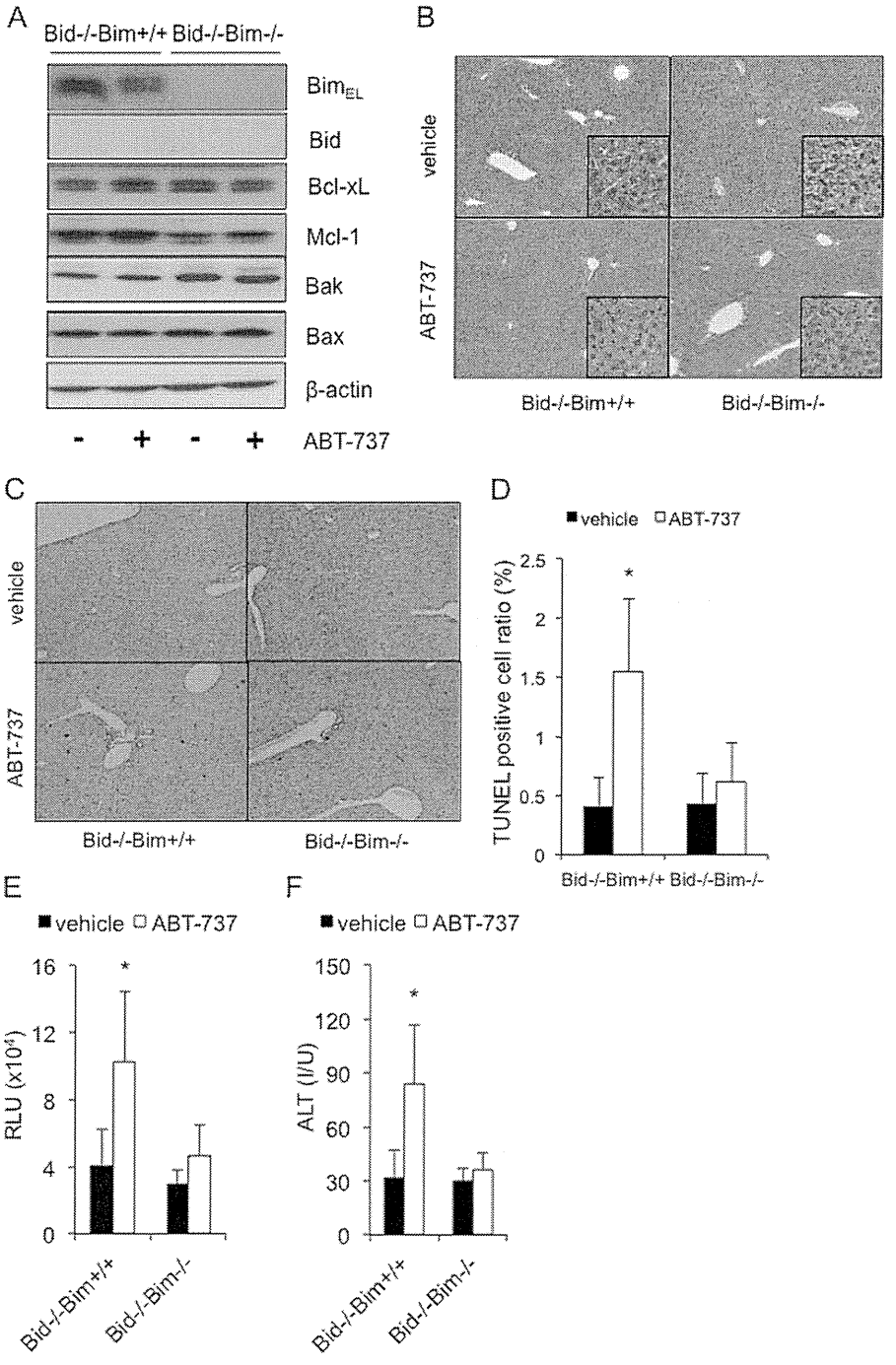


FIGURE 5. **The presence of Bim- and Bid-induced constant BH3 stress in the healthy liver causes hepatotoxicity with the use of anti-cancer agents that target anti-apoptotic Bcl-2 family proteins.** The offspring from *bim*$^{+/-}$*bid*$^{-/-}$ mating pairs were given an intraperitoneal injection of ABT-737 (100 mg/kg) or vehicle and were examined after 6 h. *A*, Western blot analysis of whole liver lysates for the expression of Bim$_{EL}$, Bid, Bcl-xL, Mcl-1, Bak, Bax, and β-actin. *B* and *C*, representative images of liver histology stained with hematoxylin-eosin and TUNEL (original magnifications, ×100 (*large panels*) and ×400 (*insets*)). *D*, TUNEL-positive cell ratio; $n = 5–6$ mice/group; *, $p < 0.05$ *versus* all. *E*, serum caspase-3/7 activity; more than 5 mice/group; *, $p < 0.05$ *versus* all. *F*, serum ALT levels; more than 5 mice/group; *, $p < 0.05$ *versus* all. *Error bars*, S.D. *RLU*, relative light units; *I/U*, international units.

cooperative involvement in the adult liver. In the present study, the combination of genetically engineered mice and *in vivo* siRNA technology enabled the investigation of their cooperative roles for the first time, and we found that the inhibition of Mcl-1 caused sublethal liver injury with massive hepatocyte apoptosis in Bcl-xL-knock-out mice. Meanwhile, we also found that sublethal apoptosis was prevented in a Bim/Bid double knock-out background, suggesting that, of the BH3-only proteins, Bim and Bid are important for activating the intrinsic pathway of hepatocyte apoptosis in the absence of anti-apoptotic Bcl-2 family proteins. It would also be interesting to determine whether other anti-apoptotic Bcl-2 family proteins or BH3-only proteins are involved in this healthy Bcl-2 rheostasis.

The anti-apoptotic Bcl-2 family proteins are often dysregulated in a variety of malignancies, and they have been recog-

nized as important oncogenes (29). ABT-737, which was recently developed to inhibit the Bcl-xL, Bcl-w, and Bcl-2 proteins, displays anti-tumor activity against lymphoid malignancies and small-cell lung carcinoma (23). These drugs were considered to selectively target tumor cells because malignant cells receive many genotoxic and environmental stress-induced BH3-only signals, so these cells are thus dependent on the anti-apoptotic Bcl-2 family members for their survival. However, we previously reported that the high-dose administration of ABT-737 (100 mg/kg) elicited hepatotoxicity via Bak/Bax-dependent apoptosis in normal hepatocytes (7), suggesting that dependence on the anti-apoptotic Bcl-2 family proteins is not a specific feature of tumor cells but is the case in healthy liver cells. In the present study, we demonstrated that the disruption of Bim and Bid completely prevented hepatocyte apoptosis and hepatotoxicity induced by high dose ABT-737 (100 mg/kg), suggesting that these proteins are responsible for this hepatotoxicity. Meanwhile, although 25 mg/kg ABT-737, which is relatively close to the clinical dose, caused moderate hepatocyte apoptosis, this apoptosis was completely blocked by Bid inhibition (supplemental Fig. 4). Therefore, it is unclear whether both Bid and Bim are truly involved in hepatotoxicity when using ABT-737 at clinically relevant doses.

This study demonstrated that Bim was also involved in the hepatocyte apoptosis caused by Mcl-1 deficiency in addition to Bid, which was noted in our previous report (13). Several previous human studies have reported that Mcl-1 proteins were down-regulated in the liver tissues of non-alcoholic steatohepatitis and primary biliary cirrhosis patients (30, 31), and experimental studies have demonstrated that Mcl-1 down-regulation by saturated fatty acids caused hepatocyte lipoapoptosis, which plays an important role in the development of fatty liver disease (32, 33). Taken together with our findings, these reports suggest the possibility that Bim- and Bid-mediated constant BH3 stresses might constitute therapeutic targets of the hepatotoxicity observed in these human liver diseases.

In conclusion, we have demonstrated that the novel rheostatic balance between the pro-apoptotic BH3-only proteins Bim and Bid and the anti-apoptotic Bcl-2 family proteins Bcl-xL and Mcl-1 regulates hepatocyte life and death in the physiological state. Our present study sheds new light on the dynamic and well orchestrated Bcl-2 networks in the healthy liver.

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Research Article

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# Carbamazepine promotes liver regeneration and survival in mice

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**Background & Aims**: Carbamazepine (CBZ), a widely used anticonvulsant and mood stabilizer, activates multiple proliferative and pro-survival pathways. Here, we hypothesize that CBZ may promote hepatocellular proliferation and ameliorate liver regeneration.

**Methods**: C57BL6/J mice were orally administered CBZ or vehicle and underwent a 70% partial hepatectomy (PHx), 85% PHx or treatment with carbon tetrachloride ($CCl_4$). Liver regeneration was determined by liver to body weight ratio, hepatocyte proliferation markers, and activation of intracellular signalling pathways.

**Results**: Two to 5 days after the 70% PHx, the liver to body weight ratio was significantly higher in the CBZ-treated mice than in the vehicle-treated mice. CBZ treatment upregulated the number of proliferative hepatocytes following PHx or $CCl_4$ treatment, as assessed by intrahepatic Ki-67 staining, BrdU uptake, and PCNA protein expression. PHx surgery induced the expression of several cyclins and activated Akt/mTOR signalling pathways, all of which were enhanced by CBZ treatment. The administration of the mTOR inhibitor temsirolimus abrogated the hepato-proliferative effect of CBZ. CBZ treatment significantly improved the survival rate of the mice that underwent lethal 85% massive hepatectomy.

