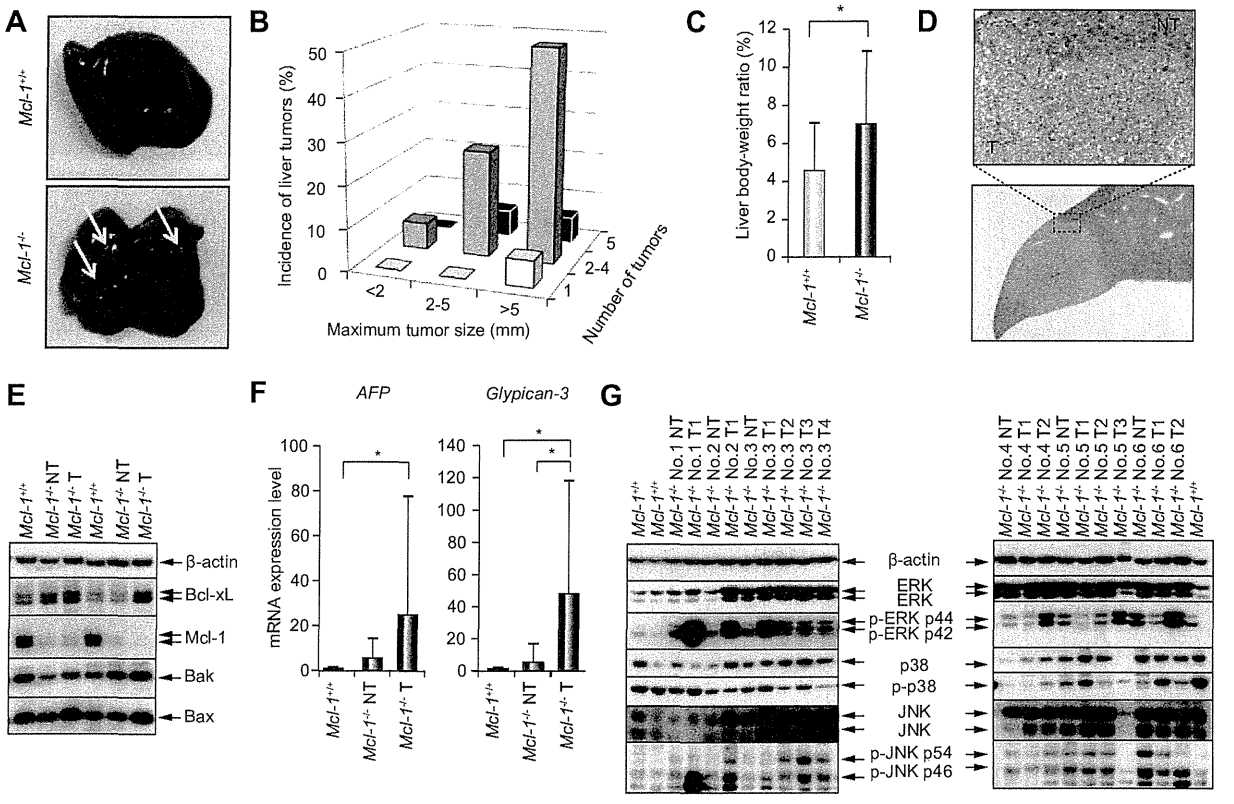

Fig. 2. Liver tumors in *Mcl-1* KO mice. (A–E) Hepatocyte-specific *Mcl-1*-deficient mice (*Mcl-1* $^{-/-}$) (N = 16) and their control littermates (*Mcl-1*$^{+/+}$) (N = 22) were sacrificed at 1.5 years of age. (A) Representative macroscopic view of the livers with arrows indicating tumors. (B) Incidence of liver tumors separated by maximum tumor size and number of tumors. (C) Liver body-weight ratio. (D) Representative histology of liver tumors in *Mcl-1* KO mice. (E) Western blot of the Bcl-2 family proteins in tumors (T) and surrounding non-cancerous livers (NT) of *Mcl-1* KO mice and livers of control mice. (F and G) Characteristics of liver tumors in *Mcl-1* KO mice. (F) Real-time RT-PCR analysis of the expression levels of α-fetoprotein (*AFP*) and *glypican-3* mRNA (N = 16 per group). (G) Expression and activation of mitogen-activated protein kinases. *$p < 0.05$.

KO mice as evidenced by TUNEL staining of liver sections, serum ALT levels and caspase-3/7 activity at 6 weeks of age (Fig. 4A–C). Weber *et al.* [12] previously described hepatocyte regeneration in the *Mcl-1* KO liver. In agreement with this, *Mcl-1* KO livers showed higher expression of cell cycle markers PCNA and ki-67, than those from control littermates (Fig. 4A, B, and D and Supplementary Fig. 4). Importantly, the levels of PCNA and ki-67 expression decreased with a *Bak* KO background in *Mcl-1* KO mice. While *Mcl-1* KO livers show a mild fibrotic change [11], the levels of col1a1 expression at 6 weeks of age and Sirius red staining at 1 year of age decreased with a *Bak* KO background in *Mcl-1* KO livers (Fig. 4E and Supplementary Fig. 5). Bak deficiency also reduced expression levels of TNF-α, MCP-1, and CD68 at 6 weeks of age (Fig. 4F). Next, we examined the impact of apoptosis inhibition by Bak deficiency on oxidative stress markers, which were increased in *Mcl-1* KO livers. Real-time RT-PCR revealed that Bak deficiency reduced the levels of HO-1 and NQO1 expression at 6 weeks of age (Fig. 4G). Consistent with these observations, *Bak* KO significantly lowered the number of 8-OHdG-positive nuclei in *Mcl-1* KO livers at 1 year of age (Fig. 4H). These results suggested that inhibition of hepatocyte apoptosis reduced oxidative stress in the liver. Finally, to examine the impact of apoptosis inhibition on liver tumor development, we compared the carcinogenetic rates in *Mcl-1* KO mice with or without *Bak* KO background at 1 year of age and found that *Bak* KO significantly suppressed liver tumor development (Fig. 5A and B and Table 1).

Discussion

Mcl-1 was first identified as a gene induced during myeloid cell differentiation. Compared with other anti-apoptotic members such as Bcl-2, Bcl-xL, Bcl-w, and Bfl-1, Mcl-1 possesses a unique N-terminus containing two PEST domains, which are found in proteins displaying rapid turnover, and its expression is tightly regulated by growth factors and a variety of other stimuli. Mice systemically deficient for Bcl-xL suffered embryonic death due to massive apoptosis in hematopoietic organs and developing neurons [22]. On the other hand, systemic *Mcl-1* KO resulted in peri-implantation lethality, but *Mcl-1* KO embryos showed no alterations in the extent of apoptosis [23], suggesting that Mcl-1 may play a role early in development that is distinct from its anti-apoptotic functions. Indeed, *in vitro* studies have shown that Mcl-1 interacts with PCNA and Cdk1 in the nucleus and inhibits proliferation [13,14]. Recently, the early responding gene *IEX-1*

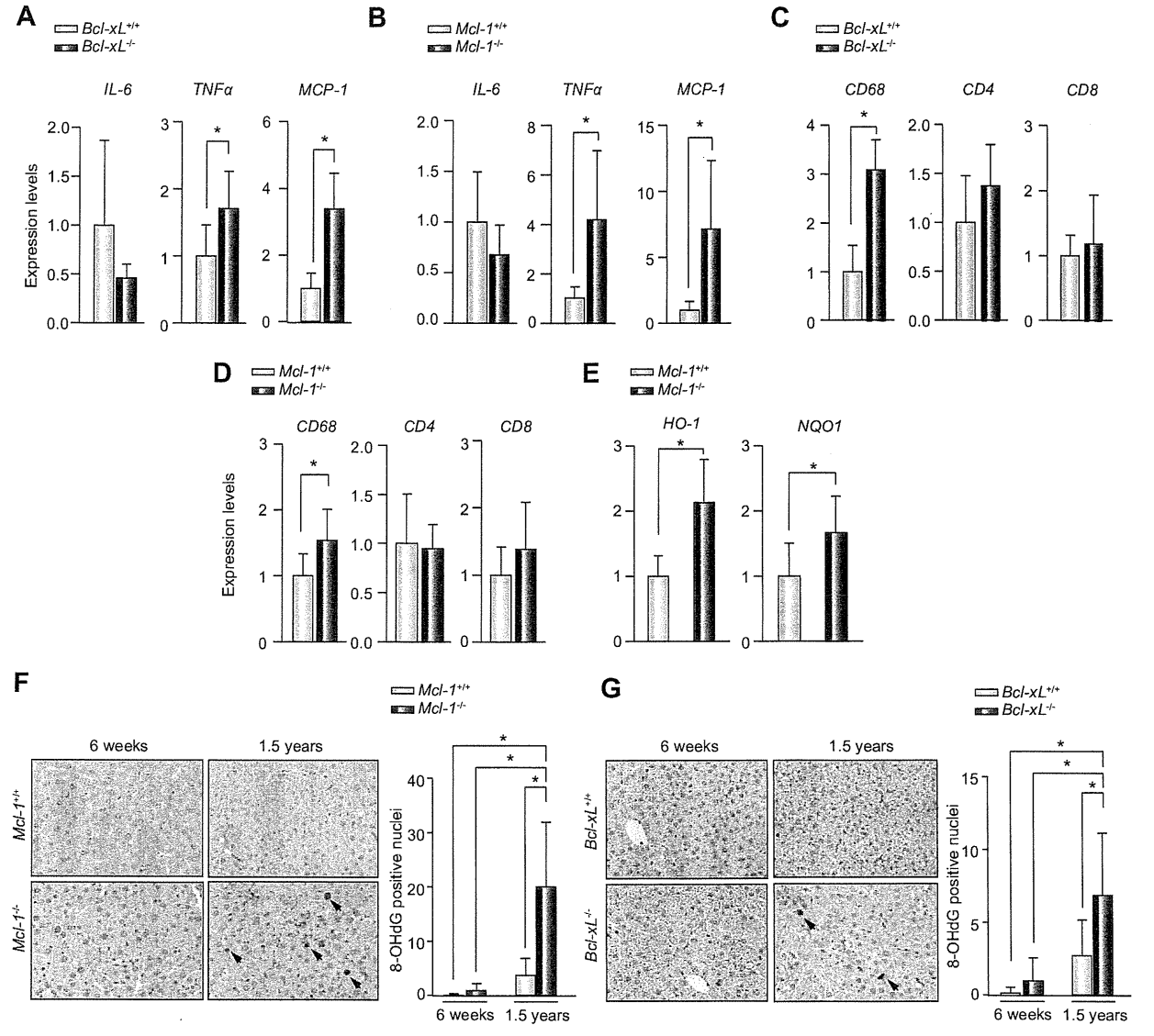

Fig. 3. Inflammatory response and oxidative stress in *Bcl-xL* or *Mcl-1* KO liver. (A–D) Inflammatory responses in KO livers. (A and C) Hepatocyte-specific *Bcl-xL* KO mice (*Bcl-xL* $^{-/-}$) and their control littermates (*Bcl-xL*$^{+/+}$) (N = 6 per group) as well as (B and D) hepatocyte-specific *Mcl-1* KO (*Mcl-1* $^{-/-}$) mice and their control littermates (*Mcl-1*$^{+/+}$) (N = 9 per group) were sacrificed at 6 weeks of age. Expression levels of (A and B) inflammatory molecules and (C and D) cell surface markers of immune cells were analyzed by real-time RT-PCR. (E–G) Oxidative injury in KO livers. (E) Real-time RT-PCR analysis of the expression levels of *HO-1* and *NQO1* of *Mcl-1* KO and control livers at 6 weeks of age (N = 9 per group). (F) Liver sections of *Mcl-1* KO or (G) *Bcl-xL* KO and the control liver at the indicated ages stained with anti-8-OHdG and statistics of the number of positive nuclei (N = 6 and more per group) (G). *p <0.05.

was found to be induced upon DNA damage and to be bound to and to transport Mcl-1 from the cytosol to the nucleus [15]. Mcl-1 was also reported to be induced upon DNA damage and to regulate the DNA damage response through activation of Chk1 [16]. These findings suggest that Mcl-1 possesses additional functions in cell cycle progression and the DNA damage response pathway. This raised concern as to whether the hepatocarcinogenesis observed in *Mcl-1* KO mice was actually related to increased apoptosis in the liver.

In the present study, we demonstrated that hepatocyte-specific destruction of Bcl-xL led to the development of liver cancer similarly to that in hepatocyte-specific *Mcl-1* KO mice. Although we could not completely exclude the possibility that Bcl-xL may have additional effects other than apoptosis, this finding clearly shows that hepatocarcinogenesis observed in the apoptosis-prone liver is not a specific finding of loss of Mcl-1 but is also observed with the knockout of other genes that are critically involved in hepatocyte integrity. Tumors observed in these murine livers frequently showed activation of ERK and JNK, similar to the activation observed in human HCC [18,19]. While 64% of *Mcl-1* KO mice (14/22) developed liver tumors within 1 year, only 27% of *Bcl-xL* KO mice (3/11) did so within 1 year (Table 1). These finding indicate that the incidence rate of carcinogenesis in *Bcl-xL* KO mice is lower than that of *Mcl-1* KO mice. This may be

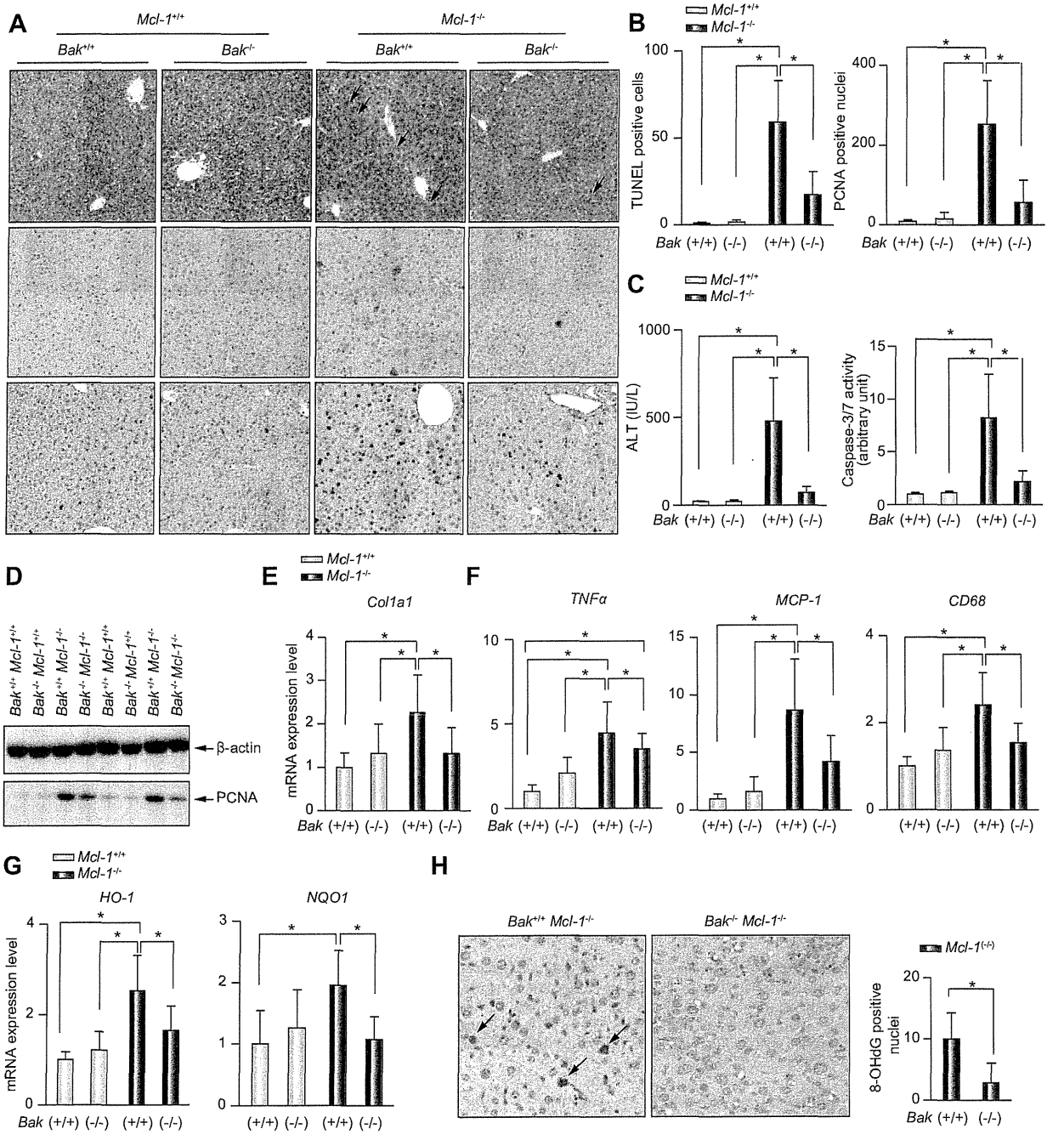

Fig. 4. Impact of Bak deficiency in *Mcl-1* KO mice. (A–G) *Bak*-deficient hepatocyte-specific *Mcl-1* KO mice (*Bak*$^{-/-}$ *Mcl-1*$^{-/-}$) were sacrificed at 6 weeks of age. (A) Representative pictures of hematoxylin–eosin with arrows indicating typical apoptotic cells (upper), TUNEL (middle) and PCNA staining (lower) and (B) statistics of TUNEL and PCNA staining of liver sections (N = 6 or 8 per group). (C) Serum levels of ALT and caspase-3/7 activity (N = 12 per group). (D) Western blot for PCNA expression. Real-time RT-PCR analysis for expression levels of (E) *Col1a1*, (F) *TNF-α*, *MCP-1*, *CD68*, (G) *HO-1* and *NQO1* in the livers at 6 weeks of age (N = 12 per group). (H) Liver sections of the *Bak*-deficient *Mcl-1* KO and control *Mcl-1* KO liver at 1 year of age stained with anti-8-OHdG. Representative images of liver sections stained with anti-8-OHdG (left) and statistics of the number of positive nuclei (N = 9 or 7 per group) (right). *p <0.05.

explained by the difference in levels of hepatocyte apoptosis and serum ALT, which are higher in *Mcl-1* KO mice than in *Bcl-xL* KO mice of the same age [10,11].

Mcl-1 executes its anti-apoptotic function by either directly or indirectly inhibiting the pro-apoptotic functions of Bak and/or Bax [24]. In the present study, we have shown that deletion of the *bak*

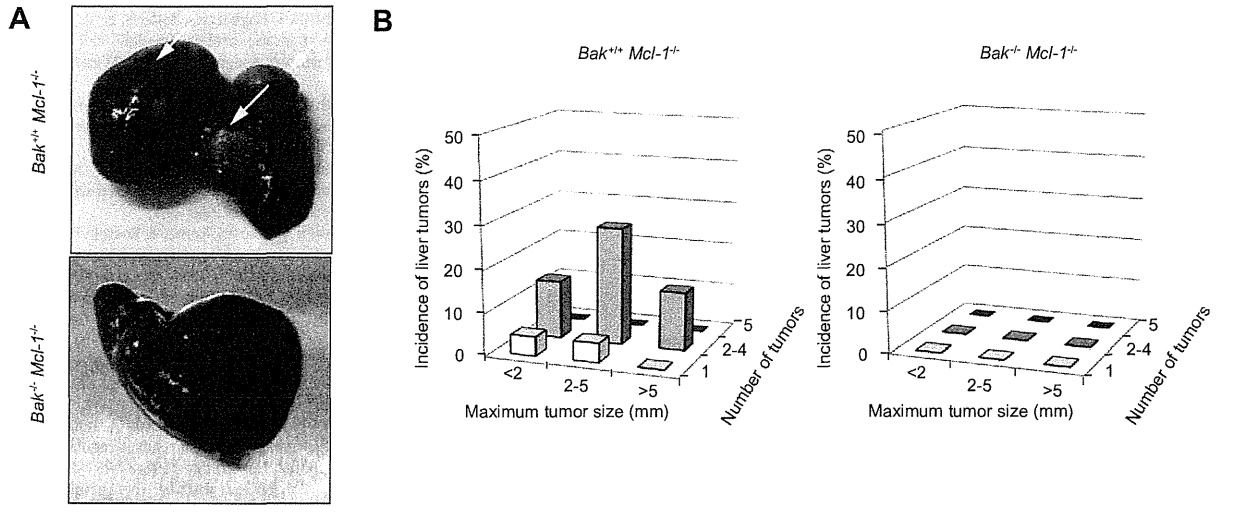

Fig. 5. Liver of aged *Bak/Mcl-1* double KO mice. (A and B) *Bak*-deficient *Mcl-1* KO mice (*Bak* $^{-/-}$ *Mcl-1* $^{-/-}$) (N = 7) and control *Mcl-1* KO mice (*Bak*$^{+/+}$ *Mcl-1* $^{-/-}$) (N = 22) were sacrificed at 1 year of age. (A) Representative macroscopic view of the livers with arrows indicating tumors. (B) Incidence of liver tumors separated by maximum tumor size and number of tumors.

gene resulted in a clear reduction in hepatocyte apoptosis in *Mcl-1* KO mice. Of importance is the finding that *bak* deletion leads to reduction of the liver regenerative response in *Mcl-1* KO mice. Bak is exclusively localized at the mitochondria in hepatocytes [25] and, upon exposure to apoptotic stimuli, undergoes oligomerization to form pores in the outer membrane of mitochondria, releasing cytochrome c, which in turn activates caspases. Since Bak is not involved in the activity of Mcl-1 in the nucleus, our present finding suggests that the regeneration observed in the *Mcl-1* KO liver is not due to loss of the Mcl-1 anti-proliferative effect but mainly to the compensatory regeneration of increased apoptosis. Most importantly, *bak* deletion clearly leads to reduced liver tumor incidence. This finding strongly suggests that the hepatocarcinogenesis observed in *Mcl-1* KO mice can be mostly ascribed to increased apoptosis in hepatocytes.

What does make hepatocytes undergo malignant transformation in the liver with increasing apoptosis? Regeneration is a physiological process in the liver like that in bone marrow or the intestine and compensatory liver regeneration itself is probably not sufficient to induce liver cancer [26]. The present study raised the possibility that TNF-α and oxidative stress are candidate factors responsible for the malignant transformation in the apoptosis-prone liver. TNF-α is reported to be a potent endogenous mutagen that promotes cellular transformation [20], and oxidative stress is reported to cause DNA damage leading to carcinogenesis [21]. Our results revealed that both TNF-α and oxidative stress were significantly increased in KO livers, and importantly, that inhibition of apoptosis by deletion of the *bak* gene reduced the levels of TNF-α and oxidative stress with a decrease in the tumorigenic rate. Some studies have shown that TNF-α induces oxidative stress in hepatocytes [27,28], while oxidative stress promotes production of inflammatory cytokines [29–31]. Taken together, oxidative stress and inflammatory cytokines may positively affect each other to turn healthy hepatocytes into malignant transformed hepatocytes in the liver of KO mice. Further studies are needed to examine the role of oxidative stress and inflammatory cytokines in apoptosis-induced hepatocarcinogenesis.

Apoptosis resistance has been established as a hallmark of cancer [32]. Indeed, accumulating evidence indicates that human HCC frequently overexpresses a variety of molecules which confer apoptosis resistance, such as anti-apoptotic Bcl-2 family proteins, Bcl-xL [33] and Mcl-1 [34,35]. Their overexpression was found to be associated with malignant phenotypes of tumors and poor prognosis of patients [36]. In the present study, tumors that developed in *Bcl-xL* or *Mcl-1* KO mice lacked expression of the respective proteins but reciprocally overexpressed Mcl-1 or Bcl-xL at high rates. We recently reported that conditional expression of Bcl-xL in tumor cells was translated into higher tumor growth in xenograft models [37], indicating that overexpression of anti-apoptotic Bcl-2 family proteins is important for tumor progression. Lack of Bcl-xL or Mcl-1 in hepatocytes generates persistent hepatocyte apoptosis leading to liver tumor development. On the other hand, reciprocal overexpression of Mcl-1 or Bcl-xL in the tumor of *Bcl-xL* or *Mcl-1* KO mice might be required for tumor progression.

Increasing evidence indicates that the serum level of ALT, a marker of hepatocyte apoptosis, is a risk factor for HCC in viral hepatitis [38] and non-alcoholic steatohepatitis [39]. A population-based study also revealed that elevated ALT levels raise the risk of liver cancer [40]. The present study provides evidence that spontaneous apoptosis in hepatocytes leads to liver cancer development and also offers genetic evidence that inhibition of apoptosis can help prevent liver cancer. Administration of caspase inhibitor was previously reported to lower serum ALT levels in patients with chronic hepatitis C [41]. It may be interesting and important, from a clinical point of view, to further determine whether pharmacological inhibition of apoptosis can be useful in preventing liver cancer development in *Bcl-xL* or *Mcl-1* KO mice.

Financial support

This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to T. Tak.) and a Grant-in-Aid for Research

on Hepatitis from the Ministry of Health, Labour, and Welfare of Japan.

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.

Acknowledgements

We sincerely thank Dr. You-Wen He (Department of Immunology, Duke University Medical Center, Durham, NC) for providing the *mcl-1* floxed mice and Dr. Lothar Hennighausen (Laboratory of Genetics and Physiology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Health, Bethesda, MD) for providing the *Bcl-x* floxed mice.
This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to T. Tak.) and Grant-in-Aid for Research on Hepatitis from the Ministry of Health, Labour and Welfare of Japan.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2012.01.027.