**Conclusions**: CBZ demonstrated a novel hepato-proliferative effect through the activation of the mTOR signalling pathway in hepatectomised mice. CBZ has the potential to be a therapeutic option for facilitating efficient liver regeneration in patients subjected to liver surgery.



Keywords: Carbamazepine; Liver regeneration; Hepatocyte proliferation; Akt; mTOR.

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*Abbreviations:* CBZ, carbamazepine; PHx, partial hepatectomy; PI-3K, phosphotidylinositol-3 kinase; MAPK, ras-mitogen-activated protein kinase; ERK, extracellular signal regulated kinase; DMSO, dimethyl sulfoxide; H&E, haematoxylin and eosin; IHC, immunohistochemistry; RT-PCR, reverse transcription PCR; JNK, c-jun N-terminal kinase; $CCl_4$, carbon tetrachloride; NPC, non-parenchymal cells; HGF, hepatocyte growth factor.

## Introduction

Hepatocyte proliferation is critically important in liver regeneration after surgical resection or living donor transplantation. It involves the recovery from loss of volume and impaired liver function [1–3]. If this fundamental proliferative ability is not sufficient to compensate for the resected liver, postoperative liver failure will occur, which is a serious complication and remains an important clinical problem [4,5]. To overcome this issue, therapeutic methods that support liver regeneration must be explored. However, few treatment options are capable of enhancing liver regeneration in a clinical setting, despite widespread interest and numerous trials [6,7]. Carbamazepine (CBZ) is FDA-approved and widely used as an anticonvulsant or a mood stabiliser in clinical settings [8,9]. Mood stabilisers have been shown to exert prosurvival and cytoprotective effects on neuronal cells through the activation of intracellular signalling pathways that involve the phosphotidylinositol-3 kinase (PI-3K)-Akt pathway and the Ras-mitogen-activated protein kinase (MAPK) cascade [10–12]. In fact, CBZ induces a rapid and prolonged phosphorylation of extracellular signal regulated kinase (ERK) in human neuroblastoma cells [13]. In addition to the close relationship of CBZ to prosurvival signalling, a recent report revealed the therapeutic potential of CBZ in treating liver fibrosis caused by α1-antitrypsin deficiency, one of the chronic liver diseases leading to cirrhosis and liver failure [14]. These findings fascinated us enough to encourage the evaluation of the favourable effect of CBZ on liver regeneration after surgical resection. In the present study, we identified a novel hepatoproliferative effect of CBZ on hepatectomised mice that is mediated through the activation of the mTOR pathway. This effect could partially protect the mice against the high lethality associated with massive liver resection. These results imply the therapeutic potential of CBZ to support liver regeneration in patients who are subjected to liver resection or living donor transplantation.

## Materials and methods

### *Mice*

Six- to eight-week-old male C57BL/6J mice were purchased from Charles River Laboratories Japan (Tokyo). The mice were maintained in a specific pathogen-free facility with a 12-hour-dark/12-hour-light cycle and received humane treatment. All animal-related procedures were approved by the Animal Care and Use committee of Osaka University Medical School.

*Surgery and materials*

The mice were anesthetised with inhaled isoflurane and subjected to sham operation or 70% partial hepatectomy (PHx) as previously described (n >3 for each group and time point) [15]. Then, the mice were euthanized at indicated time points after surgery. The 85% PHx surgical procedure was identical to 70% PHx but with the additional resection of the right lower and caudate lobes [16]. Carbamazepine (CBZ) was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in a stock solution of 50 mg/ml dimethyl sulfoxide (DMSO). The mice were orally administered 250 mg/kg of CBZ or an equivalent volume of DMSO 2 h before surgery. The CBZ dosage was determined based on a previous *in vivo* study [14]. Temsirolimus was purchased from Sigma-Aldrich and dissolved in a stock solution of 20 mg/ml DMSO. The mice were injected intraperitoneally with 5 mg/kg of temsirolimus or an equivalent volume of DMSO 4 h before surgery. The temsirolimus dosage was determined based on a previous *in vivo* study reporting its inhibitory effects on mTOR [17].

*Blood tests*

To measure serum AST and ALT levels, blood was collected from the inferior vena cava of mice and centrifuged at 10,000*g* at room temperature for 15 min. Serum AST and ALT levels were measured by a standard method at the Oriental Kobo Life Science Laboratory (Nagahama, Japan).

*Histological analyses*

The dissected livers were fixed in formalin and embedded in paraffin. The sections were stained with haematoxylin and eosin (H&E). To assess hepatocyte proliferation, the sections were further processed for immunohistochemistry (IHC) with anti-Ki-67 antibody (Sigma-Aldrich) and anti-PCNA antibody (Cell Signaling Technology, Beverly MA). For IHC, antigen retrieval was performed by steaming for 20 min in 1× Target Retrieval Solution (pH 6.0) (DAKO, Glostrup, Denmark). The quenching of the endogenous peroxidase was accomplished with a 10-min incubation in 3% hydrogen peroxide in methanol. Sections were stained using the immunoperoxidase technique and counterstained with haematoxylin. We also stained liver sections for nuclear BrdU incorporation as previously described [18].

*Western blot analysis*

A piece of frozen liver tissue was lysed in lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1× protease inhibitor cocktail [Nacalai Tesque, Kyoto Japan], 1× phosphatase inhibitor cocktail [Nacalai Tesque], phosphate-buffered saline, pH7.4). The homogenates were purified by centrifugation at 10,000*g* at 4 °C for 15 min. The protein concentrations were determined using a bicinchoninic acid protein assay (Thermo Scientific, Rockford, IL). Equal amounts of protein extract were electrophoretically separated by SDS polyacrylamide gels and transferred onto a polyvinylidene fluoride membrane. For immunodetection, the following antibodies were used: anti-cyclinE1, anti-Akt, anti-phospho Akt (Thr 308), anti-phospho Akt (Ser 473), anti-mTOR, anti-phospho mTOR (Ser 2448), anti-S6K, anti-phospho S6K (Thr 389), anti-4EBP1, anti-phospho-4EBP1 (Thr 37/46), anti-ERK, and anti-phospho ERK (Thr 202/Tyr 204), anti-JNK, anti-phospho JNK (Thr 183/Tyr 185) (Cell Signaling Technology), anti-cyclinA (Santa Cruz Biotechnology Inc., Santa Cruz, CA), PCNA and β-actin (Sigma-Aldrich).

*Real-time quantitative PCR*

Total RNA isolated from liver tissues using an RNeasy Mini Kit (QIAGEN) was reverse transcribed and subjected to real-time reverse transcription PCR (RT-PCR) as previously described [18]. The mRNA expression levels of the specific genes were quantified using TaqMan Gene Expression Assays (Applied Biosystems) as follows: murine *ccna2* (assay ID:Mm00438063_m1), murine *ccne2* (assay ID: Mm00438077_m1), murine *hgf* (assay ID:Mm01135193_m1), murine *il6* (assay ID:Mm00446190_m1) and murine *actb* (assay ID:Mm00607939_s1). The transcript levels are presented as fold change relative to the controls.

*Statistics*

Data are expressed as mean ± SD. Statistical analyses between two groups were performed by an unpaired Student's *t* test unless otherwise indicated. Multiple comparisons were performed by a one-way ANOVA, and differences in the mean values among groups were examined by a Fischer *post hoc* correction. *p* values less than 0.05 were considered to be statistically significant.

## Results

*CBZ promotes liver regeneration after PHx*

To test whether CBZ has any effect on liver regeneration, male C57BL6/J mice were orally administered CBZ or vehicle and underwent 70% PHx. The PHx procedure allows for a well-established liver regeneration model in which the liver recovers full volume after surgery. In the sham-operated mice, no difference was found in liver to body weight ratio at 48 h after drug administration between the CBZ-treated and vehicle-treated groups (Supplementary Fig. 1). In the hepatectomised mice, the ratio was significantly higher in the CBZ-treated group than in the vehicle-treated group (Supplementary Fig. 1). We then examined the liver to body weight ratio at several time points after surgery with or without one-time oral CBZ administration. After PHx, the liver to body weight ratio was rapidly recovered in the CBZ-treated mice and was significantly higher than in the vehicle-treated mice at 2, 3 and 5 days after PHx (Fig. 1A). The liver to body weight ratio reached similar levels by 14 days after surgery in both groups (Fig. 1A). These findings demonstrate that CBZ promoted liver regeneration after PHx in mice.

*CBZ enhances hepatocyte proliferation after PHx*

During liver regeneration, hepatocyte proliferation is critically important in compensating for the lost liver mass and liver function recovery. To determine whether CBZ affects hepatocyte proliferation in the hepatectomised mice, hepatocyte DNA synthesis was assessed by immunohistochemical staining of liver sections with Ki-67 and BrdU—two principal markers of DNA replication. We first confirmed that there was no difference in liver injury after PHx in the CBZ- or vehicle-treated mice, by evaluation of serum AST and ALT levels (Fig. 1B and C). H&E staining also revealed that there was no inflammatory cell infiltration or necrosis in the livers of either group (Fig. 1D). The number of Ki-67 positive cells increased to a peak at 48 h after PHx in both groups (Fig. 1E and F), but the peak value was significantly higher in the CBZ-treated livers (Fig. 1E and F). Similarly, the number of BrdU-positive nuclei was also significantly higher in CBZ-treated mice than in vehicle-treated mice at 36 h after PHx (Fig. 1G and H). Western blotting indicated higher protein expression levels for proliferating nuclear antigen (PCNA), another well-known marker of DNA replication, in CBZ-treated livers at 48 h after PHx (Fig. 1I). These findings indicate that CBZ increased the number of proliferative hepatocytes after PHx in mice. We also observed the similar hepato-proliferative effect and amelioration of liver regeneration in hepatectomized mice even after repeated CBZ administration for 3 consecutive days (Supplementary Fig. 2A and B), which is a more clinically relevant regimen since CBZ requires multiple administrations to reach steady state levels [19]. To determine whether this favourable effect of CBZ is only observed in a resected liver, CBZ-treated mice were administered a single injection of carbon tetrachloride ($CCl_4$), which causes acute liver injury, and followed compensative liver regeneration [20]. CBZ treatment did not affect the liver damage but enhanced hepatocyte proliferation (Supplementary Fig. 3A–C) suggesting that the hepato-proliferative effect of CBZ may not be limited to the hepatectomised liver.