References

[1] Malhi H, Gores G. Cellular and molecular mechanisms of liver injury. Gastroenterology 2008;134:1641–1654.
[2] Hiramatsu N, Hayashi N, Katayama K, Mochizuki K, Kawanishi Y, Kasahara A, et al. Immunohistochemical detection of Fas antigen in liver tissue of patients with chronic hepatitis C. Hepatology 1994;19:1354–1359.
[3] Mochizuki K, Hayashi N, Hiramatsu N, Katayama K, Kawanishi Y, Kasahara A, et al. Fas antigen expression in liver tissues of patients with chronic hepatitis B. J Hepatol 1996;24:1–7.
[4] Feldstein A, Canbay A, Angulo P, Taniai M, Burgart L, Lindor K, et al. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. Gastroenterology 2003;125:437–443.
[5] Kronenberger B, Wagner M, Herrmann E, Mihm U, Piiper A, Sarrazin C, et al. Apoptotic cytokeratin 18 neoepitopes in serum of patients with chronic hepatitis C. J Viral Hepat 2005;12:307–314.
[6] Papatheodoridis GV, Hadziyannis E, Tsochatzis E, Chrysanthos N, Georgiou A, Kafiri G, et al. Serum apoptotic caspase activity as a marker of severity in HBeAg-negative chronic hepatitis B virus infection. Gut 2008;57:500–506.
[7] Wieckowska A, Zein NN, Yerian LM, Lopez AR, McCullough AJ, Feldstein AE. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. Hepatology 2006;44:27–33.
[8] Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4:1065–1067.
[9] Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, et al. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. Nature 2004;431:461–466.
[10] Takehara T, Tatsumi T, Suzuki T, Rucker Er, Hennighausen L, Jinushi M, et al. Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. Gastroenterology 2004;127:1189–1197.
[11] Hikita H, Takehara T, Shimizu S, Kodama T, Li W, Miyagi T, et al. Mcl-1 and Bcl-xL cooperatively maintain integrity of hepatocytes in developing and adult murine liver. Hepatology 2009;50:1217–1226.
[12] Weber A, Boger R, Vick B, Urbanik T, Haybaeck J, Zoller S, et al. Hepatocyte-specific deletion of the antiapoptotic protein myeloid cell leukemia-1 triggers proliferation and hepatocarcinogenesis in mice. Hepatology 2010;51:1226–1236.
[13] Fujise K, Zhang D, Liu J, Yeh ET. Regulation of apoptosis and cell cycle progression by MCL1. Differential role of proliferating cell nuclear antigen. J Biol Chem 2000;275:39458–39465.
[14] Jamil S, Sobouti R, Hojabrpour P, Raj M, Kast J, Duronio V. A proteolytic fragment of Mcl-1 exhibits nuclear localization and regulates cell growth by interaction with Cdk1. Biochem J 2005;387:659–667.
[15] Pawlikowska P, Leray I, de Laval B, Guihard S, Kumar R, Rosselli F, et al. ATM-dependent expression of IEX-1 controls nuclear accumulation of Mcl-1 and the DNA damage response. Cell Death Differ 2010;17:1739–1750.
[16] Jamil S, Mojtabavi S, Hojabrpour P, Cheah S, Duronio V. An essential role for MCL-1 in ATR-mediated CHK1 phosphorylation. Mol Biol Cell 2008;19:3212–3220.
[17] Takehara T, Hayashi N, Tatsumi T, Kanto T, Mita E, Sasaki Y, et al. Interleukin 1beta protects mice from Fas-mediated hepatocyte apoptosis and death. Gastroenterology 1999;117:661–668.
[18] Ito Y, Sasaki Y, Horimoto M, Wada S, Tanaka Y, Kasahara A, et al. Activation of mitogen-activated protein kinases/extracellular signal-regulated kinases in human hepatocellular carcinoma. Hepatology 1998;27:951–958.
[19] Chen F, Beezhold K, Castranova V. JNK1, a potential therapeutic target for hepatocellular carcinoma. Biochim Biophys Acta 2009;1796:242–251.
[20] Yan B, Wang H, Rabbani ZN, Zhao Y, Li W, Yuan Y, et al. Tumor necrosis factor-alpha is a potent endogenous mutagen that promotes cellular transformation. Cancer Res 2006;66:11565–11570.
[21] Lonkar P, Dedon PC. Reactive species and DNA damage in chronic inflammation: reconciling chemical mechanisms and biological fates. Int J Cancer 2011;128:1999–2009.
[22] Motoyama N, Wang F, Roth KA, Sawa H, Nakayama K, Negishi I, et al. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. Science 1995;267:1506–1510.
[23] Rinkenberger JL, Horning S, Klocke B, Roth K, Korsmeyer SJ. Mcl-1 deficiency results in peri-implantation embryonic lethality. Genes Dev 2000;14:23–27.
[24] Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, et al. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev 2005;19:1294–1305.
[25] Hikita H, Takehara T, Kodama T, Shimizu S, Hosui A, Miyagi T, et al. BH3-only protein bid participates in the Bcl-2 network in healthy liver cells. Hepatology 2009;50:1972–1980.
[26] Aravalli RN, Steer CJ, Cressman EN. Molecular mechanisms of hepatocellular carcinoma. Hepatology 2008;48:2047–2063.
[27] Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell 2005;120:649–661.
[28] Schwabe RF, Brenner DA. Mechanisms of Liver Injury. I. TNF-alpha-induced liver injury: role of IKK, JNK, and ROS pathways. Am J Physiol Gastrointest Liver Physiol 2006;290:G583–G589.
[29] Bulua AC, Simon A, Maddipati R, Pelletier M, Park H, Kim KY, et al. Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS). J Exp Med 2011;208:519–533.
[30] Nakahira K, Haspel JA, Rathinam VA, Lee SJ, Dolinay T, Lam HC, et al. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat Immunol 2011;12:222–230.
[31] Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in NLRP3 inflammasome activation. Nature 2011;469:221–225.
[32] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–674.
[33] Takehara T, Liu X, Fujimoto J, Friedman S, Takahashi H. Expression and role of Bcl-xL in human hepatocellular carcinomas. Hepatology 2001;34:55–61.
[34] Fleischer B, Schulze-Bergkamen H, Schuchmann M, Weber A, Biesterfeld S, Müller M, et al. Mcl-1 is an anti-apoptotic factor for human hepatocellular carcinoma. Int J Oncol 2006;28:25–32.
[35] Sieghart W, Losert D, Strommer S, Cejka D, Schmid K, Rasoul-Rockenschaub S, et al. Mcl-1 overexpression in hepatocellular carcinoma: a potential target for antisense therapy. J Hepatol 2006;44:151–157.
[36] Watanabe J, Kushihata F, Honda K, Sugita A, Tateishi N, Mominoki K, et al. Prognostic significance of Bcl-xL in human hepatocellular carcinoma. Surgery 2004;135:604–612.
[37] Hikita H, Takehara T, Shimizu S, Kodama T, Shigekawa M, Iwase K, et al. The Bcl-xL inhibitor, ABT-737, efficiently induces apoptosis and suppresses

growth of hepatoma cells in combination with sorafenib. Hepatology 2010;52:1310–1321.
[38] Chen CF, Lee WC, Yang HI, Chang HC, Jen CL, Iloeje UH, et al. Changes in serum levels of HBV DNA and alanine aminotransferase determine risk for hepatocellular carcinoma. Gastroenterology 2011;141:1240–1248.
[39] Bhala N, Angulo P, van der Poorten D, Lee E, Hui JM, Saracco G, et al. The natural history of nonalcoholic fatty liver disease with advanced fibrosis or cirrhosis: an international collaborative study. Hepatology 2011;54:1208–1216.
[40] Ruhl CE, Everhart JE. Elevated serum alanine aminotransferase and gamma-glutamyltransferase and mortality in the United States population. Gastroenterology 2009;136:477–485, e411.
[41] Pockros P, Schiff E, Shiffman M, McHutchison J, Gish R, Afdhal N, et al. Oral IDN-6556, an antiapoptotic caspase inhibitor, may lower aminotransferase activity in patients with chronic hepatitis C. Hepatology 2007;46:324–329.

IJC
International Journal of Cancer

Inhibition of autophagy potentiates the antitumor effect of the multikinase inhibitor sorafenib in hepatocellular carcinoma

Satoshi Shimizu[1]*, Tetsuo Takehara[1]*, Hayato Hikita[1], Takahiro Kodama[1], Hinako Tsunematsu[1], Takuya Miyagi[1], Atsushi Hosui[1], Hisashi Ishida[1], Tomohide Tatsumi[1], Tatsuya Kanto[1], Naoki Hiramatsu[1], Naonobu Fujita[2], Tamotsu Yoshimori[2] and Norio Hayashi[3]

[1] Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan
[2] Department of Genetics, Osaka University Graduate School of Medicine, Suita, Osaka, Japan
[3] Kansai-Rosai Hospital, Amagasaki, Hyogo, Japan

Multikinase inhibitor sorafenib inhibits proliferation and angiogenesis of tumors by suppressing the Raf/MEK/ERK signaling pathway and VEGF receptor tyrosine kinase. It significantly prolongs median survival of patients with advanced hepatocellular carcinoma (HCC) but the response is disease-stabilizing and cytostatic rather than one of tumor regression. To examine the mechanisms underlying the relative resistance in HCC, we investigated the role of autophagy, an evolutionarily conserved self-digestion pathway, in hepatoma cells *in vitro* and *in vivo*. Sorafenib treatment led to accumulation of autophagosomes as evidenced by conversion from LC3-I to LC3-II observed by immunoblot in Huh7, HLF and PLC/PRF/5 cells. This induction was due to activation of autophagic flux, as there was further increase in LC3-II expression upon treatment with lysosomal inhibitors, clear decline of the autophagy substrate p62, and an mRFP-GFP-LC3 fluorescence change in sorafenib-treated hepatoma cells. Sorafenib inhibited the mammalian target of rapamycin complex 1 and its inhibition led to accumulation of LC3-II. Pharmacological inhibition of autophagic flux by chloroquine increased apoptosis and decreased cell viability in hepatoma cells. siRNA-mediated knockdown of the ATG7 gene also sensitized hepatoma cells to sorafenib. Finally, sorafenib induced autophagy in Huh7 xenograft tumors in nude mice and coadministration with chloroquine significantly suppressed tumor growth compared with sorafenib alone. In conclusion, sorafenib administration induced autophagosome formation and enhanced autophagic activity, which conferred a survival advantage to hepatoma cells. Concomitant inhibition of autophagy may be an attractive strategy for unlocking the antitumor potential of sorafenib in HCC.

Cancer Cell Biology

Sorafenib is an orally available multikinase inhibitor recently approved as the first molecular targeting compound for hepatocellular carcinoma (HCC).[1] Sorafenib inhibits Raf kinases, including Raf-1 and B-Raf, which are members of the Raf/MEK/ERK signaling pathway, and inhibits a number of receptor tyrosine kinases involved in neo-angiogenesis and tumor progression, such as vascular endothelial growth factor receptor (VEGFR) 2, platelet-derived growth factor receptor β and c-Kit. Two randomized, placebo-controlled trials revealed that sorafenib significantly prolongs the median survival of patients with advanced HCC but the response is disease-stabilizing and cytostatic rather than one of tumor regression.[2,3] Therefore, a more detailed understanding of the mechanisms underlying both the antitumor effect and the primary resistance to this compound may provide insights that can help to improve the therapeutic outcome in HCC.

Macroautophagy (hereafter referred to as autophagy) is an evolutionally conserved catabolic process that transports cellular macromolecules and organelles to a lysosomal degradation pathway.[4] It is regulated by autophagy-related (*atg*) genes that control the formation and maturation of a double-membrane vesicle, autophagosome, which sequestrates cellular proteins and organelles. Autophagosomes then fuse with lysosomes to form autolysosomes, in which lysosomal enzymes digest the sequestered content and inner membrane. Autophagy is typically induced under starvation, initially considered to be a survival strategy that recycles cellular components to meet energy requirements. Autophagy also occurs at low basal levels in virtually all cells to perform homeostatic functions such as turnover of long-lived or damaged proteins and organelles. On the other hand, autophagy can mediate cell death under certain conditions probably through over-activation of self-digestion, which is considered to be Type II programmed cell death.[5] Therefore, autophagy can promote both cell survival and death depending on the cellular context and/or initiating stimulus.

Key words: liver, HCC, mTOR, tumor, apoptosis
Grant sponsors: Ministry of Education, Culture, Sports, Science and Technology, Japan; Ministry of Health, Labor and Welfare of Japan
*S.S. and T.T. contributed equally to this work and share first authorship.
DOI: 10.1002/ijc.26374
History: Received 4 Mar 2011; Accepted 3 Aug 2011; Online 19 Aug 2011
Correspondence to: Tetsuo Takehara, Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan, Tel.: +81-6-6879-3621, Fax: +81-6-6879-3629, E-mail: takehara@gh.med.osaka-u.ac.jp

Autophagy has been shown to be involved in cancer development and progression in a variety of ways.[6] Genetic evidence supports a tumor suppressive role of autophagy in cancer development. The *Beclin 1* autophagy gene is monoallelically deleted in a subset of human sporadic breast, ovarian and prostate cancer. Heterozygous disruption of *Beclin 1* increases the frequency of spontaneous malignancies in mice.[7] On the other hand, tumor cells display autophagy or autophagic cell death under a variety of stress-inducing conditions as well as anticancer therapies.[8] Therefore, autophagy promotes or inhibits tumor progression which is also dependent on the cell types and stimuli. Recently, sorafenib has been reported to induce autophagosome accumulation, as evidenced by GFP-LC3 markers, in tumor cells.[9–11] However, its biological and clinical significance has not yet been addressed. In the present study, we examined autophagy of hepatoma cells treated with sorafenib and demonstrate that sorafenib not only induces autophagosome formation but also activates autophagic flux which is an adaptive response to this compound, and that concomitant inhibition of autophagy may be therapeutically useful for improving the anti-HCC effect.

Material and Methods

Cell lines

Hepatoma cell lines Huh7, HLF and PLC/PRF/5 were cultured with Dulbecco's modified Eagle medium (DMEM). Huh7 and HLF were obtained from the JCRB/HSRRB cell bank (Osaka, Japan) and PLC/PRF/5 was obtained from ATCC (Manassas, VA). All cell lines were cultured at 37°C in a humidified atmosphere of 5% CO_2.

Western immunoblot

Cells or tissues were lysed and immunoblotted as previously described.[12] For immunodetection, the following antibodies were used: anti-microtubule-associated protein 1 light chain (LC3) polyclonal antibody (Ab) (MBL, Nagoya, Japan); anti-ATG7 polyclonal Ab (MBL); anti-Beclin1 polyclonal Ab (CST, Danvers, MA); anti-p62 polyclonal Ab (MBL); anti-phospho-ERK polyclonal Ab (CST); anti-phospho-S6K polyclonal Ab (CST); anti-phospho-4E-BP1 polyclonal Ab (CST); anti-phospho-Akt polyclonal Ab (CST).

Transfection with fluorescent LC3 plasmid

Cells were transfected with monomeric red fluorescence protein (mRFP)-GFP tandem fluorescent-tagged LC3 expression plasmid (ptfLC3)[13] using Fugene6 (Roche Applied Science, Hague Road, IN) according to the manufacturer's instructions. At 48 hr after transfection, the medium was changed to DMEM containing sorafenib or DMSO, and the cells were further cultured and examined under a BZ8100 fluorescent microscope (Keyence, Osaka, Japan).

In vitro treatment with sorafenib

Hepatoma cells were transfected with 5 nM Silencer Select siRNAs (Ambion, Austin, TX) either of *ATG7* or negative control using RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Forty-eight hours after transfection, the medium was changed to DMEM containing sorafenib or DMSO. Cells were further cultured and assayed for cell viability by WST assay using the cell count reagent SF (Nacalai Tesque, Kyoto, Japan) and analyzed for apoptosis using Annexin V-FITC apoptosis detection kit (Biovision, Mountain View, CA). We defined apoptotic cells as Annexin V-FITC positive and propidium iodide (PI) negative cells. PI negative cells were gated and the positive cell rate of Annexin V-FITC was determined. The supernatant of the cultured cells was assayed for caspase-3/7 activity using Caspase-Glo 3/7 assay (Promega, Madison, WI) as previously reported.[12] For the treatment with a pharmacological inhibitor of autophagy, cells were cultured with DMEM containing chloroquine (Sigma-Aldrich, St. Louis, MO) or bafilomycin A1 (Sigma-Aldrich) with sorafenib or DMSO and assayed for cell viability and caspase-3/7 activity in the same manner.

Electron microscopy

Samples were fixed with 2.5% glutaraldehyde solution buffered at pH 7.4 with 0.1 M Millonig's phosphate at 4°C for 2 hr, postfixed in 1% osmium tetroxide solution at 4°C for 1 hr, dehydrated in graded concentrations of ethanol and embedded in Nissin EM Quetol 812 epoxy resin. Ultrathin sections (80 nm) cut on a Reichert ultramicrotome (Ultracut E) were stained with uranyl acetate and lead citrate, and examined with a Hitachi H-7650 electron microscope at 80 kV.

Xenograft experiments

To produce a xenograft tumor, 3–5 $\times$ 10^6 Huh7 cells were subcutaneously injected to Balb/c nude mice. Sorafenib tablets were crushed and orally administered daily with water containing 12.5% cremophor EL (Sigma-Aldrich) and 12.5% ethanol, as previously described.[14] Chloroquine was dissolved in PBS and intraperitoneally administered daily. We estimated the volume of the xenograft tumor using the following formula: tumor volume = $\pi/6 \times$ (major axis) $\times$ (minor axis)^2. Mice 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.

Statistical analysis

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

Results

In vitro treatment with sorafenib induces accumulation of autophagosomes in hepatoma cell lines

To examine the effect of sorafenib on autophagy in human HCC, we treated the hepatoma cell line Huh7 with sorafenib *in vitro*. First, we assessed the expression of LC3, a

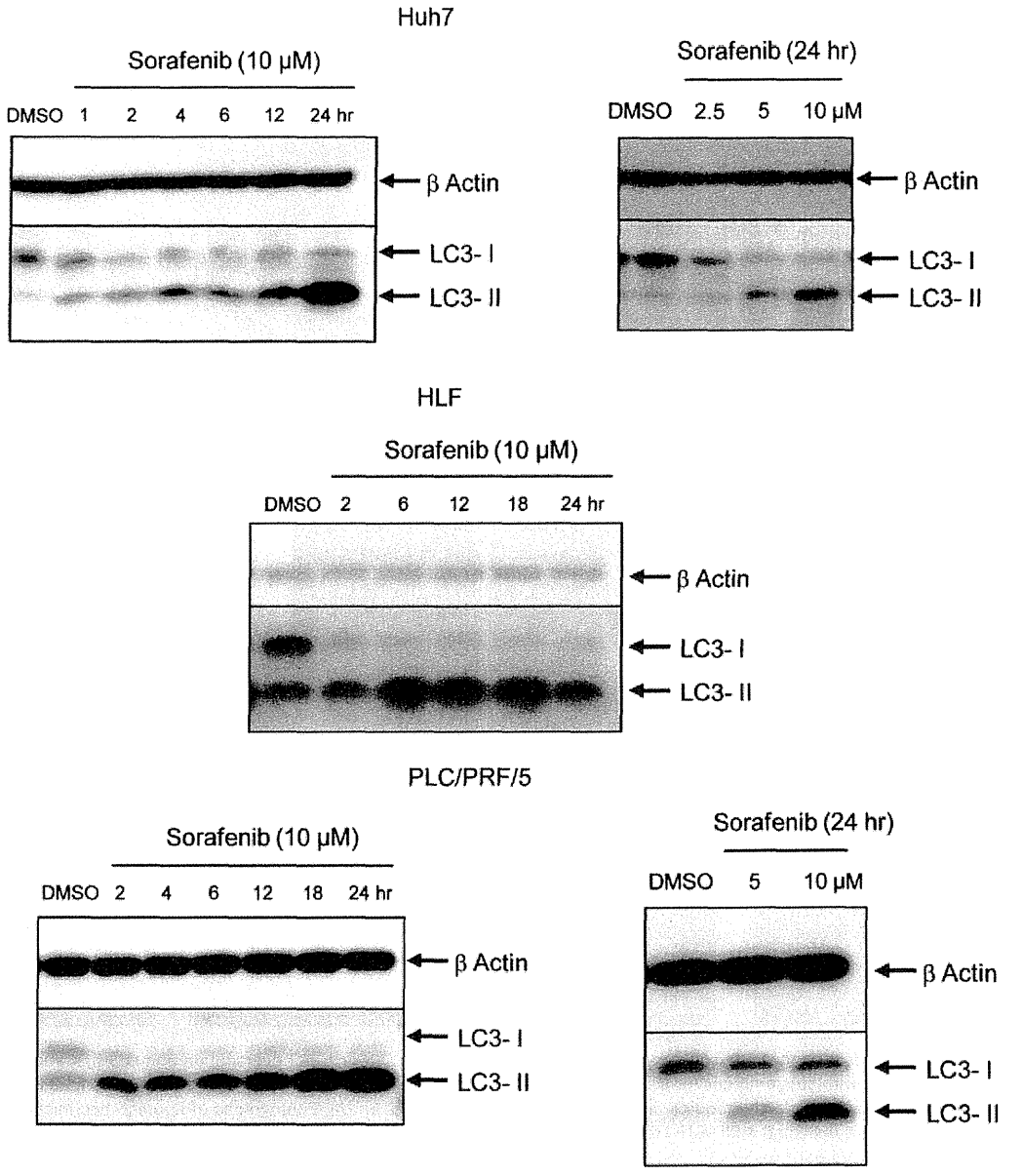

Figure 1. Sorafenib induces accumulation of autophagosomes in hepatoma cells. Western blot showing an increase in LC3-II in Huh7, HLF and PLC/PRF/5 hepatoma cells after treatment with sorafenib. Hepatoma cells were treated with 2.5, 5 or 10 μM sorafenib for the indicated times and analyzed for LC3 expression by western blot. Hepatoma cells treated with DMSO-containing media for 24 hr are shown as the control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mammalian homolog of yeast *atg8*, by immunoblot. During the progress of autophagy, the cytoplasmic form LC3-I is converted to the membrane-bound lipidated form LC3-II which is detected by a mobility shift on electrophoresis.[15] When Huh7 cells were treated with 10 μM sorafenib, LC3 conversion was observed as early as 1 hr after the treatment and gradually increased at later time points (Fig. 1). We examined the dose-dependency of this response in Huh7 cells as well. Under 2.5 μM sorafenib treatment, the amount of LC3-II did not show an obvious increase, however, the amount of LC3-I decreased which indicates modest activation of autophagosome formation. Under 5 and 10 μM sorafenib treatment, the amount of LC3-II clearly increased. Next, we investigated the effect of sorafenib on other hepatoma cell lines, HLF and PLC/PRF/5. Under sorafenib treatment, LC3 conversion was observed at 2 hr after the initiation of treatment and gradually increased until 24 hr in HLF cells and PLC/PRF/5 cells in the same manner as in Huh7 cells.