We then examined the gene expression of several cyclins, accelerators of cell cycle progression, which are important for hepatocyte proliferation in regenerating livers [21]. A real-time RT-PCR analysis revealed that the mRNA levels of *ccne2* and *ccna2* were significantly higher in CBZ-treated mice than in

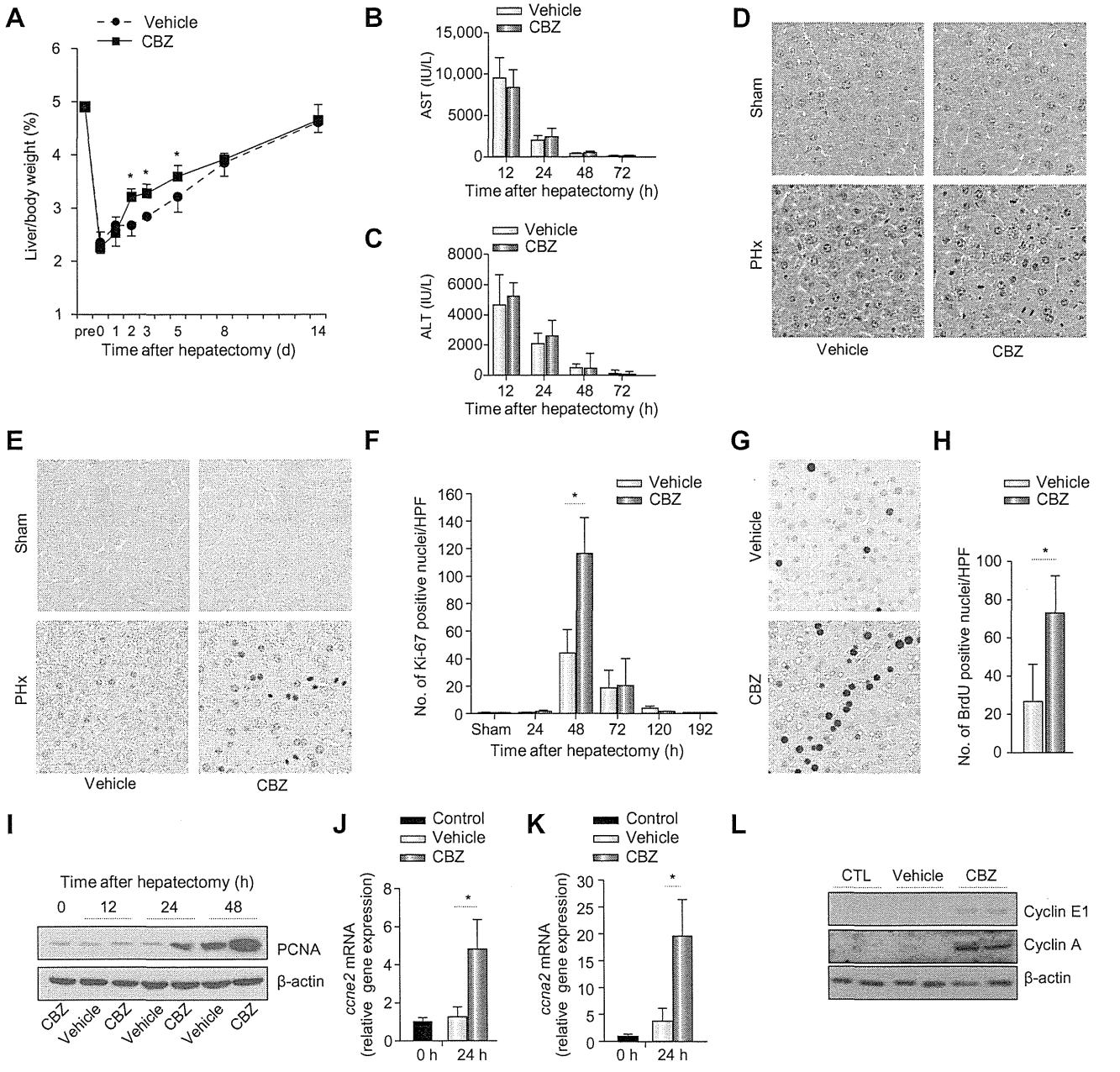


**Fig. 1. CBZ promotes liver/body weight ratio recovery and enhances hepatocyte proliferation after 70% partial hepatectomy.** Mice were administered 250 mg/kg of CBZ or DMSO orally and subjected to 70% partial hepatectomy 2 h later (3 mice per group). CBZ, PHx, and CTL stand for carbamazepine, 70% partial hepatectomy and control, respectively. (A) Changes in liver/body weight ratio over time in mice receiving PHx with vehicle or CBZ, $^{*}p$ <0.05 *vs.* vehicle. (B and C) Serum AST (B) and ALT (C) levels in vehicle- or CBZ-treated mice. (D) Liver sections at 48 h after PHx or sham operation were stained with H&E; original magnification, 400×. (E) Liver sections after surgery were evaluated for hepatocyte proliferation with anti-Ki-67 staining; original magnification, 400×. (F) The number of Ki-67 positive nuclei/high-power field (HPF) at 48 h after surgery in sham-operated mice and at indicated time in hepatectomised mice with vehicle or CBZ treatment. Six fields of view (FOVs) were counted in liver sections of individual mice, $^{*}p$ <0.05. (G) Liver sections at 36 h after PHx were stained with BrdU; original magnification, 400×. (H) The number of BrdU positive nuclei/HPF at 36 h after PHx in vehicle-treated and CBZ-treated mice. Six FOVs were counted in liver sections of individual mice, $^{*}p$ <0.05. (I) Expression of PCNA protein in liver tissue from vehicle- or CBZ-treated mice after PHx was assessed by Western blot analysis. (J and K) *ccne2* (J) and *ccna2* (K) mRNA levels in the liver were determined by real time RT-PCR at 24 h after PHx, $^{*}p$ <0.05. (L) Protein expression of cyclin E1 and cyclin A in liver tissue was assessed by Western blot analysis at 24 h after PHx.

vehicle-treated mice at 24 h after PHx (Fig. 1J and K). Evaluation by Western blot also demonstrated that protein levels of cyclin E1 and cyclin A were increased in CBZ-treated mice (Fig. 1L). Collectively, these results suggest that CBZ upregulated the cyclin levels in remnant hepatocytes, leading to an increase in the number of hepatocytes entering the cell cycle after PHx.

*CBZ strongly activates the Akt-mTOR pathway after PHx*

Mood stabilisers, including CBZ, have been reported to modulate the Akt and MAPK pathways [10–13], both of which are also involved in initiating the cell cycle progression of remaining liver cells upon liver resection [22–25]. Thus, we examined the effect