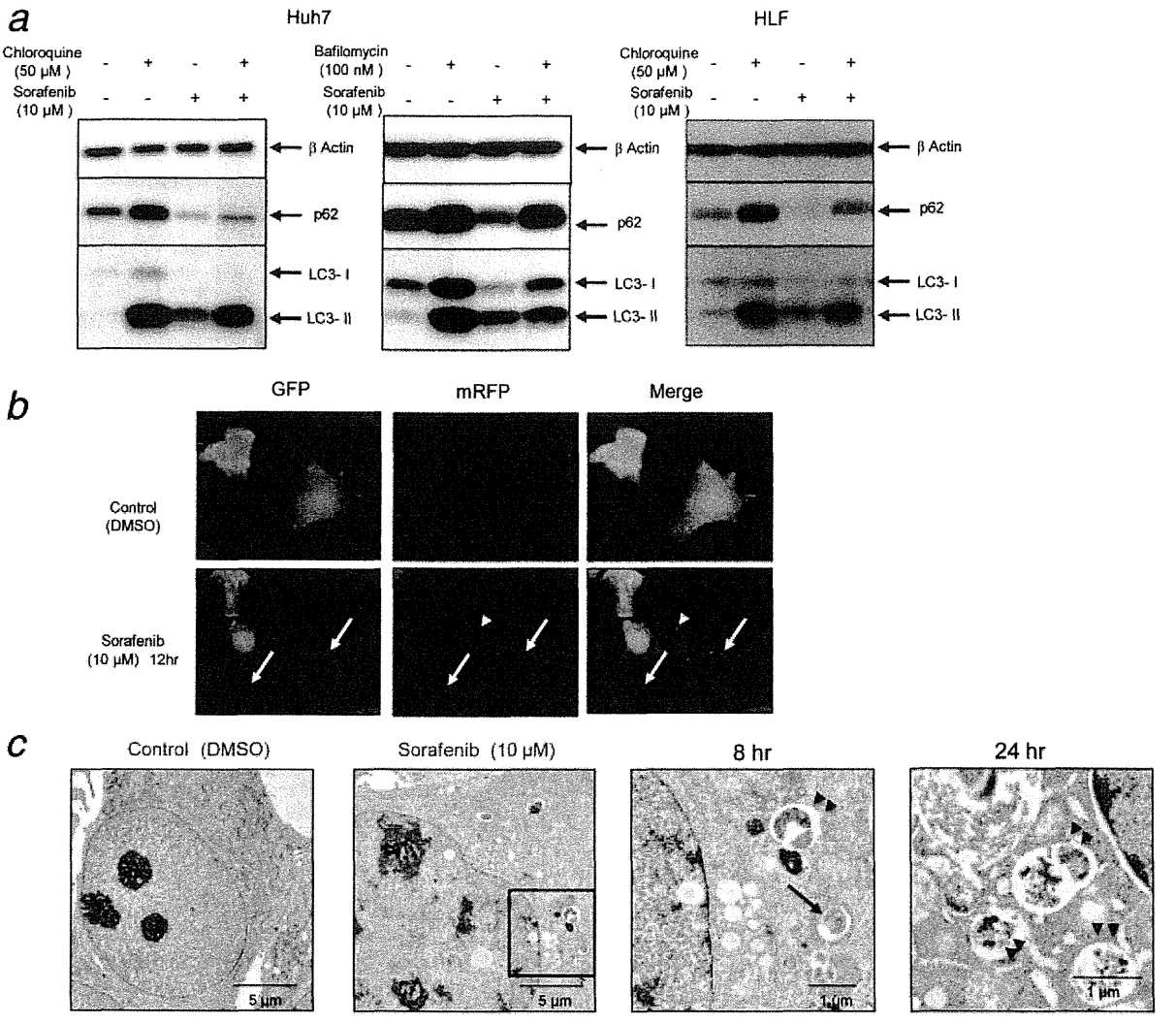

Figure 2. Sorafenib activates autophagic flux in hepatoma cells. (*a*). Western blot showing p62 degradation and LC3 lipidation in Huh7 cells and HLF cells treated with sorafenib and/or lysosomal inhibitors. Huh7 cells or HLF cells were treated with or without 10 μM sorafenib in the presence or absence of 50 μM chloroquine or 100 nM bafilomycin A1 for 12 hr. (*b*). Photographs of fluorescence microscopy of punctate fluorescence of a transfected mRFP-GFP-LC3 construct in Huh7 cells after 12-hr treatment with 10 μM sorafenib. Arrows indicate a typical example of colocalized particles of GFP and mRFP signal, while the arrowhead points to a typical example of a particle with an mRFP signal but without a GFP signal. *C*. Photographs from transmission electron microscopy showing autophagic vacuoles including autophagosomes (arrow) and probably autolysosomes (arrowhead) in Huh7 cells treated with 10 μM sorafenib.

Sorafenib activates autophagic flux in hepatoma cells

To clarify whether the accumulation of autophagosomes induced by sorafenib is a result of induction of autophagosome formation or inhibition of autophagosome degradation, we first measured the amount of p62, a selective substrate of autophagy, by immunoblot. Activation of the autophagic flux leads to a decline in p62 expression, and *vice versa*.[16] When Huh7 cells or HLF cells were treated with sorafenib, the amount of p62 decreased despite the accumulation of LC3-II implying that this accumulation of LC3-II is associated with autophagosome degradation (Fig. 2*a*). In addition, when cells were treated with both sorafenib and chloroquine, accumulation of LC3-II was further enhanced compared to the sorafenib-treated group, while the levels of p62 expression increased. We also used bafilomycin A1, which inhibits fusion of autophagosome and lysosome, and obtained similar results. Our findings indicate that the LC3-II accumulation induced by sorafenib results from activation of autophagosome formation but not from just inhibition of the autophagosome degradation steps. Second, we examined the color

change of mRFP-GFP tandem fluorescent-tagged LC3 (mRFP-GFP-LC3). When Huh7 cells were transfected with the mRFP-GFP-LC3 expression plasmid ptfLC3 and then treated with sorafenib, some punctate signals showed both GFP and mRFP signals but part of the punctate signals exhibited only mRFP signals (Fig. 2*b*). Because GFP fluorescence but not mRFP fluorescence is attenuated under lysosomal acidic condition,[13] this observation supports that autophagy induced by sorafenib proceeds to the lysosomal degradation phase. Finally, electron microscopy revealed abundant autophagic vacuoles such as autophagosomes and probably autolysosomes in sorafenib-treated Huh7 cells, but scarcely in control cells (Fig. 2*c*).

Sorafenib selectively inhibits the activity of TORC1 in hepatoma cells

Sorafenib was initially developed as a Raf kinase inhibitor, however, it can also inhibit other tyrosine kinases such as VEGR-2, Flt-3 and c-Kit.[17] The inhibitory effect of sorafenib on the Raf/MEK/ERK pathway[18] or the STAT3 pathway[19] is widely recognized in several types of cancer, but the effect of sorafenib on the PI3K/Akt pathway and the mTOR pathway has not been established yet. Because the mTOR pathway is known as a major regulatory pathway of autophagy,[20] we next examined the activity of the mTOR signaling pathway in Huh7 cells and HLF cells. Sorafenib clearly inhibited the activity of the mammalian target of rapamycin complex 1 (mTORC1), which is measured by the dephosphorylation of S6K and 4E-BP1 in Huh7 cells and HLF cells (Fig. 3*a*). 4E-BP1 is initially phosphorylated at threonine 37 and threonine 46, which promotes subsequent phosphorylation and decreases electrophoretic mobility.[21] With sorafenib administration, the upper band of phosphorylated 4E-BP1 gradually decreased and shifted to the lower band. At 24 hours after treatment initiation, the lower band diminished as well, indicating further dephosphorylation of 4E-BP1 at threonine 37 and 46. On the other hand, sorafenib treatment increased the phosphorylation of Akt at threonine 308 and serine 473 in these cells. The phosphorylation at threonine 308 suggests the activation of upstream PI3K while the phosphorylation at serine 473 suggests the activation of mTORC2.[22] Therefore, sorafenib can be presumed to possess a selective inhibitory effect on the activity of mTORC1 independent of PI3K and Akt. Administration of sorafenib clearly inhibited the phosphorylation of ERK as early as 2 hours after treatment, which is consistent with a previous report.[18] The expression of ATG7 and Beclin 1, autophagy-related gene products, did not change under sorafenib treatment. Next, we treated Huh7 cells with rapamycin or Torin1[23] to determine the impact of mTORC1 activity on autophagy induction. As expected, the levels of LC3-II increased upon rapamycin treatment in Huh7 cells (Fig. 3*b*). A similar result was obtained using another mTOR inhibitor, Torin1.

Inhibition of autophagy by siRNAs or a pharmacological inhibitor enhanced the apoptotic effect of sorafenib *in vitro*

From these results, we considered two possibilities: sorafenib-induced autophagy may be a mechanism of action of the antitumor effect of sorafenib or a stress-responsive phenomenon leading to survival of tumor cells in the presence of sorafenib treatment. To investigate the role of autophagy under sorafenib treatment, we introduced into Huh7 cells, the siRNA specific for *ATG7*. Administration of *ATG7* siRNA suppressed LC3-II expression in DMSO-treated cells and sorafenib-treated cells, indicating that autophagy is clearly suppressed under physiological conditions as well as with sorafenib treatment (Fig. 4*a*). Sorafenib treatment induced apoptosis, as determined by the elevation of caspase-3/7 activity or by the increase of Annexin V positive cells, and decreased the viability of Huh7 cells (Fig. 4*b*). Of importance is the finding that *ATG7* knockdown significantly enhanced the sorafenib-induced apoptosis and decreased cell viability in Huh7 cells. These observations imply that autophagy plays a protective role for hepatoma cells under sorafenib treatment and could be a target for enhancing its antitumor effects. We performed an ATG7 knockdown experiment using HLF cells as well and obtained a similar result (Fig. 4*c*).

Next, we treated Huh7 cells with sorafenib in combination with the pharmacological autophagy inhibitor chloroquine, which clearly blocks the downstream autophagic pathway in hepatoma cells as shown in Figure 2*a*. Chloroquine itself induced a modest activation of caspase-3/7 at a high dose under our experimental conditions (Fig. 5). However, in combination with sorafenib, chloroquine markedly enhanced the apoptotic effect of sorafenib and reduced cell viability in a dose-dependent manner. We investigated the effect of chloroquine on PLC/PRF/5 cells as well, and obtained a similar result.

Autophagy inhibitor chloroquine enhanced the anti-tumor effect of sorafenib in a xenograft model

To examine the significance of autophagy *in vivo*, nude mice were subcutaneously injected with Huh7 cells to generate xenograft tumors. To examine whether sorafenib induces autophagy in the *in vivo* setting, we administered sorafenib or vehicle for 7 days to mice bearing xenograft tumors. As we reported previously,[14] sorafenib treatment significantly suppressed tumor growth compared with the vehicle alone (data not shown). Consistent with the *in vitro* finding, xenograft tumors from sorafenib-administered mice displayed accumulation of LC3-II on immunoblot compared with those from vehicle-treated mice (Fig. 6*a*). To examine the therapeutic significance of autophagy inhibition for sorafenib therapy, mice with Huh7 xenograft were randomly assigned to two groups when the diameter of the subcutaneous tumor reached about 1 centimeter: sorafenib administration group and sorafenib plus chloroquine administration group. Coadministration of chloroquine and sorafenib for 7 days led to significant suppression of tumor growth compared with

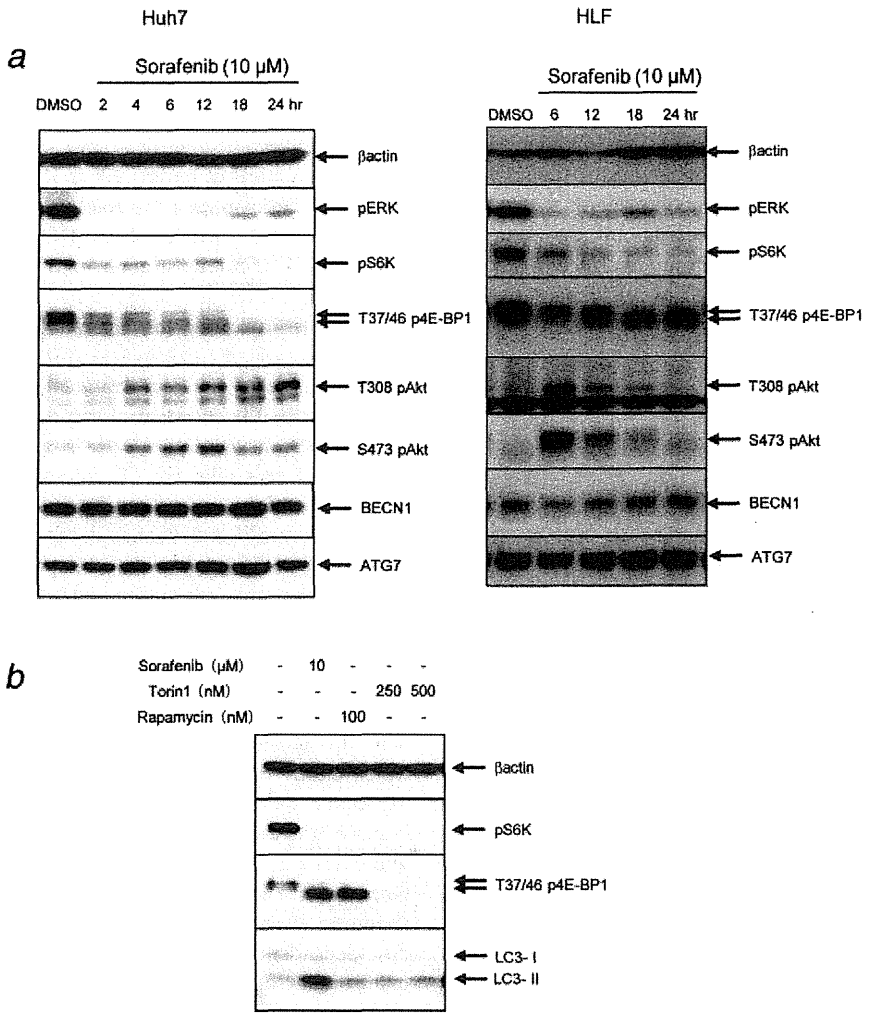

Figure 3. Raf/MEK/ERK and Akt/mTOR/S6K pathways in hepatoma cells treated with sorafenib. (*a*). Western blot showing decrease in ERK, S6K and 4E-BP1 phosphorylation, increase in Akt phosphorylation and stable expression of Beclin 1 and ATG7 in Huh7 cells and HLF cells after treatment with 10 μM sorafenib. (*b*). Western blot showing that rapamycin or Torin1 dephosphorylates both S6K and 4E-BP1 and increases the expression of LC3-II in Huh7 cells. Huh7 cells were treated with 100 nM rapamycin or the indicated concentration of Torin1 for 12 hr. Huh7 treated with sorafenib (10 μM, 12 hr) serves as a positive control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

administration of sorafenib alone (Fig. 6*b*). Administration of chloroquine alone did not affect the growth of the tumor. We performed TUNEL staining and immunohistological staining of cleaved caspase-3 of the xenograft tumor to examine the contribution of apoptosis in this xenograft model. However, nonspecific staining of the xenograft tumors treated with sorafenib interfered with an accurate evaluation of the apoptotic change (data not shown).

Discussion

Accumulating evidence indicates that cancer therapies such as irradiation and administration of cytotoxic drugs and chemicals induce autophagy and autophagic cell death in a variety of tumor cells.[8] Research has shown that autophagy induced by these treatments sometimes protects tumor cells (autophagic resistance) but promotes cell death in other settings (autophagic Type II programmed cell death). For example, temozolomide, a DNA alkylating agent,[24] and ionizing radiation[25] induce autophagy in malignant glioma cells and a variety of epithelial tumors, respectively, and this inhibition enhances antitumor effects. On the other hand, poly(dI:dC) induces endosome-mediated autophagy leading to cell death in melanoma cells.[26] Arsenic trioxide induces autophagic cell death in leukemia cells.[27] In the present study, we demonstrated that sorafenib, a recently approved molecular targeting drug for HCC, induced autophagy which appeared to

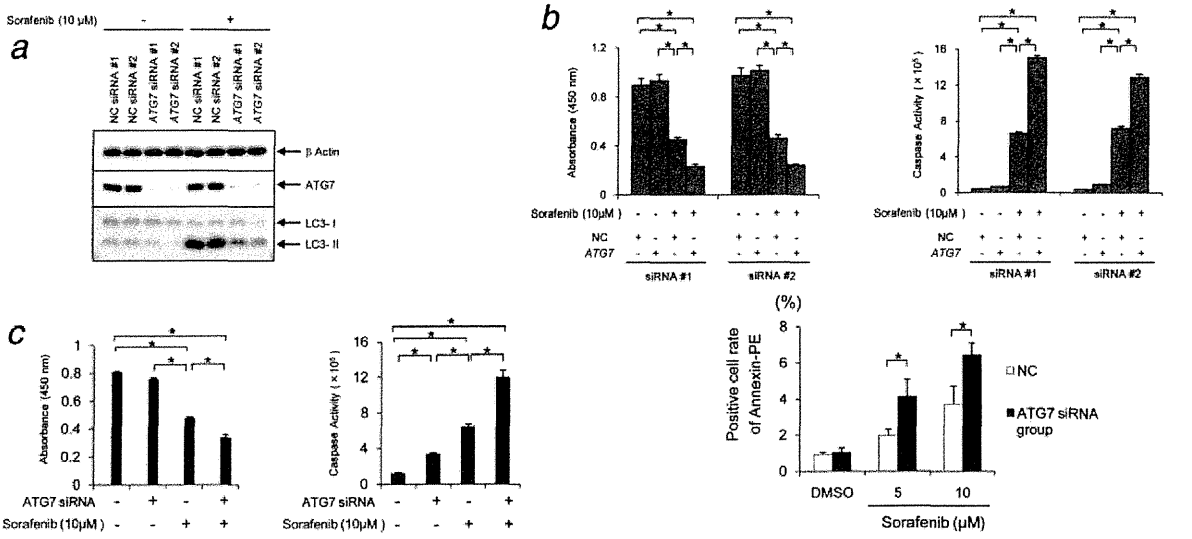

Figure 4. Genetic ablation of autophagy increases sensitivity of hepatoma cells to sorafenib. (*a*,*b*). Huh7 cells were transfected with two different sets of *ATG7* siRNA (no. 1 and 2) or control siRNA (no. 1 and 2) for 48 hr and then treated with the indicated concentration of sorafenib or vehicle for an additional 18 hr. LC3 lipidation and ATG7 expression were determined by western blot (*a*). Cell growth was determined by WST assay, while apoptosis was monitored by the activity of caspase-3/7 in the supernatant or by annexin V positive cell rate ($n = 4$) (*b*). (*c*) HLF cells were transfected with *ATG7* siRNA and examined for cell viability and caspase-3/7 activity in the same manner as Huh7 cells ($n = 4$). *$p < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

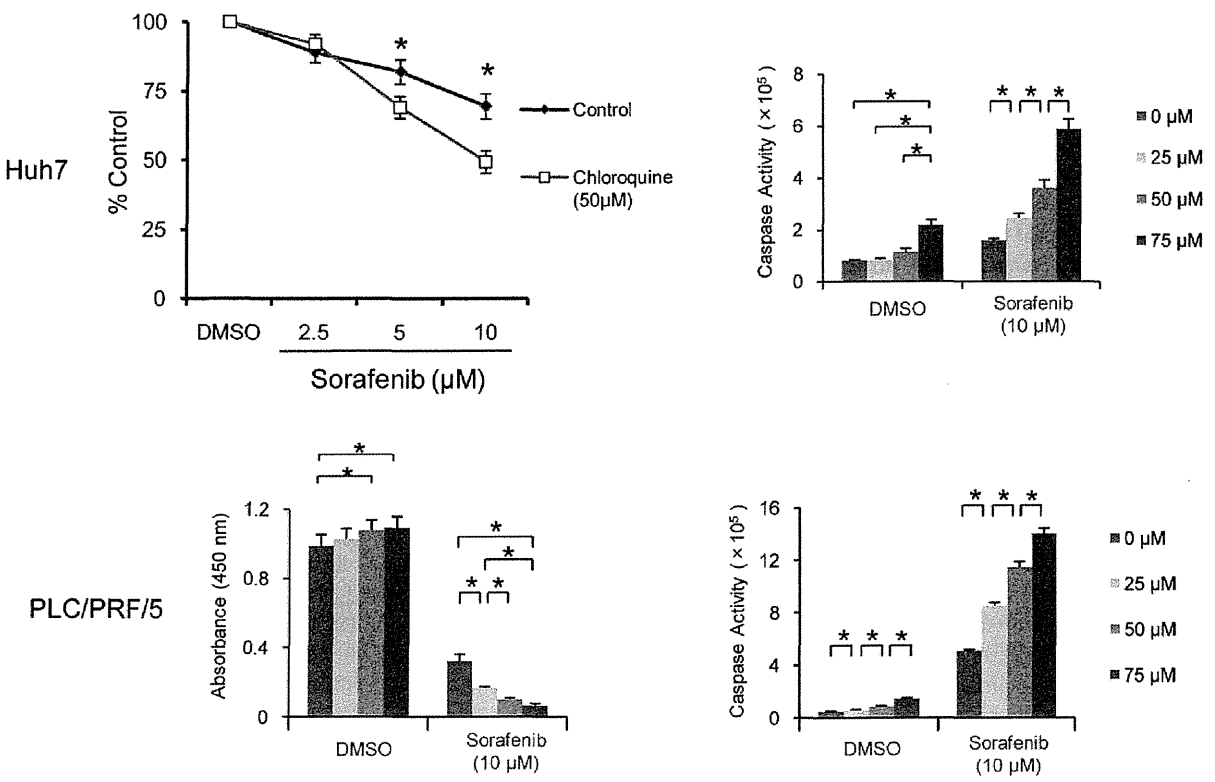

Figure 5. Pharmacological inhibition of autophagy increases sensitivity of hepatoma cells to sorafenib. Huh7 cells or PLC/PRF/5 cells were treated with or without the indicated concentration of sorafenib in the presence or absence of chloroquine for 18 hr. Capsase-3/7 activity was monitored in the supernatant, while cell growth was determined by WST assay ($n = 4$). *$p < 0.05$.

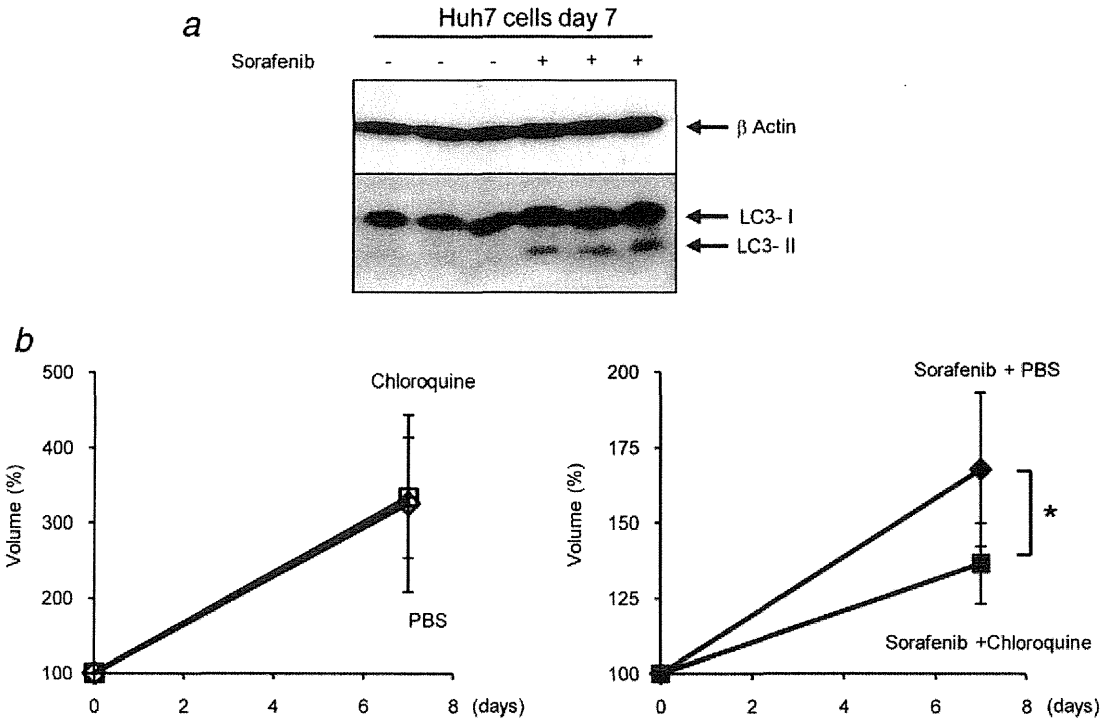

Figure 6. Inhibition of autophagy potentiates sorafenib-induced antitumor effects in Huh7 xenograft. (*a*). Western blot showing increase in LC3-II expression in Huh7 xenograft tumor after sorafenib therapy. Mice bearing xenograft tumor were administered sorafenib (30 mg kg^{-1}) or vehicle for 7 days ($n = 3$/group). (*b*). Chloroquine (60 mg kg^{-1}) itself did not affect the tumor growth of Huh7 xenograft (left panel), ($n = 7$/group), but enhanced the effect of sorafenib (30 mg kg^{-1}) in a synergistic manner (right panel), ($n = 6$/group). Mice bearing xenograft tumor were administered sorafenib and/or chloroquine for 7 days. Tumor volume at 7 days is shown as a percentage of that before initiation of the therapy. *$p < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

promote survival of hepatoma cells and thereby may be a cellular adaptive response related to primary resistance to this compound.

LC3 lipidation and its association with the isolation membranes have been established as useful signs for autophagy detectable by immunoblotting and fluorescence microscopy, facilitating research on autophagy. Previous research has shown that sorafenib induces GFP-LC3 punctate structure and LC3-II conversion in tumor cells.[9–11] However, these techniques should be analyzed more carefully, because positive results clearly indicate increased numbers of autophagosomes but do not always mean upregulation of autophagic flux.[28] For example, treatment with vinblastine or nocodazole leads to LC3 conversion and produces GFP-LC3 punctate structures, resulting from blockade of the fusion of autophagosomes and lysosomes but not from autophagy induction.[29,30] In the present study, we applied several methods including LC3 turnover assay using a lysosomal inhibitor of chloroquine or bafilomycin A1, measurement of the amount of a selective autophagy substrate p62, and observation of the mRFP-GFP color change using a fluorescent-tagged LC3 probe, to obtain evidence showing that sorafenib not only increases the number of autophagosomes but also activates the autophagic flux.

The underlying mechanisms by which sorafenib induces autophagy are not completely clear at present. In addition to the well-known target Raf/MEK/MAPK pathway, sorafenib clearly inhibited the mTORC1 pathway in the present study. Because mTOR inhibition by rapamycin or Torin1 activates autophagosome formation in hepatoma cells, sorafenib-induced inhibition of the mTORC1 pathway might be involved in sorafenib-mediated induction of autophagy. Recently, a putative tumor-suppressor gene *p53* has been shown to transactivate an autophagy-inducing gene, *dram*,[31] and *p53*-dependent induction of autophagy has been documented in response to DNA damage or reexpression of p53 in p53-negative tumor cells.[32] Because the hepatoma cells used in the present study (Huh7, HLF and PLC/PRF/5) possess mutant *p53*, sorafenib-induced adaptive autophagy could occur independently of p53. This finding may be important, because more than half of advanced HCC cases are p53-defective.[33] In such cases, our observations could be applicable and relevant.