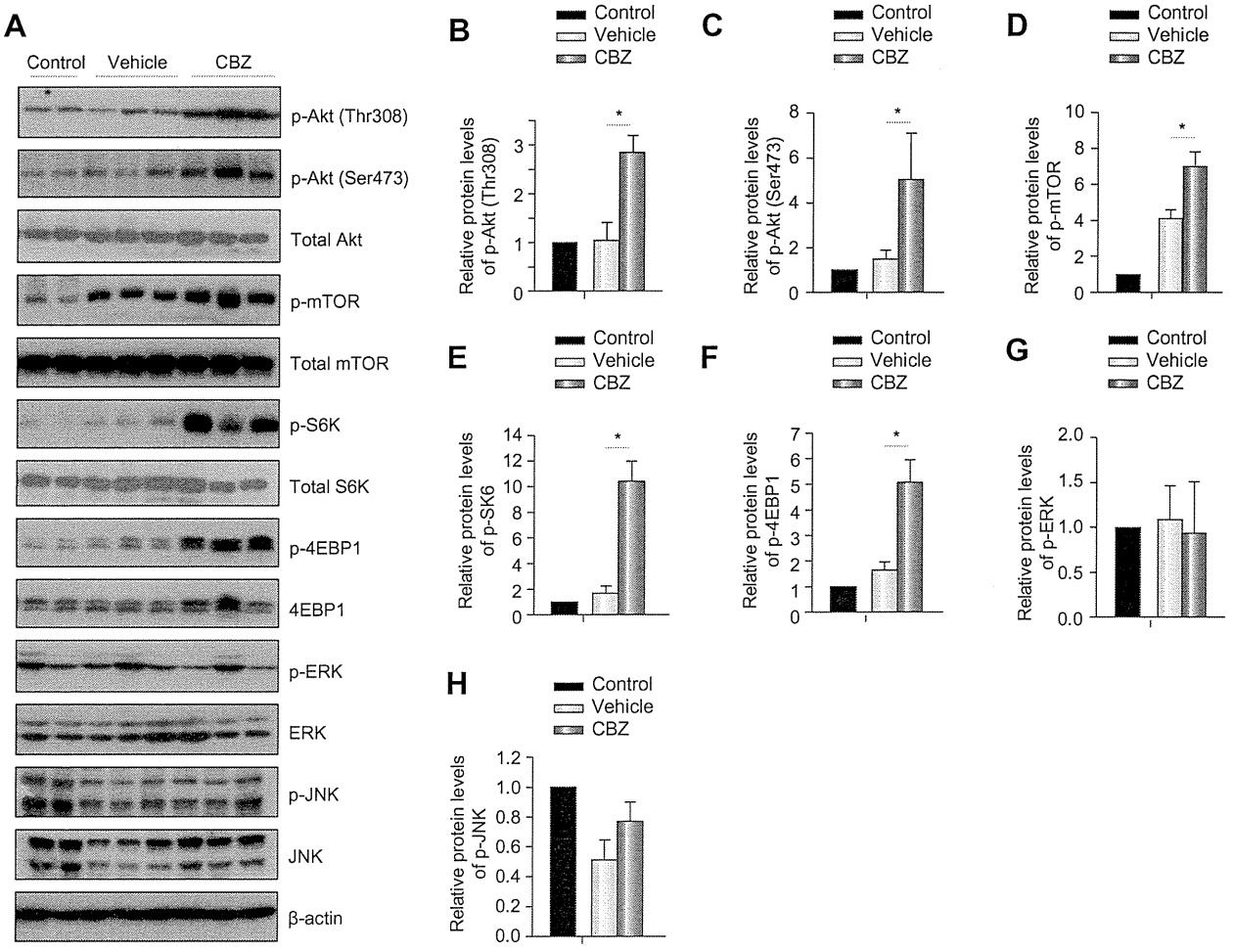


**Fig. 2. CBZ strongly activates Akt-mTOR signalling.** Mice were administered 250 mg/kg of CBZ or DMSO orally and subjected to 70% partial hepatectomy 2 h later (4 mice per group). (A) The phosphorylation status of Akt, mTOR, S6K, 4EBP1, ERK and JNK was assessed by Western blot analysis at 12 h after PHx. (B–H) Relative expression levels of phosphorylated proteins were calculated as the optical densities of their blots normalized to the β-actin blots; p-Akt (Thr308) (B), p-Akt (Ser473) (C), p-mTOR (D), p-S6 K (E), p-4EBP1 (F), p-ERK (G) and p-JNK (H). CBZ, carbamazepine; *$p$ <0.05.

of CBZ on the activation of these two pathways in the livers of hepatectomised mice. PHx induced phosphorylation of Akt (Thr308, Ser473) and activated its downstream effectors, mTOR, S6K, and 4EBP1, at 12 h after surgery. All of these signalling molecules were enhanced by CBZ treatment (Fig. 2A–F). By contrast, the phosphorylation of ERK was not different between the CBZ-treated and vehicle-treated mice (Fig. 2A and G). We also evaluated the activation of the c-jun N-terminal kinase (JNK) pathway, which is closely related to liver regeneration [26], and found no difference between the two groups (Fig. 2A and H).

*Activation of the mTOR pathway is responsible for enhanced hepatocyte proliferation in hepatectomised mice following CBZ treatment*

To investigate whether the strong activation of Akt-mTOR pathway was ascribable to the hepato-proliferative effect of CBZ after PHx, we blocked mTOR signalling by the use of the mTOR inhibitor temsirolimus. Temsirolimus administration blocked the enhancement of mTOR pathway activation in the CBZ-treated hepatectomised livers to a level similar to the vehicle-treated hepatectomised liver (Fig. 3A), while phosphorylation of Akt, an upstream signalling molecule of mTOR, was upregulated in both mice likely due to a compensative response (Fig. 3A). Under these conditions, temsirolimus abrogated the upregulation of *ccne2* and *ccna2* mRNA expression and PCNA protein expression in the CBZ-treated hepatectomised mice (Fig. 3B–E), suggesting that the hepato-proliferative effect of CBZ is attributable to the enhanced activation of the mTOR pathway. In addition, mTOR inhibition also prevented CBZ-induced acceleration of liver mass recovery 48 h after PHx (Fig. 3F). Altogether, these findings indicate that, following PHx surgery, CBZ treatment potentiated the activation of the mTOR pathway, which enhanced hepatocyte proliferation and promoted liver regeneration.

*CBZ improves the survival rate of mice that undergo 85% massive hepatectomy*

Finally, we evaluated the therapeutic significance of CBZ in regeneration of the resected liver using a severe 85% massive hepatectomy model. This PHx model typically presents extremely high mortality (82%) within 2 days after surgery [27]. Consistent

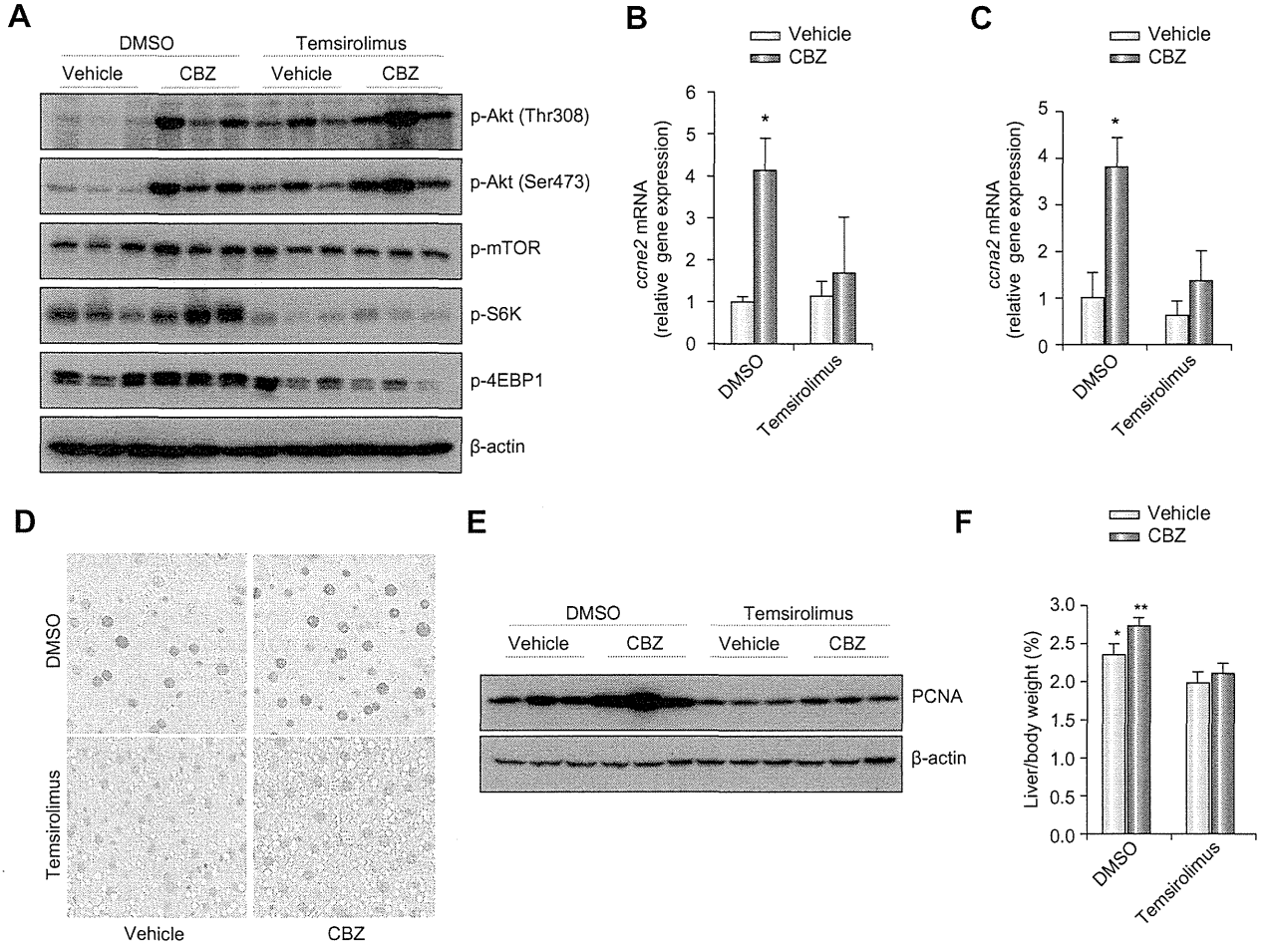


**Fig. 3. mTOR inhibitor abrogates the hepato-proliferative effect of CBZ in hepatectomised mice.** Mice were injected with temsirolimus or DMSO 4 h before PHx and orally administered 250 mg/kg of CBZ or DMSO 2 h before PHx. Then, mice were subjected to 70% partial hepatectomy and euthanized at indicated time points. (A) The phosphorylation status of Akt, mTOR, S6K, and 4EBP1 at 12 h after PHx was assessed by Western blot analysis. (B and C) Real-time RT-PCR analysis of *ccne2* (B) and *ccna2* (C) mRNA expression at 24 h after PHx, $^{*}p$ <0.05 *vs.* all. (D and E) The expression of PCNA proteins at 48 h after PHx was assessed by (D) immunohistochemistry and (E) Western blot analysis. (F) Liver/body weight ratio at 48 h after PHx in indicated groups. CBZ, carbamazepine; 3 mice per group. Statistical analyses were performed by one-way ANOVA. $^{*}p$ <0.05 *vs.* temsirolimus-vehicle group; $^{**}p$ <0.05 *vs.* all.

with the effect of CBZ observed in the 70% PHx model, CBZ did not affect liver injury but enhanced hepatocyte proliferation in the liver after the 85% PHx (Fig. 4A and B). Consequently, while only 4 of the 25 vehicle-treated mice survived for 7 days after 85% PHx, 11 of 25 CBZ-treated mice were alive at 7 days. The CBZ-treated mice survival rate was significantly higher than that of vehicle-treated mice (44% *vs.* 16%, $p$ <0.05) (Fig. 4C).