Study of rodent carcinogenesis has revealed that autophagic protein degradation is reduced in HCC.[34] In human,

malignant HCC cell lines and HCC tissue with recurrent disease display lower autophagic activity with decreased expression of Beclin 1.[35] The autophagic pathway contributes to the growth-inhibitory effect of TGF-beta in hepatoma cells.[36] Taken together, these findings suggest that defects in autophagy may promote development or progression of HCC, focusing on the tumor suppressive or antitumor effect of autophagy in the liver or HCC. In contrast, the present study clearly showed that autophagy induced by sorafenib protects hepatoma cells from apoptotic cell death, thus shedding light on the tumor-promoting effect of autophagy in HCC. Inhibition of autophagy at both an early step (by *ATG7* knockdown) and a late step (by chloroquine treatment) sensitized hepatoma cells by converting the autophagic process to an apoptotic process. Of importance are the findings that sorafenib induced autophagy in a xenograft model and that coadministration of chloroquine and sorafenib led to better suppression of xenograft tumor than sorafenib alone. Although further study is needed to elucidate the mechanism(s) involved in autophagy-mediated protection of tumor cells, the induced autophagy might degrade the damaged or harmful cellular proteins and organelles to suppress apoptosis and promote survival of hepatoma cells under sorafenib treatment.

In conclusion, the present study demonstrates both *in vitro* and *in vivo* that sorafenib induces autophagosome formation and upregulates cellular autophagy in tumor cells, which is an adaptive response to this drug, and raises the important possibility that autophagy may be a novel target for cancer treatment with sorafenib therapy.

Acknowledgements

The authors thank David Sabatini's laboratory (Whitehead Institute for Biomedical Research) and Nathanael Gray's laboratory (Dana-Farber Cancer Institute) for providing Torin1. They also thank Bayer HealthCare Pharmaceuticals Inc. (Wayne, NJ) for providing sorafenib.

References

1. Finn RS. Drug therapy: sorafenib. *Hepatology* 2010;51:1843–9.
2. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008;359:378–90.
3. Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, Luo R, Feng J, Ye S, Yang TS, Xu J, Sun Y, et al. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009;10:25–34.
4. Yoshimori T. Autophagy: a regulated bulk degradation process inside cells. *Biochem Biophys Res Commun* 2004;313:453–8.
5. Tsujimoto Y, Shimizu S. Another way to die: autophagic programmed cell death. *Cell Death Differ* 2005;12 (Suppl 2):1528–34.
6. White E, DiPaola RS. The double-edged sword of autophagy modulation in cancer. *Clin Cancer Res* 2009;15:5308–16.
7. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, Cattoretti G, Levine B. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest* 2003;112: 1809–20.
8. Kondo Y, Kanzawa T, Sawaya R, Kondo S. The role of autophagy in cancer development and response to therapy. *Nat Rev Cancer* 2005;5: 726–34.
9. Ullén A, Farnebo M, Thyrell L, Mahmoudi S, Kharaziha P, Lennartsson L, Grandér D, Panaretakis T, Nilsson S. Sorafenib induces apoptosis and autophagy in prostate cancer cells in vitro. *Int J Oncol* 2010;37:15–20.
10. Park MA, Zhang G, Martin AP, Hamed H, Mitchell C, Hylemon PB, Graf M, Rahmani M, Ryan K, Liu X, Spiegel S, Norris J, et al. Vorinostat and sorafenib increase ER stress, autophagy and apoptosis via ceramide-dependent CD95 and PERK activation. *Cancer Biol Ther* 2008;7:1648–62.
11. Park MA, Reinehr R, Häussinger D, Voelkel-Johnson C, Ogretmen B, Yacoub A, Grant S, Dent P. Sorafenib activates CD95 and promotes autophagy and cell death via Src family kinases in gastrointestinal tumor cells. *Mol Cancer Ther* 2010;9:2220–31.
12. Shimizu S, Takehara T, Hikita H, Kodama T, Miyagi T, Hosui A, Tatsumi T, Ishida H, Noda T, Nagano H, Doki Y, Mori M, et al. The let-7 family of microRNAs inhibits Bcl-xL expression and potentiates sorafenib-induced apoptosis in human hepatocellular carcinoma. *J Hepatol* 2010; 52:698–704.
13. Kimura S, Noda T, Yoshimori T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy* 2007;3:452–60.
14. Hikita H, Takehara T, Shimizu S, Kodama T, Shigekawa M, Iwase K, Hosui A, Miyagi T, Tatsumi T, Ishida H, Li W, Kanto T, et al. The Bcl-xL inhibitor, ABT-737, efficiently induces apoptosis and suppresses growth of hepatoma cells in combination with sorafenib. *Hepatology* 2010;52:1310–21.
15. Mizushima N, Yoshimori T. How to interpret LC3 immunoblotting. *Autophagy* 2007;3:542–5.
16. Bjørkøy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, Stenmark H, Johansen T. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol* 2005;171:603–14.
17. Sridhar SS, Hedley D, Siu LL. Raf kinase as a target for anticancer therapeutics. *Mol Cancer Ther* 2005;4:677–85.
18. Liu L, Cao Y, Chen C, Zhang X, McNabola A, Wilkie D, Wilhelm S, Lynch M, Carter C. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. *Cancer Res* 2006;66:11851–8.
19. Blechacz BR, Smoot RL, Bronk SF, Werneburg NW, Sirica AE, Gores GJ. Sorafenib inhibits signal transducer and activator of transcription-3 signaling in cholangiocarcinoma cells by activating the phosphatase shatterproof 2. *Hepatology* 2009;50:1861–70.
20. Díaz-Troya S, Pérez-Pérez ME, Florencio FJ, Crespo JL. The role of TOR in autophagy regulation from yeast to plants and mammals. *Autophagy* 2008;4:851–65.
21. Gingras AC, Gygi SP, Raught B, Polakiewicz RD, Abraham RT, Hoekstra MF, Aebersold R, Sonenberg N. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev* 1999;13:1422–37.
22. Foster KG, Fingar DC. Mammalian target of rapamycin (mTOR): conducting the cellular signaling symphony. *J Biol Chem* 2010;285: 14071–7.
23. Thoreen CC, Kang SA, Chang JW, Liu Q, Zhang J, Gao Y, Reichling LJ, Sim T, Sabatini DM, Gray NS. An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. *J Biol Chem* 2009;284: 8023–32.
24. Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. *Cell Death Differ* 2004;11:448–57.
25. Paglin S, Hollister T, Delohery T, Hackett N, McMahill M, Sphicas E, Domingo D, Yahalom J. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res* 2001;61:439–44.
26. Tormo D, Checińska A, Alonso-Curbelo D, Pérez-Guijarro E, Cañón E, Riveiro-Falkenbach E, Calvo TG, Larribere L, Megías D, Mulero F, Piris

MA, Dash R, et al. Targeted activation of innate immunity for therapeutic induction of autophagy and apoptosis in melanoma cells. *Cancer Cell* 2009;16:103–14.
27. Goussetis DJ, Altman JK, Glaser H, McNeer JL, Tallman MS, Platanias LC. Autophagy is a critical mechanism for the induction of the antileukemic effects of arsenic trioxide. *J Biol Chem* 2010;285: 29989–97.
28. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. *Cell* 2010;140: 313–26.
29. Seglen PO, Brinchmann MF. Purification of autophagosomes from rat hepatocytes. *Autophagy* 2010;6:542–7.
30. Bampton ET, Goemans CG, Niranjan D, Mizushima N, Tolkovsky AM. The dynamics of autophagy visualized in live cells: from autophagosome formation to fusion with endo/lysosomes. *Autophagy* 2005;1:23–36.
31. Crighton D, Wilkinson S, O'Prey J, Syed N, Smith P, Harrison PR, Gasco M, Garrone O, Crook T, Ryan KM. DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. *Cell* 2006;126:121–34.
32. Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI, Thomas-Tikhonenko A, Thompson CB. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. *J Clin Invest* 2007;117:326–36.
33. Hussain SP, Schwank J, Staib F, Wang XW, Harris CC. TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. *Oncogene* 2007;26: 2166–76.
34. Kisen GO, Tessitore L, Costelli P, Gordon PB, Schwarze PE, Baccino FM, Seglen PO. Reduced autophagic activity in primary rat hepatocellular carcinoma and ascites hepatoma cells. *Carcinogenesis* 1993;14: 2501–5.
35. Ding ZB, Shi YH, Zhou J, Qiu SJ, Xu Y, Dai Z, Shi GM, Wang XY, Ke AW, Wu B, Fan J. Association of autophagy defect with a malignant phenotype and poor prognosis of hepatocellular carcinoma. *Cancer Res* 2008;68: 9167–75.
36. Kiyono K, Suzuki HI, Matsuyama H, Morishita Y, Komuro A, Kano MR, Sugimoto K, Miyazono K. Autophagy is activated by TGF-beta and potentiates TGF-beta-mediated growth inhibition in human hepatocellular carcinoma cells. *Cancer Res* 2009;69:8844–52.

Hepatology Research

Hepatology Research 2012; 42: 627–636 doi: 10.1111/j.1872-034X.2012.00998.x

Special Report

Prevention of hepatitis B virus reactivation in patients receiving immunosuppressive therapy or chemotherapy

Makoto Oketani, Akio Ido, Hirofumi Uto and Hirohito Tsubouchi

Department of Digestive and Lifestyle-related Diseases, Health Research Course, Human and Environmental Sciences, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan

With the increasing use of potent immunosuppressive therapy, reactivation of hepatitis B virus (HBV) in endemic regions is becoming a clinical problem requiring special attention. A recent annual nationwide survey clarified that HBV reactivation related to immunosuppressive therapy has been increasing in patients with malignant lymphoma, other hematological malignancies, oncological or rheumatological disease. In the survey, rituximab plus steroid-containing chemotherapy was identified as a risk factor for HBV reactivation in hepatitis B surface antigen (HBsAg) negative patients with malignant lymphoma. In this setting, HBV reactivation resulted in fatal fulminant hepatitis regardless of the treatment of nucleoside analog. The Intractable Hepatobiliary Disease Study Group and the Study Group for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis jointly developed guidelines for preventing HBV reactivation. The essential features of the guideline are as follows. All patients should be screened for HBsAg by a sensitive method before the start of immunosuppressive therapy. Second, hepatitis B core antigen (HBcAb) and hepatitis B surface antibody (HBsAb) testing should be performed in HBsAg negative patients, especially those receiving intensive immunosuppressive therapy. Prophylaxis with nucleoside analogs is essential for preventing HBV reactivation in HBsAg positive patients. In contrast, HBsAg negative with HBcAb and/or HBsAb positive patients should be monitored monthly for an increase in serum HBV DNA during and 12 months after completion of chemotherapy. Nucleoside analogs should be administrated immediately when HBV DNA becomes positive during this period. This strategy facilitates commencement of nucleoside analogs at an early stage of HBV reactivation and results in prevention of severe hepatitis.

Key words: de novo hepatitis, fulminant hepatitis, hepatitis B virus reactivation, immunosuppressive therapy, rituximab

INTRODUCTION

HEPATITIS B VIRUS (HBV) is the most frequently identified agent that causes acute or chronic hepatitis in Eastern Asia. In Japan, approximately 970 000 people are infected with HBV, as estimated by hepatitis B surface antigen (HBsAg) testing in blood donors.[1] Chronic HBV carriers have a 15–40% lifetime risk of developing serious complications of chronic liver disease.[2] However, most carriers remain clinically silent for extended periods and some carriers will lose HBsAg over a long lifetime. In adults, most acute HBV infections are self-limited, and recovery occurs naturally. Seroconversion from acute HBV infection with HBsAg to antibody to hepatitis B surface antibody (HBsAb) is believed to represent viral clearance. Clearance of HBsAg and appearance of antibody to hepatitis B core antibody (HBcAb) with or without HBsAb provides evidence of resolved infection in patients. However, with the advent of sensitive polymerase chain reaction techniques for detecting HBV DNA in serum and liver, it has been shown that most HBsAb/HBcAb positive patients have HBV DNA in the liver and/or serum. It is estimated that 2 billion people worldwide have been infected with HBV.[3] In Japan, it is reported that 23.2% of blood donors are positive for HBcAb and/or HBsAb.[4]

Reactivation of HBV is a well-recognized complication in HBsAg positive patients who are undergoing immunosuppressive chemotherapy for cancer. The clinical manifestation ranges from subclinical hepatitis to severe, potentially fatal fulminant hepatitis. In this decade, HBV reactivation has been observed in patients

Correspondence: Dr Makoto Oketani, Department of Digestive and Lifestyle-related Diseases, Health Research Course, Human and Environmental Sciences, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan. Email: oketani@m2.kagoshima-u.ac.jp
Received 5 December 2011; revision 1 March 2012; accepted 4 March 2012.

with resolved infection (HBsAg negative and HBcAb and/or HBsAb positive) who have undergone intensive immunosuppressive chemotherapy such as rituximab plus steroid-containing chemotherapy. Previous reports have clarified that this combination therapy can lead to fulminant hepatitis and even death. For this reason, clinicians need to be aware of HBV reactivation not only in patients with current infection but also in those with resolved infection who are undergoing intensive immunosuppressive therapy.