## Discussion

Liver regeneration after surgical resection or injury is a complex phenomenon primarily dependent on hepatocyte proliferation. In the present study, we identified a new aspect of CBZ, increasing hepatocyte proliferation after partial resection of the liver in mice. We also clarified the involvement of the mTOR signalling pathway in this hepato-proliferative effect. mTOR and its downstream effectors S6K and 4EBP1, all of which were intensively upregulated by CBZ treatment, have been shown to stimulate cell cycle progression via modulation of the expression of several cyclins, such as cyclin E and cyclin A [28]. In fact, in our hepatectomised mice, CBZ enhanced upregulation of their mRNA levels, which were dependent on mTOR activation. These findings suggest that mTOR activation may produce a profound effect on cell cycle progression via upregulating cyclin expression in CBZ-treated remnant livers. In this study, we also found that CBZ enhanced Akt phosphorylation following PHx, which might be an event that is upstream of mTOR activation. As mood stabilising drugs have been described to trigger activation of PI-3K and subsequent phosphorylation of Akt in neuronal cells by generating lipid second messengers (i.e., PI-3,4,5-P3 or PI-3,4-P2) [10,13], such a mechanism might be relevant to CBZ-mediated Akt activation in resected livers. Further studies are necessary to elucidate the exact mechanism by which CBZ activates the mTOR signalling pathway.

Given that CBZ has complicated pharmacokinetic properties, a variety of mechanisms other than those involving the mTOR pathway could be related to the enhanced liver regeneration in

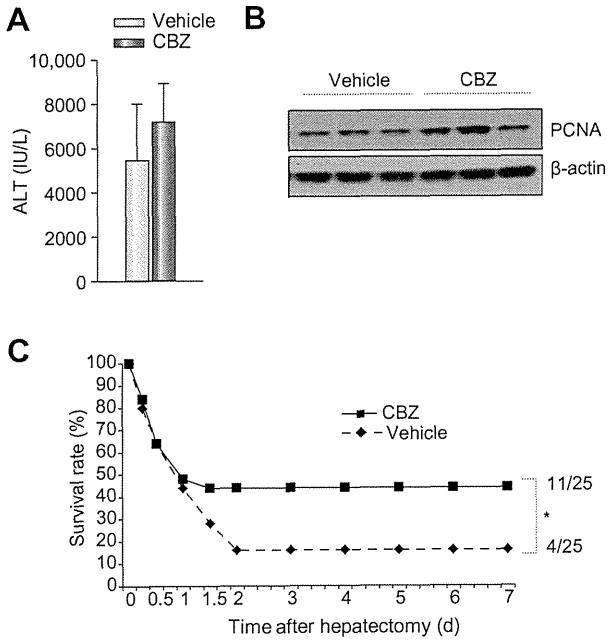


**Fig. 4. CBZ improves survival of mice that undergo 85% massive hepatectomy.** Mice were orally administered 250 mg/kg of CBZ or DMSO and subjected to 85% partial hepatectomy 2 h later. (A and B) Mice were euthanized 24 h after PHx (4 mice per group). (A) Serum ALT levels. (B) Expression of PCNA protein in liver tissue from vehicle- or CBZ-treated mice was assessed by Western blot analysis. (C) The survival rate was assessed at 7 days after surgery (25 mice per group). Statistical analysis was performed using Chi-square test. CBZ, carbamazepine; *$p$ <0.05.

CBZ-treated mice. To further investigate underlying mechanisms, we performed microarray analysis of the mouse liver tissues collected after CBZ administration. Pathway analysis of microarray data revealed activation of PXR/RXR and FXR/RXR pathways (data not shown), both of which have been reported to be involved in liver regeneration [29–31]. These pathways might be also involved in the hepato-proliferative effect of CBZ.

Following PHx, both hepatocytes and non-parenchymal cells (NPCs) are activated and integrate multiple signals originating from immune, hormonal, and metabolic networks to induce hepatocyte proliferation [24]. In particular, after PHx, hepatic stellate cells and Kupffer cells produce hepatocyte growth factor (HGF) and IL-6, respectively, both of which contribute to liver regeneration partially through modulating the intrahepatic signalling pathways focused on in this study [3,32]. Therefore, we investigated the involvement of NPCs in the CBZ-induced hepato-proliferative effect in hepatectomised mice. Neither *HGF* nor *IL6* gene expression levels were different between the CBZ-treated livers and vehicle-treated livers following PHx (Supplementary Fig. 4A and B). By contrast, in the *in vitro* study, primary hepatocytes presented sustained phosphorylation of Akt (Ser473) with transient and moderate activation of mTOR in response to the administration of CBZ (Supplementary Fig. 5). These findings support the idea that CBZ may directly activate intracellular signalling pathways in hepatocytes contributing to enhanced liver regeneration. Meanwhile, in this *in vitro* setting, primary hepatocytes did not show a proliferative response to CBZ administration (Supplementary Fig. 6). This may be because hepatocytes require additional priming stimulus to start proliferation *in vitro*, same as our *in vivo* finding that CBZ administration did not start liver regeneration in the sham-operated mice (Fig. 1E and F, and Supplementary Fig. 1). We cannot exclude the possibility that CBZ does not primarily target hepatocytes, but affects other cell types in the liver to promote liver regeneration. Actual targets of CBZ in the liver will be determined in future studies.

In rodents, 70% hepatectomy is well tolerated, but beyond 70%, resection is accompanied by higher mortality due to acute liver failure despite the inherent ability of the liver to recover to full size. This suggests that insufficient functional compensation of the remnant liver fails to maintain homeostasis in the animal [16,27]. In clinical settings, extended liver resection is reportedly associated with severe hepatic dysfunction, leading to a significant increase in postoperative mortality [33,34]. In this context, the promotion of the recovery of impaired liver function is critically important for any therapeutic drug potentially used to aid in liver regeneration. In the present study, CBZ treatment significantly improved the survival rate of the mice that underwent lethal 85% massive hepatectomy. This result elucidates the therapeutic potential of CBZ to prevent postoperative liver failure after major hepatectomy or living donor liver transplantation with extended criteria.

When considering the therapeutic application of this study, it is important to apply clinically relevant doses of CBZ to obtain relevant physiological serum levels of CBZ (4-12 μg/ml) [35]. In the present study, 2 h after oral administration of 250 mg/kg of CBZ, its serum level reached 22.9 μg/ml (Supplementary Fig. 7A) and was relatively higher than the therapeutic range in humans. It is known that repeated administration of CBZ shortens its half-life, and therefore consecutive administration is required to acquire steady state levels [19]. Thus, we evaluated CBZ serum levels after repeated administration at 250 mg/kg for 3 consecutive days. This administration method acquires physiological levels of CBZ (4.8 μg/ml) (Supplementary Fig. 7B), and importantly, the favorable effect on liver regeneration was retained in the subsequently performed 70% PHx (Supplementary Fig. 2A and B). This result may support the potential therapeutic use of CBZ. We also studied the influence of hepatectomy on serum levels of CBZ because it reduces the total amount of metabolizing cells in the liver. Serum levels of CBZ were not different between the hepatectomized mice and the sham operated mice 3 h after the surgery (Supplementary Fig. 7C), suggesting that CBZ treatment may be applicable after liver resection.

In conclusion, we demonstrated that CBZ promoted hepatocyte proliferation via the mTOR signalling pathway, resulting in early liver regeneration in mice. We also demonstrated the therapeutic implications of this drug in an 85% massive hepatectomy model. Despite a large number of basic studies searching for novel therapeutic agents to enhance liver regeneration, few options are currently available for clinical use [6,7]. Our study suggests the possibility that CBZ may enhance liver regeneration in a clinical setting, leading to a reduction in postoperative liver failure and improving survival.

## Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2013.07.018.

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***Original Article***

# Managing hepatitis B virus carriers with systemic chemotherapy or biologic therapy in the outpatient clinic

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*Aim:* The number of outpatients receiving systemic chemotherapy in Japan has recently increased. We retrospectively examined whether hepatitis B virus (HBV) carriers were safely treated and managed with systemic chemotherapy or biologic agents as outpatients at our oncology center.

*Methods:* A total of 40 115 consecutive infusion chemotherapy or biologic therapies were administrated to 2754 outpatients in the Chemotherapy and Oncology Center at Osaka University Hospital from December 2003 to March 2011. We first studied the prevalence of outpatients with hepatitis B surface antigen (HBsAg), and then retrospectively evaluated a database to determine the frequencies of testing for other HBV-related markers and the incidence of developing hepatitis or HBV reactivation in patients positive for HBsAg. As a control for comparison, we also examined these same factors in patients with hepatitis C virus antibody (anti-HCV).

*Results:* The majority of physicians at our hospital screened for HBsAg (95%) and anti-HCV (94%) prior to administrating chemotherapy. Of the 2754 outpatients, 46 (1.7%) were positive for HBsAg and 90 (3.3%) were positive for anti-HCV. Fifteen patients that were HBsAg positive were treated with lamivudine or entecavir prior to chemotherapy. None of the patients with HBsAg taking a prophylactic antiviral developed hepatitis, and only one breast cancer patient without prophylactic antiviral treatment (1/31 [3.2%]) developed hepatitis due to HBV reactivation.

*Conclusion:* HBV reactivation occurred in outpatients without prophylactic antiviral treatment, but the incidence was relatively low.

**Key words:** biologic therapy, chemotherapy, hepatitis B virus reactivation, outpatient

## INTRODUCTION

HEPATITIS B IS one of the world's most common and serious infectious diseases. It is estimated that more than one-third of the world's population has been exposed to the hepatitis B virus (HBV) and that there are approximately 350 million chronic carriers worldwide, 75% of whom live in South-East Asia and the Western Pacific regions.[1–4] In Japan, approximately 26 million people have been exposed to HBV. Of those who have been exposed, 1.5 million people are estimated to be chronic carriers.[5] Generally, one-fifth of all HBV carriers develop chronic hepatitis, cirrhosis and primary hepatocellular carcinoma. The majority of HBV patients are, however, clinically inactive.

Among HBV-related liver diseases, HBV reactivation is now a well-recognized complication in HBV inactive carriers who receive cytotoxic chemotherapy for cancer. HBV reactivation was first described in patients with lympho- and myeloproliferative disorders by Wands *et al.*[6] in 1975. Wands *et al.*[6] demonstrated that patients with hepatitis B antigen (HBAg) developed hepatitis with a marked increase in the HB-Ag titer during chemotherapy. The reactivation condition ranges from asymptomatic self-limiting anicteric hepatitis to severe, potentially fatal, progressive decompensated hepatitis. In addition, HBV reactivation during or after chemotherapy or other immunosuppressive therapy

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was recently reported as de novo HBV-related hepatitis even in previously exposed HBV patients without hepatitis B surface antigen (HBsAg), particularly in cases using rituximab.[7]

Based on this background, a guideline for preventing HBV reactivation during and after cytotoxic or immunosuppressive therapies was proposed in 2009 and revised in 2011 by two collaborative study groups from the Japanese Ministry of Health, Labor and Welfare, which included measures not only for HBV carriers, but also for patients without HBsAg.[8] The guideline was intended to identify patients with the potential for HBV reactivation. Therefore, HBsAg screening is recommended for all patients scheduled for chemotherapy or other immunosuppressive therapy. If a patient is positive for HBsAg, prophylaxis is recommended, in addition to testing for hepatitis B e-antigen (HBeAg), antibody to hepatitis B e-antigen (anti-HBe) and HBV DNA. On the other hand, if a patient is negative for HBsAg, testing for anti-hepatitis B core (HBc) and anti-HBs is recommended. If a patient is positive for either or both anti-HBs and anti-HBc, then testing for HBV DNA is recommended. If a patient is positive for HBV DNA, prophylaxis is recommended. If a patient is negative for HBV DNA, monthly monitoring of HBV DNA and aspartate aminotransferase (AST)/alanine aminotransferase (ALT) is recommended, and should be continued for at least 12 months after the end of chemotherapy.[8]

The number of outpatients undergoing cancer chemotherapy has recently increased due to the advances in cytotoxic agents and supportive therapies. Moreover, there has been an increase in the number of patients with inflammatory bowel disease or rheumatoid arthritis requiring immunosuppressive therapy, such as biologic agents (e.g. anti-tumor necrosis factor agents). In Japan, the increase in immunosuppressive therapies has led to a shift in hospital care to outpatient therapy since 2002 for heath insurance reasons. The corresponding data for HBsAg positive outpatients requiring these immunosuppressive therapies are, however, not known. In this study, we retrospectively examined whether asymptomatic HBV carriers were safely treated and managed with systemic chemotherapy or immunosuppressive therapies in the outpatient setting.

## METHODS

### Patients

THIS WAS A retrospective study in a single institute. A total of 40 115 consecutive infusion treatments in 2754 outpatients (1122 men, 1632 women) with cancer or autoimmune disease, such as rheumatoid arthritis or Crohn's disease, treated with cytotoxic or biologic agents in the Chemotherapy and Oncology Center for outpatients at Osaka University Hospital from December 2003 to March 2011 were enrolled. Patients receiving second-line or more chemotherapy were also included.

### Methods

The cytotoxic or biologic infusion agents were administrated to each patient according to the standard protocol for the specific tumor type or disease commonly treated within health insurance parameters in Japan. Oncology center staff and pharmacists basically reviewed all protocols before treatment. Medical records of all patients with HBsAg were retrospectively reviewed for this study. As a control, the records of patients with hepatitis C virus antibody (anti-HCV) were examined. If the patients were positive for HBsAg or anti-HCV, their medical records were additionally reviewed to determine whether they were tested for anti-HBs, anti-HBc, HBeAg, anti-HBe and HBV DNA, or administrated antiviral drugs before treatment. HBsAg, anti-HBs, anti-HBc, HBeAg and anti-HBe were measured by chemiluminescent immunoassay, but both HBeAg and anti-HBe were measured by chemiluminescent enzyme immunoassay until 5 May 2005. HBV DNA was measured by polymerase chain reaction (PCR) until 30 September 2009 and then real-time PCR. For the antiviral drugs, data collected included not only cases that received the drug for prophylaxis, but also cases in which treatment for chronic hepatitis was already administrated before treatment. Collected data were entered into a database that did not include any identifying information about the respondents. The follow-up period was defined as the period from the first visit in our center for outpatients to the last visit at Osaka University Hospital.

The study was approved by the Clinical Investigation and Research board of Osaka University Hospital (#11202, 10 December 2011). The study was performed in accordance with the Declaration of Helsinki, as revised in 2008.

### Definitions of hepatitis and HBV reactivation

Hepatitis was defined as a more than threefold increase in serum ALT of the upper limit of normal on two consecutive determinations. Patients who had been clinically diagnosed with hepatitis due to drug or tumor involvement were excluded from this study. HBV reactivation was defined as an increase of more than 1 log

copy/mL of serum HBV DNA, or the serum HBV DNA turned from negative to positive.

### Statistical analysis

Statistical analysis was performed with JMP software ver. 9.02 (SAS Institute). Data are expressed as the mean ± standard deviation and probability value. The $\chi^2$-test was used for the analysis of categorical variables. Probability values of less than 0.01 were considered statistically significant.

## RESULTS

### Baseline characteristics

THE MAJORITY OF physicians treating patients in our outpatient clinic screened for HBsAg (2607/2754, 95%) and anti-HCV (2586/2754, 94%) prior to administrating treatments. Of 2754 outpatients, 46 patients (1.7%) were positive for HBsAg and 90 (3.3%) were positive for anti-HCV. Two patients were positive for both HBsAg and anti-HCV. Table 1 shows the patient characteristics and Table 2 shows the laboratory data for patients with HBsAg or anti-HCV at the first infusion treatment at our outpatient clinic. The median follow-up period was 21 months (range, 2–102). Of 46 patients positive for HBsAg, 35 (76%), 14 (30%), 19 (41%), 24 (52%) and 25 (54%) patients were tested for anti-HBs, anti-HBc, HBe-Ag, anti-HBe and HBV DNA, respectively. Of 90 patients positive for anti-HCV, 24 (27%), 19 (21%), 23 (26%), seven (8%) and two (2%) patients were tested for anti-HBs, anti-HBc, HBe-Ag, anti-HBe and HBV DNA, respectively (Table 4). Two patients with both HBsAg and anti-HCV were tested for HBV DNA.

Table 1 Patient characteristics

| | Patients with HBsAg ($n = 46$) | Patients with anti-HCV ($n = 90$) |
|---|---|---|
| Age | 59 ± 10 | 66 ± 10 |
| Sex (M/F) | 16/30 | 55/35 |
| Number of treatments | 10 (1–210) | 11 (1–62) |
| Agents for treatment | | |
| Cytotoxic agents | 44 | 87 |
| Immunosuppressive agents | 2 | 3 |
| Type of cancer or basic disease | | |
| Breast cancer | 20 | 13 |
| Gastrointestinal cancer | 8 | 26 |
| Hepato-biliary-pancreatic cancer | 7 | 22 |
| Hematologic malignancy | 7 | 10 |
| Lung cancer | 2 | 7 |
| Renal cancer | 1 | 1 |
| Rheumatoid arthritis | 1 | 1 |
| Prostatic cancer | 0 | 5 |
| Gynecologic cancer | 0 | 2 |
| Others | 0 | 3 |
| Tumor infiltration of the liver | 17 | 18 |

HBsAg, hepatitis B surface antigen; anti-HCV, hepatitis C virus antigen.

Table 2 Patients' baseline laboratory data at first visit

| | Patients with HBsAg ($n = 46$) | Patients with anti-HCV ($n = 90$) |
|---|---|---|
| WBC (/μL) | 5110 ± 2015 | 4920 ± 1825 |
| Hb (g/dL) | 12.2 ± 2.1 | 12.0 ± 1.7 |
| Plt (/μL) | 20.3 ± 7.9 | 19.9 ± 9 |
| AST (U/L) | 23 ± 9 | 34 ± 32 |
| ALT (U/L) | 20 ± 11 | 27 ± 30 |
| T.Bil (mg/dL) | 0.3 ± 0.04 | 0.7 ± 0.3 |

ALT, alanine aminotransferase; AST, aspartate aminotransferase; Hb, hemoglobin; HBsAg, hepatitis B surface antigen; anti-HCV, hepatitis C virus antigen; Plt, platelets; T.Bil, total bilirubin; WBC, white blood cells.