This report summarizes the important issues related to HBV reactivation and suggests a guideline for preventing this condition in the clinical setting.

HBV REACTIVATION IN HBsAg POSITIVE CARRIERS

LIFE IN HBV carriers can generally be divided into four distinct phases: (i) immune tolerance phase; (ii) immune active phase; (iii) low-replication phase; and (iv) resolved phase. Inactive carriers in the low-replication phase are frequently associated with antibodies to hepatitis B e antigen (anti-HBe) seroconversion with a low viral load (<4.0 log copies/mL).[5] Sustained host immune control over viral replication in the low-replication phase may lead to HBsAg seroclearance. During the low-replication phase, 20–30% of patients may develop spontaneous HBV reactivation.[6] Patients with chronic HBV infection receiving immunosuppressive chemotherapy usually have impaired host immunity that may allow active HBV replication to occur. Following the completion of therapy, restoration of host immunity against HBV occurs, resulting in extensive cytotoxic T-cell-mediated lysis of the infected hepatocytes and clinical hepatitis flares. Some patients experience severe hepatitis with HBV reactivation, with fatality rates ranging 5–40%.[7–10]

The risk of HBV reactivation is mainly related to the underlying disease, intent of the immunosuppression and HBV replicative state. The risk is particularly high in patients with lymphoma.[11,12] Patients with other hematological malignancy such as multiple myeloma and B-cell chronic lymphocytic leukemia are also at risk.[13] Patients receiving intensive cytoreductive therapy and high-dose chemotherapy are highly susceptible to HBV reactivation. In non-hematological tumors, the rate of HBV reactivation is high in patients with breast cancer.[14,15] HBV reactivation can also occur in patients with non-malignant disease, such as rheumatological disease and collagen disease.[13]

The use of chemotherapy regimens containing corticosteroids or anthracycline increases the risk of HBV reactivation.[7,11,12,16,17] HBV DNA contains a glucocorticoid responsive element that has been suggested to facilitate HBV replication.[18,19] Anthracycline has also been demonstrated *in vitro* to stimulate HBV DNA replication.[20] Recently, the use of the anti-CD20 monoclonal antibody, rituximab, appears to be an independent risk factor of HBV reactivation.[21] This agent causes profound and long-lasting immunosuppression, reflecting a decrease in CD20 cells and HBsAb titer.[22] There are many reports of HBV reactivation following the use of rituximab as monotherapy[23] or in combination with other types of chemotherapy.[24–27]

REACTIVATION IN HBsAg NEGATIVE PATIENTS WITH HBcAb AND/OR HBsAb

IN PATIENTS WITH resolved HBV infection (HBsAg negative, HBsAb and/or HBcAb positive), HBV replication has been shown to persist in the liver and in peripheral blood mononuclear cells.[28,29] Cellular and humoral immune surveillance suppresses viral replication.[30,31] In the life cycle of HBV replication, covalently closed circular DNA (cccDNA) is formed in the nuclei of infected hepatocytes. cccDNA is the main template for the transcription of viral mRNA and has been shown to persist in the liver.[21] With impairment of the host defense system, cccDNA can evade host immunity and actively replicate again. This scenario of HBV reactivation in patients with resolved infection of so-called "de novo hepatitis B" appears to be a new clinical issue.

Hepatitis B virus reactivation has been reported in this setting after transplantation and allogenic and autologous hematopoietic stem-cell transplantation with the reappearance of HBsAg.[32–34] In recent years, the incorporation of rituximab with standard chemotherapy is associated with HBV reactivation in patients with non-Hodgkin's lymphoid malignancies. Hui *et al.* reported that the incidence of HBV reactivation with a combination of rituximab plus steroids was higher (12.2%, 6/49) compared with other combinations of therapy (1.0%, 2/195).[35] Recently, Yeo *et al.* reported that 23.8% of HBsAg negative/HBcAb positive lymphoma patients receiving rituximab plus steroid combination therapy developed HBV reactivation.[27] It is notable that there are a number of case reports of fatal hepatitis in HBcAb positive patients who received rituximab-containing chemotherapy for lymphoma.[24–27,36] Umemura *et al.* reported that the rate of fulminant hepatitis and

Table 1 Causes of HBV-related fulminant hepatitis and late-onset hepatic failure (LOHF)

	Total	Years					
		2004	2005	2006	2007	2008	2009
All patients	194 (9)	26 (2)	42 (2)	27	37 (1)	23 (2)	39 (2)
Transient infection	91 (1)	12 (1)	23	13	17	11	15
Carrier	72 (7)	9 (1)	11 (1)	9	14 (1)	11 (2)	18 (2)
Inactive carrier	35 (1)	6	7	3	5	3	11 (1)
Reactivation (inactive carrier)	20 (5)	2	3 (1)	1	4 (1)	5 (2)	5 (1)
Reactivation (resolved infection)	17 (1)	1 (1)	1	5	5	3	2
Undetermined	31 (1)	5	8 (1)	5	6	1	6

Data shown indicate the number of patients, and those in parentheses indicate the number of patients with LOHF.

mortality following de novo hepatitis B is high compared with acute hepatitis B in Japan.[37]

FULMINANT HEPATITIS CAUSED BY HBV REACTIVATION IN JAPAN

THE INTRACTABLE HEPATOBILIARY Diseases Study Group in Japan annually performs a nationwide survey of patients with fulminant hepatitis and late-onset hepatic failure (LOHF).[38] A recent annual nationwide survey from 2004 to 2009 revealed that HBV infection prevailed in 39.8% (194/488) of patients with fulminant hepatitis and LOHF. It is noteworthy that 19.1% (37/194) of HBV related-hepatitis was caused by HBV reactivation following immunosuppressive therapy or chemotherapy (Table 1). Furthermore, almost half of these patients have evidence of HBV reactivation from resolved infection (HBsAg negative before the start of therapy and HBsAg positive and HBcAb and/or HBsAb positive at the onset of hepatitis). The total number of patients with HBV reactivation has been increasing since 2004. We first compared the clinical features of 37 patients with HBV reactivation with those of transient infection and those with acute exacerbation (Table 2). The age of the patients was higher in the HBV reactivation group than that in the transient infection

Table 2 Clinical characteristics of patients with hepatitis B virus (HBV) reactivation, compared with those of patients with transient infection and HBV carriers who developed spontaneous acute exacerbation

	Transient infection ($n = 91$)	Acute exacerbation in HBV carriers ($n = 35$)	HBV reactivation ($n = 37$)
Age, years, median (range)	46 (17–72)	53 (15–89)	64 (29–86)**††
Male/female	58/33	23/12	22/15
Disease types (F-A/F-SA/LOHF)	80/10/1	14/20/1**	4/27/6**††
Prognosis (alive/died/LT)	40/36/15	7/18/10*	2/33/2**††
ALT, IU/L (mean ± SD)	4207 ± 2725	989 ± 1183**	902 ± 1380**
Total bilirubin, mg/dL (mean ± SD)	10.8 ± 8.2	15.2 ± 10.3*	15.8 ± 7.7**
Prothrombin time (%), median (range)	18.4 (3.1–58.6)	24.9 (2.2–58.1)**	29.8 (8.0–48.0)**
HBV DNA level, log copies/mL (mean ± SD)	5.6 ± 1.4	6.3 ± 1.7*	7.2 ± 1.4**†
Treatment			
Lamivudine	57 (63)	19 (54)	22 (59)
Entecavir	29 (32)	16 (46)	18 (49)
Interferon	28 (31)	11 (31)	11 (30)

Unless otherwise indicated, data indicate the number of patients, and those in parenthesis indicate percentages of patients.
Laboratory data are at the onset of hepatic encephalopathy of coma grade greater than II. HBV DNA levels are at the onset of hepatitis.
Significant difference among group was assessed by Student's *t*-test, Mann–Whitney *U*-test and χ^2-test.
Values significantly difference from patients with transient infection; $*P < 0.05$, $**P < 0.01$.
Values significantly different from patients with acute exacerbation in HBV carriers; $\dagger P < 0.05$, $\dagger\dagger P < 0.01$.
ALT, alanine aminotransferase; F-A, acute type fulminant hepatitis; F-SA, subacute type fulminant hepatitis; LOHF, late-onset hepatic failure; LT, liver transplantation; SD, standard deviation.

and acute exacerbation groups. There was a tendency for the reactivation group to show clinical manifestation of the subacute type or LOHF. The reactivation group had lower alanine aminotransferase (ALT) levels and higher bilirubin and HBV DNA levels. Of the 37 cases of HBV reactivation, 33 (89%) resulted in liver-related death, two (5%) survived and two(5%) received living-donor liver transplantation. We then compared the clinical features of the 20 patients with HBV reactivation in HBsAg positive carrier status with those of the 17 patients with HBsAg negative resolved HBV infection status (Table 3). The resolved infection group was older than the carrier group. Most of the resolved infection group showed clinical manifestation as subacute type. The resolved infection group had lower ALT levels and higher bilirubin levels than those in the carrier group. It is noteworthy that all patients with resolved infection who developed HBV reactivation died despite nucleoside analog treatment. Concerning underlying disease, non-Hodgkin's lymphoma or mucosa-associated lymphoid tissue lymphoma was most prevalent in 50% of the carrier group and in 76% of the resolved infection group, respectively. In the carrier group, there were patients with oncological, rheumatological or collagen disease. HBV reactivation occurred more frequently after immunosuppressive therapy in patients with resolved infection, as previously reported.[39] Rituximab plus steroids combination chemotherapy was administrated to 35% of patients in the carrier group and to 59% of patients in the resolved infection group, respectively. Corticosteroid was used as monotherapy or in combination therapy in approximately three quarters of both groups. Methotrexate and anthracycline antitumor agent were given in 10% in the carrier group.

PREVENTION OF HBV REACTIVATION FOLLOWING IMMUNOSUPPRESSIVE CHEMOTHERAPY

HBsAg positive patients

BECAUSE VIRAL REPLICATION precedes clinical evidence of hepatitis, it is efficacious to use nucleoside analogs in a prophylactic manner before the start of chemotherapy. Previous retrospective and prospective studies have shown that the risk of HBV reactivation can be greatly reduced by the use of prophylactic nucleoside analog therapy for susceptible patients.[15,40–43] In Japan, currently, there are three oral nucleoside analogs approved for the treatment of chronic hepatitis B (lamivudine, adefovir and entecavir). Concerning lamivudine, the drug has proven efficacy and safety in preventing HBV reactivation related to chemotherapy. However, a major problem with its prolonged use is the possibility of viral breakthrough following the emergence of treatment-resistant HBV variants with YMDD mutations.[44] Given their high potency and extremely low rates of drug resistance, new generation oral nucleoside analogs, such as entecavir or tenofovir, are anticipated to be effective for HBV reactivation. The incidence of entecavir resistance in nucleos(t)ide analog-naive patients is reported to be 1.2% at 3 years.[45–47] A recent report demonstrated that entecavir is effective in the prevention of HBV reactivation in cancer patients.[48,49]

The American Association for the Study of Liver Disease (AASLD) guidelines recommend that if the anticipated duration of treatment is less than 1 year and baseline serum HBV DNA is not detectable, lamivudine or telbivudine are desirable and in other cases, entecavir or tenofovir are desirable.[50] The European Association for the Study of the Liver (EASL) guidelines also recommend the use of lamivudine for patients with low HBV DNA and entecavir or tenofovir for patients with high HBV DNA.[51] A consensus of the Japan Society of Hepatology recommends the use of entecavir as the first-line drug when patients with chronic hepatitis B are treated.[52]

Although the optimal time point for the initiation of antiviral prophylaxis has not been clearly established, nucleoside analogs should ideally be started as early as possible before chemotherapy.[53] This strategy can prevent any increase in viral replication, reduce the likelihood of drug resistance, allow chemotherapy to be completed and minimize the risk of hepatitis flare-up once chemotherapy is stopped.

Another concern with the use of lamivudine has been the occurrence of hepatic flares upon cessation of the antiviral compound. The optimal duration of antiviral prophylaxis in HBsAg carriers receiving immunosuppressive chemotherapy has only partly been clarified and is under active investigation. Several cases of HBV reactivation and even fatal fulminant hepatitis have been reported when lamivudine was stopped less than 3 months after the completion of chemotherapy.[54] AASLD guidelines recommend that prophylaxis is discontinued 6 months after completion of chemotherapy in patients with baseline HBV DNA of less than 2000 IU/mL, otherwise prophylaxis continues. EASL guidelines recommend the same treatment as that for AASLD guidelines for 12 months after completion of chemotherapy.