Of the 46 patients positive for HBsAg, 15 had been treated with lamivudine or entecavir prior to chemotherapy or biologic therapies (33%). Of these 15, nine had been treated prophylactically (cases 1–9; Table 3), and the others had already been treated for chronic hepatitis B (case 10–15; Table 3) before their fist visit to the oncology center. They were all tested for HBV DNA before treatment and then monitored for HBV DNA. The method of monitoring for HBV DNA, however, basically depended on each physician and was not uniform. On the other hand, 31 patients (67%) with HBsAg underwent chemotherapy or biologic therapy without antiviral prophylaxis (Table 4). Of these 31, 10 were tested for HBV DNA before treatment and five of the 10 tested positive for HBV DNA.

Of the 46 patients positive for HBsAg, 20 patients had breast cancer, six of whom were treated with prophylactic antiviral medication (30%) and five of the six patients were positive for HBV DNA prior to chemotherapy. Of the other 14 patients without prophylaxis, four were tested for HBV DNA and 10 were not. Of the four patients tested for HBV DNA, one was positive. One of the 10 not tested developed HBV reactivation (case 35; Tables 3 and 5). There were eight patients with gastrointestinal cancer, none of whom was treated with prophylactic antiviral medication, although four were

**Table 3** Details of patients with HBsAg

| Case | No. of treatments | Follow-up period (months) | Sex | Age, years | Type of cancer or basic disease | First agent at the center | HBsAg | Anti-HBs | HBeAg | Anti-HBe | Anti-HBc | HBV DNA, log copies/ml | Anti-HCV | Corticosteroid use | Antiviral prior to chemotherapy of biologics | Hepatitis | Reactivation |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 8 | 2 | M | 56 | Malignant lymphoma | Rituximab | + | – | † | + | + | 4.3 | – | Present | Lamivudine | None | None |
| 2 | 20 | 11 | F | 47 | Breast cancer | Paclitaxel | + | – | – | + | † | 3.3 | – | Present | Entecavir | None | None |
| 3 | 10 | 32 | F | 44 | Leukemia | Rituximab | + | – | – | + | + | 3.3 | – | Present | Entecavir | None | None |
| 4 | 4 | 31 | M | 66 | Malignant lymphoma | Rituximab | + | – | + | † | † | Negative | – | Present | Entecavir | None | None |
| 5 | 11 | 28 | F | 62 | Breast cancer | Paclitaxel | + | – | – | + | + | Negative | – | Present | Entecavir | None | None |
| 6 | 23 | 26 | F | 79 | Breast cancer | Navelbine | + | – | † | + | + | 2.1 | – | Present | Entecavir | Present | None |
| 7 | 21 | 25 | F | 66 | Breast cancer | Docetaxel | + | – | – | + | + | 2.3 | – | Present | Entecavir | None | None |
| 8 | 14 | 22 | F | 43 | Breast cancer | FEC | + | – | † | + | † | <2.1 | – | Present | Entecavir | None | None |
| 9 | 9 | 16 | F | 60 | Breast cancer | Paclitaxel | + | – | † | + | + | 3.5 | – | Present | Entecavir | None | None |
| 10 | 19 | 15 | M | 71 | Bile duct cancer | Gemcitabine | + | – | † | + | † | 2.1 | – | Present | Lamivudine | None | None |
| 11 | 6 | 33 | F | 60 | Malignant lymphoma | Rituximab | + | – | – | + | † | 3 | – | Present | Lamivudine + adefovir | None | None |
| 12 | 8 | 60 | F | 73 | Malignant lymphoma | VDS + MTX | + | – | † | † | † | Negative | – | Present | Entecavir | None | None |
| 13 | 5 | 44 | F | 35 | Malignant lymphoma | CHOP | + | – | † | † | + | Negative | – | Present | Entecavir | None | None |
| 14 | 4 | 33 | F | 69 | Macroglobulinemia | Rituximab | + | – | – | + | + | Negative | – | Present | Entecavir | None | None‡ |
| 15 | 6 | 2 | M | 60 | Bile duct sarcoma | CDDP + gemcitabine | + | – | – | – | – | Negative | – | Present | Entecavir | None | None |
| 16 | 6 | 102 | M | 65 | Esophageal cancer | Paclitaxel | + | – | † | † | † | † | – | Present | None | Present | None |
| 17 | 210 | 19 | M | 61 | RCC | IL-2 | + | – | – | † | † | † | – | None | None | None | None |
| 18 | 8 | 4 | F | 56 | Breast cancer | FEC | + | † | † | † | † | † | – | Present | None | None | None |
| 19 | 18 | 15 | F | 52 | Colon Cancer | FOLFIRI | + | † | – | † | † | † | – | None | None | None | None |
| 20 | 12 | 85 | F | 51 | Breast cancer | Paclitaxel | + | – | † | † | † | Negative | – | Present | None | None | None |
| 21 | 16 | 7 | M | 49 | Gastric cancer | Paclitaxel | + | † | † | † | † | † | – | Present | None | None | None |
| 22 | 14 | 5 | F | 51 | Brest cancer | Paclitaxel | + | † | † | † | † | † | – | Present | None | None | None |
| 23 | 14 | 69 | F | 74 | Bile duct cancer | Gemcitabine | + | † | † | † | † | † | † | None | None | None | None |
| 24 | 3 | 61 | F | 64 | Lung cancer | Paclitaxel | + | † | † | † | † | † | – | Present | None | None | None |
| 25 | 5 | 66 | F | 59 | Breast cancer | FEC | + | – | – | + | + | Negative | – | Present | None | None | None |
| 26 | 8 | 4 | M | 68 | Gastric cancer | Paclitaxel | + | – | – | + | + | Negative | – | Present | None | None | None |
| 27 | 20 | 11 | F | 36 | Pancreatic NET | Dacarbazine | + | – | † | + | + | 4.4 | – | None | None | None | None |
| 28 | 3 | 4 | M | 55 | Gastric cancer | Paclitaxel | + | – | † | + | † | 3.2 | – | Present | None | None | None |
| 29 | 18 | 52 | M | 58 | Colon cancer | 5-FU + LV | + | – | – | + | † | † | – | None | None | None | None |
| 30 | 14 | 53 | F | 59 | Breast cancer | Paclitaxel | + | † | † | † | † | † | – | Present | None | None | None |
| 31 | 25 | 9 | F | 52 | Breast cancer | Paclitaxel | + | – | – | † | † | † | – | Present | None | None | None |
| 32 | 198 | 53 | F | 44 | Breast cancer | Paclitaxel/herceptin | + | – | – | + | † | 3.9 | – | Present | None | None | None |
| 33 | 70 | 20 | F | 59 | Breast cancer | 5-FU + MTX | + | – | – | + | + | † | – | Present | None | None | None |
| 34 | 11 | 13 | F | 72 | Gastric cancer | Paclitaxel | + | – | † | + | † | † | – | Present | None | None | None |
| 35 | 23 | 48 | F | 46 | Breast cancer | FEC | + | – | † | † | † | † | – | Present | None | Present | Present |
| 36 | 22 | 47 | M | 60 | Reumatoid arthritis | Infliximab | + | – | † | † | † | <2.1 | – | None | None | None | None |
| 37 | 4 | 45 | F | 68 | Breast cancer | FEC | + | † | † | † | † | † | – | Present | None | None | None |
| 38 | 11 | 8 | M | 47 | Bile duct cancer | Gemcitabine | + | + | – | + | † | 7.2 | – | Present | None | None | None |
| 39 | 4 | 39 | F | 58 | Breast cancer | Paclitaxel | + | – | † | + | † | Negative | – | Present | None | None | None |
| 40 | 14 | 16 | M | 70 | Bile duct cancer | Gemcitabine/CDDP | + | – | † | † | † | † | + | Present | None | None | None |
| 41 | 7 | 21 | M | 52 | Lung cancer (NSCLC) | Pemetrexed/CBDCA | + | – | † | † | † | † | – | Present | None | None | None |
| 42 | 2 | 4 | M | 65 | Esophageal cancer | Docetaxel | + | – | – | + | † | Negative | – | Present | None | None | None |
| 43 | 3 | 17 | M | 64 | HCC | 5-FU | + | – | – | + | + | † | + | None | None | None | None |
| 44 | 12 | 15 | F | 64 | Breast cancer | Herceptin | + | † | † | † | † | † | – | Present | None | None | None |
| 45 | B | 8 | F | 71 | Breast cancer | Docetaxel | + | † | † | † | † | † | – | Present | None | None | None |
| 46 | 14 | 12 | F | 69 | Breast cancer | Abraxane | + | † | † | † | † | † | – | Present | None | None | None |

†Untested.
‡Case 14: past history of HBV reactivation.
Corticosteroid use: as chemotherapeutic regimens (including use for anti-emetics).
HBV DNA: before prophylactic antiviral or start at chemotherapy.
5-FU, 5-fluorouracil; CDDP, cisplatin; CBDCA, carboplatin; CHOP, cyclophosphamide/adriamycin/vindesine/predonine; FEC, 5-FU/epirubicin/cyclophosphamide; FOLFIRI, 5-FU/levofolinate/irinotecan; HBc, hepatitis B core; HBeAg, hepatitis B e-antigen; HBs, hepatitis B surface; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IL-2, interleukin-2; LV, levofolinate; MTX, methotrexate; NET, neuroendocrine tumor; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma; VDS, vindesin.

**Table 4** Patients' hepatitis viral marker

| Viral marker status | Patients with HBsAg ($n = 46$) | Patients with anti-HCV ($n = 90$) |
|---|---|---|
| HBsAg | | |
| Positive/negative/untested | 46/0/0 | 2/86/2 |
| (%) | (100/0/0) | (2/96/2) |
| Anti-HBs | | |
| Positive/negative/untested | 1/34/11 | 8/16/66 |
| (%) | (2/74/24) | (9/18/76) |
| Anti-HBc | | |
| Positive/negative/untested | 13/1/32 | 8/11/71 |
| (%) | (28/2/70) | (9/12/79) |
| HBeAg | | |
| Positive/negative/untested | 1/18/27 | 0/23/67* |
| (%) | (2/39/59) | (0/26/74) |
| Anti-HBe | | |
| Positive/negative/untested | 23/1/22 | 4/3/83* |
| (%) | (50/2/48) | (4/3/93) |
| HBV DNA | | |
| <2.1/≤2.1 log copies/mL /untested | 12/13/21 | 1/1/88* |
| (%) | (26/28/46) | (1/1/98) |
| Anti-HCV | | |
| Positive/negative/untested | 2/40/4 | 90/0/0 |
| (%) | (4/87/9) | (100/0/0) |
| HCV RNA | | |
| Positive/negative/untested | 0/0/46 | 21/6/63* |
| (%) | (0/0/100) | (23/7/70) |

*$P < 0.001$. Frequency of antibody testing between patients with HBsAg vs anti-HCV.
HBc, hepatitis B core; HBeAg, hepatitis B e-antigen; HBs, hepatitis B surface; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus.

tested for HBV DNA and two of those tested positive. Seven patients had hepato-biliary-pancreatic cancer, and two of these had already received antiviral drugs before being treated for cancer (cases 10 and 15; Table 3). The other five, however, were not treated with prophylactic antiviral drugs, even though two of these were tested for HBV DNA and both were positive (cases 27 and 38; Table 3).

Seven patients positive for HBsAg had hematologic malignancies, and all were treated with antiviral drugs. Three of them were started on antiviral drugs as prophylaxis against HBV reactivation before treatment, but four patients had already received antiviral drugs before treatment for hematologic malignancies (cases 11–14; Table 3). One patient had a past history of HBV reactivation before this chemotherapy (case 14; Table 3).

## Hepatitis and HBV reactivation (Tables 3 and 5)

There were three patients with HBsAg who had hepatitis during and after chemotherapy (cases 6 [ALT, 188 U/L], 16 [ALT, 205 U/L] and 35 [ALT, 487 U/L]; Table 3 [6.5%]), two of whom (cases 16 and 35 [4.6%]) showed more than fivefold increases in serum ALT of the upper limit of normal. None of them met the diagnostic criteria for acute liver failure in Japan.[9] Two of them (cases 6 and 16) were clinically judged to be caused by drugs or alcohol from history taking and laboratory data, one of whom did not show an increase of serum HBV DNA. Only one breast cancer patient (a 47-year-old woman) without prophylactic antiviral treatment (1/31 [3.2%]), however, developed hepatitis and was clinically diagnosed with hepatitis due to HBV reactivation (case 35; Tables 3 and 5), although the definition of HBV reactivation was not strictly applied because her HBV DNA level was not tested before visiting our outpatient clinic. She underwent surgery for breast cancer, including a sentinel lymph node biopsy, on April 2008, and then received adjuvant chemotherapy for breast cancer on May 2008. Serological examination indicated that she was positive for HBsAg, but negative for HBeAg, and anti-HBs, anti-HBc, anti-HBe and HBV-DNA were not tested before chemotherapy. Her chemotherapeutic regimen comprised FEC (5-fluorouracil, 500 mg/m$^2$; epirubicin, 100 mg/m$^2$; cyclophosphamide,

**Table 5** Viral reactivation

| | Patients with HBsAg ($n = 46$) | Patients with anti-HCV ($n = 90$) |
|---|---|---|
| With prophylactic antiviral | 15 | 0 |
| Without prophylactic antiviral | 31 | 90 |
| Development of hepatitis related to viral reactivation | 1† (without antiviral) | 0 |

†Case 35.
HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus.

500 mg/m$^2$) with administration of corticosteroids. She received six cycles of FEC every 3 weeks on schedule. On day 40 after she finished the last cycle, she was aware of general fatigue and jaundice. On day 46, she was admitted to the hospital with hepatitis B. Blood tests on admission showed: AST, 508 U/L; ALT, 487 U/L; total bilirubin, 8.5 mg/dL; direct bilirubin, 6.7 mg/dL; prothrombin time, 79% (International Normalized Ratio, 1.10), $NH_3$ 122 μg/dL; and HBV DNA, 5.3 log/copies. She received glycyrrhizinic acid by i.v. injection and then entecavir (0.5 mg/day). A liver biopsy was performed on day 11 after admission and pathologically proven viral hepatitis; her Histological Activity Index (HAI) score was 10 (interface hepatitis, 3; intralobular degeneration, 3; portal inflammation, 1; fibrosis, 3). Her liver function gradually improved and she was discharged from the hospital on day 18 after admission. The liver function tests returned to normal within 6 weeks and HBV DNA was negative 8 weeks after admission.

## DISCUSSION

HEPATITIS B VIRUS reactivation is now a well-recognized complication associated with the use of immunosuppressive chemotherapy in HBV carriers. HBV reactivation depends on both the intensity of the immunosuppressive agents and factors related to HBV or a host's immune balance. Therefore, the clinical consequences vary from asymptomatic elevation of hepatic enzymes to severe hepatitis and death from fulminant hepatitis. The prevalence of HBV reactivation ranges widely and is reported to occur in 20–78% of infected patients who undergo systemic chemotherapy for non-hepatic malignancies.[10,11] Initiation of antiviral prophylaxis prior to chemotherapy and its continuation until restitution of normal host immunity is important to prevent hepatitis B reactivation.[12]

Hepatitis B virus reactivation can occur by different mechanisms. First, glucocorticoids directly stimulate HBV gene expression *in vitro*[13] because the HBV genome has a specific glucocorticoid response element.[14] Second, steroid, cytotoxic or immunosuppressive agents induced the breakdown of the host's immune balance, leading to HBV replication and sometimes severe hepatitis.

In fact, HBV reactivation may occur during or after completion of the full course of chemotherapy. Several anticancer immunosuppressive agents have been associated with HBV reactivation. Corticosteroids and anthracyclines are most frequently associated with HBV reactivation.[15–17] Anthracycline has been demonstrated *in vitro* to stimulate HBV DNA secretion from HepG2-derived 2.2.15 cells in a dose-dependent manner.[18] Until recently, most of the cases with HBV reactivation were reported in patients with hematological malignancies, particularly lymphoma. HBV reactivation, however, is increasingly observed in patients with solid tumors, particularly breast cancer. Kim *et al.*[19,20] and Yeo *et al.*[19] reported that patients with HBsAg and breast cancer during adjuvant anthracycline-based chemotherapy developed acute hepatitis related to HBV reactivation (20.7% and 24%, respectively). A previous multivariate analysis indicated that a diagnosis of lymphoma or breast cancer was significantly related to HBV reactivation.[15]

The most important precaution to prevent HBV reactivation is the oncologist's knowledge of HBV reactivation. In Japan, a recommendation for the prevention of HBV reactivation was published in January 2009[8] and revised in 2011. The guideline is intended to identify patients with the possibility of developing HBV reactivation. The guideline recommends that all patients scheduled for chemotherapy or other immunosuppressive therapy be screened for HBsAg and tested further for anti-HBc and anti-HBs, even if negative for HBsAg. The present study demonstrates a consensus for oncologists in our institute to test for HBV or HCV in the serum of patients scheduled for chemotherapy. In fact, around 95% patients were tested for HBsAg or anti-HCV, even before this recommendation, but HBV DNA was only tested in 52% patients positive for HBsAg. This finding suggests that little attention is paid to HBV reactivation.

It is reported that 20% of oncologists in the USA do not check HBV serology, and 30% of oncologists test for HBV serology only when liver tests are abnormal.[21] These findings are consistent with another study of HBV reactivation among oncologists in Canada. Some chemotherapeutic agents such as anthracyclines are well known to induce cardiotoxicity. Lee *et al.*[22] reported that all patients scheduled for cardiotoxic chemotherapy underwent left ventricular function testing (100%), but only 14% of them were tested for HBsAg. Based on these reports, HBV reactivation is not commonly tested for by oncologists throughout the world, even though the percentage of HBV carriers was less in the USA and Canada compared to that in Japan.

In our retrospective study, HBV reactivation was relatively less frequent than in previous reports. The HBV reactivation might be less frequent in outpatient clinic patients than previously speculated. We speculated that

some bias might cause relatively less frequent HBV reactivation in this study due to its nature as a retrospective study. First, as many as 46% of patients with HBsAg were not examined for HBV DNA before treatment and then some patients were not regularly monitored for HBV DNA. Although the Japanese guideline recommended measuring serum HBV DNA monthly for at least 12 months after the discontinuation of chemotherapy,[8] there was a lack of data after the discontinuation of chemotherapy in some cases because of changing hospitals for palliative therapy. These may affect relatively less frequent HBV reactivation. This finding is, however, reasonable considering that oncologists have not been sufficiently aware of HBV reactivation until recently.

In conclusion, none of the patients with HBsAg who were treated with antiviral therapy developed hepatitis. HBV reactivation occurred in HBsAg positive outpatients without prophylactic antiviral treatment, but the incidence was relatively low in selected patients with non-hematological malignancies. Educational intervention is needed to prevent reactivation of HBV, and screening for HBV viral markers should be performed before starting chemotherapy.

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